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SYNTHESIS OF SILVER NANOPARTICLES AND ANTIMICROBIAL ACTIVITIES OF METHANOLIC EXTRACT OF *AZADIRACHTA INDICA* LEAF AGAINST *ESCHERICHIA COLI, STAPHYLOCOCCUS AUREUS* AND *CANDIDA ALBICANS*

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ABSTRACT

Azadirachta indica is a plant commonly known as neem. It is native to the Indian subcontinent and most countries in Africa, including Nigeria. The aim of this study was to synthesise silver nanoparticles and examine antimicrobial activities of crude methanolic extract of *Azadirachta indica* leaves on clinical isolates of *Staphylococcus aureus, Escherichia coli* and *Candida albicans*. Methanol was the solvent used for the extraction. The characterization of synthesized AgNPs was carried out using the UV-Visible spectrophotometer. Antimicrobial activity was conducted using disc diffusion method while the Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) were determined using broth dilution method. The antimicrobial activity of the synthesized *Azadirachta indica* AgNPs was carried out using standard agar well diffusion method. Qualitative phytochemicals analysis revealed presence of tannins, phenols, alkaloids, phytosteroid, and terpenoid while flavonoids, steroids and saponin were absent. Antibacterial activities of the extracts using the ditch method (concentrations of 100mg/mL, 50mg/mL, 25mg/mL, 12.5mg/mL, and 6.25mg/mL) recorded no zone of inhibition for *Staphylococcus aureus* and *Escherichia coli.* Antimicrobial activity of synthesized silver nanoparticles (AgNPs) from methanolic extract showed no zone of inhibition for *Staphylococcus aureus.* The zone of inhibition against *Escherichia coli* was 5.33mm while for *Candida albicans* the zone of inhibition was 15.33mm. Based on the results obtained from this study, it could be said that methanolic extract of *Azadirachta indica* leaves contains phytochemicals of pharmacological significance which could be exploited as antifungal agent.

Keywords: *Azadirachta indica*, Methanolic, Synthesis, Silver nanoparticles, MIC, MBC

INTRODUCTION

Food preservation and food storage are parts of the efforts that are geared towards avoiding food spoilage and extending the shelf life of food items. Food storage has been practiced for the purpose of achieving food security (Tucker, 2022). Silver was used for food storage by Persians, Egyptians, Romans and Greeks about 5000 years ago (Mody *et al.*, 2010). The use of silver in the manufacture of utensils that are used in kitchens and for drinking was attributed to its antibacterial potency. Nano-materials the dimension ranging from 1–100 nm have high surface to volume ratio. This makes them a preferred material in the production of materials used in photonics, electronics, and biomedical devices (Saha *et al.*, 2017).

Plant extracts from leaf, root, stem, bark, leaf, fruit and bud have been used in the synthesis of silver nanoparticles (Willner *et al.*, 2007). Green synthesis of silver nanoparticles with the use of microorganisms such as bacteria and fungi, including plants has also been carried out successfully (Ahmed *et al*., 2015).

Nanotechnology is a current field of science that deals with handling of materials at the nanoscale. This branch of science has been evolving intensely and plays a central role in daily life as a result of the influence of their vast applications in all circles of human life. It is a field that deals with several structures of matter that have sizes in the order of a billionth (10^{-9}) of a metre (Saratale *et al.*, 2018).

Nanotechnology comprises the creation, characterization including manipulation of components that have in any case one of its dimensions in the region of 1-100 nm in length. When the sizes of particles decrease lower than this dimension, the resultant material has its physical and chemical attributes transformed to make it different greatly from macroscale components (Jassim *et al.*, 2019).

Nanomaterials represent the major tools of nanotechnological applications, and such include nanowires, nanotubes, nanoflowers, nanocomposites, and nanoparticles which have been extensively studied, and fabricated through various processes involving manipulations to various sizes and shapes to fit into specific applications of desire (Peralta-Videa *et al.*, 2016).

Nanomaterials have properties that have made them extremely useful in biosensing, biological labelling, catalysis, antibacterial activity, antiviral activity, drug delivery, antioxidant applications, DNA sequencing and gene therapy in the recent years (Vasantharaj *et al.*, 2018). Owing to these valuable properties, nanomaterials have been employed for applications in electronics, catalysis, photonics, information technology, environmental remediation, cosmetics, drug delivery, biomedical, optics, chemical industries, mechanics, space industries, light emitters, energy science, non-linear optical tools and single electron transistors. Functionalization of nanomaterials enables directed delivery, transportation and distribution of nanoparticles to specific cell types, and this makes them particularly useful in gene delivery, bioimaging, and other diagnostic and therapeutic applications (Vijayaraghavan and Ashokkumar, 2017).

