**Gazi Mühendislik Bilimleri Dergisi**
2021, 7(3): 320-329
Araştırma Makalesi/Research Article
*https://dergipark.org.tr/gmbd*

# Myrtus communis (Mersin) ve Üzüm Çekirdeği Özütü Kullanılarak Gümüş Nanoparçacık Biyosentezi ve Karakterizasyonu

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**MAKALE BİLGİSİ**

Alınma: 13.07.2021
Kabul : 11.11.2021

***Anahtar Kelimeler***:
Biyosentez,
Gümüş,
Nanoparçacık,
Myrtus communis,
Üzüm çekirdeği,
Antibakteriyel

**ÖZ**

Bitkisel malzemeler ve özütlerin, metal nanoparçacıkların biyosentezinde önemleri giderek artmaktadır. Biyosentez, nanoparçacık sentezinde hem ekonomik hem de çevreye zararsız bir yolak sunmaktadır. Bitki özütleri ile yapıldığında, faydalı fitokimyasallar nanoparçacık yüzeyine tutunmakta ve nanoparçacığa işlevsellik kazandırmaktadır. Bu çalışmada, mersin yaprağı ve üzüm çekirdeğinin basit sulu özütleri kullanılarak gümüş nanoparçacıkları sentezlenmiştir. Nanoparçacıklar UV-Vis spektrofotometri, XRD, SEM ve Raman spektrofotometrisi ile karakterize edilmiştir. Ortalama boyu 10-30 nm olan küre parçacıklar elde edilmiştir. Nanoparçacıkların antibakteriyel etkisi Gram-pozitif ve Gram-negatif model organizmalarda test edilmiştir. Biyosentezle üretilen nanoparçacıkların, geleneksel yöntemle sentezlenen nanoparçacıklara veya bitki özütlerinin sulu çözeltilerine göre çok daha yüksek antibakteriyel aktivite gösterdikleri belirlenmiştir.

https://dx.doi.org/10.30855/gmbd.2021.03.14

# Biosynthesis and Characterization of Silver Nanoparticles Using Myrtus Communis (Myrtle) and Grape Seed Extract

**ARTICLE INFO**

Recevied: 13.07.2021
Accepted: 11.11.2021

***Keywords:***
Biosynthesis,
Silver,
Nanoparticles,
Myrtus communis,
Grapeseed,
Antibacterial

**ABSTRACT**

Plant based materials and extracts have gained an increased importance for biosynthesis of metallic nanoparticles. Biosynthesis offers an economical and environmentally friendly pathway for nanoparticle synthesis. When done with plant extracts, beneficial phytochemicals get absorbed on the nanoparticles and provide functionality. In this study, we have used simple water extracts of myrtle and grape seed to facilitate the synthesis of silver nanoparticles. The nanoparticles were characterized via UV-Vis spectroscopy, XRD, SEM and Raman spectroscopy. Average size of 10-30 nm spherical particles was obtained. The antibacterial activity of the nanoparticles was tested on Gram-positive and Gram-negative model organisms. Biosynthesized nanoparticles showed superior antibacterial activity compared to conventionally synthesized nanoparticles or soluble plant extracts.

https://dx.doi.org/10.30855/gmbd.2021.03.14

## 1. INTRODUCTION *(GİRİŞ)*

Silver nano particles are being utilized in various fields, ranging from medical devices to textiles [1] Due to their unique inherent antibacterial properties, they have been used as antibacterial agents since ancient times [2]. In fact, before the introduction of modern antibiotics, silver in the form of aqueous colloidal suspensions were used orally and topically until the first half of the 20$^{th}$ century [2, 3]. Silver

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**To cite this article**: M. Güngörmüş "Synthesis and Characterization of Silver Nanoparticles Using Myrtus Communis (Myrtle) and Grape Seed Extract", *Gazi Journal of Engineering Sciences*, vol.7, no.3, pp. 320-329, 2021.
doi:10.30855/gmbd.2021.03.14

nanoparticles exert their antibacterial effects mainly by releasing silver cations. Silver cations are electron-pair acceptors with affinity to sulfur and nitrogen. Therefore, they can interact with the sulfur-containing thiol groups and nitrogen-containing amino groups of proteins, nucleic acids and membrane lipids, and disrupt their functions [4]. Because of the wide range of possibilities for silver cations to interact with biological processes, a generalized description of the antibacterial action of silver has not yet been possible [5].

