eISSN: 2564-6524
ISSN: 1015-3918 (1971-2010)

ANKARA ÜNİVERSİTESİ

ECZACILIK FAKÜLTESİ DERGİSİ

JOURNAL OF FACULTY OF PHARMACY OF ANKARA UNIVERSITY

Cilt / Vol : 46
Sayı / No : 3
Yıl / Year : 2022

eISSN: 2564-6524
ISSN: 1015-3918 (1971-2010)

ANKARA ÜNİVERSİTESİ

ECZACILIK FAKÜLTESİ DERGİSİ

JOURNAL OF FACULTY OF PHARMACY OF ANKARA UNIVERSITY

Cilt / Vol: 46
Sayı / No: 3
Yıl / Year: 2022

ANKARA ÜNİVERSİTESİ ECZACILIK FAKÜLTESİ DERGİSİ

(*Ankara Ecz. Fak. Derg.*) **eISSN: 2564-6524**
ISSN: 1015-3918 (1971-2010)

Sahibi:

Prof. Dr. Asuman BOZKIR

Baş Editör:

Prof. Dr. İlkay YILDIZ

Ankara Üniversitesi, Eczacılık Fakültesi, Farmasötik Kimya Anabilim Dalı,
06560 Yenimahalle-ANKARA,
Tel: 0 312 203 30 69
Faks: 0 312 213 10 81
e-posta: iyildiz@pharmacy.ankara.edu.tr
efd.editor@ankara.edu.tr

Yardımcı Editörler:

Prof. Dr. Canan HASÇİÇEK
Ankara Üniversitesi, Eczacılık Fakültesi, Farmasötik Teknoloji Anabilim Dalı
e-posta: cogan@pharmacy.ankara.edu.tr

Doç. Dr. Işıl ÖZAKÇA GÜNDÜZ
Ankara Üniversitesi, Eczacılık Fakültesi, Farmakoloji Anabilim Dalı
e-posta: ozakca@ankara.edu.tr

Dr. Aysu SELÇUK
Ankara Üniversitesi, Eczacılık Fakültesi, Klinik Eczacılık Anabilim Dalı
e-posta: aysuselcuk@ankara.edu.tr

Dr. M. Mesud HÜRKUL
Ankara Üniversitesi, Eczacılık Fakültesi, Farmasötik Botanik Anabilim Dalı
e-posta: mhurkul@ankara.edu.tr

Alan Editörleri:

Editoryal Danışma Kurulu:

Ankara Üniversitesi Eczacılık Fakültesi Dergisi (*Ankara Ecz. Fak. Derg.*) Ankara Üniversitesi Eczacılık Fakültesi'nin resmi bilimsel bir dergisidir. 1971 ve 2010 yılları arasında basılı olarak yayımlanmıştır.

Ankara Üniversitesi Eczacılık Fakültesi Dergisi yılda 3 sayı olarak (Ocak-Mayıs-Eylül) yayımlanır. Bu dergi açık erişim, hakemli bir dergi olup, Türkçe veya İngilizce olarak farmasötik bilimler alanındaki önemli gelişmeleri içeren orijinal araştırmalar, derlemeler ve kısa bildiriler için uluslararası bir yayın ortamıdır. Yayımlanan yazıların sorumluluğu yazar(lar)ına aittir. Dergiye gönderilen makalelerin daha önce tamamen veya kısmen başka bir yerde yayımlanmamış veya yayımı için başka bir yere başvuruda bulunulmamış olması gereklidir. Makaleler derginin yazım kurallarına uymalıdır.

Tarandığı İndeksler

- Scopus
- Google Scholar (GS)
- Excerpta Medica Database (EMBASE)
- Scimago Journal & Country Rank (SJR)
- OpenAIRE
- UDLEdge (i-Focus, i-Future, i- Journals)
- TR Dizin

Web adresi: http://journal.pharmacy.ankara.edu.tr/

JOURNAL OF FACULTY OF PHARMACY OF ANKARA UNIVERSITY

(*J. Fac. Pharm. Ankara*) **eISSN: 2564-6524**
ISSN: 1015-3918 (1971-2010)

Editor-in-Chief:

Prof. Dr. İlkay YILDIZ

Ankara University Faculty of Pharmacy, Department of Pharmaceutical Chemistry
06560 Yenimahalle-Ankara/TURKEY
Tel: 0 312 203 30 69
Fax: 0 312 213 10 81
e-mail:iyildiz@pharmacy.ankara.edu.tr
efd.editor@ankara.edu.tr

Editorial Advisory Board:

Journal of Faculty of Pharmacy of Ankara University (*J. Fac. Pharm. Ankara*) is official scientific journal of Ankara University Faculty of Pharmacy. It was published between 1971 and 2010 as a print.

Journal of Faculty of Pharmacy of Ankara University is published three times (January-May-September) a year. It is an international medium, an open access, peer-reviewed journal for the publication of original research reports, reviews and short communications in English or Turkish on relevant developments in pharmaceutical sciences. All the articles appeared in this journal are published on the responsibility of the author(s). The manuscript submitted to the journal should not be published previously as a whole or in part and not be submitted elsewhere. The manuscripts should be prepared in accordance with the requirements specified.

Indexing and Abstracting

- Scopus
- Google Scholar (GS)
- Excerpta Medica Database (EMBASE)
- Scimago Journal & Country Rank (SJR)
- OpenAIRE
- UDLEdge (i-Focus, i-Future, i- Journals)
- TR Dizin

Web address: http://journal.pharmacy.ankara.edu.tr/

İÇİNDEKİLER / CONTENTS 46(3), 2022

Özgün Makaleler / Original Articles ***Sayfa / Page***

Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 46(3): 664-683, 2022
Doi: 10.33483/jfpau.1084463

ÖZGÜN MAKALE / ORIGINAL ARTICLE

TÜRKİYE'DE 4 VE 5 YILLIK ECZACILIK EĞİTİMİNİN KARŞILAŞTIRILMASI

COMPARISON OF 4 YEAR AND 5 YEAR PHARMACY DEGREE EDUCATION IN TURKEY

Büşra YILDIZ[1], Muammer ÇALIKUŞU[2*], Gülbin ÖZÇELİKAY[2]

[1]Ege Eczanesi, 21100, Diyarbakır, Türkiye

[2]Ankara Üniversitesi, Eczacılık Fakültesi, Eczacılık İşletmeciliği Anabilim Dalı, 06560, Ankara, Türkiye

ÖZ

Amaç: *Bu çalışmada, 4 ve 5 yıllık eczacılık programından mezun olan eczacıların aldıkları eğitim hakkındaki görüşleri karşılaştırılarak; 5 yıllık eczacılık eğitiminin amacına ulaşıp ulaşmadığı tartışılmaktadır.*

Gereç ve Yöntem: *Araştırmada karma bir metot kullanılmış; nicel araştırmaya anket tekniği ile sorulan sorulara farklı eczacılık fakültelerinden mezun 160 eczacı katılmıştır. Nitel araştırmada ise 3 ayrı oturumda 24 eczacı ile odak grup görüşmesi gerçekleştirilmiştir.*

Sonuç ve Tartışma: *Araştırma sonucunda 5 yıllık eğitimde 4 yıllık eğitime göre olumlu yönde değişim gözlenmekle birlikte halen geliştirilmesi gereken alanların olduğu görülmüştür. Örneğin; hasta odaklı eğitim artmış, ancak yeterli olmamıştır. Klasik öğretim yöntemleri yanında, daha akılda kalıcı, probleme dayalı yöntemlerin kullanılmasının uygun olacağı, staj uygulamalarının ve denetiminin daha fazla yapılarak devam etmesinin önemi ve son olarak kariyer yönetiminin 5 yıllık eğitimde daha iyi yapıldığı belirlenmiştir. Tespit edilen eksikliklerin tamamlanması ve araştırmaların artırılması önerilmektedir.*

Anahtar Kelimeler: *Eczacılık, eczacılık eğitimi, eğitim içeriği, eğitim verimliliği*

ABSTRACT

Objective: *In this study, by comparing the views of pharmacists who graduated from 4-year pharmacy degree and 5-year pharmacy degree is discussed whether the 5-year pharmacy degree education achieves its purpose.*

Material and Method: *A mixed method was used in the research. In the quantitative research, 160 pharmacists graduated from different Pharmacy Faculties participated in the questions asked by the questionnaire technique. In the qualitative research, focus group discussions were held with 24 pharmacists in 3 separate sessions*

* **Sorumlu Yazar / Corresponding Author:** Muammer Çalıkuşu
e-posta / e-mail: mcalikusu@ankara.edu.tr, **Tel. / Phone:** +903122033129

Gönderilme / Submitted: 08.03.2021 **Kabul / Accepted:** 09.05.2022

Result and Discussion: *As a result, it has been shown that nanocrystals can perform similar imaging by using less radiocontrast materials with their unique properties. In this way, it is likely to cause fewer side effects and/or toxic effects due to the lower dosage. In the light of these successful results, it is planned to detail this study with clinical trials in the future. As a result of the research, although statistically positive changes were observed in 5-year education compared to 4-year education, it was seen that there are still areas that need improvement. Such as patient-oriented education has increased, but not enough. In addition to classical teaching methods, it was determined that it would be appropriate to use more catchy and problem-based methods, the importance of continuing internship practices and supervision, and finally, career management was better done in 5-year education. It is recommended to complete the identified deficiencies and increase the research.*

Keywords: *Educational content, educational efficiency, pharmacy, pharmacy education*

GİRİŞ

Son yıllarda ülkemizdeki eczacılık fakültelerinin sayısı hızlı bir şekilde artmaktadır. Bu artış, yeni kurulan eczacılık fakültelerinde kaliteli eczacılık lisans eğitimi için gerekli alt yapı olanaklarının ve eğitim standartlarının değerlendirilmesi ihtiyacını ortaya çıkarmıştır [1].

Eczacılık fakültesinde lisans eğitimi 1938 yılından 2005–2006 eğitim-öğretim yılına kadar 4 yıl olarak sürdürülmüş ve mezunlara eczacılık lisans diploması ile eczacı unvanı verilmiştir [2]. Avrupa Birliği'nin 85/432/EEC ve 85/433/EEC direktifleri, eczacıların Avrupa'da serbest dolaşımını ve diplomaların, sertifikaların karşılıklı tanınmasına ilişkindir [3,4]. Bu bağlamda, Türkiye'de Dekanlar Konseyi ve meslek örgütü tarafından eczacılık eğitiminin taşıması gereken koşullar ve eğitim süresi yeniden gözden geçirilmiş ve değişiklikler yapılmasına karar verilmiştir. Eczacılık eğitim programı 2005–2006 eğitim-öğretim yılından itibaren 5 yıl olarak uygulanmaya başlanmıştır. Eczacılık eğitiminde yaşanan bu gelişmeler, eczacının görev ve sorumluluklarının hem ürün hem de hasta odaklı hizmetler olarak genişletilmesi gerekliliği dolayısıyla eczacılık fakülteleri tarafından verilen eğitim içeriğinde yeniliklere yol açmıştır [1].

Avrupa Komisyonu Eczacılık Eğitimi Tavsiye Komitesi'nin öngörüleri göz önüne alınarak hazırlanmış program esas alınarak daha iyi bir eczacılık hizmeti verebilecek eczacıların yetiştirilebilmesi amacıyla ders programlarına yeni bazı zorunlu ve seçmeli dersler ilave edilmiş, bazı derslerin ders saati düşürülmüştür. Beş yıllık eczacılık programı kapsamında ilk 4 yılda çekirdek bir müfredat programı uygulanmaktadır. Beşinci yılda ise öğrenciler; eczane, hastane, endüstri eczacılığı alanlarından bir tanesini tercih ederek, alanda açılan seçmeli derslerden tercihlerine bağlı olarak alabilmektedir. Buna ek olarak son sınıfta bir mezuniyet projesi hazırlamaktadır. 5 yıllık eğitim süresinde 6 aylık staj zorunludur. Stajın bir kısmı 2., 3. ve 4. yıl eğitim-öğretim dönemlerini takiben yaz aylarında periyotlar halinde; son kısmı ise dokuzuncu ve/veya onuncu yarıyılda eğitim-öğretim dönemi sırasında olmak üzere serbest eczanelerde, hastanelerde, ilaç fabrikaları ve ar-ge merkezlerinde yapılabilmektedir [5].

2015 yılında eczacılık fakültelerinin fiziksel koşulları ve alt yapısının eğitim kalitesini asgari düzeyde sağlamak amacıyla Ulusal Eczacılık Çekirdek Eğitim Programı, Yüksek Öğretim Kurulu (YÖK) tarafından kabul edilmiştir [6]. Ulusal çekirdek eğitim programlarında yer verilen yeterlilikler, YÖK'ün 2 Şubat 2008 tarih/26775 sayılı Resmi Gazete'de yayınlanan ve 31 Aralık 2009 tarih/27449 sayılı ve 25 Mayıs 2018 tarih/30431 sayılı Resmi Gazete'de güncellenen "Doktorluk, Hemşirelik, Ebelik, Diş Hekimliği, Veterinerlik, Eczacılık ve Mimarlık Eğitim Programlarının Asgari Eğitim Koşullarının Belirlenmesine Dair Yönetmelik", 12 Nisan 2014 tarih/28970 sayılı Resmi Gazete'de yayınlanan "Eczacılar ve Eczaneler Hakkında Yönetmelik" ile 22 Mayıs 2014 tarih/29007 sayılı Resmi Gazete'de yayınlanan yönetmeliğin "Sağlık Meslek Mensuplarının İş ve Görev Tanımları" ekinde eczacının görev, yetki ve sorumlulukları dikkate alınarak Ulusal Eczacılık Eğitimi Akreditasyon Kurulu (ECZAK) tarafından yayınlanan Türkiye Ulusal Eczacılık Lisans Eğitimi Programı Akreditasyon Standartları ve Kılavuzları, dünyada ve ülkemizde eczacılık alanındaki gelişmeler ve Türkiye Yüksek Öğrenim Yeterlilikler Çerçevesi esas alınarak hazırlanmıştır [7-13].

Bu çalışmada, 4 ve 5 yıllık eczacılık programından mezun olan eczacıların aldıkları eğitim hakkındaki görüşleri karşılaştırılarak; 5 yıllık eczacılık eğitiminin amacına ulaşıp ulaşmadığı tartışılmaktadır. Günümüzde bazı Avrupa ülkelerinde eczacılık eğitiminin 6 yıl olması gündemde iken Türkiye'de ilk kez bu konu ortaya konmaya çalışılmıştır.

GEREÇ VE YÖNTEM

Araştırmada karma bir metod kullanılmıştır. Araştırmanın ilk bölümü niceliksel anket yöntemi, ikinci bölümü niteliksel yapılandırılmış odak grup görüşmeleri şeklinde yapılmıştır.

Anket ve odak grup görüşmeleri için hazırlanan sorular, YÖK tarafından kabul edilen ulusal eczacılık programı yeterlilikleri, eczacılık eğitim içeriği ve süresinin 5 yıla çıkması konusunun tartışıldığı Türk Eczacıları Birliği tarafından 1995 yılında yapılan 1. Eczacılık Eğitimi Kurultayı sonuç raporunda belirtilen konu başlıkları esas alınarak araştırmacılar tarafından hazırlanmıştır.

Çalışma için Ankara Üniversitesi Rektörlüğü Etik Kurulu'ndan 26.04 2021 tarih ve 82 sayılı kararı ile etik kurul onayı alınmıştır. Etik kurul onayından sonra 30.04.2021-15.05.2021 tarihleri arasında nicel araştırma verileri; buna paralel olarak nitel araştırma verileri ise 03.05.2021-17.05.2021 tarihleri arasında elde edilmiştir. Anket için öncelikle soru/madde havuzu oluşturulmuş, hazırlanan sorular hakkında 10 uzman görüşü alınmış ve ön uygulama yapılmıştır. Anket formları, çevrimiçi olarak çalışmaya gönüllü olarak katılmak isteyen eczacılara uygulanmıştır. Türkiye'de bulunan yaklaşık 36.000 eczacıyı temsil edecek örneklem büyüklüğü, 0.10 örneklem hata payı ve olayın görülme/görülmeme sıklıkları p:0.50, q:0.50 olması durumuna göre 96 olarak belirlenmiştir [14,15].

Çalışmadaki soruların ana başlıkları; genel eğitim içeriği, hasta ve ürün odaklı hizmetler için kazanılan beceriler, öğretim yöntemleri, sosyal ve kültürel yön geliştirici faaliyetler, staj, kariyer planlama ve bitirme projesinden oluşmaktadır. Anket formu 5'li likert ölçeği şeklindedir (Kesinlikle Katılmıyorum=1, Katılmıyorum=2, Kararsızım=3, Katılıyorum=4, Kesinlikle Katılıyorum=5). Anket sonucu elde edilen veriler IBM SPSS Statistics 25 programı kullanılarak analiz edilmiştir. Bağımsız iki grubun karşılaştırılmasında bağımsız örneklem t-testi, üç veya daha fazla bağımsız grup arasında ortalamalar arası farklılıkları tespit edebilmek için ise ANOVA (Analysis of Variance-Varyans Analizi) testi uygulanmıştır. ANOVA bulgularının değerlendirilmesinde varyansların homojenliği sağlandığından post-hoc testlerinden Scheffe kullanılmıştır. Araştırmada yapılan analizlerde anlamlılık düzeyi (α) 0,05 olarak belirlenmiştir.

Araştırmanın ikinci bölümü niteliksel araştırma tipinde yapılandırılmış odak grup görüşmeleri şeklinde uygulanmıştır [16,17]. Odak grup görüşmeleri 'COREQ Checklist' dikkate alınarak yürütülmüştür [18]. Odak grup görüşmelerinde de 4 ve 5 yıllık eğitimi karşılaştırmak üzere anket sorularına paralel sorular araştırıcılar tarafından hazırlanmış ve Covid-19 pandemisi nedeniyle çevrimiçi platformda gönüllü katılımcılarla görüşmeler gerçekleştirilmiştir. Kartopu yöntemi kullanılarak her bir grupta 4 farklı sektörde çalışan (kamu, hastane, ilaç sanayi ve toplum eczacıları), 4 ve 5 yıllık eczacılık fakültesi mezunu 8 kişiden oluşan 3 grup ile yapılandırılmış görüşmeler toplam 24 kişi ile gerçekleşmiştir. Her grupta farklı sektörlerde çalışan 4 tane 4 yıllık, 4 tane 5 yıllık mezun bulunmuştur. Görüşmeler 3 saatte tamamlanmış, kodlamalar araştırmacılar tarafından yapılmıştır. Odak grup görüşmelerinden elde edilen görüşlere içerik analizi yapılmıştır [19-22]. Odak grup görüşmesinde yöneltilen sorular;

- 4 yıllık/5 yıllık müfredattan mezun olduğunuzda çalıştığınız alanla ilgili ihtiyacınız olan bilgi, tutum ve beceriler hakkındaki düşüncelerinizden bahseder misiniz?
- Eksik yer verildiğini düşündüğünüz hususlar varsa 5 yıllık müfredata geçişle birlikte bu konular hakkında ne gibi değişimler gözlemlediniz? (Yeni dersler, staj, kariyer planlama, bitirme projeleri değerlendirilerek) (Karşılanmayan durumlar 4 yıllık mezunlardan öğrenilerek sadece 5 yıllık mezunlara sorulmuştur.)
- 5 yıllık mezunlarda müfredatta karşılanmayan durumlar nelerdi?
- 4/5 yıllık müfredatta kullanılan öğrenme yöntemleri nelerdi? Bilgilerinizin kalıcı olabilmesi için neler yapıldı?
- 4/5 yıllık müfredatta staj eğitimi ile ilgili düşünceleriniz nelerdir?
- Bitirme Projesi dersi hakkındaki görüşlerinizi paylaşır mısınız?
- Eğitiminiz sırasındaki Kariyer Planlama etkinliklerinin kariyerinizi planlama noktasındaki etkilerinden bahseder misiniz?
- Mesleki Branşlaşma hakkında ne düşünüyorsunuz? Branşlaşmanın mevzuatta yer almasının eczacılık mesleğine olası etkileri hakkında neler söyleyebilirsiniz?

- Fakültede branşlara yönelik farkındalık oluşturucu seçmeli dersleriniz nelerdi?
- Akreditasyonun eğitim kalitesine etkileri hakkında ne düşünüyorsunuz?
- Eczacılık eğitiminin verimliliği için eklemek istedikleriniz nelerdir?

SONUÇ VE TARTIŞMA

Bu çalışmada, eczacılık eğitimi dört yıldan beş yıla dönüştürüldükten sonra daha verimli olup olmadığı, eksikliklerin giderilip giderilemediği araştırılmış; elde edilen araştırma sonuçları aşağıda verilmiştir.

Araştırmacılar tarafından geliştirilen ölçek için Cronbach-alpha güvenirlik katsayısı 0,935 olarak bulunmuş olup ölçeğin yüksek güvenirliğe sahip olduğu söylenebilmektedir [23]. Araştırmaya, 19 farklı üniversiteden mezun olan 160 eczacı katılmıştır. %21.3 oranıyla en çok katılım Ankara Üniversitesi Eczacılık Fakültesi'nden olmuştur. Katılımcıların %51.9'u beş yıllık eğitim alırken dört yıllık eğitim alan eczacıların oranı %48.1'dir. Katılımcıların büyük bir kısmını %55.6'yla toplum eczacıları oluşturmaktadır. Bunu sırasıyla hastane, kamu, endüstri ve akademi eczacıları takip etmektedir. Tablo 1' de katılımcıların mezuniyet yılı, eğitim süreleri ve çalışmakta oldukları alanlara göre dağılımları gösterilmektedir.

Tablo 1. Katılımcıların Mezuniyet Yılı, Eğitim Süresi ve Çalışmakta Oldukları Alanlara Göre Dağılımları

Mezuniyet Yılı	**Sıklık**	**Yüzde**
1990 ve öncesi	24	15.0
1991-2000	28	17.5
2001-2010	46	28.7
2011-2020	62	38.8
Eğitim Süresi		
4 Yıl	77	48.1
5 Yıl	83	51.9
Çalışılmakta Olan Alan		
Toplum Eczacısı	89	55.6
Hastane Eczacısı	30	18.8
Kamu Eczacısı	20	12.5
Endüstri Eczacısı	12	7.5
Akademi Eczacısı	9	5.6
Toplam	160	100.0

Ankette yer alan, eğitim içeriği, kazanılan beceriler, kariyer planlama, sosyal ve kültürel yön geliştirici faaliyetler, öğretim yöntemleri, staj, bitirme projesini değerlendiren ifadeler ve katılımcıların bu sorulara verdikleri yanıtların ortalama ve standart sapmaları Tablo 2'de verilmiştir. Katılımcılar, *'Fakülteden mesleğim ile ilgili mevzuata hakim bir şekilde mezun oldum.', 'Fakültede, bilgiye ulaşma ve çalışma amacıyla kütüphane ortamı sağlanmıştır.'* ve *'Fakültede öğrenim süresince alınan tüm*

dersler meslek uygulamaları ile son derece uyumludur.' sorularına en yüksek oranda katıldıklarını belirtmiştir.

Tablo 2. Ankette Yer Alan Sorular ve Katılımcıların Sorulara Verdikleri Yanıtların Ortalamaları

EĞİTİM	**Ortalama**	**Standart Sapma**
1.Fakültede ağırlıklı olarak ürün/ilaç odaklı bir eczacılık eğitimi aldım.	3.67	1.395
2.Fakültede ağırlıklı olarak hasta odaklı bir eczacılık eğitimi aldım.	2.94	1.326
3.Fakültede, hastalıkların tedavisinde hastanın klinik durumuna uygun ilaç seçimi, doz ayarlaması, yan etkiler ve kontrendikasyonlar konusundaki bilgiler yeterli düzeyde verilmiştir.	3.48	1.317
4.Fakültede, ilacın üretiminden güvenli ve uygun kullanımına kadar kapsamlı bir farmakoterapi bilgisi edindim.	3.74	1.348
BECERİ		
5.Aldığım eğitim sayesinde, halk sağlığı ile ilgili durumlarda danışmanlık yapabilirim.	4.14	1.098
6.Aldığım eğitim sayesinde, sağlık personelleriyle mesleki bakımdan özgüvenli iletişim kurabiliyorum.	3.81	1.231
7.Fakültede, yeterli düzeyde verilen eğitim ile iletişim becerilerine sahip olarak mezun oldum.	3.74	1.255
8.Aldığım eğitim sayesinde, liderlik yapabilme, disiplin içi ve disiplinler arası çalışmalarda etkin biçimde yer alabilme, karar verebilme becerisini edindim.	3.73	1.192
9.Fakülteden mesleğim ile ilgili mevzuata hakim bir şekilde mezun oldum.	4.65	0.693
10.Aldığım eğitim sayesinde, ilaçları ve etki mekanizmalarını sınıflandırabiliyorum.	3.74	1.042
11.Aldığım eğitim sayesinde, ilaç analiz yöntemlerini yardım almadan kullanabiliyorum.	3.56	1.287
12.Fakültede, terapötik kullanım için uygun olan yeni kimyasal bileşiklerin sentez ve geliştirilme becerisini edindim.	4.21	0.974
13.Aldığım eğitim sayesinde, ilaçların güvenli ve rasyonel kullanımı konusunda diğer sağlık personellerine rahatlıkla tavsiye verebiliyorum.	3.74	1.320
14.Aldığım eğitim sayesinde, çalıştığım alanda hastaya rahatlıkla Farmasötik Bakım/Klinik Eczacılık hizmeti sunabilirim.	3.61	1.365
15.Fakültede, klinik eczacılık temel ilkelerini öğrenir, eczacılığın uygulandığı tüm alanlarda hasta-odaklı eczacılık uygulamalarının ve farmasötik bakımın esası olan akılcı ilaç kullanımı sağlanmasına yardımcı olabilirim.	4.19	1.106
16.Aldığım eğitim sayesinde, ilaç/tıbbi cihaz/kozmetik/bitkisel tıbbi ürünlerin saklanma, stoklanma, dağıtım ve imha süreçlerini yönetebilirim.	4.02	1.073
17.Aldığım eğitim sayesinde, reçeteye tâbi olmayan ilaçların ve sağlığa ilişkin tüm ürünlerin hastanın gereksinimlerine uygun, güvenli ve akılcı bir şekilde tedarik**ğ**ini sağlayabilir, bu konuda rahatlıkla danışmanlık hizmeti verebilirim.	4.49	0.847

*5 yıllık eğitim alanlar cevaplamıştır.
**4 yıllık eğitim alanlar cevaplamıştır.

Tablo 2 (*devamı*). Ankette Yer Alan Sorular ve Katılımcıların Sorulara Verdikleri Yanıtların Ortalamaları

EĞİTİM	**Ortalama**	**Standart Sapma**
18.Fakültede aldığım eğitim, meslek hayatımda bağımsız olarak bilgi edinme becerisi kazandırmıştır.	4.27	0.963
19.Aldığım eğitim sayesinde, sık karşılaşılan semptomları değerlendirebilir ve gerekli yönlendirmeleri yapabilirim.	3.66	1.268
20.Aldığım eğitim sayesinde, kronik hastalıklarda kullanılan tıbbi cihazların akılcı ve güvenli kullanımı konusunda hastayı bilgilendirebilirim.	3.93	1.117
21.Aldığım eğitim sayesinde, özel hasta gruplarında akılcı ve güvenli ilaç kullanımı konularında hastayı ve ilgili sağlık personelini bilgilendirebilirim.	4.03	1.154
22.Aldığım eğitim sayesinde, ilacın farmakolojik etkisinin devamlılığı ile emniyeti, etkililiği ve maliyeti bakımından gözetimini sağlayabiliyorum.	4.03	1.107
23.Fakülteden, eczacılık hizmetlerindeki uzman rolümü ve görevlerimi anlayarak mezun oldum.	4.13	1.068
24.Aldığım eğitim sayesinde, olağanüstü hâl veya kriz dönemlerinde her türlü ilaç tedarik süreçlerinde etkin rol alabilir ve kamu kurum ve kuruluşlarına yardımcı olabilirim.	3.66	1.219
25.Aldığım eğitim sayesinde, farmakoekonomik analiz için gerekli yöntemleri değerlendirebiliyorum.	4.17	1.047
26.Aldığım eğitim sayesinde ilaçların kullanımı, saklanması ve imhası ile ilgili potansiyel zararlılık risklerine karşı hasta, toplum ve çevre bilinci oluşturulmasını sağlayabilir ve sorunların çözümünde aktif görev alabilirim.	4.45	0.783
27.Aldığım eğitim sayesinde, özel reçete ilaçlarını sunabilirim.	4.01	1.110
28.Aldığım eğitim sayesinde, majistral preparatları yardım almadan hazırlayabiliyorum.	3.96	1.063
29.Aldığım eğitim sayesinde, gıda takviyelerinin akılcı ve güvenli kullanımında danışmanlık yapabilirim.	4.09	0.983
30.Aldığım eğitim sayesinde, bitkisel ürünlerle ilgili danışmanlık yapabilirim.	3.48	1.303
31.Aldığım eğitim sayesinde, tanısal testleri değerlendirebilir ve laboratuvar bulgularını yorumlayabilirim.	3.78	1.292
32.Aldığım eğitim sayesinde, farmakovijilans uygulamalarına katılabilirim.	4.09	1.103
33.Aldığım eğitim sayesinde, tedavi süresince hasta uyuncunu izleyebilir ve değerlendirebilirim.	2.99	1.416
KARİYER PLANLAMA		
34.Fakültede, mesleki bakış açımızı geliştirmek amacıyla kariyer planlamaya önem verilmiştir.	2.89	1.443
35.Fakültede, son sınıflar için mesleki yönlendirmeler yapılmıştır.	2.49	1.203

Tablo 2 (*devamı*). Ankette Yer Alan Sorular ve Katılımcıların Sorulara Verdikleri Yanıtların Ortalamaları

EĞİTİM	**Ortalama**	**Standart Sapma**
SOSYAL VE KÜLTÜREL YÖN GELİŞTİRİCİ FAALİYETLER		
36.Fakültede, sosyal ve kültürel seçmeli derslere yeterince ağırlık verildi.	2.87	1.374
37.Aldığım eğitim süresince yeterli sosyal ve kültürel aktiviteye ayıracak zamanım oldu.	4.28	1.198
ÖĞRETİM YÖNTEMİ		
38.Fakültede, bilgiye ulaşma ve çalışma amacıyla kütüphane ortamı sağlanmıştır.	4.56	0.791
39.Fakültede ezbere dayalı bir eczacılık eğitimi aldım.	3.44	1.248
40.Fakültede eğiticiler**,** öğrenim süresi boyunca araştırmaya yönelik ödevler verdi.	3.61	1.259
41.Fakültedeki eğiticiler, öğrencinin derse katılımını sağladılar.	3.61	1.239
42.Fakültedeki eğiticiler, dersin başında dersin hedef ve amaçlarını açık bir şekilde belirlerdi.	2.96	1.253
43.Fakültede öğrenim süresince alınan tüm dersler meslek uygulamaları ile son derece uyumludur.	4.50	0.971
STAJ		
44.Stajlarımı titizlikle yaptım.	2.83	1.430
45.Stajlar denetlenmekteydi.	4.01	1.216
46.Staj süreleri bilgilerimi pekiştirip hastalara aktarmak için yeterliydi.	4.10	1.219
47.Staj süresince işveren ve çalışanlar bana her konuda destek oldu.	3.69	1.289
48.Bilgilerimi stajlarda kolaylıkla hastaya aktarabildim.	3,42	1.443
49.Yaptığım stajların şu an bulunduğum alanı seçmemde çok büyük katkısı olmuştur.	4.04	1.238
50.Yaptığım stajlar, meslek hayatımda daha özgüvenli ve doğru iletişim kuran bir birey olmamı sağladı.	3.88	1.230
51.Yaptığım stajlar, fakültede edindiğim akademik bilgileri pekiştirmemi sağladı.	3.54	1.391
BİTİRME PROJESİ		
*52.Bitirme projesi, bilgi kaynağı bulma, veri toplama, değerlendirme, yorumlama ve elde edilen bilgileri bir konu bütünlüğü içinde rapor haline getirme becerisi kazandırmıştır.	3.95	1.132
*53.Bitirme projesi, eczacılığın temel çalışma alanlarına bakış açımı genişletmiştir.	3.62	1.126
*54.Bitirme projesi, eczacılıkla ilgili konulara akademik bir bakış açısı kazandırmıştır.	3.78	1.097
*55.Bitirme projesi boyunca danışmanımla rahatlıkla iletişim kurabildim.	3.73	1.169
**56.Dört yıllık eczacılık fakültesinden mezun olduğum için Bitirme Projesi hazırlamadım. Hazırlamak isterdim.	3.70	1.253

Eğitim verimliliği için katılımcılardan elde edilen toplam ölçek puanları, alt başlıklar için ise her alt başlıktan elde edilen toplam puanlar ile analizler yapılmıştır.

Eğitim Verimliliği

Katılımcıların aldıkları lisans eğitimi hakkındaki genel görüşlerinde anlamlı bir farklılık olup olmadığını test etmek amacıyla bağımsız örneklem t-testi uygulanmıştır. Analiz sonucunda müfredat yıllarına göre genel eğitim içeriği ile ilgili olarak anlamlı bir farklılık bulunmuş ($p<0.05$); beş yıllık mezunların, dört yıllık mezunlara göre aldıkları eğitimi verimli buldukları anlaşılmıştır (Tablo 3). Çalışılmakta olan alanların verimlilik karşılaştırılması için ANOVA testi uygulanmış ancak anlamlı bir fark olmadığı sonucu elde edilmiştir ($p=0.771$).

Tablo 3. Katılımcıların Aldıkları Genel Eğitim İçeriği Hakkındaki Görüşleri İçin Yapılan t-Testi Sonuçları

Eğitim Süresi	Sıklık	Ortalama Puan	Standart Sapma	Sig. (2-tailed)
4	77	208.86	27.55	0.007
5	83	222.21	33.57	

Eğitim İçeriği

Katılımcıların aldıkları eğitimin içeriği hakkındaki görüşleri ANOVA testi ile incelendiğinde (Tablo 4):

- ✓ 2011-2020 arası mezunlar, 1991-2010 arasındaki mezunlara göre aldıkları eğitimin içeriğini daha verimli bulmuşlardır ($p<0.05$).
- ✓ Toplum eczacıları ve endüstri eczacıları, akademi ve hastane eczacılarına göre aldıkları eğitimin içeriğini daha verimli bulmuşlardır ($p<0.05$).

Tablo 4. Katılımcıların Eğitim İçeriği Hakkındaki Görüşleri İçin Yapılan ANOVA Testi Sonuçları

Mezuniyet Yılı	Sıklık	Ortalama Puan	Standart Sapma	Sig. (2-tailed)
1990 ve öncesi	24	18.04	4.83	0.002
1991-2000	28	16.39	3.13	
2001-2010	46	16.80	4.20	
2011-2020	62	19.39	3.95	
Çalışılan Alan				
Toplum Eczacısı	89	18.45	4.25	0.041
Hastane Eczacısı	30	16.63	4.19	
Kamu Eczacısı	20	17.50	4.11	
Endüstri Eczacısı	12	19.75	3.05	
Akademi Eczacısı	9	15.44	3.75	

Odak grup görüşmelerinde katılımcılara ‘’Şu anki işinizde, ilaç ve/veya hasta odaklı aldığınız eğitimin yeterliliklerini değerlendirebilir misiniz?’’ sorusu sorulmuş; bu soruya verilen yanıtlara göre dört yıllık mezunların eğitimlerinin odağının ilaç olduğu görülmüştür (Benzer ifadeler bir kere yazılmıştır).

$E_{2,KE,4}$: Kamuda çalıştığım için bitkilerle ilgili aldığım eğitimimi kullanamıyorum. Liderlik, yönetim, iletişim konusunda eğitim alabilmeyi isterdim.

$E_{1,TE,5}$: İlaç ve hasta odaklı eğitimi iyi derecede aldığımızı düşünüyorum.

$E_{1,TE,4}$: Teorik bilgileri sentezleyip pratik hayatta kullanmada zorluk çektim.

$E_{2,TE,4}$: İlaç odaklı eğitim aldık. Hasta odaklı eğitimin çok zayıf kaldığını düşünüyorum

$E_{2,TE,5}$: İlk iki sene ilaç odaklı eğitim aldık. Son iki senede klinik derslere ağırlık verildi. Hasta odaklı eğitim de aldım.

$E3_{,TE,5}$: Farmasötik Bakım, Klinik Biyokimya, Farmakoterapi gibi derslerin çok faydasını görüyorum.

Kazanılan Beceri

Katılımcıların eğitimleri sırasında kazandıkları mesleki beceriler hakkındaki görüşleri ANOVA testi ile incelendiğinde (Tablo 5):

- ✓ 1990 ve öncesi mezunlar ile 2011-2020 arası mezunların, 1991-2000 arası mezunlara göre eğitimleri sırasında daha çok mesleki beceri kazandıkları belirlenmiştir ($p<0.05$).
- ✓ Çalışılmakta olan alanlar açısından ise anlamlı bir fark bulunmamıştır ($p>0.05$).

Tablo 5. Katılımcıların Eğitimleri Sırasında Kazandıkları Mesleki Beceriler Hakkındaki Görüşleri İçin Yapılan ANOVA Testi Sonuçları

Mezuniyet Yılı	Sıklık	Ortalama Puan	Standart Sapma	Sig. (2-tailed)
1990 ve öncesi	24	119.25	20.63	0.037
1991-2000	28	107.07	18.55	
2001-2010	46	111.52	18.10	
2011-2020	62	117.85	19.61	
Çalışılan Alan				
Toplum Eczacısı	89	115.98	20.61	0.736
Hastane Eczacısı	30	112.77	17.76	
Kamu Eczacısı	20	114.00	18.10	
Endüstri Eczacısı	12	111.75	15.79	
Akademi Eczacısı	9	107.89	22.86	

Kariyer Planlama

Katılımcıların eğitimleri sırasında kariyerlerini planlama konusunda aldıkları danışmanlık hakkındaki görüşleri ANOVA testi ile incelendiğinde (Tablo 6):

- ✓ 2011-2020 arası mezunların, 2010 ve öncesi mezunlara göre kariyerlerini planlama konusunda daha fazla danışmanlık aldıkları belirlenmiştir ($p<0.05$).
- ✓ Çalışılmakta olan alanlar açısından ise anlamlı bir fark tespit edilmemiştir ($p>0.05$).

Tablo 6. Katılımcıların Eğitimleri Sırasında Kariyerlerini Planlama Konusunda Aldıkları Danışmanlık Hakkındaki Görüşleri İçin Yapılan ANOVA Testi Sonuçları

Mezuniyet Yılı	Sıklık	Ortalama Puan	Standart Sapma	Sig. (2-tailed)
1990 ve öncesi	24	5.00	2.17	0.000
1991-2000	28	4.46	1.62	
2001-2010	46	4.67	2.27	
2011-2020	62	6.48	2.28	
Çalışılan Alan				
Toplum Eczacısı	89	5.46	2.28	0.774
Hastane Eczacısı	30	5.23	2.14	
Kamu Eczacısı	20	5.20	2.53	
Endüstri Eczacısı	12	6.00	3.10	
Akademi Eczacısı	9	4.78	1.86	

Odak grup görüşmelerinde dört yıllık mezunlar, beş yıllık mezunlara göre kariyer planlama ile ilgili yeteri kadar yönlendirilmediklerini, buna ihtiyaç duyduklarını belirtmişlerdir.

$E_{2,TE,4}$: Kariyer planlaması ile şekillendirilmiş branşlaşmanın çok önemli olduğunu düşünüyorum.

$E_{3,TE,4}$: Kariyer planlama etkinlikleri yapılmadı. Mezun olduğumda hangi alanda çalışacağıma karar vermede zorlandım.

$E_{3,TE,5}$: Kariyer planlama eğitimi yapıldı, zorunlu olarak girdik. O zaman zorunlu girmek hoşumuza gitmemişti ama çok yararı oldu.

$E_{2,HE,5}$: Kariyer planlama etkinlikleri vardı, ama yeterli değildi. 1.sınıftan itibaren, daha erken başlaması gerekiyordu.

$E_{1,KE,4}$: Çok nadir kariyer planlaması ile ilgili etkinlikler oluyordu.

$E_{2,İSE,5}$: Kariyer günleri ve ilaç sanayinde yapılabilen stajlar çok faydalı oldu.

Sosyal ve Kültürel Yön Geliştirici Faaliyetler

Bu alanda gruplar arasında istatistiksel açıdan anlamlı fark bulunmamıştır.

Odak grup görüşmelerinde, kariyer planlama konusunda seçmeli derslerin önemi de katılımcılar tarafından vurgulanmıştır.

$E_{1,TE,4}$: Liderlik, iletişim, yönetim becerisi geliştirici seçmeli derslerin olmasını isterdim.

$E_{3,TE,5}$: Bazı seçmeli derslerimizi çok akılcı alamadık. Ders günü ve saati, dersin içeriğinden daha önemli oldu.

$E_{2,HE,5}$: Klinik eczacılığa özgü daha fazla seçmeli ders olsaydı.

$E_{3,HE,5}$: Eczacı-Hekim ilişkisinin kalitesi ile ilgili seçmeli ders ya da etkinlik çok faydalı olurdu.

$E_{2,KE,4}$: Denetim görevi yapacak eczacılara yönelik seçmeli dersler olmalı.

$E_{3,İSE,4}$: Branşlaşma ile ilgili seçmeli derslerin olması çok faydalı olurdu.

$E_{3,İSE,5}$: Seçmeli dersler, bazı kongrelere katılım ve stajların çalışma alanımızı belirlemede büyük rolü oldu.

Öğretim Yöntemi

Katılımcıların eğitimleri sırasında uygulanan öğretim yöntemlerinin verimliliği hakkındaki görüşleri ANOVA testi ile incelendiğinde (Tablo 7):

- ✓ 2011-2020 arası mezunların 1991-2010 arası mezunlara, 1990 ve öncesi mezunların ise 2001-2010 arası mezunlara göre öğretim yöntemlerini daha verimli buldukları belirlenmiştir ($p<0.05$).
- ✓ Çalışılmakta olan alanlar açısından ise anlamlı bir fark bulunmamıştır ($p>0.05$).

Tablo 7. Katılımcıların Eğitimleri Sırasında Uygulanan Öğretim Yöntemlerinin Verimliliği Hakkındaki Görüşleri İçin Yapılan ANOVA Testi Sonuçları

Mezuniyet Yılı	Sıklık	Ortalama Puan	Standart Sapma	Sig. (2-tailed)
1990 ve öncesi	24	24.04	4.13	
1991-2000	28	21.36	3.38	
2001-2010	46	21.02	4.01	
2011-2020	62	24.00	4.00	0.000
Çalışılan Alan				
Toplum Eczacısı	89	22.34	2.81	0.394
Hastane Eczacısı	30	21.38	2.19	
Kamu Eczacısı	20	21.79	3.56	
Endüstri Eczacısı	12	22.67	3.10	
Akademi Eczacısı	9	22.04	2.65	

Odak grup görüşmelerinde, katılımcılara mesleki beceri kazanmada yüksek önemi olan 'Öğretim yöntemi nasıldı?' sorusu sorulmuş; verilen yanıtlar eczacılık eğitiminin öğretici merkezli ve büyük ölçüde ezbere dayalı olduğunu kanıtlar nitelikte olmuştur:

$E_{3,TE,4}$: Ezberci bir öğretim yöntemi uygulandı.

$E_{1,TE,5}$: Bazen ödev verilerek sunum yaptırılıyordu, o bilgiler daha kalıcı oluyordu.

$E_{1,HE,4}$: Ezberci bir eğitim vardı. Stajda bilgileri pekiştiriyorduk.

$E_{1,KE,5}$: Ezberci bir öğretim yöntemi vardı ama bunun olması gerekiyordu.

$E_{1,İSE,4}$: Pek çok dersimizi ezberleyerek geçtik ama akılda kalıcı olmadı. Keşke tüm derslerde ödev sunumu olsaydı.

Staj

Çalışmanın nicel kısmındaki sonuçlara göre staj konusunda dört ve beş yıllık eczacılar arasında anlamlı bir fark bulunmamıştır. Odak grup görüşmelerinde ise büyük ölçüde stajların yararlı olduğu belirtilmiş; ancak, staj yapan öğrenci ve staj yapılan yerlerin fakülteler tarafından daha fazla denetlenmesi önerilmiştir.

$E_{2,TE,4}$: Stajlarımız bir denetim mekanizması olmadığı için son derece verimsiz geçerdi. Stajın gerekliliğine ve faydasına inanıyorum.

$E_{3,TE,5}$: Staj çok önemli. Verimli geçti. Okul tarafından kontrol edilmesi gerektiğini düşünüyorum.

$E_{1,HE,4}$: Stajlar çok faydalı. Fakülteler doğru yönlendirmeleri yapmalı.

$E_{3,İSE,4}$: Stajımı ilaç firmasında yapabildiğim için bu alanı seçtim.

$E_{3,İSE,5}$: Stajlar bilgilerin pekişmesi için çok faydalı.

Bitirme Projesi

Katılımcıların eğitimleri sırasında bitirme projesi hazırlanmasının verimliliği hakkındaki görüşleri ANOVA testi ile incelendiğinde (Tablo 8):

- ✓ 2011-2020 arası mezunların, 2010 ve öncesi mezunlara göre bitirme projesi hazırlanmasını daha verimli buldukları belirlenmiştir ($p<0.05$).
- ✓ Çalışılmakta olan alanlar açısından ise anlamlı bir fark tespit edilmemiştir ($p>0.05$).

Tablo 8. Katılımcıların Eğitimleri Sırasında Bitirme Projesi Hazırlanmasının Verimliliği Hakkındaki Görüşleri İçin Yapılan ANOVA Testi Sonuçları

Mezuniyet Yılı	Sıklık	Ortalama Puan	Standart Sapma	Sig. (2-tailed)
1990 ve öncesi	24	16.71	4.55	0.000
1991-2000	28	15.96	3.33	
2001-2010	46	17.41	4.64	
2011-2020	62	21.87	3.47	
Çalışılan Alan				
Toplum Eczacısı	89	15.67	3.15	0.344
Hastane Eczacısı	30	16.01	2.97	
Kamu Eczacısı	20	16.76	3.12	
Endüstri Eczacısı	12	15.23	2.10	
Akademi Eczacısı	9	16.44	4.65	

Odak grup görüşmelerinde beş yıllık eğitimde son sınıfta hazırlanan bitirme projesinin çok faydalı olduğu belirtilmiştir. Bu konuyla ilgili görüşler aşağıda yer almaktadır:

$E_{2,TE,5}$: Bitirme projesi bana özgüven sağladı. Çok faydası olduğunu düşünüyorum.

$E_{2,HE,4}$: İlk işe girdiğimde bazı araştırmalar yapmam istenmişti, zorlandım. Keşke biz de bitirme projesi hazırlasaydık.

$E_{2,HE,5}$: İyi yürütülürse çok faydalı olduğunu düşünüyorum.

$E_{2,KE,5}$: Bir bilimsel araştırma nasıl yapılır bunu araştırma projesi hazırlarken öğrendim.

$E_{1,İSE,4}$: Keşke biz de bitirme projesi hazırlasaydık. Çok faydalı olurdu.

Odak grup görüşmelerine katılan eczacılara beş yıllık eğitimde toplum eczanesi, hastane eczanesi ve ilaç sanayiinde branşlaşmayı sağlayan seçmeli dersler de alındıktan sonra diplomaların bu branşlara özgü verilmesi konusundaki görüşleri sorulmuş; yanıtları aşağıda sunulmuştur:

$E_{1,TE,4}$: Branşlara özgü seçmeli dersler güzel bir uygulama ancak bunu belgelendirerek, tek bir alanda çalışma zorunluluğu kişileri mutsuz edebilir.

$E_{2,TE,5}$: Tek bir branşta çalışma zorunluluğu uygun olmazdı. Çünkü öğrencilikteki koşullar zamanla değişir, fikir de değişir.

$E_{1,HE,4}$: Öğrenciye çok fazla seçmeli ders sunulmalı, ancak tek bir alanda çalışma zorunluluğu getirilmemeli.

$E_{2,KE,5}$: Branşlaşma sınırlayıcı anlamda olmamalı ama kişinin ilgi duyduğu alandaki yeterlilikleri bu sayede geliştirilebilir.

Odak grup görüşmelerinde son olarak 'Eczacılık eğitiminin verimliliği için eklemek istedikleriniz nelerdir?' diye sorulduğunda ise:

$E_{3,TE,4}$: Liderlik ve iletişim konusunda daha fazla ders olabilir.

$E_{1,TE,5}$: Eczane yönetimi ile ilgili daha fazla uygulamaya yönelik dersler olabilir.

$E_{2,HE,4}$: Müstahzar isimleri ve jenerik ilaçlarla ilgili daha fazla bilgi verilmeli.

$E_{3,HE,5}$: Eczacı-Hekim ilişkisinin geliştirilmesi ile ilgili eğitim verilmeli.

$E_{3,İSE,5}$: Stajlar daha kaliteli yapılabilir.

Odak grup görüşmelerinde son olarak akreditasyonun önemi sorulduğunda, tüm katılımcılar tarafından gerek eğitimde gerekse istihdam alanlarında akreditasyonun yapılan işin belli standartlara göre yapıldığı ve denetlenebilir ölçütleri olması nedeniyle çok faydalı olacağı ve kaliteyi arttıracağı görüşü paylaşılmıştır.

2005 yılında eczacılık eğitim süresi 5 yıla çıkartılırken, hasta odaklı eğitimin de geliştirilmesi amacıyla mevcut ders müfredatına Farmasötik Bakım, Farmakoterapötikler, Klinik Biyokimya, Akılcı İlaç Kullanımı, İlaç Etkileşmeleri gibi zorunlu ve seçmeli olarak öğrencilerin alabileceği hasta odaklı derslerin ilave edildiği görülmektedir. Ayrıca, öğrencilerin mezun olduğunda çalıştıkları alan seçiminde kararlı ve yetkin olabilmeleri için, daha önce de var olan 6 aylık zorunlu stajların daha etkin

yapılabilmesi için bazı düzenlemeler yapılmış, 5.sınıfta tercih edilen alanla ilgili bir bitirme projesi hazırlanması istenmiş ve öğrencilerin tercih ettikleri alanla ilgili çok sayıda seçmeli ders programa ilave edilmiştir [1].

Ülkemizde bilimsel anlamda eczacılık eğitimi 1839 yılında iki yıllık eğitim süresi ile başlamıştır. Eğitim süresi daha sonra 3, 1934 yılında 4 yıl olmuştur [2]. Eczacılık lisans eğitimi 2005 yılında dört yıldan beş yıla çıkarılmış ve eğitim programında bazı değişiklikler yapılmıştır. Bu değişikliklerin verimli olup olmadığı bugüne kadar hiç araştırma konusu olmamıştır.

Türkiye'de eczacılık eğitimi ile ilgili yapılan çalışmalar, eczacıların ilaç üretim süreçlerindeki yani ilaç odaklı görev ve sorumlulukları, ilacın hastalarca akılcı kullanımı ve sağlığın en iyi biçimde sürdürülebilmesini sağlamaya yönelik hasta odaklı bakım uygulamaları ve bununla ilgili derslerin/ yeterliliklerin arttırılması yönünde öneriler sunmaktadır [24, 25]. Buna paralel olarak Amerika Birleşik Devletleri (ABD)' nde eczacılık eğitimi incelenmiş ve son 45 yılda ilaç üzerine yoğunlaşan eğitimden hasta odaklı eğitime geçiş üzerine değişiklikler yapılmıştır [26].

Bu çalışmada Türkiye'de 4 ve 5 yıllık eczacılık eğitim programı; eğitim içeriği, hasta ve ürün odaklı eczacılık hizmetleri ile ilgili kazanılan beceriler, öğretim yöntemleri, sosyal ve kültürel yön geliştirici faaliyetler, staj, kariyer planlama ve bitirme projesi konuları açısından karşılaştırılmıştır. Genel olarak beş yıllık programdan mezun eczacıların dört yıllık programdan mezun eczacılara göre aldıkları eğitimden daha memnun oldukları söylenebilmektedir.

Çalışma sonucunda tüm katılımcıların %63.7'si, dört yıllık eczacıların %77'si, 5 yıllık eczacıların %52.4 'ü ürün/ilaç odaklı eğitimi daha yoğun aldıklarını belirtmiş; 4 yıllık eczacıların %21'i, 5 yıllık eczacıların ise %58'i hasta odaklı eğitimi daha yoğun aldıklarını ifade etmişlerdir.

Dört yıllık eğitimle kıyaslandığında beş yıllık eğitimin ilaç odaklıdan hasta odaklı eğitime evrildiği anlaşılmaktadır. Eczacılık eğitiminin önemli bir çıktısı da hasta merkezli eczacılık hizmeti verebilecek mezunlar yetiştirmektir. Hasta merkezli bakım, bireysel olarak hastaların refahına net bir şekilde odaklanmak anlamına gelmektedir [27]. Bu doğrultuda, beş yıllık eğitime Farmakoterapötikler, Farmasötik Bakım, Klinik Biyokimya gibi dersler eklenmiş; ancak, 5 yıllık programdan mezun olan katılımcıların verdikleri yanıtlardan hareketle (%58) bu konuda eksiklerin olduğu, bu konuya daha fazla yoğunlaşılması gerektiği anlaşılmıştır.

Çalışmada öğretim yöntemleri sorgulanmış; tüm katılımcıların %91.8'i, dört yıllık mezunların %97'si, beş yıllık mezunların ise %90'ı ezbere dayalı eğitim yöntemi uygulandığını ifade etmiştir. Buradan hareketle ezberci öğretim yönteminin değişmediği, halen eczacılık eğitiminde baskın olduğu söylenebilmektedir.

Günümüzde toplumların kalkınması ve rekabete dayalı ekonomik düzende nitelikli bireyler yetiştirmek için eğitim daha da önem kazanmıştır. Bilişsel alanda yapılan araştırmalar, öğrenme sürecine aktif olarak katılan öğrencilerin daha iyi öğrendiklerini göstermektedir. Bu nedenle öğrencilere bilginin kaynağı ve bu bilgileri nasıl elde edecekleri, bunları nasıl değerlendirecekleri ve problemi çözmek için bu bilgiyi nasıl kullanacakları öğretilmelidir. Bu becerilerin kazandırılmasında probleme dayalı öğrenme yaklaşımının etkili olduğu yapılan birçok çalışmada ortaya konulmuştur [28-38]. Uygulama derslerinde probleme dayalı öğrenme yöntemleri kullanılmasının mesleki becerilerin kazandırılmasında etkili olacağı düşünülmektedir.

Çalışmada mezunlar, iletişim ve liderlik becerilerine yönelik yeterliliklerin önemli olduğunu vurgulamaktadır. Liderlik, uzun zamandır eczacılık mesleği için gerekli bir nitelik olarak önerilmiştir. ABD'de eczacılık öğrencileri arasında yapılan bir çalışmada, liderlik becerisi kazandırılan öğrencilerde eleştirel düşünme becerilerinin önemli ölçüde daha yüksek olduğu belirlenmiştir [39].

Eczacılık eğitimi ve uygulamasında iletişim becerileri önemi artan bir bileşen olarak kabul edilmektedir. Eczacılık eğitimi içinde genel iletişim becerileri eğitiminin etkilerini değerlendirmek amacıyla yapılan bir meta analiz çalışmasında, 2 veya daha fazla dönem haftada 5 saat ve üzeri genel iletişim becerileri eğitiminin öğrencilerin iletişim yetkinliğini geliştirmek için etkili bir yol olduğu belirlenmiştir [40].

Katılımcıların tamamı staj eğitimin önemini vurgulamıştır. Staj programlarının daha fazla denetlenmesi gerektiği düşünülmektedir. Bu konuda meslek örgütünün desteği kaçınılmazdır. Eczane stajları, dünya çapında eczacılık lisans eğitiminin önemli bir parçasıdır. Finlandiya örneğinde stajlar, dersler ile çalışma hayatı arasındaki ilişkinin daha iyi anlaşılması için ikinci ve üçüncü yıl boyunca profesyonel eğitime entegre edilmiştir [41].

Çalışmanın kariyer planlaması ile ilgili kısmında beş yıllık eğitimle beraber kariyer planlaması konusunda öğrencilere düzenlenen faaliyetlerin arttığı gözlemlenmekle birlikte eczacılık eğitiminde bu konu üzerine daha fazla yoğunlaşılması gerektiği anlaşılmaktadır. Eczacılık öğrencilerini eczacılık alanında kariyer yapmaya yönlendiren etmenleri belirlemek üzere yapılan bir çalışmada, öğrencilerin aile üyeleri, eczacılar ve kişisel deneyimlerinin yanı sıra "kariyer günü" etkinliklerinin de etkili olduğu belirlenmiştir [42].

Ülkemizde dört yıllık eczacılık eğitim müfredatında bitirme projesi dersi bulunmamaktaydı. Beş yıllık eğitime geçişle beraber son yılda bitirme projesi dersleri zorunlu olmuştur. Beş yıllık mezunların neredeyse tamamı bitirme projesi derslerinin kendilerine olumlu katkısı olduğunu ifade etmiştir.

Sonuç olarak dört yıllık eğitim ile beş yıllık eğitim karşılaştırıldığında; hasta odaklı eğitim çerçevesinde verilen dersler, kariyer planlama faaliyetleri ve bitirme projesi dersinin olumlu sonuçları

belirlenmiştir. Bununla birlikte hasta odaklı eğitimin hala geliştirilmeye ihtiyacı olduğu, stajların çok önemli olduğu, öğrencinin bunu kabul ederek staj eğitimine önem vermesinin sağlanması ve denetimlerinin yapılması gerektiği, farklı öğretim yöntemlerinin kullanılarak ezberci eğitim yöntemlerinden uzaklaşılması gerektiği de bu çalışma sonucunda ortaya çıkmıştır. Beş yıllık eğitim müfredatında yapılan değişikliklerle geliştirilen alanlar yanında eksik kalan yerler de belirlenmiştir. Eğitim içeriği yönünden hasta odaklı eğitime geçiş çabası görülmekle birlikte halen tam olarak uygulamada karşılık bulamamıştır. Farmasötik bakım, farmakoterapi gibi derslerin yanında sosyal içerikli mevzuat, iletişim, liderlik konulu derslerin etkisinin artırıldığı ancak yeterli olmadığı belirlenmiştir. Öğretim yöntemlerinden olan probleme dayalı çözüm tekniği eczacılık eğitiminde yetersizdir. Ezbere dayalı öğretim yöntemi hala baskındır. Stajların fakültelerce yeterince denetlenmediği, bu konuda fakültelere destek olmak amacıyla meslek örgütünden yardım alınabileceği; staj yapılan alandaki eczacının sorumluluğunun arttırılması gibi çözümlerin getirilebileceği önerilmektedir. Kariyer planlama faaliyetlerinin dört yıllık eğitimde çok yetersizken, beş yıllık eğitimle beraber faaliyetlerin artırılmasına yönelik çalışmaların olduğu gözlenmektedir. Bilimsel anlamda bir araştırma yapma ve raporlama ile ilgili bitirme projesi hazırlamanın önemi beş yıllık mezunların görüşleriyle ortaya konmuştur.

Son olarak, dört yıllık eğitimden beş yıllık eğitime geçişle iyileştirmelerin yapıldığı ancak eksikliklerin de bulunduğu açıktır. Eğitim verimliliğini değerlendirmek üzere daha çok araştırma yapılmalı ve eczacılık eğitiminin içeriği, dünyada ve ülkemizdeki eczacılık hizmetlerinin kalitesini attıracak şekilde güncel tutulmalıdır.

YAZAR KATKILARI

Kavram: *B.Y., M.Ç., G.Ö.*; Tasarım: *M.Ç., G.Ö.*; Denetim: *G.Ö.*; Kaynaklar: *B.Y., M.Ç., G.Ö.*; Malzemeler: *B.Y., M. Ç.*; Veri toplama ve/veya işleme: *B.Y., M.Ç., G.Ö.*; Analiz ve/veya yorumlama: *M.Ç., G.Ö.*; Literatür taraması: *B.Y., G.Ö.*; Makalenin yazılması: *M.Ç., G.Ö.*; Kritik inceleme: *M.Ç., G.Ö.*; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu yazı için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan etmişlerdir.

ETİK KURUL ONAYI

Çalışma için Ankara Üniversitesi Rektörlüğü Etik Kurulu'ndan 26.04.2021 tarih ve 82 sayılı karar

ile etik kurul onayı alınmıştır.

KAYNAKLAR

1. Eczacılık Fakülteleri Dekanlar Konseyi Web site. (2015). Retrieved January 23, 2022, from https://docplayer.biz.tr/570123-Eczacilik-fakulteleri-icin-kapsamli-mufredat.html

2. Baylav, N. (1968). Eczacılık Tarihi, Yörük Matbaası, İstanbul.

3. T.C. Sağlık Bakanlığı Web site. (2001). Retrieved January 31, 2022, from https://dosyasb.saglik.gov.tr/Eklenti/1409,serbestdolasimpdf.pdf?0

4. European Union Web site. (1985). Retrieved January 31, 2022, from https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:31985L0432

5. Türk Eczacıları Birliği Web site. (1995). Retrieved January 27, 2022, from http://e-kutuphane.teb.org.tr/arsiv.php?anabelge_no=289

6. Yüksek Öğretim Kurulu Web site. (2019). Retrieved January 27, 2022, from https://www.yok.gov.tr/Documents/Kurumsal/egitim_ogretim_dairesi/Ulusal-cekirdek-egitimi-programlari/eczacilik_cep.pdf.

7. Doktorluk, Hemşirelik, Ebelik, Diş Hekimliği, Veterinerlik, Eczacılık ve Mimarlık Eğitim Programlarının Asgari Eğitim Koşullarının Belirlenmesine Dair Yönetmelik. T.C. Resmi Gazete, 2 Şubat 2008, sayı: 26775.

8. Doktorluk, Hemşirelik, Ebelik, Diş Hekimliği, Veterinerlik, Eczacılık ve Mimarlık Eğitim Programlarının Asgari Eğitim Koşullarının Belirlenmesine Dair Yönetmelikte Değişiklik Yapılmasına Dair Yönetmelik. T.C. Resmi Gazete, 31 Aralık 2009, sayı: 27449.

9. Doktorluk, Hemşirelik, Ebelik, Diş Hekimliği, Veterinerlik, Eczacılık ve Mimarlık Eğitim Programlarının Asgari Eğitim Koşullarının Belirlenmesine Dair Yönetmelikte Değişiklik Yapılmasına Dair Yönetmelik. T.C. Resmi Gazete, 25 Mayıs 2018, sayı: 30431.

10. Eczacılar ve Eczaneler Hakkında Yönetmelik. T.C. Resmi Gazete, 12 Nisan 2014, sayı: 28970.

11. Sağlık Meslek Mensuplarının İş ve Görev Tanımları Yönetmeliği. T.C. Resmi Gazete, 22 Mayıs 2014, sayı: 29007.

12. Eczacılık Eğitimi Programlarını Değerlendirme ve Akreditasyon Derneği Web site. (2021). Retrieved January 27, 2022, from https://www.eczakder.org.tr/turkiye-ulusal-eczacilik-lisans-egitimi-programi-akreditasyon-standartlari-ve-kilavuzlari-i-640.html.

13. Yüksek Öğretim Kurulu Web site. (2011). Retrieved January 27, 2022, from http://www.tyyc.yok.gov.tr/

14. Büyüköztürk, Ş., Kılıç Çakmak, E., Akgün, Ö.E., Karadeniz Oran, Ş., Demirel, F. (2019). Bilimsel Araştırma Yöntemleri, Pegem Akademi, Ankara.

15. Çalıkuşu, M., Özçelikay, G. (2020). Türkiye'de Eczacılık Mesleğinin Gelecek İşgücü Kapasitesinin Analizi. In: G. Özçelikay and İ. Yıldız (Eds.), Eczacılıkta Yenilikler-3, (pp. 145-156). Ankara: Ankara Üniversitesi Basımevi.

16. Şahsuvaroğlu, T., Ekşi, H. (2008). Odak grup görüşmeleri ve sosyal temsiller kuramı. *Marmara Üniversitesi Atatürk Eğitim Fakültesi Eğitim Bilimleri Dergisi*, *28*(1), 127-139.

17. Koçak, A., Özgür, A. (2006). İçerik analizi çalışmalarında örneklem sorunu. *Selçuk İletişim Dergisi*, *4*(3), 21-28.

18. Tong, A., Sainsbury, P., Craig, J. (2007). Consolidated criteria for reporting qualitative research (COREQ): A 32-item checklist for interviews and focus groups. *International Journal For Quality In Health Care*, *19*(6), 349-357. [CrossRef]

19. Becerikli, S. (2013). Kuşaklararası iletişim farklılığı: Bilim teknoloji ve yenilik haberleri üzerinden bir odak grup çalışması. *Selçuk İletişim Dergisi, 8*(1), 5-18.

20. Seçer, İ., Ay, İ., Ozan, C., Yılmaz, B. (2014). Rehberlik ve psikolojik danışma alanındaki araştırma eğilimleri: Bir içerik analizi. *Türk Psikolojik Danışma ve Rehberlik Dergisi*, *5*(41), 49-60.

21. Eskici, G.Y., Özsevgeç, T. (2019). Yaşam becerileri ile ilgili çalışmaların tematik içerik analizi: bir meta-sentez çalışması. *International e-Journal of Educational Studies*, *3*(5), 1-15. [CrossRef]

22. Çokluk, Ö. (2011). Nitel bir görüşme yöntemi: Odak grup görüşmesi. *Kuramsal Eğitimbilim, 4* (1), 95-107.

23. Karakoç, F.Y., Dönmez, L. (2014). Ölçek geliştirme çalışmalarında temel ilkeler. *Tıp Eğitimi Dünyası*, *13*(40), 39-49. [CrossRef]

24. Gülpınar, G. (2019). Pharmacy education in Turkey from professional socialization perspective. *Literatür Eczacılık Bilimleri Dergisi*, *8*(3), 196-205. [CrossRef]

25. Kara, E., Okuyan, B., Demirkan, K., Sancar, M. (2021). Türkiye'de klinik eczacılık hizmetlerinin yerleştirilmesi ve geliştirilmesi aşamasında cevap bekleyen bir soru: Klinik eczacı kimdir?. *Literatür Eczacılık Bilimleri Dergisi*, *10*(1), 109-118. [CrossRef]

26. Rapp, R.P. (2007). A perspective on 45 years in clinical pharmacy education. *Annals of Pharmacotherapy*, *41*(6), 1047-1049. [CrossRef]

27. Sánchez, A.M. (2011). Teaching patient-centered care to pharmacy students. *International Journal of Clinical Pharmacy*, *33*(1), 55-57. [CrossRef]

28. Kaptan, F., Korkmaz, H. (2001). Fen eğitiminde probleme dayalı öğrenme yaklaşımı. *Hacettepe Üniversitesi Eğitim Fakültesi Dergisi*, *20*, 191-192.

29. Torp, T. (1997). What is problem-based learning? *Wingspread Journal*, 1-5.

30. Saban, A. (2000). Öğrenme Öğretme Süreci, Nobel Yayın Dağıtım, Ankara.

31. Peterson, F., Eaguest, D. (1998). Learning to teach primary science through problem based learning. *Science Education*, *82*, 215-237. [CrossRef]

32. Hmelo, C., Silver, E. (2004). Problem based learning; what and how do students learn? *Educational Psychology Review*, *16*(39), 235-263.

33. Major, C.H., Baden, M.S. (2000). Issues in problem-based learning: A message from guest editors. *Journal on Excellence In College Teaching*, 1-14.

34. Dahlgren, M.A., Castensson, R., Dahlgren, L.O. (1998). PBL from the teachers' perspective, conceptions of the tutor's role within problem based learning. *Higher Education*, *36*, 437-444.

35. Rhem, J. (1998). Problem-based learning: An introduction. *The National Teaching & Learning Forum*, *8*(1), 1-4.

36. Duch, B. (1996). Problems: A key factor in PBL. *About Teaching*, *50*, 7-8.

37. Yu-chen, H. (1999). Evaluation theory in problem based learning approach. *The National Convention of the Association for Educational Communications and Technology*, *21*, 458-468.

38. Winning, T., Skinner, V., Townsend, G., Drummond, B., Kieser, J. (2004). Developing problem-based learning packages internationally: An evaluation of outcomes. *Innovations in Education and Teaching International*, *41*(2), 125-144. [CrossRef]

39. Dykhne, M., Hsu, S.Y., McBane, S., Rosenberg, E., Taheri, R. (2021). Differences in learning styles, critical thinking skills, and peer evaluations between students with and without leadership engagement. *Currents in Pharmacy Teaching and Learning*, *13*(6), 659-664. [CrossRef]

40. Jin, H.K., Choi, J.H., Kang, J.E., Rhie, S.J. (2018). The effect of communication skills training on patient-pharmacist communication in pharmacy education: A meta-analysis. *Advances in Health Sciences Education*, *23*(3), 633-652. [CrossRef]

41. Pitkä, K., Löfhjelm, U., Passi, S., Airaksinen, M. (2014). Integrating internships with professional study in pharmacy education in Finland. *American Journal of Pharmaceutical Education*, *78*(9), 173. [CrossRef]

42. Anderson Jr, D.C., Sheffield, M.C., Hill, A.M., Cobb, H.H. (2008). Influences on pharmacy students' decision to pursue a doctor of pharmacy degree. *American Journal of Pharmaceutical Education*, *72*(2), 22. [CrossRef]

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 684-693, 2022
Doi: 10.33483/jfpau.1092181

ORIGINAL ARTICLE / ÖZGÜN MAKALE

THE EVALUATION OF AWARENESS AND LEVEL OF KNOWLEDGE RELATED TO TUBERCULOSIS OF PHARMACY FACULTY STUDENTS: A FOUNDATION UNIVERSITY EXAMPLE

ECZACILIK FAKÜLTESİ ÖĞRENCİLERİNİN TÜBERKÜLOZA İLİŞKİN FARKINDALIK VE BİLGİ DÜZEYLERİNİN DEĞERLENDİRİLMESİ: BİR VAKIF ÜNİVERSİTESİ ÖRNEĞİ

Ozgul KISA[1*], Huseyin IKIZLERLI[2], Hayriye ERTEM VEHID[3]

[1]Ufuk University, Faculty of Medicine, Department of Medical Microbiology, 06510, Ankara, Turkey

[2]Altınbaş University, Fifth grade student of Faculty of Pharmacy 34147, Istanbul, Turkey

[3]Demiroglu Bilim University, Faculty of Medicine, Department of Medical Education and Informatics, 34394, Istanbul, Turkey

ABSTRACT

Objective: *Tuberculosis (TB) is an infectious disease that is among the leading causes of death. TB can easily spread by aerosols from pulmonary TB patients. Pharmacists are unique among healthcare professionals because patients can easily and directly access them. Therefore, they have to take responsibility for management and early detection of TB. The aim of this study was to evaluate TB related awareness and level of knowledge of pharmacy students in a foundation university.*

Material and Method: *This cross-sectional study was conducted to evaluate the knowledge and attitudes of undergraduate pharmacy students about TB. A face-to-face survey was made to students in their classrooms after ethical committee's approval was received and verbal consent of participants obtained. A total of 238 students answered the survey. Descriptive statistical methods and Pearson Chi-Square test are used for the evaluation of the data.*

Result and Discussion: *We found that all of the students knew tuberculosis, but some of them lacked knowledge on some topics such as the symptoms of the disease, risk factors, definitive diagnosis, and drug resistance rates. Pharmacists who have an important role in public health should be informed and advised to be updated about TB during their undergraduate education. In addition, it should be ensured that they take responsibility to raise public awareness of TB which causes thousands of deaths each year.*

Keywords: *Awareness, pharmacy students, survey, tuberculosis*

[*] **Corresponding Author / Sorumlu Yazar:** Ozgul Kısa
e-mail / e-posta: kisaozgul@gmail.com, **Phone / Tel.:** +905322214793

Submitted / Gönderilme: 23.03.2022 **Accepted / Kabul:** 12.05.2022

ÖZ

Amaç: *Tüberküloz (TB), önde gelen ölüm nedenleri arasında yer alan bir enfeksiyon hastalığıdır. TB, akciğer tüberkülozlu hastalardan aerosoller aracılığı ile kolayca yayılabilir. Eczacılar, hastaların onlara doğrudan ve kolayca ulaşabildiği tek sağlık çalışanlarıdır. Bu nedenle, TB'nin yönetimi ve erken teşhisi için sorumluluk almak zorundadırlar. Bu çalışmanın amacı, bir vakıf üniversitesindeki eczacılık öğrencilerinin tüberküloz ile ilgili farkındalık ve bilgi düzeylerini değerlendirmektir.*

Gereç ve Yöntem: *Bu kesitsel çalışma, eczacılık lisans öğrencilerinin tüberküloz hakkındaki bilgi ve tutumlarını değerlendirmek amacıyla yapılmıştır. Etik kurul onayı ve katılımcıların sözlü onamları alındıktan sonra öğrencilere sınıflarında yüz yüze uygulanmıştır. Toplam 238 öğrenci anketi cevapladı. Verilerin değerlendirilmesinde tanımlayıcı istatistiksel yöntemler ve Pearson Ki-Kare testi kullanılmıştır.*

Sonuç ve Tartışma: *Öğrencilerin tamamının tüberkülozu bildiğini ancak bir kısmının hastalığın belirtileri, risk faktörleri, kesin tanı, ilaç direnç oranları gibi konularda bilgi eksikliği olduğunu bulduk. Halk sağlığında önemli bir yere sahip olan eczacılar, lisans eğitimleri sırasında TB hastalığı hakkında bilgilendirilmeli ve bilgilerini güncel tutmaları tavsiye edilmelidir. Ayrıca her yıl binlerce ölüme neden olan tüberküloz hastalığı konusunda toplumu bilinçlendirmek için sorumluluk almaları sağlanmalıdır.*

Anahtar Kelimeler: *Anket, eczacılık öğrencileri, farkındalık, tüberküloz*

INTRODUCTION

Tuberculosis (TB) is transmitted from person to person by inhalation of infected aerosols (particles called droplet nuclei) generated by coughing, sneezing, or speaking of the active TB patients [1,2]. The people who are in close contact with patients with active TB (e.g., family members or friends living in the same household with infected people, health-care professionals directly involved in their treatment) are at higher risk [3].

Antituberculosis drugs are essentially divided into two groups as first-line drugs (e.g., rifampin, isoniazid, ethambutol, and pyrazinamide) and second-line drugs consisting of four subgroups; aminoglycosides (e.g., kanamycin, amikacin, and capreomycin), fluroquinolones (e.g., ofloxacin, levofloxacin, moxifloxacin, and ciprofloxacin), oral bacteriostatic second-line anti- TB drugs (e.g., ethionamide, prothionamide, cycloserine, and P- aminosalicylic acid) and anti-TB drugs with limited data on efficacy (e.g., bedaquiline, delamanid, and linezolid) [3,4]. However, drug-resistant *Mycobacterium tuberculosis* strains have emerged, especially in TB endemic areas. The emergence and spread of drug resistant strains are one of the primary problems in combating TB. Multi-drug-resistant (MDR) TB is defined as TB resistant to first-line drugs such as isoniazid and rifampin, and extensive drug-resistant (XDR) TB is defined as resistance to MDR-TB plus any fluoroquinolone and at least one injectable second-line drug [5,6].

In 2019, the estimate of the new cases was 10 million (range, 8.9–11.0 million) and there were almost 1.2 million deaths among HIV-negative people [7]. The people with HIV have a much higher risk of developing TB disease due to weak immune systems. Additionally, 208.000 deaths among HIV-positive people reported. The incidence rate of TB was 130 cases per 100,000 population in 2019. The incidence of TB varies among countries due to their healthcare services, economic conditions, and

policies of governments about TB disease [7]. According to Tuberculosis Report of Turkish Ministry of Health (2019), estimated incidence rate of TB was 17 cases per 100,000 population for 2017 year. While MDR-TB strains constitute 3,2% of TB cases in Turkey, only two TB strains are XDR-TB [8].

As the most accessible health counselors, pharmacists play a vital role in the delivery of the primary health care, including the management of the chronic diseases. They have a face-to-face contact with people who may have many infectious diseases such as tuberculosis [9-11]. Although the studies were conducted to investigate the knowledge and attitudes of medical doctors and the other Healthcare professionals or nursing and medical students about TB, no studies were found with pharmacists or pharmacy students in Turkey [12-17]. The aim of this study is to evaluate the awareness and knowledge levels of the Pharmacy Faculty students about the basic information such as the transmission of TB disease, its symptoms, risk factors and protection from the disease.

MATERIAL AND METHOD

Place of Study

We implemented this cross-sectional study at a Pharmacy Faculty in İstanbul in 2019-2020 education year.

Study Population

Education in pharmacy faculties is five years and the first four years are spent with taking theoretical courses and internship. In the last year, the again internship and the graduation thesis must be completed. The survey was conducted in the period of December 2019-March 2020. The students in the first four grades were included in the study. However, fifth-year students could not be included as they are often out of school in this term due to their graduation projects or internships (e.g., hospital pharmacy, community pharmacy, and industry internships).

Data Collection

The data were collected using the “Assessment of Awareness and Knowledge Level of TB" Questionnaire. It consists of a total of 26 questions, 15 of which contain information about TB and 11 to obtain information about the personal characteristics of the participants. Previously, Enginyurt et al. [12] carried out a study for TB awareness assessment with healthy workers in 2016 in Turkey. After permission to use and modify, this questionnaire was obtained from authors and modified by reviewing current reports and literature about transmission, symptoms, risk factors, diagnosis, prevention, and treatment of TB disease. As the incidence of TB changes from year to year in the TB Dispensary report, only choices of one of TB related questions was changed. The other 11 questions were also revised

according to students. Identity information of participants were not requested, and no question related to personal privacy or daily life was present in the survey form. The privacy principle was adhered while preparing the survey questions.

The questionnaire forms were given to the participants who gave informed verbal consent after explanation of the aims, objectives, and methodology of this study. The questionnaire was administered by face-to-face interview method. The data in the forms in which all questions were answered, were evaluated.

Statistical Analysis

The data collected were analyzed by using descriptive statistical methods and Pearson Chi-Square test. P values < 0.05 were considered statistically significant.

Table 1. Answers of pharmacy students to survey questions about tuberculosis (n 238)

Characteristics		% of Respondents				
		First grade students	Second grade students	Third grade students	Fourth grade students	Total
Have you ever had an education about tuberculosis in school?		19,6%	31,3%	19,2%	22,2%	23,5%
Have you ever had a role in monitoring or treatment of a tuberculosis patient in your internships?		16,1%	14,9%	19,2%	12,7%	15,5%
Have you ever encountered with a tuberculosis patient in your clinical or pharmacy internships?		12,5%	7,5%	11,5%	7,9%	9,7%
Do you know a family member who had tuberculosis?		8,9%	10,4%	3,8%	3,2%	6,7%
Do you see tuberculosis as a professional disease of Healthcare professionals? Why?	Healthcare professionals encounter more likely with tuberculosis patients.	96,4%	88,1%	82,7%	81,0%	87,0%
	It is highly contagious in contacts (close contact during examination and treatment) since it is transmitted by respiratory tract.	100,0%	100,0%	98,1%	95,2%	98,3%
What would your thoughts be if you had tuberculosis in the following years?	I used to take my medications regularly according to my doctor's recommendations.	16,1%	13,4%	9,6%	12,7%	13,0%
	I would go to a health institution for vaccination.	78,6%	82,1%	86,5%	85,7%	83,2%
	I would be sad that the treatment of the disease took a long time.	5,4%	4,5%	3,8%	1,6%	3,8%
How many years have you been studying in Pharmacy Faculty?		23.5%	28.2%	21.8%	26.5%	-
Your sex?	Female	69,6%	65,7%	69,2%	69,8%	68,5%
	Male	30,4%	34,3%	30,7%	30,2%	31,5%
Your age?	17-19	39,3%	7,5%	0,0%	3,2%	12,2%
	20-22	58,9%	77,6%	75,0%	66,7%	69,7%
	23-25	0,0%	10,4%	19,2%	30,2%	15,1%
	26 or more	0,4%	1,3%	0,8%	0,0%	2,5%
Do you smoke?	Yes	19,6%	22,4%	25,0%	17,5%	21,0%
If yes, how many years have you been smoking?	0-2 years	8,9%	11,9%	1,9%	4,8%	7,1%
	3-5 years	5,4%	7,5%	15,4%	6,3%	8,4%
	6-8 years	1,8%	6,0%	5,8%	4,8%	4,6%
	9-11 years	0,0%	0,0%	1,9%	1,6%	0,8%
	12 years or more	3,6%	0,0%	0,0%	0,0%	0,8%

Table 2. Assessment of pharmacy students' tuberculosis related knowledge (n 238)

Questions		% of Respondents Who Answered Correctly					
		First grade students	**Second grade students**	**Third grade students**	**Fourth grade students**	**χ^2 (df)**	***p* value**
How is tuberculosis transmitted?		16,1%	49,3%	67,3%	39,7%	70.30 (15)	<0.001
Which type of tuberculosis is contagious?		3,6%	34,3%	63,5%	38,1%	69.10 (15)	<0.001
Which organs may affect by tuberculosis?		32,1%	59,7%	69,2%	57,1%	49,69 (15)	<0.001
How to make a definitive diagnosis of tuberculosis Infection?		16,1%	16,4%	11,5%	23,8%	22.47 (12)	0.033
Is tuberculosis a disease must be reported?		76,8%	94,0%	90,4%	81,0%	13,59 (6)	0.034
How often tuberculosis is seen in one year in Turkey?		8,9%	1,5%	13,5%	15,9%	25,98 (12)	0,011
What is DOT (Directly Observed Treatment=DOT) in Tuberculosis?		12,5%	22,4%	13,5%	19,0%	29,10 (12)	0,004
Which is the most important for the treatment of tuberculosis?		30,4%	52,2%	48,1%	44,4%	22,67 (12)	0,031
Which of the followings are features of Tuberculosis Dispensary? (you may mark more than 1 answer)	Keeps records of tuberculosis patients and notifies the ministry of health	82,1%	68,7%	36,5%	60,3%	25,41(3)	<0.001
	Provides follow-up and treatment of tuberculosis patients with or without health insurance	60,7%	47,8%	46,2%	52,4%	2,89 (3)	0,409
	Obtains tuberculosis drugs to the certain participation fee	78,6%	82,1%	78,8%	74,6%	1,08 (3)	0,781
	It is main component of the tuberculosis control program in our country	76,8%	76,1%	80,8%	66,7%	5,69 (6)	0,458
What do you think is the most common risk factor for Tuberculosis?		5,4%	11,9%	21,2%	34,9%	41,67 (12)	<0.001
When do you think tuberculosis should be vaccinated first?		14,3%	16,4%	21,2%	15,9%	20,64 (12)	0,056
What are the symptoms of pulmonary tuberculosis?		14,3%	34,3%	48,1%	55,6%	65,84 (13)	<0.001
How to diagnose pulmonary tuberculosis?		12,7%	6,0%	9,6%	27,0%	44,40 (15)	<0.001
Which of the following is a way of preventing Healthcare professionals from tuberculosis?		21,4%	32,8%	40,4%	22,2%	61,18 (15)	<0.001
What would be the precautions to be taken when providing health care in a hospital environment to a patient with pulmonary tuberculosis found to be infectious?		10,7%	13,6%	30,8%	34,9%	43,60 (18)	<0.001

RESULT AND DISCUSSION

During the period of the study, a total of 389 students were in either their first, second, third, or fourth years. Majority of these students (238 of 389) participated voluntarily the survey. Of the 238 participants, 163 (68.5%) were female students, while male students were 75 (31,5%). The characteristics of them were summarized in Table 1. All participants expressed having heard about TB

earlier. Although 15,5% of them received information on monitoring or treatment of a TB patients in their internships, only 9,7% had encountered with a TB patient. Majority of participants had acknowledged TB disease as a professional disease of healthcare professionals because Healthcare professionals encounter more likely with TB patients, and it is highly contagious in contacts (close contact during examination and treatment) since it is transmitted by respiratory tract (Table 1).

Participants' TB-related knowledge and attitudes are shown in Table 2. According to results of survey, 42.9% of the students knew that TB is transmitted airborne and 34.5% of them said that pulmonary TB is contagious. More than half of participants, (54.6%) explained that both lungs and many organs may be affected by TB. There was a significant difference in the answers given to first three questions in students having different study years (grades) (Tablo 2). The minority of participants (16,1%, 16,4%, 11,5% and 23,8%, from 1st to 4th grade respectively and mean 17,2%) knew that TB was diagnosed by examination of sputum whereas 38.7% of them knew the tuberculin s*kin* test (TST) as a diagnostic tool. Also, 85.7 % of students accurately answered that when a person was diagnosed with TB, it was compulsory to report to the Health Ministry of Turkey whereas 13.9 % of students had no idea if it must be reported or not. More than half of students (55%) estimated high incidence of TB in Turkey (25 or 50 people per 100,000). Less than half of the students (44.1%) expressed that they must use antituberculosis drugs for at least six months in the treatment of TB patients, students did not know that directly supervised treatment means administration of TB drugs by a trusted person. Most of the participants knew the duties -except for one option- of the TB Dispensary, no difference was observed between the study years in terms of their answers. The question “what is the most important risk factor for TB disease” was correctly answered as Acquired Immune Deficiency Syndrome (AIDS) by the students at the rate of 18.5%. Many students (41.2% and 51.7% respectively) reported that they had no idea about two questions "When do you think people should be vaccinated first for TB?" and "What is the most important risk factor for TB disease?". More than one of third of students (38.2%) correctly explained pulmonary symptoms as blood in the mouth/sputum, fever, night sweats, and prolonged cough. The correct answers were increased from the first to the fourth grades and statistical differences were observed between the grades. Although 29.0% of the students said that Healthcare professionals should take measures to prevent transmission as a way of protection from TB such as wearing a mask, 23.9% of them stated that the BCG vaccine can provide lifelong protection. Some students also considered getting a chest x-ray or vaccination after they got in touch with a pulmonary TB patient.

Healthcare professionals are more likely to encounter TB patients. Especially, community pharmacists frequently encounter individuals with respiratory diseases such as TB. So, it is important to determine and increase the knowledge level of the pharmacists about TB disease for protecting themselves and inform the patients. Pharmacy faculty students basically get information about TB from

the lectures during their undergraduate education. After the students complete their 2nd year theoretical and practical courses, their internship programs start in community or hospital pharmacies. So, they also increase their level of knowledge about many diseases during internships. The results of this study indicated that almost all pharmacy students have heard about TB disease. But they had inaccuracies in their knowledge related to: How does TB transmit, which type of TB is contagious and which organs are affected by tuberculosis (Table 2). In the answers given to these questions, there were differences between grades. The definitive diagnosis of TB is made by bacteriological examination of sputum. In different studies conducted with doctors, this question was answered correctly by 83.6% [13], 68.4% [15] and 31.9% [16] of them. While 17.2% of our students stated that the definitive diagnosis of TB is made by bacteriological sputum examination, 38.7% of them had the wrong information that this can be done with the TST. Similar studies were found in which participants stated that the definitive diagnosis of TB was made with the TST [17,18]. The 75.6% of our students correctly knew the symptoms of TB disease. The awareness rate about TB symptoms was higher in upper-class students (Table 2) and was statistically significant. Although 41.2% of the students stated that they had no idea about the risk factors of TB, 18.5% of them stated that AIDS patients had a high risk of developing TB disease. It was observed that the understanding of the relationship among AIDS and TB in upper-class students increases and this difference was statistically significant. In our country, it is obligatory to report a person diagnosed with TB disease and directly observed treatment (DOT) is applied for TB treatment. TB drugs are given to patients free of charge by TB dispensaries. TB patient records are kept and Turkey' Tuberculosis Report is annually published [8]. It is known by 44.1% (105/238) of students that it is important to use medications for at least six months in the treatment of TB disease for effectiveness. On the other hand, they (85.7 %) stated that it is mandatory to report a person diagnosed with TB. At the same time, 187 of 238 students (78.6%) students said TB drugs were given free of charge by dispensaries. When these above summarized results were evaluated statistically, no significant difference was found between the grades. Interestingly, the students estimated that the number of incidences of TB is high in Turkey. Essentially, minority of students (9.7%) have encountered TB patients during their internships yet. Only15.5% students stated that they took an active role in the monitoring and treatment of TB patients during their internships. Since many students did not encounter TB patients during their internship, this emphasizes the importance of covering TB during their undergraduate education and sharing information about TB on television or on social media in order to increase their awareness of TB. Students consider TB as an occupational disease of Healthcare professionals because of close contact during the examination and treatment of patients and because the disease is transmitted through the respiratory tract. In other respects, it is interesting that 83,2% of the students said that they would get vaccinated if they had TB. The fact that some students think that the BCG vaccine can provide lifelong protection or that some get vaccinated after encountering a patient

with TB shows that they do not have enough information about the prevention measures against TB and the effectiveness of the vaccine. In a previous study in Japan, physicians of 80 medical school hospitals answered TB-related questionnaire consisting of two sets. Authors emphasized that additional training is required for physicians and medical students [19]. Nakanishi et al. [20] conducted a study in 2002 and showed that less than 50% of physicians gave correct answers to questions about TB. Kara et al. [12] evaluated the knowledge, attitudes, and behaviors of 110 pediatric residents from three different centers and determined that physicians lacked knowledge about TB diagnosis, treatment, and follow-up. The results of our study also showed that there is a lack of knowledge about TB disease among pharmacy students. Healthcare professionals are more likely to encounter patients with respiratory infection such as TB. So, it is important to determine and increase of the knowledge level of all of healthcare professionals about TB disease to protect themselves and to inform the patients. Therefore, TB disease should be emphasized in detail in the courses given during the undergraduate education of health-related occupational groups. The level of knowledge and awareness of TB should be increased with educational programs conducted by specialists working on TB control of healthcare professionals working with TB patients after graduation.

Our research has several limitations. First, the survey was carried out only with students of the first four grades. Since we included all volunteering students in our survey, there were 238 participants from the first four grades. Therefore, our results may not represent awareness and knowledge about TB of all students including 5th grade. Second, students were almost never encountered with TB patients during their internships. Consequently, the majority of the students did not have experience in monitoring or care for TB patients. Since there were no TB patients (except 6.7%) in their families, they could not have knowledge and experience about TB from their social circles. Finally, the students participating in the survey had not yet completed their fifth year's internship.

Pharmacists are frontline Healthcare professionals, and in most countries, they are the first to come into contact with people with TB. In order to combat TB effectively, it is necessary to improve the TB knowledge level and awareness of all healthcare teams, including pharmacists.

ACKNOWLEDGEMENTS

We would like to thank Associate Professor Özgür ENGİNYURT who allowed us using and modification of questionnaire form of their study. We also want to thank all students who accepted to participate in our study. Ozgul Kisa (Corresponding Author) was working at Department of Pharmaceutical Microbiology, Faculty of Pharmacy, A Foundation University when this study was performed.

AUTHOR CONTRIBUTIONS

Concept: *O.K.*; Design: *O.K.*; Control: *O.K.*; Sources: *O.K., H.İ.*; Data Collection and/or processing: *H.İ.*; Analysis and/or interpretation: *O.K., H.E.V.*; Literature review*: O.K., H.İ.*; Manuscript writing: *O.K.*; Critical review: *O.K.*; Other: *O.K., H.E.V.*

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS COMMITTEE APPROVAL

The ethics committee approval has been received from Altınbaş University Clinical Research Ethics Committee (Decision No:03, Date: 29 Nisan 2020).

REFERENCES

1. Nardell, E.A. (2016). Transmission and Institutional Infection Control of Tuberculosis. *Cold Spring Harb Perspect Medicine*, *6*(2), a018192. [CrossRef]

2. World Health Organization (WHO) guidelines on tuberculosis infection prevention and control, 2019 update, Geneva: World Health Organization; 2019. License: CC BY-NC-SA 3.0 IGO. From https://apps.who.int/iris/bitstream/handle/10665/311259/9789241550512-eng.pdf. Accessed date 27 January 2022.

3. World Health Organization (WHO) operational handbook on tuberculosis. Module 1: prevention-tuberculosis preventive treatment. Geneva: World Health Organization; 2020. License: CC BY-NC-SA 3.0 IGO. From https://www.who.int/publications/i/item/9789240002906. Accessed date 27 January 2022.

4. Rendon, A., Tiberi, S., Scardigli, A., D'Ambrosio, L., Centis, R., Caminero, J.A., Migliori, G.B. (2016). Classification of drugs to treat multidrug-resistant tuberculosis (MDR-TB): evidence and perspectives. *Journal Thoracic Disease, 8*(10), 2666–2671. [CrossRef]

5. Khare, N., Khare, P., Singh, D.A. (2018). A Review: History, Structure, Diagnosis and Treatment of Tuberculosis Disease. *Mycobacterial Disease, 8*, 263. [CrossRef]

6. Lange, C., Aarnoutse, RE., Alffenaar, J.W.C., Bothamley, G., Brinkmann, F., Costa, J., Chesov, D., Creve, R., Dominguez, J., Duarte, R., Grobbel, H.P., Günther, G., Guglielmetti, L., Heyekendorf, J., Kay, A.W., Kirakosyan, O., Kirk, O., Koczulla, R.A., Kudriashov, G.G., Kuksa, L., Leth, F., Magis-Escurra, C., Mandalakas, A.M., Molina-Moya, B., Reimann, M., Rumetshofer, R., Schaaf, H.S., Schön, T., Tiberi, S., Valda, J., Yablonski, K., Dheda, K. (2019). Management of patients with multidrug-resistant tuberculosis. *International Journal Tuberculosis Lung Disease, 23*, 645-662. [CrossRef]

7. World Health Organization (WHO) Global Tuberculosis Report 2019. From https://www.who.int/publications/i/item/9789241565714. Accessed date 27 January 2022.

8. Türkiye'de Verem Savaşı 2018 Raporu. From https://toraks.org.tr/site/community/news/5074. Accessed date 27 January 2022.

9. Agomo, C.O. (2012). The role of community pharmacists in public health: A scoping review of the literature. *Journal of Pharmaceutical Health Services Research, 3*, 25–33. [CrossRef]

10. Weiss, M.C., Booth, A., Jones, B., Ramjeet, S., Wong, E. (2010). Use of simulated patients to assess the clinical and communication skills of community pharmacists. *Pharmacy World & Science*, *32*, 353–361. [CrossRef]

11. Bell, J., Dziekan, G., Pollack, C., Mahachai, V. (2016). Self-Care in the Twenty First Century: A Vital Role for the Pharmacist. *Advances in Therapy*, *33*, 1691–1703. [CrossRef]

12. Enginyurt, O., İşcanlı, I., Kılıç, M., Çakır, L., Öztürk, E.H., Depe, Y., Kelem, D., Tomakin, M., Çamkaya, S., Güreşçi, N., Aydın, F., Sönmez, B. (2016). Tuberküloz Farkindalik Değerlendirmesi. *Klinik Tıp Aile Hekimliği, 8*, 25-35.

13. Kara, A., Doğar, O., Yuksek, S.K., Apa, H., Bayram, N., Güneş, T., Tezer, H., Devrim, İ. (2015). The evaluation of awareness and level of knowledge about tuberculosis among pediatric residents. *İzmir Dr. Behçet Uz Çocuk Hastanesi Dergisi*, *5*, 48-53.

14. Akin, S., Gorak, G., Unsar, S., Mollaoglu, M., Ozdilli, K., Durna, Z. (2011). Knowledge of and attitudes toward tuberculosis of Turkish nursing and midwifery students. *Nurse Education Today*. *31*, 774-779. [CrossRef]

15. Mumcu, H.K. (2013). Trabzon'da Hekimlerin Tüberküloz Kontrolü ve Doğrudan Gözetimli Tedavi Stratejisi ile İlgili Bilgi ve Uygulamaları. *Sürekli Tıp Eğitimi Dergisi*. *22*, 131-137.

16. Karahan, A., Çalı, Ş. (2005). Ümraniye'de hekimlerin tüberküloz kontrolü ve DOTS hakkındaki bilgi ve tutumları. *Sürekli Tıp Eğitimi Dergisi*. *14*, 195-201. [CrossRef]

17. Değer, V., İnanç, B.B., Çiftçi, S. (2015). Knowlodge of Tuberculosis Among Health Higher School Students. *Journal of Clinical and Analytical Medicine*, *6*, 358-362.

18. Deveci, S.E., Turgut, T., Acik, Y., Deveci, F., Muz, M.H. (2003). The Knowledge, attitude and behavior related to the tuberculosis and approache s of tuberculosis therapy by the physicians providing first step service. *Tüberküloz ve Toraks*, *51*, 40-47.

19. Kurane, S., Kudoh, S. (2003). The importance of the examination of education on, and infection control of tuberculosis in medical school hospitals in Japan. *Kekkaku*, *78*, 573-580.

20. Nakanishi, Y., Izumi, M., Abe, K., Inoue, K., Wataya, H., Minami, T. Horiuchi, Y., Ishibashi, R.,Hara, N. (2002). Questionnaire about impression and knowledge of tuberculosis in employees and students in a university hospital. *Kekkaku, 77*, 457- 463.

Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 46(3): 694-702, 2022
Doi: 10.33483/jfpau.1100161

ÖZGÜN MAKALE / ORIGINAL ARTICLE

PTILOSTEMON CHAMAEPEUCE (L.) LESS.'İN SİTOTOKSİK VE ANTİMİKROBİYAL AKTİVİTESİ

CYTOTOXIC AND ANTIMICROBIAL ACTIVITIES OF PTILOSTEMON CHAMAEPEUCE (L.) LESS.

Serdar DEMİR[1*], Yalçın ERZURUMLU[2], İsmail ÖZTÜRK[3], Petek BALLAR KIRMIZIBAYRAK[4], Canan KARAALP[1]

[1]Ege Üniversitesi, Eczacılık Fakültesi, Farmasötik Botanik Ana Bilim Dalı, 35040, İzmir, Türkiye

[2]Süleyman Demirel Üniversitesi, Eczacılık Fakültesi, Biyokimya Ana Bilim Dalı, 32260, Isparta, Türkiye

[3]İzmir Katip Çelebi Üniversitesi, Eczacılık Fakültesi, Farmasötik Mikrobiyoloji Ana Bilim Dalı, 35620, İzmir, Türkiye

[4]Ege Üniversitesi, Eczacılık Fakültesi, Biyokimya Ana Bilim Dalı, 35040, İzmir, Türkiye

ÖZ

Amaç: *Bu çalışmanın amacı, P. chamaepeuce (L.) Less. (Asteraceae)'nin topraküstü kısımlarından hazırlanan n-hekzan, kloroform ve metanol ekstrelerinin antimikrobiyal ve sitotoksik aktivitelerinin araştırılmasıdır.*

Gereç ve Yöntem: *Ekstrelerin antimikrobiyal aktiviteleri, standart bakteri kökenleri (Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Salmonella enterica, Pseudomonas aeruginosa, Bacillus subtilis ve Streptococcus pneumoniae) ve mantar kökenleri (Candida albicans ve C. parapsilosis) üzerinde mikrodilüsyon metodu ile araştırılmış ve minimum inhibitör konsantrasyon (MİK) değerleri belirlenmiştir. Ekstrelerin sitotoksik aktivitesi, WST-1 reaktifi kullanılarak hücre proliferasyon analiz yöntemi ile HeLa, U2OS, PC3, MCF-7 ve A549 olmak üzere farklı kanser hücre hatları üzerinde incelenmiştir.*

Sonuç ve Tartışma: *Bitkiden elde edilen n-hekzan, kloroform ve metanol ekstrelerinin MİK değerlerinin S. aureus, E. faecalis ve B. subtilis'e karşı 250-1000 µg/mL konsantrasyon aralığında olduğu saptanmıştır. n-hekzan ve kloroform ekstrelerinin tüm kanser hücre hatlarında ise değişen düzeyde sitotoksik aktiviteye sahip oldukları belirlenmiştir (IC_{50}: 21.0-67.1 µg/mL).*

Anahtar Kelimeler: *Antimikrobiyal aktivite, Ptilostemon chamaepeuce, sitotoksisite*

* **Sorumlu Yazar / Corresponding Author:** Serdar Demir
e-posta / e-mail: serdar.demir@ege.edu.tr, **Tel. / Phone:** +905542396183

Gönderilme / Submitted: 11.04.2022 **Kabul / Accepted:** 16.05.2022

ABSTRACT

Objective: *The aim of this study is to investigate the antimicrobial and cytotoxic activities of n-hexane chloroform and methanol extracts prepared from the aerial parts of P. chamaepeuce (L.) Less. (Asteraceae).*

Material and Method: *The antimicrobial activities of the extracts on standard bacterial strains (Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Salmonella enterica, Pseudomonas aeruginosa, Bacillus subtilis and Streptococcus pneumoniae) and fungal strains (Candida albicans and C. parapsilosis) were examined by microdilution method and minimum inhibitory concentration (MIC) values were determined. The cytotoxic activity of the extracts was investigated by cell proliferation assay using WST-1 reagent on various cancer cell lines including HeLa, U2OS, PC3, MCF-7, and A549 cancer cell lines.*

Result and Discussion: *The MIC values of n-hexane, chloroform and methanol extracts were between 250-1000 µg/mL against S. aureus, E. faecalis and B. subtilis. n-hexane and chloroform extracts had varying levels of cytotoxic activity in all tested cancer cell lines (IC_{50}: 21.0-67.1 µg/mL).*

Keywords: *Antimicrobial activity, cytotoxicity, Ptilostemon chamaepeuce*

GİRİŞ

Ptilostemon Cass. (Asteraceae) cinsi, dünyada 15 tür ile, Türkiye florasında da ikisi endemik olmak üzere 6 taksonla temsil edilmektedir. *P. chamaepeuce* (L.) Less. (Sect. Ptilostemon), Kırım Yarımadası'nda ve Türkiye'nin Marmara, Ege ve Akdeniz Bölgeleri'nden, İber Yarımadası ve Fas'a kadar uzanan Akdeniz havzasında doğal yayılış gösteren, mor çiçekli, çalı şeklinde çok yıllık bir takson olup ülkemizde halk arasında bozlanotu adı ile bilinmektedir [1,2]

P. chamaepeuce var. *cyprius*'un, Kıbrıs'ta halk arasında kas ve iskelet sistemi ile ilgili rahatsızlıklarda, *P. casabonae*'nin ise Sardunya Adası'nda barsak spazmlarını gidermede kullanıldığı, ayrıca haşlanmış taze çiçek ve filizlerinin gıda olarak tüketildiği kayıtlıdır [3,4]. Çeşitli *Ptilostemon* türleri üzerinde yapılan fitokimyasal çalışmalar, *P. afer, P. diacanthum* [5], *P. gnaphaloides* [6], *P. greuteri* [7], *P. niveus* [8] ve *P. strictus*'tan [9] guayanolit tip seskiterpenler; *P. greuteri*, *P. afer*, *P. diacanthum* [5] ve *P. strictus*'tan [9] asetilenik bileşikler, lignanlar ve triterpenlerin izolasyonu ile sonuçlanmıştır [6-8]. Bir başka çalışmada *P. casabonae*'nin fenolik bileşik içerikleri belirlenmiştir [10]. Uçucu yağ çalışmaları incelendiğinde ise *P. gnaphaloides* [11] ve *P. greuteri*'de δ-kadinen [7], *P. gnaphaloides* subsp. *pseudofruticosus*'ta ise germakren D'nin [3] ana bileşenler olduğu görülmektedir. Türkiye'de yayılış gösteren endemik *P. afer* subsp. *eburneus*'un meyvelerinin, total fenolik ve flavonoit içerikleri belirlenmiş, ana yağ asidi bileşeninin linoleik asit olduğu saptanmıştır (%58.52±0.10) [12].

Ptilostemon taksonları üzerinde yapılan biyoaktivite çalışmaları ile *P. casabonae*'nın antienflamatuvar [13], *P. afe*r'in antitümoral ve antimikrobiyal [14], *P. afer* subsp. *eburneus*'un antioksidan [12] ve *P. hispanicus*'un sitotoksik [15] etkileri incelenmiştir.

Literatürde *P. chamaepeuce* üzerinde yapılan fitokimyasal çalışmalar sınırlı olup, köklerinden 2 bisabolen tipte seskiterpen (ptilostemonol ve ptilostemonal) [16], topraküstü kısımlarından ise guayanolit tip bir seskiterpen (dezaçilsinaropikrin) ve sitosterol-β-*D*-glukozit [17] izole edilmiştir.

Ayrıca gerçekleştirdiğimiz literatür taramaları kapsamında *P. chamaepeuce*'nin biyoaktivitesi ile ilgili tek veriye rastlanmıştır; Girit Adası'nda yayılış gösteren bitkinin topraküstü kısımlarından hazırlanan diklorometan, metanol ve su ekstrelerinde 100 µg/mL dozda antileişmanyal etki, 47.6 µg/ mL dozda ise antimalaryal etki gözlenmemiştir. Vero hücreleri ile gerçekleştirilen denemelerde de 47.6 µg/ mL doza kadar herhangi bir sitotoksik etki saptanmadığı rapor edilmiştir [18].

Bu çalışmada, Türkiye'de yayılış gösteren *P. chamaepeuce*'nin toprak üstü kısımlarından hazırlanan *n*-hekzan, kloroform ve metanol ekstrelerinin antimikrobiyal aktivitesi, mikrodilüsyon metodu ile araştırılmıştır. Ekstrelerin sitotoksik aktivitesi ise WST-1 [2-(4-iodofenil)-3-(4-nitrofenil)-5-(2,4-disülfofenil)-2H-tetrazolyum] reaktifi kullanılarak hücre proliferasyon analiz yöntemi ile HeLa, U2OS, PC3, MCF-7 ve A549 olmak üzere çeşitli kanser hücre hatları üzerinde incelenmiştir.

GEREÇ VE YÖNTEM

Bitkisel materyal

P. chamaepeuce bitkisinin toprak üstü kısımları Haziran 2013'te çiçeklenme döneminde, Muğla, Marmaris, İçmeler mevkiinden toplanmıştır. Bitkinin teşhisi yapılarak, herbaryum örneği Ege Üniversitesi Eczacılık Fakültesi Herbaryumu'na kaydedilmiştir (İZEF-6045).

Ekstraksiyon

Gölgede kurutulup mekanik olarak öğütülen bitki (266 g) üzerine 3 tekrarlı olmak üzere sırasıyla 3'er L *n*-hekzan, kloroform ve metanol ilave edilerek 15 dk ultrasonik su banyosunda, 24 saat 100 rpm çalkalayıcıda tutulmuş ve tekrar 15 dk ultrasonik su banyosunda tutularak filtre kağıdından süzülmüştür. Süzülen ekstreler ayrı ayrı, 40 C°'de vakum altında evaporatör ile yoğunlaştırılmış ve vial içerisine alınarak vakum konsantratör sisteminde kuruluğa kadar uçurulmuştur. Hazırlanan ekstreler -20 C°'de muhafaza edilmiştir.

Antimikrobiyal aktivite tayini

Antimikrobiyal aktivite çalışmalarında Amerikan tipi kültür koleksiyonu (ATCC) ve Refik Saydam Kültür Koleksiyonu (RSKK) standart bakteri ve mantar kökenleri kullanılmıştır (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Salmonella enterica* RSKK 04059, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* RSKK 02021, *Streptococcus pneumoniae* ATCC 49619, *Candida albicans* ATCC 90028 ve *Candida parapsilosis* ATCC 22019). Bakteriyel kökenler Mueller-Hinton agarda (Merck) 37°C'de 24 saat, *Candida* kökenleri Sabouraud dekstroz agarda (Oxoid) 37°C'de 48 saat inkübe edilerek canlandırılmıştır. Taze koloniler serum fizyolojik ile süspanse edilmiş ve süspansiyonlar dansitometre cihazı (McFarland Densitometer, DEN-1 BIOSAN) ile 0.5 McFarland'a ayarlanmıştır. 96'lık mikroplak

kuyucuklarına bakteri kökenleri için 50 µl Mueller-Hinton broth (MHB) ve *Candida* kökenleri için 50 µl 3-morfolinopropan-1-sulfonik asit (MOPS) ile tamponlanmış Roswell Park Memorial Institute (RPMI) (Sigma) besiyeri ilave edilmiştir. Her bir köken için ilk kuyucuklara 50 µl olmak üzere, ekstre çözeltisi ilave edilerek seri dilüsyonları yapılmıştır. Kontrol amacıyla, %25 DMSO, antibakteriyel ajan olarak ampisilin ve antifungal ajan olarak flukonazol kullanılmıştır. Son olarak tüm kuyucuklara 1/100 oranında dilüe edilmiş olan mikrobiyal süspansiyonlardan 50 µl ilave edilmiştir. 37°C'de bakteriyel kökenlerin 24 saat ve *Candida* kökenlerinin 48 saat inkübasyonunun ardından, gözle görülebilen üremenin önlendiği en düşük konsantrasyon minimum inhibitör konsantraston (MİK) değeri olarak kabul edilmiştir [19].

Hücre kültürü çalışmaları ve sitotoksik aktivite tayini

Ekstrelerin sitotoksik etkilerinin değerlendirilmesi amacıyla WST-1 (Roche) reaktifi kullanılarak üretici firmanın önerdiği protokole uygun olarak hücre canlılık testi yapılmıştır. Çalışmalarda ATCC bankasından temin edilen hücre hatları kullanılmıştır. Kanser hücre hattı olarak insan epitelyal serviks adenokarsinoma (HeLa), insan epitelyal kemik osteosarkoma (U2OS), insan metastatik epitelyal prostat adenokarsinoma (PC3), insan epitelyal meme adenokarsinoma (MCF-7) ve insan epitelyal akciğer karsinoma (A549) hücre hatları kullanılırken sağlıklı hücre hattı olarak Afrika yeşil maymunu böbrek (Vero) hücre hattı kullanılmıştır. Tüm hücre hatları 37°C 'de %5 CO_2 içeren kültür koşullarında, %10 fetal sığır serumu (Fetal Bovine Serum, FBS) ve 2mM L-glutamin içeren DMEM (Dulbecco's Modified Eagle Medium) besi ortamı içerisinde büyütülmüştür [20].

Sitotoksisite çalışmalarında her bir hücre grubu için kuyucuk başına 5000 hücre olacak şekilde 96 kuyucuklu hücre kültür kaplarına ekim gerçekleştirilmiştir. 24 saat sonra *P. chamaepeuce* bitkisinden elde edilen ekstreler hücrelere 6 farklı dozda (200, 100, 50, 25, 10 ve 2 µg/mL) ve 3 tekrar şeklinde uygulanmıştır. Kontrol uygulaması olarak hücrelere eş hacimde çözgen uygulanmıştır. Takiben geçen 48 saatin sonunda ekstrelerin hücreler üzerindeki sitotoksik etkilerinin belirlenmesi amacıyla WST-1 çözeltisi uygulanarak 440nm'de birer saat arayla mikroplaka okuyucuda (Molecular Devices VersaMax Tunable Multiplate Reader) spektrofotometrik olarak ölçüm alınmıştır. Ekstrelerin hücreler üzerindeki IC_{50} değerlerinin hesaplanması amacıyla Graphpad Prism 5.0 programı kullanılmış olup non-lineer regresyon analizi yapılarak hesaplamalar gerçekleştirilmiştir [20, 21].

SONUÇ VE TARTIŞMA

P. chamaepeuce toprak üstü kısımlarından elde edilen ekstre miktarları ve kuru drog miktarına göre % verimleri Tablo 1'de sunulmuştur. En yüksek verim metanol ekstresinde elde edilmiştir (% 7.84).

P. chamaepeuce'nin topraküstü kısımlarından hazırlanan ekstrelerin antimikrobiyal aktiviteleri mikrodilüsyon metodu ile incelendiğinde, *n*-hekzan ekstresinin *S. aureus*'a karşı ve kloroform ekstresinin *B. subtilis*'a karşı MİK değerlerinin 250 µg/mL olduğu belirlenmiştir (Tablo 2). Diğer mikroorganizmalara karşı ekstrelerin 1000 µg/mL ve altı konsatrasyonlarında antibakteriyel/antifungal aktivitesi saptanamamıştır. DMSO'nun çalışılan konsantrasyonlarda bakteri ve mantarların üremelerine etki etmediği, ayrıca ampisilin ve flukonazolün MİK değerlerinin CLSI kriterlerine göre istenen sınır değerler içinde olduğu saptanmıştır. Bakteri ve mantar kökenleri üzerinde antimikrobiyal etkiler değerlendirildiğinde, ekstrelerin Gram-negatif bakteri kökenlerine ve maya kökenlerine kıyasla, Gram-pozitif bakteri kökenlerine karşı daha etkili oldukları ve MİK değerlerinin 250-1000 µg/mL konsantrasyon aralığında olduğu belirlenmiştir.

Tablo 1. *P. chamaepeuce* toprak üstü kısımlarından elde edilen ekstre miktarları ve % verimleri

Ekstre	**Miktar**	**% Verim**
***n*-hekzan**	9.46 g	%3.55
Kloroform	7.88 g	%2.96
Metanol	20.85 g	% 7.84

Tablo 2. *P. chamaepeuce* ekstrelerinde mikrodilüsyon yöntemi ile elde edilen MİK değerleri (µg/mL)

	***n*-hekzan**	**Kloroform**	**Metanol**	**Ampisilin**
S. aureus	250	1000	500	0.48
B. subtilis	250	250	500	TE
E. faecalis	1000	1000	500	TE

TE: test edilmedi

Ekstrelerin sitotoksik aktivitesi ise HeLa, U2OS, PC3, MCF-7 ve A549 kanser hücre hatlarında hücre proliferasyon yöntemi ile araştırılmış, en yüksek sitotoksik aktivitenin PC3 ve U2OS hücre hatları üzerinde kloroform ekstresine ait olduğu belirlenmiştir (IC_{50}: 21.00±1.10 ve 24.80±1.26 µg/mL, sırasıyla) (Tablo 3). Ancak aynı ekstrenin sağlıklı hücre hattı olarak kullanılan Vero'da IC_{50} değeri 18.85±1.11 µg/mL olarak saptanmıştır. Diğer yandan metanol ekstresinde herhangi bir sitotoksik etkiye rastlanmamıştır.

Tablo 3. *P. chamaepeuce* ekstrelerinin farklı konsantrasyonlarda uygulanması sonucu elde edilen IC_{50}±SS değerleri (µg/ml).

	HeLA	**U2OS**	**PC3**	**MCF-7**	**A549**	**Vero**
***n*-hekzan**	39.68±1.07	31.56±1.12	40.38±1.22	41.40±1.11	67.10±1.10	24.20±1.75
Kloroform	33.87±1.31	24.80±1.26	21.00±1.10	44.80±1.16	39.58±1.34	18.85±1.11
Metanol	-	-	-	-	-	-

- : Aktivite saptanmamıştır

Türkiye'de yayılış gösteren *P. afer*'den hazırlanan su, etanol ve metanol ekstreleri, *Staphylococcus epidermidis*, *S. pyogenes*, *Klebsiella pneumonia* ve *Enterobacter cloacae*'ye karşı inaktif bulunmuştur [14]. Bir başka çalışmada, *P. afer*'den elde edilen su ekstresinin balık patojeni olan *Lactococcus garvieae* üzerindeki antibakteriyal etkisi disk difüzyon metodu ile incelenmiş ancak etkili bulunmadığı belirtilmiştir (10.1 mm± 0.29) [22]. İspanya'da yayılış gösteren *P. hispanicus*'un topraküstü kısımlarından hazırlanan ekstrenin sitotoksik aktivitesi, A549 ve MRC-5 (insan akciğer normal hücresi) hücre hatları üzerinde incelenerek IC_{50} değerleri belirlenmiştir (sırasıyla, 276.6±13.6 ve 285.7±15.8 µg/mL) [15]. Türkiye'de yayılış gösteren *P. afer*'den hazırlanan su, etanol ve metanol ekstrelerinin, patates disk tümör indüksiyonu modelinde düşük antitümoral aktivite gösterdiği saptanmıştır (tümör inhibisyon oranları sırasıyla, % 44.4, 61.1 ve 58.3) [14].

Özellikle Asteraceae familyasında yaygın bir sekonder metabolit grubu olan seskiterpenler, kanser tedavisinde geniş bir terapötik potansiyele sahip moleküllerdir. Yapılan çalışmalarla, seskiterpen türevi moleküllerin çeşitli kanser türlerine karşı aktiviteleri, preklinik ve klinik çalışmalarla ortaya konmaktadır [23]. *P. chamapeuce*'den daha önce izole edilmiş olan dezaçilsinaropikrinin A549, SK-OV-3 (insan ovaryum adenokarsinoma), SK-MEL-2 (insan cilt malign melanom), XF498 (insan merkezi sinir sistemi solid tümörü) ve HCT15 (insan kolorektal adenokarsinoma) kanser hücre hatlarında sitotoksik aktivite gösterdiği saptanmıştır (ED_{50}: 13.29, 11.93, 3.62, 3.91ve 6.03 µg/mL, sırasıyla) [24].

Sonuçlarımız *P. chamaepeuce'*nin topraküstü kısımlarından çeşitli ekstraksiyon yöntemleri ile elde edilen ekstrelerin *S. aureus* ve *B. subtilis* üzerinde düşük antibakteriyel aktivite sergilediğini, özellikle kloroform ekstrelerinin PC3 ve U2OS kanser hücre hatları üzerinde yüksek sitotoksik aktiviteye sahip olduğunu göstermiştir. Ayrıca Fokialakis ve arkadaşları tarafından gerçekleştirilen çalışmadaki sonuçlara benzer şekilde *P. chamaepeuce'*nın topraküstü kısımlarından hazırlanan metanol ekstresinin Vero hücreleri ile birlikte test edilen kanser hücre hatları üzerinde herhangi bir sitotoksik etkiye sahip olmadığı belirlenmiştir [18].

Gerçekleştirdiğimiz literatür araştırmasına göre, bu çalışma Türkiye'de doğal yayılış gösteren *P. chamaepeuce* üzerinde gerçekleştirilen ilk biyoaktivite taramasıdır. Elde edilen veriler doğrultusunda, *P. chamaepeuce'*nin topraküstü kısımlarından hazırlanan ekstrelerde, sitotoksik aktiviteden sorumlu sekonder metabolitlerin karakterizasyonuna yönelik fitokimyasal araştırmaların yapılmasına gereksinim duyulmaktadır.

YAZAR KATKILARI

Kavram: *S.D., C.K.*; Tasarım: *S.D., C.K., P.B.K., İ.Ö., Y.E.*; Denetim: *C.K., S.D.*; Kaynaklar: *C.K., P.B.K., S.D., İ.Ö., Y.E.*; Malzemeler: *C.K., P.B.K., S.D., İ.Ö., Y.E.*; Veri toplama ve/veya işleme: *C.K., P.B.K., S.D., Y.E., İ.Ö.*; Analiz ve/veya yorumlama: *C.K., P.B.K., S.D., Y.E., İ.Ö.*; Literatür taraması: *C.K.,*

P.B.K., S.D., Y.E., İ.Ö.; Makalenin yazılması: *C.K., P.B.K., S.D., Y.E., İ.Ö.*; Kritik inceleme: *C.K., P.B.K.;* Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

ETİK KURUL ONAYI

Yazarlar bu çalışma için etik kurul onayının zorunlu olmadığını beyan etmektedir.

KAYNAKLAR

1. Ekim, T. (2012). *Ptilostemon* Cass. In: Guner, A., Aslan, S., Ekim, T., Vural, M., Babac, M.T., (Eds.), Türkiye Bitkileri Listesi (Damarlı Bitkiler), (pp. 193). İstanbul: Nezahat Gökyiğit Botanik Bahçesi ve Flora Araştırmaları Derneği.

2. Vilatersana, R., Garcia-Jacas, N., Garnatje, T., Molero, J., Sonnante, G., Susanna, A. (2010). Molecular phylogeny of the genus *Ptilostemon* (Compositae: Cardueae) and its relationships with *Cynara* and *Lamyropsis*. *Systematic Botany*, *35*(4), 907-917. [CrossRef]

3. Badalamenti, N., Modica, A., Ilardi, V., Bruno, M. (2022). The chemical composition of the essential oil of *Ptilostemon gnaphaloides* subsp. *pseudofruticosus* (Asteraceae) growing in Kythira Island, Greece. *Natural Product Research*, *36*(18), 4740-4744. [CrossRef]

4. Gonzalez-Tejero, M.R., Casares-Porcel, M., Sanchez-Rojas, C.P., Ramiro-Gutierrez, J.M., Molero-Mesa, J., Pieroni, A., Giusti, M.E., Censorii, E., de Pasquale, C., Della, A., Paraskeva-Hadijchambi, D., Hadjichambis, A., Houmani, Z., El-Demerdash, M., El-Zayat, M., Hmamouchi, M., ElJohrig, S. (2008). Medicinal plants in the Mediterranean area: Synthesis of the results of the project Rubia. *Journal of Ethnopharmacology*, *116*(2), 341-357. [CrossRef]

5. Bohlmann, F., Ziesche, J. (1980). Neue guajanolide und acetylenverbindungen aus *Ptilostemon*-arten. *Phytochemistry*, *19*(4), 692-696. [CrossRef]

6. Menichini, F., Di Benedetto, R., Delle Monache, F. (1996). A triterpene epoxide and a guaianolide from *Ptilostemmon gnaphaloides*. *Phytochemistry*, *41*(5), 1377-1379. [CrossRef]

7. Di Stefano, V., Pitonzo, R. (2012). Phytochemical studies on *Ptilostemon greuteri* Raimondo & Domina (Compositae). *Records of Natural Products*, *6*(4), 390-393. [CrossRef]

8. Menichini, F., Di Benedetto, R., Delle Monache, G., Delle Monache, E. (1986). Sesquiterpenes from *Ptilostemon niveus*. *Fitoterapia*, *57*, 458-459.

9. Janackovic, P., Tesevic, V., Marin, P.D., Milosavljevic, S.M., Petkovic, B., Sokovic, M. (2002). Polyacetylenes and a sesquiterpene lactone from *Ptilostemon strictus*. *Biochemical Systematics and Ecology*, *30*(1), 69-71. [CrossRef]

10. Marengo, A., Maxia, A., Sanna, C., Mandrone, M., Bertea, C.M., Bicchi, C., Sgorbini, B., Cagliero, C., Rubiolo, P. (2019). Intra-specific variation in the little-known Mediterranean plant *Ptilostemon casabonae* (L.) Greuter analysed through phytochemical and biomolecular markers. *Phytochemistry*, *161*, 21-27. [CrossRef]

11. Djordjevic, I., Tesevic, V., Milosavljevic, S., Menkovic, N., Vajs, V., Jadranin, M., Jovanovic, A., Djokovic, D. (2008). Composition of the essential oil of *Ptilostemon gnaphaloides*. *Chemistry of Natural Compounds*, *44*(5), 668-669. [CrossRef]

12. Kurt, A., Ozcan, M., Colak, N., Ozogul, Y., Glew, R., Ozogul, F., Ayaz, F.A. (2019). Fatty acids of oil and antioxidant capacity of phenolics from fruits of 11 Cardueae (Carduoideae, Asteraceae) taxa from northeast Anatolia (Turkey). *Botanica Serbica*, *43*(1), 31-45. [CrossRef]

13. Marengo, A., Fumagalli, M., Sanna, C., Maxia, A., Piazza, S., Cagliero, C., Rubiolo, P., Sangiovanni, E., Dell'Agli, M. (2018). The hydro-alcoholic extracts of Sardinian wild thistles (*Onopordum* spp.) inhibit TNF alpha-induced IL-8 secretion and NF-kappa B pathway in human gastric epithelial AGS cells. *Journal of Ethnopharmacology*, *210*, 469-476. [CrossRef]

14. Turker, A.U., Yildirim, A.B. (2013). Evaluation of antibacterial and antitumor activities of some Turkish endemic plants. *Tropical Journal of Pharmaceutical Research*, *12*(6), 1003-1010. [CrossRef]

15. Calderon-Montano, J.M., Martinez-Sanchez, S.M., Burgos-Moron, E., Guillen-Mancina, E., Jimenez-Alonso, J.J., Garcia, F., Aparicio, A., Lopez-Lazaro, M. (2019). Screening for selective anticancer activity of plants from Grazalema Natural Park, Spain. *Natural Product Research*, *33*(23), 3454-3458. [CrossRef]

16. Bohlmann, F., Rao, N., Schwarz, H. (1974). Naturally occuring terpene derivatives XXIX. Two new sesquiterpenes from *Ptilostemon chamaepeuce* (L.) Less. *Chemische Berichte*, *107*, 650-653. [CrossRef]

17. Bruno, M., Maggio, A., Paternostro, M.P., Rosselli, S., Arnold, N.A., Herz, W. (2001). Sesquiterpene lactones and other constituents of three Cardueae from Cyprus. *Biochemical Systematics and Ecology*, *29*(4), 433-435. [CrossRef]

18. Fokialakis, N., Kalpoutzakis, E., Tekwani, B.L., Khan, S.I., Kobaisy, M., Skaltsounis, A.L., Duke, S.O. (2007). Evaluation of the antimalarial and antileishmanial activity of plants from the Greek island of Crete. *Journal of Natural Medicines*, *61*, 38-45. [CrossRef]

19. Clinical and Laboratory Standards Institute (CLSI). (2013). Performance Standards for Antimicrobial Susceptibility Testing, 23rd Informational Supplement. CLSI document M100-S23. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA.

20. Baykan Erel, S., Senol, S.G., Aydın Köse, F., Ballar, P. (2011). *In vitro* cytotoxic properties of six *Artemisia* L. species. *Turkish Journal of Pharmaceutical Sciences*, *8*(3), 247-252. [CrossRef]

21. Thavamani, B.S., Mathew, M., Dhanabal, S.P. (2013). *In vitro* cytotoxic activity of menispermaceae plants against HeLa cell line. *Ancient Science of Life*, *33*(2), 81-84. [CrossRef]

22. Turker, H., Yildirim, A.B. (2015). Screening for antibacterial activity of some Turkish plants against fish pathogens: a possible alternative in the treatment of bacterial infections. *Biotechnology & Biotechnological Equipment*, *29*(2), 281-288. [CrossRef]

23. Abu-Izneid, T., Rauf, A., Shariati, M.A., Khalil, A.A., Imran, M., Rebezov, M., Uddin, M.S., Mahomoodally, M.F., Rengasamy, K.R.R. (2020). Sesquiterpenes and their derivatives-natural anticancer compounds: An update. *Pharmacological Research*, *161*, 105165. [CrossRef]

24. Choi, S.Z., Choi, S.U., Lee, K.R. (2005). Cytotoxic sesquiterpene lactones from *Saussurea calcicola*. *Archives of Pharmacal Research*, *28*(10), 1142-1146. [CrossRef]

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 703-711, 2022
Doi: 10.33483/jfpau.1013722

ORIGINAL ARTICLE / ÖZGÜN MAKALE

IMMUNOMODULATORY POTENTIAL OF *CLAUSENA EXCAVATA* LEAVES FRACTIONS VIA DECREASING THE PRODUCTION OF REACTIVE OXYGEN SPECIES FROM IMMUNE CELLS

CLAUSENA EXCAVATA YAPRAK FRAKSİYONLARININ BAĞIŞIKLIK HÜCRELERİNDE REAKTİF OKSİJEN TÜRLERİNİN ÜRETİMİNİ AZALTARAK GÖSTERDİĞİ IMMÜNOMODÜLATÖR ETKİNLİK

Shaymaa Fadhel Abbas ALBAAYIT[1*], Rukesh MAHARJAN[2]

[1]Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

[2]H.E.J Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi-75270, Karachi, Pakistan

ABSTRACT

Objective: *Clausena excavata is known to possess anti-oxidant property. However, this property through which mechanism it affects the immune cells and suppresses the production of reactive oxygen species (ROS) has not been explored.*

Material and Method: *This study evaluated the immunomodulatory activities of ethyl acetate, petroleum ether, chloroform, and methanol C. excavata leaf extracts by decreasing the production of ROS from whole blood, polymorphonuclears (PMNs) cells and macrophages.*

Result and Discussion: *Among the fractions tested, ethyl acetate C. excavate extract (EACE) showed potent anti-oxidant property and significantly ($p < 0.001$) suppressed intracellular and extracellular phagocytic oxidative ROS burst produced by the zymosan and PMA-activated whole blood, PMNs, and macrophages cells with 50% inhibitory concentration (IC_{50}) values of 5.7 ± 0.01, 1.3 ± 0.01, and 0.7 ± 0.03 µg/mL respectively. This study provides information regarding the mechanism behind its anti-oxidant property and its herbal use in treating various higher oxidative stress associated diseases.*

Keywords: *Clausena excavata, inflammation, macrophage, ROS*

ÖZ

* **Corresponding Author / Sorumlu Yazar:** Shaymaa Fadhel Abbas Albaayit
e-mail / e-posta: shaymaa_albaayit@yahoo.com, **Phone / Tel.:** +9647808430086

Submitted / Gönderilme: 23.10.2021 **Accepted / Kabul:** 01.06.2022

Amaç: *Clausena excavate'ın anti-enflamatuvar etkinlik gösterdiği bilinmektedir. Bununla birlikte, bu özelliği, bağışıklık hücrelerine hangi mekanizma ile etki ettiği ve reaktif oksijen türlerinin (ROS) üretimini baskıladığı araştırılmamıştır.*

Gereç ve Yöntem: *Bu çalışmada, C. excavate yapraklarının etil asetat, petrol eteri, kloroform ve methanol ekstrelerinin tam kan, polimorfonkleer (PMN) hücreler ve makrofajlardan ROS üretimini azaltarak gösterdikleri immunomodülatör etki değerlendirilmiştir.*

Sonuç ve Tartışma: *Test edilen fraksiyonlar arasından, C. excavate etil asetat ekstrresi en güçlü antienflamatuvar etkiyi göstermiş ve zimosan ve PMA tarafından active edilmiş tam kan, PMN ve makrofaj hücrelerinde, intraselüler ve ekstraselüler fagositik oksidatif ROS üretimini anlamlı oranda ($p < 0.001$) baskılamış ve %50 inhibisyon konsantrasyonları (IC_{50}) sırasıyla 5.7 ± 0.01, 1.3 ± 0.01, and 0.7 ± 0.03 µg/mL olarak tespit edilmiştir. Bu çalışma, bitkinin antienflamatuvar aktivitesinin mekanizması ve çeşitli enflamatuvar rahatsızlıkların tedavisinde kullanımına dair bilgi sağlamaktadır.*

Anahtar Kelimeler: *Clausena excavata, enflamasyon, makrofaj, ROS*

INTRODUCTION

Prolonged inflammation plays detrimental role in the progression of various chronic diseases such as gastritis, atherosclerosis, cancer, diabetes, and other various diseases. Phagocytes, known for its role in first line of immune defense system provide protection in the site of inflammation by releasing sudden burst of reactive oxygen species so that it could counteract with various types of invading agents [1,2]. During higher oxidative stress, the antioxidant system of host cells and tissues could not counteract over produced ROS due to which excess ROS react with DNA, protein consequently leading to the progression of chronic inflammatory related diseases. In natural products, there are many compounds which possess antioxidant property therefore these can prevent oxidative stress and prevent damage to cells caused by foreign xenobiotics and thereby balancing oxidative stress level and prevent progression of inflammatory diseases [3-6].

Clausena excavata Burm .f. can be found in tropical and subtropical Asian regions [7]. In many countries, still the leaves of *C. excavata* are being practiced in traditional medicine to treat wound, abdominal pain, headache, diarrhea, and snake-bite [8]. Some of the major bioactive compounds like alkaloids, flavonoid, carbazole, glycosides, coumarins etc are reported to be present in the leaves of *C. excavata* [9]. Even though, many biological activities of *C. excavata* like anticancer, antiinflammatory, antioxidant, and antiulcer properties has been reported but till now [10], the immunomodulatory ability of this plant to decrease oxidative stress has not been reported yet. In pursuance to this unreported activity, we investigated the ROS inhibiting ability of *C. excavata* by using chemiluminescence assay and further gave more evidence regarding its potent antioxidant property.

MATERIAL AND METHOD

Extraction of *C. excavata*

C. excavata plant was collected and submitted to Biodiversity Unit, Institute of BioScience, Universiti Putra Malaysia. It was thoroughly evaluated by Dr. Shamsul Khamis, botanist and after authentication, a specimen voucher no: TI-013201-CE was issued. At room temperature, fresh leaves were dried, powdered, and extracted according to the procedure described previously [11]. Briefly, the extraction was done using 5:1 petroleum ether: dried plant (weight to volume) suspension for 4 days. Thus, the filtrate collected was subjected to further extraction with chloroform, ethyl acetate, and methanol. Filtrates of all extracts were dried by evaporating in a rotary evaporator under reduced pressure to obtain the crude extract.

Immunomodulatory studies

Estimation of intracellular ROS production

Fresh human blood approximately 2-3 mL was withdrawn from a healthy person with consent which was approved from independent ethics committee with protocol no (IEC-047-HB-2019 /PROTOCOL/1.0), University of Karachi. Ficoll-hypaque density gradient centrifugation method was used to isolate polymorphonuclear cells (PMNs). Equal volume (2 mL) of blood, lymphocytes separation medium, and Hank's Balance Salt solution (HBSS--) were added and mixed. RBCs were lysed using hypotonic solution for 1 min incubation so that lymphocytes purified will be freed from contaminating RBCs. Lysis process was stopped by adding HBSS solution. Thus, obtained PMNs were resuspended in (HBSS++) solution and adjusted to $0.5\text{-}1\times10^6$ cells/ mL [12].

Estimation of extracellular ROS production

One Balb/c mice (25 g) was taken from animal house facility of ICCBS, with approved animal study protocol from animal house facility of ICCBS, University of Karachi. It was important to immunize mice therefore 1 mL of fetal bovine serum (FBS) was injected intraperitoneally. After 3 days, animal was sacrificed by cervical dislocation. Whole animal was dipped in 70% ethanol to sterilize whole body. After sterilization process, 5 mL of 10% complete RPMI medium was injected into the peritoneal cavity, massaged for 2 min and peritoneal cavity was exposed by cutting abdominal skin from lower side. Previously injected RPMI containing peritoneum exudate cells having macrophage was collected and centrifuged at 500 rpm for 5 min at 4°C. Cell pellet was formed at bottom, discarded supernatant and again cells were resuspended in incomplete RPMI medium containing HBSS++ solution. Equal volume (10 µL) of macrophages and trypan blue was mixed and counted cells using hemocytometer, and cells concentrations adjusted to $2x10^6$ cells/mL [13].

Chemiluminescence assay

The protocol of Mesaik et al., 2012 was performed for luminol enhanced chemiluminescence assay. 1 mL of whole blood was diluted 20 times in sterile HBSS++. Briefly, 25 µL of this diluted blood

suspension, 25 µL of PMNs ($1x10^6$ cells/mL), 25 µL of mice macrophage ($2x10^6$ cells/mL) in different white 96 well plate, were mixed with 25 µL of different concentration of plant extract (1, 10, and 100 µg/mL) in triplicate and incubated at 37ºC. Ibuprofen was used as a drug control. After 15 min incubation, 25 µL of 0.3% serum opsonized zymosan and 25 µL of luminol dye ($7x10^{-5}$ M) were added to those wells containing PMNs and whole human blood. Whereas 25 µL of PMA dye and 25 µL lucigenin dye were added to those wells containing macrophages. The final volume in each well became 100 µL. Plate was inserted inside the luminometer and chemiluminescence was monitored as relative light units (RLU). The level of the ROS was recorded and inhibition of ROS production (%) was calculated using following formula and IC_{50} values were determined. Experiment was carried out in six wells per concentration and was done in triplicate in three different days.

$$\% \text{ inhibition of ROS production} = 100 - \frac{\text{Average reading of test plant extracts}}{\text{Average reading of positive control}} \text{X } 100.$$

Statistical analysis

One way (ANOVA) with Tukey's post-hoc test ($p<0.05$) was applied to compare the data (mean ± SD) of treated samples with untreated control. All calculations were calculated by using GraphPad prism 6.0 statistical software.

RESULT AND DISCUSSION

Since many centuries, different medicinal plants present in different geographical locations had been used locally as medicinal agent to treat various diseases, therefore, medical practitioners, chemical and biological scientists have been focusing in the area of ethnopharmacology to further explore undiscovered benefits of those plants, especially natural drug with potent immunomodulation activities by inhibiting the production of ROS from immune cells in inflammatory processes [14-24]. Previous studies reported that *C. excavata* can modulate the immune cell through different mechanism [7, 10]. In pursuance of those studies, this current study intends to evaluate the immunomodulation effects of *C. excavata* that could be potential treatment for chronic inflammatory diseases. Activator agents such as PMA/zymosan are always used for preliminary screening of the immunomodulatory efficiency of a product. Luminol and lucigenin chemiluminescence dye are used in measuring extracellular and intracellular ROS, respectively [12-14].

Methanol, chloroform, and ethyl acetate extracts exhibited a significant oxidative burst inhibition in whole blood, PMNs and macrophages. Table 1 shows the potent inhibition of whole blood and PMNs cells generated ROS. Among the tested extracts on whole blood for inhibiting ROS production, the ethyl acetate extract showed potent activity ($p < 0.01$,**) with the lowest IC_{50} of < 10 µg/mL, followed by

methanol (20.2 ± 0.3 µg/mL) (p <0.05,*) and chloroform (27.9 ± 0.4 µg/mL) (p <0.05,*) fractions. In another experiment, ethyl acetate and chloroform showed significant (p < 0.001,***) inhibition of oxidative burst generated from zymosan-activated PMNs at lower concentrations (IC_{50} of 1.3 ± 0.3 and 2.1 ± 0.1 µg/mL respectively).

Table 1. Intracellular ROS production after treatment with *C. excavata* fractions.

Fraction/Drug	WB ROS/IC_{50} ± SD (µg/mL)	PMNs ROS/IC_{50} ± SD (µg/mL)
Methanol	20.2 ± 0.3 (*)	7.1 ± 0.05 (*)
Ethyl acetate	<10 (**)	1.3 ± 0.3 (***)
Chloroform	27.9 ± 0.4 (*)	2.1 ± 0.1 (***)
Petroleum ether	93.1 ± 0.5	33.1 ± 2.1
Standard (Ibuprofen)	10.1 ± 1.7	3.0 ± 0.5

WB: Whole Blood, ROS: Reactive Oxygen Species, PMNs: Polymorphonuclear cells (*p<0.05, **p<0.01, ***p<0.001)

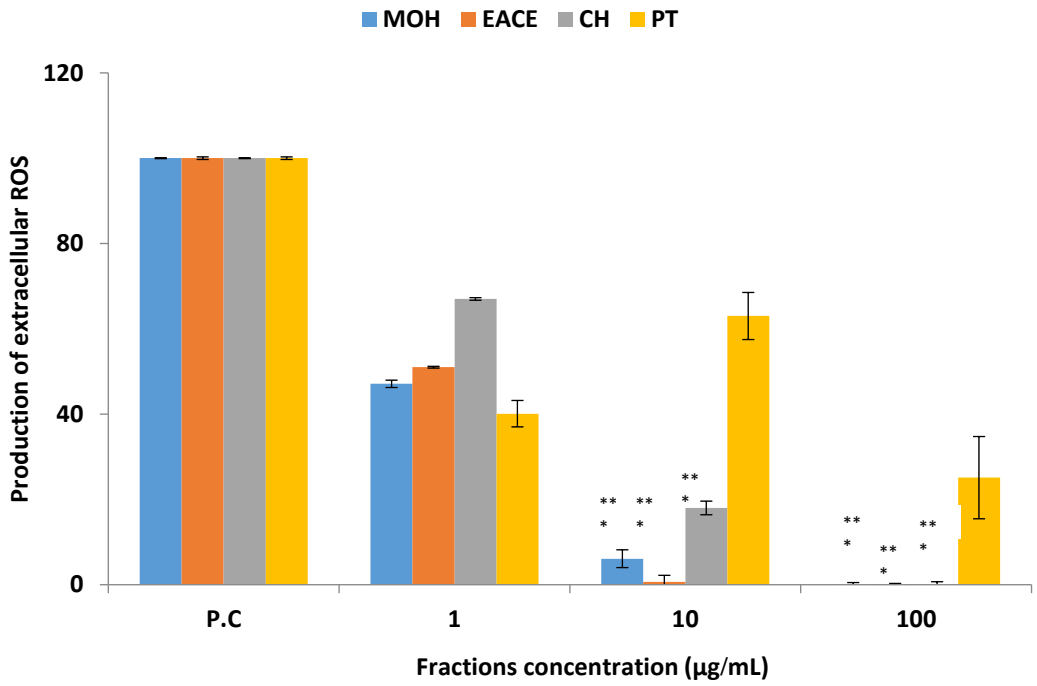


Figure 1. Extracellular ROS production after treatment with *C. excavata* fractions

Abbreviations: P.C, positive control; MOH, methanol; EA, ethyl acetate; CH, chloroform; PT, petroleum ether. (*p<0.05, **p<0.01, ***p<0.001)

The immunomodulatory activity of different fractions of *C. excavata* leaves to suppress extracellular ROS production from macrophages is presented in Fig. 1. Chloroform, methanol and ethyl acetate of *C. excavata* showed significant (p < 0.001) decreasing of oxidative burst generated from PMA-activated macrophages (IC_{50} 2.3 ± 0.01, 1.7 ± 0.01, and 0.7 ± 0.03 µg/mL respectively).

The inhibited level of different extracts might be related to high phenolic contents that could be main contributor to antioxidant and immunomodulatory capacity of the extract, as previously reported publication [11]. The phenolic compounds can inhibit protein kinase C (PKC) and complement system; therefore, it is possible that these compounds present in this plant decrease ROS production by inhibiting NADPH oxidase enzyme [21-28]. Phenolic compounds also block the mitochondrial respiratory chain and ATPase [29]. Different polyphenolic compounds like curcumin [30], resveratrol [31], quercetin [32], ellagic acid [33], chlorogenic acid [34], exert their antioxidant properties by regulating the antioxidant enzyme genes through PKC signaling. The phytochemical analysis of essential oil obtained from the leaves of *C. excavata* through GC-MS showed anethole, and estragole as the major constituents and these are reported for their strong antioxidant properties [35]. Current findings of this study are in agreement with a previous outcome [11,36,37], in which LCMS/MS analysis of methanolic and ethyl acetate leaf extracts of *C. excavata* contained higher total phenolic contents, total flavonoid content, quercetin, furocoumarin, 8-geranyloxy psoralen, myricetin glucoside conjugate, kaempferol conjugate, caffeic acid and showed anti-inflammatory and antioxidant activities. Based on the outcomes in this study and those reported earlier, the leaves extract of *C. excavata* especially ethyl acetate extract has strong therapeutic to be developed into a controlling inflammation agent.

In conclusion, the ethyl acetate, chloroform, and methanol extract possess significant immunomodulatory activity as evidenced from decreased ROS production from activated immune cells. Thus, these extracts have potential to retard the progression of acute and chronic inflammatory conditions, support, and encourage for its traditional use in the folklore herbal medicine to treat inflammatory-related conditions.

ACKNOWLEDGEMENTS

The corresponding author is thankful to Prof. Dr. M. Iqbal Choudhary for providing NAM-ICCBS fellowship, 2018 in International Center for Chemical and Biological Sciences, University of Karachi, Karachi, Pakistan

AUTHOR CONTRIBUTIONS

Conception: *S.F.A.A.*; Design: *S.F.A.A.*; Supervision: *S.F.A.A.*, *R.M.*; Resources: *S.F.A.A.*, *R.M.*; Data Collection and/or processing: *S.F.A.A.*; Analysis and/or interpretation: *S.F.A.A.*; Literature search: *S.F.A.A.*; Writing manuscript: *S.F.A.A.*, *R.M.*; Critical review: *S.F.A.A.*, *R.M.*; Other: *R.M.*

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

ICCBS/IEC-028-HB-2017/PROTOCOL/1.0, University of Karachi

REFERENCES

1. Bryan, N., Ahswin, H., Smart, N., Bayon, Y., Wohlert, S., Hunt, J.A. (2012). Reactive oxygen species (ROS) –a family of fate deciding molecules pivotal in constructive inflammation and wound healing. *European Cells and Materials*, *24*(249), e65. [CrossRef]

2. Albaayit, S.F.A., Al-Khafaji, A.S.K., Alnaimy, H.S. (2019). *In vitro* macrophage nitric oxide and interleukin-1 beta suppression by *Moringa peregrina* seed. *Turkish Journal of Pharmaceutical sciences. 16*(3), 362–365. [CrossRef]

3. Poljsak, B. (2011). Strategies for reducing or preventing the generation of oxidative stress. *Oxidative medicine and cellular longevity*, 2011. [CrossRef]

4. Albaayit, S.F.A., Abdullah, R., Abdullah, N. (2020). Zerumbone-loaded nanostructured lipid carrier gel facilitates wound healing in rats. *Revista Brasileira de Farmacognosia*, *30*(2), 272–278. [CrossRef]

5. Mohammed, E. S., Al-Taie, W. F., Numan, I. T., Hussain, S. A. (2012). Changes in Serum Levels of Tumor Necrosis Factor-Alpha and Antioxidant status in Different Stages of Malignant Prostate Cancer Patients in Iraq. Iraqi Journal of Pharmaceutical Sciences, *21*(1), 56-60. [CrossRef]

6. Ali, S. H., Hamadi, S. A., Al-Jaff, A. N. (2007). Effect of ergotamine and its combination with vitamin E or melatonin on total antioxidant status in migraine patients. *Iraqi Journal of Pharmaceutical Sciences*, *16*(2), 27-33. [CrossRef]

7. Manosroi, A., Saraphanchotiwitthaya, A., Manosroi, J. (2003). Immunomodulatory activities of *Clausena excavata* Burm. f. wood extracts. *Journal of Ethnopharmacology*, *89*(1), 155–160. [CrossRef]

8. Rahman, M.T., Alimuzzaman, M., Shilpi, J.A., Hossain, M.F. (2002). Antinociceptive activity of *Clausena excavata* leaves. *Fitoterapia*, *73*(7-8), 701–703. [CrossRef]

9. Huang, L., Zhe-Ling, F.E.N.G., Yi-Tao, W.A.N.G., Li-Gen, L.I.N. (2017). Anticancer carbazole alkaloids and coumarins from Clausena plants: A review. *Chinese Journal of Natural Medicines*, 15(12), 881–888. [CrossRef]

10. Albaayit, S.F.A., Rasedee, A., Abdullah, N., Abba, Y. (2020). Methanolic extract of *Clausena excavata* promotes wound healing via antiinflammatory and anti-apoptotic activities. *Asian Pacific Journal of Tropical Biomedicine*, *10*(5), 232-238. [CrossRef]

11. Albaayit, S.F.A., Khan, M., Rasedee, A., Noor, M.H.M. (2021). Ethyl acetate extract of *Clausena excavata* induces growth inhibition of non-small-lung cancer, NCI-H460, cell line via apoptosis. *Journal of Applied Biomedicine*, *19*(1), 40–47. [CrossRef]

12. Orhan, I. E., Mesaik, M. A., Jabeen, A., & Kan, Y. (2016). Immunomodulatory properties of various natural compounds and essential oils through modulation of human cellular immune response. *Industrial Crops and Products*, 81, 117-122. [CrossRef]

13. Albaayit, S.F.A., Maharjan, R. (2018). Immunomodulation of zerumbone via decreasing the production of reactive oxygen species from immune cells. *Pakistan Journal of Biological Sciences, 21*(9), 475–479. [CrossRef]

14. Majdalawieh, A.F., Fayyad, M.W. (2015). Immunomodulatory and anti-inflammatory action of *Nigella sativa* and thymoquinone: A comprehensive review. *International Immunopharmacology, 28*(1), 295–304. [CrossRef]

15. Albaayit, S.F.A. (2021). Evaluation of anti-methicillin resistant *Staphylococcus aureus* property of *Clausena excavata* leaves by using atomic force microscopy and flowcytometry techniques. *Pakistan Journal of Agricultural Sciences, 58*(1), 315–320. [CrossRef]

16. Albaayit, S.F.A., Maharjan, R., Khan, M. (2021). Evaluation of hemolysis activity of Zerumbone on RBCs and brine shrimp toxicity. *Baghdad Science Journal, 18*(1), 65–69. [CrossRef]

17. Al-Bahrani, R. M., Radif, H. M., Albaayit, S. F. A. (2020). Evaluation of potent silver nanoparticles production from Agaricus bisporus against Helicobacter pylori. *Pakistan Journal of Agricultural Sciences, 57*(4), 1197–1201. [CrossRef]

18. Albaayit, S. F. A., Mariam, K. H. A. N., & Abdullah, R. (2021). Zerumbone induces growth inhibition of Burkitt's lymphoma cell line via apoptosis. *Natural Volatiles and Essential Oils, 8*(3), 56-63. [CrossRef]

19. Al-Ani, L. K. T., Yonus, M. I., Mahdii, B. A., Omer, M. A., Taher, J. K., Albaayit, S. F. A., Al-Khoja, S. B. (2018). First record of use Fusarium proliferatum fungi in direct treatment to control the adult of wheat flour Tribolium confusum, as well as, use the entomopathogenic fungi Beauveria bassiana. Ecology, *Environment and Conservation, 24*(3), 29-34.

20. Albaayit, S. F. A. (2021). Enzyme inhıbıtory propertıes of zerumbone. *Pakistan Journal of Agricultural Sciences, 58*(3), 1207-1209. [CrossRef]

21. Albaayit, S.F.A. (2020). *In Vitro* Evaluation of Anticancer Activity of *Moringa Peregrina* Seeds on Breast Cancer Cells. *The Eurasia Proceedings of Science Technology Engineering and Mathematics, 11*,163-166. http://www.epstem.net/en/pub/issue/58065/838264.

22. Albaayit, S.F.A., Maharjan, R., Abdullah, R., Noor, M.H.M. (2022). Evaluation of anti-methicillin-resistant Staphylococcus aureus property of zerumbone. *Journal of Applied Biomedicine, 20*(1), 22–36.

23. Albaayit, S. F. A., Abba, Y., Abdullah, R., & Abdullah, N. (2016). Prophylactic effects of *Clausena excavat*a Burum. f. leaf extract in ethanol-induced gastric ulcers. *Drug Design, Development and Therapy.*

24. Albaayit, S. F. A., Abba, Y., Abdullah, R., & Abdullah, N. (2015). Effect of *Clausena excavata* Burm. f.(Rutaceae) leaf extract on wound healing and antioxidant activity in rats. *Drug Design, Development and Therapy.*

25. Yahfoufi, N., Alsadi, N., Jambi, M., Matar, C. (2018). The immunomodulatory and anti-inflammatory role of polyphenols. *Nutrients, 10*(11), 1618. [CrossRef]

26. Das, J., Ramani, R., Suraju, M.O. (2016). Polyphenol compounds and PKC signaling. *Biochimica et Biophysica Acta (BBA)-General Subjects, 1860*(10), 2107–2121. [CrossRef]

27. Albaayit, S. F. A., Maharjan, R., Abdullah, R., Noor, M. H. M. (2021). Anti-Enterococcus Faecalis, Cytotoxicity, Phytotoxicity, and Anticancer Studies on Clausena excavata Burum. f.(Rutaceae) Leaves. *BioMed Research International, 2021*. [CrossRef]

28. Albaayit, S.F.A., Khan, M., Rasedee, A., Noor, M.H.M. (2022). Zerumbone-Loaded Nanostructured Lipid Carrier Gel Enhances Wound Healing in Diabetic Rats. *BioMed Research International, 2022*.

