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Green Synthesis of Silver Nanoparticles using Aqueous Leaf Extract of *Ocimum basilicum* and Investigation of their Potential Antibacterial Activity

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ABSTRACT

Bacterial resistance to antibiotics has emerged as a common medical problem that requires urgent attention. Thus, investigation on seeking for a better alternative is a paramount issue. Recently, silver nanoparticles (SNPs) have shown promising results as bactericidal agent against both gram positive and gram negative bacteria without cytotoxicity. The objectives of this study were to synthesize SNPs using *Ocimum basilicum* leaf extract, characterize the nanoparticles and investigate their antibacterial activity. SNPs were prepared by mixing $AgNO_3$ precursor solution with the leaf extract of *O. basilicum*. The biologically synthesized SNPs were characterized using UV-Visible spectral, transmission electron microscopic and fourier-transform infrared spectroscopic analyses. Bactericidal efficiency of the biologically synthesized SNPs was examined against certain bacteria known for their resistance against conventional antibiotics. The results indicated that the synthesized SNPs were crystalline, spherical in nature and their size ranged between 18.7-26.2 nm. Also, the antibacterial sensitivity testing showed a great inhibitory effect of SNPs against the test bacterial pathogens which included *Proteus vulgaris*, *Escherichia coli*, *Klebsiella pneumonia* and *Bacillus* sp. Our finding suggest that biologically synthesized SNPs using aqueous leaf extract *of O. bascilicum* has a promising antimicrobial activity against some pathogenic bacteria with proven resistance to conventional antibiotics.

Keywords: Silver nanoparticles, *Ocimum basilicum*, Extract, Bacterial resistance.

Introduction

Nanotechnology is the science of creating and applying nanostructures or nanomaterials and investigating the relationship between numerous properties of materials on their nanometer unit dimensions.[1] Currently, metal-based nanomaterials have attracted interest in various applications like medicine, biotechnology, and nano-chemistry.[2] Several nanomaterials such as titanium, copper, zinc, alginate, gold, metallic elements as well as silver were used.[3,4] Silver nanoparticles (SNPs) were effectively applied in numerous industries like biomedical, pharmaceutical, textile, agricultural, etc due to their distinctive and particular characteristics, especially antimicrobial activity and non-toxicity.[5] There are different methods of synthesizing SNPs; however, greener and eco-friendly synthesis is the best. It produces non-toxic, efficient, eco-friendly and stable materials.[6-7]
Biological synthesis using plants and microorganisms such as bacteria and fungi, as well as algae, has attracted immense attention. By using plant extracts, the metal nanoparticles are synthesized with no impurities, which reduce the venturous solvents and decrease the number of agents and stabilizers.[8-9] Some plant extracts are known to be abundant bioreducing agents which can convert the silver ions into SNPs.[7] Biomolecules such as alkaloids, terpenoids, co-enzymes and phenolic compounds of plants behave as reducing agents by changing the metal ions into nanoparticles.[10] Earlier reports on green synthesis of SNPs utilized numerous plant leaf extracts such as leafy parts from *Zingber officinale*,[11] *Volkameria inermis*,[12] *Ocimum basilicum*, and Thai basil.[13] These parts have the ability to convert silver ions into SNPs.[14]
The aim of the present study was to synthesize SNPs using *Ocimum basilicum* leaf extract, characterize the synthesized nanoparticles and investigate their antibacterial activity.

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Materials and Methods

Sample collection and isolation of bacteria

Clinical samples were collected from burn infections from patients referred to Salah al-Din General Hospital, Tikrit, Iraq, during the period from March 2019 to October 2019. The bacteria were isolated using culture media and identified by biochemical tests as described by Forbes *et al*.[15]

Plant collection

The entire *Ocimum baccilicum* plant (voucher no. HCU/23451) was collected from a local vegetable farm in October 2019.

Preparation of basil leaf extract

Leaves of *O. basilicum* weighing 25 g were washed in sterilized H_2O, chopped into small parts and crushed into fine powder. Then 100 ml of sterile distilled-water were added, after which the solution was filtered using Whatman filter paper (pore size of 25 micrometre). The filtrate was again filtered using filter paper (pore size of 0.6 micrometre). At the end, the final filtrate was collected and kept at 4°C until required for experiments.[13]

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Preparation of precursor solution

Precursor solution, 1 mM of silver nitrate ($AgNO_3$) was prepared by adding 0.0421 g of $AgNO_3$ to 100 mL of double distilled water in a clean and sterile flask. The contents were mixed well and stored in an opaque bottle to prevent silver auto-oxidation.[18]

Biological synthesis of silver nanoparticles

All chemicals were purchased from Merk Company. The synthesis of SNPs was performed as previously mentioned,[18] with some modifications. An aliquot of 90 ml of aqueous solution of $AgNO_3$ (1 mM) was added to 10 mL of aqueous extract of *O. basilicum* and placed in a shaker incubator which rotated constantly for 8 hours at room temperature. Colour change to dark brown was taken as an indication of synthesis of SNPs.

