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# Effect of bellis (fungicide) on total oxidant / antioxidant and total sialic acid levels in fishes

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**Abstract:** In this study, it was aimed to investigate the effect of Bellis, a fungicide widely used in agriculture, on plasma total antioxidant level (TAS), total oxidant level (TOS) and total sialic acid (TSA) levels in *Cyprinus carpio*. A total of 30 (250-300 g) *C. carpio* were used, 10 in each group. Fish were divided into 3 groups as control group, Bellis I group (0.025 mg/L Bellis) and Bellis II group (0.050 mgl/L Bellis). After 14 days of fungicide application to Belllis groups, blood samples were taken from the fish and their plasmas were obtained. TAS, TOS and TSA levels were measured in the obtained plasma samples. According to the findings obtained in the study, the TAS level in the control group was found to be statistically significantly higher ($p<0.001$) compared to the Bellis-I and Bellis-II groups. Similarly, TOS and TSA levels in the control group were found to be statistically significantly lower ($p<0.05$) compared to the Bellis-administered (Bellis I and Bellis II) groups. According to these results, it was concluded that Bellis, which has become widespread in agricultural areas in recent years, may disrupt the antioxidant/oxidant balance in fish in favor of oxidants, depending on the increasing dose. For this reason, it is important for public health to pay attention to the consumption of fish, which is an important link of the food chain, from aquatic environments close to agricultural areas where pesticides are used intensively.

**Keywords:** Bellis, *Cyprinus carpio*, Total sialic acid, Total oxidant/antioxidant level

## *Bellis (fungisit)'in balıklarda total oksidan / antioksidan ve total sialik asit seviyeleri üzerine etkisi*

**Özet:** Bu çalışmada tarım alanında yaygın olarak kullanılan bir fungisit olan Bellis'in *Cyprinus carpio*'da plazma total antioksidan seviye (TAS), total oksidan seviye (TOS) ve total sialik asit (TSA) seviyelerine etkisinin araştırılması amaçlandı. Her grupta 10 adet olmak üzere toplam 30 adet (250-300g) *C. carpio* kullanıldı. Balıklar kontrol grubu, Bellis I grubu (0.025 mg/L Bellis) ve Bellis II grubu (0.050 mgl/L Bellis) olarak 3 gruba ayrıldı. Bellis gruplarına 14 günlük fungisit uygulamasının ardından balıklardan kan örnekleri alınarak, plazmaları elde edildi. Elde edilen plazma örneklerinde TAS, TOS ve TSA seviyeleri ölçüldü. Çalışmada elde edilen bulgulara göre kontrol grubunda TAS seviyesinin Bellis-I ve Bellis-II grubuna göre istatistiki olarak anlamlı derecede ($p<0.001$) yüksek olduğu tespit edildi. Benzer şekilde kontrol grubunda TOS ve TSA seviyelerinin Bellis uygulanan (Bellis I ve Bellis II) gruplara göre istatistiki olarak önemli derecede ($p<0.05$) düşük olduğu tespit edildi. Elde edilen bu sonuçlara göre son yıllarda tarım alanlarında kullanımı yaygınlaşan Bellis'in artan doza bağlı olarak balıklarda antioksidan/oksidan dengeyi oksidanlar lehine bozabileceği sonucuna varıldı. Bu nedenle besin zincirinin önemli bir halkası olan balıkların besin olarak tüketiminde, zirai ilaçlarının yoğun kullanıldığı tarım alanlarına yakın sucul ortamlardan yakalanıp yakalanmadığına dikkat edilmesi toplum sağlığı açısından önemlidir.

**Anahtar Kelimeler:** Bellis, *Cyprinus carpio*, Total sialik asit, Total oksidan/antioksidan seviye

## 1. Giriş

Dünyada hızlı nüfus artışı ve endüstrideki gelişmeler pek çok çevre sorununu da beraberinde getirmiştir. Meydana gelen bu çevre sorunlarının başında gelen su kirliliği, sucul ekosistemi ve insan sağlığını tehdit eder duruma gelmiştir. Suların kirlenmesine endüstriyel atıklar, deterjanlar, ilaçlar, ağır metaller, petrol ürünleri ve pestisitler neden olmaktadır (Özkan ve ark. 2009; Ahuja 2013; Deveci ve ark. 2021; Berber ve ark. 2021). Yirminci yüzyılın başlarından itibaren aşırı pestisit kullanımı doğal çevreyi ve insan sağlığını olumsuz yönde etkilenmeye başlamıştır. Tarım zararlılarıyla mücadelede kullanılan pestisitler, toprakta uzun süre bozulmadan kalabilmekte ve çevredeki diğer canlılar üzerinde olumsuz etkilere yol açabilmektedir (Abdollohi ve ark. 2004; Deveci ve ark. 2017; Deveci ve ark. 2021). Toprakta bozunmadan kalan bu pestisitler besin zinciri yoluyla birçok canlıya geçerek biyokonsantrasyona neden olmaktadır. Besin zinciri aracılığıyla vücuda alınan pestisitler ve bunların yıkım ürünleri zamanla doku ve organlarda çeşitli olumsuzluklara neden olmaktadır (Deveci ve ark. 2015; Strungaru ve ark. 2019; Kayhan 2020)

Tarım alanlarında genellikle mısır, buğday, arpa, zeytin, Antep fıstığı, çeşitli sebze ve meyvelerde bitki hastalık etkeni mantarlara karşı sıklıkla fungisitler kullanılmaktadır (Chen ve ark. 2008; Avenot ve ark. 2008; Temiz 2019). Son yıllarda Türkiye'de özellikle Gaziantep ve çevresinde Antep fıstığı, zeytin ve nar zararlıları ile mücadelede kullanımı artan fungisitlerden biri de Bellis®'tir. Bellis® (%25.2 Boscalid + %12.8 Pyraclostrobin, WG), anilid ve strobilurin birleşiminden meydana gelmektedir. Boscalid, anilid fungisitlere ait yeni bir geniş spektrumlu fungisittir. Strobilurinlerden ve diğer çoğu fungisitten etki şekli ve etki alanı yönünden farklılık göstermektedir. Boscalid, mitokondriyal elektron taşıma zincirinde kompleks II'yi inhibe ederken, piraklostrobin, kompleks III'ü inhibe eder (Avenot ve ark. 2008; Lagunas-Allué ve ark. 2015; Özkılınç 2018; Aksakal 2020).

Balıklar sucul ekosistemdeki kirliliğin (pestisit, ağır metaller ve diğer kimyasallar) ve bu kirliliğin diğer canlılar üzerindeki etkilerinin tespit edilmesinde bir biyobelirteç olarak kullanılmaktadır (Van der Oost ve ark. 2003, Işık ve Celik 2008, Deveci ve ark. 2017, Nur ve Deveci 2018, Temiz 2019). Birçok çevresel faktör canlılarda reaktif oksijen türlerini (ROT) uyararak hücre ve dokularda oksidan moleküllerin sayısını arttırmaktadır (Li ve ark. 2011, Deveci ve ark. 2021). Pestisitlerin toksikasyonu sonucu hücre ve dokularda meydana gelen oksidan moleküller antioksidan savunma sistemini olumsuz yönde etkilemektedir. (Kaya ve ark. 2014; Deveci ve ark 2017; Nur ve Deveci 2018; Temiz 2019). Kanda ve diğer dokularda hem antioksidan hem de oksidan moleküllerin tek tek ölçümünden ziyade TAS ve TOS ölçümünün, organizmanın oksidan/antioksidan dengesinin belirlenmesinde daha uygun olabileceği bildirilmektedir (Erel 2004; Erel 2005).

Yapılan bu çalışmada, son yıllarda Gaziantep yöresinde tarım alanlarında mantar hastalıklarına karşı kullanımı artan fungisit Bellis'in *Cyprinus carpio*'da plazma total antioksidan seviye (TAS), total oksidan seviye (TOS) ve total sialik asit (TSA) seviyeleri üzerine etkisinin araştırılması amaçlandı.

## 2. Materyal ve Metot

Bu çalışmada İslahiye Tahtaköprü Barajı'ndan yakalanan, 250-300 g ağırlığındaki toplam 30 adet *Cyprinus carpio* kullanıldı. Çalışmaya başlanılmadan önce kullanılan polietilen tanklar dinlendirilmiş su ile yıkanarak su doldurulmuş ve su dinlendirildikten sonra balıklar tanklara alınmıştır. Su kalitesini izlemek için her tanktaki su örnekleri günlük olarak analiz edildi. Balıklar daha sonra laboratuvara getirilerek polietilen tanklar içerisinde 5 gün boyunca yeni ortamlarına uyum sağlamaları için bekletildi. Yeni ortama uyum sağlamalarının ardından her bir grupta 10 balık olmak üzere 3 gruba ayrıldı. Kontrol grubundaki balıkların tanklarına hiçbir madde eklenmeksizin normal su konuldu. Bellis I grubu balıklar 0.025 mg/L, Bellis II grubu balıklar ise 0.050 mg/L oranında Bellis® (%25.2 Boscalid + %12.8 Pyraclostrobin, BASF TURK) uygulanan tanklarda bekletildi. Fungusitin balıklar tarafından absorbsiyonu ve buharlaşma faktörleri düşünülerek, her gün suyun değiştirilmesi (APHA, 1981) ve yeni doz uygulamalarının yapılması sağlandı. 14 günün sonunda balıklardan kan örnekleri kaudal venadan alındı. Alınan bu kan örnekleri 3000 rpm'de 10 dakika santrifüj edilmek suretiyle plazmaları elde edildi ve analizler başlayıncaya kadar -20 °C de muhafaza edildi. Plazma TAS ve TOS analizi ticari kitler (REL Assay Diagnostics, Gaziantep, Türkiye) kullanılarak spektrofotometrede yapıldı (Erel 2004; Erel 2005). Plazma TSA analizi ise Sydow (1985)'un metoduna göre yapıldı.

### *İstatistiksel Analiz*

Elde edilen verilerin istatistiksel analizi SPSS 21.0 paket programıyla gerçekleştirildi. Gruplar arasında TAS, TOS ve TSA açısından farklılık olup olmadığını belirlemek için tek yönlü varyans analizi (One Way ANOVA) kullanıldı. Anlamlı farklılık olduğu görülen gruplarda hangi gruplar arasında farklılık olduğunu belirlemek için Post-Hoc (Tukey LSD) analizi yapıldı. Elde edilen sonuçlar ortalama ± standart sapma (X±SD) şeklinde verildi. $p<0.05$ değeri istatistiksel olarak anlamlı kabul edildi.

## 3. Bulgular

Deneysel çalışma için yaşadıkları tanklara 0.025 ve 0.050 mg/L Bellis fungisit eklenen (Bellis I grubu, Bellis II grubu) ve herhangi bir şey eklenmeyen kontrol grubu balıklardan elde edilen plazma analiz sonuçları ve bunlara ait parametrelerdeki değişim Tablo 1.'de verilmiştir.

**Tablo 1.** Bellis uygulanan ve uygulanmayan *Cyprinus carpio*'da TAS, TOS ve TSA seviyelerinin karşılaştırılması.

| PARAMETRELER | Kontrol grubu | Bellis I grubu | Bellis II grubu | p |
|---|---|---|---|---|
| **TAS** (mmol Trolox eq/L) | 0.56±0.048[a] | 0.46±0.040[b] | 0.45±0.060[b] | * |
| **TOS** (μmol $H_2O_2$ eq /L) | 4.93±0.33[a] | 5.84±0.58[b] | 6.17±0.60[b] | * |
| **TSA** (mg/dL) | 35.87±3.18[a] | 42.93±3.96[b] | 40.99±3.23[b] | ** |

[a, b] Aynı satırda farklı harflerle gösterilen değerler istatistiksel olarak farklıdır (*: p< 0.001 , **: p< 0.05).

Tanklarda kullanılan su kalitesi balıkların toksik maddeye karşı geliştirdiği tepki üzerinde etkili fiziko-kimyasal bir parametre olduğundan günlük olarak izlenmiştir. Kullanılan tanklardaki su kalitesi incelendiğinde, su sıcaklığı ortalama 20±2 °C, çözünmüş oksijen 8.6±0.7 mg/L ve total sertlik 228±9.9 $CaCO_3$ mg/L olarak tespit edilmiştir.

Herhangi bir uygulama yapılmayan kontrol grubu balıklara ait plazma örnekleri, 0.025 mg/L Bellis fungisit uygulanan Bellis-I grup ve 0.050 mg/L Bellis fungisit uygulanan Bellis-II grup balıklara ait plazma örnekleri ile karşılaştırıldı. Buna göre kontrol grubu balıklarda TAS'ın diğer gruplara göre anlamlı derecede ($p<0.001$) yüksek olduğu, TOS ve TSA seviyelerinin ise istatistiki olarak önemli derecede ($p<0.05$) düşük olduğu belirlendi.

## 4. Tartışma

Dünyada olduğu gibi ülkemizde de tarım sektöründe ürün kaybına yol açan zararlılarla mücadelede pestisitlerin kullanımı giderek yaygınlaşmaktadır Pestisitler tarım zararlılarıyla mücadele sonucu ürün miktarında artış sağlasalar da bunların uzun süreli ve bilinçsiz kullanımları sonucu ekosistem ve insan sağlığı olumsuz yönde etkilemektedir (Kaya ve ark. 2012; Deveci ve ark. 2021). Son yıllarda sucul ekosistemde toksisitenin belirlenmesinde ve tarımda kullanılan kimyasalların diğer canlılar üzerindeki olası etkilerinin öngörülmesinde balıklar yaygın olarak kullanılmaktadır (Selvi ve ark. 2004; Kaya ve ark. 2014; Yılmaz ve ark. 2017; Deveci ve ark. 2017).

Pestisite maruz bırakılan balıklarla yapılan bazı çalışmalarda, pestisit maruziyetinin balık hücre ve dokularında antioksidan moleküllerin miktarını azalttığı, oksidan moleküllerin miktarını arttırdığı ve oksidatif strese yol açtığı bildirilmektedir (Kaya ve ark. 2014; Mirvaghefi ve ark. 2016; Deveci ve ark. 2017; Yılmaz ve ark. 2017; Nur ve Deveci, 2018). Bellis'in etken maddesi olan boscalid'in kullanıldığı bir çalışmada, etken maddenin lipit peroksidasyonuna neden olduğu ve artan dozuna bağlı olarak malondialdehit (MDA) düzeyinin arttığı, antioksidan enzim aktivitelerinin ise azaldığı bildirilmiştir (Aksakal, 2020). Zang ve ark. (2017) zebra balığı üzerinde Bellis'in etken maddelerinden boscalid'in toksik etkisine baktıkları çalışmada katalaz, glutatyon peroksidaz, süperoksit dismutaz aktivelerinin ve MDA düzeylerinin boscalid konsantrasyonuna ve maruz kalma sürelerine göre değişiklik gösterdiğini bildirmiştir. Buna göre çalışmanın 7. gününde antioksidan enzimlerin aktivitelerinin artmasının nedenini meydana gelen oksidatif strese bir yanıt olduğunu, 21 günlük uygulamada ise oksidan molekül miktarında önemli bir artış olduğunu bildirmiştir. Farklı pestisitlere maruz bırakılan balıklarla yapılan oksidatif stres çalışmalarında balıkların kan ve farklı doku örneklerinde total oksidan seviyenin arttığı, total antioksidan seviyenin ise azaldığı bildirilmiştir (Kaya ve ark 2014; Mirvaghefi ve ark. 2016; Yılmaz ve ark. 2017; Deveci ve ark. 2017; Azimzadeh ve Amniattalab 2017; Nur ve Deveci 2018; Şahinöz 2019). Yapılan bu çalışmada ise yukarıda farklı pestisitlerle yapılan balık çalışmalarına benzer şekilde farklı dozlarda Bellis fungisit uygulanan balıklarda total oksidan seviyenin arttığı, total antioksidan seviyenin ise azaldığı tespit edildi.

Sialik asit (SA), nöraminik asitin açillenmiş türevi olup biyolojik membranların önemli yapıtaşlarındandır. Sialik asit akut faz proteini olarak da görev yapmaktadır. Total sialik asit (TSA) ise serbest, proteine ve lipide bağlı sialik asidin toplamı olarak ifade edilmektedir (Karapehlivan ve ark. 2007; Merhan ve Özcan 2004). Oksidatif stresin hücre yüzeyindeki oligosakkaritlerden sialik asit salınımını başlatabileceği, bunun da TSA düzeylerini arttıracağı bildirilmektedir (Eguchi ve ark. 2005; Kaya ve ark. 2014). Yılmaz ve ark. (2017) pestisit (Dimethoate) maruziyetine bıraktıkları balıklarla yaptıkları bir çalışmada, pestisit maruziyetine kalan balıklarda kontrol grubu balıklara oranla TSA seviyelerinin önemli derecede arttığını, askorbik asitin ise artan TSA seviyelerini düşürdüğünü bildirmişlerdir. Benzer şekilde pestisite maruz kalmış balıklarla yapılan birçok çalışmada pestisit maruziyetinin oksidatif strese neden olabileceği, bunun da balıklarda TSA seviyelerini arttırabileceği bildirilmiştir (Kaya ve ark. 2012; Deveci ve ark. 2016; Azimzadeh ve Amniattalab 2017).

## 5. Sonuç

Tarımsal alanlarda yoğun olarak kullanılmakta olan Bellis'in balıklarda TAS, TOS ve TSA seviyelerinde önemli değişiklikler meydana getirebileceği, bu yeni fungisitin balıklardaki oksidatif stres parametrelerini etkilemesi sonucu bu balıkları tüketen diğer canlıların antioksidan savunma sistemini zayıflatabileceği gözlemlenmiştir. Buna göre pestisitlerin sık kullanıldığı tarım alanlarına yakın dere, göl, ırmak ve barajlardan yakalanan balıkların tüketiminin insan sağlığını tehdit edebileceği sonucuna varıldı. Yapılan geniş literatür taramasına rağmen önemli bir fungisit olan Bellis'in balıklarda TAS, TOS ve TSA seviyelerini araştıran herhangi bir çalışmaya rastlamadık. Bu yönüyle bu çalışmanın daha sonra yapılacak olan çalışmalara önemli bir referans olacağını düşünmekteyiz.

### Yazar katkıları

Tüm yazarların makaleye katkısı eşit olup, yazarlar makalenin son halini okuyup incelemiştir.

### Çıkar çatışması

Yazarlar arasında herhangi bir çıkar çatışması yoktur.

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# *Drosophila melanogaster*'in somatik hücrelerinde kobalt nanopartiküllerinin indüklediği genotoksisiteye karşı resveratrol'ün antigenotoksik etkisi

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**Özet:** Son yıllarda gelişen teknoloji ile birlikte yeni teknolojik ürün olarak nanopartiküllerin (NP, <100 nm) üretimi ve farklı alanlardaki kullanımları hemen her alanda giderek yaygınlaşmıştır. Sahip oldukları birçok avantaj sayesinde çok farklı alanlarda kullanılan NP'lerin insan sağlığına ve çevreye etkileri hakkındaki bilgiler hala çok yetersizdir. Bu nedenle son teknoloji ürünü bu maddelerin genotoksik potansiyellerinin tespiti bu maddelerin biyogüvenilirliği bakımından önemlidir. Resveratrol (3, 4', 5-trihidroksi-stilben: RSV) üzüm çekirdeğinde bol miktarda bulunan ve son yıllarda farklı mekanizmalardaki yardımcı etkileri üzerinde yoğun çalışılan doğal bir antioksidandır. Bu çalışmada, *Drosophila melanogaster*'de somatik mutasyon ve rekombinasyon testi (SMART) ve tek hücre alkali jel elektroforezi testi (KOMET) kullanılarak Kobalt nanopartiküllerinin (Co NP) genotoksisitesine karşı RSV'ün antigenotoksik etkisi araştırılmıştır. SMART genetik değişimleri geniş bir spektrumda hızlı ve ucuz şekilde belirlemeye yarayan güvenilir bir yöntemdir. KOMET ise özgün hücrelerde değişen alkali lezyonları ve DNA'daki tek iplik kırıklarını tespit eden farklı genotoksik ajanlar ile indüklenen genetik hasarın belirlenmesinde güçlü ve duyarlı bir teknik tir. Yapmış olduğumuz çalışma kapsamında SMART ve KOMET yöntemleri ile değerlendirilen ve somatik hücreler olan kanat imajinal disk hücrelerinde ve *Drosophila* hemositlerinde genotoksik etkiye sahip olduğunu tespit ettiğimiz Co NP'lerinin (10mM) RSV ile hem ön uygulamalı hem de eş zamanlı çalışmalarında Co NP'lerinin genotoksik etkisine karşı RSV'ün uygulanan tüm dozlarda (0.1, 0.5 ve 2.5 mM) antigenotoksik etkiye sahip olduğu gözlenmiştir.

**Anahtar Kelimeler:** *Drosophila melanogaster*, Kobalt nanopartikülü, Resveratrol, Antigenotoksisite, SMART, KOMET

## *Antigenotoxic effect of resveratrol against genotoxicity induced by cobalt nanoparticles in somatic cells of Drosophila melanogaster*

**Abstract:** With the developing technology in recent years, the production of nanoparticles (NP, <100 nm) as a new technological product and their use in different fields have become increasingly widespread in almost every field. Although NPs are widely used in many different fields due to their many advantages, the information about their effects on human health and the environment is still very insufficient. Therefore, the determination of the genotoxic potential of these state-of-the-art substances is important for their biosafety. Resveratrol (3, 4', 5-trihydroxy-stilbene: RSV) is a natural antioxidant that is abundant in grape seeds and its auxiliary effects in different mechanisms have been studied intensively in recent years. The antigenotoxic effect of RSV against genotoxicity of Cobalt Nanoparticles (Co NPs) was investigated using single cell alkaline gel electrophoresis test (KOMET). SMART is a reliable method for quickly and inexpensively detecting a wide spectrum of genetic changes. KOMET, on the other hand, is a powerful and sensitive technique for the detection of genetic damage induced by different genotoxic agents that detect altered alkaline lesions and single strand breaks in DNA in specific cells. Within the scope of our study, Co NPs (10mM) which were evaluated by SMART and KOMET methods and we found to have genotoxic effects in wing imaginal disc cells which are somatic cells and *Drosophila* hemocytes, were able to resist the genotoxic effect of Co NP in both pre-application and simultaneous studies with RSV. It was observed that RSV had an antigenotoxic effect at all doses (0.1, 0.5 and 2.5 mM).

**Keywords:** *Drosophila melanogster*, Cobalt nanoparticle, Resveratrol, Antigenotoxicity, SMART, COMET

## 1. Giriş

"Nanoteknoloji" terimi Nobel ödüllü bilim insanı Richard P. Feynman tarafından sunulduğu andan itibaren bir araştırma alanı olarak tanımlanmıştır ve günümüzde de kullanılmaktadır (Feynman 1960, Khan ve ark. 2019). Bu gelişmeyi takiben nanoteknoloji alanında yapılan araştırmalar ile nano ölçek düzeyinde malzemeler üretilerek çok önemli bir aşamaya geçilmiştir (Khan ve ark. 2019). Nanopartiküller (NP'ler) olarak tanımlanan bu malzemeler, en az bir boyutu 100 nm'den küçük olan partikülat maddeleri içeren geniş bir malzeme sınıfı olarak tanımlanmıştır (Laurent ve ark. 2008). NP'ler farklı kriterlere göre sınıflandırılabilmektedirler. Bunlar; kökenlerine göre; doğal ve antropojenik, boyutlarına göre; 1-10 nm, 10-100 nm ve 100 nm'den büyük olanlar ve kimyasal bileşenlerine göre; inorganik maddeler, organik maddeler ve elementler şeklindedir (Strambeanu ve ark. 2015). NP'ler laboratuvar koşullarında sentetik olarak üretilmesinin yanı sıra doğal olaylar sonucunda da meydana gelebilmektedir. NP'lerin doğal kaynakları arasında volkanik kül, çöl tozları, aerosoller örnek olarak verilebilmektedir (Bernhardt ve ark. 2010, Strambeanu ve ark. 2015).

NP'ler çok farklı çeşitte ve boyutta olabilmeleri sayesinde, ilaçların farmakolojik ve terapötik etkilerini geliştirme, moleküler görüntüleme, ilaç dağıtımı ve ayrıca NP'lerin yüzeylerine bağlanabilecek fonksiyonel gruplar sayesinde de tümörlerle mücadele için yeni yöntemler olmak üzere çeşitli alanlarda sıklıkla kullanılmaktadırlar (Krishna ve ark. 2017). NP'lerin üretimi ve tüketici kullanımı sonrasında çevre, ekosistem ve sular bu materyaller ile kontamine olmaktadır (Barker ve ark. 2006). Bu çevresel kontaminasyonun yanı sıra insanlar, gıda ürünlerinde nanoteknoloji kullanımının yaygınlaşması sonucunda da bu materyallere maruz kalabilmektedir (Theodore ve Kunz 2005).

Ayrıca NP'lerin endüstriyel teknolojideki kullanımı artmasına rağmen bu partiküllerin insan sağlığındaki olası zararlı etkileri tartışılmaktadır (Colvin 2003, Gogotsi 2003, Nel ve ark. 2006). İnsanların özellikle metal bazlı NP'lere olan maruziyeti, NP'lerin doğal olarak ortaya çıktıkları su, hava ve NP'lerle kontamine olmuş gıda ürünlerinde bir kirletici madde olarak bulunmaları veya antropojenik faktörlerdeki giderek artan aktivite nedeniyle önemli ölçüde artmaktadır (Mahmoud ve ark. 2016).

Özellikle son yıllarda yapılan pek çok çalışma, dışarıdan yiyecekler yoluyla veya ek olarak koruma amacıyla alınan pek çok koruyucu molekülün savunma sisteminin yetersiz kaldığı durumlarda organizmayı çeşitli kimyasalların ve serbest radikallerin zararlı etkilerine karşı koruduğunu göstermiştir (Gökpınar ve ark. 2006, Demir ve ark. 2008a, b, 2009, 2010, 2013a, 2013b). Bundan dolayı bu çalışma kapsamında laboratuar koşullarında Co NP'lerinin (10mM) indüklediği genotoksik hasara karşı resveratrolün (RSV) anti-genotoksik etkileri *in vivo Drosophila* SMART ve KOMET yöntemleri kullanılarak araştırılmıştır. Son yıllarda NP'lerin endüstriyel teknolojideki kullanımının artmasına rağmen önemli hedeflerden olan genetik materyal ve çevreye olası zararlı etkileri tartışılmaktadır (Lux Report 2008). Bu bağlamda yapmış olduğumuz çalışma literatüre katkıda bulunmaktadır.

## 2. Materyal ve Metot

### *2.1. Kimyasallar*

Co NP'leri (CAS No: 7440–48-4) ve RSV (CAS No: 501-36-0), Sigma Aldrich (USA) firmasından temin edilmiştir.

### *2.2. Nanopartikül Karakterizasyonu*

Co NP'leri, Tecnai G2 F30 model cihaz ile Transmisyon Elektron Mikroskobu (TEM) ile görüntülenmiştir. Ayrıca partikül büyüklük dağılımı ve Zeta potansiyel ölçümleri Malvem Zetasizer Nano-ZS model cihaz ile yapılmıştır.

### *2.3. Drosophila Kanat Somatik Mutasyon ve Rekombinasyon Testi (SMART)*

*Drosophila* SMART yöntemi, *Drosophila* kanat imajinal disk hücrelerinde oluşan genetik değişimler sonucu heterozigotluğun kaybolması ve farklılığın fenotipte gözlenmesi esasına dayandığı ve nokta mutasyon, delesyon, kromozomlarda ayrılmama ve rekombinasyon gibi birçok genetik sonucun belirlenebildiği bir test sistemidir (Graf ve ark. 1984). Bu çalışmada, normal metabolik aktiviteye sahip *mwh / mwh ve flr³ / TM3, Bd*$^{s}$ bireylerin çaprazlanması ile elde edilen transheterozigot larvalar kullanılmıştır. Yapılan çaprazlamalar aşağıdaki gibidir:

$$♀ flr^{3} \;/\; TM3, Bd^{S} \;\; X \;\; ♂ mwh \;/\; mwh \qquad (1)$$

Kuru halde bulunan *Drosophila* hazır besininin (*Drosophila* Instant Medium) yaklaşık 4.5 gramı hazırlanan test edilecek madde derişimlerinin 9 mL'si ile ıslatılarak farklı uygulamalar gerçekleştirilmiştir. Bütün çalışmalar sabit sıcaklık ayarlı inkübatörde (25±1 $^{o}$C) yapılmıştır. Uygulamalar sonunda elde edilen bireylerin kanat preparatları Graf ve ark. (1984)'nin metoduna göre stereo mikroskop altında hazırlanmıştır. Hazırlanan preparatlar ışık mikroskobunda 40X büyütmede incelenmiş ve elde edilen veriler Kaya ve ark. (2000)'nın metoduna göre sınıflandırılarak bu test için kullanılan Microsta istatistik paket programı ile değerlendirilmiştir (Frei ve Würgler 1988, Kaya ve ark. 2000).

### *2.4. Alkali Tek Hücre Jel Elektroforezi Testi (KOMET)*

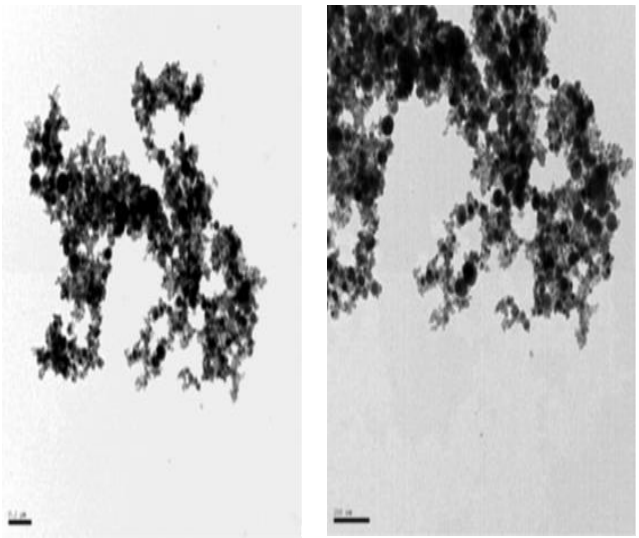
Bu çalışma kapsamında incelenecek olan Co NP, RSV ve birlikte uygulamalarının *Drosophila* hemositlerinde muhtemel DNA hasarı etkisi KOMET testi ile belirlenmiştir. *Drosophila* hemosit izolasyonu Irving ve ark. (2005) metoduna göre gerçekleştirilmiştir. Hücreler fosfat tamponu (PBS) ile süspanse edilerek düşük erime ısısına sahip agaroz (LMA) ile hızlı bir şekilde karıştırılmış ve normal erime ısısına sahip agaroz (NMA) ile kaplanmış lamlar üzerine yayılmıştır. Lamlar soğuk plakada bekletilmiş ve sonrasında içerisinde lizing solüsyonu (2.5 M NaCl, 100 mM $Na_2EDTA$, 10 mM Tris, %1 Triton X-100 ve %1 N-lauroylsarcosine sodium salt solution, pH = 10) bulunan etrafı ışık almayan şalelere yerleştirilmiştir. Lizing işlemi sonrasında preparatlar elektroforez tankında bulunan solüsyon (1 mM $Na_2EDTA$ ve 300 mM NaOH, pH = 13) içerisine konularak burada 30 dakika beklemesi

sağlanmıştır. Daha sonra, 25 V, 300 mA'de 30 dakika elektroforez yürütme işlemi gerçekleştirilmiştir. Elektroforezden sonra lamlar, içerisinde nötralizasyon solüsyonu (400 mM Tris buffer, pH = 7.5) bulunan şale içerisine alınmıştır. Her bir doz için *Drosophila* hemositlerinde 50 hücre 40X büyütmede Floresan (Nikon Eclipse E200) mikroskopta sayılarak deney tamamlanmıştır. Tüm ölçümler Comet-IV (Version 4.11) programı ile otomatik ölçüm şeklinde yapılmıştır.