29. Ahmad, Z., Hassan, S.S., Azim, S. (2017). A therapeutic connection between dietary phytochemicals and ATP synthase. *Current Medicinal Chemistry, 24*(35), 3894–3906. [CrossRef]

30. Pérez-Lara, Á., Corbalán-García, S., Gómez-Fernández, J. C. (2011). Curcumin modulates PKCα activity by a membrane-dependent effect. *Archives of Biochemistry and Biophysics*, 513(1), 36-41. [CrossRef]

31. Menard, C., Bastianetto, S., Quirion, R. (2013). Neuroprotective effects of resveratrol and epigallocatechin gallate polyphenols are mediated by the activation of protein kinase C gamma. *Frontiers in Cellular Neuroscience*, *7*, 281. [CrossRef]

32. Ferriola, P. C., Cody, V., Middleton Jr, E. (1989). Protein kinase C inhibition by plant flavonoids: kinetic mechanisms and structure-activity relationships. *Biochemical Pharmacology*, 38(10), 1617-1624. [CrossRef]

33. Mishra, S., Vinayak, M. (2015). Role of ellagic acid in regulation of apoptosis by modulating novel and atypical PKC in lymphoma bearing mice. *BMC Complementary and Alternative Medicine*, *15*(1), 1-8. [CrossRef]

34. Szaefer, H., Kaczmarek, J., Rybczyńska, M., Baer-Dubowska, W. (2007). The effect of plant phenols on the expression and activity of phorbol ester-induced PKC in mouse epidermis. *Toxicology*, *230*(1), 1-10. [CrossRef]

35. Tanruean, K., Poolprasert, P., Suwannarach, N., Kumla, J., Lumyong, S. (2021). Phytochemical Analysis and Evaluation of Antioxidant and Biological Activities of Extracts from Three Clauseneae Plants in Northern Thailand. *Plants*, *10*(1), 117. [CrossRef]

36. Albaayit, S. F. A., Abba, Y., Abdullah, R., Abdullah, N. (2014). Evaluation of antioxidant activity and acute toxicity of *Clausena excavata* leaves extract. *Evidence-Based Complementary and Alternative Medicine, 2014*, 1-10. [CrossRef]

37. Elumalai, K., Id, K. (2016). Antioxidant activity and phytochemical screening of different solvent extracts *Clausena excavata* burm F. Rutaceae) *MOJ Ecology & Environmental Sciences*, *1*(1), 1. [CrossRef]

Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 46(3): 712-727, 2022
Doi: 10.33483/jfpau.1085314

ÖZGÜN MAKALE / ORIGINAL ARTICLE

TOFİSOPAM'IN OPİOİDERJİK SİSTEM ARACILIKLI ANTİNOSİSEPTİF ETKİNLİĞİ

OPIOIDERGIC SYSTEM-MEDIATED ANTINOCICEPTIVE ACTIVITY OF TOFISOPAM

Nazlı TURAN YÜCEL[1], Umut İrfan ÜÇEL[2*], Cevşen YAZICI[3], Ümide DEMİR ÖZKAY[1], Özgür Devrim CAN[1]

[1] Anadolu Üniversitesi, Eczacılık Fakültesi, Farmakoloji Ana Bilim Dalı, 26470, Eskişehir, Türkiye

[2] Bayburt Üniversitesi, Sağlık Hizmetleri Meslek Yüksekokulu, 69000, Bayburt, Türkiye

[3]Anadolu Üniversitesi, Sağlık Bilimleri Enstitüsü, Farmakoloji Ana Bilim Dalı, 26470, Eskişehir, Türkiye

ÖZ

Amaç: *Bu çalışmada Tofisopam'ın antinosiseptif etkinlik potansiyelinin araştırılması ve bu etkiye opioiderjik sistemin olası katılımının aydınlatılması amaçlanmıştır.*

Gereç ve Yöntem: *Tofisopam'ın (25 ve 50 mg/kg) antinosiseptif etkinlik potansiyeli sıcak plaka ve asetik asit ile indüklenen kıvranma testleri ile araştırılmış; farelerin motor koordinasyonları üzerindeki olası etkileri ise Rota-rod testi ile değerlendirilmiştir.*

Sonuç ve Tartışma: *Tofisopam 50 mg/kg dozda, farelerin sıcak plaka testlerindeki reaksiyon sürelerini anlamlı ölçüde uzatmış; kıvranma testlerinde ise kıvranma davranışlarının sayılarını azaltmıştır. Bu bulgular Tofisopam'ın santral ve periferik mekanizmalar aracılıklı antinosiseptif aktiviteye sahip olduğuna işaret etmiştir. Tofisopam, uygulandığı dozlarda, farelerin motor aktivitelerinde anlamlı bir değişikliğe neden olmamıştır. Antinosiseptif etkiye opioid reseptörlerin olası katılımını araştırmak amacı ile yapılan nalokson ön-uygulamaları Tofisopam'ın antinosiseptif aktivitesini ortadan kaldırmıştır. Etkiye aracılık eden opioid reseptör alt-tiplerinin aydınlatılması amacıyla naloksonazin (μ-opioid reseptör blokörü), naltrindol (δ-opioid reseptör blokörü) ve nor-binaltorfimin (κ-opioid reseptör blokörü) ile mekanistik çalışmalar yapılmıştır. Her üç ajan da Tofisopam'ın antinosiseptif etkisini antagonize etmiştir. Elde edilen bu bulgular Tofisopam'ın 50 mg/kg dozdaki antinosiseptif etkinliğine μ-, δ- ve κ-oipiod reseptörlerin aracılık ettiğini ortaya koymuştur.*

Anahtar Kelimeler: *Analjezi, asetik asit ile indüklenen kıvranma testi, opioiderjik reseptör, sıcak plaka testi, tofisopam*

* **Sorumlu Yazar / Corresponding Author:** Umut İrfan Üçel
e-posta / e-mail: umutucel@bayburt.edu.tr, **Tel. / Phone:** +904582111171/1836

Gönderilme / Submitted: 08.03.2022 **Kabul / Accepted:** 03.06.2022

ABSTRACT

Objective: *In this study, it was aimed to investigate antinociceptive activity potential of Tofisopam and to elucidate the possible involvement of opioid system in this effect.*

Material and Method: *The antinociceptive efficacy potential of Tofisopam (25 and 50 mg/kg) was evaluated by hot-plate and acetic acid-induced writhing tests; while possible effects of this drug on the motor coordinations of mice were evaluated with the Rota-rod tests.*

Result and Discussion: *Tofisopam at a dose of 50 mg/kg significantly prolonged the reaction times of mice in hot-plate tests and reduced the number of writhing behaviors in writhing tests. These findings indicated that Tofisopam has antinociceptive activity mediated by central and peripheral mechanisms. Tofisopam did not change the motor activities of mice at administrated doses. Pre-administration of naloxone to investigate the possible involvement of opioid receptors in the antinociceptive effect abolished the antinociceptive activity of Tofisopam. To elucidate the opioid receptor subtypes mediating the effect, mechanistic studies were carried out with naloxonazine (μ-opioid receptor blocker), naltrindole (δ-opioid receptor blocker), and nor-binaltorphimine (κ-opioid receptor blocker). All agents antagonized the antinociceptive effect of Tofisopam. Obtained findings revealed that Tofisopam at a dose of 50 mg/kg has antinociceptive activity mediated by μ-, δ- and κ-opioid receptors.*

Keywords: *Analgesia, acetic acid-induced writhing test, hot-plate test, opioidergic receptor, tofisopam*

GİRİŞ

Geleneksel 1,4-benzodiazepin'lerin kimyasal yapılarındaki nitrojen atomlarının yerlerinin değiştirilmesi ile elde edilen 2,3-benzodiazepin yapılı moleküllere homofitalazinler adı verilmektedir [1,2]. Bu grubun ilk ilacı olan ve 1976'da Grandaxin® ticari adı ile ruhsatlandırılan Tofisopam, Macaristan, Çek Cumhuriyeti, Japonya ve Rusya gibi ülkelerde anksiyolitik etkisi nedeniyle reçete edilmektedir [3,4]. 2,3-benzodiazepin yapısına sahip olan "girisopam" ve "nerisopam" da Tofisopam gibi anksiyolitik etkinliğe sahip olan moleküllerdir [1,2,5,6].

Tofisopam'ın psikofarmakolojik etkinlik profili klasik 1,4-benzodiazepinlerden farklıdır. Bu ilacın anksiyolitik etkisi selektiftir; mutad dozlarda antikonvülzan, sedatif-hipnotik ya da kas gevşetici etki göstermez [6,7,8]. Tofisopam'ın vejetatif modülatör ve antiparkinson etkileri de rapor edilmiştir [1, 7]. Ayrıca psikozun adjuvant tedavisinde; bilhassa negatif ve kognitif bulguların ortadan kaldırılmasında fayda sağlayabileceği öne sürülmüştür [8]. Araştırma grubumuz tarafından yapılan bir çalışmada da Tofisopam'ın sıçanlarda skopolamin ile oluşturulan bilişsel bozukluklara karşı antiamnezik etkinlik gösterdiği ve bozulan hipokampal sinaptogenezi, nörogenezi ve glial plastisiteyi anlamlı ölçüde iyileştirdiği bildirilmiştir [4].

Tofisopam'ın etki mekanizmasına ilişkin bazı çalışmalar dekstofisopam'ın (R-enantiomeri) geleneksel benzodiazepin bağlanma yöreleri üzerine kayda değer bir afinite göstermediğini ileri sürmüştür. Hatta bu molekülün santral sinir sisteminde kendisine özel bir bağlanma bölgesine sahip olabileceği ve "2,3-benzodiazepin reseptörü" olarak karakterize edilebilecek olan bu bölgenin geleneksel 1,4- ya da 1,5-benzodiazepin reseptörlerinden farklı olduğu rapor edilmiştir [9].

Yapılan çalışmalar 2,3-benzodiazepinlerin santral sinir sisteminde GABAerjik, dopaminerjik ve opioid sistemleri etkilediğine işaret etmiştir [2,6,10-12]. Bu moleküllerin, klasik benzodiazepinlerin kendi

bağlanma bölgelerine olan afinitelerini güçlendirdikleri gösterilmiştir. Bununla birlikte musimol'ün GABA reseptörlerine bağlanma potansiyelini de artırdıkları rapor edilmiştir [2,10]. Diğer yandan 2,3-benzodiazepinlerin dopamin reseptörleri üzerinde karışık tip agonist/antagonist-benzeri etkilere neden oldukları [12] ve opioid agonistlerinin etkinliklerini selektif olarak artırdıkları ileri sürülmüştür [2,6,11].

2,3-benzodiazepinlerin etki mekanizmalarına ilişkin diğer bulgular da bu moleküllerin sinyal iletiminde rol oynayan çeşitli proteinlerin fosforilasyon süreçlerini değiştirdiklerine ilişkindir [6]. Bu moleküllerin özellikle fosfodiesterazların (PDE) çeşitli izoenzimlerine yüksek afinite ile bağlandıkları ve onları inhibe ettikleri rapor edilmiştir [8,13]. Literatürde PDE inhibitörlerinin akut ve kronik ağrıda etkili bulunduğuna dair çok sayıda çalışma mevcuttur [14-19]. Örneğin rolipram, teofilin ve sildenafil'in deney hayvanlarında asetik asitle indüklenmiş kıvranma testi yanıtlarını iyileştirdikleri [20-22]; zaprinast, ibudilast ve rolipram'ın ise nöropatik ağrıda faydalı etkiler gösterdikleri rapor edilmiştir [17, 23].

Tüm bu bilgiler ışığında bu çalışmada Tofisopam'ın akut antinosiseptif etkinlik potansiyelinin araştırılması ve bu etkiye ağrı ve analjezi ile son derece yakından ilişkili oluğu bilinen opioiderjik sistemin olası katılımının incelenmesi amaçlanmıştır.

GEREÇ VE YÖNTEM

Deney Hayvanları

Deneylerde 30-35 g ağırlığında erişkin erkek Balb/c fareler kullanılmıştır. Deney hayvanları iyi havalandırılan, 24±1ºC sıcaklıktaki odalarda tutulmuştur. 12 saat aydınlık 12 saat karanlık döngüsü (08:00-20:00) uygulanmıştır. Standart hayvan yemi ile beslenen hayvanlara testler boyunca yem veya su kısıtlaması uygulanmamıştır. Bu çalışmada kullanılan protokol ve prosedürler Anadolu Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu tarafından onaylanmıştır (10/3/2020 Karar No: 2020/13).

Kullanılan Kimyasal Maddeler

Tofisopam (Grandaxin®, Egis PLC, Budapeşte, Macaristan) ticari olarak temin edilmiştir. Morfin sülfat, nalokson hidroklorür dehidrat, naloksonazin dihidroklorür ve nor-binaltorfimin dihidroklorür Sigma-Aldrich'ten (St Louis, MO, ABD); naltrindol hidroklorür Tocris Cookson'dan (Ballwin, MO, ABD) ve asetik asit de Merck'ten (Darmstadt, Almanya) satın alınmıştır.

Farelerin Motor Koordinasyonlarının Değerlendirildiği Testler

Rota-rod Testi

Farelerin motor koordinasyonlarını değerlendirmek üzere Rota-rod cihazı (Ugo-basile, 47600, Verase, İtalya) kullanılmıştır [24]. Rota-rod testi "alıştırma" ve "test" olmak üzere iki aşamalı bir testtir. Üç gün süren alıştırma aşamasında fareler 24 saat ara ile üç kez 16 rpm'lik sabit hızda dönen milin

üzerinde yürütülmüştür. Deneyler için üçüncü günün sonunda milde en az 180 s kalabilen hayvanlar seçilmiştir. Test aşamasında ise milin üzerine tekrar yerleştirilen hayvanların düşme süreleri kaydedilmiştir. Otomatik olarak kaydedilen bu süreler hayvanların motor performanslarının göstergesi olarak değerlendirilmiştir [25].

Antinosiseptif Etkinin Değerlendirildiği Testler

Antinosiseptif etkinliğin araştırılması için Tofisopam hayvanlara 25 ve 50 mg/kg dozlarda oral yolla uygulanmış ve nosiseptif testler uygulamalardan 60'ar dakika sonra yapılmıştır [8, 26].

Sıcak Plaka Testi

Sıcak plaka testi, 55±1.0ºC sıcaklığa ayarlanmış olan Sıcak Plaka Cihazı (Ugo-basile, 7280, Verase, İtalya) kullanılarak gerçekleştirilmiştir [27]. Bu testte, termal ağrılı uyaran uygulanan hayvanların ayaklarını yalamaya başlamaları ve/veya plaka üzerinden atlama süreleri kaydedilmiştir. Deneye başlamadan önce, hayvanlara duyarlılık testi uygulanmış ve testler için 15 s içerisinde yanıt veren hayvanlar seçilmiştir. Testler sırasında pençenin hasar görmemesi için uyarı maksimum 30 s süreyle uygulanmıştır [28].

Ölçülen reaksiyon süreleri aşağıda verilen formül yardımı ile mümkün olan maksimum etkinin yüzdesi (Maximum Possible Effect, MPE) değerlerine çevrilmiştir.

$$\%\ \mathrm{MPE} = \frac{\text{Uygulama sonrası süre} - \text{Uygulama öncesi süre}}{\text{Maksimum uygulama süresi} - \text{Uygulama öncesi süre}}\ \mathrm{x}\ 100$$

Asetik-asit ile İndüklenen Kıvranma Testi

Kıvranma testi için Koster ve Williamson'un geliştirmiş olduğu yöntem uygulanmıştır [29]. Farelerde abdominal ağrı, asetik asit çözeltisinin (%0.6) intraperitonal yolla uygulanması ile oluşturulmuştur. Enjeksiyondan sonra 5 dakika beklenmiştir. Daha sonra, farelerin kıvranma davranışlarının yani, ayaklarını uzatıp gerilmelerinin ve karınlarını yere sürtmelerinin sayısı 10 dakika boyunca kaydedilmiştir [30]. Her iki test için de morfin sülfat (10 mg/kg, *i.p.*) referans ilaç olarak kullanılmıştır [28, 30].

Mekanistik Çalışmalar

Mekanistik çalışmalar için Tofisopam'ın 50 mg/kg dozu seçilmiştir. Tofisopam'ın bu çalışmada ortaya koyulmuş olan akut antinosiseptif etkisine opioiderjik sistemin katılımının araştırılması amacıyla non-selektif opioid reseptör antagonisti bir ajan olan nalokson (5.48 mg/kg, *i.p.*) kullanılmıştır [30]. Antinosiseptif etkiye aracılık eden opioid reseptör alt-tiplerinin aydınlatılması amacı ile de mekanistik çalışmalara, opioid reseptörlerin alt-tiplerine selektif ajanlar olan naloksonazin (μ-opioid reseptör blokörü; 7 mg/kg, *s.c.*), naltrindol (δ-opioid reseptör blokörü; 0.99 mg/kg, *i.p.*) ve nor-binaltorfimin (κ-opioid reseptör blokörü; 1.03 mg/kg, *i.p.*) kullanılarak devam edilmiştir [28,30]. Antagonizma

çalışmalarında kullanılan dört ajan da kontrol solüsyonu ve Tofisopam uygulamalarından 15'er dakika önce uygulanmıştır.

İstatistiksel Analiz

Her bir deney grubu için 7 adet fareden alınan veriler Graphpad Prism ver. 8.4.3 paket programı kullanılarak analiz edilmiştir. Rota-rod testlerinden ve nosiseptif deneylerden elde edilen veriler, Tek Yönlü Varyans Analizi ile değerlendirilmiştir. Çoklu karşılaştırmalar Tukey HSD Testleri ile yapılmıştır. Mekanistik çalışmalardan elde edilen veriler ise Çift-Yönlü Varyans Analizi ile değerlendirilmiş ve çoklu karşılaştırma için Bonferroni Testleri uygulanmıştır.

$P < 0.05$ değeri anlamlı kabul edilmiş ve bulgular ortalama ± ortalamanın standart hatası olarak sunulmuştur.

SONUÇ VE TARTIŞMA

Bu çalışmada, 2,3-benzodiazepin türevi bir molekül olan Tofisopam'ın antinosiseptif etkinlik potansiyeli ve bu etkiye opioid reseptörlerin olası katılımı *in vivo* yöntemler kullanılarak araştırılmıştır.

Deney hayvanlarının motor performansını bozan ajanların antinosiseptif etki tarama testlerinde yanlış pozitif sonuçlara yol açabildiği bilgisinden hareketle [28,31], Tofisopam'ın antinosiseptif etkinliği araştırılmadan önce, bu ilacın farelerin motor koordinasyonları üzerindeki olası etkisi Rota-rod testleri kullanılarak değerlendirilmiştir. Rota-rod testlerinde, kontrol solüsyonunun, 25 ve 50 mg/kg dozlarda uygulanan Tofisopam'ın ve referans ilaç diazepam'ın farelerin "dönen milin üzerinden düşme süreleri" üzerine etkileri Tablo 1'de gösterilmiştir [$F(3, 24) = 8.31$; $p < 0.001$].

Tablo 1. Rota-rod testinde kontrol solüsyonu, Tofisopam ve diazepam uygulamalarının farelerin "düşme süreleri" üzerine etkileri

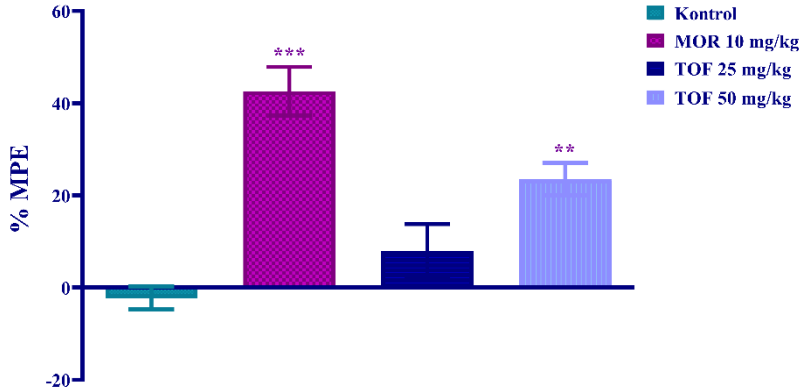
	Düşme Süresi (s)
Kontrol	600±0
Diazepam (2 mg/kg)	383.43±37.07***
TOF (25 mg/kg)	543.86±31.71
TOF (50 mg/kg)	495.86±41.18

***p < 0.001: Kontrole göre anlamlı farklılık. Tek Yönlü Varyans Analizi, takiben Tukey HSD Çoklu Karşılaştırma Testi, n=7 (TOF: Tofisopam).

Tofisopam uygulandığı 25 ve 50 mg/kg dozlarda farelerin motor koordinasyonlarında anlamlı bir değişikliğe neden olmamıştır (Tablo 1). Bu bulgular, antinosiseptif aktivite testlerinde elde edilen sonuçların hayvanların motor performanslarındaki herhangi bir değişiklikten etkilenmediğini göstermiş olması açısından önemlidir.

Tofisopam'ın antinosiseptif aktivitesi sıcak plaka ve asetik asit ile indüklenen kıvranma testleri ile değerlendirilmiştir.

Akut Tofisopam (25 ve 50 mg/kg) ve referans ilaç morfin uygulamalarının, sıcak plaka testinde hesaplanan % MPE değerleri üzerine etkileri Şekil 1'de sunulmuştur [$F(3, 24) = 18.96$; $p < 0.001$]. Çoklu karşılaştırma testleri, Tofisopam'ın 50 mg/kg dozu ile indüklenen % MPE değerlerinin, kontrol grubunun karşılık gelen değerlerine göre anlamlı ölçüde yüksek olduğunu göstermiştir ($p < 0.01$). İlaç 25 mg/kg'lık dozda etkisizdir. Referans ilaç olarak kullanılan morfin (10 mg/kg, *i.p.*), sıcak plaka testleri için hesaplanan % MPE değerlerinde beklenen artışı göstermiştir ($p < 0.001$).

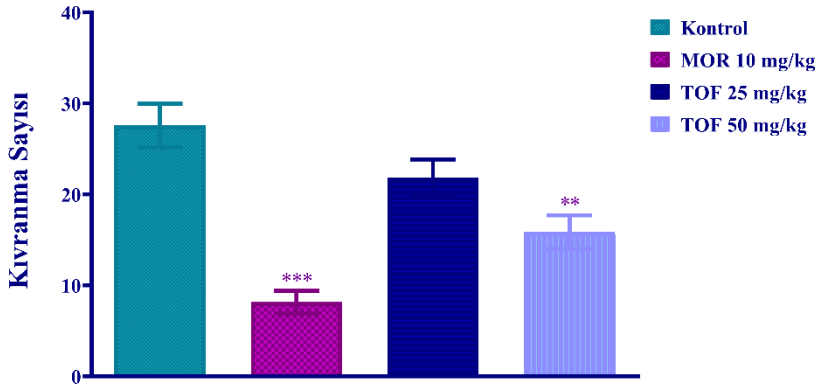


Şekil 1. Sıcak plaka testinde morfin (10 mg/kg) ve Tofisopam (25 ve 50 mg/kg) ile indüklenen % MPE değerleri. $^{**}p < 0.01$, $^{***}p < 0.001$: Kontrole göre anlamlı farklılık. Tek Yönlü Varyans Analizi, takiben Tukey HSD Çoklu Karşılaştırma Testi, n=7 (% MPE: Maksimum Etkinin Yüzdesi (Maximum Possible Effect); TOF: Tofisopam; MOR: Morfin)

Sıcak plaka testi antinosiseptif etkinin araştırılması için sıklıkla tercih edilen, geçerliliği ve güvenilirliği yüksek bir testtir [30,32,33]. Yöntemin esası, 55°C'ye ısıtılmış bir plaka üzerine yerleştirilen hayvanların, uygulanan ağrılı uyarana karşı gösterdikleri "pençe yalama" ya da "atlama" tepkilerinin sürelerinin saptanmasıdır [32]. Bu çalışmada, Tofisopam'ın 50 mg/kg dozda, farelerin termal nosiseptif uyarana karşı reaksiyon sürelerini anlamlı ölçüde uzatmış olması, bu molekülün antinosiseptif etkinliğini termal ağrılı uyaranları taşıyan nöronal yolaklar üzerinde gösterdiğini ortaya koymuştur. Bununla birlikte, sıcak plaka testinin özellikle supraspinal yolaklarda entegre edilen nosiseptif iletimi yansıttığı bilgisinden hareketle [32,34,35], Tofisopam'ın antinosiseptif etkisini supraspinal düzeyde gösterdiği ileri sürülebilir.

Asetik asit ile indüklenen kıvranma testi ise antinosiseptif etkinin taranması amacıyla sıklıkla kullanılan bir viseral ağrı modelidir [36]. Kontrol solüsyonu, akut Tofisopam (25 ve 50 mg/kg) ve referans ilaç morfin uygulamalarının, asetik asit ile indüklenen kıvranma testinde farelerin kıvranma davranışlarının sayıları üzerine etkileri Şekil 2'de sunulmuştur [$F(3, 24) = 18.94$; $p < 0.001$]. Çoklu

karşılaştırma testleri, Tofisopam'ın 25 mg/kg'lık dozda etkili olmadığını, ancak 50 mg/kg'lık dozun farelerin kıvranma sayılarını, kontrol grubunun kıvranma sayılarına göre anlamlı biçimde azalttığını ortaya koymuştur ($p < 0.01$). Referans ilaç olarak kullanılan morfin (10 mg/kg, *i.p.*) beklendiği üzere, farelerin kıvranma davranışlarının sayılarını anlamlı ölçüde azaltmıştır ($p < 0.001$).



Şekil 2. Morfin (10 mg/kg) ve Tofisopam (25 ve 50 mg/kg) uygulamalarının asetik asit ile indüklenen kıvranma sayıları üzerine etkileri. $^{**}p < 0.01$, $^{***}p < 0.001$: Kontrole göre anlamlı farklılık. Tek Yönlü Varyans Analizi, takiben Tukey HSD Çoklu Karşılaştırma Testi, n=7 (TOF: Tofisopam; MOR: Morfin)

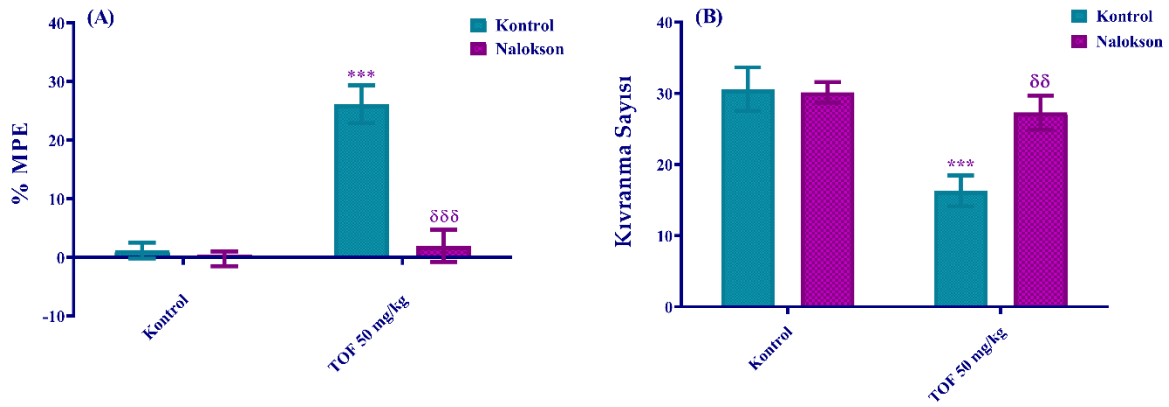
Farelere kimyasal nitelikli nosisepsiyona neden olan asetik asitin intraperitonal yolla uygulanmasının, bu hayvanlarda abdominal kasların kasılması, arka bacakların ekstensiyonu ve vücudun geriye doğru uzatılması şeklinde gözlenebilen bir "kıvranma" yanıtına sebep olduğu bilinmektedir [37]. Kıvranma davranışı, asetik asit enjeksiyonuna bağlı olarak asetilkolin, histamin, P maddesi, prostaglandinler ve kininler gibi endojen maddelerin salınmasındaki artış sonucu tetiklenmektedir. Söz konusu endojen maddeler vasküler geçirgenliği artırmakta, ağrı eşiğini azaltmakta ve nonsteroidal anti-inflamatuvar ve/veya opioid ilaçlara duyarlı nosiseptif nöronları uyarmaktadır [38-40]. Bu çalışmada, 50 mg/kg dozda akut Tofisopam uygulamasının farelerde asetik asit ile indüklenen kıvranma davranışlarının sayısını azaltmış olması, bu ilacın periferal düzeydeki antinosiseptif etkinliğini sözü edilen mekanizmaların en azından bazılarını etkileyerek göstermiş olabileceğini düşündürmektedir. Tofisopam antinosiseptif etkisini inflamatuvar mediyatörlerin salıverilmesini azaltarak; bu endojen maddelerin reseptörlerini antagonize ederek; ağrı eşiği değerlerini değiştirerek veya ağrı stimulusunun nöronal iletimini engelleyerek gerçekleştirmiş olabilir [30].

Bu çalışma kapsamında, Tofisopam'ın antinosiseptif aktivitesinin belirlenmesinden sonra, bu etkiye analjezi ile yakından ilişkili olduğu bilinen opioiderjik sistemin olası katılımının araştırılması planlanmıştır. Bu kapsamda non-selektif opioiderjik reseptör blokörü bir ajan olan nalokson ile mekanistik çalışmalar gerçekleştirilmiştir.

Şekil 3A, Tofisopam'ın (50 mg/kg) sıcak plaka testindeki antinosiseptif etkisinin nalokson ön-

uygulaması varlığındaki değişimini göstermektedir. İstatistiksel analizler % MPE değerleri üzerinde hem tedavi faktörünün [F (1, 24) = 34.46; p < 0.001] hem de antagonist faktörünün [F (1, 24) = 30.57; p < 0.001] etkili olduğunu ve tedavi faktörü ile antagonist faktörü arasında anlamlı bir etkileşim [F (1, 24) = 24.21; p < 0.001] bulunduğunu ortaya koymuştur. Çoklu karşılaştırma için uygulanan Bonferroni Testleri % MPE değerlerinde Tofisopam ile indüklenen artışın (p < 0.001) nalokson ön-uygulaması ile geri döndüğünü (p < 0.001) göstermiştir.

Tofisopam'ın (50 mg/kg) asetik asit ile indüklenen kıvranma testindeki antinosiseptif etkisinin nalokson ön-uygulaması varlığındaki değişimi Şekil 3B'de sunulmuştur. İstatistiksel analizler kıvranma sayıları üzerinde tedavi faktörünün de [F (1, 24) = 13.42; p < 0.01] antagonist faktörünün de [F (1, 24) = 5.11; p < 0.05] etkili olduğuna işaret etmiştir. Bununla birlikte, bu faktörler arasında anlamlı bir etkileşim söz konusudur [F (1, 24) = 5.97; p < 0.05]. Çoklu karşılaştırma için uygulanan Bonferroni Testleri, kıvranma sayılarında Tofisopam ile indüklenen azalmanın (p < 0.001) nalokson ön-uygulaması ile kontrol seviyesine geri döndüğünü (p < 0.01) göstermiştir.



Şekil 3. Tofisopam'ın (50 mg/kg) sıcak plaka (A) ve asetik asit ile indüklenen kıvranma (B) testlerindeki antinosiseptif etkisinin nalokson ön-uygulaması varlığındaki değişimi. $^{***}p < 0.001$: Serum fizyolojik ön-uygulaması yapılan kontrol grubuna göre anlamlı farklılık; $^{\delta\delta}p < 0.01$; $^{\delta\delta\delta}p < 0.001$: Serum fizyolojik ön-uygulaması yapılan 50 mg/kg Tofisopam grubuna göre anlamlı farklılık. Çift Yönlü Varyans Analizi, takiben Bonferroni Çoklu Karşılaştırma Testi, n=7 (% MPE: Maksimum Etkinin Yüzdesi (Maximum Possible Effect); TOF: Tofisopam).

Nalokson ön-uygulamaları her iki testte de Tofisopam'ın (50 mg/kg) antinosiseptif etkinliğini ortadan kaldırmıştır. Bu bulgular opioid mekanizmaların, bu ilacın hem santral hem de periferik düzeydeki antinosiseptif etkinliğine aracılık ettiğine işaret etmiştir. Nitekim literatürde 2,3-benzodiazepinlerin farmakodinamik etkilerinin opioiderjik sistem ile ilişkili olduğuna işaret eden araştırmalar bulunmaktadır. Bu moleküllerin opioid agonistlerinin etkilerini selektif biçimde güçlendirdikleri çeşitli *in vitro* ve *in vivo* yöntemler ile gösterilmiştir [2,6,11]. Diğer yandan, Tofisopam

ve ilişkili moleküllerin opioidlerin antinosiseptif etkinliklerini artırabildikleri de rapor edilmiştir [6]. 2,3-benzodiazepinlerin farmakolojik etkilerinin morfin toleransı olan hayvanlarda görülmemesi de, bu moleküllerin etki mekanizmalarının opioiderjik sistem ile ilişkili olabileceğini düşündürmektedir [11].

Bu çalışmada Tofisopam'ın opioiderjik sistem aracılıklı antinosiseptif etkinliği ortaya koyulduktan sonra, etkiye aracılık eden opioid reseptör alt-tiplerinin de aydınlatılması planlanmıştır. Bu amaçla sırasıyla μ-, δ- ve κ-opioid reseptöre selektif blokörler olan naloksonazin, naltrindol ve nor-binaltorfimin ile mekanistik çalışmalar yapılmıştır.

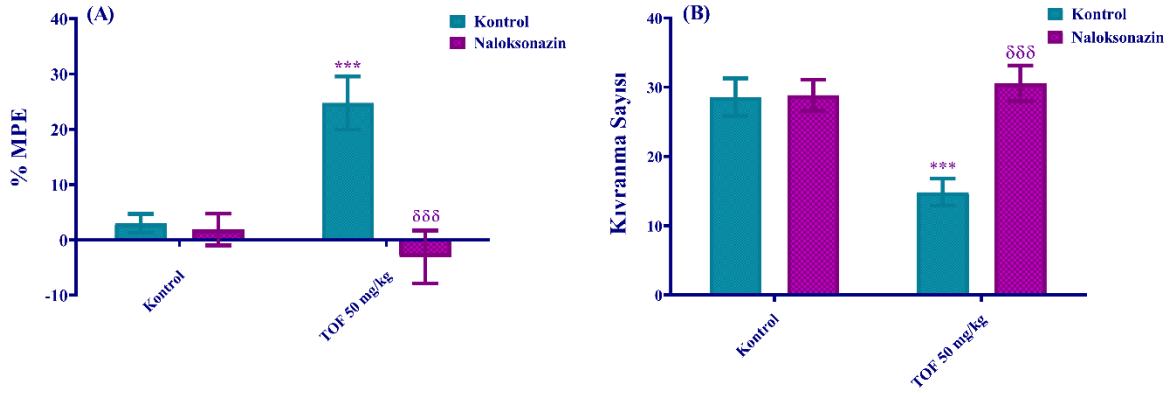
Şekil 4A, Tofisopam'ın (50 mg/kg) sıcak plaka testindeki antinosiseptif etkisinin naloksonazin ön-uygulaması varlığındaki değişimini göstermektedir. İstatistiksel analizler % MPE değerleri üzerinde hem tedavi faktörünün [$F(1, 24) = 4.94$; $p < 0.05$] hem de antagonist faktörünün [$F(1, 24) = 14.66$; $p < 0.001$] etkili olduğunu ve tedavi faktörü ile antagonist faktörü arasında anlamlı bir etkileşim [$F(1, 24) = 12.49$; $p < 0.01$] bulunduğunu ortaya koymuştur. Çoklu karşılaştırma için uygulanan Bonferroni Testleri % MPE değerlerinde Tofisopam ile indüklenen artışın ($p < 0.001$) naloksonazin ön-uygulaması ile geri döndüğünü ($p < 0.001$) göstermiştir.

Tofisopam'ın (50 mg/kg) asetik asit ile indüklenen kıvranma testindeki antinosiseptif etkisinin naloksonazin ön-uygulaması varlığındaki değişimi Şekil 4B'de sunulmuştur. İstatistiksel analizler kıvranma sayıları üzerinde tedavi faktörünün de [$F(1, 24) = 6.32$; $p < 0.05$] antagonist faktörünün de [$F(1, 24) = 11.23$; $p < 0.01$] etkili olduğuna işaret etmiştir. Bununla birlikte, bu faktörler arasında anlamlı bir etkileşim söz konusudur [$F(1, 24) = 10.45$; $p < 0.01$]. Çoklu karşılaştırma için uygulanan Bonferroni Testleri, kıvranma sayılarında Tofisopam ile indüklenen azalmanın ($p < 0.001$) nalokson ön-uygulaması ile kontrol seviyesine geri döndüğünü ($p < 0.001$) göstermiştir.

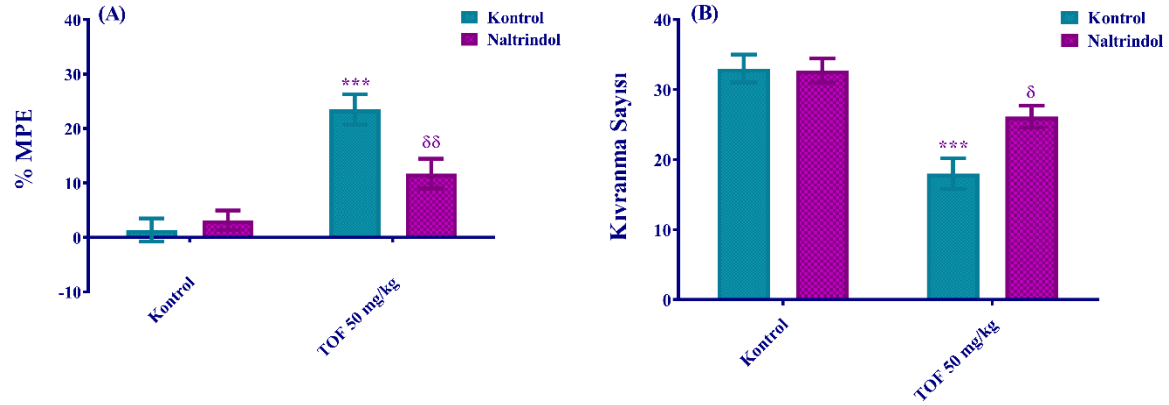
Şekil 5A, Tofisopam'ın (50 mg/kg) sıcak plaka testindeki antinosiseptif etkisinin naltrindol ön-uygulaması varlığındaki değişimini göstermektedir. İstatistiksel analizler % MPE değerleri üzerinde hem tedavi faktörünün [$F(1, 24) = 40.99$; $p < 0.001$] hem de antagonist faktörünün [$F(1, 24) = 4.33$; $p < 0.05$] etkili olduğunu ve tedavi faktörü ile antagonist faktörü arasında anlamlı bir etkileşim [$F(1, 24) = 8.04$; $p < 0.01$] bulunduğunu ortaya koymuştur. Çoklu karşılaştırma için uygulanan Bonferroni Testleri % MPE değerlerinde Tofisopam ile indüklenen artışın ($p < 0.001$) naltrindol ön-uygulaması ile geri döndüğünü ($p < 0.01$) göstermiştir.

Tofisopam'ın (50 mg/kg) asetik asit ile indüklenen kıvranma testindeki antinosiseptif etkisinin naltrindol ön-uygulaması varlığındaki değişimi Şekil 5B'de sunulmuştur. İstatistiksel analizler kıvranma sayıları üzerinde tedavi faktörünün de [$F(1, 24) = 32.56$; $p < 0.001$] antagonist faktörünün de [$F(1, 24) = 4.32$; $p < 0.05$] etkili olduğuna işaret etmiştir. Bununla birlikte, bu faktörler arasında anlamlı bir etkileşim söz konusudur [$F(1, 24) = 4.97$; $p < 0.05$]. Çoklu karşılaştırma için uygulanan Bonferroni Testleri, kıvranma sayılarında Tofisopam ile indüklenen azalmanın ($p < 0.001$) naltrindol ön-uygulaması

ile geri döndüğünü ($p < 0.05$) göstermiştir.



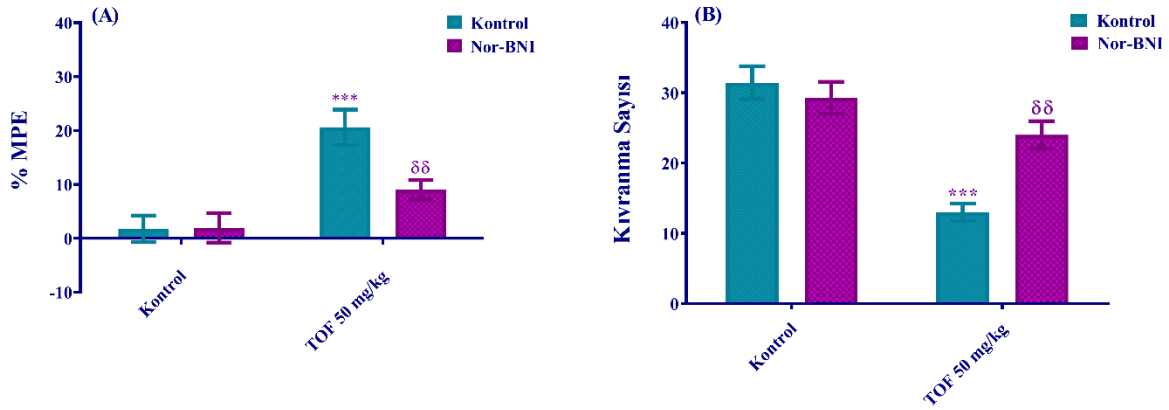
Şekil 4. Tofisopam'ın (50 mg/kg) sıcak plaka (A) ve asetik asit ile indüklenen kıvranma (B) testlerindeki antinosiseptif etkisinin naloksonazin ön-uygulaması varlığındaki değişimi. $^{***}p < 0.001$: Serum fizyolojik ön-uygulaması yapılan kontrol grubuna göre anlamlı farklılık; $^{δδδ}p < 0.001$: Serum fizyolojik ön-uygulaması yapılan 50 mg/kg Tofisopam grubuna göre anlamlı farklılık. Çift Yönlü Varyans Analizi, takiben Bonferroni Çoklu Karşılaştırma Testi, n=7 (% MPE: Maksimum Etkinin Yüzdesi (Maximum Possible Effect); TOF: Tofisopam)



Şekil 5. Tofisopam'ın (50 mg/kg) sıcak plaka (A) ve asetik asit ile indüklenen kıvranma (B) testlerindeki antinosiseptif etkisinin naltrindol ön-uygulaması varlığındaki değişimi. $^{***}p < 0.001$: Serum fizyolojik ön-uygulaması yapılan kontrol grubuna göre anlamlı farklılık; $^{δ}p < 0.05$, $^{δδ}p < 0.01$: Serum fizyolojik ön-uygulaması yapılan 50 mg/kg Tofisopam grubuna göre anlamlı farklılık. Çift Yönlü Varyans Analizi, Bonferroni Çoklu Karşılaştırma Testi, n=7 (% MPE: Maksimum Etkinin Yüzdesi (Maximum Possible Effect); TOF: Tofisopam)

Şekil 6A, Tofisopam'ın (50 mg/kg) sıcak plaka testindeki antinosiseptif etkisinin nor-binaltorfimin ön-uygulaması varlığındaki değişimini göstermektedir. İstatistiksel analizler % MPE değerleri üzerinde hem tedavi faktörünün [$F(1, 24) = 24.26$; $p < 0.001$] hem de antagonist faktörünün [$F(1, 24) = 4.71$; $p < 0.05$] etkili olduğunu ve tedavi faktörü ile antagonist faktörü arasında anlamlı bir etkileşim [$F(1, 24) = 5.03$; $p < 0.05$] bulunduğunu ortaya koymuştur. Çoklu karşılaştırma için uygulanan Bonferroni Testleri % MPE değerlerinde Tofisopam ile indüklenen artışın ($p < 0.001$) nor-binaltorfimin ön-uygulaması ile geri döndüğünü ($p < 0.01$) göstermiştir.

Tofisopam'ın (50 mg/kg) asetik asit ile indüklenen kıvranma testindeki antinosiseptif etkisinin nor-binaltorfimin ön-uygulaması varlığındaki değişimi Şekil 6B'de sunulmuştur. İstatistiksel analizler kıvranma sayıları üzerinde tedavi faktörünün de [$F(1, 24) = 35.19$; $p < 0.001$] antagonist faktörünün de [$F(1, 24) = 4.91$; $p < 0.05$] etkili olduğuna işaret etmiştir. Bununla birlikte, bu faktörler arasında anlamlı bir etkileşim söz konusudur [$F(1, 24) = 10.81$; $p < 0.01$]. Çoklu karşılaştırma için uygulanan Bonferroni Testleri, kıvranma sayılarında Tofisopam ile indüklenen azalmanın ($p < 0.001$) nor-binaltorfimin ön-uygulaması ile geri döndüğünü ($p < 0.01$) göstermiştir.



Şekil 6. Tofisopam'ın (50 mg/kg) sıcak plaka (A) ve asetik asit ile indüklenen kıvranma (B) testlerindeki antinosiseptif etkisinin nor-binaltorfimin ön-uygulaması varlığındaki değişimi. $^{***}p < 0.001$: Serum fizyolojik ön-uygulaması yapılan kontrol grubuna göre anlamlı farklılık; $^{\delta\delta}p < 0.01$: Serum fizyolojik ön-uygulaması yapılan 50 mg/kg Tofisopam grubuna göre anlamlı farklılık. Çift Yönlü Varyans Analizi, takiben Bonferroni Çoklu Karşılaştırma Testi, n=7 (TOF: Tofisopam; Nor-BNI: Nor-binaltorfimin)

Naloksonazin, naltrindol ve nor-binaltorfimin ön-uygulamaları hem sıcak plaka hem de asetik asit ile indüklenen kıvranma testlerinde Tofisopam'ın (50 mg/kg) antinosiseptif etkinliğini ortadan kaldırmıştır. Bu bulgular μ-, δ- ve κ-opioid reseptör alt-tiplerinin üçünün de bu ilacın santral ve periferik düzeylerdeki antinosiseptif etkilerine katkıda bulunduğuna işaret etmiştir. Antinosiseptif etkinin her üç

ajanın ön-uygulaması ile geri dönmüş olması Tofisopam'ın antinosiseptif etkisinin, non-spesifik bir reseptör aktivasyonundan ziyade endojen opioidlerin salınımındaki bir artışla ya da opioid reseptörlerinin allosterik modülasyonu ile ilişkili olabileceğini düşündürmektedir. Ayrıca, bu molekülün opioid post-reseptör mekanizmalar üzerine non-selektif bir etkinlik göstermiş olması da mümkün olabilir. Tüm bu varsayımların daha kapsamlı mekanistik çalışmalarla araştırılması gerekmektedir. Diğer yandan, Tofisopam tarafından indüklenen antinosiseptif etkiye nosiseptin/orfanin reseptörlerinin olası katılımının araştırılması da Tofisopam'ın antinosiseptif etki mekanizmasının daha ileri düzeyde aydınlatılmasına katkı sağlayacaktır.

Ağrı ve analjezi süreçlerinde, pek çok regülatör yolağın ve endojen mekanizmanın rol oynadığı [41,42] düşünüldüğünde, Tofisopam'ın etki mekanizmasının kesin olarak aydınlatılabilmesi için, bu molekülün antinosiseptif etkisine örneğin GABAerjik, nitrerjik, kannabinoiderjik, kolinerjik ya da adenozinerjik sistemlerin olası katılımlarının da araştırılmasının gerektiği açıktır.

Bu araştırmanın en önemli bulgusu, Tofisopam'ın ağrı ile ilişkili hastalıkların tedavisi için terapötik bir potansiyele sahip olabileceğinin ilk kez ortaya koyulmuş olmasıdır. Bu çalışmanın preklinik bulgularının klinik araştırmalar ile doğrulanması durumunda, Tofisopam "analjezik" ya da "adjuvan" bir ajan olarak değerlendirilebilecektir.

TEŞEKKÜR

Bu araştırma, Anadolu Üniversitesi Bilimsel Araştırma Projeleri Komisyonu tarafından desteklenmiştir (Proje Numarası: 1808S277).

YAZAR KATKILARI

Kavram: *Ö.D.C., Ü.D.Ö.*; Tasarım: *Ö.D.C., N.T.Y.*; Denetim: *Ö.D.C., U.İ.Ü.*; Kaynaklar: *Ö.D.C.*; Malzemeler: *C.Y.*; Veri Toplama ve/veya İşleme: *N.T.Y., U.İ.Ü.*, *C.Y.*; Analiz ve/veya yorumlama: *Ö.D.C., Ü.D.Ö.*; Literatür taraması: *N.T.Y., U.İ.Ü, C.Y.*; Makalenin yazılması: *Ö.D.C., Ü.D.Ö.*; Kritik inceleme: *Ö.D.C., Ü.D.Ö., U.İ.Ü, N.T.Y., C.Y.*; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

ETİK KURUL ONAYI

Bu çalışmanın deneysel protokolü Anadolu Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu tarafından onaylanmıştır (10/3/2020 Karar No: 2020/13).

KAYNAKLAR

1. Szénási, G., Hársing Jr, L.G. (2004). Pharmacology and prospective therapeutic usefulness of negative allosteric modulators of AMPA receptors. *Drug Discovery Today: Therapeutic Strategies, 1*(1), 69-76. [CrossRef]

2. Hamed, A., Skórzewska, A., Lehner, M., Płaźnik, A. (2007). Tofizopam. W poszukiwaniu mechanizmów działania 2,3-benzodiazepin. *Farmakoterapia w Psychiatrii i Neurologii, 2*, 109-117.

3. Rx Media Pharma® (2022). İnteraktif İlaç Bilgi Kaynağı. Erişim tarihi: 20.02.2022.

4. Üçel, U.İ., Can, Ö.D., Özkay, Ü.D., Ulupinar, E. (2020). Antiamnesic effects of tofisopam against scopolamine-induced cognitive impairments in rats. *Pharmacology Biochemistry and Behavior, 190*, 172858. [CrossRef]

5. Horváth, E.J., Salamon, C., Bakonyi, A., Fekete, M.I., Palkovits, M. (1999). [(3)H]girisopam, a novel selective benzodiazepine for the 2, 3-benzodiazepine binding site. *Brain Research Protocols, 4*(2), 230-235. [CrossRef]

6. Horváth, E.J., Horváth, K., Hámori, T., Fekete, M.I., Sólyom, S., Palkovits, M. (2000). Anxiolytic 2,3-benzodiazepines, their specific binding to the basal ganglia. *Progress in Neurobiology, 60*(4), 309-342. [CrossRef]

7. Szegó, J., Somogyi, M., Papp, E. (1993). Szemelvények a Grandaxin klinikai-farmakológiai és klinikai vizsgálataiból [Excerpts from the clinical-pharmacologic and clinical studies of Grandaxin]. *Acta Pharmaceutica Hungarica, 63*(2), 91-98. [CrossRef]

8. Rundfeldt, C., Socala, K., Wlaź, P. (2010). The atypical anxiolytic drug, tofisopam, selectively blocks phosphodiesterase isoenzymes and is active in the mouse model of negative symptoms of psychosis. *Journal of Neural Transmission (Vienna), 117*(11), 1319-1325. [CrossRef]

9. Leventer, S.M., Raudibaugh, K., Frissora, C.L., Kassem, N., Keogh, J.C., Phillips, J., Mangel, A.W. (2008). Clinical trial: dextofisopam in the treatment of patients with diarrhoea-predominant or alternating irritable bowel syndrome. *Alimentary Pharmacology and Therapeutics, 27*(2), 197-206. [CrossRef]

10. Petócz, L. (1993). Pharmacologic effects of tofizopam (Grandaxin). *Acta Pharmaceutica Hungarica, 63*(2), 79-82.

11. Fekete, M.I., Horváth, K., Kedves, R., Máté, I., Székely, J.I., Szentkuti, E. (1997). Selective interaction of homophtalazine derivatives with morphine. *European Journal of Pharmacology, 331*(2-3), 175-183. [CrossRef]

12. Pellow, S., File, S.E. (1986). Is tofisopam an atypical anxiolytic?-*Neuroscience & Biobehavioral Reviews, 10*, 221-227. [CrossRef]

13. Bernard, P., Dufresne-Favetta, C., Favetta, P., Do, Q.T., Himbert, F., Zubrzycki, S., Scior, T., Lugnier, C. (2008). Application of drug repositioning strategy to tofisopam. *Current Medicinal Chemistry, 15*(30), 3196-3203. [CrossRef]

14. Levy, R.A., Goldstein, B.D. (1981). Analgesia following microinjection of phosphodiesterase inhibitors at brainstem sites. *Pharmacology Biochemistry and Behavior, 15*(3), 501-504. [CrossRef]

15. Araiza-Saldaña, C.I., Reyes-García, G., Bermúdez-Ocaña, D.Y., Pérez-Severiano, F., Granados-Soto, V. (2005). Effect of diabetes on the mechanisms of intrathecal antinociception of sildenafil in rats. *European Journal of Pharmacology, 527*(1-3), 60-70. [CrossRef]

16. Salehi, F., Hosseini-Zare, M.S., Aghajani, H., Seyedi, S.Y., Hosseini-Zare, M.S., Sharifzadeh, M. (2017). Effect of bucladesine, pentoxifylline, and H-89 as cyclic adenosine monophosphate analog, phosphodiesterase and protein kinase A inhibitor on acute pain. *Fundamental & Clinical Pharmacology, 31*(4), 411-419. [CrossRef]

17. Fujita, M., Tamano, R., Yoneda, S., Omachi, S., Yogo, E., Rokushima, M., Shinohara, S., Sakaguchi, G., Hasegawa, M., Asaki, T. (2018). Ibudilast produces anti-allodynic effects at the persistent phase of peripheral or central neuropathic pain in rats: Different inhibitory mechanism on spinal microglia from minocycline and propentofylline. *European Journal of Pharmacology, 833*, 263-274. [CrossRef]

18. Mehanna, M.M., Domiati, S., Nakkash Chmaisse, H., El Mallah, A. (2018). Antinociceptive effect of tadalafil in various pain models: Involvement of opioid receptors and nitric oxide cyclic GMP pathway. *Toxicology and Applied Pharmacology, 352*, 170-175. [CrossRef]

19. Rojewska, E., Piotrowska, A., Jurga, A., Makuch, W., Mika, J. (2018). Zaprinast diminished pain and enhanced opioid analgesia in a rat neuropathic pain model. *European Journal of Pharmacology, 839*, 21-32. [CrossRef]

20. Kumar, A., Jain, N.K., Kulkarni, S.K. (2000). Analgesic and anti-inflammatory effects of phosphodiesterase inhibitors. *Indian Journal of Experimental Biology, 38*(1), 26-30. [CrossRef]

21. Jain, N.K., Patil, C.S., Singh, A., Kulkarni, S.K. (2001). Sildenafil-induced peripheral analgesia and activation of the nitric oxide-cyclic GMP pathway. *Brain Research, 909*(1-2), 170-178. [CrossRef]

22. Jain, N.K., Patil, C.S., Singh, A., Kulkarni, S.K. (2003). Sildenafil, a phosphodiesterase-5 inhibitor, enhances the antinociceptive effect of morphine. *Pharmacology, 67*(3), 150-156. [CrossRef]

23. Kim, H.K., Kwon, J.Y., Yoo, C., Abdi, S. (2015). The analgesic effect of rolipram, a phosphodiesterase 4 inhibitor, on chemotherapy-induced neuropathic pain in rats. *Anesthesia & Analgesia, 121*(3), 822-828. [CrossRef]

24. Dunham, N.W., Miya, T.S. (1957). A note on a simple apparatus for detecting neurological deficit in rats and mice. *Journal of the American Pharmaceutical Association (Scientific ed.), 46*(3), 208-209. [CrossRef]

25. Adzu, B., Amos, S., Dzarma, S., Wambebe, C., Gamaniel, K. (2000). Effect of Zizypus spina-christi wild aqueous extract on the central nervous system in mice. *Journal of Ethnopharmacology, 79*(1), 13-16. [CrossRef]

26. Ozawa, M., Nakada, Y., Sugimachi, K., Yabuuchi, F., Akai, T., Mizuta, E., Kuno, S., Yamaguchi, M. (1994). Pharmacological characterization of the novel anxiolytic beta-carboline abecarnil in rodents and primates. *Japanese Journal of Pharmacology*, *64*(3), 179-188. [CrossRef]

27. Woolfe, G., Mcdonald, A.D. (1944). The evaluation of analgesic action of pethidine hydrochloride (Demerol). *Journal of Pharmacology and Experimental Therapeutics*, *80*(3), 300-307. [CrossRef]

28. Kasap, M., Can, Ö.D. (2016). Opioid system mediated anti-nociceptive effect of agomelatine in mice. *Life Sciences*, *163*, 55-63. [CrossRef]

29. Koster, R., Anderson, M., Beer, E.J. (1959). Acetic acid for analgesic screening. *Federation Proceedings*, *18*, 412-418.

30. Demir Özkay, U., Can, O.D. (2013). Anti-nociceptive effect of vitexin mediated by the opioid system in mice. *Pharmacology Biochemistry and Behavior, 109*, 23-30. [CrossRef]

31. Cartmell, S.M., Gelgor, L., Mitchell, D. (1991). A revised rotarod procedure for measuring the effect of antinociceptive drugs on motor function in the rat. *Journal of Pharmacological Methods, 26*(2), 149-159. [CrossRef]

32. Le Bars, D., Gozariu, M., Cadden, S.W. (2001). Animal models of nociception. *Pharmacological Reviews*, *53*(4), 597-652. [CrossRef]

33. López-Canul, M., Comai, S., Domínguez-López, S., Granados-Soto, V., Gobbi, G. (2015). Antinociceptive properties of selective MT2 melatonin receptor partial agonists. *European Journal of Pharmacology, 764*, 424-432. [CrossRef]

34. Wong, C.H., Day, P., Yarmush, J., Wu, W., Zbuzek, U.K. (1994). Nifedipine induced analgesic after epidural injections in rats. *Anesthesia & Analgesia*, *79*(2), 303-306. [CrossRef]

35. Gabra, B.H., Sirois, P. (2003). Beneficial effect of chronic treatment with the selective bradykinin B1 receptor antagonists, R-715 and R-954, in attenuating streptozotocin-diabetic thermal hyperalgesia in mice. *Peptides*, *24*(8), 1131-1139. [CrossRef]

36. De Souza, M.M., Pereira, M.A., Ardenghi, J.V., Mora, T.C., Bresciani, L.F., Yunes, R.A., Delle Monache, F., Cechinel-Filho, V. (2009). Filicene obtained from Adiantum cuneatum interacts with the cholinergic, dopaminergic, glutamatergic, GABAergic, and tachykinergic systems to exert antinociceptive effect in mice. *Pharmacology Biochemistry and Behavior, 93*(1), 40-46. [CrossRef]

37. Park, S.H., Sim, Y.B., Kang, Y.J., Kim, S.S., Kim, C.H., Kim, S.J., Seo, J.Y., Lim, S.M., Suh, H.W. (2012). Hop extract produces antinociception by acting on opioid system in mice. *Korean Journal of Physiology & Pharmacology*, *16*(3), 187-192. [CrossRef]

38. Coelho, L.P., Reis, P.A., de Castro, F.L., Gayer, C.R., da Silva Lopes, C., da Costa e Silva, M.C., de Carvalho Sabino, K.C., Todeschini, A.R., Coelho, M.G. (2005). Antinociceptive properties of

ethanolic extract and fractions of Pterodon pubescens Benth. seeds. *Journal of Ethnopharmacology, 98*(1-2), 109-116. [CrossRef]

39. Pinheiro, M.M., Bessa, S.O., Fingolo, C.E., Kuster, R.M., Matheus, M.E., Menezes, F.S., Fernandes, P.D. (2010). Antinociceptive activity of fractions from Couroupita guianensis Aubl. leaves. *Journal of Ethnopharmacology, 127*(2), 407-413. [CrossRef]

40. Pinheiro, M.M., Boylan, F., Fernandes, P.D. (2012). Antinociceptive effect of the Orbignya speciosa Mart. (Babassu) leaves: evidence for the involvement of apigenin. *Life Sciences, 91*(9-10), 293-300. [CrossRef]

41. Millan, M.J. (2002). Descending control of pain. *Progress in Neurobiology, 66*(6), 355-474. [CrossRef]

42. Argoff, C. (2011). Mechanisms of pain transmission and pharmacologic management. *Current Medical Research and Opinion, 27*(10), 2019-2031. [CrossRef]

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 728-741, 2022
Doi: 10.33483/jfpau.1115491

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY OF DIFFERENT PARTS OF *SAMBUCUS EBULUS* L.

SAMBUCUS EBULUS L. BİTKİSİNİN FARKLI KISIMLARININ ANTİOKSİDAN VE ANTİ-ENFLAMATUVAR AKTİVİTELERİ

Büşra CUMHUR[1*], Sezen YILMAZ SARIALTIN[2], Safa GUMUSOK[1], Tülay COBAN[2], Ceyda Sibel KILIC[1]

[1]Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Botany, 06100 Ankara, Turkey

[2]Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, 06100 Ankara, Turkey

ABSTRACT

Objective: *Sambucus L. genus of Adoxaceae (Elderberry) family was investigated in previous studies for its antioxidant, anti-inflammatory, antiviral and antibacterial activities. Moreover, previous in vivo and in vitro studies performed on the leaves showed that the plant possesses anti-inflammatory activity. Our study aims to investigate the in vitro antioxidant and anti-inflammatory potential of the stem, fruit and leaf extracts of Sambucus ebulus L. plant.*

Material and Method: *The antioxidant activity was evaluated in a biological assay using Sambucus ebulus, whereas the radical scavenging activity was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ABTS methods.*

Result and Discussion: *The maximum anti-inflammatory effect was observed in stem extracts followed by leaf and fruit extracts, respectively. Stem extracts exhibited the highest ABTS and DPPH free radical scavenging activity (FRSA) similar to the results of anti-inflammatory activity. As a conclusion, stem extracts were the most potent extracts among the others regarding the FRSA and anti-inflammatory activities.In our study, the biological activity potential of the extracts was demonstrated, thus providing data supporting the traditional use of Sambucus ebulus L.*

Keywords: *Adoxaceae, anti-inflammatory activity, antioxidant activity, dwarf elder, Sambucus ebulus*

* **Corresponding Author / Sorumlu Yazar:** Büşra Cumhur
e-mail / e-posta: cmhr.busrra@gmail.com, **Phone / Tel.:** +05436988981

Submitted / Gönderilme: 11.05.2022 **Accepted / Kabul:** 28.06.2022

ÖZ

Amaç: *Adoxaceae (Mürver) ailesinin Sambucus L. cinsi, önceki çalışmalarda antioksidan, anti-enflamatuar, antiviral ve antibakteriyel aktiviteleri nedeniyle araştırılmıştır. Ayrıca, yapraklarda yapılan önceki in vivo ve in vitro çalışmalar, bitkinin anti-enflamatuar aktiviteye sahip olduğunu göstermiştir. Çalışmamız, Sambucus ebulus L. bitkisinin kök, meyve ve yaprak ekstrelerinin in vitro antioksidan ve anti-enflamatuar potansiyelini araştırmayı amaçlamaktadır.*

Gereç ve Yöntem: *Antioksidan aktivite, Sambucus ebulus kullanılarak biyolojik bir tahlilde değerlendirilirken, radikal süpürücü aktivite 2,2-difenil-1-pikrilhidrazil (DPPH) ve ABTS + yöntemleri kullanılarak ölçülmüştür.*

Sonuç ve Tartışma: *Maksimum anti-enflamatuvar etki, sırasıyla yaprak ve meyve ekstrelerinin ardından kök ekstrelerinde gözlendi. Kök ekstreleri, anti-enflamatuvar aktivitenin sonuçlarına benzer şekilde en yüksek ABTS ve DPPH serbest radikal süpürme aktivitesini (FRSA) sergiledi. Sonuç olarak, kök ekstresi, FRSA ve antiinflamatuar aktivite açısından diğerleri arasında en güçlü ekstre olmuştur.*

Anahtar Kelimeler: *Adoxaceae, anti-enflamatuvar aktivite, antioksidan aktivite, cüce mürver, Sambucus ebulus*

INTRODUCTION

Sambucus L. genus, under the Dipsacales order, was previously considered to be a member of Caprifoliaceae (Honeysuckle) family, but recently was transferred to the Adoxaceae (Elderberry) family [1]. In Turkey, *Sambucus* species are represented by two tree-like herbaceous species having drupe type fruits [2]. Among these, *S. ebulus* L. is a perennial herbaceous plant growing up to 2 m having an unpleasant odor [3]. This species is mostly found in the southern and rarely in the northwestern parts of Turkey, and grows naturally in Europe, Syria, Iraq and Lebanon, as well [2]. The general appearance and flowers of the plant are given in Figure 1.

Figure 1. *S. ebulus*; a: general view, b: fruits (Photos by B. Cumhur).

Aerial regions of the plant are used to clear up mastitis in cows [4], gastrointestinal disorders caused by inflammation, in the treatment of kidney and lung diseases, in the treatment of pathological

conditions due to oxidative stress, and furthermore, the fruit is an immune system stimulant [5]. Local names and provinces in which the plant is being used traditionally are given in Table 1 in which ethnobotanical uses of the plant are also specified.

Table 1. Ethnobotanical usages of *S. ebulus*.

Province	Local name	Used part	Purpose	Reference
Kastamonu	Hekimana	Fruit and leaves	Against stomach pain	[6]
		Leaves	Wound healing	
Kocaeli	Buzka Yivdin	Aerial parts	Relief of rheumatic pain	[7, 8]
		Ripe fruits	Hemorrhoid treatment	
		Buds	Wool dyeing	
Manavgat	Ayı Otu Mürver	Leaves	Furuncle treatment	[9]
Yalova	Şahmelik Sultanotu Bazeotu	Leaves and roots	Against scorpion and bee stings	[10]
		Ripe fruits	Anti-hemorrhoid	
Edirne	Sultan otu	Aerial parts	Against ticks	[11]
		Seeds	Against constipation	
Şile (İstanbul)	Şahmelik	Aerial parts	Cold	[12]
		Leaves	Urticaria	
			Knee pain	

It has been reported that a blue dye is obtained from the fruits and used in textile dyeing in Bosnia and Gorani [13]. In a study completed in the Kırklareli district of Turkey, it was reported that the plant was used in veterinary medicine, however both, *S. ebulus* and *Chelidonium majus* L. are known with the same local name [14].

Various biological effects of *Sambucus* species have been reported in prior research, such as anti-inflammatory [15-17], antibacterial [18], anti-Helicobacter pylori [19], antiviral [20] and radical scavenging (RS) [21]. Yeşilada et al. (1997) also documented the inhibitory effects of *S. ebulus* and *S. nigra* on interleukin (IL)-1α, 1β and tumor necrosis factor- α. Süntar et al. (2010) reported that the methanolic extract of the leaves collected from the Abant region (Bolu, Turkey) was demonstrated significant wound healing potential. Ahmadiani et al. (1998) reported that the anti-inflammatory effect of the plant might be due to its steroid or flavonoid content, however *n*-hexane extract of the leaves was reported to lack the aforementioned activity. Previous studies, both *in vitro* and *in vivo*, performed on the leaves showed that the plant had anti-inflammatory activity [16,17,22].

In this study, we aimed to examine *in vitro* antioxidant and anti-inflammatory potential of methanol extracts prepared from different parts of *S. ebulus*.

MATERIAL AND METHOD

Plant material

Plant materials were collected from Düzce, Akçakoca, Döngelli village, at landsides at an altitude of 96 m in June 2018, and fruit materials were collected from the same locality in October 2018 and voucher specimens are kept in AEF (Herbarium of Ankara University Faculty of Pharmacy) with herbarium numbers AEF 30759 and 30760, respectively.

Preparation of the plant extracts

Dried leaves (2.5 g), stems (5.5 g) and fruits (29.9 g) were grounded and macerated with methanol at room temperature (3 days). Extracts were filtered, evaporated with a rotary evaporator (Heidolph VV2000, Germany) and stored in a refrigerator until used.

Anti-inflammatory activity

Blood samples were collected from healthy individuals who were not administered any anti-inflammatory or steroidal drugs 15 days before the assay. The blood samples were centrifuged at 3000 rpm for 10 min and the packed cells were isolated. Then the cells were washed with isosaline at least three times (0.85%, pH 7.2) and the volume was determined. 10% v/v was made with isosaline. The anti-inflammatory effect of the samples was evaluated by measuring their membrane protection capacity against heat-induced hemolysis using the methods of Shinde et al. (1999) and Gunathilake et al. (2018). 10% cell suspension was mixed with the sample/standard and this mix was incubated at 56ºC for 30 min. Following the incubation period, the tubes were cooled to room temp. Then, centrifuge was applied at 2500 rpm for 5 min, and the absorbance was measured at 560 nm. Only the solvent was used as the control instead of the test sample, while ASA was the standard compound. Results were presented as stabilization percentage (%), defined by the formula: $[(A_o - A_s)/A_o] \times 100$. A_o: The absorbance of the control. A_s: The absorbance of the sample/standard. IC_{50} values were calculated by linear regression analysis for each sample. Each assay was run three times. The results were presented as mean IC_{50} values±standard deviation (SD).

Antioxidant activity

The DPPH free radical scavenging activity

The antioxidant effect of the plant extracts was determined by their scavenging ability on DPPH free radical [23]. 100 μM DPPH solution was made and maintained in dark at room temperature. Several concentrations of the crude extracts were added to each well containing DPPH solution. The mix was incubated for 30 min at room temp. Then the absorbance was measured at 517 nm. BHT was served as the standard compound. Results were calculated as inhibition %, using the following formula: [(A_o

$-A_s)/A_o] \times 100$. A_o: The absorbance of the control. A_s: The absorbance of the sample/standard. IC_{50} values were calculated by linear regression analysis for each sample. Each assay was run three times. The results were expressed as mean IC_{50} values±SD.

The ABTS $^+$ free radical scavenging activity

The antioxidant effect of the plant extracts was determined measuring their ABTS free radical scavenging potential [24] 7mM ABTS $^+$ aqueous solution and 2.45 mM potassium persulfate was reacted to get $ABTS^{\bullet+}$ radical solution. This solution was maintained at at room temp overnight in the dark before usage. At the end of the time, intensely-colored $ABTS^{\bullet+}$ radical cation was obtained. This dark solution was diluted with ethanol until to get 0.700±0.02 at 734 nm (pH=7.4). Fresh radical solution was produced every 5 days due to avoid the self-degradation. Various concentrations of the crude extracts were added to each well containing radical solution and left to kept for 6 min. Then the absorbance was measured at 734 nm. Trolox was served as the reference compound. Results were calculated as inhibition %, using the following formula: $[(A_o - A_s)/A_o] \times 100$. A_o: The absorbance of the control. A_s: The absorbance of the sample/standard. IC_{50} values were calculated by linear regression analysis for each sample. Each assay was run three times. The results were presented as mean IC_{50} values±SD.

Statistical analysis

All tests were done three times and the results were presented as mean value±SD. Statistical analyses were performed with SPSS V.25.0 using one-way analysis of variance (ANOVA) following by post-hoc Tukey's test. A *p*-value less than 0.05 was accepted as statistically significant.

RESULT AND DISCUSSION

Anti-inflammatory activity of different parts of *S. ebulus* were analyzed by measuring their red blood cell membrane stability. Reference compound showed higher anti-inflammatory activity than the plant extracts ($p<0.05$). *S. ebulus* stem extracts displayed the most potent anti-inflammatory effect followed by leaf and fruit extracts, respectively (Table 2).

Table 2. *S. ebulus* plant parts' anti-inflammatory activity

Plant extract	IC_{50} (mg/mL)
Stem	1.0879±0.0856
Fruit	6.3439±0.7177
Leaves	2.400±0.0511
ASA	0.2915±0.001
Data were expressed as mean IC_{50} ± SD.	

Antioxidant activity of different regions of *S. ebulus* was investigated by measuring ABTS and DPPH FRSA and the results are presented in Table 3. The highest ABTS and DPPH FRSA were observed in stem extracts of *S. ebulus*. Fruit extracts of *S. ebulus* exhibited greater antioxidant potential than leaf extracts in both ABTS and DPPH FRSA methods. But still the reference compounds showed higher RS potentials than the plant extracts ($p<0.05$).

Table 3. DPPH and ABTS $^{+}$ FRSA

Plant extract	IC_{50} (mg/ml)	
	DPPH	**ABTS**
Stem	0.9145±0.0250	0.0188±0.001
Fruit	1.3801±0.0187	0.3969±0.035
Leaves	2.2345±0.1591	0.6681±0.064
BHT	0.0219±0.004	-
Trolox	-	0.0171±0.001
Data were expressed as mean IC_{50} ± SD.		

Medicinal plants have long been used in ethnobotany in the treatment of inflammatory conditions [25]. When we search the literature, we can see that extract of *S. australis* Cham. & Schltdl. That have been used in Brazilian traditional medicine to treat inflammatory ailments [25,26] exhibited antibacterial activity against *Klebsiella pneumoniae* (MIC 250 lg/mL) and *Salmonella typhimurium* (MIC 250 lg/mL). Chlorogenic acid and rutin were reported to be the main compounds as revealed by LC-MS/MS and this study also established that ethanolic extract of this plant possessed significant anti-inflammatory activity.

The IC_{50} for DPPH radical scavenging activity of *S. ebulus* (SE) collected from Mazandaran Forest was found to be 202.50 ± 1.38 for water extract (SW) and 723.62 ± 3.36 μg ml^{-1} for methanol extract (SM). The tested extracts exhibited poor Fe^{2+} chelating ability. Both extracts possessed significant antioxidant activity. SW extracts exhibited a better activity pattern than BHA and Vitamin C at different incubation period. SE extracts had a good reducing ability for nitric oxide scavenging and anti-lipid peroxidation activity. The aqueous extract was found to have higher total phenol and flavonoid content than the methanol extract [27].

In another study that included 21 weel volunteers, aged 20 to 59 years, with a BMI of 23.12 ± 1.31, who consumed 200 ml of SE ripe fruit infusion per day for 30 days, blood samples were drawn before and at the end of the intervention. At the end of the study, significant decreases were found in triglycerides (14.92%), total cholesterol (15.04%) and LDL-C (24.67%). In addition, the HDL-C/LDL-C ratio was increased by 42.77% [28]. This study by Ivanova et al. (2014) is the first human intervention

study with SE fruit infusion, and the results demonstrated the plant's potential to enhance the lipid profile and serum antioxidant capacity in humans.

Total phenolic content of different extracts (petroleum ether, distilled water, ethyl acetate, acetone and methanol) obtained from stems, fruits, roots and leaves of SE (collected region of Šumarice, Kragujevac in central Serbia) were evaluated using Folin-Ciocalteu. Their total phenolic content ranged from 29.87 to 126.10 mg GAE/g, while the flavonoids ranged from 4.50 to 97.65 mg RUE/g. Their antioxidant activity was investigated by DPPH assay and IC_{50} values were ranged from 47.37 to 710.94 µg/ml. The ethyl acetate extracts of fruits and methanol extracts of leaves contain the highest level of phenolics and exhibited powerful antioxidant activity [29].

Total phenolic, proanthocyanidin, phenolic acid and flavonoid content analyses of SE (dwarf elder) fruit extracts were examined together with several antioxidant activity methods such as metal reduction and FRSA. High-performance thin-layer chromatography was performed to measure the quantity of chlorogenic acid, the bioactive metabolite of the plant sampling. It was shown that methanolic and aqueous extracts of SE fruits were affected by simulated human digestion, with changes in antioxidant activity consistent with changes in phenolics (collected Bolu province, Turkey) [30]. Table 4 shows collected region, used parts, ingredients and study methods of *Sambucus sp*.

Table 4. Studies on different *Sambucus sp*.; composition, used parts and collection localities.

Species	Collected location	Used part	Composition	Solvent	Study		References
					Method	Result	
S. australis	Brazil	aerial parts	-	methanol	Inhibition of (NF-kB) *in vitro*	anti-inflammatory activity (inhibition of of the transcription factor NF-kB activation and reducing levels of inflammatory cytokines and NO)	[25]
	South Brazil	leaf and bark	-	ethanol	FRAP DPPH NO radical scavenging assays	DPPH (IC50 43.5 and 66.2 lg/mL), FRAP (IC50 312.6 and 568.3 lg/mL) and NO radical scavenging assays (IC50 285.0 and 972.6 lg/mL) were observed	[27]
	Northeast Brazil	aerial parts	ursolic acid	ethanol	MIC	Antibacterial activity against *Escherichia coli*	[31]
					DPPH	significant activity.	

Table 4 (*continued*). Studies on different *Sambucus sp*.; composition, used parts and collection localities.

Species	Collected location	Used part	Composition	Solvent	Study		References
					Method	Result	
					DPPH	significant activity.	
S. ebulus	Romania	fruits	phenolic acids and flavonoids (flavonols and anthocyanins) (LC-PDA-MS) hyperoside, quercetin-3-*O*-glucoside, 3-quercetin-3-O-rutinoside, 4- quercetin, 5-quercetin, 6-kaempherol (HPLC-MS); 5- Cyanidin-3-Osambubioside-5-glucoside; 6-Cyanidin-3,5-diglucoside, 7-Unknown; 8-Cyanidin-3-Osambubioside; 9- Cyanidin-3-O-glucoside, 10-Cyanidin-3-O-glucoside; 11-Pelargonidin; 12-cyanidin. (HPLC-DAD)	acidified methanol (0.3% HCl, v/v)	DPPH	antioxidant capacity	[32]
	Turkey	flowers, fruits and leaves	-	methanol	DPPH Folin–Ciocalteu	strongest antioxidant activity	[33]
					micro-broth dilution	moderate effect against *Candida albicans* by fruit extract	
					L929 (ATCC, CCL-1) mouse fibroblast cell line, HeLa (ATCC, CCl-2) human cervix adenocarcinoma cell line	highest anticarcinogenic activity with leaf extract (10µg/mL)	
	Bulgaria	fruits	-	methanol	-	anti-herpes simplex virus type 1 and antioxidant (in ORACFL and HORACFL) activities	[34]

Table 4 (*continued*). Studies on different *Sambucus sp*.; composition, used parts and collection localities.

S. ebulus	Southern Romania	fruit	-	ethanol 70%	spectrophotometric	antioxidant activity EC_{50} (μg/mL) 68.45 ± 0.441	[35]
					disc diffusion method and the well diffusion method	antimicrobial activity (significant results against *Pseudomonas fluorescens* and *Enterococcus fecalis*)	
	Kordkail Kola Sofla	Leaf and fruit	-	Hexane and methanol respectively	repellant activity	repellant activity (73.4% for leaf and 78% for fruit extracts)	[36]
S. nigra	Romania	fruits	phenolic acids and flavonoids (flavonols and anthocyanins) (LC-PDA-MS) quercetin-3-O-glucoside, 3-quercetin-3-O-rutinoside, 4- quercetin, 5-quercetin, 6-kaempherol, 7- p-coumaric acid (HPLC-MS); 5-Cyanidin-3-Osambubioside-5-glucoside; 6-Cyanidin-3,5-diglucoside, 7-Unknown; 8-Cyanidin-3-Osambubioside; 9- Cyanidin-3-O-glucoside, 10-Cyanidin-3-O-glucoside; 11-Pelargonidin; 12-Cyanidin.	acidified methanol (0.3% HCl, v/v)	DPPH	antioxidant capacity	[32]

Table 4 (*continued*). Studies on different *Sambucus sp*.; composition, used parts and collection localities.

S. nigra	-	fruits	Anthocyanins, Cyanidin, Pelargonidin 3-glucoside, Cyanidin 3-sambubioside-5-glucoside, Cyanidin 3,5-diglucoside, Cyanidin 3-sambubioside, Cyanidin 3-glucoside, Quercetin, Quercetin 3-O-rutinoside, Quercetin 3-O-glucoside, Proanthocyanidins	-	-	-	[37]
		flowers	Quercetin 3-O-rutinoside, Quercetin 3-O-glucoside, Kaempferol 3-O-glucoside, Isorhamnetin 3-O-rutinoside,				
	garden of the Urmia University	fruit, leaf and bark	-	methanol	DPPH iron chelating ability nitric oxide and hydrogen peroxide radical scavenging activities.	leaf extract showed highest activity (IC50 = 21.6 ± 1.1 µg/ml) and also a higher NO radical scavenging activity	[38]

When we searched the literature for antioxidant and anti-inflammatory studies performed on different *Sambucus* species along with the ones performed with the plant species that we have also examined, we have seen that similar results were obtained in previous studies. Our results indicate that stems of SE can be served as a source of natural anti-inflammatory components since this biological activity that we have observed is compatible with the studies found in the literature. Concurrently, our results also indicate that the extract prepared from the stems of *S. ebulus* can be used as a source of antioxidant components. New studies should be performed to examine the responsible compound(s) for these biological activity activities.

ACKNOWLEDGEMENTS

Plant materials were identified by Prof. Dr Hayri DUMAN from Gazi University, Faculty of Science, Department of Biology. We appreciate his help in this matter.

AUTHOR CONTRIBUTIONS

Concept: *C.S.K., B.C.*; Design: *B.C.;* Control: *C.S.K., T.Ç.*; Sources: *B.C.;* Materials: *B.C., S.Y.S.*; Data Collection and/or processing: *B.C.*; Analysis and/or interpretation: *B.C., S.Y.S., S.G.*; Literature review: *B.C.*; Manuscript writing: *B.C.*; Critical review: *C.S.K.;* Other: *S.G.*

CONFLICT OF INTEREST

The authors declare that there is no real, potential or perceived conflict of interests for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

REFERENCES

1. Jabbari, M., Daneshfard, B., Emtiazy, M., Khiveh, A., Hashempur, M.H. (2017). Biological effects and clinical applications of dwarf elder (*Sambucus ebulus* L). *Journal of Evidence-Based Complementary & Alternative Medicin*; *22*(4), 996-1001. [CrossRef]

2. Chamberlain, D.F., Davis, P.H. (1972). Flora of Turkey and the East Aegean Islands, Edinburgh University Press, Edinburgh, p.540-41.

3. Seçmen, Ö., Gemici, Y., Görk, G., Bekat, L., Leblebici, E. (2000). Tohumlu Bitkiler Sistematiği, Ege Üniversitesi Basımevi, İzmir.

4. Ecevit-Genç, G., Özhatay, N. (2006). An ethnobotanical study in Çatalca (European part of Istanbul) II. *Turkish Journal of Pharmaceutical Sciences*, *3*(2), 73-89.

5. Tasinov, O., Kiselova-Kaneva, Y., Ivanova, D. (2013). *Sambucus ebulus* - from traditional medicine to recent studies. *Scripta Scientifica Medica*, *45*, 36-42. [CrossRef]

6. Sezik, E., Zor, M., Yesilada, E. (1992). Traditional medicine in Turkey II. Folk medicine in Kastamonu. *International Journal of Pharmacognosy*, *30*(3), 233-239. [CrossRef]

7. Şimşek, I., Aytekin, F., Yeşilada, E., Yıldırımlı, Ş., Anadolu'da Halk Arasında Bitkilerin Kullanılış Amaçları Üzerinde Etnobotanik Bir Çalışma, 14. Bitkisel İlaç Hammaddeleri Toplantısı, Bildiriler, Başer K.H.C., Kırımer N., (Eds.), 29-31 Mayıs 2002, Eskişehir.

8. Yeşilada, E., Sezik, E., Honda, G., Takaishi, Y., Takeda, Y., Tanaka, T. (1999). Traditional medicine in Turkey IX: folk medicine in north-west Anatolia, *Journal of Ethnopharmacology*, *64*, 195–210. [CrossRef]

9. Bulut, Y. (2006). Master Thesis. Manavgat (Antalya) Yöresinin Faydalı Bitkileri. Biyoloji Ana Bilim Dalı, Fen Bilimleri Enstitüsü, Süleyman Demirel Üniversitesi, Isparta, Türkiye.

10. Koçyiğit, M., Özhatay, N. (2006). Wild plants used as medicinal purpose in Yalova (Northwest Turkey). *Turkish Journal of Pharmaceutical Sciences*, *3*(2), 91-103.

11. Tuzlaci, E., Işbilen-Alparslan, D.F., Bulut, G. (2010). Turkish Folk Medicinal Plants, VIII: Lalapaşa (Edirne). *Marmara Pharmaceutical Journal*, *14*, 47-52. [CrossRef]

12. Tuzlaci, E., Tolon, E. (2000). Turkish folk medicinal plants, part III: Sile (Istanbul). *Fitoterapia*, *71*, 673-685. [CrossRef]

13. Mustafa, B., Hajdari, A., Pieroni, A., Pulaj, B., Koro, X., Quave, C.L. (2015). A cross-cultural comparison of folk plant uses among Albanians, Bosniaks, Gorani and Turks living in south Kosovo. *Journal of Ethnobiology and Ethnomedicine*, *11*(1), 1-26. [CrossRef]

14. Kültür, S. (2007). Medicinal plants used in Kırklareli Province (Turkey). *Journal of Ethnopharmacology*, *111*(2), 341–364. [CrossRef]

15. Yeşilada, E. (1997). Evaluation of the anti-inflammatory activity of the Turkish medicinal plant *Sambucus ebulus*. *Chemistry of Natural Compounds*, *33*, 539–540. [CrossRef]

16. Ahmadiani, A., Fereidoni, M., Semnanian, S., Kamalinejad, M., Saremi, S. (1998). Antinociceptive and anti-inflammatory effects of *Sambucus ebulus* rhizome extract in rats. *Journal of Ethnopharmacology*, *61*(3), 229–235. [CrossRef]

17. Ebrahimzadeh, M.A., Mahmoudi, M., Salimi, E. (2006). Antiinflammatory activity of *Sambucus ebulus* hexane extracts. *Fitoterapia*, *77*, 146–148. [CrossRef]

18. Yeşilada, E., Gurbuz, I., Shibata, H. (1999). Screening of Turkish anti-ulcerogenic folk remedies for anti-Helicobacter pylori activity. *Journal of Ethnopharmacology*, *66*(3), 289–293. [CrossRef]

19. Martinez, M.J.A., Del Olmo, L.M.B., Benito, P.B. (2005). Antiviral activities of polysaccharides from natural sources. *Studies in Natural Products Chemistry*, *30*, 393–418. [CrossRef]

20. Neto, C.C., Owens, C.W., Langfield, R.D., Comeau, A.B., Onge, J.S., Vaisberg, A.J., Hammond, G.B. (2002). Antibacterial activity of some Peruvian medicinal plants from the Callejon de Huaylas. *Journal of Ethnopharmacology*, *79*, 133–138. [CrossRef]

21. Dawidowicz, A.L., Wianowska, D., Baraniak, B. (2006). The antioxidant properties of alcoholic extracts from *Sambucus nigra* L. (antioxidant properties of extracts). *LWT-Food Science and Technology*, *39*(3), 308–315. [CrossRef]

22. Yeşilada, E., Ustun, O., Sezik, E., Takaishi, Y., Ono, Y., Honda, G. (1997). Inhibitory effects of Turkish folk remedies on inflammatory cytokines: interleukin-1alpha, interleukin-1beta and tumor necrosis factor alpha. *Journal of Ethnopharmacology*, *58*(1), 59–73. [CrossRef]

23. Blois, M.S. (1958). Antioxidant determination by the use of a stable free radical. *Nature*, *181*(4617), 1199–1200. [CrossRef]

24. Yalçin, C.Ö., Yilmaz Sarialtin, S., Çiçek Polat, D. (2020). Quantification of phenolic and flavonoid contents and some biological activities of Ornithogalum sigmoideum Freyn & Sint. *Journal of Research in Pharmacy*, *24*(4), 487-496. [CrossRef]

25. Carneiro, N.V.Q., Da Silva, H.B.F., Da Silva, R.R., Carneiro, T.C.B., Costa, R.S., Pires, A.O., Marques, C.R., Velozo, E.S., Conceição, A.S., Da Silva, T.M.S., Da Silva, T.M.G., Alcântara-Neves, N.M., Figueiredo, C.A. (2022), *Sambucus australis* modulates inflammatory response via inhibition of nuclear factor kappa B (NF-kB) in vitro. *Anais da Academia Brasileira de Ciências 11*(7), e12911729567. [CrossRef]

26. Bahiense, J.B., Marques, F.M., Figueira, M.M., Vargasa, T.S., Kondratyuk, T.P., Endringer, D.C., Scherer, R., Fronzaa, M. (2017). Potential anti-inflammatory, antioxidant and antimicrobial activities of *Sambucus australis*. *Pharmaceutical Biology*, *55*(1), 991–997. [CrossRef]

27. Ali, E.M., Somayyeh, E., Bahman, E. (2009), *Sambucus ebulus* elburensis fruits: A good source for antioxidants. *Pharmacognosy Magazine*, *5*(19), 213-218.

28. Ivanova, D., Tasinov, O., Kiselova-Kaneva, Y. (2014). Improved lipid profile and increased serum antioxidant capacity in healthy volunteers after *Sambucus ebulus* L. fruit infusion consumption. *International Journal of Food Sciences and Nutrition*, *65*(6), 740-744. [CrossRef]

29. Topuzović, M.D., Stanković, M.S., Jakovljević, D.Z., Bojović, B.M. (2016). Plant part variability of *Sambucus ebulus* L. secondary metabolites content and antioxidant activity. *Agro FOOD Industry Hi Tech, 27*(2), 60-63.

30. Barak, T.H., Celep, E., ˙Inan, Y., Yeşilada, E. (2020). *In vitro* human digestion simulation of the bioavailability and antioxidant activity of phenolics from *Sambucus ebulus* L. fruit extracts. *Food Bioscience*, *37*, 100711. [CrossRef]

31. Nascimento, P.G.G. do, Lemos, T.L.G, Bizerra, A.M.C., Arriaga, Â.M.C., Ferreira, D.A., Santiago, G.M.P., Braz-Filho, R., Costa, J.G.M. (2014). Antibacterial and antioxidant activities of ursolic acid and derivatives, *Molecules*, *19*(1), 1317-1327. [CrossRef]

32. Anton, A.M., Pintea, A.M., Rugină, D.O., Sconţa, Z.M., Hanganu, D., Vlase, L., Benedec, D. (2013). Preliminary studies on the chemical characterization and antioxidant capacity of polyphenols from *Sambucus sp.*. *Digest Journal of Nanomaterials and Biostructures, 8*(3), 973 – 980.

33. Meriç, Z.İ., Bitiş, L., Birteksöz-Tan, S., Özbaş Turan, S., Akbuga, J. (2014). Antioxidant, antimicrobial and anticarcinogenic activities of *Sambucus ebulus* L. flowers, fruits and leaves. *Marmara Pharmaceutical Journal*, *18*(1), 22-25. [CrossRef]

34. Zahmanov, G., Alipieva, K., Denev, P., Todorov, D., Hinkov, A.., Shishkov, S., Simova, S., Georgiev, M.I. (2015). Flavonoid glycosides profiling in dwarf elder fruits (*Sambucus ebulus* L.) and evaluation of their antioxidantand anti-herpes simplex activities. *Industrial Crops and Products, 6(3),* 58–64. [CrossRef]

35. Rodino, S., Butu, A., Petrache, P., Butu, M., Dinu-Pîrvu, C., Cornea, C.P. (2015). Evaluation of the antimicrobial and antioxidant activity of *Sambucus ebulus* extract. *Farmacia*, *63*(5), 751-754.

36. Motevalli-Haghi, S.F., Fathi, H., Ebrahimzadeh, M.A., Eslami, S., Karamie, M., Eslamifar, M., Dehghan, O. (2020). Evaluation of phytochemical, total phenolic and flavonoid content, antioxidant activities, and repelling property of *Sambucus ebulus*. *Journal of Medicinal Plants and By-products*, *9*, 97-105.

37. Sidor, A., Gramza-Michałowska, A. (2015). Advanced research on the antioxidant and health benefit of elderberry (*Sambucus nigra*) in food – a review. *Journal of functional foods, 18(B),* 941–958. [CrossRef]

38. Azari, B., Siami, A., Ebrahimzadeh, M.A., Khan, B.A. (2015). Antıoxidants activity of extracts from *Sambucus nigra*, efficiency of different extraction methods. *Latin American Applied Research, 45*(3), 139-144. [CrossRef]

39. Gunathilake, K.D.P.P., Ranaweera, K.K.D.S., Rupasinghe, H.P. (2018). In vitro anti-inflammatory properties of selected green leafy vegetables. *Biomedicines, 6*(4), 107. [CrossRef]

40. Shinde, U.A., Phadke, A.S., Nair, A.M., Mungantiwar, A.A., Dikshit, V.J., Saraf, M.N. (1999). Membrane stabilizing activity—a possible mechanism of action for the anti-inflammatory activity of *Cedrus deodara* wood oil. *Fitoterapia, 70*(3), 251-257. [CrossRef]

41. Süntar, İ.P., Kupeli-Akkola, E., Yalçin, F.N., Koca, U., Keleş, H., Yeşilada, E. (2010). Wound healing potential of *Sambucus ebulus* L. leaves and isolation of an active component, quercetin 3-O-glucoside. *Journal of Ethnopharmacology, 129*(1), 106–114. [CrossRef]

Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 46(3): 742-754, 2022
Doi: 10.33483/jfpau.1084639

ÖZGÜN MAKALE / ORIGINAL ARTICLE

ARCTİUM MİNUS METANOL EKSTRESİNİN ÇEŞİTLİ KANSER HÜCRE HATLARI ÜZERİNDE SİTOTOKSİK ETKİLERİ

CYTOTOXIC EFFECTS OF ARCTIUM MINUS METHANOL EXTRACT ON VARIOUS CANCER CELL LINES

Ayşe Arzu SAYIN ŞAKUL[1*], Yasemin YOZGAT BYRNE[2], Ayşe Esra KARADAĞ[3], Ebrar ALTINALAN[4], Şeyma ÇİMEN[5], Mehmet Evren OKUR[6]

[1]İstanbul Medipol Üniversitesi, Sağlık Bilim ve Teknolojileri Araştırma Enstitüsü (SABITA), Tıp Fakültesi, Tıbbi Farmakoloji Ana Bilim Dalı, 34810, İstanbul, Türkiye

[2]İstanbul Medipol Üniversitesi, Sağlık Bilim ve Teknolojileri Araştırma Enstitüsü (SABITA), Tıp Fakültesi, Tıbbi Biyoloji Ana Bilim Dalı, 34810, İstanbul, Türkiye

[3]İstanbul Medipol Üniversitesi, Sağlık Bilim ve Teknolojileri Araştırma Enstitüsü (SABITA), Eczacılık Fakültesi Fakültesi, Farmakognozi Ana Bilim Dalı, 34810, İstanbul, Türkiye

[4]İstanbul Medipol Üniversitesi, Sağlık Bilim ve Teknolojileri Araştırma Enstitüsü (SABITA), Tıp Fakültesi Fakültesi, Tıbbi Farmakoloji Ana Bilim Dalı, 34810, İstanbul, Türkiye

[5]İstanbul Medipol Üniversitesi, Sağlık Bilim ve Teknolojileri Araştırma Enstitüsü (SABITA), 34810, İstanbul, Türkiye

[6]Sağlık Bilimleri Üniversitesi, Eczacılık Fakültesi, Farmakoloji Ana Bilim Dalı, 34668, İstanbul, Türkiye

ÖZ

Amaç: *Bu çalışmada Arctium minus (Hill) Bernh. ssp. minus'un toprak üstü kısımlarından elde edilen metanol ekstresinin kanser hücre hatları üzerindeki sitotoksik etkilerinin değerlendirilmesi amaçlanmıştır.*

Gereç ve Yöntem: *Arctium minus (Hill) Bernh. ssp. minus'un metanol ekstresinin, iki farklı insan meme kanseri hücre hattına (MCF-7 ve MDA-MB-231) ve kontrol olarak normal insan fibroblast hücre hattına (MRC-*

* **Sorumlu Yazar / Corresponding Author:** Ayşe Arzu Sayın Şakul
e-posta / e-mail: aasakul@medipol.edu.tr, **Tel. / Phone:** +902166812114

Gönderilme / Submitted: 08.03.2022 **Kabul / Accepted:** 05.07.2022

5) uygulanması ile in vitro sitotoksik etkileri araştırılmıştır. Hücre canlılık tayini CellTiter-Blue metodu kullanılarak gerçekleştirilmiştir. İstatistiksel analiz için One-Way ANOVA ve Tukey post-hoc testi kullanılmıştır.

Sonuç ve Tartışma: *Analizlerde, MCF-7 kanser hücrelerinde hücre canlılığı %27,8 -38,7 oranında belirlenmiş olup önemli derecede sitotoksik aktivite tespit edilmiştir (1 mg/mL ekstre uygulaması için p<0.022). Ancak MDA-MB-231 kanser hücre hatlarında %47,8-59,7 oranında hücre canlılığı gözlemlenmiştir. MRC-5 normal fibroblast hücrelerinde ise sitotoksisite gözlemlenmemiştir (%92,4 – 105,4 hücre canlılığı). Bu bulgulardan yola çıkarak, MCF-7 kanser hücreleri ve MRC5 normal fibroblast hücrelerine 1,25 mg/mL Arcitum minus ekstresi ile muamele edilmiş ve flow sitometrisi metodu ile hücre ölümünün ölçümü gerçekleştirilmiştir. Arctium minus ekstresi uygulaması ile hücre ölümü, MCF-7 kanser hücrelerinde (%98) MRC5 normal fibroblast hücrelerinden (%25) çok daha yüksek oranda gerçekleşmiştir. Sonuç olarak, Arctium minus ssp. minus ekstresi uygulamasının hücre canlılığını MCF-7 hücre hattında normal fibroblast hücre hattına göre daha fazla azalttığı söylenilebilir.*

Anahtar Kelimeler: *Arctium minus ssp. minus, hücre canlılığı, hücre ölümü, kanser, sitotoksisite*

ABSTRACT

Objective: *This study aimed to evaluate the cytotoxic effects of Arctium minus (Hill) Bernh. ssp. minus methanol extract derived from aerial parts on cancer cell lines.*

Material and Method: *For cytotoxicity assays, two different human breast cancer cell lines (MCF-7 and MDA-MB-231) and healthy human fibroblast cell line (MRC-5)as a control were used. Cell viability determination was performed using the CellTiter-Blue method. One-Way ANOVA and Tukey post test were used for statistical analysis.*

Result and Discussion: *Cell viability has been detected between ratios of 27.8-38.7% for MCF-7 cancer cell line, and a significant cytotoxic activity was observed via the analysis (1 mg/mL extract treatment p< 0.022). However, 47.8-59.7% cell viability was observed for MDA-MB-231 cancer cell line, and MRC-5 healthy fibroblast cell line did not demonstrate any cell viability (92.4-105.4% cell viability). Depending on these data, MCF-7 cancer cell line and MRC-5 fibroblast healthy cell line were treated with Arcitum minus extract, then cell viability was detected by flow cytometry technique. The ratio of the cell death was higher in MCF-7 cancer cell line (98%) compared with the MRC-5 fibroblast healthy cell line (25%) after the Arctium minus extract treatment. In conclusion, Arctium minus ssp. minus extract has significantly decreased the cell viability in MCF-7 cancer cell line when compared with the MCR-5 fibroblast normal cell line.*

Keywords: *Arctium minus ssp. minus, cancer, cell death, cell viability, cytotoxicity*

GİRİŞ

Kanser, çevresel ve genetik faktörlerin etkisi ile hücrelerin büyüme özelliğinin bozulması ve kontrolsüz çoğalmaları ile gelişen bir hastalıktır. Somatik genetik hastalıklardan en sık görülen ve en komplike olanıdır [1]. Uluslararası Kanser Araştırmaları Ajansı (IARC) GLOBOCAN veritabanına göre, 2020 yılında dünya genelinde 19,3 milyon yeni kanser vakası bildirilmiştir. En yaygın kanser türü ise son yıllarda akciğer kanserinden (% 22,4) daha fazla tanı konulan meme kanseri (%47,8) olmuştur [2].

Kanser tedavisinde; cerrahi, kemoterapi ve radyoterapi gibi geleneksel yöntemler yaygın olarak kullanılmaya devam etse de ağır yan etki profilleri ve kemoresistans gelişimi gibi faktörler kanserle mücadelede engel olarak karşımıza çıkmaktadır [3].Son yıllarda bu olumsuz faktörleri azaltmak amacıyla yeni tedavi seçenekleri geliştirilmektedir. Kansere karşı koruma ve kanser tedavisi için güvenli ve etkin bileşiklere ihtiyaç duyulmaktadır [3]. Bu amaçla tıbbi bitkilerden elde edilen bileşenler, yan etkileri azaltılamış yüksek etkinliğe sahip yeni terapötik moleküllerin keşfi ve tasarımı için oldukça önemlidir [4].

Arctium türleri, Astaraceae familyasına ait bitkilerdir. Asteraceae cinsi tanımı gereği, *A. lappa* L.'nin (=syn. *A. major* Gaertn.), *A. minus* (Hill) Bernh., *A. tomentosum* Mill. ve melezleri veya bunların karışımlarından oluşmaktadır. Ana karakteristik bileşenleri olan inülin, arktigenin ve arktiin ile yapılan çalışmalar mevcuttur [5]. Terapötik endikasyonları arasında seboreik cilt, egzama, fronküller, akne, sedef hastalığı, idrar miktarını artırarak küçük idrar yolu şikayetlerinde adjuvan olarak kullanımı bulunmaktadır [6]. Türkiye'deki florasında ssp. *pubens* ve ssp. *minus* isimli iki alt türü mevcuttur [7].

Arctium türlerinde bulunan başlıca biyoaktif bileşenler; lignanlar, yağ asitleri, asetilenik bileşikler, fitosteroller, polisakaritler, kafeoilkinik asit türevleri, flavonoidler, terpenler/terpenoidler ve hidrokarbonlar, aldehitler, metoksipirazinler, seskiterpenlerdir [8,9,10]. *Arctium* türleri ayrıca antikanser, antidiyabetik, antioksidan, hepatoprotektif, gastroprotektif, antibakteriyel, antiviral, antimikrobiyal, antialerjik ve antiinflamatuar etkiler gibi biyoaktif özelliklere sahiptir [5,11,12] . Avrupa-Akdeniz bölgesinde altı ana tür bulunur: *A. atlanticum* (Pomel) H. Lindb., *A. lappa* L., *A. minus, A. nemorosum* Lej., *A. palladini* (Marcow) R.E.Fr. & Soderb. ve *A. tomentosum* Mill [5]. *Arctium minus,* Türkiye'de yetişen, Isparta ve Doğu Anadolu'da yayılış gösteren bir *Arctium* türüdür. [13,14]. Ülkemizde daha çok "löşlek" olarak bilinen bu tür, Avrupa'dan güney ve doğuya doğru Kuzey Afrika ve Kafkasya'ya kadar doğal yayılış gösterir. *A. minus* ülkemizde ''kabalak, büyük kabalak, acıkabalak" veya "gabalak" isimleriyle de bilinmektedir [15].

A. minus yapraklarının sulu ekstresi ile yapılan bir çalışmada 12,5 mg üzerindeki konsantrasyonunun oral candida türlerine karşı fungastik ve fungasidal etkinliği gösterilmiştir [16]. Ayrıca geleneksel Türk halk tıbbında, *A. minus* yaprakları hem güneş çarpması hem de güneş yanığı tedavisinde kullanılmaktadır [17]. Ayrıca geleneksel tedavide *A. minus'*un taze yapraklarının tuzla birlikte veya suyun içinde kaynatılarak eklemlere uygulanması romatizmal ağrıların tedavisinde kullanılır [18].

Avrupa İlaç Ajansı (EMA) uzun süreli kullanımda *A. lappa, A. minus* ve *A. tomentosum* köklerinin minör üriner sistem şikayetlerinde, geçici iştahsızlıkta ve seboreik cilt rahatsızlıklarında adjuvan olarak kullanımını onaylamaktadır [19].

*A. minus'*un antiinflamatuvar, antinosiseptif ve antioksidan etkileri önceki çalışmalarda deneysel olarak incelenmiş olsa da henüz antikanser etkinliği yeterince araştırılmamıştır [20]. Bir çalışmada akut gut atak fare modellerine *A. minus*' un tohumlarından elde edilen ekstre uygulanmış ve ekstrenin antinosiseptif etkisinin gut ağrısının tedavisinde yan etkilere neden olmadan destekleyici tedavi olarak kullanılabileceği bildirilmiştir [21]. Ancak, çalışmamızda kullanmış olan *A. minus* ssp. *minus* alt türleri ile yapılmış mevcut deneysel çalışmalar sınırlıdır. *Arctium* türlerinin sekonder metabolit olarak arktiin içerdiği bilinmektedir [22,23].

GEREÇ VE YÖNTEM

Bitki Materyali

A. minus ssp. *minus*'un çiçekli toprak üstü kısımları 25 Temmuz 2020 tarihinde Kırklareli Demirköprü'den toplanmıştır. Bitki, Ayşe Esra Karadağ tarafından teşhis edilmiş ve herbaryum örneği (IMEF 1191) İstanbul Medipol Üniversitesi Eczacılık Fakültesi Herbaryumu'nda muhafaza edilmiştir.

Ekstraksiyon

Bitki materyalinin toprak üstü kısımları açık havada gölgede kurutulmuştur ve ardından laboratuvar ölçekli bir değirmen kullanılarak uygun bir boyuta kadar toz haline getirilmiştir. Bitki materyali, metanol ile oda sıcaklığında üç kez ayrı ayrı ekstre edilmiştir. Süzüldükten sonra, metanol ekstresi 45 °C'de vakumla rotary evoparatör yardımı ile çözücüsünden kurtarılmıştır.

HPLC Analizi

HPLC çalışmasında, *A. minus* ssp. *minus* 'un toprak üstü kısmından hazırlanan metanol ekstresi, 0.22 µm membran filtrelerden süzülmüştür. HPLC analizi, Agilent 1100 LC Sistemi ile çalışılmıştır. Analiz Agilent C18 kolonu (4.6x250mm 5um) üzerinde gerçekleştirilmiş ve sıcaklık 35 °C'de tutulmuştur. Mobil faz metanol:su (55:45) kullanılarak izoktarik akış ile çalışılmıştır. Zhou ve arkadaşlarının (2011) yöntemine göre, 280 nm dalga boyunda pikler tespit edilmiştir [24]. Enjeksiyon hacmi 10 µL ve akış hızı 1,0 mL/dk olarak ayarlanmıştır. Arktiin, alıkonma süresi eşleştirilerek tanımlanmış ve ekstre de aynı koşullar altında analiz edilmiştir.

***İn vitro* Sitotoksisite Testleri**

Hücre Kültürü

Sitotoksisite deneyleri için, iki farklı insan meme kanseri hücre hattı (MCF-7 ve MDA-MB-231) ve kontrol olarak insan normal fibroblast hücre hattı (MRC-5) kullanılmıştır. MDA-MB-231 hücreleri MCF-7 hücrelerine kıyasla daha yüksek metastatik özelliğe sahip triple-negatif meme kanseri (TNBC) hücrelerdir [25]. Bu iki hücre hattının sitotoksik aktiviteleri doza bağımlı olarak karşılaştırılmıştır. Hücreler ATCC'den alınmıştır. Tüm hücre dizileri, %10 Fetal Sığır Serumu (FBS), %1 L-glutamin ve %1 antibiyotik (penisilin/streptomisin) ile takviye edilmiş yüksek glukoz içeren Dulbecco's Modified Eagle's Medium'da (DMEM) kültüre edilmiştir. İnkübasyon, 37 °C ve %5 CO_2 atmosfer ortamında gerçekleştirilmiştir. Hücreler sub-konfluent tutulmuştur ve ortam iki günde bir değiştirilmiştir.

Hücre Canlılık Analizi

MCF-7, MDA-MB-231 ve MRC-5 hücreleri, %0,25 Tripsin/EDTA (Gibco) ile flaskdan çıkarılarak hücre canlılığı deneyleri için 96 siyah kuyucuklu plaklara (Corning) $1,2x10^4$ hücre/çukur yoğunluğunda ekim yapılmıştır. Ön denemeler sonucu 0,5 mg/mL altında hücre ölümü gözlemlenmesi ve 5 mg/mL konsantrasyonda ise hücre canlılığının %1'in altına düşmesi sebebiyle analizde

uygulanacak ekstrenin konsantrasyon aralığı 0,5-4 mg/mL olarak belirlenmiştir. Gece boyunca yapışmayı takiben hücreler, tek başına ortam ile inkübe edilmiş veya farklı ekstre konsantrasyonuna (0,5; 1; 1,25; 1,5; 1,75; 2; 2,5; 3; 4 mg/mL) maruz bırakılmıştır. Stok solüsyonu hazırlamak için *A. minus* ssp. *minus* ekstresi, DMSO içerisinde çözündürülmüş ve tam DMEM ortamı kullanılarak art arda seyreltme yapılmıştır. 48 saatlik muameleden sonra, her bir kuyucuğa CellTiter Blue reaktifi (Promega) eklenmiş ve hücre canlılığı analizi üretici firmanın protokolüne göre gerçekleştirilmiş, floresan okuyucu SpectraMax i3x MultiMode Detection Platform ile belirlenmiştir. Her koşul için hücre canlılığı ölçümü, sadece medyumla muamele edilen hücrelere göre standardize edilip yapılmıştır. Üç bağımsız deney, üçer defa yapılmıştır. Her hücre için %50 inhibitör konsantrasyon (IC_{50}) değerleri GraphPad Prism sürüm 9.1 programı ile hesaplanmıştır.

Hücre Ölümü Değerlendirmesi

Ekstreye maruz kalan hücre apoptozunu tespit etmek için propidium iyodür (PI) kullanılmıştır. Hücre ölümünün değerlendirilmesi için her üç hücre hattında da ölüm başlatan ve hücreler arasındaki farkların gözlenebileceği ortak bir konsantrasyon belirlenmesi amaçlanmıştır. Üç hücre hattının ortalama IC_{50}'sine (1,31 mg/mL) en yakın değer olan 1,25 mg/mL dozu tercih edilmiştir. MCF-7, MDA-MB-231 ve MRC-5 hücrelerine, 1,25 mg/mL'lik bir konsantrasyonda *A. minus* ssp. *minus* ekstresi ile muamele edilmiş veya kontrol olarak çıkarılmıştır. Hücreler, muameleden 48 saat sonra toplanmış ve 1 x 10^6 hücre/mL konsantrasyonunda FACS tamponu içinde süspanse edilmiştir. 100 µL hücre süspansiyonu, 5 µL 100 µg/mL PI ile karanlıkta, oda sıcaklığında 15 dakika boyunca inkübe edilmiştir. İnkübasyon döneminden sonra hücreler, 1 saat içinde FACS AriaII flow sitometrisi ile analiz edilmiştir ve FlowJo yazılımı kullanılarak işlenmiştir [26].

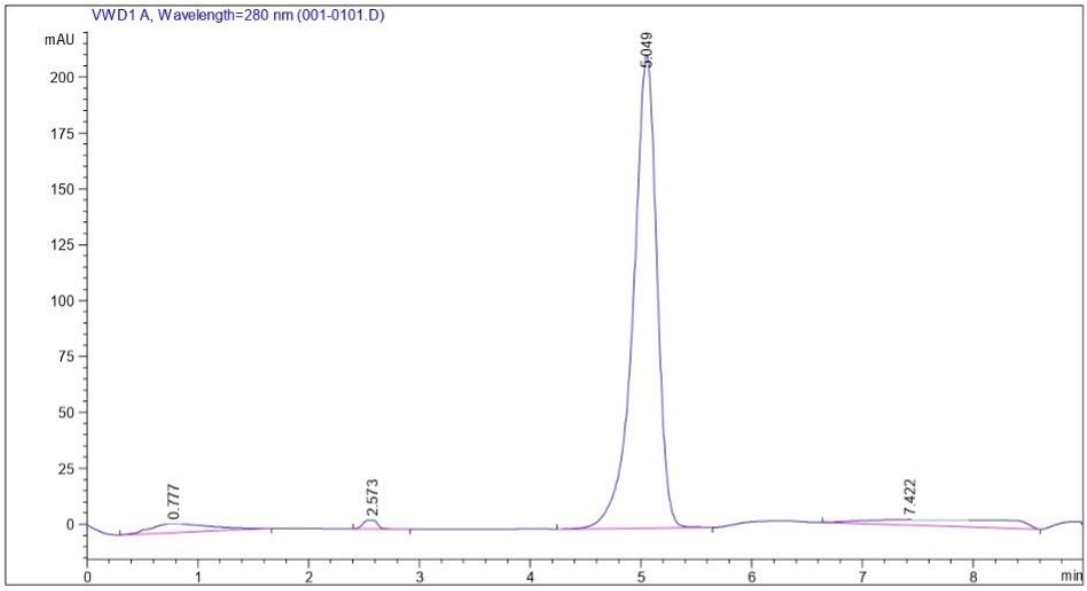
İstatistiksel Analiz

Tüm analizler üç tekrar olarak yapılıp sonuçların ortalaması alınmıştır. İstatistiksel analiz GraphPad Prism sürüm 9.1 programı ile hesaplanmış ve One-Way ANOVA ve Tukey post-hoc testi kullanılmıştır. $p<0,05$ değerlerindeki bir fark istatistiksel olarak anlamlı olarak değerlendirilmiştir (*$p<0,05$, **$p<0,01$, ***$p<0,001$).

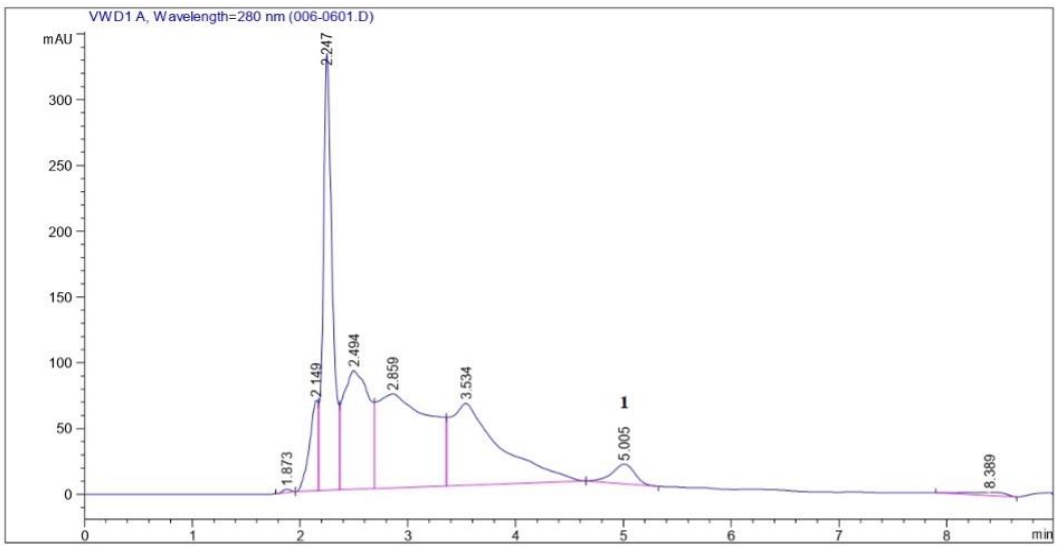
SONUÇ VE TARTIŞMA

A. minus ssp. *minus'* un toprak üstü kısımlarından arktiin analizi ilk kez bu çalışmada yapılmış olup *in vitro* sitotoksik aktiviteleri araştırılmıştır. *A. minus* ssp. *minus*'un toprak üstü kısımlardan hazırlanan metanollü ekstrenin fitokimyasal bileşen analizi, HPLC tekniği ile gerçekleştirilmiştir. Yapılan analizlerde ekstre içerisinde var olan arktiin varlığı ortaya konulmuştur. Daha önceki çalışmalarda farklı *Arctium* türlerinde türe özgü sekonder metabolit olan arktiin farklı yöntemlerle analiz edilmiştir [24,27,28]. Ancak bu çalışma, bu kapsamda *A. minus* ssp. *minus* ile yapılan ilk çalışmadır. *A.*

minus ssp. *minus* için arktiin standart kromotogramı Şekil 1 ‘de verilmiştir. Bu çalışmada *A. minus* ssp. *minus* toprak üstü kısımlarının metanol ekstrelerine HPLC ile arktiin analizi yapılmıştır (Şekil 2). Şekil 1’de elde edilen kromatogramda yer alan pikin Şekil 2’e verilen kromatogramda da yer alması ekstre içerisindeki arktiin varlığını göstermektedir. Arktiin daha önce sitotoksik etkisi ortaya konulmuş bir bileşiktir [29]. Bu nedenle bu çalışmada da arktiin içeriği açısından zengin bir ekstrenin antikanser etkisi araştırılmış ve sonuçlar literatürle bağlantılı olarak tartışılmıştır.

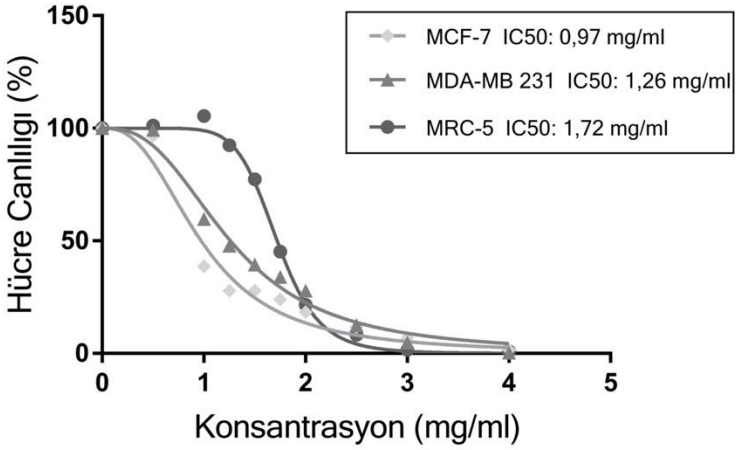


Şekil 1. Arktiin'in Standart Kromatogramı (R.T.:5.049)



Şekil 2. *A. minus* ssp. *minus* ekstresinin HPLC Kromatogramı (1: Arctin RT:5.005)

A. minus ssp. *minus* ekstresi uygulamasının, meme kanseri hücrelerini (MCF-7 ve MDA-MB-231) duyarlı hale getirip getiremeyeceği araştırılmıştır. *A. minus* ssp. *minus* ekstresinin antikanser etkilerini analiz etmek için hücre canlılığı deneylerine kontrol hücre hattı olarak MRC5 hücre hattı dahil edilmiştir. *A. minus* ssp. *minus* ekstresi için gerçekleştirilen hücre canlılığı deneylerinde, MCF-7 hücreleri (0,97 mg/mL) ve MDA-MB-231 hücreleri (1,26 mg/mL), kontrol grubu MRC5 hücrelerine (1,72 mg/ mL) göre daha düşük IC_{50} değeri göstermiştir (Şekil 3). Ek olarak, MCF-7 hücrelerinin aksine, MDA-MB-231 hücrelerinin oldukça agresif, istilacı ve çeşitli kanser önleyici ajanlara dirençli TNBC hücre hattı olduğu bilinmektedir. [30].



Şekil 3: *A. minus* ssp. *minus* ekstresinin MCF-7 ve MDA-MB-231 ile MRC5 hücreleri üzerinde sitotoksik etkileri ve hücrelerin IC_{50} değerleri

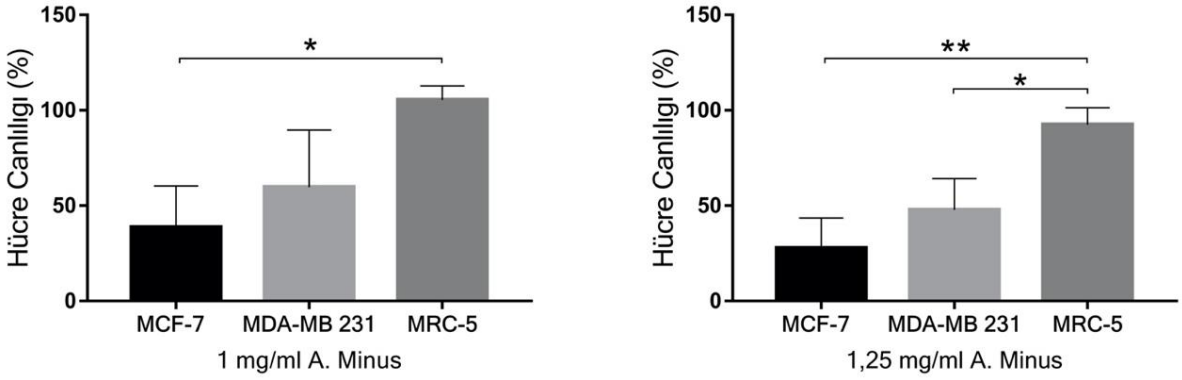
Tablo 1. Farklı hücre hatları üzerinde *A. minus* ssp. *minus* ekstresinin hücre canlılığına etkisi

***A. minus* ssp. *minus* ekstresi konsantrasyonu (mg/ml)**	**Hücre Canlılığı *(%, Ort ± SS)***		
	MRC-5	**MDA-MB 231**	**MCF-7**
0	100,00 ± 0,00	100,00 ± 0,00	100,00 ± 0,00
0,5	101,24 ± 5,48	99,26 ± 1,56	96,64 ±1,89
1	105,39 ± 7,47	59,73 ± 30,00	38,68 ±21,67
1,25	92,42 ± 8,90	47,77 ± 16,42	27,83 ±15,69
1,5	77,31 ± 12,26	39,50 ± 5,69	27,79 ±6,58
1,75	45,18 ± 24,37	33,88 ± 6,94	23,91 ± 4,17
2	21,60 ± 28,53	27,79 ± 8,32	18,31 ± 6,48
2,5	8,22 ± 12,27	12,57 ± 18,83	11,71 ± 15,05
3	1,57 ± 0,70	4,77 ± 7,18	6,84 ± 9,47
4	0,96 ± 0,26	0,36 ± 0,82	1,57 ± 0,97

3 farklı hücre hattının farklı *A. minus* ssp. *minus ekstre* konsantrasyonlarındaki canlılık oranları (%) *Ortalama ± Standart sapma* şeklinde gösterilmiştir. Tüm ortalama ve standart sapmalar 3'er tekrarlı 3 farklı deneyden elde edilmiştir. Tüm değerler ekstre verilmeyen kontrol gruplarına göre normalize edilmiştir. En yüksek konsantrasyondaki çözücü (DMSO) miktarının dahi toksik etkisi olmadığı yapılan denemelerde belirlenmiştir.

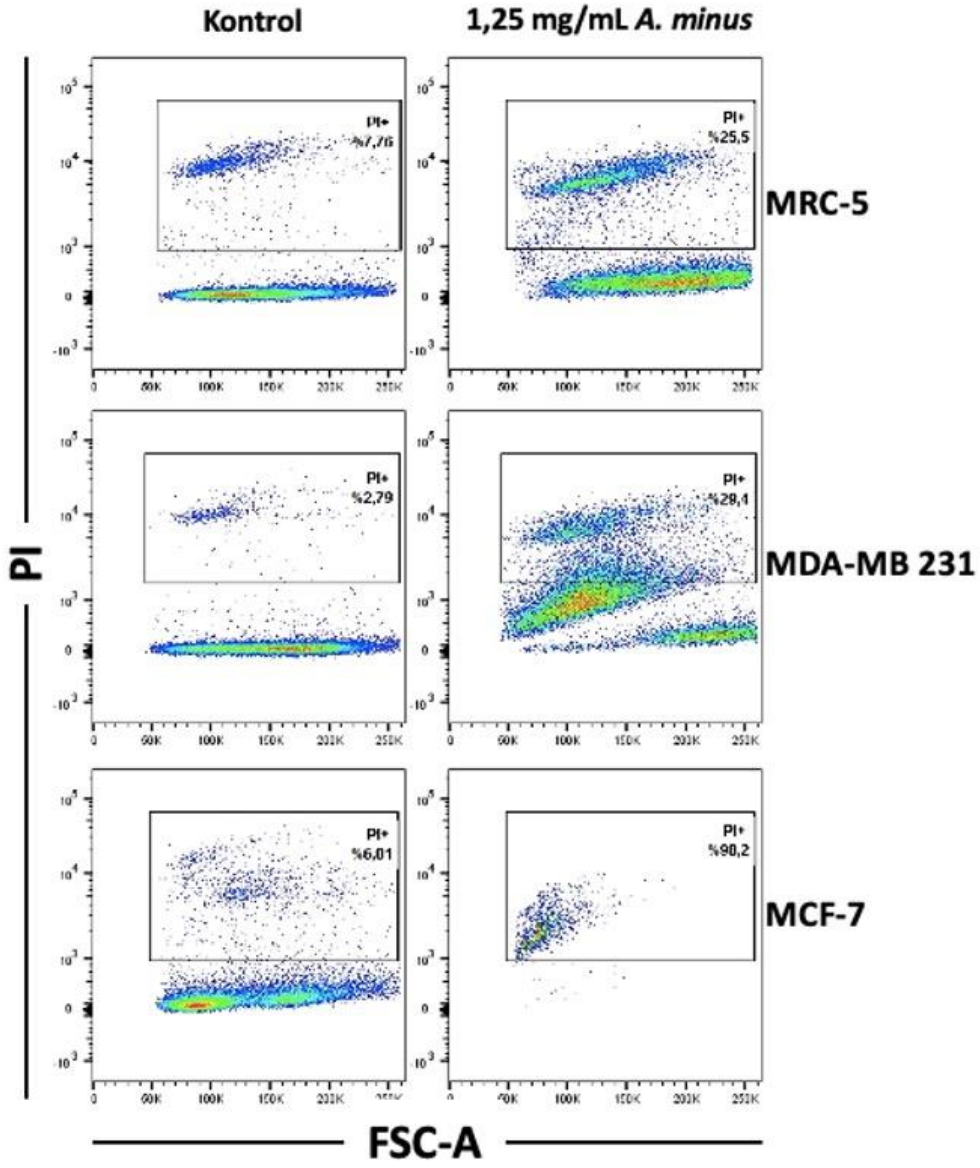
1 mg/mL *A. minus* ssp. *minus* ekstresi uygulaması, MCF-7 hücrelerinde %38,7 (en düşük) oranında hücre canlılığı ile sonuçlanırken, MDA-MB-231 hücrelerinde %59,7 oranında hücre canlılığı ile sonuçlanmıştır. Ayrıca ekstre uygulaması sonucu görülen en yüksek hücre canlılığı, MRC5 hücre hattında %105,4 oranında belirlenmiştir. 1,25 mg/mL konsantrasyonda *A. minus* ssp. *minus* ekstresi uygulaması ise sırasıyla, MCF-7 hücrelerinde %27,8 (en düşük) hücre canlılığı ile sonuçlanırken, MDA-MB-231 hücre hattında %47,8 oranında hücre canlılığı tespit edilmiştir. Ekstre uygulaması sonucu MRC5 hücre hattı %92,4 oranı ile en yüksek hücre canlılığı tespit edilen hücre hattıdır (Tablo 1).

MRC5 hücre hattı, MCF-7 hücre hattının IC_{50} değerinden yaklaşık iki kat daha yüksek olan 1,72 mg/mL IC_{50} değeri göstermiştir (Şekil 3). Bu nedenle MRC-5 hücre hattına 1,25 mg/mL ekstre ile muamele edildiğinde hücre canlılığında önemli bir azalma görülmemiştir. 1 mg/mL *A. minus* ssp. *minus* ekstresi uygulaması sonucu MCF-7 ve MRC-5 hücre hatlarının hücre canlılığı değerlendirildiğinde anlamlı bir fark gözlemlenmiştir ($p<0,022$). 1,25 mg/mL *A. minus* ssp. *minus* ekstresi uygulaması sonucunda MCF-7 ve MDA-MB-231 hücre hatları için hücre canlılığı kontrol grubu ile kıyaslandığında anlamlı bir fark gözlemlenmiştir (MRC-5 ile MCF-7 için $p< 0,0033$ ve MRC-5 ile MDA-MB-231 için $p< 0,0191$) (Şekil 4). Hücre ölümünün değerlendirilmesinde her üç hücre hattının ortalama IC_{50}'sine (1,31 mg/mL) en yakın değer olan 1,25 mg/mL dozu tercih edilmiştir. Bu bulgulardan yola çıkarak, MCF-7 ve MRC5 hücrelerine 48 saat boyunca 1,25 mg/mL *A. minus* ssp. *minus* ekstresi ile muamele edilmiştir. Hücre ölümünün flow sitometrisi ölçümü PI boyaması ile gerçekleştirilmiştir. *A. minus* ssp. *minus* uygulaması ile hücre ölümü, MCF-7 hücrelerinde (%98) MRC5 hücrelerine göre (%25) çok daha yüksek oranda artmıştır (Şekil 5).



Şekil 4. *A. minus* ssp. *minus* metanol ekstresinin farklı hücre hatlarında sitotoksisite analizi

Veriler, kontrole kıyasla ortalama ± SS olarak ifade edildi. İstatistiksel olarak anlamlı fark *$p<0,05$, **$p<0,01$, ***$p<0,001$ kabul edildi.



Şekil 5. 1,25 mg/mL *A. minus* ssp. *minus* ekstresi uygulamasının MRC-5, MDA-MB 231 ve MCF-7 hücre hatlarında flow stimoterisi ölçümü

Kanser, kesin tedavisinin henüz bulunmamış olması ve insidansının her geçen gün artması sebebiyle sık çalışılan ve ciddi bütçeler ayrılan bir araştırma konusudur. Kadınlarda en sık görülen kanser türü olması ve yüksek mortalite profili sebebiyle meme kanseri tedavisi oldukça önemlidir. Meme kanseri çalışmalarında dikkate alınması gereken en önemli özelliklerden biri hastalığın hücresel düzeyde heterojen olması ve farklı moleküler alt tiplerinin bulunmasıdır [31,32]. TNBC; tüm meme kanseri türleri arasında en agresif olanı olduğundan tedavisi daha zordur ve hastada yayılma olasılığı daha yüksektir [33]. *A. minus* ssp. *minus* metanol ekstresinin, meme kanseri modeli MCF-7 ve MDA-MB-231 hücre dizileri üzerindeki sitotoksik etkileri doza bağlı olarak araştırılmıştır.

Günümüzde mevcut çalışmalar doğrultusunda *A. minus* 'un antidiyabetik, hepatoprotektif, gastroprotektif, antibakteriyel, antiviral, antimikrobiyal, antialerjik ve antinflamatuar etkileri bilinmektedir [5]. Bununla birlikte, kanser hücrelerine karşı *A. minus* ssp. *minus* ekstresinin sitotoksisitesi yeterince çalışılmamıştır. Çalışmamızda *A. minus* ssp. *minus* metanol ekstresi uygulanması sonucu meme kanserinin farklı hücre dizilerinde *in vitro* sitotoksik etkileri değerlendirilmiştir. *İn vitro* sitoksisite deneylerimize göre, *A. minus* ssp. *minus* ekstresinin 1,25 mg/mL metanol ekstresi ile muamele edildiğinde hücre canlılığı sonuçları MCF-7 hücrelerinde %27,8 (en düşük), MDA-MB-231 hücrelerinde %47,8 ve kontrol grubu MRC5 hücre hattında ise %92,4 olarak belirlenmiştir. Bu bulgular, *A. minus* ssp. *minus* ekstresinin MCF-7 meme kanseri hücre hatları üzerinde ciddi sitotoksik etkileri olduğunu, ancak istilacı ve dirençli MDA-MB-231 meme kanseri hücreleri için sitotoksik etkisinin çok daha düşük oranda olduğunu göstermiştir. Ayrıca *A. minus* ssp. *minus* ekstresinin sitotoksik etkisinin normal fibroblast hücrelerinde kanser hücre hatlarına kıyasla oldukça düşük olduğu gözlemlenmiştir. Bu bağlamda *A. minus* ssp. *minus* ekstresinin MCF-7 hücre hattı için umut verici bir tedavi olabileceği, kanserin diğer moleküler alt tipleri için ise daha fazla çalışma yapılması gerektiği söylenebilir. Bu çalışmanın, *A. minus* ssp. *minus* ekstresinin antikanser etkisine dikkat çekeceğini, bu bitkinin antikanser etkileri araştırılmak üzere *in vivo* hayvan deneyleri ve klinik çalışmalara öncülük edeceğini düşünmekteyiz.

YAZAR KATKILARI

Kavram: *A.A.S.Ş, M.E.O*; Tasarım: *A.A.S.Ş, M.E.O.*; Denetim: *A.A.S.Ş, M.E.O;* Kaynaklar: *Y.Y.B, A.E.K.; A.A.S.Ş, M.E.O.;* Malzemeler: *Y.Y.B., A.E.K.;* Veri Toplama ve/veya işleme: *Ş.Ç., E.A., Y.Y.B., A.E.K.;* Analiz ve/veya yorumlama: *A.A.S.Ş., Y.Y.B., M.E.O.;* Literatür taraması: A.E.K., *E.A., Ş.Ç.;* Makalenin yazılması*: E.A., A.A.S.Ş.;* Kritik inceleme: *A.A.S.Ş., M.E.O., Y.Y.B., E.A., A.E.K., Ş.Ç.;* Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

ETİK KURUL ONAYI

Yazarlar bu çalışma için etik kurul onayının zorunlu olmadığını beyan etmektedir.

KAYNAKLAR

1. Futreal, P. A., Kasprzyk, A., Birney, E., Mullikin, J. C., Wooster, R., Stratton, M. R. (2001). Cancer and genomics. *Nature*, *409*(6822), 850-852. [CrossRef]

2. Global Cancer Observatory Web Site. (2020) From: https://gco.iarc.fr/today/home Erişim Tarihi: 28.04.2022

3. Banik, K., Harsha, C., Bordoloi, D., Lalduhsaki Sailo, B., Sethi, G., Leong, H. C., Arfuso, F., Mishra, S., Wang, L., Kumar, A. P., Kunnumakkara, A. B. (2018). Therapeutic potential of gambogic acid, a caged xanthone, to target cancer. *Cancer Letters*, *416*, 75-86. [CrossRef]

4. Top, R., Erden, Y., Tekin, S. (2019). Bazı önemli tıbbi bitkilerin antioksidan ve antikanser etkilerinin araştırılması. *Bitlis Eren Üniversitesi Fen Bilimleri Dergisi*, *8*(2), 435-442. [CrossRef]

5. Wang, D., Bădărau, A. S., Swamy, M. K., Shaw, S., Maggi, F., da Silva, L. E., López, V., Yeung, A. W. K., Mocan, A., Atanasov, A. G. (2019). Arctium Species Secondary Metabolites Chemodiversity and Bioactivities. *Frontiers in Plant Science*, *10*, 834. [CrossRef]

6. Avrupa Fitoterapi Bilimsel Kooperatifi-Bitkisel Tıbbi Ürünler Bilimsel Vakfı (2016). Arctii radix (Burdock Root) üzerine ESCOP Monografisi. From: http://escop.com/downloads/arctii/ Erişim Tarihi: 28.04.2022

7. Kupicha FK. (1975). Arctium minus (Hill) Bernh. Içinde *Flora of Turkey and the East Aegean Islands*.

8. Washino, T., Yoshikura, M., Obata, S. (1986). New Sulfur-containing Acetylenic Compounds from Arctium lappa. *Agricultural and Biological Chemistry*, *50*(2), 263–269. [CrossRef]

9. Xiao, W., Chuange, C., Yutao, Y., Chengchao, Z. (2004). Analysis of the essential oil from Arctium lappa L. *Natural Product Research and Development*, *16*(1), 33–35.

10. Washing, T., Kobayashi, H., Ikawa, Y. (1987). Structures of Lappaphen-a and Lappaphen-b, new guaianolides linked with a sulfur-containing acetylenic compound, from Arctium lappa L. *Agricultural and Biological Chemistry*, *51*(6), 1475-1480.

11. Lin, S., Chung, T., Lin, C., Ueng, T.-H., Lin, Y., Lin, S., Wang, L. (2000). Hepatoprotective Effects of Arctium Lappa on Carbon Tetrachloride- and Acetaminophen-Induced Liver Damage. *The American Journal of Chinese Medicine*, *28*(02), 163-173. [CrossRef]

12. Ahangarpour, A., Heidari, H., Oroojan, A. A., Mirzavandi, F., Nasr Esfehani, K., Dehghan Mohammadi, Z. (2017). Antidiabetic, hypolipidemic and hepatoprotective effects of Arctium lappa root's hydro-alcoholic extract on nicotinamide-streptozotocin induced type 2 model of diabetes in male mice. *Avicenna Journal of Phytomedicine*, *7*(2), 169-179. [CrossRef]

13. Altundağ, E., Ozturk, M. (2011). Ethnomedicinal studies on the plant resources of east Anatolia, Turkey. *Procedia - Social and Behavioral Sciences*, *19*, 756-777 [CrossRef]

14. Tuzlacı, E., Erol, M. K. (1999). Turkish folk medicinal plants. Part II: Eğirdir (Isparta). *Fitoterapia*, *6*, 593-610. [CrossRef]

15. Güner, A., Aslan, S., Ekim, T., Vural, M., Babaç, M. T. (2012). *Türkiye Bitkileri Listesi (Damarlı*

Bitkiler). Nezahat Gökyiğit Botanik Bahçesi ve Flora Araştırmaları Derneği.

16. Lubian, C. T., Teixeira, J. M., Lund, R. G., Nascente, P. S., Del Pino, F. (2010). Antifungal activity of the aqueous extract from Arctium minus (Hill) Bernh. (Asteraceae) on oral Candida species. *Brazilian Journal of Medicinal Plants*, *12*(2), 157-162. [CrossRef]

17. Altay, V., Karahan, F. (2017). Medicinal Plants Used to Sunstroke and Sunburn Treatment in Anatolian Traditional Medicine. *Erzincan University Journal of Science and Technology, 2017*, 124–137. [CrossRef]

18. Fujita, T., Sezik, E., Tabata, M., Yesilada, E., Honda, G., Takeda, Y., Tanaka, T., Takaishi, Y. (1995). Traditional medicine in Turkey VII. Folk medicine in middle and west Black Sea regions. *Economic Botany*, *49*(4), 406. [CrossRef]

19. European Medicines Agency web site (2011). From: https://www.ema.europa.eu/documents /herbal-monograph/final-community-herbal-monograph-arctium-lappa-l-radix_en.pdf Erişim Tarihi 03.03.2021

20. Erdemoglu, N., Turan, N. N., Akkol, E. K., Sener, B., Abacioglu, N. (2009). Estimation of anti-inflammatory, antinociceptive and antioxidant activities of Arctium minus (Hill) Bernh. ssp. minus. *Journal of Ethnopharmacology*, *121*(2), 318-323. [CrossRef]

21. Fischer, S. P. M., Brusco, I., Camponogara, C., Piana, M., Faccin, H., Gobo, L. A., de Carvalho, L. M., Oliveira, S. M. (2018). Arctium minus crude extract presents antinociceptive effect in a mice acute gout attack model. *Inflammopharmacology*, *26*(2), 505-519. [CrossRef]

22. Boldizsár, I., Füzfai, Z., Tóth, F., Sedlák, E., Borsodi, L., Molnár-Perl, I. (2010). Mass fragmentation study of the trimethylsilyl derivatives of arctiin, matairesinoside, arctigenin, phylligenin, matairesinol, pinoresinol and methylarctigenin: their gas and liquid chromatographic analysis in plant extracts. *Journal of Chromatography. A*, *1217*(10), 1674-1682. [CrossRef]

23. Predes, F. S., Ruiz, A. L. T. G., Carvalho, J. E., Foglio, M. A., Dolder, H. (2011). Antioxidative and in vitro antiproliferative activity of Arctium lappa root extracts. *BMC Complementary and Alternative Medicine*, *11*(1), 25. [CrossRef]

24. Zhou, X., Zhang, H., Ge, L., Gong, H., Tian, S. (2011). Determination of Arctiin and Arctigenin Contents in Arctium Tomentosum Mill. by HPLC Method. *Journal of Chemistry*, *8*, S372-S376. [CrossRef]

25. Eroğlu Köksal, E. (2019). Yüksek Lisans Tezi. *Meme Kanserinde Doğal Biyoaktif Bileşenlerin Antikanser Etkilerinin Karşilaştirmali Araştırılması*. Yıldırım Beyazıt Üniversitesi, Ankara, Türkiye.

26. Riccardi, C., Nicoletti, I. (2006). Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nature Protocols 1*, 1458–1461. [CrossRef]

27. Ge, L., Liu, F., Hu, Y., Zhou, X. (2020). Qualitative and quantitative analysis of arctiin and arctigenin in Arctium tomentosum Mill. by high-performance thin-layer chromatography. *JPC - Journal of Planar Chromatography - Modern TLC*, *33*, 1–8. [CrossRef]

28. Liu, S., Chen, K., Schliemann, W., Strack, D. (2005). Isolation and identification of arctiin and arctigenin in leaves of burdock (Arctium lappa L.) by polyamide column chromatography in combination with HPLC-ESI/MS. *Phytochemical Analysis : PCA*, *16*(2), 86–89. [CrossRef]

29. Lee, C.-Y., Hsin, M.-C., Chen, P.-N., Lin, C.-W., Wang, P.-H., Yang, S.-F., Hsiao, Y.-H. (2022). Arctiin Inhibits Cervical Cancer Cell Migration and Invasion through Suppression of S100A4 Expression via PI3K/Akt Pathway. *Pharmaceutics*, *14*(2), 365. [CrossRef]

30. Gest, C., Joimel, U., Huang, L., Pritchard, L.-L., Petit, A., Dulong, C., Buquet, C., Hu, C.-Q., Mirshahi, P., Laurent, M., Fauvel-Lafève, F., Cazin, L., Vannier, J.-P., Lu, H., Soria, J., Li, H., Varin, R., Soria, C. (2013). Rac3 induces a molecular pathway triggering breast cancer cell aggressiveness: differences in MDA-MB-231 and MCF-7 breast cancer cell lines. *BMC Cancer*, *13*, 63. [CrossRef]

31. Libson, S., Lippman, M. (2014). A review of clinical aspects of breast cancer. *International Review of Psychiatry (Abingdon, England)*, *26*(1), 4–15. [CrossRef]

32. Weigelt, B., Peterse, J. L., Van 't Veer, L. J. (2005). Breast cancer metastasis: markers and models. *Nature Reviews. Cancer*, *5*(8), 591–602. [CrossRef]

33. LaPorta, E., Welsh, J. (2014). Modeling vitamin D actions in triple negative/basal-like breast cancer. *The Journal of Steroid Biochemistry and Molecular Biology*, *144 Pt A*, 65–73. [CrossRef]

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 755-763, 2022
Doi: 10.33483/jfpau.1115035

ORIGINAL ARTICLE / ÖZGÜN MAKALE

A CROSS-SECTIONAL STUDY: PERCEPTION AND CONSUMPTION BEHAVIOR ABOUT IMMUNITY BOOSTER *SAMBUCUS* SP. PRODUCTS (AZERBAIJAN, GEORGIA, UZBEKISTAN)

IMMUN GÜÇLENDİRİCİ SAMCUBUS TÜRLERİ İÇEREN ÜRÜNLERİN ALGISI VE TÜKETİMİ HAKKINDA KESİTSEL BİR ÇALIŞMA (AZERBAYCAN, GÜRCISTAN, ÖZBEKISTAN)

Melek ULUSOYLU-DUMLU[1*]

[1]Asfarma International Pharma Marketing, Istanbul, Turkey

ABSTRACT

Objective: *The aim of this cross-sectional study was to measure and determine the difference between perception and consumption changes of elderberry products in Azerbaijan (AZ), Georgia (GEO) and Uzbekistan (UZB) countries.*

Material and Method: *A questionnaire based, cross-sectional, multi country study was conducted by using an online based with consumers of pharmacies, drug stores and shops where had food supplement category between April and August 2021.*

Result and Discussion: *The majority of responders belong to 31-50 age group, and higher gender profile was on female part, 85%. The results showed that end users of consumed products were children (51%) and older family members (39%) in the average data of 3 countries. Elderberry products were classified as expensive products but were consumed high amount during the pandemic. During the pandemic, immune boosting had been in prior place of individual healthcare, and prophylactic approach had become more important than earlier. This is the first study in Azerbaijan, Georgia and Uzbekistan as a digital survey based, cross- sectional study.*

Keywords: *Behaviour, coronavirus, immune booster, Sambucus sp., social media*

ÖZ

Amaç: *Bu kesitsel çalışmanın amacı Mürver içerikli ürünlerin Azerbaycan (AZ), Gürcistan (GEO), Özbekistan (UZB) ülkelerindeki algısı ve tüketimindeki değişimi belirlemek ve ölçmektir.*

Gereç ve Yöntem: *Anket temelli, kesitsel, çoklu ülkede, dijital ortamda; eczane ve gıda takviyesi ürünleri kategorisi bulunan eczanelerin, sağlık ürünleri mağazalarının tüketicileri ile, 2021 yılının Nisan ve Ağustos ayları arasında gerçekleştirilmiştir.*

Sonuç ve Tartışma: *Ankete katılanların büyük kısmı 31-50 yaş aralığında olup ve %85'lik kısmını kadınlar oluşturmaktaydı. 3 ülkenin ortalama verilerine göre ürünlerin nihai kullanıcıları çocuklar (%51) ve yaşlı aile üyeleridir (%39). Mürver içeren ürünler pandemi süresinde yüksek miktarda tüketilmelerine ragmen, pahalı*

* **Corresponding Author / Sorumlu Yazar:** Melek Ulusoylu-Dumlu
e-mail / e-posta: ulusoylumelek@hotmail.com, **Phone / Tel.:** +90 5335024789

Submitted / Gönderilme: 10.05.2022 **Accepted / Kabul:** 05.07.2022

ürünler olarak sınıflandırılmıştır. Pandemi döneminde, immun sistemi güçlendirmek bireysel sağlıkta önceliklenmiş ve daha önceki dönemlere göre önleyici yaklaşımlar daha önemli hale gelmiştir. Bu çalışma Azerbaycan, Gürcistan ve Özbekistan'da gerçekleştirilmiş, dijital anket temelli, kesitsel ilk çalışmadır.

Anahtar Kelimeler: *Davranış, immun güçlendirici, koronavirus, Sambucus sp, sosyal medya*

INTRODUCTION

The *Sambucus* genus consists of 5 to 30 species, mostly in the Northern hemisphere, including study countries, although they have become naturalized throughout much of the temperate and subtropical regions. Because its fruits are highly desirable for birds, elderberry rapidly colonizes moist and sunny areas along railways, roadways, forest edges, and fence lines [1]. Various parts of the elderberry have been used in traditional medicine as a diaphoretic, diuretic, astringent, laxative, and emetic. The berries were traditionally used in nutrition, such as elderberry wine and pies, and as a flavoring or dye. Currently, extracts of the berries are used primarily as antiviral agents for colds, influenza and *Herpes* virus infection. Also, several clinical trials have shown that symptoms of influenza like sneezing and fever were quickly treated with elderberry including products which are both commercially produced or home recipes [2-5]. The effects of the plant and/or parts of plants come from its main natural components, which are flavonoids, catechins and proanthocyanidins. Generally, these compound classes are accepted as natural immunostimulators. Scientific literature also emphasizes that the potent antioxidant activity of the plant is related to these phenolic compounds [6-8].

Currently, elderberry is not only on scientific researchs' target, but also has gained therapeutic popularity in the commercial market over herbal supplements. The commercial products of the plant are in the top recommendations in pharmacy practices, and digital platforms, daily life person to person in consumer practices. Elderberry is a well-known medicinal plant. Elderberry use is rising in immune boosting due to the products registered as food supplements in different pharmaceutical forms and combinations. In many countries, elderberry extracts are combined with *Pelargonium* extracts, *Echinacea* extracts, Vitamin C, Zinc and Vitamin D.

The awareness of elderberry, mostly flowers of the plant, is very high in those countries. Elderberry has already been placed in their traditional medicine and daily life. Elderberry included products are available in herbal market of countries. The product portfolio range is highly extended from medicinal tea to honey. In the pandemic, some medicinal plants have been re-launched to markets for their medicinal properties with novel marketing tactics [9-10].

The study aimed the determine consumption and perception profile of those 3 countries of medical plants and their products from a known elderberry point of view.

MATERIAL AND METHOD

This is a questionnaire based, cross-sectional, multi country study. The study was conducted by using an online based with consumers of pharmacies, drug stores and shops where had food supplement category between April and August 2021 in Azerbaijan, Georgia and Uzbekistan. The period of study was identified just after the winter in period of the pandemic for to gather total perception and consumption information, after all passed diseases and their treatments in that time.

The questionnaire contained 19 questions regarding the socio-demographic profile of medicinal plant consumption, knowledge of elderberry, choice of immunity booster if there has any, source of information and/or recommendation regarding the immunity support of the participant etc. The form consisted of yes or no answers and multiple-choice questions.

The inclusion criteria were to be a customer (a random consumer - in food supplement category was established pharmacies, drug stores and shops) in targeted locations and the ability to understand and answer online Google form questionnaires. Personal information was not requested for participation. Only google sign-in was kept compulsory to ensure that respondents would fill out the form only one time and confidentiality of the data was assured. The language of the questionnaire was simple and written in Russian. The response related to statistics gathered from the system and analyzed with the use of Excel software. The data was exported to SPSS (Version 17). Descriptive statistics such as Chi-square, t-test, Pearson and Exact test were used for analysis. p values < 0.05 were considered as statistically significant in all conducted analysis [11-13].

RESULT AND DISCUSSION

Questionnaires were distributed over digital platforms in each country, out of which 771 (296, 121, 354 Azerbaijan, Georgia, Uzbekistan respectively) responses were obtained and included to the study. The responses were shown in below Tables.

Demographic profile of population

The majority of total responders, 85% were female and 15% were males. 83% were university degree and postgraduates. The maximum number of responders was in 31-50 age group (Table 1). The mean age of responders was 40.76 (SD ± 7.44).

Immunity booster consumption profile before and during the pandemic

The main part of responders, 90%, were no COVID-19 infected during the period of the pandemic outbreak. The percentage was calculated from personal statements, not cross-checked with public

personal healthcare results. There was no correlation between herbal product consumption and COVID-19 infection. The detailed consumption profile is shown below.

"During illness" period consumptions were not changed before or in the pandemic in total data. The drastic change was on "Never" responder side. Before the pandemic 49% of responders were in "never" consumer but, during the pandemic the number decreased to 3.11%, ($p<0.05$). Besides, "everyday" and "twice a day" consumer percentages were increased. Everyday rate was increased from 0.5% to 13.62%. The rate of "twice a week" was increased from 0.9% to 34.5%, ($p<0.05$). This outcome showed that herbal product consumption was raised in the pandemic for immunity boosting and prophylaxis (Table 2). In the pandemic "everyday" and "twice a week" consumption rates were drastically increased in Azerbaijan; because of this, "During illness" consumption rates were significantly decreased (Table 2). Triggering of consumption was not related to COVID-19 infection because there is no correlation between COVID-19 infected responders' rate and increased consumption.

Table 1. Socio-demographic profile of population

Variables	AZE (n)	GEO (n)	UZB (n)	Total (n)	Percentages (%)
Age					
18-20	3	2	2	7	0.85%
21-30	50	24	43	117	15.18%
31-50	148	68	269	485	62.95%
51-60	86	23	36	145	18.81%
>60	9	4	4	17	2.20%
Gender					
Female	255	90	315	660	85.60%
Male	41	31	39	111	14.40%
Education					
Till high school	20	10	86	116	15.05%
High school	90	31	95	216	28.01%
University	160	74	150	384	49.80%
Master or PhD	26	6	23	55	7.14%

Differentiation on awareness of herbal product consumption

This study showed that the outbreak of COVID-19 pandemic increased the awareness of herbal product consumption. The results indicated that recommendations were taken from multiple sources. The main advisors were healthcare professionals (HCPs) (Doctor and Pharmacist). 84.57% of responders were taken advice from HCPs ($p<0.05$) (Table 3).

Table 2. The consumption changing profile of herbal products outbreak of the pandemic

	Variables	AZE(n)	GEO(n)	UZB(n)	Total (n)	Percentages (%)
herbal product consumption *before* the pandemic	Everyday	2(1.54) [0.14]	1(0.63) [0.22]	1(1.84) [0.38]	4	0.51%
	Twice a week	5(2.69) [1.99]	1(1.10) [0.01]	1(3.21) [1.53]	7	0.90%
	During illness	148(146.66) [0.01]	78(59.95) [5.43]	156(175.39) [2.14]	382	49.54%
	Never	141(145.12) [0.12]	41(59.32) [5.66]	196(173.56) [2.90]	378	49.02%
herbal product consumption *during* the pandemic, if any	Everyday	57(40.31) [6.91]	13(16.48) [0.73]	35(48.21) [3.62]	105	13.62%
	Twice a week	147(102.12) [19.72]	27(41.75) [5.21]	92(122.13) [7.43]	266	34.50%
	During illness	87(144.35) [22.79]	78(59.01) [6.11]	211(172.64) [8.52]	376	48.77%
	Never	5(9.21) [1.93]	3(3.77) [0.16]	16(11.02) [2.25]	24	3.11%

Table 3. Consume advisor profile

Variables	AZE (n)	GEO (n)	UZB (n)	Total (n)	Percentages (%)
Doctor	95(116.33) [3.91]	48(47.55) [0.00]	160(139.12) [3.13]	303	39.30%
Pharmacist	146(133.99) [1.08]	60(54.77) [0.50]	143(160.24) [1.86]	349	45.27%
Family Member	37(26.11) [4.55]	6(10.67) [2.05]	25(31.22) [1.24]	68	8.81%
Friend	18(19.58) [0.13]	7(8.00) [0.13]	26(23.42) [0.29]	51	6.62%

The primary end users were children (50.97%). Old family members were on the second line as 39.43%, $p<0.05$, Table 4.

Table 4. "Become consumer" end user profile in family members

Variables	AZE (n)	GEO (n)	UZB (n)	Total (n)	Percentages (%)
Children	170(150.88) [2.42]	69(61.68) [0.87]	154(180.44) [3.88]	393	50.97%
Wife/Husband	21(24.57) [0.52]	11(10.04) [0.09]	32(29.39) [0.23]	64	45.27%
Old Family Members	102(116.71) [1.85]	39(47.71) [1.59]	163(139.58) [3.93]	304	8.81%
Others	3(3.84) [0.18]	2(1.57) [0.12]	5(4.59) [0.04]	10	6.62%

Elderberry perception

Elderberry - Black Elderberry - Бузина чёрная (belongs to *Sambucu*s sp.) is a well-known plant. Meanwhile, during the pandemic, people became more fragile to increasing immune system with "novel" recommendations. Specifically, elderberry related perception was questioned in survey. Responders received information, recommendations and advice from social media about the registered (as food supplement), marketed, and "immune booster" products containing elderberry. The primary information sources were social media 40.96% and HCPs 38.26%, $p<0.05$.

During this period, the frequency of digital advertising dramatically increased. The results indicated that social media advice, advertisements, informative blogs, etc. positively influenced perception development. Consumers followed social media advice much more than traditional advice, as seen in Table 5.

Table 5. Source of information for perception development

Variables	AZE (n)	GEO (n)	UZB (n)	Total (n)	Percentages (%)
Family	36(43.77) [1.38]	22(17.89) [0.94]	56(52.34) [0.26]	114	14.78%
HCPs	99(113.26) [1.79]	46(46.30) [0.00]	150(135.45) [1.56]	295	38.26%
Social Media	155(121.32) [9.35]	47(49.59) [0.14]	114(145.09) [6.66]	316	40.96%
Do not know	6(17.66) [7.70]	6(7.22) [0.21]	34(21.12) [7.85]	46	6.00%

Preferred consumption of elderberry

Consumption preference of natural immunity booster agents/supplements/medicinal plants are still in developing from the ancient time to date. The most familiar way that to be a follower of the family heritage. The dilemma of today is traditional remedies/recipes and modern supplements. Table 6 indicates that the majority of the population (66%) preferred to use "elderberry" as a food/herbal supplement.16% of the population has been using the plant as food. The main gathered information from this is that even being a well-known medicinal plant, preference for consumption has been derived from the pharmaceutical form of supplements.

Table 6. Preferred consumption type of elderberry

Variables, as	AZE(n)	GEO(n)	UZB(n)	Total(n)	Percentages (%)
dried food	6	4	4	14	1.82%
food	65	37	18	120	15.56%
home remedy	56	18	57	131	17%
pharmaceutical food supplement	169	62	275	506	65.63%

The ratios between willingness to use after the pandemic and affordability perception

Most of the responders were not dedicated chronic-long term users of elderberry products. Also, the responders almost agreed on high priced profile of the products. There was no significant association between education and age for willingness and affordability. Another main outcome was that willingness responders were 100% female.

The use of food/herbal/dietary supplements has increased significantly in the last decade. Both in developed and developing countries, healthcare providers have suffered from raised pharmaceutical expenditures. A governmental reimbursement system is not common in many parts of the world, even if there is no coverage for food supplements, known as supportive care agents or called "anonymous" therapeutics in some countries. In all parts of the world, societies deeply understood that prophylactic

approaches are very important to be on the safe-side for wellbeing [14]. Phytopharmaceuticals, food supplements, vitamin-mineral combinations have potential from this point of view.

This multi country based, cross-sectional study, investigated pandemic related immune booster use and re-profiled elderberry perception and consumption.

The study demonstrated that the majority of responders belong to 31-50 age group in all 3 countries (63%). And, gender profile also had higher ratio on female part (average 86%, 74%, 89% Azerbaijan, Georgia and Uzbekistan respectively). The results have shown that the pandemic enormously increased consumption of herbal products. Habitual consumption was on during the illness (cold, flu, etc.) period before the pandemic (50%, 64%, 44% Azerbaijan, Georgia and Uzbekistan respectively). Still, during the pandemic the average ratios were changed such as every day 13%, twice a week 34.50% and illness period 48.77%. The main outcomes of this result; immune boosting had been in prior place of individual healthcare and prophylactic approach had become more important than earlier. The mentioned above ratio was almost in same pattern in each country ($p < 0,05$).

The results also indicated that buyer or payer of the products was not always “consumer”. The median age is 40.76, who have more responsibility for children and aged family members. The results showed that the consumer of products was 51% for children 39% for older family members in the average of 3 countries’ data ($p < 0,05$). However, elderberry products were classified as high priced but consumed in high volume during the pandemic.

A variety of supplements are utilized during the pandemic, use of any nutritional-food-dietary supplements remains the traditional experience of medicinal herbs. Future investigation should be into the effects of food supplements on supportive care and whether optimal supplementation strategies for immune boosting are required.

In COVID-19 pandemic, science literature and key opinion leaders underline the importance of having a strong immune system. This well known, heritage information proved its again and again in the pandemic waves in many parts of the world. This study is the first investigation in those countries where still follow habitual recipes in their daily life to be healthy. The mentioned cultures ensured the engagement of family-based attitudes to protection of diseases. Today, countries realize that the protective advantages of traditional folk medicine, and the novel forms-dosages of food supplements with their known ingredients are being involved in daily life. They are asked to engage in preventive practices to protect against possible diseases; exploring their perceptions is important to orient them towards this change from traditional to current perspective.

