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Phytochemical Analysis and Antimicrobial Activity of Methanolic, Ethanolic and Acetonic Extracts of Stem Bark and Leaf of Neem Plant (*Azadirachta indica*)

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Abstract: This study was carried out on phytochemicals and in vitro screening of antibacterial potentials of ethanolic, methanolic and acetonic extracts of stem bark and leaves of Neem plant (*Azadirachta indica*) by using the methods of AOAC; and agar diffusion technique. The extracts of the leaves and the stem bark were prepared and screened for the presence of different phytochemicals. The results obtained showed that both the leaf and stem bark extracts contain alkaloid, flavonoid, reducing sugar, tannin, saponin and polyphenol. The extracts were tested against selected pathogens; *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Aspergillus niger, Aspergillus fumigatus and Candida albicans* by using agar well diffusion technique. In this present research work, the acetonic, ethanolic and methanolic leaves and bark extracts of Neem plant were investigated for antimicrobial activity against these selected pathogens. The Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. The MIC for the bacterial isolates was 25 mg / ml of the leaf extracts and that for stem bark was 6.25 mg / ml. The MBC was 25 mg / ml. Results showed that the bark extract exhibited strongest antimicrobial activity against bacteria and fungi at different concentrations when compared with the activity of the leaf extract. The acetonic stem bark extract had the highest antibacterial activity with a zone of inhibition of 22 mm, and then followed closely by the stem bark's ethanol extract with a zone of inhibition of 21 mm. More so, the methanolic stem bark extract had the highest antifungal activities with a zone of inhibition of 22.50 mm. Thus, this work showed that both leaf and stem bark extracts had some phytochemicals and antimicrobial activity.

Keywords: Antimicrobial Activity, Bactericidal, Concentration, Alcoholic Extracts, Inhibitory, Minimum, *Azadirachta indica*, Phytochemical

1. Introduction

In recent years, secondary plant metabolites (photochemical), with unknown pharmacological activities have been extensively investigated as a source of medicinal agents been produced [4,5,6]. According to World Health Organization (WHO) medicinal plants would be the best source to obtain a variety of drugs [22]. Many parts of this plant (leaves, stem bark, and latex) have reported to exhibit

antibacterial activity [21]. There are more than 35,000 plants species with various phytochemicals in them being used in various human cultures and veterinary around the world for medicinal purposes. About 80 % of individuals from developed and underdeveloped countries use traditional medicine, which has compound derived from medicinal plants in various form of therapies [13,11,10]. There are more than a thousand known preventive chemicals in plants that ward off diseases; these are known as phytochemicals [24]. Phytochemical is a word derived from Greek. Phyto means plant. Any plant derived chemical is called a Phytochemical. Phytonutrient is synonym to Phytochemical. These phytonutrients differ from traditional nutrients, because they are not essential for life. They are primarily called Phytochemical for clarity [4,25,23].

The plant Neem (*Azadirachta indica*) Meliaceae, commonly known as Neem is native of India and naturalized in most tropical and sub-tropical countries is of great medicinal value and distributed wide spread in the world. Neem is an omnipotent tree and as acrid gift of nature. The *A. indica* is a very useful traditional medicinal plant in the African sub-continent and each part of the tree has some medicinal properties [19,1,8]. Neem tree is a tree in the mahogany family Meliaceae, is evergreen tree found in most tropical countries. Neem has been used extensively by human kind to treat various ailments before the availability of written records which recorded the beginning of history [14,17,23]. Since pre-historic times, Neem has been used by human kind. Neem trees (leaf, stem, bark and seed) are known to antibacterial, antifungal activities against different pathogenic microorganisms and antiviral activity against Vaccinia, Chikungunya, measles and Coxsakie B viruses. Neem also contain biologically active principles isolated from different parts of the plant include: azadirachtin, meliacin; gedunin, salanin, nimbin, valassin, and many other derivatives of these principles. Meliacin forms the bitter principles of Neem seed oil; the seed also contain tignic acid (5-methyl-2-butanicacid) responsible for the distinctive odour of the oil [17]. These compounds belong to natural products called triterpenoids (limonoids). The active principles are slightly hydrophilic, but freely lipophilic and highly soluble in organic solvent like hydrocarbon, alcohols, ketones and esters. Also, Neem twigs are used as tooth brushes in some tropics [8].