There exist a variety of nanoparticles but majorly metallic and non-metallic nanoparticles are widely recognized. Examples of such non-metallic nanoparticles are carbon, silicon, nitric oxide, chitosan, fullerenes, and graphene oxide nanoparticles among others while some of the widely investigated metallic

and metal oxide nanoparticles include cobalt, titanium, aluminium oxide, copper, silver, gold, palladium, magnesium, manganese oxide, platinum, zinc oxide, magnetite and cerium dioxide (Lateef *et al.,* 2016a).

The biological mode of creation of nanomaterials has led to the emergence of the subdiscipline of 'green nanotechnology'. Green nanotechnology is described as the use of green chemistry, green engineering, and sustainability codes to eradicate or decrease the application and generation of lethal substances in the field of nanotechnology. The emergence of green nanotechnology leading to biofabrication of biocompatible and less toxic nanomaterials has led to the upsurge in their applications in biomedicine (Lateef *et al.,* 2016b).

Silver nanoparticles (AgNPs) are increasingly used in various fields, including medical, food, health care, consumer, and industrial purposes, due to their unique physical and chemical properties. These include optical, electrical, and thermal, high electrical conductivity, and biological properties (Gurunathan *et al.,* 2015). Due to their peculiar properties, they have been used for several applications, including as antibacterial agents, in industrial processes, household, and healthcare-related products, in consumer products, medical device coatings, optical sensors, and cosmetics, in the pharmaceutical industry, in food industry, in diagnostics, orthopaedics, drug delivery, as anticancer agents, and they have ultimately enhanced the tumour-destroying effects of anticancer drugs and biopharmaceuticals (Chernousova and Epple, 2013). Recently, AgNPs have been frequently used in many textiles, keyboards, wound dressings, and biomedical devices (Li *et al.,* 2014). Nano sized metallic particles are unique and can considerably change physical, chemical, and biological properties due to their surface-to-volume ratio; therefore, these nanoparticles have been exploited for various purposes (Sharma *et al.,* 2009).

Azadirachta indica contain biologically active compounds including azadirone, promeliacin, limonoids, gedunin, vilasinin, C-secomeliacins, azodirachtin, nimbin, salanin and protein/amino acids, polysaccharides, sulphurous compounds, polyphenolics such as flavonoids, glycosides, dihydrochalcone, coumarins, tannins, and aliphatic compounds (Biswas *et al.,* 2002).

The biologically most active compound is azadirachtin, which is actually a mixture of seven isomeric compounds labelled as azadirachtin A-G and azadirachtin E which is more effective (Verkerk and Wright, 2003). The plant extracts exhibit some antimicrobial, anti-inflammatory and antipyretic properties. Also, the anti-fertility effects of the neem oil due to the potential spermicidal effect have been recorded (Lai *et al.,* 2005; Riar *et al.,* 2007).

Neem has also been used as abortifacient, analgesic, antihelminthic, antibacterial, antiyeast, antiulcer, antifilarial, antifungal, antihyperglycemic, anti-inflammatory, antiviral, antimalarial, diuretic, antinematodal, antipyretic, antispasmodic, insecticidal, antispermatogenic, antitumor, hypercholesteremic, hypoglycaemic, and immunomodulator. Similarly, neem is also useful in chickenpox, it has been used to boost immunity of body, to reduce fever caused by malaria, it has also been used for treating various fungal infections and it is also useful against termites and in curing neuromuscular pains. It is an established fact that polyphenolic compounds possess remarkable antioxidant and antimicrobial activities which are present quite commonly in the plant family Meliaceae. *Azadirachta indica* is well known in Nepal for more than 2000 years as one of the most versatile medicinal plants having a wide spectrum of biological activity (Parotta, 2001).

Azadirachta indica was used in folklore medicine for the treatment of Diabetes (Shravan *et al.,* 2011). Aqueous extract of Neem leaf has a good therapeutic potential as anti hyperglycemic agent in Insulin Dependent Diabetes Mellitus (IDDM) and Non-Insulin Dependent Diabetes Mellitus (NIDDM) (Sonia and Srinivasan, 2009). Neem leaves has antibacterial properties and could be used for controlling airborne bacterial contamination. Neem seeds in traditional medicine are used to treat infections especially those involving the eye and ear (Saseed and Khan, 2008).