ACKNOWLEDGEMENTS

The author is grateful for market access of the survey to Asfarma Azerbaijan, Georgia and Uzbekistan marketing team members. The company has no *Sambucus* extract included product in the period of study in those markets.

AUTHOR CONTRIBUTIONS

Concept: *M.U.D;* Design: *M.U.D*; Control: *M.U.D*; Sources: *M.U.D*; Materials: *M.U.D.*; Data Collection and/or processing: *M.U.D.*; Analysis and/or interpretation: *M.U.D.*; Literature review: *M.U.D*; Manuscript writing: *M.U.D.*; Critical review: *M.U.D*; Other:-

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The study was approved by the Farmatsevtika Tarmog'ini Rivojlantirish Agentligi ethical committee (No: 00/88-344, Date: 10.03.2021).

REFERENCES

1. Schmitzer, V., Veberic, R., Stampar, R. (2012). European elderberry (*Sambucus nigra* L.) and American Elderberry (*Sambucus canadensis* L.): Botanical, chemical and health properties of flowers, berries and their products. Chapter VI, In Berries: Properties, Consumption and Nutrition; C. Tuberoso (Ed), (p.127-144). New York: Nova Science Publishers Inc.

2. Tiralongo, E., Wee, E.S, Lea, A.R. (2016). Elderberry supplementation reduces cold duration and symptoms in air-travellers: A randomized, double-blind, placebo-controlled clinical trial. *Nutrients Nutrients, 8*, 182-197. [CrossRef]

3. Porter, S.R., Bode, F.R. (2017). A review of the antiviral properties of Black Elder (*Sambucus nigra* L.) Products. *Phytotheraphy Research*, *31*(4), 533- 554. [CrossRef]

4. Wieland, L.S., Piechotta, V., Feinberg, T., Ludeman, E., Hutton, B., Kanji, S., Seely, D. (2021). Elderberry for prevention and treatment of viral respiratory illnesses: a systematic review. *BMC Complementary Medicine and Therapies, 21*, 112-127. [CrossRef]

5. Kinoshita, E., Hayashi, K., Katayama, H., Hayashi, T., Obata, A. (2012). Anti-Influenza virus effects of elderberry juice and its fractions. *Bioscience, Biotechnology. and Biochemistry*, *76*(9), 1633-1638. [CrossRef]

6. Lee, J., Finn, C.E. (2007). Anthocyanins and other polyphenolics in American Elderberry (*Sambucus canadensis*) and European Elderberry (*S. nigra*) cultivars. *Journal of the Science of Food and Agriculture*, *87*(14), 2665-2675. [CrossRef]

7. Veberic, R., Jakopic, J., Stampar, F., Schmitzer, V. (2009). European Elderberry (*Sambucus nigra* L.) rich in sugars, organic acids, anthocyanins and selected polyphenols. *Food Chemistry*, *114*(2), 511-515. [CrossRef]

8. Ferreira, S.S., Silva, P., Silva, A.M., Nunes, F.M. (2020). Effect of harvesting year and Elderberry cultivar on the chemical composition and potential bioactivity: A three-year study. *Food Chemistry*, *302*, 125345-125366. [CrossRef]

9. Adams, K.K., Baker, W.L., Sobieraj, D.M. (2020). Myth Busters: Dietary supplements and COVID-19. *Annals of Pharmacotherapy*, *54*(8), 820-826. [CrossRef]

10. Salamon, I. (2021). Can medicinal plants help in the treatment of the new coronavirus? Some R&D aspects in Slovak Republic. *Current Perspectives on Medicinal and Aromatic Plants*, *4*(1), 58-65. [CrossRef]

11. Nanavaty, D.P., Sanghvi, A.A., Mehta, M.S., Gupta, S., Dumra, G. (2020). A cross-sectional study for determining the perception and preferences of immunity boosters for protection against COVID-19. *International Journal of Advances in Medicine*, *7*(10), 1498-1505. [CrossRef]

12. Wode1, K., Henriksson, R., Sharp, L., Stoltenberg, A., Nordberg, J.H. (2019). Cancer patients' use of complementary and alternative medicine in Sweden: A cross-sectional study. *BMC Complementary and Alternative Medicine, 19,* 62-65. [CrossRef]

13. Fuad, F.N.Y., Ching, S.M., Dzulkarnain, D.H.A., Cheong, A.T., Zakaria, Z.A. (2020). Complementary alternative medicine use among postpartum mothers in a primary care setting: a cross-sectional study in Malaysia. *BMC Complementary Medicine and Therapies*, *20*, 197-206. [CrossRef]

14. Khabour, O.F., Hassanein, S.F.M. (2020). Use of vitamin/zinc supplements, medicinal plants, and immune boosting drinks during COVID-19 pandemic: A pilot study from Benha city, Egypt. *Heliyon*, *7*(3), e06538. [CrossRef]

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 764-780, 2022
Doi: 10.33483/jfpau.1137486

ORIGINAL ARTICLE / ÖZGÜN MAKALE

PREPARATION AND CHARACTERIZATION OF COMBINED SALICYLIC ACID AND POVIDONE-IODINE CONTAINING NANOEMULGELS: A PRELIMINARY STUDY

KOMBİNE SALİSİLİK ASİT VE POVİDON-İYOT İÇEREN NANOEMÜLJELLERİN HAZIRLANMASI VE KARAKTERİZASYONU: ÖN ÇALIŞMA

Rukiye SEVİNÇ ÖZAKAR[1*], Şeyma ASAN[2], Azra Elisa ÖZKAN[2], Emrah ÖZAKAR[1]

[1]Atatürk University, Faculty of Pharmacy, Department of Pharmaceutical Technology, 25240, Erzurum, Turkey

[2]Atatürk University, Faculty of Pharmacy, 25240, Erzurum, Turkey

ABSTRACT

Objective: *The aim of this preliminary study is to prepare and characterize combined salicylic acid and povidone-iodine-containing nanoemulgels for use in disease models such as wounds and burns in the future.*

Material and Method: *Within the scope of the study, first of all, analytical method validation of salicylic acid was performed. Then, oil solubility studies were carried out and nanoemulsions and nanoemulgels were prepared. Morphology, zetasizer analysis, type and pH determination, FTIR analysis, spreadability, and in vitro release studies were performed to determine the characterization of the formulations.*

Result and Discussion: *Nanoemulsions and nanoemulgels have been prepared successfully. Nanoemulsions with spherical droplet structure and outer phase water were obtained, and their morphology and zeta sizer results were compatible. In the 1-month stability study, only the F1 formulation did not decompose. There was not much change in pH after holding. At the end of the FTIR analysis, it was seen that there was no interaction between the items. In the release study performed with pH 5.5 phosphate buffer, approximately 40% of the release occurred after 8 hours. This study is a preliminary study, and formulations with long-term stability and release rate can be developed by conducting more detailed studies in the future. Salicylic acid and povidone-iodine were used in combination for the first time. This combination can be translated into formulations that may be beneficial for skin diseases in the future.*

Keywords: *Characterization, nanoemulgel, povidone-iodine, salicylic acid, skin diseases*

* **Corresponding Author / Sorumlu Yazar:** Rukiye Sevinç Özakar
e-mail / e-posta: rukiyeso@atauni.edu.tr, **Phone / Tel.:** +904422315247

Submitted / Gönderilme: 29.06.2022 **Accepted / Kabul:** 19.07.2022

ÖZ

Amaç: *Bu ön çalışmanın amacı, gelecekte yapılması planlanan yara ve yanık gibi hastalık modellerinde kullanılmak üzere kombine salisilik asit ve povidone-iyot içeren nanoemüljeller hazırlamak ve karakterize etmektir.*

Gereç ve Yöntem: *Çalışma kapsamında öncelikle salisilik asitin analitik yöntem validasyonu yapılmıştır. Daha sonra yağda çözünürlük çalışması yapılıp, nanoemülsiyonlar ve nanoemüljeller hazırlanmıştır. Hazırlanan formülasyonların karakterizasyonunu belirlemek için morfoloji, zetasizer analizi, tip ve pH tayini, FTIR analizi, yayılabilirlik ve in vitro salım çalışmaları yapılmıştır.*

Sonuç ve Tartışma: *Nanoemülsiyonlar ve nanoemüljeller başarıyla hazırlanmıştır. Küresel damlacık yapısına sahip dış fazı su olan nanoemülsiyonlar elde edilmiştir ve morfolojileri ile zetasizer sonuçları uyumlu çıkmıştır. Yapılan 1 aylık stabilite çalışmasında sadece F1 formülasyonunda ayrışma gerçekleşmemiştir. Bekletme sonrasında pH'larda fazla değişim olmamıştır. FTIR analizi sonunda maddeler arasında etkileşimin olmadığı görülmüştür. pH 5.5 fosfat tamponuyla yapılan salım çalışmasında 8 saat sonunda yaklaşık % 40 oranında salım gerçekleşmiştir. Bu çalışma bir ön çalışma olup ilerde daha detaylı çalışmalar yapılarak uzun süreli stabiliteye ve salım oranına sahip formülasyonlar geliştirilebilir. Salisilik asit ve povidon-iyot ilk defa kombine halde kullanılmıştır. Bu kombinasyon gelecekte cilt hastalıkları için faydalı olabilecek formülasyonlara dönüştürülebilir.*

Anahtar Kelimeler: *Cilt hastalıkları, karakterizasyon, nanoemüljel, povidon-iyot, salisilik asit*

INTRODUCTION

Human skin diseases are one of the most important public health problems, and there has been a severe increase in skin diseases recently. Between 30 and 70% of people worldwide are affected by these problems, which are the most common reason for consultation in general practice. More than 3000 skin diseases, both acute and chronic, have been described, affecting people of all age groups [1, 2]. Since the history of ancient medicine, the skin has been the oldest organ widely used in administering many drugs. Similarly, in modern medical practices in recent years, dermal drug delivery provides an alternative to oral drug delivery and makes essential contributions to health services [3]. The dermal delivery of drugs has always been both attractive and challenging to research. Advances in modern technologies allow dermal delivery of both hydrophobic and hydrophilic small molecule and large molecule drugs. Dermal drug administration, which is a comfortable and painless way for patients, has many advantages compared to other administration ways, making it one of the most preferred. Among the important advantages are the avoidance of hepatic first-pass metabolism and the gastrointestinal tract for drugs with low bioavailability [4].

Dermal drug delivery systems can come in different physical forms, from liquid to powder, and the most popular of these are preparations in semi-solid forms, such as creams and ointments. Among semi-solid products, the use of gels has increased considerably in both cosmetic and pharmaceutical preparations. Gels are dosage forms formed by entrapping large amounts of aqueous or hydroalcoholic liquid in a colloidal network that can be created using natural or synthetic polymers. In particular, they have a high water content that allows the active ingredients to dissolve more. Compared to ointment or cream bases, gels enable the active ingredient to dissolve easily through the liquid carrier because of

having a higher aqueous component. In addition, gels are superior in terms of ease of application and patient compliance. However, gels show a significant limitation in delivering hydrophobic active substances. Therefore, studies on different gel types have increased recently to overcome these limitations. Especially with the inclusion of oil-containing systems into gels, different dosage forms have started to be developed. Among the various nanolipoidal delivery systems such as solid lipid nanoparticles, liposomes, microemulsions, and nanoemulsions, which have a very important place among the new generation oil-containing systems, especially nanoemulsions are among the most successful delivery systems for lipophilic and hydrophilic active substances applied in various ways, including the topical route [3, 5].

Nanoemulsions are oil-in-water, or water-in-oil biphasic dispersions of two immiscible liquids stabilized using a suitable surfactant and they can typically be formed with less surfactant than other colloidal dispersions and have more excellent kinetic stability properties than coarse emulsions [6]. Loading poorly water-soluble drugs into nanoemulsions increases their wettability and/or solubility, improving their pharmacokinetics and pharmacodynamics by different routes of administration. The advantages of nanoemulsions such as optimum drug release, long-term efficacy, drug intake control, low side effects, and drug protection from enzymatic or oxidative processes have been reported in recent years [7, 8].

Nanoemulgels are emulsion-based topical gel formulations in which nano-sized emulsion droplets are gelled by adding a suitable gelling agent. Because nanoemulgels contain both nanoemulsion and gel base, they are among the suitable options as drug delivery systems. The nanoemulsion component of the nanoemulgel protects the active substance from enzymatic degradation and reactions such as hydrolysis, and the gel base provides thermodynamic stability to the emulsion by increasing the viscosity of the aqueous phase by reducing the interface and surface tension. In the presence of suitable penetration enhancers, the droplet size in nano form can increase the formulation's effectiveness by improving the permeability and spreadability of the drug [3, 9].

In this study, salicylic acid (SA) and povidone-iodine (PI) were used as active ingredients. SA is a natural ingredient derived from the bark of the willow tree (*Salix alba*). It has been used worldwide for centuries for its analgesic, antipyretic and anti-inflammatory properties. SA is highly irritating to the gastric mucosa when taken orally, so topical use is preferred. The absorption of SA in the topical application is variable. The systemic effects of SA in topical applications are minimal when applied in low to moderate doses to intact skin. However, if there is deterioration in the structure of the stratum corneum, measurable levels of SA may be present in the body. SA can be used topically as a keratolytic, bacteriostatic, fungicide, and photoprotective. Today, it is frequently used to treat warts, calluses, localized hyperkeratosis, plaque psoriasis, actinic keratosis, ichthyosis, and comedonal acne [10]. PI is

a complex formed with iodine with antiseptic properties and povidone, a synthetic carrier polymer that does not have microbicidal activity. In an aqueous medium, free iodine is released from the PI complex into the solution. The antiseptic activity increases and iodine release continues until an equilibrium is established [11]. PI is also a broad-spectrum antiviral agent against enveloped and non-enveloped viruses such as adenovirus, rotavirus, rhinovirus, human immunodeficiency virus, herpes virus, and measles, polio, rubella, measles, and influenza viruses [12]. This preliminary study aims to prepare and characterize combined SA and PI-containing nanoemulgels for use in disease models such as wounds and burns in the future. SA and PI were combined for the first time.

MATERIAL AND METHOD

Materials

SA was purchased from Riedel-de-Haën (Germany). Olive oil and mineral oil were purchased from Doğa İlaç (Turkey). Sesame oil, linseed oil (LSO), and ethanol were purchased from Sigma (Germany and USA). Sunflower oil and hexane were purchased from Hasyalçın Dış Tic. (Turkey) and J. T. Baker (Holland), respectively. Tween 20 (T20), Tween 60 (T60), Span 80 (S80), and polyethylene glycol (PEG) 400 were purchased from Merck (Germany). PI and hydroxypropyl methylcellulose (HPMC) E15 were kindly received as a gift from BASF (Turkey) and Santa Farma İlaç A.Ş (Turkey).

Development of Quantification Method for Salicylic Acid

A stock solution of SA in ethanol was prepared at a concentration of 100 µg/mL in a multipoint magnetic stirrer (2mag, MIX 15 eco, Germany). After finding the wavelength at which SA gave maximum absorbance, the absorbance of the series was measured in a UV-VIS spectrophotometer (Beckman Coulter DU 730, USA) by dilution from this stock solution. The calibration equation with the calibration curve was found, and then the method was validated. The validation parameters like accuracy, precision, LOD, LOQ, and selectivity were studied (n=6) [13].

Solubility of Salicylic Acid in Different Oils and Phosphate Buffer

The saturation solubility study of SA in different oils and pH 5.5 phosphate buffer was found by stirring in a magnetic stirrer for a long time at room temperature. For this purpose, sesame oil, olive oil, LSO, sunflower oil, and mineral oil were used. Concentrated suspensions of SA in oils/phosphate buffer (4 g) were prepared and stirred for 72 hours on a multipoint magnetic stirrer (1200 rpm) at room temperature. Afterward, the samples were centrifuged (Hettich Micro 200, Germany) at 12500 rpm for 30 min, and the supernatants were diluted at certain ratios with the validation medium and/or hexane. The amounts of dissolved SA were determined by the validated UV-VIS spectrophotometric method (n=3) [14].

Preparation of Salicylic Acid Nanoemulsions

SA-containing nanoemulsions were prepared by the ultrasonication method at room temperature. First, SA (50 mg) was dissolved at a certain ratio in the oil medium (1000 mg), where it was maximum soluble via the ultrasonicator (Bandelin Sonopuls HD 2070, Germany). Then, an appropriate surfactant/co-surfactant (T20 and S80 and/or PEG 400) was added and homogenized with the ultrasonicator. Finally, ultrapure water (1650 mg) was added to the homogeneous oil solution, and nanoemulsion formation was carried out with the ultrasonicator at cycle 3 and power of 100% for 4 minutes (minimum n=6) [15]. Nanoemulsions are coded with the letter E.

Morphology of Nanoemulsions

Morphology of nanoemulsions was determined by optical microscope (Zeiss Primo Star, Germany) and transmission electron microscopy (TEM, Hitachi HighTech HT7700, Japan). For TEM determination, nanoemulsions were dispersed in ultrapure water, and one drop of diluted nanoemulsions was placed on a 400-mesh carbon-coated copper grid. The grid was then dried at room temperature overnight. The TEM imaging was conducted at 120 kV. This analysis was carried out at the East Anatolian High Technology Research and Application Center (DAYTAM) of Atatürk University [14].

Zetasizer Analysis of Nanoemulsions

Droplet size distribution, zeta potential, polydispersity index, and conductivity of nanoemulsions were determined with the zetasizer device (Malvern Zetasizer Nano ZSP, United Kingdom). Nanoemulsions were diluted with ultrapure water at a ratio of 1:9. Zeta sizer measurements were taken in the DTS1070 cell at 25°C, and 3x12 measurements were taken with a measurement angle of 173° utilizing a laser of 633 nm and 10 mW. This analysis was carried out at the East Anatolian High Technology Research and Application Center (DAYTAM) of Atatürk University [16].

Type Determination of Nanoemulsions

Type determination of nanoemulsions was made according to the dilution method. Nanoemulsions were diluted with ultrapure water at a ratio of 1:9 on the watch glass [17]. The outer phase of the nanoemulsions, which form a homogeneous mixture with water and no phase separation is observed, was accepted as the aqueous phase.

Preparation of Nanoemulgels with Povidone-Iodine

Preformulation studies have been made with natural and synthetic polymers such as sodium alginate, pectin, and HPMC E15. As a result of the preformulation studies, it was decided to use HPMC E15. HPMC E15 (500 mg) was swollen in ultrapure water at room temperature. Then, a specific concentration of PI (100 mg) and T60 (400 mg) were added and mixed manually until homogeneous.

Then, nanoemulsions containing SA were added to these gel bases and mixed manually until homogeneous again (minimum n=6) [18]. Nanoemulgels are coded with the letter F. In order to determine the phase separation stability, the nanoemulgels were kept at room temperature and in the refrigerator (4±2 °C) for 1 month. The caps of the vials are tightly closed with parafilm in an airtight manner.

pH Determination of Nanoemulgels

The pH of the freshly prepared nanoemulsions and nanoemulgels was measured with a pH meter (WTW inoLab, Germany). When the nanoemulgels' pH was lower than 5.5, the pH was adjusted to 5.5 with NaOH solution. After the pH was adjusted to 5.5, the nanoemulgels were kept at room temperature and in the refrigerator for 1 month to determine the pH stability [14].

Spreadability of Nanoemulgels

The spreadability of the nanoemulgels was determined using two transparent plastic plates. For this purpose, a circle with a diameter of 1 cm was drawn inside one of the plates, and 0.5 g of each nanoemulgel was weighed on this circle. The second plate is placed on top of the first plate. Finally, a weight of 1 kg was placed on the upper plate and left for 5 minutes. The spreadability of the nanoemulgel was evaluated by measuring the increasing diameter at the end of time [19]. In order to determine the spreadability stability, the nanoemulgels were kept at room temperature for 1 month.

***In Vitro* Drug Release of Nanoemulgels**

The dialysis membrane method of Topal et al. was used for *in vitro* drug release studies. For this purpose, a dialysis membrane (MWCO: 20 kD, Spectra/Por®Biotech, USA), pH 5.5 phosphate buffer, and a shaking water bath (Memmert WNB 14, Germany) were used. pH 5.5 phosphate buffer was prepared by adding HCl into pH 5.8 phosphate buffer (USP 30/NF 25). The dialysis membrane was incubated in the release medium for 15 minutes. 0.5 g of accurately weighed nanoemulgels and 0.5 g of pure SA's were placed in the middle of the dialysis bags. Then, dialysis bags and 50 mL pH 5.5 phosphate buffer at 37±0.5 ºC were added to each amber vial. At the specified time intervals, 2 mL samples were taken, and the same amount of release medium was placed in the bottles to maintain the sink conditions. The amounts of SA in the samples were determined by the validated UV-VIS spectrophotometric method (n=3) [20].

FT-IR Analysis of Nanoemulgels

FT-IR spectra were taken to evaluate whether there is an interaction between the active ingredients (SA, PI), the excipients (LSO, S80, T20, T60, HPMC E15), and the nanoemulgels (F1-F6). This analysis was carried out at the East Anatolian High Technology Research and Application Center (DAYTAM) of Atatürk University [21].

Statistical Analysis

The statistical analysis between the samples were evaluated with the "One-Way Analyses of Variance (ANOVA)" test (according to the homogeneity of the variances and the size of the population). Results at the $p<0.05$ level were considered significant.

RESULT AND DISCUSSION

Development of Quantification Method for Salicylic Acid

The quantification method and validation studies for SA were successfully completed quickly and easily with the UV-VIS spectrophotometric method. The calibration curve, equation, and validation study results are given in Figure 1 and Table 1. The maximum absorbance was seen at 302 nm. The desired linearity was achieved, and the validation study was carried out within the desired limits. LOD and LOQ values were found to be 0.294 µg/mL and 0.892 µg/mL, respectively. In the study of Ahmad et al., in the presence of combined benzoic acid and SA in ethanol in a UV-VIS spectrophotometer, SA gave an absorbance at 303 nm [14]. In the validation study of Sinha et al. with methanol in a UV-VIS spectrophotometer, SA gave an absorbance at 301 nm, and linearity was obtained in the range of 1-10 µg/mL [22]. Our results were similar to the results of these studies.

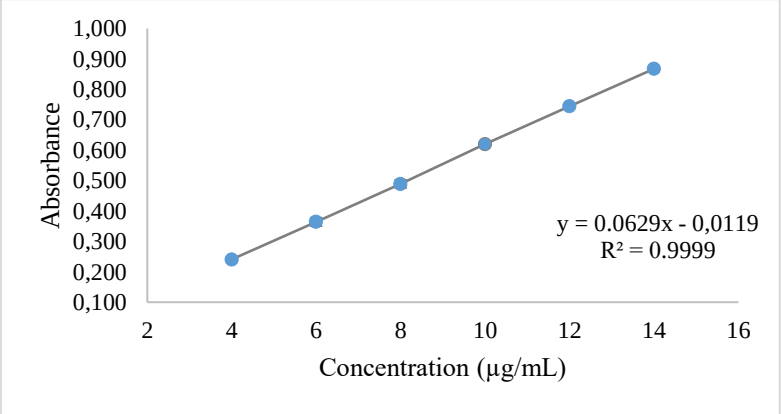


Figure 1. Calibration curve and equation of salicylic acid

Table 1. Validation study results of salicylic acid (mean±standard deviation)

	Theoretical Concentration (µg/mL)	Accuracy (% Relative Error)	Precision (% Variation Coefficient)
Inter-day	5	0.33±1.41	1.49±0.73
	9	0.79±0.77	0.99±0.38
	13	0.29±0.85	1.11±0.36
Intra-day	5	0.67±2.07	1.09±0.64
	9	1.71±0.23	1.02±0.75
	13	1.57±0.36	1.19±0.29

Solubility of Salicylic Acid in Different Oils and Phosphate Buffer

When preparing oil-containing formulations of water-insoluble active substances, it is essential that they are soluble in oil. For this reason, the solubility study was carried out to find the oil with the highest capacity to dissolve the SA which is insoluble in water. The solubility study results are given in Table 2 below. There are not many studies on the solubility of SA in oils in the literature. Many oils have been tested in the solubility study of SA in oils by Ashara et al. [23]. The study was carried out in a shaking incubator at 30 °C for 1 day. The results were evaluated by UV-VIS spectrophotometric method. Olive oil and mineral oil were used together in both our study and theirs. In Ashara et al.'s study, solubility results were obtained at 9.66±0.03 mg/g in olive oil, 2.5±0.07 mg/g in mineral oil, and 10.65±1.30 mg/mL and 0.56±0.03 mg/mL in our study, respectively. Although data showed similar results in olive oil, lower results were obtained in our study in mineral oil. However, when all the results were examined, the highest solubility value was found in LSO. For this reason, LSO was used as the oil phase in the preparation of nanoemulsions. Low solubility was also obtained in the solubility study of SA with pH 5.5 phosphate buffer. Similar low solubility data were obtained in the study of Teng et al., and the results were found to be 2.205±0.020 mg/mL in water and 5.208±0.010 mg/mL in pH 5.0 phosphate buffer at 25 °C [24].

Table 2. Solubility results of salicylic acid (mean±standard deviation).

Oil	**Sesame Oil**	**Olive Oil**	**Linseed Oil**	**Sunflower Oil**	**Mineral Oil**	**pH 5.5 Phosphate Buffer**
Solubility (mg/mL)	10.62±0.36	10.65±1.30	13.66±0.66	11.52±0.85	0.56±0.03	1.65±0.06

Preparation of Salicylic Acid Nanoemulsions

Many modifications have been made while preparing nanoemulsions. The ratios of the formulation components are given in Table 3 below. When SA studies in the literature are examined, it is seen that SA is used at ratios of 0.5% to 40% [10, 25, 26]. For this reason, the dose of SA (50 mg) in our study was chosen to be 1% in nanoemulgels. In our study, the use of dual surfactants was preferred, and Tween 20 and either Span 80 or PEG 400 were used. These surfactants are frequently used to form nanoemulsions [27, 28].

Table 3. Formulation components of nanoemulsions (mg)

Formulation Code	**Salicylic Acid**	**Linseed Oil**	**Tween 20**	**Span 80**	**PEG 400**
E1	50	1000	100	200	-
E2	50	1000	150	150	-
E3	50	1000	200	100	-
E4	50	1000	200	-	100
E5	50	1000	150	-	150
E6	50	1000	100	-	200

Morphology of Nanoemulsions

In addition, the optical microscope images of the nanoemulsions are given in Figure 3 below. As can be seen from the images, nanoemulsions with very homogeneous size distribution have been successfully prepared by the ultrasonication method.

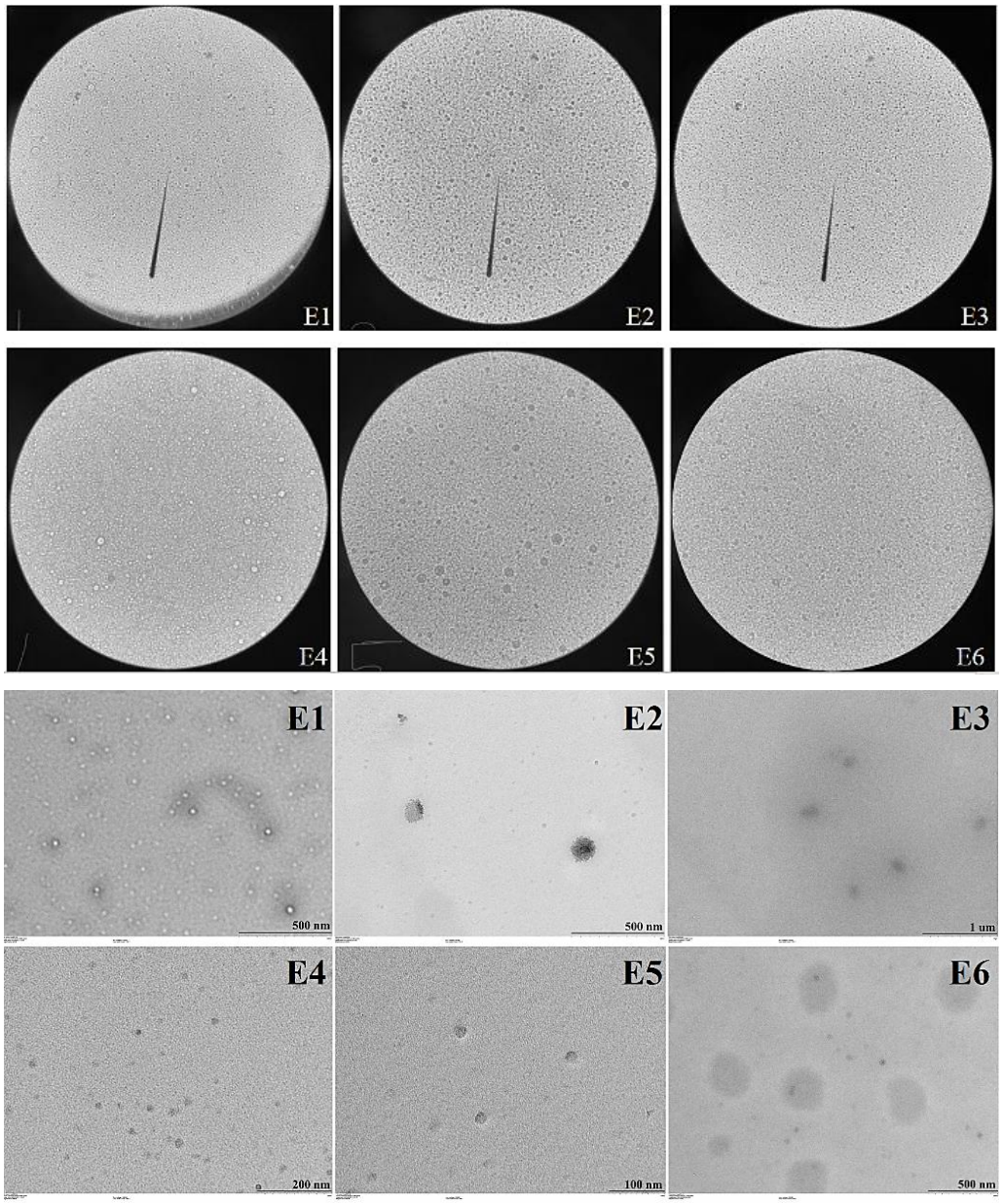


Figure 3. The optical microscope (scale: 100x) and TEM images of the nanoemulsions

Zetasizer Analysis of Nanoemulsions

The droplet size, zeta potential, polydispersity index, and conductivity results of the nanoemulsions are given in Table 4. Relatively small droplet sizes were obtained. When the droplet size

was evaluated statistically, E4, E5, and E6 were found to be significant (p<0.05). This significant difference was thought to be caused by PEG 400 found in E4- E5 and E6. Zeta potential determines the surface charge of nanoemulsions and it is very important for their physical stability [14]. When our zeta potential results were examined, our values were found in the range of (-)11.7±4.04-(-)23.5±3.08 mV. All our zeta potential values were negative. This is because non-ionic surfactants were used when developing formulations [29]. In a study by Acharya et al., nanoemulsions were developed using tweens. In the stability study conducted at room temperature and 2-8 °C for 3 months, it was stated that the stability of the formulations was quite good even at approximately -12.8 mV [14]. The highest zeta potentials were seen in the E3 and E4 formulations. When these formulations are evaluated in terms of content, it can be said that the amount of Tween 20 is the highest (200 mg) in both of them, and the increase in the amount of Tween 20 also increases the zeta potential. When the zeta potential values were evaluated statistically, E1, E3, and E4 were found to be significant (p<0.05). This significant difference was thought to be caused by Tween 20, which is high in E3 and E4, and Span 80, which is high in E1. PDI gives information about the width of the particle size distribution, and the large size distribution can cause stability problems. A PDI value of 0.1-0.25 indicates a narrow distribution, while a PDI value greater than 0.5 indicates an extensive size distribution [30, 22]. When our PDI results were examined, our values in the range of 0.181±0.019-0.228±0.006 were below 0.25, which means that the distribution is narrow. This result was also found to be compatible with the optical microscope and TEM images. Very high conductivity values were obtained in the range of 245±2-333±0 µS/cm. The high electrical conductivity values indicate that the outer phase of the nanoemulsions is water. Similar results were found in the nanoemulsion study of Arbain et al. [31].

Table 4. The droplet sizes, zeta potentials, polydispersity indexes, and conductivity results of nanoemulsions (mean±standard deviation)

Formulation Code	Droplet Size (nm)	Zeta Potential (mV)	Polydispersity Index	Conductivity (µS/cm)
E1	269.9±2.07	-11.7±4.04	0.199±0.016	333±0.00
E2	308.2±1.04	-17.5±5.47	0.207±0.005	283±2.08
E3	295.2±1.65	-23.5±3.08	0.228±0.006	245±2.08
E4	248.2±1.27	-23.0±1.71	0.182±0.006	292±2.52
E5	249.6±1.39	-15.2±3.90	0.181±0.019	318±3.61
E6	315.0±4.46	-17.8±3.59	0.195±0.012	291±3.00

Type Determination of Nanoemulsions

The images obtained by diluting the nanoemulsions with water are given in Figure 4. The fact that they are immediately miscible with water and a homogeneous mixture was obtained. This means that their outer phase is water. This result was also compatible with the electrical conductivity results.

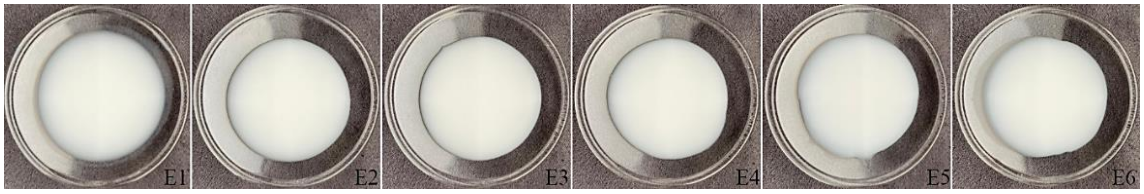


Figure 4. The images obtained by diluting the nanoemulsions with water

Preparation of Nanoemulgels with Povidone-Iodine

Nanoemulgels containing both SA and PI (100 mg) combined have been successfully prepared. The images of the nanoemulgels are given in Figure 2 below. When the PI studies in the literature are examined, it is seen that PI is generally used at the rate of 0.5% to 10% [12, 32, 33]. Therefore, in our study, the dose of PI was chosen as 2% in nanoemulgels. Since the color of PI was yellowish-brown/reddish-brown, the color of the nanoemulsion was as seen in the figure. Separation was observed in all nanoemulgels, except F1, which were subjected to 1-month stability at both room and refrigerator temperatures. The most separation was observed in F6 and decreased towards F2.

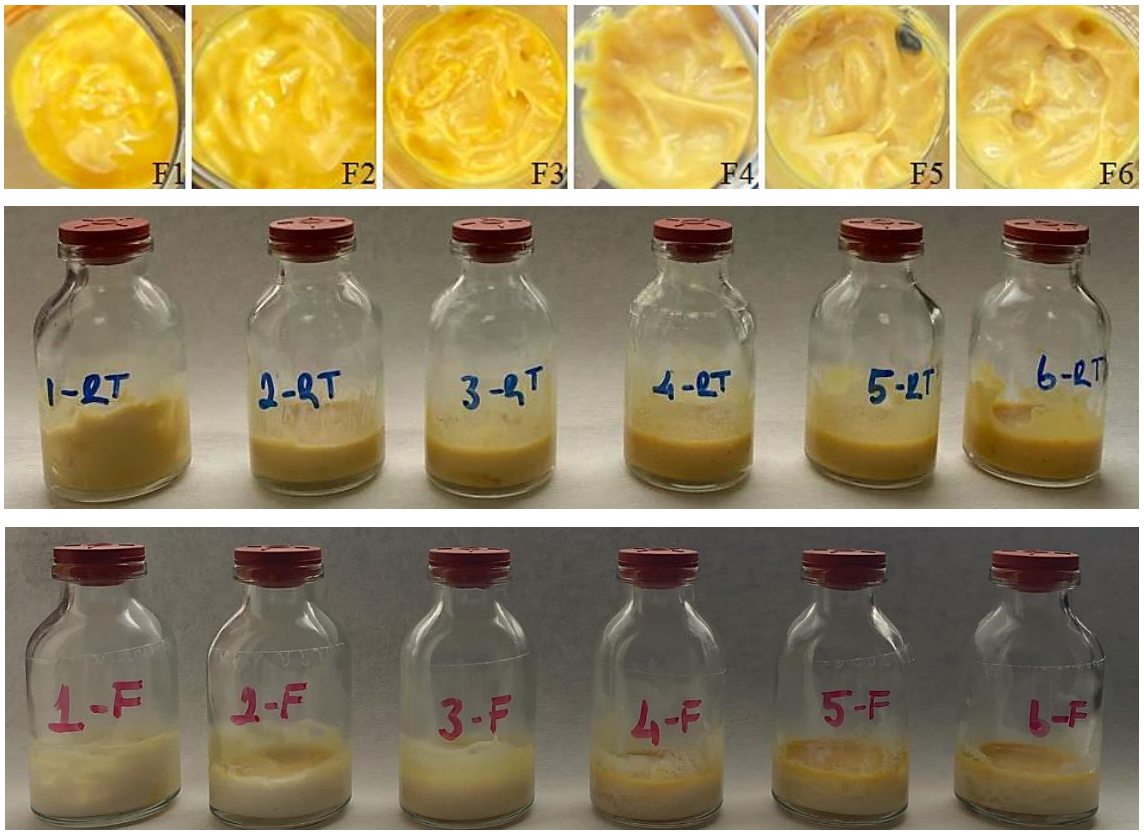


Figure 2. The images of freshly prepared (top) and kept for 1 month-nanoemulgels (RT: room temperature (middle) and F: refrigerator temperature (down))

pH Determination of Nanoemulgels

The pH measurement results of the nanoemulsions and nanoemulgels are given in Table 5. When the results are examined, it is seen that the pH's of both nanoemulsions and nanoemulgels are acidic. In addition, it was observed that the pH decreased more by gelling the nanoemulsions. The pH of the nanoemulgels was adjusted to 5.5 by the addition of NaOH and subjected to 1-month stability at room and refrigerator temperature. Normal skin pH is between 4.5 and 6.0. In the 1-month stability study, the pH values of the nanoemulgels were found in this range. Although separations were observed in most of the nanoemulgels after 1 month, the pH changes were not too much. There were no significant differences in pH between freshly prepared nanoemulsions and nanoemulgels, and nanoemulgels that were kept for 1 month at room and refrigerator temperatures ($p>0.05$).

Table 5. The pH measurement results of the nanoemulsions and nanoemulgels.

Formulation Code	Freshly Prepared-Room Temperature	Formulation Code	Freshly Prepared-Room Temperature	After 1 Month-Room Temperature	After 1 Month-Refrigerator Temperature
E1	2.80	**F1**	2.14	5.50	5.62
E2	2.78	**F2**	2.01	5.39	5.42
E3	2.76	**F3**	2.12	5.23	5.28
E4	2.68	**F4**	2.08	5.01	5.10
E5	2.64	**F5**	2.01	4.96	5.08
E6	2.62	**F6**	2.05	4.82	4.90

Spreadability of Nanoemulgels

Spreadability results of nanoemulgels which are freshly prepared and kept for 1 month are given in Table 6. Nanoemulgels are easily spreadable by applying a small force. When all the results were examined, it was observed that the spread was more in the F2 and F5 formulations where surfactants were used equally. On the contrary, these formulations showed minimal spread after 1 month of storage. It is thought that the reason for this is the higher water loss in these formulations. Similar results were seen in the study by Sharma and Tailang [34].

Table 6. Spreadability results of nanoemulgels which are freshly prepared and kept for 1 month.

Formulation Code	Spreadability (cm)	
	Freshly Prepared	After 1 Month-Room Temperature
F1	2.9	3.1
F2	4.1	2.5
F3	3.4	3.7
F4	1.9	4.0
F5	3.0	2.9
F6	2.1	4.2

In Vitro Drug Release of Nanoemulgels

As a result of the 1-month stability study performed, the release study was carried out with the F1 formulation since decompositions were observed in other formulations except for the F1 formulation. Pure SA was also evaluated in the release study. *In vitro* drug release profile of F1 and pure SA is given in Figure 6. The release medium had no further effect on the solubility of SA. It was found to be compatible with the results obtained from the solubility study. When the release profile is examined, it is seen that the pure SA is released slightly more than the nanoemulsion. However, this is a normal situation seen in water-insoluble active substances. When SA in the formulation passes from the oil phase in which it is soluble to the high viscosity aqueous phase in which it is insoluble, its release is slowed due to decreased solubility [35]. The polymer concentration that forms the hydrogel outside the droplets causes the release to be delayed due to the network-like structures formed in the gel [19]. In our study, after 8 hours, F1 released approximately 40%, while pure SA released approximately 45%. Sinha et al. prepared nanoemulsions of SA and conducted a release study using a dialysis membrane in a pH 7.4 phosphate buffer. At the end of 8 hours of the release study, approximately 50% of the SA nanoemulsion was released. However, pure SA solution in methanol showed about 99% release after 2 hours [23]. When compared with our study, it is quite clear that entrapping the SA nanoemulsion in a hydrogel slows the release of SA a little more. There are other studies in the literature that found similar results to ours [36].

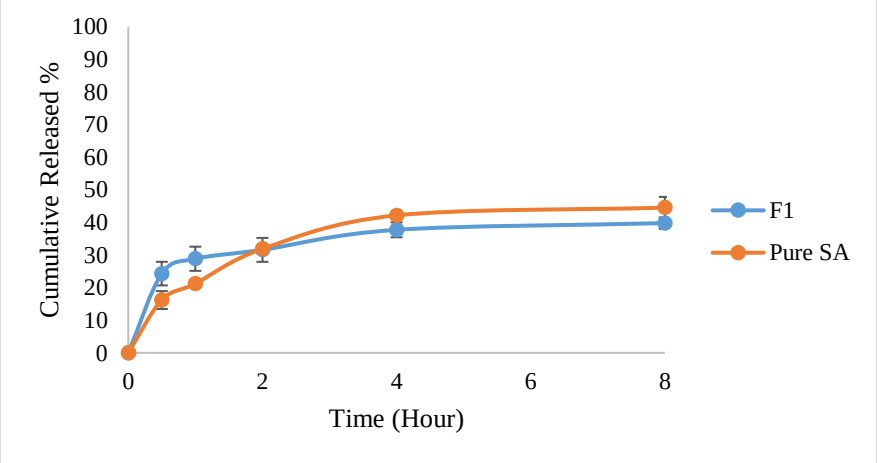


Figure 6. The release profile of F1 and pure salicylic acid

FT-IR Analysis of Nanoemulgels

The FT-IR spectra of the active substances, the nanoemulgels, and all the excipients used in the nanoemulgels are given in Figure 5 below. FTIR analysis of SA and PI is not very common in the literature. Since PI also contains groups similar to SA, it gave a similar FTIR spectrum [37]. SA has two specific functional groups, a carboxylic acid, and a phenol group. According to the chemical structure

of SA, the unique O-H phenol group gives specific peaks in the range of 3250-2750 cm^{-1} (O-H and C-H stretching 3233 cm-1 and 2999-2831 cm^{-1}), the O-H carboxylic acid group in the ranges of 2750-2250 cm^{-1} and 750-500 cm^{-1} (=C-H bending 760-669 cm^{-1}), and the C=O carboxylic acid group in the range of 1700-1500 cm^{-1} (C=O (COO-) asymmetric stretching 1652-1670 cm^{-1}) [38, 39]. When the results are examined, it is seen that the active substances and excipients in the formulations do not interact, and there is no change in the spectrum of the active substances.

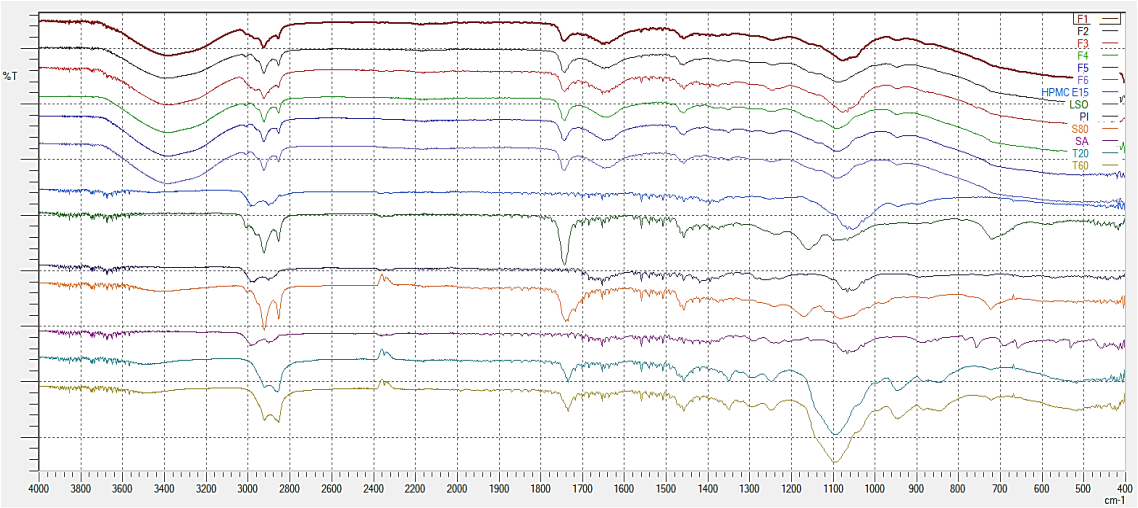


Figure 5. The FT-IR spectra of the active substances, the nanoemulgels, and all the excipients

ACKNOWLEDGEMENTS

The authors thank financial support for this work from the Atatürk University Scientific Research Project Foundation (Grant Number: TLP-2021-10099). Also, The authors are grateful BASF Turkey for PI support and Santa Farma İlaç A.Ş (Turkey) for HPMC E15 support.

AUTHOR CONTRIBUTIONS

Concept: *R.S.Ö., Ş.A., A.E.Ö., E.Ö.*; Design: *R.S.Ö., E.Ö.*; Control: *R.S.Ö., E.Ö.*; Sources: *R.S.Ö., Ş.A., A.E.Ö.*; Materials: *R.S.Ö., Ş.A., A.E.Ö., E.Ö.*; Data Collection and/or processing: *R.S.Ö., Ş.A., A.E.Ö., E.Ö.*; Analysis and/or interpretation: *R.S.Ö., Ş.A., A.E.Ö., E.Ö.*; Literature review: *R.S.Ö., Ş.A., A.E.Ö.*; Manuscript writing: *R.S.Ö., E.Ö.*; Critical review: *R.S.Ö., E.Ö.*; Other: -

CONFLICT OF INTEREST

The authors declare that there are no actual, potential, or perceived conflicts of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

REFERENCES

1. Richard, M.A., Paul, C., Nijsten, T., Gisondi, P., Salavastru, C., Taieb, C., Trakatelli, M., Puig, L., Stratigos, A., EADV burden of skin diseases project team. (2022). Prevalence of most common skin diseases in Europe: A population-based study. *Journal of the European Academy of Dermatology and Venereology, 36*(7), 1088-1096. [CrossRef]

2. Gupta, M., Agrawal, U., Vyas, S.P. (2012). Nanocarrier-based topical drug delivery for the treatment of skin diseases. *Expert Opinion on Drug Delivery*, *9*(7), 783-804. [CrossRef]

3. Anand, K., Ray, S., Rahman, M., Shaharyar, A., Bhowmik, R., Bera, R., Karmakar, S. (2019). Nano-emulgel: Emerging as a smarter topical lipidic emulsion-based nanocarrier for skin healthcare applications. *Recent Patents on Anti-infective Drug Discovery*, *14*(1), 16-35. [CrossRef]

4. Paudel, K.S., Milewski, M., Swadley, C.L., Brogden, N.K., Ghosh, P., Stinchcomb, A.L. (2010). Challenges and opportunities in dermal/transdermal delivery. *Therapeutic Delivery*, *1*(1), 109-131. [CrossRef]

5. Phad, A.R., Dilip, N.T., Ganapathy, R.S. (2018) Emulgel: A comprehensive review for topical delivery of hydrophobic drugs. *Asian Journal of Pharmaceutics, 12*(2), S382. [CrossRef]

6. Singh, Y., Meher, J.G., Raval, K., Khan, F.A., Chaurasia, M., Jain, N.K., Chourasia, M.K. (2017). Nanoemulsion: Concepts, development and applications in drug delivery. *Journal of Controlled Release, 252*, 28-49. [CrossRef]

7. Yukuyama, M.N., Kato, E.T., Lobenberg, R., Bou-Chacra, N.A. (2017). Challenges and future prospects of nanoemulsion as a drug delivery system. *Current Pharmaceutical Design*, *23*(3), 495-508. [CrossRef]

8. Tayeb, H.H., Sainsbury, F. (2018). Nanoemulsions in drug delivery: Formulation to medical application. *Nanomedicine (London, England)*, *13*(19), 2507-2525. [CrossRef]

9. Aithal, G.C., Narayan, R., Nayak, U.Y. (2020). Nanoemulgel: A promising phase in drug delivery. *Current Pharmaceutical Design*, *26*(2), 279-291. [CrossRef]

10. Madan, R.K., Levitt, J. (2014). A review of toxicity from topical salicylic acid preparations. *Journal of the American Academy of Dermatology*, *70*(4), 788-792. [CrossRef]

11. Bigliardi, P.L., Alsagoff, S.A.L., El-Kafrawi, H.Y., Pyon, J.K., Wa, C.T.C., Villa, M.A. (2017). Povidone iodine in wound healing: A review of current concepts and practices. *International Journal of Surgery*, *44*, 260-268. [CrossRef]

12. Sarma, P., Kaur, H., Medhi, B., Bhattacharyya, A. (2020). Possible prophylactic or preventive role of topical povidone iodine during accidental ocular exposure to 2019-nCoV. *Graefe's Archive for Clinical and Experimental Ophthalmology*, *258*, 2563-2565. [CrossRef]

13. Ahmad, I., Vaid, F.H. (2009). Determination of benzoic acid and salicylic acid in commercial benzoic and salicylic acids ointments by spectrophotometric method. *Pakistan Journal of Pharmaceutical Sciences*, *22*(1), 18-22.

14. Acharya, S.D., Tamane, P.K., Khante, S.N., Pokharkar, V.B. (2020). QbD based optimization of curcumin nanoemulsion: DoE and cytotoxicity studies. *Indian Journal of Pharmaceutical Education and Research*, *54*(2), 329-336. [CrossRef]

15. Abdelmonem, R., Younis, M.K., Hassan, D.H., Ahmed, M.A.G.S., Hassanein, E., El-Batouty, K., Elfaham, A. (2019). Formulation and characterization of chlorhexidine HCl nanoemulsion as a promising antibacterial root canal irrigant: In-vitro and ex-vivo studies. *International Journal of Nanomedicine*, *2019*(14), 4697-4708. [CrossRef]

16. Dalmolin, T.L., Lopez, R.F.V. (2018). Nanoemulsion as a platform for iontophoretic delivery of lipophilic drugs in skin tumors. *Pharmaceutics*, *10*(4), 214. [CrossRef]

17. Kale, S.N., Deore, S.L. (2017). Emulsion micro emulsion and nano emulsion: A review. *Systematic Reviews in Pharmacy*, *8*(1), 39-47. [CrossRef]

18. Sultan, M.H., Javed, S., Madkhali, O.A., Alam, M.I., Almoshari, Y., Bakkari, M.A., Sivadasan, D., Salawi, A., Jabeen, A., Ahsan, W. (2022). Development and optimization of methylcellulose based nanoemulgel loaded with nigella sativa oil for oral health management: Quadratic model approach. *Molecules*, *27*(6), 1796. [CrossRef]

19. Gopalan, K., Jose, J. (2020). Development of amphotericin b based organogels against mucocutaneous fungal infections. *Brazilian Journal of Pharmaceutical Sciences*, *56*, e17509. [CrossRef]

20. Topal, G.R., Kıymacı, M.E., Özkan, Y. (2022). Preparation and in vitro characterization of vancomycin loaded PLGA nanoparticles for the treatment of Enterococcus faecalis infections. *Journal of Faculty of Pharmacy of Ankara University*, *46*(2), 350-363. [CrossRef]

21. Sevinç Özakar, R., Özakar, E. (2022). Different biopolymers' effects on the evaluation and characterization of floating tablets prepared by lyophilization technique to improve the quality control parameters. *Polymer-Korea*, *46*(2), 145-158. [CrossRef]

22. Sinha, P., Srivastava, N., Rai, V.K., Mishra, R., Ajayakumar, P.V., Yadav, N.P. (2019). A novel approach for dermal controlled release of salicylic acid for improved anti-inflammatory action: Combination of hydrophilic-lipophilic balance and response surface methodology. *Journal of Drug Delivery Science and Technology*, *52*, 870-884. [CrossRef]

23. Ashara, K.C., Shah, K.V. (2017). Nuts and bolts of oil and temperature in topical preparations of salicylic acid. *Folia Medica*, *59*(3), 279-288. [CrossRef]

24. Teng, X., Wang, F., Li, K., Chen, S., Ye, P., He, L., Li, G. (2017). Determination of solubility and apparent oil/water partition cofficient of salicylic acid by RP-HPLC. *Herald of Medicine*, *12*, 186-189.

25. Şanlı, H., Kalay Yıldızhan İ. (2020). Topikal tedaviler. *Turkderm-Turkish Archives of Dermatology and Venerology*, *54*, 23-29. [CrossRef]

26. Bowe, W.P., Shalita, A.R. (2008). Effective over-the-counter acne treatments. *Seminars in Cutaneous Medicine and Surgery*. *27*(3), 170-176. [CrossRef]

27. Malik, M.R., Al-Harbi, F.F., Nawaz, A., Amin, A., Farid, A., Mohaini, M.A., Alsalman, A.J., Hawaj, M.A.A., Alhashem, Y.N. (2022). Formulation and characterization of chitosan-decorated multiple nanoemulsion for topical delivery in vitro and ex vivo. *Molecules*, *27*(10), 3183. [CrossRef]

28. Khani, S., Keyhanfar, F., Amani, A. (2016) Design and evaluation of oral nanoemulsion drug delivery system of mebudipine. *Drug Delivery*, *23*(6), 2035-2043. [CrossRef]

29. Kassem, M.A., Ghalwash, M.M., Abdou, E.M. (2020) Development of nanoemulsion gel drug delivery systems of cetirizine; factorial optimisation of composition, in vitro evaluation and clinical study. *Journal of Microencapsulation*, *37*(6), 413-430. [CrossRef]

30. Koca, M., Özakar, E., Sevinç Özakar, R. (2021). Triiyodoanilin'in sentezlenmesi, nanosüspansiyonlarının hazırlanması, in vitro karakterizasyonu ve radyokontrast özelliklerinin incelenmesi. *Journal of Faculty of Pharmacy of Ankara University*, *45*(2), 265-284. [CrossRef]

31. Arbain, N.H., Basri, M., Salim, N., Wui, W.T., Abdul Rahman, M.B. (2018). Development and characterization of aerosol nanoemulsion system encapsulating low water soluble quercetin for lung cancer treatment. *Materials Today: Proceedings*, *5*, S137-S142. [CrossRef]

32. Kim, S.S., Yu, S.B., Kim, J.D., Ryu, S.J. (2013). Comparison of disinfective power according to application order of 70% isopropyl alcohol and 10% povidone-iodine. *Korean Journal of Anesthesiology*, *65*(6), 519-524. [CrossRef]

33. Sızlan, A., Dündaröz, R., Dakak, M., Yıldıran, T., Dağlı, G., Özyurt, M. (2001). Povidon-iyot solüsyonların güvenilirliği. *SDÜ Tıp Fakültesi Dergisi*, *8*(1), 14-17.

34. Sharma P., Tailang, M. (2020). Design, optimization, and evaluation of hydrogel of primaquine loaded nanoemulsion for malaria therapy. *Future Journal of Pharmaceutical Sciences*, *6*, 26. [CrossRef]

35. Dhawan, B., Aggarwal, G., Harikumar, S.L. (2014). Enhanced transdermal permeability of piroxicam through novel nanoemulgel formulation. *International Journal of Pharmaceutical Investigation*, *4*(2), 65-76. [CrossRef]

36. Woo, J.O., Misran, M., Lee, P.F., Tan, L.P. (2014). Development of a controlled release of salicylic acid loaded stearic acid-oleic acid nanoparticles in cream for topical delivery, *The Scientific World Journal*, *2014*, 205703. [CrossRef]

37. Hashemi, A., Bahari, A. (2017). Structural and dielectric characteristic of povidone-silica nanocomposite films on the Si (n) substrate. *Applied Physics A*, *123*, 535. [CrossRef]

38. Gundlach, M., Paulsen, K., Garry, M., Lowry, S. Yin and Yang in Chemistry Education: The Complementary Nature of FT-IR and NMR Spectroscopies. *Thermo Fisher Scientific*, No:52742.

39. Trivedi, M.K., Branton, A., Trivedi, D., Shettigar, H., Bairwa, K., Jana, S. (2015). Fourier transform infrared and ultraviolet-visible spectroscopic characterization of biofield treated salicylic acid and sparfloxacin. *Natural Products Chemistry & Research*, *3*(5), 1000186. [CrossRef]

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 781-792, 2022
Doi: 10.33483/jfpau.1069607

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ANTIOXIDANT ACTIVITY AND ANTI-CANCER EFFECTS OF BILBERRY (*VACCINIUM MYRTILLUS* L.) FRUIT EXTRACT ON GASTRIC CANCER, AGS CELL LINE

YABANMERSİNİ (VACCİNİUM MYRTİLLUS L.) MEYVE ÖZÜ'NÜN MİDE KANSERİ, AGS HÜCRE HATTI ÜZERİNDE ANTİOKSİDAN AKTİVİTESİ VE ANTİ-KANSER ETKİLERİ

Nihal KARAKAŞ[1,2], Mehmet Evren OKUR[3], Tuğba SAĞIR[4], Damla ULUDAĞ[2], Derya Çiçek POLAT[5], Ayşe Esra KARADAĞ[6,7*]

[1]İstanbul Medipol University, School of Medicine, Department of Medical Biology, 34810, Istanbul, Türkiye

[2]İstanbul Medipol University, Institute for Health Sciences and Technologies (SABITA), Cancer Research Center, 34810, İstanbul, Türkiye

[3]University of Health Sciences, Faculty of Pharmacy, Department of Pharmacology, 34668, Istanbul, Turkey

[4]Pim Grup Cosmetics Consultancy, Göktürk, 34077, İstanbul, Türkiye

[5]Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Botany, 06560, Ankara, Türkiye

[6]İstanbul Medipol University, School of Pharmacy, Department of Pharmacognosy, 34810, Istanbul, Türkiye

[7]Anadolu University, Institue of Health Sciences, Department of Pharmacognosy, 26470, Eskişehir, Türkiye

ABSTRACT

Objective: *Vaccinium myrtillus L. fruits are consumed as food. This research was aimed to evaluate V. myrtillus methanol extract antioxidant and cytotoxic activities and determine its anti-cancer potential to further*

* **Corresponding Author / Sorumlu Yazar:** Ayşe Esra Karadağ
e-mail / e-posta: ayseesraguler@gmail.com, **Phone / Tel.:** +902124448544

Submitted / Gönderilme: 01.03.2022 **Accepted / Kabul:** 22.07.2022

study against gastric cancer.

Material and Method: *V. myrtillus fruit (Bilberry) methanol extract was examined for its antioxidant activities by ABTS•+ and DPPH• assays. The phytochemical analysis of the extract was studied by HPLC method. The cytotoxic effect of V. myrtillus fruit methanol extract on gastric cancer cell line AGS was measured by Cell Titer-Glo assay. Additionally, as healthy control, fibroblast like human mesenchymal stem cell line was used for testing anti-cancer efficacy.*

Result and Discussion: *V. myrtillus fruit methanol extract showed 0.1413 and 0.0439 mg/mL IC50 values as antioxidant activity by ABTS•+ and DPPH• assays, respectively. Malvidin-3-O-Glucoside was detected as an anthocyanin compound by HPLC method. Cytotoxicity analysis showed that among different concentrations (0.5-10 mg/ml), the most significantly, 2 mg/ml of Bilberry extract treatment decreased the viability of AGS gastric cancer cells while sparing healthy MSC cells. This data suggests the further analysis of Bilberry extract on several cancer cell lines as well as the determination of a potential active substance in the extract.*

Keywords: *Anti-cancer, antioxidant activity, bilberry, gastric cancer, Vaccinium myrtillus*

ÖZ

Amaç: *Vaccinium myrtillus L. meyvesi Türkiye'de gıda olarak tüketilmektedir. Bu araştırma V. myrtillus metanol ekstresinin antioksidan ve sitotoksisite aktivitelerini ve mide kanser hücresindeki potansiyel etkilerini belirlemeyi amaçlamıştır.*

Gereç ve Yöntem: *V. myrtillus meyvesi metanol ekstresinin antioksidan aktiviteleri spektrofotometrik olarak ABTS+ ve DPPH• yöntemleriyle gerçekleştirilmiştir. Fitokimyasal analiz HPLC yöntemi ile araştırılmıştır. V. myrtillus metanol ekstresinin mide kanseri hücre hattı AGS üzerindeki sitotoksik etkisi, Cell Titer-Glo testi ile ölçülmüştür. Ek olarak, anti-kanser etkinlik analizi için fibroblast benzeri insan mezenkimal kök hücre hattı sağlıklı kontrol hücreleri olarak kullanılmıştır. Morfolojik değişiklikler, faz kontrast mikroskobu kullanılarak incelenmiştir.*

Sonuç ve Tartışma: *V. myrtillus meyve metanol ekstresinde ABTS+ ve DPPH• testleri ile sırasıyla 0.1413 ve 0.0439 mg/mL IC50 değerleri hesaplanmıştır. Bir antosiyanin bileşiği olarak malvidin-3-O-glukozit HPLC yardımıyla tespit edilmiştir. Sitotoksite analizi, farklı konsantrasyonlar (0.5-10 mg/ml) arasında 2 mg/ml V. myrtillus meyve metanol ekstresinin, sağlıklı MSC hücrelerini korurken AGS mide kanseri hücrelerinin canlılığını azalttığını göstermiştir. Bu veriler, Yaban mersini özünün çeşitli kanser hücre dizileri üzerinde daha fazla analiz edilmesini ve ekstresindeki potansiyel aktif maddelerin belirlenmesini önermektedir.*

Anahtar Kelimeler: *Anti-kanser, antioksidan aktivite, mide kanseri, Vaccinium myrtillus, yaban mersini*

INTRODUCTION

Vaccinium myrtillus L. belongs to the Ericaceae family and is known as bilberry, blueberry, whortleberry, and huckleberry; it is called "yaban mersini" and "ayı üzümü" in Turkey [1, 2]. *V. myrtillus* is a type of shrub that grows naturally in the Black Sea region in Turkey; native to mountain and forest areas in Northern and Central Europe [3]. This species is a small plant with a height of up to 30 cm, shed its leaves in the winter. The fruit berry type is dark red - purple color [1].

Prepared from *V. myrtillus* leaves infusion (5%) is used for constipation, antiseptic, strengthening, and diabetes in Turkey [1]. In general herbal tea of leaves and stems of *V. myrtillus* are used for antioxidant activity, and also alcoholic extracts of these leaves and stems have been shown antibacterial activity [2, 4].

Fruits of *V. myrtillus* are consumed as food worldwide. The fruits are consumed for their health protection properties, as anti-inflammatory, anti-hypertensive, anti-microbial, and anti-cancer agents [1, 5]. The fruits include considerable quantities of antioxidant and micronutrients compounds, such as

polyphenols. These polyphenols, anthocyanins (delphinidin, cyanidin, petunidin, pelargonidin, peonidin and malvidin with the sugar part consists of glucose, arabinose, and galactose) the main ones, which belong to the flavonoid group and are responsible for the pigmentation of these fruits. Phenolic acids (caffeic, p-coumaric, ferulic, chlorogenic acids), flavonols (quercetin, myricetin), and flavonol (catechin) have been determined in fruits [5–7]. Because of these polyphenols, the fruits have antioxidant, astringent, antibacterial, and antiseptic properties. Also included are the ability to reduce the permeability and fragility of capillaries, inhibition of platelet aggregation, inhibition of urinary infection, and strengthening of collagen matrices through cross-links [8–11]. In recent studies have shown that extracts of fruit can be used in the prevention and treatment of chronic pathologies such as diabetes, cardiovascular disease, and obesity and confirm that high antioxidant and antiradical activity of bilberry fruits [2, 5, 12, 13]. Moreover, anthocyanin containing fruit extracts have the capacity to inhibit tumor formation and reduce cancer cell proliferation. It was observed that fruits inhibited MCF-7 cells by 50% at a concentration of 0.3-0.4 mg/ml. [14–21].

In this study, the total phenolic content (TPC) of methanol extract of *V. myrtillus* fruit has been determined. The extracts were investigated *in vitro* antioxidant and cytotoxic activities. To analyze cytotoxicity, cell viability was measured on the gastric cancer cell line, AGS and healthy MSC cell line treated with different doses (0.5-1-2-5-10 mg/ml) of methanol extract of *V. myrtillus* fruit. According to cell viability assay, at 2 mg/ml concentration significant cytotoxic effects of methanol extract of *V. myrtillus* fruit were determined in gastric cancer cell line while no toxicity was recorded in healthy MSC cells.

MATERIAL AND METHOD

Materials

Ascorbic acid, Trolox, 3-O-Glucoside, $ABTS^{\bullet+}$ *ve* $DPPH^{\bullet}$ radicals were purchased from Sigma, Germany. Methanol was supplied by Merck, Germany. Dulbecco's Modified Eagle's Medium (DMEM), 10% Fetal bovine serum, 1% Penicillin-Streptomycin were purchased from Gibco. To examine the effects of All other reagents used were of analytical grade.

Preparation of Samples

Fruits of *V. myrtillus* were purchased from a local market in Ankara, Turkey, and authenticated by Dr. Derya Çiçek Polat from Ankara University. Samples were pureed and they were extracted with methanol using a stirrer (250 g sample, 400 mLx3) (Heidolph MR3001). After being filtered, the extracts were concentrated in a vacuum at 40°C (Heidolph WB2000). The methanol extract yield of *V. myrtillus* was calculated as 9.32%.

Antioxidant Activity

The reaction mixture contains 100 μM DPPH• in methanol and different concentrations of *V. myrtillus* fruit extract (1-0.5 and 0.25 mg/mL). Free radical capture by measuring the absorbance value at 517 nm after 30 min at room temperature. The procedure was developed based on Blois's method [22]. Ascorbic acid was used as a positive control and the trials were carried out in triplicate.

The reaction mixture consists of 2.45 mM potassium persulfate and 7mM $ABTS^{\bullet+}$ aqueous solution. This mixture is allowed to stand in the dark overnight at room temperature. The $ABTS^{\bullet+}$ solution was diluted with ethanol. Samples were diluted 1/100 with $ABTS^{\bullet+}$ solution. 6 min later at 734 nm, the inhibition rates were determined by measuring the absorbance value [23]. Trolox was applied as a positive control and the trials were carried out in triplicate.

Determination of Phenolic Content

Folin Ciocalteu technique was used to detect total phenolics (gallic acid equivalent) of *V. mytrtillus* fruit methanol extract. The reaction mixture with extract was allowed incubate at 45°C and the absorbance was determined at 765 nm at room temperature. A linear calibration curve ($R^2 = 0.9913$) was used to determine the phenolic content [24].

HPLC Analysis

HPLC study was completed with an Agilent C18 column (250 × 4.6 mm, i.d. 5 μm). The mobile phases are A: Water:Formic acid (95:5); B: Acetonitrile and the gradient elution set up in the time frame 0–40 min, B%95–80 and the flow rate 1-0.5 mL/min. The detection wavelength was 520 nm and the injection volume was 20 μL. Malvidin-3-O-Glucoside was used for reference substance and injected at 0.2 mg/mL concentration [25].

Cell Viability Assay

In the study, AGS (ATCC®CRL-1739 ™; gastric adenocarcinoma) and MSC (UE7T-13 cells, # RBRC-RCB2161; RIKEN, Japan) lines were used. Growing and expansion media of AGS and MSC; DMEM with 2 mM L-Glutamine, 10% Fetal bovine serum, 1% Penicillin-Streptomycin. To examine the effects of *V. myrtillus* fruit methanol extract, AGS and MSC cells were seeded in 96 black well plates at a density of $5x10^3$-$1x10^4$, respectively. After 24 h the media of the cells were discarded. AGS and MSC cell lines were treated with *V. myrtillus* fruit methanol extract at various concentrations (0.5-1-2-5-10 mg/ml) and incubated for 48 h under standard culture conditions (37°C and 5% CO_2). The Cell Titer-Glo viability assay was performed according to (cat no: G7570, Promega) manufacturer's instructions. SpectraMax i3x Multi-Mode Detection Platform was used to determine the percentage of viable cells. The MSC cell line was used as a healthy control cell. Control groups of both cells were treated with the extract solvent (DMEM media). Each concentration was prepared in triplicate.

Statistical Analysis

Significance was determined according to *unpaired student t-test* using GraphPad (GraphPad Prism 7.0 program) for two group comparisons (*p* < 0.05, and p** < 0.001*).

RESULT AND DISCUSSION

Antioxidant Activity and Total Phenolic Content

Bilberry is a fruit known for its antioxidant properties and is widely consumed as a food. In addition, in many previous studies, the high antioxidant capacity of the fruit was revealed many times. In the results obtained from this study, it is seen that the antioxidant capacity of the bilberry extract used is high. The IC_{50} findings were presented in Table 1. When the obtained activity results are compared with the results obtained in previous studies, it can be considered that the results are consistent. In a previous study, the fruit extract was able to inhibit lipid peroxidation (IC_{50} = 50.28 μg/mL) and to scavenge superoxide anion (IC_{50} < 25 μg/mL). [25–28]. Studies explaining that the high antioxidant capacity of bilberry extracts is related to the phenolic compounds they contain [9, 29–31]. In another previous study, the changes in antioxidant capacity of bilberry extracts according to the seasons were examined and it was revealed that fruits collected in July had the highest antioxidant capacity, although there was not much difference [2].

Table 1. $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging activities *V. myrtillus* fruit methanol extract.

	V. myrtillus	**References**
	IC_{50} ±SD (mg/mL)	
$ABTS^{\bullet+}$	0.1413 ± 0.0075	0.0112 ± 0.001 (Trolox)
$DPPH^{\bullet}$	0.0439 ± 0.0043	0.0135 ± 0.001 (Ascorbic acid)

The phenolic content of *V. myrtillus* extract was measured as 2908 mg gallic acid/100g and found to exhibit moderate antioxidant capacity on the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ analyzes.

HPLC Analysis

The phytochemistry of the *V. myrtillus* extracts was analyzed using HPLC which headed to the discovery of several compounds. The main anthocyanin component of *V. myrtillus* fruit extract was found to be the malvidin-3-O-glucoside as seen in Figure 1 and Figure 2. Malvidin is thought to be the major component in the extract.

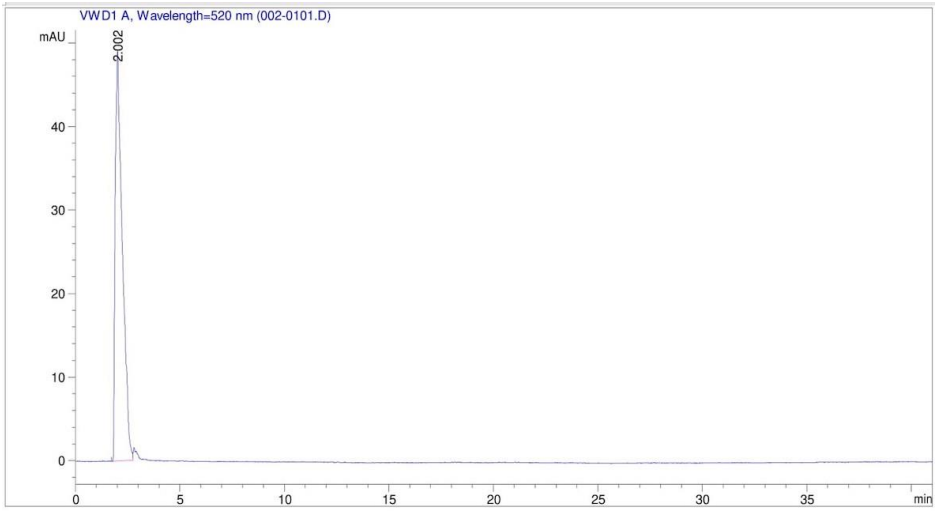


Figure 1. HPLC Standard Chromatogram

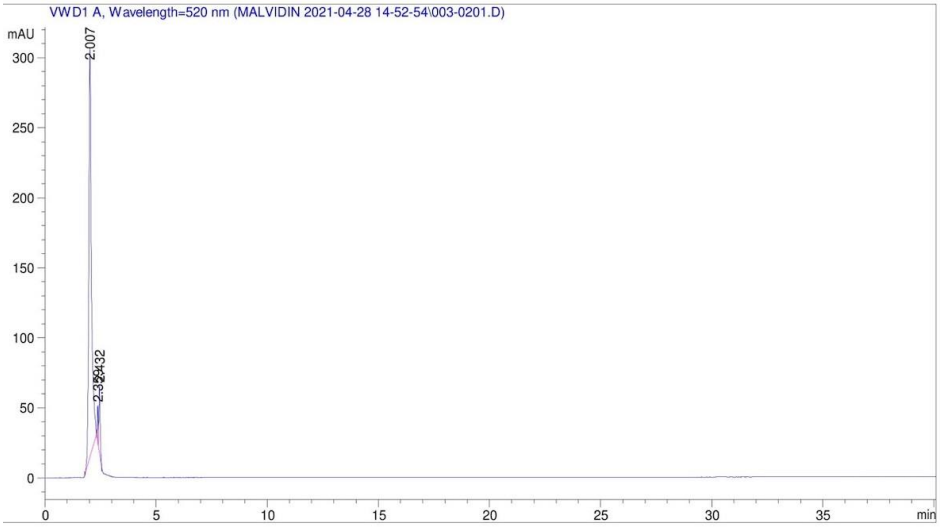


Figure 2. The HPLC chromatogram of *V. myrtillus* Methanol extract

Anti-cancer Activity

Morphological analysis displayed that 48h exposure of AGS and MSC cells with 5 or 10 mg/ml *V. myrtillus* fruit methanol extract, the cell growth was significantly inhibited, and cells became shrunken; cell blebbing and cytoplasmic degradation also observed. While there was no change in the morphology of the MSC cells treated with the 1, 2 mg/ml extract concentrations, changes in the MSC cell morphology were observed when treated 5 or 10mg/ml. The untreated (0 mg/ml) control cells preserved their healthy morphology (Figure 3A). The effects of *V. myrtillus* fruit methanol extract on the viability of the AGS cell line and MSC cells were studied after 48 h of treatment. The Cell Titer-Glo viability assay was performed. According to the result, the cell viability of the cells decreased gradually in a dose dependent manner. In comparison to the control cells, inhibition of viability of

bilberry extract treated AGS cells was ≅8% with 0.5 mg/mL, ≅33% with 1 mg/mL, ≅51% with 2 mg/mL, ≅69% with 5 mg/mL, and ≅97% with 10 mg/ml (Figure 3B). In short, cytotoxicity analysis showed that among different concentrations (0.5-10 mg/ml), 2mg/ml of Bilberry extract treatment decreased the viability of AGS gastric cancer cells the most significantly while sparing healthy MSC cells.

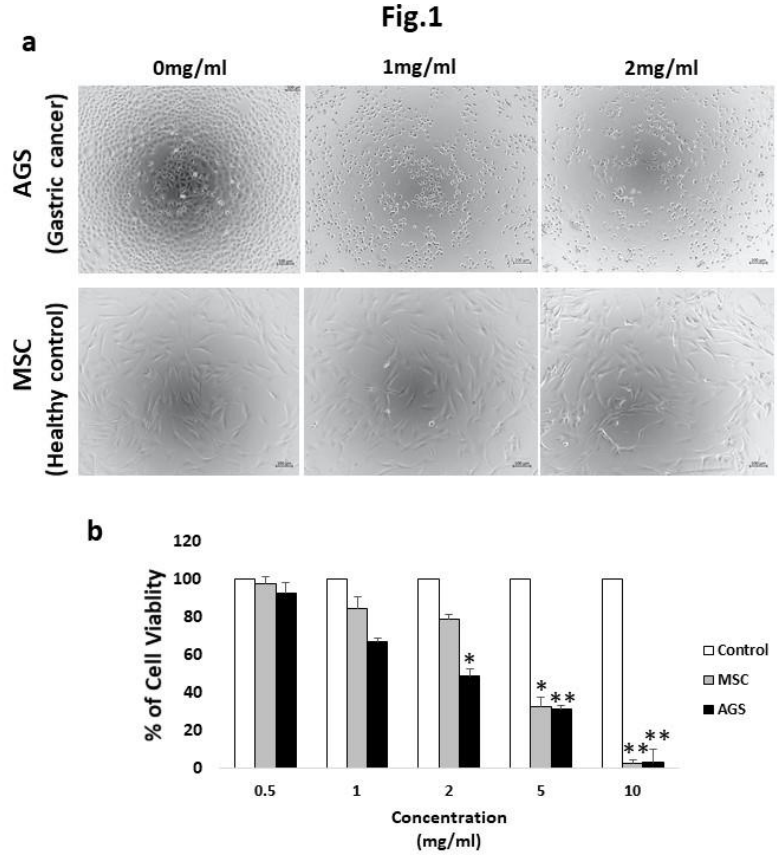


Figure 3. Cell viability results of AGS (gastric cancer) and MSC (mesenchymal stem cell; healthy control) cell lines treated with the bilberry extract for 48 h. a) Bright field image (10X Magnification). b) % Cell viability ratio. The results were expressed as the mean ± SD from triple replicates ($p^* < 0.05$, $p^{**} < 0.001$).

Bilberry (*Vaccinium myrtillus* L.) is known as one of the richest natural sources of anthocyanins especially delphinidins and cyanidins and contains other important phenolic components such as flavanols, tannins, ellagitannins, and phenolic acids [32]. In this study, the total phenolic content of the fruit extract was investigated, and an average value was determined. It is known that a significant part of the biological activities of extracts rich in phenolic substances are caused by these compounds.

Anthocyanins are the bio-flavonoid phytochemicals that give rich coloring to vegetables, flowers, and fruits, such as berries, pomegranates, and grapes. The most common anthocyanins in plants are pelargonidin, delphinidin, peonidin, petunidin, malvidin and cyanidin and are usually seen as 3-glycosides of these compounds [33–35]. Numerous studies have suggested that anthocyanins possess a powerful antioxidant, anti-inflammatory, anti-cancer activity [36, 37]. Anthocyanins have been proven to upregulate tumor suppressor genes, induce apoptosis in cancer cells, repair and protect genomic DNA integrity [33]. Anthocyanins stimulates redox-sensitive caspase 3-related apoptosis and Bad/Bcl-2 pathway dysregulation in chronic lymphocytic leukemia cells with no effect in healthy cells [38].

Several types of researches concluded that anthocyanin containing extracts significantly inhibited the growth of various cancer cells such as breast cancer, MCF-7, human colon cancer, HT-29, human cervical carcinoma, HeLa at different concentrations [16–18]. Although gastric cancer is the third cancer type worldwide [19, 20] there are no studies about the effect of the Bilberry extract on gastric cancer cells.

In recent times, a bilberry extract was proven to inhibit cell growth in MCF7-GFP-tubulin cells [16]. It seems to a similar effect with our results, but low doses inhibited cell proliferation in MCF7-GFP-tubulin breast cancer cells while higher doses inhibited cell proliferation in AGS gastric cancer cells. It can be related to gastric cancer being a more aggressive type than MCF7-GFP-tubulin breast cancer cells.

Another important point is that other studies did not look at the effects of Bilberry extract on normal cells. In this study, it was determined that blueberries are rich in phenolic compounds, and it was found that the major anthocyanin was malvidin-3-O-glucoside, which is known anti-cancer effect, by HPLC method in bilbery extract. It was demonstrated in previous studies that malvidin and its derivatives prevent oxidation in the cell, and it was thought that this may be related to cell life and its protective effect against cancer [39]. In addition, the anticancer activity of malvidin and its derivatives on some cell lines was investigated and its mechanisms were elucidated in detail [40–42]. The findings in previous studies also support the data obtained in this study. The studied bilberry extract is also rich in malvidin-3-o-glucoside, as can be understood from the HPLC results, this can explain its antioxidant and cytotoxic effects on the cancer cells.

In vitro studies have shown the significant cell killing ability of Bilberry extract at a certain concentration (2mg/ml) in AGS gastric cancer cells while no significant effect was measured for healthy human cells. Higher doses of extract increased the death cell population without cancer specificity, indicating cytotoxicity. Therefore, it was determined the optimum dose (2mg/ml) among tested, decreases cancer cell viability and did not damage normal cells. The extract can be further analyzed for

a possible active ingredient. More cancer cells can be included as a panel and response to treatments can be determined. Taken together, this study reveals a potential anti-cancer extract for a detailed analysis.

AUTHOR CONTRIBUTIONS

Concept: *N.K., M.E.O., A.E.K., D.Ç.P.;* Design: *N.K., M.E.O., A.E.K., D.Ç.P.;* Control: *M.E.O.* Sources: *N.K., M.E.O., T.S.;* Materials: *A.E.K., D.U.;* Data Collection and/or processing: *A.E.K., D.U., T.S.;* Analysis and/or interpretation: *N.K., M.E.O., A.E.K.;* Literature review: *M.E.O., A.E.K., D.Ç.P.;* Manuscript writing: *M.E.O., A.E.K., D.Ç.P.;* Critical review: *N.K., M.E.O.;* Other: -

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

REFERENCES

1. Baytop, T. (1999). Türkiyede Bitkiler ile Tedavi, Nobel Tıp Kitabevleri, Istanbul.

2. Bujor, O.C., Le Bourvellec, C., Volf, I., Popa, V.I., Dufour, C. (2016). Seasonal variations of the phenolic constituents in bilberry (Vaccinium myrtillus L.) leaves, stems and fruits, and their antioxidant activity. *Food Chemistry*, *213*, 58–68. [CrossRef]

3. Güner, A. (2012). Türkiye Bitkileri Listesi (Damarlı Bitkiler), Nezahat Gökyiğit Botanik Bahçesi Yayınları, Istanbul.

4. Rouanet, J.M., Décordé, K., Rio, D.D. , Auger, C., Borges, G., Cristol, J.P., Lean M.E.J., Crozier, A. (2010). Berry juices, teas, antioxidants and the prevention of atherosclerosis in hamsters. *Food Chemistry*, *118*(2), 266–271. [CrossRef]

5. Ochkur, O., Del Bubba, M., Orlandini, S., Kravchenko, I., Koshovyi, O., Checchini, L., Bohachik, J. (2019). Study on the Polyphenols and Antocyanins Content In the Vaccinium myrtillus L. fruits harvested in different regions of Ukraine. *Electronic Archive of the National Pharmaceutical University*, 285–287. [CrossRef]

6. Benvenuti, S., Brighenti, V., Pellati, F. (2018). High-performance liquid chromatography for the analytical characterization of anthocyanins in Vaccinium myrtillus L.(bilberry) fruit and food products. *Analytical and Bioanalytical Chemistry*, *410*(15), 3559-3571. [CrossRef]

7. Nyström, J., Östman, U.B., Mossing, T., Hed, L., Geladi, P. (2020). Solvent and Rotating Bed Reactor Extraction with One- and Two-Phase Solvents Applied to Bilberries (Vaccinium myrtillus)

for Isolating Valuable Antioxidants. *Food Analytical Methods*, *13*(4), 933–941. [CrossRef]

8. Madhavi, D.L., Bomser, J., Smith, M.A.L., Singletary, K. (1998). Isolation of bioactive constituents from Vaccinium myrtillus (bilberry) fruits and cell cultures. *Plant Science*, *131*(1), 95–103. [CrossRef]

9. Colak, N., Primetta, A.K., Riihinen, K.R., Jaakola, L., Grúz, J., Strnad, M., Torun, H., Ayaz, F.A. (2017). Phenolic compounds and antioxidant capacity in different-colored and non-pigmented berries of bilberry (Vaccinium myrtillus L.). *Food Bioscience*, *20*, 67–78. [CrossRef]

10. Machado, A.P.D.F., Pereira, A.L.D., Barbero, G.F., Martínez, J. (2017). Recovery of anthocyanins from residues of Rubus fruticosus, Vaccinium myrtillus and Eugenia brasiliensis by ultrasound assisted extraction, pressurized liquid extraction and their combination. *Food Chemistry*, *231*, 1–10. [CrossRef]

11. Satoh, Y., Ishihara, K. (2020). Investigation of the antimicrobial activity of Bilberry (Vaccinium myrtillus L.) extract against periodontopathic bacteria. *Journal of Oral Biosciences*, *62*(2), 169–174. [CrossRef]

12. Carmen Crespo, M., Visioli, F. (2017). A brief review of blue-and bilberries' potential to curb cardio-metabolic perturbations: focus on diabetes. *Current Pharmaceutical Design*, 983–988. [CrossRef]

13. Georgieva, A., Stefanova, R., Krastev, K. (2020). Exploration of The Impact of The Extraction Parameter on Biological Active Substances in The Extracts from Dried Fruit Black Blueberry (Vaccinium myrtillus L.). *Trakia Journal of Sciences*, *1*(18), 59–68. [CrossRef]

14. Matsunaga, N., Tsuruma, K., Shimazawa, M., Yokota, S., Hara, H. (2010). Matsunaga, N., Tsuruma, K., Shimazawa, M., Yokota, S., Hara, H. (2010). Inhibition of cholinesterase and amyloid-beta aggregation by resveratrol oligomers from Vitis amurensis. *Phytotherapy Research*, *24*(S1), 42–47. [CrossRef]

15. Puupponen-Pimiä, R., Nohynek, L., Alakomi, H.L., Oksman-Caldentey, K.M. (2005). Bioactive berry compounds - Novel tools against human pathogens. *Applied Microbiology and Biotechnology*, *67*(1), 8–18. [CrossRef]

16. Nguyen, V., Tang, J., Oroudjev, E., Lee, C. J., Marasigan, C., Wilson, L., Ayoub, G. (2010). Cytotoxic effects of bilberry extract on MCF7-GFP-tubulin breast cancer cells. *Journal of Medicinal food*, *13*(2), 278–285. [CrossRef]

17. Kang, S.Y., Seeram, N.P., Nair, M.G., Bourquin, L.D. (2003). Tart cherry anthocyanins inhibit tumor development in ApcMin mice and reduce proliferation of human colon cancer cells. *Cancer Letters*, *194*(1), 13–19. [CrossRef]

18. Ekşi, S., Aktura, S.Ş., Ejder, N., Şahin, K. (2019). In Vitro Cytotoxic Effect of Various Fruits on Human Cervical Carcinoma Cells. *Journal of Biotechnology and Strategic Health Research*, *2*(3), 111–116. [CrossRef]

19. Parkin, D.M. (2006). The global health burden of infection-associated cancers in the year 2002. *International Journal of Cancer*, *118*(12), 3030–3044. [CrossRef]

20. Cravo, M., Fidalgo, C., Garrido, R., Rodrigues, T., Luz, G., Palmela, C., Santos, M., Lopes, F. Maio, R. (2015). Towards curative therapy in gastric cancer: Faraway, so close! *World Journal*

of Gastroenterology, *21*(41), 11609–11620. [CrossRef]

21. Duthie, S.J. (2007). Berry phytochemicals, genomic stability and cancer: Evidence for chemoprotection at several stages in the carcinogenic process. *Molecular Nutrition and Food Research*, *51*(6), 665–674. [CrossRef]

22. Blois, M.S. (1958). Antioxidant determinations by the use of a stable free radical, Nature Publishing Group, p.1199-1200. [CrossRef]

23. Spanos, G.A., Wrolstad, R.E. (1990). Influence of processing and storage on the phenolic composition of Thompson Seedless grape juice. *Journal of Agricultural and Food Chemistry*, *38*(7), 1565-1571. [CrossRef]

24. Cesa, S., Carradori, S., Bellagamba, G., Locatelli, M., Casadei, M.A., Masci, A., Paolicelli, P. (2017). Evaluation of processing effects on anthocyanin content and colour modifications of blueberry (Vaccinium spp.) extracts: Comparison between HPLC-DAD and CIELAB analyses. *Food Chemistry*, *232*, 114-123. [CrossRef]

25. Martín-Aragón, S., Basabe, B., Benedí, J.M., Villar, A.M. (1998). Antioxidant action of Vaccinium myrtillus L. *Phytotherapy Research*, *12*(S1), S104-S106. [CrossRef]

26. Beccaro, G. L., Mellano, M. G., Botta, R., Chiabrando, V., Bounous, G. (2006). Phenolic and anthocyanin content and antioxidant activity in fruits of bilberry (Vaccinium myrtillus L.) and of highbush blueberry (V.corymbosum L.) cultivars in North Western Italy, *Acta Horticulture*, *715*, 553-557. [CrossRef]

27. Dragana, M.V., Miroslav, R.P., Branka, B.R.G., Olgica, D.S., Sava, M.V., Ljiljana, R.Č. (2013). Antibacterial and antioxidant activities of bilberry (Vaccinium myrtillus L.) in vitro. *African Journal of Microbiology Research*, *7*(45), 5130-5136. [CrossRef]

28. Burdulis, D., Šarkinas, A., Jasutiene, I., Stackevičiene, E., Nikolajevas, L., Janulis, V. (2009). Comparative study of anthocyanin composition, antimicrobial and antioxidant activity in bilberry (Vaccinium myrtillus L.) and blueberry (Vaccinium corymbosum L.) fruits. *Acta Poloniae Pharmaceutica - Drug Research*, *66*(4), 399-408.

29. Güder, A., Gür, M., Engin, M.S. (2018). Antidiabetic and Antioxidant Properties of Bilberry (Vaccinium myrtillus Linn.) Fruit and Their Chemical Composition. *Journal of Agricultural Science and Technology*, *17*(2), 401-414.

30. Ancillotti, C., Ciofi, L., Pucci, D., Sagona, E., Giordani, E., Biricolti, S., Gori M., Petrucci W.A., Giardi, F., Bartoletti, R., Chiuminatto, U., Orlandini, S., Mosti, S., Del Bubba, M. (2016). Polyphenolic profiles and antioxidant and antiradical activity of Italian berries from Vaccinium myrtillus L. and Vaccinium uliginosum L. subsp. gaultherioides (Bigelow) S.B. Young. *Food Chemistry*, *204*, 176-184. [CrossRef]

31. Martz, F., Jaakola, L., Julkunen-Tiitto, R., Stark, S. (2010). Phenolic Composition and Antioxidant Capacity of Bilberry (Vaccinium myrtillus) Leaves in Northern Europe Following Foliar Development and Along Environmental Gradients. *Journal of Chemical Ecology*, *36*(9), 1017-1028. [CrossRef]

32. Chu, W., Cheung, S.C.M., Lau, R.A.W., Benzie, I.F.F. (2011). Bilberry (Vaccinium myrtillus L.), Benzie, I.F.F., Wachtel-Galor, S. (Eds.), In Herbal Medicine: Biomolecular and Clinical Aspects, Herbal Medicine: Biomolecular and Clinical Aspects, Boca Raton (FL): CRC Press/Taylor,

Francis, pp. 55-71. [CrossRef]

33. Thibado, S., Thornthwaite, J., Ballard, T., Goodman, B. (2017). Anticancer effects of Bilberry anthocyanins compared with NutraNanoSphere encapsulated Bilberry anthocyanins. *Molecular and Clinical Oncology*, *8*(2), 330-335. [CrossRef]

34. Upton, R. (2001). Bilberry Fruit: Vaccinium Myrtillus L. : Standards of Analysis, Quality Control, and Therapeutics, American Herbal Pharmacopoeia and Therapeutic Compendium.

35. Khoo, H.E., Azlan, A., Tang, S.T., Lim, S.M. (2017). Anthocyanidins and anthocyanins: colored pigments as food, pharmaceutical ingredients, and the potential health benefits. *Food & Nutrition Research*, *61*(1), 1361779. [CrossRef]

36. Li, D., Wang, P., Luo, Y., Zhao, M., Chen, F. (2017). Health benefits of anthocyanins and molecularmechanisms: Update from recent decade. *Critical Reviews in Food Science and Nutrition*, *8*(57), 1729-1741. [CrossRef]

37. Erlund, I., Koli, R., Alfthan, G., Marniemi, J., Puukka, P., Mustonen, P., Mattila, P., Jula, A. (2008). Favorable effects of berry consumption on platelet function, blood pressure, and HDL cholesterol. *American Journal of Clinical Nutrition*, *87*(2), 323-331. [CrossRef]

38. Alhosin, M., León-González, A.J., Dandache, I., Lelay, A., Rashid, S.K., Kevers, C., Pincemail, J., Fornecker, L.M., Mauvieux, L., Herbrecht, R., Schini-Kerth, V.B. (2015). Bilberry extract (Antho 50) selectively induces redox-sensitive caspase 3-related apoptosis in chronic lymphocytic leukemia cells by targeting the Bcl-2/Bad pathway. *Scientific Reports*, *5*, 8996. [CrossRef]

39. Huang, W., Zhu, Y., Li, C., Sui, Z., Min, W. (2016). Effect of blueberry anthocyanins malvidin and glycosides on the antioxidant properties in endothelial cells. *Oxidative Medicine and Cellular Longevity*, *2016*, 1591803. [CrossRef]

40. Baba, A.B., Nivetha, R., Chattopadhyay, I., Nagini, S. (2017). Blueberry and malvidin inhibit cell cycle progression and induce mitochondrial-mediated apoptosis by abrogating the JAK/STAT-3 signalling pathway. *Food and Chemical Toxicology*, *109*, 534-543. [CrossRef]

41. Oliveira, H., Wu, N., Zhang, Q., Wang, J., Oliveira, J., De Freitas, V., Mateus, N., He, J., Fernandes, I. (2016). Bioavailability studies and anticancer properties of malvidin based anthocyanins, pyranoanthocyanins and non-oxonium derivatives. *Food and Function*, *7*(5), 2462-2468. [CrossRef]

42. Wang, Y., Lin, J., Tian, J., Si, X., Jiao, X., Zhang, W., Gong, E., Li, B. (2019). Blueberry Malvidin-3-galactoside Suppresses Hepatocellular Carcinoma by Regulating Apoptosis, Proliferation, and Metastasis Pathways in Vivo and in Vitro. *Journal of Agricultural and Food Chemistry*, *67*(2), 625-636. [CrossRef]

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 793-804, 2022
Doi: 10.33483/jfpau.1111713

ORIGINAL ARTICLE/ ÖZGÜN MAKALE

EVALUATION OF THE ASSOCIATION BETWEEN PROPER INHALER TECHNIQUE AND ANXIETY IN CHEST DISEASES OUTPATIENT CLINIC PATIENTS DURING COVID-19 PANDEMIC: A PROSPECTIVE CROSS-SECTIONAL STUDY

COVID-19 PANDEMİSİ DÖNEMİNDE GÖĞÜS HASTALIKLARI POLİKLİNİĞİ'NE BAŞVURAN HASTALARDA UYGUN İNHALER TEKNİĞİ VE ANKSİYETE İLİŞKİSİNİN DEĞERLENDİRİLMESİ: PROSPEKTİF KESİTSEL BİR ÇALIŞMA

Mefküre DURMUŞ[1], Selim GÖK[1], Ömer Faruk BAHÇECİOĞLU[1], Zeynep Ülkü GÜN[1*], Süleyman Savaş HACIEVLİYAGİL[2]

[1]Inönü University, Faculty of Pharmacy, Department of Clinical Pharmacy, 44210, Malatya, Turkey

[2]Inönü University, Faculty of Medicine, Department of Chest Diseases, 44210, Malatya, Turkey

ABSTRACT

Objective: *In this study, we aimed to investigate the association between inhaler use skills and general anxiety or coronavirus anxiety scores in patients with respiratory diseases.*

Material and Method: *Inhaler techniques of 70 asthma and chronic obstructive pulmonary disease (COPD) patients were evaluated by three clinical pharmacists. A pulmonologist assessed the patients' anxiety scores using the Hamilton Anxiety Rating Scale (HAM-A) and Coronavirus Anxiety Scale (CAS). We used IBM SPSS 25.0 as a software program for related statistical analysis*

Result and Discussion: *Proper inhaler technique wasn't found to be associated with CAS score, age, comorbidity, inhaler type. HAM-A stage (p=0.096) and educational status (p=0.074) were not found as statistically significant in affecting the proper inhaler technique. A weak correlation was found between age and CAS score (r=-0.278, p=0.02). Asthma patients have a higher rate of coronavirus anxiety than COPD patients (p=0.036). Female patients had higher HAM-A (p=0,037) and CAS scores (p=0,002) than male patients. There was a moderate correlation between HAM-A and CAS scores (r=0.407, p<0.001). The correlation between HAM-A and inhaler use skills scores were not found in statistical significance (r=-0.208, p=0.083). In conclusion, increase of CAS score wasn't found to be associated with proper inhaler technique. HAM-A score was found to affect proper inhaler technique negatively, but these results are not significant.*

* **Corresponding Author / Sorumlu Yazar:** Zeynep Ülkü Gün
e-mail / e-posta: ulku.duzgun@inonu.edu.tr, **Phone / Tel.:** +90532508040

Submitted / Gönderilme: 20.04.2022 **Accepted / Kabul:** 25.07.2022

Keywords: *Anxiety, asthma, COVID-19, COPD, inhaler technique*

ÖZ

Amaç: *Bu çalışmadaki amacımız respiratuvar hastalıkları olan hastaların inhaler kullanma becerileri ile koronovirüs anksiyete skorları ve genel anksiyete skorları arasındaki ilişkiyi belirlemektir.*

Gereç ve Yöntem: *Yetmiş astım ve kronik obstruktif akciğer hastalığı (KOAH) hastasının inhaler kullanma teknikleri üç klinik eczacı tarafından değerlendirildi. Göğüs Hastalıkları Uzmanı tarafından Hamilton Anksiyete Ölçeği (HAM-A) ve Koronavirüs Anksiyete Ölçeği (CAS) kullanılarak hastaların anksiyete skorları ölçüldü. IBM SPSS 25.0 programı kullanılarak ilgili istatistikler yapıldı.*

Sonuç ve Tartışma: *Doğru inhaler tekniği ile CAS skoru, yaş, komorbidite, veya inhaler tipi arasında ilişki bulunamamıştır. Doğru inhaler tekniği ile HAM-A seviyesi (p=0,096) ve eğitim durumu (p=0.074) arasında istatistiksel anlamlılık bulunamamıştır. Yaş ile CAS skoru arasında zayıf bir korelasyon bulunmuştur (r=-0.278, p=0.02). Astım hastalarının koronovirüs anksiyete oranı KOAH hastalarına göre anlamlı derecede yüksekti (p=0.036). Kadın hastaların HAM-A (p=0,037) ve CAS skorları (p=0,002) erkek hastalardan anlamlı derecede yüksekti. HAM-A ile CAS skorları arasında orta dereceli bir korelasyon bulunmuştur (r=0.407, p<0.001). HAM-A seviyesi ve inhaler kullanma beceri skorları arasındaki korelasyon anlamlı bulunmamıştır. (r=-0.208, p=0.083)Sonuç olarak, hastaların artmış CAS skorları inhaler kullanma becerilerini etkilememiştir. HAM-A skorları inhaler kullanma becerilerini negatif yönde etkilemiştir fakat sonuç anlamlı değildir.*

Anahtar Kelimeler: *Anksiyete, astım, COVID-19, inhaler tekniği, KOAH*

INTRODUCTION

The European Society for Patient Adherence, Compliance and Persistence (ESPACOMP) define medication nonadherence (MNA) in three categories as initiation, implementation, persistence. Implementation defined as taken prescribed dose correctly and this comprises patients' unintentional behaviour [1]. Although maintenance treatment of inhaled medication is vital for patients with asthma and chronic obstructive pulmonary disease (COPD), the majority of the patients do not adhere to therapy. The causes of MNA are complex and multifactorial. Improper inhaler technique is a component of unintentional MNA. Inhaler medications complicate drug adherence and patients have insufficient use skills of inhaler drugs because of various reasons [2]. Patients should be educated on how to use their inhaler devices, however many patients are unable to do so due to cognitive and physical limitations. [1]. Anxiety and depression have a negative impact on cognitive function and health behaviors. In a study which evaluated the association with different types of MNA and depressive and anxious symptoms has shown that these symptoms caused to unintentional and a greater extend of intentional MNA. They state that while depression has been identified as a risk factor for MNA in previous studies, research results on anxiety and comorbidity are confusing. They explained that anxiety symptoms are heterogeneous. An anxious patient could be more aware of his or her health and concerned about his or her physical ailment, which may reduce MNA, but even that adverse drug reactions may still cause MNA. [3]. Improvement in depressive symptoms had a positive effect on MNA in a study in cardiac patients, but there was no independent correlation between anxiety and MNA. They concluded that anxiety symptoms may have a lesser impact on the adherence outcomes measured. Besides, patients

with anxiety may be highly anxious about health concerns, leading to greater vigilance regarding recommendations about their health [4].

Coronavirus disease-2019 (COVID-19) has affected millions of people around the world. Clinicians estimate greater mortality rates among individuals with lung disease due to COVID-19's respiratory effects. Patients with COPD, on the other hand, have a decreased rate of COVID-19-induced hospitalizations. This lower rate could indicate that COVID-19 patients with COPD are taking effective COVID-19 preventative action [5]. But anyway respiratory effects of COVID-19 are worried among COPD patients [6].

There is evidence for an association between MNA and anxiety in asthma and COPD patients but there is no evidence of a correlation between anxiety and improper inhaler technique which is a key component of inhaler MNA according to our knowledge. As reported from the studies we could think that anxious patients endeavour to have more advanced knowledge and skills of their inhaler medication for getting rid of their symptoms [3, 4].

We aimed to investigate general anxiety and coronavirus anxiety scores in patients with COPD and asthma during COVID-19 pandemic and we wonder if moods of patients with COPD and asthma are influenced by COVID-19, there are any improve awareness of using their inhaler medication with proper technique due to increased anxiety about their health.

MATERIAL AND METHOD

This two-month cross-sectional study was conducted in the Chest Disease Outpatient Clinic at the İnönü University Turgut Özal Medical Center from December 2020 to February 2021.

Demographic data consist of patients' age, sex, diagnosis, educational level, smoking history, comorbidities, types of inhalers. Patients with asthma or COPD, who were 18 and over years old, applied to our hospital and taken at least one inhaler medication were included in the study. Patients over the age of 80 who could not self-administer their medication, patients who had difficulty speaking Turkish, patients with other respiratory diseases, acute organ failure, advanced stage cancer, and serious neurologic, mental or psychiatric diseases were excluded. During our research we conducted all face-to-face interviews within the scope of COVID-19 measures.

All the included patients' inhaler technique were assessed by three clinical pharmacists using either placebo inhalers or patients' own inhaler while providing the appropriate overdose warnings.

To assess patients' use skills, our pulmonologist first selected one inhaler medication. This inhaler medication was chosen to be used on a regular basis as a maintenance treatment, which was appropriate for their disease stage.

Inhaler techniques were evaluated in two ways; according to previous articles we evaluated use skills in nine main steps, which were given in supplementary data [7]. We scored one point for complete steps and zero points for those that were not performed. Each patient's total score was calculated and documented.

Based on published researchs and manufacturer instructions, we categorized inhaler types into four groups according to the following usage steps: metered dose inhaler (MDI), turbohaler, handihaler and diskus. We determined critical usage steps according to these literature and these steps were given in supplementary data [8, 9]. We documented the inhaler technique of patients who made one or more mistakes in critical steps as improper technique, and those who performed these steps correctly as proper technique, because inappropriate performance of critical steps would result in little or no medication reaching the lungs.

All the included patients were assessed by the same clinician using the Hamilton Anxiety Rating Scale (HAM-A). This is an asessment tool that measures anxiety in people according to 14 items. We did the rating as follows according to previous study; the score 6-14 indicated mild anxiety, 15-28 indicated moderate anxiety, 29-52 indicated severe anxiety [10]. Turkish validity and reliability of this scale has been made [11]. We divided the patients into three groups according to their scores and calculated total scores for each patient then documented them in these ways.

COPD Assesment Test (CAT) is an 8-items unidimensional measure of health status impairment in COPD. It was developed to be applicable worlwide and validated translations are available in a many languages [12]. Turkish validity and reliability of this scale has been made [13]. All COPD patients were assessed by the same clinician using CAT scale and calculated total scores then documented in this way.

Coronavirus Anxiety Scale (CAS) developed for identify COVID-19 related probable dysfunctional anxiety cases. Patients with score ≥9 are classified as having dysfunctional anxiety associated with COVID-19 crisis. Turkish validity and reliability of this scale has been made [14]. All patients were assessed by the same clinician. Then we divided patients into two group CAS-positive or negative according to their score and also, we calculated each patients' total score then documented in these ways. We evaluated patients who were diagnosed COPD and asthma by pulmonologists and we didn't assess the severity of the disease.

We used IBM SPSS 25.0 as a software program for statistical analysis. The analysis of qualitative data between the groups were done with the Pearson chi-square test or Fischer's exact test. Analysis of the quantitative data between the two groups were performed using the Independent Samples T test or the Mann-Whitney U test, depending on whether the data were normally distributed or not. Analysis of quantitative data between more than two groups were done by the Kruskal Wallis H test. Pearson (for normally distributed data) or Spearman's rho (for non-normally distributed data) correlation tests were used to determine the correlation of quantitative data.

RESULT AND DISCUSSION

Thirty-two asthma and 38 COPD patients, whose ability to use inhaler and anxiety scores were evaluated, were included in the study. The mean age of the patients was 54.33±14.44; 54.2% **(**38) of the patients were male and 45.7% (32) were female. The characteristics, anxiety scores, and the evaluation of inhaler techniques of the patients are summarized in Table 1. As expected, asthma patients were younger and had less comorbidities than COPD patients ($p<0.05$). While HAM-A scores of asthma and COPD patients were similar ($p=0.953$), CAS score was higher in asthma patients than in COPD patients ($p=0.01$).

Only 32.9% of the patients included in the study were using their inhalers properly. There were only 10 (14.3%) patients who had all steps correct.

Table 1. Characteristic features and other data of the patients

	Asthma (n=32)	**COPD (n=38)**	***p* value**
Age (mean±SD)	43.7±12	63.3±9.4	<0.001*
Gender			<0.001**
Female	27 (84.4%)	5 (13.2%)	
Male	5 (15.6%)	33 (86.8%)	
Marital status			0.130**
Single	6 (18.8%)	2 (5.3%)	
Married	26 (81.2%)	36 (94.7%)	
Smoking			0.001**
Current smoker	3 (9.4%)	10 (26.3%)	
Former smoker	11 (34.4%)	23 (60.5%)	
Never smoker	18 (56.2%)	5 (13.2%)	
Level of education			
Illiterate	4 (12.5%)	4 (10.5%)	0.906**
Primary or secondary school graduate	19 (59.4%)	25 (65.8%)	0.306**
High school graduate	6 (18.8%)	8 (21.1%)	0.952**
University graduate	3 (9.3%)	1 (2.6%)	0.325**
Comorbidity			0.002**
Present	7 (21.9%)	22 (57.9%)	
Absent	25 (78.1%)	16 (42.1%)	
Inhaler type			
MDI	4 (12.5%)	3 (7.9%)	0.695**
Diskus	12 (37.5%)	19 (50%)	0.294**
Handihaler	14 (43.8%)	15 (39.5%)	0.717**
Turbuhaler	2 (6.2%)	1 (2.6%)	0.589**
HAM-A score (mean±SD)	23.1±8.6	23.1±10.5	0.953*
CAS score (median)	1.5 (0-18)	0 (0-16)	0.01***
CAS positivity			0.036**
Positive	8 (25%)	2 (5.3%)	
Negative	24 (75%)	36 (94.7%)	
The evaluation of inhaler technique			0.448**
Proper	12 (37.5%)	11 (29%)	
Improper	20 (62.5%)	27 (71%)	
Inhalation skills score (median)	6.5 (2-9)	5 (1-9)	0.142***

***:** Independent Samples t test
****:** Chi-square test
*****:** Mann Whitney U test

There are no differences in CAT score, CAS score, age, comorbidity and inhaler type between patients who use the inhaler properly or not properly (Table 2). Only the HAM-A stage and educational status have approached statistical significance in affecting the proper inhaler technique. According to the HAM-A score, the rate of using inhalers' properly in patients with mild anxiety (n=16) is 50%, while in those with moderate (n=31) and severe anxiety (n=23) this rate is 32.2% and 21.7% respectively (p=0.096). Whilst the rate of proper use of inhalers was 50% for high school and university graduates, it was 26.9% for illiterate and primary or secondary school graduates (p=0.072). In addition, it was found that the inhalation skills scores of different education level groups were different (p=0.037). Mean inhalation skills score was 5.9 ± 2.6 for illiterates and was 7.9 ± 1.9 for university graduates (p=0.231).

Coronavirus anxiety rate of our population was 14.2% (10/70). A negative weak correlation was found between age and CAS score (r=-0.278, p=0.02). Coronavirus anxiety rate (p=0.036) and median CAS score (p=0,01) were higher in asthma patients than COPD patients. While the rate of coronavirus anxiety in female patients was 25%, it was 5.2% in male patients (p=0.036).

According to HAM-A score, the anxiety rate of our patients was 77.1%. While the anxiety rate in asthma patients was 78.1%, the anxiety rate in COPD patients was 76.3%. Although no association was found between anxiety rates according to HAM-A score and gender (p=0.453), total HAM-A scores of female patients higher than male patients (p=0.037). Similarly, there was no association between anxiety and age (p=0.181). There was a strong correlation between HAM-A score and CAT score (r=0.774, p<0.001), and a moderate correlation between HAM-A score and CAS score (r=0.407, p<0.001). There was a moderate correlation between the CAS score and the HAM-A score in both groups, asthma patients (r=0.468, p=0.007) and COPD patients (r=0.469, p=0.003). In addition, the correlation between HAM-A score and inhalation skills score were not found as statistically significant (r=-0.208, p=0.083).

Patients with COPD may experience a high prevalence of anxiety and depression. Anxiety symptoms were reported as 6% to 74 % of COPD patients[15, 16].

In a more recent study, the best estimate of anxiety prevalence in COPD patients was 36% (31-41%). Because of the social isolation induced by the current pandemic, there are little data available to show whether COPD patients are more likely to engage in negative behaviours such as excessive drinking and active smoking compared to other chronic conditions. These negative social behaviours may have a detrimental effect on the emotional health status of COPD patients [17]. In the present study, the prevalence of anxiety symptoms was shown to be present in 76.3% of patients with COPD.

Anxiety was shown to be more frequent in our study than in previous studies. The high prevalence of anxiety among COPD patients in our study was thought to be linked to the present pandemic. There was a moderate correlation between HAM-A score and CAS score in patients with COPD (r=0.469, p=0.003).

Table 2. Comparison of patients who use their inhalers properly and those who use them improperly

	Proper (n=23)	**Improper (n=47)**	***p* value**
Age (mean±SD)	54.0±15.32	54.5±14.2	0.74*
Gender			0.804**
Female	11 (47.8%)	21 (44.7%)	
Male	12 (52.2%)	26 (55.3%)	
Primary disease			0.448**
Asthma	12 (52.2%)	20 (42.6%)	
COPD	11 (47.8%)	27 (57.4%)	
Level of education			0.072**
Illiterate and primary or secondary school graduate	14 (60.9%)	38 (80.9%)	
High school and university graduate	9 (39.1%)	9 (19.1%)	
Comorbidity			0.202**
Present	12 (52.2%)	17 (36.2%)	
Absent	11 (47.8%)	30 (63.8%)	
Inhaler type			
MDI	3 (13.1%)	4 (8.5%)	0.676**
Diskus	7 (30.4%)	24 (51.1%)	0.102**
Handihaler	11 (47.8%)	18 (38.3%)	0.447**
Turbuhaler	2 (8.7%)	1 (2.1%)	0.249**
HAM-A stage			
Mild	8 (34.8%)	8 (17%)	0.096**
Moderate	10 (43.5%)	21 (44.7%)	0.924**
Severe	5 (21.7%)	18 (38.3%)	0.165**
CAT score (mean±SD)	21.8±10.1	21.2±10	0.784*
CAS score (median)	0 (0-18)	0 (0-18)	0.908***
CAS positivity			0.719**
Positive	4 (17.4%)	6 (12.8%)	
Negative	19 (82.6%)	41 (87.2%)	

***:** Independent Samples t test
****:** Chi-square test
*****:** Mann Whitney U test

In clinical studies, the prevalence of depression and anxiety in asthma patients varies. Psychological distress was found to be present in 7.5% to 80% [18, 19]. In the present study, anxiety symptoms were shown to be prevalent in 78.1% of asthma patients. People with chronic respiratory illness are disproportionately affected by COVID-19 and the measures taken to address it [20]. In our study the current pandemic might have been to cause for the high prevalence of anxiety in asthma patients. In asthma patients, there was a moderate correlation between HAM-score and the CAS score (r=0.468, p=0.007). Meanwhile, when compared to the COPD group, the asthma group had a higher CAS score positivity (p=0.036). In the trials, which had found relationship between higher CAT scores and anxiety and/or depression likely our study, several screening tools such as Beck Depression Inventory (BDI) [21], Hospital Anxiety and Depression Scale (HADS-A, HADS-D) [22], Hamilton Depression and Anxiety Rating Scale (HAM-A, HAM-D) [23], Patient Health Questionnaire-9 (PHQ-9) [24] were utilized to define depression and anxiety in patients with COPD. Patients with anxiety in the COPD group, according to a previous research, are mostly female patients [23]. Our findings support previous studies on anxiety-female gender association in asthma and COPD groups (p=0.037).

CAT is a dependable measure of COPD-specific health status [25]. It has been indicated in many trials that CAT correlates with anxiety symptoms. In the current study, this evidence was approved due to the strong correlation between the HAM-A and CAT scores ($r=0.774$, $p<0.001$).

The results demonstrated that 67.1% of the patient sample could not use their inhaler device as properly. Only 10 patients were successful in performing all the steps correctly. In previous studies, rates of inappropriate technique ranged from 4% to 97% depending on the patient sample and type of inhaler device [26, 27]. In one study, COPD patients with inappropriate inhaler device technique had a higher CAT score than patients with correct inhaler device technique ($p=0.02$) [28]. Meanwhile, one study showed that a remarkable association between CAT score and improper inhaler technique in patients with COPD [27]. However, in the present study, we could not indicate statistically significant assocation between CAT score and improper inhaler technique in COPD patients.

The type of inhaler device is a remarkable indicative of improper inhalation technique [9, 26-29]. Several studies have shown that MDI users had significantly more failures than users of other inhaler types [9, 27]. In contrast, a study indicated that patients using an MDI had a better inhalation technique [29]. Despite the two aspects, we were unable to discover the association between inhaler type and proper inhalation technique.

Previous studies evaluating the effects of age on inhaler technique have indicated that older patients make remarkably more mistakes in inhaler technique [26, 29]. However, two studies demonstrated that age was not significantly concerned to the patients' improper inhaler technique [26, 27]. Likewise, we could not show an association between patients age and proper inhalation technique.

The important factor that affected proper inhaler technique is level of education. One study demonstrated that patients with a higher educational level had significantly lower rate of mistakes [27]. However, it was found that there was no association between education level and proper inhaler technique in another study [26]. In the present study, while the rate of proper inhaler technique 39.1% for high school and university graduates and 60.9% for illiterate and primary or secondary school graduates ($p=0.072$) were not different, the total inhalation skills scores of different education level groups were found different ($p=0.037$). The mean inhalation skills score for illiterates was 5.9, while it was 7.9 for university graduates.

In this study in which both groups were included, the HAM-A scores weren't affect the proper inhaler technique. According to the HAM-A score, the rate of proper inhaler technique in patients with mild anxiety is 50%, while this rate is 27.7% in those with moderate and severe anxiety ($p=0.096$). In addition, the correlation between HAM-A and inhalation skills scores approached statistical significance ($r=-0.208$, $p=0.083$). In a study which was evaluated the association between inhaler MNA and anxiety in patients with asthma, it has reported that poor medication compliance in patients with asthma were

shown higher mean score for anxiety than good compliance group but these results were not significant [30]. In a parallel study involving patients with COPD, researchers could not determine a statistically significant difference between the inhaler MNA and anxiety [31]. Both studies [30, 31] included a single patient group (asthma or COPD), and only one inhaler device was evaluated for MNA (turbuhaler or diskus). Four inhaler device types were also included in our study, and we found patients with anxiety have more improper inhaler technique, but the results are not significant.

In a research study 84 COPD patients' treatment status, medication adherence and, and several factors that influence were evaluated over two months during COVID-19 pandemic. They discovered that 27.3% were predisposed to treatment reduction or discontinuation due to concerns about the COVID-19 pandemic, and that 31% of patients had possible depression, which was an independent risk factor for poor medication adherence. One of their study's limitations was that they couldn't meet with patients face to face [32]. Unlike this study, we had a face-to-face meeting with each patient. We didn't measure directly medication adherence, but our results showed that COVID-19 epidemic and mood disorders like as apprehensive status were not affect the inhaler technique.

This study has some limitations that may have reduced the generality of the results. If only we evaluated more patients, we'd be able to find a significant association between anxiety and inhaler technique. The number of patients admitted to chest diseases outpatient clinics is not at the expected level because to the social isolation experienced during the COVID-19 pandemic. So, the study's patient enrollment is not at the desired level. One of the limitations was assessing each patient's ability with a single inhaler type to keep the interviews short due to the pandemic**.**

The present study concludes that most COPD and asthma patients use their inhalers improperly. There wasn't any statistically significant correlation between proper inhaler technique and any parameter (CAT score, HAM-A score, CAS score, age, education level, comorbidity, inhaler device type) examined. However, patients with lower HAM-A stage and higher level of education used inhaler more properly, but it was not found statistically significant. Therefore, more attention should be paid to inhaler use skills training, especially in the low education level group and moderate-severe anxiety group.

In the present study, CAS score was not found to be associated with proper inhaler technique. HAM-A score was found to affect proper inhaler technique negatively, but these results are not significant. More studies are needed to shed light on the association between anxiety scores and proper inhaler technique.

ACKNOWLEDGEMENTS

We would like to thank our co-workers at the chest diseases outpatient clinic for making this study possible.

AUTHOR CONTRIBUTIONS

Conception: *M.D., S.G.;* Design: *M.D., S.G.;* Control: *M.D., S.G., Ö.F.B., Z.Ü.G., S.S.H.;* Sources: *M.D., S.G., Ö.F.B., Z.Ü.G., S.S.H.;* Materials: *M.D., S.G., Ö.F.B.;* Data Collection and/or processing: *M.D., S.G., Ö.F.B.;* Analysis and/or interpretation: *M.D., S.G., Ö.F.B.*; Literature review: *M.D., S.G., Ö.F.B.;* Manuscript writing: *M.D., S.G., Ö.F.B., Z.Ü.G., S.S.H.;* Critical review: *M.D., S.G., Ö.F.B., Z.Ü.G., S.S.H.*; Other:-

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS COMMITTEE APPROVAL

The study was approved by the ethics committee of the Inonu University on 24.11.2020 (decision number: 2020/1231).

REFERENCES

1. Jardim, J.R., Nascimento, O.A. (2019). The importance of inhaler adherence to prevent COPD exacerbations. *Medical Science (Basel), 7*(4), 54-64. [CrossRef]

2. George, M., Bender, B. (2019). New insights to improve treatment adherence in asthma and COPD. *Patient Preference and Adherence, 13*, 1325-1334. [CrossRef]

3. Sundbom, L.T., Bingefors K. (2013). The influence of symptoms of anxiety and depression on medication nonadherence and its causes: a population based survey of prescription drug users in Sweden. *Patient Preference and Adherence, 7*, 805-811. [CrossRef]

4. Bauer, L.K., Caro M.A., Beach S.R., Mastromauro, C.A., Lenihan, E., Januzzi, J.L., Huffman, J.C. (2012). Effects of depression and anxiety improvement on adherence to medication and health behaviors in recently hospitalized cardiac patients. *The American Journal of Cardiology, 109*(9), 1266-1271. [CrossRef]

5. Venkata, V.S, Kiernan, G. (2020). Covid-19 and COPD: pooled analysis of observational studies. *Chest Annual Meeting, 158*(4), A2469. [CrossRef]

6. Leung, J.M, Niikura, M., Yang, C.W.T., Sin, D.D. (2020). COVID-19 and COPD. *The European Respiratory Journal, 56*(2), 2002108. [CrossRef]

7. Sancar, M., Sirinoğlu, Y., Okuyan, B., Karagoz, T., Izzettin, F.V. (2015) The effect of pharmacist-led education on inhaler use skills in hospitalised patients with chronic obstructive pulmonary disease. *European Journal of Hospital Pharmacy, 22*, 366-368. [CrossRef]

8. Bryant, L., Bang, C., Chew, C., Baik, S.H., Wiseman, D. (2013) Adequacy of inhaler technique used by people with asthma or chronic obstructive pulmonary disease. *Journal of Primary Health Care, 5,* 191-198. [CrossRef]

9. Batterink, J., Dahri, K., Aulakh, A., Rempel, C. (2012). Evaluation of the use of inhaled medications by hospital inpatients with chronic obstructive pulmonary disease. *The Canadian Journal of Hospital Pharmacy, 65,* 111-118. [CrossRef]

10. Bech, P. (2011). Measuring states of anxiety with clinician-rated and patient-rated scales. In: S. Selek (Ed), Different views of anxiety disorders, (pp.169-84) Crotia: InTech.

11. Yazıcı, M., Demir, B., Tanrıverdi, N., Karaağaoğlu, E., Yolaç, P. (1998). Hamilton Anksiyete Değerlendirme Ölçeği, değerlendiriciler arası güvenirlik ve geçerlik çalışması. *Türk Psikiyatri Dergisi, 9,*114-117.

12. GOLD. Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Pulmonary Disease. (2020) https://goldcopd.org/wp-content/uploads/2019/12/GOLD-2020-FINAL-ver1.2- Accessed 03.12.2019.

13. Yorgancıoğlu, A., Polatlı, M., Aydemir, Ö., Yılmaz Demirci, N., Kırkıl, G., Naycı Atış, S., Köktürk, N., Uysal, A., Akdemir, S.A., Özgür, E.S., Günakan, G. (2012). Reliability and validity of Turkish version of COPD assessment test. *Tuberkuloz ve Toraks, 60*(4), 314-320. [CrossRef]

14. Biçer, İ., Çakmak, C., Demir, H., Kurt, M.E. (2020). Koronavirüs Anksiyete Ölçeği Kısa Formu: Türkçe Geçerlik ve Güvenirlik Çalışması. *Anadolu Kliniği Tıp Bilimleri Dergisi, 25,* 216-225. [CrossRef]

15. Kühl, K., Schürmann, W., Rief, W. (2008). Mental disorders and quality of life in COPD patients and their spouses. *International Journal of Chronic Obstructive Pulmonary Disease, 3,* 727-736. [CrossRef]

16. Carvalho, N.S., Ribeiro, P.R., Ribeiro, M., Nunes, P.T., Cukier A., Stelmach R. (2007). Comparing asthma and chronic obstructive pulmonary disease in terms of symptoms of anxiety and depression. *Jornal Brasileiro de Pneumologia, 33,* 1-6. [CrossRef]

17. Yohannes A.M. (2021). COPD patients in a COVID-19 society: depression and anxiety. *Expert Rewiev of Respiratory Medicine*, *15*(1), 5-7. [CrossRef]

18. Oraka, E., King, M.E., Callahan, D.B. (2010). Asthma and serious psychological distress: prevalence and risk factors among US adults, 2001-2007. *Chest Journal, 137,* 609-616. [CrossRef]

19. Kunik, M.E., Roundy, K., Veazey, C., Souchek J., Richardson, P., Wray, N.P., Stanley, M.A. (2005). Surprisingly high prevalence of anxiety and depression in chronic breathing disorders. *Chest Journal, 127,* 1205-1211. [CrossRef]

20. Philip, K.E.J., Lonergan, B., Cumella, A., Farrington-Douglas, J., Laffan, M., Hopkinson, N.S. (2020). COVID-19 related concerns of people with long-term respiratory conditions: a qualitative study. *BioMedCentral Pulmonary Medicine, 20,* 1-10. [CrossRef]

21. Júnior, J.L.R.S., Conde, M.B., de Sousa Corrêa, K., da Silva, C., da Silva Prestes, L., Rabahi, M.F. (2014). COPD Assessment Test (CAT) score as a predictor of major depression among

subjects with chronic obstructive pulmonary disease and mild hypoxemia: a case–control study. *BioMedCentral Pulmonary Medicine, 14,* 186-192. [CrossRef]

22. Hilmarsen, C.W., Wilke, S., Engan, H., Spruit, M.A., Rodenburg J., Janssen, D.J.A., Steinshamn, S., Jones, P.W., Wouters, E.F.M., Oldervoll L., Franssen, F.M.E. (2014). Impact of symptoms of anxiety and depression on COPD Assessment Test scores. *European Respiratory Journal, 43,* 898-900. [CrossRef]

23. Long J, Ouyang Y, Duan H, Xiang Z., Ma, H., Ju, M., Sun, D. (2020). Multiple factor analysis of depression and/or anxiety in patients with acute exacerbation chronic obstructive pulmonary disease. *International Journal of Chronic Obstructive Pulmonary Disease, 15,* 1449-1464. [CrossRef]

24. Lee Y.S., Park S., Oh Y-M., Lee S-D., Park S-W., Kim, Y.S., In, K.H., Jung, B.H., Lee, K.H., Ra, S.W., Hwang, Y.I., Park, Y-B., Jung, K-S. (2013). Chronic obstructive pulmonary disease assessment test can predict depression: a prospective multi-center study. *Journal of Korean Medical Science, 28,* 1048-1054. [CrossRef]

25. Jones, P., Harding, G., Berry, P., Jones, P.W., Harding, G., Berry, P., Wiklund, I., Chen, W-H., Leidy, N.K. (2009) Development and first validation of the COPD Assessment Test. *The European Respiratory Journal, 34,* 648-654. [CrossRef]

26. E. Hesselink BWP, Hanneke AH Wijnhoven, Didi MW Kriegsman, Jacques TM van Eijk, Arlette. (2001). Determinants of an incorrect inhalation technique in patients with asthma or COPD. *Scandinavian Journal of Primary Health Care, 19,* 255-260. [CrossRef]

27. Amini, S., Ghasemi, A., Solduzian, M., Rahimi, B., Heidari, M.K., Hadjibabaie, M., Kargar, M. (2020). Is Inhaler Technique Associated with Quality of Life in Patients with Chronic Obstructive Pulmonary Disease? *Current Therapeutic Research, 93,* 608-614. [CrossRef]

28. Gregoriano, C., Dieterle, T., Breitenstein, A-L., Dürr, S., Baum, A., Maier, S., Arnet, I., Hersberger, K.E., Leuppi, J.D. (2018). Use and inhalation technique of inhaled medication in patients with asthma and COPD: data from a randomized controlled trial. *Respiratory Research, 19,* 1-15. [CrossRef]

29. van Beerendonk, I., Mesters, I., Mudde, A.N., Tan, T. (1998). Assessment of the inhalation technique in outpatients with asthma or chronic obstructive pulmonary disease using a metered-dose inhaler or dry powder device. *The Journal of Asthma, 35,* 273-279. [CrossRef]

30. Bosley, C., Fosbury, J., Cochrane, G. (1995). The psychological factors associated with poor compliance with treatment in asthma. *The European Respiratory Journal, 8,* 899-904.

31. Sulaiman, I., Cushen, B., Greene, G., Seheult, J., Seow, D., Rawat, F., MacHale, E., Mokoka, M., Moran, C.N., Sartini Bhreathnach, A., MacHale, P., Tappuni, S., Deering, B., Jackson, M., McCarthy, H., Mellon, L., Doyle, F., Boland, F., Reilly, R.B., Costello, R.W. (2017). Objective assessment of adherence to inhalers by patients with chronic obstructive pulmonary disease. *American Journal of Respiratory and Critical Care Medicine, 195,* 1333-1343. [CrossRef]

32. Zhang, H-Q., Lin, J-Y., Guo, Y., Pang, S., Jiang, R., Cheng, Q-J. (2020). Medication adherence among patients with chronic obstructive pulmonary disease treated in a primary general hospital during the COVID-19 pandemic. *Annals of Translational Medicine, 8,* 1179-1188. [CrossRef]

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 805-816, 2022
Doi: 10.33483/jfpau.1081893

ORIGINAL ARTICLE / ÖZGÜN MAKALE

AN INVESTIGATION ON THE EFFECTS OF SIRT5 MODULATORS ON SIRT5 AND CYTOCHROME C PROTEIN EXPRESSIONS IN K562 CHRONIC MYELOID LEUKEMIA CELL LINE

K562 KRONİK MİYELOİD LÖSEMİ HÜCRE HATTINDA SIRT5 MODÜLATÖRLERİNİN SIRT5 VE SİTOKROM C PROTEİN EKSPRESYONLARI ÜZERİNE ETKİLERİNİN ARAŞTIRILMASI

Tulin OZKAN[1], Asli KOC[2*], Arzu Zeynep KARABAY[2], Yalda HEKMATSHOAR[1,3], Asuman SUNGUROGLU[1]

[1] Ankara University, Faculty of Medicine, Department of Medical Biology, Ankara, Turkey
[2]Ankara University, Faculty of Pharmacy, Department of Biochemistry, Ankara, Turkey
[3] Altınbas University, Faculty of Medicine, Department of Medical Biology, İstanbul, Turkey

ABSTRACT

Objective: *SIRT5 is a mitochondrial protein that removes acetyl, malonyl and succinyl groups from lysine moieties in target proteins and interacts with cytochrome c and causes its deacetylation. There is no study on the effects of SIRT5 in K562 chronic myeloid leukemia cells. Resveratrol and Suramin are known to play a role in modulating the deacetylase and desuccinylase activities of SIRT5. It has been reported that Resveratrol induces apoptosis of K562 cells but effects of Suramin on the apoptosis of K562 cells are largely unknown. In this study, it was aimed to elucidate the effects of SIRT5 modulators Resveratrol and Suramin on proliferation and apoptosis of K562 cells and on SIRT5 and cytochrome c protein, a known target of SIRT5.*

Material and Method: *K562 chronic myeloid leukemia cells were treated with increasing concentrations of suramin and resveratrol, cell proliferation was determined by MTT assay and BrdU incorporation. Apoptosis was determined with Annexin V staining by Flow cytometry. Western Blot analysis was performed to determine the effect of resveratrol and suramin on SIRT5 and Cytochrome c protein expression levels.*

Result and Discussion: *Our results showed that suramin did not affect SIRT5 and cytochrome c protein expressions significantly and resveratrol decreased SIRT5 and increased cytochrome c expression. Suramin did not cause any changes on the apoptosis of K562 cells. Resveratrol decreased cell proliferation and induced*

* **Corresponding Author / Sorumlu Yazar:** Asli Koc
e-mail / e-posta: akoc@ankara.edu.tr, **Phone / Tel.:** +903122033296

Submitted / Gönderilme: 02.03.2022 **Accepted / Kabul:** 28.07.2022

apoptosis of K562 cells in accordance with the literature. The SIRT5-lowering effect of Resveratrol may have mediated its apoptotic effects.

Keywords: *Apoptosis, K562, Resveratrol, SIRT5, Suramin*

ÖZ

Amaç: *SIRT5, hedef proteinlerdeki lizin rezidülerinden, asetil, malonil ve süksinil gruplarını uzaklaştıran ve sitokrom c ile etkileşerek, onun deasetilasyonuna neden olan bir mitokondriyal proteindir. SIRT5'in K562 kronik miyeloid lösemi hücrelerindeki etkilerine ilişkin bir çalışma bulunmamaktadır. Resveratrol ve Suramin'in SIRT5'in deasetilaz ve desüksinilaz aktivitelerini modüle etmede rol oynadığı bilinmektedir. Resveratrol'ün K562 hücrelerinin apoptozunu indüklediği bildirilmiştir. Ancak Suramin'in K562 hücrelerinin apoptozu üzerindeki etkileri büyük ölçüde bilinmemektedir. Bu çalışmada, SIRT5 modülatörleri Resveratrol ve Suramin'in K562 hücrelerinin proliferasyonu ve apoptozu ile SIRT5 ve SIRT5'in bilinen bir hedefi olan sitokrom c proteini üzerindeki etkilerinin aydınlatılması amaçlanmıştır.*

Gereç ve Yöntem: *K562 kronik miyeloid lösemi hücrelerine artan konsantrasyonlarda Suramin ve Resveratrol uygulandı. Hücre proliferasyonu MTT analizi ve BrdU inkoporasyon yöntemi ile belirlendi. Apoptoz, Akım sitometrisi ile Annexin V boyaması ile belirlendi. Resveratrol ve Suramin'in SIRT5 ve Sitokrom c protein ekspresyon seviyeleri üzerindeki etkisini belirlemek için Western Blot analizi yapıldı.*

Sonuç ve Tartışma: *Sonuçlarımız, Suramin'in SIRT5 ve sitokrom c protein ekspresyonlarını önemli ölçüde etkilemediğini ve Resveratrol'ün SIRT5'i azalttığını ve sitokrom c ekspresyonunu artırdığını göstermiştir. Suramin, K562 hücrelerinin apoptozunda herhangi bir değişikliğe neden olmamıştır. Resveratrol, literatüre uygun olarak hücre proliferasyonunu azaltmış ve K562 hücrelerinin apoptozunu indüklemiştir. Resveratrolün, SIRT5 protein ekspresyonunu azaltıcı etkisi ile apoptotik etkilerine aracılık etmiş olabileceği düşünülmektedir.*

Anahtar Kelimeler: *Apoptoz, K562, Resveratrol, SIRT5, Suramin*

INTRODUCTION

Chronic myeloid leukemia (CML) has been characterized by Philadelphia chromosome and Bcr-Abl protein [1]. Although tyrosine kinase inhibitors are used in the treatment of this disease, resistance to these inhibitors limits the treatment of the disease. Identification of new therapeutic agents and molecular pathways are promising strategies for the treatment of chronic myeloid leukemia [2].

Sirtuins (SIRT1-7) which require nicotinamide adenine dinucleotide (NAD(+)) for their activity are protein deacetylase enzymes and their functions are related with molecular pathways involved in tumorogenesis [3]. Although Sirtuin 5 (SIRT5) is mainly located in the mitochondrial matrix, it is also present in the cytosol, peroxisomes and nucleus. SIRT5 has vital roles in the regulation of mitochondrial proteins, mitochondrial respiration, nitrogen metabolism, glycolysis, pentose phosphate pathway and apoptosis [4-6]. However, increased SIRT5 mRNA expression has been shown in many cancer types including mammary tumors, gastric, colorectal and prostate cancer [7-9] and SIRT5 may exhibit tumor promoter functions and leads cisplatin resistance of ovarian cancer cells [10]. Several studies have reported decreased SIRT5 expression in glioblastoma tissues and hepatocellular carcinoma [3, 11]. However, there isn't any study in the literature that determines the expression of SIRT5 protein in chronic myeloid leukemia cells.

It has been shown that Resveratrol induces apoptosis of K562 chronic myeloid leukemia cell line [12-16] and one study reports the effects of Suramin on K562 cell proliferation [17]. There is no study in the literature investigating the effects of Resveratrol and Suramin on the protein expression levels of SIRT5 in K562 chronic myeloid leukemia cells. In this study, we explored the effects of SIRT5

modulators (Resveratrol and Suramin) on the cell viability and apoptosis of K562 chronic myeloid leukemia cells and also on the protein expression levels of SIRT5 and cytochrome c.

MATERIAL AND METHOD

Cell Culture

K562 chronic myeloid cell line was cultured in RPMI 1640 medium (Sigma) supplemented with 1% L-glutamine (Capricorn Scientific), 1% penicilin-streptomycin (Capricorn Scientific), 10% heat inactivated fetal bovine serum (Capricorn Scientific) at 37°C incubator which has %5 CO_2 and %95 O_2.

Cell Viability

In order to detect the effects of Suramin (Calbiochem) or Resveratrol (Sigma) on the proliferation of K562 cells, firstly cells (20.000 cells/well) were seeded to 96 well plates and treated with Suramin (0.1-100 μM) and Resveratrol (0.1-100 μM) for 72h. All wells (Suramin and Resveratrol treated and untreated) contained equal amounts of DMSO. Imatinib mesylate (IMA) was used as a positive control. Following incubation, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution was added to the wells then incubated for 4h. To dissolve formazan crystals, Sodium Dodecyl Sulfate-Hydrochloric acid (SDS-HCl) (0.01M) was used. Absorbance at 550 nm was measured using Microplate Reader (Molecular Devices-Spectra Max spectrophotometer, Sunnyvale, CA, USA). Cells which weren't treated with any substances were used as negative controls and viability of control cells was accepted as %100.

5-Bromo-2'-deoxyuridine (BrdU) Incorporation

Proliferation of K562 cells was determined by 5-bromo-2'-deoxyuridine (BrdU) incorporation assay according to BrdU Elisa kit (Cell Signalling) protocol. Birefly, cells (20.000 cells/well) were seeded to 96-well plates and SIRT5 modulators (0.1-100μM) were applied. IMA was used as a positive control. After 72h incubation, BrdU solution was added. Following 24 h incubation, cells were centrifuged (300g, 10 min) and supernatant was discarded. Fixing/denaturating solution 1X detection antibody solution was added. After washing steps, 1X HRP seconder antibody solution was added and after HRP substrate (TMB) substrate and the stop solution addition absorbance (at 450nm) was measured.

Western Blot Analysis

Western Blot analysis was performed to determine the effect of SIRT5 modulators on SIRT5 and Cytochrome c protein expression levels. K562 chronic myeloid leukemia cells were seeded to 6 well plates (500.000 cells/well) and incubated with Suramin (100μM) and Resveratrol (100μM) for 72 h. Total protein isolation was performed according to the manufacturers' recommendations (Active Motif). Protein concentrations of cell lysates were determined with Bradford Method (Thermo). Anti-beta actin primary antibody was used as housekeeping loading control. Proteins were separated by SDS

poliacrylamide gel electrophoresis and were transferred to nitrosellulose membrane and incubated with anti-SIRT5 (Cell Signaling Technology) and anti-Cytc (Cell Signalling Technology) primary antibodies. Proper secondary antibodies (Cell Signaling Technology) were used and protein bands were visualized with chemiluminesans imager (Licor CLX). Protein bands were analysed by Image J program.

Apoptotic Analysis

To determine the apoptotic effect of Suramin and Resveratrol, K562 cells were harvested in 6 well plates, and exposed to Suramin (100μM) and Resveratrol (100μM) for 72h. Cells were stained with Annexin V-7AAD and apoptosis was assessed with Flow cytometry. Cells treated with IMA were used as positive control.

Statistical Analysis

Statistical Analysis was performed by GraphPad Prism 7 software. Data were expressed as mean ± standart deviation. Differences between groups were determined by One Way Anova variance analysis and Tukey post hoc analysis was used. One Way Anova variance analysis was chosen to determine whether there are any statistically significant differences between the means of three or more independent (unrelated) groups. Tukey test was used since it is the most commonly used and powerful post-hoc test. $p<0.05$ is accepted as statistically significant.

RESULT AND DISCUSSION

Determination of the effects of SIRT 5 modulators on the proliferation of K562 cells by MTT Assay

To analyse the effects of SIRT5 modulators (Suramin and Resveratrol) on the viability of K562 cell line, cells were incubated with these modulators for 72h and MTT and BrdU Incorporation assays were performed. According to our results, Resveratrol (40-100μM) decreased the viability of cells significantly ($p<0.001$), whereas Suramin (0.1-100μM) did not exhibit any significant effect on the viability of K562 cells (Figure 1a and figure1b).

Determination of the effects of SIRT 5 modulators on the proliferation of K562 cells by BrdU incorporation

While there was no significant difference between the cells treated with Suramin (0.1-100μM) and the control group, a significant difference was observed between the control group and the cells treated between 40-100 μM Resveratrol ($p <0.05$). IMA (0.5μM) was used as a positive control (Figure 2a and 2b).

Determination of SIRT5 and Cytochrome c protein expression by Western Blot Analysis

Western blot analysis was performed to determine the effect of SIRT5 modulators on the expression of SIRT5 and Cytochrome c protein. According to our results, Resveratrol (100µM) decreased SIRT5 protein significantly ($p<0.05$), but Suramin did not show any significant effect on SIRT5 protein expression (Figure 3a and 3b). Moreover, Resveratrol significantly increased Cytochrome c protein expression ($p <0.05$), but Suramin did not show any significant effect on Cytochrome c protein expression (Figure 3c and 3d).

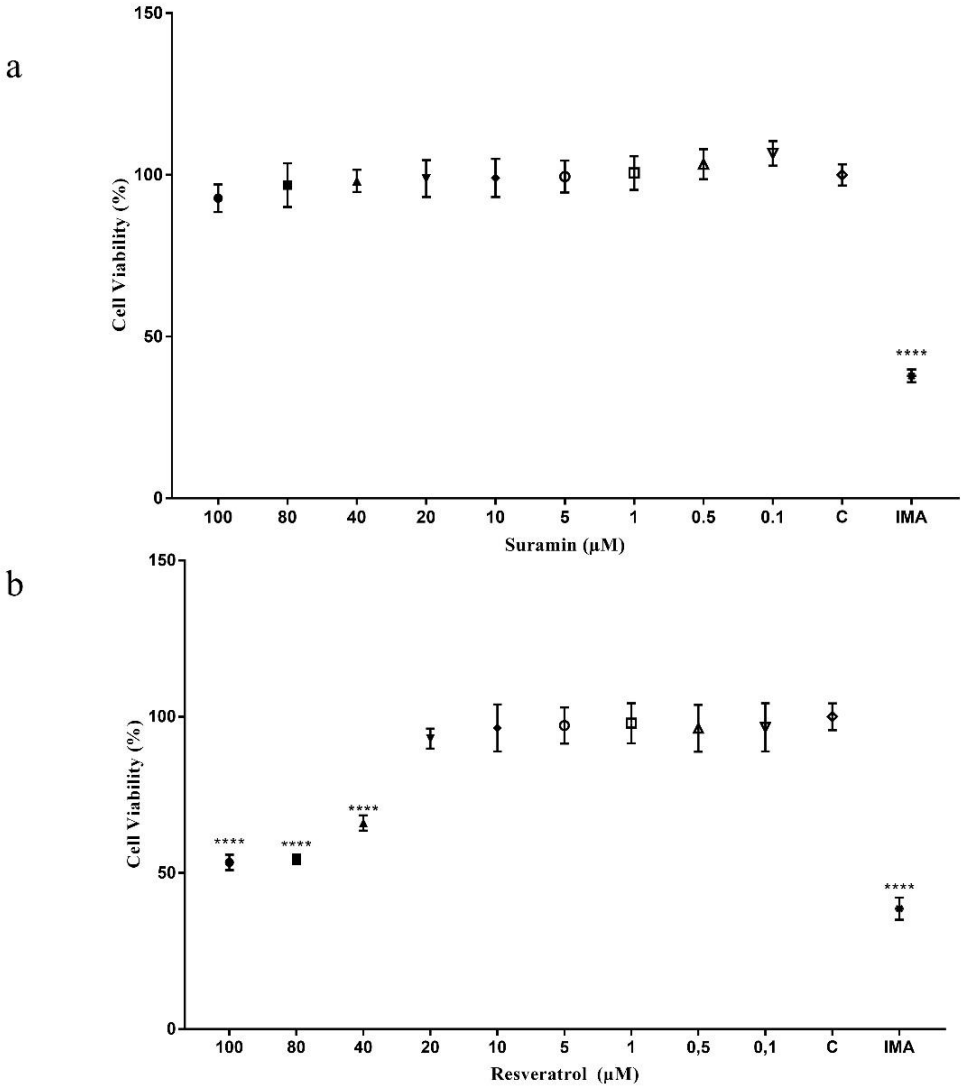


Figure 1. Effect of Suramin (a) and Resveratrol (b) on the viability of K562 cells by MTT Assay. Cells without Suramin or Resveratrol were used as a control group which is denoted by C (Control) and contains equal amounts of DMSO with the cells treated with different concentrations of Suramin or Resveratrol. IMA was used as a positive control. While Suramin did not affect cell viability significantly between 0.1-100µM concentration range, Resveratrol decreased cell viability between 40-100 µM concentration range significantly ($p<0.0001$). **** $p<0.0001$ compared to control.

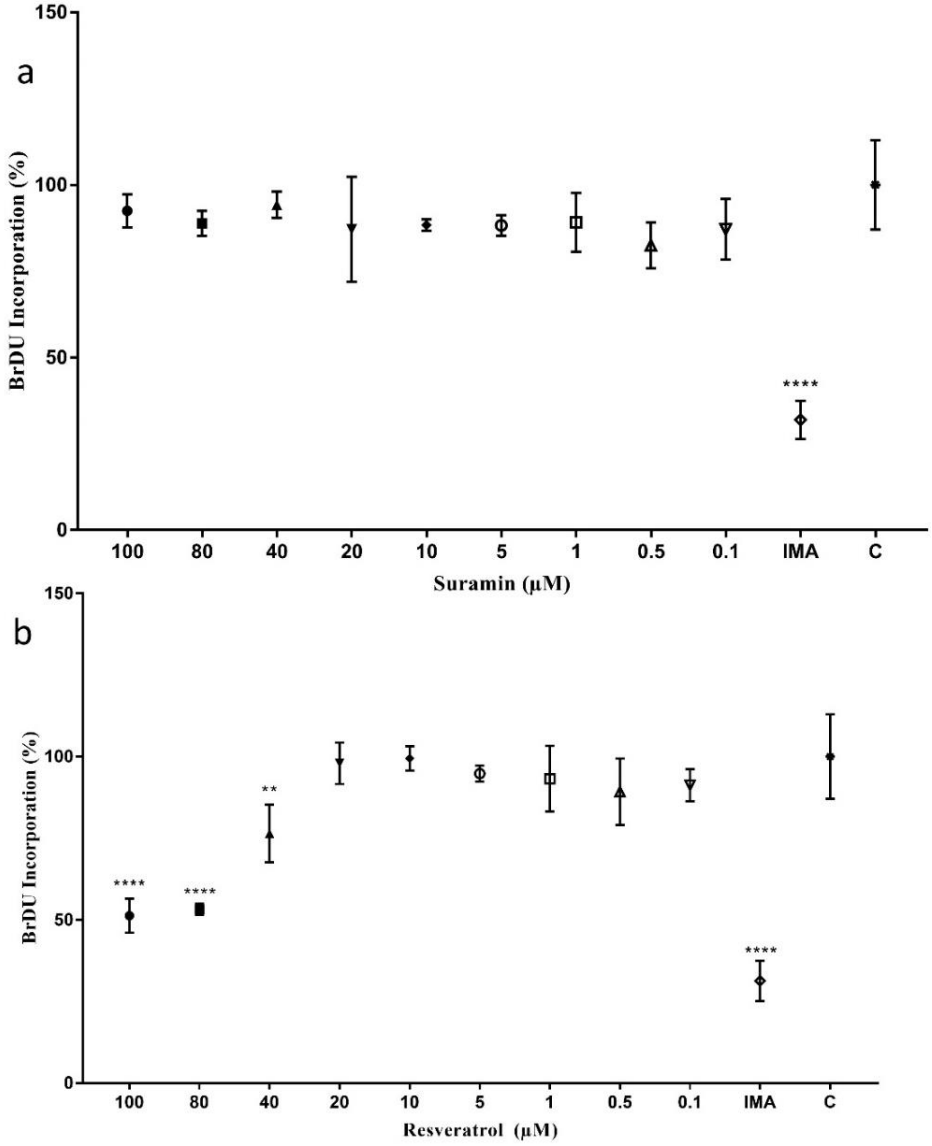


Figure 2. Effect of Suramin (A) and Resveratrol (B) on the viability of K562 cells by BrdU Assay. Cells without suramin or Resveratrol were used as control group which is denoted by C (Control) and contains equal amounts of DMSO with the cells treated with diffrent concentrations of suramin or Resveratrol. IMA was used as a positive control. While suramin did not affect cell viability significantly between 0.1-100µM concentration range, Resveratrol decreased cell viability between 40-100 µM concentration range significantly ($p<0.05$). *** $p<0.001$ and ****$p<0.0001$ compared to control.

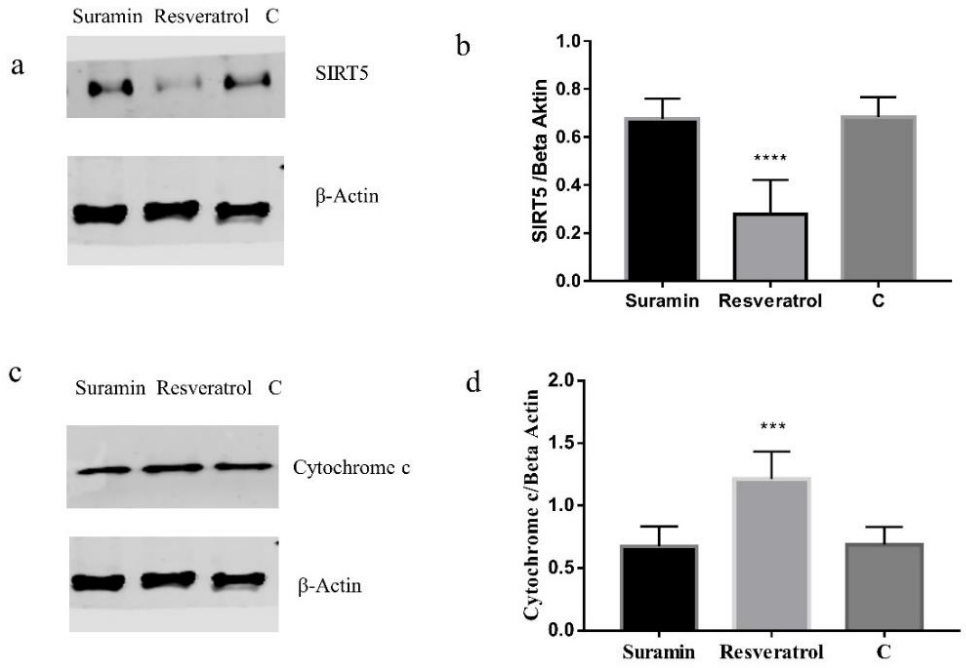


Figure 3. Effect of Suramin (100µM) and Resveratrol (100µM) on SIRT5 (a and b) and Cytochrome C (c and d) protein expression in K562 cell line. Resveratrol decreased SIRT5 and increased Cytochrome c protein expression significantly ($p<0.05$). Suramin did not effect significantly both SIRT5 and Cytochrome c protein expressions. *** $p<0.001$ and ****$p<0.0001$ compared to control.

Effects of Suramin and Resveratrol on Apoptosis of K562 cells

As a result of our analysis, it was determined that Resveratrol induces apoptosis of K562 cells ($p<0.05$) and Suramin did not exhibit any significant apoptosis inducing effect in K562 cells (Figure 4a and 4b).

In this study, we investigated the effects of Suramin and Resveratrol on the protein expressions of SIRT5 and its target Cytochrome c in K562 chronic myeloid leukemia cell line. We aimed to determine the correlations between the alterations of the SIRT5-cytochrome c axis and K562 cell apoptosis. Sirtuins which are NAD+ dependent protein deacetylases and mono-ADP-ribosyltransferases play important roles in cell proliferation, apoptosis, aging, and caloric restriction [18]. Association between sirtuins and cancer was revealed in recent studies [19, 20].

Cytochrome c is the target of SIRT5 protein and it is released from mitochondria to cytoplasm and plays an important role in apoptosis [21]. Guan et al reported that SIRT1/SIRT5 can cause deacetylation of tumor suppressor promyelocytic leukemia protein (PML) [22] and Zhang et al. showed deacetylation of Cytochrome c by SIRT5 and co-localization of SIRT5 and Cytochrome c [23].

Resveratrol is a natural phenol found in a variety of foods including grapes and berries. It was reported that it regulates many molecular targets and exhibits antitumor effects in many types of cancer [13, 24]. Resveratrol induces apoptosis of K562 cells via Bax and cytochrome c release [25] and its therapeutic effects has been shown in IMA resistant and IMA sensitive K562 cells [12]. In this study,

the role of Resveratrol on K562 cell apoptosis is evaluated together with its effect on SIRT5 protein expression. We found that Resveratrol decreases the viability of K562 cells and increases apoptosis significantly. It has been shown that in endothelial cells, there is a relationship between the anti-inflammatory effects of Resveratrol and SIRT1 and SIRT5 proteins [26]. Although it has been shown that Resveratrol activates deacetylase activity of SIRT5, Resveratol did not impact SIRT5 protein expressions in H295R cells [27, 28]. This is the first study investigating the effects of Resveratrol on SIRT5 protein expression in K562 cells. In our study, we found that Resveratrol decreases SIRT5 protein expression and increases cytochrome c expression significantly.

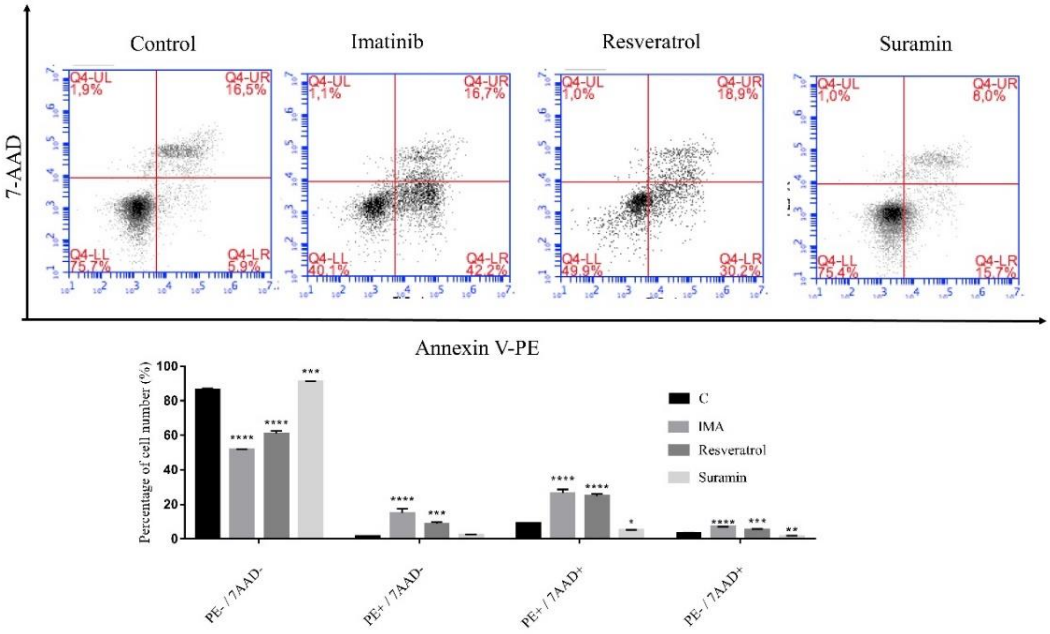


Figure 4. Effect of Suramin and Resveratrol on the apoptosis of K562 cell line. IMA was used as positive control. Cells without Suramin or Resveratrol were used as negative controls (C). (a) According to flow cytometry results, Resveratrol (100µM) increased apoptosis of K562 cells significantly ($p<0.05$) and Suramin (100µM) did not show any effect on apoptosis significantly. (b) Flow cytometry results were represented as bar graph. Viable cells are Annexin V-PE -/7AAD -, early apoptotic cells are Annexin V-PE +/7AAD-), late apoptotic cells are Annexin V-PE +/7AAD +. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ compared to control

Suramin, polysulfonated naphthylurea, has an antiproliferative and antiviral activity [29]. Suramin has also been considered as a pharmacological inhibitor of SIRT5 deacetylase activity [30, 31]. Suramin has broad range functions from antiparasitic/antiviral to anticancer [32]. In studies on the role of Suramin in cancer, it was shown that Suramin leads to growth inhibition of human stomach cancer cell lines (SNU-5 and SNU-16) [33] and inhibition of the proliferation of pancreatic cancer cells [34]. While Suramin has marked inhibitory effect on cell proliferation of KMS-11, 12, KMM-1, KMS5 myeloma cells and Raji, KMS-9 and HL-60 hematopoietic cell lines, it hasn't any singnificant

antiproliferative effect on RPMI 8226 and K562 cell lines [17]. There is no study in the literature investigating the effects of Suramin on apoptosis in K562 cells. In our study, we found that Suramin did not affect the proliferation and apoptosis of K562 cells as well as it did not affect SIRT5 and cytochrome c protein expression significantly. According to Schuetz at al., Suramin inhibited NAD (+) dependent decetylation activity of SIRT5 via binding to SIRT5 [31]. On the other hand, in our study another SIRT5 modulator Resveratrol was found to increase cytochrome c protein expression significantly in accordance with the literature [35]. This study reveals that two different SIRT5 modulators may have different effects on the protein expression of SIRT5 and apoptosis.

