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ORIGINAL: Synergistic Effects of Silver Nanoparticles with Ethanolic Extract of *Eucalyptus globules* on Standard Pathogenic Bacteria in Vitro

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ABSTRACT

Introduction: Nowadays, with the increase of resistance due to overuse of synthetic chemical antibiotics, it seems necessary to find alternative drugs. The aim of this study was to compare the effects of silver nanoparticles and *Eucalyptus globules* (eucalyptus) ethanolic extract on standard bacteria of *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Escherichia coli*.

Material and Methods: In this experimental study, aerial parts of Eucalyptus plant were collected from Marand city and identified as Eucalyptus plant by botanists of Islamic Azad University, Ahar Branch. In this study, eucalyptus ethanolic extract was prepared by Soxhlet method and the antibacterial effects of eucalyptus extract at concentrations of 20, 30, 50 and 400 mg / ml and silver nanoparticles at concentrations of 10, 20, 40 and 80 µg / ml with agar well diffusion methods and tubular dilution were investigated.

Results: The results showed that the ethanolic extract of Eucalyptus had more antibacterial properties compared to silver nanoparticles. Eucalyptus extract and silver nanoparticles had a greater effect on gram-positive bacteria. The effect of the combination of eucalyptus extract and silver nanoparticles was much greater than the effect of either.

Conclusion: The results showed that silver nanoparticles in combination with eucalyptus extract have good antimicrobial activity against pathogenic bacteria. Therefore, this extract along with silver nanoparticles can be a good option for future studies in vivo to prepare antibacterial drugs.

Introduction

The biggest challenge facing the medical community in the face of human pathogens is the resistance of these pathogens to common antibiotics, which has been created due to the continuous and indiscriminate use of these drugs. By creating this phenomenon, the effect of drugs is weakened or neutralized and finally increases the amount of drug consumption and the tendency to use compounds with newer and stronger formulations (1), Therefore, the tendency to use herbal active ingredients or natural compounds with antibiotic effect has increased and the use of medicinal plants as a natural alternative to synthetic chemical drugs has been suggested in antimicrobial treatment (2). In this way, plants can be considered as a source of

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potentially useful chemicals, only part of which has been exploited. These potentially useful chemicals can be used not only as medicine but also as a unique model as a starting point for the manufacture of drug analogues (3-5) and it was also used as an interesting tool to better understand biological phenomena (6, 7). Plant extracts and essential oils have a very high power to make medicinal compounds in the field of health and treatment of human and animal diseases and with its antimicrobial compounds, antioxidant, free radical scavenging and anti-cancer agents, it has been suggested as one of the sources of important natural medicinal compounds. Also, these herbal medicines are more popular among the people. These reasons have been the reason for the increase in extensive global studies and the introduction of antibacterial effects of various plants in recent years (8-12). In addition to the use of medicinal properties of plants, today the application of nanotechnology in medicine and the unique properties of metal nanostructures have attracted much attention. Nanotechnology researchers have identified a wide range of applications of nanoparticles that will play a major role in medicine, prevention and treatment of diseases (13). Gold, silver and copper have been used to make nanoparticles with antimicrobial activity (14). One of these nanoparticles in the field of nanotechnology is the use of silver nanotechnology. Nano-sized silver affects the metabolism, respiration and reproduction of microorganisms (15). The main properties of silver nanoparticles include non-toxicity, high stability, hydrophilicity, thermal resistance, non-creation and increase of resistance in microorganisms (16). In various studies, the antimicrobial properties of these nanoparticles and their useful use in the field of biotechnology and specific inhibition of microbes have been investigated.