Escherichia coli is a Gram-negative facultatively anaerobic rods with both fermentative and respiratory metabolism that do not involve the production oxidase. *Escherichia coli* has strain-specific O lipopolysaccharide antigens on its cell wall (175 O antigens currently recognized) and flagella or H antigens if present (56 H types are recognized). There are also 80 different capsular polysaccharide (K) antigens. *Escherichia coli* is serotyped based on the combination of O, H and K antigens, although generally only the O and H types are listed, e.g. *E. coli* O157:H7. Serotyping of *E. coli,* together with molecular and phage typing, has being a useful epidemiological tool (Desmarchelier and Fegan, 2002).

Staphylococcus aureus has been isolated from human skin and nasopharynx. It has been implicated in a wide range of infections on the skin, soft tissues, internal organs and endovascular sites. *Staphylococcus aureus* has adapted rapidly and become resistant to many antibiotics that are available in the market. Many nosocomial infections are caused by methicillin-resistant *Staphylococcus aureus* (Foster, 2002).

Candida albicans is an opportunistic fungal pathogen that exists as a harmless commensal in the gastrointestinal and genitourinary tracts in about 70% of humans and about 75% of women suffer from Candida infection at least once in their lifetime (Ruhnke and Maschmeyer, 2002). However, it becomes opportunistic pathogen for immune-compromised patients, for some immunologically weak individuals, or even for healthy persons. The infection caused by *Candida albicans* is commonly known as candidiasis (Sexton *et al.,* 2007).

Antibiotic resistance has been described as an increasing phenomenon and there is need for the development of new and more effectual drugs against microbes (Lateef *et al.,* 2016a). Resistance in bacteria can be attributed to horizontal gene transfer of the antibiotic resistance genes, adjustment in the antibiotic target, mutational changes in the biofilm formation and efflux pumps (Qayyum and Khan, 2016). Antibiotic resistance patterns of microorganisms have led to the dread about the emergence and re-emergence of multidrug resistant (MDR) parasites and pathogens. Antibiotic resistance is rising to dangerously high levels in all parts of the world. New resistance mechanisms are emerging and spreading globally, threatening our ability to treat common infectious diseases. A growing list of infections such as pneumonia, tuberculosis, blood poisoning, gonorrhea, and foodborne diseases are becoming harder, sometimes impossible to treat as antibiotics become less effective.

Research has shown that the use of antibiotics can be harmful because it disrupts many body functions and causes damage to the body. Some of these antibiotics cause damage either to the liver, kidney, lungs, or other vital organ. The search for newer drugs that can be used to combat infectious diseases and avoid these side effects has become imperative. The knowledge of antimicrobial activity of *Azadirachta indica* leaf extracts against selected pathogens will provide relevant information on the need to develop these extracts into drugs that can be used as alternative therapy against infectious

diseases. The aim of this study was to synthesize silver nanoparticles by using methanolic crude extract of *Azadirachta indica* leaves and then examine its antimicrobial activities.

MATERIALS AND METHODS

Procurement of Fresh *Azadirachta indica* Leaves

The fresh *Azadirachta indica* leaves were collected at Pakata, Ilorin West Local Government Area, (Latitude: 8.4975º North; Longitude: 4.5242º East) Ilorin, Kwara State, Nigeria. It was characterized and identified at the Herbarium Unit of Plant Biology Department, University of Ilorin, Ilorin, Nigeria and was assigned UILH/006/1280/2021 as accession number.

Preparation of media

All culture media were prepared according to manufacturers' instructions and autoclaved at 121ºC for 15 minutes. The media used were nutrient agar, Sabouraud dextrose agar and Mueller Hinton agar.

Procurement of Test Organisms

The test organisms were two bacteria (*Staphylococcus aureus* and *Escherichia coli)* and a yeast (*Candida albicans*). They were obtained from the Microbiology Department, University of Ilorin Teaching Hospital, Ilorin, Kwara State, Nigeria. The organisms were collected on sterile agar slants and incubated appropriately. They were kept as stock cultures in the refrigerator and stored at 4ºC until required for study.

