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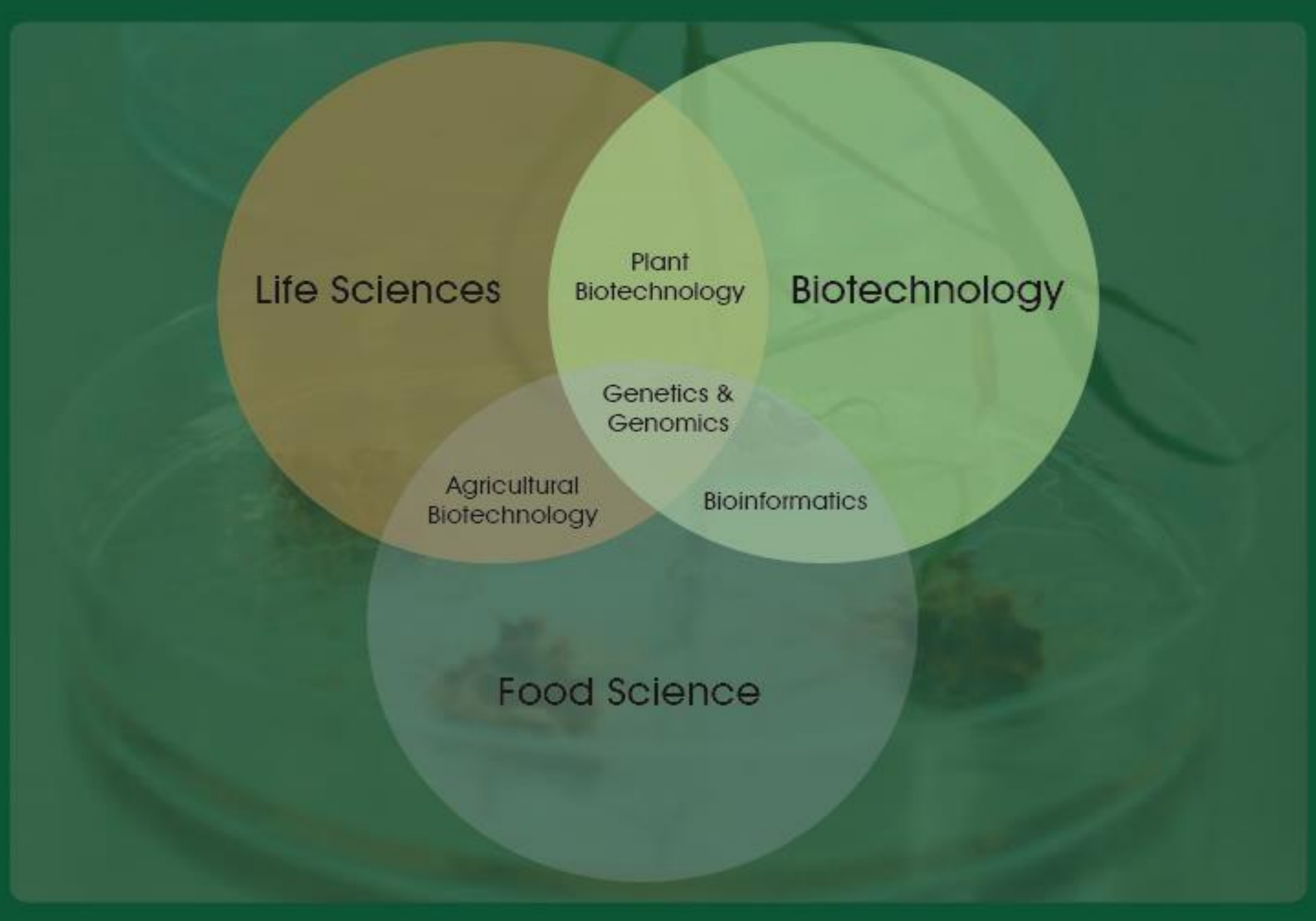

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Bu düşüncelerle 2022 yılı ikinci sayısını yayınladığımız “International Journal of Life Sciences and Biotechnology” dergisini, makaleleri ile onurlandıran akademisyenlere, Fikir / Görüş / Öneri / Katkı ve Eleştirileri ile değerlendirme süreçlerine katkılarından dolayı hakem ve yayın kurullarında yer alan kıymetli bilim insanlarına yürekten teşekkür ediyoruz. Bir sonraki sayıda görüşmek ümidiyle…

**15.08. 2022**
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**Dear Readers and Authors,**

As “International Journal of Life Sciences and Biotechnology”, we are pleased and honored to present the 13th issue of the journal. "International Journal of Life Sciences and Biotechnology" is an international double peer-reviewed open access academic journal published on the basis of research- development and code of practice.

The aims of this journal are to contribute in theoretical and practical applications in relevant researchers of Life Sciences, Biology, Biotechnology, Bioengineering, Agricultural Sciences, Food Biotechnology and Genetics institutions and organizations in Turkey, and to publish solution based papers depending on the principle of impartiality and scientific ethics principles, focusing on innovative and added value work, discussing the current and future.

With these thoughts, We are especially thankful to academicians honoring with the articles, valuable scientists involved in editorial boards and reviewers for their contributions to the evaluation processes with through their opinions/ideas/contributions/criticisms in the second issue of 2022 "International Journal of Life Sciences and Biotechnology". Hope to see you in the next issue…

**15. 08. 2022**

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**Research article**

Beyhan-Yılmaz, M., E. Seçginli, and F. Zemheri-Navruz, *Drosophila melanogaster*'de Antidepresan (Sitalopram) Kullanılarak Oluşturulan Strese Karşı E ve C Vitamininin Koruyucu Etkisi. International Journal of Life Sciences and Biotechnology, 2022. 5(2). p. 131-144. DOI: 10.38001/ijlsb.1037734

# *Drosophila melanogaster*'de Antidepresan (Sitalopram) Kullanılarak Oluşturulan Strese Karşı E ve C Vitamininin Koruyucu Etkisi

**Melisa Beyhan-Yılmaz[1,2]*, Ecem Seçginli[2], Fahriye Zemheri-Navruz[2]**

**ÖZET**

Bu çalışmada antidepresan etken maddesi olan Sitalopram'ın *D. melanogaster* ömür uzunluğuna ve eşey oranına olası etkileri ve buna karşı C ve E vitamininin koruyuculuğu araştırıldı. Sitalopram'ın 3 farklı dozu (0-1mM) ve C-E vitaminlerinden (25mg/L) oluşturulan gruplara beslenme yolu ile verildi. Döl sayısı deneyi 7 gün süreyle, ömür uzunluğu deneyi 39 gün süreyle uygulandı. Bu çalışmada yer verilen döl sayısı deneylerinde, uygulama gruplarında döl veriminin azalması beklenirken, erişkin sineklerin oluşturduğu döl sayısının kontrol grubu ile yakın sayılarda olduğu gözlemlendi. Ayrıca vitaminli gruplarda ise döl sayısı kontrole kıyasla düşüşe geçti. Bununla birlikte ömür uzunluğu deneyinde kontrol grubunda hayatta kalma yüzdesi %80'lere düşerken Sitalopram'lı gruplarda hayatta kalma yüzdesi %80'nin üzerinde kaldı. Ancak Sitalopram ve vitaminlerin birlikte uygulandığı grupların hayatta kalma oranları %60'lara inerek kontrol grubuna göre düşük seyretti. Sonuç olarak; Sitalopram'ın sineklerin döl sayısını değiştirmediği, ömür uzunluğu deneylerinde ise vitamin ile birlikte alınan ilacın kontrol grubuna kıyasla ömrü olumsuz etkilediği belirlendi.

**MAKALE GEÇMİŞİ**
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17 Aralık 2021
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**ANAHTAR KELİMELER**
Sitalopram,
E vitamini,
C vitamini,
yavru döl sayısı,
ömür uzunluğu

# Protective Effect of Vitamins E and C Against Stress Generated Using Antidepressant (Citalopram) in *Drosophila melanogaster*

**ABSTRACT**

In the study, the negative effects of Citalopram, which is an antidepressant active ingredient, on *D. melanogaster* lifespan and sex ratio, and the protection of vitamins C and E against this were investigated. Three different doses of Citalopram (0-1mM) and vitamins C and E (25mg/L) were given to the groups by diet. The number of progeny test was carried out for 7 days, and the longevity test was performed for 39 days. In the progeny number experiments included in this study, it was observed that while the fertility of the treatment groups was expected to decrease, the number of offspring formed by adult flies was close to the control group. In addition, the number of offspring in the vitamin group decreased compared to the control. However, in the longevity experiment, the survival rate in the control group decreased to 80%, while the survival rate in the Citalopram groups remained above 80%. However, the survival rate of the groups in which Citalopram and vitamins were administered together was lower than the control group, decreasing to 60%. As a result; It was determined that Citalopram did not change the number of offspring of the flies, and in the longevity experiments, the drug taken together with the vitamin affected the lifespan negatively compared to the control group.

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Citalopram,
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[1] Tekirdağ Namık Kemal Üniversitesi, Sağlık Bilimleri Enstitüsü, Tümör Biyolojisi ve İmmünolojisi Bölümü, Tekirdağ/Turkey
[2]Bartın Üniversitesi, Fen Fakültesi, Moleküler Biyoloji ve Genetik Bölümü, Bartın/Turkey
* Correspondence: Melisa Beyhan-Yılmaz; mlsa.byhn@gmail.com

## Giriş

Stres insanların sürekli maruz kaldığı bir faktördür. Stresin sürekli olması durumunda bağışıklık sistemi, kardiyovasküler sistem fizyolojisi, merkezi sinir sistemi ve sinir iletimi yolları negatif etkilenebilir [1, 2, 3]. Bununla birlikte stres depresyona yol açabilmektedir ve bu durum insanları antidepresan kullanımına yöneltebilir [1, 3].

Marasine ve ark., (2020) [4] hastalar üzerinde yaptıkları bir çalışmada hastaların neredeyse hepsinin kullandıkları antidepresanların yan etkilerinden olumsuz etkilendikleri ve hücresel düzeyde incelendiğinde ise hücrenin normal metabolizmasını bozduğu, hücreye anormal sinyallerin gelmesi ve bunları takiben hücrede oksidatif stres gözlendiğini ortaya koymuşlardır. Antidepresanlar büyük bir ilaç grubunu temsil etmektedir [1, 5]. Antidepresanlar Monoamin Oksidaz İnhibitörleri (MAOI), Trisiklik Antidepresanlar (TSA), Seçici Noradrenerjik Gerialım İnhibitörleri (NRI), Noradrenalin ve Dopamin Gerialım İnhibitörleri (NDGI) ve Seçici Serotonin Gerialım İnhibitörleri (SSRI) gibi gruplardan oluşmaktadır. Bunlardan SSRI grubu en az yan etkiye sahip olduğu için daha çok tercih edilmektedir [6].

SSRI grubunda bulunan ilaçların başında Sitalopram gelmektedir. Bu antidepresanlar içinde bulunan Sitalopram'ın son zamanlarda yapılan bazı çalışmalarda serbest radikal oluşumu ve antioksidan savunma sistemini bozulması birbirleriyle bağlantılı olacağı için serbest radikal oluşturarak antioksidan savunma sistemini bozabileceği belirtilmiştir [7, 8, 9, 10, 11]. Sitalopram'ın uzun süreli kullanımı sonucu oluşabilecek olan reaktif oksijen türleri (ROS) ve reaktif nitrojen türlerinde (RNS) meydana gelen artışların hücredeki protein, lipit ve DNA'nın yapısını olumsuz etkileyebileceği ve bununla birlikte diyabet, kanser vb. hastalıklara yol açabileceği bildirilmiştir [12, 13].

ROS'un zararını azaltabilmek ya da tamamen ortadan kaldırabilmek için hücresel antioksidanlar kullanılmaktadır. Bu hücresel antioksidanlar ROS üretimini durduramadığı zamanlarda devreye diyet kaynaklı antioksidanlar girmektedir. Askorbik asit (C vitamini), α-tokoferol (E vitamini) gibi antioksidanlar serbest radikalleri inaktive etmektedir ve bu özellikleri ile organizmada strese bağlı yaşlanmayı geçiktirebileceğine dair bazı kanıtlar elde edilmiştir [14, 15, 16].

*D. melanogaster;* hastalıklar, gelişim fizyolojisi ve genetik gibi birçok alanda kullanılan model organizmadır [17, 18]. *D. melanogaster'*in model organizma olarak kullanılmasının en önemli sebebi genomu insan genomuna %60 homolog olmasıdır ve insan hastalıklarına neden olan genlerin %75'i ile benzerlik göstermesidir [19]. Aynı zamanda boyutunun 2-3 mm civarında olması, kısa yaşam döngüsü, laboratuvarda üretiminin kolay olması, yalnızca sekiz adet kromozomunun olması, araştırmalar için en uygun hayvan modellerinden biri olarak kullanılmasının önünü açmıştır [17]. Yapılan bu çalışmada da bu özellikleri dolayısıyla meyve sineği olarak bilinen *Drosophila melanogaster* tercih edilmiştir.

Bu çalışmanın amacı doğrultusunda Sitalopram'ın olası olumsuz etkilerini azaltmak veya ortadan kaldırmak için C ve E vitamini birlikte kullanıldı. Çalışmamızda E vitamininin kullanılma sebebi, hücrede stres sonrası oluşan ROS'ların ve doymamış yağ asitlerinin ortadan kaldırılmasını sağlarken serbest radikallerin oluşumunu da engellemesidir [20, 21].

Bununla birlikte E vitamininin oksidatif hasara karşı koruyucu etkisi [19, 22, 23, 24, 25, 26] ve C vitamininin, sitoplazma ve hücre dışı sıvıda bulunan serbest radikallerle reaksiyona girebildiği yapılan çalışmalarla ortaya konulmuştur [22, 27,28]. Bu da çalışmada C ve E vitamininin kullanılabilirliğinin düşünülmesini sağlamıştır.

Yapılan çalışmalarda C ve E vitamininin bir arada kullanılması antioksidan etkiyi artırarak strese bağlı hasarın tek kullanıldıklarda ki etkisi göre daha güçlü yanıt sağladığı gösterilmiştir [22]. Bu sebeple bu çalışmada E vitamininin etkisinin artırılabilmesi için C ve E vitamini kombinasyonunu kullanıldı. Çalışmada *D. melanogaster*'de Sitalopram'a bağlı stres oluşturup bu stresin fiziksel etkilerini tespit etmek ve bu etkileri E ve C vitaminleri kombinasyonuyla ortadan kaldırmak amaçlandı ve bu amaç doğrultusunda yavru döl sayısı ve ömür uzunluğundaki değişimi belirlemek üzere deneyleri yapıldı.

## Materyal ve Yöntem

### Stok model organizma materyali

Bu çalışmada Bartın Üniversitesi, Fen Fakültesi, Moleküler Biyoloji ve Genetik Bölümü, Genetik Araştırma Laboratuvarı'nda uzun yıllardan beri kullanılan genetik olarak homozigot, mutant karakter taşımayan ve laboratuvar kültür stoğu olan *D. melanogaster'*in Oregon-R yabanıl soyu kullanıldı.

*D. melanogaster* kültürleri, 24°C sabit sıcaklığa ayarlı havalandırmalı etüvde uygun laboratuvar koşullarında yetiştirildi [29]. *D. melanogaster*'lerin beslenme ortamı için klasik *Drosophila* besi yeri 300 ml olacak şekilde hazırlandı [29, 30].

Stok kültürden alınan *D. melanogaster*'ler, çalışmada gerekli olan F1 dölünün elde edilmesi için 10 dişi ve 10 erkek birey kullanılarak 200 ml'lik cam şişelerde standart *Drosophila* besiyerlerine konularak beslenmeleri sağlandı. Standart *Drosophila* besi yerine alınan sinekler uygun yaşam koşullarını sağlamak amacıyla 25°C'de gelişmeleri için etüve yerleştirildi [29]. Sineklerin döllenme sonucu oluşan larvalarından her deney setinde 20 adet olmak üzere yavru döl deneyleri için seçildi. Ömür uzunluğu deneylerinin uygulaması için ise oluşan larvalardan birkaçı başka bir besiyeri ortamına alındı ve bu larvalardan aynı gün erginliğe ulaşan sineklerden 10 tanesi seçildi [31].

**Yöntem**

Bu çalışmada üç farklı doz Sitalopram (0.01 mM, 0.1 mM, 1.0 mM) (Citalopramhydrobromide, Sigma-Aldrich, MO, USA; CAS Number 59729-32-7) Kasture ve ark., (2019)'dan [32] uyarlanarak yükselen dozlar şeklinde uygulandı. Üç farklı doz Sitalopram ile birlikte E (Sigma-Aldrich, MO, USA; CAS Number: 10191-41-0) ve C (Sigma-Aldrich, MO, USA; CAS Number: 50-81-7) vitamini (25 mg/L) uygulama grupları olarak belirlendi [33].

Her deney seti için 8 farklı grup olmak üzere 1. grup kontrol, 2. grup 0.01 mM Sitalopram, 3. grup 0.1 mM Sitalopram, 4. grup 1.0 Mm Sitalopram, 5. grup E + C (25 mg/L) vitamin, 6. grup 0.01 mM Sitalopram ile E + C (25 mg/L) vitamin, 7. grup 0.1 mM Sitalopram ile E + C (25 mg/L) vitamin ve 8. grup 1.0 mM Sitalopram ile E + C (25 mg/L) vitamin olarak oluşturuldu.

Ömür uzunluğu deneyleri için oluşturulan gruplara aynı zamanda erginliğe ulaşmış 10 adet erkek sinek bırakıldı. Yapılan deneylerde gruplardaki dişi sineklerin yumurtlama sorunu olabileceğinden deney istenilen doğrultuda ilerletilebilmesi için erkek bireyler ile deney düzeneği kuruldu. Deneye dahil edilen sinekler karanlık ortamda her gün gözlemlendi ve bu gözlemler sonucunda gruplardaki ölüm oranları ve sağkalım süreleri hesaplandı.

Yavru döl sayısı deneyleri için ise, stoklardan alınan larvalar her gruba 20 adet eklendi ve oluşan F1 dölünden 5'er adet erkek ve dişi sinekler seçilerek yeni deney setleri oluşturuldu. Daha sonra standart besi yerinde beslenmiş erkek sineklerle antidepresanlı

ve antidepresan + vitaminli besi yerlerinde (uygulama grupları) beslenmiş dişi sinekler taze besi yerine alınarak yeni bir deney seti oluşturuldu. Aynı zamanda standart besi yerinde beslenmiş dişi sinekler ile antidepresanlı ve antidepresan + vitaminli besi yerinde beslenmiş erkek sinekler taze besi yerine alınarak bir başka deney seti daha oluşturuldu. Bu yeni deney setlerindeki sineklerin çiftleşip larva oluşturması beklendi ve oluşan F2 dölündeki bireyler gözlendi. Deneyler iki tekrar sonrası sonlandırıldı.

**İstatistiksel Analizi**

İstatistiksel analiz SPSS (Statistical Package for Social Sciences) 20.0 (20.0.1.0 (142)) programı kullanılarak yapıldı. Ömür uzunluğu deneyi ve yavru döl sayısı deneyinde kontrol ve uygulama gruplarına ait sonuçlar, oneway (tek yönlü varyans analizi) ANOVA (Scheffe, Duncan, Tukey HSD) ile analiz edilip $p < 001$ anlamlı kabul edildi.

## Bulgular ve Tartışma

### Bulgular

***Yavru döl sayısı deneyi için;***

Oluşturulan deney setinin 1. gününde dişi bireylerde kontrole göre sadece vitamin ve Sitalopram içeren grupların (5., 6., 7. ve 8. grup) yavru döl sayısı tablo 1'de gösterildiği gibi normal seyretti. En düşük doz olan 0.01 mM Sitalopram içeren grupta (2. grup) ise kontrole göre yavru döl sayısında azalma görüldü. Diğer gruplardaki döl sayısı ve erişkin birey oluşma sayıları tablo 1' de gösterildi.

Aynı zamanda sadece vitamin ve vitamin + Sitalopram içeren gruplarda (5., 6., 7. ve 8. grup) pupadan erişkine geçme süresi kontrole göre kısaldı, sadece Sitalopram (2., 3. ve 4. grup) içeren gruplarda erişkin birey görülmezken sadece vitamin içeren grupta (5. grup) 6 kat, vitamin + 0.1 mM (7. grup) Sitalopram içeren grupta 3 kat artış gözlenirken, vitamin + 1.00 mM Sitalopram içeren grupta (8. grup) kontrol grubuyla aynı sayıda erişkin gözlendi (Tablo 1).

Erkek bireylerde ise Sitalopram içeren gruplar (2., 3. ve 4. grup) kontrol grubuna göre normal seyretmekteyken sadece vitamin (5. grup) ve vitamin + 1 mM (8. grup) Sitalopram içeren gruplarda kontrol grubuna göre artış gözlendi fakat diğer vitamin + Sitalopram (6. ve 7. grup) gruplarında azalma görüldü (Tablo 1).

**Tablo 1** Yavru Döl Sayısı Deneyinin Birinci ve Yedinci Günlerinde Erişkin ve Pupa Sayıları

**Table 1** Adult and Pupa Numbers on the First and Seventh Days of the Juvenile Progeny Experiment

| **Gözlem günleri** | | **Grup 1** | **Grup 2** | **Grup 3** | **Grup 4** | **Grup 5** | **Grup 6** | **Grup 7** | **Grup 8** |
|---|---|---|---|---|---|---|---|---|---|
| **Dişi bireyler için deneyin 1. günü (uygulama grubu dişi ve normal erkek)** | | 92 pupa + 1 erişkin dişi | 45 pupa | 110 pupa | 90 pupa | 99 pupa+ 6 erişkin dişi | 93 pupa | 87 pupa+ 3 erişkin dişi | 96 pupa + 1 erişkin dişi |
| **Dişi bireyler için deneyin 7. günü (uygulama grubu dişi ve normal erkek)** | | 57 pupa + 68d. + 54e. | 29 pupa + 15e. + 10d. | 102 pupa + 44d. + 33e. | 106 pupa + 61e. + 49d. | 51 pupa + 49e. + 48d. | 67 pupa + 23d. + 26e. | 92 pupa + 66e. +64d. | 81 pupa 64e+ 55d. |
| **Toplam** | **pupa** | 149 | 74 | 212 | 196 | 150 | 160 | 179 | 177 |
| | **erişkin** | 122 | 25 | 77 | 110 | 97 | 49 | 150 | 119 |
| **Erkek bireyler için deneyin 1. günü (uygulama grubu erkek ve normal dişi)** | | 92 pupa + 1 erişkin dişi | 81 pupa | 67 pupa | 93 pupa | 108 pupa | 105 pupa | 56 pupa | 53 pupa |
| **Erkek bireyler için deneyin 7. günü (uygulama grubu erkek ve normal dişi)** | | 57 pupa + 68d. + 54e. | 86 pupa + 60e. + 65d. | 118 pupa + 57e. + 56 d. | 87 pupa + 79e. + 64d. | 101 pupa + 61d. + 60e. | 75 pupa + 82e. + 60d. | 90 pupa + 62d. + 61e. | 105 pupa + 54e. + 60d. |
| **Toplam** | **pupa** | 149 | 167 | 185 | 180 | 209 | 180 | 146 | 158 |
| | **erişkin** | 122 | 125 | 113 | 143 | 121 | 142 | 123 | 114 |

***e: Erkek sinek**

***d: Dişi sinek**

Gruplar karşılaştırıldığında ise, uygulama grubu dişi bireyler ve normal erkek bireylerin F2 döllerini içeren deney seti ile uygulama grubu erkek bireyler ile normal dişi

bireylerin F2 döllerini içeren gruplardaki oluşan yavru döl sayılarında istatistiksel olarak anlamlı farklılıklar belirlendi ($p<001$'e göre) (Tablo 2). Bu deney setlerinde oluşan yavruların pupaya dönüşmesi ve yeni ergin sineklerin oluşumuyla ilgili verilerde ise istatistiksel olarak anlamlı bir fark bulunamadı ($p<001$'e göre)(Tablo 2).

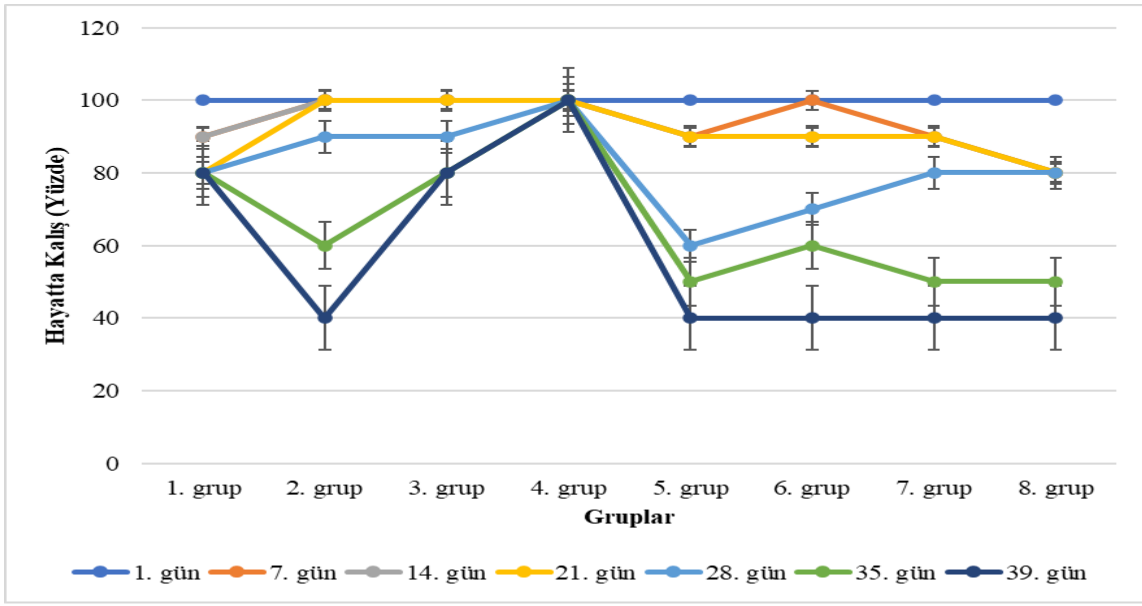
**Tablo 2** Yavru döl sayısı deneylerinin ANOVA testine göre elde edilmiş sonuçları ($^{*}p < 001$)
**Table 2** Results of offspring progeny experiments according to ANOVA test ($^{*}p < 001$)

| Varyansın Kaynağı | | Karelerin Toplamı | Karelerin Ortalaması | Df (serbestlik derecesi) | F (frekans) | Sig. (önem derecesi) |
|---|---|---|---|---|---|---|
| **Dişi** | Gruplar arası | 7656,250 | 7656,250 | 1 | 38,882* | <,001* |
| | Grup içi | 2756,750 | 196,911 | 14 | | |
| | Toplam | 10413,000 | - | 15 | | |
| **Erkek** | Gruplar arası | 6123,063 | 6123,063 | 1 | 24,155* | <,001* |
| | Grup içi | 3548,875 | 253,491 | 14 | | |
| | Toplam | 9671,938 | - | 15 | | |
| **Pupa** | Gruplar arası | 21389,063 | 21389,063 | 1 | 20,251 | 1,000 |

***Ömür uzunluğu deneyleri için;***

Kontrol grubunda ilk sineğin ölümünden 23 gün sonra Sitalopram içeren gruplarda (2., 3. ve 4. grup) ilk ölümler gözlendi. Bununla birlikte vitamin grubunda (5. grup) kontrol ile orantılı sayıda sinek ölümü gözlendi. Sitalopram içeren vitamin gruplarında ölüm oranında hızlı bir artış gözlemlenmedi (Şekil 1). Kontrol ve uygulama gruplarında hayatta kalış oranları gözlemlendiğinde beklenenin tam tersi en yüksek doz olan 1.0 mM (4. grup) Sitalopram içeren grup deneyin yapıldığı 39 gün boyunca tüm bireyler hayatta kalmayı başardı. 0.1 mM (3. grup) Sitalopram içeren grup ise kontrol grubu ile eşit hayatta kalma yüzdesine sahip olduğu görüldü bunun aksine diğer tüm gruplar (2., 5., 6., 7. ve 8. grup) kontrole göre daha az hayatta kalış gösterdi. Bu da yüksek iki dozun hayatta kalış oranlarını olumlu etkilerken en düşük doz ve etken madde ile vitamin gruplarının hayatta kalış oranlarını olumsuz şekilde etkilediğini ortaya koymaktadır (Şekil 1). Aynı zamanda vitamin içeren gruplar kontrole daha yakın

hareketler sergiledi, vitamin + Sitalopram içeren gruplarda normal hareketlere ek olarak besi yeri yüzeyinde gezinmeler görüldü.




**Şekil 1** Kontrol grubu ve uygulama gruplarının ömür uzunluğu deneylerinde hayatta kalış yüzdelerine göre karşılaştırılması

**Fig 1** Comparison of control group and treatment groups according to survival percentages in longevity experiments

Yavru döl sayısı deneylerinde olduğu gibi ömür uzunluğu deneylerinde de istatistiksel olarak karşılaştırmalar yapıldı. Bu karşılaştırmalarda grupların kendi içinde ve gruplar arasındaki hayatta kalış yüzdelerine göre yapılan karşılaştırmalarda Sitalopram + vitamin içeren grupların (6-8) istatistiksel olarak anlamlı farklılık içerdiği gözlendi (p<001'e göre) (Tablo 3). Sadece Sitalopram içeren gruplar istatistiksel olarak anlamlı bulunmadı (p<001'e göre).

**Tablo 3** Ömür uzunluğu deneylerinin ANOVA testine göre elde edilmiş sonuçları ($^{*}p < 001$)

**Table 3** Results of longevity experiments according to ANOVA test (*p < 001)

| Varyansın Kaynağı | | Karelerin Toplamı | Karelerin Ortalaması | Df (serbestlik derecesi) | F (frekans) | Sig. (önem değeri) |
|---|---|---|---|---|---|---|
| **Kontrol** | **Gruplar arası** | 11,143 | 1,857 | 6 | - | - |
| | **Grup içi** | ,000 | ,000 | 14 | | |
| | **Toplam** | 11,143 | - | 20 | | |
| **Sitalopram** | **Gruplar arası** | 17,619 | 2,937 | 6 | 1,927 | ,146 |
| | **Grup içi** | 21,333 | 1,524 | 14 | | |
| | **Toplam** | 38,952 | - | 20 | | |
| **Vitamin** | **Gruplar arası** | 80,571 | 13,429 | 6 | - | - |
| | **Grup içi** | ,000 | ,000 | 14 | | |
| | **Toplam** | 80,571 | - | 20 | | |
| **Sitalopram + Vitamin** | **Gruplar arası** | 75,238 | 12,540 | 6 | 29,259* | <,001* |
| | **Grup içi** | 6,000 | ,429 | 14 | | |
| | **Toplam** | 81,238 | - | 20 | | |

## Tartışma

Bu çalışma, antidepresanların yan etkilerinin hücrede meydana getirdiği değişikliklerin fenotipe olumsuzlukları ve bu olumsuzlukların vitamin takviyesiyle elimine edilip edilemeyeceğini tespit etmek için yapıldı ve model organizmalardan biri olan *D. melanogaster* kullanıldı. Antidepresanlı besi yerinde beslenmiş sinekler ve antidepresan ile birlikte vitamin eklenmiş besi yerinde beslenmiş sinekler karşılaştırıldı bu karşılaştırmalarda ise döl verimi ve yaşam süresine bakıldı.

Günümüzde en çok kullanılan antidepresanlardan Sertelin ve sitolapramın somatik mutasyon ve rekombinasyon testi ile *Drosophila* üzerinde genotoksik etkisi bilindiği için ve Sitalopram'ın da Sertelin'e göre daha genotoksik olduğunu otaya koyan çalışmalardan dolayı [34] bu çalışmada Sitalopram tercih edilmiştir. Sitalopram'ın neden olduğu stres antioksidanlarla önlenebildiği yapılan çalışmalarca gösterilmiştir [35]. Çalışmada antidepresan kullanımına bağlı olarak oluşabilecek strese karşı en çok

tercih edilen antioksidan vitaminler olan E ve C vitaminleri tercih edilerek *D. Melanogaster* sineklerinde denendi. Zemheri-Navruz (2018)'de [41] yaptığı çalışmada arsenik ile doyurulmuş besi yerinde beslenmiş *D. melanogaster*'lerde DNA hasarının oluştuğunu göstermiş ve bu DNA hasarını sineklerin besi yerin C ve E vitamini takviye edildikten sonra DNA hasarının azaldığını göstermiştir. Bu çalışmaya bakarak çalışmamızda Sitalopram'ın oluşturacağı strese karşı E vitamini ve E vitaminin antioksidan özelliğini artıracağı için C vitamini ile birlikte verilmesi uygun olacağı düşünüldü.

Çalışmalara başladıktan sonra besi yerine Sitalopram ilavesi yapılmış bireylerin fiziksel davranışlarının kontrole göre anormal olduğu gözlemlendi. Sitalopram içeren gruplardaki bireyler deney şişesi içerisinde hızlı uçuşlar sergiledi.

Gelegen ve ark. (2000) [36] yaptıkları çalışmalarda kadmiyum nitrat ile besledikleri larva ve sineklerde döl veriminin etkilenmediğini göstermişlerdir. Bu da *D. melanogaster* toksik bir maddeye maruz kalmış olsa bile döl veriminin etkilenmeyeceğini düşündürmektedir. Ancak bu çalışmada kullanılan en yüksek doz olan 1.0 mM Sitalopram (4. grup) içeren grupta yavru döl sayısı üzerine olumsuz sonuçlar alındı (tablo 1). Bu da Sitalopram'ın yüksek dozunun toksisite oranıyla ilgili literatürde yeterli kanıt ve yapılmış deney olmamakla birlikte *D. melanogaster*'de yavru oluşumunu engellediğini göstermektedir. Aynı deneylerde Sitalopram'ın tüm dozlarıyla birlikte vitamin E ve vitamin C verildiğinde döl sayısına olumlu yönde etkileri olurken; ömür uzunluğuna olumsuz yönde etki ettiği belirlenmiştir (Şekil 1). Driver ve Georgeou (2003)'te [40] yaptığı bir çalışmada E vitamininin *D. melanogaster*'lerde yaşam süresini uzattığı fakat aynı zamanda toksik etki de gösterebileceğini ortaya koymuştur. Bu çalışmaya dayanarak dişi sineklerde C vitaminin E vitamini ile kullanıldığında ömür uzunluğuna olumsuz yansıma sebebinin E vitaminin toksik etki gösterebileceğini düşündürmekle birlikte yavru döl sayısında ise olumlu sonuçların elde edilmesinde antioksidan özelliğinin arttığını düşündürmüştür. Erkek bireylerde ise ömür uzunluğu ve döl verimi üzerine vitaminli grupların pek bir etkisi olmadığı görülmüş bu da bireylerdeki cinsiyet farklılığına göre E ve C vitamini kombinasyonunun etkilerinin değişiklik gösterebileceği ortaya çıkmaktadır. Ries ve ark. (2017) [37] yaptıkları çalışmada sineklerde depresyon oluşturabilmek için yürüttükleri çalışmada öğrenilmiş çaresizlik deneyleri yapmışlardır. Bu deneyler sonucunda sineklerin bu stres karşısında

davranışlarının değiştiğini gözlemlemişlerdir. Bizim çalışmamızda ise stresi fiziksel bir eylem değil de bir ilaç hammaddesi oluşturdu. Sineklerin besi yeri üzerinde yavaş yürümeleri ve çoğunlukla besi yeri üzerinde kalmaları bu hammaddenin sineklerde stres oluşturduğunu düşündürmektedir. Bununla birlikte Sitalopram'lı gruplarda hem dişi sineklerde hem de erkek sineklerde hacim artışı ve uçuşlarında anormallikler de gözlemlendi. Bu durum stres altında sineklerde fiziksel özelliklerin değiştiğini gösterir. Ancak bu stresin yanında hayatta kalma oranlarında bariz bir düşüş gözlemlenmemesiyle birlikte fiziksel olarak görülen sıkıntıların organizmada olumsuz bir etki gösterip göstermeyeceği belirsizdir.

Yapılan diğer çalışmalarda meyve sineklerine uygulanan antioksidanlı besin takviyesinin oksidatif stresten koruyabildiği ve yaşam sürelerinin artışını sağladığı gösterilmiştir [38]. Bahadorani ve ark (2008) 'de [35] yaptıkları çalışmada C vitamini takviyesinin normoxia şartları altında *Drosophila*'ların yaşam süresini artırdığını göstermiştir. Bununla birlikte Massie ve ark. (1991)'de [39] *Drosophila*'la ile yaptıkları bir çalışmada sinekler tarafından C vitamini emiliminin yaşa bağlı olarak azaldığını göstermiş, C vitamini emiliminin azalması yaşlılığın bir belirtisi olduğunu bildirmiştir. Yine Bahadoroni ve ark. (2008)'de [35] yaptıkları çalışmada *D. melanogaster*'lerin diğer memelilere göre ortamdan E vitamini tutma oranının az olduğunu ve bunun da sineklerin ömür uzunluğuna etki etmediğini göstermiştir. Ancak stres altındayken E vitamini takviyesinin ömrü önemli ölçüde uzattığını göstermiştir. Yine aynı çalışmada C vitaminine maruz bırakılan sineklerde antioksidan kapasitesi artmakta fakat ömür uzunluğu değişmemektedir. Bu çalışma hipoksi gibi stres altındayken sineklerin C vitamin takviyesinde ise ömür uzunluğunun azaldığını göstermiştir. Yaptığımız çalışmada ise, C ve E vitaminini ayrı ayrı denemeyip kombine şekilde denememiz ömür uzunluğunu etkilemedi. Bahadoroni ve ark.'ların çalışmasına dayanarak sineklerin E vitaminini ortamdan yeteri kadar alamadıklarını ve C vitamininin de stres altında ömür uzunluğuna bir etkisi olmadığını düşündürmüştür.

## Sonuç ve Öneriler

Majör depresif bozukluk, anksiyete bozukluğu gibi hastalıkların tedavisinde kullanılan antidepresanların kullanımı oksidatif strese neden olabilmektedir [42]. Bu stres DNA'da bozukluklara neden olabilmekle birlikte bu bozuklukları yatıştırmak için güçlü antioksidanlar işe yarayabilmektedir [41]. İnsanların antidepresanlar ile birlikte

alabileceği vitamin takviyeleri ile bu zararlardan kurtulabilmesinin mümkün olabileceği düşünülmektedir. Bu çalışmada da uygun model organizma *D. melanogaster*'de antidepresanlardan biri olan Sitalopram'ın oluşturduğu stres ve bu stresi ortadan kaldırmak için C ve E vitamini kombinasyonu kullanıldı. Genetik testlerin yapılmaması nedeni ile Sitalopram'ın DNA üzerindeki etkisi tam olarak bilinmemekle birlikte bu çalışmanın sonucunda Sitalopram'ın sineklerde fiziksel değişikliklere yol açtığı gözlendi. Bu antidepresan hammaddesinin sineklerde oluşturduğu olumsuz etkileri yatıştırmak için C ve E vitaminli gruplar oluşturuldu fakat bu iki güçlü antioksidan vitaminin çalışmalarda pek bir etkisinin olmadığı sonucuna varıldı. Bu da E vitamininin oluşturacağı etkiyi C vitamininin baskıladığı yönde bir düşünce oluşturdu (Tablo 1, Tablo 2). Bu sebeple yapılacak diğer çalışmalarda E ve C vitamininin kombinasyonu yerine bu vitaminler tek tek çalışılması gerektiği düşülmektedir. Aynı zamanda *D. melanogaster* ile yapılacak çalışmalarda E vitaminin kullanılmasının bir faydası olmayacağı düşünülmektedir çünkü bu sinekler E vitaminini besi yerinden yeterli miktarda absorbe edememektedir. Yapılacak yeni çalışmalarda DNA hasarının belirlenmesi ve sitotoksisite testleriyle desteklenmesi önerilmektedir. Bu çalışmanın gelecek çalışmalara yol gösterebileceği düşünülmektedir.

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**Research Article**

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# Identification and characterization of potential druggable targets among Essential Hypothetical Proteins of A. Baumannii

**Bydaa Atron[1*]**

**ABSTRACT**

Acinetobacter baumannii is a critical pathogen responsible for a wide range of infections. A. baumannii exhibits resistance to a variety of antibiotic classes, emphasizing that new therapeutic targets are urgently needed. In A. baumannii, ATCC 179778. Among 458 essential genes, 47 are uncharacterized and considered to be an essential hypothetical protein (EHPs). In this study, the functional characterization of EHPs was conducted utilizing variable computational tools. The physicochemical parameters, subcellular localization, domain identification, 3D structure, and virulence capabilities were predicted for the EHPs. According to our results, they were shown to be of a different functional category such as: transporters, enzymes, binding proteins, and virulence factors. Enzymes made up around 47% of the total and 17.6% were predicted as virulence factors. BLASTP analysis against human proteome was tested to identify proteins that is found exclusively in pathogen. The druggable property of the proteins was examined. Of 34, 27 essential pathogen-specific proteins could be considered as new pharmacological targets. In draggability analysis, one EHP turned out to be druggable while the others were novel. Our findings might assist in the innovation of new drugs for *Acinetobacter baumannii* infections.

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## Introduction

*Acinetobacter baumannii* is an important bacterium that can induce a vast scope of diseases, including hospital-acquired infections. Antibiotic overuse and misuse have been increasing as a result, antibiotic-resistant strains of A. baumannii have arisen [1]. World Health Organization labeled the multidrug-resistant (MDR) A. baumannii (resistance to at least three separate classes of antimicrobial drugs) as one of three "critical priority pathogens" that in urgent need of novel therapeutics. [2]. A. baumannii is characterized by possessing intrinsic antimicrobial resistance mechanisms that can be expressed continuously or in response to antibiotic stress. The regulating of antibiotic transport via bacterial membranes, mutation of the antibiotic target site, and enzyme alterations that result in antibiotic neutralization are all types of antimicrobial resistance mechanisms.

[1] Sivas Cumhuriyet University, Faculty of Medicine, Department of Medical Microbiology, Sivas / Turkey
*Corresponding Autor: Bydaa Atron, e-mail: bidaahamad@gmail.com

[3]. More worryingly, A. baumannii can quickly develop resistance to all clinically available medicines, making illness caused by resistant strains exceedingly difficult to treat. Colistin-resistant isolates are spreading at an alarming rate throughout the world, making it critical to discover alternative therapeutics [4]. A proportion of genes is definitely vital for an organism. These genes are called essential genes. The proteins that they encode are called essential proteins (EPs). Essential genes and proteins are theoretically possible therapeutic targets since their deletion or inactivation causes the organism's death. As a result, predicting the essentiality of a gene in a pathogenic microbe might aid in the prospective therapeutics for the development of antimicrobial medicines. Database of Essential Genes (DEG) is a collection of essential elements from several organisms [5]. A large number of essential genes for A. baumannii have been identified using antisense RNA technique. However, some of them are hypothetical (uncharacterized) essential proteins (EHPs)[5]. Functional and structural characterization of these proteins will provide information on their structures, functionalities, and metabolic roles. [6]. There is evidence for the successful use of computational tools for the annotation of HPs of various pathogens, including *Vibrio parahaemolyticus* [7], *Candida dubliniensis* [8], *Chlamydia trachomatis* [9], *Leptospira interrogans* [10], *Mycobacterium tuberculosis* [11], *Haemophilus influenza* [12], *Neisseria meningitidis* MC58 [13], *Mycobacterium leprae* Br492 [14], and *Borrelia burgdorferi* [15].
Till now, no experimental information on EHPs of *A. baumannii*, the present attempt was made to annotate the function of these proteins using computational approach.

## Material and Methods

### Sequence retrieval and analysis

*A.baumannii* ATCC 179778 has 3587 coding genes, 458 (13%) of which are identified as essential genes in Database of Essential Genes. Out of the 458 essential genes, about 47 proteins with unknown function, and thus need characterization. Pseudo genes which are proteins having less than 100 amino acid residues were omitted in this study to reduce the misperceptions in functional annotation workflow (13 sequences were found with < 100 amino acids in this study), the remaining 34 were selected for the analysis.