Silver nanoparticles inhibit the respiratory system of bacteria without increasing drug resistance. This element has special properties in disinfection and its preparation is easy and its price is cheap (13, 17). Eucalyptus is a medicinal plant with antimicrobial properties that is used to treat inflammation of the respiratory tract such as bronchitis. The leaves or essential oils of some eucalyptus species are used to treat certain fevers, such as malaria and typhoid fever, and to treat some skin inflammations, such as burns and ulcers. Aqueous extracts of various species of eucalyptus are also used to treat tuberculosis, bacterial dysentery, joint pain and similar cases in Western and Eastern medicines (18). General disinfection, anti-inflammatory, expectorant, antispasmodic, hypoglycemic, antipyretic, stimulant, wound healing, parasitic, treatment of urinary tract infections, diabetes, rheumatism and intestinal parasites have been reported as the main uses of eucalyptus (19). This plant is a rich source of polyphenols and terpenoids and its main leaf composition is eucalyptol or cineole (70 to 80%) (20). The antibacterial effect of eucalyptus extract and essential oil on different bacteria has been evaluated and it has been shown that in all cases of use against different species of bacteria, the plant extract has been able to prevent bacterial growth in different concentrations (21-23). Eucalyptus has a variety of phloroglucinol and tannins. Some of these compounds have biological activities such as antioxidants (24) and antifungals (25). Synergy between two drugs is a positive interaction and enhances the effect of two drugs, when combined with each other, which has a greater inhibitory effect than the total effect of each of these substances alone and reduces the dose of one or both substances (26). Therefore, in this study, the antibacterial effects of eucalyptus ethanolic extract and silver nanoparticles were studied combined together and separately on standard strains of *S. aureus*, *B. cereus*, *E. coli* and *P. aeruginosa*.

Methods

This experimental study was performed in 2019 in the microbiology laboratory of Ahar Azad University. Plant samples were prepared from natural areas of Marand city and carefully collected so that all samples were collected from one area, then the samples were identified and approved by botanists and

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herbarium of Ahar Islamic Azad University Botanical Laboratory according to genus and species. After complete drying of the samples and separation of aerial organs (stems and leaves) from the roots of the plant, Soxhlet method was used for extraction. 60 g of dried plant powder with 300 ml of ethanol as a solvent was placed in Soxhlet extractor for 8 hours. This solvent was slowly evaporated at 40 ° C using a rotary apparatus and the concentrated extract was obtained from it. Extracts concentrated with 5% solvent DMSO (DimethyLsulfoxide) at concentrations of 20, 30, 50 and 400 mg / ml were prepared for use in MIC (Minimum inhibitory Concentration) and Disc diffusion assays. The micro-organisms tested included *B. cereus* (ATCC: 1247), *S. aureus* (ATCC: 25923), *P. aeruginosa* (ATCC: 27853) and *E. coli* (ATCC: 25922) prepared from the microbial collection of the University of Tehran. Microbial samples were regenerated according to standard methods. In order to evaluate the antimicrobial effect of ethanolic extract 4 concentrations of 20, 30, 50 and 400 mg / ml of ethanolic extract of the plant in 5% DMSO solvent were prepared. In this study, the antimicrobial effect of ethanolic extract was investigated by two methods, Agar Well Diffusion and Dilution Test. In the agar well diffusion method, 500 ml of microbial suspension of 1.5×10^6 cfu/ml was transferred to Mueller hinton agar medium and cultured with sterile swab in 3 directions. Then wells with a diameter of 6 mm and a distance of 2.5 cm were created on the agar surface. Then 100 µl of concentrations of 20, 30, 50 and 400 mg / ml of ethanolic extract were injected into each well. Negative control was obtained using a solution used to dissolve the extracts (5% DMSO) and chloramphenicol antibiotic was used as a positive control. The plates were then incubated at 37 ° C for 24 hours and after a certain period of time, microbial cultures were measured in terms of formation or non-formation of growth inhibition zone in millimeters. Using tubular dilution method, the minimum inhibitory concentration and the minimum bactericidal concentration of ethanolic extract were determined. In this method, to determine the MIC of ethanolic extract prepared, dilution serials of 6.25, 12.5, 25, 50, 100 and 200 mg / ml were obtained in Mueller hinton broth. Then 1 ml of active bacterial suspension 1.5×10^6 cfu/ml was added to each dilution. Positive control (culture medium containing bacteria without extract) and negative control (culture medium without bacteria) were used next to the tubes. Finally, the tubes were incubated for 24 hours at 37 ° C. After the incubation period, the tubes were examined for turbidity due to the growth of inoculated bacteria and the last dilution in which no turbidity was observed (no growth) as MIC was considered. After that, all tubes in which no bacterial growth was observed were sampled and the MBC was determined by culturing in the plate. The plates were then incubated at 37 ° C for 24 hours. The tube containing the lowest concentration of the extract, in which no bacterial growth was observed, was considered as the MBC of the substance. To perform antibacterial tests on silver nanoparticles, silver nanoparticles with dimensions of 20 nm were prepared from Nano Sany Engineers. The dilution series used were 10, 20, 40 and 80 µg/ml. In this test, agar well diffusion methods and MIC determination test were used in Mueller hinton agar and Mueller hinton broth media. The method of preparation of culture media, bacterial strains and method of work were the same as the methods used in the ethanolic extract test of eucalyptus. In order to investigate the synergistic effect of simultaneous use of silver nanoparticles and ethanolic extract of Eucalyptus, the dilute series mentioned in the previous two tests were added together and used as a concentration. The method of this part of the study was the same as the previous tests. To reduce the test error, each of the above experiments was repeated 5 times. In order to investigate the existence of significant differences in the results, analysis of variance and Chi-square were used and the differences between the groups were determined at a significant level of $p < 0.001$.

