Green Synthesis and Antibacterial Activity of Silver Nanoparticles From Extract of Leaves of Croton Zambesicus

Ahmed, R.N., Sani, A., Oluwo, O.N, Sule, I.O and Zakariyah, R.F
Department of Microbiology, University of Ilorin, P.M.B 1515, Ilorin, Nigeria, Tel no: +2348063109301

Abstract: Silver nanoparticles (AgNPs) was synthesized using a combination of aqueous extract of *Croton zambesicus* and silver nitrate ($AgNO_3$) solution to obtain various concentrations of 100mg/ml, 200mg/ml, 300mg/ml and 400mg/ml at 10mM. Characterization of the synthesized silver nanoparticle was done by UV-visible spectroscopy and Fourier Transform Infrared Spectroscopy (FTIR). Antimicrobial activity against four bacterial isolates was determined by standard method of agar-well diffusion assay. The activity of two standard antibiotics was compared with the AgNPs of *C. zambesicus* using the disc diffusion method. The Minimum Inhibitory Concentration (MIC) was achieved using microbroth dilution technique. Test tubes that showed low turbidity in the MIC assays were reinoculated on sterile agar plates and this was taken as Minimum Inhibitory Concentration (MBC). The presence of phytochemical constituent was examined using standard methods. The total yield of the AgNPs of the plant extract was 10.94g. The characterization by UV-visible revealed that at a wavelength of 429.0nm the particle has peak absorbance of 2.003, while the FTIR showed the presence of five (5) functional groups*E. coli* demonstrated reduction in activity as concentration increased with zone diameter of 24mm at 100mg/ml and 10mm at 400mg/ml. The analysis of MIC and MBC revealed inhibitory and bactericidal effects at the same concentration of 30mg/ml. The mode of action of the AgNPs at 100mg/ml showed a total cell lysis of all test isolates. Following the results of the phytochemical analysis, the presence of six phytochemicals were observed. It is evident from this study that AgNPs synthesized from extract of leaf of *C. zambesicus* is a very effective antibacterial agent that can compare favourably with conventional antibiotics, hence considering it as an alternative in the elimination of the tested isolates and infections caused by them.

Keywords: Croton Zambesicus, agar diffusion, FTIR, UV- Vis, Antibacterial

Introduction

The growing menace of resistance of pathogenic microorganisms to conventional medicines has being a subject of concern over the years and natural product scientists have been combing the earth for alternative remedies to combart this scourge threatening human existence. Indigenous trado-medical practice is an ancient form of treatment in human history and considered one of the earliest sources of modern pharmaceutical trade. Currently, an eco friendly green mediated synthesis of inorganic nanoparticles has become a fast growing research in the limb of nanotechnology Sathya and Ambikapathy, (2012)). Nanotechnology is the manipulation of matter on an atomic, molecular and supramolecular scale (Drexler, 1992). Over the past few decades, nanoparticles of noble metals especially silver has shown distinct physical, chemical and biological properties when compared with their bulk counterparts (Kholoud *et al*., 2010).The synthesis of nanoparticles can be by physical, chemical or biological means, however, biological methods are safer and much inexpensive compared to the other two methods. Biosynthetic methods can employ either microbial cells or plant extract for nanoparticles production (Richardson *et al*., 2006). The presence of bioactive principles possessing minimal or no toxic effect makes plants a natural candidate useful as a major raw material for the green synthesis of nanoparticles. The main mechanism considered for the process is plant-assisted reduction due to the presence of phytochemicals (Jha *et al.*, 2009).

Croton zambesicus is a medicinal plant grown in tropical west and central Africa particularly in Nigeria and used to treat fever, dysentery and convulsions (Ngadjui *et al*., 2002).. As most Euphorbiaceae, *Croton* species may contain latex, which is red-colored in some species, a characteristic usually associated with medicinal properties (Sandoval *et al*., 2002). Studies have revealed the antimicrobial properties of various parts of the plant (Abo *et al* **.,** 1999; Okokon and Nwafor, 2010).

Materials and Methods

Collection and identification of plant materials

Fresh leaves of *Croton zambesicus* were obtained from Buhari village along igbaja road, Kwara state, Nigeria. They were identified and authenticated at the herbarium unit of the Department of Plant biology, University of Ilorin.

