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ANTIBACTERIAL AND ANTI-BIOFILM EFFECT OF SILVER NANOPARTICLES SYNTHESIZED FROM *ORIGANUM MAJORANA* AND *ECHINACEA PURPUREA* (L.) *MOENCH* PLANTS VIA GREEN SYNTHESIS

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Abstract

Objective: Nanotechnology may have a role in the fight against epidemics and antibiotic resistance and has the potential to reduce the rate of develpoment of multidrug-resistant bacterial species. The aim of this study was to determine the antimicrobial and anti-biofilm activities of *Origanum majorana* and *Echinacea purpurea* silver nanoparticles (AgNPs) against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* 25922, *Pseudomonas aeruginosa* 27853, and *Klebsiella pneumoniae* 700603.

Methods: The Minimum Inhibitory Concentration (MIC) values of AgNPs were determined by microdilution method in ELISA plates. The anti-biofilm effect of AgNPs was investigated using the crystal violet method, also in microplates.

Results: The MIC value of three isolates was 128 µg/mL for the two nanoparticles. MIC value, 256 µg/ml and 512 µg/ml were found for only *Staphylococcus aureus*. The in vitro antibiofilm effect of the AgNPs was evaluated in a dose-dependent manner. A concentration of 512 µg/ml of *Origanum majorana* AgNPs reduced 24-h biofilm formation of *Staphylococcus aureus* by 92%. An *Echinacea purpurea* (L.) AgNPs concentration of 512 µg/ml reduced the 24-hour biofilm formation of *Staphylococcus aureus* by 85%. Two different nanoparticles significantly inhibited the biofilm mass of gram-negative and gram-positive bacteria.

Conclusion: AgNPs showed antimicrobial and antibiofilm effects for standard bacterial strains. The use of AgNPs as antimicrobial agents may have promise but further research is required.

Keywords: *Silver nanoparticles, antibiotic resistance, Origanum majorana, Echinacea purpurea*.

Introduction

Nanotechnology has a wide range of application in science due to the morphology of newly synthesized nano-sized molecules and their easy dispersion in cell. Additionally, various studies in different fields have focused on the use of molecules close to atoms.[1,2] It has a wide range of uses in medicine. Nanotechnology is a potential way to prevent the spread of multidrug-resistant bacterial strains.[3] The use of silver as an antibacterial agent dates back to ancient times with historical stories.[4] In general, silver is a notorious toxic ion that causes oxidative stress and damages diverse cellular components, including cell membranes, proteins, and DNA. In contrast, elemental AgNPs are effective at minimal doses and are characterized by a slower dissolution rate.[4] AgNPs are the most widely used medical nanoparticles in diagnostic, coating and sensor applications. Additionally, AgNPs have become a promising tool for the production of new antimicrobial drugs.[3] AgNPs can be synthesized by physical, chemical and biological methods. Biological synthesis is a simple, fast, inexpensive, energy-efficient and environmentally friendly method. The synthesis of AgNPs from plant extracts can reduce side effects in terms of medical applications.[5,4]

Antibiotic resistance of bacteria has become an alarming public health problem. All societies in the world are faced with antibiotic resistance. In particular, superorganisms are predicted to kill more people than the current COVID-19 epidemic by 2050.[6] Recent efforts have been directed towards preventing new epidemics and combating antimicrobial resistance.

The biofilm is an organized community of microorganisms that adhere to various surfaces and live in a matrix of self-produced extracellular polymeric material. These microbial structures are present in almost every environment. Biofilms can develop on biological or abiotic surfaces.[7] Biofilms consist of homogeneous or heterogeneous bacterial communities surrounded by extracellular polymeric substances (EPS). Bacterial cells use EPS stick together. It binds to a surface or other cells during developmental stages. Besides polysaccharides, EPS also contain other biomolecules such as proteins, lipids and nucleic acids.