Silver nanoparticles are synthesized by the reduction of silver cations. The reduction reaction can be facilitated through physical, chemical, and biological means. Physical methods include evaporation-condensation, pyrolysis, and spark discharging [6-8]. Physical methods have been shown to be environmentally safe since they include no hazardous chemicals. However, they also have low yields, require high energy consumption, and the size/shape of the particles are difficult to control [1].

Chemical methods involve a metal precursor in the form of soluble salt and a reducing agent. The reduction of the silver ions is followed by nucleation and growth of nanoparticles. Usually a capping-agent is also added to control the size and to increase the stability of the particles [9]. Chemical methods are usually simple, low cost and high yield methods, however, the reducing agents used, such as borohydride or β-mercaptoethanol, are hazardous to living organisms [10]. The toxic chemicals create environmental concerns, especially with large scale manufacturing, and medical concerns, as the toxic chemicals adsorbed to the nanoparticles poses risks to living organisms [11].

Biosynthesis and/or green synthesis methods have emerged as alternatives to the methods mentioned above. The term "green synthesis" is usually used when the reduction of metallic ions is achieved via mild or environmentally safe reducing agents, such as polysaccharides, aldehydes, or irradiation [12-15]. Biosynthesis, on the other hand, includes use of biological organisms (microbial synthesis), such as bacteria, fungi, algae, etc., or molecules obtained from biological organisms [16]. Microbial synthesis can be achieved via utilizing the natural biochemical processes within a microorganism or via genetically engineering the organism to tailor the nanoparticle formation [17, 18]. Microbial synthesis has been shown to be suitable for removing heavy metal ions from environment in the form of metallic nanoparticles [17]. However, when large scale manufacturing of nanoparticles are considered, microbial synthesis poses challenges such as requirement of specialized bioreactors and aseptic working conditions [16]. Therefore, biosynthesis of nanoparticles using plant extracts have gained increased attention. In this method, various active biomolecules, such as proteins, sugars, alkaloids, terpenoids, phenolics, etc., present in the plant extracts act as the reducing- and capping-agent during particle formation.

Biosynthesis using plant extracts have several advantages compared to the techniques discussed above. First, the extracts used are considered environmentally safe and non-hazardous [19]. The extraction and synthesis procedures are simple compared to physical or microbial techniques. There is also an economical advantage since extracts of waste products can be used as well [20]. One of the most important premises of biosynthesis is the potential of beneficial biomolecules being adsorbed on the synthesized nanoparticles [21, 22]. This results in nanoparticles that are already loaded with beneficial biomolecules without the need for immobilization or functionalization steps.

In this study, we have used extracts from *Myrtus communis* leaves and grape seeds to investigate their efficiency in synthesizing silver nanoparticles. *Myrtus communis* leaves contain high amounts of sugars, tannins, flavonoids, and volatile oils, making the extract from the leaves a suitable reducing agent for metallic nanoparticle synthesis [23]. Major components of the leaf extracts have been shown to have antibacterial, anticancer, antiviral, and antioxidant properties [24]. The major component of grape seed extract is gallic acid and it has been shown to inhibit growth and promote apoptosis of cancerous cells [25].

