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Lemon Peel Extract for Synthesizing Non-Toxic Silver Nanoparticles through One-Step Microwave-Accelerated Scheme

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ABSTRACT

In this study, biofunctionalized globular or oval shaped silver nanoparticles were obtained by using aqueous extract of lemon peel (*Citrus limon*) via microwave-accelerated heating system. While UV–visible spectroscopy, FTIR and XRD analyses were applied to recognize the formation of nano-silver, TEM and Zeta analysis were employed to reveal their morphological features. UV–vis spectrum of fabricated AgNPs indicated its characteristic maximum absorbance at 445 nm. Phytosynthesized silver nanoparticles were poly-dispersed with Z-average value of 41.86 nm, and showed excellent stability for several months with no aggregation and agglomeration. The non-toxic nature of the developed Ag nanoparticles was further confirmed by applying on healthy mouse fibroblast L929 cell line, which may expand their potentials for further studies related to medical science and other biological applications.

Research Article

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Mikrodalga Hızlandırmalı Yöntemi İle Limon Kabuk Ekstraktsiyonu Kullanarak Non-toksik Gümüş Nanopartiküllerin Sentezlenmesi

ÖZET

Bu çalışmada, mikrodalga hızlandırmalı ısıtma sistemi yöntemi ile limon kabuk (*Citrus limon*) ekstraktsiyonu kullanarak biyofonksiyonel küre şeklinde veya oval şekilli gümüş nanopartiküller elde edilmiştir. Çalışmada gümüş nanopartiküllerin oluşumunu tanımak için UV–vis absorpsiyon spektroskopi, FTIR ve XRD analizleri uygulanırken, nanopartiküllerin morfolojik özelliklerini ortaya çıkarmak için TEM ve Zeta analizi uygulanılmıştır. Üretilmiş AgNP'lerin UV-vis spektrumu, 445 nm'de karakteristik maksimum absorbansını göstermiştir. Fitosentezlenmiş gümüş nanopartiküller, Z-ortalama değeri 41,86 nm olan poli-dispersiyon haline getirilmesi ile birlikte herhangi bir agregasyon ve agglomerasyon olmadan birkaç ay boyunca mükemmel stabilite sergilemiştir. Daha sonra, sağlıklı fare fibroblast hücreleri (L929 hücre çizgisi) üzerindeki biyosentezlenmiş bu Ag NP'lerin non-toksik özelliği doğrulanmıştır. Bu durum, tıp bilimlerin yanı sıra çeşitli biyolojik uygulamalarda da bu nanopartiküllerin potansiyellerini göstermektedir.

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INTRODUCTION

Nanotechnology based researches are generating an emergent sense of great enthusiasm in every aspect of science and technology in today's world (Mohanpuria et al., 2008). Due to improved magnetic, optical, catalytic, and electrical properties as well as precise and unique morphological features, noble metallic nanoparticles and their nano oxides are being utilized in multidisciplinary processes for producing diverse merchandises (Tsuji et al., 2005). Among all metallic NPs, nano-silver (AgNPs) is one of the most attractive nanomaterials which have different types of profitable applications. They have particularly been exploited for many antimicrobial purposes as well as the applications of anti-inflammatory and antiseptic treatments (Ahamed et al., 2010). Besides, several studies also observed the *in vitro* anti-carcinogenic effects of nano silver (Ahamed et al., 2010). They further are being used as a valuable product of electronic manufacturing, for optical devices and

various kinds of sensors as well as in the purpose of food packaging and storing, in textile sectors during coating process (Furno et al., 2004; Murphy et al., 2008; Zhang et al., 2016). Referring the production of silver and other metallic nanoparticles, most conventional physical schemes are costly and demand enormous consumption of energy, whereas chemical approaches sometime require toxic solvents such as sodium borohydride, dimethylformamide (DMF), paraffin wax, polyvinylpyrrolidone (PVP) etc., which might cause serious damage to the surrounding environment (Lee et al., 2007; Iravani et al., 2014). Hence, exploitation of green, non-hazardous, biocompatible, and environmentally benign biological synthesis methods using lower organisms and plant materials for nanoparticle production earns advantages over other established approaches. However, plant-mediated synthesis of nanoparticles could be the best choice since the use of lower group of organisms for NP production is very complex and time consuming due to the fact that isolation and maintenance of microbes requires multiple steps, which might also increase the cost of whole process (Lin et al., 2010). Besides, a wide range of microorganisms are pathogenic or harmful to human being that limits their feasibility (Silver, 2003; Ahmed et al., 2003). On the other hand, vast quantities of plants and plant parts boasted with various primary and secondary metabolites are easily accessible and abundant naturally, and therefore, plant extracts can provide an inexpensive, fast and competent synthesis scheme for the production of crystalline metallic nanoparticles with desirable shapes and sizes (Thakkar et al., 2010; Rai and Yadav, 2013).

