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Sahibi:

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Baş Editör:

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06560, Yenimahalle, Ankara, Türkiye
Tel: +90 312 203 31 52
Faks: +90 312 213 10 81
*e-pos*ta: *ctsengel@pharmacy.ankara.edu.tr*
efd.editor@ankara.edu.tr

Yardımcı Editörler:

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

Dil Editörü:

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e-posta: serim@pharmacy.ankara.edu.tr

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Alan Editörleri:

Prof. Dr. Marcello LOCATELLI
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Owner:

Prof. Dr. Asuman BOZKIR
Ankara University Faculty of Pharmacy, Department of Pharmaceutical Technology
06560 Yenimahalle, Ankara, Türkiye

Editor-in-Chief:

Prof. Dr. Ceyda Tuba ŞENGEL TÜRK
Ankara University Faculty of Pharmacy, Department of Pharmaceutical Technology
06560 Yenimahalle, Ankara, Türkiye
Phone: +90 312 203 31 52
Fax: +90 312 213 10 81
e-mail: ctsengel@pharmacy.ankara.edu.tr
efd.editor@ankara.edu.tr

Associate Editors:

Assoc. Prof. Dr. Işıl ÖZAKÇA GÜNDÜZ
Ankara University, Faculty of Pharmacy, Department of Pharmacology
e-mail: ozakca@ankara.edu.tr

Assoc. Prof. Dr. Aslı KOÇ
Ankara University, Faculty of Pharmacy, Department of Biochemistry
e-mail: akoc@ankara.edu.tr

PhD. Fatıma DOĞANÇ
Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry
e-mail: doganc@ankara.edu.tr

Language Editor:

Res. Assist. Tuğrul Mert SERİM
Ankara University Faculty of Pharmacy, Department of Pharmaceutical Technology
e-mail: serim@pharmacy.ankara.edu.tr

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Section Editors:

Prof. Dr. Marcello LOCATELLI
University "G.d'Annunzio" of Chieti-Pescara, Department of Pharmacy
e-mail: marcello.locatelli@unich.it
Prof. Dr. Aylin ÜSTÜNDAĞ
Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology
e-mail: dur@pharmacy.ankara.edu.tr
Assoc. Prof. Dr. İlker ATEŞ
Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology
e-mail: iates@pharmacy.ankara.edu.tr
Assoc. Prof. Dr. Banu KAŞKATEPE
Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology
e-mail: bkaskatepe@ankara.edu.tr
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Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry
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e-mail: ergene@pharmacy.ankara.edu.tr
Assist.Prof.Dr.Belma PEHLIVANOVIC KELLE
University of Sarajevo, Fac. of Pharmacy, Depart. of Pharmacology and Clinical Pharmacy
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Assist. Prof. Dr. Berna GÜVEN ÇİLOĞLU
Ankara University, Faculty of Pharmacy, Department of Pharmacology
e-mail: bguven@ankara.edu.tr
PhD. Rafal Jerzy KOPIASZ
Warsaw University of Technology, Department of Polymer Chemistry and Technology
e-mail: rafal.kopiasz@pw.edu.pl
Res. Assist. Murat Sefa KARAASLAN
Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology
e-mail: mskaraaslan@ankara.edu.tr
Res. Assist. Selenay SADAK
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e-mail: ssadak@ankara.edu.tr
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University of Messina, Department of Chemical, Biological, Pharm. and Environmental Sciences
e-mail: natalizia.miceli@unime.it
Assoc. Prof. Dr. Marco Lucio LOLLI
University of Turin, Department of Science and Drug Technology
e-mail: marco.lolli@unito.it
Assoc. Prof. Dr. Arzu Zeynep KARABAY
Ankara University, Faculty of Pharmacy, Department of Biochemistry
e-mail: akarabay@ankara.edu.tr
Assoc. Prof. Dr. Mehmet Gökhan ÇAĞLAYAN
Ankara University, Faculty of Pharmacy, Department of Analitical Chemistry
e-posta: gcaglayan@ankara.edu.tr
Assoc. Prof. Dr. Gülin AMASYA-ÇELİKTEN
Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Technology
e-mail: gamasya@pharmacy.ankara.edu.tr
Assoc. Prof. Dr. Aysu SELÇUK
Ankara University, Faculty of Pharmacy, Department of Clinical Pharmacy
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Ankara University, Faculty of Pharmacy, Department of Pharmacy Business Administration
e-mail: calikusu@ankara.edu.tr
Res. Assist. Sevgi TEKTAŞ
Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Technology
e-mail: stektas@ankara.edu.tr
Res. Assist. Şerife Tuğçe TÜRKMEN
Ankara University, Faculty of Pharmacy, Department of Pharmacognosy
e-mail: stturkmen@ankara.edu.tr
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Ankara University, Faculty of Pharmacy, Department of Pharmacy Business Administration
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ÖZGÜN MAKALE / ORIGINAL ARTICLE

TETRABENAZİNİN BAZİK ORTAMDAKİ ÇÖZÜNME ÖZELLİKLERİNİN KATI DİSPERSİYON TEKNOLOJİSİ İLE ARTIRILMASI

INCREASING THE SOLUBILITY PROPERTIES OF TETRABENAZINE AT BASIC MEDIUM BY USING SOLID DISPERSION TECHNOLOGY

Hilal BAŞ[1,2], Fırat YERLİKAYA[2,3], Füsun ACARTÜRK[1]*

[1]Gazi Üniversitesi, Eczacılık Fakültesi, Farmasötik Teknoloji Anabilim Dalı, 06330, Ankara, Türkiye
[2]Elixir İlaç Araştırma ve Geliştirme A.Ş., 06930, Ankara, Türkiye
[3]Lokman Hekim Üniversitesi, Eczacılık Fakültesi, Farmasötik Teknoloji Anabilim Dalı, 06520, Ankara, Türkiye

ÖZ

Amaç: *Bu çalışmanın amacı, suda düşük çözünürlüğe sahip bir etkin madde olan tetrabenazinin (TBZ) bazik ortamda çözünmesini artırmak için katı dispersiyon teknolojisinin kullanılmasıdır. Katı dispersiyonların karakterizasyon çalışmaları sonucunda elde edilen bulgular ile katı dispersiyon teknolojisinin TBZ'nin çözünmesi üzerindeki etkileri değerlendirilmiştir. Katı dispersiyon teknolojsinin TBZ'nin bazik ortamda çözünmesini artırmada etkili olduğu sonucuna varılmıştır.*

Gereç ve Yöntem: *TBZ'nin çözünmesini artırmak amacıyla üç farklı katı dispersiyon formülasyonu hazırlanmıştır. Bu amaçla polimerik taşıyıcılar olarak Soluplus® ve Kollidon® VA 64, yüzey aktif madde olarak Gelucire® 50/13 kullanılmıştır. Bu polimerik taşıyıcılar ve yüzey aktif madde kullanılarak hazırlanan katı dispersiyon formülasyonları sırasıyla KD-1, KD-2 ve KD-3 olarak isimlendirilmiştir. Tüm katı dispersiyonlar, etkin madde ve polimerin 1:1 oranında karıştırılmasıyla elde edilmiştir. Hazırlama işlemi, çözücü buharlaştırma yöntemi ile gerçekleştirilmiş ve çözücü olarak aseton kullanılmıştır. Asetonun, rotavaporda düşük basınç altında ve 55°C su banyosunda buharlaştırılması ile katı dispersiyonlar elde edilmiştir. TBZ miktar tayini yöntemi, yüksek basınçlı sıvı kromatografisi (HPLC) kullanılarak 230 nm dalga boyunda geliştirilmiştir ve analitik yöntem validasyonu yapılmıştır. Katı dispersiyonların termal özellikleri diferansiyel taramalı kalorimetre (DSC) ile 25-250°C aralığında analiz edilmiş; Fourier Dönüşümlü Kızılötesi (FT-IR) spektroskopisi kullanılarak polimer ve etkin madde arasındaki olası etkileşimler incelenmiştir. Katı dispersiyonların TBZ'nin çözünmesini artırmaya olan etkisi, pH 1.2 ve pH 6.8 ortamlarında yapılan çözünme hızı çalışmaları ile değerlendirilmiş, elde edilen numuneler HPLC ile analiz edilmiştir. TBZ'nin katı dispersiyon formülasyonlarından salım kinetiklerinin belirlenmesi için çözünme hızı verilerinden hareketle DDSolver yazılımı kullanılmıştır.*

Sonuç ve Tartışma: *Katı dispersiyonlar için yapılan TBZ miktar tayini analiz sonuçları, her üç katı dispersiyonda ortalama TBZ miktarlarının %98.31 ile %99.19 arasında değiştiğini göstermiştir. Tüm miktar tayini analizlerinde düşük standart sapma değerleri gözlenmiş ve bu durum ölçümlerin tutarlılığını göstermiştir. Termal analizlerde, TBZ'nin endotermik pikinin 130ºC'de ortaya çıktığı ve amorf yapıya dönüşen katı dispersiyonların endotermik pikin şiddetinde azalmaya yol açtığı belirlenmiştir. FT-IR spektroskopisi ile elde edilen spektrumlar, TBZ'nin farklı taşıyıcı polimerlerle fiziksel veya kimyasal etkileşiminin olduğunu göstermiştir. Katı dispersiyonların TBZ'nin çözünmesini artırmaya olan etkisi çözünme hızı çalışmaları ile pH 1.2 ve pH 6.8 ortamlarında*

* **Sorumlu Yazar / Corresponding Author:** Füsun Acartürk
e-posta / e-mail: acarturk@gazi.edu.tr, **Tel. / Phone:** +903122210045

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incelenmiştir. TBZ, pH 1.2 ortamında hızlı çözünme göstermiş ve ilk 1 saatte tamamen çözünmüştür. 24 saatin sonunda pH 1.2 ortamında etkin maddenin %98.8'i çözünmüştür, pH 6.8 ortamında ise çözünme daha yavaş (%28.8) gerçekleşmiştir. TBZ'nin pH 6.8 ortamındaki çözünürlüğü düşük olduğu için hazırlanan tüm katı dispersiyonların çözünme hızı çalışmaları bu ortamda gerçekleşmiştir. Hazırlanan tüm katı dispersiyon formülasyonlarının, TBZ'nin pH 6.8 ortamındaki çözünme hızını artırdığı gözlenmiştir. Özellikle Gelucire® 50/13 ile hazırlanan katı dispersiyon formülasyonu (KD-3), 24 saatin sonunda en yüksek çözünme oranına (%85.6) ulaşmıştır. In vitro çözünme hızı çalışmaları sonucunda KD-1, KD-2 ve KD-3 formülasyonlarının salım kinetikleri DDSolver yazılımı ile belirlenmiştir. Değerlendirme sonuçlarına göre her üç formülasyon için en uyumlu modeller Korsmeyer-Peppas ve Weibull kinetikleri olarak belirlenmiştir. KD-1, KD-2 ve KD-3 için Korsmeyer-Peppas modeline ait parametreler sırasıyla, n=0.3351, n=0.3511 ve n=0.3015 olarak belirlenmiştir. Weibull modeline ait parametreler sırasıyla, β=0.4303, β=0.4369 ve β=0.5422 olarak hesaplanmıştır. n değeri 0.5'ten, β değeri 0.75'ten düşük olduğundan, salım mekanizmasının Fick difüzyonuna benzer şekilde gerçekleştiği ve katı dispersiyon teknolojisinin TBZ'nin bazik ortamda çözünmesini artırmada etkili olduğu sonucuna varılmıştır.
Anahtar Kelimeler: *Çözünme hızı, DDSolver, düşük çözünürlük, katı dispersiyon, tetrabenazin*

ABSTRACT

Objective: *The aim of this study is to use solid dispersion technology to increase the solubility of tetrabenazine (TBZ) at basic medium. The effect of solid dispersions on the solubility of TBZ, which has low solubility in water, was evaluated by characterization studies of solid dispersions. It was concluded that solid dispersion technology was efficient for the increasing the dissolution of TBZ at basic medium.*

Material and Method: *In order to increase the solubility of TBZ, three different solid dispersion formulations were prepared. For this purpose, Soluplus® and Kollidon® VA 64 were used as polymeric carriers and Gelucire® 50/13 was used as surfactant. The solid dispersion formulations prepared using these polymeric carriers and surfactant were named as KD-1, KD-2 and KD-3, respectively. All solid dispersions were obtained by mixing the active substance and polymer in a 1:1 ratio. The preparation process was carried out by solvent evaporation method and acetone was used as the solvent. Solid dispersions were obtained by evaporating acetone under low pressure in a rotavapor and in a 55°C water bath. TBZ quantification method was developed using high pressure liquid chromatography (HPLC) at 230 nm wavelength and analytical method validation was performed. Thermal properties of solid dispersions were analyzed by differential scanning calorimetry (DSC) in the range of 25-250°C; possible interactions between the polymer and the active substance were investigated using Fourier Transform Infrared (FT-IR) spectroscopy. The effect of solid dispersions on increasing the solubility of TBZ was evaluated by dissolution rate studies in pH 1.2 and pH 6.8 media, and the obtained samples were analyzed by HPLC. DDSolver software was used to determine the release kinetics of TBZ from solid dispersion formulations based on dissolution rate data.*

Result and Discussion: *The results of TBZ quantification analysis for solid dispersions showed that the average TBZ amounts in all three solid dispersions varied between 98.31% and 99.19%. Low standard deviation values were observed in all quantification analyses, demonstrating the consistency of the indicating. In thermal analyzes, it was determined that the endothermic peak of TBZ appeared at 130ºC and that solid dispersions transforming into an amorphous structure caused a decrease in the intensity of the endothermic peak. Spectra obtained by FT-IR spectroscopy showed that TBZ has physical or chemical interactions with different carrier polymers. The effect of solid dispersions on increasing the solubility of TBZ was investigated in pH 1.2 and pH 6.8 media with dissolution rate studies. TBZ showed rapid dissolution in pH 1.2 medium and was completely dissolved in the first 1 hour. At the end of 24 hours, 98.8% of the drug was dissolved in pH 1.2 medium, while dissolution occurred slower (28.8%) in pH 6.8 medium. Since the solubility of TBZ in pH 6.8 environment is low, the dissolution rate analysis of all prepared solid dispersions was carried out in this environment. It was observed that all prepared solid dispersion formulations increased the dissolution of TBZ in pH 6.8 environment. Especially, the solid dispersion prepared with Gelucire® 50/13 (KD-3) reached the highest dissolution rate (85.6%) at the end of 24 hours. As a result of in vitro dissolution studies, the release kinetics of KD-1, KD-2 and KD-3 formulations were assessed by DDSolver software. According to the evaluation of the results, the most fitted models for all three formulations were determined as Korsmeyer-Peppas and Weibull kinetics. The parameters of the Korsmeyer-Peppas model for KD-1, KD-2 and KD-3 were calculated as*

n=0.3351, n=0.3511 and n=0.3015, respectively, and the parameters of the Weibull model were calculated as β=0.4303, β=0.4369 and β=0.5422, respectively. Since the n value is less than 0.5 and the β value is less than 0.75, it is concluded that the release mechanism occurs similar to Fick diffusion and solid dispersion technology is efficient for the increasing the dissolution of TBZ at basic medium.

Keywords: *DDSolver, dissolution, low solubility, solid dispersion, tetrabenazine*

GİRİŞ

TBZ, Amerika İlaç ve Gıda İdaresi (FDA) tarafından Huntington hastalığı (HD) ile ilişkili kore ve diğer hiperkinetik hareket bozukluklarının tedavisinde onaylanmış bir ilaçtır [1]. HD; ilerleyici motor, bilişsel ve davranışsal semptomlarla karakterize nörodejeneratif bir hastalıktır [2]. HD'de görülen hareket bozuklukları, istemsiz hareketlerden (kore ve distoni) ve istemli hareketlerin bozulmasından (örneğin; yürüme, konuşma, yutma, ince motor görevler) kaynaklanmaktadır. Kore ve istemli hareketlerin bozulması, HD'nin ilerlemesinde rol oynamaktadır [3].

HD, tam penetrasyon ile otozomal baskın bir şekilde aktarılmaktadır. Amerika Birleşik Devletleri'ndeki yaygınlık oranı 100.000'de 5-10 olarak tahmin edilmektedir. Venezuela'daki Maracaibo Gölü bölgesinde çok yüksek bir HD prevalansı ve insidansı olan ailelerin genetik çalışmaları, kromozom 4'teki gende bulunan kararsız bir trinükleotid (CAG) tekrarının keşfedilmesine yol açmıştır. Normal kişilerde CAG tekrarlarının sayısı 29 iken, 36 veya daha fazla CAG tekrarının varlığı HD'nin oluşmasını sağlamaktadır. HD hastaları, 15-20 yıl içinde yavaş yavaş hareket edemeyecek hale gelmektedir. İleri evrelerde disfaji, dizartri, motor impersistanslı belirgin kore ve daha ileri evrelerde demans, depresyon ve psikozun eşlik ettiği fonksiyonel yetersizlik gelişmektedir. Bu hastalığın seyrini iyileştiren veya yavaşlatan bir tedavi yoktur. Bu hastalığa sahip olanlar için yalnızca semptomatik tedavi seçenekleri mevcuttur ve bunlar çoğunlukla depresyon, psikoz ve koreyi iyileştirmeye odaklanmıştır [4].

Birçok dopamin reseptör blokerleri (DRB) veya dopamin depletorleri dahil olmak üzere pek çok tedavi yöntemi yıllar içinde değerlendirilmiş, çoğu açık etiketli çalışmalarda veya vaka raporlarında sunulmuştur [4]. Kore üzerinde etkili olan bazı ilaçlar; istemli hareketleri kötüleştirebilir, yürüyüşü veya yutmayı tehlikeye atabilir, depresyon, sedasyon ve geç diskineziye (TD) neden olabilir [3]. DRB'lerin yerine TBZ kullanılmasının ana nedenlerinden biri, TBZ'nin TD'ye neden olduğunun hiçbir zaman belgelenmemiş olmasından dolayı diğer tedavi edici ajanlara göre göreceli güvenliğidir. Bu durum, tipik nöroleptiklere göre TBZ'nin önemli bir avantajıdır, çünkü kronik olarak DRB'ler ile tedavi edilen hastaların %25 ila %40'ı sonunda TD geliştirmektedir [4]. Ayrıca TBZ'nin, Tourette sendromu ile ilişkili tikleri ve TD ile ilişkili hareketleri azaltmada etkili olduğu bulunmuştur [5].

Sentetik bir benzokinolizin türevi olan TBZ, bir monoamin tüketicisi ve DRB'dir. TBZ, veziküler monoamin taşıyıcı 2'yi (VMAT-2) inhibe ederek çalışan, seçici, geri dönüşümlü ve merkezi etkili bir dopamin tüketici ilaçtır. TBZ presinaptik dopamin, norepinefrin ve serotonin depolarını tüketir ve postsinaptik dopamin reseptörlerini antagonize eder [6].

TBZ, yetim ilaç statüsündedir [7]. 2006 yılında HD'de görülen kore tedavisi için TBZ'nin güvenliğini, etkililiğini ve doz toleransını incelemek için bir çalışma yapılmıştır. Çalışma kapsamında HD'li 84 hasta 12 hafta boyunca TBZ (n=54) veya plasebo (n=30) ile tedavi edilecek şekilde randomize edilmiştir. TBZ dozu, istenen antikoreik etki oluşana veya dayanılmaz yan etkiler ortaya çıkana kadar 7 hafta boyunca maksimum 100 mg/gün'e arttırılmıştır. Sonuç olarak günlük 100 mg'a kadar ayarlanmış dozlarda TBZ'nin, HD hastalarındaki koreyi etkili bir şekilde azalttığı gözlenmiştir [8].

TBZ etkin maddesinin kimyasal adı 1,3,4,6,7,11b-hekzahidro-9,10-dimetoksi-3-(2-metilpropil)-2H-benzo(a)kinolizin-2-on'dur. Moleküler formülü $C_{19}H_{27}NO_3$'tür ve moleküler ağırlığı 317.43 g/mol'dür [9]. TBZ'nin kimyasal yapısı Şekil 1'de gösterilmiştir.

Şekil 1. Tetrabenazin kimyasal yapısı

TBZ, acı bir tada sahip, beyaz ila açık sarı renkli kristal tozdur. Suyla iyi karışmaz [9] ve suda zor çözünür [10]. TBZ sudaki düşük çözünürlüğü nedeniyle sınırlı oral biyoyararlanıma sahiptir [11]. TBZ, %4.9 ± 3.2'lik düşük bir biyoyararlanım gösterir.

TBZ, Biyofarmasötik Sınıflandırma Sistemi'ne (BCS) göre sınıf 4 bir etkin maddedir [12,13]. Oral dozaj formları, tüm farmasötik dozaj formlarının %50'den fazlasını oluşturur [14]. Oral yoldan alım; uygulama kolaylığı, yüksek hasta uyuncu, düşük maliyet ve dozaj formunun tasarımındaki esneklik nedeniyle ilaç uygulamasında yaygın olarak kullanılan bir yoldur. Ancak oral dozaj formlarının tasarımındaki en büyük zorluk oral yolla alınan ilaçların düşük biyoyararlanımları olarak değerlendirilir. Oral biyoyararlanım, sudaki çözünürlük, permeabilite, çözünme hızı ve ilk geçiş metabolizması gibi çeşitli faktörlere bağlıdır [15]. Oral yoldan uygulanan bir ilacın biyoyararlanımı, öncelikle gastrointestinal sistemdeki çözünürlüğü ve hücre zarları boyunca geçirgenliğine bağlıdır. İlaç moleküllerinin biyolojik zarlardan taşınabilmeleri için çözünmüş bir formda bulunmaları gerekir. Bu nedenle, sudaki düşük çözünürlük ilaç emilimini geciktirebilir veya sınırlayabilir [16]. Birçok ilaç adayı suda düşük çözünürlüğe sahiptir ve bu durum oral emilimini yetersiz hale getirebilir. Piyasadaki ilaçların yaklaşık %40'ı suda düşük çözünürlüğe sahiptir, ayrıca BCS'ye göre yeni ilaç adaylarının yaklaşık %40-90'ı suda düşük çözünürlüğe sahiptir [17].

Düşük çözünürlüğe sahip bir bileşiğin çözünürlüğünü artırmak için çeşitli stratejiler geliştirilmiştir. Çözünürlüğü artırmak için sıklıkla kullanılan yaklaşımlar arasında, mikronizasyon, tuz formlarının geliştirilmesi, siklodekstrin kompleksleri ve katı dispersiyon teknolojileri yer alır [18]. Farklı stratejiler arasından, oral yoldan uygulanan ilaçların çözünürlüğünü ve çözünme hızını arttırma, dolayısıyla biyoyararlanımını iyileştirme potansiyeline sahip etkili bir yöntem olarak katı dispersiyonlar öne çıkmakta ve bu amaçla yaygın olarak kullanılmaktadır [19].

Katı dispersiyonlar, etkin maddenin polimer matriks içinde amorf veya moleküler olarak dağınık halde bulunduğu sistemlerdir. Bu teknoloji, ilacın çözünürlüğünü artırarak daha hızlı ve etkili bir terapötik yanıt elde edilmesine olanak tanır [19-25]. Katı dispersiyon formülasyonları ilacın salımını ve stabilitesini artırmak için polimer veya polimer yerine yüzey aktif maddeler içerebilir [24].

Katı dispersiyonlarda kullanılan polimerler ve yüzey aktif maddeler, genellikle etkin maddelerin çözünürlüklerini ve biyoyararlanımlarını artırmak için kullanılır. Bu polimerler arasında polisakkaritler (maltodekstrin, pektin), selüloz türevleri (mikrokristalize selüloz, hidroksipropil selüloz) ve sentetik polimerler (polivinilpirolidon, polietilen glikol) öne çıkar. Bu polimerler, etkin maddelerin amorf bir yapıda dağılmasına yardımcı olarak çözünürlüklerini ve stabilitelerini artırır [24].

Soluplus®, polivinil kaprolaktam-polivinil asetat-polietilen glikolden oluşan bir kopolimerdir [26]. Hidrofilik kısım olarak polietilen glikol omurgasına ve lipofilik kısım olarak vinilkaprolaktam/vinil asetat yan zincirlerine sahiptir [26,27]. Polimerik bir çözücü olarak kullanılabilen yeni bir amfifilik polimerdir. Soluplus®'ın suda az çözünen ilaçların çözünürlüğünü, çözünme hızını ve dolayısıyla biyoyararlanımını artırdığı gösterilmiştir [28,29]. Soluplus®, katı dispersiyon formülasyonları için taşıyıcı olarak kullanılabilmektedir. Yüzey aktif madde olarak düşük de olsa etki göstermektedir [30]. Soluplus® maddesinin kimyasal yapısı Şekil 2'de verilmiştir.

Şekil 2. Soluplus® kimyasal yapısı

Kollidon® VA 64, kopovidon (Avrupa Farmakopesi, Amerikan Farmakopesi (USP)) veya kopolividon (Japon Farmakopesi) farmakope adlarıyla bilinmektedir. N-vinilpirolidon (NVP) ve vinil asetat (VAc) olmak üzere 2 monomerin zincir yapısından oluşan bir kopolimerdir. Bu monomerler, radikal polimerizasyon kullanılarak yaklaşık 6:4'lük bir mol oranında birleştirilir. Dolayısıyla, ticari isimdeki 64 sayısı, bu monomerler arasındaki mol oranına, karşılık gelir. Kopovidon, amfifilik bir yapıya sahip olmasa da suda düşük çözünürlüğe sahip ilaçların çözünürlüğünü artırdığı kanıtlanmıştır. Kopovidon ile stabil katı dispersiyon formülasyonları elde edilebilmektedir [31]. Kollidon® VA 64 maddesinin kimyasal yapısı Şekil 3'te verilmiştir.

Şekil 3. Kollidon® VA 64 kimyasal yapısı

Gelucire®, amfifilik karakterde olan ve değişen fiziksel özelliklere sahip inert, yarı katı, mumsu bir materyaldir. Gelucire® bileşimleri genellikle gliserol ve PEG esterlerinin yağ asidi esterleri veya poliglikolize gliseritler olarak tanımlanır [32]. Gelucire® ürünleri, yaklaşık erime noktası ve hidrofilik-lipofilik denge (HLB 1-18) değerine göre numaralarla tanımlanan, yarı katı lipit yardımcı maddelerden oluşan materyallerdir. Düşük HLB değerine sahip maddeler kısmi gliseritlerden oluşurken, HLB değeri

10'un üzerinde olanlar, kısmi doymuş gliseritler ve polietilen glikol (PEG) esterlerinin karışımlarından oluşur [33,34]. Gelucire® 50/13 iyi karakterize edilmiş PEG esterleri, küçük bir gliserit fraksiyonu ve serbest PEG'den oluşan, iyonik olmayan, suda dağılabilir bir yüzey aktif maddedir. Kimyasal adı stearoil makrogol-32 gliseritleridir. Sulu ortamla temas ettiğinde kendi kendine emülsifiye olabilir ve ince bir dispersiyon yani mikroemülsiyon oluşturur. Hem *in vitro* hem de *in vivo* koşullarda etkin maddelerin çözünürlüğünü ve ıslanabilirliğini iyi derecede artıran yüzey aktif maddelerdir. *İn vivo* ilaç çözünürlüğünü artırır ve emilimi kolaylaştırır [35,36]. HLB değeri 11 ve erime noktası 50°C'dir [36]. Gelucire® 50/13, suda düşük çözünürlüğe sahip ilaçların çözünürlüğünü ve dolayısıyla biyoyararlanımlarını arttırabilir [33,34]. Gelucire® 50/13 maddesinin kimyasal yapısı Şekil 4'te verilmiştir.

Şekil 4. Gelucire® 50/13 kimyasal yapısı

Katı dispersiyon hazırlamak için çeşitli yöntemler kullanılabilir. Bu yöntemler; eritme veya füzyon, çözücü buharlaştırma, sıcak eriyik ekstrüzyon, süper kritik sıvı, yoğurma tekniği, ortak çöktürme, ortak öğütme, jel tutma, sprey kurutma, liyofilizasyon, elektro-çekim, damlatma çözeltisi ve eriyik aglomerasyondur [37]. Çözücü buharlaştırma yöntemi, katı dispersiyonların hazırlanmasında etkin ve yaygın olarak kullanılan bir yöntemdir. Bu yöntem, öncelikle ilaç ve taşıyıcı materyalin uygun bir çözücü içinde çözünmesini içerir. Yöntemin temel prensibi, iki bileşenin aşırı doymuş çözeltisinden çözücü maddenin buharlaştırılmasıyla polimerik matris içinde etkin maddenin çöktürülmesidir [38,39].

Daha önce yapılan bir çalışmada TBZ'nin ağızda dağılabilen filmleri (ADF), film oluşturucu bir polimer kullanılarak katı dispersiyon teknolojisi ile hazırlanmıştır. Bu çalışmada TBZ ve polimerden oluşan bir formülasyon ve TBZ, polimer ve sitrik asitten oluşan ayrı bir formülasyon olmak üzere iki farklı formülasyon geliştirilmiştir. Çalışmada polimer olarak hidroksipropil metil selüloz (HPMC), hidroksietil selüloz (HEC), pullulan ve polivinilpirolidon (PVP) kullanılmıştır. Formülasyonlarda yer alan maddeler karıştırılarak jel oluşturulmuş ve bu jelin kurutulması ile ADF formülasyonları hazırlanmıştır. Hazırlanan formülasyonların karakterizasyon çalışmaları (DSC, FT-IR, camsı geçiş sıcaklığı tayini, çözünme hızı analizleri) yapılmıştır. Çalışma sonucunda ADF formülasyonlarının TBZ'nin amorf forma geçerek çözünürlüğünü artırmada etkili olduğu gözlenmiştir [19]. Literatürde TBZ'nin katı dispersiyonlarının hazırlanmasına dair çalışmalar bulunmaktadır. Ancak ilk defa bu çalışma kapsamında TBZ'nin Soluplus®, Kollidon® VA 64 ve Gelucire® 50/13 kullanılarak çözücü buharlaştırma tekniğiyle katı dispersiyon formülasyonları hazırlanarak bu formülasyonların TBZ'nin

çözünmesi üzerine etkisi değerlendirilmiştir.

Bu çalışmanın amacı, suda düşük çözünürlüğe sahip bir etkin madde olan TBZ'nin bazik ortamdaki çözünme özelliklerini iyileştirmek amacıyla katı dispersiyonlarının hazırlanması ve karakterize edilmesidir.

GEREÇ VE YÖNTEM

Materyaller

TBZ etkin maddesi Enaltec Labs Pvt. Ltd. (Hindistan) firmasından temin edilmiştir. Yüzey aktif madde olarak kullanılan Gelucire® 50/13, Gattefossé SAS (Fransa) firmasından sağlanmıştır. Polimer olarak kullanılan Kollidon® VA 64 ve polimer taşıyıcı olarak kullanılan Soluplus®, BASF SE (Almanya) firmasından temin edilmiştir. Çözücü olarak kullanılan aseton ve reaktif olarak kullanılan asetonitril, metanol, hekzilamin, orto-fosforik asit (%85), hidroklorik asit (%37), susuz sodyum karbonat (Na_2CO_3), potasyum dihidrojen fosfat (KH_2PO_4) ve sodyum hidroksit (NaOH) Merck KGaA (Almanya) firmasından tedarik edilmiştir. HPLC'de kullanılan kolonlardan; X-Bridge (C18, 150 x 4.6 mm, 5 µm partikül boyutu) Waters Corporation (Amerika Birleşik Devletleri-ABD) firmasından, Purospher Star (C18, 55 x 4 mm, 3 µm partikül boyutu) Merck KgaA (Almanya) firmasından tedarik edilmiştir.

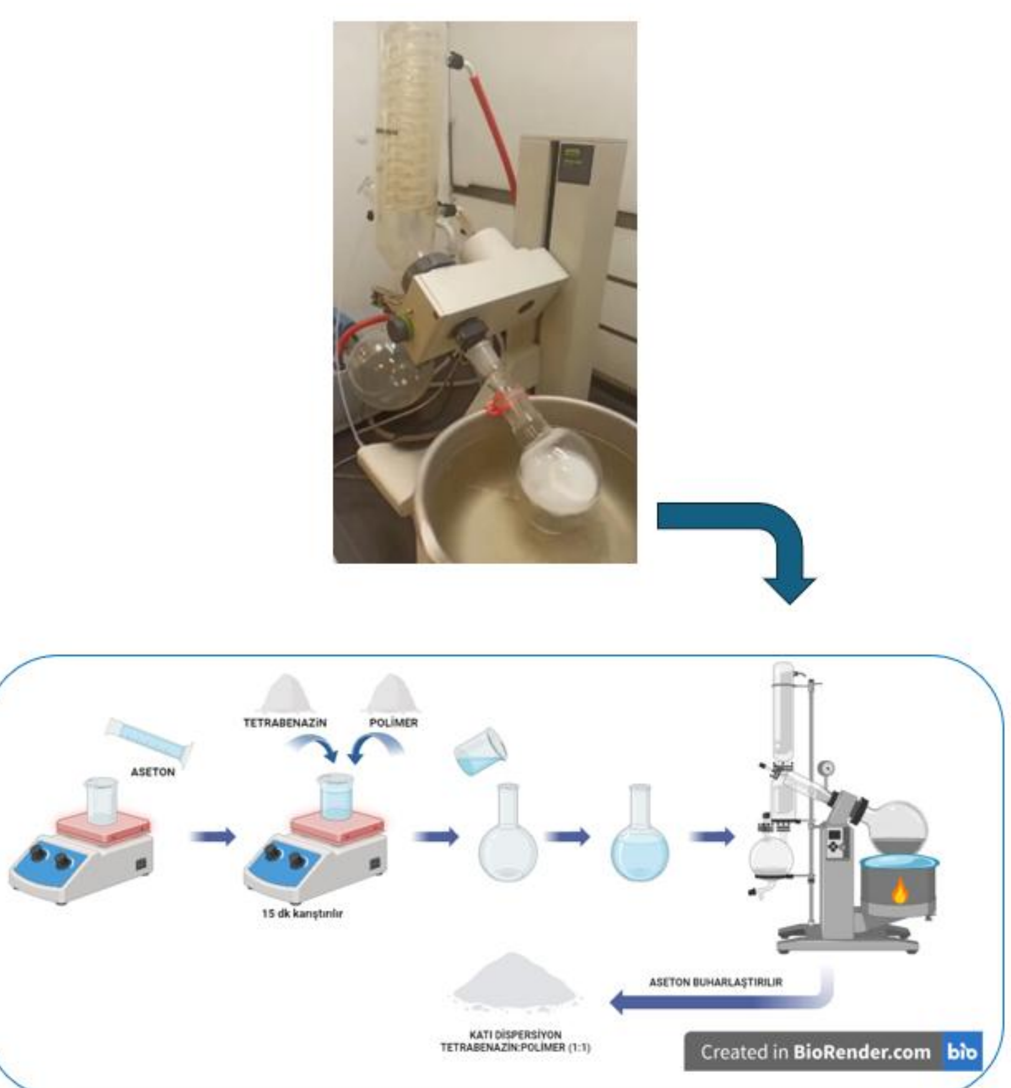
Katı Dispersiyonların Hazırlanması

Üç farklı katı dispersiyon formülasyonu hazırlanmıştır. Hazırlanan tüm katı dispersiyonlarda etkin madde ve polimer 1:1 oranında karıştırılmıştır. Hazırlanan katı dispersiyonların içerikleri Tablo 1'de yer almaktadır.

Tablo 1. Hazırlanan katı dispersiyonların formülasyon kodları ve içerikleri

Formülasyon Kodu	Katı Dispersiyon İçeriği
KD-1	Tetrabenazin:Soluplus® (1:1)
KD-2	Tetrabenazin:Kollidon® VA 64 (1:1)
KD-3	Tetrabenazin:Gelucire® 50/13 (1:1)

Her üç katı dispersiyon çözücü buharlaştırma yöntemiyle hazırlanmıştır. Çözücü buharlaştırma işlemleri için rotavapor (Buchi, İsviçre) kullanılmıştır. Çözücü olarak aseton kullanılmıştır. 4 g etkin madde ile 4 g polimerin 70 ml aseton içerisinde manyetik karıştırıcı yardımıyla 15 dakika karıştırılarak çözünmesi sağlanmıştır. Daha sonra etkin madde ve polimeri içeren çözelti rotavapor cihazına aktarılarak bu cihazda düşük basınç ve sıcaklık yardımıyla çözücünün buharlaşması sağlanmıştır. Çözücü buharlaştıkça, etkin madde ve polimer, ince bir film tabakası oluşturmuş ve böylece amorf formda bir katı dispersiyon elde edilmiştir. Rotavaporun su banyosundaki su sıcaklığı 55°C olarak ayarlanmıştır. Çözücünün buharlaştırılması sırasında vakum uygulanması, çözücünün kaynama noktasını düşürerek buharlaşmayı hızlandırmıştır [40]. Çözücü buharlaştırma işleminin ayrıntıları Şekil 5'te yer almaktadır.



Şekil 5. Katı dispersiyonların hazırlanışı

Katı Dispersiyonların Karakterizasyonu

Tetrabenazin Miktar Tayini

Katı dispersiyonlardaki TBZ miktar tayini, HPLC (Agilent 1260 Infinity II, Agilent Technologies, ABD) kullanılarak gerçekleştirilmiştir. Kullanılan analitik metot parametreleri Tablo 2'de verilmiştir.

Tablo 2. Miktar tayini analizine ait analitik metot parametreleri

Kolon	C18, 55 x 4 mm, 3 µm (Merck, Purospher Star)
Dedeksiyon	UV, 230 nm
Akış Hızı	0.8 ml/dk
Enjeksiyon Hacmi	20 µl
Kolon Sıcaklığı	40°C
Analiz Süresi	10 dk

Analizlerde mobil faz olarak, pH 7.5 tampon çözelti ve asetonitril karışımı (55:45 (h/h)) kullanılmıştır. Mobil faz, 0.45 µm filtreden süzülerek ve 5 dakika boyunca sonikasyon uygulanarak degaze edilmiştir. Katı dispersiyonların çözünmesi ve seyreltilmesi için, pH 7.5 tampon çözelti ile asetonitril karışımından oluşan bir çözelti (20:80 (h/h)) kullanılmıştır. Miktar tayini analizi için katı dispersiyonlardan 100'er mg tartılarak TBZ konsantrasyonu 50 µg/ml olacak şekilde test çözeltileri hazırlanmıştır. Bu çözeltiler vorteks yardımıyla 15 dakika boyunca karıştırılmıştır. HPLC ile elde edilen pik alanlarına karşı çözelti konsantrasyonları (25-75 µg/ml) grafiğe geçirilerek kalibrasyon doğrusu çizilmiştir. Elde edilen kalibrasyon doğrusu kullanılarak TBZ miktarı hesaplanmıştır. Kullanılan metot için analitik metot validasyonu yapılmıştır. Validasyon kapsamında doğrusallık, doğruluk (geri elde), kesinlik, seçicilik, stabilite parametreleri çalışılmıştır.

Katı Dispersiyonların Termal Analizi

Katı dispersiyonların termal analizi DSC cihazı (Mettler Toledo DSC 3, İsviçre) kullanılarak yapılmıştır. Numunelerin azot gazı altında 10 °C/dakika ısıtma hızında 25-250°C arasında DSC termogramları alınmıştır [19,41]. Saf TBZ, Soluplus®, Kollidon® VA 64, Gelucire® 50/13 ve hazırlanan katı dispersiyonlar analiz edilmiştir. Analiz için 4 mg numune tartılmış, alüminyum örnek tablası içinde basınç altında sıkıştırılmış ve DSC cihazının numune bölmesine yerleştirilerek DSC analizi gerçekleştirilmiştir.

Katı Dispersiyonların Fourier Dönüşümlü Kızılötesi (FT-IR) Spektroskopisi ile Analizi

Saf TBZ, Soluplus®, Kollidon® VA 64, Gelucire® 50/13 ve hazırlanan katı dispersiyonların FT-IR spektroskopisi analizleri, polimerler ve etkin madde arasındaki olası etkileşimleri değerlendirmek için bir ATR problu (Agilent Cary 630, ABD) FT-IR spektrometresi kullanılarak 1 cm^{-1} çözünürlükle 650 ile 4000 cm^{-1} dalga boyları arasında gerçekleştirilmiştir.

***İn Vitro* Çözünme Hızı Çalışmaları ve Salım Kinetiklerinin Değerlendirilmesi**

Hazırlanan katı dispersiyonların TBZ'nin çözünmesini artırmadaki etkisinin incelenmesi amacıyla Agilent 708-DS Çok Hücreli Çözünme Sistemi (Agilent Technologies, ABD) kullanılarak *in vitro* çözünme hızı çalışmaları yapılmıştır. Bu amaçla TBZ için pH 1.2 ve pH 6.8 ortamlarında, hazırlanan üç farklı katı dispersiyon için ise TBZ'nin düşük çözünürlük gösterdiği pH 6.8 ortamında *in vitro* çözünme hızı çalışmaları yapılmıştır. pH 1.2 (0.1 N HCl Çözeltisi) ve pH 6.8 ortamları Amerikan Farmakopesi'ne (USP 41) göre hazırlanmıştır [42,43]. Yapılan tüm *in vitro* çözünme hızı çalışmalarında USP Aparat II (Palet) kullanılmıştır. Paletin dönüş hızı 50 rpm, çözünme ortamının hacmi 900 ml ve sıcaklığı 37°C olarak belirlenmiştir. Örnekler otomatik olarak 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 15, 18 ve 24. saatlerde alınmıştır.

TBZ'nin çözünmesinin belirlenmesi için 50 mg TBZ doğrudan çözünme ortamına eklenmiştir. Önceden belirlenen zaman noktalarında numune alınmıştır.

Katı dispersiyonların TBZ'nin çözünmesi üzerine olan etkisinin değerlendirilmesi için çözünme hızı çalışmaları sadece pH 6.8 ortamında yapılmıştır. Çözünme hızı çalışmalarında 50 mg TBZ içeren 100 mg katı dispersiyon kullanılmıştır. Önceden belirlenen zaman noktalarında numune alınmıştır.

Alınan örnekler, HPLC (Agilent 1260 Infinity II, Agilent Technologies, ABD) kullanılarak analiz edilmiştir. Çözünme hızı analizinde kullanılan HPLC metodu miktar tayini metodunda kullanılan metottan farklıdır. Çözünme hızı analizine ait numunelerin analizinde kullanılan analitik metot parametreleri Tablo 3'te verilmiştir.

Mobil faz olarak, pH 10 tampon çözelti, metanol ve asetonitril karışımı (40:30:30 (h/h/h)) kullanılmıştır. Mobil faz, 0.45 µm filtreden süzülmüş ve 5 dakika boyunca degaze edilmiştir. Çözünme hızı cihazından alınan örneklerin seyreltilmesi için, pH 11.3 tampon çözelti ile asetonitril karışımından oluşan bir çözelti (20:80 (h/h)) kullanılmıştır.

Tablo 3. Çözünme hızı HPLC analizine ait analitik metot parametreleri

Kolon	C18, 150 x 4.6 mm, 5 µm (Waters, X-Bridge)
Dedeksiyon	UV, 230 nm
Akış Hızı	1.3 ml/dk
Enjeksiyon Hacmi	100 µl
Kolon Sıcaklığı	40 °C
Örnekleyici Sıcaklığı	5 °C
Analiz Süresi	11 dk

Katı dispersiyonların çözünme hızı testi tamamlandığında, her bir zaman noktasında alınan örnekler, 0.45 µm enjektör ucu filtre ile süzüldükten sonra HPLC analizi öncesi seyreltme işlemi yapılmıştır. Çözünme hızı cihazından alınan tüm örnekler aynı seyreltme oranında (1/5) seyreltilerek test çözeltileri hazırlanmıştır. Bu çözeltiler vorteks yardımıyla 15 dakika boyunca karıştırılmıştır. TBZ'nin %100 oranında çözünme göstermesi için hesaplanan konsantrasyon 11.11 µg/ml olmalıdır. Hesaplama yapılabilmesi için HPLC ile elde edilen pik alanlarına karşı çözelti konsantrasyonları (0.11-111 µg/ml) grafiğe geçirilerek kalibrasyon doğrusu çizilmiştir. Elde edilen kalibrasyon doğrusu kullanılarak TBZ'nin çözünen miktarları hesaplanmıştır. Stok çözeltiler 0.11-111 µg/ml konsantrasyon aralığında, etkin maddenin çözündüğü tek çözünme ortamı olan 0.1 N HCl'de hazırlanmış ve seyreltme işlemleri pH 11.3 tampon çözelti ve asetonitril karışımından oluşan bir çözelti (20:80 (h/h)) ile yapılmıştır. Kullanılan metot için analitik metot validasyonu yapılmıştır. Validasyon kapsamında doğrusallık, doğruluk (geri elde), kesinlik, seçicilik, stabilite parametreleri çalışılmıştır.

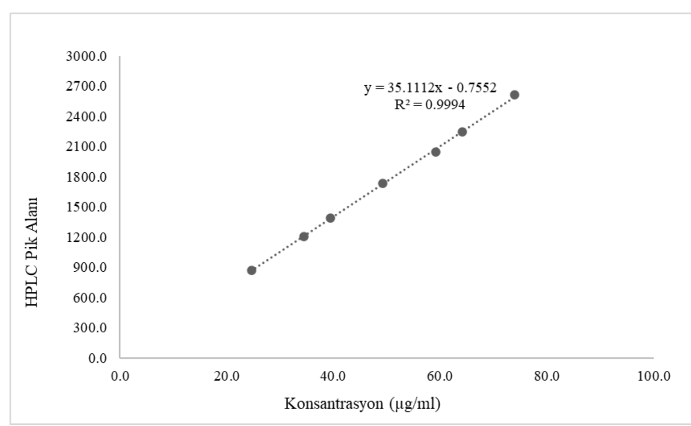
Katı dispersiyonlar için çözünme hızı analizinde elde edilen profillerin salım kinetiklerinin belirlenebilmesi için DD Solver yazılımı kullanılmıştır [44].

SONUÇ VE TARTIŞMA

Bu çalışma, suda düşük çözünürlüğe sahip bir ilaç olan TBZ'nin çözünme hızını katı dispersiyon teknolojisi kullanarak artırmayı amaçlamaktadır. Elde edilen bulgular, katı dispersiyon teknolojisinin TBZ'nin çözünmesini arttırdığını ve TBZ'nin farmasötik performansını iyileştirmede önemli bir potansiyele sahip olduğunu göstermiştir.

Tetrabenazin Miktar Tayini

Katı dispersiyonlarda TBZ miktarını hesaplamak için kullanılan kalibrasyon doğrusu ve hesaplamada kullanılan denklem Şekil 6'da verilmiştir.



Şekil 6. Katı dispersiyonlardaki tetrabenazin miktarını hesaplamak için kullanılan kalibrasyon doğrusu

Kalibrasyon doğrusu, HPLC'nin TBZ'nin miktar tayini için hassas bir yöntem olduğunu ve katı dispersiyonlardaki etkin madde içeriğini doğru bir şekilde tayin etmek için etkili bir analiz tekniği olduğunu göstermiştir. Kalibrasyon doğrusunun doğrusallığını değerlendirmek için r^2 olarak tanımlanan belirleme katsayısı kullanılmıştır. r^2 değeri 1'e yaklaştıkça grafiğin doğrusallığı artmaktadır [45]. Hesaplanan r^2 değerinin 1'e yakın olması, TBZ çözünürlüğünün konsantrasyonlara uygun şekilde ölçülebildiğini ve bu analitik yöntemin doğrusal ve tekrarlanabilir olduğunu göstermiştir. Analitik metot validasyonu kapsamında çalışılan doğruluk (geri elde) ve kesinlik parametrelerine ait değerlendirme ICH Q2 (R1) kılavuzuna göre yapılmıştır. Elde edilen tüm % bağıl sapma değerlerinin %2'nin altında olduğu görülmektedir. Böylece yöntemin doğruluğu ve kesinliği gösterilmiştir. Stabilite parametresi kapsamında hazırlanmış olan çözeltilerin 25ºC'de 32 saat boyunca stabil olduğu görülmüştür.

Katı dispersiyonlar için yapılmış olan miktar tayini analiz sonuçları Tablo 4'te verilmiştir.

Tablo 4. Katı dispersiyonlarda tetrabenazin miktar tayini sonuçları

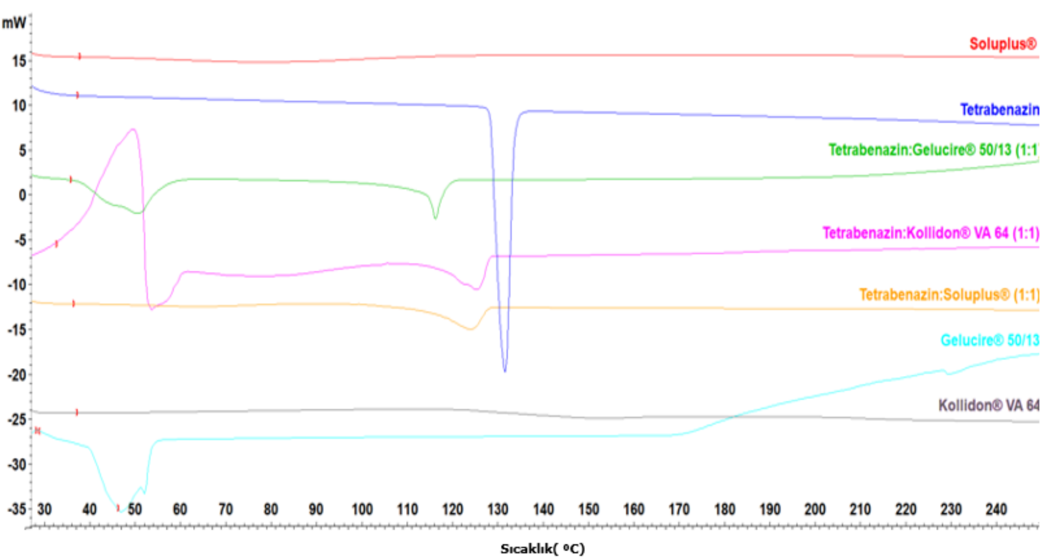
	KD-1		**KD-2**		**KD-3**	
	Miktar Tayini (mg)	Miktar Tayini (%)	Miktar Tayini (mg)	Miktar Tayini (%)	Miktar Tayini (mg)	Miktar Tayini (%)
Test-1	49.36	98.72	50.05	100.10	50.42	100.84
Test-2	49.64	99.28	49.87	99.74	49.48	98.96
Test-3	50.23	100.46	48.25	96.50	49.88	99.76
Test-4	48.91	97.82	48.59	97.18	48.55	97.10
Test-5	49.12	98.24	49.46	98.92	49.31	98.62
Test-6	48.81	97.62	48.72	97.44	49.94	99.88
Ortalama	49.35	98.69	49.16	98.31	49.60	99.19
Standart Sapma	0.53	1.06	0.74	1.48	0.64	1.29
Bağıl Standart Sapma (%)	1.07	1.07	1.50	1.50	1.30	1.30

Hazırlanan katı dispersiyon formülasyonlarının TBZ miktar tayini analiz sonuçları incelenmiştir. Tablo 4'e göre katı dispersiyonların TBZ miktar tayini sonuçlarına ait bağıl standart sapma değerlerinin düşük olması, analiz edilen örneklerde TBZ miktarının yüksek doğruluk ve tekrarlanabilirlikle tayin edildiğini göstermiştir. Her bir dispersiyon formülasyonunda altı farklı analiz gerçekleştirilmiş ve sonuçlar mg ve yüzde cinsinden raporlanmıştır. Elde edilen sonuçlara göre, KD-1 formülasyonunun ortalama 49.35 mg ve %98.69, KD-2 formülasyonunun ortalama 49.16 mg ve %98.31, ve KD-3 formülasyonunun ortalama 49.60 mg ve %99.19 TBZ içerdiği hesaplanmıştır. Bu değerler hedeflenen değere (50 mg veya %100) oldukça yakın bulunmuş olup TBZ'nin katı dispersiyonlara etkin bir şekilde yüklendiği tespit edilmiştir.

Her bir formülasyon için hesaplanan standart sapma değerleri, analizin tekrarlanabilirliği hakkında bilgi vermektedir. KD-1 formülasyonu için 0.53 (%1.06), KD-2 için 0.74 (%1.48) ve KD-3 için 0.64 (%1.28) standart sapma değerleri elde edilmiştir. Bu değerler, KD-1 formülasyonunun daha düşük bir varyasyon gösterdiğini ve bu nedenle daha güvenilir sonuçlar sunduğunu göstermiştir. KD-2 formülasyonundaki daha yüksek standart sapma, bu formülasyonun içeriğinde bazı tutarsızlıklar olabileceğini düşündürmüştür. Bağıl standart sapma değerleri ise sırasıyla %1.07, %1.50 ve %1.30 olarak hesaplanmıştır. Bu değerler, formülasyonların genel olarak iyi bir tekrarlanabilirlik sağladığını ancak KD-2'nin daha fazla optimizasyona ihtiyaç duyabileceğini göstermiştir. Tüm sonuçlar, katı dispersiyonların doğru bir şekilde analiz edilebildiğini ve formülasyonların tutarlılığını, standart sapma ve bağıl standart sapma değerleri de analizlerin güvenilirliğini göstermiştir.

Katı Dispersiyonların Termal Analizi

Yapılan analizlere ait DSC termogramları Şekil 7'de gösterilmiştir. TBZ, 130°C'de endotermik bir pik vermiştir. Hazırlanmış olan her 3 katı dispersiyon formülasyonunda, kristal yapı amorf yapıya dönüştüğü için endotermik pikin şiddeti azalmıştır.



Şekil 7. Tetrabenazin, polimerik taşıyıcılar, yüzey aktif madde ve katı dispersiyonların karşılaştırmalı DSC termogramları

DSC analizi, TBZ ve katı dispersiyonlarının termal özelliklerini incelemek için önemli bir yöntemdir. Grafik üzerinde ayrı ayrı TBZ, Soluplus®, Kollidon® VA 64, Gelucire® 50/13 ve hazırlanan 1:1 oranındaki katı dispersiyonların termogramları gösterilmiştir. Bu çalışmada, TBZ'nin ve katı dispersiyonlarının termal özellikleri farklı taşıyıcılarla (Soluplus®, Kollidon® VA 64, Gelucire® 50/13) birlikte değerlendirilmiştir.

DSC analizinin sonuçları, TBZ'nin saf formunun yaklaşık 130°C civarında belirgin bir erime noktasına sahip olduğunu göstermiştir. Elde edilen sonuç literatürde yer alan verilerle uyumlu bulunmuştur [41]. Saf TBZ'nin kristal yapısı gözlemlenen karakteristik endotermik pik ile doğrulanmış, katı dispersiyonlarda ise bu pikin şiddeti azalmıştır. Bu durum, TBZ'nin kristal yapısının başarıyla amorf forma dönüştürüldüğünü göstermiş, bu durum çözünürlüğün artmasında önemli bir faktör olarak değerlendirilmiştir. Kristal formdaki ilaç moleküllerinin daha stabil ve çözünmesi daha zor yapılar olması nedeniyle amorf formun, ilaçların çözünürlüğünü artırma potansiyeli daha yüksektir. Katı dispersiyonların hazırlanmasıyla kristal yapı bozulmuş ve ilaç molekülleri daha yüksek çözünme kapasitesine sahip düzensiz bir amorf yapıya geçmiştir. Kristallenme, ilacın biyoyararlanımını olumsuz etkileyebilmektedir. Bu nedenle katı dispersiyonlarda yeniden kristallenme gözlenmemiş olması olumlu bir sonuçtur [46,47].

Soluplus® polimeri için termogramda herhangi bir belirgin erime piki gözlenmemesi polimerin amorf yapıya sahip olduğunu ve belirgin bir erime sıcaklığının bulunmadığını göstermiştir. Kollidon® VA 64 polimeri de benzer şekilde kristal yapıya dair belirgin bir erime piki göstermemiştir. Gelucire® 50/13 polimeri, 40-50°C arasında geniş bir endotermik pik vermiştir, bu durum Gelucire®'in yarı kristal yapıya ve düşük erime sıcaklığına sahip olduğunu göstermiştir. Bu sonuçların Gelucire®'in yapısındaki yağ asitlerinin düşük erime sıcaklıklarından kaynaklandığı düşünülmüştür [32,48].

KD-1 formülasyonunun DSC termogramında TBZ'nin erime noktasında gösterdiği pikte kayma gözlenmiştir. Bu durumun, Soluplus® ile etkileşimin TBZ'nin kristal yapısını değiştirerek amorf bir yapı oluşturmasından kaynaklandığı düşünülmüştür. Soluplus®'ın yüksek çözünürlük özellikleri, TBZ'nin biyoyararlanımını artırma potansiyeli taşımaktadır. Çalışmalar, Soluplus® kullanılarak hazırlanan katı

dispersiyonların, kristal bir etkin maddenin çözünme özelliklerini önemli ölçüde artırabileceğini göstermiştir [49,50,51]. Ayrıca, amorf formların fiziksel stabiliteyi artırdığı gözlenmiş ve suda düşük çözünürlüğe sahip ilaçların taşınması ile ilişkili zorlukların aşılabileceği düşünülmüştür [52].

KD-2 formülasyonunun DSC termogramında endotermik piklerin şiddetinde TBZ'ye göre azalma gözlenmiştir. Bu durumda, polimerin kristal yapılı etkin maddenin amorf formunu stabil tutma yeteneğinin sınırlı olduğu, yani polimerin etkin maddenin kristal yapıdan amorf yapıya dönüşümünü tam anlamıyla engelleyemediği veya bu dönüşüm sonrasında amorf formun stabil kalmasını yeterince sağlayamadığı düşünülmüştür. Bu formülasyon, polimer miktarının amorf yapıların kararlı hale gelmesinde önemli olduğunu göstermiştir ve literatür ile uyumludur [53]. Ancak yine de hazırlanan katı dispersiyonda, TBZ'nin erime pikinin şiddetinin düştüğü ve amorf bir yapı oluştuğu gözlenmiştir. Kollidon® VA 64, katı dispersiyonların oluşmasında etkili bir polimerdir ve bu tür etkileşimlerin biyoyararlanımı artırdığı bilinmektedir [54,55].

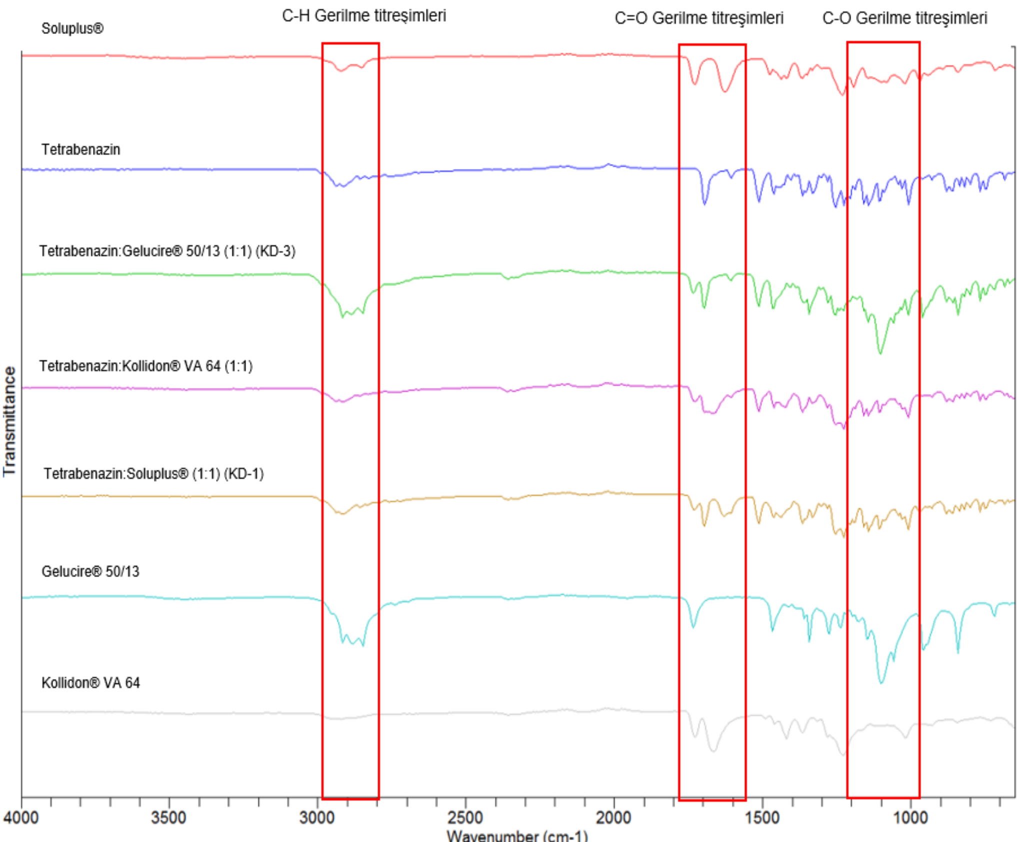
KD-3 formülasyonunun DSC termogramında endotermik piklerin şiddetinin azaldığı gözlenmiştir. Bu durum, TBZ'nin kristal yapısının kısmen bozulmuş olduğunu, ancak tamamen amorf hale geçmediği göstermiştir. Bunun nedeni, polimerin TBZ molekülleri ile yeterince güçlü bir etkileşim kuramaması olabilir. Gelucire® 50/13'ün, özellikle hidrofobik yapıdaki bileşenlerin çözünmesi üzerindeki olumlu etkileri olduğu bilinmektedir. Ayrıca Gelucire® 50/13, amfipatik özellikleri sayesinde iyi bir amorf yapı sağlamakta ve bu durum TBZ'nin çözünme özelliklerini artırma potansiyeli taşımaktadır [56,57]. Gelucire® 50/13 polimerinin düşük erime sıcaklıklarına rağmen çözünme ortamında erimeye başlamaması ve çözünme süreci boyunca yapısını koruması TBZ'nin termal stabilitesi açısından avantaj sağlar. Gelucire® 50/13 yüzey aktif maddesinin çözünme ortamında erimemesi, çözeltide hidrofobik mikroçevreler oluşturarak etkin maddeyle yüzey aktif maddenin daha uzun süre etkileşimini sağlar. Bu durum, çözünürlük artışını ve etkin maddenin kontrollü salımını sağlayabilir. Böylece daha dengeli bir biyoyararlanım sağlanabilir. Yüzey aktif maddenin çözünme ortamında erimeye başlamaması, salım kinetiklerinin değerlendirilmesinde önemli bir faktördür. Bu özellik, polimerin çözünmeye başlamadan yüzeyde bir bariyer gibi davranarak etkin maddenin kontrollü salımını sağlayabilir. Kinetik modeller açısından bakıldığında, bu durum daha çok sıfırıncı veya birinci dereceden kinetik bir model yerine Higuchi ya da Korsmeyer-Peppas gibi difüzyon kontrollü modellerin kullanımını destekleyebilir.

Katı Dispersiyonların Fourier Dönüşümlü Kızılötesi (FT-IR) Spektroskopisi ile Analizi

Yapılan analizlere ait FT-IR spektrumları Şekil 8'de gösterilmiştir. FT-IR spektrumları, TBZ'nin farklı taşıyıcı polimerlerle etkileşimlerini göstererek hazırlanan katı dispersiyonların karakteristik fonksiyonel gruplar üzerindeki etkilerini göstermiştir.

FT-IR spektroskopisi, maddelerin yapısındaki fonksiyonel grupların tayin edilmesi için kullanılan kimyasal etkileşimler, özellikle hidrojen bağları veya diğer moleküler etkileşimler hakkında bilgi veren bir yöntemdir [58,59].

TBZ'nin FT-IR spektrumunda, 2886 cm^{-1} dalga boyunda belirgin C-H gerilme pikleri gözlenmiştir. Bu pikler, metil ($-CH_3$) veya metilen ($-CH_2-$) gruplarındaki C–H bağlarından kaynaklanmıştır. Ayrıca, 1700 cm^{-1} dalga boyunda C=O gerilme titreşimleri gözlemlenmiştir. Bu pikler, molekülde karbonil grubunun varlığını göstermiştir. Bunun yanında, 1008 cm^{-1} dalga boyunda C-O gerilme titreşimleri gözlenmiştir. Bu pikler, TBZ'nin molekül yapısındaki eter veya ester bağlarından kaynaklanmıştır. Bu bölgelerdeki pikler, TBZ molekülünün karakteristik yapılarını ortaya koyarak hidrojen bağlanması gibi olası etkileşimler hakkında bilgi vermiştir. Elde edilen veriler literatür ile uyumludur [19].



Şekil 8. Tetrabenazin, polimerik taşıyıcılar, yüzey aktif madde ve katı dispersiyonların karşılaştırmalı FT-IR spektrumları

Soluplus® polimerinde, 2920 cm^{-1} ve 2853 cm^{-1} dalga boylarında C-H gerilme titreşimleri ve 1729 ve 1628 cm^{-1} dalga boylarında belirgin C=O gerilme pikleri gözlenmiştir [60]. Bu pikler, Soluplus®'ın yapısında ester ve karbonil yapıları bulunduğunu göstermiştir. Ayrıca, 1231 cm^{-1}, 1192 cm^{-1} ve 1019 cm^{-1} dalga boylarında C-O gerilme titreşimleri de Soluplus® yapısında eter veya ester gruplarının bulunduğunu göstermiştir [61]. Kollidon® VA 64'ün FT-IR spektrumunda, 2920 cm^{-1} dalga boyunda belirgin bir C-H gerilme pikinin yanı sıra, 1729 cm^{-1} ve 1668 cm^{-1} dalga boylarında C=O gerilme pikleri gözlenmiştir. Bu piklerin varlığı, Kollidon® VA 64'ün yapısında karbonil gruplarının bulunduğunu göstermiştir. Ayrıca, 1231 cm^{-1} ve 1019 cm^{-1} dalga boylarında gözlenen C-O gerilme titreşimleri, yapıda eter veya ester gruplarının bulunduğunu göstermiştir [62]. Gelucire® 50/13'ün FT-IR spektrumunda, 2886 cm^{-1} dalga boyunda bir C-H gerilme titreşimi, 1735 cm^{-1} dalga boyunda belirgin bir C=O gerilme piki ve 1103 cm^{-1} dalga boyunda C-O gerilme titreşimleri gözlenmiştir. Bu pikler, Gelucire®'nin ester yapısında olduğunu ve polimer içinde yağ asitlerinin bulunduğunu göstermiştir [48,63].

KD-1 formülasyonunda, 2920 cm^{-1} ve 2853 cm^{-1} dalga boylarındaki C-H gerilme piklerinin belirgin bir şekilde azaldığı gözlenmiştir. Bu durum, TBZ ile Soluplus® arasında bir etkileşim olduğunu, özellikle hidrojen bağlarının zayıfladığını düşündürmüştür. C=O bölgesinde, 1700 cm^{-1} dalga boyunda kaymalar gözlenmiştir. Bu durum, TBZ ile Soluplus® arasında kimyasal veya fiziksel bir etkileşim olduğunu düşündürmüştür. Ayrıca 1000-1100 cm^{-1} dalga boyu aralığında C-O gerilme titreşimlerinde değişiklikler gözlenmiş ve bu durum moleküller arasında etkileşimlerin varlığını desteklemiştir.

KD-2 formülasyonunda, 2920 cm^{-1} dalga boyunda C-H gerilme titreşimlerinde çok belirgin bir değişiklik gözlenmemiştir. Bu durum, Kollidon® VA 64 ile TBZ arasındaki etkileşimin diğer polimerlerle kıyasla daha zayıf olduğunu göstermiştir. C=O bölgesinde, 1700 cm^{-1}'de gözlenen küçük değişiklikler, TBZ ile Kollidon® VA 64 arasında zayıf etkileşimler olduğunu düşündürmüştür. 1231

cm^{-1} ve 1000 cm^{-1} dalga boylarında Kollidon® VA 64'te belirgin bir C-O piki gözlenmiş olması polimerdeki eter veya ester yapısının varlığını göstermiştir, ancak TBZ ile etkileşimi sınırlı kalmıştır.

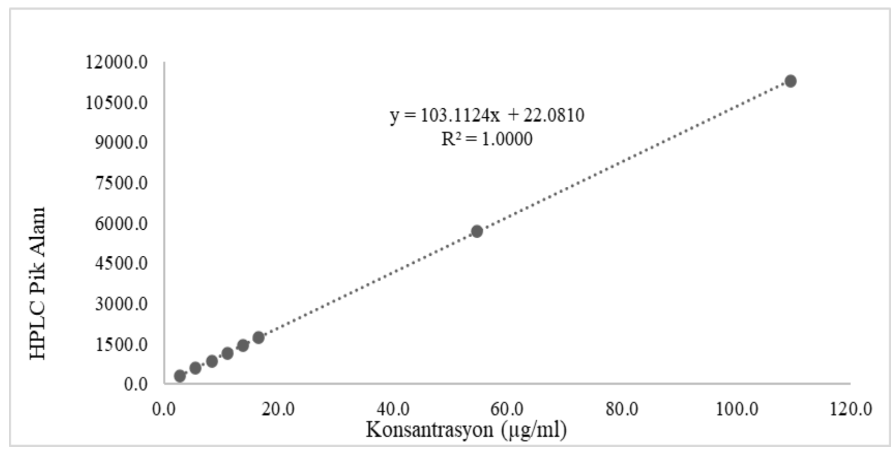
KD-3 formülasyonunda, 2886 cm^{-1} dalga boyunda C-H gerilme pikinde hafif bir değişim gözlenmiş ve bu durum iki madde arasında bir etkileşimi göstermiştir. C=O bölgesinde, 1700 cm^{-1}'deki küçük kaymalar, Gelucire®'nin yağ asitleri ve ester yapısının TBZ ile etkileşimde olduğunu düşündürmüştür. Bu durum, Gelucire® ile TBZ arasındaki fiziksel bağların varlığını düşündürmüştür. Ayrıca, 1000-1100 cm^{-1} dalga boyu aralığında gözlenen C-O titreşimlerinin değişimi de TBZ ve Gelucire® 50/13 arasında etkileşim olduğu göstermiştir.

1:1 oranında hazırlanan katı dispersiyonların spektrumlarında, özellikle C-H, C=O ve C-O bölgelerinde kaymalar görülmüştür. Bu pik kaymaları, TBZ'nin polimerlerle fiziksel veya kimyasal etkileşiminin olduğunu göstermiştir. KD-1 formülasyonunda, hidroksil gruplarının etkileşimi azalırken karbonil bölgelerinde belirgin kaymalar gözlenmiştir. Bu durum, iki madde arasında güçlü bir etkileşim olduğunu göstermiştir. Benzer şekilde, KD-3 formülasyonunda hidrojen bağları azalmış ve karbonil gruplarında kaymalar gözlenmiştir. KD-2 formülasyonunda ise nispeten daha zayıf etkileşimler gözlenmiştir.

Sonuç olarak, bu spektral analizler TBZ ile farklı taşıyıcı polimerler arasında önemli etkileşimler olduğunu göstermiştir. Özellikle Soluplus® ve Gelucire® 50/13, TBZ ile güçlü hidrojen bağları ve karbonil etkileşimleri sergilemiştir. Bu etkileşimler, katı dispersiyonların TBZ'nin çözünmesini artırarak biyoyararlanımı artırabileceğini göstermektedir. Kollidon® VA 64'ün nispeten daha zayıf etkileşimler göstermiş olması, bu polimerin çözünmeyi artırmada daha az etkili olduğunu göstermiştir.

İn Vitro Çözünme Hızı Çalışmaları ve Salım Kinetiklerinin Değerlendirilmesi

Katı dispersiyonlardaki TBZ etkin maddesinin çözünen miktarını hesaplamak için kullanılan kalibrasyon doğrusu ve hesaplamada kullanılan denklem Şekil 9'da verilmiştir.

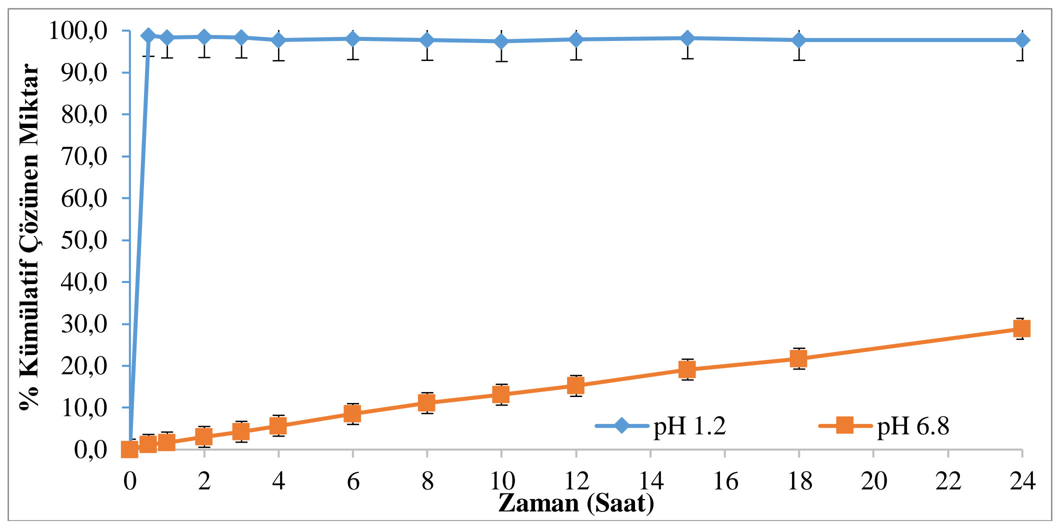


Şekil 9. Tetrabenazinin çözünen miktarını hesaplamak için kullanılan kalibrasyon doğrusu

Elde edilen sonuçlar, kullanılan analitik metot ile TBZ'nin çözünme hızı çalışması analizleri ile elde edilen konsantrasyonların doğru bir şekilde hesaplandığını göstermiştir. Bu metot, farklı formülasyonlar arasındaki çözünme hızı profillerinin karşılaştırılmasını ve TBZ konsantrasyonlarının doğru bir şekilde ölçülmesini sağlamıştır. Kalibrasyon doğrusunun doğrusallığını değerlendirmek için r^2 olarak tanımlanan belirleme katsayısı kullanılmıştır. r^2 değeri 1'e yaklaştıkça grafiğin doğrusallığı artmaktadır [45]. Hesaplanan r^2 değerinin 1 olması, TBZ'nin çözünmesinin konsantrasyonlara uygun şekilde ölçülebildiğini ve bu analitik yöntemin tekrarlanabilir olduğunu göstermiştir. Analitik metot validasyonu kapsamında çalışılan doğruluk (geri elde) ve kesinlik parametrelerine ait değerlendirme ICH Q2 (R1) kılavuzuna göre yapılmıştır. Elde edilen tüm % bağıl sapma değerlerinin %2'nin altında olduğu görülmektedir. Böylece yöntemin doğruluğu ve kesinliği gösterilmiştir. Stabilite parametresi kapsamında hazırlanmış olan çözeltilerin 5ºC'de 24 saat boyunca stabil olduğu görülmüştür.

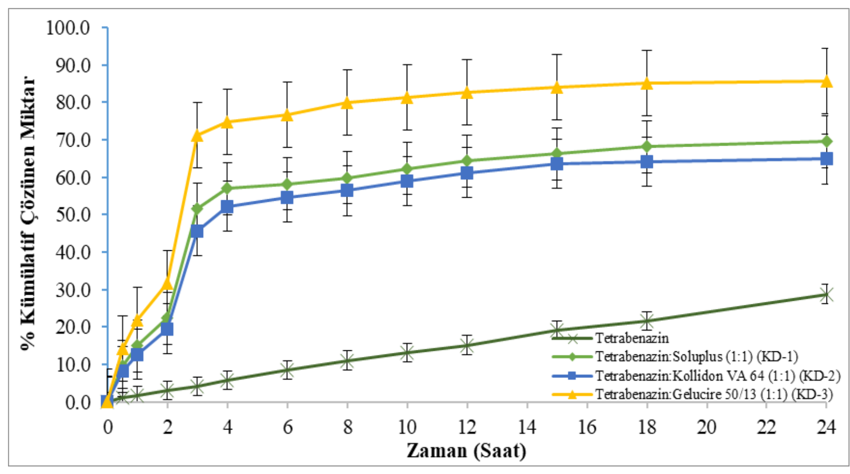
Saf TBZ etkin maddesinin pH 1.2 ve pH 6.8 ortamlarında 24 saatin sonunda çözünen miktarları sırasıyla %97.7 ve %28.8 olarak hesaplanmıştır.

TBZ'nin çözünmesini tayin etmek için pH 1.2 ve pH 6.8 ortamlarında elde edilen *in vitro* çözünme hızı profilleri Şekil 10'da yer almaktadır.



Şekil 10. Tetrabenazin etkin maddesinin pH 1.2 ve pH 6.8 ortamlarındaki *in vitro* çözünme hızı profilleri (n=6, Ortalama ± SD)

Katı dispersiyonlardan, TBZ'nin pH 6.8 ortamında, 24 saatin sonunda çözünen miktarı KD-1, KD-2 ve KD-3 formülasyonları için sırasıyla %69.5, %64.5 ve %85.6 olarak hesaplanmıştır. TBZ ve hazırlanan katı dispersiyonların pH 6.8 ortamında yapılan *in vitro* çözünme hızı profilleri Şekil 11'de yer almaktadır.



Şekil 11. Tetrabenazin ve hazırlanan katı dispersiyonların pH 6.8 ortamındaki karşılaştırmalı *in vitro* çözünme hızı profilleri (n=6, Ortalama ± SD)

Bu çalışmada, TBZ'nin çözünmesi ve katı dispersiyonların bu çözünmeye etkisi pH 1.2 ve pH 6.8 ortamlarında incelenmiştir. TBZ, merkezi sinir sistemi hastalıklarının tedavisinde kullanılan bir ilaç olup, sudaki düşük çözünürlüğü nedeniyle biyoyararlanımı zayıftır [19]. Dolayısıyla, katı dispersiyon teknolojisi, çözünürlük ve biyoyararlanımı artırmak için etkili bir yöntem olarak öne çıkmaktadır.

Çözünme hızı analizlerinde TBZ, pH 1.2 ortamında hızlı çözünme göstermiş ve ilk 1 saatte tamamen çözünmüştür. 24 saatin sonunda pH 1.2 ortamında etkin maddenin %98.8'i çözünmüştür. Bu yüksek çözünme oranı, TBZ'nin asidik ortamda, özellikle mide ortamında daha iyi çözündüğünü ve dolayısıyla biyoyararlanımının artırılabileceğini göstermiştir. Asidik ortamın, TBZ'nin iyonize olmasını sağlayarak daha iyi çözünmesini sağladığı düşünülmüştür. Buna karşın, 24 saatin sonunda pH 6.8 ortamında TBZ'nin %28.8'i çözünmüştür. Bu durum, TBZ'nin bağırsak ortamında sınırlı bir çözünürlüğe sahip olduğunu göstermiştir.

Farklı polimerik taşıyıcılar kullanılarak hazırlanan katı dispersiyonlar, TBZ'nin çözünmesini artırmayı amaçlamıştır. Bu çalışmada, Soluplus®, Kollidon® VA 64 polimerleri ve Gelucire® 50/13 yüzey aktif maddesi ile hazırlanan katı dispersiyonlar TBZ çözünürlüğünün düşük olduğu pH 6.8 ortamında incelenmiş ve tüm formülasyonların çözünme üzerinde olumlu etkileri gözlenmiştir. Özellikle Gelucire® 50/13 ile hazırlanan katı dispersiyon, pH 6.8 ortamında, 24 saat sonunda %85.6 oranında çözünme sağlamıştır. Hazırlanan 3 katı dispersiyon arasından en yüksek çözünme bu formülasyon ile elde edilmiştir. Bu durumun, Gelucire® 50/13'ün hem yüzey aktif madde hem de çözücü ajan özelliklerinden kaynaklandığı düşünülmüştür. Gelucire® 50/13, etkin maddenin daha iyi ıslanmasını ve dolayısıyla daha iyi çözünmesini sağlamıştır. Soluplus® ve Kollidon® VA 64 ile hazırlanan katı dispersiyonlar ise 24 saatin sonunda sırasıyla %69.5 ve %64.9 çözünme oranları ile çözünmeyi artırmış ancak Gelucire® kadar etkili olmamıştır. Elde edilen sonuçlar, katı dispersiyonların, TBZ'nin çözünmesini artırmada etkili bir strateji olduğunu göstermiştir.

Soluplus® hidrofobik ilaçların çözünürlüğünü artırmada yardımcı olan ve çözünmeyi hızlandırıcı özelliklere sahip bir taşıyıcıdır, hem yüzey aktif madde özellikleriyle etkin maddenin daha iyi ıslanmasını sağlayarak çözünme hızını artırmaktadır. Ancak elde edilen sonuçlar Soluplus®'ın çözünme oranını Gelucire® 50/13 kadar artırmadığını göstermiştir. Kollidon® VA 64, literatürde "paraşüt etkisi" olarak da bilinen, çözünen etkin maddenin doygunluk konsantrasyonunda yeniden kristallenmesini önleyici bir etkiye sahiptir. Bu etki, TBZ çözünürlüğünde bir artış sağlamakla birlikte, salım oranının düşük kalmasını açıklamaktadır [64]. Gelucire® sahip olduğu fizikokimyasal özelliklerden dolayı etkin maddelerin çözünürlüğünü hem *in vitro* hem de *in vivo* olarak arttıran etkili bir yüzey etkin maddedir [32-36]. Çözünürlüğü düşük ilaçların ıslanabilirliğini artırarak farmasötik formülasyonlarda geniş bir kullanım alanı bulmasına olanak tanımıştır. Gelucire® 50/13'ün farmasötik formülasyonlarda emilimi kolaylaştırarak ilaçların biyoyararlanımını artırma kapasitesi, bağırsak ortamında dikkat çekmiştir. Dolayısıyla, Gelucire® 50/13'ün kullanılması, TBZ'nin biyoyararlanımını artırmanın yanı sıra, tedavi edici etkinliğinin de yükselmesine katkı sağlayabilir. Bu çalışma, katı dispersiyon teknolojisinin etkinliğini ve Gelucire® 50/13 gibi farklı taşıyıcı polimerlerin kullanımının farmasötik formülasyonlardaki potansiyelini göstermektedir. Her üç taşıyıcının farklı mekanizmalarla etkin maddenin çözünmesini artırdığı görülmüştür.

Bu çalışmada, polimerlerin etkin maddenin çözünmesi üzerindeki etkisi net bir şekilde gözlenmiştir. Üç farklı katı dispersiyonun sonuçlarını karşılaştırdığımızda, taşıyıcı polimer seçiminin TBZ'nin çözünme davranışını belirlemede kritik bir rol oynadığı gözlenmiştir. Yüzey aktif madde ve çözücü ajan özelliklerine sahip olan Gelucire® 50/13, TBZ'nin çözünmesini artırmada Soluplus® ve Kollidon® VA 64'ten daha etkili bulunmuştur. Bu bulgu, yüzey aktif madde özelliklerine sahip yardımcı maddelerin, TBZ gibi çözünürlüğü düşük ilaçlar için katı dispersiyonların çözünmeyi önemli ölçüde artırabileceğini göstermiştir. Ayrıca, Soluplus® ve Kollidon® VA 64 gibi polimerlerin de çözünme artışı sağladığı, ancak yüzey aktif maddelerin ilave avantajlar sunduğu görülmüştür.

İn vitro çözünme hızı çalışmaları sonucunda KD-1, KD-2 ve KD-3 formülasyonları için DDSolver yazılımı ile zamana karşı % çözünme değerleri kullanılarak salım kinetikleri belirlenmiştir. Formülasyonların farklı salım kinetiklerine göre değerlendirme sonuçları Tablo 5'te yer almaktadır.

Tablo 5. KD-1, KD-2 ve KD-3 formülasyonlarından tetrabenazin salımının farklı salım kinetiklerine göre değerlendirme sonuçları

Formülasyon Türü	**Tetrabenazin:Soluplus (1:1)**		**Tetrabenazin:Kollidon VA 64 (1:1)**		**Tetrabenazin:Gelucire 50/13 (1:1)**	
Matematiksel Modeller	Parametreler		Parametreler		Parametreler	
0. Derece	r^2	0.0849	r^2	0.1581	r^2	-0.1113
	r^2adj	0.0849	r^2adj	0.1581	r^2adj	-0.1113
	AIC	116.8853	AIC	114.6430	AIC	125.2649
	MSC	-0.4032	MSC	-0.2992	MSC	-0.6338
	Modele Özgü Katsayı	K_0=4.3757	Modele Özgü Katsayı	K_0=4.1183	Modele Özgü Katsayı	K_0=5.5762
1. Derece	r^2	0.6912	r^2	0.6875	r^2	0.8805
	r^2adj	0.6912	r^2adj	0.6875	r^2adj	0.8805
	AIC	102.7647	AIC	101.758	AIC	96.2799
	MSC	0.6830	MSC	0.6920	MSC	1.5958
	Modele Özgü Katsayı	K_1=0.1066	Modele Özgü Katsayı	K_1=0.0905	Modele Özgü Katsayı	K_1=0.2526
Higuchi	r^2	0.7840	r^2	0.8066	r^2	0.7087
	r^2adj	0.7840	r^2adj	0.8066	r^2adj	0.7087
	AIC	98.1191	AIC	95.5233	AIC	107.8566
	MSC	1.0403	MSC	1.1716	MSC	0.7053
	Modele Özgü Katsayı	K_H=18.1156	Modele Özgü Katsayı	K_H=16.9943	Modele Özgü Katsayı	K_H=23.3228
Korsmeyer-Peppas	r^2	0.8674	r^2	0.8713	r^2	0.8434
	r^2adj	0.8554	r^2adj	0.8596	r^2adj	0.8291
	AIC	93.7721	AIC	92.2281	AIC	101.7912
	MSC	1.3747	MSC	1.4251	MSC	1.1719
	Modele Özgü Katsayı	K_{KP}=27.2364 n=0.3351	Modele Özgü Katsayı	K_{KP}=24.5646 n=0.3511	Modele Özgü Katsayı	K_{KP}=38.0472 n=0.3015
Hixson-Crowell	r^2	0.5615	r^2	0.5650	r^2	0.7897
	r^2adj	0.5615	r^2adj	0.5650	r^2adj	0.7897
	AIC	107.3218	AIC	106.0578	AIC	103.6228
	MSC	0.3324	MSC	0.3612	MSC	1.0310
	Modele Özgü Katsayı	K_{HC}=0.0284	Modele Özgü Katsayı	K_{HC}=0.0243	Modele Özgü Katsayı	K_{HC}=0.0579
Weibull	r^2	0.9276	r^2	0.9291	r^2	0.9302
	r^2adj	0.9132	r^2adj	0.9150	r^2adj	0.9162
	AIC	87.8983	AIC	86.4712	AIC	93.2864
	MSC	1.8265	MSC	1.8679	MSC	1.8261
	Modele Özgü Katsayı	α=2.7580 β=0.4303 T_i=0.4674	Modele Özgü Katsayı	α=3.120 β=0.4369 T_i=0.4700	Modele Özgü Katsayı	α=1.9911 β=0.5422 T_i=0.4274

* AIC: Akaike bilgi ölçütü, MSC: istatistiksel model seçim kriteri

KD-1, KD-2 ve KD-3 formülasyonlarının olası kinetik mekanizmalarını değerlendirebilmek için çözünme hızı analizleri sonucunda elde edilen çözünme hızı profillerinin 0. derece, 1. derece, Higuchi, Korsmeyer-Peppas, Hixson-Crowell ve Weilbull kinetik modellerine uyumlulukları DDSolver yazılımı kullanılarak analiz edilmiştir. DDSolver, ilaç çözünme hızı profillerinin modellenmesi ve karşılaştırılması için kullanılan bir eklenti programıdır [44]. Yapılan analizlerde değerlendirme kriterleri olarak r^2, r^2adj, AIC, MSC ve modellere özgü katsayılar kullanılmıştır.

Değerlendirme kriterlerinden r^2 belirleme katsayısı olarak tanımlanır ve r^2 değerinin 1'e yakın olması matematiksel modelin profile uyumunu artırmaktadır. Ancak genellikle r^2 değeri modele eklenen değişkenden bağımsız olarak daha fazla model parametresi eklenmesiyle artma eğilimindedir. Bu nedenle r^2 yerine r^2adj parametresinin kullanılması önerilir. r^2 parametresi ile benzer şekilde r^2adj parametresinin de 1'e yakın olması modele uyumu artırmaktadır [60,65,66]. AIC parametresi, Akaike bilgi ölçütü olarak tanımlanır ve daha düşük AIC değerine sahip modelin daha uyumlu olduğu söylenebilir [65]. MSC parametresi, istatistiksel model seçim kriteri olarak tanımlanmaktadır. Farklı modeller arasında, en yüksek MSC değerine sahip model en uygun model olarak değerlendirilmektedir. Modelin MSC değerinin genellikle 3'ten büyük olması iyi bir uyumu gösterir [60,66].

KD-1, KD-2 ve KD-3 formülasyonları 0. derece kinetik açısından değerlendirildiğinde r^2, r^2adj ve MSC değerlerinin oldukça düşük, AIC değerlerinin ise yüksek olduğu görülmüştür. Katı dispersiyonların bu model ile uyumlu olmadığı görülmüştür. 1. derece kinetik açısından değerlendirme yapıldığında, KD-1 ve KD-2 için hesaplanan düşük r^2, r^2adj ve MSC değerleri KD-1 ve KD-2'nin bu model ile uyumlu olmadığını, KD-3 için hesaplanan r^2 ve r^2adj değerlerinin 1'e yakın olması ve nispeten daha yüksek MSC değeri KD-3'ün bu model ile uyumlu olabileceğini göstermiştir. Higuchi kinetiği açısından değerlendirme yapıldığında, KD-1 ve KD-2 için hesaplanan r^2 ve r^2adj değerlerinin 1'e yakın ve KD-3 için hesaplanan r^2 ve r^2adj değerlerinden yüksek olduğu belirlenmiştir. Ek olarak, KD-1 ve KD-2'nin KD-3'e göre daha yüksek hesaplanan MSC ve daha düşük hesaplanan AIC değerleri KD-3'ün bu model ile uyumlu olmadığını ancak KD-1 ve KD-2'nin uyumlu olabileceğini göstermiştir. Korsmeyer-Peppas kinetiği açısından değerlendirme yapıldığında her üç katı dispersiyonun hesaplanan yüksek r^2, r^2adj ve MSC değerleri ve nispeten düşük AIC değerleri katı dispersiyonların bu model ile uyumlu olabileceğini göstermiştir. Bu kinetiğe uyumluluk analiz sonuçları değerlendirildiğinde KD-1, KD-2 ve KD-3 için modele özgü katsayılar sırasıyla n=0.3351, n=0.3511 ve n=0.3015 olarak belirlenmiştir. Bu kinetik modelde n değerinin 0,5'ten küçük olması yarı-Fick difüzyon, 0.5 değeri Fick difüzyon, 0,5 ile 1 arasında olması ise non-Fick transport olarak değerlendirilmektedir [67]. Hixson-Crowell kinetik açısından değerlendirme yapıldığında, KD-1 ve KD-2 için hesaplanan düşük r^2, r^2adj ve MSC değerleri KD-1 ve KD-2'nin bu model ile uyumlu olmadığını, KD-3 için hesaplanan r^2 ve r^2adj değerlerinin 1'e yakın olması ve nispeten daha büyük MSC değeri KD-3'ün bu model ile uyumlu olabileceğini göstermiştir. KD-1, KD-2 ve KD-3 formülasyonlarının *in vitro* çözünme hızı testi sonucunda elde edilen % kümülatif çözünen değerlerine göre hesaplanan parametreler değerlendirildiğinde en yüksek r^2, r^2adj ve MSC değerleri ile en düşük AIC değerine sahip olan Weibull kinetiğinin her üç formülasyon için uyumlu bir model olabileceği düşünülmüştür. KD-1, KD-2 ve KD-3 formülasyonları için Weibull kinetiğine ait modele özgü katsayı değerleri sırasıyla β=0.4303, β=0.4369 ve β=0.5422 olarak hesaplanmıştır. Bu kinetik modelde β değerinin 0.75'ten küçük olması Fick difüzyon, 0.75 ile 1 arasında olması birleşik salım ve 1'den büyük olması kompleks salım mekanizması olarak değerlendirilmektedir [60]. Her üç katı dispersiyondan TBZ salımı Korsmeyer-Peppas ve Weibull kinetikleriyle uyumlu ve Fick difüzyona benzer şekilde gerçekleşmiştir.

Sonuç

Bu çalışmada, TBZ'nin bazik ortamdaki çözünme özelliklerini iyileştirmek amacıyla katı dispersiyon formülasyonları hazırlanmış ve bu formülasyonların karakterizasyon çalışmaları yapılmıştır. Çalışmada suda düşük çözünürlük gösteren TBZ'nin çözünme özelliklerinin katı dispersiyon teknolojisi kullanılarak iyileştirildiği gözlenmiştir. Bu çalışma katı dispersiyon teknolojisinin, TBZ'nin çözünmesini, biyoyararlanımını ve dolayısıyla tedavi etkinliğini artırma potansiyeli olduğunu göstermiştir.

YAZAR KATKILARI

Kavram: H.B., F.Y., F.A.; Tasarım: H.B., F.Y., F.A.; Denetim: H.B., F.A.; Kaynaklar: H.B., F.A.; Malzemeler: H.B., F.Y., F.A.; Veri Toplama ve/veya İşleme: H.B., F.Y., F.A.; Analiz ve/veya Yorumlama: H.B., F.A.; Literatür Taraması: H.B., F.A.; Makalenin Yazılması: H.B., F.Y., F.A.; Kritik İnceleme: H.B., F.A.; 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.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

PHENOLIC CONTENT AND ANTIBACTERIAL ACTIVITY OF *COTA PESTALOZZAE* BOISS. AERIAL PARTS

COTA PESTALOZZAE BOISS TOPRAK ÜSTÜ KISIMLARININ FENOLİK İÇERİĞİ VE ANTİBAKTERİYEL AKTİVİTESİ

Özge YILMAZ[1,2], Suna Sibel RIZVANOGLU[3], Özlem BAHADIR ACIKARA[1]*

[1]Ankara University, Faculty of Pharmacy, Department of Pharmacognosy, 06560, Ankara, Turkey
[2]Ankara University, Graduate School of Health Science, 06110, Ankara, Turkey
[3]Ankara University, Department of Pharmaceutical Microbiology, 06100, Ankara, Turkey

ABSTRACT

Objective: *The genus Cota was once considered a subgenus of Anthemis but is now recognized as an independent genus. The species belonging to Cota, including several endemic to Turkey, have been less extensively studied in the literature than other plants in the Asteraceae family. The current study aimed to examine the phenolic content and antibacterial activity of C. pestalozzae Boiss., an endemic species from Turkey.*

Material and Method: *The phenolic content of the methanol extract prepared from the aerial parts of C. pestalozzae Boiss. was analysed qualitatively and quantitatively using high-performance liquid chromatography (HPLC) analysis. This study calculated the limit of detection (LOD) and quantification (LOQ) for each detected compound. The antibacterial activity of the extract was also investigated against some Gram (+) and (-) bacteria strains.*

Result and Discussion: *In the current study, chlorogenic acid, 4,5-O-dicaffeoyl quinic acid, 3,5-O-dicaffeoyl quinic acid, rutin, hyperoside and isoquercetin were qualitatively and quantitatively detected in the methanolic extract of the aerial parts of C. pestalozzae Boiss. Extract of the aerial parts exhibited antibacterial activity only against Staphylococcus aureus ATCC 43300 (MRSA) with a MIC value of 10000 µg/ml. The research indicates that C. pestalozzae contains phenolic compounds and is effective against Gram (+) bacteria.*

Keywords: *Antibacterial activity, Asteraceae, Cota, flavonoids, HPLC, phenolic acids*

ÖZ

Amaç: *Cota cinsi, Anthemis cinsinin alt türleri arasında sınıflandırılırken günümüzde ayrı bir cins olarak kabul edilmektedir. Türkiye'de de endemik türleri bulunan Cota cinsine ait türler literatürde diğer Asteracea bitkilerine kıyasla daha az çalışılmıştır. Bu çalışmada, Türkiye'de endemik bir tür olan C. pestalozzae Boiis. türünün fenolik içeriği ve antibakteriyel aktivitesinin araştırılması amaçlanmıştır.*

Gereç ve Yöntem: *C. pestalozzae Boiss. türünün toprak üstü kısmından hazırlanan metanollü ekstrenin fenolik içeriği Yüksek Performanslı Sıvı Kromatografisi (HPLC) analizi ile kalitatif ve kantitatif olarak analiz edilmiştir. Bu çalışmada, tespit edilen bileşiklerin her biri için tespit limiti ve tayin limiti hesaplanmıştır. Ayrıca ekstrenin antibakteriyel aktivitesi bazı Gram (+) ve (-) bakteri türlerine karşı araştırılmıştır.*

*__Corresponding Author / Sorumlu Yazar:__ Özlem Bahadır Acıkara
e-mail / e-posta: obahadir@ankara.edu.tr, **Phone / Tel.:** +903122033090

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Sonuç ve Tartışma: *Yapılan çalışma, C. pestalozzae Boiss. bitkisinin toprak üstü kısmının metanollü ekstresinde klorojenik asit, 4,5-O-dikafeoil kinik asit, 3,5-O-dikafeoil kinik asit, rutin, hiperozit ve izokersetin kalitatif ve kantitatif olarak tespit edilmiştir. C. pestalozzae toprak üstü kısmı ekstresi sadece Staphylococcus aureus ATCC 43300'e (MRSA) karşı 10000 µg/ml MIC değeri ile antibakteriyel aktivite sergilemiştir. Çalışma sonuçları C. pestalozzae'nin fenolik bileşikler içerdiğini ve Gram (+) bakterilere karşı etkili olduğu göstermiştir.*
Anahtar Kelimeler: *Antibakteriyel aktivite, Asteraceae, Cota, fenolik asitler, flavonoitler, HPLC*

INTRODUCTION

The *Cota* J. Gay genus, also known as *Anthemis*, was previously classified as a subspecies of the *Anthemis* genus but is now recognised as a distinct genus within the Asteraceae family [1].The most important difference between the two morphologically similar genera is the shape of the achene, and *Cota* species are distinguished from *Anthemis* species by their dorsoventrally flattened, invertedly conical-shaped achenes with prominent ribs, either straight or with 3-10 ribs on each side [2]. *Cota* species are distributed in Europe (except for North European areas), North Africa, Central Asia, the Caucasus and Southwest Asia [3,4]. The genus *Cota* comprises 63 taxa worldwide, with 22 distinct taxa in Turkey. Notably, 9 of these are endemic, highlighting Turkey's significant role in preserving global biodiversity [2]. Studies on *Cota* species are limited, and as indicated in the literature, the flowers of *Cota* species are antiseptic and are used as healing plants [4]. In Europe, *Anthemis* species, the synonym of the genus *Cota*, are known to be used in tea, ointment, extract or tincture forms for sedative, antibacterial, anti-inflammatory and antispasmodic purposes [5]. The importance of *Cota* species in the pharmaceutical, food and cosmetic areas is increasing, with flavonoids and essential oils as major constituents [6]. In a study, the essential oil and fixed oil components of the endemic plant *C. hamzaoglui* Özbek & Vural were analysed by gas chromatography (GC). The essential oil was found to be composed of 59 components. The main structures of the compounds were fatty acids (34.7%), oxygenated sesquiterpenes (17.7%), alkanes (14%) and aliphatic aldehydes (8.3%). In a thorough analysis of lipid content, unsaturated fatty acids were identified as the most prevalent. The key fatty acids were found to be linoleic acid at 26.9%, linolenic acid at 13.2%, palmitic acid at 22.2%, and oleic acid at 20.9%. The study constructively pointed out the promising potential of both the plant's essential oil and fixed oil for practical applications in medical and cosmetic industries, paving the way for future exploration and development in these areas.[4]. A study investigated the antioxidant, antidiabetic, anti-inflammatory, and antimelanogenic properties, as well as the phytochemical composition, of the essential oil and methanolic extract derived from *the* aerial parts of *C. fulvida*. The essential oil's primary components were identified as follows: hexadecanoic acid is the predominant fatty acid; the most abundant sesquiterpenes include caryophyllene oxide, humulene epoxide, and spathulenol; and the major monoterpenes consist of camphor, 1,8-cineol, and α-pinene.). In the study, *C. fulvida* essential oil was found to have anti-inflammatory and antidiabetic effects. The essential oil also showed a weak antioxidant effect compared to the methanol extract. The extract has been shown to contain phenylpropenoid dimers, phenolic acids and flavonoids, and has been shown to have antioxidant, antidiabetic, anti-inflammatory and antimelanoma activity through tyrosinase inhibition [7]. In one study, *C. pestalozzae* showed antioxidant activity and the IC_{50} value in the DPPH (2,2-diphenyl-1-picrilhydrazyl) radical scavenging activity test was calculated as 18.66 mg/ml. The total phenol content of the plant, calculated in gallic acid equivalent, was found to be 42.59 milligrams of gallic acid equivalent per millilitre. The observed antioxidant activity was associated with the plant's phenol content [8]. Another study found *C. altissima* extracts to have antidiabetic, antioxidant, anti-inflammatory and antibacterial activity [9]. A study investigated the components of the essential oil obtained from *C. tinctoria* var. *tinctoria* and the antibacterial and lipase inhibitory activities of the essential oil and its extracts prepared with different solvents (*n*-hexane, acetonitrile, water and methanol). The study identified the essential oil's primary components as terpenes, with the predominant terpenes being borneol, camphor, and *β*-pinene. The study demonstrated that *C. tinctoria* var. *tinctoria* exhibits antibacterial and lipase enzyme inhibitory effects [10]. The study of the biological activities of *C. tinctoria* by methanol and aqueous infusion indicated that the plant exhibited cholinesterase inhibitory,

tyrosinase inhibitory and antidiabetic activities in addition to its potent antioxidant activity [11]. In a further study, the hydroalcoholic extract of *C. tinctoria* demonstrated cytotoxic activity against gastric and liver cancer cell lines and the cytotoxic activity depended on the dose. The mechanism underlying this cytotoxic effect was associated with oxidative stress, cancer cell cycle and apoptosis. Furthermore, it was emphasised that the phenolic compounds present in the plant may play a role in the observed cytotoxic effect [6]. The *C. palaestina* subsp. *syriaca* is an endemic species of *Cota* genus in the Eastern Mediterranean region. The isolation and purification of a compound from the plant, identified as 1-β,10-epoxy-6-hydroxy-1,10H-inunolide, a sesquiterpene lactone, has demonstrated anti-inflammatory, cytostatic and antimetastatic effects when tested on breast cancer cell lines [12]. This study sought to explore the antibacterial properties and the phytochemical composition of a methanol extract derived from the aerial parts of the endemic plant *C. pestalozzae* Boiss. This research aimed to uncover the potential medicinal benefits of this species, examining its ability to inhibit bacterial growth and the specific chemical compounds present in the extract.

MATERIAL AND METHOD

Plant Material and Extraction

C. pestalozzae Boiss. was collected from Konya Ermenek Road on 19/05/2023, and Prof. Dr. A. Mine Gençler-Özkan identified the plant from the Pharmaceutical Botany Department of the Faculty of Pharmacy. The herbarium specimen is stored in AUEF Herbarium (AEF31016).

To perform HPLC analysis, 1 gram of the aerial plant material was extracted with 25 ml of methanol for 30 minutes in an ultrasonic water bath. To assess the antibacterial activity, a methanolic extract was prepared from the aerial parts of the plant (32 g). The plant material was extracted with 300 ml methanol three times using an ultrasonic water bath for 30 min. The obtained methanol extract was evaporated to dryness under a vacuum to obtain 1.95 g of methanol extract (yield was calculated as 6.09%).

HPLC Chromatography Conditions for Qualitative and Quantitative Analysis

In our study extract and standard compounds were analysed by HPLC (Agilent LC 1260 Cromatograph) with gradient elution. The analysis conditions are given in Table 1. The diode array detector (DAD) was configured to a wavelength of 210 nm, after which peak areas were automatically integrated by the computer using Agilent Software. Different concentrations were prepared of each standard compound to obtain calibration curves (1; 0.5; 0.4; 0.2; 0.1; 0.05 mg/ml). To make the calibration curve tested, compounds and 20 mg/ml concentration of extract were injected into HPLC three times. Peak areas were used against the concentrations; calibration curves and equations were obtained. Absorbances were measured at 210 nm for extract and standard compounds. The limit of detection (LOD) and the limit of quantitation (LOQ) values were calculated for each compound by employing the "signal/noise x 3" and the "signal/noise x 10" equations, respectively. The compounds were then injected into the HPLC 6 times.

Table 1. HPLC analysis conditions

Column	ACE 5 C18, 250mm; 4.6mm; 5µm		
Detector	Diode Array Detector		
Column temperature	40°C		
Flow rate	1 ml/min		
Injection volume	10µl		
Analyse time	40 min. with 5 min. post-time		
Mobil phase	Time (min)	Water with %0.2 phosphoric acid (%)	Acetonitrile (%)
	0	90	10
	36	70	30
	36.01	10	90
	40	10	90

Antibacterial Activity Assay

In the antibacterial activity test, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213 (methicillin-susceptible, MSSA) and *S. aureus* ATCC 43300 (methicillin-resistant, MRSA) were used as test bacteria. The *C. pestalozzae* extract (40 mg) was dissolved in dimethyl sulfoxide (10% DMSO, 1 ml). The extract's minimum inhibitory concentration (MIC) values were determined by broth microdilution [13]. Serial two-fold dilutions of the extract ranging from 10000 to 78.125 µg/ml were prepared in Mueller Hinton Broth (Merck, Germany). The inoculums were prepared from subcultures maintained for 24 hours. Subsequently, the final test concentration of the test bacteria was adjusted to $5x10^5$ cfu/ml, after which the microplate was incubated at 35°C for 18-24 hours. The last well that demonstrated complete inhibition of visual microbial growth was identified as the MIC value (µg/ml). To establish a baseline for comparison, the solvent (10% DMSO) was used as the negative control, and ciprofloxacin and gentamicin were employed as standard antibiotics.

RESULT AND DISCUSSION

Results of HPLC Analysis

In the present study, HPLC analysis results have revealed that the methanol extract contains chlorogenic acid, caffeoyl quinic acid, rutin, hyperoside, and isoquercitrin. Figure 1 gives chromatograms and retention times (Rt) of the standard compounds.

The primary objective of this study was a thorough qualitative and quantitative analysis of these compounds. Figure 2 clearly displays the HPLC chromatogram of the extract, highlighting the identified peaks that correspond to the standard compounds. The quantities of the detected compounds were accurately determined by injecting solutions of standard compounds at various concentrations. Several standards were prepared for this analysis, each at a different concentration to create a comprehensive dataset. Following this preparation, these standard solutions were injected into the analytical system, and a calibration curve was constructed based on the measured peak areas corresponding to each concentration. These calibration curves are used to calculate the amount of each compound as µg/ 100 mg plant material. The average content of each standard compound and standard deviations are presented in Table 2 together with the equations of the calibration curves of the standards. Table 2 also displays LOD and LOQ values indicating the validation of the analysis.

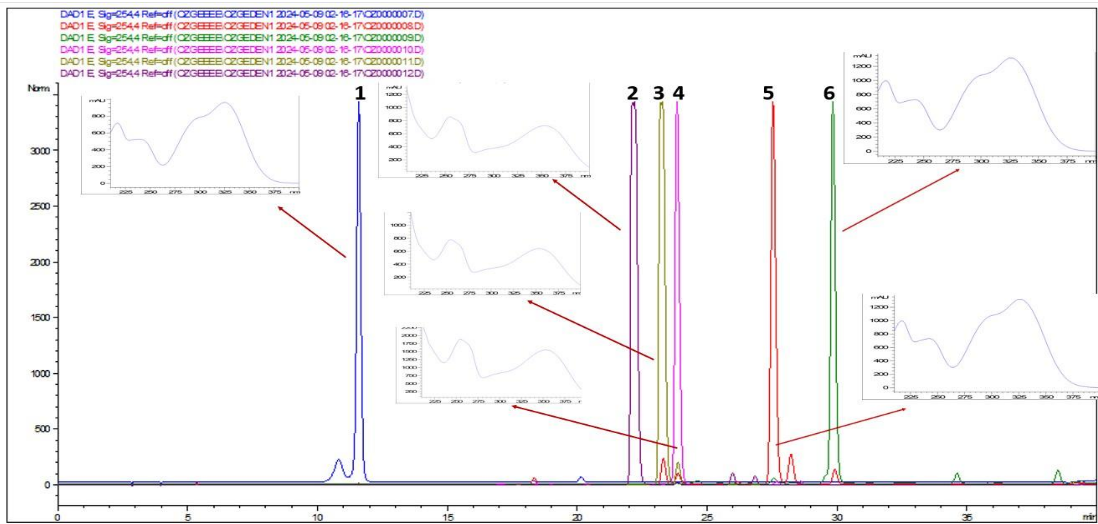

Figure 1. Chromatograms, UV spectrums and Rt (min.) of the detected compounds. Chlorogenic acid (1) Rt: 11.591, rutin (2) Rt: 22.236, hyperoside (3) Rt: 23.284, isoquercetin (4) Rt: 23.840, 3,5-*O*-dicaffeoyl quinic acid (5) Rt: 27.539, 4,5-*O*-dicaffeoyl quinic acid (6) Rt: 29.842

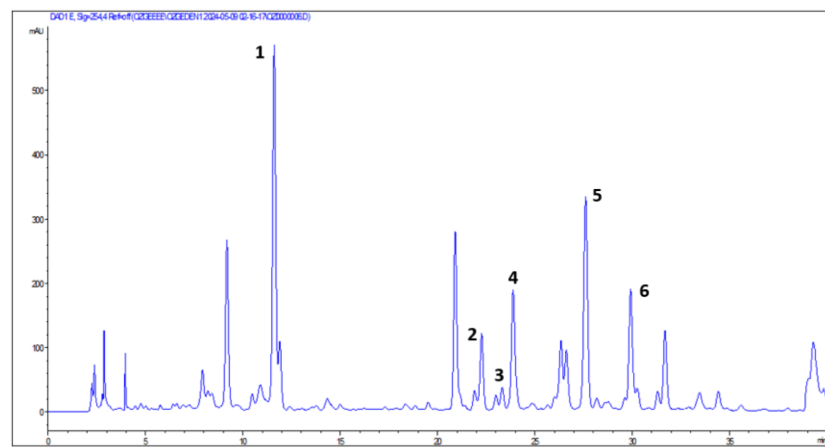

Figure 2. HPLC chromatogram of the extract and the peaks identified as belonging to the standard compounds

In a previous study, the phytochemical content of a methanol extract prepared from the aerial part of *C. fulvida* was analysed by LC-MS/MS. The results demonstrated the presence of several compounds, including 3,5-*O*-dicaffeoylquinic acid, syringic acid, 4,5-*O*-dicaffeoylquinic acid, caffeic acid derivatives, chicoric acid, quercetin glycosides and luteolin derivatives [7]. Another study revealed that methanol extract prepared from *C. tinctoria* comprises various phenolic acids and flavonoids. These include *p*-hydroxybenzoic acid, protocatechuic acid, benzoic acid, chlorogenic acid, caffeic acid, and p-coumaric acid. Additionally, flavonoids such as rutin, quercetin, kaempferol, hesperidin, and apigenin are present, along with catechin derivatives, including (+)-catechin and epicatechin. The observed potent antioxidant activity in the study was associated with the phenolic content of the plant [11]. The analysis of the phytochemical content of another *Cota* species, *C. altissima*, utilizing LC-MS/MS revealed that the main components were 3,5-*O*-dicaffeoylquinic acid, 4,5-*O*-dicaffeoylquinic acid, quercetin, and isorhamnetin glycoside [9]. The present analysis indicates the phytochemical structure of the methanol extract from *C. pestalozzae* utilizing high-performance liquid chromatography (HPLC). Drawing inspiration from previous studies on *Cota* species, this research has revealed intriguing compounds such as caffeoylquinic acid derivatives, caffeic acid derivatives, and distinct flavonoid derivatives.

Table 2. Amounts of the standard compounds in the extract, LOD and LOQ values of the compounds

Standard Compounds	**Calibration Equations and R^2 Values**	***C. pestalozzae* Aerial Part (µg/100 mg plant material) Mean±SD**	**LOD (µg/ml)**	**LOQ (µg/ml)**
Isoquercetin	y = 38.873x + 78.959 R^2 = 0.9998	544.98± 5.03	0.9	3
Chlorogenic acid	y = 16.86x + 226.21 R^2 = 0.9981	4084.967±165	0.2	0.6
4,5-*O*-dicaffeoyl quinic acid	y = 13.613x – 9.7593 R^2 = 1	1696.12±74.77	0.6	2
3,5-*O*-dicaffeoyl quinic acid	y = 11.271x – 28.347 R^2 = 0.9999	3570.00±89.95	1.2	4
Rutin	y = 25.152x – 45.754 R^2 = 0.9999	56.34±9.11	0.3	1
Hyperoside	y = 34.803x – 550.74 R^2 = 0.9988	125.52±11.28	1.2	4

Results of Antibacterial Activity Assay

The results of the current study demonstrate the *C. pestalozzae* aerial part extract exhibited antibacterial activity against *S. aureus* ATCC 43300 (MRSA) with a MIC value of 10000 µg/ml. However, no antimicrobial activity was observed against the other tested bacteria within the 10000-78.125 µg/ml concentration range (Table 3). A study investigating the antimicrobial activity of essential oil and extracts obtained from *C. tinctoria* var. *tinctoria* revealed that the essential oil and extracts were effective against gram (+) bacteria. The study indicated that the extract prepared with acetonitrile demonstrated antibacterial activity against the *Mycobacterium smegmatis* (MIC value 548 µg/ml), *S. aureus* (MIC value 274 µg/ml) and *Bacillus cereus* (MIC value 274 µg/ml) strains. In addition, it was observed that the extracts did not show any effect against *E. coli*, *P. aeruginosa*, and *Enterococcus faecalis* [10]. In another study, it was observed that n-hexane extract prepared from the *C. altissima* plant showed an antibacterial effect against the *S. aureus* strain and the MIC value was calculated as 312.5 µg/ml [9]. The findings corroborate the literature data and demonstrate that the methanol extract of *C. pestalozzae* exhibits no antibacterial activity against the test strains of Gram-(-) bacteria.

Table 3. MIC values (µg/ml) of the *C. pestalozzae* extract against test bacteria

	Gram-positive Test Bacteria		**Gram-negative Test Bacteria**		
Sample	***S. aureus* ATCC 25923 (MSSA)**	***S. aureus* ATCC 43300 (MRSA)**	***E. coli* ATCC 25922**	***K. pneumoniae* ATCC 13383**	***P. aeruginosa* ATCC 27853**
***C. pestalozzae* extract (40 mg/ml)**	-	10000	-	-	-
Ciprofloxacine	<0.00025	0.0005	<0.00025	<0.00025	0.0625
Gentamicin	0.0005	<0.00025	0.0005	<0.00025	NT

NT: not tested, (-): no activity

The present study provides evidence of the limited antibacterial activity of *C. pestalozzae* and identifies the presence of phenolic acids and flavonoids in the methanolic extract derived from the plant's aerial parts, both qualitatively and quantitatively. The results indicate that *C. pestalozzae* may serve as a promising source of naturally occurring secondary metabolites. Further research is warranted to isolate and identify these compounds in order to elucidate the phytochemical structure of the plant.

AUTHOR CONTRIBUTIONS

Concept: Ö.B.A.; Design: Ö.B.A.; Control: Ö.B.A.; Sources: Ö.Y., Ö.B.A.; Materials: Ö.Y., S.S.R., Ö.B.A.; Data Collection and/or Processing: Ö.Y.; Analysis and/or Interpretation: Ö.Y., S.S.R., Ö.B.A.; Literature Review: Ö.Y., Ö.B.A.; Manuscript Writing: Ö.Y., S.S.R., Ö.B.A.; Critical Review: Ö.Y., S.S.R., Ö.B.A.; 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.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

DEVELOPMENT AND VALIDATION OF GREEN RP-HPLC AND SPECTROPHOTOMETRIC METHODS FOR DETERMINATION OF ALPELISIB IN BULK AND PHARMACEUTICAL DOSAGE FORMS

FARMASÖTİK DOZAJ FORMLARINDA ALPELİSİB MİKTAR TAYİNİ İÇİN VALİDE EDİLMİŞ YÜKSEK PERFORMANSLI SIVI KROMATOGRAFİK VE SPEKTROFOTOMETRİK YÖNTEM GELİŞTİRİLMESİ

Wiem BOUALI[1,2]*, Mariem BOUALI[3], Asena Ayse GENC[1,2], Nevin ERK[1]*

[1]Ankara University, Faculty of Pharmacy, Department of Analytical Chemistry, 06560 Ankara, Turkey
[2]Ankara University, Graduate School of the Health Sciences, 06110 Ankara, Turkey
[3]Akdeniz University, Faculty of Agriculture, Department of Agricultural Biotechnology, 07058 Antalya, Turkey

ABSTRACT

Objective: *This study aims to develop and validate green analytical methods, specifically UV spectrophotometry, first-order derivative spectrophotometry, and reverse-phase HPLC (RP-HPLC), for determining Alpelisib (ALP) in bulk and pharmaceutical formulations. By comparing the methods using GAPI and AGREE metrics, the study evaluates their environmental friendliness, precision, and applicability.*
Material and Method: *The UV spectrophotometric and RP-HPLC analyses were conducted using Shimadzu UV 1800 and Agilent 1100 HPLC systems, respectively. The mobile phase for HPLC comprised 0.1% trifluoroacetic acid in water, acetonitrile, and methanol (50:25:25 v/v/v). ALP tablets were prepared and analyzed after dissolution in methanol/water (50:50 v/v) and filtration. Validation was conducted according to ICH guidelines.*
Result and Discussion: *The developed methods showed high precision, robustness, and sensitivity. UV and HPLC methods were effective in determining ALP in both bulk drug and tablet formulations, with detection limits of 0.078 µg/ml for direct UV spectrophotometry and 14 µg/ml for RP-HPLC. Greenness evaluation highlighted the methods' environmental compatibility, making them suitable for sustainable pharmaceutical analysis.*
Keywords: *Alpelisib, anticancer drug, greenness evaluation, RP-HPLC, spectrophotometric, validation*

ÖZ

Amaç: *Bu çalışma, Alpelisib'in (ALP) yığın ve farmasötik formlarda tayini için çevreci UV spektrofotometri, birinci derece türev spektrofotometri ve ters faz HPLC (RP-HPLC) yöntemlerini geliştirmeyi ve doğrulamayı amaçlanmıştır. Çalışma yöntemlerin çevre dostu olduğunu, doğruluklarını ve uygulanabilirliklerini değerlendirmek için GAPI ve AGREE metrikleri ile karşılaştırılmıştır.*

* **Corresponding Author / Sorumlu Yazar:** Nevin Erk
e-mail / e-posta: nevin.erk@pharmacy.ankara.edu.tr, **Phone / Tel.:** +903122033174

* **Corresponding Author / Sorumlu Yazar:** Wiem Bouali
e-mail / e-posta: wbouali@ankara.edu.tr, **Phone / Tel.:** +905380622411

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Gereç ve Yöntem: *UV spektrofotometrik ve RP-HPLC analizleri sırasıyla Shimadzu UV 1800 ve Agilent 1100 HPLC sistemleri ile gerçekleştirilmiştir. HPLC için mobil faz, %0.1 triflorasetik asit suda, asetonitril ve metanolün (50:25:25 h/h/h) karışımından oluşmaktadır. ALP tabletleri, metanol/su (50:50 h/h) içinde çözülüp filtrelendikten sonra hazırlanmış ve analiz edilmiştir. Doğrulama, ICH kılavuzlarına göre yapılmıştır.*
Sonuç ve Tartışma: *Geliştirilen yöntemler yüksek hassasiyet, dayanıklılık ve duyarlılık göstermiştir. UV ve HPLC yöntemleri kullanılarak ilaç formülasyonlarında ALP'nin tayini yapılmış ve doğrudan UV spektrofotometri için 0.078 µg/ml, RP-HPLC için ise 14 µg/ml olarak tespit limitlerine ulaşılmıştır. Çevreci değerlendirme, yöntemlerin sürdürülebilir farmasötik analiz için çevre dostu uyumluluğunu vurgulamaktadır.*
Anahtar Kelimeler: *Alpelisib, antikanser ilaç, çevreci değerlendirme, doğrulama, RP-HPLC, spektrofotometrik*

INTRODUCTION

In 2019, a pivotal achievement marked the pharmaceutical landscape with the approval of Alpelisib (ALP), the maiden alpha-specific phosphoinositide 3-kinase (PI3K) inhibitor, by the United States Food and Drug Administration (FDA) [1]. Developed by Novartis, ALP garnered recognition for its application in treating hormone receptor (HR)c-positive, human epidermal growth factor receptor 2 (HER2)-negative, PIK3CA-mutated, advanced, or metastatic breast cancer, particularly in combination with Fulvestrant. Branded as "Piqray," this groundbreaking medication, administered in oral tablet form, signifies a profound leap in targeted cancer therapy [2].

The PI3K pathway, a critical signaling cascade, assumes a central role in various cancer types, influencing essential cellular processes such as growth, survival, and metabolism [3]. Particularly noteworthy is the involvement of the Class II PI3K alpha isoform (p110α) in insulin-mediated glucose uptake and cell growth [4]. Genetic aberrations in the PIK3CA gene, encoding p110α, and modifications in the phosphatase enzyme PTEN, integral to the dephosphorylation of PIP3 to PIP2, are prevalent in diverse cancers [5]. The perturbation of the PI3K pathway is deeply implicated in cancer development, progression, and resistance to antineoplastic therapies [6,7].

The approval of ALP by the FDA represents a significant change in cancer treatment by targeting PI3K. Current research is exploring its potential use beyond breast cancer, including treatments for colorectal and ovarian cancers [8,9]. This evolution in pharmaceutical progress underscores the profound impact of targeted PI3K inhibition in reshaping the landscape of cancer therapeutics.

Developing robust analytical methodologies for the determination of ALP with high sensitivity, precise, and reliable results remains a significant challenge. A few methods including high-performance liquid chromatography combined with a fluorescence detector (HPLC-FLD) [10], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [11], and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) [12], methods for the determination and analysis of ALP have been published in the literature. Also, electrochemical methods for the ALP determination in bulk and biological fluids samples have been reported [13,14].

To the best of our knowledge, no previously published papers have addressed the use of UV spectrophotometry for the determination of ALP. UV-spectrophotometry is a versatile technique widely used for the quantitative analysis of many pharmaceutical compounds [15]. Its simplicity and time efficiency, combined with low solvent consumption, make it more economical and environmentally friendly compared to other analytical methods. Although spectral overlaps can occur when analyzing mixtures with UV-spectrophotometry, this issue can be resolved using post-mathematical processing algorithms [16].

In addition, High-Performance Liquid Chromatography (HPLC) stands as a pivotal analytical technique with widespread applications across diverse fields including pharmaceuticals, food, cosmetics, biochemistry, and environmental analysis [17]. This technique is instrumental in delivering efficient, reliable, and precise analytical methods, playing a crucial role in the characterization and quantification of compounds in complex samples [18]. HPLC's versatility and sensitivity make it an indispensable tool for scientists and researchers seeking to unravel the composition of substances in different industries, contributing significantly to advancements in analytical chemistry and related

disciplines [19]. The incorporation of HPLC coupled with a Diode Array Detector (DAD) emerges as a cost-effective strategy for therapeutic drug monitoring [20]. This approach, despite the absence of stable isotope internal standards commonly utilized in mass spectrometry, offers reliable results. The utilization of HPLC with DAD presents a viable alternative, demonstrating efficacy in drug quantification while contributing to overall cost reduction in analytical methodologies. This pragmatic application underscores the versatility and economic advantages of HPLC-DAD for therapeutic drug monitoring in the absence of stable isotope internal standards [21].

Notably, to our knowledge, no published analytical method exists for the determination of ALP in bulk and tablet formulations using an RP-HPLC system equipped with a DAD and UV spectrophotometry. This underscores the uniqueness and importance of this research endeavor. The main aim of this study is to develop novel analytical methods that are sensitive, simple, economical, accurate, and reliable for the determination of ALP in bulk and tablet formulations. To evaluate their efficacy and practicality, all developed approaches were statistically compared using analysis of variability. Additionally, we assessed the environmental effect of the novel UV spectrophotometric and HPLC methods by conducting a greenness profile assessment using AGREE and GAPI metrics.

MATERIAL AND METHOD

Instrument and Software

Shimadzu UV 1800 double-beam UV-VIS spectrophotometer with Shimadzu UV Probe 2.7 system software was used for the spectrophotometric measurements. The samples' absorbance was recorded by placing them into 1 cm quartz cuvettes. The chromatographic analyses were conducted utilizing an HPLC system, specifically the Agilent 1100 Technologies model. This HPLC system was equipped with a quaternary pump featuring an automated membrane eluent degasser unit, an autosampler, a column oven, and a DAD to enable comprehensive detection and quantification of analytes. System operation and data acquisition were proficiently overseen by employing the Agilent Chemstation Plus Software, headquartered in Palo Alto, California, USA. To achieve optimal separation and analysis of target compounds, an Inertsil C8-3 column with dimensions of 4.6 x 150 mm and particle size of 3 µm, manufactured by Supelco, based in the United States, was utilized. To ensure the pH measurements throughout the experiments, a Mettler Toledo pH meter featuring a high-quality glass electrode was employed.

Chemicals and Solvents

ALP standard and pharmaceutical preparation was provided by FARMANOVA Health Services (Istanbul, Turkey). The chemicals and reagents utilized were of gradient-grade quality suitable for chromatography analyses. Methanol, acetonitrile, and analytical grade trifluoracetic acid were obtained from Merck (Darmstadt, Germany). Millipore Milli-Q system (Milford, MA, USA) was used to provide ultrapure water for the preparation of mobile phase solutions and sample dilutions. Before HPLC analysis, all mobile phases were meticulously passed using a 0.22 µm membrane filtration process, employing a vacuum pump for rapid and efficient filtration. Furthermore, to eliminate any potential sources of air bubbles or other inconsistencies, the filtered mobile phase was subjected to sonication.

Preparation of ALP Sample Solution for UV, and HPLC Method Development

The ALP tablets with Batch No. 1704007B are produced in various formulations, including 150, 200, and 250 mg of ALP. For the experiments conducted in this study, the pharmaceutical preparation containing 200 and 250 mg of ALP was utilized. The choice of this particular formulation allows for a focused investigation into the characteristics and stability of the ALP drug at the specified dosage. In that case, 10 tablets were further weighed and then dissolved in methanol/water (50:50 v/v) within a 500 ml volumetric flask after allowing it to rest in an ultrasonic bath for 20 minutes. The obtained solution was then filtered using a 0.45 µm syringe filter. Subsequently, the filtered solution was transferred to volumetric flasks and further diluted with the mobile phase for the determination of the pharmaceutical formulation.

Preparation of ALP Standard Solution for UV Spectrophotometric, and HPLC Method Development

To create the standard stock solution for developed methods, the ALP standard was dissolved in methanol/water (50:50 v/v) at a concentration of 1000 µg/ml. To achieve the working standard with different concentrations, further, dilute the previously prepared standard stock solution with the mobile phase made from the working standard. The mixture of methanol and water (50:50 v/v) was chosen as a solvent for preparing the standard stock solution for UV spectrophotometry. For the HPLC method, the mobile phase, composed of 0.1% trifluoroacetic acid in water, was meticulously prepared by dissolving 1 ml of trifluoroacetic acid in 1000.0 ml of ultrapure water. This carefully formulated mobile phase serves as a critical component in chromatographic analysis, providing an optimal environment for the separation and quantification of ALP in subsequent experiments.

Method I (Direct UV Spectrophotometric Method)

The principle of UV-spectroscopy is the most straightforward method for conducting various analyses. A blank solution for the mobile phase was maintained. The standard ALP solution was scanned within the 200-400 nm range. An absorption spectrum was identified at 313.6 nm, which was chosen as the analytical wavelength for subsequent analysis. The recorded spectrum is displayed in Figure 1.

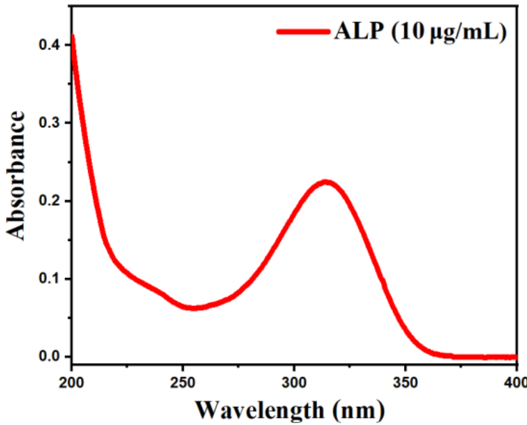

Figure 1. UV spectrum of ALP standard solution (λ_{max} = 313.6 nm)

Method II (First-Order Spectrophotometric Method)

The first-order spectrophotometric method can extract both qualitative and quantitative information from unresolved band spectra. A blank solution served as the mobile phase. Measurements were taken within the 200-400 nm range. Using the instrument's inbuilt software, zero-order spectra were converted into first-order derivative spectra (delta lambda 8.0, scaling factor 100). Analysis of the data for linearity revealed a λmax of 294.8 and 336.2 nm.

Method III (RP-HPLC Method)

The quantification of ALP was achieved through a rigorous developed and optimized process using an RP-HPLC system equipped with a DAD. To ensure accurate separation and analysis, an Inertsil C8-3 column (4.6 x 150 mm, 3 µm) was selected as the stationary phase. Various parameters such as flow rates, column temperature, and mobile phase composition were meticulously optimized to achieve optimal results. The mobile phase composition chosen for this analysis consisted of 0.1% trifluoroacetic acid, acetonitrile, and methanol in a volumetric ratio of 50:25:25 (v/v/v). The entire chromatographic run was 10 minutes, with a consistent flow rate of 1 ml/min. The injection volume was set at 10 µl, and the column temperature was maintained at a stable 20°C. In contrast, the samples were stored at a lower

temperature of 5°C to prevent any degradation or unwanted reactions before analysis. Peaks in the chromatogram were detected at a wavelength of 310 nm for the detection of ALP.

Optimization of Chromatographic Method

In the pursuit of developing and establishing a suitable RP-HPLC approach for the determination of ALP in both standard (A) and tablet forms (B), a series of meticulous preliminary tests were conducted. Various chromatographic conditions were systematically explored and scrutinized, ultimately leading to the development of optimized parameters detailed below. The conclusive analysis was executed utilizing a mobile phase consisting of 0.1% trifluoroacetic acid, acetonitrile, and methanol in a volumetric ratio of 50:25:25 (v/v/v) at a constant flow rate of 1 ml/min. The analytical procedure employed a detector wavelength of 310 nm, and an injection volume of 10 µl was introduced into the chromatographic system, facilitating a runtime of 10 minutes. The finalized method demonstrated its efficacy through the achievement of well-defined and distinct peaks, ensuring both sharpness and resolution. The optimized chromatogram, visually depicted in Figure 2, serves as a testament to the method's precision and suitability for the targeted analysis of both ALP standard and pharmaceutical formulations.

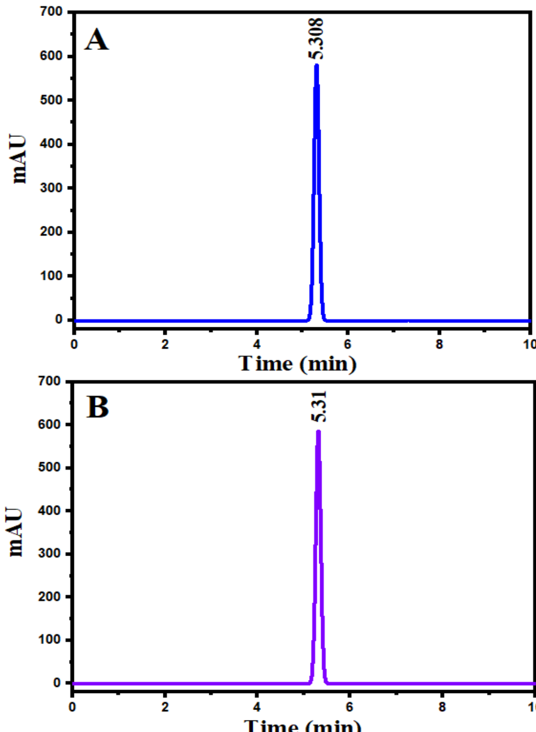

Figure 2. Chromatograms of ALP standard (A), and ALP tablet forms (B)

Method Validation

The constructed UV spectrophotometry and RP-HPLC analytical approaches of ALP have undergone validation in terms of robustness, accuracy, precision, linearity, sensitivity, and stability following ICH (Q2B, 1996) (Committee, 1996).

Robustness

The robustness of the analytical method was comprehensively assessed by subjecting it to various deliberate variations in critical parameters. In the case of the HPLC method, these parameters included the mobile phase composition, flow rate, column temperature, and detection wavelength. Two distinct mobile phase ratios were systematically investigated to ascertain the optimal conditions for

chromatographic analysis. The first composition, denoted as 50:25:25 (v/v/v), encompassed 0.1% trifluoroacetic acid, acetonitrile, and methanol. Simultaneously, an alternative ratio of 40:30:30 (v/v/v) with the same composition was explored to assess its chromatographic performance. The flow rate, another pivotal parameter, was intentionally varied over a range of 0.9, 1.0, and 1.1 ml/min, while the column temperature was modified at three distinct settings of 15, 20, and 25°C. Additionally, different detection wavelengths were explored, encompassing 308, 310, and 312 nm. For the spectrophotometric method, the absorption maxima shifted by ± 2 nm for the 25 µg/ml ALP solution. The relative standard deviation (RSD%) was calculated over five measurements.

Accuracy

The accuracy parameter was carried out using the recovery study method. The proposed method's accuracy was quantified using the standard addition method at 25 and 37.5 µg/ml for spectrophotometry. At the same time, the HPLC method, three different ALP standard concentrations, 200, 400, and 600 µg/ml, were tested to suit the low, medium, and high measurement points of the calibration chart. Then standard solutions were incorporated into the tablet solution at the mount levels of 50%, 100%, and 150% of the nominal standard concentration. The standards and prepared tablet solutions were injected into the RP-HPLC device three times and the mean, % relative standard deviation (RSD %), and recovery values were calculated from the results obtained. The recovery was evaluated by measuring the percentage recovery and quantifying the drug in three separate preparations at each concentration level. This approach provided a comprehensive assessment of the method's accuracy and its ability to produce results that are both precise and close to the true values across a wide concentration range.

Precision

The precision of Methods I and II was assessed by examining intraday precision (within one day) and interday precision (six consecutive days). For the HPLC method, precision intraday was calculated from the coefficient of variance for six replicates of injecting the standard. The ALP standard solution with the concentration of 400 µg/ml was injected in RP-HPLC six times and the area, theoretical number of plates, and tailing factor, for all six injections were compared. To evaluate the intermediate precision of the method, the standard solution at 400 µg/ml concentration and the pharmaceutical preparation with the same ALP concentration were injected for six days and the areas for all six injections were compared. The solutions were kept in amber-colored vials at 2-8°C after each injection. The change percentage between days for the ALP standard and pharmaceutical preparation was calculated. In the case of the spectrophotometric method, a concentration of 20 µg/ml of standard drug solution is evaluated for intraday and interday precision, and variations are investigated. The corresponding average absorbance (UV) and peak area (HPLC) were noted, and the results were reported as % RSD, with an acceptable limit set at below 2%.

Linearity and Sensitivity

In this study, ALP standard solutions were injected into Method I and Method II at concentrations ranging from 10 to 40 µg/ml, and into Method III at concentrations ranging from 100 to 600 µg/ml. The mean and RSD % were calculated from the results obtained. Subsequently, calibration curves and linear regression analysis were performed at the chosen wavelength for developed methods. The sensitivity assessment of the developed methods encompassed the determination of both the LOD and LOQ. The LOD represents the lowest concentration at which the analyte can be detected but not precisely quantified, while the LOQ signifies the lowest concentration within the linear range where precise and reliable quantification is achievable. Following ICH guidelines, these parameters were calculated using the following equations derived from the calibration curve: $LOD = 3.3\sigma/S$ and $LOQ = 10\sigma/S$. Here, σ represents the standard deviation of the y-intercept of the regression line, and S denotes the slope of the calibration curve. These calculations were integral to establishing the method's sensitivity and quantifying its capability to detect and quantify ALP at low concentrations.

Stability

The stability of ALP was studied by the HPLC method using 400 µg/ml ALP standard and

pharmaceutical preparation at several storage conditions for 24 hours. Both solutions were subjected to various storage conditions for 24 hours. The tested storage conditions included a dark environment at room temperature and refrigeration at 2-8°C. At hourly intervals within the 24-hour duration, the solutions were meticulously retrieved, and the areas of the chromatographic peaks were precisely determined. Subsequently, change percentages were then calculated by comparing the obtained areas before and after storage, providing a quantitative measure of any alterations in the stability of the solutions.

RESULT AND DISCUSSION

Validation of Developed Analytical Methods

Following the International Conference on Harmonization ICH guidelines, the developed I, II, and III methods were validated for robustness, accuracy, precision, linearity, sensitivity, and stability.

Robustness

The assessment of robustness, as an indicative measure of the method's resilience against small yet purposeful modifications in chromatographic parameters, was meticulously conducted. This involved a systematic exploration of the impact of small changes in mobile phase composition, flow rate, column temperature, and detection wavelength on the analytical performance. The pivotal parameters of retention time, theoretical plate count, and tailing factor were judiciously scrutinized and found to consistently adhere to predefined criteria. Consequently, the method demonstrated robustness in the face of variations across all tested conditions, substantiated by the empirical data presented in Table 1. Moreover, the robustness of the spectrophotometric method was evaluated by deliberately altering the maximum absorption wavelength. The relative standard deviation (RSD%) was found to be 1.2%, confirming the method's robustness and indicating no significant variations in absorbance values.

Table 1. Assessment of the robustness of ALP

		Retention time		**Tailing factor**		**Theoretical plate count**	
		Standard	**Sample**	**Standard**	**Sample**	**Standard**	**Sample**
Detection wavelength	**308**	5.283	5.274	1.1	1.1	10373	10088
	310	5.286	5.292	1.1	1.1	10409	10491
	312	5.282	5.301	1.1	1.1	10262	10413
Column temperature	**15**	5.309	5.305	1.1	1.1	10695	10251
	20	5.286	5.292	1.1	1.1	10409	10491
	25	5.282	5.287	1.1	1.1	9054	10654
Flow rate	**0.9**	5.862	5.857	1.1	1.1	11890	11059
	1.0	5.286	5.292	1.1	1.1	10409	10491
	1.1	4.838	4.839	1.1	1.1	9870	9960
Mobile phase composition	**40/30/30**	3.449	3.446	1.1	1.1	8875	8905
	50/25/25	5.286	5.292	1.1	1.1	10409	10491

Accuracy

The proposed method’s accuracy was quantified using the standard addition method at 25.0 and 37.5 μg/ml for direct (I) and First-order spectrophotometric (II) methods. At the same time, the accuracy of the RP-HPLC method was ascertained through a percent recovery study involving the addition of ALP standard solutions of known concentrations (50%, 100%, and 150%) to a tablet solution. Different replicate measurements using different methods I, II, and III, showed that the percent recovery was within the allowed ranges (Tables 2 and 3). It is noteworthy that all obtained RSD % values for methods I, II, and III are well within the ICH-approved limits, underscoring the robust accuracy of the developed analytical methodology. These results affirm the method's capability to yield accurate and reliable quantitative results across a range of concentrations.

Table 2. Recovery values obtained for the determination of ALP for the direct (I) and First-order spectrophotometric (II) methods

Set	ALP added (µg/ml)	ALP recovered (µg/ml)			Mean recovery %			RSD %		
		Method I	Method II (294.8 nm)	Method II (336.2 nm)	Method I	Method II (294.8 nm)	Method II (336.2 nm)	Method I	Method II (294.8 nm)	Method II (336.2 nm)
1	25.0	24.71	24.35	23.78						
2	25.0	24.89	24.35	23.91						
3	25.0	24.75	24.37	24.00	99.2	97.6	95.8	0.33	0.38	0.67
4	25.0	24.84	24.54	24.16						
1	37.50	37.47	37.23	36.66						
2	37.50	37.52	37.19	36.70	100.2	99.2	98.0	0.45	0.13	0.29
3	37.50	37.52	37.21	36.80						
4	37.50	37.84	37.16	36.90						

Table 3. Recovery values obtained for the determination of ALP for the RP-HPLC method

Recovery Levels %	Set	ALP added, mg/ml	ALP recovered		Mean recovery	RSD %
			mg/ml	%		
50	1	0.2	0.199983	99.9	99.9	0.05
	2	0.2	0.20034	99.9		
	3	0.2	1.99827	99.8		
100	1	0.4	0.406281	101.5	101.1	0.32
	2	0.4	0.403893	100.9		
	3	0.4	0.40212	101.0		
150	1	0.6	0.599941	100	99.9	0.1
	2	0.6	0.599763	99.9		
	3	0.6	0.598956	99.8		

Precision

In this study, six standard solutions of ALP were analyzed using RP-HPLC and spectrophotometric methods to evaluate intraday and interday precision. Under inter-day, the % RSD, calculated within the ICH limit of ± 2%, was found to be 0.09% for RP-HPLC and 1.06% for the spectrophotometric method, significantly assuring the precision of the proposed methods. The performance of intra-day precision and the percent RSD for the response of six replicate measurements in the spectrophotometric method were within the acceptable ranges (0.96%). In the intermediate precision analysis conducted after a six-day interval, notable differences in stability were observed between the ALP standard and the pharmaceutical preparation. The ALP standard exhibited a minimal 0.3 percent change over the six days, indicating a high degree of stability under the specified storage conditions. Conversely, the ALP in the pharmaceutical preparation demonstrated a more substantial 9.6 percent change, signifying a comparatively lower sensitivity to environmental factors when compared to the standard. These findings underscore the importance of considering formulation-specific stability, as variations in the drug solution may be influenced by additional excipients or interactions. The minimal change in the ALP standard reaffirms its robust stability, while the observed alteration in the drug solution warrants further investigation into factors affecting its stability under intermediate precision conditions.

Linearity and Sensitivity

Linearity samples were prepared through multiple dilutions of a standard stock solution. The measured responses of ALP at different concentrations using the developed methods were analyzed to create linear regression equations. Table 4 summarizes the key statistical parameters for each process. The results show an excellent correlation between the recorded responses and drug concentrations within the specified range, as indicated by high correlation coefficients. Moreover, the linearity calibration curves, visually represented in Figures 3-5, provide a clear illustration of the method's ability to generate

consistent and proportional responses across the specified concentration range. The sensitivity of the methods was successfully determined by assessing the limit of detection (LOD) and the limit of quantification (LOQ). The calculated LOD values were 0.078 µg/ml for Method I, 0.096 µg/ml for Method II, and 14 µg/ml for Method III. These results highlight the high sensitivity of the methods, demonstrating their capability to reliably detect ALP at low concentrations. This level of sensitivity is essential for the effectiveness of the methods in pharmaceutical and chemical analyses, particularly when dealing with samples containing low analyte concentrations.

Table 4. Linearity data for ALP by direct UV Spectrophotometry (I), first-order spectrophotometric (II), and RP-HPLC methods

Parameters	Method I	Method II		Method III
		(294.8 nm)	(336.2 nm)	
Linearity Range (µg/ml)	10-40	10-40	10-40	100-600
Calibration equation	y = 0.0221 x - 0.001	y = 0.047 x -0.004	y = -0.071 x + 0.023	y =11.73 x +5.122
Correlation coefficient (R^2)	0.9997	0.9996	0.9990	0.9996
LOD (µg/ml)	0.078	0.096	0.084	14.0
LOQ (µg/ml)	0.261	0.323	0.281	43.0

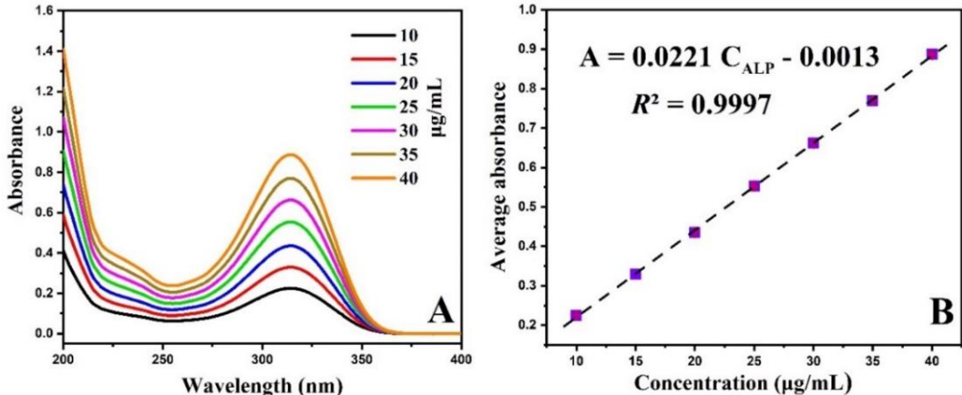

Figure 3. Spectrum of ALP for direct UV spectrophotometric method (10-40 µg/ml)

Stability

Following a meticulous examination of the stability profiles for both the standard and pharmaceutical preparation over 24 hours, including storage conditions of 2-8°C and room temperature, it was established that ALP demonstrated stability under these conditions. Throughout these trials, the highest recorded RSD% was 0.2%. This minimal variability indicates a high level of stability for ALP in both the standard and pharmaceutical preparation under the specified conditions over 24 hours. These findings affirm the robustness of the analytical method and its ability to maintain stability for ALP in various formulations and storage conditions over a short duration.

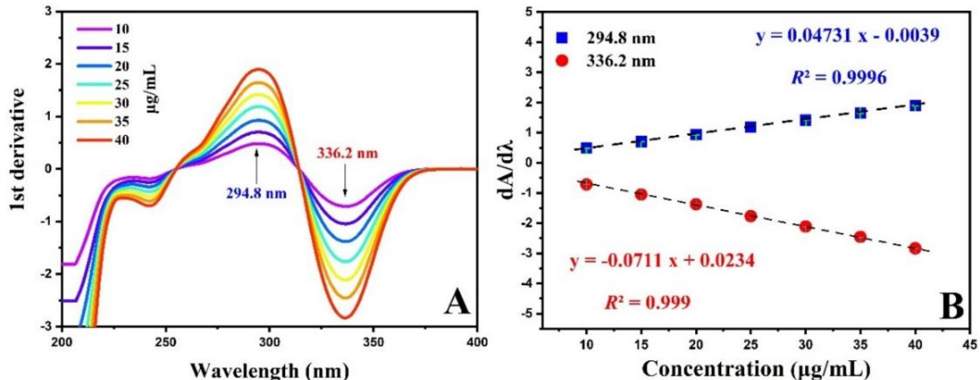

Figure 4. Overlay first-order derivative spectra with the linearity of ALP concentrations ranging from 10 to 40 µg/ml

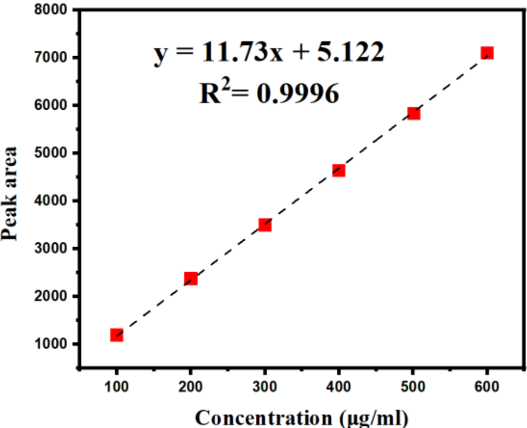

Figure 5. Calibration curve for ALP (100-600 µg/ml) for the RP-HPLC method

Greenness Assessment and Comparison of the Developed Methods

In this study, the environmental impact of both the UV spectrophotometric and HPLC approaches was evaluated in the context of green analytical chemistry principles using the Green Analytical Procedure Index (GAPI) and Analytical Greenness Measure (AGREE) tools.

Figure 6 illustrates the GAPI and AGREE evaluation of the proposed methods for determining ALP. The GAPI tool provides a comprehensive evaluation of the environmental impact of analytical methods through 5 pentagrams, each representing key steps in the process: sample collection, sample preparation, chemicals and solvents used, apparatus, and the goal of the method. GAPI employs a three-color system to reflect the environmental risk, with red indicating high risk, yellow indicating moderate risk, and green representing a lower risk and greater sustainability [22]. As can be seen in Figure 6, the green HPLC method demonstrates 4 green, 9 yellow, and 2 red pentagrams, with the red indicating the use of non-greener solvents such as methanol and trifluoroacetic acid (TFA) and the absence of waste treatment. The inclusion of TFA, along with the energy-intensive preparation steps, further reduces the method's overall greenness. Conversely, the UV spectrophotometric method shows 7 green, 7 yellow, and only 1 red pentagram, with the red similarly indicating no waste treatment. Overall, the UV spectrophotometric method is considered greener than the RP-HPLC method due to its lower energy consumption, reduced solvent use, and minimal waste generation.

Moreover, the AGREE tool was employed to assess the environmental effect of the constructed methods, providing a numerical representation of their greenness. AGREE is an accessible software that evaluates methods against the twelve key principles of green analytical chemistry. Each principle is rated on a scale from 0 to 1 and visualized using a red-yellow-green color gradient [23]. The AGREE

analytical scores of the developed spectrophotometric and HPLC methods were 0.77 and 0.69, respectively. According to the AGREE scale, scores below 0.50 indicate poor greenness, scores between 0.50 and 0.75 are acceptable, and scores above 0.75 represent excellent greenness. While both methods demonstrate environmentally friendly characteristics, the spectrophotometric method shows a higher level of greenness compared to the RP-HPLC method.

Based on green chemistry assessment tools including GAPI, and AGREE, the UV spectrophotometric method exhibited a higher green score due to its simpler, more eco-friendly sample preparation, making it a preferable choice from a sustainability perspective.

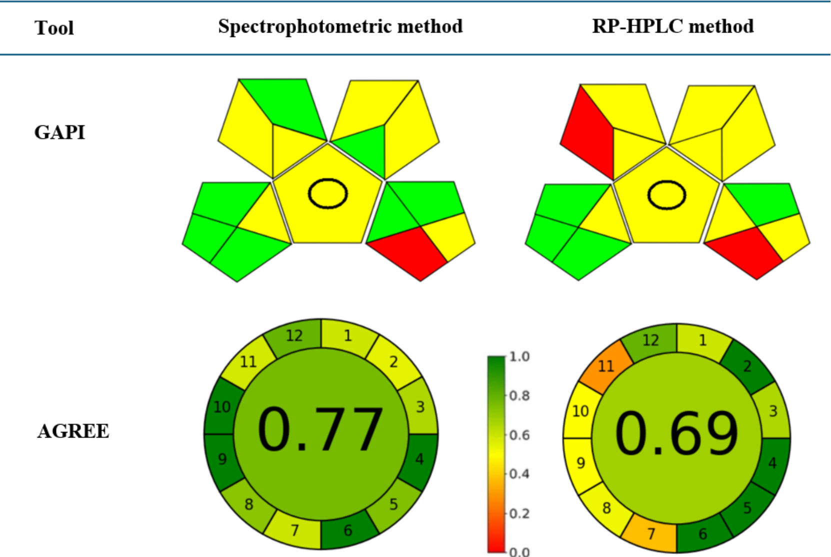

Figure 6. Comprehensive performance assessment of the developed spectrophotometric and RP-HPLC methods by GAPI and AGREE tools.