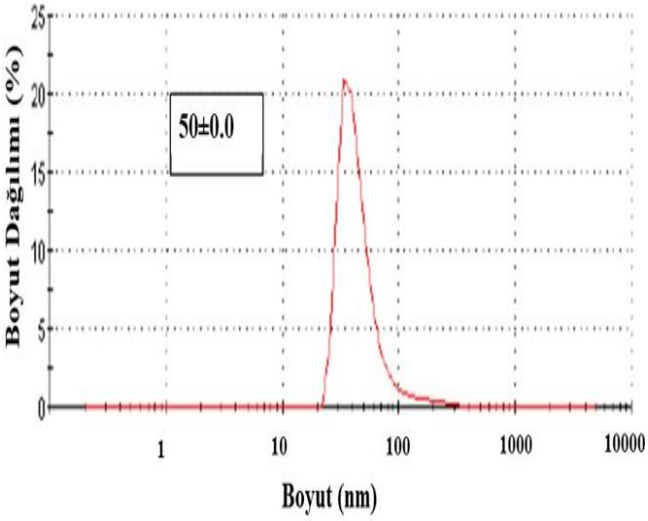
## 3. Sonuçlar

### *3.1. Nanopartikül Karakterizasyonu*

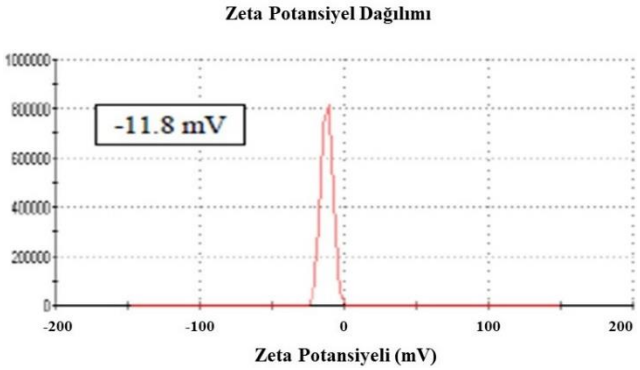
Co NP'lerinin ortalama boyutu 50 nm olarak belirlenmiştir. Ayrıca zeta potansiyel değeri -11.8 mV olarak bulunmuştur. Co NP'lerinin özellikleri Şekil 1, 2 ve 3'te özetlenmiştir.



**Şekil. 1** Co NP'lerinin TEM görüntüleri




**Şekil. 2** Co NP'lerinin Partikül Büyüklük Dağılımı




**Şekil. 3** Co NP'lerinin Zeta Potansiyeli

### *3.2. Drosophila melanogaster Kanat Somatik Mutasyon ve Rekombinasyon Testi (SMART)*

72±4 saatlik distile su uygulamasında, 80 kanat sayılan preparatta 13 adet küçük tek tip klon, 2 adet büyük tek tip klon bulunurken ikiz klon saptanmamıştır. Toplamda 15 adet klon sayılmıştır. Toplam *mwh* klon sayısı 15 adet bulunmuştur. Distile su uygulamasında klon indüksiyon frekansı ise 0.77 olarak hesaplanmıştır. Pozitif kontrol grubu olarak kullanılan EMS, negatif kontrol grubu olan distile su ile karşılaştırıldığında tüm klon tiplerinde pozitif sonuç elde edilmiştir. RSV'ün tüm dozları distile su ile karşılaştırıldığında istatistiksel anlamda bir fark bulunmamıştır. Co NP'leri (10mM) ise çözücüleri olan etanol (%1) ile karşılaştırıldığında istatistiksel olarak anlamlı pozitif sonuç ($p<0.05$) verdiği görülmüştür. Ayrıca Co NP'leri ile RSV'ün eş zamanlı ve ön uygulama sonuçları kontrol grupları ile karşılaştırıldığında istatistiksel olarak genotoksisitenin indirgenmesi açısından anlamlı ($p<0.05$) bir azalmanın olduğu gözlenmiştir. Özellikle RSV ön uygulamasının eş zamanlı uygulamaya göre Co NP toksisitesini daha fazla indirgediği tespit edilmiştir. SMART yöntemi sonuçları Tablo 1 ve Tablo 2'de gösterilmiştir.

### *3.3. Alkali Tek Hücre Jel Elektroforezi (KOMET)*

Elde edilen sonuçlara göre pozitif kontrol grubu olan konsantrasyonu 5 mM olan EMS'nin tüm parametreler (kuyruk yoğunluğu ve kuyruk momenti) açısından kontrol grubu olan distile suya göre istatistiksel olarak anlamlı ($p<0.001$) tek iplik DNA hasarı gözlenmiştir. Bunun yanında RSV'ün tüm konsantrasyonlarının (0.1, 0.5 ve 2.5 mM) bütün parametreler açısından kontrol grubu olan distile suya oranla istatistiksel olarak anlamlı tek iplik DNA hasarı gözlenmemiştir. Co NP'lerinin (10 mM) çözücüsü olan etil alkole göre istatistiksel olarak anlamlı ($p<0.001$) tek iplik DNA hasarına yol açtığı gözlemlenmiştir. Ayrıca Co NP'leri ve RSV'ün birlikte uygulamalarının tümü Co NP'leri çözücüsü olan etil alkol ve RSV çözücüsü olan distile suya göre kıyaslandığında tüm parametreler açısından istatistiksel olarak tek iplik DNA hasarına yol açmadığı tespit edilmiştir. KOMET yöntemi sonuçları Şekil 4 ve Şekil 5'de gösterilmiştir.

**Tablo 1.** *D. melanogaster*'in Normal Kanatlı Bireylerinde (*mwh/flr*$^3$) Co NP Genotoksisitesine Karşı Resveratrol'ün Ön Uygulamasının *in vivo* Antigenotoksik Etkileri

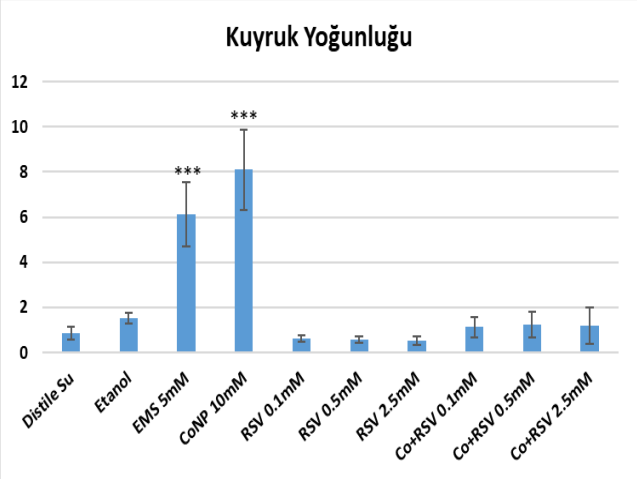
| Derişimler (mM) | Kanat Sayısı | Küçük tek tip klonlar (1-2 hücre) (*m*=2) | | | Büyük tek tip klonlar (> 2 hücre) | | | İkiz klonlar (*m*=5) | | | Toplam mwh klonlar (*m*=2) | | | Toplam klonlar (*m*=2) | | | Klon İndüsiyon Frekansı ($10^5$ hücre) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | No. | Fr. | D. | No. | Fr. | D. | No. | Fr. | D. | No. | Fr. | D. | No. | Fr. | D. | |
| Normal Kanat | | | | | | | | | | | | | | | | | |
| Distile Su (48h) | 80 | 12 | 0.15 | | 2 | 0.02 | | 0 | 0 | | 14 | 0.18 | | 14 | 0.18 | | 0.72 |
| Etanol %1 (48h) | 80 | 5 | 0.06 | - | 3 | 0.04 | i | 0 | 0 | i | 8 | 0.10 | - | 8 | 0.10 | - | 0.41 |
| EMS (1mM) | 32 | 71 | 2.29 | + | 27 | 0.87 | + | 9 | 0.29 | + | 105 | 3.39 | + | 108 | 3.48 | + | 13.45 |
| 48±4 h | | | | | | | | | | | | | | | | | |
| Resveratrol 0.1mM | 80 | 17 | 0.21 | i | 3 | 0.04 | i | 0 | 0 | i | 20 | 0.25 | i | 20 | 0.25 | i | 1.02 |
| Resveratrol 0.5mM | 80 | 19 | 0.24 | i | 5 | 0.06 | i | 0 | 0 | i | 24 | 0.30 | i | 24 | 0.30 | i | 1.23 |
| Resveratrol 2.5mM | 80 | 18 | 0.22 | i | 3 | 0.04 | i | 1 | 0.01 | i | 22 | 0.28 | i | 22 | 0.28 | i | 1.13 |
| CoNP (50nm) 10mM | 80 | 36 | 0.45 | + | 5 | 0.06 | i | 0 | 0 | i | 40 | 0.5 | + | 41 | 0.51 | + | 2.05 |
| 72±4 h 10mM CoNP (50nm) + 48±4 h Resveratrol (mM) | | | | | | | | | | | | | | | | | |
| 0.1 | 80 | 28 | 0.35 | - | 3 | 0.04 | - | 1 | 0.01 | i | 32 | 0.4 | - | 32 | 0.40 | - | 1.64 |
| 0.5 | 80 | 24 | 0.30 | - | 2 | 0.02 | - | 1 | 0.01 | i | 27 | 0.34 | - | 27 | 0.34 | - | 1.38 |
| 2.5 | 80 | 19 | 0.24 | - | 6 | 0.08 | - | 2 | 0.02 | i | 27 | 0.34 | - | 27 | 0.34 | - | 1.38 |

Fr., frekans; D., istatistik sonuçlarının gösterimi; +, pozitif; –, negatif; i, önemsiz fark; m= çarpım faktörü; olasılık düzeyi= 0.05.

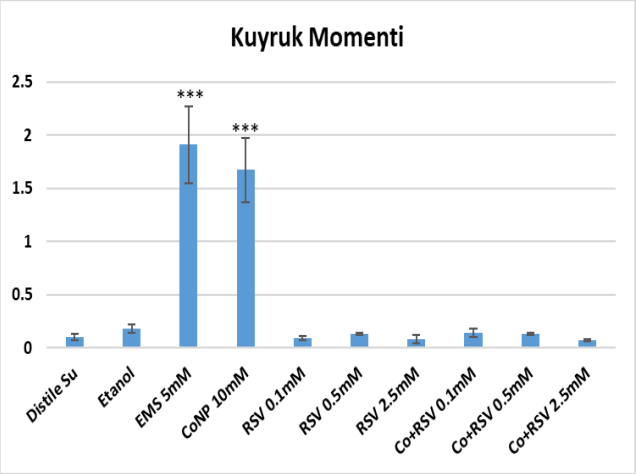
**Tablo 2.** *D. melanogaster*'in Normal Kanatlı Bireylerinde (*mwh/flr*$^3$) Co NP Genotoksisitesine Karşı Resveratrol'ün Eş Zamanlı Uygulamasının *in vivo* Antigenotoksik Etkileri

| Derişimler (mM) | Kanat Sayısı | Küçük tek tip klonlar (1-2 hücre) (*m*=2) | | | Büyük tek tip klonlar (> 2 hücre) | | | İkiz klonlar (*m*=5) | | | Toplam mwh klonlar (*m*=2) | | | Toplam klonlar (*m*=2) | | | Klon İndüsiyon Frekansı ($10^5$ hücre) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | No. | Fr. | D. | No. | Fr. | D. | No. | Fr. | D. | No. | Fr. | D. | No. | Fr. | D. | |
| Normal Kanat | | | | | | | | | | | | | | | | | |
| Distile Su (72h) | 80 | 13 | 0.16 | | 2 | 0.02 | | 0 | 0 | | 15 | 0.19 | | 15 | 0.19 | | 0.77 |
| Etanol %1 (72h) | 80 | 14 | 0.18 | i | 2 | 0.02 | i | 3 | 0.04 | i | 19 | 0.24 | i | 19 | 0.24 | i | 0.97 |
| EMS (1mM) | 32 | 71 | 2.29 | + | 27 | 0.87 | + | 9 | 0.29 | + | 105 | 3.39 | + | 108 | 3.48 | + | 13.45 |
| 72±4 h | | | | | | | | | | | | | | | | | |
| Resveratrol 0.1mM | 80 | 2 | 0.02 | - | 1 | 0.01 | i | 0 | 0 | i | 3 | 0.04 | - | 3 | 0.04 | - | 0.15 |
| Resveratrol 0.5mM | 80 | 8 | 0.10 | - | 2 | 0.02 | i | 0 | 0 | i | 10 | 0.12 | - | 10 | 0.12 | - | 0.51 |
| Resveratrol 2.5mM | 80 | 4 | 0.05 | - | 1 | 0.01 | i | 2 | 0.02 | i | 7 | 0.09 | - | 7 | 0.09 | - | 0.36 |
| CoNP (50nm) 10mM | 80 | 36 | 0.45 | + | 5 | 0.06 | i | 0 | 0 | i | 40 | 0.50 | + | 41 | 0.51 | + | 2.05 |
| 72±4 h 10mM CoNP (50nm) + 72±4 h Resveratrol (mM) | | | | | | | | | | | | | | | | | |
| CoNP 10mM+0.1mM RSV | 80 | 27 | 0.34 | - | 8 | 0.10 | - | 2 | 0.02 | i | 36 | 0.45 | - | 37 | 0.46 | - | 1.84 |
| CoNP 10mM+0.5mM RSV | 80 | 26 | 0.32 | - | 6 | 0.08 | - | 0 | 0 | i | 32 | 0.40 | - | 32 | 0.40 | - | 1.64 |
| CoNP 10mM+2.5mM RSV | 80 | 28 | 0.35 | - | 2 | 0.02 | - | 1 | 0.01 | i | 31 | 0.39 | - | 31 | 0.39 | - | 1.59 |

Fr., frekans; D., istatistik sonuçlarının gösterimi; +, pozitif; –, negatif; i, önemsiz fark; m= çarpım faktörü; olasılık düzeyi= 0.05.




**Şekil. 4** KOMET Testinde *Drosophila* Larvalarına Co NP, RSV ve Co NP ile RSV'ün Birlikte Uygulanmasından Sonra *Drosophila* Hemositlerinde Meydana Gelen DNA Hasarı (Kuyruk Yoğunluğu %)[[a]:Üç bağımsız deneyden elde edilen ±standart hata (her deney setinde 50 hücre sayılmıştır)]




**Şekil. 5** KOMET Testinde *Drosophila* Larvalarına Co NP, RSV ve Co NP ile RSV'ün Birlikte Uygulanmasından Sonra *Drosophila* Hemositlerinde Meydana Gelen DNA Hasarı (Kuyruk Momenti μm[a])[[a]:Üç bağımsız deneyden elde edilen ±standart hata (her deney setinde 50 hücre sayılmıştır)]

## 4.Tartışma

Son yıllarda geliştirilen ve çok farklı alanlarda kullanılan nanopartiküllerin genotoksisitesi hakkındaki veriler; ürünlerin çok yeni olması, her nanoboyutta farklı etkilere sahip olmasından dolayı, hem yetersiz hem de farklı test sistemlerinde çelişkili sonuçlar yaratmaktadır. Nanopartiküllerin fizikokimyasal özellikleri teknoloji ve endüstriyel uygulamalar için oldukça elverişlidir, biyolojik uygulamalardaki kullanımı ise canlı hücrelerin biyouyumluluğuna bağlıdır (Lappas 2015). Ancak bu fizikokimyasal özellikleri sağlık risklerini de beraberinde taşır ki bunlardan birisi de toksik etki göstermeleridir. Nanopartiküller özellikle küçük boyutlarda olmaları nedeniyle kolayca hücre zarını geçerek nükleusa ulaşabilmekte ve nükleer porlardan da girerek DNA ile etkileşime geçip DNA hasarına sebep olabilmektedir. Genotoksisitede nanopartiküllerin indüklediği toksik ve genotoksik mekanizmalar sadece DNA ile etkileşime geçerek değil mitozda görev alan proteinleri etkileyerek, reaktif oksijen türleri (ROS) üreterek ve hücrelerdeki antioksidan bariyerinin (süperoksit dismutaz (SOD) ve glutatyon (GSH)) kırılmasına neden olarak da DNA hasarına yol açabilirler (Carmona ve ark. 2015a, 2015b).

RSV, üzüm çekirdeğinde bol miktarda bulunan ve son yıllarda farklı mekanizmalardaki yardımcı etkileri üzerinde yoğun çalışılan doğal bir antioksidandır. RSV biyotik ve abiyotik stres koşullarına karşı, üzümlerde sentezlenen stilben grubu bir fitoaleksindir (Zhang ve ark. 2013). RSV güçlü antioksidan ve antiinflamatuar özelliklere sahiptir ve koruyucu etkisi hem *in vitro* hem de *in vivo* deneylerle kanıtlanmıştır (Lee ve ark. 2012). RSV antioksidan etkisini; koenzim Q ile yarışıp oksidatif zincir kompleksini azaltarak, mitokondride oluşan süperoksit radikalini yakalayarak ve fenton reaksiyonu ürünleri tarafından indüklenen lipid peroksidasyonunun inhibisyonuna neden olarak göstermektedir (Sayın ve ark. 2008). Bunun yanında antiaging, antidiyabetik, antiplatelet özelliklere sahiptir ve lipoproteinlerin oksidasyonunun inhibisyonunu sağladığı yapılan çalışmalarla gösterilmiştir (Mallebrera ve ark. 2015). Antiplatelet özelliği sayesinde RSV'ün, nitrik oksit sentaz üretimini etkileyerek (Das ve Maulik 2006), indüklenen oksidatif strese karşı hücre içi indirgenmiş GSH düzeyini arttırarak ve miyokardiyumdaki katalaz etkinliğini arttırarak kalpte koruyucu bir etki gösterdiği saptanmıştır (Sayın ve ark. 2008). Son zamanlarda yapılan çalışmalar RSV'ün çeşitli hücre tiplerinde intrasellüler serbest radikalleri ve diğer oksidanları etkili olarak temizlediğini göstermiştir (Zhang ve ark. 2013). Görüldüğü üzere son zamanlarda pek çok araştırmacı tarafından RSV'ün koruyucu etkileri hakkında çeşitli çalışmalar yapılmıştır. Fakat gerçekleştirilen literatür incelemesi sonucunda Co NP'lerinin genotoksisitesine karşı, RSV'ün olası koruyucu etkilerinin *Drosophila* KOMET ve SMART yöntemleri ile araştırılmadığı tespit edilmiştir. Gerçekleştirmiş olduğumuz çalışma kapsamında SMART yöntemi ile değerlendirilen ve somatik hücreler olan kanat imajinal disk hücrelerinde genotoksik etkiye sahip olduğu bilinen Co NP'leri, RSV ile eş zamanlı ve ön uygulama şeklinde çalışılmıştır. Co NP'lerinin genotoksik etkisine karşı RSV'ün uygulanan tüm dozlarda (0.1, 0.5 ve 2.5 mM) antigenotoksik etkiye sahip olduğu gözlenmiştir. Özellikle RSV'ün Co NP'e karşı ön uygulamasının birlikte uygulamaya göre toksisiteyi daha fazla indirgediği belirlenmiştir. Bu durum da RSV'ün güçlü bir koruyucu olarak genotoksisiteyi indirgeyebileceğine işaret etmektedir. KOMET yöntemi ile değerlendirilen hemositlerde Co NP'lerinin etkisiyle meydana gelen DNA hasarına karşı iki parametre açısından da (kuyruk yoğunluğu, kuyruk moment) RSV'ün tüm derişimlerinin koruyucu etki gösterdiği belirlenmiştir. Bu çalışmadan RSV'ün koruyucu etkisine dair elde edilen veriler önceki çalışmaların sonuçları ile de parelelik göstermektedir ve RSV'ün genotoksisiteye karşı güçlü bir koruyucu olabileceği fikrini güçlendirmektedir.

## 5.Sonuç

Çalışmamız sonucunda elde ettiğimiz *Drosophila* KOMET ve SMART verileri literatürü destekler şekilde Co NP'lerinin sahip olduğu yüksek genotoksisiteyi ve bu yüksek genotoksisiteyi RSV'ün güçlü bir koruyucu olarak düşürebildiğine işaret etmektedir. Ancak bu çalışmada indüklenen genetik hasara karşı RSV'ün gösterdiği koruyucu etkinin altında yatan mekanizmanın aydınlatılması için farklı mekanizmalarla çalışan test sistemleri ve farklı model organizmalarla yeni çalışmaların yapılması gerekmektedir.

## Teşekkür

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# Production of biological hydrogen and bacterial carotenoids from sugar beet molasses with *Rhodobacter sphaeroides* in a biorefinery concept

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**Abstract:** In this study, the goal was to produce biohydrogen and bacterial carotenoids with *Rhodobacter sphaeroides* O.U.001, a purple non-sulfur photosynthetic bacterium, utilizing sugar beet molasses in the context of biorefinery. First, media with different sugar concentrations (10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L) were prepared for bacterial growth. Then, hydrogen production was carried out using these media in anaerobic conditions in 100 ml bioreactors. After hydrogen gas was collected from the bioreactors, carotenoid extraction was performed from the remaining bacteria. As a result of the analyzes, it was found that the amount of biohydrogen and the amount of bacterial carotenoids obtained were inversely proportional to the increased sugar concentrations. The maximum hydrogen formation was detected in the medium containing 10 g/L of sugar (19.18 mL). According to the results of gas chromatography analysis, the quantity of hydrogen in the total gas was found to be around 23.6%. The highest yield of carotenoids was again obtained from bacteria reproduced in a medium containing 10 g/L of sugar (3.12 mg/g, carotenoid/dry biomass). As a conclusion, this study provides an example for the successful realization of two high value-added products within a biorefinery approach by using molasses obtained at an affordable cost.

**Keywords:** Carotenoid, Hydrogen, Molasses, *Rhodobacter sphaeroides*

## *Biyorafineri konseptiyle şeker pancarı pekmezinden Rhodobacter sphaeroides ile biyolojik hidrojen ve bakteriyel karotenoid üretimi*

**Özet:** Bu çalışmada biyorafineri konsepti ile şeker pancarı melasından mor kükürtsüz fotosentetik bir bakteri olan *Rhodobacter sphaeroides* O.U.001 ile biyohidrojen ve bakteriyel karotenoidin üretilmesi amaçlanmıştır. İlk önce, bakteri büyütmek için farklı şeker konsantrasyonlarında (10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L) besiyerleri hazırlanmıştır. Daha sonra, hidrojen üretimi bu besiyerlerini kullanarak 100 mL'lik biyoreaktörlerde anaerobik koşullarda çalışılmıştır. Biyoreaktörden hidrojen gazı toplandıktan sonra kalan bakterilerden karotenoid ekstraksiyonu gerçekleştirilmiştir. Analizler sonucunda, artan şeker konsantrasyonu ile elde edilen biyohidrojen miktarı ve bakteriyel karotenoid miktarının ters orantılı olduğu bulunmuştur. En yüksek hidrojen üretimine 10 g/L şeker içeren besiyerinde görülmüştür (19,18 mL). Gaz kromatografisi analiz sonuçlarına göre toplam gaz içerisinde hidrojen miktarı %23,6 civarında tespit edilmiştir. En yüksek karotenoid verimi yine 10 g/L şeker içeren besiyerinde çoğaltılan bakterilerden elde edilmiştir (3,12 mg/g, karotenoid/kuru biyokütle). Sonuç olarak, bu çalışma uygun maliyetle elde edilen melasın kullanılmasıyla yüksek katma değerli iki ürünün biyorafineri yaklaşımı ile başarıyla gerçekleştirilmesi adına bir örnek sunmaktadır.

**Anahtar Kelimeler:** Karotenoid, Hidrojen, Melas, *Rhodobacter sphaeroides*



## 1. Introduction

The vast majority of the world's energy needs are provided from non-renewable energy sources such as fossil-based energy sources. But the use of these energy sources seriously harms nature. The global population growth also increases the need for energy. For this reason, it will become very important from a strategic point of view for countries to develop their own alternative energy sources. Examples to alternative renewable energy sources are solar energy, wind energy, wave energy, hydrogen energy, hydro power and geothermal energy. Hydrogen is a renewable and clean energy source among the energy sources of the future because it is seen as one of the alternative energy sources of

the future (Sagir et al. 2018). Hydrogen can be used as a fuel, and the combustion reaction of hydrogen is called clean combustion. In the clean combustion reaction, hydrogen reacts with oxygen and water is released and it does not harm the nature in any way. Furthermore, even though hydrogen is the lightest element in the universe, it was said to have 2.75 times more energy content (123 kJ/g) when compared to the hydrocarbon-based sources (Dursun and Gülşen 2019). Hence, it is recognized as the energy carrier of the future.

Today, hydrogen is usually produced by thermochemical means. However, biological hydrogen production is also possible through dark fermentation and photobiologically with the help of various microorganisms using various waste streams like sugar beet molasses (Kars and Alparslan 2020) and waste black cumin (Dursun and Gülşen, 2021). And, since biological hydrogen production takes place at lower temperatures and pressures, it requires less energy input than thermochemical methods and production is more advantageous in terms of side products. Among others, photobiological hydrogen production can be carried out by microorganisms in a photoautotrophic or photoheterotrophic way. Various purple non-sulfur bacteria were used in photoheterotrophic hydrogen production (Özsoy Demiriz et al. 2019; Kars and Gündüz, 2010). *Rhodobacter sphaeroides* (*R. sphaeroides*), a purple non-sulfur (PNS) photosynthetic bacteria, can produce hydrogen in photosynthetic conditions (under light and under anaerobic conditions) using various carbon sources as substrate (Kars and Gündüz 2010). The enzyme responsible for the production of hydrogen is nitrogenase, and this enzyme catalyzes the production of hydrogen using ATP during the conversion of nitrogen to ammonia. So, biological hydrogen production occurs as a result of an enzyme driven reaction and it is affected from physiological conditions such as vitamins and minerals (Kars and Alparslan 2020). It is also possible to produce high value-added products such as bacterial carotenoids, coenzyme Q10, vitamin B12, 5-ALA by *R. sphaeroides*. Although hydrogen production can be done both thermochemically and biologically, the amount of hydrogen obtained from these methods has not yet reached a commercial scale. For this reason, in recent years biorefinery approach has been applied to increase the efficiency of hydrogen production processes. In this approach, in addition to an energy source, the production of some marketable products (carotenoids, and valuable chemicals such as polymer) were also aimed and this route was defined as the process of biorefining (Cherubini and Jungmeier 2010).

Carotenoids are fat-soluble, antioxidant pigments found in photosynthetic organisms (such as bacteria, plants and algae (Liu et al. 2015). The task of carotenoids is to absorb light for use in photosynthesis and also to protect chlorophylls from the harmful effect of light. Studies have shown that carotenoids prevent various types of cancer, cardiovascular diseases, and aging-related ailments with their antioxidant activities and increase antibody production (Young and Lowe 2001; Kiokias and Oreopoulou 2006; Liu et al. 2015). Additionally, previous studies have reported that a carotenoid called capsanthin obtained from pepper can be used to prevent multidrug resistance in cancer cells (Motohashi et al. 2003). Therefore, these pigments are very promising to be used for medical purposes.

It is very important that the substrate to be used in the biorefinery concept should be sustainable and renewable. For this reason, sustainable, affordable and easily obtainable sugar beet molasses was used as a substrate in this study. Then, several sets of media with varying sugar beet molasses concentrations were formulated to disclose the optimal molasses concentrations for concurrent production of biohydrogen and bacterial carotenoid with *R. sphaeroides* in a cost-effective manner within the context of the biorefinery concept. Sugar beet molasses contains about 50% sucrose and therefore can be a rich source of nutrients for bacteria. Thus, optimal conditions for the production of hydrogen and carotenoids will be determined and a significant contribution will be made to the development of a cost-effective bioprocess within a frame of biorefinery approach.

## 2. Materials and Method

### *2.1. General cultivation circumstances*

In this study, *R. sphaeroides* was used in the production of biological hydrogen and bacterial carotenoids. Bacteria can use various carbon sources as substrates. In the study, sugar beet molasses (Konya Şeker, Turkey) was used as a carbon source and L-glutamate (15 mM/2 mM) was used as a nitrogen source. The sugar concentration in the medium was increased gradually (10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L) from 10g/l to 50g/L. In addition, trace element, iron sulfate and vitamin solutions were put into the cultures as documented earlier (Kars and Ceylan 2019). According to the previous findings, the amount of Na was found to be very high after elemental investigation of molasses by ICP-MS (Kars and Alparslan 2013). For this reason, extra NaCl was not added to the medium. $KH_2PO4$ (0.5 g/L), $MgSO_4.7H_2O$ (0.2 g/L) and $CaCl_2.2H_2O$ (0.025 g/L) were added to the medium. 50 µL of iron solution (13.2 mM $FeSO_4$), 1 mL of molybdenum containing trace element solution (0.83 mM $Na_2MoO_4.2H_2O$) and 2.5 µL of vitamin solution (Niacin 500 mg/L, Biotin 15 mg/L and Thiamine 500 mg/L) were added to the prepared media. The initial pH of the media was fixed to 6.9.

After adding the components, the media were sterilized by autoclaving at 121°C for 15 minutes. Since the structure of the vitamin will deteriorate due to temperature, the vitamin solution was added to the media after autoclaving. The gas phases of the bioreactors were passed through argon gas for 3 minutes in order to form anaerobic environs. 100-watt tungsten filament incandescent bulbs were used as the light source for the photosynthetic condition. The cultures were incubated at approximately 29 °C for growth.

### *2.2. Biomass and pretreatment*

Molasses is a by-product that occurs as a result of the refining of sugar beet. Sugar beet molasses possesses a

sucrose (w/w) content of about 50% (Sagir et al. 2018). In addition to sucrose, molasses is rich in many types of carbons like organic acids (Kars and Alparslan 2013). This feature makes sugar beet molasses a prosperous nutrient for many bacterial cells. Sugar beet molasses obtained from Konya Sugar Factory was utilized as a substrate for both biohydrogen and bacterial carotenoid production. However, molasses was not used directly due to its dark color and high sugar content. Before preparing molasses and different sugar concentrations media, they were centrifuged for 20 minutes at 10.000 rpm due to the particles in their contents. After centrifugation, the solutions were filtered via 0.22 µm filters and diluted with water and media were prepared in 100 ml bioreactors.