We have compared the physical and antimicrobial characteristics of the particles synthesized using borohydride and the plant extracts as reducing agents. The physical properties were characterized via UV-Vis. spectroscopy, X-Ray diffractometry (XRD), Raman spectroscopy and scanning electron microscopy (SEM). The antimicrobial activity was tested against a gram negative (*E. coli*) and a gram positive (*S. mutans*) strain in liquid media.

## 2. MATERIALS AND METHONDS *(MATERYAL VE METOT)*

### 2.1. Preparation of Plant Extracts *(Bitki Özütlerinin Hazırlanması)*

Dried *Myrtus communis* leaves and grape seed powder from *Vitis vinifera* were obtained commercially. The extracts were prepared using water extraction [26]. Briefly, 100 g of plant material was weighed and added to 500 ml of distilled water at 60°C. The mixture was stirred via a magnetic stirrer on a temperature controlled hot plate for 1 hr., cooled to room temperature and filtered with Whatman filter number 41 (Merck & Co., Kenilworth, NJ, USA) The extracts were prepared freshly for each nanoparticle synthesis batch.

### 2.2. Synthesis of Nanoparticles with Sodium Borohydride *(Nanoparçacıkların Sodyum Borohidrür ile Sentezlenmesi)*

Nanoparticles synthesized via chemical method using sodium borohydride ($NaBH_4$) [27] as the reducing agent were used as control group. 2 mM $NaBH_4$ (Sigma Aldrich, St. Louis, MO, USA) and 20 mM tri-sodium citrate ($Na_3C_6H_5O_7$) (Merck & Co., Kenilworth, NJ, USA) mixture was prepared in 250 ml of ice-cold distilled water. The mixture was kept in an ice bath to prevent degradation of $NaBH_4$. 1 mM silver nitrate ($AgNO_3$) (Merck & Co., Kenilworth, NJ, USA) prepared in 2 ml distilled water was added on top of the mixture by dripping at ~1 drop/sec. The mixture was stirred via a magnetic stirrer for 30 min in an ice bath. At the end of 30 min, the mixture was centrifuged at 5000 rcf for 1 hr. The supernatant was discarded, and the pellet was resuspended in 25 ml distilled water. The centrifugation and resuspension was repeated two more times. After the last centrifugation step, the pellet was dried under vacuum for 24 hours.

### 2.3. Synthesis of Nanoparticles with Plant Extracts *(Nanoparçacıkların Bitki Özütleri ile Sentezlenmesi)*

1 mM $AgNO_3$ Merck & Co., Kenilworth, NJ, USA) prepared in 2 ml distilled water was added on top of the plant extracts at 60°C by dripping at ~1 drop/sec. The mixture was stirred via a magnetic stirrer for 30 min on a temperature controlled hot plate. At the end of 30 min, the mixture was centrifuged at 5000 rcf for 1 hr. The supernatant was discarded, and the pellet was resuspended in 25 ml distilled water. The centrifugation and resuspension were repeated two more times. After the last centrifugation step, the pellet was dried under vacuum for 24 hours.

### 2.4. UV-Vis Spectroscopy *(UV-Vis Spektroskopisi)*

UV-Vis spectroscopy was performed using a Varioskan Flash micro-plate reader (Fischer Scientific Inc., Hampton, NY, USA). When the synthesis of the particles was finished, 200 µl aliquots of the synthesis solutions were transferred to a 96 well plate in triplicates. Absorbance values between 300 – 600 nm wavelength were recorded and the average of the three measurements were taken.

### 2.5. XRD Analysis *(XRD Analizi)*

The XRD analyses were performed using a Miniflex 600 diffractometer (Rigaku Corp., Tokyo, Japan) employing 0.154056 nm Cu Kα radiation at a 40 kV accelerating voltage and 15 mA current. Data were collected in the range $2\theta = 20°–80°$ using a 0.01° step size and 1°/min scan speed.

### 2.6. SEM Analysis *(Taramalı Elektron Mikroskopi Analizi)*

SEM analyses on dried nanoparticles were performed using a Hitachi SU5000 field emission SEM (Hitachi High Technologies Corp., Tokyo, Japan) at 10 kV accelerating voltage.

