

Enhancing the antibacterial activity of the biosynthesized silver nanoparticles by "püse"

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ABSTRACT

Background and Aims: The silver nanoparticles (Ag NPs) synthesized by green synthesis have antimicrobial properties and their potential to be used in the medical field is quite high. Püse, also called "black doctor", is widely used as a treatment for the health problems of animals and in folk medicine in Turkish nomad culture. It has been reported that Ag NPs biosynthesized with *Juglans regia* leaf extracts have antibacterial activity. The aim and importance of this study is the preparation of a mixture of "püse" and Ag NPs for the first time and the investigation of its antibacterial activity.

Methods: In this paper, Ag NPs were synthesized by using *J. regia* leaves and morphological characteristics of the NPs obtained were determined using TEM. The formation of NPs was identified using an UV-Vis spectrophotometer. The antibacterial activity was investigated by combining the obtained and characterized Ag NPs with the "püse". The antibacterial activity of the mixture was tested on Gram (+) bacteria and Gram (-) bacter.

Results: The mixture that was prepared with püse and Ag NPs showed higher antibacterial activity than antibiotics.

Conclusion: The positive results showed that this mixture has the potential to be used as a preparation especially in the treatment of animal wounds and the skin infections caused by bacteria.

Keywords: Biosynthesized, silver nanoparticles, antibacterial activity, *Juglans regia*, püse

INTRODUCTION

It is a well-known fact that nanosilvers, which are obtained from silver particles with a size of 1-100 nm, have antibacterial, antiviral, and antifungal properties and the potential to be used in common areas such as food packaging, textile, electronic devices, cosmetics, medical devices, and materials. (McShan, Ray, & Yu, 2014; Luther, Schmidt, Diendorf, Epple, & Dringen, 2012; You et al., 2012). The antimicrobial properties of nanosilvers are described via the release of Ag⁺ ions and inhibition of the transcription which means survival for the living (Wang et al., 2013). The release of Ag⁺ ions from AgNPs into cells is called the "Trojan horse" effect and the smaller the nanosilver particles are, the higher the surface area to volume ratio and the more Ag⁺ ions that are released (Cameron, Hosseinian, & Willmore, 2018).

Humankind have used plants as a source of medicine and treatment since ancient times and the methods found through trial and error were transferred from generation to generation (Gurib-Fakim, 2006). Ethnobotany was defined by Schultes in 1967 as the relationship between man and his ambient vegetation (Gurib-Fakim, 2006). In other words, ethnobotany is a discipline that takes place in the natural and social sciences and deals with relationships between humans and plants (Garnatje, Peñuelas, & Val-lès, 2017). There are numerous studies conducted with plants for human health and well-being. Drugs of plant origin are based

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on ethnobotany information. In fact, ethnobotany-oriented approaches have become more powerful than random tests for the detection and identification of bioactive molecules from plants (Garnatje et al., 2017). Ethnobotany helps to find the when, where, and how of plants with different medicinal properties (de la Parra & Quave, 2017).

Pinus nigra is a species that grows in Southern Europe and Anatolia, especially in the Mediterranean Basin. It can grow from anywhere between 100-1800 meters above sea level, but mostly from 500-1600 meters. It is considered one of the most important coniferous species (Topacoglu, 2013). The various parts of the *Pinus* species derived from resin, cones, tar, and pine have ethnobotany and ethnomedical uses for skin problems, asthma, bronchitis, colds, coughs, and wounds (Kızıllarlan & Sevgi, 2013).

In nomadic culture (Turks having nomadic life in Anatolia and Rumelia), "püse" which is an important drug in every situation and obtained from pine, cedar and juniper, also known as the "black doctor", is used for the treatment of health problems such as abdominal pain, diarrhea, nausea, gas pains, bullet wounds, scabies, fissures in mumps, hands, and feet (Ak, 2017).