Preparation of Leaf Extract

Collected fresh *Azadirachta indica* leaves were washed using running tap water to remove adhering impurities and with distilled water. The leaves were air dried under shade in order to remove moisture (Kalainila *et al*., 2014). The leaves were grounded to fine powder using electric blender and stored in an air-tight container until further usage.

Extraction was done following the procedure described by Varghese *et al*. (2013). Fifty grams (50 g) of powdered leaves were weighed using a weighing balance into a clean and dry beaker after which 500 mL of methanol was poured into the beaker. It was stirred with a sterile rod and allowed to soak for 48 hours. After 48 hours, the content of the beaker was filtered using a muslin cloth and later with Whatman filter paper No. 1. The extractant was evaporated using water bath. The extracts were then stored until needed for analyses (Varghese *et al*., 2013).

Phytochemical Analysis

The extracts were subjected to phytochemical analysis after filtration before concentration to determine their phytoconstituents.

Detection of Tannin

Methanolic leaf extract weighing 0.2g was stirred with 5 mL distilled water in a test tube and filtered, 2-3 drops of dilute ferric chloride solution was added. A green or blue-black colouration indicated the presence of tannin (Parekh and Chinda, 2008).

Detection of Phenol

Methanolic leaf extract weighing 0.2g was dissolved in 3-4 drops of ferric chloride solution. Formation of blue-black or brown colour indicated the presence of phenol (Del-rio *et al.,* 1997).

Detection of Alkaloid

Methanolic leaf extract (0.5 g) was dissolved in 5 mL diluted HCl in a steam bath and filtered. About 1 mL of the filtrate was treated with few drops of Mayer's reagent, giving rise to a cream or pale yellow precipitate, indicating the presence of alkaloid (Parekh and Chinda, 2008).

Detection of Flavonoid

The methanolic extract of the leaf weighing 0.05 g was dissolved in dilute NaOH. On addition of HCL a yellow coloration was formed, indicating the presence of flavonoid (Okwu, 2004).

Detection of Phytosteroid

Methanolic leaf extract weighing 0.05 g was transferred into a sterile tube and 2 mL of chloroform was added to dissolve the extract, the solution was filtered using filter paper and two drops of concentrated sulphuric acid was added. The content was shaken properly and left for some minutes, the presence of a golden yellow colour indicated the presence of phytosteroid (Okwu, 2004).

Detection of Steroid

Methanolic leaves extract weighing 0.05 g was transferred into a sterile tube and 2ml of concentrated sulphuric acid was added to dissolve the extracts, 2ml of acetic anhydride was added to the solution and observation of colour change from violet to blue green was made which indicated the presence of steroid (Cowman, 1999).

Test for Saponin

Methanolic extract of the leaf weighing 0.2 g was dissolved in 5mL of distilled water, 2mL of the resulting solution was then taken in a test tube and shaken vigorously for 15 minutes. Frothing was taken as an evidence of the presence of saponin (Salah *et al*., 1995).

Tests for Terpenoid

The extract weighing 0.05 g was dissolved in 1mL of chloroform, 1mL of acetic anhydride and 2 mL of H_2SO_4 was added. Formation of reddish violet colour indicated the presence of terpenoid (Salah *et al*., 1995).

Dilution of the Extract

Using sterile dilution technique, 0.2 g of each of the extracts was dissolved separately in 2mL of the solvent to give concentration of 100mg/mL followed by serial dilution to give various concentrations of 50 mg/mL, 25 mg/mL, 12.5 mg/mL, and 6.25 mg/mL. The test tubes containing the various concentrations were labelled (Banso and Ayodele, 2001).

Standardization of Test Organisms

The spectrophotometer was powered on to warm it up for 20 minutes. After which the wavelength was set to 500 nm an uninoculated sterile broth medium was poured in a cuvette and it was blanked. The medium in which samples will be taken from were inoculated with each organism in different test tubes containing the sterile medium. The inoculated medium was pipette into the cuvette and placed in the spectrophotometer and the reading was taken within the range of 0.08-0.1. The inoculated medium which was above this range were adjusted by adding sterile medium to it while the inoculated medium which fall below this range were adjusted by inoculating it with the test organism. This reading was adjusted to 0.5 McFarland turbidity ($1.5x10^8$ CFU/mL).