### *2.3. Hydrogen production with R. sphaeroides using molasses*

Initially, hydrogen productions were carried out in the study. Hydrogen production setup was established after the cultures (5 different media with different sugar contents) were incubated for about 3 days. Hydrogen production setup was based on the principle of collecting the hydrogen gas produced by *R. sphaeroides* in bioreactors in glass tubes with 0.5 cm markings. Changes in the water levels in tubes were observed at 24-hour intervals. After collecting hydrogen gas in tubes, gas chromatography (GC) analysis was performed. An Rt-Msieve 5A column and a thermal conductivity detector were used to examine the constituents of the accumulated gas using GC (Shimadzu GC-2010 Plus, Japan). The detector and column were heated to 250 and 50 degrees Celsius, respectively. Argon gas ran inside the column with a flow rate of 4 mL per minute to carry the gas samples.

### *2.4. Carotenoid production from R. sphaeroides*

After the completion of hydrogen production, bacterial cultures were centrifuged for 20 minutes at 10 000 rpm to obtain the cells. Then, the upper phase was discarded and the precipitate was dissolved in 2 mL of distilled water to wash each cell pellet. Cell suspension was re-centrifuged at 10 000 rpm for 20 minutes and the washing process was repeated. After final centrifugation, the remaining liquid was thrown away and the entire precipitate was transferred to a beaker and dried in an oven at 65°C for one night. Bacterial cells were lysed by adding 4 mL (3 M) HCl onto the dry bacterial mass obtained as a result of drying. The cell lysate was mixed at 28°C, 100 rpm for 30 minutes. The solution was spinned at 10 000 rpm for 20 minutes. Extraction of the carotenoid was done by adding 5 mL of acetone onto the precipitate and vortexing briefly. To this extract, another 35 mL of acetone was added to reach up to 40 mL of acetone. At 30°C, 40 minutes of extraction of the carotenoids at 150 rpm was performed, and then whole extract was spinned at 10 000 rpm for 20 minutes. The carotenoids were dissolved in the supernatant which was then was transferred to a clean 50 ml tube. The optical density of the carotenoid solution was measured at 480 nm and the amount of carotenoid was calculated using the Equation 1 (Kars et al. 2020; Gu et al. 2008).

$$Carotenoid\ yield\left(\frac{\mu g\ carotenoid}{g\ dry\ cell\ weight}\right) = \frac{A \times D \times V}{0{,}16 \times W} \qquad \text{(Equation 1)}$$

A: Absorbance (480 nm)

D: Dilution coefficient

V: Volume of solvent (acetone)

W: Mass of dry bacteria (g)

## 3. Results

### *3.1. Hydrogen production with R. sphaeroides using molasses*

Biological hydrogen production was tested at five different sugar concentrations. The tubes where hydrogen gas was collected were observed at 24-hour intervals and graphs were created using these data. During the study, the experiments were repeated two times and error bars were added to the graph. The results of the experiments were figured out in a single graph. The gas chromatography (GC) analysis was carried out to reveal the total amount of pure hydrogen collected in these tubes. According to the results of GC analysis, the amount of hydrogen in the total gas was assigned to be circa 23.6%. Taking into account the results of hydrogen production (Figure 1), it was found that hydrogen production decreases as the concentration of sugar (molasses amount) increases. The highest hydrogen production occurred in the medium containing 10 g/L sugar (19.18 mL gas accumulation). It was noticed that increasing sugar concentration did not lead to an increase in hydrogen production and hydrogen production was the highest at the lowest sugar concentration. In order to raise the sugar concentration in the medium, the amount of molasses was also augmented. And, it was realized that increasing the amount of molasses darkened the color of the medium and increased its density. This, in turn, can reduce the penetration of light through the medium and prevent bacterial cells from reaching the light they need. In the end, it was anticipated that this adversely affected the amount of hydrogen produced.

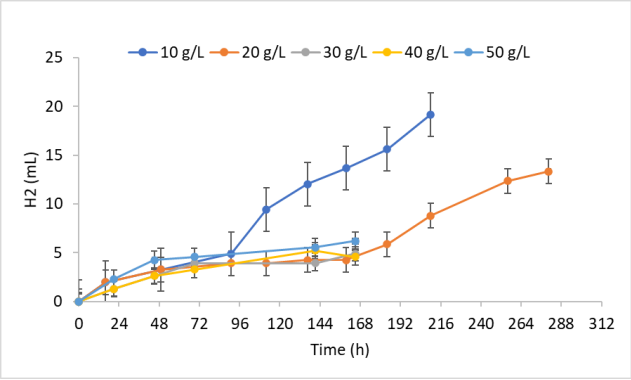


**Figure 1.** Hydrogen production at different molasses concentrations.

Molasses has long been used as substrate by our group and others and studies have been done for years to find the most efficient hydrogen production process. Examples of these

studies were gathered and shown in Table 1. It was observed that different hydrogen production yields were obtained using different bacterial strains in various hydrogen production processes. We basically thought that these variations arise from different physiological parameters (bacterial strains, media composition and processs type) implemented in these processes. The amount of hydrogen obtained from a concentration of 10 g/L (0.27 mol $H_2$/mol sucrose) was also included in the table. As can be observed from the table, the greatest amount of hydrogen production was achieved by successive dark and photo fermentation with the mutant progeny of *R. capsulatus* (13.7 mol $H_2$/mol sucrose) (Özgür et al. 2010). Among photofermentative hydrogen generation processes, the greatest amount was attained with *R. sphaeroides* O.U.001 (0.5 mol $H_2$/mol sucrose) (Kars and Alparslan 2013).

**Table 1.** Examples of biohydrogen production using molasses as substrate.

| Microorganism | Process | Yield (mol $H_2$/mol sucrose) | Reference |
|---|---|---|---|
| *Rhodobacter sphaeroides* O.U.001 | Photofermantative | 0.27 | This study |
| *Rhodobacter sphaeroides* O.U.001 | Photofermantative | 0.5 | (Kars and Alparslan 2013) |
| *Rhodobacter capsulatus* JP91 | Photofermantative | 10.5 | (Keskin and Hallenbeck 2012) |
| Mixed anaerobic culture | Dark fermentation | 2.8 | (Chang et al. 2011) |
| *Caldicellulosiruptor saccharolyticus* and *Rhodobacter capsulatus hup*-YO3 | Dark and Photofermantation | 13.7 | (Özgür et al. 2010) |

### *3.2. Carotenoid production from R. sphaeroides*

In this study, it was aimed to produce bacterial carotenoids besides hydrogen generation in a biorefinery frame. The source of carotenoids is *R. sphaeroides*. The lack of immunity and pathogenicity of *R. sphaeroides* makes it a valuable source of carotenoids. There are two types of carotenoids in the *R. sphaeroides*; spheroidene (SE) and spheroidenone (SO) (Kars et al. 2020). These carotenoids have the potential to be used as antioxidants by effectively neutralizing the surrounding singlet oxygen species. There are two possible ways to increase the current bacterial carotenoid production. These are either the application of recombinant DNA technology or the optimization of culture conditions. Genetic methods for *R. sphaeroides* are well established, but gene manipulations require significant effort and there are many targets to improve. Therefore, in this study, five distinct sugar concentrations (molasses amount) were tested to reveal the best substrate concentration for the highest carotenoid production. Based on previous experience and literature findings, it is known that sugar beet molasses is a rich medium that supports bacterial growth very well (Kars and Alparslan 2013).

In the current work, sugar beet molasses harvested in Konya was used as the substrate for the cultivation of bacteria for the production of carotenoids. In this way, locally produced molasses was utilized for the production of value-added products contributing to the circular economy. *R. sphaeroides* was cultured in media with various sugar concentrations (10 g/L, 20 g/L, 30 g/L, 40 g/L and 50 g/L). The bioprocess was run in intermittent mode using a 100 mL bioreactor. After hydrogen production was ceased, the remaining cells were collected and bacteria were dried to obtain cell dry weights (Table 2). Carotenoids were extracted from dry cells using acetone as a solvent. Optical densities were measured after 8 times dilution at 480 nm. The maximum carotenoid yield was calculated as 3.12 mg/g (carotenoid/dry biomass), taking into account the dilution factor and solvent volume (40 mL). Similar to the hydrogen generation results, it was noted that the amount of carotenoids obtained decreased as the sugar ratio (molasses amount) increased.

**Table 2.** The amounts of carotenoids obtained from cultures after hydrogen production

| Sugar (g/L) | OD (480 nm) | Dry weight (g) | Carotenoid yield (µg/g) |
|---|---|---|---|
| 10 | 5,0296 | 0,4029 | 3120,87 |
| 20 | 6,1904 | 0,5927 | 2611,10 |
| 30 | 2,4392 | 0,5702 | 1069,45 |
| 40 | 0,6584 | 0,4132 | 398,35 |
| 50 | 0,3184 | 0,369 | 215,72 |

## 4. Discussion

Sugar beet molasses used in the preparation of growth media is a waste product of the sugar factory and contains organic acids, phenolic substances and ammonium in addition to sucrose (Kars and Alparslan 2013). The organic and inorganic composition of sugar beet molasses is very diverse and rich; therefore, it supports the growth of bacteria very well. In the present study, as a common trend, as the amount of molasses in the media increased, the amounts of hydrogen and carotenoid decreased. There are many factors which might have led to this result. For instance, it is a well-known fact that phenolic substances and ammonium negatively affect hydrogen production (Kars and Alparslan 2013). This is basically due to the fact that ammonia reversibly inactivates the nitrogenase enzyme that is the enzyme functioning in hydrogen evolution. Moreover, phenolics substances also thought to poison the hydrogen production metabolism. Taking into account the previous findings, the results (decrease in hydrogen production as a result of elevated levels of molasses) obtained here is consistent with previous studies. Another common factor is the light whose sufficient amounts are needed for an efficient hydrogen generation (Uyar et al. 2007; Kars and Alparslan 2013). Because of the darkish color and density of molasses, the delivery of light through the medium and reaching the cells is blocked. As the amount of molasses

increases, the culture darkens and the light penetration efficiency decreases. This causes the cells to absorb less light and therefore less hydrogen production occurs.

Like hydrogen generation results, the biggest carotenoid yield was achieved at the lowest sugar concentration in the study. This shows that bacteria proliferate best at this concentration. So, considering both hydrogen and carotenoid production results, it seems that the most suitable sugar (sugar beet molasses) concentration for *R. sphaeroides* is 10 g/L. The amount of hydrogen production by using molasses might be less when compared to minimal media, but molasses may be preferred over synthetic media in biotechnological processes because it can be obtained at much cheaper cost compared to synthetic carbon sources. The processes carried out using molasses are described as sustainable and cost-effective. In the current work, considerable quantities of both hydrogen and carotenoids were obtained in a single process. Thus, an exemplary study was implemented for the purpose of developing a sustainable and cost-effective processes within a biorefinery concept.

Considering previous findings and present results, it was noticed that the physical features (viscosity, color etc.) of the sugar beet molasses used so far differ from each other. It is also suspected that the composition of molasses used in different times may also change. Therefore, the differences in physical and chemical properties of molasses may result in deviations in the results. Moreover, the use of inorganic iron sources like $FeSO_4$ instead of organic iron sources like ferric citrate probably led to variations in both hydrogen and carotenoid productions. These experiences demonstrated that design and composition of growth media strongly influence the hydrogen production and bacterial reproduction. So, it is of great importance to find out best medium formulations for better yields.

## 5. Conclusion

*R. sphaeroides* is a versatile microorganism able to grow in different growth modes and capable of producing many marketable products such as biopolymers, vitamins and carotenoids. It proliferates very well in media prepared by using sugar beet molasses which is an affordable, renewable and sustainable substrate. Molasses was shown to be a very efficient substrate in the production of biohydrogen as a fuel and carotenoid as a marketable product within a biorefinery concept. *R. sphaeroides'* being a non-immunogenic and non-pathogenic bacterium makes it a unique cell factory for the production of many marketable products in addition to biohydrogen as shown in the present work.

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## Authors' contributions

Planning, funding applications, experimental work and manuscript preparation were done by GK. KD contributed to experimental work and manuscript preparation. BNB contributed to the experimental work.

## Conflict of interest disclosure

There is no conflict of interest.

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# Asetil Salisilik Asit Solüsyonlarında Ön Çimlendirmenin Havuç Tohumlarının Tuz Stresi Altında Çimlenme ve Çıkışı Üzerine Etkileri

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**Özet:** Bu araştırmada asetil salisik asit (ASA) solüsyonun farklı dozlarında ön çimlendirmenin "Nantes" havuç çeşidi (*Daucus corata* L.) tohumlarının tuz stresi altında çimlenme ve çıkış performansları üzerine etkileri laboratuar koşullarında araştırılmıştır. Tohumlar, ASA solüsyonları içerisinde 24 saat süreyle iklim dolabında ön çimlendirme işlemine tabi tutulmuştur. Bu işlemden sonra ön çimlendirme ve kontrol grubu olarak iki gruba ayrılmıştır. Her iki grup 0, 50, 100 ve 150 mM NaCl stresine maruz bırakılarak, ASA uygulamasının tuzluk sterine tepkileri incelenmiştir. Araştırma sonucunda yüksek tuz konsantrasyonunun (150 mM NaCl) havuç tohumlarının çimlenmesini azalttığı, yüksek dozdaki ASA ön uygulamalarının tohumların çimlenme ve çıkış parametrelerini etkilediği belirlenmiştir. Sonuçlara göre ön uygulamalar arasında doz x tuz interaksiyonunda 150 mg/L ASA uygulmasında 0 ve 50 mM NaCl interaksiyonunda %93 ile en yüksek çimlenme oranı belirlenmiştir. ASA uygulamalarının, düşük ve yüksek tuzluluğa sahip yetiştirme ortamlarında tohumların çimlenme oranında artış sağladığı ve başarılı bir şekilde kullanılabileceği ön görülmüştür.

**Anahtar Kelimeler:** Asetil salisilik asit (ASA), Ön çimlendirme, Havuç tohumu, Tuz stresi

## *Effects of Pre-Germination in Acetyl Salicylic Acid Solutions on Germination and Emergence of Carrot Seeds Under Salt Stress*

**Abstract:** In this study, the effects of pre-germination at different doses of acetyl salicylic acid (ASA) solution on the germination and emergence performance of "Nantes" carrot cultivar (*Daucus corata* L.) seeds under salt stress were investigated in the laboratory conditions. Seeds were pre-germinated in a climate cabinet for 24 hours in 0, 50, 100 and 150 mg $L^{-1}$ ASA solutions in hydropiriming at 15ºC. After this process, they were divided into two groups as pre-germination and control groups. Both groups were exposed to the stress of 0, 50, 100 and 150 mM NaCl, and the response of ASA application to salt salt ster was investigated. As a result of the research, it was determined that high salt concentration (150 mM NaCl) decreased the germination of carrot seeds, and high dose ASA pre-applications affected the germination and emergence parameters of the seeds. According to the results, the highest germination rate was determined with 93% in the 0 and 50 mM NaCl interaction in the 150 mg/L ASA application in dose x salt interaction. It has been predicted that ASA applications increase the germination rate of seeds in low and high salinity growing environments and can be used successfully.

**Keywords:** Acetyl salicylic acid (ASA), Priming, Carrot seed, Salt stress

## 1. Giriş

Tohum bitkisel üretimde kullanılan en önemli üretim mataryeldir. Birçok sebze, tarla bitkileri, endüstri bitkileri ve süs bitkilerinin üretiminde yoğun olarak kullanılan tohumların heterojen çimlenme ve çıkışı, üretimde önemli sorunlara neden olabilmektedir. Özellikle olumsuz çevre koşulları, düşük ve yüksek toprak sıcaklıkları, toprak tuzluluğu ve toprak kaymak tabakası gibi faktörler tohumların çimlenmesi ve fide çıkışını olumsuz etkilemektedir. Ancak bu olumsuz çimlenme koşullarına karşı tohumlara birtakım ön uygulamalar (priming) yapılarak, tohumlarda çimlenme ve çıkış problemleri önlenebilmektedir (Bhanuprakash ve ark., 2016). Bu işlemlerde ise farklı kimyasallar, tuzlar veya büyüme düzenleyiciler kullanılmaktadır. Son yıllarda artan sıcaklıklar, tuzluluk ve kuraklık gibi çevresel etkenlerin bitkilerde vegetatif ve generatif gelişim dönemlerinde olumsuz etkiler gösterdiği bildirilmektedir (Blumwald 2003; Karni ve ark. 2010). Generatif aksamlardan olan tohum bir bitkinin oluşmasında ve çoğaltılmasında kullanılan en önemli üretim materyalidir. Tohum olumsuz çevre koşullarına maruz kalması durumunda çimlenme yeteneğini hızla yitirmektedir. Tohumculuk sektöründe üretim esnasında veya daha sonraki süreçlerde tohumların olumsuz koşullara maruz kalması durumunda önemli çimlenme sorunları ile karşılaşıldığı bilinmektedir. Bu durum önemli kayıpların olmasına neden olmaktadır. Örneğin; çimlenme yüzdesini hızlı kaybeden türlerden olan havuç tohumları farklı stres koşullarına maruz kalması durumunda önemli çimlenme ve çıkış kayıplarıyla karşı karşıya kalabilmektedir. Tuz stresi de bunlardan birisidir. Genel olarak tuz stresi tohumlarda çimlenme, çıkış ve fide gelişimini olumsuz yönde etkilemektedir (Kara ve ark. 2011; Yıldız ve ark. 2011). Tohumda çimlenme, kökçüğün tohum kabuğundan çıkması şeklinde tanımlanmaktadır (Rajjou ve ark., 2011)). Tohumlarda çimlenme su alımı ile başlamaktadır. Ancak tuzun varlığı su alımını azaltmaktadır (Othman 2005). Tohumun çevresindeki Na+ ve Cl- iyonlarının yüksek oranda birikmesi ile meydana gelen iyon toksisitesi ve osmotik basınç, tohumlarda su alımının azalmasına neden olmakta ve tuzlu koşullarda çimlenmeyi engellemektedir (Murillo-Amodor ve ark. 2002). Çimlenme sürecinde abiyotik bir tehlike olarak tuzluluk, yüksek seviyelerde tohum çimlenmesini inhibe edebilmekte veya daha düşük seviyelerde dormansinin başlamasına neden olabilmektedir (De Villiers ve ark. 1994). Bu olumsuz etkiler, düşük osmotik potansiyelden dolayı imbibisyonun (sıvının katı madde tarafından emilmesi) azalması (Poljakoff-Mayber ve ark. 1994), toksisite nedeni ile enzimatik aktivitenin değişmesi (Jisha ve ark., 2013)) protein metabolizmasının engellenmesi, bitki büyüme regülatörlerinin dengesinin bozulması, tohumdaki besi kullanımının azalması (Ibrahim, 2016) veya hücrelerin mitoz bölünmesinin engellenmesi (Baranova 2021) şeklinde gerçekleşmektedir. Ayrıca tuzluluk gerek osmotik, gerekse toksik iyon etkileri yoluyla bitki gelişmesini olumsuz olarak etkilemektedir (Kantar ve Elkoca 1998; Aktas 2002; Karni ve ark. 2010).

Sebze türleri içinde genellikle tohumla çoğaltılan havuç bitkisinin tuza hassasiyeti oldukça fazladır. Toprak saturasyon eriyiği elektriksel iletkenliğinin 1 dS/m 'nin üzerine çıkmasıyla havuç bitkisinde verim kayıpları da başlamaktadır (Bolton ve Simon, 2019). Osmotik potansiyelin -0,5 MPa'a kadar düşmesi çimlenme ve fide gelişimi üzerine etkili olmamakla beraber, toprak su potansiyelinin -0,01 MPa'a düşmesi havuçta çimlenme ve fide büyümesini oldukça azaltmaktadır. Bu bakımdan özellikle su stresinin artması havuç yetiştiriciliğini kısıtlamaktadır (Shannon ve Grieve 1999). Birçok araştırma bitkilerde tuzluluğun tohum çimlenmesi, büyümesi, enzim aktivitesi ve mitotik aktivite üzerinde olumsuz etkisini azaltmak için bitki büyüme düzenleyicileri ve farklı bitki besin elementlerinin kullanımını önermektedir (Çavuşoğlu ve Kabar 2008; Tabur ve Demir 2008, 2009, 2010; Çavuşoğlu ve Tabur 2015). Bitkiler çimlenme ve ilk gelişme evrelerinde tuza daha duyarlıdır. Sebzelerin tuz toleransı diğer kültür bitkilerine oranla daha düşüktür (Öztürk 2002). Tohumların çimlenmesi de bu olumsuz çevre koşullarından negatif etkilenebilmektedir. Bu durum çimlenme yüzdesi üzerine ya da çıkış üzerine olumusuz etki şeklinde görülmektedir. Ancak abiyotik stres koşullarında çimlenme ve çıkış sorunlarına karşı tohumlara; hormon ve büyüme düzenleyici madde solüsyonlarına daldırma veya bu maddelerin ön çimlendirme solüsyonuna ilave edilmesi şeklinde de uygulamalar yapılmaktadır (Kenanoğlu 2016). Böylece bu sorunların üstesinden gelinmeye çalışılmaktadır. Asetil salisilik asit (ASA) bileşiğide bunlardan biri olup, abiyotik strese karşı savunma mekanizmalarını da harekete geçirdiği bilinmektedir (Rivas-San ve Plasencia 2011; Hara ve ark. 2012). Asetil salisilik asit'in kuraklık (Miura ve Tada 2014), tuzluluk (Fahad ve Bano 2012), üşüme (Yang ve ark. 2012) ve yüksek sıcaklık (Fayez ve Bazaid 2014) gibi streslerle ilişkili olduğu yapılan araştırmalar ile ortaya konulmuştur. Ayrıca ASA'nın tohum çimlenmesi, çıkışı ve tuz stresi gibi durumlara karşı olumlu etkiler gösterdiği, bununla beraber bitkide bakteri, mantar ve viral enfeksiyonlara karşı sistemik kazanılmış (SAR) direnci uyardığı ve bazı bitkilerde nitrat redüktaz aktivitesini ve kuru madde miktarını arttırması gibi fizyolojik etkilere neden olduğu bildirilmektedir (Kaydan ve Yağmur 2006).

Bu araştırmada tuz stresine oldukça hassas olan havuç tohumlarının tuz stresi altında çimlenme performanslarının farklı dozlarda uygulanan asetil salisilik asit (ASA) ile olan ilişkileri belirlenmeye çalışılmıştır. Elde edilen bu sonuçlara göre tuzluluk stresine karşı hassasiyeti olan ve tohumla üretimi yaygın olan havuç gibi türlerde ASA uygulamalarının etkilerinin belirlenmesinde örnek teşkil etmesi hedeflenmiştir.

## 2. Materyal ve Metod

Araştırmada, bitkisel materyal olarak "Nantes" tipi havuç (*Daucus carota* L.) tohumları kullanılmıştır. Tohum ön çimlendirme uygulamaları laboratuvar ortamında gerçekleştirilmiş olup; tohumlar, 0, 50, 100 ve 150 mg L-1 ASA solüsyonlarına tabi tutulmuş, daha sonra 0, 50,100 ve 150 mM NaCl tuz dozlarına maruz bırakılararak, aşağıda detaylı açıklanan işlemler gerçekleştirilmiştir.

*Tohum nem tayini;* Nantes havuç (*Daucus carota* L.) tohumlarının nem tayini Uluslararası Tohum Deneme

Birliği (ISTA) "Yüksek Sabit Sıcaklık Yöntemi" kullanılarak gerçekleştirilmiştir. Tohumlar ön çimlendirme öncesi, 2 tekrarlı ve her tekrarda 4.5 g tohum olacak şekilde yüksek sabit sıcaklıkta "130-133 ºC" 60±3 dk. boyunca kurutulmuştur. Etüvden çıkarılan örnek, kapağı kapatılarak içinde nem çekici silika jel bulunan desikatör içine konulmuş ve oda sıcaklığında soğutulduktan sonra tartılarak, kurutma sonrası meydana gelen ağırlık kaybı orijinal (yaş) ağırlığına oranlanarak % tohum nemi belirlenmiştir. Buna göre Nantes havuç çeşidi tohumlarının % nem tayini aşağıdaki formül kullanılarak belirlenmiştir.

% nem = [(M2-M3) / M1] X 100
M1: kurutma öncesi tohum ağırlığı
M2: kurutma öncesi tohum + dara ağırlığı
M3: kurutma sonrası tohum + dara ağırlığı

Buna göre havuç tohumlarının nem kapsamlarının %10,51 olduğu tespit edilmiştir (ISTA 2009).

### *2.1. Ön çimlendirme uygulamaları*

Tohumlar saf su içinde 0 (kontrol), 50, 100 ve 150 mg L-1 ASA solüsyonlarında bekletildikten sonra 15 ºC sıcaklıkta iklim dolabında 24 saat ön çimlendirmeye bırakılmıştır. Her bir uygulama için, 10 g havuç tohumu 20 ml uygulama solüsyonu içinde (1:2 tohum solüsyon) petri kaplarında ön çimlendirme işlemine tabi tutulmuştur. Uygulama sonrası tohumlar filtre kağıdına serilerek uygulama öncesi nem ağırlıklarına kadar oda sıcaklığında kurutulmuştur. Araştırmada kontrol tohumları ile birlikte uygulama gören tohumlar çimlenme ve çıkış testine alınmıştır. Bütün uygulamalarda çimlenen tohumların sayımı günlük olarak yapılmış ve çimlenen tohumların oranı yüzde olarak belirlenmiştir. Ön çimlendirme uygulamaları gören tohumlar ile kontrol tohumlarında, standart tohum çimlenirme kurallarına göre 20 ºC sıcaklıkta kum ortamında çimlenme, çıkış ve fide büyüme testlerine tabi tutulmuşlardır.

### *2.2.Standart çimlenme testi*

Ön çimlendirme uygulamalarının NaCl stresi altında çimlenme performansı üzerine etkisinin belirlenmesi amacıyla havuç tohumları, 0, 50, 100 ve 150 mM NaCl solüsyonlarında ön çimlendirmeye alınmıştır. Ön çimlendirme öncesi ve sonrasında tohumlarda canlılık oranlarını belirlemek için, ISTA kurallarına göre her bir uygulama 4 tekerrür ve her bir tekerrürde 50 adet tohum olacak şekilde petri kaplarına ekilmiş, 20 ºC sıcaklıktaki inkübatörde 14 gün süre ile çimlendirme testine tabi tutulmuştur (ISTA 2009). Her bir petriye 4 ml tuz (NaCl) solüsyonu ilave edilmiştir. Çimlendirme ortamında nem azaldığında yeniden hazırlanan tuz solüsyonlarından ortama ilave yapılmıştır. Çimlendirme testinde günlük sayımlar yapılarak kökçük uzunluğu ≥2 mm olan tohumlar çimlenmiş kabul edilmiştir. Yapılan günlük sayım değerlerinden aşağıdaki formül kullanılarak ortalama çimlenme zamanı (gün) hesaplanmıştır. (Pedersen ve ark. 1993). Ayrıca günlük çimlenen tohumlar sayılarak ortamdan uzaklaştırılmamıştır ve test sonunda normal ve anormal çim (Şekil 1) oranları belirlenmiştir.


**Şekil 1.** Normal ve anormal çimlenen tohumlar

Ortalama çimlenme zamanı = ∑(g*n) / Sn
g: sayımın yapıldığı gün
n: sayımın yapıldığı gün çimlenen tohum sayısı
Sn: test sonunda toplam çimlenen tohum sayısı

### *2.3.Çıkış testi*

Ön çimlendirme uygulanmış tohumlar, 4 tekerrürlü ve her tekerrürde 50 adet tohum olacak şekilde steril kum ile doldurulan 500 cc plastik kaplara ekilmiştir. Çimlendirme testlerinde tohum fizyologlarının kabul ettiği tohumdan kökçük çıkışı (2 mm) canlılık kriteri olarak kabul edilmektedir. Ancak agronomik açıdan bakıldığında çimlenebilen her tohumun eksiksiz ve kusursuz organlara sahip normal bir bitki oluşturması beklenemez. Bu nedenle tohumların çimlenme testinden farklı olarak, fide oluşturabilme gücünü belirlemek için kumda çıkış testleri yapılmıştır. Çimlendirme testinde olduğu gibi her bir uygulama için 4 tekerrür ve her bir tekerrür de 50 adet tohum olacak şekilde içinde kum bulunan plastik kaplara ekimi gerçekleştirilerek 15 ºC sıcaklıktaki iklim dolabında 21 gün boyunca çıkış testine tabi tutulmuştur. Sulama suyu olarak 0, 50, 100 ve 150 mM NaCl solüsyonu kullanılmıştır. Test sonunda kum üzerine çıkan ve kotiledon yaprakları kum yüzeyine paralel hale gelen çimler sayılarak % çıkış oranı belirlenmiştir. Bu testlerde de günlük sayımlar yapılmış ve bu değerler kullanılarak standart çimlendirme testinde ifade edilen formül kullanılıp ortalama çıkış zamanı belirlenmiştir.

### *2.4.Fide kalitesindeki değişim*

Uygulamaların fide kalitesi üzerine etkisini belirlemek amacıyla, her bir uygulamadan 50 adet tohum içinde torf bulunan (Klasmann-Deilman GmbH, PotgrandH, Germany) saksılara ekilmiştir. Ekim sonrası saksılar 20 ºC sıcaklığındaki iklim odasında tutulmuştur. Saksılar 0, 50, 100 ve 150 mM NaCl solüsyonları ile sulanmıştır. Saksılarda yetiştirilen fideler 2-4 yapraklı oldukları dönemde (yaklaşık 35-40 gün), her bir uygulamadan rastgele seçilen 10 fidede, kumpas yardımı ile fide boyu (rozet gövde ile büyüme ucu arası cm olarak) ve fide çapı (rozet gövde) ölçülmüştür..

### *2.5. İstatiksel analiz*

Elde edilen veriler faktöriyel düzende SPSS programı kullanılarak varyans analizine tabi tutulmuş, ortalamalar arasındaki farklılıkların karşılaştırılmasında Varyans analizi, faktörlerin seviye ortamları arasındaki farklılıklar TUKEY testi kullanılarak belirlenmiştir ($p< 0.05$).

## 3. Bulgular

Ön çimlendirme uygulamalarında ön çimlendirme uygulanmış ve kontrol havuç tohumlarında 20°C yapılan standart çimlenme testi sonuçları değerlendirildiğinde tuz (NaCl) konsantrasyonu arttıkça tohumların çimlenme oranlarında bir azalış meydana gelirken, ASA uygulamasında ise çimlenme oranlarında artışlar gerçekleşmiştir. Ayrıca ön çimlendirme uygulamalarının çimlenme oranı üzerine etkisi istatistiki olarak anlamlı bulunmuştur. Uygulamalarda Doz x Tuz ikili interaksiyonu istatistiki önemli bulunmadığı için ikili interaksiyon hataya dahil edilmiştir. Toplam çimlenme özelliği bakımından elde edilen verilerle yapılan varyans analizi sonucunda Doz x Tuz interaksiyonu istatistiki olarak önemli bulunmuştur ($p<0.05$). Elde edilen sonuçlar incelendiğinde; 0 mg/L (kontrol) ASA ön uygulamasında en yüksek çimlenme oranı % 89 ile 0 mM NaCl tuz dozunda gözlenmiştir. Ön uygulamalar arasında en yüksek çimlenme oranına %93,0 ile 50 mM NaCl ile 50 mg/L ASA interaksiyonu 150 mg/L ASA ile 50 mM NaCl dozlarında belirlenmiştir. Tuz stresi çimlenme oranı incelendiğinde uygulamalar (ASA) ne olursa olsun tuz stresi artıkça çimlenme oranında azalmalar tespit edilmiştir (Tablo 1). Çimboyu değerleri bakımdan kontrol (0 mM NaCl) koşullarında 5.2, 5.4, 5.3, 5.8 cm değerlerini sırasıyla 0, 50,100 ve 150 ASA dozlarında en yüksek değerlere ulaştığı belirlenirken, tuz artıkça çim boyunda azalmalar oluğu belirlenmiştir. En düşük değerler 150 mM NaCl uygulamasında 50 mg/L ASA uygulamasında 0.3 ile belirlenmiştir. Ancak 50 ve 100 mM NaCl uygulamarında ASA uygulamalarının oldukça etkili olduğu gözlemlenmişitir (Şekil 2).