**Functional annotation and domain identification**

Numerous publicly accessible bioinformatics tools and databases were utilized to assign functions to all A. baumannii EHPs. Tools involved: Pfam, NCBI-BLAST, Conserved domain database, and InterProScan [16].

**BLASTp: Basic Local Alignment Search Tool**

BLASP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) is used to predict identical or similar proteins through the identification of the homologous sequences with known functions from the non-redundant NCBI protein database [16]. BLASP tool compares the amino acid chain of proteins and finds the best local alignment [17].

**Pfam**

Pfam ( http://pfam.xfam.org/ ) is a comprehensive database of conserved protein families. This tool predicts protein families based on multiple sequence alignments and hidden Markov models [18]. The seed alignments are used to build profile hidden Markov models (HMMs) that can be used to search any sequence database for homologues in a sensitive and accurate fashion. Those homologues that score above the curated inclusion thresholds are aligned against the profile to make a full alignment [19].

**InterProScan**

The InterPro database (https://www.ebi.ac.uk/interpro/) provides an integrative families, domains and motif classification. InterProScan integrates various protein traditional authentication techniques from the InterPro project for motif identification [20]. InterPro also dispenses additional information such as descriptions, literature references and Gene Ontology (GO) terms, to produce a comprehensive resource for protein classification [21].

**Conserved Domain Database (CDD)**

The Conserved Domain Database ( https://www.ncbi.nlm.nih.gov/cdd/ ) is a protein annotation resource that consists of a collection of well-annotated multiple sequence alignment models for ancient domains and full-length proteins. These are available as position-specific score matrices (PSSMs) for fast identification of conserved domains in protein sequences via RPS-BLAST. CDD content includes NCBI-curated domains, which use 3D-structure information to explicitly define domain boundaries and provide insights into sequence/structure/function relationships, as well as domain models

imported from a number of external source databases (Pfam, SMART, COG, PRK, TIGRFAMs) [22].

**Physicochemical characterization**

Physicochemical characterization of the EHPs was performed using ExPASy PROTPARAM proteomics tools ( https://web.expasy.org/protparam/ ). The physicochemical characteristics of HP can contribute to the understanding of its products and the development of additional in vitro assays. ProtParam predicts the molecular weight (MW, given in Da), isoelectric point (IP), number of AAs (No of AA), instability index (II) and atomic composition of the targets. This tool also calculates other parameters, as the extinction coefficient (EC, given in $M^{-1}cm^{-1}$), useful in spectrophotometry experiments, aliphatic index (AI), related with thermostability and grand average of hydropathy (GRAVY), that indicates the interaction of proteins with water [23].

**Subcellular localization analysis**

The functions of proteins are mainly determined by their subcellular localizations in cells. SOSUI predicts subcellular localization of proteins together with the prediction of transmembrane helices [24]. SOSUI is a web-based tool with its Internet address (https://harrier.nagahama-i-bio.ac.jp/sosui/mobile/). The performance of this tool is 99% for the discrimination between two types of proteins (membrane against soluble protein) and 96% for the prediction of transmembrane helices [25].

**Virulence factor prediction**

The web server VICMpred (http://www.imtech.res.in/raghava/vicmpred/) has been used to predict virulence factors among EHPs. According to the sequences patterns and amino acid compositions VICMpred can categories gram-negative bacterial proteins into information molecules, proteins involved in the cellular process, and virulence factors [26]. Virulence factors allow the pathogens to establish themselves in the host. They include adhesions, toxins, and hemolytic molecules [27].

**Host non-homology analysis**

Here, proteins found exclusively in the pathogen were identified in this step. This analysis is crucial to identify proteins that are unique to bacteria and do not have any similarity with the host, in order to design specific antimicrobials. Protein BLAST search against

the human proteome with the e-value was adjusted to 0.0001 and cut-off to 100. Protein that showed no significant hits passed through this level [28,29].

**Target Identification**

A target is considered if proven to show the potentiality to bind with drug-like substances with high affinity. Following the prior step, the pathogen-specific proteins were subjected to a similarity search against the DrugBank targets repertoire. The existence of similar proteins with the same function in the DrugBank target list provides evidence for their draggability. The absence demonstrates the protein's novelty, and it is thus termed as a "novel target." [30].

**Secondary structure prediction:**

Determination of protein structure is essential for the understanding of protein function. The secondary structure of our models for HPs was estimated by SOPMA server (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html ). SOPMA predicts a three-state description of the secondary structure: alpha-helix, beta-sheet and coil [31].

**Homology Modeling and Validation**

In order to recognize the protein functions of proteins at a molecular level, it is sometimes necessary to determine their 3D structure accurately and reliably. First, the 3D models of the EHPs (pdb format) were predicted by CPH model 3.2 server (https://services.healthtech.dtu.dk/service.php?CPHmodels-3.2). CPH model 3.2 is a protein homology modeling server. The template recognition is based on profile-profile alignment guided by secondary structure and exposure predictions. This tool was previously accessible through (http://www.cbs.dtu.dk/) [32]. UCSF Chimera package used to visualize the 3D structure of predicted proteins. UCSF Chimera is a program for the interactive visualization and analysis of molecular structures and related data, including density maps, trajectories, and sequence alignments [33].

**Model refinement**

To test the correctness of 3D proteins mode, The structure results were cross checked by ERRAT and PROCHECK tools were used. [34]. ERRAT verifies protein structures determined by crystallography. Error values are plotted as a function of the position of a sliding 9-residue window. The error function is based on the statistics of non-bonded atom-atom interactions in the reported structure; compared to a database of reliable high-

resolution structures [34]. PROCHECK tool checks the stereochemical quality of a protein structure by analyzing residue-by-residue geometry and overall structure geometry [35].

## Results and Discussion

Understanding the functions of essential hypothetical proteins is important since it facilitates the further comprehension of their role in physiological and pathological processes and the identification of novel classes of therapeutic targets and ultimately to combat with the infection. The present study utilized various available bioinformatics to identify drug and vaccine targets from essential hypothetical proteins of *A. baumannii*. In this paper, out of 458 *A. baumannii* essential genes, 47 EHPs were retrieved. After eliminating the pseudogenes, 34 EHPs were chosen for functional analysis. Bioinformatics methods were employed to assign functions, and they were found to be present in many functional groups (Table1).

**Table 1** functionally annotated EHPs in A. baumannii ATCC 179778

| EHP | DEG Accession Number | Function | Type | Pathogen specific |
|---|---|---|---|---|
| **1** | DEG10430024 | Iron-sulfur cluster insertion protein ErpA | Metal binding | ✕ |
| **2** | DEG10430040 | Uracil reductase RibD | Oxidoreductases | ✔ |
| **3** | DEG10430041 | LPS export ABC transporter permease LptG | Permease | ✔ |
| **4** | DEG10430066 | ABC transporter C-terminal domain | transporter | ✔ |
| **5** | DEG10430083 | Ton b family C-terminal domain protein | transporter | ✔ |
| **6** | DEG10430087 | Cytoskeletal protein RodZ | DNA binding | ✔ |
| **7** | DEG10430131 | Biotin--[acetyl-CoA-carboxylase] ligase | ligase | ✕ |
| **8** | DEG10430134 | 3-oxoacyl-ACP reductase FabG | Oxidoreductases | ✔ |
| **9** | DEG10430136 | hydroxymethylpyrimidine/phosphomethylpyrimidine kinase | kinase | ✔ |
| **10** | DEG10430158 | Solute carrier families 5 and 6-like | transporter | ✕ |
| **11** | DEG10430166 | RNase H | endoribonucleases | ✔ |
| **12** | DEG10430174 | LPS export ABC transporter periplasmic protein LptC | transporter | ✔ |
| **13** | DEG10430212 | Putative integral membrane protein | transporter | ✔ |
| **14** | DEG10430249 | Trna threonylcarbamoyl adenosine modification protein | hydrolase | ✔ |
| **15** | DEG10430253 | Hypothetical protein | transporter | ✔ |
| **16** | DEG10430256 | DUF3465 domain-containing protein | transporter | ✔ |
| **17** | DEG10430271 | DUF3465 domain-containing protein | DUF | ✔ |

**Table 1** functionally annotated EHPs in A. baumannii ATCC 179778 (Continued)

| | | | | |
|---|---|---|---|---|
| **18** | DEG10430275 | DIP1984 family protein | DUF | ✔ |
| **19** | DEG10430278 | DUF3465 domain-containing protein | DUF | ✔ |
| **20** | DEG10430290 | high frequency lysogenization protein HflD | hydrolase | ✔ |
| **21** | DEG10430321 | DUF3465 domain-containing protein | DUF | ✔ |
| **22** | DEG10430344 | Serine--trna ligase | ligase | ✕ |
| **23** | DEG10430346 | Folate-binding protein | Transferases | ✕ |
| **24** | DEG10430349 | Putative integral membrane protein | transporter | ✔ |
| **25** | DEG10430256 | TamB, inner membrane protein subunit o | Secretory protein | ✔ |
| **26** | DEG10430361 | membrane protein insertase | insertases | ✕ |
| **27** | DEG10430370 | Glutathione-dependent formaldehyde-activating enzyme | Lyases | ✕ |
| **28** | DEG10430372 | 23S rRNA (adenine(2030)-N(6))-methyltransferase | transferases | ✔ |
| **29** | DEG10430374 | rRNA maturation RNase YbeY | Ribosomal protein | ✔ |
| **30** | DEG10430415 | p)ppGpp synthase/hydrolase | hydrolase | ✕ |
| **31** | DEG10430429 | GNAT family N-acetyltransferas | transferases | ✔ |
| **32** | DEG10430446 | DUF4175 domain-containing protein | DUF | ✔ |
| **33** | DEG10430451 | Nucleotidyl transferase | transferases | ✔ |
| **34** | DEG10430452 | GlmU superfamily N-acetylglucosamine-1-phosphate-uridyltransferase | transferases | ✔ |

Around 16 (47%) of EHPs were enzymes,6 (17%) transporters,1(2%) binding protein, 5(14%) miscellaneous proteins, 4 (11%) domain of unknown function containing protein (DUF). Among EHPs 6 (17%) found to be virulence factors (Fig.1).

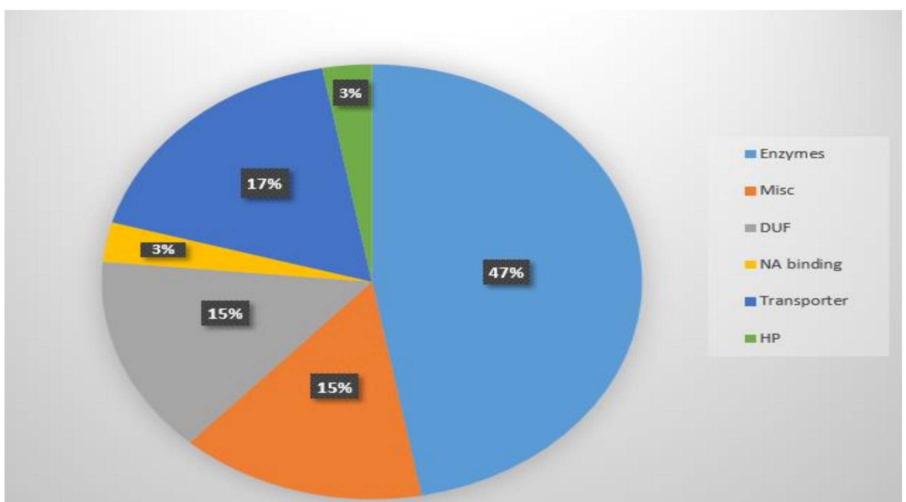



**Fig 1** Predicted Essential Proteins

Exploring pathogenicity at the molecular level requires in-depth knowledge of these functional groups. The descriptions of the protein groups are shown below.

**Functional annotation**

**Hydrolases**

Catalyzes the hydrolysis of a bonds. Bacterial hydrolase enzymes tin the splitting of distinct peptidoglycan bonds. Hydrolases are also participated in a range of additional processes, notably peptidoglycan development, autolysis, and septal cleavage on cell division [36]. Two of the proteins in this study were identified to be hydrolases. Threonylcarbamoyl adenosine biosynthesis protein TsaE and ysogenization regulator HflD are hydrolyses. The TsaE family of enzymes is engaged in the creating of threonylcarbamoyl adenosine (t(6)A) [37].

High frequency lysogenization protein (HflD) is a membrane protein that suppresses lambda phage by binding to and degrading CII and, as a result, limiting its interactions with DNA [38].

Bifunctional ppGpp (guanosine 3'-diphosphate 5'-diphosphate), a rigorous response mediator that regulate a range of cellular processes in response to nutritional availability alterations. This enzyme catalyzes both the production of pppGpp, which is afterwards hydrolyzed to create ppGpp which has a synthase and hydrolase activity), and the hydrolysis of ppGpp [39].

**Oxidoreductases**

Oxidoreductases catalyze the transfer of electrons between electron donor and acceptor, generally with cofactors such as nicotinamide adenine dinucleotide phosphate (NADP) or nicotinamide adenine dinucleotide (NAD) [40]. Two EHPs were discovered to be members of the oxidoreductase family. Uracil reductase, RibD, and 3-oxoacyl-ACP reductase. Riboflavin synthesis is supported by uracil reductase [41]. Animals can obtain riboflavin from nutrition, whereas bacteria synthesize it from scratch. This vital part of bacterial metabolism is absent in humans, indicating that this biosynthetic route might be a source of antimicrobial therapeutic targets. Dawson et al. designed methods for the purification and crystallization of RibD. They were successful in obtaining two ordered crystal forms with probable binding sites that were ready for ligand discovery [42].

**Ligases**

In the current study, two EHPs were predicted to be ligases. Ligase enzyme catalyzes the creation of a new chemical link between two large molecules. Biotin-dependent acetyl-CoA carboxylase enzyme catalyzes ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA. This process offers the required active substrate for fatty acid production [43]. This enzyme is well known to be a major target of antifungal and antibiotic medications [44,45]. The second anticipated ligase in this investigation is serine-tRNA ligase, which acts as a catalyst to the attachment of serine to tRNA (Ser). Which involved in in glycine, serine, and threonine metabolism and aminoacyl-trna biosynthesis.

**Lyases**

One EHP was predicted as lyase which is Glutathione-dependent formaldehyde-activating enzyme. In bacteria it serves in the pathway of formaldehyde detoxification [46].

**Transferases**

Six EHPs were predicted as transferases. Transferases catalyze the transfer of specific functional groups between molecules. They are involved in hundreds of different biochemical pathways throughout biology [47].

Aminomethyltransferase (T-protein) is part of the glycine cleavage system, along with P-protein, L-protein, and H-protein. This system is involved in glycine and serine catabolism pathways.

Ribosomal RNA large subunit methyltransferase methylates the adenine in 23S rRNA during ribosome biogenesis. The usage of extracellular DNA as neutrient is also mediated by this enzyme [48].

The N-acetyltransferases (NAT) is an enzyme that uses acetyl coenzyme A (CoA) to transfer an acetyl group to a substrate. This enzyme mediate pantothenate and CoA biosynthesis. Acetyl coenzyme A transfer activity is responsible for resistance to Aminoglycoside antibiotics [49].

UDP-N-acetylglucosamine diphosphorylase/glucosamine-1-phosphate N-acetyltransferase is part of the pathway for Amino sugar, nucleotide sugar, and O antigen nucleotide sugar biosynthesis [50]. The enzyme found in several bacteria such as *Escherichia coli, Bacillus subtilis*, and *Haemophilus influenza*, and has similar activity of glucosamine-1-phosphate N-acetyltransferase [51].

N-Acetylglucosamine-1-phosphate uridyltransferase abbreviated as GlmU, is a bifunctional enzyme exclusive to prokaryotes. GlmU is involved in the peptidoglycan and lipopolysaccharide biosynthesis [52]. Several studies were conducted to identify inhibitors specific to target this enzyme. [53,54].

**Permeases**

One permease was predicted. Permeases are membranous transporters that facilitate molecules diffusion around the membrane [55]. LPS export ABC transporter permease (LptG), an enzyme that carry out the function of lipopolysaccharide transport. This transport system uses energy from ATP hydrolysis in the cytoplasm to facilitate extraction of LPS from the outer face of the cytoplasmic membrane prior to transport to the cell surface. [56,57]. LptG system comprises seven different proteins (LptA, LptB, LptC, LptD, LptE, LptF, and LptG) that assemble into a complex spanning from the cytoplasm to the outer membrane [58].

**Nucleic acid Binding Proteins**

The bacterial nucleoid showes a multi-level hierarchical structural organization similar to eukaryotic chromatin [59]. This hierarchical structure maintains the global nucleoid organization and ensures the accessibility of particular chromosomal regions for DNA-dependent processes, such as replication, transcription, DNA repair, and recombination. The organization of the highly compacted yet dynamic nucleoid structure reflects the input of many different factors, including molecular crowding, depletion forces, DNA supercoiling, and nucleic acid binding proteins [60].

In this study one binding proteins was identified, which is cytoskeleton protein from RodZ family. It's a cytoskeletal protein that controls cell-shape by the manipulation of the length of the long axis [61].

**Transporters**

Six EHPs were identified as transporters. ABC transporter of C-terminal domain plays a role in ATP-binding cassette (ABC) transporter-dependent pathway. Polysaccharides O-antigenic (O-PSs) and Surface glycoconjugates are synthesized via this pathway [62]. Surface glycoconjugates are essential for cell envelope structure integrity, protection against host immunological responses, signaling cascades, and biofilm development [63]. ABC transporters act as antimicrobial targets [64]. TonB is a protein abundantly found in

many Gram-negative bacteria, and thought to regulate nutritional imports [65]. TonB system may have a function not just in protein import as well as in protein secretion [66]. Putative membrane protein Solute carrier 5 family and 6-like family; solute binding domain (SLC5). SLC5s co-transport sodium ions with other ions and sugers. [67].

Two EHPs were predicted to be membrane-associated proteins belong to EamA-like transporter family, which is the only drug/metabolite transporter that can be found in both prokaryote and eukaryotes [68].

LPS export ABC transporter periplasmic protein LptC, LptC is involved in lipopolysaccharide-assembly of gram-negative bacteria, which takes place in the outer membrane [69].

**Endoribonucleases**

It can split both single-stranded and double-stranded RNA. One protein was predicted to be Endoribonucleases. RNase H endonucleolytically cleaves RNA in RNA-DNA hybrid molecules. This activity is present in almost all organisms [70].

**Virulence proteins**

Pathogenic bacteria possess virulence factors that enable them to bring damage to the host. VICMpred predicted that among the EHP: 22 are related to cellular process, 1 in storage and information, 5 in metabolism, and 6 in virulence. (Table 2).

Antivirulence drugs are targeting virulence factors, renders the pathogen nonpathogenic (avirulent). In comparison to conventional antibiotics, it has been theorized that antivirulence agents have less resistance serendipity [71].

**Table 2** VICMpred Result

| Function Prediction | No |
|---|---|
| Cellular Process | 22 |
| Metabolism Molecule | 5 |
| Virulence factor | 6 |
| Information and storage | 1 |

**Physiochemical characterization**

Physiochemical properties for the proteins are essential for understanding the protein's function and interaction with other proteins. The physicochemical characteristics involve the molecular weight, positive and negative residues, amino acids, the theoretical isoelectric point (pI), extinction coefficient, which is important in studying protein interactions, is calculated from amino acids composition and grand average of

hydropathicity (GRAVY). The GRAVY value is used to determine whether the molecules are hydrophobic or hydrophilic. The results are depicted in the supplementary file.

**Cellular localization**

Determining protein subcellular localization is vital for understanding the role of proteins in a cell, and it also aids in the process of drug discovery and delivery. Through which it facilitates the classification of them as drug and vaccine targets. Possible drug target is cytoplasmic protein. while the membranous one considered a vaccine target. 24 were found to be cytoplasmic proteins and 10 were membrane proteins. The membrane regions were classified as primary and secondary, and the length of the transmembrane regions is predicted as well. and if the protein is rich in hydrophobic amino acids (Table 3).

**Potential Targets**

An ideal drug target should not show any similarity to human proteome. Hence, a host non-homology analysis was performed. Functionally annotated 34 hypothetical proteins subjected to host non-homology analysis using a BLASTp search against the human database. Out of 35 proteins, 27 were found to be non-homologous (potential drug targets). A druggability analysis was performed. Out of 27, one protein was found to be previously reported as a drug target. 3-Oxo-acyl-acyl carrier protein (ACP) reductase (FabG) is significant in bacterial fatty acid synthesis and has been identified as a target [72,73].

**Table 3** Prediction of cellular localization

<table>
<tr><th>EHP</th><th>Region</th><th>Transmembrane sequence</th><th>Type</th></tr>
<tr><td>1</td><td colspan="3">Soluble</td></tr>
<tr><td>2</td><td colspan="3">Soluble</td></tr>
<tr><td rowspan="4">3</td><td>10 --32</td><td>RIVGWVMRSALLLIVLSFALSEW</td><td>Primary</td></tr>
<tr><td>184--206</td><td>KVASPFSLITLVLVACSFIFGPL</td><td>Primary</td></tr>
<tr><td>209--231</td><td>QSMGFRLVIALFIGLGFYYLQDF</td><td>Secondary</td></tr>
<tr><td>237--259</td><td>LVYAPSPAWFVLMPIILMFGAGS</td><td>Primary</td></tr>
<tr><td>4</td><td colspan="3">Soluble</td></tr>
<tr><td>5</td><td colspan="3">Soluble</td></tr>
<tr><td>6</td><td>87--105</td><td>ALLAILIIAVVSLIVMGVQ</td><td>Primary</td></tr>
<tr><td>7</td><td colspan="3">Soluble</td></tr>
<tr><td>8</td><td colspan="3">Soluble</td></tr>
<tr><td>9</td><td colspan="3">Soluble</td></tr>
</table>

**Table 3** Prediction of cellular localization (Continued)

| EHP | Region | Transmembrane sequence | Type |
|---|---|---|---|
| **10** | 11--33 | SPLMAFCLSFVIMATLAPTMGIQ | Primary |
| | 40--62 | FWLLWFGTMLLLALPVCYLEIAL | Primary |
| | 80--110 | RVVGWLAVVFIPFLAGNVLSTAS | Primary |
| | 116--138 | QFAPSISGQIIFAGLAVAALVLS | Primary |
| | 143--165 | QILILLMTLGVIASIVLANTMGS | Primary |
| | 179--199 | EWGNATVLALVASGLGLGLYW | Primary |
| | 213--235 | TKTVLPIWLAQLIAVVAFGFFSL | Primary |
| | 239--261 | LPVLTWIFTGVMTSALFVQLARE | Primary |
| | 266--288 | RQLMPVLQWVIIVVAIAVWAVPE | Primary |
| | 294--316 | TLILMLWGLLICLIYAVFAGWIM | Primary |
| | 335--357 | LWRIAVRIVLPLSIIVAMIAVIG | Primary |
| **11** | Soluble | | |
| **12** | Soluble | | |
| **13** | 1--23 | MQMIAWNYASASLLCYLWFKPDI | Secondary |
| | 32--54 | PWWLIVALGVILPSIFLCLAKSL | Primary |
| | 59--81 | IVKTEIAQRLSVVLSLLAAYFFF | Secondary |
| | 87--109 | SLKLLGIGLGIFAVLLILLGHF | Primary |
| | 119--142 | AIFALMSVWFGYAAVDILLKYTS | Primary |
| | 147--169 | FTLTLNLIFITAFVLSIIYLIFQ | Primary |
| **14** | Soluble | | |
| **15** | Soluble | | |
| **16** | Soluble | | |
| **17** | Soluble | | |
| **18** | Soluble | | |
| **19** | Soluble | | |
| **20** | Soluble | | |
| **21** | Soluble | | |
| **22** | Soluble | | |
| **23** | Soluble | | |
| **24** | 1--23 | MQMIAWNYASASLLCYLWFKPDI | Secondary |
| | 32--54 | PWWLIVALGVILPSIFLCLAKSL | Primary |
| | 59--81 | IVKTEIAQRLSVVLSLLAAYFFF | Secondary |
| | 87--108 | SLKLLGIGLGIFAVLLILLGHF | Primary |
| | 119--141 | AIFALMSVWFGYAAVDILLKYTS | Primary |
| | 147--169 | FTLTLNLIFITAFVLSIIYLIFQ | Primary |

**Table 3** Prediction of cellular localization (Continued)

| EHP | Region | Transmembrane sequence | Type |
|---|---|---|---|
| **25** | Soluble | | |
| **26** | 39--61 | SGCLPLLLQMPIFLALYWVLMES | Primary |
| | 82--104 | WFILPLIMGATMFAQQMLNPQPA | Secondary |
| | 116--138 | PIMFTVFMLFFPAGLVLYWIVNN | Primary |
| **27** | Soluble | | |
| **28** | Soluble | | |
| **29** | Soluble | | |
| **30** | Soluble | | |
| **31** | Soluble | | |
| **32** | 92--114 | WLAILASLFLHVLLWLAYRAIAR | Primary |
| **33** | Soluble | | |
| **34** | Soluble | | |

In drug and vaccine design the determination of the 3D structure is important. The most common prediction method is the Homology approach. CPH model as modeling was used in this study for EHPs. Visualization of these predictions is performed by Chimera software. Validation of all structures was performed using the ERRAT program (Figure 2)

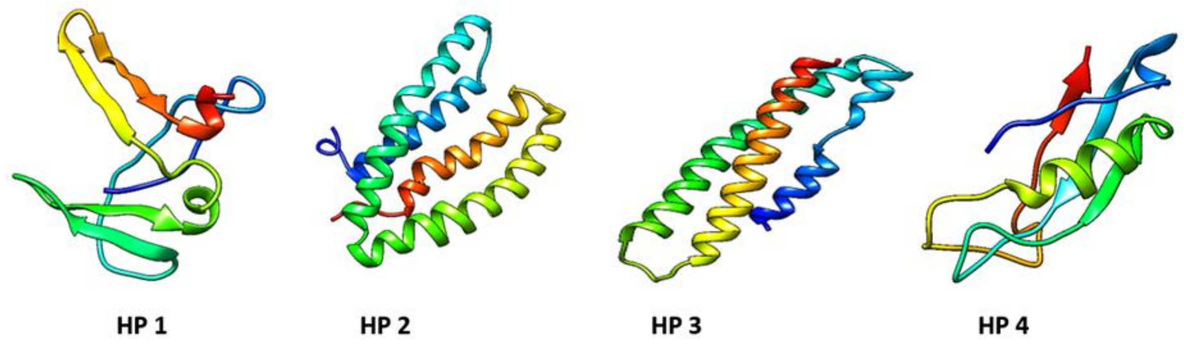
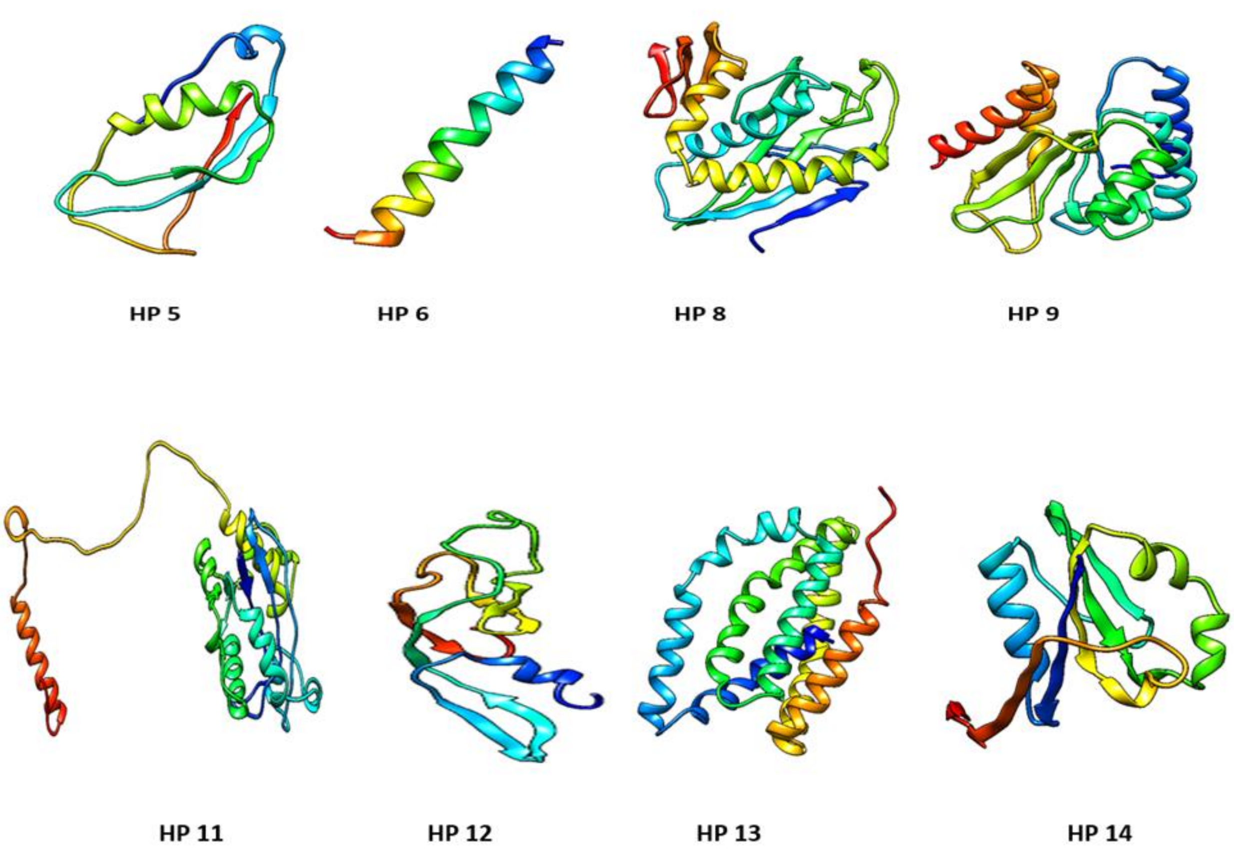



**Fig 2** Predicted 3D Structure



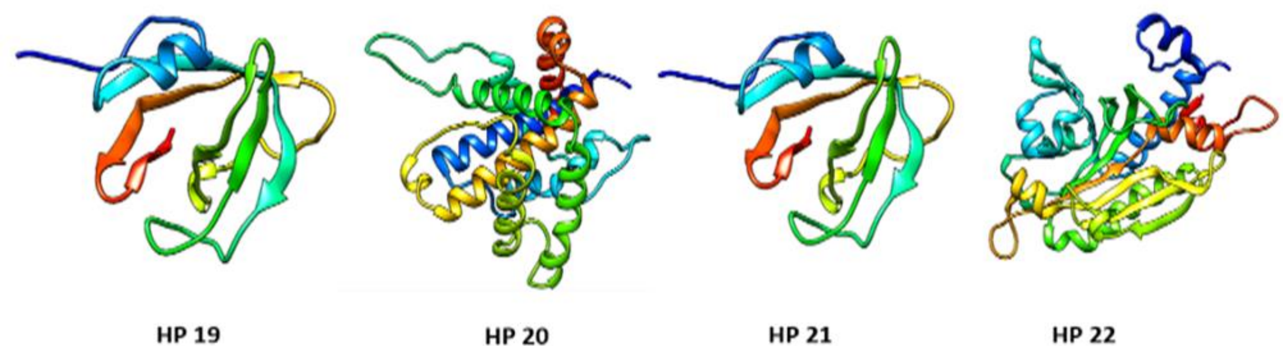
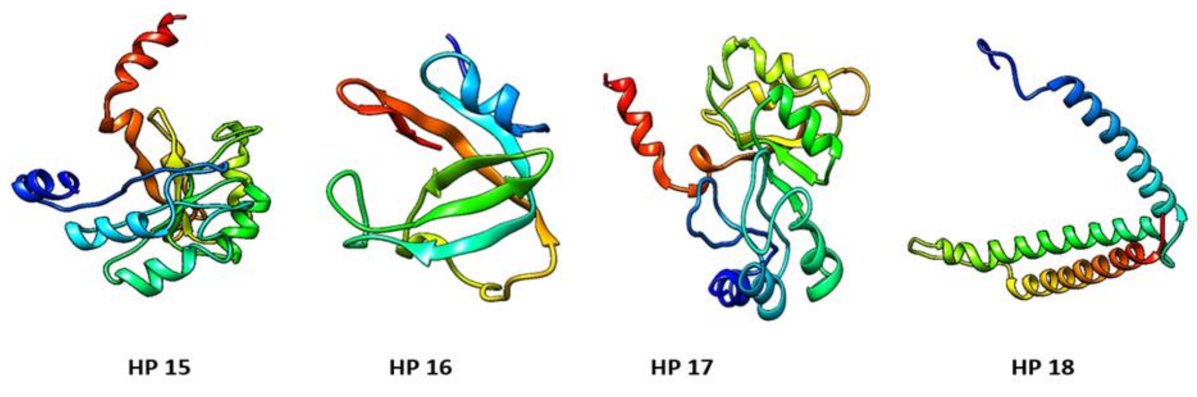




**Fig 2** Predicted 3D Structure (Continued)



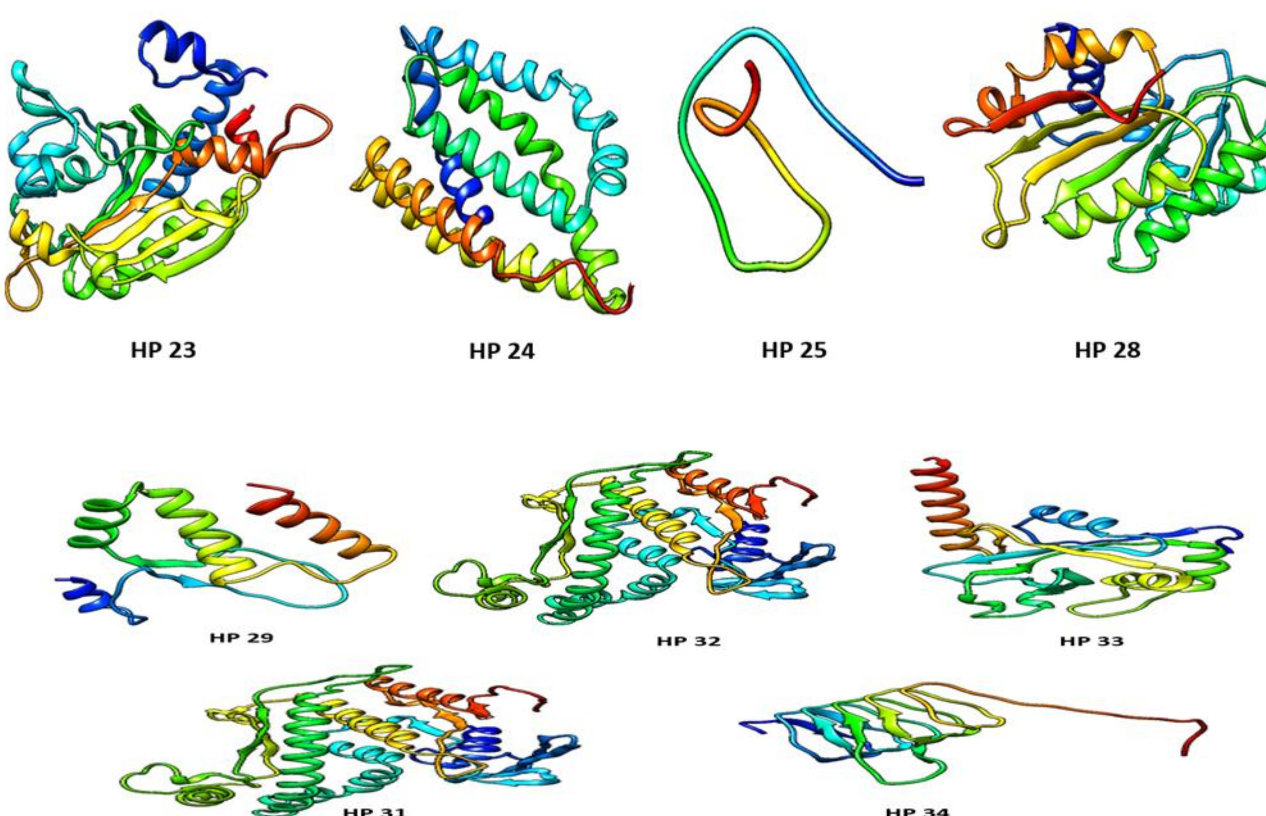




**Fig 2** Predicted 3D Structure (Continued)

## Conclusion

Exploring pathogenic microorganism's essential gene's function is crucial in medical research. The use of robust Insilco approaches, have been highlighted to prove the immense utilities of the knowledge databases and tools, toward characterization of

essential hypothetical proteins from *A. baumannii* ATCC 179778, whose genome wide information is elusive due to the presence of vast number of uncharacterized sequences. Characterizing these proteins will enhance our understanding of their virulence capacity and pathogenicity.

In this study, a variety of bioinformatic tools was employed to functionally characterize essential hypothetical proteins from *A. baumannii* ATCC 179778. The strategy applied predicted the function of more than 85% of hypothetical proteins pertaining to several key functional domains. Yet, some of EHPs predictions were not achievable due to scarcity of data. The cellular localization of these proteins was predicted using subcellular localization analysis. Subcellular localization helps in differentiating drug targets from vaccine targets. Furthermore, six of them were virulence factors. Host non-homology analysis shown that 27 proteins were specific to pathogen, so they are potential to be drug target candidates. 3-oxoacyl-ACP reductase is a known target [72]. The other proteins were identified as 'novel targets,' which require requires additional experimental validation. Hence, this study may facilitate future studies on the predicted EHPs as novel therapeutic targets for the drug and vaccine development.

**Abbreviations**

*A. baumannii*: *Acinetobacter baumannii*, CDD: Conserved domain database, BLAST: Basic Local Alignment Search Tool

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Please contact the corresponding author for any data request.

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# Çeşitli evsel atıkların ve bayat ekmeğin *Aureobasidium pullulans* tarafından melanin sentezinde kullanılması

**Gamze Nur Müjdeci[1]***

**ÖZET**
Bu çalışmada katma değeri yüksek bir pigment olan melaninin fermantasyon yolu ile üretimi için, soğan, patates, elma, armut ve havuç kabukları ile bayat ekmeklerin potansiyel kullanımı araştırılmıştır. Bu amaçla, belirtilen evsel atıklar ve bayat ekmek çeşitli ön işlemlerden geçirildikten sonra özütleri elde edilmiş ve bu özütler fermantasyon ortamı olarak kullanılmışlardır. Fermantasyon çalışmalarında inokulum olarak ayrı ayrı yerli bir suş olan *Aureobasidium pullulans* AZ-6 ile *A. pullulans* NBRC 100716 suşu kullanılmıştır. Fermentasyon deneyleri, 150 mL fermentasyon ortamı içeren 300 mL'lik pamuk tıkaçlı Erlenmeyer'lerde, 100 rpm'lik çalkalama hızında, çalkalamalı bir inkübatör kullanılarak 30°C'de gerçekleştirilmiştir. Melanin derişimleri (g/L) hücre içi ve hücre dışı olarak tespit edilmiştir. Çalışmada, *A. pullulans* AZ-6 suşunun hücre gelişimini en çok elma kabuğu özütü desteklerken, *A. pullulans* NBRC 100716'nın hücre gelişimini en fazla havuç kabuğu özütü desteklemiştir. Çalışılan substratlar ile *A. pullulans* AZ-6'nın sadece hücre dışı melanin üretebildiği ancak *A. pullulans* NBRC 100716'nın hem hücre içi hem de hücre dışı melanin üretebildiği belirlenmiştir. *A. pullulans* AZ-6 en yüksek miktarda melanin üretimini (1.34 ± 0.2 g/L) elma kabuğu özütü ile gerçekleştirmiştir. Soğan ve armut kabuklarının özütleri, *A. pullulans* AZ-6 suşunun hücre dışı melanin üretimini hiç desteklemezken, *A. pullulans* NBRC 100716'nın hücre dışı melanin üretimi için elverişli bir ortam olmuştur. Patates kabuğu özütünde *A. pullulans* AZ-6 suşu hiç melanin üretemezken, *A. pullulans* NBRC 100716 suşu diğer substratlara göre düşük miktarda (0.22 ± 0.04 g/L) hücre dışı melanin üretebilmiştir. Kuru ekmeğin substrat kaynağı olarak kullanıldığı deneylerde ise, çalışılan her iki suş tarafından da sadece hücre dışı melanin üretiminin gerçekleştiği belirlenmiştir. Bu araştırmada, en yüksek toplam (hücre içi + hücre dışı) melanin üretimi, 3.71 g/L olarak, *A. pullulans* NBRC 100716 tarafından havuç kabuğu özütü ile gerçekleşmiş; elma ve soğan kabuklarının özütleri ile de buna yakın bir sonuç elde edilmiş ve sırasıyla, 3.28 ve 3.02 g/L toplam melanin elde edilmiştir. Bu çalışma ile havuç, elma ve soğan kabuklarının melanin üretimi için önemli doğal substrat kaynakları olabilecekleri ortaya konmuştur.

**MAKALE GEÇMİŞİ**
**Geliş**
6 Aralık 2021
**Kabul**
25 Ocak 2022

**ANAHTAR KELİMELER**
Evsel atık,
bayat ekmek,
melanin,
*Aureobasidium pullulans*
fermantasyon

[1] Hitit University, Faculty of Engineering, Department of Food Engineering, Corum/ Turkey
*Corresponding Author: Gamze Nur Müjdeci, e-mail: gnurmujdeci1@gmail.com

# The use of various household wastes and stale bread in the synthesis of melanin by *Aureobasidium pullulans*

**ABSTRACT**

In this study, the potential use of onion, potato, apple, pear, and carrot peels, and stale bread for the production of melanin, a high-value-added pigment, by fermentation was investigated. For this purpose, the extracts of domestic waste and stale bread were obtained after various pre-treatments and these extracts were used as fermentation media. In the fermentation studies, a native strain Aureobasidium pullulans AZ-6 and A. pullulans NBRC 100716 strain were used separately as inoculum. Fermentation experiments were performed at 30°C using a shaking incubator at a shaking speed of 100 rpm in 300 mL cotton-plugged Erlenmeyers containing 150 mL of fermentation medium. The melanin concentrations (g/L) were determined as intracellular and extracellular. In the study, while apple peel extract supported the cell growth of A. pullulans AZ-6 strain the most, carrot peel extract supported the cell growth of A. pullulans NBRC 100716 the most. With the studied substrates, it was determined that A. pullulans AZ-6 could only produce extracellular melanin, but A. pullulans NBRC 100716 could produce both intracellular and extracellular melanin. A. pullulans AZ-6 produced the highest amount of melanin (1.34 ± 0.2 g/L) with apple peel extract. Onion and pear peel extracts did not support the extracellular melanin production of A. pullulans AZ-6 strain, while they were favorable media for the extracellular melanin production of A. pullulans NBRC 100716. While A. pullulans AZ-6 strain could not produce melanin in potato peel extract, A. pullulans NBRC 100716 strain was able to produce a low amount (0.22 ± 0.04 g/L) extracellular melanin compared to other substrates. In experiments where stale bread was used as a substrate source, it was determined that only extracellular melanin production was realized by both strains studied. In this study, the highest total (intracellular + extracellular) melanin production was achieved by A. pullulans NBRC 100716 in the carrot peel extracts as 3.71 g/L, and similar results were obtained with apple and onion peel extracts as 3.28 and 3.02 g/L, respectively. This study revealed that carrot, apple, and onion peels can be important natural substrate sources for melanin production.