*Corresponding author:
anrisikat@gmail.com ; Ahmed, R.N

Collection and maintenance of microorganisms

Test isolates were obtained from the culture collection unit of the Department of Microbiology, University of Ilorinand maintained on appropriate agar slants.They were sub cultured periodically to maintain purity and stored at refrigeration temperature. The test isolates were: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Proteus vulgaris*.

Preparation of Plant extract and Silver nanoparticles (AgNPs)

The plant leaves were thoroughly rinsed and cut into pieces to facilitate boiling with 2000ml of distilled water for 10 mins at 100^{o}C. Upon cooling the resulting mixture was filtered using a muslin cloth (Ayanniyi and Wannang, 2008). Silver nitrate (1.69g) was dissolved in 1000ml of deionized water to obtain a standard solution of 10mM. This solution was heated with the resulting filtrate of plant extract (250ml) at 100^{o}C for 5mins and allowed to precipitate for 24hrs (Tamasa, 2013). This was filtered with the aid of a vacuum pump and residue was scrapped and kept for further analysis.

Characterisation of Nanoparticles

The optical property of the particles was determined by UV- Vis spectrophotometer between wavelengths of 200 - 800nm. The resulting spectra were observed and recorded (Tamasa, 2013). Chemical composition of the particles was studied by Fourier transform infra red spectroscopy (FTIR) also as described by Tamasa (2013).

Antibacterial Sensitivity Assay

This was carried out using the agar well diffusion technique as described by Thombre *et al*. (2012). Inoculums of test isolates were separately swabbed on the surface of sterile nutrient agar plates. With the aid of sterile cork borer wells were made at the centre of the agar plates and 1ml of the silver nanoparticles (100 – 400mg/ml) of *Croton zambesicus* was introduced into the wells and plates were incubated over a 24hr period. The reference antibiotics used for the purpose of comparison were gentamicin and tetracyclines.

Determination of minimum inhibitory concentration

Broth dilution method was used. Broth (9.5ml) was separately dispensed into test tubes containing 0.3ml of varying concentrations of the nanoparticles. Inoculums (0.2ml) were separately introduced into each of the tubes and these were incubated at 37oC for 24hours. The turbidity of the tubes was observed with a spectrophotometer (NCCLS, 2000).

Minimum bactericidal concentration

One milliliters of content of test tubes that showed no growth in the MIC assay were streaked onto freshly prepared sterile nutrient agar plates and incubated at 37°C for 24hours, after which the plates were observed for growth. The lowest concentration that showed no growth on the recovery plate was regarded as minimum bactericidal concentration or minimum lethal concentration (Black, 1996).

Effect of AgNPs of *croton zambesicus* leaf extract on test isolates

Formalin (0.2 mL) was dispensed into four (4) test tubes, to each of the tubes, 1ml of the synthesized AgNPs and 1ml each of test isolates were added and left for 5 mins. This was done separately for each test isolates. The content was centrifuged at 12,168x10^{3} g (MSE Minor 35 Centrifuge) for 15 min. The cells in tubes were resuspended in 0.1 mL demineralised water. Smears of these were made on glass slides, dried and stained with dilute carbolfuschin for 30 sec, it was rinsed in water, air-dried and examined under a light microscope. Photomicrographs were taken at a magnification of X400 (Alli *et al*., 2011). A control experiment was carried out with test isolates without the addition of nanoparticles but Gram staining reagents as described by Fawole and Osho (2007). This was done to compare the cellular architecture of normal cells of test isolates with cells treated with nanoparticles.

Qualitative Phytochemical Analysis of leaf extract of *Croton zambesicus*

The crude extract of Croton zambesicus was analysed for the presence of secondary metabolites such as : tannins, saponins, flavonoids, steroids, anthraquinones and terpenoids as described by Sofowora (1993).

RESULT

Synthesis of silver nanoparticles from extract of leaf of *Croton zambesicus*

A total yield of 10.94g was recovered as crystals from the synthesis of silver nanoparticles from the extract of leaf of *Croton zambesicus*. The colour of the crystal gave a dry black appearance.

Evaluation of physical and chemical characteristics of silver nanoparticles

The analysis of the extent of absorbance of light through the synthesized AgNPs was determined and a peak value was obtained to be 2.003 at a wavelength of 429.0nm. The spectrum of absorbance is presented in Figure 1.