J. regia, a member of the Juglandaceae family, more commonly known as walnut, is a forest tree grown in temperate regions (Faraz, Islam, & Rehman, 2012; Alam, Islam, Kamal, & Gan, 2018). Walnut leaves are used as a source of health materials and bioactive molecules (Santos et al., 2013). Antibacterial and biofilm-inhibiting effects of walnuts on *Pseudomonas aeruginosa* have been shown by Dolatabadi, Moghadam, & Mahdavi-Ourtakand in 2018. The green husks, bark, leaves, and fruit of walnut are used in pharmacology for a variety of purposes and studies have shown that products of walnut and special compounds of juglone have antimicrobial properties (Dolatabadi et al., 2018; Zhao, Jiang, Liu, & Li, 2014; Shah, Ganesh, & Akthar, 2013; Moravej et al., 2016; Kaur, Michael, Arora, Härkönen, & Kumar, 2003).

The aim of this study was to investigate the antibacterial activity of the mixture obtained by combining püse, used as ethnomedical in the nomadic culture, with synthesized NPs. For this purpose, synthesized AgNPs from the *J. regia* leaf was used. The morphological characteristics of the obtained mixture were determined using a Transmission Electron Microscopy (TEM) and particular size analyzer. The formation of kinetics from the mixture was identified using a UV-Vis spectrophotometer. The antibacterial activity of synthesized AgNPs from the *J. regia* leaf and "püse" mixture was tested on various Gram (+) and Gram (-) bacteria strains.

MATERIALS AND METHODS

Preparation of "Püse"

The resinous parts of *P. nigra* wood was divided into thin pieces of 30-40 cm, placed on top of a smooth and curved surface, and covered with soil so that there was no hole. A fire was lit on the soil and a small exit path opened on the side of the slope of the device. The resin that melted with the temperature of the burning fire was isolated from the wood flow into the pit and collected from there as "püse".

AgNPs synthesis

One g of *J. regia* leaves, purchased from an herbal store, were mixed with 100 ml of distilled water (pH= 6.9), then boiled using a magnetic stirrer for 30 minutes, and the resulting plant extract was cooled at room temperature. The mixture brought to room temperature (25 °C) was filtered with Whatman No 1 filter paper, then 50 ml of the prepared leaf extract was taken, and 2 mM 50 ml AgNO₃ solution was added. The final solution was incubated for about 24 hours under room conditions to reduce the colloidal NPs.

Characterization of Ag NPs

The UV-vis absorbance spectrum of the final concentration was recorded in the wavelength range of 300-700 nm by the Eon™ Microplate Spectrophotometer operating at a resolution of 10 nm. On the spectra, the *J. regia* leaves extract diluted at a ratio of 1:1 with distilled water was taken as the reference solution, and the extract-AgNO₃ mixture was examined in the presence of this reference solution. A 96 well plate was used in the spectrophotometer. The morphological characteristics of the Ag NPs were determined by the ZEIS-LEO 906E Brand-Model Transmission Electron Microscopy (TEM).

Preparation of Ag NPs and püse mixture

Water molecules were completely removed by an evaporation method from the Ag NPs which were biosynthesized by *J. regia* leaves in an aqueous solution. Then the extract of püse was added to the Ag NPs. The mixture was kept in an ultrasonic bath for 30 minutes to prevent agglomeration.

Antibacterial activity

The antibacterial activity of the synthesized Ag NPs using an extract of *J. regia* leaves and "püse" (local name) was carried out in Gram (+) bacteria (*S. aureus* ATCC 29213,) and Gram (-) bacteria (*E. coli* ATCC 25922,) by a disc diffusion method on solid media (Russell & Furr, 1977; Irobi, Moo-Young, Anderson, & Daramola, 1994). The bacterial strains were grown for 12 hours at 37 °C in Müller Hinton Agar (MHA, pH= 7.3). The density of the bacterial suspensions were adjusted to 0.5 McFarland turbidity standard (1.5 x 10⁸ CFU/mL) by diluted 1:100 with MHA. Subsequently, 100 µl of bacteria cells were spread onto MHA Petri dishes using a sterile spreader. Then, sterile Whatman filter papers (6-mm-diameters) were placed over the medium using sterile forceps and impregnated with 50 µl (8.5 µg/ disc) of the Ag NPs and *J. regia* leaves extract and pure püse as the mixture. The *J. regia* leaves extract was used as the negative control and doxycycline 30 µg/disc and amoxicillin + clavulanic acid 20 µg/disc were used as the positive control. The plates were placed for 24 hours at 37°C in an incubator. The growth inhibition zones of each disc were measured in millimeters. All experiments were duplicated.

