INTERNATIONAL JOURNAL OF PHYTOFUELS AND ALLIED SCIENCES
(A Journal of the Society for the Conservation of Phytofuels and Sciences)
(http://www.phytofuelsciences.com) (ISSN 2354 1784)

Effect of Silver Nanoparticle Synthesized from *Chrysophyllum albidum* Leaf Extract on Selected Bacteria

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ABSTRACT

The increase in antibiotic resistant properties of pathogens has led to alternative search for new sources of effective antibiotics. This study investigated the antibacterial effect of *Chrysophyllum albidum* mediated silver nanoparticles against selected bacteria. The formation and stabilization of AgNP monitored on UV-Vis spectrophotometer indicated that the absorption spectrum peaked at 478.0 nm. Identity of biomolecules responsible for capping and reducing silver particles was determined by Fourier Transform Infrared (FTIR) spectroscopy. The FTIR spectra revealed the presence of functional groups which were; amines, amides and alcohols. Antibacterial activity was evaluated using agar well diffusion assay and activity compared with gentamicin and tetracycline. Three AgNP concentrations (100, 200 and 300 mg/ml) were tested against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. The antibacterial effect of the extract were concentration dependent showing maximum activity (23 mm) with the highest concentration (300 mg/ml) against *P. aeruginosa* and minimum acticity (7.5 mm) against *S. aureus* at 100 mg/ml. The activity of AgNP of *Chrysophyllum albidum* against *E. coli* (20mm) and *P. aeruginosa* (23mm) were higher than those of gentamicin (21mm & 14mm) and tetracycline (17mm & 17mm) respectively. Minimum inhibitory concentration (MIC) was carried out by microbroth dilution assay, and minimum bactericidal concentration (MBC) by reinoculation on agar plate. MIC and MBC values were 60 mg/ml and 100 mg/ml respectively for all test bacteria except for *S. aureus* at 55 mg/ml and 100 mg/ml respectively. The effect of AgNP on bacterial cell wall was investigated by direct exposure of test bacteria to AgNP. Disruption of cellular architecture of bacterial cells was observed which may have resulted from AgNP intrusion causing increased cell wall permeability. It is evident from this study that AgNP synthesized from *C. albidum* leaves has high antimicrobial activity against the selected organisms and can be utilized in the field of nanobiotechnology.

Key words: agar well diffusion, antibiotics, cell wall, *Chrysophyllum albidum*, permeability, silver nanoparticle

INTRODUCTION

The growing menace of resistance of pathogenic microorganisms to conventional antimicrobials has being a subject of concern over the years. This is owing to the wide use and abuse of antibiotics which has led to genetical and morphological evolutionary processes in microorganisms (wang *et al*., 2017). In view of this challenge, natural product scientists have been combing the earth for alternative remedies to combat this scourge threatening human existence. Herbal therapeutics is one of the oldest forms of medical treatment in human history and could be considered one of the forerunners of the modern pharmaceutical trade. Currently, an eco-friendly green-mediated synthesis of inorganic nanoparticles has become a fast growing aspect in nanotechnology research (Sathya and Ambikapathy, 2012). Nanotechnology is the application of nanoscale material structures, usually ranging from 1 to 100 nm in biotechnological research. The particles possess very high surface to volume ratios, a property that can be utilized in the extraction of active components from crude plants where high surface area is required (Jiang *et al*., 2004). These nanoparticles are able to

exert a more profound effect than conventional antibiotics on bacterial cell wall upon direct contact even without penetration inside the cell (Edmundson *et al*., 2013)

Silver nanoparticles are nanoparticles of silver possessing unique properties as a result of their versatility and potential to work in synergy with conventional antimicrobials towards multidrug resistance bacteria (Aziz *et al*., 2016). The synthesis of nanoparticles can be by physical, chemical or biological means. However, biological methods are safer and much inexpensive compared to other methods. Biosynthetic methods can employ either microbial cells or plant extracts (Singh *et al*., 2016). The use of plant extracts is more advantageous, as they are easily available and have a broad variety of metabolites that can aid in the reduction of silver ions during synthesis. The main mechanism considered for the process is plant-assisted reduction due to the presence of phytochemicals (Ahmed *et al*., 2016).