Conclusion

The creation and validation of green RP-HPLC and spectrophotometric methods for the determination of ALP in bulk drug and pharmaceutical dosage forms have been successfully accomplished. The study demonstrated the efficacy of three distinct analytical methods: direct UV spectrophotometry, first-order UV spectrophotometry, and RP-HPLC. Each approach was meticulously optimized and validated according to ICH guidelines, encompassing parameters including robustness, accuracy, precision, linearity, sensitivity, and stability. Direct UV spectrophotometry and first-order UV spectrophotometry provided simple, rapid, and environmentally friendly approaches for the quantification of ALP, with limits of detection as low as 0.078 µg/ml and 0.096 µg/ml, respectively. The RP-HPLC method, employing a Shimadzu Inertsil C8-3 column and a mobile phase composed of 0.1% trifluoroacetic acid, acetonitrile, and methanol, achieved reliable and precise results with a limit of detection of 14 µg/ml. The greenness assessment using AGREE and GAPI metrics underscored the environmental sustainability of the developed methods, highlighting their minimal solvent and chemical consumption and reduced waste production. Overall, the validated methods exhibit excellent potential for routine quality control analysis of ALP in pharmaceutical formulations, offering robust, precise, and environmentally benign alternatives to existing analytical techniques. This research not only advances the analytical methodologies for ALP determination but also reinforces the importance of green chemistry principles in pharmaceutical analysis. The successful implementation of these methods can significantly contribute to enhancing the efficiency, accuracy, and sustainability of drug quality assessment processes in the pharmaceutical industry.

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AUTHOR CONTRIBUTIONS

Concept: W.B., M.B., A.A.G., N.E.; Design: W.B., M.B., A.A.G., N.E.; Control: W.B., M.B., A.A.G., N.E.; Sources: W.B., M.B., A.A.G., N.E.; Materials: W.B., M.B., A.A.G., N.E.; Data Collection and/or Processing: W.B., M.B., A.A.G., N.E.; Analysis and/or Interpretation: W.B., M.B., A.A.G., N.E.; Literature Review: W.B., M.B., A.A.G., N.E.; Manuscript Writing: W.B., M.B., A.A.G., N.E.; Critical Review: W.B., M.B., A.A.G., N.E.; 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.

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ÖZGÜN MAKALE / ORIGINAL ARTICLE

KÜÇÜK HÜCRELİ VE KÜÇÜK HÜCRELİ OLMAYAN AKCİĞER KANSERLERİNDE ROL OYNAYAN MİRNA'LARIN BİYOİNFORMATİK ANALİZLERLE KARŞILAŞTIRILMASI

THE COMPARISON OF MICRORNAS INVOLVED IN SMALL CELL LUNG CANCER AND NON-SMALL CELL LUNG CANCER THROUGH BIOINFORMATICS ANALYSES

Yalda HEKMATSHOAR[1], Aynur KARADAĞ GÜREL[2]*, Adem AYDOĞAN[3]

[1]Altınbaş Üniversitesi, Tıp Fakültesi, Tıbbi Biyoloji Ana Bilim Dalı, 34147, İstanbul, Türkiye
[2]Uşak Üniversitesi, Tıp Fakültesi, Tıbbi Biyoloji Ana Bilim Dalı, 64200, Uşak, Türkiye
[3]Uşak Üniversitesi, Tıp Fakültesi, 64200, Uşak, Türkiye

ÖZ

Amaç: *Akciğer kanseri, dünya çapında karsinomla ilişkili ölümlerin önemli bir nedenidir. Küçük hücreli ve küçük hücreli olmayan 2 ana akciğer kanseri türü bulunmaktadır. İyi prognozun elde edilmesi, hedefe yönelik tedavinin geliştirilmesi ve potansiyel biyobelirteçleri belirlenmesi amacıyla yeni yöntemler bulmak, akciğer kanserinin klinik etkinliğini geliştirilmesi için büyük önem taşımaktadır. Çalışmanın amacı 2 alt tipte farklı ifade olan miRNA'ları bularak patogenezi ve potansiyel moleküler belirteçlerini incelemektir.*
Gereç ve Yöntem: *miRNA verisi içeren GSE19945 ve GSE135918 nolu veri seti GEO veri tabanından indirilmiştir. GEO2R çevrimiçi analiz aracı ile $P<0.05$ ve log kat değişimi $|(FC)|\geq 1$ alınarak analiz edilmiştir. Farklı ifade edilen miRNA'ların hedef genleri tanımlanmıştır. Cytoscape PPI kullanılarak ağ görselleştirme ve modül tanımlaması yapılmıştır. miRNA hedef genlerinden 3 adet seçilerek genlerin validasyonu akciğer kanseri hücre hattı A549'da yapılmıştır.*
***Sonuç ve Tartışma*:** *17 ifadesi azalan ve 2 ifadesi artan ortak miRNA'lar arasında hsa-miR-1249, hsa-miR-326, hsa-let-7c, hsa-miR-199a-5p, hsa-miR-940, hsa-miR-139-3p, hsa-miR-142-3p, hsa-miR-142-5p, hsa-miR-455-5p, hsa-miR-146b-5p,hsa-miR-152hsa-miR-133b, hsa-miR-498, hsa-miR-199b-5p, hsa-miR-140-3p, hsa-miR-203 ve hsa-miR-139-5p yer almaktadır. miRNA'ların moleküler fonksiyonlarının ve sinyal yollarının tanımlanması, 2 kanser tipinin moleküler mekanizmalarının mevcut anlayışını derinleştirebilir ve, tedavi seçeneklerinin geliştirilmesine katkıda bulunabilir.*
Anahtar Kelimeler: *Akciğer kanseri, biyoinformatik analiz, küçük hücreli akciğer kanseri, küçük hücreli olmayan akciğer kanseri, miRNA*

ABSTRACT

Objective: *Lung cancer is a major cause of cancer-related deaths worldwide. There are two main types of lung cancer, small cell and non-small cell. Finding new methods for achieving a good prognosis, developing targeted therapy and identifying potential biomarkers is crucial for improving the clinical efficacy of lung cancer. The aim of this study was to investigate the pathogenesis and potential molecular markers by finding differentially expressed miRNAs in 2 subtypes of lung cancer.*

* **Sorumlu Yazar / Corresponding Author:** Aynur Karadağ Gürel
e-posta / e-mail: aynur.karadag@usak.edu.tr, **Tel. / Phone:** +902762212121/6240

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Materials and Methods: *The datasets GSE19945 and GSE135918 containing miRNA data were downloaded from the GEO database. Analyzed with GEO2R online analysis tool with P<0.05 and log fold change |(FC)|≥ 1. Target genes of differentially expressed miRNAs have been identified. Network visualisation and module identification were performed using Cytoscape PPI. Three of the miRNA target genes were selected and validation of the genes was performed in the non-small cell lung cancer cell line A549.*

Results and discussion: *17 common miRNAs with decreased expression and 2 with increased expression include hsa-miR-1249, hsa-miR-326, hsa-let-7c, hsa-miR-199a-5p, hsa-miR-940, hsa-miR-139-3p, hsa-miR-142-3p, hsa-miR-142-5p, hsa-miR-455-5p, hsa-miR-146b-5p,hsa-miR-152hsa-miR-133b, hsa-miR-498, hsa-miR-199b-5p, hsa-miR-140-3p, hsa-miR-203 and hsa-miR-139-5p. Defining the molecular functions and signaling pathways of miRNAs may deepen the current understanding of the molecular mechanisms of the 2 cancer types and contribute to the development of treatment options.*

Keywords: *Bioinformatics analysis, lung cancer, microRNA, non-small cell lung cancer, small cell lung cancer*

GİRİŞ

Akciğer kanseri, dünya çapında karsinomla ilişkili ölümlerin önemli bir nedenidir ve akciğer kanserine ilişkin en son Dünya sağlık örgütü verilerine göre 2022'de bu hastalığın yaklaşık 3 milyon yeni vakası tahmin edilmektedir. Gen mutasyonları ve hücre ortamı değişiklikleri, tümörlerin oluşumunu, büyümesini ve metastazını etkileyebilir. Nasıl tedavi edildikleri, nerede büyümeye başladıkları ve ne kadar hızlı yayıldıkları gibi çeşitli şekillerde farklılık gösteren 2 ana akciğer kanseri türü vardır. Akciğer kanseri, küçük hücreli olmayan (KHDAK) (~%85) ve küçük hücreli akciğer kanseri (KHAK) ~%15] dahil olmak üzere iki ana histolojik gruba ayrılır. Akciğer kanseri tipik olarak bronşları veya akciğerin bronşiyoller veya alveoller gibi kısımlarını kaplayan hücrelerde büyümeye başlar. KHDAK ve küçük hücreli akciğer kanseri KHAK akciğerlerin bu bölümlerinde gelişmeye başlar, ancak KHAK en sık olarak trakeadan akciğerlere giden hava yolları olan bronşlarda bulunur [1]. KHAK, pulmoner karsinomun ana histolojik formudur. Geleneksel tedavi yöntemleri kemoterapi, radyoterapi ve cerrahiyi içerir. Kemoterapi, KHAK için en önemli tedavi yöntemidir ancak yüksek ilaç direnci ve nüks oranları gibi sorunlar etkinliğini sınırlamaktadır. Çoğu hasta, tanı anında genellikle hastalığın ileri evrelerinde olduğundan, KHAK genellikle düşük sağkalım oranları ve düşük yaşam kalitesi ile karakterizedir. Aslında, KHAK'ın 5 yıllık sağkalım oranı <%6 ve yüksek mortaliteye sahiptir; dahası, oldukça invazivdir ve lenfatik metastaza eğilimlidir [2]. Bugüne kadar, moleküler hedefli hiçbir ilacın hasta sağkalımını önemli ölçüde uzattığı henüz gösterilmemiştir [3]. Daha da önemlisi, KHAK'ın oluşumu, gelişimi ve metastazının altında yatan moleküler mekanizmalar tam olarak anlaşılamamıştır. Bu nedenle, bir KHAK prognozu ve hedefe yönelik tedavi için potansiyel biyobelirteçleri belirlemek için yöntemler bulmak, akciğer kanserinin klinik etkinliğini geliştirmek için büyük önem taşımaktadır. KHDAK, akciğer kanseri vakalarının yaklaşık %85'ini oluşturan en yaygın akciğer kanseri tipidir. KHDAK'nin ana alt tipleri akciğer adenokarsinomu, akciğer skuamöz hücreli karsinom ve büyük hücreli karsinomdur [4]. KHDAK'nin patogenezi karmaşıktır ve genetik ve immünolojik değişiklikleri içerir. Bir yandan tümör hücrelerinin göç, invazyon ve çoğalma yetenekleri varken; diğer yandan tümör hücreleri, anti-tümör ilaçlara karşı direnç geliştirir [4]. Erken evre KHDAK'li hastalarda genellikle belirgin semptomlar yoktur; bu nedenle çoğu hasta, tanı anında yaşam kalitelerini ve sonraki tedaviyi ciddi şekilde etkileyen evre III veya IV KHDAK'ye sahiptir [5-6]. Sürekli tıbbi teknoloji gelişmeleri ile, tümör immünoterapisi ve hedefe yönelik tedavi gibi yeni tedavi yöntemleri klinik ortamlarda yaygın olarak geliştirilmiştir. Ayrıca, tümörler için kesin ve kişiselleştirilmiş tedavi planları yavaş yavaş gelişmektedir. Ancak, erken tanı belirteçlerinin olmaması nedeniyle, KHDAK hastaları sıklıkla erken tedavi için en iyi fırsatı kaçırır ve 5 yıllık sağkalım oranları sadece %23'tür [7]. Bu nedenle, akciğer kanseri tanı ve tedavisi için belirteçlerin belirlenmesi, konu erken KHDAK tanı oranını artırmak ve uygun tıbbi tedavileri seçmek söz konusu olduğunda önemli klinik öneme sahiptir.

Mikrodizin ve biyoinformatik teknolojiler, hastalık araştırmalarında, özellikle genom boyu mRNA'yı ve miRNA'yı tanımlamanın yanı sıra bunların moleküler mekanizmaların aydınlatılmasında geniş uygulamalar göstermektedir [8-10]. Birçok küçük hücreli olmayan akciğer kanseri gen ekspresyon

profili çalışması, mikrodizin teknolojisi kullanılarak yürütülmüştür ve çok sayıda KHAK ve KHDAK ile ilişkili DEG tanımlanmıştır [11-12]. Mao et al. KHAK'ın patogenezini ve potansiyel moleküler belirteçlerini araştırmak için KHAK dokuları ve normal akciğer dokuları arasındaki mRNA ve mikroRNA'nın diferansiyel ekspresyonlarını karşılaştırmak için biyoinformatik yöntemler kullanmıştır [13]. Çalışmanın amacı 2 alt tipte farklı ifade olan miRNA'ları ve hedef genleri tanımlayarak hastalığın patogenezi altında yatan mekanizmaları ve potansiyel moleküler belirteçlerini incelemektir.

GEREÇ VE YÖNTEM

Mikrodizi Verilerinin Elde Edilmesi ve Analizi

Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo) veri tabanı kullanılarak miRNA dataları taranaraki KHAK ve KHDAK gen ekspresyon profili içeren veri kümeleri GSE19945 ve GSE135918 GEO'dan indirilmiştir. GSE19945 veri seti, 35 KHAK numunesi ve 8 normal akciğer dokusu numunesi dahil olmak üzere cerrahi rezeksiyon ile 43 numune içermektedir. miRNA'nın ifadesinde, Agilent Human 0.6K miRNA Microarray G4471A platformu kullanılmıştır. KHDAK'ın miRNA GSE135918 Exiqon miRCURY LNA microRNA array, 7th generation platformu kullanılarak analiz edilen veri seti, 5 KHDAK numunesi ve 5 normal akciğer dokusu numunesi dahil olmak üzere cerrahi rezeksiyon ile 10 numune içermektedir (Tablo 1). Örnekler arasındaki farklar, GEO'nun yerleşik çevrimiçi aracı olan GEO2R ile analiz edildi. Farklı ifade edilen genlerin (DEG'lerin) önemi, adjp değeri ve | log kat değişimi| (| log FC|) ile değerlendirildi ve adjp < 0.05 ve | log FC| > 1 tarama kriteri olarak kullanıldı.

miRNA Hedef Genlerinin Tahmin Edilmesi

MIRDB ve TargetScan (http:// www.targetscan.org), DEM hedef genlerini ve miRNA-gen çiftlerini tahmin etmek için bir biyoinformatik bir platformdur. Bu çalışmada bu veri tabanları kullanarak, ortaya çıkan miRNA'ların hedef genleri tahmin edilmiştir. Venny 2.1 çevrimiçi aracı (http://bioinfogp.cnb.csic.es), DEG'ler ve DEM'lerin öngörücü genleri arasında örtüşen genleri bulmak için kullanılmıştır. Her veri setinden ifadesi en çok artan ve azalan 5 miRNA'nın hedef genleri çıkarılmış ve bu genler karşılaştırılmıştır.

miRNA Hedef Genlerinin Fonksiyonel Zenginleştirme Analizi

Veri açıklama analizi için yaygın olarak kullanılan web tabanlı bir genomik işlevsel açıklama aracı olan DAVID (https://david.ncifcrf.gov/) kullanılmıştır. Bu çalışmada miRNA'ların hedef genleri, Gene Ontology (GO) analizi ve Kyoto Genler ve Genomlar Ansiklopedisi (KEGG) yol analizi ile moleküler fonksiyon ve yolak analizleri yapılmıştır.

Protein-Protein Etkileşimi (PPI) Ağlarının Oluşturulması ve Modül Araştırması

DEG'lerin PPI ağı, anahtar sinyal yolakların ve hücresel aktivitelerin moleküler mekanizmalarını tanımlamak için Cytoscape yazılımı (sürüm 3.6.0; www.cytoscape.org) kullanılarak oluşturulmuş ve önemli ağları belirlemek için >0.9'lük bir etkileşim puanı kabul edilmiştir. Cytoscape yazılımının Network Analyzer eklentisi kullanılarak genler arasındaki ilişki, ağın kümelenme katsayısı, düğüm derecesi dağılımı ve en kısa yol gibi ağ topolojisi özelliklerine göre analiz edilmiştir. Daha sonra, hub genlerini doğrulamak için moleküler kompleks tespiti (MCODE) kullanılmıştır. Tarama eşikleri 'derece kesme=2', 'düğüm puanı kesme=0.2', 'k-çekirdek=2' ve 'maks·derinlik=100' olarak alınmıştır

A549 Akciğer Kanseri Hücre Kültürü ile miRNA Hedef Genlerinin *İn-Vitro* Validasyonu

Hücreler pasajlanarak her kuyuda 100.000 hücre olacak şekilde 6 kuyulu kültür kaplarına ekildi. Ertesi gün hücreler, %80 oranında kuyuları kaplayınca, büyüme medyumu %1 FBS %1 penisilin/streptomisin içeren açlık medyumu ile değiştirildi. Hücreler %80 yoğunluğa ulaştıklarında santrifüj yapılarak hücre pelletleri toplanıp trizol eklenerek -80°C'de saklanmıştır. Daha sonra toplanan örneklerden RNA izolasyonu yapılmıştır.

RNA İzolasyonu ve RT-PCR ile Gen İfade Analizi

Mevcut analizimizi doğrulamak için miRNA hedef genlerinden 3 genin doğrulaması yapılmıştır. Invitrogen Purelink RNA Mini kit kullanılarak RNA izolasyonu gerçekleştirilmiştir. İzole edilen RNA'ların miktarı spektrofotometre ile ölçüldü. Ardından RNA'nın kalitesine %1'lik agaroz jel elektroforezi yapılarak bakıldı. 3 tekrar için 3 ayrı kuyudan yaklaşık 500 ng RNA'lardan, Transcriptor High Fidelity cDNA sentez kiti kullanılarak cDNA sentezi gerçekleştirildi. Elde edilen cDNA'lar nanodrop cihazında ölçülerek 500 ng olarak hesaplandı. cDNA kalıplarının eldesi ve kalitesin %1'lik agaroz jel elektroforezi yapılarak kontrol edildi. cDNA kalıpları bir süre +4°C'de bırakıldıktan sonra qRT-PCR'da yapılıncaya kadar -20°C'de saklandı. Kullanılan primer dizileri Tablo 1'de gösterilmiştir. qRT-PCR reaksiyonu, Syber Green Master mix karışımı ile yapıldı. PCR reaksiyonu, üretici firmanın Syber Green Master mix ile tamamlandı. Biorad CFX96 cihazına yerleştirilerek qRT-PCR reaksiyonu gerçekleştirildi. Bağıl kantifikasyon, $2^{-\Delta Ct}$ değerlerinden şu denklemle hesaplandı: ΔCt = Ct Gen -Ct GADPH .

Tablo 1. Primer listesi

Gen adı	Primer dizisi
GAPDH	Reverse: 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3'
	Forward: 5′-TGGTATCGTGGAAGGACTCATGAC-3'
MAPK1	Reverse: 5′-GCCTGGCCCCACCTGTG-3'
	Forward: 5′-CACGGAAGGTCCTGAGGGG-3'
KRAS	Reverse: 5′-TCCTGTAGGAATCCTCTATTG-3'
	Forward: 5′-GCCTGCTGAAAATGACTG-3'
NUP98	Forward: 5′-TTTTAGAGGGCCATACCGATTTC-3′
	Reverse: 5′-AAACCGGATTGTTCCATTCTTCT-

İstatistiksel Analiz

P değerleri ve FDR, GEO2R'nin yerleşik t- testi, Benjamini ve Hochberg (yanlış saptama oranı) ve hasta ve kontrollerin karşılaştırılmasıyla elde edilen DEM'ler ve DEG'leri belirlemek için diğer yöntemler kullanılarak hesaplanmıştır.

SONUÇ VE TARTIŞMA

Mikrodizin Verilerinin Elde Edilmesi ve Analizi

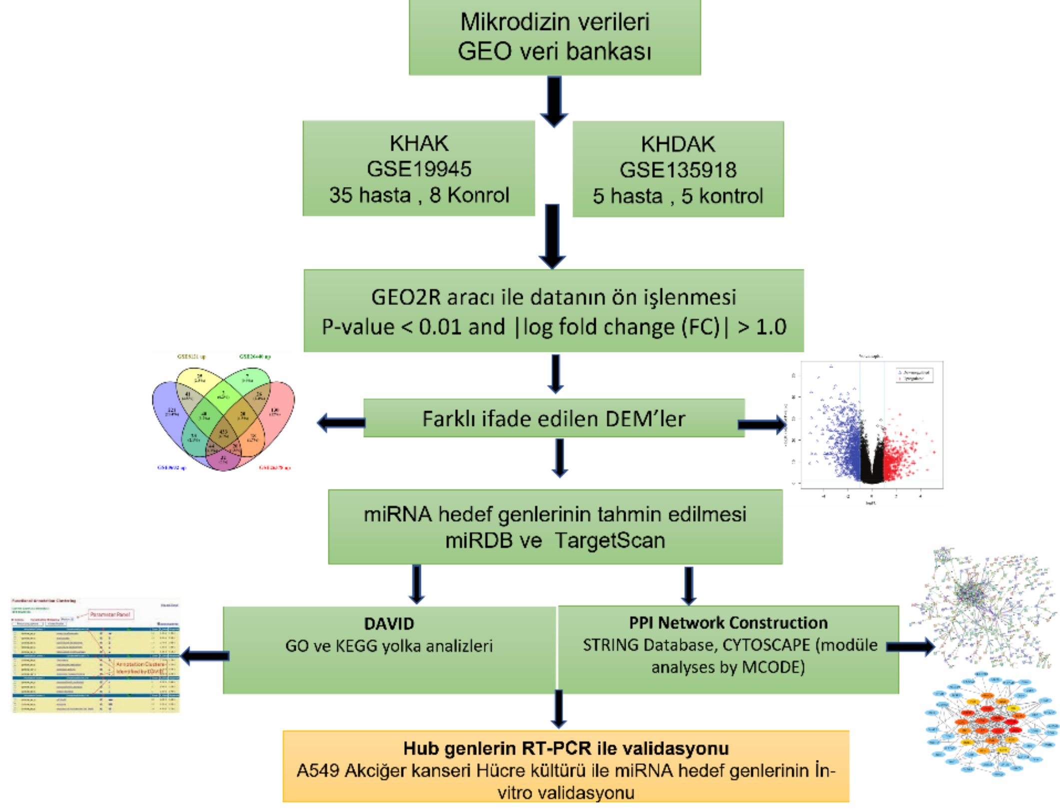
Erişime açık bir genomik veri tabanı olan Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo) veri tabanı kullanılarak miRNA verileri tarandı. İnsan KHAK ve KHDAK numunesi gen ekspresyon profili genel veri kümeleri ***GSE19945 ve GSE135918*** GEO veri tabanından indirildi. Küçük Hücreli Akciğer kanseri (KHAK) GSE19945 veri seti, 35 KHAK örneği ve 8 normal akciğer dokusundan oluşmaktadır. miRNA'nın ifadesinde, Agilent Human 0.6K miRNA Mikroarray G4471A platformu kullanılmıştır. Küçük Hücreli olmayan Akciğer kanseri (KHDAK) miRNA GSE135918 Exiqon miRCURY LNA mikroRNA array, 7th generation platformu kullanılarak analiz edilen veri seti, 5 KHDAK örneği ve 5 normal akciğer dokusu örneği içermektedir. Örnek sayıları ve çalışılan platformlar Tablo 2'de gösterilmiştir. Çalışmanın ilerleyişini gösteren iş akış şeması Şekil 1'de gösterilmiştir.

Diferansiyel olarak eksprese edilen miRNA'ların taranması 2 profilin verileri, R dili kullanarak analiz yapan GEO2R'yi temel alan bir çevrimiçi analiz programı kullanılarak analiz edildi. Profillerdeki diferansiyel miRNA'ları karşılaştırmak ve farklı olanları analiz etmek için Venn yazılımı kullanıldı ve genellikle up ve down regüle edilen diferansiyel miRNA'lar elde edildi (Şekil 2). GSE19945 ve GSE 135918 nolu verilere ait Volkano plots ve hasta ve kontrol örneklerinin gruplandığı UMAP grafikleri oluşturulmuştur. Volcano plot grafiklerinde her grafik, yatay eksende log2 (kat değişimi) ve dikey

eksende log10 (q değeri) ile çizilen genin ifadesini temsil eder. Kırmızı, up regülasyonu, mavi down regülasyonu anlamlı bir fark olduğunu gösterirken siyah noktalar ise anlamsız olanları temsil eder.Örnek dağılımı açısından bakıldığında ise kanserli örneklerin birbirine benzer yapıda olup gruplandığı yine aynı şekilde kontrol gruplarınında birbiriyle ortak gruplandığı gösterilmiştir. Yeşil noktalar kanser örneklerini mor olanlar kontrol gruplarını temsil eder (Şekil 2).

Tablo 2. Analizi yapılacak GEO veri setleri

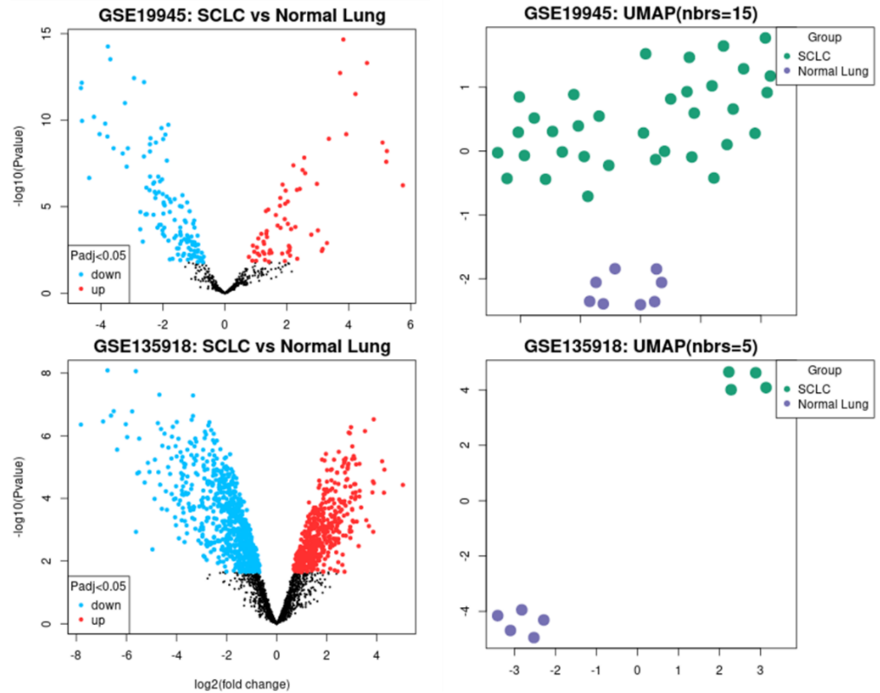
Veri seti	Hasta sayısı	Kontrol sayısı	Platform	Tipi
GSE19945	35	8	Agilent Human 0.6K miRNA Microarray G4471A platformu kullanılmıştır	KHAK
GSE135918	5	5	Exiqon miRCURY LNA microRNA array, 7th generation	KHDAK



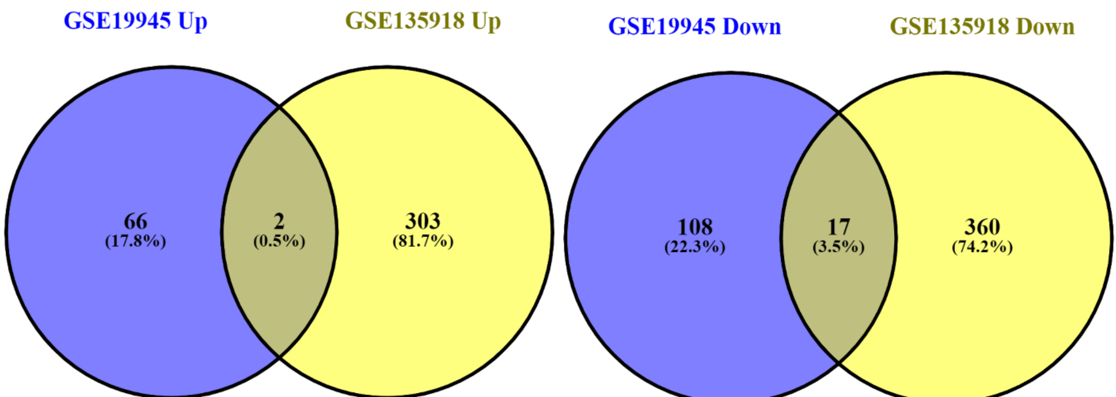
Şekil 1. İş akış planı

Küçük Hücreli ve Küçük Hücreli Olmayan Akciğer Kanserlerinde Rol Oynayan miRNA'ların DEM'lerin Tanımlanması

İki veri seti GEO2R analizi ile analiz edildi ve 1 numaralı veri seti, GSE19945'te 68 up regüle edilmiş gen ve 125 down regüle edilmiş gen, GSE135918'de ise 305 yukarı regüle edilmiş gen ve 377 aşağı regüle edilmiş miRNA bulunmuştur. 2 veri setinin farklı gen ekspresyon modellerine göre bir Venn diyagramı oluşturuldu ve 17 azalan ve 2 artan ortak diferansiyel olarak eksprese edilmiş miRNA tesbit edildi (Şekil 1). 17 azalan ortak miRNA'lar hsa-miR-1249, hsa-miR-326, hsa-let-7c, hsa-miR-199a-5p, hsa-miR-940, hsa-miR-139-3p, hsa-miR-142-3p, hsa-miR-142-5p, hsa-miR-455-5p, hsa-miR-146b-5p, hsa-miR-152hsa-miR-133b, hsa-miR-498, hsa-miR-199b-5p, hsa-miR-140-3p, hsa-miR-203 ve hsa-miR-139-5p'dir. Artan ortak 2 miRNA ise hsa-miR-758 ve hsa-miR-210'dur. Her iki grupta da ifadesi en çok artan ve azalan miRNA'lar ise Şekil 3 ve Tablo 3-4'de verilmiştir.



Şekil 2. GSE19945 ve GSE135918 nolu verilere ait Volkano plots ve hasta ve kontrol örneklerinin gruplandığı UMAP grafikleri. Her grafik, yatay eksende log2 (kat değişimi) ve dikey eksende log10(q değeri) ile çizilen genin ifadesini temsil eder. Kırmızı, up regülasyonu, mavi down regülasyonu, anlamlı bir fark olmadığını temsil eder



Şekil 3. Venn yazılımı tarafından tanımlanan artan azalan ve ortak değişen miRNA sayıları (logFC >1 ve logFC <−1, p<0,05)

Tablo 3. GSE19945 nolu küçük hücreli akciğer kanseri (KHAK) veri setinde ifadesi en çok artan ve azalan 20 miRNA (adjpval<0.05, pvalue<0.05)

İfadesi artan miRNA'lar			İfadesi azalan miRNA'lar		
miRNA_ID	**P.Value**	**logFC**	**miRNA_ID**	**P.Value**	**logFC**
hsa-miR-9*	5.86E-07	5.7	**hsa-miR-1**	1.4E-12	-4.6
hsa-miR-1290	6.17E-09	5.2	**hsa-miR-144***	6.94E-13	-4.6
hsa-miR-9	2.57E-08	5.2	**hsa-miR-144**	1.11E-10	-4.6
hsa-miR-7	1.98E-09	5	**hsa-miR-30a***	2.19E-07	-4.3
hsa-miR-130b	3.09E-12	4.2	**hsa-miR-486-5p**	6.45E-10	-4
hsa-miR-301b	6.48E-10	3.9	**hsa-miR-145**	1.6E-10	-3.8
hsa-miR-182	1.89E-13	3.7	**hsa-miR-338-3p**	8.86E-10	-3.7
hsa-miR-18a	1.2E-09	3.3	**hsa-miR-126**	5.57E-15	-3.7
hsa-miR-410	0.00363	3.1	**hsa-miR-126***	3.02E-14	-3.6
hsa-miR-592	0.000237	3	**hsa-miR-30a**	4.24E-09	-3.5
hsa-miR-200a*	4.83E-07	2.9	**hsa-miR-143**	8.45E-09	-3.2
hsa-miR-196b	0.000413	2.7	**hsa-miR-145***	1.03E-11	-3.2
hsa-miR-18b	1.17E-07	2.5	**hsa-miR-223**	4.86E-08	-3.1
hsa-miR-200b	1.48E-08	2.5	**hsa-miR-572**	4.24E-09	-3.1
hsa-miR-429	7.63E-08	2.5	**hsa-miR-638**	3.72E-13	-2.9
hsa-miR-181c*	9.47E-07	2.4	**hsa-miR-551b**	0.000203	-2.7
hsa-miR-216a	0.0103	2.3	**hsa-miR-139-5p**	2.03E-05	-2.7
hsa-miR-1287	0.00015	2.2	**hsa-miR-203**	0.00105	-2.6
hsa-miR-301a	4.1E-08	2.2	**hsa-miR-29a**	1.25E-08	-2.6
hsa-miR-30c-2*	6.37E-13	2.6	**hsa-miR-200b***	0.000197	-2.1

Tablo 4. GSE135918 nolu küçük hücreli olmayan akciğer kanseri (KHDAK) veri setinde ifadesi en çok artan ve azalan 20 miRNA (adjPval<0.05 Pvalue<0.05)

İfadesi artan miRNA'lar			İfadesi azalan miRNA'lar		
miRNA_ID	**P.Value**	**logFC**	**miRNA_ID**	**P.Value**	**logFC**
hsa-miR-665	0.000037	5.0	**hsa-miR-20a-5p**	4.37E-07	-7.8
hsa-miR-548l	0.0000655	4.2	**hsa-miR-126-5p**	8.15E-09	-6.7
hsa-miR-4470	0.0000065	4.2	**hsa-miR-126-3p**	2.26E-07	-6.6
hsa-miR-3925-5p	0.000761	3.6	**hsa-miR-199b-5p**	2.77E-06	-6.3
hsa-miR-369-3p	0.000000708	3.5	**hsa-miR-20b-5p**	4.26E-07	-6.0
hsa-miR-558	0.000194	3.3	**hsa-miR-101-3p**	1.1E-06	-5.9
hsa-miR-4485	0.00331	3.2	**hsa-miR-195-5p**	1.66E-07	-5.7
hsa-miR-1247-3p	0.000545	3.1	**hsa-miR-142-3p**	0.00117	-5.6
hsa-miR-4636	0.0000349	3.1	**hsa-miR-30a-3p**	1.59E-05	-5.5
hsa-miR-4524b-5p	0.000104	3.1	**hsa-miR-451a**	1.46E-05	-5.5
hsa-miR-224-3p	0.00000534	3.1	**hsa-miR-660-5p**	1.23E-06	-5.4
hsa-miR-4488	0.000035	3.0	**hsa-miR-15a-5p**	0.000031	-5.2
hsa-miR-4516	0.00000443	3.0	**hsa-miR-16-5p**	7.3E-06	-5.1
hsa-miR-1228-5p	0.00056	3.0	**hsa-miR-30b-5p**	1.43E-05	-5.0
hsa-miR-5011-5p	0.000067	3.0	**hsa-miR-26b-5p**	0.000103	-4.8
hsa-miR-4732-5p	0.00000217	3.0	**hsa-miR-335-5p**	1.45E-05	-4.7
hsv2-miR-H10	0.000216	3.0	**hsa-miR-26a-5p**	3.64E-05	-4.6
hsa-miR-3175	0.0000922	2.9	**hsa-miR-4536-3p**	8.53E-07	-4.4
hsa-miR-125b-1-3p	0.000000532	2.9	**hsa-miR-181c-5p**	5.3E-06	-4.2
hsa-miR-381	0.00183	2.9	**hsa-miR-144-3p**	7.21E-05	-4.1

MiRNA Hedef Genlerinin Tahmin Edilmesi

Her veri setinden ifadesi en çok artan ve azalan 5 miRNA’nın hedef genleri çıkarılmış ve bu genler karşılaştırılmıştır. GSE19945 nolu KHAK veri setinde ifadesi en çok azalan 5 miRNA’nın toplam

hedef gen sayısı 2719, ifadesi en çok artan 5miRNA'nın toplam hedef gen sayısı ise 3181'dir. GSE135918 nolu küçük hücreli olmayan akciğer kanseri (KHDAK) veri setinde ifadesi en çok azalan 5 miRNA'nın toplam hedef gen sayısı 2948, ifadesi en çok artan 5miRNA'nın toplam hedef gen sayısı ise 3894' dür. Daha sonra yapılan analizlerde bu hedef genler üzerinden yolak ve PPI analizleri yapılmıştır ($p<0.05$) (Şekil 4-5).

	hsa-miR-1290 Up (945)	hsa-miR-9 Up (1236)	hsa-miR-7 Up (875)	hsa-miR-130b Up (917)
hsa-miR-9* Up (991)	110	164	94	129
hsa-miR-1290 Up (945)		142	90	92
hsa-miR-9 Up (1236)			122	142
hsa-miR-7 Up (875)				95

	hsa-miR-144* Down (240)	hsa-miR-144 Down (1254)	hsa-miR-30a* Down (1390)	hsa-miR-486-5p Do wn (331)
hsa-miR-1-3p Down (945)	28	148	150	30
hsa-miR-144* Down (240)		30	39	9
hsa-miR-144 Down (1254)			230	62
hsa-miR-30a* Down (1390)				62

Şekil 4. GSE19945 nolu KHAK veri setinde ifadesi en çok azalan 5 miRNA'nın ortak hedef gen sayıları

	hsa-miR-548l Up (1201)	hsa-miR-4470 Up (861)	hsa-miR-3925-5p Up (1066)	hsa-miR-369-3p Up (712)
hsa-miR-665 Up (911)	69	64	55	47
hsa-miR-548l Up (1201)		150	202	125
hsa-miR-4470 Up (861)			120	73
hsa-miR-3925-5p Up (1066)				86

	hsa-miR-126-5p Do wn (1362)	hsa-miR-126-3p Do wn (19)	hsa-miR-199b-5p Do wn (556)	hsa-miR-20b-5p Do wn (1325)
hsa-miR-20a-5p Down (1381)	222	3	86	1321
hsa-miR-126-5p Down (1362)		2	78	212
hsa-miR-126-3p Down (19)			1	3
hsa-miR-199b-5p Down (556)				84

Şekil 5. GSE135918 nolu KHAK veri setinde ifadesi en çok artan 5 miRNA'nın ortak hedef gen sayıları

Hem KHAK ile KHDAK arasında farklı ifade edilen miRNA'lara hem de ortak miRNA'lara bakmak önemlidir, çünkü her iki yaklaşım da kanserin biyolojik yapısını anlamak ve uygun tedavi stratejileri geliştirmek açısından farklı avantajlar sağlar. Farklı ifade edilen miRNA'lara bakmak tanı ve

ayırıcı teşhis için önemlidir. KHAK ve KHDAK, klinik seyirleri ve tedavi yaklaşımları bakımından oldukça farklıdır. Bu iki kanser tipi arasında farklı şekilde ifade edilen miRNA'ları incelemek, her iki kanser tipini daha iyi ayırt etmeye ve erken teşhis için biyomarker geliştirmeye yardımcı olabilir. Örneğin, bazı miRNA'lar KHAK'de yüksek seviyelerdeyken, KHDAK'de düşük seviyelerde olabilir, bu da doğru tanıyı koymada kritik olabilir. Hedefe Yönelik Tedavi için farklı şekilde ifade edilen miRNA'lar, her kanser türünde farklı biyolojik süreçleri yönlendirebilir. Bu, spesifik tedavi hedeflerinin belirlenmesinde önemli olabilir. Örneğin, KHAK'de tümör büyümesini artıran bir miRNA, KHDAK'de baskılanıyor olabilir, bu da tedavi yaklaşımlarında önemli farklılıklar yaratır. Prognostik Biyomarkerlar açısından bakıldığında ise farklı şekilde ifade edilen miRNA'lar, her iki kanser tipinde hastalığın seyrini ve prognozunu anlamada kritik olabilir. Örneğin, KHAK'de belirli bir miRNA'nın yüksek ekspresyonu daha kötü prognozla ilişkili olabilirken, KHDAK'de bu durum farklı olabilir.

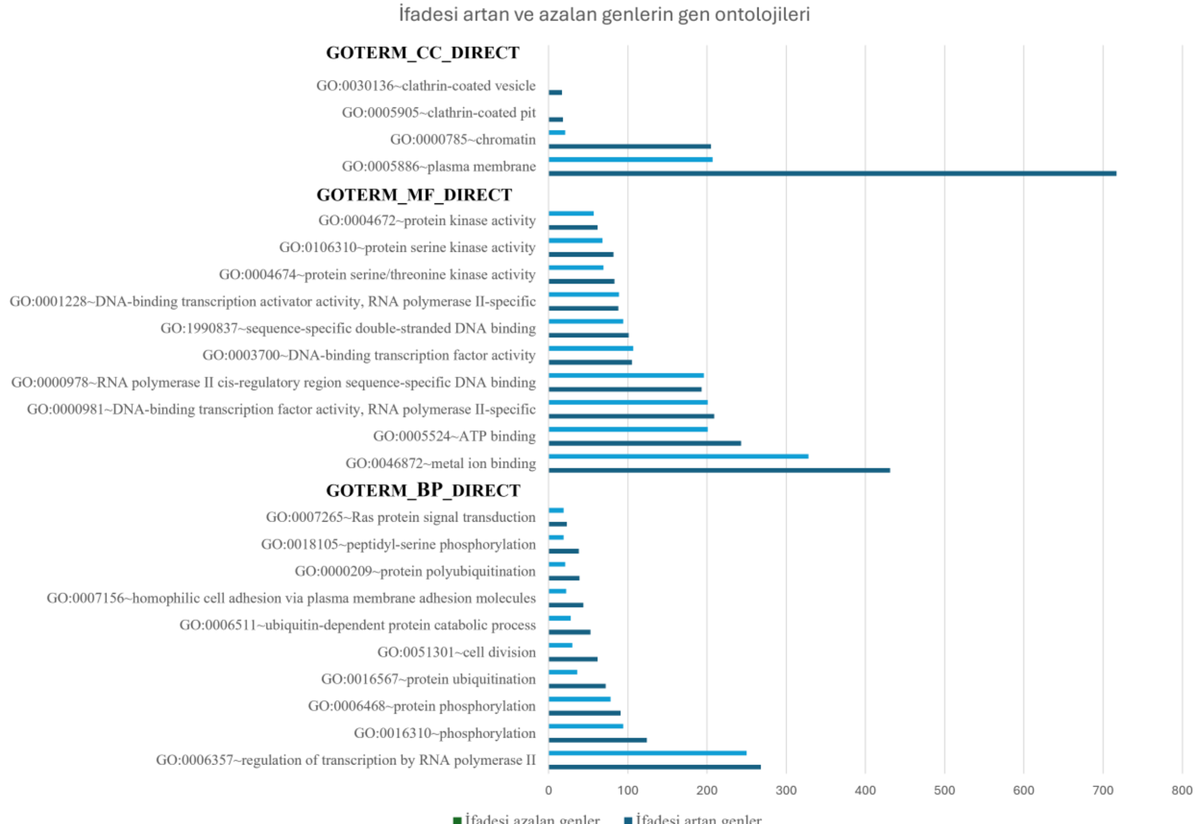
Ortak miRNA'lara bakmanın önemi ise ortak patolojik mekanizmalar için önemlidir. KHAK ve KHDAK arasında ortak şekilde ifade edilen miRNA'lar, her iki kanser türünde de benzer biyolojik süreçleri ve tümör gelişimini düzenleyen temel mekanizmaları işaret edebilir. Bu ortak miRNA'lar, tümör progresyonu, metastaz veya ilaç direnci gibi kritik süreçleri anlamada yol gösterici olabilir. Geniş spektrumlu tedavi stratejileri için ortak miRNA'lar, her iki kanser türüne de etki edebilecek geniş spektrumlu tedavi yaklaşımlarını hedeflemek için önemlidir. Eğer bir miRNA her iki kanser türünde de ortak olarak düzenlenmişse, bu miRNA'yı hedefleyen tedaviler hem KHAK hem de KHDAK için etkili olabilir. Direnç Mekanizmalarında ise ortak miRNA'lar, özellikle kemoterapi veya radyoterapiye direnç mekanizmalarını anlamada önemli olabilir. Eğer bir miRNA her iki kanser türünde de terapötik yanıtta rol oynuyorsa, bu miRNA'yı hedeflemek direnç gelişimini önlemede faydalı olabilir. Farklı miRNA'lar, tanı, tedavi ve prognozun kişiselleştirilmesi için kritik olabilir. KHAK ve KHDAK'yi birbirinden ayırmaya ve spesifik tedavi hedefleri bulmaya yardımcı olur. Ortak miRNA'lar ise ortak biyolojik süreçleri ve tedavi direncini anlamak için önemli olup, geniş spektrumlu tedavi stratejileri geliştirmede yol gösterici olabilir. Her iki grubu incelemek, hem kanser biyolojisinin derinlemesine anlaşılmasını sağlar hem de daha etkili ve kişiselleştirilmiş tedavi stratejilerinin geliştirilmesine katkıda bulunacağı düşünülmeltedir.

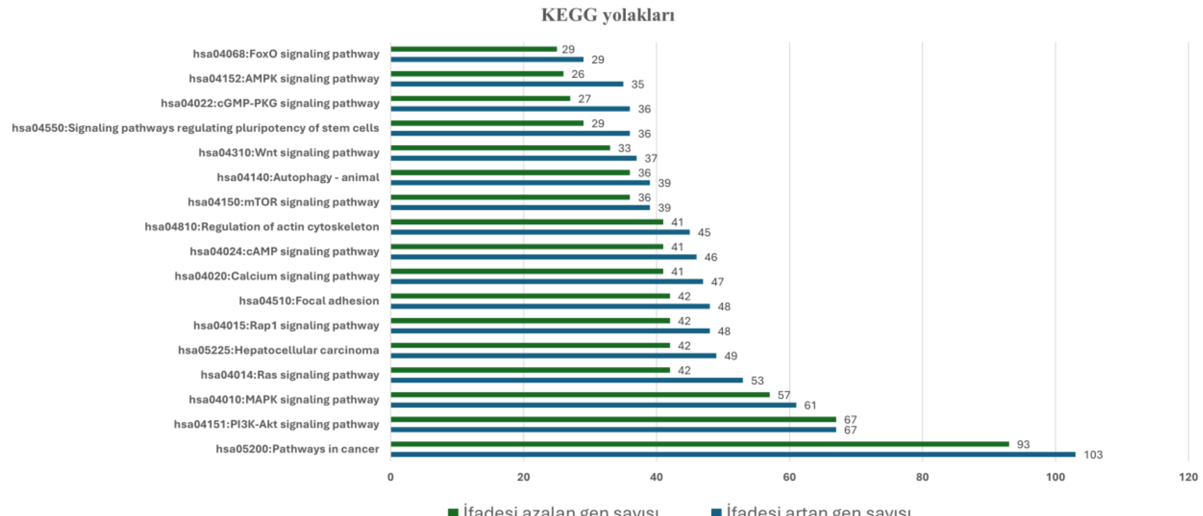
miRNA Hedef Genlerinin Fonksiyonel Zenginleştirme Analizi

Veri açıklama analizi için yaygın olarak kullanılan web tabanlı bir genomik işlevsel açıklama aracı olan DAVID (https://david.ncifcrf.gov/) kullanılacaktır. Bu çalışmada miRNA'ların hedef genleri, Gene Ontology (GO) analizi ve Kyoto Genler ve Genomlar Ansiklopedisi (KEGG) yol analizi ile moleküler fonksiyon ve yolak analizleri yapılmıştır (Şekil 6).

Protein-Protein Etkileşimi (PPI) Ağlarının Oluşturulması ve Modül Araştırması

DEG'lerin PPI ağı, anahtar sinyal yolakların ve hücresel aktivitelerin moleküler mekanizmalarını tanımlamak için Cytoscape kullanılarak oluşturmuştur. Cytoscape, biyoinformatik analizde ağ verilerini görselleştirmek ve analiz etmek için kullanılan güçlü bir araçtır. MCODE ve CytoHubba gibi eklentiler (plug-in'ler) özellikle biyolojik ağlarda anahtar genleri ve proteinleri belirlemek için kullanılır. MCODE, ağlarda modüller ya da yoğun alt ağları (cluster) tespit etmek için kullanılan bir algoritmadır. Bu modüller genellikle fonksiyonel olarak ilişkili gen ya da protein kümeleri içerir. [14]. Çalışmamızda MCODE ve cytohubba analizleri soncu önemli olan aday genleri gösterdik her iki veri seti için ayrı ayrı sonuçlar çıkarılmış ve Tablo 5-6'da ve Şekil 7-8'de belirtilmiştir.





Şekil 6. miRNA'ların hedef genleri, Gene Ontology (GO) analizi ve Kyoto Genler ve Genomlar Ansiklopedisi (KEGG) yolakları

Tablo 5. GSE19945 nolu veri setinde ifadesi en çok azalan ve artan 5 miRNA'nın ortak hedef genlerin MCODE analiz sonucu öne çıkan önemli genler ($p<0.05$)

İfadesi azalan hedef genler				
Küme	**Skor**	**Düğüm**	**Köşe**	**Genler**
1	10	10	46	BCL11A, BCL7A, ARID1A, SMARCB1, SMARCA2, SMARCC1, SS18, ARID2, BICRA, SMARCA4
2	7	7	22	YWHAZ, YWHAE, YWHAQ, WWTR1, YWHAB, CSNK1E, YWHAG
3	6.3	7	19	CTTNBP2, MOB4, SLMAP, PDCD10, STRN3, CTTNBP2NL, STRN
4	6.3	7	19	MED4, CDK8, CDK19, MED6, MED13, MED14, MED12L
5	5	5	13	BRD8, MRGBP, MEAF6, YEATS4, MBTD1
6	5	5	10	SMAD1, BMPR1B, SMAD2, BMPR1A, SMAD9
7	5	5	11	WDR61, CCNT2, AFF4, ELOC, CDC73
İfadesi artan hedef genler				
Küme	**Skor**	**Düğüm**	**Köşe**	**Genler**
1	12.5	13	75	ARID1B, SMARCC1, SMARCA2, ACTL6A, BCL7A, ARID2, DPF3, SMARCE1, MARCD2, ARID1A, PBRM1, BICRAL, SMARCD1
2	9.4	11	47	PLCG1, PTK2, PTEN, PIK3R1, PIK3R3, PIK3CD, PIK3CA, EGFR, ERBB4, ITGB1, PDGFRB
3	5.6	6	14	PPP2R1B, PPP2CA, STK24, STRN3, STRN, STRIP2
4	5	5	11	PCGF6, CBX7, ASXL1, PCGF5, PHC1
5	5	5	10	VAMP3, STX1A, SNAP23, STX6, VAMP7
6	5	5	10	CDK6, E2F1, RB1, CCNA2, CCNE1

Tablo 6. GSE135918 nolu veri setinde ifadesi en çok azalan ve artan 5 miRNA'nın ortak hedef genlerin MCODE analiz sonucu öne çıkan önemli genler ($p<0.05$)

İfadesi artan hedef genler				
Küme	**Skor**	**Düğüm**	**Köşe**	**Genler**
1	5.6	6	14	CPEB3, CNOT4, TNKS1BP1, CNOT6, CNOT7, CNOT6L
2	4.8	6	12	BHLHE41, NPAS2, CRY2, RORA, NFIL3, CLOCK
İfadesi azalan hedef genler				
Küme	**Skor**	**Düğüm**	**Köşe**	**Genler**
1	8	8	28	NUP205, NUP58, NUP133, NDC1, NUP160, AHCTF1, NUP98, NUP37
2	7	7	21	AFF1, AFF4, MLLT3, SUPT4H1, ELL, MLLT1, ELL2
3	5	5	10	SMAD7, SMAD6, SMAD1, SMAD4, SMAD5
4	4.5	5	9	LSM5, WBP4, SF3B3, LSM8, SNRPC

CytoHubba, ağlardaki en önemli düğümleri (hub) ya da genleri/proteinleri tanımlamaya yardımcı olan bir eklentidir. Farklı algoritmalar kullanarak ağdaki hub genlerini ya da proteinlerini puanlar ve sıralar. Bu analiz ile anahtar Genler/Proteinler bulunur. CytoHubba, bir biyolojik ağda kritik rol oynayan hub genleri ya da proteinleri tespit eder. Bu hub'lar genellikle ağın merkezi unsurlarıdır ve hastalık süreçlerinde veya biyolojik işlevlerde önemli rol oynayabilir. Hedef Gen Adaylarını bulmamızda yol gösterir. Bu analiz, yeni biyolojik araştırmalar veya ilaç hedeflemeleri için aday genleri belirlemek için kullanılabilir. MCODE ile fonksiyonel modüller belirlenirken, CytoHubba ile bu modüllerdeki veya ağın genelindeki en önemli genler keşfedilir. Hastalık Mekanizmalarının Aydınlatılmasında özellikle hastalıklarla ilişkili ağlarda, kritik düğümler (hub'lar) veya modüller belirlenerek hastalık süreçlerinin mekanizmaları anlaşılabilir. Biyolojik ağlardaki önemli genler ve proteinler, potansiyel tedavi hedefleri olabilir. CytoHubba, bu tür hub genlerin keşfi için kullanılır. Bu analizler, biyolojik ağların daha derinlemesine anlaşılmasını sağlar ve hücre içi etkileşimlerde kritik olan gen/protein gruplarını tespit etmeye yardımcı olur [14-20].

GSE19945 nolu küçük hücreli akciğer kanseri veri setinde ifadesi en çok artan ARID1A, SAMARCA2, SMARCD2, SMARCC1, SMARCE1, ACTL6A, ARD1B, DPF3, BICRAL, BCL7A, PIK3CA, PIK3R1, PDCFRB, EGFR ve PTEN genleri hub genler olarak tanımlanmıştır (Şekil 7A).

ARID1A, SMARCA2, SMARCD2, SMARCC1, SMARCE1, ACTL6A, ARID1B, DPF3, BICRAL, BCL7A hem ifadesi artan hemde azalan miRNA'ların hedef genleri arasında çıkmıştır. PIK3CA, PIK3R1, PDGFRB, EGFR ve PTEN genleri, potansiyel miRNA hedefleri olarak dikkat çekmektedir. Literatürde ARID1A ve SMARCA2 gibi kromatin düzenleme ile ilgili genlerin, KHAK'ın epigenetik düzenlenmesinde rol oynadığı rapor edilmiştir. Bu genlerin fonksiyon kaybı mutasyonları, hücre proliferasyonu ve metastaz ile ilişkilendirilmiştir [21]. Bununla birlikte, miRNA aracılı mekanizmalar yoluyla bu genlerin düzenlenmesine dair özellikle KHAK'daki rolü hakkında literatürde sınırlı bilgi bulunmaktadır. Ancak bu genlerin miRNA aracılı hub hedefler olarak özellikle KHAK'da fonksiyonlarının daha iyi anlaşılması için ileri düzey deneysel çalışmalar gereklidir. Literatürde bazı genler üzerine elde edilen veriler umut verici olmakla birlikte, bu genlerin tamamının miRNA aracılı düzenlenmesi hakkında kapsamlı ve sistematik veriler hala sınırlıdır. Bu durum, gelecekteki çalışmalara yön verebilecek önemli bir bilgi eksikliğine işaret etmektedir.

İfadesi azalan genler arasında ise CSNK1E, YWHAZ, YWHAG, YWHAB, CSNK1E, CREBBP, EP300, STRN (Şekil 7A) genleri bulunmaktadır (Şekil 8A-B). Protein Kinaz ve Adaptor Protein Genleri (CSNK1E, YWHAZ, YWHAG, YWHAB, CREBBP, EP300, STRN) CSNK1E (Casein Kinase 1 Epsilon), YWHA ailesi (YWHAZ, YWHAG, YWHAB), CREBBP, EP300 ve STRN genleri, hücre sinyal iletimi, apoptoz, epigenetik düzenleme ve sirkadiyen ritim gibi çeşitli biyolojik süreçlerde kritik rol oynamaktadır [22-24].

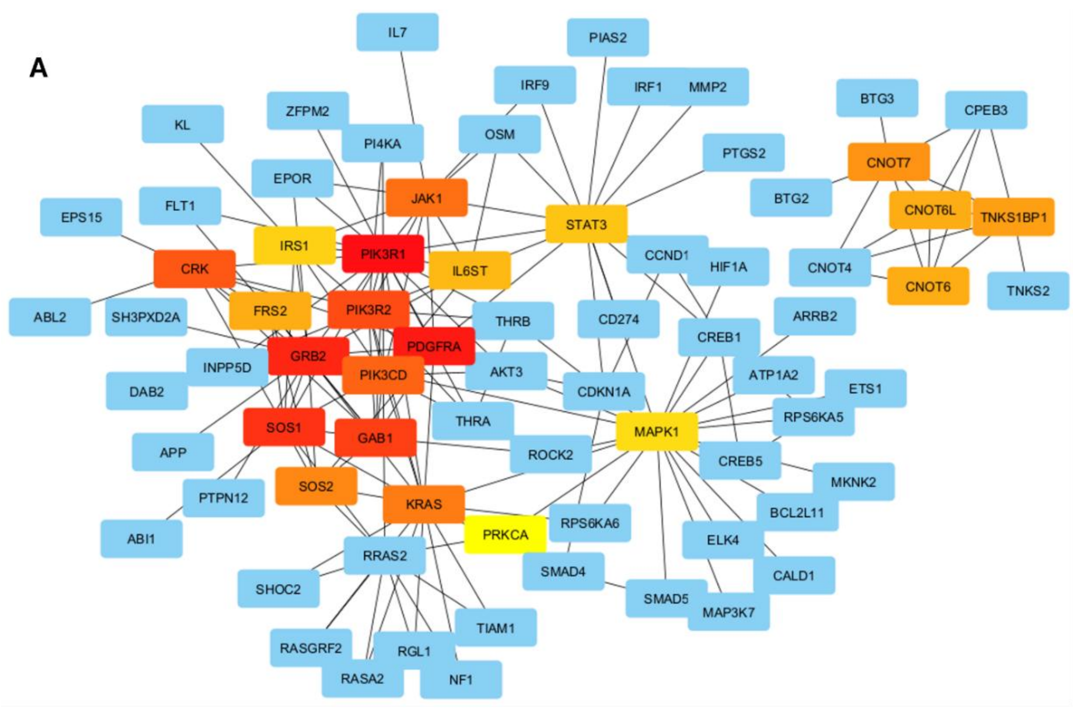
GSE135918 nolu veri setinde ifadesi en çok artan PDGFRA, PIK3R1, GRB2, SOS1 GAB1, KRAS PIK3R2, PIK3CD, CRK, JAK1, STAT3, MAPK1 genleri hub genler olarak tanımlanmıştır. Bu genler, küçük hücreli olmayan akciğer kanseri gibi kanser türlerinde hücre sinyallemesi, büyüme faktörü yanıtları ve tümör gelişiminde kritik roller oynar. PDGFRA (Platelet-Derived Growth Factor Receptor Alpha) Hücre büyümesi, farklılaşma ve migrasyon süreçlerinde rol oynar. KHDAK'da aşırı ekspresyonu veya aktivasyonu, tümör büyümesini destekleyebilir [25]. PIK3R1, PIK3R2, PIK3CD (Phosphoinositide 3-Kinase Regulatory/ Catalytic Subunits) PI3K/AKT/mTOR yolaklarının düzenlenmesinde görev alır. Bu yolak, kanser hücrelerinde proliferasyonun artışı ve apoptozun baskılanmasıyla ilişkilidir. GRB2 (Growth Factor Receptor-Bound Protein 2) hücre sinyallemesini başlatan adaptor proteinlerden biridir. EGFR mutasyonları ile ilişkili olarak KHDAK'da hücre büyümesi ve metastazı teşvik edebilir [26].

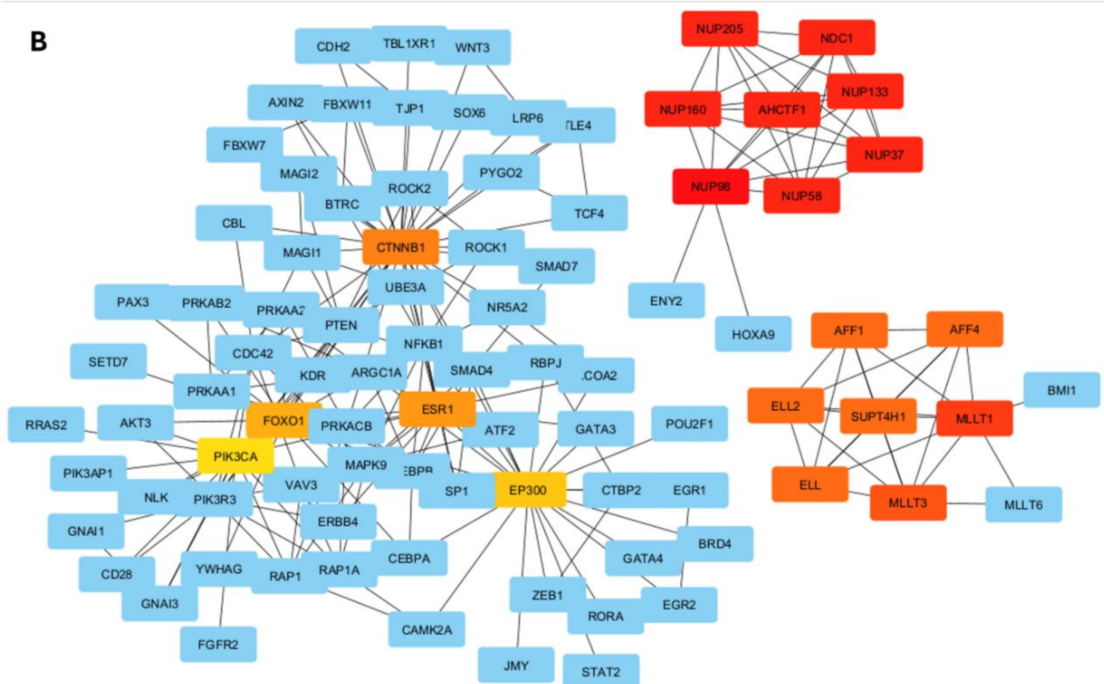
SOS1 (Son of Sevenless Homolog 1), Ras-MAPK yolaklarının aktivasyonunda görev alır ve tümör hücrelerinin büyüme sinyallerine duyarlılığını artırır. GAB1 (Grb2-Associated Binder 1) Hücre içi sinyal iletiminde önemli bir adaptor proteindir ve özellikle EGFR mutasyonlarının aktive ettiği yolaklarda rol oynar. KRAS, KHDAK'da sıkça mutasyona uğrar ve bu mutasyonlar, hücre büyüme ve hayatta kalma mekanizmalarını aktive eder. CRK (v-Crk Avian Sarcoma Virus CT10 Oncogene Homolog) hücre iskeletinin yeniden düzenlenmesi ve migrasyon süreçlerinde görev alır. CRK'nin KHDAK'daki aşırı ekspresyonu metastaz ile ilişkilendirilmiştir. JAK1 ve STAT3 büyüme faktörleri ve sitokin sinyallemesi yoluyla hücre proliferasyonu ve apoptozun baskılanmasını kontrol eder. Bu yolakların aşırı aktivasyonu KHDAK'ın ilerlemesine katkıda bulunur. MAPK1 (Mitogen-Activated Protein Kinase 1) Hücre büyümesi ve farklılaşmasında rol oynar. MAPK yolaklarının aktivasyonu, KHDAK'ın tümör progresyonu ile bağlantılıdır [27].

Ifadesi azalan hub genler ise NUP205, NUP58, NUP37, NUP133, NUP160, NUP58, AHCTF1, MLLT1, MLLT3, AFF1, AFF4, SUPT4H1, ELL, ELL2, CTNNB1, ESR1, FOXO1, PIK3CAgenleridir. Nükleoporin Genleri (NUP205, NUP58, NUP37, NUP133, NUP160, AHCTF1), hücre çekirdeği ile sitoplazma arasındaki madde alışverişini düzenleyen nükleer por kompleksinin bileşenleridir. KHDAK'da, bu genlerin disfonksiyonu hücre sinyal yollarının bozulmasına, hücre bölünmesinin düzensizleşmesine ve tümör progresyonuna yol açabilir [28].

Translokasyon ile İlişkili Genler (MLLT1, MLLT3, AFF1, AFF4), genellikle hematolojik kanserlerle ilişkilendirilen füzyon proteinleri ile bilinse de, NSCLC'de kromatin düzenlenmesi ve transkripsiyonel kontrol süreçlerinde görev alabilir. Bu genlerdeki anormallikler, hücre büyümesini ve tümör agresifliğini artırabilir [29]. Bu genlerin ifade düzeylerindeki azalmalar, genellikle hücre içi sinyal yollarının bozulmasına, tümör baskılayıcı mekanizmaların etkisizleşmesine ve tümör progresyonuna katkıda bulunur. Bu genler, KHDAK tedavisinde potansiyel hedefler olarak değerlendirilebilir.

Şekil 7. GSE19945 nolu veri setinde ifadesi en çok A. Artan ve B. azalan 5 miRNA'nın ortak hedef genlerin Cytohubba analiz sonucu öne çıkan 20 önemli gen. Kırmızıdan sarıya renkli CytoHubba düğümleri ($p<0.05$), uygulanan topolojik yaklaşıma göre en yüksek ila en düşük sıralı düğümleri temsil eder



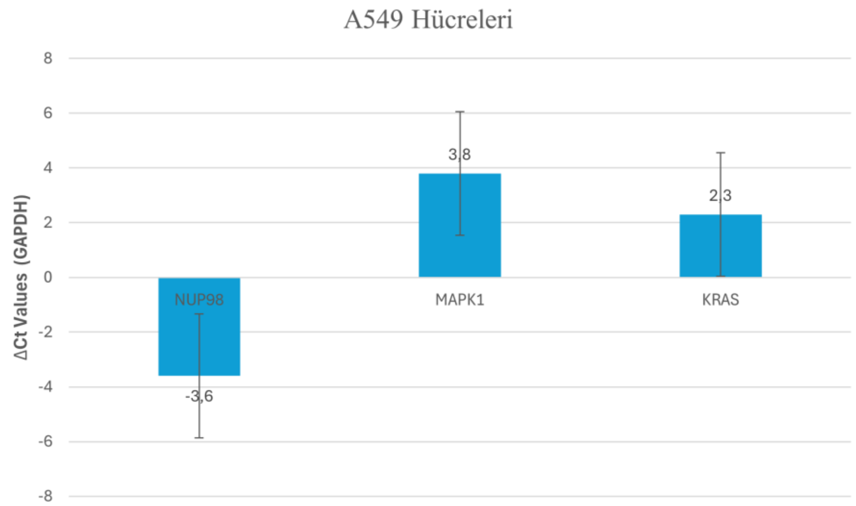


Şekil 8. GSE135918 nolu veri setinde ifadesi en çok A. artan ve B. azalan 5 miRNA'nın ortak hedef genlerin Cytohubba analiz sonucu öne çıkan 20 önemli gen. Kırmızıdan sarıya renkli CytoHubba düğümleri ($p<0.05$), uygulanan topolojik yaklaşıma göre en yüksek ila en düşük sıralı düğümleri temsil eder

A549 Akciğer Kanseri Hücre Kültürü ile miRNA Hedef Genlerinin *İn-Vitro* Validasyonu

Çalışmamız veri analizine dayandığından, sonuçlarımızı doğrulamak için biyolojik deneylere ihtiyaç vardır. Küçük hücreli olmayan akciğer kanseri hücre hattı A549'da etkilerinin araştırılması amaçlanmıştır. Mevcut analizimizi doğrulamak için miRNA hedef genlerinden 3 genin doğrulaması

yapılmıştır. MAPK1 ve KRAS ifadesi artan NUP98 geni ise ifadesi azalan gen olarak seçilmiştir. Sonuçlar yapılan analizlerle uyumlu çıkmıştır (Şekil 9).



Şekil 9. Farklı şekilde ifade edilen 3 genin ifade seviyeleri (p<0.05)

Kanser biyolojisindeki önemli ilerlemeler, özellikle kanser ilerlemesi ve gelişimi ile bağlantılı olan ve bu nedenle "biyobelirteçler" olarak adlandırılan çeşitli biyomoleküllerin keşfiyle sonuçlanmıştır. Biyobelirteçler temel olarak normal, anormal veya sadece biyolojik bir süreci tanımlamak veya izlemek için kullanılabilen hücresel, biyokimyasal ve moleküler değişiklikler olan değişikliklerdir. Patojenik süreçleri, normal biyolojik süreçleri ve bir tedavi müdahalesine verilen farmakolojik yanıtı objektif olarak test etmek ve değerlendirmek için kullanılırlar. Biyobelirteçler, kimyasal yapıları ve transkriptomik, metabolomik, genomik ve proteomik kullanılarak tanımlanabilen işlevselliklerine göre sınıflandırılabilir [15,17-20].

miRNA düzensizliği KHDAK'ta sıklıkla bulunur ve miRNA'ların anormal ifadeleri, hedef genlerini düzenleyerek KHDAK'ın proliferasyonu, istilası ve metastazında önemli bir rol oynar [16]. Bu çalışmada, KHAK ve KHDAK'taki anormal miRNA imzalarının biyoinformatik analizi yapılarak ve miRNA'ların hedef genlerini çıkararak her iki kanser tipindeki farklılıkları ve benzerlikleri ortaya koymayı planlamaktayız. Ayrıca ortaya çıkacak miRNA'ların terapötik hedefler veya biyobelirteçler olma olasılığı araştırılacaktır. miRNA'lar tarafından düzenlenen anahtar genler, akciğer kanserinin artan prognozu ve teşhisi için potansiyel biyobelirteçler hakkında bazı ipuçları sağlayabilir.

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

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

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

A COMPREHENSIVE ANALYSIS OF THE CHEMICAL PROFILE OF VOLATILE COMPOUNDS IN *NARCISSUS TAZETTA* L. SUBSP. *TAZETTA* THROUGH HIERARCHICAL CLUSTERING METHODS

NARCISSUS TAZETTA L. SUBSP. *TAZETTA UÇUCU BİLEŞİKLERİNİN KİMYASAL PROFİLİNİN HİYERARŞİK KÜMELEME YÖNTEMLERİYLE KAPSAMLI ANALİZİ*

Damla KIRCI[1]*, Osman TUGAY[2], Deniz ULUKUŞ[3], Betül DEMİRCİ[4]

[1]İzmir Katip Çelebi University, Faculty of Pharmacy, Department of Pharmacognosy, 35600, İzmir, Türkiye
[2]Selçuk University, Faculty of Pharmacy, Department of Pharmaceutical Botany, 22040, Konya, Türkiye
[3]Selçuk University, Faculty of Science, Department of Biotechnology, 22040, Konya, Türkiye
[4]Anadolu University, Faculty of Pharmacy, Department of Pharmacognosy, 26470, Eskişehir, Türkiye

ABSTRACT

Objective: *This study aimed to analyze the chemical profile of volatile and non-volatile components of Narcissus tazetta subsp. tazetta (daffodil) is grown in Türkiye. The chemical profiles of fresh and dried flowers were analyzed using Headspace Solid-Phase Microextraction (HS-SPME) and Gas Chromatography-Mass Spectrometry (GC-MS) methods. The study also aimed to investigate the effects of the drying process on the chemical profile and contribute to the plant's pharmacological, commercial, and ecological potential.*

Material and Method: *Fresh and dried flower samples collected from the Muğla region were analyzed using the HS-SPME method. Fresh samples were immediately analyzed to prevent the loss of volatile compounds, while dried samples were air-dried at room temperature until they reached a constant weight and stored at 4°C. The mass spectra of volatile compounds were evaluated using GC/MS in conjunction with the Başer Library and other reference databases. Hierarchical Cluster Analysis (HCA) was applied for statistical analysis and clustering.*

Result and Discussion: *The main volatile compounds identified in fresh flowers were (E)-β-ocimene (62.8%), 1,8-cineole (12.9%), and linalool (7.6%). In dried flowers, (E)-β-ocimene (43.1%), (Z)-3-hexenal (18.7%), and (Z)-3-hexenyl acetate (9.8%) were prominent. Significant changes were observed in volatile compounds after drying, with monoterpene hydrocarbons decreasing and new aldehyde and ester compounds emerging. The influence of regional and environmental factors on the chemical profile was emphasized. The Muğla samples showed differences compared to samples from İzmir, Siirt, and Şırnak. HCA successfully grouped the compounds based on structural and functional similarities, statistically highlighting the chemical differences between fresh and dried flowers.*

Keywords: *Chemical profile, hierarchical cluster analysis, Narcissus tazetta, volatile compounds*

ÖZ

Amaç: *Bu çalışmada, Türkiye'de yetişen Narcissus tazetta subsp. tazetta (nergis) bitkisinin uçucu ve uçucu olmayan bileşenlerinin kimyasal profili incelenmiştir. Taze ve kurutulmuş çiçeklerin kimyasal profilleri, Tepe Boşluklu- Katı Faz Mikroekstraksiyon (TB-KFME) ve Gaz Kromatografisi-Kütle Spektrometresi (GK-KS) yöntemleriyle analiz edilmiştir. Çalışma, kurutma işleminin kimyasal*

* **Corresponding Author / Sorumlu Yazar:** Damla Kırcı
e-mail / e-posta: damla.kirci@ikc.edu.tr, **Phone / Tel.:** +902323293535

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profil üzerindeki etkilerini araştırmayı ve bitkinin farmakolojik, ticari ve ekolojik potansiyeline katkı sağlamayı amaçlamaktadır.

Gereç ve Yöntem: *Muğla bölgesinden toplanan taze ve kurutulmuş çiçek örnekleri, HS-SPME yöntemiyle analiz edilmiştir. Taze örnekler uçucu bileşik kaybını önlemek için hemen analiz edilirken, kurutulmuş örnekler gölgede, oda sıcaklığında sabit ağırlığa gelene kadar kurutulmuş ve 4°C'de saklanmıştır. Uçucu bileşiklerin kütle spektrumu, GK-KS ile Başer Library ve diğer referans kütüphaneler kullanılarak değerlendirilmiştir. Verilerin istatistiksel analizi ve kümelenmesi için Hiyerarşik Kümeleme Analizi (HCA) uygulanmıştır.*

Sonuç ve Tartışma: *Taze çiçeklerde ana uçucu bileşikler arasında (E)-β-osimen (%62.8), 1,8-sineol (%12.9) ve linalol (%7.6) bulunmuştur. Kurutulmuş çiçeklerde, (E)-β-osimen (%43.1), (Z)-3-hekzenal (%18.7) ve (Z)-3-hekzenil asetat (%9.8) ön plana çıkmıştır. Kurutma işlemiyle uçucu bileşiklerde önemli değişimler gözlemlenmiş, özellikle monoterpen hidrokarbonlar azalırken, yeni aldehit ve ester bileşikleri ortaya çıkmıştır. Bölgesel ve çevresel faktörlerin kimyasal profil üzerindeki etkisi vurgulanmıştır. Muğla örnekleri, İzmir, Siirt ve Şırnak'taki örneklerle karşılaştırıldığında farklılık göstermiştir. HCA, bileşiklerin yapısal ve işlevsel benzerliklerine göre gruplandırılmasını sağlayarak, taze ve kurutulmuş çiçekler arasındaki kimyasal farklılıkları istatistiksel olarak ortaya koymuştur.*

Anahtar Kelimeler: *Hiyerarşik kümeleme analizi, kimyasal profil, Narcissus tazetta, uçucu bileşikler*

INTRODUCTION

Narcissus tazetta L., commonly known as daffodil, belongs to the Amaryllidaceae family and is widely distributed across various regions, including Türkiye. It is represented in Türkiye by two subspecies, *N. tazetta* L. subsp. *tazetta* and *N. tazetta* L. subsp. *aureus* (Jord. & Fourr.) Baker [1]. This species holds great ethnobotanical significance due to its historical applications in traditional medicine, ornamental use, and the unique aroma of its flowers, which is valued in perfumery and aromatherapy [2,3].

N. tazetta has been extensively used in traditional medicine for its antispasmodic, emetic, analgesic, and antitumor properties [2]. In East Asian countries such as China and Japan, the bulbs and flowers of *N. tazetta* are traditionally applied to treat inflammation, wounds, and skin disorders [4]. In Türkiye, while its medicinal applications are less documented, the plant plays a cultural role as an ornamental species, and its aromatic extracts are used in folk remedies and fragrance formulations [5].

The therapeutic value of *N. tazetta* primarily stems from its rich alkaloid content, a characteristic feature of the Amaryllidaceae family. Among its key alkaloids, galanthamine is widely recognized for its acetylcholinesterase inhibitory activity and is clinically utilized in Alzheimer's disease and other cognitive disorders [3]. Lycorine exhibits significant antiviral, anti-inflammatory, and antitumor properties, with prominence for its ability to inhibit cancer cell proliferation [6]. Similarly, narciclasine demonstrates potent anti-inflammatory and immunomodulatory effects while being actively studied for its antitumor potential [4]. Although less studied, another notable alkaloid, tazettine shows emerging evidence for its neuroprotective and cytotoxic activities [3]. Together with phenolic and flavonoid compounds, these alkaloids form the chemical foundation underlying the pharmacological properties of *N. tazetta*.

The volatile profile of *N. tazetta* is dominated by monoterpenes such as *(E)-β*-ocimene, linalool, and 1,8-cineole, contributing to its distinct aroma and bioactivity These monoterpenes are known for their significant roles in plant defense mechanisms, particularly in deterring herbivores and attracting pollinators [3]. Non-volatile constituents include alkaloids, phenolic acids, and flavonoids, enhancing the plant's antioxidant capacity and therapeutic potential. For example, phenolic compounds and flavonoids contribute to the plant's antioxidant activity and exhibit anti-inflammatory and neuroprotective properties [5]. These findings emphasize the dual importance of volatile and non-volatile components in defining the pharmacological and ecological value of *N. tazetta*.

N. tazetta has notable commercial value as an ornamental plant and a source of essential oils. Its aromatic compounds are widely utilized in perfumery, while its alkaloid content positions it as a potential raw material for pharmaceutical applications [6]. Moreover, recent advancements in analytical

methods have paved the way for a deeper understanding of its chemical diversity, opening new opportunities for its utilization in various industries.

This study aims to comprehensively analyse of the volatile and non-volatile compounds of *N. tazetta subsp. tazetta*, grown in Türkiye, uses advanced analytical techniques such as Headspace Solid-Phase Microextraction (HS-SPME) and Gas Chromatography-Mass Spectrometry (GC-MS). By examining fresh and dried flowers, this study seeks to understand the impact of drying on the plant's chemical profile, contributing to its broader pharmacological, commercial, and ecological context.

MATERIAL AND METHOD

General Experimental Procedures

N. tazetta subsp. *tazetta* fresh flowers were collected from the Datça (Muğla) region of Türkiye during their natural flowering season (O.Tugay 17.983 & D.Ulukuş; KNYA Herbarium no: 29.972). Fresh flowers were analyzed immediately to preserve their volatile profiles. At the same time, dried samples were prepared by air-drying at room temperature in a shaded environment until a constant weight was reached. The samples were stored in airtight containers at 4°C prior to analysis to prevent loss or degradation of volatile compounds.

Analysis of Volatile Compounds Using HS-SPME

The fresh and dried flowers of the plant were directly analyzed using HS-SPME. A manual SPME device (Supelco, Taufkirchen, Germany) equipped with a fibre coated with a 65 µm-thick layer of polydimethylsiloxane/divinylbenzene (PDMS/DVB) was employed to extract the volatiles. For the analysis, 100 mg of fresh plant material was placed in a 10 ml vial, sealed with parafilm to allow saturation of the headspace with volatiles. The fibre was then inserted through the parafilm and exposed to the material's headspace for 15 min at room temperature [7].

Determination of Volatile Components Mass Spectra Using GC/MS

Gas Chromatography-Mass Spectrometry (GC/MS) was used to determine the mass spectra of volatile oils. The study utilized an Agilent 5975 GC/MSD system with an HP-Innowax polar column (60 m × 0.25 mm i.d., 0.25 µm film thickness) and helium as the carrier gas (flow rate: 0.8 ml/min). The injection port temperature was set to 250 °C. Analyses were performed using 70 eV electron energy within a mass range of 35-450 m/z.

A total temperature program of 80 min was applied: starting at 60°C for 10 min, increasing at a rate of 4°C/min to 220°C, holding at 220°C for 10 minutes, and then increasing at a rate of 1°C/min to 240°C. Data evaluation was conducted using the "Başer Library of Essential Oil Components" as well as Wiley and MassFinder 4 Library Search Software [8]. The results are presented in Table 1.

Application of Hierarchical Cluster Analysis (HCA)

Minitab 19 (State College, PA, USA) was used for statistical analysis. The number of clusters was determined by using a cutoff point (Euclidean distance) that enables the formation of consistent clusters and by examining the rescaled distances in the dendrogram. Hierarchical Cluster Analysis (HCA) was employed to assess the similarity between the chemical compositions of volatile compounds [9].

RESULT AND DISCUSSION

Volatile Components in *Narcissus tazetta* Flowers

In this study, the volatile compound profile of both fresh and dried flowers of *N. tazetta* subsp. *tazetta* was comprehensively analyzed. Using the Solid-Phase Microextraction (SPME) method, significant differences were observed in the chemical profiles of the flowers before and after the drying process. The aroma profile of fresh flowers was notably altered post-drying, with some volatile compounds decreasing in concentration or disappearing entirely. Monoterpene hydrocarbons and oxygenated monoterpenes were identified as the most dominant compound groups in both fresh and

dried samples, highlighting the natural aromatic richness of the plant, particularly in its monoterpenes and their oxygenated derivatives. Additionally, the findings indicated that these compounds, which were abundant in fresh flowers, decreased or nearly vanished after the drying process. These results provide crucial insights into the effects of drying on the volatile compound profile of the plant.

These analyses revealed that the major volatile compounds identified in the fresh flowers were *(Z)-β*-ocimene (62.8%), 1,8-cineole (12.9%), and linalool (7.6%), while in the dried flowers, *(Z)-β*-ocimene (43.1%), (Z)-3-hexenal (18.7%), and (Z)-3-hexenyl acetate (9.8%) were found (Table 2).

The analysis further revealed that *(E)-β*-ocimene, the most abundant compound in fresh flowers (62.8%), was followed by a group of oxygenated monoterpenes. The high *(E)-β*-ocimene levels and other monoterpenes in fresh samples suggest a distinctive and intense aromatic structure. In dried flowers, however, the concentration of these compounds notably declined while new compounds categorized as "other" emerged. The proportion of monoterpene hydrocarbons decreased to 43.1% in dried samples, and oxygenated monoterpenes were reduced to a low 0.3%. These changes indicate that the drying process reshapes the volatile profile of the plant, leading to a marked transformation compared with the fresh flower profile. The substantial reduction of oxygenated monoterpenes specifically underscores the chemical transformations occurring throughout the drying process.

Table 1. The volatile components of *N. tazetta* subsp. *tazetta*

RRI[a]	Compound	Fresh flowers (%)[b]	Dried flowers (%)[b]
1032	*α*-Pinene	1.1	-
1093	Hexanal	-	3.6
1136	Isoamyl acetate	4.0	-
1174	Myrcene	1.8	-
1213	1,8-Cineole	**12.9**	-
1225	*(Z)*-3-Hexenal	-	**18.7**
1246	*(Z)- β*-Ocimene	1.0	-
1266	*(E)- β*-Ocimene	**62.8**	**43.1**
1327	*(Z)*-3-Hexenyl acetate	0.6	**9.8**
1340	*(E)*-2-Hexenyl acetate	-	1.3
1360	1-Hexanol	-	0.3
1382	*cis*-Alloocimene	0.8	-
1391	*(Z)-3*-Hexen-1-ol	-	4.0
1409	*trans*-Alloocimene	0.2	-
1412	*(E)*-2-Hexen-1-ol	-	0.9
1496	2-Ethyl hexanol	-	0.1
1553	Linalool	**7.6**	0.3
1706	*α*-Terpineol	0.6	-
1747	Benzyl acetate	3.9	1.6
1838	2- Phenylethyl acetate	0.9	0.5
1896	Benzyl alcohol	-	0.9
1935	Phenyl ethyl alcohol	-	0.4
Monoterpene hydrocarbons		67.7	43.1
Oxygenated monoterpenes		21.1	0.3
Sesquiterpene hydrocarbons		-	-
Oxygenated sesquiterpenes		-	-
Fatty acid		-	-
Others		9.4	42.1
Total		**98.2**	**85.5**

[a]: Relative retention indices calculated against *n*-alkanes; [b]: calculated from FID data

The major volatile compounds identified in this study, particularly *(E)-β*-ocimene, 1,8-cineole, and linalool, align with findings from previous research on *N. tazetta*. Studies conducted by Chen et al. (2013) and Melliou et al. (2007) have similarly reported *(E)-β*-ocimene as the predominant compound,

with concentrations exceeding 60% [4,10]. The presence of linalool, observed in our fresh flower samples at 7.6%, is consistent with the findings of Seleem and Salem (2020), who reported similar concentrations using ethyl ether extraction [6]. These alignments validate the robustness of the HS-SPME method employed in our study and confirm its suitability for analyzing the volatile compounds of *N. tazetta* species.

Table 2. Studies on the volatile compounds of *Narcissus tazetta*

HCA no	Plant name	Country	Plant parts	Method	Major compounds	Ref.
1	*N. tazetta* subsp. *tazetta*	Muğla/ Türkiye	Fresh flowers	HS-SPME	*(E)*- *β*-Ocimene (62.8%), 1,8-cineole (12.9%), linalool (7.6%)	Our study
2	*N. tazetta* subsp. *tazetta*	Muğla/ Türkiye	Dried flowers	HS-SPME	*(E)*- *β*-Ocimene (43.1%), *(Z)*-3-hexenal (18.7%), *(Z)*-3-hexenyl acetate (9.8%)	Our study
3	*N. tazetta*	İzmir/ Türkiye	Flowers	HS-SPME	Benzyl acetate (30.4%), *β*-cimene (10.9%), 3-hexenyl acetate (8.5%)	[5]
4	*N. tazetta*	Siirt/ Türkiye	Flowers	HS-SPME	3-Hexenyl acetate (37.5%), benzyl acetate (30.1%), *β*-cimene (10.3%)	[5]
5	*N. tazetta*	Şırnak/ Türkiye	Flowers	HS-SPME	Benzyl acetate (34.1%), *β*-cimene (33.6%), 3-hexenyl acetate (12.9%)	[5]
6	*N. tazetta*	Egypt	Flowers	Ethyl ether extract	*α*-Pinene (15.38%), *α*-terpinene (15.27%), ethyl cinnamate (13.68%), and linalool (11.60%)	[6]
7	*N. tazetta*	Egypt	Flowers	Ethyl ether extract	Linalool (19.34%), methyl cinnamate (11.91%), ethyl cinnamate (10.61%), and limonene (8.31%)	[6]
8	*N. tazetta*	Egypt	Flowers	Ethyl ether extract	*α*-Pinene (22.24%), ethyl cinnamate (15.89%), *α*-terpineol (14.86%), and linalool (13.42%)	[6]
9	*N. tazetta* subsp. *tazetta*	Greece	Flowers	Water distillation	*(E)*-*β*-Ocimene (61.12%), 3-phenylpropyl acetate (6.4%), and benzyl acetate (6.04%)	[10]
10	*N. tazetta* var. *chinensis*	China	Single and double flowers	HS-SPME	*(E)*-*β*-Ocimene (62.73%, 66.06%) and benzyl acetate (11.65%, 25.02%), respectively	[4]

However, distinct differences were observed in the composition of dried flowers, where *(Z)*-3-hexenal (18.7%) and *(Z)*-3-hexenyl acetate (9.8%) were prominent. This shift in chemical profile is rarely addressed in existing literature, as most studies have predominantly focused on fresh flowers. The present work fills this gap, demonstrating the impact of drying on volatile compound transformation, likely influenced by enzymatic and oxidative processes.

Additionally, the total volatile content of fresh flowers (98.2%) and dried flowers (85.5%) reported in this study is notably higher compared to previous works [5]. This finding emphasizes our methodology's analytical precision while suggesting that regional and environmental factors unique to the Muğla region of Türkiye may contribute to these variations.

Another unique aspect of this study is the regional diversity of volatile profiles among *N. tazetta* samples from Türkiye. The identification of *(E)*-*β*-ocimene as the dominant compound aligns with findings from studies conducted in Egypt, Greece, and China, suggesting that this compound is a key chemotaxonomic marker for the species. However, the variations in secondary compounds such as benzyl acetate and 3-hexenyl acetate indicate potential influences of regional climatic and soil conditions, which merit further ecological investigation.

Furthermore, the comparison of results from the Muğla region with those from İzmir, Siirt, and Şırnak, as reported by Zarifikhosroshahi et al. (2021), highlights a significant variation in compound distribution [5]. These regional differences underscore the role of local environmental factors and cultivation conditions, which may profoundly affect volatile compounds' biosynthesis.

A significant strength of this study lies in applying of hierarchical clustering analysis (HCA), which enabled the classification of compounds into distinct groups based on their structural and functional similarities. This analytical approach, rarely implemented in previous studies on *N. tazetta*, enhances our understanding of the relationships between compounds and provides a more comprehensive overview of the volatile profile (Figure 1).

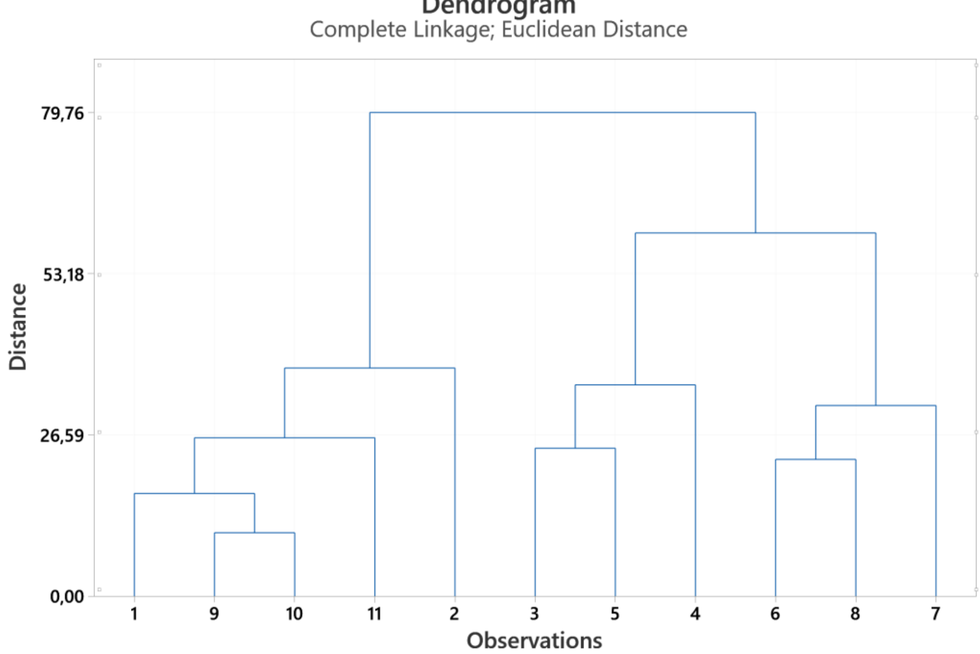

Figure 1. Hierarchical clustering dendrogram showing the similarity relationships among volatile compounds of *N. tazetta* subsp. *tazetta* fresh and dried flowers. The analysis highlights the clustering of compounds based on structural and functional similarities, with notable distinctions between fresh and dried flower profiles

The volatile compound profiles of *N. tazetta* subsp. *tazetta* fresh and dried flowers were analyzed using hierarchical clustering analysis (HCA). The results highlighted notable differences and similarities in the chemical composition of the samples. Compounds in clusters 9 and 10 exhibited the highest similarity (86.88%), indicating a close chemical relationship between these groups. In contrast, no statistical similarity was observed among clusters in the range of 1 to 3, showcasing clear compositional divergence. Furthermore, cluster 1 was found to be most like cluster 9 (78.79%), while cluster 2 demonstrated a lower similarity with cluster 1 (37.61%).

The chemical profiles revealed significant differences between fresh and dried flowers. Fresh flowers were dominated by *(E)-β*-ocimene (62.8%) and 1,8-cineole (12.9%), while dried flowers contained *(Z)*-3-hexenal (18.7%) and *(Z)*-3-hexenyl acetate (9.8%) as major components. The total volatile content was higher in fresh flowers (98.2%) than in dried flowers (85.5%), reflecting the impact of drying processes. These results suggest that enzymatic activity and oxidative processes during drying lead to substantial chemical transformations in the volatile profile.

Regional comparisons demonstrated significant variations in chemical profiles across different locations in Türkiye. The samples from the Muğla region showed a distinct composition when compared to previous studies conducted in İzmir, Siirt, and Şırnak. On an international scale, the dominance of *(E)-β*-ocimene aligns with findings from Greece, China, and Egypt, confirming its role as a

chemotaxonomic marker for *N. tazetta*. However, variations in secondary compounds, such as benzyl acetate and 3-hexenyl acetate, underscore the influence of environmental factors, including soil composition and climatic conditions in Muğla.

Drying significantly influenced the chemical profiles, with the emergence of aldehydes and esters such as *(Z)*-3-hexenal and *(Z)*-3-hexenyl acetate, which were absent in fresh flowers. These findings highlight the chemical transformations caused by drying, a process that has been underexplored in previous research. The study demonstrates how drying alters the biosynthesis and stability of specific volatile compounds, offering new insights into their potential applications.

A key strength of this study is the application of HCA. This advanced method allowed for the classification of compounds based on structural and functional similarities, providing a deeper understanding of their relationships. Compared to traditional approaches, HCA offered a statistically robust and visually effective framework for comparing the chemical profiles of fresh and dried flowers, thus enhancing the interpretative power of the findings.

(E)-β-Ocimene, as one of the main volatile compounds identified in *Narcissus tazetta* subsp. *tazetta*, plays a significant role in both ecological and pharmacological contexts. In the literature, this compound has been reported to exhibit diverse biological activities. Its antimicrobial properties are particularly noteworthy, with demonstrated efficacy against various bacterial and fungal pathogens, suggesting its potential as a natural antimicrobial agent. Furthermore, *(E)-β*-ocimene possesses antioxidant capacity, as it effectively neutralizes free radicals, thereby offering protection against oxidative stress-related conditions. Additionally, its anti-inflammatory effects are associated with the inhibition of pro-inflammatory cytokine production.

Moreover, *(E)-β*-ocimene has shown cytotoxic activity in several studies, with evidence of its effectiveness against specific cancer cell lines. These properties highlight the pharmacological relevance of this compound, reinforcing its potential applications in therapeutic and industrial contexts.

The findings of this study underscore the dual importance of *(E)-β*-ocimene, not only as a major contributor to the volatile profile of *N. tazetta* but also as a bioactive compound with significant pharmacological implications. Future studies focusing on the biological activities of this compound could provide deeper insights into its therapeutic potential and further expand the utility of *N. tazetta* in pharmacology and industry [11-14].

In conclusion, the findings of this study provide a robust framework for understanding the chemical diversity of *N. tazetta* and its potential applications. The high concentrations of *(E)-β*-ocimene and linalool suggest possible uses in perfumery and aromatherapy, while the identification of unique compounds in dried flowers could open new avenues for exploring their biological activities.

Future studies could focus on investigating the ecological and environmental factors that influence the volatile compound profiles in different regions. Understanding these variations could provide insights into the role of local conditions in shaping the chemical composition of *Narcissus tazetta*. Additionally, exploring the enzymatic pathways involved in the transformation of compounds during the drying process could shed light on the biochemical mechanisms driving these changes. Furthermore, conducting bioactivity assays to evaluate the pharmacological potential of the identified compounds would help uncover their therapeutic applications and pave the way for their utilization in pharmaceutical and industrial contexts.

In conclusion, this study not only confirms the chemical richness of *N. tazetta subsp. tazetta* but also introduces novel findings related to dried flowers and regional variations. By integrating advanced analytical methods such as HCA, it sets a benchmark for future research on the chemical ecology of this and related species.

AUTHOR CONTRIBUTIONS

Concept: D.K., B.D.; Design: D.K., B.D.; Control: B.D.; Sources: D.K., B.D, O.T., D.U.; Materials: O.T., D.U.; Data Collection and/or Processing: D.K, B.D.; Analysis and/or Interpretation: D.K. B.D.; Literature Review: D.K; Manuscript Writing: D.K., O.T., D.U., B.D.; Critical Review: D.K., O.T., D.U., B.D.; Other: D.K., O.T., D.U., B.D.

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.

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ÖZGÜN MAKALE / ORIGINAL ARTICLE

YENİ BİR SENOMORFİK İLAÇ ADAYI OLARAK HİDROKLOROTİYAZİD

HYDROCHLOROTHIAZIDE AS A NEW SENOMORPHIC DRUG CANDIDATE

Yaprak Dilber ŞİMAY DEMİR[1]*, Senem AKGÜL[2]

[1]Gazi Üniversitesi, Eczacılık Fakültesi, Farmakoloji Anabilim Dalı, 06330, Ankara, Türkiye
[2]Gazi Üniversitesi, Eczacılık Fakültesi, 06330, Ankara, Türkiye

ÖZ

Amaç: *Konvansiyonel kanser tedavisinde kullanılan kemoterapötiklerin hem kanser hem de kanser olmayan hücrelerde hücresel senesensi indüklediği bilinmektedir. Senesent hücrelerin önemli özellikleri, hücre morfolojilerinde belirgin değişikliklerin olması ve bu hücrelerden senesens ilişkili sekretuvar fenotip (SASP) olarak adlandırılan çeşitli sitokin, kemokin, büyüme faktörü ve matriks metalloproteinazların salgılanmasıdır. SASP faktörlerinin parakrin etki ile tümör mikroçevresinde kemoterapötik ilaç direncine, kanser hücre proliferasyonuna ve migrasyonuna neden olduğu gösterilmiştir. Bu nedenle, seçici olarak senesent hücre sekresyonunu baskılayan senomorfik etkili ilaçların geliştirilmesi, kanser tedavisinde yeni bir adjuvan tedavi olarak önem kazanmıştır. Rho kinaz enzim inhibisyonunun senesent hücrelerin sekretuvar aktivitesini inhibe ettiği gösterilmiştir. Yakın zamanlı bir çalışmada ise hipertansiyon tedavisinde kullanılan diüretik ilaçlardan biri olan hidroklorotiyazidin Rho kinaz enzim inhibisyonu yaptığı gösterilmiştir. Bu çalışmamızda Rho kinaz inhibisyonu yaptığı gösterilen hidroklorotiyazidin yeni bir senomorfik ilaç olma potansiyelinin değerlendirilmesi amaçlanmıştır.*

Gereç ve Yöntem: *Önceki çalışmalarımızda senesensi indüklediği belirlenen bir kemoterapötik olan doksorubisin ile HeLa hücrelerinde senesens indüklenmiştir. Daha sonra hidroklorotiyazid içeren ve içermeyen gruplarda senesent hücre sekretomu toplanarak bu sekretomlarda en belirgin SASP faktörlerinden biri olan IL-6'nın ölçülmesi ile senomorfik etkinlik değerlendirilmiştir.*

Sonuç ve Tartışma: *Bu çalışmamızda elde ettiğimiz veriler hidroklorotiyazidin, doksorubisin ile indüklenen senesent HeLa kanser hücrelerinin hem hücre boyutunu hem de sekrete edilen IL-6 düzeyini azalttığını göstermektedir. Bu verilerimiz hidroklorotiyazidin senomorfik etkiye sahip olduğuna işaret etmektedir. Kemoterapötiklerin yanında onların istenmeyen etkilerini engelleyebilecek adjuvan tedavi olarak senomorfik ilaçların kullanılması, daha efektif bir kanser tedavisi sağlanması açısından önemlidir.*

Anahtar Kelimeler: *Hidroklorotiyazid, ilaç yeniden konumlandırma, kanser, senesens, senomorfik ilaç*

ABSTRACT

Objective: *It is known that chemotherapeutics used in conventional cancer treatment induce cellular senescence in both cancer and non-cancerous cells. Important features of senescent cells include prominent changes in cell morphology and the secretion of various cytokines, chemokines, growth factors, and matrix metalloproteinases from these cells, called senescence-associated secretory phenotype (SASP). It has been shown that SASP factors cause chemotherapeutic drug*

* **Sorumlu Yazar / Corresponding Author:** Yaprak Dilber Şimay Demir
e-posta / e-mail: yapraksimay@gazi.edu.tr, **Tel. / Phone:** +903122023126

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resistance, cancer cell proliferation, and migration in the tumor microenvironment through a paracrine effect. Therefore, developing senomorphic drugs that selectively suppress senescent cell secretion has gained importance as a new adjuvant therapy in cancer treatment. Rho kinase enzyme inhibition has been shown to inhibit the secretory activity of senescent cells. A recent study showed that hydrochlorothiazide, one of the diuretic drugs used to treat hypertension, inhibits the Rho kinase enzyme. In this study, we aimed to evaluate the potential of hydrochlorothiazide, which has been shown to inhibit Rho kinase, as a new senomorphic drug.

Material and Method: *Senescence was induced in HeLa cells with doxorubicin, a chemotherapeutic that was determined to induce senescence in our previous studies. Then, senescent cell secretomes were collected in groups with and without hydrochlorothiazide, and senomorphic activity was evaluated by measuring IL-6, one of the most prominent SASP factors in these secretomes.*

Result and Discussion: *The data obtained in this study show that hydrochlorothiazide reduces both cell size and secreted IL-6 levels in doxorubicin-induced senescent HeLa cancer cells. These data indicate that hydrochlorothiazide has a senomorphic effect. The use of senomorphic drugs as adjuvant therapy to prevent their undesirable effects is important for providing more effective cancer treatment.*

Keywords: *Cancer, drug repositioning, hydrochlorothiazide, senescence, senomorphic drug*

GİRİŞ

Hücresel senesens, hücrelerin kalıcı olarak hücre siklusundan çıkması olarak tanımlanmaktadır [1]. Çoğalma yeteneklerini kaybeden bu hücreler (senesent hücre), metabolik ve transkripsiyonel olarak aktiftir. Hücresel senesens hücrelerin replikatif ömürlerinin sonunda ortaya çıktığı gibi çeşitli stres faktörleri ile de indüklenebilmektedir. Bu stres faktörlerine, radyasyon, oksidatif stres, onkogen aktivasyonu ve ilaç tedavisi örnek olarak verilebilir [2-5]. Senesens indükleyici etkene göre farklı senesens sınıflandırmaları ve adlandırmaları yer almaktadır. Örneğin; tedavi-ile indüklenen senesens çeşitli ilaç uygulamalarına bağlı olarak hücrede meydana gelen senesens yanıtı olarak tanımlanabilir. Senesent hücrelerin ayırıcı özellikleri arasında genişlemiş ve düzleşmiş hücre morfolojisi, artmış lizozomal senesens ilişkili beta galaktozidaz (SA-β-Gal) aktivitesi ve senesens-ilişkili sekretuvar fenotip olarak adlandırılan SASP'ın gelişimi yer almaktadır [6]. SASP, çok sayıda sitokin, kemokin, büyüme faktörü ve matriks metalloproteinazlar gibi güçlü parakrin etkilere yol açan biyoaktif bileşenler içermektedir. Yapılan çalışmalarla SASP'ın hücre tipine ve senesens indükleyici etkene bağlı olarak farklılıklar gösterdiği belirlenmiştir [7]. Farklı hücre tipi ve farklı etkenlerle indüklenen senesent hücreler için SASPAtlas adı verilen online platform oluşturulmuştur [7]. Böylece senesent hücrelerden sekrete edilen faktörlerle ilgili daha ayrıntılı bilgiler elde edilmiştir. Senesent hücreler sekrete ettikleri SASP aracılığıyla çevresindeki hücrelerde proliferatif/antiproliferatif/migratuvar etkiler oluşturmaktadırlar [6,8,9]. Yapılan çalışmalar yaşlanma-ile ilişkili birçok patolojinin (kardiyovasküler hastalık, demans, osteoporoz, kanser, Tip 2 diyabet, idiyopatik pulmoner fibroz ve glokom gibi) hücresel senesens ve SASP ile ilişkili olduğunu göstermiştir [10-15].

Klinikte kullanılmakta olan farklı kemoterapötik ajanların hücresel senesensi tetiklediği ve senesens sekretomunun (SASP) gelişimine neden oldukları gösterilmiştir [7]. Kemoterapötik ilaçlarla indüklenen senesent hücre sekretomunun, mikroçevrede yer alan hücrelerde tümör gelişimini indükledikleri ve/veya kemoterapötik ilaçlara karşı direnç gelişimine neden oldukları gösterilmiştir [16,17]. Bu nedenle SASP'ı baskılayan adjuvan tedavilerle daha etkin bir kanser tedavisi sağlanabileceği düşünülmektedir.

Senesensi temel alan tedaviye senoterapi denmektedir. Senoterapi iki temel bakış açısıyla alt dallara ayrılmaktadır. Bunlardan ilki senolitiklerdir. Senolitikler, seçici olarak senesent hücrenin ortadan kaldırılmasını amaçlamaktadır. Diğer yaklaşım olan senomorfikler ise senesent hücrelerden sekrete edilen SASP'ı hedefleyen tedavi yaklaşımıdır. Yakın zamanda yapılan çalışmalar yeni senolitik ve senomorfik ilaç adaylarını bulmayı amaçlamaktadır [18]. Özellikle kanser tedavisinde kemoterapötiklerle birlikte kullanılabilecek SASP'ın zararlı etkilerini önleyebilecek yeni adjuvan senomorfiklerin keşfedilmesi klinik öneme sahiptir. Metformin, glukokortikoidler, apigenin, simvastatin, rapamisin gibi birçok senomorfik ilaç adayı belirlenmiştir [19-24]. Daha önce yapmış

olduğumuz bir çalışmada Rho kinaz enzim inhibitörlerinin de senomorfik etkinliğe sahip olduğunu gösterdik [25]. Diğer taraftan Mondaca-Ruff ve arkadaşları ise tiyazid grubu bir diüretik olan hidroklorotiyazidin Rho kinaz enzimini inhibe ettiğini göstermişlerdir [26]. Bu iki çalışma birlikte değerlendirildiğinde hidroklorotiyazidin senomorfik etkinliğinin olabileceğini düşündürmektedir.

İlacın yeniden konumlandırılması ruhsatlı ilaçlar için yeni bir endikasyon belirleyerek daha az maliyet ve süre ile ilacın piyasaya sürülmesini içeren bir süreçtir. Bu nedenle günümüzde hipertansiyon tedavisinde sıklıkla kullanılan ve terapötik penceresi dar olmayan hidroklorotiyazidin yeni bir senomorfik ilaç adayı olabilmesi yeniden konumlandırma ile kanser tedavisinde adjuvan olarak kullanılabileceğine işaret edecektir. Literatürde hidroklorotiyazidin senomorfik etkinliğini değerlendiren bir çalışma yer almamaktadır.

GEREÇ VE YÖNTEM

Hücre Kültürü ve Senesens Indüksiyonu

Çalışmamızda serviks kanser hücre dizisi olan HeLa hücreleri kullanılmıştır. Daha önceki çalışmamızda HeLa hücrelerinde doksorubisin ile senesens indüksiyonu ve SASP gelişimini gerçekleştirdiğimiz deneysel şartları optimize ettiğimiz için mevcut çalışmamızda da bu hücre hattı ile çalışılmıştır [25]. Hücreler %10 fötal sığır serumu ve 100 U/ml penisilin-streptomisin içeren DMEM'li besiyerinde kültüre edilmiştir. Deneysel çalışmalarımızda hücreler, hücre kültür kabının zeminini %70-80 dolduruncaya kadar 37°C'de %5 CO_2 içeren inkübatörde (*Sanyo*) çoğaltılmıştır.

Kemoterapi ile indüklenen senesens modelini oluşturmak için, daha önce grubumuz tarafından çalışılmış olan doksorubisin-ile senesens indüksiyonu gerçekleştirilmiştir [25]. Bu çalışma modelinde %70-80 konfluensiye ulaşmış HeLa hücrelerine 300 nM doksorubisin uygulanmış ve hücreler 72 saat boyunca inkübe edilmiştir. 72 saatlik inkübasyon sonrasında hücrelerde senesens gelişimi, oluşan morfoloji ve SA-β-gal boyaması ile doğrulanmıştır.

SA-β-Gal Boyaması

Hücresel senesens gelişiminin belirlenmesi için, senesent hücrenin en yaygın kullanılan belirteci olan SA-β-Gal boyaması yapılmıştır. Bu amaçla Senesens β-Galaztozidaz Boyama Kiti (*Cellular signaling*, 9860) kullanılmıştır. Doksorubisin ile 72 saatlik inkübasyon sonrasında hücrelerin besiyeri uzaklaştırılmış ve hücreler 1 kez fosfat tamponu ile yıkanmıştır. Daha sonra kit içerisinde yer alan fiksasyon çözeltisi eklenmiş ve 10 dk oda sıcaklığında beklenmiştir. Hücrelerin üzerindeki fiksasyon çözeltisi uzaklaştırılmış ve 1 kez fosfat tamponuyla yıkanmıştır. pH'sı 6.0'a ayarlanmış beta-galaktozidaz boyama çözeltisi ilave edilmiş ve hücreler CO_2'siz kuru inkübatörde (*JSL, JSSI-100C*) yaklaşık 16 saat boyunca inkübe edilmiştir. Daha sonra hücrelerin görüntüleri ışık mikroskopu (*Leica, DFC 420C*) yardımıyla elde edilmiştir ve her bir görüntüdeki SA-β-Gal boyaması pozitif olan ve olmayan hücreler sayılmıştır. % SA-β-Gal pozitif hücre sayısı hesaplanarak gruplar arasındaki farklılıklar değerlendirilmiştir.

Senesent Hücrelerin Faz Kontrast ve Holografik Mikroskopi ile Değerlendirilmesi

İlaç inkübasyonu sonrası hücrelerde meydana gelen morfolojik değişiklikler iki boyutlu olarak faz kontrast mikroskopu *(Leica, DMI8)* ve üç boyutlu olarak da holografik mikroskop *(HoloMonitor M4)* ile incelenmiştir. Holografik mikroskop görüntüleri üzerinden *HStudio M4 2.7* yazılımı kullanılarak hücre alanı, hücre hacmi, ortalama ve maksimum hücre kalınlığı gibi morfolojik parametreler hesaplanmıştır [25]. Her iki mikroskopta da elde edilen görüntüler ve analizler 4 ayrı biyolojik tekrar ile elde edilmiştir. Yapılan ölçümler üzerinden gruplar arasındaki farklılıklar istatistiksel olarak değerlendirilmiştir.

Hücre Canlılığının Belirlenmesi ve Hidroklorotiyazid Konsantrasyonu Seçimi

Hidroklorotiyazidin olası senomorfik etkinliğinin değerlendirilmesi amacıyla öncelikle hücre ölümüne yol açmayan en yüksek HCTZ konsantrasyonu belirlenmiştir. Bu amaçla hücre proliferasyonunun ve canlılığının biyokimyasal olarak belirlenmesinde, esası mitokondriyal dehidrojenaz enzim aktivitesi ölçümüne dayanan WST-1 Hücre Proliferasyon Testi Reaktifi (*Cell*

biolabs, CBA-253) kullanılmıştır [26]. 96 çukurlu plakaların her bir çukuruna 10.000 HeLa hücresi ekilmiş ve 24 saat boyunca inkübe edilmiştir. Daha sonra HCTZ logaritmik olarak artan konsantrasyonlarda (3, 10, 30, 100 ve 300 µM) hücrelere uygulanmış ve 48 saat inkübe edilmiştir. Senomorfik etki değerlendirmesinde optimize ettiğimiz deneysel şartlarda HCTZ'in 48 saatlik etkisi değerlendirileceği için hücre canlılığı deneyleri de 48 saatlik süre ile gerçekleştirilmiştir [27]. İnkübasyondan sonra, her bir kuyucuğa 10 µg WST-1 reaktifi ilave edilmiş ve 4 saat boyunca inkübasyona bırakılmıştır. Son olarak plakaların 450 nm'deki absorbans değerleri okunarak her bir konsantrasyon için % hücre canlılığı parametresi hesaplanmış ve bu parametre üzerinden gruplar arasındaki farklılıklar istatistiksel olarak değerlendirilmiştir.

Senesent Hücre Sekretomunun Toplanması ve IL-6 Ölçümü

Doksorubisin ile senesens indüksiyonu sonrasında senesent hücre sekretomunun elde edilmesi amacıyla 72 saatlik doksorubisin uygulaması sonrasında hücrelerin besiyeri uzaklaştırılmış ve hücreler 2 kez fosfat tamponu ile yıkanmıştır. Daha sonra hücrelere serumsuz ve fenol kırmızısı içermeyen besiyeri ilave edilmiştir. 48 saatlik inkübasyon sonrasında hücrelerin üzerindeki besiyeri mikrosantrifüj tüplerine alınmıştır. Besiyeri içeren bu tüpler 10 dk 800 x g'de +4ºC'de santrifüj edilmiştir. Daha sonra süpernatant 0.22 µm'lik steril filtrelerden geçirilmiş ve böylece senesent hücre sekretomu (SASP) elde edilmiştir [25,28].

Ayrıca sekretomu toplanan hücreler tripsinize edilerek hücre kültür kaplarından toplanmış ve hücre sayım cihazı *(İnvitrogen*, *TALI*) kullanılarak her bir gruptaki hücre sayıları belirlenmiştir. Böylece sekretomların toplamda kaç hücreden sekrete edildiği tespit edilmiştir. Toplanan sekretomlar hücre sayısına göre normalize edilmiştir. SASP'ın oluşup oluşmadığı sekretomda IL-6 ölçümü ile değerlendirilmiştir [28].

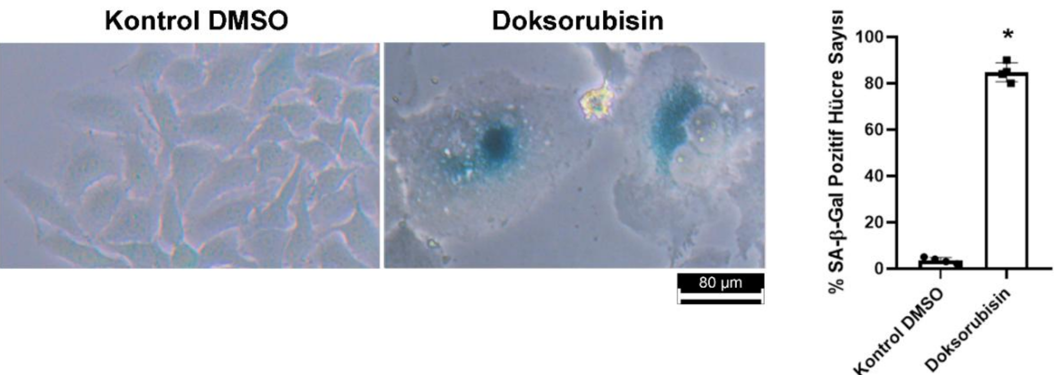
Sekretomda IL-6 ölçümü için insan IL-6 elisa kiti (*Thermo Scientific,* KHC0061) kullanılmış ve ölçüm kitin kullanma kılavuzuna uygun olarak gerçekleştirilmiştir. Kit içerisinde yer alan 96 çukurlu plakanın her bir çukuruna 50 µl biyotinli antikor solüsyonu ve 100 µl hücre sayısına göre normalize edilmiş sekretom eklenmiştir. Oda sıcaklığında 2 saat inkübasyona bırakılan plaka 3 kez yıkama çözeltisiyle yıkanmış ve her bir çukura 100 µl streptavidin-HRP çözeltisi eklenmiştir. 30 dk oda sıcaklığında inkübasyon sonrasında plaka 3 kez yıkama çözeltisiyle yıkanmış ve her bir çukura 100 µl substrat çözeltisi eklenmiştir. 30 dk karanlık ortamda inkübasyon sonrasında çukurlara kit içerisindeki reaksiyonu durdurucu çözeltiden 100 µl ilave edilmiştir. Daha sonra plaka okuyucu (*SpectraMax i3x*) kullanılarak 450 nm'deki absorbans değerleri ölçülmüştür.

İstatistiksel Analiz

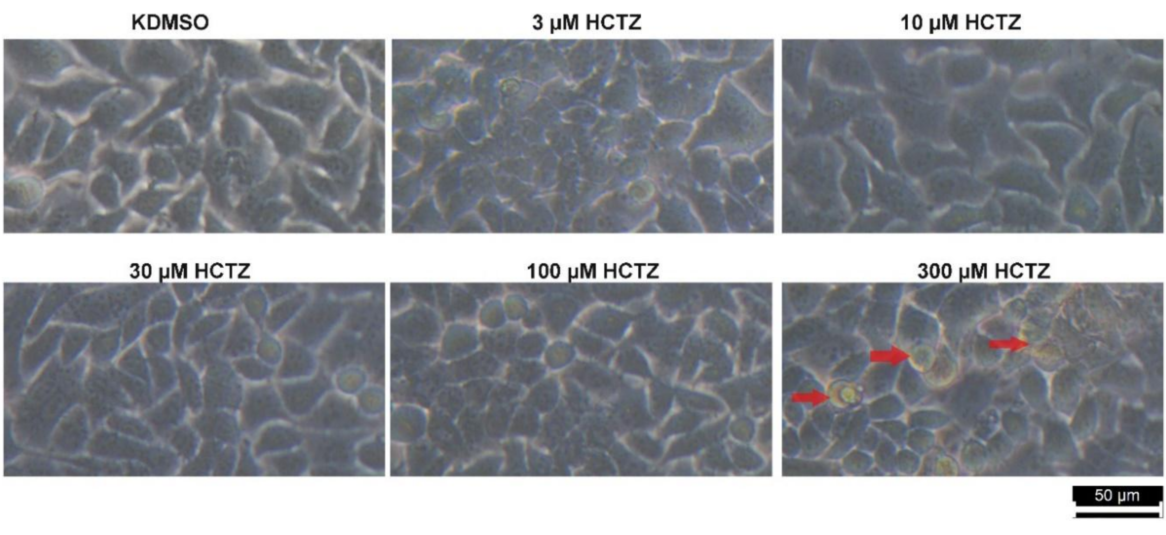
Deney sonucunda elde edilen verilerde, gruplar arasındaki farkın değerlendirilmesi için tek yönlü varyans analizi ve *post hoc Tukey* kullanılmıştır. Analizler *GraphPad Prism Version 8.0.1* programı ile gerçekleştirilmiştir. $P<0.05$ istatistiksel olarak anlamlı kabul edilmiştir.

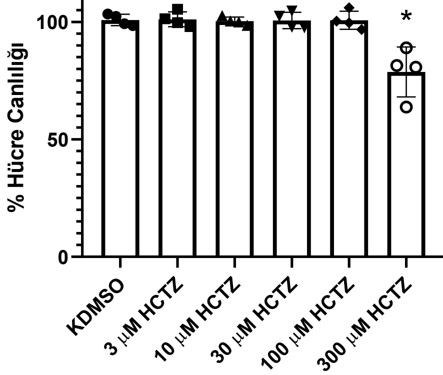
SONUÇ VE TARTIŞMA

Hücresel senesensin indüklenmesi amacıyla, HeLa hücre hattında 72 saatlik 300 nM doksorubisin uygulaması gerçekleştirilmiştir [25]. Çalışmamızda doksorubisin uygulanan grupta SA-beta gal pozitif boyaması kontrol grubuyla karşılaştırıldığında istatistiksel olarak anlamlı düzeyde artmıştır (Şekil 1). Bu sonucumuz daha önceki çalışmalarımızla tutarlı olarak belirlenen konsantrasyon ve sürede doksorubisin ile senesens indüksiyonunun gerçekleştiğini göstermektedir [25].



Şekil 1. Doksorubisin ile indüklenen senesensin SA-beta gal boyaması ile doğrulanması. Skala Bar: 80 µm. *: Kontrol grubundan istatistiksel olarak anlamlı farklılığı ifade etmektedir, n=4

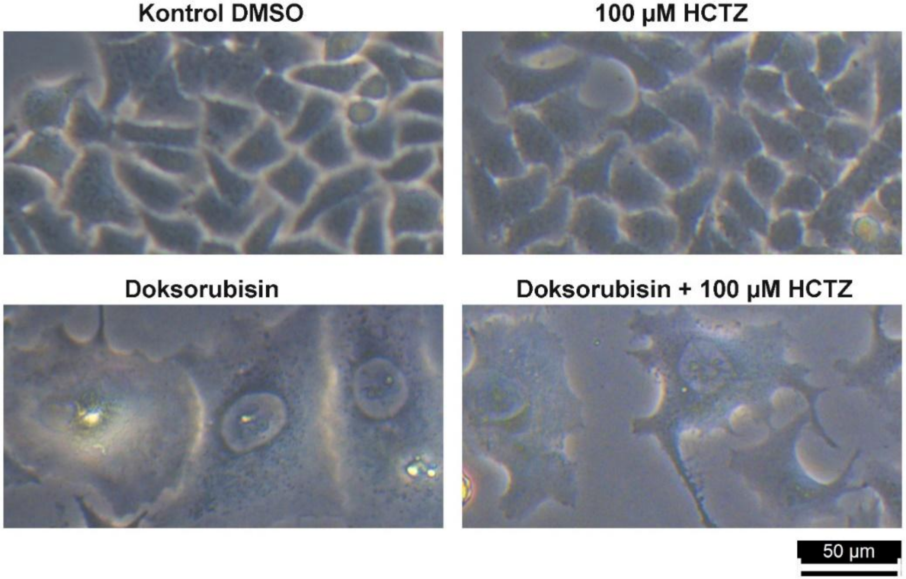




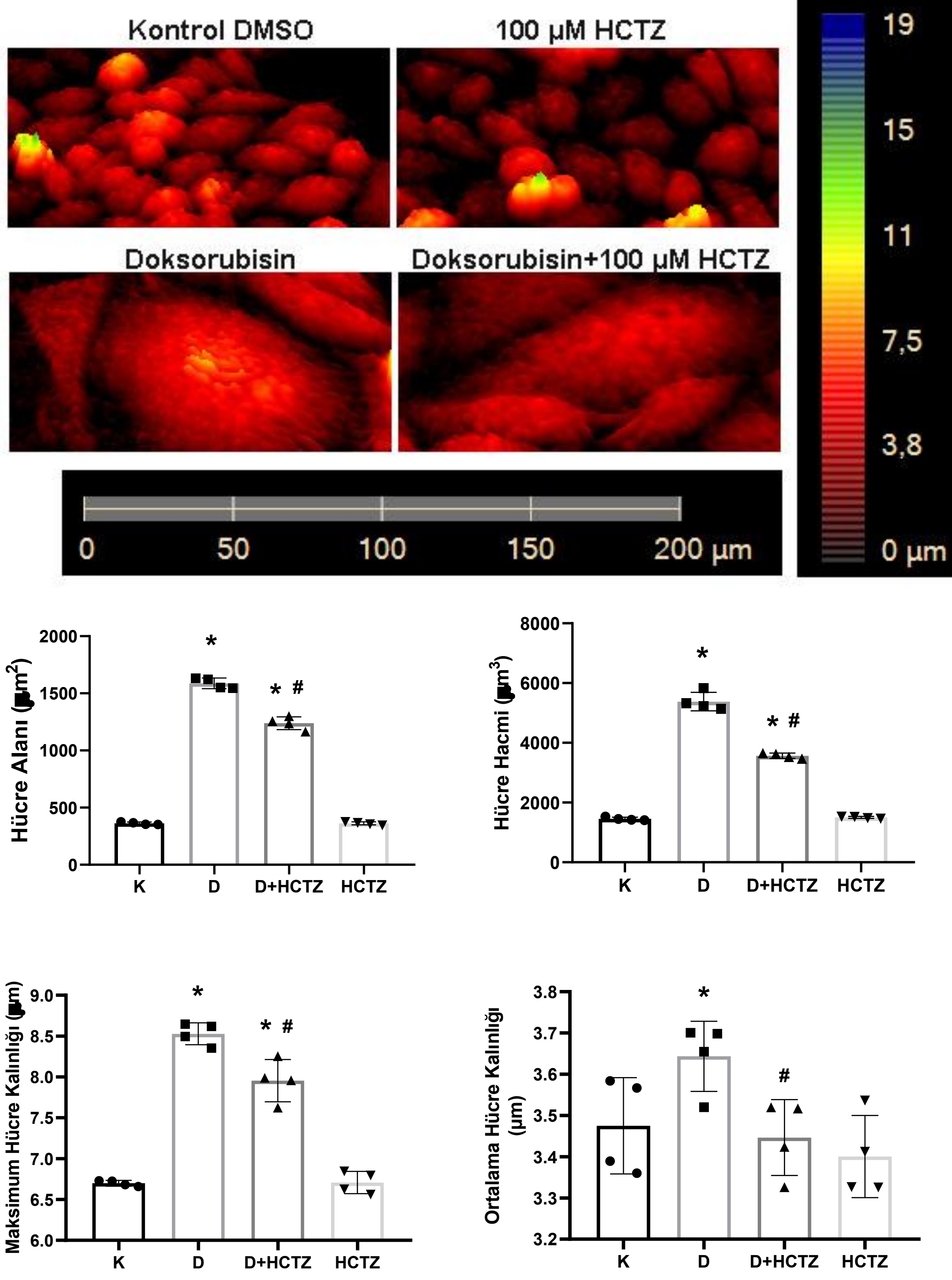
Şekil 2. HeLa hücre dizisine artan konsantrasyonlarda HCTZ uygulaması sonucu elde edilen faz kontrast görüntüleri ve WST-1 proliferasyon sonuçlarının istatistiksel analizi, KDMSO: Kontrol DMSO, HCTZ: hidroklorotiyazid. Kırmızı ok: ölü hücreleri (hücre kültür zemininden ayrılan ve küreselleşmiş hücreler) göstermektedir. *: Kontrol DMSO grubundan istatistiksel olarak anlamlı farklılığı ifade etmektedir, n=4. Skala Bar: 50 µm

Hidroklorotiyazidin senomorfik etkisinin değerlendirilmesi amacıyla öncelikle konsantrasyon seçimi yapılmıştır. Bunun için HeLa hücrelerine logaritmik olarak artan konsantrasyonlarda hidroklorotiyazid (HCTZ) uygulanmış ve hücreler 48 saat inkübe edilmiştir. Deney bitiminde hücre ölümü, mikroskobik olarak faz kontrast görüntüleme ile, biyokimyasal olarak ise WST-1 testi ile değerlendirilmiştir (Şekil 2). Çalışmamızda kullandığımız HeLa hücreleri tutunarak çoğalan aderent hücrelerdir. Bu nedenle mikroskobik olarak hücre kültür kabının zemininden ayrılan ve küreselleşen hücreler ölü hücre olarak değerlendirilmiştir (Şekil 2). Diğer taraftan WST-1 testi ile elde edilen veriler, mikroskobik gözlemlerimiz ile tutarlı olarak, en yüksek konsantrasyon olarak seçilen 300 µM HCTZ'nin hücrelerde hücre canlılığını istatistiksel olarak anlamlı düzeyde azalttığını göstermektedir (Şekil 2). Bu nedenle sekretom çalışmalarında kullanılmak üzere hücre ölümünü tetiklemeyen en yüksek konsantrasyon olan 100 µM HCTZ seçilmiştir.

HCTZ'in sekretom üzerindeki etkisinin incelenmesi amacıyla, senesent ve senesent olmayan hücrelere 100 µM HCTZ uygulanmıştır. 48 saatlik HCTZ inkübasyonu sonrasında hücrelerin hem faz kontrast hem de holografik görüntüleri elde edilmiştir (Şekil 3 ve Şekil 4). Holografik görüntülerden elde edilen görüntülerden hesaplanan morfolojik parametrelere göre, doksorubisin ile indüklenen senesent hücrelerde artan hücre alanı, hücre hacmi, maksimum hücre kalınlığı ve ortalama hücre kalınlığı hidroklorotiyazid uygulaması ile azalmıştır. Hidroklorotiyazidin senesent hücre morfolojisinde oluşturduğu bu değişiklikler daha önceki Rho/ROCK yolağı inhibisyonu ile elde ettiğimiz verilerle benzerlik göstermektedir [25].



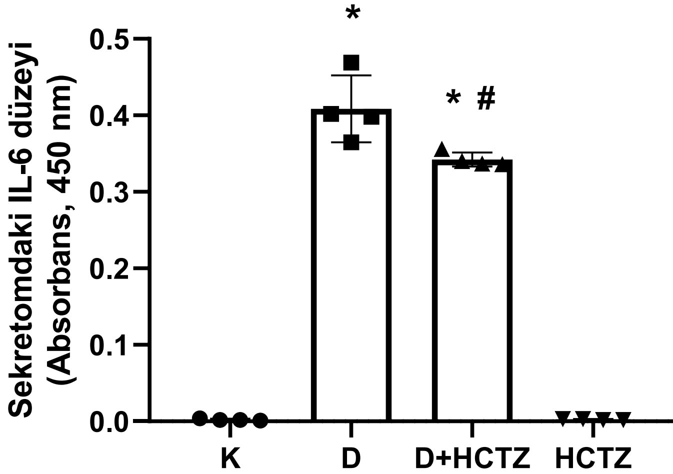
Şekil 3. Senesent ve senesent olmayan HeLa hücrelerinin morfolojisinde HCTZ'in etkisinin faz kontrast mikroskopisi ile incelenmesi. KDMSO: Kontrol DMSO, HCTZ: hidroklorotiyazid. Skala Bar: 50 µm



Şekil 4. Senesent ve senesent olmayan HeLa hücrelerinin morfolojisinde HCTZ'in etkisinin holografik mikroskopi ile incelenmesi. K: kontrol, D: 300 nM Doksorubisin, D+HCTZ: 300 nM Doksorubisin + 100 µM hidroklorotiyazid, HCTZ: 100 µM hidroklorotiyazid. *: Kontrol grubundan, #: doksorubisin grubundan istatistiksel olarak anlamlı farklılığı ifade etmektedir, n=4

Hidroklorotiyazidin doksorubisin ile indüklenen senesent hücre sekretomu üzerindeki etkinliğini değerlendirmek için hücrelerin üzerindeki sekretom toplanmış ve bu sekretomda en yaygın kullanılan

SASP belirteci olan IL-6 ölçümü gerçekleştirilmiştir. Elde ettiğimiz veriler doksorubisin ile indüklenen senesent hücrelerin sekretomundaki IL-6 düzeylerinin anlamlı olarak arttığını ve hidroklorotiyazid uygulamasının ise bu artışı istatistiksel olarak anlamlı bir düzeyde azalttığını göstermektedir (Şekil 5).



Şekil 5. Senesent ve senesent olmayan HeLa hücrelerinin sekretomunda HCTZ'in etkisinin IL-6 ölçümü ile değerlendirilmesi. K: kontrol, D: 300 nM Doksorubisin, D+HCTZ: 300 nM Doksorubisin + 100 µM hidroklorotiyazid, HCTZ: 100 µM hidroklorotiyazid. *: Kontrol grubundan, #: doksorubisin grubundan istatistiksel olarak anlamlı farklılığı ifade etmektedir, n=4

Sonuç olarak elde ettiğimiz veriler HCTZ'nin HeLa kanser hücre hattında doksorubisin ile indüklenen senesent hücre morfolojisini ve sekretomunu istatististiksel olarak anlamlı düzeyde değiştirdiğini göstermiştir. Senesent sekretomunun en yaygın olarak kullanılan belirteci olan IL-6 sekresyonunun HCTZ ile azalması, HCTZ'in senomorfik etki potansiyelini göstermektedir [29,30]. HCTZ'in bu etkilerinin daha önceki çalışmalarımızda Rho/ROCK yolağının inhibisyonunun senesent hücre morfolojisi ve sekretomunda ortaya çıkardığı etkilere benzer olduğu görülmektedir [25,31]. Mondaca-Ruff ve arkadaşları gerçekleştirdikleri çalışmada hidroklorotiyazidin Rho kinaz enzim aktivayonunu inhibe ettiğini göstermiştir [27]. Bu çalışma ve mevcut verilerimiz birlikte değerlendirildiğinde HCTZ'nin senomorfik etkinliğinin altında yatan mekanizmanın ROCK inhibisyonu olabileceğine işaret etmektedir. Bununla birlikte; Aloud ve arkadaşları HCTZ ile tedavi edilen spontan hipertansif ratların daha düşük konsantrasyonlarda TNFα ve IFNγ ürettiklerini belirlemişlerdir [32]. Diğer taraftan Mitini-Nkhoma ve arkadaşları HCTZ, diazoksit, digoksin gibi iyon taşıyıcı modülatörlerinin immünbaskılayıcı etkinliklerinin olduğunu göstermiştir [33]. Bu çalışmalar HCTZ'in senomorfik etkisinin, ROCK inhibisyonundan bağımsız olarak doğrudan sitokin üretimini azaltması ile de ilişkili olabileceğine işaret etmektedir. Daha ileri çalışmalarla hidroklorotiyazidin senomorfik özelliğinin altında yatan mekanizmaların aydınlatılması ve bu özelliğin kanser mikroçevresindeki öneminin belirlenmesi senoterapötik olma potansiyelini gösterecektir.

Senomorfik etkiye sahip olan ilaç adaylarının klinikte kullanımları, SASP'ın sürekli olarak baskılanmasını sağlamak için kronik kullanımı gerektirmektedir. Bu nedenle senomorfik ilaç adaylarının iyi güvenlik profillerine sahip olmaları gerekmektedir [34]. Bu durum, yıllardır kullanılan ve güvenliliği bilinen hidroklorotiyazidin yeniden konumlandırma ile senomorfik kullanım potansiyelini güçlendirmektedir. Diğer taraftan SASP bileşenlerinin heterojenitesi dikkate alındığında, senomorfik etkili bir ilaç adayının farklı tipteki senesent hücrelerde senomorfik etkinlik göstermeme ihtimalini de ortaya çıkarmaktadır. Bu nedenle daha ileri çalışmalarla hidroklorotiyazidin senomorfik kullanımının ayrıntılı bir şekilde değerlendirilmesi gerekmektedir.

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

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

ANALYSIS OF HOUSEKEEPING GENE STABILIZATION IN ISOLATED MITOCHONDRIA

İZOLE MİTOKONDRİLERDE HOUSEKEEPING GEN STABİLİZASYONUNUN ANALİZİ

Oner ULGER[1,2]*, Tugba FATSA[3], Sema OREN[3], Pinar M. ELCI[4]

[1]University of Health Sciences, Gulhane Institute of Health Science, Department of Mitochondria and Cellular Research, 06010, Ankara, Türkiye
[2]Gulhane Training and Research Hospital, University of Health Sciences, 06010, Ankara, Türkiye
[3]University of Health Sciences, Gulhane Institute of Health Science, Molecular Research Laboratory, 06010, Ankara, Türkiye
[4]University of Health Sciences, Gulhane Institute of Health Science, Stem Cell Laboratory, 06010, Ankara, Türkiye

ABSTRACT

Objective: *The mitochondria isolation method is used to investigate mitochondrial processes. RT-qPCR measurement of gene transcription levels is frequently used in these studies. In this method, it is important that the housekeeping genes used in the normalisation of the results are stable. The study aimed to determine which of two housekeeping genes is more stable in isolated mitochondria.*
Material and Method: *Mitochondria were isolated from FHC cells. Membrane integrity and functionality were measured by mitotracker and JC-1 stainings and ROS ratio by flow cytometry. Housekeeping genes GAPDH and β-actin were used in RT-qPCR, and gene stability was calculated using ΔCq method and percentage coefficient of variance. For gene validation, CAT and SOD1 transcriptions were calculated by the $2^{-\Delta\Delta}$Cq method. These genes were selected because oxidative stress status has been detected.*
Result and Discussion: *Isolated mitochondria membrane integrity was preserved and the membrane potential ratio was found 91%. ROS ratio was 1.6% and there was no oxidative stress that would affect the antioxidant enzymes levels. ΔCq values were 4.54±0.06 for β-actin and 0.69±0.16 for GAPDH, with 1.33% and 23.95% percentage coefficient of variance respectively. CAT transcription levels were 25.7±2.6 and 1.8±0.33, while SOD1 levels were 70.1±12.7 and 4.8±0.6 correspondingly. It was concluded that β-actin may be more stable than GAPDH. The potential impact of housekeeping gene selection on outcomes should be considered.*
Keywords: *Housekeeping gene stabilization, isolated mitochondria, RT-qPCR*

ÖZ

Amaç: *Mitokondri izolasyonu metodu mitokondriyal süreçlerin araştırılmasında kullanılır. Bu araştırmalarda gen transkripsiyon seviyelerinin RT-qPCR ölçümü sıklıkla kullanılmaktadır. Bu yöntemde sonuçların normalizasyonunda kullanılan housekeeping genlerin stabil olması önemlidir. Bu çalışma, izole mitokondride iki housekeeping genden hangisinin daha stabil olduğunu belirlemeyi amaçlamıştır.*
Gereç ve Yöntem: *Mitokondriler FHC hücrelerinden izole edilmiştir. İzole mitokondrilerin membran bütünlüğü ve fonksiyonelliği mitotracker ve JC-1 boyamaları ile ROS oranı flow*

* **Corresponding Author / Sorumlu Yazar:** Oner Ulger
e-mail / e-posta: oner.ulger@sbu.edu.tr, **Phone / Tel.:** +903123043717

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sitometriyle ölçülmüştür. RT-qPCR analizinde housekeeping gen olarak GAPDH ve β-aktin kullanılmış, gen stabilitesi ise ΔCq metodu ve yüzde varyans katsayısına göre hesaplanmıştır. Gen validasyonu için CAT ve SOD1 transkripsiyonları $2^{-\Delta\Delta}$Cq yöntemiyle hesaplanmıştır. Bu genler oksidatif stres durumunun tespit edilmiş olması nedeniyle seçilmiştir.

Sonuç ve Tartışma: *İzole mitokondrilerin membran bütünlüğünü koruduğu görülmüş, membran potansiyeli oranı ise %91 olarak bulunmuştur. ROS oranı %1.6 olarak ölçülmüştür ve buna göre antioksidan enzim düzeyini etkileyecek bir oksidatif stres olmadığı değerlendirilmiştir. ΔCq değerleri β-aktin için 4.54±0.06 ve GAPDH için 0.69±0.16 iken, yüzde varyans katsayısı sırasıyla %1,33 ve %23.95 idi. CAT transkripsiyon seviyesi, β-actin'e göre 25.7±2.6 ve GAPDH'a göre 1.8±0.33 iken, SOD1 seviyeleri β-aktin'e göre 70.1±12.7 ve GAPDH'a göre 4.8±0.6 olarak hesaplanmıştır. Sonuçlara göre β-aktin'in GAPDH'a göre daha stabil olabileceği kanısına varılmıştır. Analizlerde housekeeping gen seçiminin sonuçlar üzerindeki potansiyel etkisi dikkate alınmalıdır.*

Anahtar Kelimeler: *Housekeeping gen stabilitesi, izole mitokondri, RT-qPCR*

INTRODUCTION

Mitochondrial isolation is a widely used method in scientific research [1]. It is an important method for investigating the role of mitochondria in physiological and pathophysiological conditions. In addition, mitochondrial transplantation (MT), which uses isolated mitochondria as a therapeutic agent, is a method that has attracted much attention in recent years and has been the subject of many studies in this context.

Although mitochondria are known as the energy source of the cell, their properties are not limited to this. It is an important player in several complex processes such as maintaining the ionic balance of the cell, synaptic transmission, immune system functions, hem metabolism, intercellular communication, and cell death [2]. Mitochondria are known to be transferred between cells in the body during physiological processes. Several features, such as a double-layered membrane allow it to be transferred [3]. It is also possible to transfer these mitochondria from outside the body. This method, called MT, has been shown to help treat important diseases such as toxic organ damage, ischemic heart damage, and neurodegenerative diseases [4].

Both the better understanding of mitochondrial processes and the increasing research on the MT method have led to a greater interest in isolated mitochondria. Mitochondria are organelles that have their own genetic material (mtDNA) and in this sense are partially independent of the nucleus [5]. Even if not encoded by mtDNA, proteins associated with mitochondrial functions can be found in mitochondria, and their mRNAs have been reported to localize together with mitochondria [6,7].

Gene expression analysis is an important method used in many areas of biological research. Polymerase chain reaction (PCR) is one of the commonly used methods in these analyses, allowing gene sequences to be duplicated and quantified. It helps to make decisions about biological processes by understanding the genes that are expressed and how they interact [8].

In PCR analysis, the transcript level of the gene under investigation is calculated using the internal housekeeping gene, which is always expressed at a consistent level. Housekeeping genes are defined as the basic group of genes necessary for the organism to survive. Another important feature is that these are genes that are consistently expressed in all cells. The stability of this gene expression is usually tested in a small number of cells and conditions. However, these tests are not yet sufficient for a large number of possibilities used in many different types of research [9]. Therefore, it is necessary to show which housekeeping gene is best suited for normalization in PCR analyses under different conditions. The fact that this gene is not affected by the interventions to be applied in the research will also allow the results to be calculated in the most accurate way.

On this basis, RT-qPCR was performed on total RNA from isolated mitochondria to determine which of two different housekeeping genes is more stable. In this study, GAPDH and β-actin were selected as housekeeping genes. These genes are among the most widely used reference genes. CAT and SOD1 enzymes were selected as target genes. These target genes were used to evaluate the effect of the calculations on the results by normalizing the transcription levels according to different housekeeping genes. The calculation of housekeeping gene stabilisation is contingent upon the selection

of a target gene for inclusion in the calculation. In the present study, the selection of these genes was facilitated by the identification of the oxidative stress status of isolated mitochondria by reactive oxygen species (ROS) analysis. ROS was measured to demonstrate that there was no oxidative stress in healthy cells and that these enzymes were at physiological levels. The objective of this research is to highlight the necessity of identifying the most suitable reference gene for use with isolated mitochondria.

MATERIAL AND METHOD

Cell Culture

Fetal human colon epithelial (FHC) cells, used as a source of mitochondria, were grown in line with basic cell culture principles. Briefly, 1×10^6 cells were grown in Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12) with 10% fetal bovine serum, 1% l-glutamine and 1% penicillin-streptomycin in T75 culture flasks at 37°C and 5% CO_2. The medium was changed every 2 days until 70-80% confluence was reached.

Mitochondria Isolation

Mitochondria were isolated using a commercially available kit (Mitochondria Isolation Kit, ScienCell) according to the kit protocol. Approximately 2×10^7 cells were used for isolation. The isolated mitochondria were resuspended in phosphate buffered saline (PBS).

Membrane Integrity Analysis of Isolated Mitochondria

Isolated mitochondria were stained with Mitotracker (MitoSpy Green FM, Biolegend). Staining was performed by incubating 100 µl of isolated mitochondria with 200 nM mitotracker in a preheated oven at 37°C for 10 min. Then, 10 µl of stained isolated mitochondria were spread on slides and immediately visualized with a fluorescence microscope (Nikon i80, Japan) to show mitochondrial mass. The images were acquired through the utilization of a FITC filter, with a magnification of x1000 [10,11].

Flow Cytometry Analysis of Isolated Mitochondria

Flow cytometry analyses were performed using the BD Accuri™ C6 Plus. Samples were read at medium flow rate using SSC and FSC modes and 50.000 events were collected per sample. Unstained isolated mitochondria were used as a negative control. In addition, vehicle buffer was used to separate debris from mitochondria to detect the mitochondrial population. These data were used to obtain gates from the mitochondrial population. Analyses were performed after obtaining gates from individual mitochondria using the FSC vs. FSC-W graph. Subsequently, stained mitochondrial samples were analysed according to their dye properties as described below.

Mitochondrial Membrane Potential Measurement: After 100 µl of isolated mitochondria were added to 900 µl of PBS, the membrane potential was measured according to the kit protocol using a commercial kit (mitochondrial membrane potential (MMP) assay kit, Thorvacs). Samples were read at 488 nm excitation and 535 nm emission.

ROS Measurement: After 100 µl of isolated mitochondria in 900 µl PBS, ROS measurement was performed using a kit (ROS Analysis Kit, Thorvacs) according to the kit protocol. Samples were read at 485 nm excitation and 535 nm emission in the FL1 channel.

Reverse Transcription-Quantitative PCR (RT-qPCR) Analysis

The isolation of RNA from isolated mitochondria was conducted using a total RNA isolation kit (Total RNA Kit, Eco-Tech). The polymerase chain reaction was conducted using the SYBR Green dye detection method (CYBRFast™ qPCR Lo-ROX Master Mix, TONBO Bioscience) and an ABI 7500 PCR instrument (Applied Biosystem) under the default conditions. The temperature was maintained at 95°C for three minutes, followed by 40 cycles of 95°C for five seconds and 60°C for 30 seconds.

The FHC cells were employed as a control in calculation. As the genes under investigation are encoded by nuclear DNA, these cells were selected as the control for the calculation. The total mRNA was isolated from the mitochondria of these cells by the same method.

The ΔCq method was employed for the calculation of housekeeping gene expression stability [12-

14]. In this approach, the variance of a gene across all samples is calculated collectively. A constant ΔCq value between two genes indicates stable expression, whereas fluctuations indicate variable expression. In other words, the gene with the least variation between ΔCq measurements is considered to be the most stable, and this method can be used to select a suitable housekeeping gene. Furthermore, the coefficient of variance (CV) was calculated for these values. The gene exhibiting the lowest values was thus deemed to be the most stable [15].

Gene expression levels were analyzed using the $2^{-\Delta\Delta}$Cq method. GAPDH and β-actin were employed as housekeeping genes in the analysis. These genes are among the most widely utilized housekeeping genes in the domain of scientific research. They are also among the most frequently employed genes for normalization purposes in studies involving isolated mitochondria. For instance, these are among the most commonly used genes in mitochondrial transplantation studies using viable mitochondria, which is one of the most popular research areas of recent years [16-19]. However, a comprehensive study to determine housekeeping gene stabilization in mitochondrial transplantation research has not been found in the literature. Consequently, the selection of these genes was based on their relevance to ongoing research. In addition to measuring the expression levels of GAPDH and β-actin mRNA, the expression levels of CAT and SOD1, which are target genes, were also measured in both FHC cells and isolated mitochondria. The expression levels of CAT and SOD1 were calculated separately based on both housekeeping genes. In the calculation, the target genes were normalized to their own expression levels. The lowest $2^{-\Delta\Delta}$Cq value was selected as the basis for normalization.

The primer sequences employed in the RT-qPCR analysis are presented in Table 1. The primer designs were created using the Primer-BLAST® (Basic Local Alignment Search Tool) sequence matching analysis tool on the National Center for Biotechnology Information Gene Bank (NCBI-Gene Bank) database. The designs were then evaluated and refined with the Primer3 (https://primer3.ut.ee/) software.

Tablo 1. Table of primer sequences

Oligo Name	Base Sequence: 5′-3′	Reference ID
GAPDH	F: TTTTGCGTCGCCAGCC R: ATGGAATTTGCCATGGGTGGA	NCBI Reference Sequence: NM_002046.7 (208 BP)
β-actin	F: CTTCGCGGGCGACGAT R: CCACATAGGAATCCTTCTGACC	NCBI Reference Sequence: NM_001101.5 (104)
SOD1	F: AAAGATGGTGTGGCCGATGT R: CAAGCCAAACGACTTCCAGC	NCBI Reference Sequence: NM_000454.5 (167 BP)
CAT	F: ACTTCTGGAGCCTACGTCCT R: AAAGTCTCGCCGCATCTTCA	NCBI Reference Sequence: NM_001752.4 (210 BP)

RESULT AND DISCUSSION

Fluorescence microscopy images of isolated mitochondria labeled with Mitotracker demonstrate that the mitochondria retain their membrane integrity (Figure 1). Flow cytometry analysis of the membrane potential of isolated mitochondria demonstrated that 91% of the mitochondria retained their membrane potential (Figure 2). Accordingly, it is concluded that isolated mitochondria are viable. The ratio of ROS in isolated mitochondria, as determined by flow cytometry, was 1.6% (Figure 3). ROS measurement indicated a low level of ROS production and no evidence of oxidative stress.

RT-qPCR is one of the most widely used and accepted methods for quantitative gene expression analysis. The potential problems associated with the analysis of gene transcription changes can be mitigated using appropriate reference genes for normalization. One of the most used of these genes is GAPDH. However, its stability is susceptible to changes due to variations in tissue type and cellular metabolism [20]. In one study, GAPDH was identified as the housekeeping gene with the highest stability. The study assessed the stability of common housekeeping genes in porcine alveolar macrophages following stimulation with lipopolysaccharide and lipoteichoic acid. Different algorithms were used to assess the stability of these genes. The combination of SDHA, YWHAZ, and RPL4 was

identified as a potentially optimal housekeeping gene combination for precise normalization of gene expression levels in porcine alveolar macrophages [20]. However, another study investigated the IGF family gene expression at different stages of pregnancy in the feline uterus and identified GAPDH as the most stable housekeeping gene within the 8-gene design [20]. Similarly, stability analyses for β-actin and other housekeeping genes have been performed in studies using different research models [21].

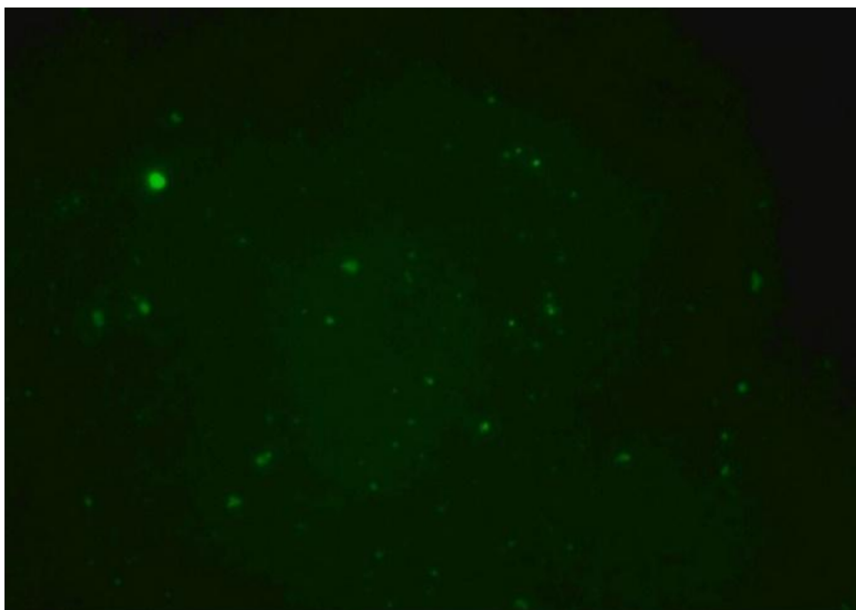

Figure 1. Fluorescence microscope image of isolated mitochondria

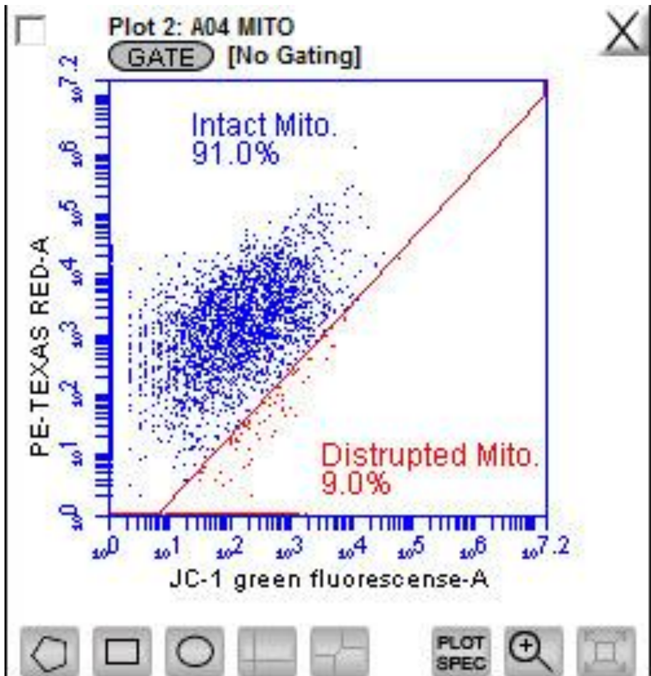

Figure 2. Flow cytometry analysis of isolated mitochondria membrane potential

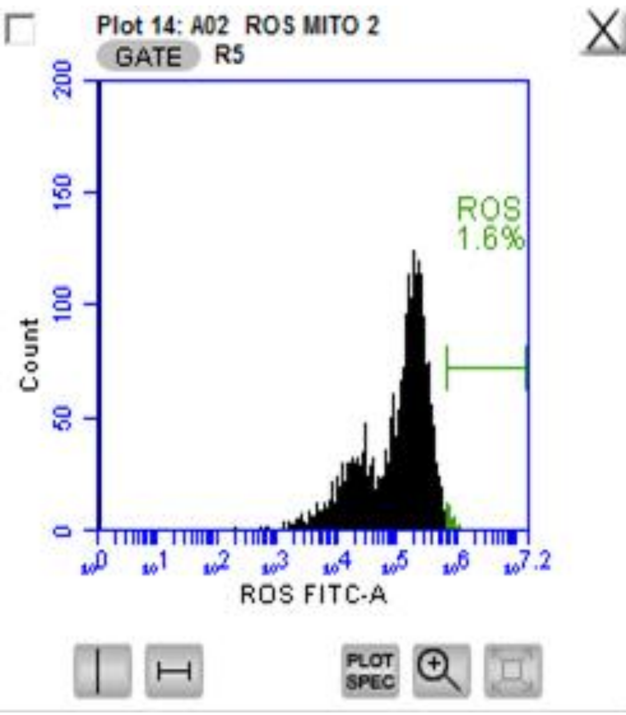

Figure 3. Flow cytometry analysis of isolated mitochondria ROS level

The objective of this study was to ascertain which of the two most commonly used genes is more stable. According to the ΔCq method used for housekeeping gene stabilization, the mean ΔCq value of β-actin was determined to be 4.54, with a standard deviation of 0.06. The mean value for GAPDH was 0.69, with a standard deviation of 0.16. The coefficient of variation (CV) was calculated to be 1.33% for β-actin and 23.95% for GAPDH (Table 2). The results demonstrated lower values in β-actin. It was therefore concluded that β-actin may be a more stable option than GAPDH among the housekeeping genes that could be used for normalization in PCR analyses.