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Results

The results showed that there was a significant difference between *S. aureus* and *B. cereus* and ethanolic extract of Eucalyptus ($P < 0.001$). With increasing the concentration of ethanolic extract of Eucalyptus, the average inhibition for *S. aureus* and *B. cereus* increased from about 7.8 and 6.8 mm to 20.4 and 18.4 mm (***Table 1***). Therefore, *S. aureus* and *B. cereus* have the highest microbial susceptibility to ethanolic extract and this inhibitory effect increased with increasing concentration of ethanolic extract on these two bacteria which was seen as an increase in growth inhibition zone. Also, the results obtained from the diameter of the growth inhibition zone show that the growth inhibitory effects of eucalyptus ethanolic extract on the gram-negative bacteria tested were so small that they had no growth inhibitory effect on *P. aeruginosa*. The values related to the MIC and the MBC of the ethanolic extract of Eucalyptus against the four tested bacteria are specified in ***Table 1***. The results show that concentrations of 25 mg / ml and 12.5 mg / ml of ethanolic extract of Eucalyptus plant have a lethal effect on *S. aureus* and *B. cereus*, respectively. In other words, *S. aureus* had the highest sensitivity to the ethanolic extract of Eucalyptus and the lowest sensitivity to *P. aeruginosa*.

The effect of silver nanoparticles concentrations on pathogenic bacteria showed that the inhibitory effects of silver nanoparticles on gram-positive bacteria were more than gram-negative bacteria. The results of the effect of different concentrations of silver nanoparticles by agar well diffusion method are shown in ***Table 2***. The results of this test show that silver nanoparticles had the greatest effect on *S. aureus* and the least effect on *E. coli*.

By mixing the prepared dilutions of silver nanoparticles and eucalyptus ethanolic extract in the previous tests, a combined concentration was obtained. The results of antibacterial test of this compound are given in ***Tables 3*** which were performed by agar well diffusion methods and MIC test. In the well diffusion method, the results showed that there was a sharp increase in the size of the growth inhibition zone of bacteria, which was mostly seen in gram-positive bacteria.

The results of this test show that by combining nano-silver and eucalyptus extract, all bacteria were affected by this compound, so that the MIC and MBC of bacteria increased significantly compared to previous tests.

Table 1. Mean diameter of growth inhibition zone and values of MIC and MBC of eucalyptus ethanolic extract on standard bacteria tested

Bacterial strain	Extract concentration (mg / ml)							
	20	30	50	400	Negative control	Positive control	MIC	MBC
S. aureus	7.8±0.83	10±1.22	13.8±0.83	20.4±1.14	--	19	12.5	25
B. cereus	6.8±1.30	9±0.70	11.6±1.51	18.4±1.67	--	18	6.25	12.5
E. coli	--	5.2±1.80	9±0.70	11±0.70	--	25	100	200
P.aeruginosa	--	--	6.2±0.44	9.6±1.81	--	21	--	--

Table 2. Mean diameter of growth inhibition zone and values of MIC and MBC of silver nanoparticles on standard bacteria tested