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## Giriş

Melanin, tüm alemlerde yer alan organizmalar tarafından üretilebilen, fenolik ve indolik bileşiklerin oksidatif polimerizasyonu ile oluşturulan, yüksek molekül ağırlıklı hidrofobik bir polimer olarak tanımlanmaktadır [1]. Tipik olarak melaninin, koyu kahverengi veya siyah renkli olduğu ve yüksek molekül ağırlığına sahip olduğu belirtilmektedir. Negatif yüklü bir pigment olan melaninin, su ve organik çözücülerde çözünemediği ifade edilmektedir. Yapısal olarak, melaninin yüksek moleküler ağırlıklı polimerlerin bir karışımını temsil ettiği ve bu yapının söz konusu pigmenti kararlı ve oksidan ajanlar, kuruma, aşırı sıcaklık, UV ışığı, ağır

metaller ve ilaçlar gibi çeşitli fizikokimyasal işlemlere karşı dirençli kıldığı vurgulanmaktadır [2].

Birçok maya ve fungus türünün melanin pigmentini üretebildiği ve bu pigmentin mikroorganizmayı çeşitli olumsuz çevresel koşullardan koruduğu belirtilmektedir. En iyi bilinen melanin üretici fungus, maya ve maya benzeri funguslar arasında; *Kluyveromyces marxianus*, *Streptomyces chibanensis*, *Aspergillus* sp., *Wangiella dermatitidis*, *Burkholderia cepacia*, *Cry. neoformans*, *Exophiala dermatitidis*, *Fonsecaea pedrosoi*, *Exophiala dermatitidis*, *Sporothrix schenckii*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Candida albicans*, *Pneumocystis jirovecii*, *Scytalidium dimidiatum* ve *Aureobasidium pullulans* yer almaktadır [3].

*A. pullulans*, funguslar alemindeki *Ascomycota* grubu içerisinde yer alan; Dothideales takımı, *Dothideaceae* familyası ve *Aureobasidium* cinsine ait polimorfik bir tür olarak tanımlanmaktadır [4]. İlk geliştiklerinde krem, açık pembe ya da açık kahverengi olan *A. pullulans* kolonilerinin, daha sonra ürettikleri melanin pigmenti nedeni ile kahverengi ya da siyah renge dönüştükleri, bu nedenle de siyah maya olarak da adlandırıldıkları ifade edilmektedir [5].

Son yıllarda dünya çapında, sentetik pigmentler yerine mikroorganizmalar tarafından üretilen doğal kaynaklardan elde edilen pigmentlere olan ilgi artmaktadır [6]. Bunun sebebi olarak ise; doğal kaynaklardan elde edilen pigmentlerin daha güvenli, çevreye zarar vermeyen, çabuk bozunabilir ve zararlı etkileri olmayan pigmentler olması gösterilmektedir. Bunun yanı sıra sentetik pigmentlerin insanlara, hayvanlara ve çevreye karşı zararlı etkilerinin olduğu da ifade edilmektedir [7]. Mikroorganizmalar kullanılarak pigment üretiminin, kimyasal yollarla sentezlenmesinden daha verimli ve düşük maliyetli bir işlem olduğu vurgulanmaktadır. Mikroorganizmaların ayrıca, mevsimsel kısıtlamalarının olmaması, ucuz ve yüksek verimli üretim gerçekleştirmeleri nedeniyle bitkiler ve hayvanlara kıyasla daha uygun pigment kaynakları oldukları ifade edilmektedir. Melanin pigmentinin tarım, kozmetik ve ilaç endüstrilerinde çok geniş uygulama potansiyeline sahip olması ve bu pigmente artan talep göz önüne alındığında, mikrorganizmalar kullanılarak melanin üretimi üzerine yapılan çalışmaların artması gerektiği vurgulanmaktadır [8,9].

Fermantasyon yolu ile melanin üretiminde kullanılan substratlar, üretilen ürünün maliyetini önemli ölçüde etkilediğinden, prosesi endüstriyel ölçekte ekonomik hale getirmek için ucuz ve verimli substratların kullanılmasına ihtiyaç duyulduğu bilinmektedir. Bu amaçla biyoproseslerde ucuz substrat kaynağı olarak çeşitli tarımsal sanayi artıklarının kullanılmaları önem kazanmaktadır. Bu atıkların, tarladan sofraya tüm gıda zincirinde ortaya çıktığı ve dünya genelinde büyük sorun oluşturduğu bilinmektedir. Türkiye'de yaklaşık olarak yılda 45 bin ton soğan kabuğu ve 542 bin ton bayat ekmek ortaya çıkmaktadır [10]. Patatesin tarladaki atık (sap) miktarının yıllık yaklaşık 455 bin ton, elma ve armudun bahçedeki budama atığının ise yıllık yaklaşık 122 bin ve 27 bin ton olduğu belirtilmiştir [11]. Yalnızca Ankara'da meydana çıkan yıllık toplam mutfak atığının yaklaşık 5.35 megaton olduğu da bilinmektedir [12]. Geleneksel atık bertaraf yöntemlerinin çevresel, ekonomik ve sosyal sorunlara neden olduğu, bu nedenle de organik atıklarının katma değeri yüksek biyoteknolojik ürünlere dönüştürülmelerinin daha sürdürülebilir, küresel ihtiyacı karşılamaya yönelik ve kârlı olduğu da ortadadır. Bu şekilde, tarımsal sanayi artıklar ile organik evsel atıklara katma değer kazandırılmakta ve günümüz problemlerinden biri olan çevre kirlenmesinin de önüne geçilebilmektedir. Bu kapsamda yapılan çalışmalarda kullanılan doğal substratlar arasında; ananas, portakal ve nar atıkları, mısır likörü, üzüm atığı, mısır maserasyon sıvısı, jack meyvesi çekirdeği, buğday kepeği, hindistan cevizi posası, pirinç kepeği, pirinç unu, melas, ayçiçeği kabuğu, darı, muz sapı ve şeker kamışı küspesi hidrolizatının yer aldığı görülmüştür [7,9].

Bu çalışmanın amacı; literatürde ilk defa soğan, patates, elma, armut ve havuç kabukları ile bayat ekmek kullanılarak *A. pullulans* suşları ile melanin üretiminin gerçekleştirilmesidir.

## Materyal ve Metot

### *Aureobasidium pullulans* suşları

Bu çalışmada melanin üretimi için *A. pullulans* AZ-6 ve *A. pullulans* NBRC 100716 suşları kullanılmıştır. Suşlar, Hacettepe Üniversitesi Gıda Mühendisliği Bölümü (Ankara) öğretim üyesi Prof. Z. Yeşim Özbaş tarafından sağlanmıştır. *A. pullulans* AZ-6 suşu, yazarın yüksek lisans tezi çalışmaları sırasında, taze Gemlik zeytinlerinden izole edilirken, *A. pullulans* NBRC 100716 suşu Japonya'dan temin edilmiş bir çilek izolatıdır.

**Evsel atıklar ve ön işlemler**

Çalışmada, soğan, patates, elma, armut ve havuç kabukları ile bayat ekmekler fermantasyon ortamı hazırlamak için doğal substrat kaynakları olarak kullanılmışlardır. Araştırmada soğan, elma, armut ve havuç kabukları, Tarangini ve Mishra [9] tarafından önerilen yönteme göre ayrı ayrı ön işlemlerden geçirildikten sonra fermantasyon ortamı olarak kullanılmışlardır. Bu amaçla, 1000 g kabuk, bir blender yardımıyla parçalandıktan sonra üzerine 2 L saf su ilave edilmiş, elde edilen karışım 100°C'de 30 dakika buyunca kaynatıldıktan sonra özüt kaba filtrasyon ile ayrılmıştır. Elde edilen özütler 300 mL'lik Erlenmeyer'lere 150 mL hacimde dağıtıldıktan sonra otoklavda, 121°C'de 15 dakika sterilize edilmiş ve doğrudan fermantasyon ortamı olarak kullanılmışlardır.

Çalışmada kullanılan bayat ekmek ve patates kabuğundaki şekerler ise, asit hidrolizi ile özütlenmişlerdir. Bu amaçla, doğal substratlar bir blender yardımı ile ayrı ayrı parçalanmışlar ve ardından üzerlerine derişimi, substrat (g): asit çözeltisi (mL); 10:100 (w/v) olacak şekilde önceden hazırlanmış olan sülfürik asit çözeltisi ilave edilmiştir. Asit çözeltisi ise; 0.5 mL, %98'lik derişik $H_2SO_4$ (Merck, Almanya) çözeltisinin, 100 mL saf suya eklenmesiyle hazırlanmıştır. Elde edilen karışımlar oda sıcaklığında, rutin olarak karıştırma işlemi yapılarak yarım saat bekletilmiş ve daha sonra nişastanın etkin parçalanması amacıyla otoklavda 121°C'de 15 dakika tutulmuşlardır.

Otoklavdan çıkarılan karışımlar soğutulduktan sonra, temiz bir tülbent yardımıyla süzülmüş ve elde edilen süzüntü, 6000 rpm'de 30 dakika boyunca santrifüjlenmiştir. Daha sonra ise elde edilen homojen hidrolizat 300 mL'lik Erlenmeyer'lere 150 mL hacimde dağıtılmış ve otoklavda, 121°C'de 15 dakika sterilize edilmiştir. Steril hidrolizatlar doğrudan fermantasyon ortamı olarak kullanılmışlardır.

Fermantasyon ortamlarındaki başlangıç glukoz ve/veya fruktoz derişimleri, Dinitrosalisilik asit (DNS) yöntemi kullanılarak [13], toplam şeker derişimleri ise, sakkaroz cinsinden, fenol sülfürik asit yöntemi kullanılarak [14] tayin edilmişlerdir.

**Aşı kültürlerinin hazırlanması**

Fermantasyon deneylerinden önce, her bir suş için aşı kültürü hazırlanmıştır. Bu amaçla ilk olarak bileşimi (g/L): glukoz; 10, maya özütü; 3, malt özütü; 3 ve pepton; 5 olan steril Yeast ekstrakt Malt ekstrakt (YM) broth ve YM agar besiyerinde 30°C'de 48 saat geliştirilerek

aktifleştirilmiş olan *A. pullulans* kültüründen, 250-300 mL'lik erlenler içerisinde bulunan 100 mL hacmindeki steril Tryptic Soy Broth (Merck, Almanya) besiyerine aşılanmıştır. Sıcaklığı ve çalkalama hızı ayarlanabilen çalkalamalı inkübatörde, 30°C'de, 100 rpm çalkalama hızında, 48 saat geliştirilen aşı kültürü, yine erlenler içerisinde hazırlanan 150 mL çalışma hacmindeki doğal fermantasyon ortamlarına ayrı ayrı %5 (v/v) oranında inoküle edilmiştir.

Bu aşamada *A. pullulans* suşlarının, fermantasyon ortamlarındaki ayrı ayrı başlangıç inokülasyon derişimlerinin belirlenebilmesi amacıyla ilk olarak, üreme ortamından alınan kültürün %0.85'lik (w/v) serum fizyolojik içerisinde ardışık seyreltileri hazırlanmış, daha sonra, bu seyreltilerden petri kaplarında, önceden hazırlanmış YM agar besiyerlerine, yüzeye sürme yöntemi ile ekimler gerçekleştirilmiştir. Ekimleri yapılan besiyerlerinin 30°C'de 48 saat inkübasyonlarının ardından, oluşan kültürlerde koloni sayımları yapılmış ve başlangıç *A. pullulan*s sayıları; kob/mL cinsinden hesaplanmıştır.

**Fermantasyon deneyleri**

Fermentasyon deneyleri, 150 mL fermentasyon ortamı içeren 300 mL'lik pamuk tıkaçlı Erlenmeyer'lerde, 100 rpm'lik bir çalkalama hızına sahip çalkalamalı bir inkübatör (NB-203QMS, N-Bitech) kullanılarak 20 gün boyunca, 30°C'de gerçekleştirilmiştir.

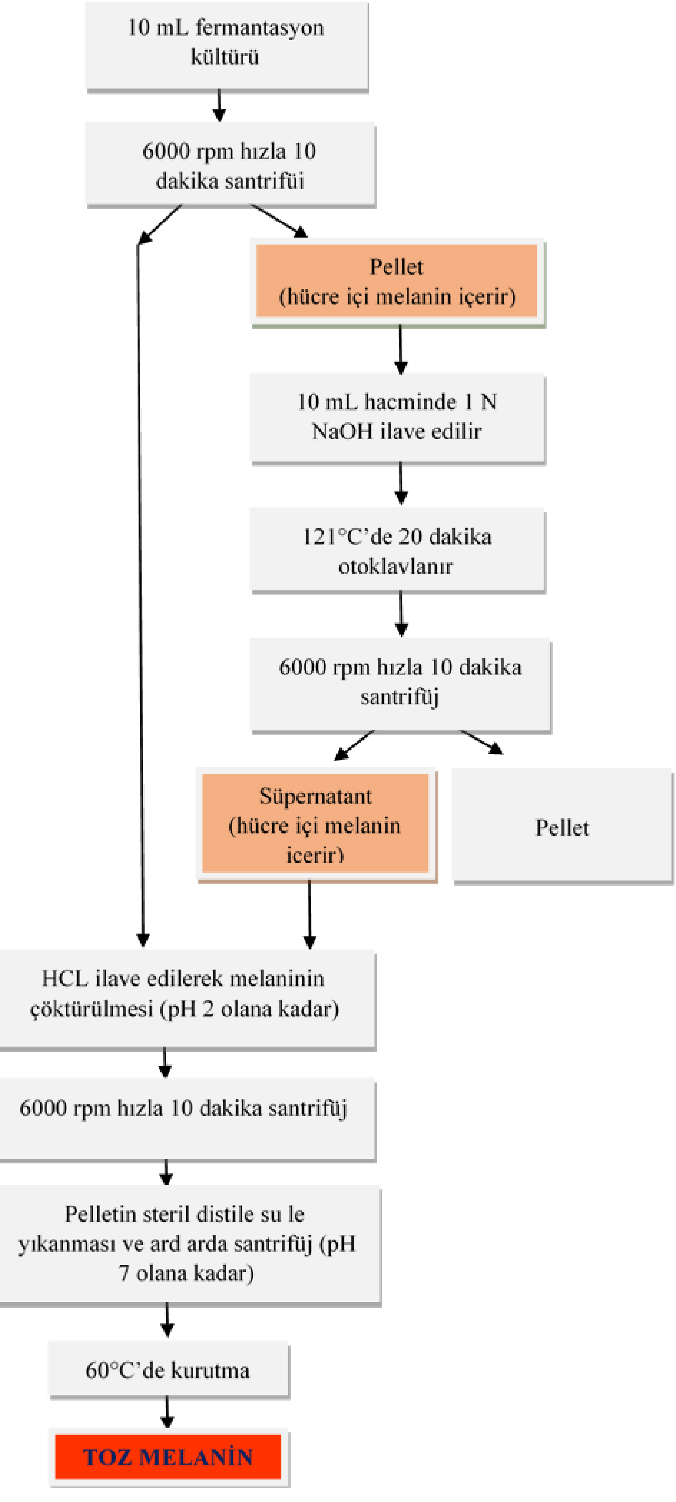
**Analitik Testler**

Fermantasyon ortamlarında *A. pullulans* AZ-6 ve *A. pullulans* NBRC 100716 suşları tarafından üretilen melanin derişimleri, fermantasyon boyunca yaklaşık her 24 saatte bir 10 mL kültür örneği alınarak tayin edilmiştir. Kültür örnekleri, 6000 rpm hızla 10 dakika santrifüjlenmiş ve hücre dışı melanin içeren süpernatant pelletten ayrılıp bir falkon tüpüne aktarılmıştır. *A. pullulans* hücreleri ile hücre içi melanin içeren pellet üzerine 10 mL hacminde 1 N NaOH ilave edilmiş ve oluşan karışım 121°C'de 20 dakika otoklavlanmıştır. Ardından karışım 6000 rpm'de 10 dakika santrifüjlenip, hücre içi melanin içeren süpernatant kısmı bir falkon tüpü içerisine ayrılmıştır. Hücre içi ve hücre dışı melanin içeren süpernatantlara, pH'ları 2 olana kadar ayrı ayrı derişik HCl ilave edilmiş ve melaninin çökmesi sağlanmıştır. Çöken melanin santrifüj (6000 rpm, 10 dakika) ile ayrılmış, ardından saf su ile pH'ı 7 olana kadar tekrar tekrar yıkanmıştır [15]. Elde edilen melanin 60°C'de sabit tartıma gelene kadar kurutularak toz haline getirildikten sonra -18°C'de muhafaza edilmiştir

(Şekil 1). Fermantasyon deneylerinde belirli aralıklarla alınan örneklerdeki melanin derişimlerinin yanı sıra biyokütle derişimleri de Mujdeci [16] tarafından önerilen yönteme göre tayin edilmiştir.

**İstatistiksel Analiz**

İstatistiksel analizler için SPSS 13.0 paket programı (SPSS, Chicago, IL, ABD) kullanılmış, iki tekrarlı olarak elde edilen veriler ortalama ± standart sapma olarak sunulmuştur. Çalışmada bağımlı değişkenlerin "maksimum biyokütle derişimi" veya "maksimum hücre dışı melanin derişimi" olduğu durumda, bağımsız değişkenler; "*A. pullulans* suşları" ile "doğal substrat kaynakları" olarak belirlenmiştir. Bağımsız değişken grupları arasındaki farkın anlamlı olup olmadığı çift yönlü ANOVA testi ile incelenmiştir. Çift yönlü ANOVA ile ayrıca bağımsız değişkenlerin birbiri ile etkileşimlerinin anlamlı olup olmadıkları da değerlendirilmiştir. Çalışmada yalnızca *A. pullulans* NBRC 100716 suşunun hücre içi melanin üretebildiği belirlendiğinden, "maksimum hücre içi melanin derişimi"nin bağımlı değişken olduğu durumda doğal substrat kaynakları arasında önemli bir fark olup olmadığı tek yönlü ANOVA testi ile değerlendirilmiştir. Genel olarak, satır ve sütunlar için elde edilen $p < 0.05$, istatistiksel olarak anlamlı kabul edilmiştir.




**Şekil 1** Fermantasyon ortamından melanin eldesinde izlenen yöntem

**Fig 1** The method followed in obtaining melanin from the fermentation medium

## Sonuçlar ve Tartışma

Soğan, patates, elma, armut ve havuç kabukları ile bayat ekmeklerin çeşitli ön işlemlerden sonra fermantasyon ortamı olarak kullanıldıkları deneylerdeki *A. pullulans* AZ-6 ve *A. pullulans* NBRC 100716'nın maksimum biyokütle derişimleri ve bu suşların ürettikleri maksimum hücre içi (hiMN) ve hücre dışı melanin (hdMN) derişimleri Tablo 1'de verilmiştir.

**Tablo 1** Farklı gıda atıkları/yan ürünler kullanılarak hazırlanan fermantasyon ortamlarında elde edilen en yüksek *A. pullulans* AZ-6 ve *A. pullulans* NBRC 100716 biyokütleleri ve bu suşların ürettikleri en yüksek hücre içi ve hücre dışı melanin derişimleri

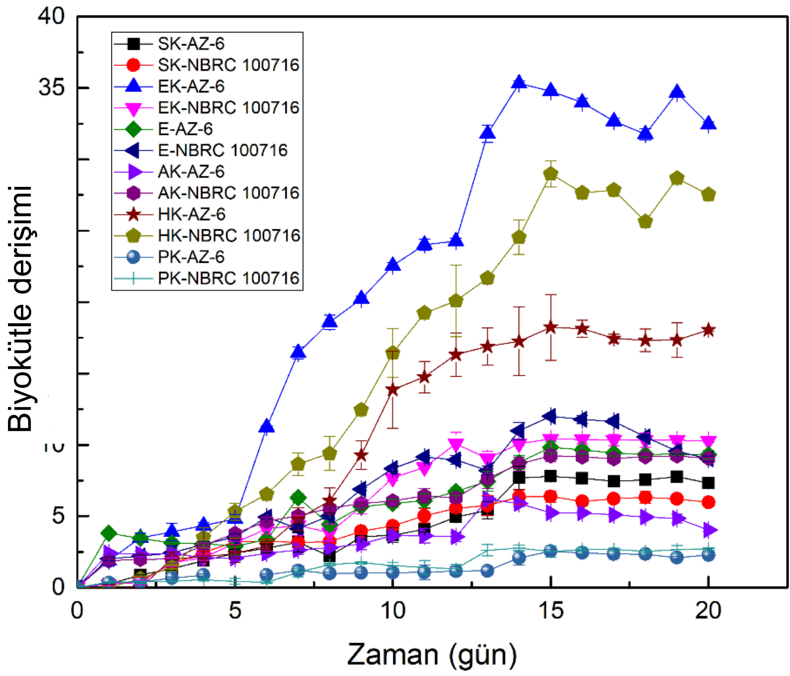
**Table 1** The highest *A. pullulans* AZ-6 and *A. pullulans* NBRC 100716 biomass obtained in fermentation media prepared using different food wastes/by-products and the highest intracellular and extracellular melanin concentrations produced by these strains

| Gıda atığı | Maksimum biyokütle derişimi (g/L) | | Maksimum hücre dışı melanin derişimi (g/L) | | Maksimum hücre içi melanin derişimi (g/L) |
|---|---|---|---|---|---|
| | AZ-6 | NBRC 100716 | AZ-6 | NBRC 100716 | NBRC 100716 |
| Soğan kabuğu | 7.80± 0.4 | 6.38 ± 0.4 | 0 ± 0.0 | 2.64 ± 0.1 | 0.38 ± 0.1 |
| Elma kabuğu | 35.31 ± 0.2 | 10.42 ± 0.1 | 1.34 ± 0.2 | 1.19 ± 0.2 | 2.09 ± 0.2 |
| Bayat ekmek | 9.83 ± 0.4 | 12.00 ± 0.1 | 0.13 ± 0.02 | 0.87 ± 0.04 | 0 ± 0.0 |
| Armut kabuğu | 6.19 ± 0.2 | 9.24 ± 0.4 | 0 ± 0.0 | 1.49 ± 0.1 | 0 ± 0.0 |
| Havuç kabuğu | 18.23 ± 2.3 | 28.98 ± 0.9 | 0.92 ± 0.2 | 3.52 ± 0.3 | 0.19 ± 0.02 |
| Patates kabuğu | 2.55 ± 0.1 | 2.76 ± 0.2 | 0 ± 0.0 | 0.22 ± 0.04 | 0 ± 0.0 |

Maksimum biyokütle ve hdMN derişimleri için yapılan çift yönlü ANOVA, farklı suş ve doğal sustrat kaynakları için elde edilen ortalamaların önemli ölçüde farklı olduğunu ($p < 0.05$) ve doğal substrat kaynakları ile suşlar arasındaki etkileşimin de önemli olduğunu ($p < 0.05$) göstermiştir. Maksimum hiMN derişimleri için yapılan tek yönlü ANOVA ise, doğal substrat kaynakları arasındaki farkın istatistiksel olarak anlamlı ($p < 0.05$) olduğunu ortaya koymuştur.

Yapılan çalışmada soğan ve elma kabuğu özütlerinin fermantasyon ortamı olarak kullanıldığı deneylerde en yüksek biyokütle derişimi sırasıyla; 7.8±0.4 ve 35.31±0.2 g/L olarak *A. pullulans* AZ-6 ile elde edilmiştir. Çalışmada bayat ekmek ile armut, havuç ve patates kabuğu özütlerinin *A. pullulans* NBRC 100716'nın gelişimini daha fazla desteklediği belirlenmiş ve bu ortamlarda en yüksek biyokütle derişimleri sırasıyla; 12.00 ± 0.1, 9.24 ± 0.4, 28.98 ± 0.9 ve 2.76 ± 0.2 g/L olarak belirlenmiştir.

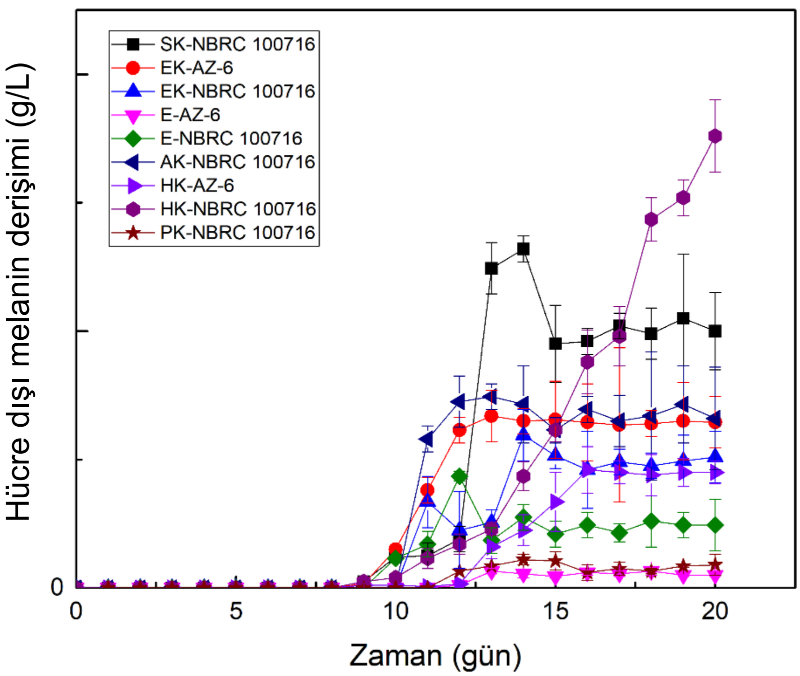
Şekil 2'de, çalışılan suşların farklı fermantasyon ortamlarındaki biyokütle derişimlerinin zamanla değişimi sunulmuştur. Söz konusu şekilde de görülebileceği gibi, soğan kabuğu, bayat ekmek ve havuç kabuğu özütlerinin ayrı ayrı fermantasyon ortamı olarak kullanıldıkları deneylerde *A. pullulans* AZ-6 ve *A. pullulans* NBRC 100716 için en yüksek biyokütle derşiminlerine fermantasyonun 15. gününde ulaşılmıştır. Elma kabuğu özütünün fermantasyon ortamı olarak kullanıldığı deneyde en yüksek biyokütle derişiminin *A. pullulans* AZ-6 ve *A. pullulans* NBRC 100716 için sırasıyla; 14 ve 15.; armut kabuğunun kullanıldığı deneylerde sırasıyla, 13 ve 15. ve patates kabuğunun kullanıldığı deneylerde ise sırasıyla, 15 ve 14. günlerde elde edildiği belirlenmiştir.




**Şekil 2** Biyokütle derişimlerinin zamanla değişimleri (**SK-AZ-6:** Gıda atığı (GA); soğan kabuğu, Suş (S); *A. pullulans* AZ-6, **SK-NBRC 100716**: GA; soğan kabuğu, S; *A. pullulans* NBRC 100716, **EK-AZ-6**: GA; elma kabuğu, S; *A. pullulans* AZ-6, **EK-NBRC 100716**: GA; elma kabuğu, S; *A. pullulans* NBRC 100716, **E-AZ-6**: GA; bayat ekmek, S; *A. pullulans* AZ-6, **E-NBRC 100716**: GA; bayat ekmek, S; *A. pullulans* NBRC 100716, **AK-AZ-6**: GA; armut kabuğu, S; *A. pullulans* AZ-6, **AK-NBRC 100716**: GA; armut kabuğu, S; *A. pullulans* NBRC 100716, **HK-AZ-6**: GA; havuç kabuğu, S; *A. pullulans* AZ-6, **HK-NBRC 100716**: GA; havuç kabuğu, S; *A. pullulans* NBRC 100716, **PK-AZ-6**: GA; patates kabuğu, S; *A. pullulans* AZ-6, **PK-NBRC 100716**: GA; patates kabuğu, S; *A. pullulans* NBRC 100716)

**Figure 2** Changes of biomass concentrations over time (**SK-AZ-6:** Food waste (FW); onion peel, Strain (S); *A. pullulans* AZ-6, **SK-NBRC 100716**: FW; onion peel, S; *A. pullulans* NBRC 100716, **EK-AZ-6**: FW; apple peel, S; *A. pullulans* AZ-6, **EK-NBRC 100716**: FW; apple peel, S; *A. pullulans* NBRC 100716, **E-AZ-6**: FW; stale bread, S; *A. pullulans* AZ-6, **E-NBRC 100716**: FW; stale bread, S; *A. pullulans* NBRC 100716, **AK-AZ-6**: FW; pear peel, S; *A. pullulans* AZ-6, **AK-NBRC 100716**: FW; pear peel, S; *A. pullulans* NBRC 100716, **HK-AZ-6**: FW; carrot peel, S; *A. pullulans* AZ-6, **HK-NBRC 100716**: FW; carrot peel, S; *A. pullulans* NBRC 100716, **PK-AZ-6**: FW; potato peel, S; *A. pullulans* AZ-6, **PK-NBRC 100716**: FW; potato peel, S; *A. pullulans* NBRC 100716)

*A. pullulans* suşlarının ürettikleri hdMN derişimlerinin zamanla değişimleri Şekil 3'de sunulmuştur.

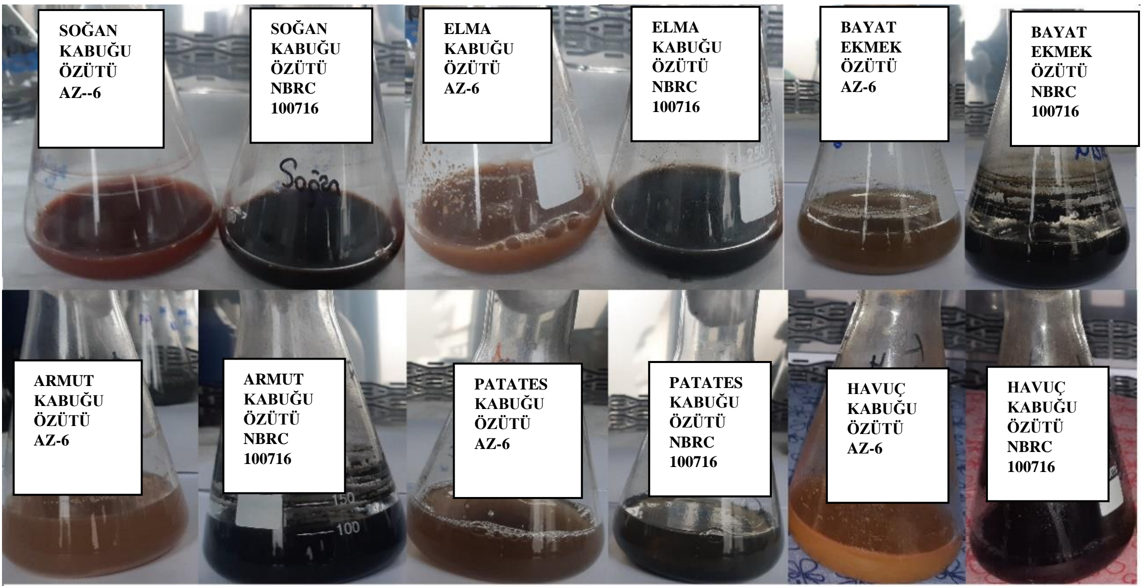



**Şekil 3** Hücre dışı melanin derişimlerinin zamanla değişimleri (**SK-AZ-6**: Gıda atığı (GA); soğan kabuğu, Suş (S); *A. pullulans* AZ-6, **EK-AZ-6**: GK; elma kabuğu, S; *A. pullulans* AZ-6, **EK-NBRC 100716**: GA; elma kabuğu, S; *A. pullulans* NBRC 100716, **E-AZ-6**: GA; bayat ekmek, S; *A. pullulans* AZ-6, **E-NBRC 100716**: GA; bayat ekmek, S; *A. pullulans* NBRC 100716, **AK-NBRC 100716**: GA; armut kabuğu, S; *A. pullulans* NBRC 100716, **HK-AZ-6**: GA; havuç kabuğu, S; *A. pullulans* AZ-6, **HK-NBRC 100716**: GA; havuç kabuğu, S; *A. pullulans* NBRC 100716, **PK-NBRC 100716**: GA; patates kabuğu, S; *A. pullulans* NBRC 100716)

**Figure 3** Changes of extracellular melanin concentrations over time (**SK-AZ-6**: Food Waste (FW); onion peel, Strain (S); *A. pullulans* AZ-6, **EK-AZ-6**: FW; apple peel, S; *A. pullulans* AZ-6, **EK-NBRC 100716**: FW; apple peel, S; *A. pullulans* NBRC 100716, **E-AZ-6**: FW; stale bread, S; *A. pullulans* AZ-6, **E-NBRC 100716**: FW; stale bread, S; *A. pullulans* NBRC 100716, **AK-NBRC 100716**: FW; pear peel, S; *A. pullulans* NBRC 100716, **HK-AZ-6**: FW; carrot peel, S; *A. pullulans* AZ-6, **HK-NBRC 100716**: FW; carrot peel, S; *A. pullulans* NBRC 100716, **PK-NBRC 100716**: FW; potato peel, S; *A. pullulans* NBRC 100716)

*A. pullulans* AZ-6 suşu maksimum hdMN üretimini, 1.34 ± 0.2 g/L olarak, elma kabuğu özütünün fermantasyon ortamı olarak kullanıldığı deneyde gerçekleşmiştir. *A. pullulans* AZ-6 suşunun soğan, armut ve patates kabuklarının kullanıldıkları deneylerde hdMN üretmediği kaydedilmiştir (Tablo 1). Çalışmanın maksimum hdMN derişimi (3.52 ± 0.3 g/L) ise, *A. pullulans* NBRC 100716 tarafından havuç kabuğu özütü ile elde edilmiştir. *A. pullulans* NBRC 100716 suşunun hdMN üretimine, soğan, elma ve patates kabukları için fermantasyonun 14. gününde, bayat ekmek, armut ve havuç kabukları için ise

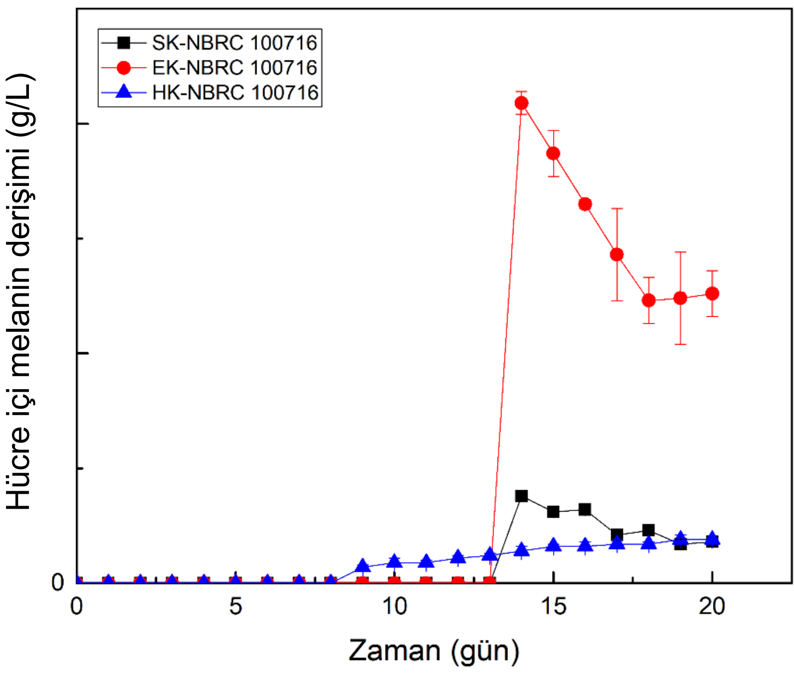
fermantasyonun sırasıyla 12, 13 ve 20. günlerinde başladığı kaydedilmiştir. Hücre dışı melanin üretiminin gözlendiği fermantasyon ortamlarının rengi orjinal renklerine kıyasla koyulaşmaya başlamış, kahverengi-siyaha dönüşmüştür (Şekil 4).




**Şekil 4** Melanin üretimine bağlı olarak fermantasyon ortamlarında görülen siyah renk oluşumları

**Figure 4** Black color formations seen in fermentation media due to melanin production

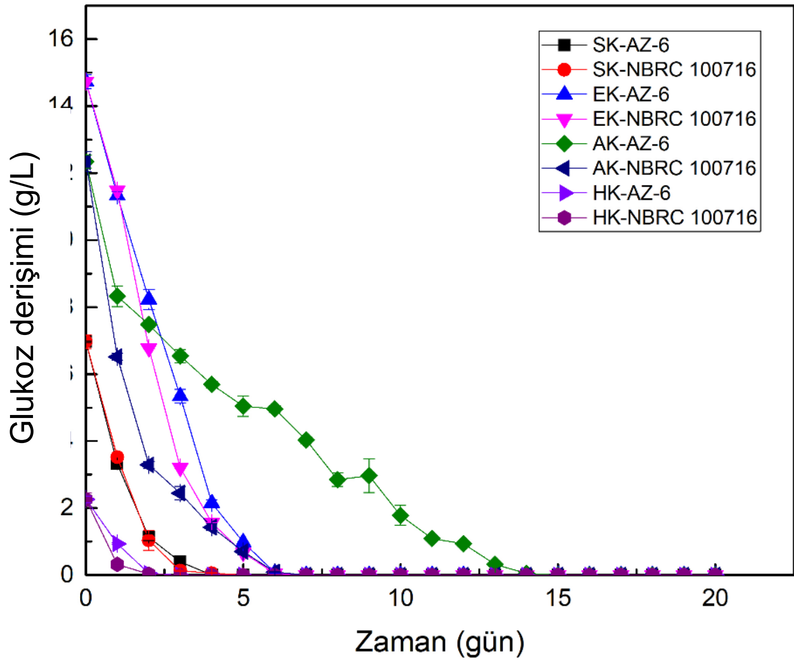
Araştırmada çalışılan suşlar arasında yalnızca *A. pullulans* NBRC 100716 suşunun hücre içi melanin üretebildiği tespit edilmiştir. *A. pullulans* NBRC 100716 suşunun hiMN üretiminin ise, yalnızca soğan, elma ve havuç kabuğu özütlerinin fermantasyon ortamı olarak kullanıldıkları deneylerde gerçekleştiği belirlenmiştir (Şekil 5). *A. pullulans* NBRC 100716 suşunun en yüksek miktarda hiMN'i soğan, elma ve havuç kabuğu özütleri için sırasıyla; 0.38, 2.09 ve 0.19 g/L olarak fermantasyonun 14, 14 ve 19. gününde ürettiği kaydedilmiştir.




**Şekil 5** Hücre içi melanin derişimlerinin zamanla değişimleri (**SK-NBRC 100716**: Gıda atığı (GA); soğan kabuğu, Suş (S); *A. pullulans* NBRC 100716, **EK-NBRC 100716**: GA; elma kabuğu, S; *A. pullulans* NBRC 100716, **HK-NBRC 100716**: GA; havuç kabuğu, S; *A. pullulans* NBRC 100716)

**Figure 5** Changes of intracellular melanin concentrations over time (**SK-NBRC 100716**: Food Waste (FW); onion peel, Strain (S); *A. pullulans* NBRC 100716, **EK-NBRC 100716**: FW; apple peel, S; *A. pullulans* NBRC 100716, **HK-NBRC 100716**: FW; carrot peel, S; *A. pullulans* NBRC 100716)

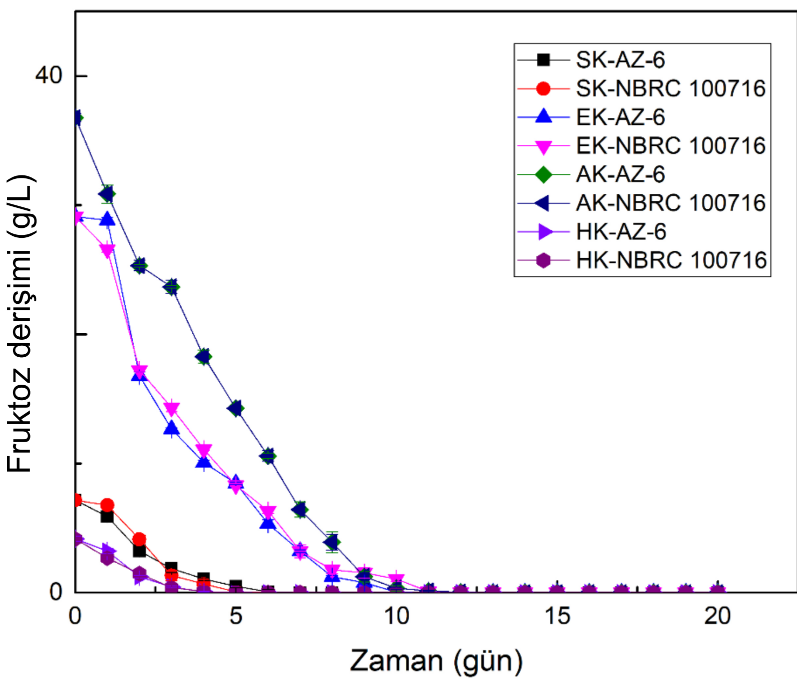
Araştırmada soğan, elma, armut ve havuç kabuğu çözeltilerinin fermantasyon ortamı olarak kullanıldıkları deneylerde ortamdaki glukoz ve fruktoz derişimlerinin zamanla değşimlerini gösteren grafikler sırasıyla Şekil 6 ve Şekil 7'de verilmiştir. Soğan kabuğu özütünün fermantasyon ortamı olarak kullanıldığı deneylerde, hem *A. pullulans* AZ-6 hem de *A. pullulans* NBRC 100716 tarafından, ortamdaki glukozun fermantasyonun 4. gününde, fruktozun ise, fermantasyonun 5. gününde tamamen tüketildiği belirlenmiştir. Elma kabuğunun kullanıldığı deneylerde, *A. pullulans* AZ-6 ve *A. pullulans* NBRC 100716 suşları tarafından glukoz daha erken (6. gün) tüketilirken, fruktoz tüketimi daha uzun (10. gün) sürmüştür.




**Şekil 6** Fermantasyon ortamlarındaki glukoz derişimlerinin zamanla değişimleri (**SK-AZ-6**: Gıda atığı (GA); soğan kabuğu, Suş (S); *A. pullulans* AZ-6, **SK-NBRC 100716**: soğan kabuğu, S; *A. pullulans* NBRC 100716, **EK-AZ-6**: GA; elma kabuğu, S; *A. pullulans* AZ-6, **EK-NBRC 100716**: GA; elma kabuğu, S; *A. pullulans* NBRC 100716, **AK-AZ-6**: GA; armut kabuğu, S; *A. pullulans* AZ-6, **AK-NBRC 100716**: GA; armut kabuğu, S; *A. pullulans* NBRC 100716, **HK-AZ-6**: GA; havuç kabuğu, S; *A. pullulans* AZ-6, **HK-NBRC 100716**: GA; havuç kabuğu, S; *A. pullulans* NBRC 100716)

**Figure 6** Changes of glucose concentrations in fermentation media over time (**SK-AZ-6**: Food Waste (FW); onion peel, Strain (S); *A. pullulans* AZ-6, **SK-NBRC 100716**: onion peel, S; *A. pullulans* NBRC 100716, **EK-AZ-6**: FW; apple peel, S; *A. pullulans* AZ-6, **EK-NBRC 100716**: FW; apple peel, S; *A. pullulans* NBRC 100716, **AK-AZ-6**: FW; pear peel, S; *A. pullulans* AZ-6, **AK-NBRC 100716**: FW; pear peel, S; *A. pullulans* NBRC 100716, **HK-AZ-6**: FW; carrot peel, S; *A. pullulans* AZ-6, **HK-NBRC 100716**: FW; carrot peel, S; *A. pullulans* NBRC 100716)

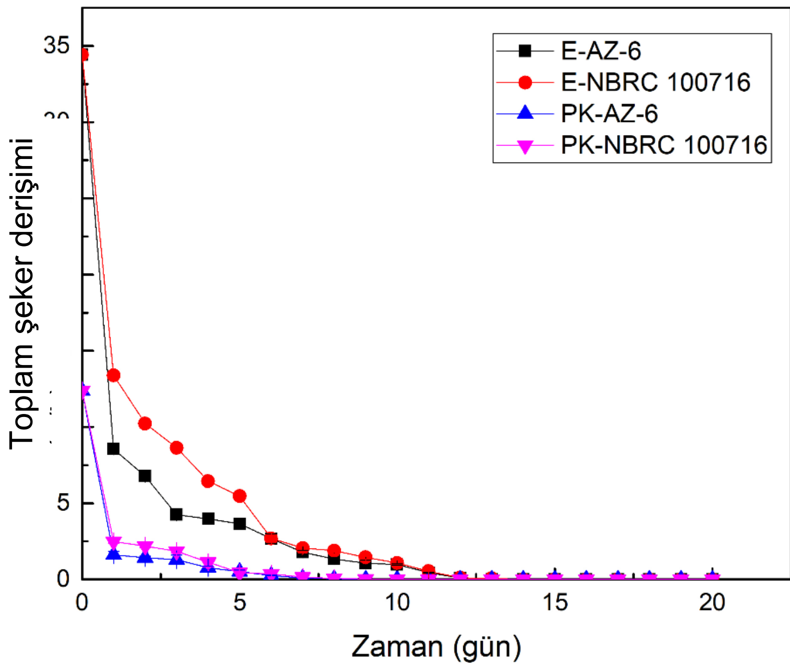
Armut kabuğunun kullanıldığı deneylerde ise, *A. pullulans* AZ-6 ve *A. pullulans* NBRC 100716 suşları ortamdaki glukozu sırasıyla; fermantasyonun 15 ve 7. günlerinde, fruktozu ise fermantasyonun 13. gününde tüketmişlerdir. Havuç kabuğu özütünde yer alan glukoz, çalışılan suşlar tarafından fermantasyonun 3. gününde tamamen tüketilirken, fruktoz fermantasyonun 5. gününde tüketilmiştir.