Tablo 2. The data presented herewith pertains to the stability calculations of the housekeeping genes

	β-actin	**GAPDH**
ΔCq values	4.55	0.88
	4.48	0.61
	4.6	0.58
Standard Deviation	0.06	0.16
Mean	4.54	0.69
Coefficient of Variance (CV)	0.013	0.239
CV%	1.33	23.95

Subsequently, the transcription levels of CAT and SOD1 enzymes were calculated separately according to both housekeeping genes for validation. According to the $2^{-\Delta\Delta}$Cq method, the CAT transcription level of isolated mitochondria was calculated as 25.7 ± 2.6 according to housekeeping gene β-actin, and 1.8 ± 0.33 according to GAPDH. SOD1 transcription levels were 70.1 ± 12.7 according to β-actin, and 4.8 ± 0.6 according to GAPDH (Figure 4). Gene transcription results are presented as the mean ± standard deviation. The results indicated a 14.3-fold difference in CAT levels and a 14.5-fold difference in SOD1 levels according to the selected housekeeping gene. The findings were evaluated as calculations based on different housekeeping genes could potentially influence the results. Consequently, it reiterates the importance of selecting appropriate housekeeping genes.

Figure 4. CAT and SOD1 enzyme transcription levels of isolated mitochondria according to different housekeeping genes. Calculations were made according to the $2^{-\Delta\Delta}$Cq method. (*) significant difference between GAPDH and β-actin in CAT calculation ($p<0.05$), (**) significant difference between GAPDH and β-actin in SOD1 calculation ($p<0.05$). Student's t-test was used in statistical analysis

It should be noted that there may be other genes that are more stable than the two housekeeping genes that were analyzed in this study. Given that mitochondria are the primary metabolic actors within cells and can influence the stability of enzymes such as GAPDH in a range of mitochondrial processes [22]. Similarly, mitochondria are highly dynamic organelles. They are closely related to other organelles and various cellular structures, both in terms of their physical and functional relationships. In general, cytoplasmic actin has been demonstrated to regulate cell morphology and dynamics, as well as exerting an influence on mitochondrial function. β-actin is essential for mitochondrial quality control, mtDNA transcription and the maintenance of mitochondrial membrane potential [23]. It can be hypothesized that these characteristics may act as factors affecting gene stability depending on the period of the cell. No comprehensive study on housekeeping gene selection in isolated mitochondria has been found in the literature. However, various studies have presented reports on the expression levels of mitochondrial genes, and in some of these studies, housekeeping gene stabilization has been determined. For instance, in a study on *Cryptolestes ferrugineus*, where mitochondrial gene expression levels were investigated, it was found that RPS13, EF1α, and γ-TUB were among the most stable genes, while α-TUB, CycA and GAPDH were among the least stable genes [24]. In another study, it was reported that ACTB, CyC1, YWHAZ and SDHA were among the stable genes for isolated and cultured limbal cells, and ATP5B, CyC1, EIF4A2, RPL13A, TOP1 and UBC were among the stable genes for isolated and cultured conjunctival cells. In this study, however, GAPDH and 18s rRNA were not identified as stable genes [25]. Mitochondrial transplantation is one of the most widely studied methods in mitochondrial research in recent years. In these studies, GAPDH and B-actin are among the most frequently used genes for normalization [16-19]. Furthermore, various genes such as 18s RNA, RPL and RNU6B have been utilized [26-28]. However, a comprehensive definition of housekeeping gene stabilization remains to be reported in these studies. Therefore, further comprehensive studies using a larger number of samples and housekeeping genes will help to select the most stable gene for mitochondrial research.

Conclusion

Mitochondria isolation is a method used in mitochondria research, which has emerged as a topic of interest in recent years. The currently defined protocols facilitate the efficient isolation of

mitochondria. These mitochondria can be employed for transplantation and subjected to analyses, including protein and ATP measurements, oxygen consumption rates, and complex analyses, with the objective of enhancing comprehension of mitochondrial processes. Moreover, gene transcription analyses of a range of mitochondria-related proteins can be conducted using isolated mitochondria. In the PCR method, which is frequently employed in gene transcription analyses, the selection of the most appropriate housekeeping gene during the analysis of the data represents a crucial step in the method. It is important to note that the selection of a housekeeping gene can potentially lead to erroneous results. Therefore, it is essential to perform analyses using the most appropriate gene. Despite the current limitations in our understanding of this subject, it is evident that further research is required on the selection of housekeeping genes in PCR analyses, particularly in the context of mitochondrial research.

AUTHOR CONTRIBUTIONS

Concept: O.U.; Design: O.U., T.F.; Control: O.U., T.F., S.O., P.M.E.; Sources: O.U., T.F., S.O., P.M.E.; Materials: O.U., T.F., S.O., P.M.E.; Data Collection and Processing: O.U., T.F., S.O., P.M.E.; Analysis and Interpretation: O.U., T.F., S.O., P.M.E.; Literature Review: O.U.; Manuscript Writing: O.U.; Critical Review: O.U., T.F., S.O., P.M.E.; 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.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

NEUROPHARMACOLOGICAL EVALUATION OF AQUEOUS EXTRACTS OF FRUIT PEELS AND THEIR POLYHERBAL FORMULATION: ANTIDEPRESSANT AND MUSCLE RELAXANT POTENTIAL

MEYVE KABUKLARININ SULU EKSTRELERİ VE POLİBİTKİSEL FORMÜLASYONUNUN NÖROFARMAKOLOJİK DEĞERLENDİRİLMESİ: ANTİDEPRESAN VE KAS GEVŞETME POTANSİYELİ

Aruna Kumari DOKKADA[1], Vijay Srinivas POTHULA[2], Gana Manjusha KONDEPUDI[1]*

[1]Vignan Institute of Pharmaceutical Technology, Department of Pharmacognosy, 530049, Visakhapatnam, India
[2]Stira Pharma LLC, Department of Analytical R & D, 07004, Fairfield, USA

ABSTRACT

Objective: *This study aims to assess the neuropharmacological properties of peels from specific fruits, namely Annona squamosa, Cucumis melo, Actinidia deliciosa, Malus pumila.*
Material and Method: *Wistar albino rats (weighing 150 g), regardless of gender, were allocated into six groups for forced swim tests, locomotion assessments, and muscle coordination evaluations. Diazepam and imipramine were employed as reference standards. At doses of 200 and 400 mg/kg, all peel extracts showed a reduction in immobility time, improved rotarod performance, and a notable increase in locomotor activity.*
Result and Discussion: *The efficacy ranking for immobility time was as follows PHF>APMP>APCM>APAS>APAD. In terms of skeletal muscle relaxation, the order of effectiveness was: PHF>APCM>APMP>APAD>APAS and the treatment groups' locomotor activity followed this sequence: PHF>APMP>APCM>APAD>APAS. All the selected peel extracts exhibited noteworthy effects on immobility, locomotor activity, and muscle relaxation. However, a more comprehensive investigation is required to elucidate the precise mechanisms underlying the antidepressant effects of the selected peel extracts and PHF.*
Keywords: *Fruit peels, neurological activity, polyherbal mixture*

ÖZ

Amaç: *Bu çalışma, Annona squamosa, Cucumis melo, Actinidia deliciosa, Malus pumila gibi belirli meyvelerin kabuklarının nörofarmakolojik özelliklerini değerlendirmeyi amaçlamaktadır.*
Gereç ve Yöntem: *Wistar albino sıçanlar (ağırlıkları 150 g) cinsiyete bakılmaksızın zorunlu yüzme testleri, hareket değerlendirmeleri ve kas koordinasyonu değerlendirmeleri için altı gruba ayrıldı. Referans standartlar olarak diazepam ve imipramin kullanıldı. 200 ve 400 mg/kg dozlarında, tüm kabuk ekstraktları hareketsizlik süresinde azalma, rotarod performansında iyileşme ve lokomotor aktivitede kayda değer bir artış gösterdi.*
Sonuç ve Tartışma: *Hareketsizlik süresine ilişkin etkinlik sıralaması şu şekildeydi: PHF>APMP>APCM>APAS>APAD. İskelet kası gevşemesi açısından etkinlik sırası şu*

* **Corresponding Author /SorumluYazar:** Gana Manjusha Kondepudi
e-mail / e-posta: manjusha0988@gmail.com, **Phone / Tel.:** +919885574803

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şekildeydi: PHF>APCM>APMP>APAD>APAS ve tedavi gruplarının lokomotor aktivitesi şu sırayı takip etti: PHF>APMP>APCM>APAD>APAS. Seçilen tüm kabuk özleri hareketsizlik, lokomotor aktivite ve kas üzerinde kayda değer etkiler sergiledi.
Anahtar Kelimeler: *Meyve kabukları, nörolojik aktivite, polibitkisel karışım*

INTRODUCTION

Oxidative stress poses a significant challenge to cells by inducing the production of reactive oxygen species (ROS) and antioxidants that influence signaling pathways. The byproducts of this process play a crucial role in brain pathology and function across various neurological conditions. Since oxidative stress is a primary therapeutic target in neurological diseases, it is essential to investigate diverse strategies that can effectively repair ROS-induced damage and address neurodegenerative disorders (NDDs) [1]. These conditions have been linked to the detrimental effects of free radicals and oxidative damage, which underlie the pathogenesis of several neurological diseases. Antioxidants, as free radical scavengers, hold the promise of preventing, delaying, or alleviating the burden of these disorders.

Free radicals are molecules with one or more unpaired electrons [2,3]. These radicals can cause various cellular alterations, such as DNA mutations, lipid peroxidation in cell membranes, changes in enzymatic activity, and even cell death [4]. Aerobic metabolism in the body produces oxygen free radicals, including hydroxyl radicals, superoxides, and reactive oxygen species (ROS) [5-7]. The excessive generation of free radicals can damage biomolecules like DNA, lipids, and proteins, increasing the risk of chronic diseases such as rheumatoid arthritis, cancer, diabetes, neurological disorders, and atherosclerosis [8]. Various neurological disorders are linked to oxidative injury or free radicals. Free radical scavengers are commonly used to prevent or delay neurological disorders by mechanisms such as scavenging activity, metal chelation, or inhibiting lipid peroxidation [9].

Antioxidants are natural compounds with low molecular weights, typically consisting of polyhydroxylated phenolic structures. These antioxidants include flavonoids, phenolic acids, tannins, lignans, stilbenes, catechins, and carotenoids, found in many fruits and vegetables. Certain cellular enzymes, located within specific cellular compartments, also exhibit strong antioxidant properties, neutralizing reactive radicals. By removing electrons or hydrogen atoms from substances, antioxidants can prevent oxidative damage within cells. The antioxidant activity is significantly influenced by the number and position of hydroxyl groups on their aromatic rings. Antioxidants act as scavengers of reactive radicals, mitigating the oxidative damage caused by reactive oxygen species (ROS), and can originate from either endogenous or exogenous sources [10,11].

In recent years, there has been a growing attention in exploring the antioxidant properties of phytoconstituents found in everyday food items like fruits and vegetables in particular. It is well-established that many of these phytochemicals, renowned for their antioxidant capabilities, are highly concentrated in the outer layers, such as peels. Regrettably, a substantial quantity of fruit and vegetable peels is discarded as waste, contributing to environmental challenges. Waste valorisation centers on transforming by-products or residues into valuable raw materials by using discarded items as energy sources or inputs in manufacturing. This process can also involve incorporating waste into final products. One common approach is recycling, where waste is recovered and repurposed into new, functional products. However, this often reduces the material's properties, leading to lower-quality applications, a process called downcycling. In contrast, upcycling enhances the value, quality, or functionality of waste materials, making it the preferred method for waste valorization [12-17].

Researchers uncover valuable bioactive compounds in these peels, demonstrating their potential health benefits, including antioxidative, anti-inflammatory, anti-cancer, antiviral, and cardio-protective activities, among others [18,19]. Numerous studies have consistently shown that the phenolic content in fruit peels often surpasses that found in the pulp [20].

Malus pumila (M. pumila) commonly referred to as the apple and belonging to the Rosaceae family, is renowned globally for its health-promoting attributes. Originally hailing from central Asia, the apple has now become a staple worldwide, with every part, including the skin, being edible.

Apples are rich in essential nutrients such as vitamin C, vitamin B12, calcium, phosphorous, and are a valuable source of carbohydrates [21]. The processing of canned apples and apple sauce generates apple peel waste, which, interestingly, has been found to be rich in polyphenols with demonstrated antioxidant and antiproliferative activities [22].

Annona squamosa (A. squamosa), a medium-sized tree belonging to the Annonaceae family, has a history of traditional use for its various parts, including fruits, seeds, leaves, and barks, to address a multitude of health issues [23]. Research on *A. squamosa (Custard apple)* peels has unveiled their antimicrobial and antioxidant properties [24].

Actinidia deliciosa (A. deliciosa), commonly known as kiwifruit and belonging to the Actinidiaceae family carries substantial global significance. Its fruits have been celebrated for their medicinal properties, containing phytoconstituents such as triterpenoids, flavonoids, phenylpropranoids, quinines, and steroids. Traditional Chinese medicine employs different parts of *A.deliciosa* to address various ailments, including hepatitis, pyorrhea, gingivitis, edema, rheumatoid arthritis, and various forms of cancer. Kiwi seeds are used as natural blood thinners, and kiwi fruit is a rich source of vitamins, often employed as a mild laxative [25,26].

Cucumis melo (C. melo) Linn, a member of the Cucurbitaceae family, is valued for different parts, including the pulp, root, seeds, and seed oil. It is associated with properties such as diuretic, emmenagogue, cooling, demulcent, aphrodisiac, galactagogue, and astringent. Over centuries, it has been used to treat kidney disorders, urinary tract issues, and various conditions like cough, bilious diseases, inflammation of the liver, liver and bile obstructions, eczema, and more [27]. Research has demonstrated the antioxidant potential of *C. melo* (musk melon) peels [28].

The current study investigates the CNS effects of aqueous peel extracts (AP) from *Malus pumila* (APMP), *Annona squamosa* (APAS), *Actinidia deliciosa* (APAD), and *Cucumis melo* (APCM). These peels utilized as a polyherbal formulation (PHF) and individually, were evaluated for their antidepressant and muscle relaxant potential. Additionally, the study sought to address the lack of research on fruit peel utilization for mood disorders, employing recognized experimental models and considering clinical relevance.

MATERIAL AND METHOD

Collection and Preparation of Peel Extracts

Fresh fruits were procured, and their peels were separated, cleaned, and shade-dried for one month. The dried peels were ground into a fine powder and extracted via cold maceration using water and ethanol (80:20). Extracts were concentrated, converted to powder, and stored for further use. A polyherbal formulation (PHF) was prepared by combining the extracts in equal proportions.

Preliminary Phytochemical Analysis

The aqueous peel extracts were tested for various phytochemicals using the standard preliminary phytochemical screening methods [29].

Dose Selection

Acute toxicity studies following OECD Guideline 423 confirmed safety up to 2000 mg/kg. Experimental doses of 200 mg/kg and 400 mg/kg were chosen, equivalent to 1/10th and 1/5th of the maximum non-toxic dose.

Experimental Animals

Male Wistar albino rats (180-200 g) were used. Gender differences were not assessed in this study due to resource constraints; however, future studies should include both sexes to ensure comprehensive results. Rats were acclimatized for two weeks before experiments.

Experimental Design

Rats were divided into 11 groups (n=6):

1. Control: Distilled water.
2. Standard: Imipramine (3 mg/kg).
   3–10 Peel Extract Groups: Individual extracts at 200 mg/kg and 400 mg/kg.
3. PHF: 200 mg/kg.
4. The extracts were reconstituted in distilled water and administered orally to the animals using a calibrated gavage. The administration was performed once daily during the study period.

Experimental Models

Forced Swim Test (FST): To evaluate antidepressant activity. Immobility time was recorded during the last 4 min of a 6-min swim session.
Locomotor Activity: Assessed using an actophotometer to measure alertness.
Rotarod Test: Evaluated muscle coordination and relaxant properties**.**

Forced Swim Test

The Forced Swim Test, a widely employed behavioral model for evaluating central nervous system depressant activity in rodents, was performed following the methodology proposed by Porsolt et al. in 1977 [30]. The test procedure closely followed established protocols in the literature.

Rats were individually placed in a transparent glass chamber measuring 25 × 15 × 25 cm filled with fresh water to a height of 15 cm, and the water temperature was maintained at 26 ± 1°C. At this water level, the rats were unable to touch the bottom or the chamber's side walls with their hind paws or tail. After each rat's turn, the water in the chamber was replaced with fresh water. During the initial 2-min phase of the 6-min testing period, each animal exhibited vigorous movement. Subsequently, the duration of immobility was manually recorded over the remaining 4 min.

Rats were considered immobile when they ceased active struggling and remained afloat in the water without any significant motion, except for the minimal movements required to keep their heads above water. After the swimming session, the rats were gently towel-dried and returned to their respective housing conditions.

Locomotor Activity

The independent measurement of spontaneous locomotor activity for each mouse was conducted over duration of 10 min using an actophotometer. Prior to the test, the peel extracts and the polyherbal formulation (PHF) were administered 60 minutes in advance, while the standard drug Imipramine hydrochloride was administered 60 minutes before the test [31].

Muscle Co-ordination Test

The rotarod test was conducted utilizing a specialized rotarod apparatus. This apparatus featured a metal rod with a diameter of 3 cm, coated with rubber, and connected to a motor set to rotate at a speed of 20 rotations per min. The length of the rod was 45 cm, and it was divided into three sections by metallic discs, which enabled the concurrent testing of three rats. The rod was positioned approximately 50 cm above the tabletop to deter the animals from leaping off the roller. Cages positioned beneath the sections were in place to confine the movements of the animals in case they fell from the roller. Prior to the formal test, albino rats

underwent a pre-test on the apparatus. Only those animals that demonstrated their capability to remain on the revolving rod (at 20 rpm) for duration of 5 minutes were selected for the test [32].

RESULT AND DISCUSSION

Preliminary Phytochemical Analysis

The preliminary phytochemical screening of aqueous extracts of the four peels revealed compounds mentioned in Table 1.

Table 1. Preliminary phytochemical analysis of aqueous extracts from selected fruit peels

Phytoconstituentsts	Tests	*A. squamosa*	*A. deliciosa*	*C. melo*	*M. pumila*
Alkaloids	Dragondorff's test Mayer's test	+ +	-	-	-
Glycosides	Shinoda test for Flavonoids	-	-	-	+
Phenolics	5% $FeCl_3$	+	+	+	+
Carbohydrates	Molisch's Test	+	-	+	-
Amino acids	Ninhydrin test	-	-	-	-
Saponins	Foam test	-	-	+	-
Tannins	Gelatin test	+	-	+	-

"+" denotes present, "-"denotes absent

Acute Toxicity

The rats were normal and no toxic signs were seen after administration of the peel extracts. There was no mortality at the highest dose of 2000 mg/kg. Hence, dosages for pharmacological studies were selected as 1/5th and 1/10th of the highest dose (2000 mg/kg).

Neuropharamcological Activity

In this study, the peel extracts significantly reduced immobility time compared to the control group. The effectiveness of the treatments was ranked as follows: PHF > APMP > APCM > APAS > APAD (Table 2 and Figure1).

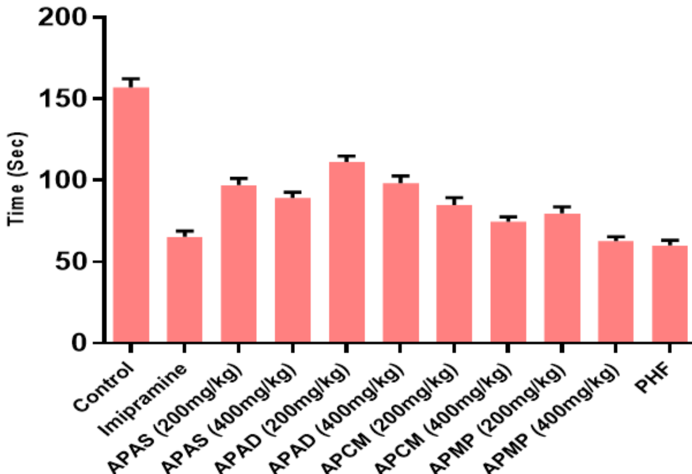

Figure 1. Impact of the chosen peel extracts and polyherbal formulation on immobility duration in the forced swim test

Table 2. Impact of the chosen peel extracts and polyherbal formulation on immobility duration in the forced swim test

Groups	Immobility Time (Sec)						Mean ± SEM
	R1	R2	R3	R4	R5	R6	
Control	164	172	154	136	150	166	157.00 ± 5.33
Imipramine	59	66	71	55	62	79	65.33 ± 3.54
APAS (200 mg/kg)	94	86	106	99	87	111	97.16 ± 4.12*
APAS (400 mg/kg)	83	76	89	91	94	102	89.16 ± 3.66*
APAD (200 mg/kg)	123	112	118	108	97	110	111.33 ± 3.64*
APAD (400 mg/kg)	99	103	87	111	107	82	98.16 ± 4.66*
APCM (200 mg/kg)	88	69	77	82	99	94	84.83 ± 4.52*
APCM (400 mg/kg)	64	75	82	68	79	80	74.66 ± 2.94*
APMP (200 mg/kg)	77	82	65	74	88	92	79.66 ± 4.00*
APMP (400 mg/kg)	61	72	60	54	59	70	62.66 ± 2.82*
PHF	66	54	62	71	58	49	60.00 ± 3.27*

$p<0.05^*$ significance followed by one way ANOVA followed by DUNNETT's multiple comparison test

The rotarod test is employed to assess skeletal muscle relaxant activity. This test is utilized to evaluate the effects of drugs on motor coordination. Dunham and Miya (1957) proposed that the muscle relaxation induced by a test compound could be assessed by measuring the rats' ability to remain on a rotating rod. The selected peel extracts showed a highly significant reduction in the time spent by the animals on the rotating rod when compared to the control ($p< 0.05$). The standard drug (diazepam) also exhibited a highly significant effect when compared to the control (Table 3 and Figure 2). The order of potency was found to be PHF>APCM>APMP>APAD>APAS. The selected extracts displayed dose-dependent effects on muscle coordination, as assessed by the rotarod method. The results from the rotarod test clearly demonstrated the significant muscle relaxation activity induced by the extracts in the tested animals.

Table 3. Impact of the chosen peel extracts and polyherbal mixture on the duration of time spent in the rotarod apparatus

Groups	Time (Sec)						Mean ± SEM
	R1	R2	R3	R4	R5	R6	
Control	175	152	134	154	143	161	153.16 ± 5.80
Diazepam(2 mg/kg)	44	56	39	52	61	71	53.83 ± 4.72
APAS (200 mg/kg)	123	114	116	130	104	106	115.50 ± 4.04*
APAS (400 mg/kg)	111	99	85	121	101	100	102.83 ± 4.96*
APAD (200 mg/kg)	102	94	116	111	102	99	104.00 ± 3.29*
APAD (400 mg/kg)	92	101	87	117	101	94	98.66 ± 4.27*
APCM (200 mg/kg)	92	89	87	102	92	80	90.33 ± 2.95*
APCM (400 mg/kg)	62	71	79	80	61	79	72.00 ± 3.57*
APMP (200 mg/kg)	89	102	111	94	88	102	97.66 ± 3.63*
APMP (400 mg/kg)	79	84	69	97	84	76	81.50 ± 3.85*
PHF	38	52	41	50	49	34	44.00 ± 3.00*

$p<0.05^*$ significance followed by one way ANOVA followed by DUNNETT's multiple comparison test

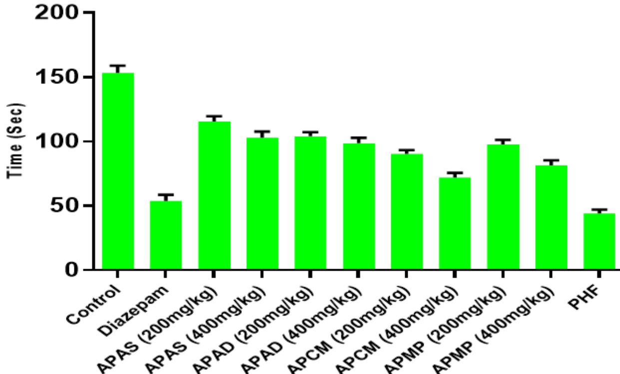

Figure 2. Impact of the chosen peel extracts and polyherbal mixture on the duration of time spent in the rotarod apparatus

Locomotor activity serves as an indicator of alertness, and a decrease in activity is indicative of sedative effects. The decreased mobility observed in the control rats may be attributed to the monoamine theory of depression, leading to depleted monoamines in the brain, consequently resulting in hypothermia and reduced mobility. However, both the standard drug and the selected peel extracts exhibited significant antidepressant activity, as evidenced by increased alertness and heightened mobility across the cell beams. Table 4 and Figure 3 sillustrated that the locomotor activity in the treatment groups followed this order: PHF>APMP>APCM>APAD>APAS.

Table 4. Impact of the chosen peel extracts and polyherbal mixture on the locomotor activity in the actophotometer

Groups	Locomotors activity (Sec)						Mean ± SEM
	R1	R2	R3	R4	R5	R6	
Control	94	77	80	93	89	76	84.83 ± 3.32
Imipramine	120	103	96	88	109	111	104.50 ± 4.65
APAS (200 mg/kg)	98	100	110	97	100	90	99.16 ± 2.63*
APAS (400 mg/kg)	113	104	98	111	92	117	105.83 ± 3.91*
APAD (200 mg/kg)	99	104	98	96	89	86	95.33 ± 2.72*
APAD (400 mg/kg)	118	100	104	111	102	99	105.66 ± 3.01*
APCM (200 mg/kg)	99	101	110	89	104	99	100.00 ± 2.87*
APCM (400 mg/kg)	120	113	99	104	101	107	107.33 ± 3.23*
APMP (200 mg/kg)	105	89	102	104	100	90	98.33 ± 2.88*
APMP (400 mg/kg)	121	111	106	99	127	117	113.50 ± 4.17*
PHF	131	124	127	104	119s	126	121.83 ± 3.91*

$p<0.05^*$ significance followed by one way ANOVA followed by DUNNETT's multiple comparison test

Various medications, including tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), selective reversible inhibitors of monoamine oxidase A (RIMAs), and specific serotonin–noradrenaline reuptake inhibitors (SNRIs), are commonly prescribed for therapeutic purposes. However, these drugs are associated with a range of side effects, such as cardiac toxicity, hypotension, sexual dysfunction, weight gain, and sleep disturbances.

Depression has a high incidence rate within communities, and it is directly or indirectly linked to morbidity and, to some extent, mortality. In the Forced Swim Test (FST), rats compelled to swim within a confined space eventually cease swimming and become motionless. This behaviour is

referred to as immobility and signifies a state of lowered mood. Agents that diminish this despondent behaviour is classified as antidepressant drugs.

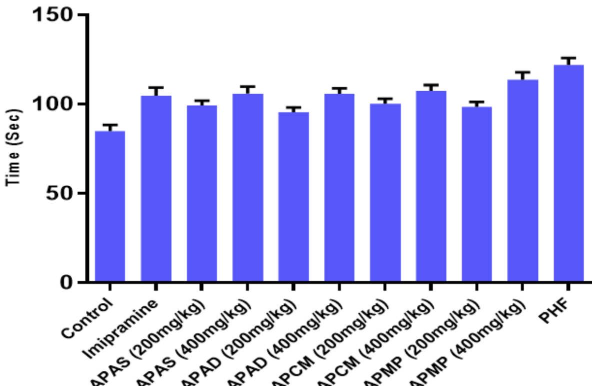

Figure 3. Impact of the chosen peel extracts and polyherbal mixture on the locomotor activity in the actophotometer

The FST serves as a highly sensitive test for assessing alterations in monoamines and represents a specific cluster of stress-induced behaviours not necessarily related to depressive symptoms in humans. Nonetheless, it is remarkably responsive to manipulations of monoaminergic systems. Furthermore, it offers a valuable model for investigating the neurobiological and genetic mechanisms underpinning stress and responses to antidepressants. These screening tests are highly sensitive and widely utilized for the assessment of rodent behaviours to predict antidepressant potential, as indicated by a decrease in immobility time [30].

The Forced Swim Test (FST) results highlighted the capacity of these extracts to reduce immobility time in rats, indicative of their potential antidepressant properties. Notably, the polyherbal mixture (PHF) exhibited the highest potency in this regard, followed by the individual fruit peel extracts. The Rotarod test, which assessed muscle coordination and relaxation, further underscored the potential therapeutic value of these extracts. The selected extracts demonstrated a notable reduction in the time spent by animals on the rotating rod, indicating their ability to induce muscle relaxation. Again, the polyherbal mixture (PHF) exhibited remarkable efficacy in this aspect [30].

Furthermore, the locomotor activity results indicated that the selected peel extracts, as well as PHF, exhibited antidepressant activity by increasing alertness and mobility, which contrasted with the reduced mobility observed in the control group [31,32].

This study sheds light on the neuropharmacological properties of fruit peel extracts from *A. squamosa*, *C. melo*, *A. deliciosa*, and *M. pumila*. The extracts demonstrated significant activity in behavioral models such as the Forced Swim Test, Rotarod Test, and locomotion assessment, suggesting their potential as antidepressant, neuromuscular, and stimulatory agents. These activities can be attributed to the presence of bioactive compounds, including polyphenols and flavonoids, known for their antioxidant and neuroprotective effects.

Notably, there are no prior studies reported in the literature investigating the neuropharmacological activities of these specific fruit peels or the fruits themselves, highlighting the novelty of this research. To strengthen the discussion, future studies should compare these findings with similar investigations on other fruit peels or plant-derived compounds that have demonstrated neuropharmacological potential. This would provide a more comprehensive context for the observed results and facilitate a deeper understanding of their pharmacological mechanisms.

This work emphasizes the need for further exploration of agro-industrial by-products, such as fruit peels, as cost-effective, sustainable sources of therapeutic agents. It also opens avenues for future research focused on standardization of extraction methods, dose optimization, and clinical validations

to establish these extracts as viable candidates for treating neuropsychiatric and neurodegenerative disorders. Such efforts would align with global initiatives to reduce waste while harnessing the untapped potential of natural resources for pharmaceutical innovation.

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

All aspects related to the housing, handling, and experimental procedures involving the animals strictly adhered to the regulations set forth by the Institutional Animal Ethics Committee (Registration No. 516/01/A/CPSCEA).

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

COMPARATIVE EVALUATION OF BIOLOGICAL EFFECT OF FOUR SUNFLOWER (*HELIANTHUS ANNUUS L.*) GENOTYPES: AGRO-FOOD BYPRODUCTS AS PROMISING NATURAL NEW FOOD ADDITIVES

DÖRT AYÇİÇEĞİ (HELIANTHUS ANNUUS L.) GENOTİPİNİN BİYOLOJİK ETKİLERİNİN KARŞILAŞTIRMALI DEĞERLENDİRMESİ: TARIMSAL GIDA YAN ÜRÜNLERİNDEN UMUT VERİCİ DOĞAL YENİ GIDA KATKI MADDELERİ

Zühal BAYRAKÇEKEN GÜVEN[1]*, Aysema TAZEGÜL ÇAVUŞOĞLU[2]

[1]Erzincan Binali Yıldırım University, Faculty of Pharmacy, Department of Pharmacognosy, 24002, Erzincan, Türkiye
[2]Eastern Anatolia Agricultural Research Institute 25100, Erzurum, Türkiye

ABSTRACT

Objective: *Food additives are widely used in industry to improve the appearance, quality and safety of food during processing, storage and packaging. The sunflower (Helianthus annuus L.) is an important agricultural product that is cultivated worldwide for seeds. Its seeds are one of the largest sources of vegetable oil. In this study, the biological activity of sunflower seeds as well as various plant parts, which are agricultural by-products and mostly treated as waste, was tested and their potential for use as a food additive was determined.*
Material and Method: *The antiproliferative effect of six different plant parts (seeds, ray florets, disc florets, leaves, stems and receptacle) of four sunflower varieties (DERAY, SY GRANIT, P64 LP 130, TR 2242 CL) against the healthy cell line L929 was investigated using the MTT method and the concentration range that can be safely used was determined. The antioxidant capacity was determined using the DPPH, TEAC and CUPRAC methods. Inhibition of the enzyme tyrosinase was investigated to prevent enzymatic browning of food.*
Result and Discussion: *It was found that even at concentration of 400 and 800 µg/ml, safe use is possible. In all methods, disc floret and ray floret showed a strong antioxidant effect. In the DPPH free radical scavenging effect of the ray floret of the TR 2242 CL showed the highest value with 101.40 mg gallic acid/g extract. Disc and ray floret showed strong inhibition of the tyrosinase enzyme in all varieties. The enzyme inhibition of methanol extracts of TR 2242 CL ray and disc floret was found to be 60.42 and 151.25 mg KAE/g extract, respectively. The lack of cytotoxicity against healthy cells, the high antioxidant capacity and the strong anti-browning activity suggest that sunflower agro-food byproducts may be a new, non-toxic, cost-effective and recyclable source to be used in the food industry instead of food additives that have negative side effects on health.*
Keywords: *Antioxidant, antiproliferative, food additives, Helianthus annuus L., tyrosinase inhibition*

* **Corresponding Author / Sorumlu Yazar:** Zühal Bayrakçeken Güven
e-mail / e-posta: zbayrakceken@erzincan.edu.tr, **Phone / Tel.:** +904462245344

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ÖZ

Amaç: *Gıda katkı maddeleri, işleme, depolama ve paketleme sırasında gıdanın görünümünü, kalitesini ve güvenliğini iyileştirmek amacıyla endüstride yaygın olarak kullanılmaktadır. Ayçiçeği (Helianthus annuus L.), dünya çapında tohumluk olarak yetiştirilen önemli bir tarım bitkisidir. Tohumları en büyük bitkisel yağ kaynaklarından biridir. Bu çalışmada tarımsal yan ürün olan ve çoğunlukla atık olarak nitelendirilen ayçiçeğinin tohumlarının ve çeşitli bitki kısımlarının biyolojik etkileri test edilmiş ve gıda katkı maddesi olarak kullanım potansiyelleri belirlenmiştir.*

Gereç ve Yöntem: *Dört ayçiçeği çeşidinin (DERAY, SY GRANIT, P64 LP 130, TR 2242 CL) altı farklı bitki kısmının (tohum, dil çiçekleri, disk çiçekleri, yaprak, gövde ve tabla) L929 sağlıklı hücre hattına karşı antiproliferatif etkisi MTT yöntemi kullanılarak araştırıldı ve güvenle kullanılabilecek konsantrasyon aralığı belirlendi. Antioksidan kapasite DPPH, TEAC ve CUPRAC yöntemleri kullanılarak belirlendi. Gıdanın enzimatik esmerleşmesini önlemek için tirozinaz enzim inhibisyonu araştırıldı.*

Sonuç ve Tartışma: *400 ve 800 µg/ml konsantrasyonlarda dahi güvenli kullanımın mümkün olduğu tespit edildi. Tüm yöntemlerde disk çiçeği ve dil çiçeği güçlü antioksidan etki gösterdi. DPPH serbest radikal süpürücü etki tayininde TR 2242 CL'nin dil çiçeği 101.40 mg gallik asit/g ekstrakt ile en yüksek etkiyi gösterdi. Disk ve dil çiçeği tüm çeşitlerde en güçlü tirozinaz enzim inhibisyonu gösterdi. TR 2242 CL dil ve disk çiçeği metanol ekstraktlarının enzim inhibisyonu sırasıyla 60.42 ve 151.25 mg KAE/g ekstrakt olarak bulundu. Ayçiçeği tarım-gıda yan ürünlerinin sağlıklı hücrelere karşı sitotoksisitesinin olmaması, yüksek antioksidan kapasitesi ve güçlü enzimatik kararma önleme aktivitesi; gıda endüstrisinde kullanılabilecek, sağlığa olumsuz yan etkileri olan gıda katkı maddelerinin yerine geçebilecek yeni, toksik olmayan, uygun maliyetli ve geri dönüştürülebilir bir kaynak olabileceğini düşündürmektedir.*

Anahtar Kelimeler: *Antioksidan, antiproliferatif, gıda katkı maddeleri, Helianthus annuus L., tirozinaz inhibisyonu*

INTRODUCTION

Food additives and colorants are important ingredients that ensure that the taste, appearance and quality of food remain unchanged throughout the entire process from preparation to consumption [1,2]. Food additives can be divided into different groups depending on their function, e.g. bleaching agents, sweeteners, antioxidants, preservatives, colorants and thickeners [3]. Tyrosinase is a key enzyme responsible for enzymatic browning and melanogenesis in mammals. It is found in many plants such as mushrooms, apples, bananas, potatoes and avocados as well as in shrimps. This metalloenzyme, which carries copper in its core, is responsible for the enzymatic browning in foods [4]. Enzymatic browning leads to color changes, softening, texture deterioration and taste changes in foods. This negatively affects the taste and appearance of food. Inhibitors targeting the tyrosinase enzyme are used as food additives to prevent enzymatic browning. As a result, foods such as vegetables, fruit and shrimp retain their fresh taste and appearance without browning [1].

Colorants are additives that are added to foods to correct the colors of foods that change during the production process or to make them look more attractive and bright. There are both natural colorants, which are obtained from animals, plants or minerals, and artificial colorants in synthetic form [5].

Synthetic food dyes are often used for coloring because of their low cost. However, many of them have toxic side effects with long-term use and cause numerous health problems such as anemia, asthma, eczema, urticaria, pathological lesions in various organs, cancer and mental retardation [6]. Tartrazine is a synthetic azo dye that is often used in the food industry to produce a bright yellow color. Its use in large quantities has negative effects on human health. Studies have reported negative effects such as increased oxidative stress, damage to cells, carcinogenic, mutagenic and reproductive toxicity [7].

Natural and synthetic antioxidants are used in the food industry to extend the shelf life of foods without them losing their appearance and nutritional value. Vitamins C, E and spices such as basil, rosemary, pepper and thyme are used as natural antioxidants. Butylated hydroxylanisole, butylated hydroxyltoluene and propyl gallate, which are synthetic antioxidants, are among the most commonly used and very effective food additives. However, various studies show that synthetic antioxidants in particular can lead to obesity, excessive sweating, asthma, stomach, eye and skin diseases with

prolonged use [8]. Rodents exposed to high levels of butylated hydroxyanisole (BHA) in the diet developed forestomach tumors classified by the IARC (International Agency for Research on Cancer) as Group 2B ("possibly carcinogenic to humans") [9]. Sulphites are synthetic food additives that are widely used in the food industry for their anti-browning, color-stabilizing, antimicrobial and antioxidant properties and are added to fruits and vegetables, seafood, some beverages and meat products. However, a high dietary intake of sulphites can cause various negative side effects such as allergic diseases and vitamin deficiencies and have a negative impact on the microbiota [10].

In view of all these negative side effects, it seems that synthetic food additives in particular are not welcome by consumers. Natural products, which are a good alternative to increase the safety, quality and attractiveness of food, can also be used to support physiological functions with their high nutritional and mineral content. This situation has shown that there is a need to replace synthetic compounds used as food additives with natural bioactive sources.

The sunflower (*Helianthus annuus* L.) is a plant of global importance, as it is consumed as food and animal feed. Although it is native to South America, the sunflower is cultivated worldwide as it adapts to different climates and soils. Its seeds are consumed as food due to their high nutritional value, used in the kitchen to produce cooking oil or offered to consumers as nuts [11].

In this study, the antioxidant, anti-browning and antiproliferative effects against healthy fibroblast cells of extracts obtained from different parts of 4 registered sunflower plants grown within the scope of the adaptation project were examined and their potential as an effective, low-cost, new food additive that can be used safely was determined.

MATERIAL AND METHOD

Plant Material

DERAY, P64 LP 130, TR 2242 CL and SY GRANIT genotypes used in the study were obtained from Edirne Trakya Agricultural Research Institute within the scope of the project "Determination of Adaptations of Oil and Snack Sunflower Genotypes Suitable for Eastern Anatolia Region" and tested at Erzurum Eastern Anatolia Agricultural Research Institute sites in 2023. The plant material was provided by the project leader, agricultural engineer, M.sc Aysema Tazegul Cavusoglu.

Preparation of Extracts from Different Parts of 4 Varieties of *Helianthus annuus L.*

H. annuus seeds, ray florets, disc florets, leaves, stems and receptacle were dried and extracted separately with MeOH three times at 40℃. The extracts were combined and evaporated under vacuum to obtain the main extracts. After freeze-drying, they were stored at 4 °C to be used for biological activity studies.

Antiproliferative Effect

To evaluate the effect of the extracts on the viability of the L929 cell line (mouse fibroblasts), cells were plated at a density of 1×10^5 cells per well and cultured for 24 hours under appropriate conditions in an incubator. Then the cells were incubated for 48 hours with extracts in the concentration range of 0-800 µg/ml. After incubation, 10 µl of MTT solution (5 mg/ml in PBS) was added and incubated for 4 hours. Formazan crystals were then dissolved in 100 µl DMSO and the absorbance values were measured at 570 nm using a microplate reader. The antiproliferative effect was expressed as percentage viability [12].

DPPH Radical Scavenging Effect

The DPPH radical scavenging effect was determined by spectroscopic evaluation of the color change of the methanolic 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution, which changed from purple to yellow [13]. The analyzes were repeated three times with gallic acid as standard. The results were expressed as gallic acid equivalents.

Trolox Equivalent Antioxidant Capacity (TEAC)

The ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] radical scavenging activity of

methanol extracts obtained from different parts of *H. annuus* was evaluated by spectrophotometric measurement of color change at 734 nm. The unit of total antioxidant activity was expressed as mg Trolox/g extract [14].

Copper Reducing Antioxidant Capacity (CUPRAC)

The antioxidant capacity, which reduces copper ions, was determined according to the method of Özyürek et al. The change in absorbance values was measured at 450 nm [15]. A standard curve was prepared with different concentrations of the standard compound Trolox. The unit of total antioxidant activity was expressed as mg Trolox/g extract.

Mushroom Tyrosinase Enzyme Inhibition Assay

The anti-browning effect of the extracts was determined by tyrosinase inhibition test. The method developed by Kim et al. was modified in some respects [16-18]. In this method, in which kojic acid was used as a standard compound (positive control), L-tyrosine was determined as a substrate and the dopachrome extinction was measured spectrophotometrically at a wavelength of 475 nm, which results from the reaction of substrate and enzyme.

Statistical Analysis

All experiments were performed in triplicate and data are presented as mean ± SD. Statistical analysis of the results was performed using one-way ANOVA (analysis of variance) followed by Duncan's test using SPSS-22 software, and $p < 0.001$ was considered significant.

RESULT AND DISCUSSION

Antiproliferative Effect Evaluation of the Extracts

The antiproliferative effect of methanol extracts from seeds, ray florets, disc florets, leaves, stems and receptacles of the 4 registered varieties *H. annuus* (DERAY, SY GRANIT, P64 LP 130, TR 2242 CL) on normal L929 fibroblast cells was investigated using the MTT method and the maximum non-toxic concentration range was determined. The extracts were applied in a concentration range of 0-800 µg/ml, and cell viability was close to 100% even at the highest concentration of 800 µg/ml. Receptacle methanol extract of TR 2242 CL methanol extract inhibited cell viability by 12.04% only at the highest concentration (800 µg/ml), but was found not to affect cell viability as the concentration decreased. Cell viability at the highest concentration of methanol extracts from the DERAY and SY GRANIT were 93%. Leaf methanol extracts were found to reduce cell viability at 800 µg/ml in all varieties. However, the cytotoxic effect was found to disappear 400 µg/ml (Figure 1-4).

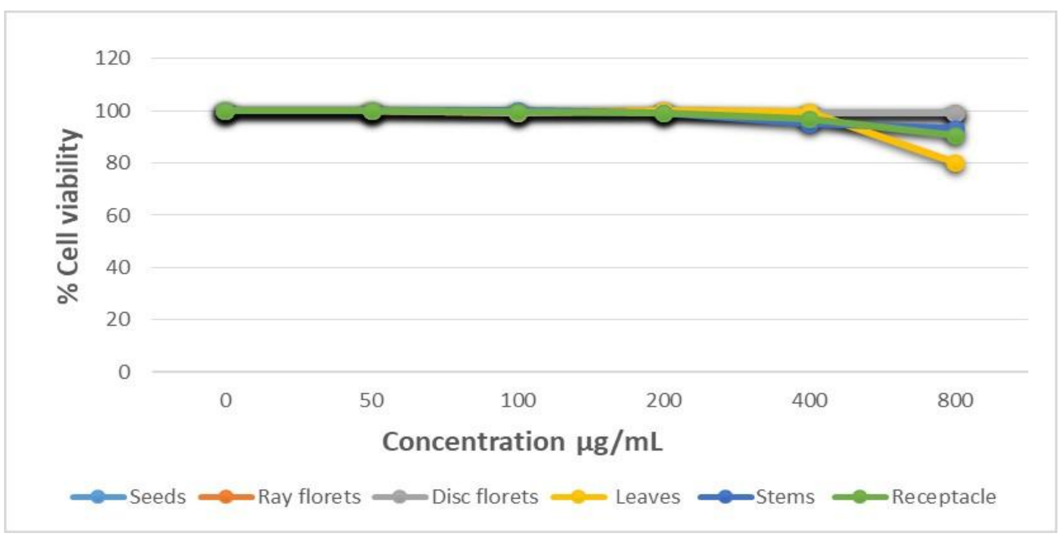

Figure 1. Antiproliferative effect of the DERAY extracts against L929

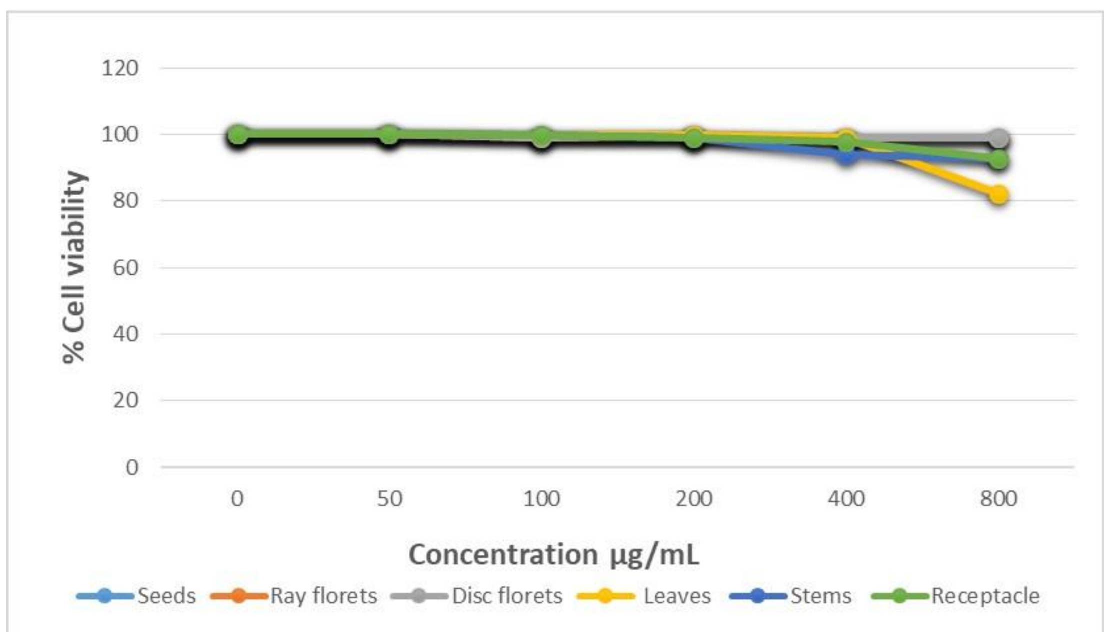

Figure 2. Antiproliferative effect of the SY GRANIT extracts against L929

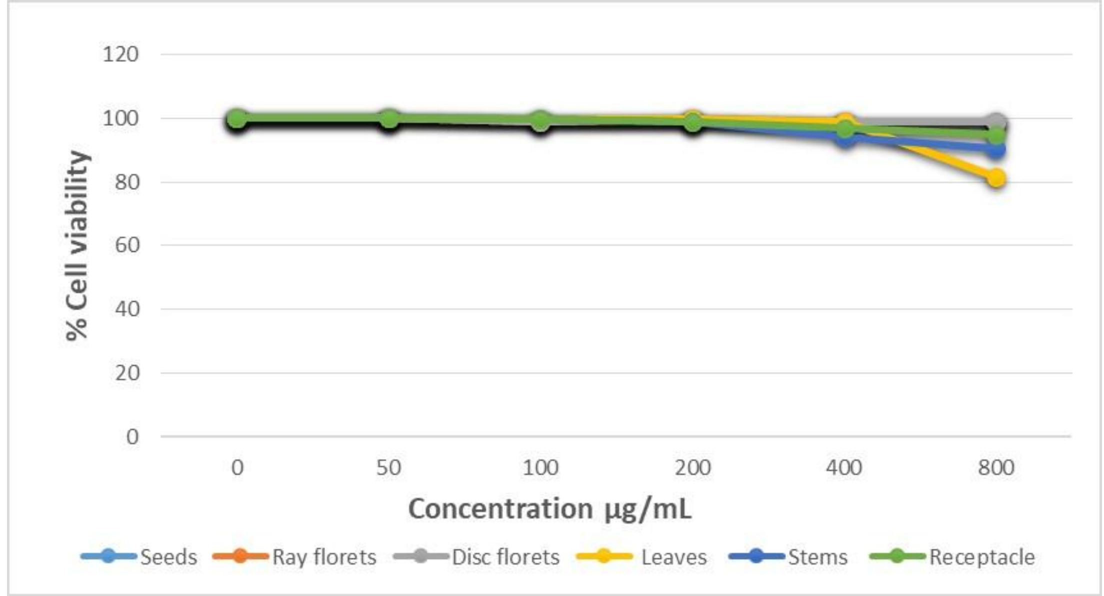

Figure 3. Antiproliferative effect of the P64 LP 130 extracts against L929

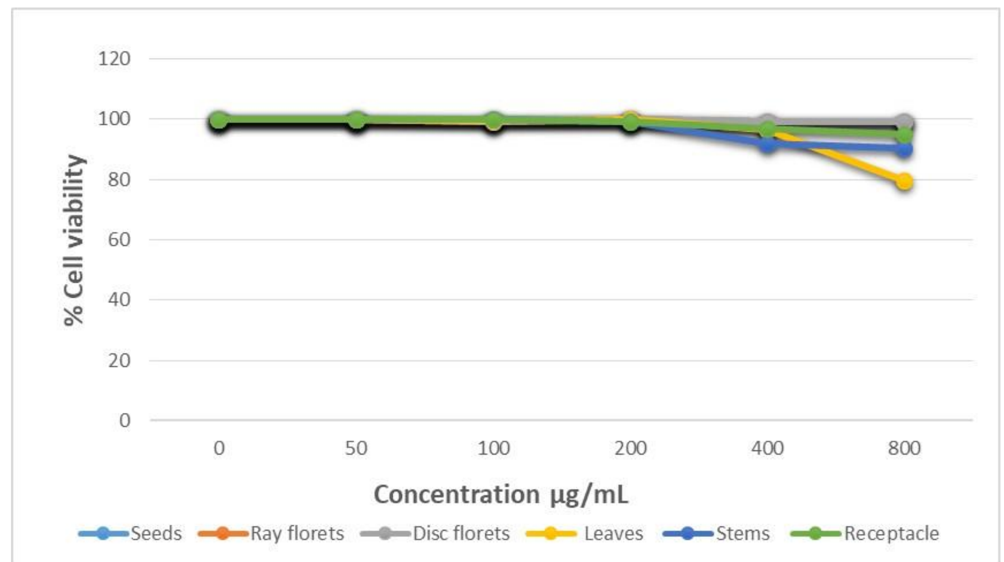

Figure 4. Antiproliferative effect of the TR 2242 CL extracts against L929

Antioxidant Capacity of the Extracts

The antioxidant capacity of methanol extracts of 4 different *H. annuus* varieties was determined by DPPH, TEAC and CUPRAC analyses. While all three types of methods, disc floret and ray floret, were found to be the most effective, the weakest effect was seen in stem methanol extracts (Table 1-4). In the radical scavenging effect of DPPH, where gallic acid was used as the standard compound, ray floret of the TR 2242 CL variety showed the highest effect with a value of 101.40 mg gallic acid/g extract. DERAY disc floret with 98.12 mg trolox/g extract value in the CUPRAC method and SY GRANIT disc floret with 83.80 mg trolox/g extract value in the TEAC method were found to be the most effective extracts.

Table 1. Antioxidant capacity of DERAY extracts

Plant Part	DPPH[a]	CUPRAC[b]	TEAC[b]
Seeds	48.32 ± 0.94	62.29 ± 1.84	53.57 ± 1.46
Ray floret	98.16 ± 0.90	86.24 ± 1.24	79.60 ± 1.82
Disc floret	94.24 ± 1.84	98.12 ± 1.28	73.22 ± 2.34
Leaves	58.14 ± 2.40	60.25 ± 1.84	69.46 ± 1.42
Stems	13.42 ± 0.62	19.18 ± 0.64	19.68 ± 0.84
Receptacle	24.32 ± 0.99	20.55 ± 0.94	26.56 ± 1.02

Data are presented as mean ± SD, n=3 experiments, ($p < 0.001$)
a: mg gallic acid/g extract, b: mg trolox/g extract

Table 2. Antioxidant capacity of SY GRANIT extracts

Plant Part	DPPH[a]	CUPRAC[b]	TEAC[b]
Seeds	58.122 ± 1.06	84.15 ± 2.01	69.05 ± 1.26
Ray floret	99.14 ± 1.96	77.26 ± 1.68	75.43 ± 1.22
Disc floret	92.26 ± 1.44	78.80 ± 1.28	83.80 ± 1.64
Leaves	60.18 ± 1.80	63.49 ± 1.84	59.05 ± 1.42
Stems	14.12 ± 0.84	10.17 ± 0.94	14.07 ± 0.94
Receptacle	28.12 ± 0.98	24.81 ± 0.74	24.75 ± 1.22

Data are presented as mean ± SD, n=3 experiments, ($p < 0.001$)
a: mg gallic acid/g extract, b: mg trolox/g extract

Table 3. Antioxidant capacity of P64 LP 130 extracts

Plant Part	DPPH[a]	CUPRAC[b]	TEAC[b]
Seeds	62.02 ± 1.46	63.81 ± 2.01	53.71 ± 1.64
Ray floret	98.20 ± 2.24	66.58 ± 1.88	70.01 ± 1.42
Disc floret	99.88 ± 1.04	68.88 ± 2.08	78.28 ± 1.84
Leaves	54.28 ± 1.84	47.02 ± 1.14	59.14 ± 1.02
Stems	11.08 ± 0.94	9.07 ± 0.84	12.07 ± 0.98
Receptacle	30.82 ± 0.98	32.04 ± 0.94	31.99 ± 1.20

Data are presented as mean ± SD, n=3 experiments, ($p < 0.001$)
a: mg gallic acid/g extract**,** b: mg trolox/g extract

Table 4. Antioxidant capacity of TR 2242 CL extracts

Plant Part	DPPH[a]	CUPRAC[b]	TEAC[b]
Seeds	60.32 ± 1.86	44.66 ± 1.81	46.66 ± 1.24
Ray floret	101.40 ± 2.64	76.72 ± 1.08	70.01 ± 2.42
Disc floret	99.08 ± 1.08	78.73 ± 2.68	75.61 ± 1.64
Leaves	58.24 ± 1.64	36.70 ± 1.40	53.71 ± 2.02
Stems	14.28 ± 0.96	11.90 ± 0.88	15.69 ± 1.02
Receptacle	34.84 ± 1.02	30.81 ± 0.98	31.81 ± 1.20

Data are presented as mean ± SD, n=3 experiments, ($p < 0.001$)
a: mg gallic acid/g extract, b: mg trolox/g extract

Mushroom Tyrosinase Inhibition Activity

By studying the inhibition of the enzyme tyrosinase, which is responsible for the enzymatic browning of food, the potential of the extracts for use as food additives was determined. The results were calculated using the standard compound kojic acid and are given as mg KAE/g extract. The highest activity to inhibit the mushroom tyrosinase enzyme was observed in methanol extracts of disc floret, followed by ray floret. While no activity was observed in the leaf extracts of all varieties, low inhibition was observed only in SY GRANIT and P64 LP 130 in the stem extracts (Table 5).

Table 5. Mushroom tyrosinase inhibition of *H. annuus* extracts (mg KAE/g extract)

Varieties	Ray floret	Leaves	Stems	Disc floret	Seeds	Receptacle
DERAY	60.01 ± 0.98	na	na	125.31 ± 1.44	55.70 ± 0.82	30.61 ± 1.68
SY GRANIT	55.48 ± 1.42	na	8.57 ± 0.84	106.12 ± 1.86	49.59 ± 1.06	41.02 ± 1.24
P64 LP 130	50.43 ± 0.88	na	5.46 ± 0.86	147.23 ± 1.64	46.53 ± 0.98	40.06 ± 1.06
TR 2242 CL	60.42 ± 0.98	na	na	151.25 ± 1.20	30.68 ± 1.04	34.32 ± 0.86

* Data are are means±S.D. of three parallel measurements ($p < 0.05$). KAE, kojic acid equivalents. na: not active

The growing population and changing lifestyles are leading to significant changes in the composition of food. In addition, changing eating habits have led to a significant change in the demand for food. The food industry is striving to increase the shelf life and quality of food by using additional food additives and preservatives [3,19].

In the food industry many compounds used as additives to extend the taste, appearance and shelf life of food have undesirable side effects on health. All these negative effects show that agricultural food by-products, which are renewable raw materials, can be one of the possible solutions.

The sunflower is one of the oilseeds that play an important role worldwide as a source of first-class oil and fiber, which are of great benefit to human health. The products of the sunflower (*H. annuus*), a plant cultivated worldwide, are mostly consumed as food in the kitchen or marketed as animal feed. Thanks to the sunflower's adaptation to different climatic and soil conditions, its importance as an oilseed crop has increased worldwide [11].

In Türkiye, about half of the vegetable oil demand is covered by sunflowers. Some of the main reasons why production is so high are its drought and cold tolerance compared to other oil-producing crops, its good adaptability to all soil types and its adaptability to different ecosystems [20].

In this study, the antioxidant, antityrosinase and antiproliferative effects of 4 different sunflower genotypes were investigated and the use of different plant parts as potential food additives and the seeds used as food were tested. Since both safe use and efficacy were sought, the cytotoxic effects of 24 different extracts on L929 healthy fibroblast cells were investigated. The antiproliferative activity of the extracts at different concentrations (0-800 µg/ml) was investigated, and the viability of all extracts was calculated to be over 90% at a concentration of 400 µg/ml.

The antioxidant effect was tested using 3 different methods. Disc floret and ray floret extracts showed the highest activity in all methods. Compared to other varieties in ray floret of TR 2242 CL cultuvar showed the highest antioxidant capacity with 101.40 mg gallic acid/g extract.

Enzymatic browning, one of the biggest problems in the food industry, was tested with the inhibition of the mushroom tyrosinase enzyme. The results were parallel to the results of the determination of antioxidant capacity. The TR 2242 CL varieties, which has the highest antioxidant effect, was also found to have the highest enzyme inhibition (151.25 mg KAE/g extract).

In a study by Özcan et al., the antioxidant effect of DERAY seeds was examined using the DPPH method and a value of 1.73 mmol Trolox (TE)/kg extract was determined [21].The antioxidant activity of various extracts from the disc and ray florets of *H. annuus* was investigated by a few methods, including the free radical scavenging effects of DPPH and ABTS. It was found that 90% (v/v) aqueous methanol floret extracts had the highest phenolic content and antioxidant capacity [22]. In a study comparing the antioxidant and enzyme-inhibiting activities of oils from the seeds of seven new sunflower (*Helianthus annuus* L.) lines, only the seed oils of the APO41, APO42, APO43 and BOH3 lines showed tyrosinase enzyme inhibition [23]. In the seed methanol extracts of the same lines, all lines showed a significant inhibition of the tyrosinase enzyme in the range of 52.94–60.43 mg kojic acid equivalent/g and considerable antioxidant activity. The antioxidant capacity of the methanol extracts of the seeds of the lines ranged between 22.60-40.42 mg TE/g in the DPPH method, 19.46-31.90 mg TE/g in the ABTS method and 59.98-117.86 mg TE/g in the CUPRAC method. [24]. Our antioxidant capacity results were similar to the previous studies and the tyrosinase enzyme inhibition of the seeds was found to be in the range of 30.68-55.7 mg kojic acid equivalent/g extract.

In a study by Mutiah et al. the antiproliferative effect of sunflower leaf, stem and root extracts against HeLa cells was determined using the MTT method. While the IC_{50} value of the root and stem extracts was >1000 µg/ml, it was found to be 153.76 µg/ml and 126.6 µg/ml for the seeds and leaves, respectively [25]. In another study, the antiproliferative effect of 3 different meal extracts obtained from sunflower seeds on L929 cells in the concentration range of 6.25-100 mg/ml was investigated and it was found that they did not damage healthy cells in this concentration range. The ABTS radical scavenging activity was 709.48-736.40 mg Trolox/100 g and the DPPH radical scavenging activity was 1320.12-1597.60 mg Trolox/100 g [26]. In our results, the antiproliferative effect against L929 cells in the range of 50-800 µg/ml was investigated, and similar to the studies of Adascălului et al., it was found that the healthy viability of the cells was continued almost without damage.

Sunflower is cultivated worldwide to meet nutritional, medicinal and industrial needs. The seeds, which play an important role in the food industry and nutrition, are rich in antioxidants, proteins, vitamins and trace elements and are used for oil extraction. Usually, only the seeds of the plant are in the foreground, while other parts are considered as agricultural by-products. In our study, the antioxidant, the antibrowning and the antiproliferative effect were carried out for the first time on six different parts of sunflowers belonging to four genotypes and the potential of the plant parts as food additives was investigated. Thus, by-products were obtained that may contain value-added compounds with high functionality and/or bioactivity for the agriculture- food industry.

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AUTHOR CONTRIBUTIONS

Concept: Z.B.G., A.T.Ç.; Design: Z.B.G.; Control: Z.B.G.; Sources: Z.B.G., A.Ç.T.; Materials: Z.B.G., A.Ç.T.; Data Collection and/or Processing: Z.B.G.; Analysis and/or Interpretation: Z.B.G.; Literature Review: Z.B.G., A.T.Ç.; Manuscript Writing: Z.B.G.; Critical Review: Z.B.G., A.T.Ç.; 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.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITY OF *PHOLIOTA AURIVELLA* (BATSCH) P. KUMM.

PHOLIOTA AURIVELLA (BATSCH) P. KUMM.'NİN KİMYASAL BİLEŞİMİ VE BİYOLOJİK AKTİVİTELERİ

Cenker YAMAN[1]*, Gizem GÜL[1], Atakan BENEK[4], Dilay TURU[1], Selime Deniz BOZKURT[1], Ilgaz AKATA[3], Kerem CANLI[2,4]

[1]Dokuz Eylül University, Graduate School of Natural and Applied Sciences, Departmant of Biology, 35390, İzmir, Türkiye
[2]Dokuz Eylül University, Faculty of Science, Departmant of Biology, 35390, İzmir, Türkiye
[3]Ankara University, Faculty of Science, Departmant of Biology, 06560 Ankara, Türkiye
[4]Dokuz Eylül University, Research and Application Center of Fauna and Flora (FAMER), 35390, İzmir, Türkiye

ABSTRACT

Objective: *This study aims to determine the chemical composition of the mushroom Pholiota aurivella by investigating its antimicrobial, antibiofilm, and antioxidant activities. The potential effects of the ethanol extract of the mushroom on a total of 27 different microorganisms, including strains with multidrug resistance (MDR), were examined. Additionally, the biofilm inhibition capacity and free radical scavenging activities of the mushroom extract were tested.*
Material and Method: *The ethanol extract obtained from the P. aurivella sample was subjected to various tests to evaluate its biological activities. Antimicrobial activity was analyzed using disk diffusion and Minimum Inhibitory Concentration (MIC) tests. Antibiofilm activity was assessed based on its capacity to inhibit biofilm formation, while antioxidant activity was tested using the DPPH method. The chemical composition was determined by Gas Chromatography-Mass Spectrometry (GC-MS) analysis.*
Result and Discussion: *Our study reveals that P. aurivella is a promising natural agent, especially in terms of its antimicrobial and antibiofilm activities. The extract exhibited antimicrobial activity against 16 different Gram-positive and Gram-negative microorganisms. Expanding zones of inhibition were observed with increasing extract amounts in strains such as Klebsiella pneumoniae and Enterobacter aerogenes, which show multidrug resistance (MDR). In biofilm inhibition tests, the strongest effects were observed in strains of Listeria innocua and Bacillus subtilis DSMZ 1971. On the other hand, the antioxidant activity tested by the DPPH method was quite low; this can be explained by the absence of phenolic compounds in the chemical composition of the extract. GC-MS analysis identified the major components as linoleic acid (59.20%) and ethyl linoleate (17.13%). These findings indicate that P. aurivella, with its antibiofilm and antimicrobial properties, has potential for pharmaceutical applications and may offer an important natural resource for developing treatment options even against MDR pathogens.*
Keywords: *Antibiofilm activity, antimicrobial activities, antioxidant activity, GC-MS, Pholiota aurivella*

* **Corresponding Author / Sorumlu Yazar:** Cenker Yaman
e-mail / e-posta: cenkeryaman@gmail.com, **Phone / Tel.:** +905419592277

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ÖZ

Amaç: *Bu çalışma, Pholiota aurivella mantarının antimikrobiyal, antibiyofilm ve antioksidan aktivitelerini araştırarak kimyasal kompozisyonunu belirlemeyi amaçlamaktadır. Çalışmada, mantarın etanol ekstraktının, çoklu ilaç direncine sahip suşlar da dahil olmak üzere toplam 27 farklı mikroorganizma üzerindeki potansiyel etkisi incelenmiştir. Ayrıca, mantar ekstraktının biyofilm inhibisyon kapasitesi ve serbest radikal süpürme aktiviteleri test edilmiştir.*
Gereç ve Yöntem: *P. aurivella örneğinden etanol ile elde edilen ekstrakt, biyolojik aktivitelerini değerlendirmek amacıyla çeşitli testlere tabi tutulmuştur. Antimikrobiyal etkinlik, disk difüzyon ve Minimum İnhibitör Konsantrasyon (MİK) testleri ile analiz edilmiştir. Antibiyofilm aktivitesi, biyofilm oluşumunu inhibe etme kapasitesiyle değerlendirilirken, antioksidan aktivite DPPH yöntemi ile test edilmiştir. Kimyasal kompozisyon ise Gaz Kromatografisi-Kütle Spektrometrisi (GC-MS) analiziyle belirlenmiştir.*
Sonuç ve Tartışma: *Çalışmamız, P. aurivella'nın özellikle antimikrobiyal ve antibiyofilm aktiviteleri açısından umut verici bir doğal ajan olduğunu ortaya koymaktadır. Ekstrakt, 16 farklı Gram-pozitif ve Gram-negatif mikroorganizmaya karşı antimikrobiyal etkinlik gösterirken, çoklu ilaç direnci (MDR) gösteren Klebsiella pneumoniae ve Enterobacter aerogenes gibi suşlarda da artan ekstrakt miktarı ile genişleyen inhibisyon alanları gözlemlenmiştir. Biyofilm inhibisyonu testlerinde de en güçlü etkiler, Listeria innocua ve Bacillus subtilis DSMZ 1971 suşlarında gözlemlenmiştir. Öte yandan, DPPH yöntemiyle test edilen antioksidan aktivite oldukça düşüktür; bu durum, ekstraktın kimyasal bileşiminde fenolik bileşiklerin bulunmamasıyla açıklanabilir. GC-MS analizinde, majör bileşenlerin linoleik asit (%59.20) ve etil linoleat (%17.13) olduğu tespit edilmiştir. Bu bulgular, P. aurivella'nın antibiyofilm ve antimikrobiyal özellikleriyle farmasötik uygulamalar açısından potansiyele sahip olduğunu ve MDR patojenlere karşı dahi tedavi seçenekleri geliştirilmesinde önemli bir doğal kaynak sunabileceğini göstermektedir.*

Anahtar Kelimeler: *Antibyiofilm aktivite, antimikrobiyal aktivite, antioksidan aktivite, GC-MS, Pholiota aurivella*

INTRODUCTION

The search for antimicrobial agents began with Alexander Fleming's discovery of penicillin in 1928, and the need for new antibiotics - and consequently, research in this area - has continued to grow exponentially. Despite the leaps made in medical science over the past decades, infectious diseases remain an uncontainable problem. The indiscriminate use of antibiotics has led bacteria to develop resistance to existing drugs, strengthening their resistance mechanisms. For example, before the antibiotic era, the mortality rate of infections caused by *Staphylococcus aureus* was around 80% [1]. With the introduction of penicillin into our lives in the early 1940s, patients' chances of recovery increased significantly; however, as early as 1942, penicillin-resistant staphylococci began to appear first in hospitals and then in the community [2]. By the late 1960s, 80% of staphylococcal strains obtained from both hospitals and the community were resistant to penicillin [3]. This rapid resistance pattern, which first emerged in hospitals and then spread to the community, is not limited to penicillin. Almost every antibiotic faces this model of resistance. Unfortunately, unless our habits regarding antibiotic use change, new drugs will meet the same fate as the existing ones [4]. A study reports that 700,000 people die annually due to antimicrobial resistance, and this number is projected to exceed 10 million by 2050 [5].

An examination of all drugs produced between 1981 and 2010 shows that 118 new antibacterial drugs were developed, and 77 of them were derived from natural compounds [6]. There are many reasons why naturally obtained compounds are used more frequently and effectively in antimicrobial studies. Firstly, the compounds used are isolated from organisms that have survived to the present day by exhibiting resistance to pathogens through natural selection. In other words, they are products of existing resistance mechanisms. Additionally, because they exhibit lower toxicity, they offer a safer route with fewer side effects compared to synthetic antibiotics.

Mushrooms have been part of the human diet since ancient times, both as a nutritious food and for medicinal purposes [7,8]. Examples of their medical use include their effects against diseases such as hypertension, hypercholesterolemia, and cancer [9]. It has been shown that a large portion of

mushrooms possess many important bioactivities, including antioxidant, antiviral, anti-angiogenic, anti-tumor, anti-inflammatory, anti-obesity, and immunomodulatory properties [10]. Furthermore, today mushrooms are used in various fields such as pharmacology, the textile industry, wastewater purification for ecosystem recovery, and the manufacture of cosmetic and hygiene products [11-14].

In this study, *Pholiota aurivella* (Batsch) P. Kumm., one of the mushrooms that have been part of the human diet for centuries, was selected. *P. aurivella* typically grows in clusters on live tree trunks during the summer and autumn months [15]. This mushroom, which has a sticky texture, golden yellow or orange color, and a spicy odor, has been reported in the literature as both edible and inedible [16,17].

Studies on *P. aurivella*, the subject of our research, are limited, and the disk diffusion, minimum inhibitory concentration (MIC), and antibiofilm tests conducted using its ethanol extract are pioneering. In this study, the antimicrobial activity of the mushroom was investigated using disk diffusion and MIC methods, along with its antibiofilm and antioxidant activities. Its chemical composition was determined through Gas Chromatography-Mass Spectrometry (GC-MS) analysis. This allowed us to gain insights into which compounds might play a role in the observed biological activities.

MATERIAL AND METHOD

Macrofungus Sample

P. aurivella specimens were collected from the Yomra region of Trabzon by Prof. Dr. Ilgaz Akata and, after being completely dried, were preserved under the herbarium code MA0015 at the Fauna and Flora Research and Application Center (FAMER) of Dokuz Eylül University.

Extraction Procedure

The extraction procedure was performed as described by Canlı et al. [18]. An ethanol extract containing 0.495 g of material in 15 ml was prepared for use in disk diffusion testing and GC-MS analysis. For the antioxidant test, the ethanol extract was diluted at a ratio of 1 mg/ml. For antibiofilm and MIC tests, the ethanol was removed from the extract, and the resulting residue was dissolved in 1% DMSO (dimethyl sulfoxide) to prepare a water-based extract.

Microorganisms

In this study, a total of 27 strains were analyzed, including 7 Food Isolates (FI), 12 Standard Isolates (ST), one of which is a yeast, 1 Clinical Isolate (CI), and 7 Multi-Drug Resistant (MDR) strains. The microorganisms were obtained from the microbiology laboratory of the Department of Biology, Faculty of Science, Dokuz Eylül University (Table1-4).

Preparation of Inocula

The bacteria were enriched by incubation at 37°C for 24 hours, while the yeast was incubated at 28°C for 48 hours. Subsequently, to standardize inoculum concentrations, each bacterial and yeast sample was adjusted to 0.5 McFarland standard using sterile 0.9% NaCl solution, corresponding to approximately 10^8 cfu/ml for bacteria and 10^7 cfu/ml for *Candida albicans*. The experiments were conducted with these standardized microorganism samples [19,20].

Disc Diffusion Method

The antimicrobial activity of the ethanol extract of *P. aurivella* was evaluated using the disk diffusion method as described by Andrews [20]. Three different volumes of the extract (50 µl, 100 µl, and 200 µl) were loaded onto 6 mm antimicrobial susceptibility test disks. Instead of applying the entire volume at once, these volumes were loaded incrementally in 10 µl steps. For the higher volumes, the disks were allowed to dry between applications to ensure complete evaporation of ethanol. The disks were left to dry overnight to evaporate the remaining ethanol, resulting in disks containing 2.88 mg, 5.77 mg, and 11.54 mg of the extract, respectively. Subsequently, pre-prepared microorganisms suspended in sterile saline solution were inoculated onto petri dish surfaces to ensure uniform coverage. Following inoculation, extract-loaded disks were placed on the agar surface, and the plates were

incubated. The diameters of the inhibition zones formed were measured in millimeters (mm) using a caliper and recorded.

Sterile blank disks and the extraction solvent (ethanol) were used as negative controls, while Gentamicin and Tobramycin antibiotic disks served as positive controls. All tests were conducted in triplicate, and the results are presented as means with standard errors [21].

Minimum Inhibitory Concentration Test

MIC is defined as the lowest extract concentration that inhibits visible bacterial growth. For the MIC test, the serial dilution method described by Baldas and Altuner [22] was applied, resulting in a concentration range of 0.055–7.067 mg/ml. For bacterial growth controls, wells without extract were used as positive controls, and wells without bacteria were used as negative controls. All tests were performed in triplicate.

Antibiofilm Activity

In this study, the antibiofilm test was adapted from [23]. The procedure consisted of two parts: determining biofilm formation conditions and evaluating antibiofilm activity.

Five strains were used in the study: *Bacillus subtilis* DSMZ 1971, *Listeria monocytogenes* ATCC 7644, *Escherichia coli* ATCC 25922, *Listeria innocua* (FI), and *Escherichia coli* (CI). The strains, adjusted to 0.5 McFarland standard, were transferred into microplates containing different concentrations of glucose monohydrate and incubated at 37°C for 24 and 48 hours. After incubation, the microplates were washed, stained with crystal violet, and allowed to sit. The biofilm stained with crystal violet was dissolved using an ethanol-acetone solution, and absorbance was measured at 550 nm using a microplate reader. The results showed that the optimal biofilm formation conditions for all tested strains were determined to be 48 hours of incubation with 1.5% glucose monohydrate concentration.

The *P. aurivella* DMSO-water extract, whose antibiofilm activity was to be assessed, was loaded into the microplates at a concentration range of 0.055–7.067 mg/ml and incubated under the previously determined optimal conditions. Negative controls, consisting of wells containing both extract and broth without microorganisms, were included to account for any background absorbance. Positive controls, consisting of wells containing both microorganisms and broth without extract, were used to ensure that biofilm formation occurred in the absence of the extract. Following incubation, the same procedures described in the biofilm formation stage were applied, and antibiofilm activities were determined by measuring absorbance at 550 nm with a microplate reader.

GC-MC Analysis

The analyses were performed with modifications based on the study by Benek et al. [24]. The experiments were conducted using an Agilent GC 8890-Agilent GC/MSD 5977B (Agilent Technologies Inc., Santa Clara, CA, USA) device equipped with an HP5-MS capillary column (30 m x 0.25 mm; film thickness 0.25 µm).

Analytical conditions were as follows: the injector temperature was set to 350°C, with helium used as the carrier gas at a flow rate of 1 ml/min. The injection mode was split, with a split ratio of 10:1, and the injection volume was 1 µl of the ethanol extract. The oven temperature was programmed to increase from 40°C to 350°C at a rate of 4°C/min, with a 10-minute hold at 350°C. MS scan conditions were set with a transfer line temperature of 280°C, interface temperature of 280°C, and ion source temperature of 230°C. Component identification was achieved by matching retention times with the Wiley-NIST MS libraries.

Antioxidant Activity

The extract's antioxidant activity was measured by observing its scavenging effects on the DPPH radical. The DPPH test was performed as described by Turu et al. [25]. The samples' absorbance was measured at 515 nm using a spectrophotometer. All tests were conducted in triplicate, with ascorbic acid prepared in ethanol at a concentration of 1 mg/ml used as the control.

Statistics

All tests were conducted in triplicate. One-way analysis of variance (ANOVA), a parametric method, was performed with a significance level of P = 0.05. The Pearson correlation coefficient was calculated to evaluate any potential correlation between antimicrobial activity intensity and concentration. Statistical analyses were performed using R Studio, version 2024.09.1 [26].

For antibiofilm results, the mean inhibition values were reported with ± SD based on nine replicates. The results obtained from each antioxidant assay were reported as mean ± SD, based on three independent experimental replicates. EC_{50} values were calculated and expressed as 95% confidence intervals using Four-Parameter Logistic Regression.

RESULT AND DISCUSSION

Antimicrobial Activity

The diameters of the inhibition zones were measured in millimeters, and the results were presented in Table 1, 2, 3 and 4 alongside the positive controls, gentamicin and tobramycin. No growth was observed on the negative control discs.

Table 1. The antimicrobial activity of *P. aurivella* on standard microorganisms (Inhibition zones in mm)

Standard Isolated Microorganisms	50 µl	100 µl	200 µl	Gentamicin (10 µg)	Tobramycin (10 µg)
***Bacillus subtilis* DSMZ 1971**	8.00 ± 0.00	9.00 ± 0.00	9.33 ± 0.57	30	26
***Candida albicans* DSMZ 1386**	-	-	-	12	13
***Enterobacter aerogenes* ATCC 13048**	-	-	-	24	18
***Enterococcus faecalis* ATCC 29212**	-	7.00 ± 0.00	7.33 ± 0.57	12	8
***Escherichia coli* ATCC 25922**	-	-	-	22	20
***Listeria monocytogenes* ATCC 7644**	7.00 ± 0.00	9.00 ± 0.00	10.00 ± 0.00	28	24
***Pseudomonas aeruginosa* DSMZ 5071**	-	-	7.00 ± 0.00	15	22
***Pseudomonas fluorescens* P1**	-	-	-	13	12
***Salmonella enteritidis* ATCC 13076**	-	-	7.00 ± 0.00	21	-
***Salmonella typhimurium* SL 1344**	-	-	-	24	15
***Staphylococcus aureus* ATCC 25923**	7.00 ± 0.00	8.00 ± 0.00	9.00 ± 0.00	21	14
***Staphylococcus epidermidis* DSMZ 20044**	8.00 ± 0.00	9.66 ± 0.57	11.00 ± 0.00	22	20

* Strains exhibiting no antimicrobial activity were indicated with a "–"

The only study investigating the antimicrobial activity of *P. aurivella* was conducted by Dyakov et al. (2011) [27], in which the antibiotic biosynthesis capacity of cultured fungi against various pathogens present in the same medium was examined. The study reported that *P. aurivella* did not

exhibit any antimicrobial activity against any strain. In the same study, other species such as *P. squarrosa* and *P. lenta* were also examined, and only *P. lenta* was observed to possess antibiotic biosynthesis capability.

Table 2. The antimicrobial activity of *P. aurivella* on food isolated microorganisms (Inhibition zones in mm)

Food Isolated Microorganisms	50 µl	100 µl	200 µl	Gentamicin (10 µg)	Tobramycin (10 µg)
Enterococcus durans	-	8.00 ± 0.00	8.33 ± 0.57	11	13
Enterococcus faecium	9.00 ± 0.00	10.00 ± 0.00	11.00 ± 0.00	28	15
Klebsiella pneumoniae	7.00 ± 0.00	7.33 ± 0.57	9.00 ± 0.00	19	23
Listeria innocua	8.00 ± 0.00	8.00 ± 0.00	9.00 ± 0.00	13	15
Salmonella infantis	-	-	-	17	14
Salmonella kentucky	-	-	7.00 ± 0.00	12	16
Escherichia coli	-	-	7.00 ± 0.00	20	-

* Strains exhibiting no antimicrobial activity were indicated with a "–"

Table 3. The antimicrobial activity of *P. aurivella* on clinically ısolated microorganism (Inhibition zones in mm)

Clinically Isolated Microorganism	50 µl	100 µl	200 µl	Gentamicin (10 µg)	Tobramycin (10 µg)
Staphylococcus aureus	8.00 ± 0.00	9.00 ± 0.00	10.00 ± 0.00	22	18

* Strains exhibiting no antimicrobial activity were indicated with a "–"

Table 4. The antimicrobial activity of *P. aurivella* harboring multi-drug resistance microorganisms (Inhibition zones in mm)

Multi-Drug Resistance Microorganisms	50 µl	100 µl	200 µl	Gentamicin (10 µg)	Tobramycin (10 µg)
Escherichia coli	-	-	-	8	9
Klebsiella pneumoniae	7.00 ± 0.00	8.00 ± 0.00	9.00 ± 0.00	15	20
Acinetobacter baumannii	-	-	-	-	-
Enterobacter aerogenes	8.66 ± 0.57	9.00 ± 0.00	10.00 ± 0.00	16	18
Serratia odorifera	-	-	-	7	9
Streptococcus pneumoniae	-	-	-	10	8
Proteus vulgaris	-	-	-	11	11

* Strains exhibiting no antimicrobial activity were indicated with a "–"

Although different methods were used in the studies, a common finding was that no effect was observed for any *Pholiota*species, including *P. aurivella*, against *E. coli* ATCC 25922. In this regard, the two studies are consistent. However, while Dyakov et al. found no activity against numerous strains, our study clearly demonstrates that *P. aurivella* acquired antimicrobial activity against a total of 16 different strains, including multidrug-resistant ones, after undergoing the maceration process.

The observation of zone formation in all *Staphylococcus* strains studied is a noteworthy finding. In 2019, *S. aureus*, along with *E. coli*, *S. pneumoniae*, *K. pneumoniae*, and *P. aeruginosa*, accounted for 30.9% of the 7.7 million deaths related to infections, making it the leading bacterial cause of death in 135 countries. That same year, *S. aureus* was associated with 1.105.000 deaths, while methicillin-resistant strains (MRSA) were the most common cause of antimicrobial-resistant infection-related deaths in 27 countries [28]. Such data highlight the significant threat posed by staphylococci. The

effectiveness of *P. aurivella* against the *Staphylococcus* strains used in this study also offers promising potential for the treatment of other *Staphylococcus* strains.

As observed with many other strains, the inhibition zones for multidrug-resistant *K. pneumoniae* and *E. aerogenes* were found to expand with increasing extract concentrations. This suggests that higher extract quantities may yield greater efficacy against these pathogens. Considering the significant burden these pathogens, which do not respond to conventional antibiotic therapies, place on the healthcare sector, the importance of these findings becomes even more evident.

Table 5. Minimum inhibitory concentrations (MIC)

Microorganisms	MIC
***Listeria innocua* (FI)**	0.442 mg/ml
***Listeria monocytogenes* ATCC 7644**	1.767 mg/ml
***Enteroccus faecalis* ATCC 29212**	1.767 mg/ml
***Staphylococcus aureus* ATCC 25923**	3.533 mg/ml
***Staphylococcus aureus* (CI)**	3.533 mg/ml
***Escherichia coli* (FI)**	3.533 mg/ml
***Enterococcus durans* (FI)**	7.067 mg/ml
***Enterococcus faecium* (FI)**	>7.067 mg/ml
***Klebsiella pneumoniae* (FI)**	>7.067 mg/ml
***Salmonella kentucky* (FI)**	>7.067 mg/ml
***Pseudomonas aeruginosa* DSMZ 5071**	>7.067 mg/ml
***Salmonella enteritidis* ATCC13076**	>7.067 mg/ml
***Bacillus subtilis* DSMZ 1971**	>7.067 mg/ml
***Staphylococcus epidermidis* DSMZ 20044**	>7.067 mg/ml
***Enterobacter aerogenes* (MDR)**	>7.067 mg/ml
***Klebsiella pneumonnii* (MDR)**	>7.067 mg/ml

For *P. aurivella*, the MIC study was conducted for the first time, and the minimum inhibitory concentrations for seven strains were determined. Among these, the lowest inhibitory concentration was measured at 0.442 mg/ml for *L. innocua*. Conversely, the strain requiring the highest extract concentration for inhibition was *E. durans*, with a value 7.067 mg/ml (Table 5).

Of the seven strains yielding results in MIC tests, six were gram-positive, clearly indicating that the extract is more effective against gram-positive bacteria compared to gram-negative ones. The thick peptidoglycan layer of gram-positive bacteria allows hydrophobic fatty acids and esters in the extract to easily penetrate the cell wall. In contrast, the outer membrane of gram-negative bacteria, containing a lipopolysaccharide (LPS) layer, restricts the passage of hydrophobic molecules, making them more resistant [29]. Therefore, the higher efficacy observed in gram-positive strains can be attributed to the composition of the extract. The extract was particularly effective against *L. innocua* and *L. monocytogenes* strains, even at very low concentrations. Bacteria of the genus *Listeria* pose a significant contamination risk in the food industry during both production and storage stages, as they can continue to proliferate at low temperatures, such as 4°C [30-32]. Even a minimal amount of the mushroom used in this study was capable of inhibiting the growth of *Listeria* species. While further studies are required, *P. aurivella* could potentially provide a novel solution to such challenges in this field.

In the study by Skalicka-Woźniak et al. (2010) [33], the MIC value of linoleic acid for *S. aureus* ATCC 25923 was reported as 500 µg/ml. In comparison, the MIC value of *P. aurivella* ethanol extract for the same strain was determined to be 3.533 µg/ml. Considering that 59.2% of the extract is composed of linoleic acid, the extract is believed to exhibit a strong synergistic antimicrobial effect.

Additionally, a study by Islek et al. (2021) [34] determined MIC values for *P. adiposa*, *P. lubrica*, and *P. squarrosa* species against nine different strains. Four microorganisms (*S. aureus, E. faecalis, E. coli, P. aeruginosa*) were common between the two studies and demonstrated antimicrobial activity in both cases, whereas *A. baumannii* and *C. albicans* showed no antimicrobial effect with *P. aurivella.*

Based on GC-MS analysis, approximately 88% of *P. aurivella* ethanol extract was found to consist of hydrophobic substances, such as linoleic acid esters, linoelaidic acid, palmitic acid, myristic acid, tetradecanal, 3-tetradecen (E)-, and other fatty acids and esters. The lack of effect observed in MIC tests against strains that showed inhibition in the disk diffusion test is thought to be due to the high content of hydrophobic compounds in the extract, which are less soluble in liquid media. Hydrophobic compounds may experience solubility issues in liquid environments, limiting the access of active compounds to bacterial cells and reducing or eliminating antimicrobial activity. In contrast, in disk diffusion tests, hydrophobic compounds can diffuse more effectively in agar, achieving locally high concentrations that inhibit bacterial growth. Previous studies in the literature support this possibility. Van Vuuren (2008) [35] noted that plant extracts may yield different results in disk diffusion and MIC tests, which can be attributed to the physicochemical properties of the extract.

Antioxidant Activity

The tests revealed that the antioxidant effect of the mushroom extract was quite limited. Even at the highest concentration, its effect was limited to just 20% in scavenging DPPH radicals, while the positive control, ascorbic acid, demonstrated a scavenging rate of 94.7% (Table 6).

Table 6. Antioxidant values

Concentration (µg/ml)	*P. aurivella* % scavenging	Ascorbic acid % scavenging
1000	20.80 ± 1.66	94.70 ± 0.002
500	16.90 ± 1.11	94.30 ± 0.059
250	14.80 ± 0.35	92.40 ± 0.010
125	13.20 ± 0.88	91.00 ± 0.017
62.5	10.50 ± 0.91	73.00 ± 0.041
31.25	9.00 ± 0.33	40.20 ± 0.071
15.62	8.26 ± 0.44	23.21 ± 0.273

Previously, linoleic acid was tested for antioxidant activity using the DPPH method, but no significant results were observed [36]. GC-MS analysis of the extract, which is composed of 59% linoleic acid, revealed that it predominantly consists of fatty acids and ethers, with no phenolic compounds detected. Considering that antioxidant activity is typically associated with phenolic compounds, this result is not unexpected.

In a study by Regeda et al. (2021) [37], the antioxidant activity of seven different *Pholiota* species was compared using the DPPH radical scavenging method. Methanol was used as the solvent to prepare biomass and culture liquid extracts, yielding DPPH scavenging rates ranging from 83.6% to 7.37%, with *P. aurivella* recording a value of 82.37%. Contrary to these findings, the low antioxidant activity observed in our study is thought to be due to the solvent used. Methanol, being more polar than ethanol, has a greater potential to effectively extract phenolic compounds.

When the statistical results were evaluated, the EC_{50} value of the mushroom extract was found to be 1.8160 ± 2.0329 mg/ml. However, since the highest observable scavenging activity in the test was around 20%, this value represents a statistical estimation. The EC_{50} value of ascorbic acid, used as the positive control, was determined to be 0.0400 ± 0.0005 mg/ml. Based on the overall results, the p-value of the ANOVA test comparing the extract and ascorbic acid was calculated as 1.094×10^2, indicating no statistically significant difference between the extract and the positive control. Furthermore, the Pearson correlation coefficient between the increasing dose of the extract and its effect was calculated as 0.8833, which indicates a strong positive correlation.

Since the primary focus of this study was not on antioxidant activity, other solvents were not considered for extraction. However, to comprehensively evaluate and understand the antioxidant capacity of *P. aurivella*, it is necessary to extract its phenolic compounds using solvents of varying polarities. This approach could contribute to a better understanding of the mushroom's potential antioxidant capacity.

Although mushrooms are known for their low-fat content, the fats they contain predominantly consist of polyunsaturated fatty acids [51]. This study aligns with that observation. The GC-MS analysis revealed that 59.2% of the extract consists of linoleic acid, an Omega-6 fatty acid; 17.13% consists of linoleic acid ethyl ester; 8.14% consists of hexadecanoic acid (palmitic acid), a common saturated fatty acid; and 2.75% consists of hexadecanoic acid ethyl ester. The extract was found to be predominantly composed of fatty acids. Notably, this is the first such analysis reported for *Pholiota aurivella*. Fatty acids are known to exhibit toxic effects on bacterial cells by destabilizing their membranes, disrupting respiratory processes, and ultimately leading to cell death [52]. The antimicrobial activity observed in this study is likely attributable to the high fatty acid content of the extract.

GC-MS Analysis

Linoleic acid has long been recognized for its antimicrobial and antifungal properties [53]. It has also been demonstrated through both *in vivo* and *in vitro* studies that it possesses anti-inflammatory and anti-atherogenic effects and may be effective against major health issues affecting a large portion of the population, such as cancer, diabetes, and obesity [54].

Hexadecanoic acid, also known as Palmitic acid, is recognized as a natural and essential component of human skin and has been shown to exhibit antimicrobial activity against *S. aureus* strains [55,56]. However, a study revealed that *S. aureus* strains can rapidly develop resistance to hexadecanoic acid within a few hours. While the mortality rate of colonies without resistance development was 99%, this rate dropped to only 12% in treated and resistant strains [57]. This finding highlights the remarkable ability of *S. aureus* strains to develop resistance mechanisms against external threats. In addition, Palmitic acid is also known to have hemolytic, hypocholesterolemic, and nematicidal effects [43].

The composition of 89.02% of the ethanol extract of *P. aurivella* was elucidated through GC-MS analysis (Table 7). This analysis revealed that the extract predominantly contains polyunsaturated and saturated fatty acids. The antimicrobial activity exhibited against Gram-positive bacteria is thought to result from the ability of these fatty acids to penetrate bacterial cell membranes and disrupt membrane stability. Major components of the extract, such as linoleic acid and palmitic acid, are known to exert antibacterial effects by altering bacterial membrane permeability and impairing cellular functions [58]. This analysis represents the first report on *P. aurivella* in the literature, providing a comprehensive foundation for exploring its biological activities. The chemical profile obtained suggests that this mushroom has the potential to be evaluated as a natural antimicrobial agent in future pharmacological applications.

Table 7. Biochemical screening of *Pholiota aurivella*

Retention Time	Area%	Compound Name	Formula	Molecular Weight (g/mol)	Known Activity
25.868	0.15	3-Tetradecene, (E)-	$C_{14}H_{28}$	196.37	Antimicrobial and antifungal activity [38]
35.464	0.28	Tetradecanal	$C_{14}H_{28}O$	212.37	Bioluminescence reactions [39]
37.227	0.56	Tetradecanoic acid	$C_{14}H_{28}O_2$	228.37	Antiurease, antielastase and antioxidant [40], insecticide [41, 42]
42.410	8.14	Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	Antimicrobial activity [42] and nematicide [43]
42.517	2.75	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284.5	Antioxidant, hemolytic, hypocholesterolemic, anti-androgenic activity, nematicide and flavor [43]
44.924	0.24	Linoleic acid, methyl ester	$C_{19}H_{34}O_2$	294.5	Antioxidant [44] and anticancer activity [45]
46.590	17.13	Ethyl Linoleate	$C_{20}H_{36}O_2$	308.5	Anti-inflammatory [46] and antioxidant activity [47]

Table 7 (*continue*). Biochemical screening of *Pholiota aurivella*

46.736	59.20	Linoleic acid	$C_{18}H_{32}O_2$	280.4	Anticancer [48], antioxidant [49] and anti-inflammatory activity [50]
62.576	0.32	2-Methyl-3-(3-Methyl-2-Butenyl)-2-(4-Methyl-3-Pentenyl)oxetane	$C_{15}H_{26}O$	222.37	-
67.122	0.25	Fumaric acid, pent-4-en-2-yl tridecyl ester	$C_{22}H_{38}O_4$	366.5	-

* Compounds with no known activity in the literature are indicated with "-"

Antibiofilm Activity

This study represents the first investigation into the antibiofilm activity of *P. aurivella*. A significant increase in biofilm formation was observed in *B. subtilis* DSMZ 1971, *E. coli* ATCC 25922, *L. monocytogenes* ATCC 7644, and *L. innocua* strains, which correlated positively with the increase in extract concentration. In contrast, a positive but weak correlation was observed in the *E. coli* strain, known to possess an efflux pump system (Table 8).

Table 8. Correlation results of mushroom extract and biofilm effect

Microorganisms	Correlation (R^2)
***Bacillus subtilis* DSMZ 1971**	0.942559
***Escherichia coli* ATCC 25922**	0.921367
***Listeria monocytogenes* ATCC 7644**	0.916541
***Listeria innocua* (FI)**	0.956585
***Escherichia coli* (CI)**	0.693696

Although the extract maintained its ability to reduce biofilm formation, its effect was weaker compared to the other strains. This is thought to be due to the intracellular efflux pump system. While the exact mechanism is not fully understood, it is known that these pump systems are specialized to protect bacterial cells from toxic substances, such as antibiotics, that could potentially damage the cell, and they are highly effective in this role [59].

The absence of antimicrobial activity of *P. aurivella* against *E. coli* ATCC 25922, along with the strong biofilm activity enhancement, supports this hypothesis. The concentrations at which the strongest biofilm inhibition was observed are presented in Table 9. For each microorganism, the value of 0.220 mg/ml is indicated as the concentration where the highest inhibition was achieved.

Table 9. The most effective biofilm inhibition values found against microorganisms

Microorganisms	Biofilm Inhibition Concentration (mg/ml)	Biofilm Inhibition (%)	OD 550
***Bacillus subtilis* DSMZ 1971**	0.220	20.97 ± 1.29	0.304 ± 0.03
***Escherichia coli* ATCC 25922**	-	-	-
***Listeria monocytogenes* ATCC 7644**	0.220	50.65 ± 2.57	0.131 ± 0.06
***Listeria innocua* (FI)**	0.220	52.75 ± 1.36	0.135 ± 0.04
***Escherichia coli* (CI)**	0.220	46.27 ± 3.22	0.112 ± 0.02

B. subtilis exhibited moderate biofilm inhibition rates (approximately 21%), while *E. coli* showed no biofilm inhibition under the tested conditions. These findings highlight the potential of the extract to address biofilm formation in certain strains, such as *B. subtilis*, while suggesting strain-specific variability in its activity against *E. coli*. Taken together, these results demonstrate the extract's selective

antimicrobial and antibiofilm activity, emphasizing its potential for applications targeting specific bacterial pathogens. (Figure 1-3)

Biofilm inhibition tests demonstrated strong effects with inhibition rates exceeding 50% against biofilm-forming microorganisms, including *L. innocua* and *L. monocytogenes* (Figure 4 and Figure 5). Additionally, antibiofilm activity was observed at sub-MIC concentrations below the MIC value of 0.442 mg/ml against the food isolate *L. innocua*. The observation of antibiofilm activity at sub-MIC values, where antimicrobial effects were not present, highlights the true antibiofilm potential of the extract. Furthermore, it is believed that this activity is attributed to linoleic acid, the major component of the extract. As previously demonstrated by Kim et al. (2019) [60] in a study on *P. aeruginosa*, linoleic acid can inhibit biofilm formation without affecting bacterial cell growth. These findings, consistent with our study, highlight the potential applications of linoleic acid in biofilm inhibition.

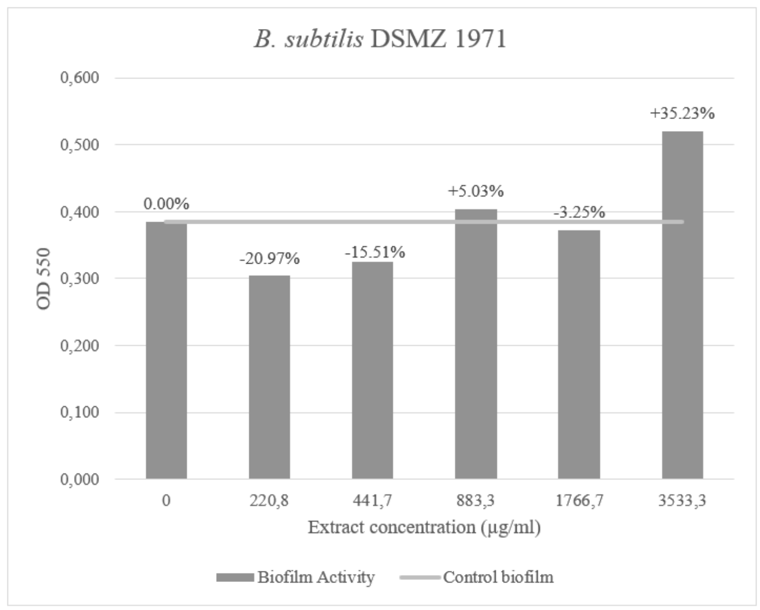

Figure 1. Antibiofilm effect of mushroom extract on *Bacillus subtilis* DSMZ 1971

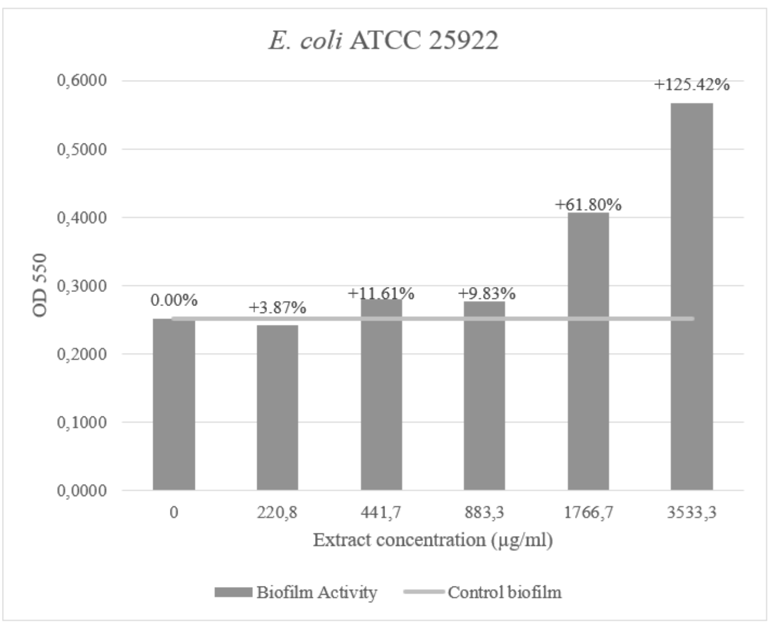

Figure 2. Antibiofilm effect of mushroom extract on *Escherichia coli* ATCC 25922

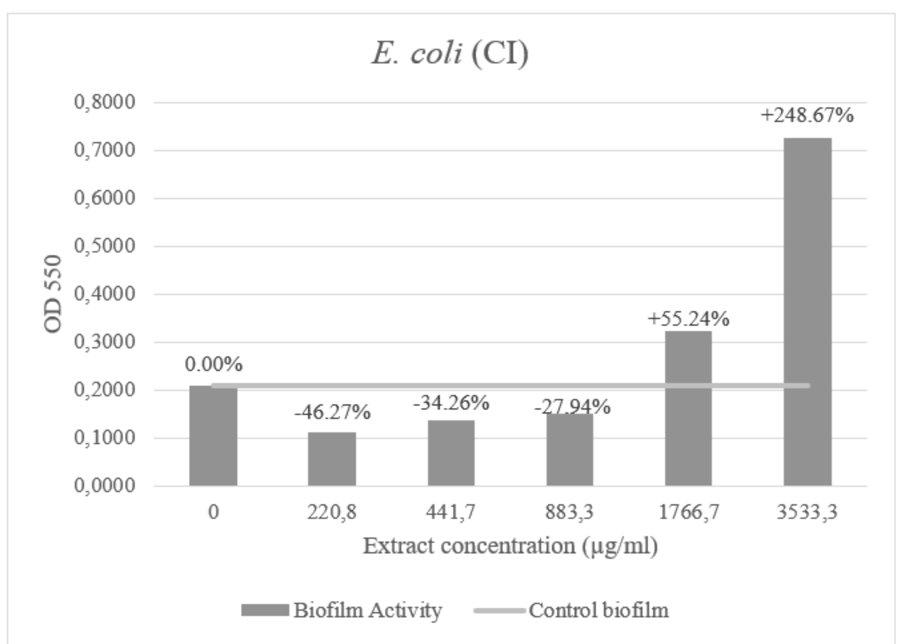

Figure 3. Antibiofilm effect of mushroom extract on *Escherichia coli* (CI)

When compared to a study [61] in which the natural compounds cinnamaldehyde (160 µg/ml) and resveratrol (100 µg/ml) were found to be effective against *L. monocytogenes* biofilm, the result of the mushroom extract, composed of complex compounds, showing activity at a concentration of 220.8 µg/ml is notable. The study concludes that the sub-MICs of these natural antimicrobial compounds reduce biofilm formation by suppressing the quorum sensing system rather than inhibiting flagellum formation. Further studies need to be conducted to investigate the mechanism of action of *P. aurivella*.

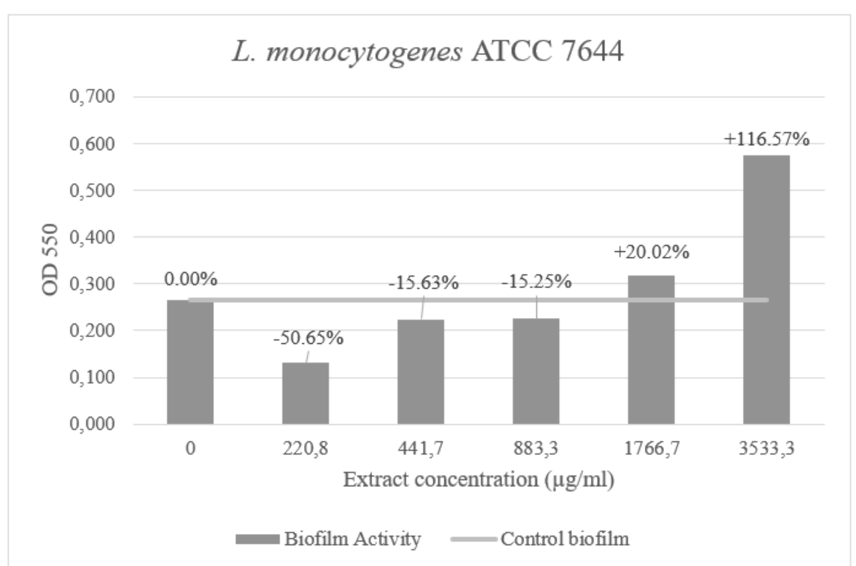

Figure 4. Antibioiofilm effect of mushroom extract on *Listeria monocytogenes* ATCC 7644

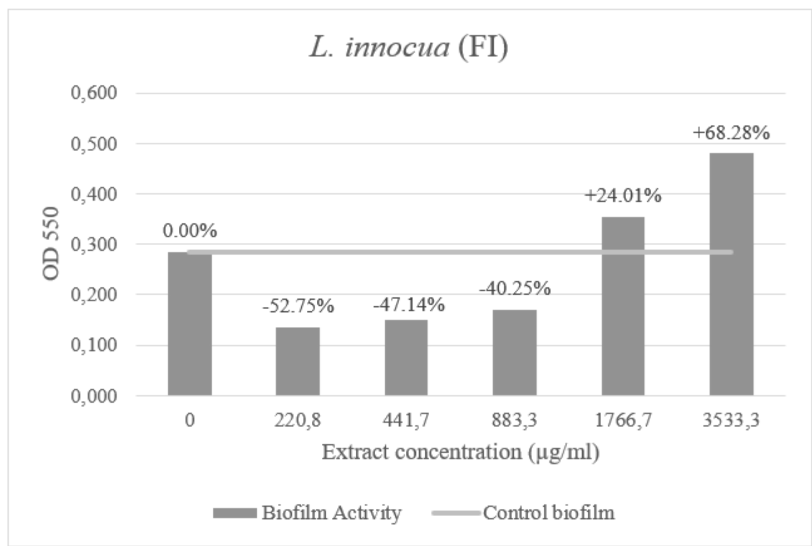

Figure 5. Antibiofilm effect of mushroom extract on *Listeria innocua* (FI)

Listeriosis, caused by *L. monocytogenes*, is a serious foodborne infection primarily affecting vulnerable groups. The bacterium's ability to form biofilms enables environmental persistence, making disinfection in the food industry challenging. This increases the need for advanced prevention strategies against biofilm formation and cell communication [62]. Particularly, the consistency of antimicrobial and antibiofilm activities in *Listeria* species highlights the dual functionality of the extract, not only against planktonic cells but also in combating biofilm-related challenges, making it a promising candidate for pharmaceutical applications targeting multidrug-resistant pathogens.

The results obtained demonstrate that the extract possesses antibiofilm activity against both Gram-positive and Gram-negative bacteria. Consistent with the MIC test, these findings indicate that the extract not only inhibits bacterial growth but also prevents biofilm formation, suggesting a promising potential for controlling persistent infections. These results emphasize that *P. aurivella* could be utilized as a potential antimicrobial agent in pharmaceutical applications and should be further supported by comprehensive research in the future.

AUTHOR CONTRABIBUTIONS

Concept: C.Y., G.G., K.C.; Design: C.Y., G.G., A.B.; Control: A.B., D.T., I.A., K.C.; Sources: A.B., I.A.; Materials: S.D.B., I.A., K.C.; Data Collection and/or Processing: C.Y., G.G., A.B., D.T.; Analysis and/or Interpretation: C.Y., A.B., D.T.; Literature Review: C.Y., G.G., S.D.B.; Manuscript Writing: C.Y., G.G., D.T., S.D.B.; Critical Review: A.B., D.T., K.C.; 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.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

BOX-BEHNKEN DESIGN APPROACH (BBDA) IN DEVELOPMENT AND OPTIMIZATION OF METFORMIN EXTENDED RELEASE TABLETS (MERT)

METFORMİN UZATILMIŞ SALIMLI TABLETLERİN (MERT) GELİŞTİRİLMESİ VE OPTİMİZASYONUNDA BOX-BEHNKEN TASARIM YAKLAŞIMI (BBDA)

Amaresh PRUSTY[1]*, **Sanjit Kumar SENAPATI[1]**, **Gyanranjan BEHERA[1]**

[1]Department of Pharmaceutics, Royal College of Pharmacy and Health Sciences, Berhampur, Odisha, 760002, India

ABSTRACT

Objective: *The present research outlines the use of quality by design (QbD) method to formulate MERT using Box-Behnken Design approach (BBDA). Based on quality target product profile (QTPP) to achieve tablets hardness and % cumulative Drug Release (% CDR) (at 2 hour and 10 hour), Critical quality attribute (CQA)were identified and selected as independent variable. In this present work, HPMC K 100M, Eudragit RL 100, and excipients MCC are selected as independent variables at their high and low levels in development of MERT.*

Material and Method: A*s per Design-Expert® prediction, total 19 formulations are prepared where each tablets of weight of 850 mg prepared by direct compression method. For each formulation, responses are determined and analyzed to find most optimized concentration.*

Result and Discussion: *HPMC K 100 M, Eudragit RL 100 and MCC have antagonistic effects on the % CDR after 2 hour and 10 hours. From diagnostic plot it has been observed normal distribution of all data points near to straight line for normal plot of residuals, and predicted vs. actual. The desirability cube and the contour graph showing maximum desirability for optimized values of 76.75 mg, 203 mg and 58 mg for HPMC K 100M, Eudragit RL 100 and MCC respectively which are selected as independent factors in formulation of MERT. Prepared optimized tablets of MERT releases drug for more than 10 hr.*

Keywords: *Box Behnken Design, extended release tablets, QbD, metformin*

ÖZ

Amaç: *Bu çalışma, tasarımla kalite (QbD) yaklaşımı kullanılarak MERT formülasyonunun geliştirilmesini ve Box-Behnken Tasarım (BBD) yöntemiyle optimize edilmesini ele almaktadır. Tabletlerin sertliği ve kümülatif etkin madde salım yüzdesi (% CDR) (2. ve 10. saatlerde) hedef ürün kalite profiline (QTPP) dayalı olarak belirlenmiştir. Kritik kalite özellikleri (CQA) tanımlanmış ve bağımsız değişkenler olarak seçilmiştir. Bu doğrultuda, MERT formülasyonunun geliştirilmesinde HPMC K 100M, Eudragit RL 100 ve yardımcı madde olarak MCC yüksek ve düşük seviyelerde bağımsız değişkenler olarak değerlendirilmiştir.*

Gereç ve Yöntem: *Design-Expert® programı tahminlerine göre toplam 19 farklı formülasyon hazırlanmıştır. Her biri 850 mg ağırlığında olan tabletler doğrudan basım yöntemiyle üretilmiştir. Her bir formülasyon için yanıtlar belirlenmiş ve en uygun konsantrasyonun belirlenmesi amacıyla analiz edilmiştir.*

* **Corresponding Author / SorumluYazar:** Amaresh Prusty
e-mail / e-posta: amareshprusty@gmail.com, **Phone / Tel.:** +917978413243

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Sonuç ve Tartışma: *HPMC K 100M, Eudragit RL 100 ve MCC'nin, 2. ve 10. saatlerdeki % CDR üzerinde antagonist etkiler gösterdiği tespit edilmiştir. Tanısal grafiklerden elde edilen veriler, normal artıklar grafiği ve tahmin edilene karşı gerçek değerler doğrultusunda tüm veri noktalarının doğruya yakın bir dağılım sergilediğini göstermektedir. Optimizasyon çalışmaları sonucunda belirlenen istenen değerlere göre en yüksek uygunluğu sağlayan HPMC K 100M, Eudragit RL 100 ve MCC konsantrasyonları sırasıyla 76,75 mg, 203 mg ve 58 mg olarak belirlenmiştir. Optimum formülasyon ile hazırlanan tabletler, 10 saatten daha uzun süre boyunca etkin madde salımı gerçekleştirebilmiştir.*

Anahtar Kelimeler*: Box Behnken Tasarımı, QbD, metformin, uzatılmış salımlı tabletler*

INTRODUCTION

In 21st century the prime objective is to formulate quality pharmaceutical preparationsnot only involving minimum number of man-hours but also without wasting raw materials used in formulations, which can save time. Therefore, nowadays research uses statistical tools such as factorial design to achieve this objective [1].

Quality by Design (QbD) approach first introduced by Dr. Joseph M. Juran [2]. Out of different stastical design based on QbD analysis, Box-Behnken design Approach (BBDA) uses lesser experimental runs during optimization making it more cost-effective technique without affecting quality of products [3-9]. This approach generates contour plots where independent factors are shown in vertical and horizontal axis. In QbD quadratic or cubic model generated by software represents complete description of process behavior. The confirmation of quality end product can be determined by design Space which is the operating ranges of input variables [10].

Metformin used in type II diabetes treatment as it lowers blood glucose concentrations without causing hypoglycaemia. It is frequently referred to as a "insulin sensitizer", resulting in a decline in insulin resistance and a considerable fall in plasma fasting insulin levels with therapeutic significance. A further well recognized advantage of this medication is its ability to cause modest weight loss, making it a very useful option for obese individuals with type II diabetes [11-14].

Study of CQA of Formulations and Process

During formulations of Pharmaceutical products, if parameters like CQA and Critical Material attributes (CMA) are controlled, then it helps to prepare a quality product which is safety, efficacy and stable. CQAs are qualities (physical, chemical, biological, or microbiological) that should fall within acceptable ranges to ensure the product meets quality standards. Whereas CMAs are characteristics of raw materials that must remain within predetermined parameters in order to produce consistent, high-quality drug compounds, excipients, or intermediates. Critical Process Parameters (CPP) are production process variables that may affect finished product, for which it has to be monitored and controlled properly for obtaining quality product [15].

Initial Risk Assessment of Formulation Variables

The failure mode and effects analysis (FMEA) was used to determine the majority of risk factors and their levels in order to prepare pharmaceutical formulations development [16]. These factors are selected based on their CMAs and CPPs which have an impact on drug release. Various research works suggest in preparation of extended release tablets, polymers role is vital for extending drug release [17]. QbD principles help to study the risk levels of different polymers and excipients and helps in optimization and in formulation of MERT. In this present work Polymer levels (HPMC K 100 M, Eudragit RL) along with MCC levels, are selected significant variables that affects % CDR and tablet hardness which are selected as responses in formulation of MERT.

Design Space

Design space is generated from the chosen initial set point by considering appropriate limits different independent factors which is then used in formulating an optimized formulation of MERT.

MATERIAL AND METHOD

Materials

The pharmaceutical company Pfizer India Healthcare Limited supplied the metformin. All the polymers like HPMC K 110 M, Eudragit RL 100, microcrystalline cellulose (MCC) along with talc and magnesium stearate used in this research work purchased from Rolex Pharmaceuticals, Bhubaneswar, Odisha.

Methods

Box-Behnken Experimental Design Approach (BBDA) used in optimization of independent factors and in analysis of responses [18-19].

Preparation and Optimization of MERT

MERT were prepared by direct compression method. Based on initial risk assessment in formulation of extended release tablets of Metformin, parameters like HPMC K 100 M amount (A), Eudragit RL amount (B), MCC amount (C) have highest influence on the dissolution profiles. So based on the above outcomes a screening design was constructed taking the independent factors like HPMC K 100 M amount (A), Eudragit RL 100 (B), MCC (C) at their low and high levels to study on selected responses like hardness (Y1) and % cumulative drug release (CDR) at 2 hour (Y2) and at 10 hour (Y3). The powder mixture then compressed in 10 mm tablet press punching machine. The weight of tablets was 860 mg. Optimization done by using Design-Expert® software (version 13; Stat-Ease Inc., Minneapolis, MN, USA). QbD Approach in Selection of Independent Variables at their High and low Levels for formulation of MERT mentioned in Table 1. Similarly the selected dependent responses are shown in Table 2. Based on the results of responses are analyzed by ANOVA and diagnostic plots are plotted ho evaluate factors effect [20-21]. The model also predict quadratic equation, which is used to identify the effect of factors on responses. The positive and negative sign on the magnitude of regression coefficients represent synergistic and antagonistic effect respectively [22].

$Y=b0+b1A+b2B+b3C+b4AB+b5BC+b6AC + b7A^2+b8B^2+b9C^2$
Where Y=dependent variable or response;
A, B, and C= independent variable coded levels;
b0=intercept; b1 to b9=regression coefficients

Table 1. QbD approach in selection of independent variables at their high and low levels for formulation of MERT

Factor	Name	Units	Minimum	Maximum
A	HPMC K 100M	mg	65.00	100.00
B	EUDRAGIT RL 100	mg	200.00	215.00
C	MCC	mg	50.00	70.00

Table 2. Responses (dependent variables)

Response	Name	Units
Y1	HARDNESS	kg/cm2
Y2	% CDR (after 2hour)	%
Y3	% CDR(after10 hours)	%

Compatibility Study

Fourier Transform Infrared Spectroscopy (FTIR) Study

In FTIR study, 10 milligrams of the sample and four hundred milligrams of potassium bromide (KBr) were triturated in a mortar. Next, a tiny amount of the triturated mixture was put into a pellet maker and compacted with a hydraulic press at a pressure of 10 kg/cm^2. A Shimadzu FTIR Spectrophotometer was used to scan the resultant pellet from 4000 cm^{-1} to 400 cm^{-1} after it was placed on the sample holder.

DSC Study

The thermal behavior of a drug or a polymer can be measured by DSC (Schimadzu, DSC-60, Japan). Samples weighing 5mg were sealed in aluminum pans and heated to 300ºC at a rate of 40ºC per minute.

Characterization of Pre Compression Parameters of Tablets

Pre-compression parameters like bulk density, tapped density, angle of repose and Compressibility index and percentage porosity were calculated [23].

Bulk Density

In bulk density determination, volume occupied is determined by transferring 25 gm powder samples into 100 ml graduated cylinder and the ratio between weights of sample to volume gives bulk density value.

Tapped Density

In tapped density, 25 gm of powder samples transferred to a 100 ml graduated cylinder and tapped to get tapped volume reading and ratio between weigh to tapped volume gives tapped density.

Compressibility Index (CI)

It is determined by using following formula.

$CI= (\rho t - \rho 0)/(\rho t)x\ 100$
ρ_t =tapped density, ρ_0=bulk density

Percentage Porosity

It was determined by liquid displacement method by applying formula

% Porosity= (True Density-Bulk Density)/True Density X 100

Angle of Repose

Angle of repose was calculated by following funnel method using the equation.

$\theta= \tan -1h/r$,
Where “h” and “r” are the height of pile and radius of the pile.

Characterization of Post Compression Parameters of Tablets

Characterization of MERT

From experimental batches as suggested by software, different tablet batches are prepared and evaluated post compression parameters. Tablets parameters like thickness, hardness (measured with Pfizer hardness tester and units in Kg/cm^2) and percentage friability were determined [24]. Roche Friabilator (Labindia) was used to determine friability, where 10 tablets were weighed initially by placing in the friabilator for 4 min giving 100 rpm and after that final tablet weight was measured.

The percent friability (PF) = (Initial Weight – Final Weight) / Initial Weight X 100.

Weight variation test is performed as per USP guidelines to calculate average weight which compared with % deviation.

Drug Content

To determine drug content of MERT, five tablets of each formulation were weighed and finely powdered. About 0.1 gm equivalents were accurately weighed completely dissolved in buffer and was filtered. About 1ml of the filtrate was further diluted to 100ml with buffer. The solution's absorbance was measured at 282 nm using a UV-visible spectrophotometer.

In Vitro Dissolution Studies

It was carried out by USP Type II paddle type dissolution apparatus (Disso 2000, Labindia) by taking 900 ml of 0.1 N HCl (pH 1.2) media by maintaining temperature at 37±0.5°C. MERT are immersed in medium by setting the paddle to rotate at 100 rpm [25]. At regular intervals 10 ml samples were removed and replaced with same media of the same volume. The samples were examined for drug concentration using a double beam UV-Visible spectrophotometer (Genesis-2, USA) at a wavelength of 282 nm, to calculate the percentage of cumulative drug release.

RESULT AND DISCUSSION

FTIR Study

The characteristic absorption of the Metformin shows peak at 3367.71 cm^{-1}, which is assigned to the stretching vibration of Primary amine group of Metformin HCl and another characteristics bands at 1623, 1560 and 1068.78 cm^{-1} assigned to C=N stretching band at is due to C=N symmetric vibration. FTIR spectrum of Eudragit RL 100 showed the peak at 3432.1 cm^{-1} due to the presence of tertiary amine, at 1731.4 cm^{-1} due to the presence of C = O (ester), and at 1450.2 cm^{-1} due to –CH3 bend. The FTIR of drug and drug with excipients shown in Figure 1 and 2 which showed that compatibilities occurred between the drug and polymers used.

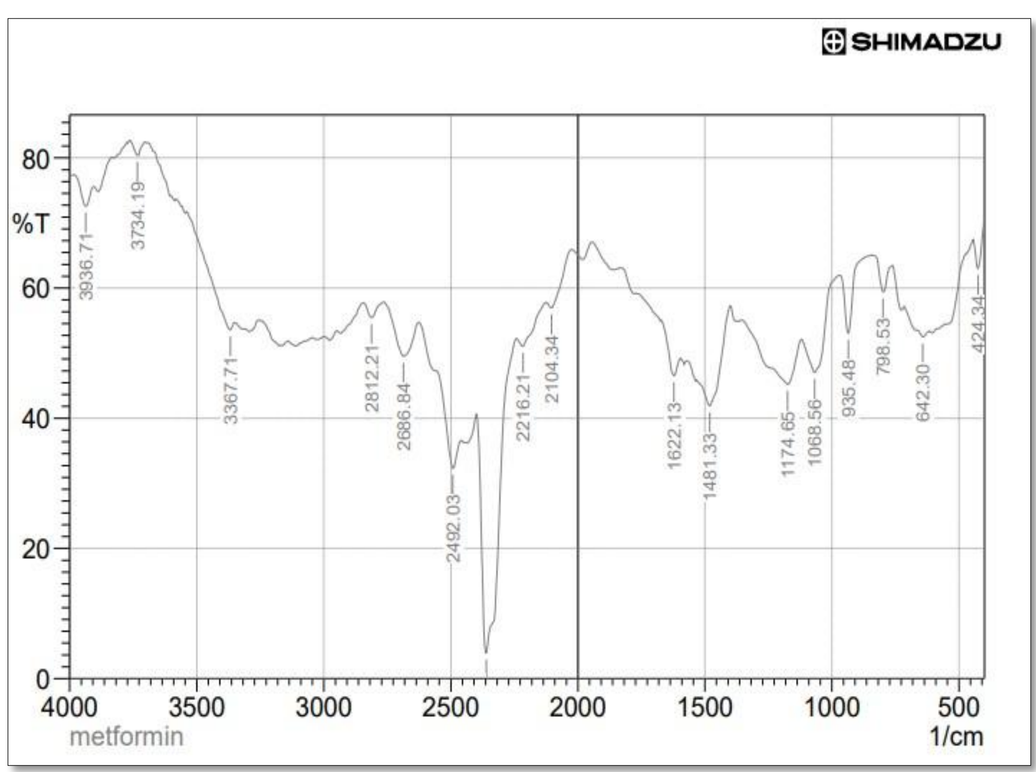

Figure 1. FTIR Spectrum of metformin

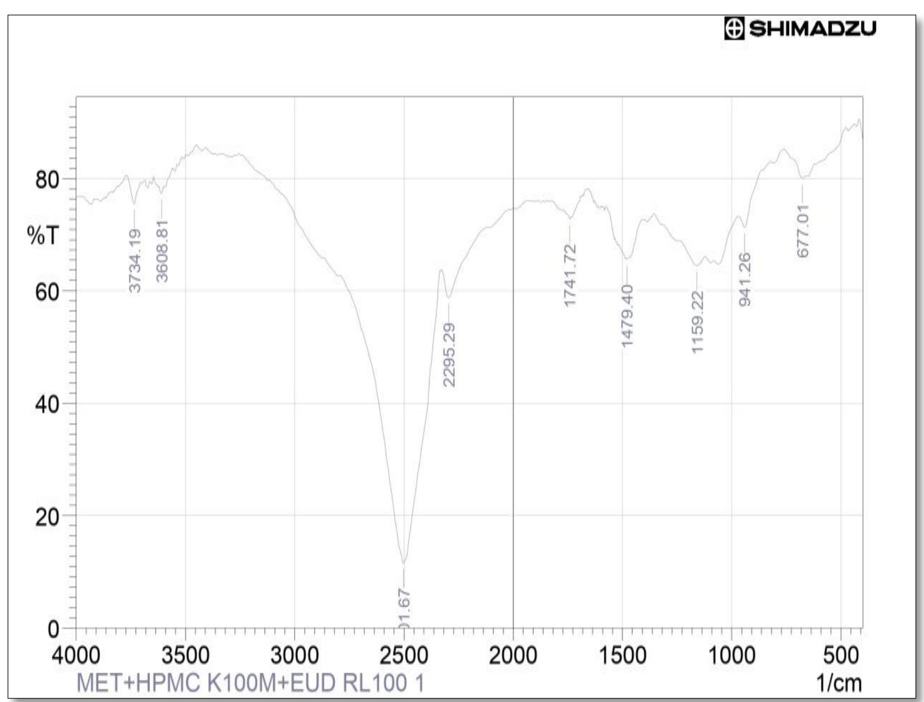

Figure 2. FTIR Spectrum of physical mixture of metformin, HPMC K 100 M and Eudragit RL 100

DSC Study

In DSC curve of Metformin showed a sharp endothermic peak at 251.61°C corresponding to its melting point. There was no significant change in the endothermic peak between drug and formulation which shows peak at 248.12°C and use of polymer HPMC K 100 M does not affect the stability of the drug confirming the formulation thermodynamically stable nature (Figure 3 and Figure 4).

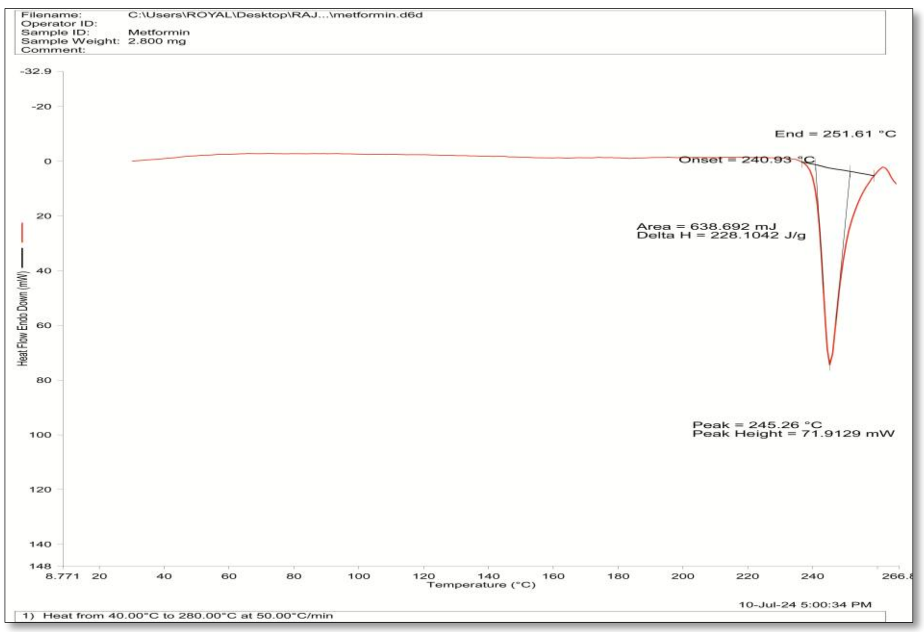

Figure 3. DSC thermogram of drug metformin

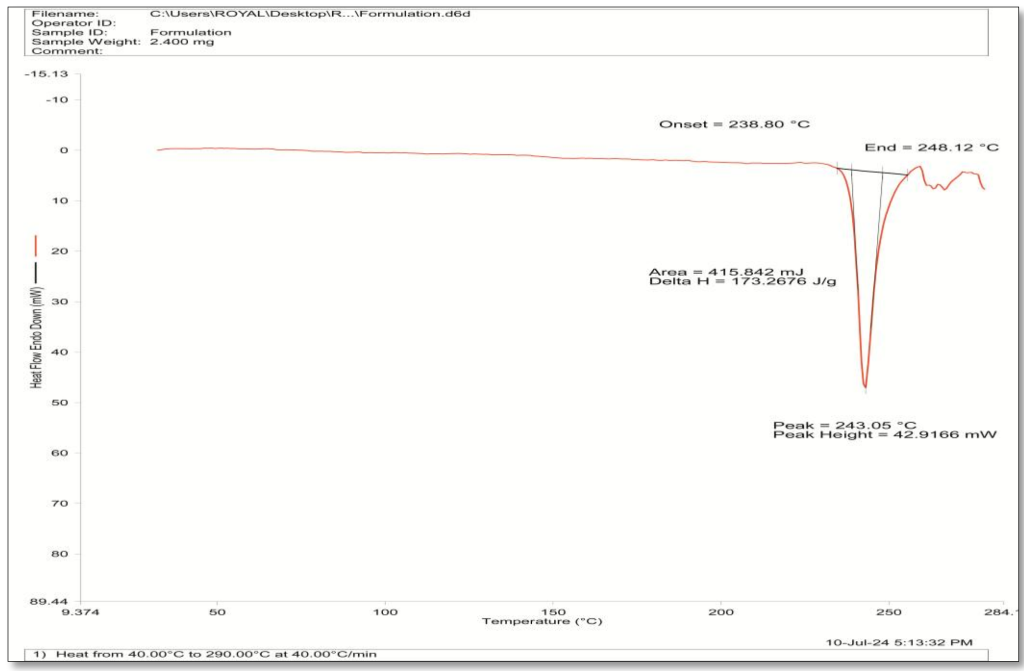

Figure 4. DSC thermogram of tablet

Results of Experimental Formulation of MERT

The total 19 formulations of MERT were prepared experimentally in triplicate and all dependent responses (Y1, Y2 and Y3) are calculated as per prescribe procedure. Results are shown in Table 3 for all experimental formulations. Data obtained for responses were analyzed by Design Expert software.

Table 3. Results of different responses at different level of independent variables

Run	Factor A (HPMC K M)	Factor B (EUDRAGIT RL 100)	Factor C (MCC)	HARDNESS kg/cm^2	% CDR (After 2 hours)	% CDR (After 10 hours)
1	65	200	60	5	55.052	99.98
2	100	200	60	6	33.09	95.65
3	65	215	60	6	57.272	99.12
4	100	215	60	7	33.09	92.73
5	65	207.5	50	5	57.272	99.12
6	100	207.5	50	6	42.102	91.75
7	65	207.5	70	6	51.052	96.87
8	100	207.5	70	7.5	33.09	92.36
9	82.5	200	50	5	55.052	98.65
10	82.5	215	50	7.5	57.272	97.16
11	82.5	200	70	7.5	55.052	98.36
12	82.5	215	70	7.5	39.82	95.98
13	82.5	207.5	60	6	57.272	98.36
14	82.5	207.5	60	7.5	37.32	94.12
15	82.5	207.5	60	7.5	42.102	96.09
16	82.5	207.5	60	7	33.09	94.36
17	82.5	207.5	60	7	47.272	97.65

Data Interpretation for Response Y1

The obtained data for response Y1 as shown in Table 4is analyzed by ANOVA by fitting to the appropriate models (linear, 2-FI, and quadratic) and results are shown in Table 4 to 9. The model is significant when $p<0.05$, and lack of fit is non significant if $p>0.05$ [26]. The quadratic equation generated by software helps to find out the effect of dependent variables (A, B, and C) on the responses Y1, Y2, Y3. Table 4 and 5 indicate summary of results on Hardness. As the results indicate P-values of less than 0.0500 indicate model terms are significant and factors A, B, C, BC, A^2 are more significant to affect response Y1. The Lack of Fit F-value of 0.05 ($p>0.05$) implies model is not significant relative to the pure error and Model F-value of 7.11 implies the model is significant for response Y1.

Table 4. Results of p value and lack of fit p value for different equation on effect of independent variables on hardness (ResponseY1)

Factors (Types of Equation)	p-value	Lack of fit p-value	Adjusted R^2	Predicted R^2	
Linear	0.0101	0.3507	0.4701	0.2646	
2FI	0.3777	0.3418	0.4873	0.0433	
Quadratic	***0.0026***	***0.9824***	***0.7746***	***0.7929***	***Suggested***
Cubic	0.9824		0.6202		**Aliased**

Table 5. Results of ANOVA for responses Y1

Source	Sum of squares	F-value	p-value	
Model	0.5292	7.11	0.0085	significant
A-HPMC K 100M	0.1040	12.58	0.0094	
B-EUDRAGIT RL 100	0.1040	12.58	0.0094	
C-MCC	0.1263	15.27	0.0058	
AB	0.0001	0.0089	0.9275	
AC	0.0014	0.1732	0.6897	
BC	0.0631	7.63	0.0280	
A^2	0.1238	14.97	0.0061	
B^2	0.0031	0.3695	0.5625	
C^2	0.0001	0.0071	0.9354	
Residual	0.0579			
Lack of Fit	0.0022	0.0516	0.9824	not significant
Pure Error	0.0557			
Cor Total	0.5871			

The model also predicts that there is very less difference of 0.2 between Predicted R^2 (value of 0.7929) and Adjusted R^2 (value of 0.7746) as shown in Table 5. Similarly the selected independent variables for response Y1 shows Adeq Precision of 7.704 (desirable value is greater than 4) which indicates an adequate signal and can be used to navigate the design space and results are shown in Table 7.

Quadratic equation:

$$Y_{1}=+2.64+0.1256\,A+0.1140\,B+0.1140C-0.0043\,AB+0.0189\,AC-0.1256\,BC-0.1715\,A^2-0.0269B^2-0.0037\,C^2$$

Above equation shows the impact of the independent factors that affect response Y1. So from equation it is concluded that higher polymers concentration increases hardness of MERT. The result is

similar to earlier reported study where It was observed that tablets containing HPMC K100M as the intra granular polymer increase hardness, this could be due to the higher binding capacity of HPMC K100M [27-28]. Eudragit concentration also affects drug release from tablets. Eudragit at higher levels causes reduction in the permeation of water inside the powder granules causing slower drug release [29]. Similar effect also derived for MCC, which explain synergistic effect on hardness of tablets.

Data Interpretation for Response Y2

In Table 6 and 7 which explain the effect of independent variables on response Y2, shows p value of 0.0028 indicating significant of model. The lack of fit F value of 0.3843 which indicates model is non significant ($p>0.05$), and is adequate for prediction of the response. The Model F-value of 6.16 also confirms the model is significant for response Y2.

Table 6. Results of p value and lack of fit p value for different equation on effect of independent variables on % CDR at 2 hours (Response Y2)

Factors (Types of Equation)	p-value	Lack of fit p-value	Adjusted R^2	Predicted R^2	
Linear	0.4431	0.8967	0.4207	0.1431	
2FI	0.7178	0.8321	0.4189	0.1237	
Quadratic	***0.0028***	***0.8925***	***0.4916***	***0.3735***	***Suggested***
Cubic	0.8967		0.1139		**Aliased**

Table 7. Results of ANOVA for responses Y2

Source	Sum of squares	F-value	p-value	
Model	933.67	6.16	0.0078	significant
A-HPMC K 100M	785.59	15.54	0.0017	
B-EUDRAGIT RL 100	14.56	0.2880	0.6005	
C-MCC	133.53	2.64	0.1281	
Residual	657.04			
Lack of Fit	304.67	0.3843	0.8925	not significant
Pure Error	352.37			
Cor Total	1590.71			

Similarly as per results shown in Table 10, the Predicted $R^2$and adjusted value are 0.3735 and 0.4916 respectively andAdeq Precision of 8.117 indicates model can be used to predict the design space.

Coefficients in Terms of Coded Factors:

$$Y2=+46.25-9.91 A -1.35 B -4.09C-0.097AB +0.0731 AC -0.1256 BC -4.1715 A^2-2.0269B^2-0.1213 C^2$$

Data Interpretation for Response Y3

In Table 8 and 9 which explain the effect of independent variables on response Y3, shows p value of 0.0012 which demonstrates that the model is significant ($p<0.05$) . The lack of fit F value of 0.5376 and Model F-value 9.82 confirm that the proposed model is model is significant and adequate for prediction of the response.

Table 8. Results of p value and lack of fit p value for different equation on effect of independent variables on % CDR at 10 hours (Response Y3)

Factors (Types of Equation)	p-value	Lack of fit p-value	Adjusted R^2	Predicted R^2	
Linear	0.1717	0.9968	0.6794	0.7569	
2FI	0.7665	0.6946	0.5607	0.1575	
Quadratic	***0.0012***	***0.7982***	***0.6231***	***0.4956***	***Suggested***
Cubic	0.9968		0.4456		**Aliased**

Table 9. Results of ANOVA for responses Y3

Source	Sum of Squares	F-value	p-value	
Model	72.37	9.82	0.0012	significant
A-HPMC K 100M	63.85	25.98	0.0002	
B-EUDRAGIT RL 100	7.32	2.98	0.1081	
C-MCC	1.21	0.4920	0.4954	
Residual	31.94			
Lack of Fit	17.49	0.5376	0.7982	not significant
Pure Error	14.46			
Cor Total	104.31			

Similarly as per results shown in Table 11, Adeq Precision of 9.9457 indicates model can be used to predict the design space.

$$Y3= +96.37-2.83 A -0.9563 B -0.3888C-0.373 AB -1.190 AC -0.634 BC -0.775 A^2-0.3269B^2-1.037 C^2$$

So for response Y2 and, Y3 the equation indicates higher level of polymer concentration causes decrease in drug release in extended release tablets. Polymer HPMC K100Mat higher level prevents water uptake and form a thick and turbid gel that shows resistant to erosion by forming a protective gel layer causing the slower drug dissolution from the tablet surface [25]. As suggested by the polynomial equations, Eudragit RL 100 has negative or antagonistic effect on drug release. At higher levels Eudragit retards the release of drug and can be used in the sustained release tablet formulation due to its property of formation of a matrix system. Above results are in agreement with previous research report which shows Eudragit polymer along with HPMC can be used in formulation of extended release tablets [30]. Similarly at higher levels MCC retards the release of drug as presence of MCC causes less disentanglement or increased binding, resulting in a lower percentage of medication release during that time period.

Diagnostic Plots for Different Responses Predicted by BBDA

In order to study the effect of independent variables on selected response Y1, Y2 and Y3, diagnostic plots and contour plots are designed by software based on BBDA and are shown in Figure 5 to 8. From figure it has been observed for Normal plot of residuals, and Predicted vs. Actual, normal distribution of all data points near to straight line. Similarly in plots of Residuals *vs*. Predicted, and Residuals *vs*. Run between predicted and actual response, all data points are placed within the limits, which indicates the fit of the model.

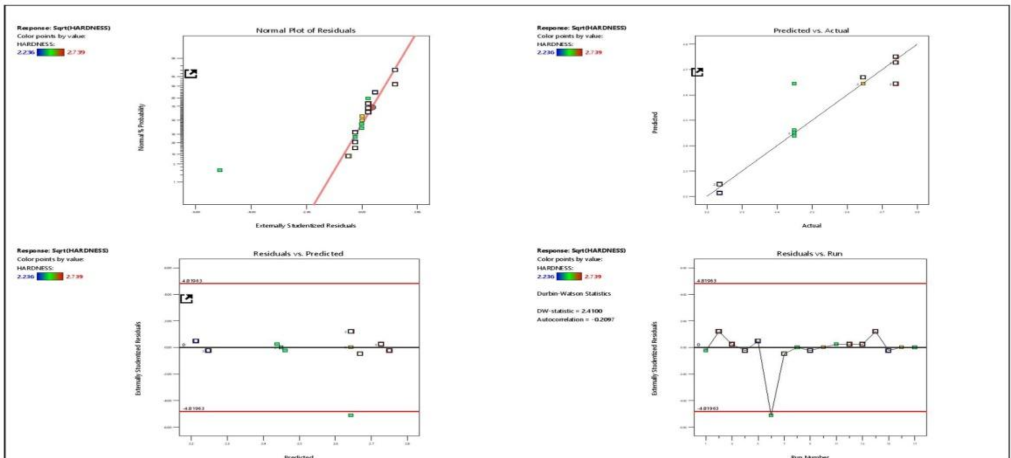

Figure 5. Diagnostic plots showing the effect of independent variables on hardness of tablets (Y1) for (a) Normal plot of residuals, (b) Predicted vs. Actual (c) Residuals *vs*. Predicted, (d) Residuals *vs*. Run between predicted and actual response

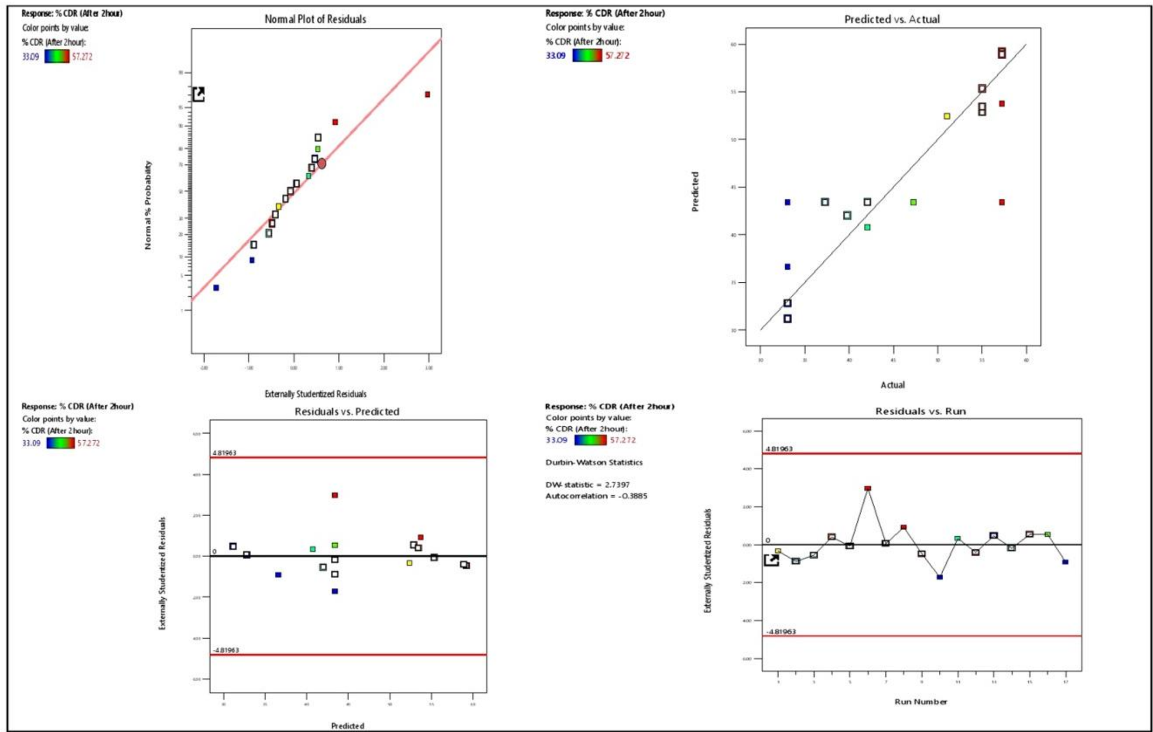

Figure 6. Diagnostic plots showing the effect of independent variables on % CDR at 2 hr (Y2) (a) Normal plot of residuals, (b) Predicted vs. Actual (c) Residuals *vs*. Predicted, (d) Residuals *vs*. Run between predicted and actual response

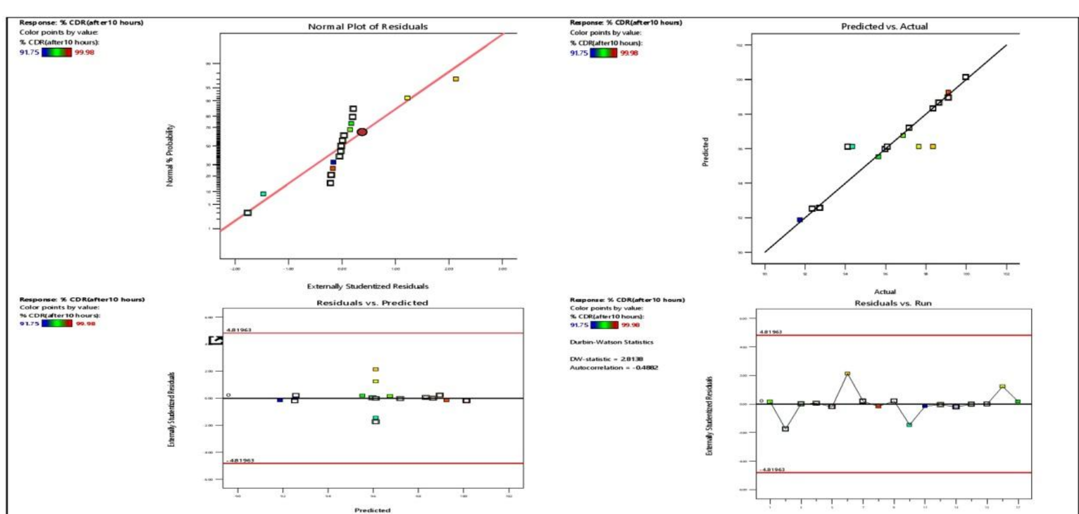

Figure 7. Diagnostic plots showing the effect of independent variables on % CDR at 10 hr (Y3) (a) Normal plot of residuals, (b) Predicted vs. Actual (c) Residuals *vs*. Predicted, (d) Residuals *vs*. Run between predicted and actual response

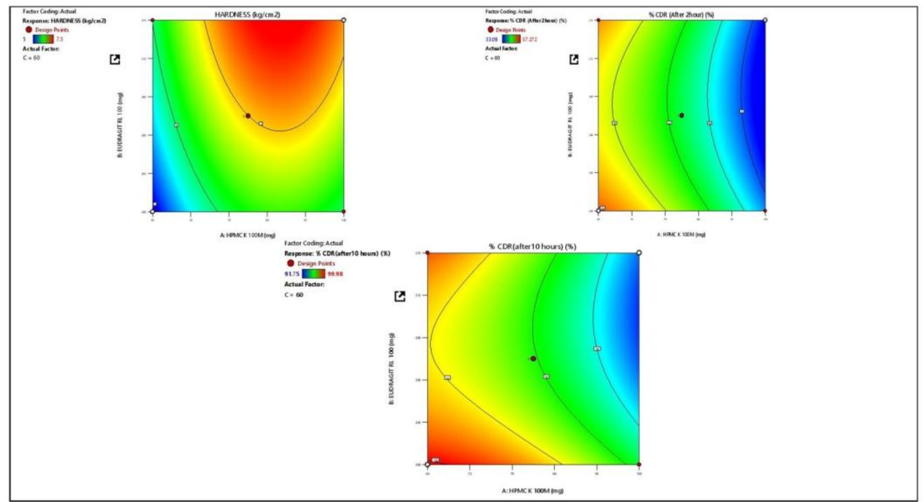

Figure 8. Contour Plots showing effect of independent variables on the hardness (Y1), drug release at 2 hour (Y2) and drug release at 10 hours (Y3)

Cook's Distance

It can be used to prioritize which runs to investigate first. It is a measure of how much the regression would change if the case is omitted from the analysis [31-32]. Relatively large values are associated with cases with high leverage and large studentized residuals. Large values should be investigated as they could be caused by recording errors or form an incorrect model, or a design point far from the remaining cases. In our cases all values are less than 1 indicating feasibility of responses and cook's plot for all variables against responses Y1, Y2, and Y3 are mentioned in Figure 9.

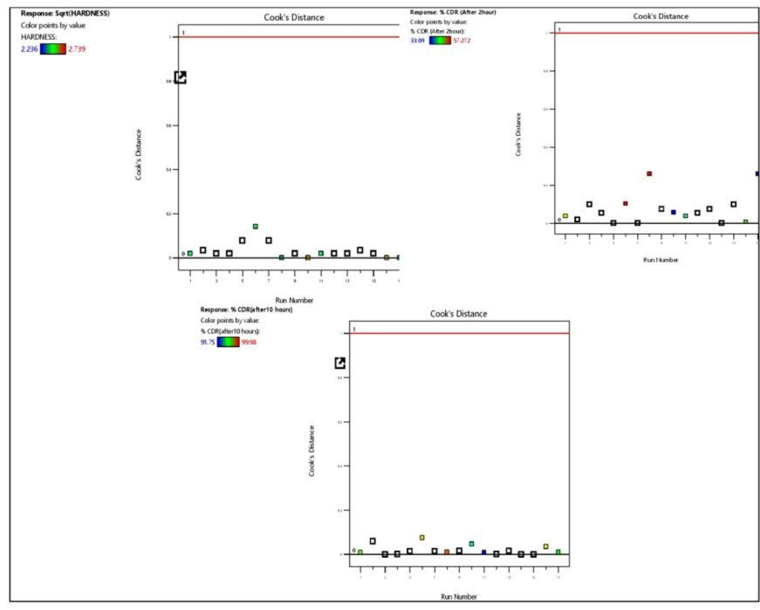

Figure 9. The diagnostic plots showing Cook's distance plot obtained by the Box-Behnken Design using Cook's distance versus run number by the different independent factors against responses

Optimization of Data in Defining Design Space

From prediction by design expert, the optimized values are76.75 mg, 203 mg and 58 mg for HPMC K 100M, Eudragit RL 100 and MCC respectively which are selected as independent factors in formulation of MERT and the values are shown in Figure 10 was designed for preparing MERT to generate space based on contour plots [24]. The desirability cube and the contour graph showing maximum desirability of 1 for optimized MERT is shown in Figure 11 and 12. For constructing a satisfying fit of the model for the optimized formulation, result analysis was carried out for predicted and observed response and results are shown in Table 10. The results are shown in cube plot and in contour graph showing how three factors combine to affect the different responses. This demonstrated the reliability method using BBDA in predicting the optimized formula for MERT.