However, the presence of the phytochemical constituents such as alkaloids, flavonoids, tannins, and phenolic compounds has been reported to be important compounds in many other medicinal plants [14]. These secondary metabolites are organic compounds that are not directly involved in the normal growth, development and reproduction of organisms. Absence of secondary metabolites does not result in immediate death, but long term impairment of organisms. They play an important role in plant defense. The secondary metabolites are used for medicine, flavourings and recreational drugs. These compounds after possible chemical manipulation provide and improved drugs to treat the infectious diseases [19].

With the increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial agents has led to the screening of several medicinal plants for their potential antimicrobial activity. Plants produce a diverse range of bioactive molecules, making them rich sources of different types of medicines; therefore, such plants with possible antimicrobial activities need to be tested. This study is aimed at screening for phytochemical properties and investigating the antimicrobial activity of methanolic, ethanolic and acetonic extracts of stem bark and leaf of Neem against some pathogenic bacteria and fungi.

2. Materials and Methods

The following standard materials were required and used in the cause of this scientific research study and Standard Operation Procedures (SOP) are absolutely been observed. Materials and reagents (Biotec, H&B Warners, Merck and Pfizer product) for this study were of analytical grade and were obtained commercially.

2.1. Sampling and Samples Collection

The samples of plant parts (leaves and stem bark) of Neem plant (*Azadirachta indica*) were collected in the month of January, 2015 from the tree growing in side environs of Nnamdi Azikiwe University, Awka. They were identified by a Botanist, Maxwell Nwata from the Department of Botany, (the plant is locally called Mbritem or native name given by Igbo language) the voucher specimen were deposited in herbarium (ASC Number 221) within the same department and University, and finally transported to the Research Laboratory, Department of Applied Microbiology and Brewing, in the same University.

2.2. Preparation of the Samples

The plant parts materials obtained were prepared and standard operation procedures (SOP) are absolutely being observed and as described by Gwana *et al*, (2014).

2.2.1. Pulverization of the Samples

After the collection and authentication of the samples, the leaves were destalked carefully. The leaves and the stems bark were separately washed under running tap water. Each sample was washed with distilled water and finally with deionised water in order to eliminate dust and other foreign particles. But the stems bark was chopped in to pieces with a sharp knife before washing them. They were shade dried for 12 days at room temperature. After which the barks were grinded in to powder by using a blender (homogenizer) and the leaves were grinded in to fine powder by using a mortar and pestle, and subsequently by a blender. Each sample was transferred and packed in to clean, grease free and sterilized plastic bottle. They were labeled, B-leaf and C-bark for the leaves powder and the stems bark powder respectively. The labeled plastic bottles containing the plant powders samples were airtight screwed and capped, stored at dry and cool condition, kept away from light and under temperature of

18°C to 25°C ready for extraction.

2.2.2. Preparation of Crude Extracts of the Samples

About 10 grams of the each sample (B-leaf and C-bark) were electronically weighed in to six 250 ml conical flasks (3 flasks for each sample). One hundred ml of each solvent (absolute acetone, ethanol and methanol) were added in to each flask, and shaken with a vibrator - shaker (that can house six conical flasks) for 4 hours at room temperature. The movement of the vibrator – shaker serves to disrupt the plant tissue so that the solvents were allowed in to the tissue resulting in adequate extraction.

On completion of homogenization, the mixture was filtered using Whatman filter paper No1 at room temperature (30°C). The extracts were labeled appropriately as followed; B acetone, BA; B ethanol, BE; B methanol, BM for the sample B – leaf and C acetone CA, C ethanol CE and C methanol, CM for sample C – bark respectively. Then each sample was dispensed in to a sterile beaker and placed in the water bath for evaporation at the boiling point of each solvent. After evaporation, the residual masses obtained individually were measured and dimethyl-sulfoxide (DMSO) was used to prepare a starting concentration of 100 mg / ml for all the extracts. All the extracts were stored in refrigerator at 4°C until when needed. Another 10 grams of each sample were weighed individually in to six 250 ml conical flask and 100 ml of each solvent were added and shaken with a vibrator – shaker for 4 hours at room temperature. On the completion of homogenization, filtration, the extracts were labeled and kept in a refrigerator at 4°C for phytochemical screening on each of the solvent extract.