Bacterial strain	Nanoparticle concentration (µg / ml)							
	10	20	40	80	Negative control	Positive control	MIC	MBC
S. aureus	11.8±0.83	13±0.70	15.4±0.54	18±0.70	-	20	6.25	12.5
B. cereus	9.2±0.83	11.6±0.89	13.6±0.89	17±0.70	-	18	12.5	25
E. coli	7.6±0.89	10.6±0.89	12.4±0.54	15.6±0.89	-	24	50	50
P.aeruginosa	5.4±0.54	9.8±0.83	11±0.70	14.8±0.83	-	22	50	100

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Table 3. Mean diameter of growth inhibition zone and values of MIC and MBC of silver nanoparticles and eucalyptus ethanolic extract on standard bacteria tested

Bacterial strain	Extract concentration (mg / ml) and Nanoparticle concentration (µg / ml)							
	20mg/ml +10µg/ml	30mg/ml +20µg/ml	50mg/ml +40µg/ml	400mg/ml +80µg/ml	Negative control	Positive control	MIC µg/ml, mg/ml	MBC µg/ml, mg/ml
S. aureus	12.4±0.89	13.6±0.89	16±1.22	21.4±1.14	-	19	6.25	12.5
B. cereus	10.8±1.83	12.4±0.89	14.2±0.83	19±0.70	-	17	6.25	12.5
E. coli	8.8±0.83	10.6±1.51	12.8±0.83	16.8±1.30	-	24	6.25	15.5
P.aeruginosa	6.8±1.64	8±1.58	11±1.22	14.6±1.14	-	21	12.5	25

Discussion

Due to the increasing resistance of bacteria to various antibiotics, efforts have been made to obtain more information about the effective use of compounds in plants and their application in the treatment of various diseases (27). Due to the effect of antibiotics in very small amounts and in the amount of micrograms on pathogenic bacteria in plants, it is also tried to screen plants that have a stronger antimicrobial effect to use them in lower concentrations than take advantage of more desirable antimicrobial properties. In this study, the effect of ethanolic extract of Eucalyptus on standard strains of *S. aureus*, *B. cereus*, *P. aeruginosa* and *E. coli* was evaluated. The results of ethanolic extract of Eucalyptus indicate that the ethanolic extract of this plant has a significant effect on *S. aureus* and *B. cereus* strains. So that with increasing concentration, the amount of this effect increases. Nagata et al. In 2006 studied the antibacterial effects of eucalyptus and showed that this extract has significant effects on gram-positive and gram-negative bacteria (28). Bachir and Benali in a Nagata research study showed that eucalyptus essential oil has a very high inhibitory effect on Gram-negative *E. coli* and Gram-positive *S. aureus*, although the antibacterial effect on Gram-negative bacteria is greater than Gram-positive (29). The antimicrobial properties of eucalyptus extract are due to the presence of compounds such as citronellol, cineole 1.8, p-cymene and citronellyl acetate (30). ِDakov in 2011 by examining the antibacterial effects of eucalyptus essential oil against various microorganisms showed that the diameter of the inhibitory halo was very significant compared to the control. This halo was observed in S. pyogenes and S. aureus strains of 25-51 and 48-22 mm, respectively, and in E. coli strain (23-47 mm) (31). In another similar study, Eucalyptus leaf extract with concentrations of 64, 32 and 16 mg / ml showed a significant inhibitory effect on *S. aureus*, *S. pyogenes*, *S. pneumoniae* and *H. influenzae*, respectively (32). The results of this study differ in the amount of concentration used with the results of other studies, so that the best concentration determined in this study is in the range of 50-400 mg / ml. The reason for this increase can be partly due to changes in the active ingredient of plant extracts, which can vary depending on the habitat of different plants and different climatic conditions, and of course have different effects on microorganisms but the main factor in the variability of the data is the lack of use of similar bacterial strains. According to the above results, it is clear that the highest level of inhibition is related to gram-positive bacteria. Due to the type of cell wall structure in gram-positive and gram-negative bacteria, the reaction between peptidoglycan constituents in the wall can be involved in inhibition. Another study showed that eucalyptus methanolic extract isolated from warm regions of Mexico had a bactericidal and inhibitory effect of 11 and 5 mg / ml, respectively, on *E. coli* and *S. aureus* (32). Silver nanoparticles act against bacteria in several possible ways: by the interaction of silver ions with thiol groups of enzymes and proteins that are important for the respiratory chain of bacteria and the transport of important substances out of the cell membrane into and out of the cell (33) or the binding of silver ions to the cell wall or