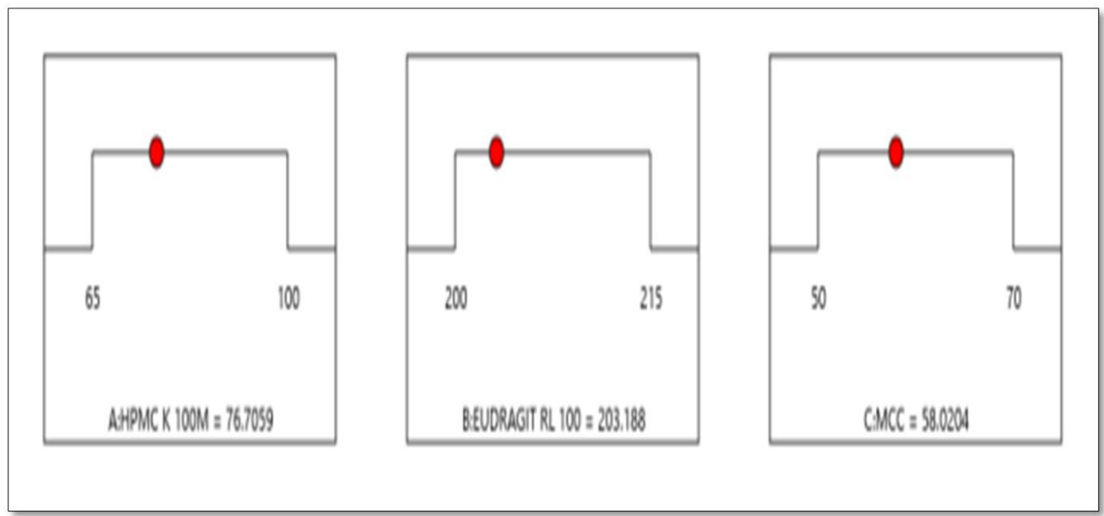

Figure 10. Optimized values of independent factors in formulation of MERT

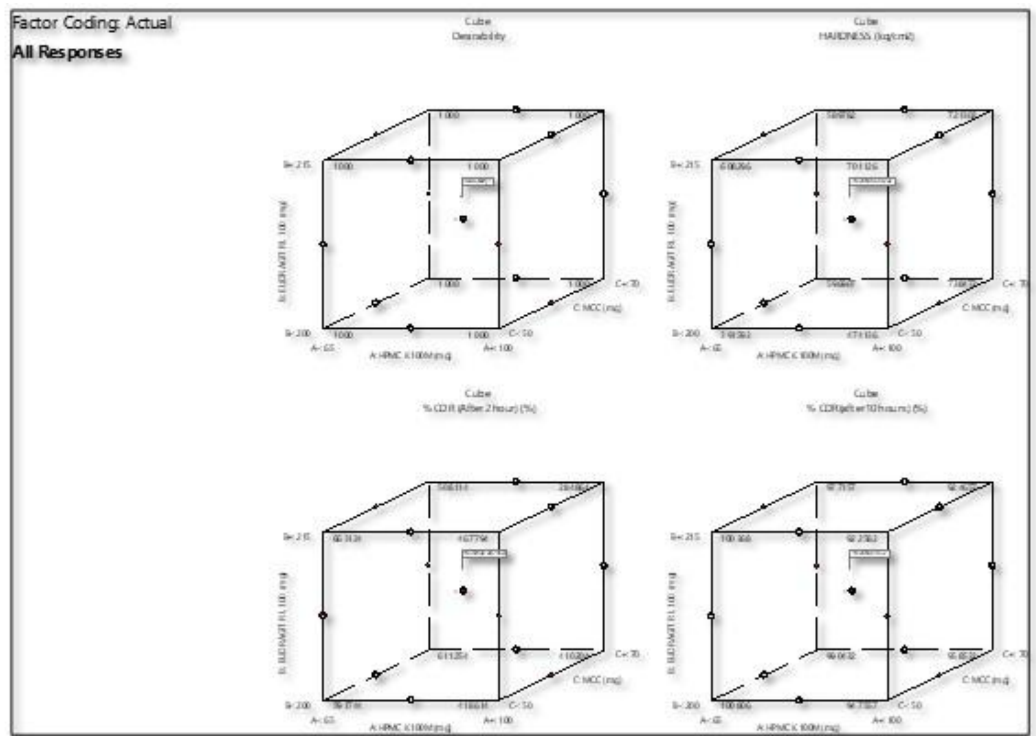

Figure 11. The optimized formulation with maximum desirability shown in cube

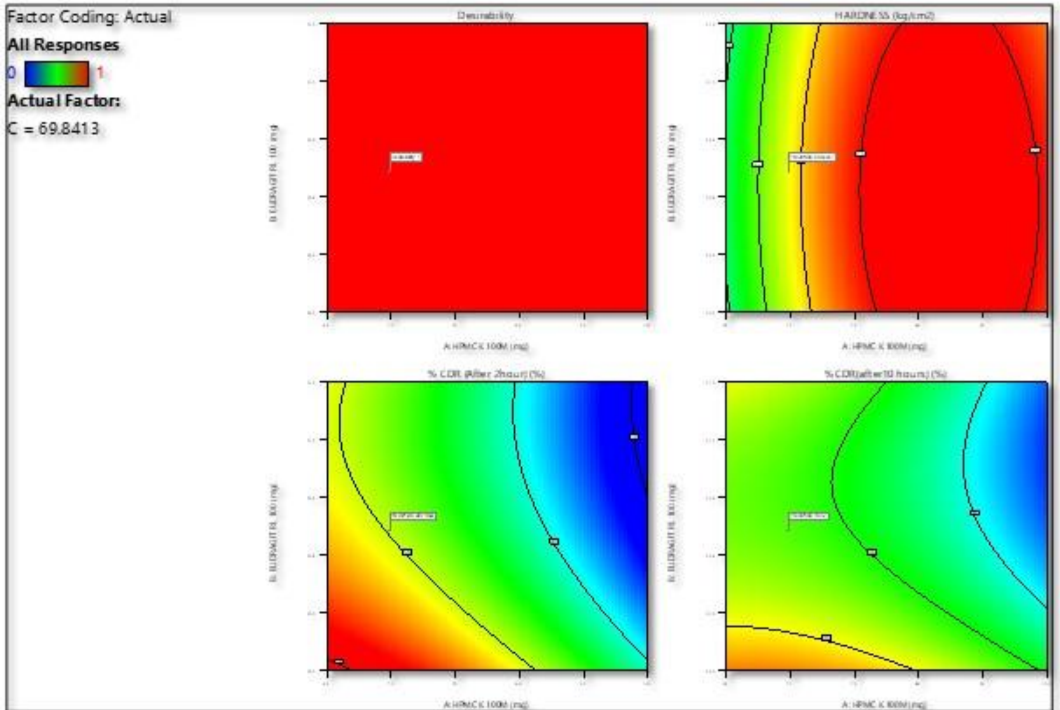

Figure 12. The optimized formulation with maximum desirability has shown in contour graph showing how independent factors combine to affect the different responses

Table 10. The result of predicted and observed response for the optimized formulation of MERT with maximum desirability

Analysis of Selected Responses	Predicted Mean	Predicted Median	Observed
HARDNESS	6.99712	6.98885	7
% CDR (After 2hour)	46.2513	46.2513	47.329
% CDR (after10 hours)	96.3712	96.3712	96.439

The desirability cube and the contour graph showing maximum desirability for optimized values of 76.75 mg, 203 mg and 58 mg for HPMC K 100M, Eudragit RL 100 and MCC respectively which are selected as independent factors in formulation of MERT. The prepared MERT are evaluated for different parameters like weight variation, tablet hardness, % Friability and Percentage of drug content along with drug release and the results are shown in Table 11. All the results are within the acceptable limit as per official compendia available. The results of drug release shows prepared MERT release drug which extended for more than 10 hr and dissolution profile is shown in Figure 13.

Table 11. Results of pre compression parameters and post compression parameters of tablets for optimized formula

Parameters	Results
Angle of repose(Θ)	25.3±0.76
Compressibility index (%)	16.02± 0.36
Hausners ratio	1.12± 0.02
Thickness (mm)± S.D	3.1 ± 0.2
Hardness(kg/cm^2) ±S.D	8.4±0.12
%Friability± S.D	0.11±0.01
Weight variation ±S.D	860±1.22
%Drug content± S.D	99.98±1.5

n= 6; SD-standard deviation

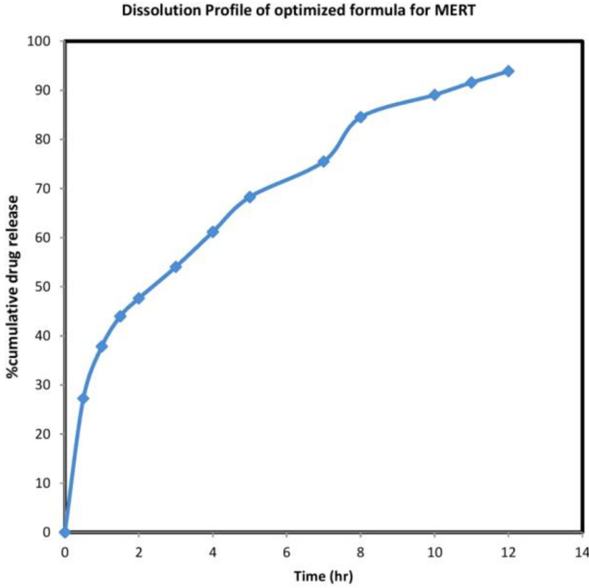

Figure 13. Dissolution profile of optimized formula for MERT

So QbD based BBDA represents a systematic and science-based strategy to optimize polymer concentration by a thorough understanding of formulation and process variables. In this research work QbD helps to formulate MERT by optimizing different Polymers and excipients levels to give hardness, drug release, as per QTTP. The desirability cube and the contour graph showing maximum desirability for optimized MERT. So the present experimental work prepared MERT, which shows extended drug release of more than 10hour thereby reducing the frequency of dosing and improved patient compliance.

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AUTHOR CONTRIBUTION RATE STATEMENT

Concept: A.P.; Design: A.P., S.K.S., G.B.; Control: A.P., S.K.S., G.B.; Sources: A.P., S.K.S., G.B.; Materials: A.P., S.K.S., G.B.; Data Collection and/or Processing: A.P., S.K.S., G.B.; Analysis and/or Interpretation: A.P., S.K.S., G.B.; Literature Review: A.P., S.K.S., G.B.; Manuscript Writing: A.P.; Critical Review: A.P., S.K.S., G.B.; Other: -

CONFLICT OF INTEREST

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

ETHICS COMMITTE APPROVAL

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

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ÖZGÜN MAKALE / ORIGINAL ARTICLE

KORNEA AMELİYATLARI SONRASINDA KULLANILABİLECEK GÖZ KURULUĞUNU ÖNLEYİCİ *İN-SİTU* HİDROJEL FORMÜLASYONLARININ TASARIMI VE *İN VİTRO* KARAKTERİZASYONU

DESIGN AND CHARACTERIZATION OF IN-SITU HYDROGEL FORMULATIONS TO PREVENT DRY EYE AFTER CORNEAL SURGERIES

Şevval DEMİR[1], Aylin DELJAVAN GHODRATI[1,2], Tansel ÇOMOĞLU[1]*, Kader ÇÖMLEKÇİ[3]

[1]Ankara Üniversitesi, Eczacılık Fakültesi, Farmasötik Teknoloji Anabilim Dalı, 06560, Ankara, Türkiye
[2]Ankara Üniversitesi, Sağlık Bilimleri Enstitüsü, 06110, Ankara, Türkiye
[3]Argis İlaç Sanayi ve Tic. A.Ş., 06370, Ankara, Türkiye

ÖZ

Amaç: *Göz kuruluğunu önlemek için kullanılan göz damlalarının gözde kalış süresinin kısa olmasıyla alternatif dozaj şekillerinin araştırılmasını gerektirmiştir. İn-situ hidrojeller, saklama sırasında sol fazında olup çevresel koşullarda jel fazına geçerek hedef bölgede daha uzun süre kalabilmektedir. Bu çalışmada, doğal ve biyouyumu polimerler kullanılarak göz sıcaklığında sol-jel geçişi yapabilen in-situ hidrojel formülasyonları geliştirilmiş ve karakterizasyonları gerçekleştirilmiştir.*
Gereç ve Yöntem: *HA, CMC, PAA ve sitrik asit, fiziksel karıştırma yöntemi esas alınarak 37°C sıcaklıkta distile su içerisine serpilerek homojen dağılımı sağlanmıştır. Göz fizyolojisine uygunluğu karakterizasyon çalışmaları ile kontrol edilmiştir. pH ölçümü, şişme/erozyon ve sol-jel geçiş sıcaklığı değerlendirilmesi ve viskozite ölçümü karakterizsyon çalışmaları altında gerçekleştirilmiştir. Tüm veriler değerlendirilerek optimum özelliklere sahip en uygun formülasyon/lar seçilerek hızlandırılmış stabilite çalışmaları gerçekleştirilmiştir.*
Sonuç ve Tartışma: *Su tutma kapasitesi yüksek, biyouyumlu, gözde uzun süre kalabilen in-situ hidrojel formülasyonları için hyaluronik asit, poliakrilamid ve karboksimetil selüloz kombinasyonu uygun bulunmuştur. 7 günlük hızlandırılmış in vitro stabilite çalışmasında, formülasyonların pH ve viskozite değerlerinde önemli bir değişiklik gözlenmemiştir.*
Anahtar Kelimeler: *Hyaluronik asit, in-situ hidrojel, karboksimetil selüloz, kornea, poliakrilamid*

ABSTRACT

Objective: *The short retention time of eye drops used to prevent dryness has necessitated the exploration of alternative dosage forms. İn-situ hydrogels remain in a sol phase during storage but transition to a gel phase under environmental conditions, allowing prolonged retention at the target site. In this study, in-situ hydrogel formulations capable of sol-gel transition at ocular temperature were developed using natural and biocompatible polymers, and their characterizations were performed.*

* **Sorumlu Yazar / Corresponding Author:** Tansel Çomoğlu
e-posta / e-mail: comoglu@pharmacy.ankara.edu.tr, **Tel. / Phone:** +903122033164

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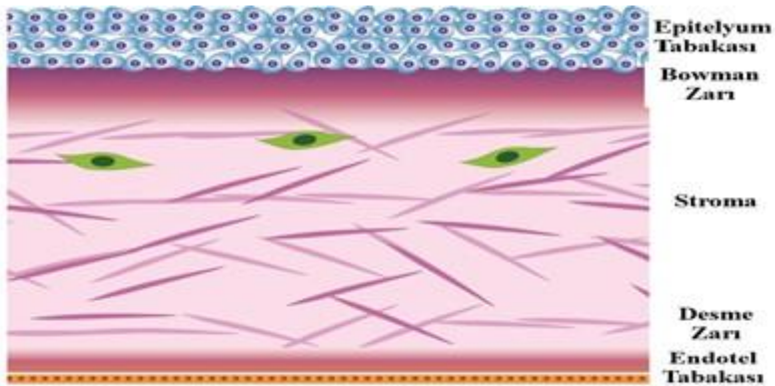
Material and Method: *HA, CMC, PAA, and citric acid were homogeneously dispersed in distilled water at 37°C by the physical mixing method. The suitability for ocular physiology was verified through characterization studies. pH measurement, swelling/erosion assessment, sol-gel transition temperature evaluation, and viscosity measurement were conducted as part of the characterization studies. Based on the evaluation of all data, the most suitable formulation(s) with optimal properties were selected, and accelerated stability studies were performed.*
Result and Discussion: *The combination of hyaluronic acid, polyacrylamide, and carboxymethyl cellulose was found suitable for in-situ hydrogel formulations with high water retention capacity, biocompatibility, and prolonged ocular residence time. During the 7-day accelerated in vitro stability study, no significant changes were observed in the pH and viscosity values of the formulations.*
Keywords: *Carboxymethyl cellulose, cornea, hyaluronic acid, in-situ hydrogel, polyacrylamide*

GİRİŞ

Kornea, göz küresinin ön kısmında yer alan şeffaf, damar bulunmayan, göz içeriğini koruyan, ışığın kırıldığı ilk tabakadır. Şekil 1'de gösterilen beş farklı katmandan meydana gelen doğal bir bariyer görevi görmektedir [1]. Özellikle cerrahi işlem sonrasında bu yapının iyileşmesi ve kendini onarım sürecinde göz saydamlığını koruyarak bulanık görmeyi önleyecek, kornea üzerini nemli tutarak üzerinde oluşabilecek kırılma ve basıncın önüne geçebilecek tedavi yaklaşımları epitelizasyonu hızlandırmaktadır. Bu yapının nemli kalması için güncel tedavi yaklaşımları arasında gözyaşı filminin viskozitesini artırmak ve nem kaybını önlemek yer almaktadır. Bu doğrultuda, yapay gözyaşı ürünlerinin kullanımı, viskozite artırıcı maddeler, yağlı maddeler gibi bileşenlerden oluşan ürünlerin kullanımı karşımıza çıkmaktadır [2,3]. Geleneksel topikal uygulama olarak göz damlalarının göz kırpma ve nazolakrimal drenaj gibi nedenlerle göz yüzeyinden hızla akarak uzaklaşması, gözyaşı ile seyrelerek istenilen konsantrasyonda kalamaması, enzimatik bozulmaya uğraması gibi birçok dezavantajları bulunmaktadır. Bu dezavantajlardan dolayı deneysel çalışmalar göz yüzeyinde daha uzun süre kalabilen, hastaya minimum rahatsızlıkla daha az dozda ilaç uygulamayı hedefleyen yenilikçi sistemler üzerine yoğunlaşmaktadır [4].



Şekil 1. Korneayı oluşturan 5 tabakanın anatomik görseli [1]

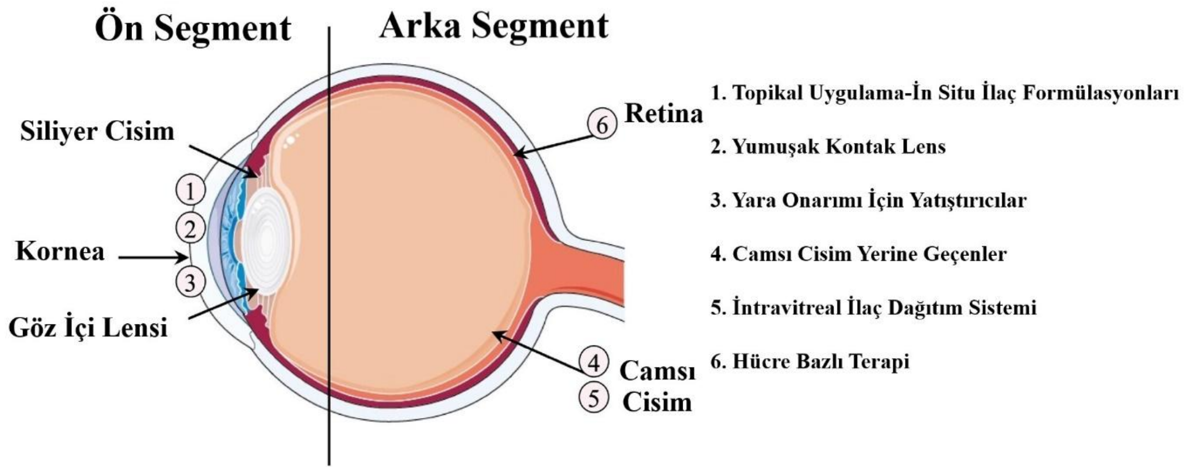
Hidrojeller yapısında önemli miktarda suyu muhafaza edebilen, üç boyutlu hidrofilik polimerik ağ yapısına sahip, çeşitli özelliklerine göre birçok alt gruba ayrılan, hedeflenmiş ve kontrollü ilaç salımı sağlayan ilaç taşıyıcı sistemlere entegre edilebilen jel yapıdadır [5,6]. En önemli avantajları arasında yumuşak ve esnek yapıları ile kolay şekillenebilmeleri, canlı dokuya benzer profil oluşturmaları, şişme davranışlarının pH, sıcaklık, iyonik/elektromanyetik kuvvetler gibi faktörlerle kontrol edilebilmeleri yer almaktadır. Böylelikle hidrojellerin şekil, mekanik ve kimyasal özellikleri uygulanması istenilen hedef bölgeye göre ayarlanabilmektedir [6,7]. Geniş uygulama alanına sahip olan hidrojeller, farklı fiziksel ve kimyasal yöntemlerle elde edilebilmektedirler. Fiziksel yöntemlerle oluşturulan hidrojeller kovalan olmayan etkileşimlerle (hidrojen bağları, van der waals kuvvetleri, iyonik etkileşimler gibi) meydana

geldiğinden tersinir jeller olarak da kabul edilmektedirler [8]. Kimyasal yöntemlerle oluşturulan hidrojeller kovalan çapraz bağlı ağlardan oluşmasından ötürü kalıcı/kimyasal jeller olarak da isimlendirilmektedirler [9].

Hidrojeller, "Önceden Jelleştirilmiş Sistemler" ve "İn-situ Jelleşen Sistemler" olmak üzere iki temel mekanizma ile jelleşme göstermektedirler. Önceden jelleştirilmiş sistemler, hali hazırda jelleşmiş bir yapının basit viskoz özelliklere sahip jeller halinde paketlenmesiyle oluşturulmaktadır [10]. İn situ jelleşen hidrojeller ise uygulama sırasında pH, sıcaklık, iyon dengesi, enzim gibi faktörler ile karşılaşması sonucu jelleşen sistemlerdir. İn-situ jelleştirme sisteminin parenteral olmayan yollardan (oküler, nazal, bukkal, vajinal, gastrointestinal, intravezikal) uygulanan lokal veya sistemik ilaçların etkinliğinin artırılması, bunların etki/absorpsiyon bölgesinde kalış sürelerinin uzatılması gibi faktörlerde oldukça başarılı olduğu kanıtlanmıştır [11].

Günümüzde biyomedikal uygulamalar için biyobozunur ve biyouyumlu polimerlerin kullanımı üzerine araştırma ve geliştirme çalışmalarına yoğunlaşıldığı görülmektedir. Bu çalışmalardan biri de *in-situ* hidrojellerin ilaç taşıyıcı sistemlere entegre edilmesidir. İn-situ implantlar, biyolojik olarak parçalanabilen biyouyumlu çözücü sistemlerde polimer çözeltisi içerisinde çözünen veya süspanse edilen aktif madde taşıyan sistem olarak tanımlanmaktadır [12]. Hidrojellerin düşük invaziv yöntemler kullanılarak yüksek ilaç etkinliği göstermeleri ve istenilen hedef bölgede sürekli salım sağlamalarıyla implante sistemlerde sıklıkla kullanıma sahip olmaktadırlar. Ayrıca biyolojik sıvıda parçalanmaları sonucu toksik madde oluşturmamaları da onları diğer sistemlere göre daha fazla ön plana çıkartmaktadır [13].

İn-situ jelleşen ilaç taşıyıcı sistemlerin sıklıkla kullanımı tercih edilen uygulama bölgelerinden biri oküler bölgedir. Uygulamadan önce sol halinde bulunan jel, oküler bölgedeki sıcaklık, gözyaşı sıvısındaki iyonlar ve pH gibi çevresel uyaranlar ile faz geçişine uğrayarak o bölgede kalış süresinin uzun olduğu viskoelastik jellere dönüşmektedir [14]. Akıllı hidrojellerin Şekil 2'de göz anatomisi üzerinde uygulama bölgeleri sunulmaktadır [15].



Şekil 2. Akıllı hidrojellerin göz anatomisi üzerinde uygulama bölgeleri [15]

Hidrojellerin oküler tedavide topikal uygulama, intrakameral enjeksiyon, intravitreal enjeksiyon gibi çoklu uygulama yollarıyla farklı etki bölgelerine uygulanabilmektedir [16]. Araştırma ve geliştirme çalışmalarının ilerlemesiyle birlikte hidrojel içerisine hem hidrofilik hem de hidrofobik etkin maddelerin eş zamanlı yüklenmesine imkân tanıyan kapsüllenebilir nanokompozit hidrojel oluşturulmuş böylelikle farklı ilaçların eş zamanlı verilebilmesine olanak tanıyan sistemler geliştirilmiştir [17].

Hidrojellerin biyolojik sıvıda uyumlu olmaları onları doku mühendisliğinde enjekte edilebilir hidrojeller olarak gelişmesine de olanak tanımaktadır. Enjekte edilebilir hidrojeller, uygulama alanına uyum sağlarken minimum rahatsızlıkla vücudun çeşitli bölgelerine verilebilmekte, böylece ilaç salımı ve doku mühendisliği uygulamaları için çok yönlü bir sistem sağlamaktadır [18].

Ahsan ve arkadaşlarının geliştirmiş oldukları disülfiram taşıyan ısıya duyarlı kitosan bazlı hidrojeller, kanser tedavisi için enjekte edilebilir antikanser bir formülasyon örneği olarak sunulmuştur [19]. Kemoterapötik ajanların tümörlere uygulanmasında hidrojel bazlı ilaç taşıyıcıların kullanılması hedef dışı olan sağlıklı hücreler ve dokular üzerindeki olumsuz etkilerini azaltmak için umut verici adaylar arasında yer almaktadır [20].

Fathi ve arkadaşları kitosanın ısıya ve pH'a duyarlı enjekte edilebilir doksorubisin yüklü hidrojel formülasyonu meme kanseri tedavisi için enjekte edilebilir ilaç taşıyan bir diğer örnek olarak verilebilir [21].

Ren ve arkadaşları Parkinson hastalığının tedavisi için dopamin ve bir anti-inflamatuar ilaç olarak enkapsüle halde metronidazol içeren uzun etkili hidrojel sistemini parenteral kullanım için hazırlamışlardır [22].

FDA/EMA tarafından onaylı enjekte edilebilir hidrojel sistemleri arasında SpaceOAR® Hidrojel, Vantas®, Radiesse®, Artefill®, Sculptra®, Belotero Balance® (+) Lidokain, Teosyal® RHA, Belotero Balance® gibi preparatlar yer almaktadır [18].

Göz yapısındaki gözyaşı içeriği, pH'sı ve göz içi sıcaklığı gibi fizyolojik faktörlerden dolayı *in-situ* jelleşen hidrojellerden sıcaklığa, iyona ve pH'ya duyarlı olan hidrojeller oküler tedavide sıklıkla tercih edilmektedir [23].

Yukarıda açıklanan bilgiler doğrultusunda, çalışmanın temel hedefi; farklı *in-situ* jelleşen polimer gruplarından seçilen bileşenlerle, kornea ameliyatları sonrasında gözün nem dengesini sağlayarak oküler onarıma destek olabilecek hidrojel formülasyonlarının geliştirilmesidir. Bu kapsamda, HA, PAA ve CMC polimerleri kullanılarak, göz pH'sına ve izotonisite değerlerine uygun, steril bir *in-situ* hidrojel ürününün formüle edilmesi amaçlanmıştır. Ayrıca, geliştirilen formülasyonların *in vitro* karakterizasyon çalışmalarıyla viskozite, şişme/erozyon davranışı ve jelleşme kapasitesi gibi önemli parametrelerinin değerlendirilmesiyle, optimum özelliklere sahip formülasyonun belirlenmesi hedeflenmiştir.

Çalışmamızın yenilikçi yönü ise, oküler yüzeyde jelleşme yeteneğine sahip *in-situ* hidrojel formülasyonlarının tasarlanmasıdır. Bu formülasyonlar, göze damlatıldığında sıvıdan jel fazına geçerek göz yüzeyinde koruyucu bir tabaka oluşturmakta ve gözyaşı kaybını önlemeye yardımcı olmaktadır. Çalışma kapsamında, kornea ameliyatlarından sonra ortaya çıkan göz kuruluğunun önlenmesi ve hastaların nem ihtiyacının uzun süreli karşılanması için yenilikçi *in-situ* hidrojel formülasyonları geliştirilmesi planlanmaktadır. Bununla birlikte, bu çalışmada kullanılan polimerlerin gözyaşının doğal biyoaktif bileşenlerinden olması nedeniyle, hazırlanan hidrojel formülasyonlarının göz kuruluğu kaynaklı rahatsızlıkları azaltacağı ve göz yüzeyinin iyileşmesine destek sağlayacağı düşünülmektedir.

GEREÇ VE YÖNTEM

Çalışma kapsamında kullanılan malzemeler; Hyaluronik asit (HA) Bloomage BioTechnology Corporation Limited; Sitrik asit Vasa Pharmachem Pvt. Ltd; Karboksimetil selüloz (CMC) Yiğitoğlu Kimya A.Ş.; Poliakrilamid (PAA) Sigma Aldrich'ten proje sanayi ortağımız Argis İlaç San. & Tic. A.Ş. tarafından tarafımıza temin edilmiştir. Hazırlanan tüm *in-situ* hidrojellerde distile su kullanılmıştır.

Fiziksel karıştırma esas alınarak hazırlanan *in-situ* hidrojellerin göz fizyolojisine uyumluluğu pH ve viskozite ölçümü, sol-jel geçiş sıcaklığı ve şişme/erozyon değerlendirmesi çalışmaları yapılarak kontrol edilmiştir. Göz yapısına en yakın ve kalış süresi en uzun olabilecek optimum özelliklere sahip formülasyon/ların hızlandırılmış *in vitro* stabilite çalışmaları gerçekleştirilerek saklama koşulları belirlenmiştir.

***İn-Situ* Hidrojel Hazırlanışı**

Çalışmada HA %0.1, %0.25, %0.5; CMC %0.5; %1.0; PAA %0.1; %0.2; Sitrik asit %0.01 konsantrasyonlarında olacak şekilde 12 adet *in-situ* hidrojel formülasyonu Tablo 1'de gösterildiği gibi tasarlanmıştır. Karakterizasyon çalışmalarında kullanılmak üzere her bir formülasyondan 50 ml olacak miktarda fiziksel karıştırma yöntemi esas alınarak hazırlanmıştır [24].

Darası alınmış beher içerisine hesaplı miktarda distile su alınmıştır ve 37°C sıcaklığa getirilmiştir. Hesaplanan HA beher içerisine serpiştirilerek manyetik karıştırıcıda homojen karışması sağlanmıştır. Homojen karışım gerçekleştikten sonra CMC, sitrik asit ve PAA aynı şekilde sırayla beher içerisine

serpiştirilmiş ve tekrar karıştrılmıştır. Karışım sırasında su kaybını minimuma indirmek için saat camı ile beherin ağzı kapatılmıştır [25].

Tablo 1. Hidrojel formülasyonların içerik tablosu

Formülasyon Kodu	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
Hyaluronik asit (%)	0.1	0.1	0.1	0.1	0.25	0.25	0.25	0.25	0.5	0.5	0.5	0.5
Karboksimetil Selüloz (%)	0.5	1.0	1.0	0.5	0.5	1.0	1.0	0.5	0.5	1.0	1.0	0.5
Poliakrilamid (%)	0.1	0.1	0.2	0.2	0.1	0.1	0.2	0.2	0.1	0.1	0.2	0.2
Sitrik asit (%)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Distile su	y.m	y.m	y.m	y.m	y.m	y.m	y.m	y.m	y.m	y.m	y.m	y.m

İn Vitro Karakterizasyon Çalışmaları

Hazırlanan *in-situ* hidrojellerin pH ölçümü, viskozite ölçümü, sol-jel geçiş sıcaklığı ve şişme/erozyon değerlendirmesi gibi literatürde tanımlanan karakterizasyon çalışmaları gerçekleştirilmiştir. Deneysel çalışmalar sonucunda göze uygulanabilecek optimum özellikteki formülasyonlar seçilerek hızlandırılmış *in vitro* stabilite değerlendirmeleri yapılmıştır.

pH Ölçümü

F1 formülasyonundan başlayarak, her formülasyon için pH ölçümü dijital pH metre (Mettler Toledo) ile üç kez tekrarlanmış ve bu tekrarlardan elde edilen değerlerin ortalamaları alınmıştır (n=3). Ölçüm sonuçlarına göre formülasyonların pH değerlerinin, belirlenen 6.5-7.5 aralığına uyum sağlaması için nötralizasyon işlemi gerçekleştirilmiştir. Formülasyonların pH değerlerini 6.5-7.5 aralığına getirmek amacıyla, 1N NaOH çözeltisi kullanılarak nötralize edilen hidrojellerin pH ölçüm işlemi için 3:10 (3 g hidrojel:10 g distile su) oranında seyreltilmiş numune hazırlanmıştır. Seyreltilen formülasyonların pH değerleri tekrar ölçülmüş ve elde edilen değerler Tablo 2'de verilmiştir [26].

Viskozite Ölçümü

İn-situ jelleşen hidrojel formülasyonlarının viskoziteleri, 22-23°C sıcaklık aralığında Brookfield DVI Prime viskometre cihazı kullanılarak ölçülmüştür. Ölçüm sırasında S14 disk spindle ile sırayla önce 5, 10, 20, 50, 100 kayma hızında (rpm) daha sonra tekrar sırayla 100, 50, 20, 10, 5 rpm'de viskozite (cP) değerlerinin okuması gerçekleştirilmiştir. Elde edilen okuma değerlerine dayanarak, viskozite (cP) ve kayma hızı (rpm) arasındaki ilişkiyi gösteren grafikler Şekil 3,4 ve 5'te sunulmuştur [26,27].

Sol-Jel Geçiş Sıcaklığı

İçerisinde *in-situ* hidrojel formülasyonu bulunan bir test tüpü eğildiğinde, çözeltinin 30 saniye boyunca akış göstermesi durumunda sol fazı, akışın gözlemlenmediği durumda ise jel fazı olarak tanımlanmaktadır [28,29]. Santrifüj tüplerine alınan 1 mL'lik hidrojeller 25 °C sıcaklıktaki su banyosuna daldırılmış ve sıcaklık 2°C kademeli bir şekilde göz sıcaklığı olan 34 °C'ye kadar kontrollü bir şekilde artırılarak sol-jel geçişi gözlemlenmiştir [29,30]. Deneyin sol-jel geçiş sonuçları Şekil 6,7, 8, 9 ve 10'da sunulmuştur.

Şişme/Erozyon Değerlendirmesi

Darası alınmış flakonlara 1 ml sol-jel geçişi yapabilen hidrojel formülasyonları eklenmiş ve 60°C etüvde (NUVE FN500) ters çevrildiğinde akışın olmadığı ana kadar kurutulmuştur. Ardından, tartım yapılarak kuru jel ağırlığı not edilmiştir (W_0). Bu değer, T_0 anındaki ağırlık miktarı olarak tanımlanmaktadır [26]. Her bir flakon üzerine 1 ml pH 7.4 (PBS) tamponu akıtılarak, su banyosunda göz içi sıcaklık değeri olan 34°C'de bekletilmiştir. Belirlenen zaman aralıklarında (0, 3 ve 7. gün) üstte kalan pH 7.4 fosfat tamponu çözeltisi alınarak hidrojellerin etüvde kuruması sağlanmıştır. Daha sonra tartım yapılarak zamana karşı % jel erozyon grafiği oluşturulmuştur [31,32].

% Ağırlık Kaybı (Jel Erozyonu): $\%100 \times (W_0 - W_t)/ W_0$ eşitliği kullanılarak hesaplanmıştır [33,34]. Elde edilen sonuçlar Tablo 3 ve Şekil 11,12 ve 13'te sunulmuştur.

Optimum Özelliklere Sahip Formülasyon(lar)ın Seçimi

Karakterizasyon çalışmaları ve değerlendirmeler sonucunda, hazırlanan 12 *in-situ* hidrojel formülasyonunun pH ve viskozite değerleri, akış özellikleri, sol-jel geçiş sıcaklıkları ile şişme ve erozyon davranışları incelenmiş ve en uygun formülasyonlar F9 ile F12 olarak belirlenmiştir. Her iki formülasyon da göz ile uyumlu pH aralığında (6.5-7.5) kalarak oftalmik uygulamalar için kritik bir gerekliliği karşılamaktadır. Bu pH aralığı, gözde irritasyon riskini en aza indirerek uygulama sonrası hasta konforunu artırmaktadır. Ayrıca, F9 ve F12'nin viskozite değerleri, hem yüksek mekanik dayanıklılık sağlamakta hem de düşük kayma hızında yüksek viskozite göstererek gözde daha uzun süre kalabilme potansiyeline işaret etmektedir. Bu özellik, formülasyonların etkinliğini artırmaktadır. Akış özelliklerinin incelenmesi, uygulama sırasında rahatlık ve etkinlik açısından önemli bir diğer parametredir; F9 ve F12, uygun akış profilleri ile oftalmik kullanımda kolaylık sağlayacak şekilde değerlendirilmiştir. Bunun yanı sıra, bu formülasyonların göz sıcaklığında jel fazına geçiş yapabilme yetenekleri, tedavi etkinliğini artıran bir diğer önemli özelliktir. Şişme ve erozyon davranışlarının değerlendirilmesi, formülasyonların stabilitesini ve uzun süreli etkilerini belirlemek açısından kritik bir rol oynamış ve F9 ile F12'nin bu yönlerden de uygun sonuçlar verdiği görülmüştür. Bu nedenle, seçilen formülasyonların stabiliteleri, oda sıcaklığında karanlıkta ve gün ışığında, ayrıca buzdolabında (4-8°C) muhafaza edilerek değerlendirilecektir. Stabilite testleri, formülasyonların fiziksel ve kimyasal özelliklerinin zamanla nasıl değiştiğini belirlemek açısından önem taşımaktadır. Özellikle oftalmik uygulamalarda, formülasyonların stabilitesinin sağlanması, etkinliğin ve güvenliğin korunması açısından büyük önem taşımaktadır. Bu testler sonucunda, F9 ve F12 formülasyonlarının hangi koşullarda en iyi performansı gösterdiği belirlenerek, gelecekteki uygulamalar için en uygun muhafaza koşulları tanımlanacaktır.

Stabilite Çalışmaları

Göze uygulanacak preparatların, raf ömrü boyunca fizyolojik sınırlar içinde kalması arzu edilmektedir [27]. Bu doğrultuda, karakterizasyon çalışmaları sonucunda en iyi performansı gösteren F9 ve F12 *in-situ* hidrojel formülasyonlarının stabilitesini değerlendirmek amacıyla, 1. gün (t_1) ve 7. gün sonunda (t_7) görünüm, viskozite ve pH ölçümleri yapılmıştır. Bu ölçümler, formülasyonların farklı saklama koşullarına maruz bırakılmasının ardından gerçekleştirilmiştir. Örnekler, karanlıkta 25°C'de, gün ışığında 25°C'de ve buzdolabında (4-8°C aralığında) saklanarak stabiliteleri değerlendirilmiştir [27,35].

F9 ve F12 hidrojel formülasyonlarının farklı saklama koşulları altındaki davranışlarının değerlendirilmesi, hangi saklama ortamının formülasyonların daha iyi performans göstermesine olanak sağladığını anlamak açısından önemlidir. Bu testler sayesinde, formülasyonların pH ve viskozite gibi temel parametrelerinin zamanla nasıl değişim gösterdiği izlenerek, preparatların güvenilirliğini destekleyecek değerli veriler elde edilmiştir. Özellikle pH seviyesinin korunması, göz dokularında tahrişe yol açmamak için kritik bir unsur olurken, viskozitenin uygun aralıkta kalması ise preparatın göz yüzeyinde tutunma süresi ve uygulanabilirliği açısından gereklidir. Elde edilen sonuçlar, formülasyonların stabilitesini korumak için en uygun saklama koşullarının belirlenmesine rehberlik etmiş ve klinik uygulamalarda hasta güvenliği ile etkinliğini artırmayı hedeflemiştir. Bu veriler, oftalmik ürünlerin raf ömrü boyunca etkinliğini ve güvenliğini optimize etmek için temel bir kaynak oluşturmaktadır.

Her bir formülasyondan, gün ışığında, karanlıkta ve buzdolabında saklanmak üzere ayrı ayrı 50 ml olacak şekilde toplamda 150 ml'lik numuneler hazırlanmıştır. Bu numunelerde, saklama koşullarına göre gerekli viskozite ve pH ölçümleri gerçekleştirilmiş, elde edilen veriler doğrultusunda formülasyonların stabilitesi ve saklama koşulları hakkında kapsamlı değerlendirmeler yapılmıştır. Bu yaklaşım, formülasyonların stabilitesini ve performansını etkileyebilecek çevresel faktörlerin etkisini anlamak adına önemli bir adım olarak geliştirilmiştir. Stabilite değerlendirmesi, numunelerin 1. ve 7. günlerinde alınan pH ve viskozite ölçüm sonuçlarına dayandırılmıştır. Elde edilen bulgular, formülasyonların saklama koşullarına bağlı olarak nasıl değişiklikler gösterdiğini ortaya koymak amacıyla Tablo 4, 5, 6 ile Şekil 14-17'da sunulmuştur. Bu bulgular, özellikle oftalmik formülasyonların raf ömrü boyunca etkinlik ve güvenliğini koruyabilecek saklama koşullarının belirlenmesinde rehber

niteliği taşıyacak şekilde değerlendirilmiştir. Ayrıca, saklama koşullarının formülasyonların fiziksel özellikleri üzerindeki potansiyel etkilerini ve en ideal saklama koşullarının güvenilirliğe katkısını netleştirmek amacıyla değerli bilgiler sağlanmıştır.

SONUÇ VE TARTIŞMA

Bu bölümde, *in vitro* karakterizasyon çalışmalarından elde edilen bulgular detaylı bir şekilde değerlendirilmiş ve bu sonuçların formülasyonların etkinliği, stabilitesi ve klinik potansiyeli üzerindeki etkileri tartışılmıştır. Çalışma kapsamında formülasyonların pH, viskozite, akış özellikleri, sol-jel geçiş sıcaklıkları, şişme ve erozyon davranışları incelenmiş ve sonuçlar belirli kriterler doğrultusunda analiz edilmiştir.

pH Ölçümü Verilerinin Değerlendirilmesi

Formülasyonların göz ile uyumlu olması ve tahriş ya da rahatsızlık hissi oluşturmaması hedeflenmiştir. Bu bağlamda, hazırlanan hidrojellerin pH değerlerinin gözle uyumlu bir aralık olan 6.5-7.5 değerlerinde olması beklenmektedir [30]. Nötralizasyon işlemi sonrasında *in-situ* hidrojellerin pH değerleri 6.5-7.5 aralığında olduğu sonucu Tablo 2'de sunulmuştur. Yapılan bu ölçümler, formülasyonların göz ile uyumlu olmasını sağlayarak, göz sağlığını koruyacak şekilde tasarlandığını ve gözde tahriş veya rahatsızlık hissi oluşturmayacak nitelikte olduğunu göstermektedir. Bu tür bir uyum, özellikle oftalmik uygulamalar için kritik öneme sahiptir, zira gözün hassas yapısı nedeniyle pH dengesinin sağlanması, tedavi etkinliğinin artırılması ve kullanıcı konforunun temin edilmesi açısından son derece önemlidir [27].

Tablo 2. *İn-situ* hidrojel formülasyonlarının pH değerleri (n=3)

Formülasyon Kodu	pH±SS
F1	6.75±0.021
F2	7.36±0.007
F3	7.40±0.113
F4	6.59±0.035
F5	6.69±0.085
F6	6.68±0.042
F7	6.53±0.078
F8	6.67±0.007
F9	6.58±0.042
F10	6.83±0.021
F11	7.68±0.028
F12	7.07±0.092

*SS: Standard Sapma

Viskozite Ölçümü Verilerinin Değerlendirilmesi

Yapılan viskozite ölçümleri, formülasyonların akış özelliklerini ve jelleşme davranışını değerlendirmek amacıyla gerçekleştirilmiştir. Bu ölçümler, hidrojel formülasyonlarının uygulama esnasında nasıl davrandığını anlamak için kritik öneme sahiptir. Özellikle, viskozite değerlerinin belirlenmesi, *in-situ* jelleşen sistemlerin hedef bölgeye uygulanabilirliğini ve bu süreçte kontrol edilebilirliği artırmaktadır [36-38].

Hidrojel formülasyonlarının viskozitesinin sıcaklık değişimi ile nasıl etkilendiği de göz önünde bulundurulmalıdır; çünkü sıcaklık artışı genellikle viskoziteyi azaltırken, sıcaklık düşüşü viskoziteyi artırabilir. Bu özellik, oftalmik uygulamalarda ve diğer hedeflendirilmiş tedavi yöntemlerinde, formülasyonun uygun viskozitede olup olmadığını ve gerektiğinde jelleşme sürekliliğini sağlamaktadır [35].

Tüm *in-situ* jelleşen hidrojel formülasyonları, tiksotropik özellikler sergileyerek kayma ile incelen (shear-thinning) davranış göstermektedir. Bu akış durumunda, görünür viskozite değerleri belirli zaman aralıklarında azalma göstermektedir. Ayrıca, kayma durduktan bir süre sonra dinlenmiş akışkanlar, başlangıçtaki yapılarına kısmen veya tamamen geri dönebilirler [26,36,37]. Şekil 3,4 ve 5'te formülasyonların HA yüzdelerine göre kayma hızına göre viskozite değerlerini içeren reogramları gösterilmektedir.

Tüm hidrojel formülasyonlarının karşılaştırmasının daha kolay ve anlaşılır olması açısından grafikler HA yüzdesi değişimine göre gruplandırılmıştır. Hidrojel formülasyonlarının sınıflandırılmasında temel kriter olarak HA yüzdesinin temel alınmasının nedeni, CMC ve PAA yüzdesindeki değişimlerin viskozite değerlerine olan etkisinin, HA yüzdesindeki değişimlere kıyasla daha sınırlı olmasıdır.

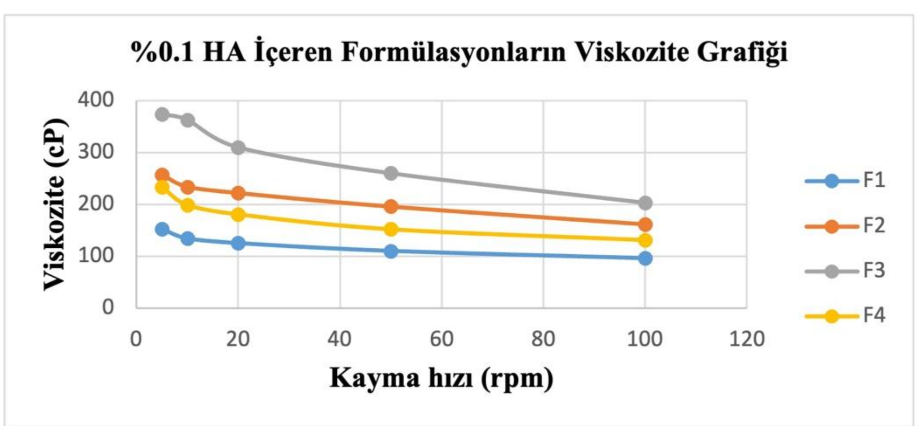
F1 ve F2 formülasyonlarının kıyaslanmasında, F1'in viskozitesi 152 cP iken, F2'nin viskozitesi 257 cP olarak ölçülmüştür. Bu fark, CMC oranının %0.5'ten %1.0'a çıkarılmasından kaynaklanmaktadır. Benzer şekilde, F1 ve F4 formülasyonlarının karşılaştırılması sonucunda, F1'in viskozitesi 152 cP, F4'ün viskozitesinin ise 243 cP olduğu belirlenmiştir ve bu artış, PAA oranının %0.1'den %0.2'ye yükseltilmesiyle ilişkilidir. F1 ve F5 formülasyonlarının değerlendirilmesinde ise, F1'in viskozitesi 152 cP, F5'in viskozitesi ise 562 cP olduğu gözlemlenmiştir ve bu büyük fark, HA oranının %0.1'den %0.25'e çıkarılması sonucunda meydana gelmiştir.

Yapılan analizler, hidrojel formülasyonlarının viskozite değerlerindeki değişimlerin en güçlü şekilde HA yüzdesindeki artışlarla ilişkilendirildiğini ortaya koymaktadır. CMC ve PAA oranlarındaki değişikliklerin de viskozite üzerinde etkisi bulunmakla birlikte, bu etkiler HA yüzdesindeki değişimlerle karşılaştırıldığında sınırlı kalmaktadır. Bu durum, HA'nın hidrojel yapısındaki kritik rolünden ve polimer ağlarının su tutma kapasitesine olan etkisinden kaynaklanmaktadır. HA, yüksek molekül ağırlığına sahip bir biyopolimer olarak, hidrojel matrisinde su molekülleriyle güçlü etkileşimler kurarak viskozitenin artmasını sağlar. HA'nın hidrofilik yapısı, suyu daha fazla hapsederek polimer zincirlerinin arasındaki kayganlığı azaltır ve böylece viskozite değerlerinin daha keskin şekilde yükselmesine neden olur. Bu etki, özellikle düşük HA oranlarında bile belirgin bir fark yaratmaktadır.

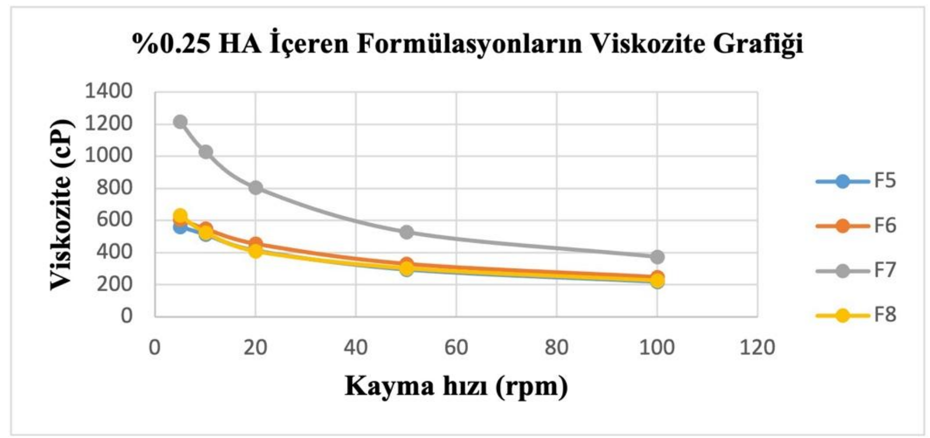
CMC, PAA ve HA oranlarındaki değişimlerin viskozite üzerindeki etkisi, formülasyonların kıyaslanmasıyla net bir şekilde ortaya konmuştur. F1 ve F2 formülasyonları arasında, CMC oranının %0.5'ten %1.0'a çıkarılması viskoziteyi 152 cP'den 257 cP'ye yükseltmiştir. Benzer şekilde, F1 ve F4 formülasyonlarında PAA oranının %0.1'den %0.2'ye çıkarılması viskoziteyi 152 cP'den 243 cP'ye yükseltmiştir. Her iki durumda da viskozitedeki artış önemli olmakla birlikte, sınırlı bir seviyede kalmıştır.

Buna karşılık, F1 ve F5 formülasyonlarının kıyaslanmasında, HA oranının %0.1'den %0.25'e çıkarılması, viskozitede dramatik bir artışa neden olmuş ve değer 152 cP'den 562 cP'ye ulaşmıştır. Bu sonuç, CMC ve PAA değişimlerine kıyasla HA'nın viskozite üzerinde çok daha belirgin bir etkisi olduğunu göstermektedir. HA'nın bu belirgin etkisi, polimer ağının reolojik özelliklerini değiştirme kapasitesine bağlanabilir. HA, yüksek moleküler ağırlığı ve dallanmış yapısı sayesinde çözelti içinde daha yoğun bir polimer ağı oluşturur ve bu ağın su tutma kapasitesini artırır. HA molekülleri, çözelti içindeki serbest su molekülleriyle hidrojen bağları oluşturarak hareketliliklerini sınırlar. Bu durum, polimer zincirlerinin birbirine yakınlaşmasını ve daha sıkı bir yapı oluşturmasını sağlar.

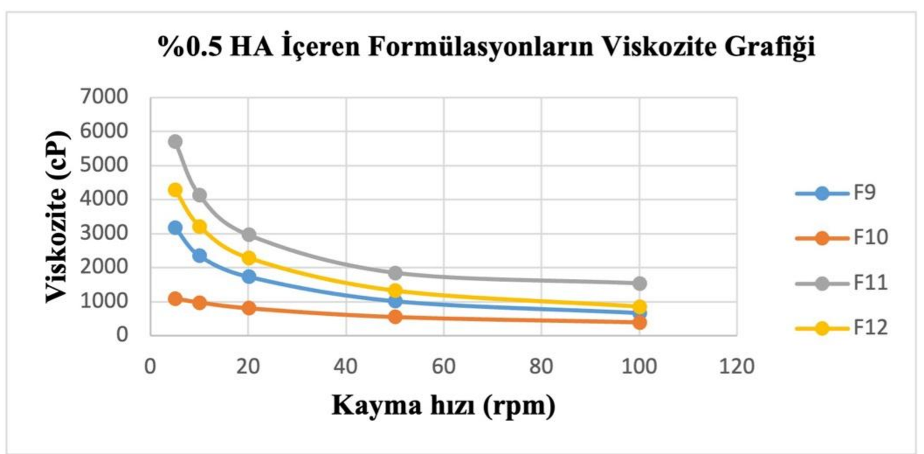
CMC ve PAA gibi diğer polimerler de hidrojel viskozitesine katkıda bulunmakla birlikte, bu polimerlerin düşük konsantrasyonlardaki etkileri daha sınırlıdır. Özellikle PAA, yüksek iyonik yapısı nedeniyle pH ve iyonik güç gibi çevresel faktörlere daha duyarlıdır. Ancak HA'nın viskozite üzerindeki etkisi daha doğrudan ve güçlüdür, çünkü HA yalnızca çözeltinin viskozitesini artırmakla kalmaz, aynı zamanda çözeltinin elastikiyet ve kayma direnci gibi reolojik özelliklerini de önemli ölçüde değiştirir.



Şekil 3. % 0.1 HA içeren formülasyonların reogramları



Şekil 4. % 0.25 HA içeren formülasyonların reogramları



Şekil 5. % 0.5 HA içeren formülasyonların reogramları

Oftalmik preparatların göze uygulandığında, gözyaşı filminin akış etkinliğini olabildiğince az etkilemesi beklenmektedir. Bu nedenle, düşük kayma hızında yüksek viskoziteye, yüksek kayma hızında ise düşük viskoziteye sahip viskoelastik sıvılar tercih edilmektedir. Bu özellikler, formülasyonların göz ile uyumlu olmasını sağlarken, aynı zamanda terapötik etkinliklerini de

artırmaktadır. Dolayısıyla, hidrojel formülasyonlarının tiksotropik davranışları, oftalmik uygulamalar için önemli bir parametre olarak öne çıkmaktadır [39].

Sol-Jel Geçiş Sıcaklığı Verilerinin Değerlendirilmesi

İn-situ hidrojel sistemlerinde sıcaklıkla jelleşme mekanizması, polimer-su etkileşimlerinin sıcaklık değişimine bağlı olarak yeniden düzenlenmesi esasına dayanmaktadır. Sıcaklık arttıkça polimer zincirlerinin konformasyonu değişir ve su molekülleri polimerden ayrılarak çözelti fazından jel fazına geçiş gerçekleşir. Bu süreçte PAA, belirli bir kritik jelleşme sıcaklığında çapraz bağlanma ile fiziksel bir ağ yapısı oluşturur ve hidrojelin viskozitesini artırırken mekanik dayanıklılığını güçlendirir [21]. HA, su molekülleri ile hidrojen bağları kurarak hidrojelin nem tutma kapasitesini artırır ve jel yapısının stabilitesine katkıda bulunur. Bu sayede formülasyonun elastikiyeti ve biyolojik yüzeylere yapışma potansiyeli artar [30,32,39]. CMC ise çözeltinin viskozitesini artırarak jelleşmenin fiziksel dayanıklılığına destek sağlar [40].

Yapılan çalışmada CMC ve PAA yüzdesindeki değişimlerin viskozitede artışa neden olduğu görülse de bu değişimlerin etkisi, HA yüzdesindeki artışla kıyaslandığında sınırlı kalmıştır. HA yüzdesindeki artış, hidrojelin viskozitesinde belirgin bir yükselişe yol açtığından, formülasyonlar arasındaki karşılaştırmanın daha net yapılabilmesi ve sonuçların daha anlaşılır hale getirilmesi amacıyla gruplandırma kriteri olarak HA yüzdesi tercih edilmiştir.

Formülasyonların sol-jel geçiş durumlarına bakıldığında, HA yüzdesi yüksek olan formülasyonlarda jel geçişinin daha belirgin bir şekilde gözlemlendiği görülmektedir. HA yüzdesinin en düşük olduğu (% 0.1) F1, F2, F3 ve F4 formülasyonlarında jelleşme, akışın azalması ile gözlemlense de, Şekil 6'da da görüldüğü gibi tam bir jel fazına geçiş söz konusu değildir. Bu sonuçlar, HA formülasyonların jelleşme özellikleri üzerindeki etkisini vurgulamakta ve göz uygulamaları için gereken jel fazına geçişin sağlanmasında HA oranının önemini göstermektedir.

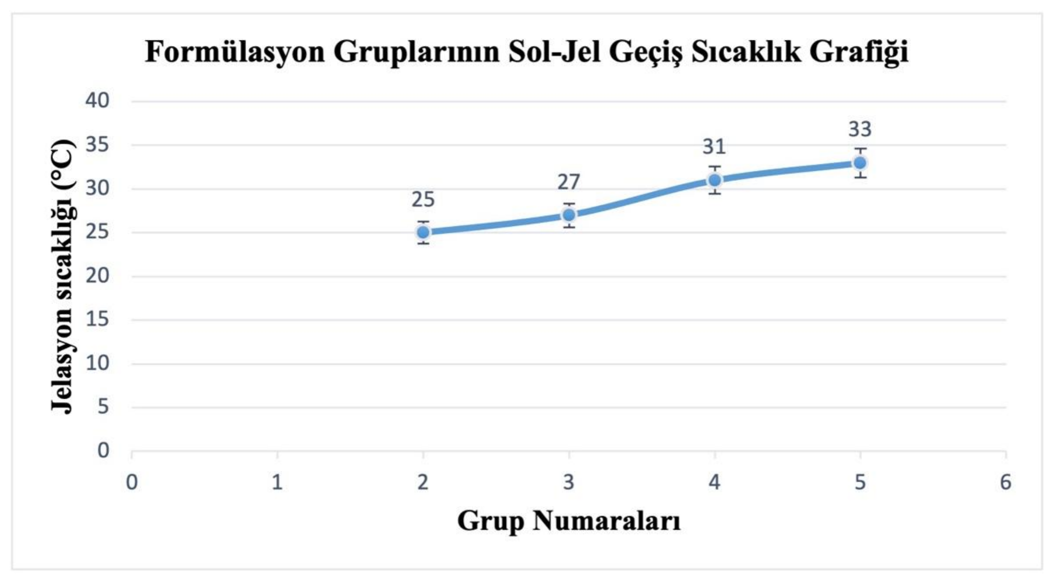
HA yüzdesinin % 0.25 olduğu F5, F6, F7 ve F8 formülasyonlarında ise % 0.1 olan formülasyonlara göre daha fazla jelleşmenin olduğu görülse bile net bir jel geçiş durumundan bahsedilememektedir. Şekil 7'de F5, F6, F7 ve F8 formülasyonlarının ters tüp yöntemi ile sol-jel durumları gösterilmiştir. HA yüzdesinin % 0.5 olduğu F9, F10, F11 ve F12 formülasyonları, Şekil 8'de görüleceği üzere belirli sıcaklıklarda sol fazında kalırken, sıcaklığın değişmesi ile jel fazına geçiş yapabilmektedir. Bu durum, yüksek hyaluronik asit içeriğinin, formülasyonların sıcaklığa bağlı jelleşme özelliklerini olumlu bir şekilde etkilediğini göstermektedir.

Şekil 6. F1, F2, F3 ve F4 Formülasyonların ters tüp yöntemi ile sol-jel durumları

Şekil 7. F5, F6, F7 ve F8 Formülasyonların ters tüp yöntemi ile sol-jel durumları

Şekil 8. F9, F10, F11 ve F12 Formülasyonların ters tüp yöntemi ile sol-jel durumları

Şekil 9'da gösterilen geçiş, jelleşmenin sıcaklıkla tetiklenebildiğini ve dolayısıyla bu formülasyonların belirli bir sıcaklık aralığında etkili bir jel yapısı oluşturduğunu ortaya koymaktadır. Özellikle oftalmik preparatlarda, sıcaklık değişimlerinin gözdeki jelleşme ve kalış süreleri üzerindeki etkisi önemlidir. Gözdeki sıcaklık genellikle 34°C civarında olduğundan, bu formülasyonların sıcaklık değişimleriyle jel fazına geçiş yapabilmesi, gözde kalış sürelerini artırma potansiyeli taşımaktadır [35].



Şekil 9. Formülasyon gruplarının jelasyon sıcaklıkları

***Grup1: F1, F2, F3, F4, F5, F6, F7, F8, F10** (Hiçbir sıcaklıkta jel fazına dönüşmeyen grup)
***Grup 2: F11** (25°C'de jel fazındaki formülasyon grubu)
***Grup 3: F9, F11, F12** (27°C'de jel fazındaki formülasyon grubu)
***Grup 4: F9, F11** (31°C'de jel fazındaki formülasyon grubu)
***Grup 5: F11, F12** (33°C'de jel fazındaki formülasyon grubu)

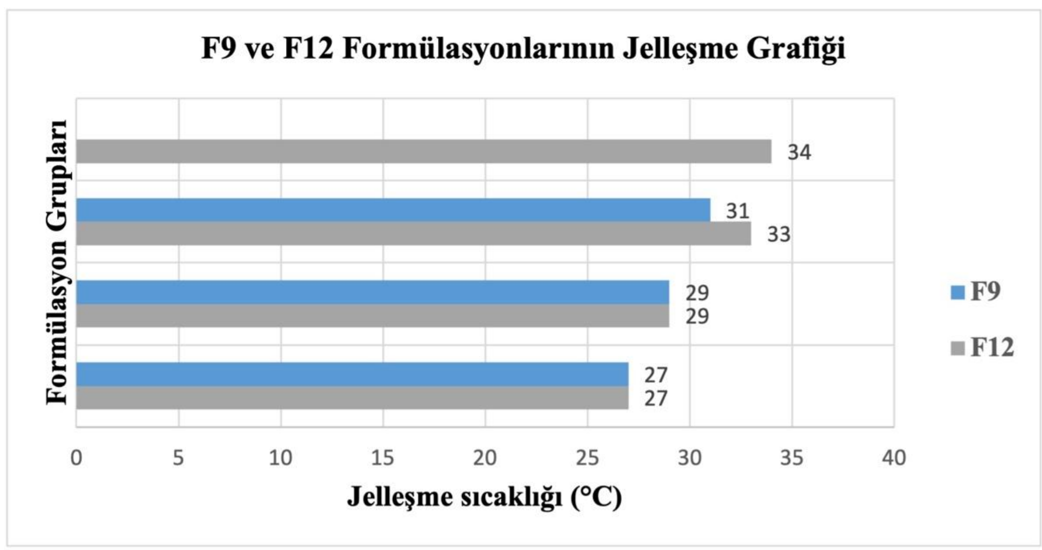
Grup 1'de yer alan formülasyonlar, belirli sıcaklıklarda oldukça yoğun bir viskozluğa sahip olmalarına rağmen, ters çevrilen tüplerde 30 saniye bekletildiğinde yavaş da olsa bir akış gözlemlenmiştir. Bu durum, söz konusu formülasyonların jel fazında olmadığını göstermektedir. Ayrıca, bu formülasyonların viskoziteleri, tam bir sol faz olarak tanımlanabilmesi için yeterince düşük değildir.

F11 formülasyonu, 25-34°C sıcaklık aralığında yapılan tüm deney basamaklarında jel fazında kalmaktadır. Bu durum, F11'in jelleşme özelliklerinin etkili olduğunu ve belirli bir sıcaklık aralığında stabil bir jel yapısı oluşturabildiğini göstermektedir. Göz uygulamaları için gerekli olan jel fazının elde edilmesi, formülasyonun gözde kalış süresini uzatabilir ve terapötik etkinliği artırabilir. Ayrıca, F11'in

bu sıcaklık aralığında jel fazında kalması, oftalmik preparatların gözde konforunu ve etkinliğini artırmak için önemli bir avantaj sunmaktadır. Yüksek sıcaklıkta bile jelleşme sağlanması, formülasyonun gözyaşı filmi ile etkileşimini optimize edebilir ve muhtemel irritasyon veya rahatsızlık hissini azaltabilir. Dolayısıyla, formülasyon içeriği Tablo 1'de belirtilmiş F11 formülasyonunun göz uygulamaları için uygun bir aday olduğunu söylemek mümkündür. Ancak, uzun süreli stabilite ve etkinlik açısından daha fazla inceleme yapılması gerekmektedir.

F9 ve F12 formülasyonları, belirli sıcaklıklarda sol fazında kalırken, belirli bir sıcaklığa ulaştıklarında jel fazına geçiş yapmaktadır. Bu durum, her iki formülasyonun sıcaklığa bağlı jelleşme özelliklerinin etkili olduğunu göstermektedir. Sıcaklık değişiminin jelleşme sürecindeki rolü, oftalmik preparatların etkinliği açısından kritik öneme sahiptir, çünkü gözde kalış süresini artırmak için sıcaklık etkisi ile uyumlu bir jelleşme sağlanması gerekmektedir.

Şekil 10'da belirtilen jelasyon sıcaklık değerleri, Tablo 1'de içeriği belirtilen F9 ve F12 formülasyonlarının hangi sıcaklık aralıklarında etkili bir jel yapısı oluşturduğunu göstermektedir. Bu bilgiler, formülasyonların tasarımında, özellikle göz uygulamaları için optimal sıcaklık aralığının belirlenmesine yardımcı olabilir. Ayrıca, bu formülasyonların jelleşme özelliklerinin sıcaklıkla nasıl etkileşimde bulunduğunu anlamak, formülasyonların stabilitesini ve etkinliğini artırma potansiyelini de ortaya koymaktadır. Sonuç olarak, F9 ve F12 formülasyonlarının sıcaklığa bağlı jelleşme özellikleri, oftalmik uygulamalar için önemli bir avantaj sunmakta olup, bu formülasyonların daha fazla araştırılması, göz tedavileri için potansiyel uygulamalarını geliştirebilir.



Şekil 10. F9 ve F12 formülasyonlarının jelleşme sıcaklık değerleri

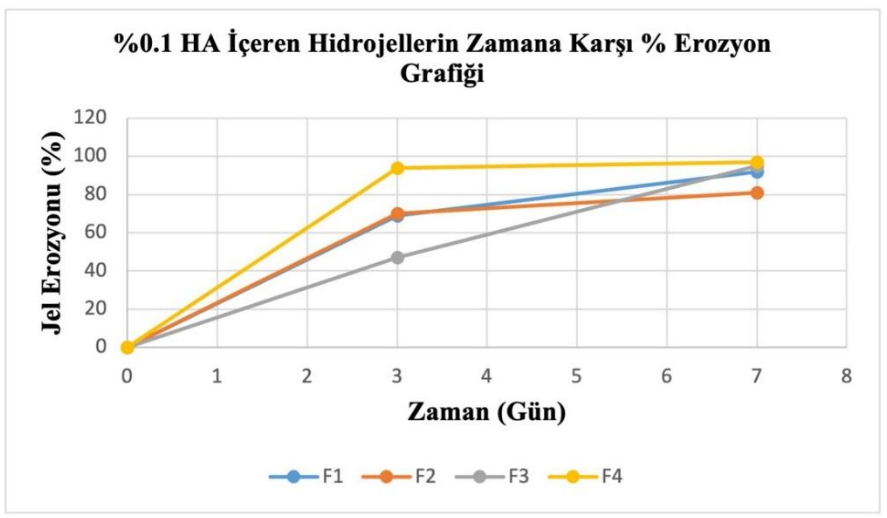
Şişme/Erozyon Verilerinin Değerlendirilmesi

Bu deney, *in-situ* jelleşen hidrojellerin erozyon dayanıklılığını değerlendirmek amacıyla tasarlanmıştır. pH 7.4 fosfat tamponu ortamında jellerin zamanla ne ölçüde ağırlık kaybettiği, erozyon süreçlerinin ve jelleşme stabilitesinin anlaşılmasına yardımcı olacaktır. Ağırlık kaybının zamanla izlenmesi, hidrojellerin biyouyumluluk ve etkinlik özelliklerini anlamak için kritik bir parametredir. Elde edilen veriler, farklı formülasyonların performansını karşılaştırarak, oftalmik uygulamalar için en uygun hidrojellerin seçilmesine yönelik bilgi sağlayacaktır. Ayrıca, zaman aralığına göre ağırlık kaybını incelemek, formülasyonların gözde kalış süresi ve etkinliğini artırmak için gerekli optimizasyonların yapılmasına olanak tanıyacaktır [33,34].

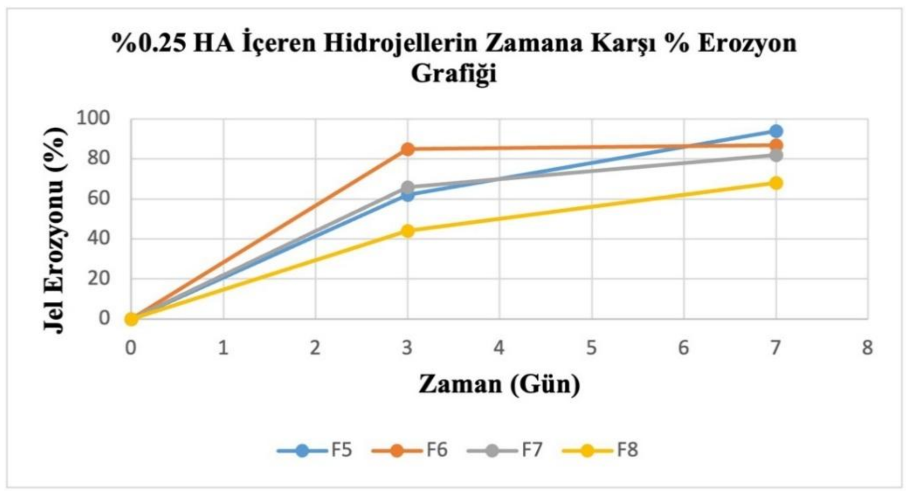
Tablo 3. Hidrojel formülasyonlarının % jel erozyon değerleri

Formülasyon Kodu	T_0 (Başlangıç) % Jel Erozyonu	T_1 (3.gün) % Jel Erozyonu	T_2 (7.gün) % Jel Erozyonu
F1	0	69	92
F2	0	70	81
F3	0	47	95
F4	0	94	97
F5	0	62	94
F6	0	85	87
F7	0	66	82
F8	0	44	68
F9	**0**	**66**	**86**
F10	0	79	88
F11	0	43	49
F12	**0**	**64**	**84**

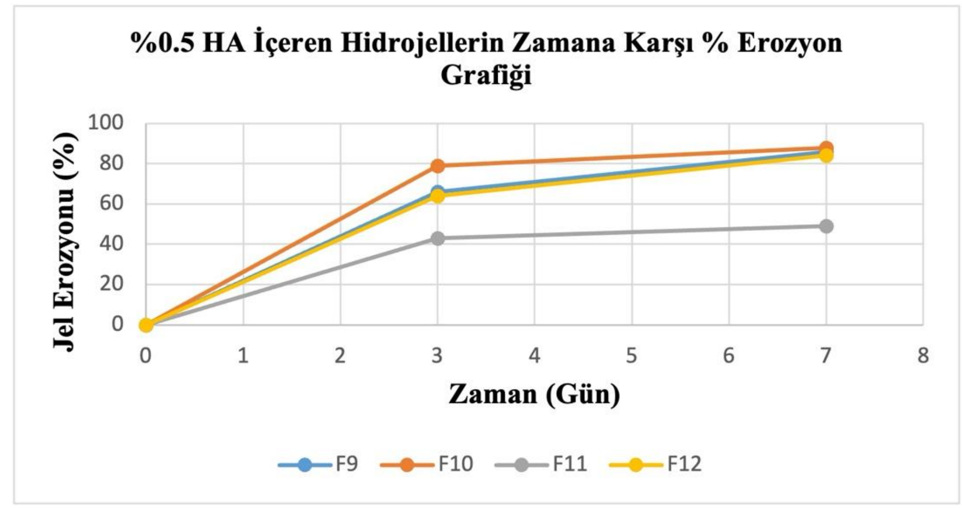
Tablo 3'te yer alan veriler ile Şekil 11,12 ve 13'te yer alan zamana karşı % jel erozyon grafiği oluşturulmuştur.



Şekil 11. % 0.1 HA içeren hidrojellerin zamana karşı % erozyon grafiği



Şekil 12. % 0.25 HA içeren hidrojellerin zamana karşı % erozyon grafiği



Şekil 13. % 0.5 HA içeren hidrojellerin zamana karşı % erozyon grafiği

Hyaluronik asit (HA) konsantrasyonunun artmasıyla birlikte hidrojellerin viskozitelerinin de arttığı bilinmektedir. Bu artış, hidrojelin moleküler yapısının yoğunlaşmasına ve daha karmaşık bir ağ yapısı oluşturmasına neden olur. Grafikler incelendiğinde, viskozitesi artan bir hidrojelin bozunma miktarının azaldığı, yani dayanıklılığının arttığı gözlemlenmektedir. Bu durum, yüksek viskoziteli hidrojellerin, mekanik dayanıklılıkları ve dış etkenlere karşı dirençleri ile göz önünde bulundurulduğunda, oftalmik uygulamalarda daha iyi bir performans sergileyebileceğini göstermektedir. Dayanıklılığı artan hidrojeller, gözde kalış süresini uzatabilir ve terapötik etkinliği artırabilir. Ayrıca, yüksek viskoziteli hidrojellerin, uygulama sırasında gözde oluşabilecek rahatsızlık hissini azaltma potansiyeli bulunmaktadır [32].

Stabilite Çalışmaları

Formülasyonların stabilitesi, hızlandırılmış bir haftalık *in vitro* stabilite testleriyle değerlendirilmiştir. Bu testlerde, her bir formülasyondan gün ışığında, karanlıkta ve buzdolabında (+4°C ile +8°C aralığında) saklanmak üzere 50 ml'lik numuneler hazırlanarak toplamda 150 ml elde edilmiştir. Saklama koşullarına göre dağıtılan numunelerde gerekli viskozite ve pH ölçümleri gerçekleştirilmiştir.

7 günlük hızlandırılmış *in vitro* stabilite çalışmasında, pH ve viskozite değerlerinde anlamlı bir değişiklik görülmemiştir. Ancak Şekil 17'de görsel değerlendirmeler sonucunda, F9 formülasyonunun gün ışığında daha az stabil olduğu anlaşılmaktadır. Verilere dayanarak, F9 formülasyonunun stabilitesini korumak için en uygun saklama koşullarının +4°C ile +8°C aralığında buzdolabında, UV ışığından koruyan amber renkli cam şişelerde saklanması olduğu belirlenmiştir. F12 formülasyonu da benzer koşullarda muhafaza edilebilir; ancak bu formülasyonun homojenliğini korumakta zorlandığı göz önüne alınarak, serin ve karanlık ortamlarda saklanması önerilmektedir. Bu saklama koşulları, formülasyonların uzun vadeli stabilitesini sağlayarak klinik uygulamalarda güvenilirliğini ve etkinliğini korumaya katkıda bulunacaktır.

Tablo 4. 25°C 'de karanlık ortamda bekletilen formülasyonların pH sonuçları (n=3)

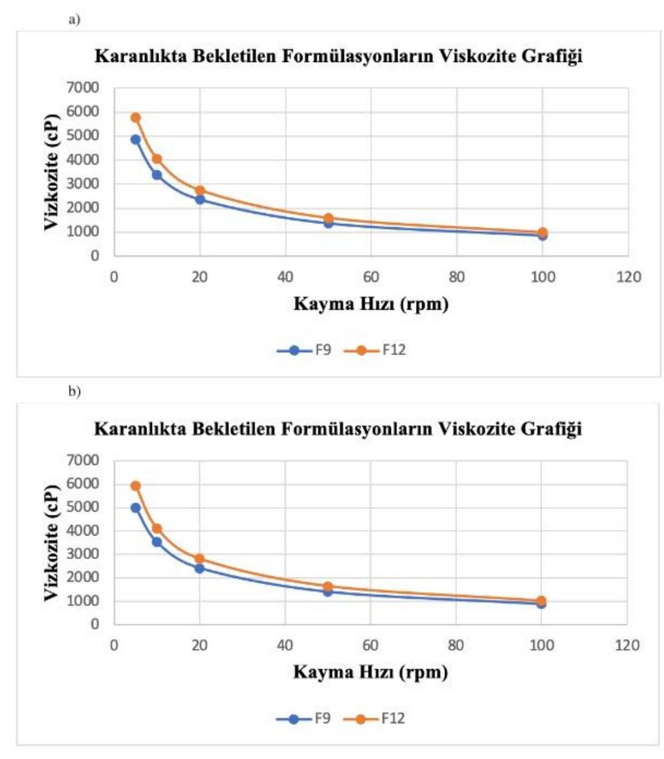
	1.GÜN	**7.GÜN**
Formülasyon Kodu	**pH (Ortalama±SS)**	**pH (Ortalama± SS)**
F9	6.44±0.03	6.54±0.03
F12	6.51±0.03	6.63±0.01

Tablo 5. 25°C'de gün ışığında bekletilen formülasyonların pH sonuçları (n=3)

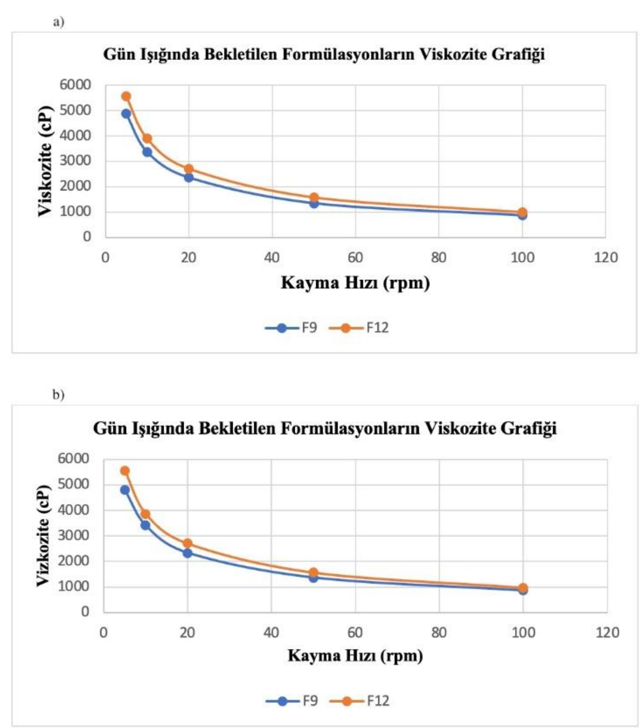
	1.GÜN	**7.GÜN**
Formülasyon Kodu	**pH (Ortalama±SS)**	**pH (Ortalama± SS)**
F9	6.44±0.02	6.62±0.03
F12	6.50±0.03	6.53±0.02

Tablo 6. 4-8°C'de buzdolabında bekletilen formülasyonların pH sonuçları (n=3)

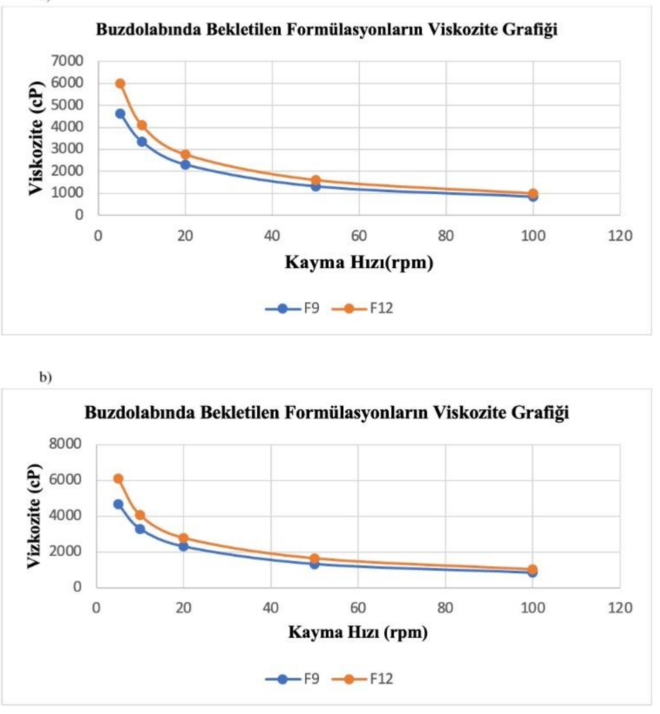
	1.GÜN	**7.GÜN**
Formülasyon Kodu	**pH (Ortalama±SS)**	**pH (Ortalama± SS)**
F9	6.45±0.11	6.46±0.01
F12	6.60±0.01	6.50±0.02



Şekil 14. 25°C'de karanlıkta bekletilen formülasyonların viskozite grafiği a) 1. gün, b) 7. gün



Şekil 15. 25°C'de gün işığında bekletilen formülasyonların viskozite grafiği a) 1. gün, b) 7. Gün



Şekil 16. 4-8°C'de buzdolabında bekletilen formülasyonların viskozite grafiği a) 1. gün, b) 7. Gün

Şekil 17. 25°C'de gün ışığında bekleyen F9 ve F12 formülasyonların görseli a) 1. gün, b) 7. gün

SONUÇ

Çalışmamız kapsamında, kornea ameliyatları sonrasında göz kuruluğunu önlemek amacıyla *in-situ* hidrojel formülasyonlarının geliştirilmesi hedeflenmiştir. İnsan vücudunda doğal olarak bulunan ve yüksek su tutma kapasitesine sahip HA, suda çözünebilen ve şişme özellikleri gösteren PAA ile biyouyumlu ve biyobozunur bir selüloz türevi olan CMC, *in-situ* hidrojel oluşturmak için seçilmiştir. Bu polimerlerin kombinasyonu, göz sıcaklığında sol-jel geçişi yapabilen, uzun süre göz yüzeyinde kalabilen ve gözün nemli kalmasına katkıda bulunan göz dostu hidrojellerin hazırlanmasına imkân tanımaktadır.

Formülasyon çalışmalarında, polimerlerin göze uygulanabilir güvenli konsantrasyon aralıkları belirlendikten sonra pH ölçümü, şişme/erozyon çalışmaları, viskozite ölçümleri ve sol-jel geçiş sıcaklık değerlendirmesi yapılmıştır. Bu testler sonucunda, optimum özellikleri taşıyan *in-situ* hidrojel formülasyonları seçilmiştir. Göz uygulamaları için düşük kayma hızında yüksek, yüksek kayma hızında ise düşük viskozite sergileyen viskoelastik sıvılar tercih edilmektedir. Çalışma kapsamında hazırlanan *in-situ* hidrojellerin tiksotropik özellik gösterdiği, yani shear-thinning (kayma ile incelme) davranışı sergilediği gözlenmiştir. Bu özellik, formülasyonların gözle uyumunu sağlarken, uygulama kolaylığını ve terapötik etkinliği artırmaktadır.

Çalışmada test edilen 12 *in-situ* hidrojel formülasyonun bileşimini Tablo 1'de verilmiştir. Karakterizasyon çalışmaları sonucunda, geliştirilen formülasyonlar arasından F9 ve F12 formülasyonun istenilen özelliklere sahip olduğu belirlenmiştir. Bu formülasyonlar göz yapısının fizyolojik koşulları altında hedeflenen sıcaklık aralıklarında (34-37°C'de) sol-jel geçişi yapabilmesi, istenilen pH aralığında bulunmaları, viskoelastik bakımdan ve diğer formülasyonlara göre şişme/erozyon profillerinin daha uygun olması açısından seçilmiştir.

Saklama koşulları hakkında fikir edinebilmek için gerçekleştirilen hızlandırılmış *in vitro* stabilite çalışmasında F9 ve F12 formülasyonları gün ışığında, karanlıkta ve buzdolabında (+4°C ile +8°C aralığında) 7 gün boyunca bekletilmiştir. Formülasyonların pH ve viskozite değerlerinde anlamlı bir değişiklik gözlenmemiştir. Gün ışığında saklanan F9 formülasyonunun saydamlığında farklılaşma belirlenmiş, F12 formülasyonunda ise jel orta tabakasında halka şeklinde dumanlaşma oluşarak homojen yapı bozulmuştur. Bu bulgu, jel formülasyonlarının stabilitesinin UV ışığı ile olumsuz etkilendiğini kanıtlamaktadır. F9 ve F12 formülasyonlarının stabilitesini korumak için en uygun saklama koşulunun, +4°C ile +8°C aralığında buzdolabında, UV ışığından koruyan amber renkli cam şişeler olduğu belirlenmiştir.

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YAZAR KATKILARI

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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.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

FRUITFUL REMEDIES: ANALYZING THERAPEUTIC POTENTIALS IN ESSENTIAL AND FATTY OILS, AND AQUEOUS EXTRACTS FROM *PRUNUS CERASIFERA*, *MALUS SYLVESTRIS*, AND *CORNUS MAS* USING LC-MS AND GC-MS

MEYVELERDEN ŞİFAYA: PRUNUS CERASIFERA, MALUS SYLVESTRIS VE CORNUS MAS TÜRLERİNDEN ELDE EDİLEN UÇUCU YAĞLAR, YAĞ ASİTLERİ VE SU EKSTRELERİNİN LC-MS VE GC-MS YÖNTEMLERİYLE TERAPÖTİK POTANSİYELLERİNİN ANALİZİ

Bilge AYDIN[1], Hafize YUCA[2], Gözde ÖZTÜRK[3], Enes TEKMAN[4,5], Songül KARAKAYA[5]*, Gamze GÖGER[6], Mehmet ÖNAL[7], Betül DEMİRCİ[3], Zühal GÜVENALP[2], Oksana SYTA[8]

[1]Erzincan Binali Yıldırım University, Faculty of Pharmacy, Department of Pharmacognosy, 24100, Erzincan, Türkiye
[2]Atatürk University, Faculty of Pharmacy, Department of Pharmacognosy, 25240, Erzurum, Türkiye
[3]Anadolu University, Faculty of Pharmacy, Department of Pharmacognosy, 26210, Eskişehir, Türkiye
[4]Ankara University, Graduate School of Health Sciences, 06110, Ankara, Türkiye
[5]Atatürk University, Faculty of Pharmacy, Department of Pharmaceutical Botany, 25240, Erzurum, Türkiye
[6]Afyonkarahisar Health Sciences University, Faculty of Pharmacy, Department of Pharmacognosy, 03218, Afyonkarahisar, Türkiye
[7]Eastern Anatolia Forestry Research Institute, 25050, Erzurum, Türkiye
[8]Slovak University of Agriculture, Institute of Plant and Environmental Sciences, 94976, Nitra, Slovakia

ABSTRACT

Objective: *The chemical composition and bioactive properties of essential oils, fatty oils and aqueous extracts of Prunus cerasifera, Malus sylvestris and Cornus mas were investigated.*
Material and Method: *Antidiabetic, antimicrobial, anticholinesterase and antioxidant activities of P. cerasifera, M. sylvestris and C. mas were reported. The quantitative determination of some secondary metabolites was also analysed by LC-MS/MS. The chemical composition of the essential oils was also investigated by GC-MS.*
Result and Discussion: *Fatty oils' major compounds were oleic acid (77.1%) in P. cerasifera, palmitic acid (32.5%) in M. sylvestris fruits, and linoleic acid (43.2%) in C. mas seeds. Benzaldehyde (70.1%), nonacosane (30.4%), (E,E)-2,4-Decadienal (43.3%) were found as major compounds of P. cerasifera, M. sylvestris fruits, and C. mas seed essential oils, respectively. Quinic acid was predominant compound in all extracts, ranging from 11262.2996 to 18179.6260 ng/ml. C. mas fatty oil was showed antimicrobial activity against Candida albicans and C. parapsilosis with MIC =625-1250 µg/ml. P. cerasifera, C. mas, and M. sylvestris hold potential as α-glucosidase inhibitors, with varying degrees of potency.* IC_{50} *values further underscore effectiveness of aqueous extracts, especially in cases of C. mas and M. sylvestris with <10 and 399 µg/ml.*

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* **Corresponding Author / Sorumlu Yazar:** Songül Karakaya
e-mail / e-posta: snglkarakaya@yahoo.com, **Phone / Tel.:** 0442 231 5250

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ÖZ

Amaç: *Prunus cerasifera, Malus sylvestris ve Cornus mas'ın uçucu yağları, yağ asitleri ve sulu ekstrelerinin kimyasal bileşimi ve biyoaktif özellikleri araştırılmıştır.*
Gereç ve Yöntem: *P. cerasifera, M. sylvestris ve C. mas'ın antidiyabetik, antimikrobiyal, antikolinesteraz ve antioksidan aktiviteleri rapor edilmiştir. Ayrıca bazı sekonder metabolitlerin kantitatif tayini LC-MS/MS ile analiz edilmiştir. Uçucu yağların kimyasal bileşimi de GC-MS ile araştırılmıştır.*
Sonuç ve Tartışma: *Yağların ana bileşikleri P. cerasifera'da oleik asit (%77.1), M. sylvestris meyvelerinde palmitik asit (%32.5) ve C. mas tohumlarında linoleik asit (%43.2) olarak bulunmuştur. Benzaldehit (%70.1), nonakozan (%30.4), (E,E)-2,4-dekadienal (%43.3) sırasıyla P. cerasifera, M. sylvestris meyveleri ve C. mas tohum uçucu yağlarının ana bileşikleri olarak bulunmuştur. Kinik asit, 11262.2996 ila 18179.6260 ng/ml arasında değişen tüm ekstrelerde en çok bulunan bileşik olmuştur. C. mas yağı Candida albicans ve C. parapsilosis türlerine karşı MIC =625-1250 µg/ml ile antimikrobiyal aktivite göstermiştir. P. cerasifera, C. mas ve M. sylvestris, değişen derecelerde etki gücüne sahip α-glukozidaz inhibitörleri olarak potansiyel taşımaktadır. Özellikle <10 ve 399 µg/ml olan C. mas ve M. sylvestris örneklerinde IC_{50} değerleri sulu ekstrelerinin etkinliğini daha da vurgulamaktadır.*
Anahtar Kelimeler: *Antidiyabetik, antikolinesteraz, Cornus mas, LC-MS/MS, Malus sylvestris, Prunus cerasifera*

INTRODUCTION

Diabetes mellitus (DM) presents a significant and growing challenge in global healthcare, with treatment and management complexities. Recent data indicate a troubling rise in DM prevalence among adults aged 20 to 79 worldwide, projected to reach 439 million by 2030, up from 285 million in 2010. Type 2 diabetes ranks as the fourth or fifth leading cause of mortality in many developed nations, with indications of epidemic proportions in several developing regions. Dietary patterns characterized by high glycemic index and low fiber content have been associated with increased DM risk. Furthermore, specific dietary fatty acids may impact insulin resistance and DM risk differently. Certain food categories and dietary constituents, including fatty acids, fruits, vegetables, whole grain cereals, dietary fiber, fish, magnesium, and nuts, have demonstrated potential in improving insulin sensitivity [1]. Diet plays a crucial role in managing diabetes mellitus (DM), and traditional medicinal practices continue to hold relevance, particularly in developing countries, where approximately 80% of the population relies on them. These traditional remedies often incorporate plant-based elements that may not be prevalent in typical diets. Herbs, spices, and vegetables are not only integrated into daily meals but are also consumed in various medicinal forms [2]. Choosing a diet abundant in fruits, vegetables, and whole grains has been associated with a decreased likelihood of developing DM [3].

Alzheimer's disease (AD) is characterized by the presence of amyloid beta (Aβ) plaques, neurofibrillary tangles, diffuse cortical neuronal loss, and cognitive decline. Notably, studies have revealed reduced brain insulin receptor sensitivity and expression in postmortem AD brains. Diabetes mellitus (DM), associated with insulin resistance, is a recognized risk factor for AD. Recent meta-analyses of long-term population studies have shown a 50% increased risk of AD in individuals with diabetes. Intranasal insulin has been shown to reach physiological levels in the brain in humans, mirroring findings from rodent studies where brain insulin influences appetite and energy metabolism. In humans, intranasal insulin has been linked to reduced body fat, changes in food preferences, decreased food intake, and improved peripheral insulin signaling. These findings deepen our understanding of the complex interplay between insulin, cognitive function, and metabolism in AD [4]. Globally, more than 26 million individuals are affected by AD, the most prevalent form of dementia. A hypothesis proposes that even a modest delay of approximately 2 years in the onset of AD could lead to a considerable reduction in the projected worldwide prevalence by 2050. This delay could potentially prevent around 22 million cases and result in significant cost savings. Certain dietary factors have shown protective

effects against cardiovascular diseases, obesity, hypertension, DM, and hypercholesterolemia—all of which are closely associated with the risk of developing AD [5]. Four cholinesterase inhibitors (AChEIs) treat AD. Tacrine, approved in 1993, is rarely used due to hepatotoxicity. Donepezil, rivastigmine, and galantamine are now standard. Donepezil is approved for all AD stages; the others for mild to moderate cases. AChEIs improve neurotransmission by inhibiting acetylcholine breakdown. Clinical trials show modest, temporary benefits, lasting up to 24 months. Despite limitations, AChEIs remain the primary AD treatment [6]. The crucial involvement of insulin in the central nervous system is widely recognized and firmly established [7]. A constellation of risk factors linked to Type 2 diabetes and vascular disease, including elevated blood glucose levels, obesity, hypertension, elevated blood triacylglycerols, and insulin resistance, are interrelated with an increased susceptibility to developing AD and vascular dementia. These common features shared between DM and different types of dementia underscore the complex interplay between metabolic disorders and cognitive well-being [8]. Oxidative stress (OS) arises from an imbalance between the production of reactive oxygen species (ROS) within cells and the body's ability to detoxify them. This phenomenon acts as a catalyst for the initiation and advancement of various diseases, including cardiovascular disease, atherosclerosis, DM, pulmonary disorders, and cancer. The detrimental effects of ROS on cellular and tissue integrity disrupt normal cellular function, providing a fertile ground for the pathogenesis of these diseases [9]. Systemic inflammation, marked by IL-6 and acute-phase reactants, has been linked to type 2 diabetes since 1997. Recent findings also reveal local inflammation in pancreatic islets, with immune cell infiltration, amyloid-associated macrophages, and complement activation. These processes, resembling AD, highlight the role of localized immune responses in diabetes pathogenesis [10].

Plums have a rich historical background dating back to ancient civilizations and have maintained their popularity and commercial significance over the years. In Turkey, a variety of plum species are cultivated, including *Prunus spinosa* L., *P. cerasifera* Ehrh., *P. domestica* L., and *P. insititia* L., The origins of plum cultivation can be traced back to regions around the Caucasus and Caspian Sea, including Turkey, which served as its ancestral homeland before its spread worldwide. Plums are cultivated extensively throughout Turkey, spanning diverse eco-geographical zones from Southeastern Anatolia to the Mediterranean and Aegean regions, and reaching across Central Anatolia. It's worth noting that while plums thrive in many Turkish regions, they are less common in the elevated plateaus of Eastern Anatolia and the arid, warm zones of Southeastern Anatolia [11]. *P. cerasifera,* also referred to as cherry plum or Myrobalan plum, is a versatile species encompassing various subspecies and natural variations. It serves multiple purposes, including being utilized as a rootstock for grafting fruit trees and grown for ornamental purposes. Moreover, it is considered one of the ancestral progenitors of the cultivated garden plum *(P. domestica* L.*)* [12]. *Cornus mas* L., colloquially known as "Cornelian cherry" or "dogwood," is a deciduous plant indigenous to eastern and southern regions of Europe, as well as West Asia. Typically, these trees can attain heights ranging from 7 to 8 meters when cultivated in temperate climates with well-drained soil. The fruits of *C. mas* (Cornaceae) are edible, exhibiting an oval or pear-like shape and showcasing colors that vary from red to purple hues. Renowned for its abundant content of vitamin C and polyphenols, this plant species is esteemed for its nutritional value. The fruits and leaves of *C. mas* harbor substantial quantities of iridoids, anthocyanins, and flavonoids, which contribute to their robust antioxidant and anti-tumor properties. Beyond its nutritional significance, Cornelian cherry finds application as a traditional ingredient in the crafting of liquors, jams, confections, and an array of fruit-based delicacies. The diverse array of bioactive compounds present in *C. mas* renders it not only a flavorful culinary addition but also a potential source of health-promoting benefits [13]. *C. mas* finds its origin in Southern Europe and Southwest Asia. Fruit extracts from this plant are harnessed in Europe for cosmetic purposes, serving as a natural alternative to synthetic astringents, and are reputed to enhance skin complexion. Moreover, the cornelian cherry enjoys popularity as an ornamental plant, celebrated for its striking foliage and profuse, charming blossoms. It is frequently adorned in small gardens and parks, cherished for its aesthetic allure [14]. *Malus sylvestris* (L.) Mill., commonly known as the European crab apple or simply crab-apple, is a member of the Rosaceae family. As the solitary native wild apple species in central Europe, it boasts extensive distribution throughout the European landscape, ranging from southern Scandinavia to the Iberian Peninsula and from the Volga to the British Isles. Typically inhabiting woodlands, scrublands,

and hedgerows, the crab apple thrives in habitats characterized by sparse forests or along the fringes of wooded areas, where ample sunlight is readily available [15]. A small deciduous tree, typically reaching heights between 4 to 10 meters, characterized by its petite spherical fruit, measuring approximately 2.5 x 2.8 cm, and displaying flattened ends. The fruit, glossy and pale green, adorned with sizable white dots, transitions to a flushed or crimson-spotted hue during autumn. Rich in carbohydrates, dietary fats, sugars, proteins, and minerals, this fruit also contains an abundance of polyphenols such as tannins and anthocyanins, alongside saponins, alkaloids, and flavonoids including procyanidin, quercetin, phloretin, myricetin, and epicatechin. Renowned for its diverse pharmacological applications, it serves as a nerve sedative, anxiety reliever, blood pressure regulator, carminative, digestive aid, emollient, hypnotic, laxative, refrigerant, antioxidant, and antibacterial agent. In traditional medicine, crab apples are revered for their effectiveness in treating various ailments, including cancer, malaria, warts, dysentery, fever, scurvy, and spasms. The crushed fruits are applied topically to alleviate inflammation, minor wounds, and sore throats [16].

This study aimed to quantitatively analyze 35 phenolic compounds present in the fruits of three distinct plant species-*Prunus cerasifera* (fruit), *Malus sylvestris* (fruit), and *Cornus mas* (seed)-employing LC-MS/MS methodology. Additionally, the research sought to explore the inhibitory effects of aqueous and hexane extracts derived from these fruits against enzymes such as α-glucosidase, α-amylase, acetylcholinesterase, and butyrylcholinesterase. The antimicrobial activity was assessed by determining the minimum inhibitory concentration (MIC), while antioxidant properties were evaluated using the DPPH and ABTS methods. Moreover, the compositions of fatty acids and essential oils were scrutinized via GC-MS analysis.

MATERIAL AND METHOD

Plant Materials

P. cerasifera, *M. sylvestris* and *C. mas* specimens were collected from natural habitats in Ormanagzi district, Olur, Erzurum-Turkey in 2021. The collection and identification of plants were carried out by Mehmet ÖNAL, who is the Chief Engineer of the Eastern Anatolia Forestry Research Institute. Herbarium specimens identified as M. Önal 114, M. Önal 268, and M. Önal 1019 are meticulously preserved in the Artvin Çoruh University Herbarium (ARTH), respectively. The photos of *P. cerasifera*, *M. sylvestris*, and *C. mas* were presented in Figure 1.

Extraction

To extract the compounds from the *P. cerasifera* (fruit)*, M. sylvestris* (fruit)*,* and *C. mas* (seed), the dried plant material was first ground and then subjected to extraction using a Soxhlet apparatus with hexane under a reversing cooler for 3 hours. Subsequently, the obtained extract was subjected to evaporation until complete dryness, after which the resulting residue was carefully weighed [17].

For the aqueous extract, the dried fruit material was macerated in water at room temperature for 3 days (8 hours per day), filtered, and frozen at -80 degrees Celsius. The frozen extract was then subjected to lyophilization and weighed [18].

Essential Oil Extraction and Analysis

The extraction of essential oils from *P. cerasifera* (fruit), *M. sylvestris* (fruit), and *C. mas* (seed) involved the use of a Clevenger apparatus, incorporating water addition throughout the 3-4 hour extraction process. Further details regarding this methodology can be referenced in our previously published work by Karakaya et al. (2023) [19].

Preparation of Fatty Acid Methyl Esters

The process for preparing Fatty Acid Methyl Esters entails a series of sequential steps. Initially, the residual substance (extracted oil via the Soxhlet apparatus) is subjected to reflux with a solution containing 0.5 N sodium hydroxide in methanol for a duration of 10 minutes. Following this, a solution comprising 14-20% BF_3 in methanol is introduced through the condenser, and the mixture is boiled for an additional 2 minutes. Subsequently, 5 ml of n-hexane is added to the mixture, followed by another

minute of boiling. The solution is then allowed to cool, and 5 ml of saturated NaCl solution is incorporated. Gentle agitation of the flask ensures thorough blending of the constituents. Further addition of saturated NaCl solution facilitates the separation of the hexane solution, which floats into the neck of a 1 ml flask. The upper hexane solution is then cautiously transferred into a vial for subsequent application [20].

Figure 1. The photos of *Prunus cerasifera, Malus sylvestris*, and *Cornus mas* by Mehmet Önal

Quantitative Determination of Secondary Metabolites

The quantitative analysis of secondary metabolites in the most effective extracts was conducted using an Agilent 6460 Triple Quadrupole Liquid Chromatography-Tandem Mass Spectrometer (LC-MS/MS) system at the Ataturk University East Anatolia High Technology Application and Research Center (DAYTAM). The separation of analytes was achieved using an Agilent Poroshell 120 EC-C18 column (4.6 × 100 mm, 3.5 µm) coupled with an Agilent 1260 HPLC system. The HPLC system was operated in positive ion mode with electrospray ionization (ESI). For detection and quantification, the protonated product ion $[M + H]^+$ of each compound, prepared at a concentration of 1 mg/mL, was determined using the standard scan mode. The dual mobile phase consisted of phase A (0.5% formic acid in water) and phase B (0.5% formic acid in acetonitrile). The injection volume was 5 µl, and the analysis was performed in Multiple Reaction Monitoring (MRM) mode.

Antimicrobial Activity (MIC, µg/ml)

The antimicrobial efficacy of the extracts was assessed against pathogenic microorganisms including *E. coli* ATCC 8739, *Salmonella enterica* ATCC 14028, *B. subtilis* ATCC 19659, *Pseudomonas aeruginosa* ATCC 9027, *Klebsiella aerogenes* ATCC 13048, *Staphylococcus aureus* ATCC 6538, *Candida albicans* ATCC 10231, and *Candida parapsilosis* ATCC 22019. Further details regarding this methodology can be referenced in our previously published work by Karakaya et al. (2023) [19].

***α*-Glucosidase Inhibition Assay**

The α-glucosidase enzyme inhibition assay was carried out, following the procedure described by Bachhawat et al. (2011) with modifications as proposed by Yuca et al. (2021) [21,22].

***α*-Amylase Inhibition Assay**

The α-amylase enzyme inhibition assay was carried out, following the procedure described by Nampoothiri et al. (2011) with modifications as proposed by Yuca et al. (2021) [22,23].

Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) Inhibition Assay

The assays for inhibiting acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were performed, utilizing a customized protocol derived from the methodology described by Ingkaninan et al. (2000), with additional enhancements as refined by Karakaya et al. (2023) [19,24].

ABTS$^{•+}$ Scavenging Activity

The evaluation of ABTS$^{•+}$ scavenging activity was conducted in accordance with the methodology established by Re et al. (1999). For more comprehensive information on this methodology, readers are encouraged to consult our prior publication by Karakaya et al. (2023) [19,25].

DPPH$^{•}$ Scavenging Activity

The evaluation of DPPH$^{•}$ scavenging activity adhered to the methodology devised by Blois (1958). For additional insights into this methodology, readers are directed to our earlier publication by Karakaya et al. (2023)[19,26].

Total Phenolic Content

The quantification of total phenolic content in the extracts was conducted using a modified approach based on the method initially established by Folin and Denis (1912) and later improved by Slinkard and Singleton (1977), with minor adjustments [27,28]. For a deeper understanding of this methodology, readers are encouraged to consult our earlier research by Karakaya et al. (2023) [19]. The gallic acid standard graph was given in Figure 2.

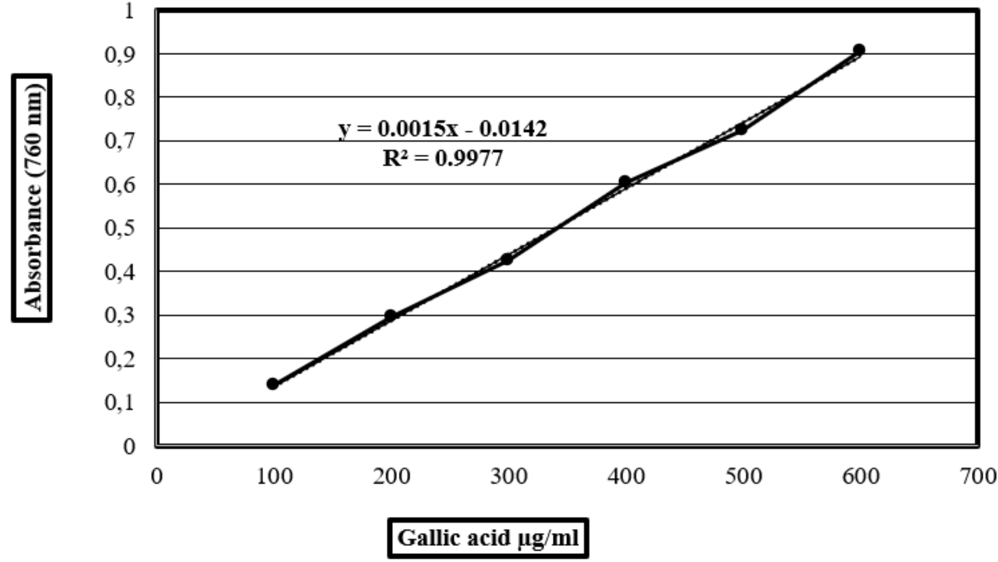

Figure 2. The gallic acid standard graph. [(760 nm) Absorbance = 0,0015 × Gallic acid – 0.0142]

Total Tannin Content

The assessment of total tannin content in aqueous extracts and fatty oils from the species involved a customized method derived from the Folin-Ciocalteu method, as outlined by Makkar (2003) [29]. For additional insights into this methodology, readers are directed to our prior publication by Karakaya et al. (2023) [19]. The tannic acid standard graph was given in Figure 3.

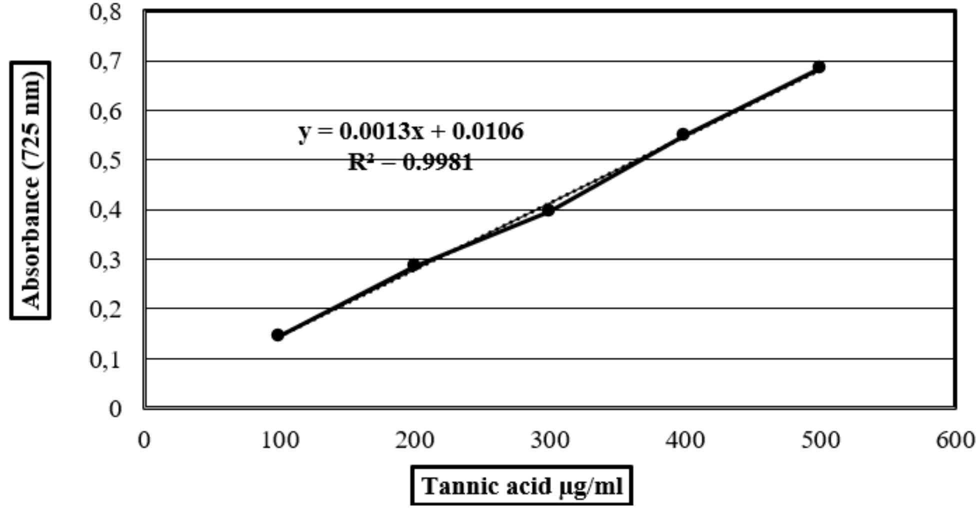

Figure 3. The tannic acid standard graph. [(725 nm) Absorbance = 0,0013 × Tannic acid + 0.0106]

RESULT AND DISCUSSION

Extraction

The aqueous extract of *P. cerasifera* had the highest yield among the all extracts (66%), while *M. sylvestris* hexane extract had the least yield (5.99%). The dried powdered of samples and yields of extracts were given in Table 1.

Table 1. The dried powdered of samples and yields of extracts

Samples		Dried powdered (g)	Yield (%)
P. cerasifera	Hexane	16	7.25
	Aqueous	50	66.00
M. sylvestris	Hexane	12	5.99
	Aqueous	50	29.47
C. mas	Hexane	15	6.27
	Aqueous	50	12.00

Common solvents for polyphenol extraction include methanol, water, ethanol, acetone, and others, each with varying polarity affecting their efficiency. Organic solvents, especially in aqueous mixtures, often enhance extraction yields. For instance, acetone outperformed methanol, water, and ethanol in extracting polyphenols from lychee flowers, while water was more effective for walnut husks. Generally, solvents with higher polarity are more effective due to the greater solubility of polyphenols [30]. Methanol has shown marginally greater efficiency than ethanol in extracting polyphenols and anthocyanins [31].

Fatty and Essential Oils Analysis

A total of nine compounds, constituting 100% of the fatty oil in *P. cerasifera* fruit, were identified. The predominant compound was oleic acid, representing 77.1% of the composition. In the fatty oil of *M. sylvestris* fruit, twelve compounds, totaling 99.9%, were identified, with palmitic acid as the major compound at 32.5%. *C. mas* seed fatty oil, composed of nine compounds and representing 97.1% of the oil, was primarily composed of linoleic acid at 43.2%. The fatty acid compositions of *P. cerasifera*, *M. sylvestris*, and *C. mas* are detailed in Table 2.

Earlier investigations aimed at assessing the oil content and fatty acid composition in kernels from 15 varieties of *Prunus* species in Turkey. The oil yields obtained from these kernels ranged from 46.3% to 55.5%. Oleic acid was identified as the predominant fatty acid in *Prunus* kernel oils, varying from 43.9% to 78.5%, followed by linoleic acid in the range of 9.7% to 37%, and palmitic acid ranging from 4.9% to 7.3% [32]. Oils extracted from by-products of *Malus* spp., particularly cv. "Ola," exhibit rich fatty acid content, including linolenic acid (57.8%), α-linolenic acid (54.3%), and oleic acid (25.5%) (Radenkovs et al. 2018). For *C. mas*, the supercritical CO2 (SC-CO2) extraction technique yielded a seed oil with concentrations ranging from 2.35% to 5.18%. The fatty acid profile of the seed oil included predominantly linoleic acid (65.73%), followed by oleic acid (23.69%), palmitic acid (8.05%), stearic acid (1.92%), erucic acid (0.48%), and arachidic acid (0.13%) [33]. Omega-3 (N-3) polyunsaturated fatty acids (PUFAs) are indispensable for proper neuronal and brain function. These fatty acids serve as vital components of cell membranes and play critical roles in various physiological processes, including inflammation and oxidative stress modulation. The main N-3 PUFAs encompass docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3). While both can be synthesized internally from alpha-linolenic acid (18:3n-3), the conversion rate in humans is notably limited. Hence, dietary intake remains the primary source of DHA and EPA [5].

The fatty acid composition of the fruits from *P. cerasifera*, *M. sylvestris*, and *C. mas* reveals significant variations that underscore the unique biochemical profiles of these species. Each fruit displays a distinct fatty acid profile, highlighting the diversity in bioactive lipids that could contribute to their therapeutic potential. In *P. cerasifera*, oleic acid was the predominant fatty acid, constituting 77.1% of the total fatty acid composition. Oleic acid, a monounsaturated omega-9 fatty acid, is well-known for its beneficial effects on human health, particularly its role in cardiovascular health by improving lipid profiles and reducing inflammation. Its high concentration in the fatty oil of *P. cerasifera* suggests that this fruit could be a valuable source of bioactive lipids, potentially contributing to the prevention of cardiovascular diseases, as well as possessing antioxidant and anti-inflammatory properties. This result is consistent with previous studies that have highlighted the health-promoting effects of oleic acid, especially in the context of olive oils and other plant-based oils. In contrast, *M. sylvestris* exhibited a fatty acid profile with palmitic acid as the major component (32.5%). Palmitic

acid, a saturated fatty acid, has been widely studied due to its potential implications for human health. While saturated fatty acids are generally considered to contribute to atherosclerosis and other metabolic disorders when consumed in excess, recent studies have suggested that their effects may be context-dependent, influenced by factors such as the overall fatty acid composition of the diet. In this case, the relatively moderate concentration of palmitic acid in *M. sylvestris* fruit oil may not have the same negative impact as higher concentrations found in other sources. Additionally, the presence of other unsaturated fatty acids in the oil may balance the potential adverse effects of palmitic acid. *C. mas* seed oil was dominated by linoleic acid, an essential polyunsaturated omega-6 fatty acid, which comprised 43.2% of the oil. Linoleic acid is recognized for its beneficial effects on skin health, inflammation modulation, and potential anti-cancer properties. Its abundance in *C. mas* seed oil suggests that this species could serve as a promising source of essential fatty acids, contributing to various therapeutic applications, including dermatological and anti-inflammatory treatments. Furthermore, linoleic acid is known to support the integrity of cellular membranes, enhance skin barrier functions, and improve the overall lipid profile, adding to the potential health benefits of *C. mas* oil.

The diversity of fatty acids observed in these fruit oils-monounsaturated oleic acid, saturated palmitic acid, and polyunsaturated linoleic acid-illustrates the potential of these species as sources of nutraceuticals. Each species offers distinct advantages depending on the specific health outcomes sought. The predominance of oleic acid in *P. cerasifera* highlights its suitability for cardiovascular health, while the linoleic acid content in *C. mas* seeds positions it as a valuable oil for inflammatory and dermatological applications. The presence of palmitic acid in *M. sylvestris* may require more careful consideration, especially in the context of overall fat consumption, but its moderate concentration may still provide nutritional benefits when consumed as part of a balanced diet.

In conclusion, the fatty acid compositions of these three species highlight their potential as sources of bioactive lipids with diverse health benefits. Further studies on the antioxidant, anti-inflammatory, and other biological properties of these oils, particularly in vivo models, are warranted to fully understand their therapeutic potentials and to guide their use in functional foods and medicinal applications.

Table 2. The fatty acid compositions of *P. cerasifera, M. sylvestris,* and *C. mas*

Compound	*P. cerasifera* %	*M. sylvestris* %	*C. mas* %
Caprylic acid (C8:0)	-	1.2	-
Pelargonic acid (C9:0)	-	0.6	-
Capric acid (C10:0)	-	-	-
Myristic acid (14:0)	tr	1.1	0.1
Palmitic acid (16:0)	3.0	32.5	10.4
Margaric acid (17:0)	-	1.0	-
Stearic acid (18:0)	3.0	19.8	3.1
Oleic acid (18:1)	77.1	18.5	36.3
Elaidic acid (18:1)	1.8	-	1.6
Linoleic acid (18:2)	11.1	1.2	43.2
Linolenic acid (18:3)	1.1	-	1.2
Nonadecanoic acid (20:0)	-	1.4	-
Arachidic acid (20:0)	1.5	13.0	tr
Heneicosanoic acid ** (21:0)	-	1.8	-
Behenic acid (22:0)	1.4	7.8	1.2
Total	100	99.9	97.1

% calculated from FID data
tr Trace (< 0.1 %)
** Tentative identification

The essential oil extraction from *P. cerasifera* fruit yielded 0.11%, and a total of 39 compounds, constituting 86.3% of the essential oil, were identified. The predominant component was benzaldehyde, representing 70.1%. *M. sylvestris* fruit essential oil had a yield of 0.09%, with 32 compounds identified, making up 79.6% of the essential oil. Nonacosane (30.4%) and hexadecanoic acid (19.1%) were the

major constituents. *C. mas* seed essential oil had a yield of 0.14%, with 17 compounds identified, constituting 83.4% of the essential oil. The primary components were (E,E)-2,4-Decadienal (43.3%) and (E,Z)-2,4-Decadienal (12.1%).

Prior research has indicated that the principal constituents found in *P. armeniaca* leaf essential oil consist predominantly of (Z)-phytol (27.18%), pentacosane (15.11%), nonacosane (8.76%), and benzaldehyde (7.25%) [34]. Benzaldehyde plays a crucial role in the flavor industry, with its demand primarily satisfied through synthetic manufacturing processes. However, in the leaf essential oil of *P. persica*, extracted across various seasons and subjected to analysis via GC-FID and GC-MS, notably elevated levels of benzaldehyde (ranging from 63.1% to 98.3%) were observed [35]. In the oil extracted from *M. domestica* leaves, the primary constituents were identified as eucalyptol (43.7%), phytol (11.5%), α-farnesene (9.6%), and pentacosane (7.6%) [36]. Meanwhile, in *M. sylvestris* fruits, the predominant sesquiterpenes detected included E-β-Farnesene (35.03%), E-caryophyllene (7.17%), and germacrene D (5.76%). Noteworthy sesquiterpene oxides comprised bisabolol oxide B (4.82%), spathulenol (4.78%), α-eudesmol (4.52%), caryophyllene oxide (4.46%), 2Z, 6 E-Farnesol (4.34%), and Z-dihydro-apofarnesol (2.22%) [37]. In the dehydrated fruit of *C. officinalis*, prominent constituents included palmitic acid (11.1%), benzyl cinnamate (10.2%), isobutyl alcohol (9.6%), isoamyl alcohol (9.2%), furfural (9.2%), methyl eugenol (7.4%), isoasarone (7.1%), β-phenylethyl alcohol (4.1%), trans-linalool oxide (3.3%), and elemicine (3.2%) [38]. The essential oil extracted from *C. mas* flowers was distinguished by the abundance of monoterpenoids, including camphor, verbenone, borneol, α-terpineol, β-thujone, carvone, and 1,8-cineole [39]. Based on our literature review, this is the first study to comprehensively compare both fatty and essential oil contents of *P. cerasifera, M. sylvestris,* and *C. mas* species.

The essential oil profiles of *P. cerasifera*, *M. sylvestris*, and *C. mas* provide valuable insights into the chemical composition and potential applications of these oils, showcasing distinct variations in their primary constituents and their respective yields. These differences highlight the unique therapeutic potentials and industrial applications of the essential oils from these species. In *P. cerasifera*, the essential oil yield was 0.11%, with 39 compounds identified, contributing to 86.3% of the oil. Benzaldehyde was the dominant compound, comprising 70.1% of the essential oil. Benzaldehyde is a volatile aromatic compound commonly associated with a pleasant, almond-like aroma, and has demonstrated various bioactive properties, including antimicrobial and antioxidant activities. Its high concentration in *P. cerasifera* essential oil suggests that it may offer significant therapeutic potential, particularly in applications related to fragrance, antimicrobial agents, and food preservation. The dominance of a single compound like benzaldehyde also indicates that *P. cerasifera* could provide a relatively straightforward and potent source of bioactive volatiles for pharmaceutical and cosmetic industries. In contrast, *M. sylvestris* fruit essential oil showed a yield of 0.09% with 32 identified compounds, accounting for 79.6% of the oil. The major components, nonacosane (30.4%) and hexadecanoic acid (19.1%), are both long-chain hydrocarbons. Nonacosane, a saturated hydrocarbon, is often found in plant waxes and has been reported to possess antimicrobial and insecticidal properties. Hexadecanoic acid, a saturated fatty acid, is known for its anti-inflammatory and potential antimicrobial effects. The significant presence of these compounds in *M. sylvestris* essential oil suggests its potential use in applications requiring both antimicrobial properties and stable long-chain molecules, such as in the development of functional food additives or natural preservatives. *C. mas* seed essential oil yielded 0.14%, and the oil was composed of 17 identified compounds, which made up 83.4% of the oil. The two predominant components were (E,E)-2,4-Decadienal (43.3%) and (E,Z)-2,4-Decadienal (12.1%), both of which are unsaturated aldehydes with distinct aromatic profiles. These compounds have been shown to possess antimicrobial, antifungal, and antioxidant properties, which makes *C. mas* essential oil promising for therapeutic applications, particularly in natural antimicrobial formulations. The high concentration of (E,E)-2,4-Decadienal, in particular, could also offer strong potential for use in perfumery and flavoring due to its characteristic aroma. The unsaturated nature of these aldehydes may also contribute to the oil's stability and potency as a bioactive agent. The yield differences observed between the three species-*P. cerasifera* (0.11%), *M. sylvestris* (0.09%), and *C. mas* (0.14%)-highlight the variability in essential oil production even within the same plant family, suggesting that extraction methods, plant variety, and environmental conditions may play significant roles in determining oil

yields. The variation in chemical composition further emphasizes the importance of species selection for specific applications, as each oil shows a distinct profile of bioactive compounds (Table 3).

In conclusion, the essential oils of *P. cerasifera*, *M. sylvestris*, and *C. mas* each present unique chemical signatures with substantial potential for various applications. *P. cerasifera* is particularly notable for its high concentration of benzaldehyde, making it a promising candidate for use in antimicrobial and fragrance-based industries. *M. sylvestris* oil, with its predominance of nonacosane and hexadecanoic acid, offers opportunities for applications in food preservation and antimicrobial formulations. Finally, *C. mas* essential oil, rich in unsaturated aldehydes, holds promise for both medicinal and industrial uses, especially in antimicrobial and flavoring applications. These findings suggest that these oils may offer diverse and valuable bioactive properties, deserving further investigation for their potential therapeutic and industrial uses.

Table 3. The composition of the essential oil *P. cerasifera, M. sylvestris,* and *C. mas*

RRI	Compound	*P. cerasifera*	*M. sylvestris*	*C. mas*
1244	2-Pentyl furan	0.1	-	-
1360	1-Hexanol	0.4	0.1	-
1374	4-Hydroxy-4-methyl-2-pentanone	-	0.1	-
1400	Nonanal	0.4	0.1	-
1400	Tetradecane	-	0.4	2.9
1452	1-Octen-3-ol	-	-	0.4
1479	Furfural	0.3	0.7	0.3
1541	Benzaldehyde	70.1	5.9	-
1542	Vitispirane	-	2.3	-
1543	Ethyl vitispirane	-	0.6	-
1548	(*E*)-2-Nonenal	-	-	tr
1553	Linalool	0.1	-	-
1600	Hexadecane	tr	0.5	3.2
1655	(*E*)-2-Decenal	-	0.2	0.6
1661	Safranal	0.2	-	-
1694	*p*-Vinylanisole	0.1	-	-
1706	*α*-Terpineol	0.2	-	-
1751	Carvone	tr	-	-
1765	(*E*)-2-Undecanal	-	-	0.6
1773	*δ*-Cadinene	tr	-	-
1779	(*E,Z*)-2,4-Decadienal	-	0.1	12.1
1800	Octadecane	0.1	0.4	2.9
1827	(*E,E*)-2,4-Decadienal	0.2	1.0	43.3
1838	(*E*)-*β*-Damascenone	tr	-	-
1853	Ethyl dodecanoate	0.4	-	-
1868	(*E*)-Geranyl acetone	0.2	-	-
1900	Nonadecane	-	0.7	-
1958	(*E*)-*β*-Ionone	0.3	-	-
1973	1-Dodecanol	tr	-	-
2000	Eicosane	0.1	1.1	1.9
2050	(*E*)-Nerolidol	-	tr	-
2056	Ethyl tetradecanoate (=*E. myristate*)	0.8	0.6	-
2100	Heneicosane	0.8	0.4	0.7
2131	Hexahydrofarnesyl acetone	0.2	1.2	1.1
2226	Methyl palmitate	0.8	0.3	-

RRI Relative retention indices calculated against *n*-alkanes
% calculated from FID data
tr Trace (< 0.1 %)
Unknown I: EIMS, 70 eV, m/z (rel. .int.): 117[M]+ (0.5), 115(10.2), 81 (52.8), 54 (38.9), 41 (100), 39 (47.4)

Table 3 *(continue).* The composition of the essential oil *P. cerasifera, M. sylvestris,* and *C. mas*

RRI	Compound	*P. cerasifera*	*M. sylvestris*	*C. mas*
2262	Ethyl palmitate	1.5	0.7	-
2300	Tricosane	0.4	1.8	-
2369	(*2E,6E*)-Farnesol	-	4.4	
2380	*epi*-Manoyl oxide	-	-	1.3
2384	Farnesyl acetone	0.1	tr	-
2400	Tetracosane	-	-	1.3
2438	Kaur-16-ene	-	-	4.7
2456	Methyl oleate	0.3	1.1	-
2467	Ethyl stearate	0.2	-	-
2492	Ethyl oleate	0.7	-	-
2500	Pentacosane	tr	-	-
2503	Dodecanoic acid	1.4	tr	-
2509	Methyl linoleate	0.2	tr	-
2538	Ethyl linoleate	0.8	tr	-
2583	Methyl linolenate	0.3	-	-
2613	Ethyl linolenate	0.5	-	-
2670	Tetradecanoic acid	1.1	2.5	-
2700	Heptacosane	-	2.9	-
2900	Nonacosane	0.4	30.4	6.1
2931	Hexadecanoic acid	2.6	19.1	-
	TOTAL	**86.3**	**79.6**	**83.4**

RRI Relative retention indices calculated against *n*-alkanes
% calculated from FID data
tr Trace (< 0.1 %)
Unknown I: EIMS, 70 eV, m/z (rel. .int.): 117[M]+ (0.5), 115(10.2), 81 (52.8), 54 (38.9), 41 (100), 39 (47.4)

Quantitative Determination of Secondary Metabolites

A comprehensive examination was conducted on three aqueous extracts derived from *P. cerasifera*, *M. sylvestris*, and *C. mas*, revealing the presence of 35 unique phenolic compounds using LC-MS/MS. These compounds encompass a diverse array, including cyanidin-3-O-glucoside, quinic acid, caffeic acid, fumaric acid, p-coumaric acid, gallic acid, pyrogallol, chlorogenic acid, catechin, peonidin-3-O-glucoside, 4-OH-benzoic acid, luteolin, syringic acid, epicatechin, rosmarinic acid, epigallocatechin gallate, taxifolin, vanillic acid, vanillin, vitexin, naringin, ellagic acid, naringenin, hesperidin, ferulic acid, resveratrol, keracyanin chloride, quercetin, myricetin, apigenin, isorhamnetin, chrysin, sinapic acid, curcumin, and galangin.

Among these compounds, quinic acid, fumaric acid, gallic acid, cyanidin-3-O-glucoside, chlorogenic acid, catechin, epicatechin, ellagic acid, and quercetin were detected in all three extracts. Notably, quinic acid exhibited the highest concentration across all three extracts, with values of 11262.2996 ng/ml, 14802.2687 ng/ml, and 18179.6260 ng/ml for *P. cerasifera*, *M. sylvestris*, and *C. mas* extracts, respectively. Detailed results of the quantitative analysis of these phenolic compounds are outlined in Table 4.

The research investigated the combined effects of quercetin and quinic acid on a streptozotocin (STZ)-induced diabetic rat model. Diabetic rats were administered single and combined doses of quercetin and quinic acid over a period of 45 days, followed by analysis of their impact on liver, kidney, and pancreatic tissues. The findings revealed that treatment with quercetin and quinic acid led to increased levels of insulin and C-peptide, while also reducing hyperglycemia and oxidative stress. These results indicate a potential therapeutic role of quercetin and quinic acid in managing diabetes and its associated complications [40]. The study explored the neuroprotective properties of four derivatives of quinic acid sourced from Aster scaber in mitigating toxicity induced by amyloid Aβ in PC12 cells. The results demonstrated a significant reduction in cell toxicity caused by Aβ when cells were pre-treated with these quinic acid derivatives. Notably, among the derivatives, (−)4,5-dicaffeoyl quinic acid

emerged as the most effective in protecting against Aβ-induced cell toxicity. This underscores the potential of (−)4,5-dicaffeoyl quinic acid as a promising candidate for neuroprotection against Aβ-induced toxicity in PC12 cells [41]. Peaches, rich in polyphenols, protect against various health issues like obesity and diabetes. It was analyzed methanol, ethanol, and hexane extracts from fresh red peaches in Mersin, Turkey. Thirteen compounds, including quinic acid and chlorogenic acid, were identified in the ethanol extract by LC-MS/MS, which showed the highest antioxidant, antibacterial, and enzyme inhibition activities. These results highlight ethanol extract as a potential ingredient for antidiabetic and antibacterial formulations [42]. It was analyzed 14 *Malus* spp. genotypes for polyphenol content and composition, with concentrations ranging from 560 to 4860 mg/L. Using RP-HPLC and LC-MS/MS, 19 polyphenols were quantified, with hydroxycinnamates and flavan-3-ols being the most abundant. The 'Kola' hybrid showed the highest total polyphenol concentration (1429 mg/l), mainly composed of chlorogenic acid. 'Kaz 95 18–06' had a high level of flavan-3-ols, while 'Zapta' contained the highest phlorizin. LC-MS identified 120 polyphenols, including some with potential health benefits [43]. The Cornus fructus was treated with varying osmotic pressures, pH, heat, and ethanol concentrations. The highest gallic acid extraction (1.57 mg/g) was achieved using 100% ethanol for 1 hour. Extracts with 70% ethanol for 24 and 48 hours resulted in 1.35 and 1.50 mg/g of gallic acid, respectively. The analysis was performed using HPLC and LC-MS/MS [44].

Table 4. Quantitative assessment of 35 distinct phenolic compounds in aqueous extracts utilizing LC-MS/MS

NO	Compound	Samples	Final Conc (ng/ml)
1.	Quinic Acid	*P. cerasifera*	11262.2996
		M. sylvestris	14802.2687
		C. mas	18179.6260
2.	Fumaric Acid	*P. cerasifera*	144.3326
		M. sylvestris	32.8088
		C. mas	165.3011
3.	Gallic Acid	*P. cerasifera*	0.0000
		M. sylvestris	0.0000
		C. mas	4987.8455
4.	Cyanidin-3-*O*-glucoside	*P. cerasifera*	80.3648
		M. sylvestris	192.1741
		C. mas	40.7629
5.	Chlorogenic Acid	*P. cerasifera*	3861.2059
		M. sylvestris	3234.2241
		C. mas	15.4365
6.	Catechin	*P. cerasifera*	316.6816
		M. sylvestris	260.3998
		C. mas	0.0000
7.	Epicatechin	*P. cerasifera*	230.0535
		M. sylvestris	162.3451
		C. mas	0.0000
8.	Ellagic Acid	*P. cerasifera*	0.0000
		M. sylvestris	0.0000
		C. mas	1590.5162
9.	Quercetin	*P. cerasifera*	2.0335
		M. sylvestris	2.3522
		C. mas	0.1642

This study identified 35 phenolic compounds in the aqueous extracts of *Prunus cerasifera*, *Malus sylvestris*, and *Cornus mas* using LC-MS/MS, highlighting their potential therapeutic value. Compounds like quinic acid, fumaric acid, gallic acid, cyanidin-3-O-glucoside, chlorogenic acid, catechin, epicatechin, ellagic acid, and quercetin were found in all three extracts, suggesting they may play a key

role in the plants' biological activities. Quinic acid was present in the highest concentrations across all extracts, indicating its potential importance in the therapeutic effects of these plants. It is known for its antioxidant, anti-inflammatory, and hepatoprotective properties. Other identified compounds, such as catechins, caffeic acid, and gallic acid, are also recognized for their antioxidant and anti-inflammatory effects, which could contribute to the health benefits of these plants. The variations in concentrations of these compounds across the different species suggest that each plant may offer unique health benefits. For example, *C. mas* had the highest concentration of quinic acid, which may point to its stronger antioxidant effects compared to the others. Overall, the diverse phenolic profiles of these plants indicate their potential as sources of bioactive compounds with therapeutic properties, warranting further research into their biological effects and potential health applications.

Antimicrobial Activity

The fatty oils of fruit and aqueous extracts *of P. cerasifera, M. sylvestris,* and *C. mas* were evaluated against some of Gram (+), Gram (-), and yeasts. MIC values were presented in Table 5. Generally, the MIC value was observed between 1250-5000 µg/ml.

Table 5. Minimum inhibitory concentrations (µg/ml)

Samples	*E. coli*	*S. enterica*	*S. aureus*	*B. subtilis*	*P. aeruginosa*	*K. aerogenes*	*C. albicans*	*C. parapsilosis*
Fatty oil of fruit from *M. slyvestris*	2500	1250	>2500	2500	1250	1250	5000	5000
Fatty oil of fruit from *P. cerasifera*	2500	2500	>2500	2500	2500	1250	5000	2500
Fatty oil of fruit from *C. mas*	2500	>2500	>2500	>2500	>2500	>2500	625	1250
Aqueous extract of *P. cerasifera*	>2500	>2500	>2500	>2500	>2500	ND	2500	2500
Aqueous extract of *M. sylvestris*	>2500	>2500	>2500	>2500	>2500	ND	2500	2500
Aqueous extract of *C. mas*	625	>2500	312.5	312.5	>2500	ND	625	5000
Moxifloxacin	0125>	0125>	0125>	0125>	0125>	ND	-	-
Ampicilline	0.125>	1.0	0.125>	0.125>	>64	32	-	-
Terbinafine	-	-	-	-	-	-	4.0	32
Fluconazole	-	-	-	-	-	-	1	1

ND: Not detected

Fatty oil of fruit from *M. slyvestris* was found effective against *Salmonella enterica* ATCC 14028, *Pseudomonas aeruginosa* ATCC 9027 and *Klebsiella aerogenes* ATCC 13048 with MIC= 1250 µg/ml. Fatty oil of fruit from *P. cerasifera* was more effective against *Klebsiella aerogenes* ATCC 13048. Fatty oil of seed from *C. mas* was showed antimicrobial activity against *Candida* species with MIC =625-1250 µg/ml. Generally, fatty oil of fruit from *C. mas* was found effective MIC=> 2500 µg/ml against Gram (+) and Gram (-).

Generally, the MIC value was observed between >2500 µg/ml for the aqueous extracts of *P. cerasifera, M. sylvestris,* and *C. mas.* The aqueous extract of *C. mas* was found more effective againts *S. aureus* and *B. Subtilis* with MIC=312.5 µg/ml. Also the exract of *C. mas* was more effective against *C. albicans* at MIC = 625 µg/ml than *C. parapsilosis.*

In scientific literature, the antimicrobial efficacy of leaf extracts from *P. divaricata* subsp. *divaricata* was assessed using petroleum ether, dichloromethane, methanol, and distilled water against various bacterial strains including *Bacillus subtilis* NRS-744, *Staphylococcus aureus* NRRL B-767, *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 7644, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* NRRL B-4420, and *Klebsiella pneumonia* ATCC 700603. The results revealed that the petroleum ether extract exhibited antimicrobial activity against *E. faecalis*, with an inhibition zone of 11 mm [45]. The antimicrobial potential of methanol extracts from the fruit of *P.*

divaricata subsp. *divaricata* was assessed against eight bacterial strains and two yeast strains. Among the clinical isolates, the most pronounced antimicrobial effects against Gram-negative organisms were observed for *E. coli* and *K. pneumonia*, whereas *C. albicans* and *C. parapsilosis* exhibited the least susceptibility to the extracts [46].

The antibacterial efficacy of peel extract derived from *M. sylvestris* was examined against *Salmonella typhi*. The findings of this investigation revealed a MIC of 12.5%, accompanied by an average diameter of inhibition zone measuring 13.67 mm [47]. The peel extract of *M. sylvestris* in various solvent formulations containing ethanol concentration demonstrated inhibitory effects on the growth of *Streptococcus agalactiae* and *E. coli* [48].

Ethanol or methanol extracts derived from various parts of C. mas, including the bark, fruits, leaves, and seeds, were evaluated for their efficacy against a spectrum of pathogens including *S. aureus, S. pyogenes, E. coli, P. aeruginosa, C. albicans, Aspergillus fumigatus,* and *Trichophyton mentagrophytes*. Utilizing the disc-diffusion method, leaf and seed extracts exhibiting inhibition zone diameters ranging between 10-15 mm demonstrated the most potent antibacterial activity against *S. aureus* and *C. albicans* [49]. To evaluate the antimicrobial efficacy, water and methanol extracts of fruit from *C. mas* were tested against 93 clinical isolates of human pathogenic strains, including C. *albicans, E. coli, Proteus mirabilis, P. aeruginosa, S. aureus, C. glabrata, C. krusei, C. parapisilosis,* and *C. tropicalis*. The methanol and water extracts of the fruit exhibited potent antibacterial activity against *S. aureus*, yielding a notable 25 mm inhibition zone and a MIC value of 0.156 mg/ml [50]. In our investigation, the fatty oil extracted from *C. mas* fruits demonstrated superior efficacy against *C. albicans.* The observed activity can be attributed to the bioactive compounds in the extracts, such as phenolics and fatty acids, known for their antimicrobial properties. These extracts showed broad-spectrum activity, effective against both Gram (+) and Gram (-) bacteria, as well as yeasts. These results suggest that the plant extracts have potential as natural antimicrobial agents. Further studies are needed to explore their mechanisms of action and optimize extraction methods for enhanced efficacy.

α-Glucosidase and α-Amylase Inhibition Assay

Based on the assays, the results indicate noteworthy *α*-glucosidase inhibition activity in various samples, including *P. cerasifera*, *C. mas*, and *M. sylvestris.* The inhibitory effects on α-glucosidase were evaluated by measuring both the percentage inhibition at a concentration of 5000 µg/ml and the determination of IC_{50} values.

At a concentration of 5000 µg/ml, the aqueous extract from *P. cerasifera* exhibited significant inhibition of α-glucosidase, showing a notable inhibitory activity of 67.73%, albeit slightly lower compared to acarbose (77.61%). Similarly, the aqueous extract of *C. mas* and *M. sylvestris* exhibited notable *α*-glucosidase inhibition activities, with values of 81.58% and 70.11%, respectively. In comparison, the fatty oil of *P. cerasifera*, *C. mas*, and *M. sylvestris* demonstrated relatively lower *α*-glucosidase inhibition activities, with values of 0.22%, 18.73%, and 51.17%, respectively.

Furthermore, the IC_{50} values for *α*-glucosidase inhibition were calculated, with acarbose serving as a reference. The IC_{50} value of acarbose was 2434 µg/ml. Among the samples, the aqueous extract of *P. cerasifera* exhibited an IC_{50} value of 650 µg/ml, indicating significant *α*-glucosidase inhibitory potential. *C. mas*'s aqueous extract displayed an even more potent inhibition, with an IC_{50} value of less than 10 µg/ml. Additionally, *M. sylvestris*'s aqueous extract demonstrated an IC_{50} value of 399 µg/ml. However, the fatty oil samples did not yield IC_{50} values, suggesting that their inhibitory effects were not as pronounced.

In terms of *α*-amylase inhibition activity, acarbose exhibited an inhibition of 65.79% at a concentration of 5000 µg/ml. Among the samples, *P. cerasifera*'s fatty oil displayed an *α*-amylase inhibition of 36.23%, while *C. mas* and *M. sylvestris* exhibited lower inhibitions of 11.36% and 20.72%, respectively. The aqueous extracts of all samples did not exhibit significant *α*-amylase inhibition (Table 6).

In a prior investigation, we assessed the antidiabetic properties of a 70% methanolic extract obtained from *P. cerasifera* fruit. Intriguingly, the extract exhibited no inhibitory effects against both α-glucosidase and α-amylase enzymes [51]. In another study, health benefits of various traditional *Prunus* fruits cultivated in Serbia were investigated. The study focused on assessing their inhibitory activities

against *α*-glucosidase and *α*-amylase. For the extracts used in the study, a 50% ethanol preparation method was employed. Noteworthy results showed that the white cherry plum (*P. cerasifera*) extract had a substantial inhibitory effect on *α*-glucosidase (6.18 mg/ml), while it exhibited no detectable inhibitory effect on *α*-amylase. In comparison, the reference compound acarbose showed an inhibitory effect of 0.11 mg/ml on *α*-amylase and 3.73 mg/mL on *α*-glucosidase [52].

Table 6. The results of *α*-glucosidase and *α*-amylase inhibition assays

***α*-Glucosidase Inhibition Activity (% Inhibition of 5000 µg/ml ± standard deviation)**			
Acarbose	77.61 ± 4.70		
Samples	***Prunus cerasifera***	***Cornus mas***	***Malus sylvestris***
Fatty oil	0.22 ± 2.31	18.73 ± 5.75	51.17 ± 3.89
Aqueous extract	67.73 ± 2.72	81.58 ± 1.29	70.11 ± 2.79
***α*-Glucosidase Inhibition Activity (IC_{50} values, µg/ml)**			
Acarbose	2434		
Samples	***Prunus cerasifera***	***Cornus mas***	***Malus sylvestris***
Fatty oil	-	-	3887
Aqueous extract	650	<10	399
***α*-Amylase Inhibition Activity (% Inhibition of 5000 µg/ml ± standard deviation)**			
Acarbose	65.79 ± 3.03		
Samples	***Prunus cerasifera***	***Cornus mas***	***Malus sylvestris***
Fatty oil	36.23 ± 5.13	11.36 ± 9.10	20.72 ± 3.41
Aqueous extract	ND	ND	ND

ND: Not Determined

A study aimed to investigate the potential of hydroalcoholic (80% ethanol) extract derived from cornelian cherry fruit as inhibitors of key carbohydrate digestive enzymes, pancreatic α-amylase, and intestinal α-glucosidase. The findings revealed that the cornelian cherry extract exhibited inhibition of α-glucosidase and α-amylase, with IC_{50} values of 6.87 and 6.05 mg/ml, respectively. In comparison, the reference compound acarbose demonstrated IC_{50} values of 0.023 mg/ml against α-amylase and 0.043 mg/mL against α-glucosidase [53]. A study aimed to evaluate the bioactivity of extracts obtained from red and yellow Cornelian cherry fruits. These extracts were investigated for their inhibitory effects against α-glucosidase. The results indicated that at concentrations of 25.7 and 30.0 µg/ml, the extract derived from red fruits displayed a more pronounced inhibitory effect on α-glucosidase activity compared to the extract from yellow fruits at equivalent concentrations. Specifically, the red fruit extract exhibited an IC50 value of 25.68 µg/ml, whereas the yellow fruit extract showed a slightly higher IC_{50} value of 28.46 µg/ml. In contrast, the reference compound acarbose demonstrated a significantly higher IC_{50} value of 5.68 × 103 µg/ml [54]. A research study was undertaken to explore the inhibitory effects on α-amylase and α-glucosidase activities using aqueous extracts from three apple varieties: *M. sylvestris* (green apple), *M. pumila* (red apple), and *Syzygium samarangense* (wax apple). The findings revealed that all apple varieties exhibited dose-dependent inhibition of α-amylase (with IC_{50} values ranging from 12.66 to 16.98 µg/ml) and α-glucosidase (ranging from 13.55 to 16.23 µg/ml) activities. Notably, green apple demonstrated the most potent inhibitory activity, while wax apple exhibited the least inhibitory effect [55]. These results highlight the potential of aqueous extracts, particularly from *C. mas*, as effective α-glucosidase inhibitors. However, the fatty oils' limited inhibitory activity suggests that they may not be as effective for inhibiting carbohydrate-digesting enzymes. Further studies are required to explore the specific compounds responsible for these effects and their potential use in diabetes management.

Anticholinesterase Assays

In terms of acetylcholinesterase inhibition activity at 100 µg/ml concentration, the reference compound donepezil demonstrated a strong inhibitory effect of 99.20%. Among the samples tested, the *Prunus cerasifera* extract displayed an inhibition of 11.56%, *Cornus mas* had an inhibition of 10.70%,

and *Malus sylvestris* exhibited an inhibition of 11.71% for the fatty oil. For the aqueous extract, *P. cerasifera* showed an inhibition of 10.02%, *C. mas* had an inhibition of 9.31%, and *M. sylvestris* displayed an inhibition of 8.13%.

In terms of butyrylcholinesterase inhibition activity at 1000 µg/ml, donepezil exhibited complete inhibition with a value of 100%. Among the samples, *P. cerasifera* extract showed an inhibition of 10.39%, *C. mas* exhibited an inhibition of 8.96%, and *M. sylvestris* displayed an inhibition of 6.39% for the fatty oil. For the aqueous extract, *P. cerasifera* had an inhibition of 1.13%, *C. mas* showed an inhibition of 11.28%, and *M. sylvestris* exhibited an inhibition of 8.93% (Table 7).

Table 7. The results of acetylcholinesterase and butyrylcholinesterase inhibition assays

Acetylcholinesterase Inhibition Activity (% Inhibition of 100 µg/ml ± standard deviation)			
Donepezil	99.20 ± 0.22		
Samples	***Prunus cerasifera***	***Cornus mas***	***Malus sylvestris***
Fatty oil	11.56 ± 7.70	10.70 ± 1.82	11.71 ± 4.26
Aqueous extract	10.02 ± 0.87	9.31 ± 4.58	8.13 ± 2.32
Butyrylcholinesterase Inhibition Activity (% Inhibition of 1000 µg/ml ± standard deviation)			
Donepezil	100 ± 0.49		
Samples	***Prunus cerasifera***	***Cornus mas***	***Malus sylvestris***
Fatty oil	10.39 ± 9.64	8.96 ± 3.19	6.39 ± 1.75
Aqueous extract	1.13 ± 2.69	11.28 ± 1.37	8.93 ± 3.85

Our study stands as the inaugural exploration into the anticholinesterase potential of *Prunus cerasifera*, in line with our existing knowledge. When examining research conducted on the *Prunus* genus, a study aimed to assess the anticholinesterase property of *P. mahaleb*. Results indicated that the methanolic extract's lower IC_{50} values ($p<0.05$) for acetylthiocholinesterase (AChE), and butyrylcholinesterase (BChE) (52.1 and 86.2 µg/ml, respectively) compared to the hexane extract (85.2 and 110.7 µg/ml, respectively). In comparison to the standard drug galantamine (with IC_{50} values of 12.8 and 10.7 µg/ml, respectively), both extracts exhibited very limited activity [56]. Another study investigated the inhibitory effects of sweet and bitter apricot (*P. armeniaca*) kernel extracts on cholinesterase enzymes utilizing Ellman's method. The aqueous extract of the bitter variety exhibited the most prominent AChE inhibitory activity (IC_{50}= 134.93 µg/ml). Notably, none of the extracts displayed inhibitory activity against BChE. Comparatively, the reference compound rivastigmine showed AChE inhibitory activity with an IC_{50} value of 2.77 µg/ml and BChE inhibitory activity with an IC_{50} value of 1.93 µg/ml [57].

Our research represents the first investigation into the potential anticholinesterase properties of *Cornus mas*, aligning with our current understanding. Upon reviewing studies carried out within the *Cornus* genus, the objective of a study was to explore the anticholinesterase activity of methanol extracts from *C. sanguinea* leaves and fruits. Specifically, leaves extract exhibited dose-dependent AChE inhibitory effects, ranging from 16.84% to 50.89% at concentrations of 12.5 to 100 µg/ml. In contrast, fruits extract displayed no AChE inhibition at 12.5 and 25 µg/ml, but demonstrated inhibitory effects of 11.59% and 24.58% at 50 and 100 µg/ml, respectively. The IC_{50} values for both extracts were determined as 93.64 and > 100 µg/ml, respectively. Notably, both extracts exhibited lower inhibitory effects compared to the positive control, galantamine [58].

A study was conducted to evaluate the potential of fresh fruit juice derived from *M. domestica* x *M. sylvestris* in mitigating AD symptoms in mice. Cognitive enhancement was assessed through behavioral tests including the Morris water maze (MWM) and Passive shock avoidance paradigm (PSAP), alongside the estimation of AChE activity. Two doses (1 ml/kg and 1.5 ml/kg, b.w, p.o) of juice were administered against AD induced by scopolamine (0.4 mg/kg, i.p), with piracetam (400 mg/kg, i.p) used as the standard. Prolonged administration of both low and high doses of juice notably reduced transfer latency (TL) and escape latency time (ELT) in PSAP and MWM ($P < 0.01$ and $P < 0.05$, respectively). Enhanced memory retention was evidenced by higher time spent in the target quadrant (TSTQ) values in the MWM model. Furthermore, the higher dose of fruit juice significantly ($p < 0.01$)

lowered AChE activity in the brain, indicating improved learning and memory retention [59]. These findings suggest that while these extracts have some cholinesterase inhibitory effects, they are far less potent than donepezil. However, they may still hold potential for future research, particularly in combination with other treatments for neurodegenerative diseases. Further studies are needed to identify the specific compounds responsible for these effects.

ABTS•+ and DPPH• Scavenging Activity

Table 8 summarizes the antioxidant activity of aqueous extracts and fatty oils from the investigated species. In ABTS cation radical scavenging tests, the analysis of % inhibition values at a concentration of 100 µg/ml indicated that the *C. mas* aqueous extract showed significantly higher % inhibition on $ABTS^{\cdot+}$ compared to the standards (α-tocopherol (TC) and trolox (TR)). Other samples exhibited antioxidant capacities that were generally similar to the standards.

Table 8. Antioxidant activity test results

$ABTS^{\bullet+}$ Scavenging Activity of Standards and Aqueous Extracts (% Inhibition of 100 µg/ml ± standard deviation)			
***α*- Tocopherol**	94.099 ± 0.0017		
Trolox	99.614 ± 0.0012		
Samples	***Prunus cerasifera***	***Cornus mas***	***Malus sylvestris***
Fatty oil	0.949 ± 0.0106	6.546 ± 0.0141	5.775 ± 0.0229
Aqueous extract	8.491 ± 0.0037	**98.693 ± 0.0026**	12.442 ± 0.0093
$DPPH^{\bullet}$ Scavenging Activity of Standards and Aqueous Extracts (% Inhibition of 100 µg/ml ± standard deviation)			
***α*- Tocopherol**	88.886 ± 0.001		
Trolox	91.285 ± 0.0004		
Samples	***Prunus cerasifera***	***Cornus mas***	***Malus sylvestris***
Fatty oil	ND	5.338 ± 0.0566	0.823 ± 0.0345
Aqueous extract	2.726 ± 0.0797	**89.515 ± 0.0002**	9.492 ± 0.0142

ND: Not detected

Similarly, in DPPH radical scavenging assays at a concentration of 100 µg/ml, the *C. mas* aqueous extract demonstrated a superior % inhibition value on $DPPH^{\bullet}$, closely approaching that of the standards. This underscores the substantial antioxidant potential of the *C. mas* aqueous extract. The results from both antioxidant activity tests were consistent with each other.

In a separate investigation evaluating the total antioxidant capacity, phenolic composition, organic acid, and vitamin C content of three plum species, it was observed that *P. spinosa*, characterized by the highest phenolic content, organic acids, and vitamin C composition, exhibited stronger antioxidant capacity compared to *P. domestica* and *P. cerasifera*. Remarkably, a positive correlation was identified between the vitamin C content and the overall antioxidant capacity [10].

Through ferric reducing ability of plasma and ABTS radical scavenging tests conducted to evaluate the *in vitro* antioxidant capacity of *C. mas*, it was evident that the plant exhibits significant antioxidant properties. As a result, it was deduced that *C. mas* possesses the capability to mitigate acute inflammation [60].