### 2.7. Raman Spectroscopy Analysis *(Raman Spektroskopisi Analizi)*

The Raman spectra of the nanoparticles was recorded using a NRS 4500 confocal Raman microscope (JASCO Co., Tokyo, Japan). The data was collected at a spectral range of 120–1900 $cm^{-1}$ at 784 nm laser wavelength and 2 mW laser power.

### 2.8. Antibacterial Activity Tests *(Antibakteriyel Aktivite Testleri)*

Antibacterial activity of the plant extracts themselves and the nanoparticles synthesized using the plant extracts were tested in liquid media. *E. coli* (ATCC 25922) and *S. mutans* (ATCC 25175) were used as model organisms. Frozen stocks of the bacteria were grown overnight in Luria-Bertani (LB) Broth (Sigma Aldrich, St. Louis, MO, USA) at 37°C. Fresh passages from the overnight cultures were grown until mid-log phase.

LB broth containing 1:10 and 1:100 dilutions of the plant extracts were used to test the plant extracts themselves. LB broth containing 60 ng/ml silver nanoparticles was used to test the nanoparticles. LB broth containing no additives was used as negative control. 300 µl of the prepared broths were placed in a 96-well microtiter plate and the bacteria were added to their respective broths to a final concentration of $5x10^4$ CFU/ml. Blanks were prepared for each group with the same type of media with no bacteria added. The bacteria were grown at 37°C with 200 rpm orbital

shaking. The growth of the bacteria was followed at 90 minutes intervals using a Varioskan Flash micro-plate reader (Fischer Scientific Inc., Hampton, NY, USA) at 600 nm wavelength for 16 hours. The absorbance values from the blanks of each group were subtracted from the absorbance values obtained from the respective groups.

Growth rates of the bacteria were calculated using Eq. 1 [28]

$$\mu = \frac{(\ln N_t - \ln N_{t0})}{t - t_0} \quad (1)$$

where μ is the growth rate (hour$^{-1}$), $t_0$ and $t$ are the onset and end time of the log phase, respectively (hour), and $N_{t0}$ and $N_t$ are the number of bacteria at t0 and t, respectively (bacteria/ml). Conversion of OD to bacteria/ml was done using previously reported conversion factors [29, 30]

### 2.9. Statistical Analysis (*İstatiatik Analiz)*

For antibacterial activity tests, one-way repeated measures analysis of variance (RM-ANOVA) test was performed, using SPSS 25.0 (IBM, NY, USA). *P* values < 0.05 were regarded to be statistically significant.

## 3. RESULTS AND DISCUSSION *(BULGULAR VE TARTIŞMA)*

### 3.1. UV-Vis Spectroscopy *(UV-Vis Spektroskopisi)*

UV-Vis spectroscopy yielded characteristic absorbance peaks for silver nanoparticles. The $\lambda_{max}$ for nanoparticles synthesized with $NaBH_4$, Myrtus communis and grape seed were measured as 382 nm, 378 nm and 402 nm, respectively. (Fig. 1)

UV-Vis spectroscopy is the first and easiest step for the verification and characterization of nanoparticles. The absorbance arises from the localized surface plasmon resonance created by the oscillations of free electrons in the nanoparticles excited by light [31]. The position of the peak is an indicator of the size of the particles, the intensity of the peak is an indicator of the number of particles and the overall peak width is an indicator of the size distribution of the particles. The peaks obtained in this study indicate particles were in between 10-30 nm [32]. The size of the particles was further investigated by SEM.