**Tablo 1**. Farklı tuz (NACl) ve ASA uygulamalarının standart çimlendirmeye etkileri

| ASA (mg/L) | NaCl (mM) | Çimlenme oranı (%≥2mm) | Normal çimlenme Oranı (%) | Anormal çimlenme | Çim boyu (cm) |
|---|---|---|---|---|---|
| 0 | 0 | 89,0 Ba | 80,0 Aa | 9,0 Aab | 5,2 Aa* |
| | 50 | 87,0 Ba | 77,5 Aa | 9,5 Aa | 4,4 Bb |
| | 100 | 81,5 Bb | 72.0 Aa | 9,5 Aa | 3,3 Cab |
| | 150 | 54,0 Ac | 46,0 Bb | 8,0 Ab | 1,8 Da |
| 50 | 0 | 92,0 Aa | 82,0 Aa | 10,0 Aba | 5,4 Aa |
| | 50 | 93,0 Aa | 83,0 Aa | 10,0 Aba | 5,0 Aab |
| | 100 | 88,0 Ab | 75,0 Aa | 13,0 Aa | 3,2 Bb |
| | 150 | 45,0 Bc | 32,0 Bc | 13,0 Aa | 0,3 Cb |
| 100 | 0 | 92,5 Aa | 86,5 Aa | 6,0 Cb | 5,3 Aa |
| | 50 | 92,0 Aa | 85,0 Aa | 7,0 Ca | 5,0 Aab |
| | 100 | 85,0 Ab | 68,0 Ba | 17,0 Ba | 3,5 Bab |
| | 150 | 43,0 Cc | 24,0 Cc | 19,0 Aa | 0,7 Cb |
| 150 | 0 | 93,0 Aa | 87,0 Aa | 6,0 Bb | 5,8 Aa |
| | 50 | 93,0 Aa | 86,0 Ba | 7,0 Aba | 5,4 Aa |
| | 100 | 88,0 Ab | 77,0 Ba | 11,0 Aa | 3,9 Ba |
| | 150 | 46,5 Bc | 40,0 Cb | 6,5 Bb | 0,6 Cb |

*Büyük harfler her bir tuz dozunda ASA dozları arası farklılığı her küçük harfler ise her bir ASA dozunda tuzların seviye ortalamaları arası farklılığı göstermektedir. $p<0.05$ **

Araştırma sonuçlarına göre ön çimlendirme uygulamaları fide boyu ve fide çapı üzerine etkileri olumlu etkide bulunurken, NaCl uygulamaları fide boyu ve çapında azalmalara neden olmuştur. Tablo 2'de gösterilen verilere göre tuz stresi artıkça fide boyu ve fide çapı azalmıştır. En yüksek fide boyu 0 NaCl dozunda 150 mg/L ASA uygulmasında 11.9 cm ile ilk sırada yer alırken, bunu yine aynı tuz dozunda 11.5 ve 11.4 cm ile sırasıyla 50 ve 100 mg/L ASA uygulamaları takip etmiştir. En kısa fide boyu 50 mg/L ASA uygulamasında 100 mM NaCl dozunda 3.8 cm ile elde edilmiştir. Fide çapı değerleri incelendiğinde en yüksek değer 1.17 mm ile 100 mg/L ASA uygulamasında ve 0 mM NaCl tuz dozundan elde edilmiştir (Tablo 2). En düşük değerler ise 0 mg/L ASA uygulamarında 0.25 ve 0.29 mm ile sırasıyla 100 ve 150 mM NaCl tuz dozlarında görülmüştür (Tablo 2).

**Tablo 2**.Ön çimlendirme uygulamalarının tuz stresi altında havuç tohumlarının fide boyu ve fide çapı üzerine etkileri

| ASA (mg/L) | NaCl (mM) | Fide boyu (cm) | Fide çapı (mm) |
|---|---|---|---|
| 0 | 0 | 10,6 Ab | 0,78 Ab |
| | 50 | 8,0 Ba | 0,49 Bb |
| | 100 | 5,2 Cab | 0,25 Cb |
| | 150 | 4,2 Da | 0,29 Cb |
| 50 | 0 | 11,5 Aa | 0,94 Ac |
| | 50 | 7,0 Bb | 0,68 Bb |
| | 100 | 4,7 Cb | 0,43 Cab |
| | 150 | 3,8 Da | 0,49 Ca |
| 100 | 0 | 11,4 Aa | 1,17 Aba |
| | 50 | 7,3 Bb | 0,86 Ba |
| | 100 | 5,4 Ca | 0,54 Ca |
| | 150 | 4,0 Da | 0,43 Cab |
| 150 | 0 | 11,9 Aa | 0,74 Ac |
| | 50 | 7,1 Bb | 0,67 Aa |
| | 100 | 5,7 Ca | 0,53 Ba |
| | 150 | 4,4 Da | 0,43 Cab |

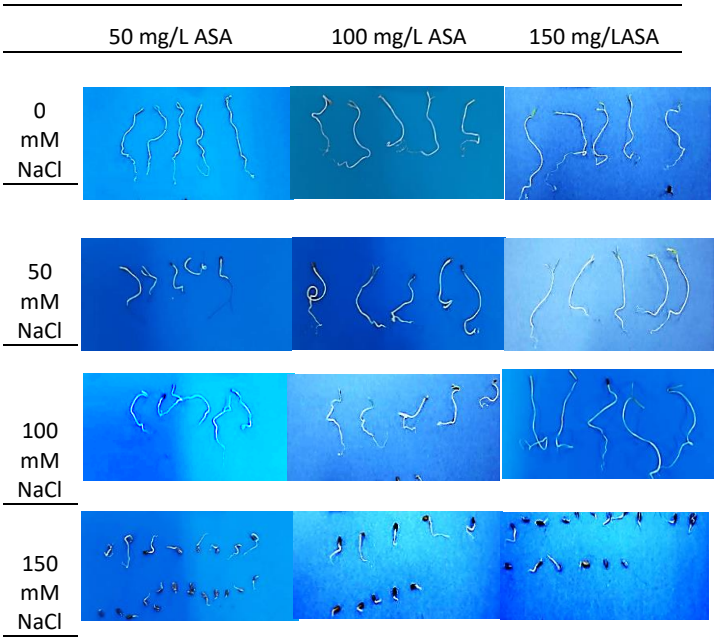
*Büyük harfler her bir tuz dozunda ASA dozları arası farklılığı her küçük harfler ise her bir ASA dozunda tuzların seviye ortalamaları arası farklılığı göstermektedir. $p<0.05$ **

ASA uygulamalarının tuz stresi altında havuç tohumlarının ortalama çıkış zamanı üzerine etkileri denemede araştırılmış olup Tablo 3'te gösterilmiştir. Buna göre tuz dozu artıkça ortalama çıkış süresininde artığı ayırıca ASA uygulmalarının bu çıkış süresi üzerine çok olumlu bir etkisinin olmadığı belirlenmiştir. Örneğin 150 mg/L ASA uygulması ve 150 mM NaCl uygulmasında çıkışlar 12.5 gün ile en uzun sürede çimlenmiştir. 0 mM NaCl (konrtol) uygulmalarında 7.7, 9.0, 8.7, 8.3 gün sırasıyla 0, 50, 100, 150 mg/L ASA uygulamaları ile bir artış gösterdiği tespit edilmiştir.

**Tablo 3.** ASA uygulamalarının tuz stresi altında havuç tohumlarının ortalama çıkış zamanı üzerine etkileri

| ASA (mg/L) | NaCl (mM) | Ort. Çıkış Zamanı (gün) |
|---|---|---|
| 0 | 0 | 7,7 Cb |
| | 50 | 9,1 Aba |
| | 100 | 10,0 Aa |
| | 150 | 8,6 BCc |
| 50 | 0 | 9,0 Ba |
| | 50 | 8,4 Ba |
| | 100 | 11,2 Aa |
| | 150 | 11,3 Aa |
| 100 | 0 | 8,7 Bab |
| | 50 | 8,6 Ba |
| | 100 | 9,9 Abb |
| | 150 | 11,0 Ab |
| 150 | 0 | 8,3 Cab |
| | 50 | 9,5 Bca |
| | 100 | 10,0 Bab |
| | 150 | 12,5 Aa |

*Büyük harfler her bir tuz dozunda ASA dozları arası farklılığı her küçük harfler ise her bir ASA dozunda tuzların seviye ortalamaları arası farklılığı göstermektedir. $p<0.05$ **




**Şekil 2**. ASA ve tuz kombinasyonlarının tohum çimlenmesi üzerine etkileri

## 4. Tartışma

Bu çalışmada son yıllarda yapılan birçok araştırmada yaygın olarak kullanılan; Asetil Salisilik Asitin (ASA) tuz stresi altında havuç tohumlarının çimlenme ve çıkışı üzerine etkileri araştırılmıştır

Tuz stresi altında havuç bitkisinin çimlenme ve çıkışını iyileştirmek amacıyla yapılan denemede standart çimlendirme, düşük ve yüksek sıcaklık çimlendirme, çıkış testi, fide kalitesindeki değişimi gösteren parametreler incelenmiş ve istatistiki olarak önemli bulunmuştur. Çalışmada, tuzluluk, konsantrasyonuna bağlı olarak havuç tohumlarının çimlenmesini engellemiştir (Tablo 1). Tuzun tohumlarda çimlenmeyi engelleyici etkisi, pek çok araştırmada ortaya konmuştur (Ghoulam ve Fores 2001; Gulzar ve Khan 2002; Çavuşoğlu ve ark. 2007; Ibrahim 2016). Çimlenme oranının azalmasına yüksek tuz konsantrasyonunun su alımını engellemesi, tuzun toksik etki yapması ve çimlenme sırasında gerekli olan enzimlerin tuz stresinden dolayı aktif hale gelememesinin neden olduğu bildirilmektedir (Essa 2002; Sadeghian ve Yavari 2004; Läuchli ve Grattan, 2007; Khayamim ve ark., 2014).

Yapılan bu araştırmada tuz stresi ile çimlenme yüzdesi düşmüştür. Ancak ASA uygulamaları tuz stresi altında çimlenme yüzdesini artırmıştır (Tablo 1). Örneğin; 0 mg/L ASA uygulamasında 100 mM tuz stresinde çimlenme %81,5 oranında gerçekleşirken aynı doz tuz stresinde 150 mg/L ASA uygulamasında %88 çimlenme gözlenmiştir. Buna göre uygulanan asitlerin çimlenme oranı üzerine %6,5 oranında olumlu etkide bulundukları tespit edilmiştir. Benzer bir araştırmada yine havuç bitkisinde abiotik stres altında ASA uygulamalarının çimlenme üzerine olumlu etkide bulunduğu belirlenmiştir (Rajasekaran ve ark. 2002).

Araştırma sonucunda elde edilen bulgulara göre, 0 mg/L (kontrol) ASA dozunun ön uygulamasında en yüksek çimlenme oranı %89 ile 0 mM NaCl uygulamasında elde edilmiştir. Ön uygulamalar arasında en yüksek çimlenme oranına %93.0 ile 50 mg/L asit ile 50 mM NaCl dozu ve 150 mg/L asit ile 50 mM NaCI dozunda belirlenmiştir. Ayrıca çim boylarının ASA uygulamaları iyleştiği belirlenmiştir (Şekil 2). Benzer bir çalışmada Maş fasulyesinde tuzlu koşullarda uygulanan SA ve $KNO_3$ uygulamalarının çimlenme ile igili özellikleri iyileştirdiği ve tuz stresine karşı çimlenme aşamasında dayanıklılığı artırdığı gözlenmiştir (Entasari ve ark. 2012).

Normal çimlenme sonuçları incelendiğinde ise yüksek normal çimlenme oranının %87 ile 150 mg/L ASA ile 0 mM NaCl dozu kombinasyonundan elde edildiği belirlenmiştir (Tablo 1).

Denemede elde edilen standart çimlendirme testi bulgularına yapılan varyans analizi sonucuna göre havuç tohumlarına yapılan ön uygulamaların ortalama çimlenme zamanı üzerine etkisi NaCl doz interaksiyonu önemli bulunmuştur. En düşük ortalama çimlenme zamanı 3.9 gün ile 0 mM NaCl dozunda gözlenmiştir. En yüksek ortalama çimlenme zamanı 150 mM NaCl tuz dozunda 9.0 gün olmuştur (Tablo 3). Sonuçlar göre tuz dozu artıkça ortalama çimlenme zamanı uzamıştır. Havuç bitkisi tuza tolerans düzeyi düşük olan bir bitki olduğu için tuzluluk ortalama çimlenme zamanını olumsuz etkilemiştir. Araştırmacıların yaptığı çalışmalar ile bu denemede elde edilen sonuçlar paralellik göstermiş olup; tuzluluğun çimlenme üzerine olumsuz etkiler gösterdiğini belirtilmiştir (De Villiers ve ark. 1994). Ancak tohuma yapılan ön uygulamaların hem

çimlenme hemde çim boyu üzerine olumlu etkilerinin olduğu söylenebilir nitekim yapılan bir araştırmada ASA uygulanmış biber tohumlarının çimlenme ve çıkış performanslarını artırdığı belirlenmiştir (Korkmaz 2005). Bu durum ASA'nın abiyotik stres koşullarına karşı toleransın artırılmasında önemli rol alabileceğini göstermektedir.

Fide boyu ve fide çapı denemede yapılan istatistiki analizler sonucu ASA x NaCl interaksiyonu önemli bulunmuştur. Tespit edilen değerler incelendiğinde en yüksek fide boyu değeri 150 mg/L ASA uygulamasının 0 mM NaCl dozunda 11,9 cm olarak elde edilmiştir, en düşük fide boyu ise 50 mg/L asit uygulamasının 150 mM NaCl dozunda 3,8 cm olarak ölçülmüştür. Diğer uygulamalar arasında ASA x NaCl interaksiyonlarına bakıldığı zaman 0 mM tuz dozu oranlarında asit dozu artırıldığında fide boyu üzerinde olumlu etki gözlenmiştir (Tablo 2). Fide çapı bakımndan ise 100 mg/L ASA dozunda 1.17 mm ile en yüksek fide çapı değeri 0 mM NaCl dozunda belirlenmiştir. ASA x NaCl interaksiyonunda asit dozu artıkça fide çapı değeri artmıştır ve tuz dozu artıkça fide çapı değeri azalmıştır. En düşük fide çapı değeri 0 mg/L (kontrol) ASA dozunun 100 mM NaCl uygulamasında 0,25 mm olarak ölçülmüştür. ASA uygulamalarında asit dozları tuzluluk stresine karşı pozitif etki sağladığı belirlenmiştir (Tablo 2). Havuç gibi çimlenmesi geç ve zor olan küçük embriyolu bazı sebze tohumlarının olumsuz toprak koşullarındaki çimlenmesini iyileştirmek ve homojen fide çıkışını sağlamak amacıyla yapılan araştırmalarda (Brocklehurst ve ark. 1984; 1987a,b; Yanmaz ve Özdil 1992) ekim öncesi bazı ön uygulamalar sonunda çimlenme ve çıkış hızı oranlarını arttığı, erken ve homojen fide çıkışı sağladığı belirlenmiştir (Duman ve Eşiyok 1998).

Ön çimlendirme uygulamalarının tuz stresi altında ortalama çıkış zamanına etkisi incelendiğinde ASA x NaCl doz interaksiyonu istatistiki olarak önemli bulunmuştur. Denemede sonuçlara bakıldığı zaman 0 mg/L ASA (kontrol) ön uygulamasında en düşük ortalama çıkış zamanı 7,7 gün ile 0 mM NaCl uygulamasında gözlenmiştir. Araştırma sonuçlarına göre tuz dozunun artması havuç tohumlarının ortalama çıkış zamanını uzatmıştır, 150 mg/L ASA doz ön uygulamasında 150 mM tuz dozunda 12,5 gün ortalama çıkış zamanıyla en yüksek değere ulaşmıştır.

## 5. Sonuç

Bu araştırmadan elde edilen sonuçlar genel olarak değerlendirildiğinde Nantes havuç tohumlarına uygulanan tuz çözeltisi genel olarak bitki büyümesini olumsuz yönde etki ederken ASA ön uygulaması tuzun bu olumsuz etkilerinin açığa çıkmasını geciktirmiş ve tohumlarda tuz stresi belirtilerinin daha geç görülmesine yol açmıştır. Bu açıdan ASA çimlenme oranı, çıkış testi ve fide kalitesi üzerine tuz stresinin olumsuz etkilerini düzeltici etkiler yapmıştır. ASA uygulamasının bitki gelişimini olumlu yönde etkilediğini ve tuzun olumsuz etkisini tolere ettiğini göstermiştir. Streslere toleransı arttırmak için ASA gibi ön uygulamaların kullanımı alternatif yaklaşımlar olarak görülmektedir. Yapılan bu çalışmanın havuç üretiminde tuzluluk stresine karşı olumsuz etkileri en aza indirerek üreticiye ışık tutacağı ve ülke ekonomisine katkı sağlayabileceği düşünülmektedir.

## Teşekkür

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# Biochemical laboratory findings on COVID-19 patients: pathogen-disease relationship

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**Abstract:** The COVID-19 process, which started in Wuhan, China, is one of the most significant viral diseases characterized by high mortality and catching millions of people around the world since it appears. In this study, a total of 189 patients, 85 outpatients, and 104 inpatients were diagnosed with COVID-19 with positive PCR tests examined, admitted to the COVID 1-2-3 services of the Faculty of Medicine of Kafkas University between November 1 and November 16, 2020, has been examined. The relationship between laboratory findings and pathogen disease in the diagnosis, treatment and course of the disease has been tried to be revealed. The majority of patients with fever, reflux diabetes and tumours are over the age of 65 (61.7%), and 68 (36.2%) of them are women and 120 (63.8%) are men. Demographic characteristics of the patients, biochemical parameters such as serum ferritin, iron, troponin T, D-dimer levels, and hemogram and coagulation results were evaluated. Findings will contribute to clinicians and biochemists about the prognosis and mortality of COVID-19, its course in some other diseases, and the ways to be followed in treatment.

**Keywords:** COVID-19, biochemical parameters, chronic diseases.

## *COVID-19 hastalarında biyokimyasal laboratuvar bulguları: patojen ve hastalık ilişkisi*

**Özet:** Çin'in Wuhan kentinde başlayan COVID-19 süreci; ortaya çıkışından bu yana dünya üzerinde milyonlarca insanın yakalandığı ve yüksek mortalite ile karakterize edilen en önemli viral hastalıklarının başında yer almaktadır.1-16 Kasım 2020 zaman aralığında Kafkas Üniversitesi Tıp Fakültesi Covid 1-2-3 servislerine başvuran PCR testleri pozitif çıkan Covid-19 tanısı almış ayakta tedavi edilen 85, yatarak tedavi edilen 104 hasta olmak üzere 189 hasta alındı. Hastalığın tanı, tedavi ve seyrinde laboratuvar bulgularının patojen hastalık ilişkisi ortaya konmaya çalışıldı. Araştırmada ateş, reflü, diyabet ve tümörlü hastaların büyük çoğunluğunu 65 yaşın üzerinde (%61.7) olup bunların 68 (%36.2)'i kadın, 120 (%63.8)'si erkekti. Hastaların demografik özellikleri, serum ferritin, demir, troponin T, D-dimer düzeyleri gibi biyokimyasal parametreler ile hemogram ve koagülasyon sonuçları değerlendirildi. Bulgular; Covid-19'un prognozu ve mortalitesi ve diğer bazı hastalıklardaki seyri hakkında klinisyenlere ve biyokimyacılara tedavide izlenecek yollarda katkı sağlayacaktır.

**Anahtar Kelimeler:** Covid-19, biokimyasal parametreler, kronik hastalıklar.



## 1. Introduction

Coronaviruses causing COVID-19 are of the Orthocoronavirinae subfamily in the Coronaviridae family. They are enveloped (zoonotic) RNA viruses that can infect humans and a wide variety of animal species (Mac-Lachlan and Dubovi 2017). They are included in the same virus family as SARS CoV and MERS CoV. The subtypes circulating in humans (HCoV-229E, HCoV-OC43, HCoV-NL63 and HKU1-CoV) constitute a large virus family that mostly causes the common cold (Inal 2016). SARS-CoV-2 shares 79.6% of its genome sequences with SARS-CoV. Thanks to the spike (S) glycoprotein encoded, both viruses enter the host cell by binding to the transmembrane serine protease (TMPRSS) and ACE2 (angiotensin converting enzyme (Inal 2016) receptors released from the host cell

membrane. Once inside the cell, SARS-CoV-2 causes cellular death and injury in the airway epithelial cells through various processes such as pyroptosis. Increased proteases and reactive oxygen species cause further damage and hyperinflammation (Lu 2020; Lan et al. 2020). SARSCoV-2 has been found to show quite high affinity to the ACE2 receptor. Several organs and especially the lung possess ACE2 receptors on cell surfaces (Xu et al. 2020). The new type of coronavirus has been shown to bind to the ACE2 receptor ten times more strongly (Qi et al. 2020; Kappert et al. 2020).

Many studies have been conducted to identify the predictors of laboratory findings related to the course of the disease since the start of the coronavirus disease pandemic in 2019. The oxygen level decreases due to the virus-related alveolar collapse in most of the studies, but the condition advances with shortness of breath since CO2 exchange continues (Dreher et al. 2020; Kappert) et al. 2020; Wang. Et al.2020) In other words, all tissues and organs, and especially the lung, are affected by the pathogen. In addition to the physiological, clinical, hematological and homeostasis-related findings, biochemical parameters also provide partial but important information about the course of the disease in order to determine the pathogen's effects. These chemical markers not only reflect the severity of the disease, but also shed light on the prognosis and the pathological mechanisms.

The elevation of proinflammatory cytokines in the disease indicates that the serum concentration acute phase proteins are the cause of the increased CRP in particular (Ruan et al 2020; Banerjee et al. 2020)). while high ferritin levels indicate advanced disease and increased mortality (Chan et al. 2020; Guo et al. 2020). Cardiovascular disease (CVD), diabetes mellitus type 2 (DMT2), hypertension, malignancy and chronic obstructive pulmonary disease (COPD) can be present among Covid-19 patients. This is due to the fact that there may be oxidative species (ROS) formed during reactive release with an imbalance in the endogenous antioxidant capacity (Wang et al. 2010). However, increased coagulation abnormalities and a high incidence of thrombotic events together with changes in the activated partial thromboplastin time (aPTT) and prothrombin time, increased D-dimer levels, and thrombocytopenia have been found in these patients (Yılmaz and Eren 2020).

One of the most striking laboratory results in COVID-19 is high serum ferritin levels. Understanding how iron metabolism and viral infection interact may be important in developing new methods of keeping the disease under control. The extracellular iron found in normal healthy lungs is reported to cause oxidative damage and make the organism particularly vulnerable to viral infections (Memikoğlu and Genç 2021). COVID-19 has been reported to potentially cause long-term and progressive hypoxia by binding to the heme groups in the hemoglobin in erythrocytes, in addition to multi-organ damage by decreasing the oxygen saturation (Khot and Nadkar 2020).

The iron-related severe fatigue seen in the patients at the onset and later stages of COVID-19 can be considered a hypoxic state due to the decrease in oxygen. Biochemical examinations of 99 patients with coronavirus have revealed a significant increase in serum ferritin, erythrocyte sedimentation rate, C-reactive protein, albumin, and lactate dehydrogenase index values, while most of the hemoglobin and neutrophil counts were decreased. This clinical picture indicates that the pneumonia is associated with hemoglobin (Guo et al. 2020). The aim of this study was to understand how the iron metabolism and viral infection interact, in order to possibly guide the development of other studies and new methods, take the disease under control, and make new recommendations. A total of 189 patients with positive COVID-19 PCR tests were included in the study. These patients' demographic characteristics, laboratory values, and the relationship of the pathogen with oxygen and iron were investigated. Based on these data, we tried to determine whether there was a significant relationship between the biochemical values of the patients and compensation, and whether these parameters could be used as prognostic indicators.

## 2. Materials and Method

The records of 189 patients attending the Kafkas University Faculty of Medicine Research Hospital's COVID department and intensive care unit during the 1-16 November, 2020 period were retrospectively reviewed. Taking the WHO guidelines into account, patients diagnosed with SARS-CoV-2 RNA with the polymerase chain reaction (PCR) test were included in the study with a diagnosis of COVID-19, regardless of whether they had symptoms or not. The study was conducted according to the Helsinki Declaration. Approval for the study was obtained from the Kafkas University Faculty of Medicine's Non-Interventional Research Ethics Committee, dated April 04, 2021 and numbered 80576354-050-991/62. The patients were divided into two groups as inpatients and outpatients. Thorax CT and a PCR test were performed in every patient. The patients who were positive and whose vital functions had been adversely affected by the COVID-19 symptoms were hospitalized and COVID-19 treatment was started. Asymptomatic patients who were diagnosed with COVID-19 and did not have direct chest x-ray or lung tomography images and also did not have pneumonia were followed up as outpatients.

Roche Cobas C501 brand device with urea (kinetic test, ureaz and glutamate dehydrogenase), creatinine (Jaffe method), lactate dehydrogenase(LDH), aspartate aminotransferase(AST), alanine aminotransferase (ALT) (absorbance), gamma glutamyltransferase(GGT) (enzymatic colorimetric), alkaline phosphatase(ALP) (colorimetric), bilirubin (diazo method), CK, Ca, Mg, P, Hb (photometric), albumin, D-dimer (immunoturbidimetric), C-reactive protein(CRP) (turbidimetric), Na, Cl (ion selective electrode (ISE)), ferritin (electrochemiluminescence), troponin (electrochemiluminescence). Coagulation markers were measured with the Ceveron Alpha device, using the activated partial thromboplastin time(APTT), prothrombin time(PT): clotting test method. Hemogram was performed

with Abxpentra Dx 120 and leukocytes, red blood cell (RBC) and defined platelet(PLT) with double hydrodynamic sequential system (DHSS) cytometry, impedance and absorbance methods.

### *2.1. Statistical Analysis*

The descriptive data (mean, standard deviation, minimum, maximum, percentage) of the variables were calculated. The Mann-Whitney U test was used for two independent groups and the Kruskal-Wallis test for more than two independent groups as the nonparametric test for the intragroup comparison of the parameters not showing a normal distribution. A confidence interval of 95% and a statistical significance level of $p<0.05$ were used.

## 3. Results

A total of 189 patients diagnosed with COVID-19, including 85 outpatients and 104 inpatients were included in the study. Most of the patients (61.7%) were aged over 65 years; 68 (36.2%) were female and 120 (63.8%) were male (Table 1).

**Table 1.** Demographic characteristics of patients

| **Variable** | | | |
|---|---|---|---|
| **Age** | 35-49 years | 35 - 49 years | 50 - 64 years |
| Number(n) | 23 | 14 | 49 |
| Percentage (%) | 12.2 | 7.4 | 26.1 |
| **Gender** | | | Total |
| Famale | 68 | 121 | 189 |
| Male | 36.2 | 63.8 | 100.0 |

**Table 2.** Chronic disease distribution of patients

| **Variable** | **None** | **Present** | **Total** | | **None** | **Present** | **Total** |
|---|---|---|---|---|---|---|---|
| **Tumor** | | | | **Cough** | | | |
| Number | 182 | 6 | 188 | Number | 116 | 72 | 188 |
| Precentage(%) | 96.8 | 3.2 | 100.0 | Precentage(%) | 61.7 | 38.3 | 100.0 |
| **Dabet** | | | | **Fever** | | | |
| Number | 152 | 36 | 188 | Number | 185 | 3 | 188 |
| Precentage(%) | 80.9 | 19.1 | 100.0 | Precentage(%) | 98.4 | 1.6 | 100 |
| **HT** | | | | **KH** | | | |
| Number | 147 | 39 | 186 | Number | 165 | 23 | 188 |
| Precentage(%) | 79.0 | 21.0 | 100.0 | Precentage(%) | 87.8 | 12.2 | 100.0 |
| **Variable** | None | Present | Total | | None | Present | Total |
| **Tumor** | | | | **Cough** | | | |

A tumor was present in 3.2% 0f the patients in 19.1% HT in 21.0%, cardiac failure in 12.2% symptoms consistent with gastrointestinal reflux in 18.6%, acough in 38.3% and fever (above 37.4 degrees 0C) in 1.6% (Table 2). The blood biochemical parameters, hemogram and coagulation results evaluated in the study are given in Table 3 and 4.