In conclusion, SIRT5 modulators exhibited different effects on the proliferation and apoptosis of K562 cells. Suramin did not show any significant antiproliferative and apoptosis inducing effect on K562 cells. On the other hand, Resveratrol was found to reduce cell proliferation and induce apoptosis significantly. Suramin had no significant effect on SIRT5 and Cytochrome c protein expressions, whereas Resveratrol decreased SIRT5 protein expression and increased cytochrome c protein expression significantly. This study will contribute to future studies on the role of SIRT5 protein in the treatment of chronic myeloid leukemia.

ACKNOWLEDGEMENTS

This study was supported by Ankara University Scientific Research Projects Coordination Unit with the project number 18H0237008.

AUTHOR CONTRIBUTIONS

Concept: *A.K., T.O., A.Z.K*; Design: *A.K., A.Z.K., T.O.*; Control: *A.K., A.Z.K., T.O., Y.H.*; Sources: *A.K., T.O., A.Z.K., Y.H., A.S.;* Materials: A.K., *T.O., A.Z.K., Y.H., A.S.*; Data Collection and/or processing: *A.K., T.O., A.Z.K., Y.H., A.S.*; Analysis and/or interpretation: *A.K., T.O., A.Z.K., Y.H., A.S.*; Literature review: *A.K., T.O., A.Z.K., Y.H., A.S.*; Manuscript writing: *A.K., T.O., A.Z.K., Y.H., A.S.*; Critical review: *A.K., T.O., A.Z.K., Y.H., A.S.*; Other: *A.K., T.O., A.Z.K., Y.H.*

CONFLICT OF INTEREST

Authors confirm no declaration of interest.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

REFERENCES

1. Deininger, M.W., Goldman, J.M., Melo, J.V. (2000). The molecular biology of chronic myeloid leukemia. *Blood*, *96*(10), 3343-3356. [CrossRef]

2. Pophali, P.A., Patnaik, M.M. (2016). The Role of New Tyrosine Kinase Inhibitors in Chronic Myeloid Leukemia. *The Cancer Journal*, *22*(1), 40-50. [CrossRef]

3. Chen, X., Xu, Z., Zeng, S.,Wang, X., Liu,W.,Qian, L.,Wei, J., Yang, X., Shen, Q., Gong, Z., Yan, Y. (2019). SIRT5 downregulation is associated with poor prognosis in glioblastoma. *Cancer Biomarkers*, *24*(4), 449-459. [CrossRef]

4. Bringman-Rodenbarger, L.R., Guo, A.H., Lyssiotis, C. A., Lombard, D.B. (2018). Emerging roles for SIRT5 in metabolism and cancer. *Antioxidants & Redox Signaling*, *28*(8), 677-690. [CrossRef]

5. Kumar, S., Lombard, D. B. (2018). Functions of the sirtuin deacylase SIRT5 in normal physiology and pathobiology. *Critical Reviews in Biochemistry and Molecular Biology*, *53*(3), 311-334. [CrossRef]

6. Wang, C. H., Wei, Y.H. (2020). Roles of mitochondrial sirtuins in mitochondrial function, redox homeostasis, insulin resistance and type 2 diabetes. *International Journal of Molecular Sciences*, *21*(15), 5266. [CrossRef]

7. Greene, K. S., Lukey, M. J., Wang, X., Blank, B., Druso, J. E., Lin, M. J., Stalnecker, C.A., Zhang, C., Abril, Y. N., Erickson, J.W., Wilson, K.F., Lin, H., Weiss, R.S., Cerione, R. A. (2019). SIRT5 stabilizes mitochondrial glutaminase and supports breast cancer tumorigenesis. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(52), 26625-26632. [CrossRef]

8. Guan, J., Jiang, X., Gai, J., Sun, X., Zhao, J., Li, J., Li, Y., Cheng, M., Du, T., Fu, L., Li, Q. (2020). Sirtuin 5 regulates the proliferation, invasion and migration of prostate cancer cells through acetyl-CoA acetyltransferase 1. *Journal of Cellular and Molecular Medicine*, *24*(23), 14039-14049. [CrossRef]

9. Wang, K., Hu, Z., Zhang, C., Yang, L., Feng, L., Yang, P., Hu, H. (2020). SIRT5 Contributes to Colorectal Cancer Growth by Regulating T Cell Activity. *Journal of Immunology Research*, *2020*, 3792409. [CrossRef]

10. Sun, X., Wang, S., Gai, J., Guan, J., Li, J., Li, Y., Zhao, J., Zhao, C., Fu, L., Li, Q. (2019). SIRT5 Promotes Cisplatin Resistance in Ovarian Cancer by Suppressing DNA Damage in a ROS-Dependent Manner via Regulation of the Nrf2/HO-1 Pathway. *Frontiers in Oncology*, *9*, 754. [CrossRef]

11. Guo, D., Song, X., Guo, T., Gu, S., Chang, X., Su, T., Yang, X., Liang, B., Huang, D. (2018). Vimentin acetylation is involved in SIRT5-mediated hepatocellular carcinoma migration. *American Journal of Cancer Research*, *8*(12), 2453-2466.

12. Can, G., Cakir, Z., Kartal, M., Gunduz, U., Baran, Y. (2012). Apoptotic effects of resveratrol, a grape polyphenol, on imatinib-sensitive and resistant K562 chronic myeloid leukemia cells. *Anticancer Research*, *32*(7), 2673-2678.

13. Chakraborty, P.K., Mustafi, S.B., Ganguly, S., Chatterjee, M., Raha, S. (2008). Resveratrol induces apoptosis in K562 (chronic myelogenous leukemia) cells by targeting a key survival protein, heat shock protein 70. *Cancer Science*, *99* (6), 1109-1116. [CrossRef]

14. Kartal, M., Saydam, G., Sahin, F., Baran, Y. (2011). Resveratrol triggers apoptosis through regulating ceramide metabolizing genes in human K562 chronic myeloid leukemia cells. *Nutrition and Cancer, 63*(4), 637-644. [CrossRef]

15. Wang, B., Liu, J., Gong, Z. (2015). Resveratrol induces apoptosis in K562 cells via the regulation of mitochondrial signaling pathways. *International Journal of Clinical and Experimental Medicine, 8*(9), 16926-16933.

16. Yan, H.W., Hu, W.X., Zhang, J.Y., Wang, Y., Xia, K., Peng, M. Y., Liu, J. (2014). Resveratrol induces human K562 cell apoptosis, erythroid differentiation, and autophagy. *Tumour Biology, 35*, 5381-5388. [CrossRef]

17. Bai, L., Naomoto, Y., Miyazaki, M., Orita, K., Namba, M. (1992). Antiproliferative effects of suramin on human cancer cells in vitro and in vivo. *Acta Medica Okayama, 46*(6), 457-463. [CrossRef]

18. Carafa, V., Rotili, D., Forgione, M., Cuomo, F., Serretiello, E., Hailu, G. S., Jarho, E., Lahtela-Kakkonen, M., Mai, A., Altucci, L. (2016). Sirtuin functions and modulation: from chemistry to the clinic. *Clinical Epigenetics, 8*, 61. [CrossRef]

19. Costa-Machado, L. F., Fernandez-Marcos, P. J. (2019). The sirtuin family in cancer. *Cell Cycle, 18*(18), 2164-2196. [CrossRef]

20. O'Callaghan, C., Vassilopoulos, A. (2017). Sirtuins at the crossroads of stemness, aging, and cancer. *Aging Cell, 16*, 1208-1218. [CrossRef]

21. Lim, M.L., Lum, M.G., Hansen, T.M., Roucou, X., Nagley, P. (2002). On the release of cytochrome c from mitochondria during cell death signaling. *Journal of Biomedical Science, 9*, 488-506. [CrossRef]

22. Guan, D., Lim, J.H., Peng, L., Liu, Y., Lam, M., Seto, E., Kao, H.Y. (2014). Deacetylation of the tumor suppressor protein PML regulates hydrogen peroxide-induced cell death. *Cell Death & Disease, 5*, e1340. [CrossRef]

23. Zhang, R., Wang, C., Tian, Y., Yao, Y., Mao, J., Wang, H., Li, Z., Xu, Y., Ye, M., Wang, L. (2019). SIRT5 Promotes Hepatocellular Carcinoma Progression by Regulating Mitochondrial Apoptosis. *Journal of Cancer, 10*, 3871-3782. [CrossRef]

24. Chao, S. C., Chen, Y. J., Huang, K. H., Kuo, K. L., Yang, T. H., Huang, K. Y., Wang, C.C., Tang, C.H., Yang, R.S., Liu, S.H. (2017). Induction of sirtuin-1 signaling by resveratrol induces human chondrosarcoma cell apoptosis and exhibits antitumor activity. *Scientific Reports, 7*, 3180. [CrossRef]

25. Luzi, C., Brisdelli, F., Cinque, B., Cifone, G., Bozzi, A. (2004). Differential sensitivity to resveratrol-induced apoptosis of human chronic myeloid (K562) and acute lymphoblastic (HSB-2) leukemia cells. *Biochemical Pharmacology, 68*(10), 2019-2030. [CrossRef]

26. Yu, H., Pan, W., Huang, H., Chen, J., Sun, B., Yang, L., Zhu, P. (2019). Screening Analysis of Sirtuins Family Expression on Anti-Inflammation of Resveratrol in Endothelial Cells. *Medical Science Monitor, 25*, 4137-4148. [CrossRef]

27. Gertz, M., Nguyen, G.T.T., Fischer, F., Suenkel, B., Schlicker, C., Fränzel, B., Tomaschewski, J., Aladini, F., Becker, C., Wolters, D., Steegborn, C. (2012). A molecular mechanism for direct sirtuin activation by resveratrol. *PLoS One, 7*(11), e49761. [CrossRef]

28. Marti, N., Bouchoucha, N., Sauter, K. S., Flück, C. E. (2017). Resveratrol inhibits androgen production of human adrenocortical H295R cells by lowering CYP17 and CYP21 expression and activities. *PLoS One, 12*, e0174224. [CrossRef]

29. Perabo, F. G., Müller, S. C. (2005). New agents in intravesical chemotherapy of superficial bladder cancer. *Scandinavian Journal of Urology and Nephrology, 39*(2), 108-116. [CrossRef]

30. Gertz, M., Steegborn, C. (2010). Function and regulation of the mitochondrial sirtuin isoform Sirt5 in Mammalia. *Biochimica et Biophysica Acta (BBP)- Proteins and Proteomics, 1804*(8), 1658-1665. [CrossRef]

31. Schuetz, A., Min, J., Antoshenko, T., Wang, C. L., Allali-Hassani, A., Dong, A., Loppnau, P., Vedadi, M., Bochkarev, A., Sternglaz, R., Plotnikov, A.N. (2007). Structural basis of inhibition of the human NAD+-dependent deacetylase SIRT5 by suramin. *Structure, 15*(3),377-389. [CrossRef]

32. Wiedemar, N., Hauser, D. A., Mäser, P. (2020). 100 years of suramin. *Antimicrobial Agents and Chemotherapy, 64*(3), e01168-19. [CrossRef]

33. Choe, G., Kim, W. H., Park, J. G., Kim, Y. I. (1997). Effect of suramin on differentiation of human stomach cancer cell lines. *Journal of Korean Medical Science, 12*(5), 433-442. [CrossRef]

34. Bhargava, S., Hotz, B., Hines, O.J., Reber , H.A., Buhr, H. J., Hotz, H. G. (2007). Suramin inhibits not only tumor growth and metastasis but also angiogenesis in experimental pancreatic cancer. *Journal of Gastrointest Surgery, 11*, 171-178. [CrossRef]

35. Gogada, R., Prabhu, V., Amadori, M., Scott, R., Hashmi, S., Chandra, D. (2011). Resveratrol induces p53-independent, X-linked inhibitor of apoptosis protein (XIAP)-mediated Bax protein oligomerization on mitochondria to initiate cytochrome c release and caspase activation. *Journal of Biological Chemistry, 286*(33), 28749-28760. [CrossRef]

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 817-826, 2022
Doi: 10.33483/jfpau.1122581

ORIGINAL ARTICLE / ÖZGÜN MAKALE

EVALUATION OF THE ATTITUDES AND PRACTICES OF PATIENTS REGARDING THE USE OF HERBAL PRODUCTS

HASTALARIN BİTKİSEL ÜRÜN KULLANIMINA İLİŞKİN TUTUM VE UYGULAMALARININ DEĞERLENDİRİLMESİ

Songül TEZCAN[1*], Melike BUTUR[1]

[1]Marmara University, Faculty of Pharmacy, Department of Clinical Pharmacy, 34854, İstanbul, Turkey

ABSTRACT

Objective: *Herbal products are commonly used in public as a part of complementary and alternative medicine. The aim of our study is to evaluate the patients' attitudes and practices towards herbal products' uses.*

Material and Method: *This study was a cross-sectional descriptive study conducted at two community pharmacies in Istanbul (Turkey) in December 2017-February 2018. Sociodemographic characteristics of the participants were recorded and a self-structured questionnaire was applied.*

Result and Discussion: *A total of 200 patients were included in the study. The mean age of the patients was 34.6 years. Over sixty percent of the patients were using herbal products and approximately half of them believed that herbal products use was completely harmless ($p<0.05$). It was determined that the rate of using herbal products was statistically higher in female, adults, patients with low educational level, patients with chronic diseases, and non-smokers ($p<0.05$). Patients with chronic illnesses commonly used herbal products and most of the patients believed that these products were harmless. It is important to provide reliable information for the community via pharmacists as health counselors.*

Keywords*:* *Attitudes, herbal products, patient, pharmacist*

ÖZ

Amaç: *Bitkisel ürünler, tamamlayıcı ve alternatif tıbbın bir parçası olarak toplumda yaygın olarak kullanılmaktadır. Çalışmamızın amacı, hastaların bitkisel ürünlerin kullanımına yönelik tutum ve uygulamalarını değerlendirmektir.*

* **Corresponding Author / Sorumlu Yazar:** Songül Tezcan
e-mail / e-posta: songulbutur@hotmail.com, **Phone / Tel.:** +905052449918

Submitted / Gönderilme: 28.05.2022 **Accepted / Kabul:** 28.07.2022

Gereç ve Yöntem: *Bu çalışma, Aralık 2017-Şubat 2018 tarihlerinde İstanbul'da (Türkiye) iki serbest eczanede gerçekleştirilen kesitsel tanımlayıcı bir çalışmadır. Katılımcıların sosyodemografik özellikleri kaydedilmiş ve kendi kendine yapılandırılmış bir anket uygulanmıştır.*

Sonuç ve Tartışma: *Çalışmaya toplam 200 hasta dahil edillmiştir. Hastaların ortalama yaşı 34,6'dır. Hastaların yüzde altmışından fazlası bitkisel ürün kullanmakta olup, yaklaşık yarısı bitkisel ürün kullanımının tamamen zararsız olduğuna inandığını belirtmiştir (p<0,05). Kadın, yetişkin, eğitim düzeyi düşük, kronik hastalığı olan ve sigara içmeyen hastalarda bitkisel ürün kullanım oranının istatistiksel olarak daha yüksek olduğu belirlenmiştir (p<0,05). Kronik hastalığı olan hastaların yaygın olarak bitkisel ürünleri kullandığı ve hastaların çoğunun bu ürünlerin zararsız olduğuna inandığı tespit edilmiştir. Sağlık danışmanları olarak eczacılar aracılığıyla topluma güvenilir bilgi sağlanması önemlidir.*

Anahtar Kelimeler: *Bitkisel ürünler, eczacı, hasta, tutum*

INTRODUCTION

Herbal products are a part of complementary and alternative medicine (CAM) and have been used over thousands of years [1]. Approximately 40% of adults in the United States (U.S.) use CAM therapy and 1 in every 5 adults report taking an herbal product [2-5]. In Turkey, it was found that 48.8% of people use herbal medicine in a study conducted in a hospital [6]. In another study in Turkey, it was showed that almost half of women use at least one herbal product during pregnancy [7]. Also, Tulunay et al. (2015) found approximately one-third of patients with chronic diseases had used herbal products for treatment [8].

Pharmacists have critical roles in prevention of adverse effects of short- and long-term use of herbal products via patient education, counseling and monitoring. The perspectives of patients and pharmacists regarding the use of herbal products can be different. According to Gelayee et al. (2017) majority of pharmacists think that use of herbal products has beneficial effects. In the same study most of pharmacists reported that there is a significant interaction between drug and herbal products [9]. In another study the most of pharmacists were stated that they dispense herbal products in their pharmacies and 36% of pharmacists are "always" counseling patients about the use of herbal drugs [10]. According to Shraim et al. (2017); pharmacists' recommendations on the use of herbal products include the following issues; herbal drug use in pregnancy and children, consultation about herb- drug interactions, side effects, and evidence-based effectiveness of herbal drugs. [11].

Bhat et al. (2019) reported that patients mostly prefer herbal products over conventional medicines since they think that these products are more efficacious and have minimal side effects. In the same study, patients' major sources of information regarding herbal medicines were friends and advertisements [12]. A study from Turkey showed that participant's knowledge on herbal products depends on advertisements through media and herbal products are usually purchased from herb sellers [13]. In the same study, it was revealed that most of the participants (84%) did not know that the herbal products could interact with other drugs.

Peltzer et al. (2017) reported that 43.6% percent of patients with chronic diseases were using herbal products and their sources of information about the herbal products were family members [14]. In a cross-sectional study, 44% of participants were found to be using herbal drugs and 45% of those were also using conventional drugs [15].

Herbal products are frequently used and easily accessible to the public. Evaluation of the profile of people who use these products may contribute to their rational and safe use. Therefore, the aim of our study is to evaluate the attitudes and practices of the patients regarding the use of herbal products.

MATERIAL AND METHOD

This cross-sectional descriptive study was conducted in two community pharmacies in Istanbul (Turkey) between December 2017-February 2018. The ethical approval was taken from a local ethic committee (Marmara University Medicine Faculty School of Medicine Ethics Committee Protocol code: 09.2017.644). All respondents were asked for their consent before participation in the study. All data was collected by the authors vis a vis.

Data Collection Method for the Patients to Evaluate the Attitudes and Practices of Patients Regarding the Use of Herbal Products

The patients' sociodemographic characteristics (age, gender, graduation status, smoking, alcohol consumption, chronic diseases and herbal product use) were recorded via a standard form.

A structured questionnaire was adapted from previous studies [13,14]. The questionnaire consists a total of 9 questions (closed-ended) for evaluating the patients' attitudes and practices towards the use of herbal products (belief on herbal medicines are harmless; where they obtained; reasons to use herbal products; expectations from the use of herbal products; when to use herbal products; whether or not they informed their doctors/pharmacists about using herbal products; recommendation for the use of herbal products to relatives with similar complaints; the most reliable information sources regarding the use of herbal product and the factors that affect the use of herbal products).

Statistical Analysis

Statistical Package for Social Sciences (SPSS) version 15.0 for windows (SPSS Inc., Chicago, IL) was used for analysis of data. Descriptive statistics (frequencies, percentages, and means), Pearson’s Chi-square test of independence and Fisher’s exact test were used to assess correlations between sociodemographic factors and items of the questionnaire. *P* value <0.05 was considered significant.

RESULT AND DISCUSSION

Sociodemographic Characteristics of Patients

The mean age of the patients was 34.6±1.1 (18-83) and 68% of patients were female. The other sociodemographic characteristics of patients are given on Table 1.

Table 1. Characteristics of patients

	N	%
Gender		
Female	136	68
Male	64	32
Educational level*		
Low educational level	112	56
High educational level	88	44
Chronic disease status		
No	68	34
Yes	132	66
Smoking status		
No	140	70
Yes	60	30
Alcohol consumption		
No	184	92
Yes	16	8
Herbal products use		
No	65	32
Yes	13	68

N: number of patients; * Educational level: Low educational level (illiterate, Primary school, Secondary school, High school); High educational level (University and postgraduate degree)

Sixty-eight percentage of the patients stated that they had used herbal products. It was determined that the rate of using herbal products was statistically higher in patients with female gender, younger adults, low educational level, patients with chronic diseases and non-smokers ($p<0.05$) (Table 2).

Attitudes and Practices Towards the Use of Herbal Products

Sixty two percentage of patients believe that the use of herbal products is not completely harmless, 74% of patients stated that they buy herbal products from herbalists, 69% of patients stated that they use herbal products due to believe that they are beneficial, 38% of patients stated that they use the herbal drugs to reduce of the symptoms, 46% of the patients stated that they started using herbal products when the symptoms first appeared, 52% of the patients stated that they notify the doctor or pharmacist that the usage herbal products report the information about previous or current herbal product use to the doctor or pharmacist and 76% of the patients stated that they recommend the use of herbal products to relatives with similar complaints (Table 3).

Table 2. Comparison of sociodemographic characteristics of patients using herbal products

	n*	%	*P* value
Gender			
Female	95	70	>.05
Male	41	30	
Age			
<26	49	36	<.05
26-45	40	29	
>45	47	35	
Educational level**			
Low educational level	90	66	<.05
High educational level	46	34	
Chronic disease status			
No	36	26.5	<.05
Yes	100	73.5	
Smoking status			
No	87	64	<.05
Yes	49	36	
Alcohol consumption			
No	13	90	>.05
Yes	123	10	

n*: number of patients using herbal products; ** Educational level: Low educational level (illiterate, Primary school, Secondary school, High school); High educational level (University and postgraduate degree)

A majority of patients (68%) stated that they use herbal products and about half of these patients (use herbal products) stated that they believed that herbal products were completely harmless (48%) ($p<0.05$).

According to the report of World Health Organization (WHO), traditional and complementary medicine continues to be widely used in most countries [1]. Majority of the world population use or benefit from herbal products as primary health care applications. Although herbal products are widely used for different reasons in the community, the attitudes and practices towards the use of herbal products could be differed between the patients and the pharmacists. This study is the one of the studies which evaluates the attitudes and practices of both towards the use of herbal products.

In our study it was found that the prevalence of the use herbal products as 68%. The frequency of using herbal products was determined as 35-85% in other studies [6, 13, 15, 16, 17]. Additionally, it was emphasized that the herbal medicine uses in Africa reaches up to 90%, in Saudi Arabia 94% and in India 70% of the population [18].

It was determined that the majority of the patients using herbal products were women (70%) in our study. In many studies it was found that the use of herbal products is higher in women than in men [3, 6, 13, 15, 16, 19].

In our study, the rate of herbal products use is higher in patients with low education levels (%66). It was determined that similar results (respectively 70% and 40%) were obtained in other studies [13, 15].

The majority of patients (69%) stated that they use herbal products due to the belief that herbal products were beneficial. This ratio is higher than the other similar studies (respectively, 58% vs 57%) [13, 18]. Approximately half of the patients believed that herbal products were harmless in our study while Nur (2010) reported this ratio as 21% [13].

Table 3. Patients' attitudes and practices towards the use of herbal products

	N	%
Believing that the use of herbal products is completely harmless		
No	123	62
Yes	77	38
Patients buy herbal products from		
Herbalist	149	74
Gathering from nature	73	36
Pharmacy	43	21
Websites	8	4
Television	3	1.5
Reasons to use herbal products		
Believe in they are beneficial	139	69
Fail in treatment with conventional drugs	21	10.5
Public influence	28	14
Feeling Desperate	9	4.5
Not believe in effects of drugs	5	2.5
Don't want to go to the doctor	3	1.5
Other	23	11
Expectations from the use of herbal products		
To reduce of symptoms	76	38
To cure the disease/s	73	36
To support the treatment	52	26
To prevent recurrence	31	15
Starting time to use herbal products		
When symptoms appear	93	46
Using even though there is no symptom	60	30
After the using prescription drugs	26	13
When the symptoms increase	26	13
Notifying the doctor or pharmacist about the use of herbal products		
No	96	48
Yes	105	52
Recommending the use of herbal products to relatives with similar complaints	49	24
No	151	76
Yes		

n: number of patients (some patients have given more than one answers)

Forty-two percentage of the patients stated that the most reliable source of information regarding the use of herbal products was the recommendation of their relatives while 19% of patients stated that the pharmacist (Figure 1). In parallel with this result, the most common factor that affect herbal product use was determined as "the relatives" with a rate of 46% (Figure 2).

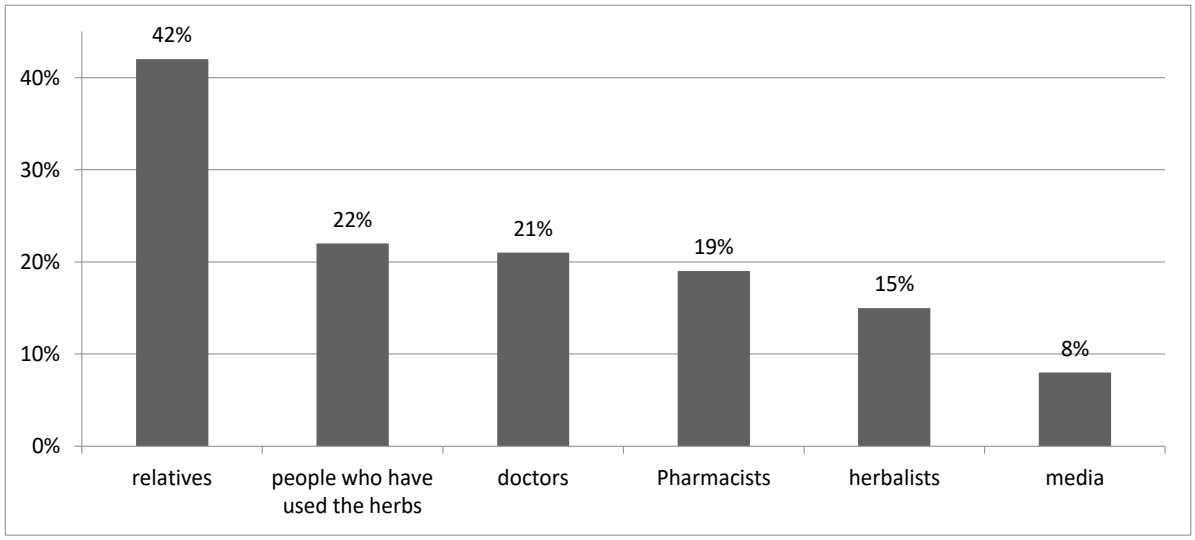


Figure 1. Percentage of the information sources regarding the use of herbal products

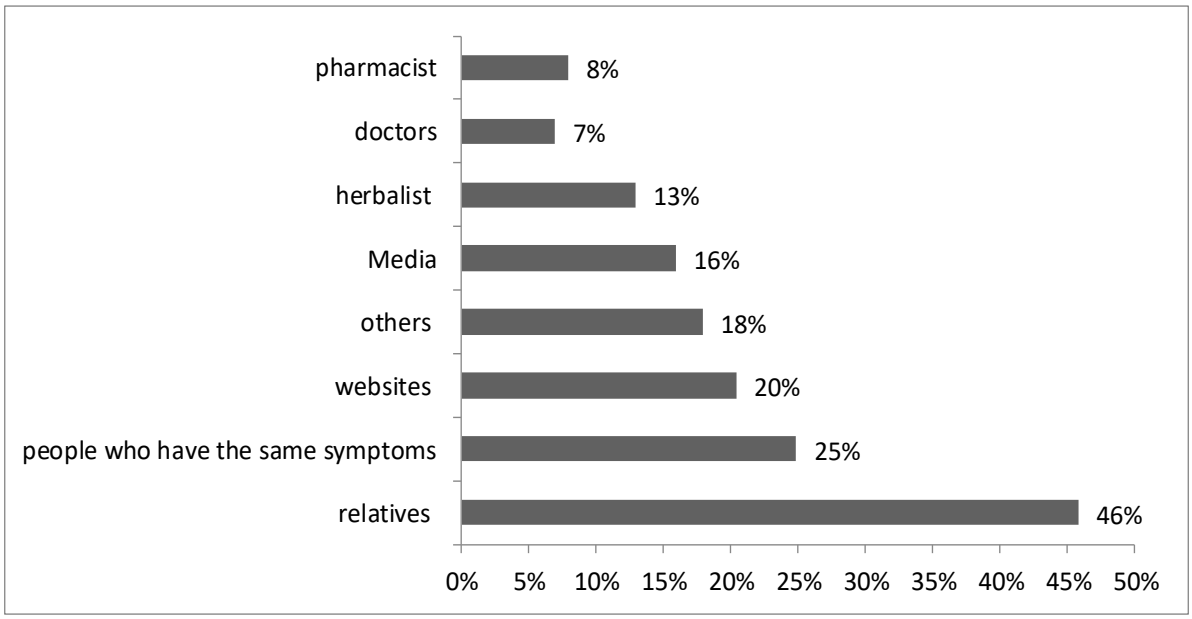


Figure 2. Percentage of factors that affect patients' use of herbal products

In this study, over half of the patients (52%) stated that they gave information about using herbal products to the doctor or pharmacist while Nur (2010) reported a lower rate of 37.9% [13].

In our study it was found that approximately 73.5% of the patients with chronic disease use herbal products. Peltzer et al. (2019) and Tulunay et al. (2015) reported lower ratios compared to our study (44% and 29%, respectively) [8, 20]. In another two studies it was determined that approximately 40% of patients with chronic disease used herbal medicine [16,20]. On the other hand, about 40% of patients with chronic diseases stated that they use herbal products due to the benefices [8].

In the literature, it has been determined that the 34-62% of patients believe that relatives are the reliable information source about herbal products [8, 19]. Similarly, in our study it was found that 44% of the patients stated that the most reliable source of information regarding the use of herbal products was the recommendation of their relatives.

Twenty percent of patients stated that they get information about using herbal products from the doctors in our study. This result is similar to the study of Nur (2010) with thirty percent [13].

The rate of informing the doctor or pharmacist about the use of herbal products was found to be 52% in our study. This ratio was found to be in the range of the other studies (50-80%) [15, 21].

In our study, approximately half of the patients (42%) stated that they used herbal products with the advice of their relatives, and the majority (76%) said that they recommended herbal products to people with the same symptoms. Less than half of the patients (46%) stated that they started the use of herbal products via recommendations of relatives. Several studies revealed similar results between the range of 44-75% [13, 15, 20].

In our study, the approaches towards on using herbal products of patients were evaluated. This study showed that a majority of patients use herbal products and advise the use of these products to their relatives. In addition, majority of patient believe that the use of herbal products is completely harmless and approximately half of the patients stated that they did not give information about using herbal products to the doctor or pharmacist. According to the results of our study, patients prefer herbal products especially in minor ailments. For this reason, we think that pharmacists, who are the closest health consultants, will contribute positively to the rational and safe use of these products, especially through patient education.

AUTHOR CONTRIBUTIONS

Conception: *S.T., M.B.*; Design: *S.T.*; Supervision: *S.T.*; Resources: *S.T., M.B.*; Materials*: S.T., M.B.*; Data Collection and/or processing: *S.T., M.B.*; Analysis and/or interpretation: *S.T., M.B.*; Literature search: *S.T.*; Writing manuscript: *S.T.*; Critical review: *S.T.*; Other: -

CONFLICT OF INTEREST

The authors declare that there are no actual, potential or perceived conflicts of interest for this article.

ETHICS COMMITTEE APPROVAL

The ethical approval was taken from a local ethic committee (Marmara University Medicine Faculty School of Medicine Ethics Committee Protocol code: 09.2017.644).

REFERENCES

1. WHO Traditional Medicine Strategy 2014-2023, WHO 2013 http://apps.who.int /iris/bitstream/handle/10665/92455/9789241506090_eng.pdf;jsessionid=C5293D0A3BA3A637 B909B0093487A5B0?sequence=1. (Accessed date: 23 Jan 2022)

2. Mosihuzzaman, M. (2012). Herbal medicine in healthcare-an overview. *Natural Product Communications*, *7*(6), 807–812.

3. Mbizo, J., Okafor, A., Sutton, M. A., Leyva, B., Stone, L. M., Olaku, O. (2018). Complementary and alternative medicine use among persons with multiple chronic conditions: results from the 2012 National Health Interview Survey. *BMC Complementary and Alternative Medicine*, *18*(1), 281. [CrossRef]

4. Williams, C. T. (2021). Herbal supplements: Precautions and safe use. *The Nursing Clinics of North America*, *56*(1), 1–21. [CrossRef]

5. Chopra, B., Dhingra, A. K. (2021). Natural products: A lead for drug discovery and development. *Phytotherapy Research: PTR*, *35*(9), 4660–4702. [CrossRef]

6. Soner, B.C., Şahin, A.S., Şahin, T.K. (2013). A survey of Turkish hospital patients' use of herbal medicine. *European Journal of Integrative Medicine, 5(6),* 547-52. [CrossRef]

7. Kıssal, A., Çevik Güner, Ü., Batkın Ertürk, D. (2017). Use of herbal product among pregnant women in Turkey. *Complementary Therapies in Medicine*, *30*, 54–60. [CrossRef]

8. Tulunay, M., Aypak, C., Yikilkan, H., Gorpelioglu, S. (2015). Herbal medicine use among patients with chronic diseases. *Journal of Intercultural Ethnopharmacology*, *4*(3), 217–220. [CrossRef]

9. Gelayee, A. D., Binega Mekonnen, G., Asrade Atnafe, S., Birarra, M. K., Asrie, A. B. (2017). Herbal medicines: personal use, knowledge, attitude, dispensing practice, and the barriers among community pharmacists in Gondar, Northwest Ethiopia. *Evidence-Based Complementary and Alternative Medicine: eCAM*, *2017*. [CrossRef]

10. Alsayari, A., Almghaslah, D., Khaled, A., Annadurai, S., Alkhairy, M. A., Alqahtani, H. A., Alsayed, B. A., Alasiri, R. M., Assiri, A. M. (2018). Community pharmacists' knowledge, attitudes, and practice of herbal medicines in Asir Region, Kingdom of Saudi Arabia. *Evidence-Based Complementary and Alternative Medicine: eCAM*, *2018*. [CrossRef]

11. Shraim, N. Y., Shawahna, R., Sorady, M. A., Aiesh, B. M., Alashqar, G. S., Jitan, R. I., Abu Hanieh, W. M., Hotari, Y. B., Sweileh, W. M., Zyoud, S. H. (2017). Community pharmacists' knowledge, practices and beliefs about complementary and alternative medicine in Palestine: a cross-sectional study. *BMC Complementary and Alternative Medicine*, *17*(1), 429. [CrossRef]

12. Bhat, B. B., Udupa, N., Ligade, V. S., Khan, S., Sreedhar, D. (2019). Assessment of knowledge and attitude of patients on herbal medicine use in Udupi Region, Karnataka, India. *Tropical Journal of Pharmaceutical Research*, *18*(1), 117-121. [CrossRef]

13. Nur, N. (2010). Knowledge and behaviours related to herbal remedies: a cross-sectional epidemiological study in adults in Middle Anatolia, Turkey. *Health Social Care in The Community*, *18*(4), 389–395. [CrossRef]

14. Peltzer, K., Huu, T.N., Ngoc, N.B., Pengpid, S. (2017). The use of herbal remedies and supplementary products among chronic disease patients in Vietnam, *Studies on Ethno-Medicine*, *1*(2), 137-145. [CrossRef]

15. Djuv, A., Nilsen, O. G., Steinsbekk, A. (2013). The co-use of conventional drugs and herbs among patients in Norwegian general practice: a cross-sectional study. *BMC Complementary and Alternative Medicine*, *13*, 295. [CrossRef]

16. Rashrash, M., Schommer, J. C., Brown, L. M. (2017). Prevalence and predictors of herbal medicine use among adults in the United States. *Journal of Patient Experience*, *4*(3), 108–113. [CrossRef]

17. Elolemy, A. T., Albedah, A. M. (2012). Public knowledge, attitude and practice of complementary and alternative medicine in riyadh region, saudi arabia. *Oman Medical Journal*, *27*(1), 20–26. [CrossRef]

18. Alkhamaiseh, S. I., Aljofan, M. (2020). Prevalence of use and reported side effects of herbal medicine among adults in Saudi Arabia. *Complementary Therapies in Medicine*, *48*, 102255. [CrossRef]

19. Kristoffersen, A. E., Stub, T., Salamonsen, A., Musial, F., Hamberg, K. (2014). Gender differences in prevalence and associations for use of CAM in a large population study. *BMC Complementary and Alternative Medicine*, *14*, 463. [CrossRef]

20. Peltzer, K., Pengpid, S. (2019). The use of herbal medicines among chronic disease patients in Thailand: a cross-sectional survey. *Journal of Multidisciplinary Healthcare*, *12*, 573–582. [CrossRef]

21. Carr, A., Santanello, C. (2019). Pharmacists' knowledge, perceptions, and practices regarding herbal medicine. *Innovations in Pharmacy*, *10*(3), 15. [CrossRef]

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 827-838, 2022
Doi: 10.33483/jfpau.1133615

ORIGINAL ARTICLE / ÖZGÜN MAKALE

INVESTIGATION OF PHYTOCHEMICALS IN METHANOLIC HERBA EXTRACT OF *SILENE RUSCIFOLIA* BY LC-QTOF/MS AND GC/MS

SILENE RUSCIFOLIA METANOLİK HERBA EKSTRESİNDEKİ FİTOKİMYASALLARIN LC-QTOF/MS VE GC/MS İLE İNCELENMESİ

Kenan Can TOK[1], Muhammed Mesud HÜRKUL[2*], Nazmiye Neslihan BOZKURT[1], Ayhan İbrahim AYSAL[3], Şeyda YAYLA[2]

[1]Ankara University, Institute of Forensic Sciences, Department of Forensic Toxicology, Ankara, Türkiye

[2]Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Botany, Ankara, Türkiye

[3]Gazi University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Ankara, Türkiye

ABSTRACT

Objective: *Silene L. (Caryophyllaceae) species are traditionally used for the treatment of inflammation, urinary inflammation, eye trouble, skin problem, stomach ache, dysentery, tooth decay, fever, headache, malaria, pimples and backache. Chemical ingredients of Silene species consist of flavonoids, anthocyanidins, terpenoids, triterpene saponins, phytoecdysteroids, benzenoids, vitamins, and show antioxidant, anti-inflammatory, antitumor and antiviral activities. Silene ruscifolia (Hub.-Mor. & Reese) Hub.-Mor. is called "gizli nakıl" in Türkiye.*

Material and Method: *The plant material was collected from Beynam Forest (Ankara/Türkiye). The aerial parts of the plant were extracted with methanol in an ultrasonic bath. HPLC system (Agilent 1260 Series) with an autosampler, binary pump, column oven, and a UV detector was coupled with an iFunnel Quadrupole Time-of-Flight LC-MS system (Agilent G6550A) with a Dual Spray Agilent Jet Stream electrospray ionization source. Agilent TC C-18 (4.6 mm x 150 mm x 5 µm) column was used for the separation of the compounds. The GC-MS analysis of extract was performed using an Agilent 6890 gas chromatograph equipped with an Agilent 5973N quadrupole mass spectrometer (Agilent, USA). Mass Hunter software (Qualitative Analysis B.07.00) and the NIST Mass Spectral Library (2014) were used to determine and identify the compounds.*

Result and Discussion: *LC-MS Q-TOF analysis showed that S. ruscifolia contained rutin, narcissin, luteolin, isorhamnetin, rhamnetin and quercetin dimethyl ether. GC-MS analysis showed that the extract had*

* **Corresponding Author / Sorumlu Yazar:** Muhammed Mesud Hürkul
e-mail / e-posta: huerkulmm@gmail.com, **Phone / Tel.:** +903122033035

Submitted / Gönderilme: 21.06.2022 **Accepted / Kabul:** 01.08.2022

the highest content of sugar (50.5%) and sugar alcohols (46.39%). It also contains carboxylic acid (0.47%), fatty acid (0.64%), sugar acid (0.42%), glycoside (0.48%), carotenoids (0.61%) and benzoic acid ester (0.49%). D-pinitol has the highest content in the extract with 41.14%.

Keywords: *GC/MS, Herba, LC-QTOF/MS, Silene ruscifolia*

ÖZ

Amaç: *Silene L. (Caryophyllaceae) türleri geleneksel olarak iltihap, idrar yolu iltihabı, göz rahatsızlığı, cilt sorunu, mide ağrısı, dizanteri, diş çürümesi, ateş, baş ağrısı, sıtma, sivilce ve sırt ağrısı tedavisinde kullanılmaktadır. Silene türlerinin kimyasal bileşenleri flavonoidler, antosiyanidinler, terpenoidler, triterpen saponinler, fitoekdisteroidler, benzenoidler, vitaminlerden oluşur ve antioksidan, antiinflamatuar, antitümör, antiviral aktivite gösterirler. Silene ruscifolia (Hub.-Mor. & Reese) Hub.-Mor. Türkiye'de "gizli nakıl" olarak adlandırılır.*

Gereç ve Yöntem: *Bitki materyali Beynam Ormanı'ndan (Ankara/Türkiye) toplandı. Bitkinin toprak üstü kısımları ultrasonik banyoda metanol ile ekstre edildi. Otomatik örnekleyici, ikili pompa, kolon fırını ve bir UV dedektörüne sahip HPLC sistemi (Agilent 1260 Serisi), Çift Sprey Agilent Jet Stream elektrosprey iyonizasyon kaynağına sahip bir iFunnel Quadrupole Time-of-Flight LC-MS system (Agilent G6550A) ile birleştirildi. Bileşiklerin ayrılması için Agilent TC C-18 (4.6 mm x 150 mm x 5 µm) kolonu kullanıldı. Ekstrenin GC-MS analizi, bir Agilent 5973N dört kutuplu kütle spektrometresi (Agilent, ABD) ile donatılmış bir Agilent 6890 gaz kromatografı kullanılarak yapıldı. Bileşiklerin belirlenmesi ve tanımlanması için Mass Hunter yazılımı (Qualitative Analysis B.07.00) ve NIST Mass Spectral Library (2014) kullanıldı.*

Sonuç ve Tartışma: *LC-MS Q-TOF analizi, S. ruscifolia'nın rutin, narsissin, luteolin, izoramnetin, ramnetin ve kersetin dimetil eter içerdiğini gösterdi. GC-MS analizi, ekstrenin şeker (%50.5) ve şeker alkolleri (%46.39) olarak en yüksek içeriğe sahip olduğunu göstermiştir. Ayrıca karboksilik asit (%0.47), yağ asidi (%0.64), şeker asidi (%0.42), glikozit (%0.48), karotenoidler (%0.61) ve benzoik asit esteri (%0.49) içerir. D-pinitol, %41.14 ile ekstredeki en yüksek içeriğe sahiptir.*

Anahtar Kelimeler: *GC/MS, Herba, LC-QTOF/MS, Silene ruscifolia*

INTRODUCTION

The Caryophyllaceae consists of herbaceous or mostly semi-shrub plants, usually with simple, entire and exstipulate leaves. Actinomorphic and solitary or in cymes flowers have anthophore or perigynous. Fruits are baccate or opening capsule [1-3]. The family contains 101 accepted genera worldwide [4].

Silene L. is a large and polymorphic genus. Subarctic-temperate zone to tropical mountains are the distribution areas of the genus in the world and includes 887 species [4]. Annual, biennial and perennial herbs (mostly semi-shrub) consist of this genus. The calyx is tube-shaped and vascularization is seen on it very clear. Fruit an opening capsule. Petals, stamens and ovary rise on anthophore [2,4]. *Silene ruscifolia* (Hub.-Mor. & Reese) Hub.-Mor. is perennial-herbaceous and has a strong woody stem at the base. Basal leaves are oblanceolate and cauline leaves are defined as ovate-cordate. Flowers in dichasia, and white petals rise on glabrous anthophore. Fruit is a capsule [2]. *S. ruscifolia* is called "*gizli nakıl*" in Türkiye [5]. Previous taxonomical studies have reported that *S. ruscifolia* is very similar to the Iranian *Silene commelinifolia* Boiss. [2]. According to current studies, *S. ruscifolia* is accepted as a synonym of *S. commelinifolia* [4].

Silene species are traditionally used against inflammation, urinary inflammation, eye trouble, skin problem, stomach ache, dysentery, tooth decay, fever, headache, malaria, pimples and backache [6-14]. Chemical ingredients of *Silene* species consist flavonoids, anthocyanidins, terpenoids, triterpene saponins, phytoecdysteroids, benzenoids, vitamins [15-19] and show antioxidant, anti-inflammatory, antitumor, antiviral activities [20-24].

MATERIAL AND METHOD

Plant materials

The plant material was collected from Beynam Forest (Ankara/Türkiye). A voucher specimen was deposited in the Herbarium of Ankara University Faculty of Pharmacy (AEF30851). Plant samples were dried in the shade and powdered with a grinder.

Extraction process

The aerial parts of the plant were extracted with methanol in ultrasonic bath for 30 min. The extract was filtered from 0.45 μm filters before injection to the LC-QTOF/MS and GC/MS systems [25].

LC-QTOF/MS analysis

HPLC system (Agilent 1260 Series) with an autosampler, binary pump, column oven, and a UV detector coupled with an iFunnel Quadrupole Time-of-Flight LC-MS system (Agilent G6550A) with a Dual Spray Agilent Jet Stream electrospray ionization source (Agilent Technologies, Inc., CA, USA) was used for analysis of the extracts of the sample. The instrument was operated with negative and positive electrospray ionization in a 2 GHz extended dynamic range mode. During the run of the samples, all ions mode with collision energies 0 eV, 10 eV, and 40 eV were used with the acquisition rate was 1 spectra/second. Agilent TC C-18 (4.6 mm x 150 mm x 5 μm) column was used for the separation of the compounds. Agilent MassHunter software B 06.00 was used for the analysis of the data. Metlin Metabolite database, Massbank.eu, and Pubchem databases were used for the identification of compounds [26-27]. The detailed instrumental conditions are tabulated in Table 1.

GC/MS analysis

The two-step derivatization procedure was performed before the GC/MS analysis. During the procedure, methoxymation followed by silylation was applied before the analysis [28-29]. Methoxymation was used to prevent split or duplicated signals of sugars onto the chromatogram.

Methoxyamine HCl (Merck, Darmstadt, Germany) solution was prepared daily as 25 mg/ml pyridine. After the sample (100 μl) was evaporated the nitrogen flowed gently, and 50 μl methoxyamine HCl solution was added to the dried sample. After waiting for 1.5 hours at about 30°C, 50 μl of

Bis(trimethylsilyl)trifluoroacetamide + 1% Trimethylchlorosilane (BSTFA + 1% TMCS) (Sigma-Aldrich, Germany) was added, and kept for another 45 min. at 70°C for a complete silylation. GC/MS system was used for the rest of the analysis (Agilent 6890/5973N model), (Santa Clara, USA). Crossbonded diphenyl dimethyl polysiloxane phase-coated Restek Rtx-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 µm) (Bellefonte, USA) was preferred for the analysis with the carrier gas (99.999%) helium at a 1.5 ml/min flow rate. 1 µl of the derivatized sample solution was injected in the splitless mode. The transfer line, as well as the injection port, were set at 280°C. Quadrupole was arranged at 150°C while the ion source was maintained at 230°C. The temperature program initially started at 50ºC and was held for 2 min, then increased to 280°C at the rate of 3°C/min. In this stage, it was held for 12 min. to complete the analysis (90 min.). Mass Hunter software (Qualitative Analysis B 07.00) and the NIST Mass Spectral Library (2014) were used for the determination and identification of the compounds.

Table 1. Instrumental parameters for LC-QTOF/MS system

Column	TC C-18 (4.6 mm x 150 mm x 5 µm)
Column Temperature (°C)	30
Injection volume (µl)	10
Run Time (min.)	82
Mobile Phase A	0.1% acetic acid in water
Mobile Phase B	0.1% acetic acid in acetonitrile
Flow rate (ml/min)	0.65
Gradient (time-B%)	0 min-5 % B; 4 min-5 % B; 12 min-10 % B; 15 min-10 % B; 28 min-20 % B; 48 min-40 % B; 60 min.-60 % B; 65 min-70 % B; 66 min-90 % B; 72 min-90 % B; 72.1 min-5 % B
UV (nm)	280
Ionization mode	Negative and positive electrospray ionization mode with jet stream technology
Drying gas temp (°C)	200
Drying gas flow (l/min)	14 l/min
Nebulizer pressure (psi)	40
Sheath gas temperature (°C)	350
Sheath gas flow (l/min)	11
Capillary Voltage (V)	1500
Nozzle Voltage (V)	1000
Mass range (amu)	30-1700
Reference ions	980.0147, 1033.9881 for negative run, 922.0098 for positive run

RESULT AND DISCUSSION

LC-MS Q-TOF systems were used for the identification of compounds using mass spectra of both precursor and product ions within a mass error of 5 ppm. Using different collision energies (0-10-40 eV) precursor ions were fragmented and product ions gave information about the compounds' functional groups and allowed to structure elucidation. Sample extract was analyzed using in gradient elution LC

program to get enough resolution to identify each compound. The UV chromatogram at 280 nm showed main composition of the extract and the most abundant 7 compounds were identified using reference standards and database search via Metlin and Pubchem. MassHunter generated molecular formula and product ions were used for identification. In case of compound 3 in Table 2, m/z 665 produced 315 ion (Isorhamnetin), so the compound can be classified as a glycoside of Isorhamnetin. The same product ion pattern was achieved for Narcissin as well. Isorhamnetin/Rhamnetin seperation on column (retention time 51.5 min. and 52.8 min. respectively) and product ion spectrum of compounds were used identification of compounds. Rhamnetin spectra showed m/z 165 product ion as a base peak while Isorhamnetin had m/z 151. All identified compounds were confirmed both by generated precursor ions and their fragments with mass error less than 5 ppm. The main components of the sample were eluted between 40-60 min. interval. Total ion chromatogram (A) and UV chromatogram (B) at 280 nm are shown below.

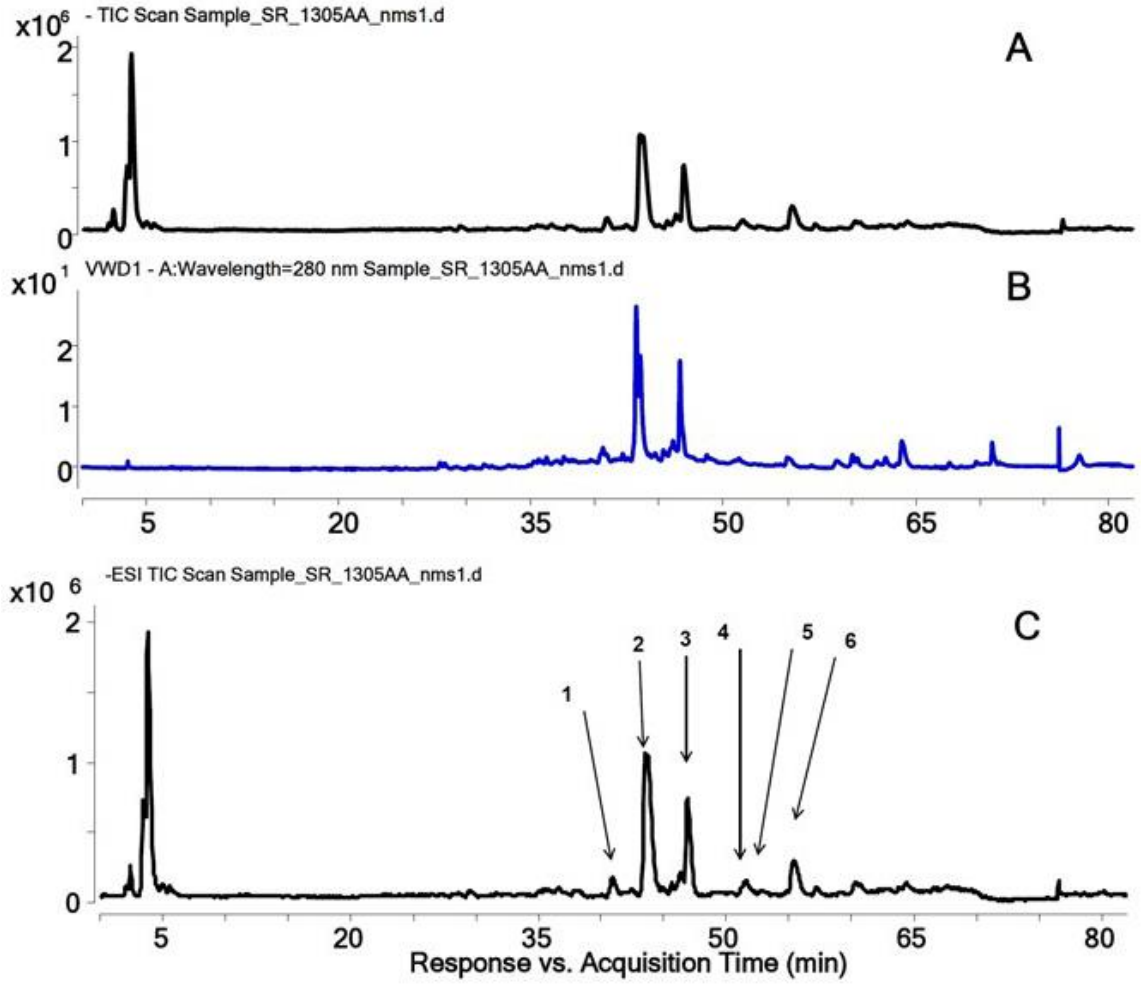


Figure 1. Total ion chromatogram of sample, negative mode MS (A) and 280 nm UV chromatogram (B) and the identified compounds (C)

LC-MS Q-TOF analysis showed that *S. ruscifolia* contained rutin, narcissin, luteolin, isorhamnetin, rhamnetin, and quercetin dimethyl ether. Phenolic compounds are produced as secondary metabolites in plants and are common [30-32]. They show sedative, hepatoprotective, antibacterial, antifungal, anticonvulsant, wound healing and antiviral activities [33-44]. Moreover, they also have antidepressant, diuretic, antiulcer, antidiabetic, antioxidant, anti-hypercholesterolemic, anticancer, and cardioprotective effects [45-60].

Table 2. Details of identified compounds in the extract

No.	Name	Formula	Calculated ion	Found ion	Error,ppm	rt, min
1	Rutin	$C_{27}H_{30}O_{16}$	609.1461	609.1441	3.28	41.2
2	Narcissin	$C_{28}H_{32}O_{16}$	623.1618	623.1615	0.48	43.4
3	Isorhamnetin glycoside compound	$C_{30}H_{34}O_{17}$	665.1723	665.1706	2.56	46.9
4	Luteolin	$C_{15}H_{10}O_6$	285.0405	285.0396	3.16	51.2
5	Isorhamnetin	$C_{16}H_{12}O^7$	315.0510	315.0512	-0.63	51.5
6	Rhamnetin	$C_{16}H_{12}O_7$	315.0510	315.0513	-0.95	52.8
7	Quercetin dimethylether	$C_{17}H_{14}O_7$	329.0667	329.0673	-1.82	55.4

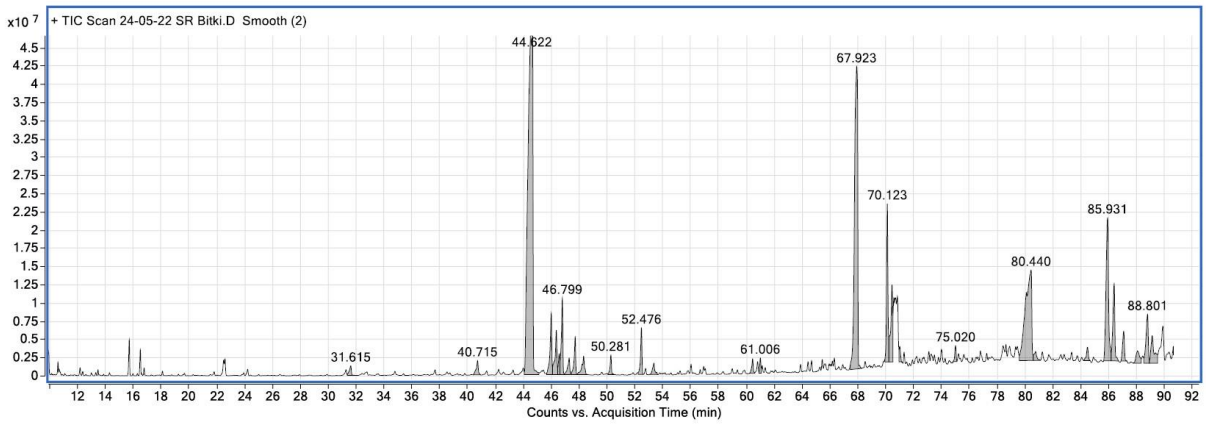


Figure 2. GC-MS Chromatogram for the extract

As a result of GC/MS analysis, 38 compounds were detected and 23 of them were identified (Table 3). These 23 compounds constitute 70.19% of the total peak area. GC-MS analysis showed that the extract had the highest content of sugar (50.5%) and sugar alcohols (46.39%). It also contains carboxylic acid (0.47%), fatty acid (0.64%), sugar acid (0.42%), glycoside (0.48%), carotenoids (0.61%) and benzoic acid ester (0.49%). D-pinitol has the highest content in the extract with 41.14%. D-pinitol, first isolated from *Pinus monticola*, is the most common inositol ether in plants, and also has anti-inflammatory, antidiabetic, antitumoral, antioxidant and cancer chemopreventive effects [61].

Table 3. Identified compounds in the extract by GC/MS.

#	RT (min)	Identified Compounds	Area Sum % of Identified Compounds	Area Sum % of Detected Compounds	Classification
1	31.615	Malic acid	0.47	0.33	Carboxylic acid
2	40.715	Xylitol	0.69	0.49	Sugar alcohol
3	44.622	D-Pinitol	41.14	29.3	Sugar alcohol
4	46.002	D-Fructose	2.52	1.8	Sugar
5	46.369	D-(-)-Fructose	2.01	1.43	Sugar
6	46.572	D-Allose	0.69	0.49	Sugar
7	46.799	D-(+)-Talose	2.71	0.97	Sugar
8	47.289	D-Allose	0.81	0.58	Sugar
9	47.719	D-Sorbitol	1.37	0.97	Sugar alcohol
10	48.331	D-Pinitol	1.13	0.81	Sugar alcohol
11	50.281	Palmitic Acid	0.64	0.46	Fatty acid
12	52.476	Myo-Inositol	1.55	1.1	Sugar alcohol
13	53.367	D-Allose (isomer 1)	0.6	0.42	Sugar
14	60.453	Dulcitol	0.51	0.37	Sugar alcohol
15	61.006	D-(+)-Galacturonic acid	0.42	0.3	Sugar acid
16	67.923	Sucrose	22.79	16.23	Sugar
17	70.123	D-(+)-Trehalose	5.04	3.53	Sugar
18	71.014	Aucubin	0.48	0.34	Glycoside
19	75.02	Methyl 2,4-dihydroxybenzoate	0.49	0.35	Benzoic Acid Ester
20	80.761	Lycopene	0.61	0.42	Carotenoids
21	85.931	Lactose	8.52	6.07	Sugar
22	88.801	D-(-)-Fructofuranose	2.62	1.87	Sugar
23	89.133	Maltose (isomer 1)	2.19	1.56	Sugar

AUTHOR CONTRIBUTIONS

Conception: *K.C.T., M.M.H.*; Design: *K.C.T., M.M.H.*; Supervision: *M.M.H.*; Resources: *K.C.T., M.M.H., N.N.B., A.İ.A., Ş.Y.*; Materials: *M.M.H., Ş.Y.*; Data collection and/or processing: *K.C.T., M.M.H., N.N.B., A.İ.A., Ş.Y.*; Analysis and/or interpretation: *K.C.T., M.M.H., N.N.B., A.İ.A., Ş.Y.*; Literature search: *K.C.T., M.M.H., N.N.B., A.İ.A., Ş.Y.*; Writing manuscript: *M.M.H.*; Critical review: *K.C.T., M.M.H., N.N.B., A.İ.A., Ş.Y.*; Other: -

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

REFERENCES

1. Bittrich, V. (1993). Caryophyllaceae. In: Flowering Plants, Dicotyledons, (pp. 206-236). Berlin, Heidelberg: Springer.

2. Cullen, J., Coode, M. (1967). *Silene* L. In: P.H. Davis (Ed.), Flora of Turkey and the East Aegean Islands, Vol. 2, (pp.179). Edinburgh: Edinburgh University Press.

3. Stevens, P.F. (2001). Angiosperm Phylogeny Website. Version 14, July 2017. http://www.mobot.org (Accessed 17.06.2022).

4. POWO (2022). Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. Published on the Internet; http://www.plantsoftheworldonline.org (Accessed 17.06.2022).

5. Güner, A., Aslan, S., Ekim, T., Vural, M., Babaç, M.T. (2012). Türkiye bitkileri listesi (damarlı bitkiler). Nezahat Gökyigit Botanik Bahçesi Yayınları Flora Dizisi I.

6. Rigat, M., Bonet, M.À., Garcia, S., Garnatje, T., Valles, J. (2007). Studies on pharmaceutical ethnobotany in the high river Ter valley (Pyrenees, Catalonia, Iberian Peninsula). *Journal of Ethnopharmacology, 113*(2), 267-277. [CrossRef]

7. Kayani, S., Ahmad, M., Sultana, S., Shinwari, Z.K., Zafar, M., Yaseen, G., Bibi, T. (2015). Ethnobotany of medicinal plants among the communities of Alpine and Sub-alpine regions of Pakistan. *Journal of Ethnopharmacology, 164*, 186-202. [CrossRef]

8. Cakilcioglu, U., Khatun, S., Turkoglu, I., Hayta, S. (2011). Ethnopharmacological survey of medicinal plants in Maden (Elazig-Turkey). *Journal of Ethnopharmacology, 137*(1), 469-486. [CrossRef]

9. Cornara, L., La Rocca, A., Terrizzano, L., Dente, F., Mariotti, M.G. (2014). Ethnobotanical and phytomedical knowledge in the North-Western Ligurian Alps. *Journal of Ethnopharmacology, 155*(1), 463-484. [CrossRef]

10. Özdemir, E., Alpınar, K. (2015). An ethnobotanical survey of medicinal plants in western part of central Taurus Mountains: Aladaglar (Nigde-Turkey). *Journal of Ethnopharmacology, 166*, 53-65. [CrossRef]

11. Leto, C., Tuttolomondo, T., La Bella, S., Licata, M. (2013). Ethnobotanical study in the Madonie Regional Park (Central Sicily, Italy)-Medicinal use of wild shrub and herbaceous plant species. *Journal of Ethnopharmacology, 146*(1), 90-112. [CrossRef]

12. Rehman, K., Khan, M.A., Ullah, Z., Chaudhary, H.J. (2015). An ethno botanical perspective of traditional medicinal plants from the Khattak tribe of Chonthra Karak, Pakistan. *Journal of Ethnopharmacology, 165*, 251-259. [CrossRef]

13. Polat, R., Cakilcioglu, U., Satıl, F. (2013). Traditional uses of medicinal plants in Solhan (Bingöl-Turkey). *Journal of Ethnopharmacology, 148*(3), 951-963. [CrossRef]

14. Said, O., Khalil, K., Fulder, S., Azaizeh, H. (2002). Ethnopharmacological survey of medicinal herbs in Israel, the Golan Heights and the West Bank region. *Journal of Ethnopharmacology, 83*(3), 251-265. [CrossRef]

15. Mamadalieva, N.Z. (2012). Phytoecdysteroids from *Silene* plants: distribution, diversity and biological (antitumour, antibacterial and antioxidant) activities. *Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas, 11*(6), 474-497.

16. Mamadalieva, N.Z., Lafont, R., Wink, M. (2014). Diversity of secondary metabolites in the genus *Silene* L. (Caryophyllaceae)-Structures, distribution, and biological properties. *Diversity, 6*(3), 415-499. [CrossRef]

17. Gaidi, G., Miyamoto, T., Laurens, V., Lacaille-Dubois, M.A. (2002). New acylated triterpene saponins from *Silene fortunei* that modulate lymphocyte proliferation. *Journal of Natural Products, 65*(11), 1568-1572. [CrossRef]

18. Dötterl, S., Wolfe, L.M., Jürgens, A. (2005). Qualitative and quantitative analyses of flower scent in *Silene latifolia*. *Phytochemistry, 66*(2), 203-213. [CrossRef]

19. Eshmirzaeva, N.E., Khidyrova, N.K., Khodzhaeva, M., Mezhlumyan, L.G., Shakhidoyatov, K.M. (2005). Chemical composition of *Silene viridiflora*. *Chemistry of Natural Compounds, 41*(4), 451-453.

20. Chandra, S., Rawat, D.S. (2015). Medicinal plants of the family Caryophyllaceae: a review of ethno-medicinal uses and pharmacological properties. *Integrative Medicine Research, 4*(3), 123-131. [CrossRef]

21. Lacaille-Dubois, M.A., Hanquet, B., Cui, Z.H., Lou, Z.C., Wagner, H. (1995). Acylated triterpene saponins from *Silene jenisseensis*. *Phytochemistry, 40*(2), 509-514. [CrossRef]

22. Karamian, R., Ghasemlou, F. (2013). Screening of total phenol and flavonoid content, antioxidant and antibacterial activities of the methanolic extracts of three *Silene* species from Iran. *International Journal of Agriculture and Crop Sciences, 5*(3), 305.

23. Mamadalieva, N.Z., Ul'chenko, N.T., Yuldasheva, N.K., Zhanibekov, A.A., Egamberdieva, D.R., Glushenkova, A.I. (2010). Neutral lipids and biological activity of the $CHCl_3$ extract of the aerial part of *Silene guntensis*. *Chemistry of Natural Compounds, 46*(4), 621-622. [CrossRef]

24. Mamadalieva, N.Z., Egamberdieva, D., Zhanibekov, A.A., Triggiani, D., Tiezzi, A. (2009). Chemical components of *Silene viridiflora* and their biological properties. *Chemistry of Natural Compounds, 45*(4), 589-591. [CrossRef]

25. Kurtul, E., Eryilmaz, M., Sarialtin, S.Y., Tekin, M., Acikara, Ö.B., Çoban, T. (2022). Bioactivities of *Alchemilla mollis*, *Alchemilla persica* and their active constituents. *Brazilian Journal of Pharmaceutical Sciences, 58*, e18373. [CrossRef]

26. Guijas, C., Montenegro-Burke, J.R., Domingo-Almenara, X., Palermo, A., Warth, B., Hermann, G., Koellensperger, G., Huan, T., Uritboonthai, W., Aisporna, A.E., Wolan, D.W., Spilker, M.E., Benton, P., Siuzdak, G. (2018). METLIN: a technology platform for identifying knowns and unknowns. *Analytical Chemistry, 90*(5), 3156-3164. [CrossRef]

27. Pinheiro, P.F., Justino, G.C. (2012). Structural analysis of flavonoids and related compounds-a review of spectroscopic applications. In: V. Rao (Ed.), Phytochemicals: A Global Perspective of Their Role in Nutrition and Health, (pp. 33-56). Rijeka, Croatia: InTech.

28. Kıymacı, M.E., Tok, K.C., Hürkul, M.M. (2022). A study on phytochemical analysis and antibacterial activity of *Quercus macranthera* subsp. *syspirensis* (K. Koch) Menitsky branch and leaf extracts. *Journal of Faculty of Pharmacy of Ankara University, 46*(1), 160-169. [CrossRef]

29. Tok, K.C., Yayla, Ş. (2022). Gas Chromatography-Mass Spectrometry (GC-MS) analysis of *Consolida thirkeana* extract. *Journal of Faculty of Pharmacy of Ankara University, 46*(2), 444-449. [CrossRef]

30. Shen, N., Wang, T., Gan, Q., Liu, S., Wang, L., Jin, B. (2022). Plant flavonoids: Classification, distribution, biosynthesis, and antioxidant activity. *Food Chemistry*, 132531. [CrossRef]

31. Gökbulut, A., Özhan, O., Karacaoğlu, M., Şarer, E. (2012). Radical scavenging activity and vitexin content of *Vitex agnus castus* leaves and fruits. *FABAD Farmasötik Bilimler Dergisi*, 35(2), 85-91.

32. Ağca, A.C., Ekici, A.N.Y., Sarıaltın, S.Y., Çoban, T., İşcan, G.S., Yılmaz, B.S. (2021). Antioxidant, anti-inflammatory and antidiabetic activity of two *Sternbergia* taxons from Turkey. *South African Journal of Botany*, 136, 105-109. [CrossRef]

33. Fernández, S.P., Wasowski, C., Loscalzo, L.M., Granger, R.E., Johnston, G.A., Paladini, A.C., Marder, M. (2006). Central nervous system depressant action of flavonoid glycosides. *European Journal of Pharmacology, 539*(3), 168-176. [CrossRef]

34. Schwingel, T.E., Klein, C.P., Nicoletti, N.F., Dora, C.L., Hadrich, G., Bica, C.G., Lopes, T.G., Da Silva, V.D., Morrone, F. B. (2014). Effects of the compounds resveratrol, rutin, quercetin, and quercetin nanoemulsion on oxaliplatin-induced hepatotoxicity and neurotoxicity in mice. *Naunyn-Schmiedeberg's Archives of Pharmacology, 387*(9), 837-848. [CrossRef]

35. Arima, H., Ashida, H., Danno, G.I. (2002). Rutin-enhanced antibacterial activities of flavonoids against *Bacillus cereus* and *Salmonella enteritidis*. *Bioscience, Biotechnology and Biochemistry, 66*(5), 1009-1014. [CrossRef]

36. Pimentel, R.B.D.Q., Da Costa, C.A., Albuquerque, P.M., Junior, S.D. (2013). Antimicrobial activity and rutin identification of honey produced by the stingless bee Melipona compressipes manaosensis and commercial honey. *BMC Complementary and Alternative Medicine, 13*(1), 1-14. [CrossRef]

37. Araruna, M.K., Brito, S.A., Morais-Braga, M.F., Santos, K.K., Souza, T.M., Leite, T.R., Costa, J.G.M., Coutinho, H.D. (2012). Evaluation of antibiotic & antibiotic modifying activity of pilocarpine & rutin. *The Indian Journal of Medical Research, 135*(2), 252-254.

38. Han, Y. (2009). Rutin has therapeutic effect on septic arthritis caused by *Candida albicans*. *International immunopharmacology, 9*(2), 207-211. [CrossRef]

39. Johann, S., Mendes, B.G., Missau, F.C., Resende, M.A.D., Pizzolatti, M.G. (2011). Antifungal activity of five species of Polygala. *Brazilian Journal of Microbiology, 42*, 1065-1075. [CrossRef]

40. Nieoczym, D., Socała, K., Raszewski, G., Wlaź, P. (2014). Effect of quercetin and rutin in some acute seizure models in mice. *Progress in Neuro-Psychopharmacology and Biological Psychiatry, 54*, 50-58. [CrossRef]

41. Carvalho, O.V., Botelho, C.V., Ferreira, C.G.T., Ferreira, H.C.C., Santos, M.R., Diaz, M.A.N., Oliveira, T.T., Soares-Martins, J.A.P., Almeida, M.R., Junior, A.S. (2013). *In vitro* inhibition of canine distemper virus by flavonoids and phenolic acids: implications of structural differences for antiviral design. *Research in Veterinary Science, 95*(2), 717-724. [CrossRef]

42. Ibrahim, A.K., Youssef, A.I., Arafa, A.S., Ahmed, S.A. (2013). Anti-H5N1 virus flavonoids from *Capparis sinaica* Veill. *Natural Product Research, 27*(22), 2149-2153. [CrossRef]

43. Tran, N.Q., Joung, Y.K., Lih, E., Park, K.D. (2011). *In situ* forming and rutin-releasing chitosan hydrogels as injectable dressings for dermal wound healing. *Biomacromolecules, 12*(8), 2872-2880. [CrossRef]

44. Almeida, J.S., Benvegnú, D.M., Boufleur, N., Reckziegel, P., Barcelos, R.C.S., Coradini, K., De Carvalho, L.M., Bürger, M.E. Beck, R.C.R. (2012). Hydrogels containing rutin intended for cutaneous administration: efficacy in wound healing in rats. *Drug Development and Industrial Pharmacy, 38*(7), 792-799. [CrossRef]

45. Machado, D.G., Bettio, L.E., Cunha, M.P., Santos, A.R., Pizzolatti, M.G., Brighente, I.M., Rodrigues, A.L.S. (2008). Antidepressant-like effect of rutin isolated from the ethanolic extract from *Schinus molle* L. in mice: evidence for the involvement of the serotonergic and noradrenergic systems. *European Journal of Pharmacology, 587*(1-3), 163-168. [CrossRef]

46. Alarcón-Alonso, J., Zamilpa, A., Aguilar, F.A., Herrera-Ruiz, M., Tortoriello, J., Jimenez-Ferrer, E. (2012). Pharmacological characterization of the diuretic effect of *Hibiscus sabdariffa* Linn (Malvaceae) extract. *Journal of Ethnopharmacology, 139*(3), 751-756. [CrossRef]

47. Abdel-Raheem, I.T. (2010). Gastroprotective effect of rutin against indomethacin-induced ulcers in rats. *Basic & Clinical Pharmacology & Toxicology, 107*(3), 742-750. [CrossRef]

48. La Casa, C., Villegas, I., De La Lastra, C.A., Motilva, V., Calero, M.M. (2000). Evidence for protective and antioxidant properties of rutin, a natural flavone, against ethanol induced gastric lesions. *Journal of Ethnopharmacology, 71*(1-2), 45-53. [CrossRef]

49. Kappel, V.D., Zanatta, L., Postal, B.G., Silva, F.R.M.B. (2013). Rutin potentiates calcium uptake via voltage-dependent calcium channel associated with stimulation of glucose uptake in skeletal muscle. *Archives of Biochemistry and Biophysics, 532*(2), 55-60. [CrossRef]

50. Hsu, C.Y., Shih, H.Y., Chia, Y.C., Lee, C.H., Ashida, H., Lai, Y.K., Weng, C.F. (2014). Rutin potentiates insulin receptor kinase to enhance insulin-dependent glucose transporter 4 translocation. *Molecular Nutrition & Food Research, 58*(6), 1168-1176. [CrossRef]

51. Kozachok, S., Pecio, Ł., Kolodziejczyk-Czepas, J., Marchyshyn, S., Nowak, P., Mołdoch, J., Oleszek, W. (2018). γ-Pyrone compounds: flavonoids and maltol glucoside derivatives from *Herniaria glabra* L. collected in the Ternopil region of the Ukraine. *Phytochemistry, 152*, 213-222. [CrossRef]

52. Al-Rejaie, S.S., Aleisa, A.M., Sayed-Ahmed, M.M., Al-Shabanah, O.A., Abuohashish, H.M., Ahmed, M.M., Al-Hosaini, K.A., Hafez, M.M. (2013). Protective effect of rutin on the

antioxidant genes expression in hypercholestrolemic male Westar rat. *BMC Complementary and Alternative Medicine, 13*(1), 1-9. [CrossRef]

53. Kalgaonkar, S., Gross, H.B., Yokoyama, W., Keen, C.L. (2010). Effects of a flavonol-rich diet on select cardiovascular parameters in a Golden Syrian hamster model. *Journal of Medicinal Food, 13*(1), 108-115. [CrossRef]

54. Mouria, M., Gukovskaya, A.S., Jung, Y., Buechler, P., Hines, O.J., Reber, H.A., Pandol, S.J. (2002). Food-derived polyphenols inhibit pancreatic cancer growth through mitochondrial cytochrome C release and apoptosis. *International Journal of Cancer, 98*(5), 761-769. [CrossRef]

55. Lima Gonçalves, C.F., De Souza dos Santos, M.C., Ginabreda, M.G., Soares Fortunato, R., Pires De Carvalho, D., Freitas Ferreira, A.C. (2013). Flavonoid rutin increases thyroid iodide uptake in rats. *PLoS One, 8*(9), e73908. [CrossRef]

56. Alonso-Castro, A.J., Domínguez, F., García-Carrancá, A. (2013). Rutin exerts antitumor effects on nude mice bearing SW480 tumor. *Archives of Medical Research, 44*(5), 346-351. [CrossRef]

57. Ali, M., Mudagal, M.P., Goli, D. (2009). Cardioprotective effect of tetrahydrocurcumin and rutin on lipid peroxides and antioxidants in experimentally induced myocardial infarction in rats. *Die Pharmazie-An International Journal of Pharmaceutical Sciences, 64*(2), 132-136. [CrossRef]

58. Annapurna, A., Reddy, C.S., Akondi, R.B., Rao, S.R. (2009). Cardioprotective actions of two bioflavonoids, quercetin and rutin, in experimental myocardial infarction in both normal and streptozotocin-induced type I diabetic rats. *Journal of Pharmacy and Pharmacology, 61*(10), 1365-1374. [CrossRef]

59. Panche, A., Diwan, A., Chandra, S. (2016). Flavonoids: An overview. *Journal of Nutritional Science, 5*, E47. [CrossRef]

60. Havsteen, B.H. (2002). The biochemistry and medical significance of the flavonoids. *Pharmacology & Therapeutics, 96*(2-3), 67-202. [CrossRef]

61. Sánchez-Hidalgo, M., León-González, A.J., Gálvez-Peralta, M., González-Mauraza, N.H., Martin-Cordero, C. (2021). D-Pinitol: A cyclitol with versatile biological and pharmacological activities. *Phytochemistry Reviews, 20*(1), 211-224. [CrossRef]

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 839-852, 2022
Doi: 10.33483/jfpau.1129575

ORIGINAL ARTICLE / ÖZGÜN MAKALE

INHIBITION OF IRE1α/XBP-1 BRANCH OF UPR BY GSK2850163 DRIVES THE SENSITIVITY TO TAMOXIFEN IN BREAST CANCER CELLS

UPR'NİN IRE1α/XBP-1 DALININ GSK2850163 ARACILI İNHİBİSYONU MEME KANSERİ HÜCRELERİNDE TAMOKSİFENE DUYARLILIĞI ARTIRIR

Yalcin ERZURUMLU[1*], Hatice Kubra DOGAN[2], Deniz CATAKLI[3]

[1]Suleyman Demirel University, Faculty of Pharmacy, Department of Biochemistry, 32260, Isparta, Turkey

[2]Suleyman Demirel University, Institute of Science, Department of Bioengineering, 32260, Isparta, Turkey,

[3]Suleyman Demirel University, Faculty of Medicine, Department of Pharmacology, 32260, Isparta, Turkey

ABSTRACT

Objective: *Tamoxifen is used as the leading treatment against breast cancer and has been broadly applied for the last 40 years. However, resistance development against tamoxifen is one of the major limitations in the effective treatment of breast cancer. The aim of our study was to investigate whether blockage of the IRE1α/XBP-1 branch of UPR by GSK2850163 efficiently limited the carcinogenic ability of tamoxifen-resistant MCF-7 cells.*

Material and Method: *Firstly, tamoxifen-resistant breast cancer cells were obtained by regularly exposing MCF-7 cells to tamoxifen. The biochemical activity of GSK2850163 was confirmed by immunoblotting and qRT-PCR. The possible effect of combined treatment of GSK2850163 and tamoxifen on proliferation, invasion, migration, and colony formation abilities of tamoxifen-resistant breast cancer cells were evaluated by using WST-1 based proliferation assay, Boyden-chamber invasion test, wound-healing assay, and plate colony formation methods, respectively.*

Result and Discussion: *Here, we showed that specific blockage of the IRE1α/XBP-1 by GSK2850163 efficiently limited the carcinogenic ability of tamoxifen-resistant MCF-7 cells. Moreover, co-treatment with*

* **Corresponding Author / Sorumlu Yazar:** Yalçın Erzurumlu
e-mail / e-posta: yalcinerzurumlu@sdu.edu.tr, **Phone / Tel.:** +902462110345

Submitted / Gönderilme: 12.06.2022 **Accepted / Kabul:** 02.08.2022

tamoxifen and GSK2850163 significantly reduced the invasion, migration, and colony formation abilities of breast cancer cells through improved the anti-cancer property of tamoxifen. Our results strongly suggested that IRE1α/XBP-1 inhibitors may be potent therapeutics in breast cancer treatment.

Keywords: *Breast Cancer, GSK2850163, IRE1α/XBP-1, Tamoxifen, UPR*

ÖZ

Amaç: *Tamoksifen meme kanserine karşı önde gelen tedavi olarak kullanılmaktadır ve son 40 yıldır yaygın olarak uygulanmaktadır. Ancak tamoksifene karşı direnç gelişimi meme kanserinin etkin tedavisindeki en büyük sınırlamalardan birisidir. Çalışmamızın amacı, UPR'nin IRE1α/XBP-1 dalının GSK2850163 tarafından bloke edilmesinin, tamoksifene dirençli MCF-7 hücrelerinin kanserojen yeteneğini verimli bir şekilde sınırlayıp sınırlamadığını araştırmaktır.*

Gereç ve Yöntem: *İlk olarak, MCF-7 hücrelerinin düzenli olarak tamoksifene maruz bırakılmasıyla tamoksifene dirençli meme kanseri hücreleri elde edildi. GSK2850163'ün biyokimyasal aktivitesi, immünoblotlama ve qRT-PCR ile doğrulandı. GSK2850163 ve tamoksifenin kombine tedavisinin tamoksifene dirençli meme kanseri hücrelerinin proliferasyon, invazyon, migrasyon ve koloni oluşturma yetenekleri üzerindeki olası etkileri sırasıyla WST-1 tabanlı proliferasyon testi, Boyden-chamber invazyon testi, yara iyileştirme testi ve plaka koloni oluşturma yöntemleri ile değerlendirildi.*

Sonuç ve Tartışma: *Çalışmamızda IRE1α/XBP-1'in GSK2850163 tarafından spesifik blokajının, tamoksifene dirençli MCF-7 hücrelerinin kanserojen yeteneğini verimli bir şekilde sınırladığını gösterdik. Ayrıca, tamoksifen ile GSK2850163'ün eş uygulaması tamoksifenin anti-kanser özelliğini geliştirerek meme kanseri hücrelerinin istila, göç ve koloni oluşturma yeteneklerini önemli ölçüde azalttı. Sonuçlarımız, IRE1α/XBP-1 inhibitörlerinin meme kanseri tedavisinde güçlü terapötikler olabileceğini önermektedir.*

Anahtar Kelimeler: *Meme Kanseri, GSK2850163, IRE1α/XBP-1, Tamoksifen, UPR*

INTRODUCTION

Breast cancer is the second reason of cancer-related deaths among women around the globe [1]. The receptor expression profiles of breast cancer cells directly affect the developmental profile in the carcinogenesis process. Today, we know that up to 75% of breast cancer cells possess estrogen receptors (Er) and/or progesterone receptors (PR) [2]. Selective estrogen modulator, tamoxifen behaves as an estrogen antagonist and prevents the binding of estrogens to the Er in breast tissue. In hormone receptor-positive breast tumors, tamoxifen is one of the most frequently used and effective chemotherapeutic agents [3]. It is used as a leading treatment against breast cancer and has been broadly applied for the last 40 years [4]. However, 20-30% of breast cancer patients develop de novo or acquired resistance to tamoxifen for various reasons [3,4]. Today, patients can have the chance of hormonal treatment according to the receptor status of the primary tumor. However, resistance development against tamoxifen is the main limitation in the effective treatment of breast cancer.

Recent studies have pointed out that Endoplasmic reticulum (ER) stress and the Unfolded Protein Response (UPR) mechanism are crucial key regulators in cancer progression and acquired drug resistance [5]. ER comprises 30% of the newly synthesized proteins in eukaryotic cells, therefore maturation processes of newly synthesized polypeptide chains are tightly controlled by protein quality mechanisms organized in the ER. Additionally, ER coordinates a variety of metabolic processes, such as synthesis of lipids, phospholipids, and steroids, biogenesis of autophagosomes and peroxisomes, and

gluconeogenesis [6,7]. Under altered physiological conditions, ER directly responds to adapt and protect the eukaryotic cells. Besides, various stressful conditions such as malformed protein accumulation, imbalance protein synthesis, and hypoxic conditions are caused by the disruption of the ER homeostasis, which is termed ER stress [6]. To readjust the ER homeostasis, the UPR signaling mechanism is activated, which is regulated through the ER-membrane localized three transmembrane proteins, activating transcription factor 6 alpha (ATF6α), protein kinase RNA-like ER kinase (PERK) and inositol requiring-enzyme 1 alpha (IRE1α) [6]. Recent studies have pointed out that IRE1α/XBP-1 branch of UPR is an important key regulator in the process of carcinogenesis, including prostate and breast cancer [8–10]. Notably, increased IRE1α/XBP-1 activity has been associated with the resistance of breast cancer cells to tamoxifen [11].

X-box binding protein-1 (XBP-1) (also known as unspliced XBP-1, XBP-1u) is an effector protein of IRE1α, which is activated through excising a 26-nucleotide-long intron in the XBP1 mRNA by the endoribonucleolytic activity of IRE1α. Activated XBP-1 termed spliced XBP1 (XBP-1s) function as a transcription factor and induces a specialized transcriptional program of UPR target genes under ER stress [12]. Previous studies have shown elevated expression levels of XBP-1 in therapy-resistant breast cancer cells [13,14]. Moreover, XBP-1s overexpression in Er (+) breast cancer enables estrogen-independent growth and less sensitivity to growth inhibition caused by tamoxifen and Faslodex independent of p53 [15]. Furthermore, activated XBP-1 has been shown to play a critical role in the tumorigenicity and progression of triple-negative breast cancer and also other cancer types [9,16–19]. Furthermore, higher ratios of XBP-1s/XBP-1u mRNA were determined in 100 primary breast cancer patients who received tamoxifen treatment [16]. Lastly, Ming et al. have determined that the modulation of IRE1α by STF083010 which inhibits the endonuclease activity of IRE1α, significantly increased tamoxifen sensitivity of MCF-7 cells, and progressively reduced breast carcinogenesis [11].

In this study, we evaluate the effect of the highly selective inhibitor of IRE1α, GSK2850163 effect on tamoxifen sensitivity and tumorigenic abilities of tamoxifen-resistant MCF-7 cells (termed MCF-7(R) hereafter). Our results suggest that GSK2850163 efficiently reverses tamoxifen resistance of MCF-7(R) cells. Moreover, tumorigenic abilities including proliferation rate, invasion-migration, and colony formation capability of MCF-7(R) cells are strongly limited by GSK2850163.

MATERIAL AND METHOD

Materials

Cell culture grade reagents including Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and additional growth requirements were obtained from Biological Industries. Tamoxifen was obtained from Santa Cruz Biotechnology. GSK2850163 was obtained from Sigma Aldrich. Rabbit

polyclonal antibodies XBP-1u (25997-1-AP) and XBP-1s (24868-1-AP) were obtained from Proteintech and Mouse monoclonal beta-actin (#A5316) and polyclonal anti-phospho-IRE1α (#SAB5700519) were purchased from Sigma Aldrich. Rabbit monoclonal anti-IRE1α (#3294) was purchased from Cell Signaling Technology. HRP-conjugated anti-mouse or anti-rabbit IgG was purchased from Pierce.

Cell culture and treatments

MCF-7, a human breast cancer cell line was obtained from American Type Culture Collection (ATCC, USA). The MCF-7 cell lines were cultured and passaged in DMEM media supplemented with 10% FBS at 37°C and 5% CO_2. All the treatment compounds were prepared as 1000x in the DMSO or ethanol. The final concentration of solvent did not exceed 0.1%.

Establishment of Tamoxifen-resistant MCF-7 cells

To develop the tamoxifen-resistant MCF-7 cells were cultured at conventional cell culture conditions and integrities. Additionally, cell culture media was supplemented with 1 μM tamoxifen and then cells were cultured for 30 days.

Reverse Transcription PCR (RT-PCR)

Total RNA was extracted from cell pellets using Aurum™ Total RNA Mini Kit (Bio-Rad Laboratories, Hercules, California). Total RNA (1 μg) was subjected to reverse transcription reaction using iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, California). Specific primers were designed against XBP-1u/s and RPLP0. Sequence information of PCR primers is available upon request. The expression of RPLP0 was used for normalization. Target regions were amplified by Polymerase Chain Reaction (PCR). PCR products were separated by 3% agarose gel and visualized by Gel-Doc UV-transilluminator (Bio-Rad).

Western blot analysis

MCF-7 cell lysates were prepared by homogenizing cultured cells in RIPA buffer. After removal of the insoluble phase by centrifugation at 14.000 r.p.m. for 20 minutes at 4 °C, protein concentrations were determined using the Bicinchoninic acid assay (BCA) kit (Takara). Samples were resolved by SDS-PAGE and electroblotted to a PVDF membrane. Protein bands were visualized using enhanced chemiluminescence (Bio-Rad) by ChemiDoc XRS+ System (Bio-Rad). Protein bands were densitometrically analysed by Image Studio™ Lite (LI-COR®).

Proliferation assay

The cell proliferation rate of MCF-7(R) cells was evaluated in triplicates by WST-1 cell proliferation assay according to the manufacturer's protocol (Takara). 5000 cells/well were seeded into a 96-well plate. After 24h incubation period compounds were applied to the cells for 48h. Following 20

µl of WST-1 were added to the cells, and the cells were incubated under conventional cell culture conditions for 2h. The plate was read using a microplate reader (BioTek, Epoch 2) by measuring the absorbance of the dye at 450nm, with 600nm set as the reference wavelength. Averages of the absorbance values were calculated.

Colony formation assay

MCF-7(R) cells were seeded in a 6-well plate (1000 cells/ml). 24h later cells were treated with compounds and kept at 37 °C in a CO_2 incubator for 72h. The media was removed, and the plate was washed with 1xPBS solution twice. Colonies were fixed and stained with 0,05% crystal violet (Alpha Chemika)

Invasion assay

Matrigel (BD Biosciences) and serum-free DMEM were gently mixed (1:8) in a tube. A volume of 45 µl mixed Matrigel was used to cover the bottom of the transwell insert with an 8-µm pore size (Sarstedt). All chambers were placed at 37 °C in a CO_2 incubator for 3h. The residual liquid in the upper chamber was removed, 100 µl serum-free DMEM was added, and the chamber was placed in the incubator for 30 min hydration. MCF-7(R) cells (10000 cells) were seeded into the Transwell basket before the addition of 600 µl complete medium containing 20% FBS to the lower chamber. The cells were kept at 37 °C in a CO_2 incubator for 72h. Invaded cells were fixed and stained with crystal violet. Invaded cells were observed using Sunny SopTop microscope and camera system. The invasion was quantified by counting stained cells.

Wound-healing assay

MCF-7(R) cells were seeded in a 12-well plate ($3,5x10^5$ cells/well) and propagated at 37 °C in a CO_2 incubator for 24h. The following scratch was made in the 12-well plate with a 200 µl micropipette tip. Cell culture media was removed and cells were washed off for removing the detached cells with preheated PBS. Cells were treated with compounds for 72h. Scratch width ratios were monitored, and images were taken by microscope. Wound closure (%) was analysed by ImageJ software (http://imagej.nih.gov/ij/).

Statistics

Results were presented as means ± standard deviation (SD). The statistical significance of differences between groups was determined by a two-tailed equal variance Student's t-test with a confidence interval, minimum, of 95% using GraphPad Prism 5 software. Values of $p < 0.05$ were considered significant.

RESULT AND DISCUSSION

Obtaining Tamoxifen-resistant MCF-7 cells

To achieve tamoxifen-resistant MCF-7, conventional MCF-7 cells were continuously exposed to 1μM tamoxifen for 30 days and then cell viability of these cells and non-exposed MCF-7 cells were compared at 2μM, 5μM, and 10μM tamoxifen doses. Our data indicated that the MCF-7(R) cells exhibited significantly less sensitivity to tamoxifen at all three concentrations compared to the conventional MCF-7 cells (Figure 1A). Besides, tamoxifen-resistant MCF-7 cells are known to exhibit higher XBP-1s expression [15,16] thus we evaluated the endogenous XBP-1s levels by qRT-PCR and immunoblotting. To analyze the mRNA level of XBP-1s, XBP1s/ XBP1u ratio was used as a measure of XBP1 splicing activity. Our results showed that MCF-7(R) cells have significantly higher mRNA expression levels of XBP-1s compared to conventional MCF-7 cells (Figure 1B). Consistently with these data, XBP-1s protein levels were higher in MCF-7(R) compared to conventional MCF-7 cells (Figure 1B). Moreover, cell proliferation assay data confirmed the resistance of MCF-7(R) cells to tamoxifen compared to regularly propagated MCF-7 cells (Figure 1C).

Inhibition of the IRE1α branch of UPR by GSK2850163 sensitizes the tamoxifen-resistant MCF-7 cells to tamoxifen

GSK2850163 is a novel IRE1α inhibitor, which is specifically inhibited IRE1α kinase activity at 20nM and it also blockage IRE1α RNase activity affecting its endonuclease activity at 200nM [20]. In this assay system, we used well-known ER stress inducer, Thapsigargin as a positive control. Our results show that GSK2850163 treatment efficiently reversed Thapsigargin-induced splicing of XBP-1 at 20nM and 200nM doses (Figure 2A). In addition, kinase activity blockage of IRE1α at 20nM dose compared to 200nM treatment is less efficient for inhibition on XBP-1s (Figure 2A).

GSK2850163 significantly decreased the proliferation rate of MCF-7(R) cells

To evaluate the anticarcinogenic ability of IRE1α inhibition by GSK2850163 in breast cancer, we tested the tumorigenic features of MCF-7(R) cells by proliferation rate. Our results showed that GSK2850163 significantly decreased the proliferation rate of MCF-7(R). A combination of tamoxifen and GSK2850163 showed a more efficient regressive effect in MCF-7(R) ($p < 0.05$ for all tested groups) (Figure 2C).

GSK2850163 and tamoxifen synergistically reduce the invasion-migration and colony formation capability of MCF-7(R) cells

In these assay systems, we specifically aimed to investigate the effects of IRE1α inhibition on breast cancer cells by colony formation, invasion, and migration abilities. To evaluate the effect of

GSK2850163 on the invasion capability of MCF-7(R) cells, we modelized the invasion assay by using a Boyden-chamber assay. Our data showed that GSK2850163 and tamoxifen applied groups more effectively delimitate the invasion of breast cancer cells than only the GSK2850163 treated group (Figure 3B). Furthermore, we tested the migration ability of MCF-7 (R) cells by scratching assay, our data indicated that similar to the invasion assay, GSK2850163 significantly decreased the migration capacity of breast cancer cells (Figure 3C). Similar to migration assay results, the colony formation abilities of MCF-7(R) cells were significantly limited by GSK2850163 (Figure 3A). Moreover, more effective results were obtained with the combined application of GSK2850163 and tamoxifen for all cell-based assays (Figure 3A, B, C).

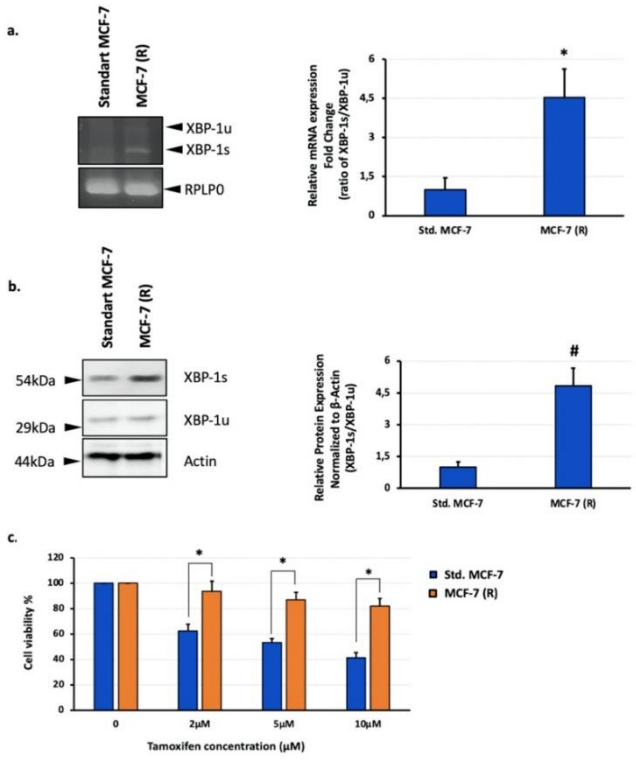


Figure 1. Confirmation of high expression level of XBP-1s in obtained tamoxifen-resistant MCF-7 cells. MCF-7 cells were exposed to 1μM tamoxifen for 30 days. a. Endogenous XBP-1s mRNA levels were detected by RT-PCR. RPLP0 gene is used as a housekeeping gene. b. Protein expression levels of XBP-1s were determined by immunoblotting and beta-actin was used as a loading control. Representative results are shown. p-values were calculated concerning vehicle-treated cells by two-tailed equal variance Student's t-test (*$p < 0.05$). c. The efficiency of tamoxifen resistance was evaluated with a WST-1 based cell growth assay of three biological and six technical replicates. Cells were treated with tamoxifen (2, 5, 10μM) for 48h. p-values were calculated concerning control group cells by two-tailed equal variance Student's t-test (*$p < 0.05$).

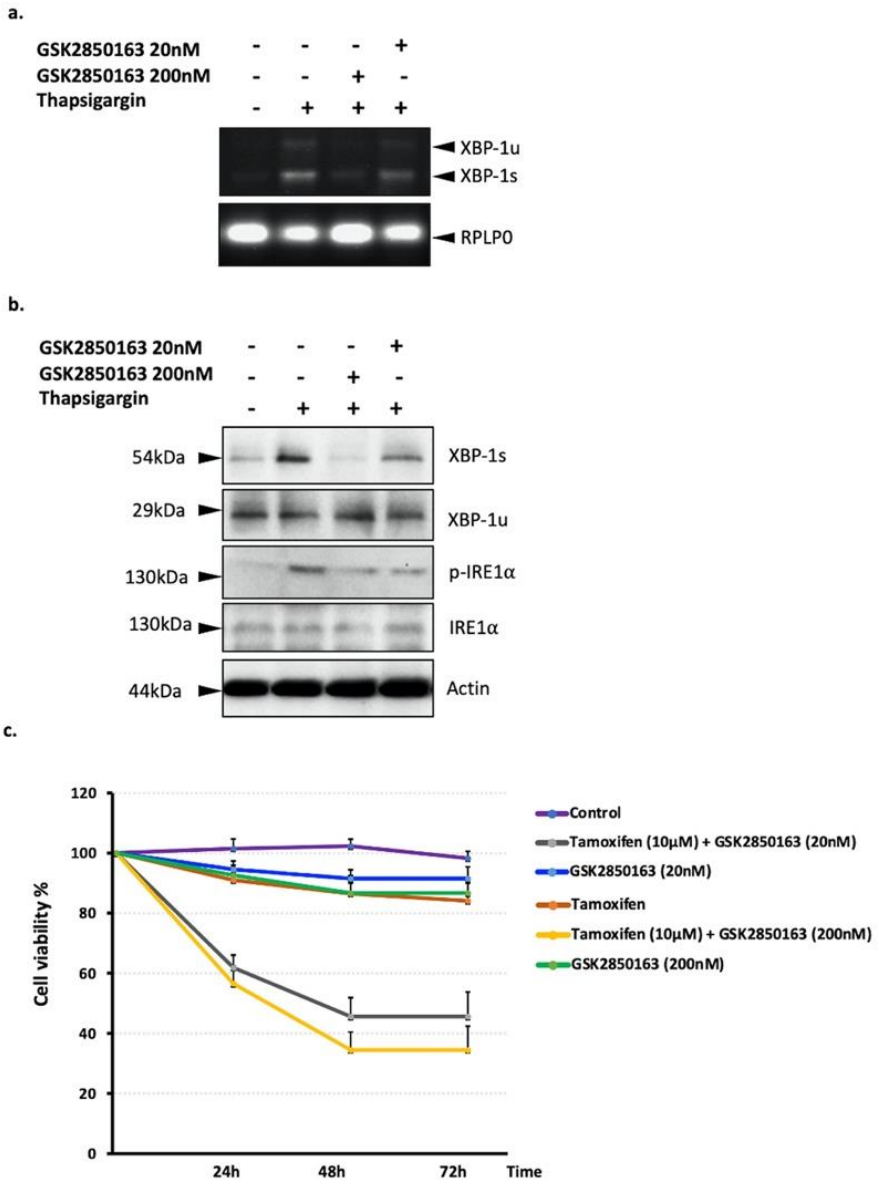


Figure 2. IRE1α/XBP-1 inhibitor GSK2850163 reverses the tamoxifen resistance of MCF-7(R). Cells were treated with 20 or 200nM GSK2850163 and Thapsigargin or combined with Thapsigargin and indicated doses of GSK2850163. a. XBP-1s levels were analyzed as in Fig. 1a. b. The expression levels of the related proteins were evaluated by immunoblotting. Beta-actin was used as a loading control. Representative results are shown. Thapsigargin was used as a positive control. c. The effect of GSK2850163 on tamoxifen sensitivity of MCF-7(R) cells was determined with cell viability assay, WST-1. Cells were treated with GSK2850163, tamoxifen, and co-treatment of GSK2850163 and tamoxifen as indicated doses. Results from three different experiments are presented in a graph.

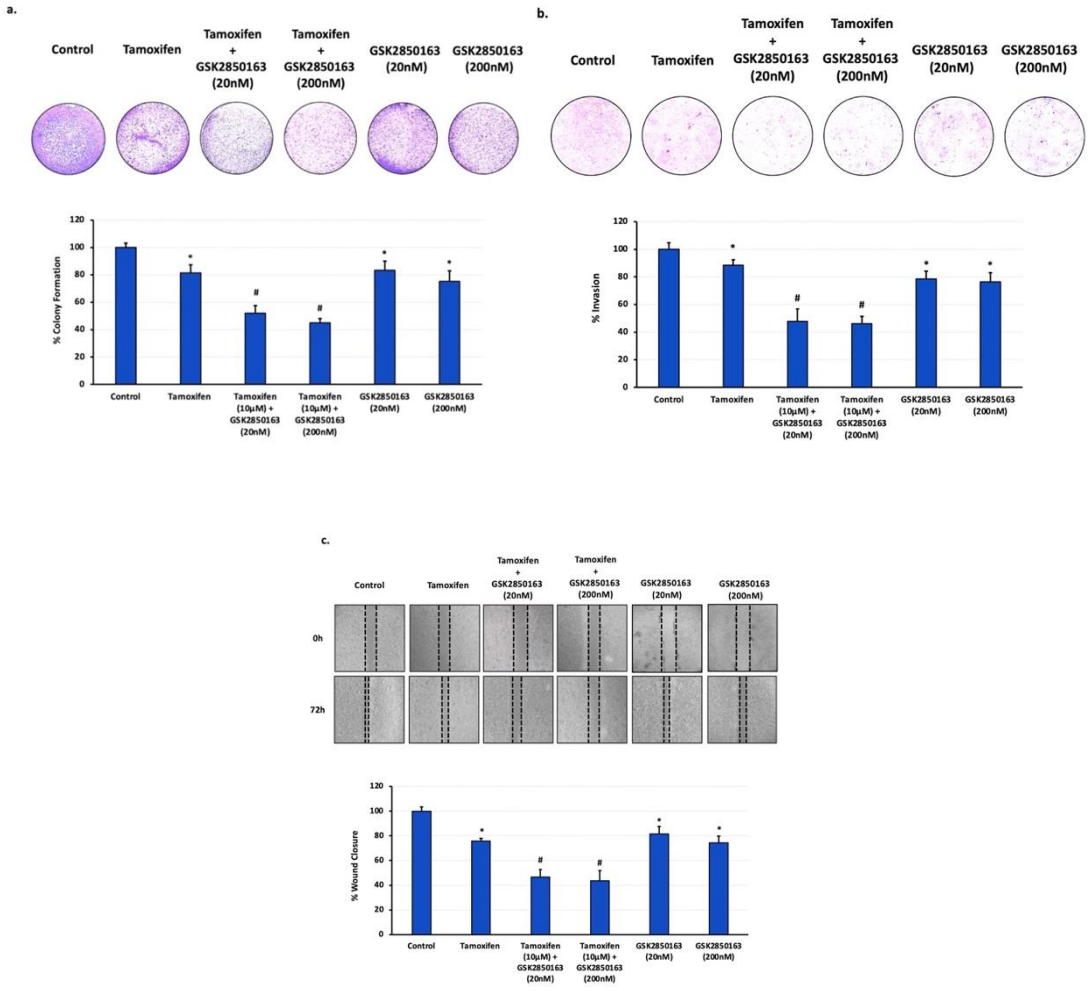


Figure 3. GSK2850163 significantly suppresses the tumorigenic properties of MCF-7(R) cells by acting synergistic effect with tamoxifen. a. MCF-7(R) cells were treated with 20 or 200nM GSK2850163 and tamoxifen or combined with tamoxifen and indicated doses of GSK2850163. Representative microscope images are presented. Quantification was performed with Image J software (*$p < 0.05$, #$p < 0.005$). b. The invaded MCF-7(R) cells on the lower surface of the membrane filter were fixed and stained with 0.2% crystal violet. Representative images are shown. The efficiency of invasion was quantified by counting stained cells with an inverted microscope. The mean percentage of invaded cells compared to control groups was given using the data obtained from two independent biological replicates in triplicates (*$p < 0.05$, #$p < 0.005$). c. A wound-healing assay was performed using MCF-7(R) cells. The closure of the gap created by the removal of the insert was monitored for 72h. The analysis of wound closure % was determined using the ImageJ software. Representative images are presented. p-values were calculated with respect to control group cells by two-tailed equal variance Student's t-test (*$p < 0.05$, #$p < 0.005$).

UPR is a key adaptive cytoprotective mechanism and mediates the adaptation of cells to changing physiological conditions [7]. In mammals, ER-resident three transmembrane proteins; IRE1α, PERK, and ATF6 are controlled the UPR [17]. Several researchers reported that the highly active IRE1α/XBP-1 is correlated with hepatocellular carcinoma, prostate, pancreatic, as well breast cancer [17–19]. Moreover, previous studies have shown that UPR activity is strictly associated with various features of tumor cells including invasion, migration, and angiogenesis. In particular, the IRE1α/XBP-1 has a critical role in implicated in the development of drug resistance in breast cancer [9]. Here, we indicated that GSK2850163, IRE1α dual inhibitor molecule which specifically inhibited IRE1α kinase activity RNase activity, is an important therapeutic molecule against drug-resistance development in breast cancer cells.

Firstly, we established tamoxifen-resistance MCF-7 cells by regularly exposing cells to a low dose of tamoxifen for 30 days. To validate the tamoxifen resistance, we performed cell viability tests and our results showed that MCF-7 (R) cells have less sensitivity to tamoxifen than standard MCF-7 cells by up to 60%. The previous study has demonstrated that tamoxifen-resistant MCF-7 cells exhibit a higher XBP-1s expression profile than standard cells [11]. Next, we investigated the expression level of XBP-1s and phospho IRE1α in MCF-7(R) and standard cells by immunoblotting assay, our results confirmed higher expression level of interested proteins in MCF-7(R) cells (Figure 1A, B). Collectively, these results showed that MCF-7(R) cells successfully mimic tamoxifen-resistant breast cancer.

GSK2850163 was discovered in an attempt to identify IRE1α-selective inhibitors of XBP-1 splicing that could regulate multiple myeloma cancer cells. It selectively inhibits the kinase and RNase activities of IRE1α [20]. In studies where we tested the efficiency of GSK2850163 treatment, we showed that thapsigargin-induced phospho-Ire1α and XBP-1s levels were significantly reduced compared to the thapsigargin group. As expected, we determined that 20nM dose of GSK2850163, which blocks the kinase activity of IRE1α, suppresses XBP-1s levels less efficiently than 200nM GSK2850163 administration (Figure 2B).

To investigate the limiting role of IRE1α/XBP-1 in tamoxifen-resistant breast carcinogenesis, we disrupted the IRE1α/XBP-1 by a specific inhibitor of IRE1α, which has a dual role inhibit IRE1α kinase activity and RNase activity for dose-dependent manner and evaluated the cell proliferation, invasive, migrative, and colony formation ability of tamoxifen-resistant breast cells. To evaluate the possible effect of the combined treatment of GSK2850163 and tamoxifen, MCF-7(R) cells were exposed and sensitivity to tamoxifen was determined by WST-1 based proliferation assay. Cell viability assay data showed that the GSK2850163 for both doses significantly reverses the tamoxifen resistance of MCF-7(R) cells. Besides, we observed that a higher dose of GSK2850163 treatment, which inhibits the

ribonuclease activity of IRE1α, is more efficient than a low dose of GSK2850163 in improving the tamoxifen sensitivity (Figure 2C).

Recent work has associated the IRE1α/XBP-1 branch of UPR with promoting tumor growth [19–22]. IRE1α/XBP-1 signaling is proposed as a functional mechanism of survival and adaptation for cancer cells. To investigate the possible effects of GSK2850163 on the tumorigenic abilities of tamoxifen-resistance breast cancer cells, we treated the MCF-7(R) cells either with the GSK2850163 at 20nM and tamoxifen, or GSK2850163 at 200nM and tamoxifen. Our data showed that combined treatment of GSK2850163 and tamoxifen synergistically affected the colony formation capability of MCF-7(R) cells. Continuity of clonogenic formation ability is an important limitation of cancerous tissues and cell-based assays evaluate the adhesion-independent cell proliferation of cancer cells [22,23]. Our results suggested that the administration of GSK2850163 in tamoxifen-resistant breast cancer cells significantly suppresses the colony-forming ability (Figure 3A).

Highly active migration and invasion ability are recognized as hallmarks of aggressive cancer [24]. We analyzed the limiter effect of GSK2850163 on the invasion of MCF-7(R) cells by using the matrigel-modified Boyden Chamber assay. Our data indicated that reducing IRE1α/XBP-1 significantly decreased the invasion ability of MCF-7(R) cells (Figure 3B). We also drastically obtained similar results in the wound-healing assay (Figure 3C).

The downstream target of IRE1α, XBP-1 has been suggested as a candidate oncogenic gene and is overexpressed in various cancers including prostate, oral squamous cell carcinoma, chronic lymphocytic leukemia as well as breast cancer [10,25–27]. Previous studies have shown that ectopically overexpressed XBP-1 resulted in metastasis in breast cancer [28,29]. Moreover, XBP-1 was reported to be significantly correlated with clinical outcomes in various tumors, such as breast carcinoma [28]. In addition, XBP-1s has been suggested as a critical regulator in drug resistance in certain subtypes of breast cancers [28].

The previous study has demonstrated that co-treatment with STF083010 inhibits the endonuclease activity of the IRE1α/XBP-1, without affecting its kinase activity, and tamoxifen significantly reduced breast cancer progression in a xenograft mammary tumor model [11]. Consistently with these results, our data suggest that targeting of kinase or endonuclease activity of IRE1α by GSK2850163 can restore the tamoxifen sensitivity of MCF-7(R) cells.

In conclusion, our study suggests that therapeutic approaches involving GSK2850163 may offer a potential therapeutic approach against breast cancer by overcoming tamoxifen resistance.

ACKNOWLEDGEMENTS

We thanks to Suleyman Demirel University-Innovative Technologies Application and Research Center. This study was supported by grants from the Suleyman Demirel University internal funds (TSG-2021-8302, TAB-2020-8253).

AUTHOR CONTRIBUTIONS

Conception: *Y.E.;* Design: *Y.E.;* Supervision: *Y.E.;* Resources: *Y.E.;* Materials: *-;* Data Collection and/or processing: *Y.E., H.K.D., D.C.;* Analysis and/or interpretation: *Y.E.;* Literature search: *Y.E., H.K.D., D.C.;* Writing manuscript: *Y.E.;* Critical review: *Y.E., H.K.D., D.C.;* Other: -

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

REFERENCES

1. Momenmovahed, Z., Salehiniya, H. (2019). Epidemiological characteristics of and risk factors for breast cancer in the world. *Breast Cancer, 11*, 151–164. [CrossRef]

2. Clarke, R., Liu, M.C., Bouker, K.B., Gu, Z., Lee, R.Y., Zhu, Y., Skaar, T.C., Gomez, B., O'Brien, K., Wang, Y., Hilakivi-Clarke, L.A. (2003). Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling. *Oncogene, 22*(47), 7316–7339. [CrossRef]

3. Shang, Y., Brown, M. (2002). Molecular determinants for the tissue specificity of SERMs. *Science, 295*(5564), 2465–2468. [CrossRef]

4. Lee, W.L., Cheng, M.H., Chao, H.T., Wang, P.H. (2008). The role of selective estrogen receptor modulators on breast cancer: from tamoxifen to raloxifene. *Taiwanese Journal of Obstetrics & Gynecology, 47*(1), 24–31. [CrossRef]

5. Avril, T., Vauléon, E., Chevet, E. (2017). Endoplasmic reticulum stress signaling and chemotherapy resistance in solid cancers. *Oncogenesis, 6*(8), e373. [CrossRef]

6. Adams, C.J., Kopp, M.C., Larburu, N., Nowak, P.R., Ali, M.M.U. (2019). Structure and molecular mechanism of ER stress signaling by the Unfolded Protein Response signal activator IRE1. *Frontiers in Molecular Biosciences, 6*, 11. [CrossRef]

7. Hetz, C. (2012). The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nature Reviews - Molecular Cell Biology*, *13*(2), 89–102. [CrossRef]

8. Zhao, N., Cao, J., Xu, L., Tang, Q., Dobrolecki, L.E., Lv, X., Talukdar, M., Lu, Y., Wang, X., Hu, D.Z, Shi, Q., Xiang, Y., Wang, Y., Liu, X., Bu, W., Jiang, Y., Li, M., Gong, Y., Sun, Z., Ying, H., Yuan, B., Lin, X., Feng, X.H., Hartig, S.M, Li, F., Shen, H., Chen, Y., Han, L., Zeng, Q., Patterson, J.B., Kaipparettu, B.A., Putluri, N., Sicheri, F., Rosen, J.M., Lewis, M.T., Chen, X. (2018). Pharmacological targeting of MYC-regulated IRE1/XBP1 pathway suppresses MYC-driven breast cancer. *Journal of Clinical Investigation*, *128*(4), 1283-1299. [CrossRef]

9. Chen, X., Iliopoulos, D., Zhang, Q., Tang, Q., Greenblatt, M. B., Hatziapostolou, M., Lim, E., Tam, W.L., Ni, M., Chen, Y., Mai, J., Shen, H., Hu, D.Z., Adoro, S., Hu, B., Song, M., Tan, C., Landis, M. D., Ferrari, M., Shin, S. J., Brown, M., Chang, J.C., Liu, X.S., Glimcher, L.H. (2014). XBP1 promotes triple-negative breast cancer by controlling the HIF1α pathway. *Nature*, *508*(7494), 103–107. [CrossRef]

10. Sheng, X., Nenseth, H.Z., Qu, S., Kuzu, O.F., Frahnow, T., Simon, L., Greene, S., Zeng, Q., Fazli, L., Rennie, P.S., Mills, I. G., Danielsen, H., Theis, F., Patterson, J.B., Jin, Y., Saatcioglu, F. (2019). IRE1α-XBP1s pathway promotes prostate cancer by activating c-MYC signaling. *Nature Communications*, *10*(1), 323. [CrossRef]

11. Ming, J., Ruan, S., Wang, M., Ye, D., Fan, N., Meng, Q., Tian, B., Huang, T. (2015). A novel chemical, STF-083010, reverses tamoxifen-related drug resistance in breast cancer by inhibiting IRE1/XBP1. *Oncotarget*, *6*(38), 40692–40703. [CrossRef]

12. Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., Mori, K. (2001). XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell*, *107*(7), 881–891. [CrossRef]

13. Ding, L., Yan, J., Zhu, J., Zhong, H., Lu, Q., Wang, Z., Huang, C., Ye, Q. (2003). Ligand-independent activation of estrogen receptor α by XBP-1. *Nucleic Acids Research*, *31*(18), 5266–5274. [CrossRef]

14. Fang, Y., Yan, J., Ding, L., Liu, Y., Zhu, J., Huang, C., Zhao, H., Lu, Q., Zhang, X., Yang, X., Ye, Q. (2004). XBP-1 increases ERalpha transcriptional activity through regulation of large-scale chromatin unfolding. *Biochemical and Biophysical Research Communications*, *323*(1), 269–274. [CrossRef]

15. Gomez, B.P., Riggins, R. B., Shajahan, A.N., Klimach, U., Wang, A., Crawford, A. C., Zhu, Y., Zwart, A., Wang, M., Clarke, R. (2007). Human X-box binding protein-1 confers both estrogen independence and antiestrogen resistance in breast cancer cell lines. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *21*(14), 4013–4027. [CrossRef]

16. Davies, M.P.A., Barraclough, D.L., Stewart, C., Joyce, K.A., Eccles, R.M., Barraclough, R., Rudland, P.S., Sibson, D.R. (2008). Expression and splicing of the unfolded protein response gene XBP-1 are significantly associated with clinical outcome of endocrine-treated breast cancer. *International Journal of Cancer. Journal International Du Cancer*, *123*(1), 85–88. [CrossRef]

17. Madden, E., Logue, S.E., Healy, S.J., Manie, S., Samali, A. (2019). The role of the unfolded protein response in cancer progression: From oncogenesis to chemoresistance. *Biology of the Cell / under the Auspices of the European Cell Biology Organization*, *111*(1), 1–17. [CrossRef]

18. Robinson, C.M., Talty, A., Logue, S.E., Mnich, K., Gorman, A.M., Samali, A. (2021). An Emerging role for the Unfolded Protein Response in pancreatic cancer. *Cancers, 13*(2). [CrossRef]

19. Romero-Ramirez, L., Cao, H., Regalado, M.P., Kambham, N., Siemann, D., Kim, J.J., Le, Q. T., Koong, A.C. (2009). X box-binding protein 1 regulates angiogenesis in human pancreatic adenocarcinomas. *Translational Oncology*, 2(1), 31–38. [CrossRef]

20. Concha, N.O., Smallwood, A., Bonnette, W., Totoritis, R., Zhang, G., Federowicz, K., Yang, J., Qi, H., Chen, S., Campobasso, N., Choudhry, A.E., Shuster, L.E., Evans, K.A., Ralph, J., Sweitzer, S., Heerding, D.A., Buser, C.A., Su, D.S., Phillip DeYoung, M. (2015). Long-Range Inhibitor-Induced Conformational Regulation of human IRE1α endoribonuclease activity. *Molecular Pharmacology, 88*(6), 1011–1023. [CrossRef]

21. Bujisic, B., De Gassart, A., Tallant, R., Demaria, O., Zaffalon, L., Chelbi, S., Gilliet, M., Bertoni, F., Martinon, F. (2017). Impairment of both IRE1 expression and XBP1 activation is a hallmark of GCB DLBCL and contributes to tumor growth. *Blood, 129*(17), 2420–2428. [CrossRef]

22. Drogat, B., Auguste, P., Nguyen, D.T., Bouchecareilh, M., Pineau, R., Nalbantoglu, J., Kaufman, R.J., Chevet, E., Bikfalvi, A., Moenner, M. (2007). IRE1 signaling is essential for ischemia-induced vascular endothelial growth factor-A expression and contributes to angiogenesis and tumor growth in vivo. *Cancer Research, 67*(14), 6700–6707. [CrossRef]

23. Franken, N.A.P., Rodermond, H.M., Stap, J., Haveman, J., van Bree, C. (2006). Clonogenic assay of cells in vitro. *Nature Protocols, 1*(5), 2315–2319. [CrossRef]

24. Wittekind, C., Neid, M. (2005). Cancer invasion and metastasis. *Oncology*, *69*(l), 14–16. [CrossRef]

25. Yang, J., Cheng, D., Zhou, S., Zhu, B., Hu, T., Yang, Q. (2015). Overexpression of X-Box Binding Protein 1 (XBP1) Correlates to Poor Prognosis and Up-Regulation of PI3K/mTOR in Human Osteosarcoma. *International Journal of Molecular Sciences, 16*(12), 28635–28646. [CrossRef]

26. Tang, C.-H.A., Ranatunga, S., Kriss, C.L., Cubitt, C.L., Tao, J., Pinilla-Ibarz, J.A., Del Valle, J. R., Hu, C.-C. A. (2014). Inhibition of ER stress-associated IRE-1/XBP-1 pathway reduces leukemic cell survival. *The Journal of Clinical Investigation, 124*(6), 2585–2598. [CrossRef]

27. Sun, Y., Jiang, F., Pan, Y., Chen, X., Chen, J., Wang, Y., Zheng, X., Zhang, J. (2018). XBP1 promotes tumor invasion and is associated with poor prognosis in oral squamous cell carcinoma. *Oncology Reports, 40*(2), 988–998. [CrossRef]

28. Andres, S.A., Wittliff, J.L. (2011). Relationships of ESR1 and XBP1 expression in human breast carcinoma and stromal cells isolated by laser capture microdissection compared to intact breast cancer tissue. *Endocrine, 40*(2), 212–221. [CrossRef]

29. Li, H., Chen, X., Gao, Y., Wu, J., Zeng, F., Song, F. (2015). XBP1 induces snail expression to promote epithelial- to-mesenchymal transition and invasion of breast cancer cells. *Cellular Signalling, 27*(1), 82–89. [CrossRef]

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 853-858, 2022
Doi: 10.33483/jfpau.1098785

ORIGINAL ARTICLE / ÖZGÜN MAKALE

THE COMPARISON OF VOLATILE COMPONENTS OF *AJUGA ORIENTALIS* L. COLLECTED FROM FOUR DIFFERENT LOCATIONS IN TURKEY

TÜRKİYE'DE DÖRT FARKLI LOKASYONDAN TOPLANAN AJUGA ORIENTALIS L.'İN UÇUCU BİLEŞENLERİNİN KARŞILAŞTIRILMASI

Ayla KAYA[1*], Betül DEMİRCİ[2]

[1]Anadolu University, Faculty of Pharmacy, Department of Pharmaceutical Botany, 26470 Eskisehir, Turkey

[2]Anadolu University, Faculty of Pharmacy, Department of Pharmacognosy, 26470 Eskisehir, Turkey

ABSTRACT

Objective: *This study aimed to determine and compare the volatile compositions of A. orientalis collected from Antalya, Balıkesir, Erzurum, and Samsun in Turkey.*

Material and Method: *Microdistilled volatile samples of A. orientalis were determined by GC-FID and GC-MS systems simultaneously.*

Result and Discussion: *o-Cresol is also present in the volatiles of three provinces (except Erzurum province) as a common main compound. The main compounds of the volatiles were methyl hexadecanoate (15.8%), linalool (11.4%) and hexahydrofarnesyl acetone (9.0%) for Erzurum province; o-cresol (43.7%), methyl oleate (10.1%) and methyl linoleate (9.1%) for Samsun province; o-cresol (75.2%) for Antalya province; hexadecanoic acid (20.3%), o-cresol (10.8%) and dihydroactinidiolide (10.8%) for Balıkesir province. The samples are rich in fatty acid+esters in all localities, except Erzurum province.*

Keywords: *Ajuga orientalis, GC-FID, GC-MS, Lamiaceae, volatile component*

ÖZ

Amaç: *Bu çalışmada, Türkiye'de Antalya, Balıkesir, Erzurum ve Samsun'dan toplanan A. orientalis'in uçucu bileşenlerinin belirlenmesi ve karşılaştırılması amaçlamıştır.*

Gereç ve Yöntem: *A. orientalis'in mikrodistilasyonla elde edilen uçucu bileşenleri GC-FID ve GC-MS sistemleri ile eş-zamanlı olarak belirlenmiştir.*

* **Corresponding Author / Sorumlu Yazar:** Ayla Kaya
e-mail / e-posta: aykaya@anadolu.edu.tr, **Phone / Tel.:** +902223350580

Submitted / Gönderilme: 05.04.2022 **Accepted / Kabul:** 03.08.2022

Sonuç ve Tartışma: *o-Krezol üç ilin (Erzurum ili hariç) uçucu yağlarında ortak ana bileşik olarak bulunmaktadır. Uçucu maddelerin ana bileşikleri Erzurum ili için metil hekzadekanoat (%15.8), linalol (%11.4) ve hekzahidrofarnesil aseton (%9.0); Samsun ili için o-krezol (%43.7), metil oleat (%10.1) ve metil linoleat (%9,1); Antalya ili için o-kresol (%75.2); Balıkesir ili için hekzadekanoik asit (%20.3), o-krezol (%10.8) ve dihidroaktinidiolid (%10.8). Erzurum ili hariç tüm lokaliteler yağ asidi+ester yönünden zengindir.*

Anahtar Kelimeler: *Ajuga orientalis, GK-AİD, GK-KS, Lamiaceae, uçucu bileşen*

INTRODUCTION

The genus *Ajuga* L. (Lamiaceae), comprising about 300 species, is mainly distributed in Asia, Africa, Australia, Europe and North America. In the Flora of Turkey, the genus *Ajuga* is represented by 23 taxa, seven of which are endemic [1]. The genus of *Ajuga* contains many important bioactive compounds are used in traditional medicine against some illnesses such as gout, rheumatism and gastrointestinal diseases. Also, they have antibacterial, antitumor [2], neuroprotective effects [3] and antioxidant [4] activities among others biological effects.

A. orientalis is a perennial herbaceous plant with 6-30 cm long stems. The cauline leaves are 1-2-paired, obovate-oblong shaped. Verticillasters are crowded with violet-blue and cream-colored flowers [5]. The plant is usually grows in every region of Turkey and it is called "dağmayasılı" in the areas where it grows wildly [1].

Previous studies reported that *A. orientalis* was characterized with germacrene D (24.7%), β-cubebene (18.3%) and β-caryophyllene (16.9%) by Sajjadi and Ghannadi [6]; phytol (36.7%), n-hexadecanoic acid (14.2%) and dodecanoic acid (12.2 %) by Kücükbay et al. [7]. Volatile compounds of *A. orientalis* from natural habitat and cultivars in the first and second vegetation periods isolated by SPME analyzed by GC-MS by Dönmez [8]. 2-Hexen-1-al and 1-octen-3-ol were determined as the main components in the natural habitat and cultivars of the samples. In addition, the amount of limonene in *A. orientalis* volatile compounds was found to be another main compound [8].

In this survey, we report chemical compounds of *A. orientalis* growing in four different regions (Erzurum, Samsun, Antalya and Balıkesir) of Turkey.

MATERIAL AND METHOD

Plant Material

A. orientalis (Fig. 1) was collected from Erzurum (ESSE 14461), Samsun (ESSE 10209), Antalya (ESSE 9369) and Balıkesir (ESSE 14462) provinces of Turkey (Fig. 2). A voucher specimen is kept at the herbarium of the Faculty of Pharmacy at Anadolu University, Turkey.

Figure 1. *A. orientalis*

Figure 2. Collection areas

Isolation and Analysis of the Volatiles

The volatiles were obtained by microdistillation of the dried, ground plant materials (~500 mg) using an Eppendorf MicroDistiller®. The volatiles were analyzed by GC-FID and GC-MS systems, simultaneously. All processes were performed with reference to Demirci et. al. [9].

Identification of the Components

Identification of the volatile components was carried out with reference to Demirci et. al. [9].

RESULT AND DISCUSSION

Comparisons of the volatile components for the four populations are given in Table 1. Thirteen, fifteen, eleven and eight components were identified from the volatiles of Erzurum, Samsun, Antalya and Balıkesir localities, respectively, accounting for 72.6%, 95.2%, 97.1% and 64.9% of the oil. The major constituents of the volatiles were methyl hexadecanoate (15.8%), linalool (11.4%), hexahydrofarnesyl acetone (9.0%) and methyl linoleate (7.5%) for Erzurum province; *o*-cresol (43.7%), hexadecanoic acid (11.6%), methyl oleate (10.1%) and methyl linoleate (9.1%) for Samsun province; *o*-cresol (75.2%) for Antalya province; hexadecanoic acid (20.3%), *o*-cresol (10.8%) and dihydroactinidiolide (10.8%) for Balıkesir province. *O*-cresol is also present in three provinces as a common main compound. The volatiles are rich in fatty acid+esters and others (except Balıkesir province) while they are poor in oxygenated monoterpenes (except Erzurum province) in all localities according to Table 1. Oxygenated sesquiterpenes and sesquiterpene hydrocarbones are identified from only two localities, respectively, Erzurum and Samsun.

Table 1. The composition of the volatiles of *Ajuga orientalis*

RRI	Compound	E %	S %	A %	B %	IM
1202	3-Hexanol	-	-	-	0.8	MS
1452	1-Octen-3-ol	tr	-	0.6	5.1	MS
1479	(*E*,*Z*)-2,4-Heptadienal	-	-	-	-	MS
1496	2-Ethyl hexanol	6.1	-	-	4.2	MS
1553	Linalool	**11.4**	-	-	-	MS
1562	Octanol	-	0.3	tr	-	MS
1600	Hexadecane	4.2	-	-	-	RRI, MS
1683	*trans*-Verbenol	-	0.6	-	-	MS
1703	Salicylaldehyde	-	1.0	0.6	-	RRI, MS
1706	α-Terpineol	5.8	-	-	-	RRI, MS
1719	Borneol	-	0.5	0.3	-	RRI, MS
1725	Verbenone	-	1.6	-	-	MS
1856	*m*-Cymen-8-ol	-	0.7	-	-	MS
1864	*p*-Cymen-8-ol	-	0.9	-	-	RRI, MS
2004	*o*-Cresol	-	**43.7**	**75.2**	**10.8**	RRI, MS
2131	Hexahydrofarnesyl acetone	**9.0**	2.2	1.5	6.2	MS
2226	Methyl hexadecanoate	**15.8**	6.7	4.0	-	RRI, MS
2239	Carvacrol	-	2.8	1.9	**7.5**	RRI, MS
2255	α-Cadinol	5.8	-	-	-	MS
2329	Methyl heptadecanoate	-	3.4	-	-	RRI, MS
2380	Dihydroactinidiolide	-	-	-	**10.0**	MS
2456	Methyl oleate	4.9	**10.1**	4.2	-	RRI, MS
2509	Methyl linoleate	**7.5**	**9.1**	4.6	-	RRI, MS
2583	Methyl linolenate	1.5	-	-	-	RRI, MS
2613	Ethyl linolenate	0.5	-	-	-	RRI, MS
2931	Hexadecanoic acid	0.1	**11.6**	4.2	**20.3**	RRI, MS
	Oxygenated Monoterpenes	17.2	5.5	2.2	7.5	
	Sesquiterpene Hydrocarbones	-	1.6	-	-	
	Oxygenated Sesquiterpenes	5.8	-	-	-	
	Fatty acid+esters	19.3	47.2	77.9	54.9	
	Others	30.3	40.9	17	2.5	
	Total	**72.6**	**95.2**	**97.1**	**64.9**	

RRI: Relative retention indices calculated against *n*-alkanes; %: calculated from FID data
Locality: E: Erzurum, S: Samsun, A: Antalya, B: Balıkesir
IM: Identification method based on the relative retention indices (RRI) of authentic compounds on the HP Innowax column; MS, identified on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and comparison with literature data

In an earlier study, the essential oil composition of *A. orientalis* growing Iran was studied by Sajjadi and Ghannadi [6] and the main constituents were found as germacrene D (24.7%), β-cubabene (18.3%) and β-caryophyllene (16.9%). Iran oil was rich in hydrocarbon sesquiterpenes. Contrary to the earlier report that fatty acid+esters and others were present as main compounds in Turkish oil. In another study, thirty components were identified and phytol (36.7%), *n*-hexadecanoic acid (14.2%) and dodecanoic acid (12.2%) were reported as the main components in the essential oil of the aerial parts of

A. orientalis from Erzurum by Kücükbay et al. [7]. However, the essential oil composition of our samples was found to be quite different from those already reported. Later, Dönmez [8] analyzed the leaves and flowers of *A. orientalis*, which were taken from both their natural habitat and cultivars. 2-hexen-1-al (30.89% in the natural area and 23.12% cultivated plants) was found as the dominant component in the first vegetation period of *A. orientalis* however, there was a nearly 80% decrease during the second vegetation period. In the first and second vegetation periods of the samples, 1-octen-3-ol (26.30-28.16% in natural habitat and 26.79-28.34% in cultivars) was determined as the highest amount of another component. In addition, the amount of limonene (12.26% in natural habitat and 11.69% in cultivars) in *A. orientalis* essential oil increased in the second vegetation period compared to the first.

In the literature, some papers have reported on the variation in the essential oil composition induced by environmental, physiological, and edaphic factors which can cause changes in biosynthesis accumulation or metabolism of given compounds of the essential oil [10].

AUTHOR CONTRIBUTIONS

Concept: *A.K.*; Design: *A.K.*; Control: *A.K.*; Sources: *A.K., B.D.*; Materials: *A.K., B.D.*; Data Collection and/or processing: *A.K.*; Analysis and/or interpretation: *B.D.*; Literature review: *A.K., B.D.*; Manuscript writing: *A.K.*; Critical review: *A.K., B.D.*; Other: -

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study

REFERENCES

1. Güner, A., Aslan, S., Ekim, T., Vural, M., Babaç, M. (2012). Türkiye Bitkileri Listesi (Damarlı Bitkiler). Nezahat Gökyiğit Botanik Bahçesi ve Flora Araştırmaları Derneği Yayını: Türkiye

2. Ben Jannet, H., Chaari, A., Bakhrouf, A., Mighri, Z. (2006). Structure antibacterial activity relationship of secondary metabolites from *Ajuga pseudoiva* Rob. leaves. *Natural Product Research. 203*, 299-304. [CrossRef]

3. Guo, P., Li, Y., Xu, J., Liu, C., Ma, Y., Guo, Y. (2011). Bioactive neo-clerodane diterpenoids from the whole plants of *Ajuga ciliata* Bunge. *Journal of Natural Products. 747*, 1575-1583. [CrossRef]

4. Makni, M., Haddar, A., Kriaa, W., Zeghal, N. (2011). Antioxidant, free radical scavenging, and antimicrobial activities of *Ajuga iva* leaf extract. *International Journal of Food Properties. 164,* 756-765. [CrossRef]

5. Davis, P.H. (1982). *Ajuga* L. In: P.H. Davis (Ed.) Flora of Turkey and the East Aegean Islands. Vol. 7. UK: Edinburgh University Press.

6. Sajjadi, S.E., Ghannadi, A. (2004). Volatile oil composition of the aerial parts of *Ajuga orientalis* L. from Iran. *Zeitschrift für Naturforschung. 59c*, 166-168. [CrossRef]

7. Küçükbay, F.Z., Kuyumcu, E., Yıldız, B. (2013). Essential oil composition from the aerial parts of *Ajuga orientalis* L. growing in Turkey. *Asian Journal of Chemistry. 25,* 9126-9128. [CrossRef]

8. Dönmez, Ş. (2019). Volatile oil composition of *Ajuga* species of natural and cultivated origin in the lake district of Turkey. *Applied Ecology And Environmental Research, 17*(2), 3859-3866. [CrossRef]

9. Demirci, B., Küçükboyaci, N., Adıgüzel, N., Başer, K.H.C., Demirci, F. (2010). Characterization of *Szovitsia callicarpa* volatile constituents obtained by micro- and hydrodistillation. *Natural Product Communications 5*, 297-300. [CrossRef]

10. Senatore, F., De Fusco, R., De Feo, V. (1997). Essential oils from *Salvia* spp. (Lamiaceae). I. chemical composition of the essential oils from *Salvia glutinosa* L. growing wild in southern Italy. *Journal of Essential Oil Research 9*, 151-157. [CrossRef]

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 859-871, 2022
Doi: 10.33483/jfpau.1107620

ORIGINAL ARTICLE / ÖZGÜN MAKALE

DETERMINATION OF pK_a VALUES OF TENOXICAM, PIROXICAM AND MELOXICAM BY RP-HPLC AT 25°C AND 37°C IN THF-WATER BINARY MIXTURES

TENOKSİKAM, PİROKSİKAM VE MELOKSİKAM'IN pK_a DEĞERLERİNİN RP-HPLC YÖNTEMİYLE 25°C VE 37°C'DE THF-SU İKİLİ ORTAMINDA TAYİNİ

Dilara BAŞAT DERELİ[1*]

[1]Bitlis Eren University, Tatvan Vocational School, Department of Chemistry and Chemical Processing Technologies, 13100, Bitlis, Turkey

ABSTRACT

Objective: *In this study, the ionization constant (pK_a) values of oxicam group drug active ingredients, tenoxicam, piroxicam and meloxicam, were investigated both because of their effectiveness in reducing pain and inflammation and because of their pharmaceutical importance.*

Material and Method: *pK_a values were determined by RP-HPLC method in tetrahydrofuran-water binary mixture (30%-40%(v/v)) at 25°C and 37°C. The pK_a values of these compounds in water were evaluated by mole fraction and Yasuda-Shedlovsky extrapolation methods.*

Result and Discussion: *This study is the first pK_a determination study for tenoxicam, piroxicam and meloxicam in tetrahydrofuran-water media and also at 37°C, which is body physiological temperature. For tenoxicam, piroxicam and meloxicam, the pK_a values calculated by the mole fraction method at 25°C were 5.067 ± 0.037; 5.237 ± 0.065; 4.027 ± 0.144; pK_a values at 37°C are 5.166 ± 0.017; 5.197 ± 0.084; 4.161 ± 0.116. By Yasuda-Shedlovsky extrapolation, pK_a values calculated at 25°C were 5.061 ± 0.035; 5.232 ± 0.063; 4.021 ± 0.141; pK_a values at 37°C are 5.161 ± 0.013; 5.192 ± 0.053; 4.155 ± 0.094. The results are in agreement with previous studies with different methods and different solvents at 25°C.*

Keywords: *Oxicam, RP-HPLC, THF-water binary mixtures*

ÖZ

Amaç: *Bu çalışmada, oksikam grubu ilaç etken maddeleri olan tenoksikam, piroksikam ve meloksikamın hem ağrı ve iltihabı azaltmadaki etkinlikleri hem de farmasötik önemi nedeniyle iyonizasyon sabiti (pK_a) değerleri araştırıldı.*

[*] **Corresponding Author / Sorumlu Yazar:** Dilara Başat Dereli
e-mail / e-posta: dbasat@beu.edu.tr, **Phone / Tel.:** +904342220096/9618

Submitted / Gönderilme: 22.04.2022 **Accepted / Kabul:** 03.08.2022

Gereç ve Yöntem: *pK_a değerleri, 25°C ve 37°C'de tetrahidrofuran-su ikili karışımında (%30-40(h/h)) RP-HPLC yöntemiyle belirlendi. Bu bileşiklerin sudaki pK_a değerleri, mol kesri ve Yasuda-Shedlovsky ekstrapolasyon yöntemleri ile değerlendirildi.*

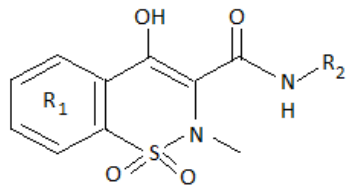
Sonuç ve Tartışma: *Tenoksikam, piroksikam ve meloksikam için 25°C'de mol kesri yöntemiyle hesaplanan pK_a değerleri 5.067 ± 0.037; 5.237 ± 0.065; 4.027 ± 0.144; 37°C'de pK_a değerleri 5.166 ± 0.017; 5.197 ± 0.084; 4.161 ± 0.116. Yasuda-Shedlovsky ekstrapolasyonu ile 25°C'de hesaplanan pK_a değerleri 5.061 ± 0.035; 5.232 ± 0.063; 4.021 ± 0.141; 37°C'deki pK_a değerleri 5.161 ± 0.013; 5.192 ± 0.053; 4.155 ± 0.094. Sonuçlar, 25°C'de farklı yöntemler ve farklı çözücüler ile yapılan önceki çalışmalarla uyumludur. Bu çalışma, tetrahidrofuran-su ortamında ve ayrıca vücut fizyolojik sıcaklığı olan 37°C'de tenoksikam, piroksikam ve meloksikam için yapılan ilk pK_a belirleme çalışmasıdır.*

Anahtar Kelimeler: *Oksikam, RP-HPLC, THF-su ikili karışımı*

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the best-selling over-the-counter medicines in the world, accounting for 5% of prescription medicines used for the treatment of patients suffering from chronic pain and inflammatory conditions [1]. They cover a wide range of drugs according to their structural and functional properties. NSAIDs are classified according to their chemical properties as major derivatives of salicylic acid, acetic acid, enolic acid, anthranilic acid or propionic acid. In general, they show weak organic acid properties because they have an acidic part such as carboxylic acid or enol attached to an aromatic functional group [1, 2]. Ionization constants (pK_a) range from 3 to 6 [2, 3].

Oxicams are enolic acid derivatives and are widely used NSAID drugs in the pain and inflammation of rheumatic diseases [2, 4]. Tenoxicam, meloxicam and piroxicam are oxicam group drugs, which are enolic acid derivatives with similar structural properties [2, 5] (Figure 1).



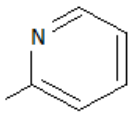
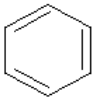
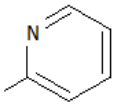
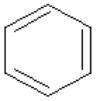
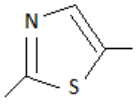
Compound Name	R_1	R_2
Tenoxicam		
Piroxicam		
Meloxicam		

Figure 1. Chemical structure of tenoxicam, meloxicam and piroxicam

When their chemical structures are examined, it is seen that while R_1 groups are different in tenoxicam and piroxicam, R_2 groups are different in meloxicam and piroxicam. Although they have different therapeutic activities depending on these similarities and differences in their structures, their mechanisms of action are similar. At the same time, due to their high lipophilicity, they cause side effects such as various gastrointestinal complications [1, 4]. Absorption, distribution, metabolism and excretion (ADME) mechanisms of these drugs in the body need to be improved in order to reduce their side effects and facilitate their use. In order to improve ADME properties, it is necessary to know the pK_a value, which affects on the properties of drugs such as solubility, lipophilicity, permeability and protein binding [6, 7]. With the pK_a value, it is possible to comment on the effect on the biological behavior of the molecule, depending on the pH of the media [6]. In particular, it is necessary to know the pK_a value in order to develop and design new drug active molecules, because with this value, it can be interpreted about the movement of drugs into cells and other membranes [8].

When the literature studies are examined, it is seen that there are comprehensive studies that include the methods used for the determination of pK_a [8, 9, 10, 11, 12]. In these methods, pK_a can be calculated based on the measurement of a physical property of the analyte. At the same time, there are studies on computer programs developed for the estimation of pK_a and their comparison with experimental results [9, 13, 14, 15, 16]. Among these methods, HPLC method is one of the preferred methods, especially in determining the pK_a of drugs that are poorly soluble in water, in addition to its features such as obtaining fast results with a small sample, high sensitivity and precision [8, 9, 17]. An organic solvent-water mixture is generally used to determine the pK_a of poorly water-soluble drugs [18, 19]. By performing several experiments in different organic solvent-water mixtures, the pK_a values in these media ($^{s}_{s}pK_a$) are determined and the pK_a values in water ($^{w}_{w}pK_a$) can be estimated by an extrapolation method such as Yasuda-Shedlovsky [8, 12, 14, 20, 21, 22]. The selected organic solvents consist of alcohols such as dimethyl sulfoxide, dimethylformamide, acetonitrile, acetone, dioxane, tetrahydrofuran (THF), which are widely used with this extrapolation method and have the ability to dissolve ionizable compounds [12]. Although there is no universal organic solvent-water binary mixture for the determination of pK_a, the most preferred solvent mixtures are methanol-water, acetonitrile-water and THF-water mixtures [12, 23].

When the literature is examined for the determination of pK_a values of oxicams, it is stated that the pK_a values of the compounds are determined by different methods. In these literatures, it was generally studied at 25°C for the determination of pK_a. The biorelevant temperature in humans is 37°C. Therefore, it is known that studies at this temperature are more beneficial in biologically occurring mechanical dissolution studies, and in elucidating intercellular transmission mechanisms by ionizable molecules [24].

In this study first time, pK_a values were calculated at both 25℃ and 37℃ by RP-HPLC method using THF-water organic solvent mixture (30%-40% (v/v)), which has not been used before in the pK_a determination of meloxicam, piroxicam and tenoxicam compounds. From the ${}^{s}_{s}pK_a$ values calculated for each media, ${}^{w}_{w}pK_a$ values were calculated using the Yasuda–Shedlovsky equation and the mole fraction-pK_a extrapolation method.

MATERIAL AND METHOD

Chemicals and Reagents

Meloxicam, piroxicam and tenoxicam, whose pK_a was determined by RP-HPLC, were obtained from Sigma Aldrich (Darmstadt, Germany). THF, the organic solvent used for the mobile phase, and uracil, the unretained species in the column, were supplied from Sigma Aldrich (Darmstadt, Germany). Sodium hydroxide used for adjusting the mobile phase pH was purchased from Merck (Darmstadt, Germany), and ortho-phosphoric acid was obtained from Riedel-de Haën (Seelze, Germany). Potassium hydrogen phthalate (KHP) used for pH meter calibration, were supplied from Merck (Darmstadt, Germany).

Apparatus

Agilent 1260 Infinity (Santa Clara, United States) brand High Performance Liquid Chromatograph device was used to determine the pK_a values of the studied compounds. The system includes 1260 Quat Pump VL pump, 1260 DAD VL detector, 1260 ALS automatic sample injection part.

Hanna HI 221 pH meter (Carrollton, Texas) device was used for pH measurements of RP-HPLC mobile phase and Hanna HI 1131 glass electrode was used for measurements. During the adjustment of the mobile phase pH, the temperature was kept constant at 25 °C.

As a column, ACE C18 (5 µm, 4.6 x 250 mm) column with low silanol activity was used. Thanks to this feature, it removes the negative effects on the chromatographic separation of silanols, and is preferred for the separation of both acidic and basic compounds. In addition, due to its high stability, its repeatability and peak shapes are also very good. The fact that it can work up to 100℃ in the pH range of 2.0-8.0 is one of the other reasons for preference of the column.

Chromatographic Procedures

The electrode was calibrated by keeping it in the working water-organic solvent mixture for 15-20 minutes so that the readings could be stable. THF-water binary mixtures were prepared with pH in the range of 3.0-7.0 for each water-organic solvent mixture. In these adjustment processes, 50 mM

phosphoric acid was used, taking into account the buffer range.

100 ppm stock solutions were prepared by weighing 1 mg of each of the compounds provided in analytical purity and dissolving them in 10 ml of mobile phase. 20 µl of these prepared solutions were injected into the liquid chromatography device. In the RP-HPLC study, the required to (dead time) value for the determination of the capacity factors was determined using uracil. In this study, the average retention factors of both uracil and compounds were determined by injecting twice for each compound. For the determination of the ionization constants of the compounds, THF-water binary mixture was used, the column temperature was determined as 25°C and 37°C, and the flow rate was 1 ml/min. The studied wavelenghts were 355 nm for compounds and 254 nm for uracil. For the determination of the ionization constant of the compounds, 30%, 35% and 40% (v/v) THF-water binary mixtures were used.

Data Treatment

The t_o value used to calculate the capacity factors of the compounds was calculated with the formula $k=(t_R-t_0)/t_0$ using uracil. The variation of the capacity factor values of the compounds with pH shows sigmoidal behavior. The pH value at the midpoint of this sigmoidal gives the pK_a value of that compound on the working media. The ${}^{s}_{s}pK_a$ values of the compounds were calculated by evaluating the capacity factor –pH relationship in 30%, 35% and 40% (v/v) THF-water binary media with the NLREG program. NLREG is a nonlinear regression program specially developed for calculating pK_a values from capacity factors and pH values [25]. Mole fraction extrapolation method and Yasuda–Shedlovsky equation were used for the transition from ${}^{s}_{s}pK_a$ values calculated from THF-water mixtures to ${}^{w}_{w}pK_a$ values in water.

In the mole fraction extrapolation method, a linear equation is obtained by plotting the pK_a values against the mole fraction values calculated depending on the volume percent of the organic solvent used in the mobile phase. The ${}^{s}_{s}pK_a$ value is calculated from the intercept value of this equation. Mole fraction values for THF-water mixtures were obtained from Barbosa, 1999.

Yasuda-Shedlovsky equation is another extrapolation method that helps to calculate ${}^{s}_{s}pK_a$ value from different organic solvent-water mixtures with Equation 1 based on Born electrostatic model [12, 21, 22].

$$ {}^{s}_{s}pK_a + \log[H_2O] = a_\varepsilon\, \varepsilon^{-1} + b_\varepsilon \qquad \text{Equation 1} $$

Here ${}^{s}_{s}pK_a$ gives the pK_a value of the mobile phase, ε the dielectric constant of the organic solvent in the water-organic solvent binary mixture, aε the slope value obtained from the linear equation, and bε the intercept value obtained from the linear equation. In order to calculate the ${}^{s}_{s}pK_a$ values in each media, the dielectric (ε) and autoprotolysis (pK_w) constants must be known. These values were obtained from literature sources [23, 26].

RESULT AND DISCUSSION

${}^{s}_{s}pK_a$ Values Determined by RP-HPLC

Acetonitrile-water, methanol-water and THF-water media, which are more preferred by RP-HPLC method in pK_a determination, were tested with both Agilent Zorbax Eclipse Plus C18 column and ACE C18 column at 25°C and 37°C. ACE C18 column, peak shapes and retention times in THF-water media were found suitable when evaluated in terms of both temperature conditions, since it provides a short retention time and the peak shapes are good.

In this study, 30%, 35% and 40% (v/v) THF-water mobile phase composition was studied. Considering the pK_a values of the enolic acid group in the structure of the compounds, the retention factors were determined in the pH range of 3.0-7.0. Chromatograms showing the retention times of the compounds at pH 3.0 and 7.0 are given in Figure 2. The relationship between the pH value and capacity factors in 30% (v/v) THF-water media obtained with the NLREG program gives a sigmoidal curve, as can be seen in Figure 3.

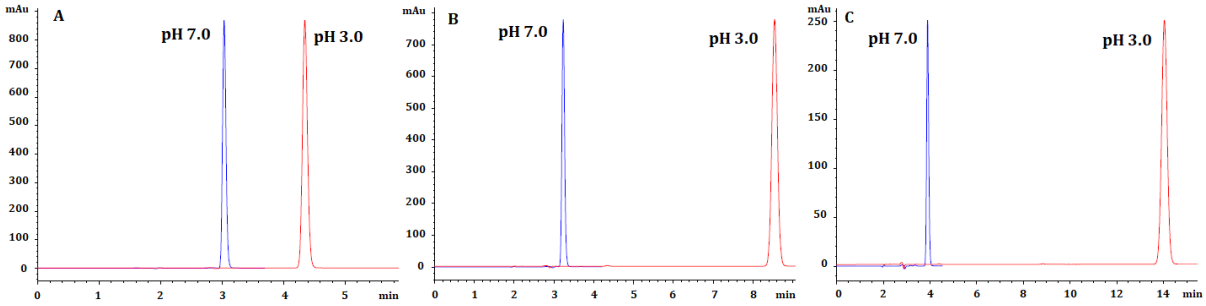


Figure 2. Chromatograms showing the retention times of the compounds at pH 3.0 and 7.0 in 35% (v/v) THF-water media at 37°C (A: tenoxicam, B: piroxicam, C: meloxicam)

The condensation of the benzene ring or thiophene ring in the structure of tenoxicam, meloxicam and piroxicam compounds with the heterocyclic system and also the presence of the amide group in the structure gives the enolic group acidic properties [5]. While tenoxicam has a thiophene ring that replaces the benzene ring in piroxicam; meloxicam is an analog of piroxicam and has 5 methylthiazole groups in the amidic part of the molecule instead of the pyridyl ring. (Figure 1). In this study, pK_a values of enolic acid group were determined for tenoxicam, piroxicam and meloxicam. The pK_a values of the compounds obtained from this study in THF-water media are given in Table 1. As can be seen from Table 1, an increase was observed in pK_a values as THF content increased. This can be explained by the increase in THF content, which has a low dielectric constant (Ɛ (H_2O)=78.36 and Ɛ (THF)=7.6), a decrease in the dissociation of electrolytes and a decrease in conductivity [26].

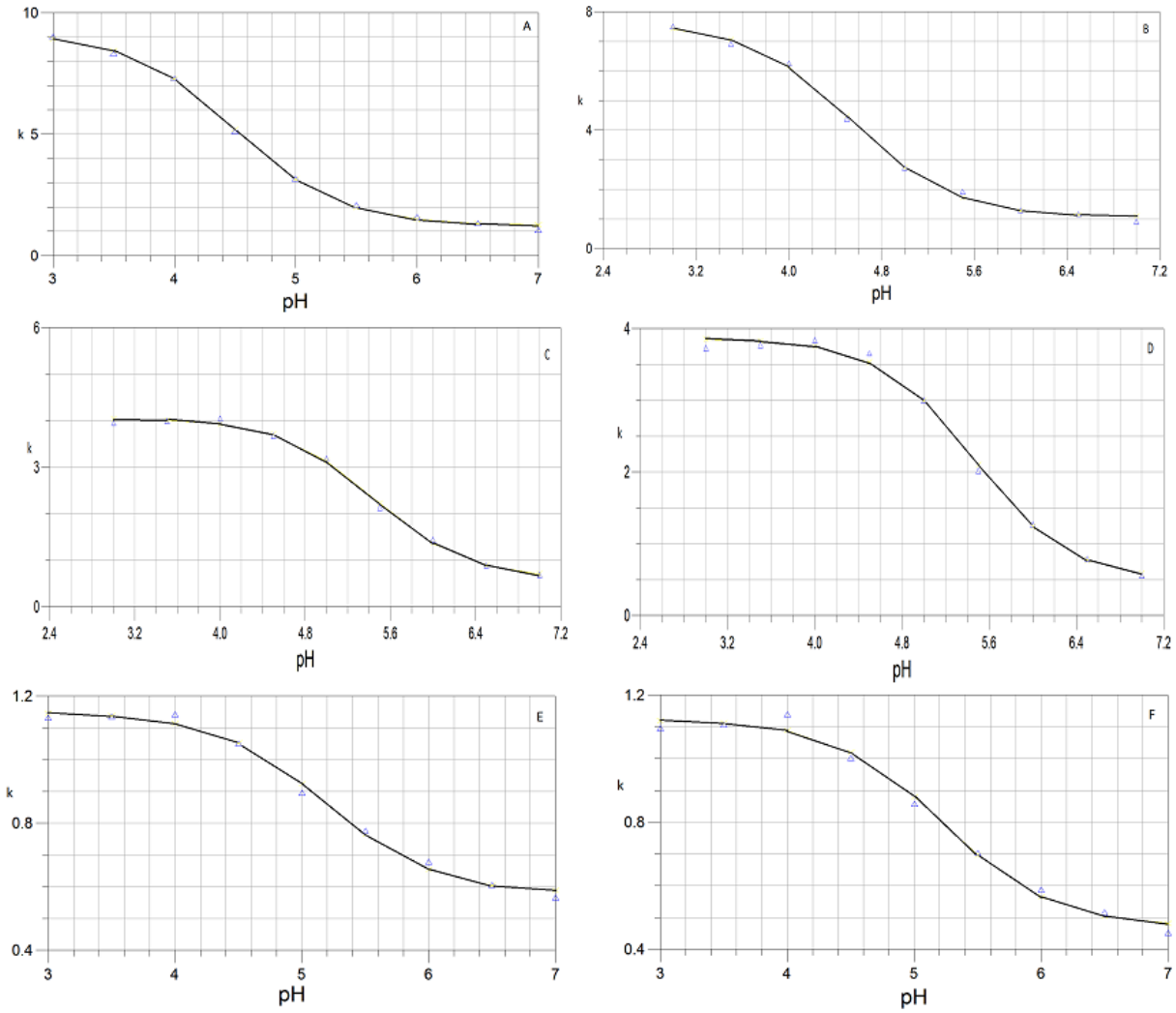


Figure 3. NLREG plots of compounds in 30% THF-water media of compounds (A: meloxicam at 25°C, B: meloxicam at 37°C, C: piroxicam at 25°C, D: piroxicam at 37°C, E: tenoxicam at 25°C, F: tenoxicam at 37°C.)