Chrysophyllum albidum G. Don (African star apple) is a tropical edible fruit tree of the family Sapotaceae which has up to 800 species (Ehiagbonare *et al*., 2008). It is primarily a forest tree species found in diverse ecozones in Nigeria, Uganda, Niger Republic, Cameroon and Cote d'Ivoire (Bada, 1997). Some reports have revealed the medicinal potentials of this plant. The roots and leaves are used as a remedy for yellow fever and malaria as well as in the treatment of skin eruptions, diarrhea and stomach ache (Adewusi, 1997). Studies have also examined the anti-inflammatory and antioxidant activities of eleagnine, an alkaloid isolated from the seed cotyledons (Idowu *et al*., 2006). However, not much is known about the exact mechanisms by which nanoparticles synthesized from this plant act in the elimination of infectious agents which is a vital component of drug pharmacokinetics. This study investigated the functional chemical properties and antibacterial activity of AgNP of *C. albidum* leaf extract on selected bacteria.

MATERIALS AND METHODS

Collection and Identification of Plant Material

Fresh young leaves of *C. albidum* were collected from a natural habitat at Ogbomosho, Oyo state, Nigeria in the month of July, 2017 and authenticated at the herbarium unit of Plant Biology Department, University of Ilorin (voucher specimen number UIH001/1170).

Collection and Maintenance of Test Bacteria

Three bacteria *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* were obtained from the Culture Collection Unit of the Department of Microbiology, University of Ilorin, Nigeria. The organisms were maintained on Mueller-Hinton agar slants and sub-cultured periodically for the purpose of purity.

Plant Preparation and Extraction

The leaves of *C. albidum* were thoroughly washed with deionized water; air-dried at room temperature for 10 days and thereafter pulverized using electric blender. Crude aqueous extract of the plant leaves was obtained using the method used by Kamba and Hassan (2011) with slight modification. Measured pulverized leaves (100 g) was soaked in 1 litre of deionized water and heated at 80 ^{o}C for 1 hour. The solution was filtered and stored at 4 ^{o}C until further use.

Preparation of Silver Nanoparticles

Silver nanoparticles were synthesized by adding leaf extract of *C. albidum* to 10 mM silver nitrate solution in the ratio 1:10 respectively. The mixture was heated at 30 ^{o}C for 4 hours and aged for 24 hours at room temperature for formation of AgNP crystals which was filtered with the aid of a vacuum pump (Richardson *et al.*, 2006).

Characterization of Silver Nanoparticles

Ultra-violet/visible (UV-Vis) spectrophotometry: The formation and stabilization of the reduced AgNP was monitored by UV-Vis spectrophotometer (BeckamCouler DU®730) using wavelengths in the range of 200 to 800 nm, the resulting spectra was observed and recorded (Cheon and Park, 2016).

Fourier transform infrared spectroscopy (FTIR) analysis: The chemical compositions of the

synthesized AgNP were identified using Shimadzu (8400S) Fourier-transform infrared (FTIR) spectrometer at a resolution of 4 cm^{-1} in the range of 500–4000 cm^{-1} using KBr pellets (Kurian*et al*., 2016).

Antibacterial Susceptibility Assay of Silver Nanoparticle against Test Bacteria

The antibacterial screening of AgNP of *C. albidum* was carried using three concentrations (100, 200 and 300 mg/ml) following the agar well diffusion method as described by NCCLS (2000) protocols. Each concentration of AgNP (1 ml) was separately introduced into wells of inoculated agar plates and incubated at 37 ^{o}C for 24 hours. Plates were observed for the presence of clear zones around agar wells. Diameter of zones of inhibition was measured to the nearest millimeter and recorded.