**Table 3** Biochemical laboratory results

| **Variable** | **n** | **Mean** | **Sd** | **Median** | **Min** | **Max** |
|---|---|---|---|---|---|---|
| Albumin | 12 | 4.09 | 0.77 | 4.09 | 2.83 | 5.59 |
| **Urea** | 184 | 44.48 | 23.18 | 39.00 | 0.89 | 152.00 |
| **Creatinine** | 182 | 1.05 | 0.77 | 0.92 | 0.38 | 10.07 |
| **ALP** | 112 | 85.71 | 28.61 | 80.00 | 40.00 | 192.00 |
| **ALT** | 184 | 31.79 | 45.87 | 21.00 | 5.00 | 526.00 |
| **AST** | 184 | 32.23 | 26.13 | 26.00 | 11.00 | 213.00 |
| **D-bilirubin** | 181 | 0.22 | 0.28 | 0.16 | 0.00 | 2.40 |
| **GGT** | 135 | 45.99 | 52.82 | 31.00 | 6.00 | 440.00 |
| **LDH** | 178 | 299.97 | 114.28 | 273.00 | 108.00 | 814.00 |
| **CK** | 170 | 110.53 | 109.59 | 75.50 | 0.50 | 855.00 |
| **LDH** | 178 | 299.97 | 114.28 | 273.00 | 108.00 | 814.00 |
| **Ca** | 178 | 9.05 | 0.67 | 9.00 | 7.20 | 12.50 |
| **Mg** | 16 | 1.88 | 0.35 | 1.91 | 1.09 | 2.54 |
| **P** | 30 | 3.99 | 0.65 | 4.19 | 2.06 | 5.09 |
| **Na** | 185 | 135.58 | 13.62 | 137.00 | 9.80 | 154.00 |
| **Cl** | 175 | 101.36 | 11.26 | 102.20 | 4.31 | 117.70 |

**Table 4.** Hemogram and coagulations tests

| Variable | n | Mean | Sd | Median | Min | Max |
|---|---|---|---|---|---|---|
| **RBC** | 184 | 5.02 | 0.59 | 5.02 | 3.49 | 6.90 |
| **WBC** | 184 | 5.97 | 2.69 | 5.25 | 2.10 | 20.50 |
| **HGB** | 184 | 14.68 | 1.76 | 14.70 | 9.60 | 19.30 |
| **HCT** | 184 | 43.56 | 4.98 | 43.60 | 30.90 | 60.70 |
| **MCV** | 184 | 86.98 | 5.97 | 87.00 | 65.00 | 107.00 |
| **MCH** | 184 | 29.28 | 2.51 | 29.30 | 19.90 | 38.60 |
| **MCHC** | 184 | 33.64 | 1.49 | 33.60 | 28.50 | 36.70 |
| **RDV** | 184 | 14.41 | 1.95 | 14.00 | 4.00 | 22.40 |
| **Hb** | 184 | 14.68 | 1.76 | 14.70 | 9.60 | 19.30 |
| **NEU** | 183 | 4.61 | 6.48 | 3.20 | 1.00 | 58.00 |
| **LYM** | 184 | 1.95 | 3.65 | 1.30 | 0.37 | 34.10 |
| **MON** | 183 | 0.86 | 1.64 | 0.50 | 0.10 | 12.10 |
| **BAS** | 106 | 0.06 | 0.17 | 0.02 | 0.00 | 1.60 |
| **EOS** | 147 | 0.13 | 0.33 | 0.10 | 0.00 | 3.70 |
| **NEU%** | 182 | 60.99 | 12.38 | 62.75 | 0.00 | 89.30 |
| **PLT** | 184 | 210.92 | 96.27 | 198.00 | 13.80 | 602.00 |
| **PDW** | 184 | 23.15 | 35.50 | 16.00 | 10.00 | 236.00 |
| **Activated PTT** | 175 | 34.71 | 19.15 | 31.40 | 12.00 | 262.20 |
| **Ferritin** | 175 | 384.23 | 331.27 | 296.20 | 6.01 | 2000.00 |
| **D-Dimer** | 81 | 2309.95 | 4179.33 | 736.00 | 205.00 | 18800.00 |
| **PT(SN)** | 176 | 12.75 | 3.69 | 12.20 | 1.01 | 40.90 |

**Table 5.** Relationship between fever, reflu, diabetes and tumor laboratory findings

| | | Temperature | | | | Reflux | | | | Diabetes | | | | Tumor | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | None | | Present | | None | | Present | | None | | Present | | None | | Present | |
| Parameters | | Hospitalization | Discharged | Hospitalization | Discharged | Hospitalization | Discharged | Hospitalization | Discharged | Hospitalization | Discharged | Hospitalization | Discharged | Hospitalization | Discharged | Hospitalization | Discharged |
| CRP | r | .203* | .152* | 0.87 | 1.000 | .246* | .226* | 0.07 | -0.005 | .250* | .187* | -0.03 | 0.01 | .261* | .202* | -0,68 | -0.41 |
| | p | 0.01 | 0.05 | 0.33 | N/A | 0.00 | 0.01 | 0.69 | 0.98 | 0.00 | 0.03 | 0.86 | 0.94 | 0.00 | 0.01 | 0.14 | 0.42 |
| Ferritin | r | 0.03 | 0.02 | -0.87 | -0.50 | 0.06 | 0.08 | -0.09 | -0.19 | 0.07 | 0.03 | -0.09 | 0.07 | 0.05 | 0.05 | -0.72 | -0.76 |
| | p | 0.70 | 0.75 | 0.33 | 0.67 | 0.46 | 0.34 | 0.62 | 0.30 | 0.39 | 0.76 | 0.62 | 0.70 | 0.49 | 0.53 | 0.11 | 0.08 |
| D-Dimer | r | 0.18 | 0.17 | N/A | N/A | 0.18 | 0.17 | 0.15 | 0.06 | 0.20 | 0.22 | 0.04 | -0.20 | 0.13 | 0.13 | 0.87 | 0.87 |
| | p | 0.11 | 0.13 | N/A | N/A | 0.15 | 0.17 | 0.62 | 0.84 | 0.11 | 0.07 | 0.90 | 0.54 | 0.25 | 0.26 | 0.33 | 0.33 |
| WBC | r | 0.09 | 0.10 | 0.00 | -0.50 | 0.07 | 0.10 | 0.14 | 0.10 | 0.09 | 0.12 | 0.01 | -0.04 | 0.11 | 0.12 | -0.48 | -0.53 |
| | p | 0.24 | 0.18 | 1.00 | 0.67 | 0.42 | 0.22 | 0.42 | 0.56 | 0.29 | 0.15 | 0.94 | 0.84 | 0.13 | 0.11 | 0.34 | 0.28 |
| RBC | r | -.181* | -0.14 | 0,00 | 0.50 | -.212* | -.170* | -0.03 | 0.05 | -.177* | -0.09 | -0.18 | -0.30 | -.186* | -0.14 | 0.09 | -0.20 |
| | p | 0.02 | 0.07 | 1.00 | 0.67 | 0.01 | 0.04 | 0.89 | 0.78 | 0.03 | 0.27 | 0.30 | 0.09 | 0.01 | 0.07 | 0.87 | 0.70 |
| HGB | r | -0.14 | -0.06 | -0.87 | -0.50 | -.164* | -0.08 | -0.07 | -0.01 | -.164* | -0.05 | -0.01 | -0.04 | -.158* | -0.07 | 0.48 | 0.09 |
| | p | 0.06 | 0.45 | 0.33 | 0.67 | 0.05 | 0.32 | 0.69 | 0.97 | 0.05 | 0.53 | 0.94 | 0.82 | 0.04 | 0.36 | 0.34 | 0.87 |
| PT (Sec) | r | 0.06 | 0.13 | 1.000 | 0.87 | 0.08 | .166* | 0.02 | 0.06 | 0.02 | 0.08 | 0.22 | 0.32 | 0.03 | 0.09 | 0.72 | .971* |
| | p | 0.44 | 0.10 | N/A | 0.33 | 0.36 | 0.05 | 0.90 | 0.75 | 0.83 | 0.34 | 0.21 | 0.07 | 0.73 | 0.26 | 0.11 | 0.00 |
| PT (%) | r | 0.03 | -0.04 | -1.000 | -0.87 | 0.03 | -0.08 | -0.02 | -0.05 | 0.09 | 0.03 | -0.21 | -0.32 | 0.05 | -0.02 | -0.15 | -0.41 |
| | p | 0.66 | 0.60 | N/A | 0.33 | 0.74 | 0.37 | 0.91 | 0.79 | 0.26 | 0.77 | 0.23 | 0.07 | 0.55 | 0.82 | 0.78 | 0.42 |
| PT (INR) | r | -0.02 | 0.02 | 1.000 | 0.87 | -0.02 | 0.05 | 0.02 | 0.06 | -0.08 | -0.05 | 0.23 | 0.32 | -0.03 | 0.00 | 0.15 | 0.41 |
| | p | 0.77 | 0.80 | N/A | 0.33 | 0.85 | 0.59 | 0.90 | 0.75 | 0.33 | 0.56 | 0.20 | 0.07 | 0.66 | 0.97 | 0.78 | 0.42 |

Table 5 shows the Mann-Whitney U test analysis results for the association between reflux, diabetes and tumor and the C-reactive protein, Ferritin, D-Dimer, white blood cells (WBC), red blood cells(RBC), hemoglobin (HGB), prothrombin time (PT) (Sec), PT (%) and PT (INR) values. A significant relationship was present between reflux and the PT (Sec) value (p=0.014). The median PT (Sec) value was 12.25 (1.01-39.50) in the absence of reflux and 12 (11-21.80) in the presence of reflux. A significant relationship was also found between reflux and the PT (%) value (p=0.032). The median PT (%) was 89.3 (0.88-120.8) in the absence of reflux and 95 (45.50-108) in the presence of reflux. The relationship between reflux and the PT (INR) variable was found to be significant (p=0.03). No significant relationship was found between fever, diabetes, and tumor presence and the CRP, Ferritin, D-Dimer, WBC, RBC, HGB, PT (Sec), PT (%), and PT (INR) values.

## 4. Discussion

The COVID-19 pandemic threatens the population's health, weakens the economy, and destabilizes the society worldwide (Legido et al. 2020). Although various treatment methods are used in COVID-19 patients, there is no currently proven definitive treatment and the search for an effective treatment continues. The oxygen level decreases due to the virus-related alveolar collapse, but the severity of the shortness of breath increased since the $CO_2$ exchange continues (Dreher et al. 2020). This means that the organism is exposed to oxygen saturation. Respiratory distress has been accepted as an oxygen saturation level below 93% in our country. Detection of decreased oxygen saturation, platelet count, and lymphocyte count, in addition to increased CRP, ESR, prothrombin time, and D-iron in the early stage of viral infections could save lives. Our results indicate that normal or low WBC is generally associated with lymphopenia but a lymphocyte count below 1000/mcL can be associated with more serious diseases. The platelet count is usually normal or slightly low. Diffuse alveolar and endothelial damage associated with thrombus has been reported in the pulmonary small arteries in this condition (Kuno et al. 2020). CRP and ESR are generally high. The prothrombin time and D-dimer elevation can be associated with serious diseases, and have been found to high in diabetes patients (Khot and Nadkar 2020). The most common comorbid disease was found to be cardiovascular disease with 28.2% of the cases having hypertension, 8.6% coronary artery disease, and 6.9% heart failure (HF) in a study from the United States of America including 8438 COVID-19 (Palmieri et al. 2020). We similarly found COVID-19 to affect the cardiovascular system, directly or indirectly. The morbidity and mortality seem to increase when COVID-19 is accompanied by CVD. The mean age of the 59304 COVID-19 patients who died was 80 years and the death rate was higher in men in the December 2020 report from Italy, the country with the highest death rate (Drakesmith and Prentice, 2008). Besides, some studies report those elderly men are more likely to be infected and rapidly develop acute respiratory distress syndrome (ARDS), creating a life-threatening situation (Guo et al. 2020).

Iron is important for both biological and physiological events. In case of disease, both the pathogen and the infected person need iron. RNA gains active multi-reactive capacity and the ability to catalyze new chemical reactions in limited oxygen respiration or in the presence of iron in the environment in disease states. People with congenitally or acquired strong immune systems can carefully regulate the iron metabolism to limit the presence of iron at times of infection. Iron can increase virulence and pro-oxidant reactions and contribute to oxidative stress in the lungs. The result is peroxidation, which leads to cellular damage in the blood and tissues. Viruses target the same channel used for iron transfer during pH-dependent cellular entry into the intracellular acidic compartments. The inflammatory effect has been seen to cause disturbed gas exchange between the alveoli and blood vessels, leading to pulmonary fibrosis and organ failure, in the pathogenesis (Drakesmith and Prentice 2008). The incidence of oxidative stress and acute respiratory distress syndrome (ARDS) in COVID-19 patients has been reported as 17-29% and the rate for intensive care need in these patients as 23-32% (Goh et al. 2020). ARDS patients are exposed to severe oxidative stress during this process. COVID-19 glycoproteins bind to the heme in the erythrocyte and the toxic oxidative iron within the specific structure is separated. Free iron circulates freely when outside the cell. Hemoglobin can no longer bind to oxygen without the iron ion. In other words, erythrocytes lose their biological activity when hemoglobin deteriorates. Erythrocytes circulate without oxygen only with the porphyria-related COVID-19 virus. The insufficient oxygen in the tissues causes severe hypoxia and a decrease in blood oxygen saturation. When the iron ion is released from the heme structure of millions of erythrocytes in patients carrying the pathogen, this iron increases prooxidant reactions and causes oxidative stress in the lung (Thell 2003). In contrast, there is oxidant-antioxidant balance in the organism under normal conditions. One of these defense systems consists of the small macrophages that circulate and clean free radicals such as this oxidative iron, while endogenous antioxidant enzymes constitute another (Güven and Kaya, 2005). The transferrin iron saturation is reported to increase to high levels in viral pneumonia and accordingly a deterioration to be present in the level of antioxidant protection. (Thomas and Thomas 2017). The presence of excess iron outside healthy lung cells decreases the defenses against viral infections. In the presence of severe oxygen deficiency in patients with COVID-19, the RBC values in the patient's circulation provide direct or indirect information about the severity of the disease (Ruddell et al. 2009). Whether serum ferritin, which indicates the status of iron storage in humans, is released from the cell with an active mechanism or by cell leakage is not certain. However, while many studies report that it can function as a proinflammatory cytokine responsible for cell damage (Guo et al. 2020). It also protects the cells during the oxidation of Fe (II) to Fe (III). Besides, after SARS-CoV-2 enters the host cell, it encodes the production of structural and non-structural proteins during the viral replication stage and invades hemoglobin, which is one of the non-structural proteins, and removes the iron atom, and

also prevents the transport of the oxygen by binding to the relevant region. Such hemoglobin types are considered risky hemoglobins. This clinical picture may explain the rapidly developing hypoxia. Theoretically speaking, the viral load would be primarily responsible for the severity of the disease, since patients with certain diseases have risky hemoglobin (Liu et al. 2020).

While C-reactive protein (CRP) values were observed to increase in patients with a ground glass image by thorax CT in the lungs infected with COVID-19 after detecting a positive PCR test in the blood, lymphopenia together with increased creatinine phosphokinase (CPK), ferritin, D-iron (>1 mcg/ml), and prothrombin time values were found, possibly as a result of kidney damage (Kappert et al. 2020). The similar situation in our study strengthens the view that the CRP value can be considered an indicator of disease severity. Wang et al. (2020) have reported that increased severity of the disease is associated with increased iron levels. The biochemistry laboratory results of 63 COVID-19 patients have revealed that the blood iron values were parallel to the classification of the disease severity in the patients as light, moderate, severe and critical (Sun et al. 2020). Increased iron concentrations in the body cause tissue damage, inflammation, and impaired organ function. The pathological changes caused by the irregularities in serum ferritin are also important (Lan et al. 2020). Certain biochemical values are reported to undergo more significant changes in covid-19 patients with diabetes mellitus and reach the level of hyperferritinemia syndrome (Mehta et al. 2020). and this could increase the mortality rate due to immunosuppression (Mehta et al. 2020; Shoenfeld 2020). Monitoring of the serum iron level is recommended in other studies and regulators of iron homeostasis such as serum ferittin and hepadin are stated as a possible reason for the elevated serum ferritin. It can therefore be said that D-iron and ferritin have a strong correlation with the diagnosis and disease severity (Kurz et al. 2011). The infection symptoms and signs and the test values in the diabetic COVID-19 patients in our study were similar to classic COVID-19 cases.

Advanced age itself is seen to be a factor in decreasing the immune response and the prevalence of this weakened state increases markedly in this age group. Considering the data obtained in the study and the age of the patients, it is necessary to address the possible COVID-19 biochemical parameters that increase disease severity singly or in combination, analyze by which mechanisms the negative effects occur, and implement the relevant practices in diagnosis and treatment. For example, markers such as iron should be investigated in addition to the possible mechanisms mediated by ACE2. These findings could be very important and provide clues to clinicians and biochemists about the prognosis and mortality of COVID-19. We therefore recommend conducting specific studies that further reveal the metabolic pathways of virus-iron interaction and focusing on decreasing the iron levels to enable more specific treatment in viral infections. Hypercoagulopathy and thrombosis were common in this infection and the major reason for many complications seen in COVID-19. Creatine kinase, cardiac troponins, myoglobin were used to assess cardiac status; aspartat aminotransferase, albumin, lactate dehydrogenase were used broadly to assess liver function and fibrin degradation products, activated partial thromboplastin time and D-dimer were used to determine the thrombotic state.

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# Thymoquinone Prevents Valproic Acid-Induced Nephrotoxicity in Rat Kidney

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**Abstract:** Valproic acid (VA), widely used as an antiepileptic, causes structural and functional kidney disorders. Whether thymoquinone (TQ) has a beneficial effect on VA-induced nephrotoxicity has been investigated. Twenty-one male Spraque Dawley rats were grouped into control, VA, and VA + TQ groups (n=7 for per group). VA (500 mg/kg/day) and TQ (50 mg/kg/day) were applied to the rats orally for 14 days. They were euthanized on the 15th day of the treatment. The cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) gene expression levels, biochemical parameters, total antioxidant/oxidant statuses (TAS/TOS), oxidative stress index (OSI), histological and immunohistochemical analysis were performed to evaluate kidney toxicity. In the VA + TQ group, COX-1 expression levels increased, while COX-2 expression levels decreased. While the creatinine (Cr) and blood urea nitrogen (BUN) levels, production of caspase-3 (CAS-3) and NADPH oxidase-4 (NOX-4) were increased in the VA-treated group, they were decreased in VA + TQ group. Treatment with TQ against VA administration decreased TOS and OSI levels while increasing TAS. TQ protects the kidney against the toxic effects of VA.

**Keywords:** Apoptosis, COX-1, COX-2, Oxidative stress, Thymoquinone, Valproic acid



## 1. Introduction

Valproic acid (VA), an antiepileptic agent, has been used for the treatment of different types of seizure and psychiatric disorders for over 30 years. Although it is used as a safe drug in the treatment of diseases, high doses and long-term use bring some adverse effects e.g. weight gain, liver toxicity, gastrointestinal problems, pancreatitis, thrombocytopenia, encephalopathy, hematological abnormalities, and renal injury (Knights and Finlay 2014; Gezginci-Oktayoglu et al. 2016; Heidari et al. 2018). The renal injury in human and animal models caused by VA has been shown by previous studies but the mechanisms remain unclear yet (Altunbaşak et al. 2001; Endo et al. 2010; Gezginci-Oktayoglu et al. 2016; Heidari et al. 2018).

Toxic intermediates like VA can cause renal injury as a consequence of the formation of reactive oxygen species (ROS), inflammation, and fibrosis, and can disrupt the structure of the kidney (Chang and Abbott 2006; Sher et al. 2015). Studies have shown that VA causes oxidative stress, inflammation, carnitine and mitochondrial deficiencies, and fibrosis in kidney tissue (Hamed 2017). In particular, renal dysfunction and mitochondrial damage decrease ATP production and increase oxidative stress, impair apoptosis, and cause necrosis by leading to microvascular loss and fibrosis, thereby risking kidney function (Eirin et al. 2017). Oxidative stress in patients with acute renal failure is associated with an imbalance between the antioxidant system and ROS production (Ragheb et al. 2009).

The kidneys have important roles in the regulation of water, electrolyte, nitrogen, and acid-base balances to maintain the body's homeostasis (Faria et al. 2019). The kidney has abundant blood flow and a large capillary surface area. Due to the kidney's role as an excretory pathway and detoxifying effect of many drugs, it is vulnerable to drugs, and 20% of total acute renal failure cases are drug-related (Fanos and Cataldi 2002). Kidney dysfunction, which is often associated with drug use, can lead to accumulation and clinical toxicity of antiepileptic drugs, prolonging the elimination period (Asconapé 2014). The mechanisms of kidney injury include a wide network of signaling pathways driven by the interaction of ROS, apoptotic factors, and inflammatory cytokines/chemokines (El Sabbahy and Vaidya 2011). VA treatment induces the formation of ROS (Tung and Winn 2011). Increasing levels of ROS which are

also important secondary messengers in cellular signaling lead to the oxidation of DNA, proteins, and lipids that cause cellular damage in the kidney (Irazabal and Torres 2020).

Previous studies indicate that prototypical inflammatory cytokines e.g. TNF-α, interleukin 1β (IL-1β), IL-6, and prostaglandins (PGs), especially PGE 2, play an important role among the diverse inflammatory mediators that lead to epilepsy (Cole-Edwards and Bazan 2005; Vezzani et al. 2008). Cyclooxygenase (COX) is the main target of nonsteroidal anti-inflammatory drugs (NSAIDs), of which VA is one of them, and is a rate-limiting enzyme in PG synthesis (Takemiya et al. 2007; Blumenfeld et al. 2012). Neuroinflammation has a critical role in brain disorders, and COX is one of the major drug targets for reducing neuroinflammation (Dhir 2019). There are at least two COX isoenzymes: COX-1 and COX-2. COX-1 is a builder and produces PGs that protect the stomach and kidneys from damage (Vane and Botting 1998). COX-1 maintains the homeostatic functions of the body and is constitutively expressed in a variety of cells and tissues such as parenchymal cells of some organs, e.g. kidneys, endocrine glands, and neurons observed in the neuroendocrine system, and reproductive system (Zidar et al. 2009; Dhir 2019). COX-2 produces PGs that contribute to pain and swelling of inflammation (Vane and Botting 1998). In addition, COX-2 is a subtype that is upregulated by stimuli such as pain, inflammation, or cancer proliferation (Dhir 2019). PGs produced by COX-1 are responsible for the regulation of renal blood flow, mucous membrane protection, and homeostasis, while COX-2 is responsible for high prostanoid production at sites of disease and inflammation (Oksuz et al. 2016).

Nephrotoxicity is associated with mortality and morbidity, and the use of natural products has a very important place in investigating drug-induced nephrotoxicity and eliminating the side effects of the drug. Therefore, in this study, thymoquinone (TQ), a natural antioxidant, was used against nephrotoxicity caused by VA. TQ, as the most abundant component of the essential oil of black cumin (*N. sativa*) seeds, has properties such as antimicrobial, anti-nociceptive, anti-epileptic, hypoglycemic, hypolipidemic, and bronchodilator properties (Tavakkoli et al. 2017). TQ exhibits a variety of pharmacological activities including nephroprotection, gastroprotection, anti-hepatocellular carcinoma, cardioprotection, neuroprotection, anti-allergy, retinal protection, bladder protection, reproductive system protection, and respiratory protection (Talebi et al. 2021; Tastemir Korkmaz et al. 2021).

In this study, we aimed to investigate whether the antioxidant and anti-inflammatory features of TQ have ameliorative effects against oxidative stress-induced damage of VA in kidney cells.

## 2. Materials and Method

### *2.1. Study groups and experimental design*

Twenty-one 3-4 month-old and 250-300 g weighted Sprague Dawley albino male rats were obtained from Fırat University Experimental Research Center in Elazığ/Turkey. They were kept in polycarbonate cages with free access to food and water at 24°C, 42% ± 5% relative humidity, and a 12/12 hour light/dark cycle. They were randomly separated into 3 groups: Control (n=7), VA (n=7), and VA + TQ (n=7). Nothing was applied to the rats in the control group. The VA group received daily doses of VA (500 mg/kg) for 14 days whereas TQ (50 mg/kg) was given in addition to VA (500 mg/kg) to the VA + TQ group for 14 days orally (Atta et al. 2017; Barrett et al. 2017). When the study was completed, all rats were sacrificed through cervical dislocation under anesthesia. Venous blood samples were centrifuged at 5000 x g after collection and serums were obtained for biochemical analysis. Besides, the kidneys were taken for genetic, histological, and immunohistochemical analysis, and both the serums and kidneys were stored at -80 °C until use. This study was approved by Animal Experiments Local Ethics Committee (Protocol no 2017/135).

### *2.2. Biochemical Analysis*

#### *2.2.1. Serum Levels of Creatinine (Cr) and Blood Urea Nitrogen (BUN)*

Serum Cr and BUN levels were measured to assess kidney function. For this purpose, Roche Diagnostics kits (Mannheim, Germany) and Hitachi automatic biochemical analyzer 7060 c (Japan) were used according to the picric acid method (Junge et al. 2004). Cr and BUN levels were analyzed as mg/dL serum.

#### *2.2.2. Serum levels of Total Antioxidant/Total Oxidant Statuses (TAS/TOS) and Oxidative Stress Index (OSI)*

Serum obtained with the automated colorimetric kit Erel (REL Assay Diagnostics, Gaziantep, Turkey) was used to determine the total antioxidant/total oxidant statuses (TAS/TOS). The method applied by Bilgic et al. (2017) was used to determine TAS and TOS levels. Oxidative stress index (OSI) was calculated according to the formula OSI = TOS / TAS (Gul et al. 2017).

### *2.3. COX1 and COX2 Gene Expression Analysis*

#### *2.3.1. RNA Extraction and cDNA Preparation*

Fresh frozen rat kidneys were processed according to the manufacturer's instructions under RNAse-free conditions using total RNA extraction solution (Bioneer) for RNA extraction and its purity was measured in a NanoDrop spectrophotometer (Denovix DS-11) at 260/230 nm and 260/280 nm, and stored at -80 °C. For qRT-PCR, the first 5 µg of total RNA was reverse transcripted using RT PreMix (Bioneer) using appropriate controls to ensure the absence of genomic DNA contamination.

#### *2.3.2. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Assay*

The GreenStar qRT-PCR PreMix (Bioneer) using the ExiCyclerTM96 qRT- PCR system (Bioneer) was used for detecting the expression levels of COX-1 and COX-2 genes. The GAPDH gene was amplified as an internal control. The primers used were taken from previous studies (Kis et al. 2003; Langnaese et al. 2008; Qiao et al. 2013). PCR

conditions were 95 °C for 1 min, followed by 45 cycles at 95 °C for 5 sec, and 55 °C for 40 seconds. The $2^{\wedge-\Delta\Delta Ct}$ method was used to calculate the relative mRNA expression.

### *2.4. Histopathological and Immunohistochemical Analysis*

All kidney tissues preserved in 10% neutral formaldehyde were used for histological and immunohistochemical studies. Binocular light microscopy (ECLIPSE Ni-U, Nikon, Tokyo, Japan) was used to detect damage and healing rate in kidney tissues. Approximately 4-5 µm-thick tissue was obtained from paraffin-wax embedded kidney tissue with microtome stained with hematoxylin-eosin for detecting structural changes (vascular congestion, dilatation, and degeneration of proximal-distal tubules, glomerular degeneration, tubular dilatation, hemorrhagic areas, and mononuclear cell infiltration in the medulla) in all groups. The results were evaluated according to the scoring made by Abdel-Wahhab et al. (1999). The method mentioned by Savran et al. (2020) was used for detecting and scoring the activity of Caspase 3 (CAS-3) and NADPH oxidase-4 (NOX-4).

### *2.5. Statistical Analysis*

Statistical Package for the Social Sciences (SPSS) 25.0 Software program was used for all comparisons. All data are presented as mean ± SEM. The normality was identified by the Shapiro-Wilk test. One-way ANOVA after LSD posthoc was used for the comparison of parametric values in genetic parameters. A semi-qualified evaluation of the histopathological scores was evaluated with the Mann Whitney U test to determine significant differences between groups. A p-value less than 0.05 was considered statistically significant.

## 3. Results

### *3.1. Serum Levels of Oxidative Stress Biomarkers*

Cr and BUN levels are shown in Table 1. They were increased in the VA group against the control and VA + TQ groups and these increases were found statistically significant ($p < 0.05$). Additionally, the levels of TOS and OSI were increased significantly in the VA group when compared with the control and VA + TQ groups ($p < 0.05$) (Table 1).

It was observed that TAS levels increased with TQ applied against VA, while TOS and OSI levels decreased ($p < 0.05$). TAS level was found to be significantly higher in the control group compared to the VA group ($p < 0.05$). While TAS level increased significantly in VA + TQ group compared to VA ($p < 0.05$), TOS level increased in the VA group when compared to the control and VA + TQ groups, and these differences were found statistically significant ($p < 0.05$). OSI level was found higher in the VA group than control and VA + TQ groups significantly ($p < 0.05$) (Table 1, Figure 1).

**Table 1** Serum biochemical and renal tissue oxidative stress biomarkers of the experimental groups.

| | STUDY GROUPS | | |
|---|---|---|---|
| | **Control** | **VA** | **VA + TQ** |
| **BUN (mg/dL)** | $30.600 \pm 1.039^{b}$ | $56.00 \pm 5.263^{a,c}$ | $41.00 \pm 4.313^{b}$ |
| **Cr (mg/dL)** | $0.530 \pm 0.018^{b}$ | $0.613 \pm 0.031^{a,c}$ | $0.548 \pm 0.043^{b}$ |
| **TOS (µmol/L)** | $3.42 \pm 0.62^{b}$ | $6.41 \pm 0.42^{a,c}$ | $4.31 \pm 0.56^{b}$ |
| **TAS (mmol/L)** | $1.45 \pm 0.15^{b}$ | $0.61 \pm 0.05^{a,c}$ | $1.15 \pm 0.28^{b}$ |
| **OSI (AU)** | $0.25 \pm 0.06^{b}$ | $1.13 \pm 0.14^{a,c}$ | $0.57 \pm 0.23^{b}$ |

Each group represents the mean ± SEM for seven rats. a: Significant from Control; b: Significant from VA; c: Significant from VA + TQ. $p < 0.05$. Abbreviations: Cr, creatinine; BUN, blood urea nitrogen; TAS, total antioxidant status; TOS, total oxidant status; OSI, Oxidative stress index; VA: valproic acid; TQ: thymoquinone; VA: 500 mg/kg VA; VA + TQ: 500 mg/kg VA + 50 mg/kg TQ. AU: Arbitrary Units

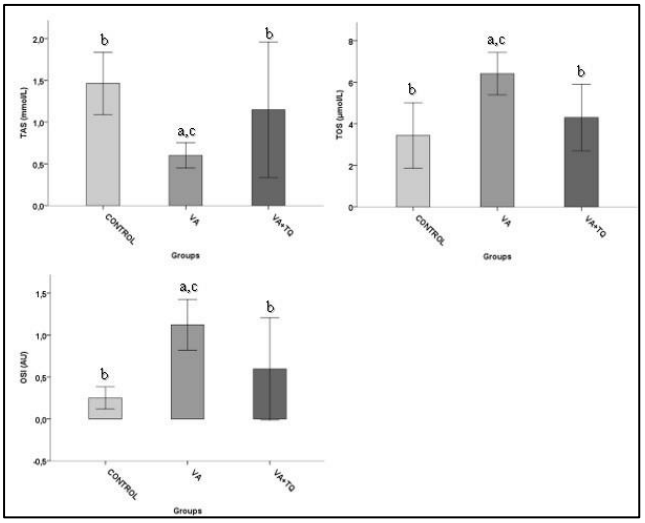


**Fig. 1** Effects of VA, TQ, and their coadministration on the serum level of TAS, total antioxidant status; TOS, total oxidant status; OSI, Oxidative stress index in rats after two weeks. Values are expressed as mean ± SEM of seven animals. ANOVA followed by the LSD post hoc test were used. a $p<0.05$ versus control; b $p<0.05$ versus VA treated rats; c $p<0.05$ versus VA + TQ treated rats. Abbreviations: VA: valproic acid; TQ: thymoquinone; VA: 500 mg/kg VA; VA + TQ: 500 mg/kg VA + 50 mg/kg TQ. AU: Arbitrary Units.

### *3.2. COX1 and COX2 Gene Expression Levels*

Table 2 shows the expression levels of COX-1 and COX-2 genes in control, VA, and VA + TQ groups. Statistically, COX-1 expression levels were decreased in the VA group, while COX-2 expression levels were increased ($p < 0.05$). In the VA + TQ group, COX-1 expression levels increased, while COX-2 expression levels decreased ($p < 0.05$).

