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Antimicrobial activity of biogenic silver nanoparticles synthesized with the aid of *Citrus* peel

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Abstract :Silver nanoparticles (AgNPs) have gained considerable attention as antimicrobial agents. The present study aimed at synthesizing AgNPs using navel orange (*Citrus sinensis* L.) fruit peel. *Citrus* peel (CP) was extracted in water using different methods. The ability of CP extracts to mediate AgNPs synthesis from $AgNO_3$ was monitored by visual observation, ultraviolet-visible spectral analysis and electron microscopy. The change in reaction mixture color from yellow to brown primarily indicated AgNPs formation. In addition, absorption bands at about 450 nm confirmed the formation of AgNPs. Transmission electron microscopy revealed that AgNPs were almost spherical with average size < 30 nm. Antimicrobial activity of AgNPs was tested against *Bacillus subtilis* and *Staphylococcus epidermidis* using disk diffusion method. Clear zones were formed with inhibition diameter of 44.1 mm for *B. subtilis* and 35.2 mm for *S. epidermidis*. Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of AgNPs were found to be 0.3 mg/ml for the two microbes. AgNPs could inhibit respiratory chain dehydrogenases and induce cellular sugar and protein leakage as well as lipid peroxidation of the two microbes. However, the effect of AgNPs on the activity of dehydrogenases and lipid peroxidation of *B. subtilis* was more pronounced than that on *S. epidermidis*. The reverse was recorded for the change in cellular sugar and protein leakage where the effect of AgNPs was more pronounced on *S. epidermidis*. Therefore, biogenic AgNPs synthesized with the aid of CP extract can be recommended as potent antimicrobial agent against *B. subtilis* and *S. epidermidis*.

keywords: antimicrobial, *Citrus*, nanoparticles, peel, silver

1.Introduction

Recently, nanotechnology has been emerged as an interesting field of research with countless applications in different fields. As an outcome of nanoscience, nanoparticles could be developed as ultra-fine objects with size ranging from 1 to 100 nm. These nanoparticles possess unique features because of their high surface area to volume ratio (Nikzamir *et al*., 2021). Nanoparticles can be classified as organic or inorganic; the organic being either carbon based or non-carbon based, while the inorganic include metallic and metal oxide nanoparticles. Among metallic inorganic nanoparticles, silver nanoparticles (AgNPs) have gained great attention in the past few years **(10)**. Applications of AgNPs are versatile; but their employment in biomedical field is outstanding. In this regard, a review by Arif and Uddin (2021) referred to the reasonable chemical stability along with the good biocompatibility of AgNP; and how these merits qualify AgNPs to be potent antibacterial, antiviral, antifungal, anti-plasmodial, anti-inflammatory, antioxidant and anticoagulant agents.

The broad-spectrum antibacterial activity of AgNPs against various Gram-positive and negative bacteria is well documented. To combat deadly bacterial diseases, especially those arising from multidrug-resistant strains, AgNPs began to be involved and their efficacy in this aspect is noticeably promising. The

mechanism of AgNPs antibacterial activity generally includes adhesion of nanoparticles to the microbial cell wall or membrane followed by cell penetration and damage of macromolecules like proteins and DNA **(24).** Moreover, AgNPs were recorded to cause oxidative stress by stimulating overproduction of free radicals and reactive oxygen species leading to apoptosis of microbial cells **(21).**

Usually, AgNPs can be synthesized by chemical, physical and biological methods. Chemical synthesis of AgNPs includes various routes like pyrolysis, condensation, sputtering and sol-gel processes **(16).** Meanwhile, when physical methods are followed to synthesize AgNPs, certain physical force has to be involved like gamma irradiation, laser ablation, ball milling and ultrasonication **(12)**. For biological synthesis, either micro-organisms or plant extracts are involved; and the obtained biogenic AgNPs are thus more eco-friendly and biologically safer than those synthesized by chemical or physical methods (Eid *et al*., 2020). In this context, various plant species were utilized to mediate biogenic synthesis of AgNPs.