2.3. Phytochemical Analysis

The extracts were analyzed to test for the presence of the active chemical constituents such as alkaloid, tannin, saponins, steroid, flavonoids, anthraquinones, hydroxyl methyl anthraquinones, reducing sugar, polyphenol, terpenoid and cardiac glycoside. The phytochemical analysis was done on the two samples, leaves (B) and bark (C) using the following solvent extract - acetone, methanol, and ethanol by using the methods of AOAC, (1990); Egan *et al*, (1981).

2.3.1. Mayer's Test for Alkaloids

The following procedures were performed.

i. *Procedure:-*
   2ml of acetonic leaf extract was added in to a test tube and the mixture was heated for 20 minutes using water bath. The heated mixture was filtered and 1ml of the filtrate was measured in to a test tube and 0.5 ml of Wagner's reagent was added to it. A reddish brown coloration was observed.

ii. Frothing test

Procedure:-

3 ml of the acetonic leaf extract was pipette in to a test tube; 2 ml of distilled water was added to it. Then it was shaken vigorously. A persistence frothing movement was observed.

iii. Emulsion test

*Procedure:-*3 ml of the acetonic leaf extract was pipette out in to a test tube and 5 drops of olive oil was also incorporated in to it and then it was shaken vigorously emulsification was observed (tiny droplets incorporated in to the body of the extract).

2.3.2. Lieberman – Buchard's Test for Steroids

*Procedure:-*1 ml of the extract was treated with 0.5 ml of acetic acid, 0.5 ml of chloroform and 1 ml of concentrated H_2SO_4 was also added to it. A reddish brown ring was formed at the separating level of the two liquids indicating the presence of steroids.

2.3.3. Sodium Hydroxide's Test for Flavonoids

Procedure:-

3 ml of the acetonic leaf extract was pipette out and 10 ml of distilled water was added to it and it was shaken and 1 ml of 10% NaOH was also added in to the mixture. A yellow coloration was observed showing the presence of flavonoid.

2.3.4. Ferric chloride's Test for Tannins

*Procedure:-*1 ml of the extract was measured in to a test tube and it was heated. One drop of 10% ferric chloride was added to it. The mixture showed a green coloration.

2.3.5. Free / Combined Anthraquinones Test

*Procedure:-*2 ml of leaf extract was shaken with 5 ml of 10% ammonia solution. The mixture was shaken and the presence of a pink - red to violet colour in the ammoniacal (lower) phase indicated by the presence of anthraquinones.

2.3.6. Bourn stranger's Test for Hydroxyl Methyl **Anthraquinones**

Procedure:-

2 ml of acetonic extract was treated with 5 ml of 10% ammonia solution. The formation of a red coloration or precipitate indicates the presence of hydroxyl methyl anthraquinones.

2.3.7. Free Reducing Sugar's Test for Reducing Sugar

Procedure:-

2 ml of acetonic extract in a test tube was added to 5 ml of Fehling solutions and heated in a water bath at 80°C for 10 minutes. The formation of a brick red precipitate or solution was taken; as an evidence for the presence of reducing compounds.

2.3.8. Test for Polyphenol

*Procedure:-*To 2 ml of extract was added 5 ml of distilled water and heated in a water bath for 10 minutes. 1 ml of ferric chloride was added to the mixture followed by 1 ml of 1% potassium ferricyanide. The formation of a green – blue coloration indicated the presence for polyphenol.

2.3.9. Salkowski's Test for Terpenoid

Procedure:-

5 ml of each extract was mixed with 2 ml of chloroform ($CHC1_3$) in a test tube. 3 ml of concentrated H_2SO_4 was carefully added to the mixture to form a layer. An interface

with reddish brown coloration was formed, if terpenoid constituent is present.

2.3.10. General Test for Cardiac Glycoside

Procedure:-

0.5 g of each extract was dissolved in 2 ml of chloroform. Concentrated (2 ml) sulphuric acid was carefully added to it to form a lower layer; a reddish – brown colour at the interface indicated the presence of a steroidal ring (a glycogen portion of the cardiac glycoside).