Antibiotic Susceptibility Testing of Standard Antibiotics against Test Bacteria

Antibiotic susceptibility was determined using modified Kirby-Bauer (1966) method. Gentamicin and tetracycline were the antibiotics of choice. Antibiotic discs were placed on inoculated agar plates with the aid of sterile forceps. Plates were incubated at 37^{o}C and diameter of zones of inhibition recorded after 24 hrs.

Minimum Inhibitory Concentration of Silver Nanoparticle against Test Bacteria

Broth dilution assay was employed to determine the minimum inhibitory concentration (MIC). Broth (9.5 ml) was dispensed into nine test tubes containing 0.3 ml of various concentrations (100, 90, 80, 70, 60, 55, 50, 45, 30 mg/ml) of AgNP. To the test tubes, 0.2 ml each of test bacteria was separately added. The turbidity of the tube contents was taken using a spectrophotometer, after which they were incubated at 37^{o}C for 18–24 hrs. Subsequently, turbidity of the samples was taken again and recorded (NCCLS, 2000).

Minimum Bactericidal Concentration of Silver Nanoparticle against Test Bacteria

The method of Gumgumjee *et al.* (2012) was adopted. The test tubes from the MIC assay that showed reduced turbidity were streaked onto freshly prepared sterile nutrient agar plates and incubated at 37 ^{o}C for 24 hrs. The lowest concentration that showed no growth on the recovery plate was regarded as MBC.

Effect of Silver Nanoparticle on Cell Wall of Test Bacteria

The action of the AgNP on cell walls of test bacteria was determined is briefly described. Formalin (0.2 ml) was dispensed into each of three test tubes, and 1 ml each of synthesized AgNP and inoculum of each test bacterium were added to each tube and left for 5 mins. The contents of the tubes were centrifuged at 12,000 ×*g* (MSE Minor 35 Centrifuge) for 15 mins. Cells in the tubes were re-suspended in 0.1 ml demineralised water. Smears of from the suspensions were prepared on glass slides, dried and stained with dilute carbol fuschin for 30 sec. This was rinsed in water, air-dried and examined under the light microscope. Photomicrographs were taken at magnification of ×400 (Alli *et al*., 2011). A control experiment was made to compare the architecture of the untreated test bacteria with those treated with the synthesized AgNP. Gram stain reaction of test bacteria was carried out as described by Fawole and Osho (2007).

Statistical Analysis

The means and standard error of means of the inhibition zones were determined using MS Excel 2013. The statistical significance was calculated using SigmaStat version 3.5 (IBM SPSS Inc. NY, US).

RESULTS AND DISCUSSION

Synthesis of Silver Nanoparticles from Extracts of Leaves of C. albidum

A total yield of 14.9 g of AgNP was obtained as crystals from *C. albidum* leaf extract with a black dry appearance indicating formation of AgNP.

Evaluation of Physical and Chemical Characteristics of AgNP

The analysis of UV-Vis spectrophotometry data in Fig. 1 showed an appearance of surface plasmon resonance (SPR) peak at 478 nm

wavelength, which corresponds to AgNP production. AgNP absorbs radiation intensely at a wavelength of 400 nm due to the transition of electrons (Okafor *et al*., 2013). No other peak was observed in the spectrum which confirms that the synthesized particles are Ag only.

FTIR measurement was carried out to identify the possible biomolecules from aqueous extract of leaves of *C. albidum* responsible for capping and reducing the AgNP. Three obvious infrared bands were observed at 3437 cm^{-1}, 1629 cm^{-1} and 1033 cm^{-1} (Fig. 2). The intense broad band at 3437 cm^{-1} is due to N−H and O−H stretching mode in the linkage of proteins. This corresponds to the presence of amides and alcohol, which is in line with the functional groups of compounds established by the work of Ajitha *et al*. (2015) on *Lantana camara* mediated silver nanoparticles. The medium intense band at 1629 cm^{-1} arises from the C=O and H–N–H stretching mode in primary amines which is commonly found in proteins, indicating the presence of proteins as capping agent for AgNP. This increases the stability of AgNP synthesized and agrees with findings of Wojciechowski and Brzezinski (2002). Absorption bands at 1033 cm^{-1} can be assigned to the C–O stretching vibrations in alcohols, phenols esters, ethers and carboxylic acids. This supports an allusion by Pavia *et al*. (2001) that absorption bands at 1033 cm^{-1} and 1388 cm^{-1} are typical of C–O stretching vibrations in alcohols, phenols esters, ethers and carboxylic acids. To obtain good signal to noise ratio of AgNP the readings were taken in the range 500–4000 cm^{-1}.