Navel orange (*Citrus sinensis* L.) is one of the most commonly grown fruit crops all over the world. Fruit of navel orange is consumed by eating its flesh or by being processed into juice after peeling **(18)**. *Citrus* peel (CP) is rich in phytochemicals of different classes; so it was used as reducing and antioxidant agent for the synthesis of various nanoparticles **(3)**. Nevertheless, the choice of the solvent and method adopted for CP extraction is of great importance when targeting an economic approach. Therefore, the current study aimed at testing the ability of CP extracts prepared by different methods to mediate the synthesis of AgNPs. Afterward, the obtained AgNPs would be screened for their antimicrobial activity against *Bacillus subtilis* and *Staphylococcus epidermidis*. The mechanism of AgNPs antibacterial activity would be also addressed.

2. Materials and methods

Plant material and extraction methods

Ripe fruits of navel orange (*Citrus sinensis* L.) were locally purchased, washed and peeled. Five methods were followed to prepare 5% aqueous extracts of CP. Dry CP powder was blended, extracted in water bath at 60°C or at 100°C for half an hour followed by centrifugation and filtration. These single-stepped extraction methods were marked as "blending", "heating" or "boiling". Another two methods were followed by taking the residue left after blending or heating to be further extracted at 100°C for another half an hour followed by centrifugation and filtration; with combining the filtrates resulting from the first and second steps. These double-stepped methods were marked as "blending & boiling" and "heating & boiling".

Synthesis and characterization of AgNPs

Of each of the five CP extracts, 20 ml was incubated with 80 ml $AgNO_3$ (1 mM) in firmly plugged flaks for 24 hours in dark with shaking at 120 rpm. As positive control, the same mixture was prepared with CP replaced by distilled water. Negative control was also prepared as the same but $AgNO_3$ was replaced by distilled water. After incubation, color of the reaction mixture was monitored and spectral analysis was performed at 200-900 nm using ultraviolet-visible spectrophotometer (Jenway 6705, England). The obtained AgNPs were further characterized by transmission electron microscopy (TEM) using electron microscope (JEOL JEM-2100, Japan) **(14).**

Antimicrobial activity of AgNPs

The most promising AgNPs (those with the minimum size and the most regular shape) were surveyed for their antibacterial activity against *Bacillus subtilis* (ATCC® 6633™) and *Staphylococcus epidermidis* (ATCC® 12228™) following disk diffusion method **(25).** The tested bacteria were spread on agar plates with Luria-Bertani (LB) medium. Sterile paper disks saturated with AgNPs, gentamicin (positive control) and sterilized water (negative control) were used. After being incubated at 37°C for 24 hours, diameters of clear zones were measured. For the two microbes, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of AgNPs were determined (Andrews, 2001). MIC was determined by the standard spectrophotometric dilution method on LB broth medium using AgNPs at 0.3, 0.15, 0.075, 0.0375 and 0.01875 mg/ml. For MBC, the

standard agar method was followed using LB agar medium.

Effect of AgNPs on microbial cells

The effect of AgNPs on respiratory chain dehydrogenases activity of the tested microbes was determined by iodonitrotetrazolium chloride **(15)**. Also, the effect of AgNPs on cellular sugar and protein leakage was determined by dinitrosalicylic acid and coomassie brilliant blue methods; respectively (Sadasivam and Manickam, 1996). In addition, the effect of AgNPs on lipid peroxidation of the microbes was determined by malondialdehyde method **(1).** For these analyses, one set of the tested microbes was treated with AgNPs sample at MIC, while another set was left untreated. After incubation at 37°C for 12 hours, 1 ml of the cultures was centrifuged and the supernatants were subjected to the analyses. Percent change in each parameter was calculated and graphically represented.

3. Results and Discussion

Synthesis and characterization of AgNPs

By incubating each of the five CP extracts with $AgNO_3$ for 24 hours, the color of the reaction mixture was changed from yellow to reddish brown (Figure 1A). The intensity of the resulted brown color varied based on the extraction method of CP. The darkest color was recorded for the reaction mixture in which CP was extracted by boiling (Figure 1A). As recorded in other studies, the change of color into brown primarily indicates the formation of AgNPs **(22).** Moreover, spectral analysis of the reaction mixtures revealed absorption bands at about 448 nm; a further characteristic confirming the formation of AgNPs. The maximum absorbance of beaks was recorded herein for CP extracted by boiling (Figure 1B). This may indicate that the maximum concentration of AgNPs was formed in the reaction mixture with CP extracted by boiling. The recorded absorption band in the visible light range is a feature distinctive to AgNPs because of their surface plasmon resonance **(11).**