In a literature-registered study, 38 extracts derived from fruits and leaves of various species were investigated for their antioxidant properties. Comparing the DPPH radical scavenging activities of 50% methanolic extracts from the fruits, it was observed that C. mas exhibited more potent antioxidant effects compared to P. divaricata (with EC_{50} values on DPPH radical of 1.078 and 1.692, respectively) [61]. This suggests that *C. mas* is a strong natural antioxidant, potentially more effective than some common standards. Other samples showed antioxidant activity similar to TC and TR, indicating their moderate effectiveness. These results highlight *C. mas* as a promising source of antioxidants for potential applications in food and pharmaceuticals to combat oxidative stress-related diseases. Further studies on the active compounds are needed to confirm their benefits.

Total Phenolic and Tannin Content

The test results for total phenolic and tannin content of the samples were provided in Table 9. Upon evaluating the studies conducted on these samples, it was determined that the aqueous extract of *Cornus mas*, in particular, exhibited high levels of total phenol and total tannin content. Additionally, this extract demonstrated the highest antioxidant activity. Consequently, the findings of our study are consistent and mutually supportive.

Table 9. Total phenolic and tannin content test results

Total phenolic content (µg GAE/mg extract ± standard deviation)			
Samples	***Prunus cerasifera***	***Cornus mas***	***Malus sylvestris***
Fatty oil	14.511 ± 0.0017	24.777 ± 0.0008	18.866 ± 0.0019
Aqueous extract	34.666 ± 0.0007	**198.511 ± 0.0041**	41.755 ± 0.001
Total tannin content (µg TAE/mg extract± standard deviation)			
Samples	***Prunus cerasifera***	***Cornus mas***	***Malus sylvestris***
Fatty oil	ND	9.512 ± 0.0008	2.692 ± 0.0019
Aqueous extract	20.923 ± 0.0007	**209.974 ± 0.0041**	29.102 ± 0.001

GAE: Gallic acid equivalent, TAE: Tannic acid equivalent

Previous research has reported the total phenolic contents of *Cornus* genotypes to be within the range of 25.90–74.83 mg gallic acid equivalent per gram of fresh weight [62,63]. However, in the investigation conducted by Hamid et al., the total phenol content was determined to be within the range of 1097.19–2695.75 mg gallic acid equivalent per 100 grams of fresh weight. The discrepancy in these values compared to previous studies was attributed to variations in total phenolic content influenced by environmental factors and post-harvest processes [64]. In our research, we observed that the total phenolic content of the aqueous extract from *C. mas* was notably higher. Another study investigating the phenolic content of 11 wild fruit species, including *P. spinosa, C. mas,* and *M. sylvestris*, using the HPLC-UV method, revealed that catechin hydrate was most abundant in *C. mas*, with a concentration of 268.16 mg per 100 grams of dry weight [65]. It was also demonstrated the strongest antioxidant activity, which correlates well with its bioactive content. These findings suggest that the extraction method plays a significant role in the quantity of bioactive compounds extracted, and this, in turn, affects the antioxidant potential of the extract.

Conclusions

In conclusion, this study highlighted the relationship between the chemical content and bioactive activities of the essential oils, fatty oils, and aqueous extracts of *P. cerasifera*, *M. sylvestris*, and *C. mas*. The fatty oils from these species were primarily composed of oleic acid (*P. cerasifera*), palmitic acid (*M. sylvestris*), and linoleic acid (*C. mas*), which are known for their potential health benefits, including anti-inflammatory and antioxidant properties. The essential oils revealed key compounds such as benzaldehyde in *P. cerasifera*, nonacosane in *M. sylvestris*, and (E,E)-2,4-Decadienal in *C. mas*, which may contribute to their therapeutic effects. Quinic acid, the most predominant compound in all extracts, was associated with antioxidant and antidiabetic activities, supporting its role as a bioactive agent in these plants. Notably, *C. mas* showed strong antimicrobial activity against *Candida* species, with MIC values indicating its potential as an antifungal agent. Furthermore, the aqueous extracts demonstrated promising α-glucosidase inhibition, with particularly potent activity observed in *C. mas* and *M. sylvestris*, as reflected in their low IC_{50} values. These findings demonstrate that the chemical composition of these extracts directly correlates with their bioactivity, underlining the potential of these plants for future therapeutic applications, especially in the management of diabetes, infections, and oxidative stress.

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CONFLICTS 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.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

GRAPHENE QUANTUM DOTS AND ITS DERIVATIVES AS A POTENTIAL INHIBITOR OF GLUTATHIONE *S*-TRANSFERASE PI-1 ISOENZYME: A COMPUTATIONAL STUDY

GLUTATYON S-TRANSFERAZ PI-1 İZOENZİMİNİN POTANSİYEL BİR İNHİBİTÖRÜ OLARAK GRAFEN KUANTUM NOKTALARI VE TÜREVLERİ: HESAPLAMALI BİR ÇALIŞMA

Murat KILIÇ[1]*, Bahadır BOYACIOĞLU[1]

[1]Ankara University, Vocational School of Health Services, 06290, Ankara, Türkiye

ABSTRACT

Objective: *Phase II detoxification enzymes called Glutathione S-Transferases (GSTs) protect our bodies from the harmful effects of xenobiotics. The GSTP1 isoenzyme not only detoxifies toxic substances but also contributes to cancer treatment resistance. The earliest and most potent GST inhibitor is ethacrynic acid (EA). This work compares graphene quantum dots (GQDs) with EA that has been shown to be beneficial in anticancer investigations, using molecular docking analysis in order to offer the idea of a possible inhibitor of GSTP1.*
Material and Method: *The density functional theory (DFT) method was applied to theoretical calculations on the GQDs compound using Gaussview 5.0 software. The application Gaussian 09 was used to refine the geometry. Calculations of molecular electrostatic potential (MEP) were used to identify the compounds' reactive sites. PyRx Tools and AutoDock Vina software were used to conduct molecular docking studies between the optimized EA and the GQDs molecule with GSTP1. The receptor-ligand interactions were visualized using Discover Studio Visualizer 4.0.*
Result and Discussion: *GQDs were found to interact with the H Site residues of GSTP1, as in EA. However, their electrophilicity was much lower than EA. We think that they can be GSTP1 inhibitors by increasing their electrophilicity with surface modifications.*
Keywords: *Density functional theory, glutathione S-transferase, graphene quantum dots, molecular docking*

ÖZ

Amaç: *Glutatyon S-Transferazlar (GST'ler) adı verilen Faz II detoksifikasyon enzimleri vücudumuzu ksenobiyotiklerin zararlı etkilerinden korur. GSTP1 izoenzimi yalnızca toksik maddeleri detoksifiye etmekle kalmaz, aynı zamanda kanser tedavisi direncine de katkıda bulunur. En erken ve en etkili GST inhibitörü etakrinik asittir (EA). Bu çalışma, GSTP1'in olası bir inhibitörü fikrini sunmak için moleküler yerleştirme analizini kullanarak antikanser araştırmalarında faydalı olduğu gösterilen EA ile grafen kuantum noktalarını (GQD'ler) karşılaştırmaktadır.*
Gereç ve Yöntem: *Gaussview 5.0 yazılımı kullanılarak, yoğunluk fonksiyonel teorisi (DFT) yöntemi GQDs bileşiği üzerindeki teorik hesaplamalara uygulandı. Geometriyi iyileştirmek için Gaussian 09 uygulaması kullanıldı. Bileşiklerin reaktif bölgelerini belirlemek için moleküler elektrostatik potansiyel (MEP) hesaplamaları kullanıldı. Optimize edilmiş EA ve GQDs molekülü ile GSTP1*

* **Corresponding Author / Sorumlu Yazar:** Murat Kılıç
e-mail / e-posta: muratkilic_genetic@yahoo.com, **Phone / Tel.:** +903123573242

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arasında moleküler yerleştirme çalışmaları yürütmek için PyRx Tools ve AutoDock Vina yazılımı kullanıldı. Reseptör-ligand etkileşimleri Discover Studio Visualizer 4.0 kullanılarak görselleştirildi.
Sonuç ve Tartışma: *GQD'lar, EA'da olduğu gibi GSTP1'in H Site rezidüleriyle etkileşimde olduğu bulundu. Ancak, elektrofiliklik kapasitesi EA'ya göre çok daha düşüktü. Yüzey modifikasyonları ile elektrofilikliği artırılarak, GSTP1 inhibitörü olabileceği düşüncesindeyiz.*
Anahtar Kelimeler: *Glutatyon S-transferaz, grafen kuantum noktaları, moleküler kenetlenme, yoğunluk fonksiyonel teorisi*

INTRODUCTION

With their multifunctional and broad-spectrum substrate specificity, Glutathione *S*-transferases (GSTs), found in many human tissues and organs, play a defensive role in organisms exposed to harmful substances [1,2]. To carry out this function, GSTs catalyze the thiol (-SH) group's nucleophilic attack on electrophilic substrates. This attack originates from the cysteine residue (Cys) of glutathione (GSH). Therefore, this conjugation reaction facilitates the synthesis of water-soluble conjugates that are less harmful and can be expelled from cells and tissues than the initial toxic molecule [3].

The three classifications of GSTs found in human cells and tissues, based on their primary structures and amino acid sequences, structural features, enzymatic properties, antibody reactions, chemical affinities, and behaviors, are categorized as cytosolic, microsomal, and mitochondrial GSTs. There are 11 distinct GST isoenzymes known to be present in humans: cytosolic GSTs have seven isoenzymes, including GST Alpha (GSTA), GST Mu (GSTM), GST Pi (GSTP), GST Sigma (GSTS), GST Theta (GSTT), GST Zeta (GSTZ), and GST Omega (GSTO); microsomal GSTs have three isoenzymes, MGST1, MGST2, and MGST3, which are membrane-bound proteins involved in eicosanoid and glutathione metabolism, part of the membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG); and mitochondrial GST has one member, GST Kappa (GSTK) [2,4].

The coding gene of GSTP, the most comprehensive, most studied and even the first isoenzyme studied in this enzyme family, is located on chromosome 11 and consists of seven exons. Two identical dimer subunits, each with 210 amino acids and two binding sites, typically make up human GSTP. The G-Site, which binds GSH, and the H-Site, which binds the electrophilic substrate, are two examples of these binding positions [5,6]. To enhance catalytic activity, GSTP can selectively bind to GSH or GSH analogs through the G-site, thereby facilitating the interaction between GST amino acid residues and GSH sulfhydryl groups and ubiquitous electrophilic substances at the H-sites [7].

To defend cells against oxidative stressors like reactive oxygen species (ROS), GSH and other related metabolic enzymes are essential. Redox (oxidation and reduction) processes offer this defense [5,8,9]. Research investigating the activities of antioxidant enzymes γ-Glutamyl Cysteine Synthase (γ-GCS) and Glutathione Reductase (GR) in tumors and peripheral normal tissues revealed that the GSTP1 isoenzyme has a strong positive connection with these antioxidant enzymes. Accordingly, by catalyzing glutathione's interaction with intracellular ROS and/or other electrophiles, GSTP1 may contribute to the control of cellular redox status, enhancing tumor cells' antioxidant capacity and assisting them in fending against externally harmful stimuli [10]. GSTP1, in particular, is involved in the intracellular transport of a wide range of substances, including bile acids, thyroid hormone, and steroid hormones, which are lipophilic and do not function as substrates for conjugation reactions, along with detoxifying reactive metabolites, GSTP1 in particular, which has the ability to bind non-enzymatic ligands. Furthermore, through their roles in protein-protein interactions, a number of kinases, including c-Jun N-terminal kinase (JNK), Mitogen-Activated Protein Kinase (MAP-Kinase), Protein Kinase C (PKC) and Protein Kinase A (PKA), and Epidermal Growth Factor Receptor (EGFR), directly alter the activities of these proteins. These kinases regulate a variety of cellular processes, such as cell proliferation, apoptosis, autophagy, and inflammation [11]. For instance, when GSTP1 is overexpressed or when it develops a reaction against ROS, interacts with and inhibits c-Jun N-terminal kinase-1 (JNK-1), which is crucial for the apoptotic pathway and cell proliferation, when it is overexpressed or when it develops a reaction against ROS. In this instance, GSTP1 seems to play a "non-enzymatic" antiapoptotic role in addition to influencing a cellular redox status [6,7]. Many malignancies, including those of the gastrointestinal tract [12], lung [4,13], breast [1,14], pancreatic [15], liver [16], ovary [17], lymphoma [18], and melanoma

[8], exhibit overexpression of GSTP1. Tumor cells also use GSTP1 to create a complex with GSH and chemotherapeutic drugs, which aids in detoxification and prevents apoptosis in malignant tissues or cells. P glycoprotein 1 (P-gp 1), which is encoded by the MDR1 (Multidrug Resistance) gene, causes an increase in their expression in tumor tissues. They collaborate with multidrug resistance proteins (MRP), which belong to the ATP-binding cassette (ABC) carrier protein family, to promote drug resistance [12,14,19,20].

For all of these reasons, scientists are looking for potent inhibitors of GSTP1 because they believe it is a good target for overcoming treatment resistance in cancer [6,21,22]. Accordingly, GSTP1 inhibitors are classified into two classes, α and β, based on their GSH conjugates formed with GSH and unsaturated carbonyl derivatives. It is known that GSTs catalyze the chemical conjugation of ethacrynic acid (EA) ([2,3-Dichloro-4(2-methylene-butyryl)phenoxy] acetic acid), a diuretic derived from α and β unsaturated carbonyl derivatives, to GSH [23]. Through covalent binding, EA and the EA-GSH conjugate are both strong reversible inhibitors of GSTs, especially those of the GSTA, GSTM, and GSTP classes [23,24]. Early investigations on EA's inhibitory action revealed that it significantly enhanced the toxicity of chlorambucil in resistant cells and increased the sensitivity of human leukemia, lymphoma, and myeloma cells to doxorubicin and nitrogen mustards [25]. EA has also been demonstrated to enhance the cytotoxicity of several alkylating drugs, including melphalan, carmustine, and mitomycin C, and to have anti-proliferative effects on tumor cells [26]. Despite all of these benefits, EA's low specificity, lack of isoenzyme specificity, and clinically significant diuretic side effects have restricted its usage as a GST inhibitor target [27-29]. To lessen the aforementioned negative effects, researchers have either synthesized several EA analogs [23,26] or have been investigating and testing various GST inhibitors [30].

Research on nanotechnology has grown in importance in the treatment of cancer. An example of this is graphene quantum dots (GQDs), a subclass of quantum dots (QDs) that are made from graphene sheets and have characteristics similar to both graphene and carbon dots. Because of these characteristics, GQDs are now increasingly frequently utilized in fields such as energy storage, drug delivery, and sensing and imaging. Furthermore, the ability to intentionally alter their properties by heteroatom doping accounts for their utilization in a variety of cutting-edge applications. They have unique qualities as a result [31]. Nanocomposites incorporating graphene have started to emerge as viable carriers for medicinal applications in research [32]. GQDs can enter cells and interact with the proteins and genetic material there. They alter the nucleus and cytoplasm of the cell, which eventually results in cytotoxic effects [33,34]. After a sequence of metabolic events from the mitochondria to the cytoplasm, exposure to graphene and graphene-based nanomaterials increases intracellular Reactive Oxygen Species (ROS), such as the superoxide radical generated by the cell's mitochondria, following a sequence of metabolic events from the mitochondria to the cytoplasm [35,36]. Furthermore, some research has attempted to combine GQDs with antibodies specific to cancer cells or integrate them into a carrier system to lessen their cytotoxicity in healthy cells [37]. Furthermore, because of their small size, high surface area, superior conductivity, and optical qualities, GQDs-a significant research topic in the field of nanotechnology-have a wide range of potential applications such as energy storage, sensors, and biomedical applications. This is due to their unique physical and chemical properties as well as their biological activity [38,39]. On the other hand, little is known about their possible characteristics in relation to cancer medication resistance.

In light of this, the purpose of this study was to compare the inhibitory potential of GQDs with ethacrynic acid using molecular docking analysis. Additionally, the study aimed to provide theoretical information that the molecule can also contribute to resistance against certain factors through various surface modifications. This was based on literature studies where analyses were conducted and the molecule's current use as a drug carrier. The potential results were supported by density functional theory (DFT) calculations.

MATERIAL AND METHOD

Density Functional Theory (DFT) Calculations

The GQDs molecule, whose molecular geometry was retrieved from the PubChem database and the model specified in Ref.[40], was subjected to theoretical calculations using the density functional theory (DFT) method [41] utilizing Gaussview 5.0 software [42]. The Gaussian 09 software was used to make geometric optimizations in the ground state in order to produce a high-quality theoretical approach [43]. The LanL2DZ (Los Alamos National Laboratory 2 double-ζ) basis set and the B3LYP (Becke's three-parameter hybrid exchange correlation function) functional were employed in these optimizations [44,45]. Then, in order to determine the reactive sites of the molecules and investigate their electrophilic and nucleophilic fields, molecular electrostatic potential (MEP) calculations were also carried out.

Molecular Docking Calculations

Molecular docking studies between the optimized Ethacrynic Acid and GQDs compound and GSTP1 protein (retrieved from the Protein Data Bank, PDB ID: 2GSS [25]) were performed via PyRx Tools [46] using AutoDock Vina software [47]. UCSF Chimera 1.18 software was used to remove water molecules and other heteroatoms from the protein prior to the docking process [48]. Using PyRx Tools, binding energies (kJ/mol) were computed as vina scores to assess the materials' affinity for binding to the protein. Software called Discover Studio Visualizer 4.0 was used to visualize receptor-ligand interactions [49]. The purpose of these analyses was to understand how the materials might interact with biological targets.

RESULT AND DISCUSSION

The binding affinities of ethacrynic acid and GQDs to GSTP1 protein are shown in Table 1. The result with the lowest binding affinity was ethacrynic acid. The reference ligand had a comparatively poor interaction with the GSTP1 protein, as seen by the maximum binding affinity value of -6.5 kcal/mol. With binding affinity values ranging from -11.6 to -10.9, GQDs show the strongest interaction. This indicates that GQDs and the GSTP1 protein have a high potential for binding. Furthermore, the stability of the reference ligand's binding sites is indicated by the fact that ethacrynic acid's consistently low RMSD (Root Mean Square Deviation) values, which range from 0 to 20 units. It is evident that the values of GQDs span a broad range from 0 to 30. This demonstrates the possibility of both stable and changing binding locations (Table 1).

Protein-ligand interaction analysis is one of the key computations in the molecular docking analysis approach. The binding location of EA in the three-dimensional GSTP1 protein crystal structure is displayed in Fig. 1a. The structural order is shown by color representations of the protein's helical, beta-sheet, and alpha-helix structures (green and yellow, respectively). The red dashed circle indicates the binding pocket where EA is placed. This position implies that EA has a potent ability to bind to the protein's active site. The specific interactions between the EA molecule and the amino acid residues in the binding pocket are depicted in two dimensions in Fig. 1b. Conventional hydrogen bonds, carbon-hydrogen bonds, π-π stacking, and van der Waals interactions are present. Through π-π interactions and traditional hydrogen bonding, aromatic amino acids like TYR (tyrosine) and PHE (phenylalanine) greatly enhance the binding stability specifically. Additionally, residues like ARG, GLY, ILE, and ASN are stabilized by van der Waals forces and are compatible with the binding site of EA. Fig. 1c shows a superficial visualization of the donor and acceptor sites of hydrogen bonds in the binding pocket. The hydrogen bond donor sites, represented in magenta, and the acceptor sites, shown in green, indicate strong hydrogen bond interactions that support the positioning of EA in the binding site. These bonds allow EA to be stably positioned in the binding site. The binding pocket's hydrophobicity map is displayed in Figure 1d. The binding site's apolarity and the strong hydrophobic interactions that EA has with those sites are represented by the dark patches. The parts that are open to polar interactions are indicated by the blue areas, which represent the hydrophilic portions. EA's flexibility and stability in the binding mechanism are enhanced by its capacity to engage with both polar and apolar locations in the binding site.

Table 1. Binding affinities of ethacrynic acid and GQDs to GSTP1 protein

	Ligand Model	Binding Affinity (kcal/mol)	RMSD* (ub)**	RMSD (lb)***
Ethacrynic Acid	1	-6.5	0	0
	2	-6.4	3.753	2.634
	3	-6.4	19.939	16.96
	4	-6.2	6.597	1.528
	5	-6.2	6.541	2.213
	6	-6.0	19.56	16.424
	7	-5.9	6.508	2.548
	8	-5.6	20.461	17.167
	9	-5.6	2.743	1.881
GQDs	1	-11.6	0	0
	2	-11.4	5.403	0.781
	3	-11.4	30.149	25.314
	4	-11.3	4.208	0.695
	5	-11.2	28.941	24.49
	6	-11.0	2.775	2.645
	7	-11.0	28.984	25.11
	8	-10.9	29.891	25.296
	9	-10.9	4.341	2.615

*:RMSD (Root Mean Square Deviation) values; **: ub (upper bound) and ***: lb (lower bound)

According to Oakley et al.'s earlier crystallographic structure elucidation work, ethacrynic acid interacts with the GSTP1 protein's Tyr7, Phe8, Val10, Arg13, Trp38, Ile104, Tyr108, and Gly205 residues [25]. In our work, EA interacts with the GSTP1 residues Val35, Gly205, Arg13, Gly12, Ile104, Asn204, Pro9, and Trp38 through van der Waals interactins, with Val10 through π-π stacking; with Phe8 through Pi-sigma interactions,; and with Tyr108 and Tyr7 residues through conventional hydrogen bonding, much like in the study of Oekley et al. The reference study stated that the inhibitor was situated in a hydrophobic pocket that was bordered by the aliphatic moieties of Arg13, Val35, Ile104, and Tyr108 as well as the side chains of Tyr7, Phe8, Pro9, and Val10. The loop portion of the inhibitor was positioned between the aromatic side chains of Tyr108 and Phe8 [25]. Additionally, our investigation showed that EA interacts with the binding sites through strong hydrophobic interactions. The findings of our docking analysis can be considered reliable based on data from the literature.

The three-dimensional binding of GQDs to the GSTP1 protein is depicted in Fig. 2a. Here, the colors green, yellow, and orange stand in for the protein's alpha-helix and beta-sheet structures. The GQDs molecule is situated in the protein's active site and in the binding location indicated by a red dashed circle. This suggests that GQDs are firmly attached to the binding pocket specific to the protein, and this association might be biologically significant. The GQDs molecules' interactions with the GSTP1 protein are shown in two dimensions in Fig. 2b. The key amino acid residues in the GQD binding regions, as well as the types of interactions and their distances, are described in detail here. Thus, van der Waals contacts are formed by residues Gly205, Asn204, Tyr108, Tyr7, Gly12, and Arg13; π-alkyl interactions by residues Val10, Val35, Trp38, and Ile104; and π-π stacking interactions by Phe8. We can better understand the strength and manner in which GQDs molecules attach to GSTP1 because the residues form hydrogen bonds, hydrophobic contacts, and π-π interactions with GQDs. The hydrogen bond donor (magenta) and acceptor (green) sites in the GSTP1 binding area are seen on the surface in Fig. 2c. GQDs form strong hydrogen bonds when they bind to these areas, and these interactions make the binding region more stable. The proper positioning and interaction of GQDs in the binding area suggest possible pathways that could influence the protein's biological functions. Lastly, the binding site's hydrophilic and hydrophobic regions are displayed in Fig. 2d. Whereas the blue color denotes the hydrophilic regions, the brown hydrophobic regions show that the GQDs molecules forms strong connections with these regions. This suggests that GQDs interact with both polar and apolar regions of the protein to help it bind and secure a stable place in the binding pocket. Our research is the first to

compare the potential inhibitory effects of GQDs based on EA, showing that the residues to which GQDs and EA bind GSTP1 are quite comparable. Furthermore, the strength of the connection is highlighted by the fact that interactions such as hydrogen bonds, hydrophobic interactions, van der Waals, and π-π stacking interactions are responsible for the contact with these residues in both the reference study [25] and our docking investigation. It should be noted, nevertheless, that GQDs exhibit van der Waals interactions with the GSTP1 protein's Gln51 residue, but EA does not. Furthermore, our data indicate that GQDs, such as EA, bind to the hydrophilic and hydrophobic domains of GSTP1, demonstrating that GQDs help the protein bind by interacting with both polar and apolar areas and achieve stability in the binding pocket.

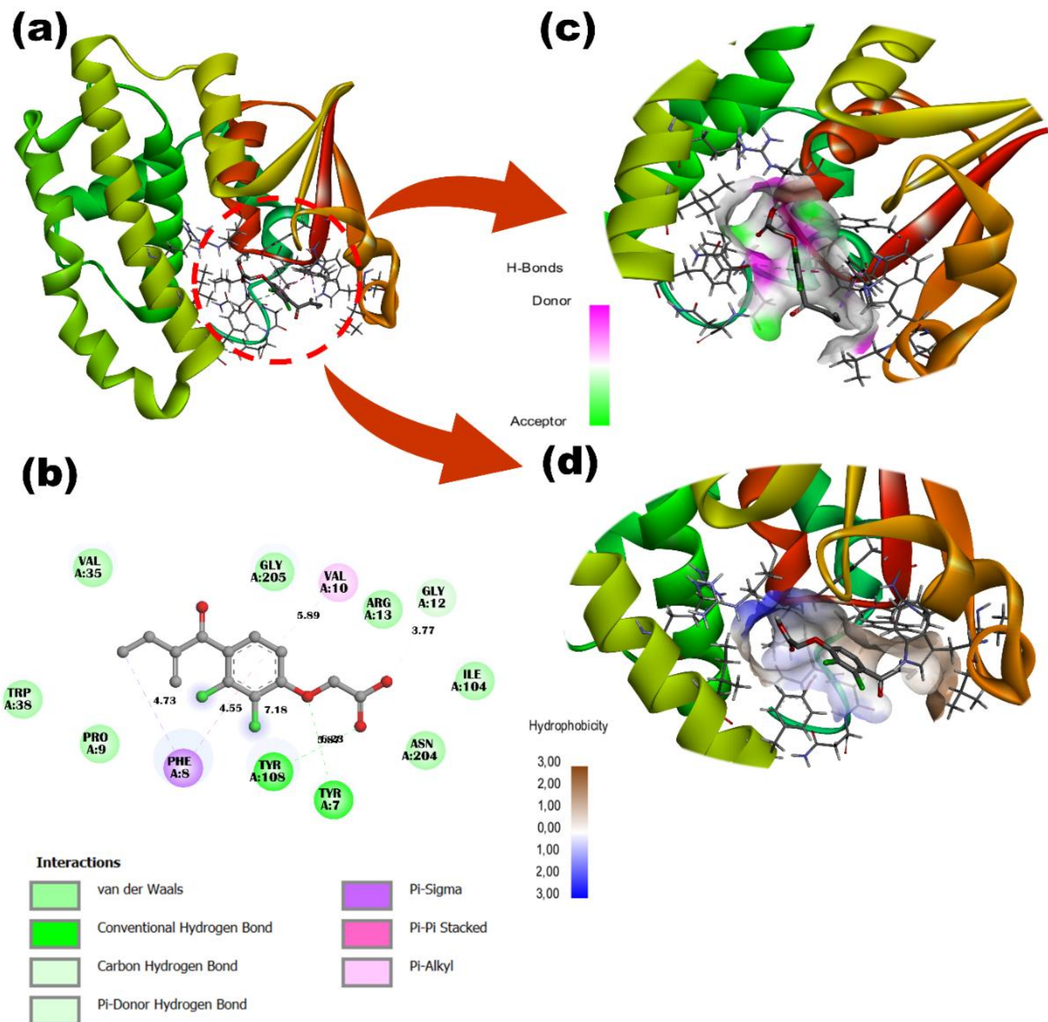

Figure 1. (a) A three-dimensional illustration that shows how the EA molecule binds to the GSTP1 active site and the interaction sites (red dashed circle); **(b)** a two-dimensional interaction map that displays the binding pocket residues that interact with EA; **(c)** a surface visualization of the GSTP1 binding pocket; **(d)** a map of the GSTP1 binding site's hydrophobicity, which shows the hydrophilic (blue) and hydrophobic (brown) areas surrounding the EA molecule

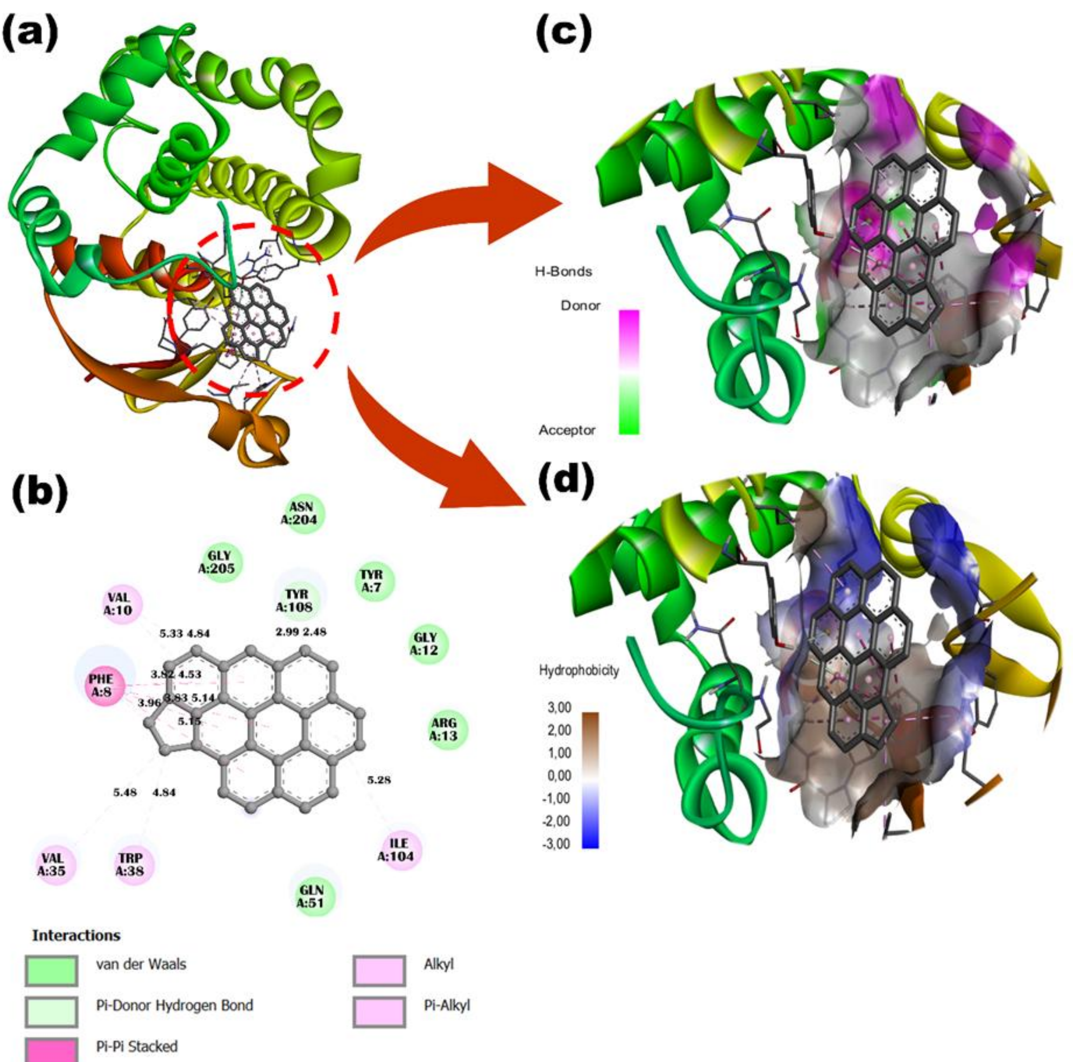

Figure 2. **(a)** A three-dimensional illustration of the GQDs molecule's binding to the GSTP1 active site and interaction sites (red dashed circle); **(b)** A two-dimensional interaction map showing the residues in the binding pocket interacting with the GQDs; **(c)** A visualization of the surface of a GSP1 binding pocket; **(d)** A map of the hydrophobicity of the GQDs molecule, showing the hydrophilic (blue) and hydrophobic (brown) regions

One crucial technique commonly employed for this goal is the development of molecular electrostatic potential (MEP) maps, which are an effective way to view a molecule's electrophilic and nucleophilic regions and assess the contributions of these regions to protein-ligand interactions [50]. By examining the electrophilic and nucleophilic areas of molecules, MEP maps offer crucial information for understanding biological processes and protein-ligand interactions. In this regard, MEP maps acquired for GQDs and EA were assessed. For both compounds, the MEP range is between -0.04 a.u. (red) and 0.04 a.u. (blue) (Fig 3). The red spots on the MEP map of EA (Fig. 3a) are locations that have carbonyl groups and other electrophilic characteristics. These areas may interact with protein regions that form hydrogen bonds or are positively charged. However, as Fig. 1b illustrates, the blue patches in and around the aromatic ring of EA show electrophilic sites that interact with nucleophilic amino acids. Strong and precise binding to the active sites of proteins like GSTP1 is made possible by these characteristics of EA.

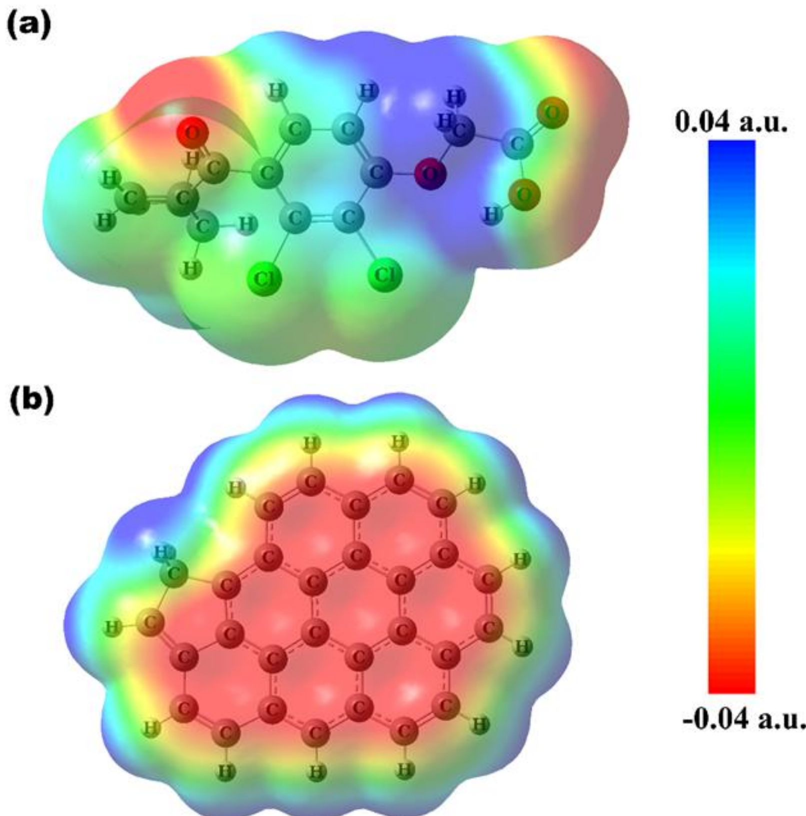

Figure 3. MEP maps of (a) EA and (b) GQDs

The red color in the GQDs' MEP map (Fig. 3b) highlight their nucleophilic characteristics. Examining the map reveals that the red spots represent the graphene structure's double bonds, or aromatic rings; these locations have a greater electron density and are thus nucleophilic. These nucleophilic regions may interact with the positively charged regions of proteins or hydrogen bond donors. The blue colored areas indicate that the carbon atoms are more electrophilic; in particular, the surface-bound sp^2 hybridized carbon atoms can interact with protein nucleophiles. Since GQDs are made entirely of hydrogen and carbon atoms, this highlights the hydrophobic nature of the molecule and raises the possibility of attaching through aromatic interactions—like π-π stacking—instead of polar interactions. This indicates that in biological settings, GQDs can establish potent π-π or π-doped interactions with proteins, particularly amino acids with aromatic rings.

The H Site, one of GSTP1's two binding sites, is often where electrophilic substrates bind to provide their inactivating effects [5-7]. The hexyl moiety of the S-hexyl-GSH complex crystal structure is similar to the location where EA, an inhibitor of GSTs, attaches to the enzyme's H site [51]. Reinemer et al. state that residues Tyr7, Phe8, Val10, Val35, Tyr108, and Gly205 are present in the electrophile-binding site H-Site. Ji et al., based on crystallization findings using S-hexylglutathione or 9-(6-glutathionyl)-10-hydroxy-9,10-dihydrophenanthrene, include the additional residues Arg13 and Asn204 in this area [51,52]. Nevertheless, certain chemicals have also been shown to bind to the residues of the active site, or G Site, and inhibit the enzyme. For instance, TER-117 (g-glutamyl-(S-benzyl)cysteinyl-D-phenylglycine) is the most selective hGST P1-1 inhibitor, according to Oakley and Bello et al. (1997). According to their research, the GSH conjugate bound to hGST P1-1 and the binding of TER-117 to GSTP1 had many structural similarities to that of EA. However, they also noted that this inhibitor differs from EA in that the phenyl ring in the TER-117 structure attaches to the G-site of GSTP [53]. In addition, another study highlighted that Ezatiostat (TLK199), also known as Telintra, inhibits the enzyme by forming significant contacts with amino acid residues in the G Site region of the enzyme, including Tyr7, Lys44, Arg13, and Ser11 [54]. Nevertheless, there is no biological effects or molecular support

for these ideas. It is our belief that both compounds interact with GSTP1 from its active site and alter the enzyme's activity rather than directly inhibiting it. Although GQDs also demonstrated binding and interaction with the GSTP1 protein's H Site residues, as evident in the literature, our results suggest that the core of the GQDs has greater nucleophilic characteristics when the MEP map is analyzed. It is possible to argue that the surface is more electrophilic than the interior. Given that the GSTP1 isoenzyme's surface is more electrophilic than its center, it makes sense that GQDs have attached to the H Site.

Nonetheless, our investigations and data from the literature have shown that EA has potent electrophilic qualities as a GSTP1 inhibitor. Therefore, we think that by making GQDs more electrophilic through various and biocompatible surface modifications, an alternative to EA as a GSTP1 inhibitor can be created. The goal of choosing biocompatible modifications to surface is to minimize toxicity while delivering the desired inhibitory effects. To improve the electrophilic qualities of the GQD structure, heteroatoms like phosphorus (P), boron (B), and nitrogen (N) can be added [53]. For instance, hydrothermal techniques can be used to create N-doped GQDs (N-GQDs), and this process is biocompatible [31,40]. The addition of oxygen-containing compounds, such as carboxyl or carbonyl groups, to the surface of GQDs can improve their interaction with GSTP1 by increasing the GQDs' electrophilia [55]. GQDs can be coated with biomolecules like protein nanofibers [56] or folic acid [57,58] to improve their electrophilic qualities and give them targeting capability. Because it improves the ability to target cells, this strategy is especially well-suited for cancer treatment. Furthermore, the surface electron density of GQDs can be altered by electrochemically adding active groups, and biocompatible outcomes can be obtained [55]. Without a doubt, in vitro and later in vivo experimental research should be conducted to examine the roles of GQDs and biocompatible surface modifications that will increase their electrophilia in preventing drug resistance.

In conclusion, our research has demonstrated for the first time whether GQDs, which are crucial in physics, chemistry, and biology research, can potentially inhibit the GSTP1 isoenzyme. Therefore, our study followed the same methodology as the first studies on the topic in the literature. Using the docking method, we first demonstrated the molecular interaction between EA and GSTP1 and provided a standardization. Then, we compared GQDs with this standard and examined their potential as a GSTP1 inhibitor. As shown in the computational analyses performed in our study, it can be said that GQDs bind to the H Site of the enzyme, interact strongly with similar residues in the H Site of the enzyme like EA, and therefore they can be an effective inhibitor like EA. However, since the electrophilic properties of GQDs are lower than EA, we think that this inhibitory effect should be shown with evidence-based experimental studies by increasing their electrophilicity with biocompatible surface modifications.

AUTHOR CONTRIBUTIONS

Concept: M.K.; Design: M.K., B.B.; Control: M.K., B.B.; Sources: M.K., B.B.; Materials: M.K., B.B.; Data Collection and/or Processing: M.K., B.B.; Analysis and/or Interpretation: M.K., B.B.; Literature Review: M.K., B.B.; Manuscript Writing: M.K., B.B.; Critical Review: M.K., B.B.; 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.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

EXAMINATION OF THE RELATIONSHIP OF SERUM ADIPOKINE HORMONE LEVELS WITH THE DEGREE AND SOME MARKERS OF OBESITY IN OBESE CHILDREN

OBEZ ÇOCUKLARDA SERUM ADİPOKİN HORMON DÜZEYLERİNİN OBEZİTE DERECELERİ VE BAZI BELİRTEÇLERİ İLE İLİŞKİSİNİN İNCELENMESİ

Merve DEMİRBİLEK[1], Dolunay Merve TOPUZ[1], Ayşe Derya BULUŞ[2], Erhan FAKIOĞLU[3], Fatih Mehmet KIŞLAL[3], Sevgi YARDIM-AKAYDIN[1]*

[1]Gazi University, Faculty of Pharmacy, Department of Biochemistry, 06330, Ankara, Türkiye
[2] University of Health and Sciences, Department of Pediatric Endocrinology, Kecioren Training and Research Hospital, 06290, Ankara, Türkiye
[3]University of Health and Sciences, Kecioren Training and Research Hospital, Department of Pediatrics, 06290, Ankara, Türkiye

ABSTRACT

Objective: *Obesity is defined as excessive increase in adipose tissue in the body. Adipose tissue also provides the secretion of adipokine hormones such as leptin, adiponectin, asprosin and apelin, which play an important role in metabolism and energy homeostasis. For this reason, changes in the levels of these adipokine hormones secreted from the adipose tissue, not the increase in adipose tissue alone, are effective in determining childhood obesity and explaining the pathological mechanisms. Evaluation of these polypeptides will be effective in the diagnosis of obesity and elucidating the pathological mechanisms associated with obesity.*

Material and Method: *In our study, it was conducted to reveal the relationship of obese children with some markers of obesity such as serum leptin, adiponectin, asprosin and apelin levels, and blood lipids, fasting blood glucose and insulin resistance index (HOMA-IR) values. 105 obese children and 38 normal weight children participated in our study. The demographic information of the children was determined by the face-to-face survey method. Leptin, adiponectin, asprosin and apelin levels were determined in morning fasting blood samples from obese and normal weight children with the help of commercial Elisa kit.*

Result and Discussion: *Leptin and adiponectin levels were not different in obese and normal weight children ($p>0.05$). Asprosin and apelin levels were found to be significantly higher in obese children compared to normal weight children ($p<0.001$). Positive correlations were observed between adipokine hormones in both obese and normal children. The ratios of leptin/asprosin, leptin/apelin, adiponectin/asprosin, adiponectin/apelin, asprosin/apelin were found to be statistically significantly lower in obese children when compared with normal weight children ($p<0.05$). In our study, high levels of asprosin and apelin, which have conflicting results in the literature, and positive correlations between adipokine hormones can be considered as important findings.*

Keywords: *Adiponectin, apelin, asprosin, childhood obesity, leptin*

* **Corresponding Author / SorumluYazar:** Sevgi Yardim-Akaydin
e-mail / e-posta: sevgiy@gazi.edu.tr, **Phone / Tel.:** +903122023155

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ÖZ

Amaç: *Obezite, vücuttaki yağ dokusunun aşırı artışı olarak tanımlanır. Yağ dokusu ayrıca metabolizma ve enerji homeostazında önemli rol oynayan leptin, adiponektin, asprosin ve apelin gibi adipokin hormonlarının salgılanmasını sağlar. Bu nedenle, sadece yağ dokusundaki artış değil, yağ dokusundan salgılanan bu adipokin hormonlarının düzeylerindeki değişiklikler çocukluk çağı obezitesini belirlemede ve patolojik mekanizmaları açıklamada etkilidir. Bu polipeptitlerin değerlendirilmesi obezitenin tanısında ve obeziteyle ilişkili patolojik mekanizmaların aydınlatılmasında etkili olacaktır.*

Gereç ve Yöntem: *Çalışmamız, obez çocukların serum leptin, adiponektin, asprosin ve apelin düzeyleri gibi bazı obezite belirteçleri ile kan lipitleri, açlık kan şekeri ve insülin direnci indeksi (HOMA-IR) değerleri arasındaki ilişkiyi ortaya koymak amacıyla yapıldı. Çalışmamıza 105 obez çocuk ve 38 normal kilolu çocuk dahil edildi. Çocukların demografik bilgileri yüz yüze anket yöntemi ile belirlendi. Leptin, adiponektin, asprosin ve apelin düzeyleri obez ve normal kilolu çocuklardan alınan sabah açlık kan örneklerinde ticari Elisa kiti yardımıyla tayin edildi.*

Sonuç ve Tartışma: *Leptin ve adiponektin düzeyleri obez ve normal kilolu çocuklarda farklı değildi (p>0,05). Asprosin ve apelin düzeyleri obez çocuklarda normal kilolu çocuklara göre anlamlı olarak yüksek bulundu (p<0,001). Hem obez hem de normal kilolu çocuklarda adipokin hormonları arasında pozitif korelasyonlar gözlendi. Leptin/asprosin, leptin/apelin, adiponektin/asprosin, adiponektin/apelin, asprosin/apelin oranları obez çocuklarda normal kilolu çocuklara göre istatistiksel olarak anlamlı şekilde düşük bulundu (p<0,05). Çalışmamızda literatürde çelişkili sonuçlar bulunan asprosin ve apelin düzeylerinin yüksek olması ve adipokin hormonları arasındaki pozitif korelasyonlar önemli bulgular olarak değerlendirilebilir.*

Anahtar Kelimeler: *Adiponektin, apelin, asprosin, çocukluk çağı obezitesi, leptin*

INTRODUCTION

Childhood obesity is a growing global health problem all over the world. Obesity also cause an economic burden on the health system [1,2]. Children at risk need to be carefully identified and treated to effectively prevent obesity. The causes of childhood obesity are highly complicated and multifactorial. These factors include the genetic, environmental and ecological factors that can be associated with the family environment, the society in which they are raised, and particularly the school where they spend most of their days. Some endocrine disorders, insufficient sleep and some medications can also be considered as causes of obesity [1,3,4].

Obesity in children and adolescent is related with metabolic diseases such as psychological problems, obstructive sleep apnea, orthopedic disorders, polycystic ovarian diseases, type 2 diabetes, cardiovascular diseases and metabolic disorders [5]. Nowadays, the relationship of obesity with chronic diseases and the fact that the foundations of adult obesity are laid in childhood become substantial in the prevention, particularly in the diagnosis and treatment of childhood obesity.

By definition, obesity is defined as an overgrowth of adipose tissue, and adipose tissue is the main site where triacylglycerols are stored as energy [6,7]. However, adipose tissue does not only serve as a storage, but also secretes hormones called adipokines, which play an essential role in metabolism and energy homeostasis, such as leptin, adiponectin, apelin and asprosin [7,8]. These adipokines have a significant impact on obesity-related comorbidities and complications. Many studies have found increase plasma apelin and asprosin levels in obese individuals, and this increase has been associated with gain in adipose tissue [1,6,9,10]. These secretions of adipose tissue play a predispose role in diseases such as insulin resistance, metabolic syndrome and type 2 diabetes [8].

Understanding the pathophysiological relationship between the serum adipokine levels of obese individuals and obesity-related comorbidities may enable more accurate identification of high-risk patients. This definition comes to the fore more in child groups where the progression of obesity and obesity-related complications is important. Unlike the current data in this study on childhood obesity; not only one but several of the adipokines that may be associated with obesity have been evaluated together.

MATERIAL AND METHOD

Study Groups

In this study, the relationship between serum adipokine hormone levels and the degree of obesity and some markers in obese children was examined cross-sectionally. The study included 105 obese children (54 females and 51 males) between the ages of 10-17 who applied to the Pediatric Endocrinology Polyclinic of Keçiören Training and Research Hospital (KAEH) and 38 healthy and normal weight children (14 females and 24 males) who applied to the Healthy Child Polyclinic. The obese group is between the ages of 10-17 and has a BMI of ≥95 according to the WHO's 5-19 age and sex-specific references. Additionally, children were classified as I. degree obese (mild; 100-120% of Body Mass Index (BMI) corresponding to the 95th percentile), II. degree obese (moderate; 120-140% of BMI corresponding to the 95th percentile), III. degree obese (severe; BMI >140 corresponding to the 95th percentile) and this was considered as an inclusion criterion [11]. The control group included normal-weight children aged 10-17 years with a BMI between the 5th and 85th percentile according to the WHO (2007) 5-19 age and sex-specific references.

Local ethics committee approval (dated 11.05.2021 and numbered 2012-KAEK-15/2289) was obtained before sample collection for the study. Children and their families were informed verbally and in writing about the details of the study before they were included in the study. Along with that, consent was obtained from both parents and children by signing an informed consent form.

Blood samples were taken from the children participating in the study after at least 8 hours fasting. The blood sample was centrifuged and the serum part was separated and stored at -20°C.

Anthropometric Measurements

Demographic information were determined by face-to-face survey method before the children accepted to the study. Then, body weight (kg), height (cm), waist circumference (cm), hip circumference (cm), BMI (kg/m²), neck circumference (cm), waist/hip ratio were taken in accordance with the specified methods. Fat percentages of obese children were measured by the Bioimpedance Current (BIA) method (Tanita).

Experimental Measurements

The amounts of serum leptin, adiponectin, asprosin and apelin (Human ELISA kits, SunRedBio, China) were determined with a commercial kit. According to these measurement results, apelin/adiponectin ratio, leptin/adiponectin ratio and asprosin/adiponectin ratios were evaluated. In addition, all children's fasting blood glucose, triglyceride (TG), total cholesterol (TC), LDL-C, HDL-C, insulin and HbA1C measurements were also made with routine laboratory tests.

Statistical Analysis

The data obtained as a result of the study are indicated as the mean (standard deviation). Data analysis was performed using IBM SPSS Statistics 22.0 software (IBM Corporation, Armonk, NY, USA). Whether the distributions of continuous variables were normal or not was determined by Kolmogorov-Smirnov test. Chi-square test was used for comparison of qualitative data in comparison of demographic data. While the mean differences between groups were compared using Student's t test, the Mann–Whitney U test was applied for the comparisons of the continuous variables, in which the parametrical test assumptions were not met. Spearman-correlation test was used to examine the relationship between the parameters for the non-normally distributed data. P values of less than 0.05 were regarded as statistically significant.

RESULT AND DISCUSSION

In the Kolmogorov-Smirnov test (significance level was taken as 0.05), the adipokine hormone levels of the obese and normal weight children in our study did not show a normal distribution. For this reason, non-parametric tests, which do not require the normality condition, were applied in the statistical analysis of the findings.

Table 1 shows the demographic information of obese and normal weight children. The study groups were compared statistically according to age, age range, gender, family obesity status, familial

chronic disease history and mode of delivery, and the difference between other criteria was found statistically significant ($p<0.05$). Waist circumference, neck circumference, hip circumference, BMI and waist/hip circumference ratios were compared and a significant difference was found between these anthropometric measurements ($p<0.05$) (Table 1).

Table 1. Main characteristics of obese and normal weight children

Variable	Obese Child (N= 105)	Normal Child (N=38)	P
Age, Mean (Standard Deviation)	13.6 (2.1)	12.8 (1.9)	0.031
Age Range			
≤11 age, N (%)	23 (21.9)	10 (26.3)	
12 age, N (%)	11 (10.5)	10 (26.3)	
13 age, N (%)	16 (15.2)	8 (21.1)	0.028
14 age, N (%)	16 (15.2)	5 (13.2)	
≥15, age N (%)	39 (37.1)	5 (13.2)	
Gender			
Girls, N (%)	54 (51.4)	14 (36.8)	--
Boys, N (%)	51 (48.6)	24 (63.2)	
Obesity Status of the Family			
Yes, N (%)	87 (82.9)	15 (39.5)	0.000
No, N (%)	18 (17.1)	23 (60.5)	
Family History of Chronic Disease			
Yes, N (%)	71 (67.6)	17 (44.7)	0.013
No, N (%)	34 (32.4)	21 (55.3)	
Mode of Delivery			
Vaginal Delivery, N (%)	56 (53.3)	23 (60.5)	0.566
Cesarian Section, N (%)	49 (46.7)	15 (39.5)	
Anthropometric Measurements			
Waist Circumference (cm)	98.1 (11.9)	69.8 (8.6)	0.000
Neck Circumference (cm)	37.2 (3.2)	30.7 (2.3)	0.000
Hip Circumference (cm)	112.9 (11)	83.7 (10)	0.000
Fat percentage (%)	37.3 (7)	--	--
BMI (kg/m^2)	31.5 (4.7)	19.4 (2.8)	0.000
Waist/Hip ratio	0.86 (0.075)	0.83 (0.072)	0.020
Obesity Degree			
1th Degree, N (%)	51 (48.6)	--	--
2th Degree, N (%)	45 (42.9)	--	--
3th Degree, N (%)	9 (8.6)	--	--

Table 2 includes routine biochemistry tests of obese and normal weight children. TC, TG, LDL-C, HDL-C, fasting blood glucose, insulin level and HOMA-IR values were compared. Mean TG and fasting insulin levels of obese children were found to be significantly higher than those of normal weight children ($p<0.05$). The HDL-C level of the normal-weight children was found to be significantly higher than the obese children ($p<0.05$). There was no significant difference between obese and normal weight children in terms of mean TC, LDL-C, fasting blood glucose, and HbA1c levels ($p>0.05$) (Table 2).

Adipokine hormones such as leptin, adiponectin, asprosin, apelin, resistin, and visfatin secreted from adipose tissue [9,10,12,13] play an important role in metabolism and energy homeostasis [9]. Depending on these hormones, disorders such as insulin resistance, metabolic syndrome and diabetes may develop [10]. In determining childhood obesity and explaining the mechanisms, not only the increase in adipose tissue, but also the evaluation of the changes in the levels of these adipokines secreted from the adipose tissue will be effective. In our study, serum leptin, adiponectin, asprosin and apelin levels and their ratios to each other were evaluated in obese and normal weight children, and the correlations between them and their relationship with the degree of obesity and some of its markers were

examined. When the hormone levels of children were evaluated, leptin and adiponectin levels did not show a statistically significant difference between obese and normal weight children (p>0.05) (Table 3).

Table 2. Routine biochemistry tests of obese and normal weight children

Parameter	Obese Child (N= 105)	Normal Weight Child (N=38)	P
	Mean (SD)		
Total Cholesterol (mg/dl)	158.1 (45.8)	155.7 (35)	0.776
Triglyceride (mg/dl)	141.4 (92.5)	87.1 (50.5)	0.000
HDL-C	41.8 (12.3)	52.3 (11)	0.000
LDL-C	90.6 (31.1)	85.9 (29.5)	0.422
Glucose (mg/dl)	90.5 (23.6)	92.1 (7.9)	0.545
Insulin	19.8 (13.5)	7.9 (3.3)	0.000
HbA1c (%)	5.1 (1.2)	5 (0.4)	0.503
HOMA-IR	4.99 (3.8)	1.8 (0.7)	0.000

Table 3. Adipokine hormone levels and rates of hormone levels in obese and normal weight children

Adipokine Levels	Obese Child* Mean (SD)	Normal Weight Child** Mean (SD)	P
Leptin (ng/l)	4.25 (4.65)	3.59 (1.83)	0.265
Adiponectin (ng/l)	10.46 (12.01)	11.31 (9.62)	0.443
Asprosin (ng/l)	22.58 (18.33)	11.15 (11.17)	**0.000**
Apelin (ng/l)	19.36 (14.55)	10.66 (13.94)	**0.001**

*N= 105, **N=38

Leptin is associated with fat storage, which reflects energy status in the body [14]. Obese individuals with high fat content have high circulating leptin levels. However, when leptin promotes satiety, it was thought that obese individuals might develop a type of central leptin resistance that impairs the satiety response [15]. In our study, leptin levels did not show a significant difference between obese and normal weight children. However, the slight increase in leptin levels observed in obese children can be considered as a reflection of the development of leptin resistance in childhood. In a study conducted; obese children have been reported to have higher serum leptin levels than controls [16]. In our study, a negative correlation was observed between serum leptin levels and TC and LDL-C levels in obese children (p=0.008, p=0.002). Unlike our study, according to a study that reported that leptin levels may be determinative in the evaluation of blood lipids, leptin levels were found to be higher in obese children, and a positive correlation was reported between leptin levels and TG and LDL-C levels, and a negative correlation between HDL-C levels [17]. However, the effect of leptin on blood lipids is still unclear.

Adiponectin can be considered as a therapeutic approach to reduce the burden of chronic diseases such as obesity, diabetes and heart diseases [18]. In our study, although there was no significant difference between the adiponectin levels of obese and normal weight children, it was observed to be lower in obese children. Other studies have also reported lower adiponectin levels in obese children compared to controls [16,19]. In our study, adiponectin levels were observed to be lower in obese children aged 11 years and younger. In addition, we found a positive correlation between serum adiponectin levels and HOMA-IR values in children with normal weight (p=0.005). This may suggest that there may be a mechanism to prevent the development of obesity in children.

Asprosin is a fasting-related adipokine hormone that regulates glucose release from the liver [20]. Asprosin has been shown to be significantly associated with glucose and insulin release in the liver during fasting. It has been reported that increased asprosin levels may be a risk factor for insulin resistance. It is also thought that reducing asprosin levels may prevent hyperinsulinemia associated with metabolic syndrome [21]. Consistent with hepatic glucose release during fasting, serum asprosin levels increase with fasting and rapidly decrease with re-intake of food. Studies on asprosin suggest that

asprosin may be a potential target for the treatment of diabetes and obesity [20]. In our study, serum asprosin levels were found to be significantly higher in obese children than in normal-weight children. When both obese and normal weight children were evaluated together, it was observed that serum asprosin levels showed a positive correlation with insulin levels and HOMA-IR values (R=0.200; p=0.027 and R=0.225; p=0.013, respectively). A positive correlation was observed between serum asprosin level and TC in normal weight children (p=0.035). High levels of asprosin, a hormone that regulates glucose secretion from the liver and is released during fasting, in obese children can be considered not only as a result of obesity but also as a cause. In a study by Wang et al. [20] serum asprosin levels were found to be significantly higher in obese children compared to controls. In addition, asprosin levels were found to be higher in children with insulin resistance. According to these results; it has been reported that serum asprosin levels may be a new marker for predicting obesity and obesity-related diseases [20]. In another study, it was reported that asprosin levels were significantly higher in obese children compared to controls and that asprosin may be a predictor of obesity [21]. However, Long et al. [8] reported that obese children had lower asprosin levels compared to normal weight controls. Although it is accepted that these low asprosin levels will cause weight loss, it has been stated that this may be related to compensatory adaptations in energy metabolism. It has also been reported that this may be related to the degree of obesity [8]. However, in our study, no significant relationship was found between the degree of obesity and adipokine hormone levels.

Apelin, synthesized from adipose tissue, is one of the newly discovered bioactive peptides. Apelin plays an important role in feeding mechanisms by stimulating the secretion of cholecystokinin and in lowering blood pressure through the nitric oxide mechanism. Apelin synthesis is stimulated by insulin. It is known that plasma levels may increase especially in association with insulin resistance and hyperinsulinemia [22]. Apelin secretion decreases with fasting and increases with food intake similar to insulin. Apelin is effective in regulating fluid balance, blood pressure, heart contraction and stimulating the release of ACTH by the pituitary gland. Many studies have shown that there may be a relationship between serum apelin levels and metabolic diseases [23]. In our study, positive correlations were observed between apelin, insulin and HOMA-IR in normal weight children (p=0.011 and p=0.016, respectively). At the same time, a positive correlation was observed between apelin, TC and LDL-C in normal-weight children in our study (p=0.020, p=0.007, respectively). Studies show that there may be a relationship between serum apelin levels and metabolic diseases [9,22]. In our study, serum apelin levels were found to be significantly higher in obese children, supporting these findings. The fact that apelin is an insulin-stimulated hormone [22] and the association between obesity and hyperinsulinemia, insulin resistance, and diabetes [4] suggests that apelin may be elevated as a result of obesity. In a study, serum apelin levels were found to be significantly higher in obese children compared to controls, and it was stated that this may play a role in childhood obesity [9]. Similarly, in another study, serum apelin levels were found to be significantly higher in obese children compared to controls. In addition, a positive correlation was observed between serum apelin level and fasting blood glucose, serum insulin, HOMA-IR, TG and TC values in obese children. According to these results, it has been reported that serum apelin levels may play a role in the development of obesity-related complications such as insulin resistance, hypertension and metabolic syndrome in children [22]. Increased serum apelin levels with adipose tissue increase may play a role in the pathological mechanism of obesity. In this case, apelin can be used in clinical applications as a biomarker or a therapeutic target. However, in another study; It has been reported that obese children have lower plasma apelin levels than controls, and there is a negative correlation between plasma apelin levels and BMI, insulin levels, and HOMA-IR values [19]. The findings of studies investigating the level and role of apelin in obesity are inconsistent and more studies are needed.

The ratios of hormones were compared with each other, leptin/asprosin, leptin/apelin, adiponectin/asprosin, adiponectin/apelin and asprosin/apelin levels in obese children, it was found to be significantly lower ($p<0.05$) to normal weight children (Figure 1). Studies have calculated that the leptin/adiponectin ratio is higher in obese individuals than in normal-weight individuals [24,25]. In our study, although not statistically significant, the leptin/adiponectin ratio was found to be slightly higher in obese children. However, in our study, the ratios of all hormones were evaluated for the first time, and significant differences were observed between the ratios of leptin/asprosin, leptin/apelin,

adiponectin/asprosin, adiponectin/apelin, asprosin/apelin in obese children compared to normal-weight children. In one study, it was reported that the leptin/adiponectin ratio in obese children was more strongly associated with insulin resistance and cardiometabolic comorbidities than leptin and adiponectin separately [24]. In another study; It has been reported that the leptin/adiponectin ratio may be a better predictor of insulin sensitivity than leptin and adiponectin levels [25]. According to these results, the evaluation of the ratios of these interrelated hormones may reveal stronger results. In our study, positive correlations were observed between leptin and adiponectin and asprosin, between adiponectin and asprosin, and between asprosin and apelin in obese children. In normal weight children, positive correlations were observed between adiponectin and asprosin and between asprosin and apelin. In this case, the positive correlations between these hormones may be effective on the complex mechanism of obesity. Especially the fact that these hormones increase and decrease together can be effective in increasing or decreasing food intake. Our study was the first to examine the relationships between these adipokine hormones. However, considering the relationship between these hormones, more research is needed.

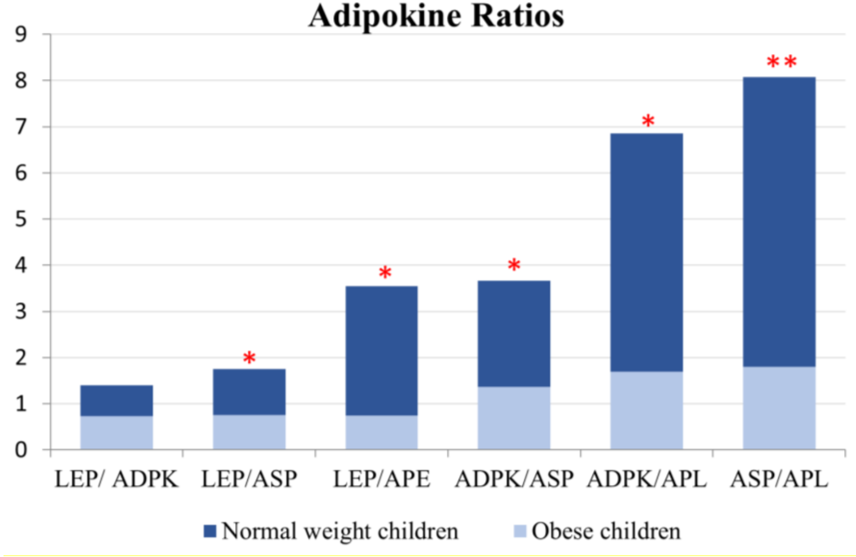

Figure 1. Adipokine hormone ratios in obese and normal weight children (* $p<0.001$, ** $p<0.05$)

When compared by gender, asprosin and apelin levels in obese girls were found to be significantly higher than in normal-weight girls. Whereas obese and normal weight children were evaluated separately, asprosin hormone levels in normal weight girls [22.39 (15.79) ng/l] were found to be significantly lower than those in normal weight boys [6.98 (8.09) ng/l] ($p<0.001$), and apelin hormone levels in obese girls [21.63 (14.76) ng/l] were significantly higher than those in obese boys [3.58 (5.33) ng/l] ($p<0.005$). Asprosin and apelin levels in girls in the obese group were observed to be higher than in girls in the normal weight group. However, in the obese group, asprosin levels did not differ depending on gender, while it was found to be higher in boys in the normal weight group. Apelin levels were found to be higher in obese girls. In our study, when obese and normal weight children were considered together, apelin levels were found to be higher in girls than in boys ($p<0.05$). In a study, apelin levels were found to be significantly higher in girls compared to controls, similar to our study [9]. Another study reported lower serum asprosin levels in boys than girls and a negative association between asprosin and male gender in all groups [26]. The relationship between apelin and asprosin levels and gender differences has not been clearly investigated and explained. In a study, it was reported that apelin and adiponectin levels were independent of gender differences. However, significant differences in apelin hormones in the pubertal period and adiponectin in the pre- and post-pubertal periods suggested that there may be a relationship between sex hormones and adipokine hormone levels [19]. In another study, it was reported that the hormone levels of adipokines secreted from adipose tissue may increase

in girls because of hormonal differences during adolescence, because more adipose tissue and non-fatty tissue increase in boys [27].

When there was no statistically significant difference in hormone levels between the groups in terms of obesity degree, adiponectin and apelin levels showed a linear increase, while asprosin levels showed a linear decrease (p>0.05).

When the correlations between adipokine hormones were examined in obese children, positive correlations were observed between leptin and adiponectin and asprosin, between adiponectin and asprosin, and between asprosin and apelin (Table 4). Considering the correlations between adipokine hormones in normal weight children, positive correlations were observed between adiponectin and asprosin and apelin, and between asprosin and apelin (Table 4). When obese and normal weight children were evaluated together, positive correlations were observed between adiponectin and leptin, asprosin and apelin, and between asprosin and apelin (Table 4).

Tablo 4. The relationship between adipokine hormone relationship in obese and normal weight children

			R/P*	
		Leptin	**Adiponectin**	**Asprosin**
Obese Child	**Adiponectin**	**0.354 (0.000)**	--	
	Asprosin	**0.227 (0.035)**	**0.411 (0.000)**	--
	Apelin	-0.045 (0.670)	0.174 (0.098)	**0.254 (0.023)**
Normal Weight Child	**Adiponectin**	0.206 (0.275)	--	
	Asprosin	0.181 (0.339)	**0.379 (0.021)**	--
	Apelin	0.222 (0.446)	**0.628 (0.003)**	**0.603 (0.005)**
Total	**Adiponectin**	**0.319 (0.000)**	--	
	Asprosin	0.118 (0.205)	**0.400 (0.000)**	**--**
	Apelin	-0.107 (0.276)	**0.192 (0.043)**	**0.394 (0.000)**

*R/P: Correlation coefficient/Significance

Although there are conflicting results in the literature, high asprosin and apelin levels in obese children can be considered as important findings in our study. High levels of asprosin, an adipokine that increases food intake, may lead to the development of obesity. It could also be a therapeutic approach for the treatment of obesity. High apelin, a hormone that increases with food intake and insulin secretion, may be a result of obesity. However, more comprehensive studies are needed on these issues.

The study had two main limitations. One of these is that the number of children in the study and control groups was not equal, which limited the homogeneous evaluation of the data obtained. The other is the lack of standard measurements of adipokine hormone levels, which makes evaluation difficult.

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AUTHORSHIP CONTRIBUTIONS

Concept: M.D., S.Y.A.; Design: M.D., D.M.T., S.Y.A.; Control: M.D., S.Y.A.; Sources: S.Y.A.; Materials: D.M.T., A.D.B., E.F., F.M.K., S.Y.A.; Data Collection and/or Processing: D.M.T., A.D.B., E.F., F.M.K.; Analysis and/or Interpretation: M.D., A.D.B., E.F., F.M.K., S.Y.A.; Literature Review: M.D., S.Y.A.; Manuscript Writing: M.D., D.M.T., A.D.B., E.F., F.M.K., S.Y.A.; Critical Review: S.Y.A.; 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 protocol was approved by the Keçiören Clinical Research Ethics Committee (date: 11 May 2021, number: 15/2289).

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

PHARMACIST-DRIVEN MEDICATION REVIEW SERVICE IN PATIENTS WITH HEART FAILURE: A PROSPECTIVE STUDY

KALP YETERSİZLİĞİ HASTALARINDA ECZACI ODAKLI İLAÇ İNCELEME HİZMETİ: PROSPEKTİF BİR ÇALIŞMA

Sena USTAÖMER[1,2]*, Nurten SAYAR[3], Murat SÜNBÜL[3], Mesut SANCAR[4]

[1]Department of Clinical Pharmacy, Institute of Health Sciences, Marmara University, 34854, Istanbul, Türkiye
[2]Department of Clinical Pharmacy, Faculty of Pharmacy, Karadeniz Technical University, 61080, Trabzon, Türkiye
[3]Department of Cardiology, Faculty of Medicine, Marmara University, 34854, Istanbul, Türkiye
[4]Department of Clinical Pharmacy, Faculty of Pharmacy, Marmara University, 34854, Istanbul, Türkiye

ABSTRACT

Objective: *This study was conducted to detect and prevent drug-related problems (DRP) and potentially inappropriate medication (PIM) in patients with heart failure (HF) through a medication review service provided by clinical pharmacists and to increase drug prescription rates according to guideline-directed medical therapy (GDMT).*

Material and Method: *In this prospective study, which included observation and intervention periods, medication review services were provided to patients with HF between September 2023 and March 2024 by two clinical pharmacists. DRPs were classified according to Hepler-Strand and PIMs were evaluated according to Beers criteria®.*

Result and Discussion: *A total of 162 DRPs (1.8 per patient) were detected in 90 patients. The most common cause of DRPs was untreated indication (66.05%). In the observation period, no recommendations were offered, whereas in the intervention period, recommendations were offered to cardiologists, and 63.3% of them were implemented. DRPs were prevented and decreased by recommendations from two clinical pharmacists (from 1.76 to 0.64; $p < 0.001$). The prescription rates of sodium-glucose co-transporter 2 inhibitors and mineralocorticoid receptor antagonists increased ($p<0.05$). However, there was no difference in the number of PIMs per patient after the intervention ($p>0.05$). Our results provide compelling evidence that clinical pharmacists' assessment of medication use in patients with HF has made a crucial contribution to treatment management aligning treatment management with current guidelines and reducing DRPs.*

Keywords: *Clinical pharmacist, drug-related problems, heart failure, potentially inappropriate medications*

ÖZ

Amaç: *Bu çalışma, klinik eczacılar tarafından sağlanan ilaç inceleme hizmeti aracılığıyla kalp yetersizliği (KY) hastalarında ilaçla ilişkili sorunları (İLİS) ve olası uygunsuz ilaçları tespit etmek*

* **Corresponding Author / Sorumlu Yazar:** Sena Ustaömer
e-mail / e-posta: ustaomersena@gmail.com, **Phone / Tel.:** +904623778847

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*ve önlemek ve kılavuza dayalı tıbbi tedaviye göre ilaç reçete oranlarını artırmak amacıyla yürütülmüştür***.**

Gereç ve Yöntem: *Gözlem ve müdahale dönemlerini içeren bu prospektif çalışmada, Eylül 2023 ile Mart 2024 tarihleri arasında KY hastalarına iki klinik eczacı tarafından ilaç inceleme hizmeti sunulmuştur. İLİS'ler Hepler-Strand'a göre sınıflandırılmış ve olası uygunsuz ilaçlar Beers kriterlerine® göre değerlendirilmiştir.*

Sonuç ve Tartışma: *90 hastada toplam 162 İLİS (hasta başına 1.8) tespit edildi. İLİS'lerin en sık nedeni tedavi edilmemiş endikasyondu (%66.05). Gözlem döneminde herhangi bir öneri sunulmamışken, müdahale döneminde kardiyologlara öneriler sunulmuş ve bunların %63.3'ü uygulanmıştır. Ancak müdahaleden sonra tespit edilen hasta başına düşen olası uygunsuz ilaç sayısında bir fark yoktu. İki klinik eczacının önerileriyle İLİS'ler önlendi ve azaltıldı (1.76'dan 0.64'e; p < 0.001). Sonuçlarımız, klinik eczacılar tarafından KY hastalarında ilaç kullanımının değerlendirilmesinin, tedavi yönetimini güncel kılavuzlarla uyumlu hale getirerek ve İLİS'leri azaltarak tedavi yönetimine önemli bir katkı sağladığına dair ikna edici kanıtlar sunmaktadır.*

Anahtar Kelimeler: *İlaçla ilişkili sorunlar, kalp yetersizliği, klinik eczacı, olası uygunsuz ilaçlar*

INTRODUCTION

Heart failure (HF) is a progressive syndrome caused by functional and/or structural changes in the heart that impair its ability to fill or eject blood from the ventricle. HF is considered a global public health issue, affecting approximately 64 million people worldwide, and its prevalence is rapidly increasing owing to population aging [1-3]. It is known that the prevalence of HF is between 1-2% in developed countries. In Türkiye, this rate is 2.9% according to the HAPPY study [4].

It is known that the incidence of HF increases with age. Because more than 80% of patients with HF are individuals aged 65 and older. The prognosis is more severe in geriatric patients than in younger patients due to geriatric syndromes.

Angiotensin receptor neprilysin inhibitors (ARNI), angiotensin-converting enzyme inhibitors (ACEi), angiotensin receptor blockers (ARB), beta-blockers, sodium-glucose cotransporter-2 inhibitors (SGLT-2i), mineralocorticoid receptor antagonists (MRA) are pharmacotherapy options shown in current guidelines to improve hospitalization and mortality in patients with HF [5].

In addition to standard therapy, patients receive many different medications to treat their comorbidities. Due to the polypharmacy common in patients with HF, these patients are at higher risk of potential drug-drug interactions and adverse events. DRPs associated with inappropriate medication management play an important role in the increased prevalence of hospitalization in patients with HF. Data from different healthcare systems show that inappropriate HF treatment according to current guidelines is a global problem [6,7]. Due to pharmacokinetic and pharmacodynamic changes observed particularly in older adults, it is important to assess the appropriateness of medication use in geriatric patients with HF according to geriatric criteria such as Beers®, START/STOPP® and TIME-to-START/TIME-to-STOPP®.

International guidelines recommend a multidisciplinary approach for the management of patients with multiple comorbidities. Therefore, more recent recommendations include the inclusion of clinical pharmacists in the HF multidisciplinary team [8-10]. Clinical pharmacists have a key role in the review of medications in patients with HF. Clinical pharmacists review prescription drugs to verify whether a drug is necessary or appropriate for the treatment of a patient and contribute significantly to the improvement of patient care [11-13]. Studies in the literature where pharmacists evaluate the treatment of patients with HF according to current guidelines are still needed [8,14-16]. This study was aimed to detect and resolve DRPs observed in patients with HF through the medication review service provided by clinical pharmacists, to increase the prescription rates of medications that HF patients should use according to guideline-directed medical therapy (GDMT), and to determine PIMs observed in geriatric patients with HF according to Beers criteria®. The study describes the impact of the role of clinical pharmacists in the care system of patients with HF in a cardiology service in Istanbul.

MATERIAL AND METHOD

Study Design

This prospective study, in which two clinical pharmacists and two cardiologists participated, was conducted between 11 September 2023 and 11 March 2024 in the cardiology service of a 600-bed training and research hospital in Istanbul. The study was completed in two periods: a 3-month observation and a 3-month intervention period. To more accurately interpret the impact of the medicines review service provided by clinical pharmacists, we separately assessed the intervention period before and after the recommendation.

Participants of Study

The sample size was calculated using G*Power (Version 3..9.7) [Computer software] with an alpha of 0.05 and a power of 90%, based on the data in the literature that DRPs can be reduced from an average of 5 (SD 3) to 3 (SD 1) (approximately 40%) per patient in HF patient groups recommended by the clinical pharmacist and was determined as at least 28 patients in each group. Considering a drop-out rate of 15%, a total of 64 patients were included, with at least 32 patients in each group [15,16].

Patients with HF over the age of 18 who presented to the cardiology service and who gave informed consent were included in the study. Pregnant and breastfeeding women, patients younger than 18, and patients with HF in cardiology outpatient clinics were excluded from this study. The first 45 patients with HF, whose written and oral approval was obtained in both periods, were included in the study.

Medication Review Services Provided by Clinical Pharmacists

During the study period, there was a clinical pharmacist who was in the cardiology ward three days a week (Monday, Tuesday, and Wednesday) to provide medical review services to patients with HF and attended physician visits. The second clinical pharmacist contributed to the recommendations submitted for the solution of DRPs detected by the clinical pharmacist in the cardiology ward.

The socio-demographic characteristics of the patients, laboratory parameters, and the medications they used were recorded to account for confidentiality. During the medication review service, patients were assessed for polypharmacy. In our study, polypharmacy was defined as the daily use of five or more medications. The CKD-EPI formula was used to evaluate patients' renal function, and Uptodate® was used for the patient-specific evaluation of the drug dose prescribed during hospitalization. Lexicomp® was used as a primary tool for the detection of potential drug-drug interactions (pDDIs). X, D level potential drug-drug interactions with moderate, major; good, and excellent reliability ratings and clinically significant potential drug-drug interactions were accepted as DRP, and recommendations were presented to physicians for these pDDIs. According to UptoDate®, clinically important pDDIs were also checked on Medscape® before making recommendations to physicians. Only patients were monitored for level C interactions. A and B-level interactions were not considered as DRP.

The '2022 American Heart Association (AHA)/American College of Cardiology (ACC)/Heart Failure Association of America's (HFSA) Heart Failure Management Guidelines' and the '2023 Focused Update of the 2021 ESC Guidelines for the Diagnosis and Treatment of Acute and Chronic Heart Failure' were used to evaluate medications prescribed to patients with HF [17]. UpToDate® (Wolters Kluwer Health A.Sh., 2022) was used to improve informed clinical decision-making. The American Geriatrics Society 2023 Beers Criteria® was used to assess the appropriateness of treatment for patients with HF aged 65 years and older. The Beers Criteria® consist of 5 main sections: potentially inappropriate medications for use in geriatric adults, potentially inappropriate medications for use in certain diseases or syndromes, drugs that should be used with caution in geriatric adults, potentially clinically important drug-drug interactions that should be avoided in geriatric adults, medications that require dose adjustment according to renal function in geriatric adults [18].

During the medicine review service provided by the clinical pharmacists, the DRPs detected in patients with HF evaluated in both periods were categorized according to the Hepler and Strand classification system. During the observation period, no intervention was performed by the clinical pharmacist in the treatment process of the patients included in the study, unless there was a vital risk.

When DRPs that could threaten a patient's life were detected, the relevant physician was informed, and the patient was excluded from the study. To prevent and resolve DRPs detected during the intervention period, recommendations on adding and stopping medications, drug dose adjustments, switching to appropriate treatment alternatives, management of potential drug-drug interactions and possible side effects, and drug administration were presented orally and in writing by clinical pharmacists to the two professors of cardiology responsible for the ward (Figure 1).

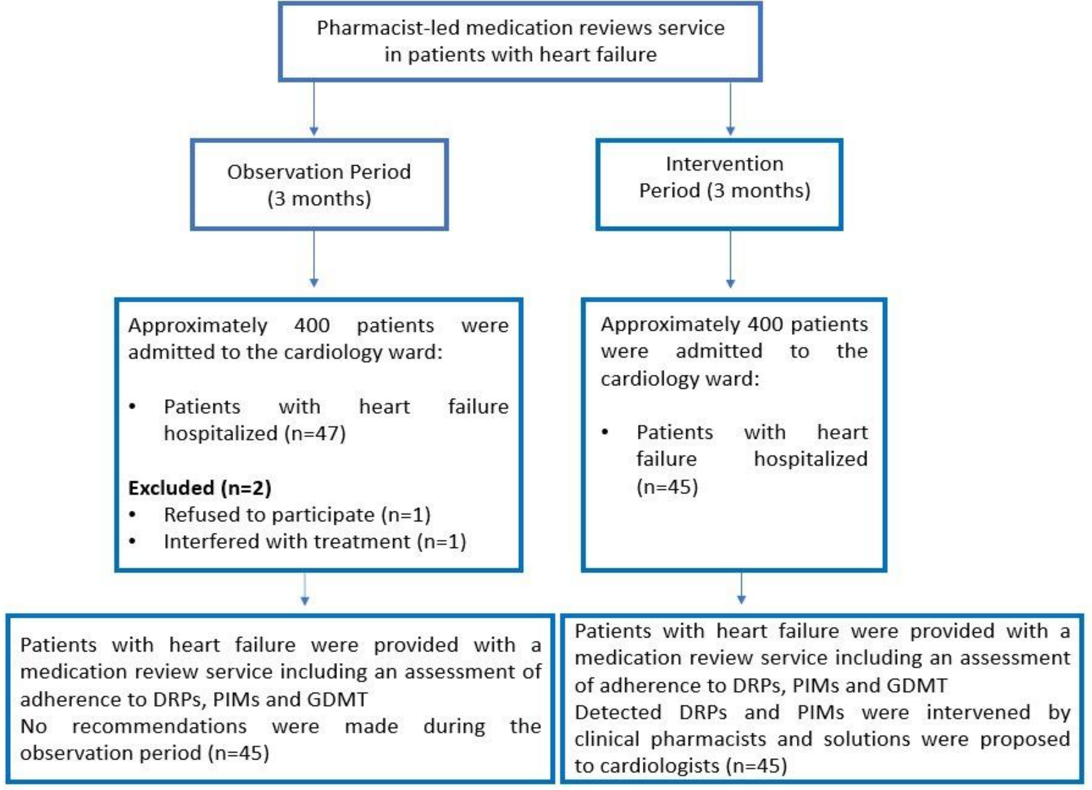

Figure 1. Flowchart for the observation and intervention period of the study

Outcome Measures

Our primary outcome in the medicine review service offered by clinical pharmacists is to determine the observed DRPs in patients with HF and to prevent and resolve these DRPs by consensus with cardiologists. The secondary outcome of our study is to determine the prescribing rates of the medication groups that should be prescribed to patients with HF according to GDMT, to increase these rates by the recommendations provided by the clinical pharmacist, and to detect and reduce PIM according to Beers criteria® in patients over the age of 65. The total and per capita number of DRPs and the number of PIMs according to Beers criteria®, the number of recommendations presented and accepted by physicians, and the prescription rates of medication groups are among the outcome measures of our study.

Data Analysis

All data from the study were analyzed using the Statistical Package for Social Sciences (SPSS) for Windows 23.0 software. Chi-square analysis and Fisher's exact test were used for the analysis of categorical variables. For comparisons of quantitative data between the observation and intervention groups, the Student's t-test was used for normally distributed data, and the Mann–Whitney U test was applied for non-normally distributed data. The change in the prescription rates of medications prescribed

for HF before and after the recommendation was determined using McNemar's analysis. Data were considered statistically significant in all analyses at a p-value < 0.05 in the 95% confidence interval.

RESULT AND DISCUSSION

A total of 90 patients were included, 45 in each period. The mean age of all patients was 68.26 ± 11.22 (41-89) years. Fifty-nine (65.6%; 59/90) patients were male. The characteristics of the patients in both periods are shown in Table 1.

Table 1. Patient characteristics

Characteristic variables	**Observation period (n=45)**	**Intervention period (n=45)**	***P* value**
Age (year)	71.1 ± 11.3 Max-min (41-89)	65.4 ± 10.5 Max-min (43-89)	**0.016****
Height (cm)	170 IQR (160-175)	170 IQR (160-175)	0.758*
Weight (kg)	77 IQR (68,5-85)	77 IQR (68-83)	0.878*
Body weight index (kg/m^2)	26.4 IQR (23.6-29.8)	26.4 IQR (24.9-28.3)	0.879**
Gender n, (%) Female Male	 16 (35.6%) 29 (64.4%)	 15 (33.3%) 30 (66.7%)	1.000***
Educational status n, (%) No education Primary school Middle school High school University	 3 (6.7%) 25 (55.6%) 7 (15.6%) 7 (15.6%) 0 (0%)	 3 (6.7%) 28(62.2%) 1 (2.2%) 5 (11.1%) 1 (2.2%)	0.213***
History of cigarette use n, (%) Yes No	 8 (19.0%) 34 (81.0%)	 12 (28.5%) 30 (71.5%)	0.443***
History of alcohol use n, (%) Yes No	 3 (7.1%) 39 (92.9%)	 2 (4.8%) 40 (95.2%)	1.000***
Heart failure class of patients according to LVEF n, (%) HFpEF HFmrEF HFrEF	 6 (13.6%) 8 (18.2%) 30 (68.2%)	 5 (11.6%) 11 (25.6%) 27 (62.8%)	0.701***
Comorbidities Hypertension Hyperlipidemia Coronary artery disease Myocardial infarction	 25 (55.6%) 4 (8.9%) 22 (48.9%) 5 (11.1%)	 22 (48.9%) 5 (11.1%) 20 (44.4%) 19 (42.2%)	 0.673*** 1.000*** 0.833*** **0.020*****
The number of comorbidities	3 IQR (2-4)	3 IQR (2-4)	0.532*

LVEF: Left ventricular ejection fraction, HFpEF: Heart failure with preserved ejection fraction, HFmrEF: Heart failure with mildly reduced ejection fraction, HFrEF: Heart failure with reduced ejection fraction
Mann–Whitney U test*, Student's t test**, chi-square test***
$p < 0.05$ indicates statistical significance.
$p < 0.001$ indicates high statistical significance. IQR: Interquartile range

Table 1 (*continue*). Patient characteristics

Characteristic variables	Observation period (n=45)	Intervention period (n=45)	*P* value
Use of medication			
Diuretics	24 (53.3%)	19 (42.2%)	0.399***
Beta-blocker	39 (86.6%)	41 (91.1%)	0.737***
Angiotensin-converting enzyme inhibitor (ACEi)	24 (53.3%)	25 (55.6%)	1.000***
Angiotensin receptor blocker (ARB)	3 (6.7%)	4 (8.9%)	1.000***
Angiotensin receptor-neprilysin inhibitor (ARNI)	9 (20.0%)	3 (6.7%)	0.121***
Mineralocorticoid receptor antagonists (MRA)	20 (44.4%)	16 (35.6%)	0.519***
Sodium-glucose cotransporter receptor 2 inhibitor (SGLT2i)	9 (20.0%)	10 (22.2%)	1.000***
The number of drugs	9 IQR (7-10)	8 IQR (7-10)	0.788*

LVEF: Left ventricular ejection fraction, HFpEF: Heart failure with preserved ejection fraction, HFmrEF: Heart failure with mildly reduced ejection fraction, HFrEF: Heart failure with reduced ejection fraction
Mann–Whitney U test*, Student's t test**, chi-square test***
$p < 0.05$ indicates statistical significance.
$p < 0.001$ indicates high statistical significance. IQR: Interquartile range

Drug-Related Problems (DRPs)

A total of 162 DRPs were detected in 90 patients, 79 in the intervention period and 83 in the observation period. Eighty-six patients (95.5%) had at least one DRP. During the intervention period, 79 recommendations were made by clinical pharmacists to cardiologists, of which 69 (87.3%) were accepted and 50 (63.3%) were implemented. The median number of DRPs detected per patient in the intervention period decreased from 2 IQR (1-2) to 1 IQR (0-1) ($p < 0.001$). The distributions of the relationships detected during the observation and intervention periods according to DRP class are shown in Table 2 and Table 3.

Prescription Rates of Medications

β blockers, ARNIs, ACEis/ARBs, MRAs, SGLT2is, and diuretics are among the groups of drugs prescribed for the treatment of HF. The frequencies of the medication groups found in the orders of 90 patients included in the study were as follows: β blockers (80/90; 88.9%), ARNI (12/90; 13.3%), ACEi (49/90; 54.4%), ARB (7/90; 7.8%), MRA (36/90; 40%), SGLT2i (19/90; 21.1%), and diuretics (43/90; 47.8%). The drug prescription rates for the intervention period are shown in Table 4.

Table 2. The number of DRPs detected during the observation and intervention periods by DRP class

DRPs detected according to Hepler-Strand's DRPs classification system	Observation period n (%)	Intervention period n (%)	*P* value
Drug use without an indication	2 (2.41)	2 (2.53)	1.000
Untreated indication	50 (60.24)	57 (72.15)	0.377
Subtherapeutic dosage	8 (9.64)	3 (3.80)	0.110
Overdosage	3 (3.61)	3 (3.80)	0.669
Improper drug selection	7 (8.43)	9 (11.40)	0.583
Failure to receive drugs	9 (10.84)	1 (1.26)	**0.014***
Adverse drug reactions	0 (0)	1 (1.26)	0.317
Drug interactions	4 (4.82)	3 (3.80)	0.696

Mann–Whitney U test; *$p < 0.05$ indicates statistical significance

Table 3. The number of DRPs detected before and after recommendation according to DRP class

DRPs detected according to Hepler-Strand's DRPs classification system	Intervention period		*P* value
	Before the recommendation, n (%)	After the recommendation, n (%)	
Drug use without an indication	2 (2.53)	1 (3.45)	0.317
Untreated indication	57 (72.15)	26 (89.65)	**< 0.001****
Subtherapeutic dosage	3 (3.80)	1 (3.45)	0.157
Overdosage	3 (3.80)	0 (0)	0.102
Improper drug selection	9 (11.40)	1 (3.45)	**0.005***
Failure to receive drugs	1 (1.26)	0 (0)	0.317
Adverse drug reactions	1 (1.26)	0 (0)	0.317
Drug interactions	3 (3.80)	0 (0)	0.083

*Wilcoxon t test, $p < 0.05$ indicates statistical significance
**Wilcoxon t test, $p < 0.001$ indicates high statistical significance

Potentially Inappropriate Medications (PIMs)

There were 60 geriatric patients in the observation (n=31) and intervention (n=29) periods. A total of 63 PIMs were identified according to the Beers Criteria®, including at least one PIM in 39 patients (65%) during the observation and intervention period. Medications causing PIMs detected in geriatric patients with HF during the intervention period: Diuretics (12; 44.2%), SGLT2is (6; 22.2%), antipsychotics (2; 7.4%), antidepressants (2; 7.4%), antiplatelet (1; 3.7%), anticoagulant (1; 3.7%), digoxin (1; 3.7%), cilostazol (1; 3.7%), and hyoscine n-butylbromide (1; 3.7%). After the recommendations were presented during the intervention period, there was no significant change in the number of inappropriate criteria according to Beers® (from 27 to 29 $p > 0.05$).

In this study, we evaluated the impact of a medication review service provided by clinical pharmacists on identifying, preventing, and resolving PIMs and DRPs observed in patients with HF.

The characteristics of the patients, except for age, were similar during both the observation and intervention periods. A difference was observed because the patients hospitalized in the cardiology ward during the intervention period were younger than those in the observation period. The patients with HF included in the study all had polypharmacy, as they were taking medications that both affected mortality and treated comorbidities. According to Goyal et al., polypharmacy is observed in at least 75% of

outpatients with HF, and at least 25% are taking 10 medications. It was reported that 96% of HF patients used at least 5 medications after discharge, and 57% used at least 10 medications [19-21].

Table 4. Prescription rates of medication groups prescribed for the treatment of heart failure before and after the recommendation

Groups of medication prescribed for the treatment of heart failure	Intervention period		*P* value
	Before the recommendation, n (%)	After the recommendation, n (%)	
Diuretics	19 (42.2)	19 (42.2)	1.000
β blocker	41 (91.1)	42 (93.3)	1.000
Angiotensin-converting enzyme inhibitor (ACEi)	25 (55.6)	30 (66.7)	0.125
Angiotensin receptor blocker (ARB)	4 (8.9)	3 (6.7)	1.000
Angiotensin receptor-neprilysin inhibitor (ARNI)	3 (6.7)	4 (8.9)	1.000
Mineralocorticoid receptor antagonist (MRA)	16 (35.6)	26 (57.8)	**0.006***
Sodium-glucose cotransporter receptor 2 inhibitor (SGLT2i)	10 (22.2)	22 (48.9)	**< 0.001****

McNemar analysis; * $p < 0.05$ indicates statistical significance
** $p < 0.001$ indicates high statistical significance

Hypertension and coronary artery disease were among the most common comorbidities in patients with HF. However, there was no significant difference in these comorbidities between the observation and intervention periods, whereas the number of patients with a history of myocardial infarction was significantly higher during the advice period. According to Jaber et al., hypertension (78.1%), coronary artery disease (69.8%), and diabetes (43.3%) were among the most common comorbidities associated with HF [22].

In studies conducted with patients with HF, it has been reported that the number of DRPs per patient varies between 1-2. The numbers of DRPs and the most frequently observed DRP classes vary among reported studies because DRPs are detected by different methods. The median value of DRP per patient in patients with HF who received medication review services by clinical pharmacists in the pre-discharge period was 2 IQR (1-2), similar to the literature [23-26]. The difference between the median DRP value per patient in the observation period and the before-recommendation period of the intervention period was not significant (1 IQR(1-3); 2 IQR(1-2) $p > 0.05$). The high number of DRPs supports the need for a clinical pharmacist in the healthcare team of patients with HF. According to the literature, DRPs are most commonly observed during the prescribing process and the most common DRP identified during this process is the untreated indication. During our study, the most common DRP in patients was an untreated indication (66.05%) and this supports the literature [13,24]. DRP categories other than the untreated indication did not differ for the observation period and before the recommendation intervention period. The medication group that caused this DRP most frequently was SGLT2i, which cardiologists hesitated to prescribe due to reimbursement conditions.

DRP was detected during the observation and intervention periods, and DRP numbers were compared in studies in the literature, but after the recommendations were presented to the physicians, DRP control could not be performed due to insufficient time spent in the clinical environment and the implementation rate of the recommendations could not be calculated. In our study, the acceptance rate of the recommendations as well as the implementation rate were determined. In our study, the

acceptance and implementation rate of the recommendations were also determined. In the studies, the acceptance rate of recommendations to cardiologists was at least 70% and more than 90% [25-27]. Similarly, 87.3% of the recommendations presented during the intervention period were accepted and 63.3% were implemented. The high acceptance rate of clinical pharmacists' recommendations regarding HF treatment indicates their critical role in optimizing HF treatment. Reimbursement conditions of the drugs (65.52%), hypotension (10.34%), hyponatremia (6.9%), other reasons (6.88%), and high bleeding risk (3.44%) were among the reasons for the recommendations that could not be implemented.

The secondary outcome of our study is the provision of appropriate treatment to patients with HF according to the GDMT through the drug review service provided by clinical pharmacists. Although ACEis/ARBs, β-blockers, and MRAs have been the first-line treatments for patients with HF, ARNIs, and SGLT2is have replaced other drugs in the current treatment of HF because of the evidence that these novel medications reduce hospitalization and mortality rates in patients. According to the '2023 Focused Update of the 2021 ESC Guidelines for the Diagnosis and Treatment of Acute and Chronic Heart Failure', SGLT2is, whose effect on hospitalization and mortality was demonstrated in the EMPEROR-Preserved study (ClinicalTrials.gov number, NCT03057951), should be prescribed to all patients with HF, regardless of class [28-30]. Patil et al. (2022), have shown a significant increase in ARNI (from 22.5% to 48.8%), MRA (from 22.5% to 38.8%), and SGLT2i (from 7.5% to 32.5%) prescribing rates as a result of optimization service according to GDMT offered to patients with HF ($p<0.001$). According to Campbell et al. (2024) in the study, ACEi, ARB or ARNI (from 22% to 92%); beta-blockers (from 72% to 92%); MRA (from 54% to 88%); and SGLT2i (from 67% to 94%) the rate of prescribing inhibitors has increased ($p<0.001$) [5,13,31]. Within the scope of the medication review service, the prescription rates of MRA (from 35.6% to 57.8%, $p < 0.05$) and SGLT2i (from 22.2% to 48.9%, $p < 0.001$) in orders issued according to GDMT increased significantly compared to the recommendations of clinical pharmacists. Although SGLT2i like ARNI, is not reimbursed, the prescription rate of SGLT2i can be increased with more affordable equivalent drugs in the market, while no significant change was observed in the prescription rate of ARNI. Despite higher rates of prescription at discharge, β-blockers, ACEs/ARBs, and MRAs are under-prescribed in many patients and are the most common drug groups causing DRP. However, SGLT2i was not evaluated in these studies [15,32].

In the study by Jaber et al., the prevalence of PIM in geriatric patients with HF was found to be 61.1%, and the main drugs causing PIM were proton pump inhibitors (PPIs) and amiodarone [22]. In this study, the prevalence of PIM prescriptions according to the Beers Criteria® in geriatric patients with HF was 65%. Of the PIMs, 23 (85.19%) were PIMs that should be used with caution in geriatric adults, 3 (11.11%) were PIMs used in geriatric adults, and 1 (3.7%) was PIM used in geriatric adults due to drug-disease interactions that may exacerbate the disease or syndrome. Furosemide (12; 44.4%), dapagliflozin (4; 14.8%) and empagliflozin (2; 7.4%), quetiapine (2; 7.4%), sertraline (1; 3.7%), citalopram (1; 3.7%) and ticagrelor (1; 3.7%) were used by patients during the pre-recommendation intervention period and should be used with caution in geriatric adults according to 2023 Beers Criteria®. For medications in this group, other than ticagrelor, there is only a follow-up recommendation. Furosemide was monitored with caution because of the risk of hyponatremia, SGLT2i because of the risk of euglycaemic diabetic ketoacidosis in older adults, and antipsychotics and antidepressants because of the risk of inappropriate ADH release, and these PIMs were not considered DRPs. The Beers Criteria® recommends clopidogrel instead of ticagrelor for the risk of bleeding in adults 75 years and older. This PIM is also viewed as a DRP and the relevant recommendation is presented to physicians. PIMs used in geriatric adults and medications that may exacerbate the disease due to drug-disease interaction were considered improper drug selection from the DRP categories.

The patients' laboratory parameters were monitored during furosemide and SGLT2i use, but no events requiring intervention occurred. Although the number of PIMs detected per patient was similar in the observation and intervention periods, no significant change was observed after the recommendations were presented in the intervention period, and the total number of PIMs increased owing to the increase in SGLT2i prescription rates (total number of PIMs from 27 to 29, $p > 0.05$).

Strengths and Limitations

The study has strengths, including the identification of DRPs through the consensus of two clinical pharmacists and two cardiologists, the identification of DRPs, the presentation of proposed solutions and subsequent implementation rates, and the fact that the prescription rates of SGLT2is, which should be prescribed to all patients with HF regardless of HF class, have not been evaluated in previous studies. However, there are some limitations to our study. First of all, our study was conducted in a single center with a small sample size. DRPs were detected with the Hepler-Strand DRP classification system. However, the Hepler-Strand classification system does not systematically evaluate the causes and relationship status. The effects of interventions during hospitalization in the cardiology ward on patients' quality of life and the treatment process were not assessed. According to the Beers criteria®, medications requiring caution in older adults were considered as PIMs. Therefore, the number of PIMs was high.

Conclusion

Patients with HF frequently encounter DRPs during hospitalization. The main reason for these DRPs is the low rate of SGLT2i prescription. The fact that SGLT2is are not within the scope of reimbursement for HF indications in our country causes this rate to be low. However, with the consensus of clinical pharmacists and cardiologists, DRPs have decreased and the prescription rates of MRAs and SGLT2is have increased. Clinical pharmacists play a key role in the healthcare process of patients with HF.

Randomized controlled trials with larger samples in which clinical pharmacists are included in the HF multidisciplinary team will further clarify the role of clinical pharmacists and provide updated consensus reports. Future studies should determine the impact of the medication review service provided by clinical pharmacists to patients with HF on economic and clinical outcomes such as mortality and rehospitalization rates.

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AUTHOR CONTRIBUTIONS

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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 with protocol code 09.2023.866 was approved by the Clinical Research Ethics Committee of Marmara University Faculty of Medicine. This study was conducted in accordance with the Declaration of Helsinki and the Clinical Research Ethics Committee. Written informed consent was obtained from all participants included in this study.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

THE EFFECT OF EMOTIONAL LABOR ON PHARMACISTS' JOB SATISFACTION AND THE MEDIATING ROLE OF EMOTIONAL EXHAUSTION

DUYGUSAL EMEĞİN ECZACILARIN İŞ TATMİNİ ÜZERİNDEKİ ETKİSİ VE DUYGUSAL TÜKENMİŞLİĞİN ARACILIK ROLÜ

Nur ÇAMLICA ŞENDEMİR[1]*, Ozan BÜYÜKYILMAZ[2]

[1]Karabük University, Social Sciences Vocational School, Department of Office Services and Secretariat, 78050, Karabük, Türkiye
[2]Karabük University, Faculty of Business, Department of Business Administration, 78050, Karabük, Türkiye

ABSTRACT

Objective: *This study investigates the complex interplay among emotional labor strategies, emotional exhaustion, and job satisfaction, with a specific focus on examining the mediating role of emotional exhaustion. The primary objective of the research is to empirically evaluate the mediating influence of emotional exhaustion on the relationships between pharmacists' emotional labor behaviors (surface acting and deep acting) and their job satisfaction.*
Material and Method: *The data for the study was collected through a survey of 186 pharmacists employed in various organizations in Karabük, Türkiye. The research hypotheses were tested using Partial Least Squares Structural Equation Modeling (PLS-SEM), a robust analytical technique suited for examining complex multivariate relationships.*
Result and Discussion: *The findings of the study reveal that deep acting, whereby pharmacists genuinely express their true emotions, has a direct negative effect on emotional exhaustion and a direct positive effect on job satisfaction. In contrast, the results indicate that surface acting, in which pharmacists suppress their authentic emotions and display artificial emotional responses, has a significant positive effect on emotional exhaustion, while its direct impact on job satisfaction is insignificant. Importantly, the study demonstrates that emotional exhaustion plays a partial mediating role in the relationships between both surface acting and job satisfaction, as well as between deep acting and job satisfaction. This suggests that the depletion of pharmacists' emotional resources is a crucial mechanism through which their emotional labor strategies influence their job satisfaction levels.*
Keywords: *Deep Acting, emotional exhaustion, emotional labor, job satisfaction, surface acting*

ÖZ

Amaç: *Bu çalışma, duygusal emek stratejileri, duygusal tükenme ve iş tatmini arasındaki karmaşık ilişkileri, özellikle duygusal tükenmişliğin aracılık rolü kapsamında araştırmaktadır. Araştırmanın amacı, duygusal tükenmişliğin eczacıların duygusal emek davranışları (yüzeysel rol yapma ve derin rol yapma) ile iş tatminleri arasındaki ilişkiler üzerindeki aracılık etkisini ampirik olarak değerlendirmektir.*
Gereç ve Yöntem: *Çalışma kapsamında veriler, Karabük ilindeki farklı eczanelerde çalışan 186 eczacıya uygulanan bir anket aracılığıyla toplanmıştır. Araştırma hipotezleri, karmaşık çok*

* **Corresponding Author / Sorumlu Yazar:** Nur Çamlıca Şendemir
e-mail / e-posta: nurcamlica@karabuk.edu.tr, **Phone / Tel.:** +903704188883

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değişkenli ilişkileri incelemek için uygun bir teknik olan Kısmi En Küçük Kareler Yapısal Eşitlik Modellemesi (PLS-SEM) kullanılarak test edilmiştir.
Sonuç ve Tartışma: *Çalışmanın bulguları, eczacıların gerçek duygularını samimi bir şekilde ifade ettikleri derin rol yapmanın duygusal tükenmişlik üzerinde doğrudan negatif, iş tatmini üzerinde ise doğrudan pozitif bir etkiye sahip olduğunu ortaya koymuştur. Bunun aksine, sonuçlar, eczacıların gerçek duygularını bastırdığı ve yapay duygusal tepkiler sergilediği yüzeysel rol yapmanın duygusal tükenmişlik üzerinde önemli bir pozitif etkiye sahip olduğunu, ancak iş tatmini üzerindeki doğrudan etkisinin anlamsız olduğunu göstermektedir. Daha da önemlisi, bu çalışma duygusal tükenmişliğin hem yüzeysel rol yapma ile iş tatmini arasındaki hem de derin rol yapma ile iş tatmini arasındaki ilişkilerde kısmi bir aracılık rolü oynadığını belirlemektedir. Bu durum, eczacıların duygusal emek stratejilerinin duygusal kaynaklarının tükenmesi üzerinden iş tatmini düzeylerini etkileyen önemli bir mekanizma olduğunu göstermektedir.*
Anahtar Kelimeler: *Derin rol yapma, duygusal emek, duygusal tükenmişlik, iş tatmini, yüzeysel rol yapma*

INTRODUCTION

Employees' emotions and job responsibilities are interwoven in today's changing work environment. Employees are increasingly required to manage their emotions professionally in order to fulfill their job duties more efficiently, in addition to completing tasks utilizing their technical abilities. Consequently, employees are no longer viewed as just cogs in a machine, but rather as critical components of an organization's total success. Because of its crucial role in establishing pleasant relationships and improving customer experiences, the notion of emotional labor has arisen as an important subject of study that has piqued the interest of scholars, practitioners, and policymakers [1,2].

The concept of emotional labor was first introduced by sociologist Arlie Hochschild in 1983. It refers to the “process of managing and regulating one’s emotions in accordance with the expectations of the job role” [1]. Emotional labor involves managing and displaying emotions to fulfill the emotional requirements of a job [2]. This concept suggests that employees should regulate their emotions to meet organizational expectations and satisfy the emotional needs of service recipients, customers, or colleagues [3].

Pharmacists are frequently seen as hidden heroes in the healthcare industry, providing not just clinical skill but also empathy, compassion, and assistance to patients in need [4]. A study by Rahme et al. [5] highlighted that pharmacists often engage in significant emotional labor during patient interactions, which is essential for effective communication and patient care. Therefore, emotional labor has an undeniable value for pharmacists, whose job requires them to balance human emotions with medical science. Emotional labor for pharmacists requires not only dispensing medicines accurately, but also interacting with patients and their families in moments of vulnerability, distress, and sometimes deep joy. It is important for pharmacists to use their emotional intelligence to connect with patients, offering comfort and guidance on their healthcare journey [6-9].

Emotional labor is a crucial aspect of the healthcare industry, as the quality of interaction between pharmacists and patients can significantly impact medication adherence, treatment outcomes, and overall satisfaction with the healthcare experience [10]. Therefore, the emotional labor behavior exhibited by pharmacists is not only a tool but also the foundation of patient-centered care.

However, beneath the surface of these emotionally charged interactions, pharmacists face a complex array of emotional experiences that they must navigate. Balancing their own emotions while addressing the diverse emotional needs of patients can take a toll on their well-being. This strain often manifests as burnout, characterized by emotional exhaustion, fatigue, and a depletion of emotional resources [11,12]. It is increasingly recognized by researchers that emotional exhaustion is a prevalent issue in contemporary workplaces and has significant implications for both individual well-being and organizational effectiveness. A study by Katsogiannis et al. [13] emphasized that high levels of emotional exhaustion among pharmacists correlate with decreased job satisfaction and emotional well-being.

The application of emotional intelligence by pharmacists in establishing rapport with patients and providing them with comfort and guidance throughout their medical journey is of utmost importance

[14-19]. In this context, various studies have demonstrated that emotional exhaustion significantly affects several employee attitudes and behaviors, including job performance [20], employee engagement [21], and turnover intention [22]. However, emotional exhaustion affects one of the crucial organizational factors, namely job satisfaction, which is regarded as the foundation of employee well-being and organizational achievement [23-25].

Job satisfaction is a critical employee attitude that reflects the contentment with the role and may impact motivation and overall performance [26,27]. In the case of pharmacists who are responsible for their patients' health and well-being, job satisfaction is a crucial indicator of their ability to provide consistent, high-quality care. Satisfied pharmacists are more likely to exhibit organizational commitment and lower turnover intentions, which are crucial for maintaining a stable workforce in healthcare settings [28-30]. Accordingly, a crucial question arises: How does emotional exhaustion, resulting from emotional labor, impact pharmacists' job satisfaction?"

Based on the mentioned rationale, this study examines the complex relationship between emotional labor, emotional exhaustion, and job satisfaction with a particular focus on the mediating role of emotional exhaustion. The study aims to determine the mediating role of emotional exhaustion in the impact of pharmacists' emotional labor behaviors on job satisfaction. This study explores the less-explored facets of a pharmacist's professional life, shedding light on the emotional labor inherent in their duties, which can yield both positive and negative outcomes. By scrutinizing the interrelationships among emotional labor, emotional exhaustion, and job satisfaction within the realm of pharmacy practice, this study seeks to enhance our understanding of the challenges that pharmacists encounter and the strategies that can be employed to enhance their job satisfaction. Additionally, it provides valuable insights for healthcare institutions and organizations to offer the necessary support and resources to pharmacists, fostering an environment where they can excel in their critical role while safeguarding their emotional well-being.

MATERIAL AND METHOD

Hypotheses Development

Employee job satisfaction is a critical facet of organizational well-being and productivity. Job satisfaction pertains to an individual's general attitude towards their job, encompassing affective and cognitive components [31]. Various factors contribute to job satisfaction, including personality [32], perceived organizational support [33], leader-member exchange [34] and work-life balance [35]. One of the factors affecting job satisfaction is thought to be emotional labor strategies employed by individuals in the workplace [23-25]. On the other hand, it was determined that the effect of emotional labor on job satisfaction was bidirectional. Studies show that surface acting and depth acting, which constitute the two dimensions of emotional labor, affect job satisfaction in different directions.

Surface acting involves the outward display of emotions that are not genuinely felt. The Conservation of Resources Theory posits that persistent threats to valued resources, like job security, can negatively impact work quality. This can lead to emotional dissonance, where individuals engage in surface acting (faking emotions) to conceal their true feelings. This dissonance depletes personal resources and requires increased emotional effort, hindering the replenishment of intrinsic emotional resources. Ultimately, this can result in decreased job satisfaction and performance [36].

Studies highlight the potential downsides of surface acting. Hochschild [37] argued that suppressing genuine emotions and displaying inauthentic expressions can lead to emotional dissonance and exhaustion, ultimately decreasing job satisfaction. In particular, jobs with high emotional demands and low control over emotional expressions have been found to contribute to decreased satisfaction and increased burnout [23]. In a study by Hülsheger et al. [25], employees who engaged in frequent surface acting reported lower levels of job satisfaction compared to those who expressed genuine emotions at work. Furthermore, Grandey et al. [38] found that surface acting can lead to emotional dissonance, where employees experience a misalignment between their displayed emotions and inner feelings. This incongruence has been linked to lower job satisfaction and increased burnout. Hence, existing literature provides empirical support for the notion that surface acting is associated with decreased levels of job satisfaction among employees. This suggests that the emotional toll of suppressing one's own feelings

while adhering to expected displays can outweigh the positive aspects of emotional labor. Therefore, it was hypothesized that pharmacists' surface acting behaviors would be negatively related to job satisfaction.

H1: Surface acting has a negative effect on job satisfaction

In contrast to surface acting, depth acting involves the authentic experience and expression of emotions. Depth acting comprises aligning one's internal feelings with the organization's desired emotional display by altering personal interpretations of emotional situations. This alignment can create a state of psychological balance and harmony, potentially even generating genuine positive emotions. Consequently, depth acting can enhance individual emotional resources and lead to increased job satisfaction [39].

Studies have indicated a positive association between emotional labor and job satisfaction. For example, a study by Xu and Fan [40] on nurses found a positive correlation between emotional labor and job satisfaction, mediated by the quality of nurse-patient relationships. Similarly, Lee [41] demonstrated a positive link between emotional intelligence, which facilitates effective emotional labor, and job satisfaction among public service employees. Hochschild [37] proposed that depth acting, when employees genuinely feel and express emotions congruent with organizational expectations, may lead to positive outcomes such as enhanced job satisfaction. This suggests that possessing the skills to manage emotions and project positivity can enhance job satisfaction, particularly in service-oriented roles. Research by Huang et al. [42] supports the positive relationship between depth acting and job satisfaction. In their study, employees who engaged in depth acting reported higher levels of job satisfaction over time compared to those who relied on surface acting. This suggests that the genuine expression of emotions in the workplace is conducive to a more satisfying work experience. Additionally, Humphrey [43] found that depth acting is associated with a sense of authenticity and emotional congruence, leading to higher levels of job satisfaction. The findings indicate that when individuals are able to express their true emotions at work, they experience greater job satisfaction and well-being. Therefore, it was hypothesized that pharmacists' surface acting behaviors would be positively related to job satisfaction.

H2: Depth acting has a positive effect on job satisfaction

Many researchers have identified that emotional labor of employees within the context of organizational norms can affect individual well-being [43,44]. More specifically, emotional labor was closely related to emotional exhaustion, which is defined as a lack of energy and a feeling that one's emotional resources are depleted [14,16,19]. Emerging research has revealed that emotional labor, the act of regulating emotions to meet job demands, can lead to emotional exhaustion [43, 45-47]. Notably, the manner in which emotions are displayed plays a crucial role. Studies suggest that surface acting, where employees feign desired emotions while suppressing their true feelings, carries a significantly higher risk of exhaustion compared to deep acting, where genuine emotional alignment with the job occurs [37,39,48].

The reasoning behind this disparity lies in the inherent demands of each strategy. Surface acting, like putting on a performance, requires sustained effort and cognitive resources to maintain a facade, leading to emotional dissonance – the conflict between displayed and genuine emotions. This persistent discrepancy creates a psychological strain, depleting emotional reserves and ultimately contributing to exhaustion [11].

The existing body of research further substantiates the detrimental consequences of surface acting. In their comprehensive meta-analysis, Hülsheger and Schewe [44] found that when employees engage in surface acting, they are more likely to experience emotional dissonance – a psychological state characterized by the incongruence between their felt emotions and the emotions they are required to express. This emotional dissonance creates a significant strain, which ultimately leads to emotional exhaustion. Moreover, surface acting demands substantial psychological effort from the individual, as they must comply with organizational display rules and suppress their authentic emotions. This effortful emotional labor, coupled with the emotional dissonance experienced, depletes the individual's emotional resources, contributing to the development of depersonalized relationships and, consequently, emotional

exhaustion [11]. Building on this, Martínez-Iñigo et al. [49] suggested that the excessive expenditure of mental and emotional energy associated with surface acting is a key contributor to job burnout among employees. Similarly, a study by Kim et al. [50] demonstrated that surface acting amplifies emotional dissonance, directly leading to emotional exhaustion. Informed by these empirical findings, the current study hypothesizes that pharmacists' engagement in surface acting behaviors will be positively associated with their level of emotional exhaustion.

H3: Surface acting has a positive effect on emotional exhaustion

Depth acting, in contrast, fosters a greater sense of congruence between internal and displayed emotions. By genuinely embracing the desired emotional state, employees experience less inner conflict and expend less energy on emotional regulation [11]. This alignment facilitates emotional well-being and helps mitigate the risk of exhaustion [25]. Grandey [48] argued that while depth acting, where individuals genuinely adopt the desired emotional state, requires effort to manage negative emotions, it generates minimal emotional dissonance. This dissonance arises from the conflict between felt and displayed emotions. Depth acting aligns internal feelings with outward expressions, minimizing the effort needed and potential for exhaustion. This aligns with empirical findings demonstrating a negative association between depth acting and emotional exhaustion [43,49,51-54]. Therefore, it was hypothesized that pharmacists' depth acting behaviors would be negatively related to emotional exhaustion.

H4: Depth acting has a negative effect on emotional exhaustion

Emotional exhaustion has been posited to mediate the relationship between various job demands (including emotional labor) and job outcomes like job satisfaction [55]. The negative impact of emotional exhaustion on job satisfaction is well-documented, suggesting that diminished emotional resources can lead to a less favorable evaluation of one's job [25,56]. Furthermore, the strain experienced from prolonged emotional labor, particularly surface acting, can deteriorate job satisfaction by depleting emotional resources, aligning with the Conservation of Resources Theory [36].

Janssen et al. [57] investigated the specific relationships between job demands, job resources, and psychological outcomes, highlighting the mediating role of negative work–home interference in the context of emotional exhaustion and job satisfaction. Karatepe [58] examined the effects of work overload and work-family conflict on job embeddedness and job performance, finding that emotional exhaustion fully mediates these relationships, underscoring the detrimental effects of emotional labor on job satisfaction. Lin and Chang [59] explored the relationship between emotional labor and job performance among physicians, identifying emotional exhaustion as a mediator that significantly impacts job satisfaction and performance. Bakker et al. [60] delved into the daily ripple effects of emotional labor, demonstrating that work-related exhaustion partially mediates the relationship between surface acting at work and at home, further affecting relationship satisfaction.

The aforementioned studies collectively support the hypothesis that emotional exhaustion mediates the relationship between surface acting and job satisfaction. This mediation underscores the importance of managing emotional labor in the workplace to mitigate the adverse effects on employee well-being and job satisfaction. Therefore, it was hypothesized that pharmacists' emotional exhaustion would be mediates the relationship between surface acting and job satisfaction.

H5: Emotional exhaustion mediates the relationship between surface acting and job satisfaction

Deep acting, a form of emotional labor wherein individuals strive to align their internal emotional states with outward expressions, stands in contrast to surface acting, which entails adjusting external emotional displays without corresponding changes in internal feelings [37]. Scholars posit that deep acting is less detrimental to psychological well-being compared to surface acting because it involves authentic emotion regulation [48]. Additionally, deep acting necessitates proactive efforts from individuals to manage their emotions, requiring the active transformation of emotional perceptions, cognitions, and perspectives [11,25]. Over time, employees come to perceive the display of positive emotions in their professional roles as natural, effectively mitigating negative emotions by internalizing positive outlooks and cognitions [43,54]. This process has the potential to reduce emotional exhaustion,

consequently contributing indirectly to increased job satisfaction.

Research has shown that emotional exhaustion can mediate the relationship between emotional labor and relevant outcome variables. For instance, Zhao et al. [61] demonstrated that depth acting exerted its effect on task performance and customer loyalty through the mediating effects of emotional exhaustion. In the hotel and catering industry in China, Peng and Li [62] found that customer service employees' emotional exhaustion played a fully mediating role in the relationship between depth acting and employees' withdrawal behavior. Huang et al. [42] found that deep acting was less emotionally exhausting for service providers when they viewed their tasks as challenging, suggesting that the context of emotional labor significantly influences its outcomes on emotional exhaustion and job satisfaction. Therefore, it was hypothesized that pharmacists' emotional exhaustion would be mediates the relationship between depth acting and job satisfaction (Figure 1).

H6: Emotional exhaustion mediates the relationship between depth acting and job satisfaction

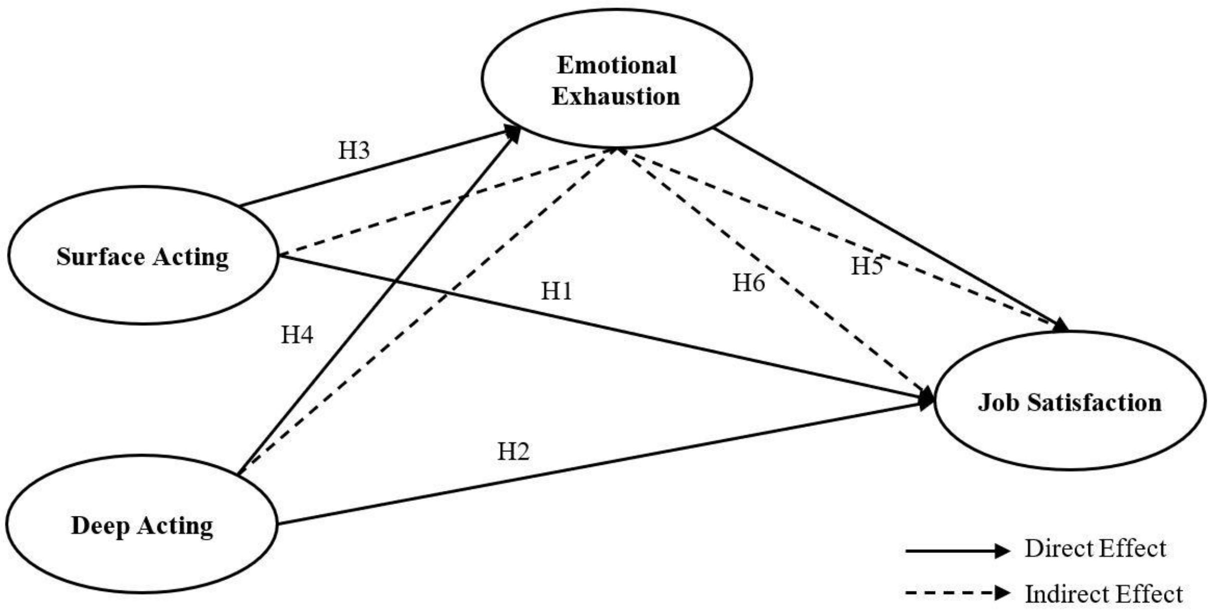

Figure 1. Research model

Respondents

This research study focuses on investigating the prevalence of emotional labor in the service industry, specifically in the pharmacy sector in Karabuk, Turkey. The participants of the study were employees who directly interacted with customers in the pharmacy industry. All pharmacists with customer contact were invited to take part in the survey.

According to information gathered from the Kastamonu Chamber of Pharmacists Karabuk Representative Office, there are 78 pharmacies in Karabuk, employing a total of 273 individuals. The primary population for this study comprises these 273 employees. Due to various factors, including response rates, willingness to participate, time constraints, and budget limitations, a convenience sampling method was employed. The minimum sample size required to accurately represent the primary population of 273 individuals, considering a 5% margin of error, was established as 162 participants [62]. Consequently, 228 employees were interviewed face-to-face and invited to complete an online questionnaire in Turkish via the Google Forms platform. To ensure the reliability and confidentiality of the collected data, the purpose of the survey was clearly explained to the participants, emphasizing that the questionnaire information would be used solely for research purposes and would be treated as strictly confidential. Data collection was conducted in November 2023.

A total of 186 valid questionnaires were collected for analysis and the demographic distribution

of the participants is presented in Table 1.

Table 1. Demographic distribution

Variable	Category	N	%	Variable	Category	N	%
Gender	Male	106	57.0%	Job Tenure	5 and under	53	28.5%
	Female	80	43.0%		6-10 years	59	31.7%
Marital Status	Married	112	60.2%		11 and over	74	39.8%
	Single	74	39.8%	Education	High school	48	25.8%
Age (Average=37)	30 and under	46	24.7%		Associate degree	38	20.4%
	31-40 years old	87	46.8%		Bachelor's degrees	80	43.0%
	41 and over	53	28.5%		Postgraduate degree	20	10.8%

Among the participants, 106 were male (57.0%) and 80 were female (43.0%). In terms of marital status, 112 were married (60.2%) and 74 were single (39.8%). Regarding age distribution, 46 respondents (24.7%) were under 30 years old, 87 (46.8%) were between 31 and 40 years old, and 53 (28.5%) were above 40 years old. The age range of the participants varied from 22 to 72 years, with an average age of 37. Concerning job tenure, 53 participants (28.5%) had less than 5 years of experience, 59 (31.7%) had 6 to 10 years of experience, and 74 (39.8%) had over 10 years of experience. In terms of educational background, 48 participants (25.8%) had a high school degree, 38 (20.4%) had an associate degree, 80 (43.0%) held bachelor's degrees, and 20 (10.8%) held postgraduate degree.

Measures

In the study, all the variables were assessed using established scales, ensuring the reliability and validity of the measurements. The questionnaire design employed a five-point Likert scale, ranging from "strongly disagree" to "strongly agree," with assigned values of 1 to 5, respectively.

Emotional Labor

The measurement of emotional labor utilized the scale developed by Diefendorff et al. [3]. Emotional labor was assessed through two dimensions: surface acting and deep acting. Surface acting was evaluated using a seven-item scale, which included statements such as “I put on a ‘show’ or ‘performance’ when interacting with customers.” and “I show feelings to customers that are different from what I feel inside.” The reliability of the surface acting scale, as indicated by Cronbach's alpha, was found to be 0.920. Deep acting, on the other hand, was measured using a four-item scale. Sample items included “I make an effort to actually feel the emotions that I need to display toward others.” and “I work at developing the feelings inside of me that I need to show to customers.” The deep acting scale demonstrated high internal consistency, with a Cronbach's alpha coefficient of 0.934. The scale has been adapted into Turkish by Basim and Begenirbas [63]. The internal consistency of the scale was determined to be α=0.844 for the surface acting dimension and α=0.863 for the deep acting dimension.

Emotional Exhaustion

The assessment of employees' emotional exhaustion in this study employed a nine-item scale developed by Maslach and Jackson [19]. The scale is unidimensional, meaning it measures emotional exhaustion as a single construct. The adaptation of the emotional burnout scale into Turkish was carried out by Ergin [64]. The reliability coefficient for the scale was established at α=0.930. The specific items used in the scale included statements such as “I feel fatigued when I get up in the morning and have to face another day on the job.” and “I feel emotionally drained from my work.” The internal consistency of the scale, determined by Cronbach's alpha coefficient, was found to be 0.941 in the present study.

Job Satisfaction

Job satisfaction in this study was assessed using a five-item scale developed by Hackman and Oldham [27]. The Turkish adaptation of the job satisfaction scale was conducted by Şeşen [65]. The reliability value for the scale was determined as α=0.840. The scale represents a unidimensional measure, focusing on overall job satisfaction. Sample items from the scale include statements such as

"In general, I am satisfied with my work." and "I think I am happier in my work than many other people." The internal consistency of the scale, as measured by Cronbach's alpha coefficient, was found to be 0.934 in the current study.

Data Analysis

Data analysis for this study was conducted using SPSS Statistics and SmartPLS software programs. Demographic characteristics of the participants were summarized using SPSS Statistics, and descriptive statistics were calculated. For reliability and validity analyses of the variables, as well as hypothesis testing, Partial Least Squares Structural Equation Modeling (PLS-SEM) was employed using the SmartPLS 3 program.

PLS-SEM is a statistical modeling technique used to analyze the relationships between latent variables in a model, similar to Covariance-Based Structural Equation Modeling (CB-SEM) [66]. However, PLS-SEM differs from CB-SEM in that it performs variance-oriented calculations instead of covariance-oriented calculations. It is considered a component-based estimation method [67].

There are two stages in the PLS-SEM. The first stage includes the evaluation of the measurement model, and the second stage includes the evaluation of the structural model [67-70]. The first stage of the measurement model includes analyses of construct validity (factor analysis), convergent and discriminant validity and reliability. The structural model, which is the second stage, includes path analysis. Path analysis includes analyses related to the hypotheses to be tested in the research.

PLS-SEM offers several advantages to researchers. Firstly, it is particularly suitable for studies that adopt a predictive modeling approach. Therefore, it is an appropriate method when the research aims to explain relationships between variables. Secondly, PLS-SEM provides robust predictions even when the data used in the study does not meet certain assumptions for analysis, such as a small sample size or non-normal distribution of the data [68,69,71]. In this study, the PLS-SEM method was chosen due to the suitability of the data structure for structural equation modeling, and the limited sample size that couldn't be increased due to population constraints.

RESULTS AND DISCUSSION

Factor Analysis (Construct Validity)

The concept of construct validity concerns the extent to which the results obtained from the use of a measurement instrument are consistent with the underlying theoretical construct that the instrument is intended to capture [72]. A key method of assessing construct validity is to examine the factor loadings of individual measurement items [68].

The premise is that all items designed to measure a particular theoretical construct should show strong factor loadings on the target construct when subjected to factor analysis. Conversely, if an item is found to load more strongly on a construct other than the one it was designed to reflect, this suggests that the item may not be a reliable indicator of the intended construct. In such cases, the recommended course of action is to remove or eliminate the problematic item from the measurement instrument [68]. This helps to ensure that the remaining items more accurately capture the essence of the target construct, thereby strengthening the overall construct validity of the measurement approach.

Establishing robust construct validity is crucial as it provides evidence that the measurement is actually assessing the theoretical concept it was designed to measure, rather than something else entirely [72].

Table 2. Factor loadings and cross loadings

	Surface Acting	Deep Acting	Emotional Exhaustion	Job Satisfaction
surf_act1	**0.701****	0.039	0.324	-0.121
surf_act2	**0.919****	0.082	0.433	-0.083
surf_act3	**0.780****	0.145	0.368	0.007
surf_act4	**0.813****	0.090	0.378	-0.087
surf_act5	**0.868****	0.074	0.403	-0.113
surf_act6	**0.765****	0.017	0.376	-0.042
deep_act1	0.063	**0.912****	-0.236	0.565
deep_act2	0.038	**0.892****	-0.239	0.553
deep_act3	0.147	**0.779****	-0.203	0.469
deep_act4	0.090	**0.939****	-0.279	0.562
exhaust1	0.350	-0.249	**0.833****	-0.362
exhaust2	0.260	-0.321	**0.768****	-0.344
exhaust3	0.439	-0.245	**0.915****	-0.352
exhaust4	0.355	-0.174	**0.721****	-0.281
exhaust5	0.285	-0.251	**0.683****	-0.267
exhaust6	0.387	-0.249	**0.811****	-0.293
exhaust7	0.410	-0.113	**0.735****	-0.273
exhaust8	0.435	-0.138	**0.791****	-0.290
exhaust9	0.448	-0.218	**0.907****	-0.352
safisf1	-0.041	0.577	-0.327	**0.905****
safisf2	-0.034	0.469	-0.317	**0.774****
safisf3	-0.061	0.507	-0.326	**0.829****
safisf4	-0.098	0.534	-0.349	**0.883****
safisf5	-0.147	0.531	-0.368	**0.901****

*p<0.05; **p<0.01; N=186

The factor loadings for all latent variables in the analysis are greater than 0.70, and the cross-loadings are below the 0.70 threshold. This indicates the measurement scales used in the study have demonstrated construct validity [68,73].

Convergent and Discriminant Validity Analysis

For convergent and discriminant validity, average variance extracted (AVE), square root of AVE, and correlation values are needed. All values are presented in Table 3.

Table 3. AVE's and correlations

Constructs	AVE	Correlation			
		1	2	3	4
1.Surface Acting	0.657	**(0.811)** [a]			
2.Deep Acting	0.780	0.093	**(0.883)** [a]		
3.Emotional Exhaustion	0.639	0.471**	-0.272**	**(0.800)** [a]	
4.Job Satisfaction	0.740	-0.090	0.610**	-0.393**	**(0.860)** [a]

*p<0.05; **p<0.01; N=186; AVE: Average variance extracted
Note: Values denoted by "a" are square root values of AVE

The analysis of the AVE values shown in Table 2 indicates that all constructs have AVE measures that exceed the recommended threshold of 0.50. This indicates that the scales used in the measurement model have sufficient convergent validity, meaning that the items strongly converge to represent the intended latent constructs [68,74-76].

Furthermore, analysis of the correlations between constructs compared to the square root of each construct's AVE indicates that the square root of the AVE for each construct is greater than its

correlations with all other constructs. This pattern of results supports the discriminant validity of the measurement scales, suggesting that the constructs are distinct and that items are more strongly associated with their own constructs than with other constructs in the model [75,76].

Overall, the convergent and discriminant validity assessments based on AVE and interconstruct correlations provide strong evidence that the measurement scales used in this study have the necessary validity to accurately capture the intended latent constructs.

Reliability Analysis

The reliability of the measurement model was evaluated using both Cronbach's alpha (α) and composite reliability (CR) metrics, as presented in the findings shown in Table 4.

Table 4. Cronbach alfa (α) and composite reliability (CR)

Constructs	Cronbach Alfa (α)	Composite Reliability (CR)
1.Surface Acting	0.920	0.919
2.Deep Acting	0.934	0.934
3.Emotional Exhaustion	0.941	0.941
4.Job Satisfaction	0.934	0.934

The analysis results reveal that the Cronbach's alpha and composite reliability values for all the variables under examination surpass the recommended threshold of 0.70. The high internal consistency reliability, as evidenced by the Cronbach's alpha and composite reliability scores, suggests the scale items are closely interrelated and effectively capture the underlying constructs they are intended to measure. This finding indicates that the measurement scales employed in the study demonstrate strong reliability [68,77].

Path Analysis

PLS-SEM approach involves a two-step process. The first step is to validate the measurement model, ensuring the reliability and validity of the constructs. Once the measurement model is accepted, the second step is to test the structural model. Within the structural model, path analysis is conducted to evaluate the hypothesized relationships between the constructs. The path analysis provides a visual representation of the structural model and allows for the testing of the proposed hypotheses. The general view of the path analysis applied with the SmartPLS program is given in Figure 2.

The initial step in the structural model analysis involves assessing whether multicollinearity exists among the exogenous (independent) variables. To test for multicollinearity, the variance inflation factor (VIF) is evaluated. According to Hair et al. [68], if the VIF values are 5 or less, it can be concluded that multicollinearity is not a concern. An examination of the VIF values from the analysis reveals the following: surface acting has a VIF of 1.376, deep acting has a VIF of 1.118, and Emotional exhausting has a VIF of 1.474. Since all of these VIF values are less than the recommended threshold of 5, it can be inferred that there is no issue of multicollinearity among the predictor variables [68].

The path analysis conducted within the structural model framework provides insights into the explained variance (R^2), standardized regression coefficients (β), and dimensional effects (f^2) of the exogenous (independent) variables on the endogenous (dependent) variable. These findings are then utilized to interpret the results of the hypothesis testing. Regarding the interpretation of the R^2 values, Chin [74] suggests that an R^2 of 0.67 is considered high, 0.33 is medium, and 0.19 is low. Similarly, for the f^2 values, Cohen [78] proposes that 0.35 is a large effect, 0.15 is a medium effect, and 0.02 is a small effect.

The specific findings from the path analysis are presented in Table 5 of the study. These results provide a comprehensive understanding of the relationships between the exogenous and endogenous variable, which is then used to draw conclusions about the hypotheses tested within the structural model.

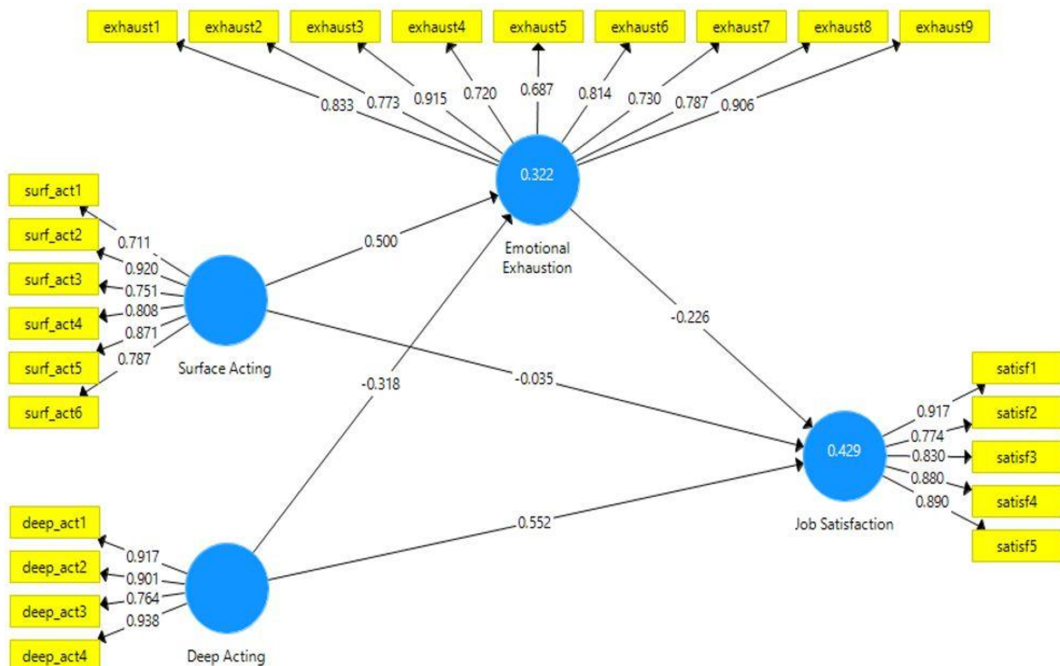

Figure 2. Structural model

Table 5. Findings of path analysis

Hypotheses				β	Std. Error	p	t-value	R^2	f^2	VIF	Decision
H1	Surface acting	→	Job Satisfaction	-0.035	0.098	0.725	0.352	0.429	0.002	1.376	Not Supported
H2	Deep acting	→	Job Satisfaction	**0.552****	0.072	0.001	7.630		0.461	1.158	**Supported**
	Emotional Exhaustion	→	Job Satisfaction	**-0.226***	0.112	0.043	2.022		0.061	1.474	Not Hypothesized
H3	Surface acting	→	Emotional Exhaustion	**0.500****	0.077	0.001	6.525	0.322	0.365	1.008	**Supported**
H4	Deep acting	→	Emotional Exhaustion	**-0.318****	0.060	0.001	5.322		0.148	1.008	**Supported**

*p<0.05; **p<0.01; N=186

The path analysis results, as presented in Table 5, reveal several key findings regarding the hypotheses tested within the structural model. First, the effect of surface acting on job satisfaction, although negative, is not statistically significant (H1: $\beta = -0.035$, $p > 0.05$). In contrast, deep acting was found to have a positive and significant effect on job satisfaction (H2: $\beta = 0.552$, $p < 0.01$). Based on these results, hypothesis H1 is rejected, while hypothesis H2 is accepted. Additionally, the analysis indicates that emotional exhaustion has a significant and negative effect on job satisfaction.

The path analysis further demonstrates that both surface acting (H3: $\beta = 0.500$, $p < 0.01$) and deep acting (H4: $\beta = -0.318$, $p < 0.01$) have a significant impact on emotional exhaustion. Specifically, surface acting is positively related to emotional exhaustion, while deep acting is negatively associated with emotional exhaustion. Consequently, hypotheses H3 and H4 are accepted.

In summary, the findings suggest that while surface acting does not significantly influence job satisfaction, deep acting has a positive effect on job satisfaction among pharmacists. Additionally, emotional exhaustion is found to have a negative impact on job satisfaction. Furthermore, surface acting behaviors contribute to increased emotional exhaustion, whereas deep acting behaviors are associated

with reduced emotional exhaustion.

Mediation Analysis

This study established a serial mediation analysis, examining the path from surface acting to job satisfaction via emotional exhaustion, as well as the path from deep acting to job satisfaction via emotional exhaustion. The study utilized the bootstrapping method proposed by Preacher and Hayes [79] to assess the indirect effects of the variables, examining these effects across 5,000 samples. The variance accounted for (VAF) approach, which is considered one of the best methods for testing mediation effects in PLS-SEM was utilized to examine the mediation effect [68, 69]. The VAF value is calculated by dividing the indirect effect by the total effect. A VAF value greater than 0.80 indicates full mediation, a VAF value between 0.20 and 0.80 suggests partial mediation, and a value less than 0.20 represents no mediation [68]. The mediation paths and their respective VAF values are presented in Table 6.

Table 6. Findings of mediation analysis

Hypotheses						**Direct Effect**	**Indirect Effect**	**Total Effect**	**Std. Error**	**p**	**t-value**	**VAF Value**	**Decision**
H5	Surface acting	→	Emotional Exhaustion	→	Job Satisfaction	-0.035	**-0.113***	-0.148*	0.055	0.041	2.051	0.764	Partial Mediation
H6	Deep acting	→	Emotional Exhaustion	→	Job Satisfaction	0.552**	**0.172***	0.724**	0.036	0.046	2.002	0.238	Partial Mediation

*p<0.05; **p<0.01; N=186

The fifth hypothesis investigates the effect of surface acting on job satisfaction through the role of emotional exhaustion. Although the direct effect of surface acting on job satisfaction is insignificant, the effect of surface acting on job satisfaction with the mediating effects of emotional exhaustion is found to be significant with the VAF value of 0.764 and confirms the partial mediation effects between surface acting and job satisfaction (H5: $\beta = -0.113$, $p < 0.05$). Thus hypothesis H5 is accepted.

The sixth hypothesis investigates the effect of deep acting on job satisfaction through the role of emotional exhaustion. The results of mediation analysis are positive and significant with the VAF value of 0.238, which is a partial mediating effect on their relationship (H6: $\beta = -0.172$, $p < 0.05$). Thus, the hypothesis H6 is also confirmed.

This study examines the complex interplay between emotional labor, emotional exhaustion, and job satisfaction among pharmacists. Specifically, it investigates the direct effects of two types of emotional labor - surface acting and deep acting - on pharmacists' job satisfaction, as well as the mediating role of emotional exhaustion in these relationships. The researchers collected survey data from 186 pharmacists in the Karabuk province and analyzed the data to uncover these dynamics.

Firstly, the analysis revealed that while surface acting (i.e., hiding true emotions while displaying customer-oriented behaviors) had a negative effect on pharmacists' job satisfaction, this effect was not statistically significant. This finding diverges from previous research that has shown surface acting to directly reduce job satisfaction, as it creates an internal dissonance for employees [23,25,37]. It is suggested that this contradictory finding may be attributed to the unique characteristics of the pharmacy profession. Specifically, Diefendorff et al. [3] posit that if employees are more experienced in surface acting, this behavior may not negatively impact their job satisfaction. In professions where customer service is paramount, such as pharmacy, employees may be more accustomed to surface acting and therefore it does not diminish their job satisfaction. The current study's findings indicate that for pharmacists, surface acting does not directly translate to reduced job satisfaction, unlike what has been reported in the broader literature on emotional labor.

The analysis also revealed that deep acting (i.e., pharmacists aligning their true emotions with the required emotional display) had a positive and statistically significant effect on their job satisfaction. This finding aligns with previous research [7,39,40,42,43], which indicates that when employees engage in deep acting, they are able to provide more genuine and empathetic service to customers. This, in turn,

enhances their own job satisfaction. For pharmacists, the ability to reflect their authentic emotions while serving customers appears to increase their job satisfaction. By engaging in deep acting, pharmacists can establish more empathic interactions with patients, which positively contributes to their overall satisfaction with their work. This contrasts with the non-significant effect found for surface acting, suggesting that for pharmacists, aligning internal feelings with outward emotional displays is more impactful on job satisfaction than merely hiding their true emotions.

The analysis further revealed that surface acting had a positive and significant effect on pharmacists' emotional exhaustion. This finding aligns with prior research [8,9,11,43,44,47,50], indicating that the display of inauthentic emotional behaviors towards customers, where pharmacists hide their true feelings, depletes their emotional resources over time. This suggests that the artificial emotional labor required in surface acting negatively impacts the emotional and psychological well-being of pharmacists.

In contrast, the analysis determined that deep acting decreased pharmacists' emotional exhaustion. When pharmacists are able to align their genuine emotions with the required emotional display, it enables them to preserve their emotional capacity. This finding aligns with prior studies [9,11,43,49,51-54]. Reflecting true empathy and care when interacting with customers appears to mitigate the risk of emotional exhaustion for pharmacists, in contrast to the depleting effects of surface acting.

The analysis also revealed that while the direct effect of surface acting on job satisfaction was not statistically significant, emotional exhaustion was found to play a partial mediating role in this relationship. This finding suggests that emotional exhaustion is an important factor in the decrease of job satisfaction stemming from pharmacists' display of artificial emotional behaviors towards customers. In line with prior research [37,48], surface acting indirectly decreases job satisfaction by increasing pharmacists' levels of emotional exhaustion. The act of suppressing their true emotions and exhibiting inauthentic emotional responses takes a toll on pharmacists over time, leading to heightened feelings of emotional exhaustion, which in turn negatively impacts their job satisfaction.

Conversely, the analysis determined that deep acting decreases pharmacists' emotional exhaustion and increases their job satisfaction. When pharmacists are able to genuinely activate and exhibit their real emotions during customer interactions, they can engage in a more authentic manner. This reduces their feelings of emotional exhaustion and, as a result, enhances their overall job satisfaction. This finding aligns with previous studies [11,43] on the beneficial effects of deep acting for employees' wellbeing and positive work outcomes.

Theoretical Implications

This study makes an important contribution to the limited body of research examining the relationships between emotional labor, job satisfaction, and emotional exhaustion among pharmacists. The key findings provide valuable insights that extend the existing literature in several ways.

Firstly, the insignificant direct effect of surface acting on job satisfaction, coupled with the partial mediating role of emotional exhaustion, offers a new perspective on the inconsistent results reported in prior studies. This suggests that the detrimental impact of surface acting on job satisfaction is primarily channeled through its ability to increase pharmacists' feelings of emotional exhaustion.

Secondly, the finding that deep acting positively influences job satisfaction and reduces emotional exhaustion provides evidence supporting the beneficial consequences of displaying authentic emotions among service sector employees, such as pharmacists. This aligns with and extends the validity of emotional labor theories proposed by Grandey [48] and Morris and Feldman [2] by demonstrating their applicability in the context of pharmacists' work experiences.

Overall, this study contributes to the limited research on the emotional labor dynamics of pharmacists, revealing the divergent effects of surface acting and deep acting on their job satisfaction and emotional exhaustion. These findings hold important implications for understanding and promoting the emotional well-being and positive work outcomes of pharmacists in the healthcare service industry.

Practical Implications

The findings of this study suggest that pharmacy managers should consider pharmacists' emotional labor strategies and provide appropriate support to enhance their well-being and

organizational performance. Specifically, the results indicate that pharmacy managers should take proactive measures to address the adverse consequences of pharmacists' surface acting behaviors.

First, the study reveals that pharmacists' surface acting, whereby they suppress their true emotions and display artificial emotional responses to customers, increases their emotional exhaustion, which in turn indirectly decreases their job satisfaction. Consequently, pharmacy managers should implement supportive interventions to protect pharmacists' emotional resources. This could involve providing emotional intelligence training, developing burnout coping strategies, and implementing mentoring programs to help pharmacists reduce their reliance on superficial role-playing and instead encourage the use of deep acting.

Conversely, the study finds that when pharmacists are able to genuinely reflect their true emotions while providing services to customers, it not only increases their job satisfaction but also reduces their emotional exhaustion. Therefore, pharmacy managers should strive to cultivate an organizational climate that supports and motivates pharmacists to engage in deep acting behaviors. This may involve implementing policies, practices, and leadership approaches that enable and empower pharmacists to authentically express their emotions during customer interactions.

By adopting these evidence-based strategies, pharmacy managers can effectively address the emotional labor challenges faced by pharmacists, ultimately enhancing their job satisfaction, reducing emotional exhaustion, and improving overall organizational performance.

Limitations and Future Directions

This study acknowledges several limitations that warrant consideration when interpreting its findings and implications. Firstly, the research was conducted solely on pharmacists employed in the Karabuk province, which may constrain the generalizability of the results. It is possible that conducting similar investigations in different organizational contexts or with more diverse samples of service employees could yield varying findings.

Secondly, the cross-sectional design of the study, whereby data was collected at a single point in time, restricts the ability to draw definitive conclusions about the causal relationships between the examined variables. To provide more compelling causal evidence, future research could benefit from adopting a longitudinal approach or implementing experimental research designs.

These limitations underscore the need for cautious interpretation of the current study's findings and highlight opportunities for further research to expand the knowledge base in this area. Investigations that replicate and extend the current study across different settings, populations, and methodological approaches would contribute to a more comprehensive understanding of the emotional labor dynamics and their impacts on job-related outcomes among service professionals, such as pharmacists.

By acknowledging these limitations upfront, this study demonstrates a commitment to methodological rigor and set the stage for subsequent studies to build upon the existing knowledge in a systematic and robust manner. Addressing these limitations through future research can help refine and strengthen the theoretical and practical implications derived from the present study.

In summary, the key findings of this study reveal the distinct impacts of pharmacists' surface acting and deep acting behaviors on their job satisfaction, as well as the mediating role of emotional exhaustion in these relationships. Notably, the insignificant direct effect of surface acting on job satisfaction, coupled with the partial mediation by emotional exhaustion, suggests that the depletion of pharmacists' emotional resources plays a critical role in diminishing their job satisfaction when they engage in inauthentic emotional displays towards customers.

Conversely, the study demonstrates that when pharmacists are able to genuinely reflect their true emotions (deep acting), it positively influences their job satisfaction and, importantly, reduces their emotional exhaustion. These findings underscore the importance of pharmacy managers taking proactive measures to protect their employees' emotional well-being and facilitate the adoption of deep acting strategies.

Collectively, the results of this investigation highlight the need for pharmacy managers to prioritize the implementation of interventions and policies that support pharmacists' emotional resources and encourage the expression of their authentic emotions when interacting with customers. By doing so, they can foster improved job satisfaction among pharmacists, which is essential for enhancing overall

organizational performance and service quality.