### 3.2. XRD Analysis *(XRD Analizi)*

XRD analyses of the particles showed that the resulting particles were a mixture of Ag, AgO and $Ag_2O$ nanoparticles. $NaBH_4$ yielded Ag and $Ag_2O$ only, while *Myrtus communis* and grape seed extracts yielded a mixture of the three. (Fig. 2)

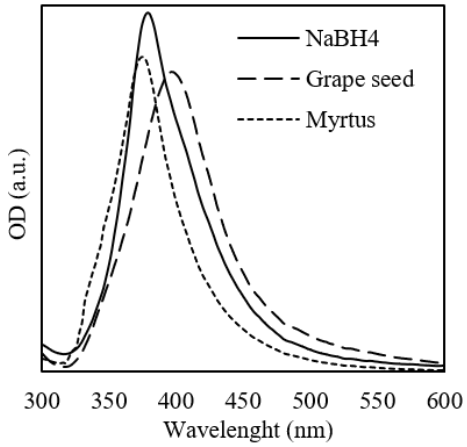


Figure 1. UV-Vis absorption spectra of the nanoparticles *(Nanoparçacıkların UV-Vis absorpsiyon spektraları)*

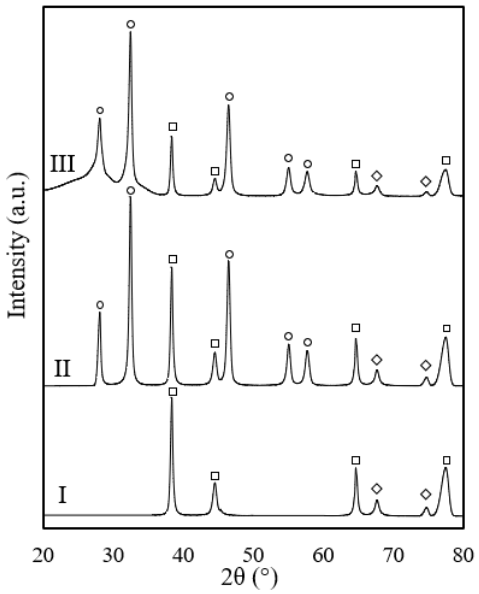


Figure 2. XRD patterns of the nanoparticles (I: $NaBH_4$, II: *Myrtus communis*, III: Grape seed. □: Metallic AgNP, ○: AgO, ◊:$Ag_2O$) *(Nanoparçacıkların XRD paternleri (I: NaBH4, II: Myrtus communis, III: Üzüm çekirdeği. □: Ag, ○: AgO, ◊:Ag2O)*

AgO and $Ag_2O$ are the two stable phases of silver oxide. These phases can be deoxidized and decomposed into metallic Ag [33]. Silver oxides also possess strong antibacterial properties like metallic AgNPs [34, 35]. In fact, it is suggested that the antibacterial activity of silver oxides is higher compared to metallic AgNPs [36]. Therefore, the lack of ultrapure metallic AgNPs was not a concern within the context of this study.

### 3.3. SEM Analysis *(Taramalı Elektron Mikroskopi Analizi)*

SEM analyses showed that roughly spherical particles with a wide size distribution was obtained in all cases. (Fig. 3) The size of the particles ranged from 5-30 nm with majority of the particles being on the larger side. These findings were in accordance with the UV-Vis spectroscopy findings (Fig. 1).