Compared to planktonic cells, biofilms are highly resistant to antimicrobial agents and disinfectants. This feature makes biofilms 100-1000 times less sensitive to antibiotics. For this reason, antibiofilm agents have become a subject of research today. Biofilms can cause significant environmental and health problems. Biofilms can cause many infections. It plays a major role, especially in nosocomial infections. It is thought that one of the reasons for biofilm resistance is the complex structure of the biofilm matrix and its diffusion barrier. The biofilm matrix makes it difficult for antimicrobial agents to reach microorganisms. AgNPs are potential therapeutic agents for biofilm inhibition.[8,9,10] These challenges have given rice to significant interest in the scientific society in developing plant-based therapeutics with antimicrobial activity as a safer, green alternative to antibiotics.[11]

Origanum majorana, also known as marjoram, is a delicate perennial herb belonging to the Lamiaceae family in the world. In addition to being endemic to Cyprus, it is also common in the Mediterranean region. Hippocrates first used marjoram as an antiseptic. It is a popular herb used as a home remedy for treating infectious diseases. *Origanum majorana* essential oil contains major metabolites such as sabinene, trans-sabinene hydrate, cis-sabinene hydrate, p-cymene and terpinen-4-ol. Various phytochemical tests performed on ethanol extracts of *Origanum majorana* revealed flavonoids, hesperidin, oleanolic acid, amentoflavone, ursolic acid, arbutin, routine, phenolic acids, rosmarinic acid, caffeic acid, catechin, coumaric acid and gallic acid.[12] Research on *Origanum majorana* has been promising because it is rich in phenolic compounds that reduce silver nitrate.[3]

Echinacea purpurea, also known as purple elderberry, belongs to the Asteraceae family and is a flowering plant native to eastern and central North America. For centuries, *E. purpurea* has been used in traditional medicine by Native American tribes. This herb is known for its potential medicinal properties, which are said to boost the immune system and reduce inflammation. Some people take Echinacea supplements to help prevent or relieve symptoms of colds and respiratory infections. They consume it as tea or extract, but scientific evidence needs to be clarified. Echinacea has been shown to reduce the severity of gingivitis and dental plaque. This herbal medicine generally does not have any side effects, it is claimed that even ingestion does not cause side effects.[13]

This study focused on the antimicrobial and anti-biofilm activities of green synthesized AgNPs from aqueous leaf extracts of *Origanum majorana* and *Echinacea purpurea* against *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Klebsiella pneumoniae* (*K. pneumoniae*) strains.

Methods

Silver Nanoparticles

In this study, two types of nanoparticles were used. AgNPs@om and AgNPs@ep were synthesized from *Origanum majorana* and *Echinacea purpurea* respectively.[2,14] The synthesis method and spectroscopic results were reported in the given references.

Bacteria Isolation

In this study, standard strains of *S. aureus* ATCC 25923, *P. aeruginosa* 27853, *E. coli* 25922, *K. pneumoniae* 700603 were used. Standard strains were removed from -80°C and inoculated onto %5 sheep blood agar and EMB agar (Oxoid, Basingstoke, UK). Incubated at 37°C for 24 hours. For experiments, the bacteria removed from the stock was passaged twice.

Antimicrobial Effect of Silver Nanoparticles

The antimicrobial activity of AgNPs synthesised from different plant extracts by green synthesis method was evaluated by broth microdilution method. MIC (Minimum Inhibitory Concentration) values of AgNPs for *S. aureus*, *E. coli*, *P. aeruginosa* and *K. pneumoniae* strains were determined by the microdilution method in 96-well ELISA plates. 100 microliters of Brain Heart Infusion (BHI) were added to the wells. 100 microliters of AgNPs prepared at a concentration of 1024 µg/ml were added to the wells and two-fold serial dilutions were made. A 0.5 McFarland turbidity standard was prepared for each microorganism and inoculated into the wells. The first well with no growth after incubation was accepted as the MIC value.

Antibiofilm Effect of Silver Nanoparticles detected by Crystal Violet Method

The effect of AgNPs on biofilm was performed in 96-well flat-bottom microplates. 180 µl of tryptic soy broth (TSB) was transferred to the wells. From a concentration of 512 µg/ml up to 32 µg/ml, AgNPs were diluted. A bacterial

suspension with 0.5 McFarland turbidity was prepared from 24-hour cultures grown on 5% sheep blood agar medium. 20µl were added. The prepared microplates were incubated for 24 hours at 37°C. The next day, microplates were emptied by inverting and washed 3 times with 200 µl phosphate buffered saline (PBS) to remove non-adherent bacteria. The plates were inverted to allow them to air dry, and the biofilm was fixed for 15 minutes by adding 200 µl of methanol. Following fixation, it was stained with 150µl of 0.1% crystal violet for 15 minutes at room temperature. The dye was drained by inverting the plates. It was washed 3 times with 200 µl of sterile physiological saline. 95% ethanol was placed on the air-dried plates and incubated for 15 minutes at room temperature. Then, the results were taken on an ELISA reader at 630 nm. The biofilm inhibition rate was calculated with the formula given below.[15]