AUTHOR CONTRIBUTIONS

Concept: N.Ç.Ş., O.B.; Design: N.Ç.Ş.; Control: O.B.; Sources: N.Ç.Ş.; Materials: N.Ç.Ş., O.B.; Data Collection and/or Processing: N.Ç.Ş., O.B.; Analysis and/or Interpretation: O.B.; Literature Review: N.Ç.Ş., O.B.; Manuscript Writing: N.Ç.Ş., O.B.; Critical Review: O.B.; Other: -

CONFLICT OF INTEREST

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

ETHICS COMMITTEE APPROVAL

Karabuk University Social and Human Sciences Research Ethics Committee granted approval for the questionnaire application. This approval was documented in a decision dated June 23, 2022, and numbered 2022/05-4.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

STRATEGIC QBD IMPLEMENTATION IN RPHPLC-PDA METHOD FOR SIMULTANEOUS QUANTIFICATION OF CYSTIC FIBROSIS DRUGS TEZACAFTOR AND IVACAFTOR

KİSTİK FİBROZİS İLAÇLARI TEZACAFTOR VE IVACAFTOR'UN EŞ ZAMANLI KANTİFİKASYONU İÇİN RPHPLC-PDA YÖNTEMİNDE STRATEJİK QBD UYGULAMASI

Anusha GANDI[1]*, Srinivasa Rao YARGUNTLA[1], Varaprasada Rao KOLLABATHULA[1]

[1]Department of Pharmaceutical Analysis, Vignan Institute of Pharmaceutical Technology, Besides VSEZ, Duvvada, Visakhapatnam, Andhra Pradesh 530049, India

ABSTRACT

Objective: *A novel RP-HPLC PDA method was developed using a Quality by Design (QbD) approach for the simultaneous quantification of Tezacaftor and Ivacaftor medications employed in the management of cystic fibrosis.*

Material and Method: *Optimization was performed by Central Composite Design by selecting mobile phase ratio of methanol, pH of buffer and flow rate as factors and evaluating responses namely retention time and tailing factor. This technique makes use of an Inertial ODS C18 column (250 x 4.6 mm, 5 µm particle size) in conjunction with a Waters module fitted with a photo diode array detector. The chromatographic conditions including a flow rate of 1.0 ml/min, a mobile phase composed of methanol and buffer in a 45:55 ratio, and a detection wavelength of 210 nm, were thoughtfully designed to effectively separate Tezacaftor and Ivacaftor.*

Result and Discussion: *The method demonstrated remarkable accuracy, with average recoveries of 99.69% for ivacaftor and 100.06% for tezacaftor. The % assay results for system suitability, method precision, and intermediate precision consistently fell within the range of 99.91% to 100.37%. Linearity data exhibited correlation coefficient values of one for both Tezacaftor and Ivacaftor. The LOD and LOQ values for Tezacaftor and Ivacaftor were determined to be 0.56, 0.57, 1.69, and 1.74, respectively. The results obtained from the validation parameters demonstrate that this RP-HPLC method, developed using the QbD approach, is robust and dependable. It serves as a valuable tool for routine analysis and plays a pivotal role in bioanalytical and bioequivalence research within the realm of cystic fibrosis treatment. This method ensures precise and accurate quantification of Tezacaftor and Ivacaftor in combination tablet formulations.*

Keywords: *Central composite design, ivacaftor, method development, method validation, RP-HPLC, tezacaftor*

ÖZ

Amaç: *Kistik fibrozis tedavisinde kullanılan Tezacaftor ve Ivacaftor ilaçlarının eş zamanlı ölçümü için Tasarıma Göre Kalite (QbD) yaklaşımı kullanılarak yeni bir RP-HPLC PDA yöntemi geliştirildi.*

* **Corresponding Author / Sorumlu Yazar:** Anusha Gandi
e-mail / e-posta: gandianusha11@gmail.com, **Phone / Tel.:** +917330809694

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Gereç ve Yöntem: *Optimizasyon, metanolün mobil faz oranı, tamponun pH'ı ve akış hızının faktör olarak seçilmesi ve tepkilerin, yani alıkonma süresi ve kuyruk faktörünün değerlendirilmesi yoluyla Merkezi Bileşik Tasarım ile gerçekleştirildi. Bu teknik, bir foto diyot dizisi dedektörü ile donatılmış bir Waters modülü ile birlikte bir Inertial ODS C18 kolonundan (250 x 4,6 mm, 5 µm parçacık boyutunda) yararlanır. 1.0 ml/dak'lık bir akış hızı, 45:55 oranında metanol ve tampondan oluşan bir mobil faz ve 210 nm'lik bir tespit dalga boyunu içeren kromatografik koşullar, Tezacaftor ve Ivacaftor'u etkili bir şekilde ayırmak için iyi bir şekilde tasarlanmıştır.*

Sonuç ve Tartışma: *Yöntem, ivacaftor için %99.69 ve tezacaftor için %100.06'lık ortalama geri kazanımlarla dikkate değer bir doğruluk gösterdi. Sistem uygunluğu, yöntem kesinliği ve ara kesinlik için % miktar tayini sonuçları sürekli olarak %99.91 ile %100.37 aralığında tespit edildi. Doğrusallık verileri, hem Tezacaftor hem de Ivacaftor için bir korelasyon katsayısı değeri gösterdi. Tezacaftor ve Ivacaftor için LOD ve LOQ değerleri sırasıyla 0.56, 0.57, 1.69 ve 1.74 olarak belirlendi. Doğrulama parametrelerinden elde edilen sonuçlar, QbD yaklaşımı kullanılarak geliştirilen bu RP-HPLC yönteminin sağlam ve güvenilir olduğunu göstermektedir. Rutin analizler için bu teknik değerli bir araç olarak hizmet eder ve kistik fibroz tedavisi alanında biyoanalitik ve biyoeşdeğerlik araştırmalarında önemli bir rol oynar. Bu yöntem, kombinasyon tablet formülasyonlarında Tezacaftor ve Ivacaftor'un kesin ve doğru miktarının belirlenmesini sağlar.*

Anahtar Kelimeler: *Merkezi kompozit tasarım, ivacaftor, RP-HPLC, tezacaftor, yöntem geliştirme, yöntem validasyonu*

INTRODUCTION

Ivacaftor is identified by the molecular formula $C_{24}H_{28}N_2O_3$ and is chemically described as N-(2,4-di-tert-butyl-5-hydroxyphenyl)-4-oxo-1,4-dihydroquinoline-3-carboxamide [1]. Enhances the movement of chloride by boosting the likelihood of the G551D-CFTR protein channel to open, thereby facilitating chloride transport (see figure 1B) [2]. Genetic mutations affecting the CFTR gene, responsible for controlling the transport of ions like chloride and water in the body, are the root cause of cystic fibrosis [3]. The molecular formula of Tezacaftor is $C_{26}H_{27}F_3N_2O_6$, and its chemical structure is represented by 1-(2,2-Difluoro-1,3-benzodioxol-5-yl)-N-[1-[(2R)-2,3-dihydroxypropyl]-6-fluoro-2-(2-hydroxy-1,1-dimethylethyl)-1H-indol-5-yl]cyclopropane carboxamide. It functions as a corrector by aiding in the proper folding and presentation of the protein on the cell surface, ultimately enhancing its functionality in individuals with the F508 delmutation (Figure1A) [4].

Analytical Quality by Design (AQbD) is the practice of applying the Quality by Design (QbD) principle specifically to the development of analytical methods and procedures. It claims that rather than determining quality merely by testing of end results, quality should be integrated into the design of the analytical process. The foundation of this approach is the Quality Target Method Profile (QTMP), which begins the process. The term "ATP" stands for Analytical Target Profile, describing the method itself. This method profile outlines the desired outcome and directs decision-making throughout the research and development phases of a project [5]. Adhering to the definition of QTMP can aid in the identification of CAAs. CAAs are similar to critical quality attributes (CQA) in the context of product development. To ensure the required product quality, limits, ranges, or distributions for CQAs, which the ICH Q8 (R2) defines as chemical, physical, biological, or microbiological qualities, must be established.

According to a literature review, there are few developed RP-HPLC PDA methods for determining tezacaftor and ivacaftor simultaneously in a fixed dose combination [6-13]. Despite the extensive analytical methods available for individual quantification of Tezacaftor and Ivacaftor, there is a scarcity of robust, validated RP-HPLC methods developed using the QbD approach for their simultaneous estimation. Existing methods often lack systematic optimization strategies, leading to variability in retention time, peak symmetry, and sensitivity. Conventional HPLC methods often do not account for critical factors such as mobile phase composition, buffer pH, and flow rate in a systematic manner, leading to variability in retention time, resolution, and peak symmetry. Furthermore, few studies have provided comprehensive validation data, including system suitability, method precision, intermediate precision, and sensitivity parameters such as LOD and LOQ. Additionally, limited research has focused on the application of QbD-based RP-HPLC methods for

routine pharmaceutical analysis and bioequivalence studies in cystic fibrosis treatment. This study addresses these gaps by employing a QbD approach to develop a novel, highly accurate, and validated RP-HPLC method for the simultaneous estimation of Tezacaftor and Ivacaftor.We couldn't find any earlier studies that used design of experiments approach to build an AQbD-based HPLC method for quantitatively analysing Tezacaftor and Ivacaftor in commercially available formulations and pure medication. As a result, our current study represents a successful attempt to develop an AQbD-driven HPLC technique for measuring Tezacaftor and Ivacaftor in pure drug and formulations utilising Design of Experiments principles (DoE). The validated method can be applied in bioanalytical research to assess drug concentration profiles in biological matrices, essential for regulatory approval of generic formulations. With a run time optimized for efficiency, this method is suitable for routine quality control analysis in pharmaceutical industries.

A) B)

Figure 1. A) Structure of Tezacaftor, B) Structure of Ivacaftor

MATERIAL AND METHOD

Instrumentation

1.HPLC Waters Model series No .2690/95 with PDA using Empower software.
2.Electronic balance (SARTORIOUS)
3.Sonicator (FASTCLEAN).

Material and Reagents

Tezacaftor and Ivacaftor sample with 99.8% w/w purity was obtained from a Hetero labs Pvt.Ltd. Analytical grade potassium dihydrogen phosphate, orthophosphoric acid, HPLC grade methanol and Mill-Q water was procured from Merck. Drug combination tablets of dose 100mg Tezacaftor and 150mg Ivacaftor with a brand name Symkevi were procured from local pharmacy.

Table 1. Experimental factors and levels used in design

Factor	Lower Limit(-1)	Upper Limit(+1)
A:Mobile phase of methanol	40	50
B: pH of Buffer	2.40	4.40
C: Flow rate	0.80	1.20

Optimisation

The Design-Expert software employed a numerical optimisation method to evaluate the model's accuracy. Based on desirability 1.0, the software chose one of 100 solutions. Design Expert specified the optimal conditions for the experiment. The chromatographic settings recommended by Design-Expert were mobile phase, pH, and flow rate [14-16]. As shown in Figure 2, the model projected a method response of retention time and tailing factor. The HPLC equipment was used in the same experiment under the same conditions and optimized conditions are tabulated in Table 3. Figure 3, 4, 5, 6 and Table 2 shows that the predicted and observed values have a 0.999 correlation. As illustrated

in the Figure 2, all of these variables have been demonstrated to have a considerable impact on retention time and tailing factor. The chromatogram of optimized condition by HPLC is represented in Figure 7.

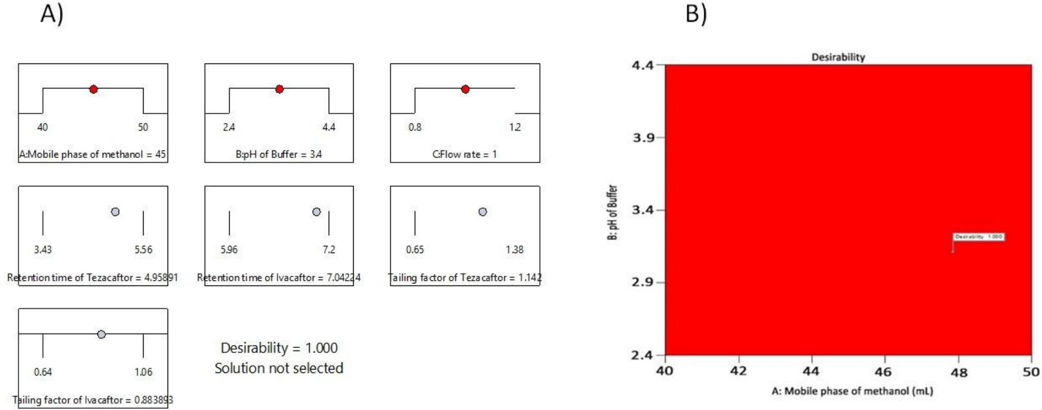

Figure 2. A), B) Optimisation and prediction of method responses by model

Table 2. Summary of central composite design 3 factors; 20 runs

			F 1	F 2	F 3	R 1	R 2	R 3	R 4
S.No.	**Std**	**Run**	**A:Mobile phase of methanol**	**B:pH of Buffer**	**C:flow rate**	**RT of Tezacaftor**	**RT of Ivacaftor**	**Tailing factor of Tezacaftor**	**Tailing factor of Ivacaftor**
			ml	ml	mL/min	mins	mins		
1	5	1	40	2.5	1.2	3.9	6.39	0.77	0.75
2	8	2	50	4.4	1.2	5.19	7.2	1	0.99
3	1	3	40	2.5	0.8	3.88	6.63	0.66	0.72
4*	16	4	45	3.4	1	4.97	7.07	1.15	0.89
5	3	5	40	4.4	0.8	4.06	6.54	0.85	0.7
6	10	6	53.4	3.4	1	5.56	6.8	1.26	1.06
7	11	7	45	1.8	1	4.35	6.9	1	0.88
8	7	8	40	4.4	1.2	3.87	6.3	0.95	0.72
9	12	9	45	5	1	4.62	7.02	0.99	0.83
10	6	10	50	2.5	1.2	5.27	7.2	1.15	1
11*	17	11	45	3.4	1	4.97	7.07	1.15	0.89
12*	15	12	45	3.4	1	4.89	6.91	1.1	0.85
13	4	13	50	4.4	0.8	5.42	7.1	1.2	0.95
14	13	14	45	3.4	0.7	4.89	6.98	1.12	0.84
15*	20	15	45	3.4	1	4.97	7.07	1.15	0.89
16*	19	16	45	3.4	1	4.97	7.07	1.15	0.89
17	9	17	36.6	3.4	1	3.43	5.96	0.65	0.64
18	14	18	45	3.4	1.3	4.99	7.1	1.07	0.92
19*	18	19	45	3.4	1	4.97	7.07	1.15	0.89
20	2	20	50	2.5	0.8	5.19	7.1	1.38	0.97

*F1,F2,F3- Factors of 1,2,3
*R1,R2,R3,R4- Response of 1,2,3,4

Table 3. Optimized chromatographic conditions

Parameters	Method
Stationary phase	Inertsil-ODSC$_{18}$(250x4.6mm,5µ)
Mobile Phase	Methanol:Buffer(45:55)
Flowrate	1.0ml/min
Runtime	12min
Column temperature	Ambient
Volume of injection loop	20µl
Detection wavelength	210nm
Drug RT(min)	4.977min for Tezacaftor and 7.077 for Ivacaftor.

Method Development

In our present study, we fine-tuned the chromatographic parameters of the HPLC method by employing the Central Composite Design. This design was selected because it provides the flexibility to modify experimental parameters whenever necessary. Our main objective was to create and confirm a Quality by Design approach based on HPLC. Table 1 outlines the recommended values for the low, medium, and high levels of the variables under consideration as suggested by the software. We conducted experimental runs to evaluate the impact of these factors on the Critical Analytical Attributes (CAAs).

Preparation of Diluent

Creating the standard and sample solutions required diluting them with the mobile phase.

pH 3.4 Phosphate Buffer Preparation

To produce a pH 3.4 phosphate buffer, begin by measuring 2.7218 grams of KH_2PO_4. Add this measured amount of KH_2PO_4 to a 1000ml beaker with 1000ml of HPLC water. Use orthophosphoric acid to adjust the solution's pH to 3.4.

Mobile Phase

Methanol degassed and buffered in a 45:55v/v ratio.

Preparation of Standard Stock Solution

Place 10 milligrams of each drug into a 10-ml volumetric flask, and then add 7 ml of ethanol and sonicate for half an hour. After half an hour add remaining 3ml upto the mark and sonicate it to 5 mins (i.e.,1000 ppm).

Preparation of Working Standard Preparation

Add 1 ml of Tezacaftor standard solution and 1ml of Ivacaftor in a 10 ml volumetric flask. Add methanol upto the mark, then sonicate it for five minutes (100 ppm).

Method Validation

The method parameters are validated as per ICH guidelines.

RESULT AND DISCUSSION

Response Surface Modeling by Central Composite design (CCD)

The Central Composite design was used to screen and optimise the chromatographic conditions. The mobile phase of methanol, pH of Buffer, and flow rate were all varied in the 40-50 ratio, 2.4-4.4, and 0.8-1.2 ml/min respectively. The levels of the selected method responses are shown in Table 1. Table 2 displays the outcomes of 20 runs conducted with the response surface method's . The results of utilising Design of Experiments software to build a quadratic model of ANOVA regression parameters

for retention time are shown in Table 1. The model's F-value 29.82, 117.52, 259.34, 201.24 indicates its importance. Model terms are considered significant if their p-value is less than 0.0500.

Retention Time of Ivacaftor

A quadratic model represented the built-in ANOVA. The model is crucial. Table 4 provides an overview of the model i.e., quadratic and lack of the fit test. The drug's retention time's built-in value suggested that it is substantial. The coded equation =+7.04+0.3035A+0.0037B-0.0080C+0.0258AB+0.0850AC-0.0015BC-0.2291A2-0.0273B2+0.0078C2 with the factors coded. The contour plot is depicted by the built-in model graph in Figure 3A, while the specifics of the 3D surface design points are shown in Figure 3B.

Table 4. Model summary statistics and lack of fit test

	Response	Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	F-value	p-value
Ivacaftor	RT	Quadratic	0.0883	0.9641	0.9317	0.7860	29.82	<0.0001
Ivacaftor	Tailing Factor	Quadratic	0.0147	0.9906	0.9822	0.9654	117.59	<0.0001
Tezacaftor	RT	Quadratic	0.0536	0.9957	0.9919	0.9738	259.34	<0.0001
Tezacaftor	Tailing Factor	Quadratic	0.0195	0.9945	0.9896	0.9759	201.24	<0.0001

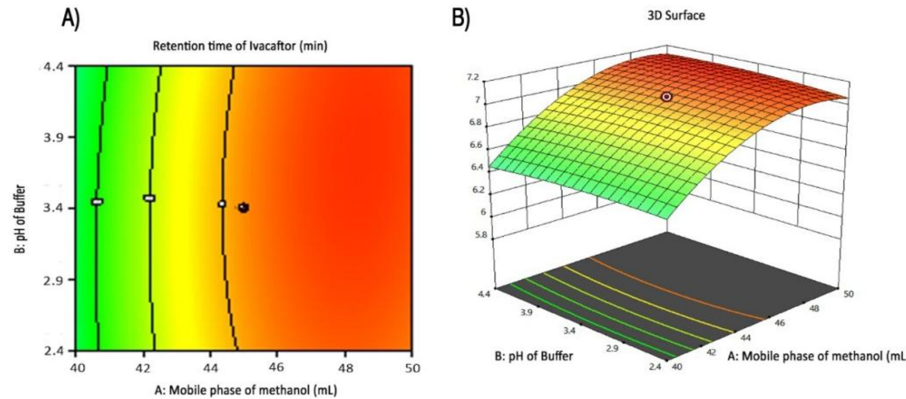

Figure 3. A) Contour plot for retention time of Ivacaftor, B) 3D Response surface retention time of Ivacaftor

Tailing Factor of Ivacaftor

The tailing factor was optimized using the built-in quadratic ANOVA model in the CCD programme to verify the peak summary. The lack-of-fit test and the quadratic model are summarized in Table 4. $+0.08839+0.1264A-0.0120B+0.0192C+0.0027AB+0.0025AC-0.0002BC-0.0137A^2-0.0130B^2-0.0044C^2$ is the final coded equation. Figure 4A built-in model graph shows a contour plot and Figure 4B specifies the 3D surface design points.

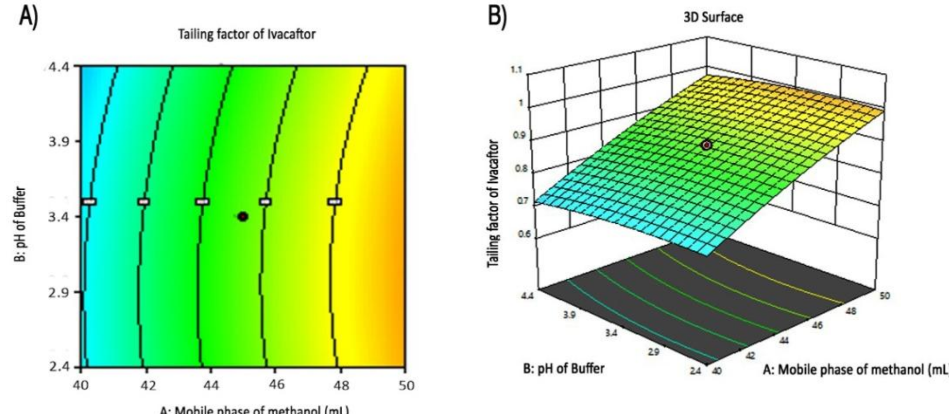

Figure 4. A) Contour plot for tailing factor of Ivacaftor, B) 3D Response surface tailing factor of Ivacaftor

Retention Time of Tezacaftor

Quadratic modelling was used for the built-in ANOVA. It is important to use the model. The quadratic model is summarised in Table 4 along with the "lack of the fit test." The retention duration for Tezacaftor was predetermined to be important based on its built-in value. The solution contained the coded factors $+4.96+0.6551A+0.0690B-0.0114C+0.0008AB+0.0025AC-0.0698BC-0.1687A^2-0.1904B^2-0.0152C^2$. The built-in model graph in Figure 5A shows the contour plot, whereas Figure 5B shows the specifics of the 3D surface design points.

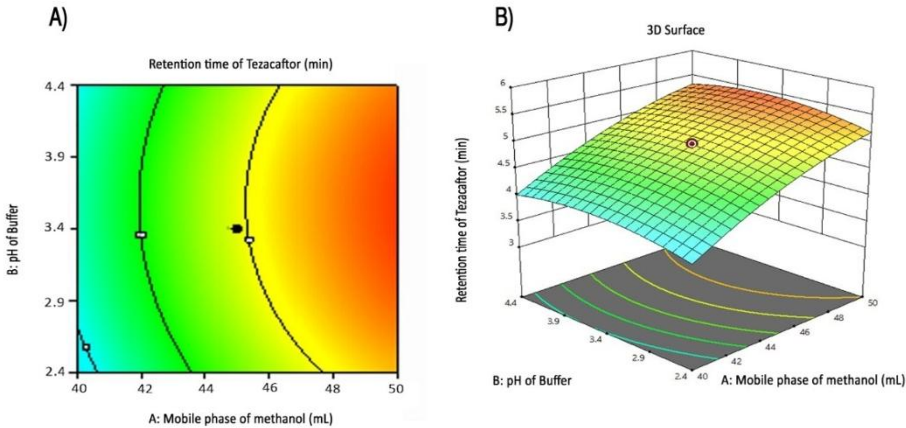

Figure 5. A) Contour plot for retention time of Tezacaftor, B) 3D Response surface retention time of Tezacaftor

Tailing Factor of Tezacaftor

To optimise the tailing factor and verify the peak summary, the CCD application's built-in quadratic ANOVA model was employed. The lack-of-fit test and the quadratic model are summarised in Table 4. The final coded equation is $+1.14+0.1877A+0.0051B-0.0237C-0.0919AB-0.0800AC+0.0024BC-0.0678A^2-0.0588B^2-0.0233C^2$.The contour plot is depicted by the built-in model graph in Figure 6A, while the 3D surface design points are described in more depth in Figure 6B.

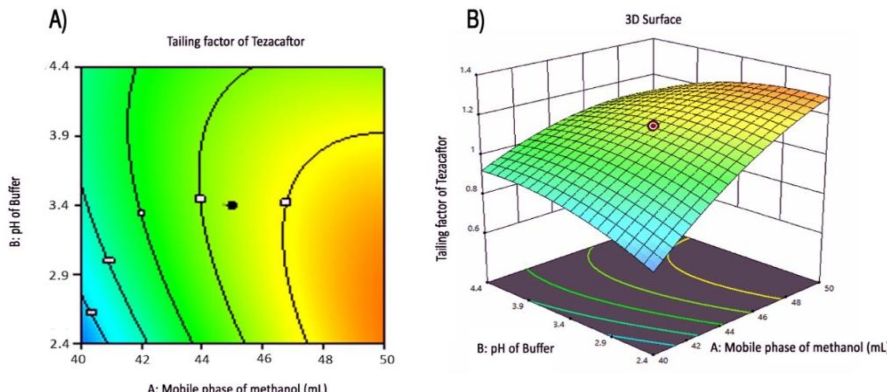

Figure 6. A) Contour plot for tailing factor of Tezacaftor, B) 3D Response surface tailing factor of Tezacaftor

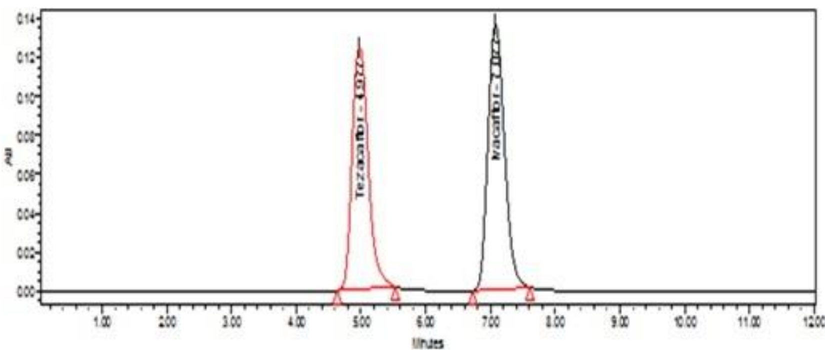

Figure 7. Optimized chromatogram

Method Validation

System Suitability

The system suitability results are presented in Table 5 and Table 6, and chromatogram of system suitability is represented in Figure 8.

Table 5. Data on Tezacaftor system suitability

Injection	RT	Peak Area	USP Plate count	USP Tailing
1	4.975	674753	10953.6097	1.15
2	4.976	674261	10951.0146	1.15
3	4.974	675298	10003.2730	1.15
4	4.975	679221	10986.9427	1.15
5	4.979	688636	10946.8723	1.15
6	4.972	674326	10964.9081	1.15
Mean	**4.975167**	**677749.2**	**10768.3467**	**1.15**
SD	**0.002115**	**5156.873**	**-------**	**-------**
%RSD	**0.0425**	**0.76**	**-------**	**-------**

Table 6. Data on Ivacaftor system suitability

Injection	RT	Peak Area	USP Plate count	USP Tailing
1	7.075	1218805	9478.3171	0.899633
2	7.076	1214014	9452.1967	0.893423
3	7.074	1215474	9569.9285	0.894443
4	7.070	1227655	9619.6337	0.882222
5	7.075	1267019	9749.9072	0.892316
6	7.072	1225625	9620.7336	0.889233
Mean	**7.0736**	**1228099**	**9573.9971**	**0.892407**
SD	**0.002055**	**18098.07**	**-------**	**-------**
%RSD	**0.029**	**1.47**	**-------**	**-------**

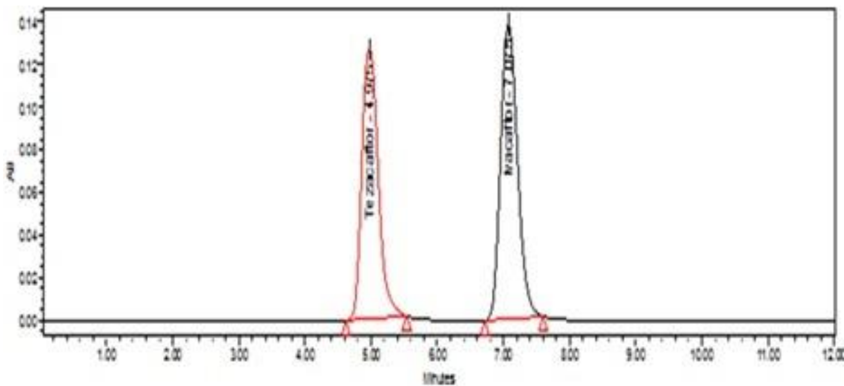

Figure 8. Chromatogram of system suitability

Specificity

By comparing the drug with the blank solution and analyzing for drug and blank solution interference, the specificity of this created approach is assessed [17-23]. The chromatogram of Blank and standard is represented in Figure 9A and 9B.

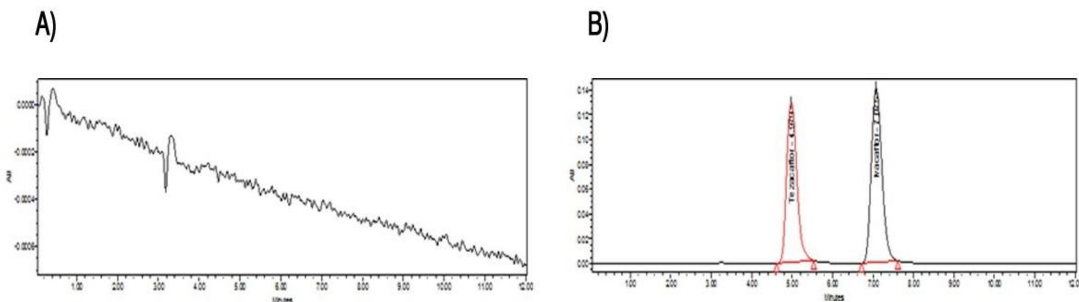

Figure 9. A) Blank chromatogram, B) Standard chromatogram

Precision

There are three types of precisions which are used in the development of HPLC those three precisions were system precision, intermediate precision and method precision [24-27]. It is measured at the concentration at 100 ppm, the peak area and the %assay were calculated based on the data. The results of precision is tabulated in Table 7-9.

Table 7. Results of system precision

Injection no.	Peak Area	
	Tezacaftor	**Ivacaftor**
1	678433	1228593
2	675498	1215374
3	679321	1226655
4	676341	1216454
5	679642	1224568
6	677541	1226548
Mean	677796	1223032
S.D	1505.65	5175.06
%RSD	0.22	0.42

Table 8. Results for intermediate precision

Inj.No.	Analyst-I		Analyst-II	
	Tezacaftor	**Ivacaftor**	**Tezacaftor**	**Ivacaftor**
1	644614	1206333	644607	1206333
2	645622	1216481	645245	1203264
3	642361	1205632	643216	1206513
4	647413	1216548	646648	1215484
5	647614	1205632	647012	1206513
6	645622	1213245	645146	1204516
Mean	645541	1210645	645312.3	1207104
SD	1769.49	4907.783	1264.72	3936.173
%RSD	0.27	0.40	0.19	0.32

Table 9. Results for method precision

Injection no.	Peak Area	
	Tezacaftor	**Ivacaftor**
1	637312	1202687
2	635732	1204628
3	634623	1205416
4	633214	1213268
5	637216	1202846
6	636632	1205416
Mean	635788.2	1205710
S.D	1475.349	3554.527
%RSD	0.23	0.29

Accuracy

Different concentrations (50%, 100%, 150%) of spiked solutions containing Tezacaftor and Ivacaftor were prepared and injected into the HPLC system [28-30]. The percentage recovery was calculated for each concentration. The accuracy results derived from these experiments are documented and presented in Table 10.

Table 10. Tezacaftor and Ivacaftor data with accuracy

	Concentration % of spiked level								
	50%			100%			150%		
	Amount added (ppm)	Amount found (ppm)	% recovery	Amount added (ppm)	Amount found (ppm)	% recovery	Amount added (ppm)	Amount found (ppm)	% recovery
Tezacaftor	20	19.93	99.69	40	39.93	99.83	60	59.98	99.97
Ivacaftor	20	20.19	100.06	40	40.01	100.04	60	60.01	100.02

Limit of Detection and Limit of Quantification

The values for Limit of Detection (LOD) and Limit of Quantification (LOQ) have been tabulated and are presented in Table 11. These values represent the sensitivity and lowest detectable and quantifiable levels of the substances under study.

Table 11. LOD and LOQ

Drug	LOD (µg/ml)	LOQ (µg/ml)
Tezacaftor	0.56	1.69
Ivacaftor	0.57	1.74

Linearity

The method's linearity was assessed by preparing concentration levels ranging from 20 to 70 ppm of Tezacaftor and Ivacaftor, which were subsequently injected into the HPLC system. Figures 10A and 10B depict the linearity plots of Tezacaftor and Ivacaftor, respectively. Tables 12 and 13 present the linearity data for Tezacaftor and Ivacaftor, showcasing the relationship between concentration levels and the analytical response, demonstrating the method's ability to provide accurate and consistent results across a range of concentrations.

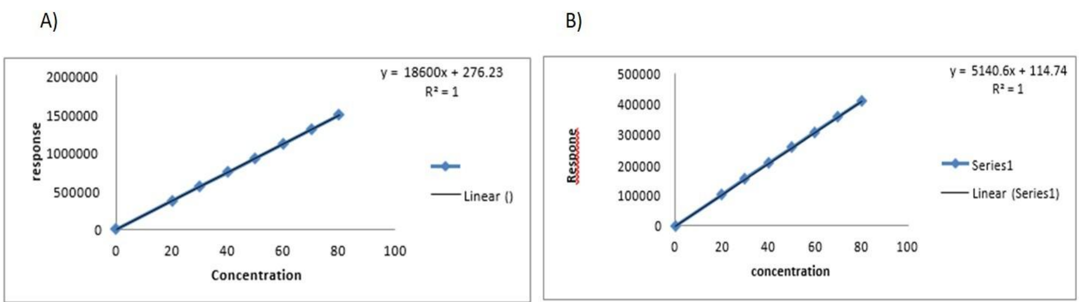

Figure 10. A) Tezacaftor's linearity plot, B) Ivacaftor's linearity plot

Table 12. Linearity data on Tezacaftor

Conc.(ppm)	Average Area
0	0
20	632546
30	658296
40	694400
50	730308
60	916282
70	9402046

Table 13. Linearity data on Ivacaftor

Conc.(ppm)	Average Area
0	0
20	1202965
30	1254371
40	1295856
50	1297167
60	1308577
70	1359903

During the development of the QbD analytical method various parameters were explored. Initially, Tezacaftor showed the highest absorbance at 241 nm, while Ivacaftor exhibited its peak absorbance at 254 nm. Consequently, 254 nm was chosen as the standard wavelength due to its excellent purity in peak. An injection volume of 20 µl was selected, providing a satisfactory peak area. The Inertsil C18 column (ODS) was chosen for its ability to generate well-defined peaks. The pharmaceutical solution proved suitable for analysis at room temperature. A flow rate of 1.0 ml/min was established offering acceptable peak area, retention time, and good resolution. Multiple mobile phase ratios were examined with the methanol:buffer ratio of 45:55v/v ultimately selected for its superior peak symmetry and strong resolution. This mobile phase was then applied in the suggested analysis. Both the system and method precision demonstrated adherence to acceptable limits. Successful curve fitting, correlation coefficient, and linearity studies indicated the linear behavior of the analytical methods for both Tezacaftor and Ivacaftor across the desired concentration range of 20-70 ppm. Accuracy tests also showed successful results, with excellent percentage recovery at all concentration levels, affirming the dependability of the analytical method for both Tezacaftor and Ivacaftor.

The RP-HPLC method developed through the QbD approach is a robust and reliable analytical tool. Its successful optimization chromatographic conditions and comprehensive validation parameters make it a valuable asset for the pharmaceutical industry and research community. This method not only ensures the accurate quantification of Tezacaftor and Ivacaftor but also contributes to the advancement of knowledge in the field of cystic fibrosis treatment and pharmaceutical quality assurance.

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AUTHOR CONTRIBUTIONS

Concept: A.G., S.R.Y., V.R.K.; Design: A.G.; Control: S.R.Y., V.R.K.; Sources: A.G., S.R.Y., V.R.K.; Materials: A.G., S.R.Y., V.R.K.; Data Collection and/or Processing: A.G.; Analysis and/or Interpretation: A.G., S.R.Y., V.R.K.; Literature Review: A.G.; Manuscript Writing: A.G.; Critical Review: S.R.Y., V.R.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.

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ÖZGÜN MAKALE / ORIGINAL ARTICLE

SAKLAMA KOŞULLARININ İZOLE LENFOSİT CANLILIĞI ÜZERİNE ETKİSİ

THE EFFECT OF STORAGE CONDITIONS ON ISOLATED LYMPHOCYTE VIABILITY

Seniha KERİMOĞLU[2], Seda TEKNECİ[1,2], Aylin ÜSTÜNDAĞ[1]*, Yalçın DUYDU[1]

[1]Ankara Üniversitesi, Eczacılık Fakültesi, Farmasötik Toksikoloji Ana Bilim Dalı, 06560, Ankara, Türkiye
[2]Ankara Üniversitesi, Sağlık Bilimleri Enstitüsü, 06110, Ankara, Türkiye

ÖZ

Amaç: *Saklama koşullarının izole lenfosit canlılığı üzerindeki etkisi, hücrelerin uzun süreli saklanabilmesi için kritik bir faktördür. Lenfositler, bağışıklık sisteminin önemli hücreleri olup, çoğunlukla kanser gibi kronik hastalıkların değerlendirilmesi veya farklı araştırma konularında kullanılmak üzere laboratuvar ortamında izole edilip saklanırlar. Bu saklama işlemi, hücrelerin fizyolojik ve biyokimyasal özelliklerini koruyabilmesi için uygun koşullarda yapılmalıdır. Bu bağlamda, saklama koşullarının izole lenfositlerin canlılığı üzerindeki etkileri belirlemek, yapılacak araştırmalarda doğru sonuç alabilmek için çok önemlidir.*

Gereç ve Yöntem: *Bu makalede, insan lenfosit örneklerinde saklama koşullarının DNA hasarını etkileyip etkilemediği araştırılmıştır. Tam kan ve İzole lenfositler 1, 3, 6 ve 12 aylık süreler boyunca 2 farklı sıcaklık (-20°C ve -80°C) altında saklanmıştır. Ayrıca, her bir zaman aralığı için farklı sıcaklık koşullarında saklanan lenfositlerin bir kısmı %10 DMSO içeren kriyoprezervasyon çözeltisinde, diğer bir kısmı ise doğrudan saklanmıştır. Bu çalışmayla, farklı sürelerde ve farklı saklama koşulları altında lenfosit canlılıklarını değerlendirerek insan biyoizleme çalışmaları gibi lenfositlerin kullanıldığı farklı çalışma alanlarına elde ettiğimiz veriler ile katkıda bulunmak amaçlanmıştır.*

Sonuç ve Tartışma: *Çalışmamızda elde edilen bulgulara göre lenfositlerin hayatta kalma oranı -80°C'deki saklama koşullarında daha yüksek bulunmuştur. Ayrıca, lenfositlerin -80°C'de kriyoprezervasyon çözeltisi ile (%10 DMSO) saklanmasının lenfosit bütünlüğünü korumak için daha uygun bir strateji sağladığı söylenebilir.*

Anahtar Kelimeler: *Canlılık, DNA hasarı, lenfosit, saklama koşulları*

ABSTRACT

Objective: *The effect of storage conditions on isolated lymphocyte viability is a critical factor for the long-term preservation of cells. Lymphocytes are essential cells of the immune system and are often isolated and stored in laboratory environments for different research topics such as chronic diseases, including cancer. This storage process must be conducted under suitable conditions to preserve the cells' physiological and biochemical properties. In this context, determining the effects of storage conditions on the viability of isolated lymphocytes is crucial for obtaining accurate results in research.*

Material and Method: *This research investigates whether storage conditions affect DNA damage in human lymphocyte samples. Whole blood and isolated lymphocytes were stored at two different*

* **Sorumlu Yazar / Corresponding Author:** Aylin Üstündağ
e-posta / e-mail: dur@pharmacy.ankara.edu.tr, **Tel. / Phone:** +903122033114

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temperatures (-20°C and -80°C) for 1, 3, 6, and 12 months. Additionally, for each time point, some of the lymphocytes stored under different temperature conditions were preserved in a cryopreservation solution containing 10% DMSO, while others were stored directly. The aim of this study is to contribute to various research areas, such as human biomonitoring studies, where lymphocytes are used, by evaluating lymphocyte viability under different storage conditions and time periods.

Result and Discussion: *According to the findings of our study, the survival rate of lymphocytes was higher under storage conditions at -80°C. Additionally, it can be stated that storing lymphocytes at -80°C with a cryopreservation solution (10% DMSO) provides a more suitable strategy for preserving lymphocyte integrity.*

Keywords: *DNA damage, lymphocyte, storage conditions, viability*

GİRİŞ

Kanser gibi kronik hastalıkların toksikolojik yönden değerlendirilmesinde epidemiyolojik ve *in vitro* araştırmalar önemli bir yer tutmaktadır. Bu araştırma türlerinde biyolojik örnekler kullanılmaktadır. En çok kullanılan ve tercih edilen biyolojik örnek ise insan kanıdır. İnsan kanı venöz kandan kolaylıkla alınmakta ve tam kandan izole edilen lenfositler insan biyoizlem çalışmalarında sıklıkla kullanılmaktadır. Biyoizlem çalışmalarında organizmanın oksidatif stres düzeyinin belirlenmesinde, enfeksiyonel hastalıklarda tedavi yöntemlerinin geliştirilmesinde, aşı geliştirme çalışmalarında lenfositlerin kullanılması, bu hücrelerin saklama koşulları ve saklama sürecindeki ve sonrasındaki adımların değerlendirilmesini, lenfositlerin kullanıldığı çalışmalardan elde edilen sonuçların doğru bir şekilde yorumlanmasıı açısından önemli hale getirmektedir [1,2].

Yapılacak olan çalışmanın amacına göre katılımcılardan alınan kan; tam kan, serum, plazma ve lenfosit şeklinde oda sıcaklığında, +4ºC'de veya -80ºC sıcaklıkta dondurularak saklanabilir [3]. Ancak bu hücrelerin çalışmanın kapsamına göre aynı gün toplanması ve aynı gün içerisinde saklanması her zaman mümkün olmamaktadır [4].

Tam kandan izole edilen lenfositlerin saklanmaları laboratuvarlar arası deneyler arası farklılığı azaltmak için tercih edilen bir prosedürdür. Ancak bu yöntem lenfositlerde yapısal ve/veya fonksiyonel önemli değişikliklere neden olabilmektedir [5]. Lenfositler saklama süresi boyunca apoptozis sürecine girebilmekte ve bu durum saklama süresine ve sıcaklık koşullarına bağlı olarak değişiklik gösterebilmektedir [3,4]. Lenfositlerin izolasyonundan itibaren başlayan saklama koşulları lenfosit DNA bütünlüğünde de olumsuz sonuçlar oluşturabilmektedir [1,6,7]. Saklama koşullarına bağlı olarak gelişebilecek etkiler DNA hasarının değerlendirildiği çalışmalarda, klinik araştırmalarda veya biyoizleme çalışmalarında sonuçların yanlış yorumlanmasına neden olmaktadır. Lenfositlerdeki DNA hasarının değerlendirilmesi organizmanın oksidatif stres düzeyi hakkında önemli bilgi sağlamaktadır. Ancak lenfositleri saklama sürecindeki izolasyon, kriyoprezervasyon, saklama, saklama sonrası hücreleri çözme ve yıkama işlemleri, saklama sonrasındaki DNA hasar seviyesinin saklama öncesindeki DNA hasar seviyesini tam olarak temsil etmemesi ile sonuçlanabilmektedir [1]. Ancak lenfositlerde saklama koşullarının DNA hasarı üzerine etkisinin değerlendirilmesi çalışmamızın kapsamı dışında olup, DNA hasarının öncesinde hücre canlılığının belirlenmesi amaçlanmıştır.

Farklı saklama koşullarının lenfositlerin hücre canlılığı ve proliferasyonu üzerine etkisi olduğu bilinmektedir [3,8,9,10]. Özellike uzun süreli saklama için kullanılan kriyoprezervasyon yönteminde hücrelerin donması sırasında meydana gelen buz kristallerinin neden olabileceği ekstra hücre hasarını önlemeye yönelik kullanılan dimetil sülfoksitin (DMSO) hücrelerin çözünmesi sırasında sitotoksisiteye neden olabileceği belirtilmiştir [11].

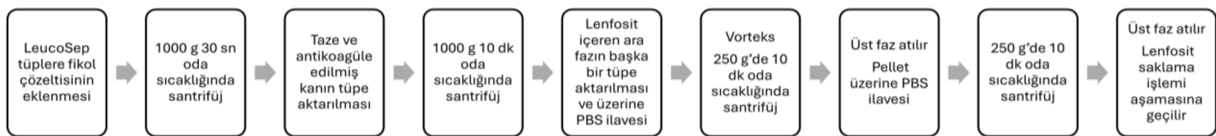
Tam kandan izole edilen lenfositlerin saklama koşullarının hücre sayısı ve canlılığı üzerine etkisi kanser araştırmaları, biyoizlem çalışmaları, enfeksiyonel hastalıklarda tedavi yöntemlerinin geliştirilmesi, aşı çalışmaları gibi önemli ve kapsamlı araştırmalarda sonuçların yanlış şekilde değerlendirilmesine ve DNA hasarının belirlendiği durumlarda da hücrelerin IC_{50} (inhibitör konsantrasyon) değerinin üzerinde olması gerektiği göz önünde bulundurularak çalışmamızda farklı sürelerde ve saklama koşulları altında lenfosit canlılıklarının değerlendirilmesi amaçlanmıştır.

GEREÇ VE YÖNTEM

34 yaşında sağlıklı ve ilaç kullanmayan 1 kadın gönüllüden toplam 10 ml kan örneği heparinli bir tüpe alınarak çalışmamıza başlanmıştır. 10 ml kanın 5 ml'si ile LeucoSep (Greiner bio-one, 12 ml) tüpler kullanılarak lenfosit izolasyonu yapılmıştır. Hücrelerin sayım işlemi TC20 Otomatik Hücre Sayım Cihazı (Bio-Rad TC20, Singapur) kullanılarak gerçekleştirilmiştir. Alınan kanın diğer 5 ml'si tam kan çalışmasında kullanılmıştır.

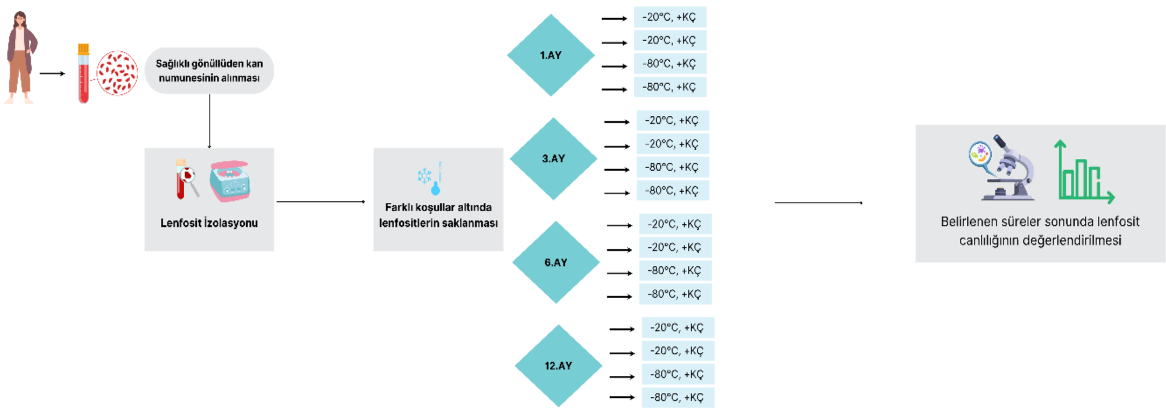
Lenfosit İzolasyonu ve Saklanması

Lenfositlerin izolasyon işlemi Şekil 1'de gösterilmiştir.



Şekil 1. Lenfosit izolasyon aşamaları

İzole lenfositler 1, 3, 6 ve 12 aylık süreler boyunca 2 farklı sıcaklık (-20°C ve -80°C) altında saklanmıştır. Ayrıca, her bir zaman aralığı için farklı sıcaklık koşullarında saklanan lenfositlerin bir kısmı %10 DMSO içeren kriyoprezervasyon çözeltisinde, diğer bir kısmı ise kriyoprezervasyon çözeltisi olmadan doğrudan saklanmıştır. Farklı koşulları altında saklanan lenfositlerin canlılığını değerlendirmek için izlenen süreç ve yöntem Şekil 2'de gösterilmiştir.

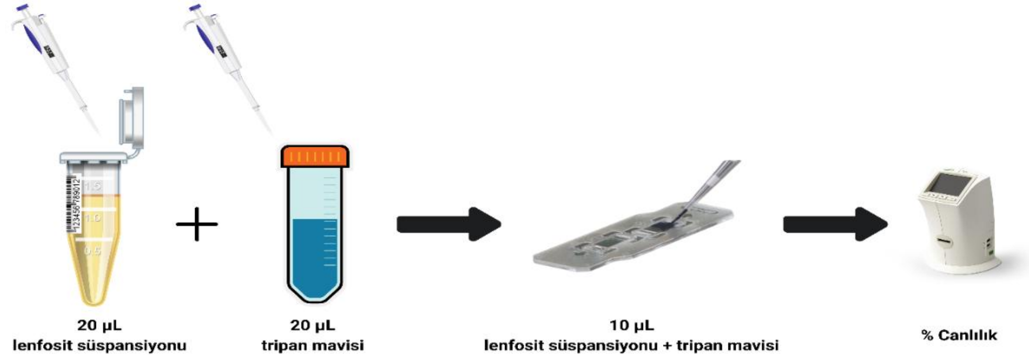


Şekil 2. Lenfosit canlılığının değerlendirilmesi için izlenen yöntem ve süreç (KÇ: %10 DMSO içeren kriyoprezervasyon çözeltisi). Sağlıklı gönüllüden alınan tam kandan lenfosit izolasyonu Şekil 1'e göre gerçekleştirilmiştir. Lenfositlerin saklanmasında farklı koşullar olarak süre (1, 3, 6 ve 12 ay), sıcaklık (-20°C ve -80°C) ve %10 DMSO içeren kriyoprezervasyon çözeltisi ve doğrudan saklama lenfosit canlılığının değerlendirilmesi için seçilen parametrelerdir

Hücre Canlılığının Belirlenmesi

Sitotoksisite test yöntemlerinden biri olan boyama yöntemi hücre canlılığını belirlemede uygulama kolaylığı ve hızlı sonuç vermesi açısından tercih edilen yöntemlerden biridir. Bu yöntem içerisinde tripan mavisi ile hücre canlılığının belirlenmesi, bir azo boyası olan tripan mavisinin hücre zarı bütünlüğü korunan canlı hücrelerin zarından geçememesi, zar bütünlüğünü bozulan hücreleri ise boyaması prensibine dayanmaktadır. Çalışmamızda lenfositlerin canlılığını belirlenen süreler sonunda değerlendirmek için bir sitotoksisite test yöntemi olan tripan mavisi ile boyama yöntemi kullanılmıştır.

Tripan mavisi yönteminde, 20 µl lenfosit süspansiyonu 20 µl tripan mavisi çözeltisi içerisine ilave edilmiştir. Hazırlanan tripan mavisi + lenfosit süspansiyonundan 10 µl alınarak hücre sayım lamı üzerine yayılmıştır. Hücre sayım lamı TC20 Otomatik Hücre Sayım Cihazına yerleştirildikten sonra % canlı hücre sayısı cihaz ekranında okunan değer ile belirlenmiştir (Şekil 3).



Şekil 3. Tripan mavisi yöntemi ile hücre canlılığının belirlenmesi. TC20 otomatik hücre sayım cihazı ile, cihaza özgü hücre sayım lamı yerleştirilerek hücre süspansiyonsiyonu içerisindeki % canlılık değerleri elde edilmiştir. Sayım cihazının ekranında 1 ml'deki toplam hücre sayısı, canlı hücre sayısı ve % canlılık verileri gösterilmektedir

İstatiksel Analiz

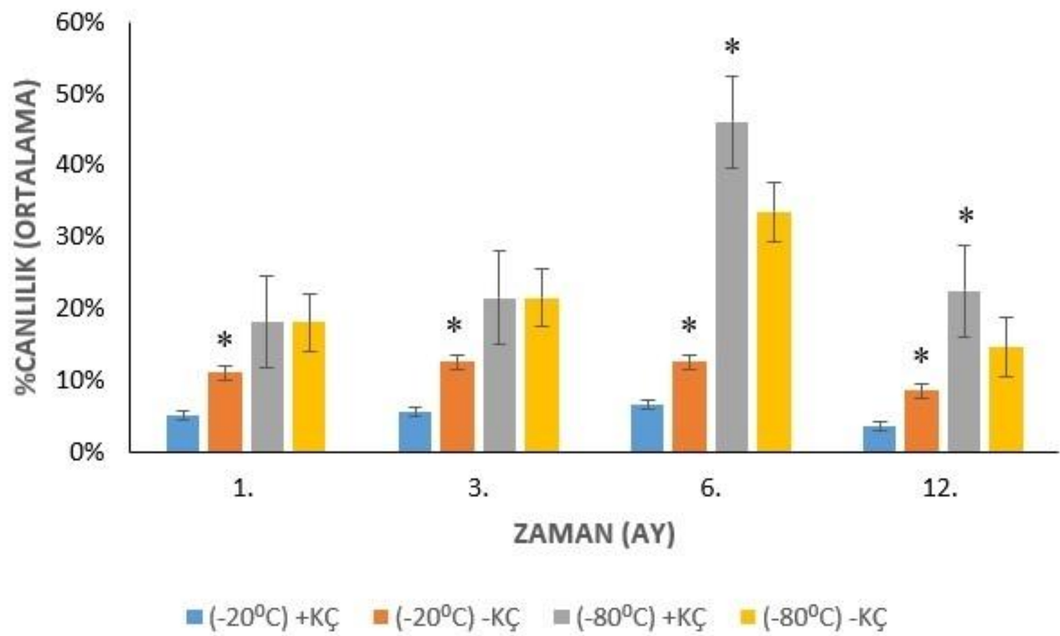
Çalışmamızda lenfositlerin canlılık değerleri bir sitotoksisite test yöntemi olan tripan mavisi yöntemi ile belirlenmiş ve istatiksel analizi IBM SPSS programı (SPSS programı (SPSS Windows Release 22.0) *t-test* kullanılarak yapılmıştır. Istatiksel olarak anlamlılık sınırı $p<0.05$ kabul edilmiştir.

SONUÇ VE TARTIŞMA

Tam kan ya da kan fraksiyonları (serum, plazma, lenfosit) popülasyon bazlı farklı çalışma amaçları için sıklıkla kullanılan biyolojik bir materyaldir. Kan/fraksiyonları yapılacak olan çalışmanın amacına göre farklı koşullar altında saklanmaktadır. Uygun bir saklama ortamı; analiz türü, amacı ve süresi gibi birçok faktöre bağlıdır [8]. Farklı saklama koşulları ve süreleri altında lenfositlerin canlılığının değerlendirilmesi ise planlanan çalışmanın amacına uygun sonuçların elde edilmesi ve yorumlanması açısından önem taşımaktadır. Ayrıca, lenfosit canlılıklarının değerlendirilmesi özellikle izole lenfositlerin bir yerden bir yere gönderilmesi ve saklanması ile ilgili prosedürlere katkı sağlaması açısından önemlidir [6].

Farklı saklama koşulları ve sürelerinde lenfosit canlılığını değerlendirdiğimiz çalışmamızdan elde ettiğimiz bulgulara göre, belirlenen her bir sürede lenfosit canlılığında azalma görülmüş ve bu azalma -20°C ve %10 DMSO içeren kriyoprezervasyon çözeltisi ile saklanan lenfositlerde daha yüksek bulunmuştur (Şekil 4). -20°C'de doğrudan saklanan lenfositlerin aynı sıcaklıkta kriyoprezervasyon çözeltisi içerisinde saklanan lenfositlere göre hücre canlılığı istatiksel olarak daha yüksek bulunmuştur ($p<0.05$; *t-test*). Her iki saklama koşulları altında 12. ayda lenfosit canlılığında azalma meydana gelmiştir (Şekil 4).

Şekil 4'te verilen zaman - % canlılık grafiğine göre -80°C sıcaklık altında %10 DMSO içeren kriyoprezervasyon çözeltisinde saklanan ve aynı sıcaklıkta bir kriyoprezervasyon çözeltisi olmadan saklanan lenfositlerin canlılıkları 1. ve 3. ayda eşit olarak bulunmuştur. 6. ve 12. ayda ise kriyoprezervasyon çözeltisi içerisinde saklanan lenfositlerin canlılık yüzdesi daha yüksek olarak tespit edilmiştir ($p<0.05$; t-test).



Şekil 4. Aylara ve saklama koşullarına göre lenfosit canlılığı grafiği. Mavi bar -20°C'de %10 DMSO içeren kriyoprezervasyon çözeltisinde saklanan lenfositleri, turuncu bar -20°C'de saklama çözeltisi olmadan doğrudan saklanan lenfositleri, gri bar -80°C'de %10 DMSO içeren kriyoprezervasyon çözeltisinde saklanan lenfositleri, sarı bar ise -80°C'de doğrudan saklanan lenfositlerin % canlılık değerlerini göstermektedir. * % canlılık değerleri istatiksel olarak anlamlı ($p<0.05$; t-test)

Konu ile ilişkin literatür çalışmaları tarandığında Belloni ve arkadaşlarının (2008) gerçekleştirdiği bir çalışmada artan sıcaklık ve saklama sürelerinde lenfosit canlılığında azalma meydana gelmiş ve saklama sürecinde lenfostilerin apoptozise uğradıkları belirtilmiştir. Yaptıkları çalışmada lenfosit canlılığının ve proliferasyonun saklama koşullarına bağlı olduğu gösterilmiştir. Hücrelerin stimülasyonunu sağlayan fitohemaglütinin kullanılması ve 96 saat boyunca +4ºC sıcaklıkta saklanması lenfosit canlılığının korunması için uygun bir koşul olduğu bildirilmiştir. Benzer şekilde, yapılan bir çalışmada, izole lenfositlerin 24 saate kadar olan kısa sürelerde oda sıcaklığında saklanabileceğini, daha uzun süreli çalışmalar için ise soğukta saklamananın tercih edilmesi gerektiği belirtilmiştir [8]. Belirtilen çalışmalarda artan sıcaklık ve saklama sürelerinde lenfosit canlılığında azalma meydana gelmesi bizim de çalışmamızdan elde ettiğimiz sonuç ile benzerlik göstermektedir. -20°C sıcaklık altında kriyoprezervasyon çözeltisi olsun ya da olmasın saklanan lenfositlerin canlılıkları -80°C'de saklananlara göre daha düşük bulunmuştur. Bir başka çalışmada lenfosit alt türlerinin (T-hücreleri, B-hücreleri ve doğal katil hücreler) canlılığı saklama sıcaklığı ve süresine göre değerlendirilmiştir. HIV pozitif hastalardan alınan kan örneklerinden elde edilen lenfosit alt türlerinin 21ºC sıcaklıkta önemli ölçüde canlılığını kaybettiği ve inflamasyon sürecinde görevli olan CD8+ ve CD3+ hücre canlılıklarında da azalma olduğu belirtilmiştir [12]. Radyasyonun neden olduğu kromozomal hasarın insan lenfosit hücreleri üzerinde doğru bir şekilde değerlendirilmesi için fitohemaglütinin varlığında 48 saat boyunca lenfositlerin +4°C'de saklanmasının, lenfositleri apoptozise karşı koruduğu belirtilerek, kromozomal hasarın doğru bir şekilde değerlendirilebileceği belirtilmiştir [7].

Çalışmamızdan elde edilen bulgulara göre lenfositlerin hayatta kalma oranı -80°C'deki saklama koşullarının test edilen tüm zaman aralıklarında istatistiksel olarak daha yüksek bulunmuştur ($p<0.05$; t-test) (Şekil 4). Saklama süresi uzadığında (6. ve 12. aylarda) -80°C'de saklanan numunelerin lenfositlerin kriyoprezervasyon çözeltisi ile (%10 DMSO) saklandığında istatistiksel olarak canlılıklarının daha yüksek olduğu bulunmuştur ($p<0.05$; t-test). Çalışmamızdan elde ettiğimiz tüm sonuçlar değerlendirildiğinde lenfositlerin bütünlüğünü korumak için -80°C'de saklamanın -20°C'ye göre daha uygun olduğu, -80°C'de de lenfositleri direkt saklamak yerine kriyoprezervasyon çözeltisi ile

saklamanın lenfosit bütünlüğünün daha uzun süre korunması için daha uygun bir strateji sağladığı söylenebilir.

Saklama süresi ve koşullarına göre lenfosit canlılığının değerlendirildiği literatür çalışmaları az sayıda olup, farklı saklama koşulları altında konu ile ilgili yapılacak olan daha fazla sayıda çalışmaya ihtiyaç duyulmaktadır. Bu sayede insan biyoizlem çalışmaları gibi birçok alanda en çok tercih edilen biyolojik materyallerden biri olan kan numunesinin ve fraksiyonlarının gerçekleştirilecek olan çalışmanın amacına göre saklanması, depolanması, transfer edilmesi gibi süreçlerine katkı sağlayacaktır.

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

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REVIEW ARTICLE / DERLEME MAKALE

IMPACT OF PLANT METABOLITES IN GLUCOSE METABOLISM FOR REGULATION OF BLOOD GLUCOSE LEVEL IN DIABETES: AN EXCLUSIVE UPDATE

DİYABETTE KAN GLUKOZ SEVİYESİNİN DÜZENLENMESİ İÇİN GLUKOZ METABOLİZMASINDA BİTKİ METABOLİTLERİNİN ETKİSİ: ÖZEL GÜNCELLEME

Pulak MAJUMDER[1]*, Amrutanand TRIPATHY[1], Swathi K B[1]

[1]Adichunchanagiri University, Faculty of Pharmacy- Sri Adichunchanagiri College of Pharmacy, Department of Pharmacognosy, 571448, Mandya, Karnataka, India

ABSTRACT

Objective: *To explore the molecular targets of plant chemicals and their role in regulating hyperglycemia, providing insights into potential strategies for developing new treatment for this condition.*
Result and Discussion: *Understanding the biochemical pathways involved in sugar regulation is crucial for developing treatments for conditions such as hyperglycemia. Phytochemicals derived from plants have shown promise in regulating blood sugar levels through various molecular targets. By targeting specific enzymes and pathways involved in glucose metabolism, these phytochemicals offer potential therapeutic benefits for managing hyperglycemia. Plant chemicals have demonstrated the ability to influence key enzymes and pathways in glucose metabolism. Phytochemicals have been found to modulate glycolysis, the Krebs cycle, and gluconeogenesis, offering the potential for regulating blood sugar levels. Additionally, these plant extracts have shown effects on processes such as cholesterol synthesis, glycogen synthesis and degradation, carbohydrate metabolism and absorption, as well as insulin production and release. The diverse impact of these medicinal plants on multiple physiological processes highlights their potential to address hyperglycemia through a multi-faceted approach. In this review, we will further explore the molecular targets and mechanisms of action of these plant chemicals, which can provide valuable insights for developing novel treatments for hyperglycemia.*
Keywords: *Biochemical pathways, diabetes, medicinal plants, phytochemicals*

ÖZ

Amaç: *Bu makalenin amacı, bitki kimyasallarının moleküler hedeflerini ve hiperglisemiyi düzenlemedeki rollerini araştırmak, bu durum için yeni tedaviler geliştirmeye yönelik potansiyel stratejiler hakkında fikir vermektir.*
Sonuç ve Tartışma: *Şeker regülasyonunda yer alan biyokimyasal yolların anlaşılması, hiperglisemi gibi durumlara yönelik tedavilerin geliştirilmesi açısından çok önemlidir. Bitkilerden elde edilen fitokimyasallar, çeşitli moleküler hedefler aracılığıyla kan şekeri seviyelerinin düzenlenmesinde umut vaat etmektedir. Glikoz metabolizmasında yer alan spesifik enzimleri ve yolakları hedef alan bu fitokimyasallar, hipergliseminin yönetilmesinde potansiyel terapötik faydalar sunar. Bitki kimyasalları, glikoz metabolizmasında yer alan anahtar enzimleri ve yolakları etkileme yeteneğini göstermiştir. Fitokimyasalların glikolizi, Krebs döngüsünü ve glukoneogenezi*

* **Corresponding Author / Sorumlu Yazar:** Pulak Majumder
e-mail / e-posta: pulakmajumder@accp.co.in, **Phone / Tel.:** +07829807212

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modüle ettiği ve kan şekeri seviyelerini düzenleme potansiyeli sunduğu bulunmuştur. Ek olarak, bu bitki özlerinin kolesterol sentezi, glikojen sentezi ve bozulması, karbonhidrat metabolizması ve emiliminin yanı sıra insülin üretimi ve salınımı gibi süreçler üzerinde de etkileri olduğu gösterilmiştir. Bu şifalı bitkilerin çoklu fizyolojik süreçler üzerindeki farklı etkisi, bunların hiperglisemiyi çok yönlü bir yaklaşımla ele alma potansiyelini vurgulamaktadır. Bu derlemede, bu bitki kimyasallarının moleküler hedeflerinin ve etki mekanizmalarının daha fazla araştırılması, hiperglisemiye yönelik yeni tedavilerin geliştirilmesi için değerli bilgiler sağlayabilir.
Anahtar Kelimeler: *Biyokimyasal yolaklar, diyabet, fitokimyasallar, tıbbi bitkiler*

INTRODUCTION

In the Indian traditional system Ayurveda, "Madhumeha" or diabetes mellitus, was extensively described in classical literatures such as "*Charaka-Samhita*," "*Sushruta-Samhita*," and "*Bhrigu-Samhita*". Herbal remedies for diabetes are described in the "*Shushruta-Samhita*," along with the distinctions between acquired and inherited types of the disease [1].

Ethnobotanically, more than 1200 plant species have been found to have hypoglycemic properties. Due to their various beneficial medicinal qualities conferred by various plant parts as well as the presence of host of multi-componential compounds, medicinal plants are of great interest to contemporary medicine for the treatment of hypoglycemia and hyperglycemia. All those herbs are enriched with numerous phytochemicals, including alkaloids, glycosides, flavonoids, polysaccharides, hypo glycans, guanidine, steroids, carbohydrates, terpenoids, amino acids, inorganic ions, saponins, etc. All those secondary metabolites play an active role in the regulation of blood sugar levels in diabetic conditions, either directly or indirectly [1].

This overview, aims to pinpoint the metabolic pathways that phytochemicals most likely influence to determine a patient's blood sugar level. A thorough literature search was carried out to find pertinent research publications in the English language. We searched PubMed, Science Direct, and Google Scholar, three electronic databases, for publications published between 2000 and 2024. More or less all published data are likely to have a superficial approach to the mechanism of action of hyperglycemia, but accurate mechanisms still need to be uncovered. Hence, this review will provide a comprehensive idea about the mechanism of medicinal plants in diabetes management.

Influence of Phytochemicals on Glucose Regulation in the Body

Both simple and complex sugars belong to the family of carbohydrates. Simple sugars include glucose and fructose, whereas complex sugars include cellulose, starch, and glycogen. Multi-monosaccharide molecules combine to form complex sugars, also known as polysaccharides. Polysaccharides function as structural elements and energy storage molecules, i.e., starch and glycogen.

Blood glucose levels are regulated by biochemical metabolic processes such as glycolysis, gluconeogenesis, glycogenesis, and glycogenolysis, wherein the glucokinase (GK) enzyme is a key player in maintaining glucose homeostasis. Any element that alters the above-stated metabolic processes is damaging.

Glycolysis and Kreb's Cycle

Glycolysis is the primary biological pathway in the human body where glucose is converted into pyruvic acid by the oxidation process and starts energy production for the cell. The entire process of conversion is catalyzed or regulated by various enzymes like hexokinase, phosphofructokinase, pyruvate kinase, etc. [2]. The central pathway of energy production is known as the Krebs cycle. The pyruvate produced after glycolysis, is further oxidized into carbon dioxide and water via Acetyl-CoA with the production of energy and NADH, which further produces ATP through the electron transport chain. This cycle is catalyzed or regulated by various enzymes, like succinate dehydrogenase and malate synthase [2]. Various herbs have been reported to control glycolysis and the Krebs cycle by regulating various catalysts through their phytochemicals. The descriptions of their targeted sites are indicated in Figure 1 and 2 and the reported plants with this regulatory activity and their indicated dose have been listed in the Table 1 and 2, respectively.

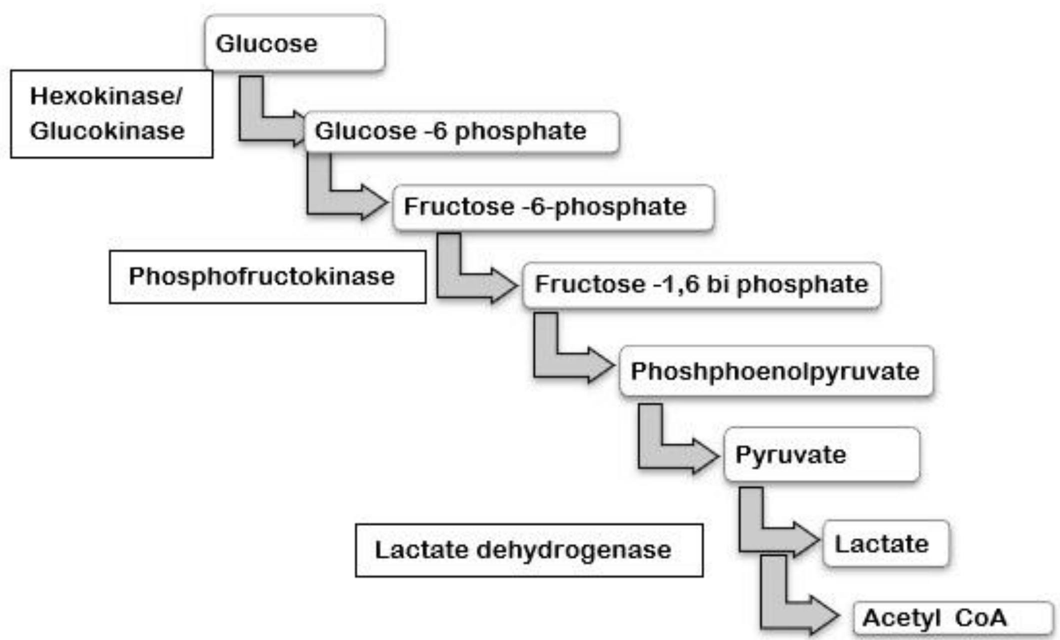

Figure 1. Metabolic pathway of Glycolysis

Table 1. Medicinal plants regulate Glycolysis by activating the enzymes lactate dehydrogenase

Medicinal plant	Family	Major chemical constituents	Dose	Reference
***Aegle marmelos* (L.) Correa**	Rutaceae	Aegelin, β & ϒ-Sitosterol, Marmelosin, Marmesin	1g/kg/day	3,4
Allium cepa L. var. aggregatum Don.	Liliaceae	S-methyl cysteine sulfoxide, S-allyl cysteine sulfoxide.	100-200 mg/kg	5,6
Allium sativum L.	Alliaceae	Allicin, Apigenin, Alliin, S-allyl cysteine sulfoxide.	200 mg/kg	7,8
***Curcuma longa* L.**	Zingiberaceae	Curcumin, Turmerone, β -Sitosterol, Zingiberene.	0.08 g/kg	9,10
***Trigonella foenum-graecum* L.**	Leguminosae	Trigonelline, Choline, Galactomannan	1g/kg	11,12
***Piper betle* L.**	Piperaceae	β-phenol, Chavicol, Cadinene.	75 mg/kg	13
***Mucuna pruriens* (L.) DC.**	Fabaceae	Mucunine, Mucunadine, β-Sitosterol, Mucuadinine.	200 mg/kg /day	14
***Eugenia jambolana* Lam.**	Myrtaceae	Malic acid, Gallic acid, Oxalic acid, Tannins	100 mg/kg	15,16
***Momordica charantia* L.**	Cucurbitaceae	Charantin, Momordicoside	200mg/kg /day	17,18,19, 20
***Panax quinquefolius* L.**	Araliaceae	Quinquenoside L3 & L9, Vina-Ginsenoside R3.	300mg/kg/day	21

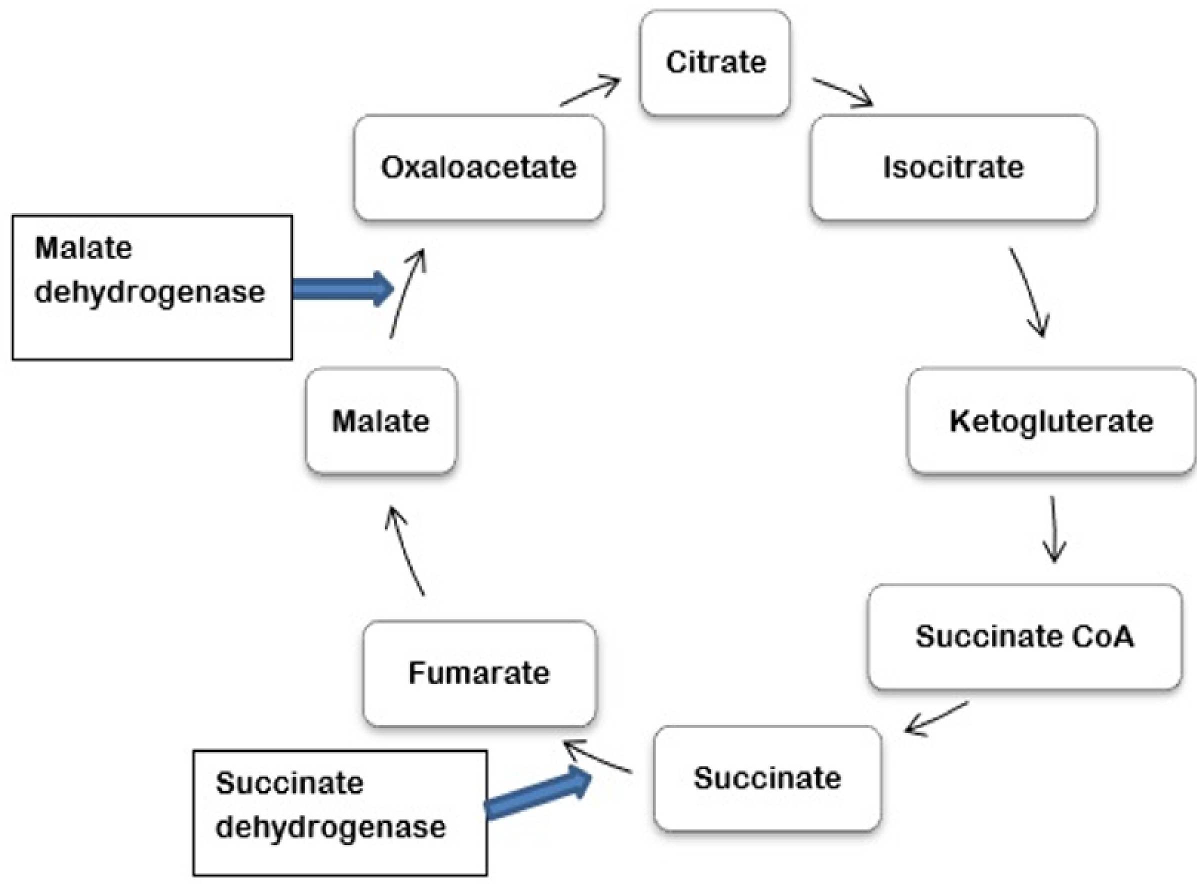

Figure 2. Metabolic pathway of Kreb's cycle

Table 2. Medicinal Plants regulate Kreb's cycle by activating the enzymes Malate dehydrogenase and Succinate dehydrogenase

Medicinal plant	Family	Major chemical constituents	Reported dose	Reference
***Aegle marmelos.* (L.) Corrêa**	Rutaceae	Aegelin, β & ϒ-Sitosterol, Marmelosin, Marmesin	1 g/kg/day	3,4
***Catharanthus roseus* (L.) G. Don**	Apocyanaceae	Vinblastine, Vineristine, Vinine, Vincamine, Alstonine.	500 mg/kg	22,23
***Panax quinquefolius* L.**	Araliaceae	Ginsenosides, peptides, polyacetylenic alcohols, fatty acids	15mg/kg	24

Gluconeogenesis

The generation of glucose from non-sugar carbonated substrates, i.e., pyruvate, lactate, glycerol, and glucogenic amino acids, etc., by the biological pathway known as gluconeogenesis (Figure 3). In this process, the four enzymes catalyze the entire process, i.e., pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose-1, 6-bisphosphatase and glucose-6-phosphatase. Those catalysts are inhibited by various plant phytochemicals, and as a result, glucose formation is altered. Table 3 has a list of medicinal plants that influence or regulate gluconeogenesis.

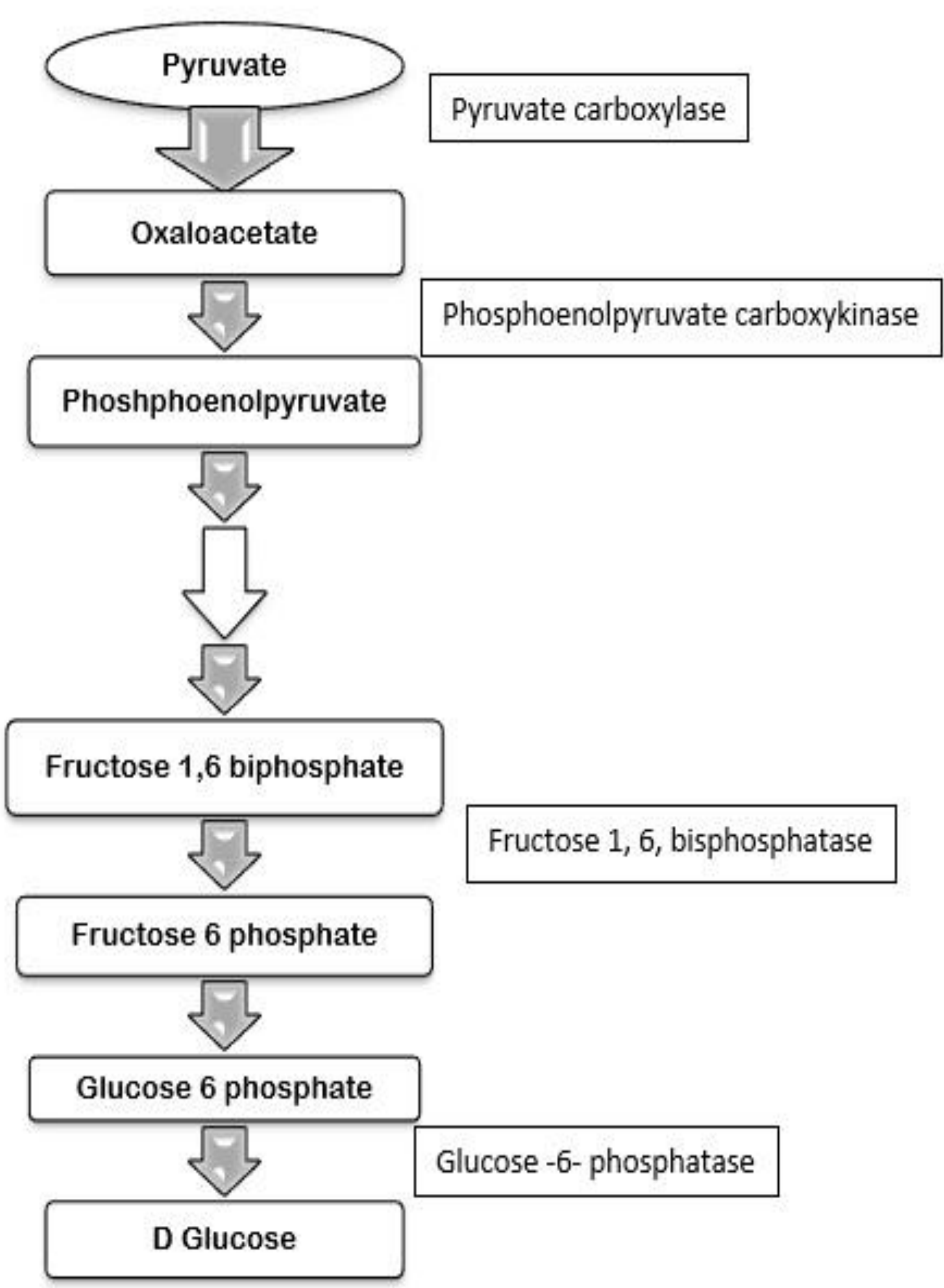

Figure 3. Metabolic pathway of gluconeogenesis

Table 3. Medicinal plants that gegulate gluconeogenesis

Medicinal plant	Family	Major chemical constituents	Reported dose	Reference
Inhibits phosphoenol pyruvate carboxykinase enzyme				
Eucommia ulmoides var.	Eucommiaceae	Isoquercitrin, Quercetin3-O-α-l arabinopyranosyl -(1, 2)-β-d glucopyranoside, Astragalin.	0.187g/ 100g std. diet	25
***Gongronema latifolium* Benth.**	Asclepiadaceae	Phytate, anthranoids, anthraquinones, cyanogenic glycoside, glycides, phblobatannins, hydroxymethyl anthraquinones, polyphenols.	100 mg/Kg	26
***Ocimum sanctum* Linn.**	Lamiaceae	Eugenol, Carvacrol, Linalool, Caryophylline, β-Sitosterol.		27,28
***Panax quinquefolius* L.**	Araliaceae	Quinquenoside L3 & L9, Vina-Ginsenoside R3.	200 mg/kg/day	29
Syzygium aromaticum (L.) Merr. & L.M.Perry	Myrtaceae	Isoflavones	50 mg/kg	30,31
***Hericium erinaceus* (Persoon). (Fungi)**	Hericiaceae	hericenones, erinacines, hericerins, erinarols, 2,6-diethylpyrazine, 2-methyl-3-furanthiol, corallocin	100 mg/kg	32
Inhibits fructose-1, 6-bisphosphatase				
***Aegle marmelos.* (L.) Corrêa**	Rutaceae	Aegelin, β & ϒ-Sitosterol, Marmelosin, Marmesin	1 g/kg/day	3,4
***Casearia esculenta* Roxb.**	Flacourtaceae	Leucopelargonidin, Dulcitol, Beta sitosterole.	250 mg/kg	33
***Eclipta alba* (L.) Hassk.**	Asteraceae	Wedelolactone, Demethyl wedelolactone, Eclipticine.	250 mg/ kg	34
***Murraya koenigii* (L.) Spreng.**	Rutaceae	3-carene, caryophyllene, thujene, allyl(methoxy)dimethyl silane, myrcene, terpinene	80 mg/kg /day	35,36
***Ocimum sanctum* Linn.**	Lamiaceae	Eugenol, Carvacrol, Linalool, Caryophylline, β-Sitosterol.		37,38
***Piper betle* L.**	Piperaceae	β-phenol, Chavicol, Cadinene	75 mg/kg	39
***Trigonella foenum-graecum* L.**	Leguminosae	Trigonelline, Choline, Galactomannan	1g/kg	40,41
***Coccinia grandis* (L.) Voigt**	Cucurbitaceae)	Taraxerone, Taraxerol	2 gm/kg	42,43
Inhibits glucose-6- phosphatase				
***Aegle marmelos.* (L.) Corrêa**	Rutaceae	Aegelin, β & ϒ-Sitosterol, Marmelosin, Marmesin	1g/kg/day	3,4
***Allium sativum* L.**	Alliaceae	Allicin, Apigenin, Alliin, S-allyl cysteine sulfoxide.	200 mg/kg	7,8
***Piper betle* L.**	Piperaceae	β-phenol, Chavicol, Cadinene.	75 mg/kg	13
***Eugenia jambolana* Lam.**	Myrtaceae	Malic acid, Gallic acid, Oxalic acid, Tannins	100 mg/kg	15,16
***Momordica charantia* L.**	Cucurbitaceae	Charantin, Momordicoside	200mg/kg /day	17,18,19,20
***Panax quinquefolius* L.**	Araliaceae	Quinquenoside L3 & L9, Vina-Ginsenoside R3.		44
Coccinia indica	Cucurbitaceae	Taraxerone, Taraxerol	2g/kg	45

Hexose Monophosphate (HMP) Shunt

The glucose is oxidized by alternative pathways called HMP Shunt or Hexose Monophosphate Shunt other than glycolysis and Krebs cycle. In this pathway, with six Carbone structures, glucose produces five-carbon sugars. This entire process is catalyzed by an enzyme called glucose-6-phosphate dehydrogenase. The regulation of this enzyme is one of the vital parts of diabetic control. Numerous plants are found to be potent in this context of enzyme regulation (Table 3) with their secondary chemicals.

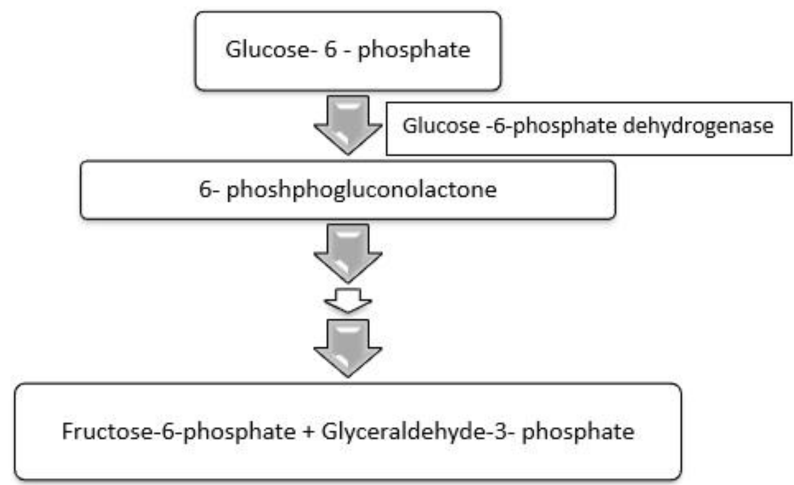

Figure 4. Metabolic pathway HMG shunt

Table 4. Medicinal plants that regulate HMG shunt

Medicinal plant	Family	Major chemical constituents	Reported dose	Reference
***Balanites roxburghii* Planch**	Balanitaceae	Sapogenin, Diosgenin, Yamogenin, β-sitosterol.	1.5g/kg	46
***Casearia esculenta* Roxb.**	Flacourtaceae	Resin, Sterol, Flavonoid.	300mg/kg	47
***Catharanthus roseus* (L.) G. Don**	Apocyanaceae	Vinblastine, Vineristine, Vinine, Vincamine, Alstonine.	500 mg/kg	48,49
***Dioscorea cayenensis* Lam.**	Dioscoreaceae	Hydro-Q chromene, tocopherol-9, 1-feruloylglycerol, dioscorin, cyanidine-3-glucoside, peonidin3-gentiobioside	200mg/kg	50,51
***Aconitum carmichaelii* Debx.**	Ranunculaceae	Songoramine, Hypaconitine, Karakanine, Songorine	12.5 - 50 mg/kg	33,34
Coccinia indica	Cucurbitaceae	Taraxerone, Taraxerol	2 g/kg	42,43
***Curcuma longa* L.**	Zingiberaceae	Curcumin, Turmerone, β-Sitosterol, Zingiberene.	0.08 g/kg	52,53
***Ocimum sanctum* L.**	Lamiaceae	Eugenol, Carvacrol, Linalool, Caryophylline, β-Sitosterol		54,55

Glycogen Synthesis

The role of glycogen synthase is to synthesize glycogen from unused glucose, which involves multiple steps in the liver.

The enzyme UDP-glucose pyrophosphorylase acts to activate glucose, which further undergoes the synthesis of glycogen (Figure 5). The improper conversion of glucose to glycogen leads to a rise in sugar levels in the blood in diabetic conditions. There are several plants that act on the enzyme glycogen synthase to regulate the blood sugar level in elevated conditions. Some reported findings are listed in Table 5.

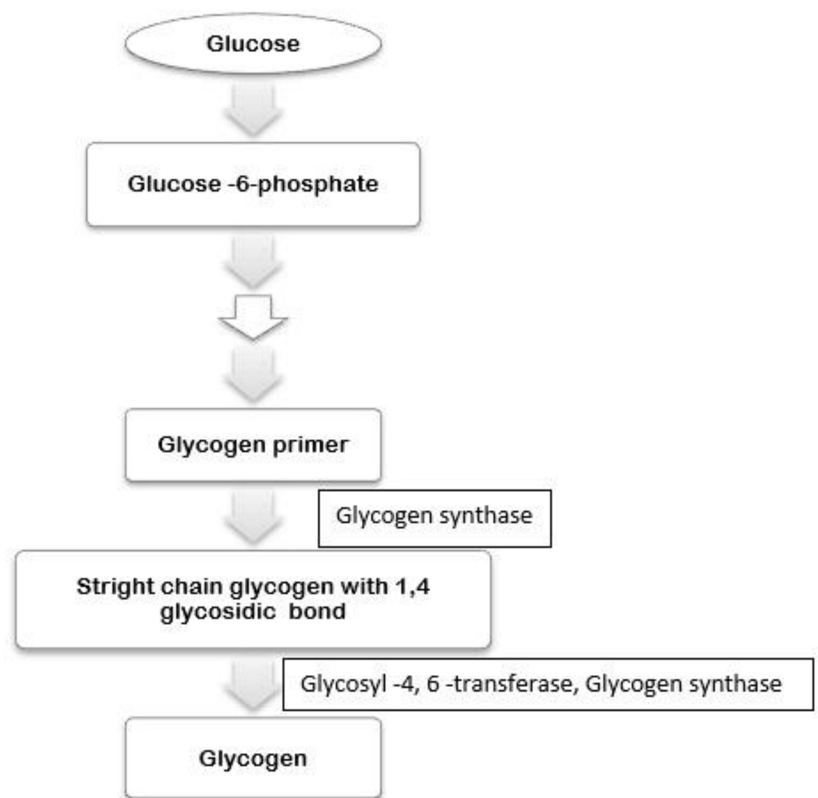

Figure 5. Metabolic pathway of glycogen synthesis

Table 5. Medicinal plants that regulate glycogen synthesis

Medicinal plant	Family	Major chemical constituents	Reported dose	Reference
Coccinia indica	Cucurbitaceae	Taraxerone, Taraxerol	2 g/kg	56,57
***Catharanthus roseus* (L.) G. Don**	Apocyanaceae	Vinblastine, Vineristine, Vinine, Vincamine, Alstonine.	500 mg/kg	22,58
***Ocimum sanctum* L.**	Lamiaceae	Eugenol, Carvacrol, Linalool, Caryophylline, β-Sitosterol.	**-**	27,28,59
***Piper betle* L.**	Piperaceae	β-phenol, Chavicol, Cadinene	75 mg/kg	39,60
***Curcuma longa* L.**	Zingiberaceae	Curcumin, Turmerone, β-Sitosterol, Zingiberene.	0.08 g/kg	9,10,61,62
***Momordica charantia* L.**	Cucurbitaceae	Charantin, Momordicoside.	200 mg/kg /day	18,19,63

Glycogenolysis

The process of glycogen degradation into glucose is known as glycogenolysis, which results in an increase in blood glucose levels in the body. The primary enzyme called Glycogen phosphorylase is responsible for the breakdown of glycogen in the body (Figure 6). Various plants have been reported

like *Aegle marmelos.* (L.) Corrêa, *Murraya koenigii* (L.) Spreng., *Ocimum sanctum* L., *Brassica juncea* L. etc. that can exhibit the regulation of glycogen degradation by inhibiting the enzyme Glycogen phosphorylase. Hence, the glucose levels decrease in the blood (Table 6). Even in some special cases where it was observed that the peripheral utilization of glucose or the process of glycogenolysis has been blocked by the plant extract like *Azadirachta indica* A. Juss [64].

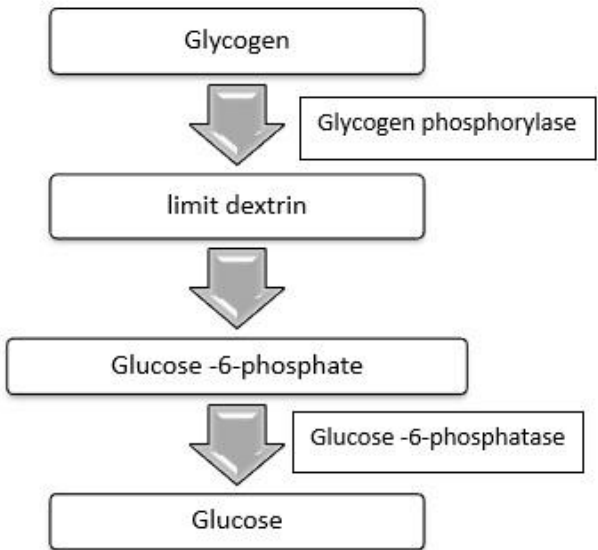

Figure 6. Metabolic pathway of glycogenolysis

Table 6. Medicinal plants that regulate glycogenolysis

Medicinal plant	Family	Major chemical constituents	Reported dose	Reference
***Murraya koenigii* (L.) Spreng.**	Rutaceae	Polyphenols and flavonoids	80 mg/kg /day	36,65,66
***Ocimum sanctum* L.**	Lamiaceae	Eugenol, Carvacrol, Linalool, Caryophylline, β-Sitosterol	-	27,28,67
***Aegle marmelos.* (L.) Corrêa**	Rutaceae	Aegelin, β & ϒ-Sitosterol, Marmelosin, Marmesin.	1g/kg/day	3,4,68

Dilatory Carbohydrate Digestion and Absorption

A lion's share of the energy supply in the body directly comes from the diet, especially from carbohydrate- enriched foods, through its metabolism. Generally, starch and sugar, i.e., sucrose, are the major chemical constituents in carbohydrates. In preliminarily, starch decomposition in the presence of a digestive enzyme called α-amylase, the starch is converted to oligosaccharides. This enzyme, α-amylase, is commonly found in the salivary glands and also in pancreatic juice. On the other hand, another membrane-bound enzyme, i.e., α-Glucosidase, is located in the small intestinal epithelial region. This enzyme actively catalyzed the process of converting glucose from disaccharides and oligosaccharides. Such converted glucose is absorbed through the walls of the intestine, reaching into the bloodstream and ultimately reaching the hepatic system (Figure 7). Eventually, various digestive enzymes will actively participate in the process of carbohydrate digestion. It includes α -Glucosidase, maltase, sucrase, α-amylase, lactase and isomaltase etc. Among all, α-amylase and α -Glucosidase play the most crucial role in carbohydrate metabolism. That is why herbs with these enzyme-inhibitory actions become the indirect choice for diabetes treatment. There are several medicinal plants reported to suppress this enzymatic activity during carbohydrate metabolism, even several plants are also been found (Table 7) which can decrease the absorption of carbohydrates through the deactivation of the

intestinal brush border cells. Currently, Miglitol and Acarbose are two commercially available synthetic drugs which inhibit the activity of these digestive enzymes in the body.

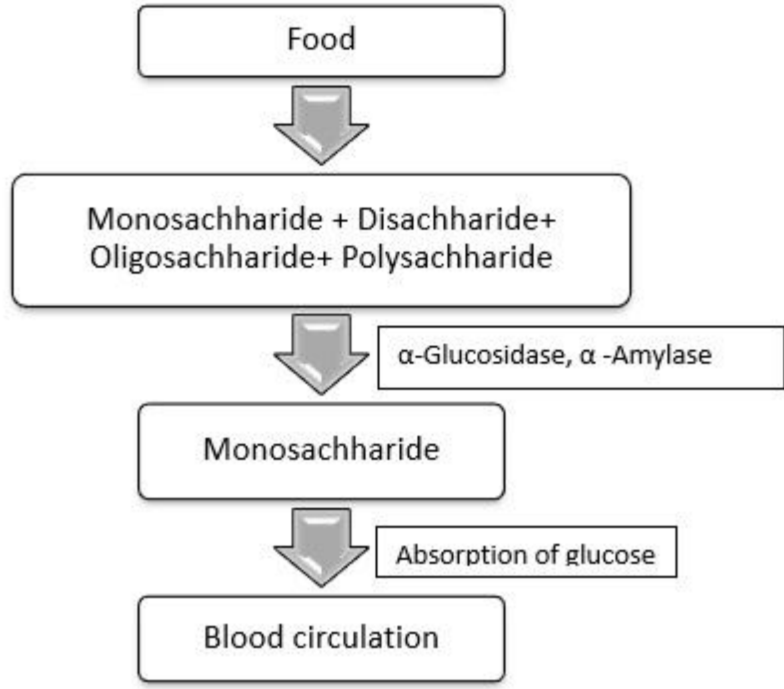

Figure 7. Metabolic pathway of carbohydrate

Table 7. Medicinal plants that regulate the inhibition of digestive enzyme and absorption of carbohydrate

Medicinal plant	Family	Major chemical constituents	Reported dose	Reference
Inhibition of α-glucosidase, α -amylase enzyme action				
***Artemisia pallens* Wall. ex-DC**	Compositae	T-Cadinol, α-urunene, β-Eudesmol, β -Ubebene.	100 mg/kg	69
***Morus alba* L.**	Moraceae	Isoquercitrin, Astragalin, Scopolin, Roseoside II.	200 mg/kg	70
***Salacia reticulata* Wight**	Celastraceae	Mangiferin, Salacinol, Kotalanol, Epigallocatechin	1 ml/day/rat	71
***Salacia oblonga* Wall. ex Wight & Arn.**	Celastraceae	Mangiferin, Salacinol, Kotalanol, Epigallocatechin	250 mg/kg	72
***Urtica dioica* L.**	Urticaceae	Quercetin, Kaempferol, Glucoquinone.	-	73
***Morus bombycis* Koidz.**	Moraceae	3-Epifagomine, Fagomine, Castanospermine	IC50 = 0.1 mg/ml	74
Decrease the absorption of glucose				
***Artemisia pallens* Well.**	Compositae	T-Cadinol, α-urunene, β-Eudesmol, β -Ubebene.	100 mg/kg	75
***Bauhinia variegata* L.**	Leguminosae	Astragalin, Kaempferitrin, Astragalin, Bauhinoside.	400 mg/kg	76,77

Natural Herbs with Insulin Mimetic Action

Typically, Insulin is a peptide hormone produced due to the sensitization of the islet of Langerhans of pancreatic beta cells for the regulation of glucose levels in the bloodstream by regulating the carbohydrate metabolism in the body. It is the main anabolic hormone in the body for the promotion of glucose uptake, stimulation of lipogenesis, diminished lipolysis, and increasing amino acid transport into the cells.

The mechanism of insulin release is a complex process (Figure 8). It involves the process of ATP-gated potassium channels closer and voltage-gated calcium channels activation for the release of insulin from cellular granules. A vast number of medicinal plants are reported for their action to alter insulin expression along with its synthesis and degradation (Table 8). Some plants also reported their action on Sulphonylurea binding site 1 (SUR1). Due to the cellular membrane depolarization, the Ca^{2+}influx takes place which leads to the close down of the ATP-sensitive potassium channel [78]. The medicinal plants directly act on the Ca^{2+} channels for insulin secretion and also those plants decrease the degradation by inhibiting insulinase enzyme are listed in Table 8**.**

There is a group of nuclear receptor transcription factors responsible for the Peroxisome proliferators and cellular metabolism of carbohydrates along with protein and lipids known as Peroxisome proliferator-activated receptors or (PPARs) [78]. There are three types of PPAR (Figure 8).

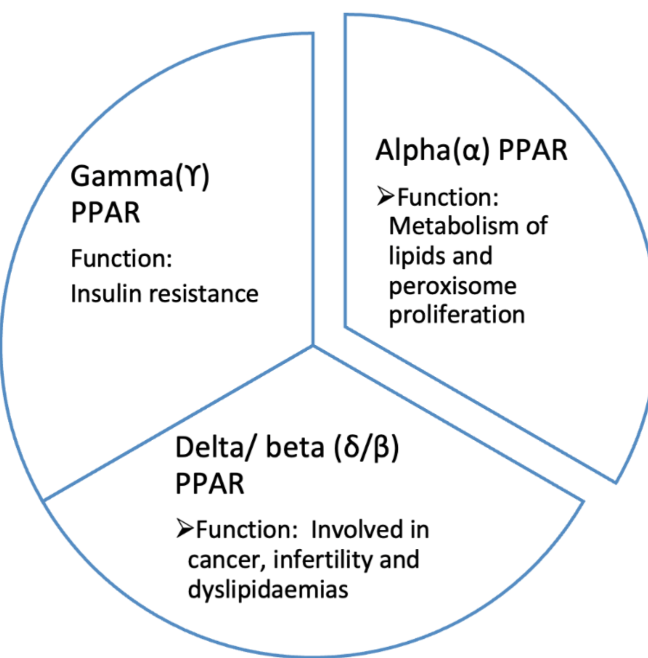

Figure 8. Types and functions of PPARs

Many medicinal herbs are reported for their elevated expression of PPAR gamma and decreased insulin resistance. For example, the phytoconstituents Curcumin from *Curcuma longa* L. and 6-gingerol from *Zingiber officinale* Roscoe are likely to target PPAR gamma. Even synthetic antidiabetic molecules like the Thiazolidinedione class of drugs, including roziglitazone, troglitazone and pioglitazone also target PPAR gamma.

The alteration of cellular signaling and metabolic process is also triggered by a messenger called cyclic AMP which naturally mediates the cellular network of signaling. It leads to suppress the intensity of insulin by decreasing the action of cAMP phosphodiesterase. Several medicinal plants which include *Betula alnoides* Buch.-Ham. ex D. Don, *Hiptage benghalensis* (L.) Kurz, *Leea indica* (Burm. f.) Merr.

and *Senna surattensis* (Burm. f.) H.S. Irwin & Barneby [79] possess cAMP phosphodiesterase inhibitory activity, as a result of which the action of insulin is retained**.**

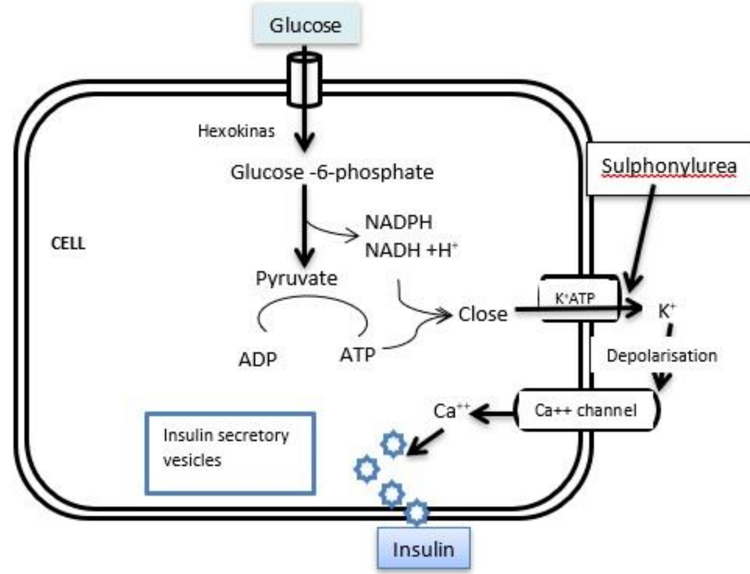

Figure 9. Exocytosis mechanism of insulin and sulfonylurea

Table 8. Medicinal plants used to increase insulin secretion

Medicinal plant	Family	Major chemical constituents	Reported dose	Reference
***Aloe barbadensis* miller.**	Aloaceae	Isobarbalin, Aloe-emodin, Aloetic acid, Barbaloin	500 mg/kg	80
***Caesalpinia bonduc* (L.) Roxb.**	Caesalpiniaceae	Bargenin, Caeselpinine A, α & β-Amyrin, Lupeol.	30 mg/kg	81
***Agaricus campestris* L.**	Agaricaceae	α -Terpineol, Hexanol, Furfural, Captylic acid	1 mg/mL	82
***Acacia arabica* Willd. var. indica Benth.**	Mimosaceae	m-Digallic acid, Chlorogenic acid, (+)- Catechin	4 g/kg	83,84
***Abelmoschus moschatus* Medik.**	Malvaceae	β -Sitosterol, Ambrettelide, Myricetin-3-glucoside.	1.0 mg/kg	85
***Helicteres isora* L.**	Sterculiaceae	Cucurbitacin B, Isocucurbitacin B.	100 mg/kg	86
***Musa sapientum* L.**	Musaceae	2-heptyl acetate, 2-methylbutyl acetate	150 mg/kg	87
***Swertia chirayita* (Roxb.) H. Karst.**	Gentianaceae	Amarogentin, Swerchirin, Chirantin, Gentiopicrin	100 mg/kg	88
***Catharanthus roseus* (L.) G. Don**	Apocyanaceae	Vinblastine, Vincristine, Reserpine, Vinceine	500 mg/kg	89,90
***Xanthocercis zambesiaca* (Baker) Dumaz-le-Grand.**	Leguminoceae	Castanospermine, Fagomine, Epifagomine, α-Homono jirimycin, Deoxynojirimycin	50 mg/ml	91
Potentiates insulin action by inactivating insulinase enzyme				
***Arctostaphylos uva-ursi* (L.) Spreng.**	Ericaceae	Arbutin, Ericolin, Ellagic acid, Myricetin,Ursone.	6.25% by weight	92
***Ocimum canum* Sims**	Lamiaceae	Camphor, Eugenol, Juvocimene I & II, Trans ß-ocimene, Linalool.	0.03 mg/ml	93

Herbs Helps in the Effective Transportation of Glucose Transporters Glut (GLUT)

The cytoplasmic vesicles include glucose transporters (GLUT), which aid in the movement of glucose into and out of cells. GLUT travels to the membrane in response to glucose stimulation and carries out its necessary activity. In the instance of DM, GLUT is not transported to the plasma membrane (Figure 9). GLUT is effectively transported to the plasma membrane by a group of medicinal plants, including *Syzygium cumini* L. Skeels, *Aegle marmelos.* (L.) Corrêa, *Allium sativum* L. [94-96], *Canna indica* L. [97], *Lagerstroemia speciosa* (L.) Pers. [98], and *Cornus officinalis* Siebold. & Zucc. [99]. This allows glucose to be transported into the cells and its concentration in the blood decreases (Table 9).

Table 9. Phytochemicals and their glucose uptake inhibition potentials

Phytochemicals	Mechanisms of action	References
Cyanidin	↓ glucose uptake in monoblast U937 cell lines	100
Genistein	↓ glucose uptake monoblast U937 cell lines and binds on GLUT1 surface.	101
Apigenin	↓ glucose uptake monoblast U937 cell lines and MC3T3-G2/PA6 cells and inhibits activation of GLUT4.	102
Daidzein	↓ glucose uptake in U937 cell lines.	103
Fisetin	↓ glucose uptake in U937 and MC3T3-G2/PA6 cells	104
Catechin	↓ glucose uptake in monoblast U937 cell lines.	105
Hesperetin	↓ glucose uptake in human myelocytic cell lines.	106
(−)-Epigallocatechin gallate	↓ insulin-stimulated glucose uptake in MC3T3-G2/PA6 cell lines (mouse).	107
Kaempferol	↓ insulin-stimulated glucose uptake in mouse MC3T3-G2/PA6 cells and inhibits activation of Akt and translocation of GLUT4	108
Naringenin	↓ glucose uptake in human U937 cells	109,110
Silybin	↓ insulin-stimulated glucose uptake in mouse MC3T3-G2/PA6 cells	111,112
Myricetin	↓ glucose uptake in human U937 cell line.	113
Quercetin	↓ glucose uptake in U937 and MC3T3-G2/PA6 cell lines and GLUT4.	114,104
Luteolin	↓ insulin-stimulated phosphorylation of IR-β, and translocation of GLUT4.	115

Various classes of phytochemicals also been reported as potent glucose uptake inhibitors such as hesperetin (*Citrus aurantium* L.), catechin (*Camellia sinensis* (L.) Kuntze), cyanidin (*Viburnum rafinesquianum* J.A. Schultes), myricetin (tomatoes, oranges), genistein (*Genista tinctoria* L.), quercetin, apigenin, daidzein (soy phytoestrogens) etc. few phytochemicals name and their effect are listed in Table 9.

RESULT AND DISCUSSION

Many plants are known to decrease the synthesis of glucose, increase its consumption, and address subsequent problems. Merely 1% of the approximately 25 billion plants have undergone pharmacological screening, with a small portion of those screenings being evaluated for diabetes [116]. As such, it makes sense to investigate herbal medication a choice of treatment for diabetes. Therefore, it is established that medicinal plants have the potential to be helpful in the treatment of diabetes and that phytochemicals play a significant role in diabetes management. The Presence of phytochemicals such as flavonoids and phenolic compounds in biological systems makes them strong antioxidants and scavengers. According to the research, diabetes mellitus causes free radicals to increase and may even lower the antioxidant capacity of cells, which can result in oxidative stress in both insulin-dependent and independent forms of the disease. For instance, the *Cassia auriculata L., Cinnamomum tamala* (Buch. -Ham.) T. Nees & Eberm. *Ficus benghalensis* L, *Mangifera indica* L., *Trichosanthes dioica*

Roxb. are the well-versed hypoglycemic plants acting on various pathways to control the blood sugar levels with their high antioxidant potentials [117].

Kinsenoside (*Anoectochilus roxburghii* (Wall.) Lindl) functions against oxidative stress and NO factor, as well as regulating antioxidant enzymes that scavenge free radicals and aid in the regeneration of injured β cells in the pancreas [118]. In addition to protecting against oxidative damage, bacosine (*Bacopa monnieri* (L.) Wettst.) functions similarly to insulin and may affect peripheral glucose consumption due to its antihyperglycemic action [119]. Several mechanisms are known to be involved in the action of berberine (*Berberis aristata* DC.), including insulin-mimetic activity, AMPK (5′ adenosine monophosphate-activated protein kinase)-induced improvement of insulin action, AMPK-dependent up-regulation of insulin receptor expression in reducing insulin resistance, and glycolysis [120]. Even though, both piceatannol and scirpusin B (*Callistemon rigidus*) exhibit significant repressive effects on α-amylase-related activities. Moreover, scirpusin B has antidiabetic activity via controlling α-amylase [121]. The most notable isolated antidiabetic compound chamaemeloside, 3-hydroxy-3-methylglutaric acid (*Chamaemelum nobile* (L.) All.) revealed hypoglycemic activity by slowing down digestion and lowering the rate of absorption of carbohydrates [122]. Most of the scientific reports, stated plants for their antidiabetic properties but the responsible phytoconstituent or constituents need to be better reported in broader pharmaceutical management of diabetes. To achieve greater success in this arena of diabetes, we need more focus on the area of identification and isolation of biomolecules in specified targets of glucose regulation such as 1.5-dideoxy-1.5-imino-D-sorbitol (DNJ) in *Morus alba* L. [123], shogaol, gingerol in *Zingiber officinale* Roscoe [124], ginsenoside in *Panax ginseng* Meyer [125], galactomannans, 4-hydroxyisoleucine in *Trigonella foenum-graecum* L. [126,127] etc. are been highlighted with great specifications for the purpose.

Targeting cellular and molecular pathways involved in carbohydrate metabolism has been demonstrated by plant metabolites such as alkaloids (broussonetine, radicamine, catharanthine, vindoline and vindolinine etc.), flavonoids (Rutin, Silymarin, Kaempferol, puerarin etc.), and saponins (Arjunolic Acid, Christinin-A, Senegasaponins A, B and C, Gymnemic Acid, and Platyconic Acid etc.). These substances have the ability to lessen oxidative stress, restore insulin signalling, shield pancreatic beta cells, and prevent the breakdown and absorption of carbohydrates [128]. Finding safe, natural, and effective antidiabetic drugs to supplement or improve existing diabetes treatment is the goal of this review, which aims to comprehend these processes.

This comprehensive study may lead the researchers to get equipped with the basic molecular mechanism of various plant chemicals towards the regulation of blood glucose level in blood. Further research is required to meet the demand for the development of pharmaceuticals and nutraceuticals derived from natural plant resources. To fully understand the pharmacological actions of herbal treatments used to treat diabetes mellitus, more study is obligatory.

Furthermore, few studies have looked at combination therapy, including prescription and naturally occurring plant-based medicines [129]. Without the proper identification of constituent/constituents acting on elevated blood sugar levels and knowledge upon their specific mechanistic pathways, the further advances in the field of diabetes management are quite inadequate. It is advisable to increase the number of clinical studies by using bigger populations in the diabetes segment for better therapeutic outcome. Overall, this review contributes in understanding how natural plant-based compounds can be incorporated in to nutraceuticals that can have significant effect on countering oxidative stress and subsequently into diabetes management strategies, alongside, supporting the development of new therapies that leverage the health benefits of plant metabolites.

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AUTHOR CONTRIBUTIONS

Concept: P.M.; Design: P.M.; Control: P.M.; Critical Review: P.M., A.T., S.K.B.; Literature Review: A.T., S.K.B.; Data Collection and Processing: P.M., A.T., S.K.B.; Analysis and/or Interpretation: P.M., A.T.; Literature Review: A.T., S.K.B.; Manuscript Writing: P.M.; Critical Review: P.M., A.T., S.K.B.; Other: A.T.

CONFLICT OF INTEREST

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

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REVIEW ARTICLE / DERLEME MAKALE

AN OVERVIEW OF THE CHEMICAL COMPOSITION, BIOLOGICAL ACTIVITIES AND TRADITIONAL USES OF GENUS *ALKANNA* TAUSCH

ALKANNA TAUSCH CİNSİNİN KİMYASAL BİLEŞİMİ, BİYOLOJİK AKTİVİTELERİ VE GELENEKSEL KULLANIMLARINA GENEL BİR BAKIŞ

Sadi YUSUFBEYOGLU[1,2], Ayşe BALDEMIR KILIÇ[1]*

[1]University of Health Sciences, Faculty of Gulhane Pharmacy, Department of Pharmaceutical Botany, 06018, Ankara, Turkey
[2]Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Botany, 06560, Ankara, Turkey

ABSTRACT

Objective: *This study summarizes some scientific studies conducted on the chemical contents and biological activities of the Alkanna genus, which is thought to be medically important and has various traditional uses among the public.*
Result and Discussion: *Alkanna spp. have been used among the public since ancient times for the treatment of dermatological disorders, jaundice, kidney stones and for its wound healing, antinociceptive, anti-inflammatory, antibacterial, antiseptic and astringent effects. It has been observed that there are important biological activity studies on the chemical contents and active ingredients of Alkanna spp. and effective results have been obtained. According to the literatüre data, the Alkanna spp. may be an important candidate for drug development potential in the future.*
Keywords: *Alkanna, biological activity, Boraginaceae, chemical content, traditional use*

ÖZ

Amaç: *Bu çalışmada, tıbbi açıdan önemli olduğu düşünülen ve halk arasında çeşitli geleneksel kullanımları bulunan Alkanna cinsinin kimyasal içerikleri ve biyolojik aktiviteleri üzerine yapılan bazı bilimsel çalışmalar özetlenmiştir.*
Sonuç ve Tartışma: *Alkanna türleri halk arasında eski çağlardan beri dermatolojik rahatsızlıklar, sarılık, böbrek taşı tedavisi ve yara iyileştirici, antinosiseptif, antiinflamatuar, antibakteriyel, antiseptik ve büzücü etkileri nedeniyle kullanılmaktadır. Alkanna türlerinin kimyasal içerikleri ve aktif bileşenleri üzerinde önemli biyolojik aktivite çalışmaları yapıldığı ve etkili sonuçlar elde edildiği gözlemlenmiştir. Literatür verilerine göre, Alkanna türleri gelecekte ilaç geliştirme potansiyeli için önemli bir aday olabilir.*
Anahtar Kelimeler: *Alkanna, biyolojik aktivite, Boraginaceae, geleneksel kullanım, kimyasal içerik*

INTRODUCTION

The use of traditional medicine has spread internationally and grown in popularity. Herbal medicine is the most popular in usage on the big scale [1]. Plants are a rich natural source of chemical compounds that exhibit biological activities. They serve as reservoirs and sources for a wide range of

* **Corresponding Author / Sorumlu Yazar:** Ayşe Baldemir Kılıç
e-mail / e-posta: aysebaldemir.kilic@sbu.edu.tr, **Phone / Tel.:** +903123046071

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secondary metabolites with therapeutic properties, such as alkaloids, flavonoids, tannins, and terpenoids [2,3]. Medicinal plants are still widely used by rural communities, with increasing popularity for treating or preventing various infections. The therapeutic efficacy of these products and their fewer side effects make them a good alternative source to synthetic drugs. Therefore, there is a great need for studies providing scientific data on the pharmacological and therapeutic effects of these traditional medicines [4-6].

The *Alkanna* genus has been used in traditional medicine since ancient times for its medicinal, pharmaceutical, and therapeutic properties. Additionally, plants from this genus have found applications in cosmetics, dye production, and food industries due to their bioactive compounds and natural pigments [7,8]. The genus has been cited as having great significance as a source of compounds that benefit health and are used for traditional medicine [3]. Especially, the root of the *Alkanna* is the most useful portion, and because of its intense red color, it is frequently used for cosmetic and beauty purposes. It has been demonstrated to cure skin damage beyond those uses; however, they may cause allergic and irritating reactions when applied topically. *Alkanna* root has been used for stomach ulcers, cancer, and diarrhea. In these situations, oral dosing was advised [4,5]. The root extract has also shown radical scavenging activity, which suggests it could have antiaging properties, however, in the scope there is very little clinical data available [9].

Alkanna root is used to treat and cure several ailments, according to the USDA (United States Department of Agriculture) National Nutrient [3]. Herein, information on the chemical content, various biological activity studies, and traditional uses of *Alkanna* spp., which are thought to have significant pharmaceutical potential, has been compiled. To date, such a comprehensive compilation study on *Alkanna* sp. has not been conducted. Chemical and biological activity studies on *Alkanna* spp. are generally summarized in the manuscript using the Web of Science, Mendeley, PubMed, and Scopus databases. In the literature, studies on traditional uses, chemical contents, and biological activities of *Alkanna* spp. and extracts have been compiled.

Distribution of *Alkanna* Tausch Genus

Alkanna Tausch, a Boraginaceae family member, commonly grows in the Mediterranean, Europe, and Western Asia and has spread worldwide to regions including the Caucasus, Iran, and Saudi Arabia. The genus is one of the large genera represented by approximately 60 spp. in the family [10] The genus Alkanna is represented by 36 spp. and 41 taxa in Türkiye. 30 of the *Alkanna* spp. are endemic, so it can be assumed that the Anatolian Peninsula is the gene center of the genus [11-13].

History and Traditional Uses of *Alkanna* spp.

Among the *Alkanna* spp., the use of *Alkanna tinctoria* Tausch as a red root dye dates back to 4000 BC. *Alkanna* spp. have a long history of usage in traditional Chinese medicine. The first known use of *Alkanna* roots was in the writings of the Greek physician and philosopher Hippocrates in the 4th and 5th century BC, who employed plant roots to heal ulcers [3,14]. In a study by Papagerogiou et al. (1999) alkannin was isolated for the first time from *A. tinctoria* root extracts and the wound-healing and antimicrobial properties of this extract were reported [12]. Greek physician Hippocrates also noted that the root of *A. tinctoria* could be used to treat various dermatological diseases . The botanist Theophrastus also indicated that it could be used as a medicine and dye. In addition, Dioscorides recommended *A. tinctoria* for treating multiple dermatological diseases [15]. Among the healing properties of the *Alkanna* spp., it has also been reported used in preparing traditional ointments and pastes for wound dressing. For example, Iranian folk medicine has mentioned its antinociceptive and anti-inflammatory activities for pain-related ailments. *A. tinctoria* has also been reported to be used ulcers, inflammatory wounds, jaundice, and kidney stones. It also has a deworming effect. Extracts of the root possess antibacterial, antiseptic, and astringent properties, so they are also used for varicose veins, bed sores, and itchy rashes [16,17]. Purple flowers of *Alkanna trichophila* var. *mardinensis* Hub. - Mor. are consumed as food in some regions of Türkiye [18]. In Table 1, different traditional uses of *Alkanna* spp. are mentioned in detail.

Table 1. Traditional uses of *Alkanna* sp.

Plant name	Plant part	Preparation	Method of application	Usage	Ref.
***Alkanna orientalis* Boiss.**	Root	Boiling with oil	External	*Tinea barbae* induced Dermatophyte	[19]
	Aerial part	Crushed, cooked with butter, and added beeswax		Wound healing Scar treatment Burn treatment Inflamed wounds Leg ulcer	
		Cooked with butter	External	Festering sore, ambustion	
		Boiled	External with pulp for one day	Ophthalmia	
	Leaves	Crushed	External	Ophthalmia	
		Decoction	Internal before breakfast	Asthma, Bronchitis, Stomach ailments	
***Alkanna cappadocica* Boıss. & Balansa**	Root	The roots are roasted in butter and made into an ointment	External	Wound healing	[16]
***Alkanna tinctoria* Boiss.**	Root	The roots are roasted in butter and made into an ointment	External	Kidney stone Bed sores Itching Rash	[6, 15, 20]
	Aerial parts	Cooking in oil Vinegar Decoction	Internal	Wound and burn treatment Inflamed wounds	
			External	Leprosy Antihelmintic Jaundice	
***Alkanna trichophila* Hub.-Mor.**	Base leaves, Flowers	Decoction	Internal	Anti-inflammatory	[21]
***Alkanna orientalis* (L.) Boiss.**	Root	Boiled	Internal	Anti-inflammatory	[22]
	Root	Boiled	External	Wounds Conjunctivitis Ambustion Scabies Earache	
	Aerial parts	Infusion	Internal	Diabetes Hypercholesteremia Analgesic Hemorrhoid	

Chemical Contents and Pharmacological Activities of *Alkanna* Sp.

Since ancient times, the genus *Alkanna* has been extensively known for its medical and pharmacological capabilities, based on its plant parts' chemical contents. The bark of the root, which contains dyeing chemicals, is the most essential portion of the plant [6,9,12,14,23]. Chemical and biological activity studies on *Alkanna* spp. are generally summarized in Figure 1. The chemical examination of these molecules revealed that the most prevalent components were aromatic diketones called naphthoquinones, also known as alkannins and shikonins. The double ring structure of these compounds, which give the deep red pigments, gives them their colour. These are lipophilic hydroxy

naphthoquinone red pigments that are widely employed in a variety of applications [9,12]. Various flavonoids, pyrrolizidine alkaloids, alkannin derivatives (isohexenylnaphtazarine), and fatty acids have been identified in phytochemical studies on the genus of *Alkanna* [24-27]. *Alkanna*'s roots and aerial parts were used as the primary materials for developing potentially effective extracts. Maceration and infusion techniques were employed for extraction, utilising solvents with varying polarities. The chemical contents of the extracts were analysed using HPLC, LC/MS GC/MS, and other spectrophotometric methods, identifying approximately 50 bioactive compounds, including phenolic acids, flavonoids, coumarins, and terpenes [8,28,29]. The chemical composition of *Alkanna* sp. is shown in Table 2.

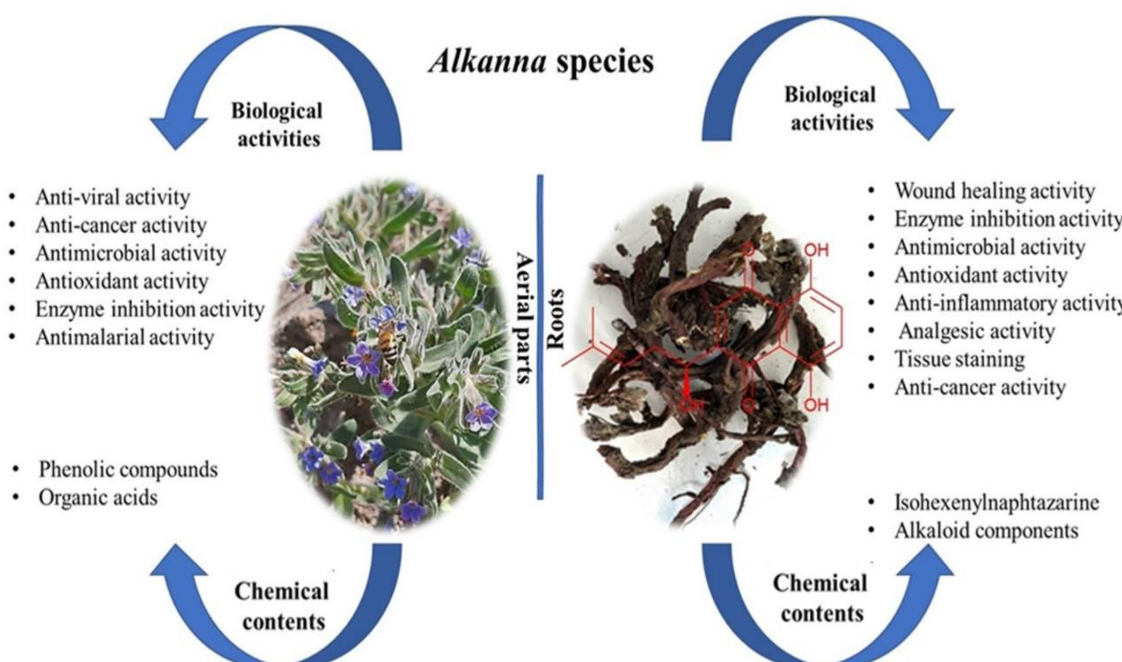

Figure 1. Highlights of chemical contents and biological activity studies of *Alkanna* spp.

Table 2. Chemical contents of *Alkanna* sp.

Plant name	Plant part	Active ingredient	Method	Extract	Ref.
***Alkanna corcyrensis* Hayek**	Root	Acetyl Alkannin Isobutyl Alkannin Angelic Alkannin ß-ß dimethylacryl Alkannin Isovaleryl - α-methyl -*n*-butyl Alkannin	HPLC-PDA-MS	Hexane extract	[23]
***Alkanna tinctoria* Tausch**	Root	Alkannin Acetyl Alkannin Isobutyl Alkannin Angelic Alkannin ß-ß dimethylacryl Alkannin Isovaleryl – α- methyl -*n*-butyl Alkannin	HPLC-PDA-MS	Hexane extract	

HPLC: High-Performance Liquid Chromatography; MPLC: Medium Pressure Liquid Chromatography; GLC: Gas-Liquid Chromatography; GC: Gas Chromatography; LC: Liquid chromatography; TLC: Thin-Layer Chromatography; VLC: Vacuum Liquid Chromatography; MS: Mass Spectrometry; H-NMR: Proton Nuclear Magnetic Resonance; DAD: Diode-Array Detection; FIB: Flame Ionization Detection PDA: Flame Ionization Detection

Table 2 ***(continue).*** Chemical contents of *Alkanna* sp.

Plant name	Plant part	Active ingredient	Method	Extract	Ref.
***Alkanna methanaea* Hausskn.**	Root	Alkannin Acetyl Alkannin Isobutyl Alkannin Angelic Alkannin ß-ß dimethylacryl Alkannin Isovaleryl - α -methyl -*n*-Butyl Alkannin	HPLC-PDA-MS	Hexane extract	[23]
***Alkanna orientalis* (L.) Boiss.**	Root	Alkannin Acetyl Alkannin Isobutyl Alkannin Angelic Alkannin ß-ß dimethylacryl Alkannin Isovaleryl - α -methyl -*n*-Butyl Alkannin	HPLC-PDA-MS	Hexane extract	
***Alkanna calliensis* Heldr. ex Boiss.**	Root	Alkannin Acetyl Alkannin Isobutyl Alkannin Angelic Alkannin ß-ß dimethylacryl Alkannin Isovaleryl – α- methyl -*n*-butyl Alkannin	HPLC-PDA-MS	Hexane extract	
***Alkanna graeca* Boiss. & Spruner**	Root	Alkannin Acetyl Alkannin Isobutyl Alkannin Angelic Alkannin ß-ß dimethylacryl Alkannin Isovaleryl - α methyl -n-Butyl Alkannin	HPLC-PDA-MS	Hexane extract	
***Alkanna primuliflora* Griseb.**	Root	Acetyl Alkannin Isobutyl Alkannin Angelic Alkannin ß-ß dimethylacryl Alkannin Isovaleryl - α methyl -n-Butyl Alkannin	HPLC-PDA-MS	Hexane extract	
***Alkanna stribrnyi* Velen.**	Root	Alkannin Acetyl Alkannin Isobutyl Alkannin Angelic Alkannin ß-ß dimethylacryl Alkannin Isovaleryl - α methyl -n-Butyl Alkannin Teracryalkannin	HPLC-PDA-MS	Hexane extract	

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Table 2 ***(continue).*** Chemical contents of *Alkanna* sp.

Plant name	Plant part	Active ingredient	Method	Extract	Ref.
***Alkanna sieberi* A. DC.**	Root	Alkannin Acetyl Alkannin Isobutyl Alkannin Angelic Alkannin ß-ß dimethylacryl Alkannin Isovaleryl - α methyl -n-Butyl Alkannin Teracryalkannin	HPLC-PDA-MS	Hexane extract	[23]
***Alkanna corcyrensis* Hayek**	Root	Alkannin Shikonin	HPLC-DAD	Hexane extract	[30]
***Alkanna methanaea* Hausskn.**	Root	Alkannin Shikonin	HPLC-DAD	Hexane extract	
***Alkanna pindicola* Hausskn.**	Root	Alkannin Acetyl Alkannin Isobutyl Alkannin Angelic Alkannin ß-ß dimethylacryl Alkannin Isovaleryl - α methyl -n-Butyl Alkannin	HPLC-PDA-MS	Hexane extract	
***Alkanna pindicola* Hausskn.**	Root	Alkannin Shikonin	HPLC-DAD	Hexane extract	
***Alkanna graeca* Boiss.& Spruner**	Root	Alkannin Shikonin	HPLC-DAD	Hexane extract	
***A. graeca* var. *versicolor* Boiss.**	Root	Alkannin Shikonin	HPLC-DAD	Hexane extract	
***A. graeca* ssp. *Baeotica* (A.DC.) Nyman**	Root	Alkannin Shikonin	HPLC-DAD	Hexane extract	
***Alkanna primuliflora* Griseb.**	Root	Alkannin Shikonin	HPLC-DAD	Hexane extract	
***Alkanna stribrnyi* Velen.**	Root	Alkannin Shikonin	HPLC-DAD	Hexane extract	
***Alkanna sieberi* A. DC.**	Root	Alkannin Shikonin	HPLC-DAD	Hexane extract	
***Alkanna calliensis* Heldr. ex Boiss.**	Root	Alkannin Shikonin	HPLC-DAD	Hexane extract	

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Table 2 ***(continue).*** Chemical contents of *Alkanna* spp.

Plant name	Plant part	Active ingredient	Method	Extract	Ref.
Alkanna tinctoria **Tausch**	Root	Alkannin Shikonin	HPLC-DAD	Hexane extract	[30]
Alkanna noneiformis **Griseb.**	Root	Alkannin Shikonin	HPLC-DAD	Hexane extract	
Alkanna tinctoria **Tausch**	Culture medium	Alkannin Shikonin Acetyl Alkannin ß-ß dimethylacryl Alkannin	HPLC TLC	Culture medium	[31]
Alkanna tinctoria **Tausch subsp.** ***tinctoria***	Root	Alkannin	HPLC-DAD LC-MS	Hexane extract Methanol extract Chloroform extract	[32]
Alkanna tinctoria **Tausch subsp.** ***anatolica*** **Hub.-Mor.**	Root	Alkannin	HPLC-DAD LC-MS	Hexane extract Methanol extract Chloroform extract	[32]
Alkanna tinctoria **Tausch subsp.** ***subleiocarpa*** **(Hub.-Mor.) Hub.-Mor.**	Root	Alkannin	HPLC-DAD LC-MS	Hexane extract Methanol extract Chloroform extract	
Alkanna pseudotinctoria **Hub.-Mor.**	Root	Alkannin	HPLC-DAD HPLC-PDA LC-MS	Hexane extract Methanol extract Chloroform extract	[33]
Alkanna areolate **Boiss. var.** ***areolata***	Root	Alkannin	HPLC-DAD HPLC-PDA LC-MS	Hexane extract Methanol extract Chloroform extract	
Alkanna pinardii **Boiss.**	Root	Alkannin	HPLC-DAD HPLC-PDA LC-MS	Hexane extract Methanol extract Chloroform extract	
Alkanna aucheriana **A.DC.**	Root	Alkannin	HPLC-DAD HPLC-PDA LC-MS	Hexane extract Methanol extract Chloroform extract	
Alkanna mughlae **H.Duman,Güner& Cagban**	Root	Alkannin	HPLC-DAD HPLC-PDA LC-MS	Hexane extract Methanol extract Chloroform extract	
Alkanna saxicola **Hub.-Mor.**	Root	Alkannin	HPLC-DAD HPLC-PDA LC-MS	Hexane extract Methanol extract Chloroform extract	

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Table 2 *(continue).* Chemical contents of *Alkanna* sp.

Plant name	Plant part	Active ingredient	Method	Extract	Ref.
***Alkanna macropjylla* Boiss.& Heldr.**	Root	Alkannin	HPLC-DAD HPLC-PDA LC-MS	Hexane extract Methanol extract Chloroform extract	[33]
***Alkanna hirsutissima* (Bertol.) A.DC.**	Root	Alkannin	HPLC-DAD HPLC-PDA LC-MS	Hexane extract Methanol extract Chloroform extract	
***Alkanna cordifola* K.Koch**	Root	Alkannin	HPLC-DAD HPLC-PDA LC-MS	Hexane extract Methanol extract Chloroform extract	
Alkanna tinctoria* (L.) Tausch subsp. *subleiocarpa (Hub.-Mor.) Hub.-Mor.	Root	Alkannin	HPLC-DAD HPLC-PDA LC-MS	Hexane extract Methanol extract Chloroform extract	
***Alkanna trichophila* Hub.-Mor.**	Aerial parts	Gallic acid 3-Caffeoylquinic acid 5-Caffeoylquinic acid Vanilla acid Caffeic acid Syringic acid *p*- Coumaric acid Ferulic acid 3,5-Dicaffeoylquinic acid Naringin Rutin Hyperoside Kaempferol-3-glucoside Quercetin Quercitrin	HPLC-MS/MS	Methanol extract	[8]
***Alkanna orientalis* (L). Boiss**	Root	α-methyl-*n*- butyl Alkannin Alkannin acetate	TLC	Hexane extract	[34]
***Alkanna orientalis* (L.) Boiss.**	Aerial parts	ß- eudesmol α- eudesmol Isophorone β- caryophyllene	GC/GC-MS	Essential oil	[35]
***Alkanna primuliflora* Griseb.**	Aerial parts	7-Angeloylretronecine 9-Angeloylretronecine 7-Tigloylretronecine 9-Tigloylretronecine Triangularine Triangularicine Dihydroxytriangularine Dihydroxytriangularicine	GC-MS	Methanol extract	[27]

HPLC: High-Performance Liquid Chromatography; MPLC: Medium Pressure Liquid Chromatography; GLC: Gas-Liquid Chromatography; GC: Gas Chromatography; LC: Liquid chromatography; TLC: Thin-Layer Chromatography; VLC: Vacuum Liquid Chromatography; MS: Mass Spectrometry; H-NMR: Proton Nuclear Magnetic Resonance; DAD: Diode-Array Detection; FIB: Flame Ionization Detection PDA: Flame Ionization Detection

Table 2 *(continue).* Chemical contents of *Alkanna* sp.

Plant name	Plant part	Active ingredient	Method	Extract	Ref.
***Alkanna graeca* Boiss. & Spruner**	Aerial parts	7-Angeloylretronecine 9-Angeloylretronecine 7-Tigloylretronecine 9-Tigloylretronecine Triangularine Triangularicine Dihydroxytriangularine Dihydroxytriangularicine	GC-MS	Methanol extract	[27]
***Alkanna orientalis* (L.) Boiss.**	Aerial parts	Kaempferol-3 - glucoside Kaempferol-3-rutinoside Quercetin-3-glucoside Quercetin-3- rutinoside Kaempferol-3,6-dimethyl ether 7-glucoside Kaempferol-3,6- dimethyl ether	Column Chromatogra phy UV-Enzymatic Activity	Ethanol extract (70%)	[36]
***Alkanna orientalis* (L.) Boiss.**	Aerial parts	7- angeloylretronicin 9-angeloylretronicin Dihydroxytriangularin triangularine	H-NMR, ^{13}C-NMR, MS	Ethanol extract	[37]
***Alkanna orientalis* (L.) Boiss.**	Aerial parts	7-Angeloylretronecine 9-Angeloyretronecine 7-Senecioylretronecine 7-Angeloyl-9 (hydroxypropenoyl) retronecine Triangularicine Dihydroxytriangularicine Dihydroxytriangularicine	GLC-MS	0.5N HCl – with ultra thorax extraction	[38]
***A. tuberculate* Greuter**	Aerial parts	7-Angeloylretronecine 7-Tigloylretronecine 7-Angeloyl-9-(hydroxypropenoyl) retronecine 7-Tigloyl-9-(hydroxypropenoyl) Retronecine Triangularine Triangularicine Dihydroxytriangularicine Dihydroxytriangularicine	GLC-MS	0.5N HCl - with ultra thorax extraction	
***Alkanna bracteosa* Boiss.**	Aerial parts	β- eudesmol α- eudesmol Pulegone 1,8-cineole Farnesyl acetate Terpinyl acetate	GC-FID	Essential oil	[39]

HPLC: High-Performance Liquid Chromatography; MPLC: Medium Pressure Liquid Chromatography; GLC: Gas-Liquid Chromatography; GC: Gas Chromatography; LC: Liquid chromatography; TLC: Thin-Layer Chromatography; VLC: Vacuum Liquid Chromatography; MS: Mass Spectrometry; H-NMR: Proton Nuclear Magnetic Resonance; DAD: Diode-Array Detection; FIB: Flame Ionization Detection PDA: Flame Ionization Detection

Table 2 ***(continue).*** Chemical contents of *Alkanna* sp.

Plant name	Plant part	Active ingredient	Method	Extract	Ref.
***Alkanna sfikasiana* Tan, Vold and Strid**	Root and aerial parts	IsobutyrylAlkannin α- methylbutyrylalkanine Lanosterol Linoleic acid Rosmarinic acid Quercetin 3-O-rutinoside Quercetin 3-O-glucoside	-	-	[40]
***Alkanna tinctoria* Tausch**	Roots	Alkannin Acetyl-alkanine- 1H-benzotriazole, 4-nitro, 2-chloroethyl (methylsulfonyl) methanesulfonate and 2, 5-cyclohexadien-1-one, 4-diazo- (napthoquinone)	GC-MS	Ethanol (96%)	[41]
Alkanna tinctoria* Tausch subsp. *tinctoria		Pulegone 1,8-cineole α- terpinyl acetate Isophytol	GC-MS	Essential oil	[42]
***Alkanna sfikasiana* Tan, Vold and Strid**	Roots and aerial parts	Disodium rhabdosine Rosmarinic acid Linoleic acid Quercetin 3-O-β-D-Glucoside Quercetin 3-O-β-D-Rutinoside	VLC MPLC	Cyclohexane Dichloromethane Distilled water Methanol	[29]
***Alkanna orientalis* (L.) Boiss.** ***Alkanna* tinctoria Tausch** ***Alkanna kotschyana* A.DC.**	Aerial parts	Organic acid (Gluconic acid, Malic acid) Phenolic acid (Caffeic acid, Rosmarinic acid) Flavonol (Kaempferol-3-rutinoside, Quercetin-3-glucopyranoside) Flavone (Luteolin-7 rutinoside)	LC/Q-TOF-MS	Methanolic Extract	[28]
***Alkanna orientalis* (L.) Boiss**	Aerial parts	Sarothrin	HPLC	Methanol: water extract	[43]

HPLC: High-Performance Liquid Chromatography; MPLC: Medium Pressure Liquid Chromatography; GLC: Gas-Liquid Chromatography; GC: Gas Chromatography; LC: Liquid chromatography; TLC: Thin-Layer Chromatography; VLC: Vacuum Liquid Chromatography; MS: Mass Spectrometry; H-NMR: Proton Nuclear Magnetic Resonance; DAD: Diode-Array Detection; FIB: Flame Ionization Detection PDA: Flame Ionization Detection

Table 2 *(continue).* Chemical contents of *Alkanna* sp.

Plant name	Plant part	Active ingredient	Method	Extract	Ref.
***Alkanna tubulosa* Boiss.**	Aerial parts	Salvianic acid A 9-Angeloylretronecine Jacoline Benzoic acid Caffeic acid Senecionine N-oxide Caffeoylshikimic acid Caffeoylshikimic acid Yunnaneic acid Rutin Rosmarinic acid Luteolin Naringenin Salvianolic acid C Dihydroxy-trimethoxy(iso)flavone derivates	HPLC LC-MS/MS	Ethyl acetate extract Ethanol extract Infusion Ethanol/Water extract	[44]
***Alkanna tinctoria* Tausch**	Root	Butanoic acid 3-hydroxy-3-methyl Arnebin 7 Dioctyl phthalate	GC-MS/MS	Ethanol extract Water extract	[45]
***Alkanna tinctoria* Tausch**	Root	1,4 naphthoquinones Alkannins Acetyl alkannin Isobutyl alkannin β,β-dimethyl acryl alkannin	HPLC	Methanol extract	[46]

HPLC: High-Performance Liquid Chromatography; MPLC: Medium Pressure Liquid Chromatography; GLC: Gas-Liquid Chromatography; GC: Gas Chromatography; LC: Liquid chromatography; TLC: Thin-Layer Chromatography; VLC: Vacuum Liquid Chromatography; MS: Mass Spectrometry; H-NMR: Proton Nuclear Magnetic Resonance; DAD: Diode-Array Detection; FIB: Flame Ionization Detection PDA: Flame Ionization Detection

According to Table 2, Alkannin derivatives (Acetylalkannin, Propionylalkannin, Isobutylalkannin, Angelylalkannin, Dimethylacryl-alkannin, Isovalerylalkannin, α-Methyl-n-Butylalkannin, and Hydroxyisovalerylalkannin etc.) were detected in chemical studies on *Alkanna* spp. These derivatives and the amounts that were detected varied between spp. and even within the same spp. when it was cultivated in various climates and altitudes [23]. Isohexanylnaphthazarine derivative substances are used as natural colorants for food, cosmetics, and textiles. The interest in isohexenylnaphtazarins has been increasing recently because of the strong anti-inflammatory [47,48], antifungal [49,50], antioxidant [39,40,51], cytotoxic [52–55], radical scavenging [48,52], and enzyme inhibitor properties. Besides these activities, scientific studies have shown that derivatives of isohexenylnaphtazarins play a role in topoisomerase I inhibition and are potential anticancer agents [40, 52]. *Alkanna* roots also include wax, tannins, and alkaloids. In addition, 11-deoxyalkannin and its derivatives (5-O-methyl-11-deoxyalkannin, 8-O-methyl-11-deoxyalkannin, 5-O-methyl-11-O-acetylalkannin, and 5-O-methyl-dimethylacrylalkannin) were isolated from *Alkanna cappadocica* Boiss. et Bal. and have been shown to have cytotoxic effects [52,53] .

Biological activities according to the part of the plant used (root, leaf, and aerial parts) and extraction method are shown in Table 3.

Table 3. Biological activity studies on *Alkanna* spp.

Plant name	Plant part	Extract	Biological activity	Method	Ref.
***Alkanna frigida* Boiss.**	Root	Ethanol extract	Anti-inflammatory Antinociceptive	Claw oedema from carrageenan Formalin test	[47]
***Alkanna orientalis* (L.) Boiss.**	Root	Ethanol extract	Anti-inflammatory Antinociceptive		
***Alkanna bracteosa* Boiss.**	Root	Methanol extract	Anti-inflammatory Analgesic	Claw oedema from carrageenan Formalin test	[48]
***Alkanna trichophila* Hub.-Mor.**	Root	Methanol extract	Anti-inflammatory Analgesic		
***Alkanna bracteosa* Boiss.**	Root	Methanol-water (8:2) extract	Antioxidant	DPPH FTC TBA	[24]
***Alkanna trichophila* Hub.-Mor.**	Root	Methanol-water (8:2) extract	Antioxidant		
***Alkanna frigida* Boiss.**	Root	Methanol-Water (8:2) extract	Antioxidant		
***Alkanna orientalis* (L.) Boiss.**	Root	Methanol-water (8:2) extract	Antioxidant		
***Alkanna orientalis* (L.) Boiss.**	aerial parts	Ethanol extract (70%)	Venom prophylaxis	Liver function test Hematological parameters Evaluation of internal hemorrhages in the rat abdominal cavity	[56]
***Alkanna orientalis* (L.) Boiss.**	Aerial parts	Ethanol extract	Anti-viral	*In vitro* Coxsackievirus	[37]
***Alkanna tinctoria* Tausch**	Root	-	*Staphylococcus aureus* induced eczema	Molecular docking	[57]
***A. cappadocica* Boiss. et. Bal.**	Aerial parts	Methanol extract	Anti-cancer Topoisomerase-I inhibition	*In vitro* cytotoxicity	[53, 58]
***A. pseudotinctoria* Hub.-Mor.**	*In vitro* culture medium	*In vitro* culture medium	Antibacterial (*Staphylococcus aureus, Mycobacterium smegmatis, Acinetobacter baumanii , Escherichia coli, Pseudomonas aeruginosa , Klebsiella pneumonia , Proteus spp ., Enterococcus hirae , Pichia guilliermondii ,Debaryomyces hansen*)	*In vitro* MIC	[59]

DPPH: 2,2-diphenyl-1-picrylhydrazy; FTC: ferric thiocyanate; TBA: Thiobarbituric Acid; MIC: Minimum Inhibitory Concentration; LDL: Low Density Lipoprotein; MBC: Minimal Bactericidal Concentration; PCR: Polymerase Chain Reaction; PV: Peroxidase Activity; CUPRAC: Cupric Reducing Antioxidant Capacity; FRAP: Ferric Reducing Antioxidant Power Assay; PPE: Porcine Pancreatic Elastase; SPF: Sun Protection Factor; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ABTS: 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt

Table 3 *(continue).* Biological activity studies on *Alkanna* spp.

Plant name	Plant part	Extract	Biological activity	Method	Ref.
***A. cordifolia* K.Koch**	Roots	60% Methanol	LDL oxidation inhibitor	*In vitro* LDL oxidation and lipid peroxidation	[60]
***A. tinctoria* Tausch subsp. *subleicarpa* (Hub.-Mor.) Hub.-Mor.**	Leaves	Water extract Chloroform extract Ethanol extract Hexane extract	Antibacterial (A. *baumannii* , *E. coli* , *P. aeruginosa* , *S. aureus*)	Disk diffusion, MIC, MBC	[61]
***A. tubulosa* Boiss.**	Aerial parts and Roots	Methanol: water extract	Antimicrobial or efflux pump inhibitory activity (*Mycobacterium smegmatis,* *S. aureus*)	MIC	[43]
***Alkanna orientalis* (L.) Boiss.**	Roots	Butanol extract Petroleum ether extract	Antimutagenic	Ames *Salmonella* test	[62]
***Alkanna tinctoria* Tausch**	Roots	Ethanol extract (96%)	Antibacterial (*P. areuginosa* , *Bacillus subtilis* , *E. coli* and *S. aureus*	Disk diffusion and MIC	[41]
***Alkanna bracteosa* Boiss.**	Aerial parts	80% Methanol extract	Anticancer (Gastric cancer cell line)	Real-time PCR and Flow cytometry	[63]
***Alkanna tinctoria* Tausch**	Roots	Topical cream	Wound healing	Randomized, blinded, placebo-controlled study	[64]
***Alkanna tinctoria* Tausch**	Roots	%95 Ethanol extract	Antiproliferative Apoptosis induction	Cell proliferation analysis Cell cycle analysis Apoptotic analysis	[4]
***Alkanna orientalis* (L.) Boiss.**	Roots and aerial parts	Cyclohexane extract Dichloromethane extract Water extract Methanol extract	Antioxidant Enzyme inhibition activity	DPPH CUPRAC FRAP Phosphomolybdenum method Acetylcholine esterase inhibition Butyrylcholine esterase inhibition α -amylase inhibition	[29]
***Alkanna tinctoria* Tausch**	Roots	Ethanol extract (70%)	Histological staining of liver tissue	*In vivo* staining	[65]
***Alkanna orientalis* (L.) Boiss.**	Roots	Olive oil-Beeswax	Clinical trials	It was carried out in the burn unit with 64 patients.	[66]

DPPH: 2,2-diphenyl-1-picrylhydrazy; FTC: ferric thiocyanate; TBA: Thiobarbituric Acid; MIC: Minimum Inhibitory Concentration; LDL: Low Density Lipoprotein; MBC: Minimal Bactericidal Concentration; PCR: Polymerase Chain Reaction; PV: Peroxidase Activity; CUPRAC: Cupric Reducing Antioxidant Capacity; FRAP: Ferric Reducing Antioxidant Power Assay; PPE: Porcine Pancreatic Elastase; SPF: Sun Protection Factor; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ABTS: 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt

Table 3 ***(continue).*** Biological activity studies on *Alkanna* spp.

Plant name	Plant part	Extract	Biological activity	Method	Ref.
Alkanna tinctoria **Tausch**	Aerial parts	Methanol extract	Antimicrobial, Antioxidant	Disk diffusion test β-Carotene-linoleic acid antioxidant test	[7]
Alkanna tinctoria **Tausch**	Roots	-	Venous ulcer	Pressure-induced venous rabbit model	[67]
Alkanna tinctoria **Tausch**	Aerial parts	Water extract Ethanol extract Chloroform extract	Prostate cancer	Cell proliferation analysis Isobologram analysis Apoptotic analysis	[68]
Alkanna tinctoria **Tausch**	Roots	Water extract Methanol exrtact Hexane extract Acetone extract	Antioxidant Anti- elastase Sunscreen	DPPH PPE SPF calculation Antielaestase activity	[15]
Alkanna sfikasiana **Tan, Vold and Strid**	Aerial parts	Water extract Ethanol extract Methanol extract	Antimalarial activity	Brine shrimp method *In vivo* antimalarial test	[69]
Alkanna tinctoria **Tausch**	Root	Hexane extract	Wound healing activity	Clinical trial	[70]
Alkanna tinctoria **Tausch**	Root	Dichloromethane extract	Antioxidant activity	PV	[9]
Alkanna tinctoria **Tausch**	Root	Hexane extract	Cytotoxic and apoptotic activity	MTT test Hoechst and AO/EB staining	[54]
Alkanna trichophila **Hub.-Mor.**	Aerial parts	Methanol extract	Antioxidant activity Enzyme inhibition activity	DPPH CUPRAC FRAP Phosphomolybdenum method Tyrosinase inhibition α-glucosidase inhibition Choline esterase inhibition α -amylase inhibition	[8]
Alkanna tinctoria **Tausch**	Roots	Hexane extract	Tissue Staining	*In vivo* staining	[71]
Alkana orientalis **(L.) Boiss**	Roots	Ointment with chloroform extract	Keratinolytic activity Wound healing.	Case-control, single-center, and pilot study that enrolled 60 patients	[72]
Alkanna tinctoria **Tausch**	Roots	Ointment from extract	The burn was infected with *P. aeruginosa*	*In vivo* wound healing.	[73]

DPPH: 2,2-diphenyl-1-picrylhydrazy; FTC: ferric thiocyanate; TBA: Thiobarituric Acid; MIC: Minimum Inhibitory Concentration; LDL: Low Density Lipoprotein; MBC: Minimal Bactericidal Concentration; PCR: Polymerase Chain Reaction; PV: Peroxidase Activity; CUPRAC: Cupric Reducing Antioxidant Capacity; FRAP: Ferric Reducing Antioxidant Power Assay; PPE: Porcine Pancreatic Elastase; SPF: Sun Protection Factor; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ABTS: 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt

Table 3 *(continue).* Biological activity studies on *Alkanna* spp.

Plant name	Plant part	Extract	Biological activity	Method	Ref.
***Alkanna tinctoria* Tausch**	Aerial parts	Water extract	Antimicrobial activity (Gram-negative and Gram-positive)	*In vitro* MIC	[74]
			DNA gyrase gene expression, cytotoxicity	*In vitro* MIC	
***Alkanna tubulosa* Boiss.**	Aerial parts	Ethyl acetate extract Ethanol extract Infusion Ethanol/Water extract	Antioxidant activity	DPPH ABTS CUPRAC FRAB MH PBD	[44]
			Enzyme inhibition activity	AChE activity BChE activity Tyrosinase inhibition α-Amylase inhibition α-Glucosidase inhibition Inhibition of carbonic anhydrases	
			Cytotoxic activity	DU-145 (Prostate carcinoma) HGC-27 (Gastric carcinoma) DU-145 (Prostate carcinoma) MDA-MB- 231 (Breast carcinoma) HELA (Over carcinoma)	
***Alkanna tinctoria* Tausch**	Root	Methanol extract	Antioxidant activity Antidiabetic activity	DPPH ABTS H_2O_2 activity α-amylase activity	[5]
***Alkanna tinctoria* Tausch**	Root	Ethanol extract	Antibacterial activity Wound Healing	Agar well diffusion method Wistar rats model	[75]
***Alkanna orientalis* (L.) Boiss**	Aerial parts	Aqueous ethanol extract (80%)	Anti-viral activity	*In vitro* (SARS-CoV M^{pro})	[76]
***Alkanna tinctoria* Tausch**	Root	Methanol extract	Antibacterial and antifungal activity Wound healing activity	Agar-well diffusion method *In vivo* (burn wound)	[46]
***Alkanna orientalis* (L.) Boiss**	Roots	Ointment	Clinical trials management of burn wounds	External application	[77]
***Alkanna strigosa* Boiss. & Hohen.**	Root	Hexane extract	Wound healing activity	*In vivo* wound	[78]

DPPH: 2,2-diphenyl-1-picrylhydrazy; FTC: ferric thiocyanate; TBA: Thiobarbituric Acid; MIC: Minimum Inhibitory Concentration; LDL: Low Density Lipoprotein; MBC: Minimal Bactericidal Concentration; PCR: Polymerase Chain Reaction; PV: Peroxidase Activity; CUPRAC: Cupric Reducing Antioxidant Capacity; FRAP: Ferric Reducing Antioxidant Power Assay; PPE: Porcine Pancreatic Elastase; SPF: Sun Protection Factor; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ABTS: 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt

RESULT AND DISCUSSION

Medicinal plants are rich sources of natural chemical products used as traditional medicines and they produce many modern medicines from these plants. Secondary metabolites produced by plants are often responsible for the biological activities of plant spp. used worldwide. Nowadays, the medical and pharmaceutical importance of Alkanna has been understood and in this context, it has attracted the attention of many scientists and pharmaceutical companies. The main components of *Alkanna* spp. are Alkannin and its derivatives [53,78,79], as well as alkaloids, flavonoids, and other secondary compounds [24,28]. According to the scientific studies conducted on *Alkanna* spp. so far, it is thought that they can be a promising source for the discovery of a new herbal drug as an antimicrobial, antitumor, antioxidant, and anti-inflammatory agent [80]. In this context, focusing on standardization studies and supporting them with toxicity and clinical studies would be appropriate.