**Şekil 7** Fermantasyon ortamlarındaki fruktoz derişimlerinin zamanla değişimleri (**SK-AZ-6**: Gıda atığı (GA); soğan kabuğu, Suş (S); *A. pullulans* AZ-6, **SK-NBRC 100716**: soğan kabuğu, S; *A. pullulans* NBRC 100716, **EK-AZ-6**: GA; elma kabuğu, S; *A. pullulans* AZ-6, **EK-NBRC 100716**: GA; elma kabuğu, S; *A. pullulans* NBRC 100716, **AK-AZ-6**: GA; armut kabuğu, S; *A. pullulans* AZ-6, **AK-NBRC 100716**: GA; armut kabuğu, S; *A. pullulans* NBRC 100716, **HK-AZ-6**: GA; havuç kabuğu, S; *A. pullulans* AZ-6, **HK-NBRC 100716**: GA; havuç kabuğu, S; *A. pullulans* NBRC 100716)

**Figure 7** Changes of fructose concentrations in fermentation media over time (**SK-AZ-6**: Food Waste (FW); onion peel, Strain (S); *A. pullulans* AZ-6, **SK-NBRC 100716**: onion peel, S; *A. pullulans* NBRC 100716, **EK-AZ-6**: FW; apple peel, S; *A. pullulans* AZ-6, **EK-NBRC 100716**: FW; apple peel, S; *A. pullulans* NBRC 100716, **AK-AZ-6**: FW; pear peel, S; *A. pullulans* AZ-6, **AK-NBRC 100716**: FW; pear peel, S; *A. pullulans* NBRC 100716, **HK-AZ-6**: FW; carrot peel, S; *A. pullulans* AZ-6, **HK-NBRC 100716**: FW; carrot peel, S; *A. pullulans* NBRC 100716)

Bayat ekmek ve patates kabuğu özütlerinin fermantasyon ortamı olarak kullanıldıkları deneylerde ortamdaki toplam şeker derişimlerinin (sakkaroz cinsinden) zamanla değişimlerini gösteren grafikler Şekil 8'de sunulmuştur. Bayat ekmeğin kullanıldığı deneylerde ortamdaki substratın, *A. pullulans* AZ-6 ve *A. pullulans* NBRC 100716 suşları tarafından fermantasyonun 13. gününde tamamen tüketildiği belirlenmiştir. Patates kabuğu özütünün fermantasyon ortamı olarak kullanıldıkları deneylerde ise, ortamdaki toplam şekerin *A. pullulans* AZ-6 ve *A. pullulans* NBRC 100716 suşları tarafından fermantasyonun sırasıyla; 9 ve 10. günlerinde tüketildiği tespit edilmiştir.




**Şekil 8** Fermantasyon ortamlarındaki toplam şeker derişimlerinin zamanla değişimleri (**E-AZ-6**: Gıda atığı (GA); bayat ekmek, Suş (S); *A. pullulans* AZ-6, **E-NBRC 100716**: GA; bayat ekmek, S; *A. pullulans* NBRC 100716, **PK-AZ-6**: GA; patates kabuğu, S; *A. pullulans* AZ-6, **PK-NBRC 100716**: GA; patates kabuğu, S; *A. pullulans* NBRC 100716)

**Figure 8** Changes of total sugar concentrations in fermentation media over time (**E-AZ-6**: Food Waste (FW); stale bread, Strain (S); *A. pullulans* AZ-6, **E-NBRC 100716**: FW; stale bread, S; *A. pullulans* NBRC 100716, **PK-AZ-6**: FW; potato peel, S; *A. pullulans* AZ-6, **PK-NBRC 100716**: FW; potato peel, S; *A. pullulans* NBRC 100716)

Genel olarak değerlendirildiğinde; *A. pullulans* AZ-6'nın hücre gelişimini en fazla destekleyen fermantasyon ortamı elma kabuğu özütü olmuş bunu ise sırasıyla; havuç kabuğu özütü ve bayat ekmek hidrolizatı izlemiştir. *A. pullulans* NBRC 100716 suşunun hücre gelişimi için ise en elverişli ortamların sırasıyla; havuç kabuğu özütü, bayat ekmek hidrolizatı ve elma kabuğu özütü olduğu belirlenmiştir. Çalışılan doğal substrat kaynakları arasında en düşük biyokütle derişimleri ise, her iki suş için de patates kabuğu hidrolizatında elde edilmiştir. Söz konusu substrat, göz ardı edilemeyecek bir şeker kaynağı olsa da, bileşimindeki fenolik maddelerin bu çalışmadaki suşların gelişimlerini ve melanin üretimlerini baskılayabileceği sonucuna varılmıştır. Gebrechristos vd. [17] tarafından yapılan bir çalışma da bu sonucu destekler niteliktedir. Söz konusu çalışmada, patates kabuğu

özütünün bileşiminde kafeik, klorojenik ve neoklorojenik asitlerin oladuğu ve bu fenolik bileşenlerin *Escherichia coli*, *Salmonella enterica* ve *Staphylococcus aureus*'un gelişimini inhibe ettiği vurgulanmıştır. Benzer şekilde, Noushad vd. [18] tarafından yapılan çalışmada da patates kabuğu özütünün *Enterococcus faecalis* ATCC 29212 suşu üzerine antibakteriyel etkiye sahip olduğu belirtilmiştir.

Çalışılan suşlar arasında en yüksek miktarda toplam melanin (3.71g/L) üreticisi suşun *A. pullulans* NBRC 100716 olduğu tespit edilmiştir. Bu üretim ise havuç kabuğu özütünün fermantasyon ortamı olarak kullanıldığı deneyde gerçekleşmiştir. Elma ve soğan kabuklarının doğal substrat kaynağı olarak kullanıldıkları deneylerde ise, *A. pullulans* NBRC 100716 suşunun ürettiği toplam melaninin sırasıyla; 3.28 ve 3.02 g/L olduğu belirlenmiştir. Bu araştırmada, *A. pullulans* NBRC 100716 suşu ile melanin üretimi için elverişli olan ilk üç ortamın havuç, elma ve soğan kabuklarının özütleri olduğu tespit edilmiştir. Genel olarak pigment üretiminin, fermantasyon ortamındaki besin maddelerinin azalması ve artan inkübasyon zamanı ile arttığı ifade edilmektedir. Melanin üreten organizmaların bu pigmenti kendilerini çevresel strese karşı korumak için ürettikleri rapor edilmektedir [19]. Bu çalışmada da havuç kabuğu özütündeki, diğer substrat kaynaklarına kıyasla, kısıtlı şeker derişiminin (bkz. Şekil 6 ve 7) hücre gelişimi için yeterli ve melanin üretimi için elverişli olabileceği sonucuna varılmıştır. Çalışılan tüm doğal substrat kaynakları için ortamdaki şekerin tamamen tükenmesinden sonra melanin üretiminin başladığı tespit edilmiştir.

Soğan ve elma kabuğunda bulunan temel fenolik bileşenlerden biri olan kuersetinin, sıcak su ekstraksiyonunda da bir miktar özüte geçtiği bildirilmiştir [20]. Kuersetinin melanin biyosentezini baskıladığı bilinse de [21–23], bunun tam tersini savunan çalışmalar da mevcuttur [24,25]. Bu çalışmada ise, sterilizasyon aşamasında kuersetinin büyük oranda parçalandığı [26] ve melanin üretimini engellemediği düşünülmektedir

Mujdeci (2021) tarafından daha önce yapılmış olan bir çalışmada, havuç, kavun ve karpuz kabuklarının özütleri ile peynir altı suyu ve melasın fermantasyon ortamı olarak kullanıldıkları deneylerde, *A. pullulans* NBRC 100716 ile en yüksek toplam melanin derişimine bu çalışmada olduğu gibi havuç kabuğu özütü ile ulaşılmıştır. Söz konusu çalışmada ikinci sırada en yüksek melanin derişimi (0.78 g/L) karpuz kabuğu özütü ile elde edilmiştir [16]. Meyve atıklarının melanin üretiminde substrat kaynağı olarak kullanıldıkları

bir çalışmada, ananas, portakal ve nar atığı karışımının (portakal karpelleri, ananas çekirdekleri ve ezilmiş nar taneleri) özütü fermantasyon ortamı olarak kullanılmış ve bu ortamda *Bacillus safensis*'in melanin üretimi araştırılmıştır. Söz konusu çalışmada, femantasyon ortamının başlangıç pH'ının 6.84 ve sıcaklığın 30.7°C olduğu koşulda, melanin derişiminin 6.96 mg/mL olduğu belirtilmiştir [9].

Melanin üretimi için düşük maliyetli substratların denendiği bir başka çalışmada ise, pirinç kepeği, buğday kepeği, hindistan cevizi kabuğu, muz sapı, pirinç unu ve koyu darı gibi çeşitli tarımsal atıkların denendiği belirtilmiştir [7]. Çalışmaya göre, *Streptomyces griseorubens* DKR4 suşu tarafından en yüksek pigment üretimi muz sapı ve hindistan cevizi kabuğunun kullanıldığı katı hal fermantasyonunda üretilirken en düşük pigment üretimi koyu darı kullanılan durumda elde edilmiştir. Tarangini ve Mishra [27] tarfından yapılan bir araştırmada, *Pseudomonas* sp. olarak tanımlandığı belirtilen bir izolatın melanin üretim özelliklerinin saf deniz suyu ve atık lahana özütü gibi ortamlarda incelendiği belirtilmiştir. Saf deniz suyunda elde edilen melanin derişiminin 5.35 mg/mL olduğu, lahana atığı suyunda ise üretimin gerçekleşmediği ifade edilmiştir. Ancak deniz suyunun aşı ortamı olarak kullanıldığı durumda, lahana atığı suyunda yaklaşık 2.79 mg/mL melanin üretiminin olduğu vurgulanmıştır [27].

Dünyada gıdaya ulaşımın her geçen gün zorlaşması ve bu durumun ölümlere dahi neden olması, "sıfır açlık" ve "sıfır atık" hedefleri ile çeşitli çalışmaların yaygınlaşmasına neden olmaktadır. Bununla birlikte henüz bu hedeflere ulaşılamaması, halen oldukça yüksek miktarlarda atıkların var olması anlamına gelmektedir. Evsel atıkların da içerisinde yer aldığı gıda atıkları, insan sağlığı ve çevreye zarar vermelerinin yanı sıra ekonomik kayıplara da neden olmaktadır. Bu nedenle de gıda atıklarının katma değeri yüksek ürünlere dönüştürülmesi dünyadaki önemli hedeflerden biridir. Bu hedef doğrultusunda, bu çalışmada, *A. pullulans* NBRC 100716 ve AZ-6 suşları ile melanin üretmek için soğan, patates, elma, armut ve havuç kabukları ile bayat ekmeğin potansiyel kullanımı araştırılmıştır. Çalışmadaki gıda atıklarından en yüksek toplam melanin üretimi (3.71 g/L) *A. pullulans* NBRC 100716 tarafından havuç kabuğu özütü ile gerçekleşmiş, elma kabuğu ve soğan kabuğu özütleri ile de sırasıyla; 3.28 ve 3.02 g/L olarak yakın sonuçlar elde edilmiştir.

**Teşekkür**
Yazar, bu çalışmanın MUH19001.20.012 nolu genel araştırma projesi ile desteklenmesinden dolayı, Hitit Üniversitesi'ne teşekkür eder.

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**Research Article**

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# New Approach to Inducing Rat Tcell Activation Against Colon Cancer by Using Cancer Mediate Exosome: An *in vitro* Study

**Aysegul Yilmaz[1], Irmak Ferah Okkay[2], Ali Taghizadehghalehjoughi[3*]**

**ABSTRACT**

Although exosomes were first described as cellular waste in the late 1980s, their role in cellular communication has been revealed by recent studies. In addition to components such as DNA, RNA, and protein, it is thought that it may also be associated with the immune system, as it contains tetraspanins such as CD9, CD81, and major histocompatibility complex (MHC) molecules. Therefore, it has been seen as a new source for immunotherapy. Immunotherapy is one of the methods used for colon cancer, which is one of the most common and deadly cancers, where traditional treatments are insufficient. In our study, we first performed exosome isolation from the CaCo-2 cell line, then lymphocyte (T lymphocyte) activation by the exosome. Then, we counted the activated lymphocytes (10,000, 20,000, 40,000, and 80,000 cells) and applied them to the CaCo-2 cell line in vitro. After 48 hours, we performed viability (MTT), antioxidant (TAC), oxidant (TOS) and lactate dehydrogenase (LDH) analyzes. Exosome characterization was demonstrated with TEM, SEM, and AFM images. According to our results, it was seen that the lymphocytes activated by exosomes act at similar rates with the lymphocytes activated by IL-2. In the groups given 80,000 cells, a significant decrease was observed in the viability and antioxidant level of the cancer line, while an increase in oxidant and lactate levels was observed. The tumor-suppressive properties of exosomes obtained from immune cells have been demonstrated in the literature. We have successfully produced this study with our own experience and knowledge of the literature. We have successfully produced this study with our own experience and knowledge of the literature.

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## Introduction

Exosomes are type of microvesicles with endosomal origin. The earliest identification of exosomes in the extracellular space was in the late 1980s. Until this date, exosomes secreted from cells were thought to be cellular wastes resulting from cell damage or by-products of

[1] Department of Medical Pharmacology, Faculty of Medicine, Ataturk University, Erzurum, Turkey
[2] Department of Pharmacology, Faculty of Pharmacy, Ataturk University, Erzurum, Turkey
[3] Department of Medical Pharmacology, Faculty of Medicine, Bilecik Seyh Edebali University, Bilecik, Turkey
[*]Corresponding Author: Ali Taghizadehghalehjoughi, E-mail: ali.tgzd@bilecik.edu.tr

cell homeostasis. However, recently it has been shown that these vesicles have structures such as proteins, lipids, nucleic acids and have important effects on intercellular communication [1]. Exosomes exert very important effects on many cellular processes such as immune response, signal transduction, and antigen presentation. In addition, exosomes contain structures such as tetraspanins (CD9, CD63, CD81, etc.), heat shock proteins (such as HSP70 and HSP90), and major histocompatibility complex (MHC) molecules and play a role in antigen presentation [2]. It is thought that due to increased growth rates or stressful conditions, cancer cells tend to release more exosomes than healthy cells. The use of exosomes as an immune activator together with the above feature and ability to present antigen makes our study very valuable in terms of specificity [3-5]. It is now clear that exosomes play important roles in various diseases, including cancer, infection [6], cardiovascular and autoimmune diseases [7]. Due to the MHC molecules in the structure of exosomes, their effects on the immune system including maturation, antigen presentation, activation and differentiation of immune cells are becoming increasingly interesting [8]. It has also been shown that exosomes secreted by cancer cells can promote cancer cell growth [9], as well as trigger anti-tumor responses dependent on CD8+ T cells by antigen presentation and stimulation of T cells [10].

The main purpose of cancer immunotherapy is to increase the activity of intracellular cytotoxic T cells (CTL) and to create effective and permanent anti-cancer immunity. Therefore, CD8+ T cells play a key role in controlling cancer [11]. The fact that exosomes released from cancer cells can also respond to CD8+ T cells shows that they can be used in immunotherapy.

Surgical resection has traditionally been the first choice in the treatment of colorectal cancer. It is effectively preferred with chemotherapy, radiation therapy or both to increase the patient survival rate. However, 25% of patients in the metastatic stage have a five-year survival rate of only 10% [12]. For all these reasons, more effective therapeutic strategies in the treatment of the disease are still being investigated. Various difficulties have been observed in the application of immunotherapy, which is one of these therapeutic approaches. Disadvantages are seen, such as insufficient immune effector response, limited recognition of tumor targets, or introduction of foreign components into the body.

In our study, we aim to activate T lymphocytes by using exosomes obtained from colon cancer, and to reveal the effects of activated T cells on colon cancer in vitro as cell viability, oxidant and antioxidant.

## Material and Methods

### Cell culture and doses

The Caucasian colon adenocarcinoma (CaCo-2) cell line was obtained from Ataturk University, Department of Medical Pharmacology. It was allowed to grow to sufficient density (80%) in 25 $cm^3$ flasks (incubation medium at 37°C and 5% $CO_2$). After the CaCo cells were grown, their medium was removed and exosomes were isolated according to the procedure. Blood was cultured from animals, and white blood cell isolation was performed in vitro. The exosome was applied at a dose of 10 µg/ml to each blood culture medium and the isolated T cells were activated.

Exosome-activated T lymphocytes were applied to the CaCo-2 cell line as 10,000, 20,000, 40,000 and 80,000 cells.

### Lymphocyte (T lymphocyte) isolation

Whole blood from the donor is collected in heparinized blood tubes, then diluted with double volume of sterile PBS. Pipette the density gradient solution into a separate centrifuge tube. An equal amount of diluted blood is added onto the density gradient solution for phase separation. Centrifuge for 20 minutes at 1200 x g. Then, the Peripheral Blood Mononuclear Cell (PBMC) layer containing lymphocytes is carefully removed with a sterile pipette. Add 3-4 times the volume of PBMCs in PBS and mix gently. Centrifugation at 200 x g for 10 minutes is repeated 3 times and the cells at the bottom are lymphocyte cells. Isolated lymphocyte cells are allowed to grow in a medium. After the cells reach a sufficient density, activation is performed with the exosome 10 µg/ml dose and the doses are adjusted as described above.

### MTT tetrazolium assay concept

MTT analysis was performed with a purchased kit (Sigma Aldrich, USA). MTT reagent was added to each well of the 96-well plate and the plate was incubated for 4 hours (%5 $CO_2$; 37°C). The medium was removed after 4 hours and 100 µL of dimethylsulfoxide (DMSO)

(Sigma, USA) was added to dissolve the formazan crystals. Cell viability (%) was measured using a spectrophotometer reader based on optical density at 570 nm (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific, Canada, USA). The control group was accepted as 100, other groups were calculated according to the formula below [13].

Viability Rate (%) = (O.D of groups/Control O.D) X 100

**Total antioxidant capacity (TAC) assay**

Total antioxidant assay testing was performed with commercially purchased kit (Rel Assay Diagnostics® Company (Gaziantep, Turkey)). Briefly, to determine the antioxidant level, 30 µL samples followed by 500 µL Reagent 1 solution were added to the wells and the first absorbance value was measured at 660nm. For the second absorbance measurement, 75 µL Reagent 2 solution was added to the same wells, and measurement was made at 660nm after 10 minutes. Absorbance values were applied according to the formula below and antioxidant values were calculated as Trolox Equiv mmol/$L^{-1}$ [14].

A2-A1= Δ Absorbance (Standard, sample or $H_2O$)

$$\text{Result} = \frac{(H_2O\ \Delta\ \text{Abs} - \text{Sample}\ \Delta\ \text{Abs})}{(H_2O\ \Delta\ \text{Abs} - \text{Standard}\ \Delta\ \text{Abs})}$$

**Total oxidant status (TOS) assay**

Total oxidant status (TOS) is the evaluation of color intensity in the spectrophotometer based on the number of oxidants in the sample (from Rel Assay Diagnostics® Company (Gaziantep, Turkey)). To determine the TOS level, 500 µL of reagent 1 was added to the wells containing 75 µL of sample and the first absorbance value was measured at 530 nm. Then, 25 µL of Reagent2 solution was added to the same wells, incubated for 10 minutes at room temperature, and the second absorbance value was measured. Absorbance values were changed according to the procedure as stated below, and TOS values were calculated as $H_2O_2$ Equiv/mmol $L^{-1}$.

A2-A1= Δ Absorbance (Standard or sample)

$$\text{Result} = \frac{\text{Sample}\ \Delta\ \text{Abs}}{\text{Standard}\ \Delta\ \text{Abs}} \times 10$$

**Lactate dehydrogenase (LDH) analyze**

Lactate dehydrogenase level was determined according to the LDH detection kit (Cayman Chemicals, USA) and manufacturer's instructions. OD values were determined after the measurement made at 440 nm with the spectrophotometric method.

**Exosome isolation and characterization**

When the cell line (CaCo-2) grown in flasks reached the desired density (80%), the cell medium was removed from the medium. Ultracentrifugation method was applied to the cell medium collected for exosome isolation. First, the cell medium is centrifuged for 10 minutes at 300 x g at 4°C. The resulting supernatant was filtered through a 0.22 µm filter to remove microparticles and cell debris. Centrifugation was then repeated at 100,000 x g at 4°C for 90 minutes. The supernatant was carefully removed and the pellet portion containing the exosomes was resuspended in cold PBS. Finally, it was centrifuged a second time at 100,000 x g for 90 minutes at 4°C and pure exosomes were suspended in 500 µL of PBS.

**Scanning electron microscopy (SEM)**

SEM, Exosome were seeded on glass slides. Three glass slides were selected. For inspection, the specimens were commissioned to the Eastern Anatolia High Technology Application and Research Center (DAYTAM).

**Transmission electron microscopy (TEM)**

TEM was used to observe the Exosome. The Exosomes were fixed on clean glassed then kept at 4°C until further analysis. The specimens were subsequently commissioned to the Eastern Anatolia High Technology Application and Research Center (DAYTAM) for post-processing.

**Statistical analysis**

Results were calculated as mean ± standard error. Statistical comparison between groups was calculated using One-way ANOVA and Tukey LSD method. For statistical analyzes, all calculations were performed using SPSS 20 software and statistically $P<0.05$ was considered to be a significant difference in all tests.

## Results

### MTT tetrazolium assay concept results

In our study on the CaCo-2 cell line, after the cells reached the appropriate density (80%), we applied the lymphocytes activated with exosome and IL-2 to our Caco-2 cells in vitro and showed their effects on cell viability after 48 hours in figure 1. According to our results, we revealed that cell viability decreased as the number of cells increased in lymphocyte groups activated with exosome and IL-2. The most significant results were seen in the exosome/40,000 and exosome/80,000 groups, while the exosome/80,000 group, which reduced viability by 35%, was more effective. Another important result of the exosome/80.000 group is that the interleukin-2 (IL-2) cytokine, which is known to play a role in the stimulation of lymphocytes (Ross & Cantrell, 2018), has similar results with the IL-2/80.000 group. Thus, we have shown that lymphocytes activated by exosome, which is the main purpose of our study, are effective.

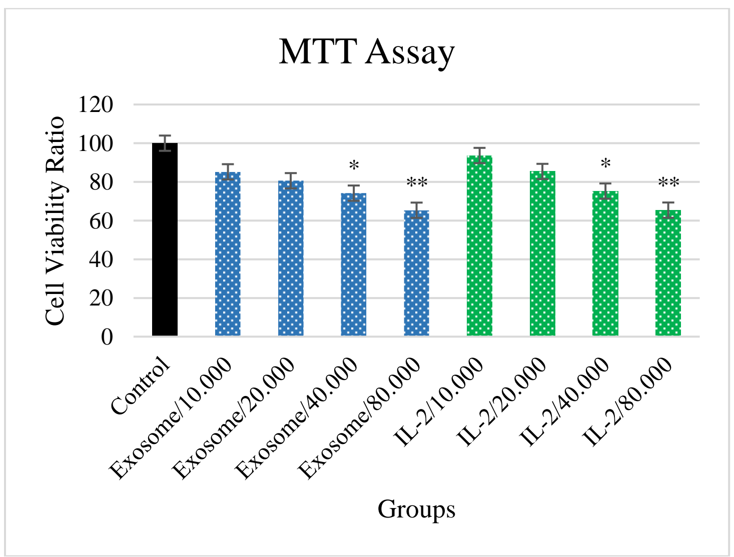


**Fig 1** MTT assay results for CaCo-2 cell line after 48h exosome and IL-2 activated lymphocytes (T lymphocyte) treatment. (*P < 0.05 compared to control group; **P < 0.001 compared to control group)

### Total antioxidants capacity (TAC) and total oxidant status (TOS) results

When our results in figures 2 and 3 were examined, it was found that there was no significant difference between exosome/10,000 and exosome/20,000 groups and IL-2/10,000 and IL-

2/20,000 groups in antioxidant and oxidant levels. The exosome/40,000 and exosome/80,000 groups, which reduced the antioxidant level to 4.1 and 3.8 Trolox equiv mmol/$L^{-1}$, respectively, showed a significant difference. In the effects of the same groups on the oxidant level, it was observed that the oxidant level increased in parallel with the antioxidant results (exosome/40.000, 1.6-time increase; exosome/80.000, 1.8-time increase).

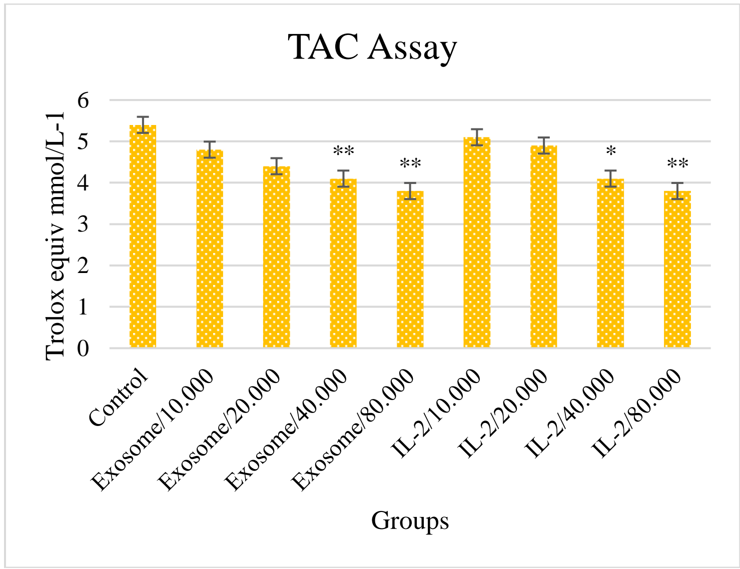


**Fig 2** TAC Assay for CaCo-2 cell line after 48h exosome and IL-2 activated lymphocytes (T lymphocyte) treatment. (*P < 0.05 compared to control group; **P < 0.001 compared to control group)

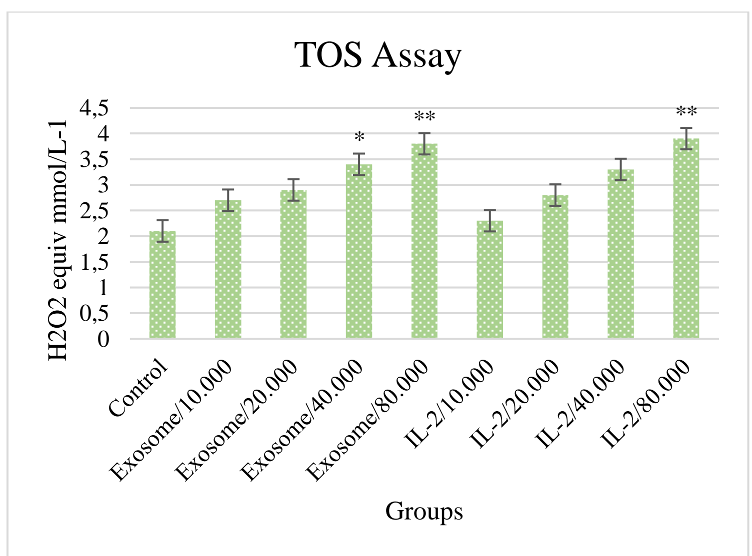


**Fig 3** TOS Assay for CaCo-2 cell line after 48h, exosome and IL-2 activated lymphocytes (T lymphocyte) treatment. (*P < 0.05 compared to control group; **P < 0.001 compared to control group)

### Lactate dehydrogenase (LDH) result

Lactate dehydrogenase (LDH) can be used as a marker for cell death both in vitro and in vivo. We can say that cellular viability decreases due to the increased LDH value in vitro. When we examine our results in Figure 3 based on all this information, we see that lymphocytes activated by exosome and IL-2 increase the LDH level due to the increasing number of cells. The most significant result was the exosome/80.000 group, which increased the LDH level 2.2 times and produced results close to the IL-2/80.000 group.

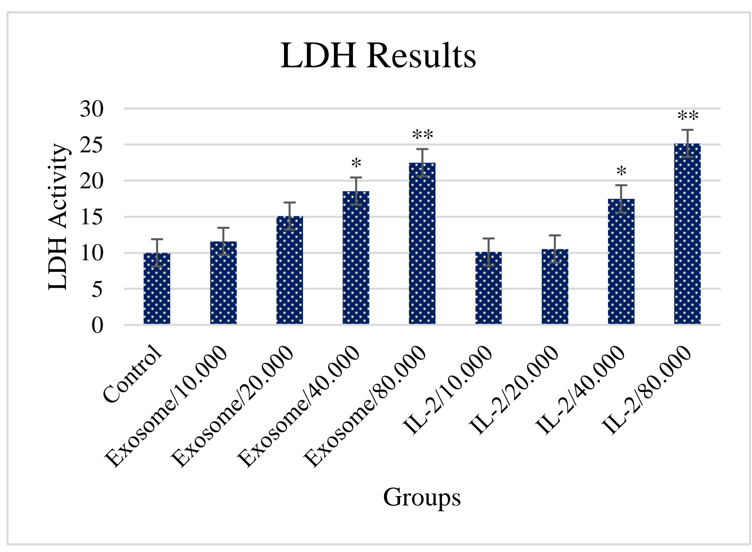


**Fig 4** LDH Results for CaCo-2 cell line after 48h exosome and IL-2 activated lymphocytes (T lymphocyte) treatment. (*P < 0.05 compared to control group; **P < 0.001 compared to control group)

### Atomic force microscopy (AFM) results

The microstructure of exosomes was visualized in figure 4 by atomic force microscopy (AFM). The vesicle sizes in the indicated images confirm that they consist of typical exosome-sized populations of vesicles, consistent with exosome AFM images reported in the literature (Di Noto et al., 2014; Raposo and Stoorvogel 2013; Sharma et al., 2010).

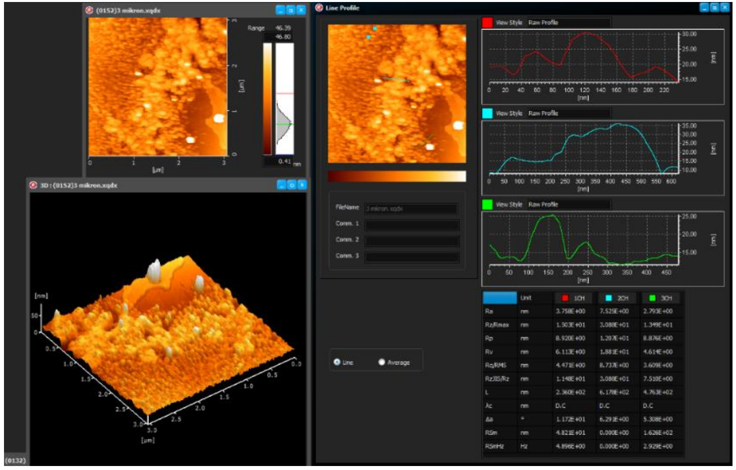

**Fig 5** Atomic force microscopy (AFM) images of isolated exosomes

**Transmission electron microscopy (TEM) and scanning electron microscopy (SEM)**

Electron microscopy (EM) is a widely used technique to characterize and visualize a variety of samples. In biological sample studies, two types of EM are commonly used, namely transmission electron microscopy (TEM) and cryo-electron microscope (cryo-EM). In our study, we obtain TEM images with the hitachi TEM system to visualize exosomes and determine their diameter and show them in figure 5A. We also support the exosome characterization with the results we obtained with the ZEISS device at 200 nm magnification. With the scanning electron microscope (SEM) image in Figure 5B, we show vesicles with a size of 115 nm. In summary, the structures of colon cancer-derived exosomes were revealed by both TEM and SEM results. It is shown in figure 5 that they are round or oval in shape and their size ranges from 40-150 nm.

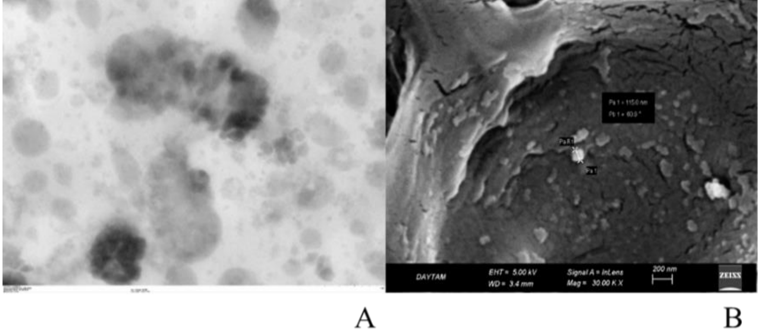


**Fig 6** Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) images of isolated exosomes

## Discussion

In studies on exosomes and their biological functions, it has been revealed that exosomes obtained from different cell types provide intercellular communication [1]. The main cell types are fibroblasts, endothelial cells and immune cells that interact with the signals of exosomes in the tumor microenvironment (TME). As a result of these interactions, different pathways are triggered [15]. For example, exosomes in the TME can act as a cargo in carrying information, and may enable tumor cells to proliferate and cancer to progress. These exosomes are completely tumor-focused and can induce metastasis by silencing and altering antitumor immune responses Peinado et al., (2012) demonstrated in their study that exosomes derived from metastatic melanoma cells can program the bone marrow into an environment that supports the development of invasive melanoma cells.

Exosomes in the TME can also stimulate immune cell functions or suppress antitumor activity. Factors such as the origin, capacity and load of exosomes are important in these stimulating and inhibitory functions. For example, exosomes in the TME may be the main source of activated immune cells in early-stage tumor progression, but not in advanced cancers. On the contrary, exosomes in TME are secreted to suppress the immune system [16]. Liu et al., (2006) showed that in vivo breast cancer-derived exosomes exert immunosuppressive effects by inhibiting NK cell proliferation by tumor-mediated exosomes. It has been shown that tumor-mediated exosomes can also target myeloid cells to modulate their function. Valenti et al., (2006) demonstrated that exosomes originating from human colon cancer cells exert a suppressive effect on T cells in vitro through TGF-β1.

Immunotherapies, which use immune cells for treatment, aim to increase the activity of the immune system to destroy cancerous cells [17]. In colon cancer, T cell infiltration into the tumor site suggested that immune-regulation may play a role in tumor progression [18]. In the 1900s, Paul Ehrlich suggested that the human body constantly produces neoplastic cells that are destroyed by the immune system, and today, cancer immunotherapy has re-emerged and made significant progress [19]. While Halliday et al., (1995) showed the ability of T cells to fight cancerous tissue in their studies, Teng et al., (2015) proved that natural killer (NK) and active cytotoxic T cells (CD8+T) recognize and eliminate cancer cells. Again, in different

studies, it has been revealed how CD8 + T cells prevent the development of many cancers [20-22].

T lymphocytes effectively control almost all parts of the body to capture foreign substances. Eventually, naive and active T cells become migratory cells, which are highly skilled in enhancing immunity against undesirable conditions such as infection and cancer. In particular, CD8+ T cells are very important in immunotherapy as they are the main killers of pathogens and neoplastic cells. CD4+ T cells, on the other hand, are effective in maintaining the effect of CD8+ T cells and preventing depletion [23].

The main purpose of our study, it is revealed by studies that exosomes increase their function by adding a new perspective to immunotherapy and their importance is increasingly understood. Seo et al., (2018) showed that tumor mesenchymal cells were depleted and tumor progression stopped by applying exosomes obtained from activated CD8+T cells to the cancer site in vivo. When we examined the in vitro viability results in our study, we revealed that lymphocytes activated by exosomes obtained from the CaCo-2 cancer line also affected the viability of the CaCo-2 cell line. Similarly, Klibi et al., (2009) showed that tumor-mediated exosomes affect T cell proliferation, activation and apoptosis. They showed that exosomes obtained from human nasopharyngeal carcinoma (NPC) stimulate regulatory T cell (Treg) formation via miRNAs and inhibit T cell proliferation. On the other hand, we demonstrate that in addition to vitality, activated lymphocytes increase the oxidant level by decreasing the antioxidant level in the CaCo-2 cell line. Tokuda et al., (2021) also investigated the development of cancer by administering cancer-mediated exosomes in vivo intraperitoneally. As a result, they observed that cancer development was inhibited and NK cell stimulation increased. Various studies have shown that IL-2, which we used as a control point in our study, is effective in T cell proliferation and activation [24-26].

## Conclusion

In our study, when we examined the effects on the CaCo-2 line of lymphocytes activated by exosomes against lymphocytes activated by IL-2, we found that both groups were effective, especially when 80,000 cells were applied. We demonstrated that exosomes can be as effective in activating as IL-2. The effective results found have been a source of hope for the

development of these studies, especially in the field of immunotherapy and in the treatment of a very important cancer type such as colon cancer. The idea of combining immunotherapy with exosomes, which is increasingly important, has been studied recently, but still no effective results have been obtained. We think that our work will be an important start in this sense.

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**Research article**

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# Isolation and Characterisation of the Degradation Potential of 2,2-Dichloropropionate (2,2-Dcp) By *Bacillus amyloliquefaciens* From Gebeng

**Firdausi Aliyu[1,2], Aliyu Adamu[1,3], Roswanira Abdul Wahab[4], Fahrul Huyop[1]**

**ABSTRACT**

Most halogenated compounds such as α-halocarboxylic acid (αHCA) are potential carcinogens that are toxic and widely liberated in both aquatic and terrestrial ecosystem. 2,2-dichloropropionate (2,2-DCP) is a synthetic halogenated compound commonly used in agricultural activities as an herbicide. This study is focused on the isolation and characterization of dehalogenase producing bacteria capable of utilizing 2,2-dichloropropionate as the sole carbon and energy source from soil sample obtained from the industrial hub of Gebeng (Pahang), Malaysia. An enrichment culture technique supplemented with 2,2-DCP was used to isolate the bacteria. A pure culture strain designated as strain FDS grew well in media containing 20 mM 2,2-DCP at a temperature of 30˚C exhibiting a cell doubling time of 22.21hours and a maximum rate of chloride ion release of 0.345mmol/L suggesting this is the optimum concentration for growth. Based on microscopic observations, biochemical characteristics and phylogenetic analysis of the 16S rRNA gene sequence, FDS was identified as *Bacillus amyloliquefaciens* with 98% homology. A literature survey revealed no report regarding the role of *B. amyloliquefaciens* on pesticide biodegradation as well as on 2,2-DCP, therefore the current isolate was designated *Bacillus amyloliquefaciens* FDS.

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## Introduction

The advancement in technology has resulted in the formation of various man-made organic compounds. These compounds (xenobiotics) are produced in enormous quantities which also benefit our life. Among these xenobiotics are halogenated compounds. These

[1] Department of Biotechnology, Faculty of Biosciences and Bioengineering, Universiti Teknologi Malaysia, 81310 Johor Bahru, Malaysia
[2] Department of Biotechnology, Faculty of Science, Nigerian Defence Academy (NDA),Kaduna PMB2109,Nigeria
[3] Department of Microbiology, Faculty of Science, Kaduna State University, Tafawa Balewa way, Kaduna PMB 2339, Nigeria.
[4] Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Malaysia.
*Corresponding author: Firdausi Aliyu, afirdausi@nda.edu.ng

compounds are extensively used as antibiotics (chloramphenicol, vancomycin), anticancer (iododoxorubicin, salinosporamide) and as agrochemicals (herbicides, pesticides, soil fumigants) and solvents (lubricants, silicones) [1]. Halogenated compounds have been reported to cause serious health issues such as liver necrosis, dissociation of hepatocytes, fat disintegration [2], hormonal imbalance, lower respiratory problems, skin and lung cancer etc. [3], in addition to causing serious environmental pollution owing to their toxicity, their toxic breakdown products, persistence and bioaccumulation in the food chain and water bodies [4]. These compounds are poisonous and very difficult to degrade [5].

2,2 -Dichloropropionate (2,2-DCP) is a chlorinated aliphatic acid also known as Dalapon. It is used in agriculture as an herbicide and a plant growth regulator used on certain annual and perennial grasses. It kills only certain plants while sparring non-target plants thereby preventing lipid synthesis, stops cell division, reduces wax production by leaves, affects carbohydrate, lipid and nitrogen metabolism [6].

Microorganisms play an important role in the degradation of xenobiotics, they help transform toxic compounds into non-hazardous substances. Some groups of bacteria use halogenated organic compounds as their main carbon and energy source, these microorganisms detoxify or remove pollutants owing to their diverse metabolic capabilities thereby helping reverse the effect of environmental halogen- associated pollution [7].

These bacteria produce enzymes known as dehalogenase which catalyze the cleavage of carbon-halogen bonds in halogenated organic compounds thereby producing some environmental friendly products by a process known as dehalogenation [8], [9]. Some examples of aerobic degradative bacteria include; *Pseudomonas, Bacillus, Micrococcus* and *Rhodococcus* while anaerobic degradative bacteria are *Syntrophus*, *Methanospirillum*, *Syntrophobacter*. Among them *Pseudomonas* and *Bacillus* species are the most studied [10], [11]. The present study is focused on the isolation and characterization of 2,2-DCP degrading bacteria from Gebeng industrial area using enrichment techniques.

## Materials and Methods

### Isolation and identification of 2,2-DCP degrading bacteria

The soil sample was collected from the industrial area of Gebeng (Pahang), Malaysia. The industries around the are generate a lot of chemical waste containing halogen, which contaminate soil in the environment.

### Preparation of medium for isolation

Varying concentrations (10 mM, 20 mM, 30 mM and 40 mM) of 2,2-DCP liquid minimal medium were prepared according to Table 1. tenfold of trace metal salts which comprised of Nitriloacetic acid (1.0g/L), $MgSO_4.7H_2O$ (2.0g/L), $FeSO_4.7H_2O$ (0.12g/L), $MnSO_4.4H_2O$ (0.03g/L), $CoCl_2.6H_2O$ (0.01g/L), $ZnSO_4.H_2O$ (0.03g/L) were dissolved in distilled water. The basal salts were prepared as a tenfold concentration of $K_2HPO_4.3H_2O$ (42.5g/L), $NaH_2PO4.2H_2O$ (10.0g/L), $(NH_4)_2SO_4$ (20.0g/L) in distilled water. The medium for isolation contained 10ml of 10x trace metal salts and 10ml of 10x basal salts per 100ml distilled water were autoclaved (121˚C, for 15min at 15psi). Then 2ml of 2,2-DCP (1M) was filter sterilized using a nylon filter (0.2µm pore size) and added aseptically into the media and made up to the desired final concentration the plates were prepared by adding bacteriological agar (1.5% w/v) prior to sterilization[12].