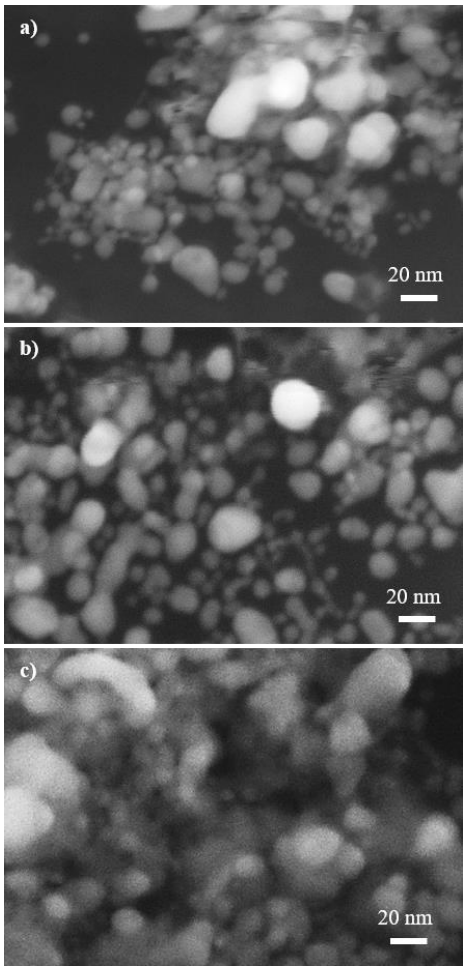


Figure 3. SEM images of the nanoparticles synthesized with borohydrite (a), Myrtus communis extract (b) and grape seed extract (c) *(Borohidrür (a), Myrtus communis özütü (b) ve üzüm çekirdeği özütü (c) ile sentezlenen nanoparçacıkların SEM görüntüleri)*

### 3.4. Raman Spectroscopy Analysis *(Raman Spektroskopisi Analizi)*

Raman spectroscopy was performed to investigate possible phytochemicals absorbed on the silver nanoparticles. The Raman spectra obtained from the nanoparticles used in the study are shown in Fig. 4. The observed Raman spectra bands and their tentative assignments are shown in Table 1. Spectral differences and some common bands were observed. The ~220 cm-1 band attributed to Ag-O or Ag-N bands were observed in all samples. Bands attributed to in-plane bending C=O bonds and out of plane bending C-H bonds were also observed in all specimens. As expected, bands attributed to plant biomass and pigments were only observed in nanoparticles synthesized with plant extracts.

Raman spectroscopy has become increasingly popular for investigating biomolecules and their orientations on metallic surfaces [37]. The origin of the observed spectra is believed to originate by the surface plasmon resonance and the charge transfer interactions between the molecules on the surface [38]. Our findings indicate that phytochemicals present in the plant extracts have indeed been absorbed on the silver nanoparticles. Slight variations observed in the spectra are expected due to the differences in the compositions of the two different plants.

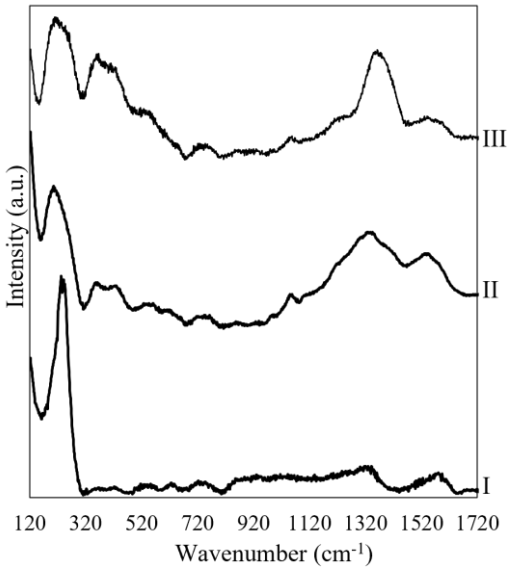


Figure 4. Background-corrected Raman spectra of the nanoparticles synthesized with borohydride (I), *Myrtus communis* extract (II) and grape seed extract (III) *(Borohidrür (I), Myrtus communis özütü (II) ve üzüm çekirdeği özütü (III) ile sentezlenen nanoparçacıkların zemindeğer düzeltmesi yapılmış Raman spektraları)*