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

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

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DERLEME MAKALE / REVIEW ARTICLE

KARDİYOVASKÜLER SİSTEM HASTALIKLARINDA KALSİYUM KANAL BLOKÖRLERİ

CALCIUM CHANNEL BLOCKERS IN CARDIOVASCULAR SYSTEM DISEASES

İrem HARMANŞAH[1], Sümeyye GÜNEY KALKAN[1,2], Meltem CEYLAN ÜNLÜSOY[1]*

[1]Ankara Üniversitesi, Eczacılık Fakültesi, Farmasötik Kimya Ana Bilim Dalı, 06560, Ankara, Türkiye
[2]Ankara Üniversitesi, Sağlık Bilimleri Enstitüsü, 06110, Ankara, Türkiye

ÖZ

Amaç: *Kalsiyum kanal blokörü bileşiklerin (KKB'ler) kalsiyum girişini engelleyerek ve voltaja bağlı kalsiyum kanallarında tanımlanan bağlanma bölgeleriyle etkileşime girerek arterlerin kasılmasını azalttıkları gösterilmiştir. Bu bileşikler toplam periferik direnci azaltarak arteriyel basıncı düşürür. Kalbin yükünü boşaltarak ve koroner kan akışını artırarak miyokard oksijenlenmesini iyileştirir. Bu çalışmada kalsiyum kanal yapısı, alt tipleri ve özellikleri; KKB'lerin sınıflandırılması, yapı-aktivite ilişkileri, kimyasal yapısı, metabolizması, farmakolojik özellikleri, bunların kardiyovasküler bozukluklar için yerleşik terapötik kullanımları ve genel terapötik endikasyonları hakkında bilgi verilmesi amaçlanmıştır.*
Sonuç ve Tartışma: *Hipertansiyonun etkili tedavisi, hipertansiyona bağlı kardiyovasküler ve böbrek hastalıklarının yükünü azaltmak için önemli bir stratejiyi temsil eder. Rasyonel, entegre ve sinerjik kombinasyon tedavileri, birinci basamak strateji olarak önerilen kan basıncı hedeflerine ulaşmayı amaçlamıştır. Hipertansiyonun klinik tedavisi için halihazırda mevcut olan olası antihipertansif ilaç sınıfları içinde, hem monoterapi hem de kombinasyon terapisinde, renin-anjiyotensin sistemini inhibe eden ilaçların ve kalsiyum kanal blokörlerinin (KKB'ler), kan basıncı seviyelerini düşürmede ve hedefe ulaşmada etkili aynı zamanda güvenli olduğu gösterilmiştir. İyi bir tolere edilebilirlik profiline sahip olan KKB'ler kan basıncını düşürmedeki etkinlikleri, hipertansiyonun kardiyovasküler ve renal sonuçlarını azaltmaya yönelik çok sayıda bilimsel kanıt nedeniyle son 20 yılda en yaygın kullanılan antihipertansif sınıflarından biri olmuştur.*
Anahtar Kelimeler: *Hipertansiyon, kalsiyum kanalları, kalsiyum kanal blokörleri, kalsiyum kanal tipleri*

ABSTRACT

Objective: *Calcium calcium channel blockers (CCBs) have been shown to reduce arterial constriction by blocking calcium entry and interacting with binding sites identified on voltage-gated calcium channels. These compounds reduce arterial pressure by reducing total peripheral resistance. They improve myocardial oxygenation by disloading the heart and increasing coronary blood flow. In this study, an exploration has been undertaken concerning the makeup, variations, and attributes of calcium channels. Furthermore, an examination has been conducted regarding the categorization of compounds that block calcium channels, encompassing their molecular structure, mechanism of aciton, structure-activity relationships, metabolic pathways, recognized therapeutic applications in cardiovascular conditions, and broader therapeutic implications.*

* **Sorumlu Yazar / Corresponding Author:** Meltem Ceylan Ünlüsoy
e-posta / e-mail: munlusoy@pharmacy.ankara.edu.tr, **Tel. / Phone:** +903122033079

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Result and Discussion: *Hypertension represents a substantial strategy to reduce the burden of cardiovascular and renal diseases associated with hypertension. Rational, integrated, and synergistic combination therapies which are recommended as a first-line strategy aimed at achieving the blood pressure targets. Among the currently available classes of antihypertensive drugs for the clinical treatment of hypertension, both in monotherapy and combination therapy, it has been shown that drugs inhibiting the renin-angiotensin system and calcium channel blockers (CCBs) are effective and safe in lowering and reaching the target blood pressure levels. Due to extensive scientific evidence demonstrating their efficacy in mitigating cardiovascular and renal complications linked to hypertension, calcium channel blockers have gained widespread use as a preferred class of antihypertensive medications over the last two decades, primarily attributed to their favorable tolerability profile.*
Keywords: *Calcium channels, calcium channel blockers, hypertension, types of calcium channels*

GİRİŞ

Dünya Sağlık Örgütüne göre kardiyovasküler sistem hastalıkları kalp ve damarlardaki bozulmalardan kaynaklanan hastalıkları kapsamaktadır. Sağlıksız beslenme, fiziksel olarak hareketsizlik, sigara ve alkol kullanımı bu hastalıklar için risk faktörlerini oluşturmaktadır. Bu risk faktörleri kan basıncında yükselme, kan glukoz ve lipid seviyelerinde artışa sebep olabilmektedir. Bu etkilerin sonucu olarak kalp krizi, felç, kalp yetmezliği gibi rahatsızlıklar ortaya çıkmaktadır [1].

Kardiyovasküler hastalıklara bağlı ölümlerin önemli bir kısmı hipertansiyon ve inmeye bağlı olarak gerçekleşmektedir [2]. Hipertansiyon; belirli aralıklarla tekrarlanan klinik ölçümler ile sistolik kan basıncının 140 mmHg, diyastolik kan basıncının 90 mmHg üzerinde olması hali olarak tanımlanmaktadır. Hipertansiyon önlenebilir, tedavi edilebilir bir hastalıktır. Hastanın hipertansiyonu sistolik kan basıncı değerine, yaşına, kardiyovasküler risk faktörlerine bağlıdır [3]. Hipertansiyonun primer ve sekonder olmak üzere iki farklı türü vardır. Esansiyel hipertansiyon olarak da adlandırılan primer hipertansiyon en yaygın olanıdır ve hipertansif hastaların %90-%95' ini etkilemektedir. Sekonder hipertansiyon, ilaç tedavisi veya altta yatan bir tıbbi durum gibi faktörlerden, çoğunlukla da kronik böbrek hastalığı gibi böbrek sisteminin patolojilerinden kaynaklanmaktadır. Hipertansif hastaların %5 ile %10' unu etkilemektedir [4].

Gün geçtikçe hipertansiyon prevalansı dünya genelinde artmaya devam etmektedir. Hipertansiyonun 60 yaş ve üzeri kişilerde görülme sıklığı %60'tan fazladır [5].

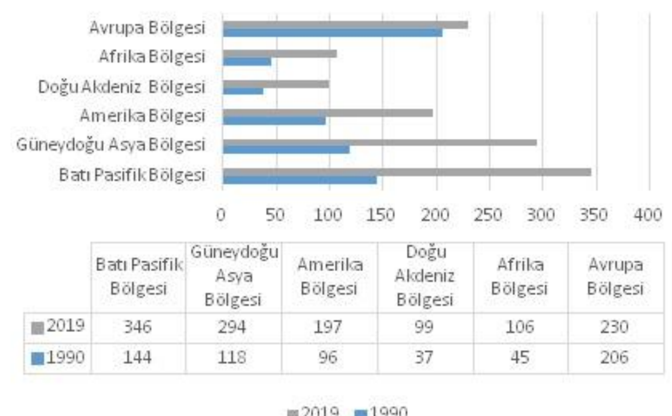
Dünya Sağlık Örgütü'nün 2023 yılında yayınladığı rapora göre 30-79 yaş arası yetişkinlerin %33'ünü etkilediği tahmin edilmektedir. Bununla birlikte nüfus artışının bir sonucu olarak nüfusun yaş dağılımı değiştiğinden dolayı dünyadaki toplam yetişkin sayısı artmaktadır. Hipertansiyon oranı da yaşla birlikte artan bir eğilim göstermektedir. 1990 yılında 650 milyon olan yetişkin hipertansiyon hastası sayısı 2019 yılında iki katına çıkarak 1.3 milyara ulaşmıştır (Şekil 1) [6].

Hipertansiyonun tedavisinde yaşam tarzı değişiklikleri, ilaç veya kombine tedavi uygulanmaktadır. Yaşam tarzı değişiklikleri olarak günlük sodyum alımının 2-2.4 gram ile sınırlandırılması, kilonun ve fiziksel aktivitenin optimizasyonu, dengeli ve düzenli beslenme, tütün ve tütün ürünlerinin bırakılması, alkol alımının kısıtlanması, stres yönetimi önerilmektedir [7].

Hipertansiyonun etkili tedavisi, kardiyovasküler hastalık riskini ve kardiyovasküler hastalıklara bağlı toplam mortaliteyi azaltır. Klinik bulgular kan basıncının düzenli kontrolünün kalp yetmezliği, miyokard enfarktüsü ve felç insidansını sırasıyla %50, %25 ve %40 oranında azaltabileceğini göstermektedir [8].

Diüretikler, kalsiyum kanal blokörleri (KKB'ler), anjiyotensin dönüştürücü enzim (ACE) inhibitörleri veya anjiyotensin reseptörü blokörleri (ARB'ler) birlikte tedavide kullanılabilir [9].

Hipertansiyonu olan hastaların %20 ile %30'unun tedavisi tek bir sınıfa ait antihipertansif ilaçlarla sürdürülebilir. Ancak önerilen kan basıncı hedeflerine ulaşmak için hastaların %70 ile %80'inin en az iki sınıfın kombinasyon tedavisini alması gerekmektedir. Bu kombine tedavi KKB'ler yanısıra ARB'ler, ACE inhibitörleri veya tiyazid dahil diüretikler ile yapılmaktadır [10].



	Batı Pasifik Bölgesi	Güneydoğu Asya Bölgesi	Amerika Bölgesi	Doğu Akdeniz Bölgesi	Afrika Bölgesi	Avrupa Bölgesi
2019	346	294	197	99	106	230
1990	144	118	96	37	45	206

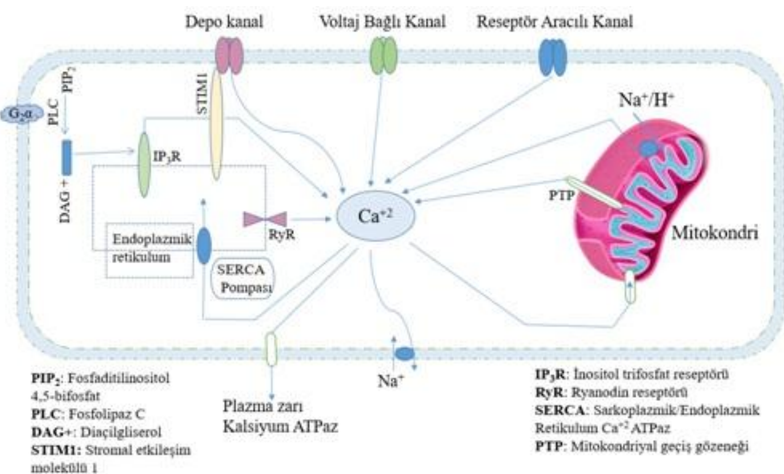
*Değerler milyon olarak ifade edilmiştir

Şekil 1. Bölge ve 1990-2019 yıllarına göre 30-79 yaş arası yetişkin hipertansiyon hasta sayısı

Kalsiyum Kanalları ve Kalsiyum Kanalları Üzerine Etkili Bileşikler

Kalsiyum Kanalları

Organizmadaki kalsiyum dengesi voltaj bağımlı kanallar, reseptör aracılı kanallar ve depo kanallar aracılığıyla sağlanmaktadır. Hücre içi kalsiyum seviyeleri kalsiyumun giriş, çıkış ve tutma mekanizmalarıyla kontrol edilmektedir. Kalsiyumun hücre içine girmesi veya dışarı çıkması, hücre içi ve hücre dışı sodyum, kalsiyum oranına bağlıdır. Bu iyonların giriş, çıkış ve tutma süreçleri uyarılma-kasılma döngüsü için gereklidir. Kalsiyumun depolanması, salınması sarkoplazmik retikulum ve mitokondri tarafından gerçekleştirilmektedir (Şekil 2). Depolama mekanizmaları, hücre içindeki kalsiyum seviyelerinin uygun düzeyde kalmasını sağlamaktadır [11].



Şekil 2. Hücresel kalsiyum akış homeostazı [11]

Voltaj bağımlı kalsiyum kanalları; hücre yüzey zarının depolarizasyonu, kasılması, sekresyonu, nörotransmisyonu ve gen ekspresyonu gibi hücre içi olayları regüle etmektedir. Kalsiyum akımları ilk olarak depolarizasyonun kalsiyum için oldukça seçici olduğu kalp kasında kaydedilmiştir. Voltaj bağımlı kalsiyum (Ca_V) kanalları birçok uyarılabilir hücre tipinde aksiyon potansiyeliyle aktive olmaktadırlar [12].

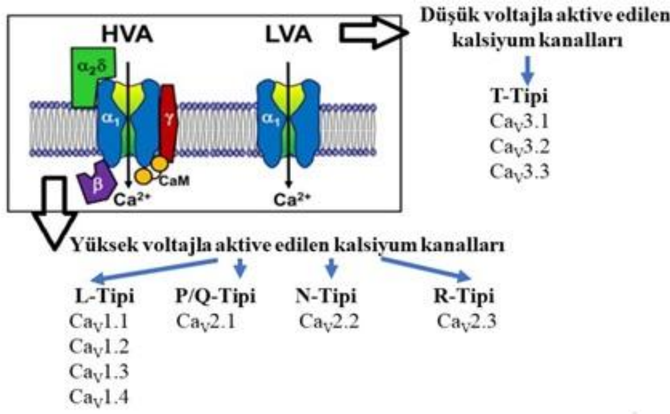
Kardiyak elektriksel aktivite, çeşitli kanallar aracılığıyla akımın toplanması sonucu oluşmaktadır. Özellikle kalsiyum kanalları, kardiyak fonksiyonun düzenlenmesinde önemli bir rol üstlenmektedir.

Kalsiyum kanalının işlevinde görülen değişiklikler kardiyak aksiyon potansiyelinde değişimlere ve kardiyak fonksiyonda patolojik durumlara yol açabilmektedir [13]. Bu nedenle, kardiyak aritmi bozukluklarının nedenlerini anlamak için bu kalsiyum kanallarının tanımlanması ve karakterize edilmesi önem arz etmektedir.

Kalsiyum Kanallarının Sınıflandırılması

Kalsiyum kanalları ilk olarak Fatt ve Katz tarafından 1953 yılında tanımlanmıştır. Aktive edilme yollarına göre; reseptöre duyarlı ve voltaja duyarlı kalsiyum kanalları olarak sınıflandırılmaktadırlar. Reseptöre duyarlı kalsiyum kanalları, adrenalin ve noradrenalin gibi kateşolaminlerle aktive edilmektedir. Alfa (α)-reseptör ve beta (β)-reseptör blokörleri, bu kanalları farmakolojik olarak etkileyebilirler. Voltaja duyarlı kalsiyum kanalları ise aktivasyon hızlarına, farmakolojik duyarlılıklarına, toksinlere duyarlılıklarına ve voltaj aktivasyon seviyesinin düşük veya yüksek olmasına bağlı olarak sınıflandırılmaktadır [14].

Memelilerde voltaj bağımlı kalsiyum kanalları Ca_V1 ($Ca_V1.1$-$Ca_V1.4$), Ca_V2 ($Ca_V2.1$- $Ca_V2.3$) ve Ca_V3 ($Ca_V3.1$-$Ca_V3.3$) olmak üzere başlıca üç tipe ayrılmaktadır [15]. Düşük voltajla aktive edilen kalsiyum kanalları T-tipi ($Ca_V3.1$, $Ca_V3.2$, $Ca_V3.3$) voltaj kanallarıdır. Yüksek voltajla aktive edilen kalsiyum kanallarını ise L-tipi ($Ca_V1.1$, $Ca_V1.2$, $Ca_V1.3$, $Ca_V1.4$), P/Q-tipi ($Ca_V2.1$), N-tipi ($Ca_V2.2$), R-tipi ($Ca_V2.3$) voltaj kanalları oluşturmaktadır (Şekil 3) [16].



Şekil 3. Kalsiyum kanalları ve alt tipleri [16]

T-Tipi (Ca_V3) Voltaj Bağımlı Kalsiyum Kanalları

T-tipi (Ca_V3) voltaj bağımlı kalsiyum kanalı düşük voltajla aktive olan bir kanaldır. Bu kanal çoğunlukla nöronlarda bulunmaktadır. Ancak kardiyak miyositler, glia hücreleri, fibroblastlar, osteoblastlar, pacemaker hücreleri, adrenokortikal hücreler ve retinal hücrelerde de bulunmaktadır [17].

$Ca_V3.1$, $Ca_V3.2$ ve $Ca_V3.3$ adlı üç alfa alt birimi kodlayan üç bağımsız genin farklı ve heterojen ifadesinden kaynaklanmaktadır. Bu genler sırasıyla CACNA1G, CACNA1H ve CACNA1I'dır ve aynı zamanda a1G, a1H ve a1I olarak da adlandırılmaktadır [18].

$Ca_V3.1$ kalsiyum kanalı özellikle uyku-uyanıklık ve beslenme döngüsünde rol oynarken, $Ca_V3.3$ ise serebral arterlerde $Ca_V1.2$ ile birlikte kas hücrelerinin kasılmasına aracılık etmektedir. $Ca_V3.2$ kanalları aferent eksitabilite ile spinal dorsal kökteki sinaptik fonksiyonları düzenlemektedir. $Ca_V3.2$ kanallarının ağrı sırasındaki etkin fonksiyonu nedeniyle, bu kanalın bloke edilmesi analjezik etkiye neden olmaktadır [19, 20, 21]. Kalpte, T-tipi (Ca^{2+}) akımı başlıca olarak $Ca_V3.1$ ve $Ca_V3.2$ alt tipleri tarafından taşınmaktadır [22].

T-tipi kalsiyum kanalları, nöronal ateşleme, noziseptif his, kan damarı daralması ve genişlemesi, lenfatik damar kasılması, düz kas kasılması, miyoblast füzyonu, nörotransmitter salınımı, döllenme, hücre büyümesi, diferansiyasyon ve proliferasyon gibi çeşitli fizyolojik süreçlerde rol oynamaktadır. T-

tipi kalsiyum kanalları farmakolojik ve kinetik düzeylerde uyarılabilir ve uyarılabilir olmayan birçok dokuda belirgin davranışların sergilerlenmesinde görev almaktadır [22].

L-Tipi (Ca_V1) Voltaj Bağımlı Kalsiyum Kanalları

L-tipi (Ca_V1) voltaj bağımlı kalsiyum kanalları, özellikle dihidropiridine duyarlı olan ve moleküler genetik araştırmalarında yüksek afiniteli bileşiklerin varlığıyla tanımlanmıştır [13].

$Ca_V1.1$, $Ca_V1.2$, $Ca_V1.3$ ve $Ca_V1.4$ olmak üzere 4 alt tipi bulunmaktadır. Bunların dağılımı, farklı uyarılabilir hücrelerdeki işlev farklılığından dolayı alt tipler arasında geniş çapta değişiklik göstermektedir. $Ca_V1.1$ esas olarak iskelet kasında ve kas kasılmasında rol oynamaktadır. $Ca_V1.2$ ve $Ca_V1.3$ birçok dokuda örtüşen bir ekspresyon gösterir ve çoğunlukla adrenal kromaffin hücreleri, kalp ve nöron hücreleri gibi aynı hücre tiplerinde bulunmaktadırlar. $Ca_V1.2$ ve $Ca_V1.3$ ağırlıklı olarak hücre somasında postsinaptik olarak, nöronlardaki dendritlerin omurgasında ve gövdesinde yer almaktadır. Ayrıca sinoatriyal düğümde, atriyal kardiyomiyositlerde de eksprese edilir ve kalbin pacemaker aktivitesinde rol oynamaktadır. Kardiyomiyositlerde $Ca_V1.2$ esas olarak uyarılma-kasılma eşleşmesinde görev alır. $Ca_V1.3$ pankreas ve böbrekte endokrin sekresyonla ilişkili olarak bulunmaktadır. Ayrıca işitsel iletimi düzenlemek için kokleada da bulunmaktadır. $Ca_V1.4$ ise esas olarak retinal hücrelerde eksprese edilir ve görme fonksiyonlarına yardımcı olmaktadır [23,24].

N-Tipi ($Ca_V2.2$) Voltaj Bağımlı Kalsiyum Kanalları

N-tipi ($Ca_V2.2$) voltaj bağımlı kalsiyum kanalı, yüksek gerilimli bir kalsiyum kanalıdır. Daha önce keşfedilen L-tipi ve T-tipi akımlara ek olarak, daha önce bilinmeyen bir üçüncü tür olan "N" adlı bir iletkenlik türü olarak tanımlanmıştır. N-tipi voltaj bağımlı kalsiyum akımı, bir L-tipinden daha negatif voltaj eşiğinde akımın inaktivasyonu ve dihidropiridinlere karşı duyarsızlık ile ayırt edilmiştir. Bir T-tipi voltaj bağımlı kalsiyum akımından daha pozitif voltaj eşiğinde aktivasyonu ile farklılık göstermektedir [25].

Presinaptik sinir terminallerinde, nöronal dendrit şaftlarda ve nöroendokrin hücrelerde bulunarak sinir uçlarından nörotransmitter salımına neden olmaktadır. N-tipi kalsiyum kanalları, G-proteinler gibi reseptöre bağlı sekonder haberci sistemler tarafından modifiye edilebilmektedirler. Bu sebeple bu kanallar hipokampal piramidal nöronların hücre gövdesi, dendritleri ve dendritik uçlarında bulunmaktadırlar [26].

R-Tipi ($Ca_V2.3$) Voltaj Bağımlı Kalsiyum Kanalları

R-tipi ($Ca_V2.3$) voltaj bağımlı kalsiyum kanalları, başlangıçta "E-tipi" olarak adlandırılan L- ve P- tipi kanallardan daha hızlı inaktive olan yüksek voltajlı aktivasyona sahip kalsiyum kanallarıdır [27]. $Ca_V2.3$ kanalları beyin, kalp, iskelet ve düz kaslar gibi uyarılabilir hücre zarlarında bulunmaktadırlar. Membran potansiyelindeki değişikliklere yanıt olarak gelişen açılıp kapanma aşamaları ile kalsiyum iyonlarının hücrelere girmesi gerçekleşmektedir [28].

P/Q-Tipi ($Ca_V2.1$) Voltaj Bağımlı Kalsiyum Kanalları

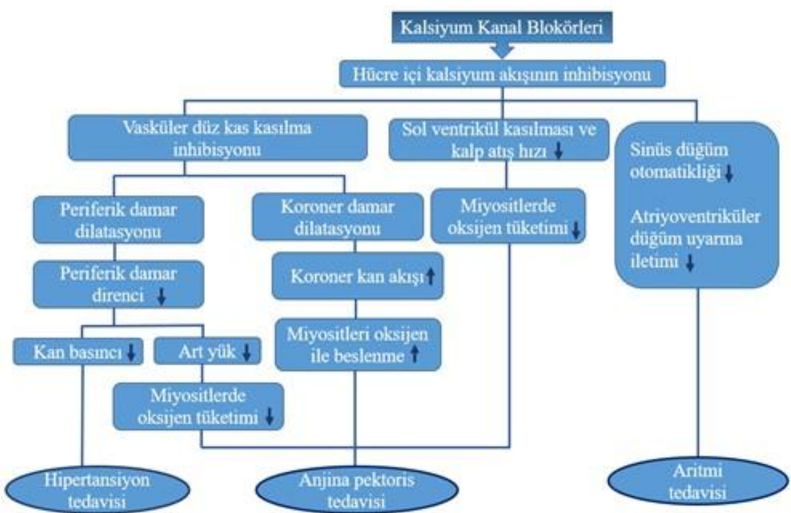
P/Q-tipi ($Ca_V2.1$) voltaj bağımlı kalsiyum kanalı, yüksek gerilimli bir kalsiyum kanalıdır. Purkinje hücrelerinde, -30 ile -20 mV arasındaki voltajlarda zirve yapan ve depolarizasyonu süresince yavaş inaktive olan bir akım gözlenmiştir [29].

$Ca_V2.1$, merkezi ve periferik sinir sisteminde nörotransmitter salınımı için önemlidir. $Ca_V2.1$ kalsiyum kanalları santral ve periferik nöronların akson uçları ile somatodendritik kısımlarında bulunmaktadır [30].

Kardiyovasküler Sistemde Kalsiyum Kanal Blokörü Bileşikler

Kalsiyum kanal blokörü bileşikler, hücre duvarı boyunca uzanan iyona özgü kanallar yolu ile hücre dışı kalsiyum akışını inhibe eden bileşiklerdir. Kalsiyumun akışı engellendiğinde, damar düz kas hücrelerinde vazodilatasyon gözlenir ve kan basıncında düşüş meydana gelmektedir. Kalp kasında kasılma azalırken sinüs pacemaker ile atriyoventriküler iletim hızları yavaşlamaktadır (Şekil 4) [31,33].

Kalsiyum kanal blokörü bileşikler, özellikle kalp hücrelerindeki hızlı ateşlenebilen hasar görmüş kardiyomiyositlerin kalsiyum kanalları üzerindeki etkilerini arttıran güçlü frekans bağımlı blokör etkiye sahiptirler [32].



Şekil 4. Kalsiyum kanal blokörlerinin etki mekanizması [31,33]

Kalsiyum kanal blokörü bileşikler hipertansiyon, konjestif kalp yetmezliği, anjina pektoris, sessiz miyokardiyal iskemi, akut koroner yetmezlik ve miyokardiyal enfarktüs başlıca olmak üzere birçok kardiyovasküler bozuklukların tedavisinde kullanılmaktadırlar. Günümüzde ise kanser tedavisinde (T-tipi KKB'ler), merkezi sinir sistemi bozukluklarında (L-tipi KKB'ler), ağrı tedavisinde (N-tipi KKB'ler) ve tokolitik etkisi nedeniyle obstetrikte yaygın olarak uygulanmaktadırlar. Ayrıca KKB'ler için yeni terapötik endikasyonlar da çalışılmaya devam etmektedir. Dünya Sağlık Örgütü'nün Anatomik Terapötik Kimyasal sınıflandırmasına göre farklı fonksiyonel etkilerine ve klinik kullanımlarına bağlı olarak kalsiyum kanal blokörü bileşikler bulunmaktadır (Şekil 5). Bu bileşikler, kimyasal olarak dört ana sınıfta toplanmaktadır [11]:

- 1,4-Dihidropiridinler
- Fenilalkilaminler
- Benzotiyazepinler
- Diaminopropanol eterler

1,4-Dihidropiridinler

Hipertansiyon ve koroner arter hastalığının tedavisinde dihidropiridin türevi kalsiyum kanal blokörü ilaçlar yaygın olarak tercih edilmektedir. Bu grup ilaçlar, yüksek affiniteli bağlanma tarafından yönlendirilen güçlü voltaj bağımlı blokör etkiye sahiptir. Dihidropridinler kan damarlarının kasılmasını sürdüren, sürekli depolarize olan vasküler düz kas hücrelerinde kalsiyum kanallarını inhibe etmektedir [34].

1,4-Dihidropiridin, piridin halkasının kısmi redüksiyonuyla oluşan bir halka sistemidir. Teorik olarak beş tane dihidropiridin türevi izomerin oluşumu söz konusudur (Şekil 6) [35]. 1,4-DHP halkasının Nikotinamid Adenin Dinükleotid (NADH) koenziminin "hidrojen transferi" özelliklerinden sorumlu olduğunun anlaşılmasıyla bu kimyasal bileşik sınıfına olan ilgi artmıştır. 1970'lerin başına kadar 1,4-DHP'lerin farmakolojik özellikleri tam olarak aydınlatılamamıştır [11].

Şekil 5. Farklı kimyasal yapıdaki kalsiyum kanal blokörlerinin temsilcileri

Şekil 6. Farklı dihidropiridin yapıları

Dihidropiridin iskeleti çok yönlülüğü ve geniş terapötik faydaları nedeniyle özellikle klinik uygulamalardaki etkinliği incelenmiştir. Öncü molekül nifedipinin kimyasal yapısı üzerinde önemli değişiklikler yapılmıştır. Bu değişiklikler yapı-aktivite ilişkilerini anlamak ve kalsiyum modülasyon etkilerini artırmak amacıyla gerçekleştirilmiştir [36]. Sınıflandırması Tablo 1'de sunulmuştur [37]. Ülkemizde bu sınıfa ait bileşiklerden 8 tanesi piyasada bulunmaktadır (Şekil 7).

Tablo 1. Dihidropiridin türevi bileşiklerin sınıflandırılması

NESİL	BİLEŞİK	TİCARİ ADI	NESİL	BİLEŞİK	TİCARİ ADI
1.Nesil	Nifedipin	Procardia, Adalat Crono*	3.Nesil	Barnidipin	Libradin, Vasexten
2.Nesil	Felodipin	Plendil*		Benidipin	Coniel, Benipin*, Benitide*
	İsradipin	Lomir		Efonidipin	Landel
	Nikardipin	Ninax*, Perdipine		Lasidipin	Motens, Lacipil*
	Nimodipin	Nimotop*, Myodipine		Lerkanidipin	Zanidip, Lercadip*, Lercix*
	Nisoldipin	Baymycard, Sular, Syscor		Manidipin	Calslot, İperten
	Nitrendipin	Nitrel, Bayotensin		Nilvadipin	Nivadil
	Amlodipin	Norvasc*,Amlodis*		Silnidipin	Atelec
3.Nesil	Aranidipin	Sapresta		Klevidipin	Cleviprex
	Azelnidipin	Calblock, Rezaltas		Levamlodipin	Conjupri

* Ülkemizde müstahzarı bulunanlar

Şekil 7. Türkiye'de müstahzarı bulunan 1,4-dihidropiridinler

Amlodipin

Amlodipin; en uzun etkili, lipofilik ve üçüncü nesil bir dihidropiridin türevi L-tipi kalsiyum kanal blokörü bileşik olarak 1987 yılında Amerikan Gıda ve İlaç Dairesi (FDA) tarafından onaylanmıştır [38]. Klinik uygulamada yaygın olarak kullanılan farmasötik form, *S(-)*-amlodipin ve *R(-)*-amlodipinin rasemik karışımıdır [39,40].

Amlodipin kalsiyumun vasküler düz kas hücrelerine ve miyokardiyal hücrelere akışını inhibe ederek periferik vasküler direncin azalmasına sebep olmaktadır. Hipertansiyon, kronik stabil anjina ve vazospastik anjina tedavisinde endikedir. Bir dizi randomize çalışma sonucu hipertansiyonun yanı sıra anjina pektoriste de kullanımı kanıtlanmıştır [41,42].

Periferik ödem, amlodipinin yaygın yan etkilerinden biridir. Ayrıca baş dönmesi, yorgunluk, kızarıklık, vertigo, baş ağrısı, kalp çarpıntısı, bulantı yan etki olarak görülebilmektedir [43].

Klaritromisin veya eritromisinle birlikte kullanımının, CYP3A4 tarafından metabolizmasını azaltması nedeniyle hipotansiyon ve akut böbrek hasarı riskini artırdığı bildirilmiştir. Yüksek dozda statinlerle birlikte kullanıldığında, miyopati ve rabdomiyoliz riski artış göstermektedir [44,45].

Felodipin

Felodipin, hipertansiyon tedavisinde yaygın olarak kullanılan 1978 yılında patent almış ve 1988 yılında klinikte kullanımı onaylanmış ikinci nesil bir bileşiktir. Türkiye'de Ocak 2000 tarihinde onaylanmıştır. L-tipi voltaja bağlı kalsiyum kanallarını bloke ederek depolarizasyon sırasında kalsiyum iyonlarının vasküler düz kas ve kalp kası hücrelerine akışını inhibe etmektedir. Bu da arteriyel vazodilatasyonun oluşmasına, kardiyak yükün ve oksijen tüketiminin azalmasına neden olmaktadır [46,47].

Felodipinin doza bağlı yaygın yan etkileri kızarma, çarpıntı ve baş ağrısıdır. Kullanımında en sık görülen klinik yan etkisi ise periferik ödemdir. Felodipinin dozdan bağımsız yan etkileri arasında yorgunluk, bulantı ve diş eti hiperplazisi yer almaktadır. Diş eti hiperplazisi hastaların %1'inden azında görülür ve genellikle felodipinin kullanımının bırakılmasıyla geçmektedir [48,49].

Benidipin

Benidipin, 1981 yılında patent almış ve 1991 yılında klinikte kullanımı onaylanmıştır [46]. Benidipin, sadece L-tipi Ca^{2+} kanalını değil aynı zamanda N-tipi ve T-tipi Ca^{2+} kanallarını da inhibe ederek etki göstermektedir. Vazospastik anjina tedavisinde önemli ölçüde faydalı olduğu bildirilmiştir. Ayrıca aldosteron üretimini ve aldosteron kaynaklı mineralokortikoid reseptör aktivasyonunu doğrudan inhibe ettiği de gösterilmiştir [50].

Geniş spektrumlu bir kalsiyum kanal blokörü olan benidipinin, ayrıca kalsiyumun nöronlara girişini bloke ederek nörodejenerasyonu önlemede etkili olduğu bilinmektedir. Bu durum benidipinin epilepsideki nörodejenerasyon üzerinde de olumlu etkileri olabileceğini ortaya koymaktadır [51].

DHP'den türetilen birçok KKB bileşik, afferent arteriyolleri genişletmektedir. Ancak benidipin hem afferent hem de efferent arteriyolleri genişletir. Efferent arteriyolleri genişletme etkisi benidipinin karakteristik özelliği olarak bilinmektedir ve bu özelliği ilacın böbrek koruyucu etkilerine katkıda bulunmaktadır [52].

İsradipin

İsradipin, 1978 yılında patent almıştır ve 1989 yılında klinikte kullanımı onaylanmıştır [46]. Voltaja bağlı L-tipi kalsiyum kanallarını hedef almaktadır. İsradipin, kardiyak ve arteriyel düz kas hücrelerine kalsiyum akışını engellemek için spesifik ve yüksek affiniteli olarak kalsiyum kanallarına bağlanır. Özellikle anjina pektorisin profilaktik tedavisinde ve hipertansiyon tedavisinde kullanılmaktadır. Bunların yanı sıra, güncel bazı çalışmalar Parkinson hastalığının tedavisinde de kullanılabileceğini göstermektedir [53]. Ayırca isradipinin serebral kan akışını iyileştirdiğinin gözlenmesi isradipinin kokain bağımlılığının yaygın iskemik sonuçları için potansiyel bir tedavi olabileceğini göstermiştir [54].

Levamlodipin

Amlodipinin *S* enantiyomeridir, *S*-amlodipin olarak da bilinmektedir. Amlodipine göre farmakokinetik profili ve tolere edilebilirliğinin iyi olması, daha az periferik ödem insidansı göstermesi nedeniyle anjina ve hipertansiyonda kullanılmaktadır [55].

Nifedipin

Nifedipin 1969 yılında geliştirilmiş ve 1972 yılında hipertansiyon, anjina ve kardiyak aritmilerin tedavisinde kullanılmak üzere onaylanmıştır. Nifedipin, voltaja bağlı L- tipi kalsiyum kanallarını inhibe eder, bu da vasküler düz kasların gevşemesine, kalp üzerinde negatif inotropik ve kronotropik etkilerin gözlenmesine neden olmaktadır [56, 57]. Nifedipin, oksitosin veya prostaglandin tarafından indüklenen kasılmaların genliğini ve sıklığını azaltmaktadır. Nifedipin ayrıca spontan erken doğum tehdidini geciktirmek için kullanılan bir bileşiktir ancak etkinliği sınırlıdır [58].

İdiyopatik chilblains (Pernio); nemli, soğuk havaya maruz kalma ile tetiklenen enflamatuvar bir hastalıktır. Ayırıcı tanısı Raynaud fenomeni, soğuk pannikülit ve chilblain lupustur. Pernio tedavisi öncelikle önleyici tedbirleri içerir, ancak şiddetli veya geçmeyen vakalarda farmakolojik tedavide oral nifedipin kullanılabilmektedir [59].

Nifedipin düz kas gevşemesine neden olduğu için anal fissür tedavisinde %0.2 oranında topikal olarak tercih edilmektedir [60].

Aranidipin

Aranidipin, nifedipininkine benzer hipotansif aktivite sergileyerek güçlü ve uzun süreli damar genişletici etkisi göstermektedir [61].

Şimdiye kadar geliştirilen diğer dihidropiridinlerden farklı olarak aranidipinin metabolitlerinden M-1(α) [(R*)-2-hidroksipropilmetil(R*)-1,4-dihidro-2,6-dimetil-4-(2-nitrofenil)-3,5-piridindikarboksilat] ve M-1(β) [(R*)-2-hidroksipropilmetil(S*)-1,4-dihidro-2,6-dimetil-4-(2-nitrofenil)-3,5-piridindikarboksilat], kalsiyumun indüklediği kasılmayı inhibe etmektedir. Ancak bu metabolitlerin hipotansif aktiviteleri aranidipininkinden daha düşük gözlenmiştir [62].

Nilvadipin

Hipertansiyon tedavisi için kullanılan ikinci nesil bir dihidropiridin türevi kalsiyum kanal blokörüdür. Retina ve optik sinir başı (ONH) gibi oküler nöral dokulara ulaşmada yüksek lipofilitesi nedeniyle avantajlıdır. Bu yüzden glokom tedavisinde de tercih edilmektedir [63].

Nilvadipin merkezi sinir sistemine diğer KKB'lerden daha yüksek konsantrasyonlarda ulaşarak serebral kan akışını artırabilmektedir. Aynı zamanda hücre içi kalsiyum iyonu konsantrasyonundaki aşırı artışları doğrudan engelleyerek nöronları da korumaktadır [64].

Azelnidipin

Azelnidipin, 2003 yılında Calblock olarak Japonya'da pazara sunulan üçüncü nesil dihidropiridin türevi bileşiktir [65]. Amlodipin ve nifedipin gibi birinci ve ikinci nesil KKB'ler ağırlıklı olarak L- tipi kalsiyum kanallarını bloke ederken azelnidipin hem afferent hem de efferent arteriyollerde bulunan T-tipi kalsiyum kanallarını da bloke etmektedir. Etkisini düz kasların hücre membranlarındaki voltaja bağımlı kanallar yoluyla transmembran Ca^{2+} akışını inhibe ederek göstermektedir [66].

Klinik çalışmalar azelnidipinin hipertansif hastalarda sempatik sinir aktivitesini baskılayarak kalp atım hızını ve proteinüriyi önemli ölçüde azalttığını göstermiştir. Diğer dihidropiridin türevlerinden farklı olarak azelnidipin, kan basıncında kademeli bir düşüşe neden olduğundan refleks taşikardiye neden olmaz [67].

Azelnidipinin güvenilir ve genellikle iyi tolere edilebilir olduğu görülmüştür. Hafif baş ağrıları, baş dönmesi ve kızarma gibi sınırlı yan etkilerle ilişkilendirilmiştir [68].

Efonidipin

Efonidipin, hem T- hem de L-tipi voltaja bağlı kalsiyum kanallarını bloke eden antihipertansif ve antianjinal bir ilaçtır [69].

Diğer dihidropiridin KKB bileşiklerden farklı olarak efonidipin, hipertansiyon ve böbrek hastalığı olan hastalarda plazma aldosteronunu azaltmaktadır. Bu etkiyi adrenal hücreler tarafından aldosteron üretiminin doğrudan inhibisyonu ile sağlamaktadır. Bu nedenle efonidipinin; ACE inhibitörleri, ARB'ler ve mineralokortikoid reseptör blokörleri gibi renin-anjiyotensin aldosteron sisteminin (RAAS) inhibitörlerinden farklı bir mekanizma ile aldosteronun zararlı etkilerinden kardiyovasküler koruma sağlamaktadır [70].

KKB bileşikler arasında verapamil ve efonidipinin diyabetik hastalarda böbrek hastalığının ilerlemesini geciktirmede etkili olduğu rapor edilmiştir [71].

Nimodipin

1971 yılında patent almış ve 1988 yılında Amerika Birleşik Devletleri (ABD)'nde klinik kullanımı için onaylanmıştır [46]. Etkisini düz kas hücrelerinin depolarizasyonunu takiben transmembrandaki Ca^{2+} akışını inhibe ederek ve Ca^{2+}'ya bağlılığı azaltarak göstermektedir. L- tipi kalsiyum kanal blokörü olarak başlangıçta hipertansiyon tedavisi için geliştirilmiş ancak yalnızca subaraknoid kanamada kullanım için onaylanmıştır [31,72].

Nimodipin, subaraknoid kanamada vazospazmın önlenmesi amacıyla nörolojide yaygın olarak kullanılmaktadır. Nimodipinin Alzheimer hastalığı veya kronik organik beyin sendromu olarak adlandırılan hastalık tablosunda hem bilişsel hem de davranışsal semptomları iyileştirdiği rapor edilmiştir [73].

Yapılan kontrollü çalışmalar bölünmüş dozlarda günlük 120 mg oral nimodipinin, klasik veya yaygın migreni olan hastalarda migren ataklarının sıklığını, şiddetini ve süresini azalttığı görülmüştür [74].

Nisoldipin

Nisoldipin, 1975 yılında patent almış ve 1990 yılında klinikte kullanım için onaylanmış L-tipi kalsiyum kanal blokörü bileşiktir. Kalsiyum akışını engellenmesiyle hücreler üzerinde bulunan voltaj bağımlı kalsiyum kanallarını bloke ederek sistemik düz kas hücreleri üzerine etki etmektedir. Kalsiyumdaki azalma, koroner ve sistemik arterlerin genişlemesine neden olmaktadır. Bu da sistemik kan basıncının düşmesine ve miyokardiyal oksijen talebinin azalmasına neden olmaktadır. Böylece hem antihipertansif hem de antianjinal etki gözlenmektedir [75,76]. Ayrıca nisoldipin KKB bileşiklerin

gösterdiği periferik ödem, baş dönmesi, baş ağrısı, yorgunluk, yüzde kızarma, hipotansiyon gibi yan etkiler göstermektedir [77].

Nitrendipin

Nitrendipin; T-tipi kalsiyum kanallarını inhibe eden dihidropiridin türevi bir bileşiktir. Hipertansiyonun klinik tedavisinde kullanılmaktadır. Nitrendipinin kullanımı hipertansiyona bağlı ölümcül olmayan felcin ve tüm kardiyak sorunların görülme insidansında azalmayla ilişkilendirilmiştir [78]. 1971 yılında patent almış ve 1985 yılında klinikte kullanım için onaylanmıştır [46].

Nitrendipin kullanan hipertansiyon hastalarında demans prevalansı azalmıştır. Bu da nitrendipinin nöroprotektif bir etkisi olduğunu göstermektedir [73].

Nitrendipinin yarı ömrü 8 saattir. Vücuda alındıktan sonra ilk geçiş etkisi göstererek karaciğerde inaktive olur. Bu yüzden de nitrendipinin dilaltı mukozal uygulaması, hipertansif acil durumların tedavisi için uygundur. Oral tablet olarak alındığında daha yavaş antihipertansif aktivite göstermektedir [79].

Manidipin

Manidipin; kalp atış hızında veya kalp debisinde önemli bir artış olmadan, 24 saat boyunca sürekli bir etkiyle kan basıncını etkili bir şekilde azaltan üçüncü nesil bir dihidropiridin türevi kalsiyum kanal blokörü bileşiktir [80]. 1982 yılında patent almış ve 1990 yılında klinikte kullanım için onaylanmıştır [46].

Manidipin, adipositlerin oluşumunu ve farklılaşmasını uyarmanın yanı sıra peroksizom proliferatör aktive edici reseptör gama (PPAR-γ) aktivitesini koruyarak insülin duyarlılığını arttırmaktadır. Ayrıca oksidatif stres üzerinde de olumlu etkileri gözlenmiştir [81].

Hipertansif hastalarda manidipinin, tek başına veya bir RAAS inhibitörleri ile kombinasyon halinde kan basıncında amlodipine benzer düşme görülmesine rağmen amlodipin ile karşılaştırıldığında daha yüksek bir böbrek koruması sergilediği görülmüştür. Bu da manidipinin L- ve T- tipi kalsiyum kanallarını birlikte bloke etmesiyle açıklanmıştır [82].

Diyabet, metabolik sendrom veya diyabet geliştirme riski taşıyan hipertansif hastalarda RAAS inhibitörleri kullanılmaktadır. Ancak ikinci bir antihipertansif ilaca ihtiyaç duyulduğunda kalsiyum kanal blokörü olarak manidipin tedavide kullanılmalıdır [83]. Manidipin kullanan hastalarda yan etki olarak periferik ödem, baş dönmesi, baş ağrısı, yorgunluk, yüzde kızarma görülebilmektedir [84].

Nikardipin

Nikardipin; vasküler bozuklukların tedavisinde kullanılan L-tipi voltaja duyarlı bir kalsiyum kanal blokörüdür. 1973 yılında patent almış, Aralık 1988 yılında FDA tarafından klinik kullanımı onaylanmıştır. Serebral ve koroner kan damarları için daha seçicidir ve iskemik inme, travmatik beyin hasarı ve subaraknoid kanama dahil olmak üzere akut beyin hasarı sonrası şiddetli hipertansiyonun tedavisinde yaygın olarak kullanılmaktadır [46,85].

Kardiyak iletim yollarını etkilemeden periferik arteriyel vazodilatasyon üretir ve dolayısıyla bradikardi riski düşüktür. Bu nedenle, akut serebrovasküler acil durumlarda tedavide birinci basamak olarak önerilmektedir. İntravenöz olarak uygulanmaktadır. Yarı ömrü kısa ve yan etkileri azdır [86].

Silnidipin

Silnidipin klinik uygulamada renoprotektif, nöroprotektif ve kardiyoprotektif etkileri nedeniyle kullanılmaktadır. Hem L- hem de N- tipi voltaja bağlı kalsiyum kanallarını bloke ederek etki göstermektedir [87]. 1984 yılında patent almış ve 1995 yılında klinikte kullanımı Japonya'da onaylanmıştır [46].

Silnidipin, periferik sempatik sinir uçlarında inhibisyon sağlayarak kan basıncını düşürmektedir. Silnidipin, sabah yüksek tansiyonu olan hastalarda sempatik sinirlerin aşırı aktivasyonunun potansiyel rol oynadığı durumları da tedavi etmektedir. Silnidipin, vasküler endotel disfonksiyonunu düzelterek kardiyovasküler hastalıkların uzun vadeli tedavisinde fayda göstermektedir [87,88].

Oral uygulamadan sonra ilaç konsantrasyonları 1.8 ile 2.2 saat arasında pik yapar ve yarı ömrü 7.5 saattir. İlaç, %98 oranında proteine bağlanır ve bu nedenle yarılanma ömrü kısa olmasına rağmen

nifedipin ve nikardipine göre daha yavaş etki gösterdiği fakat daha uzun süre etkili olduğu gösterilmiştir [88].

Silnidipin, N-tipi kalsiyum kanallarını diğer KKB'lere göre daha güçlü bir şekilde inhibe etmektedir. Ayrıca glukoz homeostazisinde önemli bir rol oynamaktadır. Tip 2 diyabetli hipertansif hastalarda katekolamin düzeylerini önemli ölçüde azaltır ve insülin direncini iyileştirir. Magnezyum takviyesiyle birlikte kullanımı enerji metabolizması ve oksidatif durum üzerine ek fayda sağlamaktadır [89].

Günde bir kez uygulanmasının, aşırı hipotansiyon veya refleks taşikardi olmadan kan basıncını etkili bir şekilde düşürdüğü gösterilmiştir. Ancak baş dönmesi, kızarma, baş ağrısı, uyuşukluk, depresyon, titreme, ateş, döküntü, miyalji, gastrointestinal rahatsızlıklar, periferik ödem, çarpıntı, anormal karaciğer fonksiyonu gibi yan etkiler gözlenebilmektedir [90,91].

Flordipin

Flordipin, nifedipine benzer bir yapıya ve kardiyovasküler etkilere sahip kalsiyum kanal blokörüdür. Ancak diğer kalsiyum kanal blokörlerinin aksine negatif inotropik etkisi yoktur. Nifedipinden farklı olarak aktivasyonu için hepatik metabolizma gerekmektedir [92]. Yapılan bir çalışmada flordipinin düşük kan basıncı sağlarken refleks taşikardiye sebep olabileceği gösterilmiştir [93].

Klevidipin

Çok kısa etkili bir ilaç olan klevidipin, kardiyak veya kardiyak olmayan müdahalede şiddetli hipertansiyon durumunda intravenöz infüzyon yoluyla uygulanan bir lipit emülsiyonudur. Yeni nesil dihidropiridin türevi L-tipi kalsiyum kanalı blokörü olarak perioperatif kullanım için FDA tarafından onaylanmıştır. Esterazlarla hidrolizi nedeniyle yarılanma ömrü yaklaşık 2 dakikadır [69,94].

Klevidipin periferik arterleri genişletir, vasküler direnci azaltır ve ardından kan basıncını düşürmektedir. Ayrıca arteriyel düz kas depolarizasyonu sırasında kalsiyum akışına aracılık ederek kardiyovasküler sistemdeki uyarılma ve kasılmayı birleştirir [95]. Klevidipin atriyal fibrilasyon başlangıcı, baş ağrısı, kızarma, ateş, mide bulantısı, kusma gibi yan etkiler göstermektedir [96].

İlaçlarla yiyecekler arasındaki etkileşimi önlemek için klevidipin tedavisi sırasında flavonoid açısından zengin içecek ve yiyeceklerin alımı azaltılmalıdır [97].

Lasidipin

Lasidipin; günde bir kez oral olarak uygulanan, lipofilik dihidropiridin kalsiyum kanal blokörüdür. 1984 yılında patent almış ve 1991 yılında klinikte kullanımı onaylanmıştır. Uzun bir etki süresi ve yüksek vasküler seçiciliği vardır. Voltaja bağlı L-tipi kalsiyum kanallarını bloke ederek vazodilatasyon sağlar ve böylece toplam periferik vasküler direnci zayıflatarak kan basıncının azalmasını sağlamaktadır. Kalsiyum kanalıyla modüle edilen vazodilatasyona ek olarak lasidipin, diğer dihidropiridin KBB'lerden daha fazla antioksidan aktivite göstermektedir [46,98].

Amlodipin ve lasidipinin çeşitli biyolojik aktiviteleri dikkate alındığında Leishmania *donovani*'nin neden olduğu Visseral leishmaniasis (Karahumma) hastalığında etkili olabileceği gösterilmiştir. Nifedipin ise hastalığın başlangıcında *Leishmania*-makrofaj tutunmasını inhibe edebilmektedir [99].

Barnidipin

Barnidipinin klinik antihipertansif etkinliği, nitrendipin ve amlodipin gibi diğer 1,4-DHP türevi KBB'ler ile ve atenolol ve enalapril gibi diğer ilaç sınıflarına ait antihipertansiflerle benzer olan L-tipi kalsiyum kanalı blokörüdür. Kardiyovasküler kalsiyum reseptörlerine karşı seçici antagonist etkiye sahip olduğu ve antihipertansif etkisini kalsiyum iyonu akışının seçici olarak bloke edilmesiyle göstermekte olup yaşlı hastalarda hipertansiyon tedavisinde hidroklorotiyazid kadar etkili ve iyi tolere edildiği bilinmektedir [100].

Lerkanidipin

Lerkanidipin, hidroklorür formunda kullanılan üçüncü nesil bir dihidropiridin kalsiyum kanal blokörüdür. İlk kez 1997 yılında Hollanda'da satışa sunulmuş olup şu anda hafif-orta şiddette hipertansiyonun tedavisinde kullanılmaktadır. Hipertansif hastalarda nitrik oksit biyoyararlanımını ve endotel bağımlı vazodilatasyonu artırmaktadır. Aynı zamanda plazmada bulunan lipoperoksit, izoprostan ve miyeloperoksidaz gibi oksidatif stres belirteçlerini de azaltmaktadır [101].

İntraglomerüler kılcal basınçta herhangi bir değişiklik olmadan hem afferent hem de efferent glomerüler arterleri doğrudan genişleterek diğer türevlerden farklı etki göstermektedir. Bu etkisi L-tipi ve T-tipi kalsiyum kanalının inhibisyonundan kaynaklanmaktadır. Yüksek lipofilitesi nedeniyle lipit membranlara bağlanarak L-tipi kalsiyum kanalıyla uzun süreli etkileşime girmektedir. Böylelikle diğer DHP'lere kıyasla daha uzun etki süresi oluşmaktadır [102].

Benzotiyazepin Türevi Bileşikler

1970'lerin başında aromatik heterosiklik halkada çeşitli sübstitüsyonlar ile geliştirilmiş olan ilk analog bileşikler 1980'lerde popüler hale gelmiştir [11,103].

Diltiazem

Diltiazem; 1982 yılında ABD'de klinikte kullanım için onaylanmış, L- tipi voltaja bağlı kalsiyum kanal blokörüdür. Kalp hızını azaltarak miyokardiyal oksijen tüketimini azaltmaktadır. Bu etki, antianjinal etkilerine katkıda sağlamaktadır. Benidipin ve diltiazemin kombinasyon tedavisi, anjina pektoriste tercih edilmektedir. Çünkü diltiazem, miyokardın oksijen ihtiyacını benidipine göre daha etkili bir şekilde azaltır [52].

Diltiazem vazodilatasyona neden olarak damar direncini azaltırken optik sinir başındaki kılcal kan hızını arttırmaktadır. Bu nedenle düşük tansiyonlu glokomda kullanılmaktadır [104].

Fenilalkilamin Türevi Bileşikler

Fenilalkilamin yapısına sahip L-tipi kalsiyum kanal blokörler olan verapamil ve gallopamil günümüzde kardiyak aritmi, hipertansiyon ve anjina pektoris tedavisinde kullanılmaktadır (Şekil 4) [105]. Fendilin, lidoflazin, perheksilin diğer benzer ilaçlar arasında sayılmaktadır [11]. Fenilalkilamin türevi KKB bileşikler vasküler sistem ve kalp ile nodal yapılar üzerinde etki ederken, dihidropiridin türevi KKB bileşikler esas olarak vasküler dilatasyon oluşturarak etki etmektedirler [106].

Verapamil

Verapamil, 1981 yılında ABD'de klinikte kullanım için onaylanmıştır [46]. Verapamil, kalsiyumun kardiyak iletim sistemine, kardiyak miyositlere ve vasküler düz kaslara hızlı akışını bloke ederek iletim süresinin uzamasına, miyokardiyal kontraktilitenin azalmasına ve vasküler gevşemeye neden olmaktadır [31].

Supraventriküler taşikardide verapamilin diltiazemden daha etkili olduğu görülmüştür. İntravenöz verapamil, reetran supraventriküler taşikardinin akut dönüşümü için etkilidir. Ayrıca özellikle yaşlı hastalarda amiodaron veya flekainidle tedavide verapamil eklenmesinin atriyal fibrilasyon nükslerini önemli ölçüde azalttığı gözlenmiştir [71].

Tip 1 diyabetli bireylerde sürekli oral verapamil kullanımı diyabetin ilerlemesini geciktirir. Endojen beta hücre fonksiyonunu desteklerken insülin gereksinimini azaltmaktadır. Ayrıca verapamil, tiyoredoksin sistemini düzenlemektedir. Verapamil Langerhans adacıklarında antioksidatif, anti-apoptotik ve immünomodülatör gen ekspresyon profilini desteklemektedir [107].

Kabızlık, verapamilin bağırsak düz kasları üzerindeki inhibitör etkisi nedeniyle sık görülen bir yan etkidir. Ayrıca verapamil için kaydedilen en yaygın advers reaksiyonlar arasında baş dönmesi, baş ağrısı ve yorgunluk yer almaktadır [108].

Gallopamil

Gallopamil, verapamilin kimyasal yapısında bulunan fenil halkasına bir metoksi grubunun daha ilave edilmesi ile elde edilen türevidir ve bir fenilalkilamin kalsiyum kanal blokörüdür. Hücre zarı üzerinde kalsiyuma bağlı akımı inhibe ederek L-tipi voltaja bağlı kalsiyum kanallarına etki etmektedir.

Akut miyokard iskemisi ve reperfüzyon ile ilişkilendirilen metabolik değişiklikleri ve iyon değişikliklerini azaltarak kardiyoprotektif etki göstermektedir [106, 109].

Lidoflazin

Lidoflazin bir piperazin türevidir. Janssen Pharmaceutica'da 1964 yılında keşfedilen ve anjina pektoris, akut koroner sendromu, atriyal fibrilasyon tedavisinde kullanılan bir kalsiyum kanal blokörüdür [11].

Edinilmiş uzun QT sendromu (aLQTS) vakalarının neredeyse tamamı, hERG kanalının kimyasal blokajıyla ilişkilendirilmiştir. hERG ile etkileşime giren diğer proteinlerdeki tek nükleotid polimorfizmlerinin küçük moleküller tarafından inhibe edilebileceğini ve dolayısıyla aLQTS'ye neden olabileceği bildirilmiştir. Bu yüzden aLQTS vakaları lidoflazin bileşiğinin kullanımını sınırlandırmıştır [110].

Fendilin

Fendilin, L-tipi kalsiyum kanallarını inhibe eden antianjinal bir bileşiktir (Şekil 4). Kalsiyum aracılı kardiyak aksiyon potansiyellerini inhibe ederek etki gösterir [111]. Fendilin ve sisplatinin birlikte uygulanmasıyla indüklenen nöroblastoma büyümesinin inhibisyonu bir sinerjizm ile karakterize edilmiştir. Fendilin, sisplatinin pro-apoptotik aktivitesini güçlü bir şekilde arttırmaktadır. Kombine tedavi ile nöroblastoma hastalarının hayatta kalma oranı artmakta ve nüks riski azalmaktadır [112].

Perheksilin

Bir piperidin türevi olan perheksilin (Şekil 4), ilk olarak 1970'lerde bir antianjinal ilaç olarak geliştirilmiştir. Anjina için etkili bir bileşik olmasına rağmen şiddetli hepatoksisite ve nörotoksisite raporları 1980'lerin sonlarına doğru küresel kullanımın azalmasına neden olmuştur. Bununla birlikte perheksilin, dirençli anjina pektorisli hastaların veya diğer tedavilerin kontrendike olduğu anjinalı hastaların tedavisinde onaylandığı Avustralya ve Yeni Zelanda dahil olmak üzere birçok ülkede reçete edilmeye devam etmektedir. Ayrıca kalp yetmezliğinde ve hipertrofik kardiyomiyopatide miyokardiyal enerjiyi arttırmaktadır [113,114,115].

Diaminopropanol Eterler

Bepridil

Bepridil daha önce önemli antianjinal aktivitesi nedeniyle kullanılmış olan uzun etkili, nonselektif bir amin türevi kalsiyum kanal blokörüdür (Şekil 4). Bepridilin kardiyak iyon kanalı akımları üzerinde birçok etkisi olduğu gösterilmiştir. Ancak antiaritmik etkileri tam olarak tanımlanmamıştır. Atriyal fibrilasyonun tedavisi için olası bir seçenek olarak tartışılmaktadır. Bepridil şu an yalnızca Japonya'da mevcuttur [11,116].

Bepridil, düşük biyoyararlanımı (<%60), büyük dağılım hacmi (8 l/kg) ve uzun yarı ömrü (48-80 saat) nedeniyle plazma konsantrasyonlarında bireyler arası değişkenliğe neden olmaktadır. Bepridil, esas olarak hepatik CYP2D6 ve kısmen de CYP3A tarafından metabolize edilmektedir [117]. McN-A-2600 (2-[[3-(fenil)(fenil-metil)amino]-2-(1-pirolidinil)propoksi]metil]-2-propanol) ve McN-6303 (4-[[2-(2-metil-propoksi)-1-[[(fenil)(fenilmetil)amino]metil]etil]amino]bütanoik asit) majör metabolitleri olarak bilinmektedir [118].

Bepridil'in COVID-19'un patojeni olan SARS-CoV-2'nin temel ana proteazını inhibe etme açısından düşük mikromolar EC_{50} değerlerine sahip olduğu gösterilmiştir. Bu da bepridilin antiviral etkisi olabileceğini düşündürmektedir [119]. Ayrıca Haziran 2015'te, Ebola için potansiyel farmasötik tedavileri araştıran bir deney sırasında Ebola'ya maruz kalan fareler için bepridilin %100 hayatta kalma oranıyla sonuçlandığını ortaya koyan ve gelecekteki Ebola tedavisinde kullanılabileceğini gösteren bir araştırma yayınlanmıştır [120].

Diğer Bileşikler

Mibefradil

Mibefradil tetralol türevi bir organik bileşiktir (Şekil 4). Hoffmann LaRoche'taki araştırmacılar tarafından geliştirilen ilk L- ve T-tipi kalsiyum kanal blokörü olarak Posicor ticari adıyla 1997 yılında ABD'de piyasaya çıkmıştır. Mibefradil kimyasal olarak diğer T-tipi KKB bileşiklerden farklıdır ve nanomolar konsantrasyonlarda T-tipi Ca_V kanallarına karşı L-tipi kanallara göre 30 kat daha etkilidir. Klinik çalışmalar antihipertansif, antianjinal ve antiiskemik etkinliğinin bazı L-tipi kalsiyum kanal blokörlerine eşit veya hatta onlardan daha üstün olduğunu göstermektedir. Bazı L-tipi kalsiyum kanal blokörlerinin aksine, mibefradilin refleks taşikardiyi veya negatif inotropizmi tetikleme eğilimi daha azdır [121,122].

Klinik uygulama sonrası gözlemler, özellikle yaşlı hastalarda mibefradil ile beta blokörler, digoksin, verapamil ve diltiazem arasında ciddi bir potansiyel etkileşim olduğunu ortaya çıkarmıştır. 8 Haziran 1998 yılında Roche firması tarafından ilaç-ilaç etkileşiminin olumsuz kardiyovasküler yan etkileri nedeniyle ilaç piyasadan gönüllü olarak çekilmiştir [11,123].

T-tipi kalsiyum kanalları, kanser hücresi çoğalmasının tümör büyümesinin temel düzenleyicileri olduğundan son zamanlarda antikanser etkinliği açısından FDA tarafından bir "yetim ilaç" olarak onaylanan mibefradil farmakolojik açıdan yeniden tasarlanıp kullanılmaya başlanmıştır. Yumurtalık ve pankreas kanseri ile glioblastoma tedavisinde yer almaktadır. Ayrıca çeşitli kanser hücre dizileri üzerinde antiproliferatif etkiye sahip olduğu da gösterilmiştir [122].

SONUÇ VE TARTIŞMA

Bu çalışma kapsamında ele alınan kalsiyum kanal blokörü bileşiklerinin (Tablo 2) kalsiyum kanallarını inhibe ederek etki göstermesi nedeniyle öncelikle bu kanalların özellikleri hakkında bilgi verilmiş olup detaylı literatür verileri ile ele alınan KKB'ler incelenip değerlendirilmiştir. Kalsiyum kanallarının kalp atış hızında, elektriksel kalp iletimi ve kan basıncı düzenlenmesinde önemli olduğu bilinmektedir. Böylece bu kanalların blokajı çeşitli kardiyovasküler hastalıkların tedavisinde önemli bir strateji olarak kullanılmaktadır. Bununla birlikte, çeşitli KKB türleri arasındaki farklılıklar, hastaların bireysel ihtiyaçlarına göre tedavide yerini almaktadır. Başlıca L-tipi kalsiyum kanalları üzerine etkili 1,4-dihidropridinler antihipertansif etkilerini vazodilatör olarak gösterirken 1,4-DHP ve diğer gruplara ait bazı türevler N- tipi ve T-tipi kalsiyum kanallarını da etkileyerek hem antihipertansif etki hem de organ koruyucu ve analjezik aktivite göstermektedir. T-tipi kalsiyum kanallarının kardiyovasküler, endokrin ve sinir sisteminde bulunması, bu dokuların gelişiminde ve onarımında görev alması T-tipi kalsiyum kanal blokörlerinin organ koruyucu etkilerini ortaya çıkarmıştır. Bunun dışında N-tipi kalsiyum kanalların ağrı sinyallerinin periferden merkezi sinir sistemine taşınmasında görev alması N-tipi kalsiyum kanal blokörü bileşiklerin analjezik aktivite ve nöropatik ağrıda da etkili olabileceğini göstermiştir. Bu bağlamda benidipin, efonidipin, nitrendipin, azelnidipin, manidipin, silnidipin, lerkanidipin gibi kalsiyum kanal blokörü bileşiklerin L-tipi kalsiyum kanallarının dışında N-tipi ve/veya T-tipi kalsiyum kanallarını da etkilemesi ile beraber renoprotektif, nöroprotektif ve kardiyoprotektif etkileri ortaya çıkmaktadır. Bu bileşiklerden benidipin her üç kanalı da bloke etme özelliği nedeniyle geniş spektrumlu KKB olarak nitelendirilmektedir. Genel olarak, L-tipi kalsiyum kanalları dışında T-tipi kalsiyum kanallarını da bloke ederek etki gösteren bileşikler, olumlu yan etki profili göstermekte ve KBB'lerin sebep olabileceği refleks taşikardiyi, sempatik sinir sistemi aktivitesini baskılayarak ortadan kaldırmaktadır. L-tipi kalsiyum kanalı dışında T- ve N-tipi kalsiyum kanalları üzerine etki gösteren bileşiklerin organ koruyucu etkileri kronik olarak kardiyovasküler sistem hastalıklarından muzdarip olan hastalar için önemli bir olguyu meydana getirmektedir. Bu organ koruyucu etkilerin neticesi uzun vadede kalp damar sistemi hastalıklarının sebep olabileceği hasarı en aza indirmekte veya ortadan kaldırmakta etkili olacaktır.

Etki olarak farklılaşan KKB türevlerinin yan etki profillerindeki farklılıklar da tedavi sırasında yan etkilerin yönetilmesi açısından kritik öneme sahiptir. Örneğin, periferik ödem riskini en aza indirmek için dihidropiridinler yerine fenilalkilaminler tercih edilebilir. Bu nedenle, bu ilaçların farmakolojisini optimize etmeye daha fazla ihtiyaç vardır. Dahası, bu ilaçların uzun vadeli etkilerinin ne olduğu da belirsizdir. Bu yüzden klinik deneylerde ayrıntılı olarak çalışılması gerekmektedir. Olumsuz kardiyovasküler sonuçların önlenmesinde kan basıncının düşürülmesi birincil öneme sahiptir.

Tablo 2. Kalsiyum kanal blokörlerinin etki yöresi, endikasyonları ve yan etkileri

Bileşik	Bloke Ettiği Ca^{2+} Kanal Tipi	Endikasyon	Yan Etki
Amlodipin	L-Tipi	Hipertansiyon Koroner arter hastalıkları Kronik stabil anjina	Periferik ödem, Baş dönmesi, Baş ağrısı, Yorgunluk, Yüzde kızarma, Çarpıntı
Felodipin	L-Tipi	Hipertansiyon	Periferik ödem, Yorgunluk, Yüzde kızarma, Çarpıntı, Diş eti hiperplazisi
Benidipin	L-, N- ve T-Tipi	Hipertansiyon Kronik stabil anjina	Periferik Ödem, Baş dönmesi, Baş ağrısı, Çarpıntı, Kabızlık
İsradipin	L-Tipi	Hipertansiyon	Periferik ödem, Baş ağrısı, Sıcak basması
Levamlodipin	L-Tipi	Hipertansiyon	Amlodipine göre daha az periferik ödem insidansı
Nifedipin	L-Tipi	Hipertansiyon Kronik stabil anjina Vazospastik anjina	Baş dönmesi, Baş ağrısı, Yorgunluk, Yüzde kızarma , Titreme, Kas krampları, Refleks taşikardi
Aranidipin	L-Tipi	Hipertansiyon Kronik stabil anjina	Periferik ödem, Baş dönmesi, Baş ağrısı, Yüzde kızarma, Hipotansiyon
Nilvadipin	L-Tipi	Hipertansiyon Kronik stabil anjina Vazospastik anjina	Periferik ödem, Baş dönmesi, Baş ağrısı, Yorgunluk, Yüzde kızarma
Azelnidipin	T-Tipi	Hipertansiyon	Periferik ödem, Baş dönmesi, Baş ağrısı
Efonidipin	L- ve T-Tipi	Hipertansiyon	Baş ağrısı, Sıcak basması, Yüzde kızarma
Nimodipin	L-Tipi	Subaraknoid kanama sonrası nörolojik sonuçları iyileştirmede	Periferik ödem, Baş dönmesi, Baş ağrısı, Yüzde kızarma
Nisoldipin	L-Tipi	Hipertansiyon	Periferik ödem, Baş dönmesi, Baş ağrısı, Yorgunluk, Yüzde kızarma, Hipotansiyon
Nitrendipin	T-Tipi	Hipertansiyon	Periferik ödem, Baş dönmesi, Baş ağrısı, Yorgunluk, Yüzde kızarma
Manidipin	L- ve T-Tipi	Hipertansiyon	Periferik ödem, Baş dönmesi, Baş ağrısı, Yorgunluk, Yüzde kızarma
Nikardipin	L-Tipi	Hipertansiyon Kronik stabil anjina Travmatik beyin hasarı ve subaraknoid kanama sonrası nörolojik sonuçları iyileştirmede	Baş dönmesi, Baş ağrısı, Yorgunluk, Yüzde kızarma, Kas krampları, Kabızlık
Silnidipin	L- ve N-Tipi	Hipertansiyon Uç-organ hasarı	Periferik ödem, Baş dönmesi, Baş ağrısı, Yüzde kızarma, Diş eti hiperplazisi
Flordipin	L- ve T-Tipi	Hipertansiyon	Refleks taşikardi
Klevidipin	L-Tipi	Hipertansiyon	Atriyal fibrasyon başlangıcı, Baş ağrısı, Kızarma, Ateş, Mide bulantısı, Kusma
Lasidipin	L-Tipi	Hipertansiyon	Baş dönmesi, Baş ağrısı, Yorgunluk, Yüzde kızarma, Taşikardi, Bulantı, Poliüri
Barnidipin	L-Tipi	Hipertansiyon Kronik stabil anjina	Periferal ödem, Baş ağrısı, Yüzde kızarma, Çarpıntı
Lerkanidipin	L- ve T-Tipi	Hipertansiyon Kronik stabil anjina Raynaud sendromu	Periferik ödem, Baş dönmesi, Yorgunluk, Yüzde kızarma, Çarpıntı, Diş eti hiperplazisi
Mibefradil	L- ve T-Tipi	Hipertansiyon Kronik stabil anjina	Normal olmayan QT uzaması
Diltiazem	L-Tipi	Hipertansiyon Kronik stabil anjina Vazospastik anjina	Periferik ödem, Baş dönmesi, Baş ağrısı, Mide bulantısı, Döküntü
Verapamil	L-Tipi	Hipertansiyon Kronik stabil anjina Vazospastik anjina Stabil olmayan anjina Supraventrikuler taşiaritmilerde	Periferik ödem, Baş dönmesi, Baş ağrısı, Mide bulantısı, Kabızlık
Gallopamil	L-Tipi	Hipertansiyon Kronik stabil anjina Kardiak aritmi	Periferik ödem, Mide bulantısı
Lidoflazin	L-Tipi	Hipertansiyon Kronik stabil anjina	Periferik ödem, Baş ağrısı, Yüzde kızarma, Düzensiz kalp atımı
Fendilin	L-Tipi	Hipertansiyon Kronik stabil anjina	Periferik ödem, Baş dönmesi, Baş ağrısı, Kabızlık
Perheksilin	L-Tipi	Kronik stabil anjina	Baş dönmesi, Mide bulantısı, Uyuşukluk, Titreme, Nörotoksisite, Hepatotoksisite
Bepridil	L-Tipi	Hipertansiyon Kronik stabil anjina	Baş ağrısı, Döküntü, Mide bulantısı, Renal fonksiyon testlerinde bozukluk, Karaciğer fonksiyon testlerinde bozukluk, İdrar testlerinde bozukluk

Çok sayıda desteklenen veri ile KKB'lerin hipertansiyon tedavisinde kan basıncını etkili şekilde azalttığı bilinmektedir. Bu yüzden KKB'ler, bazı ulusal komiteler tarafından kan basıncını düşürmek için tek başına veya diğer tedavilerle kombinasyon halinde başlangıç tedavisi için önerilmektedir. Ayrıca diğer ilaç gruplarıyla kombine tedavi, tedavinin etkinliğini artırabilir ve yan etki riskini azaltabilir. Bu tür kombinasyon tedavilerde optimal dozaj ve kombinasyon stratejilerinin belirlenmesi için daha fazla klinik çalışmaya ihtiyaç vardır.

Kalsiyum kanal blokörlerinin mevcut kullanım alanlarının ötesinde nöroprotektif etkileri, antikanser özellikleri ve metabolik bozukluklarda potansiyel faydaları üzerine yapılan araştırmaların umut verici sonuçları her geçen gün artmaktadır. Özellikle, KKB'lerin nörolojik hastalıklar ve onkoloji alanındaki potansiyel terapötik etkileri, araştırılması ve üzerinde durulması gereken bir durumdur.

Terapötik önemleri göz önüne alındığında, kalsiyum kanallarının ve kalsiyum kanal blokörü ilaç araştırmalarının daha iyi anlaşılması, gelecek için büyük bir potansiyel barındırmaktadır. Yeni nesil KKB'ler ve bu bileşiklerin farklı kimyasal modifikasyonları, daha spesifik ve etkili tedavi seçeneklerinin geliştirilmesinin yanısıra yan etki profilinin iyileştirilmesine yönelik çalışmalara ışık tutmaktadır.

YAZAR KATKILARI

Kavram: M.C.Ü.; Tasarım: İ.H., S.G.K., M.C.Ü.; Denetim: İ.H., S.G.K., M.C.Ü.; Kaynaklar: İ.H., S.G.K., M.C.Ü.; Malzemeler: - ; Veri Toplama ve/veya İşleme: - ; Analiz ve/veya Yorumlama: - ; Literatür Taraması: İ.H., S.G.K., M.C.Ü.; Makalenin Yazılması: İ.H., S.G.K., M.C.Ü.; Kritik İnceleme: İ.H., S.G.K., M.C.Ü.; Diğer: -

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

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DERLEME MAKALE / REVIEW ARTICLE

ÖZEL TIBBİ AMAÇLI GIDALARIN DEĞERLENDİRİLMESİ

EVALUATION OF FOODS FOR SPECIAL MEDICAL PURPOSES

Duygu YILMAZ USTA[1]*

[1]Gazi Üniversitesi, Eczacılık Fakültesi, Farmasötik Teknoloji Anabilim Dalı, 06330, Ankara, Türkiye

ÖZ

Amaç: *Bu çalışmada özel tıbbi amaçlı gıdaların tanımı ve kapsamı, tercih edildiği durumlar, ilaç ve takviye edici gıdalardan farkı, Türkiye ve dünyada özel tıbbi amaçlı gıdalara yönelik yasal düzenlemeler ve uygulanması, küresel pazarda payı ve pazarın önümüzdeki beş yıllık dönem içinde değişimi hakkındaki durum incelenmektedir.*

Sonuç ve Tartışma: *Özel tıbbi amaçlı gıdalar, tıbbi gözetim altında ağızdan tüketilmek veya uygulanmak üzere özel olarak formüle edilen, bilimsel ilkelere dayalı olarak farklı beslenme gereksinimlerinin tıbbi değerlendirmeyle belirlendiği bir hastalık veya durumun spesifik diyet yönetimi için tasarlanan ürünlerdir. Bu ürünler ilaç ve gıda desteklerinden ayrı bir grup olarak değerlendirilmesi gerekmektedir. Özel tıbbi amaçlı gıdalar için dünya genelinde yasal düzenlemeler açısından benzer yaklaşımlar bulunmaktadır. Bununla birlikte Türkiye 2023 yılında yayımladığı ruhsat yönetmeliği ile özel tıbbi amaçlı gıdaların ilaçlarda olduğu gibi ruhsatlandırma prosedürüne tabi tutulacağını, sadece eczanelerde ve hekim reçetesi ile satılacağını beyan etmiştir. Bu uygulama ile Türkiye Cumhuriyeti Sağlık Bakanlığı Türkiye İlaç ve Tıbbi Cihaz Kurumu özel tıbbi amaçlı gıdalar için ruhsat uygulayıcı ilk otorite olarak diğer ülkelere de örnek teşkil etmektedir. Özel tıbbi amaçlı gıda pazarının 2021-2030 döneminde büyük bir büyüme yaşayacağı, 2021-2028 dönemi için %5.7 bileşik bazda yıllık büyüme oranı ile pazar artışı göstereceği ve pazarın gelişmekte olduğu ülkelerde artan yeniliklerle birlikte daha fazla ürün tanıtım faaliyetlerinin pazarın gelişimini artıracağı öngörülmektedir. Yaşam boyu süren kronik hastalıkların artan yaygınlığı, çeşitli kardiyovasküler, kronik ve nöropati hastalıkları yaşayan geriatrik nüfusun hızla büyümesinden dolayı beslenmeye yönelik çok sayıda tıbbi gıda ve besin desteğinin piyasaya çıkacak olmasının da pazarın önümüzdeki yıllarda büyümesine katkıda bulunacağı beklenmektedir.*

Anahtar Kelimeler: *Enteral beslenme, özel tıbbi amaçlı gıda, yasal düzenlemeler*

ABSTRACT

Objective: *In the present study, the definition and scope of food for special medical purposes, the situations in which they are preferred, their difference from drugs and supplementary foods, the legal regulations and implementation of foods for special medical purposes in Turkey and the world, their share in the global market and the situation about the change of the market in the next five years will be examined.*

Result and Discussion: *Foods for special medical purposes are specially formulated to be consumed or administered orally under medical supervision, are designed for the specific dietary management of a disease or condition, where different nutritional requirements are determined by medical evaluation based on scientific principles. They should be evaluated as a separate group from medicines and food supplements. There are similar approaches to legal regulations around the world for foods for special medical purposes. However, with the licensing regulation published*

* **Sorumlu Yazar / Corresponding Author:** Duygu Yılmaz Usta
e-posta / e-mail: yilmazduyguusta@gazi.edu.tr, **Tel. / Phone:** +903122023052

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in 2023, Turkey declared that foods for special medical purposes will be subject to the licensing procedure as in drugs and will be sold only in pharmacies and subject to a physician's prescription. With this regulation, the Ministry of Health of the Republic of Türkiye, the Turkish Medicines and Medical Devices Agency, sets an example for other countries as the first legal authority to apply licenses for foods for special medical purposes. It is anticipated that the foods for special medical purposes market will experience major growth in the 2021-2030 period, the market will increase with a compound annual growth rate of 5.7% for the 2021-2028 period. It is thought that more product launch activities along with increasing innovations in countries where the market is developing will increase the development of the market. Due to the increasing prevalence of lifelong chronic diseases and the rapid growth of the geriatric population suffering from various cardiovascular, chronic and neuropathy diseases, it is expected that many medical foods and nutritional supplements for nutritional management will come to the market, contributing to the growth of the market in the coming years.
Keywords: *Enteral nutrition, food for special medical purposes, legal regulations*

GİRİŞ

Beslenmenin yaşamsal aktiviteyi sağlayan ve yaşam için temel bir gereksinim olan ana faktörlerden biri olduğu bilimsel olarak kanıtlanmış ve yaygın olarak kabul edilmektedir. Günümüzde beslenme sağlıklı olma durumunun sürdürülmesinde olduğu kadar hastalıkların ortaya çıkması, önlenmesi ve tedavisinde de kilit mekanizmalardan biri olarak önemli bir rol oynamaktadır [1].

Beslenme ve sağlıkla ilişkili hastalıklara dair bilgi birikimimiz son 50 yılda oldukça genişlerken, beslenme araştırmaları ve beslenmeye yönelik özel ürünlerin geliştirilmesi konusundaki çalışmalarda bu bilgilerle hız kazanmıştır.

Bazı kronik ve/veya doğuştan gelen kalıtsal hastalıklar ve/veya bazı tıbbi durumlar (kilo kontrolü ve hastanede yatan hastalar gibi) normal gıdaların tüketilmesini, emilmesini veya metabolize edilmesini engelleyebilir, yutmada zorluklara yol açabilir. Bununla birlikte yetersiz beslenme de sadece gelişmekte olan ülkelerde önemli bir sağlık sorunu değil, aynı zamanda dünyada ve Avrupa'da da çeşitli şekillerde görülmektedir. Dünya Sağlık Örgütü (World Health Organization-WHO)'ne göre yetersiz beslenme, "bir kişinin enerji ve/veya besin alımındaki eksiklikler, fazlalıklar veya dengesizlikler" anlamına gelir ve yetersiz beslenmenin yanı sıra aşırı kilo, obezite veya mikro besin öğelerinin yetersiz alımını da içerir [2]. Hastalıkla ilişkili yetersiz beslenme durumunda ise altta yatan sorun, etkilenen bireylerin normal gıda alımının mümkün olmaması veya vücutlarının beslenme talebini karşılamak için dengeli bir beslenmeden yeterince yararlanamaması şeklinde olabilir.

Yetersiz beslenme enfeksiyon, yara iyileşmesinin bozulması ve ölüm riskini artırırken iyileşmenin gecikmesi, hastanede kalma süresinin uzaması ve sağlık bakım masraflarının artışına da neden olabilir. Tüm bu sorunların farkına varılması, sadece halk sağlığı açısından değil, ekonomik açıdan da önleyici strateji ve çözümlerin oluşturulması için önem taşımaktadır.

Metabolik ihtiyaçların eksiksizliği ve karşılanması, acil durumdaki insanlar için, özellikle duygusal ve fiziksel aşırı yük, stres, hastalık ve yaralanmalar gibi olaylarda çok önemlidir. Bu gibi durumlarda, belirli ihtiyaçlara göre uyarlanmış özel yiyecekler, hastalığa bağlı yetersiz beslenmeye karşı korunmak için değerli bir çözüm olabilir [3]. Bu nedenle gıda ürünlerinin özel tıbbi amaçlarla tüketilmesi (kullanılması) oldukça yaygınlaşmıştır. Bu ürünler, özel ihtiyaçları karşılamak ve fizyolojik durumu normalleştirmek için bilimsel temelli ve amaca uygun olarak seçilmiş besin maddeleri ve biyolojik olarak aktif maddelerin bileşimlerinden oluşmaktadır [4,5].

Özel tıbbi amaçlı ürün pazarına ait ürün çeşitlerinin her düzeyde istikrarlı bir büyüme gösterdiği tespit edilmiştir. Bu durum, hem tedavi ve iyileşme koşullarını iyileştirmeye yönelik genel eğilim, işlevsellik odaklı ürünlerin kullanımı hem de koronavirüs enfeksiyonu (COVID-19) yayılmasının bir sonucu olarak halkın kendi sağlığı konusunda artan endişesi ile açıklanmaktadır [6].

1972'den önce özel amaçlı tıbbi gıdalar öncelikle kalıtsal metabolik hastalıkları olan hastaların tedavisine yönelik formüllerdi ve çoğunlukla sınırlı nüfus için olduğundan "yetim" ürünler ve kullanımlarının tıbbi gözetim altında olmasını sağlamak için ilaç olarak kabul edilmekteydi. Üretim ve satış için Amerika Birleşik Devletleri (ABD) Gıda ve İlaç İdaresi'nden (The US Food and Drug Administration-FDA) onay almadan önce diğer tüm yeni ilaçlar için gerekli olan kimyasal, biyolojik

etkiler, görünür etkinlik ve toksisite ile ilgili aynı kapsamlı belgelere tabi tutulması gerektiği anlamına geliyordu. 1972'de FDA, özel amaçlı tıbbi gıdaların gelişimini ve bulunabilirliğini artırmak için bu ürünleri ilaç grubundan çıkararak özel diyet kullanımına yönelik gıdalar olarak yeniden sınıflandırdı [7]. Aradan geçen yıllarla "tıbbi gıdalar" olarak sınıflandırılan çok çeşitli ürünler geliştirilmiş, halihazırda pazarlanan çok çeşitli iddialara sahip tıbbi gıdalar, tüm yaş gruplarında kritik hastaların, kronik ve kalıtsal hastaların tedavisinde ve rutin yaşamında destek yöntemi olarak yaygın şekilde kullanılmaktadır.

Bu çalışma ile günümüzde gittikçe önem kazanan özel tıbbi amaçlı gıdalar, bunların Türkiye ve dünya üzerindeki yasal düzenlemeleri, değerlendirmeleri ve küresel pazar durumu hakkındaki bilgileri derlenmiştir.

Özel Tıbbi Amaçlı Gıda

Özel tıbbi amaçlı gıda tanımına baktığımızda sağlık çalışanları, üreticiler ve yasal düzenleyici otoriteler tarafından pek çok farklı tanım yapıldığı ve terminolojinin oldukça karmaşık olduğu görülmektedir [8]. Ayrıca tıbbi gıda pazarının karşılaştığı en büyük zorluklardan biri sağlık hizmeti sağlayıcıları ve tüketiciler arasındaki farkındalık eksikliğidir. Birçok sağlık hizmeti sağlayıcısı tıbbi gıdalara aşina değildir ve bunları hastalara ne zaman önereceklerini bilmeyebilir. Tüketiciler arasında tıbbi gıdalar ile besin takviyeleri arasındaki fark konusunda da bilgi eksikliği vardır ve bu da kafa karışıklığına yol açmaktadır. Sağlık hizmeti sağlayıcıları ve düzenleyiciler için tıbbi gıdalara ilişkin farkındalığın ve eğitimin artırılması ile hastalara mümkün olan en iyi bakımı alma fırsatının oluşturulacağı düşünülmektedir [9].

Özel tıbbi amaçlı gıdalar (Foods for Special Medical Purposes-FSMP) hastalığa bağlı yetersiz beslenmeye ve bunun sonuçlarına karşı korunmak amacıyla, ilgili hasta grupları için hayati öneme sahiptir. Farklı form ve bileşimlerde, çeşitli beslenme sorunları olan tüm yaş gruplarını kapsayan birçok farklı hasta grubunda beslenmeyi desteklemek için kullanılmaktadır [3,10]. "Tıbbi gıdalar" terimi, hastalara verilen tüm gıdalar için geçerli değildir. Tıbbi gıdalar (doğal haliyle kullanılan, doğal olarak oluşan gıda maddelerinin aksine) destekleyici veya ana tedavi yöntemi olarak ürüne ihtiyaç duyan hastalar için özel olarak formüle edilmiş ve işlenmiş gıdalardır. Tıbbi gıdalar, özel diyet kullanımına yönelik daha geniş gıda kategorisinden, besin desteklerinden ve sağlıkla ilgili iddialarda bulunan gıdalardan tıbbi gözetim altında kullanılması gerekliliğiyle ayrılır. Özel tıbbi amaçlı gıdalar, besin destekleri ve reçeteli ilaçlar arasındaki farklar Tablo 1'de verilmektedir [11].

Tablo 1. Özel tıbbi amaçlı gıdalar, besin destekleri ve reçeteli ilaçlar arasındaki farklar

	Tıbbi amaçlı gıda	Besin destekleri	Reçeteli ilaçlar
Oral kullanım	✓	✓	
Medikal gözlem gerekliliği	✓		✓
Hastalığa spesifik besinsel eksikliği karşılaması	✓		
Sağlıklı insanların kullanımına yönelik		✓	
Klinik çalışmalar ile etkinliğinin kanıtlanması	✓		✓

Yukarıda verilen bilgiler doğrultusunda özel amaçlı tıbbi gıdalar:

- Bir doktorun gözetiminde enteral (ağızdan) olarak tüketilmek veya uygulanmak üzere özel olarak formüle edilmiş
- Kanıtlanmış bilimsel ilkelere dayalı olarak farklı beslenme gereksinimlerinin tıbbi değerlendirmeyle belirlendiği bir hastalık veya durumun spesifik diyet yönetimi için tasarlanmış ürünler olarak tanımlanabilir.

Özel tıbbi amaçlı gıdaların uygulanmasını tanımlayan klinik terim ise enteral beslenme olup, vücudun besin ihtiyacının tamamının veya bir kısmının karşılanmasına katkıda bulunmak amacıyla

besin solüsyonunun ağız yoluyla veya beslenme kateteri aracılığıyla uygulanması olarak bilinmektedir [12]. Oral beslenme takviyeleri ve tüple beslenme enteral beslenme için veriliş yolları olup bu terimin doğru kullanımına ilişkin literatürde çok fazla değişkenlik vardır. Birçok araştırmacı "enteral" teriminden bahsederken yalnızca tüple beslenmeyi düşünmektedir. Ancak en son geliştirilen Avrupa kılavuzları (European Society for Clinical Nutrition and Metabolism-ESPEN) "Enteral Beslenme"yi hem oral takviye hem de tüple beslenmeyi içerecek şekilde tanımlamaktadır.

Özel tıbbi amaçlı gıdaların sınıflandırılması ve içeriklerinde sıklıkla kullanılan bileşenler Tablo 2'de verilmiştir [7,13].

Tablo 2. Özel tıbbi amaçlı gıdaların sınıflandırılması ve sıklıkla içeriklerinde kullanılan bileşenler

Beslenme açısından tam formüller	Üreticinin bildirdiği talimatlara uygun olarak kullanıldığında, kullanan kişilerin beslenme kaynağını tek başına oluşturabilen, standart besin ögelerini içeren, beslenme yönünden tam olan ürünler / bir hastalık ya da sağlık sorununa özel geliştirilmiş, beslenme yönünden tam olan ürünlerdir. Et, süt, kazeinat, soya, laktoz veya lif içeren ve içermeyen, önceden sindirilmiş protein veya kristal amino asitler, şeker, hidrolize mısır nişastası, orta zincirli yağlar, mısır ve soya fasulyesi yağı, vitaminler, mineraller ve elektrolitler.
Kullanımdan önce diğer ürünlerle karıştırılabilen besin açısından eksik formüller	Tek başına beslenme kaynağı olarak kullanımı uygun olmayan, standart formüllü veya bir hastalık ya da sağlık sorunu için besin ögeleri içeriği özel uyarlanmış, beslenme yönünden tam olmayan ürünlerdir. Sadece protein, sadece karbonhidrat, sadece vitamin, sadece mineral, emülsifiye yağ.
Metabolik (genetik) bozukluklara yönelik formüller	Belirli besinlerin çıkarıldığı formüller (Fenilalanin, dallı zincirli amino asitlerin bulunmaması gibi)
Oral rehidrasyon ürünleri	Sodyum, klorür, potasyum sitrat, dekstroz, su

Bilinen ilk özel tıbbi amaçlı gıda Lofenalac olup, 1957 yılında Mead Johnson firması tarafından geliştirilmiştir ve vücudun fenilalanin adı verilen bir amino asidi parçalamasını önleyen nadir bir durum olan fenilketonüri hastası bebekler için ilk ticari formül olarak piyasaya çıkmıştır.

Yasal Otoritelere Göre Özel Tıbbi Amaçlı Gıdalar

Türkiye'de Özel Tıbbi Amaçlı Gıdaların Değerlendirilmesi ve Ruhsatlandırma Süreçleri

Türkiye'de özel tıbbi amaçlı gıdalar, Türkiye Cumhuriyeti Sağlık Bakanlığı Türkiye İlaç ve Tıbbi Cihaz Kurumu Bitkisel ve Destek Ürünler Dairesi Başkanlığı tarafından denetlenmekte ve sıkı düzenlemelere tabidir. Üretim, etiketleme ve pazarlama süreçlerinde belirli standartlar ve yönergeler uygulanmakta, bu süreçler ile kullanıcıların güvenliğini ve sağlığını korumayı hedeflemektedir.

28 Ocak 2023 tarihinde Resmî Gazetede yayımlanan "Özel Tıbbi Amaçlı Gıdalar Ruhsatlandırma Yönetmeliği" ile (Resmî Gazete, Sayı: 32087, 2023) [14]:

- Özel tıbbi amaçlı gıdalarda beslenme ve sağlık beyanları kullanılamayacağı
- Özel tıbbi amaçlı gıdalarda ilaçlarda olduğu gibi ruhsatlandırma prosedürüne tabi tutulacağı, sadece eczanelerde ve hekim reçetesine tabi olarak satılacağı
- Kurum tarafından ruhsat düzenlenmeyen hiçbir özel tıbbi amaçlı gıdanın piyasaya sunulamayacağı
- Yönetmeliğin yürürlüğe girdiği tarihten önce Sağlık Bakanlığı Türkiye İlaç ve Tıbbi Cihaz Kurumu tarafından onaylanarak ithal veya imal izni düzenlenmiş ve bu şekilde piyasaya arz edilmiş ürünler için en geç 31 Aralık 2025 tarihine kadar bu yönetmelik hükümlerine uygun olarak ruhsat alınmasını zorunlu kılınmıştır.

Bu uygulama ile Türkiye, dünya genelinde özel tıbbi amaçlı gıdalar için ruhsat gerekliliği arayan ilk resmi otorite olarak diğer ülkelere örnek teşkil etmektedir. Bu yönetmelikle özel tıbbi amaçlı

gıdaların istenen etkililik, güvenlilik ve gereken kaliteye sahip olmalarını sağlamak üzere ruhsatlandırma işlemlerinde ve ruhsatlandırılmış ürünlere ilişkin iş ve işlemlerde uygulanacak usul ve esasları belirlenmesi amaçlanmıştır.

Yönetmelik kapsamında özel amaçlı tıbbi gıda, besin ögelerini veya metabolitlerini vücuda alma, sindirme, absorbe etme, metabolize etme ve vücuttan atma kapasitesi sınırlı, azalmış veya bozulmuş olan ve diyet yönetimleri normal diyetin düzenlenmesi ile sağlanamayan hastaların diyetlerini düzenlemek amacıyla tıbbi gözetim altında kullanılmak üzere geliştirilen, özel olarak formüle edilip endüstriyel yöntemlerle elde edilen, beslenme yönünden tam veya tam olmayan ürünleri ifade eder. Buna göre özel tıbbi amaçlı gıdalar aşağıdaki özelliklere sahip olmalıdır [15]:

- Formülasyonları uluslararası geçerliliği olan tıp ve beslenme prensiplerine dayanmalı, üreticinin bildirdiği talimatlara uygun olarak kullanıldığında kullanan kişilerin özel beslenme gereksinimlerini karşılamada güvenli, yararlı ve etkili olduğu bilimsel verilerle desteklenmelidir.
- Bebeklerin beslenme gereksinimlerini karşılamak için geliştirilen özel tıbbi amaçlı gıdalar ve bunun dışındaki özel tıbbi amaçlı gıdalar ile ilgili hususlar ilgili kılavuzda belirtilen bileşim özelliklerine uygun olmalıdır.
- Üretimlerinde, besin ögelerine ihtiyacı karşılamak amacıyla yalnızca ilgili kılavuzda listelenen vitaminler, mineral maddeler, amino asitler ve diğer azot içeren bileşikler, diğer besin ögeleri kullanılabilir.

Bununla birlikte “Özel Tıbbi Amaçlı Gıdalar Yönetmeliği” kapsamında:

- “Türk Gıda Kodeksi Bebek ve Küçük Çocuklara Yönelik Gıdalar ile Vücut Ağırlığı Kontrolü İçin Diyetin Yerini Alan Gıdalar Yönetmeliğinde” tanımlanan sağlıklı bebek ve küçük çocuklar için üretilen bebek formüllerini, devam formüllerini, bebek ve küçük çocuk ek gıdalarını, vücut ağırlığı kontrolü için diyetin yerini alan gıdaları,
- “Türk Gıda Kodeksi Gıda Etiketleme ve Tüketicileri Bilgilendirme Yönetmeliğine” göre glutenin gıdadaki yokluğu veya azaltılmış varlığı hakkında ifade içeren gıdaları,
- “Türk Gıda Kodeksi Sporcu Gıdaları Tebliği (Tebliğ No: 2003/42)”nde tanımlanan sporcu gıdaları,
- “Türk Gıda Kodeksi Takviye Edici Gıdalar Tebliği (Tebliğ No: 2013/49)”nde tanımlanan takviye edici gıdalar yer almamaktadır.

Ürünlerin iç ve dış ambalajlarında “Özel Tıbbi Amaçlı Gıda” ifadesi, bileşenler listesi, ürünün kullanılması amaçlanan hangi hastalık ya da sağlık sorunuyla ilgili olduğu, yaş grubu, tıbbi gözetim altında kullanılması gerektiği gibi yönetmelikte yer verilen açıklama ve bilgileri yer almalıdır. Ambalaj, hijyen kriterleri, katkı maddeleri, aroma vericiler ve bulaşanlar, mikrobiyolojik kriterler, numune alma ve analiz metotları gibi özel tıbbi amaçlı gıdalar ile ilgili kriterler için “Özel Tıbbi Amaçlı Gıdaların Stabilite Çalışmalarına İlişkin Kılavuz”u, “Özel Tıbbi Amaçlı Gıdaların Ambalaj Bilgilerine ve Okunabilirliklerine İlişkin Kılavuz”u, hüküm bulunmayan hususlarda ise Türk Gıda Kodeksi ilgili mevzuat hükümleri dikkate alınmalıdır [16-18].

Dünya’da Özel Tıbbi Amaçlı Gıdalar

Dünya genelinde birden fazla paydaşın ihtiyaçlarını karşılayan özel tıbbi amaçlı gıda/tıbbi gıda düzenlemelerinin ortak unsurları bulunmaktadır ve genel anlamda özel tıbbi amaçlı gıda veya tıbbi gıda düzenlemeleri için tanımları, kısıtlamaları ve fırsatları, birçok ülke arasında dikkate değer benzerlikler göstermektedir.

Sağlık profesyonellerinin, hangi hastaların hangi ürünlerden fayda sağlayacağı ve özel olarak formüle edilmiş beslenme ürünlerinin hangi tıbbi durumları ele aldığına ilişkin klinik verileri desteklemek için açık, bilimsel olarak geçerli ve ayrıntılı ürün bilgilerine; üreticilerin, ürünü güvenle üretmek ve pazarlamak için hasta/hastalık uygunluk kriterlerine, iyi üretim uygulamaları (Good Manufacturing Practice-GMP) standartlarını ve her türlü kayıt veya onay sürecini belirleyen iyi tanımlanmış düzenlemelere ihtiyacı vardır.

Düzenleyici kurumlar, basit diyet değişikliklerinin/düzenlemelerinin başaramadığı durumu telafi etmek için özel olarak formüle edilmiş özel tıbbi amaçlı gıdalar gerektiren çeşitli hastalıkların farklı beslenme gereksinimlerini karakterize etmek için araştırmalara ve tıp bilimcilerine güvenmektedir. Ancak, daha önce de belirtildiği gibi, diyetteki “biyoaktif bileşenlerin” sağlığa nasıl katkıda bulunduğu

ve hatta hastalıktan kaynaklanan sağlıksız durumları nasıl yeniden dengelediği konusunda daha bütünleşmiş bir fizyolojik, metabolik ve hücresel anlayışa ihtiyaç vardır [19]. Bu, genel "sağlıklı nüfus" için belirlenen günlük gereksinimlerle birlikte önceden tanımlanmış besinleri de içermelidir; ancak kapsamlı araştırma sonuçları artık enzimlerin, substratların ve metabolik olarak aktif bileşenlerin diyet kaynaklarının da dikkate alınması gerektiğini göstermektedir. Devlet veya üçüncü taraf sağlık hizmeti ödeyenler, tipik "gıda" ile karşılaştırıldığında özel içeriklerin daha yüksek maliyetinin geri ödenmesini haklı çıkarmak için özel tıbbi amaçlı gıdaları hastalık yönetiminin önemli bir bileşeni olarak düşünmelidir.

Sağlık ve hastalık yönetimindeki potansiyel destekleri nedeniyle ilgi çeken özel tıbbi amaçlı gıdalar için dünya genelinde prensipte GMP dahil olmak üzere genel gıda ve bazı özel mevzuatlara (yeni gıda, kirletici maddeler, katkı maddeleri ve paketleme gibi konularda) tabidir ve kullanım amaçlarına göre etiketlendiği gibi güvenli olmalı, kaliteli olmalı, yanıltıcı iddialarda bulunmamalıdır [19].

Bu derleme makalede FDA, Kodeks Alimentarius komisyonu ve Avrupa Birliği (AB)'nin özel tıbbi amaçlı gıdalara ilişkin görüşlerine kapsamlı olarak değinilirken, ülke bazındaki değerlendirmeler genel anlamda bu üç yasal düzenleyici tarafından sağlandığından daha sınırlı yer verilecektir.

FDA

İlaçlardan ayrı olarak bir hastalığı veya özel durumu olan hastaların spesifik beslenme yönetimine yönelik bir tıbbi gıda kategorisi oluşturulmakta zorlanılmış, "tıbbi gıda" terimi ilk kez 1988'de değiştirilen "Yetim İlaç Yasası" ile tanımlanmıştır. Bu Yetim İlaç Yasası'nın (21 U.S.C. 360ee(b)(3)) 5(b)(3) bölümünde tanımlandığı gibi tıbbi gıda: "Bir doktorun gözetimi altında enteral olarak tüketilmek veya uygulanmak üzere formüle edilen, kanıtlanmış bilimsel ilkelere dayalı olarak, tıbbi değerlendirme ile belirlenen farklı beslenme gereksinimlerinin olduğu bir hastalık veya durumun özel diyet tedavisi için verilmesi amaçlanan bir gıdadır" [20].

Tıbbi gıdalar, semptomları yönetmek veya bir hastalık veya durumun riskini azaltmak için genel bir diyetin parçası olarak bir doktor tarafından basitçe tavsiye edilen gıdalar değildir. Diyet yönetimi gerektiren hastalıklar da dahil olmak üzere, bir hastalığı olan hastalara verilen gıdaların tümü tıbbi gıda değildir. Tıbbi gıdalar, özel diyet kullanımına yönelik daha geniş gıda kategorisinden, tıbbi gıdaların bir hastalık veya durumun kendine özgü beslenme gereksinimlerini karşılaması, tıbbi gözetim altında kullanılması ve bir hastalık veya durumun özel diyet yönetimine yönelik olması gerekliliğiyle ayrılır. FDA, tıbbi gıdaların yasal tanımını, bu gıda kategorisine giren ürün türlerini dar bir şekilde sınırlamak için dikkate almaktadır (21 CFR 101.9(j)(8)) [20].

FDA tarafından genel olarak bir ürünün tıbbi gıda olarak kabul edilebilmesi için aşağıdaki kriterleri karşılaması gerekir:

- Ürünün ağızdan veya tüple beslenmeye uygun bir gıda olması
- Ürün tıbbi bir bozukluğun, hastalığın veya durumun diyet yönetimine yönelik olarak verilmesi
- Ürün tıbbi gözetim altında kullanılmak üzere ve öncelikli olarak hastaneler, klinikler ve diğer tıbbi ve uzun süreli bakım tesislerinde kullanılması
- Güvenli ve etkili olması
- Tıbbi gıdalardaki bileşenlerin, en yüksek güvenlik standardı olan FDA tarafından Genel Olarak Güvenli (Generally Recognized as Safe-GRAS) sınıflandırmasına dahil olması
- Diyet takviyelerinin (vitaminler ve nutrasötikleri içerebilen) aksine, tıbbi gıdalar kapsamlı güvenlik verileri, yayınlanmış, hakemli insan çalışması verileri ile desteklenmelidir.

FDA'ya göre tıbbi gıdalar için reçete zorunluluğu yoktur. Bu nedenle özellikle ilaçlara uygulanan herhangi bir düzenleme gerekliliğine tabi değildir. Ancak, Amerika Birleşik Devletleri'nde tıbbi gıdaların üretimi, işlenmesi, paketlenmesi veya tüketilmesi için saklanması ile uğraşan herhangi bir tesisin FDA'ya kaydolması gerekir. Özel tıbbi amaçlı gıdalar hakkında daha ayrıntılı bilgiler FDA'nın internet sayfasında "Medical Foods Guidance Documents & Regulatory Information" bölümünde bulunmaktadır [21].

Kodeks Alimentarius Komisyonu

Kodeks Alimentarius veya "Gıda Yasası", gıda, gıda üretimi, gıda etiketleme ve gıda güvenliği ile ilgili olarak Birleşmiş Milletler Gıda ve Tarım Örgütü (Food and Agriculture Organization-FAO) ve

WHO tarafından kurulan Kodeks Alimentarius Komisyonu tarafından yayınlanan uluslararası kabul görmüş standartlar, uygulama kuralları, kılavuzlar ve diğer tavsiyelerin bir koleksiyonudur [22].

Kodeks Alimentarius Komisyonu 1963 yılında çalışmalarına başlamış, 2019 yılı itibariyle komisyonda 188 üye devlet bulunmaktadır [23]. Komisyonun görevi tüketicilerin sağlığını korumak ve gıda ticaretinde adil uygulamaları sağlamak için uluslararası düzeyde uyumlaştırılmış gıda güvenliği ve kalite standartları geliştirmektir [24]. Her ne kadar Kodeks Alimentarius standartları ve yönergeleri prensip olarak isteğe bağlı olarak takip edilse de bunların, özellikle gelişmekte olan ülkelerde, düzenlemelerin oluşturulması ve yorumlanmasına ilişkin dünya çapındaki süreçler üzerinde önemli bir etkisi vardır.

Kodeks Alimentarius Standart 180-1991, özel tıbbi amaçlı gıdaları, hastaların diyet yönetimi için özel olarak işlenmiş veya formüle edilmiş ve sunulan ve yalnızca tıbbi gözetim altında kullanılabilen, özel diyet kullanımlarına yönelik bir gıda kategorisi (Foods for Special Dietary Uses-FSDU; Kodeks Standart 146-1985) olarak tanımlar [25,26]. Sıradan gıda maddelerini veya bunların içerdiği belirli besin maddelerini alma, sindirme, absorbe etme veya metabolize etme kapasitesi sınırlı veya zayıf olan hastaların özel veya kısmi beslenmesi veya diyet yönetimi yalnızca normal diyetin değiştirilmesi, diğer FSDU'lar veya ikisinin kombinasyonu ile sağlanamayan tıbbi olarak belirlenmiş başka özel besin gereksinimleri olan kişiler için tasarlanmıştır.

Kullanımlarının, amaçlanan hasta kişilerde beslenme gereksinimlerini karşılamada güvenli ve yararlı olduğu bilimsel kanıtlarla gösterilmelidir. Özel tıbbi amaçlı her türlü gıdanın etiketleri, beraberindeki broşürler ve/veya diğer etiketleme ve reklamlar, gıdanın doğası ve amacı hakkında yeterli bilginin yanı sıra bunların kullanımına ilişkin ayrıntılı talimat ve önlemleri de içermelidir. Ayrıca bu ürünlerin kamuya reklamının yapılması yasaktır. Verilen bilgilerin formatı, hedeflenen hasta grubuna uygun olmalıdır.

Avrupa Birliği

Özel tıbbi amaçlı gıdaların AB'de uzun bir geçmişi ve deneyimi bulunmaktadır. 2013 yılında özel tıbbi amaçlı gıdalara ilişkin yasal çerçeve, AB üye ülkeleri arasında uyumlaştırılan Belirli Gruplara Yönelik Gıdalar Tüzüğü (AB) 609/201313 (Foods for Specific Groups Regulation (EU)) kapsamında yeniden gözden geçirilmiş, yönetmelik (AB) 2016/12816 ile özel tıbbi amaçlı gıdalara yönelik özel bileşim ve bilgi gereklilikleri revize edilmiştir [27]. Tüm üye devletlerden gelen uzman girdilerinin bir sonucu olan AB Özel Tıbbi Amaçlı Gıda Tüzüğü bileşim kriterlerine, pazardaki ürünlerin etiketlenmesine, bilgilendirilmesine ve izlenmesine ilişkin özel ve ilgili hükümleri içeren iyi tanımlanmış bir çerçeve sağlar.

Bu düzenlemelere göre özel tıbbi amaçlı gıdalar "ihtiyaçları normal gıdalarla karşılanamayan kişilerin özel veya kısmi beslenmesi amacıyla tıbbi gözetim altında kullanılacak ürünler" olarak tanımlanmıştır ve pazarlama öncesi onaya tabi değildir. Mevzuat, bu tür ürünlerin hastalıkların, bozuklukların veya tıbbi durumların diyet yönetimine yönelik kullanım amaçlarını karşılamak üzere sağlam tıbbi ve beslenme ilkelerine (örneğin, ESPEN kılavuzları) dayalı olarak formüle edilmesinde esnekliğe izin vermektedir [28].

Çoğu AB üye ülkesinde, özel tıbbi amaçlı gıda olarak bildirilen birçok ürün, yerel sağlık sistemlerinin (kamu veya özel ödeme yapanlar) bir parçası olarak geri ödenebilir. Bu, tipik olarak, tek besin kaynağı olarak hizmet edebilen, beslenme açısından eksiksiz özel tıbbi amaçlı gıdaları içerir. Geri ödeme seviyeleri ayrıca belirli kategorilere (örneğin bebekler/yetişkinler, hidrolize/hidrolize olmayan proteinler) göre belirli bileşim kriterlerine (örneğin protein, enerji düzeyi [Kcal/KJ], lif) veya ulusal kılavuzlara göre belirlenebilir. Hastalıkla ilişkili yetersiz beslenme için tasarlanan ürünlerin yanı sıra, inek sütü proteinlerine karşı bebek alerjisi veya çoklu gıda alerjileri, malabsorbsiyon sendromları (örneğin, kısa bağırsak sendromu), karbonhidrat, yağ veya protein metabolizmasında kalıtsal doğuştan hatalar (örneğin, fenilketonüri) ve epilepsi (ketojenik diyet formülleri) gibi hastalıkları veya tıbbi bozuklukları yöneten belirli özel tıbbi amaçlı gıdalar için de geri ödeme mümkün olabilir:

Tablo 3'te FDA, Kodeks Alimentarius Komisyonu ve AB'ye göre küresel özel tıbbi amaçlı gıda/tıbbi gıdalar için yasal düzenlemeler çerçevesinde genel bir bakış sunmaktadır [19]. Tablo 4'te ise özel tıbbi amaçlı gıdalara/tıbbi gıdalara ilişkin ülkelere göre düzenleyici özete yer verilmiştir.

Tablo 3. FDA, Kodeks Alimentarius Komisyonu ve AB’ye göre özel tıbbi amaçlı gıdalara/tıbbi gıdalara ilişkin düzenleyici özet

	FDA	**Kodeks alimentarius**	**AB**
Geçerli yasal tanım	Bölüm 5(b) Yetim İlaç Kanunu (21 U.S.C. 360ee(b)(3)	Kodeks Alimentarius Standart 180-1991	Regülasyon 178/2002
Tıbbi gıda düzenleme tanımı	21 CFR 101.9(j)(8)		-
Özel tıbbi amaçlı gıda (FSMP) tanımı	-		Regülasyon 609/2013
Mikrobesin bileşenleri	Spesifik olarak belirtilmemiş		Regülasyon 2016/128, Ek I
Etiketleme	21 CFR 101.9(j)(8)	Kodeks Standart 146-1985 Kodeks Standart 180-1991	Regülasyon 1169/2011 Regülasyon 2016/128
Besinler	Spesifik olarak belirtilmemiş	CAC/GL 10-1979	Regülasyon 609/2013 Regülasyon 2016/128
Katkı maddeleri	Spesifik olarak belirtilmemiş	Gıda Katkı Maddeleri için Kodeks Standart 192-1995 Gıda Kategori No. 13.3	Regülasyon 1333/2008

FSMP: Foods for Special Medical Purposes

Tablo 4. Özel tıbbi amaçlı gıdalara/tıbbi gıdalara ilişkin ülkelere göre düzenleyici özet

Ülke	**Yasal düzenlemeler**
Çin	Gıda Güvenliği Yasası [29-31] Özel tıbbi amaçlı gıda (FSMP) tanımı-GB 25596-2010 Mikrobesin bileşenleri-GB 29922-2013 Etiketleme-GB 7718-2010 / GB 13432-2010 Besinler-GB 14880-2012 Katkı maddeleri-GB 2760-2010
Brezilya	ANVISA Özel Amaçlı Gıda Yönetmeliği (Portaria 29/1998) [32] Enteral beslenme formülleri (RDC 21-2015) [33] Enteral beslenme formüllerine eklenebilecek besin bileşikleri ve diğer maddeler (RDC 22-2015) [34] Enteral beslenme formüllerine yönelik spesifik gıda katkı maddelerine ilişkin yönetmelik (RDC 160-2017) [35]
Kanada	Health Canada Kanada Gıda Denetleme Kurumu Bölüm 24'ün ve diğer ilgili gıda düzenlemelerinin uygulanmasından sorumludur. Kanada'da tıbbi gıdalar veya FSMP'ler için düzenleyici hükümler bulunmamaktadır. Çok düşük enerjili diyetlerde kullanılmak üzere sunulan gıdalar haricinde, Kanada'da bir FSDU'yu piyasaya sürmek için herhangi bir bildirim süreci yoktur; bu durumda 90 günlük bir pazarlama öncesi bildirim gereklidir. Kanada FSDU çerçevesi, kapalı kategorik tanımları, mevcut beslenme önerileriyle uyumlu olmayan dar ve esnek olmayan bileşim gereklilikleri ve diğer FSMP çerçeveleriyle uluslararası uyumdan farklılığıyla karakterize edilir [36].
Güney Afrika	Kodeks Alimentarius Gıda maddelerinin etiketlenmesi ve reklamı ile ilgili düzenlemeler (No. R.146-2010) Bebekler için özel beslenme yönetimine yönelik bebek veya takip formülleri, bebekler ve küçük çocuklara yönelik gıda maddelerine ilişkin yerel düzenlemeler (No. R. 991-2012) [37]
Avustralya ve Yeni Zelanda	Avustralya Yeni Zelanda Gıda Standartları (FSANZ) Kodunun 2.9.5 Standardı [38] Bölüm 2.9.5-9 ve 2.9.5-10

FSMP: Foods for Special Medical Purposes/Özel tıbbi amaçlı gıda; FSDU: Foods for Special Dietary Uses/Özel diyet kullanımlarına yönelik gıda

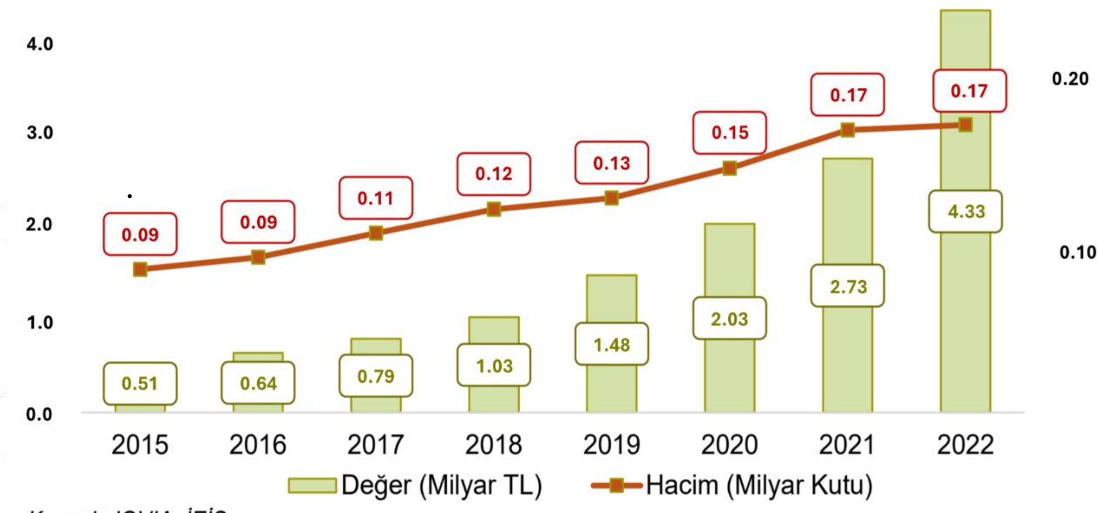
Tablo 4 *(devamı)*. Özel tıbbi amaçlı gıdalara/tıbbi gıdalara ilişkin ülkelere göre düzenleyici özet

Ülke	Yasal düzenlemeler
Japonya	Japon gıda yönetmeliğindeki FSMP standardı, Kodeks Alimentarius standardı 180-1991'den önce oluşturulmuştur ve bu nedenle uluslararası standarttan önceliklidir. FSMP ürünleri, özel beslenme amaçlı kullanıma (Food for Special Dietary Uses-FOSDU) yönelik gıda kategorisine girer. Bir ürünü FOSDU olarak tescil ettirmek için otorite onayına ihtiyaç vardır; bu süreç yaklaşık 2-3 yıl sürer ve kullanım amacını destekleyecek bilimsel kanıt gerektirir.
Hindistan	Kodeks Alimentarius Standardı 180-1991 Gıda Güvenliği ve Standartları (Sağlık Takviyeleri, Nutrasötikler, Özel Diyet Amaçlı Gıdalar, Özel Tıbbi Amaçlı Gıdalar ve Probiyotik ve Prebiyotik Gıdalar) Yönetmeliğinin (2022), FSS (Nutra) Yönetmeliği [39]
Orta Doğu Birleşik Arap Emirlikleri (BAE), Umman, Kuveyt, Katar, Suudi Arabistan Krallığı ve Bahreyn'in de aralarında bulunduğu Körfez İş birliği Konseyi	Özel tıbbi amaçlı gıdaları standartı (GSO 1366-2021) [40] Gıda takviyelerinde yeni standart (GSO 2571-2021) [41] Bebek maması, takip maması ve bebeklere yönelik özel tıbbi amaçlı mama (GSO 2106-2021) [42]
ASEAN Üye Ülkeleri Endonezya, Malezya, Filipinler, Singapur, Tayland ve daha yeni üyeler Vietnam, Brunei Sultanlığı, Kamboçya, Laos ve Myanmar'dan	Birçoğunda FSMP kategorisi henüz net bir şekilde tanımlanmamıştır, ancak bazılarında bazı düzenleme çalışmaları başlamıştır. Kodeks Alimentarius Standardı 180-1991 herkes için önemli ve saygı duyulan bir referanstır [25]. Bununla birlikte ülke bazında bazı aşağıda da belirtilen bazı özel düzenlemelerde bulunmaktadır. **Endonezya** Özel tıbbi amaçlı gıdaların dağıtımı (Eczaneler, hastane eczaneleri ve diğer kamu sağlığı merkezleri)-1-2018 sayılı Yönetmelik Madde 16) [43] Özel tıbbi ihtiyaçlara yönelik bebek maması-Yönetmelik HK.03.1.52.08.11.07235-2011 Madde 1[44] Özel tıbbi ihtiyaçlara yönelik bebek ve çocuk gıdaları-24-2020 Sayılı Değişiklik Yönetmeliği [45] **Vietnam** Özel tıbbi amaçlı gıdaları veya tıbbi gıdalar tanımı-43-2014 Sayılı Genelge Madde 2 [46] Bebeklere yönelik özel tıbbi amaçlı formüller-21-2012 Sayılı Genelge Madde 3 [47] Menşei ülkedeki yetkili makamlar tarafından onaylanmamış FSMP'ler için insan etkinliği klinik çalışmaları-43-2014 Sayılı Genelge Madde 4 [46] **Tayland** Özel amaçlı gıdalar-238 ve 357 Sayılı Tayland Tebliği [48,49] **Malezya** Özel amaçlı gıdalar -Yönetmelik 388 [50] **Singapur** Özel amaçlı gıdalar-Yönetmelik 247 [51] **Brunei Sultanlığı** Özel amaçlı gıdalar-Yönetmelik 318 [52] Düzenleme ve uygulamalar Malezya ve Singapur ile uyumludur. **Filipinler** Özel tıbbi amaçlı gıdalara ilişkin özel bir düzenleme veya tanım bulunmamaktadır. Özel tıbbi amaçlı gıdalar, Kodeks Alimentarius Standardı 180-1991'e uygun olmalıdır ve "yüksek riskli gıda" olarak düzenlenmiştir [53]. **Kamboçya**, **Laos** ve **Myanmar**'ın özel tıbbi amaçlı gıdalara ilişkin özel düzenlemeleri yoktur ancak Kodeks standartlarını takip etmektedirler.

FSMP: Foods for Special Medical Purposes/Özel tıbbi amaçlı gıda; FSDU: Foods for Special Dietary Uses/Özel diyet kullanımlarına yönelik gıda

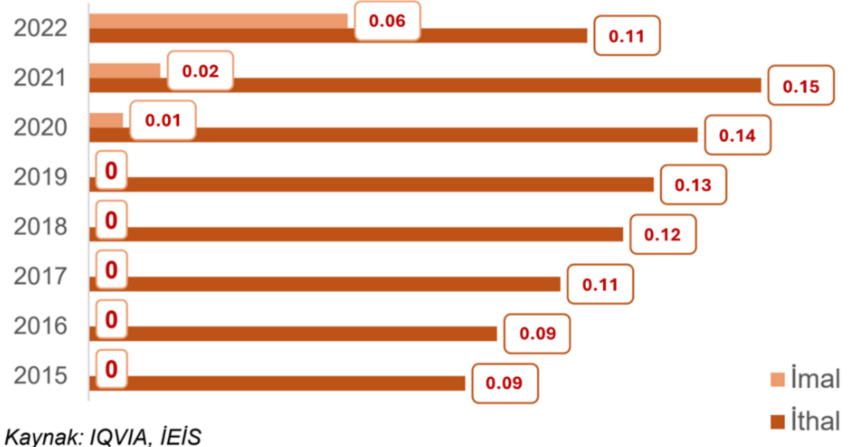
Türkiye'de Özel Tıbbi Amaçlı Gıdalar İçin Pazar Durumu

Türkiye'de pazarın tamamı özel tıbbi amaçlı gıdalar için referans ürünlerden oluşmakta olup geri ödeme kapsamında %98.4'ü yer almaktadır ve 2022 yılında özel tıbbi amaçlı gıdalar 4.3 milyar TL'ye ulaşarak değerde %58.4 büyüme göstermiştir (Şekil 1). 2015-2022 yılları arasında toplam büyüme %742 seviyesinde olup bileşik bazda yıllık büyüme (Compound Annual Growth Rate-CAGR) %35.6 iken, enflasyon etkisinden bağımsız reel büyüme oranı %3.9 olmuştur [54].



Şekil 1. Türkiye için özel tıbbi amaçlı gıda pazarı verileri. IQVIA: Quintiles and IMS Health, Inc. (I (IMS Health), Q (Quintiles), and VIA (by way of)), İEİS: İlaç Endüstrisi İşverenler Sendikası [54]

Özel tıbbi amaçlı gıdalar kutu bazında incelendiğinde 2015-2022 yılları arasındaki büyüme %101 seviyesindedir (Şekil 2). Son yıllara kadar tamamı ithal referans ürünlerden oluşan pazarda, ülkemizde kurulan üretim tesislerinin faaliyete geçmesi ile imal ürünlerin payı hızlı bir yükseliş içine girmiştir. İlaç Endüstrisi İşverenler Sendikası "Haziran 2024 Pazar Analiz Raporu"nda Haziran 2023-Haziran 2024 dönemi için özel tıbbi amaçlı gıdaların kutu bazında (yaklaşık 12 milyon) önemli bir değişiklik gözlenmezken, 816 milyon TL'ye ulaşarak değerde yaklaşık %70 büyüme göstermiştir [55].



Şekil 2. Türkiye için kutu bazında imal-ithal özel tıbbi amaçlı gıda pazarı (milyar kutu) verileri IQVIA: Quintiles and IMS Health, Inc. (I (IMS Health), Q (Quintiles), and VIA (by way of)),İEİS: İlaç Endüstrisi İşverenler Sendikası [54]

Dünyada Özel Tıbbi Amaçlı Gıdalar İçin Pazar Durumu

Özel tıbbi amaçlı gıdaların dünya pazarındaki durumunun değerlendirilmesi için kamuya açık istatistiksel veriler ve raporlar, uzman tahminleri, devlet ve kamu kuruluşlarının internet kaynaklarında yayınlanan bilgiler kullanılmıştır. Pek çok serbest araştırma grubunun yaptığı çalışmalar yeni trendleri, ürün gelişmelerini, pazardaki son haberleri ve pazarın tahmini gelirlerini ortaya koyarken; küresel pazarı veriliş yolu, ürün türü, uygulamaya, üretici firma ve dağıtım yapılan ülkeler gibi farklı parametrelere göre değerlendirme sunmaktadır (Şekil 3) [56,57].

Şekil 3. Küresel pazar etkenleri

Özel tıbbi amaçlı gıda ürünlerinin küresel pazar hacminin 2023 yılında 14 milyar ABD dolarını aştığı ve son yıllarda pazarın bu segmentinin istikrarlı bir büyüme göstermesi ile 2024-2030 tahmin dönemi için %5.2'lik bir bileşik bazda yıllık büyüme oranı ile 2030 yılına kadar 33.5 milyar ABD dolarına ulaşması beklenmektedir (Şekil 4) [58]. Avrupa pazarının hacminin ise 2023 yılında 3.3 milyar ABD dolarını aştığı, son 3 yılın ortalama bileşik bazda yıllık büyüme oranının neredeyse %5.6 olduğu belirlenmiştir.

Şekil 4. Uluslararası düzeyde özel tıbbi amaçlı gıda pazarının 2019-2023 yılları arasında değişimi [59,60]

Özel amaçlı tıbbi gıda pazarı için yasal düzenleyicilerle yaşanan sıkıntılar, sınırlı bilgi ve buna bağlı ürün geliştirmedeki zorluklar, maliyet kaygıları, geri ödeme kapsamı ve küresel ekonomik değişkenler gibi çeşitli faktörler kısıtlama oluştursa da yapılan pazar araştırma analizlerinin sonuçları özel amaçlı tıbbi gıda pazarının büyümesinde aşağıdaki ana faktörlerin etkili olacağını göstermektedir [59-61]:

- Ortaklıklar ve iş birliklerin artması
- Teknolojik ve tıbbi araştırmalardaki gelişmeler
- Enteral beslenmeye olan popülerliğin değişmesi
- Akılcı beslenmeye olan eğilim, tüketici farkındalığının artması
- Kişiselleştirilmiş ilaca olan ihtiyaçtaki artış
- Modernleşme ve şehirleşme artışı ile sedanter yaşam tarzının getirdiği hastalıkların artması
- AB'de ve çoğu Avrupa ülkesinde doğum oranlarındaki düşüş, toplam yaşam beklentisindeki (önümüzdeki yıllarda da artmaya devam edecek olan) artış ve buna bağlı kronik hastalıkların (diyabet, kalp-damar hastalıkları, kanser dahil) görülme oranlarının artması

Uluslararası pazarda özel tıbbi amaçlı gıdaların ana üreticilerinin Nestle, Danone Nutricia, Abbott, Bayer, Mead Johnson, Lenus Pharma, Gruppo FarmaImpresa ve Galen Limited gibi çok uluslu şirketlerdir ve bu şirketlerin pazardaki toplam payının yaklaşık %65 olduğu bilinmektedir [6]. Bu firmalar dışında özel tıbbi amaçlı gıda ürünleri için küresel pazarın diğer önemli katılımcıları Fresenius Kabi, Targeted Medical Pharma, Inc., Primus Pharmaceuticals Inc., Ajinomoto, EnterNutr şirketleridir [59, 62]. Ürünleri Avrupa ülkelerinde pazarda geniş çapta temsil edilen ana üreticiler ise Nestle, Danone Nutricia, Gruppo Farmaimpresa, Lenus Pharma GesmbH, Fresenius Kabi, Abbott ve Bayer'dir. Bu durum uluslararası düzeydeki duruma hemen hemen benzemektedir [62-64].

Yapılan çalışmalar ve analizler, beslenme açısından tam olmayan ürünlerin özel tıbbi amaçlı gıda tüketiminin küresel pazarda ilk sırada yer aldığını göstermektedir ve toplam tüketimde pazar payları %65 civarındadır (Şekil 5). Beslenme açısından tam olmayan ürünleri, hastalığa yönelik spesifik ürünler ve beslenme açısından tam ürünler takip etmektedir.

Şekil 5. Özel tıbbi amaçlı gıda türlerinin 2023 küresel pazar payı [59]

Bu özel ürünlerin hedeflediği tüketici kategorileri arasında yetişkinler %55 ile ilk sırada yer alırken, bunu bebekler ve küçük çocuklar ile yaşlılar izlemektedir. En az sayıdaki grubu hamile ve emziren anneler oluşturmaktadır (Şekil 6) [59].

Şekil 6. Özel tıbbi amaçlı gıdaların tüketici gruplarına göre 2023 küresel pazar dağılımı [59]

Dünyanın çeşitli bölgelerinde 2023 yılı boyunca özel tıbbi amaçlı gıdaya yönelik mevcut talep durumu ve tüketim hacimlerine ilişkin verilerin analizi sonuçlarına dayanarak pazardaki paylarının dağılımı Şekil 7'de gösterilmiştir [60,62]. Uluslararası özel tıbbi amaçlı gıda pazarının söz konusu düzeydeki gelişiminin dinamiklerini analiz ederken, bu malların en büyük dolaşım hacminin Kuzey Amerika ülkelerinde, en küçüğünün ise Güney Amerika ülkelerinde olduğu görülmektedir.

Şekil 7. Dünyanın belirli bölgelerinde özel tıbbi amaçlı gıda tüketim payları [59,60,62]

AB'de kabul edilen yatay ve dikey mevzuat terminoloji, sınıflandırma, bileşim, gıda işletmecilerinin sorumlulukları, ürünlerin etiketlenmesi ve bunların pazarda uygun şekilde tanıtılmasına ilişkin gereklilikleri tanımlayan teknik düzenlemelerin dünyadaki en gelişmiş düzenlemelerden biri olduğu kabul edilmektedir [62,65]. Bu nedenle özel tıbbi amaçlı gıdalara yönelik dünya pazarının yanında Avrupa pazarının durumu da dikkate alınmalı, bu pazarın özel tıbbi amaçlı gıda üretimi ve tüketimi (kullanımı) açısından en büyük ve en aktif katılımcılarının Almanya, İngiltere, Fransa, İtalya,

İspanya, Avusturya, Benelüks ülkeleri (Belçika, Hollanda), Lüksemburg) ve İskandinavya (Danimarka, İzlanda, Norveç, Finlandiya, İsveç) gibi AB ülkeleri olduğu unutulmamalıdır [63,64].

SONUÇ VE TARTIŞMA

Bu derleme makalesi ile özel tıbbi amaçlı gıdalar ve kapsamı, Türkiye ve dünyadaki güncel yasal düzenlemeler ve küresel pazar durumu genel hatları ile incelenmiştir. Tıbbi gözetim altında, bilimsel veriler ile etkinliği kanıtlanmış olan özel amaçlı tıbbi gıdalar hastaların beslenme durumunu iyileştirmeyi veya sürdürmeyi amaçlamaktadır. Bu ürünlere ait mevzuatlar, ihtiyaç sahibi bireylerin sağlığını koruyan veya geliştiren hayati çözümlerin yüksek kalitesini ve etkinliğini sağlamak amacıyla faaliyet göstermesi son derece zorlu ve dinamik bir alandır. Özel amaçlı tıbbi gıdalar normal gıda ve tıbbi ürünler arasında yasal bir kategori olarak hem gıda hem de ilaç endüstrisi için gelişime açık cazip bir grubu oluşturmaktadır. Dinamik ve artan pazar hacmi ile gelişmeye açık bir alan olan özel tıbbi amaçlı gıda pazarı için yeni formülasyonlar, dağıtım yöntemleri ve uygulamalar geliştiren şirketlerin tıbbi gıda pazarındaki büyümenin önemli bir itici gücü olacağı düşünülmektedir.

YAZAR KATKILARI

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REVIEW ARTICLE / DERLEME MAKALE

MARKET ACCESS FOR PHARMACEUTICALS IN TÜRKİYE: A COMPREHENSIVE OVERVIEW

TÜRKİYE'DE İLAÇLARIN PAZARA ERİŞİMİ: KAPSAMLI BİR İNCELEME

Ebru ERDAĞ[1]*, Selen YEĞENOĞLU[1]

[1]Hacettepe Üniversitesi, Eczacılık Fakültesi, Eczacılık İşletmeciliği Anabilim Dalı, 06100, Ankara, Türkiye

ABSTRACT

Objective: *This study provides a detailed analysis of the market entry processes for pharmaceuticals in Türkiye, examining the key factors shaping these processes and their alignment with international standards. The aim is to highlight the significance of regulatory mechanisms, economic strategies, and health technology assessment (HTA) in ensuring patients' access to high-quality medicines.*

Result and Discussion: *The pharmaceutical sector in Türkiye has evolved into a dynamic and growing industry, supported by significant contributions from both domestic and international stakeholders. Regulatory advancements, such as the establishment of the Turkish Medicines and Medical Devices Agency (TİTCK), have improved licensing, pricing, and reimbursement processes, aligning them with international practices. Additionally, HTA has emerged as a critical tool in evidence-based decision-making, optimizing the efficient use of healthcare resources and supporting sustainable access to innovative treatments. However, to maintain their effectiveness, these processes require continuous updates and improvements. Such efforts will provide significant benefits not only to the industry but also to patients and society as a whole.*

Keywords: *Health technology assessment (HTA), market access, pharmaceutical, pricing, reimbursement*

ÖZ

Amaç: *Bu çalışma, Türkiye'deki ilaçların piyasaya giriş süreçlerini ayrıntılı bir şekilde inceleyerek, bu süreçleri şekillendiren ana unsurları ve uluslararası standartlarla olan uyum düzeylerini ele almaktadır. Çalışmanın hedefi, düzenleyici mekanizmaların, ekonomik stratejilerin ve sağlık teknolojisi değerlendirmesinin (STD), hastaların kaliteli ilaçlara erişimini sağlamadaki önemini ortaya koymaktır.*

Sonuç ve Tartışma: *Türkiye'deki ilaç sektörü, yerli ve yabancı paydaşların önemli katkılarıyla dinamik ve gelişen bir endüstri haline gelmiştir. Türk İlaç ve Tıbbi Cihaz Kurumu'nun (TİTCK) kurulması gibi düzenleyici yenilikler, ruhsatlandırma, fiyatlandırma ve geri ödeme süreçlerini geliştirerek uluslararası standartlarla uyumlu hale getirmiştir. Bunun yanı sıra, sağlık teknolojisi değerlendirmesi (HTA), kanıta dayalı karar alma süreçlerinde önemli bir araç olarak öne çıkmış, sağlık kaynaklarının verimli kullanımıyla yenilikçi tedavilere sürdürülebilir erişimi desteklemiştir. Ancak, bu süreçlerin etkili kalabilmesi için, sürekli olarak güncellenmesi ve iyileştirilmesi gerekmektedir. Bu tür çabalar yalnızca sektöre değil, aynı zamanda hastalara ve topluma da önemli faydalar sağlayacaktır.*

Anahtar Kelimeler: *Fiyatlandırma, geri ödeme, ilaç, pazara erişim, sağlık teknolojisi değerlendirmesi (STD)*

* **Corresponding Author / Sorumlu Yazar:** Ebru Erdağ
e-mail / e-posta: ebru.erdag@hacettepe.edu.tr, **Phone / Tel.:** +903123052349

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INTRODUCTION

Pharmaceutical industry is a part of medical sector that discovers, develops, produces, and markets pharmaceutical products intended to be used for the good of patients in terms of curing, preventing, or alleviating symptoms of diseases [1-3]. In Türkiye, the pharmaceutical industry is a dynamic sector where both domestic and foreign companies operate, with ensuring patient access to medicines being a primary goal [4-6]. According to the data from Pharmaceutical Industry Employers' Association (İEİS), as of the end of 2023, the industry comprises 870 organizations, including 109 pharmaceutical and radiopharmaceutical manufacturing facilities operating at international standards, 4 facilities producing specialized dietary foods for medical purposes, and 13 active ingredient manufacturing facilities [7]. This recent phase progress in pharma indusstry has made market access even more crucial than ever.

On the other hand, market access in Turkish pharmaceutical industry is influenced by various factors such as regulatory frameworks, economic policies, and the competitive environment [3].

The foundation of Turkey's organized healthcare system dates back to 1920s with the establishment of the Ministry of Health (MoH), aimed at post-war restoration and laying the system's essential structures. The 1961 Constitution recognized healthcare services as a core responsibility of the government toward its citizens. In 2003, supported by the World Bank (WB), the "Health Transformation Program" initiated a series of comprehensive reforms, fundamentally altering the financing structure, service provision, and accessibility of healthcare, resulting in significant improvements across the system [8-10].

In Türkiye, the Ministry of Health (MoH) serves as the primary health authority. Until 2011, the regulatory functions for pharmaceuticals and pharmacy services in Türkiye were conducted by the General Directorate of Pharmaceuticals and Pharmacy under the MoH. In 2011, these responsibilities were transferred to the newly established Turkish Medicines and Medical Devices Agency (TİTCK), an entity with a special budget and legal body under the MoH [8,11,12]. The Turkish Medicines and Medical Devices Agency (TİTCK) is responsible, on behalf of the MoH, for overseeing the pharmaceutical and medical devices sectors, including clinical trial approvals, licensing, and pricing for pharmaceuticals and cosmetics. This agency conducts in other words conducts the Pharmaceuticals Electronic Tracking System (İTS), registers medical devices, operates the pharmacovigilance system, and evaluates applications for off-label medicine use, among other tasks. Additionally, the agency oversees market supervision and the surveillance of medicines. [11,13].

Further more with this article we aimed to detail the market access process of the pharmaceutical industry in Türkiye and the factors influencing these processes.

Definition of Market Access

In its broadest sense, "market access" refers to the degree to which a country's markets are open to foreign goods and services [14]. The World Trade Organization (WTO) initially introduced the concept of market access to define the competitive relationship between domestic and imported products within a country. This competitive relationship is governed by a series of measures designed to facilitate or restrict trade. In healthcare, market access aims to ensure that pharmaceuticals, in particular, achieve a reimbursable price through a health insurance system and can be prescribed [15].

In today's world, market access can be defined as the set of strategies, and processes developed by pharmaceutical and medical technology companies to ensure that their medicines or other medical technologies are accessible at affordable prices within a specific healthcare system [15,16]. In other words, market access can be defined as the comprehensive set of processes aimed at delivering a product to appropriate patients who are likely to benefit from it, in a cost-effective, timely, and sustainable manner.

Market access in the pharmaceutical industry is a global process which involves making medicines available for patients worldwide. This process requires taking into account regulatory rules, healthcare systems, and reimbursement policies in different countries [10,16,17].

The field of Market Access is no longer just about transforming a market authorization into health technology assessment (HTA) and reimbursement applications using health economic models. The

increasing focus on rare diseases and the shift to biotechnological solutions have brought Market Access departments to the forefront of product launch strategies, creating a need for Access Excellence. The target patient groups for innovative drugs are smaller, and prices are higher, while public health systems face limited resources in an aging society. Market access is becoming a leading interface that influences clinical development, regional marketing activities, patient engagement, and compliance with post-market access requirements [18].

Scope of Market Access

A comprehensive market access strategy at both global and local levels forms the backbone of successful product launches and sustainable long-term efficiency. An effective global strategy should serve as a guiding framework for sub-strategies such as health economics and outcomes research (HEOR), pricing, and patient advocacy. These components must be integrated into a cohesive market access plan to ensure the successful introduction of global pharmaceutical products [10,18]. Market access encompasses a broad range of activities, including health economics, pricing, and reimbursement processes, which are critical for ensuring efficient resource allocation and patient access to innovative treatments. Specifically, in the pharmaceutical industry, these processes occur predominantly after a drug receives market authorization, making them integral to market access strategies [1,14-16].

Economics, at its core, addresses the scarcity of resources and the need for their efficient utilization across all sectors. Health economics applies these fundamental economic principles, theories, and practices to the healthcare domain. It plays a vital role in evaluating the cost-effectiveness of healthcare interventions, with pricing and reimbursement forming essential components of this discipline [19].

In many countries, pricing and reimbursement decisions are centralized at the national level. The growing prevalence of high-cost medicines has posed challenges even for well-resourced nations, prompting the development of policies aimed at enhancing access to medicines in a cost-effective manner [14,20]. Drug pricing policies are central to healthcare regulations, encompassing both price setting and reimbursement processes. These policies are shaped by economic and political considerations, with a primary objective of ensuring that patients have access to effective, safe, and affordable medications. The introduction of a drug to the market and its inclusion in reimbursement lists typically involves three key stages: registration, price determination, and reimbursement evaluation [12,20].

Globally, the market access process for drugs and devices can be categorized into three primary steps. First, companies submit a dossier containing efficacy, safety, and tolerability data to regulatory agencies to obtain market authorization (Figure 1) [15]. Following approval, applications for pricing and reimbursement are prepared to establish the drug's financial and accessibility frameworks. Lastly, logistics and supply chain activities are organized to ensure the product is marketed and distributed without interruption [16].

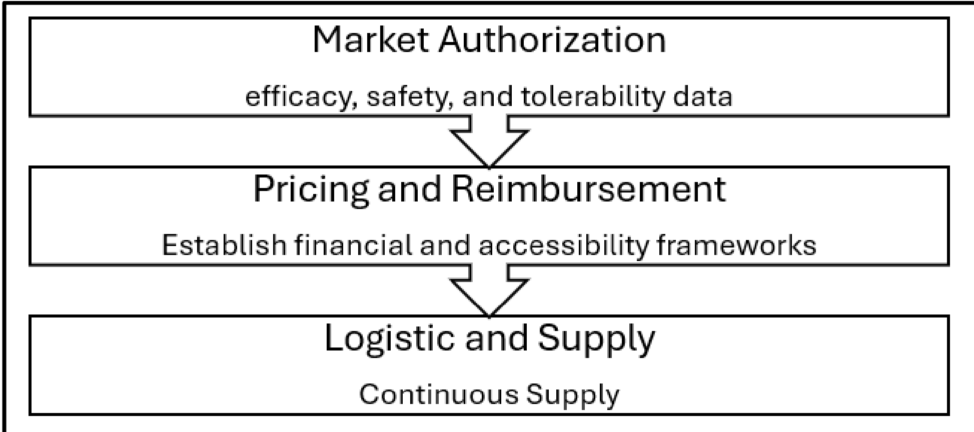

Figure 1. Market access process

The ultimate goal of market access is to ensure that all eligible patients who can benefit from a new product have continuous access to it at reasonable prices. This objective underscores the importance of aligning regulatory, economic, and logistical strategies to create a sustainable healthcare system that balances innovation with affordability.

Factors Affecting Market Access

Licencing

In Türkiye, the drug authorization process is carried out in accordance with European standards. This process is supported by International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines and memberships, and it is standardized using the Common Technical Document (CTD). Licensing applications include Good Manufacturing Practices (GMP) inspections of manufacturing facilities and priority evaluation processes [1,11,21,22].

During the licensing process, GMP inspections of products are conducted first. For conventional products, only the finished product GMP inspection is required, while for biological or biotechnological products, both active ingredient and finished product GMP inspections are required. Inspection processes consist of pre-assessment, scientific evaluation, and administrative evaluation steps. In the pre-assessment, the integrity of the documentation is examined; in the scientific evaluation, quality, efficacy, and safety are assessed; and in the administrative evaluation, common controls are done [5,21,22].

Pricing

External price referencing (EPR) serves as a widely utilized strategy for regulating pharmaceutical costs, aiming to contain expenditures by referencing drug prices across different markets. This approach provides payers and regulators with a reference point for setting or negotiating the price of identical products within their own market [23-25].

In Türkiye, The Turkish Medicines and Medical Devices Agency (TİTCK) is responsible for pricing of pharmaceuticals on behalf of the Ministry of Health. Since 2004, the Reference Price System is being used for drug pricing in Türkiye. The countries (Spain, France, Greece, Italy, Portugal) taken as reference in the Reference Price System are selected from EU member states those show similar socioeconomic status like Türkiye. Pricing is done using a fixed Euro value and varies according to whether the product is imported or locally manufactured and whether it is original or generic. Special condition product groups are also considered in pricing [5,11,12,20,26-30].

Pricing for locally manufactured products can be done using the card of manufacturing costs. Original products are priced at 100%, while generic products are priced at 60%. The price of the original product is also reduced to 60%. For price-protected products, this rate is set at 80%. Price updates are made by updating the fixed Euro value within the first 45 days of each year. Drug price research is conducted annually during the real source price change period, and increases or decreases are applied to the products [11,12,27,31].

The Pricing Evaluation Commission, coordinated by the MoH with representatives from the Social Security Institution (SGK), the Ministry of Treasury and Finance, and the Presidency of Strategy and Budget, makes decisions on increasing, decreasing, or maintaining prices when deemed necessary [12,31].

Reimbursement

Reimbursement can be defined as the full or partial payment of the cost of a medical product by the insurance institution. In Türkiye, Social Security Institution (SGK) is the governmental institution responsible for reimbursement [9,11]. Regulations on which drug expenses used in treatment are covered and to what extent are included in the Drug Reimbursement Regulation. The deadline for reimbursement applications for each year is the last day of March and July [32].

The drug must first be licensed, published on the price list, and have a sales permit to apply for the consideration to reimbursement [9]. Since 2005, the reimbursement system has been based on a positive list, which categorizes medicines into groups according to their active ingredient,

pharmaceutical form, and dosage [5,11]. The reimbursement status and discounts for licensed drugs in our country are updated every Wednesday on the Institution's official website under the Announcements section as the positive (Attachment-4A) list (Figure 2). For products on overseas drug lists, the reimbursement status is shared with Attachment-4C lists [5,32,33].

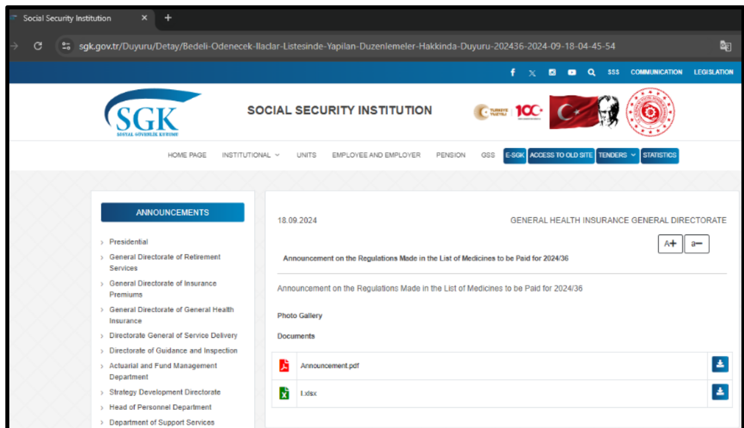

Figure 2. Positive List on Social Security Institution Website (taken from sgk.gov.tr)

When considering reimbursement, it is important to take into consideration several key terms. The Therapeutic Reference (TR) Group is the grouping of products containing the same active ingredient and the same indication for price comparison purposes. The TR Band Range (Base price application) is the public payment of up to 5% more than the unit price of the lowest-priced drug. The yellow band is used for products that have not achieved a 1% market share and are not considered in the base price/band calculation. The public sector statutory rebate is the discount rate determined according to the wholesaler's (ex-factory) price of the drug and its reference/equivalent status. The Public Price (Reimbursed price) is the price obtained by applying the public sector statutory rebate to the retail sales price [29,34].

The Drug Reimbursement Commission is chaired by the General Director of General Health Insurance, with representatives from the General Health Insurance Directorate, the Ministry of Treasury and Finance, the Ministry of Health, and the Presidency of Strategy and Budget. The Medical and Economic Evaluation Commission is responsible for scientific and economic evaluation. The Alternative Reimbursement Commission is chaired by the General Director of General Health Insurance, with representatives from the General Health Insurance Directorate, the Ministry of Treasury and Finance, the Ministry of Health, and the Presidency of Strategy and Budget. The commission process and the decisions made by the commission are confidential for all times [5,12,29,32].

Health Technology Assessment (HTA)

A key aspect of market access is the clinical, economic and humanistic evaluation of drugs, which involves analyzing their effectiveness and cost-effectiveness using real-world data. Clinical evaluation relies on scientific evidence to confirm a drug's safety and efficacy, while economic evaluation assesses its cost-effectiveness and impact on health economics [15,35,36].

According to the definition on the official HTA Glossary website, operated in collaboration with Health Technology Assessment International (HTAi) and the International Network of Agencies for Health Technology Assessment (INAHTA), Health Technology Assessment (HTA) is defined as a multidisciplinary process that uses explicit methods to determine the value of a health technology at different points in its lifecycle. In HTA process the aim is to inform decision-making in order to promote and provide an equitable, efficient, and high-quality healthcare services [37,38]. HTA is a multidisciplinary field that is integral to this process, evaluating drugs from clinical, economic, ethical, and social perspectives. HTA informs pricing and reimbursement decisions, emphasizing cost-effectiveness and health outcomes [14,15,35,38-40].

According to the Directorate of Research, Development, and Health Technology at the General Directorate of Health Services, which operates under the Ministry of Health, Health Technology Assessment (HTA) is defined as "the examination and interpretation of various aspects of technologies used in healthcare. While this assessment is primarily conducted to inform decision-making mechanisms, it also aims to ensure that all stakeholders are adequately informed about the technology in question" [38,40].

In other words, HTA allows governments to indirectly control drug prices. Although its influence on pricing can be vague, there is a clear pattern: a lower assessment value increases the likelihood that a drug will be either not purchased or bought at a lower price. This system prioritizes valuable and cost-effective medications for procurement. [1,14,15,39,40].

Since the late 1980s, health technology assessments have rapidly developed and became institutionalized in advanced economies. In our country, such assessments came into the agenda starting in 2003 with the implementation of the "Health Transformation Program" and the associated health reforms. The institutionalization of HTA in Türkiye began and continues with the support and leadership of the Evidence-Based Medicine Association, established in late 2007. The first legal regulation regarding HTA's institutional framework was introduced in 2011 with Decree Law No. 663 [41].

Türkiye acknowledges the necessity of conducting Health Technology Assessments (HTA) to evaluate and improve the capacity of health technologies. The country has established three key HTA bodies: the Turkish Medicines and Medical Devices Agency (TITCK), which operates under the Ministry of Health and focuses on health policy; the Directorate General for Health Research, also affiliated with the Ministry of Health, specializing in clinical processes; and the Social Security Institution (SGK), under the Ministry of Labor and Social Security, which has yet to publish any reports. [11,40].

Logistics and Supply

Considering the definition of market access, it is evident that logistics play a crucial role in the success of a market access strategy. In the drug supply process, coordination with stakeholders is essential to address shortages of unavailable medications. Conducting a thorough research and maintaining effective stock control are the key elements to ensure patients receive necessary drugs promptly [15].

In Türkiye, MoH works efficiently to make sure sustainability by using Electronic Tracking System (ETS, ITS) to control stock in the market [11,42]. ITS ensures the procurement of original and reliable pharmaceuticals by preventing drug counterfeiting and trafficking [42]. The adoption of ITS has resulted in numerous benefits, notably the optimization of pharmacy operations and the improvement of medication safety and accessibility for patients [36]. Additionally, it is possible to facilitate access to medications not licensed in our country.

The Overseas Medicine Supply (Medicines Brought From Abroad, MBFA) is used for unlicensed drugs and licensed molecules that are unavailable in the market due to various reasons. It is standard policy that drugs can only be marketed after receiving marketing authorization from the Turkish Medicines and Medical Devices Agency. However, the MBFA pathway allows access to specific medicines with high medical need, even in the absence of marketing authorization or when a marketing authorization has been granted, but the drug is not commercially available [9,43]. Through close collaboration with international suppliers and the MoH, these medications are delivered to patients efficiently. The Ministry ensures that all aspects, including the source of the medication and compliance with Good Distribution Practices (GDP), are meticulously monitored and approved [43].