**Table1** Composition of minimal media

| **2,2-DCP concentration** | **10mM** | **20mM** | **30mM** | **40mM** |
|---|---|---|---|---|
| Distilled water | 79 | 78 | 77 | 76 |
| Trace metal | 10 | 10 | 10 | 10 |
| Basal Salt | 10 | 10 | 10 | 10 |
| V1, 1M | 1 | 2 | 3 | 4 |
| Total (ml) | 100 | 100 | 100 | 100 |

### Isolation procedure

5g of sample was added into a 250ml shaker flask containing 100ml minimal salts and supplemented with 20 Mm 2,2-DCP as the sole carbon and energy source. Following 10

days incubation with shaking at 200rpm at 37˚C, an aliquot (0.1ml) was pipette out and spread on plates supplemented with 20 Mm 2,2-DCP and incubated at 37˚C. The resulting colonies were selected and screened. Pure cultures, the resulting colonies were repeatedly sub cultured in the same medium to confirm carbon source utilizing ability.

**Identification of isolate**

To ascertain the identity of the isolate, Gram staining, motility, spore staining and biochemical test were carried out: Oxidase, catalase, gelatin liquefaction, lactose fermentation, citrate test, Nitrate reduction, indole, urease and starch hydrolysis test [12].

**Growth profile**

The growth profile of the bacterial culture in different concentrations of 2,2-DCP (10mM-40mM) were monitored by taking 1ml of the sample from the growth medium at 2 hours interval over 20 hours growth period and measured at $A_{680nm}$ using Jenway 6305 UV Spectrophotometer (Staffordshire, UK).

**Halide ion assay**

The utilization of 2,2-DCP by the isolates results in the release of halide ion during dehalogenation reaction as carried out from adaptation of [13]. A sample (1ml) from the liquid growth medium, 100µl of Reagent I (0.25M ferric ammonium sulfate in 9M nitric acid) was added and thoroughly mixed. To this, Reagent II (100µl of mercuric thiocyanate dissolved in saturated ethanol) was also added and the solution was mixed by shaking. Color could develop for 10 minutes and measured at $A_{460nm}$.

**16S rRNA genomic analysis**

The genomic DNA was extracted from an overnight bacterial culture using Promega® Wizard® Genomic DNA Purification Kit (Promega,Madison, WI, USA). The purification and merging the DNA concentration was measured by Nanodrop machine.The Polymerase chain reaction (PCR) was carried out using universal primers, Fd1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rP1 (5'-ACG GTC ATA CCT TGT TAC GAC TT-3') to amplify the DNA fragment as suggested by [14], [15]. The PCR cycle for DNA amplification was performed for 30 cycles and set as initial denaturation at 95˚C for 5 minutes; cooling, denaturation at 94˚C for 1 minute; annealing at 55˚C for 1 minute; final

elongation at 72˚C for 10 minutes. The PCR products were purified using QIA quick PCR purification kit and sequenced by 1ST Base Laboratory, Malaysia.

**Phylogenetic study**

The bacterial 16S rRNA gene sequences were compared with other sequences of dehalogenase producing bacteria in GenBank database using BLASTn. The FASTA format of all 16S rRNA sequences obtained were aligned together by MUSCLE using MEGA7 [16]. The topology of the tree was constructed using neighbor-joining method with bootstrap test values above 50% based on 500 resampling.

## Results

### Isolation of 2,2-DCP degrading bacteria

Following 5 days incubation of the soil sample obtained from Gebeng industrial area lead to the isolation of a bacterium designated strain FDS. The Size, morphology (Table 2) while Gram staining showed the microorganism to be Gram positive rod shape (figure 1). However, to fully ascertain the identity of strain FDS, biochemical tests (Table 3) were performed according to Bergy's Manual of Systematic Bacteriology [17].

**Table 2** Morphological characteristics of Strain FDS on solid media

| Parameters | Results |
|---|---|
| Pigmentation (pure colony) | Creamy |
| Elevation | Flat |
| Colony | Rough circular |
| Margin of the colony | Undulate |
| Microscopic shape | Rod |
| Size | 3.40µm |
| Spore formation | Negative |
| Oxygen requirement | Aerobic |

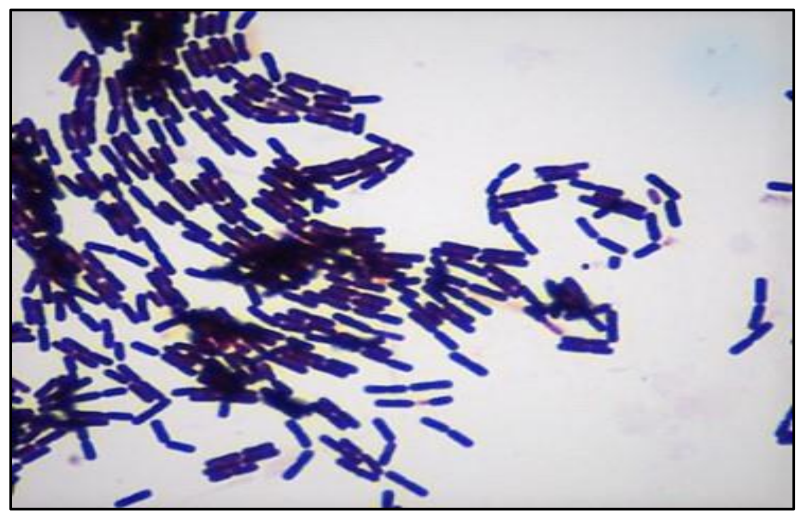

**Fig 1** Microscopic observation showing the Gram-stain of strain FDS (1000 X magnification

**Table 3** Results from the Partial biochemical tests

| Test | Results |
|---|---|
| Oxidase | Positive |
| Catalase | Positive |
| Indole | Positive |
| Gelatin liquefaction | Positive |
| Motility | Negative |
| Citrate | Negative |
| Urease | Positive |
| Nitrate reduction | Positive |
| Starch hydrolysis | Positive |
| Lactose utilization | Negative |

**Growth profile and Halide ion assay**

The growth profile of FDS in different concentrations (10mM-40mM) of 2,2-DCP are shown (Figure2). 20mM 2,2-DCP exhibited good bacterial growth. 10mM and 30mM 2,2-DCP showed low growth, while 40mM 2,2-DCP exhibited no growth which suggests that the concentration was toxic. Following this observation, the bacterium FDS was grown in

liquid minimal medium supplemented with 20mM 2,2-DCP at 37˚C with agitation at 200 rpm with growth measured at 2 hours interval over 20 hours period. The doubling time of the bacterium was 22.21 hours with a maximum chloride ion of 0.345mmol/L.

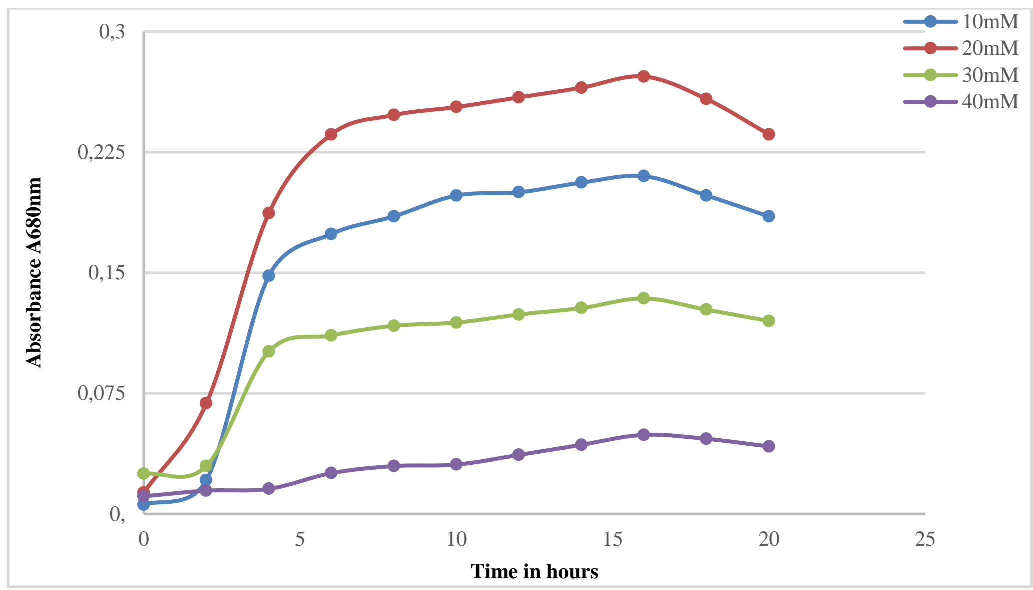


**Fig 2** Growth rate of strain FDS in different concentrations of 2, 2-DCP

**Bacterial growth on other halogenated compounds**

The bacterium FDS was grow in 10mM trichloroacetic acid (TCA), 10mM 2-chloropropionate (2-CP) and 10mM 2-bromopropionate (2-BP) liquid minimal medium. The results (Table 4) showed growth in 10mM TCA while no growth was observed in 2-CP and 2-BP which suggests the substrate is not suitable for growth. The cell doubling time of FDS grown in 10mM TCA was 14.09 hours and a maximum chloride ion of 0.223mmol/L (Figure 3).

**Table 4** Growth properties of strain FDS in different substrate

| Growth Substrate (10mM) | Maximum Medium Turbidity ($A680_{nm}$) | Doubling time(hours) |
|---|---|---|
| Trichloroacetic acid | 0.184 | 14.09 |
| 2-bromopropionate | NG* | NG* |
| 2-chloropropionate | NG* | NG* |

*NG: No Growth

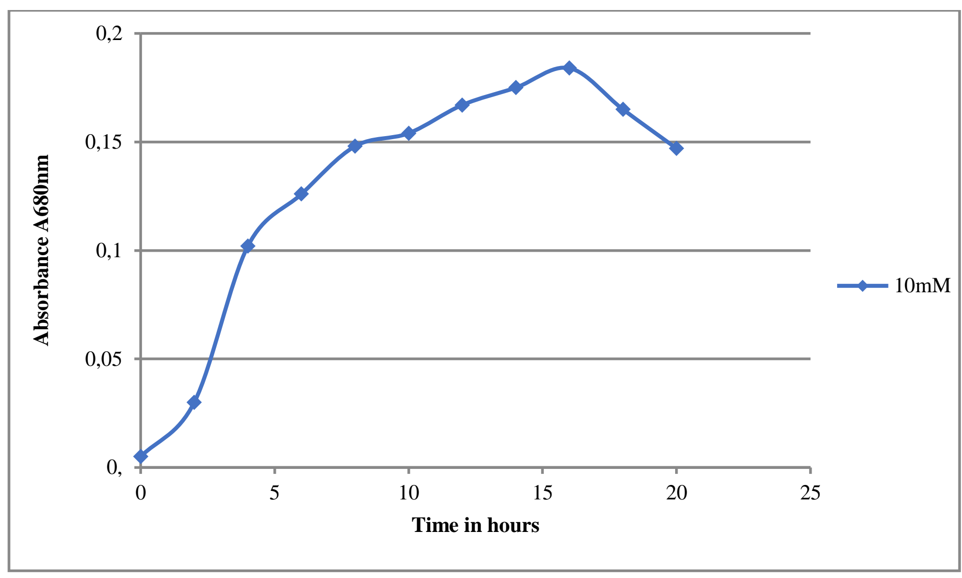


**Fig 3** Growth rate of strain FDS in 10mM concentration of TCA

**Identification of 2,2-DCP degrading bacteria using 16S rRNA analysis**

The genomic DNA of FDS was successfully extracted and amplified using universal primers Fd1 and rP1. Gel electrophoresis of the PCR product revealed a single fragment of approximately 1500bp (Figure 4).

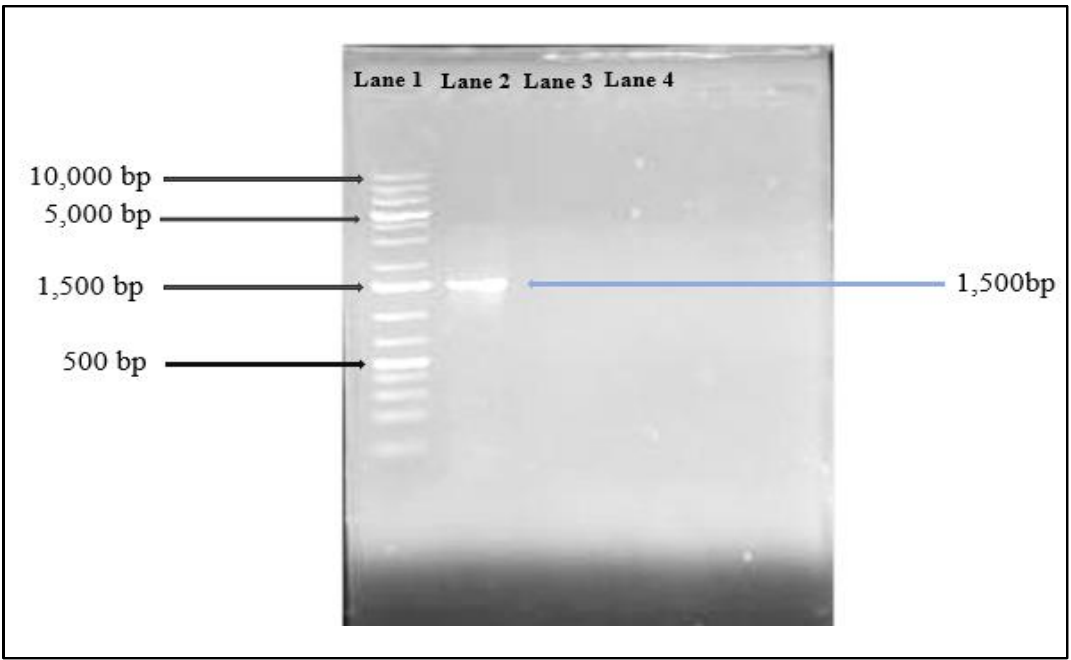


**Fig 4** PCR product for 16S rRNA under UV light

Lane 1: 1kb DNA ladder, Lane 2: PCR product of 16S rRNA, Lane 3: Control ($dH_2O$ + Fd1+ PCR mix), Lane 4: Control ($dH_2O$ + rP1+ PCR mix).

**Sequencing and analysis of 16S rRNA gene**

The outcome of the PCR product sequenced by 1st Base Company was received in ". abl'' format. The files were viewed using Sequence scanner software. The BLASTn results showed that bacterium FDS shared 98% identity to *Bacillus amyloliquefaciens* (Table 5).

**Table 5** The top ten BLAST search result of strain FDS

| Accession number | Description | Maximum score | Query coverage (%) | Identity (%) |
|---|---|---|---|---|
| NC 014551.1 | *Bacillus amyloliqueficiens* DSM7 | 1995 | 97 | 98 |
| NC 000964.3 | *Bacillus subtilis* strain 168 | 1973 | 97 | 98 |
| NZ CP007640.1 | *Bacillus atrophaeus* subsp. globiqii strain BSS | 1962 | 97 | 97 |

**Phylogenetic study**

The top dehalogenase producing bacteria obtained from BLASTn analysis as obtained from NCBI database were selected for the construction of phylogenetic tree using MEGA 7 by neighbour-joining with bootstrap value (Figure 5). The results inferred that the bacterium FDS may be designated as *Bacillus amyloliquefaciens* FDS.

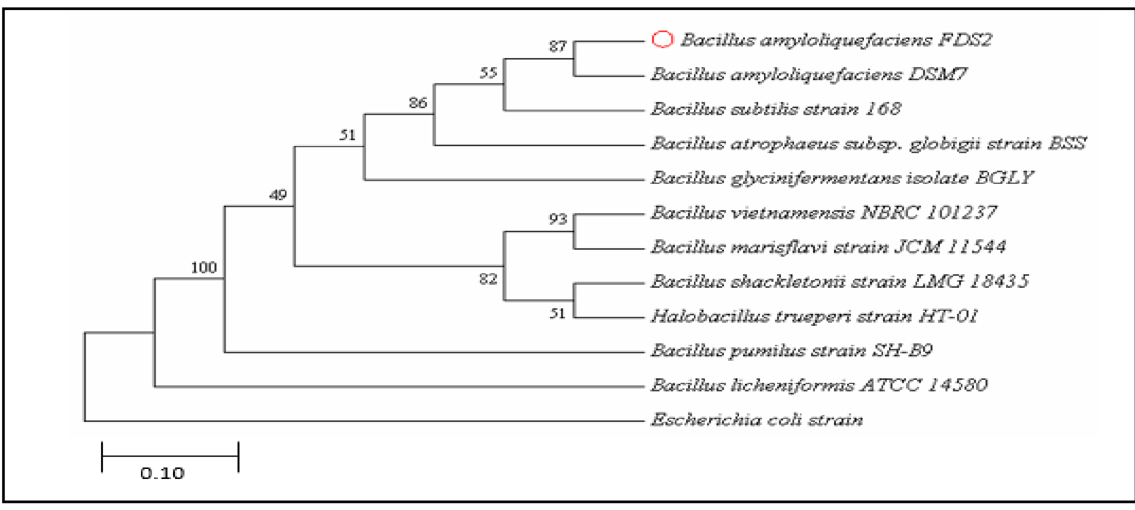


**Fig 5** Phylogenetic relationships between *Bacillus amyloliquefaciens* FDS and other dehalogenase producing bacteria16S rRNA sequences

## Discussion

In this study, Soil sample obtained from Gebeng industrial area which is synonymous with the manufacturing of various chemical and petrochemical products was used as the source for the isolation of bacteria capable of degrading 2,2-DCP and TCA as the sole carbon and energy. The isolation procedure employed in this study entailed the enrichment of minimal salts with the carbon sources (2,2-DCP and TCA), followed by plating on a similar agar medium. The two procedures help ensures that the isolation is that of bacteria only capable of utilizing the carbon sources. The successful isolation of a strain designated FDS was observed to have various morphological (Table 4.1) and biochemical characteristics (Table 4.2). However, to properly characterize and identify the strain a method of bacterial taxonomy known as 16S rRNA was employed this routine procedure in prokaryotic taxonomy results in large and growing databases which improve identification results, phylogeny reconstructions and primer specificity evaluations[18]. The 16S rRNA gene sequence analysis suggests that strain FDS gene sequence shared at least 98% identity to the sequence of *Bacillus amyloliquefaciens* which suggests strain FDS belongs to the *Bacillus* sp. (Figure 4.9). Thus the 2, 2-dichloropropionate degrading bacteria isolated belong to *Bacillus* sp. This was further supported by staining properties and biochemical analysis and in accordance with Bergy's Manual of Systematic Bacteriology [17]. *Bacillus amyloliquefaciens* is a gram positive, rod-shape, catalase positive and aerobic soil bacteria. As with other members of the *Bacillaceae* they have the ability to form endospore allowing them to survive for a prolonged period time, thereby increasing their soil shelf life [19], [20]. Previous studies have shown that several strains of the bacteria belonging to the genus *Bacillus* particularly *B.subtilis* and *B.amyloliquefaciens* species were known to be effective for the biocontrol of many plant-diseases caused by soil borne pathogens [21], [22] or post-harvest pathogens [23]–[25]. Their ability to colonize plants rhizosphere thereby suppressing competing phytopathogenic bacteria and fungi these classifies them as microbial biopesticides. They represent about half of the commercially available bacterial biocontrol agents [26]. However, there has not been any report of *Bacillus amyloliquefaciens* that is able to degrade 2,2-dichloropropionate and trichloroacetate

reported so far. Therefore, this strain of *Bacillus amyloliquefaciens* that can degrade 2,2-dichloropropionate deserves more research.

They are four basic criteria for a given halogenated compound to be utilized by an organism as a sole carbon and energy source as reported by [27]. Firstly, the organism should be able to synthesize enzyme (dehalogenase) which can remove the substituent halogen(s) from the compound. Secondly, the product of dehalogenation should be non-toxic and easily converted to an intermediate which can readily be absorbed in the organism's central metabolic pathway. Thirdly, entry of halogenated compound into the organism should be either through passive or active transport in order for the compound to be synthesized by the enzyme, and finally, the halogenated compound should not be toxic to the organism at intracellular concentrations [27]. These criteria were satisfied when *Bacillus amyloliquefaciens* FDS grew on 2,2-dichloropropionate and trichloroacetic acid as the sole carbon and energy source. The isolate grew more rapidly on 2,2-dichloropropionate compared to trichloroacetic acid, possibly because the organism was originally isolated using 2,2-dichloropropionate. 2,2-dichloropropionate failed to support growth at concentration exceeding 40mM, while trichloroacetic acid failed to support growth exceeding 10Mm; presumably the intracellular concentration of halo-aliphatic acid had reached a toxic level.

## Conclusion

The successful isolation identification and characterization of *Bacillus amyloliquefaciens* FDS capable of degrading 2,2-DCP and TCA from Gebeng would suggest that these study areas possess enormous reservoir of unexplored/unexploited microbes. In conclusion, this study provides the identity of a bacterium that can utilize halogenated compound and used in bioremediation strategies. In future, the study can help describe the diversity of microbial dehalogenases and the dehalogenation enzyme regulation.

**Abbreviations**

2,2-DCP: 2,2-dichloropropionic acid, BLAST: Basic Local Alignment Search Tool, Br: Bromine, Cl: Chloride, DNA: Deoxyribonucleic acid, EDTA: Ethylenediaminetetraacetic acid, EtBr: Ethidium Bromide, F: Flourine, I: Iodine, LB: Luria Bertani,MEGA: Molecular Evolutionary Genetics Analysis, NaCl: Sodium chloride,NADH: Nicotinamide adenine dinucleotide,NCBI: National Centre for Biotechnology Information, PCR: Polymerase chain reaction, RNA: Ribonucleic acid, sp.: Species, TAE: Tris acetate EDTA, TCA: Trichloroacetic acid, UV: Ultraviolet

**Conflict of Interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Research article**

Bakir, O. and E. B. Kurbanoglu, Investigation of antioxidant activity and urease, collagenase enzyme inhibitory effects of Hippophae rhamnoides L seeds. International Journal of Life Sciences and Biotechnology, 2022. 5(2): p. 213-224. DOI: 10.38001/ijlsb.1077292

# Investigation of antioxidant activity and urease, collagenase enzyme inhibitory effects of *Hippophae rhamnoides* L. seeds

**Ozlem Bakir [1*], Esabi Basaran Kurbanoglu[1]**

**ABSTRACT**

In this study, water and methanol extracts were obtained from sea buckthorn seeds, which have an important place in health. It was aimed to determine the DPPH, ABTS antioxidant activities and urease, collagenase enzyme inhibition activities of these extracts. The inhibitory effect of the prepared extracts on these enzymes was evaluated by in vitro method. The best DPPH and ABTS scavenging activity was observed in aqueous extracts. DPPH ($IC_{50}$ = 19.34±0.49 µg/mL) and ABTS ($IC_{50}$ = 7.24±0.14 µg/mL) were determined. According to DPPH results, while aqueous extract provided 25,85 % removal, the methanolic extract provided 20,44 % removal. In ABTS removal activity, while aqueous extract provided 25,55 % removal, the methanolic extract provided 18,23% removal. The lower the $IC_{50}$ value, the higher the free radical scavenging power. The best urease and collagenase inhibition from sea buckthorn seed extracts was seen in the aqueous extract ($IC_{50}$ = 0,23 ± 0,003µg/mL, $IC_{50}$ = 2,19 ± 1,12µg/mL). Methanol extracts did not show tyrosinase and urease enzyme inhibition. This study clearly shows that sea buckthorn the aqueous extract of can be used as an alternative source of anti-urease and anti-collagenase source. Due to the important role of these enzymes in various diseases, new drugs with inducing or inhibitory effects should be developed.

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## Introduction

Medicinal plants, which are generally used in the field of health, are the most important primary sources of naturally occurring bioactive compounds in the pharmaceutical industry. Therefore, research continues to find valuable compounds from medicinal and aromatic plants. It has been calculated that approximately 40% of the drugs in the pharmaceutical markets are obtained from plant bioactive components so far [1]. There are many medicinal plants that have not been researched by researchers. Among these plants, limited information is available on sea buckthorn (*Hippophae rhamnoides* L.). One of the important medicinal and aromatic plants grown in our country is the *Hippophae rhamnoides* plant. The high

[1] Department of Biology, Faculty of Science, Ataturk University, Erzurum, Turkey
*Corresponding Author: Ozlem Bakir, e-mail: ozlembakir@atauni.edu.tr

concentration of essential oil contained in the fruits of the *Hippophae rhamnoides* plant is anti-inflammatory and controls the formation of cancer cells. It is used in the treatment of skin, stomach and intestinal diseases [2,3]. Enzymes are the most studied biomolecules that have an essential role in every process in every aspect of life [4]. Inhibitors are substances that reduce or stop enzyme activity. They affect the catalytic activity of the enzyme. Enzyme inhibition strategy attracts attention in modern pharmacy today and it is known that an important part of antiviral, antibiotic and antiparasitic drugs show their efficacy in this way. Enzyme inhibition is not only important in terms of fighting diseases, but also in terms of keeping biotechnological processes under control. This indicates that for the near future it will be vital that the main focus of pharmaceutical research remains specific enzyme inhibition [5]. Urease (urea amidohydrolase, E.C.3.5.1.5.) is a nickel-containing metalloenzyme that catalyzes the hydrolysis of urea to ammonia and carbon dioxide. Urease is found in many plants, algae, fibrous fungi and bacteria [6]. *Helicobacter pylori* is the only microorganism that can colonize the stomach. Ammonia is formed by the hydrolysis of urea and helps colonization of *Helicobacter pylori* by neutralizing stomach acid [7]. Urease enzyme plays an important role in diseases caused by *H. pylori.* Damages caused by *Helicobacter* in the stomach due to the increase in the urease enzyme form the basis for the formation of cancer [8]. Urease enzyme inhibition is considered a treatment for infections caused by urease-producing bacteria [7,9]. With this approach, interest in the use of urease enzyme inhibitors in the treatment of chronic stomach disorders has increased in recent years. In recent years, the inhibitory effects of various plant extracts on the urease enzyme have been investigated for this purpose [10]. It is a sulfated polysaccharide found in *Euphorbia decipiens* and different brown seaweeds, an example of a natural substance with urease inhibition activities [11]. Collagen is the organic component of bones, teeth and cornea, as well as the main component of skin, tendons and cartilage. Collagen constitutes approximately 25-33% of the total protein in the mammalian organism and 80% of this collagen is found in the dermis layer of the skin and bones. Collagen is a large molecule approximately 300 nm long and carries 3 parallel polypeptide chains [12]. The collagenase enzyme obtained from *Clostridium histolyticum* also breaks down the intercellular matrix proteins. Collagenases play an important role in physiological conditions such as tissue

remodeling, normal structuring of tissues and systems, wound healing and normal developmental processes [13]. They are also involved in pathological processes such as tumor cells spreading to surrounding tissues and disrupting their function [14]. It was aimed to investigate the inhibition potentials of seed extracts for these enzymes, which have clinical and industrial importance. Due to the important role of these enzymes in various diseases, new drugs with inducing or inhibitory effects should be developed. Therefore, sea buckthorn seed may be a good candidate for further development of nutraceuticals and functional foods. Moreover, to our knowledge, the information on anti-urease and collegenase property of sea buckthorn seed has not previously been reported. Biochemical investigation of the effect of seed extracts on the inhibition of target enzymes may pave the way for their introduction to the biotechnology market as alternative drug molecules for use in different fields of pharmaceutical chemistry and industry.

## Materials and Methods

### Preparation of samples

The buckthorn fruit used in the experiments was obtained from the Erzurum region by collecting from its branch. After the seeds were removed, they were kept in an oven at 30 ºC at a constant temperature without light. After drying, they were kept in plastic containers.

**Fig 1** Fruits, wet and dried seeds of *Hippophae rhamnoides* L

### Preparation of aqueous extract

After the seeds were pulverized, 10 g were weighed and placed in a glass balloon and 100 mL of distilled water was added. The mixture was refluxed for 8 hours under reflux. After

the obtained mixture is cooled, the water will be completely removed from the mixture by filtering through filter paper and under low pressure in the rotary evaporator. The resulting aqueous extract will be stored at +4 ºC until the enzyme activities are determined.

**Preparation of methanol extract**

After taking 10 g of seeds, ground and powdered, they were extracted with 300 ml of pure methanol in a soxhlet extraction device for 24 hours. After the extraction process, the balloons with solvent-extract mixtures were removed from the soxhlet system and placed in the Rotary evaporator. The solvent was completely removed. The methanol extracts obtained will be stored at -20°C until the analysis processes are started.

**Anti-urease activity method**

Urease inhibitory activity was determined spectrophotometrically using the method of Van Slyke and Archibald [15]. 0.5 mL was taken from the prepared solutions at different concentrations. 0.5 mL of a solution of urease in 100 mM 16 mg/mL phosphate buffer (pH = 6.8) was added. This mixture was incubated for 15 minutes at room temperature. 0.5 mL of 500 mM urea solution (prepared in 100 mM phosphate buffer with pH 6.8) was taken as control solution. 0.5 mL of 16 mg/mL urease solution (prepared in 100 mM phosphate buffer with pH 6.8) was added. This mixture was incubated for 15 minutes at room temperature. 0.4 mL of urea phosphate buffer solution (100 mM, pH = 6.8) containing 1 µg of phenol red in 1 mL of sample and control solutions was added and absorbance values were read against the blank experiment at 570 nm. Thiourea was used as a positive control.

**Measurement of collagenase enzyme inhibition**

The inhibitory effect on the collagenase enzyme was determined spectrophotometrically [13]. 50 µL of the solution containing *Clostridium histolyticum* collagenase enzyme (ChC) at 0.8 units/ml was taken. 50 µL of plant extracts at different concentrations were added to it. Then 0.9 mL of 50 mM Trisin buffer solution was added. For the blank, buffer solution, water and substrate were used instead of enzyme. Blank, control and sample solutions were left for the first incubation at 25°C for 30 minutes. After this first incubation, 1 mM 0.05 mL of N-(3-[2-Furyl]acryloyl)-Leu-Gly-Pro-Ala (collagenase enzyme substrate) solution was added to all solutions and left for a second incubation for 15 minutes at 25°C. The absorbance

values of the sample solutions and control solution were read at 340 nm in the UV spectrophotometer against the blank. Epigallocatechingallate was used as a positive control.

**Identification of Radical Scavenging Potential**

**DPPH method**

For the preparation of 0.1 mM DPPH solution, it was dissolved by adding 200 ml of methanol to 4 mg of DPPH. 50µl of extract and sample was added to eppendorf tubes. It was vortexed by adding 200µl of DPPH solution on them. It was kept in the dark for 60 minutes and added to the wells of 96-well plates and absorbance was measured at 517 nm [16]. DPPH free radical scavenging activity (% inhibition) was calculated using the following equation. BHA was used as standard.

$\% \text{Inhibition} = (A_{control} - A_{Sample}) / A_{control} \times 100$

**ABTS method**

For 7 mM ABTS solution, 0.192 g ABTS in 50 ml distilled water and 0.0324 g potassium persulfate in 50 ml distilled water were added and the volume was made up to 100 ml by mixing these mixtures and kept in the dark for one night. Then, 1 ml of this mixture was taken and 39 ml of methanol was added to it and diluted to 40 ml. After dilution, 15µl of extract and sample was taken. 285 µl of ABTS solution was added on them and vortexed. After 2 hours in the dark, absorbance was measured at 734 nm [16]. ABTS activity (% inhibition) was calculated using the following equation. BHT was used as standard.

$\% \text{Inhibition} = (A_{control} - A_{Sample}) / A_{control} \times 100$

**Statistical analysis**

GraphPad Prism 6.0. Statistical analyzes of enzyme inhibition values were performed by performing one-way analysis of variance (One-way ANOVA) with the software. $IC_{50}$ values were calculated by creating dose response curves with Microsoft Excel program, cases where the p value was less than 0.05 were considered statistically significant.

## Result and Discussion

In the last few years, there have been studies on the effects of plant extracts and natural compounds isolated from plants against this enzyme. The inhibitory effects of aqueous extracts obtained from wild sea buckthorn seed against urease enzyme are shown in Table 1.

All extracts with inhibitory potential on the urease enzyme were found to be in a concentration-dependent manner. Aqueous extract concentrations of 1 µg/mL,1.5 µg/mL,1.8 µg/mL and 2.5 µg/mL inhibited the urease enzyme by 20, 25, 30, and 34%, respectively. Compared with the standard substance, thiourea (IC50 = 152.2 ± 0.63 µg/mL) appears to have the highest inhibition in the aqueous extract at a concentration of 1 µg/mL ($IC_{50}$ = 20.76 ± 1.616 µg/mL) (Fig. 2). This was followed by 1.5 µg/mL, 1.8 µg/mL, 2.5 µg/mL concentrations 25.54 ± 1.485 µg/mL, 30.14 ± 1.571 µg/mL, 34.59 ± 0.417 µg/mL. The $IC_{50}$ values of wild sea buckthorn seed aqueous extracts vary considerably, as shown in Table 1. A lower $IC_{50}$ value represents a higher anti-urease activity

They showed that it is likely to use a 1 µg/mL aqueous extract of wild sea buckthorn seed as a urease inhibitor. Edible plants are rich sources of phytochemicals such as vitamins, terpenoids, alkaloids, orgonosulfides, pigments and other phenolic compounds [17]. It has been reported that catechins in green tea extract strongly inhibit *H. pylori* urease [18]. It has been stated that mulberry leaves show high anti-urease activity ($IC_{50}$ = 72.81 ± 15.60) [9].

**Table 1** Anti-urease and anti-collagenase activity values of sea buckthorn aqueous extract and standard

| | **Concentration (µg/mL)** | **% Inhibition*** | **Anti-urease $IC_{50}$** |
|---|---|---|---|
| **Sea buckthorn (aqueous)** | 1 | 20.76 ± 1.616 | 0.23 ± 0.003 |
| | 1.5 | 25.54 ± 1.485 | |
| | 1.8 | 30.14 ± 1.571 | |
| | 2.5 | 34.59 ± 0.417 | |
| **Thiourea** | 1 | 60.06 ± 2.177 | 1.29 ± 0.65 |
| | 1.5 | 55.9 ±1.638 | |
| | 1.8 | 35.9 ± 1.976 | |
| | 2.5 | 25.4 ± 0.646 | |

| | **Concentration (µg/mL)** | **% Inhibition*** | **Anti-collagenase $IC_{50}$** |
|---|---|---|---|
| **Sea buckthorn (aqueous)** | 1.3 | 33.4 ± 2.55 | 2.19 ± 1.12 |
| | 2 | 40.7 ± 1.52 | |
| | 2.6 | 44.3 ± 1.23 | |
| | 3.3 | 48.6 ± 0.33 | |
| | 4.6 | 50.7 ± 0.01 | |
| **Epigallocatechingallate (EGKG)** | 1.3 | 15.26 ± 1.24 | 1.05± 0.002 |
| | 2 | 17.35 ± 0.22 | |
| | 2.6 | 22.5 ± 0.37 | |
| | 3.3 | 25.9 ± 1.03 | |
| | 4.6 | 28.36 ± 1.24 | |

*Values are the mean of three experiments ± standard deviation

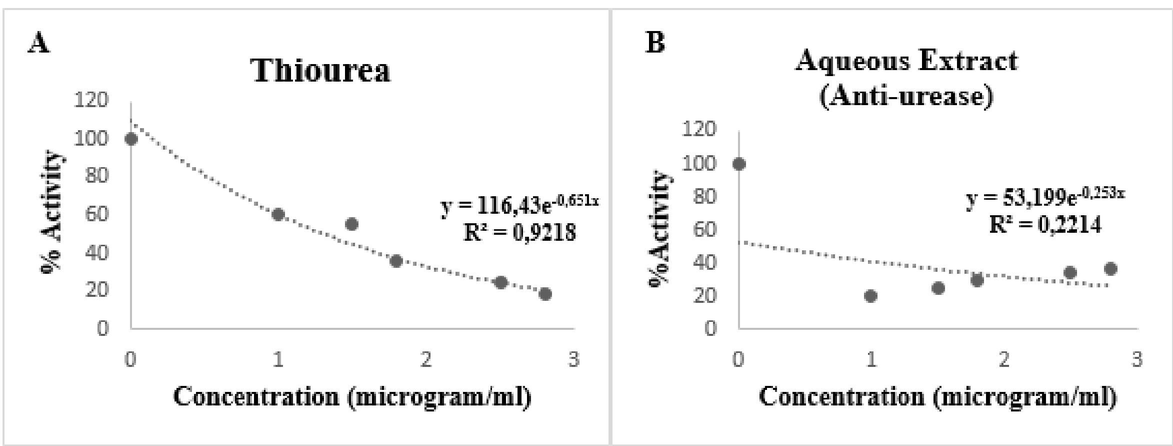


**Fig 2A** $IC_{50}$ value of thiourea to anti-urease enzyme **B** $IC_{50}$ value of sea buckthorn aqueous extract to anti-urease enzyme

The inhibitory effects of extracts obtained from wild sea buckthorn seed against the collagenase enzyme are shown in Table 1. All extracts with inhibitory potential on the collagenase enzyme were found to be in a concentration-dependent manner. Aqueous extract concentrations of 1.3 µg/mL, 2 µg/mL, 2.6 µg/mL, 3.3 µg/mL, 4.6 µg/mL inhibited the collagenase enzyme by 33.4%, 40.7, 44.3, 48.6 and 50.7%, respectively. The $IC_{50}$ values of wild sea buckthorn seed aqueous extracts vary considerably, as shown in Table 1. Compared with the standard substance, epigallocatechingallate ($IC_{50}$ = 1.05±0.002 µg/mL) appears to have the highest inhibition in the aqueous extract at a concentration of 1.3 µg/mL ($IC_{50}$ = 33.4 ± 2.55 µg/mL) (Fig. 3). This was followed by 2 µg/mL, 2.6 µg/mL, 3.3 µg/mL, 4.6 µg/mL concentrations 40.7 ± 1.52 µg/mL, 44.3 ± 1.23 µg/mL, 48.6 ± 0.33 µg/mL, 50.7 ± 0.01 µg/mL. A lower $IC_{50}$ value represents a higher anti-collagenase activity. It showed that a 1.3 µg/mL aqueous extract of wild sea buckthorn seed is likely to be used as a collagenase inhibitor. Matrix metalloproteinase enzymes have degenerative effects on structural proteins, but metalloproteinase inhibitors can reduce the severity of injury and contribute to the healing process. Therefore, inhibition of the collagenase enzyme that breaks down these components may be beneficial for the wound healing process. It has been previously reported that minimizing the level of this enzyme is vital. It has been found that locust bean gum has both hyaluronidase and collagenase inhibitory activities, which can clearly explain its wound healing potential [19]. In another study, *A. baytopae*, *A. brevicaulis*

subsp. *brevicaulis* var. *brevicaulis, A. Cilicica* 's have investigated the effects of extracts prepared from different parts on hyaluronidase, collagenase and elastase enzymes. It was found that acetone extract of *A. cilicica* (ACRAc) roots showed an inhibitory effect of 37.61% and 48.44% on collagenase and elastase enzymes, respectively. In addition, A. *brevicaulis* subsp. *brevicaulis* var. *brevicaulis* (ABrLAc) showed an inhibitory effect on collagenase and elastase enzymes with values of 31.38% and 39.39%, respectively [20]. In another scientific study with the *Triphala guggulu* plant, it was proven that 2.5 mg/mL solution of this plant inhibited the collagenase enzyme by 80%. For solutions with lower concentrations, this inhibition rate shows decreasing values such as 39% for 1.25 mg/mL and 27% for 0.625 mg/mL [21]. In another study conducted with various types of mulberry; The leaves of *Morus alba*, the white mulberry species, were used. The anti-inflammatory and antitumor properties of this herb have been proven by [22]. Based on the fact that white mulberry has these properties, the collagenase inhibition $IC_{50}$ value we obtained was found to be 33.47 ± 12.36 µg/mL. In another study conducted by pomegranate, inhibited the collagenase enzyme with an $IC_{50}$ value of 48.3 ± 0.06 µg/mL [23].

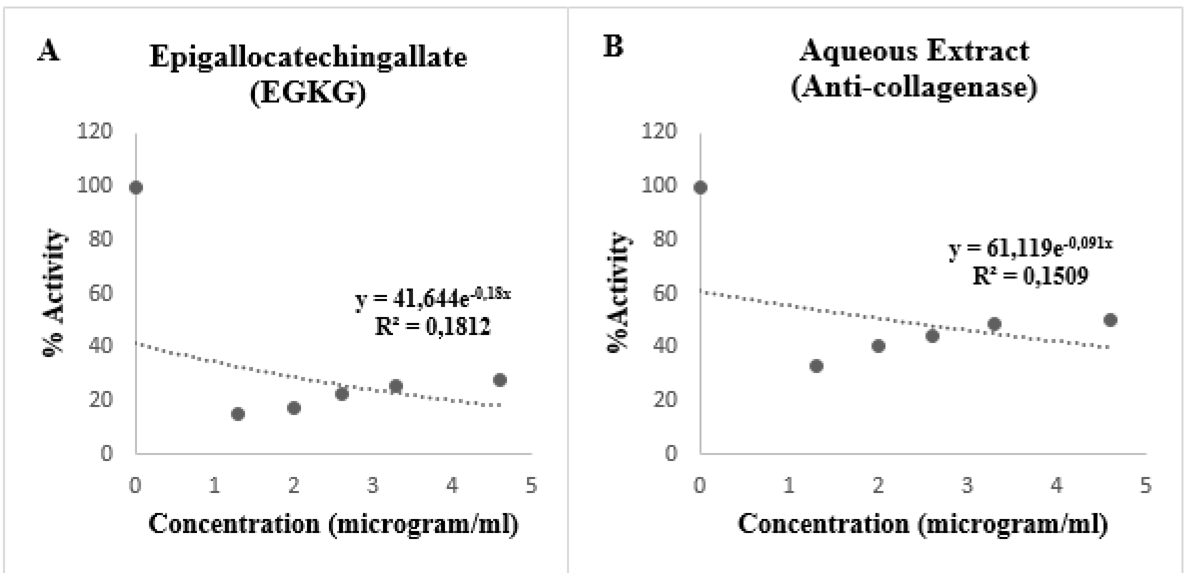


**Fig 3A** $IC_{50}$ value of epigallocatechingallate to anti-collagenase enzyme **B** $IC_{50}$ value of sea buckthorn aqueous extract to anti-collagenase enzyme

DPPH (2,2-diphenyl-1-picrylhydrazil) is an organic nitrogen radical and is a commercially available product. It is a simple and fast method used to measure the antioxidant capacity of sea buckthorn extracts. According to DPPH results, while aqueous extract provided 25,85 % removal, the methanolic extract provided 20,44 % removal (Fig. 4A). It shows that it has a synergistic effect between flavonoid and phenolic groups in buckthorn seeds, and its physical and chemical properties are also effective in DPPH removal. Butylated hydroxyl anisole (BHA) DPPH removal was 60,33 %, and it was higher than extracts. The DPPH free radical scavenging activity of the extracts is given in Table 2 as % inhibition. The $IC_{50}$ values of the BHA, aqueous extract and methanol extract of the seed for the DPPH assay were 17.03 ± 1.43 µg/mL, 19.34±0.49 µg/mL, 22.24±1.01 µg/mL (Fig. 5A). Aqueous extract ($IC_{50}$ = 19.34±0.49 µg/mL) showed the best activity in sea buckthorn seeds. In antioxidant activity, aqueous extract was found to be significantly higher than methanol extract. The lower the $IC_{50}$ value, the higher the free radical scavenging power. In the antioxidant capacity determination studies of *Rhododendron luteum*, the DPPH radical scavenging capacity of the ethyl acetate solution of the plant (mg TE/g extract) was reported as 41.94 ± 0.96 mg, the methanol extract 480.07 ± 0.85 mg, and the water solution 381.07 ± 3.08 mg. Antioxidant and activity of ethanol extract of flowers of *Rhododendron arboreum* were determined. The antioxidant activity of the flower extract for the DPPH experiment was 134.1 ± 2.34 mM TE/g [24].