This study utilized lemon (*Citrus limon*) peel extract as reducing and stabilizing agents for the production of silver nanoparticles. This is due to the fact that citrus fruits are one of the prominent fruit crops, and globally, their production is increasing day by day. Commercially, 34% of annually produced oranges and lemons are being consumed for juice production, resulting approximately 44% peel as waste product (Li et al., 2006). Lemon and other citrus peel is a rich source of various active phytochemicals such as polyphenols, carotenoids, essential oils, amino acids, dietary fibers, minerals and vitamins, with very high functional properties (Bocco et al., 1998).

For increasing reaction rate within a short period of time, microwave heating was adjusted during the synthesis based on temperature and time which was very facile and affordable. For nanoparticle synthesis, microwave-assisted heating system has become an effective and fruitful practice, lately. This is due to the fact that the microwave system supplies rapid initial heating which facilitates immediate utilization of reactants by amplifying reaction kinetics. It also offers standardized nucleation and homogenous growth conditions in the synthesis medium, which accelerate rapid reducing and capping rates, and thus, helps to yield higher production of NPs within a short period of time (Jahan et al., 2019). Moreover, it was observed that microwave heating-based synthesis reduced the aggregation rate of NPs compared to synthesizing at room temperature (Kudle et al., 2013).

Succeeding the biosynthesis of AgNPs, detail characterization was conducted through different techniques to reveal their shape, size and other morphological features. Moreover, cytotoxic effect of phytosynthesized silver nanoparticles was also investigated to measure their biocompatibility.

MATERIALS and METHODS

Plant Extract Preparation and Fabrication of Ag-Nanoparticles

In this study, silver nitrate ($AgNO_3$) and other required chemicals were analytical grade, which were collected from Sigma-Aldrich (St. Louis, MO, USA). Properly dried and autoclaved instruments were used in every step of this experiment. Fresh lemon fruits were washed thoroughly with ultra-purified deionized water. Afterwards, yellow colored fruit peel was cut off and chopped into small pieces using a sterilized kitchen paring knife.

Around 5 g of the sliced peel were put into 50 ml of ultra-purified deionized water (1:10 ratio), and placed into the laboratory-grade microwave closed vessel (Milestone Microsynth microwave labstation) system for approximately 2 minutes at the irradiation level of 700 W. Afterwards, Whatman No. 1 filter paper (pore size: 11 µm) was employed for removing debris from the extract solution was then filtered using and kept at 4°C. The ratio of plant material and water taken in this study, and selection of the shortest extraction time with highest irradiation wavelength (700 W) were due to increase the extraction yield with minimal evaporation of reagents, which also prevented the degradation of phenolic and other biocompounds (M'hiri et al., 2014).

Fabrication of silver nanoparticles was started combining 10 ml extract, 90ml ultra-purified water and 0.017 g of silver nitrate (1mM), which was placed in a laboratory-grade microwave for 25 minutes at 90°C by the highest heating level of 300 W with continuous stirring.

Following the color changing, Whatman Grade No.5 filter paper with 2.5 µm pore size was used to eliminate large discarded particles from the sample solutions; then centrifuged 3-4 times at 5000 rpm for 15 minutes at 4°C. Lastly, the purified precipitate was dried under vacuum condition; and powdered AgNPs stocked in a dark colored vial and stocked up at 4°C for further experimentation. Following the color changing, the solution was filtered using Whatman Grade No.5 filter

paper with 2.5 µm pore size to eliminate large discarded particles from the sample solutions and then centrifuged 3-4 times at 5000 rpm for 15 minutes at 4°C for further purification. Lastly, to avoid the photo-oxidation (Grillet et al., 2013), the purified precipitate was dried under vacuum condition, and using a dark colored vial, the powdered AgNPs was stocked at 4°C.

Characterization of Synthesized AgNPs

Optical property of developed NPs was investigated by UV-vis spectroscopy (UV-1700 spectrophotometer, Shimadzu, Europe) in which ultra-purified water was taken as blank and spectral peaks were collected in the range of 200 - 800 nm. Shimadzu IR Prestige-21 FTIR-ATR instrument was used to read IR spectroscopic graph. XRD patterns with a step size of 0.02 was taken at the range of 2h from 10° to 80° via X-ray diffraction (PANalytical Empyrean model) plan for recognizing crystalline nature of synthesized NPs; XRD graph was regenerated by the Origin 8.5 software. Transmission Electron Microscopy (TEM 1400, JEOL, Tokyo, Japan) at increased speed voltage of 120 kV was used for imaging to reveal morphological features of AgNPs. Finally, particle average size distribution, and potential value of developed NPs were determined using Zeta sizer (model name: Zetasizer nano ZS, Malvern Instruments Ltd., UK).