2.4. Preparation of Culture Media

The experiments were conducted under sterile condition and hygienic environment. The media used were Muller Hinton Agar (Biotec product), Sabouraud Dextrose Agar (Merck product), Nutrient Broth (Biotec), Sabouraud Dextrose Broth (Merck) and Nutrient agar (Biotec). The antimicrobial drugs used were Fluconazole (Pfizer) and ciprofloxacin (Pfizer product) for fungi and bacteria isolates respectively. The media were prepared according to the manufacturer's instructions. The Muller Hinton agar (MHA) was used for the bacteria, while the Sabouraud Dextrose Agar was used for the fungi. After preparing the two media, they were allowed to cool to 45°C before dispensing aseptically in to fourteen (14) plastic Petri dishes each. The plates were allowed to solidify before inversion.

2.4.1. Source of Microorganisms

The organisms used were *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Aspergillus niger*, *Aspergillus fumigatus* and *Candida albicans*. The organisms were obtained from Glanson Medical centre, Awka and Department of Applied Microbiology and Brewing Laboratory stock culture, Azikiwe University, Awka, Nigeria.

2.4.2. Maintenance of Organisms (Bacterial and Fungal) Isolates

The organisms were maintained in agar slant wrapped with aluminium foil and kept in the refrigerator at 4°C.

2.4.3. Fungal Isolate

They were maintained in agar slants containing Sabouraud Dextrose agar. They were carefully wrapped aluminium foil and kept in the refrigerator at 4°C.

2.4.4. Inoculums Preparation

The Bacterial and fungal inoculums were prepared by inoculating a loopful of test organisms in 10 ml of nutrient broth in to three separate Bijou bottles (for bacterial isolates) and 10 ml of Sabouraud dextrose broth (SDB) in to three separate Bijou bottles (for fungal isolates). They were incubated at 37°C and 25°C for 4 – 6 hours for bacteria and fungi respectively till a moderate turbidity were developed.

2.4.5. Standard Antibiotic

The standard drug of quality; Ciprofloxacin and Fluconazole (Pfizer product) were obtained commercially as a standard for the working concentration and antimicrobial activity test.

2.5. Determination of Antimicrobial Activity

The antimicrobial activity of the leaf and bark extracts (acetonic, ethanolic and methanolic) were determined using agar well diffusion method by the following procedure as described by Joanne *et al.*, (2011). A 2 – fold serial dilution of these extracts using Dimethyl sulfoxide (DMSO) as the diluents were prepared separately to obtain l00 mg / ml, 50 mg / ml, 25 mg / ml, 2.50 mg / ml and 6.25 mg / ml. Muller Hinton agar plates prepared earlier were inoculated with test organisms with the aid of sterile syringes and the bacterial inoculums (0.l ml) were spread on the surfaces of each media using sterile swab sticks. The plates were allowed to dry before the holes were made. Agar surfaces were cut with the help of a sterile cork borer having a diameter of 5 mm size to make appropriate wells.

Each leaf extract (acetonic, ethanolic and methanolic) had four plates and two holes each for the first two plates were made. Also 3 holes each in the other two plates were made (this represent l00 mg / ml and 50 mg / ml for the first two plates while the other two plates represent 2.5 mg / ml, 12.50 mg / ml and 6.25 mg / ml).

Different concentrations – 100 mg / ml, 500 mg / ml, 25 mg / ml, 12.50 mg / ml and 6.25 mg / ml respectively of the leaf extracts were added to the holes of *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* plates respectively.

Also, a working concentration of the ciprofloxacin (as a standard) was obtained after reconstitution and it was poured in to the wells of *E. coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* plate respectively. This was done after boring two holes on each different plate for the organisms.

Sabouraud Dextrose Agar (SDA) plates were used for the fungal isolates. The SDA was dispensed in to fourteen (14) plastic Petri dishes for each extracts (acetonic, ethanolic and methanolic) and this is a total of forty – two (42) plates. 2 – fold serial dilutions were also prepared using DMSO as the diluents.

However, the concentration obtained were l00 mg / ml, 50 mg / ml, 25 mg / ml, 12.50 mg / ml, 6.25 mg / ml, 3.13 mg / ml and 1.56 mg / ml. The SDA plates were inoculated with the organisms with the aid of sterile syringe and the fungi inoculums (0.l ml in each plate) were spread on the surfaces of each media using sterile swab sticks. The plates were allowed to dry before the holes were bored. Agar surface were cut with the help of a sterile cork borer having a diameter of 5 mm size to make appropriate wells. Each leaf extracts (i.e. acetonic, ethanolic and methanolic) had four plates and four (4) holes on each plate were made. The SDA plates for the standard drug (Fluconazole) were six – two for each organism. Two holes were bored on the six (6) plates (a total of 14 plates for each organism were used).