Inhibition (%)= [1- OD sample/OD control]×100

Results

Antimicrobial Effect of AgNPs

To evaluate the antibacterial activity of nanoparticles, MIC values of 4 standard strains and MIC values of nanoparticles were determined and the results are shown in Table 1.

Table 1. MIC values of microorganisms

Microorganisms	*Echinacea purpurea* (L.) *Moench* AgNP MIC (µg/ml)	*Origanum majorana* AgNP MIC (µg/ml)
S. aureus	256	512
E. coli	128	128
P. aeruginosa	128	128
K. pneumoniae	128	128

Anti-biofilm Activity of AgNPs

In this study, the in-vitro antibiofilm effect of AgNPs against biofilm-forming bacteria was evaluated in a dose-dependent manner. It was observed that the synthesized AgNPs prevented biofilm formation to a greater extent than the negative control of the experiment. It was observed that AgNPs (512 µg/ml) synthesized using the plant extract of *S. aureus*, *Origanum majorana*, reduced biofilm formation by 92% as a result of incubation for 24 hours. It was found that biofilm formation was reduced by 85% as a result of incubation of *S. aureus* with AgNPs (512 µg/ml) synthesized using *Echinacea purpurea* (L.) Moench plant extract for 24 hours. Two different nanoparticles were also found to significantly inhibit biofilm mass. Biofilm mass was determined quantitatively in the crystal violet method (Figure 1, Figure 2).

Discussion

There has been increased interest in nanoparticles as alternative antimicrobial agents to prevent microbial resistance problems that directly threaten public health in humans and animals. Antimicrobial activity methods popular in the scientific community include agar dilution, agar well diffusion, disc diffusion, bioautographic, microdilution, and macrodilution methods. In our study, the antimicrobial effect was determined by calculating the minimum inhibitor concentration using the broth microdilution method. In addition, the crystal violet method was used to determine the inhibition of biofilm of antibiotic resistance, which is the problem of the era.

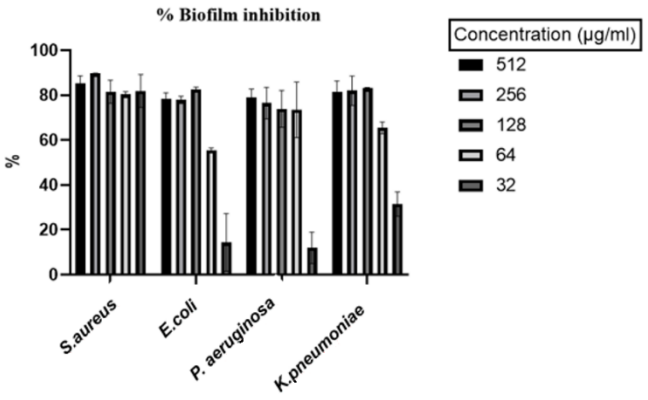

Figure 1. % Biofilm inhibition of *Echinacea purpurea* (L.) *Moench* AgNPs

It has been reported that Origanum species show different levels of antibacterial effects on food pathogens. It has been observed that the antimicrobial effect of Origanum extracts shows more inhibition against *S. aureus* than others, whereas the least inhibition occurs against *Campylobacter jejuni*.[16] AgCl-NPs were produced by the green synthesis method using *Origanum majorana* plant leaf extract. Photocatalytic antibacterial experiments revealed that AgCl-NPs have good antibacterial properties against *E. coli* bacteria under sunlight irradiation. It has been reported that *E. coli* bacteria are destroyed within 30 minutes under sunlight irradiation in the presence of AgCl-NPs.[17]