In ABTS radical scavenging activity while aqueous extract provided 25,55 % removal, the methanolic extract provided 18,23% removal (Fig. 4B). BHT (Butylated Hydroxy Toluene) provided 70,22 % ABTS removal compared to extracts. In antioxidant activity, aqueous extract was found to be significantly higher than methanol extract. The ABTS radical scavenging activity of the extracts is given in Table 2 as % inhibition. The IC50 values of the BHT, aqueous extract and methanol extract of the seed for the DPPH assay were 6.83 ± 0.73 µg/mL, 7.24±0.14 µg/mL, 8.36±0.06 µg/mL (Fig. 5B). Aqueous extract (IC50 = 6.83 ± 0.73 µg/mL) showed the best activity in sea buckthorn seeds. In antioxidant activity, aqueous extract was found to be significantly higher than methanol extract. The lower the IC50 value, the higher the free radical scavenging power [25].

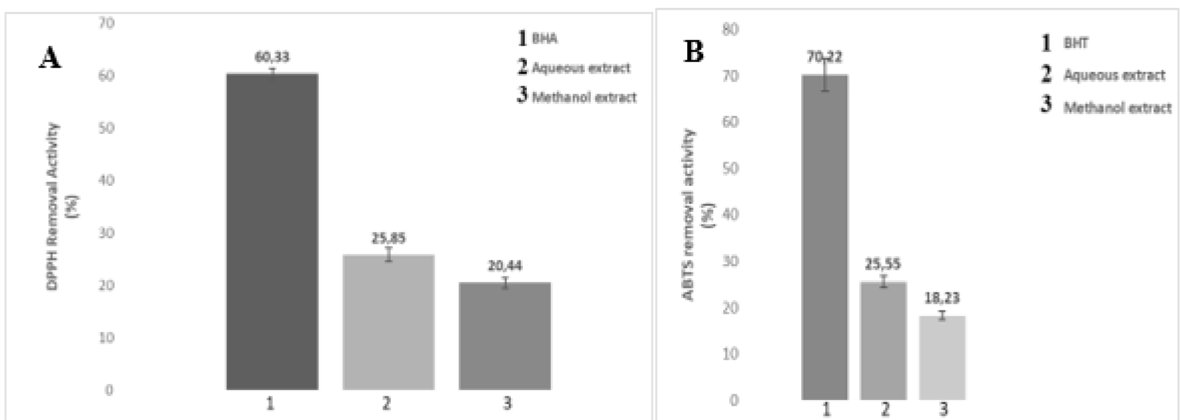


**Fig 4A** DPPH scavenging activity of aqueous and methanol extracts **B** ABTS scavenging activity of aqueous and methanol extracts

**Table 2** DPPH radical scavenging activity ($IC_{50}$), ABTS radical scavenging activity ($IC_{50}$) values and standard deviations of the extracts

| | **DPPH ($IC_{50}$)** | **ABTS ($IC_{50}$)** |
|---|---|---|
| **Aqueous extract** | 19.34±0.49 | 7.24±0.14 |
| **Methanol extract** | 22.24±1.01 | 8.36±0.06 |
| **BHA** | 17.03 ± 1.43 | - |
| **BHT** | - | 6.83 ± 0.73 |

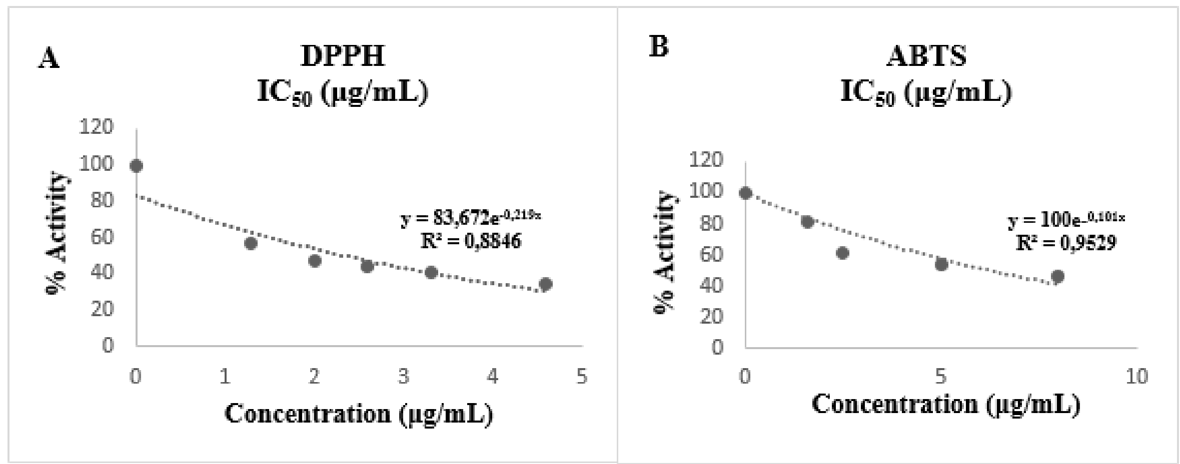


**Fig 5**A DPPH radical scavenging activity (IC50) B ABTS radical scavenging activity (IC50)

## Conclusion

This study clearly shows that sea buckthorn the aqueous extract of can be used as an alternative source of anti-urease and anti-collagenase source. Due to the important role of these enzymes in various diseases, new drugs with inducing or inhibitory effects should be developed. However, further studies are needed to prove the collagenase and urease enzyme inhibitions of these sea buckthorn extracts with in vivo experiments.

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**Research article**

Mercimek Takcı, H.A., et al., i*n vitro* Antibacterial, Antioxidant and DNA Damage Protective Activity *of* Blackberry (*Rubus fruticosus* L.*)* Root Extracts. International Journal of Life Sciences and Biotechnology, 2022. 5(2): p. 225-234. DOI: 10.38001/ijlsb.1085539

# *in vitro* Antibacterial, Antioxidant and DNA Damage Protective Activity *of* Blackberry (*Rubus fruticosus* L.*)* Root Extracts

**Hatice Aysun Mercimek Takcı[1]*, Sema Genç[2], Ayşenur Yalçın[3], Eda Özdemir[4]**

**ABSTRACT**
The aim of this study is to explore the antibacterial, antioxidant, and DNA damage protection potentials of blackberry (*Rubus fruticosus* L) root extracts. Antioxidant activity of root extracts was researched by using DPPH$^{\cdot}$ free radical scavenging and reducing capacity analysis. Methanolic extract of blackberry root system showed the maximum activity for TPC, TFC, DPPH$^{\cdot}$, and ferric reducing capacity. Antibacterial activity of blackberry root extracts was screened against clinic isolates (*Escherichia coli*, *Klebsiella* spp., *Proteus* spp., *Pseudomonas* spp. and *Staphylococcus aureus*) by the Kirby Bauer method. Although the metanolic extract possessed a significant antibacterial activity at 100 mg/mL concentration, distilled water extract did not show any inhibition to clinic isolates. The protective effects of root extracts on pBR322 plasmid DNA against the mutagenic effect of UV photolysis of $H_2O_2$ were tested. All concentrations of methanolic and distilled water extracts were observed to protect DNA damage in the presence of $H_2O_2$ and UV. These results indicated that the presence of antioxidant substances of root system extract of blackberry (*Rubus fruticosus* L) can be effective against harmful effects of free radicals.

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Antioxidant activity, antibacterial activity, blackberry, DNA damage protective, root extract

## Introduction

Blackberry (*Rubus fruticosus* L.) wild-grown and belongs to the genus *Rubus* of the Rosaceae family, is a perennial shrub [1, 2]. This bushy plant is lasting for three seasons or more. The fruit-bearing species of are naturalized throughout the world from sea level to heights up to 1100 [3]. Its edible fruits, known as blackberries and extensively used for the production of candy, dietary supplements, ice cream, jam, marmalade, and wine, are

[1] Department of Molecular Biology and Genetics, Faculty of Arts and Sciences, Kilis 7 Aralık University, Kilis, Turkey
[2] Institute of Science and Technology, Kilis 7 Aralık University, Kilis, Turkey.
[3] The Faculty of Medicine, Van Yuzuncu Yıl University, Van, Turkey
[4] Department of Molecular Biology and Genetics, Faculty of Arts and Sciences, Kilis 7 Aralık University, Kilis, Turkey
* Corresponding Author**:** Hatice Aysun Mercimek Takcı, E-mail: mersimek@hotmail.com

functional foods [2]. Blackberries including dietary fiber, vitamin C, vitamin K, and the essential mineral substances possess a high nutritional profile. The roots also contain saponins and tannins [4]. The gastroprotective and cardioprotective properties and pharmacological activities such as antioxidant, anti-carcinogenic, anti-inflammatory, antimicrobial, anti-diabetic, anti-diarrheal, and antiviral of these bioactive components in different parts of the plant are noted [1, 3]. Blackberry leaves and roots are traditionally used in herbal medicine as a regulatory remedy for menses, enteritis, chronic appendicitis, leukorrhea, diarrhea, dysentery, and anemia [4, 5]. Decoction of root bark and leaves being strongly astringent, depurative, diuretic and vulnerary are useful medicinally [6].

Despite the expressed activity of its various parts, very few published studies are on the biological activity of blackberry root extracts. Therefore, we aimed to screen phytochemical properties and pharmacological activities such as antioxidant, antimicrobial, and DNA damage protective of methanolic and aqueous root extracts of *R. fruticosus*.

## Materials and Methods

**Sample preparation**

*Rubus fruticosus* root parts were extracted by methanol and distilled water (1:10 (w/v)) at room temperature for 3 days under shaking conditions Following filtered by Wattman No. 4 paper, solvents were evaporated, and then samples were suspended in methanol at the 100 mg/mL final concentration. The extracts were kept at +4ºC for antioxidant, antimicrobial, DNA protective activity, and phytochemical analysis. Phytochemical and antioxidant analyses were continued by using 0.5, 1.0, 2.5, 5.0, and 10.0 mg/mL concentrations of blackberry root extracts.

**Total phenolic (TPC) and flavonoid content (TFC)**

For the total phenolic content in all samples, Folin-Ciocalteu colorimetric method was performed. The total phenolic content was expressed as mg of gallic acid equivalents (mg GAE)/g of extract using a standard curve [7]. The total flavonoid content of samples was determined by a method predicated by Sharm and Vig (2013) [8]. The **results were** calculated according to a standard curve prepared by using rutin and expressed as mg of rutin equivalent (mg RE)/g.

**Antioxidant activities of blackberry root extracts**

***DPPH˙ radical scavenging capacity***

Free radical scavenger method by declared Blois (1958) is based on turning colorless of the 1,1-diphenyl-2-picryl-hydrazil (DPPH) reagent solution depending on the electron or proton-transfer ability of samples [9]. For this analysis, 100 µL of the extracts was added to 3.9 mL of DPPH˙ in reagent solution prepared in methanol (0.1 mM). For allowing the chemical reaction, this mixture was incubated at room temperature in the dark for 120 min. Then incubation, the absorbance of the mixture was measured at 517 nm against methanol blank. The absorbance of control was determined by using 1 mL of methanol instead of the sample. DPPH˙ scavenging activity expressed as % inhibition was calculated by the following equation. Butylated hydroxytoluene (BHT) at 200-1000 µg/mL concentrations was used as a standard antioxidant substance.

$$\text{Inhibition } (\%) = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

***Reducing capacity assay***

In this assay, the reducing $Fe^{3+}$ to $Fe^{2+}$ capability of antioxidant substances in extracts was tested [10]. The absorbance of the Prussian blue color formed by adding $FeCl_3$ in the reaction mixture was measured. A high absorbance value is indicated a high reducing capacity of samples. In brief, blackberry root extract (1 mL) was mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium $K_3Fe(CN)_6$. This reaction solution was incubated at 50ºC for 20 min. To terminate the activity, 10% TCA was added and the mixture was centrifuged at 2500 rpm for 10 min. The equal volume of distilled water and 0.5 mL $FeCl_3$ (0.1%) were added to 2.5 mL of supernatant. The absorbance of the reaction mixture was measured at 700 nm. A beta-hydroxy acid (BHA) was evaluated as standard.

**Statistical analysis**

In order to determine significant differences between the samples, the software SPSS 22.0 for Windows (SPSS Inc., Chicago, IL, USA) was performed variance analysis (ANOVA) and Tukey multiple comparison tests. Each spectrophotometric analysis was repeated at least three times.

**Antibacterial activity**

The antibacterial activity of extracts was researched by using the Kirby-Bauer disk diffusion susceptibility test [11]. For this method, 5 clinical strains obtained from Kilis State Hospital (*E. coli*, *Klebsiella* spp., *Proteus* spp., *Pseudomonas* spp. and *S. aureus*) were tested. The density of cultures in growth Luria-Bertani (LB) broth for 24 h was adjusted to 0.5 McFarland turbidity. The antibacterial activity was performed on Mueller Hinton Agar. The sterile antimicrobial blank discs impregnated with 20 µL of the extracts were strategically placed away from each other. Methanol solvent and standard antibiotics were used as the negative and positive control, respectively. Following the incubation at 37ºC for 24 h, the clear zones around the discs were evaluated as antibacterial activity. Minimal Inhibition Concentration (MIC) of the extracts that showed antibacterial activity against test microorganisms was determined. This analysis was performed based on fact that the lowest inhibitory concentration is determined to be effective on test microorganisms. In this test, the 10, 25, 50, 75, and 100 mg/mL concentrations of extracts were investigated for their inhibitory effects against various microorganisms.

**DNA damage protective activity**

DNA damage inhibition activity of *Rubus fruticosus* root extracts was tested by using pBR322 plasmid DNA (vivantis). Oxidative DNA damage was induced by hydroxyl radicals generated from the ultraviolet (UV)/$H_2O_2$-radical system [12]. For this analysis, blackberry root extracts at different concentrations (50, 40, 20, 10, and 5 mg/mL) were prepared. The reaction mixture contained 3 µL pBR322 plasmid DNA, 5 µL of root extract, and 2 µL of 30% $H_2O_2$ in a microfuge tube. The tubes were UV irradiated at a wavelength of 230 nm using a UV lamp for 5 min at room temperature. The control tube contained only untreated DNA and DNA and $H_2O_2$ without root extract as an internal and negative control, respectively. Subsequently UV irradiation, all samples were analyzed by gel electrophoresis performed on 1% agarose gel.

## Result and Discussion

As can be seen in Table 1, the range of the total phenolic content (TPC) in blackberry root extracts was very extensive: from 0.02±0.00 and to 0.63±0.01 mg GAE/g. High TPC was

calculated as 0.63±0.01 mg GAE/g in the methanolic root extract at 10 mg/mL concentration.

**Table 1.** Total phenolic contents of blackberry root extracts (mg GAE/g)

| | **Methanol** | **Distilled water** |
|---|---|---|
| **0.5 mg/mL** | 0.14±0.01[e] | 0.02±0.00[e] |
| **1 mg/mL** | 0.24±0.00[d] | 0.02±0.00[d] |
| **2.5 mg/mL** | 0.50±0.01[c] | 0.05±0.00[c] |
| **5 mg/mL** | 0.59±0.01[b] | 0.12±0.00[b] |
| **10 mg/mL** | 0.63±0.01[a] | 0.22±0.00[a] |

*(The presented datas are mean of triplicate determinations (n=3), ± standard deviation. The difference between the values expressed by the different symbols in table (a-e) is significant ($p<0.05$)).

The lower TPC values ranging from 0.02±0.00 to 0.22±0.00 mg GAE/g were obtained from aqueous extracts. The highest total flavonoid content was identified in blackberry methanolic root extract, 0.37±0.02 mg RE/g (Table 2).

**Table 2.** Total flavonoid compounds of blackberry root extracts (mg RE/g)

| | **Methanol** | **Distilled water** |
|---|---|---|
| **0.5 mg/mL** | 0.01±0.00[d] | 0.00±0.00[c] |
| **1 mg/mL** | 0.02±0.00[d] | 0.00±0.00[c] |
| **2.5 mg/mL** | 0.05±0.00[c] | 0.01±0.00[bc] |
| **5 mg/mL** | 0.09±0.01[b] | 0.01±0.00[b] |
| **10 mg/mL** | 0.37±0.02[a] | 0.02±0.01[a] |

*(The presented datas are mean of triplicate determinations (n=3), ± standard deviation. The difference between the values expressed by the different symbols in table (a-c and a-d) is significant ($p<0.05$)).

The total flavonoid contents noted minimum in aqueous root extracts (ranging from 0.00±0.00 to 0.02±0.01 mg RE/g) stated in terms of rutin equivalents (RE). For both methanolic and deionized water extracts, TPC and TFC values in root extracts increased depending upon the increase in concentration. In this present study, all quantitative examinations of phytochemical analysis were found statistically significant ($P<0.05$). Četojević-Simin et al. (2017) predicated that the highest TPC and TFC values of blackberry extracts were 0.089±3.48 mg GAE/g and 0.045±2.16 mg RE/g [13]. Those are quite lower than our methanolic extract results. Whereas 79.1 mg GAE/g value of methanolic blackberry extract reported by Najda and Labuda (2013) [14] was dramatically higher than ours. For both methods applied as shown in Tables 3 and 4, the highest antioxidant activities were recorded in methanolic root extract at 10 mg/mL concentration. DPPH$^{\cdot}$ radical scavenging activity was evaluated compared to standard antioxidant BHT.

**Table 3.** Antioxidant activity (DPPH˙ scavenging) of blackberry root extracts (%)

| | **0.5 mg/mL** | **1 mg/mL** | **2.5 mg/mL** | **5 mg/mL** | **10 mg/mL** |
|---|---|---|---|---|---|
| **Methanol** | 81.25±1.91$^{c}$ | 87.12±0.38$^{b}$ | 88.46±0.06$^{ab}$ | 89.35±0.06$^{ab}$ | 89.60±0.06$^{a}$ |
| **Distilled water** | 48.85±0.13$^{b}$ | 50.83±0.19$^{b}$ | 54.97±5.74$^{b}$ | 78.13±3.00$^{a}$ | 85.08±0.26$^{a}$ |
| **Standard** | **200 mg/mL** | **400 mg/mL** | **600 mg/mL** | **800 mg/mL** | **1000 mg/mL** |
| **BHT** | 26.29±0.18$^{e}$ | 42.53±1.31$^{d}$ | 51.99±0.54$^{c}$ | 63.53±1.61$^{b}$ | 70.67±0.30$^{a}$ |

*(The presented data are mean of triplicate determinations (n=3), ± standard deviation. The difference between the values expressed by the different symbols in the table (a-b, a-c, and a-e) is significant (p<0.05)).

**Table 4.** Reducing Capacity of blackberry root extracts (absorbance)

| | **0.5 mg/mL** | **1 mg/mL** | **2.5 mg/mL** | **5 mg/mL** | **10 mg/mL** |
|---|---|---|---|---|---|
| **Methanol** | 0.21±0.01$^{e}$ | 0.44±0.03$^{d}$ | 0.83±0.04$^{c}$ | 1.02±0.05$^{b}$ | 1.18±0.02$^{a}$ |
| **Distilled water** | 0.03±0.00$^{d}$ | 0.04±0.00$^{d}$ | 0.12±0.02$^{c}$ | 0.22±0.01$^{b}$ | 0.51±0.01$^{a}$ |
| **Standard** | **20 mg/mL** | **50 mg/mL** | **100 mg/mL** | **200 mg/mL** | **400 mg/mL** |
| **BHA** | 0.22±0.01$^{c}$ | 0.24±0.01$^{c}$ | 0.25±0.02$^{c}$ | 0.32±0.03$^{b}$ | 0.51±0.01$^{a}$ |

*(The presented data are mean of triplicate determinations (n=3), ± standard deviation. The difference between the values expressed by the different symbols in the table (a-c, a-d, and a-e) is significant (p<0.05)).

According to results (Table 3), % inhibition values ranged from 48.85±0.13 to 89.60±0.06. Methanol root extract showed comparable % DPPH˙ activity with BHT. However, root methanol and distilled water extracts showed statistically significant antioxidant activity ($P<0.05$). % DPPH˙ removal activity (87.12±0.38%) value observed at 1000 µg/mL extract concentration was higher than this exhibited by BHT (70.67±0.30%). % DPPH˙ radical scavenging activity of distilled water extract at the same concentration was 50.83±0.19% and this value lower than BHT and methanolic extract. In terms of reducing capacity activity, BHA showed higher antioxidant activity than plant extracts. The tested extract revealed a variable value of reducing capacity activity (0.03±0.00-1.18±0.02). The highest reducing capacity among extracts was 1.18±0.02 and recorded for methanolic extract at 10 mg/mL concentration. But this value was rather lower than BHT showing a significant antioxidant activity (0.51±0.01) at 0.4 mg/mL concentration. On the other hand, 0.51±0.01 reducing capacity value for aqueous root extract could be calculated at 10 mg/mL concentration. The results obtained in our study can be discussed with other blackberry studies, Begam et al. (2018) noted as 82.42% and 0.25 abs. of the maximum DPPH radical scavenging and reducing power activity of blackberry ethanol extract, values lower than

our result [15]. Similar results were achieved by other researchers such as Stajčić et al. (2012) [16] who expressed that the DPPH˙ free radical scavenging activity of the extracts increased based on increasing concentration.

The antioxidant activity of the herbal extract is dependent on the solubility of phenolic substances in tested solvents. This is indicated that soluble phenolic compounds at a high amount are present in methanolic extract achieving high DPPH˙ radical scavenging and reducing capacity activities. Our results also expressed the effect of extraction solvents on antioxidant activity and phytochemical contents in bioactivity studies of plants.

The antibacterial activity of both extracts is represented on clinic strains isolated from patients in Table 5.

**Table 5.** MIC values of methanol extract (in terms of mm)

| | **Methanol extract** | | | | |
|---|---|---|---|---|---|
| | **100 mg/mL** | **75 mg/mL** | **50 mg/mL** | **25 mg/mL** | **10 mg/mL** |
| ***Pseudomonas* spp.** | 12.00±0.00 | 11.00±0.00 | 9.50±0.50 | -* | -* |
| ***Klebsiella* spp.** | 11.00±0.00 | 9.00±0.00 | 8.00±0.00 | -* | -* |
| ***Proteus* spp.** | 11.00±1.00 | 9.50±0.50 | 9.50±0.50 | -* | -* |
| ***S. aureus*** | 11.00±1.00 | 9.30±1.53 | 9.50±0.50 | -* | -* |
| ***E. coli*** | 8.50±0.50 | 8.00±0.00 | 6.50±0.50 | -* | -* |

-*: Any inhibition zone was not observed on MHA plates.

Distilled water root extract demonstrated no antibacterial activity against test bacteria. Negative control (methanol) showed no inhibitory effect on strains. Methanolic extract of *R. fruticosus* roots exhibited any inhibition at minimum extract level (10 and 25 mg/mL) tested against clinic strains but had an antibacterial activity of the other concentrations. The methanolic root extract of *R. fruticosus* was active to test bacteria with average zone diameters ranging from 6.50±0.50 to 12.00±0.00 mm. Methanolic root extract was given a greater zone of inhibition (12.00±0.00 mm) to *Pseudomonas* spp. This extract showed the lowest inhibition with 6.50±0.50 and 8.50±0.50 mm zone diameters against *E. coli* for all concentrations performed. Inhibition diameters of positive controls were ranging to 25±0.00 mm for Polymyxin B, 27.5±0.05 mm for Methicilin, and 16.5±0.05 mm for Tetracycline tested on *Pseudomonas* spp., *S. aureus,* and *Klebsiella* spp., respectively. The inhibitory effect recorded on *E.coli* was 9.00±0.00 mm for Tetracycline. These zones had

the most dramatic effect compared to methanolic root extracts. But the antibacterial effect of methanolic root extract observed against *Proteus* spp. (11.00±1.00 mm) had higher than that of standard tetracycline applied (9.00±0.00 mm). So, the remarkable antibacterial activity of methanolic root extract was observed on *Proteus* spp. MIC for *R. fruticosus* was observed starting at 50 mg/mL concentration against tested all clinic strains. Similar results were acquired by Yigit and Yigit (2014) who stated that the methanol extract of blackberry leaves had antibacterial potential on *S. aureus*, *Escherichia coli, Proteus mirabilis,* and *Pseudomonas aeruginosa* [17]. Similar to our result, Riaz et al. (2011) [6] declared that blackberry root extract showed comparable antibacterial activity to the standard Ampicillin used at micron dose level.

For DNA damage protective analysis, blackberry roots dried after evaporation of methanol were used by suspending with distilled water to the preparation of concentrations needed. The electrophoretic pattern of pBR322 run following UV-photolysis of $H_2O_2$ in presence of different concentrations of root extracts is represented in Figure 1. Three sharp bright bands having different molecular weights belongs to the untreated pBR322 plasmid DNA digested with Alu I, Bsn I, and Hind III enzyme showed on agarose gel electrophoresis (lane A1). OH produced after UV photolysis of $H_2O_2$ without extract is caused the breakage of DNA strand and smears are ascertained on the gel in lane A2. As can be shown in Fig. 1, methanolic and distilled root extracts at tested all concentrations were revealed to protect all of the bands in the presence of $H_2O_2$ and UV. Protective effect seen against mutagenicity of UV-photolysis of $H_2O_2$ on pBR322 plasmid DNA is associated with the highest phytochemical content of the extract. Similarly, the DNA protective activity of blackberry extracts against ultraviolet-B (UVB) radiation was declared by other studies [18]. Our findings revealed that natural antioxidant resources such as *Rubus fruticosus* L. extracts have the efficiency to preserve DNA from adverse effects of UV radiations.

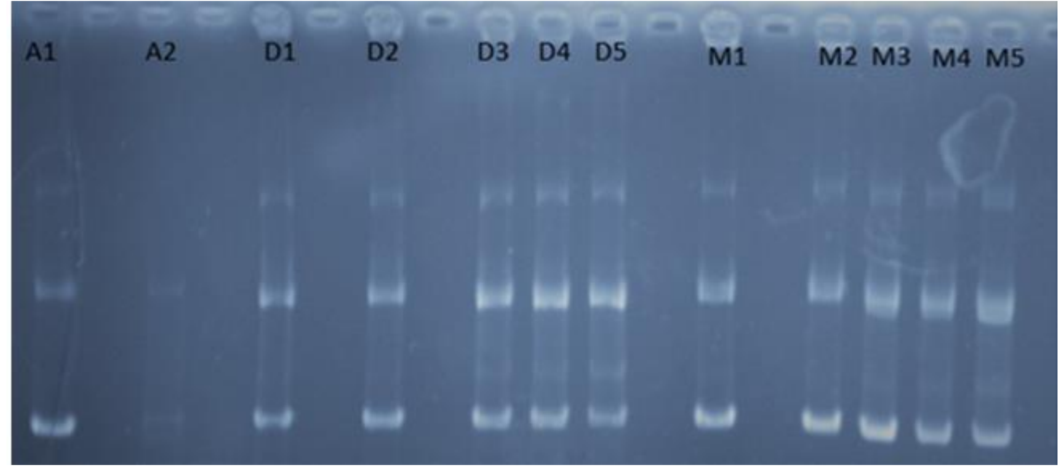


**Fig 1** DNA protective activity of the *Rubus fruticosus* L root extracts
A1 lane: pBR322 plasmid DNA+d$H_2O$, A2 lane: pBR322 plasmid DNA+d$H_2O$+ 5 min. UV+1 µl $H_2O_2$, D1 lane: pBR322 plasmid DNA + distilled water root extract (50 mg/mL) + 5 min. UV+ 1 µl $H_2O_2$, D2 lane: pBR322 plasmid DNA + distilled water root extract (40 mg/mL) + 5 min. UV+ 1 µl $H_2O_2$, D3 lane: pBR322 plasmid DNA + distilled water root extract (20 mg/mL) + 5 min. UV+ 1 µl $H_2O_2$, D4 lane: pBR322 plasmid DNA + distilled water root extract (10 mg/mL) + 5 min. UV+ 1 µl $H_2O_2$, D5 lane: pBR322 plasmid DNA + distilled water root extract (5 mg/mL) + 5 min. UV+ 1 µl $H_2O_2$, M1 lane: pBR322 plasmid DNA + methanolic root extract (50 mg/mL) + 5 min. UV+ 1 µl $H_2O_2$, M2 lane: pBR322 plasmid DNA + methanolic root extract (40 mg/mL) + 5 min. UV+ 1 µl $H_2O_2$, M3 lane: pBR322 plasmid DNA + methanolic root extract (20 mg/mL) + 5 min. UV+ 1 µl $H_2O_2$, M4 lane: pBR322 plasmid DNA + methanolic root extract (10 mg/mL) + 5 min. UV+ 1 µl $H_2O_2$, M5 lane: pBR322 plasmid DNA + methanolic root extract (5 mg/mL) + 5 min. UV+ 1 µl $H_2O_2$

## Conclusion

Recently, major researchers have exhibited the biological activity potential of substances obtained by extracting different parts of various plant spices. So, we screened phytochemical properties and pharmacological activities such as antioxidant, antimicrobial, and DNA damage protective of methanolic and aqueous root extracts of *R. fruticosus.* By this study, the methanolic extract exhibited higher activity than those of the standard, BHT and tetracycline for antioxidant and antibacterial analysis. Especially, it was revealed that both extracts possessed significant protective activity of root extracts against UV/$H_2O_2$ at all tested concentrations in the DNA damage protective test system. Now, the next step is to clarify the chemical structures of health-beneficial bioactive compounds in methanolic root extracts

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**Technical Note**

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# An *In-silico* analysis of the molecular interactions between PmCBP-VP24 and PmCBP-VP28 protein complex to understand the initial initiating events of shrimp WSSV infection

**Sk Injamamul Islam[1*], Moslema Jahan Mou[2], Saloa Sanjida[3], Sarower Mahfuj[1], Md. Ashraful Alam[4], Yeasmin Ara[5]**

**ABSTRACT**

The White Spot Syndrome Virus (WSSV) has been the most pathogenic in the shrimp and other crustacean industries across the world in terms of production and financial benefits. Invertebrate vectors, freshwater, and sediments spread it horizontally as well as vertically from diseased broodstock to post-larvae. It is an exceedingly contagious disease that may cause entire mortality within 3–10 days of an outbreak under standard culture conditions. The sequencing and characterization of several WSSV strains have begun to reveal information regarding pathogen biology, pathogenicity, and WSSV must bind to the shrimp digestive system to infect it, and failure to do so results in ineffective infection. The *Penaeus monodon* chitin-binding protein (*Pm*CBP), as well as the viral envelope proteins VP24 and VP28, are required for viruses to bind to the shrimp digestive system. In this study, we have shown the molecular interactions between PmCBP-VP24 and *Pm*CBP-VP28 complex and speculated about the first steps of virus ingression in shrimps for the first time.

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## Introduction

In cultured penaeid shrimp, White Spot Syndrome Virus (WSSV) causes white spot disease (WSD). WSSV is more likely to infect penaeid shrimps like *Litopenaeus vannamei*, *Penaeus*

[1] Department of Fisheries and Marine Bioscience, Faculty of Biological Science, Jashore University of Science and Technology, Jashore-7408, Bangladesh

[2] Department of Genetic Engineering and Biotechnology, Faculty of Earth and Life Science, University of Rajshahi, Bangladesh

[3] Department of Environmental Science and Technology, Faculty of Applied Science and Technology, Jashore University of Science and Technology, Jashore-7408, Bangladesh

[4] Faculty, Rural Development Academy (RDA)-5842, Bangladesh

[5] Department of Fisheries Management, Hajee Mohammad Danesh Science and Technology University, Dinajpur

*Corresponding author: 6378506331@student.chula.ac.th

*monodon*, *Marsupenaeus japonicus*, and *Fenneropenaeus indicus*. WSSV is a big double-stranded DNA baculovirus, and infection with the pathogen in shrimp farming can result in up to 100% mortality in 3 to 10 days [1]. The white dots on the carapace of shrimp infected with WSSV are immediately identifiable [2]. The initial symptoms, on the other hand, arise after the animal has been infected and is near death. As a result, diagnosis methods have evolved from morphological-based characterization using electron microscopy (EM) to exceedingly effective immunological and molecular methods that previously could only detect the virus in asymptomatic carriers [3]. Given the severity of WSD in captive crustaceans, it is hardly unexpected that a lot of effort has gone into figuring out the disease's basic causes and finding potential remedies for disease control or mitigation[4]. Regardless of the diverse means adopted at present (i.e., environmental control, pre-exposure of shrimp to the pathogens, herbal treatments, DNA/RNA-based vaccines), there is no absolute drug or antiviral that can prevent the ingress of the virus. The main disadvantage of the illness is that its mechanism, which includes ingression, growth, and dispersion of the virus within the host, must be thoroughly understood [5]. The cannibalistic nature of shrimp results in infecting a healthy shrimp with WSSV. As a result, WSSV infection occurs when a normal shrimp feeds a WSSV-infected live/dead shrimp. The main site from which shrimp become infected is the lining of their digestive tube. Generally, chitinous linings line the esophagus, stomach, and hindgut while semipermeable peritrophic membranes (PM) line the midgut epithelium [5]. Chitin fibrils are embedded in a collage of proteins, proteoglycans, and mucopolysaccharides to form this PM. Several host cell membranes, particularly the cell membranes of epithelial cells, have receptors that can interact with virus envelope proteins and facilitate virus entry. An often-studied protein among all receptors in the chitin-binding protein *PmCBP* (CBP in *Penaeus monodon*). The interaction of yeast two hybrids with *PmCBP* has been studied in vitro using yeast two hybrids. VP24 and VP28 are two of the most significant proteins in this cluster of at least 11 viral envelope proteins associated with *PmCBP* [6, 7]. Furthermore, a unique complex protein aggregation (dubbed 'infectome') was discovered, which was made up of proteins such as VP24, VP28, VP31, VP32, VP39B, VP53A, and VP56 [7]. By being linked with CBP, this complex performed a critical function in facilitating viral percolation over the basal membrane of the alimentary canal. Furthermore, the chitin-binding assays have

revealed the association between infectomes and CBP is facilitated by VP24, VP28, and *PmCBP* [8]. The viral envelope protein VP24 protrudes outside from the viral envelope and this feature additionally facilitates its interaction with CBP [9, 10]. A critical factor for anchoring the viral particle to the inner lining of the shrimp alimentary canal is the interaction between chitin and VP24 within the stipulated period along with the association with VP28. Ineffective attachment advancement can lead to infection failure if this interaction is hampered. In this regard, the present study aims to use in-silico approaches to explore the molecular interactions between virus envelope proteins VP24, VP28, and shrimp receptor protein *PmCBP*. To accomplish this, 3D structures of *PmCBP* were designed using molecular modeling. To understand the amino acid interactions occurring within VP24-*PmCBP* and VP28-*PmCBP* complexes, the docked 3D structure of *PmCBP* was subjected to molecular docking and dynamics approaches. *The pmCBP-VP24* complex is vital while the PmCBP-VP24 complex also aids in the entry of the virus into shrimp bodies. In one *in-silico* based study researchers predicted the interactions among the VP24-PmCBP complex [11], however, the interaction of *PmCBP* with other virion proteins particularly with VP28 has not been studied yet. Moreover, the researchers only predict the binding interaction of VP24 proteins short amino acid sequence(186-200) with PmCBP [11]. Based on their study it is very difficult to conclude the initial instigation of WSSV. Because the three-dimensional structure of the VP24 has many active binding sites. Therefore, our study aimed to predict the interaction between PmCBP-VP24 and PmCBP-VP28 complex to provide information regarding the shrimp WSSV virus. Also, it is needed to understand the other virion protein's role with PmCBP protein to better analyze the WSSV initial action on the shrimp body. Thus, the present study provides a glimpse into the initial infection and the ingression of the virus within the shrimp body. Furthermore, this study may be used to search for inhibitors that can interfere with PmCBP's activity in forming complexes with VP24 and VP28 proteins, thereby inhibiting virus entry into shrimp.

## Material and Methods

### Sequence Analysis of PmCBP Protein

The *PmCBP* protein sequence was retrieved through a review of the literature [10]. The ProtParam program was used to calculate primary structure specifications for *PmCBP* protein, including molecular weight, theoretical pI, atomic composition, extinction coefficient, estimated half-life, aliphatic index, and grand average of hydropathicity (GRAVY) [12]. The DiANNA tool was utilized to gain a more detailed understanding of the protein's secondary structure. The DiANNA tool is a neural network program that gives information about disulfide connectivity and protein fold stability. *PmCBP* protein disulfide bonds were accessed using the tool [13].

### Preparation of Molecules PmCBP, VP24, and VP28

Homology modeling was used to predict the three-dimensional structure of *PmCBP* employing BlastP and Protein Data Bank. Because an adequate template could not be found using BlastP, we used a de novo modeling strategy. Therefore, the three-dimensional structure of the target PmCBP protein was predicted using the Raptorx server (http://raptorx.uchicago.edu/) [14]. RaptorX is a protein structure prediction server developed by the Xu group, excelling at predicting 3D structures for protein sequences without close homologs in the Protein Data Bank (PDB). Given an input sequence, RaptorX predicts its secondary and tertiary structures as well as solvent accessibility and disordered regions. RaptorX also assigns the following confidence scores to indicate the quality of a predicted 3D model: P-value for the relative global quality, GDT (global distance test) and uGDT (un-normalized GDT) for the absolute global quality, and RMSD for the absolute local quality of each residue in the model. RaptorX-Binding is a web server that predicts the binding sites of a protein sequence, based upon the predicted 3D model by RaptorX. On the other hand, the 3D structure of VP24 was also predicted using the RaptorX server while the VP28 protein structure retrieves from Protein Data Bank (PDB) (2ED6). The models generated were visually analyzed using Pymol (Lill and Danielson, 2011). Although the VP24 has the 3D structure available in Protein Data Bank (PDB ID: 5HLJ), however the structure contains many other functional molecules. So, according to the objective of our

study we decided to modelled the 3D structure of VP24 protein by RaptorX server to avoid the expanded ligand-protein interaction.

### Molecular Docking and Simulation Studies

The molecular docking and simulation studies were carried out using Cluspro 2.0 web server. The algorithm running behind Cluspro 2.0 server is very robust and does not require any prior information regarding either template or binding site between the proteins. The online service can be accessed at (https://cluspro.bu.edu/login.php). The server provides a simple home page for basic use, requiring only two files in Protein Data Bank (PDB) format. However, ClusPro also offers several advanced options to modify the search; these include the removal of unstructured protein regions, application of attraction or repulsion, accounting for pairwise distance restraints, construction of homo-multimers, consideration of small-angle X-ray scattering (SAXS) data, and location of heparin-binding sites. Six different energy functions can be used, depending on the type of protein. Docking with each energy parameter set results in ten models defined by centers of highly populated clusters of low-energy docked structures [15]. The final docked PmCBP-VP24 and PmCPBP-VP28 complex were visualized in Pymol and Discovery studio. Further, the amino acid interactions occurring between protein PmCBP-VP24 and PMCBP-VP28 were tabulated using PDBsum [16]. The binding energy of the PmCBP-VP24 and PmCBP-VP28 complex was evaluated using the HawkDock server [17]. The server employs molecular mechanics Poisson–Boltzmann surface area (MM-PBSA) to estimate interaction free energies between various protein-protein complexes [17].

## Results

### Sequence Analysis of Protein PmCBP

In the study, we performed sequence analysis of *PmCBP* using ProtParam, which revealed that *PmCBP* was made of 168 amino acids. The estimated molecular weight of *PmCBP* is 17471.11 Da. The total negatively charged residues and positively charged residues are 8 and 18 respectively. The Aliphatic index of the protein is 56.43 suggesting that it is a soluble protein. The Grand average of hydropathy (GRAVY) is - 0.114 suggesting that protein is

hydrophilic. DiANNA tool predicted that the protein contained 3 disulfide linkages, which provided extracellular stability to the PmCBP (Table1).

**Table 1** Physiochemical features of *PmCBP* protein from different tools and server

| **ProtParam tool** | **Values** |
|---|---|
| Number of AA | 168 |
| MW | 17471.11 Da |
| pI | 4.45 |
| Extinction coefficients | 7825 |
| Instability index | 50.04 |
| Aliphatic index | 56.43 |
| GRAVY | -0.114 |
| Total number of negatively charged residues (Asp + Glu) | 18 |
| Total number of positively charged residues (Arg + Lys) | 8 |
| Formula | $C_{775}H_{1140}N_{198}O_{253}S_6$ |
| Total number of atoms | 2372 |

**Table 2** DIANA predict cysteine residues important in disulfide bonding

| **DIANA** | | |
|---|---|---|
| **Predicted bonds** | **Distance** | **Bonded cysteine** |
| 86 - 126 | 40 | STSFACLDRPY - QYSFLCGEGSR |
| 101 - 139 | 38 | DEENSCHIFHI - QKELTCVAESE |
| 107 - 148 | 41 | HIFHICYPALF - SEAIPCQESSN |

**Preparation of Molecules PmCBP, VP24, and VP28**

The proportions of helix content, beta-sheet content, coil content, and overall confidence value were 0 %, 1 %, 18 %, 82 %, and 72.5%, respectively, according to the PROTEUS Structure Prediction Server 2.0 study. The final 3D structure of PmCBP was designed by the RaptorX server and out of 5 models we chose Model-1. Since model 1 depicted maximum RMSD score and 90.48% of the PmCBP residues show average 3D-1D score ≥ 0.2 as per Verify3D results, therefore, it was finally selected as the best model for PmCBP (Figure 1). As predicted by the CASTp v.3.0 algorithm, the VP24 protein modeled contains 32 unique active sites (Figure 3). CASTp is a database server that can recognize regions on proteins, determine their boundaries, compute the area of the areas, and calculate the dimensions of

the areas. Vacuums concealed within proteins and pockets on protein surfaces are also involved.

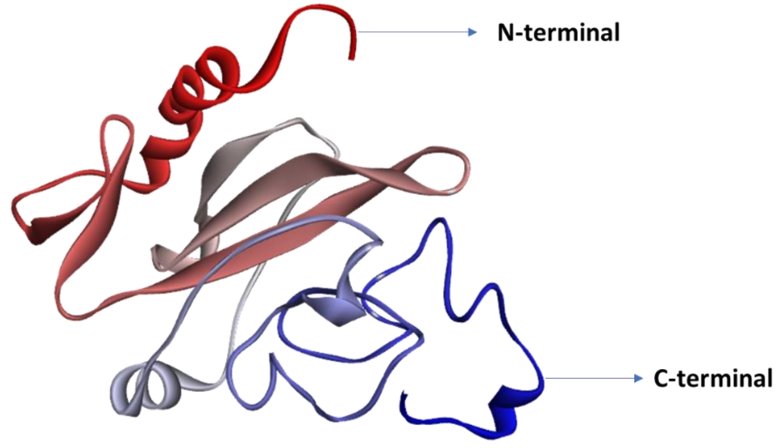


**Fig 1** 3D structure of PmCBP

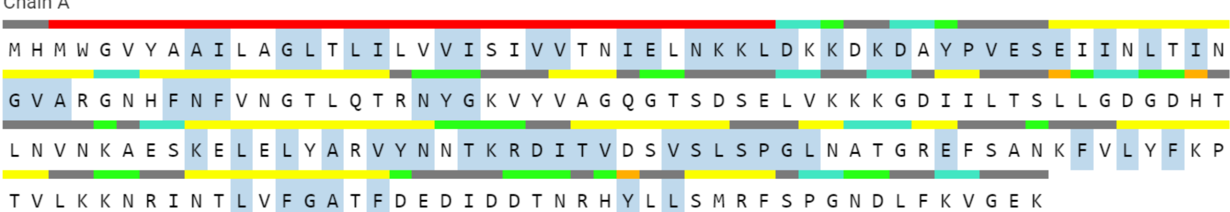


**Fig 2** The active location of the VP24 protein. The active site of amino acid residues (Blue color)

## Molecular Docking and Simulation Studies

The docking prediction performed by Cluspro 2.0 server result showed 10 models for each of the docked complexes. Model 1 (Figure 3 (A)) is considered to be the most probable model of *PmCBP*-VP24 complex because it depicted RMSD (root-mean-square deviation) value 2.48 Å whereas Model 7 (Figure 3 (B)) is considered to be the most probable model of *PmCBP*-VP28 complex which depicted RMSD value 2.53 Å. After the selection of these appropriate models, a detailed investigation was done into the amino acid sequences that interact between the protein *PmCBP*-VP24 and *PmCBP*-VP28 complex via the PDBsum web server. The amino acid interactions revealed that the PmCBP-VP24 complex is stabilized by 4 hydrogen bonds (Figure 3(A)). Within the PmCBP-VP24 complex, Ser81, Asn99, Gly48,

and Ser81 of *PmCBP* respectively interact with Asn27, Thr26, Gly103, and Thr26 of VP24 through H-bonds (Figure 3(A)). On the other hand, the amino acid interactions revealed that the *PmCBP*-VP28 complex is stabilized with 12 H-bonds. Within the PmCBP-VP28 complex, Asn51, Asn51, Asp49, Glu50, Glu50, Glu50, Asp75, Ile37, Thr41, Lys147, Ser152 and Ser153 of *PmCBP* respectively interact with His195, Thr197, Asn47, Thr46, Gly200, Thr201, Arg130, His40, His40, Glu109, Thr155 and Ser153 (Figure 3 (B)). The binding free energy of complex *PmCBP*-VP24 and *PmCBP*-VP28 was evaluated by the HawkDock server and found to be - 45.04 (kcal/mol) and -48.07 (Kcal/mol) proving that the complex is stable. Additionally, some disulfide interactions also contribute towards the stability of the *PmCBP*-VP24 complex (Figure. 3 (A, B)).