Different concentrations of the serially diluted plant extracts were added in to the holes of *Aspergillus niger, Aspergillus fumigatus* and *Candida albicans* plates respectively. Also, the working concentration of the reconstituted Fluconazole was added in to the wells of

Aspergillus niger, Aspergillus fumigatus and *Candida albicans* plates respectively.

In addition, the same procedures were used for the bark extracts (acetonic, methanolic and ethanolic) for fungi and bacteria culture. The plates were left for some time to allow for the diffusion of extracts and drugs before incubation. Bacterial cultures were incubated at 37°C for 24 hours and fungal cultures at 25°C for 48 hours. Antimicrobial activities were determined by measuring the zone of inhibition surrounding the well in millimeter (mm) using a pair of divider and a ruler. Each concentration included duplicates and also the drugs (standard). The results are average of the two independent experiments. The results were recorded on tables.

2.6. Cultural Characteristics

The bacterial isolates were cultured on different selective media. For *E. coli* on EMB agar was used, *Staphylococcus aureus* on Mannitol Salt agar and *Pseudomonas* on Centrimide agar were used for *Pseudomonas aeruginosa* and the following tests were used to confirm the bacterial isolates; Gram staining reaction, Biochemical tests: Catalase, Coagulate, Indole, Methyl red, Voges proskaeur, Urease tests, and Motility, while fungi isolates were confirmed by germ tube and slide culture tests.

2.7. Determination of Minimum Inhibitory Concentration MIC

The minimum inhibitory concentration values were determined by broth dilution assay. 2 – fold serial dilutions for MIC of each extracts (leaf and bark acetonic, ethanolic and methanolic extracts) were prepared. To perform MIC experiment, four (4) test tubes were taken, washed and dried. 0.5 ml of nutrient broth was dispensed to each test tube, plugged the mouths and sterilized at 121°C for 15 minutes. After cooling, 0.5 ml plant extract from the stock (l00 mg / ml) test tube was added to the first test tube was mixed properly and 0.5 ml mixture of this test tube was transferred to the next (second) test tube. 0.5 ml was taken from this second test tube and dispensed it to the third test tube, and then the procedure was repeated until the fourth (4^{th}) test tube. 0.5 ml from the last test tube was removed and discarded. Then 0.5 ml bacterial culture (4 – 6 hours old) were added to each test tube and incubated at 37°C for 18 – 24 hours. Controls were done simultaneously. The first control test tube contains 0.5 ml of nutrient broth and 0.5 ml of the test organism, while the other control test tube contains 0.5 ml of nutrient broth and 0.5 ml of the extract. They were also incubated under the same condition.

However, for the fungal isolates, six (6) test tubes and two control test tubes. The six test tubes and the control contained Sabouraud Dextrose Broth (SDB). After washing of test tubes, drying and sterilization (at 121°C for 15 minutes), a 2 – fold serial dilutions were also prepared. From the stock (crude extract also l00 mg / ml), 0.5 ml plant extract was added to the first test tube, was mixed properly and 0.5 ml mixture of this test tube was taken and added to the next (second) test tube. 0.5 ml from this second test tube was taken and dispensed in to the next tube; same thing was done to the last tube subsequently. 0.5 ml from the last test tube was discarded. Then 0.5 ml fungal culture (4 – 6 hours old) were added to each test tube and incubated for 25°C for 48 hours. The controls were done simultaneously. The first control contain: SDB (0.5 ml) and test organism (0.5 ml) while the other control had SDB (0.5 ml) and plant extract (0.5 ml). This procedure was done for all six (6) organisms (3 bacteria and 3 fungi) using the acetonic, ethanolic and methanolic extracts of leaves and bark respectively.

3. Results

Plants produce a diverse range of bioactive molecules, making them rich sources of different types of medicines and foods. Plant that produces such product, antimicrobial activities should be tested against appropriate microbes to confirm the activity and to ascertain the parameters associate with it, and this leads to the results obtained from this research work on phytochemicals and in vitro screening of antibacterial potentials of acetonic, ethanolic, and methanolic extracts of stem bark and leaves of Neem plant (*Azadirachta indica*), procedures of analysis were done on six organisms (3 bacteria and 3 fungi) were that:

Table 1 showed the serial dilution, the concentrations obtained after the 2 – fold serial dilutions before incubation were for quality control, the Neem extracts (acetonic, ethanolic and methanolic leaf and bark extracts) were separately cultured on Muller Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) to determine their purity. After overnight incubation, no growth of any colonies of bacteria was observed. Also for the SDA after 48 hours of incubation, no growth of fungi was observed.