Characterization of silver nanoparticles with uv- visible spectral analysis

Biologically synthesized SNPs were characterized as described by Ahmed *et al*,[19] and Wang *et al*,[20] with some modifications. Ultra violet-visible photometric analysis was performed by employing a Genesys 6 spectrophotometer (Thermo Electron Corporation, USA). An aliquot of 2 mL sample was used and SNP resonance of surface plasmon was distinguished using an ultra violet-visible photometer at a particular resolution of 200-800 nm.

Analysis of silver nanoparticles using the transmission electron microscope

Synthesized SNPs were analysed (morphology and size) by transmission electron microscope (TEM, Hitachi H-7100). The sample was prepared by pouring the SNP solution on grids of copper, covered with a carbon layer (300 mesh size). Then, the solution was left to evaporate slowly until dryness and the sample was checked.

Fourier-transform infrared spectroscopy analysis of silver nanoparticles

The biologically synthesized SNP sample was prepared for FTIR analysis by centrifuging the SNP solution at 10,000 rpm for 20 min. Then, the residue of the SNPs was washed with deionized H_2O, fully dried and the powder obtained was used for fourier-transform infrared spectroscopy (WQF-520, Biotech, England) analysis. The range of scanning was between 4000-400 cm^{-1} and a resolution of 4 cm^{-1}.

Antibacterial susceptibility testing of five antibiotics

The Kirby-Bauer methodology was used for testing the sensitivity of bacterial isolates against 5 antibiotics: erythromycin (E; mcg/disc), tetracycline (TE; 10 mcg/disc), vancomycin (VA; 30 mcg/disk), penicillin (P; 10 mcg/disk) and naldixic acid (NA; 30 mcg/disc).[16] At the end of incubation period, the inhibition zones around every antibiotic disc was calculated in mm according to the Clinical and Laboratory Standards Institute guidelines.[17]

Determination of inhibitory effect of biologically synthesized silver nanoparticles

The well diffusion method was used to explore the bactericidal effect of the biologically synthesized SNPs. A sterile cork borer was used to make wells on a pre-prepared Mueller Hinton agar with a diameter of 6 mm and of equal dimensions. An aliquot of 100 µl of the bacterial suspension (1 x 10^8) was spread on the surface of the medium. Then, 100 µl of different concentrations (25, 50, 75, and 100 %) of the SNPs were transferred into the wells. One of the wells was assigned as control in which 100 µl of sterile distilled water were poured. The cultures were incubated for 18 hours at 37 °C. At the end of incubation period, the inhibition zones were measured.[21]

Statistical analysis

All statistical analyses were performed using SPSS statistical software version 25 (IBM Corporation, USA). Descriptive statistics was used to express data of the different variables.

Results and Discussion

Methods of preparing nanoparticles have gained more attention in recent decades, and the environmentally friendly procedure of synthesis is referred to as biological methodology. Current international interests within the usage of cost-effective and eco-friendly sources induce employment of extremely praised medicinal plants to perform biological synthesis of metal nanoparticles that have numerous biological characteristics. Moreover, the synthesis of SNPs involved two steps: the first step was the combination of silver nitrate (higher bioactive component) with aqueous extract of *Ocimum* sp. (chemically effective and less toxic), while the second step included the increased surface development within the reduction reaction (the surface area is higher compared to the volume ratio) which resulted in excellent bioactivity. Spherical shaped mixture of SNPs were produced to explore the antibacterial potential of *O. basilicum*.[22]

During synthesis, a gradual color change from colourless to brownish color (Figure 1) was observed when 90 ml of silver nitrate solution (1mM) was added to the aqueous extract of basil leaf and the resulting solution was left for 8 hours at room temperature in a shaker incubator. The color change indicated the synthesis of SNPs. This color change was due to the reducing metal ions during the course of the reaction.[23] More so, the colour intensity was more intense with an increase in incubation time. Colour intensity observation is mainly because of the surface plasmon resonance excitation (SPR) in the SNPs. This observation was in agreement with a previous study,[24] indicating a conversion of silver nitrate to SNPs. The SPR absorbance is mainly affected by shape, size, and the environment at which nanoparticles are biosynthesized.[25] Plasmon bands formed are wide in the longer wavelengths, suggesting an enhancement in the synthesized NPs size.