Table 1. Raman spectra bands of the nanoparticles and their tentative assignments. (BH: Borohydride, Mc: Myrtus communis extract, Gs: Grapeseed extract) *(Nanoparçacıkların Raman bantları ve atamaları (BH: Borohidrür, Ms: Myrtus communis özütü, Gs: Üzüm çekirdeği özütü)*

| Band ($cm^{-1}$) | | | Assignment |
|---|---|---|---|
| **BH** | **Mc** | **Gs** | |
| 229 | 222 | 221 | Ag-N, Ag-O [39] |
| - | 380 | 380 | Plant biomass (cellulose) [40] |
| 560 | 560 | 560 | in-plane bending C=O bonds [41] |
| 739 | 739 | 739 | out of plane bending C-H bonds [41] |
| - | 1056 | 1056 | in-plane bending C-H bonds [41] |
| - | 1207 | 1207 | C-X stretching vibrations [41] |
| 1307 | 1310 | - | In-plane bending C-H bonds [41] |
| - | - | 1350 | CH– and $–CH_2$ deformations [42] |
| - | 1527 | 1527 | Pigments (chlorophyll and carotenoids) [40] |
| 1570 | - | - | C-COO- [43] |

### 3.5. Antibacterial Activity Tests *(Antibakteriyel Aktivite Testleri)*

Antibacterial activity tests were performed using a gram-positive and a gram-negative strain in liquid media. The growth rates and the growth curves of *S. mutans* and *E. coli* are shown in Table 2 and Fig. 5, respectively. The percent growth inhibition rates are shown in Fig. 6.

For both strains, maximum inhibition was observed with AgNPs synthesized with *M. communis* extract at ~80% inhibition. AgNPs synthesized with grape seed extract showed a slightly lower and similar inhibition at ~75% inhibition. AgNPs synthesized with borohydride showed and inhibition ~60%. The 1:10 dilution of the plant extracts showed a moderate inhibition at ~25-35% inhibition. At 1:100 dilution almost no inhibition was observed.

These results indicate that biosynthesized nanoparticles had a more potent antibacterial effect compared to the extracts alone. This finding is noteworthy since when the extracts are added to the growth media, the available concentration of phytochemicals is expected to be higher, compared to biosynthesized nanoparticles. However, as our findings indicate, biosynthesized nanoparticles had a more potent antibacterial effect compared to the extracts themselves.

One possible explanation to this observation is that when the plant extracts are added to the growth media, they may be getting inactivated via degradation, oxidation, interactions with the components of the media, or other mechanisms. Raman spectroscopy analyses showed that the nanoparticles synthesized with plant extracts carry phytochemicals through absorption to their surface. As has been reported before, activity or stability of proteins can be enhanced through absorption onto nanoparticles [44, 45]. A similar effect may be responsible for the superior antibacterial effects on nanoparticles. Absorption to the nanoparticle surface may be increasing the stability of the phytochemicals by preventing/delaying inactivation.

Another possible explanation for this observation is a synergistic antibacterial activity of silver ions and the phytocemicals. Similar synergistic effects have been reported for silver nanoparticles and conventional antibiotics, antimicrobial peptides, and other plant extracts [46-48].

Further studies elaborating the individual components of the plant extracts and the antimicrobial effect mechanisms will help to elucidate the observations reported here.

Table 2. Calculated growth rate of the bacteria (Group names are the same as indicated in Fig. 5) *(Bakterilerin hesaplanmış büyüme oranları (Grup isimleri Şekil 5'te belirtilenlerle aynıdır))*

| Group | Growth rate ($hour^{-1}$) | |
|---|---|---|
| | *E. coli* | *S. mutans* |
| I | 0.1335 | 0,1114 |
| II | 0.1786 | 0,1649 |
| III | 0.1808 | 0,1659 |
| IV | 0.2928 | 0,2603 |
| V | 0.3368 | 0,2833 |
| VI | 0.4385 | 0,3567 |
| VII | 0.4502 | 0,3765 |
| VIII | 0.4519 | 0,3911 |