Medicines brought from abroad are subject to patient-specific procedures. While MBFA differs from Compassionate Use, it can be categorized under the Named-Patient Program, which is granted in response to requests by physicians on behalf of specific or "named" patients [9,43,44].

Under European Regulation 726/2004/EC, the European Union defines Compassionate Use as the provision of unauthorized medicinal products to patients who suffer from chronic or seriously debilitating conditions or life-threatening diseases, where no satisfactory authorized treatments are available [15].

In Türkiye, Compassionate Use is a program for the application of drugs that have completed at least Phase II studies worldwide or have obtained efficacy and safety data that allow for the advancement to the later phase of the study in which Phase II and III are conducted together. These programs are used in the treatment of individuals with life-threatening or severely quality-of-life impairing diseases those have not been successful with available treatments in our country. These programs aim to ensure that drugs reach patients quickly and safely [44]. A similar program, titled the "Temporary Utilization Program (ATU)," has been implemented in France since 1992 to improve early access to medicines authorized abroad. Similarly, the United Kingdom government launched the Early Access to Medicines Scheme (EAMS) to provide patient access to new medicines before marketing authorization. EAMS is a voluntary program, and the manufacturer provides the medicine free of charge, just like the Compassionate Use Program in Türkiye [9,44].

Global Insights on Market Access

Türkiye, as a prominent emerging market, shares several characteristics with well-established pharmaceutical markets, such as those in the USA, Europe, the UK, and Japan, but also faces distinct challenges influenced by its unique economic and healthcare contexts.

The Turkish Medicines and Medical Devices Agency (TITCK) governs the pharmaceutical approval process in Türkiye, aligning it with European Medicines Agency (EMA) standards. However, the approval timelines in Türkiye tend to be longer, averaging between 18 and 24 months [5,11,21,22]. In contrast, the U.S. Food and Drug Administration (FDA) offers expedited pathways, such as Priority Review and Breakthrough Therapy Designation, which allow critical drugs to be approved within 10 months or less [45,46]. In the European Union, the EMA provides centralized approval for medicinal products, but individual member states maintain independent processes for pricing and reimbursement. Approval timelines within the EU are generally similar to those in Türkiye, averaging 1–2 years [46-48]. In the UK, the Medicines and Healthcare products Regulatory Agency (MHRA) has introduced the Innovative Licensing and Access Pathway (ILAP), which aims to accelerate drug approvals and facilitate quicker patient access [46,49]. Similarly, Japan, the world's third-largest pharmaceutical market, has a Pharmaceuticals and Medical Devices Agency (PMDA) that prioritizes expedited market access for innovative drugs, offering conditional early approvals for treatments addressing rare diseases or unmet medical needs. [46,50].

In terms of pricing, Türkiye utilizes an external reference pricing (ERP) system, which benchmarks drug prices against the lowest prices in a basket of European countries. While this system ensures affordability for patients, it restricts profitability for pharmaceutical companies, often deterring the introduction of innovative therapies [5,11,12,20,23-30]. In contrast, the U.S. follows a market-driven pricing model, allowing pharmaceutical companies to set their own prices. This results in higher drug prices but also fosters innovation and significant investment in research and development (R&D) [45,46]. Most European countries implement ERP, but many combine it with value-based pricing, which aligns the cost of drugs with their clinical benefits [46-48]. The UK utilizes the National Institute for Health and Care Excellence (NICE) to assess the cost-effectiveness and clinical benefits of treatments, ensuring affordable healthcare within the National Health Service (NHS) [46,49]. Japan, similarly, conducts biennial price reviews, regularly reducing reimbursement rates for less innovative drugs, thus maintaining affordability while incentivizing ongoing R&D [46,50].

Regarding Health Technology Assessment (HTA), its role in Türkiye is relatively limited. The Social Security Institution (SGK) oversees reimbursement decisions, primarily based on pharmacoeconomic evaluations. This centralized approach often leads to delays in market access due to lengthy negotiations and stringent cost-containment measures [11,40]. In the U.S., HTA plays a minimal role, as reimbursement decisions are primarily determined through negotiations between private insurers and manufacturers. However, in several European countries, including Germany (via IQWiG) and France (via HAS), HTA is integral to the reimbursement process, assessing both clinical outcomes and economic considerations [14,47,48]. In the UK, NICE is a global leader in HTA methodologies, combining rigorous clinical and economic evaluations to make transparent, evidence-based reimbursement decisions [14,46,49]. Japan does not yet have a formal HTA agency like UK's NICE or Germany's IQWiG. Japan's approach to HTA is more selective but is gradually gaining prominence as

a tool for balancing cost-effectiveness with the need to maintain a sustainable, universal healthcare system [46,50].

This heading has been summarized in the table below (Table 1).

Table 1. Global insights on market access factors

	Regulatory authority	Approval time	Pricing mechanism	Reimbursement	Access delays	Generic penetration
United States (USA)	FDA (Food and Drug Administration)	~10 months	Market driven	Private insurance, Medicare, Medicaid	Minimal	High (~90%)
Europe (EU)	EMA (European Medicines Agency) + National Agencies	1 – 2 years	HTA and reference pricing	Public reimbursement systems	Delays due to HTA and pricing reviews	Moderate (60–80%)
United Kingdom (UK)	MHRA (Medicines and Healthcare products Regulatory Agency	1 – 2 years	NICE-led cost effectiveness evaluations	NHS-funded reimbursement for approved drugs	Moderate delays due to NICE evaluations	Moderate (~60%)
Japan	PMDA (Pharmaceuticals and Medical Devices Agency)	~1 year	Price control via negotiations	National health insurance	Moderate post-approval delays	Moderate
Türkiye	TİTCK (Turkish Medicines and Medical Devices Agency)	~18 - 24 months	External reference pricing	SGK (Social Security Institution) centralized reimbursement	Significant delays due to reimbursement and pricing	High (~80%)

RESULT AND DISCUSSION

Market access in the pharmaceutical industry in Türkiye is a multifaceted and complex process. Many factors, such as regulations, pricing and reimbursement policies, logistics, and supply processes, affect this process. Achieving and sustaining effective market access for pharmaceuticals in Türkiye requires a multifaceted approach that balances regulatory requirements, economic constraints, and healthcare needs. The dynamic landscape of Türkiye's pharmaceutical industry, with both domestic and international stakeholders, has made structured market access not only a goal but a necessity to ensure that the patient receives high-quality medicines. For the pharmaceutical industry to respond quickly and meeting patients' needs, these processes must be managed effectively. This is significant for improving public health and the sustainable growth of the pharma industry [5,11,51]. Türkiye's regulatory advancements, including the establishment of the Turkish Medicines and Medical Devices Agency (TİTCK), have streamlined processes for licensing, pricing, and reimbursement, bringing them in line with international standards. Additionally, the integration HTA has allowed for an evidence-based approach to drug evaluation, optimizing resource allocation and supporting cost-effective healthcare

solutions.

Looking forward, Türkiye's healthcare policy and its commitment to innovation will likely enhance the role of HTA, addressing the challenges posed by the increasing prevalence of high-cost biotechnological treatments. Through coordinated regulatory frameworks and stakeholder engagement, Türkiye's healthcare system aims to not only improve accessibility but also to reinforce a sustainable model that upholds the principles of equity, efficiency, and quality in pharmaceutical access.

Türkiye has made significant steps and continues to make progress in market access in the pharmaceutical industry. However, these processes must be continuously updated and improved. This will be beneficiary not only for the industry but also for patients and society.

AUTHOR CONTRIBUTIONS

Concept: E.E., S.Y.; Design: E.E., S.Y.; Control: E.E., S.Y.; Sources: E.E., S.Y.; Materials: E.E., S.Y.; Data Collection and/or Processing: - ; Analysis and/or Interpretation: - ; Literature Review: E.E., S.Y.; Manuscript Writing: E.E., S.Y.; Critical Review: E.E., S.Y.; Other: -

CONFLICT OF INTEREST

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

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DERLEME MAKALE / REVIEW ARTICLE

YARA ÖRTÜLERİNDE GÜNCEL TEKNOLOJİLER VE FARMASÖTİK KARAKTERİZASYON YÖNTEMLERİ

CURRENT TECHNOLOGIES IN WOUND DRESSINGS AND PHARMACEUTICAL CHARACTERIZATION METHODS

Ayşe Nur BÜKE[1,2], Müge KILIÇARSLAN[1]*

[1]Ankara Üniversitesi, Eczacılık Fakültesi, Farmasötik Teknoloji Anabilim Dalı, 06560, Ankara, Türkiye
[2]Ankara Üniversitesi, Sağlık Bilimleri Enstitüsü, 06110, Ankara, Türkiye

ÖZ

Amaç: *Bu derlemede yara örtüsü üzerine yapılan çalışmalar incelenerek yara bakım prensipleri, ideal yara örtüsü özellikleri, yara örtülerinin sınıflandırmaları, hazırlanma yöntemleri ve yara örtülerinde yapılan karakterizasyon çalışmaları karşılaştırmalı olarak değerlendirerek en güncel yara örtüsü üretim teknolojilerinin araştırılması amaçlanmıştır.*
Sonuç ve Tartışma: *İdeal bir yara örtüsü temel yara bakım prensiplerini karşılamalıdır. Yara iyileşme sürecini kolaylaştırmak ve hızlandırmak için geçmişten günümüze dek pek çok çalışma yapılmıştır. Günümüzde modern teknolojilerin gelişmesiyle birlikte, gazlı bezler gibi geleneksel örtüler yerine doğal ve sentetik polimerler ve bunların kombinasyonlarından oluşan modern yara örtüleri kullanılmaya başlamıştır. Temeli polimerlere dayanan modern yara örtüleri sayesinde, bünyesinde yara iyileşmesini kolaylaştıran ve hızlandıran etkin maddeleri taşıyabilen ve farklı fiziksel formlarda uyarlanabilen yara örtüleri geliştirilmiştir. Bu derlemede modern yara örtüleri filmler ve hidrojeller, nanolifler, köpükler ve süngerler, kompozitler ve iskeleler, biyoaktif yara örtüleri ve biyolojik yara örtüleri olarak sınıflandırılmıştır. Hazırlama yöntemleri, çeşitli avantajları ve dezavantajları, onaylı piyasa preparatlarından örnekler verilerek açıklanmıştır. Hem stabilite hem etkinlik açısından yara örtülerinden beklenen özelliklerin karakterizasyon çalışmaları değerlendirilmiştir. Konuyla ilgili incelemelerimiz sonucunda, farklı yara türlerine uygun ideal tek bir yara örtüsünden bahsedilemese de özellikle hastaya özgü kronik yaraların boyutuna uygun yara örtülerinin hazırlanabildiği 3B baskı, nanolif teknolojileri gibi deriyi neredeyse birebir taklit edebilen teknolojilerin veya püskürtülebilir-partiküler yara örtüsü sistemlerinin kullanımı sayesinde kitlesel olarak üretilen terapötik sistemlerin kişiye özel hale getirilebileceği tespit edilmiştir.*
Anahtar Kelimeler: *Biyomateryal, polimerik ilaç taşıyıcı sistemler, yara iyileşmesi, yara örtüsü, yara yönetimi, in vitro karakterizasyon*

ABSTRACT

Objective: *This review aims to evaluate studies on wound dressings by comparatively analyzing wound care principles, ideal wound dressing properties, classifications of wound dressings, preparation methods, and characterization studies of wound dressings. Additionally, it seeks to investigate the latest technologies in wound dressing production.*
Result and Discussion: *An ideal wound dressing must meet the fundamental principles of wound care. Numerous studies have been performed from past to present to facilitate and accelerate the wound healing process. With the advancement of modern technologies, traditional wound dressings*

* **Sorumlu Yazar / Corresponding Author:** Müge Kılıçarslan
e-posta / e-mail: cmkilicarslan@gmail.com, **Tel. / Phone:** +903122033156

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such as gauze have been replaced by modern wound dressings made from natural and synthetic polymers or their combinations. Modern polymer-based wound dressings enable the development of products that can carry drugs facilitating and accelerating wound healing and can be adapted into various physical forms. In this review, modern wound dressings are classified into films and hydrogels, nanofibers, foams and sponges, composites and scaffolds, bioactive wound dressings, and biological wound dressings. Preparation methods, advantages, disadvantages, and examples of approved market preparations are discussed. The characterization studies of wound dressings regarding their stability and efficacy are also evaluated. As a result of our studies on the subject, although a single ideal wound dressing suitable for different wound types cannot be identified, it has been determined that mass-produced therapeutic systems can be customized where wound dressings can be prepared according to the size of patient-specific chronic wounds thanks to the use of technologies that can almost exactly mimic the skin such as 3D printing, nanofiber technologies, or use of sprayable-particulate wound dressing systems.

Keywords: *Biomaterial, polymeric drug delivery systems, wound healing, wound dressing, wound management, in vitro characterization*

GİRİŞ

Vücudun en büyük organı olan deri, ağrı, basınç, kaşıntı, sıcak/soğuk gibi duyusal fonksiyonlar, sıcaklık regülasyonu, dehidrasyonun önlenmesi, D3 vitamini sentezlemesi, esnekliği, elastikiyeti ve dayanıklılığı ile vücudun normal anatomik hareketine izin vermesi gibi pek çok fizyolojik görevinin yanı sıra mikrobiyal enfeksiyon gibi olası çevresel tehditlere karşı da fiziksel bir bariyerdir. Deri mekanik, fiziksel veya metabolizma ile ilişkili hastalıklar sebebiyle hasar gördüğünde bu bariyer ortadan kalkar ve yara oluşur. Yara oluşumuyla derinin fonksiyonlarını yerine getirememesi yanında dış çevreden gelebilecek tehlikelere karşı da vücut savunmasız kalmaktadır. Bu noktada, hasarlı bariyeri geçici olarak telafi etmek ve iyileşmeyi hızlandırmak için dışarıdan destekleyici bir uygulama olan yara örtülerine ihtiyaç duyulur. Yara örtüsü; yaralı bölgeyi dış ortamdan izole ederek yara üzerinde koruyucu bir tabaka oluşturan, kanamayı önleyen, yarayı nemli tutan, fazla eksüdayı gideren, iyileşme sürecini kolaylaştırmak ve hızlandırmak için etkin maddelerin salımına izin veren, doku ile uyumlu materyaller olarak tanımlanabilir [1-3].

Bu derlemede yara örtüsü üzerine yapılan çalışmalar derlenerek yara bakım prensipleri, ideal yara örtüsü özellikleri, yara örtülerinin sınıflandırmaları, hazırlanma yöntemleri ve yara örtülerinde yapılan karakterizasyon çalışmaları incelenecektir.

Yara Bakım Prensipleri

Yara oluşum nedenine bağlı olarak vücutta çeşitli tipte doku hasarı oluşabilir. Bunlar genetik hastalıklar, yanma, donma, elektriksel, kimyasal, radyasyon veya friksiyon gibi nedenlerle oluşan akut travmalar, diyabetik ayak ülserleri, yatak yaraları, venöz ve arteriyal bacak ülserleri gibi kronik rahatsızlıklar veya cerrahi girişimler sebebiyle meydana gelebilir. Yara bakım prensipleri yara oluşum nedenine bağlı olarak değişmekte ve yara örtülerinden beklenen özelliklerin de temelini oluşturmaktadır. Yara bakımında dikkat çeken temel faktörler nem, oklüzyon, debridman ve doku oksijenlenmesi olarak sıralanabilir.

Nem: Yara bölgesini kurutmaya yönelik uzun yıllar süren yanlış yara bakımlarının ardından yaraların nemli bir ortamda daha hızlı iyileştiği, aşırı kuru yaraların daha fazla doku ölümüyle sonuçlandığı tespit edilmiştir. Nemli ortam koşullarında yara dokusundaki epitelizasyonun hızlandığı ve hatta hastalarda ağrı rahatlaması sağladığı bildirilmektedir [4,5].

Oklüzyon: Yaranın mikrobiyal enfeksiyonlara ve çevresel faktörlere karşı etkili bariyer sağlanması olarak tanımlanır. Açık yaralarda kontaminasyon, kolonizasyon veya enfeksiyon oluşturmuş halde mikroorganizmalar bulunabilir. Enfeksiyon halinde bakteri çoğalması iyileşmeyi engeller ve yara dokusu zarar görebilir [6].

Debridman: İyileşmeye yardımcı olmak için yara dokudaki skar/kabuk gibi ölü dokuların, bozulmuş fibrinlerin, bozulmuş hücre dışı matris proteinlerinin, eksüdaların, beyaz kan hücrelerinin, bakterilerin ve bunların karışımı vs.'nin yaradan uzaklaştırılması işlemi debridman olarak tanımlanmaktadır [1,4].

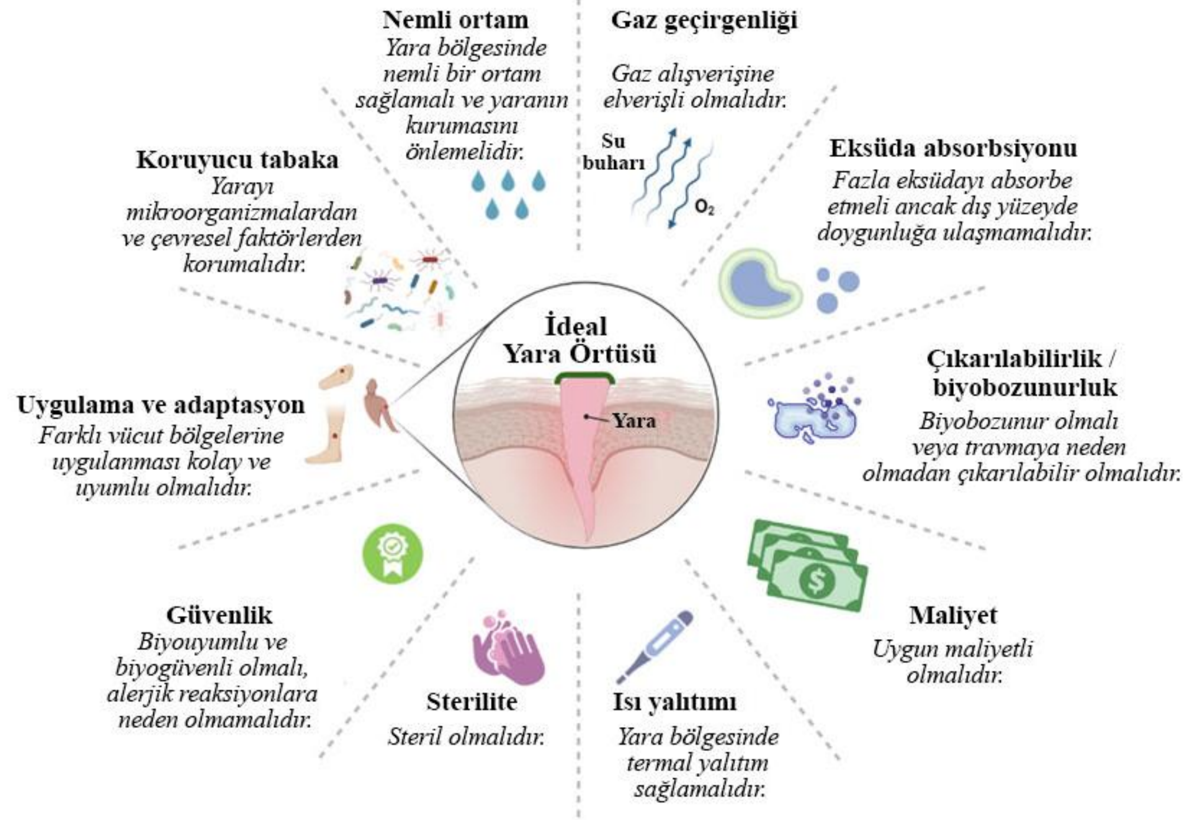
Skarlı/kabuklu yaralar gibi kuru ve fazla eksüda üretmeyen yaralarda yara iyileşmesini hızlandırmak için bu ölü hücrelerden oluşan nekrotik dokunun çıkarılması gerekmektedir [7].

Doku oksijenlenmesi: Yaralanmış dokudaki hücrelerin beslenmesi, gelişip büyümesi için dokunun oksijenlenmesi enzimatik ve hücresel pek çok olayda önemli role sahiptir [8]. Hipoksi durumu, yaralı dokunun ölümüyle sonuçlanabilir.

Venöz ülserler ve piyoderma gangrenozum gibi aşırı sıvı üreten eksüdatif yaralarda ise çevredeki sağlıklı dokuların da aşırı nem ile maserasyonunu en aza indiren absorpsiyon kapasitesi yüksek yara örtülerine ihtiyaç duyulmaktadır. Ancak, örtü veya fiziksel baskı uygulandığında yarada daha fazla eksüda oluşumu da dikkat edilmesi gereken bir noktadır. Arteriyel ülserler, diyabetik ülserler, basınç ülserleri, cerrahi işlem sonucu oluşan yaralarda derin/tünel yaralar sık görülmektedir. Yaralar bu kadar derin olduğunda yaradaki fazla eksüdayı absorbe eden ve yara boşluğunu etkili şekilde doldurabilen yara örtüleriyle iyileşmenin hızlandırılabileceği tespit edilmiştir.

İdeal Yara Örtüsü

Yaraların biyolojik karmaşıklığı da göz önüne alındığında, her yara tipi için uygun özellikte mükemmel tek bir yara örtüsünün olması mümkün değildir. Yara örtüleri doğrudan yara ile temas ettikleri için yaranın tipine ve aynı zamanda yaranın eksüdasyon derecesi gibi faktörlere göre uyarlanabilmelidir [9]. Uygun olmayan yara örtüleri yarada iyileşmenin aksine istenmeyen sonuçlara neden olabilir. Yara bakımından yola çıkarak ideal bir yara örtüsünden beklenen özellikler Şekil 1'de özetlenmiştir.



Şekil 1. İdeal bir yara örtüsünden beklenen özellikler

Yara Örtülerinin Sınıflandırılması

Yara örtüleri ile ilgili ilk yazılı belgeler olan eski Mısır Ebers Papirüslerinde (MÖ 1550), bitkisel liflerin sıvı absorpsiyon kabiliyeti, hayvansal yağların bariyer özellikleri ve balın antibakteriyel

özelliklerinden yararlanılarak keten şeritlere uygulanmış halde yara örtüleri olarak kullanımlarından bahsedilmektedir [4,10]. Sonraki dönemlerde şarap, sirke, kaynatılmış su içeren yün ve pamuk örtüler yara örtüsü olarak kullanılmıştır. Geçmişten günümüze kadar teknolojinin de ilerlemesiyle çok çeşitli yara örtüleri geliştirilmiş ve hatta piyasada yerini almıştır. Yara örtüleri yaradaki işlevlerine, üretildikleri materyallere veya fiziksel formlarına göre çeşitli şekillerde sınıflandırılmıştır. En yaygın sınıflandırma şekli geleneksel ve modern yara örtüleridir [2,3]. Modern teknolojiyle birlikte doğal veya sentetik bandajlar, hidrofil pamuk, gazlı bezler gibi geleneksel örtülerin yerine doğal ve sentetik polimerler ve bunların kombinasyonlarından oluşan film, hidrojel, iskele, nanolif, sünger gibi farklı fiziksel formlarda oluşturulabilen modern yara örtüleri kullanılmaya başlamıştır.

Geleneksel Yara Örtüleri

Bu kategorinin en tipik örneği tıbbi gazlı bezlerdir. Pamuk, suni ipek, polyester veya bunların kombinasyonlarıyla dokunmuş ya da dokunmamış liflerinden üretilen gazlı bezler, düşük maliyetleri, kolay kullanımları ve kolay üretilebilirlikleri yanında hızlı su absorbsiyon kabiliyeti ve sterilize halde bulunmaları nedeniyle yara örtüsü olarak tercih edilmektedir. Ancak ideal yara örtüsünden beklenenin aksine;

- Yaranın mikrobiyal kontaminasyondan tam olarak korunamaması,
- Fazla eksüdaları absorbe ederken hızlıca doygunluğa ulaştığı için yüzeyde biriken eksüda sebebiyle mikrobiyal atağı kolaylaştırması,
- Düşük gaz geçirgenliği,
- Kuru olması nedeniyle yarada nemli ortam sağlanamaması,
- Biyodegredable olmadığı için değiştirme veya çıkarma sırasında, yara yüzeyine yapışarak kanamaya veya yenilenen epitel florasında hasara neden olması

gibi dezavantajları kullanımlarını kısıtlamaktadır [3]. Özellikle değişim veya çıkarılma sırasında ağrı oluşumunu hafifletmek ve yara hasarını en aza indirmek amacıyla, gazlı bezlerin adezif olmayan bir iç yüzey ile kaplanması (örn; kitozan-Ag-ZnO nanokompozit kaplama) [5] veya sıvı parafin ya da vazelin emdirilmesi [4] gibi işlemlerle bu dezavantajı elimine edilmeye çalışılmıştır. Gazlı bezler, vazelin, tuzlu su, antibiyotikler ve antiseptikler gibi katkı maddelerinin taşıyıcısı olarak veya diğer yara örtüsü ürünleri ile kombinasyon halinde hazırlanmaları ile modern uygulamalara uyarlanmaya çalışılmıştır [4,11]. Vazelinli gazlı bez olarak Xeroform™, parafinli gazlı bez olarak Bactigras™, Jelonet™ ve Paratulle, yüzeysel yaralara uygun niteliğe sahip ticari olarak temin edilebilen örneklerinden bazılarıdır [10]. Ancak yara örtüsü olarak kullanılan tıbbi gazlı bezler, sargı bezleri ile karıştırılmamalıdır, yara örtüleri doğrudan yara ile temas eden ürünlerdir.

Modern Yara Örtüleri

Geleneksel yara örtüleriyle tam olarak sağlanamayan ideal yara örtüsü koşullarını sağlayabilmek için modern yara örtüleri geliştirilmiştir [2,3]. Modern yara örtüleri genellikle polimerlere dayanır, doğal ve sentetik kaynaklı pek çok polimerden hazırlanabilirler. Özellikle biyolojik olarak bozunabilen polimerlerin kullanıma girmesiyle birlikte, tedavi sonunda çıkarılma gereksinimine ihtiyaç olmayan yara örtülerinin hazırlanabilmesi, bu polimerik materyallerin bünyesinde yara iyileşmesini kolaylaştıran ve hızlandıran etkin maddeleri de taşıyabilmeleri ve uyarlanabilen özelliklere sahip farklı fiziksel formlarda hazırlanabilmeleri sayesinde modern yara örtülerine olan ilgi artmıştır. Bu derlemede modern yara örtüleri aşağıdaki şekilde sınıflandırılmıştır:

- Filmler ve Hidrojeller
- Nanolifler
- Köpükler ve Süngerler
- Kompozitler ve İskeleler
- Biyoaktif Yara Örtüleri
- Biyolojik veya Biyosentetik Yara Örtüleri

Filmler ve Hidrojeller

Modern yara örtüleri araştırıldığında ilk örneklerinin tipik olarak poliüretandan yapılmış, elastik, sentetik ince filmler olduğu görülmektedir. Hem yara bakımında beklenen özellikleri sağlayabilmeleri açısından hem de film halinde uygulanma kolaylığı sebebiyle dikkat çeken bu ürünler, Opsite™, Tegaderm™ ve Biooclusive™ ticari isimleriyle piyasada yer almaktadır (Tablo 1). Elastik ve esnek yapıları sayesinde uygulandıkları vücut bölgesinde rahat hareket sağlamaları ve şeffaf oldukları için yara bölgesinden çıkarılmadan yara kapanması takibi gibi avantajlara sahiptir. Ancak, absorbsiyon yeteneklerinin düşük olması nedeniyle yalnızca düşük eksüdalı yaralarda kullanımları ve biyodegredable olmadıkları için çıkarıldıklarında yarada hasara neden olma eğilimleri bu yara örtülerinin kullanımlarını kısıtlamıştır [2,3].

Alternatif yaklaşım ise büyük miktarda su ile şişebilen çapraz bağlı üç boyutlu ağ şeklindeki polimerik yapılar olan hidrojel yara örtüleri olmuştur. Hidrojellerin hidrofilik yapıları ile yaralara nem sağlayabilmeleri, su ile şişebilen çapraz bağlı ağ yapıları sayesinde yarayla temas ettiklerinde önemli miktarda su absorbe edebilmeleri, porlu yapılarının gaz geçişine izin vermesi ve yumuşak elastik özellikleri ile kolay uygulama ve hasara neden olmadan kolay çıkarmaya elverişli olmaları, hidrojel filmleri yara örtüsü uygulamaları için mükemmel adaylar haline getirmektedir [2,3].

Hidrojeller, basit jelleştirme ile hazırlanarak jel halinde yaralı dokuya uygulanabildikleri gibi polimerik film halinde de uygulanabilirler [4]. Ancak jel halinde uygulandıklarında, genellikle gazlı bez gibi ikincil bir sargı uygulamasına ve sık sık değiştirilme gereksinimine neden olmaktadır. Film halinde hazırlandığında bu dezavantajların üstesinden gelinmesinin yanında, yaranın etrafına oturacak şekilde kesilme olanağı ile hasta uyuncu yüksek yara örtüleri hazırlanabilmektedir. Bunun dışında gazlı bezlere emdirilmiş halde geleneksel yara örtüleriyle kombine uygulamaları da vardır [11].

Hidrojel yara örtüleri, hazırlandıkları polimerik materyallere göre hidrokolloidler, aljinatlar, hidrofiberler gibi farklı isimlerle adlandırılmaktadır. Özellikle jel oluşturucu hidrofilik kolloidal polimerlere ek olarak elastomer ve adezivler gibi ürün bileşimlerinden hazırlanan hidrojel yara örtüleri hidrokolloid olarak adlandırılmaktadır [4,10,11]. İnce filmler halinde hazırlanabilen bu hidrojellerin mekanik olarak daha dayanıklı ancak su buharı geçirgenliklerinin biraz daha düşük olduğu bildirilmektedir. Bir diğer hidrojel örneği de aljinat polimerlerinin kullanıldığı aljinat yara örtüleridir [2]. Karboksimetil selüloz (CMC) liflerinden oluşan ve özellikleri bakımından aljinat yara örtülerine benzer özelliklere sahip hidrojeller ise hidrofiberler olarak adlandırılmıştır [12]. Bunların dışında, kitozan, soy proteini, polivinil alkol (PVA), karbopol, jelatin, kolajen, hidroksi etil selüloz (HEC), hidroksi propil metil selüloz (HPMC), polivinil pirolidon (PVP), poli kaprolakton (PCL), kondroitin sülfat, glikoz amino glikanlar, fibrin, dekstran, polimetil metakrilatlar (PMMA) gibi pek çok polimer çeşitli film ve hidrojel yara örtüsü çalışmalarında kullanılmaktadır [5,11] ve ticari ürün haline gelmiş pek çok preparat piyasada mevcuttur (Tablo 1).

İdeal bir yara örtüsü alternatifi olarak en uygun formülasyonlardan biri de püskürtülebilir hidrojel yara örtüleridir. Püskürtülebilen bir yara örtüsüyle tek kullanımlık ürünler yerine çok kullanımlık ürünlerin elde edilmesi, klinik uygulama perspektifinden değerlendirildiğinde yara yüzeyine hızlı ve kolay uygulama, formülasyonun istenen boyutta yüzey temas alanı sağlaması ve daha derin yaralara daha kolay erişim gibi avantajlar elde edilebilir [13-15]. Önceden şekillendirilmiş yara örtülerine kıyasla yara defektine göre şekil alarak *in situ* oluşan hidrojeller, yara örtüsünün yeniden şekillendirilmesine ihtiyaç duyulmadan hazırlanabilmeleri yönüyle bu formülasyonların temelini oluşturmaktadır. Balakrishnan ve ark., çalışmalarında oksijenlenmiş aljinatın boraks varlığında jelatin gibi polimerlerle hızla çapraz bağlanma özelliğinden yaralanarak toksik çapraz bağlayıcı kullanmadan, çok basit bir yöntemle *in situ* oluşan hidrojel yara örtüsü hazırlanabileceğini göstermişlerdir [15]. Cheng ve ark., metakrillenmiş jelatin ve fotoçapraz bağlayıcı karışımıyla hazırladıkları polimer çözeltisini püskürttükten sonra UV ışığa maruz bıraktıklarında, uygulama yerinde hızlıca *in situ* oluşan hidrojel yara örtüleri geliştirmişlerdir [16]. Annabi ve ark. ise UV ışığın neden olabileceği doku ve DNA hasarını önlemek için görünür ışıkta çapraz bağlanabilen metakrillenmiş jelatin ve metakrillenmiş insan tropoelastininden oluşan püskürtülebilir bir yara örtüsü geliştirilebileceğini bildirmişlerdir [17]. Püskürtülebilmeleri için bu *in situ* hidrojel formülasyonlarının psödoplastik yani karıştıkça incelen bir davranış sergilemeleri gerekmektedir. Grip ve ark., yara iyileştirici beta glukan içeren püskürtülebilir

yara örtüsü formülasyonu için psödoplastik özelliği sebebiyle bir karbomer olan karbopol polimeri ile çalışmışlardır [13]. Bu yara örtüleri 1950'lerden beri mevcuttur ve bunlardan biri de yara üzerine püskürtüldüğünde *in situ* film oluşturan polihidroksi etil metakrilat ve PEG bazlı ticari bir yara örtüsüdür (Hydron®) [15]. Bunların dışında hidrojellerin çapraz bağlanma özelliklerinden yararlanarak, sık hareket eden eklem gibi vücut bölgelerinde uygulanan yara örtülerinin olası parçalanma risklerine karşı kendi kendini onaran hidrojeller sayesinde yara örtülerinin kullanım sürelerinin de büyük ölçüde uzatılabileceğini gösteren çalışmalar da yer almaktadır [18].

Tablo 1. Piyasada ticari olarak mevcut olan film ve hidrojel yara örtüleri

Ürün	Şirket	Polimer	Açıklama
Bioclusive™	Johnson & Johnson	Poliüretan	Film
Opsite™ Flexigrid	Smith & Nephew	Poliüretan	Film
Tegaderm™	3M Health Care	Poliüretan	Film
Intrasite™	Smith&Nephew	Modifiye CMC	Jel hidrojel
Nu-gel™	KCI Medical	Sodyum Aljinat	Jel hidrojel
Aquaform™	Aspen Medical	Modifiye Nişasta	Jel hidrojel
Purilon™	Coloplast	Aljinat	Jel hidrojel
Restore® Hydrogel	Hollister	Aljinat	Jel hidrojel
Geliperm®	Geistlich Sons	PMMA'lar	Jel ve film hidrojel
Sorbsan™	BerTec	Kalsiyum Aljinat	Gazlı beze emdirilmiş
Kaltostat™	ConvaTec	Sodyum-Kalsiyum Aljinat	Gazlı beze emdirilmiş
Algisite™ M	Smith&Nephew	Kalsiyum Aljinat	Gazlı beze emdirilmiş
Tegagel™	3M Health Care	Kalsiyum Aljinat	Gazlı beze emdirilmiş
Aquacel™	ConvaTec	NaCMC	Gazlı beze emdirilmiş
Granuflex™	ConvaTec	CMC, Jelatin, Pektin	Hidrokolloid yapıştırılmış poliüretan film
Comfeel™	Coloplast	NaCMC, HEC, Kalsiyum Aljinat	Hidrokolloid yapıştırılmış poliüretan film
Tegasorb™	3M Health Care	Poliizobutilen	Hidrokolloid yapıştırılmış poliüretan film

*CMC: Karboksimetil selüloz, HEC: Hidroksietil selüloz, NaCMC: Sodyum karboksimetil selüloz, PMMA: Polimetil metakrilat

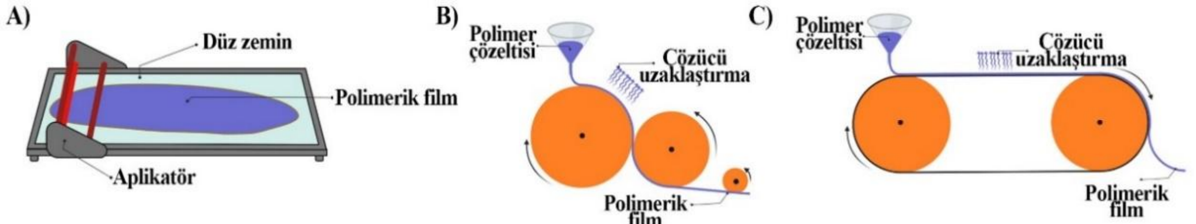
Polimerik filmler pek çok doğal ve sentetik kaynaklı polimer kullanılarak çözücü dökme yöntemi, eriyik presleme, eriyik ekstrüzyonu, kabarcık üfleme yöntemi, üç boyutlu (3B) baskı teknolojileri ve nanolif teknolojilerini içeren yöntemlerle hazırlanabilir:

Çözücü dökme yöntemi, temel olarak polimerik bir çözeltinin veya jelin petri kabı veya plakası gibi uygun bir zemine dökülerek kurutulmasını içerir [19,20]. İmalat kolaylığı ve daha ucuz işlem maliyetleri nedeniyle yaygın olarak kullanılmaktadır. Bu yöntemde, viskoz bir çözelti elde etmek için polimer uygun bir çözücü içinde çözündürülür, daha sonra düz ve yapışkan olmayan bir yüzeye dökülür. Çözücünün buharlaşmasına izin verilir ve kuru filmler bu yüzeyden çıkarılarak elde edilir.

Bu yöntemle homojen filmler hazırlayabilmek için polimer ve çözücü seçiminin önemli olduğu vurgulanmaktadır. Polimer çözeltisinde homojeniteyi bozan zayıf kümelenme oluşumunu önlemek için uygun molekül ağırlığında bir polimerle çalışılması önerilmektedir. Hazırlanan polimer çözeltisinin uygun zemine dökme işlemi sırasında bu işlemi kolaylaştırmak için, oda sıcaklığında ya da biraz üzerinde uçucu olan çözücüler tercih edilir. Filmde kabarcık veya yarı kristal çökelti oluşumlarına karşı buharlaşma hızının yavaş olması istenir. Aynı zamanda hızlı buharlaşmanın, filmin hızlı soğumasına ve ortamdaki su buharının yoğunlaşmasına neden olabileceği de bildirilmektedir. İyi kalitede filmler elde etmek için kaynama noktaları 60-100°C arasında olan çözücülerin kullanımı daha uygundur [21].

Katmanlar halinde film hazırlama olanağı ile de dikkat çeken çözücü dökme yöntemi, laboratuvar boyutlarından endüstriyel boyutlara kadar ölçeklenebilir olması ile de oldukça avantajlı bir yöntemdir [21-23]. Küçük ölçekte, homojen kalınlıkta polimerik bir film, sadece polimer çözeltisini düz bir yüzey üzerine yayarak hazırlanabildiği gibi değişen ölçeklerde otomatik film makineleri gibi sistemlere

uyarlanabilmektedir. Şekil 2'de değişen ölçeklerde çözücü dökme yöntemi ile film hazırlanması şematize edilmiştir.



Şekil 2. Laboratuvar koşullarında (A) ve daha büyük ölçeklerde (B, C) çözücü dökme yöntemiyle polimerik film hazırlanışının şematik gösterimi

Eriyik presleme yöntemi, termal olarak stabil polimerlerin kullanıldığı formülasyonlar için uygundur. Temel olarak elektrikle ısıtılan iki plaka arasına yerleştirilen toz halindeki polimerin, belirli bir süre boyunca basınç uygulaması ile film hazırlanması esasına dayanır. Soğutma işleminden sonra oluşan filmler sistemden ayrılır. Uygulama sırasında, sıcaklık ve basınç önemli kritik parametrelerdendir. Örneğin sıcaklık çok yüksek olduğunda, polimer sistemden dışarı akabilir. Çok düşük olduğunda ise, yetersiz erime nedeniyle opak veya zayıf film oluşumuyla sonuçlanabilir. Laboratuvar ölçekli üretim için uygundur, fakat filmlerin büyük ölçekte bu yöntemle hazırlanması zordur ve işlem süreksizdir [21].

Eriyik ekstrüzyon yöntemi, sürekli bir işlem olduğu için eriyik presleme işlemine göre daha avantajlıdır [21]. Bu yöntemde, polimer tozu veya pelletleri, polimeri ısıtan bir ekstrüdere verilir. Burada eriyen polimer ekstrüde edilip bir seri döner silindir ile soğutularak polimerik filmler elde edilir. Repka ve ark., eriyik ekstrüzyon yöntemi ile hazırlanan mekanik ve fiziksel olarak stabil hidroksipropil selüloz filmlerinin, topikal etkin madde salımı ve yara bakımı formülasyonlarında kullanılabileceğini göstermişlerdir [24].

Kabarcık üfleme yöntemi, eriyik ekstrüzyon yöntemine alternatif olarak geliştirilmiştir. Toz halindeki polimer, polimeri ısıtan vidalı bir ekstrüdere verilir. Vidalı ekstrüderden gelen erimiş polimer, orta kısmından gaz geçişine izin verilen halka şeklinde bir kalıptan geçirilir. Ardından inert bir gaz sisteme verilerek oluşan gaz basıncıyla kabarcıklar oluşur. Bu kabarcık, özel olarak tasarlanmış bir dizi silindir ile sarılarak düzleştirilir ve polimerik filmler elde edilir [21].

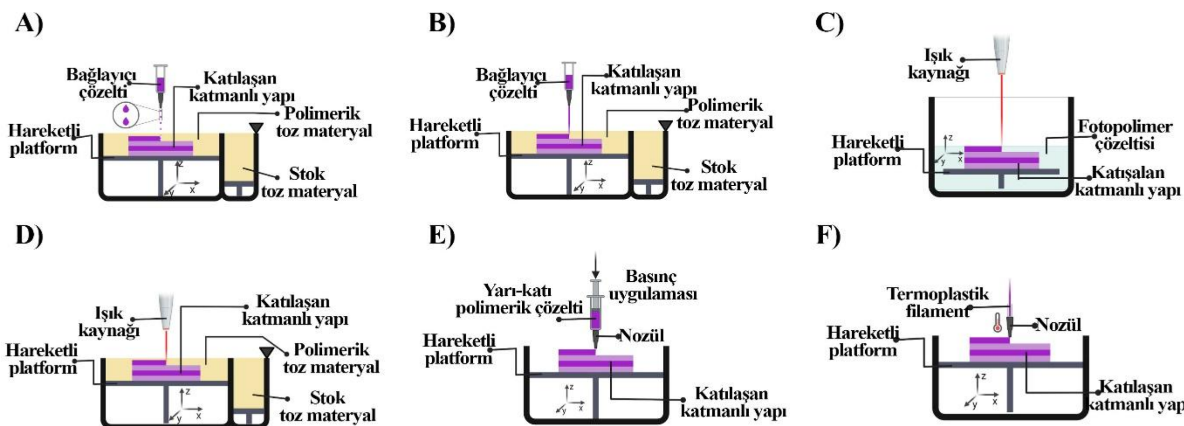
3B Baskı teknolojileri, film hazırlamada alternatif olarak tercih edilen bir yöntemdir. Fiziksel bir nesne oluşturmak için Bilgisayar Destekli Tasarım (CAD) yardımıyla materyallerin tabaka tabaka biriktirilmesi esasına dayanan bir yöntemdir. İstenen şekle sahip yapı ve bileşenlerin hızlı ve doğru bir şekilde üretimini sağlaması hem zaman hem maliyetin azaltılması açısından avantajlıdır. Farmasötik anlamda 3B baskının sağladığı en büyük avantaj ise kitlesel olarak üretilen terapötik sistemleri kişiye özel hale getirmeyi başarmasıdır. Böylece hastaların tedavi şeklini temelden değiştiren, hastaya özgü karakteristiklerle kişiye özel implantlar, protezler, yara örtüleri ve çeşitli ilaç sistemleri 3B olarak üretilebilmektedir [20,25].

3B baskı teknolojileri üç farklı mekanizma ile hazırlanabilmektedir (Şekil 3) [20,25]. Mürekkep püskürtme sistemleri (IJP; inkjet printing) bir zemin üzerine çözücü olarak bağlayıcı çözeltinin sürekli (Şekil 3A) veya isteğe bağlı dijital kontrollü olarak küçük sıvı damlaların tabakalı olarak birikmesi esasına dayanır (Şekil 3B). Nozzle esaslı birikim sistemleri, polimer ve diğer bileşenlerin 3B baskıdan önce karıştırılarak hazırlanan viskoz yarı katı veya erimiş katı karışımlarının bir nozzle başlığından ekstrüde edilmesiyle doğrudan baskı yapılan sistemlerdir. Herhangi bir ısıl işlem olmadan viskoz bir yarı katının ekstrüde edilmesiyle (SSE; Yarı katı ekstrüzyonu) (Şekil 3E) veya termoplastik bir polimerin yüksek sıcaklıkta eritilerek ekstrüzyonu (FDM; Eriyik Birikim Modelleme) ile baskı yapılabilir (Şekil 3F). Lazer esaslı baskı sistemleri ise polimerik materyallerin sistemde yer alan ışık kaynağıyla etkileşimine dayanan bir baskı yöntemidir. Fotobaşlatıcı içeren polimerlerin fotopolimerizasyonu (SLA: Stereolitografi) (Şekil 3C) veya polimerik toz materyallerin sıvılaşarak

kaynaşması ve katılaşması (SLS: Seçici lazer sinterleme) ile baskı yapılabilir (Şekil 3D). Bu mekanizmalar arasında çeşitli biyomedikal uygulamalarda yüksek sıcaklık uygulaması gerektirmeden, doğal dokuyu taklit edebilen, yeterli mekanik ve biyolojik özelliklere sahip film ve hidrojelin 3B baskıyla hazırlanmasında nozzle esaslı 3B baskı sistemleri başarıyla kullanılmıştır.

SSE sistemi kullanılan formülasyonlarda çözücü kullanıldığı için baskı işleminden sonra bir kurutma işlemi gerekir [20,25]. Oluşan filmin fazla sertleşmesi ve çatlamasına neden olması açısından seçilen çözücünün uçuculuğu, kuruma için önemli bir kriterdir. Baskı sırasında uygun birikmenin sağlanması için kontrol edilmesi gereken diğer parametre polimerin konsantrasyonu ve dolayısıyla viskozite olmaktadır. Uygun olmayan viskozluk, baskı sırasında nozzle tıkanması veya aşırı akışkanlık gibi nedenlerle baskıya engel olabilir. Baskı hızının seçimi, kullanılan nozzle çapı ve oluşan tabakanın kalınlığı da filmlerin dış görünüşü üzerinde değişikliğe neden olabilen sistem parametrelerindendir. Eğer FDM sistemi kullanılıyorsa, sıcaklığın ayarlanması, kullanılan polimer açısından önemlidir.

Hafezi ve ark., SSE mekanizmalı 3B baskı yöntemi kullanarak hazırladıkları yüksek şişme oranına sahip ve biyouyumluluk sergileyen genipin çapraz bağlı kitozan filmlerin, yara bakımı uygulamaları için yara örtüsü potansiyeli taşıdıklarını bildirmişlerdir [26]. Goyanes ve ark., akne tedavisi için salisilik asit içeren maske biçiminde filmleri hazırlamak için iki farklı 3B baskı yöntemi (FDM ve SLA) kullanmışlardır [27]. Kişiye özel anti akne ilacı yüklü burun maskesi biçimindeki filmlerin başarıyla oluşturulabilmesi, benzer şekilde hastaya özgü kronik yaraların boyutuna uygun yara örtülerinin hazırlanabilmesini desteklemektedir. Literatürlerde 3B baskı teknolojileriyle hazırlanmış, biyouyumlulukları ve *in vivo* olarak oluşturulan yaralardaki olumlu etkileriyle yara örtüsü potansiyeli gösteren çeşitli film çalışmaları yer almaktadır [26,28,29].



Şekil 3. Farmasötik alanda kullanılan 3B baskı teknolojilerinin şematik gösterimi; A) Kesintili mürekkep püskürtmeli inkjet, B) Sürekli mürekkep püskürtmeli inkjet, C) Stereolitografi (SLA), D) Seçici lazer sinterleme (Selective Laser Sintering, SLS), E) Yarı katı ekstrüzyon (Semi-solid Extrusion, SSE), F) Eriyik yığma modelleme (Fused Deposition Modeling, FDM)

Nanolifler ve Nanolif Teknolojileri

Nanolifler, elektrik yüklü bir polimer çözeltisi lifinden, birkaç nanometreden birkaç mikrometreye değişen çaplarda nanolif ağların elektrospinning (elektro-eğirme veya elektrospinleme), faz ayrılması, kendi kendine bir araya gelme, kalıpla sentezleme ve mekanik çizim gibi yöntemlerle üretilir [30]. Bu yöntemler arasında elektrospinleme nispeten kolay, uygun maliyetli, basit işlem gerekliliği ve diğer geleneksel lif şekillendirme teknikleriyle kolayca elde edilemeyen basit üretim aşamalarıyla ultra ince liflerin üretilebilmesi sayesinde en çok tercih edilen teknik olmuştur [31]. Elektrospinleme sırasında, çözelti halindeki veya erimiş haldeki polimer sıvısı bir enjektöre yüklenir ve sisteme yüksek voltajlı bir elektrik alanı uygulanır [31-33]. Enjektörün kılcal ucundaki polimer damlacıklarının sahip olduğu yüzey gerilimi ile sistemdeki elektrik alan kuvvetleri denge halinde iken,

elektrik alan kuvvetinin artması ile bu denge bozularak enjektör ucundan lifler halinde uzamaya başlar. Bu lifler ortam boyunca ilerledikçe çözücü buharlaşır ve nanolifler halinde toplayıcı üzerinde birikir.

Sentetik, doğal, biyolojik olarak bozunabilir, biyolojik olarak bozunamaz veya bunların karışımlarını içeren pek çok polimer bu teknikte kullanılabilir. Elektrospinleme kullanarak biyolojik olarak bozunabilir polimerlerle hazırlanan formülasyonların, ekstraselüler matris (ECM)'in morfolojik özelliklerini taklit edebilen, derinin ECM'sine çok benzeyen yapılar üretilebildiği keşfedilince, elektrospun nanolifler, yara bakımı materyallerinin geliştirilmesinde de son teknolojilerden biri haline gelmiştir [11,30,33,34]. Elektrospun nanolifler, sahip oldukları yüksek yüzey alanları ve gözenekli/porlu yapılarıyla yara eksüdalarını absorbe etme, gaz geçirgenliğini kolaylaştırma, aşırı dehidrasyonu ve bakteriyel enfeksiyonu önleme gibi ideal yara örtülerinden beklenen özellikleri nedeniyle yara örtüsü olarak kullanımları dikkat çekmiştir. Nanoliflerin film ve iskele halinde yara örtüsü uygulamaları araştırılmıştır. Ignatova ve ark., kitozan ve kuarternize türevi ile polilaktik asit (PLA) bazlı nanolif yara örtülerinin elektrospinleme yöntemi ile başarılı bir şekilde hazırlanabileceğini göstermişler, çözücü dökme yöntemiyle hazırlanan film formülasyonlarına kıyasla daha yüksek antibakteriyel etkiye sahip olmalarını nanoliflerin yüksek spesifik yüzey alanı ile açıklamışlardır [35].

Deri yenilenmesinde elektrospun nanolif performansının artırılması için, nanolif yüzeyine biyoaktif moleküllerin ve hücre tarafından tanınabilen ligantların kimyasal ve fiziksel olarak ilave edilmesiyle, birden fazla tabakanın ayrı ayrı üretilmesiyle veya üretim tekniğindeki değişikliklerle modifiye edilebileceği bildirilmektedir. Aynı zamanda stabilitelerini artırmak için üretim işleminden sonra kimyasal olarak çapraz bağlanma gibi ek prosesler de kullanılmaktadır [35,36]. Polimer özellikleri, çözücü özellikleri, polimer çözeltisinin konsantrasyonu, spinleyici çapı ve uygulanan voltaj gibi parametrelerde yapılan değişikliklerle, üretilen liflerin morfolojisi ve çapı üzerinde de modifikasyonlara olanak tanımaktadır [33,36,37].

Köpükler ve Süngerler

Köpükler poliüretan veya silikondan üretilmiş gözenekli materyallerdir. Köpükler, çeşitli polimer çözeltilerinin dondurularak kurutulması ile hazırlanan sünger yara örtülerinin de öncüsüdür. Bu yüksek gözenekli yapıları ile daha fazla eksüda absorbe edebilirler ve gaz geçişine elverişlidirler [4,11,30]. Bunlara ek olarak mekanik esneklikleri, yaralı dokuda yastık gibi mekanik destek sağlayabilmeleri ve iyi termal izolasyon özellikleri ile karakterize edilirler. Bu sayede özellikle kemikli bir çıkıntının üzerindeki yaralarda kullanımları oldukça avantajlıdır [7]. Köpüklerin sıvı absorpsiyon kapasitesi, köpük kalınlığı, gözenek boyutu gibi köpük özellikleri ile kontrol edilebilir [4]. Köpükler, adeziv ya da adeziv olmayan özellikte, çeşitli şekil ve boyutlarda hazırlanabildikleri gibi yara kuruduğunda yaraya yapışmayı, eksüda sızıntısını ve bakteriyel kontaminasyonu önlemek için ek olarak oklüziv polimerik bir film katmanı ile kombine halde hazırlanabilmektedir [4,10]. Film formülasyonlarına kıyasla sık değiştirme işlemi gerektirmelerine rağmen günümüzde köpük ve sünger halinde hazırlanmış çeşitli yara örtüsü çalışması mevcuttur [38-41]. Ticari ürün haline gelmiş pek çok köpük preparatı piyasada yer almaktadır (Tablo 2).

Tablo 2. Köpük yara örtüsü örnekleri

Ürün	Şirket	Polimer	Açıklama
Lyofoam®	ConvaTec	Poliüretan	Poliüretan tabaka
Lyofoam® A	ConvaTec	Poliüretan	Adeziv
Lyofoam® C	ConvaTec	Poliüretan	Aktif karbon kömürü ile emdirilmiş kumaşlı iki poliüretan tabaka
Allevyn™	Smith & Nephew	Poliüretan	Poliüretan film tabakalı
Tielle®	Johnson & Johnson	Poliüretan	Akrilik adeziv
Dermalix®	Abdi İbrahim	Kolajen-Laminin	Resveratrol yüklü mikropartiküller içeren kolajen-laminin yara örtüsü

Berce ve ark.'nın resveratrol içeren kitozan ve hyalüronik asitten hazırlanan sünger yara örtüsü çalışmasında, yara iyileştirici etkisi *in vivo* olarak değerlendirilmiştir [41]. Bu çalışmada etkin madde içeren polimerik jellerin liyofilize edilmesiyle hazırlanan süngerlerin, farelerde oluşturulan *in vivo* yara ile temas ettiğinde, eksüdayı absorbe ederek yarayı tamamen kaplayan jelatinimsi bir film oluşturduğu gözlenmiştir. Aynı zamanda yara iyileşmesindeki olumlu etkileri ve formülasyonların biyodegredable olması sayesinde çıkarılmaya gerek duymadan kullanılabilme potansiyelleri ile klasik köpük formülasyonlarında karşılaşılan yara örtüsü değiştirme dezavantajı da sünger formülasyonları ile aşılabilmektedir.

Kompozitler ve İskeleler

Yara oluşum nedenine bağlı olarak değişen yara büyüklüğü ve derinliği, farklı deri tabakalarını etkileyebilir. Derinin hücre yoğunlukları birbirinden farklı olan tabakalarındaki iyileşme hızı da farklı olacaktır. Farklı katmanların büyümesini destekleyen, uygun özelliklere sahip çok katmanlı yapıların imalatının, doku üretim hızını daha da artırabileceği düşünülerek son zamanlarda kompozit ve iskele olarak adlandırılan çok katmanlı yapılar geliştirilmektedir [11].

Kompozit veya iskele şeklindeki yara örtülerinde birden fazla katman vardır ve her katman fizyolojik olarak farklı olabilir [10]. Klasik kompozit yara örtülerinin çoğu üç katmana sahiptir. En dış katman yarayı enfeksiyondan korur, orta katman genellikle nem sağlayan ve otolitik debridmana yardımcı olan absorbe edici malzemeden oluşur, alt katman ise yaraya yapışmayı önleyen adeziv olmayan malzemeden oluşur. Katmanlar halinde hidrojellerin bir araya getirilmesi ile veya nanolif teknolojileri ve 3B baskı teknolojileri kullanılarak kompozit yara örtüleri hazırlanabilir [11].

Kompozit sistemler, katmanlarına dahil edilen bileşenlerin faydalı özelliklerini bir araya getirir. Bu nedenle, uygun polimer ve diğer bileşenlerle, hızlı yara iyileşmesi için hem fiziksel hem de biyolojik gereksinimleri karşılayan kompozitler tasarlanabilir [11,42]. Maver ve ark., bir tabakasında analjezik içeren iki katmanlı selüloz filmlerin yara örtüsü potansiyellerini araştırmışlardır [42]. Kompozit filmler elektrospinleme işlemi ile hazırlanarak etkin madde içeren tek tabakalı elektrospun filmlerle karşılaştırmışlardır. Bir ve iki katmanlı filmlerde hızlı ilk çıkışın ardından salım profillerinin farklılaştığı gözlenmiştir. Analjezik ilaçların derhal salımı ile başlayan analjezik etkinin, kompozit film sayesinde yara iyileşme sürecinin sonraki aşamalarında daha kontrollü ve daha yavaş bir salım ile hastaların yaşam kalitesini önemli ölçüde iyileştirebileceği düşünülmüştür. Bir başka çalışmada ise, 3B baskı ve elektrospinleme yöntemleri kullanılarak her tabakasında farklı salım profillerine sahip iki farklı etkin madde içeren selüloz bazlı kompozit yara örtüleri başarıyla hazırlanmıştır [34]. Bunların dışında çok sayıda kompozit yara örtüsü çalışması literatürlerde yer almaktadır [23,29].

Kompozit yara örtüleri daha az esnek olmaları ve hazırlanma teknolojileri nedeniyle daha maliyetli olmalarına rağmen, hidrojellerin şişmiş haldeki zayıf mekanik özellikleri geliştirilmek istendiğinde de birden fazla polimer içeren kompozit sistemleri tercih edilebilir [5,11].

Biyoaktif Yara Örtüleri

Yara nemini yönetebilme kabiliyeti yanı sıra kitozan, aljinat, kolajen, selüloz ve hyalüronik asit gibi pek çok polimer, yara iyileşmesini indükleyici etkilerinden dolayı yara örtüsü çalışmalarında biyoaktif malzeme olarak yer almaktadır [10]. Bu biyomateryaller biyouyumlu, biyobozunur ve toksik olmayan yapıları ile bilinir. Yaranın türüne bağlı olarak tek başına veya kombinasyon halinde kullanılabilir ve bazen yara iyileşme sürecini geliştirmek için büyüme faktörleri ve antimikrobiyaller gibi çeşitli etkin maddeleri içerebilir. Wathoni ve ark., bir siyanobakteri türü olan *Aphanothece sacrum*'dan ekstre edilen sakran polisakkaritinin biyoaktif yara örtüsü potansiyelini araştırmışlardır [43]. Fiziksel olarak çapraz bağlı ağlar oluşturabilen sakran hidrojel filmleri, çözücü dökme yöntemiyle hazırlanmış ve sonuçlar aynı yöntemle hazırlanmış aljinat filmlerle karşılaştırılmıştır. Analiz sonuçlarına göre aljinata kıyasla yüksek sıcaklık uygulamalarına daha dayanıklı, porlu yapılarıyla daha iyi şişme özelliğine sahip, sulu ortamda aljinat gibi hızla degrade olmayan, biyouyumlu ince filmler elde edilebileceği gösterilmiştir. Farelerde oluşturulan *in vivo* eksizyon yaralarına uygulandığında hızlı yara kapanma süreleri ile doğrulanan doku yenilenmesi sayesinde yara örtülerinde kullanım potansiyeline sahip yeni bir biyomateryal olabileceği sonucuna varılmıştır.

Biyolojik veya Biyosentetik Yara Örtüleri

Yara iyileşmesini hızlandırmak için kullanılan bir diğer yöntem ise, greft olarak adlandırılan insan, hayvan veya kadavraların donör bölgelerinden normal ve taze derinin nakledilmesini veya insan fibroblast ve keratinosit hücreleri içeren yaşayan deri eklerinin yaraya uygulanmasıdır [5,10,44]. Medikal olarak kullanılan en eski biyolojik örtü amniyon membranlardır [44]. Yara bölgesini koruma özellikleri ve mikrobiyal yükü azaltma etkilerine rağmen mekanik stabiliteleri zayıftır. Taze, kuru, dondurulmuş, dondurularak kurutulmuş halde hazırlanabilirler. Pek çok akut ve kronik yarada etkilidirler. Amniyon membranlar dışında epidermis, dermis veya her iki deri tabakasının yerini alabilecek çeşitli biyolojik ve biyosentetik yara örtüleri de geliştirilmiştir. Doğrudan canlı hücrelerle hazırlandıkları için hazırlanma koşullarına ve stabilite özelliklerine bağlı olarak raf ömürleri günlerden aylara varan sürelerde değişmektedir. Yüksek maliyetli olmalarına ve her yaraya uygulanabilecek ideal tek bir biyolojik yara örtüsü olmaması gibi kısıtlamalara rağmen, ticari olarak temin edilebilen pek çok ürün piyasada yer almaktadır (Tablo 3).

Tablo 3. Piyasada ticari olarak yer alan biyolojik yara örtüleri [44]

Ticari Ürün	İçerik	Taşıyıcı Membran	Endikasyon	Raf Ömrü
Epifix®	Keratinosit	Amniyotik zar	Diyabetik ülserler	>2 yıl
Epicel®	Keratinosit	Fibrin	Yanık yaraları	19 gün
CellSpray®	Keratinosit	-	Yüzeysel yaralar	5 gün
Myskin™	Keratinosit	-	Yanık yaraları, ülserler ve diğer iyileşmeyen yaralar	2-3 hafta
EpiDex®	Keratinosit	Silikon	Yanık yaraları, bacak ülserleri	1 ay
Matriderm®	Kolajen	Elastin	Yanık yaraları ve diğer kronik yaralar	-
Biobrane™	Kolajen	Silikon-Naylon	Yanık yaraları	-
OASIS® Wound Matrix	Domuz bağırsak mukozası matrisi	-	Yanık yaraları, ülserler ve diğer kronik yaralar	>2 yıl
Integra®	Kolajen	Silikon-Kondroitin sülfat	Yanık yaraları, diyabetik ayak ülserleri	-
Nevelia®	Kolajen	Silikon	Yanık yaraları, cerrahi yaralar ve travmatik yaralar	-
AlloDerm™	Bazal membranlı hücresiz dermal matris	-	Yanık yaraları ve yumuşak doku defektleri	-
dCELL®	Deri greftlerinden elde edilmiş	-	Bacak ülserleri	-
TransCyte®	Fibroblast	Silikon-Naylon	Yanık yaraları	-
Dermagraft®	Fibroblast	Poligalaktin	Diyabetik ayak ülserleri	*
Hyalomatrix®	Hyalüronik asit	Silikon	Yanık yaraları, cerrahi yaralar ve diğer kronik yaralar	-
Hyalograft 3D™	Fibroblast	Hyalüronik asit	Diyabetik ayak ülserleri	24 gün
Apligraf®	Keratinosit, Fibroblast	Kolajen	Diyabetik ayak ülseri, Venöz bacak ülseri	5 gün
StrataGraft®	Keratinosit	-	Yanık yaraları	-
OrCel®	Keratinosit, Fibroblast	Kolajen	Kronik yaralar	-

* Dondurulmuş olarak -75°C'de kullanıma hazır

Yara Örtülerinde Topikal Etkin Madde Uygulamaları

İyileşme sürecinin hızlanması ve yara bölgesindeki koşulların antimikrobiyal ajanlar ile kontrol altında tutulması yara tedavisinde tercih edilen temel tedavi yaklaşımlarındandır [4,6,45]. Biyoaktif yara

örtülerinin kullanımının [35,46] yanı sıra etkin madde taşıyıcısı olarak bünyesinde antimikrobiyal ajan içeren yara örtüsü araştırmaları da literatürlerde yer almaktadır. Egozi ve ark., soy proteinden hazırladıkları hidrojel filmlerden gentamisinin [9], Muwaffak ve ark., PCL filmlerden gümüş nitrat, bakır sülfat ve çinko oksitin [28], Azad ve ark., ile Ignatova ve ark., kitozanın antimikrobiyal etkisinden faydalanmayı amaçlamışlardır [35,46]. Yaraya topikal olarak antimikrobiyal uygulaması, kontaminasyona karşı bariyer oluşturarak daha iyi ve hızlı yara iyileşmesi sağlayabilir. Yara örtüsü çalışmalarında antibiyotikler, inorganik nanopartiküller (gümüş, bakır, çinko vb.) veya doğal kaynaklı ürünler (bal, bor, kurkumin, keratin vb) gibi çok çeşitli antimikrobiyal ajan kullanılmaktadır.

Büyüme faktörleri, hücre büyümesini, farklılaşmasını, göçünü düzenleyen ve yara iyileşmesinin tüm evrelerinde önemli biyolojik aktivitelere sahip polipeptitlerdir [1]. Dönüştürücü büyüme faktörü (TGF), trombosit türevi büyüme faktörü (PDGF), epidermal büyüme faktörü (EGF), fibroblast büyüme faktörü (FGF), insülin büyüme faktörü (IGF), keratinosit büyüme faktörü (KGF), vasküler endotelyal büyüme faktörü (VEGF), interlökinler (IL-1) ve granülosit makrofaj koloni uyarıcı faktörler (GM-CSF) klinik olarak kullanılan büyüme faktörlerindendir. Bazı klinik araştırmalarda, büyüme faktörlerinin, yan etki olmaksızın yara iyileşmesinde hızlanma ve deri yenilenmesi sağladığı bildirilmektedir.

Yara tedavisinde tercih edilen tedavi yaklaşımlarından biri de yara iyileştirici etkisi bilinen tıbbi bitkilerin kullanımıdır. Örneğin antioksidan ve antienflamatuvar etkileriyle bilinen kurkumin bitkisel bir saponindir [37]. Kurkumin içeren karboksimetil kitozan ve aljinattan oluşan hidrojeller hazırlanmıştır [47]. 72 saatten daha uzun sürelerde kontrollü etkin madde salımı ile yara iyileşmesinde kullanım potansiyeline sahip olduğu bildirilmiştir. *Centelle asiatica* ekstresi, *Biophytum sensitivum* [37], *Aloe vera* [48] ve *Hypericum perforatum* [45] da yara iyileştirici etkileriyle kullanılan bazı tıbbi bitkilerdendir.

Yara iyileşmesini ve deri yenilenmesini desteklemek amacıyla, yara örtüleri kendileri terapötik etkin madde taşıyabildikleri gibi etkin madde taşıyan polimerik nano/mikropartikül, lipit nanopartikül, lipozomlar ve niozomlar gibi çeşitli ilaç taşıyıcı sistemleri de içerebilirler. Bu ilaç taşıyıcı sistemlerin kullanımı ile yara örtüsündeki duyarlı etkin maddelerin dış çevreden koruyarak stabiliteleri artırılabilir, olası yan etkiler en aza indirilebilir. Aynı zamanda polimerik matris aracılığıyla etkin madde salımının kontrol edilebilmesiyle yara örtüsü uygulama süreleri de modifiye edilebilir [47,49,50].

Etkin madde içeren çeşitli formlarda hazırlanmış yara örtüsü çalışmaları, içerdikleri etkin madde, hazırlandıkları materyal ve üretim yöntemlerine göre Tablo 4'te listelenmiştir.

Tablo 4. Üretim yöntemleri, kullanılan malzemeler ve içerdikleri etkin maddelere göre farklı formlarda hazırlanmış yara örtüsü çalışmaları

Üretim Yöntemi	Yara Örtüsü Formu	Polimer	Etkin Madde	İlaç Taşıyıcı Sistem	Ref.
3B Baskı	Kompozit Film	Pluronic F127-PEG-PVA	5-florourasil	-	[51]
3B Baskı Elektrospinleme	Kompozit Film	CMC-Aljinat, CMC-PEO	Diklofenak, lidokain	-	[34]
3B Baskı	Hidrojel Film	Poli dimetil siloksan	Gümüş	-	[52]
3B Baskı	Film	PCL	Gümüş nitrat, bakır sülfat, çinko oksit	-	[28]
3B Baskı	Hidrojel	Metakrillenmiş kitozan	Lidokain, levofloksasin	-	[53]
3B Baskı	Film	PCL, PLA, PEG, Poliüretan	Salisilik asit	-	[27]
Çözücü Dökme	Hidrojel Film	Aljinat, Jelatin	Aloe vera	Niozom	[48]
Çözücü Dökme	Hidrojel Film	PVA-PEG, PVP, Karbopol, HEC, HPMC, NaCMC	Basitrasin	-	[54]
Çözücü Dökme	Hidrojel Film	PVP-CMC	Borik asit	-	[55]
Çözücü Dökme	Hidrojel Film	PVA, PEG	Centella asiatica	-	[56]
Çözücü Dökme	Hidrojel Film	Aljinat	Esansiyel yağlar	-	[57]

Tablo 4 (*Devam*). Üretim yöntemleri, kullanılan malzemeler ve içerdikleri etkin maddelere göre farklı formlarda hazırlanmış yara örtüsü çalışmaları

Üretim Yöntemi	Yara Örtüsü Formu	Polimer	Etkin Madde	İlaç Taşıyıcı Sistem	Ref.
Çözücü Dökme	Hidrojel Film	Soya proteini	Gentamisin	-	[9,58]
Çözücü Dökme	Hidrojel Film	Hipromelloz süksinat, kitozan	Gentamisin	-	[59]
Çözücü Dökme	Kompozit Hidrojel Film	Akrilik asit, Metilen akrilamid, Grafen oksit	Gümüş nitrat	-	[60]
Çözücü Dökme	Hidrojel Film	Kitozan, HPMC	Gümüş sülfadiazin	Lipit nanopartikül	[61]
Çözücü Dökme	Hidrojel Film	Kitozan	Hypericum perforatum	-	[62]
Çözücü Dökme	Hidrojel Film	NaCMC, PVP	Kırmızı soğan	-	[63]
Çözücü Dökme	Hidrojel Film	PVA, Kitozan	Minosiklin	-	[64]
Çözücü Dökme	Hidrojel Film	Na-Aljinat, PVA	Minosiklin	-	[65]
Çözücü Dökme	Hidrojel Film	Kitozan	Moksifloksasin	Niozom	[49]
Çözücü Dökme	Hidrojel Film	Kitozan	Mupirosin	Lipozom	[66]
Çözücü Dökme	Hidrojel Film	Na-Aljinat, PVP, PVA	Neomisin sülfat	-	[67]
Çözücü Dökme	Hidrojel Film	PVA, Aljinat	Nitrofurozon	-	[68]
Çözücü Dökme	Hidrojel Film	Jelatin, Jelatin-PEG	Siprofloksasin	-	[32]
Çözücü Dökme	Hidrojel Film	Na-Aljinat, PVP, PVA	Sodyum fusidat	-	[69]
Çözücü Dökme	Hidrojel Film	PVA, Poli akrilamid	Tetrasiklin, gentamisin	-	[70]
Çözücü Dökme	Film	Kitozan-jelatin	Timol	Polimerik mikropartikül	[71]
Çözücü Dökme	Hidrojel Film	Kolajen	Usnik asit	Lipozom	[72]
Çözücü Dökme	Hidrojel Film	Jelatin	Usnik asit	Lipozom	[73]
Çözücü Dökme	Hidrojel Film	CMC, PVP, Karbopol	Usnik asit	-	[74]
Elektrospinleme	Nanofiber Kompozit	Trimetil silil selüloz	Diklofenak	-	[42]
Elektrospinleme	Nanofiber Film	Kitozan	Gentamisin	Lipozom	[75]
Elektrospinleme	Nanofiber	PVP-PEG	Gentiopikrosit, Timokinon	-	[76]
Elektrospinleme	Nanofiber	PLGA, PEG	Gümüş nitrat	-	[77]
Elektrospinleme	Nanofiber Film	PCL	Hypericum perforatum	-	[37]
Jelleşme	Hidrojel	Metakrilatlı jelatin-Metakrilatlı insan tropoelastin	Antimikrobiyal protein	-	[17]
Jelleşme	Hidrojel	Karbopol	Beta glukan	-	[13]
Jelleşme	Hidrojel	Kitozan, Kolajen	Borik asit	-	[78]
Jelleşme	Hidrojel	Metakrilatlı jelatin	Dopamin	-	[16]
Jelleşme	Hidrojel	Polikarbofil, Pluronic	EGF	Lipid nanopartikül	[50]
Jelleşme	Hidrojel	CMC, HEC	Hypericum perforatum	Niozom	[79]
Jelleşme	Hidrojel	CMC	Hypericum perforatum	-	[80]
Jelleşme	Hidrojel	NaCMC	Hypericum perforatum	-	[81]
Jelleşme	Hidrojel	Jelatin	Kemokin	-	[14]

Tablo 4 (*Devam*). Üretim yöntemleri, kullanılan malzemeler ve içerdikleri etkin maddelere göre farklı formlarda hazırlanmış yara örtüsü çalışmaları

Üretim Yöntemi	Yara Örtüsü Formu	Polimer	Etkin Madde	İlaç Taşıyıcı Sistem	Ref.
Jelleşme	Hidrojel	Kitozan	Kloramfenikol	Lipozom	[82]
Jelleşme	Hidrojel	Karboksimetil kitozan, Aljinat	Kurkumin	Polimerik mikropartikül	[47]
Jelleşme	Hidrojel	PEG-PCL-PEG	Kurkumin	Polimerik miseller	[83]
Jelleşme	Hidrojel	Kitozan	Kurkumin	Polimerik miseller	[18]
Jelleşme	Hidrojel	Kitozan	Rodamin	Lipozom	[84]
Jelleşme	Hidrojel	Kitozan	Rutin	-	[85]
Jelleşme	Hidrojel	Karbopol	Sodyum pentaborat pentahidrat	-	[86-88]
Jelleşme	Hidrojel	Karbopol	Sülfadiazin	Niozom	[89]
Jelleşme	Hidrojel	Oksijenlenmiş CMC-Karboksimetil kitozan	Tetrasiklin	Polimerik nanopartikül	[90]
Jelleşme	Hidrojel	Kitozan dialdehit bakteriyel selüloz	Tetrasiklin HCl	-	[91]
Liyofilizasyon	Kompozit Sünger	Kolajen, Hyalüronik asit	EGF	-	[39]
Liyofilizasyon	Sünger	Aljinat, Kitozan	Siprofloksasin	-	[40]
Liyofilizasyon	Sünger	Jelatin	EGF	Polimerik mikropartikül	[38]
Liyofilizasyon	Sünger	Kitozan-Hyalüronik asit	Resveratrol	-	[41]
Liyofilizasyon	Sünger	PLGA	Gentamisin	-	[92]
Liyofilizasyon	Sünger	Kitozan-Aljinat	Smad3 antisens oligonükleotid	-	[93]

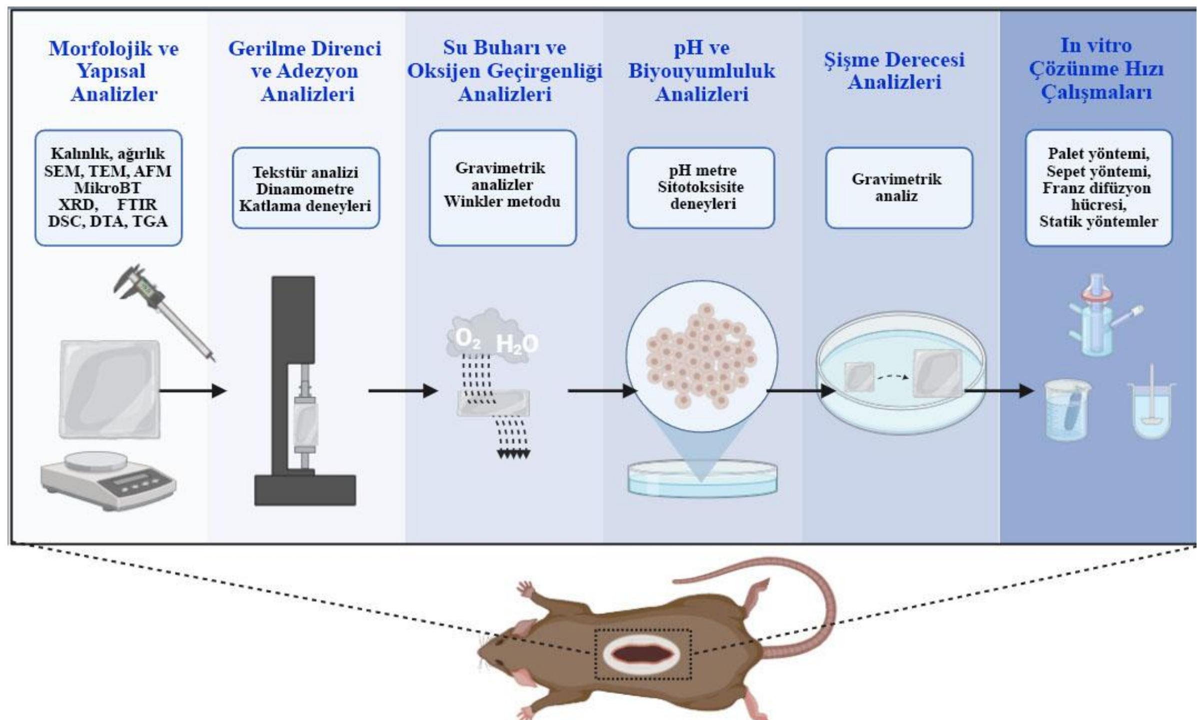
*CMC: Karboksimetil Selüloz, EGF: Epidermal Büyüme Faktörü, HEC: Hidroksietil Selüloz, HPC: Hidroksipropil Selüloz, HPMC: Hidroksipropil Metilselüloz, NaCMC: Sodyum Karboksimetil Selüloz, PCL: Polikaprolakton, PEG: Polietilen Glikol, PEO: Polietilen Oksit, PLA: Polilaktik Asit, PLGA: Polilaktik-ko-glikolik Asit, PVA: Polivinil Alkol, PVP: Polivinilpirolidon

Son Güncel Çalışmalar: Partiküler Yara Örtüleri

Yara örtüsü çalışmalarına dair en yeni çalışmalardan biri de yara sıvısı ile temas ettiğinde jelleşen partiküler yara örtüsü sistemleridir. Coyne ve ark., yara sıvısıyla temas ettiğinde başarıyla jelleşen jelatin nanopartiküler yara örtüsü geliştirmişlerdir [94]. Romic ve ark., geliştirdikleri melatonin yüklü kitozan ve poloksamer bazlı mikropartikül yara örtüsünün, sitotoksik olmaması, simüle edilmiş yara sıvısıyla temas ettiğinde hızla hidrojel oluşturması ve optimum su buharı geçirgenliği özellikleriyle yara yönetiminde kullanılabilecek potansiyele sahip olduğunu bildirmişlerdir [95]. Benzer bir çalışma gentamisin yüklü aljinat ve pektin bazlı nanopartiküler sistemle yapılmış ve sonuçlar olumlu bulunmuştur [96]. Basha ve ark., sefodril yüklü kitozan bazlı nanopartikül çalışmalarında elde edilen *in vivo* yara iyileşme sonuçlarıyla, bu partiküler sistemlerin yara örtüsü olarak uygulanabilirliklerini kanıtlamışlardır [97]. Partiküler yara örtülerinin katı-jel dönüşümü ile farklı şekil ve geometrideki yaralara uyum sağlayabilmeleri önemli bir avantaj sağlar. Ek olarak püskürtülebilir sistemlere adapte edilebildiklerinde klinik uygulama açısından uygulanabilirliği daha pratik yara örtüleri de geliştirilebilecektir.

Yara Örtülerinde Yapılan Karakterizasyon Çalışmaları

Hazırlanma aşamasından sonra elde edilen yara örtülerinin istenen özelliklerde olup olmadığı bir takım karakterizasyon çalışmaları ile hem stabilite hem etkinlik açısından değerlendirilir (Şekil 4).



Şekil 4. Yara örtülerinin özelliklerini değerlendiren çeşitli *in vitro* karakterizasyon çalışmaları

Morfolojik İncelemeler ve Yapı Analizleri

Değişen formülasyon koşullarında uniform yara örtülerinin elde edildiğinin ve hazırlama yönteminin tutarlı olduğunun bir göstergesi olarak kalınlık ve ağırlık ölçümleri yapılmaktadır [98]. Özellikle jel halinde hazırlanmayan yara örtülerinde kalınlığın tespit edilmesi önemlidir. Kalınlık ölçümleri, numunelerin birkaç farklı noktasından değişik boy ve duyarlılıkta mikrometreler kullanılarak yapılabilir. Elde edilen kalınlık verileri, yara örtülerinin gerilme direnci ve su buharı geçirgenliği hesaplamalarında da kullanılır. Belirli ebatlarda kesilen yara örtülerinin terazide ölçümüyle belirlenen ağırlıkları ise yara örtülerinin şişme derecesi tespitinde kullanılmaktadır [43,74].

İdeal bir yara örtüsünde aranan özellikler arasında yara eksüdası absorblama kabiliyeti ve gaz geçirgenliği yer almaktadır. Bu iki temel özellik, yara örtülerinin porlu yapılarıyla mümkün olmaktadır [43,98]. Yara örtülerinin porozitesi hem şişme derecesini hem de su buharı geçirgenliğini doğrudan etkilemektedir. Çalışmalarda artan poroziteyle birlikte su absorblama kapasitesinin de arttığı bildirilmektedir. Aynı zamanda porların homojen dağılım gösterip göstermemesi yukarıdaki etkilerine ek olarak bir etkin madde içermesi durumunda salım özelliklerini de etkileyecektir [62]. Bu yüzden karakterizasyon çalışmalarında porozitenin belirlenmesi önemlidir. Yara örtülerinin yüzey özelliklerinin ve porozitesinin incelenmesinde en yaygın kullanılan yöntem Taramalı Elektron Mikroskobu (SEM) yöntemidir. SEM dışında Atomik Kuvvet Mikroskobu (AFM), Geçirimli Elektron Mikroskobu (TEM) ve optik mikroskop yöntemleri de kullanılmaktadır [36,42,57].

Devi ve Dutta kitozan ve bentonit bileşiminden hazırladıkları film şeklindeki yara örtüsü çalışmasında poroziteyi belirlemek için sıvı yer değiştirme metodunu kullanmışlardır [98]. Bu metotta ağırlığı önceden tespit edilmiş film, belirli hacimdeki etanolde 24 saat bekletildikten sonra filmdeki ağırlık farkı hesaplanarak yer değiştiren sıvı miktarından porozite tayini yapılmıştır. Naseri ve ark., ise kitozan ve PEO polimerlerinden hazırladıkları nanolif film yara örtüsü çalışmasında porozite tayini için

gaz adsorbsiyon yöntemini kullanmışlardır [36]. Diğer bir araştırmada ise kitozan ve hyalüronik asit polimerleriyle hazırlanan sünger yara örtüsü çalışmasında yüzeyin 3B olarak net bir şekilde incelenmesine olanak sağlayan mikrobilgisayarlı tomografi (μCT) analizinden yararlanılmıştır [41]. Bu 3B görüntüleme sayesinde formülasyonların poroziteleri hakkında detaylı veriler elde edilebilmekte ve μCT analizi ile formülasyonlarda açık ve kapalı porların tespiti yapılabilmektedir. Bunun dışında polimerik nanopartikül gibi bileşenler içeren formülasyonlarda, bu bileşenlerin formülasyondaki dağılımları hakkında da detaylı veriler sağlanmaktadır [19,99].

Yara örtülerinin kristal yapılarının analizinde X-Işını Kırınımı (XRD) yöntemi, termal degradasyon davranışlarının analizinde ve bir ölçüde yapısal konformasyonun değerlendirilmesinde Diferansiyel Taramalı Kalorimetre (DSC), Termogravimetrik Analiz (TGA) ve Diferansiyel Termal Analiz (DTA) yöntemleri [23,63], etkin madde ile yardımcı maddeler arasındaki yapı etkileşimleri ise Fourier Dönüşümlü Kızılötesi Spektroskopisi (FTIR) yöntemi kullanılmaktadır. Renkli maddelerle çalışıldığında yara örtülerinin fiziksel görünümlerinde meydana gelebilecek değişiklikler kromometre analizleri ile tespit edilebilmektedir [62].

Gerilme Direnci Analizleri

Yara örtüsünün mekanik özellikleri, yara koruma performansında önemli bir faktördür [58]. Ne tür yaraya veya vücudun hangi bölgesine uygulanırsa uygulansın hem klinik uygulama sırasında hem de günlük aktiviteler sırasında karşılaşılan farklı streslere dayanıklı olmalı ve deforme olmamalıdır [26,58,70]. Özellikle jel halinde uygulanmayan yara örtülerini yara yüzeyinde rahat, güvenli ve dikkatli bir şekilde tutmak için bu örtülerin belirli bir esneklikte olması gerekir [98]. Bu dayanıklılık ve esneklik gibi mekanik özellikler, yara örtülerinde gerilme direnci analizleri yapılarak tespit edilir.

Gerilme direnci ölçümleri, kalınlıkları ölçülen ve belirli ebatta kesilen yara örtülerine tekstür analiz cihazları ile uygulanan gerilme kuvveti karşısındaki uzama miktarları grafiğe geçirilerek belirlenir. Bu grafikten üç önemli veri elde edilir; gerilme direnci, elastik modül ve kırılma noktası. Gerilme direnci, birim kesit alana uygulanan gerilim karşısında uzama eğrisindeki maksimum gerilim olarak tanımlanır ve yara örtülerinin dayanıklılığı hakkında bilgi verir. Elastikiyet elastik modül ile belirlenir ve grafikteki doğrusal bölgenin eğimi olarak tanımlanır. Uygulanan gerilim karşısında maksimum uzamanın olduğu nokta da kırılma noktasıdır. Ölçüm yapılan cihaza bağlı olarak değişse de, sağlıklı koşullarda insan derisinin 2.5 ile 35 MPa arasında değişen gerilme dirençlerine dayanıklı olduğu bildirilmektedir [23]. İyi bir yara örtüsü de derinin doğal deformasyonlarına dayanıklı olmalıdır.

Gerilme direnci ölçümleri yalnızca kuru haldeki yara örtülerinde analiz edilebildiği gibi bazı çalışmalarda yara eksüdasını absorladığı durumları taklit etmek amacıyla ıslak haldeki yara örtülerinde de analiz edilmektedir. Ancak şişmiş haldeki yara örtüsünde şişme dengesinin sağlanmadığı durumlarda mekanik özelliklerinin tam olarak tespit edilmesi mümkün olmayabilir [23,55].

Genellikle mekanik özellikler, yara örtüsünü oluşturan polimerler arasındaki bağ oluşumları [32], çapraz bağlanmalar [22,68], etkin madde yardımcı madde etkileşimleri [62,64] gibi yara örtülerinin mikroyapıları ve moleküllerarası kuvvetlerle ilişkilidir, formülasyon parametrelerine bağlı olarak gerilme direncinde artma veya azalma durumları gözlenebilir. Yara örtülerini hazırlamak için kullanılan biyopolimerler, kolay kullanım avantajlarına rağmen yeterli mekanik dayanıklılıkta olmadıklarında plastizer gibi yardımcı maddeler ilave edilerek yara örtülerinin esnekliklerini artırmaya yönelik çalışmalar yapılabilir [26].

Tekstür analiz cihazları dışında katlanma deneyleri gibi manuel işlemlerle de yara örtülerinin gerilme dirençleri tespit edilebilmektedir [98].

Adezyon Testleri

Topikal formülasyonların biyoadezyon özellikleri, adeziv eksipiyanlar içeren formülasyonlarda olduğu gibi yara örtülerinde de önemlidir. Çünkü yara örtüsünün yarada kalış süresini ve tedavi sonucunu etkileyebilir. Örneğin, yaraya uygulanmış bir yara örtüsünden güvenilir bir etkin madde salım profili tahmini, terapötik yanıtın oluşmasına yetecek süre boyunca yara örtüsünün uygulama bölgesinde kalmasına bağlıdır [66]. Yara örtülerinde biyoadezyon değerlendirmeleri, formülasyon ile deri veya deriyi taklit eden bir yüzey arasında oluşan adeziv bağı koparmak için gereken kuvvet ve yapılan adezyon işinin belirlenmesi esasına dayalı olarak ölçüm yapan dinamometre ve tekstür analiz cihazları

ile yapılır. Adezyon testleri domuz [63], tavşan [54], sıçan [49] ve fare [69] gibi hayvan derileri kullanılarak *ex vivo* yapılabildiği gibi, yaralı deriyi taklit eden yumurta kabuğu zarı [61] ve dondurulmuş %6,67 (a/h)'lik jelatin membranlar [26] kullanılarak da *in vitro* şekilde yapılabilir.

Yara örtülerinin adezyonu hem hidrofobik hem hidrofilik etkileşimlerin kombinasyonuna bağlı karmaşık bir mekanizmaya sahiptir [63,74]. Hidrofobik etkileşimler, yara örtüsü ile yara çevresindeki stratum korneumu arasındaki bağ oluşumlarında etkindir. Hidrofilik etkileşimler ise yaradaki eksüda ile örtü temasındaki bağ oluşumlarında etkindir. Bu etkileşimler, polimerik zincirlerin genişlemesi ve organizasyonu ile yara örtüsünde homojen dağılmamış etkin madde gibi partiküllerin varlığından etkilenebilir ve adezyon artar ya da azalır.

Su Buharı Geçirgenliği Analizleri

İdeal yara örtülerinden beklenen özellikler arasında yara bölgesinde nemli bir ortam sağlaması, yara kurumasını önlemesi ve su buharı gibi gaz alışverişine elverişli olması yer almaktadır. Bu yüzden, yara yüzeyinden su kaybının optimum düzeyde tutulması hem nemli ortam sağlanması hem de aşırı kurumanın önlenmesi açısından önemli bir parametredir. Yara örtülerinde su buharı geçirgenliği, birim zamanda birim yüzeyden su buharının ağırlıkça değişiminin tespit edilmesine dayanır. Bu analiz su buharı geçirgenlik test cihazları ile otomatik olarak ölçülebildiği gibi, belirli miktarda su veya simüle edilmiş yara sıvıları içeren, belirli ağırlık ve ebatlardaki şişelerin dairesel açıklığına uygun şekilde yerleştirilen yara örtülerinin inkübe edilmesiyle manuel olarak da ölçülebilir. İnkübasyondan sonra belirlenen sürede periyodik olarak yeniden tartılan her bir numunenin, zamanın fonksiyonu olarak yüzey alanına düşen ağırlık farkları yüzde olarak hesaplanır [23].

Sağlıklı bir deri için su buharı geçirgenliğinin 204 $g.m^{-2}.gün^{-1}$ olduğu, yaralı deri için bu değerin yara türüne bağlı olarak 279 ile 5138 $g.m^{-2}.gün^{-1}$ arasında değiştiği bildirilmektedir. Piyasada 34 ile 11000 $g.m^{-2}.gün^{-1}$ arasında değişen değerlerde su buharı iletim hızına sahip yara örtüleri olduğu tespit edilmesine rağmen [23], etkili bir yara örtüsü için su buharı geçirgenliği hızının 2000 ile 2500 $g.m^{-2}.gün^{-1}$ değerleri arasında olması önerilmektedir [54].

Oksijen Geçirgenliği Analizleri

Yara dokusu hücrelerinin beslenmesi, gelişip büyümesi için dokunun oksijenlenmesi önemli olduğu için yara örtülerinde oksijen geçirgenliği analizleri yapılmaktadır. Bu analizlerin temeli Winkler metoduna göre suda çözünmüş oksijen miktarının tespit edilmesine dayanır. Belirli miktarda su içeren belirli ağırlık ve ebatlardaki şişelerin dairesel açıklığına uygun şekilde monte edilen yara örtüleri, açık bir ortamda belirli süre bekletildikten sonra yara örtüsünden penetre olan oksijen miktarı kantitatif olarak analiz edilir. Sonuçları kıyaslamak için pozitif kontrol olarak oksijenin şişeye girerek suda çözünmesine izin veren ağzı açık şişe, negatif kontrol olarak oksijenin şişeye girmesini önleyen kapaklı bir şişe kullanılarak aynı şartlarda deney sürdürülür. İdeal bir yara örtüsü için süre sonunda analiz edilen değerin negatif ve pozitif kontrol verileri arasında olması önerilmektedir [59,70].

pH Analizleri

Hem yara yüzeyinde enfeksiyon oluşumunun engellenmesi hem de fibroblast proliferasyonunun hızlandırılması açısından yara örtülerinin pH'sı önemli bir parametredir. Derinin normal pH'sı 4.0 ile 6.8 arasında değişmektedir. Bu yüzden ideal bir yara örtüsünün yüzeyi hafif asidik olduğunda deri tarafından tolere edilebilir. Nötr ve alkali bir ortama kıyasla yara iyileşme süreci hızlandırılabilir. Piyasada mevcut olan yara örtülerinin pH değerlerinin 5.25 ile 7.90 arasında olduğu bildirilmiştir [32,54,98].

Şişme Derecesi Analizleri

Yarada fazla eksüda birikmesi, yaralı bölgede mikrobiyal enfeksiyona neden olan ve kronikleşen bakteri üremesine neden olabilir. Aynı zamanda yara çevresindeki sağlıklı dokularda maserasyona neden olabilir. Bu yüzden yara tedavisinde fazla eksüdanın uzaklaştırılması önemlidir. Ancak bu absorbsiyon, dokuda dehidrasyona neden olmadan optimum nem koşullarını sağlayacak nitelikte olmalıdır. Bu özellikler yara örtülerinin su buharı geçirgenliği ve sıvı tutma kabiliyetleri ile sağlanabilir. Yara örtülerinin sıvı absorbsiyon kapasiteleri gravimetrik olarak şişme derecesi analizleriyle tespit

edilir. Belirli ebatlarda kesilen yara örtüsü örnekleri, ağırlıkça denge durumuna ulaşana dek su veya vücut sıvılarını taklit eden sıvı bir ortamda bekletilerek elde edilen yüzde ağırlık değişimi, yara örtüsünün şişme derecesini gösterir. Ancak jel formunda hazırlanmış hidrojel yara örtülerinde, deney sırasında orijinal viskozitesini kaybetme durumu söz konusu olduğu için modifiye edilmiş yöntemler kullanılmaktadır [84]. Bir yara örtüsü için ideal şişme derecesi, esas olarak yaranın türüne ve durumuna göre değerlendirilmelidir [62]. Orta ve yüksek düzeyde eksüdalı yaraların tipik olarak 24 saatte her 10 cm^2'de yaklaşık 5 ml eksüda ürettiği bildirilmiştir [70].

Yara örtülerinin biyolojik sıvıları absorbe edebilmesi, polimerik ağlarda bulunan etkin maddelerin çözünmesi ve salınması dışında degredasyonlarında da etkilidir [63,74]. Yara örtüsü gereksinimlerinden biri de travmaya neden olmadan çıkarılma kolaylığıdır. Örneğin fazla miktarda su ile temastan sonra polimer zincirleri tamamen gevşediğinde kolaylıkla çıkarılabilir. Bu açıdan yara örtülerinin su absorbsiyon kapasiteleri belirlenirken degredasyon derecelerinin de tayin edilmesi önerilmektedir. Yara örtülerinin degredasyon dereceleri şişmiş haldeki örneklerin yeniden kurutulmasıyla hesaplanan kuru ağırlıktaki yüzde değişimin tespit edilmesine dayanır.

Biyouyumluluk Analizleri

Geliştirilen yara örtüleri, yara örtülerinden beklenen en iyi karakterizasyonlara sahip olsalar bile amaçlanan kullanım içinde deri dokusundaki hücrelere zarar vermediğinin gösterilmesi güvenlik açısından önemlidir [34,43]. Yara örtülerinin güvenirlikleri, sitotoksisite değerlendirmeleriyle yapılır. İnsan ya da hayvan kaynaklı fibroblast ve keratinosit hücreleri, bağ dokunun ana hücresel bileşenleri olmaları ve ECM oluşumundaki rolleri sebebiyle sitotoksisite çalışmalarında yaygın olarak kullanılmaktadır [62,87]. Sitotoksisite analizlerinde MTT assay, MTS assay ve WST-I assay en sık kullanılan yöntemlerdir. Temel olarak canlı hücrelerdeki dehidrogenaz enzimlerinin etkisiyle sarı renkli tetrazolyum tuzlarının mor renkli formazana dönüşümünden yararlanılan bu sitotoksisite analizlerinde, oluşan formazan ürünleri spektrofotometrik yöntemlerle analiz edilmekte ve hücre canlılığı yüzde olarak belirlenmektedir. Analiz sonucunda hücre canlılığı %70'in üzerinde olan yara örtülerinin sitotoksik olmadığı, dolayısıyla biyouyumlu olduğu kabul edilmektedir [26,56]. Nötral kırmızısı alım testi [23,29] ve su karidesi letalite testi [100] de yara örtülerinin sitotoksisitesinin belirlenmesinde kullanılmaktadır.

Yarayla doğrudan temas eden biyomalzemelerin biyouyumlu olması zorunludur. Bu yüzden yara örtülerinin kan biyouyumluluğu da analiz edilmektedir. Bu analizler hemolitik indeks tayini, gravimetrik trombojenite tayini ve yara örtüsü yüzeyine protein adsorbsiyonu testleriyle yapılabilir [59,65,70,98].

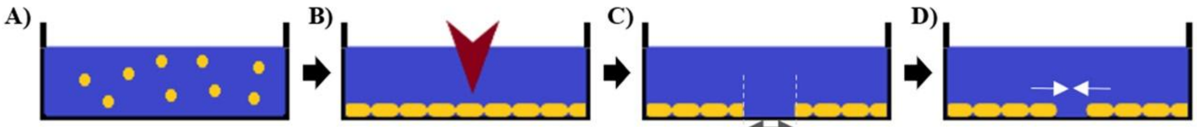
***İn vitro* Çözünme Hızı Çalışmaları**

İyileşme sürecini kolaylaştırmak ve hızlandırmak için çeşitli etkin maddeler içeren yara örtüsü çalışmalarında, polimerik matristen bu etkin maddelerin salım performansı *in vitro* çözünme hızı çalışmalarıyla belirlenir. Bu amaçla disk üzerinde palet yöntemi (USP Apparatus 5) [67,69,74] ve sepet yöntemi (USP Apparatus 1) [79] gibi ofisinal yöntemlerle, franz difüzyon hücresi [63] ve statik tüp yöntemi [26] gibi ofisinal olmayan yöntemler kullanılmaktadır. Yara örtüsünden beklenen etkiye bağlı olarak saatlerden günlere varan analiz süreleri boyunca vücut sıvılarını taklit eden sıvı ortamda salım profilleri test edilir.

***İn vitro* Yara İyileşmesi**

Yara örtülerinin yara iyileştirici etkileri genellikle *in vivo* hayvan deneyleri ile yapılmaktadır. Hayvanlarda eksizyon, insizyon veya yanık yarası oluşturulduktan sonra yara örtüsü formülasyonu yaraya uygulanır. Yara boyutundaki azalma ile yara kapanması mikroskobik olarak değerlendirildikten sonra süre sonunda hayvanlara ötenazi uygulanır. Yara bölgesinden alınan doku örnekleri, doku ülserasyonu, nekrozu ve epitelizasyonu açısından değerlendirilir. Ancak bu değerlendirmeler hem maliyet hem de zaman bakımından oldukça zorlu bir süreç içerir. Hem *in vivo* deney sonuçlarının önceden tahmin edilebilmesi hem de yara iyileşme mekanizmasına dair ayrıntılı veriler elde edilebilmesi amacıyla *in vitro* yara iyileşmesi deneyleri yapılmaktadır [74,86-88]. Sitotoksisite analizlerinde olduğu gibi bağ dokunun ana hücresel bileşenleri olmaları ve ECM oluşumundaki rolleri sebebiyle fibroblast ve keratinosit hücreleri *in vitro* yara iyileşmesi çalışmalarında yaygın olarak kullanılmaktadır. Uygun koşullarda kültüre alınan hücreler, tek bir tabaka haline geldiğinde orta hatlarından bir çizgi çizilerek *in*

vitro yarayı temsil edecek boşluk oluşturulur. Bu boşluğun hücrelerin göçü ile kapanma süreci ise *in vitro* yara iyileşmesini temsil eder. Bu süreç mikroskobik olarak izlenerek deney tamamlanır. Şekil 5'te *in vitro* yara iyileşmesi aşamaları şematik olarak gösterilmiştir. Ticari olarak temin edilebilen örneklerinden CytoSelect™ *in vitro* yara iyileşmesi kiti yara örtüsü çalışmalarında kullanılmaktadır [74].



Şekil 5. *İn vitro* yara iyileşme deneyinin şematik gösterimi; (A) hücrelerin kültüre alınması, (B,C) tek katmanlı hücrelerin orta hattında bir boşluk oluşturulması, (D) boşluğun kapanması

SONUÇ VE TARTIŞMA

Sonuç olarak günümüze kadar yapılan yara örtüsü çalışmaları incelendiğinde her yaraya uygun ideal tek bir yara örtüsünden bahsedilemese de gelişen teknolojiyle birlikte ideal yara örtülerinden beklenen özellikleri sağlayabilecek nitelikte yara örtüleri geliştirilebilmektedir. Özellikle hastaya özgü kronik yaraların boyutuna uygun yara örtülerinin hazırlanabildiği 3B baskı, nanolif teknolojileri gibi deriyi neredeyse birebir taklit edebilen teknolojilerin veya püskürtülebilir-partiküler yara örtüsü sistemlerinin kullanımı sayesinde kitlesel olarak üretilen terapötik sistemlerin kişiye özel hale getirilebileceği görülmektedir. Yara örtülerinin etkinliğini artırmak için mekanik dayanıklılık, biyouyumluluk, gaz geçirgenliği ve nem tutma kapasitesi gibi parametrelerin kapsamlı karakterizasyonu, ürünlerin klinik uygulamalarda güvenilirliğini sağlamak ve ideale en yakın yara örtüsünü üretebilmek açısından önemlidir. Çok farklı yöntemler ile farklı yara türlerine göre çeşitli yara örtüsü formülasyonlarının hazırlanabilme olasılığı ve formülasyondaki modifikasyon alternatiflerinin çokluğu, bu konuda ileride daha fazla çalışma ve onay almış ürünün piyasada yer alabileceğini düşündürmektedir.

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Yayım Koşulları

1. Ankara Üniversitesi Eczacılık Fakültesi Dergisi (Ankara Ecz. Fak. Derg. – J. Fac. Pharm. Ankara), açık erişimli, hakemli bir dergi olup yılda üç kez (Ocak-Mayıs-Eylül) yayımlanır.

2. Dergiye Eczacılığın her alanında daha önce hiç bir yerde yayınlanmamış, Türkçe veya İngilizce olarak hazırlanmış makaleler kabul edilir. Deneylerde, insan için "the Declaration of Helsinki" ve hayvan için "European Community Guidlines"'a bağlı kalınmalıdır. Etik Kurul Onayının zorunlu olduğu çalışmalarda, etik kurul onayı alınan kurumun adı ve etik kurul onay numarası, gereç ve yöntem bölümünde ve Etik Kurul Onay bölümünde belirtilmeli ve ilgili belge makale gönderim sırasında yüklenmelidir.

3. Yayın Komisyonuna gelen makaleler en az 2 danışmana gönderilir. Ankara Üniversitesi Eczacılık Fakültesi Dergisi'nin makale değerlendirme süreci çift taraflı kör hakemlik ilkesiyle yürütülür.

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 yayımlanmadan önce yazarların yayımcıya makalenin "Copyright Transfer Form'unu doldurarak telif hakkını göndermesi gerekmektedir.

6. Yayımlarda intihal olup olmadığı kontrol edilmelidir. Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne yayımlanmak üzere gönderilen makaleler intihal tarama programları (iThenticate) ile taranmalı ve çevrim içi makale gönderim sırasında makalelerin intihal içermediğine dair rapor yüklenmelidir.

7. Ankara Üniversitesi Eczacılık Fakültesi Dergisi'nin makale yayın ücreti (APC) veya abonelik ücreti yoktur.

8. Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne aşağıdaki makale türleri kabul edilir:

   a) **Özgün makaleler**: Türkçe veya İngilizce hazırlanmış, şekiller ve tablolar dahil tamamı en çok 25 A4 kağıdı sayfası olan, orjinal araştırmaların bulgu ve sonuçlarını açıklayan makalelerdir. Araştırma makalelerinin yenilikçi ve bilime katkı sağlayan çalışmalar olması beklenir. Makaleler, yazım kurallarında belirtilen ana başlıkları taşımalı ve Windows uyumlu bir program kullanılarak hazırlanmalıdır.
   b) **Derleme makaleler:** Türkçe veya İngilizce hazırlanmış, şekil ve tablolar dahil tamamı en çok 30 A4 kağıdı sayfası olan, yeterli sayıda bilimsel makale taranarak, o güne kadarki gelişmeleri özetleyerek ortaya koyan ve sonuçlarını yorumlayarak değerlendiren makalelerdir. Makaleler, yazım kurallarında belirtilen ana başlıkları taşımalı ve Windows uyumlu bir program kullanılarak hazırlanmalıdır.
   c) **Kısa bildiriler:** Devam etmekte olan bir çalışmanın bulgularını zaman kaybetmeden duyurmak için Türkçe veya İngilizce yazılan en çok 5 A4 kağıdı sayfası olan makalelerdir. Makaleler, yazım kurallarında belirtilen ana başlıkları taşımalı ve Windows uyumlu bir program kullanılarak hazırlanmalıdır.

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 1 satır aralıkla yazılmalıdır. Yayımı kabul edilen makaleler doğrudan "Microsoft Word" dosyası halinde çevrim içi 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, ORCID noları ve yazışma yapılacak yazarın açık adresi, telefon ve e-mail adresi belirtilmeli ve ortalı yazılmalıdır. İlk sayfada başlıktan önce yukarıdan 3 satır aralığı bırakılmalıdır. Başlık ile Öz/Abstract arası 1 satır aralıkla yazılmalıdır. Sorumlu yazarın soyadının üstüne (*) işareti konularak belirtilmelidir. Bu kişinin Adı Soyadı, açık adresi, 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 bir 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, 06560, 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 (**JPEG formatında**) makale içinde yerleşmiş ve **resimler 300 dpi veya daha yüksek çözünürlükte** olmalıdır. 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 ve bir satır aralıkta yazılmalıdır. Tabloya ait açıklama varsa tablonun altına 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. Şekil başlığı ve şekil arasında 6 nk aralık olmalıdır. Tablo ve Şekiller metin içine yerleştirilirken metin ile aralarında 18 nk aralık olmalıdır.

    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 başlığı ve tablo arasında 6 nk aralık olmalıdır.

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.

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 (Yazı karakteri "Times New Roman" ve 11 punto, "1" aralık, ortalı)

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 bölümleri ifade eden başlıklar (Makalenin ilk başlığı hariç) **12 punto ile koyu olarak büyük harflerle ve sayfanın solundan başlanarak** yazılmalıdır. **GİRİŞ**'ten önce ve sonra sırasıyla 18 nk ve 6 nk aralık bırakılmalıdır. Diğer ana başlıklardan önce ve sonra sırasıyla 12 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 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 (aşağıda görüldüğü gibi) halinde makalenin özeti sunulmalıdır. Her bir alt başlık 10 punto, koyu, normal yazılmalıdır. Alt başlıkların içeriğindeki metinler *italik* yazılmalıdır. **ÖZ ve ABSTRACT metni blok halinde sağdan ve soldan 1 cm boşluk bırakılarak yazılmalıdır.**

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 az 3 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, 1satır aralık, **bold/koyu** yazılmalı ve sola dayalı olmalıdır Alt başlıklarda numaralandırma sistemi **kullanılmamalıdır**. Alt başlıklardan önce ve sonra 6 nk aralık olmalıdır.

- **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.
  - Dileyen yazar, RESULT AND DISCUSSION bölümünün son paragrafı olarak "Conclusion" başlığı oluşturabilir. Ancak 11 punto Times New Roman karakterinde İlk harfi büyük diğer harfleri küçük olmalıdır.

- **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. Yazar katkısı belirtilmeyecek alanlar için "-" işareti konulmalıdır.

  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: -

- **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 belirtilmeli ve Etik Kurul Onayını makale gönderim sırasında yüklemelidir. 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 10 punto, "1" aralık, iki yana yaslı. Metinde, geçiş sırasına göre köşeli parantez içinde, örneğin: [1,6,9], [5-7] gibi numaralandırılmalı ve metin sonunda bu numaralara göre sıralanmalıdır. Alt başlıkların yanına kaynak belirtilmemelidir. Tablo içinde kaynak bildirilmesi gerekiyorsa metin içinde verildiği gibi belirtilmelidir.

  - **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 <u>kullanılmamalıdır</u>**.), makalenin tam başlığı, derginin adı, cilt no, varsa sayı no (parantez içinde), başlangıç ve bitiş sayfa numarası (veya makale 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. Lütfen https://www.crossref.org/'da yer almayan makaleleri** [CrossRef] şeklinde **belirtmeyiniz.**
https://doi.org/10.1016/0006-2952(89)90403-6

Örnekler:

1. 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]
2. 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]

- **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). Erişim adresi 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.

- **Patent için:** Yazarın soyadı, adının baş harfleri, yıl yazar isimlerinden sonra (parantez içinde) yazılıp nokta işareti konmalıdır. Patent başlığı ve patent numarası yazılmalıdır.

Örnek:
Mahoney, S., Molz, L., Narayan, S., Saiah, E. (2018). Heteroaryl RHEB Inhibitors and Uses Thereof. WO 2018/191146 A1.

ETİK İLKELER VE YAYIN POLİTİKASI

Ankara Üniversitesi Eczacılık Fakültesi Dergisi, açık erişimli, hakemli bir dergi olup Türkçe veya İngilizce olarak farmasötik bilimler alanındaki önemli gelişmeleri içeren orijinal araştırmalar, derlemeler ve kısa bildiriler için bir yayım ortamıdır. Ankara Üniversitesi Eczacılık Fakültesi Dergisi'nin makale yayın ücreti (APC) veya abonelik ücreti yoktur.

Yayın kurulu olarak dergi kapsamında önemli katkı sağlayan kaliteli yeni çalışmaların yayınlanması amaçlanmaktadır. Bu amaca ulaşmak için gönderilen makaleler, dergide yayınlanmak için bilimsel ve biçimsel gerekli kriterleri karşıladıklarından emin olmak adına baş editör ve/veya editör yardımcıları tarafından ilk değerlendirmeye tabi tutulur. Yalnızca bu ön değerlendirme sürecini geçen çalışmalar, daha ileri değerlendirme için diğer aşamalara devam ettirilir.

Ön Değerlendirme

- Çalışmanın bilimsel kalitesi ve yeniliği dergide yayınlanmak için yeterli olmalıdır.
- Dergiye gönderilen çalışmalar derginin amaç ve kapsamına uygun olmalıdır.
- Metin İngilizce veya Türkçe olarak dilbilgisi kurallarına uygun ve bilimsel olarak iyi yazılmış olmalıdır.
- Dergiye gönderilen çalışmaların benzerlik oranı %20'i geçmemelidir.
- Çalışmalar derginin yazım kurallarına ve şablonuna uygun olacak şekilde düzenlenmelidir.
- Telif hakkı devir formu, etik kurul onay belgesi, yazar katkı formu mutlaka yüklenmeli ve imzalı olmalıdır.
- Çalışmalar elektronik online başvuru sistemi aracılığı ile dergiye gönderilmiş olmalıdır.

Bu yeterlikleri taşımayan çalışmaların ileri değerlendirme süreci başlatılamaz.

Dergi yayınlanma sürecinde dergi editörleri, hakemler ve yazarlara bazı sorumluluklar düşmektedir. Bu sorumluluklar aşağıdaki şekilde açıklanmıştır.

1. Editörün Görevleri ve Etik Sorumlulukları

Editör, dergiye gönderilen makalelerden hangilerinin yayınlanması gerektiğine bağımsız olarak tek başına karar verebileceği gibi editör kurulunun üyelerine veya hakemlere de danışabilir. Derginin etik ilkeleri ve yayın politikası çerçevesinde, çalışmaların ön değerlendirme, hakem değerlendirmesi ve yayınlanma aşamalarının tarafsız, denetlenebilir, adil, çıkar ilişkisinden bağımsız ve gizlilik ilkelerine uygun şekilde yürütülmesinden sorumludur. Yayın politikası ve etik ilkeleri açısından ihlal yoksa derginin amacına ve kapsamına uygun çalışmaları, ön değerlendirme aşamasına almalıdır.

Baş editörün, editör yardımcılarının, alan editörlerinin ve editoryal danışma kurulunun görevleri ve tanımları aşağıdaki gibidir:

Baş Editör: Dergi içeriğinin yayınlanması konusunda tam yetkiye sahip kişidir. Editör yardımcıları, alan editörleri ve editöryal danışma kurulu ile birlikte çalışır.
Editör Yardımcıları: Dergi ilgili sorulara cevap vermek, dergi hakem ve kuruluna önerilerde bulunmak, makale yayın sürecinde baş editöre yardımcı olan kişilerdir.
Alan Editörleri: Çift kör hakem atamalarının gerçekleşmesi ve dergi ile ilgili sorulara cevap vermek konusunda yazarlara yardımcı olan kişilerdir.
Editoryal Danışma Kurulu: Editoryal Danışma Kurulu, Ankara Üniversitesi Eczacılık Fakültesi Dergisinin, amacına uygun ve kaliteli yayın üretilmesine ilişkin konularda Baş Editör ve Editör Yardımcılarına kılavuzluk eder.

1.1. Yayın Politikası

- Baş editör, dergiye gönderilen makalelerden hangilerinin yayımlanması gerektiği kararından tek başına sorumludur. Editörün kararı, derginin editör kurulunun prensipleri doğrultusunda olabileceği gibi, onur kırıcı yayım yapmak, telif hakkı ihlali ve intihal gibi konularla ilgili olarak yürürlükte olan yasal gereklilikler ile sınırlandırılmıştır.
- Baş editör, makale yayımlanmadan önce yazarların yayımcıya makalenin "Copyright Transfer Form" unu, doldurarak telif hakkını gönderdiğinden emin olmaktadır.
- Baş editör, yazarların makale yayımlanmadan önce "Conflict of Interest Form"unu ve "Author Contribution Form" unu doldurduğundan emin olmaktadır.
- Baş Editör, dergiye gönderilen makalelerin biçimsel olarak incelenmesi için editör yardımcılarını görevlendirmektedir. Ankara Üniversitesi Eczacılık Fakültesi Dergisinin kurallarını sağlamayan makaleler kesinlikle değerlendirmeye alınmadan reddedilmektedir.

1.2. Yayın Değerlendirmesi

- Baş editör, yayın değerlendirme sürecinin adil, tarafsız ve zamanına uygun şekilde gerçekleşmesini sağlamaktan sorumludur.
- Editör, tüm makaleleri genel olarak dışardan ve bağımsız en az iki hakem ile değerlendirilmesini sağlamaktadır. Gerek olması durumunda editör üçüncü bir hakemden ek görüş istemektedir.
- Editör, hakem seçimini makale kapsamına uygun olan uzmanları değerlendirerek yapar.
- Editör, olası çıkar çatışmaları için yapılan açıklamaları, hakemler tarafından yapılan "self-citation" önerilerini ve herhangi bir taraflılık olasılığını değerlendirmek ve karar vermek için dikkatli bir şekilde yayın sürecini gözden geçirmektedir.
- Baş editör/editörler, hakem değerlendirmesi veya değerlendirme/yayım sürecinin herhangi bir noktasında bir benzerlik tespit yazılımı (iThenticate) tarafından taratılmasını yazardan istemektedir veya kendisi yapmaktadır. Bu anlamda ifadelerin veya cümlelerin yazarın/yazarların kendileri olsa dahi metin daha önce yayınlanmış verilerle kabul edilemez bir benzerliğe sahip olmamalıdır.
- Baş editör, bir makaledeki hataları yayımlanmadan önce tespit ederse düzeltmektedir. Eğer daha sonra tespit ederse bu durumda düzeltmeleri yayımlamak zorundadır. Tüm düzeltme veya geri çekme bildirimlerini dergide belirgin bir şekilde yayımlamalıdır. Ayrıca içindekiler sayfasında listelemelidir.
- Ankara Üniversitesi Eczacılık Fakültesi Dergisinin editörleri, Yayın Etiği Komitesi (Committee on Publication Ethics (COPE)) tarafından yayınlanan "COPE Code of Conduct and Best Practice Guidelines for Journal Editors" ve "COPE Best Practice Guidelines for Journal Editors" kılavuzlarına uyarak çalışmalarını sürdürür.

1.3. Adil Değerlendirme

- Baş editör/editörler, makaleleri yazarların ırk, cinsiyet, cinsel eğilim, inanç, etnik köken, vatandaşlık ya da politik görüşlerine bakmaksızın bilimsel içeriklerine göre değerlendirmektedir. Derginin editoryal prensipleri şeffaf ve tümüyle dürüst değerlendirmeyi desteklemektedir.
- Editör, hakemlerin ve yazarların kendilerinden bekleneni tam olarak anladıklarından emin olmalıdır.
- Editör, dergi ile ilgili tüm iletişimini derginin elektronik başvuru sisteminden yapar ve kararlarında itirazlar olması halinde şeffaf ve hakkaniyetli bir yol izler.

1.4. Gizlilik İlkesi

- Baş editör/editör, dergiye yapılan başvurudaki tüm materyallerin ve hakemlerle yapılan tüm iletişimin gizliliğini (ilgili yazar ve hakemlerle aksi onaylanmadığı sürece) korumakla yükümlüdür.

- Baş editör/editör, hakemlerin isimlerinin açıklanmasını kabul etmediği sürece, hakemlerin kimliklerini ve haklarını korumakla sorumludur.
- Başvurusu tamamlanmış bir makaleye ait basılmamış materyaller, yazarın yazılı onayı alınmadan editörün kendi çalışmaları/araştırmaları için kullanılmamalıdır.
- Baş editör/editör, makale değerlendirme sürecinde edinilen tüm bilgileri veya fikirleri gizli tutmalı ve kişisel amaçlar için kullanmamalıdır.

2. Hakemlerin Görevleri ve Etik Sorumlulukları

Ankara Üniversitesi Eczacılık Fakültesi Dergisi'nin makale değerlendirme süreci çift taraflı kör hakemlik ilkesiyle yürütülmektedir. Dolayısıyla hakemler yazar/yazarlarla iletişim kuramazlar, değerlendirmeler dergipark yönetim sistemi üzerinden paylaşılır. Değerlendirme sürecinde tam metinlere ilişkin değerlendirme formları hakem yorumları editör aracılığı ile sorumlu yazara iletilir. Hakemler, değerlendirme süreci boyunca tarafsızlık, gizlilik, nesnellik, bilimsel yönden inceleme ilkelerine uygun hareket etmelidir. İlgili alanda uzman ve yetkinliğe sahip olmalıdır. Değerlendirmesine sunulan çalışmaya ilişkin raporunu belirtilen zaman aralığı içinde bitirmelidir. Zamanında sunulamayacak raporlar için gecikmeden editör ile iletişime geçilmelidir. Etik ilkeleri, telif hakkı ihlali, olası çıkar çatışması ve intihal yapıldığının fark edilmesi durumlarında editör kurulunu bilgilendirmelidir.

Ankara Üniversitesi Eczacılık Fakültesi Dergisi için makaleleri değerlendiren hakemlerin aşağıda belirtilen görevlere ve etik sorumluluklara uyması beklenmektedir.

2.1. Editöryal Kararlara Katkı

- Hakemler, yazarların sundukları çalışmaları yapıcı ve uygun şekilde değerlendirmelidirler.
- Hakemler, makalede yer alan araştırmayı değerlendirmeye yetkin olmadığını düşünüyorsa veya yeterli sürede tamamlayamayacaksa editöre durumu bildirmelidirler.
- Hakemler, yazarlara yönelik sert ve kişisel eleştirilerde bulunmamalıdırlar.
- Hakemler, makale değerlendirmesi için davet aldığında eğer kendilerini makalede çalışılan konu hakkında yetersiz hissederlerse makaleyi değerlendirmeyi reddetmelidirler.
- Hakemler, makale değerlendirmesini verilen süre içinde yapmalıdırlar.
- Hakemler, sadece çalışmanın içeriğine ilişkin değerlendirmeyi objektif olarak yapmalıdırlar.

2.2. Gizlilik

- Hakemler, değerlendirmeyi tarafsızlık ve gizlilik içerisinde yapmalıdırlar.
- Hakemler, makale hakkındaki değerlendirmelerini ya da bilgilerini üçüncü kişilerle paylaşmamalıdırlar.
- Hakemler, makale değerlendirme sürecinde edinilen bilgileri, fikirleri ve basılmamış materyal veya çalışmaları gizli tutmalı ve kişisel amaçlar için kullanmamalıdırlar.
- Hakemler, makalenin bir kopyasını elinde bulundurmamalı veya çoğaltmamalıdırlar.

2.3. Etik Sorunları Fark Etme

- Hakemler, makalede yer alan etik sorunları fark etmeli ve editörün dikkatine sunmalıdırlar.
- Hakemler, makalenin daha önce başka bir yerde basıldığını veya basılmış önceki bir makale ile önemli ölçüde benzerlik ya da örtüşme tespit ederse editöre bildirmelidirler. Daha önce yayımlanmış olan herhangi bir gözlem ve/veya argüman, ilgili referans ile birlikte verilmelidir.

2.4. Tarafsızlık ve Rekabet Standartları

- Hakemler, tarafsız olarak değerlendirmelerini yapmalı ve önyargıdan uzak şekilde değerlendirmelidirler. Yazarın kişi olarak eleştirilmesi uygun değildir. Hakemler, görüşlerini destekleyici argümanlarla ifade etmelidirler.

- Hakemler, makale değerlendirmeyi kabul etmeden önce olası çıkar çatışmasını kontrol etmelidirler. Eğer çıkar çatışmasıyla karşı karşıya olduğunu düşünüyorsa makaleyi incelemeyi reddetmeli ve editörü bilgilendirmelidirler.
- Hakemler, yazar tarafından hakemin (ya da hakemle çalışan kişilerin) çalışmalarının kaynak olarak alındığını ileri sürerse, gerçek bilimsel gerekçeler sunmalılar, bu durumun hakemin kaynak gösterilme sayısını ya da çalışmalarının görünürlüğünü artırmaya yönelik bir girişim olmamasına özen göstermelidirler.
- Hakemler, değerlendirmelerini yaparken bilimsel gerçeklikten uzaklaşmamalı ve gerekirse kaynak gösterme yoluna başvurmalıdırlar.

3. Yazarların Görevleri ve Etik Sorumlulukları

Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne gönderilen makaleler, daha önce herhangi bir yayın organında yayımlanmamış olmalıdır veya yayımlanmak üzere aynı zaman diliminde başka bir yayın organına gönderilmiş olmamalıdır. Çalışmalarda yararlanılan araştırmaların ve yayınların, alıntılarının veya atıflarının bilimsel araştırma ilkelerine uygun olarak eksiksiz yapılması ve kaynakların belirtilmesi zorunludur. Çalışmada yer alan yazar sayısı birden fazla ise, yazarların çalışmaya bilimsel ve akademik olarak somut ve yeterli düzeyde katkı sağlaması beklenir. Çalışmaya ait tüm finansal destek kaynakları açıklamalıdır. Olası çıkar çatışması durumlarını yayın kuruluna bildirmelidir.

Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne makale gönderen yazar/yazarların aşağıda belirtilen görevlere ve etik sorumluluklara uymalıdır.

3.1. Bildirim Standartları

- Yazar(lar)ın gönderdiği makale (araştırma, derleme veya kısa bildiri) özgün olmalıdır.
- Yazar(lar), çalışmanın önemine ilişkin tarafsız bir tartışma ile gerçekleştirilen araştırmayı net bir şekilde sunmalıdır.
- Yazar(lar), makalede verileri açık bir şekilde sunmalıdır.
- Yazar(lar)ın başka çalışmalardan faydalanması halinde tam ve doğru bir şekilde alıntı yapmalıdır.
- Makale, diğer araştırmacıların çalışmayı tekrar edebilmesine olanak verecek şekilde yeterli detay ve kaynak içermelidir.
- Yazar(lar), etik dışı davranarak yanıltıcı ya da net olmayan ifadeleri makalelerinde kullanmamalıdır.
- Yazar(lar), dergi kurallarına uymadıkları ve belirtilen sürede aksiyon almadıkları sürece makalelerinin dergi tarafından yayımlanmayacağını bilerek hareket etmelidir.

3.2. Veri Ulaşımı ve Saklama

- Yazarlardan editöryal değerlendirme için makalelerini destekleyici araştırma verisi istenebilir.
- Yazarlar, değerlendirme sürecinde makalelerine ilişkin ham verilerin veya makalelerini destekleyecek verilerin talep edilmesi durumunda belirtilen verileri yayın kuruluna sunmaya hazır bulunmalıdırlar.

3.3. Orijinallik, İntihal ve Kaynakların Belirtilmesi

- İntihal, yazarın başka bir makaleyi kendi çalışması olarak göstermesi, kaynak göstermeden başka birine ait çalışmanın belli bölümlerinin kopyalanması ya da başka sözcüklerle anlatılması veya başkaları tarafından yapılan çalışmanın sonuçlarının alınarak sunulması şeklinde olabilir. İntihalin her biçimi etik olmayan davranıştır ve kesinlikle kabul edilmemektedir. Yazarlar intihalden uzak durmalıdır. İntihal tanımı için buraya bakınız.
- Yazarlar çalışmalarının tümüyle orijinal olduğunu garanti etmelidirler. Yazarlar, başkalarının fikirlerini veya metinlerini kullanıyorlarsa mutlaka uygun şekilde kaynak ya da alıntı

göstermeliler ve gerekliyse izin almalıdırlar.

- Yazarlar kendilerine ait olan çalışmayı etkileyen ve çalışmaya ait uygun içeriğin oluşturulmasında katkısı olan tüm yayınları veya eserleri kaynak olarak göstermelidirler. Özel olarak (görüşme, yazışma ya da üçüncü taraflar ile tartışma) ile elde edilen bilgiler kullanılmamalı ya da kullanılacaksa izin alınarak bildirilmelidir.
- Yazarlar, Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne yayımlanmak üzere gönderdikleri makalelerini intihal tarama programları (iThenticate) ile taramalı ve dergipark sisteminde çevrim içi makale gönderim sırasında makalelerinin intihal içermediğine dair raporu yüklemek zorundadırlar.

3.4. Çoklu, Gereksiz ve Tekrar Yayınlama

- Aynı makale ile birden fazla dergiye başvuruda bulunmak etik olmayan bir davranıştır ve asla kabul edilmemektedir. Genel olarak, yazar daha önce basılmış bir yayını, özet formunda ya da yayınlanmış bir ders, akademik tez ya da elektronik ön baskının bir parçası olması dışında, değerlendirme için başka bir dergiye göndermemelidir.
- Yazarlar başvuru sırasında makaleyi başka bir dergiye daha aynı anda göndermediklerini garanti etmelidirler.
- Yazarlar, gönderilen yazının değerlendirme aşamasında olmadığını veya başka bir yerde yayımlanmak üzere kabul edilmediğini ve eğer kabul edilirse, aynı biçimde, başka bir dilde, elektronik ortam da dahil olmak üzere, yazarın yazılı izni olmaksızın başka bir yerde yayımlanmayacağını garanti etmelidir.

3.5. Yazar Katkıları

- Yazar katkıları, çalışmanın konseptine, tasarımına, gerçekleştirilmesine ya da yorumlanmasına önemli katkı sağlayan kişiler ile sınırlandırılmalıdır.
- Yazarlar, çalışmaya katkı veren yazarların listesini dikkatli bir şekilde hazırlamalıdır. Bazı durumlar eşyazar (co-author) olmayı bazı durumlar ise çalışmanın "Teşekkür" (Acknowledgement) bölümünde yer almasını hak edebilir.
- Sorumlu yazar, tüm eşyazarların çalışmada uygun şekilde yer aldığına, tüm eşyazarların çalışmayı görüp onayladıklarına ve yayınlanmak üzere başvuru yapılmasına dair verdikleri onaya ilişkin sorumluluğu üstlenmelidir.
- Sorumlu yazar, makaledeki tüm yazarların yazar sıralaması, çalışmanın kesinliği ve bütünlüğü gibi konularda fikir birliğinin sağlanmasından sorumludur ve orijinal başvuru sırasında kesin bir yazar listesi sunmalıdır.
- Çalışmanın başvurusu tamamlandıktan sonra, sadece istisna durumlarda, editör yazar listesinde ekleme, silme ya da yeniden düzenleme yapabilir. Tüm yazarlar bu şekilde yapılacak ekleme, silme ve yeniden düzenleme konusunda fikir birliği içinde olmalıdırlar. Tüm yazarlar çalışmanın ortak sorumluluğunu aldıklarını kabul ederler. Her yazar, uygun şekilde araştırılan ve karara bağlanan çalışmanın kesinliği ve bütünlüğü ile ilişkili sorulardan sorumludur.
- Sorumlu yazar, editör ile iletişime geçen kişi olarak Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne makale ile birlikte "Yazar Katkı Formu"nun da doldurulup gönderilmesinden sorumludur.

3.6. Çıkar Çatışması Beyanı

- Yazarlar, çalışmalarını uygunsuz bir şekilde etkileyebilecek olarak gördükleri diğer kişi veya organizasyonlarla çıkar çatışması oluşturabilecek her türlü durum ve ilişkileri beyan etmelidirler.
- Sorumlu yazar, editör ile iletişime geçen kişi olarak Ankara Üniversitesi Eczacılık Fakültesi Dergisi'ne makale ile birlikte "Çıkar Çatışması Beyanı Formu"nun da doldurulup gönderilmesinden sorumludur.

- Yazarlar çıkar çatışmalarının olduğu durumları mutlaka açıklamalıdırlar.

3.7. Temel Hataların Bildirimi

- Yazarlar, yayımlanmış, erken görünüm veya değerlendirme sürecinde olan bir çalışmasında önemli bir hata ya da eksiklik fark ettiğinde, acil olarak dergi baş editörüne/yayınevine veya ilgili editöre bildirmek ve editör tarafından gerekli görülmesi durumunda makaleyi geri çekmek veya düzeltmek için editörle işbirliği yapmak ile yükümlüdür.
- Editör/yayınevi yayımlanmış olan makalenin bir hata içerdiğini üçüncü bir taraftan öğrenirse, editör ile işbirliği yapmak ve gerektiğinde destekleyici kanıt sağlamak yazarın yükümlülüğüdür.

3.8. Olası Riskler ve İnsan veya Hayvan Konuları

- Yazarlar, kullanımları sırasında olağan dışı risk yaratan kimyasallar, işlemler ya da malzemeler ile çalışmışlarsa açıkça belirtmelidirler.
- Eğer çalışmada hayvan ya da insan örnekleri/gönüllüler kullanılıyorsa, araştırmacılar 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 makale içinde yer alması sağlamalıdırlar.
- Yazarlar, Etik Kurul Onayının zorunlu olduğu çalışmalarda, etik kurul onayı alınan kurumun adı ve etik kurul onay numarasını, gereç ve yöntem kısmında ve Etik Kurul Onay bölümünde belirtmelidirler. Ayrıca, kullanılan protokol ve prosedürlerin etik olarak gözden geçirildiğini ve onaylandığını, makalenin gereç ve yöntem bölümüne eklemelidirler.
- Etik kurul raporu alınması gerektiği halde, etik kurul raporu olmayan çalışmalar reddedilecektir.
- İnsanlar veya insandan elde edilen örnekler üzerinde yapılan klinik araştırmalarda bilgilendirilmiş onam formu mutlaka alınmış olmalıdır ve gereç ve yöntem kısmında belirtilmelidir. İnsan gönüllüleri ile yapılan araştırmalar için araştırma protokolüne uygun olarak hazırlanmış yazılı bilgilendirilmiş gönüllü onam formu alınmalıdır.
- Yazarlar, çalışmalarında, hayvan ya da insan örnekleri/gönüllüler kullanmışsa gerekli etik kurul izinlerini aldığından emin olmalıdır. Etik kurul izin ifadesini makalede mutlaka belirtmelidir.
- Bu anlamda yazarlar aşağıda sıralanmış olan kılavuzlara uyarak çalışmalarını gerçekleştirmiş olmalıdırlar:
  İnsanlar üzerinde gerçekleştirilen tüm araştırmalar Helsinki Bildirgesi ilkelerine göre yapılmalıdır (World Medical Association (WMA) Helsinki Declaration for Medical Research in Human Subject). İnsan gönüllülerinden bilgilendirilmiş onam formu alınmış olmalıdır. Tüm hayvan çalışmaları ARRIVE kılavuzuna uygun olmalı (Animal Research: Reporting of In Vivo Experiments (ARRIVE) Guidelines) ve "Bilimsel Amaçlı Kullanılan Hayvanların Korunmasına İlişkin Konsey Direktifi"ne (EU Directive 2010/63/EU for animal experiments), "Birleşik Krallık Hayvan Yasası"na (The U.K. Animals (Scientific Procedures) Act 1986) ve/veya "U.S. İnsan Bakımı ve Laboratuvar Hayvanlarının Kullanımına İlişkin Halk Sağlığı Hizmeti Politikası" rehberine (U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals) uygun şekilde yürütülmelidir. Bitkiler ile ilgili tüm deneysel araştırmalar, uluslararası yönergelere uygun olmalıdır.

4. Ücret Politikası

- Hiçbir ad altında yazar veya kurumundan ücret alınmaz.
- Dergi ile işleme ve yayınlama ücretsizdir. Gönderilen veya kabul edilen makaleler için makale işleme ücreti veya gönderim ücreti yoktur.

Publication Terms

1. The Journal of Faculty of Pharmacy of Ankara University (J. Fac. Pharm. Ankara) is an open-access, peer reviewed journal and 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 English. The experiments used have to be adhered to the Declaration of Helsinki for humans and European Community Guidlines for animals. In studies where Ethics Committee Approval is mandatory, the name of the institution from which ethics committee approval was obtained and the ethics committee approval number should be stated in the material and method section and the Ethics Committee Approval section, and the relevant document should be uploaded during article submission.

3. All manuscripts will be submitted to a review process by the editors and by qualified at least 2 outside reviewers. The article evaluation process of Journal of Faculty of Pharmacy of Ankara University is carried out on the principle of double-blind refereeing.

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, authors must send the copyright of the article to the Publisher by filling out the "Copyright Transfer Form".

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7. Journal of Faculty of Pharmacy of Ankara University does not have an article publication fee (APC) or subscription fee.

8. The following types of articles are accepted in the Journal Faculty of Pharmacy of Ankara University:

    a) **Original articles**: Articles written in English or Turkish in scientific format presenting original research. Articles should be printed on A4 size papers not exceeding 25 pages (including tables and figures). Research articles are expected to be innovative and contributing to science. Articles must have the main headings specified in the writing rules and must be prepared using a Windows compatible program.
    b) **Review articles:** 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 30 pages (including tables and figures). Articles must have the main headings specified in the writing rules and must be prepared using a Windows compatible program.
    c) **Short communications:** Rapid announcement of the results of a continuing research written in English or Turkish, no longer than 5, A4 size pages. Articles must have the main headings specified in the writing rules and must be prepared using a Windows compatible program.

Preparation of Manuscript

1. Texts must be written in A4 norm (21 x 29.7 cm).
2. Texts should be written with 1 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 (3 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 title of the manuscript the name/s, the full address/es and ORCID no of the author/s, and the full address, telephone number, e-mail address of the corresponding author should be written and all should be centered in the text. It should be indicated by placing (*) above the surname of the corresponding author. Name, surname, full address, 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, 06560, 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.) should be numbered below with Arabic numbers (**Figure 1., Table 2.**) 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 JPEG format**) must be placed in the article and pictures must be at least **300 dpi or in higher resolution**. 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 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. There must be **6 nk** space between the figure and figure title. There should be **18 nk** space between the text and title of figure and/or table.
    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**. There must be **6 nk** space between the table and table title.

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.

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.

Example for figure:

Figure 1. General view of *C. Nummularia* (The font size must be 11 pt with 1 line spacing and "Times New Roman" font, and must be centered in the text)

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**. 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 space before and 6 nk space after the INTRODUCTION.** For, there should be 12 nk space before and 6 nk space after the other titles. Between the chapter titles and the text, a separate space **should not be left** other than the specified in this document.

- **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 written in blocks with 1 cm margins from the right and left.

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 minimum of 3 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 **RESULT 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.
  - If the author wishes, "Conclusion" can be added as the last paragraph of the RESULT AND DISCUSSION section. The font size must be 11 pt with 1 line spacing and "Times New Roman" font and the first letter must be uppercase and the other letters must be lowercase.

- **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. If there is not any author contribution for the specified sections, "-" should be added. Please see below example for writing author contributions.

  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: -

- **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. Approval from the ethics committee should be uploaded during the manuscript submission. 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 10 font size, "1" spacing, justified. In the text, it should be numbered in square brackets according to the order of appearance, such as: [1,6,9], [5-7] 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.

  - **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 <u>should not be</u> "and" between the last author and the previous author. The "&" symbol <u>should not be</u> used**. The full title of the article should be written as the name of the journal, volume number, if available, the number (in parentheses), the beginning and ending page number (or article id), 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. Please do not add CrossRef hyperlink if the article is not listed at https://www.crossref.org/.**

https://doi.org/10.1016/0006-2952(89)90403-6

Examples:

1. 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]
2. 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]

- **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. Retrieved August 12, 2005, from ttp://www.bmj.com/cgi/content/full/317/7150/

- **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.

- **For patent:** 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. The title and number of the patent should

be indicated.

Example:

Mahoney, S., Molz, L., Narayan, S., Saiah, E. (2018). Heteroaryl RHEB Inhibitors and Uses Thereof. WO 2018/191146 A1.

ETHICAL PRINCIPLES AND PUBLICATION POLICY

Journal of Faculty of Pharmacy of Ankara University is an open-access, peer-reviewed journal and a publishing medium for original research, reviews and short communications covering important developments in the field of pharmaceutical sciences in Turkish or English. Journal of Faculty of Pharmacy of Ankara University does not have an article publication fee (APC) or subscription fee.

As the editorial board, it is aimed to publish high-quality new studies that make a significant contribution to the scope of the journal. To achieve this goal, articles submitted are subject to initial evaluation by the editor-in-chief and/or assistant editors to ensure that they meet the scientific and formal criteria to be published in the journal. Only studies that pass this preliminary evaluation process are continued to other stages for further evaluation.

Preliminary Assessment

- The scientific quality and novelty of the study must be sufficient to be published in the journal.
- Studies submitted to the journal must comply with the purpose and scope of the journal.
- The text must be written in English or Turkish, grammatically and scientifically well-written.
- The similarity rate of studies submitted to the journal should not exceed 20%.
- Studies should be arranged in accordance with the journal's writing rules and template.
- Copyright transfer form, ethics committee approval document and author contribution form must be uploaded and signed.
- Studies must be sent to the journal via the electronic online application system.

The further evaluation process of studies that do not meet these qualifications cannot be initiated.

Journal editors, reviewers and authors have certain responsibilities during the journal publication process. These responsibilities are explained below.

1. Editor's Duties and Ethical Responsibilities

The editor can independently decide which of the articles sent to the journal should be published, or can also consult with members of the editorial board or reviewers. Within the framework of the journal's ethical principles and publication policy, it is responsible for carrying out the preliminary evaluation, peer review and publication stages of the studies in an impartial, auditable, fair, independent of conflict of interest and in accordance with confidentiality principles. If there is no violation in terms of publication policy and ethical principles, studies that comply with the purpose and scope of the journal should be taken to the preliminary evaluation stage.

The duties and descriptions of the editor-in-chief, associate editors, section editors and editorial advisory board are as follows:

Editor-in-Chief: Editor in chief has full authority over the publication of the journal content. Editor in chief works with Associate Editors, Section Editors and the Editorial Advisory Board.
Associate Editors: Associate Editors are primarily responsible for answering questions about the journal, making suggestions to the journal reviewers and board, and assisting the Editor-in-Chief during the article publication process.
Section Editors: Section Editors assist authors in assigning double-blind referees and answering questions about the journal.
Editorial Advisory Board: The Editorial Advisory Board guides the Editor-in-Chief and Associate Editors on issues related to the production of quality publications that are appropriate for the purpose of Journal of Faculty of Pharmacy of Ankara University.

1.1. Publication Policy

- The editor-in-chief is solely responsible for deciding which articles sent to the journal should be published. The editor's decision may be in line with the principles of the journal's editorial

board or is limited by applicable legal requirements regarding issues such as defamatory publication, copyright infringement and plagiarism.

- The editor-in-chief ensures that the authors fill out the "Copyright Transfer Form" and send the copyright of the article to the publisher before the article is published.
- The editor-in-chief ensures that the authors fill out the "Conflict of Interest Form" and the "Author Contribution Form" before the article is published.
- The editor-in-chief assigns associate editors to formally review the articles sent to the journal. Articles that do not comply with the rules of the Journal of Faculty of Pharmacy of Ankara University are rejected without being evaluated.

1.2. Publication Review

- The editor-in-chief is responsible for ensuring that the publication evaluation process is fair, impartial and timely.
- The editor generally ensures that all articles are evaluated by at least two external and independent reviewers. If necessary, the editor requests additional opinion from a third reviewer.
- The editor selects the reviewers by evaluating experts who are suitable for the scope of the article.
- The editor carefully reviews the publication process to evaluate and decide on disclosures made for possible conflicts of interest, "self-citation" suggestions made by reviewers, and any possibility of bias.
- The editor-in-chief/other editors requests for the article to be scanned by a similarity detection software (iThenticate) at any point during the peer review or evaluation/publication process, or they do it themselves. In this sense, even if the expressions or sentences are the author(s) themselves, the text should not have an unacceptable similarity to previously published data.
- If the editor-in-chief detects errors in an article before it is published, he/she corrects them. If he/she detects it later, then he/she has to publish the corrections. All corrections or retraction notices must be prominently published in the journal. It should also be listed on the contents page.
- Journal of Faculty of Pharmacy of Ankara University's editors follow the "COPE Code of Conduct and Best Practice Guidelines for Journal Editors" and "COPE Best Practice Guidelines for Journal Editors" guidelines published by the Committee on Publication Ethics (COPE).

1.3. Fair Evaluation

- Editor-in-Chief/other editors evaluates articles according to their scientific content, regardless of the authors' race, gender, sexual orientation, belief, ethnicity, citizenship or political views. The journal's editorial principles support transparent and completely honest review.
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- The chief editor/other editors is obliged to maintain the confidentiality of all materials in the application to the journal and all communication with the reviewers (unless otherwise approved by the relevant authors and reviewers).
- The chief editor/other editors is responsible for protecting the identities and rights of the reviewers, unless the reviewers agree to their names being disclosed.
- Unpublished materials belonging to a submitted article should not be used for the editor's own studies/research without the written consent of the author.
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The article evaluation process of Journal of Faculty of Pharmacy of Ankara University is carried out on the principle of double-blind review. Therefore, reviewer cannot communicate with the author(s), evaluations are shared through the Dergipark management system. During the evaluation process, evaluation forms and reviewers' comments regarding the manuscripts are forwarded to the corresponding author through the editor. Reviewers must act in accordance with the principles of impartiality, confidentiality, objectivity and scientific review throughout the evaluation process. They must be an expert and competent in the relevant field. They must complete their report on the work submitted for evaluation within the specified time period. For reports that cannot be submitted on time, the editor should be contacted without delay. The editorial board should be informed in cases of copyright and/or ethical infringement, possible conflict of interest and plagiarism.

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2.1. Contribution to Editorial Decisions

- Reviewers must evaluate the work submitted by authors constructively and appropriately.
- If the reviewers think that they are not competent to evaluate the research in the article or cannot complete it in sufficient time, they must notify the editor.
- Reviewers should not make harsh and personal criticisms towards the authors.
- When reviewers receive an invitation to evaluate an article, they should refuse to evaluate the article if they feel inadequate about the subject studied in the article.
- Reviewers must evaluate the article within the given time.
- Reviewers should only objectively evaluate the content of the study.

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- Reviewers must make the evaluation impartially and confidentially.
- Reviewers should not share their evaluations or information about the article with third parties.
- Reviewers must keep confidential the information, ideas and unpublished materials or studies obtained during the article evaluation process and must not use them for personal purposes.
- Reviewers should not retain or reproduce a copy of the article.

2.3. Detecting Ethical Issues

- Reviewers should notice the ethical problems in the article and bring them to the attention of the editor.
- If the reviewers detect that the article has been previously published elsewhere or that there is a significant similarity or overlap with a previously published article, they must notify the editor. Any previously published observations and/or arguments should be accompanied by the relevant reference.

2.4. Impartiality and Competition Standards

- Reviewers must make their evaluations impartially and free from bias. It is not appropriate to criticize the author as a person. Reviewers must express their opinions with supporting arguments.
- Reviewers must check for possible conflict of interest before agreeing to evaluate the article. If they feel they face a conflict of interest, they should refuse to review the manuscript and inform the editor.
- If reviewers claim that the reviewers' (or people working with the reviewers) work has been taken as a source by the author, they must provide real scientific justifications and be careful that this is not an attempt to increase the reviewers' number of references or the visibility of their work.

- Reviewers should not stay away from scientific reality when making their evaluations and should resort to citing sources if necessary.

3. Authors' Duties and Ethical Responsibilities

Articles submitted to Journal of Faculty of Pharmacy of Ankara University must not have been previously published in elsewhere or should not have been sent to another publication within the same time period for publication. It is mandatory that the quotations or citations of the research and publications used in the studies are made completely in accordance with the principles of scientific research and the sources are stated. If the number of authors in the study is more than one, the authors are expected to make a concrete and sufficient scientific and academic contribution to the study. All sources of financial support for the study must be disclosed. Authors must report possible conflict of interest situations to the editorial board.

The author(s) who sent articles to Journal of Faculty of Pharmacy of Ankara University, must comply with duties and ethical responsibilities listed below:

3.1. Notification Standards

- The article (research, review or short communication) sent by the author(s) must be original.
- The author(s) should clearly present the research performed with an unbiased discussion of the significance of the study.
- The author(s) must present the data clearly in the article.
- If the author(s) uses other works, they must cite them fully and accurately.
- The article must contain sufficient detail and sources to enable other researchers to replicate the study.
- Author(s) should not act unethically and use misleading or unclear expressions in their articles.
- Authors act with the knowledge that their articles will not be published by the journal unless they comply with the journal rules and take action within the specified time.

3.2. Data Transportation and Storage

- Authors may be asked for research data supporting their articles for editorial evaluation.
- Authors must be ready to submit the specified data to the editorial board in case raw data regarding their articles or data to support their articles are requested during the evaluation process.

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- Plagiarism may occur in the form of the author representing another article as his own work, copying or paraphrasing certain parts of someone else's work without citing the source, or presenting the results of work done by others. Any form of plagiarism is unethical behavior and is completely unacceptable. Authors should avoid plagiarism. Please click here for the definition of plagiarism.
- Authors must guarantee that their work is completely original. If authors use others' ideas or texts, they must indicate appropriate sources or citations and obtain permission if necessary.
- Authors must cite as references all publications or works that influence their work and contribute to the creation of appropriate content for the work. Information obtained privately (interview, correspondence or discussion with third parties) should not be used or, if used, should be reported with permission.
- Authors must scan the articles that they send to Journal of Faculty of Pharmacy of Ankara University for publication with plagiarism scanning programs (iThenticate) and upload a report stating that their articles do not contain plagiarism during online article submission in the Dergipark system.

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- Submitting to more than one journal with the same article is unethical behavior and is never accepted. In general, the authors should not submit a previously published publication to another journal for review, except in abstract form or as part of a published lecture, academic thesis, or electronic preprint.
- Authors must ensure that they do not submit the article to another journal at the same time during the submission.
- Authors must guarantee that the submitted manuscript is not under evaluation or has been accepted for publication elsewhere, and if accepted, it will not be published elsewhere in the same format, in another language, including electronic media, without the written permission of the author.

3.5. Author Contributions

- Author contributions should be limited to individuals who have made significant contributions to the concept, design, implementation, or interpretation of the work.
- Authors should carefully prepare the list of authors who contributed to the study. In some cases, the work may deserve to be a co-author, and in some cases, the work may deserve to be included in the "Acknowledgment" section.
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- The corresponding author is responsible for ensuring that all authors on the manuscript agree on issues such as author order, accuracy and integrity of the work, and must submit a definitive author list at the time of the original submission.
- After the application of the study is completed, the editor can add, delete or rearrange the author list only in exceptional cases. All authors must agree on such additions, deletions and rearrangements. All authors acknowledge shared responsibility for the work. Each author is responsible for questions relating to the accuracy and integrity of the work that have been properly researched and adjudicated.
- The corresponding author, as the person who contacts the editor, is responsible for filling out and sending the "Author Contribution Form" along with the article to Journal of Faculty of Pharmacy of Ankara University.

3.6. Conflict of Interest Declaration

- Authors must declare any situations or relationships that may create a conflict of interest with other individuals or organizations that they deem to be inappropriately influencing their work.
- The corresponding author, as the person who contacts the editor, is responsible for filling out and sending the "Conflict of Interest Declaration Form" along with the article to Journal of Faculty of Pharmacy of Ankara University.
- Authors must disclose situations where they have conflicts of interest.

3.7. Reporting Basic Errors

- When authors notice a significant error or omission in a published work that is in the early review or evaluation process, they are obliged to immediately notify the journal editor-in-chief / publisher or the relevant editor and, if deemed necessary by the editor, cooperate with the editor to withdraw or correct the article.
- If the editor/publisher learns from a third party that the published article contains an error, it is the author's obligation to cooperate with the editor and provide supporting evidence where necessary.

3.8. Potential Risks and Human or Animal Issues

- Authors should clearly state if they have worked with chemicals, processes, or materials that pose unusual risks when used.
- If animal or human samples/volunteers are used in the study, researchers should ensure that a statement is included in the article that all procedures were carried out in accordance with the relevant laws and institutional guidelines and that these procedures were approved by the appropriate administrative board and Ethics Committee Approval was obtained.
- In studies where Ethics Committee Approval is mandatory, authors must indicate the name of the institution from which ethics committee approval was obtained and the ethics committee approval number in the materials and methods section and the Ethics Committee Approval section. They should also include in the materials and methods section of the manuscript that the protocols and procedures used have been ethically reviewed and approved.
- Although an ethics committee report is required, studies without an ethics committee report will be rejected.
- In clinical research conducted on humans or samples obtained from humans, an informed consent form must be obtained and must be stated in the materials and methods section. For research conducted with human volunteers, a written informed consent form prepared in accordance with the research protocol must be obtained.
- Authors must ensure that they obtain the necessary ethics committee permissions if they use animal or human samples/volunteers in their studies. Ethics committee permission must be stated in the article.
- In this sense, authors must have carried out their work by following the guidelines listed below: All research conducted on humans should be conducted in accordance with the principles of the Declaration of Helsinki (World Medical Association (WMA) Helsinki Declaration for Medical Research in Human Subject). Informed consent must have been obtained from human volunteers. All animal studies must comply with the ARRIVE guideline (Animal Research: Reporting of In Vivo Experiments (ARRIVE) Guidelines) and the "Council Directive on the Protection of Animals Used for Scientific Purposes" (EU Directive 2010/63/EU for animal experiments), "United Kingdom Animal (The U.K. Animals (Scientific Procedures) Act 1986) and/or "U.S. It must be conducted in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals. All experimental research on plants must comply with international guidelines.

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YAYIN İDARE MERKEZİ ADRESİ	**:** Ankara Üniversitesi, Eczacılık Fakültesi, Dekanlığı, 06560 Yenimahalle/Ankara
YAYIN İDARİ MERKEZİ ADRESİ TEL	**:** 0 (312) 203 30 01
YAYIN TÜRÜ	**:** Bilimsel Periyodik Elektronik Dergi, Yılda 3 Sayı

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