Antibacterial/Antifungal Activity of the Extracts

Agar ditch diffusion method was adapted for the antibacterial susceptibility test. Mueller-Hinton agar or Sabouraud dextrose agar was dispensed into each sterile Petri dish and allowed to set and then labelled. A sterile 6 mm cork borer was then used to punch holes (5 wells) in the already inoculated agar plates. The test organisms used were *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. The wells that were formed were filled with different concentrations of the extract which were labelled accordingly as follows; 100 mg/mL, 50 mg/mL, 25 mg/mL and 12.5 mg/mL and 6.25 mg/mL. These were then left on the bench for 1 hour for adequate diffusion of the extracts and incubated at 37°C for 24 hours for bacteria and 25°C for 48 hours for fungus. After incubation, the diameter of the zones of inhibition around each well was measured to the nearest millimetre (Del-rio *et al.,* 1997).

Determination of Minimum Inhibitory Concentration (MIC) of the Extracts

The minimum inhibitory concentration of the plant extract was determined using broth dilution method (Santosh, 2013). Varying concentrations of the extract were weighed and mixed with 3ml of Mueller-Hinton broth and Sabouraud dextrose broth in test tubes for bacteria and fungus respectively. The contents were thoroughly mixed and the tubes were inoculated with 0.2 mL of standardized isolates. The tubes were then incubated at 37ºC and 25ºC for 24 hours and 48 hours for bacterial and fungal growth respectively (Kareem *et al*., 2008). The least concentration of the extract that did not permit any visible growth of the test isolates in the broth media were regarded as the minimum inhibitory concentration (MIC) in each case (Sathiyavimal *et al.,* 2018).

Determination of Minimum Bactericidal/Fungicidal Concentration of the Extracts (MBC/MFC)

The determination of the minimum bactericidal/fungicidal concentration was followed by the determination of minimum inhibitory concentration. The minimum bactericidal/fungicidal concentration was the lowest concentration of the antibacterial/antifungal agent that kills at least 99% of the test organism. Mueller-Hinton agar was inoculated with sample from each of the tubes that shows no turbidity and the plates were incubated at 37ºC for 24 hours. Sabouraud dextrose agar was inoculated with sample from each of the tubes that shows no turbidity and the plates were incubated at 25ºC for 48 hours. The highest dilution that yielded no bacterial colony or fungal colony was taken as the Minimum Bactericidal/Fungicidal Concentration (Sathiyavimal *et al.,* 2018).

Antibiotic Susceptibility Testing using Conventional Antibiotic Disc

This was done as described by National Committee for Clinical Standards (NCCLS) 2017 using Agar disc diffusion method. Dried surfaces of Mueller-Hinton agar plates were swabbed with 0.5ml broth culture of the standardized test organisms. Commercially purchased antimicrobial discs were placed onto the surface of the inoculated agar plate. Each disc was pressed down to ensure complete contact with the agar surface and the plates were incubated at 37ºC for 24 hours without inverting. The diameter of inhibition zones was measured with a ruler in mm and compared with a standard antibiotic sensitivity table by National Committee for Clinical Standards (NCCLS, 2017).

Preparation of Silver Nitrate Solution (1mM)

Silver nitrate (0.17 g) was added to 1000 mL double distilled water and dissolved properly. The solution obtained was transferred to an amber coloured bottle to prevent autoxidation of silver (Lateef *et al*., 2016c).

Synthesis of Silver Nanoparticles

The *Azadirachta indica* leaves extracts (1 mL) was added to 9 mL of 1 mM aqueous silver nitrate. Nine millilitres (9 mL) of 10^{-3}M silver nitrate solution was considered as control. The resulting solution was kept under direct sunlight. Observation for gradual colour change was done and the result was recorded (Das *et al*., 2016).

Characterization of Synthesized AgNPs

The bioreduction of pure Ag^{+} to Ag^{O} ions was confirmed by subjecting 2mL of the synthesized AgNPs to UV-Visible spectrophotometer (Model- Shimadzu UV-1800, Japan) in the range of 190-800 nm (Das *et al*., 2016). A graph of the Absorbance readings against the Wavelength was plotted.