RESULTS AND DISCUSSION

Nowadays, nanoparticles synthesized using silver, gold, zinc, copper and hybrid Ag / ZnO draw interest especially in the biomedical field as antibacterial, antifungal, antiviral, anti-inflammatory, anti-angiogenic, and anti-cancer agents (Chaloupka, Malam, & Seifalian, 2010; Strayer, Ocoy, Tan, Jones, & Paret, 2016). The physical and chemical methods used to produce nanoparticles

have major disadvantages, as they contain expensive, raw, toxic, and dangerous chemicals (Some et al., 2019). To overcome these problems, studies have focused on the production of nanoparticles by biosynthesis. For this purpose, various organisms including plants, algae, and microorganisms are used as biosynthesis agents in the synthesis of NPs (Shah, Fawcett, Sharma, Tripathy, & Poinern, 2015; Roy, Bulut, Some, Mandal, & Yilmaz, 2019). In addition, biomolecules including DNA, enzymes, peptides, proteins, and plant extracts are also used in NP synthesis (Duman, Ocsoy, & Kup, 2016). Especially because plant extracts are very cheap, scalable, easily available, stable, and require no special storage conditions compared to other biomolecules (Ocsoy et al., 2013; Demirbas, Welt, & Ocsoy, 2016).

In this study, the *J. regia* aqueous extract was mixed with an AgNO_3 solution, and the color of the initially transparent mixture changed to brown within about 30 minutes by reducing the silver ions (Figures 1A and 1B). Color change is an indication that Ag NPs are formed, and this change is due to the superficial plasmon vibrations of nanoparticles (Forough & Farhadi, 2010). The reduction of pure Ag^+ ions to Ag^0 was monitored by measuring the UV-vis spectrum of the reaction. The absorbance of the mixtures whose color turned brown showed SPR (Surface Plasmon Resonance) peaks at 420 nm after 24 hours (Figure 2). The morphological properties of the NPs were determined by TEM. As shown in Figure 3, the sizes of the NPs are between 8 and 35 nm and the shape of the NPs is spherical.

The mechanism of how Ag NPs biosynthesized using plant extracts has not been fully clarified (Okaiyeto, Ojemaye, Hoppe,



Figure 1. A-B The images of synthesized Ag NPs, (A) *J. regia* extract, (B) Ag NPs and *J. regia* leaf mixture.

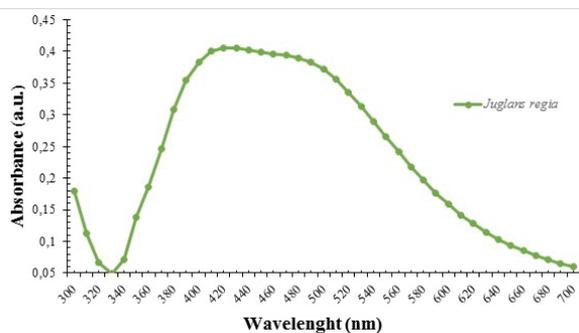


Figure 2. UV-Vis absorbance spectrums of Ag NPs and *J. regia* leaf mixture.