The result of the FTIR revealed the presence of four functional groups in the particles. They include: Amide, Carboxylic acid, Phenols, Carbonyl and

Hydroxyl. The infrared spectroscopy is presented in Figure 2.

Figure 1: UV/Vis spectroscopy of AgNPs from *C. zambesicus* leaf extract.

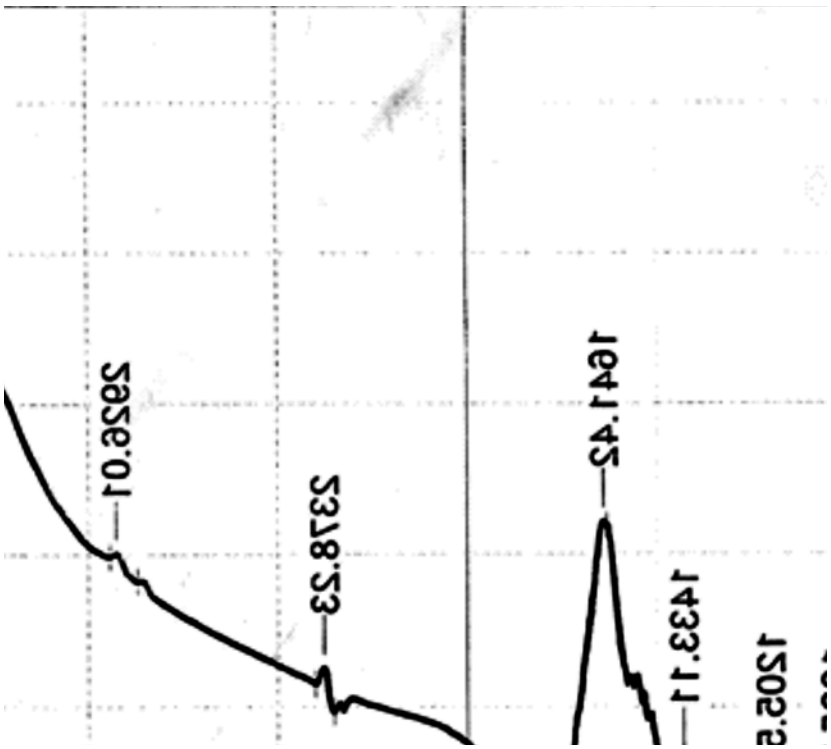


Figure 2: Fourier-Transform Infrared Spectroscopy 0f $AGNO_3$ synthesized from *C. zambesicus* extract.

Evaluation of antibacterial activity of different concentrations of AgNPs of extract leaf of *Croton zambesicus*

The screening of the AgNPs against the test isolates showed varying degree of activity. However, it was observed that only *P. aeruginosa* had a concentration dependent susceptibility pattern. This result is presented in Table 1.

Table 1: Sensitivity profile of AgNPs of *C. zambesicus* leaf extract to test isolates at different concentrations.

Concentrations (mg/ml)	**Test Isolates**			
	E. coli	*P. vulgaris*	*S. aureus*	*P. aeruginosa*
100	27.5±1.00^{c}	28.0±2.00^{c}	21.0±1.00^{a}	26.0±1.00^{a}
200	22.0±2.00^{b}	16.5±2.00^{b}	29.0±1.00^{c}	26.5±1.00^{a}
300	18.5±1.00^{ab}	10.5±2.00^{a}	24.5±1.00^{ab}	28.0±1.00^{a}
400	16.5±1.00^{a}	10.0±1.00^{a}	27.0±1.00^{bc}	28.5±1.00^{a}

Means with different superscript in each column are significantly different at $P < 0.05$.

Table 2: Comparison of effects of AgNPs of *C. zambesicus* with two conventional antibiotics against selected test isolates.