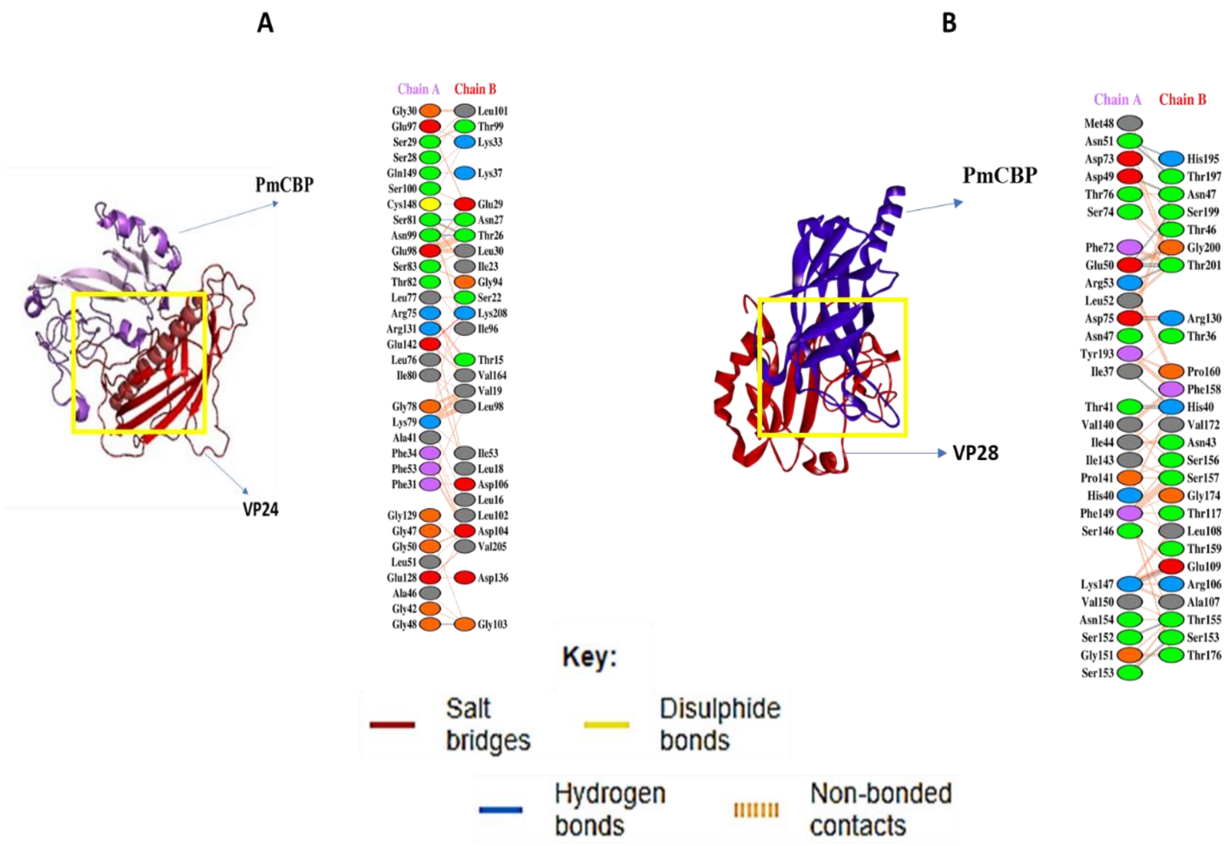


**Fig 3** Protein-Protein interaction between (A) *PmCBP*-VP24 protein complex and (B) *PmCBP*-VP28 protein complex

## Discussion

Protein-protein interactions are important to understanding the cellular function and organization of pathogens [15]. WSSV is primarily composed of its envelope and the proteins that are found within that envelop, which come in direct contact with shrimp digestive membranes and play a crucial role in the disease's propagation. WSSV envelope proteins were also considered capable of forming a protein complex dubbed an 'infectome'[18]. VP28 and VP24 are the most prevalent WSSV viral envelope proteins [19]. Previously, many studies revealed the role of *Penaeus monodon* Rab7 and chitin-binding protein-virion protein complex in WSSV infection through wet lab analysis [18, 20, 21]. Bioinformatics analysis showed stable interactions between VP24-PmCBP [11]. In their study, they only chose the amino acid sequence of VP24 from 186 to 200 to see the interaction with the PmCBP [11]. However, the interaction of the whole protein structure of VP24 with PmCBP protein needs to be brought into the light to better understand the initial instigation of WSSV infection. Moreover, the role and effect of VP28-PmCBP protein also can be the factor to analyze the initial infection of WSSV in shrimp.

To initiate infection with WSSV, dead or infected shrimp (viral particles) must be anchored properly to the shrimp alimentary canal's inner membrane. The virus traverses the shrimp alimentary canal to reach the basal membrane after accurate binding of its proteins with the shrimp receptor proteins that line the alimentary canal [5]. Upon reaching the basal membrane, the virion particles ooze into the alimentary canal before becoming plasma and eventually affecting the target organs such as eyestalks, cells of the cardiovascular system, brain, and reproductive organs such as gonads [22]. Individual shrimps become infected by the virus when it enters their nucleus and replicates itself to spread in the body of individual shrimps, resulting in their death [23]. Thus, the virus ingresses into the shrimp body when its receptor proteins make complexes with the viral envelope protein, which then anchors the virus to the shrimp. A vital role in disease initiation in shrimp is played by *PmCBP*-VP24 and *PmCBP*-VP28, which are important protein complexes in the process. Despite having no structure for *PmCBP*, we designed it by in-silico approaches. In addition, VP24 and VP28 are believed to form the envelope protein complex, the so-called 'infectome', which plays a critical role in recognizing host cells, anchoring to them, and directing WSSV into the host

cells [7]. Recent reports have shown that VP24 and VP28 interact with shrimp chitin receptors, but the exact role and molecular mechanisms involved remain unclear [24, 25]. Additionally, VP24 has been proposed as a potential monomertrimer transformation during the WSSV ingression mechanism in shrimp [9]. Therefore, when the virus enters the shrimp body, VP24 converts into its monomeric form and attaches itself to *PmCBP's* surface when its β9 is exposed. Using this PmCBP-VP24 and PmCBP-VP28 complex, we discovered that the complexes are stabilized by hydrogen bonds and disulfide interactions for the first time. The H bonds located between Ser81, Asn99, Gly48, and Ser81 of *PmCBP* respectively with Asn27, Thr26, Gly103, and Thr26 of VP24, and these bonds might play a crucial role in adhering VP24 to *PmCBP* but they have to be further validated experimentally. Similarly, all the 12-hydrogen bonds between *PmCBP*-VP28 complexes also proved stable relations among them and this needs to be further researched as well. VP24 facilitates the attachment of VP28 to the host cell, thereby promoting membrane fusion to promote the viral infection after the formation of *PmCBP*-VP24 in the shrimp. VP28 attached to VP28 and *PmCBP* forms *PmCBP*-VP24, thereby promoting anchoring of WSSV to the shrimp's alimentary canal [9]. The infective amino acid residues found sandwiched between the complexes *PmCBP*-VP24 and *PmCBP*-VP28 in this work might be crucial for shrimp-viral interaction at the cellular level. In this way, disruption of the bonding between these amino acids could have a detrimental effect on *PmCBP*-VP24 and *PmCBP*-VP28 formation, thereby preventing the cascade of events that involve viral structural proteins, especially VP24 and VP28. This would lead to a slowed spreading of the virus throughout the body since the virus cannot transit seamlessly across the digestive tract. Given the importance of the complexes associated with WSSV ingression into shrimp, we will try to predict a potential inhibitor in our future studies that can disrupt the amino acid interactions in the complexes. If such inhibitors behave in a plausible antiviral manner in the wet lab, then they could be administered as a shrimp feed to disrupt the initial instigation process of the virus in shrimps, thus resulting in disease mitigation.

## Conclusion

WSSV genome and structure have been extensively studied, but information about how WSSV infects cells is limited. The purpose of this study was to establish the biological significance of the protein-protein interaction between WSSV envelope protein (VP24 and VP28) and *Penaeus monodon* chitin-binding protein (*PmCBP*). The WSSV needs to bind to shrimp digestive tracts that *PmCBP* interacts with the viral envelope proteins, and failure to do so results in ineffective infection. Therefore, we found stable binding interactions between two major WSSV envelope proteins and *PmCBP*, and thus this study can play a vital role in producing WSSV drug inhibitors or vaccines in the future.

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**Review article**

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# Assessment of Food Safety During Covid-19 Pandemic

**Cennet Pelin Boyaci Gunduz[1]*, Mehmet Fatih Cengiz[2]**

**ABSTRACT**
SARS-CoV-2, a novel Coronavirus that causes COVID-19 disease and the World Health Organization (WHO) declared COVID-19 as a pandemic on March 11, 2020. Until now, foodborne or waterborne exposure to this virus has not been reported as the transmission route. However, the infected individual in the food production and service facility and, contaminated surfaces, may serve as the source of transmission route since Coronavirus can survive on the inanimate surfaces. Based on the available data, we reviewed the persistence of Coronaviruses on inanimate surfaces in the context of the food contact materials. Coronavirus persists on stainless steel, plastic and glass surfaces for a few days which are commonly used in food production and processing facilities. Therefore, appropriate food contact materials having fewer risk levels can be preferred. Additionally, using biocidal surfaces could help reduce the incidence of infections spread due to touching contaminated surfaces. In other parts of this review, appropriate inactivation procedures and ongoing food handling practices were explained. For prevention of virus transfer due to the contamination of food packaging material and also, food-handling by an infected person through food processing and serving, ongoing hygiene practices in food facilities should continue and inactivation procedures should be widened by taking into consideration the human Coronavirus and also, other foodborne viruses which have distinct properties compared to bacteria. Last of all, pandemics have impacts on the food supply chains, especially during harvest and logistics. Therefore, it is important to continue production and processing by raising awareness about food safety to ensure people in the food supply chain are not at risk of transmission.

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## Introduction

Food and water are essential requirements for the survival of living beings. However, due to both natural and human-based processes, an array of contaminants find their way into food and water through multiple routes and contamination of drinking water and foods consumed by people is a global food safety issue [1, 2]. Food products undergo various stages during production, processing, packaging and transportation and each stage could be a potential source of contaminants of biological, chemical and physical origins [3]. If food is contaminated with pathogenic microorganisms or their toxins or

[1] Adana Alparslan Turkes Science and Technology University, Faculty of Engineering, Food Engineering Department, 01250, Adana, Türkiye
[2] Akdeniz University, Faculty of Agriculture, Department of Agricultural Biotechnology, 07058, Antalya, Türkiye
*Corresponding Author: Cennet Pelin Boyaci Gunduz, e-mail: cennetpelinboyaci@gmail.com

chemical contaminants, it is a significant problem due to causing transmission or onset of diseases [4].

Chemical contaminants in food and water are substances that can lead to disease or injury when consumed at high concentrations. Natural toxic compounds produced by plants or marine organisms, mycotoxins, food additives, environmental contaminants, pesticides and veterinary drug residues, thermal process contaminants such as acrylamide, furan, 3-monochloropropanediol (3-MCPD) and migrants from packaging materials can be categorized in that group [2, 5, 6].

Biological hazards are the microorganisms such as bacteria, yeasts, molds, viruses and parasites. Some microorganisms including many bacteria, yeast and mold species are considered safe and used in the production of fermented foods and food ingredients due to their beneficial and functional effects [7]. On the other hand, some microbial species can cause foodborne diseases or food spoilage [8]. Microbial foodborne diseases can result in either infection by a pathogen itself or intoxications by toxins or toxic metabolites of microorganisms [4]. Most foodborne illnesses are infections caused by a variety of bacteria, viruses and parasites [9]. Pathogens cause a great number of foodborne and waterborne illness outbreaks with significant impacts on human health and also, economy [10]. Therefore, they have a noteworthy concern in the food industry.

Viruses are very different than other foodborne bacterial pathogens since they have distinct properties. Viruses are not free-living organisms and need an appropriate host for multiplication. Therefore, they only reproduce in the living cells of humans, other animals, plants and bacteria [11]. Viruses cannot replicate in foods, but they can be present; hence, they do not require food, water or air for their survival [4]. Unlike bacteria, viruses can not multiply or produce toxins in food. Therefore, foods only act as vehicles for their transfer [12]. Viruses could persist a long time as infectious particles in the environment or foods [13]. Food and water contamination can occur during different stages of the food production chain. Food may be intrinsically contaminated at any stage of pre-harvest, harvest and post-harvest production or contaminated if handled by infected food handlers under a food handling environment with poor sanitation conditions [14]. On the other hand, the transmission of zoonotic viruses could occur through the consumption of animal-origin products contaminated with viruses [15].

Viruses with a lipid envelope could be easily inactivated [16]. But viruses that do not contain a lipid envelope are more resistant and stable to even extreme conditions for a long time in foods or the environment. In food processing, a virus could resist some of the food processing techniques which destroy bacterial pathogens. Therefore, if food or water is contaminated with viruses, they could survive in foods, on food contact surfaces and on hands for extended periods [11].

Enteric viruses are an important food safety concern [11]. European Food Safety Authority (EFSA) reported that viruses accounted for 9.2% of total foodborne and waterborne outbreaks in 2015 [17]. In foodborne infections, the most frequently involved viruses human Norovirus (NoV) and Hepatitis A virus (HAV), but the other viruses such as Human Rotavirus (HRV), Hepatitis E virus (HEV), Astrovirus (AstV), Enterovirus(EV), Sapovirus, Aichivirus (AiV), Parvovirus, Coronavirus and Human Adenovirus (HAdV) could also transmit by food [12, 18-20]. Among them, NoV has been recognized as one of the most common causes of foodborne gastroenteritis worldwide [21]. NoV and Hepatitis A virus are very infectious viruses and human-to-human spread is the most common transmission route and the secondary transmission route of these viruses is through foodborne contamination [13]. Food handling is an important route of transmission. In addition, foods consumed raw or undercooked are considered at greatest risk of causing enteric viral diseases since viral foodborne outbreaks have been associated mainly with contaminated foods served and eaten raw or uncooked such as shellfish [22], fruits and vegetables [23]. Contaminated water, fruits and vegetables, shellfish, and food-handling are transmission routes of NoV. Foods commonly involved in Hepatitis A outbreaks were contaminated shellfish, fruits, vegetables, salads, dairy products, reconstituted frozen orange juice and raw or not fully cooked foods [24]. Hepatitis E outbreaks were associated with the consumption of contaminated meat and shellfish. The consumption of contaminated meat from infected animals, contaminated shellfish and vegetables has been associated with Rotavirus [25, 26]. Different from foodborne gastrointestinal viruses that cause illness through contaminated food, highly pathogenic human Coronaviruses caused important outbreaks in the world [27].

The novel Coronavirus has recently emerged as the third highly pathogenic human Coronavirus and named as the severe acute respiratory syndrome Coronavirus-2

(SARS-CoV-2) causing COVID-19 disease [28]. This review aims to discuss effects of COVID-19 disease on food safety in the light of current knowledge.

## SARS-COV-2 Virus

Coronaviruses are members of *Coronavirinae* subfamily in the *Coronaviridae* family [29]. Coronaviruses are enveloped, pleomorphic or spherical particles, including single-stranded (positive-sense) RNA associated with a nucleoprotein within a capsid comprised of matrix protein [30]. Coronaviruses are important pathogens that cause human and vertebrate diseases [31]. Highly pathogenic Coronavirus outbreaks, SARS-CoV in 2002-2003 and MERS-CoV in 2012, have occurred in the last two decades and these CoVs caused illnesses from cold to more severe diseases such as severe acute respiratory syndrome (SARS) and Middle East Respiratory syndrome (MERS). Currently, a novel Coronavirus, SARS-CoV-2 causing COVID-19 disease has recently emerged from China and then, quickly spread globally. The WHO declared COVID-19 as a pandemic on March 11, 2020 [32]. According to current evidence, WHO reported person-to-person transmission of SARS-CoV-2 causing COVID-19 disease through direct contact to the respiratory droplets of the infected person by sneezing or coughing. Another route could be indirect contact with contaminated surfaces or objects [33]. Currently, foodborne exposure to this virus has not been known yet as the route of transmission and there is no evidence of food and food packaging being associated with transmission of the COVID-19 disease [34]. However, food handling by an infected person or contamination of food packaging material should be evaluated. Because it is known that Coronaviruses can persist on inanimate surfaces [35]. Therefore, it is important to assess the possible impacts on the current knowledge.

## Food and Water Safety during COVID-19 Pandemic

COVID-19 pandemic has affected the food systems [36] from many routes including food safety, food security, contamination of foods and food contact materials, hygiene and sanitation procedures, resilience and sustainability, lockdowns of the food facilities, changes in food consumption patterns and consumer behaviors as shown in Figure 1.

Food safety is one of the four pillars of the food systems that were affected during COVID-19 pandemic and is a very important matter for preventing the spread of the SARS-CoV-2 virus, which causes COVID-19 pandemic, among consumers, producers

and retailers [37]. In the light of current knowledge, foodborne or drinking water exposure to this virus has not been reported as the transmission route. However, surfaces and fomites may serve as a source of transmission respiratory droplets of the infected individual or contaminated hands [38]. Surface contamination could occur by the direct landing of droplets expelled during infected person sneezing or coughing or due to the indirect transfer from contaminated hands. Therefore, viruses retained on surfaces could cause a risk of infection to anyone who contacts the contaminated surface.

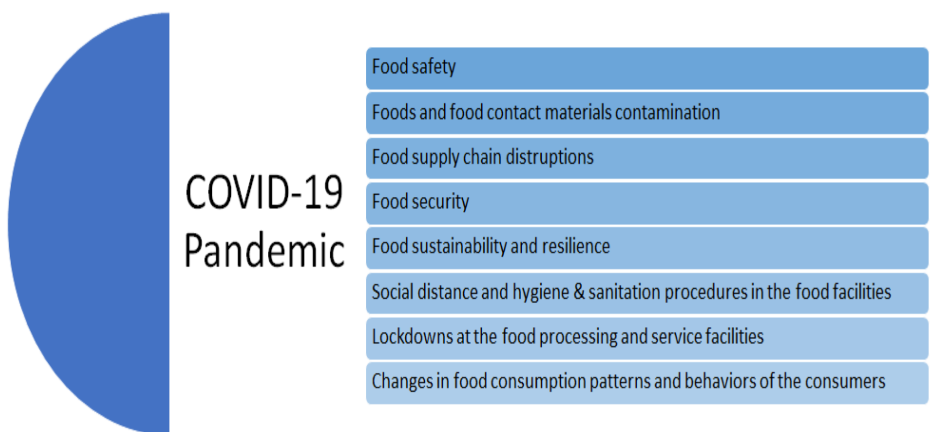


**Fig 1** Effects of COVID-19 on food systems

Ensuring the safety of food reaching consumers' plates is very important for the food sector [39]. Therefore, the possibility of virus transfers due to the contamination of food packaging material and also, food handling by an infected person through food processing and serving should be assessed for food safety and precaution measures should be implemented. First of all, four key steps of food safety, cleaning, separation, cooking and chilling, should be followed as normally done to prevent any foodborne illness [34]. Secondly, it is known that Coronaviruses can persist on inanimate surfaces [35]. While there is limited data about the survival of the enveloped COVID-19 virus, however, it is likely to be inactivated significantly more rapidly than non-enveloped human enteric viruses [38]. Therefore, food production, processing and service facilities should take appropriate infection-control measures by optimal inactivation of the virus to prevent the possible transfer from one person to another person, to the surface or food or food packaging material.

Moreover, the number of foodborne illnesses were decreased in 2020 compared to the previous year [40]. Foodborne illnesses have significant impacts from a public health and also, economical point of view [41]. Widespread public health interventions together with increased precautions and hygiene procedures in the food facilities to prevent transmission of SARS-CoV-2 for reducing the risk of COVID-19 disease might affect the number of foodborne illnesses. Besides, public health and hygiene procedures, closures of restaurants, or fewer food businesses trading could have contributed to declines. Centers for Disease Control and Prevention (CDC) reported a 26% reduction compared with 2017–2019 in the incidence of infections caused by pathogens transmitted commonly through food in the US during 2020 which is an important ratio from a food safety point of view [42].

**Contamination of Coronaviruses through food contact materials**

Surface contamination and transmission of pathogens from contaminated surfaces have recently been found that they could be more important than originally thought for the spread of illnesses [43, 44]. Food contact surfaces are typically made of stainless steel and different kinds of plastic material and also, could contain other materials like wood, ceramics, rubber or glass [45]. Among them, stainless steel surfaces and utensils are the most preferred in kitchen areas and other food production and processing facilities [46]. Glass is used as the food packaging material for making bottles and jars and also commonly used in kitchens as food serving materials. Therefore, some food contact materials could be a source of transmission in the food and drink service and preparation facilities. Also, plastics are used as food contact equipment. Viruses can persist for days and even weeks on inanimate surfaces under ambient conditions of temperature and humidity. Persistence of viruses on different surfaces, survival temperature and durations and also, some inactivation procedures are given in Table 1.

In food technology, many food packaging materials with different features are used to protect the food from physical, chemical and biological damage from the external environment, to extend shelf life, to retard deterioration and to maintain the food product's quality and safety until the food reaches to the consumer and also, provide ingredient and nutritional information to the consumers. Due to the low cost and functional advantages, multiple types of plastic polymers are used for food packaging. In the current knowledge, virus could persist on stainless steel and plastic and food

packaging material could be the source of the transmission, maybe not to food but the hands or other surfaces.

Enveloped viruses remain infectious on surfaces for several days [47, 48]. However, many of them are less stable in the environment and are more susceptible to oxidants [49]. Howie, Alfa [50], investigated the survival of two laboratory enveloped and non-enveloped viruses after dried on the surface of polyvinyl chloride test carriers in the absence or presence of an organic matrix and reported the survival of the non-enveloped reovirus during 30 days despite drying in a commercial artificial test soil (ATS; US Patent 6447990) containing worst-case levels of carbohydrate, protein, endotoxin and hemoglobin to represent the low-level nutrient surface. On the other hand, enveloped virus survived 2 days and died. They confirmed that the non-enveloped viruses could persist in the environment, especially in the presence of organic material. Recently, Kampf, Todt [35] reviewed the analysis of 22 studies of human and veterinary Coronaviruses and reported that human Coronaviruses could survive on inanimate surfaces like plastic, metal or glass for up to 9 days, but they could be inactivated by various surface disinfection procedures efficiently.

In a recent study conducted by [46], survival rates of infectious SARS-CoV-2 was investigated at 20 °C, 30 °C and 40 °C with 50% relative humidity on several common surface types and results showed that viable virus was isolated for up to 28 days at 20 °C from glass, stainless steel and paper surfaces and with increased temperature, survival rate was decreased. In the study of Duan, Zhao [51], the survival abilities of a SARS-CoV (strain P9) on the eight different surfaces and also, in water and soil, were investigated and strong persistence of Coronaviruses on the surfaces were reported since viral infectivity of the viruses persisted for 60 hours, however, after 72 to 96 h of exposure it started to drop and then became almost undetectable after 120 h. After 48 h, determined infected cells were less than 50 % except for glass surface since 51-75 % infected cells were detected on it. After 120 h, any cells were not detected on the surfaces, except filter paper, metal and cloth which still contained a few infected cells at the end of 5 days. van Doremalen, Bushmaker [52] investigated the stability of MERS-CoV on plastic and steel surfaces by comparing MERS-CoV (HCoV-EMC/2012) and A/Mexico/4108/2009 (H1N1) viruses. H1N1 virus was only stable until four hours on both surfaces. On the other hand, MERS-CoV could be recovered from both surfaces

after 48 hours at 20°C (40% relative humidity) as shown in Table 1. At 30°C, MERS-CoV virus remained viable for 8 and 24 hours with 80% and 30% relative humidity on both surfaces, respectively. These surfaces are commonly used surfaces in the food industry and also, food preparation facilities. In addition, they are used as food contact materials.

Temperature and relative humidity affect the stability of the viruses. A study conducted on an airborne enveloped human Coronavirus (HCoV-229E) reported that besides temperature, relative humidity is also important for the survival of the virus since recovery was higher at low relative humidity than at high relative humidity [53]. It was reported that at 20 °C, HCV/229E recovery was better at 30% RH and 50% RH (87% and 91%, respectively), but, at 80% RH, only 55% of the original input HCV/229E was detected. However, at 6°C, at all three relative humidity levels, the survival of aerosolized virus was significantly enhanced. But this study was conducted with a human Coronavirus and, it was reported that SARS-CoV on surfaces could be more persistent compared to human Coronavirus 229E [54, 55]. Rabenau, Cinatl [54] reported that in a dried state, HCoV-229E human Coronavirus loses its infectivity within 24 h while SARS-CoV retains its infectivity even after 6 days and it loses completely after 9 days at the dried state. Casanova, Jeon [56] investigated the effects of air temperature and relative humidity on survival of two potential surrogates on the surfaces and reported that survival was greater at low relative humidity. This study was conducted using two potential surrogate viruses, mouse hepatitis virus (MHV) and transmissible gastroenteritis virus (TGEV) due to the challenges of working with SARS-CoV and concluded that the relationship between inactivation and relative humidity was not monotonic and at low relative humidity, the survival rate was higher. It was concluded that based on the survival data for surrogate viruses, enveloped viruses could remain infectious long enough on the surfaces for people contacting them, posing a risk for exposure that causes to possible illness transmission. The same study reported the persistence on the stainless steel at 4°C for as long as 28 days. The inactivation was reported to be faster at 20°C than at 4°C at all humidity levels. Both viruses were inactivated more rapidly at 40°C than at 20°C [56]. As reported, storing in food at refrigerating conditions did not stop the survival of the foodborne [57] or human viruses [56]. Viruses can remain stable under refrigerated and frozen storage conditions [58].

Chan, Peiris [59] showed that dried SARS-CoV (HKU39849) virus on plastic maintained its viability for up to 5 days at 22–25°C and of 40–50% relative humidity and high temperature (38°C) at high relative humidity (>95%) have a synergistic effect on inactivation of SARS-CoV viability while low temperature and low humidity levels caused prolonged persistence of virus on the contaminated surfaces.
A recent study was conducted on the stability of SARS-CoV-2 (HCoV-19 nCoV-WA1-2020) and SARS-CoV-1 (Tor2) in aerosols and on various surfaces including plastic (polypropylene), stainless steel (AISI 304 alloy), copper and cardboard at 21 - 23°C and 40% relative humidity for over 7 days and reported that the stabilities of SARS-CoV-2 and SARS-CoV-1 were similar under the experimental conditions [43]. The study reported that fomite and aerosol transmission of SARS-CoV-2 is probable because the virus could stay viable and infectious in aerosol for hours and on surfaces for days. It was observed that SARS-CoV-2 remained viable in aerosols for 3 hours, with a reduction in infectious titer [43]. A similar reduction was determined SARS-CoV-1. It was reported that SARS-CoV-2 was more stable on stainless steel and plastic compared to copper and cardboard. After application on stainless steel and plastic surfaces, the viable virus was detected for up to 72 hours. SARS-CoV-1 survived 72 hours on plastic and 48 hours on stainless steel. Plastics including polypropylene form and stainless steel are commonly used in food production and processing facilities. Stainless steel (Type 304) is among the used food contact materials in the food industry. An exponential decay was observed in virus titer for both viruses during all experimental conditions, but the viable virus was still detected up to 72 hours after application to stainless steel and plastic surfaces. On the other hand, on copper, no viable SARS-CoV-2 and SARS-CoV-1 were measured after 4 and 8 hours, respectively. On cardboard, no viable SARS-CoV-2 and SARS-CoV-1 were detected after 24 and 8 hours, respectively. Therefore, the longest viability of both of the viruses was reported on stainless steel and plastic surfaces; the estimated median half-life of SARS-CoV-2 was approximately 5.6 and 6.8 hours on stainless steel and plastic, respectively. According to the determined results, inanimate surfaces, but especially plastic and stainless steel could be the source of transmission of SARS-CoV-2, since the virus can remain viable on surfaces up to days depending on the inoculum. It is very important to perform precautionary preventive measures for pandemic mitigation efforts.

**Table 1** Persistence of viruses on different surfaces

| Virus | Surface | Inoculum[2] | Survival Temperature | Survival Time[1] | Inactivation | Reference |
|---|---|---|---|---|---|---|
| SARS-CoV-2 | stainless steel | $10^{5.25}$ | 21-23 °C | 72 h | | [43] |
| | plastic | | | 72 h | | |
| | cardboard | | | 24 h | | |
| | copper | | | 4 h | | |
| SARS-CoV-1 | stainless steel | $10^{6.75-7.00}$ | | 48 h | | |
| | plastic | | | 72 h | | |
| | cardboard | | | 8 h | | |
| | copper | | | 8 h | | |
| SARS-CoV-2 | stainless steel | $4.97 \times 10^{7}$ | 20 °C | 28 d | | [46] |
| | glass | | | 28 d | | |
| | vinyl | | | 28 d | | |
| | paper | | | 28 d | | |
| | cotton | | | 7 d | | |
| | | | 30 °C | | | |
| | stainless steel | | | 7 d | | |
| | glass | | | 7 d | | |
| | vinyl | | | 3 d | | |
| | cotton | | | 3 d | | |
| | paper | | 40 °C | 21 d | | |
| | stainless steel | | | <1 d | | |
| | glass | | | <1 d | | |
| | vinyl | | | <1 d | | |
| | cotton | | | <1 d | | |
| | paper | | | <1 d | | |
| Reovirus (non-enveloped) | | | | 30 d | *2.6% glutaraldehyde 7%, %0.5 AHP[3]* | [50] |
| Sindbis virus (enveloped) | ATS[4] | ˜$10^{8}$ | Room temperature | ˜2 d | *2.6% glutaraldehyde 7%, %0.5, %0.05 AHP[3]* | |
| SARS-CoV | wood | $10^{6}$ | Room temperature | 4 d | *Heat treatment* | [51] |
| | glass | | | 4 d | *-56°C for 90 min* | |
| | mosaic | | | 3 d | *-67°C for 60 min* | |
| | metal | | | 5 d | *-75°C for 30 min* | |
| | cloth | | | 5 d | *UV irradiation for 60 min* | |
| | Press paper | | | 4 d | | |
| | Filter paper | | | 5 d | | |
| | plastic | | | 4 d | | |
| | water | | | 4 d | | |
| | soil | | | 4 d | | |
| TGEV | Stainless steel | $10^{6}$ | 4°C | ≥28 d | | [56] |
| | | | 20°C | 3-28 d | | |
| | | | 40°C | <6-120 h | | |
| MHV | | | 4°C | ≥28 d | | |
| | | | 20°C | 3-28 d | | |
| | | | 40°C | <6-120 h | | |
| SARS-CoV | plastic | $10^{7}$ | 21–25°C | 6-9 d | *Thermal inactivation* | [54] |
| H-CoV | | | | 72 h | *-56°C-60°C 30 min* | |
| SARS-COV | plastic | $10^{5}$ | 21-25 °C | 5 d | | [59] |
| MERS-CoV | steel | $10^{5}$ | 20°C | 48 h | | [52] |
| | | | 30°C | 8-24 h | | |
| | plastic | | 20°C | 48 h | | |
| | | | 30°C | 8-24 h | | |
| H-CoV | polyfluorotetraethylene (Teflon; PTFE) | $10^{3}$ | 21 °C | 5 d | | [44] |
| | polyvinyl chloride (PVC) | | | 5 d | | |
| | ceramic tiles | | | 5 d | | |
| | stainless steel | | | 5 d | | |
| | glass | | | 5 d | | |
| | silicon rubber | | | 3 d | | |
| | copper | | | <0.5h-2h | | |
| H-CoV | Aluminum | 5 *$10^{3}$ | 21 °C | 2-8 h | | [60] |

[1]h: hours; d:days, [2]Inoculum: 50% tissue-culture infectious dose ($TCID_{50}$)/ml; pfu: plaque-forming units; viral titer, [3]AHP: accelerated hydrogen peroxide, [4] ATS: artificial test soil (US Patent 6447990)

Warnes, Little reported that SARS and MERS human Coronaviruses caused increasing concern of contact transmission during outbreaks and determined that human Coronavirus (HuCoV-229E) could survive for at least 5 days on the polyfluorotetraethylene (Teflon; PTFE), polyvinyl chloride (PVC), ceramic tiles, glass and stainless steel surfaces and for 3 days on the silicon rubber at 21°C and relative humidity of 30- 40% [44]. The initial inoculum in that study was lower compared to other studies, but although low-level initial inoculum concentration, the virus maintained infectivity for 5 days on all of the surfaces, with the exception of silicon rubber. It was reported that contamination of surface material with very few Coronavirus particles can cause a considerable risk of infection spread after being touched and then transferred to facial mucosa. As the food contact material, stainless steel is preferred in the food industry due to its corrosion resistance and also, durability based on the percentage of chromium and nickel. The same study showed that nickel and stainless steel did not show any antiviral activity. However, the inactivation of human Coronavirus was reported on brass and copper-nickel surfaces at room temperature. In this study, brasses containing at least 70% copper were very effective to inactivate the studied human Coronavirus and the rate of inactivation was proportional to the copper percentage. Coronavirus was inactivated on copper nickels containing less than 70% copper in 120 min and if alloy contains >90% copper inactivation of Coronavirus in <30 min. The study concluded that incorporation of copper alloys to commonly used areas can help to decrease infection spread from touching surfaces contaminated with Coronaviruses [44]. Antimicrobial properties mainly on pathogenic bacteria of copper previously reported [61-63]. Also, viral inactivation of murine norovirus (MNV) on copper and copper alloy surfaces was reported [64]. Parra, Toro [62] suggested the potential usage of copper surfaces to control the microbiological hazards in the poultry industry based on the antimicrobial effect over pathogenic and non-pathogenic microorganisms. Geng, Zhang [63] reported that compared to stainless steel, copper could have a potential application in the field of food packaging, disinfection and piping of drinking water due to the antibacterial activity. Delgado, Quijada [65] reported that ion copper delivery plastic materials based on polypropylene with embedded copper nanoparticles could have great potential as antimicrobial agents. However, copper and copper alloys such as brass have limited usage in the food

industry due to the corrosion problem especially when it is contacted with low acidity food products. Stainless steel is a cheaper alternative compared to copper and has excellent properties to be used as a food contact material. But, using biocidal surfaces could help to decrease the incidence of infections spread by touching contaminated surfaces and more studies should be conducted for possible usage.

***Coronavirus inactivation techniques***

Viruses can persist for days and even weeks on inanimate surfaces and enveloped viruses remain infectious on surfaces during several days [47, 48]. However, many of them are more susceptible to oxidants [49]. Therefore, disinfection practices are important and should continue to be applied. Bosch, Gkogka [58] reviewed the various control procedures and different antiviral food components for the inactivation of foodborne viruses and reported that incorporating additional preservation steps to an existing process should assist in eliminating or destroying viruses in many foods.

In the current COVID-19 pandemic, foods, hands or contaminated surfaces could serve as vehicles. Therefore, disinfection should be applied. WHO [66] reported that many disinfectants are active against enveloped viruses and currently, recommended effective ones include 70% ethyl alcohol and sodium hypochlorite at 0.1%.

Recently, Ong, Tan [67] analyzed SARS-CoV-2 causing COVID-19 in the environmental samples and reported extensive environmental contamination with the virus, but post-cleaning samples were negative showing current decontamination practices are sufficient. It is already known that the virus could be easily inactivated by commonly used disinfectants [68]. Howie, Alfa [50] investigated the efficacy of glutaraldehyde and hydrogen peroxide: accelerated hydrogen peroxide (AHP) formulations on the destroying two enveloped and non-enveloped viruses during 1-20 min and reported the elimination of the enveloped test virus by diluted disinfectants. In their study, glutaraldehyde 2.6%, 7% and 0.5% AHP killed both of the test viruses within 20 min (Table 1). Kampf, Todt [35] reviewed in details all of the persistence of veterinary and human Coronaviruses on the inanimate surfaces and also, their inactivation procedures with biocidal agents applied for chemical disinfection and reported that surface disinfection procedures with various concentrations of ethanol, propanol, hydrogen peroxide or sodium hypochlorite were efficient for inactivation compared to biocidal agents such as chlorhexidine digluconate or benzalkonium

chloride which are less effective. The review evaluated the results of the biocidal agents in suspension or carrier tests to inactivate human and veterinary Coronaviruses. Among them, the biocidal agents in suspension tests inactivating infectivity of SARS and MERS Coronaviruses more than 4 $log_{10}$ include ethanol (78-95%), 2-propanol (75%), the combination of 2-propanol (45%) with 1-propanol (30%), 0.5% hydrogen peroxide, glutardialdehyde (0.5-2.5%) and povidone iodine (0.23-7.5%).

Food facilities are required to use EPA-registered sanitizer products for cleaning and sanitizing practices [34]. United States Environmental Protection Agency (US EPA) listed all the commercial products that meet EPA's criteria for using against SARS-CoV-2 on surfaces [69]. According to the update (22.04.2020), active ingredients for human Coronavirus include quaternary ammonium, hydrogen peroxide, peroxyacetic acid, octanoic acid, sodium hypochlorite, isopropanol, sodium carbonate, ethanol, triethylene glycol, l-lactic acid, glycolic acid, silver ion, citric acid, phenolic, hypochlorous acid, ammonium carbonate, ammonium bicarbonate. Different brands were listed including reported active ingredients alone or in combination. For the efficient use of disinfectants, it is very important to prepare according to the manufacturer instructions at appropriate concentration during enough time.

Chemical sanitizing is more frequently used in food production facilities. However, sanitization may be achieved through thermal or radioactive processes besides chemical disinfectants. Thermal processing is an effective strategy in inactivating viruses and different viruses could be inactivated depending on the applied temperature degree and duration [58, 70]. Irradiation is effective to preserve foods. However, most viruses could be far more resistant to irradiation and the effectiveness of the irradiation against viruses is dependent on the virus, food product characteristics and application conditions [58, 71].

Rabenau, Cinatl [54] reported that thermal inactivation at 56°C was very effective in the absence of protein; but, the addition of 20% protein exerted a protective effect in the residual infectivity. Heat treatment at 60°C for at least 30 min should be used for the inactivation of protein-containing solutions (Table 1). Duan, Zhao [51] reported that when SARS-CoV cells were exposed to higher temperatures, the infectivity was virtually eliminated at 56°C for 90 min, 67°C for 60 min and 75°C for 30 min. Exposure to UV irradiation on the virus in culture medium for 60 min destroyed viral

infectivity at an undetectable level. Leclercq, Batéjat [72] investigated the survival of MERS-CoV at 25, 56 and 65°C and reported that 56°C, the common temperature used for inactivation of enveloped viruses, for almost 25 minutes were required to decrease initial titer by 4 $log_{10}$. Raising the temperature to 65°C showed a negative effect on the viral infectivity since virucidy decreased significantly in 1 min and 15 min at 65°C was more sufficient for complete inactivation. Kampf, Voss [73] reported that a thermal disinfection at 60°C - 30 min, 65°C - 15 min and 80°C - 1 min was efficient to significantly reduce Coronavirus infectivity by at least 4 $log_{10}$. The effect of heat could be related to the thermal aggregation of SARS-CoV membrane protein [74]. It was reported that the nucleocapsid protein of SARS-CoV is completely denatured at 55°C in 10 min [75]. The survival ability of the viruses could depend on the type of surface, temperature, relative humidity and the strain of the virus. Therefore, the development of new procedures to assess the activity of new antiseptic disinfectants on the viruses should be further investigated.

**Mitigation of biological risks**

Food supply chains have paramount importance during the pandemic and every step to prevent contamination should be implemented. There is no evidence of the transmission of this virus through foods or food packaging materials but the asymptomatic food handlers that might carry the virus could be the potential transmission route to the food chain [76]. Four key steps of food safety, cleaning, separation, cooking and chilling, should be followed as normally done to prevent any foodborne illness [34]. There are already ongoing practices for food safety to prevent any foodborne illnesses such as frequent hand-washing, cleaning of the surfaces and utensils, and cooking food to the right temperature and these steps could also reduce the potential transmission of any virus particles through food.

In a food facility, ongoing applied procedures related to personal hygiene, sanitation and also, recognized food safety practices will decrease the possibility of pathogens that will threaten the safety of food supply chain [77]. As reported by French Agency for Food, Environmental and Occupational Health & Safety [78], two theoretical routes of food contamination by the SARS-CoV-2 virus could be associated with infected livestock animals and the transfer of the virus to food products of animal origin, or the handling of foods by people infected with this virus. However, there is not any evidence that the

SARS-CoV-2 virus causing COVID-19 disease is carried by the domestic food-producing animals, the consumption of foods of animal origin from infected animals was not thought as a source of infection based on the current knowledge [77, 78]. The other route, contamination of food via the infected person with the SARS-CoV-2 virus could be prevented in the food facilities through ongoing good hygiene practices. Moreover, it is evident that Coronavirus persist on inanimate surfaces, it is very important to often apply heat or sanitizers such as chlorine and hydrogen peroxide for the disinfection. Individuals could contaminate the environment and surroundings by sneezing or coughing due to the transmission of the virus through respiratory droplets. Therefore, in food-processing environments, to prevent contamination of any equipment, food contact material or food directly or through cross-contamination from surfaces or workers' hands to food and to protect other healthy workers (Figure 2), food workers experiencing clinical gastrointestinal or respiratory disease symptoms should not participate in food preparation and processing [77].

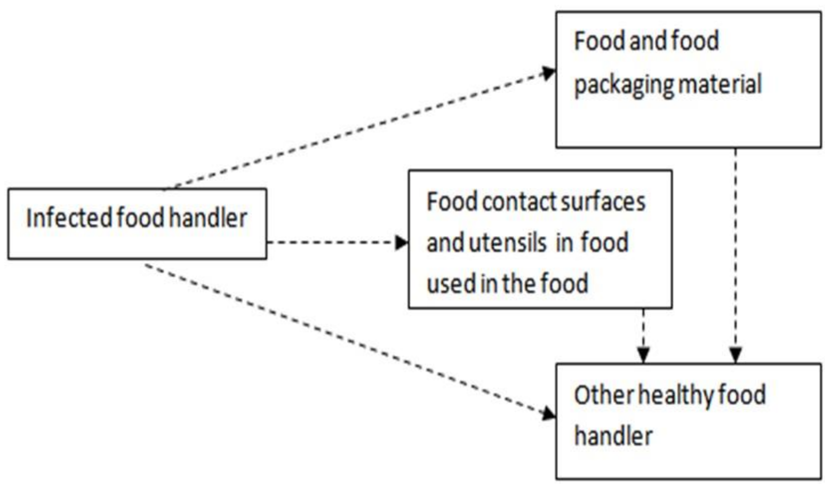


**Fig 2** Possible modes of transmission from inanimate surfaces in the food production environments based on the available data from [33, 34, 77]

### Possible reduction techniques of SARS-CoV-2 contamination for some specific food types and water

In the food industry, it is important for food or water not to be contaminated at any point during its journey along the supply chain. Foodborne exposure to SARS-CoV-2 virus has not been known to be a route of transmission [79]. However, it is very

important to be aware of the possible roles of water and fresh foods. Because viruses could be stable at many conditions and surfaces. Despite not being grown in foods, they could be stable on the surfaces and cause to contaminate other surfaces and hands. In addition, person-to-person transfer in a food facility should be taken into consideration [80].