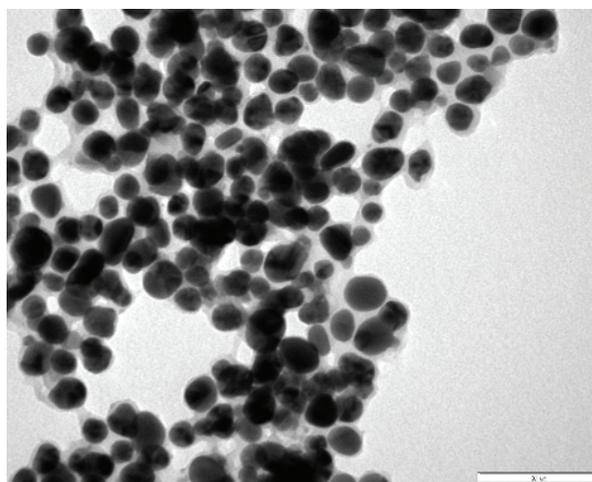


Figure 3. Transmission Electron Microscopy (TEM) imaging of Ag NPs.

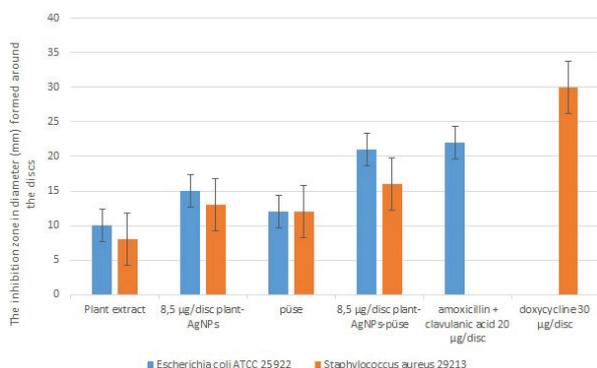
Mabinya, & Okoh, 2019). However, studies have shown that phytochemical compounds such as phenolic, flavonoids, carbohydrates, terpenoids, and proteins are responsible for the reduction and capping/stabilization of Ag NPs (Al-Sheddi et al., 2018; Ramesh, Devi, Battu, & Basavaiah, 2018; Karatoprak et al., 2017; Ocsoy et al., 2017). In other words, these compounds contain active oxygen that has the potential to donate electrons for the reduction of silver precursor into Ag NPs. Triterpenoic acid, a terpenoid type, present in plant extract has a $-\text{COO}-$ group which acts as a chelating group for capping of the synthesized Ag NPs (Ramesh et al., 2018).

Studies have shown that *J. regia* leaf extracts are rich in phenolic acids, tannins, essential fatty acids, ascorbic acid, flavonoids, caffeic acid, 26 terpenoid substances, and paracomaric acid (Mohammadi, Mirzaei, Azizi, Rouzbehi, & Delaviz, 2012; Nahrstedt, Vetter, & Hammerschmidt, 1981; Paudel, Satyal, Dosoky, Maharjan, & Setzer, 2013). Therefore, it can be said that the biomolecules responsible for the formation of NPs synthesized with *J. regia* leaf extract may be phenolic acids, flavonoids, and terpenoids.

The antibacterial activity of the synthesized Ag NPs using extracts of *J. regia* leaves and "püse" was carried out in Gram (+) bacteria (*S. aureus* ATCC 29213,) and Gram (-) bacteria (*E. coli* ATCC 25922.). The results are reported in Table 1 and Figure 4.

Table 1. The inhibition zone in diameter (mm) formed around the discs impregnated with mixtures in various volume.