### *3.3. Histopathological and Immunohistochemical Findings*

Table 3 shows the histopathological findings in the present study. No other findings than normal histological structures were found in the histological examination of the kidney tissue sections of the control group (Table 3, Figure 2: a-b). Significant structural changes including dilatation and

degeneration in proximal-distal tubules, vascular congestion, hemorrhagic areas, glomerular degeneration, tubular dilatation in the medulla, and mononuclear cell infiltrations were observed in the VA group compared with the control group ($p < 0.05$) (Table 3, Figure 2: c-c1-c2-c3). An improvement in histopathological findings was observed in the VA + TQ group compared to the VA group ($p < 0.05$) (Table 3, Figure 2: d-e).

**Table 2** Effects of VA and TQ on the expression of COX-1 and COX-2 genes in rat kidneys

| Groups | Expression value (Ct value; Mean ± SEM) | |
|---|---|---|
| | COX-1 | COX-2 |
| **Control** | 35.10 ± 1.05 [b] | 32.70 ± 0.77 [b] |
| **VA** | 30.57 ± 1.22 [a,c] | 39.07 ± 1.42 [a,c] |
| **VA + TQ** | 34.98 ± 1.64 [b] | 34.57 ± 1.51 [b] |

Each group represents the mean ± SEM of seven rats. a: Significant from control; b: Significant from VA; c: Significant from VA + TQ; $p < 0.05$

**Table 3** Histopathological changes of renal samples in the experiment groups

| Parameters/Score | Experimental groups | | |
|---|---|---|---|
| | Control | VA | VA + TQ |
| Tubular dilatations in the medulla | – | +++ [a] | ++ [b] |
| Proximal - distal tubule dilatation | – | +++ [a] | ++ [b] |
| Proximal - distal tubule degeneration | – | +++ [a] | ++ [b] |
| Glomerular degeneration | – | +++ [a] | ++ [b] |
| Vascular congestion | – | +++ [a] | ++ [b] |
| Interstitial mononuclear cell infiltration | – | +++ [a] | ++ [b] |
| Hemorrhagic area | – | +++ [a] | ++ [b] |

Scoring system is as described in the methods section. n = 7. Abbreviations: VA, Valproic acid; TQ, thymoquinone. a: valproic acid increased renal damage, $p < 0.05$ vs. control. b: thymoquinone reduced renal damage, $p < 0.05$ vs. VA.

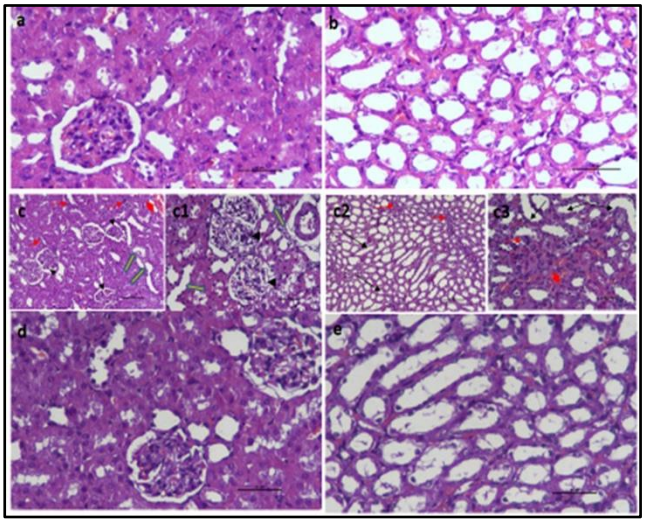


**Fig. 2** Kidney tissue sections of the control and experimental groups. a (cortex) and b (medulla); normal histological appearance is observed in the kidney tissue sections of the control group. (a; cortex; Bowman's capsule, distal, proximal tubules b; medulla; collecting tubules are observed normally (H–E, x20). c - c2 (cortex), c1 - c3 (medulla); kidney tissue sections belonging to the group given VA: red arrows; mononuclear cell infiltrates, yellow filled arrows; prochymal – distal tubule dilatations, black arrowheads; glomerular degenerations, red arrowhead; hemorrhagic area, black arrows; tubular dilatations in the medulla (c- c2; H–E, x20, c1-c3; H–E, x40 ). d (cortex) and e (medulla); Renal tissue sections of the group given VA + TQ: compared to VA group, a significant improvement is observed in the cortex and medulla (H–E, x20).

The immunochemical findings are shown in Table 4 and Figures 3 - 4. In the immunohistochemical staining of kidney tissue sections, a significant difference was found between the control group and the VA and VA + TQ groups ($p < 0.05$). In the kidney samples, CAS-3 immunoreactivity was observed intensely in the VA group but not in the control group. Interestingly, it was poorly observed in VA + TQ group (Table 4, Figure 3). At the same time, NOX-4 immunoreactivity was also observed similar to CAS-3 immunoreactivity results (Table 4, Figure 4).

**Table 4** Immunreactivity grades of CAS–3 and NOX–4 in renal tissues of experimental groups.

| Target stained immunohistochemically | Experimental groups | | |
|---|---|---|---|
| | Control | VA | VA + TQ |
| CAS–3 | – | +++ [a] | + [b] |
| NOX–4 | – | +++ [a] | + [b] |

(n = 7 for each group). Scoring system is described in the methods section shows the apoptotic renal ratio. Abbreviations: VA, valproic acid; TQ, thymoquinone. a: valproic acid increased renal damage, $p < 0.05$ vs. control. b: thymoquinone reduced renal damage, $p < 0.05$ vs. VA.

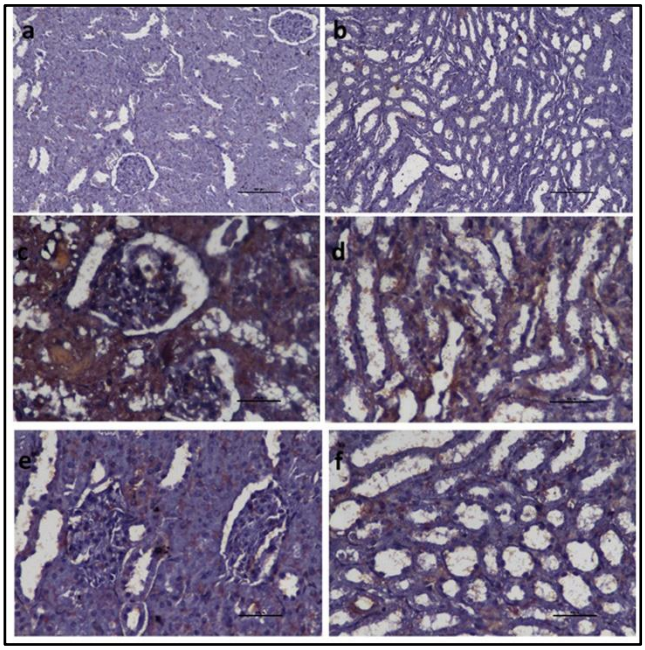


**Fig. 3** Kidney tissue section of the control and experimental groups immunostaining with CAS-3. a (cortex) and b (medulla); Control group kidney tissue sections were stained negative with CAS-3, brown areas are not observed (immunostaining, x20). c (cortex) and d (medulla) VA group kidney tissue sections stained positively with CAS-3, with no large amounts of brown areas observed (immune staining, x40). VA + TQ group kidney tissue sections e (cortex) and f (medulla) show less positive staining brown areas with CAS-3 compared to VA group (immune staining, x20).

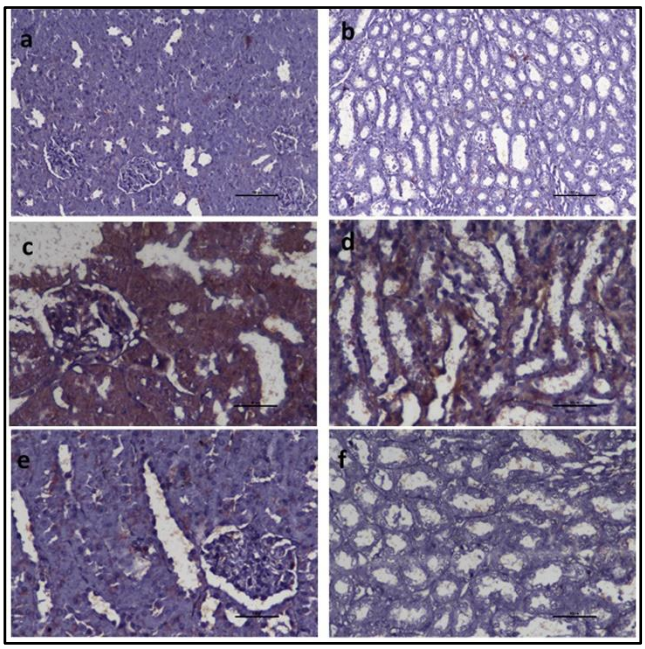


**Fig. 4** Kidney tissue section of the control and experimental groups immunostaining with NOX-4. Control kidney tissue sections a (cortex) and b (medulla) stained negatively with NOX-4 and no brown areas were observed (immune staining, x20). c (cortex) and d (medulla) VA group kidney tissue sections stained positively with NOX-4, with no large amounts of brown areas observed (immune staining, x40). VA + TQ group kidney tissue sections e (cortex) and f (medulla) show less positive staining brown areas with NOX-4 compared to VA group (immune staining, x20).

## 4. Discussion

VA is a well-tolerated, effective antiepileptic drug that is widely used as a mood stabilizer (Ornoy et al. 2020). Previous studies have reported markers of tubular and renal glomerular injury as a result of chronic use of some antiepileptic drugs such as VA (Hamed 2017). In previous studies, it was observed that kidney and liver functions were impaired, thrombocytopenia and coagulopathy developed after 3 months of VA treatment (Yaman et al. 2013). A previous study has reported that exposure to various drugs for therapeutic or diagnostic purposes causes damage and clinical manifestations in renal tubules, interstitium, glomerulus, and renal microvasculature (Malyszko et al. 2017).

In this study, BUN and Cr levels increased as a result of kidney function loss. Makris and Spanou (2016) have reported that acute kidney injury is characterized by increased blood levels of BUN and Cr, as well as oliguria and electrolyte disturbances. It was found that BUN and Cr levels were significantly higher in VA-treated rats than in the control group, indicating damage in the function and structure of the kidneys. TQ used in this study is to reduce the nephrotoxic effect of VA. It was found that VA + TQ treatment significantly reduced BUN and Cr levels. TQ is a well-known ROS scavenger and antioxidant (Karimi et al. 2019). The findings of this study demonstrate the protective effects of TQ against VA-induced nephrotoxicity in rats. Studies have reported that TQ reduces serum BUN and Cr levels and protects kidney function (Badary et al. 2000; Jalili et al. 2017).

In this study, TQ inhibited elevated TOS and OSI levels and increased TAS, ameliorated impaired kidney function, and reshaped histopathological changes in the kidney. According to the study, TOS and OSI levels increased and TAS levels decreased in VA-treated rats (Takeuchi et al. 2005). On the other hand, the results showed that TQ protects against VA-induced kidney injury by suppressing oxidative stress and apoptosis, as reported in previous studies (Tekbas et al. 2018).

It has been reported that COX-2 expression is associated with inflammation and cancer proliferation, while COX-1 plays a cleaning role in the homeostatic process (de Leval et al. 2000). In this study, COX-1 expression levels decreased in the VA group, while COX-2 expression levels increased. COX-1 inhibition has positive effects on seizure suppression, while COX-1 inhibition has been reported to cause gastrointestinal toxicity (Wallace et al. 2000; Barbalho et al. 2016). COX-2 is increased in PG production, clinical inflammation, and some types of human cancer, particularly colon cancer (Crofford 1997). In the study, while COX-1 expression levels increased in the TQ group, COX-2 expression levels decreased. COX-1 inhibition can cause side effects such as kidney and liver failure (Dhir 2019). COX-2-induced inflammation contributes to epileptogenesis and neuronal damage that develops after brain injuries (Polascheck et al. 2010). Anti-inflammatory

treatments such as selective COX-2 inhibitors can be used as anti-epileptogenesis in brain injuries such as cerebral ischemia, head injury, or status epilepticus (SE) (Polascheck et al. 2010). In the study, it was reported that the inhibition of COX-1 upregulated the expression of COX-2.

When the histological results were evaluated, normal kidney histology was observed in the control group. In the VA group, important histopathological changes such as tubular dilatations, proximal-distal tubule dilatation, proximal-distal tubule degeneration, glomerular degeneration, vascular occlusion, interstitial mononuclear cell infiltration, and hemorrhagic area were observed in the medulla. Histopathological changes were very few in the group treated with VA + TQ. According to the histological results, it can be said that TQ has a corrective effect on kidney damage.

CAS-3 is a protease with a well-known role in apoptosis (Shalini et al. 2015). CAS-3 is known to have a role in cell growth and differentiation, cytokine maturation, and apoptosis (Yan et al. 2013). Generation of ROS and upregulation of CAS-3 lead to apoptosis (Rana 2008). CAS-3 immunoreactivity, a marker of apoptosis, was not seen in the control group, whereas immunostaining of CAS-3 was strong in the VA group. CAS-3 immunoreactivity was higher in the VA group than in the VA + TQ group. From this point of view, it can be said that TQ reduces VA-induced apoptosis. TQ was found to prevent programmed cell death by inhibiting CAS-3.

NOX-4, the major isoform of NADPH in the kidney, produces $H_2O_2$ and contributes to redox processes by activating multiple signaling pathways in kidney diseases such as acute kidney injury, diabetic nephropathy, and hypertensive nephropathy (Yang et al. 2018). Upregulation of NOX-4, an important source of ROS, causes kidney damage. In the present study, NOX-4 immunoreactivity was not seen in the control group, but it was seen at high intensity in the VA group. The immunoreactivity was weaker in the VA + TQ group compared to the VA group. TQ appears to prevent VA-induced nephrotoxicity with its antioxidant properties. Previous studies have shown that TQ protects by showing anti-inflammatory and antioxidant properties in various kidney diseases such as renal ischemia-reperfusion, diabetic nephropathy, and nephrotoxicity caused by inflammation and oxidative stress (Shaterzadeh-Yazdi et al. 2018; Erdemli and Yigitcan 2020; Azirak et al. 2022).

## 5. Conclusion

This study demonstrated the beneficial effects of TQ against VA-induced nephrotoxicity by reducing oxidative stress due to its antioxidant, and anti-inflammatory effects. However, more clinical studies are needed to confirm these results.

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**Authors' contributions:**

Study design (SA, MKÖ), Biochemical analysis (SB), Genetic analysis (SA, DTK), Histological and immunohistochemical analysis (MÖ), Analysis and interpretation of the data (SA, DTK, SB, MÖ, MKÖ), The drafting of the paper (SA, DTK), Final approval of the version to be published (SA, DTK, SB, MÖ, MKÖ)

**Conflict of interest disclosure:**

There is no conflict of interest.

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# Türkiye'nin Adana ve Osmaniye illerinde *Callophrys mystaphia* Miller, 1913 (Lepidoptera: Lycaenidae: Eumaeini)'nın İlk Kaydı

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**Özet:** Adana ili Saimbeyli ilçesi ve Osmaniye ili Yarpuz Yaylası Bezelik Daz'ında 2021 yılı Nisan-Eylül ayları arasında yapılan arazi gözlem çalışmalarında Işgın Zümrütü-Minik Zümrüt (*Callophrys mystaphia*) tespit edilmiştir. Tür, Adana ve Osmaniye illeri için yeni kayıt niteliğindedir. Türün erkek bireyinin morfolojik tarifi ve bazı parametreleri ölçülerek çalışmamızda verilmiştir.

**Anahtar Kelimeler:** *Callophrys mystaphia*, Işgın Zümrütü, Minik Zümrüt, Lycanidae, Lepidoptera.

## *The First Record of Callophrys mystaphia Miller, 1913 (Lepidoptera: Lycaenidae: Eumaeini) in Adana and Osmaniye Provinces of Turkey*

**Abstrac:** In the field observation studies carried out between April and September 2021 in Adana province Saimbeyli district and Osmaniye province Yarpuz Plateau, Miller's Green Hairstreak (*Callophrys mystaphia*) was detected. The species is a new record for Adana and Osmaniye provinces. The morphological description of the male individual of the species and some parameters have been measured and given in our study.

**Keywords:** *Callophrys mystaphia,* Miller's Green Hairstreak, Lycanidae, Lepidoptera.



## 1. Giriş

Türkiye'de *Callophrys* Bilberg, 1820 (Lycaenidae: Eumaeini) cinsi altı tür ile temsil edilmektedir (Koçak ve Kemal 2018). Bu türlerden biri olan *Callophrys mystaphia* Işgın Zümrütü ya da Minik Zümrüt olarak bilinmektedir. Tür, Miller tarafından 1913 yılında ilk kez Türkiye'nin doğusunda, Ermenistan sınırı yakınlarında, Iğdır ili, Aras Nehri Vadisinde kaydedilmiştir (Krupitsky ve Kolesnichenko 2013). Dünya Doğayı ve Doğal Kaynakları Koruma Birliği (International Union for Conservation of Nature and Natural Resources, kısaca **IUCN**) tarafından tür, Tehlikede (EN) ve Endemiğe Yakın olarak değerlendirilmektedir (Karaçetin ve Welch 2011). Kemal (2008), Türkiye ve İran'dan çok az bilinen bu türün Van Gölü Havzasında yalnız Erak Dağında bulunduğunu ve konukçu bitkisi olan Işgın (*Rheum ribes*) ile birlikte yok olma tehlikesi ile karşı karşıya olduğunu bildirmektedir. Tür, Türkiye'de Adıyaman, Hakkâri, Kars, Kahramanmaraş, Siirt, Tunceli ve Van illerinde (Tshikolovets 2011; Koçak ve Kemal 2018), Güneybatı İran'da Kuh-e Dinar'daki Dena Koruma Alanında yaşamaktadır (Karaçetin ve Welch 2011).

Minik Zümrüt besin bitkisinin varlığıyla sınırlı bir dağılışa sahiptir. Küresel yaşam alanı yaklaşık olarak 24 km$^2$'dir. Işgın Zümrütü, mayıs ve haziran aylarında uçan, 2200-2800 metre yükseklikte kaydedilmiş, yılda tek nesil veren bir türdür. Larvanın beslendiği bitki Türkiye'de Işgın (*Rheum ribes*) ve İran'da *Rheum persicum*'dur. Yumurtalar besin bitkisine bırakılmakta ve tırtıllar laboratuvar ortamında yaklaşık 25 günde gelişmektedir. Pupa evresi yaklaşık 11 aydır (Karaçetin ve Welch 2011).

Küçük, yerel ve izole dört ya da beş parçalanmış alt-popülasyon şeklinde bulunduğu bilinen bu türün muhtemelen Türkiye'de yaşayan en nadir tür olduğu düşünülmektedir ve türün Türkiye'deki en büyük tehdidi, sapları Van, Erzincan ve Ağrı illerindeki yerel pazarlarda

sebze olarak satılan ışgın bitkisinin toplanmasıdır. Aşırı toplanma ışgın bitkisi popülasyonunun ciddi oranda azalmasına neden olmaktadır. Buna bağlı olarak da her ışgının üzerindeki tırtıllar popülasyon dışı kalacak ve böylece de kelebek popülasyonunda düşüşler olacaktır (Karaçetin ve Welch 2011).

## 2. Material ve Method

Çalışmamız arazi ve laboratuvar çalışmaları şeklinde yürütülmüştür. Arazi çalışmaları Adana ili Saimbeyli ilçesi ve Osmaniye ili Yarpuz Yaylası Bezelek Daz'ında 2021 yılı Nisan-Eylül ayları arasında yapılmıştır. Arazi çalışmaları gündüz saatlerinde 08 ile 18 saatleri arasında gözlem ve fotoğraf çekme şeklinde yapılmıştır. Laboratuvar çalışmalarında ise müze materyali olarak araziden alınan kelebek örnekleri değerlendirilmiştir. Atay (2006)'ın çalışmasına göre müze materyali haline getirilen kelebek örnekleri Hatay Mustafa Kemal Üniversitesi Biyoloji Bölümü Zooloji Laboratuvarında saklanmaktadır.

## 3. Bulgular ve Tartışma

**Genus:** *Callophrys* Bilberg, 1820 (Lepidoptera: Lycaenidae: Eumaeini)

***Callophrys mystaphia*** Miller, 1913

Kanat açıklığı, 28-31 mm; ön kanat uzunluğu 12 mm, arka kanat uzunluğu 10 mm'dir. Anten topuzu siyah, topuz ucu sarı renkte pullarla kaplıdır. Baş tepesi sarı, göz çevresi, yanaklar ve labial palpus yeşil pulludur. Erkek ve dişilerde ön ve arka kanat renklenmesi benzerdir. Ön ve arka kanat üzeri tamamen kahverengidir, kanat altları ise tamamen zümrüt yeşilidir. Kanat saçakları ince ve sarımsıdır. Ön ve araka kanat altlarında küçük beyaz lekelerden oluşan bir orta dış bandı benek dizisi vardır, bu bandı oluşturan lekeler ön kanat altında yuvarlak ve çok küçüktür, ancak bu benek dizisi lekeleri eksiktir. Arka kanat anal açı bölgesinde dışa doğru bir lop şeklinde çıkıntı yoktur, bu bölge az çok yuvarlaklaşmıştır (Resim 1).

Koçak (2018)'ın bildirdiğine göre Türkiye'nin kelebek çeşitliliği altı familyada 412, Osmaniye 89 ve Adana 185 türdür (Tablo 1). Minik Zümrüt (*Callophrys mystaphia*) Osmaniye ve Adana illeri için yeni kayıt niteliğindedir. Bu tür ile birlikte Osmaniye 90 ve Adana ise 186 kelebek türüne ev sahipliği yapmaktadır.

Resim 1. Işgın Zümrütü-Minik Zümrüt (*Callophrys mystaphia*)

Tablo 1. Osmaniye, Adana ve Türkiye'nin kelebek çeşitliliği.

| | Osmaniye | Adana | Turkey |
|---|---|---|---|
| Familia | Species | Species | Species |
| Hesperiidae | 6 | 24 | 43 |
| Lycaenidae | 29 | 71 | 180 |
| Nymphalidae | 16 | 24 | 57 |
| Satyridae | 20 | 32 | 81 |
| Papilionidae | 4 | 8 | 13 |
| Pieridae | 14 | 26 | 38 |
| Total | 89 | 185 | 412 |

## 4. Sonuç

Minik Zümrüt (*Callophrys mystaphia*) Osmaniye ve Adana illeri için yeni kayıt niteliğindedir. Bu tür ile birlikte Osmaniye 90 ve Adana ise 186 kelebek türüne ev sahipliği yapmaktadır. Türün neslinin devamlılığı için üzerinde larvanın beslendiği Işgın bitkisinin aşırı toplatılmaması şarttır. Ayrıca türün üzerinde beslendiği konukçu bitkinin bulunduğu arazilerde hayvan otlatılmasının azaltılması gerekmektedir. Türün yaşadığı alanlarda yeni yolların açılması ve erozyona neden olacak bitki kayıpları yaşam alanı kaybına ve tüm bunların sonucunda Işgın Zümrütünün yaşam alanı kalitesini etkileyecektir. Türün neslinin devamlılığı için alan korunmasın yapılması zorunludur. Kelebekleri zirveye yakın Işgın (Rheum ribes) bitkisinin bulunduğu alanlarda görmek mümkündür (Resimler 2, 3).

Resim 1. Işgın Zümrütü-Minik Zümrüt'ün Larvalarının Beslendiği Işgın Bitkisi (*Rheum ribes*).

Resim 3. Işgın Zümrütü-Minik Zümrüt (*C. mystaphia*) Yaşam Alanı (Osmaniye Yarpuz Yaylası Bezelek Dazı, 1250-1750 m).

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# Investigation of antioxidant properties of olive leave extracts from Hatay by different extraction methods

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**Abstract:** In this study, it was aimed to compare the antioxidant properties of the extracts obtained from the extraction of olive leaves in Hatay province prepared with different solvents. For this purpose, olive leaves were extracted using pure methyl alcohol with soxhlet and also by maceration using 60% ethanol, 70% methanol, 90% acetone (v/v) and distilled water. Total Flavonoid Content (TFC), Total Phenolic Content (TPC), Ferric Antioxidant Reducing Power (FRAP), DPPH radical scavenging avctivity, ABTS Cation Radical Scavenging activity and total sulfydryl groups by Ellman methods were used to determine the antioxidant properties of the extracts. As a result of the study, the highest TFC values per g leaf and g extract were obtained for 70% methanol maceration and soxhlet-methanol extract, respectively, while the highest TPC observed per leaf and g extract were determined in the extracts obtained with 90% acetone. In the FRAP method, the extract obtained with 60% Ethanol showed the highest activity per g leaf and g extract. Extracts obtained with Soxhlet showed the highest activity for both ABTS activity and Ellman method. In the DPPH method, the lowest EC50 value was determined in the extract obtained using 70% methanol, and it was determined that the extracts obtained with water showed the lowest performance in all antioxidant activity methods.

**Keywords:** Olive leaves, Extraction, Antioxidant activity, Soxhlet, Maceration



## 1. Introduction

It is stated that there are an estimated 500 million olive trees in the world and 90% of them are located in the Mediterranean. It has been reported that there are more than 13 million fruit bearing and approximately 3 million non-fruiting olive trees throughout the province of Hatay (2020-2021 Yield report).

Olive leaf extract is obtained from olive tree leaves by physical and chemical methods. In recent clinical studies, It has been shown that the olive leaf extract is effective in many conditions such as viral, bacterial and fungal infections, HIV-AIDS, chronic fatigue, hepatitis B, pneumonia, tuberculosis, influenza, common cold, meningitis, shingles, Epstein-barr virus, encephalitis, gonorrhea, severe diarrhea, ear, tooth, urinary tract and surgical infections (Page 2002). It has been reported that the olive leaf has been used in the treatment of malaria and febrile diseases for many years, and the oleuropein in the leaf is an antioxidant and has an antimicrobial effect against viruses, bacteria, molds, fungi and parasites (Benavente-Garcia et al. 2000). At the same time, it has been reported that olive leaves, which have biological value, are a natural, safe and inexpensive alternative antioxidant source and even have the feature of extending the shelf life in foods due to their antimicrobial effects and inhibition of lipid oxidation (Jemai et al. 2009; Boudhrioua et al. 2009; Bouaziz et al. 2010). Utilizing the phenolic compounds found in olive leaves at the highest level can only be achieved by maintaining an effective extraction and solubility properties (Mourtzinos et al. 2007).

Atoms or molecules with one or more unpaired electrons are called free radicals. Such substances are highly reactive because they have unpaired electrons. Free radicals which play an important role in biological systems gain or lose electrons through any interaction. Therefore, they can be positive, negative or neutral (Gönenç et al. 2002). Antioxidants are substances that significantly inhibit or delay oxidation when present in food or in low concentrations in the body (Aeschbach et al. 1995). Food manufacturers use synthetic food preservative antioxidants to prevent food spoilage and preserve the nutritional value of food. Antioxidants are of interest to biochemists and health professionals because they protect the body against damage caused by reactive oxygen species and degenerative diseases.

The market for medicinal and aromatic plants is estimated to be approximately US$14 billion per year and will reach more than US$5 trillion by 2050. About 3000 medicinal and aromatic plant species are traded worldwide, 2000 of which belong to European countries such as Switzerland, Germany and France (Thakur and Kumar, 2021). while India has an important position with 7000 medicinal and aromatic plant species (Prasathkumar et al. 2021). Olive leaves show antioxidant properties due to the functional bioactive compounds in their structure. Thanks to this feature, it has been reported that olive leaf extracts can be used to extend the quality and shelf life of foods (Erbay and Icier 2010; Abaza et al. 2011).

Although few literatures have been reported to reveal the different biological effects of olives regarding its anti-inflammatory, anti-allergic, antibacterial, antimycotic, immunoregulatory, antidiabetic and hypolipemic (Omar 2010) effects; studies of *in vivo* or *in vitro* effects of olive leaves that remain after the olive harvest and destroyed by burning are unsufficient. In addition, variables such as the method used in the extraction process, shredding, drying, temperature and organic solvent type also change the amount of antioxidant substances (Tsimidou and Papoti 2010).

Antioxidants are molecules that prevent or slow down the oxidation of biological macromolecules in living things. The role of antioxidants is to neutralize oxidative molecules via hydrogen atom or electron transfer. Therefore, antioxidants are generally substances with reducing, radical scavenging, oxidizing properties such as polyphenols or thiols (Adwas et al. 2019). To determine antioxidant power, besides determining the amount of phenolic, flavonoid and thiol substances, which are important in showing effectiveness as an antioxidant system, it is also essential to analyze their activities such as radical scavenging, radical quenching and reduction. The antioxidant activity properties of the olive leaves have become one of the remarkable issues. Studies in this regard have gained momentum recently. The antioxidant properties of olive leaves extracts varies according to the type of olive, the agricultural process applied, the region where it is grown and the harvest time as well as the extraction method and the type of solvent used. Antioxidant activity determination methods are important in terms of getting an idea for this purpose. Relative antioxidant capacity is an important antioxidant activity determination method for comparing the antioxidant capacity of different foods, food components measured by two or more chemical analyzes (Amensour et.al. 2010).

The TEAC analysis uses ABTS' heavily colored cation radicals to test the antioxidants' ability to quench radicals. TEAC analysis is widely used for testing antioxidant capacity in food samples (Re et al.1999). With the ABTS method, a large number of samples can be scanned in a short time.

The ferric reducing antioxidant power (FRAP) assay is a typical electron transfer-based method that measures the reduction of ferric ion ($Fe^{3+}$)-ligand complex to the intensely blue-colored ferrous ($Fe^{2+}$) complex by antioxidants in an acidic medium (Kumar S. 2018).

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in ethanol. The biggest advantage of reagent interact with all substances in the mixture, including the weakest antioxidants, and it can react with lipophilic as well as hydrophilic antioxidants. Therefore, it is an easy, reliable and fast method (Kedare and Singh 2011).

In this study, it was aimed to compare the effects of solvents used during extraction on antioxidant activity in olive leaf extracts. For this, five different methods which were reported in the literature were selected and leaf extraction was carried out by applying the soxlet or maceration, and then the total flavonoid content (TFC), total phenolic content (TPC) of these extracts and antioxidant activities were determined by FRAP, DPPH, ABTS, Ellman methods.

## 2. Materials and Method

Ethanol, methanol, acetone, sodium carbonate, aluminum chloride, $K_3[Fe(CN)_6]$ were obtained from Merck; 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), Folin – Ciocalteu's reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), trolox were purchased from Sigma. All other chemicals used are analytical grade. Spectrophotometric measurements were performed with a Perkin Elmer UV-Visible Lambda spectrophotometer.

### *2.1. Leaves sampling and extraction*

The olive leaves were collected from the land in Altınözü district of Hatay (36°08'62"N and 36°30'17"E) (Fig. 1).