Cytotoxicity Study of silver nanoparticles

The cytotoxic activity of biosynthesized AgNPs was evaluated on L929 mouse fibroblast cell lines. Using XTT assay [2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] the percentage of viable cells in culture media was determined by observing optical intensity of these viable cells. For maintaining the culture of cell line, DMEM-F12 medium was utilized supplemented with 10% fetal bovine serum and penicillium-streptomycin, which was incubated at 37°C with 5% CO_2 air flow. After incubation, completely affluent cells were detached from the upper layers of the cell containing vessels using Trypsin. Afterward, by staining with Trypan blue, the viable cells were identified, and counted from the detached cultured cells. Prior to applying silver nanoparticles into cell medium, 1 mL medium was used to adjust the density of obtained viable cells to 10^6. Aiming this adjustment, 100 µL of cell suspension was plated in every well of sterile 96-well flat bottom microplate (BD, Biosciences) within a short period of time. Before incubating at 37°C, the biosynthesized AgNPs was added to cultured cells with an increasing concentration (0, 0.1, 0.25, 0.5, 1, 2.5 and 5 µg/mL). After 24 hours of incubation, old medium was removed, and 100 µL XTT solution (with 0.5 mg/ml DMEM, which was adjusted to Phenazine methosulfate (7.5 µg/mL)) containing 100ml fresh medium was added, and again incubated for 4 hours at the same temperature. Later on, a multiplate reader (model: Lab-Line Instruments, Melrose Park, IL, USA) at 450 nm was employed for measuring optical density (OD) of active viable cells from the suspension. Lastly, the cell viability was calculated in percentage (%) following this equation (Sahu et al., 2016):

$$\text{Cell viability (\%)} = \frac{\text{OD of specimen}}{\text{OD of control}} \times 100$$

RESULTS and DISCUSSION

The main objective of this study was to synthesize silver nanoparticles by lemon peel extract using the lowest concentration of AgNO3 solution within a significantly shortest period of time. For optimizing the synthesis protocol, different synthesis cycles were designed according to the variation of the ratio of plant extract and salt solution as well as temperature at different wavelengths and time durations. The color change in the synthesis media was used as the indication of the completion of reaction (Figure 1).

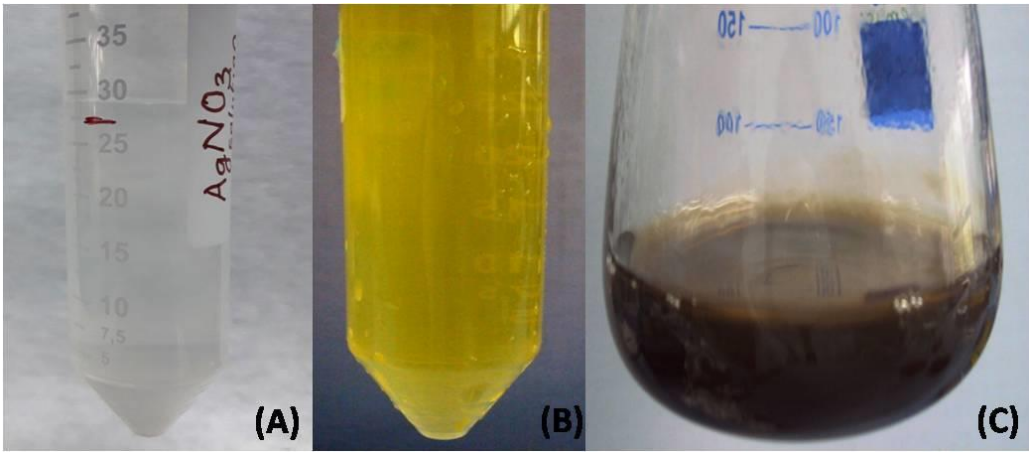

Figure 1. Silver nitrate solution (A), aqueous extract of lemon peel (B) and color changed after the synthesis of silver nanoparticles (C).

Şekil 1. Gümüş nitrat çözeltisi (A), limon kabuğu (B) 'nin sulu ekstraktı ve gümüş nanopartiküllerin (C) sentezinden sonra renk değişikliği

Since metallic nanoparticles show their characteristic and distinctive absorbance peak due to surface plasmon resonance (Iravani, 2011), the formation of AgNPs in the reaction medium was confirmed shortly after the synthesis through UV-vis spectroscopy. The UV–vis spectrum of fabricated AgNPs in aqueous colloidal solution revealed its maximum absorbance at 445 nm (Figure 2), which is expected characteristic surface plasmon of nano-silver (Yin et al., 2002; Kaviya et al., 2011).