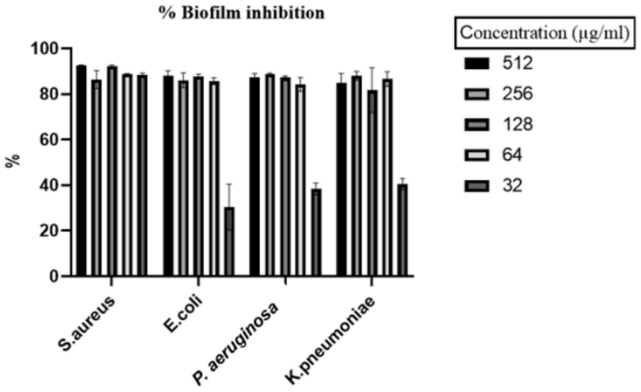

Figure 2. % Biofilm inhibition of *Origanum majorana* AgNPs

Origanum species are known to have a strong antimicrobial effect on clinical pathogens. It is claimed that there is not enough information about the antibiofilm effect. They stated that the highest effect of *Origanum majorana* essential oil on biofilm was observed against the *S. aureus* isolate, with 87.40% biofilm inhibition observed at a concentration of 50 µg/ml.[18] Our results support other studies and the highest antibiofilm effect belonged to *S. aureus.* The highest antibiofilm effect on other pathogens was determined at 512 µg/ml. The dose-dependent antibiofilm effect was determined to be 92%. In another study conducted on four clinical isolates (*S. aureus, Bacillus subtilis, P. aeruginosa, E. coli*) of *Origanum majorana* extract and AgNPs-OMs, the highest effect was reported in *E. coli* with a rate of approximately 30%.[12] The antimicrobial activity, antifungal and anti-biofilm effects of 4 plant formulations, including echinacea, were examined. Among these, they have shown very high results in terms of high antimicrobial effect and anti-biofilm degradation formation.[19]

Chitosan (CS)/pectin (PN) nanoparticles (NPs) prepared using *E. pallida* were evaluated for antibacterial and

antibiofilm activity against *S. aureus.* It has been shown to have antibacterial and antibiofilm activity against bacteria of public health concern. In line with these findings, its antibacterial activity with stability have been increased and will help the development of new plant extracts. Thus reducing the need for antibiotics.[20] It has been stated that *Echinacea purpurea* has antimicrobial and anti-biofilm activity against oral bacteria in the form of a mixture of essential oils and extracts. The lowest MIC against oral bacteria was 0.0002 mg/ml for *Streptococcus pyogenes*, and the highest MIC was 0.25 mg/ml for *Eikenella corrodens.*[21] The in vitro anti-biofilm activity of AgNPs of *Semecarpus anacardium*, *Glochidion lanceolarium* and *Bridelia retusa* was evaluated in a dose-dependent manner against the biofilm-forming bacteria *P. aeruginosa*, *E. coli*, and *S. aureus.* Regardless of the extracts, all AgNPs exhibited excellent anti-biofilm formation properties.[22]

Essential oils of Origanum and Echinacea species have been shown to be rich in phenolic compounds, which have been found to be effective reducing agents of silver nitrate for the synthesis of AgNPs. It was especially more effective on gram negative bacteria.[3,13] Essential oils show anti-biofilm activity due to both hydrophobic and hydrophilic parts in their composition. Accordingly, it is claimed that the hydrophobic components of essential oils penetrate the lipid substances of the cell membrane to reduce biofilm formation, while the hydrophilic components diffuse throughout the exopolysaccharide matrix of the biofilm.[11]

As a result, we predict that nanoparticles can be used as antimicrobial and antibiofilm agents. It is predicted that the environmentally friendly *Origanum majorana* AgNPs and *Echinacea purpurea (L.) moench* AgNPs is predicted to be an alternative therapeutic agents to current antibiotics. It is possible that nanoparticles might also have antimicrobial and antibiofilm effects on resistant strains.

Conflict of interest

There is no conflict of interest.

Compliance with Ethical Statement

In our study, standard strains purchased commercially were used. Since no biological material belonging to any living creature (patient) is used, ethics committee approval is not required.

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The authors declared that no financial support.

Author's Contributions

A.K.T., B.M.S., R.E.: Study idea/Hypothesis; A.K.T., B.M.S.: Design; A.K.T., B.M.S., R.E.: Data Collection; A.K.T., B.M.S., R.E.: Analysis; A.K.T., B.M.S.: Literature review; A.K.T., B.M.S., R.E.: Writing; A.K.T., B.M.S., R.E.: Critical review.

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