Table 2 and 3 showed the results of the phytochemical test which was done to find the presence of active chemical constituents such as terpenoid, polyphenol, cardiac glycoside, anthraquinones, flavonoids, saponins; steroids, tannins, alkaloids, reducing sugar and hydroxyl methyl anthraquinones.

Table 4 showed the characteristic and identification features of the fungal isolate being observed and examined.

Table 5 showed the physical characteristics of the bacterial isolates being examined.

Table 6 showed the biochemical test characteristics of the bacterial isolates being examined.

Table 7 showed the results of the antibacterial activity and antifungal activity of ciprofloxacin and Fluconazole against selected pathogens in order to ascertain the potency and efficacy of pathogens and the standard antimicrobial drugs.

Table 8 to 10 showed the results obtained from the study of the antibacterial and antifungal activities of acetone, ethanol and methanol extracts of *Azadirachta indica* leaf and bark which were investigated using agar well diffusion method against the selected pathogens; *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Aspergillus niger, Aspergillus fumigatus* and *Candida*

albicans. All the examined extracts showed varying degrees of antimicrobial activities against the pathogens.

Table 11, 12 and 13 showed the results that were obtained from the analysis and it revealed the minimum inhibitory concentration (MIC) values of the acetonic (leaf; 12.5 – 25 and bark; 3.13 – 12.5), ethanolic (leaf; 6.25 – 25 and bark; 6.25) and methanolic (leaf; 6.25 – 25 and bark; 6.25 - 25) of Neem Plant Parts Extracts in mg / ml respectively.

Table 14 showed the results obtained from this study that, the minimum bacterial concentration (MBC) values of acetone (0 – 25 mm), ethanol (12.5 – 25 mm) and methanol (0 – 25 mm) extracts of Neem leaf.

Table 15 showed the results that were obtained from the analysis and it revealed the minimum bacterial concentration (MBC) values of acetone (12.5 – 25 mm), ethanol (0 – 25 mm) and methanol (12.5 – 25 mm) extracts of Neem stem bark.

Figure 1, 2, 3, and 4 showed the antibacterial activity of acetonic, ethanolic and methanolic extract of Neem plant parts.

Table 1. *Concentrations of Fungi and Bacteria obtained after the 2 – fold serial dilutions.*

Type of Microbe.	Tube.	Dilution in mg / ml.
Fungi	1st	25
	2nd	12.50
	3rd	6.25
	4th	3.13
	5th	1.56
	6th	0.78
Bacteria	1st	25
	2nd	12.50
	3rd	6.25
	4th	3.13

NB: The concentrations obtained after the 2 – fold serial dilutions before incubation.

Table 2. *Qualitative Phytochemical Analysis of Acetone, Ethanol, Methanol Leaf Extract.*

Type of Phytochemical.	Test Type.	Type of Leaf Extract.		
		Acetone.	Ethanol.	Methanol
Alkaloids	Mayer's	-	+	-
Fronthing		-	-	+
Emulsification		++	-	++
Tannin	cchloride's	+	-	+
Glycoside cardiac	General	-	+	-
Cyanogenic		-	+	-
Flavonoid	Sodium hydroxide's	-	+	-
Anthraquinones	Free / Combined	-	+	-
Reducin gsugar	Free Reducing Sugar's	+	+	+++
Polyphenol		-	+++	-
Terpenoid	Salkowski's	-	+	-
Steroid	Sodium hydroxide's	-	+	-
Saponins		-	-	-

KEYS: - = Absent, + = Scanty, ++ = Moderate, +++ = Abundance.