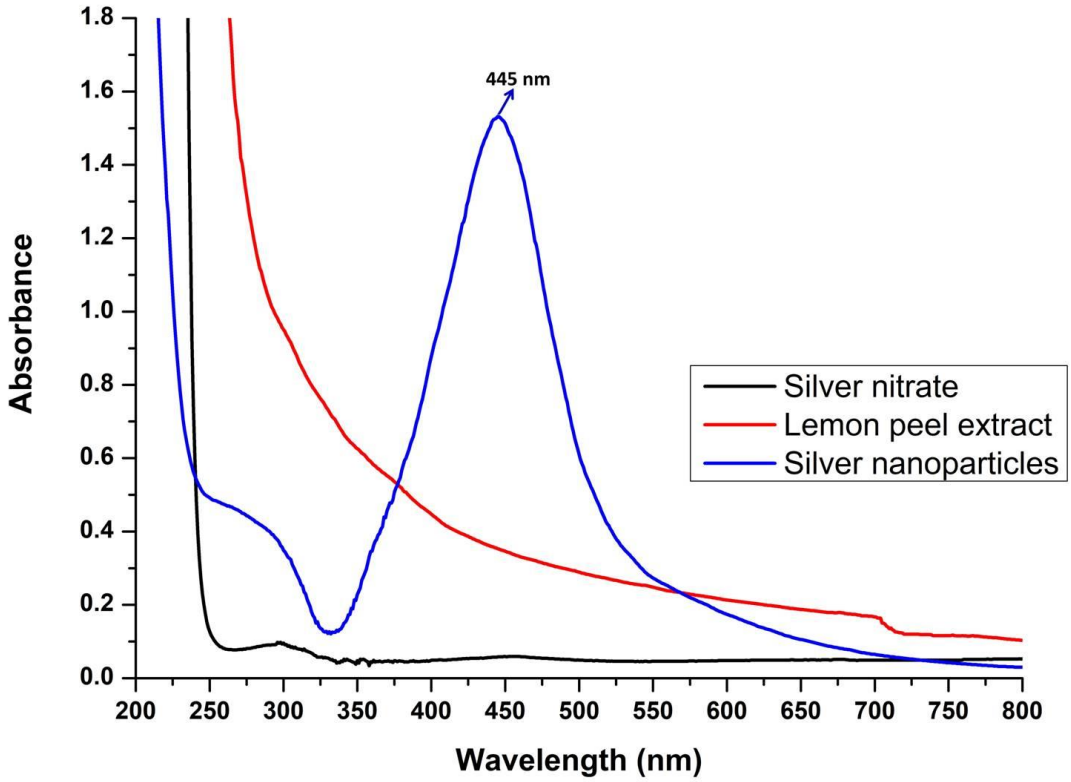

Figure 2. UV–Vis spectra of biosynthesized silver nanoparticles using lemon peel extract.
Şekil 2. Limon kabuğu ekstraktı kullanılarak biyosentezlenmiş gümüş nanopartiküllerinin UV-Vis spektrumları.

Furthermore, X-ray diffraction (XRD) study of synthesized silver NPs corroborated the presence of crystalline metallic silver as shown in Figure 3. XRD graph revealed four significant reflections at 38.11°, 44.30°, 64.54° and 77.50° which matched respectively with the characteristic Bragg's diffraction plans i.e. (111), (200), (220) and (311) for face-centered cubic crystalline structures of metallic silver, based on the database of the JCPDS file no: 04–0783 (Hamedi et al., 2017). Moreover, it is noticeable and striking that the matching of these significant peaks with standard data was found for their relevant peak positions and relative intensities (Roy et al., 2014). In addition, the existence of only distinctive peaks devoid of any additional reflections is convincing in term of confirming the presence of pure nano-silver, phytosynthesized by lemon peel extract.

Figure 4 demonstrates a TEM image that has been traced from the drop-coated specimen layer of silver nanoparticles accomplished from lemon peel extract. The AgNPs in the TEM image are dispersed as roughly globular, spherical and oval in shape with the size ranged from 7.5 nm to 69.83 nm. Besides, Figure 5 revealed particle size distribution and zeta potential measurement of biosynthesized AgNPs using lemon peel extract. Particle dimension distribution in term of Z-average value was measured as 41.86 nm, which was consistent with TEM outcomes. On the other hand, average zeta potential value that refers the surface charge of nano-silver in colloidal solution was determined as -18.70 mV. In a suspension, high negative or positive potential value confirmed excellent physical consistency of colloidal nanoparticles as a result of electrostatic repulsion of individual particles (Kumar and Dixit, 2017). Besides, z-values larger than ±30 mV indicate monodisperse nature of colloidal nano-suspension, whereas lower potentials, less ±5 mV signify the presence of aggregation and agglomeration (Gumustas et al., 2017). Based on TEM and Zeta analyses, it can be concluded that AgNPs biosynthesized using lemon peel extract showed their polydisperse nature, which were distributed with the absence of aggregation and agglomeration.