Bacterial species	The inhibition zone in diameter (mm) formed around the discs impregnated with mixtures in various volume					
	Plant extract	8,5 µg/disc plant-AgNPs	püse	8,5 µg/disc AgNPs-püse	amoxicillin + Clavulanic acid 20 µg/disc	Doxycycline 30 µg/disc
<i>E. coli</i>	10	15	12	21	22	
<i>S. aureus</i>	8	13	12	16		30

**Figure 4.** The antibacterial activity of the synthesized Ag NPs using extracts of *J. regia* leaves and "püse".

Antibacterial activities of the walnut leaf extract, which was used as the control group, on *E. coli* and *S. aureus* bacteria were investigated. According to the obtained data, the walnut leaf extract produced an inhibition zone of 8-and10-mm diameter on both bacterial species, respectively. These results are consistent with the results of Pereira et al. Flavonoids, phenolic, and other compounds contained in natural products have antibacterial effects. For example, quercetin in *J. regia* has been at least partially attributed to inhibition of DNA gyrase (Pereira et al., 2007). The antibacterial activity of the walnut-Ag NPs, which was another control group, formed inhibition zones of 15-and 13-mm diameters on both bacterial species, respectively.

P. nigra is an important plant used in the production of folk medicine in the Turkish nomadic culture. They used the resin of the pine tree produced by a special method in the treatment of various diseases that occur in both people and the animals they raised (Alptekin, 2019).

In this study, antibacterial activities of püse on *E. coli* and *S. aureus* bacteria were investigated as another control group. The results showed that there was a 12 mm diameter inhibition zone on two bacterial species.

Silver NPs were synthesized by using walnut leaves as described in the method section and a mixture was obtained with the synthesized product. The antibacterial activity of this mixture on *E. coli* and *S. aureus* bacterial species were compared to the effect of antibiotics. The mixture produced a 21 mm diameter inhibition zone in *E. coli* at a concentration of 8.5 µg / disc, while amoxicillin + clavulanic acid produced 22 mm diameter at a concentration of 20 µg / disc. Here, the

concentration of antibiotic is 2.35 times the concentration of the mixture. Thus, the mixture showed a more effective antibacterial activity than the antibiotic. Likewise, when the antibacterial activity of the mixture on *S. aureus* was investigated, Doxycycline with a concentration of 30 µg / disc produced an inhibition zone of 30 mm diameter while the mixture with a concentration of 8.5 µg / disc produced an inhibition zone of 16 mm diameter. Compared to antibiotics, the mixture with a concentration of 3.5 times less was highly effective. This is a clear result of the synergistic effects of püse and silver ions whose NPs were synthesized by using a walnut extract.

Bacteria have a genetic flexibility that can manipulate threats from the environment that is dangerous to them, including antibiotics. Bacteria that develop resistance to antibiotics usually use two genetic strategies. These are mutations in the genes related to the mechanisms that the molecule will affect and a horizontal gene transfer of DNA containing resistance determinants against these molecules from one bacterium to another (Munita & Arias, 2016).

Antibiotic resistance has become a global crisis threatening human and animal health all over the world due to its increasing and unlimited use (Qiao, Ying, Singer, & Zhu, 2018). Unconscious consumption, as well as overuse of antibiotics, accelerated the emergence of antibiotic-resistant bacterial strains and antibiotic resistant genes, thereby reducing the therapeutic effect of these molecules on bacteria that cause infection in humans and animals (Qiao et al., 2018; Wright, 2010). In general, antibiotics are poorly metabolised by humans and animals and are therefore excreted as active main chemicals with urine and feces and released into the environment through wastewater and fertilizer (Qiao et al., 2018). This means that more bacteria in our environment gain resistance.

Infections associated with health care are defined as infection of patients by infectious agents while receiving health care (Haque, Sartelli, McKimm, & Bakar, 2018; Collins, 2008). Most of these infections are surgical site infections (SSIs), pneumonia, and gastrointestinal infections (Magill et al., 2014). Around 12-17 microorganisms, including *E. coli* and *S. aureus*, cause 80-87% of hospital infections (HCAs) (Sievert et al., 2013; Hidron et al., 2008; Weiner et al., 2016). This major problem, which we will have to face in the very near future, has made it necessary to find alternative solutions to antibiotics or rather to take precautions to prevent infection.

According to the results obtained in this study, this product has the potential to be converted into a commercial product

in the field of health that can be used as a disinfectant to prevent cross-contamination and also as an antibacterial product especially in animal hospitals for the treatment of various diseases.

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