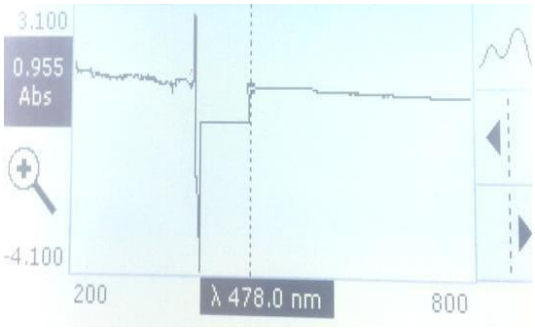

Fig 1: UV-Vis Spectrophotometry of AgNP Synthesized from *C. albidum* Leaf Extract

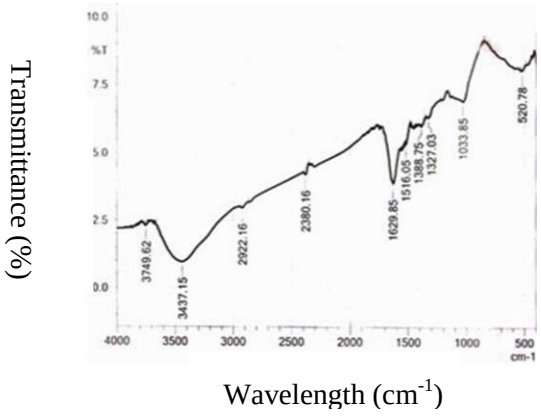

Fig. 2: FTIR Spectroscopy of AgNP Synthesized from *C. albidum* Leaf Extract

Antibacterial Activity of Different Concentrations of Silver Nanoparticles on Test Isolates

In the screening for antibacterial activity, it was observed that the test bacteria showed varying susceptibility to AgNP with significant increase ($p < 0.05$) in inhibition as concentration increased (Table 1). The susceptibility of *E. coli* to AgNP is shown in Fig. 3. The susceptibility of the organisms to AgNP may be owing to the presence of very reactive functional groups, arising from the combination of phytochemical components in *C. albidum* and the reduced Ag^{+} from silver nitrate. Jagesar and Davendra (2008) revealed the toxicity of amides to some selected Gram-positive and negative bacteria. The largest zone of inhibition was recorded when the AgNP were assayed against *P. aeruginosa* (23 mm) and *E. coli* at 300 mg/ml while the lowest zone of inhibition was observed with *S. aureus* (7.5 mm) at the lowest concentration of 100 mg/ml. According to Gogoi *et al.* (2006) the cell surface of *E. coli* is negatively charged therefore positively charged Ag^{+} easily interacts to the cell membrane, thus disabling their function and allowing permeability of AgNP. Some researchers have reported similar observations of reduced susceptibility of *S. aureus* to antibacterial substances, stating that presence of teichoic acids in Gram-positive cell wall prevent

intrusion of antimicrobial agents into such cells (Brown *et al*., 2013).

Comparison of Antibacterial Activity of Reference Antibiotics with Silver Nanoparticle of C. albidum

The degree of susceptibility of test bacteria to two conventional antibiotics as compared to AgNP is presented in Table 2. It was observed that AgNP compared favourably with both reference antibiotics. In particular, the activity produced against *E. coli* and *P. aeruginosa* suggest that the AgNP of *C. albidum* could replace the use of these antibiotics because of their demonstration of similar efficacies. The activity of AgNP compared to the antibiotics may be due to their massive surface area, which provides better contact with microorganisms (Logeswari *et al*., 2015).