Figure 2 shows the ultra violet-visible spectrum of SNPs. The attained curve has a bell-shape, which indicated that the SPR occured at 448 nm, compared with the *Ocimam basillicum* leaf extract that did not demonstrate any peak after 8 hours of synthesis. The reaction solution of the UV–Vis absorption spectrum confirmed the formation of SNPs from silver ions at 448 nm. Our result agrees with a finding by Elumalaia,[26] who revealed the sharp peak band of 450 nm of *O. basilicum* extract-synthesized SNPs. This indicated the occurrence of SNPs in the solution. Furthermore, Malapermal et al. recorded a peak band of 438 nm for SNPs that were biologically synthesised with *O. basilicum* leaf extract,[22] while Pirtarighat et al.,[27] detected a peak at 450 nm. The broad band of uv-vis absorption spectra is attributed to the existence of numerous metabolites of *O. basilicum* extract existing in reactive solution that were found in the investigational spectrophotometric limit. The nature and characteristics of NPs can be obtained from SPR peak. Sphere-shaped and uniform NPs in the solution have a sole SPR band within the absorption range.[23]

Size and shape of green synthesized silver nanoparticles as revealed by TEM analysis

TEM was utilized to investigate the shape and size of SNPs and the results showed that most of the biologically synthesized SNPs displayed a uniform round shape and the size ranged between 18.7-26.2 nm, as shown in Figure 3. This result is in accordance with that obtained by Malapermal,[22] who found in their studies that synthesized SNPs were 17 nm in size. Also, Bakht Dalir *et al*,[28] detected the diameter of SNPs and reported a range of 12–30 nm.

Functional group analysis by fourier transmission infrared spectroscopy

FTIR analysis was conducted to reveal the functional group(s) and detect biomolecules that led to the reduction of Ag ions to SNPs. The spectra of FTIR for the SNPs that were biologically synthesized (Figure 4) detected intense bands between 514.99 to 3637.75 cm^{-1} which included different absorption peaks (3294, 3120, 3169, 2891, 2666, 1683.86, 1525, 1485, 1226.66, 700, 514.99). Our results are in agreement with the study of Malapermal *et al*.[22] who showed the band frequencies from 3300.21-3297.12 cm^{-1}, suggesting the presence of an OH functional group. Moreover, the bands from 1634.78-1635.67 cm^{-1} are assigned to carbonyl group (C-H stretch), while a band at 1217 cm^{-}

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[1] is assigned to the ether linkages. The dual properties of SNPs that were biologically synthesized with *O. basilicum* were clearly indicated with the addition of C-H stretch. It is thus clear that the change of C-OH biomolecules to C,O group resulted in the conversation of Ag+ into Ag^0. Dada *et al.*[29] in a research showed that the absorption peak at 2858 cm^{-1} might be associated with the organic compound, aldehyde (–CH stretching vibrations). In addition, spectra of the FTIR indicateed bands of 1452, 1513, and 1610 cm^{-1} which were identified as a carbonyl group (C, O) for organic compound amide I and II; presence of amine, assisted by the N-H banding at 1580 cm^{-1} and; N-H waging at 707 and 808 cm^{-1} respectively.[30]

Some functional chemical groups like OH and CO within the sample played an essential role in the biosynthesis of SNPs.[31] These biologically active known particles are reducing agent of Ag ions that are accountable for the synthesis of SNPs. The FTIR spectral analysis confirmed the organic molecules that were present, such as nitro group, ethylamine, amine, alkene, ester, etc., and proved that the plant extract of *O. bascilicum* played a vital role in the green synthesis of SNPs.[32]

Biologically synthesized silver nanoparticles possess bactericidal activity

The results of the SNP-antibacterial effect on some pathogenic bacteria such as *Proteus vulgaris, Escherichia coli, Klebsiella pneumonia, Bacillus spp*. isolated from burn are presented in table 1, These bacteria showed 100 % resistant to the test antibiotics in this study. Furthermore, the isolates were tested against various concentrations (25, 50, 75 and 100 %) of SNPs.