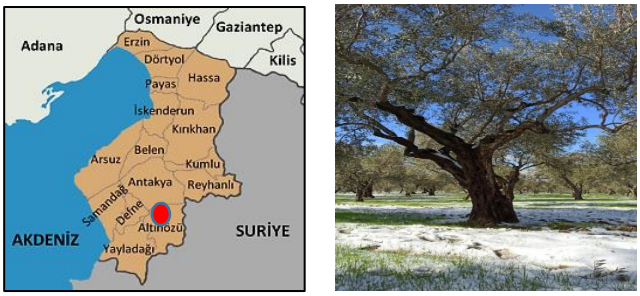



**Fig. 1** Sampling area

The leaves were collected in November of 2021, and on the same day they were sorted and washed with water and then cleaned with pure water. The filtered leaves were dried at room temperature for 7 days and then ground. In the extraction step, 60 ml of solvent per 1 gram was used for all methods. For the extraction processes, 5 methods were selected, one of which is soxlet and the other four are maceration. For Soxhlet, methanol was used as a solvent, and for maceration, 90% acetone, 60% ethanol, 70% methanol and pure water were used as solvents. Maceration was carried out in a shaker incubator at 40 °C for 4 hours; In the soxhalet extraction, 3 siphons were made. After extraction, the extracts were filtered and

stored at +4°C until use.Extraction procedure is given in Fig.2.

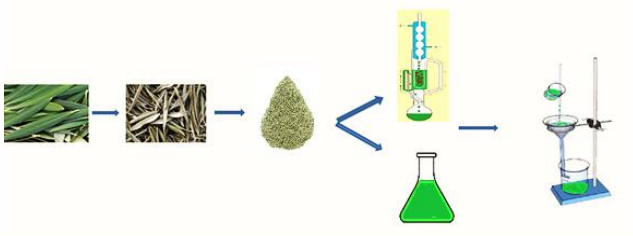


**Fig. 2** Extraction of the olive lea

### *2.2. Antioxidant activities*

Extracts were used after 4 fold dilution for TFC, TPC and FRAP assay while 60 fold diluted extracts were used for ABTS, DPPH and Ellman assays. Experimental studies were carried out in 5 parallels.

#### *2.2.1. Total flavonoid content (TFC)*

The flavonoid content (TFC) was determined by the aluminum chloride method using quercetin as the reference compound (Kumaran and Karunakaran 2006). After the extracts obtained, 0.5 ml was taken and after adding 2 ml of water and 150 µl of 5% $NaNO_2$ aqueous solution, respectively, it was waited for 5 minutes. Then, 150 µl of 10% $AlCl_3$ aqueous solution was added and waited for 1 min. Finally, after the addition of 1 ml of 1 M NaOH, the absorbance was read at 510 nm. The TFC value of the extracts was calculated as mg quercetin per gram leaf or per gram extract.

#### *2.2.2. Total phenolic content (TPC)*

The total phenolic content of the olive leaf extract was determined by the Folin Ciocalteau method (Gutfinger 1981) according to the gallic acid standard. 2.5 ml of water and 0.25 ml of Folin reagent were added to 0.5 ml of the extracts, and 750 µl of 20% $Na_2CO_3$ was added 5 minutes later. After 2 hours in dark, absorbances were read at 750 nm and TPC values were calculated as mg gallic acid equivalent per gram leaf or g extract.

#### *2.2.3. Ferric reducing antioxidant power-FRAP assay*

In this method, $Fe(CN)_6^{3-}$ is reduced and the formed $Fe(CN)_6^{4-}$ reacts with $Fe^{+3}$ to form the $Fe[Fe(CN)_6]^-$ complex, which gives maximum absorption at 700 nm (Hue et al. 2012 ). As a result of the reaction, a dark blue complex is formed, and the higher the absorbance of the complex, the higher the reducing power. 0.4 ml of 0.2 M phosphate buffer at pH 6.6, 0.4 ml of 1% of $K_3Fe(CN)_6$ were added to 1 ml of extract, respectively, and it was kept at 50 °C for 20 minutes, 1 ml of TCA was added and centrifuged for 10 minutes. 1.5 ml of supernatant was taken from the supernatant and 1.5 ml of distilled water and 0.3 ml of $FeCl_3$ were added to it, 10 minutes after the absorbance was read at 593 nm. Trolox was used as a standard and results were calculated as mg trolox equivalent reducing power per g leaf and gram extract.

#### *2.2.4. Trolox equivalent antioxidant capacity (TEAC)-ABTS assay*

This method is based on the scavenging of ABTS radical cation by antioxidants. ABTS radical is formed by mixing 7 mM ABTS and 2.45 mM $K_2S_2O_8$ in a 2:1 ratio and keeping it in the dark for 16 hours. The prepared radical is diluted with ethanol until its absorbance is fixed at 0.7 at 734 nm. Then, after adding 3 ml of the radical ready to use in the experiment to the test tubes, 5 minutes after adding 100 µl of the extracts, the absorbance (A) was read at 734 nm. Inhibition percentage was calculated using Eq.1

$$\text{Inhibition \%} = [(Ao-A)/Ao].100 \qquad \text{Eq.1}$$

TEAC was calculated as mg trolox equivalent per gram of leaf or gram of extract.

#### *2.2.5. DPPH assay*

This method is based on measuring the scavenging effects of antioxidants with a stable free radical, 1,1-diphenyl-2-picrylhydrazine (DPPH) (Molyneux 2004). DPPH radical scavenging capacity analysis methods are a frequently used method to measure the antioxidant capacity of natural extracts (Mot et al. 2011). The purple colored DPPH radical solution gives maximum absorption at 517 nm. In this method, which is based on radical reduction by adding antioxidant to ethanol or methanolic DPPH solution, a decrease in absorbance is observed at 517 nm. In the method, after adding 2 ml of DPPH solution to each of the test tubes, 5 different concentrations of the extract were added and incubated at room temperature for 30 minutes. At the end of the period, absorbance was read at 517 nm. Extract concentrations versus % inhibition values were plotted and the concentration (EC50) values corresponding to 50% inhibition were calculated and compared with the standard antioxidants BHA and BHT.

#### *2.2.6. Determination of total sulfhydryl groups by Ellman assay*

In this method, thiol groups, which have antioxidant properties, interact with DTNB (5,5'-dithio-bis(2-nitrobenzoic acid)), also known as Ellman's reagent, and an equivalent amount of 5-thio-2-nitrobenzoic acid is formed. The absorbance of the yellow product formed is measured at 412 nm and the results are evaluated by using the standard curve drawn for cysteine (Hansen et al. 2007).

Ellman's reagent was prepared as 0.1M DTNB in 0.1 M pH 8 phosphate buffer containing 1 mM EDTA. After adding 250 microliters of extract to 2.5 ml of the reagent, it was kept at room temperature and in the dark for 15 minutes, then the absorbance was read at 412 nm. Results were calculated as mg cysteine equivalents per gram leaf or gram extract.

## 3. Results and Discussion

Extracts obtained with soxleth-methanol, 90% acetone, 70% methanol, 60% ethanol and distilled water were dried

by lyophilization and 0.196, 0.194, 0.151, 0.176 and 0.275 g of extract were obtained per gram leaf, respectively.

### *3.1. Total flavonoid contents (TFC) of olive leaf extracts*

TFC of the extracts obtained as a result of the applied extraction methods is given in Fig. 3 in terms of mg quercetin per gram leaf and gram extract (mg QE/g).

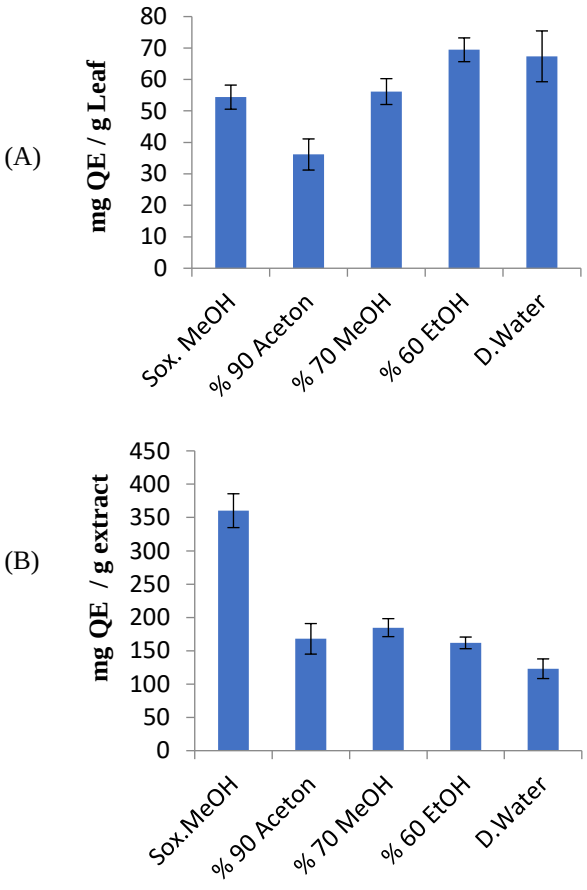



**Fig.3** TFC values per gram leaf (A) and gram extracts as mg quercetin equvialent

As seen in Figure 3, the highest TFC value per g leaf was 70% methanol as 69.44 mg quercetin; 360.21 mg of quercetin per g extract was obtained in soxhlet-methanol extraction. The TFC values of the extracts made with water were the lowest both per g leaf and g extract.
In order to determine the effect of age (Çetinkaya et al. 2016). periodically investigated the TFC values in the leaves of the 9 and 65 years old trees of Kilis Yağlık cultivar. The highest and lowest TFC values were determined as 87.24 mg QE/g in August and 65.42 mg QE/g in October, respectively, in old tree leaves. However, in young tree leaves, the highest and lowest values were determined as 84.27 mg QE/g in April and 61.05 mg QE/g in October, respectively (Khizrieva et al. 2021) obtained olive leaf extract by water-alcohol and also by subcritical water at 120, 180 and 220 °C; and they reported TFC values as 33.0, 25.4, 29.7 and 65.2 mg rutin equvalient/g, respectively.

### *3.2. Total phenolic contents (TPC) of olive leaf extacts*

The TPC values of the extracts obtained in the study were determined by the Folin method and calculated as mg gallic acid equivalent (mg GAE) per gram leaf and gram extract, and the results were presented in Fig. 4.
As seen in Fig. 4, the highest TPC values per g leaf and g extract were determined as 25.58 mg GAE in 90% acetone and as 128.86 mg GAE in Soxhlet-methanol extracts, respectively. It was determined that the lowest TPC values was the extract obtained by using water 11.31 mg GAE/g leaf and 38.47 mg GAE/g extract.

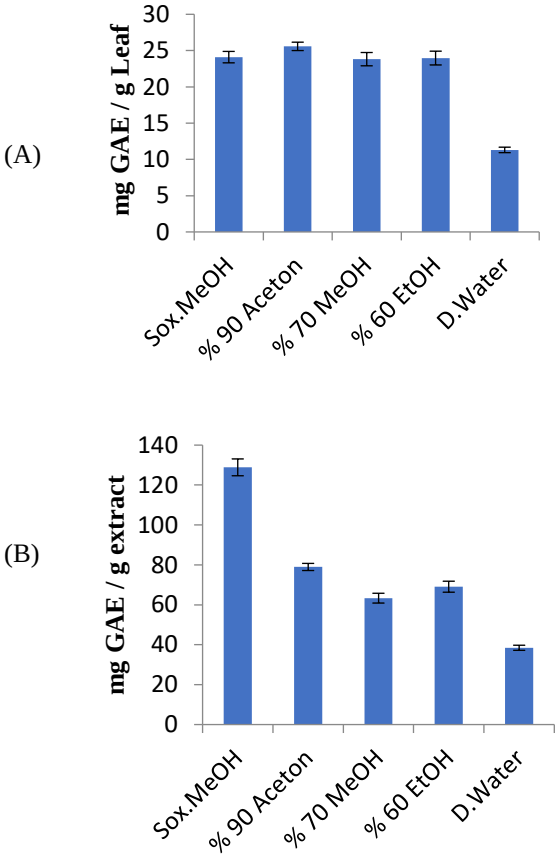



**Fig. 4** TPC values per gram of leaf (A) and per gram of extracts (B) as Gallic acid equivalent (GAE) amount

(Abaza et al. 2011) used 80% methanol, 70% ethanol, 80% acetone and deionized water as solvents to investigate the effect of solvent type on the phenolic substance and antioxidant activity values of Chetoui olive leaf extracts. According to the results obtained, they reported that the highest value of the total amount of phenolic substance in the extract prepared using 80% acetone was 24.09 ± 0.73 mg GAE /g dry matter and ( Khizrieva et al. 2021). Obtained olive leaf extract by water-alcohol and also by subcritical water at 120, 180 and 220 °C; and they reported TPC values as 42.6, 32.7, 41.8 and 70.4 mg GAE/g, respectively.

### *3.3. Ferric reducing antioxidant power (FRAP) of olive leaf extracts*

FRAP values of leaf extracts were given in Fig. 5. as mg trolox equvialent (TE). As can be seen in Fig. 5(A), the FRAP values calculated per gram leaf were the smallest in water extract (11.31 mg TE), while they were quite close to each other, approximately 25 mg TE in those of other methods. When the FRAP values per gram extract given in Fig. 5(B) were examined, it was observed that the highest reducing power belonged to methanol-soxlet extract (128.86) and the lowest reducing power belonged to water extract (38.47 mg TE) (Cho et al. 2020). investigated the effect of TPC, antioxidant and antimicrobial activity by extracting olive leaves with ethanol, methanol, 50%, 70% and 90% (v/v) aqueous solutions of acetone, as well as deionized water.

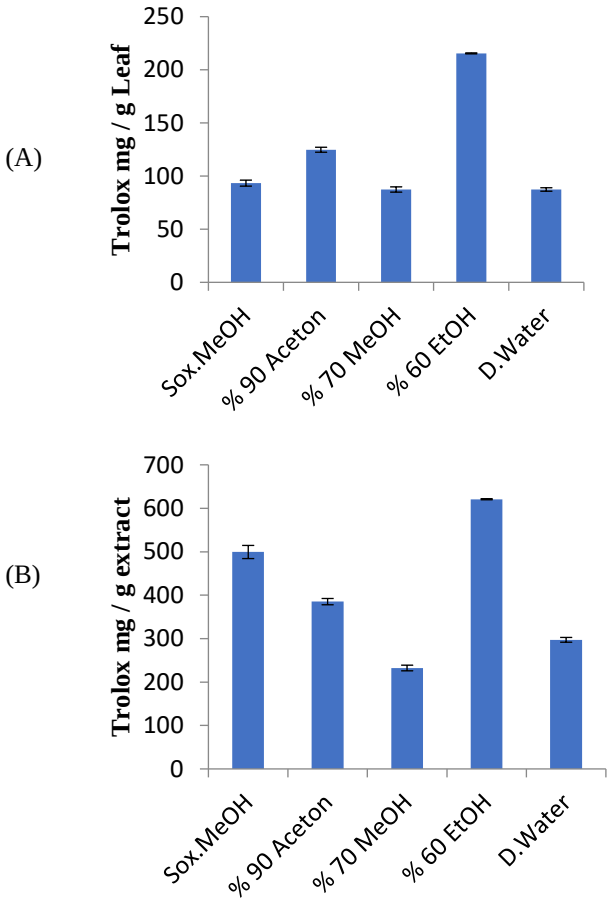



**Fig. 5** FRAP values per gram of leaf (A) and per gram of extracts (B) as trolox equivalent amount

As a result of the study, when the extracts were analyzed by HPLC for oleuropein, hydroxytyrosol and tyrosol contents, they reported that the highest extraction efficiency was obtained as 20.41% when using 90% methanol solution. The highest TPC values were obtained as 232 and 231 mg GAE/100 g for 90% methanol and 90% ethanol, respectively. Also, They determined that the highest values in DPPH, FRAP and $Fe^{2+}$-chelating activity and antioxidant activity were obtained when 90% methanol was used.

### *3.4. Trolox equivalent antioxidant capacity (TEAC)-ABTS of olive leaf extracts*

In this method, the effect of quenching ABTS cation radical of the extracts was investigated, using trolox as a standard and the results were given as trolox equivalent (TEAC) (Fig.6).

As seen in Figures 6(A) and 6(B), the highest activity per g leaf and g extract was 18.82 TEAC mg/g leaf and 60.01 TEAC mg/g extract value in the extract obtained from methanol-soxhlet extraction, whereas the lowest activity was 3.34 TEAC mg/g leaf and 10.31 TEAC mg/g extract and the extract obtained by using water. Sevim and Tuncay (2012) determined the ABTS antioxidant activity in Ayvalık and Memecik olive leaves as 825.38-1056.16 µmol TE/100 g (Martin-Garcia et al. 2022). investigated ABTS scavenging activity and reported between 26.92 and 35.7 mg TE/g extract in 7 different olive leaf extracts cultivated in Spain.

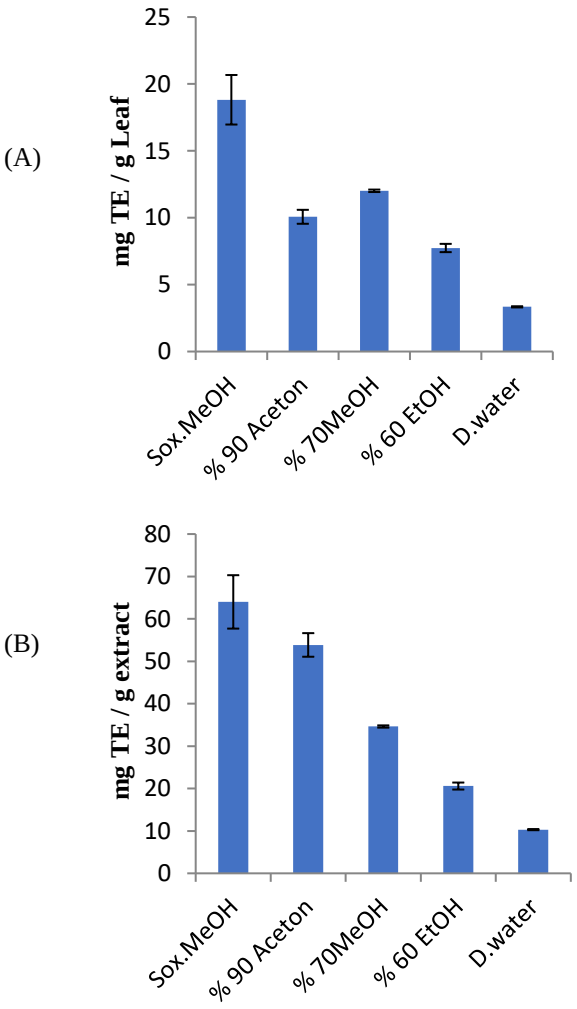



**Fig. 6** ABTS scavenging activities per gram of leaf (A) and per gram of extracts (B) as trolox equivalent amount

### *3.5.DPPH radical scavenging activities of olive leaf extracts*

The DPPH radical scavenging activities of the leaf extracts obtained per gram of leaf and gram of extract as $EC_{50}$ are given in Table 1. The results are compared with BHA and BHT. As seen in Table 1, Soxhlet-Methanol extract showed incredibly higher DPPH scavenging activity. In particular, the activity per gram extract was quite close to the BHA standard in soxlet-methanol extraction; and also , in 70% methanol and water extracts, DPPH scavenging activity as high as BHT was observed.

**Table 1** $EC_{50}$ values of extracts and BHA and BHT standards obained by DPPH assay

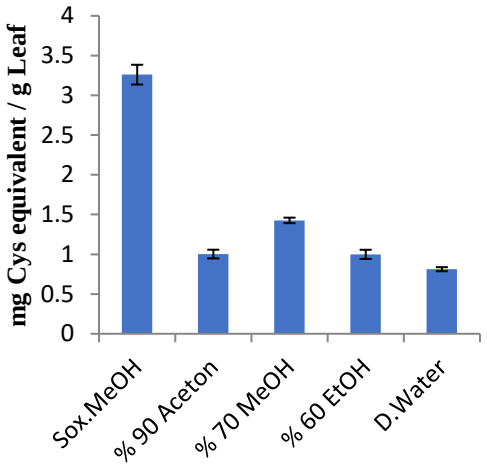
| | $EC_{50}$ (µg /ml) | |
|---|---|---|
| | **leaf** | **Extract** |
| **Sox.MeOH** | 10.34±7.49 | 2.03±1.47 |
| **% 90 Acetone** | 200.00±0.00 | 38.80±0.00 |
| **% 70 MeOH** | 150.17±0.29 | 22.67±0.04 |
| **% 60 EtOH** | 177.27±14.29 | 31.20±2.52 |
| **D.Water** | 275.90±15.38 | 20.87±4.23 |
| **BHA** | 1.00±0.35 | |
| **BHT** | 22.92±2.89 | |

Martin-Garcia et al. (2022) determined DPPH scavenging activity ranging from 33.03 to 46.8 mg TE/g extract in 7 different olive leaf extracts collected in Spain.

### *3.6. Total sulfhydryl groups of olive leaf extracts by Ellman assay*

In this method, total free sulfhydryl groups were determined spectrophotometrically by the Ellman method and the results were calculated as mg cysteine equivalents. The variation of the amounts of free sulfhydryl groups per gram leaf and gram extract is given in Figure 6. As seen in Figure 6(A), the highest values per gram leaf and gram extract were observed as 3.26 mg CysE/g leaf and 10.06 mg CyS E/g extract, respectively, in the samples obtained with soxlet-methanol. It was also observed that the results obtained with other methods are close to each other.

(A)




(B)

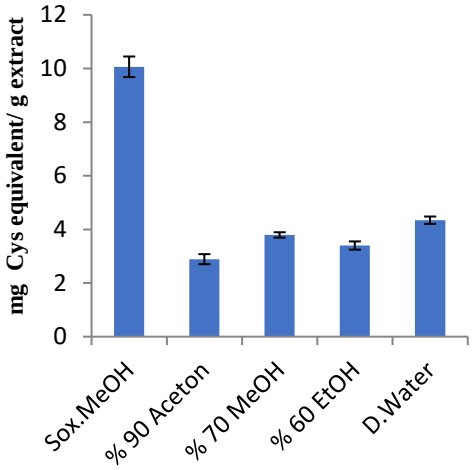



**Fig.7** Total sulfydryl group by Ellman method per gram of leaf (A) and per gram of extracts (B) as Cystein equivalent amount

Considering that the antioxidant activity of the soxlet-methanol extract is higher than the other methods, it may be associated with the high free thiol groups in this extract.

## 4. Conclusion

Olive leaf extract amounts and antioxidant activities are affected by the method and solvent used. TFC value, ABTS radical scavenging activity and total amount of sulfhydryl groups were found to be higher than the extracts obtained by other methods, especially in the extracts obtained by Soxlet-methanol extraction. According to the FRAP method, the extract prepared with 60% ethanol showed the highest reducing power based antioxidant activity. It has been revealed that deionized water is not suitable for obtaining an extract with high antioxidant activity.

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**Authors' contributions:** Seda Ağçam contributed to the experimental studies, the evaluation of the data and the preparation of the article. Gül Özyılmaz contributed to the organization of the study, the planning of the experimental studies, the evaluation of the data and the writing of the article.

**Conflict of interest disclosure:** There is no financial, commercial, legal or professional conflict of interest that could affect our research.

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# Effects of different nitrogen sources on invertase production by *Aspergillus niger*

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**Abstract:** Investigation various nitrogen sources effects on the production of invertase by *Aspergillus niger* was researched in this study. Invertase is a precious enzyme used in many industries like food, pharmacy, confectionery, invert syrup production. Taguchi design of experiment (DOE) was preferred to optimize the cultivation conditions. L16 ($4^3$) orthogonal array was selected in the current study including nitrogen source, initial pH of the medium and incubation time at four levels for statistical optimization. The data showed that optimized version of invertase production was achieved using proteose peptone, 5.5 initial pH and 3 days for incubation time. Bacto peptone had higher enzyme activity than casein and yeast extract. pH of the medium was found as the most efficient factor among nitrogen source and incubation time. Besides, percentage contribution of the nitrogen source and incubation time were indicated at similar rates (9 and 10%, respectively). The highest enzyme activity was defined as 45.87 U/ml, which was found to be closer to the predicted result (46.33 U/ml). As a conclusion, proteose peptone increased the invertase activity and use of Taguchi DOE supported quick and effective optimization.

**Keywords:** Invertase, *Aspergillus niger*, optimization, Taguchi DOE



## 1. Introduction

Invertase (EC 3.2.1.26, β-fructofuranosidase) is a valuable enzyme used in various industries that acts on the hydrolysation of sucrose and related glycosides into reducing sugars (fructose and glucose) at equal concentration. This mixture is named "invert sugar" (Kotwal and Shankar 2009; Nadeem et al. 2015; Tasar 2017). Invert sugar has higher sweetener capacity and has been widely used in many industries such as beverage, confectionary, soft-centered candy production, non-crystallizing creams (Canli et al. 2011; Kotwal and Shankar 2009; Taskin et al. 2013). Invertase enzyme is also used for the production of invert sugar for honeybees (Das et al. 2016; Shafiq et al. 2001).

Invertase is produced by *Saccharomyces cerevisiae* or *S. carlbergensis* commercially (Aranda et al. 2006). Furthermore, filamentous fungi and bacteria have been studied for invertase production (Rubio and Navarro 2006). This enzyme is inducible in filamentous fungi, however, it is structurally synthesized by the yeasts (Rubio et al. 2002). *Aspergillus niger* has been extensively used in biotechnological applications. *A. niger* has GRAS (Generally Regarded As Safe) status that was approved by US Food and Drug Administration (FDA) (Abarca et al. 2004). *A. niger* has license to produce 19 different food enzymes. This microorganism is also preferred as an efficient and excellent producer of many valuable products either in submerged or in solid state fermentation (Li et al. 2020).

Optimization of the process parameters is one of the main parts of microbial fermentation. The target product is affected from the composition of the medium. Besides, different carbon and nitrogen sources, mineral salts and other medium components strongly affect the production of the desired product. Classical and statistical optimization tools are preferred due to enhance the product yield. Both of these two optimization techniques have positive and negative aspects (Abdel-Rahman et al. 2020; Tasar 2022). Classical one variable at a time method is a simple and basic technique for optimization; on the other hand, it needs more time and labour when compared with the statistical optimization tools. The statistical optimization tools allow quick screening and effective production of the desired product (Farid et al. 2013; Tasar 2020). Taguchi design of experiment (DOE) presents a robustness optimization with less time and labour (Rao et al. 2008).

The purpose of the current study was to find out the effects of different nitrogen sources on the production of invertase enzyme by *A. niger* OZ-3 using Taguchi DOE methodology. Medium composition and fermentation conditions alter the yield of enzyme; hence the optimization of the medium ingredients and some environmental conditions were studied.

## 2. Materials and Method

All the reagents were purchased from Sigma Chemical Co. (USA).

### *2.1. Microorganism and medium*

The microorganism *A. niger* OZ-3 (GenBank accession number JX110160) was isolated and determined before (Taskin et al., 2013). The fungus was grown in potato dextrose agar (PDA) slants at 4˚C and subcultured monthly. The minimal medium composition was designated as following (g/l): 20 sucrose, 1 $KH_2PO_4$, 3 nitrogen sources. 100 ml of basal medium was placed to each 250-ml flasks and incubated at 200 rpm and 30 ˚C for all runs. The pH was adjusted to the desired value using 1 N NaOH or 1 N HCl and the flasks were sterilized in autoclave at 121 ˚C for 20 min.

### *2.2. Enzyme assay*

Extracellular invertase activity was measured in culture filtrate using a previous method that was described by Ge and Zhang (Ge & Zhang, 2005) with a few modification. The culture filtrate was used for enzyme source due to measure the reducing sugar released from sucrose. 100 µl of enzyme source was placed to the test tubes and 900 µl of 0.1 M sodium acetate buffer (pH 5.5) containing 2% of sucrose (w/v) was added. The glass test tubes were incubated at 55 ˚C for 15 min. After this step, the reducing sugar was measured using the DNS method (Miller, 1959). The preparation of DNS reagent was reported in detail before (Canli et al., 2011). 1 ml of DNS reagent was added in each test tubes and they were allowed to incubate in boiling water (90 ˚C) for 15 min and the total volume of the tubes were raised to 15 ml with distilled water. The reducing sugar released from sucrose was determined at 550 nm absorbance. For the control, a blank was prepared using distilled water instead of enzyme source. One invertase unit was determined as the enzyme catalysing the release of 1 µmol reducing sugar from sucrose per min.

### *2.3. Taguchi DOE and ANOVA test*

Taguchi DOE methodology was preferred in the current study for its advantages on optimization. For this purpose, nitrogen source, pH of the medium and incubation time factors were selected at four levels using Taguchi L16 ($4^3$) orthogonal array (Table 1).

**Table 1.** Optimization factors and preferred levels.

| Levels | Factors | | |
|---|---|---|---|
| | **Nitrogen sources** | **pH** | **Time (h)** |
| **1** | Bacto peptone | 4.0 | 24 |
| **2** | Casein peptone | 4.5 | 48 |
| **3** | Proteose peptone | 5.0 | 72 |
| **4** | Yeast extract | 5.5 | 96 |

Taguchi DOE benefits the signal/noise (S/N) ratio to interpret the situation of the target value with three different characteristics following, the larger-the better, the nominal-the better and the smaller-the better (Tan et al. 2005). It was aimed to optimize and enhance the enzyme activity in the current study, hence, the larger-the better criterion was preferred using the following equation:

$S/N = -10 \log_{10} (1/n \sum_{i=1}^{n} 1/Yi^2)$ .

The *n* symbol is for the definition of the number of repetitions for the setup conditions, Y*i* defines the situation of the *i*th experiment (Jean and Tzeng 2003). Analysis of variance (ANOVA) test was calculated to perform the quality characteristics which were derived from the selected factors. Minitab® 19.0 version Statistical Software was used for both Taguchi methodology and ANOVA test.

## 3. Results

### *3.1. Taguchi DOE L16 orthogonal array results*

Taguchi L16 ($4^3$) orthogonal array showed that the selected factors caused variation on the enzyme activity. Effects of nitrogen sources, initial pH of the medium and incubation time were defined. The data illustrated that invertase activity was highly reliant with the selected factors (Table 2). The maximum enzyme activity was existed from the 4$^{th}$ experimental set while the minimum activity was determined from the 13$^{th}$ run.

**Table 2.** Taguchi L16 orthogonal array and invertase activity

| Exp. | Factors | | | Results |
|---|---|---|---|---|
| **No.** | Nitrogen source | pH | Time (d) | Invertase (U/ml) |
| 1 | Bactopeptone, | 4.0 | 1 | 23,67 |
| 2 | Bactopeptone | 4.5 | 2 | 28,79 |
| 3 | Bactopeptone | 5.0 | 3 | 31,65 |
| 4 | Bactopeptone | 5.5 | 4 | 43,37 |
| 5 | Casein | 4.0 | 2 | 26,22 |
| 6 | Casein | 4.5 | 1 | 21,45 |
| 7 | Casein | 5.0 | 4 | 33,57 |
| 8 | Casein | 5.5 | 3 | 38,95 |
| 9 | Proteose peptone | 4.0 | 3 | 29,33 |
| 10 | Proteose peptone | 4.5 | 4 | 33,11 |
| 11 | Proteose peptone | 5.0 | 1 | 34,21 |
| 12 | Proteose peptone | 5.5 | 2 | 38,36 |
| 13 | Yeast extract | 4.0 | 4 | 21,37 |
| 14 | Yeast extract | 4.5 | 3 | 31,85 |
| 15 | Yeast extract | 5.0 | 2 | 29,67 |
| 16 | Yeast extract | 5.5 | 1 | 33,53 |

Table 3 illustrated the response table for means that indicates the individual effects of the factors. Delta value demonstrates the alteration of the highest and the lowest results for each factor. Initial pH of the medium had the highest delta rank while the nitrogen source had the lowest value.