Evaluation of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The result of the MIC and MBC of AgNP of *C. albidum* leaf extract against bacteria are shown in Table 3. It was observed that *P. aeruginosa* and *E. coli* demonstrated MIC at 60 mg/ml while *S. aureus* was at 55 mg/ml. The MBC for all test bacteria were obtainedat a much higher dose than MIC in the total extermination of the microbial cells. This result agrees with Akinpelu *et al.* (2016) reporting that MBC of extract of stem bark of C. *albidum* against *S. aureus* were achieved at much higher concentrations than MIC.

Table 1: Susceptibility Profile of Test Bacteria to Different Concentrations of AgNP Synthesized from of *C. albidum* Leaf Extract

Concentration (mg/ml)	**Bacteria**		
	E. coli	*S. aureus*	*P. aeruginosa*
100	12.0 ± 2.00^{a}	7.5 ± 2.00^{a}	11.0 ± 2.00^{a}
200	15.0 ± 2.00^{b}	10.0 ± 2.00^{b}	15.0 ± 1.00^{b}
300	20.0 ± 2.00^{c}	12.0 ± 2.00^{c}	23.0 ± 1.00^{c}

Means ± SEM with different superscript in each column are significantly different at $p < 0.05$.

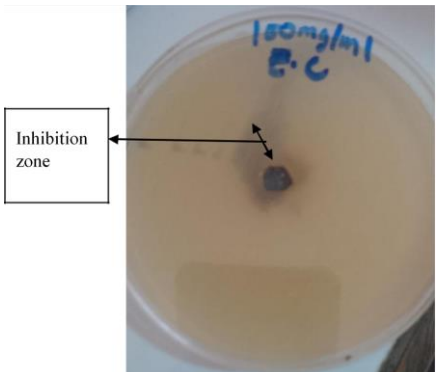

Fig. 3: Inhibition of *E. coli* by 100 mg/ml AgNP Synthesized from *C. albidum* Leaf Extract

Table 2: Comparison between Effects of AgNP of *C. albidum* and Two Conventional Antibiotics against Test Bacteria

Antimicrobial	**Inhibition zone (mm)**		
	E. coli	*S. aureus*	*P. aeruginosa*
AgNP of *C. albidum*	20	12	23
Tetracycline	17	20	17
Gentamicin	21	19	14

Table 3: Minimum Inhibitory and Minimum Bactericidal Concentrations of AgNP Synthesized from Leaf Extract of *C. albidum*

Concentrations (mg/ml)	**Test bacteria**								
	E. coli			***S. aureus***			***P. aeruginosa***		
	0 h	18 h		0 h	18 h		0 h	18 h	
100	1.210	1.100	MBC	1.024	0.702	MBC	1.300	1.264	MBC
90	1.360	1.272	I	1.987	1.448	I	1.304	1.027	I
80	1.620	1.238	I	1.053	0.916	I	0.970	0.835	I
70	0.805	0.705	I	1.924	1.162	I	1.422	1.372	I
60	0.670	0.600	MIC	0.946	0.865	I	0.940	0.703	MIC
55	0.900	1.482	NI	0.401	0.243	MIC	1.285	1.360	NI
50	0.861	0.926	NI	0.779	1.042	NI	0.892	0.951	NI
45	0.710	0.843	NI	0.718	0.863	NI	0.846	0.944	NI
30	0.774	1.192	NI	0.754	1.239	NI	0.891	1.129	NI
Control	0.232	0.711	NI	0.531	0.962	NI	0.424	1.031	NI

Keys: MIC = minimum inhibitory concentration, I = inhibitory action, MBC = minimum bactericidal concentration, NI= no inhibitory action

Effect of AgNP of C. albidum on Bacterial Cell Wall

Microscopic examination revealed that AgNP disrupted the cell walls of test bacteria on direct contact and probably resulted to the intrusion of AgNP into the cytosol of cells. The photomicrographs of treated cells showing disrupted cellular architecture as compared with untreated test bacteria (control) are shown in Figures, 4–6. This effect may be due to direct interaction of the nanoparticle with the cell membrane, and the release of ionic silver (Gugala *et al*., 2016). This is also in line with the hypothesis of Dakal *et al*. (2016) proposing that AgNPs can adhere to microbial cell membranes through electrostatic attractions between the positive charges of the nanoparticles and the negative charges of the cells or by interaction of nanoparticles with the sulfur and phosphorylated proteins present in bacterial cell wall leading to partial dissolution.