Test isolates	AgNPs of *C. zambesicus* at 100mg/ml	Antibiotics	Diameter of zone of inhibition of antibiotics(mm)
E. coli	27.5	Tetracycline	17
		Gentamicin	21
S. aureus	21	Tetracycline	20
		Gentamicin	19
P. aeruginosa	26	Tetracycline	17
		Gentamicin	14
P. vulgaris	28	Tetracycline	15
		Gentamicin	16

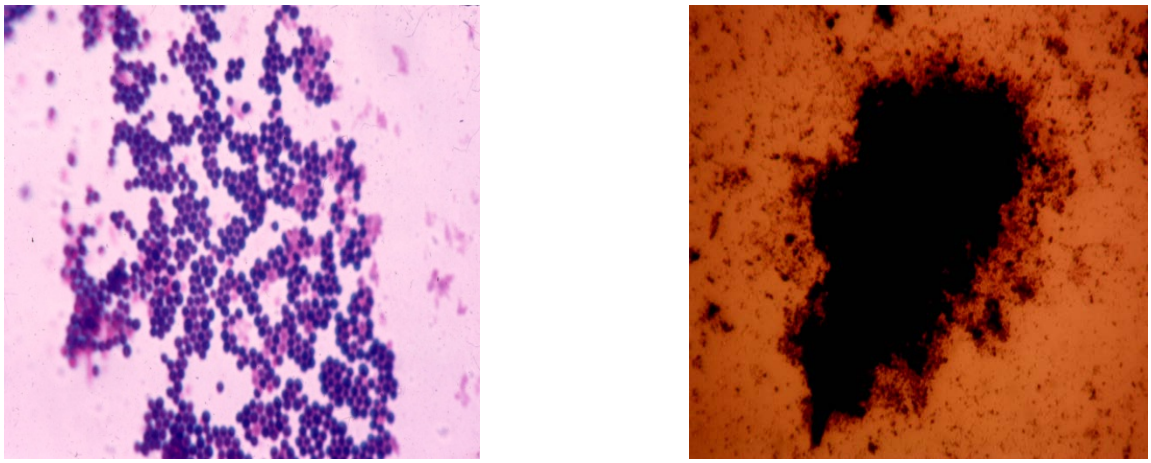
Evaluation of MIC and MBC of nanoparticles
The MIC and MBC were obtained at the same concentration of 30mg/ml as shown in Table 3.

Table 3: Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of AgNPs of *C. zambesicus*

	Escherichia coli		***S. aureus***		***P. aeruginosa***		***P. vulgaris***		**MIC/MBC**
Concentrations	**0hrs**	**24hrs**	**0hrs**	**24hrs**	**0hrs**	**24hrs**	**0hrs**	**24hrs**	
100	1.324	0.936	0.806	0.590	1.062	1.012	0.921	0.553	-
90	1.227	0.711	1.947	1.802	1.325	1.045	0.788	0.506	-
80	0.869	0.314	2.729	2.580	1.098	0.958	0.715	0.498	-
70	0.623	0.287	0.423	0.227	0.940	0.888	0.543	0.301	-
60	0.532	0.356	0.473	0.373	0.864	0.623	0.421	0.339	-
50	0.567	0.451	0.476	0.387	0.678	0.596	0.487	0.399	-
40	0.459	0.356	0.428	0.321	0.446	0.306	0.436	0.334	-
30	0.444	0.311	0.346	0.328	0.478	0.305	0.399	0.301	++
CONTROL	0.432	1.197	0.483	0.747	0.458	0.965	0.375	0.762	-

Effect of synthesized AgNPs on cell wall of isolates

The AgNPs was observed to have acted on the test isolates by a mechanism of action thought to be responsible for cell wall disruption, leading to the intrusion of the AgNPs. The photomicrograph of treated cells showed a disrupted cellular architecture when compared with the untreated control cells. This is shown in Plates 1 a and b.



a **b**

Plate 1: Photomicrograph showing normal cellular architecture of *S. aureus* without treatment with AgNPs (a) and plate b shows distorted cellular architecture of *S. aureus* as a result of exposure to 100mg/ml of AgNPs of extract of leaf of *C. zambesicus.*

Phytochemical analysis.

Phytochemical screening of the AgNPs synthesized from the extract of leaf as compared with the methanolic extract of leaf of *Croton zambesicus* is represented in Table 4.

Table 3: Comparison of phytochemicals of AgNPs synthesized extract with non-AgNPs Extract of *C. zambesicus*.