**Table 3.** Response table for means

| Level | Nitrogen source | pH | Time |
|---|---|---|---|
| 1 | 31.87 | 25.15 | 28.22 |
| 2 | 30.05 | 28.80 | 30.76 |
| 3 | 33.75 | 32.28 | 32.95 |
| 4 | 29.11 | 38.55 | 32.85 |
| Delta | 4.65 | 13.40 | 4.73 |
| Rank | 3 | 1 | 2 |

Taguchi DOE uses the main effects plot graphs (Fig. 1). This illustration showed the best conditions for selected factors at the peak levels. The optimum levels for each factor were determined as proteose peptone, 5.5 pH value and 3 days for incubation time, respectively. At the last step of the optimization, Taguchi DOE used these optimal levels to predict the highest enzyme activity. The prediction experiment was done according to the suggested levels above and the maximum invertase activity was found as 45.87 U/ml which was near to the predicted value (46.33 U/ml).

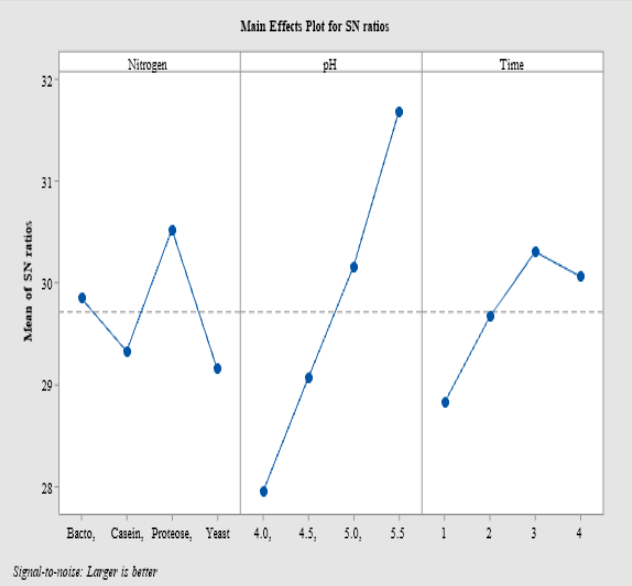


**Fig. 1** Main effects plot for S/N ratios

### *3.2. ANOVA (Analysis of variance)*

ANOVA test was performed to calculate the experimental data to evaluate the distinction for the results owing to each factor (Table 4). The P value represented the probability that was calculated from an F-distribution with the degrees of freedom (DF). The highest F value with the lowest P value were obtained from pH of the medium, on the contrary the lowest F value with the highest P value was determined by the nitrogen source. These results were related about the percentage contribution of the factors (Fig. 2).

**Table 4.** ANOVA table for means

| Source | DF | Seq SS | Adj SS | Adj MS | F | P |
|---|---|---|---|---|---|---|
| Nitrogen source | 3 | 50,73 | 50,73 | 16,91 | 1,4 | 0,30 |
| pH | 3 | 390,43 | 390,43 | 130,14 | 11,4 | 0,01 |
| Time | 3 | 59,55 | 59,55 | 19,85 | 1,7 | 0,25 |
| Residual Error | 6 | 67,95 | 67,95 | 11,32 | | |
| Total | 15 | 568,65 | | | | |

DF: Degree of freedom; Seq SS: Sequential sum of square; Adj SS: Adjusted sum of square; Adj MS: Adjusted mean of squares; F: F value; P: P value.

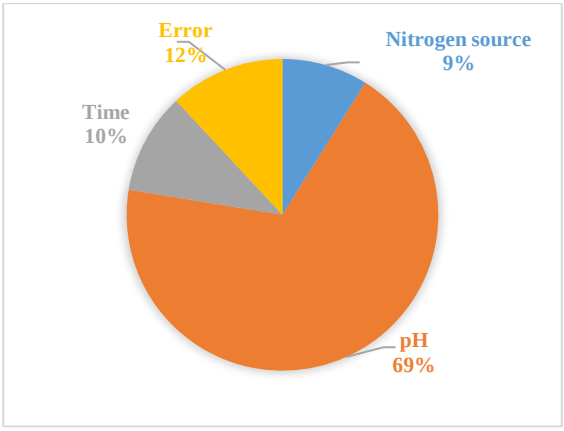


**Fig. 2.** The percentage contribution of each factor

## 4. Discussion

Invertase enzyme activity is strongly related with the medium components, the kind of the producer-microorganism and environmental conditions. The results showed that proteose peptone, pH 5.5 and 3 days for incubation time were the best choices for invertase production by *A. niger* OZ-3. Bacto peptone, casein and yeast extract had lower enzyme production. In a previous study about optimization of invertase production by *S. cerevisiae*, urea achieved higher enzyme activity than the nutrient broth and yeast extract. Besides, the optimal pH (6.0) was found closer to the current study (pH 5.5) (Ikram-Ul-Haq et al. 2005).

Medium composition has a great role in enzyme production. In a prior paper, nutrient broth, peptone and yeast extract effects on invertase production by *S. cerevisiae* were studied and the optimal nitrogen source was determined as the peptone at pH 6.0 (Shafiq et al., 2001). On the other hand, another prior studies revealed similar pH value to the current study for different *A. niger* strains for invertase production (Boddy et al. 1993; Laothanachareon et al. 2022). Besides, the same pH value was found to be optimal for invertase production by *Cryptococcus laurentii.* They found the enzyme to be thermostable up to 60˚C at pH 5.5 (Das et al., 2016).

Waste materials are preferred as substrate for microbial fermentation. Either recycling of the waste materials or low-cost production are important parameters for an effective fermentation. Various studies have been done using waste or low-cost materials as substrate for invertase production.

Use of sugar beet, enhanced the economic value of invertase production by *Rhodotorula glutinis*. The optimal conditions were determined for pH and incubation time as 4.5 and 3 days, respectively (Tasar 2017). In a prior study, about invertase production by *A. niger*, the peptone was reported to be enhancer for thermostable invertase from *A. niger* IBK1 strain. The other optimal conditions were determined as pH 5.0 and 120 h at 35˚C (Oyedeji et al. 2017). The pH value and nitrogen source were found to be like the current study; however, the incubation time was found longer from this study. It may be caused by use of a waste substrate (pineapple peel) and different strain.

The response table illustrated the difference between the highest and the lowest enzyme activities for the factors (Table 3). Initial pH of the medium was found to be the most effective factor on invertase production. The percentage contribution of each factor varied depending on the individual effects of the factors (Fig. 2). Initial pH of the medium was found to be the most efficient factor. However, nitrogen source and incubation time factors were found to be closer to each other.

## 5. Conclusion

Optimization process is the main part of an effective microbial fermentation. Use of classical and statistical optimization tools causes variety on the product yield. Taguchi DOE is one of the most preferred statistical optimization tools for biotechnological applications. In the current study, Taguchi DOE was used in the investigation of the most suitable conditions for invertase production by *A. niger* OZ-3 strain. Effects of bacto peptone, casein, proteose peptone and yeast extract were studied in detail. Initial pH of the medium and incubation time factors were also determined. The results showed that the optimal nitrogen source was proteose peptone at pH 5.5 and 3 days for incubation time. As a conclusion, an enhanced invertase production was achieved in the current study using Taguchi DOE as the statistical optimization tool.

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The author declares that she has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Ethics approval and consent to participate**

This study does not contain any studies with human participants or animals performed by the author.

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# SARS-COV-2 enfeksiyonu olan hastalarda laboratuvar parametrelerin prognoz değeri

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**Özet:** Bu çalışmada, insanlık için en son küresel biyolojik tehlike olduğu varsayılan koranavirüs-19 (COVID-19) hastalığının prognoz sürecinde önem arz eden laboratuvar parametrelerini değerlendirmek amaçlanmıştır. Bu amaç doğrultusunda, COVID-19 hastalığı ve laboratuvar parametrelerinin esas alındığı literatür taraması yapılmıştır. Bu literatür taraması sonucunda 25 adet makalenin değerlendirilmesi ile çalışma oluşturulmuştur. Laboratuvar parametreleri, üç ana başlık altında kategorize edilmiştir. Bu başlıklar, hematolojik parametreler, koagulasyon parametreleri ve inflamatuar parametrelerdir. Hematolojik parametrelerde lenfosit sayısının düşük çıkması, hastalığın her aşamasında görülen bir durum olduğu saptanmıştır. Prognozun kötüye gidişatında nötrofil sayısının yüksekliği ön plana çıkmaktadır. Hastalığın şiddetinin artması ile birlikte nötrofil/lenfosit oranında yükseklik belirtilmiştir. Buna ek olarak trombosit düşüklüğü de gözlenebilmektedir. Koagulasyon parametrelerinde ise, COVID-19 hastalığını ağır geçirenlerde artan protrombin zamanı ve D-dimer düzeylerinin yüksekliği ön plana çıkmaktadır. İnflamatuar parametrelerden C-reaktif protein (CRP) düzeylerinin yüksekliği, hastalığın her aşamasında gözlenebilen bir durumdur. Buna ek olarak, hastalığın seyri kötüye gittikçe C-reaktif protein düzeyleri de daha çok yükselmektedir. Ayrıca, prokalsitonin ve ferritin yüksekliği de hastalığın durumu ağırlaştıkça gözlenmektedir. Sonuç olarak, hastalığın seyri boyunca, lenfositopeni ve CRP düzeylerinin yüksekliği ön plana çıkarken, hastalığın daha agresif bir hale dönmesiyle birlikte, artan protrombin zamanı, nötrofil/lenfosit oranı, nötrofil sayısı, D-dimer, prokalsitonin ve ferritin düzeyleri yüksekliği ortaya çıkmıştır.

**Anahtar Kelimeler:** SARS-CoV-2, Koronavirüs, COVID-19, Laboratuvar parametreleri, Prognoz

## *Prognostic value of laboratory parameters in patients with SARS-COV-2 infection*

**Abstract:** In this study, it was aimed to evaluate the laboratory parameters that are important in the prognosis process of coranavirus-19 (COVID-19), which is assumed to be the latest global biohazard for humanity. For this purpose, a literature review was conducted based on COVID-19 disease and laboratory parameters. As a result of this literature review, the study was created by evaluating 25 articles. Laboratory parameters are categorized under three main headings. These headings are hematological parameters, coagulation parameters and inflammatory parameters. It has been determined that low lymphocyte count in hematological parameters is a condition seen at every stage of the disease. High neutrophil count is a sign of worsening prognosis. With the increase in the severity of the disease, an increase in the neutrophil/lymphocyte ratio was noted. In addition, low thrombocyte levels may also be observed. In coagulation parameters, increased prothrombin time and high D-dimer levels are prominent in patients with severe COVID-19 disease. High levels of C-reactive protein (CRP), one of the inflammatory parameters, can be observed at every stage of the disease. In addition, as the course of the disease worsens, C-reactive protein levels rise more and more. In addition, an increase in procalcitonin and ferritin levels is observed as the condition of the disease worsens. As a result, while lymphocytopenia and high CRP levels were prominent throughout the course of the disease, increased prothrombin time, neutrophil/lymphocyte ratio, neutrophil count, D-dimer, procalcitonin and ferritin levels emerged with the disease becoming more aggressive.

**Keywords:** SARS-CoV-2, Coronavirus, COVID-19, Laboratory parameters, Prognosis

## 1. Giriş

Son yirmi yılda ortaya çıkan şiddetli akut solunum sendromu (SARS) ve Orta Doğu solunum sendromu (MERS) salgınları yüzünden sırasıyla 2002 ve 2012'de koronavirüslere karşı Dünya Sağlık Örgütü acil durumla toplandı. 2019'un sonunda, Çin'in en kalabalık şehirlerinden biri olan Wuhan'da etiyolojisi bilinmeyen bir pnömoni salgını rapor edildi (Lu ve ark. 2020). Bu pnönoni salgınına yol açan virüs, muhtemelen yarasalarda bulunabilen SARS benzeri bir koronavirüsten türetilen ve spike glikoproteininde (protein S) ve nükleokapsid N proteini mutasyonların ortaya çıkmasından sonra insanlara bulaşan Coronaviridae ailesine ait yeni ortaya çıkan bir virüstür (Benvenuto ve ark. 2020). Dünya Sağlık Örgütü tarafından koronavirüs hastalığı 2019 (COVID-19) olarak adlandırılan bu zoonotik patojenin, insanlar için en son küresel biyolojik tehlike olduğu varsayılmaktadır. Mortalite oranı, SARS ve MERS'den daha düşük olmasına rağmen, uzun kuluçka süresi (2 haftaya kadar) ile birlikte SARS-CoV-2 bulaşma riskini artırmakta ve yayılmasını kolaylaştırmaktadır (Wu ve McGoogan 2020). Dünya Sağlık Örgütünün verilerine göre, 20 Eylül 2021 tarihi itibariyle COVID-19 kaynaklı 228394572 teyit edilmiş vaka ve 4690186 ölüm bildirilmiştir (https://covid19.who.int/).

COVID-19, klinik olarak kuru öksürük, nefes darlığı ve ateş gibi klinik semptomlar gösteren gizemli bir solunum ve sistemik sendromdur (Xu ve ark. 2020). COVID-19 hastalığı tanısı ve SARS-CoV-2'den genetik materyali tanımlamak için en yaygın kullanılan yöntem, gerçek zamanlı polimeraz zincir reaksiyonudur (RT-PCR). Bu yöntem, virüsün RNA genetik materyalinin tamamlayıcı DNA'ya (cDNA) ters transkripsiyonunu ve ardından cDNA'nın bazı bölgelerinin amplifikasyonunu içerir (Goudouris 2021). Klinik verilere dayanarak, onaylanmış vakalar hafif, orta, şiddetli ve kritik vakalar olarak sınıflandırılabilir. Vakaların yaklaşık %5'i kritik vaka olarak kabul edilirken, %14'ü şiddetli vaka olduğu kabul edilmektedir (Abbasi-Oshaghi ve ark. 2020). COVID-19 hastalarının klinik durumu, özellikle periferik oksijen satürasyonu seviyeleri ve eşzamanlı komorbiditeleri, yoğun bakım ünitelerine kabul edilme ihtiyacını büyük ölçüde belirlemektedir (Velavan ve Meyer 2020).

COVID-19 hastalarının bir kısmı, yoğun bakım ünitesinde, ventilasyon ve Ekstrakorporal Membran Oksijenizasyonu tedavisi gerektiren çok şiddetli bir hastalık dönemi geçirir (Skevaki ve ark. 2020). Virüsü bulaştırma ve hastalığı yayma riski yüksek olan COVID-19 hastaları, yoğun bakım ünitelerinde doluluk oranlarını artırmaktadır. Bu durum, hastaların yoğun bakım ünitelerine yatışları ve tedavilerinde büyük bir zorluk oluşturmaktadır (Velavan ve Meyer 2020). Bundan dolayı, ciddi vakaların erken tanınması, hastaların zamanında triyajı için kesinlikle gereklidir. Her ne kadar periferik oksijen satürasyonu seviyeleri ve eşzamanlı komorbiditeleri, yoğun bakım ünitelerine kabul edilmeyi büyük ölçüde belirlese de, çeşitli laboratuvar parametreleri hastalık şiddetinin değerlendirilmesini kolaylaştırabilir. Bu durum göz önünde bulundurularak çalışmamızın amacı, COVID-19 hastalarında gözlenen anormal laboratuvar parametrelerinin, hastalığın seyrinde ki önemini incelemektir.

## 2. Veri kaynakları

COVID-19 hastalığının prognozunda anormal laboratuvar parametrelerinin öneminin daha iyi anlaşılmasını sağlamak amacıyla Google Scholar, Web of Science ve PubMed tıbbi veritabanlarını kullanılarak literatür taraması yapıldı. Anahtar kelime olarak, SARS-CoV-2, Coronavirus, COVID-19, Laboratory parameters ve Prognosis kelimeleri kullanıldı. Alıntılama sayıları dikkate alınarak 25 makale seçildi. Ayrıca bu seçim, hastalığın prognozu esasına dayalı yapıldı. Buna ek olarak, seçilen makalelerde örneklem büyüklüğü de göz önünde bulunduruldu.

Labortuvar parametreleri 3 ana başlık altında kategorize edildi. Bu başlıklar sırasıyla, hematolojik parametreler, koagulasyon parametreleri ve inflamatuar parametrelerdir. Kaynak gösterilen çalışmalar dikkate alınarak, hematoljik parametrelerde, tam kan sayımı, lenfosit sayımı, nötrofil sayımı, trombosit sayımı ve nötrofil/lenfosit oranıına odaklanıldı. Aynı şekilde, koagulasyon parametrelerinde de protrombin zamanı ve D-dimer parametrelerine odaklanıldı. İnflamatuar parametrelerde ise, C-reaktif protein, prokalsitonin ve ferritin parametrelerine odaklanıldı.

## 3. Laboratuvar parametrelerinin önemi

Tüm klinik kararların alınmasında laboratuvar parametrelerinin etkinliğinin yaklaşık %70 olduğu tahmin edilmektedir. Bu laboratuvar parametreleri, tüm objektif sağlık kaydı verilerinin de yaklaşık %40 ila %94'ünü oluşturmaktadır. Şüphesiz ki bir hastalığın tanımlanması, sınıflandırılması, tedavisi ve izlenmesi için laboratuvar sonuçlarının doğruluğu esastır (Dasgupta ve Sepulveda 2013). COVID-19 hastaların tanısında altın standart olarak kabul edilen RT-PCR testinin, duyarlılığı yaklaşık %70 ve özgüllüğü ise %95 olduğu tahmin edilmektedir (Goudouris 2021). Laboratuvar parametreleri açısından COVID-19 hastalarının, hastalığın şiddetine göre sınıflandırılması, tedavisi ve izlenmesi için ise hematolojik, koagulasyon, inflamatuar bazı parametrelerden yararlanılmaktadır.

### *3.1. Hematolojik parametreler*

COVID-19, hematopoietik sistem ve hemostaz üzerinde önemli etkileri olan sistemik bir enfeksiyondur. Lenfopeni, önemli bir laboratuvar işareti olarak kabul edilebilir ve potansiyel olarak prognostiktir. Nötrofil/lenfosit ve trombosit/lenfosit oranları vakaların ciddiyetini değerlendirmeye yardımcı olabilir (Vieira ve ark. 2020). Pourbagheri-Sigaroodi ve arkadaşlarının (2020) yaptığı çalışmada, Şiddetli ve hafif vakalardan oluşan COVID-19 hastaların hematolojik parametrelerinin değerlendirildiği 15 yayınlanmış makaleden elde edilen veriler şu şekilde özetlemişlerdir. Lenfopeni çoğu hastada belirgin bir bulgu iken, bazı çalışmalarda nötrofil sayısında artış bildirilmiştir. Özellikle, toplam lökosit sayısı hastalar arasında farklılık gösterir ve bu da lenfopeni veya nötrofilinin baskınlığını yansıtabilir. Birlikte ele alındığında, hafif trombositopeninin eşlik ettiği azalmış lenfositler, COVID-19 hastalarının tam kan sayımında

dikkat çeken en yaygın anormal bulgular arasındadır (Pourbagheri-Sigaroodi ve ark. 2020). Lenfosit sayısı, ciddi hastalığı olan ve olmayan COVID-19 hastalarını doğrudan ayırt etmek için önemli bir parametredir (Wang ve ark. 2020; Ruan ve ark. 2020). Çoğu COVID-19 ölümünün daha fazla lenfopeni yaşadığı göz önüne alındığında, lenfosit sayısının COVID-19'da hastalık şiddetini öngörebilen hızlı ve yaygın olarak bulunan bir laboratuvar parametresi olduğunu varsaymak mantıklıdır (Ruan ve ark. 2020; Tan ve ark. 2020). Yang ve arkadaşlarının (2020) 94 hasta üzerinde yapılan bir çalışmada, lökositler ve nötrofiller de şiddetli bir grupta anlamlı olarak daha yüksekti. Bu çalışmalarla uyumlu olarak, Huang ve arkadaşlarının (2020) yoğun bakım ünitesinde bulunan hastaların, yoğun bakım ünitesi olmayan hastalara göre daha sık lökositoz, nötrofili ve lenfopeni yaşadığına dikkat çekerek benzer bulgular bildirmiştir Wang ve arkadaşlarının (2020) yaptığı çalışmada da yoğun bakım hastalarının, yoğun bakım ünitesinde olmayan hastalara göre daha az lenfosite ve daha fazla lökosit ve nötrofile sahip olduğunu bildirmiştir. Trombositopeni, daha yüksek bir miyokardiyal hasar riski ve daha kötü bir prognoz ile ilişkilidir. Lenfopeni, virüsün sitopatik etkisini, apoptoz indüksiyonunu, interlökin 1 aracılı piroptoz ve inflamatuar sitokinler tarafından kemik iliği baskılanmasını içeren çok faktörlü bir mekanizmadan kaynaklanır (Goudouris 2021).

Liu ve arkadaşları (2020) yaptıkları çalışmada, COVID-19 hastalığını ağır geçirenlerin daha yüksek nötrofil/lenfosit oranına sahip olma eğiliminde olduğunu gösterdiğini belirtmişlerdir. Yang ve arkadaşları (2020) yüksek nötrofil/lenfosit oranının COVID-19 prognozunu öngörebileceğini bildirmiştir. Altı çalışmanın meta-analizi sonuçları, artan nötrofil/lenfosit oranının, SARS-CoV-2 enfeksiyonu olan hastalarda kötü bir prognoz önerebileceğini göstermiştir (Lagunas-Rangel ve ark. 2020). Ayrıca, 50 yaşından büyük COVID-19 hastalarında kritik hastalık insidansının, nötrofil/lenfosit oranının 3,13'den düşük olan hastalar için % 9,1 sonucunu bulmuşlar iken, nötrofil/lenfosit oranının 3,13'den yüksek olan hastalar için %50 sonucunu saptamışlardır (Liu ve ark. 2020).

### *3.2. Koagulasyon parametreleri*

Laboratuvar testlerinin prognostik önemi basit tam kan sayımı incelemeleriyle temsil edilen değerli verilerle sınırlı değildir. Artan protrombin zamanı ve D-dimer değerleri daha kötü bir prognozun göstergesi olabilmektedir (Tang ve ark. 2020; Huang ve ark. 2020). Tang ve arkadaşları (2020), hastalıktan ölen COVID-19 hastaları arasında hayatta kalanlara kıyasla yaygın damar içi pıhtılaşma açısından koagülopatinin ortaya çıkma oranında önemli bir fark olduğunu bildirmişlerdir. Hiperkoagülabilite, COVID-19 nedeniyle hastaneye yatırılan hastalarda yaygındır. Yüksek D-dimer seviyeleri, seviyelerindeki ilerleyici artışa vurgu yapılarak, durumun kötüleştiğini gösteren tutarlı bir şekilde rapor edilmiştir. Protrombin zamanı ve aktive parsiyel tromboplastin zamanı uzaması ve şiddetli trombositopeni gibi pıhtılaşma testlerinin diğer anormallikleri, yaygınlaştırılması gereken yaygın damar içi pıhtılaşma oluşma olasılığını gösterir (Vieira ve ark. 2020). Şiddetli hastalarda D-dimerlerin ortalama değeri, şiddetli olmayan vakalardan önemli ölçüde daha yüksek bulunmuştur. D-dimer düzeyinin yükselmesinin, hastalığın olumsuz bir klinik tabloya doğru ilerlemesini yansıtmaya etkili bir şekilde katkıda bulunabileceğini vurgulanmıştır (Bashash ve ark. 2020). Huang ve arkadaşları (2020), COVID-19 hastalarda kötü prognozu tahmin etmek için D-dimer için bir eşik seviyesi olarak 0,4 µg/mL önermektedirler. COVID-19 hastalığını ağır geçiren ve hafif geçiren iki hasta grubunun karşılaştırıldığı bir çalışmada, ağır geçiren grup hafif geçiren gruba göre önemli ölçüde anormal pıhtılaşma parametrelerine sahip olduğu belirtilmişdir. Bu çalışmada, ağır geçiren grubun protrombin zamanı, INR ve D-dimer düzeylerinin daha yüksek saptandığı belirtilmiştir (Bao ve ark. 2020).

### *3.3. İnflamatuar parametreler*

COVID-19 hastaları için istenen ana rutin testler arasında tam kan sayımı, pıhtılaşma ve fibrinoliz basamaklarını araştıran testler ve inflamasyonla ilgili parametreler yer alır (Pourbagheri-Sigaroodi ve ark. 2020). Enflamasyonla ilgili parametrelerin akut fazlarda oldukça yükseldiği daha önce tespit edilmişti. COVID-19, bu kuralın bir istisnası değildir. Çünkü, bu hastaların serumlarında eritrosit sedimantasyon hızı, C-reaktif protein ve prokalsitonin farklı değerlerde de olsa yükselmektedir (Pourbagheri-Sigaroodi ve ark. 2020). COVID-19 hastalarda C-reaktif protein (CRP) düzeyinde ki değişikliklerin bilgisayarlı tomografi bulgularından daha önce ortaya çıktığı belirtilmiştir. Daha da önemlisi, CRP düzeyleri hastalık gelişimi ile ilişkilendirilmiş ve COVID-19'un erken bir aşamasında hastalığın ciddiyetini tahmin etmede iyi bir performans gösterdiği belirtilmişdir (Tan ve ark. 2020). CRP' nin tanısal değeri prokalsitoninden daha üstün olmakla birlikte, prokalsitonin, hastalığın ilerlemesini tahmin etmek için potansiyel olarak CRP' den daha büyük bir değere sahip olabilir (Pourbagheri-Sigaroodi ve ark. 2020). SARS-CoV-2 ile enfekte olan hastaların, hastalık şiddetine göre gruplandırması ile yapılan bir çalışmada, serum prokalsitonin düzeyleri ortalamalarının, şiddetli hastalarda orta düzey hastalara göre dört katın üzerinde olduğu ve kritik hastalarda ise orta düzey hastalara göre sekiz katın üzerinde olduğu saptanmıştır (Hu ve ark. 2020). COVID-19 mortalitesinin klinik belirleyicilerini araştıran Ruan ve arkadaşları (2020), COVID-19 mortalitesinin virüsle aktive olan sitokin fırtına sendromundan kaynaklanabileceğini öne sürdü. Ölümcül vakalarda C-reaktif protein seviyeleri 126,6 mg/L'ye karşı taburcu edilen vakalarda C-reaktif protein seviyeleri 34,1 mg/L olarak saptamıştır. Aynı çalışmada ölümcül vakalarda serum ferritin seviyeleri 1297 ng/mL 'ye karşı taburcu edilen vakalarda serum ferritin seviyeleri 614 ng/mL olarak saptamıştır (Ruan ve ark. 2020). Zhou ve arkadaşları (2020) tarafından yapılan bir çalışmada, serum ferritin seviyeleri, klinik seyir boyunca hastalık kötüleştikçe yükselmiştir. Özellikle, hiperferritineminin proinflamatuar sitokinlerin salgılanmasını artıran makrofajları aktive edebildiği ve müteakip inflamasyonun esas olarak organ hasarından sorumlu olduğu bildirilmiştir (Kernan ve Carcillo 2017). Ferritin pozitif bir akut faz reaktanı olmasına ve bu hücre içi proteinin serum seviyesi

inflamasyon sırasında artmasına rağmen, ölmekte olan hücreler de ferritin salgılayabilir. Bu nedenle, ciddi şekilde etkilenen COVID-19 hastalarında daha yüksek serum ferritin düzeylerinin daha büyük ölçüde organ hasarına işaret edebileceğini varsaymak mantıklıdır (Pourbagheri-Sigaroodi ve ark. 2020).

**Table 1.** Seçilen çalışmaların temel özellikleri

| Çalışma | Örneklem büyüklüğü | Ön plana çıkan parametre |
|---|---|---|
| ***Hematolojik parametreler*** | | |
| Vieira ve ark. 2020 | 150 | Lenfopeni, N/L, T/L yüksekliği |
| Pourbagheri-Sigaroodi ve ark. 2020 | Meta analiz (15 yayın) | Lenfopeni, Nötrofil yüksekliği, Lökositoz |
| Wang ve ark. 2020 | 138 | Lenfopeni |
| Ruan ve ark. 2020 | 150 | Lenfopeni, N/L yüksekliği |
| Tan ve ark. 2020 | 35 | Lenfopeni |
| Yang ve ark. 2020 | 94 | Nötrofil yüksekliği, Lökositoz |
| Huang ve ark. 2020 | 41 | Lenfopeni, Nötrofil yüksekliği, Lökositoz |
| Wang ve ark. 2020 | 60 | Lenfopeni, Nötrofil yüksekliği, Lökositoz |
| Goudouris 2021 | Derleme | Trombositopeni, Lenfopeni |
| Liu ve ark. 2020 | 61 | N/L yüksekliği |
| Yang ve ark. 2020 | 93 | N/L, T/L yüksekliği |
| Lagunas-Rangel ve ark. 2020 | Meta analiz (6 yayın) | N/L yüksekliği |
| Liu ve ark. 2019 | 270 | N/L yüksekliği |
| ***Koagulasyon parametreleri*** | | |
| Tang ve ark. 2020 | 183 | Artan protrombin zamanı ve D-dimer yüksekliği |
| Huang ve ark. 2020 | 41 | Artan protrombin zamanı ve D-dimer yüksekliği |
| Vieira ve ark. 2020 | 150 | Artan protrombin zamanı, aktive parsiyel tromboplastin zamanı |
| Bashash ve ark. 2020 | Meta analiz (21 yayın) | D-dimer yüksekliği |
| Bao ve ark. 2020 | 178 | Protrombin zamanı, INR D-dimer yüksekliği |
| ***İnflamatuar parametreler*** | | |
| Pourbagheri-Sigaroodi ve ark. 2020 | Meta analiz (15 yayın) | C-reaktif protein, prokalsitonin yüksekliği |
| Tan ve ark. 2020 | 102 | C-reaktif protein yüksekliği |
| Hu ve ark. 2020 | Derleme | Prokalsitonin yüksekliği |
| Ruan ve ark. 2020 | 150 | C-reaktif protein yüksekliği |
| Zhou ve ark. 2020 | 191 | Ferritin yüksekliği |

**Kısaltmalar:** N/L= Nötrofil/Lenfosit, T/L= Trombosit/Lenfosi**t**

## 4. Sonuç

COVID-19 hastalarında laboratuvar parametreleri, pıhtılaşma ve bağışıklık sisteminin hiper aktivasyonu, hiper inflamasyon,ve sitokin fırtınası gelişimi sonucu ortaya çıkan değişiklikleri göstermektedir. Hastalığın seyri boyunca, lenfosit sayımlarının ve CRP düzeyleri takibinin yapılması, hastalığı değerlendirmeye yardımcı olabilir. Hastalığın daha agresif bir hale dönmesinde, nötrofil/lenfosit oranı, nötrofil sayımları, protrombin zamanı, D-dimer, prokalsitonin ve ferritin düzeyleri, daha kötü prognozu gösteren önemli faktörler olarak kabul edilmektedir.

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