As revealed by TEM, the obtained AgNPs had average size in the nano-range (< 30 nm); but their size varied based on the extraction method of CP (Figure 2). TEM micrographs also revealed almost spherical shape of AgNPs. However, the minimum size and the most regular shape of AgNPs were recorded for those obtained using CP extracted by boiling (Figure 2). Thus, boiling seemed to be the most efficient method for extraction of CP as compared with the other tested single- or double-stepped extraction methods. High temperature (100°C) without blending or heating at 60°C seemed to extract the majority of CP phytochemicals with reducing and/or antioxidant capacity. Synthesis of AgNPs as mediated by plant extracts was documented to depend mainly on the reduction of Ag ions of the used precursor into Ag atoms that aggregate in definite number forming AgNPs; and the reverse is prevented by the antioxidant capacity of the plant extract involved in biogenic synthesis **(16).**

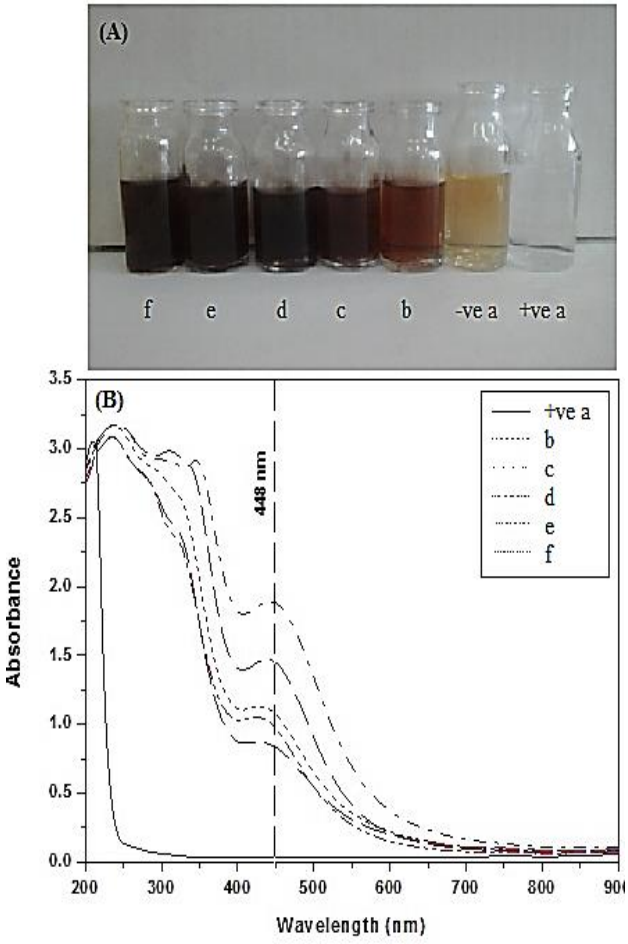

Figure 1. Visual observation (A) and ultraviolet-visible spectral analysis (B) of AgNPs solutions obtained by reacting $AgNO_3$ with CP aqueous extracts prepared by various extraction methods (a= control, b= blending, c= heating, d= boiling, e= blending & boiling, f= heating & boiling)

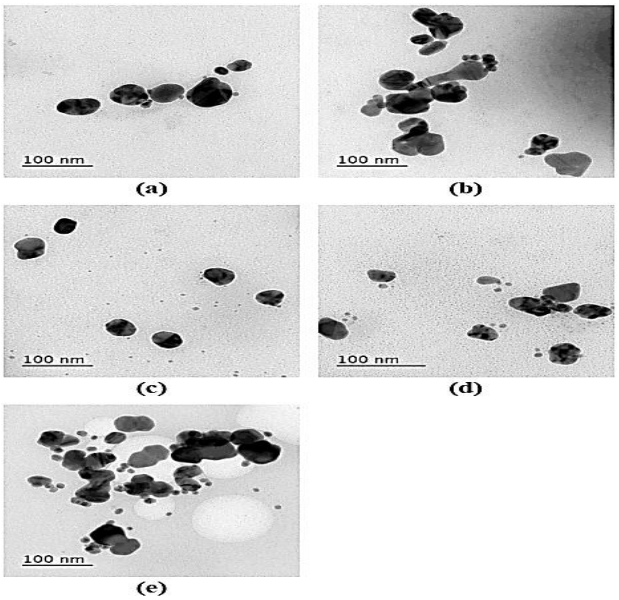

Figure 2. TEM micrographs of AgNPs obtained by reacting $AgNO_3$ with CP aqueous extracts prepared by various methods (a= blending, b= heating, c= boiling, d= blending & boiling, e= heating & boiling)