Our results showed an increase in the inhibitory activity of concentration-dependent manner. The current observations are in accordance with the study of Elumalaia,[26] who found that SNPs synthesized with *O. basilicum* had inhibitory effect on *Proteus mirabilis, Staphylococcus aureus, Proteus valgar, Klebselia pneumonia*, and *E. coli* with 8.9, 9.2, 7.8, 8.1 and 7.4 mm inhibition zone, respectively. Also, Pirtarighat,[27] revealed the antibacterial activity of SNPs against *B. subtilis* and *E. coli* with 13 and 12 mm zone of inhibition, respectively. In another study, Muthulakshmi et al.[33] showed inhibition zone of SNPs against *Bacillus cereus* (12 mm), *Streptococcus pyogenes* (12 mm), *Klebsiella pneumonia* (14 mm), *E. coli* (15 mm) and *Salmonella typhi* (13.9 mm). Other workers have reported zones of inhibition of 15 mm against *Proteus vulgaris*, 18 mm against *Klebsiella oxytoca*, 19 mm against *E. coli* and *Klebsiella pneumoniae*.[34] Karthiga depicted the area of inhibition which surround inoculated well with different suspensions of SNPs and opined that they are highly effective against Gram negative bacteria as their cell walls are weaker because of less peptidoglycan content as compared with the Gram-positive bacteria. Hence, the efficacy of SNPs was proven against both Gram positive and Gram-negative bacteria.

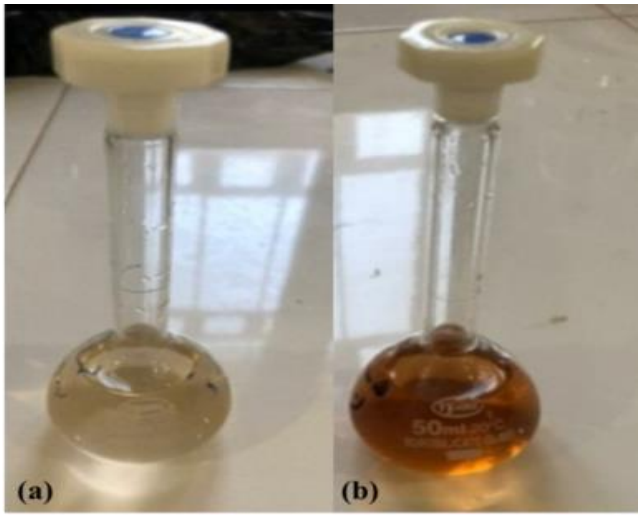

Figure 1: Change in color after reduction of Ag^+ to silver nanoparticles by *Ocimum basilicum* leaf extract. **a:** Before incubation; **b:** After 8 hrs of incubation

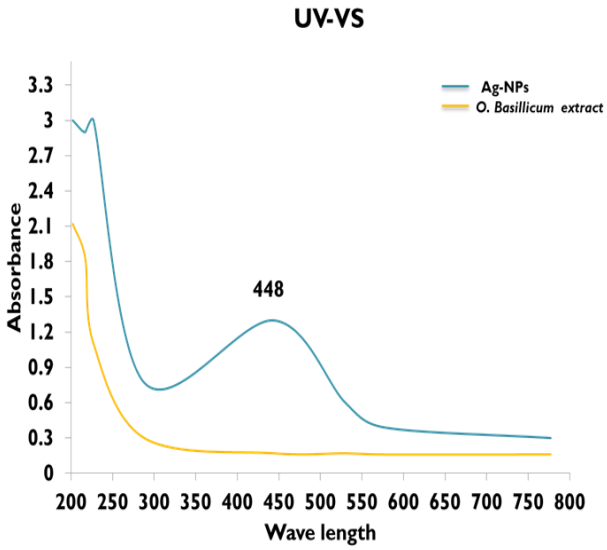

Figure 2: Ultra violet-visible spectrum of silver nanoparticles synthesized with *Ocimum basilicum* leaf extract showing surface plasmon resonance peak at 448 nm.

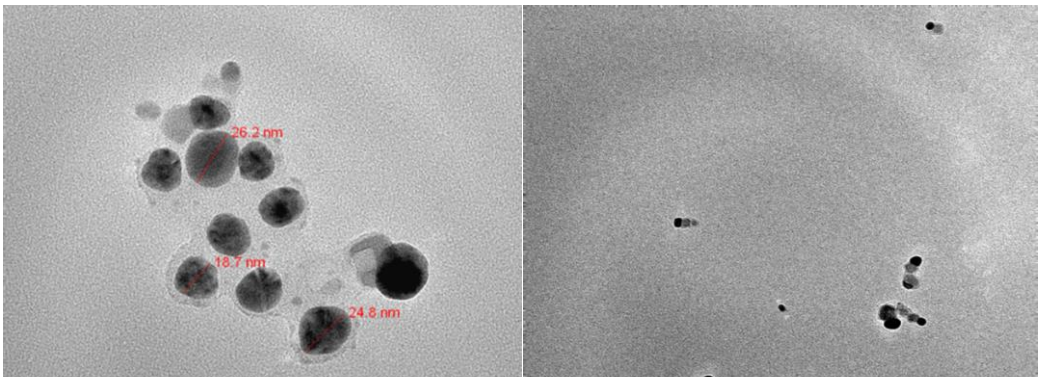

Figure 3: Transmission electron microscopic images of biologically synthesized silver nanoparticles.