Antimicrobial Activity of the Synthesized *Azadirachta indica* AgNPs

The silver nanoparticles synthesized using *Azadirachta indica* was tested for antibacterial activity by standard agar well diffusion method (Parekh and Chinda, 2008). The test organisms for antibacterial activity were *Staphylococcus aureus* and *Escherichia coli*. The pure culture of bacteria and fungus were subcultured on Mueller-Hinton agar and Sabouraud agar respectively. Fresh overnight grown cultures of the respective bacteria and fungus were spread on Mueller-Hinton agar and Sabouraud agar respectively contained in a Petri plates. Each strain was swabbed uniformly using sterile glass spreader. Wells of 6mm diameter were made on Mueller-Hinton agar and Sabouraud agar using sterile cork borer. The synthesized *Azadirachta indica* AgNPs and $AgNO_3$ were loaded into different wells. After incubation at 37ºC for 24 hours for bacteria and 25ºC for 48 hours for fungus. The zones of inhibition were measured (Kim *et al.,* 2008).

RESULTS AND DISCUSSION

Results

Different concentrations of methanolic extracts of *Azadirachta indica* (100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL and 6.5 mg/mL) were tested against three pathogenic organisms (*Staphylococcus aureus*, *Escherichia coli* and *Candida albicans)* using the agar well diffusion method. The antimicrobial activities of the leaves extracts were assessed through measuring of the zones of inhibition, observing the minimum inhibitory concentration (MIC), minimum bactericidal concentrations (MBC), minimum fungicidal concentration (MFC) as well as the antimicrobial activity of its silver nanoparticles was assessed using the agar well diffusion method. The phytochemical analysis of the extract revealed the presence of tannin, phenol, alkaloid, phytosteroid, and terpenoid.

Antibacterial activity of the extract showed that the extract was active against the test organisms. The zones of inhibition against *Escherichia coli*, and *Staphylococcus aureus* were 13.33±2.03 and 12.00±1.53 respectively using 100 mg/mL of the extract. The zone of inhibition against *Candida albicans* was 9.67±1.76 using 100 mg/mL of the extract. The minimum inhibitory concentrations (MICs) of the methanolic extract of *Azadirachta indica* against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* were 25 mg/mL, 50 mg/mL and 50 mg/mL respectively. The minimum bactericidal

concentration (MBC) of the extract against *Escherichia coli* and *Staphylococcus aureus* was 50 mg/mL while the minimum fungicidal concentration (MFC) of the extract against *Candida albicans* was 100 mg/mL.

Analysis of the antibacterial activity of synthesised silver nanoparticles (AgNPs) from the methanolic extract revealed 5.33±0.88 as the minimum inhibitory concentration against *Escherichia coli*. The synthesised silver nanoparticles (AgNPs) from the methanolic extract was not active against *Staphylococcus aureus*. The minimum inhibitory concentration of the synthesised AgNPs against *Candida albicans* was 15.33±0.88.

Results obtained when conventional antibiotics were used against the bacteria showed that *Escherichia coli* was susceptible to tetracycline, cotrimaxazole and amikacin and was resistant to gentamicin, cefuroxime, chloramphenicol, vancomycin etc. *Staphylococcus aureus* was susceptible to erythromycin, tetracycline, cotrimaxazole, gentamicin and ciprofloxacin while it was resistant to ampicillin, metropenem, cefuroxime, augmentin, vancomycin, ceftazidime and cephalexin.

Table 1: Phytochemical analysis of methanolic extracts of *Azadirachta indica* leaves

TEST	INFERENCE
Tannin	Present
Phenol	Present
Alkaloid	Present
Flavonoid	Absent
Phytosteroid	Present
Steroid	Absent
Saponin	Absent
Terpenoid	Present

Table 2: Antibacterial activity of methanolic extracts of *Azadirachta indica* leaves against *Staphylococcus aureus* and *Escherichia coli*

Extract concentration (mg/mL)	Zones of inhibition(mm)	
	Escherichia coli	*Staphylococcus aureus*
6.25	0 ± 0^{a}	0 ± 0^{a}
12.5	0 ± 0^{a}	0 ± 0^{a}
25	4.67 ± 1.45^{b}	1.67 ± 0.33^{a}
50	8.67 ± 1.45^{b}	6.33 ± 0.88^{b}
100	13.33 ± 2.03^{c}	12.00 ± 1.53^{c}

Values are mean±standard error of mean. Values with the same superscript are not significantly different across the column ($p\leq0.05$) based on Duncan's multiple range test

Table3: Antifungal activity of methanolic extract of *Azadirachta indica* leaves against *Candida albicans*

Extract concentration(mg/mL)	Zone of inhibition (mm)
6.5	0 ± 0^{a}
12.5	0 ± 0^{a}
25	0 ± 0^{a}
50	4.330 ± 0.67^{b}
100	9.670 ± 1.76^{c}