Table 1. The pK_a values of the studied compounds in THF-water mixture at 25°C and 37°C.

Temperature	THF%	Tenoxicam	Meloxicam	Piroxicam
25°C	30%	5.184 ±0.066[a]	4.497 ±0.032	5.444 ±0,040
	35%	5.225 ±0.052	4.609 ±0.041	5.512 ±0.067
	40%	5.258 ±0.176	4.782 ±0.015	5.574 ±0.064
37°C	30%	5.222 ±0.084	4.538 ±0.039	5.463 ±0.047
	35%	5.236 ±0.063	4.633 ±0.045	5.554 ±0.066
	40%	5.256 ±0.066	4.768 ±0.057	5.631 ±0.063

[a]: Standart deviation

David et al. obtained decreased pK_a values (belonging to the pyridinyl group) despite the increasing methanol percentage of oxicams in 40-60% methanol-water media by RP-HPLC method [27]. They concluded that this situation is related to the keto/enol equilibrium state of oxicam drugs in

the methanol-water media. In another study, 30-40% acetonitrile-water with RP-HPLC method and 30-45% acetonitrile-water media with potentiometric titration method, pK_a values (belonging to the enol group) increased with increasing acetonitrile percentage [20, 28]. Syatesh et al., with the spectrophotometric method, concluded that the pK_a values of both the enol group and the pyridinyl group of meloxicam increased with increasing ethanol percentage in 20-80% ethanol [29]. In this study, the pK_a values obtained in THF media are slightly higher than the values obtained in different solvent-water media in the literature. This is because in aprotic solvents such as THF, cationic forms adhere more than anionic form and pK_a values are higher in water-organic solvent media [30]. Kütt et al. show that pK_a values in THF-water media may differ by about 2-3 units [18].

Unlike other studies, the pK_a values were studied both at 25°C and at 37°C, which is the biorelevant temperature. The effect of temperature on pK_a depends on the structure of the functional group. With the increase in temperature, basic groups tend to protonate more easily, so a decrease was observed in the pK_a values of basic groups while an increase was observed in the pK_a values of acidic groups [24, 31, 32]. The pK_a values of the enolic acid group for tenoxicam, piroxicam and meloxicam increased with temperature, as seen in Table 1.

${}^{w}_{w}pK_a$ Values Obtained Using The Yasuda–Shedlovsky Equation and the Mole Fraction (X_{THF} – pK_a) Extrapolation Method

Yasuda-Shedlovsky mathematical equation and X_{THF}-pK_a extrapolation method were used to calculate ${}^{w}_{w}pK_a$ values from ${}^{s}_{s}pK_a$ values obtained from three different THF-water solvent media. The linear equations obtained from these methods are given in Table 2 and Table 3.

As can be seen in Table 2 and Table 3, positive slope values were obtained in the equations obtained by both extrapolation methods. This is because Tenoxicam, piroxicam and meloxicam are weakly acidic, so the acidity decreases with increasing THF content in the solution. Due to the similarity of the R_2 groups in Tenoxicam and Piroxicam, the slope values gave close results. Table 4 shows the ${}^{w}_{w}pK_a$ values of the compounds obtained from the X_{THF}–${}^{s}_{s}pK_a$ extrapolation method, the Yasuda Shedlovsky method, and the ${}^{w}_{w}pK_a$ values in the literature. It is seen that the ${}^{w}_{w}pK_a$ values obtained by both extrapolation methods are compatible with each other and the results are close to the literature values. The pK_a value of the enolic structure was found to be close to each other with the effect of the pyridinyl group in Tenoxicam and Piroxicam.

This is the first study in which the pK_a values of tenoxicam, piroxicam and meloxicam compounds were determined by RP-HPLC method in THF-water solvent media. The pK_a values of the enolic group in the structure of these compounds, which have low water solubility, were evaluated in THF-water solvent mixture in the range of 30-40%. ${}^{s}_{s}pK_a$ values were calculated with the NLREG program. Using these data, the ${}^{w}_{w}pK_a$ values of each compound were determined.

Table 2. Linear equations obtained from Yasuda-Shedlovsky extrapolation method of studied compounds at 25°C and 37°C.

Temperature	Compound	Yasuda Shedlovsky Equation	r
25°C	Tenoxicam	$\log[H_2O] + {}^{s}_{s}pK_a = 25.966(1/Ɛ) + 6.474$	0.995
	Meloxicam	$\log[H_2O] + {}^{s}_{s}pK_a = 105.69(1/Ɛ) + 4.418$	0.997
	Piroxicam	$\log[H_2O] + {}^{s}_{s}pK_a = 46.939(1/Ɛ) + 6.377$	0.997
37°C	Tenoxicam	$\log[H_2O] + {}^{s}_{s}pK_a = 11.111(1/Ɛ) + 6.763$	0.997
	Meloxicam	$\log[H_2O] + {}^{s}_{s}pK_a = 84.871(1/Ɛ) + 4.817$	0.998
	Piroxicam	$\log[H_2O] + {}^{s}_{s}pK_a = 61.087(1/Ɛ) + 6.157$	0.995

Table 3. Linear equations obtained from the X_{THF}–${}^{s}_{s}pK_a$ method of extrapolation of the studied compounds at 25°C and 37°C

Temperature	Compound	X_{THF}–${}^{s}_{s}pK_a$	r
25°C	Tenoxicam	${}^{w}_{w}pK_a = 27.652\ X_{THF} + 4.714$	0.997
	Meloxicam	${}^{w}_{w}pK_a = 107.37\ X_{THF} + 2.657$	0.997
	Piroxicam	${}^{w}_{w}pK_a = 48.624\ X_{THF} + 4.617$	0.997
37°C	Tenoxicam	${}^{w}_{w}pK_a = 12.796\ X_{THF} + 5.003$	0.998
	Meloxicam	${}^{w}_{w}pK_a = 86.557\ X_{THF} + 3.056$	0.998
	Piroxicam	${}^{w}_{w}pK_a = 62.772\ X_{THF} + 4.397$	0.995

Table 4. ${}^{w}_{w}pK_a$ values of studied compounds at 25°C and 37°C and literature values

Temperature	Compound	${}^{w}_{w}pK_a$ values		
		X_{THF}–${}^{s}_{s}pK_a$	Yasuda Shedlovsky	Literature values
25°C	Tenoxicam	5.067 ± 0.037	5.061 ± 0.035	5.34 [33] 5.29 [27] 5.19-5.26 [20] 5.26 [34] 4.97 [35]
	Meloxicam	4.027 ± 0.144	4.021 ± 0.141	4.08 [3, 4, 33] 4.34 [27] 4.17 [34] 4.0 [29]
	Piroxicam	5.237 ± 0.065	5.232 ± 0.063	6.3 [3, 36, 37] 5.3 [4] 5.46 [33] 4.58 [27] 5.98-6.02 [20] 5.3 [38] 5.31 [34]
37°C	Tenoxicam	5.166 ± 0.017	5.161 ± 0.013	-
	Meloxicam	4.161 ± 0.116	4.155 ± 0.094	-
	Piroxicam	5.197 ± 0.084	5.192 ± 0.053	-

RP-HPLC method has been preferred because it gives fast and reliable results in the determination of pK_a in organic solvent-water mixtures. The results obtained showed that the pK_a values of these compounds were dependent on the pH of the capacity factor for different THF contents in the mobile phase.

ACKNOWLEDGEMENTS

The author is thankful to Prof. Dr. Ebru Çubuk Demiralay, faculty member at the Faculty of Pharmacy at Süleyman Demirel University, for supplying pharmaceutical standards.

AUTHOR CONTRIBUTIONS

Concept: *D.B.D.*; Design: *D.B.D.*; Control: *D.B.D.*; Sources: *D.B.D.*; Materials: *D.B.D.*; Data Collection and/or processing: *D.B.D.*; Analysis and/or interpretation: *D.B.D.*; Literature review: *D.B.D.*; Manuscript writing: *D.B.D.*; Critical review: *D.B.D.*; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

REFERENCES

1. Bindu, S., Mazumder, S., Bandyopadhyay, U. (2020). Non-steroidal anti-inflammatory drugs (NSAIDs) and organ damage: A current perspective. *Biochemical Pharmacology, 180*, 114147. [CrossRef]

2. Starek, M., Krzek, J. (2009). A review of analytical techniques for determination of oxicams, nimesulide and nabumetone. *Talanta, 77*(3), 925-942. [CrossRef]

3. Szabó-Révész, P. (2018). Modifying the physicochemical properties of NSAIDs for nasal and pulmonary administration. *Drug Discovery Today Technologies, 27*, 87-93. [CrossRef]

4. Alves, L. P., Da Silva Oliveira, K., Da Paixão Santos, J. A., Da Silva Leite, J. M., Rocha, B. P., Lucena Nogueira, P. de, Araújo Rêgo, R. I. de, Oshiro-Junior, J. A., Damasceno, B.P.G.d.L. (2020). A review on developments and prospects of anti-inflammatory in microemulsions. *Journal of Drug Delivery Science and Technology, 60*, 102008. [CrossRef]

5. Christian, A., Iorgulescu, E. E., Mihailciuc, C. (2010). Electrochemıcal Studıes Usıng Actıvated Glassy Carbon. I. Meloxıcam. *Academia Romana, 55*(5), 329-334.

6. Cruciani, G., Milletti, F., Loriano, S., Sforna, G., Goracci, L. (2009). In silico pK_a Prediction and ADME Profiling. *Chemistry & Biodiversity, 6*, 1812-1821. [CrossRef]

7. Manallack, D. T. (2007). The pK_a Distribution of Drugs: Application to Drug Discovery. *Perspectives in Medicinal Chemistry, 1*, 25-38. [CrossRef]

8. Babić, S., Horvat, A. J., Mutavdžić Pavlović, D., Kaštelan-Macan, M. (2007). Determination of pK_a values of active pharmaceutical ingredients. *TrAC Trends in Analytical Chemistry, 26*(11), 1043-1061. [CrossRef]

9. Dardonville, C. (2018). Automated techniques in pK_a determination: Low, medium and high-throughput screening methods. *Drug Discovery Today Technologies, 27*, 49-58. [CrossRef]

10. Reijenga, J., van Hoof, A., van Loon, A., Teunissen, B. (2013). Development of Methods for the Determination of pK_a Values. *Analytical Chemistry Insights, 8*, 53-71. [CrossRef]

11. Subirats, X., Fuguet, E., Rosés, M., Bosch, E., Ràfols, C. (2015). Methods for pK_a Determination (I): Potentiometry, Spectrophotometry, and Capillary Electrophoresis. In Reference Module in Chemistry, Molecular Sciences and Chemical Engineering. Elsevier. [CrossRef]

12. Fuguet, E., Subirats, X., Ràfols, C., Bosch, E., Rosés, M. (2015). Methods for pK_a Determination (II): Sparingly Soluble Compounds and High-Throughput Approaches. In Reference Module in Chemistry, Molecular Sciences and Chemical Engineering. Elsevier. [CrossRef]

13. Trapl, D., Del Río, C.C., Kříž, P., Spiwok, V. (2020). Prediction of pK_a in a system with high orthogonal barriers: Alchemical flying Gaussian method. *Chemical Physics Letters, 760*, 138012. [CrossRef]

14. Settimo, L., Bellman, K., Knegtel, R.M.A. (2014). Comparison of the accuracy of experimental and predicted pK_a values of basic and acidic compounds. *Pharmaceutical Research, 31*(4), 1082-1095. [CrossRef]

15. Balogh, G.T., Tarcsay, A., Keserű, G.M. (2012). Comparative evaluation of pK_a prediction tools on a drug discovery dataset. *Journal of Pharmaceutical and Biomedical Analysis, 67*(68), 63-70. [CrossRef]

16. Mioduszewska, K., Dołżonek, J., Wyrzykowski, D., Kubik, Ł., Wiczling, P., Sikorska, C., Toński, M., Kaczyński, Z., Stepnowski, P., Białk-Bielińska, A. (2017). Overview of experimental and computational methods for the determination of the pK_a values of 5-fluorouracil, cyclophosphamide, ifosfamide, imatinib and methotrexate. *TrAC Trends in Analytical Chemistry, 97*, 283-296. [CrossRef]

17. Manderscheid, M., Eichinger, T. (2003). Determination of pK_a Values by Liquid Chromatography. *Journal of Chromatographic Science, 41*, 323-326. [CrossRef]

18. Kütt, A., Selberg, S., Kaljurand, I., Tshepelevitsh, S., Heering, A., Darnell, A., Kaupmees, K., Piirsalu, M., Leito, I. (2018). pK_a values in organic chemistry – Making maximum use of the available data. *Tetrahedron Letters, 59*(42), 3738-3748. [CrossRef]

19. Völgyi, G., Ruiz, R., Box, K., Comer, J., Bosch, E., Takács-Novák, K. (2007). Potentiometric and spectrophotometric pK_a determination of water-insoluble compounds: Validation study in a new cosolvent system. *Analytica Chimica Acta, 583*(2), 418-428. [CrossRef]

20. Demiralay, E. Ç., Yılmaz, H. (2012). Potentiometric pK_a Determination of Piroxicam and Tenoxicam in Acetonitrile-Water Binary Mixtures. *SDU Journal of Science, 7*(1), 34-44.

21. Yasuda, M. (1959). Dissociation Constants of Some Carboxylic Acids in Mixed Aqueous Solvents. *Bulletin of the Chemical Society of Japan, 32*(5), 429-432.

22. Shedlovsky, T. (1962). Electrolytes: The behaviour of carboxylic acids in mixed solvents. Pergamon Press.

23. Barbosa, J., Barrón, D., Butí, S. (1999). Chromatographic behaviour of ionizable compounds in liquid chromatography. Part 1. pH scale, pK_a and pH_S values for standard buffers in tetrahydrofuran–water. *Analytica Chimica Acta, 389*(1-3), 31-42. [CrossRef]

24. Sun, N., Avdeef, A. (2011). Biorelevant pK_a (37 °C) predicted from the 2D structure of the molecule and its pK_a at 25 °C. *Journal of Pharmaceutical and Biomedical Analysis, 56*(2), 173-182. [CrossRef]

25. NLREG Nonlinear Regression Analysis and Curve Fitting Program, Version 4.0 http//www.nlreg.com Accessed: 18 December 2018.

26. Muinasmaa, U., Ràfols, C., Bosch, E., Rosés, M. (1997). Ionic equilibria in aqueous organic solvent mixtures the dissociation constants of acids and salts in tetrahydrofuran/water mixtures. *Analytica Chimica Acta, 340*(1-3), 133-141. [CrossRef]

27. David, V., Albu, F., Medvedovici, A. (2004). Structure–Retention Correlation of Some Oxicam Drugs in Reversed-Phase Liquid Chromatography. *Journal of Liquid Chromatography & Related Technologies, 27*(6), 965-984. [CrossRef]

28. Demiralay, E. C., Alsancak, G., Ozkan, S. A. (2009). Determination of pK_a values of nonsteroidal antiinflammatory drug-oxicams by RP-HPLC and their analysis in pharmaceutical dosage forms. *Journal of Separation Science, 32*(17), 2928-2936. [CrossRef]

29. Shayesteh, O. H., Musavi, S. M., Mahjoub, P., Ataie, Z. (2017). Application of Chemometrics in determination of the effects of ionic and non-ionic surfactants on acid dissociation constant (pK_a) of Meloxicam using spectrophotometric method. *Iranıan Journal of Pharmacology & Therapeutıcs, 15*(1), 1-7.

30. Garrido, G., Rosés, M., Ràfols, C., Bosch, E. (2008). Acidity of Several Anilinium Derivatives in Pure Tetrahydrofuran. *Journal of Solution Chemistry, 37*(5), 689-700. [CrossRef]

31. Hartono, A., Saeed, M., Kim, I., Svendsen, H.F. (2014). Protonation Constant (pK_a) of MDEA in Water as Function of Temperature and Ionic Strength. *Energy Procedia, 63*, 1122-1128. [CrossRef]

32. Pobudkowska, A., Ràfols, C., Subirats, X., Bosch, E., Avdeef, A. (2016). Phenothiazines solution complexity- Determination of pK_a and solubility-pH profiles exhibiting sub-micellar aggregation at 25 and 37°C. *European Journal of Pharmaceutical Sciences, 93*, 163-176. [CrossRef]

33. Chakraborty, H., Banerjee, R., Sarkar, M. (2003). Incorporation of NSAIDs in micelles: implication of structural switchover in drug–membrane interaction. *Biophysical Chemistry, 104*(1), 315-325. [CrossRef]

34. Rodríguez-Barrientos, D., Rojas-Hernández, A., Gutiérrez, A., Moya-Hernández, R., Gómez-Balderas, R., Ramírez-Silva, M.T. (2009). Determination of pK_a values of tenoxicam from 1H NMR chemical shifts and of oxicams from electrophoretic mobilities (CZE) with the aid of programs SQUAD and HYPNMR. *Talanta, 80*(2), 754-762. [CrossRef]

35. Ramírez-Silva, M. T., Guzmán-Hernández, D.S., Galano, A., Rojas-Hernández, A., Corona-Avendaño, S., Romero-Romo, M., Palomar-Pardavé, M. (2013). Spectro-electrochemical and DFT study of tenoxicam metabolites formed by electrochemical oxidation. *Electrochimica Acta, 111*, 314-323. [CrossRef]

36. Dal, A.G., Oktayer, Z., Doğrukol-Ak, D. (2014). Validated method for the determination of piroxicam by capillary zone electrophoresis and its application to tablets. *Journal of Analytical Methods in Chemistry, 2014*, 352698. [CrossRef]

37. Damiani, P., Bearzotti, M., Cabezón, M., Olivieri, A. (1998). Spectrofluorometric determination of piroxicam. *Journal of Pharmaceutical and Biomedical Analysis, 17*(2), 233-236. [CrossRef]

38. Goosen, C., Du Plessis, J., Müller, D., van Janse Rensburg, L. (1998). Correlation between physicochemical characteristics, pharmacokinetic properties and transdermal absorption of NSAID's. *International Journal of Pharmaceutics, 163*(1-2), 203-209. [CrossRef]

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 872-882, 2022
Doi: 10.33483/jfpau.1134347

ORIGINAL ARTICLE / ÖZGÜN MAKALE

PREPARATION AND *IN VITRO* CHARACTERIZATION OF SOLID LIPID MICROPARTICLES FOR PROTEIN DELIVERY

PROTEİN VERİLİŞİ İÇİN KATI LİPİT MİKROPARTİKÜLLERİN HAZIRLANMASI VE İN VİTRO KARAKTERİZASYONU

Berrin KÜÇÜKTÜRKMEN[1*], Umut Can ÖZ[1], Asuman BOZKIR[1]

[1]Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Technology, 06560, Ankara, Turkey

ABSTRACT

Objective: *The aim of this research was to assess the effect of the process and formulation parameters during the preparation of solid lipid microparticles. Solid lipid microparticles (SLMs) have evident advantages such as biocompatibility, simplicity of production and characterization, prolonged release, and especially high protein loading capacity, despite being less investigated than lipid nanoparticles.*

Material and Method: *SLMs were prepared via emulsion solvent diffusion technique using glyceryl tridecanoate (GTD) as a biocompatible and biodegradable lipid. The optimum formulation conditions for producing homogenous spherical microparticles were found and represented by a triangle phase diagram area. After optimizing the particle size and encapsulation efficiency by changing the formulation parameters, the microparticles were characterized by in vitro release, morphological analysis, thermal analysis and electrophoretic analysis on the selected formulations.*

Result and Discussion: *The maximum drug loading efficiency was achieved by combining 100 mg of lipid, 60% triacetin and 3% emulsifier. The average microparticle size was observed as 8.9 µm. The in vitro drug release were analyzed in pH 7.4 phosphate buffer and were mainly completed at 8th hour.*

Keywords: *Bovine serum albumin, glyceryl tridecanoate, lipid microparticle, triacetin, triangular phase diagram*

ÖZ

Amaç: *Bu araştırmanın amacı, katı lipid mikropartiküllerinin hazırlanması sırasında proses ve formülasyon parametrelerinin etkisini değerlendirmektir. Katı lipid mikropartiküller (SLM'ler), lipid nanopartiküllerinden daha az araştırılmış olmalarına rağmen biyouyumluluk, üretim ve karakterizasyon kolaylığı, uzun süreli salım ve özellikle yüksek protein yükleme kapasitesi gibi belirgin avantajlara sahiptir.*

* **Corresponding Author / Sorumlu Yazar:** Berrin Küçüktürkmen
e-mail / e-posta: bbasaran@pharmacy.ankara.edu.tr, **Phone / Tel.:** +903122033159

Submitted / Gönderilme: 22.06.2022 **Accepted / Kabul:** 09.08.2022

Gereç ve Yöntem: *SLM'ler, biyouyumlu ve biyolojik olarak parçalanabilen bir lipid olarak gliseril tridekanoat (GTD) kullanılarak emülsiyon çözücü difüzyon tekniği ile hazırlanmıştır. Homojen küresel mikropartiküller üretmek için en iyi formülasyon koşulları belirlenmiş ve bir üçgen faz diyagram alanı ile temsil edilmiştir. Mikropartiküller, formülasyon parametreleri değiştirilerek partikül boyutu ve enkapsülasyon etkinliği optimize edildikten sonra, seçilen formülasyonlar in vitro salım, morfolojik analizler, termal analiz ve elektroforetik analiz ile karakterize edilmiştir.*

Sonuç ve Tartışma: *En yüksek etken madde yükleme etkinliği 100 mg lipid, %60 triasetin ve %3 emülgatör kullanılarak elde edilmiştir. Ortalama mikropartikül boyutu 8.9 µm olarak gözlenmiştir. İn vitro etken madde salımı pH 7.4 fosfat tampon çözeltisinde değerlendirilmiş ve 8. saatte tamamlanmıştır.*

Anahtar Kelimeler: *Gliseril tridekanoat, lipid mikropartikül, sığır serum albümini, triasetin, üçgen faz diyagramı*

INTRODUCTION

In recent years, protein delivery has emerged as a particularly interesting subject of research due to the selectivity, efficacy, and relative maturity of recombinant protein expression techniques. Solid lipid particles have shown excellent potentials against proteolysis of integrated proteins among protein delivery methods designed for parenteral administration. These particles also provide steric barriers that can contribute in the controlled release of drugs. [1]. The lipid matrix of SLM is composed of physiological lipids, which lowers the risk of both acute and long-term toxicity [2,3]. On the other hand, SLMs provide high biocompatibility and stability, and can encapsulate different protein therapeutic agents effectively [4].

One of the methods to produce lipid nanoparticles is the solvent emulsification-diffusion method, in which the lipid and active component are dissolved in an organic solvent miscible with water and dispersed in an external aqueous phase including surfactant. Emulsification is carried out by mixing at high speed. The resulting emulsion is then mixed with the aqueous phase to encourage organic solvent migration from droplets to the aqueous phase, which solidifies the lipid molecule particles [3]. While this technique is reproducible and it has the advantage of not exposing the active substance to high temperature, it has the disadvantage of diffusing especially the water-soluble active substance into the aqueous phase and lowering the encapsulation efficiency.

Several advantages of SLMs have driven numerous studies for the application of SLMs as peptide/protein carrier system for oral [5-7], nasal [8] and particularly for parenteral administrations [9-11]. The physicochemical properties and stability of SLMs also depend on the properties of the encapsulated active substance. In this context, the effect of formulation parameters on the physicochemical characteristics of SLMs should be evaluated to obtain an optimal SLM formulation for each active ingredient. With this objective, glyceryl tridecanoate and triacetin were used as lipids for SLM formulations in this research for bovine serum albumin (BSA) delivery as a model protein. The use of glyceryl tridecanoate as a matrix material for SLM formulations for peptides and proteins has rarely been reported [1, 12, 13]. Triacetin is an oil with many unique features such as non-toxicity, high

drug dissolving capacity, self-emulsifying formulation ability, and plasticizer properties. There are few studies in the literature on the use of triacetin in lipid microspheres produced by solvent diffusion method. It has been stated that the use of triacetin in the microsphere formulation allows the solvent and triacetin to diffuse easily into the polymer matrix and increases the drug retention efficiency [14, 15].

MATERIAL AND METHOD

Materials

GTD, triacetin, BSA, Pluronic F127 and ethanol were obtained from Sigma-Aldrich Co (USA). All other reagents were of analytical grade.

Pseudo-Ternary Diagram

In this research, the first step in formulating emulsion-based microparticles was to create the phase diagram used in the literature to determine the stable emulsion region in the optimization process of microparticle formulations [16]. The pseudo-ternary phase diagram was constructed based on the selected lipids and combination of surfactant at different ratios. The lipophilic phase consisted of a GTD and triacetin solution in ethanol, and Pluronic F127 was used as a surfactant. By maintaining the GTD concentration constant (100 mg) and altering the amounts of the surfactant and oil, it was possible to obtain the stable emulsion formation area in a ternary phase diagram. Appropriate amounts of lipophilic phases were weighed in the beaker and mixed for sufficient time to reach equilibrium, then mixed with the aqueous phase containing Pluronic F127 and homogenized with a high-speed homogenizer (IKA T 18 digital ULTRA-TURRAX®) at 13500 rpm for 5 min. The resulting mixture was first visually examined in terms of formation of emulsion. Afterwards, all mixtures produced in the phase diagram where no separation was observed visually were subjected to particle size analysis to confirm the microparticle formation.

Preparation of solid lipid microparticles

In order to prepare solid lipid microparticles, firstly, Pluronic F127 was mixed in a calculated amount of water and kept in the refrigerator overnight to form a solution. GTD was dissolved in 2 ml of alcohol and triacetin was added and mixed. Afterwards, the lipophilic phase was added to the aqueous phase containing Pluronic F127 and homogenized with a high-speed homogenizer at 13500 rpm for 5 min. Then, for the removal of the ethanol, the formulations were taken into a dialysis bag (Spectra/Por Biotech CE Tubing, 300 kD MWCO) and dialyzed for a total of 9 h by changing every 3 h at 350 rpm against one liter of water. The formulation taken from the dialysis bag was frozen at -40°C and

lyophilized. In order to encapsulate BSA into SLMs, 10 mg of BSA was first dissolved in 1 ml pH 7.4 PBS by keeping it in the refrigerator and added to the lipophilic phase.

Particle size measurements

A sample of 10 µL formulation was diluted with ultrapure water. The particle size and size distribution of microparticles were evaluated at 25°C using Malvern Mastersizer 3000 (Malvern Instruments, UK).

Determination of Encapsulation Efficiency (EE) and Loading Capacity (LC)

To determine the BSA encapsulation efficiency of SLM formulations, 5 mg of SLM was weighed and dissolved in 0.5 ml of ethanol. 4.5 ml of ultrapure water was added and mixed in a shaking water bath for 24 h. Then the mixture was centrifuged at 4000 rpm for 10 min and the amount of BSA in the supernatant was determined by microBCA analysis [17]. Encapsulation efficiency and loading capacity of SLM formulations were calculated with the following equations:

$$EE\ \% = \frac{\text{Total amount of BSA} - \text{Free BSA}}{\text{Total amount of BSA}} * 100 \qquad \text{Eq. (1)}$$

$$LC\% = \frac{\text{Total amount of BSA} - \text{Free BSA}}{\text{Weight of SLM formulation}} * 100 \qquad \text{Eq. (2)}$$

***In vitro* BSA release**

In vitro release of BSA from SLM formulations carried out with slight modification of the method used by Sedyakina et al. [18]. In order to determine the *in vitro* release rate of BSA, the SLM formulation containing 200 µg of BSA was placed in Eppendorf tube and 2 ml of pH 7.4 Phosphate Buffered Saline (PBS) was added. Eppendorf tubes were placed in a shaker incubator at 37°C and were shaken horizontally at 50 rpm. At certain time intervals for 24 h, 0.2 ml of sample was taken and replaced with fresh medium. The amount of BSA in the samples was determined by microBCA analysis and in vitro release graphs were created.

Morphology of the SLMs

The morphology of the SLM formulation was determined by both optical microscopy and AFM. The morphology of the F6 formulation was observed using optical microscopy (BX4, Olympus, Tokyo, Japan) with a 10× objective lens. Images were acquired using a digital camera (Q- Color 3, Olympus, Tokyo, Japan). On the other hand, for AFM analysis, F6 formulation was dispersed in distilled water and then dropped onto mica and dried under nitrogen. Then, the mica surface was scanned, and imaging was performed with the AFM device (ezAFM, Nanomagnetics Inst., UK).

DSC Analysis

The thermal properties of BSA, GTD, triacetin, and BSA loaded SLM formulation (F6) were analyzed with the Shimadzu DSC-60 device. Samples weighing 3-5 mg were placed on an aluminum pan and compressed. Samples were heated linearly at a rate of 10 °C/min between 30 and 300 ° C while being examined using nitrogen gas at a flow rate of 50 ml/min. The blank was an empty pan.

SDS-PAGE Analysis

SDS-PAGE was carried out under reducing conditions. The in vitro release samples of the F6 formulation and various amounts of BSA standards produced in PBS (pH 7.4) were analyzed. The protein solutions (20 µL) were mixed with 5 µL of sample buffer, which was boiled for 5 minutes at 100° C. The sample buffer contains 1.0 M Tris (pH 6.8), 1 % bromophenol blue, 2-mercapto ethanol, and 50% glycerol. The samples of 10 µL were loaded onto 15% SDS-PAGE gel and electrophoretically separated. Coomassie brilliant blue was used to visualize protein bands.

RESULT AND DISCUSSION

Construction of the phase diagram

All batches were made with various concentrations of the oil and surfactant and a constant GTD concentration throughout the optimization procedure. Since the GTD concentration was kept constant in the lipid phase, the pseudoternary diagram was created using the varying percentage of triacetin. Pluronic F127 was only employed below 9 % concentration because gel formation was observed above this rate. The region marked in the phase diagram is the region where the emulsion is stable and microparticle formation is observed (Figure 1). The formulations were prepared according to the ratios in this designated area and the formulation compositions are shown in the Table 1.

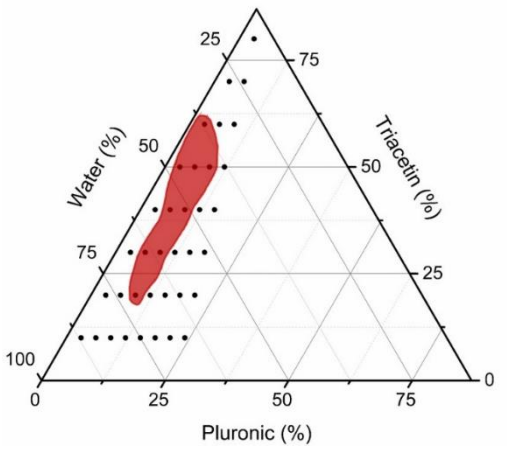


Figure 1. Triangular phase diagram of the water/oil/surfactant system. The stable region is framed by a line and represents formulations yielding spherical microparticles.

Table 1. Composition of SLM formulations

	GTD (mg)	Triacetin (%)	Pluronic F127 (%)	BSA (mg)
F1	100	30	6	10
F2	100	20	9	10
F3	100	50	3	10
F4	100	40	6	10
F5	100	30	9	10
F6	100	60	3	10
F7	100	50	6	10
F8	100	40	9	10
F9	100	50	9	10

Particle size measurements

The particle size homogeneity of the SLMs has a crucial role in the repeatability of the drug release from formulations. [16]**.** Particle sizes of SLM formulations were found within the range of 1.28–12.4 μm (Table 2). Narrow particle size distributions were obtained in this study. Among the formulations, it was observed that the F4 formulation had the smallest particle size, while the F3 formulation had the smallest particle size distribution.

Encapsulation Efficiency (EE) and Loading Capacity (LC)

Encapsulation efficiencies and loading capacities of SLM formulations were found within the range of 7.6–43.2 % and 0.182-0.996 %, respectively (Table 2). The increase in the amount of triacetin and Pluronic F127 together increased the amount of loaded protein. It is known that triacetin weakens the intermolecular forces between polymer molecules due to its plasticizer function and increases the free volume in the polymer matrix by causing the polymer relaxation [14]. Therefore, the increased encapsulation efficiency in the microspheres with the increase of triacetin concentration can be attributed to the easy diffusion of protein into the polymer matrix. Similarly, in the study of Yüksel et al. [14], the addition of triacetin increased the encapsulation efficiency of indomethacin into microspheres. It was observed that the F6 formulation had the highest encapsulation efficiency and the F3 formulation had the highest loading capacity.

***In vitro* BSA release**

The *in vitro* BSA release of the F4 formulation with the smallest particle size, the F3 formulation with the highest drug loading capacity, and the F6 formulation with the highest encapsulation efficiency were compared. Burst effect was observed in the first hour in all formulations. F6 formulation significantly slowed the release of the active substance compared to other formulations. It was thought that this was due to the higher amount of triacetin in the F6 formulation, and that triacetin increased the encapsulation of BSA into the lipid microparticle structure rather than the surface regions. Similarly, in

the study of Beck et al. [19], the addition of triacetin slowed the release of diclofenac from the nanoparticle-coated inorganic microparticles. In another study triacetin modified the release of indomethacin from the microspheres [14]. F6 was chosen as the optimum formulation in this study because of its high encapsulation efficiency and relatively slow drug release (Figure 2).

Table 2. Characterization of SLM formulations

	Triacetin (%)	**Pluronic F127 (%)**	**Mean size (µm)**	**Span**	**Encapsulation efficiency (%)**	**Loading capacity (%)**
F1	30	6	12.4±0.152	3.01	30.0±3.6	0.559±0.067
F2	20	9	3.79±0.009	1.78	11.9±0.7	0.441±0.026
F3	50	3	2.51±0.007	1.44	17.2±0.6	0.996±0.032
F4	40	6	1.28±0.004	2.41	7.6±0.5	0.510±0.036
F5	30	9	4.62±0.009	2.97	29.0±1.3	0.343±0.016
F6	60	3	8.90±0.717	15.4	43.2±2.2	0.260±0.013
F7	50	6	2.03±0.004	1.69	20.7±1.5	0.623±0.045
F8	40	9	5.87±0.046	6.00	38.8±2.5	0.380±0.025
F9	50	9	9.95±0.462	3.77	33.9±5.4	0.182±0.029

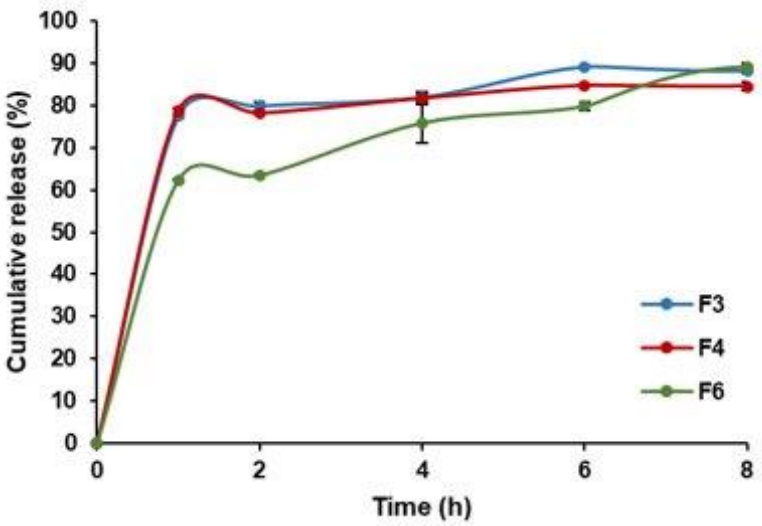


Figure 2. *In vitro* BSA release

Morphology of the SLMs

After the optimization of the process parameters in the previous section, the morphological characteristics of the F6 formulation were investigated. As shown in Figure 3, SLMs were found to be spherical and monodisperse. As a result of the AFM analysis, it was observed that the SLMs were spherical in size and the particle size was similar to the size measured by laser diffraction.

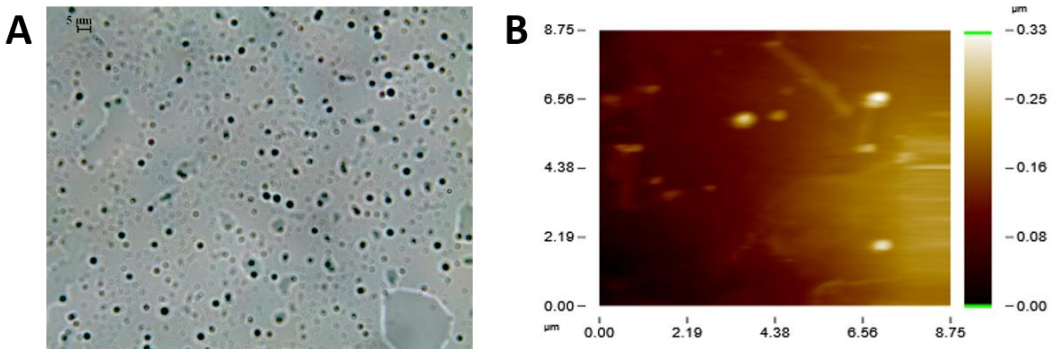


Figure 3. Optical microscope (A) and AFM (B) images of the F6 SLM formulation

DSC Analysis

As a result of DSC analysis, GTD created an endothermic peak at 36°C and is compatible with the literature [1]. Triacetin formed a broad peak at 187°C, corresponding to the flash point [14]. Characteristic peaks of BSA were observed at 89°C and 224°C [20]. DSC thermogram of F6 SLM formulation exhibited only a peak of lipid at 31°C. The absence of characteristic peaks of BSA in the DSC thermogram confirmed that BSA was encapsulated in the lipid microparticle structure (Figure 4).

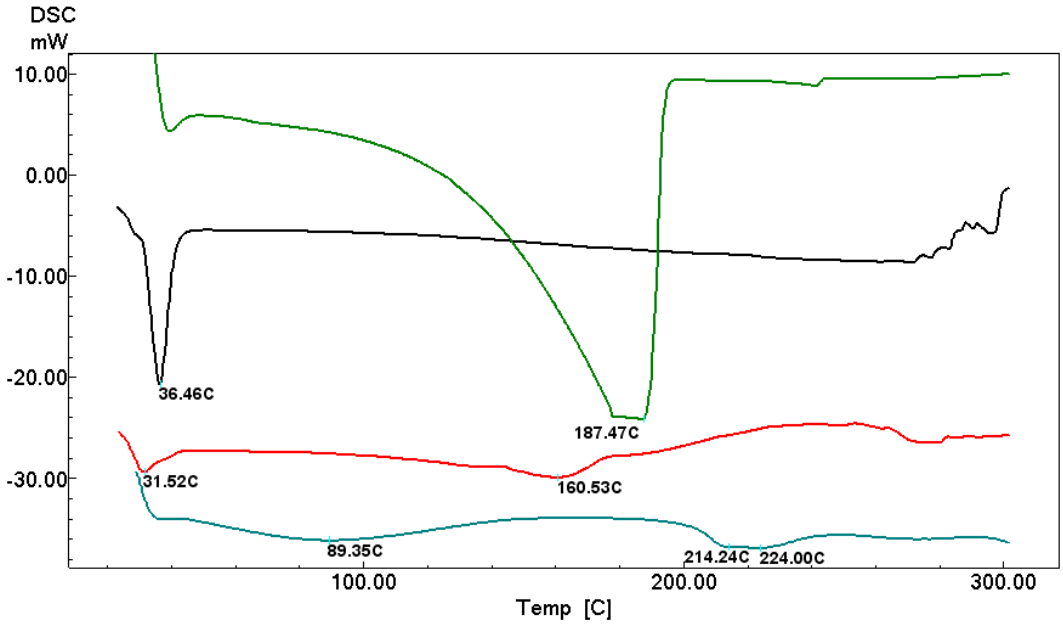


Figure 4. DSC thermograms of triacetin (green), GTD (black), F6 formulation (red) and BSA (blue).

SDS_PAGE Analysis

In the current study, BSA was loaded on SLMs and the molecular weight integrity of BSA released from the NPs was measured by SDS-PAGE whether the structure of the protein was affected during the SLM preparation procedure. As a result of SDS-PAGE analysis, *in vitro* release samples from formulation F6 and BSA standards emerged as a single chain aligned (Figure 5). Apart from these, no

additional bands were observed. This result showed that the protein in the formulation remained stable during the formulation preparation stage and during the 8-hour *in vitro* release period.

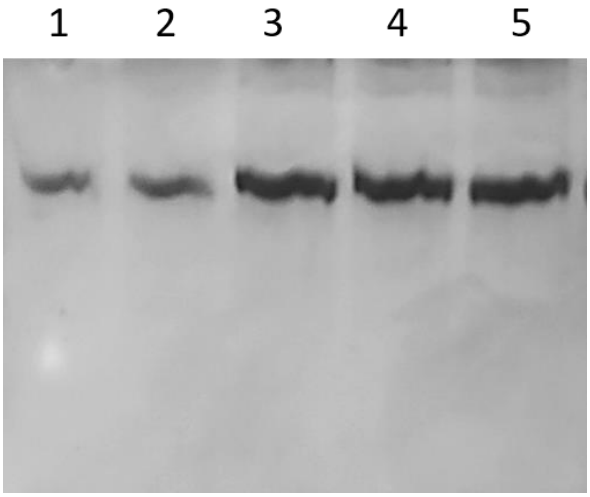


Figure 5. Results of SDS-PAGE analysis. Lane 1: BSA standard 25 µg/ml, Lane 2: BSA standard 50 µg/ml, Lane 3: 2nd hour *in vitro* release sample, Lane 4: 4th hour *in vitro* release sample, Lane 5: 8th hour *in vitro* release sample

In this research, the preparation of SLM formulation as well as its potential for application as a protein carrier system was studied. The triangle phase diagram is used to optimize the effect of emulsion components employed in the emulsion solvent diffusion method on SLM production. The formulations were characterized in detail after loading BSA into the SLM formulations in the region defined by the triangle phase diagram. The optimized F6 formulation was shown to provide BSA release for up to 8 hours and to have acceptable particle size and encapsulation efficiency. Based on these findings, SLM formulations are recommended as an effective protein delivery system.

AUTHOR CONTRIBUTIONS

Concept: *B.K., U.C.Ö., A.B.*; Design: *B.K., U.C.Ö., A.B.*; Control: *B.K., U.C.Ö.*; Sources: *B.K., U.C.Ö., A.B.*; Materials: *B.K., U.C.Ö., A.B.*; Data Collection and/or processing: *B.K., U.C.Ö., A.B.*; Analysis and/or interpretation: *B.K., U.C.Ö.*; Literature review: *B.K., U.C.Ö.*; Manuscript writing: *B.K., U.C.Ö.*; Critical review: *B.K., U.C.Ö.*, A.B.; Other: -

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS COMMITTEE APPROVAL

The authors declare that this article does not require the ethical committee's approval.

REFERENCES

1. Din, F. U., Mustapha, O., Kim, D. W., Rashid, R., Park, J. H., Choi, J. Y., Ku, S.K., Yong, C.S., Kim, J.O., Choi, H. G. (2015). Novel dual-reverse thermosensitive solid lipid nanoparticle-loaded hydrogel for rectal administration of flurbiprofen with improved bioavailability and reduced initial burst effect. *European Journal of Pharmaceutics and Biophamaceutics, 94*, 64-72. [CrossRef]

2. Trotta, M., Cavalli, R., Carlotti, M. E., Battaglia, L., Debernardi, F. (2005). Solid lipid micro-particles carrying insulin formed by solvent-in-water emulsion-diffusion technique. *International Journal of Pharmacetics, 288*(2), 281-288. [CrossRef]

3. Küçüktürkmen, B., Bozkır, A. (2018). Development and characterization of cationic solid lipid nanoparticles for co-delivery of pemetrexed and miR-21 antisense oligonucleotide to glioblastoma cells. *Drug Development and Industrial Pharmacy, 44*(2), 306-315. [CrossRef]

4. Scalia, S., Young, P. M., Traini, D. (2015). Solid lipid microparticles as an approach to drug delivery. *Expert Opinion on Drug Delivery, 12*(4), 583-599. [CrossRef]

5. Christophersen, P. C., Zhang, L., Müllertz, A., Nielsen, H. M., Yang, M., Mu, H. (2014). Solid lipid particles for oral delivery of peptide and protein drugs II--the digestion of trilaurin protects desmopressin from proteolytic degradation. *Pharmaceutical Research, 31*(9), 2420-2428. [CrossRef]

6. Bertoni, S., Tedesco, D., Bartolini, M., Prata, C., Passerini, N., Albertini, B. (2020). Solid Lipid Microparticles for Oral Delivery of Catalase: Focus on the Protein Structural Integrity and Gastric Protection. *Molecular Pharmaceutics, 17*(9), 3609-3621. [CrossRef]

7. Liu, J., Christophersen, P. C., Yang, M., Nielsen, H. M., Mu, H. (2017). The impact of particle preparation methods and polymorphic stability of lipid excipients on protein distribution in microparticles. *Drug Development and Industrial Pharmacy, 43*(12), 2032-2042. [CrossRef]

8. Saraf, S., Mishra, D., Asthana, A., Jain, R., Singh, S., Jain, N. K. (2006). Lipid microparticles for mucosal immunization against hepatitis B. *Vaccine, 24*(1), 45-56. [CrossRef]

9. Erni, C., Suard, C., Freitas, S., Dreher, D., Merkle, H. P., Walter, E. (2002). Evaluation of cationic solid lipid microparticles as synthetic carriers for the targeted delivery of macromolecules to phagocytic antigen-presenting cells. *Biomaterials, 23*(23), 4667-4676. [CrossRef]

10. Reithmeier, H., Herrmann, J., Göpferich, A. (2001). Lipid microparticles as a parenteral controlled release device for peptides. *Journal of Controlled Release, 73*(2-3), 339-350. [CrossRef]

11. Reithmeier, H., Herrmann, J., Göpferich, A. (2001). Development and characterization of lipid microparticles as a drug carrier for somatostatin. *International Journal of Pharmaceutics, 218*(1-2), 133-143. [CrossRef]

12. Choi, S. H., Jin, S. E., Lee, M. K., Lim, S. J., Park, J. S., Kim, B. G., Ahn, W.S., Kim, C. K. (2008). Novel cationic solid lipid nanoparticles enhanced p53 gene transfer to lung cancer cells. *European Journal of Pharmaceutics and Biophamaceutics*, *68*(3), 545-554. [CrossRef]

13. Jin, S. E., Kim, C. K. (2012). Long-term stable cationic solid lipid nanoparticles for the enhanced intracellular delivery of SMAD3 antisense oligonucleotides in activated murine macrophages. *Journal of Pharmacy and Pharmaceutical Sciences*, *15*(3), 467-482. [CrossRef]

14. Yuksel, N., Baykara, M., Shirinzade, H., Suzen, S. (2011). Investigation of triacetin effect on indomethacin release from poly(methyl methacrylate) microspheres: evaluation of interactions using FT-IR and NMR spectroscopies. *International Journal of Pharmaceutics*, *404*(1-2), 102-109. [CrossRef]

15. Gharbavi, M., Manjili, H. K., Amani, J., Sharafi, A., Danafar, H. (2019). In vivo and in vitro biocompatibility study of novel microemulsion hybridized with bovine serum albumin as nanocarrier for drug delivery. *Heliyon*, *5*(6), e01858. [CrossRef]

16. Rodriguez, L.B., Avalos, A., Chiaia, N., Nadarajah, A. (2017). Effect of Formulation and Process Parameters on Chitosan Microparticles Prepared by an Emulsion Crosslinking Technique. *AAPS PharmSciTech.*,*18*(4),1084-1094. [CrossRef]

17. Kraisit, P., Sarisuta, N. (2018). Development of Triamcinolone Acetonide-Loaded Nanostructured Lipid Carriers (NLCs) for Buccal Drug Delivery Using the Box-Behnken Design. *Molecules*, *23*(4), 982. [CrossRef]

18. Sedyakina, N., Kuskov, A., Velonia, K., Feldman, N., Lutsenko, S., Avramenko, G. (2020). Modulation of Entrapment Efficiency and In Vitro Release Properties of BSA-Loaded Chitosan Microparticles Cross-Linked with Citric Acid as a Potential Protein-Drug Delivery System. *Materials (Basel)*, *13*(8), 1989. [CrossRef]

19. Beck, R.C., Pohlmann, A.R., Guterres, S.S. (2004). Nanoparticle-coated microparticles: preparation and characterization. *Journal of Microencapsulation*, *21*(5), 499-512. [CrossRef]

20. Kim, D., Maharjan, P., Jin, M., Park, T., Maharjan, A., Amatya, R., Yang, J., Min, K.A., Shin, M. C. (2019). Potential Albumin-Based Antioxidant Nanoformulations for Ocular Protection against Oxidative Stress. *Pharmaceutics*, *11*(7), 297. [CrossRef]

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 883-895, 2022
Doi: 10.33483/jfpau.1112165

ORIGINAL ARTICLE / ÖZGÜN MAKALE

ANTIVIRAL ACTIVITY OF BETAFERON FOR COVID-19

BETAFERON'UN COVID-19 İÇİN ANTİVİRAL AKTİVİTESİ

Fatma BAYRAKDAR[1], Sibel A. ÖZKAN[2*], Kamil Can AKÇALI[3,4*]

[1]Ministry of Health General Directorate of Public Health, Microbiology References Laboratory, 06430, Ankara, Turkey

[2]Ankara University, Faculty of Pharmacy, Department of Analytical Chemistry, 06560, Ankara, Turkey

[3]Ankara University, School of Medicine, Department of Biophysics, 06230 Ankara, Turkey

[4]Ankara University, Stem Cell Institute, 06100 Ankara, Turkey

ABSTRACT

Objective: *SARS-CoV-2 infection has been spread worldwide since 2019 and declared a pandemic infection. Unfortunately, humanity is still trying to deal with the infection. Under these circumstances, scientists head towards drug repurposing studies as the fastest solution for combatting SARS-CoV-2 viral infection. Betaferon (Interferon beta-1b) is a member of interferons, and its mechanism of action is the same as naturally produced interferon beta-1a in the immune system.*

Material and Method: *In this study, the antiviral effect of Betaferon on SARS-CoV-2 infection in vitro and in silico was analyzed. The drug toxicity, gene expression, and docking calculations are evaluated.*

Result and Discussion: *Betaferon showed significant antiviral activity against COVID-19. Furthermore, Betaferon decreased the expression of both viral entries mediating proteins such as ACE2 and TMRPSS2. Betaferon decreases not only the expression of TMPRSS2 but also the enzymatic activity of TMPRSS2. Furthermore, in silico analyses revealed that Betaferon interacts with viral Spike protein. Hence, a decrease in the expression of viral entry mediating proteins, inhibition of the activity of TMPRSS2, and interaction with viral Spike protein indicate that Betaferon has an antiviral activity for COVID-19 virus through inhibition of viral entry pathway.*

Keywords: *Betaferon, COVID-19, drug-repurposing, SARS-CoV-2*

* **Corresponding Author / Sorumlu Yazar:** Sibel A. Özkan
e-mail / e-posta: Sibel.Ozkan@pharmacy.ankara.edu.tr, **Phone / Tel.:** +905338183635

* **Corresponding Author / Sorumlu Yazar:** Kamil Can Akçalı
e-mail / e-posta: Can.Akcali@ankara.edu.tr, **Phone / Tel.:** +905353067509

Submitted / Gönderilme: 23.05.2022 **Accepted / Kabul:** 12.08.2022

ÖZ

Amaç: *COVID-19 enfeksiyonu 2019'dan beri dünya çapında yayılmış ve pandemik enfeksiyon olarak ilan edilmiştir. Maalesef, insanlık hala bu enfeksiyonla başa çıkmaya çalışıyor. Bu koşullar altında bilim insanları, SARS-CoV-2 viral enfeksiyonu ile mücadelede en hızlı çözüm olarak ilaç yeniden konumlandırma çalışmalarına yönelmiştir. Betaferon (İnterferon beta-1b), interferon ailesinin bir üyesidir ve immün sistem tarafından doğal olarak üretilen interferon beta-1a aynı etki mekanizmasına sahiptir.*

Gereç ve Yöntem: *Betaferon'un SARS-CoV-2 enfeksiyonuna karşı antiviral etkisi in vitro ve in siliko olarak araştırıldı. Antiviral aktivitenin tayini için ilaç toksisitesi, gen ifadesi ve docking (kenetlenme) hesaplamaları yapılarak değerlendirildi.*

Sonuç ve Tartışma: *Betaferon, SARS-CoV-2 viral enfeksiyonuna karşı önemli antiviral aktivite göstermiştir. Ayrıca Betaferon, hem virüsün girişinde rol oynayan ACE2 ve TMRPSS2 proteinlerinin ifadesini azaltmıştır. Betaferon, yalnızca TMPRSS2 ifadesini değil, aynı zamanda TMPRSS2'nin proteolitik aktivitesini de doza bağlı bir şekilde azaltmıştır. Bununla birlikte Betaferon'un viral Spike protein ile etkileşime girdiği in silico analizlerle gösterilmiştir. Dolayısıyla, ACE2 ve TMPRSS2 ifadesinin azalması, TMPRSS2 aktivitesinin düşmesi ve SARS-CoV-2'nin Spike proteini ile etkileşimi, Betaferon'un viral giriş yolağını engellenmesi yoluyla SARS-CoV-2 virüsüne karşı antiviral aktiviteye sahip olduğunu göstermiştir.*

Anahtar Kelimeler: *Betaferon, COVID-19, ilaç yeniden konumlandırma, SARS-CoV-2*

INTRODUCTION

Chinese authorities found the first case of COVID-19 in Wuhan, where it was first reported in December of this year. There has been an ongoing pandemic since the disease spread around the world [1]. Globally, pre-clinical and clinical trials for the treatment of COVID-19 have been ongoing. Different therapeutic strategies have been studied, including antiviral, antimalarial, and immunomodulatory approaches [2]. However, there is still no definitive therapeutic strategy. One of the methods to introduce new therapeutics is drug repurposing which significantly reduces the time and cost of drug studies [3].

Interferon beta-1b (Betaferon), the active ingredient in Betaferon, is a member of a class of medications known as interferons. Betaferon was originally indicated for Multiple Sclerosis (MS) treatment and its active ingredient is Interferon beta-1b [4]. Interferons are naturally occurring chemicals that the body produces to aid it in the battle against attacks such as infections caused by viruses, among other things [5]. It has been shown that Interferon beta-1b has immune modulator activity, and anti-inflammatory and antiviral activity [6]. It is possible to generate interferon beta-1b by a process known as 'recombinant DNA technology. The interferon beta-1b is produced by bacteria that has been implanted with a gene (DNA) that allows it to manufacture the protein [7]. Interferon-beta-1b is the non-glycosylated version of interferon beta-1a and the replacement interferon beta-1b functions in the same way as naturally produced interferon beta-1a in the immune system [8,9].

Moreover, interferon beta-1a inhibited the replication of the SARS-CoV-2 virus [10]. Furthermore, Interferon 1 beta reduced lung fibrosis [11]. In addition, ex vivo lung tissue samples revealed that viral infection did not induce the production of interferons hence, usage of Interferon beta-1b can have antiviral

activity against the COVID-19 [12,13]. Herein, the antiviral effect of Interferon beta-1b to combat SARS-CoV-2 was studied particularly via computational and molecular biology analyses.

MATERIAL AND METHOD

In silico calculations

All reagents were analytical grade and used without processing. Betaferon was kindly provided by Bayer Pharmaceutical Company (Istanbul-Turkey). Betaferon structure (PDB ID:1au1), spike protein closed form (PDB ID:6vxx) and spike protein open form (PDB ID:6vyb) structures were used. HDock server [14] was used for docking calculations. For both forms, the 4 lowest-energy bonds were examined. The protein structures obtained from the PDB databank are visualized via BIOVIA Discovery Studio 2021 software [15].

MTT Assay

The MTT assay was carried out the determination of the toxic doses of Betaferon (Millipore, CT02). Vero E6 cells detached with trypsinization. Detached cells were counted and 10.000 cells per well were seeded on a 96-well plate and incubated for 24 hours. For the next 48 hours, the cells were treated with 0.96, 0.48, 0.24, 0.12, and 0.06µg/ml Betaferon for 48 hours. Betaferon had its solvent, it was applied as solvent control. Absorption was measured at 570 nm using EpochTM Microplate Spectrophotometer. The OD values were analyzed relative to the control hence, the percentage of cell viability was determined.

Flow Cytometry for Protein Expression

TMPRSS2 expression of Betaferon treated cells, solvent control cells, and control cells were examined by flow cytometry (NovoCyte Flow Cytometry). Cells were harvested with trypsinization. Collected cells were fixed with 4% Paraformaldehyde, permeabilize, and stained using an antibody against TMPRSS2 (Santa Cruze, sc-515727) respectively. Novo Express 1.3.0 Software (ACEA Biosciences, Inc) is used for flow cytometry.

Evaluation of Gene Expression Through qRT-PCR

TMPSS2 and ACE2 mRNA expression level alterations after Betaferon treatment were assessed with quantitative real-time PCR (qRT-PCR). RNA isolation was carried out with RiboEx (GeneAll® Ribo Ex, 301-001) by following the manufacturer's protocol. cDNA synthesized with 1µg of isolated mRNA (TONBO Biosciences, 31-5300-0100R). qRT-PCR was carried out with instructions of the manufacturer by using CYBER FastTM qPCR Hi-ROX Master Mix (TONBO Biosciences). Gene expression normalized to the expression of GAPDH. Raw data was analyzed with the $2^{-\Delta\Delta CT}$ method.

Western Blotting

The impact of Betaferon on ACE2 protein levels was evaluated through Western Blotting. Cells are harvested with a cell scraper and collected cell pellet lysed with protein lysis buffer. Concentrations of proteins were measured with BCA Protein Assay Reagent (Pierce, Rockford, IL). 60 μg protein for each treatment group loaded with polyacrylamide gel. Polyacrylamide gel was transferred to polyvinylidene difluoride (PVDF) membrane (TransBlot Turbo, Bio-Rad) with a semi-dry transfer technique due to directives of the manufacturer (Bio-Rad, Hercules, CA). Monoclonal anti-ACE2 antibody from ProSci (1:250) and goat anti-rabbit IgG-horseradish peroxidase-conjugated antibody from Santa Cruz Biotechnology (1:2500) were used as primary and secondary antibodies respectively. Monoclonal anti-β-actin antibodies from Santa Cruz and goat anti-mouse HRP conjugated from Pierce were used as primary and secondary antibodies respectively for β- actin. All of the antibodies were diluted in a 5% BSA containing TBST blocking solution. Membrane developed and images were taken with ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA).

Proteolytic Activity Assay to Evaluate the Enzymatic Activity of TMPRSS2

In Vero E6 cells, the proteolytic activity of TMPRSS2 after Betaferon treatment was examined. To assess the proteolytic activity of TMPRSS2, 20.000 cells/well were seeded into a 96-well plate in 100 μl of cell culture maintenance medium. Seeded cells were treated with varied Betaferon concentrations (0.96μg/ml, 0.48μg/ml, 0.24μg/ml and, 0.12μg/ml) for 48 hours. The cells were washed with 1X PBS and incubated with fluorogenic synthetic peptide Boc-Gln-Ala-Arg-AMC (Enzo, BML-P237-0005) at a final concentration of 200 μM at 37°C for 2 hours. Supernatants in the wells were collected individually. Low-rate centrifugation was performed at 3000 rpm, 10 min at 4°C. A new 96-well plate was used to replace the cleared supernatants. Fluorescence intensity was measured through a fluorescence spectrometer at 380 nm (excitation) and 460 (reflection).

Statistical Analysis

Each piece of the data was subjected to statistical analysis to determine its importance. We conducted in vitro experiments excluding qRT-PCR were performed in 3 biological replicates, whereas qRT-PCR was performed in 6 biological replicates to obtain the most accurate results. Paired two-tailed t-tests were used to compare the control and drug-treated group. Significance was defined as p values less than 0.05. To be considered significant p-value must fall below 0.05. Graph Pad Prism 8.01 was used to analyze the data.

RESULT AND DISCUSSION

MTT assay was carried out for the assessment of the viability of Vero E6 cells after the Betaferon treatment as a concentration gradient (0.06, 0.12, 0.24, 0.48, and 0.96 µg/ml). We did not observe a significant decrease in cell viability accompanied by an increasing concentration of Betaferon. According to these results, the concentration of 0.96 µg/ml was determined to use in the forward experiments including anti-viral as 0.96 µg/ml is the topmost dose while the viability of the cell is resembling with the control group. Vero E6 cells were infected with SARS-CoV-2 in which were pretreated with Betaferon with concentration gradient (0.06, 0.12, 0.24, 0.48, and 0.96 µg/ml) and after 48-hour viral copies in the supernatant were collected and analyzed through qRT-PCR for determination of the antiviral activity of Betaferon. Based on anti-viral activity results and MTT results (Figures 1 and 2), it was determined that 0.96 µg/ml Betaferon concentration for further molecular experiments.

We performed molecular analysis to understand the downstream impacts of Betaferon on the viral life cycle. Since TMPRSS2 and ACE2 proteins play a crucial role in enabling virus entry, we tested whether Betaferon has a repressive effect on both of these proteins. Firstly, we analyzed gene expression levels of TMPRSS2 after the 0.96 µg/ml Betaferon treatment by qRT-PCR. We verified that the application of Betaferon significantly lowers TMPRSS2 expression compared to solvent control after 48 hours (Figure 3A). To evaluate the alterations in TMPRSS2 protein expression with the application of Betaferon, we performed a flow cytometry analysis. TMPRSS2 protein level was reduced in the Betaferon treated group after 48 hours due to the control. (For Vero-E6; Control cells 91.8 ± 0.3 %, Solvent treated cells 98.8 ± 0.45%, Betaferon treated cells 84.68 ± 0.31%) (Figure 3B). These data suggest that the application of Betaferon lowers the expression of TMPRSS2 both at protein and mRNA grade in 48 hours.

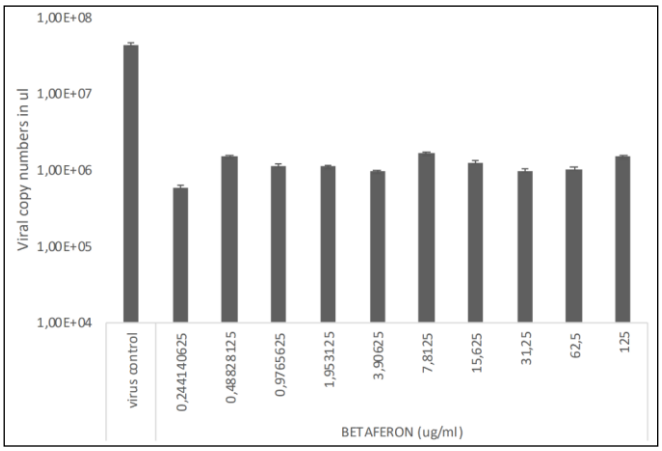


Figure 1. Antiviral activity of Betaferon. Pre-treated Vero E6 cells with Betaferon at different concentrations infected with SARS-CoV-2. After 48-hour viral copies in the supernatant were analyzed through qRT-PCR.

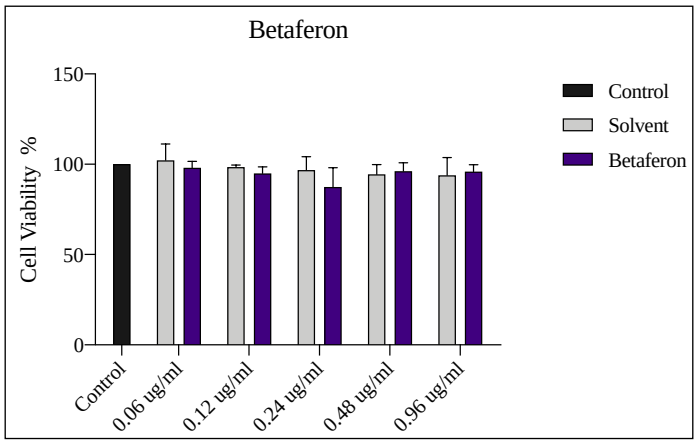


Figure 2. Cytotoxic effect of Betaferon at the concentrations of 0.06, 0.12, 0.24, 0.48, and 0.96 µg/ml on Vero E6 for 48 hours. Data show the mean and the SD of samples. The black bar represents the cells cultured with the maintenance medium (Control). The data obtained from the cells treated with the maintenance medium including Betaferon's solvent represented as grey bars in the graph (Solvent Control). Purple bars show the cells treated with Betaferon. Data was interpreted as the mean ± SD (n= 4).

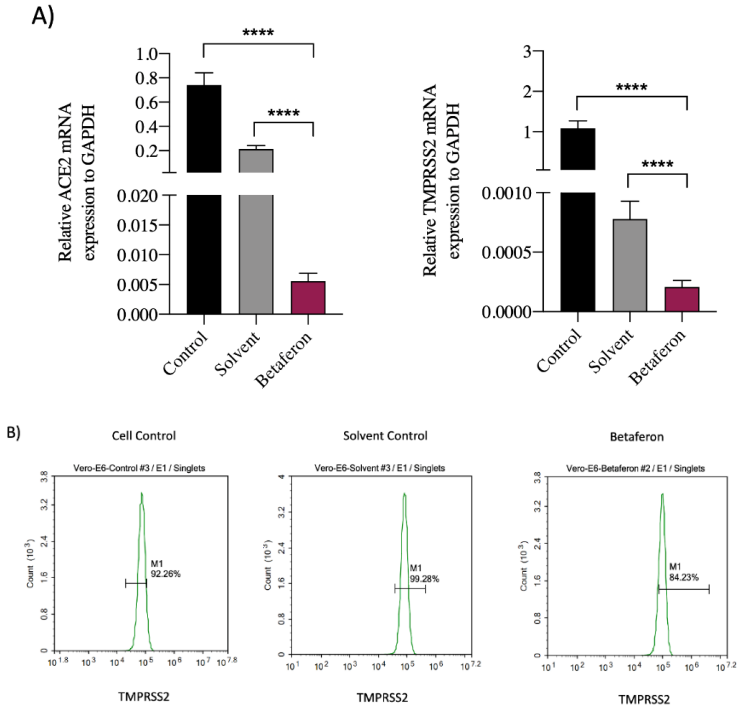


Figure 3. qRT- PCR manifests relative fold differences of TMPRSS2 mRNA expression after 48 h Betaferon treatment in **A)** Vero-E6 cells ($p<0.0005$). **** indicates $p<0.0005$. Data was interpreted as the mean ± SD (n= 6). **B)** Illustration represents flow cytometry graphs of Betaferon (0.96 µg/ml) treated cells. The Betaferon treated cells show a decrease in the TMPRSS2 expression (Paired Two-tailed T-test, $P<0.05$). * indicates $p<0.05$. Data was interpreted as the mean ± SD (n= 3).

To realize our suggestion about the anti-viral activity of Betaferon, and to reveal the impact of Betaferon in the regulation of ACE2 at protein and transcript grades, we carried out western blotting and qRT-PCR. Our qRT-PCR results showed that Betaferon significantly decreases the mRNA level of ACE2 after 48-hour treatment (Figure 3A). Consequently, we observed that Betaferon has a significantly diminishing impact on the regulation of ACE-2 protein in Vero-E6 (Figure 4).

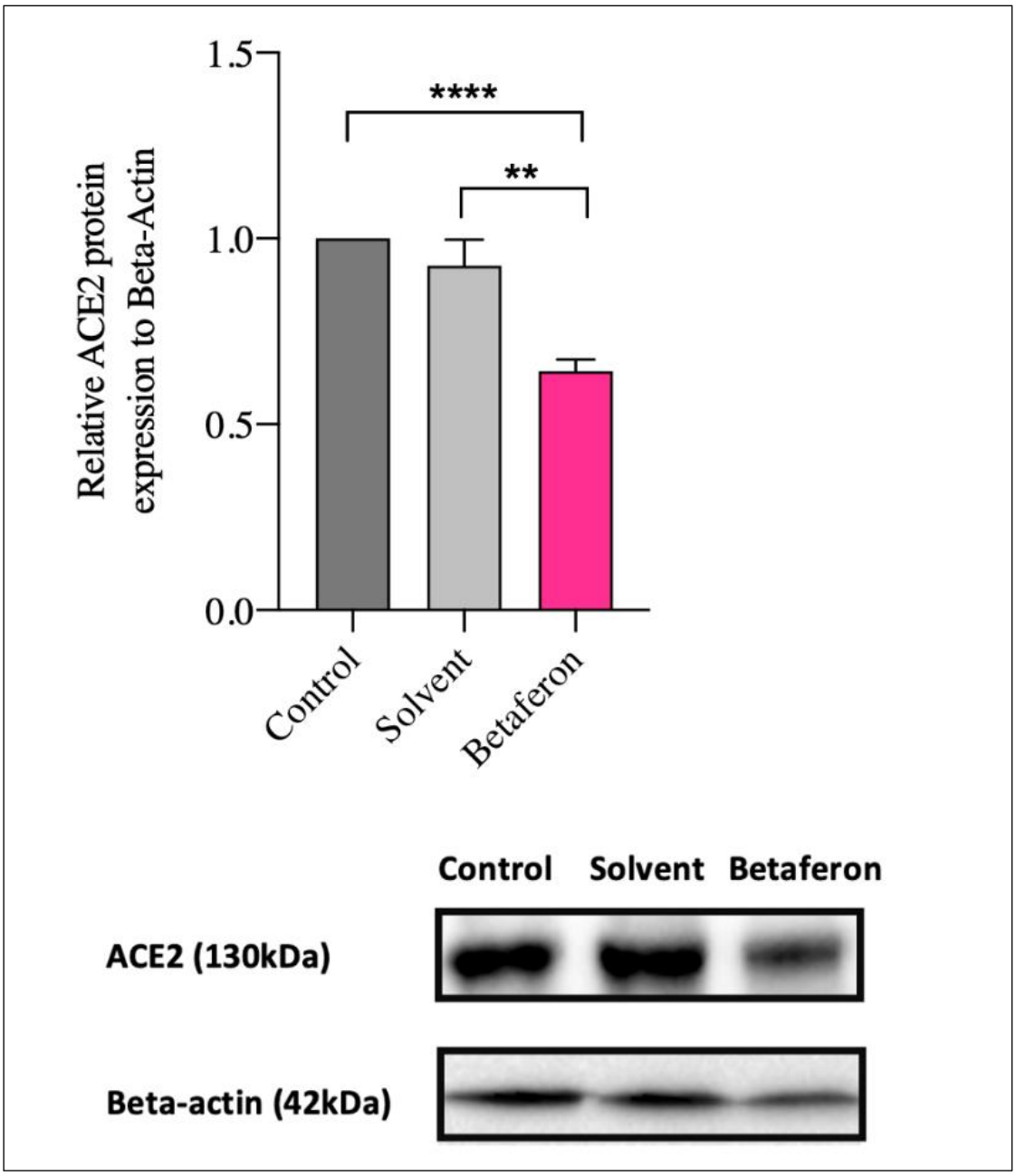


Figure 4. Betaferon decreases ACE2 protein expression in Vero E6 cells. Analysis of band intensities shows alterations in protein levels of ACE2 in Betaferon (0.96 μg/ml) treated Vero-E6 cells. The Betaferon treatment results in a significant decrease in cells. ** indicates $p<0.005$. Data was interpreted as the mean ± SD (n= 3).

As we know, SARS-CoV-2 penetrates the host cell through its surface spike protein for binding on the host cell. Moreover, SARS-CoV-2 priming with the host cell results in fusion. TMPRSS2 has a cleavage domain that cuts the spike protein to induce fusion of membrane. Our previous study about the anti-viral activity of Ribavirin on SARS-CoV-2 revealed that the inhibitory impacts on the proteolytic activity of TMPRSS2 are valuable as a symbol of the anti-viral effect of the drug at the molecular grade [17]. The TMPRSS2 proteolytic activity is measured with the addition of synthetic commercial protease substrate into treated Vero-E6 cells with 0.48 and 0.96 μg/ml Betaferon for 48 hours [17]. We determined the proteolytic activity of TMPRSS2 due to the detection of soluble forms of proteins liberated from cells. Our results indicated that the application of Betaferon significantly diminishes TMPRSS2 activity in a dose-dependent manner (Figure 5). This finding indicates that especially 0.96 μg/ml Betaferon treatment has an inhibitory impact on the proteolytic activity of TMPRSS2.

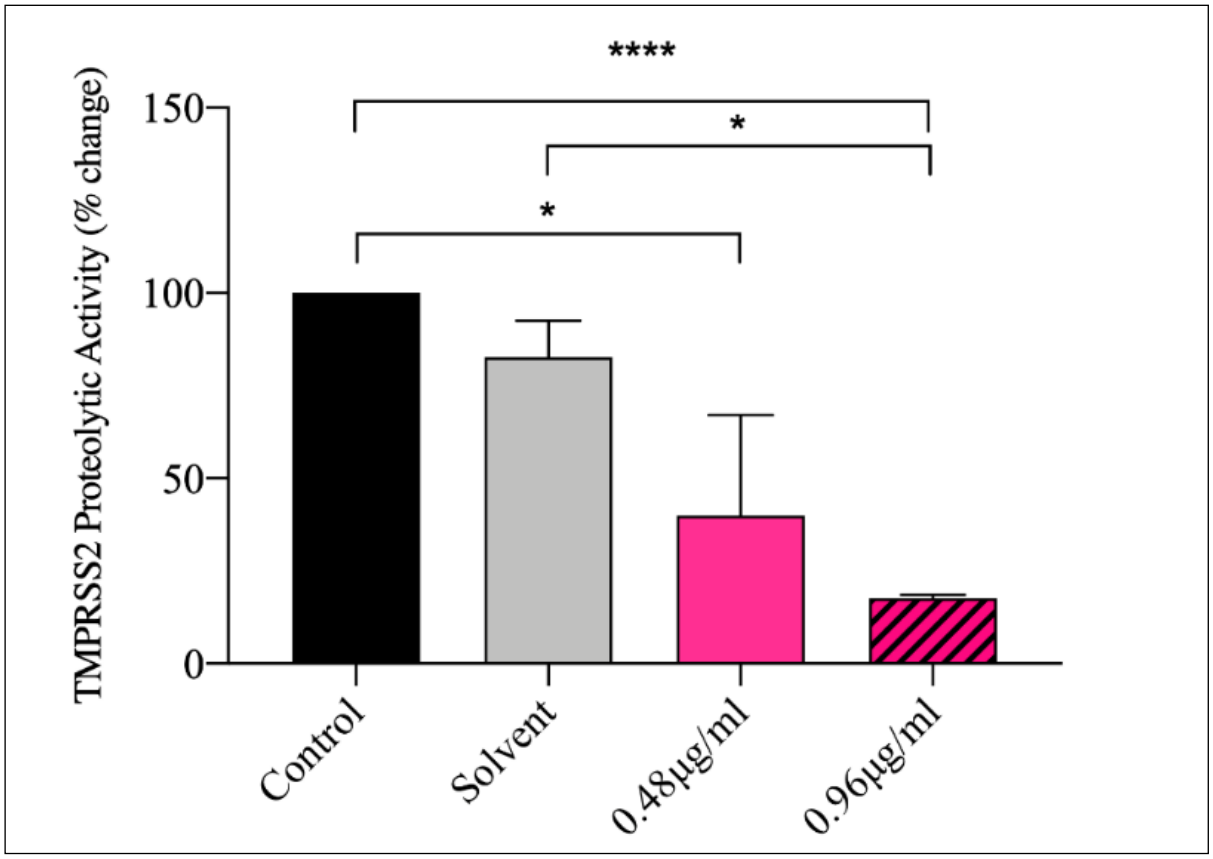


Figure 5. Examination of the proteolytic activity of TMPRSS2 carried out through substrate assay analysis. Betaferon treatment causes a remarkable reduction of the primed substrate by TMPRSS2. This data demonstrates that Betaferon suppresses the enzymatic activity of TMPRSS2. * indicates $p<0.05$; **** indicates $p<0.005$. Data interpreted as the mean ± SD (n= 3)

Concerning the analysis of the drug's mechanism of action against viruses in-depth, we performed *in silico* analyzes. *In silico* analyses revealed that the lowest energy binding score to the closed form of

Spike protein is -418.13 kcal/mol. All 4 bindings to this region are in the RBD region (Figure 6). Binding to the RBD region is critical for virus attachment to the ACE2 receptor and inactivating the mechanism of entry into the cell.

The lowest energy of the bonds to the open form of Spike protein is -339.66 kcal/mol. There are differences between the patterns of attachment to this form; 1st and 2nd pattern binding to the RBD region, 3rd pattern binding to the S2 subunit, and 4th pattern binding are to the N-terminal domain (Figure 7). Regions other than RBD are also used in antibody targets and are effective in inactivating the virus.

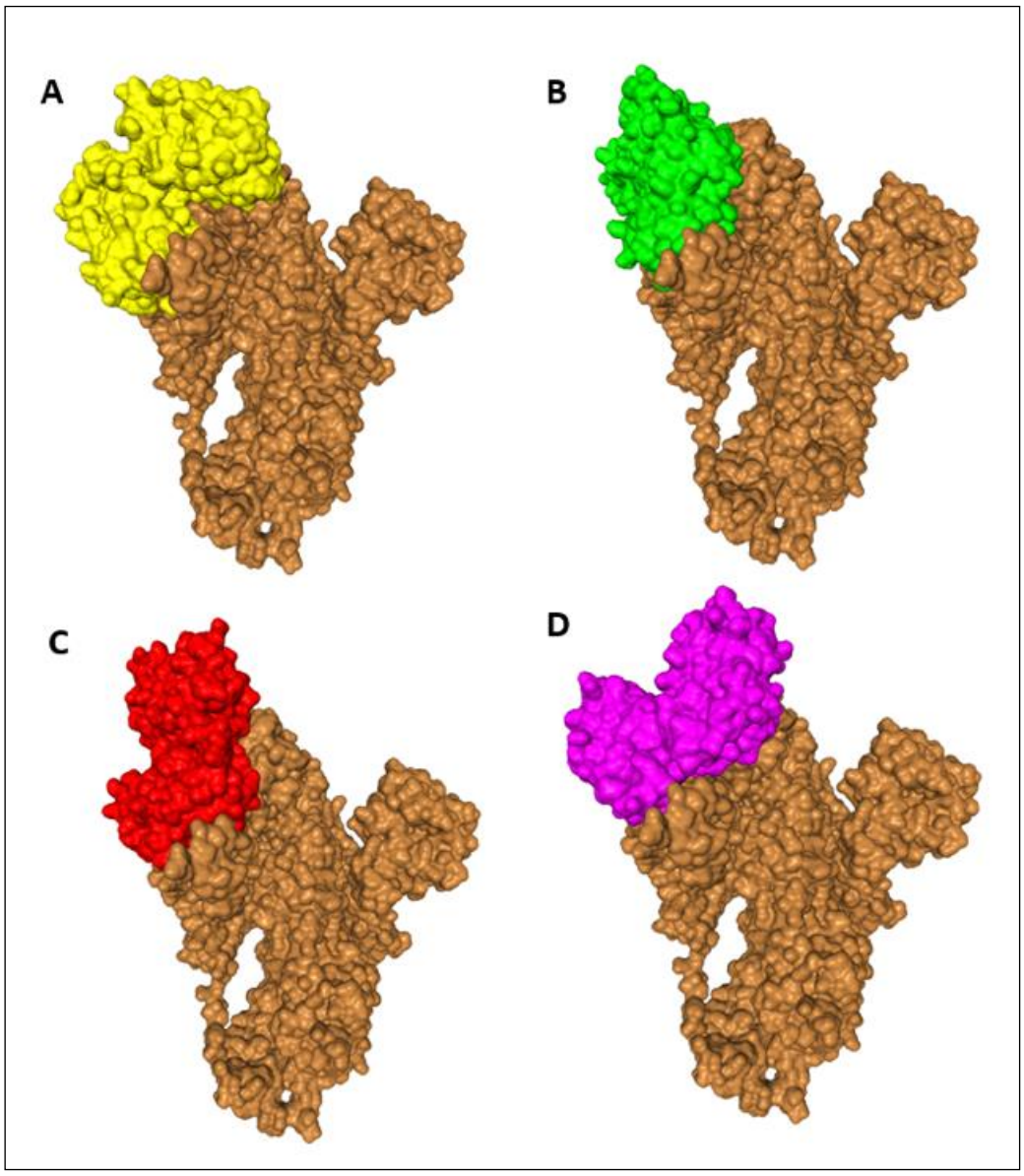


Figure 6. Docking results of Betaferon and spike protein (close form). All docking has been to the RBD region. The binding energies are respectively A: -418.13 kcal/mol, B: -337.87 kcal/mol, C: -324.85 kcal/mol, D: -322.86 kcal/mol.

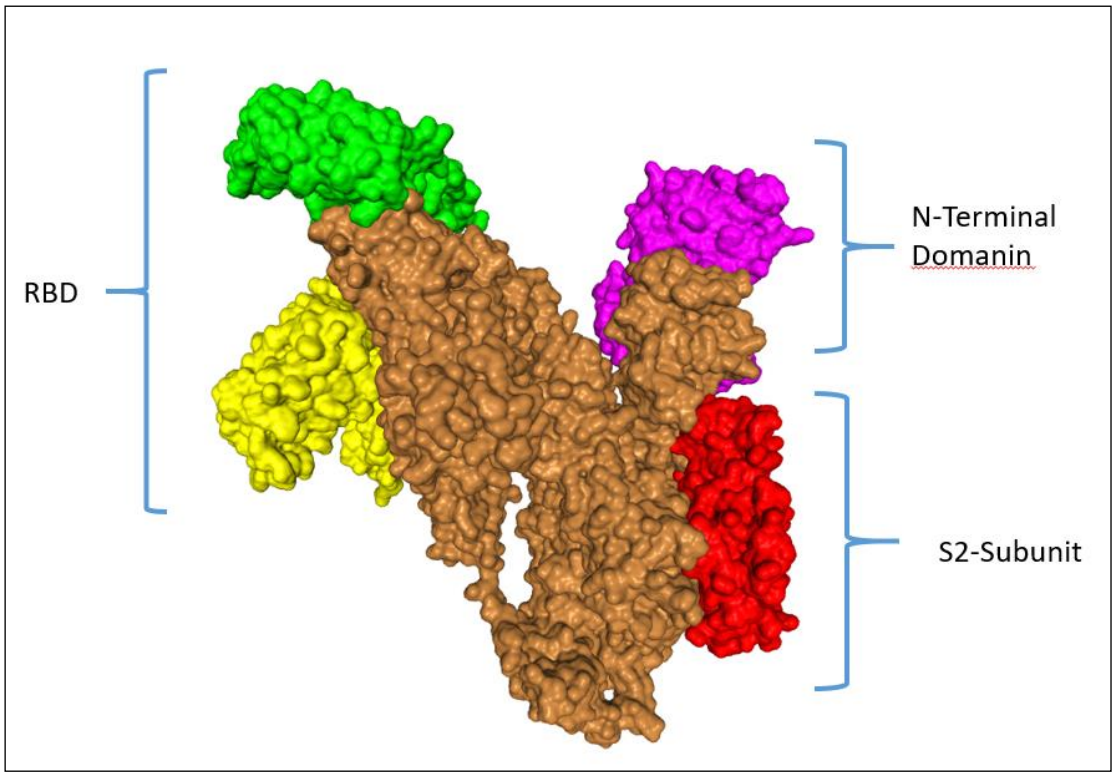


Figure 7. Docking results of Betaferon and spike protein (open form). The first two binding to the open form are to the RBD sites (binding energy: -339.66 kcal/mol and -317.65 kcal/mol), the others are the S2- subunit (binding energy: -316.42 kcal/mol) and N-Terminal (binding energy: -316.42). kcal/mol) area. These bindings to different regions of the protein are important for virus inactivation.

When the bond profile of spike protein (closed form) and betaferon is examined, it is seen that there are many hydrogen bonds. In addition to these, electrostatic interactions are also observed, albeit slightly. The interaction of betaferon with the open form was observed in 4 different regions. Hydrophobic and electrostatic interactions are observed with hydrogen bonds in the 1st region. The connection profile here shows a high similarity with the closed form. When the interactions in the 2nd region are examined, it is seen that the pi-sulfur bond forms a pi-sulfur bond with the residue CYS141. While hydrophobic interactions increase in the 3rd region, the interaction profile in the 4th region shows a high similarity with the 2nd region.

In conclusion, Betaferon showed significant antiviral activity against the SARS-CoV-2 virus. *In vitro* and computational studies revealed that Betaferon actively inhibits the viral entry pathway. It decreases in expression of ACE2 and TMPRSS2 proteins in both protein and mRNA grades. In addition to the decrease in the expression of TMPRSS2 protein Betaferon also inhibits the proteolytic activity of TMPRSS2. Furthermore, the interaction of Betaferon with the SARS-CoV-2 Spike protein indicates that Betaferon shows Betaferon is effective not only in the host but also in the SARS-CoV-2 virus. Hence,

Betaferon has significant antiviral activity against the COVID-19 virus via inhibition of the viral entry pathway by targeting both host and viral proteins. In our study, it is seen that the experimental data were confirmed by *in silico* results. The interaction of betaferon with both the closed and open form of the Spike protein increases its inhibition capacity. interaction with different regions of the open form increases the effect of the drug despite the high binding energies. The fact that it is larger than the small molecule structurally affects the binding profile considerably. In this way, it increases its affinity by establishing many hydrogen bonds with different residues. Since the spike-ace2 interface is very large, inhibiting this region is only possible with large molecules such as betaferon. The most important result of this is that the probability of being affected by mutations in the Spike protein decreases.

ACKNOWLEDGEMENTS

Betaferon was kindly supplied by Bayer Pharmaceutical Company (Istanbul-Turkey), and we are grateful to Bayer Pharmaceutical Company for their kind support.

AUTHOR CONTRIBUTIONS

Conception: *F.B., K.C.A.;* Design: *F.B., K.C.A., S.A.O;* Supervision: *K.C.A., S.A.O;* Resources: F.B., *K.C.A., S.A.O;* Materials: *F.B. K.C.A.;* Data collection and/or processing: *F.B., K.C.A., S.A.O;* Literature search: *F.B.;* Writing manuscript: *F.B.; K.C.A., S.A.O.*; Other:-

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS COMMITTEE APPROVAL

The authors declare that ethics committee approval is not required for this study.

REFERENCES

1. Merad, M., Blish, C.A., Sallusto, F., Iwasaki, A. (2022). The immunology and immunopathology of COVID-19. *Science, 375*(6585), 1122–1127. [CrossRef]

2. Lythgoe, M.P., Middleton, P. (2020). Ongoing clinical trials for the management of the COVID-19 pandemic. *Trends in Pharmacological Sciences, 41*(6), 363–382. [CrossRef]

3. Casaos, J., Gorelick, N.L., Huq, S., Choi, J., Xia, Y., Serra, R., Felder, R., Lott, T., Kast, R.E., Suk, I., Brem, H., Tyler, B., Skuli, N. (2019). The use of ribavirin as an anticancer therapeutic:

Will it go viral? Ribavirin as an anticancer therapeutic. *Molecular Cancer Therapy*, *18*(7), 1185–1194. [CrossRef]

4. Burks, J. (2005). Interferon-beta1b for multiple sclerosis. *Expert Review of Neurotherapeutic*, *5*(2), 153–164. [CrossRef]

5. Kieseier, B.C. (2011). The mechanism of action of interferon-β in relapsing multiple sclerosis. *CNS Drugs*, *25*(6), 491–502. [CrossRef]

6. Brzoska, J., Eick, H.V., Hündgen M. (2020). Interferons in the therapy of severe coronavirus infections: A critical analysis and recollection of a forgotten therapeutic regimen with interferon beta. *Drug Research*, *70*(7), 291. [CrossRef]

7. Haile, L.A., Polumuri, S.K., Rao, R., Kelley-Baker, L., Kryndushkin, D., Rajaiah, R., Israely, T., Rao, V.A., Verthelyi, D. (2017). Cell based assay identifies TLR2 and TLR4 stimulating impurities in Interferon beta. *Scientific Reports*, *7*, 10490. [CrossRef]

8. Runkel, L., Meier, W., Pepinsky, R.B., Karpusas, M., Whitty, A., Kimball, K., Brickelmaier, M., Muldowney, C., Jones, W., Goelz, S.E. (1998). Structural and functional differences between glycosylated and non-glycosylated forms of human interferon-β (IFN-β). *Pharmaceutical Research*, *15*(4), 641–649. [CrossRef]

9. Dhib-Jalbut, S. (2002). Mechanisms of action of interferons and glatiramer acetate in multiple sclerosis. *Neurology*, *58*(8), 3-9. [CrossRef]

10. Hensley, L.E., Fritz, E.A., Jahrling, P.B., Karp, C., Huggins, J.W., Geisbert, T.W. (2004). Interferon-β 1a and SARS Coronavirus Replication. *Emerging Infectious. Disease*, *10*(2), 317. [CrossRef]

11. Luckhardt, T.R., Coomes, S.M., Trujillo, G., Stoolman, J.S., Vannella, K.M., Bhan, U., Wilke, C.A., Moore, T.A., Toews, G.B., Hogaboam, C., Moore, B.B. (2011). TLR9-induced interferon β is associated with protection from gammaherpesvirus-induced exacerbation of lung fibrosis. *Fibrogenesis & Tissue Repair*, *4*(1), 1–15. [CrossRef]

12. Chu, H., Chan, J.F.W., Wang, Y., Yuen, T.T.T., Chai, Y., Hou, Y., Shuai, H., Yang, D., Hu, B., Huang, X., Zhang, X., Cai, J.P., Zhou, J., Yuan, S., Kok, K.H., To, K. K.W., Chan, I.H.Y., Zhang, A.J., Sit, K.Y., Au, W.-K., Yuen, K.Y. (2020). Comparative replication and immune activation profiles of SARS-CoV-2 and SARS-CoV in human lungs: An ex vivo study with implications for the pathogenesis of COVID-19. *Clinical Infectious Disease*, *71*(6), 1400–1409. [CrossRef]

13. Hung, I.F.N., Lung, K.C., Tso, E.Y.K., Liu, R., Chung, T.W.H., Chu, M.Y., Ng, Y.Y., Lo, J., Chan, J., Tam, A.R., Shum, H.P., Chan, V., Wu, A.K.L., Sin, K.M., Leung, W.S., Law, W.L., Lung, D.C., Sin, S. (2020). Triple combination of interferon beta-1b, lopinavir–ritonavir, and ribavirin in the treatment of patients admitted to hospital with COVID-19: an open-label, randomised, phase 2 trial. *Lancet*, *395*(10238), 1695–1704.[CrossRef]

14. Yan, Y., Zhang, D., Zhou, P., Li, B., Huang, S.Y. (2017). HDOCK: a web server for protein-protein and protein-DNA/RNA docking based on a hybrid strategy. *Nucleic Acids Research*, *3*(45), 365-373. [CrossRef]

15. BIOVIA Discovery Studio 2017. Dassault Systèmes, San Diego, California, USA. Available from http://www.3ds.com/products-services/biovia/.

16. Unal, M.A., Bitirim, C.V., Summak, G.Y., Bereketoglu, S., Zeytin, I.C., Besbinar, O., Gurcan, C., Aydos, D., Goksoy, E., Kocakaya, E., Eran, Zeynep., Murat, M., Demir, N., Aksoy Ozer, Z. B., Somers, J., Demir, E., Nazir, H., Ozkan, S.A., Ozkul, A., Azap, A., Yilmazer, A., Akcali, K.C. (2021). Ribavirin shows antiviral activity against sars-cov-2 and downregulates the activity of tmprss2 and the expression of ace2 in vitro. *Canadian Journal of Physiology and Pharmacology*, *99*(5), 449–460. [CrossRef]

17. Ko, C.J., Huang, C.C., Lin, H.Y., Juan, C.P., Lan, S.W., Shyu, H.Yi., Wu, S.R., Hsiao, P.W., Huang, H.P., Shun, C.T., Lee, M.S. (2015). Androgen-induced TMPRSS2 activates matriptase and promotes extracellular matrix degradation, prostate cancer cell invasion, tumor growth, and metastasis. *Cancer Research*, *75*(14), 2949–2960. [CrossRef]

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 896-908, 2022
Doi: 10.33483/jfpau.1150706

ORIGINAL ARTICLE / ÖZGÜN MAKALE

DRUG REPOSITIONING APPROACH FOR THE TREATMENT OF ANKYLOSING SPONDYLITIS

ANKİLOZAN SPONDİLİT TEDAVİSİNDE İLAÇ YENİDEN YERLEŞTİRME YAKLAŞIMI

Gözde YALCIN OZKAT[1*]

[1]Recep Tayyip Erdogan University, Faculty of Engineering and Architecture, Bioengineering Department, 53100 Rize, Turkey

ABSTRACT

Objective: *In this study, it was aimed to determine an FDA-approved molecule that inhibits the IL-17 receptor, which is an important target for the prevention of inflammation in Ankylosing Spondylitis (AS), using the drug repositioning approach.*

Material and Method: *Using the Drug-Gene Interaction database, 18 molecules specific to the active HLA-B gene were identified in AS. Then, the 3D structure of IL-17 was obtained from the RSCB database. I) Blind docking II) Computed Atlas of Surface Topography of Proteins web tool was used to determine the binding package. The interaction between the known inhibitor of IL-17, rhodomyrtone, and IL-17, was determined by molecular docking using grid boxes around the determined binding packages. Accordingly, configuration files were prepared with the selected grid box features, and docking was performed for 18 molecules with the AutoDock Vina program.*

Result and Discussion: *The carbamazepine molecule shows the best binding affinity and binding profile with IL-17. It was also revealed that minocycline, sulfasalazine, and thalidomide are tightly packed in the active site. It has been demonstrated that these molecules may be lead molecules for the treatment of AS disease.*

Keywords: *Ankylosing spondylitis, blind docking, drug repositioning, IL-17, molecular docking*

ÖZ

Amaç: *Bu çalışmada, AS'de inflamasyonun önlenmesinde önemli bir hedef olan IL-17 reseptörünü inhibe eden FDA onaylı bir molekülün ilaç yeniden konumlandırma yaklaşımı kullanılarak belirlenmesi amaçlanmıştır.*

Gereç ve Yöntem: "*Drug-Gene Interaction" veritabanı kullanılarak AS'de etkin HLA-B genine özgü 18 molekül belirlenmiştir. Ardından IL-17'nin 3D yapısına RSCB veri tabanından ulaşılmıştır. Bağlanma paketinin belirlenmesi için I) Kör kenetlenme II) "Computed Atlas of Surface Topography of Proteins" web aracı kullanılmıştır. Belirlenen bağlanma paketleri çevresindeki grid kutuları kullanılarak IL-17'nin bilinen*

* **Corresponding Author / Sorumlu Yazar:** Gözde Yalcin Ozkat
e-mail / e-posta: gozde.yalcin@erdogan.edu.tr, **Phone / Tel.:** +905065055074

Submitted / Gönderilme: 29.07.2022 **Accepted / Kabul:** 12.08.2022

inhibitörü rhodomyrtone ile IL-17 arasındaki etkileşim moleküler doking ile belirlenmiştir. Buna göre seçilen grid kutusu özellikleri ile konfigürasyon dosyaları hazırlanarak 18 molekül için de AutoDock Vina programı ile doking gerçekleştirilmiştir.

Sonuç ve Tartışma: *Karbamazepin molekülü, IL-17 ile en iyi bağlanma afinitesini ve bağlanma profilini göstermiştir. Ayrıca minosiklin, sülfasalazin ve talidomidin moleküllerinin de aktif bölgede sıkıca paketlendiği ortaya çıkmıştır. Bu moleküllerin AS hastalığının tedavisi için bir öncü molekül olabileceği gösterilmiştir.*

Anahtar Kelimeler: *Ankilozan spondilit, IL-17, ilaç yeniden konumlandırma, kör kenetlenme, moleküler kenetlenme*

INTRODUCTION

Ankylosing Spondylitis (AS), which usually occurs at a young age; is a painful, inflammatory type of autoimmune disease that affects the spine and the joint between the spine and hipbone [1-3]. Although the exact cause of ankylosing spondylitis is unknown, hereditary factors are known to play an important role. People who carry the HLA-B27 gene have a higher risk of developing the disease [4-6].

As a result of inflammation, these two bones combine to form a single bone. Inflammation and then ossification occurs in all edges of the disc and ligaments from the lower part of the spine to the neck. As a result, an anterior curvature occurs in the upper part of the spine. Although most patients can go on with their lives, the spinal motion may be restricted in a group of patients with advanced disease [7-10]. Although the course of the disease generally continues with periods of remission, it is sometimes exacerbated by periods of attacks. During these attacks, the disease also affects the lungs, heart, kidneys, eyes, and also affects hip joints. Most often, eye involvement occurs with uveitis, and if left untreated, this leads to loss of vision in the patient. Therefore, the treatment of the disease is of great importance [11-13].

There is no known cure for AS. The drugs used clinically for AS are mostly aimed at relieving symptoms. Non-steroidal anti-inflammatory drugs (NSAIDs) are used specifically for pain relief. In addition, specific drugs such as sulfasalazine (CVS) and methotrexate (MTX), and TNF-alpha inhibitor immunosuppressants are used to relieve seizures [4, 14, 15]. The biggest problem with these drugs is their side effects. Therefore, the identification of more specific drugs is of great importance.

As with neurological diseases such as Alzheimer's Disease and central nervous system diseases such as Multiple Sclerosis, the biggest problem in AS is inflammation [16-18]. Therefore, prevention of inflammation is the main goal. TNF-α is the primary target in the prevention of inflammation and many signaling pathways are activated through it. This signaling releases several cytokines and initiates the apoptotic pathway, resulting in target cell activation, resulting in an inflammatory and immune response [19]. Currently, five tumor necrosis factor-alpha (TNF-α) inhibitors (Infliximab, Etanercept, Adalimumab, Certolizumab, and Golimumab) are available in the treatment of AS [20]. However, these structures, which are monoclonal antibodies, are known for their intense side effects [20, 21]. For this

reason, researchers turned to studies on the development of IL-17 inhibitors, which is another target in inflammation [22]. Another important pathway for inflammation in the pathogenesis of AS is IL-23/IL-17 pathway. T-helper 17 cells are one of the largest immunological flows that are expressed in AS patients and are involved in the immune response. Both animal models and clinical studies use IL-17 cytokines in this response of T-helper 17 cells [22]. Therefore, it is possible to stop inflammation in AS by inhibiting IL-17. Although Phase 2 and Phase 3 studies are ongoing for different anti-IL-17 agents (Secukinumab, Ixekizumab, Bimekizumab, Brodalumab) for rheumatological and inflammatory diseases, there is no specific IL-17 inhibitor used clinically [22-24].

Drug repositioning, in other words, drug repurposing, is a set of methods that enable us to investigate the use of drug molecules that have been clinically approved or whose phase studies are in progress, outside of their medical indications. It provides a great advantage in terms of both time and cost compared to the detection of a completely new drug molecule, since it works with molecules that have been clinically and preclinically determined to be safe [25-27].

Within the scope of the study, which is the subject of this article, FDA-approved drug molecules suitable for the active HLA-B gene family in AS were determined and their IL-17 cytokine inhibitory properties were examined by web tools and molecular docking methods.

MATERIAL AND METHOD

Drug-Gene Interaction Research

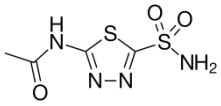
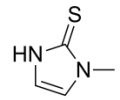
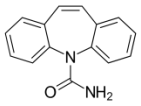
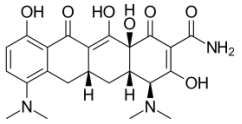
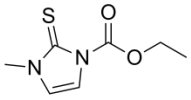
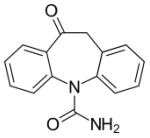
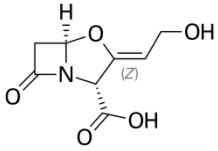
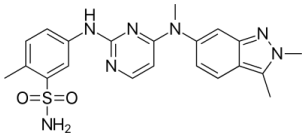
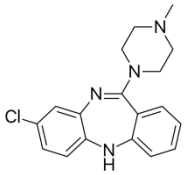
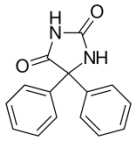
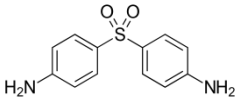
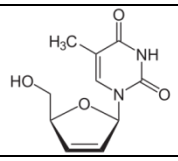
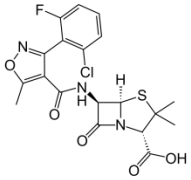
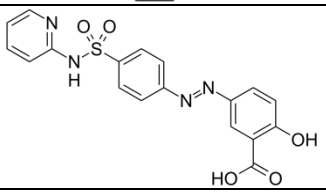
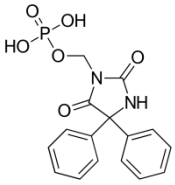
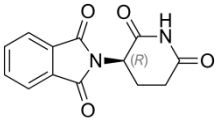
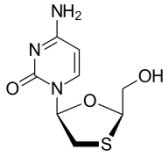
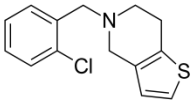
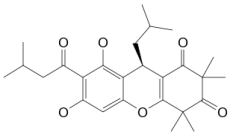
Using the Drug-Gene Interaction database (DGIdb) [28], a database containing drug molecules for genetic sources of diseases, 25 molecules associated with the HLA-B gene were identified. Eighteen of these molecules (Table 1) were downloaded from the PubChem database [29] for use in this study.

Molecular Docking

The interaction between the IL-17 receptor and 19 molecules (18 molecules from DGIdb and reference molecule, Rhodomyrtone, a known IL-17 inhibitor) was investigated by molecular docking. Molecular docking was performed using the AutoDock Vina program [30]. These 18 molecules determined were prepared by the “Ligand” module of the AutoDock Tools (ADT) [31] package, and gasteiger loads and TORSDOF parameters were also regulated by this module. The receptor structure (PDB ID: 4HR9 [32]) was downloaded from the RSCB database [33], and polar hydrogens and gasteiger charges were added to the ADT to obtain the 3D structure used in docking. However, since the receptor cannot crystallize with an inhibitor molecule, it is not possible to have a definite judgment about the binding pocket. For this reason, to determine the dimensions of the grid box be used in docking, firstly, the determination of the docking package was studied. All binding affinity values given in the article

are taken for conformations where the RMSD value is 0 Angstrom.

Table 1. Features of selected compounds for molecular docking

Compound	2D Structure	Compound	2D Structure
Acetazoleamide		Methimazole	
Carbamazepine		Minocycline	
Carbimazole		Oxcarbazepine	
Clavulanic Acid		Pazopanib	
Clozapine		Phenytoin	
Dapsone		Stavudine	
Floxacillin		Sulfasalazine	
Fosphenytoin		Thalidomide	
Lamivudine		Ticlopidine	
Rhodomyrtone (Reference)			

Binding Pocket Prediction of IL-17 Receptor

Computed Atlas of Surface Topography of Proteins (CASTp) [34], a web tool that scans for regions where molecules can interact within the receptor by examining receptor cavities and solvent access points, has been used to predict the receptor binding package. Predictions of the binding pocket are given in Table 2.

Blind Docking

Blind docking is performed when the target site containing the binding pocket at which receptor-ligand interaction will occur is unknown. The results of this process are combined by dividing the grid box into several pieces or repeating the process several times for the different pieces [35]. Another blind docking method is to specify a grid box containing all the receptors used in this study. Thus, it is predicted that the region where the greatest number of structures interact from different conformations of the molecule is the active site of the receptor. Blind docking was carried out using the AutoDock Vina program, and the dimensions of the grid box containing the whole receptor were determined by the "Grid" module of ADT (Table 2).

Grid Box Properties

Grid box parameters created by CASTp and blind docking are given in Table 2. In both methods, amino acid residues estimated to be in the active site were determined. While determining the grid box, the coordinates and dimensions of the box containing these amino acids were taken as a basis.

Table 2. Grid parameters used for molecular docking

Method	Pocket	X center	Y center	Z center	Grid Point	Dimensions
Blind Docking	-	11.572	28.93	48.65	0.375 Å	100 Å × 100 Å × 100 Å
	1	7.034	31.898	42.638	0.375 Å	40 Å × 40 Å × 40 Å
	2	21.505	23.063	49.071	0.375 Å	40 Å × 40 Å × 40 Å
	3	-7.205	28.536	65.44	0.375 Å	40 Å × 40 Å × 40 Å
CASTp Web server	1	-2.998	35.541	53.645	0.375 Å	40 Å × 40 Å × 40 Å
	2	16.385	28.426	48.287	0.375 Å	50 Å × 70 Å × 70 Å
	3	-3.586	28.31	63.124	0.375 Å	40 Å × 40 Å × 40 Å

RESULT AND DISCUSSION

Binding Pocket Prediction with CASTp

20 binding pockets were identified via the CASTp database. Among these packages, the properties of the 5 with the highest areas and the amino acid residues in the package are given in Table 3.

Table 3. Properties of pockets obtained from CASTp

Pocket	Area (SA)	Volume (SA)	Aminoacid Residues
1	434.832	256.325	LEU 53,TYR 62, PRO 63,VAL 65, ILE 66, TRP 67, ALA 69, GLN 94, GLU 95, ILE 96, LEU 97, VAL 98, LEU 99, LEU 112, LYS 114, VAL 117, SER 118, VAL 119
2	208.076	189.577	ARG 46, SER 47, THR 48, CYS 76, ILE 77, ASN 78, ALA 79, ASP 84, MET 87, CYS 123, THR 125
3	59.868	29.344	TYR 44, ASP 45, PRO 50, TRP 51, ASN 52, LEU 53, ARG 72
4	8.462	2.933	ARG 100, ARG 101, GLU 102, PRO 104, ARG 111
5	5.401	0.998	ASN 56, ARG 61, ILE 66, GLU 68, ILE 115, VAL 117

Configuration files were created with the first 3 packages for molecular docking work. The positions and sequences of the first 3 packages in the receptor are given in Figure 1.

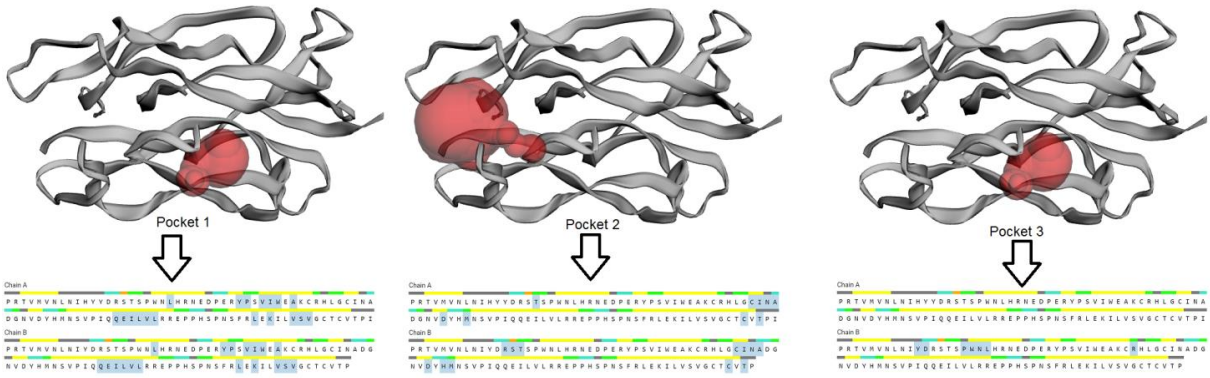


Figure 1. In the receptor shown in Cartoon style on the upper part of the figure, the parts areshown in CPK style (red balls) constitute the active regions. In the receptor sequence given at the bottom of the figure, the areas shaded in gray are the predicted binding packages.

Blind Docking

Blind docking was performed with the known inhibitor of IL-17, rhodomyrtone. The conformation of molecule 20 and its positions on the receptor was given in Figure 2. Accordingly, the 3 binding sites where the highest number of conformations is docked are given in the figure. The features of the Grid Box created by centering these regions are given in Table 2.

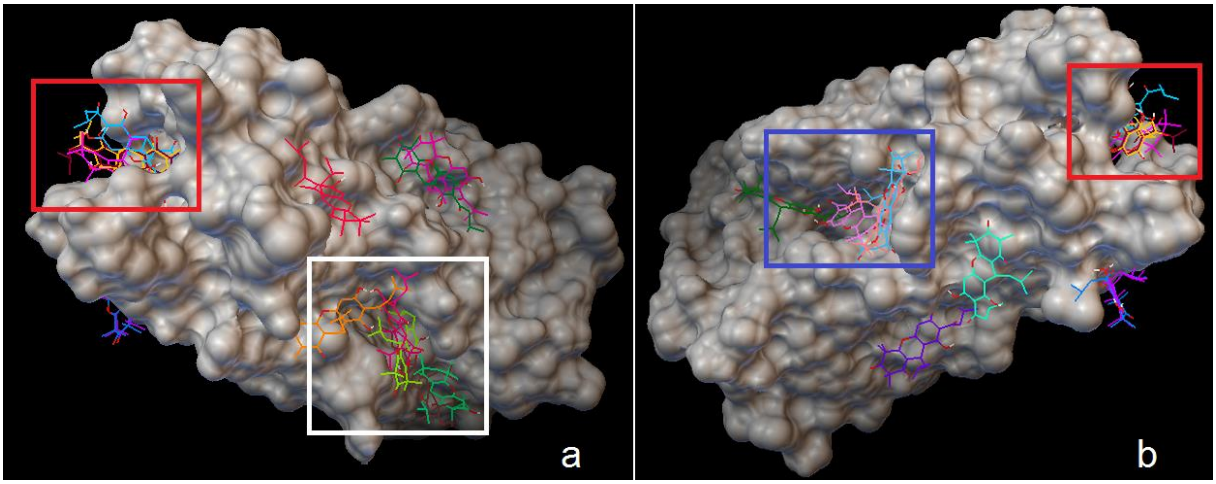


Figure 2. Blind docking conformations of rhodomyrtone. a) Front view of the surface representation of the receptor b) Back view of the surface representation of the receptor. The white rectangle represents binding pocket 1, the blue rectangle the binding pocket 2, and the red rectangle the binding pocket 3.

Molecular Docking

To determine the required docking protocol for examining the interaction of the molecules given in Table 1 and the IL-17 receptor, the interaction of rhodomyrtone was examined by using all the Grid Box features given in Table 2. Accordingly, when the obtained binding affinities are examined, it is seen that the second package obtained by blind docking and the second package obtained through the CASTp web tool give the same binding affinity (-7.6 kcal/mol) (Table 4).

Table 4. Properties of pockets obtained from CASTp

Method	Pocket	Affinity (kcal/mol)
Blind Docking	1	-6.9
	2	-7.6
	3	-5.9
CASTp Web server	1	-6.8
	2	-7.6
	3	-5.8

At the same time, it is understood from Table 2 that these 2 packages also show the same region on the receptor. For this reason, it was determined that the active site of the receptor was expressed by package 2, and other molecules were docked with the configuration file of package 2. In Figure 3, the interaction properties of rhodomyrtone in the binding package are given. Accordingly, it was determined that rhodomyrtone interacted with TRP 67 in accordance with the literature [36].

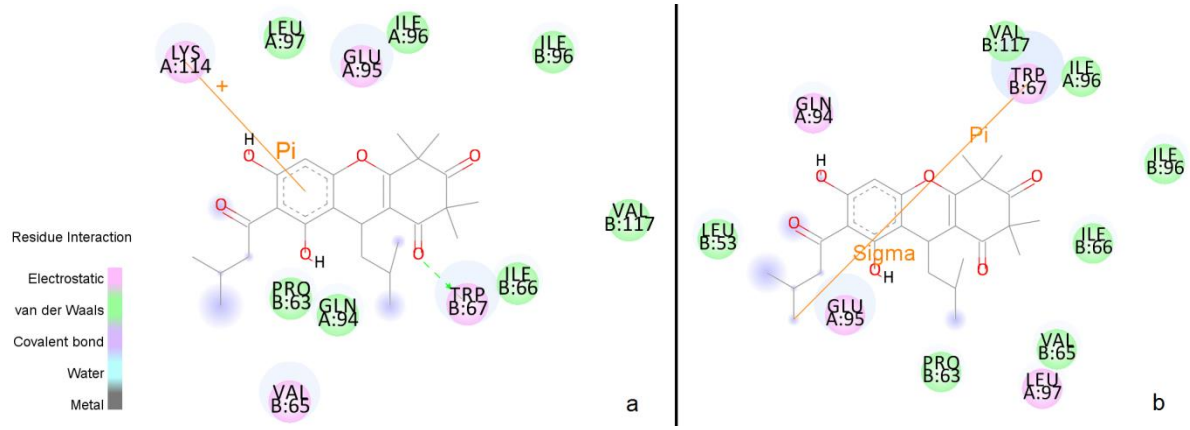


Figure 2. Analysis of interactions detected as a result of docking via Discovery Studio Visualizer program [37]. a) Interaction profile in binding packet 2 obtained via the CASTp web tool b) Interaction profile in bind pack 2 obtained with blind docking

The binding affinities of the molecules given in Table 1 with the binding package 2 are given in Table 5. Accordingly, it was determined that the highest binding affinity was obtained with carbamazepine (-7.8 kcal/mol). It was also observed that minocycline, sulfasalazine, and thalidomide gave a higher binding affinity (-7.7 kcal/mol) than rhodomyrtone (-7.6 kcal/mol).

Table 5. Molecular docking results between selected ligands and IL-17 receptor

Ligand	Affinity (kcal/mol)	Ligand	Affinity (kcal/mol)
Acetazoleamide	-5.0	Minocycline	-7.7
Carbamazepine	-7.8	Oxcarbazepine	-5.5
Carbimazole	-4.4	Pazopanib	-6.8
Clavulanic Acid	-5.5	Phenytoin	-6.8
Clozapine	-7.4	Stavudine	--5.9
Dapsone	-6.2	Sulfasalazine	-7.7
Floxacillin	-6.5	Thalidomide	-7.7
Fosphenytoin	-5.8	Ticlopidine	-4.9
Lamivudine	-5.0	Rhodomyrtone	-7.6
Methimazole	-3.2		

Figure 3 shows the interaction between carbamazepine and the receptor. Accordingly, it is observed that the molecule is in the binding package of the receptor and exhibits a similar interaction profile with rhodomyrtone and also shows a π-cation interaction with LYS 114.

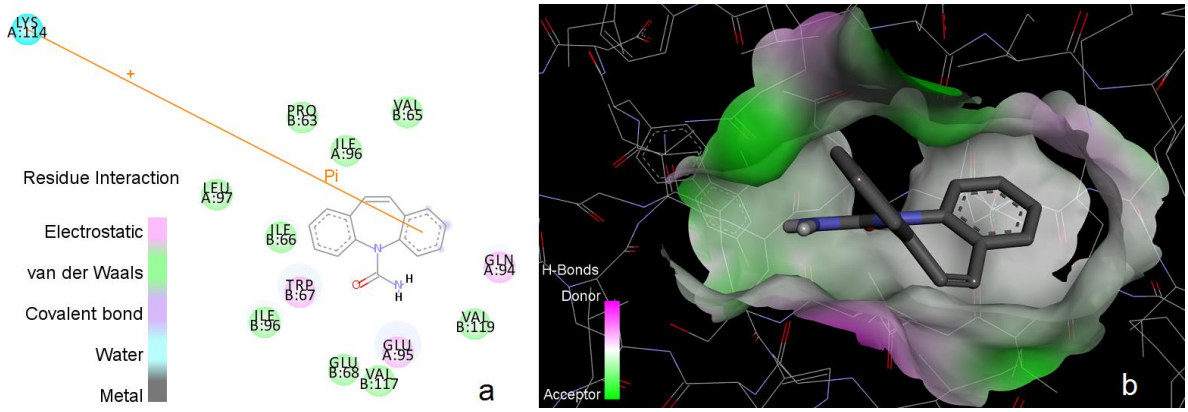


Figure 3. Binding profiles of the carbamazepine. a) 2D structure view. The orange line demonstrates *π-cation* interaction. b) 3D structure view

In Figure 4, it is observed that minocycline, sulfasalazine, and thalidomide are included in the binding package. Although minocycline does not show a similar interaction profile to the literature, it has been shown that sulfasalazine and thalidomide exhibit a similar profile to rhodomyrtone. Accordingly, it was determined that sulfasalazine made *π-cation* interaction and hydrogen bond with LYS 114, while thalidomide made hydrogen bonds with TRP 67.

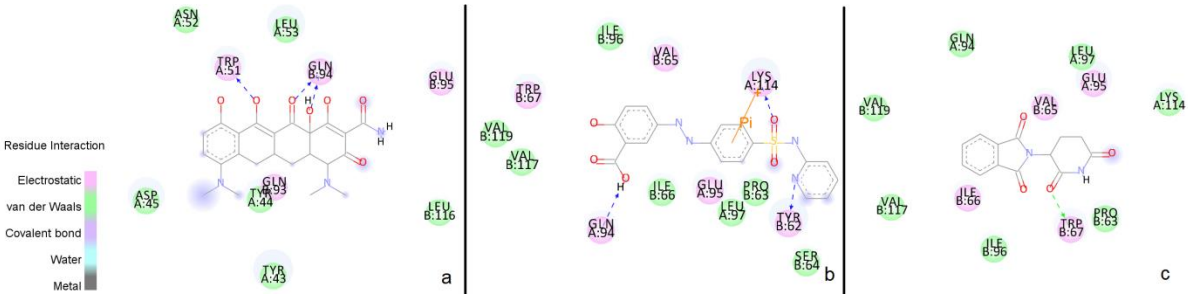


Figure 4. Binding profiles of compounds. a) minocycline b) sulfasalazine c) thalidomide. The orange line demonstrates *π-cation* interaction, blue and green discreet lines demonstrate the hydrogen bond.

In conclusion, the carbamazepine molecule shows the best binding affinity and binding profile with IL-17. It was also revealed that minocycline, sulfasalazine, and thalidomide are tightly packed in the active site. It has been demonstrated that these molecules may be lead molecules for the treatment of AS disease.

ACKNOWLEDGEMENTS

The calculations reported in this paper were partially performed at TUBITAK ULAKBIM, High Performance and Grid Computing Center (TRUBA resources).

AUTHOR CONTRIBUTIONS

Concept: *G.Y.O.*; Design: *G.Y.O.;* Control: *G.Y.O.;* Sources: *G.Y.O.;* Materials: *G.Y.O.;* Data Collection and/or processing: *G.Y.O.;* Analysis and/or interpretation: *G.Y.O.;* Literature review: *G.Y.O.;* Manuscript writing: *G.Y.O.;* Critical review: *G.Y.O.;* Other: *G.Y.O.*.

CONFLICT OF INTEREST

The author declares that there are no apparent financial or personal conflicts of interest that may affect the work in this article.

ETHICS COMMITTEE APPROVAL

The author declares that the ethics committee approval is not required for this study.

REFERENCES

1. Ebrahimiadib, N., Berijani, S., Ghahari, M., Pahlaviani, F.G. (2021). Ankylosing spondylitis. *Journal of Ophthalmic and Vision Research*, *16*(3), 462-469. [CrossRef]

2. Cornell, T. (2004). Ankylosing spondylitis: an overview. *Journal of Professional Nursing*, *19*(8), 431-432.

3. Hwang, M. C., Ridley, L., Reveille, J. D. (2021). Ankylosing spondylitis risk factors: a systematic literature review. *Clinical Rheumatology*, *40*(8), 3079-3093. [CrossRef]

4. Jung, J. H., Bang, C. H., Seok, H., Choi, S. J., Song, G. G. (2019). Clinical findings of Ankylosing Spondylitis with and without Human Leukocyte Antigen (HLA)-B27 and HLA-B51. *Annals of the Academy of Medicine of Singapore*, *48*(10), 321-329.

5. Asquith, M., Sternes, P.R., Costello, M.E., Karstens, L., Diamond, S., Martin, T. M., Li, Z., Marshall, M. S., Spector, T. D., Cao, K. A., Rosenbaum, J. T., Brown, M. A. (2019). HLA Alleles associated with risk of Ankylosing Spondylitis and Rheumatoid Arthritis influence the Gut Microbiome. *Arthritis & Rheumatology*, *71*(10), 1642-1650. [CrossRef]

6. Hu, N., Liu, D., Zhao, N., Wang, X., Bai, Y., Sun, H. (2021). Lack of association between TNF polymorphism and ankylosing spondylitis susceptibility in HLA-B27-positive population: a meta-analysis. *European Spine Journal*, *30*(8), 2401-2408. [CrossRef]

7. Simone, D., Al Mossawi, M. H., Bowness, P. (2018). Progress in our understanding of the pathogenesis of ankylosing spondylitis. *Rheumatology (Oxford)*, *57*(6), 4-9. [CrossRef]

8. Watad, A., Bridgewood, C., Russell, T., Marzo-Ortega, H., Cuthbert, R., McGonagle, D. (2018). The early phases of ankylosing spondylitis: Emerging insights from clinical and basic science. *Frontiers in Immunology***,** *9*(NOV), 1-9. [CrossRef]

9. Garcia-Montoya, L., Gul, H., Emery, P. (2018). Recent advances in ankylosing spondylitis: Understanding the disease and management [version 1; peer review: 2 approved]. *F1000Research*, *7*(0), 1-11. [CrossRef]

10. Pedersen, S.J., Maksymowych, W.P. (2019). The Pathogenesis of Ankylosing Spondylitis: An update. *Current Rheumatology Reports*, *21*(10), 58. [CrossRef]

11. Yang, P., Wan, W., Du, L., Zhou, Q., Qi, J., Liang, L., Wang, C., Wu, L., Kijlstra, A. (2018). Clinical features of HLA-B27-positive acute anterior uveitis with or without ankylosing spondylitis in a Chinese cohort. *British Journal of Ophthalmology*, *102*(2), 215-219. [CrossRef]

12. Choi, E.Y., Lee, M., Lee, C.S. (2020). Uveitis occurrence in patients with ankylosing spondylitis according to the type of tumour necrosis factor inhibitor: a cohort study of 175 patients. *Clinical and Experimental Rheumatology, 38*(6), 1132-1137.

13. The Standardization of Uveitis Nomenclature (SUN) Working Group. (2021). Classification Criteria for Spondyloarthritis/HLA-B27-Associated Anterior Uveitis. *American Journal of Ophthalmology, 228*, 117-125. [CrossRef]

14. Fan, M., Liu, J., Zhao, B., Wu, X., Li, X., Gu, J. (2020). Indirect comparison of NSAIDs for ankylosing spondylitis: Network meta-analysis of randomized, double-blinded, controlled trials. *Experimental and Therapeutic Medicine*, 3031-3041. [CrossRef]

15. Carbo, M. J. G., Spoorenberg, A., Maas, F., Brouwer, E., Bos, R., Bootsma H., Van der Veer, E., Wink, F., Arends, S. (2018). Ankylosing spondylitis disease activity score is related to NSAID use, especially in patients treated with TNF-α inhibitors. *PLoS One*, *13*(4), 1-12. [CrossRef]

16. Yalcin-Ozkat, G., Yildiz, I. (2022). *In silico* studies to develop new GSK3 Inhibitors effective in the Alzheimer's Disease. *Letters in Drug Design & Discovery*, *19*(8), 691-705. [CrossRef]

17. Huylu, B., Yalcin, G. (2022). MS hastalığının tedavisine yönelik Sfingozin-1-Fosfat Reseptör Modülatörlerin geliştirilmesi. *Konya Mühendislik Bilimleri Dergisi, 10*(1), 102-114. [CrossRef]

18. Yalcin, G., Huylu, B. (2022). Development of new Cyclophilin D Receptor Inhibitors for the treatment of Multiple Sclerosis. *Journal of Faculty of Pharmacy of Ankara University*, *46*(2), 458-473. [CrossRef]

19. Osman, M. S., Maksymowych, W. P. (2017). An update on the use of tumor necrosis factor alpha inhibitors in the treatment of ankylosing spondylitis. *Expert Review of Clinical Immunology*, *13*(2), 125-131. [CrossRef]

20. Ma, Z., Liu, X., Xu, X., Jiang, J., Zhou, J., Wang, J., Chen, D., Luo S. (2017). Safety of tumor necrosis factor-alpha inhibitors for treatment of ankylosing spondylitis. *Medicine (Baltimore)*, *96*(25), e7145. [CrossRef]

21. Nigil Haroon, N., Sriganthan, J., Al Ghanim, N., Inman, R. D., Cheung, A. M. (2014). Effect of TNF-alpha inhibitor treatment on bone mineral density in patients with ankylosing spondylitis: A systematic review and meta-analysis. *Semin Arthritis Rheumatology*, *44*(2), 155-161. [CrossRef]

22. Perrotta, F. M., Scriffignano, S., Ciccia, F., Lubrano, E. (2022). Therapeutic targets for Ankylosing Spondylitis – Recent insights and future prospects. *Open Access Rheumatology: Research and Reviews*, *14*(April), 57-66. [CrossRef]

23. Armstrong, A., Fahrbach, K., Leonardi. C, Agustin, M., Neupane, B., Kazmierska, P., Betts, M., Freitag, A., Kiri, S., Taieb, V., Slim, M., Gomez, N. N., Warren, R. B. (2022). Efficacy of Bimekizumab and other biologics in moderate to Severe Plaque Psoriasis : A systematic literature review and a network meta-analysis. *Dermatology and Therapy*, *12*(8), 1777-1792. [CrossRef]

24. Reich, K., Warren, R. B., Lebwohl, M., Gooderham, M., Strober, B., Langley, R. G., Paul, C., Cuyper, D. D., Vanvoorden, V., Madden, C., Cioffi, C., Peterson, L., Blauvelt, A. (2021). Bimekizumab versus Secukinumab in Plaque Psoriasis. *The New England Journal of Medicine*, *385*(2), 142-152. [CrossRef]

25. Low, Z. Y., Farouk, I. A., Lal, S. K. (2020). Drug repositioning: New approaches and future prospects for life-debilitating diseases and the COVID-19 pandemic outbreak. *Viruses*, *12*(9), 1058. [CrossRef]

26. Ngidi, N. T. P., Machaba, K. E., Mhlongo, N. N. (2022). *In Silico* drug repurposing approach: Investigation of Mycobacterium tuberculosis FadD32 targeted by FDA-Approved Drugs. *Molecules*, *27*(3), 668. [CrossRef]

27. Pushpakom, S., Iorio, F., Eyers, P.A., Escott, K. J., Hopper, S., Wells, A., Doig, A., Guilliams, T., Latimer, J., McNamee, C., Norris, A., Sanseau, P., Cavalla, D., Pirmohammed, M. (2018). Drug repurposing: Progress, challenges and recommendations. *Nature Reviews Drug Discovery*, *18*(1), 41-58. [CrossRef]

28. Freshour, S. L., Kiwala, S., Cotto, K.C., Coffman, A. C., McMichael, J. F., Song, J. J., Griffith, (2021). Integration of the Drug-Gene Interaction Database (DGIdb 4.0) with open crowdsource efforts. *Nucleic Acids Research*, *49*(D1), D1144-D1151. [CrossRef]

29. Kim, S., Thiessen, P. A., Bolton, E.E., Chen, J., Fu, G., Gindulyte, A., Han, L., He, J., He, S., Shoemaker, B. A., Wang, J., Yu, B., Zhang, J., Bryant, S. H. (2016). PubChem Substance and Compound databases. *Nucleic Acids Research*, *44*(D1), D1202-D1213. [CrossRef]

30. Trott, O., Olson, A. (2010). AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry*, *31*(2), 455-461. [CrossRef]

31. Steffen, C., Thomas, K., Huniar, U., Hellweg, A., Rubner, O., Schroer, A. (2009). AutoDock4 and AutoDockTools4: Automated Docking with Selective Receptor Flexibility. *Journal of Computational Chemistry*, *31*(16), 2967-2970. [CrossRef]

32. Liu, S., Song, X., Chrunyk, B.A., Shanker, S., Hoth, L. R., Marr, E. S., Griffor, M. C. (2013). Crystal structures of interleukin 17A and its complex with IL-17 receptor A. *Nature Communications*, *4*(May), 1-9. [CrossRef]

33. Berman, H. M., Westbrook, J., Feng, Z., Gilliand, G., Bhat, T. N., Weissig, H., Shindyalov, I., N., Bourne, P. E. (2000). The Protein Data Bank. *Nucleic Acids Re*search, *28*(1), 235-242. [CrossRef]

34. Tian, W., Chen, C., Lei, X., Zhao, J., Liang, J. (2018). CASTp 3.0 : Computed atlas of surface topography of proteins. *Nucleic Acids Research*, *46*(June), 363-367. [CrossRef]

35. Hassan, N. M., Alhossary, A. A., Mu, Y., Kwoh, C. K. (2017). Protein-Ligand Blind Docking Using QuickVina-W with Inter-Process Spatio-Temporal Integration. *Scientific Rep*orts, *7*(1), 1-13. [CrossRef]

36. Verma, A. K., Hossain, M. S., Ahmed, S. F., Hussain, N., Ashid, M., Upadhyay, S. K., Vıshakarma, N. K., Bhojiya A. A., Srivastava, S. K. (2022). In silico identification of ethoxy phthalimide pyrazole derivatives as IL-17A and IL-18 targeted gouty arthritis agents. *Journal of Biomolecular Structure and Dynamics*, 1-15. [CrossRef]

37. Dassault Systèmes. (2019). *Discovery Studio Visualizer* (No. 2019). BIOVIA.

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 909-919, 2022
Doi: 10.33483/jfpau.1124829

ORIGINAL ARTICLE / ÖZGÜN MAKALE

THE BENEFICIAL EFFECT OF INTRACAVERNOSAL INJECTION OF SILDENAFIL ON ERECTILE DYSFUNCTION IN DUTASTERIDE TREATED RATS

İNTRAKAVERNOSAL SİLDENAFİLİN SIÇANLARDA DUTASTERID TEDAVİSİ SONRASI GELİŞEN EREKTIL DİSFONKSİYON ÜZERİNE YARARLI ETKİSİ

Didem YILMAZ-ORAL[1,2], Serap GUR[2*]

[1]Cukurova University, Faculty of Pharmacy, Department of Pharmacology, 01330, Adana, Turkey

[2]Ankara University, Faculty of Pharmacy, Department of Pharmacology, Ankara, Turkey

ABSTRACT

Objective: *Benign prostatic hyperplasia (BPH) and erectile dysfunction (ED) are the most common illnesses in aged male patients. 5α-reductase inhibitors (5-ARIs) are suggested for the treatment of BPH. Furthermore, the association of 5ARIs with ED has been indicated. This study aimed to investigate the effect of intracavernosal injection of sildenafil on ED in 5ARI treated rats.*

Material and Method: *Sprague-Dawley rats (n=30) were divided into three groups: Control; 10-week dutasteride treatment (0.5 mg/rat/day); and 6-week durasteride treatment followed by a 4-week washout period. In vivo erectile responses were assessed before and after intracavernosal injection of sildenafil (0.3mg/kg/rat). The relaxant and contractile responses of isolated corpus cavernosum were evaluated in in vitro organ bath.*

Result and Discussion: *Prostate weight decreased after 10-week dutasteride treatment. In vivo erectile responses, endothelial and nitrergic relaxation responses were decreased in dutasteride groups. The washout period moderately normalized erectile responses. The intracavernosal injection of sildenafil increased erectile function in treatment groups. Contractile responses were augmented in 10-week dutasteride treated rats. The cessation of the treatment did not alter erectile function as well as endothelial relaxation and nitrergic relaxation. Also, intracavernosal sildenafil caused an improvement in 5ARI treatment-induced ED.*

Keywords: *5-alpha reductase inhibitor, corpus cavernosum, dutasteride, erectile dysfunction, sildenafil.*

ÖZ

Amaç: *Benign prostat hiperplazisi (BPH) ve erektil disfonksiyon (ED), yaşlı erkek hastalarda en sık görülen hastalıklardır. BPH tedavisi için 5α-redüktaz inhibitörleri (5-ARI) önerilmektedir. Ayrıca, 5ARI'lerin*

* **Corresponding Author / Sorumlu Yazar:** Serap Gür
e-mail / e-posta: serapgur@ankara.edu.tr, **Phone / Tel.:** +903122033137

Submitted / Gönderilme: 03.06.2022 **Accepted / Kabul:** 16.08.2022

ED ile ilişkisi belirtilmiştir.Bu çalışmada, 5ARI tedavili sıçanlarda gelişen ED üzerine intrakavernozal sildenafil enjeksiyonunun etkisinin araştırılması amaçlanmıştır.

Gereç ve Yöntem: *Sprague-Dawley sıçanlar (n=30) üç gruba ayrılmıştır: Kontrol; 10 haftalık dutasterid tedavili (0.5mg/kg/gün); 6 haftalık dutasterid tedavili ve 4 hafta tedavisiz. İn vivo erektil yanıtlar, sildenafilin intrakavernozal enjeksiyonundan (0.3 mg/kg) önce ve sonra değerlendirilmiştir. İzole korpus kavernozum dokularının gevşeme ve kasılma yanıtları in vitro olarak organ banyosunda değerlendirildi.*

Sonuç ve Tartışma: *10 haftalık dutasterid tedavisinden sonra prostat ağırlığı azalmıştır. Dutasterid gruplarında in vivo erektil yanıtlar, endotelyal ve nitrerjik gevşeme yanıtları azalmıştır. Tedavinin kesilmesi azalan erektil yanıtları kısmen geri döndürmüştür. Sildenafilin intrakavernozal enjeksiyonu, tedavi gruplarında erektil yanıtları artırmıştır. 10 hafta dutasterid ile tedavi edilen sıçanlarda kontraktil yanıtlar artmıştır. Tedavinin kesilmesi, erektil fonksiyonun yanı sıra endotelyal ve nitrerjik gevşemeyi iyileştirmemiştir. Ayrıca, intrakavernozal sildenafil, 5ARI tedavisinin neden olduğu ED'de bir iyileşmeye neden olmuştur.*

Anahtar Kelimeler: *5-alfa redüktaz inhibitörü, dutasterid, erektil disfonksiyon, korpus kavernozum, sildenafil.*

INTRODUCTION

Benign prostatic hyperplasia (BPH), one of the most mutual conditions in aged men, is likely to induce lower urinary tract symptoms (LUTS) which is linked to a decrement in the quality of life [1, 2]. In addition, the prevalence of BPH rises with age that affects 75% of men over 50 years of age [2, 3].

5α-reductase inhibitors (5-ARIs), α1-adrenoceptor antagonists and phosphodiesterase-5 inhibitors (PDE5Is) are suggested for the treatment of BPH/LUTS [4]. Prostate growth is induced by dihydrotestosterone (DHT), which is converted from testosterone via 5-AR [5]. Dutasteride, a 5-ARI, is frequently used as a treatment option for BPH [6]. Dutasteride blocks 5-AR enzyme resulting in a decrement in serum, intraprostatic concentration of DHT and prostate volume [6-8]. Previous data have confirmed that 5-ARIs induce side effects on sexual function, especially erectile dysfunction (ED) [9, 10]. Furthermore, in preclinical data, dutasteride treatment induced ED in rats, even after withdrawal of dutasteride [11, 12]. In addition, PDE5Is are the preferred choice, even though there are conflicting results [13-15]. Concomitant treatment with 5ARI and PDE5Is prevented ED [14, 16]. Sildenafil is a highly selective PDE5Is and the most used drug for ED [17, 18]. On the other hand, a rat model of BPH treated with sildenafil displayed a decrease in corporal smooth muscle content compared to the BPH group [13].

To the best of our knowledge, studies assessing the acute effect of sildenafil on 5ARI-induced ED are lacking. The aim of this study was to examine the effect of intracavernosal sildenafil injection on ED after 6-week and 10-week dutasteride treatment, as well as to evaluate *in vitro* relaxant and contractile responses in the corpus cavernosum (CC).

MATERIAL AND METHOD

Drugs

All drugs were purchased from Sigma Chemical Co (St. Luis, MO) except dutasteride (Avodart,

GlaxoSmithKline) and sildenafil tablets (Viagra, Pfizer).

Animals

Adult male Sprague-Dawley rats (n=30, ten weeks old, 295.9±5.8 g) were purchased from Bilkent University (Ankara, Turkey). Rats were randomly divided into three groups; control (n=10), 10 weeks of dutasteride treatment (n=10), and 6 weeks of dutasteride treatment followed by 4 weeks of washout (n=10). As in previous studies [11, 12, 19], dutasteride was administered in drinking water (0.5 mg/rat/day). Dutasteride treatment was performed during 6 and 8 weeks in a previous study [19]. However, the alterations of erectile responses were partially restored in the rats treated with dutasteride after 2 weeks of washout period [19]. Therefore, we performed 4 weeks of washout period instead of 2 weeks.

All experimental procedure was approved by Ankara University Local Ethics Committee of Animal Experiments (approval no: 2015–16-185). The rats were housed individually in artificially lit rooms (from 7:00 a.m. to 7:00 p.m.) with food and water ad libitum under controlled temperature (22 ± 1 °C).

The body weights of all rats were calculated via a precision scale before the sacrifice of animals. The penis and prostate tissues were excised and weighted by an electronic scale after sacrifice.

***In vivo* erectile function measurement**

Erectile responses were measured by cavernosal nerve (CN) stimulation and monitoring electric stimulation-induced intracavernous pressure (ICP, mmHg) and main arterial pressure (MAP, mmHg). After ten weeks, in anesthetized rats (ketamine/xylazine; 100/10 mg/kg, i.p.) [20], polyethylene-50 tubing was inserted into the right crura of the penis and the carotid artery to measure ICP and MAP with a transducer (Statham, Oxnard, CA, USA) and a data acquisition system (Biopac MP 100 System, Santa Barbara, CA, USA). Following the detection of cavernosal nerve (CN), the CN was stimulated (2.5, 5, and 7.5 V, 15 Hz, 1 ms pulse width) with a stainless-steel bipolar-hook stimulating electrode and a square pulse stimulator (Grass Instruments, Quincy, MA) [12]. After intracavernosal injection of sildenafil (0.3 mg/kg) [21-23], the measurements were repeated in dutasteride-treated animals. Sildenafil was administered via intracavernosal injection to eliminate the blood pressure-lowering effect [23, 24].

Organ Bath Experiments

Organ bath experiments were performed to measure the relaxation and contractile responses in isolated CC. Following *in vivo* experiments, the rats were killed under anesthesia (ketamine/xylazine; 100/10 mg/kg, i.p.) [20], and CC tissues were isolated for organ bath experiments. After the isolation, the CC (1 × 1 × 8 mm) was placed under a resting tension (1g) within organ bath system containing Krebs-Ringer bicarbonate solution (containing mmol/L: NaCl 118.1, $CaCl_2$ 2.5, KCl 4.7, $NaHCO_3$ 25.0, $MgSO_4$ 1.0, KH_2PO_4 1.0, glucose 11.1) at 7.4 pH and 37 °C with 95%O_2/5%CO_2. The isolated strips

were attached to platinum electrodes (Grass Instruments, Quincy, MA, USA). Electrical field stimulation (EFS) of the autonomic nerves (15 seconds duration; amplitude 40-90 V; frequency 1-40 Hz; pulse width 5 ms) was executed with platinum electrodes (Grass Instruments, Quincy, MA). All tension alterations were noted via an isometric force transducer attached to a PC-based data acquisition system (Biopac System, St. Barbara, CA, USA). After 60 minutes, endothelium-dependent relaxant response to acetylcholine (ACh, 10^{-8}-10^{-3} M), nitrergic relaxant response to EFS (1- 20 Hz), endothelium-independent relaxant response to sodium nitroprusside (SNP, 10^{-8}-10^{-3} M) and a PDE5I, sildenafil (10^{-8}-10^{-4} M)-caused relaxant responses were obtained following precontraction of CC strips with phenylephrine (Phe, 10μM). Alpha- adrenergic agonist, Phe (10^{-8} to 10^{-3}M), EFS-induced neurogenic contraction (1–40 Hz), and KCl (60 mM)-caused contractile responses in CC strips were performed and standardized to tissue weight grams [20, 25].

Data analysis

The findings were analyzed by Prism v.4 (GraphPad Software, San Diego, CA, USA) and shown as mean± standard error of the mean (SEM). Multiple groups were compared via one-way analysis of variance (ANOVA) with post hoc Bonferroni analysis. A value of $p<0.05$ was considered to be statistically significant.

RESULT AND DISCUSSION

The weight of body and penis did not change in groups (Table 1). Furthermore, the weight of prostate in 10-week treated rats was lower ($p<0.001$) than in controls, which was partially returned after the discontinuation ($p<0.05$ vs. controls, Table 1). Similarly, previous studies have shown a decrease in prostate weight [26] as well as no alteration in body and penis weights [19].

Table 1. Body, penis and prostate weights of the control, 10-week and 6-week dutasteride groups

	Control	**Dutasteride-10 week**	**Dutasteride-6 week+4 week w/o**
Body weight (g)	418.3±13.6	401.2±10.9	414.1±7.9
Penis weight (g)	0.39±0.04	0.34±0.02	0.38±0.02
Prostate weight (g)	1.0±0.1	0.5±0.1 ***	0.8±0.1*

Values are the mean ± SEM from per group (n=10). *p < 0.05, ***p < 0.001 vs control.

Rats in the 10-week dutasteride group displayed reduced ICP/MAP ($p<0.001$ at 7.5V) and ICP ($p<0.01$ at 7.5V) values compared to controls (Figure 1A and 1B). In addition, the decrease in ICP/MAP and ICP was partially returned after the washout period, but there was no significant difference between the 10-week and 6-week dutasteride groups (Figure 1A and 1B). Likewise, earlier data demonstrated that 4 and 8 weeks of

dutasteride treatment induced a decline in erectile response, and also, the washout period did not alter the erectile response [11]. Furthermore, after intracavernosal injection of sildenafil, an increase in erectile responses was noted in all dutasteride treated groups (Figure 1A and 1B). PDE5Is have been involved in European Association of Urology (EUA) guidelines for the treatment for LUTS. The underlying mechanism of PDE5I on LUTS stays unclear, and also, it is likely to be dependent on increasing nitric oxide/intracellular cyclic guanosine monophosphate resulting in decreasing muscle tone of the detrusor, urethra and prostate as well as enhancing oxygenation and blood perfusion in the lower urinary tract [27]. Furthermore, PDE5I has good clinical outcomes for 5ARIs-linked ED [14, 16]. Moreover, Munk et al. indicated that the efficacy of the combined treatment of PDE5I plus 5-ARIs is unclear and needs further research [28]. However, other data reported PDE5Is were not sufficient to treat ED in hypogonadal men [29]. Testosterone is a critical component for maintaining erectile function. Indeed, castration caused ED and diminished the effectiveness of PDE5I in rabbits [30]. Also, hypertension-induced BPH and dutasteride treatment induced detrimental morphological changes in rat penis, which was not improved by coadministration of sildenafil [13]. Unlike the castrate model or different BPH models, dutasteride treatment may conserve both testosterone and DHT which can preserve the expression of PDE5 and the activity of PDE5Is.

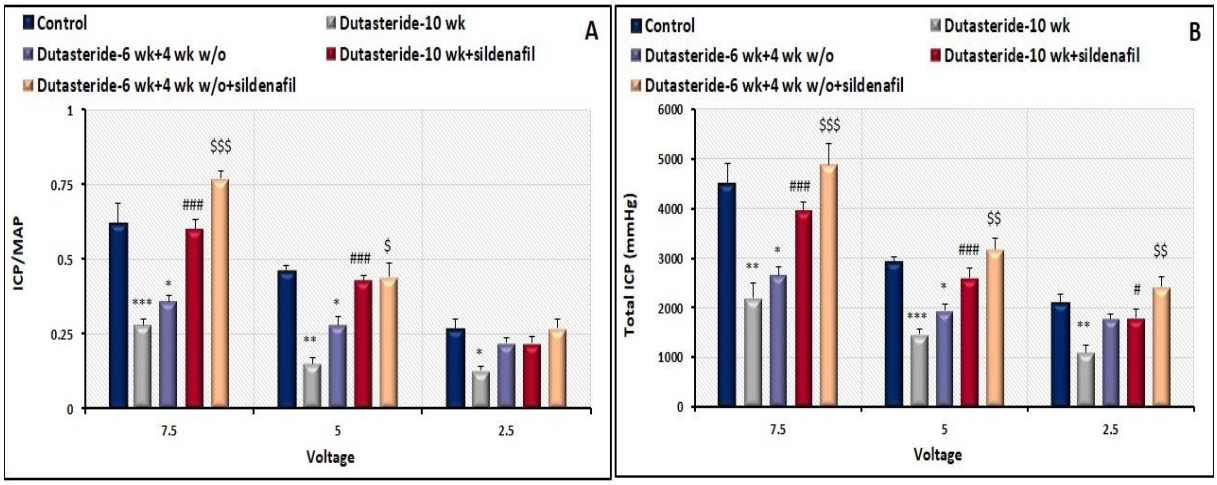


Figure 1. The effect of sildenafil on *in vivo* erectile function in all groups. Bar graphs are presenting ICP/MAP (A) and total ICP (B). Data are shown as the mean ± SEM (n=8-10). *p<0.05, **p<0.01, ***p<0.001 vs. control; # p<0.05, ###p<0.001 vs. 10-week dutasterid treated group; $ p<0.05, $$ p<0.01, $$$ p<0.001 vs. 6-week dutasterid treated group.

ACh-induced endothelium-dependent relaxation responses in both dutasteride treated rats were lower than in controls (p<0.001, Figure 2A). A previous study showed ACh-induced dose response curve shifted right without an alteration in the maximum response after 4-week dutasteride treatment [31]. In addition, the relaxation response to ACh was lowered in the 8-week dutasteride and 2-week

washout groups [19]. Furthermore, the endothelium-dependent relaxation responses were lower after both 8-week and 12-week dutasteride treatment [12]. According to the results, the reduction in the endothelium-dependent relaxation response is likely to be associated with a decreased in free and total T levels following 5-ARIs treatment [12].

EFS-caused relaxant response was considerably decreased in 10-week and 6-week dutasteride treatment groups ($p<0.001$ vs. controls), except 1 and 5V (Figure 2B). Similarly, earlier data indicated the reduction in both EFS-induced relaxation response and normal penile erection after 5ARI treatment [31]. Moreover, Oztekin et al. [19] showed that the neurogenic relaxant responses were decreased after dutasteride treatment.

The endothelium-independent relaxant response to SNP did not change in all groups (Figure 2C). Pinsky et al. [31] displayed no differences in SNP-induced relaxant responses between control and 4-week dutasteride treated rats. However, another study showed that relaxation responses to SNP were decreased after 12-week dutasteride treatment [12]. This difference was most probably due to the duration of dutasteride treatment.

Sildenafil-induced relaxation response did not change (Figure 2D). A previous study also indicated no differences in sildenafil dose-response curve between control and 4-week dutasteride treated groups [31]. Nevertheless, sildenafil-induced relaxation responses at 1 and 10 μM dosages in the 12-week dutasteride treated group was reduced compared to controls without alteration in the maximum relaxation response [12].

Phe-induced contractile responses at 100 μM and 1 mM were increased in 10-week dutasteride-treated rats (Figure 3A, $p<0.01$ vs. control). EFS-induced neurogenic contractile responses in 10-week dutasteride-treated rats were considerably increased at 15-40 Hz compared to controls ($p<0.05$, Figure 3B). Also, the increment in contractile responses was decreased after the washout period (Figure 3A-B). KCl-induced contractile responses were not different among groups (Figure 3C). Similarly, 4-week and 12-week dutasteride treatment regimens augmented α1-adrenergic agonist and neurogenic-induced contractile responses [12, 31]. Moreover, Phe-caused contraction in castrated rats was greater than in controls [32]. The current results can indicate that 5ARI treatment increased vasoconstrictor responses in the penile tissue resulting in ED.

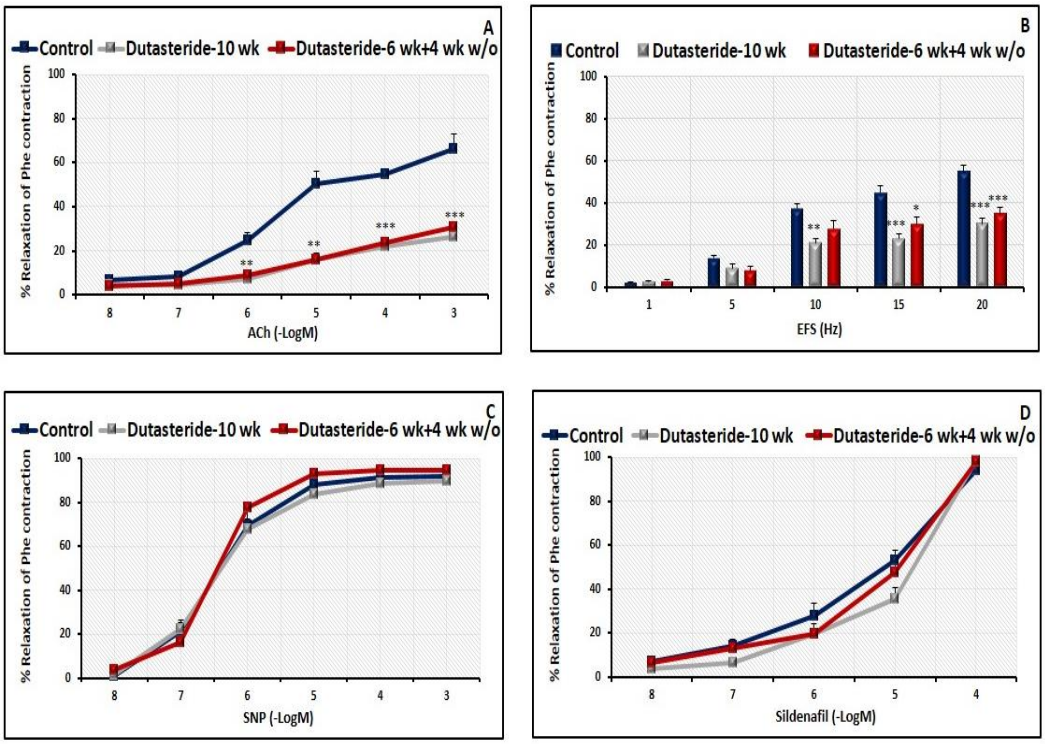


Figure 2. Dose-response curves in the isolated cavernosal strips to ACh (10^{-8}–10^{-3} M, A), EFS (1–20 Hz frequency, B), SNP (10^{-8}–10^{-3} M, C), sildenafil (10^{-8}–10^{-4} M, D)-caused relaxation responses. Data are mean ± SEM (n = 8-10). *p < 0.05, **p < 0.01, ***p < 0.001 vs. control group (ANOVA, Bonferroni post hoc).

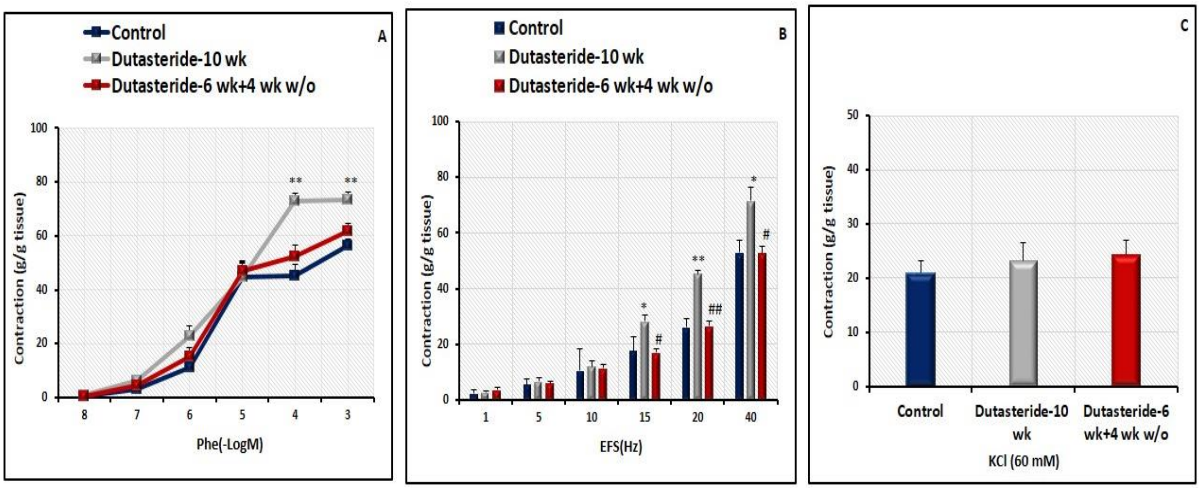


Figure 3. Dose-response curves in the isolated cavernosal strips to Phe (10^{-8}–10^{-3} M, A), EFS (1–40 Hz frequency, B) and KCl (60mM, C)-induced contractile responses. Data are mean ± SEM (n = 8-10). *p < 0.05, **p < 0.01 vs. control group; # p<0.05, ##p<0.01 vs. 10-week dutasterid treated group (ANOVA, Bonferroni post hoc).

In conclusion, the current study showed that treatment with dutasteride caused decreasing endothelial and nitrergic relaxant responses and increasing contractile responses resulting in ED in rats. The alterations in erectile function and relaxation responses except contractile responses were not ameliorated after the cessation. The cavernous injection of sildenafil improved erectile function, and induced the relaxation of the penile tissue in dutasteride treated rats. Further preclinical and clinical studies are needed to expand the knowledge of the combination treatment of sildenafil and 5ARIs which is a favorable alternative for the management of BPH and ED.