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outer membrane of the bacterium and altering the function of the bacterial cell membrane (34) can inhibit the enzymatic system of the respiratory chain and alter DNA synthesis (35, 36). The bioactivity properties of silver nanoparticles are highly dependent on physicochemical properties such as size, shape and surface charge. Recent results, however, indicate that the most important factor is the ability of nanoparticles to release silver ions, which are thought to be the main cause of bacterial toxicity. The engineering of silver nanoparticles to regulate the phenomenon of silver ion release, in addition to controlling the release process, provides a powerful pathway for the development of new antibacterial drugs (37). Gram-positive bacteria such as *S. aureus* have thick, layered peptidoglycans, but gram-negative bacteria such as *E. coli* have thinner peptidoglycans but their outer membranes contain lipopoly-saccharide, which has low permeability to antibacterial and antibacterial agents. Therefore, *E. coli* is more resistant than *S. aureus* (38). Asadi et al., by examining the antimicrobial effect of silver nanoparticles on *S.* aureus and *E. coli*, concluded that *E. coli* was more resistant to the presence of nanoparticles than *S. aureus* at low concentrations. It had the highest bactericidal concentration at 50 ppm (39). By studying the effect of silver nanoparticles on *S. aureus* and *E. coli*, Ruparelia et al., showed that *E. coli* bacteria are more resistant to silver nanoparticles than *S. aureus* (40). Silver nanoparticles kill and kill bacteria by acting on their cell wall. Salopek and Sondi consider the accumulation of silver nanoparticles on the bacterial cell wall and their penetration into the cell to cause bacterial death. They also consider the size and shape of silver nanoparticles to be very effective in killing them, as silver nanoparticles with smaller dimensions and spherical shape have higher germicidal effects (41). Cho et al. reported that the surface of the *E. coli* cell wall was severely damaged by contact with silver nanoparticles. The lack of growth of *S. aureus* and *E. coli* in test plates containing silver nanoparticles with a concentration of 50 ppm showed high and effective effectiveness of silver nanoparticles by destroying the cell wall of these two bacteria (33). Otari et al., (2014) showed that silver nanoparticles had an inhibitory effect on the growth of *E. coli* bacteria *S. aureus* and *B. cereus*; the diameter of the growth inhibition zone for these bacteria was 21, 20 and 19 mm, respectively (42). In a 2012 study, the effect of silver nanoparticles combined with eucalyptus ethanolic extract on the inhibition of *E. coli* growth was investigated by disk antibiogram method. The results showed that the highest inhibitory effect on the growth of this bacterium was obtained 6 days after treatment with a combination of nano-silver at a concentration of 50 parts per million with ethanolic extract of eucalyptus in vitro (43). In another study, the synergistic effect of eucalyptus and nano-silver alcoholic extracts on inhibiting the growth of *Aspergillus niger* was evaluated. The fungus was first treated with nano-silver and also evaluated with a mixture of nano-silver and ethanolic extract of eucalyptus. Then, the morphology of changes and the number of colonies in the case and control groups were compared. The results of this study showed that the growth rate of *A. niger* significantly decreased after 8 days of treatment. The results of this study showed that a mixture of silver nanoparticles and eucalyptus ethanolic extract would be useful in the treatment of human fungal diseases (44). Mobini et al., after examining the effect of Eucalyptus essential oil and silver nanoparticles on *P. aeruginosa*, concluded that Eucalyptus essential oil and silver nanoparticles were not very effective on *P. aeruginosa* (45).

Conclusion

From the present study, it can be concluded that the highest antibacterial effect of Eucalyptus extract is against gram-positive bacteria, so that the active compounds in this extract on *P. aeruginosa*, which has an outer membrane with purines with very small pores. It has no growth inhibitory effect. Due to the significant antibacterial effect of

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eucalyptus ethanolic extract and silver nanoparticles on pathogenic bacteria, especially gram-positive samples that are involved in causing various destructive and nosocomial infections. It can also provide a good opportunity for the pharmaceutical and nano industries to produce new drugs with high efficacy and fewer side effects.

Conflicts of interest

Authors declare that there is no conflict of Interests.

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