According to Pal *et al*. (2007) AgNPs could also be effective as an antibacterial agent upon entry into the cell, thereby releasing silver ions leading to increase in reactive oxygen species (ROS) that could damage enzymes involved in the cellular oxidation-reduction processes of cells and ultimately resulting into cell deaths.

The antimicrobial activity of AgNPs is strain and cell wall structure dependent. Therefore, the charge of the AgNPs also determines their interaction with biological environments and its cellular uptake will lead to a modulation of its antibacterial activity (Duran *et al*., 2016). In

addition to this, AgNPs have been widely used in medicine and biotechnology fields, due to their properties as antimicrobials for the treatment of several diseases, including congenital gonorrhea (Ahmed *et al.*, 2016). Another study has confirmed the effectiveness of AgNPs to inhibit the growth of pathogenic bacteria such as *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus pyogenes*, *Escherichia coli* and *Proteus vulgaris* (Abbaszadegan *et al*., 2015; Jo *et al*., 2015). Intriguingly, activity has also been demonstrated using AgNPs obtained by biological methods which are considered to reduce the negative effects associated with traditional nanoparticle synthesis commonly used in the laboratory (Singh *et al*., 2018).

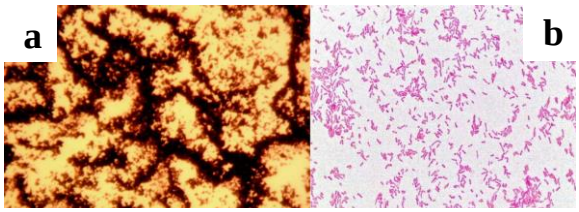

Fig. 4: Photomicrograph of (a) distorted cellular architecture of *E. coli* exposed to 100 mg/ml of AgNP synthesized from leaf extract C. *albidum* (a) and (b) normal cellular architecture of *E. coli*

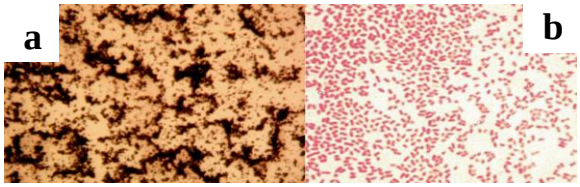

Fig. 5: Photomicrograph of (a) distorted cellular architecture of *P. aeruginosa* exposed to 100 mg/ml of AgNP synthesized from leaf extract C. *albidum* (a) and (b) normal cellular architecture of *E. coli*

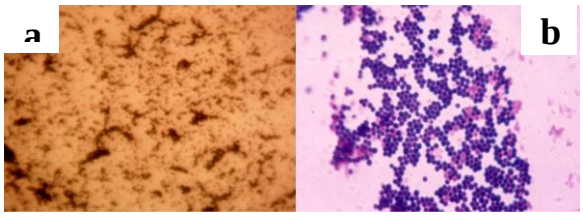

Fig. 6: Photomicrograph of (a) distorted cellular architecture of *S. aureus* exposed to 100 mg/ml of AgNP synthesized from leaf extract C. *albidum* (a) and (b) normal cellular architecture of *E. coli*

Conclusion

The *C. albidum* mediated silver nanoparticles produced in this study provides environmentally friendly, simple and efficient route for synthesis of benign nanoparticles which can play a major role in the field of nanobiotechnology. From the present study, it is evident that the extract of *C. albidum* is very suitable in the biological synthesis of AgNP possessing antibactsignificant antibacterial activity against *P. aeruginosa*, *E. coli* and *S. aureus* and thus can be used as an alternative to gentamicin and tetracycline in the treatment of infections caused by these organisms.

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