Values with the same superscript are not significantly different across the column ($p\leq0.05$) based on Duncan's multiple range test

Table 4: Minimum inhibitory concentration (MIC) of methanolic extract of *Azadirachta indica* leaves against the test organisms

Test organism	Minimum Inhibitory Concentration (MIC) of the extract (mg/mL)
Escherichia coli	25
Staphylococcus aureus	50
Candida albicans	50

Table 5: Minimum bactericidal concentration (MBC) of methanolic extracts of *Azadirachta indica* leaves against *Escherichia coli* and *Staphylococcus aureus*

Test organisms	Minimum Bactericidal Concentration (MBC) of the extract (mg/mL)
Staphylococcus aureus	50
Escherichia coli	50

Table 6: Minimum Fungicidal Concentration (MFC) of methanolic extract of *Azadirachta indica* leaves against *Candida albicans*

Test organism	Minimum Fungicidal Concentration (MFC) of the extract (mg/mL)
Candida albicans	100

Table 7: Antibacterial activity of synthesised silver nanoparticles (AgNPs) from methanolic extract of *Azadirachta indica* leaves on *Escherichia coli* and *Staphylococcus aureus*

Test organism	Zone of inhibition (mm)	
	AgNPs	$AgNO_3$
Escherichia coli	5.33±0.88	0±0
Staphylococcus aureus	0±0	0±0

Table 8: Antifungal activity of synthesised silver nanoparticles (AgNPs) from methanolic extract of *Azadirachta indica* leaves on *Candida albicans*

Test organism	Zone of inhibition (mm)	
	AgNPs	$AgNO_3$
Candida albicans	15.33±0.88	7.33±0.88

Table 9: Antibiotic susceptibility pattern of *Escherichia coli* to standard antibiotics

Antibiotics	Concentrations (µg)	Zone of inhibition(mm)	Interpretation
Tetracycline (TET)	10	$12.00±1.15^{b}$	S
Cotrimaxazole (COT)	25	$16.67±1.20^{c}$	S
Gentamicin (GEN)	10	$0±0^{a}$	R
Cefuroxime (CRX)	30	$0±0^{a}$	R
Chloramphenicol (CHL)	10	$0±0^{a}$	R
Ceftriaxone (CTR)	30	$0±0^{a}$	R
Ciprofloxacin (CIP)	5	$0±0^{a}$	R
Amikacin (AMC)	30	$34.33±1.76^{d}$	S
Cefotaxime (CTX)	30	$0±0^{a}$	R
Vancomycin (VAN)	30	$0±0^{a}$	R
Ceftazidime (CPZ)	30	$0±0^{a}$	R
Meropenem (MEM)	10	$0±0^{a}$	R

KEYS:
R=Resistance S=Sensitive I=Intermediate

Values are mean ± standard error of mean. Values with the same superscript are not significantly different across the column ($p \leq 0.05$) based on Duncan's multiple range test

Table 10: Antibiotic susceptibility pattern of *Staphylococcus aureus* to standard antibiotics

Antibiotic	Concentrations (µg)	Zone of inhibition(mm)	Interpretation
Ampicillin (AMP)	10	$0±0^{a}$	R
Meropenem (MEM)	10	$0±0^{a}$	R
Erythromycin (ERY)	5	$33.33±2.03^{e}$	S
Tetracycline (TET)	30	$22.33±2.33^{d}$	S
Cotrimaxazole (COT)	25	$13.33±2.03^{b}$	S
Cefuroxime (CRX)	10	$0±0^{a}$	R
Gentamicin (GEN)	10	$17.67±1.45^{c}$	S
Ciprofloxacin (CIP)	5	$11.33±0.88^{b}$	S
Augmentin (AUG)	30	$0±0^{a}$	R
Vancomycin (VAN)	30	$0±0^{a}$	R
Ceftazidine (CPZ)	10	$0±0^{a}$	R
Cephalexin (CP)	1.5	$0±0^{a}$	R

KEYS:
R=Resistance S=Sensitive I=Intermediate

Values are mean ± standard error of mean. Values with the same superscript are not significantly different across the column ($p \leq 0.05$) based on Duncan's multiple range test