Antimicrobial activity of AgNPs

Testing antimicrobial activity of AgNPs obtained herein with the aid of CP aqueous extract prepared by boiling indicated that the growth of *B. subtilis* and *S. epidermidis* was inhibited by AgNPs forming clear zones on agar medium (Figure 3). The diameter of inhibition zone formed by AgNPs was 44.1 mm for *B. subtilis* and 35.2 mm for *S. epidermidis* (Table 1). These values are to somewhat comparable with those recorded herein for gentamicin.

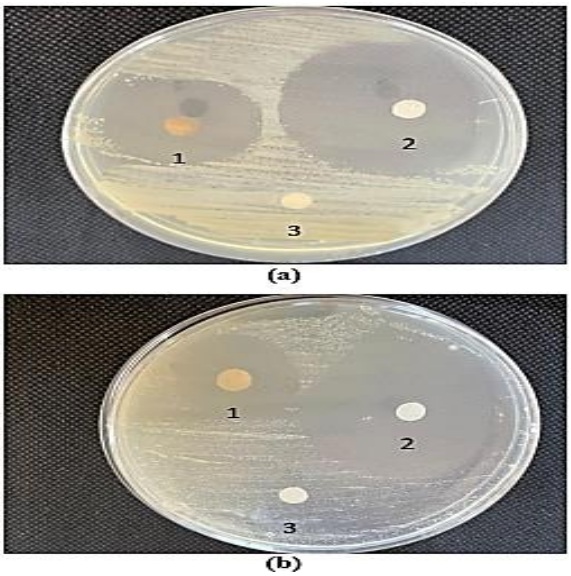

Figure 3. Disk diffusion test for AgNPs (1), gentamicin antibiotic (2) and distilled water (3) against *B. subtilis* (a) and *S. epidermidis* (b)

Antimicrobial activity of AgNPs against different Gram-positive bacteria was previously recorded **(8).** Among Gram-positive bacteria formerly tested for their susceptibility to AgNPs, *B. subtilis* was found to be sensitive to biogenic AgNPs synthesized with the aid of *Cullen tomentosum* plant extract **(5).** Also, growth of *S. epidermidis* could be inhibited by biogenic AgNPs whose synthesis was mediated by *Picria fel-terrae* plant extract **(19)**. Of special concern, both *B. subtilis* and *S. epidermidis* were intensively reported as multidrug-resistant bacteria (**7**); **(23).**

Table 1. Diameter of inhibition zone formed by AgNPs and gentamicin against *B. subtilis* and *S. epidermidis*

Microbe	**Diameter of inhibition zone (mm)**	
	AgNPs	Gentamicin
B. subtilis	44.1	54.1
S. epidermidis	35.2	47.1

Moreover, MIC and MBC of AgNPs were found to be 0.3 mg/ml for *B. subtilis* and *S. epidermidis* (Figure 4 and Table 2). These MIC and MBC values are minute and indicate high potency of AgNPs as antimicrobial agents against the two microbes. In a study conducted by Katerere and Eloff (2008), antibacterial potency of the tested material was categorized based on MIC values into weak (MIC ≥ 8 mg/ml), moderate (8 mg/ml > MIC > 1 mg/ml) or noteworthy (MIC ≤ 1 mg/ml). In this way, the biogenic AgNPs obtained in the current study can be described as noteworthy regarding their antibacterial activity against the tested microbes.