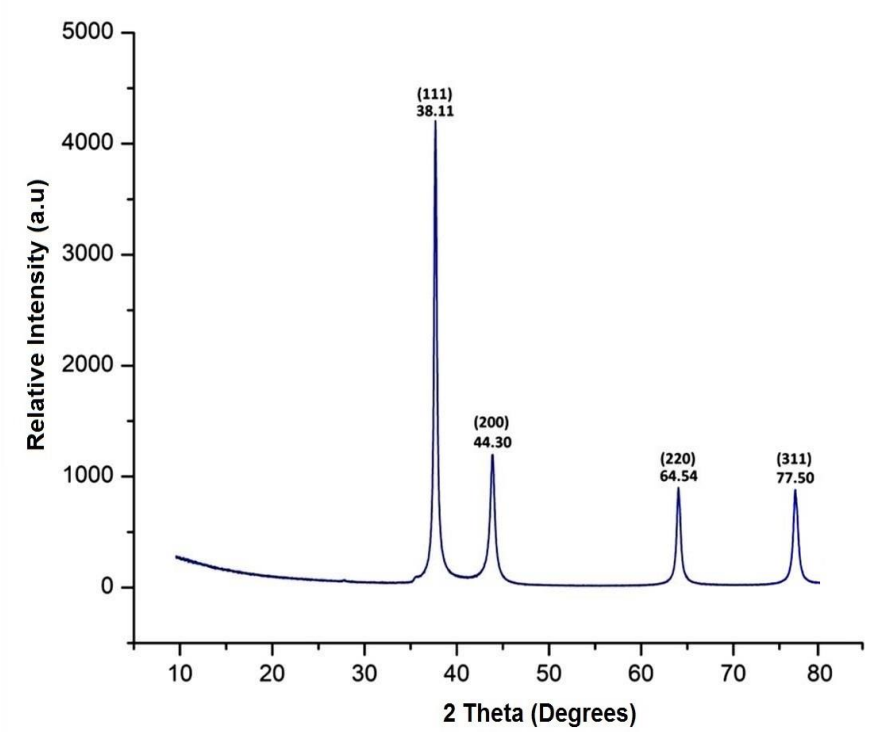

Figure 3. XRD graph of phytosynthesized silver nanoparticles.
Şekil 3. Fitosentezlenmiş gümüş nanoparçacıkların XRD grafiği

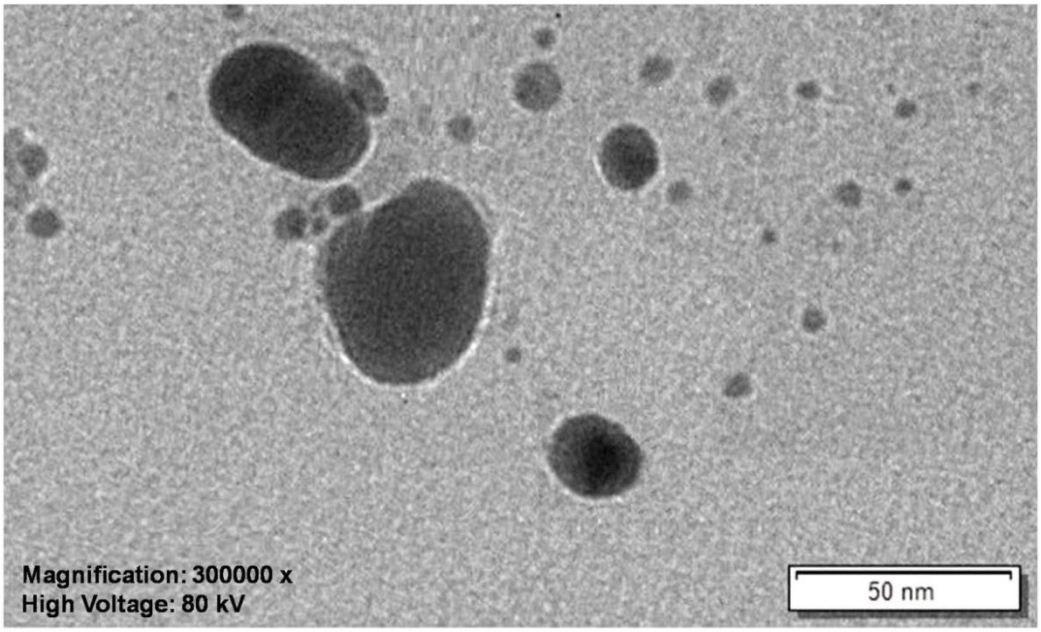

Figure 4. TEM image of biosynthesized silver nanoparticles at 50 nm scale.
Şekil 4. 50 nm ölçeğinde biyosentezlenmiş gümüş nanoparçacıkların TEM görüntüsü