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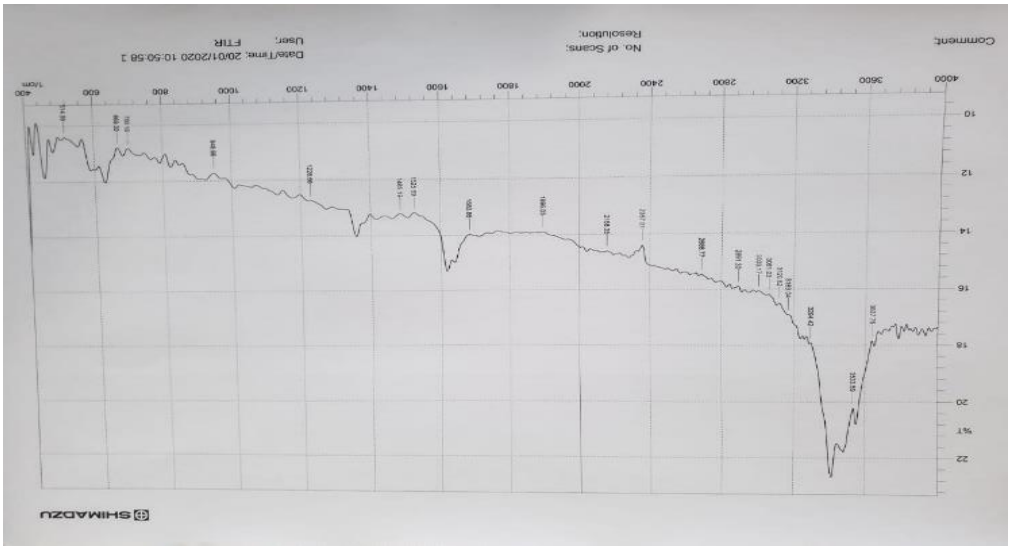

Figure 4: Fourier-transform infrared spectroscopy analysis of biologically synthesized silver nanoparticles.

Table 1: Zone of inhibition (mm) of biologically synthesized silver nanoparticles against pathogenic bacterial strains

Bacterial species	Concentration of SNPs (%)				Antibiotics				
	25	50	75	100	TE	V	VA	NA	P
P. vulgaris	16	17	19	20	R (0)	R (0)	R (0)	R (0)	R (0)
E. coli	14	15	16	17	R (0)	R (0)	R (0)	R (0)	R (0)
K. pneumonia	19	20	21	23	R (0)	R (0)	R (0)	R (0)	R (0)
B. sp	12	13	14	15	R (0)	R (0)	R (0)	R (0)	R (0)

SNPs: Silver nanoparticles; R (0): Resistance (no inhibition zone); TE: Tetracycline; E: Erythromycin; VA: Vancomycin; NA: Naldixic acid; P: Penicillin

The potent bactericidal activity which was considered against several bacteria indicates the varied mechanism of nanoparticles which interact with microorganisms.[36] Notably, the silver cations being released from SNPs result in the antibacterial activity against test pathogens.[37] The antibacterial features of SNPs could be determined by attachment of nanoparticles to the cell wall of bacteria, altering cell membrane permeability, and inhibiting respiration.[38] Also, the outer membrane being destabilized and cytoplasmic membrane degraded result in the reduction of intra cellular ATP.[39] Moreover, nanoparticles have high inclination to react with phosphorus or sulphur of biomolecules of cells and they interact with DNA in such a manner that the DNA will not be able to replicate or reproduce, leading to bacterial death.[40] There are many researches which reported down regulation of some virulence factors caused by direct impact of SNPs and TiO_2 nanoparticles against pathogenic bacteria.[41]

Conclusion

Green synthesized-SNPs using *O. basilicum* aqueous leaf extract has a promising antimicrobial activity against some pathogenic bacteria that are well-known for their resistance to traditional antibiotics. The biologically synthesized SNPs have uniform, monodispersed, crystalline and spherical shape, with an average diameter ranging between 18.7-26.2 nm. These nanoparticles are potential alternative to antibiotics after their efficacy has been tested *in vivo*.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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