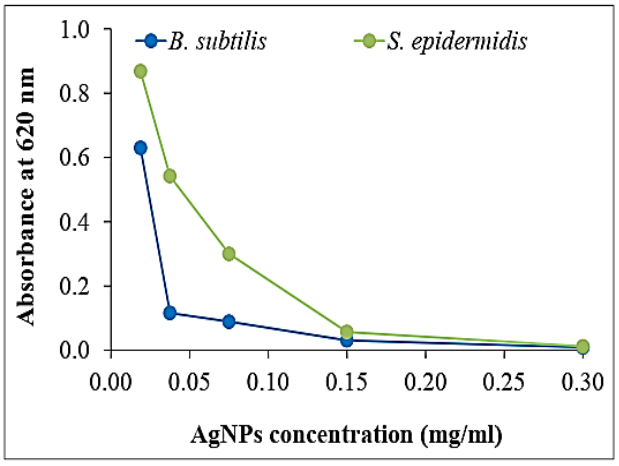

Figure 4. Absorbance of liquid cultures of *B. subtilis* and *S. epidermidis* as affected by different concentrations of AgNPs

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of AgNPs for *B. subtilis* and *S. epidermidis*

Microbe	AgNPs MIC (mg/ml)	AgNPs MBC (mg/ml)
B. subtilis	0.3	0.3
S. epidermidis	0.3	0.3

Effect of AgNPs on microbial cells

Treating *B. subtilis* and *S. epidermidis* with MIC of AgNPs resulted in reduced activity of respiratory chain dehydrogenases of microbial cells (Table 3). However, percent change in dehydrogenases activity of *B. subtilis* was slightly higher than that of *S. epidermidis* (Figure 5). Also, treating the two tested microbes with AgNPs resulted in increased leakage of reducing sugars and proteins from their cells (Table 3). The percent change in cellular sugar leakage and cellular protein leakage of *S. epidermidis* was higher than that of *B. subtilis* (Figure 5). In addition, treatment with AgNPs increased lipid peroxidation of the two microbes (Table 3); and the percent change in lipid peroxidation of *B. subtilis* was higher than that of *S. epidermidis* (Figure 5). In accordance with these results, biogenic AgNPs exerted potent antimicrobial activity via their inhibiting effect on microbial respiratory chain dehydrogenases in addition to their induction to cell membrane leakage (indicated by leakage of cellular sugars and proteins) and acceleration of lipid peroxidation **(9).**

Table 3. Activity of respiratory chain dehydrogenases, cellular sugar and protein leakage and lipid peroxidation of *B. subtilis* and *S. epidermidis* as affected by AgNPs

B. subtilis		
	Untreated	Treated
Dehydrogenases activity	0.083	0.043
Cellular sugar leakage	0.120	0.175
Cellular protein leakage	0.159	0.228
Lipid peroxidation	0.133	0.202
S. epidermidis		
	Untreated	Treated
Dehydrogenases activity	0.037	0.020
Cellular sugar leakage	0.095	0.151
Cellular protein leakage	0.158	0.252
Lipid peroxidation	0.144	0.194

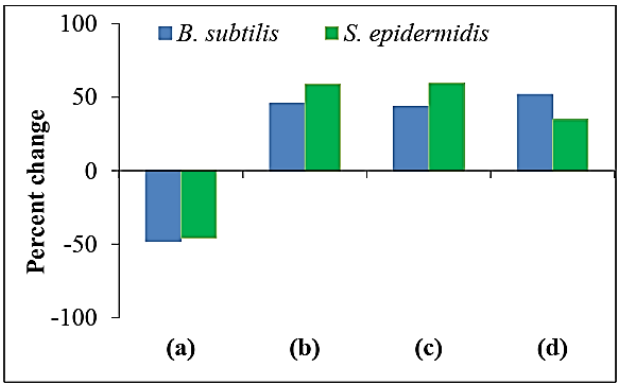

Figure 5. Percent change in the activity of respiratory chain dehydrogenases (a), cellular sugar leakage (b), protein leakage (c) and membrane lipid peroxidation (d) of *B. subtilis* and *S. epidermidis* as affected by AgNPs

Potency of AgNPs to block respiration of microbial cells is well documented; and this occurs through different strategies the most common of which is through inhibiting respiratory chain dehydrogenases **(26).** Also, antimicrobial activity of AgNPs was previously ascribed to the induction of oxidative stress via free radical formation **(21).** Free radicals accelerate lipid peroxidation and result in cellular membrane or wall damage. In consequence, greater extent of metabolites (mainly sugars and proteins) is leaked from the microbial cells. Thus, the mechanism of antibacterial activity of AgNPs becomes to somewhat clear. In consequence, the results obtained in the current study recommend utilizing CP aqueous extract simply prepared by boiling for biogenic synthesis of AgNPs. The as-synthesized AgNPs are potent antimicrobial agents against *B. subtilis* and *S. epidermidis*.

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