There are some previous studies synthesized AgNPs by using *Citrus* spp. extracts (Basavegowda and Lee, 2013; Kahrilas et al., 2014; Nisha et al., 2014; Ayinde et al., 2019). Among them, so far one study has been conducted by lemon peel extract revealing spherical and irregular shaped AgNPs with the size range between 17.3 and 61.2 nm, by keeping the synthesis medium at room temperature for 5 hours (Nisha et al., 2014). However, this study has been so far the first attempt of microwave-assisted green synthesis using lemon peel extract which is comparatively very new and faster method yielding significantly smaller AgNPs in roughly globular, spherical and oval shape; which are more functional in their application due to their high surface area.

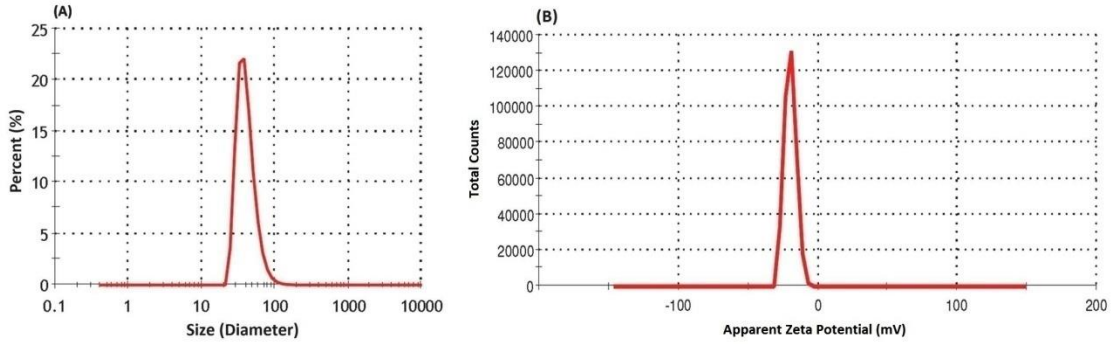

Figure 5. Particle size distribution (A) and zeta potential value (B) of fabricated silver nanoparticles.
Şekil 5. Fabrikasyon gümüş nanoparçacıkların parçacık boyutu dağılımı (A) ve zeta potansiyel değeri (B).

Fourier transform infrared (FTIR) analysis was applied to identify different functional groups of biomolecules available in lemon peel extract which played as reducer and stabilized by creating a coating layer on the surface of the NPs (Usha et al., 2017). The FTIR spectrum of AgNPs (Figure 6) showed the band at 3392.79 cm^{-1} corresponds to carboxylic acid and other intermolecular bonded strong O-H stretching. Peaks were observed mainly at 2926.01 cm^{-1} is responsible for saturated alkane (-C-H) medium stretching and the spectrum at 1641.42 cm^{-1} is responsible for strong alkene monosubstituted (C=C) stretching. A strong C-O stretching of primary alcohol bond represents at the peak of 1058.92 cm^{-1}; the spectrum at 974.05 cm^{-1} represents a strong alkene disubstituted (trans) bonds and finally, the peak at 786.96 cm^{-1} demonstrated medium alkene (C=C) trisubstituted bond. FTIR spectra therefore suggested that fabricated AgNPs were attached by various active phytochemicals of lemon peel extract, which created coating layer encapsulating the NPs to stabilize them and thereby, prevent aggregation, and agglomeration (Hind et al., 2001; Faraji et al., 2010).