AUTHOR CONTRIBUTIONS

Concept: *D.Y.O., S.G.*; Design: *D.Y.O., S.G.*.; Control: *S.G., D.Y.O* ; Sources: *S.G., D.Y.O*; Materials: *D.Y.O., S.G.*; Data Collection and/or processing: *D.Y.O.*; Analysis and/or interpretation: *D.Y.O, S.G.*; Literature review: *D.Y.O, S.G.*; Manuscript writing: *D.Y.O, S.G*; Critical review: *D.Y.O, S.G.*; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

All experimental procedure of the animals was approved by the Ethics Committee of Ankara University (approval no: 2015–16-185).

REFERENCES

1. Ng, M., Baradhi, K.M. (2022). *Benign Prostatic Hyperplasia*, in *StatPearls*. Treasure Island (FL).

2. Rowhrborm, C., McConnell, J.D. (2002). Etiology, pathophysiology, epidemiology and natural history of benign prostatic hyperplasia. *Campbell's Urology*, *37*, p. 1297-1330.

3. Committee, A.U.A.P.G. (2003). AUA guideline on management of benign prostatic hyperplasia (2003). Chapter 1: Diagnosis and treatment recommendations. *Journal of Urology*, *170*(2 Pt 1), 530-547.

4. La Vignera, S., Aversa, A., Cannarella, R., Condorelli, R.A., Duca, Y., Russo, G.I., Calogero, A.E. (2021). Pharmacological treatment of lower urinary tract symptoms in benign prostatic hyperplasia: consequences on sexual function and possible endocrine effects. *Expert Opinion on Pharmacotherapy*, *22*(2), 179-189. [CrossRef]

5. Goldenberg, L., So, A., Fleshner, N., Rendon, R., Drachenberg, D., Elhilali, M. (2009). The role of 5-alpha reductase inhibitors in prostate pathophysiology: Is there an additional advantage to inhibition of type 1 isoenzyme? *Canadian Urology Association Journal*, *3*(3 Suppl 2), 109-114. [CrossRef]

6. Kim, E.H., Brockman, J.A., Andriole, G.L. (2018). The use of 5-alpha reductase inhibitors in the treatment of benign prostatic hyperplasia. *Asian Journal of Urology*, *5*(1), 28-32. [CrossRef]

7. Shigehara, K., Miyagi, T., Nakashima, T., Izumi, K., Kitagawa, Y., Mizokami, A., Koh, E., Shimamura, M., Namiki, M. (2016). Effects of dutasteride on lower urinary tract symptoms: A prospective analysis based on changes in testosterone/dihydrotestosterone levels and total prostatic volume reduction. *Aging Male*, *19*(2), 128-33. [CrossRef]

8. Marberger, M. (2006). Drug insight: 5alpha-reductase inhibitors for the treatment of benign prostatic hyperplasia. *National Clinical Practice Urology*, *3*(9), 495-503. [CrossRef].

9. Corona, G., Tirabassi, G., Santi, D., Maseroli, E., Gacci, M., Dicuio, M., Sforza, A., Mannucci, E., Maggi, M. (2017). Sexual dysfunction in subjects treated with inhibitors of 5alpha-reductase for benign prostatic hyperplasia: a comprehensive review and meta-analysis. *Andrology*, *5*(4), 671-678. [CrossRef]

10. Gur, S., Kadowitz, P.J., Hellstrom, W.J. (2013). Effects of 5-alpha reductase inhibitors on erectile function, sexual desire and ejaculation. *Expert Opinion on Drug Safety*, *12*(1), 81-90. [CrossRef]

11. Sung, H.H., Yu, J., Kang, S.J., Chae, M.R., So, I., Park, J.K., Lee, S.W. (2019). Persistent Erectile Dysfunction after discontinuation of 5-Alpha Reductase Inhibitor therapy in rats depending on the duration of treatment. *The World Journal of Men's Health*, *37*(2), 240-248. [CrossRef]

12. Oztekin, C.V., Yilmaz-Oral, D., Kaya-Sezginer, E., Kirlangic, O.F., Ozen, F.Z., Ozdal, B., Topcu, H.O., Gur, S. (2021). Beneficial effects of Human Umbilical Cord Blood Mononuclear Cells on Persistent Erectile Dysfunction after treatment of 5-Alpha Reductase Inhibitor in Rats. *The Journal of Sexual Medicine*, *18*(5), 889-899. [CrossRef]

13. Da Silva, M.H.A., Medeiros, J.L., Jr., Costa, W.S., Sampaio, F.J.B., De Souza, D.B. (2020). Effects of the dutasteride and sildenafil association in the penis of a benign prostatic hyperplasia animal model. *Aging Male*, *23*(5), 1009-1015. [CrossRef]

14. Roehrborn, C.G., Casabe, A., Glina, S., Sorsaburu, S., Henneges, C., Viktrup, L. (2015). Treatment satisfaction and clinically meaningful symptom improvement in men with lower urinary tract symptoms and prostatic enlargement secondary to benign prostatic hyperplasia: Secondary results from a 6-month, randomized, double-blind study comparing finasteride plus tadalafil with finasteride plus placebo. *International Journal of Urology*, *22*(6), 582-587. [CrossRef]

15. Watanabe, D., Yamashita, A., Miura, K., Mizushima, A. (2020). Effects on sexual function in Japanese patients with benign prostatic hyperplasia upon switching from combination therapy with alpha1 blocker and dutasteride to combination therapy with tadalafil and dutasteride. *Aging Male*, *23*(5), 501-506. [CrossRef]

16. Serati, M., Andersson, K.E., Dmochowski, R., Agro, E.F., Heesakkers, J., Iacovelli, V., Novara, G., Khullar, V.Chapple, C. (2019). Systematic review of combination drug therapy for Non-neurogenic Lower Urinary Tract Symptoms. *European Urology*, *75*(1), 129-168. [CrossRef]

17. Scaglione, F., Donde, S., Hassan, T.A., Jannini, E.A. (2017). Phosphodiesterase Type 5 Inhibitors for the treatment of Erectile Dysfunction: Pharmacology and Clinical Impact of the Sildenafil Citrate Orodispersible tablet formulation. *Clinical Therapeutics*, *39*(2), 370-377. [CrossRef]

18. Goldstein, I., Lue, T.F., Padma-Nathan, H., Rosen, R.C., Steers, W.D., Wicker, P.A. (1998). Oral sildenafil in the treatment of erectile dysfunction. Sildenafil Study Group. *The New England Journal of Medicine*, *338*(20), 1397-1404 [CrossRef]

19. Oztekin, C.V., Gur, S., Abdulkadir, N.A., Lokman, U., Akdemir, A.O., Cetinkaya, M., Hellstrom, W.J. (2012). Incomplete recovery of erectile function in rat after discontinuation of dual 5-alpha reductase inhibitor therapy. *The Journal of Sexual Medicine*, *9*(7), 1773-1781. [CrossRef]

20. Yilmaz-Oral, D., Kaya-Sezginer, E., Oztekin, C.V., Bayatli, N., Lokman, U., Gur, S. (2020). Evaluation of combined therapeutic effects of hydrogen sulfide donor sodium hydrogen sulfide and phosphodiesterase type-5 inhibitor tadalafil on erectile dysfunction in a partially bladder outlet obstructed rat model. *Neurourology and Urodynamics*, *39*(4), 1087-1097. [CrossRef]

21. Oger-Roussel, S., Behr-Roussel, D., Caisey, S., Kergoat, M., Charon, C., Audet, A., Bernabe, J., Alexandre, L.Giuliano, F. (2014). Bladder and erectile dysfunctions in the Type 2 diabetic Goto-Kakizaki rat. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *306*(2), R108-117. [CrossRef]

22. Assaly, R., Gorny, D., Compagnie, S., Mayoux, E., Bernabe, J., Alexandre, L., Giuliano, F., Behr-Roussel, D. (2018). The Favorable Effect of Empagliflozin on Erectile Function in an Experimental Model of Type 2 Diabetes. *The Journal of Sexual Medicine*, *15*(9), 1224-1234. [CrossRef]

23. Tian, L., Xin, Z.C., Yuan, Y.M., Fu, J., Liu, W.J., Wang, L.L. (2004). Effects of icariin on intracavernosal pressure and systematic arterial blood pressure of rat. *Zhonghua Yi Xue Za Zhi*, *84*(2), 142-145.

24. McAuley, I.W., Kim, N.N., Min, K., Goldstein, I.Traish, A.M. (2001). Intracavernosal sildenafil facilitates penile erection independent of the nitric oxide pathway. *Journal of Andrology*, *22(4)*, 623-628. [CrossRef]

25. Cengiz, T., Kaya, E., Oral, D.Y., Ozakca, I., Bayatli, N., Karabay, A.Z., Ensari, T.A., Karahan, T., Yilmaz, E.Gur, S. (2017). Intracavernous Injection of Human Umbilical Cord Blood Mononuclear Cells Improves Erectile Dysfunction in Streptozotocin-Induced Diabetic Rats. *The Journal of Sexual Medicine*, *14*(1), 50-58. [CrossRef]

26. Da Silva, M.H.A., Costa, W.S., FJ, B.S., De Souza, D.B. (2018). The corpus cavernosum after treatment with dutasteride or finasteride: A histomorphometric study in a benign prostatic hyperplasia rodent model. *Asian Journal of Andrology*, *20*(5), 505-510. [CrossRef]

27. Gacci, M., Corona, G., Salvi, M., Vignozzi, L., McVary, K.T., Kaplan, S.A., Roehrborn, C.G., Serni, S., Mirone, V., Carini, M., Maggi, M. (2012). A systematic review and meta-analysis on the use of phosphodiesterase 5 inhibitors alone or in combination with alpha-blockers for lower urinary tract symptoms due to benign prostatic hyperplasia. *European Urology*, *61*(5), 994-1003. [CrossRef]

28. Munk, N.E., Knudsen, J.S., Comerma-Steffensen, S., Simonsen, U. (2019). Systematic review of Oral Combination Therapy for Erectile Dysfunction when Phosphodiesterase Type 5 Inhibitor Monotherapy fails. *Sexual Medicine Reviews*, *7*(3), 430-441. [CrossRef]

29. Gooren, L.J., Saad, F. (2006). Recent insights into androgen action on the anatomical and physiological substrate of penile erection. *Asian Journal of Andrology*, *8*(1), 3-9. [CrossRef]

30. Traish, A.M., Park, K., Dhir, V., Kim, N.N., Moreland, R.B., Goldstein, I. (1999). Effects of castration and androgen replacement on erectile function in a rabbit model. *Endocrinology*, *140*(4), 1861-1868. [CrossRef]

31. Pinsky, M.R., Gur, S., Tracey, A.J., Harbin, A., Hellstrom, W.J. (2011). The effects of chronic 5-alpha-reductase inhibitor (dutasteride) treatment on rat erectile function. *The Journal of Sexual Medicine*, *8*(11), 3066-3074. [CrossRef]

32. Reilly, C.M., Stopper, V.S., Mills, T.M. (1997). Androgens modulate the alpha-adrenergic responsiveness of vascular smooth muscle in the corpus cavernosum. *Journal of Andrology*, *18*(1), 26-31.

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 920-930, 2022
Doi: 10.33483/jfpau.1127275

ORIGINAL ARTICLE / ÖZGÜN MAKALE

SPECTROPHOTOMETRIC METHOD DEVELOPMENT AND VALIDATION FOR GLICLAZIDE QUANTITATION IN TABLETS

TABLETLERDE GLİKLAZİT MİKTAR TAYİNİ İÇİN SPEKTROFOTOMETRİK YÖNTEM GELİŞTİRME VE DOĞRULAMA

Lidiia LELEKA[1*], Svitlana VASIUK[2]

[1] Zaporizhzhia State Medical University, Pharmaceutical Faculty, Analytical Chemistry Department, 69035, Zaporizhzhia, Ukraine

[2] Zaporizhzhia State Medical University, Pharmaceutical Faculty, Analytical Chemistry Department, 69035, Zaporizhzhia, Ukraine

ABSTRACT

Objective: *A new spectrophotometric method for the gliclazide quantitation in dosage forms has been developed*

Material and Method: *The subjects of the study were modified-release tablets ("Diaglizide" 80 mg, "Diaglizide MR" 60 mg, "Diaglizide MR" 30 mg, "Diabeton" MR 60 mg, "Gliklada" 60 mg). As a reagent, bromocresol green in acetone was used. Analytical equipment: spectrophotometer Specord 200, electronic scales ABT-120-5DM.*

Result and Discussion: *It has been experimentally determined that gliclazide reacts with bromocresol green in acetone medium at room temperature to form a yellow product with maximum absorption at 411 nm. The method was validated for linearity, accuracy, precision, and robustness. The agent's optimal concentration was established and the stability of the investigated solutions was checked by measuring their optical density for 30 minutes. Subordination of Beer's law is observed in the range of 62.00 - 94.00 mg/100 ml. The limit of detection is $4.02 \cdot 10^{-6}$ g/ml, which indicates a high sensitivity of the reaction. The proposed method is validated according to the requirements of the State Pharmacopoeia of Ukraine. The results of the study show that the developed method is simple and affordable to implement and can be used to determine gliclazide in drugs in laboratories for quality control of dosage forms.*

Keywords: *Bromocresol green, gliclazide, spectrophotometry.*

ÖZ

[*] **Corresponding Author / Sorumlu Yazar:** Lidiia Leleka
e-mail / e-posta: demyanova0610@gmail.com, **Phone / Tel.:** +380674387005

Submitted / Gönderilme: 07.06.2022 **Accepted / Kabul:** 17.08.2022

Amaç: *Dozaj formlarında gliklazid miktar tayini için yeni bir spektrofotometrik yöntem geliştirilmiştir.*

Gereç ve Yöntem: *Gliklazidin oda sıcaklığında aseton ortamında bromokresol yeşili ile reaksiyona girerek 411 nm'de maksimum absorpsiyona sahip sarı bir ürün oluşturduğu deneysel olarak belirlenmiştir. Kimyasal ajanın optimal konsantrasyonu belirlendi ve araştırılan çözeltilerin stabilitesi, 30 dakika boyunca optik yoğunlukları ölçülerek kontrol edildi ve 62.00-94.00 mg/100 ml aralığında Beer yasasına uygun olduğu görüldü. Tespit limiti, reaksiyonun yüksek hassasiyetini gösteren 4.02~10-6 g/ml'dir. Önerilen yöntem, Ukrayna Devlet Farmakopesi gerekliliklerine göre doğrulanmıştır.*

Sonuç ve Tartışma: *Çalışmanın sonuçları, geliştirilen yöntemin uygulamasının basit ve ekonomik olduğunu ve dozaj formlarının kalite kontrolü için laboratuvarlarda ilaçlarda gliklazidin belirlenmesinde kullanılabileceğini göstermektedir.*

Anahtar Kelimeler: *Bromokresol yeşili, gliklazid, spektrofotometri*

INTRODUCTION

Nowadays, diabetes is one of the most common issues. As of 2019, the number of patients with type 2 diabetes was 463 million people. However, according to the IDF, by 2030 the number of patients will increase to 578 million, and by 2045 – to 700 million [1]. Therefore, there is a need to develop new and improved existing methods of hypoglycemic drug analysis.

One of the effective drugs for type 2 diabetes treatment is gliclazide 1-(Hexahydrocyclopenta[c]pyrrol-2(1H)-yl)-3-[(4-methylphenyl)sulfonyl]urea, which is a derivative of sulfonylurea (Figure 1). It lowers plasma glucose levels by stimulating insulin secretion by pancreatic β-cells. Gliclazide-based drugs on the pharmaceutical market are presented in the form of white tablets [2].

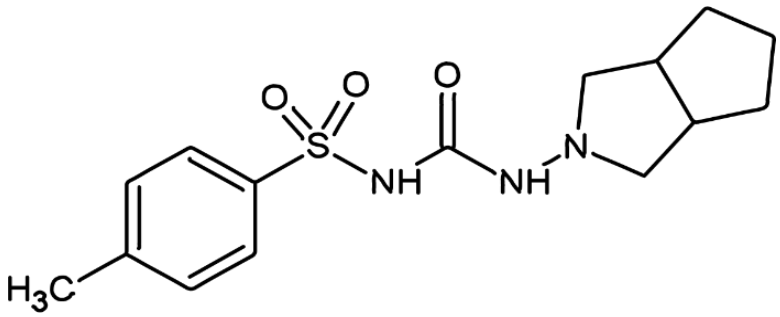


Figure 1. The structure of gliclazide

According to the literature, chromatographic [3-7], mass spectrometric [3, 8], spectrophotometric methods of analysis in UV and visible spectrum [9-12] and indicating-micellar electrokinetic chromatography method [13] are most often used for gliclazide quantitation in dosage forms. However, most of the proposed methods are characterized by either low sensitivity and selectivity, or require expensive equipment or hard-to-reach agents.

Spectrophotometry in the visible spectrum allows you to quickly and very accurately quantify, identify and determine the purity of the substance. According to the requirements of the State Pharmacopoeia of Ukraine, each method must be validated [14]. Therefore, the work aims to develop

and validate a new method for gliclazide quantitation in drugs by absorption spectrophotometry in the visible spectrum.

MATERIAL AND METHOD

The subjects of the study were modified-release tablets "Diaglizide" 80 mg (Farmak (Ukraine) series 111021), "Diaglizide MR" 60 mg (Farmak (Ukraine) series 101120), "Diaglizide MR" 30 mg (Farmak (Ukraine) series 240519), "Diabeton" MR 60 mg (Servier Ukraine LLC series 6019126) "Gliklada" 60 mg (KRKA (Slovenia) series D96697).

The used the following agents and solvent: standard sample solution (SSS) of gliclazide (Jiuzhou, series 2017-0020), bromocresol green (BCG) of analytical grade, acetone of analytical grade.

Equipment: Specord 200 spectrophotometer (Analytik Jena), 1 cm quartz cuvettes, Kern ABT-120-5DM electronic scales and Class A chemical utensils.

Gliclazide determination in general methods

Preparation of the gliclazide reference solution: 0.01950 g of gliclazide is placed in a 25.00 ml volumetric flask, dissolved in acetone and adjusted to the mark with the same solvent.

Preparation of the compensating solution: 1 ml of a 4.2% solution of BCG in acetone is transferred to a 10.00 ml volumetric flask, adjusted to the mark with acetone and mixed.

Transferred an aliquot of gliclazide solution (6.20-9.40 mg) to a 10.00 ml volumetric flask, add 1.00 ml of 4.2% BCG solution and make up to the mark with acetone. Absorption is measured against the background of a compensating solution that does not contain the test substance at a wavelength of 411 nm.

Gliclazide quantitation in tablets

The exact weight of crushed tablets "Diaglizide" 80 mg (0.02860-0.04321 g), "Diaglizide MR" 60 mg (0.07830-0.11847 g), "Diaglizide MR" 30 mg (0.07688-0.11641 g), "Diabeton MR" (0.08323-0.12609) d), "Glyclad" (0.08255-0.12505 g) is transferred into a volumetric flask with a volume of 25.00 ml and adjusted to the mark with acetone. The resulting solutions were stirred and filtered. The first portions of the filtrate are discarded because the first portions of the filtrate were cloudy. We took 1.00 ml of the solution from the filtrate and transfer it to a 10.00 ml flask and add 2.00 ml of BCG solution, make up to volume with acetone and analyze according to the general procedure. In parallel, we carried out the reaction with 1.00 ml of 0.06% SSS gliclazide. The quantitative content of gliclazide in tablets is calculated according to a typical formula.

RESULT AND DISCUSSION

In the course of the experiment, it was found that gliclazide reacts with sulfophthalein dies in acetone to form dyed reaction products (Figure 2).

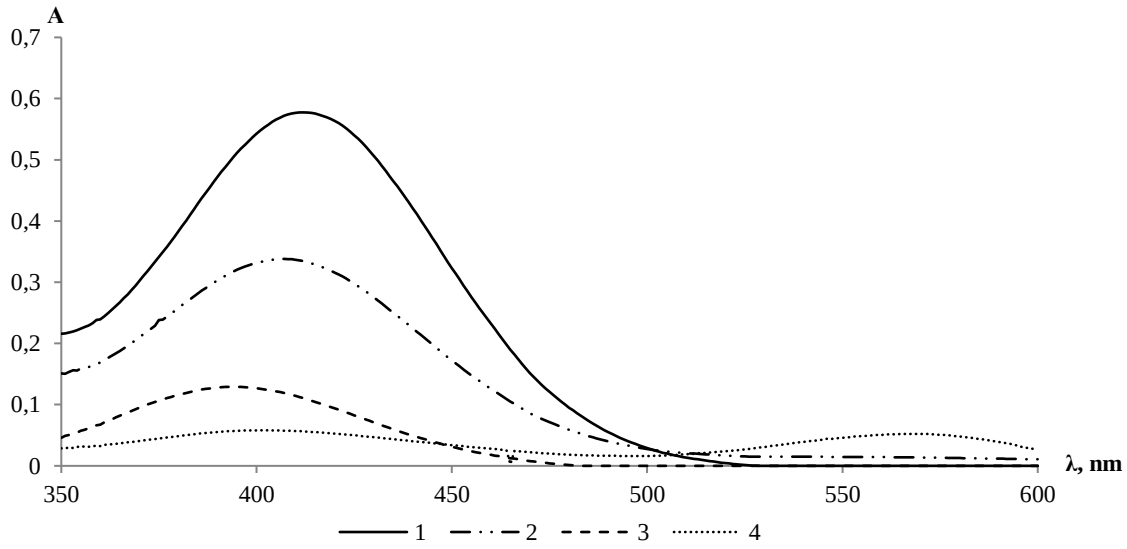


Figure 2. The absorption spectrum of gliclazide reaction products with bromocresol green (1), bromothymol blue (2), bromocresol purple (3), thymol blue (4) in acetone

As can be seen from Figure 2, the highest value of optical density was observed when using bromocresol green. When choosing a solvent, the solubility of gliclazide and BCG, as well as the maximum value of the optical density of the obtained solution, were taken into account. It was experimentally established that gliclazide interacts with BCG in an acetone environment with the formation of a colored product with a light absorption maximum at 411 nm (Figure 3).

It was experimentally determined that BCG interacts with gliclazide rapidly at room temperature and does not require temperature and time adjustment. The limit of detection under such conditions is 4.02 μg/ml.

To determine the ratio of stoichiometric coefficients between gliclazide and BCG, the method of continuous changes (isomolar series method) and the method of molar ratios ("saturation" method) were used.

As can be seen from the Figures 4 and 5, gliclazide interacts with BCG in a ratio of 1:1.

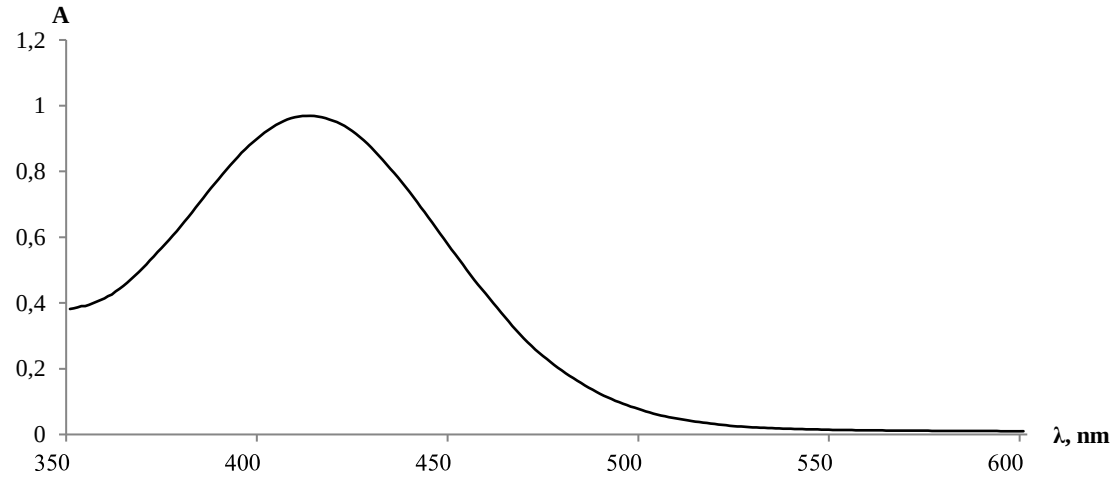


Figure 3. The absorption spectrum of gliclazide reaction product (0.06%) with bromocresol green (4.19%) in acetone

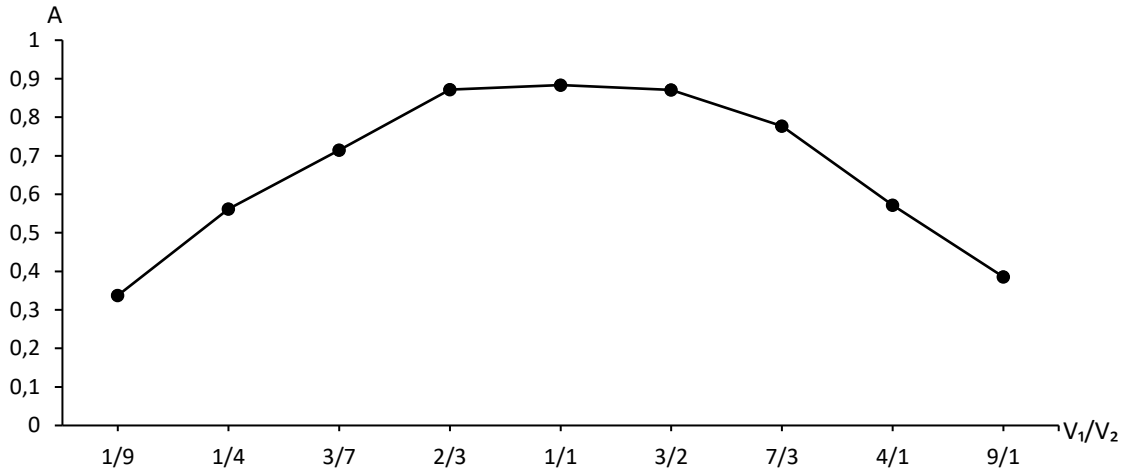


Figure 4. The graph of dependence of absorption on the ratio of components of an isomolar solution (V_1 – volume of 0.01 M BCG solution, V_2 – volume of 0.01 M solution of gliclazide)

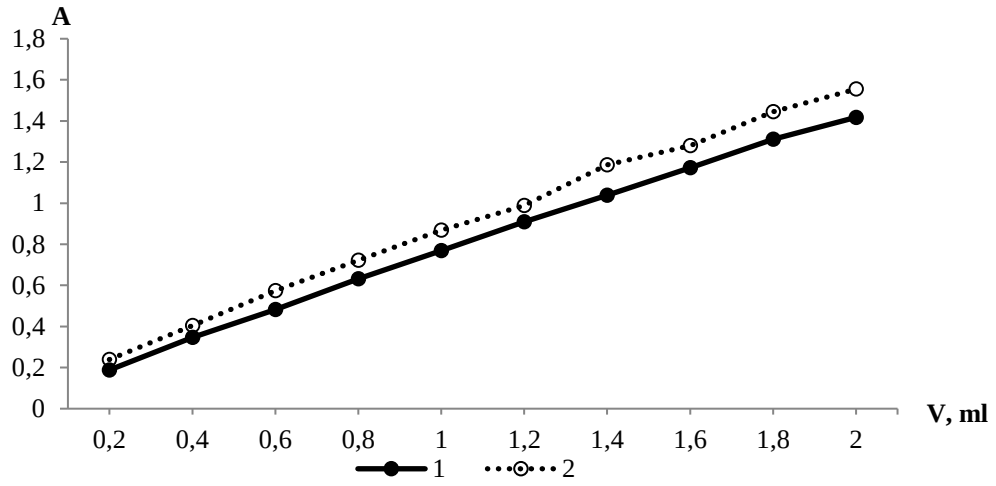


Figure 5. Saturation curves: 1 – gliclazide at constant reagent concentration (1.00 ml of 0.01 M solution); 2 – BCG at a constant concentration of gliclazide (1.00 ml of 0.01 M solution)

Proposed method validation

All validation characteristics of the proposed method are determined by the requirements of the State Pharmacopoeia of Ukraine. Parameters such as robustness, linearity, accuracy and precision were taken into account.

Robustness

The stability of the studied solutions was investigated during the robustness test. To do this, we measured the optical density of the analyzed solution of the corresponding dosage form and the working standard solution of gliclazide every 5 minutes for 30 minutes. As can be seen in Figure 6, the analyzed solution is stable for 30 minutes.

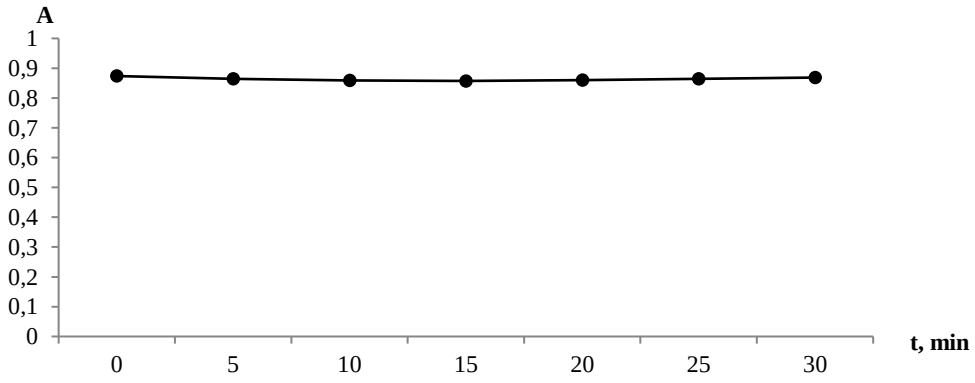


Figure 6. The dependence of the reaction product absorption on time

Linearity

To determine the linearity, 9 measurements of the absorption of gliclazide comparison solution were performed in the range of concentrations in which the subordination of Beer's law is observed, namely 62.00-94.00 mg/100 ml. The curve of absorption dependence on the gliclazide concentration in normalized coordinates is shown in Figure 7.

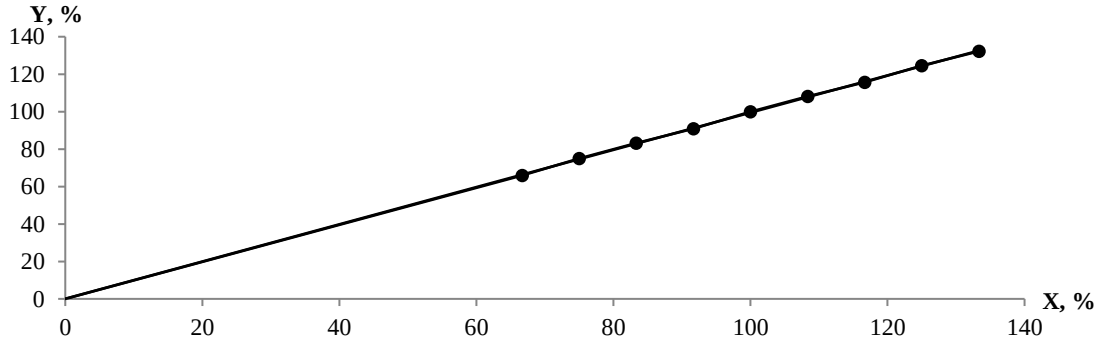


Figure 7. The curve of absorption dependence on the gliclazide concentration (in normalized coordinates) at a wavelength of 411 nm.

The linearity of the proposed method was estimated by regression analysis using the least square method. The obtained values are shown in Table 1.

Table 1. Numerical indicators of linear dependence of the method

Size	Value	Criteria	Conclusion
Equation of linear regression	Yi = bXi + a		
Correlation coefficient, r	0.9998	≥ 0.9962	meets
Residual standard deviation, $S_{x,0}$	0.4355	≤ Δ_{As} (%) /t (95%, 7) 1.689	meets
Intercept term, a ± (S*a*)	0.217 ± (0.685)	≤ t (95%, 7) · S*a* 1.298	meets
Slope, b ± (S*b*)	0.993 ± (0,00670)	—	—

Precision

The precision of the proposed method for each dosage form was determined at the level of convergence. 9 parallel measurements were performed. Three solutions were prepared from three samples, each with three parallel measurements under optimal conditions. In parallel, the absorption of the reference solution was determined and the content of the test substance was calculated. The data obtained are shown in Table 2.

Table 2. Determination of precision and accuracy of the method for the gliclazide quantitation in tablets

Dosage form	$\bar{Z}$ % (n = 9)	S_z%	Δ_{As}	$\Delta_{\%}$	$\delta \leq \Delta_{\%}/3$
Diahlizid, 80 mg	99.70	1.06	1.6	1.97	0.30 ≤ 0.66
Diahlizid MR, 60 mg	100.26	0.88	1.6	1.65	0.26 ≤ 0.55
Diahlizid MR, 30 mg	100.23	0.95	1.6	1.78	0.23 ≤ 0.59
Diabeton MR, 60 mg	99.54	1.25	1.6	2.32	0.46 ≤ 0.77
Gliklada, 60 mg	98.86	0.77	1.6	1.44	0.14 ≤ 0.48

Accuracy

The used the method of additives to establish the accuracy of the proposed method. Different volumes of SSS were added to three equal samples of the drug substance and the optical density was determined three times. As can be seen in Table 3, the results of the determinations are accurate, as the results obtained are within the established confidence interval.

Table 3. Determination of the convergence of the method for gliclazide quantitation in tablets

Dosage form	$\bar{Z}$ % (n = 9)	S_z%	$\Delta_{\%}$	$\lvert 100 - \bar{Z} \rvert$	$\sigma \leq \Delta_{\%}/3$
Diahlizid, 80 mg	99.55	0.85	1.57	0.45	0.45 ≤ 0.52
Diahlizid MR, 60 mg	100.19	0.96	1.78	0.19	0.19 ≤ 0.59
Diahlizid MR, 30 mg	99.94	0.93	1.72	0.06	0.06 ≤ 0.57
Diabeton MR, 60 mg	99.89	0.37	0.68	0.11	0.11 ≤ 0.23
Gliklada, 60 mg	99.65	0.65	1.15	0.35	0.35 ≤ 0.38

Complete uncertainty of analytical methods

To confirm that the developed method will be correctly reproduced in other laboratories, the calculation of the complete uncertainty of the method results was performed. According to the SPU, the projected complete uncertainty of the methodology should not exceed the maximum allowable value.

The forecast of complete uncertainty was calculated by the formula (1):

$$\Delta_{As} = \sqrt{\Delta_{sp}^2 + \Delta_{FAO}^2} \qquad (1)$$

Where Δ_{sp} – the uncertainty of sample preparation;

Δ_{FAO} – projected uncertainty of the final analytical operation.

The calculation of the sample preparation uncertainty of the test solution and the comparison solution is shown in Table 4. The calculations took into account the minimum weight of the finished drug sample.

Table 4. Calculation of the sample preparation uncertainty of the test solution and the comparison solution of gliclazide.

Operation of sample preparation	Uncertainty, %
The investigated solution	
Taking a sample of the finished drug	0.2 mg/28.6 mg · 100 % = 0.70
Bring the volume to the mark in a volumetric flask of 25 ml	0.23
Taking an aliquot of dilution of the finished drug with a pipette per 1 ml	0.74
Bring the volume to the mark in a 10 ml volumetric flask	0.50
The solution of comparison	
Taking a sample of gliclazide	0.2 mg/19.5 mg · 100 % = 1.03
Bring the volume to the mark in a volumetric flask of 25 ml	0.23
Taking an aliquot of dilution of the finished drug with a pipette per 1 ml	0.74
Bring the volume to the mark in a 10 ml volumetric flask	0.50
$\Delta_{sp} = \sqrt{0.70^2 + 0.23^2 + 0.74^2 + 0.50^2 + 1.03^2 + 0.23^2 + 0.74^2 + 0.50^2} = 1.80$	

When performing three parallel measurements with the extraction of the cuvette, the value of the uncertainty of the final analytical operation was equal to 0.70% [15].

Projected complete uncertainty of the analysis results:

$$\Delta_{As} = \sqrt{1.80 + 0.70} = 1.93\%$$

Thus, the projected complete uncertainty of the analysis (1.93%) does not exceed the maximum allowable uncertainty of the method (2.40%) and the method can be reproduced in other laboratories [14].

As a result of the study, a spectrophotometric method for gliclazide quantitation in tablets was developed and validated. The developed method is easy to perform, accessible and meets the requirements of the State Pharmacopoeia of Ukraine, so it can be recommended for gliclazide analysis in laboratories for quality control of drugs.

ACKNOWLEDGEMENTS

We're grateful to the Zaporizhzhia State Medical University for providing some facilities for carrying out the research.

AUTHOR CONTRIBUTIONS

Concept: *L.L., S.V.;* Design: *L.L, S.V.;* Control: *L.L, S.V.;* Sources: *L.L, S.V.;* Materials: *L.L, S.V.;* Data Collection and / or Processing: *L.L;* Analysis and / or Interpretation: *L.L;* Literature Review: *L.L;* Manuscript Writing: *L.L, S.V.;* Critical Review: *S.V.;* Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

REFERENCES

1. International Diabetes Federation. (2019). IDF Diabetes Atlas 9th Edition 2019. Retrieved June 6, 2022, from https://diabetesatlas.org/atlas/ninth-edition/

2. Compendium online directory of medicines Web site. (2022) Date added: 03.05.2022 https://compendium.com.ua/dec/266948/

3. Wang, C. Y., Zhang, W., Xiang, B. R., Yu, L. Y., Ma, P. C. (2008). Liquid chromatography–mass spectrometry method for the determination of gliclazide in human plasma and application to a pharmacokinetic study of gliclazide sustained release tablets. *Arzneimittel Forschung, 58*(12), 653-658. [CrossRef]

4. Kumar, B. V. V., Patnaik, A. K., Raul, S. K., Rao, N. N. (2013). A RP-HPLC method development and validation for the estimation of gliclazide in bulk and pharmaceutical dosage forms. *Journal of Applied Pharmaceutical Science, 3*(4), 59-62.

5. Gandhimathi, M., Anandakumar, K., Cheriyan, A., Ravi, T.K.. (2003). Simultaneous estimation of Metformin and gliclazide in tablet using reverse phase high performance liquid chromatography. *Indian Journal of Pharmaceutical Sciences. 65*, 530-531.

6. Krzek, J., Czekaj, J., Moniczewska, M., Rzeszutko, W. (2001). Determination of Gliclazide in Pharmaceutical Preparations by Capillary Gas Chromatography with Cool On-Column Injection and Elimination of the Matrix Effect. *Journal of AOAC Internatıonal, 84*(6), 1695-1702. [CrossRef]

7. Bhattacharya, K., Mathew, J. (2021). Development and validation of stability-indicating UPLC method for the determination of gliclazide and its impurities in pharmaceutical dosage forms. *Future Journal of Pharmaceutical Sciences, 7*, 95. [CrossRef]

8. Wang, X., Zhang, C. (2022) Determination of Gliclazide in Dameikang Tablets by Internal Standard Method of Nuclear Magnetic Resonance[J]. *Experiment Science and Technology*, *20*(3), 7-10.

9. Reichal, C.R., Rao, M.G. (2015). Development and validation of spectrophotometric method for simultaneous estimation of gliclazide and sitagliptin phosphate monohydrate in bulk and pharmaceutical dosage form. *International Journal of Pharmacy and Pharmaceutical Sciences, 7*. 372-376.

10. Samina, A. J., Snehal, P. M., Poonam, S. K., Yogesh, V. P., Kishor, B. B. (2011). Development and validation of UV spectrophotometric method for the determination of Gliclazide in tablet dosage form. *Der Pharma Chemica*, *3*(4), 338-343. [CrossRef]

11. Saroj, R., Bukkuru, S., Patibandla, S., Vegiraju, V. (2016). UV Spectrophotometric Method Development and Validation for the Estimation of Gliclazide in Bulk and Pharmaceutical Dosage Form. *Asian Journal of Pharmaceutical Analysis*, *6*(3) 143-146. [CrossRef]

12. Jeha, B., Salami, M. (2019). Spectrophotometric Determination of Gliclazide in Bulk and Pharmaceutical Formulation using 1, 2-naphthoquinone-4-sulfonic acid Sodium salt. *Research Journal of Pharmacy and Technology*, *12*(11), 5310-5314. [CrossRef]

13. Doomkaew, A., Prutthiwanasan, B., Suntornsuk, L. (2015) Stability indicating MEKC method for the determination of gliclazide and its specified impurities. *Journal of Pharmaceutical and Biomedical Analysis*, *102*, 119-128. [CrossRef]

14. Pharmacopoeia of Ukraine, D. (2015). SE "Ukrainian Scientific Pharmacopoeial Center for Quality of Medicines". 2nd type. Kharkiv: SE "Ukrainian Scientific Pharmacopoeial Center for Quality of Medicines, 1, 1148 p.

15. Grizodub, A.I. (2016). Standardization of the procedure for validation of methods of quality control of drugs. (pp 64-67). Kharkiv: State Enterprise "Ukrainian Scientific Pharmacopoeial Center for Drug Quality".

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 931-954, 2022
Doi: 10.33483/jfpau.1130916

ORIGINAL ARTICLE / ÖZGÜN MAKALE

PREPARATION AND *IN-VITRO* CHARACTERIZATION OF FLOATING-PULSATILE HOLLOWBEADS CONTAINING INDOMETHACIN

İNDOMETAZİN İÇEREN YÜZEN-PULSATİL İÇİ BOŞ BONCUKLARIN HAZIRLANMASI VE İN-VİTRO KARAKTERİZASYONU

Emrah ÖZAKAR[1*], Hasan Burak GÜNEŞ[2], Rukiye SEVİNÇ ÖZAKAR[1]

[1]Atatürk University, Faculty of Pharmacy, Department of Pharmaceutical Technology, 25240, Erzurum, Turkey

[2]Pharmacist, 46300, Kahramanmaraş, Turkey

ABSTRACT

Objective: *Objective: Designing matrix structured controlled release systems using polymers or waxy lipids is a popular option today. Hollowbeads are formulations characterized by the formation of an air-filled cavity inside. In our study, indomethacin was chosen as a model drug. Cetyl alcohol was selected to create the hollowbeads structure, and NaCMC was chosen to achieve long-term release. Kollicoat® MAE100P was used to reduce and/or prevent ulcer formation and control release.*

Material and Method: *The formulations were prepared using a new "wax removal" technique. Different concentrations of $ZnCl_2$ and $CaCl_2$ were used as crosslinkers. In the preformulation studies, 24 different formulations were prepared by changing the amount of NaCMC, the amount of crosslinker, and the crosslinking time. The structure, size, encapsulation efficiency, yield, hollow structure, and long-term release capacity were investigated in the formulations. These parameters were statistically evaluated depending on the amount of NaCMC, the type of crosslinker, the amount of crosslinker, and contact times with the crosslinker.*

Result and Discussion: *Hollowbeads were characterized by SEM and FT-IR. In vitro release studies, release kinetics, and release mechanisms were performed in pH 1.2 HCl and pH 6.8 phosphate buffer media. Swelling, and buoyancy studies were performed. The long-term stability, encapsulation efficiencies, drug loading efficiencies, and yields of the formulations were also evaluated. Two promising formulations (F2 and F19) were found to be able to release indomethacin in both the stomach and intestinal media for 24 hours.*

Keywords: *Controlled release, hollowbeads, indomethacin, polymer, wax removal*

* **Corresponding Author / Sorumlu Yazar:** Emrah Özakar
e-mail / e-posta: emrahozakar@atauni.edu.tr, **Phone / Tel.:** +904422315217

Submitted / Gönderilme: 15.06.2022 **Accepted / Kabul:** 18.08.2022

ÖZ

Amaç: *Polimerlerin veya mumsu lipidlerin kullanılması ile matriks yapılı kontrollü salım sağlayan sistemler tasarlamak günümüzde popüler bir seçenektir. İçi boş boncuklar (hollowbeads), içinde hava dolu bir boşluğun oluşması ile karakterize formülasyonlardır. Çalışmamızda, indometazin model ilaç olarak seçilmiştir. Hollowbeads yapısını oluşturmak için setil alkol ve uzun süreli salım elde edebilmek için de NaCMC seçilmiştir. Ülser oluşumunu azaltmak ve/veya önlemek ve salımı kontollü elde etmek için Kollicoat® MAE100P kullanılmıştır.*

Gereç ve Yöntem: *Formülasyonlar, yeni bir teknik olan "wax removol" tekniği kullanılarak hazırlanmıştır. Çapraz bağlayıcı olarak $ZnCl_2$ ve $CaCl_2$'nin farklı konsantrasyonları kullanılmıştır. Önformülasyon çalışmalarında, NaCMC miktarı, çapraz bağlayıcı miktarı ve çapraz bağlanma sürelerini de değiştirerek 24 farklı formülasyon hazırlanmıştır. Formülasyonların yapısı, boyutu, enkapsülasyon etkinliği, verimi, hollow yapısı, uzun süreli salım kapasiteleri incelenmiştir. Bu parametreler NaCMC miktarına, çapraz bağlayıcı tipine, çapraz bağlayıcı miktarına ve çapraz bağlayıcıyla olan temas sürelerine bağlı olarak istatistiksel olarak değerlendirilmiştir.*

Sonuç ve Tartışma: *Hollowbeads'ler, SEM ve FT-IR ile karakterize edilmiştir. pH 1.2 HCl ve pH 6.8 fosfat tamponu ortamlarında in vitro salım, şişme ve yüzme çalışmaları gerçekleştirilmiştir. Salım kinetikleri ve salım mekanizmaları açıklanmıştır. Formülasyonların uzun süreli stabiliteleri, enkapsülasyon etkinlikleri, ilaç yükleme etkinlikleri ve verimleri de değerlendirilmiştir. Umut vadeden iki formülasyonun (F2 ve F19), 24 saat süreyle hem mide hem de bağırsak ortamında indometazin salımı gerçekleştirebildiği tespit edilmiştir.*

Anahtar Kelimeler: *İndometazin, kontrollü salım, mum yerdeğiştirme, oyuk boncuk, polimer*

INTRODUCTION

The oral route is the most common route of drug administration as it is multifunctional and convenient. Different release systems have been developed to increase bioavailability by reducing toxicity and side effects in drugs with the advances in pharmaceutical technology [1]. Modified-release dosage forms have always been a more effective therapeutic alternative to conventional or immediate dosage forms. The application purpose of modified release dosage forms is to regulate drug absorption from the gastrointestinal (GI) tract by controlling the release of the therapeutic agent. Such a dosage form can keep the concentration of the therapeutic agent in plasma stable for a longer period of time, effectively reducing dose-related side effects [2]. Modified release systems provide great benefits for patients with reduced dosing frequency and dose amount. Also, they provide the therapeutic advantages of drugs with less fluctuation in plasma blood levels. It is a popular option today to design matrix structured controlled release systems using polymers or waxy lipids in order to achieve controlled release [3, 4]. It is now possible to change the release rate of drugs by adjusting the amount of polymer [5].

Polymers have become an integral part of drug delivery systems with their improved pharmacokinetic properties. They can be targeted more specifically to tissues. Diffusion-based drug delivery systems are an important area in investigating the use of polymers in the delivery of solvent-activated drugs. In these systems, the drug is dissolved in a fully swollen matrix that does not dissociate. These solvent-activated systems swell when exposed to aqueous media and release the drug. They are also hydrophilic by nature [6-8].

In recent years, polysaccharide-based polymers have been widely used in biomedical and pharmaceutical applications due to their biocompatibility and biodegradability. It is studied in various fields

such as chemical engineering, medicine, pharmacy, food, and agriculture [9]. Sodium carboxy methyl cellulose (NaCMC) is a water-soluble, low-cost polysaccharide derivative that swells on contact with the GI system. Their viscosity and solubility change depending on the molecular weight and the length of the polymer chains [10]. They are used in many studies in formulation development and drug delivery systems [9-11].

One of the polymers used to prepare floating systems in the stomach is acrylic acid derivative polymers. Among these polymers is Kollicoat® MAE 100P. Drug delivery systems with mucoadhesive properties and pH-dependent release can be designed using this polymer [10]. The release of the drug from the polymeric matrix is controlled by three main mechanisms: diffusion, disintegration, and swelling. Efforts continue to increase the bioavailability of drugs by adhering to the mucosa, swelling, collapsing, expanding, gas-producing or super porous systems to increase the residence time of the dosage form in the stomach [12]. In recent years, scientific and technological advances have been made in the research and development of rate-controlled drug delivery systems by overcoming the disadvantages such as short residence times in the stomach and unpredictable gastric emptying times [13, 14]. Gastroretentive systems are dosage forms that have the feature of self-retention in the stomach to increase the absorption of the drug released from the acidic media in a controlled manner. Four types of gastroretension can be achieved with high-density systems, modified systems, mucoadhesive systems, and floating systems. Floating drug delivery systems have a lower density than gastric fluid. Therefore, they manage to remain floating in the stomach for a long time without being affected by the gastric emptying rate [10, 15].

Hollowbead systems are characterized by the formation of an air-filled space inside the dried beads after preparation [16]. It has been reported that hollowbeads are obtained by combining drug-containing polymeric structures with waxy components and injecting them into the system containing the organic phase [14, 17].

Indomethacin was chosen as the model drug in our study. Indomethacin is a nonsteroidal anti-inflammatory drug (NSAID) with antipyretic, analgesic, and anti-inflammatory activity [18, 19]. However, its serious gastrointestinal side effects limit its use and its poor solubility in biological fluids requires the design of special formulations [20]. It generally has a plasma concentration of 2 to 3 μg/mL and a biological half-life of 5 to 10 hours [21]. Indomethacin has been associated with ulcers it causes in relation to its residence time in the stomach [22]. Indomethacin exerts a higher ulcerogenic effect than other NSAIDs [23]. Our aim is to design and develop indomethacin-containing hollowbeads formulations floating in stomach contents with the “Wax Removal” technique [17]. Thus, both the ulcer-forming potential of indomethacin is reduced and formulations that can provide analgesic and anti-inflammatory effects have been reached for a longer period. In this study, 24 different hollowbeads formulations were studied to determine the effect of crosslinker type, crosslinker amount, mixing times, and polymer ratio.

The hollowbead formulations containing indomethacin were characterized by SEM and FT-IR. In vitro release studies were carried out in release media containing pH 1.2 HCl (USP30-NF25) and pH 6.8 phosphate (USP30-NF25) buffer. Also, swelling and floating experiments were performed containing pH 1.2 HCl buffer media. The release kinetics of each formulation were determined and the release mechanism was explained. Long-term stability, encapsulation efficiencies (EE%), drug loading capacities (LC%), and yields (Y%) of the formulations were also evaluated.

MATERIAL AND METHOD

Materials

Indomethacin was purchased from Alfa Aesar, Germany. Kollicoat® MAE 100P and Ethanol were purchased from Sigma-Aldrich®, USA. NaCMC was purchased from Doğa İlaç, Turkey. Cethyl alcohol and $ZnCl_2$ were purchased from Merck, Germany. $CaCl_2$ was purchased from J.T Baker, Germany. Ultra-pure water with resistivity higher than 18.2 MΩ cm was used in all experiments.

Development and validation of the quantity assay method

The quantity assay method for indomethacin was developed using UV-spectrophotometer (Beckman DU 730, Germany). 100 µg/mL stock solution was prepared of indomethacin in ethanol, and UV spectra were obtained in the wavelength range of 200-400 nm by making various dilutions from this stock solution. The wavelength at maximum absorbance (λmax) was determined. A calibration curve was created, and the assay method was validated for ICH parameters. All experimental studies were conducted in the dark to protect the indomethacin from light.

Experimental design

In our study, 24 different formulations were developed and the relationship between dependent variables and independent variables was statistically examined. The formulation design and data on the variables are given in Table 1. The effects on the independent variables (size, encapsulation efficiency, drug loading efficiency, yield, buoyancy, swelling, and release mechanism) were investigated by changing the dependent variables (the amount of NaCMC, $ZnCl_2$-$CaCl_2$, and mixing times).

Table 1. Formulation design and variables

Dependent Variables			
NaCMC amount (mg)	**$ZnCl_2$ amount (g)**	**$CaCl_2$ amount (g)**	**Mixing time (h)**
50	0.5	0.5	0.5
100	1.0	1.0	1
-	1.5	1.5	-

Preformulation studies

In our study, formulations were developed using the "Wax Removal" technique [17]. For this purpose, 25 mg of indomethacin, 100 mg of Kollicoat® MAE 100P, and 100 mg of cetyl alcohol were dissolved in a flacon with 2.5 mL of ethanol. In a separate vial, it was dissolved in 2.5 mL of distilled water by adding 50-100 mg of NaCMC. The ethanolic mixture was added to the mixture containing NaCMC and vortexed at 2000 rpm for 5 min. Then, 10 mL of solution containing different amounts of $ZnCl_2$ or $CaCl_2$ was dropped dropwise with a 22G injector while mixing at 550 rpm. Stirring was continued for 5 min after dropping. At the end of this period, the hollowbeads were filtered with filter paper and dried in an oven at 50 °C for 4 hours [24]. Obtained hollowbeads in dry form were stored in a moisture-free environment for further experiments. Each formulation was studied in triplicate. The prepared formulations and formulation parameters are given in Table 2. Blank hollowbeads without indomethacin were also prepared as described above without the addition of indomethacin.

Size, EE%, LC% and Y% of hollowbeads formulations containing indomethacin

The sizes of hollowbeads formulations containing indomethacin were measured manually using a caliper. Sizes were calculated as mean ($\bar{X}$) and standard deviation (SD), over at least 50 randomly selected beads from each formulation. The images were taken with a digital camera.

100 mg of dry beads of each formulation were powdered in a mortar and mixed in a vial containing 5 mL of ethanol for 4 hours at 750 rpm. The beads were completely fragmented by passing through a mechanical homogenizer (IKA®, T-18 Digital Ultra-Turrax, Germany) for 5 minutes at 10,000 rpm. Samples filtered through 0.45 μm membrane filters were quantified using a validated assay method. EE%, LC%, and Y% of the formulations were calculated as $\bar{X}$±SD using the formulas below. Each formulation was studied in triplicate [25].

EE% = (The calculated drug amount- Experimentally determined drug amount) / (The calculated drug amount) x 100
LC% = (The calculated drug amount- Experimentally determined drug amount) / (The amount of obtained beads) x 100
Y% = (The amount of obtained beads) / (The calcuated total amount of formulation ingredients) x 100

Swelling study of hollowbeads

50 mg of each hollowbead formulations containing indomethacin was weighed and placed in a vial. 50 mL of pH 1.2 HCl (USP30-NF25) buffer was added to them, and swelling was carried out at 100 rpm in a horizontal shaker water bath (Memmert WNB 14, SV-1422, Germany) adjusted to 37 °C for 24 hours. The swelling beads in each formulation were filtered from the medium, and their wet weights were recorded by reading on a precision balance at certain time intervals (0.5, 1, 2, 4, 8, 12, 24 hours). Each sample was run in three repetitions. The results were evaluated statistically. The weights of the beads before the experiment were compared with the weights after 24 hours, and the swelling capacity was determined as percentage [26].

$$\text{Swelling Degree (\%)} = 100 \times (W_2 - W_1) / W_1$$

W_1 = Beads weight before the experiment
W_2 = Beads weight after the experiment

Table 2. Formulations and formulation development parameters

	Formulation Parameters						
	Indomethacin (mg)	**Cethyl alcohol (mg)**	**Kollicoat® MAE 100P (mg)**	**NaCMC (mg)**	**$CaCl_2$ (g)**	**$ZnCl_2$ (g)**	**Mixing time (h)**
F_1	25	100	100	50	-	0.5	0.5
F_2	25	100	100	50	0.5	-	0.5
F_3	25	100	100	50	-	0.5	1
F_4	25	100	100	50	0.5	-	1
F_5	25	100	100	50	-	1	1
F_6	25	100	100	50	1	-	1
F_7	25	100	100	50	-	1.5	1
F_8	25	100	100	50	1.5	-	1
F_9	25	100	100	50	1	-	0.5
F_{10}	25	100	100	50	-	1	0.5
F_{11}	25	100	100	50	1.5	-	0.5
F_{12}	25	100	100	50	-	1.5	0.5
F_{13}	25	100	100	100	0.5	-	0.5
F_{14}	25	100	100	100	-	1	1
F_{15}	25	100	100	100	1	-	1
F_{16}	25	100	100	100	-	1.5	1
F_{17}	25	100	100	100	1.5	-	1
F_{18}	25	100	100	100	-	0.5	0.5
F_{19}	25	100	100	100	1	-	0.5
F_{20}	25	100	100	100	-	1	0.5
F_{21}	25	100	100	100	0.5	-	1
F_{22}	25	100	100	100	-	0.5	1
F_{23}	25	100	100	100	-	1.5	0.5
F_{24}	25	100	100	100	1.5	-	0.5

Buoyancy study of hollowbeads

One hundred beads were taken from each of the hollowbead formulations containing indomethacin and placed in a vial. pH 1.2 HCl (USP30-NF25) buffer was added to them, and floating was carried out at 100 rpm in a horizontal shaking water bath adjusted to 37 °C for 24 hours. The number of floating beads in each formulation was counted and recorded at certain time intervals (0.5, 1, 2, 4, 8, 12, 24 hours). Floated beads were determined at each measurement time as percentage. The time was examined for the beads to come to the surface of the vial and the total time the beads floated. Floating behavior and the number of floating beads were statistically compared with other formulations. This experiment was run in triplicate for each formulation [12, 26].

In vitro release study of hollowbeads

10 mg of dry hollowbeads were weighed and placed in amber-colored vials. 20 mL of pH 1.2 HCl (USP30-NF25) buffer or pH 6.8 phosphate buffer (USP30-NF25) was added to them, and the release study was carried out in a horizontal shaking water bath (37±0.5 °C, 100 rpm). One mL of each release medium was withdrawn and filtered through a 0.45 µm membrane filter at certain time intervals (0.5, 1, 2, 4, 8, 12, 24 hours). The same volume of fresh pH 1.2 HCl buffer or pH 6.8 phosphate buffer was added instead of the amount taken from each formulation to maintain sink conditions. The amount of indomethacin in supernatants was calculated using the validated quantity assay method. % cumulative release results were calculated. Each formulation was studied in triplicate [27, 28].

Release kinetics of hollowbeads

Release kinetics is an essential parameter in understanding the absorption, distribution, metabolism, and excretion of the active substance. Some mathematical processes and equations were used to explain the mechanism of indomethacin release from hollowbeads. R^2 values were taken as a basis in order to determine the appropriate kinetic model. The data for release profile of all the drug lodaded formulations in pH 1.2 HCl buffer (USP30-NF25) and pH 6.8 phosphate buffer (USP30-NF25) was processed by Microsoft Office Excel program in order to determine the best fitted kinetic model (Zero Order, First Order, Korsmeyer-Peppas, and Higuchi models) [12, 29].

FT-IR analysis of hollowbeads

It was carried out in order to determine the interactions between the active substance and excipients and to determine the presence of the active substance in the prepared formulations. For this purpose, infrared spectrums of indomethacin and two formulations (F2 and F19) with the longest floating/releasing time in the stomach, were taken using an FT-IR (Bruker, VERTEX ATR 70v, Germany). FT-IR analysis were carried out in the wavenumber range 4000-400 cm^{-1} for all bead formulations [29].

Examination of morphology of hollowbeads

Shapes and surface properties of the pure indomethacin and prepared hollowbeads examined using a SEM (Zeiss Sigma 300, Germany). The formulations were fixed on metal sheets with two-sided adhesive tape. The hollowbeads were coated with 100 Å thick gold. They were carefully cut in the middle. Their interiors were examined and photographed [30].

Evaluation of the stability of hollowbeads

Stability studies are an integral part of formulation development. It gives an idea to determine the most suitable conditions for the structure of the prepared formulation. The stability study was performed according to ICH guidelines for promising formulations (F2 and F19). The stability test for 12 months

was carried out at 25±2 °C/60 ± 5% RH in a non-hygroscopic package with a certain amount of hollowbeads belonging to F2 and F19 [26]. The stability and effectiveness of the product were evaluated with various parameters at the end of the period [31].

Statistical Analysis

All analysis and experiments were statistically analysed determine whether a significant difference between formulations. Fort his purpose, One-way Analysis of Variance (ANOVA) test was applied. In addition, all experimental data were calculated as arithmetic mean and standard deviation. The confidence limit was accepted as 95%. The $p<0.05$ level was considered statistically significant in the differences between the groups.

RESULT AND DISCUSSION

Development and validation of the quantity assay method

Dilutions were made from the stock solution of indomethacin and the maximum wavelength was found to be 317 nm (Figure 1). The calibration curve was drawn from 7 points in the concentration range of 10-55 µg/mL. Method validation was provided on the parameters of specificity, sensitivity, linearity, accuracy, precision (repeatability, reproducibility) and stability in accordance with ICH guidelines. The method is specific and sensitive for indomethacin. The method was found to be linear within the range of the calibration curve. % Relative Standard Deviation (RSD%) and % Relative Error (RE%) were found below 2%. It was determined that the stock solution remained stable for 3 days at both room temperature and +4 °C. Other parameters of validation are given in Table 3.

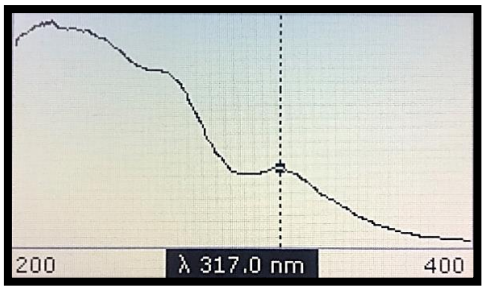


Figure 1. Indomethacin UV spectrum

Table 3. Validation parameters

Equation	y=0.0185x+0.0043
R^2	0.9993
Λ (nm)	317
LOD (µg/mL)	0.857
LOQ (µg/mL)	2.688

* Accuracy and precision calculations were found to be less than ±2%.
** Recovery (%) calculations were not found less than 98%.

Formulation development studies

Images of hollowbeads as a result of formulation development studies are given in Figure 2. It has been determined that the hollowbeads in our study were generally spherical and their sizes vary between 1330 μm and 1960 μm. The beads formed when they were dropped into solutions containing Ca^{2+} or Zn^{2+} ions. However, the beads exhibited poor mechanical strength, especially in solution containing Zn^{2+} ions. It was determined that there was no significant change in the dimensions of the hollowbeads with the increasing amount of polymer (NaCMC) ($p>0.05$).

In almost all formulations, it was observed that the use of $CaCl_2$ as a crosslinker decreased the bead size compared to the use of $ZnCl_2$, and this was found to be statistically significant ($p<0.05$). It was also found that as the amount of crosslinker increased (such as 0.5, 1 and 1.5 w/v), the size decreased and this was statistically significant between formulations prepared using the same group of crosslinkers ($p<0.05$). This situation has also been reported in similar studies in the literature. In particular, it has been reported that during the dropping of the polymeric content into the solution containing high amount of crosslinker results in spherical beads due to the excess number of ions surrounding the dripped content [32].

Size, EE%, LC% and Y% of hollowbeads formulations containing indomethacin

Size, EE%, LC% and Y% of hollowbeads formulations containing indomethacin are given in Table 4. It was observed that there was no statistically significant difference among the formulation yields with the change in the amount of crosslinker ($p>0.05$). On the other hand, upon comparison of the cross-linkers, it was determined that the formulations with better yields were those prepared using $CaCl_2$. They showed statistically significant difference ($p<0.05$). In literature, it has been reported that the type and amount of polymer changes the yield in this direction [12]. Another important point is the mixing time. The data indicated that the yield increased as the mixing time was shortened. It was observed that the highest yield values were obtained with 0.5 hour mixing time.

The formulations were evaluated in terms of EE% and LC%, it was observed that LC% and EE% increased statistically with the increase in the amount of crosslinker in the solution (0.5, 1.0, 1.5 g) ($p<0.05$). The $CaCl_2$ and $ZnCl_2$ were compared in terms of LC% and EE%, it was determined that the formulations prepared with $CaCl_2$ had statistically significantly better EE% and LC% ($p<0.05$). In addition, increasing the amount of NaCMC from 50mg to 100mg increased EE% and LC% in all formulations. This situation was found to be statistically significant ($p<0.05$). Similar results have been reported in the literature [12, 33].

Swelling study of hollowbeads

The swelling behavior of the polymer is an important factor in controlling the release of drugs from

the bead systems. The degree of swelling of the formulated beads showed that swelling was associated with different polymer ratios [26]. The swelling study results of our experiments for 24 hours are given in Table 5 as %. The swelling experiments were conducted in the gastric media since the main route of absorption of indomethacin is the stomach and the formulations do not dissolve at gastric pH. Due to the dissolution of Kollicoat® MAE 100P at the intestinal media, no swelling study was performed in the intestinal media.

Figure 2. Images of hollowbeads containing indomethacin

In literature, Giri et al. reported that no formulation swelled above 10% at the end of the first 1.5 hours in the floating study performed on the beads prepared using pectin and xanthan gum [32]. Awasthi and Kulkarni determined that the swelling rate of the beads they prepared was below 3% in the swelling study conducted in a pH 1.2 HCl media for 3 hours [12]. Taranalli et al. designed hollowbeads by experimenting with different polymer combinations. They reported that their formulations floated for 12 hours and 24 hours in a pH 1.2 HCl media, but their swelling rate could not exceed 2% [26]. In our study, it was observed that the formulations swelled in the range of 35-96% even at the end of the first half-hour. The swelling balance was reached in a short time in all formulations. With the doubling of the amount of NaCMC (100 mg), it was determined that the water absorption and swelling rates of the formulations generally showed minor swelling than the formulations prepared using less amount of NaCMC (50 mg) ($p<0.05$). This situation may be since water takes longer to enter the beads due to the increase in polymer amount. It is also thought that the water absorption rate remains low, as the surface in contact with this water erodes and begins to break down over time.

Table 4. Size, EE%, LC% and Y% of hollowbeads formulations containing indomethacin (X̄±SD)

Formulations	Size (mm)	EE%	LC%	Y%
F_1	0.178±0.011	82.15±3.25	3.42±0.41	82.42±3.61
F_2	0.152±0.012	93.68±1.33	5.87±0.78	92.21±1.33
F_3	0.180±0.014	76.73±3.67	2.15±1.11	81.13±3.49
F_4	0.163±0.017	88.19±0.22	4.92±0.78	87.01±1.95
F_5	0.172±0.015	77.68±3.13	2.42±0.55	74.65±3.15
F_6	0.141±0.009	83.41±1.88	3.66±0.71	79.18±1.01
F_7	0.133±0.012	84.56±0.51	3.91±0.58	76.10±0.22
F_8	0.156±0.012	87.75±2.27	4.01±1.03	79.77±0.36
F_9	0.148±0.013	89.13±0.44	4.28±0.54	84.41±.028
F_{10}	0.196±0.013	85.05±1.98	3.88±0.97	81.13±0.75
F_{11}	0.146±0.016	87.42±2.45	4.11±0.48	81.01±1.13
F_{12}	0.206±0.016	82.69±0.79	3.55±2.12	79.15±1.47
F_{13}	0.146±0.017	85.93±2.05	3.75±0.71	88.91±0.18
F_{14}	0.182±0.019	78.17±3.15	2.61±1.09	73.45±2.46
F_{15}	0.144±0.014	91.49±0.85	5.13±0.84	89.81±2.12
F_{16}	0.172±0.014	80.45±2.18	3.88±1.23	76.43±3.08
F_{17}	0.154±0.016	90.81±0.43	4.19±1.65	80.17±0.09
F_{18}	0.176±0.015	78.15±3.59	2.83±0.78	84.12±0.52
F_{19}	0.150±0.014	95.42±2.38	5.94±1.14	93.11±0.43
F_{20}	0.189±0.020	82.42±2.10	3.41±0.22	80.81±0.83
F_{21}	0.154±0.024	89.41±1.33	4.01±2.12	83.15±0.53
F_{22}	0.166±0.015	81.79±0.97	3.01±1.71	81.94±2.54
F_{23}	0.171±0.017	87.43±1.89	3.88±1.47	79.13±0.50
F_{24}	0.188±0.021	90.71±1.41	4.52±1.03	84.15±1.16

In our study, among the groups with the same amount of polymer (F1-F12 and F13-F25), it was determined that the formulations prepared with $CaCl_2$ crosslinker could absorb water significantly less

than the formulations prepared with $ZnCl_2$ (eg, F2 and F19) ($p<0.05$). This may have occurred as a result of the strong interaction of Ca^{+2} ions with the carboxylic end groups on the polymer. This indicates that the type of crosslinker contributes to the hardener strength and toughness on the bead structure. It has also been determined that it allows the water to enter the bead at a later time and to remain in the media for a more extended period of time without disturbing the integrity of the bead. This situation has also been reported in similar studies in the literature [1].

Table 5. Swelling results of hollowbeads formulations (%)

Formulations	**Swelling rate (%)**						
	0.5 h	**1 h**	**2 h**	**4 h**	**8 h**	**12 h**	**24 h**
F_1	94	98	74	52	47	23	0
F_2	71	81	86	87	84	75	60
F_3	83	99	65	41	19	0	0
F_4	53	62	72	73	72	65	46
F_5	Structure was degraded						
F_6	46	54	48	41	39	34	23
F_7	77	88	62	51	28	20	12
F_8	35	42	51	55	57	42	17
F_9	41	55	60	67	50	28	19
F_{10}	Structure was degraded						
F_{11}	45	26	Structure was degraded				
F_{12}	72	10	Structure was degraded				
F_{13}	63	72	71	62	39	34	34
F_{14}	76	16	Structure was degraded				
F_{15}	52	57	56	44	46	41	41
F_{16}	Structure was degraded						
F_{17}	46	41	41	36	28	22	19
F_{18}	64	27	Structure was degraded				
F_{19}	66	74	68	65	42	43	43
F_{20}	82	17	Structure was degraded				
F_{21}	63	52	49	40	33	32	29
F_{22}	96	41	34	27	18	16	0
F_{23}	Structure was degraded						
F_{24}	42	47	39	32	24	20	18

Buoyancy study of hollowbeads

The results of the buoyancy study of hollowbeads formulations containing indomethacin are given in Table 6, and the sample images of the F2 and F19 formulations at the end of the 24th hour are given in Figure 3.

Table 6. Buoyancy results of hollowbeads formulations (%)

Formulations	Buoyancy rate (%)						
	0.5 h	**1 h**	**2 h**	**4 h**	**8 h**	**12 h**	**24 h**
F_1	100	100	80	50	25	25	25
F_2	100	100	100	100	100	75	75
F_3	100	50	50	25	25	10	10
F_4	100	100	100	100	100	75	75
F_5	100	0	0	0	0	0	0
F_6	100	80	60	40	25	25	25
F_7	100	100	90	50	40	25	10
F_8	100	100	100	75	50	25	10
F_9	100	75	50	50	25	25	25
F_{10}	0	0	0	0	0	0	0
F_{11}	50	25	0	0	0	0	0
F_{12}	50	25	0	0	0	0	0
F_{13}	100	100	100	100	50	40	40
F_{14}	75	25	0	0	0	0	0
F_{15}	100	100	100	50	50	40	40
F_{16}	0	0	0	0	0	0	0
F_{17}	100	75	75	50	25	10	10
F_{18}	50	25	0	0	0	0	0
F_{19}	100	100	100	100	50	50	50
F_{20}	50	25	0	0	0	0	0
F_{21}	100	75	75	50	30	30	25
F_{22}	100	75	50	25	10	10	10
F_{23}	0	0	0	0	0	0	0
F_{24}	100	100	50	25	15	10	10

In the acidic media, the functional carboxyl groups of hollowbeads remain protonated and exert an insignificant electrostatic repulsive force. As a result, the beads swell at a very low rate. However, when higher pH values are reached in the medium, the carboxyl groups of the beads may be ionized and the osmotic pressure inside the beads increases. In this way, swelling and fragmentation of the structure can be observed by absorbing water faster [34]. Further swelling can be observed due to the exchange of positively charged ions (such as Ca^{+2} or Zn^{+2}) in the cross-linked beads with sodium ions in the phosphate buffer in alkaline media. So, it leads to water penetration and swelling [12]. Chemate et al. reported that calcium pectinate beads completed drug release after 10 hours, and the number of floating beads was below 20% at the end of 24 hours [35]. Awasthi et al. designed gastroretentive beads containing sodium alginate, pectin and HPMC. They reported that the floating rate of the formulations was between 60-70% for 12 hours [12]. Somani et al. prepared calcium pectinate hollowbeads and determined that the number of beads floating in the stomach medium was reduced by 50% after 12 hours. At the end of 16 hours, they reported that there was no floating bead [36]. Hsu et al. designed beads in a core-shell structure and reported that they obtained the best floating ratio with 1:4 chitosan:xantham gum. They reported that as the chitosan rate increased, the floating rate decreased [1].

Chauhan et al. also designed floating tablets containing indomethacin and reported that a floating study was performed at 37 °C in pH 1.2 HCl buffer for 12 hours [31].

In our study, it was determined that 13 of the 24 formulations floated even at the end of the 24th hour. It was determined that almost all of the formulations prepared using $CaCl_2$ crosslinker floated after this period. Compared to the formulations prepared using $ZnCl_2$ crosslinker, this difference was significant in terms of floating rate ($p<0.05$). In addition, it was determined that the amount of polymer used (NaCMC) did not make a significant difference ($p>0.05$). It was determined that F2 and F19 formulations floated by 75% and 50%, respectively, even after 24 hours.

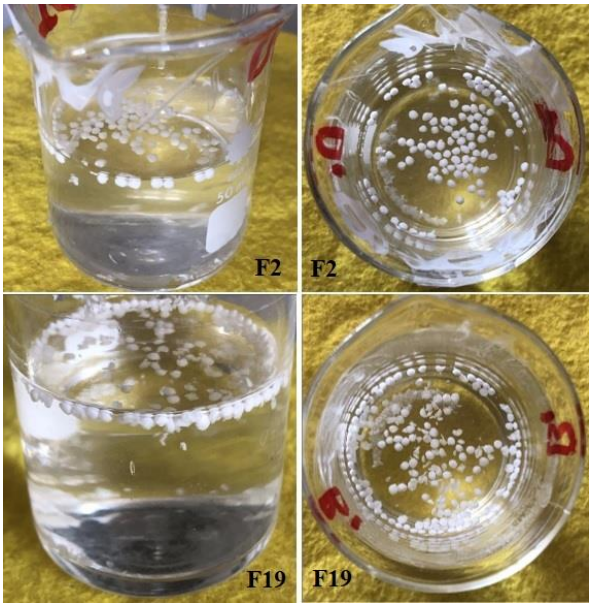


Figure 3. Buoyancy images of formulations after 24 hours (upper: F2; bottom: F19)

Among the obtained hollowbeads, two promising formulations (F2 and F19) were selected as a result of EE%, LC%, Y%, swelling and buoyancy studies. Further studies were carried out on these formulations.

In vitro release study of hollowbeads

The *in vitro* release study results for hollowbeads (F2 and F19) conducted in pH 1.2 HCl buffer and pH 6.8 phosphate buffer media are given graphically in Figure 4 below. While approximately 10% of pure indomethacin was released in pH 1.2 HCl buffer media in 24 hours, this ratio was approximately 3% in our F2 formulation developed using 50 mg NaCMC, and this ratio was approximately 2% in our F19 formulation developed using 100 mg NaCMC. These data indicate that the amount of polymer did not significantly affect the release of indomethacin. Still, both formulations produced significantly less and more extended indomethacin release than pure indomethacin ($p<0.05$).

Approximately 100% of pure indomethacin was released in pH 6.8 phosphate buffer in 4 hours. This rate was achieved at approximately 12 hours in our F2 and F19 formulations. It has been determined that our formulations release indomethacin for a long time in pH 1.2 and pH 6.8 buffer media. Especially with the F2 and F19 formulations, which can float in the stomach content even after 24 hours, the release of indomethacin in non-ionized form has been found to be sustainable for 24 hours. This also has been reported by other authors who have designed similar formulations for the gastrointestinal tract. In particular, hollowbeads underwent very little ionization (~2-3%) at pH 1.2 due to the presence of carboxylic acid-containing groups from Kollicoat® MAE 100P. Therefore, the bead structure remained significantly stable at this pH. However, ionization occurred in the pH 6.8 media. The intramolecular and intermolecular electrostatic repulsive forces of the polymer were increased, and the beads were fragmented and released faster. Since pH conditions differ in the stomach and intestinal tract, the use of beads prepared with such pH-sensitive polymers as drug carrier systems provides a significant advantage over other systems [27].

The release of hollowbeads in a pH 6.8 environment was sustained for approximately 12 hours. Although it was observed that the increase in the amount of NaCMC polymer used in F19 compared to F2 prolonged the release somewhat, it did not create a statistically significant difference ($p>0.05$). It was observed that the drug release between the polymeric matrix chains was delayed in both environments with the increase in the amount of polymer. These results have also been reported in similar studies in the literature [32]. It was determined that the F19 formulation produced a longer duration and less indomethacin release compared to the F2 formulation. However, it was determined that both formulations released indomethacin in a longer time compared to pure indomethacin ($p<0.05$). It has also been reported in studies in the literature that the release of the active substance is accelerated when the amount of polymer remains too high or too low [1]. The lower drug loading efficiency in the beads results in larger pore formation resulting in higher swelling and faster drug release. Higher the drug loading efficiency, larger the drug area formed in the beads. This causes the size of the matrix as well as the shrinkage of the pores, resulting in a decrease in drug release. A similar finding has been reported by other authors [32].

Slower drug release from the beads in pH 1.2 HCl buffer media may be due to the limited solubility of the beads in the acidic media as they contain the enteric polymer Kollicoat® MAE 100P, as well as the weak acidic nature of the drug. At higher pH values, Kollicoat® MAE 100P will dissolve and the carboxyl groups of the beads will ionize. As a result, the osmotic pressure inside the beads increases. The result is a higher rate of swelling and faster drug release. In an acidic media, the carboxyl groups cause the electrostatic repulsion to be lost, thereby reducing the swelling of the beads. This slows down the release of the drug. It was also observed that the drug release decreased as the crosslinker ($CaCl_2$) concentration increased. This may have been caused by the reduction of the free volume of the polymer matrix in the presence of higher crosslinkers, thereby inhibiting the movement of solutes through the

polymer matrix [32].

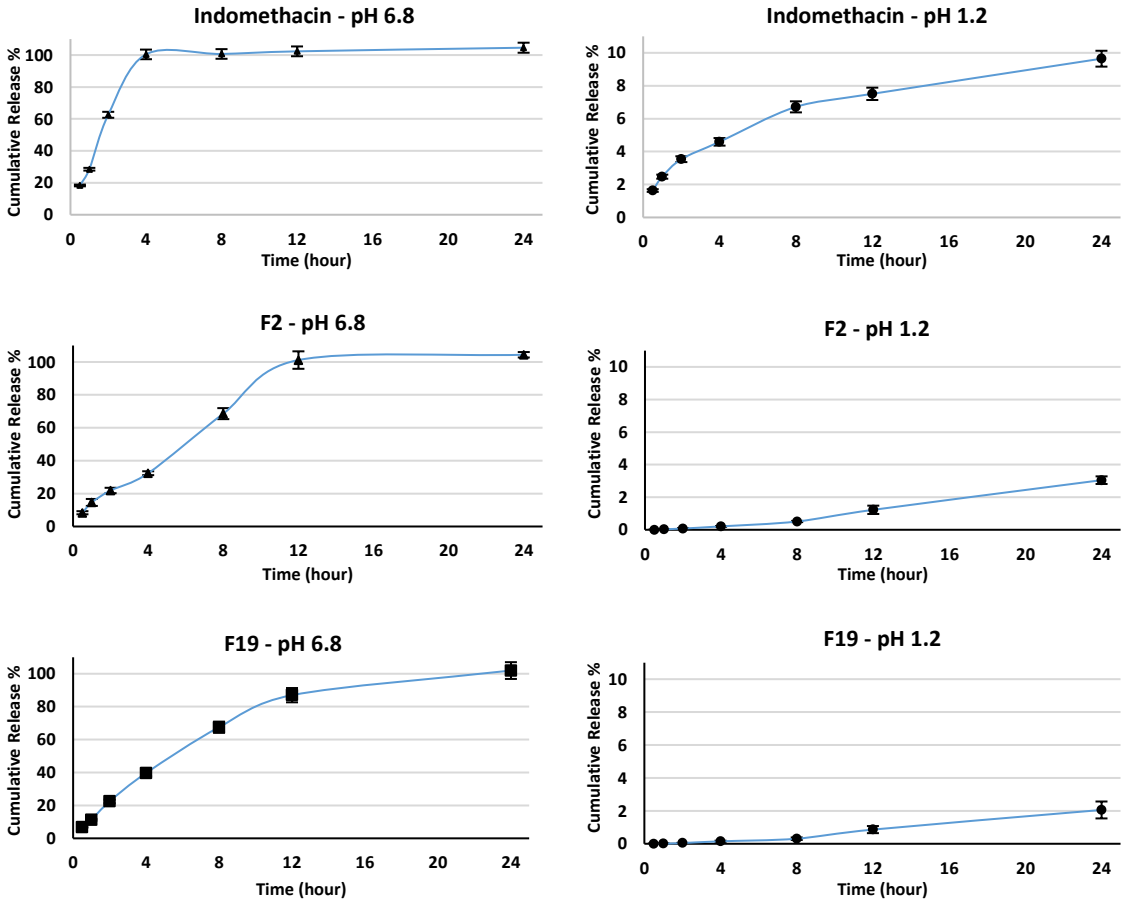


Figure 4. *In vitro* release study of indomethacin, F2 and F19 formulations

Release kinetics of hollowbeads

The release kinetics of F2 and F19 hollowbeads formulations are given in Table 7 and Table 8 respectively, as below. Drug release data were analyzed using Higuchi, Korsmeyer-Peppas, Zero-Order, and First-Order model equations to determine drug release kinetics from hollowbeads.

Table 7. Release kinetics of hollowbeads in pH 1.2 buffer media

	Zero-Order	First-Order	Higuchi	Korsmeyer-Peppas		Release Mechanism
Hollowbeads	**R^2**	**R^2**	**R^2**	**R^2**	**n**	
F2	0.987	0.770	0.867	0.666	0.940	Super Case-II Transport
F19	0.981	0.600	0.866	0.519	1.026	Super Case-II Transport

Table 8. Release kinetics of hollowbeads in pH 6.8 buffer media

	Zero-Order	First-Order	Higuchi	Korsmeyer-Peppas		Release Mechanism
Hollowbeads	**R^2**	**R^2**	**R^2**	**R^2**	**n**	
F2	0.854	0.645	0.920	0.935	0.799	Non-Fickian diffusion
F19	0.788	0.574	0.885	0.925	0.866	Non-Fickian diffusion

In vitro dissolution data of all series were calculated according to the Peppas equation versus time to find the drug release mechanism and confirm whether the diffusion mechanism is Fickian or non-Fickian. If the "n" value resulting from the Peppas equation is less than or equal to 0.5, it indicates that the drug release mechanism is by diffusion without swelling. However, if the "n" value is greater than 0.5 and less than 1, it is understood that the release is by diffusion based on swelling. If this "n" value is above 1, it indicates a (non-Fickian) release where the release mechanism occurs by abnormal diffusion and Fick's laws cannot be applied [12, 29, 37]. It was clear from the kinetic data that the drug release kinetics followed Peppas' kinetics for F2 and F19 in a pH 6.8 release medium, and the release was via swelling controlled diffusion. The calculated slope values of the Peppas equations gave a value close to but less than 1. This confirmed that the mechanism for the release of indomethacin from hollowbeads was Fickian diffusion with swelling in the intestinal medium. From the kinetic data, it was seen that the drug release kinetics followed the zero-order kinetics for F2 and F19 in the pH 1.2 release medium, and the release mechanism of indomethacin from hollowbeads was confirmed to be Super Case-II Transport in the gastric media. This indicates that indomethacin release from our formulations occurs at a constant rate in the pH 1.2 release medium. The value of "n" greater than 0.85 indicates "Super Case-II Transport" related to polymer relaxation during swelling [19, 32].

FT-IR analysis of hollowbeads

IR spectra were taken from powder samples of selected F2 and F19 hollowbeads formulations and pure indomethacin. FT-IR spectra of indomethacin and formulations are shown in Figure 5. It is seen that the characteristic peaks of indomethacin did not change and were clearly observed in the FT-IR spectra of the formulations. This showed that there was no interaction between indomethacin and excipients [38].

The characteristic peaks of pure indomethacin were compared with the peaks obtained from the formulations. C=O stretching, one of the characteristic peaks of indomethacin, was observed at the frequency of 1711.44 cm^{-1} with the same or slight differences. In the spectra were also seen characteristic peaks at 1689.05 cm^{-1} (C=O stretching vibrations), 1221.34 cm^{-1} (asymmetric aromatic O-C stretching), and 1066.31 cm^{-1} (symmetric aromatic O–H stretching) in the formulations [19, 31].

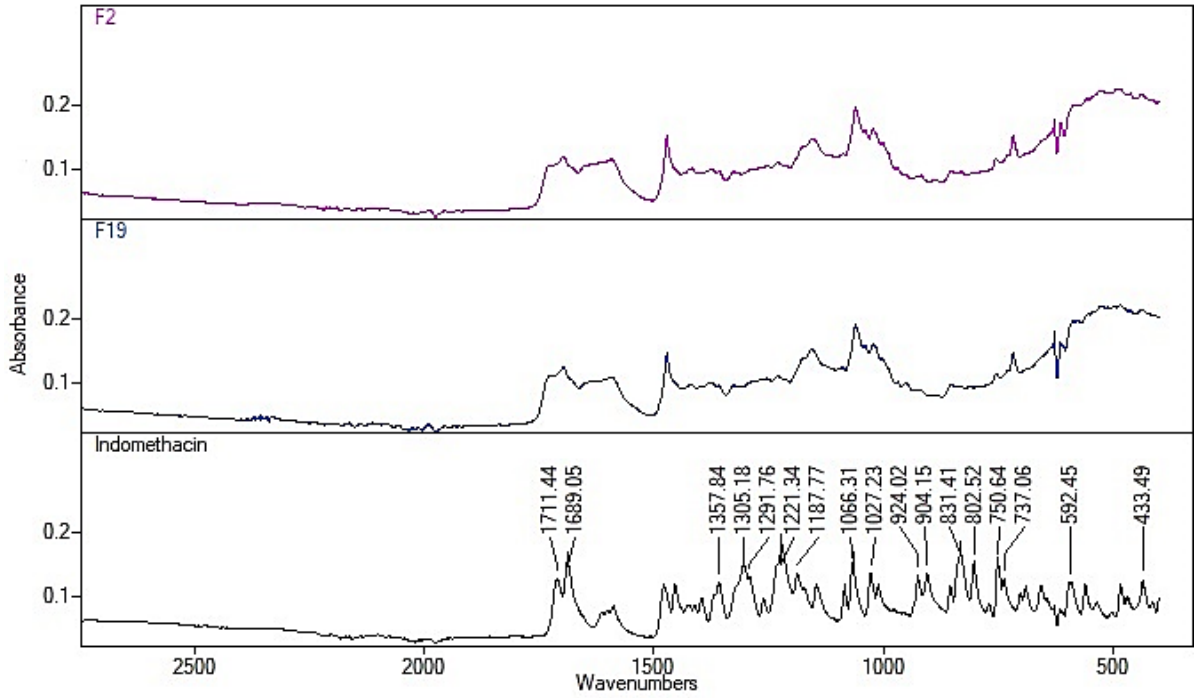


Figure 5. IR spectra of F2 (upper), F19 (middle) and indomethacin (bottom)

Examination of morphology of hollowbeads

SEM images of pure indomethacin, blank, F2, and F19 hollowbeads formulations are given in Figure 6. In the image of blank hollowbeads (upper right), it is seen a very smooth and spherical structure. In the images of F2 (bottom left) and F19 (bottom right), beads were cut in the middle. The cross-section of beads from F2 and F19 showed a hollow core and multiple small hollow pockets in the matrix. The layered structure within the bead wall is also an indication of a long-term release during *in vitro* release studies. The outer surfaces of the beads were very dense, and the inner layers were very porous and pocketed. The fact that the core was completely empty may have allowed the formulations to float for a long time. A similar situation has been reported in studies of hollowbeads and porous beads in the literature [1, 24, 27].

Evaluation of the stability of hollowbeads

Stability studies were carried out on the two promising formulations (F2 and F19) within the scope of formulation development studies. EE%, LC%, buoyancy, swelling, and release mechanisms data were evaluated and comparisons with freshly prepared hollowbeads study results were made at the end of 12 months. The results obtained are given in Table 9 below.

As a result of the stability study performed for 12 months according to the ICH guidelines on formulations that could be potential drugs, it has been shown that no significant changes were observed in the appearance of the formulations, encapsulation efficiencies, drug loading capacity, buoyancy,

swelling and *in vitro* release studies. F2 and F19 formulations were confirmed to be stable after 12 months at 25 ± 2°C/60 ± 5% RH. The obtained results were evaluated and compared to the freshly prepared formulation data. The obtained data at the end of 12 months for all the analyzes mentioned did not show a statistically significant change ($p>0.05$).

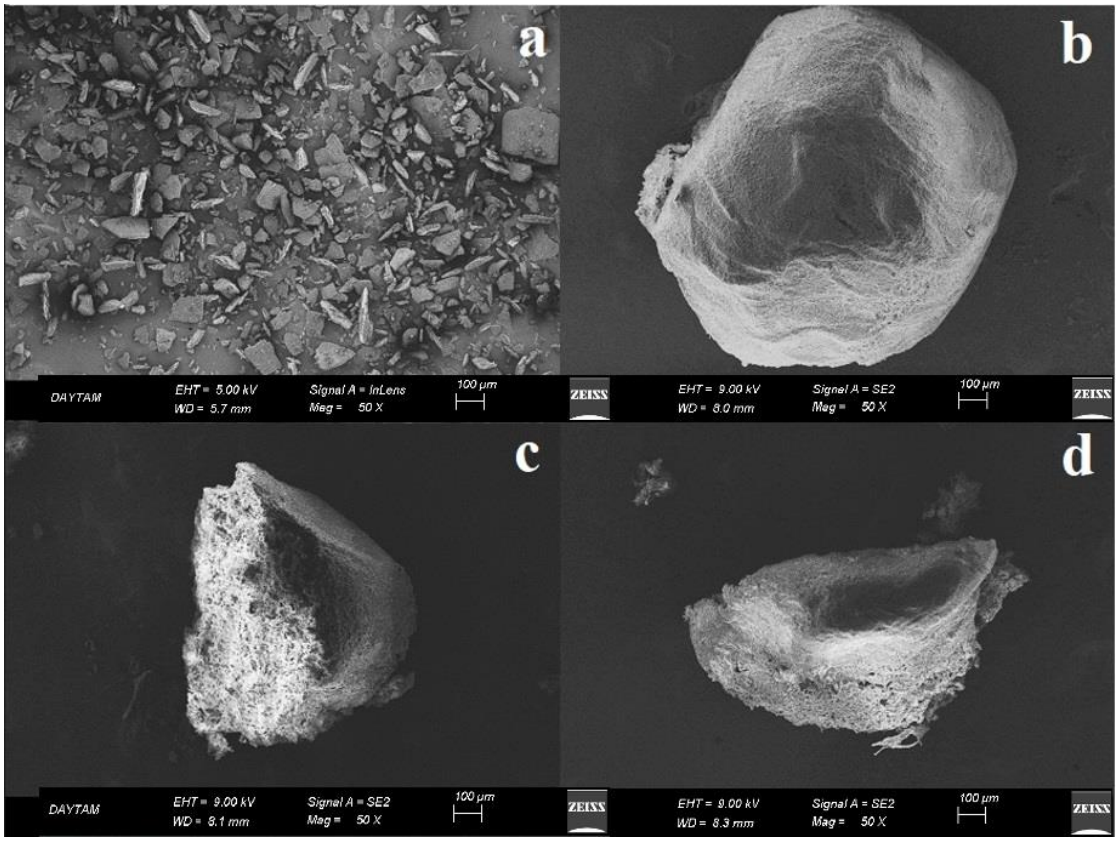


Figure 6. SEM images of hollowbeads and pure indomethacin (**a**:indomethacin, **b**:blank, **c**:F2, **d**:F19)

Table 9. Evaluation of the stability of hollowbeads for 12 months ($\bar{X}$±SD)

		EE%	**LC%**	**Buoyancy rate (%) (24 h)**	**Swelling rate (%) (24 h)**	**Release Mechanism pH 1.2**	**Release Mechanism pH 6.8**
Freshly prepared	F2	93.68±1.33	5.87±0.78	75	60	Super Case-II Transport	Non-Fickian diffusion
	F19	95.42±2.38	5.94±1.14	50	43	Super Case-II Transport	Non-Fickian diffusion
12 months later	F2	92.77±2.49	5.19±0.91	70	61.5	Super Case-II Transport	Non-Fickian diffusion
	F19	93.41±3.18	5.63±0.42	50	46	Super Case-II Transport	Non-Fickian diffusion

Modified release studies of indomethacin were examined in the literature. Similarities or differences of these studies with our study are mentioned below. Ekhodairy et al. designed colon-targeted tablets containing indomethacin and reported that the pectin-based formulation can be released for 16 hours, which is insoluble in the gastric media [19]. In another study, Abbas et al designed nanofibers containing indomethacin targeted to the colon. They were able to increase the release time up to 10 hours with formulations after passing into the intestine [37]. Damiati prepared microparticles indomethacin-loaded and conducted a release study at pH 7.4. He reported that 36% in the first 6 hours and 80% in total indomethacin release at the end of 9 days [33]. Sravani et al. developed pulsatile tablet formulations containing indomethacin. They conducted a release study of the formulations they obtained at pH 7.2 and reported that they achieved nearly 100% indomethacin release at the end of 4 hours [39]. Chauhan et al. reported that nearly 100% release occurred after 12 hours at pH 1.2 in indomethacin floating tablet formulations. The tablets were assumed to float for 12 hours and the study was terminated here [31]. In our study, we were observed that two most effective formulations floated at the rate of 75% and 50% (F2 and F19) even after 24 hours, thus maintaining the release of indomethacin for a longer period of time.

Among the anti-inflammatory drugs, indomethacin is one of the NSAIDs that exhibits the most effective inflammatory response (20 times more than acetylsalicylic acid). It exhibits side effects due to its non-ionization in the stomach medium. In particular, the potential to cause stomach ulcers, the most common side effect, limits the use of this effective NSAID. However, it is very effective in treating chronic inflammatory diseases such as ankylosing spondylitis, osteoarthritis, rheumatoid arthritis, and gout. Against the most critical side effect of indomethacin, our primary goal was to reduce the number of daily doses by prolonging the stay in the stomach (daily dose at least 2x1). Thus, we aimed to develop a formulation in which indomethacin can be used more safely and effectively with a single dose formulation that floats in gastric fluid for a long time and releases nearly 100% indomethacin in the intestinal medium for ~12 hours.

It has been determined that hollowbeads' spherical nature, size, encapsulation efficiency, efficiency, hollowness, and long-term release differ depending on the amount of NaCMC, the type of crosslinker ($ZnCl_2$, $CaCl_2$), the crosslinker concentration and the contact time with the crosslinker. Indomethacin can be released in both the stomach and intestines for at least 24 hours from our indomethacin-containing hollowbeads formulations. These formulations can stay for a long time in stomach and prevent the formation of stomach ulcers. Considering the oral dosage forms of indomethacin in the current drug market, we think that this formulation, which we have developed as an alternative, will be advantageous both in terms of reducing the dose frequency and eliminating the restriction of its use due to its side effects. It could be used safely in the future, when supported by preclinical and clinical studies, for those patients with anti-inflammatory diseases at risk of developing ulcer.

ACKNOWLEDGEMENTS

The authors are grateful to Atatürk University Scientific Research Projects (Project No: TLP-2021-8892) for financial support and Atatürk University, Eastern Anatolia High Technology Application and Research Center (DAYTAM) for technical support.

AUTHOR CONTRIBUTIONS

Concept: *E.Ö., R.S.Ö.*; Design: *E.Ö.*; Control: *E.Ö., R.S.Ö.*; Sources: *E.Ö, H.B.G.*; Materials: *E.Ö, H.B.G.*; Data Collection and/or processing: *E.Ö., H.B.G.*; Analysis and/or interpretation: *E.Ö., R.S.Ö., H.B.G.*; Literature review: *E.Ö.*; Manuscript writing: *E.Ö., H.B.G.*; Critical review: *E.Ö., R.S.Ö., H.B.G.*; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

REFERENCES

1. Hsu, Y.T., Kao, C.Y., Ho, M.H., Lee, S.P. (2021). To control floating drug delivery system in a simulated gastric environment by adjusting the Shell layer formulation. *Biomaterials Research, 25*(1), 31. [CrossRef]

2. Abdul, S., Chandewar, A.V., Jaiswal, S.B. (2010). A flexible technology for modified-release drugs: Multiple-unit pellet system (MUPS). *Journal of Controlled Release, 147*(1), 2-16. [CrossRef]

3. Uhrich, K.E., S.M. Cannizzaro, R.S. Langer, K.M. Shakesheff. (1999). Polymeric systems for controlled drug release. *Chemical Reviews, 99*(11), 3181-3198. [CrossRef]

4. Bhowmik, D., Goinath, H., Kumar, B.P., Duraivel, S., Kumar, K.P.S. (2012). Controlled Release Drug Delivery Systems. *Pharma Innovation 1*(10), 24-32.

5. Miyazaki, S., A. Nakayama, M. Oda, M. Takada, D. Attwood. (1995). Drug release from oral mucosal adhesive tablets of chitosan and sodium alginate. *International Journal of Pharmaceutics, 118*(2), 257-263. [CrossRef]

6. Ulbrich, K., Holá, K., Šubr, V., Bakandritsos, A., Tuček, J., Zbořil, R. (2016). Targeted drug

delivery with polymers and magnetic nanoparticles: covalent and noncovalent approaches, release control, and clinical studies. *Chemical Reviews, 116*(9), 5338-5431. [CrossRef]

7. Geraili, A., Xing, M., Mequanint, K. (2021). Design and fabrication of drug-delivery systems toward adjustable release profiles for personalized treatment. *View, 2*(5), 20200126. [CrossRef]

8. Srivastava, A., Yadav, T., Sharma, S., Nayak, A., Kumari, A.A., Mishra, N. (2015). Polymers in drug delivery. *Journal of Biosciences and Medicines, 4*(1), 69-84.

9. Rokhade, A.P., Agnihotri, S.A., Patil, S.A., Mallikarjuna, N.N., Kulkarni, P.V., Aminabhavi, T.M. (2006). Semi-interpenetrating polymer network microspheres of gelatin and sodium carboxymethyl cellulose for controlled release of ketorolac tromethamine. *Carbohydrate Polymers, 65*(3), 243-252. [CrossRef]

10. Kaushik, A.Y., Tiwari, A.K., Gaur, A. (2015). Role of excipients and polymeric advancements in preparation of floating drug delivery systems. *International Journal of Pharmaceutical Investigation, 5*(1), 1-12. [CrossRef]

11. Kumar, K., Dhawan, N., Sharma, H., Vaidya, S., Vaidya, B. (2014). Bioadhesive polymers: novel tool for drug delivery. *Artif Cells Nanomed Biotechnol, 42*(4), 274-283. [CrossRef]

12. Awasthi, R., Kulkarni, G.T. (2014). Development of novel gastroretentive drug delivery system of gliclazide: hollow beads. *Drug Development and Industrial Pharmacy, 40*(3), 398-408. [CrossRef]

13. Singh, B.N., Kim, K.H. (2000). Floating drug delivery systems: An approach to oral controlled drug delivery via gastric retention. *Journal of Controlled Release, 63*(3), 235-259. [CrossRef]

14. Sriamornsak, P., Asavapichayont, P., Nunthanid, J., Luangtana-Anan, M., Limmatvapirat, S., Piriyaprasarth, S. (2008). Wax-incorporated emulsion gel beads of calcium pectinate for intragastric floating drug delivery. *AAPS PharmSciTech, 9*(2), 571-576. [CrossRef]

15. Prajapati, S., Patel, L., Patel, C. (2011). Polymers for floating drug delivery system. *Systematic Reviews in Pharmacy, 2*(1), 1. [CrossRef]

16. Ramli, R.A. (2017). Hollow polymer particles: A review. *RSC Advances, 7*(83), 52632-52650. [CrossRef]

17. Huanbutta, K., Nernplod, T., Akkaramongkolporn, P., Sriamornsak, P. (2017). Design of porous Eudragit® L beads for floating drug delivery by wax removal technique. *Asian Journal of Pharmaceutical Sciences, 12*(3), 227-234. [CrossRef]

18. Lucas, S. (2016). The pharmacology of indomethacin. *Headache: The Journal of Head and Face Pain, 56*(2), 436-446. [CrossRef]

19. Elkhodairy, K.A., Elsaghir, H.A. , Al-Subayiel, A.M. (2014). Formulation of indomethacin colon targeted delivery systems using polysaccharides as carriers by applying liquisolid technique. *BioMed Research International, 2014*, 704362. [CrossRef]

20. El-Leithy, E.S., Ibrahim, H.K., Sorour, R.M. (2015). *In vitro* and *in vivo* evaluation of indomethacin nanoemulsion as a transdermal delivery system. *Drug Delivery, 22*(8), 1010-1017. [CrossRef]

21. Helleberg, L. (1981). Clinical pharmacokinetics of indomethacin. *Clinical Pharmacokinetics, 6*(4), 245-258. [CrossRef]

22. Vane, J.R., Botting, R.M. (1998). Mechanism of action of nonsteroidal anti-inflammatory drugs. *The American Journal of Medicine, 104*(3S1), 2S-8S. [CrossRef]

23. Suleyman, H., Albayrak, A., Bilici, M., Cadirci, E., Halici, Z. (2010). Different mechanisms in formation and prevention of indomethacin-induced gastric ulcers. *Inflammation, 33*(4), 224-234. [CrossRef]

24. Badve, S.S., Sher, P., Korde, A., Pawar, A.P. (2007). Development of hollow/porous calcium pectinate beads for floating-pulsatile drug delivery. *European Journal of Pharmaceutics and Biopharmaceutics, 65*(1), 85-93. [CrossRef]

25. Sevinç Özakar, R., Özakar, E. (2021). The effect of polymer amount and crosslinker ratio in polymeric hydrogel beads on characterization. *Journal of Research in Pharmacy, 25*(5). [CrossRef]

26. Taranalli, S.S., Dandagi, P.M., Mastiholimath, V.S. (2015). Development of hollow/porous floating beads of metoprolol for pulsatile drug delivery. *European Journal of Drug Metabolism and Pharmacokinetics, 40*(2), 225-233. [CrossRef]

27. Lin, H.R., Ou, L.H., Lin, Y.J., Ling, M.H. (2010). Hollow, pH-sensitive calcium–alginate/poly (acrylic acid) hydrogel beads as drug carriers for vancomycin release. *Journal of Applied Polymer Science, 118*(4), 1878-1886. [CrossRef]

28. Sevinç Özakar, R. (2022). Development and *in vitro* characterization of gastroretentive formulations as calcium pectinate hydrogel pellets of pregabalin by ionotropic gelation method. *Indian Journal of Pharmaceutical Education and Research, 56*(1), S9-S20. [CrossRef]

29. Sevinç Özakar, R., Ozakar, E. (2022). Different biopolymers' effects on the evaluation and characterization of floating tablets prepared by lyophilization technique to improve the quality control parameters. *Polymer-Korea, 46*(2), 145-158. [CrossRef]

30. Koca, M., Sevinç Özakar, R., Ozakar, E., Sade, R., Pirimoğlu, B., Özek, N.Ş., Aysin, F. (2022). Preparation and characterization of nanosuspensions of triiodoaniline derivative new contrast agent, and investigation into its cytotoxicity and contrast properties. *Iranian Journal of Pharmaceutical Research, 21*(1), e123824. [CrossRef]

31. Chauhan, Y.S., Kataria, U., Dashora, A. (2018). Formulation and evaluation of floating tablet for indomethacin. *Journal of Drug Delivery and Therapeutics, 8*(4), 338-345. [CrossRef]

32. Giri, T., Choudhary, C., Alexander, A., Badwaik, H., Tripathy, M., Tripathi, D. (2013). Sustained release of diltiazem hydrochloride from cross-linked biodegradable IPN hydrogel beads of pectin and modified xanthan gum. *Indian Journal of Pharmaceutical Sciences, 75*(6), 619. [CrossRef]

33. Damiati, S.A., Damiati, S. (2021). Microfluidic synthesis of indomethacin-loaded PLGA microparticles optimized by machine learning. *Frontiers in Molecular Biosciences, 8*, .677547. [CrossRef]

34. Verma, A., Kumar, P., Rastogi, V., Mittal, P. (2021). Preparation and evaluation of polymeric beads composed of chitosan–gellan gum–gum ghatti/-gum karaya polyelectrolyte complexes as polymeric carrier for enteric sustained delivery of diclofenac sodium. *Future Journal of*

Pharmaceutical Sciences, 7(1), 1-13. [CrossRef]

35. Chemate, S., Godge, G., Pawa, K., Rupnar, K. (2016). Preparation and evaluation of hollow calcium pectinate beads for floating-pulsatile drug delivery. *Turkish Journal of Pharmaceutical Sciences, 13*(1), 91-102.

36. Somani, V., Shahi, S., Udavant, Y., Atram, S., Satpute, R., Shinde, N. (2009). A floating pulsatile drug delivery system based on hollow calcium pectinate beads. *Asian Journal of Pharmaceutics (AJP), 3*(2). [CrossRef]

37. Akhgari, A., Heshmati, Z., Garekani, H.A., Sadeghi, F., Sabbagh, A., Makhmalzadeh, B.S., Nokhodchi, A. (2017). Indomethacin electrospun nanofibers for colonic drug delivery: *In vitro* dissolution studies. *Colloids and Surfaces B: Biointerfaces, 152*, 29-35. [CrossRef]

38. Penjuri, S.C.B., Damineni, S., Ravouru, N., Poreddy, S.R. (2017). Self-emulsifying formulation of indomethacin with improved dissolution and oral absorption. *Turkish Journal of Pharmaceutical Sciences, 14*(2), 108. [CrossRef]

39. Senthilnathan, B., Suba, V. (2013). Formulation and evaluation of pulsatile drug delivery of miglitol using pulsincap technology. *Inventi Rapid:NDDS. 12 (2)*, 557.

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 955-966, 2022
Doi: 10.33483/jfpau.1147465

ORIGINAL ARTICLE / ÖZGÜN MAKALE

PERCEPTION OF COMMUNITY PHARMACIST TOWARD WEIGHT LOSS PRODUCTS: KAHRAMANMARAŞ CITY SAMPLE

TOPLUM ECZACILARININ KİLO VERME ÜRÜNLERİNE KARŞI ALGISI: KAHRAMANMARAŞ İLİ ÖRNEĞİ

Nilay AKSOY[1*], Nazlı Gül ERDOĞAN[1]

[1]Altinbaş University, Faculty of Pharmacy, Department of Clinical Pharmacy, 34147, Istanbul, Turkey

ABSTRACT

Objective: *The purpose of this study is to better understand the role of community pharmacists in obesity management by observing their knowledge of weight loss drugs, analyzing their attitudes and behaviors toward patients, and determining their transfer patterns to patients.*

Material and Method: *After ethical approval, a cross-sectional study was conducted using an online questionnere via a Google online form. The research population consists of community pharmacists working in the province of Kahramanmaraş. The questionnaire was designed to assess Community pharmacists' knowledge and attitudes toward weight loss products. The data was then analyzed using the SPSS for Windows 26.0 program (Statistical Package for Social Sciences).*

Result and Discussion: *Eighty-six percent of the pharmacists reported always/often counselling customers who request to buy products for weigh management on the safe and effective use of the product and 69.9% always/often check for drug or food interaction while dispensing weight loss products. The majority of Community pharmacists (72.8%) agreed that continuous education of the pharmacist should include weight management and training. Pharmacists surveyed have a moderate knowledge of weight loss products. Time, staff, budget were the barriers to offering pharmacy weight management services. Continuing education, supporting pharmacists with sufficient resources, and conducting more comprehensive research can play a critical role in the fight against obesity.*

Keywords: *Community pharmacists, knowledge, obesity, perception, weight loss*

ÖZ

Amaç: *Bu çalışmanın amacı, toplum eczacıların kilo verme ilaçları hakkındaki bilgilerini gözlemleyerek, hastalara yönelik tutum ve davranışlarını analiz ederek ve hastalara aktarım şekillerini belirleyerek obezite yönetimindeki rolünü daha iyi anlamaktır.*

* **Corresponding Author / Sorumlu Yazar:** Nilay Aksoy
e-mail / e-posta: nilay.aksoy@altinbas.edu.tr

Submitted / Gönderilme: 23.07.2022 **Accepted / Kabul:** 25.08.2022

Gereç ve Yöntem: *Etik onaydan sonra, çevrimiçi Google formu aracılığıyla bir anket uygulanarak kesitsel bir çalışma yapılmıştır. Araştırma Kahramanmaraş ilinde çalışan toplum eczacılar üzerinden yürütülmüştür. Anketler, toplum eczacıların zayıflama ürünlerine yönelik bilgi ve tutumlarını değerlendirmek için tasarlanmıştır. Veriler daha sonra Windows 26.0 için SPSS programı (Statistical Package for Social Sciences) kullanılarak analiz edilmilştir.*

Sonuçlar ve Tartışma: *Yüzde seksen altı, kilo yönetimi için ürün satın almak isteyen müşterilere ürünün güvenli ve etkili kullanımı konusunda her zaman/sıklıkla danışmanlık yaptığını ve %69,9'u zayıflama ürünlerini dağıtırken ilaç-gıda etkileşimini her zaman/sıklıkla kontrol ettiğini bildirdi. toplum Eczacıların çoğunluğu (%72,8) eczacının sürekli eğitiminin kilo yönetimi ve eğitimini içermesi gerektiği konusunda hemfikirdi. Ankete katılan eczacılar, kilo verme ürünleri hakkında orta düzeyde bilgiye sahipti. Zaman, personel ve bütçe kısıtlılığı, eczacıların kilo yönetimi hizmetleri sunmalarının önünde engeldir. Eczacıların mezun olduktan sonra kilo yönetimi konusunda eğitimlerinin devam etmesi, etkili kilo yönetimi hizmetleri sunmaları için yeterli kaynaklarla eczacıların desteklenmesi ve daha kapsamlı araştırmalar yapılması obezite ile mücadelede kritik rol oynayabilir.*

Anahtar kelimeler: *Algı, bilgi, kilo verme, obezite, toplum eczacıları*

INTRODUCTION

Obesity is defined as an abnormal accumulation of fat in the body caused by excessive energy intake. It is a chronic multifaceted disorder. Numerous morbidities are linked to obesity that are progressively affecting a larger population. Many illnesses, such as type 2 diabetes (DM), cardiovascular disease, depression, and several malignancies are all made more likely by obesity. Although the Body Mass Index (BMI) is the most used parameter in obesity classification [1], other parameters such as waist circumference and body fat may be required when diagnosing obesity. In recent years, three treatment models for obesity have been used: lifestyle modifications, pharmacological treatment, and bariatric surgery. If weight reduction through lifestyle modifications is only transitory or the required goal couldn't be achieved, pharmacological treatment is required. Lorcaserin, Orlistat, Phentermine/Topiramate, and Naltrexone/Bupropion combinations are the most commonly used drugs for pharmacological treatment. Additionally, several newer promising agents are currently being evaluated. Neuropeptide Y antagonists, cannabinoid type-1 receptor blockers, lipase inhibitors, glucagon-like peptide-1 (GLP-1) analogues (Liraglutide), leptin analogues, amylin analogues, and anti-obesity vaccinations [2]. The most common surgical procedures for bariatric surgery are sleeve gastrectomy, Roux-en-Y gastric bypass, adjustable gastric band, and biliopancreatic diversion. Over-the-counter weight loss products, dietary and herbal supplements are also popular among overweight and obese patients [3].

Liraglutide is a GLP-1 receptor agonist used in in the treatment of DM and obesity. Liraglutide stimulates insulin release while inhibiting glucagon secretion, slows gastric emptying, and increases satiety after a meal. Liraglutide 3.0 mg daily subcutaneous injection was recently authorized by the FDA for the treatment of obesity or overweight as well as comorbidities associated with obesity [4]. For the most part, greater liraglutide dosages are needed to treat type 2 diabetes [5].

A scoping review stated the critical role of community pharmacists in managing weight loss programs and treating obesity [6]. An Australian pilot study that examined the impact of a pharmacist-led, non-product-centered weight control program found improvements in outcomes like weight, BMI, and waist circumference after three months. Diet, exercise, and behavioral adjustment counseling were the main interventions [7]. Data from a weight-control program created by community pharmacists in the United Kingdom were retrospectively analyzed, and the results showed that the pharmacists' efforts resulted in significant weight loss and waist circumference reduction [8]. Gómez-Martinez et al. observed that the patient-oriented weight management approach had a positive effect on the improvement of all clinical and therapeutic outcomes in research to ascertain its effects in community pharmacies [9].

MATERIAL AND METHOD

A cross-sectional study was conducted using a questionnaire administered in a Google form and distributed online via WhatsApp or e-mail to all pharmacies in the Kahramanmaraş province in Turkey. The questionnaire is divided into five sections, each of which explores the sociodemographics variables of pharmacists' and pharmacy's patient population's, community pharmacists' opinions about their role in weight management, and pharmacy's patient population's sociodemographics., evaluation of community pharmacist knowledge, community pharmacists' current practices and attitudes toward weight management services and barriers to community pharmacists providing weight management services. The questionnaire items were gathered from medical literature, translated into Turkish by two academicians from the clinical pharmacy department who are native speakers, and then tested in a pilot study before being given to community pharmacists [10,11]. The Cronbach's alpha was greater than 0.6 for the translated questionner.

The study was approved by the Altinbas University ethical committee (No: 06, Date: 06.01.2022). The questionnaires were then distributed online to all pharmacists working in Kahramanmaraş pharmacies between 15.02.2022 to 09.03.2022. According to data acquired from the Turkish pharmacist association, Kahramanmaraş has 335 pharmacies with one pharmacist at least employed there. Other pharmacists who work in fields other than community pharmacy or who are unwilling to participate in the survey were barred from taking part.

The acquired data was analyzed using the Statistical Package for Social Sciences (SPSS, version 26). The characteristics of the individuals, as well as their views, habits, barriers, and knowledge, were summarized using frequencies and proportions.

RESULT AND DISCUSSION

103 community pharmacists took part in the study with a response rate of 31%, 57.3 % were female. It is not surprising that more female pharmacists than male pharmacists participated in our study, given that women outnumber men in the pharmacy workforce globally [12] and in Turkey, particularly in Kahramanmaraş. Ages 20 to 29 made up 62.1% of the participants. 19.4 % of the participants aged 30-39, 10.7% of those aged 40-49, and 7.9 % of those aged 50-59. The majority of community pharmacists, 61.2 % had 0-5 years of experience in community pharmacy, 18.4% had 6-10 years, 7.8% had 11-15 years, 6.8% had 16-25 years while 5.8% had 26 years or more experience. The most common age range of patients visited the pharmacy is young adults' range (20-29 years) (47.6%). 67% of participants reported often/sometimes to frequency of demand for weight loss products in their pharmacy, besides that the most common age range of customers that want to buy weight loss products from participants' pharmacy is young adults (89.3%). Demographic variables of the pharmacists and their patient population were listed in Table 1.

In our study, we found a promising higher percentage of pharmacists 69.9 % always/often evaluate the drug or food interaction while dispensing weight loss products, the majority of them were always/often asking the patients to eat low calorie diet, do more exercise, and consume more soluble fiber (63.1%), these rates were 77.6 % and 78.6 %, respectively in study conducted in Lebanon [10]. However, the majority of participants (65.7%) stated that they do not provide BMI calculations, Likewise, 40.7% and 72.1% of the pharmacists in Hijazi et al study and Almukdad study rarely or never measured patients BMI [10,11]. However, the pharmacists in other countries such as United Kingdom were highly participated in practices like calculation of BMI (67.4%) in their pharmacies [13].

In contrast to the Almukdad study, which found that only 34.9 % of the community pharmacists referred patients to other healthcare providers such as dieticians [11], referral to dieticians was common in our study (75.7 %). The majority of community pharmacists (62.1 %) said they often question patients about any adverse effects or unpleasant responses they may have experienced after using weight-loss drugs, and 51.4% said they regularly report any toxicity or negative consequences of these products. Data from the survey conducted in Lebanon, however, shows comparable results, with percentages of 66.8%, 73.6%, and 35.7%, respectively [10]. Almukdad et al mentioned that 56.9% of the pharmacists were counseling their patient regarding the adverse effects of weight loss products [11]. Overall, although the weight management practices performed by community pharmacists in Kahramanmaraş are somewhat in line with those of previous studies in Qatar, Lebanon, Scotland, Australia, and Kuwait and United Kingdom, improvements in these practices are needed [10,11-13-15]. Any pharmacist should not disregard patient counseling regarding the adverse effects of weight loss products because serious adverse effects that can accompany medication use or herbal medicine use can occur at any time [16,17].

Additionally, numerous studies suggest using other anthropometric measurements to categorize obesity, such as body fat and waist circumference, and encourage the pharmacists to use these measurements while providing weight-management treatments [18]. In our investigation, community pharmacists' existing practices for offering weight control treatments are shown in Table 2.

Table 1. Demographic variables of the pharmacists and their patient population

QUESTIONS	**n=103**	**%**
Gender of the pharmacists		
Female	59	57.3 %
Male	45	43.7%
Age of the pharmacists		
20-29 years old	64	62.1%
30-39 years old	20	19.4%
40-49 years old	11	10.7%
50-59 years old	8	7.8 %
Years of experience		
0-5 years old	63	61.2%
6-10 years old	19	18.4%
11-15 years old	8	7.8%
16-25 years old	7	6.8%
26 or more years old	6	5.8%
When you consider all the patients who apply to your pharmacy with or without a prescription, what is the most common age range for patients?		
Chidren	2	1.9%
Adults	49	47.6%
Middle Age (50-65)	33	32.0%
Elder (≥65)	17	16.5%
What is the frequency of demand for weight loss products in your pharmacy?		
Always	1	1.0%
Often	24	23.3%
Sometimes	45	43.7%
Rarely	30	29.1%
No	1	1.0%
What is the age range of patients who want to buy weight loss products from your pharmacy		
Chidren	2	1.9%
Adults	92	89.3%
Middle age (50-65)	7	6.8%
Elder (≥65)	0	0%
Do you give information to your patients who will use liraglutide about how to apply or how to store the drug?		
Always	45	43.7%
Often	18	17.5%
Sometimes	19	18.4%
Rarely	8	7.8%
No	5	4.9%

Table 2. Current practice of community pharmacists in providing weight management services.

QUESTIONS	n=103	%
Do you check for drug-drug interaction or food-drug interaction while dispensing weight loss products?		
Always	32	31.1
Often	40	38.8
Sometimes	20	19.4
Rarely	7	6.8
No	0	0
Dou you give nonpharmacological recommendations to your costumers such as following low calorie diet, increasing physical activities for weight loss?		
Always	33	32.0
Often	32	31.1
Sometimes	18	17.5
Rarely	9	8.7
No	7	6.8
Do you provide weight and height, waist circumference measurements for patients?		
Always	25	24.3
Often	21	20.4
Sometimes	18	17.5
Rarely	21	20.4
No	13	12.6
Do you provide BMI calculation for your patients?		
Always	15	14.6
Often	16	15.5
Sometimes	27	26.2
Rarely	24	23.3
No	17	16.5
Do you refer your patient to dieticians when needed?		
Always	21	20.4
Often	39	37.9
Sometimes	24	23.3
Rarely	12	11.7
No	4	3.9
Do you ask patients for any adverse effect or undesirable reaction after taking weight loss products?		
Always	31	30.1
Often	33	32.0
Sometimes	24	23.3
Rarely	10	9.7
No	2	1.9
Do you report any toxicity or adverse reaction of weight loss products?		
Always	23	22.3
Often	30	29.1
Sometimes	21	20.4
Rarely	20	19.4
No	5	4.9

Most of the community pharmacists in our study have a strong belief that they play an important role in the field of weight management (65%), ongoing education of pharmacists should incorporate exercise and weight management (72.8%), weight management products should be sold only in pharmacies (82.6%) likewise agreeing or highly agreeing that consumers misuse weight-loss medications. (74.7%). According to research done in Scotland, Australia, and Lebanon, participants clearly believed that pharmacists played a crucial role in weight control. This was confirmed by pharmacists' assessments of the provision of weight management services [7,10, 13,19].

Indeed, media and advertisements have both positive and negative effects on our lives. There is no doubt that if we can standardize and validate information related to drug products, including weight loss products, we will benefit greatly from media and advertisements. The Kahramanmaraş pharmacists agreed or strongly agreed that commercials and the media are doing a good job of teaching consumers about weight reduction products and weight control [10] only 48.9% of pharmacists agreed or strongly agreed with the benefit of media and advertisements. We can explain the pharmacists' disagreement in our study and the Lebanon study by stating that the pharmacists have doubts about the standardization and validation of information published in the media. Table 3 displays community pharmacists' opinions on their involvement in weight control.

When we assessed the community pharmacists' knowledge of weight loss products, we discovered that while more than half of them answered most of the questions correctly, there was still a significant number of them who had incorrect answers, but their percentage remained consistent with previous studies. For example, 68 % of participants in our study and 51% of participants in the Lebanon study correctly answered, 'once the weight loss goal is met, there is no risk in discontinuing treatment question. When comparing Kahramanmaraş community pharmacists and Lebanon community pharmacists, the correct answers were somewhat similar for other knowledge questions except for two questions, “consuming green tea can harm liver cells, and orlistat shouldn't be used by anyone with cardiovascular problems”, which were answered correctly in the rate of 83.5% and 72.8% of pharmacists in our study, while in Lebanon study, 17.3% and 54.6% respectively [10]. Table 4 rates the knowledge of weight control among neighborhood pharmacists.

In our study the barriers to provide weight management services in community pharmacies, as ranked by the participant pharmacists limited number of staff who provide weight management services (42,8%), limited budget (39.7%), limited time (38.7%), limited knowledge (35.7%), lack of interest (21.4%). Barriers to provide weight management services in community pharmacies in our study presented in Table 5.

Table 3. Community pharmacists' beliefs towards their role in weight management

QUESTIONS	**n=101**	**%**
Continuous education of the pharmacist should include weight management and training		
Strongly Agree	30	29.1
Agree	45	43.7
Neutral	22	21.4
Disagree	3	2.9
Strongly Disagree	0	0
Do you think the public is conscious about weight loss products?		
Strongly Agree	6	5.8
Agree	2	1.9
Neutral	8	7.8
Disagree	50	48.5
Strongly Disagree	34	33.0
Do you think pharmacists give enough information to patients about weight loss products?		
Strongly Agree	3	2.9
Agree	24	23.3
Neutral	45	43.7
Disagree	25	24.3
Strongly Disagree	2	1.9
Do you believe that pharmacists have a role to play in the field of weight management?		
Strongly Agree	17	16.5
Agree	50	48.5
Neutral	20	19.4
Disagree	10	9.7
Strongly Disagree	1	1.0
Do you think that weight loss products should be sold only in pharmacies?		
Strongly Agree	63	61.2
Agree	22	21.4
Neutral	11	10.7
Disagree	2	1.9
Strongly Disagree	1	1.0
Do you think that patients are abusing weight loss products		
Strongly Agree	37	35.9
Agree	40	38.8
Neutral	19	18.4
Disagree	3	2.9
Strongly Disagree	0	0
Do you think that companies marketing weight loss products are making false promises?		
Strongly Agree	43	41.7
Agree	31	30.1
Neutral	23	22.3
Disagree	1	1.0
Strongly Disagree	1	1.0

Table 3 (***continued***). Community pharmacists' beliefs towards their role in weight management

QUESTIONS	**n=101**	**%**
Do you think that media and advertisements are playing a positive role in educating patients towards weight loss products and weight management		
Strongly Agree	51	49.5
Agree	34	33.0
Neutral	13	12.6
Disagree	1	1.0
Strongly Disagree	0	0

Table 4. Evaluation of self-knowledge towards weight management among community pharmacists

Question	**True/False**	**% of correct answer**	**% of incorrect answer**
Once the weight loss goal is achieved, there is no risk in stopping the treatment.	F	68	28.6
Laxatives are considered a beneficial method for weight loss in people with obesity or overweight individuals.	F	78.6	16.5
Herbal Laxatives (like senna, cascara, v) are recommended for pregnant or breastfeeding women.	F	78.6	16.5
Consumption of green tea can cause toxicity in liver cells.	T	83.5	10.7
Chronic use of laxatives can increase the effect of diuretics, which causes fluid and electrolyte loss.	T	81.6	12.6
Orlistat is contraindicated in patients with cardiovascular disease.	T	72.8	20.4
Liraglutide can be used for weight loss in Type 1 Diabetes Mellitus patients.	F	60.2	34

Table 5. The challenges of community pharmacists in offering weight control treatments.

QUESTIONS	**n=98**	**%**
I don't have enough staff to provide weight management services	42	42.8
I don't have budget to provide weight management services	39	39.7
I don't have enough time to provide weight management services	38	38.7
I don't have enough knowledge to provide weight management services	35	35.7
I don't have the interest to provide weight management services	21	21.4
I don't have enough space to provide weight management services	0	0

The barrier that presented in Newlands et al study, include high workload (n = 77, 92.8%) and the need for further compensation (75.9%) and insufficient staff (59.7%) [13]. Infrastructure, time, and cost-effectiveness were determined to be the key obstacles preventing community pharmacists from participating in weight management services in another study [7]. The most anticipated barriers to obesity counseling in a study done in Kuwait were a lack of patient awareness about pharmacists'

expertise in counseling these types of products (76.2%), while the pharmacists' beliefs that obese patients lack willpower and are non-adherent to weight loss interventions (71.8%) [20]. Similary, Lack of time, excessive work demands, and a lack of advertising are obstacles to running a weight loss program in a community pharmacy in England, according to Peletidi and Kayyali [21]. Age, pharmacy type, highest level of education, and work status of Malaysian community pharmacists are sociodemographic and practice variables that have an impact on attitudes, practices, and perceived barriers related to weight management services [22]. Peletidi and Kayyali Although it is outside the scope of our study, it is important to note that numerous studies in the medical literature show the significant advantages of weight-loss programs managed by community pharmacists. With these programs, significant reductions in waist circumference, systolic and diastolic blood pressure, and lipid levels were seen [23,24].

Finally, we can conclude that the Kahramanmaraş community pharmacists polled have a basic understanding of weight loss products. Additionally, there were significant obstacles to Kahramanmaraş community pharmacists providing weight control treatments in terms of time, manpower, and money. Overall, the study's conclusions shed light on community pharmacists in Kahramanmaraş's attitudes, knowledge, and beliefs towards weight loss. Continuing education for pharmacists on weight management after graduation, providing pharmacists with adequate resources to provide effective weight management services, and conducting more comprehensive research on this topic can all help in the fight against obesity. The study's findings could be used to guide future evidence-based community pharmacist-led weight control services on a national and global scale.

AUTHOR CONTRIBUTIONS

Concept: *N.A*; Design: *N.A*; Control: *N.A, N.E*; Sources: *N.A, N.E*; Materials: *N.A, N.E*; Data Collection and / or Processing: *N.A, N.E*; Analysis and / or Interpretation: *N.A, N.E*; Literature Review: *N.A, N.E*; Manuscript Writing: *N.A*; Critical Review: *N.A, N.E*; Other: *N.A, N.E.*

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The study was approved by the Altınbaş University ethical committee (No: 06, Date: 06.01.2022**).**

REFERENCES

1. Hruby, A., Hu, F. B. (2014). The epidemiology of obesity: A big picture. *PharmacoEconomics, 33*(7), 673–689. [CrossRef]

2. Bhat, S. P., Sharma, A. (2017). Current drug targets in obesity pharmacotherapy – A review. *Current Drug Targets, 18*(8), 983–993. [CrossRef]

3. Kissane, N. A., Pratt, J. S. (2011). Medical and surgical treatment of obesity. Best practice & research. *Clinical anaesthesiology, 25*(1), 11–25. [CrossRef]

4. Clements, J. N., Shealy, K. M. (2015). Liraglutide: An injectable option for the management of obesity. *The Annals of Pharmacotherapy, 49*(8), 938–944. [CrossRef]

5. Whitten, J. S. (2016). Liraglutide (Saxenda) for weight loss. *American Family Physician, 94*(2), 161-166.

6. Rosenthal, M., Ward, L. M., Teng, J., Haines, S. (2018). Weight management counselling among community pharmacists: A scoping review. *The International Journal of Pharmacy Practice, 26*(6), 475–484. [CrossRef]

7. Um, I. S., Krass, I., Armour, C., Gill, T., Chaar, B. B. (2015). Developing and testing evidence-based weight management in Australian pharmacies: A Healthier Life Program. *International Journal of Clinical Pharmacy, 37*(5), 822–833. [CrossRef]

8. Boardman, H. F., Avery, A. J. (2014). Effectiveness of a community pharmacy weight management programme. *International Journal of Clinical Pharmacy, 36*(4), 800–806. [CrossRef]

9. Gómez-Martinez, J., López-Pintor, E., Lumbreras, B. (2020). Effectiveness of a Patient-Centered Weight Management Model in a Community Pharmacy: An Interventional Study. *Patient Preference and Adherence, 14*, 1501–1511. [CrossRef]

10. Hijazi, M. A., Shatila, H., El-Lakany, A., Al Rifai, H., Aboul-Ela, M., Naja, F. (2020). Role of community pharmacists in weight management: Results of a national study in Lebanon. *BMC Health Services Research, 20*(1), 386. [CrossRef]

11. AlMukdad, S., Zaghloul, N., Awaisu, A., Mahfoud, Z. R., Kheir, N., El Hajj, M. S. (2021). Exploring the role of community pharmacists in obesity and weight management in Qatar: A Mixed-Methods Study. *Risk Management and Healthcare Policy, 14*, 2771–2787. [CrossRef]

12. Bates, I., John, C., Bruno, A., Fu, P., Aliabadi, S. (2016). An analysis of the global pharmacy workforce capacity. *Human Resources for Health, 14*(1), 61. [CrossRef]

13. Newlands, R. S., Watson, M. C., Lee, A. J. (2011). The provision of current and future Healthy Weight Management (HWM) services from community pharmacies: A survey of community pharmacists' attitudes, practice and future possibilities. *The International Journal of Pharmacy Practice, 19*(2), 106–114. [CrossRef]

14. Fakih, S., Marriott, J. L., Hussainy, S. Y. (2015). A national mailed survey exploring weight management services across Australian community pharmacies. *Australian Journal of Primary Health, 21*(2), 197–204. [CrossRef]

15. Krska, J., Lovelady, C., Connolly, D., Parmar, S., Davies, M. J. (2010). Community pharmacy contribution to weight management: Identifying opportunities. *The International Journal of Pharmacy Practice, 18*(1), 7–12. [CrossRef]

16. Lunsford, K. E., Bodzin, A. S., Reino, D. C., Wang, H. L., Busuttil, R. W. (2016). Dangerous dietary supplements: Garcinia cambogia-associated hepatic failure requiring transplantation. *World Journal of Gastroenterology, 22*(45), 10071–10076. [CrossRef]

17. Yen, M., Ewald, M. B. (2012). Toxicity of weight loss agents. Journal of medical toxicology: O*fficial journal of the American College of Medical Toxicology, 8*(2), 145–152. [CrossRef]

18. Nimptsch, K., Konigorski, S., Pischon, T. (2019). Diagnosis of obesity and use of obesity biomarkers in science and clinical medicine. *Metabolism: Clinical and Experimental, 92*, 61–70. [CrossRef]

19. Zieck, M., Um, I. S., Chaar, B. B. (2018). The future of weight management in pharmacy education - Perspectives of new generation pharmacists. *Currents in Pharmacy Teaching & Learning, 10*(5), 596–601. [CrossRef]

20. Awad, A., Waheedi, M. (2012). Community Pharmacists role in obesity treatment in Kuwait: A cross-sectional study. *BMC Public Health, 12*, 863. [CrossRef]

21. Peletidi, A., Kayyali, R. (2019). Experiences of the pharmacy-led weight management service: Views of service providers in England. *Pharmacy, 7*(3), 82. [CrossRef]

22. Verma, R. K., Paraidathathu, T., Taha, N. A., Chong, W. W. (2019). Attitudes, practices, and barriers of Malaysian community pharmacists toward provision of weight management services. *Frontiers in Pharmacology, 10*, 138. [CrossRef]

23. Ahrens, R. A., Hower, M., Best, A. M. (2003). Effects of weight reduction interventions by community pharmacists. *Journal of the American Pharmacists Association, 43*(5), 583-589. [CrossRef]

24. Clements, J. N., Albanese, N. P., D'Souza, J. J., Misher, A., Reece, S., Trujillo, J., Whitley, H. P. (2021). Clinical review and role of clinical pharmacists in obesity management: An opinion of the endocrine and metabolism practice and research network of the American College of Clinical Pharmacy. *Journal of the American College of Clinical Pharmacy, 4*(11), 1469-1484. [CrossRef]

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 967-978, 2022
Doi: 10.33483/jfpau.1139157

ORIGINAL ARTICLE / ÖZGÜN MAKALE

DETERMINATION OF ALPHA-2-MACROGLUBULIN IN SERUM SAMPLES

SERUM ÖRNEKLERİNDE ALFA-2-MAKROGLUBULİN TAYİNİ

Süreyya ÖZCAN KABASAKAL[1*]

[1]Middle East Technical University (METU), Department of Chemistry, 06880, Ankara, Turkey

ABSTRACT

Objective: *Proteomics is one of the fastest growing omics that has been extensively used in clinical studies. Proteomics involves qualitative and quantitative protein analysis in a wide range of samples starting from a single cell to complex biological samples. Protein-based biomarker studies have been applied to many diseases including metabolic diseases, cancer and neuropsychiatric diseases for both diagnostic and prognostic purposes. Alpha-2-macroglubulin (A2MG) is a clinically relevant secreted protein involving in various biological processes including blood coagulation, protein binding and protease inhibition. Current methods for A2MG analysis are limited, as they focus on either immune-specific binding through a certain protein unit or a unique peptide. As a single protein could be in different forms (complexes, modifications, etc) and the biological activity is structure specific, an extensive analysis is necessary. Here a new Mass-Spectrometry (MS) based method was developed for comprehensive A2MG analysis.*

Material and Method: *A reference human serum and A2MG protein standard were used for method development. Proteolytic protein digestion was performed using trypsin and Circular-Dichroism (CD) spectroscopy was used to ensure protein unfolding and denaturation prior to digestion. Targeted MS method was developed to monitor 12 unique peptides for A2MG in serum.*

Result and Discussion: *Monitoring multiple peptides for a single protein enabled to observe biological differences offer a robust and reliable A2MG analysis in serum. The method can also easily be implemented to other proteins. The concept of targeted-MS provides an ideal quantification and validation platform which then can be easily transferred to clinical laboratories.*

Keywords: *Alpha-2-macroglobulin, serum, targeted proteomics*

ÖZ

Amaç: *Proteomik, klinik çalışmalarda yaygın olarak kullanılan en hızlı büyüyen omiklerden biridir. Proteomik, tek bir hücreden başlayarak karmaşık biyolojik örneklere kadar geniş bir örnek yelpazesinde kalitatif ve kantitatif protein analizini içerir. Protein bazlı biyobelirteç çalışmaları, metabolik hastalıklar, kanser ve nöropsikiyatrik hastalıklar dahil olmak üzere birçok hastalığa hem tanısal hem de prognostik*

* **Corresponding Author / Sorumlu Yazar:** Sureyya Ozcan Kabasakal
e-mail / e-posta: sozcan@metu.edu.tr

Submitted / Gönderilme: 01.07.2022 **Accepted / Kabul:** 02.09.2022

amaçlarla uygulanmıştır. Alfa-2-makroglubulin (A2MG), kan pıhtılaşması, protein bağlanması ve proteaz inhibisyonu dahil olmak üzere çeşitli biyolojik süreçlerde yer alan, klinik önemi olan ve salgılanan bir proteindir. A2MG analizi için mevcut yöntemler, belirli bir protein birimi veya benzersiz bir peptit yoluyla immün spesifik bağlanmaya odaklandıklarından sınırlıdır. Tek bir protein farklı formlarda (kompleksler, modifikasyonlar, vb.) olabileceğinden ve biyolojik aktivite yapıya özel olduğundan, kapsamlı bir analiz gereklidir. Bu çalışmada kapsamlı A2MG analizi için yeni bir Kütle Spektrometresi (MS) tabanlı yöntem geliştirildi.

Gereç ve Yöntem: *Bu çalışmada, kapsamlı A2MG analizi için yeni bir Kütle Spektrometresi (MS) tabanlı yöntem geliştirilmiştir. Analitik yöntem geliştirme referans insan serumu ve A2MG protein standardı ile yapılmıştır. Proteolitik protein sindirimi için tripsin kullanılmış ve sindirimden önce ve sonra proteinin denatürasyonu Dairesel-Dikroizm (CD) spektroskopisi kullanılarak test edilmiştir. Hedefli MS yöntemi, serumda A2MG için 12 benzersiz peptidi izlemek için geliştirilmiştir.*

Sonuç ve Tartışma: *Bu çalışmada, biyolojik farklılıkları gözlemlemek için geliştirilen tek bir protein için çoklu peptitlerin ölçülmesi ile sağlam ve güvenilir serumda A2MG analizi geliştirilmiştir. Yöntem, diğer proteinlere de kolayca uygulanabilir. Hedeflenen MS konsepti, daha sonra klinik laboratuvarlara kolayca aktarılabilen ideal bir niceleme ve doğrulama platformu sağlayacaktır.*

Anahtar Kelimeler: *Alpha-2-macroglobulin, hedefli proteomik, serum*

INTRODUCTION

Proteins are the main targets for biomarkers as they are involved in biosynthesis, cell, tissue and organ signaling, cell and tissue structural stability in organisms. Rapidly evolving mass spectrometry (MS)- based technologies in proteomics enable to study proteins in a wide range of samples starting from a single cell[1] to complex biological samples such as blood [2] and tissue. Protein-based biomarker studies have been applied to various diseases including cancer [3] , inflammatory diseases [2, 3], cardiovascular diseases [6], diabetes [7] and even psychiatric disorders [6, 9].

Alpha-2-macroglobulin (A2MG, Uniprot ID: P01023) is a tetrameric glycoprotein with a molecular weight of 725 kDa. It is involved in various biological processes including blood coagulation, protein binding and protease inhibition [10]. It is synthesized by liver and secreted in various biological fluids including serum, saliva, cerebral spinal fluid, ocular fluid and tissues. The average A2MG level in human blood is 1.5-4.0 mg/ml [11].

A2MG is an acute phase protein that can be used to monitor immunological response for both inflammation and infection [12]. Thus, it is also utilized to track the progression of pathological changes in animal models to unravel the inflammatory and infectious diseases [12]. Recent studies have also shown that A2MG has increased significantly in patients who showed severe COVID-19 symptoms. Consequently, the protein is involved in critical biological processes and a valuable biomarker for clinical studies. Hence, a reliable and reproducible quantification of A2MG in serum is vital.

Current methods commonly used in proteomics are two- dimensional gel electrophoresis (2DE) [13], enzyme-linked immunosorbent assays (ELISAs) [14], protein arrays and Mass Spectrometry (MS) based technologies [18,19,20]. Although immunoassays are considered as the gold standard in clinical laboratories, they suffer from several drawbacks such as non-specific binding, batch to batch variation

and cross reactivity [15]. MS based methods have several advantages over immunoassays as they require less sample, provide better specificity due to tandem capacity, low cost and reproducibility [19 -21].

MS based protein quantification is peptide centric and only unique peptide(s) is/are used for both protein identification and quantification. However, a single protein often has more than one representative peptide but only limited number of peptides are used in common practice. In order to reflect the actual biological changes, methods should be validated and the clinical utility must be assessed for all target analytes.

Here, a new targeted MS method was developed for the simultaneous determination of A2MG unique peptides in human serum. This study is the most comprehensive method up to now covering multiple unique peptides for A2MG. The selective measurement in complex biological samples such as serum and plasma provide a reliable quantification and validation platform. Thus, it can be easily transferred to clinical laboratories and enable large scale screening.

MATERIAL AND METHOD

Materials

A reference human serum and A2MG protein standard were obtained from Sigma-Aldrich (St. Louis, MO USA). HPLC grade water, acetonitrile, and formic acid were purchased from Merck (Darmstadt, Germany). Dithiothreitol (DTT), iodoacetamide (IAA), and ammonium bicarbonate (ABC) were purchased from Sigma- Aldrich (St. Louis, MO USA). Sequencing grade trypsin was obtained from Promega (Madison, WI USA).

Protein Digestion

Proteolytic digestion was performed according to the previous study [21]. Five µL of serum sample and 5 µL of A2MG protein standard (0.1 µg/µL) were added to an Eppendorf tube containing 105 µL of 50 mM ammonium bicarbonate. Prior to enzymatic degradation, disulfide bonds were reduced with 10 µL of 45 mM dithiothreitol (DTT) and proteins were denaturated at 65 ◦C for 45 minutes. Cysteine residues were then alkylated using 10 µL of 100mM Iodoacetamide (IAA) for 30 minutes at room temperature. Enzymatic digestion was performed by trypsin at 1:20 (enzyme: protein) molar ratio for 16 hours. Samples were stored at -80 ◦C prior to MS analysis.

Circular-dichroism (CD) Spectroscopy

A 0.2 mg/mL pure A2MG dissolved in 10 mM phosphate buffer (potassium dihydrogen phosphate/disodium hydrogen phosphate) at pH 7.8 was used for CD experiments. CD spectra were recorded with an Jasco J-1500 Circular Dichroism Spectrometer between 180 and 600 nm at 1 point/sec

scan speed. Measurements were carried out at 20 ◦C, using 1 nm band-width and 0.5 nm step size, in a quartz cuvette with 10 mm optical path length. CD spectra were normalized by subtracting the signal of the blanks (10 mM phosphate buffer).

Targeted MS Analysis

Quantitation of twelve A2MG unique peptides was performed by an Agilent 1260 Infinity II HPLC system coupled to an Agilent 6470A triple-quadrupole (QQQ) system (Santa Clara, CA). Peptide separation was performed by Infinity Lab Poroshell 120 EC C18 (3.0 x 150 mm, 2.7 microns) column at 50 ºC over a gradient elution. Flow rate was 0.3 ml/min, mobile phase A was 0.1% formic acid in LC-MS grade water, and mobile phase B was 0.1% formic acid in LC-MS grade acetonitrile. The mass spectrometer was operated in positive mode. The dynamic Multiple Reaction Monitoring (MRM) acquisition was used, the cycle time was 500 ms and the dwell time was at least 20 ms. The voltage of the fragmentor was set to 135 V. The MS method was optimized using Skyline software package (version 21.1.0). The predominant charge state and interference free transitions were screened for all unique peptides. An Agilent MassHunter Quantitative Analysis software was used for data acquisition and processing.

Data Analysis

Raw MS data was processed using the Skyline software package (version 21.1.0) [22]. The peaks were integrated and the peak areas were calculated. Two-stage normalization were applied. In the first stage, the peak areas were normalized using total peak intensities. Then log 2 normalization was applied to normalize the changes that may occur during the analysis.

RESULT AND DISCUSSION

The A2MG protein was first investigated to identify unique peptides and their locations on the crystal structure. The structure has been obtained from Protein Data Bank (PDB; 4ACQ). The peptide uniqueness was confirmed via Uniprot human proteome database. The sequence yields 15 unique tryptic peptides. The position of the peptides on the protein structure is shown in Figure 1. Two peptides, VGFYESDVMGR and LVHVEEPHTETVR are unstructured in the PDB, thus they are excluded from the figure. The tryptic peptide PLLVEPEGLEK is represented as DTVIKPLLVEPEGLEK containing one mis-cleavage. The trypsin is less sufficient when the cleavage site is close to a Proline (P) residue.

The efficiency of the protein denaturation was investigated using CD spectroscopy. CD Spectroscopy was used for checking the unfoldedness of the protein upon addition of a reducing agent (DTT), and heating. It was also used for confirming the complete digestion. For this purpose, CD spectra of the protein in the buffer, after DTT and heat treatment, and after digestion were measured (Figure 2).

A2MG in the phosphate buffer before any treatment shows a CD spectrum, suggesting a secondary structure comprising alpha helices and beta sheets, as seen in its three-dimensional structure. After adding DTT, and heating of the protein, the CD spectrum changed to have features of polyproline II conformation which is a signature for the secondary structure of unfolded state of proteins. After trypsin digestion, A2MG lost all secondary structure elements, suggesting the complete degradation.

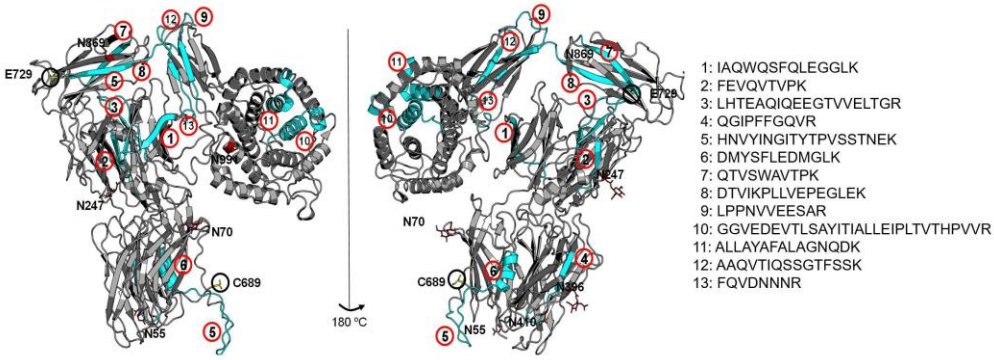


Figure 1. The A2MG protein structure and the position of unique peptides (shown in turquoise) in the protein sequence

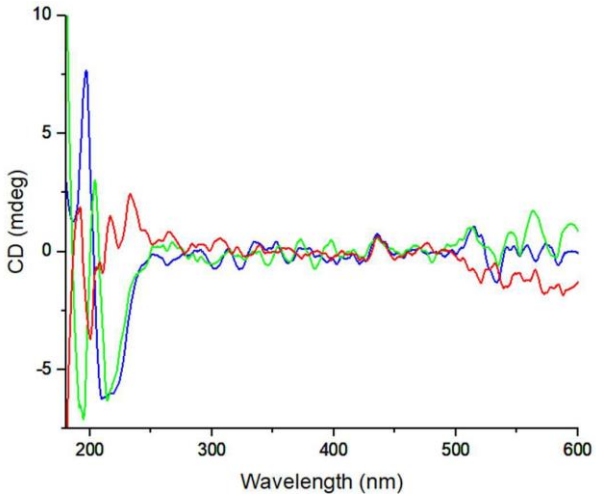


Figure 2. CD spectra of A2MG in phosphate buffer (blue), after addition of DTT and heating (green), and after digestion (red). The changes in secondary structure suggest the transition from unfolding to complete digestion.

Targeted MS method was developed to profile peptides for A2MG. Thirteen tryptic peptides representing A2MG were used for the determination of A2MG. Peptides were digested from the standard protein. Predominant charges and transitions were screened to identify qualifier and quantifier transitions. One out of thirteen peptides, QTVSWAVTPK showed poor ionization. Thus, it was not

included in the study. Remaining twelve peptides were further investigated to ensure reliable and reproducible measurements. Peptide fragmentations were optimized to yield specific and sensitive measurement.

The chromatographic separation for 12 A2MG peptides is shown in Figure 3. The method enables simultaneous quantification at different time windows. Overlapping peaks correspond to fragments of each peptide that can be used as qualifier transitions. First standard A2MG protein was digested to optimize the instrumental parameters. Figure 3a represents the corresponding peptide separation. Then the same method was applied to the serum digest to identify potential interferences that might come from biological matrix (Figure 3b). The most intense and interference free transitions were used for quantification purposes. Table 1 lists the specific precursors, charge states of each peptide, qualifier and quantifier transitions shown in Figure 3c. The same unique peptides were identified in both standard protein and serum samples however, peptide profiles were slightly different. The difference could be related to the sample matrix. Total protein concentration in serum is higher than that of pure protein standard. Therefore, digestion efficiency might be different. Future studies should focus on quantitative protein-unique peptide relationship.

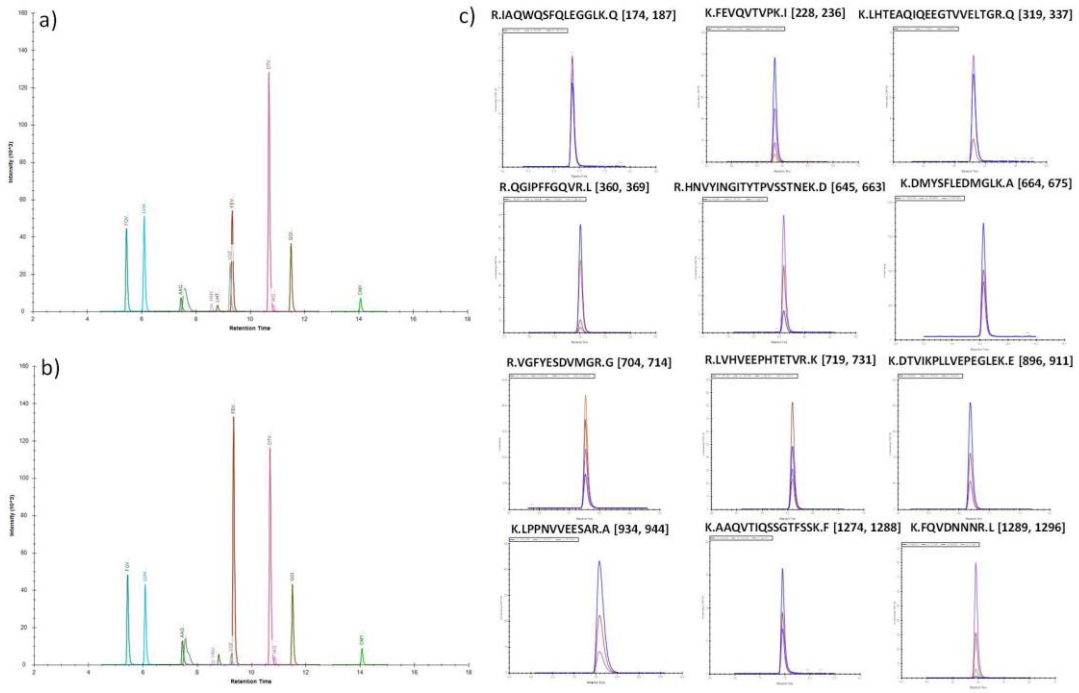


Figure 3. Chromatographic separation of A2MG peptides in the a) A2MG standard b) standard human serum digest and c) qualifier and quantifier transitions for 12 target peptides.

The reproducibility of peptide measurements was further investigated. Table 2 lists the replicates (N=6) log 2 normalized peak intensities for all 12 A2MG unique peptides.

Relative peptide abundances of A2MG proteins in the protein standard and human serum are shown in Figure 4. Each peptide was color coded and were normalized based on total intensities. All twelve A2MG peptides were detected in both samples. Peptides DTVIKPLLVEPEGLEK, FEVQVTVPK, FQVDNNNR, LPPNVVEESAR, LVHVEEPHTETVR and QGIPFFGQVR are the most intense peptides in all samples. However, relative abundances showed discrepancy between the standard protein and human serum. While the commonly used DTVIKPLLVEPEGLEK containing 1-miscleavage is the most abundant in the pure protein, FQVDNNNR is the dominant peptide in human serum.

A2MG is a clinically relevant protein associated with neurological diseases, liver fibrosis, and many inflammatory diseases. It is an abundant protein secreted in blood. Thus, it has a great potential to be utilized in clinical studies.

MS is emerging as a standard analytical method to quantify proteins in complex biological samples. The bottom-up approach involves protein digestion and quantification based on proteolytic peptides. In order to ensure the specificity of the measurements, only unique peptide(s) are used for this purpose. However, the number of unique peptides for each target protein varies depending on protein size and sequence. Often a limited number of peptides are used in clinical studies. Recently, Plubell et al. disputed using peptides from a protein can be misleading as an important biological difference may be lost while focusing on a single unit. The peptide-centric approach involves protein denaturation and proteolytic digestion using specific proteases such as trypsin. In order to verify the change on protein structure after denaturation and digestion, a CD analysis was performed. The DTT and heat treatment caused A2MG to unfold from its native state. The completeness of digestion was confirmed by measuring the CD spectrum after trypsin digestion. The spectrum showed signature signals demonstrating the degradation of the protein into peptides.

The abundance of specified fragment ions is indicative of the abundance of peptides and proteins in the sample. As there is often one unique peptide for each protein, it is important to monitor all to identify the most relevant one(s). There are two fundamental parameters affecting the peak intensities; proteolytic digestion and peptide ionization. Both parameters were investigated in this study by replicate measurements of standard A2MG protein and human serum samples. A reproducible digestion was observed in all samples. The ionization of a peptide depends on the size and the amino acid composition. The QTVSWAVTPK, GGVEDEVTLSAYITIALLEIPLTVTHPVVR and ALLAYAFALAGNQDK peptides showed poor ionization and were excluded from the method.