***Fresh fruits and vegetables***

Viruses cannot replicate in foods, but they can be present [4]. Microbial control strategies used to keep foods safe microbiologically could not be directly applicable to viruses because for viruses ‘growth’ is not a concern whereas ‘survival’ or maintaining infectivity is key [58]. Although viruses will not grow in or on foods, raw vegetables and fruits might serve as vehicles for infection [81]. Mullis, Saif [82] investigated if the contaminated vegetables may serve as a vehicle for Coronavirus transmission to humans by using bovine Coronavirus as a surrogate on lettuce surface at refrigeration conditions and determined that on lettuce bovine Coronavirus retained infectivity for at least 14 days.

During COVID-19, food consumption patterns were changed compared to previous years due to increased awareness of consumers and fruits and vegetables have significantly higher consumption scores compared to the period before the pandemic [83]. Good personal and food hygiene practices are very important during the handling of ready-to-use foods, fresh fruits and vegetables that may be consumed raw and/or without any further processing. Effective thermal treatment is very important for pathogen inactivation. Therefore, fresh food that will be consumed without heat treatment could be particularly susceptible to contamination from the environment and food handlers. It is critically important to keep food contact environments, equipment and tools clean, conduct good hand washing practices, and separate raw and cooked foods and use clean water for minimizing the risk of exposure to any foodborne bacteria and viruses [77]. Moreover, surface decontamination using sanitizers could be applied. Fresh products usually undergo a sanitization step after harvesting from the field, but commonly used sanitizers could be unsuccessful for viruses [11]. Therefore, it is important to apply formulations appropriate for the target virus inactivation and, toxicologically safe. For consumers, as normally done, it is very important to continue to wash fruits and vegetables using potable running water effectively.

***Meat and poultry***

There is no evidence that meat and poultry play a role in the spread of the SARS-CoV-2 virus causing this disease [78]. However, separating raw meats and poultry from other foods and cooking to the right temperature should be followed as normally done to prevent any foodborne illness [34]. It is important to avoid the consumption of raw or undercooked foods of animal origin including meat, milk products, eggs to reduce exposure to all viruses and also, other foodborne pathogens. Therefore, foods of animal origin should be heat-treated sufficiently before consumption [77].

On the other hand, high numbers of COVID-19 cases in meat processing facilities were reported worldwide and compared to other food sectors, outbreaks were so severe in meat companies causing some plants to shut down in the United States. However, exposure to SARS-CoV-2 was not through the meat products the workers handle in meat processing facilities. Centers for Disease Control and Prevention (CDC) reported that the reason SARS-CoV-2 spread rapidly in meat processing facilities is due to the work environments where employees have prolonged close workplace contact with each other at processing lines and other areas in busy plants for long periods. This may contribute significantly to their potential exposures [84].

***Water***

As reported by WHO [38], although the persistence of the virus in drinking water, there is no evidence about the waterborne transmission of the virus causing COVID-19. Updated report of WHO (23 April 2020) claimed that the virus could be in untreated drinking water, however, it has not been detected in drinking-water supplies yet. Based on the available virus on other Coronaviruses, the risk of Coronavirus transmission through water supplies is low [66]. CDC [85] reported that the virus was found in untreated wastewater. However, there is no evidence to date that this virus can cause disease through exposure to untreated wastewater or sewerage systems. Enveloped Coronavirus becomes inactivated considerably more rapidly than non-enveloped human enteric viruses since enveloped viruses are less stable in the environment and are more susceptible to oxidants. Standard filtration and disinfection procedures for water treatment can inactivate COVID-19 [66]. Determination of gene fragments of the SARS-CoV-2 virus in incoming sewage water and screening of the virus at municipal

waste water plants should be possible to follow the evolution of the pandemic [84, 86, 87].

## Conclusion

In the light of current knowledge and cases, the foodborne transmission of SARS-CoV-2 was not reported as the route. However, the infected individual in the food production and service facility and, contaminated surfaces, may serve as the source of transmission route since Coronavirus can survive on the inanimate surfaces. Therefore, it is important to evaluate food contact surfaces since the survival of the virus is changed depending on the surface type. In the food industry, it is important for food or water not to be contaminated at any point during its journey along the supply chain. Food supply chains have paramount importance during the pandemic and every step to prevent contamination should be implemented. Standard practices for food safety to prevent any foodborne illnesses especially hand-washing frequently, cleaning of surfaces and utensils, and cooking food to the right temperature should continue to decrease the possible transmission of any virus particles through food. It is important to efficiently apply chemical sanitation, thermal or UV treatment applications. Moreover, food facilities could plan a more frequent cleaning and sanitation schedule to prevent cross-contamination. The COVID-19 pandemic has already affected food systems. Some food chains, especially high-value commodities requiring a large amount of labor for their production, were affected more than others due to the health problems of the employees, lockdowns resulting in unable to travel of local and migrant laborers or social distancing requirements in the food processing facilities. Therefore, sustainable and also, resilient food systems to shocks, crisis and pandemics should be developed for continuity of the food production. In addition, food safety should be the high priority. It has been observed that public health interventions to prevent SARS-CoV-2 transmission influenced exposures associated with other foodborne illnesses. It is critically important to continue production and processing by raising awareness about food safety to ensure people along the food supply chain are not at risk of COVID-19 disease transmission. Prevention strategies should be well defined and applied through farm to processing plant to restaurants and homes to reduce the occurrence of infections.

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**Review Article**

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# Plant Attractants and Rewards for Pollinators: Their Significance to Successful Crop Pollination

**Nur Athirah Zariman[1], Nurul Alia Omar[1], A. Nurul Huda[1*]**

**ABSTRACT**

Plant and pollination have a mutualistic relationship where both parties offer and gain benefits for each other. The plant-pollinator interactions resulted in successful crop pollination in which the plant received pollination services by animal pollinator to increase food production that eventually increase crop economic value. Overall, ecosystem is highly dependent on pollinator thus there is a need to review potential valuation method of crop production and analyse the current understanding of the value of pollination service towards the ecosystem as well as the traits plant offer and benefits that pollinator gain from the relationship. The attractant and rewards highly depending on each other. Plant often able to attract pollinators through traits like the shape, size and colours of flower, deception, scents as well as location. In the meantime, plant would provide a reward for pollinators that visited the flower which includes food from pollen and nectar that contains high nutritional value, energetic rewards to reduce energy cost of survival, protection and shelter against predator and not to forget breeding, oviposition and mating sites inside the flower plant. This review emphasizes the ecological relationship of plant and pollinator that resulting in effective crop pollination if the attractant and incentives are significantly reliant on one another. However, there could be flaws, such as modifications to plant or environmental factors, would affect the rewards supplied and resulting in decrease crop output. With this review and current technological advancements, optimistically deeper investigations in the interaction of pollinator and flowering plant can be conducted and best pollinator management approaches can be established to secure sustainable crops production.

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## Introduction

Pollination is the main ecosystem service that responsible for the transfer of genetic information between plants through pollen that is important to support the sexual reproduction of a wide range of crops [1]. The pollination activities usually performed by two types of agents which is biotic and abiotic such as birds, insects, water, wind and gravity that transport these pollens to stigma from anther. Pollination biology is often associated with mutualistic interactions among plants and their animal pollen vectors

[1] Department of Plant Science, Kulliyyah of Science, International Islamic University Malaysia (IIUM), 25200 Kuantan, Pahang, Malaysia.
*Corresponding Author: A. Nurul Huda, anhuda@iium.edu.my

[2]. Plant-pollinator interactions work when pollinators transferring pollen to facilitate plant reproduction while they forage on flower for resources and rewards [3,4]. The relationship between plant and pollinations depends on the quantity of rewards they will receive and these usually correlates with floral trait or display [5]. The plant-pollinator communications include the display of reward attractants such as nectar, pollen, fragrances, oils, shelter, heat or reproduction [6,7] through the signalling of floral attractants with their visual, olfactory, gustatory and tactile signals to enable detection and discrimination by pollinators [8]. Plants have evolved variety of colours, shapes and sizes of flowers or inflorescences to attract animal pollinators [9]. As for the flower visitors, they developed numerous sensory capabilities to handle the floral signalling [10]. Through specific signals, innate and learnt preferences of flower visitors and sensory manipulation [11] in the selective attraction of pollinators and limitation of flower antagonists make communication between flowers and possible pollinators a highly complex and diversified relations [10]. Nevertheless, pollination is needed for the production of a variety of crops for food manufacture and human livelihoods and pollination by animals, especially insects which is a key element of the food chain. This short review highlights certain aspects involved in pollination contributions to food production and rewards gained by pollinators form the service.

## Economic Value of Pollination Services

Globally, animal pollination is a major ecosystem service since crops plants representing 35% of the world's food crops production profit from animal-mediated pollination [12]. This mutualistic interaction between plants and animals is necessary by providing welfares to humans mainly in acquiring varied seed and fruit resources, supporting the population of plant diversity and assisting other cultural values [13]. Pollinator-dependent crops are also the core source of numerous micronutrients such as vitamin A and C, calcium, folic acid and fluoride [14]. Meanwhile, pollinators benefit from this interaction by gaining essential foods such as pollen and nectar. Insects mainly bees, flies, butterflies, beetles, wasps, moths, and midges play an important role to provide pollination services worldwide [15]. Among these pollinators, pollination activities primarily provided by Apidae bee species such as honey bees (*Apis mellifera*), some bumblebee species (e.g., *Bombus terrestris* L., *Bombus ignitus* Smith) and stingless bees [16]. In Europe, some beetles pollinate oilseeds and cucurbits, butterflies

pollinate blackberry and clovers while certain flies are commercially used to pollinate sheltered crops such as chive, onion, strawberry, carrot and blackberry [17]. The existing information on this pollinator's contribution to pollination services are often inconsistent and inconclusive, as pollination requirements for fruit crops vary depending on the breeding method used. Certain crops are highly self-incompatible, while others may bear fruit with their own pollen. Despite that, there are varieties of fruits crops that depend or profit from animal pollination listed such as kiwifruit, grapefruit, blueberry, cashew, cherry, apple, pear, orange, plum, litchi and tangerine [18]. For tropical fruit, the most common crops that used pollination include citrus, starfruits, papaya, watermelon, guava, coconut, durian and mango [19].

Animal pollination of crop production is provided by both managed and wild pollinators, although most studies have highly valued the services offered by honey bee (*A. mellifera)*. A few studies have attempted to assess wild pollinators despite their ability to assist pollination with honeybees in the event of pollination shortage. Moreover, the service of pollination provided by wild and managed insects is dependent on their numbers and could be improved by diverse pollinator communities. Compared to managed pollinators, native animal species or wild pollinators and insects in certain areas capable of effectively assisting pollination in both agricultural and wild plants, where a variety of pollinators could contribute to sustainable pollination of crops and provide an insurance service to reduce the projected costs of crop failure [20]. However, in the recent years, the delivery of pollination service by wild and managed pollinators has declined progressively but steadily. Studies have shown that with the continuous decreasing in species distributions it is possible that pollination services to crops and wild plants have also reduced [21]. Although studies on pollinator decline are still inadequate, some researcher have highlighted the causes for this decline such as agricultural intensification [22], climate change [23], pesticide and viruses [24], risk from invasive alien species [25], and habitat fragmentation [26], although some of the factors may vary depending upon the type of crops and environmental conditions in the regions.

Pollination service provided from animal pollination hugely benefit crops because there is a significant surge in fruit production, improvement in fruit quality and economic value. In addition, certain fruits require insect-mediated pollination for fruit production

itself, but for other fruits, although pollination with insects is not a strict requirement for fruit production, it greatly increases yields [1]. For example, in Eastern Amazon where crops with higher dependent on animal pollinators such as cocoa, watermelon, palm and soybean provide higher crop production value [27]. They added that most crop-producing areas account for more than 10% of the gross domestic product (GDP) associated with pollination services and it is said that it depends more on pollination of crops for its economic stability. Moreover, strawberry crop that is highly cross-pollinated and fully depending on insect for fruit production shows a higher percentage of fruit set and with less malformed fruits compared to control crops [28]. In Asia, most crops depend to a large extent on pollination services provided by pollinators who naturally live in the ecosystem, as they are rich in various animal pollinators such as stingless bees, honey bees, other solitary bees, beetles and even bats. Moreover, around 70% of tropical crops appear to have at least one variation for which animal pollinators improve production [19]. For example, crop studies conducted at Gunung Tebu Forest Reserves in Besut, Terengganu, such as rambutan, durian, melon and watermelon, showed that the commercial value of the wild pollinator constructed on the pollinator dependency ratio was around RM 6,588,630.91, representing approximately 56% of the region total production values [19]. This proved that pollination services could improves certain agriculture crops economic values.

However, the assessment of pollination services has been a subject of debate because of the complexity of the system and the lack of sources to properly evaluate the services provided to the crops economic value. Besides, the idea of studies in evaluating pollination services is to apprehend the significance that will be misplaced as a result of the loss of some pollinators in a given area include at the regional, national and global levels [29]. Moreover, to support the maintenance of pollination service in agriculture, it is necessary to better understand the economic value generated by the pollination services. In most agricultural areas, pollination is provided by a combination of managed honeybees and wild insects. Many publications have tried to value the pollination of honeybees, while fewer studies have attempted to value wild pollinators. Although honeybees are widely known as economically most valuable pollinators, studies have shown that wild pollinators are often plentiful as bees on crop inflorescences [30]. Other than bee pollinators, the non-bee pollinators such as flies,

wasps, beetles, birds, butterflies executed around 25 to 50% of total number of visits of flowers. Even though non-bees were less effectual pollinators than bees per flower visit, they visit flowers more often; therefore, these two aspects compensate each other, ensuing in pollination services provided by non-bees which are the same as those offered by bees [31].

There is variety of method which has been proposed to access the economic value of pollination services in crops. The most common method used to evaluate the value of the pollination service includes the production value method that focus on the value of crop production attributable toward pollination and replacement value method which means to estimate the cost of using alternative technology or organism to attain the same function [29]. Other method used to evaluate economic value includes measuring the crop price, managed pollinator prices, dependence ratio, partial and generalised equilibrium models and stated preferences [32]. For example, the economic value of the pollination service is on the basis of three different levels using the production function method [1]. The author concluded that the value of the service varies greatly depending on the crop and market conditions at the local level. While nationally, the estimated value of the pollination service ranges from 1% to 16% of the market value of agricultural production and currently no reliable estimated value of the pollination service on a wide scale.

Nevertheless, estimates aimed at the economic value of the pollination services vary widely and there is no generally acknowledged evaluation method. Despite various doubts and differences, the present body of works mainly illustrates that pollination services are economically significant, and their forfeiture will have consequences for people around the biosphere. Moreover, even studies on the extent to which pollination services is limiting the current production of crops are still scarce, the results obtained showed that the decline of the pollinator could result directly in a decrease in yields or production for most crops [30]. A loss of pollinators might influence the manufacture of a pollinator-dependent crop by reducing yield and/or increasing producers' costs. Once pollinators are lost, the fruit set may decrease and, as a result, overall yield decreases. However, it is unclear to what extant agriculture crops could be impacted by pollinator deficits.

Not to mention, producing crops in a sustainable manner improves the system's ability to maintain long-term steady levels of food supply and quality. One of the methods to sustain crop production in a long-term includes, conservation agricultures that emphasises the preservation of a permanent soil cover, little soil disturbance, and plant diversity. This approach benefits agriculture in terms of reducing erosion, increasing water infiltration, increases soil surface aggregates, reduces soil compactness, promotes biological tilling methods, levels of surface soil organic matter as well as carbon content [33]. Moreover, Nanoremediation also able to sustain crop production by using nanoparticles for environmental remediations. The application of nanotechnology to the remediation of pollutants has yielded encouraging results that able to purify soil, air and water resources using nanoparticles as a catalyst and/or sensing systems [34]. By managing pollution as a priority, crop productions are able to sustains in a suitable environment. Similarly, biofertilizers can also increase crop productivity on a bigger scale while also saving the environment and contributing to soil sustainability [35]. Biofertilizer is an organically produced product containing specific living microorganism that provides nutrient supplies.

## Plant Attractants

As mentioned earlier, the mutualistic plant-pollinator interaction is of central importance since it results in seed and fruit production and therefore key contributors to biodiversity, ecosystem maintenance and essential to economic services. Pollination is a coexistence process between flowering plants and pollinators that involves the display of primary attractants, such as nectar, pollen, or other types of floral rewards, that essential to its survival and also secondary attractants of flowers to enable acknowledgement and discrimination by pollinators [7,10]. Some features that contribute to the attraction of pollinators are called secondary attractants, which, with their visual, olfactory, or tactile signals, constitute the signalling apparatus or advertisement directed at potential pollinators [36]. Plants attractants are able to deliver information concerning the existence, location and quality of the reward. Plants have evolved specific structures to interact with pollinators which are the flowers. Flowers send signals to particular type of pollinators that are facilitated by floral characteristics or traits known as “pollination syndrome” [37,38]. Flower traits such as shape, size, colour, scent production, electric fields and movement have been measured and

recognized to play roles in the recognition and attraction of pollinators to flowers [39–43]. Pollinator especially bees and other insects are impressively influenced by shape, outline form, length of flowers, odour, colour, pollen, nectar and other flower rewards of flowers. The biodiversity of angiosperms is largely based on a variety of traits that serve to attract pollinators [44] while repelling herbivores and excluding nectar and pollen thieves [10].

**Visual attractants**

Visual signals are the utmost studied in the context of flower trait evolution in shaping plant-animal interactions [45] where these signals aid in flower recognition and learning by pollinators [46]. Flower colour is a very essential feature that constrains the specific pollinators that visit flowmers and influences overall pollinator behaviour [47]. Insects perceived flower colour differently compared to humans which may influence the types of insects visiting flowers and the rates of visitation [48]. The colour of the flower varies between flowering plants and as well changes with the life of the flower. These colour differences do not refer to the darkening or fading of the flower, but to becomes fully bloomed and desirable flowers. The colours would provide signals toward pollinators to obtain flower and pollen location, species selection, sweetest nectar reward and ripeness [37,49]. However, colour itself is not the only factor considered in studying the attractiveness of flowers to pollinators of different plant species. In order to form a complete understanding of stimuli to pollinators based on “colour”, the intensity, wavelength content, brightness, and contrast could be deliberated in coincidence with colour vision. For certain flowering plants species, the contrast of dark spots on light background is an ordinary characteristics [50].

Changes in colour of flowers with age affects pollination behaviour and to be well pollinated, flowers must stand out from their background, as flowers developing in shady parts and against dim backgrounds be likely to be pale, whereas those flowering in open areas and against light backgrounds are darker [47]. For example, *Quisqualis indica* tend to change their flowers colour from white to pink to red, which might be referred with a change from moth to butterfly pollination. Firstly, the hawkmoths pollinated the flowers blossomed in white and when the flowers turn to pink and toward red, they droop and are pollinated by bees, flies and other possible insects [51]. Flower colour changes during development and function as visual cues for pollinators,

pollination state, and even time of the day [52] to evade old flowers and surge pollination competence. In flowers, the flower parts such as the anthers, filaments, ovary, flower bracts and pollen can also be visually eye-catching even the petals and sepals are usually the main coloured structures [49]. Flowers pollinated by bee are usually bright in colour which reflect light in the blue to purple part of the spectrum and have nectar guides that formed during the daytime. Due to their sturdy physical adaptation, flowers coloured in blue, lilac, and purple flowers are often visited by bees. Bees are driven to flowers by nectar cues that resemble a bull's-eye or stripes in the core of the flower, and they commonly involve UV coloration that humans cannot perceive [53]. The flower seems yellow to human in visible light, however, there is a gleaming yellow edge and a duller, darker centre under UV light, which only bees can sense [54]. For example, a yellow aster (*Asteraceae* sp.) were seen as yellow coloured flower by human, but appear different under UV light [53]. As mentioned by Miller [54], Yellow sorrel (*Oxalis fontana Bunge*) also visibily yellow in human eye (Fig. 1a) but has a darker center under UV light (Fig. 1b). Besides, yellow-flowered crops such as melons, oilseed rape are also often considered to interest a variety of insects due to the high reflectance of yellow [50,55]. Bright or light-coloured flowers are pollinated by moths and bats, with appropriate odour, and have nectar guides with nectar produced during the night time. Meanwhile, birds (particularly hummingbirds) are more attractive to red-coloured flowers that open during the day compared to blue-coloured flowers. The association of butterflies with pink and red flowers is well known [47]. In addition, successful pollination could result as specialized colour attractants will intensify the likelihood of pollinator constancy by guaranteeing pollinators visit one conspecific after another [56].

**Shape and size**

Equally, the flower shape and size are closely linked to pollinator attraction. Flowers are derived in many diverse forms, with different structures, and in various arrangements. This variety of flower forms has developed to perform pollination tasks. Flowers are formed mainly by natural selection from their pollinators, and the flower form varies based on the flowering plant and from the outcome of convergent evolution [37]. Flower shape can increase attraction to pollinators and facilitate pollen deposition, flower handling, and the degree of pollinator specialization, as well as influence the

electrostatic properties of pollen deposition [56]. Moreover, flower shapes could offer a hint as to what animals have the potency to perform as pollinators for a plant. Flowers that are animal-pollinated usually have larger showy petals of different sizes and shapes to attract pollinators. For example, study on two yellow melon hybrids showed that tropical hybrid has larger flowers that could be more attractive to bee species such as *A. mellifera*, [57] large solitary bees of genus *Xylocopa* by providing larger landing platform [58,59] compared to the other melon hybrids from Brazil. There is a positive connection between the size of flowers, inflorescences, or flower fields and attractiveness to insects. The smaller flowers or inflorescences are thought to suffer lower visitation rates due the insect's incompetence to detect them [50]. Meanwhile, larger flowers are easier to be detected and offer more rewards [60,61] and as an outcome some findings have acknowledged pollinator-mediated phenotypic selection for larger flowers [62,63]. Large flower such as *Cistus ladanifer* obtains great benefits in visitation rates and diversity that resulted in increasing fruit and seed production but it also increased florivory costs since they are more prone to be attacked by hostile insects like pollen-eating ants (Fig. 1c) and petal- eating beetles (Fig. 1d) that may cause damage to the flowers [61]. Nevertheless, there are some factors selecting for smaller flowers [45] as such theory predicts that when the pollinators are plentiful and competent, smaller flowers could be gainful in relations to water balance particularly. Moreover, these small, inconspicuous flowers are regularly assembled into large inflorescences to more effectively attract pollinators [7]. If not, larger flower are commonly preferred by pollinators by increasing the pollen dispersion level [64].

The aspects of floral morphology such as the arrangement of the reproductive parts and petals can be important in limiting access to floral reward [48]. Floral traits such as floral designs or corolla entrance diameter likely evolved to enable and increase the effectiveness of pollination [45]. Moreover, lipped, or labiate, flowers can provide a platform on which bumblebees can land before entering the flower, while hummingbirds visit long tubular flowers, that hover while probing the deep flowers with their long beaks [9]. For example, Alberto and others [65] determined that different types of bees such as *Apis mellifera* and native bees have different preferences of landing zone on strawberry flowers whether on top of flowers, stamen zone and on the petal zone. Besides, pollen detachment from pollinators would be facilitates by

increased style length, along with its deposition on the stigma that could accelerate pollen transmission between the contrarily-charged flower and pollinator [56]. Flowers symmetry is another main visual trait where selection performs based on pollinator perception, information processing and activity patterns. Insect pollinators detect and perceive symmetrical patterns, radial or bilateral in comparison to flowers that differ from symmetry and such floral patterns were found to receive higher visitation rates and better pollen transfer resulting in efficient pollination [7,40]. Commonly, floral colour, size and shape are closely linked to attract potential pollinator. For example, the flower form that appealing to hummingbirds tend to have a red-orange colour, a long flower tube form, a sweet scent with a nectar reward at the base of the flower tube. In addition, bee-pollinated flowers often have coloured guides on a landing podium shaped by the lower petal where the nectar is at the base of the tubular flower where bees usually enter. Certain flowers fit pollination by butterflies, moths, or hummingbirds take advantage of the insects' long mouthparts and have nectar spurs at the base of the flower [37].

**Fig 1** The diversity of plant attractant. (**a**) A yellow aster (*Asteraceae* sp.) under human vision and (**b**) under UV light [54]. (**c**) An ant species picking stamens by consuming pollen and (**d**) betteles eating petals degrade the flower attractiveness and caused damage to the flowers of *Cistus ladanifer* [64].

## Olfactory attractants

Apart from colour and shape, floral scent or fragrance also responsible for the attraction of specific pollinators. Olfactory signals advertise reward goods to pollinators often

synergistically and in concert with visual cues. Compared to colours and colour patterns, olfactory signals are adapted faster more precisely chosen [38], making them more resilient. Scent is a complex element of the floral phenotype that is primarily involved in communication amongst flowering plants and their pollinators. It encourages specialization in plant-pollinator relationships (via secretive channels with uncommon connections) and outcrossing and reproductive isolation (via flower perseverance) [66]. Floral scents are likely to be a necessary determining factor of communication network structure because they are among the most significant cues used by pollinators to trace nectar and pollen rewards from a distance [7,67]. These odorous substances are nearly always a mixture of numerous volatile organic compounds (VOCs), consist of over 100 different mixtures [66]. The VOC emission can alter quantitatively (total emission rate) and qualitatively (ratio between odorants) over the diurnal cycle in both flowers and fruits [68]. The repeated association of odour with food and the integration of gustatory and olfactory pathways may have developed neuronal structures that facilitate olfactory reactions in insects which permitting animals with food-related learning abilities[10]. Moreover, in both flowers and fruits, odour production possibly will be particularly important in plants species that lack perfect visual attraction and in species that depend on nocturnal pollinators [40]. Usually the flowers that open at night are pleasantly fragrant, attracting pollinators to pollinate and providing them with a fragrant reward and sometimes an essential oil [37]. Besides, for nectar-less crops that relatively unattractive to insect pollinators like kiwifruit, they tend to produce staminate or pistillate pollen that have odour in order to attract them [69,70]. Flowers pollinated by insects often emit fragrance, while flowers pollinated by birds are usually scentless. For moth pollination, night-blooming plants with characteristically strong and penetrating floral scents are necessary for long-distance advertising. In day-blooming plants, the floral scents are not as strong and these scents could act as attractants for landing that connected with nectar ladders [47].

At both long and short distances, the scent of flowers and fruits can attract animals. For flowers that depend on nocturnal pollinators, long-distance attraction is mutual in the surroundings where visual signals are unnoticed [56]. In addition, floral scent can also provide nuanced information about the quality of reward in nearby proximity. Studies of primate pollinator behaviour, for example, have also found that certain species

consciously smell fruit at close range [71]. Moreover, flower scent is an essential chemical trait for modifying the flower visitors' behaviour and signifies an evolutionary trade-off between attracting mutualists and deterring competitors. Both pollinators and herbivores might be attracted toward the identical odorants [72]. Flowers that normally have a pleasant-smelling fragrance use it to attract moths, butterflies, and bats. Certain flowers produce such strong scents that can be detected by insects more than half a mile away. Plants that use scent to attract pollinators may perhaps not have colourful flowers as the scent is the primary method of attracting pollinators [9]. Plants may also emit unpleasant odours that mimic the smell of rotting meat or manure that attract flies and beetles [47]. As the insect inspects the flower to trace the source of these odours, it indirectly comes in contact with pollen. This relationship is rather communalistic, with the plant benefiting from the interaction as pollen is transferred but the pollinator receives no profit at all. There are variety of crops that produce fragrant flowers to attract pollinators, for example, flies (Diptera) which are the main pollinator crops for mango are attracted to the plant due to the scent produce compared to honey bee (Apidae) that more attracted towards nectar rewards [73]. Moreover, although less is identified about the stone fruit crops pollinators, El-Sayed and others [74] showed that stone fruits plants (plum, *Prunus domestica* L.; apricot, *Prunus armeniaca* L.; peach, *Prunus persica* L.; cherry, *Prunus avium* L.) produce floral scents that perform as a common chemical attraction to a wide range of possible pollinators. Previous study investigates the effects on inbreeding in *Solanum carolinense* and resulted in altered floral traits and this caused the reduction in floral rewards (pollen) and negatively affect pollinator visitation [75]. The flower itself offers their own rewards that targets certain insects, thus, any changes to the plant may affect the rewards offered and reduce pollinators visitors.

**Floral attraction by deceit**

Not all plant species attract their animal pollinators on reward bases; instead, several mutualisms are exposed to "deception" by one partner or another, and interactions between plants and pollinators are no exception. Deceptive pollination has developed in 4-6% of angiosperms and is based on the incapability of pollinators to differentiate between a real resource such as breeding sites, mating partners, food and the flower that imitates the reward [76–78]. These plants have therefore evolved signals to deceit

insects to carry out pollination process. Mimicry is the common pollination strategy in which the flower does not provide a pollination reward (pollen or nectar) in this type of adaptation, but deceptively lures the pollinator to visit the flower [79]. Flower mimicry is a very diverse phenomenon, including Batesian mimicry, in which an unrewarding flower mimics a rewarding one, and Mullerian mimicry or signal normalization, in which two distinct rewarding flowers display similar signals [79,80]. The deception system has developed in different flowering plants families where about one-third of the species in the Orchidaceae family are well known to deceive their pollinators [80–82]. These deceiving flowers have therefore developed the capability to discharge cues that elicit essential reactions toward targeted insects [83]. One of the examples of mimicry acts in angiosperms includes the imitation of floral features. For instance, in begonia, only male flowers provide pollen rewards. To initiate visitation by animal visitors to the female flower, both twisted pistils and stigmas emit the presence of male stamens, also identified as flower automimicry [37]. In addition, the different morphologies of stamens within the same flowers in crape myrtle (Lagestroemia) serves as a signal to attract pollinators where the inner spiral of the male reproductive organ is on short filaments which assembled in the middle of the flower and the external whorl of stamens is on lengthy pigmented filaments [37]. Certain melittophilous flowers display pollen and stamens imitations include colorations on the petals that bear a resemblance to the stamens, typically as a bright yellow spot on the petals, patterns of flowers, stamen-like pistils and staminodes [79].

Meanwhile, sexually deceptive plants secure pollination by sexually attracting male insects through chemical and/or physical mimicry of the pollinator's female [84,85]. Sexual mimicry is the most widespread among the orchid genera. For example, sexually deceptive orchids like *Cryptostylis* spp, which are pollinated by haplodiploid wasps (*Lissopimpla excelsa*), enticing male to mate with flowers that provide no reward which frequently lead to sperm loss [86]. Other than that, orchid species *Ophrys heldreichii* flowers tend to resemble bees in form and coloration. The tactic is to draw male bees to undergo pseudocopulation with female bees [87]. Brood-flower mimicry is a particular sort of reproductive illusion in which the flower imitates a spot that insects mistakenly think is a place to breed. A common characteristic of brood flowers is the creation of a foul odour to appeal to carrion flies (sapromyophilia), dung beetles

(coprocantharophilia) and fungus gnat flowers (mycethophily) [78]. In addition, visual cues play an important role in attracting pollinators because a larger flower exemplify extra odour-producing features and the quantity of odour formed is positively correlated with insect attraction. For example, *Rafflesia arnoldii* which is the largest single flower in the world (up to one meter in diameter) emits a rotten, decaying flesh odour to attract potential pollinators [88]. Besides, the diurnal species of *Nymphaea*, attract beetles, flies or bees by floral trap mechanism where there is a stigmatic liquid that fills the flower cup. Visiting insects particularly bees and flies would fall into the cup and finally drown. The liquid may wash away all the pollen that the insects or pollinators visit to pollinate the plants [89,90]. Basically, flowers mimic the signals that pollinators use to find other flowers or substrates that they routinely visit for feeding or oviposition, initiating the distinctive or learned foraging behaviour to hunt for a non-existent reward.

## Rewards for Pollinator

Plant and pollinators interactions affect the morphological as well as physiological adaptations to a great extent [4]. The interactions involved a mutualistic relationship where both parties gained benefits. As previously described, pollinators rely on plant ads such as visual, chemical, or structural cues to attract them and give information to possible pollinators regarding the location of and access to flower rewards [7]. Flowering plants provides reward for pollinator to encourage them to make return visits and with the increased number of regular visits, greater awards will be provided [91]. Pollinators visits and forage on flowers mainly for food rewards such as nectar that contains variety of compounds including sugars, and pollen that provides an important protein resources, however some visits are made for non-nutritive reasons, including as breeding grounds, shelter, and gathering places [7]. Pollinators visit flowers for a variety of reasons, including caloric reward, energy, protection, and oviposition sites [10,92].

### Food rewards

Pollinators usually feed on plants to gain nutritional resources mainly pollen and nectar [93]. Pollinators generally relies on plant attractant to guide them to the food resources as the rewards are often concealed within a flower, which cannot be seen directly by pollinators [94]. Animal-pollinated flower usually provide nectar to pollinators as a reward [95]. Furthermore, the most recorded resources offered to insects to encourage

plant pollination are nectar [93]. Nectar are also an important nutritional and energy sources for pollinators [92]. Pollinators that visit flower usually rewarded with carbohydrate-rich nectar even when other rewards such pollen or wax were offered [96]. Dipterans feed on nectar to gain carbohydrates with high sugar concentrations for short terms energy needs such as mating, migration and oviposition, as well as to obtain lipids that able to provide energy, also they feed on nectar containing protein-building amino acids for longevity especially for mosquitos as amino acids may reduce the needs for blood meal and apart from that, vitamins, minerals and salts in nectar are equally important for their nutritional sources [7]. As mentioned by Prasifka [48], many pollinator-dependent fruit and vegetable crops shows a positive correlations between bee visits and volume of nectar such as blueberry, watermelon, raspberries and blackberries. According to bee behavioural research utilising nectar sugar concentrations reported in *Vicia faba*, showed a weak but significantly positive relationship where bumblebee (*Bombus terrestris*) favours 55 % w/w sugar solution over 40 % w/w sugar solution, but has no preference between 55 % w/w and 68 % w/w sugar solution [97]. In addition, the increased frequency of bee that visits zucchini flowers (*Cucurbita pepo* L.) was associated with higher nectar volumes and sucrose/hexose ratios, which seemed to be excellent markers of pollinator choices [98].

Apart from nectar, pollen also regards as the important floral reward that showing intraspecific variation [48]. Pollen contains nutritional sources like proteins, carbohydrates and lipids that benefit animal forage on them. Animal pollinator especially bees rely on pollen as the sole protein source for larvae development [3]. Besides, sterols which is a lipid in pollen are important for insects to have the ability to produce hormone or pheromone [92]. In addition, pollen proteins contains enzymes that helps pollen tube growth and undergo fertilization [99]. The enzyme responsible to support the growth of pollen tube are called proline which also helps insects pollinators to gain energy for flight [7]. Other pollinators puncture on the pollen grains to draw out the protoplasm while on the contrary, Diptera consume the entire grains and they may eat a lot of pollen until their belly becomes bloated and yellow, and the pollen digested can be seen in their excrement [7]. Pollen-foraging bees have been shown to favour certain flowers over others and are able to discern variations between pollen(-like) samples with various chemical, colour, and/or mechanosensory properties [100]. The

ability of *Eristalis tenax* L. to distinguish between pollen and nectar is studied, as well as the triggering of pollen ingestion on sunflower (*Helianthus annuus*), in behavioural preference tests extracted pollen is ingested in lesser amounts compared to than untreated pollen, demonstrating that water-soluble compounds are necessary for acceptability. Pollen that is dry is favoured over pollen that is moist where the grains clump together, implying that the pollen's mechanical features play a part in its sensory evaluation [101].

There is a significant association between pollen quality and reproductive system where pollen from insect-pollinated plants had increased protein content, while pollen from plants frequented by pollen-collecting bumblebees provided the best pollen [102]. However, a study of how floral abundance and resource quality influence pollinator choice found that bees preferred good nectar over pollen as the main driver of floral choice. Furthermore, the abundance of floral in a given area is important as a resource selection, even though the quality of rewards often influences forager choices [93].

**Warmth, heat and energy rewards**

Energy balance for pollination involves energy intake as in rewards pollinators gained from flowering plants and pollinator's own energy used while foraging. Body temperature is a measure of the amount of energy expended as heat [92]. Energy is supplied as food or as heat and most pollinators' energy requirements are determined by factors such as cost of living, locomotion, thermoregulation, and behaviour, which is primarily influenced by body size. Pollinators often forage on warmer flowers to gain net rate of energy and reduce the amount of energy needed to get their bodies to flight temperature before leaving the flower [96]. Thermogenic flowers offers energy rewards to pollinators which able to retain insect pollinators longer compared to protogynous plant and thus endothermic pollinators benefited from increased independence from environmental conditions, allowing them to forage in cooler and wetter conditions as well as enable them to have a high energy level to distribute pollen in wider weather tolerance and longer distances [2]. Researched done by Abrol [103], stated that pollinators' foraging profitability appears to be linked to the relationship between energy cost and reward in which *Apis dorsata*, which is larger in size and tongue length, forages high-energy-rewarding flowers while *Apis florea* forages low-energy-rewarding

flowers [103]. *Apis dorsata* clearly spends more energy while foraging than *Apis florea*, hence its energy requirements and foraging rate are higher.

Apart from energy, some plants also provide warmth to ectothermic pollinators. While the flower release heat to provide warmth for the pollinators, it is similar as providing extra metabolic, however, floral warming may also be a process to increases the production of flower nectar as nectar secretion often reduced at low temperatures [96]. Actively thermogenic flowers, such as the sacred lotus *Nelumbo nucifera*, may provide a consistent supply of predicted warmth [104]. Besides, when visiting the solar-heated flowers of *Narcissus longispathus*, an early-flowering montane species, the mining bee *Andrena bicolor* showed a positive correlation between visit length and floral temperature [105]. During the colder hours of the day, warm flowers are favoured by insect pollinators. Flower species that bloom at cooler times of the year or develop in colder habitats would be benefitted, where providing heat not only boosts the rewards offered to attract pollinators, but it may also be required to retain any pollinators present in the environment [96].

Not to mention, a study stated the *Oncocyclus* iris flowers that are partially or completely dark in colour do not produce nectar and have hidden pollen, so no pollinators will visit during the day and will pollinate only at night, but they can gather heat by absorbing solar radiation, and this heat acts as a reward for pollinators [5]. For example, scarab beetles, *Cyclocephala colasi* requires additional energy for activity that are 2.0-4.8 times greater outside flower *Philodendron solimoesense* (Araceae) than inside [106]. Scarab beetle spend the majority of their time inside floral chambers of heat-producing flowers, where they feed and mate at night and rest during the day, so flower heat are important energy rewards for the beetle to reduce energy expenditure for its activity [106].

**Protection, brood sites, oviposition and mating location**

Besides, plant blossoms are responsible in providing protection to most insects including thrips, beetles and flies. According to Liker [92], insects pollinators that requires protection from plant includes, *Taeniothrips ericae* that spends the majority of its life in *Colluna vulgaris* blooms, as well as mutualism between the *Tegiticula* moth and *Yucca*, and aganoid wasps and fiscus. Protective mutualisms with ants are common in the *Macaranga* genus. *Macaranga* have a type of inflorescence called ‘enclosing’ in which bracteoles

cover flowers including all the thrips- and hemipteran-pollinated species, bracteole "chambers" also protect thrips or hemipteran pollinators that use these structures as feeding and breeding sites. Furthermore, pollinators may be physically protected from natural enemies such as ants by the bracteoles of the 'enclosing' form [104,107].

During flower visitation, pollen movement may occur and resulted in flower breeding. Flower also offers rewards for pollinators in terms of breeding sites as individual flies visit multiple flowers during mating, oviposition, or it may occur concurrently with the plant reproductive systems and, for example, pollen transfer occurs while flies move from female-phase inflorescences where they mate and oviposit to male-phase inflorescences where they consume nutritional pollen, as demonstrated in the pollination of protogynous *Peltandra virginica* Kunth (Araceae) by *Elachiptera formosa Loew* (Chloropidae) [7].

Flowers also often provide benefits to pollinators in terms of oviposition and mating sites. Previous studies were done to examine the hoverfly preferences in ovipositional sites using three model flowers that exhibits attributes by real flowers in terms of colour, pollen and nectar resources and from the observations, hoverfly laid eggs on flowers that has a bright visual which means colour are important in eliciting oviposition response compared to olfactory stimuli of pollen and nectar [108]. Besides, female diptera are required to visit flowers in order to obtain nectar and pollen; therefore, flowers may be an excellent location for males to find mates [7]. Some males diptera usually lingers around and repeatedly flying near the flower and acts like a pollinator but instead searching for mate.

**Other rewards**

Some flowers also offer fatty oils as rewards for pollinators and mainly for bees where they use the oil mixed with pollen for larval provisioning and for water-resistant call lining and these bees generally has specialization to gather oils with their forelegs that equipped with special combs, brushes, and hair tufts [95]. Apart from that, weeds can also act as a source of floral reward in order to maintain the survivability of pollinators to maintain diversity and enhance crop yields by providing food sources for pollinators that requires pollen and nectar to survive and to prepare their food, this can be done letting the weeds to grow on roadside or fallow land and also through a proper planned establishment on bunds in agriculture land to support pollinators diversity [109].

## Conclusion

In conclusion, the plant-pollinator interactions may result in successful crop pollination where the attractant and rewards are heavily dependent on each other. Most pollinators attracted to plants that invest more on advertisement and/or rewards such as larger display, bright colour, good scent compared to those that does not and while visiting the flower both parties will gain benefits in pollination and plant reproduction as well as pollinators fitness. Pollinators may also revisit and return to plant that provide greater rewards based on the experienced and this indirectly ensure the pollination process to occurs effectively. Even after decades of studies, there is still a vast knowledge and great opportunity to uncover about the pollination and pollinators. Many more questions are likely to arises especially involving the pollination and pollinator relationships in environment affected by human activities. The investigation of the issues requires new approaches and methods that resulted in better understanding of the significance of insects in pollination, especially plant strategies to attract pollinators as well as how pollinator react and responds to it. It is important to study the behavioural of insects, their neurological processes and routes with regards to the attractant and rewards. Moreover, more detailed investigations must be done in determining the content of carbohydrates, lipids, proteins and other compound in pollen and nectar and how it can greatly influence pollinator's visitations. Thus, further studies into the chemical interactions between pollinators, visitors, and flowering plants with the recent advancement in technology is required in order to develop best pollinator management techniques for the cultivation of human food crops. Identifying nutrient rewards in fruits and connecting them to plant attractants may provide more information into the relationship between fruit nutrients and attractants. Although flower provide their own rewards for insects, studies such as addition of fragrance or attractant in fruits may also increase visitation by insects and improve fruit quality that may contributes to the successful of crop production and increase economic value of pollination services. Therefore, additional fragrance or attractant such as sugar solution or even palm wine may be also useful in attraction of insect's visitation.

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Arasoglu, T., et al., Synthesis, characterization, and antibacterial activity of juglone encapsulated PLGA nanoparticles. Journal of applied microbiology, 2017. 123 (6): p. 1407-1419.

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**Genetic Analysis Related To Organized Genetic Changes in Potato And Processed Potatoes**

Gulfidan Kuyumcu[1*], Muhammed Majed Abed[2]

Author Addresses: [1] Samsun Ondokuz Mayıs University, Faculty of Agriculture, Department of Agricultural Biotechnology, Samsun / Turkey
[2] Samsun Ondokuz Mayıs University, Faculty of Agriculture, Department of Agricultural Biotechnology, Samsun / Turkey

*Corresponding Autor: Gulfidan Kuyumcu, e-mail: ijlsb@intsa.org

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