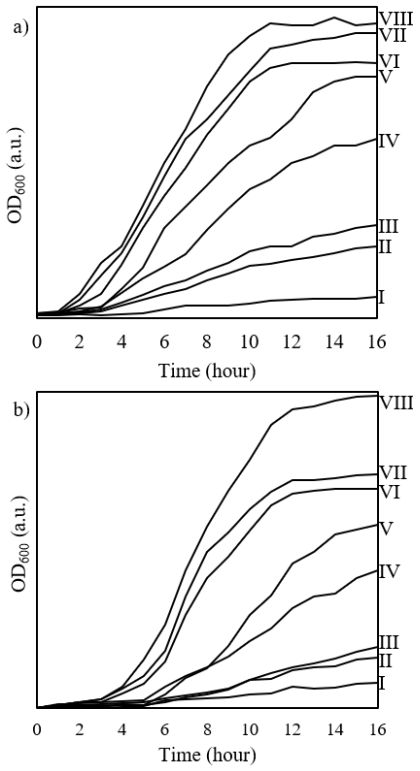


Figure 5. Growth curves of *E. coli* (a) and *S. mutans* (b) (I: *M. communis*-AgNP, II: Grape seed-AgNP, III: Borohydride-AgNP, IV: 1:10 *M. communis* extract, V: 1:10 grape seed extract, VI: 1:100 *M. communis* extract, VII: 1:100 grape seed extract, VIII: Control) *(E. coli (a) ve S. mutans (b) büyüme eğrileri (I: M. communis-AgNP, II: Üzüm çekirdeği-AgNP, III: Borohydrür-AgNP, IV: 1:10 M. communis özütü, V: 1:10 üzüm çekirdeği özütü, VI: 1:100 M. communis özütü, VII: 1:100 üzüm çekirdeği özütü, VIII: Kontrol))*

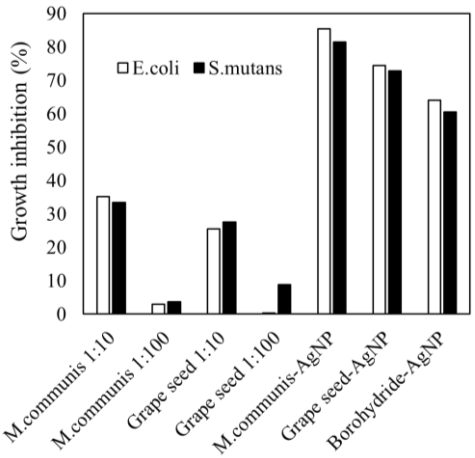


Figure 6. Percent inhibition rates of the tested agents against *E. coli* and *S. mutans*. *(Test edilen ajanların E. coli ve S. mutans'a karşı yüzde inhibisyon oranları)*

## 4. CONCLUSIONS *(SONUÇ)*

Biosynthesis of silver nanoparticles offer a myriad of advantages, such as being surfactant free, economically, and environmentally viable, and resulting in particles loaded with phytochemicals. In this study we have used two plant extracts prepared by simple water extraction for biosynthesis of silver / silver oxide nanoparticles. We have shown that silver nanoparticles and the phytochemicals absorbed on them show a synergistic effect, resulting in superior antibacterial activity compared to nanoparticles synthesized by conventional solution-based techniques. Moreover, we have shown that biosynthesized nanoparticles show superior antibacterial activity compared to the plant extracts themselves. We propose the observed effect is due to stabilization of the phytochemicals when absorbed onto the nanoparticles. Further studies elaborating the individual components of the plant extracts and the antimicrobial effect mechanisms will help to elucidate the observations reported here.

## ACKNOWLEDGMENT *(TEŞEKKÜR)*

This study was carried out using the facilities of Ankara Yıldırım Beyazıt University, Central Research Laboratory Application and Research Center (MERLAB).

## CONFLICT OF INTEREST STATEMENT *(ÇIKAR ÇATIŞMASI BİLDİRİMİ)*

The author declare that there is no conflict of interest.

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