Table 1. Transition list and method parameters for A2MG

Peptide	Precursor Ion	MS1 Res	Transition	MS2 Res	Dwell	Fragmentor	Collision Energy	Polarity
IAQWQSFQLEGGLK	535.62	Unit	744.43	Unit	20	130	14.5	Positive
IAQWQSFQLEGGLK	535.62	Unit	616.37	Unit	20	130	14.5	Positive
IAQWQSFQLEGGLK	535.62	Unit	616.31	Unit	20	130	14.5	Positive
FEVQVTVPK	523.80	Unit	770.48	Unit	20	130	14.1	Positive
FEVQVTVPK	523.80	Unit	671.41	Unit	20	130	14.1	Positive
FEVQVTVPK	523.80	Unit	543.35	Unit	20	130	14.1	Positive
FEVQVTVPK	523.80	Unit	704.36	Unit	20	130	14.1	Positive
LHTEAQIQEEGTVVELTGR	704.03	Unit	931.52	Unit	20	130	20.5	Positive
LHTEAQIQEEGTVVELTGR	704.03	Unit	773.45	Unit	20	130	20.5	Positive
LHTEAQIQEEGTVVELTGR	704.03	Unit	793.42	Unit	20	130	20.5	Positive
LHTEAQIQEEGTVVELTGR	704.03	Unit	832.91	Unit	20	130	20.5	Positive
QGIPFFGQVR	574.81	Unit	850.46	Unit	20	130	15.9	Positive
QGIPFFGQVR	574.81	Unit	753.40	Unit	20	130	15.9	Positive
QGIPFFGQVR	574.81	Unit	606.34	Unit	20	130	15.9	Positive
QGIPFFGQVR	574.81	Unit	425.73	Unit	20	130	15.9	Positive
HNVYINGITYTPVSSTNEK	713.02	Unit	962.48	Unit	20	130	20.9	Positive
HNVYINGITYTPVSSTNEK	713.02	Unit	861.43	Unit	20	130	20.9	Positive
HNVYINGITYTPVSSTNEK	713.02	Unit	431.22	Unit	20	130	20.9	Positive
HNVYINGITYTPVSSTNEK	713.02	Unit	638.82	Unit	20	130	20.9	Positive
DMYSFLEDMGLK	724.83	Unit	1039.51	Unit	20	130	21.3	Positive
DMYSFLEDMGLK	724.83	Unit	952.48	Unit	20	130	21.3	Positive
DMYSFLEDMGLK	724.83	Unit	805.41	Unit	20	130	21.3	Positive
VGFYESDVMGR	630.29	Unit	1160.50	Unit	20	130	17.9	Positive
VGFYESDVMGR	630.29	Unit	956.41	Unit	20	130	17.9	Positive
VGFYESDVMGR	630.29	Unit	793.35	Unit	20	130	17.9	Positive
VGFYESDVMGR	630.29	Unit	664.31	Unit	20	130	17.9	Positive
LVHVEEPHTETVR	515.94	Unit	667.33	Unit	20	130	13.8	Positive
LVHVEEPHTETVR	515.94	Unit	598.80	Unit	20	130	13.8	Positive
LVHVEEPHTETVR	515.94	Unit	420.22	Unit	20	130	13.8	Positive
LVHVEEPHTETVR	515.94	Unit	707.37	Unit	20	130	13.8	Positive
DTVIKPLLVEPEGLEK	594.01	Unit	672.36	Unit	20	130	16.6	Positive
DTVIKPLLVEPEGLEK	594.01	Unit	782.47	Unit	20	130	16.6	Positive
DTVIKPLLVEPEGLEK	594.01	Unit	732.94	Unit	20	130	16.6	Positive
DTVIKPLLVEPEGLEK	594.01	Unit	336.68	Unit	20	130	16.6	Positive
LPPNVVEESAR	605.82	Unit	1000.51	Unit	20	130	17.0	Positive
LPPNVVEESAR	605.82	Unit	690.34	Unit	20	130	17.0	Positive
LPPNVVEESAR	605.82	Unit	549.28	Unit	20	130	17.0	Positive
LPPNVVEESAR	605.82	Unit	500.76	Unit	20	130	17.0	Positive
AAQVTIQSSGTFSSK	756.39	Unit	1142.57	Unit	20	130	22.4	Positive
AAQVTIQSSGTFSSK	756.39	Unit	1041.52	Unit	20	130	22.4	Positive
AAQVTIQSSGTFSSK	756.39	Unit	928.44	Unit	20	130	22.4	Positive
AAQVTIQSSGTFSSK	756.39	Unit	800.38	Unit	20	130	22.4	Positive
FQVDNNNR	503.74	Unit	859.40	Unit	20	130	13.3	Positive
FQVDNNNR	503.74	Unit	731.34	Unit	20	130	13.3	Positive
FQVDNNNR	503.74	Unit	632.27	Unit	20	130	13.3	Positive
FQVDNNNR	503.74	Unit	517.25	Unit	20	130	13.3	Positive

Table 2. Replicate A2MG peptide measurements (N=6) Log 2 normalized peak intensities for all 12 unique peptides.

A2MG Peptides	**Area (Log2)**		
Peptide Sequence	**Mean**	**Stdev**	**CoV**
AAQVTIQSSGTFSSK	15.24	0.22	1.5
DMYSFLEDMGLK	15.15	0.27	1.8
DTVIKPLLVEPEGLEK	15.05	0.26	1.7
FEVQVTVPK	14.95	0.37	2.4
FQVDNNNR	14.91	0.38	2.5
HNVYINGITYTPVSSTNEK	14.87	0.37	2.5
IAQWQSFQLEGGLK	14.84	0.37	2.5
LHTEAQIQEEGTVVELTGR	14.76	0.27	1.8
LPPNVVEESAR	14.71	0.22	1.5
LVHVEEPHTETVR	14.65	0.15	1.1
QGIPFFGQVR	15.09	1.33	8.8
VGFYESDVMGR	15.52	1.73	11.2

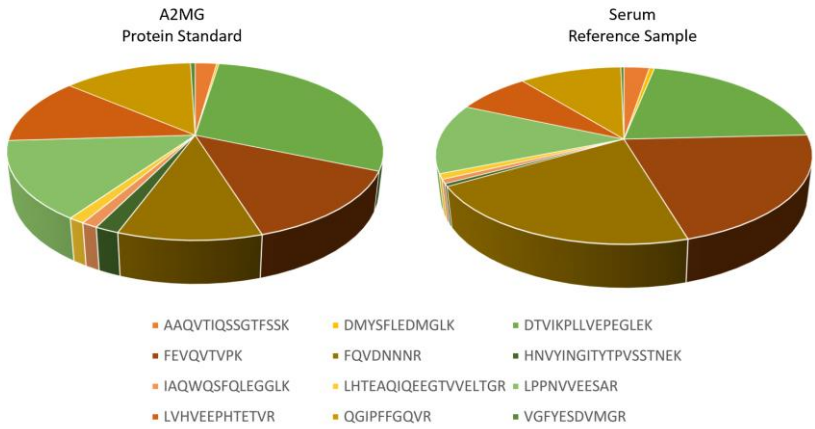


Figure 4. Relative peptide abundance of A2MG peptides in serum and protein standard respectively.

The MS method performance was developed and optimized for 12 peptides that are all specific to A2MG.The method performance was tested against a standard human protein and a reference serum sample. The comparison showed that although the top 6 peptides, DTVIKPLLVEPEGLEK, FEVQVTVPK, FQVDNNNR, LPPNVVEESAR, LVHVEEPHTETVR and QGIPFFGQVR, are the most abundant in both samples, the relative intensities of those showed differences. The difference could be associated with the sample matrix. While a standard protein is relatively simple as it is isolated and purified, the human serum involves hundreds of proteins. Therefore, the digestion performance could be different, Zhang et al. previously stated that the different digestion protocols may also affect the targeted quantification from protein-protein interaction analysis [23-24]. The change in peptide intensities should be further investigated to understand the root cause of the observed differences. Conventional methods used in clinical proteomics target either specific binding point or single/limited unique peptide(s). Proposed approach offers comprehensive screening by monitoring multiple unique

peptides for A2MG. Although results indicate reproducible measurements between replicate samples for each peptide, the relative peptide abundances show different pattern in reference serum than that of protein standard. Therefore, the variation in unique peptide responses raise a number of important questions; i) is the variation due to matrix affect? ii) are digestion efficiencies different in the standard and serum? iii) Is protein concentration directly proportional to individual peptides? iv) Are all unique peptides correlated to each other? v) If not, which unique peptide gives more reliable information and above all iv) Are the differences related to the biological complexity considering the presence of different protein forms. Consequently, monitoring multiple unique peptides for a single protein offers great advantages and provide comprehensive information. The approach can easily be implemented to other proteins and clinical studies. Future studies should focus on the gaps described above.

Here, a new method was developed to profile all the unique peptides isolated from A2MG.This method is the most comprehensive method available to date for the determination of A2MG. Monitoring multiple peptides for a single protein enables to observe unseen differences at peptide level. The concept of targeted-MS provides an ideal quantification and validation platform for clinical applications.

ACKNOWLEDGEMENTS

This work was supported by the METU BAP (AGEP-103-2019-10105).

AUTHOR CONTRIBUTIONS

Concept: *S.O.K.*; Design: *S.O.K.*; Control: *S.O.K.*; Sources: *S.O.K.*; Materials: *S.O.K.*; Data Collection and/or processing: *S.O.K.*; Analysis and/or interpretation: *S.O.K.*; Literature review: *S.O.K.*; Manuscript writing: *S.O.K.*; Critical review: *S.O.K.*; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

REFERENCES

1. Perkel, J. M. (2021). Single-cell proteomics takes centre stage. *Nature*. *597*, 580–582. [CrossRef]

2. Geyer, P. E., Voytik, E., Treit, P. V, Doll, S., Kleinhempel, A., Niu, L., Müller, J. B., Buchholtz, M., Bader, J. M., Teupser, D., Holdt, L. M., Mann, M. (2019). Plasma Proteome Profiling to detect and avoid sample-related biases in biomarker studies. *EMBO Molecular Medicine 11*(11), 1-12. [CrossRef]

3. Macklin, A., Khan, S., Kislinger, T. (2020) Recent advances in mass spectrometry based clinical proteomics: Applications to cancer research. *Clinical Proteomics*. *17*, 1–25. [CrossRef]

4. Titz, B., Gadaleta, R. M., Sasso, G. Lo, Elamin, A., Ekroos, K., Ivanov, N. V., Peitsch, M. C., Hoeng, J. (2018). Proteomics and Lipidomics in Inflammatory Bowel Disease Research: From Mechanistic Insights to Biomarker Identification. *International Journal of Molecular Sciences*, *19*, 2775. [CrossRef]

5. Okada, K., Itoh, H., Ikemoto, M. (2021). Serum complement C3 and α 2-macroglobulin are potentially useful biomarkers for inflammatory bowel disease patients. *Heliyon*. *7*(3), e06554. [CrossRef]

6. Stanley, B. A., Gundry, R. L., Cotter, R. J., Van Eyk, J. E. (2004). Heart disease, clinical proteomics and mass spectrometry. *Disease Markers*, *20*, 167–178. [CrossRef]

7. Roverso, M., Dogra, R., Visentin, S., Pettenuzzo, S., Cappellin, L., Pastore, P., Bogialli, S. (2022). Mass spectrometry-based "omics" technologies for the study of gestational diabetes and the discovery of new biomarkers. *Mass Spectrometry Reviews*, 1-38. [CrossRef]

8. Cooper, J. D., Ozcan, S., Gardner, R. M., Rustogi, N., Wicks, S., Van Rees, G. F., Leweke, F. M., Dalman, C., Karlsson, H., Bahn, S. (2017). Schizophrenia-risk and urban birth are associated with proteomic changes in neonatal dried blood spots. *Translation Psychiatry*, *7*, 1290, 1-14. [CrossRef]

9. Yeon, S., Han, S., Cooper, J. D., Ozcan, S., Rustogi, N., Penninx, B. W. J. H., Bahn, S. (2019). Integrating proteomic, sociodemographic and clinical data to predict future depression diagnosis in subthreshold symptomatic individuals. *Translational Psychiatry*, *9*, 277. [CrossRef]

10. Vandooren, J., Itoh, Y. (2021) Alpha-2-Macroglobulin in Inflammation, Immunity and Infections. *Frontiers in Immunology*, *12*, 5411. [CrossRef]

11. Birkenmeier, G., Müller, R., Huse, K., Forberg, J., Gläser, C., Hedrich, H., Nicklisch, S., Reichenbach, A. (2003). Human α2-macroglobulin: Genotype–phenotype relation. *Experimenatl Neurology*, *184*, 153–161. [CrossRef]

12. Gautreaux, M. A., Tucker, L. J., Person, X. J., Zetterholm, H. K., Priddy, L. B. (2022). Review of immunological plasma markers for longitudinal analysis of inflammation and infection in rat models. *Journal of Orthopaedic Research : Official Publication of the Orthopaedic Research Society*, *40*(6), 1251-1262 [CrossRef]

13. Gygi, S. P., Corthals, G. L., Zhang, Y., Rochon, Y., Aebersold, R. (2000). Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. *Proceedings of the National Academy of Sciences of the United States*, *97*, 9390–9395. [CrossRef]

14. Tighe, P. J., Ryder, R. R., Todd, I., Fairclough, L. C. (2015). ELISA in the multiplex era: Potentials and pitfalls. *Proteomics. Clinical Applications*, *9*, 406–422 [CrossRef]

15. Rappsilber, J., Mann, M. (2002). What does it mean to identify a protein in proteomics? *Trends*

in Biochemical Sciences, *27*, 74–78. [CrossRef]

16. Kim, M. S., Pinto, S. M., Getnet, D., Nirujogi, R. S., Manda, S. S., Chaerkady, R., Madugundu, A. K., Kelkar, D. S., Isserlin, R., Jain, S., Thomas, J. K., Muthusamy, B., Leal-Rojas, P., Kumar, P., Sahasrabuddhe, N. A., Balakrishnan, L., Advani, J., George, B., Renuse, S., Selvan, L. D. N., Patil, A. H., Nanjappa, V., Radhakrishnan, A., Prasad, S., Subbannayya, T., Raju, R., Kumar, M., Sreenivasamurthy, S. K., Marimuthu, A., Sathe, G. J., Chavan, S., Datta, K. K., Subbannayya, Y., Sahu, A., Yelamanchi, S. D., Jayaram, S., Rajagopalan, P., Sharma, J., Murthy, K. R., Syed, N., Goel, R., Khan, A. A., Ahmad, S., Dey, G., Mudgal, K., Chatterjee, A., Huang, T. C., Zhong, J., Wu, X., Shaw, P. G., Freed, D., Zahari, M. S., Mukherjee, K. K., Shankar, S., Mahadevan, A., Lam, H., Mitchell, C. J., Shankar, S. K., Satishchandra, P., Schroeder, J. T., Sirdeshmukh, R., Maitra, A., Leach, S. D., Drake, C. G., Halushka, M. K., Prasad, T. S. K., Hruban, R. H., Kerr, C. L., Bader, G. D., Iacobuzio-Donahue, C. A., Gowda, H., Pandey, A. (2014). A draft map of the human proteome. *Nature*, *509*, 575–581. [CrossRef]

17. Geyer, P. E., Holdt, L. M., Teupser, D., Mann, M. (2017). Revisiting biomarker discovery by plasma proteomics. *Molecular Systems Biology 13*, 942, 1-15. [CrossRef]

18. Glish, G. L., Vachet, R. W. (2003). The basics of mass spectrometry in the twenty-first century. *Nature Reviews Drug Discovery*, *2*, 140–150. [CrossRef]

19. Liebler, D. C., Zimmerman, L. J. (2013). Targeted Quantitation of Proteins by Mass Spectrometry. *Biochemistry*, *52*, 3797–3806. [CrossRef]

20. Mesaros, C., Blair, I. A. (2016). Mass spectrometry-based approaches to targeted quantitative proteomics in cardiovascular disease. *Clinical Proteomics*, *13*, 20, 1-18. [CrossRef]

21. Ozcan, S., Cooper, J. D., Lago, S. G., Kenny, D., Rustogi, N., Stocki, P., Bahn, S. (2017). Towards reproducible MRM based biomarker discovery using dried blood spots. *Scientific Reports*, *7*, 45178. [CrossRef]

22. MacLean, B., Tomazela, D. M., Shulman, N., Chambers, M., Finney, G. L., Frewen, B., Kern, R., Tabb, D. L., Liebler, D. C., MacCoss, M. J. (2010). Skyline: An open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics*, *26*, 966–968. [CrossRef]

23. Goldstein, F. W. (2007). Combating resistance in a challenging, changing environment. *Clinical Microbiology and Infection*, *13*, 2–6. [CrossRef]

24. Zhang, Y., Sun, H., Zhang, J., Brasier, A. R., Zhao, Y. (2017). Quantitative Assessment of the Effects of Trypsin Digestion Methods on Affinity Purification-Mass Spectrometry-based Protein-Protein Interaction Analysis. *Journal of Proteome Research*, *16*, 3068–3082. [CrossRef]

Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 46(3): 979-992, 2022
Doi: 10.33483/jfpau.1117954

ÖZGÜN MAKALE / ORIGINAL ARTICLE

HEKİM-ECZACI İŞBİRLİĞİNE YÖNELİK TUTUM ÖLÇEĞİNİN TÜRKÇE'YE UYARLANMASI: TIP VE ECZACILIK ÖĞRENCİLERİ ÖRNEKLEMİ

TURKISH ADAPTATION OF THE SCALE OF ATTITUDES TOWARD PHYSCIAN-PHARMACIST COLLABORATION: MEDICINE AND PHARMACY STUDENTS SAMPLE

Betül AKALIN[1*], Zeynep DÖRTBUDAK[2]

[1]Sağlık Bilimleri Üniversitesi, Sağlık Bilimleri Fakültesi, Sağlık Yönetimi Ana Bilim Dalı, 34668, İstanbul, Türkiye

[2] Okan Üniversitesi, Lisansüstü Eğitim Enstitüsü, 34394, İstanbul, Türkiye

ÖZ

Amaç: *Bu çalışma kapsamında meslek eğitimi alan öğrencilerde Hojat ve Gonnella (2011) tarafından geliştirilen Hekim-Eczacı İşbirliğine Yönelik Tutum Ölçeğinin Türkçe uyarlamasının yapılması amaçlanmıştır.*

Gereç ve Yöntem: *Çalışmanın örneklemini Sağlık Bilimleri Üniversitesi Tıp ve Eczacılık Fakültesi öğrencileri (N=189) oluşturmaktadır. Ölçeğin Türkçe uyarlamasının yapılması amacıyla dil, kapsam, yapı geçerliliği ve güvenilirliği sınanmıştır.*

Sonuç ve Tartışma: *Gerçekleştirilen açımlayıcı faktör analizi neticesinde toplam varyansın %60,41'ini açıklandığı ve ifadelerin iki faktör altında toplandığı ve toplam varyansın %52,59'unu birinci faktörün ve %7,82'sını ikinci faktörün açıkladığı tespit edilmiştir. Orijinal ölçekle uyumlu olarak 16 ifadeden oluşan ölçeğin alt boyutları İş Birliği ve Ekip Çalışması, Sorumluluk olarak adlandırılmış, ölçeğin iç tutarlılık katsayısı ,938 olarak bulunmuştur. Araştırmadan elde edilen bulgular neticesinde Hekim-Eczacı İşbirliğine Yönelik Tutum Ölçeğinin Türk kültürüne uygun, geçerli ve güvenilir bir ölçme aracı olduğu sonucuna varılmıştır.*

Anahtar Kelimeler: *Eczacı, hekim, mesleklerarası iş birliği*

ABSTRACT

Objective: *The aim of this study is the Turkish adaptation of the Scale of Attitudes Toward Physician-Pharmacist Collaboration-(SATP^{2}C) developed by Hojat and Gonnella (2011) in a professional education sample.*

* **Sorumlu Yazar / Corresponding Author:** Betül Akalın
e-posta / e-mail: betul.akalin@sbu.edu.tr, **Tel. / Phone:** +905325959782

Gönderilme / Submitted: 17.05.2022 **Kabul / Accepted:** 14.09.2022

Material and Method: *The study sample consists of 189 students of the Faculty of Medicine and Pharmacy at the University of Health Sciences Turkey. The Turkish adaptation of the scale was performed through language, content, reliability and construct validity testing.*

Result and Discussion: *Results of the exploratory factor analysis show that the scale items explained 60,41% of the total variance under two factors(Factor 1: 52,59%; Factor 2: 7,82%.). Concurent with the original scale, the internal consistency coefficient of the 16-item scale was ,938 and the two dimensions were were titled Collaboration and Teamwork, Accountability. The findings of this study show that SATP^{2}C is a valid and reliable instrument suitable for use in the Turkish language and culture.*

Keywords: *Interprofessional collaboration, pharmacist, physician*

GİRİŞ

Sağlık hizmeti sunumu, birçok farklı meslek disiplininin bir araya gelerek hizmet ürettikleri çalışma alanlarındandır. Bu yapıda başarının sağlanması için organizasyondaki her bir halka arasında iş birliği ve eşgüdüm gereklidir. Profesyoneller arası iş birliği "belirli rolleri olan, birbirine bağlı görevleri yerine getiren ve ortak bir hedefi paylaşan" iki veya daha fazla sağlık uzmanının hasta bakımına getirdiği uzmanlığa ve katkıya değer veren müzakere edilmiş bir anlaşma olarak tanımlanırken, gerekli düzeyde başarılı iş birliği istenmeyen olayları engeller, hasta yatış süresini kısaltır, hasta sonuçlarını iyileştirerek memnuniyet düzeyini artırır [1,2,3].

Hekimler, hasta odaklı tedaviye yönelik planlama ve karar alma sürecinde veri kaynağı olarak ihtiyaç duydukları bilgilere erişim sürecinde teorik kitaplar, akademik yayınlar, uzman görüşü gibi pek çok kaynağa başvurabilmektedirler. Hekimlerin hastaya ilaç yazdıkları reçeteler, eczacılarla doğrudan iletişim amacı taşımaktadır. Eczacılar ve Eczaneler Hakkındaki 2014 tarihli yönetmelikte eczacıların görev ve sorumlulukları hastaya ilaç sunumunun 'hastanın gereksinimleriyle uyumlu, güvenli ve akılcı' olmasını da içermekte olup, hastanın ilaç uyunç sürecinde eczacı, hekimin tedavi başarısında önemli bir rol oynamaktadır [4]. Özellikle ülkemizde, eczacıların hastayla iletişimi sosyal olarak sıcak ve hekimin yazdığı ilaçların doğru kullanımına yönelik açıklayıcı bir danışmanlığı da içermekte olup, bu empatik iletişim, bakım ve tedavi çıktılarını olumlu etkileyebilme potansiyeli içermektedir. Buna karşın, yapılan çalışmalarda hekimlerin büyük bir kısmının eczacıların aldıkları mesleki müfredat ve görevlerinin kapsamına tam olarak vakıf olmadığı ve güven eksikliği, yetersiz iletişim gibi sebeplerle eczacılara başvurmadığını göstermektedir [5,6]. Oysa eczacılar farmasötik açıdan sağlık ekibinin çok önemli bir parçası olup; hekim ve eczacılar arasındaki doğru iş birliği, hastaların doğru ilaç kullanımı ve ilaç yönetiminin doğru işletilmesi sağlık hizmeti sonuçlarının iyileştirilmesinde etkilidir [7,8]. Yapılan çalışmalar özellikle kronik hastalıkları olan ve/veya düzenli ilaç incelemeleri gerektiren hastalar üzerinde doğru antibiyotik kullanımı, enfeksiyon kontrolü, hasta uyumunun artırılması ile advers etkinin azaltılması gibi birçok alanda yüz güldürücü sonuçlar doğurduğunu göstermektedir [9,10,11]. Seostianin ve arkadaşları tarafından yayınlanan bir çalışmada, hekim ve eczacıların hasta bakımı sırasında video görüşmeleri/yüzyüze görüşmelerle sürdürebilecekleri işbirliğinin gerek farmakoterapi uygunluğu ve

tedavinin güvenliliğini arttıracağı, gerekse ilaç bağlantılı problemlerin sıklığını azaltabileceği vurgulanmıştır [12]. Türkiye'de 2018 yılında yapılan bir pilot çalışmada da 'birinci basamak sağlık hizmetlerinde yapılan yasal düzenlemeler sonrasında' serbest eczacıların %60'ının hasta bakımı sürecinde hekimlerle gerek ilaç uygulaması gerekse ilaç etkileşimleri konusunda iletişim isteği belirttikleri bulgulanmıştır [13].

Ülke literatüründe sağlık profesyonelleri arasında multidisipliner iş birliğini değerlendiren ölçekler bulunmakla birlikte çalışma ortamında veya eğitim sürecinde Hekim-Eczacı iş birliğine yönelik bir ölçeğe rastlanmamıştır. İngilizce orijinalinden dilimize uyarlanan Hekim-Eczacı İşbirliğine Yönelik Tutum Ölçeği (Scale of Attitudes Toward Physician-Pharmacist Collaboration), her iki meslekten öğrencilerin disiplinlerarası işbirliğini destekleyen eğitim müfredatlarının oluşturulmasına katkı sağlamak, hekim ve eczacıların işbirlikçi tutumları arasındaki farkları karşılaştırmak ve profesyoneller arasındaki işbirlikçi çalışmanın klinik sonuçlarını ortaya koymak amacıyla kullanılmaktadır [14,15]. Çalışmada orijinal ölçeğin Türk dili ve kültürüne uyarlanması sürecinde, orijinal soru formunun dil çevirisi ve içerik geçerliliğinin sağlanmasına ilişkin gerekli metodolojik düzen uygulanmıştır. Bu çalışmanın amacı, tıp / eczacılık öğrencileri ile hekim/ eczacılara yönelik iş birliği tutumlarını değerlendirmeye yönelik Hekim-Eczacı İşbirliğine Yönelik Tutum Ölçeğinin Türk diline uyarlanmasıdır.

GEREÇ VE YÖNTEM

Bu araştırma kapsamında, Mayıs 2019 - Mayıs 2020 tarihleri arasında Hojat and Gonnella tarafından geliştirilen 16 maddelik Scale of Attitudes Toward Physician-Pharmacist Collaboration ($SATP^2C$) ölçeğinin Türkçeye uyarlanması amacı ile geçerlilik güvenirlik çalışması yapılmıştır.

Dil Çevirisi

İlk aşamada ölçek anadili Türkçe olan ve anadiline yakın düzeyde İngilizce bilen bağımsız 3 akademisyen tarafından Türkçeye çevrildikten sonra, bu üç çeviri karşılaştırılarak tercüme edilen sözcüklerdeki anlam uyumsuzlukları ve belirsizlikler giderildi. İngilizce'ye geri tercüme işlemi iki uzman akademisyen tarafından gerçekleştirildi. Ölçek iyi derecede İngilizce bilen klinik eczacı, tıp öğrencisi ve bir akademisyen tarafından tekrar Türkçe'ye çevrildi ve çeviri süreçlerinde karşılaşılan tüm tutarsızlıklar giderildikten sonra Türkçe son halini aldı.

Örneklem

Türk diline uyarlaması yapılan ölçekte toplam 16 madde bulunmaktadır. Kaynaklarda ölçek geçerliliği süreçlerinde ölçekteki madde sayısının on katı kadar örneklem kullanılması gerektiği belirtilmektedir [16,18]. Bu doğrultuda, araştırmaya Sağlık Bilimleri Üniversitesi Tıp ve Eczacılık

Fakültesinde öğrenim gören ve çevrimiçi veri toplama aracını dolduran 200 öğrenci dahil edilmiş, çalışmada ölçeği ve soru formunu eksiksiz olarak dolduran 189 (86 eczacılık fakültesi, 103 tıp fakültesi) öğrencisine ait veriler kullanılmıştır.

Verilerin Toplanması

Ölçek formu Sağlık Bilimleri Üniversitesinde okuyan Tıp ve Eczacılık fakültesi öğrencilerinin tümüne çevrimiçi olarak gönderilmiştir. Ölçek, cinsiyet, yaş, kayıtlı oldukları bölüm ve sınıf sorgulanan demografik soruları takiben 4'lü Likert tipi (1=kesinlikle katılmıyorum, 2= katılmıyorum, 3=katılıyorum, 4= kesinlikle katılıyorum) 16 sorudan oluşmaktadır. Tüm sorular doğrudan puanlanırken 9. Soru ters (1=kesinlikle katılıyorum, 2= katılıyorum, 3= katılmıyorum, 4=kesinlikle katılmıyorum) olarak puanlanmıştır. Ölçekten elde edilecek en düşük puan 16, en yüksek puan ise 64 'dür. Herhangi bir kestirim noktası olmaksızın, ölçekten ve alt boyutlarından alınacak yüksek puan hekim ile eczacı arasındaki ilişkinin daha olumlu bir tutum gösterdiğini belirlemektedir. Tüm katılımcılar anket formunun giriş bölümünde çalışma hakkında bilgilendirilmiş; gönüllülük, vazgeçme hakkı, kişisel bilgilerin korunma güvencesi verilen onam metnini takiben çevrimiçi anketi doldurmaya devam eden katılımcılar, araştırmaya katılmaya zımni onam vermiş sayılmıştır.

Verilerin Analizi

Araştırmanın veri analizi IBM SPSS Statistics 25© paket programı kullanılarak yapılmıştır. İlk aşamada, ölçeğin iç-tutarlılığı Cronbach's Alpha testi ile sınanmış ve α=0,93 olarak bulunmuştur. Korelasyon değeri 0,30'dan büyük olan tüm ölçek maddeleri analize dahil edilmiştir. Ölçeğin yapı geçerliliği temel bileşenler yöntemi ve Varimax dönüşümü kullanılarak açıklayıcı faktör analizi ile sınanmıştır. Bu amaçla önce Kaiser-Meyer-Olkin (KMO) örneklem yeterliliği testleri ve Bartlett küresellik testi yapılarak veritabanının faktör analizine uygunluğu sınanmış; açıklayıcı faktör analizi ile ölçeğin içeriğindeki faktörler belirlenedikten sonra bu faktörler ölçeğin alt boyutları olarak tanımlanmıştır [17].

Son olarak, faktör analizi sonucunda bulunan alt boyutlar için yeni değişkenler oluşturularak katılımcıların ölçek toplam ve alt-boyut puanları hesaplanmış, araştırma örneklemi üzerinde ölçek puanları ve katılımcıların demografik bilgilerine ait tanımlayıcı analizler yapılmıştır. Katılımcıların öğrenim gördükleri fakülte ve cinsiyete göre puanları ise bağımsız gruplar t-testi ile karşılaştırılmıştır.

SONUÇ VE TARTIŞMA

Demografik Özellikler

Katılımcıların demografik bilgilerine ait frekans ve yüzde dağılımları Tablo 1'de sunulmuştur. Yaş değerine ait ortalama ve standart sapma değerleri ile minimum ve maksimum değerleri

belirtilmiştir. Buna göre olguların %65,6'sı kadın, %86,2 ile çoğunluğu TC vatandaşı olup, 103 (%54,5) tıp ve 86 (%45,4) eczacılık fakültesi öğrencisinden oluşmakta olup yaş ortalamaları 20,86±1,78 bulunmuştur.

Tablo 1. Katılımcıların demografik özellikleri

Cinsiyet	**N**	**%**	**Uyruk**			**N**	**%**
Kadın	124	65.6	**TC Vatandaşı**			163	86.2
Erkek	65	34.4	**Diğer**			26	13.8
Toplam	189	100	**Toplam**			189	100
Fakülte	**N**	**%**	**Yaş**	**Min.**	**Maks.**	**N**	**Ort. ±ss**
Eczacılık	86	45.5					
Tıp	103	54.5		18	36	189	20.86± 1.78
Toplam	189	100					

Ölçeğin Geçerlilik-Güvenirlik Analizleri

Hojat and Gonnella'nın geliştirdiği Türkçe dil uyarlanması tamamlanan Attitudes Towards Physician and Pharmacist Collaboration ($SATP^2C$) ölçeğinin maddeleri Tablo 2'de verilmiştir. Türk diline uyarlama sürecinin ardından Hekim- Eczacı İşbirliğine Yönelik Tutum Ölçeği (HEİYTÖ) olarak adlandırdığımız bu araç, 4'lü Likert tipi 16 maddeden oluşmaktadır. Ölçek hekim-eczacı işbirliğine yönelik tutum, algı ve beklentileri ifade eden cümlelerden oluşmakta olup, orijinal ölçekteki maddelerin tamamını içermektedir (Tablo 2). Tablo 2'de ölçeğe verilen yanıtların ortalama ve standart sapmaları incelendiğinde katılımcıların ağırlıklı olarak 'katılıyorum' ya da 'kesinlikle katılıyorum' şeklinde yanıt verdikleri görülmektedir (ortalama>3,00).

Ancak, ölçek maddelerine verilen yanıtlar meslek eğitimi gruplarına ayrıldığında, eczacılık öğrencilerinin yanıtları örneklemin tamamıyla uyumlu gözükmekle birlikte; tıp öğrencilerinin, eczacıların sorumluluk ve yetkinlik alanlarına yönelik maddeler olan 2,4,7,8,9,14 ve 15. maddelerin puan ortalamaları incelendiğinde 'katılmıyorum=3' yanıtına yakın düştükleri dikkat çekmektedir.

Ölçekte yer alan ifadelerin iç tutarlılığını ölçmek amacıyla 189 katılımcının yanıtları üzerinden öncelikle Cronbach's Alpha güvenilirlik analizi yapılmıştır (Tablo 3). Cronbach's Alpha testi sonucunda, ölçeğin tümü için α=0,938 olarak bulunmuştur. Bu değer 0,70'in üzerinde bulunduğu için genel kabuller doğrultusunda ölçeğin yüksek güvenilirlikte olduğu görülmektedir [18].

Tablo 2. Hekim- Eczacı İşbirliğine Yönelik Tutum Ölçeği ifadelere göre yanıtların dağılımı

		Örneklem N=189	**Eczacılık Öğr. N=86**	**Tıp Öğr. N=103**
No	**İfadeler**	**Ortalama ±standart sapma**		
1	Bir hekim bir eczacının amiri gibi değil meslektaşı ve iş ortağı olarak görülmelidir	3.57±0.65	3.86 ±0.38	3.33±0.72
2	Eczacılar, hastaların ilaç tedavisi ile ilgili ihtiyaçlarını değerlendirme ve cevaplamada niteliklidir	3.23±0.76	3.71±0.48	2.84±0.74
3	Eğitimleri sırasında eczacılık ve tıp öğrencileri, birbirlerinin rollerini anlamak için ekip çalışmasına dahil edilmelidir	3.47±0.65	3.75±0.43	3.23±0.72
4	Eczacılar, hastaları etkileyebilecek ilaç etkileşimi ile ilgili kararlarda katkıda bulunabilirler	3.24±0.80	3.74±0.47	2.83±0.78
5	Eczacılar verdikleri ilaç konusunda hastalara karşı sorumlu olmalıdır	3.42±0.64	3.65±0.55	3.23±0.66
6	Hastaların ilaç tedavisinde, eczacılar ve hekimler arasında örtüşen birçok sorumluluk alanı vardır	3.39±0.66	3.72±0.45	3.12±0.68
7	Eczacı, hastanın ilaç tedavisi ile ilgili danışmanlık yapacak özel uzmanlığa sahiptir	3.01±0.87	3.62±0.56	2.50±0.77
8	Hem eczacılar hem de hekimler hastalara verilen ilacın türü ve dozu ile ilgili kararlara katkıda bulunmalıdır	3.06±0.92	3.65±0.53	2.57±0.88
9	Eczacının birincil işlevi, hekimin reçetesini sorgulamadan yerine getirmektir (ters puanlama)	3.13±0.82	3.55±0.66	2.79±0.79
10	Eczacılar çalışma alanları olan hastane veya eczane hizmetlerindeki ilaç politikalarına yönelik verilen kararlarda söz sahibi olmalıdırlar	3.38±0.61	3.74±0.47	3.09±0.54
11	Eczacılar ve hekimler, ilaçların hastalar üzerindeki etkilerini takip etmekten sorumludurlar	3.37±0.69	3.61±0.51	3.17±0.76
12	Eczacılar hekimlerin verdiği ilacın hasta üzerinde kötü sonuçlar doğurabileceğini düşündüğünde hekimle görüşüp ilacı teyit etmelidir	3.56±0.59	3.79±0.44	3.38±0.64
13	Hekimler ve eczacılar işbirlikçi ilişkiler kuracak şekilde eğitilmelidir	3.55±0.60	3.78±0.42	3.37±0.66
14	Hekimler, ilaç tedavisine olumsuz reaksiyonu olan veya ilaç tedavisine dirençli hastalara yardımcı olmak için eczacılara danışmalıdırlar	3.16±0.83	3.67±0.50	2.75±0.84
15	Hekimler, eczacıların doğru/isabetli ilaç tedavisi konusunda kendilerine yardım edebileceklerini bilmelidir	3.32±0.73	3.76±0.45	2.95±0.71
16	Hekimler ve eczacılar arasındaki mesleklerarası iletişim konuları, üniversite eğitim programlarına dahil edilmelidir	3.40±0.70	3.67±0.52	3.18±0.74

Tablo 3. Güvenirlik analizi sonuçları

Madde	Düzeltilmiş Madde-Toplam Korelasyonu	Madde Çıkarılırsa Cronbach's Alpha	Cronbach's Alpha	Madde Sayısı
MADDE 1	0.619	0.935	0.938	16
MADDE 2	0.637	0.935		
MADDE 3	0.663	0.934		
MADDE 4	0.736	0.932		
MADDE 5	0.461	0.939		
MADDE 6	0.696	0.934		
MADDE 7	0.752	0.932		
MADDE 8	0.733	0.933		
MADDE 9	0.657	0.935		
MADDE 10	0.735	0.933		
MADDE 11	0.570	0.936		
MADDE 12	0.616	0.935		
MADDE 13	0.695	0.934		
MADDE 14	0.770	0.932		
MADDE 15	0.835	0.930		
MADDE 16	0.655	0.934		

Faktör Analizi

Çalışmanın faktör analizi aşamasında. her madde çıkarıldığında α değerlerindeki değişim incelenerek. madde-toplam korelasyon değerlerine bakılmış. korelasyon değeri 0.30'dan küçük olan madde bulunmadığı için tüm maddeler analize dahil edilerek ölçeğin içeriğindeki maddelerin hangi temel kavramlar altında kümelendiğini saptamaya yönelik açıklayıcı faktör analizi gerçekleştirilmiştir (Tablo 3). Ölçeğin yapı geçerliliği incelemesi amacıyla yapılan açıklayıcı faktör analizi sonucunda; 16 maddelik ölçeğin Kaiser-Meyer-Olkin örneklem yeterliliği testi sonucu 0.932 ve değişkenler arasında faktör analizi yapmak için yeterli düzeyde bir ilişki olup olmadığını inceleyen Bartlett küresellik testi sonucu <0.001 olarak bulunmuştur (Tablo 4). Buna göre ölçeğin faktör analizi yapmak için uygun. saha çalışmasında elde edilen ve analize esas olarak kullanılacak örneklemin ise çalışmamız amaçları doğrultusunda yeterli olduğuna karar verilmiştir [16.19].

Aşağıda. Tablo 4'de görüldüğü üzere faktör yapılarını ortaya çıkarmaya yönelik Temel Bileşen analizi ve Varimax –Kaiser Normalizasyon yöntemiyle ölçeğin iki faktörlü bir yapıda olduğu bulunmuştur. Bu iki faktöre dağılan ölçek maddeleri arasında faktör yük değeri 0.30'un altında olan madde bulunmamaktadır. Bu bulgular ışığında ölçek. orijinal ölçekte kullanılan 16 maddenin tamamı üzerinden ve iki faktörlü olarak değerlendirilmiştir. Birinci faktör toplam varyansın %52.588'ini ve ikinci faktör %7.826'sını açıklamaktadır. Ölçeğin tamamı ise toplam varyansın %60.415'ini açıklamaktadır [19] Bulunan iki faktör. orijinal ölçekle uyumlu olarak ve kullanılan madde içeriklerine uygun olarak: 1: İşbirliği ve ekip çalışması. 2: Sorumluluk olarak adlandırılmıştır. (Tablo 4).

Tablo 4. Açıklayıcı faktör analizi sonucunda maddelerin faktörlere dağılımları ve faktör yükleri

Madde No.	İfade	Faktör 1: İşbirliği ve Ekip Çalışması	Faktör 2: Sorumluluk
1	Bir hekim bir eczacının amiri gibi değil meslektaşı ve iş ortağı olarak görülmelidir	0.504	
3	Eğitimleri sırasında eczacılık ve tıp öğrencileri. birbirlerinin rollerini anlamak için ekip çalışmasına dahil edilmelidir	0.745	
6	Hastaların ilaç tedavisinde. eczacılar ve hekimler arasında örtüşen birçok sorumluluk alanı vardır	0.633	
10	Eczacılar çalışma alanları olan hastane veya eczane hizmetlerindeki ilaç politikalarına yönelik verilen kararlarda söz sahibi olmalıdırlar	0.642	
12	Eczacılar hekimlerin verdiği ilacın hasta üzerinde kötü sonuçlar doğurabileceğini düşündüğünde hekimle görüşüp ilacı teyit etmelidir	0.678	
13	Hekimler ve eczacılar işbirlikçi ilişkiler kuracak şekilde eğitilmelidir	0.856	
16	Hekimler ve eczacılar arasındaki mesleklerarası iletişim konuları. üniversite eğitim programlarına dahil edilmelidir	0.797	
2	Eczacılar. hastaların ilaç tedavisi ile ilgili ihtiyaçlarını değerlendirme ve cevaplamada niteliklidir		0.773
4	Eczacılar. hastaları etkileyebilecek ilaç etkileşimi ile ilgili kararlarda katkıda bulunabilirler		0.814
5	Eczacılar verdikleri ilaç konusunda hastalara karşı sorumlu olmalıdır		0.319
7	Eczacı. hastanın ilaç tedavisi ile ilgili danışmanlık yapacak özel uzmanlığa sahiptir		0.839
8	Hem eczacılar hem de hekimler hastalara verilen ilacın türü ve dozu ile ilgili kararlara katkıda bulunmalıdır		0.779
9	Eczacının birincil işlevi. hekimin reçetesini sorgulamadan yerine getirmektir		0.503
11	Eczacılar ve hekimler. ilaçların hastalar üzerindeki etkilerini takip etmekten sorumludurlar		0.443
14	Hekimler. ilaç tedavisine olumsuz reaksiyonu olan veya ilaç tedavisine dirençli hastalara yardımcı olmak için eczacılara danışmalıdırlar		0.673
15	Hekimler. eczacıların doğru/isabetli ilaç tedavisi konusunda kendilerine yardım edebileceklerini bilmelidir		0.644
Özdeğer		8.415	1.251
Açıklanan Varyans Yüzdesi		52.596	7.816
Kümülatif varyans		52.596	60.412
Boyut Cronbach's Alfa Güvenirlik Katsayısı		α=0.889	α=0.908

KMO: .932.
Bartlett Küresellik Testi: Ki-kare=1899.561. df=120. p>0.0001
Ölçek Cronbach's Alfa Güvenirlik Katsayısı α=0.908

'Eczacılar verdikleri ilaç konusunda hastalara karşı sorumlu olmalıdır' ifadesini içeren Madde 5. orijinal çalışmaya benzer şekilde. iki faktör altında birbirine yakın katsayılarla bulgulanmış. bu madde Türkçe içerik açısından değerlendirilerek 'Sorumluluk' alt boyutuna dahil edilmiştir [14].

Ölçek Puan Ortalamaları ve Gruplar Arası Farkların İncelenmesi

Faktör analizi sonucunda iki alt boyuttan oluştuğu belirlenen HEİYTÖ ölçeğinin. katılımcılardan elde edilen puan ortalamaları Tablo 5 de görülmektedir. Çalışma örnekleminde. 7 maddeden ve oluşan en düşük 7 en yüksek 28 puan alınabilen 'İşbirliği ve ekip çalışması' alt boyutundan ortalama 24.31 (±3.46) puan elde edilmiştir. Buna karşın 9 maddeden oluşan ve en düşük 9. en yüksek 36 puan alınabilen 'Sorumluluk' alt boyutunda ise ortalama 28.96 (±5.41) puan elde edilmiştir.

Tablo 5. Katılımcıların HEİYTÖ puan ortalamaları

Ölçek Puanları	N	Min.	Maks.	Ortalama	Standart sapma
İşbirliği ve Ekip Çalışması	189	7.00	28.00	24.31	3.46
Sorumluluk	188	15.00	36.00	28.96	5.41
Ölçek Toplam Puanı	189	22	64	53.24	8.38

Ölçek alt boyut puan ortalamalarının cinsiyet ve eğitim görülen fakülteye göre farklılıkları incelendiğinde. iki alt boyut puan ortalamasının da eczacılık öğrencilerinde (26.31±2.23) tıp öğrencilerine göre (22.70±3.46) daha yüksek olduğu. aradaki farkın istatistiksel olarak anlamlı düzeyde olduğu bulunmuştur (Tablo 6. p<.0001).

Tablo 6: Tıp ve eczacılık öğrencilerinin HEİYTÖ puan ortalamaları

Ölçek Puanları	ECZACILIK Ort. ±ss	TIP Ort. ±ss	Student's t-testi [t]	p değeri
İşbirliği ve Ekip Çalışması	26.31±2.23	22.70±3.46	8.642	<0.0001*
Sorumluluk	32.96±3.27	25.61±4.53	12.863	<0.0001*
Ölçek Toplam Puanı	59.27±5.24	48.29±7.18	12.095	<0.0001*
N	85	103		

*İstatistiksel olarak anlamlı düzeyde fark
Ort : Ortalama
ss: Standart sapma

Cinsiyete göre farklılıkların bağıntı analizlerinde ise kadın öğrencilerin HEİYTÖ alt boyut puan ortalamalarının (24.95± 3.01; 30.22 ±5.02) erkeklere (23.54± 3.44; 27.05 ±5.27) göre daha yüksek olduğu. ve bu farkların istatistiksel olarak anlamlı düzeyde olduğu gözlenmektedir (Tablo 7. p=.004. <.0001). Benzer şekilde. ölçek toplam puanı ortalamaları da kadın olgularda erkeklere göre istatistiksel olarak anlamlı düzeyde yüksek bulunmuştur (55.12 ±7.66; 50.58± 8.05).

Tablo 7: Cinsiyete göre HEİYTÖ puan ortalamaları

Ölçek Puanları	**Kadın Ort.± ss**	**Erkek Ort.± ss**	**Student's t-testi t**	**p değeri**
İşbirliği ve Ekip Çalışması	24.95 ±3.01	23.54 ±3.44	2.894	0.004*
Sorumluluk	30.22 ±5.02	27.05 ±5.27	4.026	<0.0001*
Ölçek Toplam Puanı	55.12 ±7.66	50.58 ±8.05	3.774	<0.0001*
N	120	65		

*İstatistiksel olarak anlamlı düzeyde fark (p<0.05)

Ancak. ölçek puanlarının cinsiyete göre tabakalı analiz ile incelenmesi sonucunda. Tablo 6 bulgularına yakın ve benzer şekilde gerçek farklılığın öğrencilerin eğitim gördüğü fakülteler arasında olduğu görülebilmektedir (Tablo 8).

Tablo 8: Tıp ve eczacılık öğrencileri arasında cinsiyete göre HEİYTÖ puan ortalamaları

Cinsiyet	Ölçek Puanları	Fakülte	N	Ort.± ss	Student's t-testi t	p değeri
Kadın	Ölçek Toplam Puanı	Eczacılık	70	59.76±4.67	11.27	<0.0001*
		Tıp	50	48.62±6.15		
	İşbirliği ve Ekip Çalışması	Eczacılık	70	26.44±2.03	7.469	<0.0001*
		Tıp	50	22.86±2.93		
	Sorumluluk	Eczacılık	70	33.31±2.93	11.896	<0.0001*
		Tıp	49	25.8±3.97		
Erkek	Ölçek Toplam Puanı	Eczacılık	15	57±7.09	3.890	0.001
		Tıp	50	48.66±7.34		
	İşbirliği ve Ekip Çalışması	Eczacılık	15	25.67±2.99	2.882	0.005
		Tıp	50	22.9±3.33		
	Sorumluluk	Eczacılık	15	31.33±4.32	3.993	0.005
		Tıp	50	25.76±4.86		

Bu çalışmada hekim- eczacı işbirliğine yönelik tutum ölçeğinin Türkçe geçerlilik ve güvenilirliği incelenmiştir. Tıp ve eczacılık öğrencilerinden oluşan bu örneklemde HEİYTÖ'nün özgün çalışmasıyla uyumlu şekilde 16 maddeden oluşan ve iki faktörlü bir yapı ortaya çıkmıştır. Hojat and Gonnella eczacılık öğrencilerinin hekim-eczacı işbirliğine yönelik tutumlarını ölçmek ve bu tutumları tıp

öğrencilerinin tutumlarıyla karşılaştırmak için ölçeğin geçerli ve güvenilir olduğunu göstermiştir [14]. Bu çalışmada da ölçeğin eczacılık ve tıp öğrencisi popülasyonunda geçerli ve güvenilir olduğu saptanmıştır.

Hojat and Gonnella. ölçeğin geliştirilmesine yönelik ilk yayınlarında sorumluluk ve hesap verebilirlik. iş birliği ve ekip çalışması olarak adlandırlıkları iki faktörlü çözümünü doğrulamıştır [14]. Yine aynı çalışmada eczacılık öğrencilerinin puan ortalaması (56.2 ± 4.3). tıp öğrencilerinden (52.8 ± 8.6) anlamlı düzeyde yüksek bulunmuştur. Bu çalışmada ise iki faktör 'iş birliği ve ekip çalışması'. 'sorumluluk' alt boyut başlıkları ile tanımlanmış. puan ortalamaları benzer şekilde eczacılık fakültesi öğrencilerinde anlamlı düzeyde yüksek bulunmuştur (Tablo 6). Ancak. yazarların daha sonra yayınladıkları örneklem büyüklüğü genişletilmiş çalışmada ölçek üç faktörlü olarak ve farklı alt boyut isimleriyle değerlendirilmiştir (sorumluluk ve hesap verebilirlik. paylaşılan yetki ve disiplinlerarası eğitim) [15]. Bulgular. özgün çalışmanın ikinci uygulamasıyla buçalışma arasında faktör yapısı açısından farklılıklar göstermektedir. Bunun nedeninin çalışmaların uygulandığı tıp ve eczacılık öğrencileri grupları arasındaki farklılıklar ya da ölçeğin çevrimiçi uygulanması olabileceği düşünülmektedir. Özgün çalışmada araştırmaya katılan tıp ve eczacılık öğrencilerinin müfredat gereği ortak dersler aldığı belirtilmekte. ancak bu araştırmanın örneklemini oluşturan tıp ve eczacılık öğrencileri arasında böyle bir disiplinlerarası eğitim programı bulunmamaktadır [15]. Ancak Wang ve arkadaşlarının SATP^{2}C ölçeğini kullanarak yürüttükleri bir girişimsel araştırmada müfredatta ortak ders bulunmamasına karşın disiplinlerarası bir toplum sağlığı çalışmasına katılan tıp ve eczacılık öğrencilerinin ölçek puanlarının kurs sonrası anlamlı düzeyde arttığı da bulgulanmıştır [20].

Ölçeğin üç faktörlü versiyonuyla Endonezya'da yapılan bir çalışmada da eczacılık öğrencileri ve eczacılar için ortalama SATP^{2}C toplam puanları sırasıyla 56.53 ve 56.77'dir (veya olası maksimum puan 64). bu da her iki grubun işbirliğine yönelik olumlu tutumunu gösterir. Eczacıların iki işbirliği faktörü ("işbirliği ve ekip çalışması" ve "hesap verebilirlik") için eczacılık öğrencileri ve eczacıların olumlu tutumları da rapor edilmiştir [21].

Aynı ölçeğin Brezilya'da tıp ve eczacılık öğrencilerinde uygulandığı bir başka çalışmada. ölçek maddelerinin puan ortalamaları karşılaştırılmış ve bu araştırmanın bulgularıyla uyumlu şekilde tıp öğrencilerinin. eczacıların sorumluluk alanlarının tanımlanmasına yönelik ölçek maddelerinden istatistiksel olarak anlamlı düzeyde düşük puan ortalamaları aldıkları bulgulanmıştır [22]. Cunha ve arkadaşlarının bu çalışmasında ölçek faktör yapısıyla puanlandırılmamış olup. sadece ölçek toplam puanlarının gruplara göre istatistik olarak anlamlı düzeyde farklılığı vurgulanmıştır. Ancak madde bazında değerlendirmeler bu araştırmanın bulgularıyla da uyumlu şekilde tıp öğrencilerinin eczacıların sorumluluk alanları konusunda net bir fikirleri olmadığı şeklinde sonuçlar göstermektedir. Cunha ve arkadaşlarının bulgularında da bu çalışmanın ön analizlerine benzer şekilde kadın öğrencilerin erkeklere

göre tıp ve eczacılık branşları arasındaki işbirliği konusunda daha pozitif bir tutum içerisinde olduğu bulgulanmıştır. hatta cinsiyetler arasındaki bu istatistiksel olarak anlamlı farklılık kadınların sosyal ve iletişimsel becerilerine dayandırılarak yorumlanmıştır. Ancak Brezilya'da yapılan bu çalışmada cinsiyete göre tabakalı analiz sonuçları verilmediğinden. esas ayrımın. bu araştırmada olduğu gibi tıp ve eczacılık eğitimi alan öğrencilerin arasındaki tutum farklılığına indirgenebileceği olasıdır [22].

Bu çalışmada. eczacı- hekim iş birliğine dayalı bir ölçek olan HEİYTÖ ($SATP^2C$)' nin. hekim-eczacı işbirliğine yönelik tutumlardaki grup farklılıklarını incelemede geçerli ve güvenilir bir ölçek olduğu bulunmuştur. İçinde yer alan maddelerin sayısı ve kapsamı açısından sahada kısa sürede ve kolay uygulanabilen HEİYTÖ. hekim ve eczacıların farklı ortamlarda en yüksek kalitede bakımı sağlamak için hastalar. hasta aileleri ve çevreleri ile birlikte çalışarak kapsamlı hizmetler sağladığı bir durum olarak tanımlanan profesyonel işbirliğinin uygulamaya yönelik ve doğrulanmış bir tutum ölçeğidir.

Sağlık sisteminin önemli profesyonellerinden olan hekim ve eczacıların birbirlerinin ekip içindeki rollerini bilmeleri. karşılıklı açık ve etkili ileşitim ile birbirlerini daha iyi anlamaları eğitim sürecinden itibaren önem kazanmaktadır. Hekim ve eczacıların bilgi ve becerilerin paylaşılması ile sağlık bakım hizmeti talebinde bulanan bireylerin sorunlarına bütüncül bir bakış açısı getirilerek. ortak amaç ve hedeflere ulaşılması noktasında sağlık çıktılarında önemli kazanımlar sağlayacaktır.

HEİYTÖ. farklı meslekten öğrencilerin birbirleriyle olan işbirliklerini. birbirlerinden öğrenecekleri disiplinler arası eğitim programlarının oluşturulmasını ve değerlendirilmesini ve sağlık sonuçlarını iyileştirmek için kullanılma potansiyeline sahiptir. Ölçek. mesleki eğitim sırasında ve klinik ortamda hekim-eczacı işbirliğine yönelik tutumlardaki grup farklılıklarını incelemenin yanısıra sağlık uzmanları arasındaki ekip çalışması ve işbirliğinin klinik sonuçlarını değerlendirmek amacıyla deneysel araştırmalarda da kullanılabilecektir. HEİYTÖ'nün Türkçe'ye kazandırılması. gelecekte farklı sağlık meslek grup örneklemlerinde yapılabilecek işbirlikçi tutum araştırmalarına da kapı açabilecektir.

YAZAR KATKILARI

Kavram: *B.A.*; Tasarım: *B.A., Z.D.*; Denetim: *B.A., Z.D.*; Kaynaklar: *B.A., Z.D.*; Malzemeler: *B.A.*; Veri Toplama ve/veya işleme: *B.A., Z.D.*; Analiz ve yorumlama: *Z.D.*; Literatür taraması: *B.A.*; Makalenin yazılması: *B.A., Z.D.*; Kritik inceleme: *B.A., Z.D.*; Diğer:-

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek. potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

ETİK KURUL ONAYI

Bu çalışma. T.C Sağlık Bilimleri Üniversitesi Hamidiye Bilimsel Araştırmalar Etik kurulu tarafından 20/138 kayıt numarası ile onaylanmıştır.

KAYNAKLAR

1. Epstein, N. E. (2014). Multidisciplinary in-hospital teams improve patient outcomes: A review. *Surgical Neurology International*. 5(Suppl 7). S295. [CrossRef]

2. Swarenstein, M., Goldman, J., Reeves, S. (2009). Interprofessional collaboration: Effects of practice-based interventions on professional practice and healthcare outcomes. *Cochrane Database of Systematic Reviews*. *3*. CD000072. [CrossRef]

3. Tan, E. C., Stewart, K., Elliott, R. A., George, J. (2014). Pharmacist services provided in general practice clinics: a systematic review and meta-analysis. *Research in Social and Administrative Pharmacy*. *10*(4). 608-622. [CrossRef]

4. Türkiye Cumhuriyeti Cumhurbaşkanlığı Mevzuat Bilgi Sistemi Web site. (2014). From https://www.mevzuat.gov.tr/mevzuat?MevzuatNo=19569&MevzuatTur=7&MevzuatTertip=5

5. Hasan, S. (2008). A tool to teach communication skills to pharmacy students. *American Journal of Pharmaceutical Education*. *72*. 1-6. [CrossRef]

6. Ulutaş, E., Sözen Şahne, B., Yeğenoğlu, S. (2015). Eczacılıkta İletişimin Rolü. *Marmara Pharmaceutical Journal*. *19*. 200-207. [CrossRef]

7. Borenstein, J. E., Graber, G., Saltiel, E., Wallace, J., Ryu, S., Jackson, A., Weingarten, S. R. (2003). Physician-pharmacist comanagement of hypertension: A randomized. comparative trial. Pharmacotherapy: *The Journal of Human Pharmacology and Drug Therapy*. *23*(2). 209-216. [CrossRef]

8. Boudreau, D. M., Capoccia, K. L., Sullivan, S. D., Blough, D. K., Ellsworth, A. J., Clark, D. L., Stevens, N. G. (2002). Collaborative care model to improve outcomes in major depression. *Annals of Pharmacotherapy*. *36*(4). 585-591. [CrossRef]

9. Gallagher, R. M., Gallagher, H. C. (2012). Improving the working relationship between doctors and pharmacists: is inter-professional education the answer? *Advances in Health Sciences Education*. *17*(2). 247-257. [CrossRef]

10. Fortescue, E. B., Kaushal, R., Landrigan, C. P., McKenna, K. J., Clapp, M. D., Federico, F., Bates, D. W. (2003). Prioritizing strategies for preventing medication errors and adverse drug events in pediatric inpatients. *Pediatrics*. *111*(4). 722-729. [CrossRef]

11. Hunt, J. S., Siemienczuk, J., Pape, G., Rozenfeld, Y., MacKay, J., LeBlanc, B. H., Touchette, D. (2008). A randomized controlled trial of team-based care: impact of physician-pharmacist collaboration on uncontrolled hypertension. *Journal of General Internal Medicine*. *23*(12). 1966-1972. [CrossRef]

12. Seostianin, M., Neumann-Podczaska, A., Wieczorowska-Tobis, K. (2020). Towards effective collaboration of physicians and pharmacists for the care of older people (including COVID-19 perspective). *Journal of Medical Science*. *89*(2). e424-e424. [CrossRef]

13. Bayraktar Ekincioğlu, A., Demirkan, K. (2018). Opinions of community pharmacists about collaboration with general practitioners: A descriptive pilot study after new legislation in Turkey. *Turkish Journal of Pharmaceutical Sciences. 15*. 212-218. [CrossRef]

14. Hojat, M., Gonnella, J.S. (2011) An instrument for measuring pharmacist and physician attitudes towards collaboration: Preliminary psychometric data. *Journal of Interprofessional Care*. *25*(1). 66-72. [CrossRef]

15. Van Winkle, L.J., Fjortoft, N., Hojat, M. (2011). Validation of an instrument to measure pharmacy and medical students' attitudes toward physician-pharmacist collaboration. *American Journal of Pharmaceutical Education*. *75*(9). [CrossRef]

16. Alpar, R. (2020). Uygulamalı Çok Değişkenli İstatistiksel Yöntemler. Detay Yayincilik. Ankara. 830 s.

17. Karagöz, Y. (2019). SPSS 23 ve AMOS 23 Uygulamalı İstatistiksel Analizler (2. Baskı). Nobel Akademik Yayıncılık. Ankara. 1314s.

18. Kılıç, S. (2016). Cronbach'ın alfa güvenirlik katsayısı. *Journal of Mood Disorders*. *6*(1). 47-48. [CrossRef]

19. Özdamar, K. (2016). Eğitim. Sağlık ve Davranış Bilimlerinde Ölçek ve Test Geliştirme Yapısal Eşitlik Modellemesi IBM SPSS. IBM SPSS AMOS ve MINITAB Uygulamalı. Nisan Kitabevi. 286s. Eskişehir

20. Wang, J., Hu, X., Liu, J., Li, L. (2016). Pharmacy students' attitudes towards physician–pharmacist collaboration: Intervention effect of integrating cooperative learning into an interprofessional team-based community service. *Journal of Interprofessional Care*. *30*(5). 591-598. [CrossRef]

21. Setiadi, A. P., Wibowo, Y., Irawati, S., Setiawan, E., Presley, B., Gudka, S., Wardhani, A. S. (2017). Indonesian pharmacists' and pharmacy students' attitudes towards collaboration with physicians. *Pharmacy Practice (Granada)*. *15*(4). [CrossRef]

22. Cunha, L., Neves, S., Marques, T. C., Araújo, D., Alcantara, T., Lyra, D. P. (2017). Cross-cultural adaptation and validation to Brazil of the scale of attitudes toward physician-pharmacists collaboration. *Pharmacy Practice (Granada)*. *15*(2). [CrossRef]

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 993-1008, 2022
Doi: 10.33483/jfpau.1149890

ORIGINAL ARTCLE / ÖZGÜN MAKALE

DEVELOPMENT OF *IN SITU* GEL FORMULATION CONTAINING BISPHOSPHONATE-LOADED PLGA MICROSPHERES FOR BONE REGENERATION IN MAXILLOFACIAL SURGERY APPLICATIONS; FORMULATIONS, *IN VITRO* CHARACTERIZATION AND RELEASE KINETIC STUDIES

MAKSİLLOFASİYAL CERRAHİ UYGULAMALARINDA KEMİK REJENERASYONU İÇİN BİFOSFONAT YÜKLÜ PLGA MİKROKÜRELERİ İÇEREN İN SİTU JEL FORMÜLASYONLARININ GELİŞTİRİLMESİ; FORMÜLASYONLAR, İN VİTRO KARAKTERİZASYON VE SALIM KİNETİK ÇALIŞMALARI

Heybet Kerem POLAT[1*], Sedat ÜNAL[2]

[1]Republic of Turkey Ministry of Health, Turkish Medicines and Medical Devices Agency, 06100 Ankara, Turkey

[2] Department of Pharmaceutical Technology, Faculty of Pharmacy, Erciyes University, 38280, Kayseri, Turkey

ABSTRACT

Objective: *In this study, it was aimed to locally apply the bisphosphonate-loaded microsphere drug delivery system with in situ gel formulation, which was prepared to increase bone regeneration in the implant area in maxillofacial surgery.*

Material and Method: *In order to design the combination delivery system, bisphosphonate-loaded PLGA microspheres were embedded in the prepared in situ gel formulations. In vitro drug release, pH, clarity, sol-gel transition temperature and release kinetic studies were all assessed for the developed formulations.*

Result and Discussion: *The produced formulations' in situ gelation temperatures ranged from 33 to 37°C; their pH values were in the range of 6; and they were all syringeable, which is defined as the force required to expel each formulation from a syringe equipped with a 20-gauge needle. With the preparations, the amounts of*

* **Corresponding Author / Sorumlu Yazar:** Heybet Kerem Polat
e-mail / e-posta: kerem_hybt@hotmail.com, **Phone / Tel.:** +903122183000

Submitted / Gönderilme: 27.07.2022 **Accepted / Kabul:** 19.09.2022

P407 and chitosan increased, lowering in vitro burst release while simultaneously raising viscosity. However, each in situ gel formulation releases over a period of 14 days. Consequently, Bisphosphonate loaded PLGA microspheres embedded in situ gel formulations were elucidated in detail and presented as a locally applicable drug delivery system in maxillofacial surgery, especially in dental implant applications

Keywords: *Bisphosphonate, combination delivery systems, plga microspheres, poloxamer, chitosan*

ÖZ

Amaç: *Bu çalışmada maksillofasiyal cerrahide implant bölgesinde kemik rejenerasyonunu artırmak için hazırlanan bifosfonat yüklü mikrosfer ilaç taşıyıcı sistemin in situ jel formülasyonu ile lokal olarak uygulanması amaçlanmıştır.*

Gereç ve Yöntem: *Kombinasyon taşıyıcı sistemini tasarlamak için bifosfonat yüklü PLGA mikroküreleri, hazırlanan in situ jell formülasyonlarına yüklenmiştir. Geliştirilen formülasyonlar için in vitro ilaç salım, pH, berraklık, sol-jel geçiş sıcaklığı ve salım kinetik çalışmaları değerlendirilmiştir.*

Sonuç ve Tartışma: *Üretilen formülasyonların yerinde jelleşme sıcaklıkları 33 ila 37°C arasında; pH değerleri 6 civarında ve bütün formülasyonlar 20 gauge'lik şırıngalardan uygulanabilir düzeydeydi. Preparatlar içerisinde yer alan, P407 ve kitosan miktarları arttıkça, in vitro patlama salınımını düşürürken aynı zamanda viskoziteyi yükselmiştir. Bununla birlikte, her bir in situ jel formülasyonu, 14 günlük bir süre içinde salım yapmıştır. Sonuç olarak, Bifosfonat yüklü PLGA mikroküreleri yüklü in situ jel formülasyonlarına ayrıntılı olarak değerlendirilmiş ve özellikle dental implant uygulamalarında maksillofasiyal cerrahide lokal olarak uygulanabilir bir ilaç taşıma sistemi olarak sunulmuştur*.

Anahtar Kelimeler: *Bifosfonat, kombinasyon taşıma sistemleri, plga mikroküreler, poloksamer, kitosan*

INTRODUCTION

Due to the restricted amount of bone available for implant placement and the postoperative bone's mechanical characteristics, bone regeneration is a critical component of maxillofacial surgery, particularly in implantology procedures increased osteoclastic activity, which accelerates bone resorption, may be the root cause of low bone density at the surgical site and the associated clinical implant failure [1]. In order to address peri-implant bone abnormalities, particularly for maxillofacial processes, preclinical and clinical investigations have concentrated on novel therapeutic approaches using multidisciplinary concepts [2]. Working together in disciplines like medicine, pharmaceutical sciences, biology, and materials science can help create effective and novel approaches to bone regeneration because it is an interdisciplinary and complicated process.

According to their affinity for hydroxyapatite, bisphosphonates are strong inhibitors of osteoclastic bone resorption and can improve bone density by shifting the ratio of bone production to bone resorption [3, 4]. The U.S. Food and Drug Administration (FDA) has approved a class of third-generation bisphosphonates called alendronate sodium (AS), which is well known for preventing bone loss and boosting bone mass [5, 6]. Because of bone resorption, it is sometimes difficult to employ bisphosphonates, which include AS, to repair bone abnormalities, especially in maxillofacial surgery. These issues are still being researched as current pharmaceutical issues [7].

Innovative polymeric micro drug delivery systems that can be applied locally to the site of the bone defect administer medications with a controlled release while acting as mechanical supports for

the region where bone resorption takes place. Research is still being done on methods to improve AS's stability and duration spent in the resorption zone, prolong the drug's release, and lessen side effects. Due to the aforementioned problems, AS is rarely used, particularly in procedures involving dental implants and maxillofacial surgery [8, 9].

One of the most popular synthetic biodegradable polymers in the pharmaceutical industry, Poly(lactic-co-glycolic acid) (PLGA), has received FDA and European Medicines Agency approval (EMA [10]. It is frequently employed in the creation of novel drug delivery systems (NDDS), biomedical and bioengineering applications due to its physiological inertness, biocompatibility, and biodegradability [11]. Problems with solubility and associated bioavailability of active ingredients can be overcome with the use of micro/nanoparticulate drug carrier systems comprised of PLGA copolymers. As a result, therapeutic effectiveness and side effects related to dose can be improved. Additionally, release patterns may be changed, dosage frequency may be decreased by controlled or delayed release, and the required dose may be reduced by increasing the molecule's cellular interaction [12].

The periodontal pocket, the intended location for local drug delivery, is a complicated area with a continuous flow of saliva fluid. To deliver the drug's intended effects in the challenging environment, an injectable *in situ* sustained release gel would be the rational and effective choice. An innovative technique being used at the moment to do this is the use of in-situ gel-forming formulations, which first supply drugs in a liquid dosage form before producing powerful gels at the delivery site. Among in-situ gelling polymers, thermosensitive systems like poloxamer have been researched as an acceptable dosage form for injection into periodontal pockets. In order to improve contact intimacy and lengthen the residence time of the dose form in the periodontal pocket, semisolid formulations containing mucoadhesive polymers such, hydroxyethyl cellulose (HEC), chitosan, and carbopol have also been proposed [13].

The objective of the current study is to prepare and evaluate the *in situ* gel formulation containing Bisphosphonate-loaded PLGA microspheres (BMA) (22) as a local medication with combination delivery system approach for bone regeneration in maxillofacial implant operations. These formulations contained chitosan, a mucoadhesive polymer, as well as the thermosensitive polymer poloxamer 407 (P407). For this purpose, AS loaded PLGA microspheres prepared and published within the scope of our previous study were used [14]. The present study is designed as a further study inspired by the results of the previous study. Promising results of PLGA microspheres prepared with AS, which is widely used in bone regeneration in order to prevent post-operative bone defect and bone loss in the implant area in maxillofacial surgery applications, have been extensively reported in our previous study. In this study, an *in situ* gel formulation, which allows easy application of AS-loaded PLGA microspheres

to the implant site where bone regeneration is desired, prolongs the residence time of the formulation and drug release, was developed and *in vitro* characterization studies were completed.

MATERIAL AND METHOD

Materials

Poly(lactide-co-glycolide) (PLGA) 50:50 (RG 503H), alendronate sodium trihydrate (solubility in water 10 mg/mL), poloxamer, chitosan (low molecular weight), phosphate buffered saline (PBS) tablet were purchased from SigmaAldrich.

Method

Preparation and *in vitro* characterization of BMA formulation

Within the scope of this study, AS loaded PLGA microsphere formulations embedded in gel formulations were prepared in our previous study and comprehensive *in vitro* characterization studies were completed and reported [14]. In summary, PLGA microspheres were prepared by the multiple emulsion method (W/O1/O2) and characterization parameters, morphology, release profiles, degradation studies, stability and *in vitro* release kinetics were analyzed. It was observed that the AS release could be maintained for 14 days with the prepared drug delivery system, and PLGA microspheres deteriorated over time during this period. The release kinetics of the microspheres were mathematically modeled and found to be compatible with the Weibull and Peppas-Sahlin models [14].

In conclusion, the *in vitro* properties of AS loaded PLGA microspheres, which can be used in maxillofacial surgery applications, were elucidated in detail and presented as a locally applicable microparticulate drug delivery system for dental bone regeneration. In the light of the results obtained, it is aimed to design an *in situ* gel formulation that can be applied locally to the effect area with an advanced formulation approach for the microsphere formulation, which was designed and characterized. Comprehensive *in vitro* characterization data of AS-loaded PLGA microspheres prepared within the scope of the previous study and forming the basis of this study's details are available in our previous study [14].

Preparation of the In Situ Forming Gels

The *in situ* gel formulations were prepared using a modified cold technique [15]. The polymer was combined with cold (4 °C) water to create all of the P 407 solutions (17, 19 and 21 % w/v) utilized in this work. For 24 hours, the polymer solutions were refrigerated. The chitosan (0.5, 1% w/v) solution was then made ready for formulations. Chitosan was first dissolved in a 2 % v/v acetic acid solution, after which the chitosan solution was refrigerated for 24 hours. The chitosan solution and PF127 solution

were then mixed together at 4 °C then 0.2% BMA was added to the solution. The samples are still kept at 4 ° C in preparation for auxiliary exercise.

Table 1. Ingredients of *in situ* gels

	Content of ingredients in each formulation (%, w/w)			
Code	**Poloxamer 407**	**BMA**	**Chitosan (low molecular weight)**	**Water (qs)**
A1	17	0.2	0.5	100
A2	17	0.2	1	100
A3	19	0.2	0.5	100
A4	19	0.2	1	100
A5	21	0.2	0.5	100
A6	21	0.2	1	100

pH

Using a pH meter, the pH was determined. (HANNA of Germany) A total of three measurements were made (n=3).

Clarity

After gelation, the formulation of the *in situ* gel was examined in bright light against a dark background to determine its clarity [16].

Syringeability study

The ability of the created formulations to flow easily through a syringe with a 20 gauge needle was assessed using the same method as Maheshwari et al. [17]. Before assessing the flowability of the cold gel at standard handling pressure, one cc of the gel was placed into a 20 gauge needle syringe.

Gelation Temperature

In a water bath, a magnetic stirrer was used to stir each polymer solution (10 ml). At a speed of 100 rpm and 1 °C/min, heated polymer solutions were swirled (Thermomac-TM19). Three measurements were made on each [18].

Viscosity

The viscosity of *in situ* gels was measured using a CP 52 spindle and the Brookfield, DV2T-RV viscometer (Essex, UK). Additionally offered for comparison was viscosity at 10 rpm. Viscosities of all *in situ* gels were measured at both 25°C and 37°C (Table 3). Three measurements were made on each [19].

In Vitro Release Studies

The release of *in situ* gel compositions *in vitro* was examined using the dialysis bag method. [20]. The next procedure was to seal the dialysis bags, add 100 µL of BMA loaded *in situ* gel, and then place them at 37 °C in 25 mL of an isotonic phosphate buffer with a pH of 7.4. Such is the provision of the sink condition. Equivalent amounts of the medium were removed and replaced with equivalent amounts of the new buffer media at various intervals (0, 0.5, 1, 2, 4, 7, 10, 12, and 14 days). BMA concentrations were measured using a UV-vis spectrophotometer. To create a BMA release profile, the total amount of medicine released from each formulation over time was used. Three times were run through the experiment.

Release Kinetic Studies

DDSolver software was used to evaluate the *in vitro* release kinetic of AS from *in situ* gel formulations. In this regard, various mathematical models were used to examine the kinetics of drug is released from *in situ* gel formulations (Zero order, First order, Higuchi, Korsmeyer-Peppas, Peppas-Sahlin and Weibull). In order to select the "best fit" models, data were computed using the DDSolver program after evaluating the *in vitro* AS release profiles from formulations. Four criteria were evaluated; Akaike Information Criterion (AIC), coefficient of determination (R^2), adjusted coefficient of determination (R2adjusted), and model selection criterion (MSC). The greatest R^2, $R^2_{adjusted}$, MSC, and lowest AIC values were used to choose the model that best fitted the *in vitro* release data and had a higher correlation [21].

RESULT AND DISCUSSION

Preparation and *in vitro* characterization of BMA formulation

The characterization findings of AS loaded PLGA microspheres, which were prepared in our previous study and whose comprehensive *in vitro* characterization studies were completed, and embedded in the *in situ* gel formulations prepared in this study, was briefly explained in the method section. Details are available in our previous study [14].

Characterisation of *in situ* gel formulations

The viscosity results of blank *in situ* gels are shown in table 2. Viscosity coefficients were measured at 10 rpm at both 25 °C and 37 °C for A1-A6. When compared with microsphere loaded *in situ* gels, it is seen that there is no significant difference ($p<0.05$).

All of the *in situ* forming gel formulations for BMA were found to gel between 31 and 37 °C, making them all appropriate for use in dental procedures. Table 3's data serve as evidence. All

formulations have a pH that ranges from 5.92 to 6.11. It can be syringed using a 20 gauge needle, according to research.

Table 2. Results of *in situ* gels' *in vitro* characterisation analysis

Formulation	Viscosity (centipoise) 25 °C	Viscosity (centipoise) 37 °C
A1	240±34	8904±102
A2	282±29	8994±154
A3	300±17	9187±297
A4	308±25	9372±203
A5	315±24	9888±302
A6	331±28	10057±197

As a result, viscosity coefficients were measured at 10 rpm at both 25 °C and 37 °C for all formulations. The findings demonstrated that the values of viscosity varied according to the amounts of polymer (Table 3).

Table 3. Results of *in situ* gels' *in vitro* characterisation analysis

Formulation	pH (±SD)	Gelation temperature (°C±SD)	Viscosity (centipoise) 25 °C	Viscosity (centipoise) 37 °C	Clarity
A1	6.11±0.01	37±0.7	231±21	8842±121	Clear
A2	6.02±0.02	36±0.3	275±31	8938±169	Clear
A3	5.99±0.03	35±0.1	290±22	9145±312	Clear
A4	5.95±0.02	34±0.2	301±14	9352±218	Clear
A5	5.93±0.08	33±0.4	314±38	9868±214	Clear
A6	5.92±0.02	33±0.6	326±29	10042±231	Clear

Two different temperatures-room temperature of 25°C and the application periodontal pocket site temperature of 37°C-were used in the sol-gel transition studies to determine if the formulations were suitable for in-situ application as well as storage conditions [22,23]. According on the grade, concentration, and other formulation elements used, poloxamer solutions have been observed to undergo thermoreversible gelation.

When temperatures are lower than the critical micelle temperature (CMT), at which the critical micelle concentration (CMC) occurs, poloxamer maintains each molecule's separation in an aqueous

environment. When the temperature exceeds CMT, the molecules are stimulated to organize into micelles that surround the hydrophobic core while maintaining contact with the aqueous media through hydrophilic pluronic chains. Consequently, a lower CMT value is produced by a higher poloxamer concentration [23]. The gelation temperature is also negatively impacted by this circumstance. The concentration of poloxamer's polymer is mostly associated with the gelation temperature. At lower concentrations, they produce monomolecular micelles, but at greater concentrations, multimolecular lattice structures are produced [24-26].

Our formulations use lower poloxamer concentrations so that gelation happens at corneal temperature. As a result, as the concentration of poloxamer decreased, gelation temperatures increased. The literature turned up similar results [27]. Increasing the chitosan concentration has a detrimental impact on the gelation temperature. P407's hydrophobic domains were aggregated during gelation to lessen the hydrophilic surfaces and the amount of water surrounding the hydrophobic domains [28].

Table 3 displays the *in vitro* characterization findings of *in situ* gels. The look of all formulations was clear. The pH of all formulations ranges between 5.92 to 6.11. Hypodermic syringes with gauges 19–27 are used for oral injection. An extremely viscous solution requires the use of a needle with a smaller gauge [29]. Syringeability is defined as the amount of force necessary to discharge each formulation from a syringe fitted with a 20-gauge needle. The syringeability requirements are met by all formulations.

The formulations with a larger amount of chitosan were found to be more viscous than those with a lower amount, according to the results of the viscosity test. The amount of gel that may be injected into periodontal pockets is extremely constrained, so it must be thickened for improved retention and medication release control. On the other hand, the formulation ought to have the ideal viscosity to easily infuse in the periodontal pocket.

As a result, viscosity coefficients were measured at 10 rpm at both 25 °C and 37 °C for all formulations. The findings demonstrated that the values of viscosity varied according on the amounts of polymer (Table 3). This illustration shows how viscosity is significantly influenced by the concentration of the polymer. When the findings were analyzed in literary context, the conclusions are consistent [30].

Drug Release

Experiments on the *in vitro* release of drugs were conducted on *in situ* gels at 35 °C in an isotonic phosphate buffer with a pH of 7.4 and BMA (% 0.2). *In vitro* release profiles of AS loaded MS and the BMA loaded *in situ* gels were monitored for 14 days (Figure 1).

Comparing release profiles revealed a noticeable variation between them (Fig. 1). The overall release percentage for AS loaded MS was 96 ±3.1%. When BMA loaded (A1-A6) *in situ* gels are examined, it is seen that drug release is between 88± 1.1% and 91%± 1.3. The burst effects of the formulations are clearly shown in Fig.2. It is also seen that the burst release from the microspheres is less with the gel formulation. It indicates that from 0 h to 24 h, the cumulative release percent of AS loaded MS were 31.2± 2.54% and when BMA loaded (A1-A6) *in situ* gels are examined from 0h-24h, it is seen that drug release is between 16.7 ± 0.7% and 19.6±1.2%. In comparison to *in situ* gels, AS loaded MS had a much greater release percentage.

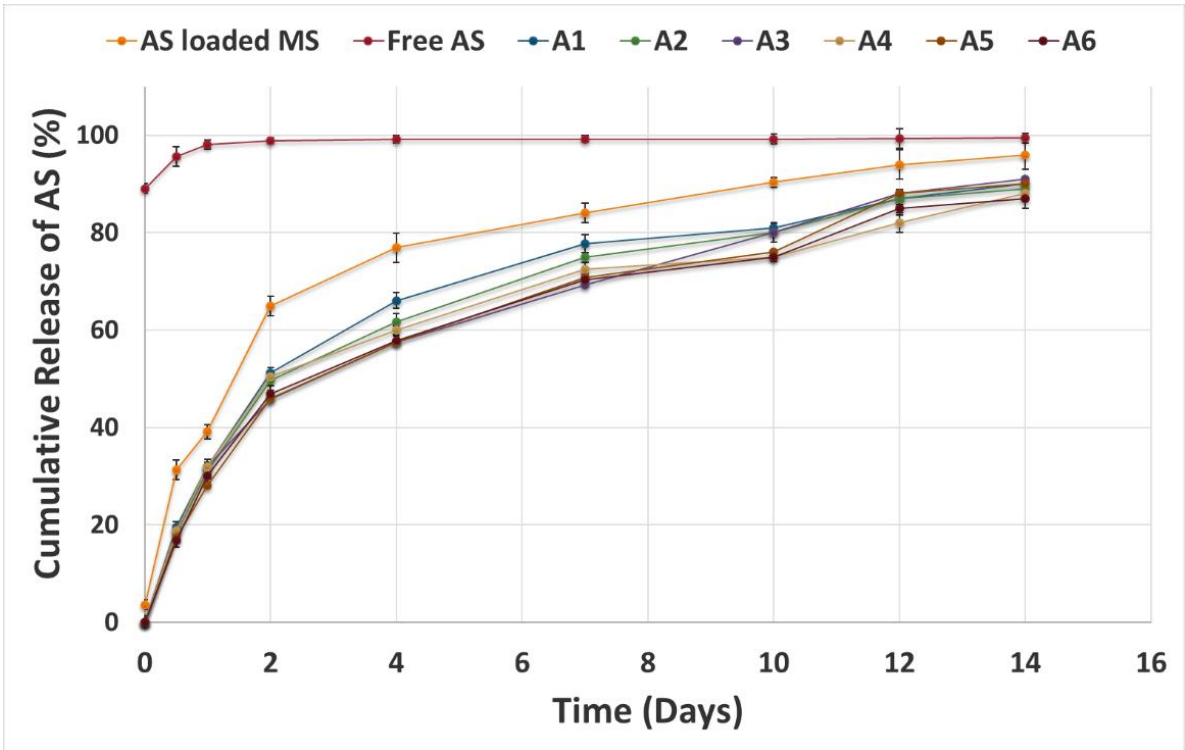


Figure 1. Cumulative release of AS from microspheres and *in situ* gel formulations containing AS-loaded microspheres (A1-A6)

Poloxamer gel deteriorated fully in 24 hours in our earlier erosion investigation, which isn't discussed in this publication. As a result, it is reasonable to assume that *in situ* gel release profiles consisted of two steps: in the first, the *in situ* gel gradually degraded while simultaneously releasing the microspheres from the skeleton carrier. The second phase involved the drug's gradual release from PLGA microspheres. This provided an explanation for the differences between the two release profiles of the two formulations. At first, the gel prevented AS loaded MS from touching the release medium. An increasing number of microspheres discharged as the gel slowly degraded. Because the release behavior of microspheres was dependent on a diffusion mechanism, they all released after the gel had

completely dissolved at 24 hours and did so at a rate that was comparable to that of AS loaded MS. When the literature is evaluated, it can be shown that the findings are consistent [31-35].

When *in situ* gel formulations are examined among themselves, the burst release diminished when poloxamer P407 concentration grew from 17% to 21%. It's possible that fewer and larger water channels and more and larger micelles inside the gel structure are the mechanisms producing this improved resistance. Because of the closer proximity of the micelles, there are more cross-links, which results in a higher viscosity and a slower rate of drug release. This hypothesis might be supported by rheology research, which shows a direct correlation between gel concentration and viscosity [15]

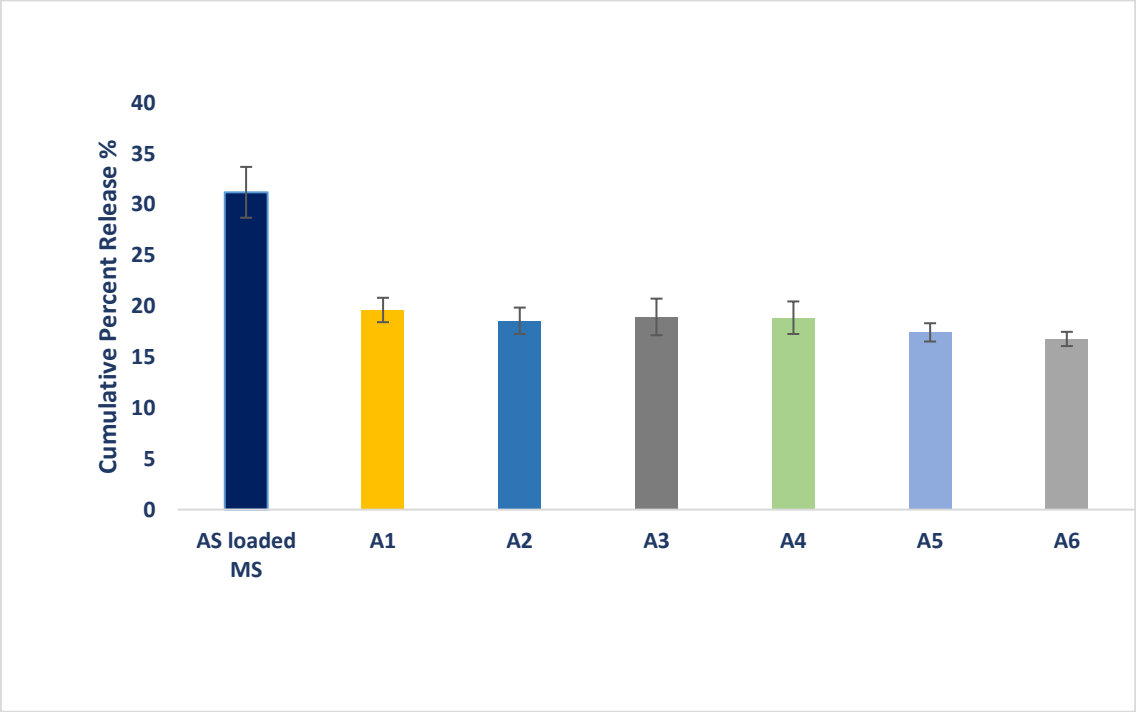


Figure 2. Comparison of *in vitro* AS release from microspheres and *in situ* gels (0h-24h)

Release Kinetic Studies

Various kinetic models were applied mathematically to the formulations in order to examine the AS release mechanism from the prepared combined delivery systems (Zero order, First order, Higuchi, Korsmeyer-Peppas, Peppas-Sahlin, and Weibull). Six different models were applied to six different combination delivery systems and microsphere formulations to examine critical parameters and comprehensively evaluate their release kinetics. Details and results on parameters that are valuable for release kinetic studies are shown in Table 4. As can be seen in the table, higher correlation was found for all formulations in Peppas-Sahlin and Weibull models compared to other models. From this point of view, it was evaluated that the kinetics of AS release from the prepared drug delivery systems fit both

Weibull and Peppas-Sahlin models, and it was seen that the release kinetics fit not only one model but more than one model. These results are consistent with the reports that the release kinetics of new drug delivery systems can fit more than one model and combined release kinetics can be observed, in line with the literature [36]. In the Weibull model, the "β" exponent is a indicator used to evaluate the release from a polymeric structures and "β"≤0.75 indicates Fickian diffusion [37]. Diffusion was considered to be the dominant mechanism in the Weibull model for all formulations. This situation was interpreted as compatible with both the release mechanism from the gel and the release mechanism from the microspheres. Considering the other compatible model, it should be evaluated in terms of the Peppas-Sahlin model. In principle, The Peppas-Sahlin model is based on Fickian diffusion and erosion of the polymeric matrix [38]. Polymeric matrix erosion is important both for microspheres and for release from the gel structure. When evaluated in the light of this information, the Peppas-Sahlin model also supports the diffusion phenomenon associated with erosion of the polymeric structure. In this context, It was observed that there was a mixed process in both the Fickian (pure diffusion phenomena) and non-Fickian models for the release of AS from formulations (due to the relaxation of the polymer chains between the networks). At the end of the release kinetic analysis, it was determined that both diffusion-based Fickian and polymeric structure degradation-based non-Fickian mechanisms were observed with the *in situ* gel combination delivery system.

In conclusions, bone loss in the surgical site caused by bone resorption is still one of the most common clinical consequences, particularly in maxillofacial surgery applications. Pharmaceutical research on the viability of medications that act locally in the surgical site and can stimulate bone growth by preventing bone resorption is ongoing for this purpose. The usage of AS, which is supposed to stop bone loss and increase bone mass, is still ineffective, particularly following dental implant placement. As part of the experiment, various polymer solutions including different concentrations of P407, the mucoadhesive polymer chitosan, and BMA were created. These formulations were all put to the test *in vitro* (pH, clarity, gelation temperatures and syringeability). The gelation temperature dropped when the poloxamer concentrations were compared to the gelation temperatures. However, it was discovered that the pH of all formulations was close to 6. The compositions would not irritate the dentition, according to research. When the drug release of formulations is examined, it is seen that busrt release decreases with increasing concentration. However, when all these *in vitro* characterization studies are evaluated, it is seen that A1 and A2 may be the most appropriate formulation. Since bone resorption is one of the most significant challenges in maxillofacial surgery applications, this formulation can be assessed as a novel medication delivery strategy that may improve bone regeneration in order to overcome the issues associated with them. In light of this, it has been deemed to be a promising drug delivery for upcoming preclinical and clinical studies.

Table 4. Release kinetic modeling and results of AS-loaded PLGA MC and BMA loaded *In Situ* Gels

Model and equation	Formulation		Evaluation criteria							
			Parameter		R^2	$R^2_{adjusted}$	AIC	MSC	n/m*
Zero-order	MS embedded in situ gel formulations	A1	k0	7.970	0.4918	0.4918	77.2040	-0.0160	-
		A2	k0	7.841	0.5470	0.5470	75.9833	0.1168	-
		A3	k0	7.796	0.6313	0.6313	74.0604	0.3398	-
		A4	k0	7.555	0.4957	0.4957	76.0188	-0.0196	-
F=k0*t		A5	k0	7.702	0.6511	0.6511	73.6385	0.4191	-
		A6	k0	7.532	0.5912	0.5912	74.4478	0.2354	-
	AS-loaded PLGA MS		k0	8.732	0.1763	0.1763	81.7092	-0.0282	-
First-order	MS embedded in situ gel formulations	A1	k1	0.265	0.9342	0.9342	58.8002	2.0289	-
		A2	k1	0.255	0.9306	0.9306	59.0998	1.9928	-
		A3	k1	0.261	0.9166	0.9166	60.6840	1.8261	-
		A4	k1	0.231	0.8833	0.8833	62.8467	1.4439	-
F=100*[1-Exp(-k1*t)]		A5	k1	0.252	0.9221	0.9221	60.1488	1.9180	-
		A6	k1	0.232	0.9068	0.9068	61.1379	1.7143	-
	AS-loaded PLGA MS		k1	0.364	0.9261	0.9261	60.0101	2.3828	-
Higuchi	MS embedded in situ gel formulations	A1	kH	26.739	0.9332	0.9332	58.9376	2.0136	-
		A2	kH	26.208	0.9506	0.9506	56.0392	2.3328	-
		A3	kH	25.877	0.9757	0.9757	49.5694	3.0610	-
		A4	kH	25.321	0.9352	0.9352	57.5535	2.0321	-
F=kH*t^0.5		A5	kH	25.534	0.9747	0.9747	50.0353	3.0417	-
		A6	kH	25.087	0.9610	0.9610	53.3071	2.5844	-
	AS-loaded PLGA MS		kH	29.697	0.8366	0.8366	67.1514	1.5893	-
Korsmeyer-Peppas	MS embedded in situ gel formulations	A1	kKP	31.538	0.9620	0.9565	55.8744	2.3540	0.434
		A2	kKP	30.111	0.9704	0.9662	53.4355	2.6221	0.446
		A3	kKP	29.724	0.9904	0.9890	43.2572	3.7624	0.442
		A4	kKP	30.559	0.9689	0.9645	52.9455	2.5441	0.422
F=kKP*t^n		A5	kKP	27.720	0.9812	0.9785	49.3660	3.1161	0.470
		A6	kKP	28.072	0.9738	0.9700	51.7317	2.7594	0.458
	AS-loaded PLGA MS		kKP	42.812	0.9622	0.9568	55.9752	2.8311	0.336
Peppas-Sahlin	MS embedded in situ gel formulations	A1	k1	**40.079**	**0.9851**	**0.9802**	**49.4194**	**3.0712**	**0.450**
		A2	k1	**37.309**	**0.9881**	**0.9841**	**47.2227**	**3.3125**	**0.450**
		A3	k1	**33.461**	**0.9953**	**0.9937**	**38.8371**	**4.2535**	**0.450**
		A4	k1	**37.571**	**0.9858**	**0.9811**	**47.8819**	**3.1067**	**0.450**
F=k1*t^m+k2*t^(2*m)		A5	k1	**32.265**	**0.9896**	**0.9862**	**45.9934**	**3.4908**	**0.450**
		A6	k1	**34.040**	**0.9879**	**0.9839**	**46.7403**	**3.3140**	**0.450**
	AS-loaded PLGA MS		k1	**52.786**	**0.9857**	**0.9809**	**49.2549**	**3.5778**	**0.450**
Weibull	MS embedded in situ gel formulations	A1	β	**0.604**	**0.9973**	**0.9964**	**34.0547**	**4.7784**	-
		A2	β	**0.609**	**0.9975**	**0.9966**	**33.2835**	**4.8612**	-
		A3	β	**0.610**	**0.9957**	**0.9959**	**37.9087**	**4.6900**	-
		A4	β	**0.565**	**0.9929**	**0.9906**	**41.5984**	**3.8049**	-
F=100*{1-Exp[-((t-Ti)^β)/α]}		A5	β	**0.632**	**0.9922**	**0.9896**	**43.4439**	**3.7741**	-
		A6	β	**0.603**	**0.9944**	**0.9925**	**39.8409**	**4.0806**	-
	AS-loaded PLGA MS		β	**0.566**	**0.9910**	**0.9881**	**45.0154**	**4.0489**	-

Best fit release kinetic models shown with gray fill; In all models, F is the fraction (%) of drug released in time t, k0: zero-order release constant, k1: first-order release constant, kH: Higuchi release constant, kKP: release constant incorporating structural and geometric characteristics of the drug-dosage form, n: is the diffusional exponent indicating the drug-release mechanism, m: diffusional exponent and similar exponent like ‘’n’’, m use in Peppas-Sahlin model equation only, β: the shape parameter which characterizes the curve as either exponential (β=1; case 1), sigmoid, S-shaped, with upward curvature followed by a turning point (β > 1; case 2), or parabolic, with a higher initial slope and after that consistent with the exponential (β < 1; case 3). Values shown in bold and with gray fill in the table are selections made according to criteria.

AUTHOR CONTRIBUTIONS

Conception: *H.K.P., S.Ü.*; Design: *H.K.P., S.Ü.*; Supervision: *H.K.P., S.Ü.*; Resources: *H.K.P., S.Ü.*; Materials: *H.K.P., S.Ü.*; Data Collection and/or processing: *H.K.P., S.Ü.*; Analysis and/or interpretation: *H.K.P., S.Ü.*; Literature search: *H.K.P., S.Ü.*; Writing manuscript: *H.K.P., S.Ü.*; Critical review: *H.K.P., S.Ü.*; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

REFERENCES

1. Haynes, D.R., Crotti, T., Zreiqat, H. (2004). Regulation of osteoclast activity in peri-implant tissues, *Biomaterials*, *25*, 4877-4885. [CrossRef]

2. Retzepi, M., Donos, N. (2010). Guided bone regeneration: biological principle and therapeutic applications, *Clinical Oral Implants Research*, *21*, 567-576. [CrossRef]

3. Brown, J.E., Coleman, R.E. (2001). The role of bisphosphonates in breast cancer: the present and future role of bisphosphonates in the management of patients with breast cancer, *Breast Cancer Research*, *4*, 1-6. [CrossRef]

4. Von Knoch, F., Jaquiery, C., Kowalsky, M., Schaeren, S., Alabre, C., Martin, I., Rubash, H.E., Shanbhag, A.S. (2005). Effects of bisphosphonates on proliferation and osteoblast differentiation of human bone marrow stromal cells, *Biomaterials*, *26*, 6941-6949. [CrossRef]

5. Miladi, K., Sfar, S., Fessi, H., Elaissari, A. (2015). Encapsulation of alendronate sodium by nanoprecipitation and double emulsion: From preparation to *in vitro* studies, *Industrial Crops and Products*, *72*, 24-33. [CrossRef]

6. Carvalho, I., Marques, C., Oliveira, R., Coelho, P., Costa, P., Ferreira, D. (2015). Sustained drug release by contact lenses for glaucoma treatment—A review, *Journal of Controlled Release*, *202*, 76-82. [CrossRef]

7. Öz, U.C., Toptaş, M., Küçüktürkmen, B., Devrim, B., Saka, O.M., Deveci, M.S., Bilgili, H., Ünsal, E., Bozkır, A. (2020). Guided bone regeneration by the development of alendronate sodium loaded in-situ gel and membrane formulations, *European Journal of Pharmaceutical Sciences*, *155*, 105561. [CrossRef]

8. Wysowski, D.K., Chang, J.T. (2005). Alendronate and risedronate: reports of severe bone, joint, and muscle pain. *Archives of Internal Medicine*, *165*, 346-347. [CrossRef]

9. Ideguchi, H., Ohno, S., Hattori, H., Ishigatsubo, Y. (2007). Persistence with bisphosphonate therapy including treatment courses with multiple sequential bisphosphonates in the real world, *Osteoporosis international*, *18*, 1421-1427. [CrossRef]

10. Dong, Y., Su, H., Jiang, H., Zheng, H., Du, Y., Wu, J., Li, D. (2017). Experimental study on the influence of low-frequency and low-intensity ultrasound on the permeability of the Mycobacterium smegmatis cytoderm and potentiation with levofloxacin, *Ultrasonics Sonochemistry*, *37*, 1-8. [CrossRef]

11. Pardeshi, S.R., Nikam, A., Chandak, P., Mandale, V., Naik, J.B., Giram, P.S. (2021). Recent advances in PLGA based nanocarriers for drug delivery system: a state of the art review, *International Journal of Polymeric Materials and Polymeric Biomaterials*, 1-30. [CrossRef]

12. Ruhe,P., Boerman, O., Russel, F., Spauwen, P., Mikos, A., Jansen, J. (2005). Controlled release of rhBMP-2 loaded poly (dl-lactic-co-glycolic acid)/calcium phosphate cement composites in vivo. *Journal of Controlled Release*, *106*, 162-171. [CrossRef]

13. Nasra, M.M., Khiri, H.M., Hazzah, H.A., Abdallah, O.Y. (2017). Formulation, in-vitro characterization and clinical evaluation of curcumin in-situ gel for treatment of periodontitis, *Drug Delivery*, *24*, 133-142. [CrossRef]

14. Ünal, S., Aktaş, Y. (2022). Bisphosphonate-loaded PLGA microspheres for bone regeneration in dental surgery: formulation, characterization, stability, and comprehensive release kinetic studies. *International Journal of Polymeric Materials and Polymeric Biomaterials*, 1-12. [CrossRef]

15. El-Kamel, A. (2002), *In vitro* and *in vivo* evaluation of Pluronic F127-based ocular delivery system for timolol maleate. *International Journal of Pharmaceutics*, *241*, 47-55. [CrossRef]

16. Polat, H.K. (2022). In situ gels triggered by temperature for ocular delivery of dexamethasone and dexamethasone/ SBE-β-CD complex., *Journal of Research in Pharmacy*, *26*(4), 873-883. [CrossRef]

17. Maheshwari, M., Miglani, G., Mali, A., Paradkar, A., Yamamura, S., Kadam, S. (2006). Development of tetracycline-serratiopeptidase-containing periodontal gel: formulation and preliminary clinical study, *AAPS PharmSciTech*, *7*, E162-E171. [CrossRef]

18. Qi, H., Chen, W., Huang, C., Li, L., Chen, C., Li, W., Wu, C. (2007). Development of a poloxamer analogs/carbopol-based *in situ* gelling and mucoadhesive ophthalmic delivery system for puerarin. *International Journal of Pharmaceutics*, *337*(1-2), 178-187. [CrossRef]

19. Alexandridis, P., Hatton, T.A. (1995). Poly (ethylene oxide) poly (propylene oxide) poly (ethylene oxide) block copolymer surfactants in aqueous solutions and at interfaces: thermodynamics, structure, dynamics, and modeling, *Colloids and Surfaces A: Physicochemical and Engineering Aspect*s, *96*, 1-46. [CrossRef]

20. Karataş, A., Sonakin, O., Kiliçarslan, M., Baykara, T. (2009). Poly (ε-caprolactone) microparticles containing Levobunolol HCl prepared by a multiple emulsion (W/O/W) solvent evaporation technique: Effects of some formulation parameters on microparticle characteristics, *Journal of Microencapsulatio*n, *26* 63-74. [CrossRef]

21. Zhang, Y., Huo, M., Zhou, J., Zou, A., Li, W., Yao, C., Xie, S. (2010). DDSolver: an add-in program for modeling and comparison of drug dissolution profiles, *The AAPS Journal*, *12*, 263-271. [CrossRef]

22. Pham, D.T., Phewchan, P., Navesit, K., Chokamonsirikun, A., Khemwong, T., Tiyaboonchai, W. (2021). Development of metronidazole-loaded *in situ* thermosensitive hydrogel for periodontitis treatment, *Turkish Journal of Pharmaceutical Sciences*, *18*, 510. [CrossRef]

23. Bohorquez, M., Koch, C., Trygstad, Pandit, N. (1999). A study of the temperature-dependent micellization of pluronic F12, *Journal of Colloid and Interface* Science, *216*, 34-40. [CrossRef]

24. Okur, N., Yozgatli, V., Okur, M.E., (2020). *In vitro–in vivo* evaluation of tetrahydrozoline-loaded ocular *in situ* gels on rabbits for allergic conjunctivitis management, *Drug Development Research, 81*, 716-727. [CrossRef]

25. Karatas, A., Boluk, A., Hilal Algan, A. (2014). Poloxamer/Chitosan *in situ* gelling system for ocular delivery of ofloxacin. *Current Drug Therapy*, *9*(4), 219-225. [CrossRef]

26. Gilbert, J. C., Richardson, J. L., Davies, M. C., Palin, K. J., Hadgraft, J. (1987). The effect of solutes and polymers on the gelation properties of pluronic F-127 solutions for controlled drug delivery. *Journal of Controlled Release*, *5*(2), 113-118. [CrossRef]

27. Edsman, K., Carlfors, J., Petersson, R. (1998). Rheological evaluation of poloxamer as an *in situ* gel for ophthalmic use, *European Journal of Pharmaceutical Sciences*, *6*, 105-112. [CrossRef]

28. Bansal, M., Mittal, N., Yadav, S.K., Khan, G., Gupta, P., Mishra, B., Nath, G.Ç (2018). Periodontal thermoresponsive, mucoadhesive dual antimicrobial loaded in-situ gel for the treatment of periodontal disease: Preparation, in-vitro characterization and antimicrobial study, *Journal of Oral Biology and Craniofacial Research*, *8*, 126-133. [CrossRef]

29. Du, L., Tong, L., Jin, Y., Jia, J., Liu, Y., Su, C., Li, X. (2012). A multifunctional in situ–forming hydrogel for wound healing. *Wound Repair and Regeneration*, *20*(6), 904-910. [CrossRef]

30. Fathalla, Z.M., Vangala, A., Longman, M., Khaled, K.A., Hussein, A.K., El-Garhy, O.H., Alany, R.G. (2017). Poloxamer-based thermoresponsive ketorolac tromethamine *in situ* gel preparations: Design, characterisation, toxicity and transcorneal permeation studies, *European Journal of Pharmaceutics and Biopharmaceutics*, *114*, 119-134. [CrossRef]

31. Chu, K., Chen, L., Xu, W., Li, H., Zhang, Y., Xie, W., Zheng, J. (2013). Preparation of a paeonol-containing temperature-sensitive *in situ* gel and its preliminary efficacy on allergic rhinitis. *International Journal of Molecular Sciences*, *14*(3), 6499-6515. [CrossRef]

32. Munot, N.M., Kishore, G., Mithila, K. (2014). Development and evaluation of biodegradable microspheres embedded in *in situ* gel for controlled delivery of hydrophilic drug for treating oral infections: *In vitro* and *in vivo* studies, *Asian Journal of Pharmaceutics (AJP)*, *8*, 3. [CrossRef]

33. Morsi, N., Ghorab, D., Refai, H., Teba, H. (2016). Ketoroloac tromethamine loaded nanodispersion incorporated into thermosensitive *in situ* gel for prolonged ocular delivery. *International Journal of Pharmaceutics*, *506*(1-2), 57-67. [CrossRef]

34. Beg, S., Dhiman, S., Sharma, T., Jain, A., Sharma, R. K., Jain, A., Singh, B. (2020). Stimuli responsive *in situ* gelling systems loaded with PLGA nanoparticles of moxifloxacin

hydrochloride for effective treatment of periodontitis. *AAPS PharmSciTech*, *21*(3), 1-18. [CrossRef]

35. Hu, C., Feng, H., Zhu, C. (2012). Preparation and characterization of rifampicin-PLGA microspheres/sodium alginate *in situ* gel combination delivery system, *Colloids and Surfaces B: Biointerfaces*, *95*,162-169. [CrossRef]

36. Yang, H., Li, J., Patel, S.K., Palmer, K.E., Devlin, B., Rohan, L.C. (2019). Design of poly (lactic-co-glycolic acid)(PLGA) nanoparticles for vaginal co-delivery of griffithsin and dapivirine and their synergistic effect for HIV prophylaxis, *Pharmaceutics*, *11*, 184. [CrossRef]

37. Papadopoulou, V., Kosmidis, K., Vlachou, M., Macheras, P. (2006). On the use of the Weibull function for the discernment of drug release mechanisms, *International Journal of Pharmaceutics*, *309*, 44-50. [CrossRef]

38. Sanchez, L.T., Pinzon, M.L., Villa, C.C. (2022). Development of active edible films made from banana starch and curcumin-loaded nanoemulsions, *Food Chemistry*, *371*, 131121. [CrossRef]

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 1009-1029, 2022
Doi: 10.33483/jfpau.1161238

ORIGINAL ARTICLE / ÖZGÜN MAKALE

PACLITAXEL-LOADED POLYCAPROLACTONE NANOPARTICLES FOR LUNG TUMORS; FORMULATION, COMPREHENSIVE *IN VITRO* CHARACTERIZATION AND RELEASE KINETIC STUDIES

AKCİĞER TÜMÖRLERİNE YÖNELİK PAKLİTAKSEL YÜKLÜ POLİKAPROLAKTON NANOPARTİKÜLLERİ; FORMÜLASYON, KAPSAMLI İN VİTRO KARAKTERİZASYON VE SALIM KİNETİK ÇALIŞMALARI

Sedat ÜNAL[1], Osman DOĞAN[2], Yeşim AKTAŞ[1*]

[1]Erciyes University, Faculty of Pharmacy, Department of Pharmaceutical Technology, 38280, Kayseri, Turkey

[2]Abdullah Gül University, Faculty of Life and Natural Sciences, Bioengineering Department, 38080, Kayseri, Turkey

ABSTRACT

Objective: *Today, cancer is still among the most common chronic diseases. Nanoparticular drug delivery systems prepared with biocompatible and biodegradable polymers such as polycaprolactone are rational solution for anticancer agents with poor solubility and low bioavailability. The aim of this study is to prepare paclitaxel-loaded polycaprolactone nanoparticles, which is known to be a potent anticancer, and to elucidate in vitro characteristics and release kinetic mechanisms.*

Material and Method: *It was aimed to prepare paclitaxel-loaded polycaprolactone nanoparticles by nanoprecipitation. Preformulation studies were carried out with different molecular weights of polycaprolactone (Mw: 14.000, Mw: 80.000). Nanoparticles were coated with Chitosan or Poly-l-lysine to obtain cationic surface charge and to increase cellular interaction. Comprehensive characterization of formulations and release kinetic studies were performed.*

Result and Discussion: *The particle size of the formulations ranged from 188 nm to 383 nm. Encapsulation efficiency increased to 77% in different formulations. SEM analysis confirmed the nanoparticles were spherical. Within the scope of in vitro release studies, the release continued for up to 96 hours and less than 50% of the therapeutic load was released in the first 24 hours. Mathematical modeling indicated that the*

* **Corresponding Author / Sorumlu Yazar:** Yeşim Aktaş
e-mail / e-posta: yaktas@erciyes.edu.tr, **Phone / Tel.:** +905057154102

Submitted / Gönderilme: 12.08.2022 **Accepted / Kabul:** 19.09.2022

release kinetics fit more than one model with the Korsmeyer-Peppas, Peppas-Sahlin and Weibull models, which show high correlation.

Keywords: *Chitosan, lung cancer, paclitaxel, polikaprolakton, poly-l-lysine*

ÖZ

Amaç: *Günümüzde kanser hala en sık görülen kronik hastalıklar arasında yer almaktadır. Polikaprolakton gibi biyouyumlu ve biyoparçalanır polimerlerle hazırlanan nanopartiküler ilaç taşıyıcı sistemler, düşük çözünürlük ve düşük biyoyararlanım gösteren birçok antikanser ajan için rasyonel bir çözümdür. Bu çalışmanın amacı, güçlü bir antikanser olduğu bilinen paklitaksel yüklü polikaprolakton nanopartiküllerinin hazırlanması ve hazırlanan nanopartiküllerin in vitro karakterizasyonlarını ve salım kinetik mekanizmalarını aydınlatmaktır.*

Gereç ve Yöntem: *Nanoçöktürme yöntemi ile paklitaksel yüklü polikaprolakton nanopartiküllerinin hazırlanması amaçlanmıştır. Polikaprolakton polimerinin iki farklı moleküler ağırlığı (Mw: 14.000 ve Mw: 80.000) ile ön formülasyon çalışmaları yapılmıştır. Hazırlanan nanopartiküller, katyonik yüzey yükü elde etmek ve hücresel etkileşimi artırmak için Chitosan (CS) veya Poly-l-lisin (PLL) ile ayrı ayrı kaplanmıştır. Formülasyonların kapsamlı karakterizasyon çalışmaları ve salım kinetik çalışmaları yapılmıştır.*

Sonuç ve Tartışma: *Formülasyonların partikül boyutu 188 nm ila 383 nm arasında değişmektedir. Enkapsülasyon etkinliği, farklı formülasyonlarda %77'ye kadar yükselmiştir. SEM analizi, nanopartiküllerin küre şeklinde olduğunu doğrulamıştır. İn vitro salım çalışmaları kapsamında 96 saate kadar salım devam etmiş ve ilk 24 saatte terapötik yükün %50'sinden azı salınmıştır. Matematiksel modelleme çalışmaları, formülasyonların salım kinetiğinin, yüksek korelasyon gösteren Korsmeyer-Peppas, Peppas-Sahlin ve Weibull modelleri ile birden fazla modele uyduğunu göstermiştir.*

Anahtar Kelimeler: *Akciğer kanseri, kitosan, paklitaksel, polikaprolakton, poli-l-lizin*

INTRODUCTION

Cancer is a leading cause of death throughout the world, characterized by metastasis and uncontrolled proliferation. Approximately, 40% of cancer cases consists of breast, lung, prostate, stomach, colon, and skin cancer [1]. Among them, lung cancer is one of the most commonly diagnosed cancers in both men and women. Lung cancer related deaths account for approximately 20% of total cancer related deaths [2]. Lung cancer is classified into two main types, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCL accounting for %87 of total lung cancer cases, is highly resistant to treatment including surgery, radiotherapy and chemotherapy [3]. Despite recent advances in cancer treatment, patients with lung cancer still have few therapeutic options and a very low survival rate which remains under 20%, due to poor prognosis, late diagnosis, development of drug resistance as well as low tumor selectivity [4]. In addition to these shortages, current traditional chemotherapeutics not only have limited efficacy but also cause systemic adverse effects which are among the most important factors in treatment discontinuation [5]. Another disadvantage of current traditional chemotherapeutic agents is that extremely high dose needs to be used in order to provide effective treatment. Thus, healthy tissue cells die more, and it is more likely to occur multi-drug resistance [6]. Given all of these drawbacks, it is obvious that a novel effective treatment method is required to increase the survival rate of lung cancer.

Paclitaxel (PCX) is a natural product isolated from North American Pacific yew tree, Taxus

brevifolia. Shortly after the discovery of PCX, it was found that PCX exhibited high anti-cancer activity [7]. Today PCX is one of the widely utilized molecules for an effective treatment of cancers namely lung, ovarian, breast and other cancer types. PCX is a classical microtubule inhibitor that acts by inhibiting the depolymerization of microtubules and block cancer cell at the G2/M phase [8]. Although PCX seems to be an acceptable candidate as anti-cancer agent, its severe side effects restrict the use of PCX in cancer therapy. PCX also adversely influences healthy cell such as immune system cells which may result in escaping tumor cells and the propagation of drug-resistant clones [9]. Another issue that relates to the drawback of PCX is the poor solubility of PCX in water (less than 1 μg/ml) [10]. Owing to its poor aqueous solubility, natural form of PCX cannot be used efficiently in treatment.

Nanomedicine provides a wide range of benefits that can overcome significant obstacles which other conventional chemotherapeutics suffer from and increase the survival rate in cancer treatment. Therefore, in recent years, enormous time and effort have been dedicated to developing nanotechnological approaches. Nanotechnology-based drug delivery systems offer longer circulation time, accumulation in targeted area, controlled release profile, resulting in more efficient therapeutic effect [11]. In particular many anti-cancer agents currently used in treatment such as paclitaxel, docetaxel, camptothecin have quite poor aqueous solubility due to their large polycyclic nature [12]. Nanoparticles (NPs) may also help to solve solubility problem of hydrophobic molecules [13]. After it has been realized that NPs is a promising carrier to encapsulate and deliver poorly soluble molecules, different types of NPs have been developed including carbon-based nanoparticles, metal nanoparticles, polymeric nanoparticles, lipid-based nanoparticles. Among them, polymeric NPs have emerged as promising carrier platform because of their capability of high drug entrapment, biocompatible properties and protection against drug degradation [14]. Polycaprolactone (PCL) is a semi-crystalline aliphatic polyester approved by FDA (Food and Drug Administration) to be used in clinical studies. High drug permeability, slow in vivo degradation as well as non-toxic properties of PCL make it a suitable polymer for drug delivery. In order to achieve an outstanding therapeutic potential, PCL has been combined with various drugs such as docetaxel, campthotecin, mitomycin C [15-17]. Various modifications can be made with a state-of-art approach in the design of PCL-based nanoparticulate drug delivery systems in order to increase the therapeutic efficacy, increase the cellular interaction through cationic properties, and revise the drug release profile in line with the objectives [17-19]. Therefore, various modification approaches can provide pharmaceutical advantages in the design of PCL-based nanoparticle drug delivery systems.

Recently, surface coating has aroused a great deal of interest. Surface modification materials have an essential role in fate and behavior of NPs between in biological environment. Despite the unique features of NPs, they cannot reach optimum point to show maximum impact. Surface coating of NPs with appropriate material enhances the adhesion and the retention time of NPs on target tissue [19].

Chitosan (CS) is a natural cationic polysaccharide consisting of two main units, glucosamine and N-acetylglucosamine [20]. CS has been widely preferred polymer as coating material mainly owing to its positive charge which increases the mucoadhesion of NPs. Besides, other properties of chitosan such as non-toxic, biocompatible, antimicrobial make it one of the most suitable materials for nanomedicine [21]. Poly-L-lysine (PLL) is another cationic biocompatible polymer. PLL significantly enhances cell adhesion as positively charged PLL causes interaction between NP and negatively charged cell membrane. Furthermore, PLL has good solubility in water, stable structure in biological environment as well as exerts antimicrobial effect in neutral or weak acid conditions [22].

In the present study, we prepared PCX-loaded PCL NPs as a new drug delivery system for PCX by nanoprecipitation method. In the scope of the study, passive targeting strategy was aimed for tumor targeting of nanoparticles. The passive targeting strategy is one of the most common tumor targeting approaches in nanotechnology studies. With the passive targeting approach realized due to the particle size being at the nanoscale, tumor site accumulation as a function of the size of the nanoparticles and due to the degeneration of the vasculature in the tumoral region was determined as a preliminary phenomenon [23]. NPs were coated by CS or PLL to increase cellular interaction because of their cationic charges. There are several studies that point out impact of molecular weight of PCL on characterization of NPs [23,24]. Therefore, two different kinds of PCL with different molecular weight, 14.000 and 80.000 were used in this study. In order to characterize NPs, polydispersity index, zeta potential, particle size, drug entrapment efficiency, and in vitro PCX release from PCL NPs and mathematic modeling of release kinetic were examined.

MATERIAL AND METHOD

Materials

Polycaprolactone (PCL) (Mw:14.000 and Mw:80.000)**,** Paclitaxel (Mw:853.91, ≥95% (HPLC)) and Poly-L-lysine solution (0.1% (w/v)) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Chitosan (Protasan UP G-113; Mw:<200 kDa) was purchased from Novamatrix, Norway. Acetone and dialysis cellulose tubing membrane (average flat width 25 mm, MWCO: 14,000 Da) were purchased from Sigma&Aldrich, USA.

Preparation of blank and PCX-loaded PCL nanoparticles

Preparation of blank and PCX-loaded PCL NPs was carried out by nanoprecipitation method. Organic phase was prepared using two different PCL types, 14.000 and 80.000 molecular weight. For PCX-loaded PCL NPs, 10 mg PCL was weighed for each batch and 1mg of PCX was added to the organic phase. Polymer and PCX were dissolved in 5 mL acetone by magnetic stirring for 45 min. To

prepare the aqueous phase, three batches were designed according to coating material, CS and PLL. 75 mg of Pluronic F-68 and 2.5 mg chitosan or 0.01% PLL (v/v) were added to 10 mL ultra-pure water and stirred at 500 rpm at room temperature. The third group consists of NPs that do not contain any coating materials. Organic phase was added dropwise to aqueous phase at 800 rpm. In order to evaporate organic phase, solutions were mixed at 500 rpm at room temperature overnight. Dispersions were centrifuged at 3500 rpm for 5 min. Finally, in order to to remove the aggregate from solution, supernatants were filtered using 0.45 μm pore size filter. Same process was conducted for blank formulations.

Characterization of nanoparticles

Determination of particle size, PDI and zeta potential

Particle size, polydispersity index and zeta potential of PCL NPs were analysed by Malvern Zetasizer Nano ZS series, UK. All measurements were conducted at an angle of 173° for particle diameter and 12.8° for zeta potential. Measurements were performed in triplicate at room temperature. Particle size distribution was calculated as mean diameter (nm) ± standard deviation (SD) and PDI. Zeta potential (mV) was expressed as the average of three subsequent measurements ± SD.

Morphological analysis

For the determination of the surface morphology of NPs, Scanning Electron Microscopy (SEM) (Zeiss evo LS-10, Germany) was employed. The lyophilized PCL NPs were embedded on metal stubs and then coated with 100 A° thick layer of gold and palladium and dried for 24 h. The shape and surface morphology of drug loaded formulations were determined by SEM.

Determination of entrapment efficiency (EE) and drug loading (DL)

The entrapment efficiency of PCX-loaded PCL NPs was quantified directly by UV spectrophotometer. Briefly, 3 mg of the freeze-dried PCL NPs were dissolved in 300μL dichloromethane (DCM) and mixed thoroughly for 10 min so that polymer structure is disrupted and entrapped drug is released from nanoparticles. To dissolve PCX, 3 mL of methanol was added, and dichloromethane was evaporated by stirring at 500 rpm for 2 hours. Final solution was centrifuged at 3000 rpm for 5 min and supernatant was tested to determine entrapment efficiency of PCL NPs (Eq. 1 and Eq. 2). Within the scope of the validation of the spectrophotometric method for PCX quantification, linearity, specificity, precision, repeatability, limit of detection (LOD), limit of quantification (LOQ) were determined. Quantitation of PCX was determined by the validated spectrophotometric method (r^2=0.9974) at 230 nm.

DL (%) = (Weight of PCX in the NPs / Total weight of NPs) × 100 (1)

EE (%) = (Weight of PTX in NPs / Initial weight of PTX used) × 100 (2)

In vitro release of PCX from PCL nanoparticles

In vitro drug release of PCX from PCL nanoparticles were determined by using dialysis membrane (MWCO: 14,000 Da). Dialysis membrane was activated with 1% w/v NaOH overnight. PCL nanoparticle formulations loaded with PCX were prepared freshly. 2 mL of PCL NPs suspension was transferred into a dialysis membrane, hermetically sealed, and subsequently incubated in a beaker containing 75 mL phosphate buffer saline (PBS) (pH: 7.4). The system was conducted under sink conditions at 37 °C stirring at 200 rpm. At predetermined time intervals (0.5, 1, 2, 4, 8, 16, 24, 36, 48, 72, 96h), 1 mL sample was collected and replaced with fresh PBS at same volume and temperature. The amount of released PCX in PBS was analysed with UV spectrophotometer at 230 nm. Release percentage over time of the PCX-loaded PCL NPs was evaluated and plotted for each formulation.

Release kinetic studies

The release profile of PCX-loaded PCL nanoparticles was examined using DDSolver, a program intended to speed up computations and avoid computational mistakes. The resulting data were fitted to several kinetic models and examined for release mechanism (Zero order, First order, Higuchi, Korsmeyer-Peppas, Peppas-Sahlin, Weibull and Baker-Lonsdale model) [25]. After the in vitro release profiles of nanoparticles were clarified, inputs were computed using the DDSolver program to determine the four most significant and meaningful criteria: coefficient of determination (R^2), coefficient of determination adjusted ($R^2_{adjusted}$) Akaike Information Criterion (AIC), and Model Selection Criterion (MSC). The highest R^2, $R^2_{adjusted}$ and MSC values and the lowest AIC values were used to determine the best fist model [25, 26]. Additionally, using the "difference (f1)" and "similarity (f2)" factors, the release variations or similarities of PCX-loaded PCL nanoparticles were evaluated [25, 27]. In order to compare the release profiles of nanoparticles, the difference factor (f1) and similarity factor (f2) were computed using a method outlined in the Guidance for Industry from the FDA's Center for Drug Evaluation and Research (CDER) [28]. These two factors can be calculated mathematically by the following equations [29]. R and T represent the dissolved percentages of the reference and test profiles, respectively whereas t represents the time point, and n is the total number of sample points. It should be noted that f1 values for 0–15 and f2 values for 50–100 show that these release profiles are similar [30, 31].

$$f1 = \{(\sum_{t=1}^{n}|R - T|)/(\sum_{t=1}^{n} R)\}\, x\, 100 \quad (3)$$

$$f2 = 50.log\left[\frac{100}{\sqrt{1+\frac{\sum_{t=1}^{t=n}[Rt-Tt]^2}{n}}}\right] \quad (4)$$

RESULT AND DISCUSSION

Determination of particle size, PDI and zeta potential

The particle size, PDI, and zeta potential of both types of PCL NPs are shown in Table 1. Particle size plays vital role in interaction between NPs and cell membrane, cellular uptake, penetration as well as determining the administration route. Particularly for intravenous administrations, nanoparticle diameter should be below a certain size since they might cause occlusion in blood capillaries. On the other hand, smaller particles are likely to have toxic effects due to their greater surface area [32]. Therefore, mean size of NPs should be at optimal range for *in vivo* delivery. The particle size of the developed PCL NPs ranged from 199 nm to 383 nm indicating that the size of the produced particles is in the acceptable range. The first group prepared with 80.000 MW PCL has greater particle size compared to second group prepared with 14.000 MW PCL. The main reason of this difference could be elucidated by viscosity resulted from high molecular weight. The increase of PCL molecular weight causes enhancement of viscosity in organic phase that hinders the diffusion of organic phase into aqueous phase. As a result, larger particles are formed. Our results showing that the increase of the particle size relies on molecular weight are in accordance with the data presented by Miladi et al. [23]. Particle size of uncoated NPs are between 188nm-230nm. While the particle size of CS-coated NPs ranged from 316nm-383nm, particle size of PLL-coated NPs ranged from 199nm-248nm. Both coating polymers caused an increase in particle size due to their settlement on the nanoparticle surface. This increase was higher in CS coated nanoparticles than in PLL. There are studies with similar results in the literature, and our findings were evaluated in accordance with previous studies [15, 17, 31]. As expected, surface modification had remarkable impact on particle size.

PDI values indicate homogeneity of NPs size in formulation. Values close to 0 indicate a monodisperse system while values close to 1 indicate that the system has a heterogeneous structure consisting of aggregate, polymer residues, particles with different size. As presented in Table 1, each PDI values were quite close to 0. There were also no notable variations between two types of PCL.

Zeta potential is among the most important factors affecting particle character and stability. In the literature, it is emphasized that NPs with positively charged are highly likely to interact with cell membrane since cell surface is charged negatively [23]. Yue et al. reported that positive charge encouraged the internalization of NPs to cells and further enhanced the cellular uptake. Additionally, while majority of positively charged NPs escape from lysosome, negatively or neutrally charged nanoparticles localize with lysosome [34]. According to this study, Ünal et al. stated that NPs coated with positively charged materials considerably increased the interaction between NPs and mucus layer [35]. PCL has negative surface charge because of terminal carboxylic groups. Coating materials used in the current study, PLL and CS, markedly shifted zeta potential of NPs from negative to positive. As

illustrated in Table 1, zeta potential values of uncoated NPs were all negative, between -20.1 and -25.8, while surface charge of coated NPs are range from +29.6 to +57.1. Coating materials not only altered surface charge positively but also provided higher zeta potential. Regardless of the charge type, NPs with low zeta potential tend to form aggregation owing to low electrostatic repulsive forces between the NPs [36]. In Table 1, it is clearly observed that surface modification with PLL and CS considerably rose the surface charge of NPs, from -21.4 mV to +57.1 mV. CS also showed superiority to PLL in terms of increase in zeta potential and encapsulation efficiency. It can be interpreted that CS coating has more influence on the properties of nanoparticles than PLL coating. There are studies with similar results in the literature, and our findings were evaluated in accordance with previous studies [15, 17, 31].

Table 1. Mean particle size, PDI and zeta potential of blank and PCX-loaded PCL nanoparticles prepared with different molecular weight of polymer (Organic solvent is acetone, PCL concentration is 0.2 % (w/v), CS concentration is 0.025 % (w/v), PLL concentration is 0.01 % (w/v), Pluronic F-68 concentration is 0.75% (w/v), organic phase: aqueous phase ratio 1:2 (v:v)) (n = 3, ± SD).

PCL Nanoparticle Formulations			**Particle Diameter ± SD (nm)**	**PDI ± SD**	**ZP ± SD (mV)**
M_w (Da)	**Formulation Code**	**Blank/PCX loaded**			
80,000	PCL NPs	Blank	209.2±1.1	0.081±0.009	-21.4±1.1
		PCX loaded	234.4±1.3	0.112±0.014	-24.9±2.1
	CS/PCL NPs	Blank	342.2±1.9	0.216±0.091	+57.1±3.1
		PCX loaded	383.8±2.4	0.253±0.122	+51.3±6.1
	PLL/PCL NPs	Blank	226.5±1.5	0.098±0.014	+34.1±3.1
		PCX loaded	248.7±1.6	0.121±0.103	+30.6±4.2
14,000	PCL NPs	Blank	188.3±1.2	0.099±0.004	-20.1±1.0
		PCX loaded	204.4±1.9	0.125±0.036	-25.8±2.3
	CS/PCL NPs	Blank	316.1±3.2	0.197±0.022	+52.3±2.3
		PCX loaded	346.2±3.9	0.224±0.018	+48.9±2.3
	PLL/PCL NPs	Blank	199.7±1.4	0.133±0.008	+31.6±1.1
		PCX loaded	227.5±1.6	0.148±0.012	+29.6±1.1

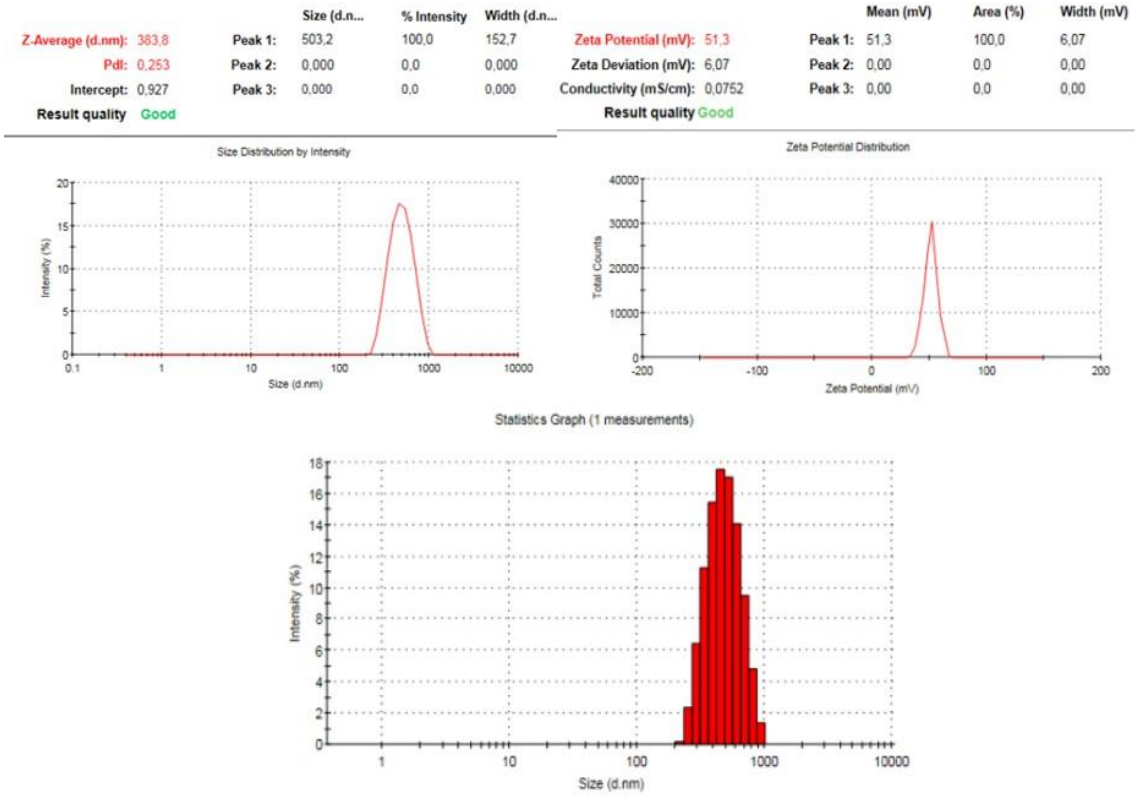


Figure 1. Analysis results of nanoparticles by Malvern zetasizer ZS. Representative images are presented for PCX-loaded CS coated PCL NPs (CS/PCX-PCL NPs) (Mw 80,000 Da) as the largest nanoparticles.

Determination of entrapment efficiency (EE) and drug loading (DL)

Table 2. summarizes the entrapment efficiency and drug loading of batches of PCX-PCL NPs prepared with two types of PCL polymer and different coating materials. Encouragingly, all prepared NPs exhibited a high entrapment rate of more than 50%. Encapsulation efficiency of NPs prepared with 80.000 MW PCL ranged from %59.4 to %64.7 while entrapment efficiency of NPs with 14.000 MW PCL varied between %51.3 and %63.1. The drug loading of PCX-loaded NPs prepared with 80.000 and 14.000 MW PCL resulted in between %6.2-8.4 and %5.2-6.6, respectively.

As it can be seen from Table 2, maximum encapsulation efficiencies were obtained with 80.000 MW of PCL. In accordance with results reported by Ali et al. and Miladi et al. NPs prepared with high molecular weight PCL showed higher encapsulation efficiency compared to NPs prepared with low molecular weight PCL [23,37]. The reason why NPs prepared with 80.000 MW PCL have higher encapsulation efficiency is the same as the reason why NPs have higher size. Increasing organic phase viscosity caused bigger particles resulting in high encapsulation efficiency. Considering the effects of coating material on encapsulation efficiency; it is clearly seen in Table 2 that minimum entrapment

ratios belonged to uncoated NPs while coating materials significantly increased encapsulation rates. This situation proves that due to the presence of coating materials, more PTX was absorbed into the surface of PCL NPs. In addition to that, NPs coated with CS had higher encapsulation efficiency than NPs coated with PLL. It can be interpreted that CS coating has more influence on the properties of nanoparticles size than PLL coating. The reason for this situation may be experimental differences, as well as the high hydrophilic character of the PLL polymer solution has a tendency to leak into the aqueous phase in the coating process. This situation was found to be compatible with similar results in previous studies [17, 31]. Also similar results regarding effect of coating material on encapsulation were presented by Badran et al. [38].

Table 2. Encapsulation efficiency (EE) and drug loading (DL) of PCX-loaded PCL nanoparticles prepared with different molecular weight of polymer.

PCL Nanoparticle Formulations		**Encapsulation efficiency % ± SD (EE)**	**Drug loading % ± SD (DL)**
M_w (Da)	**Formulation Code**		
80,000	PCX-PCL NPs	59.4±.1.6	6.2±0.3
	CS/PCX-PCL NPs	77.2±2.1	8.4±0.7
	PLL/PCX-PCL NPs	64.7±1.9	6.7±0.2
14,000	PCX-PCL NPs	51.3±1.1	5.2±0.1
	CS/PCX-PCL NPs	63.1±1.7	6.6±0.3
	PLL/PCX-PCL NPs	58.4±1.4	5.9±0.1

Morphological analysis

In order to elucidate the surface morphology of PCX-loaded NPs, SEM pictures were taken. Figure 2 and Figure 3 present SEM picture of NPs prepared with 14.000 MW and 80.000 MW PCL, respectively. In Figure2, size of NPs ranged between 200-310 nm, and they were in coincidence with results obtained from DLS. In Figure 3, size of NPs was 238-380 nm which shows coherence with data obtained from DLS. As seen in Figure 2 and 3, coated NPs are larger than uncoated NPs. These SEM pictures indicate that all NPs formulations have smooth and spherical surfaces.

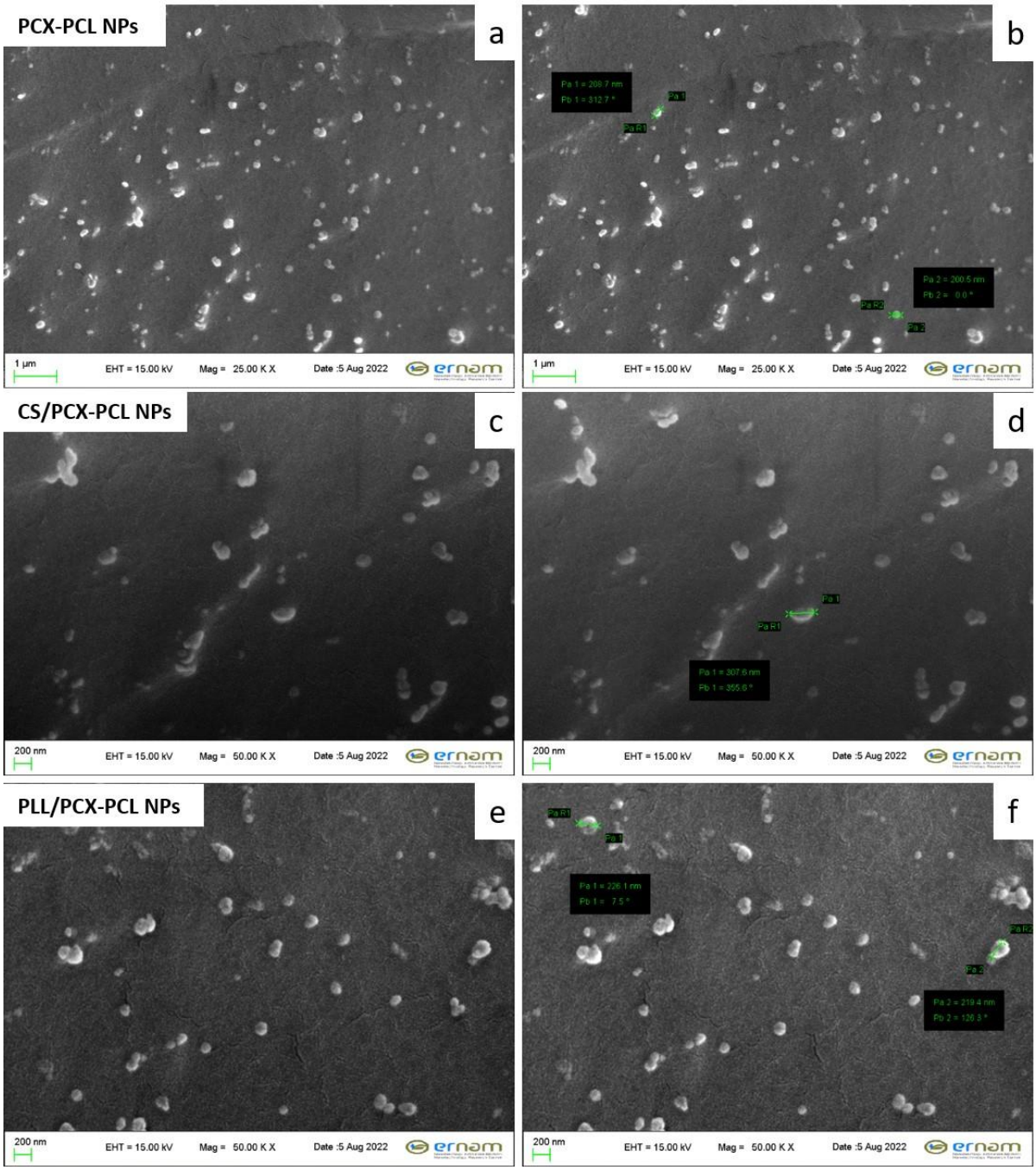


Figure 2. Scanning electron microscopy (SEM) micrographs of PCX-loaded NPs. (a, b) PCX-PCL NPs. (c, d) CS/PCX-PCL NPs. (e, f) PLL/PCX-PCL NPs. (a, b, c, d, e, f; PCL MW: 14,000Da). (b, d, f; Representative images with measuring scales)

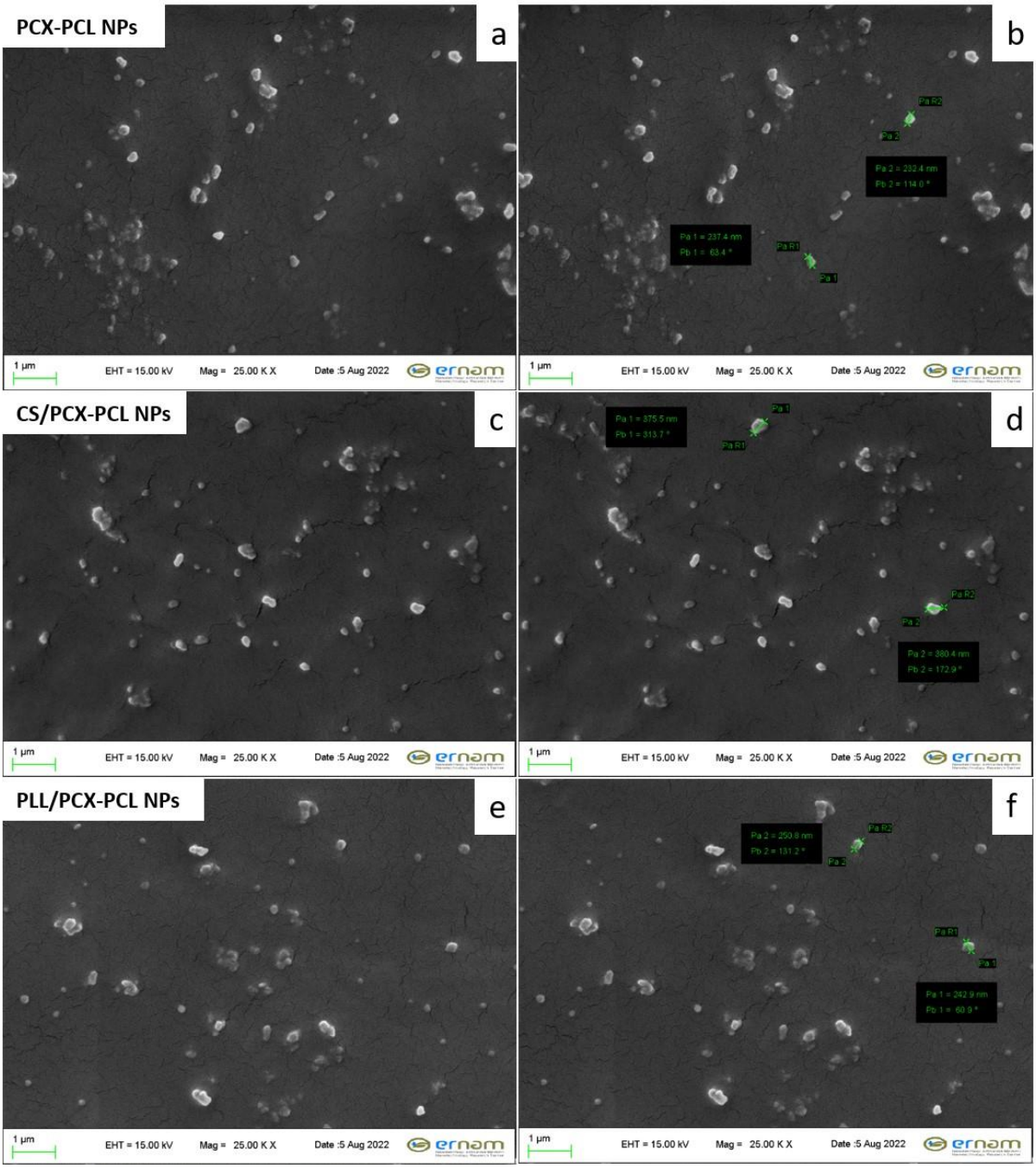


Figure 3. Scanning electron microscopy (SEM) micrographs of PCX-loaded NPs. (a, b) PCX-PCL NPs. (c, d) CS/PCX-PCL NPs. (e, f) PLL/PCX-PCL NPs. (a, b, c, d, e, f; PCL MW: 80,000Da). (b, d, f; Representative images with measuring scales).

In vitro release of PCX from PCL nanoparticles

Fig. 4 displays the *in vitro* release profiles of PCX from PCL NPs determined by using the dialysis membrane. In the light of the information obtained from the pre-formulation studies, since PCL 80.000 had higher encapsulation and drug loading, it was selected for in vitro release studies. As it can

be seen in Figure 4, PCX release from uncoated PCL formulation exhibited markedly faster profile up to 96 h in comparison with coated formulations. On the other hand, in the first 24 hour as highlighted in Figure 4, a burst release of PCX was observed for both CS and PLL coated formulations. It is believed that some of the drug which is absorbed to the coating material provides an initial fast release [39]. The reduction in the acceleration of the release profile of the coated NPs after burst effect proved this theory.

Negatively charged PCX has a stronger interaction with cationic NPS and thus, positively charged NPs extend the release time of the anionic molecules [40]. In our study, positively charged NP formulations established strong interaction with PCX and prolonged the release time of PCX from NPs. It is known that NPs with small particle size tend to exhibit faster drug release due to larger surface area [41]. As seen in Figure 4, the CS coated formulation had the slowest release profile and the uncoated PCX-PCL formulations had the faster release profile. When evaluated together with the particle sizes, according to the release profile of the formulations, smaller particles (PCX-PCL) showed a higher release rate and larger particles (CS/PCX-PCL) showed a slower release. The same is true for PLL/PCX-PCL NPs and is clearly visible. These results showed that the increase in particle size led to a decrease in the release rate, and the results were considered to be compatible with similar studies in the literature [17, 31, 41].

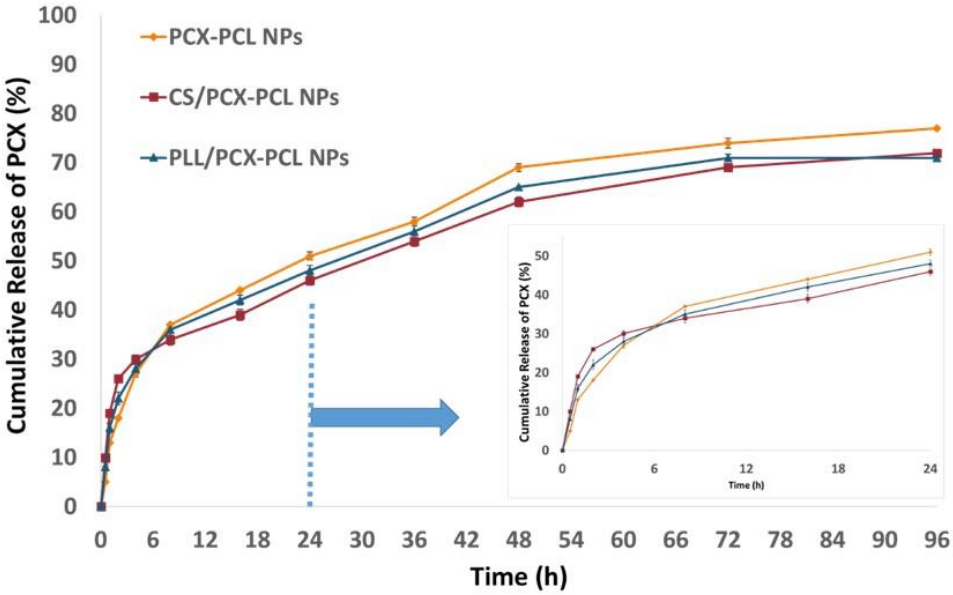


Figure 4. Release profile of PCX from PCL nanoparticles (n=3)

Release kinetic studies

In vitro dissolution test has an important role in drug development processes and quality control. It is not only a way to observe the stability of drug products but also rapid and affordable technique to

estimate fate of drug in vivo. Thus, recently quantitative examination of drug dissolution profiles has received considerable attention [25].

Kinetic modeling of PCX release from PCL NPs are demonstrated in Table 3. In order to determine the commonly used parameter (R^2, $R^2_{adjusted}$, AIC, MSC) in release kinetic, obtaining data were processed using DDSolver program. According to the results, the model having the highest R^2, $R^2_{adjusted}$, MSC as well as the lowest AIC was considered as the best-fitted one [42].

For the Weibull model, β exponent is used to explain the release from a polymeric matrix. If β is less than 0.75, it points out Fickian diffusion. $0.75< \beta <1$ means Fickian diffusion and swelling controlled release [43]. Considering the Peppas-Sahlin model, "m" represents diffusional exponent. Release exponent ("n" for Korsemeyer-Peppas) value helps to explain how the drug released from their matrix. $m \leq 0.45$ indicates Fickian diffusion. "m" value between 0.45-0.85 indicates non-Fickian diffusion and for m=0.85 the drug release occurs through case II transport [44]. The Peppas-Sahlin and Weibull model were the two-model having highest R^2, $R^2_{adjusted}$, MSC values and lowest AIC. In other words, there was a remarkable correlation between Peppas-Sahlin and Weibull model. Our results pointed out that release of PCX from PCL NPs was predominantly driven by Fickian release. In the literature, there are studies showing consistency with our results [45, 46].

Diffusional exponent value was represented using "m" in Peppas-Sahlin and "n" in Korsmeyer-Peppas model. In the case of CS-coated NPs, Korsmeyer-Peppas model exhibited superior to portray the release of PCX. The R^2 value was 0.9858 and release exponent of Korsmeyer-Peppas (n) was 0.329. The n value lower than 0.43 indicates the approach of PCX release mechanism toward Fickian diffusion-controlled release [47]. On the other hand, data obtained from CS/PCX-PCL formulations were also well fitted by Weibull model. According to the results obtained by Weibull model, PCX release kinetics from PCL NPs was found to be compatible with Fickian diffusion. Both two models showed almost same R^2 and $R^2_{adjusted}$ values. Soares et. al. reported similar results suggesting the possibility of two models for a formulation [48].

Regarding the PLL/PCX-PCL NPs, Peppas-Sahlin and Weibull were the model with the best fitting. This means that release of PCX from PLL-coated NPs, like uncoated PCX-PCL NPs, is driven by mechanism of Fickian release [45,46].

For each formulation, similarity and difference factors were evaluated. As it can be seen in Table 4, all formulations have the difference factor below 15 and similarity factor above 50. All the formulations were similar to each other in terms of release profile. This means that although different materials were used for coating resulting in changes in properties of NPs, release profile of formulations exhibited similarity due to the use of the same polymer constructing the common structure.

Table 3. Release kinetic modeling and results of PCX-loaded PCL NPs

Model and equation / Formulation		Evaluation criteria						
		Parameter		R^2	$R^2_{adjusted}$	AIC	MSC	n/m*
Zero-order	PCX-PCL	k0	1.084	0.4452	0.4452	102.8355	0.2077	-
	CS/PCX-PCL	k0	1.004	0.2343	0.2343	102.8346	-0.1918	-
F=k0*t	PLL/PCX-PCL	k0	1.023	0.3049	0.3049	103.0578	-0.0601	-
First-order	PCX-PCL	k1	0.028	0.8284	0.8284	88.7561	1.3809	-
	CS/PCX-PCL	k1	0.025	0.6319	0.6319	94.0461	0.5405	-
F=100*[1-Exp(-k1*t)]	PLL/PCX-PCL	k1	0.025	0.7202	0.7202	92.1401	0.8497	-
Higuchi	PCX-PCL	kH	9.219	0.9319	0.9319	77.6686	2.3049	-
	CS/PCX-PCL	kH	8.601	0.8650	0.8650	82.0087	1.5437	-
F=kH*t^0.5	PLL/PCX-PCL	kH	8.765	0.8932	0.8932	80.5806	1.8130	-
Korsmeyer-Peppas	PCX-PCL	kKP	11.280	0.9378	0.9316	78.5728	2.2295	0.466
	CS/PCX-PCL	kKP	16.878	0.9858	0.9844	56.9837	3.6291	0.329
F=kKP*t^n	PLL/PCX-PCL	kKP	14.467	0.9730	0.9703	66.0692	3.0223	0.380
Peppas-Sahlin	PCX-PCL	k1	15.077	0.9918	0.9899	56.3194	4.0840	0.450
	CS/DCX-PCL	k1	15.161	0.9776	0.9726	64.4759	3.0047	0.450
F=k1*t^m+k2*t^(2*m)	PLL/PCX-PCL	k1	15.395	0.9926	0.9909	52.6259	4.1426	0.450
Weibull	PCX-PCL	β	0.547	0.9930	0.9915	54.3357	4.2493	-
	CS/PCX-PCL	β	0.394	0.9830	0.9793	61.1209	3.2843	-
F=100*{1-Exp[-((t-Ti)^β)/α]}	PLL/PCX-PCL	β	0.449	0.9929	0.9914	51.9966	4.1950	-
Baker-Lonsdale	PCX-PCL	kBL	0.003	0.9631	0.9631	70.3079	2.9183	-
	CS/PCX-PCL	kBL	0.002	0.9193	0.9193	75.8300	2.0586	-
3/2*[1-(1-F/100)^(2/3)]-F/100=kBL*t	PLL/PCX-PCL	kBL	0.002	0.9498	0.9498	71.5278	2.5674	-

Best fit release kinetic models for PCX-loaded PCL NPs shown with grey; In all models, F is the fraction (%) of drug released in time t, k0: zero-order release constant, k1: first-order release constant, kH: Higuchi release constant, kKP: release constant incorporating structural and geometric characteristics of the drug-dosage form, n: is the diffusional exponent indicating the drug-release mechanism, m: diffusional exponent and similar exponent like ''n'', m use in Peppas-Sahlin model equation only, α: is the scale parameter which defines the time scale of the process; β: the shape parameter which characterizes the curve as either exponential (β=1; case 1), sigmoid, S-shaped, with upward curvature followed by a turning point (β > 1; case 2), or parabolic, with a higher initial slope and after that consistent with the exponential (β < 1; case 3), Ti: the location parameter which represents the lag time before the onset of the dissolution or release process and in most cases will be near zero. Values shown in grey in the table are selections made according to criteria.

Table 4. Calculation of the differences and similarities of the release profiles of the nanoparticle formulations with the difference (f1) and similarity (f2) factors

Nanoparticle Formulation	***Difference Factor (f1)***	***Similarity Factor (f2)***
PCX-PCL NPs and CS/PCX-PCL	*11.84*	*64.35*
PCX-PCL NPs and PLL/PCX-PCL	*6.98*	*74.21*
PLL/PCX-PCL NPs and CS/PCX-PCL	5.42	79.82

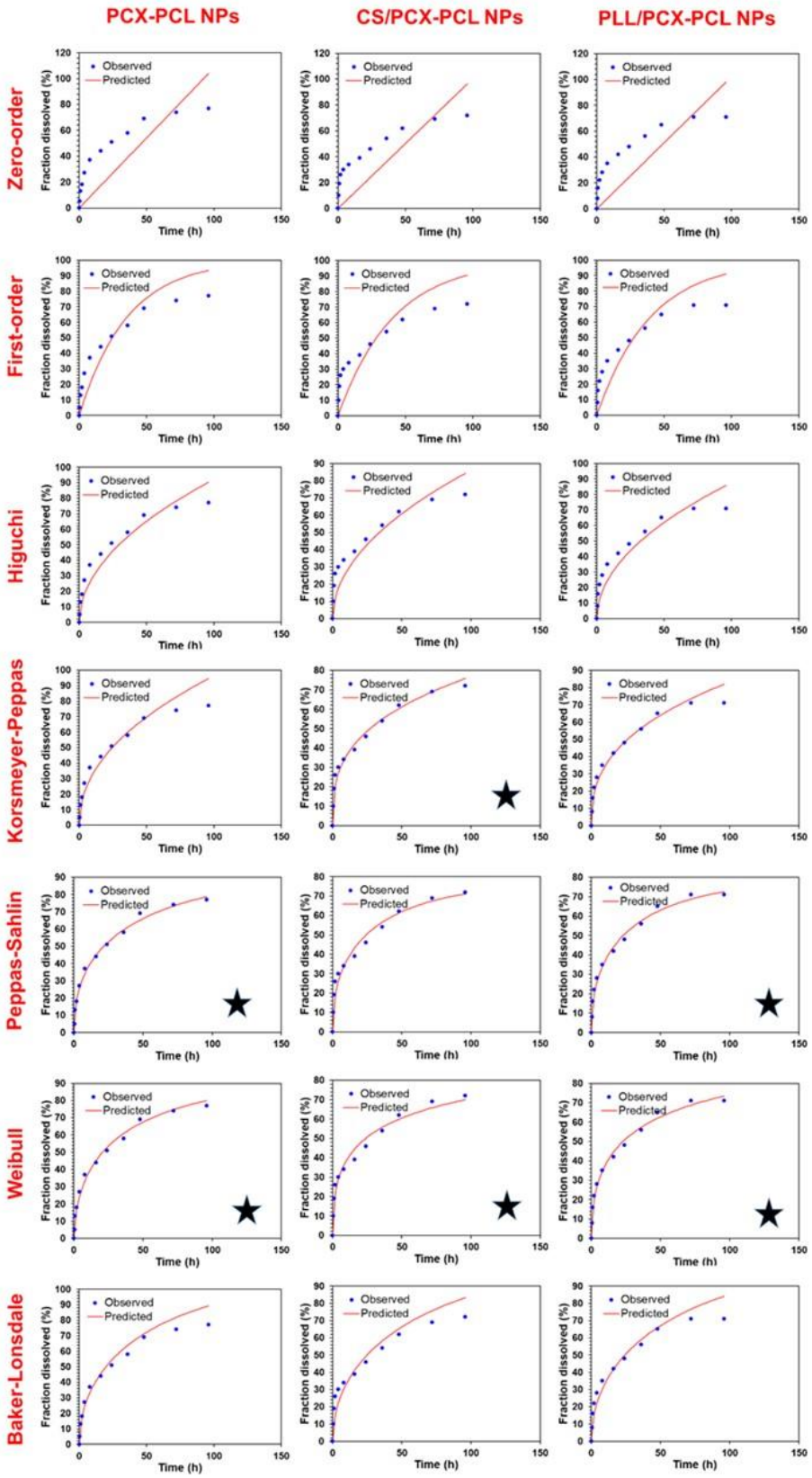


Figure 5. Mathematical model fitting of PCX release from PCL NPs for Zero-order, first-order, Higuchi, Korsmeyer-Peppas, Peppas-Sahlin, Weibull and Baker-Lonsdale models (*best fit models).