Phytochemicals	Methanolic leaf extract
Tannins	+
Flavonoids	–
Alkaloids	+
Saponins	+
Steroids	–
Phenols	+
Terpenoids	+
Anthraquinones	+
Glycosides	+

Key: + = Present = Absent

Discussion

With the increasing interest in the field of nanotechnology, as it affects various sectors of life, its study with phytomedicines has almost become a necessity (Colvin *et al*., 1994). In this study, during the synthesis of silver nanoparticles, the reduction of silver ions into silver nanoparticles upon exposure to plant extracts was determined by the appearance of a colour change. This change was due to the Surface Plasmon Resonance phenomenon (SPR). The silver nanoparticles normally possess free electrons, which give the SPR absorption band, due to the combined vibration of electrons of metal nanoparticles in resonance with light wave (Tamasa, 2013). The sharp bands of the synthesized silver nanoparticles were observed around 429 nm with an absorbance of 2.003 (Figure 1). This broad absorption peak of the sample is accompanied with the fact that absorption of light by a solution is due to the transition of electrons. The absorption ability of a sample is proportional to the concentration of absorbing molecules. This result obtained is in consonance with Tamasa (2013), which at a wavelength of 421nm, there was an absorbance value of 0.550.

As shown in Fig. 2, the FTIR revealed the presence of four functional groups, indicating the

presence of chemical bonds biomolecules for capping and efficient stabilization of silver nanoparticles. This also agrees with the findings of Block *et al*. (2004) that four functional groups were present in the AgNPs of extract of leaf of *Croton zambesicus.*

Results from the antimicrobial assay indicated that the AgNPs synthesized from the extract of leaf of *Croton zambesicus* showed high antibacterial activity against all four test isolates. The resulting activity could be as a result of the presence of the organic groups revealed by the FTIR. The highest sensitivity experienced with *E. coli* may be due to the gram-negative nature of the cells and for this reason, silver ions which are positively charged therefore can easily react with the cell (Gogoi *et al.,* 2006), rendering the cell wall permeable to the AgNPs of extracts of leaf of *C. zambesicus*, and allowing for penetration and intrusion of components into the cells (Burda *et al*., 2005).

In the comparative analysis between the conventional antibiotics and the AgNPs of the *C. zambesicus*, the presence of several bioactive components together in a matrix may be responsible for this effect as compared with the conventional antibiotics which have been purified and containing a single working principle. In addition, the efficacy of the AgNPs may be as a result of the silver present in the extract.

The minimum inhibitory concentration and minimum bactericidal concentration which indicated that the inhibitory and bactericidal property of the synthesized AgNPs of extract of leaf of *Croton zambesicus* was obtained at low concentration (30mg/ml) is indicative of the potency of the AgNPs.
The effect of AgNPs on the cell wall of *S. aureus* as shown by a photomicrograph revealed the extent of cellular architectural damage of the cells, consequent upon the ability of the AgNPs to permeate the cell walls of the isolates. *S. aureus* being a gram positive cell, the nature of their cell wall is such that they lack an outer covering, which is the critical site of attack of antimicrobial agents. This result is in line with the work of Alli *et al*.(2011) which reported distortion and elongation of cells treated with extracts of garlic. The results of the phytochemical screening revealed the presence of Tannins, Saponin, Steroids, Terpenoids, Anthraquinones, Phenols, Alkaloids, which are secondary metabolic products of plants. Past literatures have shown that terpenoids inhibit the growth of some enteric pathogenic organisms and have been classified under the beta-lactam group of antibiotics as they diffuse through the medium and destroy the integrity of the cell membrane (Elander, 2003). Tannins which are found to be dominant in food product of plant-vegetable origin such as tea and many fruits may have contributed to the inhibitory effect of the extract, as they possess the ability to shrink proteins in the cells of microorganisms which in turn destroys their cell walls (Barnabas and Nagarajan, 1988). It has been reported by Prindle and Wright (1997) that the antimicrobial activity of phenolic compounds present in extracts require higher doses for effective activity, affecting enzymatic activity related to energy production at low concentrations and causing protein precipitation at high concentrations. Phenols can be released by the hydrolysis of non toxic glycosides which have toxic effects on microbial pathogens (Aboaba and Etuwape, 2001).

From the results emanating from this study, it is evident that silver nanoparticles synthesized from extract of leaf of *Croton zambesicus* was effective against the test isolates and could be a useful alternative antimibacterial agent for the management of infections resulting from these organisms. Since the menace of antibiotic resistance has called for urgent and effective alternatives, there is the need to augment health care needs with herbal preparations incorporated with nanotechnology. Fortunately, *Croton zambesicus* is a medicinal plant grown in tropical west and central Africa particularly in Nigeria, hence making rendering its production a cost effective one in indigenous health care delivery.

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