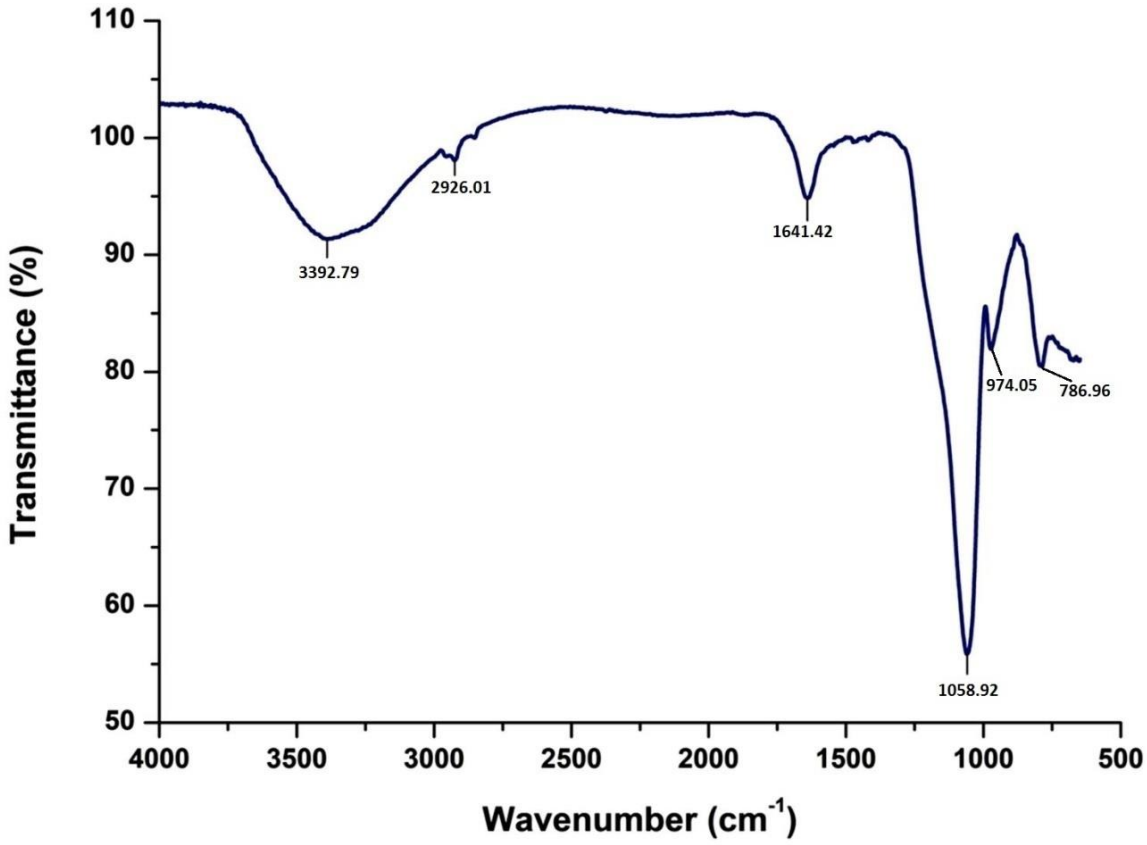

Figure 6. Graphical presentation of IR spectra of biosynthesized silver nanoparticles.
Şekil 6. Biyosentezlenmiş gümüş nanopartiküllerin IR spektrumlarının grafik sunumu

Finding the level of biocompatibility is very significant for NPs to estimation their suitability for any kind of biological application. Green synthesis of nanomaterials can offer the privilege of producing non-hazardous and biocompatible NPs since no toxic chemicals are required in this process. However, different nanomaterials especially the metallic NPs themselves can be toxic to human body or other mammal cells because of their remarkable and significant actions against diverse microbial strains attributable to their remarkable chemical, physical and biochemical properties (Dizaj et al., 2014; Seil and Webster, 2012). Aiming this, cytotoxic effect of biosynthesized AgNPs with different concentration (0, 0.1, 0.25, 0.5, 1, 2.5 and 5 µg/mL) were measured in this study by using healthy regular mouse fibroblasts cell line (L929). After treated with silver nanoparticles, pigmentation rate of functional mitochondrial enzymes of viable cells was observed by using XTT reagent. Cell viability in terms of mitochondrial enzyme activities was measured as the absorbance of optical density, which is directly proportional to the cell viability. Following the cell viability (%) formula, the percentage of cell viability was more than 90% in each concentration of nanoparticles (Figure 7), which is considered as non-toxic (López-García *et al.* 2014).