In this study, our aim was to prepare PCX-loaded PCL NPs by the nanoprecipitation method and to elucidate the *in vitro* characteristics and release kinetic mechanisms of PCL NPs. Six formulations were prepared with using PCL with two different molecular weight and different coating material in order to observe influence of both molecular weight and coating material on characterization of NPs. According to the results, high molecular weight PCL increased the particle size but increased the encapsulation efficiency. With the cationic coating, the zeta potential of the nanoparticles could be made positively charged. On the other hand, coating materials significantly increased the particle size and encapsulation efficiency, as well. Coating materials also provided longer release time compared to uncoated formulation. Considering the mathematical modeling of release kinetic, the release profile of PCX-PCL NPs and PLL/PCX-PCL were compatible with Peppas-Sahlin and Weibull model. Korsmeyer-Peppas and Weibull model was superior to describe the release of PCX from CS-coated PCL NPs. For each formulation, PCX release kinetic from PCL NPs was compatible Fickian diffusion. This study constitutes a preliminary research for the PCX-loaded PCL NPs to increase the therapeutic efficacy in lung cancer.

AUTHOR CONTRIBUTIONS

Concept: *S.Ü., O.D.*; Design: *S.Ü., O.D.*; Control: S.*Ü., O.D., Y.A.*; Sources: *S.Ü., O.D., Y.A.*; Materials: *S.Ü., O.D., Y.A.*; Data Collection and/or processing: *S.Ü., O.D., Y.A.*; Analysis and/or interpretation: *S.Ü., O.D., Y.A.*; Literature review: *S.Ü., O.D., Y.A.*; Manuscript writing: S.*Ü., O.D., Y.A.*; Critical review: *S.Ü., O.D., Y.A.*; Other: *S.Ü.*

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

REFERENCES

1. Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*, *71*(3), 209-249. [CrossRef]

2. Houston, K. A., Henley, S. J., Li, J., White, M. C., Richards, T. B. (2014). Patterns in lung cancer incidence rates and trends by histologic type in the United States, 2004-2009. *Lung Cancer*, *86*(1), 22-28. [CrossRef]

3. Zarogoulidis, K., Zarogoulidis, P., Darwiche, K., Boutsikou, E., Machairiotis, N., Tsakiridis, K., Katsikogiannis, N., Kougioumtzi, I., Karapantzos, I., Huang, H., Spyratos, D. (2013). Treatment of non-small cell lung cancer (NSCLC). *Journal of Thoracic Disease*, *5* (Suppl 4), S389-S396. [CrossRef]

4. Zappa, C., Mousa, S. A. (2016). Non-small cell lung cancer: Current treatment and future advances. *Translational Lung Cancer Research*, *5*(3), 288-300. [CrossRef]

5. Mangal, S., Gao, W., Li, T., Zhou, Q. T. (2017). Pulmonary delivery of nanoparticle chemotherapy for the treatment of lung cancers: Challenges and opportunities. *Acta Pharmacologica Sinica*, *38*(6), 782-797. [CrossRef]

6. Chandolu, V., Dass, C. R. (2013). Treatment of lung cancer using nanoparticle drug delivery systems. *Current Drug Discovery Technologies*, *10*(2), 170-176. [CrossRef]

7. Massey, A. E., Sikander, M., Chauhan, N., Kumari, S., Setua, S., Shetty, A. B., Mandil, H., Kashyap, V. K., Khan, S., Jaggi, M., Yallapu, M. M., Hafeez, B. B., Chauhan, S. C. (2019). Next-generation paclitaxel-nanoparticle formulation for pancreatic cancer treatment. *Nanomedicine: Nanotechnology, Biology, and Medicine*, *20*, 102027. [CrossRef]

8. Xiao, H., Verdier-Pinard, P., Fernandez-Fuentes, N., Burd, B., Angeletti, R., Fiser, A., Horwitz, S. B., Orr, G. A. (2006). Insights into the mechanism of microtubule stabilization by Taxol. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(27), 10166-10173. [CrossRef]

9. Shurin, G. V., Tourkova, I. L., Shurin, M. R. (2008). Low-dose chemotherapeutic agents regulate small Rho GTPase activity in dendritic cells. *Journal of Immunotherapy*, *31*(5),491-499. [CrossRef]

10. Ruttala, H. B., Ko, Y. T. (2015). Liposome encapsulated albumin-paclitaxel nanoparticle for enhanced antitumor efficacy. *Pharmaceutical Research*, *32*(3), 1002-1016. [CrossRef]

11. Bernabeu, E., Helguera, G., Legaspi, M. J., Gonzalez, L., Hocht, C., Taira, C., Chiappetta, D. A. (2014). Paclitaxel-loaded PCL-TPGS nanoparticles: In vitro and in vivo performance compared with Abraxane®. *Colloids and Surfaces. B, Biointerfaces*, *113*, 43-50. [CrossRef]

12. Lukyanov, A. N., Torchilin, V. P. (2004). Micelles from lipid derivatives of water-soluble polymers as delivery systems for poorly soluble drugs. *Advanced Drug Delivery Reviews*, *56*(9), 1273-1289.[CrossRef]

13. Joshi, K., Chandra, A., Jain, K., Talegaonkar, S. (2019). Nanocrystalization: An emerging technology to enhance the bioavailability of poorly soluble drugs. *Pharmaceutical Nanotechnology*, *7*(4), 259-278. [CrossRef]

14. Thamake, S. I., Raut, S. L., Ranjan, A. P., Gryczynski, Z., Vishwanatha, J. K. (2011). Surface functionalization of PLGA nanoparticles by non-covalent insertion of a homo-bifunctional spacer for active targeting in cancer therapy. *Nanotechnology*, *22*(3), 035101. [CrossRef]

15. Bilensoy, E., Sarisozen, C., Esendağli, G., Doğan, A. L., Aktaş, Y., Sen, M., Mungan, N. A. (2009). Intravesical cationic nanoparticles of chitosan and polycaprolactone for the delivery of Mitomycin C to bladder tumors. *International Journal of Pharmaceutics*, *371*(1-2), 170-176. [CrossRef]

16. W. Badri W., El Asbahani, A., Miladi, K., Baraket, A., Agusti, G., Nazari, Q. A., Errachid,A., Fessi, H., Elaissari, A. (2018). Poly (ε-caprolactone) nanoparticles loaded with indomethacin and Nigella Sativa L. essential oil for the topical treatment of inflammation. *Journal of Drug Delivery Science and Technology*, *46*, 234-242. [CrossRef]

17. Varan, C., Bilensoy, E. (2017). Cationic PEGylated polycaprolactone nanoparticles carrying post-operation docetaxel for glioma treatment. *Beilstein Journal of Nanotechnology*, *8*, 1446-1456. [CrossRef]

18. Jia, W., Gu, Y., Gou, M., Dai, M., Li, X., Kan, B., Yang, J., Song, Q., Wei, Y., Qian, Z. (2008). Preparation of biodegradable polycaprolactone/poly (ethylene glycol)/polycaprolactone (PCEC) nanoparticles. *Drug Delivery*, *15*(7), 409-416. [CrossRef]

19. Lima, I. A., Khalil, N. M., Tominaga, T. T., Lechanteur, A., Sarmento, B., Mainardes, R. M. (2018). Mucoadhesive chitosan-coated PLGA nanoparticles for oral delivery of ferulic acid. *Artificial Cells, Nanomedicine, and Biotechnology*, *46*(sup2), 993-1002. [CrossRef]

20. Cheung, R. C., Ng, T. B., Wong, J. H., Chan, W. Y. (2015). Chitosan: An update on potential biomedical and pharmaceutical applications. *Marine Drugs*, *13*(8), 5156-5186. [CrossRef]

21. Prabaharan, M. (2008). Review paper: Chitosan derivatives as promising materials for controlled drug delivery. *Journal of Biomaterials Applications*, *23*(1), 5-36. [CrossRef]

22. Yuan, Y., Shi, X., Gan, Z., Wang, F. (2018). Modification of porous PLGA microspheres by poly-l-lysine for use as tissue engineering scaffolds. *Colloids and Surfaces. B, Biointerfaces*, *161*, 162-168. [CrossRef]

23. Miladi, K., Sfar, S., Fessi, H., Elaissari, A. (2015). Encapsulation of alendronate sodium by nanoprecipitation and double emulsion: From preparation to in vitro studies. *Industrial Crops and Products*, *72*, 24-33. [CrossRef]

24. Jesus, S., Bernardi, N., da Silva, J., Colaço, M., Panão Costa, J., Fonte, P., Borges, O. (2020). Unravelling the immunotoxicity of polycaprolactone nanoparticles-effects of polymer molecular weight, hydrolysis, and blends. *Chemical Research in Toxicology*, *33*(11), 2819-2833. [CrossRef]

25. Zhang, Y., Huo, M., Zhou, J., Zou, A., Li, W., Yao, C., Xie, S. (2010). DDSolver: An add-in program for modeling and comparison of drug dissolution profiles. *The AAPS Journal*, *12*(3), 263-271. [CrossRef]

26. Gamal, A., Saeed, H., El-Ela, F., Salem, H. F. (2021). Improving the antitumor activity and bioavailability of sonidegib for the treatment of skin cancer. *Pharmaceutics*, *13*(10), 1560. [CrossRef]

27. Murtaza, G., Ahmad, M., Khan, S. A., Hussain, I. (2012). Evaluation of cefixime-loaded chitosan microspheres: Analysis of dissolution data using DDSolver. *Dissolution Technologies*, *19*(2), 13-19. [CrossRef]

28. FDA, U. (1997). Guidance for Industry: Dissolution testing of immediate-release solid oral dosage forms. *Food and Drug Administration, Center for Drug Evaluation and Research (CDER)*, 1-11.

29. Aldeek, F., McCutcheon, N., Smith, C., Miller, J. H., Danielson, T. L. (2021). Dissolution testing of nicotine release from OTDN pouches: Product characterization and product-to-product comparison. *Separations*, *8*(1), 7. [CrossRef]

30. Puthli, S., Vavia, P. R. (2009). Stability studies of microparticulate system with piroxicam as model drug. *AAPS Pharmscitech*, *10*(3), 872-880. [CrossRef]

31. Moore, T., Shangraw, R., Habib, Y. (1996). In dissolution calibrator tablets: A recommendation for new calibrator tablets to replace both current USP calibrator tablets. *Pharmacopeial Forum*, pp:2423-2428.

32. Yang, W., Wang, L., Mettenbrink, E. M., DeAngelis, P. L., Wilhelm, S. (2021). Nanoparticle Toxicology. *Annual Review of Pharmacology and Toxicology*, *61*(1), 269-289. [CrossRef]

33. Mailänder, V., Landfester, K. (2009). Interaction of nanoparticles with cells. *Biomacromolecules*, *10*(9), 2379–2400. [CrossRef]

34. Yue, Z. G., Wei, W., Lv, P. P., Yue, H., Wang, L. Y., Su, Z. G., Ma, G. H. (2011). Surface charge affects cellular uptake and intracellular trafficking of chitosan-based nanoparticles. *Biomacromolecules*, *12*(7), 2440–2446. [CrossRef]

35. Ünal, H., d'Angelo, I., Pagano, E., Borrelli F., Izoo A., Ungaro F., Quaglia F., Bilensoy E. (2015). Core–shell hybrid nanocapsules for oral delivery of camptothecin: Formulation development, in vitro and in vivo evaluation. *Journal of Nanoparticle Research*, *17*, 42. [CrossRef]

36. Chigumira, W., Maposa, P., Gadaga, L.L., Dube, A., Tagwireyi, D., Maponga, C. C. (2015). Preparation and evaluation of pralidoxime-loaded PLGA nanoparticles as potential carriers of the drug across the blood brain barrier. *Journal of Nanomaterials*, *2015*, 1-5. [CrossRef]

37. Ali, R., Farah, A., Binkhathlan, Z. (2017). Development and characterization of methoxy poly(ethylene oxide)-*block*-poly(ε-caprolactone) (PEO-*b*-PCL) micelles as vehicles for the solubilization and delivery of tacrolimus. *Saudi Pharmaceutical Journal : SPJ : The Official Publication of the Saudi Pharmaceutical Society*, *25*(2), 258-265. [CrossRef]

38. Badran, M. M., Alomrani, A. H., Harisa, G. I., Ashour, A. E., Kumar, A., Yassin, A. E. (2018). Novel docetaxel chitosan-coated PLGA/PCL nanoparticles with magnified cytotoxicity and bioavailability. *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*, *106*, 1461-1468. [CrossRef]

39. Ubrich, N., Bouillot, P., Pellerin, C., Hoffman, M., Maincent, P. (2004). Preparation and characterization of propranolol hydrochloride nanoparticles: A comparative study. *Journal of Controlled Release: Official Journal of the Controlled Release Society*, *97*(2), 291-300. [CrossRef]

40. Varan, G., Benito, J. M., Mellet, C. O., Bilensoy, E. (2017). Development of polycationic amphiphilic cyclodextrin nanoparticles for anticancer drug delivery. *Beilstein Journal of Nanotechnology*, *8*, 1457-1468. [CrossRef]

41. Mora-Huertas, C. E., Fessi, H., Elaissari, A. (2010). Polymer-based nanocapsules for drug delivery. *International Journal of Pharmaceutics*, *385*(1-2), 113-142. [CrossRef]

42. Azadi, A., Hamidi, M., Rouini, M. R. (2013). Methotrexate-loaded chitosan nanogels as 'Trojan Horses' for drug delivery to brain: Preparation and in vitro/in vivo characterization. *International Journal of Biological Macromolecules*, *62*, 523-530. [CrossRef]

43. Papadopoulou, V., Kosmidis, K., Vlachou, M., Macheras, P. (2006). On the use of the Weibull function for the discernment of drug release mechanisms. *International Journal of Pharmaceutics*, *309*(1-2), 44-50. [CrossRef]

44. Unagolla, J. M., Jayasuriya, A. C. (2018). Drug transport mechanisms and in vitro release kinetics of vancomycin encapsulated chitosan-alginate polyelectrolyte microparticles as a controlled drug delivery system. *European Journal of Pharmaceutical Sciences: Official Journal of the European Federation for Pharmaceutical Sciences*, *114*, 199-209. [CrossRef]

45. Öztürk, A. A., Yenilmez, E., Özarda, M. G. (2019). Clarithromycin-loaded poly (lactic-*co*-glycolic acid) (PLGA) nanoparticles for oral administration: Effect of polymer molecular weight and surface modification with chitosan on formulation, nanoparticle characterization and antibacterial effects. *Polymers*, *11*(10), 1632. [CrossRef]

46. Yang, H., Li, J., Patel, S. K., Palmer, K. E., Devlin, B., Rohan, L. C. (2019). Design of poly(lactic-*co*-glycolic Acid) (PLGA) nanoparticles for vaginal co-delivery of griffithsin and dapivirine and their synergistic effect for HIV prophylaxis. *Pharmaceutics*, *11*(4), 184. [CrossRef]

47. Supramaniam, J., Adnan, R., Mohd Kaus, N. H., Bushra, R. (2018). Magnetic nanocellulose alginate hydrogel beads as potential drug delivery system. *International Journal of Biological Macromolecules*, *118*(PtA), 640-648. [CrossRef]

48. Soares, P., Sousa, A. I., Silva, J. C., Ferreira, I., Novo, C., Borges, J. P. (2016). Chitosan-based nanoparticles as drug delivery systems for doxorubicin: Optimization and modelling. *Carbohydrate Polymers*, *147*, 304-312. [CrossRef]

Ankara Ecz. Fak. Derg. / J. Fac. Pharm. Ankara, 46(3): 1030-1043, 2022
Doi: 10.33483/jfpau.1151181

ÖZGÜN MAKALE / ORIGINAL ARTICLE

TÜRKİYE'DE YETİŞEN *ULMUS* L. TÜRLERİNİN YAPRAK, DAL VE KABUK YAPILARININ ANATOMİK AÇIDAN KARŞILAŞTIRILMASI

ANATOMICAL COMPARISON OF LEAF, BRANCH AND BARK STRUCTURES OF ULMUS L. SPECIES GROWING IN TURKEY

Neziha Yağmur DİKER[1*], İffet İrem ÇANKAYA[1], Ayşe Mine GENÇLER ÖZKAN[2]

[1]Hacettepe Üniversitesi, Eczacılık Fakültesi, Farmasötik Botanik Ana Bilim Dalı, 06100, Ankara, Türkiye

[2]Ankara Üniversitesi, Eczacılık Fakültesi, Farmasötik Botanik Ana Bilim Dalı, 06100, Ankara, Türkiye

ÖZ

Amaç: *Ulmaceae familyasına ait Ulmus cinsi Türkiye'de dört tür (U. glabra Hudson, U. minor Miller, U. canescens Melville ve U. laevis Pallas) ile temsil edilmektedir. Karaağaç olarak bilinen bu tür halk ilacı olarak kullanılmaktadır. Bu türlerin tedavi edici kısmı iç kabuğudur ve çoğunlukla haricen yaraların ve kemik kırıklarının tedavisinde kullanılır. Bu çalışmanın amacı Ulmus türlerinin yaprak, yaprak sapı, dal ve kabuğunun anatomik özelliklerini belirlemek ve karşılaştırmaktır.*

Gereç ve Yöntem: *Doğadan toplandıktan sonra %70'lik alkolde muhafaza edilen tüm örneklerin enine kesitleri ve yaprakların her iki tarafından alınan yüzeysel kesitler sartur reaktifi ile boyandıktan sonra ışık mikroskobu ile incelenmiştir.*

Sonuç ve Tartışma: *Anatomik incelemede tüm türlerin tipik Ulmaceae familyasına ait elementlere sahip olduğu belirlenmiştir. U. canescens'in diğer türlere göre anatomik yapısında gözlenen farklılıklar; yaprak sapında hilal şeklinde iletim demetleri, köşeli kollenkimanın varlığı ve dalın öz bölgesinde yer alan kalsiyum oksalat kristalleridir. Diğer taraftan U. laevis'de daldaki müsilaj kanallarının sayısı diğer türlere göre önemli ölçüde azdır. Glandular olmayan trikomlar ve kristaller tüm türlerde yoğunluk ve büyüklük bakımından farklıdır. Kabuk anatomisinde, U. minor ve U. canescens'in periderminde düzenli bir şekilde sıralanmış kristaller bulunmuştur.*

Anahtar Kelimeler: *Bitki anatomisi, dal, kabuk, Ulmus türleri, Ulmaceae, yaprak*

ABSTRACT

[*] **Sorumlu Yazar / Corresponding Author:** Neziha Yağmur Diker
e-posta / e-mail: yagmurdiker@hacettepe.edu.tr, **Tel. / Phone:** +903123101089

Gönderilme / Submitted: 30.07.2022 **Kabul / Accepted:** 21.09.2022

Objective: *The genus Ulmus belonging to the Ulmaceae represented with four species (U. glabra Hudson, U. minor Miller, U. canescens Melville, and U. laevis Pallas) in Turkey. This genus, known as "Karaağaç," is used in folk medicine. The therapeutic part of these species is the inner bark; externally, it is mainly used to treat wounds and bone fractures. This study aims to determine and compare anatomical characteristics of the leaf, petiole, branch, and bark of Ulmus species.*

Material and Method: *Cross-sections of all samples preserved in 70% alcohol after collection from the field -also superficial sections taken from both sides of the leaf- were examined with a light microscope.*

Result and Discussion: *The anatomical examination determined that all species had typical Ulmaceae elements. Differences observed in the anatomical structure of U. canescens compared to other species; a crescent-shaped vascular bundle and angular collenchyma on the petiole, and calcium oxalate crystals located in the pith region of the branch. On the other hand, in U. laevis, the number of mucilage channels in the branch is significantly less compared to other species. Non-glandular trichomes and crystals are different in density and size in all species. In the bark anatomy, crystals showing a regular arrangement were found in the periderm of U. minor and U. canescens.*

Keywords: *Bark, branch, leaf, plant anatomy, Ulmus species, Ulmaceae*

GİRİŞ

Ulmaceae familyasına ait *Ulmus* cinsinin dünyada değişik coğrafyalarda yayılış gösteren 45 türü mevcuttur [1]. Türkiye Florası'na göre bu cins Türkiye'de ise üç tür ve beş takson (*U. glabra* Hudson, *U. minor* Miller (*U. minor* ssp. *minor* ve *U. minor* ssp. *canescens* (Melville) Browicz & Zielinski), *U. laevis* Pallas) ile temsil edilmektedir [2]. *U. minor* ssp. *canescens, U. canescens* Melville'in sinonimi olarak onaylanmıştır [3]. İndirgenmiş çiçek ve meyveler, değişken vejetatif karakterler ve türler arası hibridizasyon nedeniyle türlerin sınırları ve taksonomik yakınlığı tartışmalıdır [4]. Geniş bir literatür taraması yapıldığında, Ulmaceae familyasının anatomik özelliklerini tanımlayan veya karşılaştırmalı olarak ele alan yayınlara [5-7] kolayca ulaşılabilir, ancak bildiğimiz kadarıyla *Ulmus* türlerinin kapsamlı bir anatomik incelemesi mevcut değildir. Diğer yandan, yapılan bir çalışmada Sicilya'daki *Ulmus* türlerinin ve melezlerinin yaprak anatomisi ve morfolojisi, *Ulmus* sistematiğindeki karmaşıklığı çözmek ve atipik yaprak yapısı için belirleyici karakterlerin tespiti amacıyla karşılaştırmalı olarak ele alınmıştır [8].

Ulmus türleri Anadolu'da "Karaağaç" olarak bilinir ve halk ilacı olarak kullanılan kısmı daha çok iç kabuklarıdır. *U. glabra*'nın iç kabuklarından hazırlan lapa, ateroskleroz ve kemik kırığı [9] veya apseleri [10] tedavi etmek için kullanılmaktadır. *U. canescens*'in iç kabukları, yaraları [11] ve ellerdeki çatlakları tedavi etmek için doğrudan veya kaynatma ile hazırlanarak dışarıdan uygulanmaktadır [12]. Ayrıca *U. minor*'ün iç kabuklarının kaynatılmasıyla hazırlanan lapa, haricen uyuz [13], kas ağrıları, kadın hastalıkları tedavisinde ve kemiklerin uygun şekilde iyileşmesinde kullanılmaktadır [14-15]. *Ulmus* türlerinde tedavi amaçlı kullanılan iç kabuk, kambiyumun dışında kalan tüm dokular için kullanılan kabuk yapısının bir parçası olup kabuk anatomisine göre canlı floem kısmını tanımlanmaktadır [16].

Yapılan literatür araştırmaları ise yalnızca *U. glabra* kabuğunun bütün olarak anatomik açıdan incelendiğini göstermiştir [16-18]. Ayrıca, tedavide kullanımı olan ve İngilizce "Slippery Elm" olarak

bilinen *U. rubra* Muhl iç kabuğuna ait toz drog anatomisi üzerine çalışmalar da mevcuttur [19-20]. Türkiye'de ise *Ulmus* türleri üzerinde Akkemik (1994) tarafından yapılan çok kapsamlı bir çalışmada; morfolojik karakterler (habitat, yaprak, ağaç kabuğu, sürgün, tomurcuk, çiçek ve meyve), yaprak damarlanması farklılıkları, odun anatomisi (trakeler, ışınlar, lifler ve odun parankiması) ve polen morfolojisi ele alınmıştır [21].

Bu çalışmanın amacı, Türkiye'de doğal olarak yetişen *Ulmus* türlerinin yaprak, yaprak sapı, dal ve kabuk anatomilerini belirlemek ve karşılaştırmaktır.

GEREÇ VE YÖNTEM

Bitki örnekleri 2019 ve 2021 yılları arasında Türkiye'nin farklı bölgelerine yapılan arazi çalışmalarında doğal habitatlarından toplandı. Örnekler, taksonomik olarak "Flora of Turkey and the East Aegean Islands" [22] ve Akkemik (1994) tarafından yazılan yüksek lisans tezi kullanılarak tanımlandı [21]. Türün ayrımında kullanılan tüm karakterleri taşıyan ve teşhislerinde şüphe bırakmayan örnekler ile çalışılmıştır. Çalışmada yer alan bitkilere ait şahit herbaryum örnekleri Hacettepe Üniversitesi Eczacılık Fakültesi Herbaryumu (HUEF) ve Ankara Üniversitesi Eczacılık Fakültesi Herbaryumu (AEF)'na kaldırıldı. Bu çalışmanın bitki materyallerine ait lokasyon bilgileri ve şahit örnek herbaryum kayıt numaraları Tablo 1.'de verilmiştir.

Tablo 1. *Ulmus* türlerinin toplandığı lokasyon bilgileri ve şahit örneklerine ait herbaryum numaraları

Bitki Türleri	Lokasyon Bilgileri	Herbaryum Numaraları	
***U. minor* Miller**	A4: Bolu; Merkez; Ankara-Bolu yolu üzeri, Bolu'ya 15 km kala yol kenarı, 724 m, 29.04.2019, N.Y.Diker, S.D.Erkakan, E.Erkakan	HUEF 19060	AEF 28808
***U. canescens* Melville**	C5: Adana; Yumurtalık; Yumurtalık köyü'nden Ayvalık köyü'ne doğru, Büyük Bağırsak deresini geçince, yol kenarı, 28 m, 09.04.2021, N.Y.Diker, C.Diker, M.Diker	HUEF 21008	AEF 30930
***U. glabra* Hudson**	A4: Bolu; Merkez; Yedigöller Milli parkı içi, Mengen yoluna doğru, yol kenarı, 626 m, 29.04.2019, N.Y.Diker, S.D.Erkakan, E.Erkakan	HUEF 19056	AEF 28803
***U. laevis* Pallas**	A3: Düzce; Gölyaka; Gölyaka Kültür Parkı, yaprak döken orman, 120 m, 27.04.2019, N.Y.Diker, C.Diker, M.Diker	HUEF 19062	AEF 28804

Anatomik inceleme için toplanan taze yaprak, dal ve ağaç kabuğu örnekleri %70'lik etanol içinde saklandı. Her organ için enine kesitler ve sadece yapraklar için yüzeysel kesitler keskin bir jilet kullanılarak elle alındı. Kesitler Sartur reaktifi ile boyandı [23] ve LeicaDM500 ışık mikroskobunda 4x, 10x ve 40x büyütmelerde gözlemlendi. Örneklerin fotomikrografları LeicaDM500'e entegre edilmiş bir dijital kamera kullanılarak alındı. Şekil ölçekleri, aynı optik koşullar altında (bir milimetre ölçeği) fotoğraflanarak elde edildi. Fotomikrograflar CorelDRAW X8 yazılımı kullanılarak düzenlendi. Anatomik özelliklere ait tanımlayıcı terimler standart anatomi kitabı ve makalelerden alındı [24-27].

SONUÇ VE TARTIŞMA

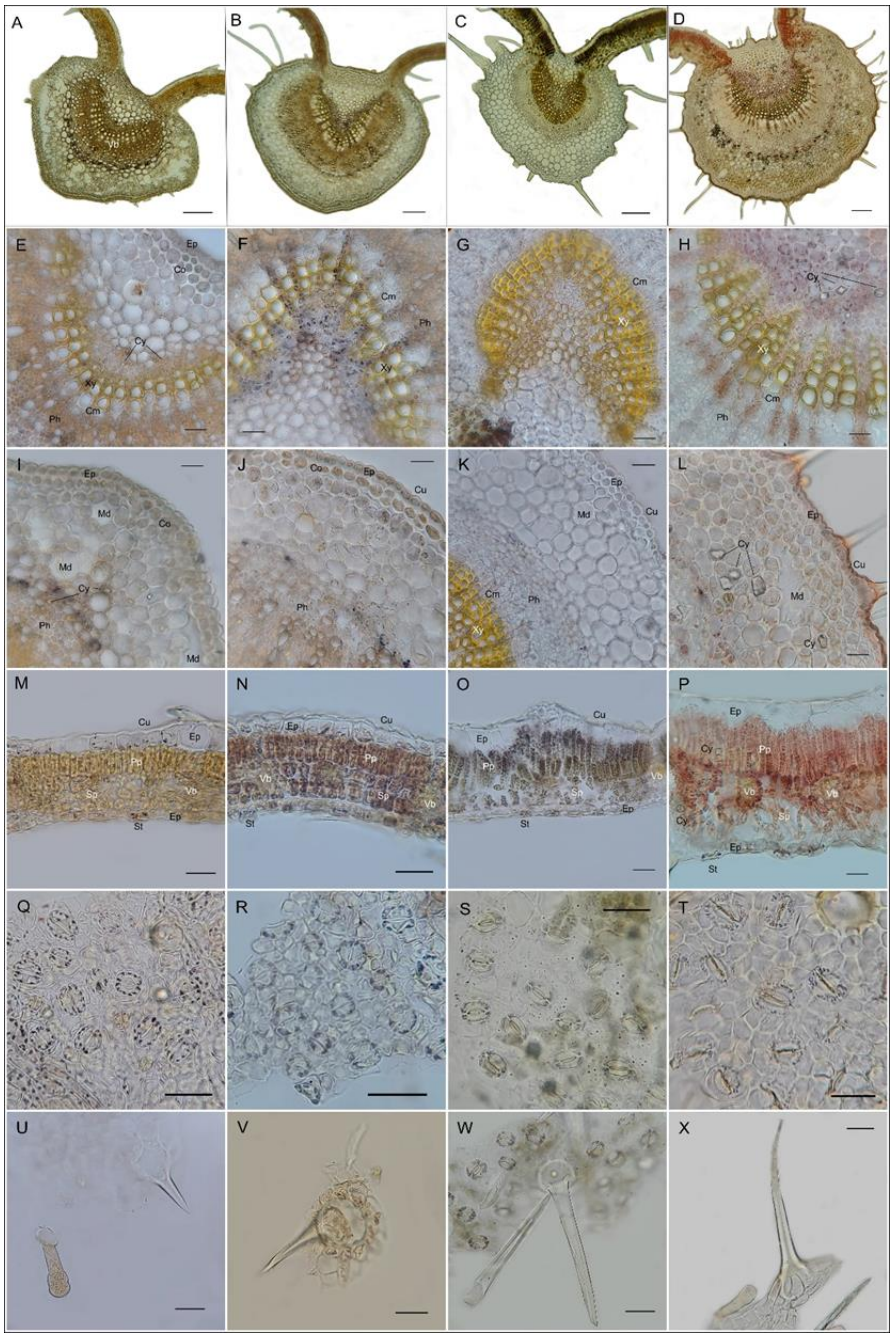
Yaprak Anatomisi

Laminanın enine kesitleri incelendiğinde, epidermis tek sıra halinde, dikdörtgen veya izodiametrik hücrelerden oluşur. Adaksiyal yüzeyde hücreler abaksiyale göre göreceli olarak daha büyüktür. *U. canescens* ve *U. minor*'ün adaksiyal epidermis hücreleri köşeli veya düzensiz bir şekle sahiptir. Her iki epidermisin üzeri düzensiz ve kırışık bir kütikula tabakası ile örtülüdür. Tüm türlerde yaprak enine kesitte bifasiyaldir ve mezofil tabakası palizat ve sünger parenkiması olmak üzere iki ayrı bölgeye ayrılmıştır. Mezofil kalınlığı türler arasında değişiklik göstermektedir. Adaksiyal epidermisin altında çoğunlukla iki tabaka halinde düzenlenmiş, uzun ve silindirik hücrelere sahip palizat parenkiması yer almaktadır. Palizat parenkiması hücrelerinin boyutları türlere göre değişiklik göstermektedir. Örneğin *U. minor* ve *U. canescens* palizat parenkima hücrelerinin boyu eninin 3 katından fazla iken *U. glabra* ve *U. laevis* hücrelerinin boyu eninin 2 katı kadardır. Sünger parenkiması çoğunlukla yuvarlak hücrelerden oluşur ve farklı büyüklükte hücreler arası boşluklara sahiptir. *U. minor*'de bu dokunun kalınlığı diğer türlere göre daha azdır. Hücreler arası boşluklar *U. minor* ve *U. canescens*'de oldukça geniş olarak gözlenirken *U. glabra* ve *U. laevis*'de boşluklar daha küçük ve dardır. İletim demetini çevreleyen demet kılıfı tek katmanlı hücrelere ve uzantılara sahip olduğundan yapraklar hem heterobarik hem de homobariktir. *U. canescens*'in mezofil tabakasında druz kristalleri bulunmaktadır (Şekil 1.: M, N, O ve P).

Yüzeysel kesitlerde ise, her iki yaprak yüzeyinde hem basit örtü tüyleri hem de salgı tüyleri bulunmaktadır. Tek hücreli basit örtü tüyleri oldukça geniş ve dairesel (bulbose-based) tabana sahiptir ama boyutları ve yoğunluğu türlere göre değişiklik göstermektedir. Buna ek olarak, alt yüzeyde yer alan basit örtü tüyleri orta damar ve ikincil damarlarda daha yoğundur. Salgı tüyleri ise daha seyrek yayılış göstermesinin yanı sıra tek hücreli bir sapa ve çok hücreli bir baş yapısına sahiptir. Nişasta taneleri içeren stomalar yalnızca abaksiyal yüzeyde, farklı büyüklüklerde (*U. minor* – 2,62-3,20 µm, *U. canescens* – 2,5-3,62 µm, *U. glabra* – 3,80-4,96 µm, *U. laevis* – 1,89-2,88 µm), dağınık bir şekilde yer almakla birlikte anomositik tiptedir (Şekil 1.: Q, R, S, T, U, V, W ve X).

Orta damar yaprağın abaksiyal yüzeyinde belirgin bir çıkıntı oluşturmaktadır. Adaksiyal epidermanın altında yer alan kollenkimatik kalınlaşma gösteren hücreler türlere göre farklı hücre sayılarına sahiptir. Abaksiyal epidermaya bitişik annular kollenkima ise *U. glabra, U. laevis* ve *U. minor*'de çoğunlukla tek veya 2-3 sıralı olarak gözlenmektedir. *U. canescens*'te ise kollenkima dokusuna ait hücreler lakunar tipte olup daha kalındır. Laminadaki palizat parenkiması orta damarda aniden bitmektedir. Kollenkima hücreleri ile iletim demeti arasında yer alan parenkima hücreleri geniş yuvarlak şekle ve daha ince duvarlara sahiptir. *U. canescens* ve *U. glabra*'da parankimatik hücreler arasında müsilaj hücreleri ve boşlukları bulunmaktadır. Aynı zamanda, büyük kristaller yalnızca *U.*

canescens'in parankimatik hücrelerinde dağılım göstermektedir. Tüm türlerde orta damarın merkezinde hilal şeklinde bir damar demeti bulunur (Şekil 1.: A, B, C, D, E, F, G, H, I, J, K ve L).

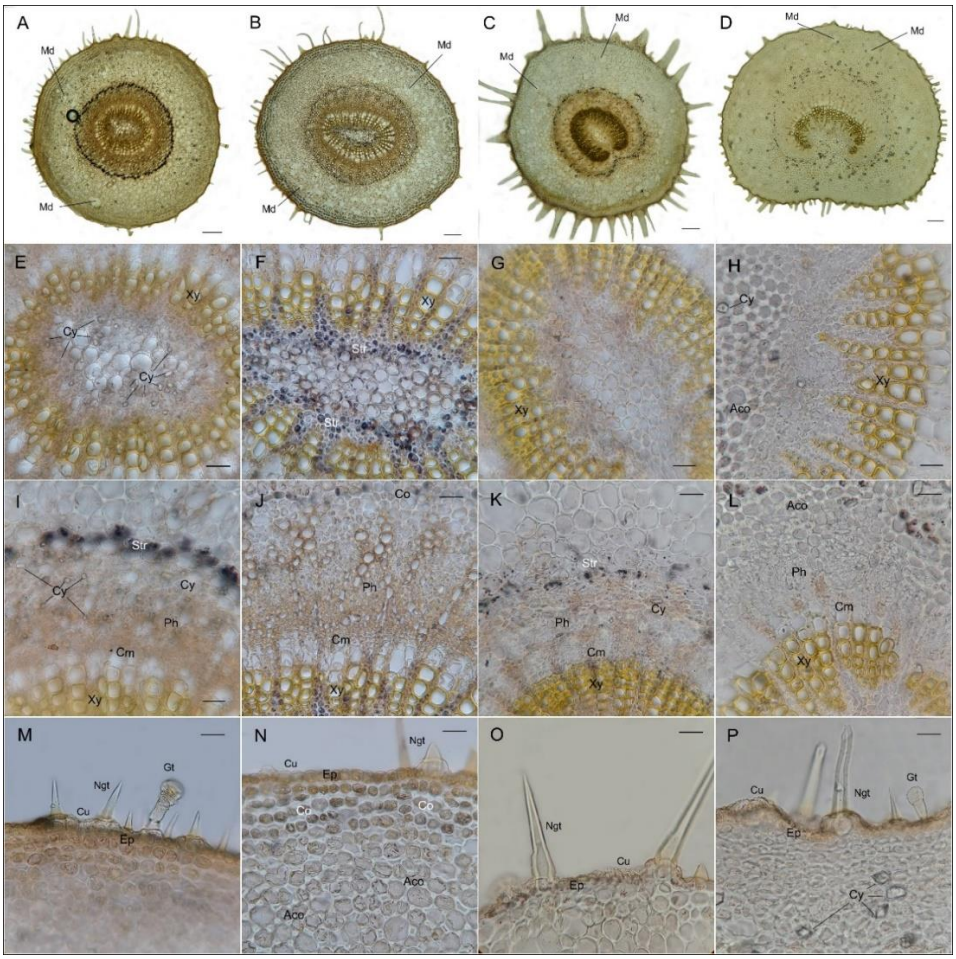


Şekil 1. *Ulmus* türlerinin yapraklarına ait enine ve yüzeysel kesitler. A, E, I, M, Q ve U. *Ulmus glabra*. B, F, J, N, R ve V. *Ulmus laevis*; C, G, K, O, S ve W. *Ulmus minor*; D, H, L, P, T ve X. *Ulmus canescens*. Cu, kütikula; Ep, epidermis; Pp, palizat parenkiması; Sp, sünger parenkiması; St, stoma; Xy, ksilem; Cy, kristal; Md, müsilaj kanalı; Cm, kambium; Vb, iletim demeti; Ph, floem; Co, kollenkima. Ölçek: (A, B, C ve D) 20µm, (E, F, G, H, I, J, K, L, M, N, O, P, Q, R, S, T, U, V, W ve X) 5µm.

İletim demeti adaksiyal epidermis yönünde ışınsal yayılan ligninleşmiş ksilem ve abaksiyal epidermis yönünde ince duvarlı ve küçük hücrelerden oluşan floem kemerinden oluşmaktadır. Vasküler kambiyum belirgin olmamakla beraber 2-3 sıralıdır. Türlerin tamamında iletim demetlerini çevreleyen sklerenkima dokusuna rastlanmamıştır. Floem ve ksilem hücreleri etrafında türlere göre değişik yoğunluk ve boyutlarda kristaller bulunmaktadır. Kesitte gözlenen örtü tüylerinin boyutları ve yoğunluğu türlere göre değişiklik göstermekte olup özellikle *U. canescens, U. minor* ve *U. laevis*'de oldukça büyük yapıda gözlenmektedir (Şekil 1.: A, B, C, D, E, F, G, H, I, J, K ve L.).

Petiol Anatomisi

Yaprak sapının enine kesiti (Şekil 2.) *U. canescens* hariç tüm türlerde silindirik bir şekle sahiptir.



Şekil 2. *Ulmus* türlerine ait yaprak sapı enine kesitleri A, E, I ve M. *U. glabra*. B, F, J ve N. *U. laevis*; C, G, K ve O. *U. minor*; D, H, L ve P. *U. canescens*. Xy, ksilem; Cy, kristal; Md, müsilaj kanalı; Cm, kambiyum; Ph, floem; Co, kollenkima hücreleri; Aco, köşeli kollenkima hücreleri; Cu, kütikula; Ep, epidermis; Ngt, örtü tüyü; Gt, salgı tüyü; Str, nişasta. Ölçek: (A, B, C ve D) 20µm, (E, F, G, H, I, J, K, L, M, N, O ve P) 5µm.

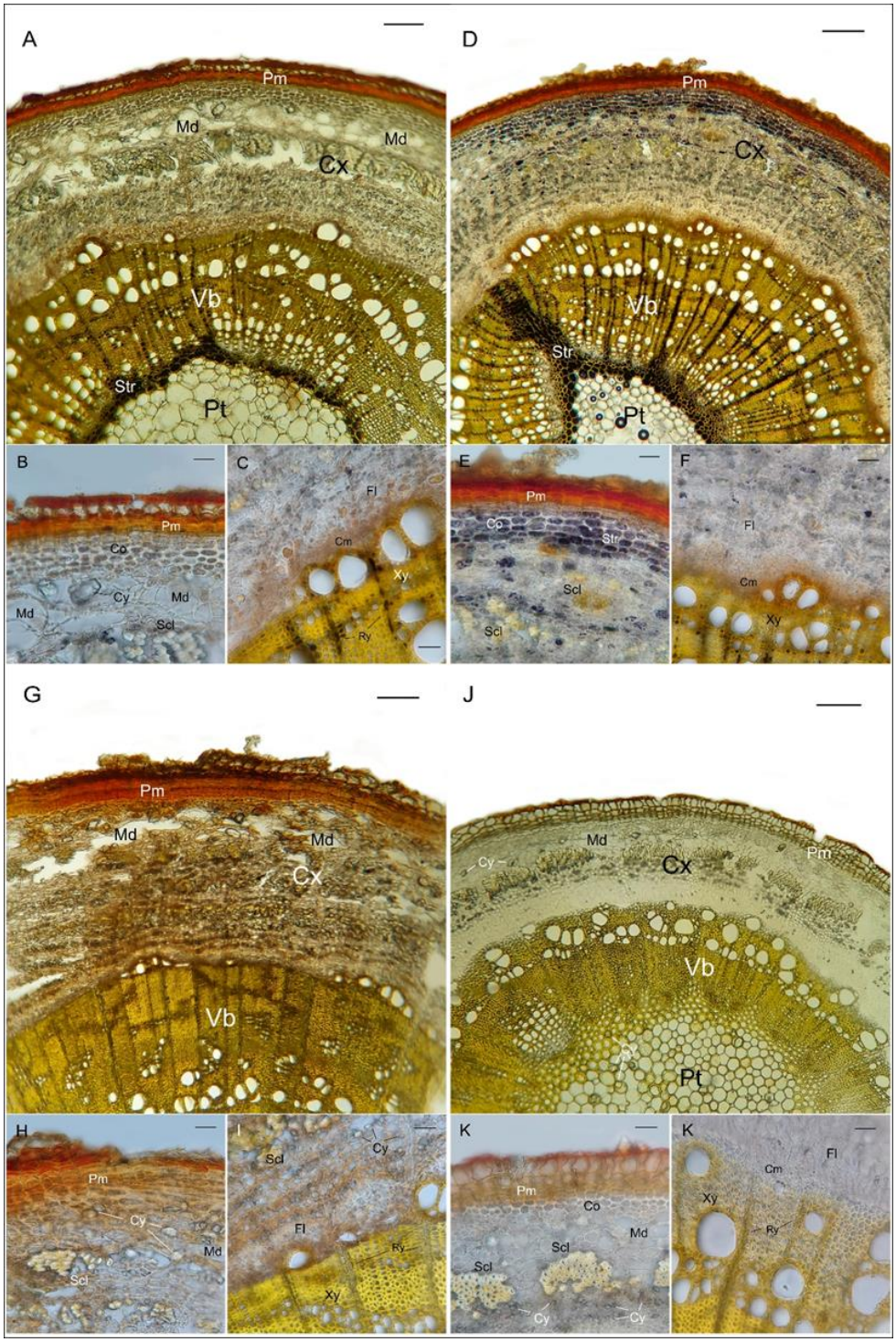
Epidermiste, tek hücreli basit, farklı boyut ve yoğunluklara sahip örtü tüyleri ve çok hücreli salgı tüyleri vardır. 1 sıralı epidermis hücreleri kutikula tabakası ile örtülüdür. Epidermanın altında 4-6 sıralı kollenkima dokusu yer alır. İri hücreli parankima dokusu içerisinde müsilaj hücreleri ile çevrelenmiş salgı kanalları ve büyük basit kristaller tüm türlerde bulunur. Kristaller *U. glabra*'da en fazla yoğunlukta olup *U. laevis*'te ise rastlanmamıştır. İletim demeti merkezde kollateral tiptedir. *U. glabra* ve *U. leavis*'de halkasal bir yapı gösterirken *U. minor* ve *U. canescens*'de neredeyse kapanmış halkasal ve at nalı şeklinde bir yapı görülmektedir. Ksilem öz bölgesi yönünde floem ise epidermis yönünde konumlamıştır. Değişken nişasta yoğunluğuna sahip 1 sıralı hücre hattı, floem ve parankima dokusu arasında bulunur. Köşeli kollenkima *U. canescens*'de damar demetleri etrafında, *U. laevis*'te ise dış tarafında yer almaktadır.

Dal Anatomisi

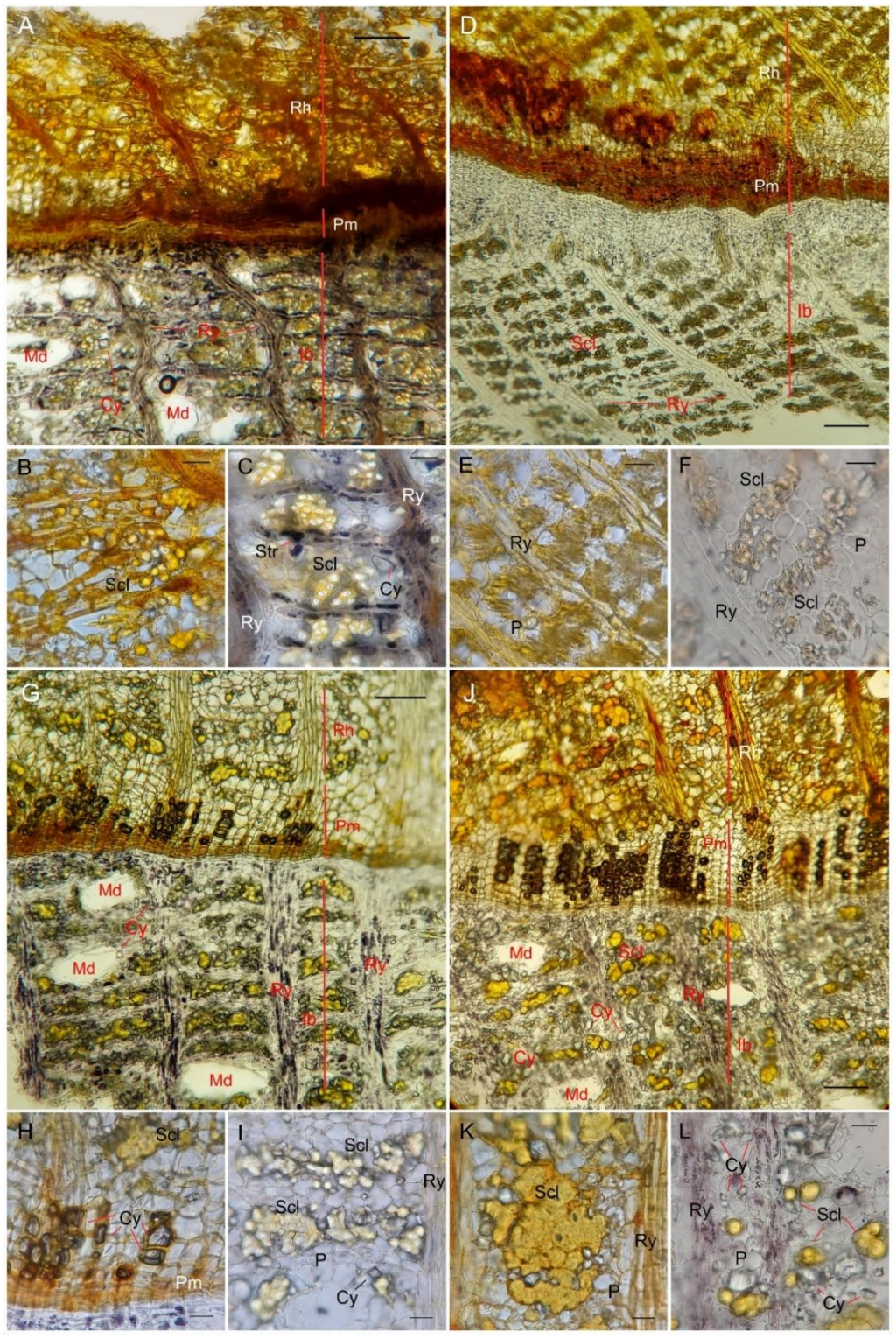
Enine kesitin en dış tabakasında köşeli hücrelerden oluşan periderm tabakası bulunur ve kalınlığı türlere göre değişmektedir. Kortekste yer alan parenkima dokusu *U. minor* ve *U. glabra*'da belirgin ve çok yoğun müsilaj kanalları içerirken *U. canescens* ve *U. laevis*'te müsilaj kanalları azdır veya yoktur. Çeşitli büyüklük ve yoğunluktaki basit kristaller *U. laevis* hariç tüm türlerde parenkima hücreleri içerisinde bulunur. Ek olarak, iletim demeti dairesel bir şekle sahiptir ve kollateral tiptedir. *U. glabra* ve *U. laevis*'te öz ve ksilem arasında yer alan değişken yoğunlukta nişasta içeren çok sıralı bir hücre hattı bulunmaktadır (Şekil 3.).

Kabuk Anatomisi

Dış kabuk veya ölü floem dokusu olarak da bilinen ritidom iç kabuk yapısı ile benzerlik göstermektedir. Periderm dokusu fotoğrafta görülmesine karşın içerisindeki fellem, fellogen ve felloderm olan 3 farklı tabakalaşma net ayırt edilememektedir. Periderm hücreleri yan hücre duvarları aynı hizada olan kare veya dikdörtgen şekle sahiptir ve türlere göre farklı kalınlıklar gösterseler de tüm türlerde görülmektedir. Diğer türlerden farklı olarak *U. minor* ve *U. canescens*'de fellem yapısı içerisinde düzenli dizilişli ve oldukça iri billurlar bulunmaktadır. Tüm türlerde iç kabuk yapısı düzenli teğetsel katmanlar şeklindedir. Öz kolları 2-3 hücreli ve kısmen dalgalanma göstermektedir. Müsilaj kanalları karakteristiktir. Parenkima hücreleri pek çok büyük prizmatik kristaller ile birlikte nişasta taneleri içerir. Morfolojik olarak fiber yapısına benzemesine karşın sklereit hücrelerinde olduğu gibi eksensel floem parenkima hücrelerinin ikincil olarak farklılaşmasından oluşan [25] fiber benzeri sklereit hücre grupları parenkima dokusunda dağılım göstermektedir (Şekil 4.).



Şekil 3. *Ulmus* türlerine ait dal enine kesitleri. A, B ve C. *U. glabra*. D, E ve F. *U. laevis*; G, H ve I. *U. minor*; J, K ve L. *U. canescens*. Xy, ksilem; Cy, kristal; Md, müsilaj kanalı; Cm, kambiyum; Pt, öz; Vb, iletim demetleri; Cx, korteks; Ph, floem; Pm, periderm; Co, kollenkima hücreleri; Ray, öz kolları; Scl, sklereitler, Str, nişasta; C. Ölçek: (A, D, G ve J) 20µm, (B, C, E, F, H, I, K ve L) 5µm.



Şekil 4. *Ulmus* türlerine ait kabuk enine kesitleri. A, B ve C. *U. glabra*. D, E ve F. *U. laevis*; G, H ve I. *U. minor*; J, K ve L. *U. canescens*. Cy, kristal; Md, müsilaj kanalı; P, Parenkima; Pm, periderm; Rh, ritidom; Ib, iç kabuk; Ray, öz kolları; Scl, sklereitler, Str, nişasta; C. Ölçek: (A, D, G ve J) 20µm, (B, C, E, F, H, I, K ve L) 5µm

Bu çalışmada, Türkiye'de doğal olarak yetişen ve halk ilacı olarak kullanılan dört *Ulmus* türünün yaprak, yaprak sapı, dal ve kabuk anatomisi karşılaştırmalı olarak incelenmiştir. Türler, Ulmaceae'nin genel karakteristik özelliklerini sergilemektedir [5]. Bu özellikler şunlardır: I) bifasiyel yapraklar; II) basit tek hücreli örtü ve çok hücreli salgı tüyleri; III) yaprağın sadece alt yüzünde bulunan, karakteristik bir düzende dizili olmayan komşu hücrelere sahip stomalar; IV) kabukta ve yaprak mezofilinde bulunan müsilaj hücreleri; V) basit kristaller.

Ulmus türlerinde yaprakta epidermal silika hücreleri [5] ve yüzeyde mineral plakları [6] olduğu bildirilmiştir. Mineral plakları özellikle *U. glabra* ve *U. laevis*'te gözlenmiştir. Ancak bu özellikler, tür ayrımı için önemli değildir. Epidermal silika hücrelerinin görevinin, bitkiyi böceklere [28] veya doğrudan güneş ışınımına karşı korumak olduğu tahmin edilmektedir [29]. Işık mikroskobu ile yapılan incelemelerde bu hücrelerin varlığının gözlenmesi mümkün değildir. Bu hücrelerin varlığının belirlenmesi için ancak bazı analiz yöntemlerinin uygulanması önerilir.

Metcalfe ve Chalk (1983) ve Sweitzer'e (1971) göre *Ulmus* cinsinde mezofilde yer alan sünger parenkiması büyük hücreler arası boşluklara sahiptir [5-6]. Ancak *U. laevis* ve *U. glabra*'nın sünger parenkima hücreleri arasında oldukça küçük boşluklar gözlenmiştir. Bitkinin bulunduğu ortamdaki ışığın şiddeti, yaprak gelişimi sırasında mezofildeki palizat ve sünger tabakalarındaki hücre yoğunluğu ve düzeni üzerinde etkilidir. Gölgede gelişen yapraklar, *U. laevis* gibi tek sıra halinde dizilmiş palizat hücrelerine ve sünger parenkimasında dar hücrelerarası boşluklara sahiptir. Aynı zaman aralığında ve Akdeniz ikliminden toplanan *U. canescens*, diğer türlere göre daha fazla ışığa maruz kalması nedeniyle kalın bir palizat tabakasına sahiptir ve sünger tabakasındaki hücreler arası boşlukları da oldukça büyüktür [27].

Bazı *Ulmus* türlerinde iletim demetleri, parenkima kılıfı veya sklerenkima hücreleri tarafından çevrelenmemiştir [5]. Şekil 1.'de görüldüğü gibi, iletim demeti klorofil içermeyen parankimatik hücrelerle çevrilidir ve mezofil boyunca uzanır. İncelenen türlerin hiçbirinde iletim demetleri etrafında sklerenkima hücrelerine veya parenkima kılıfına rastlanmamıştır. Demet kılıfı uzantısı mevcuttur [6]. Işığın mezofilin iç tabakasına ulaşmasına ve hidrolik işlevi görmesine izin verilir [30].

Bu çalışmada, tüylerin ve kristallerin yoğunluğunun *Ulmus* türleri arasında farklılık gösterdiği gözlemlenmiştir. Scialabba ve diğerleri (1997) tarafından yapılan çalışmada da yapraklarda bulunan tüy ve kristal varlığının ve dağılımının türler arası ayrım için kullanılabileceği bildirilmiştir [8]. Mezofilde yer alan kristaller, fotosentetik dokulara ışık girişini destekler. Ayrıca örtü tüyleri, yaprağı güneş ışığının zararlı dalga boylarından korur veya yaprak yüzeyinin optik özelliklerini etkiler. Genel olarak yapraklardaki tüy ve kristallerin işlevi fotosentezde ışıktan maksimum yararı sağlamaktır. Bu nedenle değişen ortam koşullarına göre yoğunlukları ve boyutları değişmektedir [30].

Petiolde genellikle yaklaşık olarak ortada yer alan ve demet aksesuarlarına sahip olmayan, tam

ya da yaklaşık dairesel şekilde iletim demetleri gözlenir [5]. Bu genelleştirmeden farklı olarak, *U. canescens* hilal şeklinde gözlenen bir iletim demetine sahiptir. Kristal yoğunluğu, *U. laevis* dışında türler arasında farklılık göstermiştir. Dal yapısı ayrı ayrı liflerden oluşan belirgin perisiklik sklerenkima formuna sahiptir; kortekse dağılmış çok sayıda müsilaj hücresinin bazen boşluklar oluşturmak üzere birleştiği gözlenir; bunun yanında basit ve bileşik kristallerin varlığı da korteks için ayırt edici bir karakterdir [5]. Bu çalışmada, *U. laevis*'in diğer türlere kıyasla daha az ve küçük müsilaj hücre boşluklarına ve basit kristallere sahip olduğu belirlenmiştir. Ayrıca, *U. canescens*'in öz bölgesinde kristaller mevcuttur.

Schweingruber (2019) tarafından yalnızca *U. glabra*'nın kabuk anatomisi incelenmiştir [16]. Bu çalışmada yapılan incelemede tanımlanan anatomik karakterler ve doku dizilimi tüm türler için ortak olup Schweingruber tarafından yapılan çalışma ile örtüşmektedir. Kabuk örneklerindeki doku kalınlıkları örneklerin yaşına bağlı olarak değişmektedir. *U. minor* ve *U. canescens*'in periderminde kristallerin varlığı belirlenmiş olup, bu özellik türler arasında görülen tek fark olarak değerlendirilmiştir. Bunun nedeni ise bu iki türün birbirine yakın türler olarak kabul edilmesidir [2]. Karaağaç kabuk anatomisi çalışmalarında hibrit türlerin belirlenmesinde bu karakterin ayırt edici bir özellik olarak değerlendirilebileceği düşünülmektedir.

Sonuç olarak, *Ulmus* cinsine ait dört türde incelenen kısımlara ait anatomik özelliklerdeki farklılığın, değişken ortam koşullarına bağlı olduğu anlaşılmıştır. Ayırt edici bir özellik olarak görülmese de türler arasında farklılık gösteren karakterler şunlardır; I) *U. canescens*'in yapraklarındaki mezofil tabakasındaki druz kristallerinin varlığı, II) aynı türün petiol enine kesitinde hilal şeklinde bir iletim demeti ve geniş bir şekilde etrafını saran lakunar kollenkima dokusunun varlığı, III) *U. laevis*'in dal kesitinde müsilaj kanalı sayısının diğer türlere kıyasla daha az olması ve kristal yapılarının gözlenmemesi, IV) *U. canescens*'de ise dal kesitinin öz bölgesinde çok sayıda kristal varlığı, V) *U. canescens* yaprak enine kesitinde ise yine kollenkima dokusuna ait hücrelerin lakunar tipte olması, VI) Başta *U. canescens* ve *U. laevis* olmak üzere tüm türlerde örtü tüylerinin ve kristallerin yoğunluk ve büyüklük bakımından farklı olmasıdır. Bununla birlikte, türleri ayırt etmek için kullanılabilecek belirgin sabit bir anatomik karakter gözlenmemiştir. *U. glabra* dışındaki tüm türlerin kabuk anatomisinin ilk kez incelendiği bu çalışma ile Türkiye'de doğal olarak yetişen *Ulmus* türlerinin karakterizasyonuna katkı sağlanmaya çalışılmıştır. Ancak anatomik karakterlerin hibritlerin ayrımında kullanımı ile ilgili olarak, belirgin karakterlere sahip türler ile birlikte hibrit örnekleri de içeren daha kapsamlı çalışmaların yapılması önerilmektedir. Tıbbi değeri olan *Ulmus* türlerinin ayrımında kullanılacak ayırt edici karakterlerin belirlenmesi için çalışmaların sürdürülmesi önemlidir. Bu bağlamda, çalışma ekibimiz tarafından bu türlerin fitokimyasal (veya kemotaksonomik) açıdan daha ayrıntılı bir şekilde araştırılması planlanmaktadır.

YAZAR KATKILARI

Kavram: *İ.İ.Ç., A.M.G.Ö.*; Tasarım: *İ.İ.Ç., A.M.G.Ö.*; Denetim: *İ.İ.Ç., A.M.G.Ö.*; Kaynaklar; *N.Y.D., İ.İ.Ç., A.M.G.Ö.*; Malzemeler: *N.Y.D.*; Veri toplama ve/veya işleme: *N.Y.D.*; Analiz ve/veya yorumlama: *N.Y.D., A.M.G.Ö.*; Literatür taraması: *N.Y.D., A.M.G.Ö.*; Makalenin yazılması: *N.Y.D., A.M.G.Ö.*; Kritik inceleme: *N.Y.D., İ.İ.Ç., A.M.G.Ö.*; Diğer: -

ÇIKAR ÇATIŞMASI BEYANI

Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

ETİK KURUL ONAYI

Yazarlar bu çalışma için etik kurul onayının zorunlu olmadığını beyan etmektedir.

KAYNAKLAR

1. Wiegrefe, S.J., Sytsma, K.J., Guries, R.P. (1994). Phylogeny of Elms (*Ulmus*, Ulmaceae): Molecular Evidence for a Sectional Classification. *Systematic Botany*, *19*(4), 590-612. [CrossRef]

2. Browicz, K., Zielinski, J. (1982). *Ulmus* L., Davis, P.H. (Ed.), Flora of Turkey and the East Aegean Islands, Cilt.7, (s.: 645-648). Edinburgh University Press: Edinburgh.

3. The Plant List (2013). A working list of all plant species. Version 1.1 Erişim adresi: [http://www.theplantlist.org/tpl1.1/search?q=Ulmus] Erişim Tarihi: 17.03.2021

4. Richens, R.H. (1983). Elm. Cambridge University Press: Cambridge.

5. Metcalfe, C.R., Chalk, L. 1983. Anatomy of the Dicotyledones: Vol 2, wood structure and conclusion of the general introduction, 2nd edn. Oxford University Press: New York.

6. Sweitzer, E.M. (1971) Comparative anatomy of the Ulmaceae. *Journal of the Arnold Arboretum, 52*, 523-585.

7. Schweingruber, F.H., Börner, A., Schulze, E.D. (2011) Atlas of Stem Anatomy in Herbs, Shrubs and Trees. Springer: Switzerland, sayfa 450-453.

8. Scialabba, A., Melati, M.R., Raimondo, F.M. (1997) Taxonomic studies on the Sicilian elms: leaf structure of their species and hybrids. *Bocconea*, *5*(2), 493-504.

9. Sezik, E., Yeşilada, E., Honda, G., Takaishi, Y., Takeda, Y., Tanaka, T. (2001) Traditional medicine in Turkey X. Folk medicine in Central Anatolia. *Journal of Ethnopharmacology*, *75*, 95-115. [CrossRef]

10. Han, M.İ., Bulut, G. (2015). The folk-medicinal plants of Kadişehri (Yozgat-Turkey). *Acta Societatis Botanicorum Poloniae, 84*(2), 237-248. [CrossRef]

11. Kültür, Ş. (2007) Medicinal plants used in Kırklareli Province (Turkey). *Journal of Ethnopharmacology, 111*, 341-364. [CrossRef]

12. Tuzlacı, E., Alparslan İşbilen, D.F., Bulut, G. (2010) Turkish folk medicinal plants, VIII: Lalapaşa (Edirne). *Marmara Pharmaceutical Journal, 14*, 47-52.

13. Yeşilada, E., Sezik, E., Honda, G., Takaishi, Y., Takeda, Y., Tanaka, T. (1999) Traditional medicine in Turkey IX. Folk medicine in North-west Anatolia. *Journal of Ethnopharmacology, 64*, 195-210. [CrossRef]

14. Ezer, N., Avcı, K. (2004). Çerkeş (Çankırı) yöresinde kullanılan halk ilaçları. *Hacettepe Üniversitesi Eczacılık Fakültesi Dergisi*, *24*(2), 67-80.

15. Elçi, B., Erik, S. (2006). Güdül (Ankara) ve çevresinin etnobotanik özellikleri. *Hacettepe Üniversitesi Eczacılık Fakültesi Dergisi*, *26*(2), 57-64.

16. Schweingruber, F.H., Steiger, P., Börner, A. (2019). Bark anatomy of trees and shrubs in the temperate northern hemisphere. Springer: Switzerland, sayfa 192-193.

17. Gregory, M. (1994). Bibliography of systematic wood anatomy of dicotyledons. IAWA Journal Supplement 1. International Association of Wood Anatomists: Netherlands.

18. Holdheide, W. (1951). Anatomie mitteleuropaischer Gehölzrinden. In: Handbuch der Mikroskopie in der Technik, ed. H. Freund vol. 1, Umschau: Frankfurt, sayfa 193-367.

19. Jackson, B.P., Snowdon, D.W. (1990). Atlas of microscopy of medicinal plants, culinary herbs and spices. Belhaven Press: London, sayfa 218-219.

20. Upton, R., Graff, A., Jolliffe, G., Langer, R., Williamson, E. (2011). American Herbal Pharmacopoeia Botanical Pharmacognosy Microscopic Characterization of Botanical Medicines. CRC Press: USA, sayfa 667-669.

21. Akkemik, Ü. (1994). Yüksek Lisans Tezi. Türkiye'nin Doğal Karaağaç (*Ulmus* L.) Taksonlarının Morfolojik ve Palinolojik Özellikleri. İstanbul Üniversitesi, İstanbul, Türkiye.

22. Davis, P.H. (eds.) (1965-1985). Flora of Turkey and The East Aegean Islands. Vol. 1-9, Edinburg University Press: Edinburgh.

23. Çelebioğlu, S., Baytop, T. (1949). Bitkisel Tozların Tekiki için Yeni Bir Reaktif. Farmakognozi Enstitüsü Yayınları, No.10, *Farmakolog (19)*, 301.

24. Metcalfe, C.R., Chalk, L. (1979). Anatomy of the Dicotyledones: Vol 1, Systematic anatomy of the leaf and stem, 2nd edn. Oxford University Press: New York.

25. Trockenbrodt, M. (1990). Survey and Discussion of the Terminology Used in Bark Anatomy. *IAWA Bulletin n.s., 11*(2), 141-166. [CrossRef]

26. Lev-Yadun, S. (1991). Terminology used in Bark Anatomy: Additions and Comments. *IAWA Bulletin n.s., 12*(2), 207-209. [CrossRef]

27. Crang, R., Lyons-Sobaski, S., Wise, R. (2018). Plant Anatomy: A Concept-Based Approach to the Structure of Seed Plants. Springer: Switzerland.

28. Massey, F.P., Ennos, A.R., Hartley, S.E. (2006). Silica in grasses as defence against insect herbivores: contrasting effects on folivores and a phloem feeder. *Journal of Animal Ecology, 75*, 595-603. [CrossRef]

29. El Zaatari, S., Grine, F.E., Ungar, P.S., Hublin, J.J. (2011). Ecogeographic variation in Neanderthal dietary habits: evidence from occlusal molar microwear texture analysis. *Journal of Human Evolution 61*, 411-424. [CrossRef]

30. Karabourniotis, G., Liakopoulos, G., Bresta, P., Nikolopoulos, D. (2021). The Optical Properties of Leaf Structural Elements and Their Contribution to Photosynthetic Performance and Photoprotection. *Plants, 10*(7), 1455. [CrossRef]

J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 46(3): 1044-1063, 2022
Doi: 10.33483/jfpau.1109583

REVIEW ARTICLE / DERLEME MAKALE

QUALITY ASSURANCE AND QUALITY CONTROL OF RADIOPHARMACEUTICALS: AN OVERVIEW

RADYOFARMASÖTİKLERİN KALİTE GÜVENCESİ VE KALİTE KONTROLÜ: GENEL BİR BAKIŞ

Meliha EKİNCİ[1], Ralph SANTOS-OLİVEİRA[2,3], Derya İLEM-ÖZDEMİR[1*]

[1]Ege University, Faculty of Pharmacy, Department of Radiopharmacy, 35040, Izmir, Turkey

[2]Brazilian Nuclear Energy Commission, Nuclear Engineering Institute, Laboratory of Synthesis of Novel Radiopharmaceuticals and Nanoradiopharmacy, Rio de Janeiro, 21941906, Brazil

[3]Zona Oeste State University, Laboratory of Nanoradiopharmaceuticals and Radiopharmacy, Rio de Janeiro, 23070200, Brazil

ABSTRACT

Objective: *Radiopharmacy is a special field of pharmacy that examines, develops, conducts quality controls, deals with distribution and application of pharmaceutical forms called radiopharmaceuticals that are used for diagnostic and therapeutic purposes, carrying pharmaceutical and radioactive properties together. Radiopharmaceuticals contain radionuclides. This is the most important difference that distinguishes radiopharmaceuticals from other conventional drugs. Since radiopharmaceuticals are administered to humans, they must be sterile, pyrogen-free, isotonic, isohydric, and subject to all quality control tests required for conventional drug. Also, additional quality control tests are required due to radionuclide they contain. In this review, quality control tests applied to radiopharmaceuticals, hospital radiopharmacy laboratory types and Good Radiopharmacy Practices (GRP) will be discussed.*

Result and Discussion: *Radiopharmaceuticals should be prepared in accordance with standards specified in relevant sections of pharmacopoeias. For each series of radiopharmaceuticals, tests prescribed in the pharmacopoeias and records must be kept. Production and preparation of radiopharmaceuticals should be carried out in accordance with Good Manufacturing Practices for sterile preparations and GRP for radioactive products. However, radiopharmaceuticals that pass quality control tests can be administered to patients after dose measurements are made in dose calibrators. Thus, the patient's safety and benefit are maximized, while the risk is minimized. Some radiopharmaceuticals with a short half-life are used before quality control tests are completed. In this case, the effectiveness and continuation of the quality assurance system should be tested at*

* **Corresponding Author / Sorumlu Yazar:** Derya İlem-Özdemir
e-mail / e-posta: deryailem@gmail.com, **Phone / Tel.:** +902323111963

Submitted / Gönderilme: 27.04.2022 **Accepted / Kabul:** 30.06.2022

appropriate intervals. According to procedures performed by hospital radiopharmacy laboratories, international standards are determined as Level IA/B, Level IIA/B and Level IIIA/B/C. Quality assurance of radiopharmaceuticals is provided by a sufficient number of trained personnel within the scope of GRP, devices that have been calibrated and controlled, appropriate substances and a working order in which tests determined at each stage are made and results are recorded accurately and regularly.

Keywords: *Good radiopharmacy practices, radiopharmaceuticals, radiopharmacy lab, quality assurance, quality control*

ÖZ

Amaç: *Radyofarmasi, radyofarmasötik adı verilen, teşhis ve tedavi amaçlı kullanılan, farmasötik ve radyoaktif özellikleri bir arada taşıyan farmasötik formları inceleyen, geliştiren, kalite kontrollerini yapan, dağıtımı ve uygulaması ile uğraşan özel bir eczacılık alanıdır. Radyofarmasötikleri diğer konvansiyonel ilaçlardan ayıran en önemli fark; radyofarmasötiklerin radyonüklid içermesidir. Radyofarmasötikler insanlara uygulandığı için steril, pirojensiz, izotonik, izohidrik olmalı ve geleneksel ilaç için gerekli tüm kalite kontrol testlerine tabi olmalıdır. Ayrıca içerdikleri radyonüklid nedeniyle ek kalite kontrol testlerinin uygulanması da gereklidir. Bu derlemede radyofarmasötiklere uygulanan kalite kontrol testleri, hastane radyofarmasi laboratuvar tipleri ve İyi Radyofarmasi Uygulamaları (GRP) ele alınacaktır.*

Sonuç ve Tartışma: *Radyofarmasötikler, farmakopelerin ilgili bölümlerinde belirtilen standartlara uygun olarak hazırlanmalıdır. Her radyofarmasötik serisi için farmakopelerde belirtilen testler ve kayıtlar tutulmalıdır. Radyofarmasötiklerin üretimi ve hazırlanması, steril preparatlar için İyi Üretim Uygulamaları ve radyoaktif ürünler için GRP uyarınca yapılmalıdır. Ancak kalite kontrol testlerini geçen radyofarmasötikler, doz kalibratörlerinde doz ölçümleri yapıldıktan sonra hastalara verilebilir. Böylece hastanın güvenliği ve faydası maksimize edilirken risk minimuma indirilir. Yarı ömrü kısa olan bazı radyofarmasötikler kalite kontrol testleri tamamlanmadan kullanılmaktadır. Bu durumda kalite güvence sisteminin etkinliği ve devamlılığı uygun aralıklarla test edilmelidir. Hastane radyofarmasi laboratuvarları tarafından yapılan işlemlere göre uluslararası standartlar Seviye IA/B, Seviye IIA/B ve Seviye IIIA/B/C olarak belirlenmişitr. Radyofarmasötiklerin kalite güvencesi, GRP kapsamında yeterli sayıda eğitimli personel, kalibre ve kontrolleri yapılmış cihazlar, uygun maddeler ve her aşamada belirlenen testlerin yapıldığı ve sonuçların doğru ve düzenli olarak kayıt altına alındığı bir çalışma düzeni ile sağlanır.*

Anahtar kelimeler: *İyi radyofarmasi uygulamaları, radyofarmasötikler, radyofarmasi laboratuvarı, kalite güvencesi, kalite kontrol*

INTRODUCTION

Radiopharmaceuticals are sterile and pyrogen-free drug formulations, which are mostly prepared to be administered intravenously to patients for diagnosis and treatment in nuclear medicine [1,2]. The most important difference from traditional drug formulations is that they contain a short half-life radionuclide in their structure. Unlike traditional drugs, they are produced, quality control tests are performed and administered to patients within the same working day [3]. Because some radionuclides have short half-life like ^{11}C, ^{13}N, ^{15}O, ^{18}F, these radiopharmaceuticals do not allow all quality control tests to be completed before being administered to patients. The quality control tests of these radiopharmaceuticals are specified in the relevant pharmacopoeias, which should be done before administration to the patient or which ones can be done retrospectively [4,5].

The purpose of quality control tests is to ensure that radiopharmaceuticals are administered to humans effectively and safely. Quality control of radiopharmaceuticals protects patients from unnecessary radiation exposure and side effects. It provides image quality and accurate diagnostic information in diagnosis, and maximizes patient benefit by providing maximum effect in treatment [6].

Quality assurance is a system that includes all the necessary components to ensure the safety, efficacy and purity of radiopharmaceuticals [7]. This system consists of many components. These are:

1. A sufficient number of personnel who have received the necessary training
2. A properly planned and traceable laboratory
3. Adequate equipment with proper calibration and maintenance
4. Documentation consisting of appropriate job descriptions and traceable regular records
5. Suitable chemicals and auxiliaries
6. Appropriate quality control tests at every stage [7]

Before the radiopharmaceuticals are administered to the patient, differential tests are performed quickly and effectively. These tests can be examined under four groups as physicochemical tests (radioactivity, radionuclidic purity, radiochemical purity and chemical purity), biological tests (sterility, pyrogenicity), pharmaceutical tests (appearance, color, pH, mean particle size, particle distribution, ionic strength, isotonicity, and osmolality) and toxicity tests [8]. In this review, quality control tests of radiopharmaceuticals, hospital radiopharmacy laboratory types and Good Radiopharmacy Practices (GRP) to ensure quality assurance of radiopharmaceuticals will be discussed.

Physicochemical Tests of Radiopharmaceuticals

Radioactivity

Radioactivity is the number of nuclear transformation per unit time in the amount of radioactive preparation. The unit of radioactivity Becquerel (Bq) expresses the number of transformations per second [9]. When working with radiopharmaceuticals, since it is a very small unit in practice, multiples such as kBq (10^3 Bq), MBq (10^6 Bq) and GBq (10^9 Bq) are used instead of Bq. To measure radioactivity, dose calibrators measure in micro Curie (μCi), mCi or MBq, GBq. There are well-type gas ionization chamber or dose calibrator systems to measure the radioactivity delivered to the patient. These devices must pass certain tests to achieve the correct activity. The dose calibrator quality control tests should be carried out at the installation stage and at the specified periodic intervals after installation. These tests are accuracy, stability, linearity, and geometry [10-12]. The acceptable limits of the dose that should be given to the patient according to the rules of the Nuclear Regulatory Commission (NRC) is ±10%. If the deviation in the dose calibrator is greater than 10%, the device must be repaired, recalibrated or replaced [13-15].

Radionuclidic identity

In order to determine the identity of the radionuclide in pharmacopoeias, some tests depending on the half-life and energy of the radionuclide are recommended [4,5]. The energy test is done by taking the gamma spectrum of the radionuclide, which is the unique identifier for identity testing [16]. It is

difficult to perform in the radiopharmacy laboratory as it requires equipment. The spectrometer system must also be calibrated for accurate measurement. In the ionization chamber linearity test proposed to determine the identity of the radionuclide, the radioactivity is measured at regular intervals for about 3 half-lives and plotted against time. The slope gives the decay constant λ. Half-life is calculated from the activity at time t with the formula $In(A_t)=In(A_0) - \lambda$ (Eq.1).

$T_{1/2} = In(2)/\lambda$ (Eq. 2)

A_t = The activity at time t

A_0 = The activity at the beginning

$T_{1/2}$ = Half-life of radionuclide

λ = Decay constant of radionuclide

Radionuclidic purity

Radionuclidic purity is defined as the ratio of the activity of the radionuclide to the total radioactivity. The undesired radionuclides can belong to the same element as the desired radionuclide or to a different element. The purpose of radionuclide purity control is to protect the patient from unnecessary radiation and to use the radiopharmaceutical effectively [17]. The radionuclide must be free from all other species. Impurities arise from the production method of the radionuclide: Ge-68 in Ga-68 eluate in $^{68}Ge/^{68}Ga$ generator, Sr-82 in Rb-82 eluate in $^{82}Sr/^{82}Rb$ generator, or Mo-99 in Tc-99m eluate in $^{99}Mo/^{99m}Tc$ generator.

Radionuclidic purity is determined by measuring the type of radiation emitted from each radionuclide and its half-life. Gamma-emitting radionuclidic impurities are measured by multi-channel analyzer systems with NaI(Tl) or Ge(Li) detectors, while pure beta-emitting radionuclides are measured with beta spectrophotometer or liquid scintillation methods [18]. Identifying pure beta emitters is not as easy as gamma emitters due to the counting problem. Mo-99 impurity in Tc-99m is practically measured by gamma photon absorption (lead shield) method. $^{99}Mo/^{99m}Tc$ generator is loaded on glass column (Al_2O_3), Mo-99 activity is adsorbed on column material in the form of MoO_4^{-2} (molybdate). The ^{99}Mo activity in the eluate should be less than 0.15 $\mu Ci.mCi^{-1}$ Tc-99m. The quantities of impurities should not exceed acceptable limits. The molybdenum impurity in the product obtained from the $^{99}Mo/^{99m}Tc$ generator at each eluating and the expiry time of Tc-99m should be determined. Expiry time should not exceed 12 h after eluating. NRC has set acceptable limits for each radionuclide [19].

Radiochemical purity

Radiochemical purity indicates the ratio of radionuclide contained in the radiopharmaceutical in the chemical compound and is expressed in percent. Radiochemical purity is calculated by the ratio of the activity in the desired chemical form to the total radioactivity. Radiochemical impurities are caused

by changes in solution temperature or pH, light, the presence of reduced or oxidized agents, and structural deterioration due to radiolysis. The distribution in the biological system and the absorbed radiation dose are directly related to the radiochemical purity. This situation causes low quality images to be taken and the patient to receive high doses of radioactivity due to the weak localization of the radiopharmaceutical in the desired area and the high ground activity from the surrounding tissues. The reason for the radiolabeled compounds to decay by radiolysis is the type and energy of radiation the radionuclide has. As a result of the absorption of the radioactivity of the radiolabeled molecule, free radicals with unshared electrons are formed and these free radicals cause other molecules to degrade. Secondly, chemical structures such as hydrogen peroxide (H_2O_2) or hydrogen dioxide (HO_2) formed as a result of the radiolysis of water (H_2O) cause the labeled molecule and other molecules to deteriorate. Particular radiation releases its energy at a shorter distance than gamma radiation. In order to ensure the stability of radiopharmaceuticals, substances such as ascorbic acid, *p*-aminobenzoic acid and gentisic acid are added to the formulations as antioxidant agents in some cases [20,21].

There are chromatography methods such as gel chromatography, gas chromatography (GC), high performance liquid chromatography (HPLC), and paper chromatography (PC) to determine radiochemical purity. In the hospital radiopharmacy laboratory, the applicable quality control method is PC or thin layer chromatography (TLC) in terms of being fast and economical [20].

Chromatography is a method of separating and purifying the components that make up the chemical mixture in a two-phase system, one of which is stationary and the other is mobile phase. It is based on the principle that the components are drifted with different speeds on the stationary phase with the help of the moving phase [22]. Radiochromatography, unlike ordinary chromatography, detects radioactive species based on their location rather than their chemical or physical properties [23]. Paper is used as the stationary phase in PC. Separation is ensured by passing a solvent that will provide separation from the paper, by making use of the different speed of the substances to be separated on the paper. The difference of TLC from PC is that instead of paper, silica gel, aluminum oxide, aluminum silicate or cellulose coated papers (Whatman 3M) are used and the separation process is completed faster and in a short time. The Relative front (R_f) value of a compound is descriptive as the distance that compound travels through a liquid through a stationary phase. The R_f value must be between 0-1. If the administered compound remained at the application point, R_f is indicated as 0, if it progressed to the extreme point, it is designated as R_f 1 [23,24]. Figure 1 shows the calculation of the R_f value on the strip. While Table 1 shows R_f values of radiopharmaceuticals labeled with Tc-99m, Table 2 shows R_f values of various radiopharmaceuticals labeled with radionuclides other than Tc-99m [25,26].

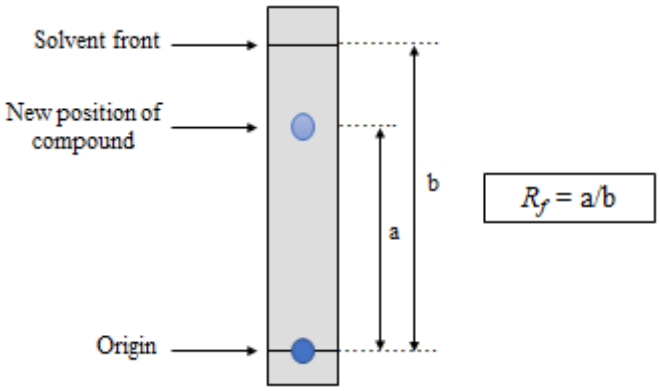


Figure 1. Determination of R_f value

Table 1. Chromatographic data of radiopharmaceuticals labeled with Tc-99m

Radiopharmaceutical	**Chromatographic System**	***R_f*** **Radiopharmaceutical**
^{99m}Tc-Sodium pertechnetate	Chromatography paper / Acetone: 2N Hydrochloric acid (80:20)	0.9
^{99m}Tc-Sodium pertechnetate	ITLC-Silica gel / 0.9% NaCl	1.0
^{99m}Tc-Sodium pertechnetate	Chromatography paper / Water: Methanol (20:80)	0.6
^{99m}Tc-(pyro and trimeta) phosphate	Chromatography paper / Physiological saline	0.0-0.1
^{99m}Tc-(pyro and trimeta) phosphate	Chromatography paper / Methyl Ethyl Ketone	0.0-0.1
^{99m}Tc-Albumin aggregate	Chromatography paper / Dilute methanol (7:10)	0.0-0.1
^{99m}Tc-Albumin aggregate	ITLC-Silica gel / 2-Butanone	0.0-0.1
^{99m}Tc-Arkitumomab	Silica gel / Acetone	0.0-0.1
^{99m}Tc-Bisisate	Silica gel / Ethyl acetate	0.4
^{99m}Tc-Bisisate	ITLC-Silica gel / Saturated NaCl	0.0-0.1
^{99m}Tc-Bisisate	ITLC-Silica gel / Methanol: Ammonacetate (50:50)	0.9-1.0
^{99m}Tc-Etifenin	Chromatography paper / Water: Acetonitrile (40:60)	0.9-1.0
^{99m}Tc-Etifenin	Chromatography paper / Methyl Ethyl Ketone	0.0-0.1
^{99m}Tc-Gluconate	Silica gel / 0.9% NaCl	0.9-1.0
^{99m}Tc-Gluconate	Silica gel / Methyl Ethyl Ketone	0.0-0.1
^{99m}Tc-Gluseptat	Chromatography paper / Acetone	0.0-0.1
^{99m}Tc-Human albumin	Silica gel / Methyl Ethyl Ketone	0.0-0.1

Table 1 (*continued*). Chromatographic data of radiopharmaceuticals labeled with Tc-99m

Radiopharmaceutical	Chromatographic System	R_f Radiopharmaceutical
^{99m}Tc-Stannous pyrophosphate	Silica gel / Sodium acetate	0.9-1.0
^{99m}Tc-Stannous pyrophosphate	Silica gel / Methyl Ethyl Ketone	0.0-0.1
^{99m}Tc-Colloidal tin	Silica gel / 0.9% NaCl	0.0-0.1
^{99m}Tc-Colloidal Rhenium sulfide	Chromatography paper / Physiological saline	0.1
^{99m}Tc-Colloidal sulfur	Chromatography paper / Physiological saline	0.1
^{99m}Tc-Lidophene	Silicylic acid impregnated fiberglass paper tape / Nitrogen-leached solution of NaCl	0.0-0.1
^{99m}Tc-Medronat	Silica gel / Sodium acetate	0.0-0.1
^{99m}Tc-Nofetumomabmer pentane	Silica gel / 0.73 N Trichloroacetic acid	0.0-0.1
^{99m}Tc-Oxydronate	Chromatography paper / Physiological saline	0.9-1.0
^{99m}Tc-Oxydronate	Silica gel plate / Methyl Ethyl Ketone	0.0-0.1
^{99m}Tc-Pentetate	Silica gel / 0.9% NaCl	0.9-1.0
^{99m}Tc-Pentetate	Silica gel plate / Methyl ethyl ketone	0.0-0.1
^{99m}Tc-Sestamibi	Octadecylsilyl silica gel plate / Tetrahydrofuran: Ammonium acetate: Methanol: Acetonitrile (10:20:30:40)	0.3-0.6
^{99m}Tc-Sestamibi	Aluminum oxide coated TLC / Ethanol	0.9-1.0
^{99m}Tc-Sucimer	Silica gel plate / Methyl Ethyl Ketone	0.0-0.1
^{99m}Tc-Sulfur colloid	Chromatography paper / Methanol	0.0-0.1
^{99m}Tc-Tetrofosmine	ITLC-Silica gel / Acetone: Dichloromethane (35:65)	0.5
^{99m}Tc-Dimercaptosuccinic acid	ITLC-Silica gel / 2-Butanone	0.0-0.1
^{99m}Tc-Diphosphonates	ITLC-Silica gel / 1M NaAcetate	0.9-1.0
^{99m}Tc-Diphosphonates	ITLC-Silica gel / 2-Butanone	0.0-0.1
^{99m}Tc-Diethylene Triamine Penta Acetate	ITLC-Silica gel / NaCl	0.9-1.0

Table 1 (*continued*). Chromatographic data of radiopharmaceuticals labeled with Tc-99m

Radiopharmaceutical	Chromatographic System	R_f Radiopharmaceutical
^{99m}Tc-Diethylene Triamine Penta Acetate	ITLC-Silica gel / 2-Butanone	0.0-0.1
^{99m}Tc-Ethyl Cysteinate Dimer	Baker Silica gel / Ethylacetate	0.9-1.0
^{99m}Tc-Hexamethylene-propyleneamine oxime	ITLC-Silica gel /2-Butanone	0.9-1.0
^{99m}Tc-Hexamethylene-propyleneamine oxime	ITLC-Silica gel / 0.9% NaCl	0.0-0.1
^{99m}Tc-Dimethyl-acetanilide-iminodiacetic acid	ITLC-Silica gel / 0.9% NaCl	0.0-0.1
^{99m}Tc-Dimethyl-acetanilide-iminodiacetic acid	ITLC-Silica gel / 50% Acetonitrile	0.9-1.0
^{99m}Tc-Ethylenedicysteine	ITLC-Silica gel / 2-Butanone	0.0-0.1
^{99m}Tc-Ethylenedicysteine	ITLC-Silica gel / 0.3M Acetic acid	0.9-1.0
^{99m}Tc-Monoclonal Antibodies	ITLC-Silica gel / 0.9% NaCl	0.0-0.1

Table 2. Chromatographic data of various radiopharmaceuticals labeled with radionuclides other than Tc-99m

Radiopharmaceutical	Chromatographic System	R_f Radiopharmaceutical
^{18}F-Alovudin	Silica gel / Water: Acetonitrile (5:95)	0.7
^{18}F-Fluorodeoxyglucose	Silica gel / Water: Acetonitrile (5:95)	0.0
^{18}F-Fluorodopa	Octadesylsilyl silica gel / Methanol: Water (50:50)	0.3
^{18}F-Fluoroethyl-L-Tyrosine	Silica gel / Acetic acid: Methanol (10:90)	0.7
^{18}F-Fluorocholine	Silica gel / Acetonitrile: NaCl solution (50:50)	0.5
^{18}F-Fluoromisonidazole	Silica gel / Water: Acetonitrile (5:95)	0.8
^{68}Ga-Edotreotide	Silica gel / 77 $g.L^{-1}$ Ammonium Acetate solution in water: Methanol (50:50)	0.8-1.0
^{68}Ga-Chloride	Silica gel / 77 $g.L^{-1}$ Ammonium Acetate solution in water: Methanol (50:50)	0.0-0.2

Table 2 (*continued*). Chromatographic data of various radiopharmaceuticals labeled with radionuclides other than Tc-99m

Radiopharmaceutical	Chromatographic System	R_f Radiopharmaceutical
^{111}In-Ibritumomab	Silica gel / 0.9% NaCl	0.0-0.1
^{111}In-Kapromab Pentetate	Silica gel / 0.9% NaCl	0.0-0.1
^{111}In-Chloride	Silica gel / NaCl solution (pH:2.3)	0.5-0.8
^{111}In-Pentetate	Silica gel / 0.9% NaCl	0.9-1.0
^{111}In-Satumomab Pentetate	ITLC-Silica gel / 0.9% NaCl	0.0-0.1
^{111}In-Octreotide	ITLC-Silica gel / 0.1M Na-Citrat pH:5	0.0-0.1
^{90}Y-Ibritumomab Tiuxetan	Silica gel / 0.9% NaCl	0.0-0.1
^{125}I-Albumin	Chromatography paper / Diluted methanol (7:10)	0.0-0.1
^{125}I-Iotalamate sodium	Chromatography paper / Methanol (adjusted to pH 3-6 with 2N Sulfuric acid): Ammonium hydroxide (100:1.5)	0.0-0.1
^{131}I-Rosebengal sodium	Chromatography paper / N Acetic acid	0.0-0.1
^{51}Cr-Edetat	Chromatography paper / Concentrated Ammonia: Ethanol: Water (1:2:5)	0.8-0.9
^{51}Cr-Sodium chromate	Chromatography paper / Ammonia: Ethanol: Water (25:50:125)	0.9
^{32}P-Chromic phosphate	Chromatography paper / Water	0.0-0.1
^{11}C-Methyl-L-Methionine	Octadecylsilyl silica gel / Methanol: Water (50:50)	0.58
$^{177}Lu^{3+}$	Silica gel / NaCl solution (pH:2.3)	0.4-0.7

Each radiopharmaceutical must be tested for radiochemical purity before being administered to the patient. In terms of radiochemical purity, only free Tc-99m is expected in eluating product obtained from generator.

The presence of Reduced/Hydrolyzed (R/H) Tc-99m in the eluating solution creates an impurity. In radiopharmaceutical kits, a Tc-99m substrate becomes captive in a special organ system by binding to the molecule. It may cause some problems in imaging by creating both free Tc-99m ($^{99m}TcO_4^-$) and R/H Tc-99m (TcO_2) radiochemical impurity that occurs during the radiopharmaceutical preparation process [20].

Radiochemical impurities may cause difficulty in evaluating images and even inaccurate clinical diagnosis. Therefore, it is important to effectively couple the radionuclide and the desired molecule to ensure the accuracy of reported disease diagnoses. The desired efficacy and safety will not be achieved

after treatment with radiopharmaceutical with low binding activity. United States Pharmacopeia (USP) has established minimum standard binding efficiencies for many radiopharmaceuticals [4,5]. The rate of radiochemical impurities can be at most 5% [27].

Chemical purity

Chemical impurities are all non-radioactive chemical structures in the radiopharmaceutical preparation and cause adverse effects directly or by radiolabeling. The most common chemical impurities are Al^{+3} for ^{99m}Tc radiopharmaceuticals and Kryptofix 2.2.2 for ^{18}F-FDG. According to USP standards, the amount of Al^{+3} ions in the Tc-99m eluate should not exceed 10 $\mu g.mL^{-1}$ [4,5]. If there is more, colloidal Tc-Al particles are formed. These particles can be accumulated in the liver and cause cell damage by aggregation, with capillary blockage when larger particles appear, and by binding to red blood cells. The amount of chemical impurities is measured by the spectrophotometric method. A simpler method to detect aluminum is that the indicator paper gives the reagent red in the presence of Al^{+3}. A standard solution of 10 $\mu g.mL^{-1}$ of aluminum is used for color analysis. All commercial kits contain stannous ions as reducing agents. If there is too much stannous ions in the kit, it may cause some problems; for example, while the bone should be visible, the liver is visible. Another problem is that the excess stannous remaining in the circulation is destroying the red blood cells and collecting in the spleen [27].

Biological Tests of Radiopharmaceuticals

Each parenterally injected product must be sterile, pyrogen-free, and non-toxic. Biological tests are performed during and after production to ensure that radiopharmaceuticals are sterile and clear of pyrogens. In order for the product to be sterile and free of pyrogens, it must be manufactured under sterile conditions, with sterile environments and materials. Toxic dose should be determined in animal studies before being used in humans.

Sterility

Sterile solution is the solution medium in which there are no pathogenic or non-pathogenic living organisms. Sterilization can be done by autoclaving at 121°C at 15 psi pressure or by 0.22 μm membrane filtration sterilization under aseptic conditions. Sterility control is performed to show that there is no live bacteria or microorganism in the radiopharmaceutical preparation. It is carried out by taking a sample from the drug and making a culture in a suitable medium. Incubate for 14 days at 30-35°C in liquid thioglycolate medium or incubate for 14 days at 20-25°C in soy-casein medium. If there is no microbial growth, the drug is sterile [28].

Many radiopharmaceuticals are produced and used on the same day. Since the sterility test is time-consuming, the radiopharmaceutical which has radionuclide with short half life, is sent to the user before the sterility test is completed, and the sterility results are recorded retrospectively. Clean environmental

conditions should be maintained during production, High Efficiency Particulate Air (HEPA) filters take particles of 0.3 μm and larger, sterile disposable materials, sterile chemicals (water for injection, NaCl, etc.) should be used. If aseptic conditions are applied correctly, the possibility of bacterial contamination is reduced. It is recommended to take samples from the radiopharmaceutical prepared periodically once a week and send it to the microbiology laboratory for bacteriological testing [3].

Pyrogenicity

Pyrogens are metabolic wastes of living organisms, or non-living organisms. Pyrogens are typical bacterial endotoxins. They cannot be destroyed in the autoclave and cannot be separated by membrane filtration. Although the solution is sterile, it may contain pyrogen. The way to prevent it is to use high quality water and chemicals. The patient experiences fever, chills, headache and chest pain 45-90 min after the injection of the pyrethetic substance, and this non-lethal situation ends after 3-4 h. Pyrogen test is determined by two different methods.

Limulus Amebocyte Lysate (LAL) test

Gram negative endotoxin is the most important source of pyrogen contamination. The LAL test is a fast and sensitive method that detects the presence of pyrogen and displays pyrogens at the $ng.mL^{-1}$ level. The protein isolated from the blood cells of the horseshoe crab (limulus polyphemus) forms a matte gel with nanograms or greater concentrations of gram negative bacterial endotoxin. The sample to be tested is incubated with lysate at 37°C for 60 min. Formation of a matt gel indicates the presence of pyrogen [29].

Rabbit pyrogenicity test

In the rabbit test, the test sample is injected into 3 rabbits. After injection, rectal temperature is recorded once an hour for three hours. It is compared with the temperature before injection. A rise in temperature of 0.6°C in each rabbit or a total temperature of 1.4°C in three rabbits is indicative of the presence of pyrogenic matter. The rabbit test method, which is decided by considering the rise in fever of rabbits, is no longer used [30].

Radioassay/radioactive dose for the patient

During the preparation of the radiopharmaceutical and before it is given to the patient, the radioactivity of the radiopharmaceutical should be known according to the dose calculation made for each patient. The amount of radioactivity in the radiopharmaceutical that is drawn into the injector to be administered to the patient is measured with a dose calibrator. The amount of radioactivity is given in Ci or Bq. The activity of the radiopharmaceutical decreases over time depending on the half-life of the radionuclide used [31]. The following formula is used to calculate the activity of the pharmaceutical over time (Eq. 3):

$A_t = A_0 \times e^{-0.693 \times t / T_f}$ (Eq. 3)

A_t = Activity (Bq or Ci) at time t

A_0 = Activity (Bq or Ci) in original sample

t = Time

T_f = Physical half-life of radionuclide

Pharmaceutical Tests of Radiopharmaceuticals

Radiopharmaceuticals must have the proper ionic structure, isotonicity, and osmolality in order to be used in humans. After each preparation, the radiopharmaceutical should be examined visually. The mean particle size and size distribution should be determined, as well as the quantity of particles to be supplied to the patient. The pH of the first product must be controlled in each batch prepared. An injectable drug's isotonicity should be the same as a 0.9% sodium chloride solution.

Appearance and color

The physical appearance of a radiopharmaceutical after preparation and during use is specified in the relevant monograph of the relevant pharmacopeia [4,5]. The radiopharmacist should check the clarity and color of the radiopharmaceuticals after each preparation. If the radiopharmaceutical is not in particle form, but in solution form, it should not contain particles.

pH

In the dilution of radiopharmaceuticals, liquids with an ionic structure and pH long-term value that will not disturb the stability and physiological compatibility are used. For the stability of the radiopharmaceutical, all radiopharmaceuticals are formulated in the optimum pH range. The most suitable pH value for parenteral use is the blood pH value of 7.4. At the same time, due to the buffering property of blood, radiopharmaceuticals can be prepared between pH 2-9. In clinical routine use, pH value measurement with pH paper is a simple and fast method [2,6].

Mean particle size and size distribution

Mean particle size and size distribution is an important test that must be controlled in order to achieve the desired distribution of the radiopharmaceutical in the biological system. The particle-containing radiopharmaceutical is heterogeneous, and the mean particle size is determined by light microscope, hemocytometer or dynamic light scattering. It contains two main types of radiopharmaceutical particulate structures: albumin microspheres [macro aggregated albumin (MAA), human albumin microspheres (HAM), human serum albumin (HSA)] for lung scintigraphy and colloids for the reticuloendothelial system. According to USP standards, at least 90% of the particles to be used for lung imaging should be 10-90 μm in diameter [4,5]. Smaller particles easily pass through the lungs

and are accumulated in the reticuloendothelial system (liver, bone marrow). No particle should exceed 150 μm. The number of particles is determined according to the condition of the disease and the child patient. Particles should be in the range of 0.1-1 μm for liver and spleen imaging and 0.01-0.3 μm for lymphoscintigraphy. Dynamic light scattering is the best methods for measuring mean particle size and size distribution [32,33].

Ionic strength, isotonicity, and osmolality

The final form of the radiopharmaceutical must be isotonic, in other words the ionic strength must be the same as the blood. Isotonic fluid has the same osmotic pressure as human serum. Radiopharmaceuticals with 250-350 $mOsm.kg^{-1}$ are considered isotonic. Isotonia test is done using an osmometer. Osmolarity controls the transitions between intracellular and extracellular. When an equal concentration of solute occurs between the inside and outside of the cell, the cells are not damaged because the pressure inside and outside the cell will be equal. Hypertonic or hypotonic fluid damages cells. 0.9% sodium chloride solution is an isotonic solution, and the applied radiopharmaceuticals are usually injected in this solution [34]. Due to the blood's high dilution and buffering capacity, the tolerances for pH and osmolality of intravenous injections are broad. However, the osmolality tolerances are critical for intrathecal administrations. The preparation and selection of radiopharmaceuticals, and diluents used for intrathecal administration requires special consideration.

Toxicity Tests of Radiopharmaceuticals

Acute and chronic toxicity test of radiopharmaceuticals is carried out by animal testing. After the autopsy performed 2-6 weeks after the radiopharmaceutical administration, toxicity limits are determined by cell culture study. The dose that kills 50% of the animals is shown as LD_{50} [3].

Hospital Radiopharmacy Laboratory Types and Required Quality Controls

The procedures performed in hospital radiopharmacy laboratories around the world are classified under 3 main categories [35-37].

Operational level I

Operational level I A

It includes radiopharmaceuticals, in the final form, purchased from authorized manufacturers, in single or multiple dose form where no combination is required. Quality control studies consist of measuring the radioactive dose of the radiopharmaceutical. The consistency of the dose calibrator should be evaluated daily with a long half-life radionuclide such as Cs-137 [35-37].

Operational level I B

This level of operation includes the preparation of ready-to-use injections of Sr-90 and Sm-153 used for therapy and pain relief therapy, and other ready-to-use open source radionuclides. Periodically, radionuclidic and radiochemical impurities should be checked [35-37].

Operational level II

Operational level II A

Routine use of the Tc-99m generator involves the reconstitution of pre-sterilized radiopharmaceutical cold kits. Quality controls are quality controls on generator and generator eluating in addition to Operational Level I. In the first eluating, radionuclidic impurity test, sterility, radiochemical impurity and aluminum ion contamination should be checked. Radiochemical impurity tests should also be performed before the radiopharmaceuticals are administered to the patient [35-37].

Operational level II B

It involves radiolabeling of blood cells taken from the patient to display infection or inflammation. In quality controls, the shelf life of ^{99m}Tc-HMPAO, ^{111}In-Oxine and Tc-99m colloids should be considered and the radiolabeling percentage of leukocytes should be calculated routinely [35-37].

Operational level III

Operational level III A

This level covers radiopharmaceuticals prepared for diagnostic applications. Procedures in Level I and II, sterility test, bacterial endotoxin test, microbiological and pyrogenicity tests, specific activity, chemical purity, HPLC, GC, TLC, radiochemical purity, stability and toxicity studies are required for quality control studies [35-37].

Operational level III B

This level involves the preparation of radiopharmaceuticals (Metaiodobenzylguanidine (MIBG) radioionidation, ^{188}Re-lipiodol, etc.) for therapeutic applications for research and development purposes. Radionuclidic purity of the radiopharmaceutical is important in quality control [35-37].

Operational level III C

This level includes the synthesis of Positron Emission Tomography (PET) radiopharmaceuticals, research and development purpose radiopharmaceuticals produced from long-life generators such as Ga-68 and Re-188 generators. Quality control of radiochemical purities must be done quickly by HPLC. Sterility, pyrogenicity and physicochemical tests should also be performed routinely [35-37].

Good Radiopharmacy Practices (GRP)

Regarding the preparation and administration of radiopharmaceuticals, GRP combines the principles of traditional Good Manufacturing Practices (GMP) of pharmaceuticals with radioprotection concepts. The components of GRP are personnel and resources, qualifications, quality assurance, facility and equipment, quality control, documentation and labeling [38,39].

Personnel and resources

In radiopharmacy lab, radiopharmacist is the responsible person which the owner of this area. Also, the number of personnel should be such that all necessary procedures are completed before the application of the prepared radiopharmaceutical. It is recommended that the duties and responsibilities of all personnel are clearly defined in written documents. In GRP, an organizational chart is created between the preparation steps and personnel responsibilities. All operations must be carried out under the control of the responsible person (radiopharmacist). All personnel in charge from the preparation to distribution of radiopharmaceuticals should be properly trained in quality systems, GRP and all regulatory requirements related to radiopharmaceuticals. Personnel exposure to radiation should be monitored by approved personnel dosimeters that are regularly checked and readings are recorded. After the radiopharmaceutical preparation is completed, it should be checked whether there is radioactive contamination in both the staff and the working area with appropriate monitors [39,40].

Qualifications:

- The radiopharmacist must establish procedures for the inspection and evaluation of incoming materials and ensure that every incoming material is examined and evaluated before it is used against specifications.
- The person responsible for quality assurance of radiopharmaceuticals should establish and manage the overall quality assurance system and verify that the documentation is written correctly.
- The person responsible for the production of radiopharmaceuticals must approve the production processes, evaluate, sign and store production records.

The person responsible for quality control must describe the specifications, test methods and other quality control procedures. It must evaluate, sign and keep quality control reports and records [39,40].

Quality assurance

It is recommended to establish a quality assurance program to comprehensively design and correctly implement a quality assurance system, taking into account the appropriate risk assessment in GRP. Risk assessment plays an important role in all stages involved in the preparation of radiopharmaceuticals. The quality assurance team should inform the persons involved in the preparation

of radiopharmaceuticals according to up-to-date information, and declare the necessary measures in writing to ensure that the radiopharmaceuticals are released, stored and transported in a manner that ensures the desired quality throughout the shelf life and in accordance with the expiry date [7].

Facility and equipment

Facilities should be designed to ensure proper handling of materials and equipment, and prevent contamination of the equipment or product according to personnel or environmental conditions. It should be such that all equipment used in production (eg particle accelerator, synthesis units or other special equipment) is located and maintained in an easily accessible manner to all work areas in the normal production stage. Access to workplaces should be limited to responsible personnel. In the preparation of radiopharmaceuticals, the same area or room can be used for multiple purposes. However, in the case of multiple radiopharmaceutical preparations, it is important to develop the appropriate level of control required to avoid confusion and contamination. In order to avoid confusion and contamination, different production areas should be clearly defined and segregated, especially with regard to unidirectional material flow, intermediate and finished products. The preparation of radiopharmaceuticals, quality control and storage of all approved ingredients, including laboratory procedures (such as release testing), containers and lids can be done in the same room. All equipment can potentially affect the quality and purity of radiopharmaceuticals or give incorrect or invalid test results when used or not performed incorrectly. It is therefore essential to demonstrate that the equipment is fit for the intended purposes, is properly installed, maintained and can produce valid results over and over again [41].

Quality control

It is recommended that any quality control method required for radiopharmaceuticals have the appropriate equipment to perform its function. For this purpose, the dose calibrator, radioactive TLC scanner, HPLC device should be checked and maintained at recommended times [8].

Documentation

Quality control and assurance is based on an appropriate documentation system, organized in written or electronic form, containing any documents, standart operating procedures (SOPs) and records regarding any relevant step in the radiopharmaceutical preparation process to ensure traceability of the entire process. Written procedures should cover how to select each material (ingredients, containers and lids), the preparation process and all controls. The processes must cover the life of a material from receipt of the material to final consumption. Records should be kept accessible and readily available to any internal or external auditor within a reasonable time during the audit. Records must be kept for at least 1 year. However, archiving time must comply with local and national regulations [42].

Labeling

Labels can be computer-generated or handwritten. It is common practice to prepare most of the labeling ahead of time due to radiation exposure concerns. For example, an empty product bottle can be pre-labeled with partial information (product name, serial number, date) prior to infiltration of the radioactive product, and upon completion of the quality control test, the outer shielded box is labeled to contain the required information (radioactivity). A final check should be performed to verify that the correct and complete label is attached to the can and container [43].

RESULT AND DISCUSSION

Quality control tests for radiopharmaceuticals are specified in pharmacopoeias. Quality control procedures should be carried out at all operational levels and accurate and regular retrospective records should be kept. Quality assurance and quality products are obtained with calibrated and controlled devices, quality suitable materials, working order in which determined tests are performed by trained personnel at every stage and a properly planned laboratory according to GRP rules.

AUTHOR CONTRIBUTIONS

Conception: *M.E., R.S.O., D.İ.Ö.*; Design: *M.E., R.S.O., D.İ.Ö.*; Supervision: *R.S.O., D.İ.Ö.*; Resources: *M.E., R.S.O., D.İ.Ö.*; Materials: *M.E., R.S.O., D.İ.Ö.*; Data collection and/or processing: *M.E.*; Analysis and/or interpretation: *M.E.;* Literature search: *M.E.*; Writing manuscript: *M.E.*; Critical review: *M.E., R.S.O., D.İ.Ö.*; Other: -

CONFLICT OF INTEREST

The authors state that there are no actual, potential, or perceived conflicts of interest for this paper.

REFERENCES

1. Ekinci, M., İlem-Özdemir, D. (2021). Radyofarmasötikler ve teranostikler. *Journal of Literature Pharmacy Sciences, 10*(1), 119-132. [CrossRef]

2. Wadsak, W., Mitterhauser, M. (2010). Basics and principles of radiopharmaceuticals for PET/CT. *European Journal of Radiology, 73*(3), 461-469. [CrossRef]

3. Shukla, J., Vatsa, R., Garg, N., Bhusari, P., Watts, A., Mittal, B.R. (2013). Quality control of positron emission tomography radiopharmaceuticals: An institutional experience. *Indian Journal of Nuclear Medicine, 28*(4), 200-206. [CrossRef]

4. Guideline on Radiopharmaceuticals, CHMP, EMEA/CHMP/QWP/306970/2007 (draft released for consultation) Web site. (2007). From http://www.emea.europa.eu/pdfs/human/qwp/30697007en.pdf. Accessed: 17.01.2022

5. European Pharmacopoeia. (2009). Radiopharmaceutical Preparations, Online 8.5.

6. Saha, G.B. (2018). Nuclear Pharmacy. In: Fundamentals of Nuclear Pharmacy, 3rd ed, (pp. 185-202).

7. Bringhammar, T., Zolle, I. (2007). Quality Assurance of Radiopharmaceuticals. In: Zolle, I. (Ed.), Technetium-99m Pharmaceuticals: Preparation and Quality Control in Nuclear Medicine, (pp. 67-75). Springer-Verlag Berlin Heidelberg.

8. International Atomic Energy Agency Web site (2018). Quality control in the production of radiopharmaceuticals. From https://www-pub.iaea.org/MTCD/Publications/PDF/TE-1856web.pdf Accessed: 17.01.2021

9. Pauwels, E.K.J. (2005). Radioactivity radionuclides radiation. *European Journal of Nuclear Medicine and Molecular Imaging, 32*, 628. [CrossRef]

10. Kowalsky, R.J., Falen, S.W. (2013). Radiopharmaceuticals in Nuclear Pharmacy and Nuclear Medicine. American Pharmacists Association. APhA, 3rd ed, pp. 875.

11. Skuldt, D.H. (2020). Nuclear Medicine. In: Webster, J.G. (Ed.), Minimally Invasive Medical Technology, 1st ed, (pp. 334). CRC Press.

12. Biersack, H.J., Freeman, L.M., Zuckier, L.S., Grünwald, F. (2007). Clinical Nuclear Medicine. Springer Berlin Heidelberg, pp. 548.

13. Saha, G.B. (2006). Physics and Radiobiology of Nuclear Medicine. 3rd ed, Springer-Verlag New York, pp. 320.

14. Zanzonico, P. (2008). Routine quality control of clinical nuclear medicine instrumentation: A brief review. *Journal of Nuclear Medicine, 49*(7), 1114-1131. [CrossRef]

15. Zimmerman, B.E., Cessna, J.T. (2000). Experimental determinations of commercial "dose calibrator" settings for nuclides used in nuclear medicine. *Applied Radiation and Isotopes, 52*(3), 615-619. [CrossRef]

16. He, J., Tang, X., Gong, P., Wang, P., Wen, L., Huang, X., Han, Z., Yan, W., Gao, L. (2018). Rapid radionuclide identification algorithm based on the discrete cosine transform and BP neural network. *Annals of Nuclear Energy, 112*, 1-8. [CrossRef]

17. Neacsu, B., Cimpeanu, C., Barna, C. (2013). Radionuclidic purity - An essential parameter in quality control of radiopharmaceuticals. *Romanian Reports in Physics, 65*(1), 155-167.

18. L'Annunziata, M. (2003*).* Handbook of Radioactivity Analysis. 2nd ed, Academic Press, pp. 1326.

19. Dash, A., Knapp, F.F., Pillai, M.R.A. (2013). ^{99}Mo/^{99m}Tc separation: An assessment of technology options. *Nuclear Medicine and Biology, 40*(2), 167-176. [CrossRef]

20. Molavipordanjani, S., Tolmachev, V., Hosseinimehr, S.J. (2019). Basic and practical concepts of radiopharmaceutical purification methods. *Drug Discovery Today*, *24*(1), 315-324. [CrossRef]

21. Vallabhajosula, S., Killeen, R.P., Osborne, J.R. (2010). Altered biodistribution of radiopharmaceuticals: Role of radiochemical/pharmaceutical purity, physiological, and pharmacologic factors. *Seminars in Nuclear Medicine*, *40*(4), 220-241. [CrossRef]

22. Hage, D.S. (2018). Chromatography. In: Rifai, N., Horvath, A.R., Wittwer, C.T., Hoofnagle, A. (eds), Principles and Applications of Clinical Mass Spectrometry: Small Molecules, Peptides, and Pathogens, (pp. 1-32), Elsevier Inc.

23. Sherma, J. (2009). Thin layer radiochromatography. In: Cazes, J. (ed), Encyclopedia of Chromatography, 3rd ed, CRC Press.

24. Kagan, I.A., Flythe, M.D. (2014). Thin-Layer Chromatographic (TLC) separations and bioassays of plant extracts to identify antimicrobial compounds. *Journal of Visualized Experiments*, *85*, 51411. [CrossRef]

25. Ullah, H., Ahmad, I., Rauf Khattak, M., Shah, S., Ahmad, S., Khan, K., Ahmad, S.Z. (2019). Evaluation of radiochemical purities of routinely used radiopharmaceuticals: Three years' experience of a single institute. *Iranian Journal of Nuclear Medicine*, *27*(1), 19-25. [CrossRef]

26. Taşçı, C., Ülker, Ö., Ertay, T., Taner, M.S., Soylu, A., Berk, F., Erdoğan, S., Yürekli, Y., Aday, S., Ünak, P. (2003). Radyofarmasötikler için kalite kontrol yöntemleri kılavuzu. *Turkish Journal of Nuclear Medicine, 12,* 137-148.

27. Wernick, M.N., Aarsvold, J.N. (2004). Emission Tomography: The Fundamentals of PET and SPECT. Elsevier Academic Press, pp. 576.

28. Sandle, T. (2013). Sterility, sterilisation and sterility assurance for pharmaceuticals. Woodhead Publishing Series in Biomedicine. Woodhead Publishing, 1st ed, pp. 362.

29. Blechová, R., Pivodová, D. (2001). Limulus amoebocyte lysate (LAL) test - an alternative method for detection of bacterial endotoxins. *Acta Veterinaria Brno*, *70*(3), 291-296. [CrossRef]

30. Putra, A.R., Lestari, E., Kurniasih, D., Karyadi, K., Sarmini, E., Aries, A. (2019). Applicability of bacterial endotoxin test (BET) for some radiopharmaceutical sterile kits by the use of tachypleus amebocyte lysate (TAL). *Journal of Pharmaceutical Sciences and Community*, *16*(1), 20-28. [CrossRef]

31. Mattsson, S., Johansson, L., Leide Svegborn, S., Liniecki, J., Noßke, D., Riklund, K. Å., Stabin, M., Taylor, D., Bolch, W., Carlsson, S., Eckerman, K., Giussani, A., Söderberg, L., Valind, S., ICRP (2015). Radiation dose to patients from radiopharmaceuticals: a compendium of current information related to frequently used substances. *Annals of the ICRP*, *44*(2 Suppl), 7-321. [CrossRef]

32. Janković, D., Maksin, T., Djokić, D., Milonjić, S., Nikolić, N., Mirković, M., Vranjes-Djurić, S. (2008). Particle size analysis: ^{90}Y and ^{99m}Tc-labelled colloids. *Journal of Microscopy*, *232*(3), 601-604. [CrossRef]

33. Persico, M.G., Lodola, L., Buroni, F.E., Morandotti, M., Pallavicini, P., Aprile, C. (2015). ^{99m}Tc-human serum albumin nanocolloids: particle sizing and radioactivity distribution. *Journal of Labelled Compounds and Radiopharmaceuticals*, *58*(9), 376-382. [CrossRef]

34. Chourasia, M.K., Ashawat, M.S., Jain, N., Chalasani, K.B., Jain, R.K., Jain, A.K., Sabitha, M., Jain, S.K. (2003). Radiopharmaceuticals: Preparation, evaluation and applications. *Indian Journal of Pharmaceutical Sciences, 65*(5), 439-449.

35. International Atomic Energy Agency Web site. (2010). Competency based hospital radiopharmacy Training. Training Course Series No. 39. Vienna. From https://www-pub.iaea.org/MTCD/Publications/PDF/TCS-39_web.pdf. Accessed: 27.01.2021

36. Ertay, T., Yürekli, Y. (2017). Radyofarmasötiklerin kalite kontrolü. In: Ünak, P., Durmuş Altun, G., Teksöz, S., Biber Müftüler, Z. (eds), Uygulamalı Temel Radyofarmasi, (pp. 117-130). İstanbul: Nobel Tıp Kitabevleri.

37. International Atomic Energy Agency Web site. (2008). Operational guidance on hospital radiopharmacy: A safe and effective approach. Vienna, pp. 1-90. From https://www-pub.iaea.org/MTCD/publications/PDF/Pub1342/Pub1342_web.pdf. Accessed: 23.01.2022

38. Duatti, A., Bhonsle, U. (2013). Strengthening radiopharmacy practice in IAEA member states. *Seminars in Nuclear Medicine, 43*(3), 188-194. [CrossRef]

39. European Association of Nuclear Medicine Web site. (2010). Guidance on current good radiopharmacy practice (cGRPP) for the small-scale preparation of radiopharmaceuticals. EANM Radiopharmacy Committee, pp. 1–14. From https://eanm.org/publications/guidelines/5_EJNMMI_Guidance_cGRPPfulltext_05_2010.pdf. Accessed: 16.01.2021

40. Norenberg, J.P., Petry, N.A., Schwarz, S. (2010). Operation of a radiopharmacy for a clinical trial. *Seminars in Nuclear Medicine, 40*(5), 347-356. [CrossRef]

41. Grecco, C.H.S., Vidal, M.C.R., Cosenza, C.A.N., Santos, I.J.A.L., Carvalho, P.V.R. (2012). New approach for safety management in radiopharmaceutical production facilities. *Latin American Journal of Pharmacy, 31*(8), 1199-1202.

42. de Blois, E., de Zanger, R.M.S., Chan, H.S., Konijnenberg, M., Breeman, W.A.P. (2019). Radiochemical and analytical aspects of inter-institutional quality control measurements on radiopharmaceuticals. *EJNMMI Radiopharmacy and Chemistry, 4*(3), 1-13. [CrossRef]

43. Taş, A., Özer, A.Y. (2020). Waste disposal and management in radiopharmaceuticals. *FABAD Journal of Pharmaceutical Sciences, 45*(1), 91-103.

Yayım Koşulları

1. Ankara Üniversitesi Eczacılık Fakültesi Dergisi (Ankara Ecz. Fak. Derg. – J. Fac. Pharm. Ankara) yılda üç kez (Ocak-Mayıs-Eylül) yayımlanır.

2. Dergiye Eczacılığın her alanında daha önce hiç bir yerde yayınlanmamış, Türkçe veya yabancı dilde (İngilizce, Fransızca, İspanyolca ve Almanca) olarak hazırlanmış makaleler kabul edilir. Deneylerde, insan için "the Declaration of Helsinki" ve hayvan için "European Community Guidlines"'a bağlı kalınmalıdır.

3. Yayın Komisyonuna gelen makaleler en az 2 danışmana gönderilir.

4. Makaleler yayına kabul ediliş sırasına göre yayınlanır.

5. Danışmanlar tarafından önerilen düzeltmelerin yapılması için yazar/ yazarlara geri gönderilen makaleler, düzeltilip yayınlanmak üzere 3 ay içinde tekrar yayın kuruluna gönderilmezse, yeni başvuru olarak işlem görür. Makale yayımlandan önce yazarların yayımcıya makalenin "Copyright Transfer Form'unu doldurarak telif hakkını göndermesi gerekmektedir.

6. Yayımlarda intihal olup olmadığı kontrol edilmelidir.

7. Dergimize aşağıdaki makale türleri kabul edilir:

    a) **Araştırma makalesi**: Türkçe veya ingilizce hazırlanmış, şekiller ve tablolar dahil tamamı en çok 20 A4 kağıdı sayfası olan, orjinal araştırmaların bulgu ve sonuçlarını açıklayan makalelerdir.
    b) **Derleme:** Türkçe veya ingilizce hazırlanmış, şekil ve tablolar dahil tamamı en çok 25 A4 kağıdı sayfası olan, yeterli sayıda bilimsel makale taranarak, o güne kadarki gelişmeleri özetleyerek ortaya koyan ve sonuçlarını yorumlayarak değerlendiren makalelerdir.
    c) **Önbilgiler:** Devam etmekte olan bir çalışmanın bulgularını zaman kaybetmeden duyurmak için Türkçe veya ingilizce yazılan en çok 5 A4 kağıdı sayfası olan makalelerdir.

Yazım Kuralları

1. Metinler, A4 normunda (21 x 29,7 cm) yazılmış olmalıdır.
2. Metinler A4 normundaki sayfanın sağ ve sol tarafından 2,5 cm., üst ve alt kenarlarından 3 cm. boşluk bırakılarak (ilk sayfada yukarıdan 5 satır aralığı) 1,5 satır aralıkla yazılmalıdır. Yayımı kabul edilen makaleler doğrudan "Microsoft Word" dosyası halinde online olarak sisteme yüklenecektir (online submission). Ana metin yazı karakteri "**Times New Roman**" ve **11 punto** olmalıdır.
3. Sayfa numaraları makalede **belirtilmemelidir**.
4. Paragraf başları **1 cm içeriden** başlamalıdır. Paragraflar arası ilave boşluk bırakılmamalıdır.
5. Başlık sayfasında yayın adı, yazar/yazarların adları ve yazışma yapılacak yazarın açık adresi, telefon ve faks numaraları, varsa e-mail adresi belirtilmeli ve ortalı yazılmalıdır. Sorumlu yazarın soyadının üstüne (*) işareti konularak belirtilmelidir. Bu kişinin Adı Soyadı, açık adresi, faks numarası, telefon numarası ve e-mail adresi başlık sayfasının en altında belirtilmelidir.
6. **Yazar Adı** (ilk harfi büyük diğerleri **küçük harf**) ve **SOYADI** (tamamı **büyük harf**) **koyu** olarak başlığın altına üç satır aralık verildikten sonra altına unvan belirtmeden yazılmalıdır. Birden çok yazar varsa virgülle ayrılıp bir boşluk bırakılarak yazılmalıdır. Yazarların soyadları üzerine konulacak rakamlarla hemen isimlerin altındaki satıra kurum adları ve posta adresleri (Örneğin: Ankara Üniversitesi Eczacılık Fakültesi, Farmasötik Kimya Anabilim Dalı, 06100, Ankara, Türkiye) açıkça yazılmalıdır.
    - **Tüm yazarlar için ORCID numarası** mutlaka beyan edilmelidir. Yazarların ORCID ID'leri ilgili logoya köprü oluşturularak URL linklerinin eklenmesiyle gerçekleştirilmelidir.
7. Uluslararası kısaltmalar kullanılabilir. Metin içinde mililitre için ml; dakika için dak. olarak belirtilen şekliyle yazılmalıdır.
8. Birimler metrik sistemi kullanılarak ifade edilmelidir.
9. Bütün tablo ve şekiller metin içindeki yerlerine yazım alanından taşmadan yerleştirilmiş olmalıdır.
10. Tablolar üstlerine, şekiller (formül, grafik, şema, spektrum, kromatogram, fotoğraf vb.) de altlarına arabik rakamlarla (**Şekil 1**., **Tablo 2**.,) numaralandırılmalı ve metin içinde yer verilmelidir. "Tablo", "Şekil" sözcükleri ile bunlara ait numaralar koyu yazılmalı ve 11 punto olmalıdır. Şekil/Resim (JPG formatında) makale içinde yerleşmiş ve resimler yüksek çözünürlükte olmalıdır. En yüksek baskı kalitesini sağlamak için şekilleriniz mümkün olan en yüksek çözünürlükte (300 dpi veya daha yüksek) JPEG formatında gönderilmelidir. Üzerinde oynanmış (parlaklık, kontrast, gama ayarı vb.) şekillerde şekil altı metninde yapılan ayarlar belirtilmelidir. Yazarlar, önceki makalelerinden alıntılanmış olsalar bile, diğer kaynaklardan herhangi bir görüntüyü çoğaltmak için ilgili yayıncılardan yazılı izin almalıdır.
11. **Tablo** başlıkları Tabloların üstüne ve iki yana yaslı ve bunların genişliğini aşmayacak şekilde 11 punto yazılmalıdır. Tabloya ait açıklama varsa tablonun altına boşluk bırakmadan 9 punto ile yazılmalıdır. Tablo içindeki metin 8-11 punto arasında yazılabilir. **Şekil** başlıkları ise Şekillerin altına birer satır aralıkla ortalı ve 11 punto yazılmalıdır. Tablo ve Şekiller metin içine yerleştirilirken metin ile aralarında net ayırımı sağlayacak kadar boşluk bırakılmalıdır.

    Örnek tablolar için bakınız.
    - Tüm satır ve sütun çizgileri yer almalı.
    - Tablo tasarımı tüm makalede tek tip ve düz olmalı, herhangi bir renklendirme/gölgelendirme kullanılmamalıdır.
    - Tablo içinde yer alan başlıklar **bold/koyu** renkte yazılmalıdır.

Tablo 1. Türlere ait morfolojik özellikler

Bitki kısmı*	***C. nummularia***	***C. integerrimus***
Yaprak	Genişçe eliptik-orbikular, 0.9-2.5-(4) x 0.5-2.5-(3-5) cm	Orbikulardan ovata kadar farklı şekillerde, 1.2-(4-5) x 0.9-3 cm
Tohum	3.5-4 x 1-2 mm, koyu kahverengi	3-4 x 1.5-2 mm, açık kahverengi

*Açıklama: 9 punto, 1 aralık olmalı. Açıklama ile tablo bitiminde boşluk bırakılmamalıdır.

Tablo 2. Hastaların özellikleri

Demografik bilgiler	**A grubu***	**B grubu**	**C grubu**
Erkek cinsiyet	10 (%30)	20 (%60)	10 (% 30)
Sigara kullanımı	20 (%60)	10 (%30)	20 (%60)

*Açıklama: 9 punto yazılmalıdır.

Örnek şekil;

Şekil 1. *C. nummularia*'nın genel görünüşü

12. Makalelerin bölümleri **BAŞLIK** (Türkçe ve İngilizce), **ÖZ**, **ABSTRACT**, **GİRİŞ, GEREÇ VE YÖNTEM, SONUÇ VE TARTIŞMA, TEŞEKKÜR** (varsa eklenmeli), **YAZAR KATKILARI**, **ÇIKAR ÇATIŞMASI**, **ETİK KURUL ONAYI** (varsa eklenmeli) ve **KAYNAKLAR** sırasına uygun olarak hazırlanmalıdır. Bu bölümler birbirlerinden 2 satır aralık ile ayrılmalıdır. Bu bölümleri ifade eden başlıklar (Makalenin ilk başlığı hariç) **12 punto ile koyu olarak büyük harflerle ve sayfanın solundan başlanarak** yazılmalıdır. Ana başlıklardan önce ve sonra sırasıyla 18 nk ve 6 nk aralık olmalıdır. Bölüm başlıkları ile metin arasında belirtilenin dışında ayrıca aralık **bırakılmamalıdır.**

- **BAŞLIK:** Türkçe ve İngilizce olarak büyük harf ve **ilk başlık** (Türkçe makalelerde Türkçe başlık, İngilizce makalelerde İngilizce başlık ilk başlıktır) **14 punto, koyu** ve ikinci başlık 12 punto, *italik* olarak yazılmalıdır. Başlık metine uygun, kısa, çalışmayı tanıtıcı ve açık ifadeli olmalıdır.

- **ÖZ** ve **ABSTRACT:** Türkçe (**ÖZ**) ve İngilizce (**ABSTRACT**) olarak makalelerin başında 200'er kelimeyi geçmeyecek şekilde 10 punto ile *italik* olarak ve **çerçeve** içinde yazılmalıdır. Yabancı dilde yazılmış makalelerde önce **ABSTRACT** daha sonra mutlaka Türkçe olarak **ÖZ** bulunmalıdır. **ÖZ** ve **ABSTRACT** başlıkları 12 punto ve koyu yazılıp kendi içlerinde alt başlıklar halinde makalenin özeti sunulmalıdır. Her bir alt başlık 10 punto, koyu, normal ve 1

cm içerden başlayarak yazılmalıdır. **ÖZ** ve **ABSTRACT** 16 cm genişliğindeki kutucukta yer almalıdır.

Özgün makalelerde;

ÖZ için kullanılacak alt başlıklar:
Amaç: *Metin italik yazılmalıdır.*
Gereç ve Yöntem: *Metin italik yazılmalıdır.*
Sonuç ve Tartışma: *Metin italik yazılmalıdır.*
Anahtar Kelimeler: *Metin italik yazılmalıdır, alfabetik sıralama gözetilmelidir*

ABSTRACT için kullanılacak alt başlıklar:
Objective: *Metin italik yazılmalıdır.*
Material and Method: *Metin italik yazılmalıdır.*
Result and Discussion: *Metin italik yazılmalıdır.*
Keywords: *Metin italik yazılmalıdır, alfabetik sıralama gözetilmelidir*

Derleme makalelerde;

ÖZ için kullanılacak alt başlıklar:
Amaç: *Metin italik yazılmalıdır.*
Sonuç ve Tartışma: *Metin italik yazılmalıdır.*
Anahtar Kelimeler: *Metin italik yazılmalıdır, alfabetik sıralama gözetilmelidir*

ABSTRACT için kullanılacak alt başlıklar:
Objective: *Metin italik yazılmalıdır.*
Result and Discussion: *Metin italik yazılmalıdır.*
Keywords: *Metin italik yazılmalıdır, alfabetik sıralama gözetilmelidir*

- **Anahtar Kelimeler (Keywords):** En fazla 5 sözcükten oluşmalı, ilgili dilde alfabetik, *italik* olarak, yalnızca ilk anahtar sözcüğün ilk harfi büyük olacak şekilde (büyük harf kullanılarak yapılan kısaltmalar hariç) aralara virgül konularak yazılmalı son anahtar sözcükten sonra ise bir imla işareti **kullanılmamalıdır.**

- **METİN:** Orijinal türkçe makalede metin kısmı **GİRİŞ, GEREÇ VE YÖNTEM, SONUÇ VE TARTIŞMA** olmak üzere 3 ana başlıktan oluşmalıdır. Bu ana başlıkların tamamı 12 punto, **büyük harflerle** ve koyu olacak şekilde yazılmalıdır. Derleme makalelerde ise **GİRİŞ** ile **SONUÇ VE TARTIŞMA ana başlıkları olmalı,** diğer başlıklar yazarın belirleyeceği şekilde her kelimenin ilk harfi büyük diğerleri küçük ve koyu olacak şekilde yazılmalıdır. Alt başlıklar 11 punto, 1,5 satır aralık, **bold/koyu** yazılmalı ve sola dayalı olmalıdır Alt başlıklarda numaralandırma sistemi **kullanılmamalıdır**.

- **GİRİŞ:** Araştırmanın amacı ve konuyla ilgili çalışmaların yer aldığı bölüm olmalıdır.

- **GEREÇ VE YÖNTEM**: Kullanılan gereç belirtilerek, uygulanan yöntem hakkında gerekli bilgiler açıkça ifade edilmelidir. **Bileşiklerin karakterizasyonu** ayrı bir paragraf ile gösterilmeli ve yeni bileşiklerin saflıkları ve yapı aydınlatılmaları sağlanmalıdır. Eğer çalışmada hayvan ya da insan örnekleri/gönüllüler kullanılıyorsa, araştırıcılar tüm işlemlerin ilgili kanun ve kurumsal kılavuzlara uygun şekilde gerçekleştirildiğine ve uygun idari kurul tarafından bu işlemlerin onaylandığına ve Etik Kurul onayı alındığına dair ifadenin çalışma içinde yer almasını sağlamalıdırlar. Etik Kurul onayının zorunlu olduğu çalışmalarda, etik kurul onayı alınan kurumun adı ve etik kurul onay numarası, gereç ve yöntem kısmında belirtilmelidir. Ayrıca, kullanılan protokol ve prosedürlerin etik olarak gözden geçirildiği ve onaylandığı, makalenin gereç ve yöntem bölümüne eklenmelidir. Detaylı bilgi için lütfen http://journal.pharmacy.ankara.edu.tr/en/ethical-principles-and-publication-policy/ web sayfasını ziyaret ediniz.

- **SONUÇ VE TARTIŞMA:** Bulguların verilerek değerlendirildiği bölümdür.
  - Metinde sonuçlar (conclusions) başlığı yer **almamalıdır.**

- **TEŞEKKÜR:** Varsa araştırmayı destekleyen kuruluşa ve katkısı olan kişilere Yazarların Katkısından önce yer alan bu bölümde kısaca teşekkür edilebilir.

- **YAZAR KATKILARI:** Makalede yer alan yazarların katkısı yazarlar tarafından imzalanan Telif Hakkı Devir Sözleşmesi (*Copyright Transfer Agreement*) uyarınca, çıkar çatışması bildiriminden hemen önce, makalede yer alan isim sırası gözetilerek yazılmalıdır. Lütfen bu bildirim için açık ad ve soyad yerine aşağıdaki örnekte olduğu gibi yazarların baş harflerini kullanınız.

  Örnek:

  YAZAR KATKILARI

  Kavram: *İ.Y., M.M.H., C.H., K.B.*; Tasarım: *İ.Y., C.H., I.Ö.G., Ö.Ü.*; Denetim: *C.H., I.Ö.G., M.M.H., K.B.*; Kaynaklar: *Ö.Ü., Z.K., K.B., M.M.H., A.K., İ.A., G.A.G., B.G., B.K.*; Malzemeler: *I.Ö.G., B.E., G.A.G., B.K., D.Ç.P.*; Veri Toplama ve/veya İşleme: *A.K., Ö.Ü., M.K., A.S., D.Ç.P., T.C.Ş.T.*; Analiz ve/veya Yorumlama: *Ö.Ü., B.G., T.C.Ş.T., E.K.S.*; Literatür Taraması: *B.K., D.Ç.P, B.G., B.E.*; Makalenin Yazılması: *A.K., İ.A. , T.C.Ş.T.*; Kritik İnceleme: *İ.Y., B.G., Ö.Ü., İ.A.*; Diğer: *İ.Y., A.K.*

- **ÇIKAR ÇATIŞMASI BEYANI**

  Çıkar çatışması varsa ne şekilde olduğu açıkça beyan edilmelidir. Eğer yok ise "Yazarlar bu makale için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler." ifadesini kullanmalıdırlar.

- **ETİK KURUL ONAYI**

  Çalışmanın sonunda kaynaklardan önce etik kurul onayı alınmışsa hangi kurumdan ve ne zaman alındığı onay numarası ile mutlaka belirtilmelidir. Etik kurul onayına gerek olmayan çalışmalarda aşağıdaki cümle yazılmalıdır.

  "Yazarlar bu çalışma için etik kurul onayının zorunlu olmadığını beyan etmektedir."

- **KAYNAKLAR:** Kaynak yazım stili Amerikan Psikoloji Derneği'ne (APA) göredir. Yazı karakteri "Times New Roman" ve 11 punto, "1" aralık, iki yana yaslı. Metinde, geçiş sırasına göre köşeli parantez içinde, örneğin: [1,2,…] gibi numaralandırılmalı ve metin sonunda bu numaralara göre sıralanmalıdır. Alt başlıkların yanına kaynak belirtilmemelidir. Tablo içinde kaynak bildirilmesi gerekiyorsa metin içinde verildiği gibi belirtilmelidir. Kaynaklar aşağıdaki örneklere uygun olarak **aralarında 1 satır boşluk bırakılarak yazılmalıdır**.

  - **Makale için:** Yazarın soyadı, adının baş harfleri (Birden fazla adı olan yazarın her bir isminin baş harfinden sonra nokta konmalı ve arada boşluk bırakılmamalıdır. Birden fazla yazarların arasında virgül yer almalıdır. **Son yazar ile bir önceki yazar arasında "ve" kelimesi veya "&" sembolü kullanılmamalıdır**.), makalenin tam başlığı *derginin adı* (italik), *cilt no* (italik), varsa sayı no (parantez içinde), başlangıç ve bitiş sayfa numarası, yıl yazar isimlerinden sonra (parantez içinde) yazılmalıdır. **Birden fazla yazar varsa hepsi yazılmalıdır.** Makalenin adı yazılırken ilk kelimenin ilk harfi büyük diğer kelimelerin ilk harfi küçük yazılmalıdır. Kaynaklarda verilen **dergi adları kısaltma yapılmadan açık olarak yazılmalıdır.**

Her bir referansın sonuna [CrossRef] **ekleyerek aşağıdaki formatta DOI numarasını köprü olarak giriniz.**
https://doi.org/10.1016/0006-2952(89)90403-6

Örnekler:

Moncada, S., Palmer, R.M.J., Higgs, E.A. (1989). Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication. *Biochemistry and Pharmacology*, *38*(11), 1709 – 1715. [CrossRef]

Macedo, T., Ribeiro, V., Oliveira, A.P., Pereira, D.M., Fernandes, F., Gomes, N.G.M., Andrade, P.B. (2020). Anti-inflammatory properties of Xylopia aethiopica leaves: Interference with pro-inflammatory cytokines in THP-1-derived macrophages and flavonoid profiling. *Journal of Ethnopharmacology*, *248*, 112312. [CrossRef]

- **Elektronik Makale için:**

Örnek:
Perneger, T.V., Giner, F. (1998). Randomized trial of heroin maintenance programme for adults who fail in convential drug treatments. *British Medical Journal, 317*, from http://www.bmj.com/cgi/content/full/317/7150/ Erişim tarihi: 14.03.2021

- **Web sitesi için:**

Örnek:
Clinical Pharmacology Web site. (2001). From http://cpip.gsm.com/ Erişim tarihi: 14.03.2021.

- **Kitap için:** Yazarın soyadı, adının baş harfleri, kitabın adı, cilt no (varsa), kitabevi, yayınlandığı şehir, sayfa no, basıldığı yıl (parantez içinde) yazılmalıdır.

Örnek:
Franke, R. (1984). Theoretical Drug Design Methods, Elsevier, Amsterdam, p.130.

- **Kitap bölümü için:** Yazarın soyadı, adının baş harfleri, bölümün başlığı, editör/editörlerin soyadı, adının baş harfleri, (Ed./Eds.) ibaresi, kitabın adı, varsa cilt no, kitabevi, yayınlandığı şehir, sayfa no, basıldığı yıl (parantez içinde) yazılmalıdır.

Örnek:
Weinberg, E.D. (1979). Antifungal Agents. In: M.E. Wolff and S.E. Smith (Eds.), Burger's Medicinal Chemistry, (pp. 531-537). New York: John Wiley and Sons.

- **Tez için:** Yazarın soyadı, adının baş harfleri, yıl yazar isimlerinden sonra (parantez içinde) yazılıp nokta işareti konmalıdır. Ne tür tez olduğu belirtildikten sonra tezin başlığı, nerde yapıldığı yazılmalıdır.

Örnek:

Ahmed, J. (2008). PhD Thesis. Pharmaceutical Botany investigations on *Prangos* Lindl. (Umbelliferae) growing in Konya province. Department of Pharmaceutical Botany, Faculty of Pharmacy, Ankara University, Ankara, Turkey.

Guide for Authors

1. The Journal of Faculty of Pharmacy of Ankara University (J. Fac. Pharm. Ankara) is published three times (January-May-September) a year.

2. The Journal of Faculty of Pharmacy of Ankara University publishes articles in every field of Pharmaceutical Sciences. The manuscript to the journal should not be published previously as a whole or in part and not be submitted elsewhere. Manuscript should be written in Turkish or in a foreign language (English, French, Spanish or German). The experiments used have to be adhered to the Declaration of Helsinki for humans and European Community Guidlines for animals.

3. All manuscripts will be submitted to a review process by the editors and by qualified at least 2 outside reviewers.

4. Manuscripts are published in order of final acceptance after review and revision.

5. If a manuscript returned to the authors for revision is not received back to the editor within 3 months it will be treated as a new article. When the article is published, the by authors are considered to transfer all rights of the manuscript to the Publisher.

6. Manuscript will be controlled using plagiarism checker.

7. Manuscripts with the following charactheristics are accepted:

    a) **Research article**: Articles written in English or Turkish in scientific format presenting original research. Articles should be printed on A4 size papers not exceeding 20 pages (including tables and figures).
    b) **Review:** An updated comprehensive review of scientific works on a particular subject. Articles written in English or Turkish should be printed on A4 size papers not exceeding 25 pages (including tables and figures).
    c) **Rapid communication:** Rapid announcement of the results of a continuing research written in English or Turkish, no longer than 5, A4 size pages.

Preparation of Manuscript

1. Texts must be written in A4 norm (21 x 29.7 cm).
2. Texts should be written with 1.5 line spacing, with 2.5 cm margins on the left and right sides of the A4 norm page, 3 cm margins each from the top and bottom edges (5 line spacing from the top on the first page). Articles accepted for publication will be directly uploaded to the system as a "Microsoft Word" file (online submission). The main text font should be **"Times New Roman"** and **11 pt**.
3. Page numbers **should not be specified** in the article.
4. Paragraph headings must **begin 1 cm inside**. Additional spaces should not be left between paragraphs.
5. On the title page, the name of the publication, the names of the author / authors, the full address of the correspondent author, telephone and fax numbers, e-mail address, if available, should be written and centered. It should be indicated by placing (*) above the surname of the corresponding author. Name, surname, full address, fax number, telephone number and e-mail address of this person should be specified at the bottom of the title page.
6. **Author's Name** (**first letter capital, others lowercase**) and **SURNAME** (**all capital letters**) should be written in bold, three lines spaced under the title, and without a title underneath. If there is more than one author, they should be written by separating them with a comma and leaving a space. The numbers to be placed on the surnames of the authors and the institution names and postal addresses (For example: Ankara University Faculty of Pharmacy, Department of Pharmaceutical Chemistry, 06100, Ankara, Turkey) should be clearly written on the line just below the names.
    - **ORCID ID number must be declared for all authors**. ORCID IDs of the authors should be created by creating a hyperlink to the relevant logo and adding URL links.
7. International abbreviations may be used. ml for milliliter in the text; min. for minutes It should be written as specified.
8. Units should be expressed using the metric system.
9. All tables and figures should be placed in their places in the text without exceeding the writing area.
10. Tables should be numbered on the top, figures (formula, graph, chart, spectrum, chromatogram, photograph, etc.) and Arabic numbers (**Figure 1., Table 2.)** below them and should be included in the text. The words "Table", "Figure" and their numbers should be written in bold and in 11 pt. Figure / Picture (in JPG format) must be placed in the article and pictures must be in high resolution (300 dpi or higher). Authors must obtain written permission to reproduce any images from other sources.
11. **Table** titles should be written in 11 font size justified on the top of the tables and not exceeding their width. If there is an explanation for the table, it should be written in 9 font size without leaving any space at the bottom of the table. The text in the table can be written between 8-11 points. **Figure titles** should be written at the bottom of the figures with a line spacing, centered and 11 pt. While placing tables and figures in the text, enough space should be left to provide clear separation from the text.

    See for below examples for tables:
    - All row and column lines should be included.
    - Table design should be uniform and straight throughout the article, no coloring / shading should be used.
    - Headings in the table should be written in **bold**.

Table 1. Morphological characteristics of the species

Plant part*	***C. nummularia***	***C. integerrimus***
Leaf	Broadly elliptical-orbicular, 0.9-2.5-(4) x 0.5-2.5-(3-5) cm	From orbicular to ovate, 1.2-(4-5) x 0.9-3 cm,
Seed	3.5-4 x 1-2 mm, dark brown	3-4 x 1.5-2 mm, light brown

* Explanation should be 9 font size, 1 range. There should not be any space between end of the table and the explanation.

Table 2. Patient demographics

Demographics	**Group A***	**Group B**	**Group C**
Male gender	10 (%30)	20 (%60)	10 (% 30)
Cigarette consumption	20 (%60)	10 (%30)	20 (%60)

* Explanation should be 9 font size, 1 range. There should not be any space between the end of the table and the explanation.

Example for figure:

Figure 1. General view of *C. nummularia*

12. The sections of the articles should be prepared in accordance with the **TITLE** (Turkish and English), **ABSTRACT, INTRODUCTION, MATERIAL AND METHOD, RESULT AND DISCUSSION, ACKNOWLEDGEMENTS** (if available), **AUTHOR CONTRIBUTIONS, CONFLICT OF INTEREST, ETHICS COMMITTEE APPROVAL** (if available) and **REFERENCES**. These sections should be separated from each other by 2 line spacing. Titles expressing these sections (except the first title of the article) should be written in **12 pt**, **bold capital letters and starting from the left of the page**. There should be 18 nk and 6 nk intervals, respectively, before and after the main headings. Between the chapter titles and the text, a separate space **should not be left** other than the specified one.

- **TITLE:** Capital letters and **first title** in Turkish and English (Turkish title is the first title in Turkish articles, English title is the first title in English articles), **14 pt, bold** and the second title should be written in 12 pt, *italic*. The title should be appropriate to the text, short, introducing the work and clearly worded.

- **ABSTRACT** and **ÖZ:** It should be written in English (**ABSTRACT**) and Turkish (**ÖZ**) at the beginning of the articles, not exceeding 200 words, 10 pt, *italic* and within a frame. In articles written in a foreign language, first **ABSTRACT** and then **ÖZ** in Turkish. **ABSTRACT** and **ÖZ** titles should be written in 12 pt. And bold and the summary of the article should be presented as subheadings. Each subtitle should be written in 10 pt, bold, normal and 1 cm indented. **ABSTRACT** and **ÖZ** should be placed in the box of 16 cm width.

For original articles;

Subheadings to be used for **ABSTRACT**:

Objective: *Text should be written in italic.*
Material and Method: *Text should be written in italic.*
Result and Discussion: *Text should be written in italic.*
Keywords:

Subheadings to be used for **ÖZ**:

Amaç: *Text should be written in italic.*
Gereç ve Yöntem: *Text should be written in italic.*
Sonuç ve Tartışma: *Text should be written in italic.*
Anahtar Kelimeler: *Text should be written in italic.*

For review articles;

Subheadings to be used for **ABSTRACT**:

Objective: *Text should be written in italic.*
Result and Discussion: *Text should be written in italic.*
Keywords:

Subheadings to be used for **ÖZ**:

Amaç: *Text should be written in italic.*
Sonuç ve Tartışma: *Text should be written in italic.*
Anahtar Kelimeler:

- **Keywords (Anahtar Kelimeler)**: It should consist of a maximum of 5 words, should be written alphabetically, italic in the relevant language, with only the first letter of the first keyword capitalized (except for abbreviations using capital letters) with commas between them and a spelling mark **should not be** used after the last keyword.

- **TEXT:** The text part of the original Turkish article should consist of 3 main headings: **INTRODUCTION, MATERIAL AND METHOD, RESULT AND DISCUSSION**. All of these main headings should be written in 12 pt, **capital letters** and bold. In review articles, there should be the main headings of **INTRODUCTION** and **CONCLUSION AND DISCUSSION**, other titles should be written with the first letter of each word capital, the others in lowercase and bold, as determined by the author. Subheadings should be written in 11 font size, 1.5 line spacing, **bold** and aligned to the left. Numbering system **should not be** used in subheadings.

- **INTRODUCTION:** There should be a section containing the purpose of the research and studies on the subject.

- **MATERIAL AND METHOD:** Required information about the method should be clearly stated by indicating the material used. **Characterization of compounds** should be shown in a separate paragraph and clarification of the purity and structure of the new compounds should be provided. If animal or human samples / volunteers are used in the study, researchers should ensure that a statement stating that all procedures are carried out in accordance with the relevant laws and institutional guidelines and that these procedures have been approved by the appropriate administrative committee and that the approval of the Ethics Committee is included in the study. In studies for which Ethics Committee approval is mandatory, the name of the institution for which the ethics committee approval was obtained and the ethics committee approval number should be specified in the materials and methods section. It should also be included in the materials and methods section of the article that the protocols and procedures used are ethically reviewed and approved. For detailed information, please visit http://journal.pharmacy.ankara.edu.tr/en/ethical-principles-and-publication-policy/ web page.

- **RESULT AND DISCUSSION:** This is the section where findings are given and evaluated.
    - The title of conclusions **should not be** included in the text.

- **ACKNOWLEDGMENTS**: If any, the organization supporting the research and the people who contributed can be acknowledged briefly in this section prior to the Authors' Contribution.

- **AUTHOR CONTRIBUTIONS:** Contribution of the authors in the article should be written just before the conflict of interest notification, in accordance with the *Copyright Transfer Agreement* signed by the authors. Please use the initials of the authors for this notice instead of the full name and surname as in the example below.

    Example:

    AUTHOR CONTRIBUTIONS

    Concept: *İ.Y., M.M.H., C.H., K.B.*; Design: *İ.Y., C.H., I.Ö.G., Ö.Ü.*; Control: *C.H., I.Ö.G., M.M.H., K.B.*; Sources: *Ö.Ü., Z.K., K.B., M.M.H., A.K., İ.A., G.A.G., B.G., B.K.*; Materials: *I.Ö.G., B.E., G.A.G., B.K., D.Ç.P.*; Data Collection and / or Processing: *A.K., Ö.Ü., M.K., A.S., D.Ç.P., T.C.Ş.T.*; Analysis and / or Interpretation: *Ö.Ü., B.G., T.C.Ş.T., E.K.S.*; Literature Review*: B.K., D.Ç.P, B.G., B.E.*; Manuscript Writing: *A.K., İ.A. , T.C.Ş.T.*; Critical Review: *İ.Y., B.G., Ö.Ü., İ.A.*; Other: *İ.Y., A.K.*

- **CONFLICT OF INTEREST**

    If there is a conflict of interest, it should be clearly declared in what form it is. If not, "The authors declare that there is no real, potential, or perceived conflict of interest for this article." They should use the expression.

- **ETHICS COMMITTEE APPROVAL**

    If the ethics committee approval is obtained before the sources at the end of the study, the approval number must be specified from which institution and when it was obtained. In studies that do not require ethics committee approval, the following sentence should be written.

    "The authors declare that the ethics committee approval is not required for this study".

- **REFERENCES:** Bibliography style is according to the American Psychological Association (APA). Typeface "Times New Roman" and 11 font size, "1" spacing, justified. In the text, it should be numbered in square brackets according to the order of appearance, such as: [1,2,…] and listed according to these numbers at the end of the text. Reference should not be given next to the subtitles. If it is necessary to provide a source in the table, it should be specified as given in the text. References should be written in accordance with the examples below, **with 1 line space between them**.

    - **For the article:** Author's surname, the initials of the name (There should be a period after the initial letter of each name of the author with more than one name, and there should not be a space in between. **There should not be "and" between the last author and the previous author. The "&" symbol should not be used**. The full title of the article should be written as the *name of the journal (italic*), *volume number (italic*), if available, the number (in brackets), the beginning and ending page number, the year after the author names (in parentheses). **If there is more than one author, all of them should be written**. While writing the name of the article, the first letter of the first word should be capitalized and the first letter of the other words should be written in

lowercase. Journal names given in references should be written clearly without abbreviation.

Add the [CrossRef] **sign at the end of each reference and enter the DOI number as a hyperlink with the right click in the format below.**
https://doi.org/10.1016/0006-2952(89)90403-6

Examples:
Moncada, S., Palmer, R.M.J., Higgs, E.A. (1989). Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication. *Biochemistry and Pharmacology*, *38*(11), 1709 – 1715. [CrossRef]

Macedo, T., Ribeiro, V., Oliveira, A.P., Pereira, D.M., Fernandes, F., Gomes, N.G.M., Andrade, P.B. (2020). Anti-inflammatory properties of Xylopia aethiopica leaves: Interference with pro-inflammatory cytokines in THP-1-derived macrophages and flavonoid profiling. *Journal of Ethnopharmacology*, *248*, 112312. [CrossRef]

- **Online articles:**

Example:
Perneger, T.V., Giner, F. (1998). Randomized trial of heroin maintenance programme for adults who fail in convential drug treatments. *British Medical Journal*, *317*, from ttp://www.bmj.com/cgi/content/full/317/7150/ Retrieved August 12, 2005

- **Web sites:**

Example:
Clinical Pharmacology Web site. (2001). Retrieved June 16, 2004, from http://cpip.gsm.com/. Accessed date 14.03.2021.

- **Books:** The surname of the author, the initials of the name, the name of the book, volume number (if any), the bookstore, the city where it was published, the page number, the year it was published (in parentheses) should be written.

Example:
Franke, R. (1984). Theoretical Drug Design Methods, Elsevier, Amsterdam, p.130.

- **Book chapters:** Author's surname, initials of the name, the title of the section, the editor / editors' surname, the initials of the name, the phrase (Ed./Eds.), The title of the book, if any, the book house, the city where it was published, the page number, the year it was published (in parentheses) should be written.

Example:
Weinberg, E.D. (1979). Antifungal Agents. In: M.E. Wolff and S.E. Smith (Eds.), Burger's Medicinal Chemistry, (pp. 531-537). New York: John Wiley and Sons.

- **For the thesis**: The surname of the author, the initials of the name, the year should be written (in parentheses) after the author's names and a full stop. After specifying the type of thesis, the title of the thesis and where it was made should be written.

Example:
Ahmed, J. (2008). PhD Thesis. Pharmaceutical Botany investigations on Prangos Lindl. (Umbelliferae) growing in Konya province. Department of Pharmaceutical Botany, Faculty of Pharmacy, Ankara University, Ankara, Turkey.

ANKARA ÜNİVERSİTESİ ECZACILIK FAKÜLTESİ DERGİSİ

YAYIN SAHİBİNİN ADI : Prof. Dr. Asuman BOZKIR
SORUMLU YAZI İŞLERİ MÜDÜR ADI : Prof. Dr. İlkay YILDIZ
YAYIN İDARE MERKEZİ ADRESİ : Ankara Üniversitesi, Eczacılık Fakültesi, Dekanlığı, 06560 Yenimahalle/Ankara
YAYIN İDARİ MERKEZİ ADRESİ TEL : 0 (312) 203 30 69
YAYIN TÜRÜ : Bilimsel Periyodik Elektronik Dergi, Yılda 3 Sayı

ANKARA ÜNİVERSİTESİ ECZACILIK FAKÜLTESİ DERGİSİ

JOURNAL OF FACULTY OF PHARMACY OF ANKARA UNIVERSITY

Cilt / Vol : 46
Sayı / No : 3
Yıl / Year : 2022
eISSN : 2564-6524
ISSN: 1015-3918 (1971-2010)

İÇİNDEKİLER / CONTENTS

Özgün Makaleler / Original Articles ***Sayfa / Page***