Discussion

The results of the phytochemical screening of methanolic extract of *Azadirachta indica* leaves show the presence of tannins, phenols, alkaloids, phytosteroid, and terpenoid and the absence of flavonoids, steroids and saponin. This agrees with a similar study by Akinjogunla *et al*. (2011). These phytochemical constituents were further reported to be responsible for many antimicrobial activities of different plant

species (Ogbuewu *et al*., 2011; Ghonmode *et al*., 2013; Ogar *et al*., 2015). Tannins have been demonstrated to possess antibacterial activities (Akiyama *et al*., 2001). Phenol was present in the methanolic extracts. This agrees with similar finding by Akter *et al*. (2013) who reported that secondary metabolites are widely found in plant. Some simple phenolic compounds serve as defence against pathogens. Terpenoids was found to be present in the methanolic extracts, it exhibits various important pharmacological activities such as anti-bacterial, anti-malarial, anti-cancer and anti-viral activities (Mahfuzul *et al.*, 2007).

The antibacterial activities of the methanolic extracts showed that there was an observable zone of inhibition for *Staphylococcus aureus* and *Escherichia coli* at concentrations of 25 mg/mL, 50 mg/mL and 100 mg/mL. Also, the antifungal activities of the extracts showed that there was an observable zone of inhibition for *Candida albicans* at concentrations of 50 mg/mL and 100 mg/mL (Akter *et al.,* 2013).

The antibacterial and antifungal activities of *Azadirachta indica* extracts were carried out using ditch method. The result of the antibacterial activity was measured in term of diameter of zone of inhibition (mm). *Staphylococcus aureus* was resistant to Ampicillin, Meropenem, Cefuroxime, Augmentin, Vancomycin, Ceftazidine and Cephalexin but it was susceptible to Erythromycin, Tetracycline, Cotrimoxazole, Gentamicin and Ciprofloxacin. *Escherichia coli* was resistant to Gentamicin, Cefuroxime, Chloramphenicol, Ceftriaxone, Ciprofloxacin, Cefotaxime, Vancomycin, Ceftazidime and Meropenem, but it was susceptible to Tetracycline, Amikacin and Cotrimoxazole. The high of level resistance recorded for *Staphylococcus aureus* and *Escherichia coli* could be associated with earlier exposure of these pathogen(s) to synthetic antibiotics which may have enhanced the development of resistance within them Banso and Ayodele, 2001).

Comparison of antibacterial activity of methanolic extracts of *Azadirachta indica* leaves, its synthesized silver nanoparticles and antibiotics against *Staphylococcus aureus* shows that the synthesized silver nanoparticles with had no observable zone of inhibition, (0mm) and the methanolic extract also had no zone of inhibition (0mm), as well as some standard antibiotics 0mm except Erythromycin 30 mm, Tetracycline 20 mm, Cotrimaxazole 10mm, Ciprofloxacin 10mm, Gentamicin 15mm. This means the *Staphylococcus aureus* was resistant to the methanolic extract of *Azadirachta indica*.

Comparison of antibacterial activity of methanolic extracts of *Azadirachta indica* leaves, its synthesized silver nanoparticles and antibiotics against *Escherichia coli* shows that the synthesized silver nanoparticles with a zone of inhibition 5mm had a higher antibacterial effect than the methanolic extract with a zone of inhibition of 0mm as well as some standard antibiotics 0mm except Amikacin 35mm, Tetracycline 10mm, Cotrimaxazole 15mm.

Comparison of antifungal activity of methanolic extracts of *Azadirachta indica* leaves and its synthesized silver nanoparticles shows that the methanolic extract with a zone of inhibition (0mm) had a lower antifungal activity than the synthesized silver nanoparticles which pretended with a zone of inhibition of 14mm.

CONCLUSION

Based on the results obtained from this study, it could be said that plant extracts contain chemical constituents of pharmacological significance. The presence of bioactive compounds (Tanin, phenol, alkaloid, phytosteroid, terpenoid) in the methanolic extract of *Azadirachta indica* extract confers antimicrobial activities against the tested pathogenic organisms.

The presence of these chemicals constituents in plants is an indication that if properly screened drugs of pharmaceutical significance could be harvested from these plant extract. Further research is therefore recommended to isolate, purify and characterize these chemical constituents with a view to supplementing conventional drug development especially in developing countries. This study shows that the methanolic extract of *Azadirachta indica* has antibacterial effect against *Staphylococcus aureus* and *Escherichia coli* indicating a potent source of new antibiotic alternative. The study recommends that further tests should be conducted in order to determine the safety in humans and animals. Effects of this plant extract on more pathogenic organisms should be tested.

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