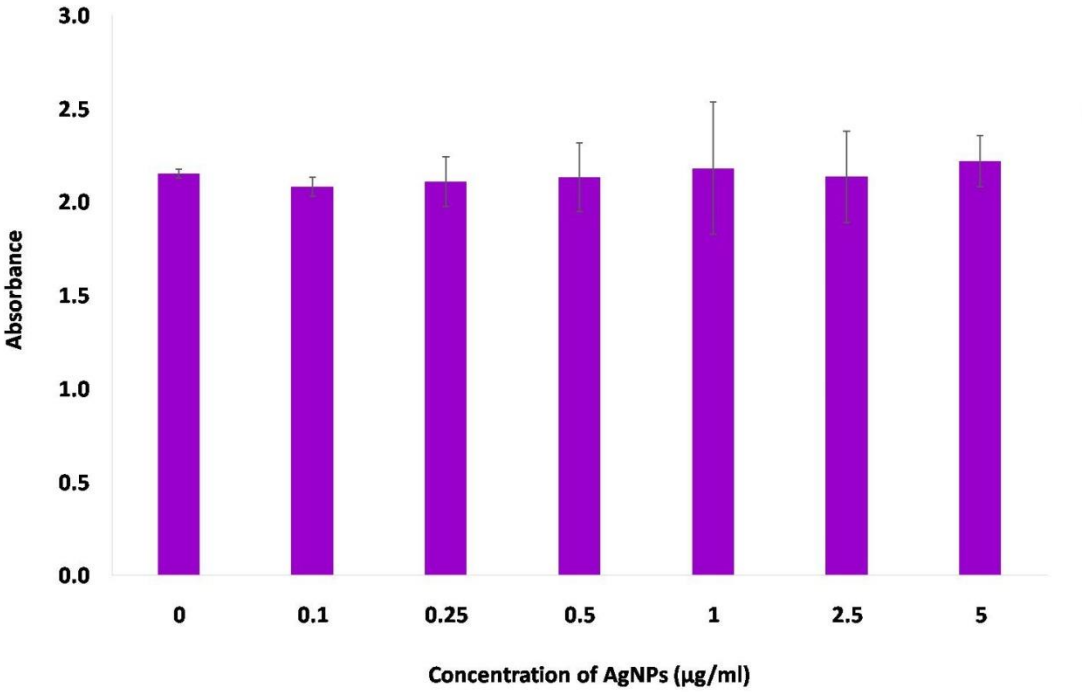

Figure 7. Graph showing the non-toxic nature of biosynthesized silver nanoparticles on healthy regular mouse fibroblasts cell line (L929) via the absorbance of viable cells.

Şekil 7. Canlı hücrelerin absorbansı yoluyla sağlıklı düzenli fare fibroblast hücre hattı (L929) üzerinde biyosentezlenmiş gümüş nanoparçacıkların toksik olmayan doğasını gösteren grafik.

The concentration-dependent cytotoxicity of silver nanoparticles can be influenced by the size and shape of the particles as well as the synthesizing method (Liu et al., 2010). For instance, in A549 cells silver nanowires showed more adverse effects on cytotoxic parameters than spherical and different other shaped silver nanoparticles (Stoehr et al., 2011). On the other hand, particle size was reported to be directly proportional to the cell viability; among 5 nm, 25 nm, 50 nm, and 110 nm AgNPs, 5 nm sized particles were the most toxic whereas 110 nm showed the least cytotoxicity on cochlear cells (HaCaT and HEI-OC1 cell lines) at the concentration below 3 µg / ml (Perde-Schrepler et al., 2019), which is even lower concentration presented in this study. Additionally, aggregated AgNPs could form hydrodynamic diameter larger than macro or bulk particles, which lead them to show fewer effects on the cellular level; and therefore, high concentration is required to confirm their cytotoxicity (Lankoff et al., 2012). Here, it is important that the obtained NPs in this study did not show any aggregation which might be one of the possible reasons for supporting its non-toxic nature at such lower concentration.

The exposure time of the NPs to the cell is also a vital factor for showing their dose-dependent toxicity. For example, the toxicity threshold (TT) of AgNPs in U937 cell line was found at 2.0 ppm after 4 hours of

treatment, but the percentage of cell viability started to decline significantly at 0.05 ppm after 24 hours of treatment (Kaba and Egorova, 2015). Since the mouse fibroblasts cell line (L929) was treated over 24 hours with different concentration (0, 0.1, 0.25, 0.5, 1, 2.5 and 5 µg/mL) and none of them showed any toxicity in this study, it could be the strong indication for non-toxic nature of the obtained AgNPs.

Considerable number of other studies also reported the toxic effect of silver nanoparticles at 5 ppm or lower concentration on different mammalian cell lines including mouse spermatogonial, mouse macrophages (RAW 264.7), healthy lung epithelial (L132), human liver cancer (HepG2), human ovarian cancer (A2780), human breast cancer (MCF-7 and MDA-MB 231) cell lines (Braydich-Stolle et al., 2005; Park et al., 2010; Faedmaleki et al., 2014; Akter et al., 2018).

CONCLUSION

This study developed a rapid, simple, inexpensive, and environment friendly approach of microwave-assisted fabrication of silver nanoparticles using lemon (*Citrus limon*) peel extract. The reduction of ionic silver to their nanoparticles and succeeding capping for stabilizing nano-silvers was suggested to happen throughout the participation of various active phytochemicals of this extract. The biosynthesized AgNPs produced in this study showed their polydisperse nature, and confirmed excellent stability for several months with the absence of aggregation and agglomeration. Besides, the non-toxic nature of this AgNPs at the given concentration may expand their relevance for further studies related to medical science, healthcare, veterinary medicine, cosmetics, food industry, nanobiotechnology etc.

Researchers Contribution Rate Declaration Summary

The authors declare that they have contributed equally to the article.

Conflicts of Interest Statement

None of the authors had any financial or personal relationships with other individuals or organizations that might inappropriately influence their work during the submission process.

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