Table 3. *Qualitative Phytochemical Analysis of Acetone, Ethanol, Methanol Stem Bark Extract.*

Type of Phytochemical.	Test Type.	Type of Leaf Extract.		
		Acetone.	Ethanol.	Methanol
Alkaloids	Mayer's	-	+	+
Fronthing		-	-	-
Emulsification		-	+	+
Tannin	Ferric chloride's	+++	-	-
Glycoside cardiac	General	-	-	-
Cyanogenic		-	-	-
Flavonoid	Sodium hydroxide's	+	-	-
Anthraquinones	Free / Combined	-	-	-
Reducing sugar	Free Reducing Sugar's	+++	++	+
Polyphenol		++	-	++
Terpenoid	Salkowski's	-	-	-
Steroid	Sodium hydroxide's		-	-
Saponins		-	-	-

KEYS: - = Absent, + = Scanty present, ++ = Moderate, +++ = Abundance.

Table 4. *Fungal Isolates (Moulds) Identification.*

Type of Fungus.	Cultural Characteristics.	Microscopic Characteristics.
Aspergillus niger.	Flat compact colonies, white at first then becoming black homogeneously with grey underside.	Septate and hyphae with erect, simple and thick walled conidiophores bearing conidial heads split in to over four (4) loose conidia columns with four (4) fragments apically.
Aspergillus fumigatus.	Flat compact colonies, white at first, then becoming then dark green underside.	Septate and hyphae with thin walled conidiophores becoming heads composed of catenucleac conidial.
Candida albicans.	Cream coloured pasty colonies usually appear after some hours (24 – 48 hours) incubation at 37°C, with distinctive yeast smell.	Budding cells can be easily seen and can be identify by the formation of pseudohyphae and chlamydospores.

Table 5. *Physical Characteristics of the Bacterial Isolates.*

Type of Isolates.	Characteristic identification of Isolates under Tests.			
	Cultural Appearance	Morphological Appearance.	Gram Stain Reaction.	Motility Test.
Escherichia coli	Green metallic sheen on EMB agar	Rod	+	-
Staphylococcus aureus	Yellow colonies on MSA	Coccus	-	+
Pseudomonas aeruginosa	Green colonies on PCA	Rod	+	-

KEYS: - = Negative, + = Positive, EMB = Eosin Methylene Blue Agar, MSA = Mannitol Salt Agar, PCA = *Pseudomonas* Centrimide Agar.

Table 6. *Biochemical Test Characteristics of the Bacterial Isolates.*

Type of Isolates.	Biochemical Test of Isolates under Tests.				
	Catalase.	Coagulase.	Indole.	Methyl Red.	Voges Proskaeur.
Escherichia coli	-	+	+	+	-
Staphylococcus aureus	+	+	+	+	+
Pseudomonas aeruginosa	+	-	-	-	-

KEYS: - = Negative, + = Positive.

Table 7. *Antimicrobial Activity of Acetonic Extract of Neem Leaf and Standard Drugs Zones of Inhibition (mm).*

Type of Concentration		Zones of Inhibition by each Microbe (mm).					
Extract.	**mg/ml.**	***Staphyloccus aureus.***	***Pseudomonas aeruginosa.***	***Escherichia coli.***	***Aspergillus niger.***	***Aspergillus fumigatus.***	***Candida albicans***
Leaf	100	No	12	10	8	12	10
	50	13.50	9.50	9.50	10.50	10.00	8.00
	25	9.75	9.00	7.00	9.00	No	9.00
	12.50	No	10.00	6.50	8.50	14.50	8.50
	6.25	No	9.00	No	8.50	13.00	15.50
	3.13	-	-	-	7.00	16.50	11.50
	1.56	-	-	-	10.00	15.50	14.00
Ciprofloxacin	100	l20	14.50	21.	-	-	-
Fluconazole	100	-	-	-	12.00	15.5.	13.40

Keys: No = no activity i.e. no zone of inhibition, - = this means not tested.

Table 8. *Antimicrobial Activity of Acetonic Extract of Neem Stem Bark and Standard Drugs Zones of Inhibition (mm).*

Type of Concentration		Zones of Inhibition by each Microbe (mm).					
Extract.	**mg/ml.**	***Staphyloccus aureus.***	***Pseudomonas aeruginosa.***	***Escherichia coli.***	***Aspergillus niger.***	***Aspergillus fumigatus.***	***Candida albicans***
Stem bark.	100	16.00	24.00	12.00	11.00	19.00	11.000
	50	12.00	21.00	18.00	10.00	11.00	11.50
	25	11.50	11.00	12.00	7.00	14.00	12.00
	12.50	10.00	13.00	9.00	12.00	12.00	13.50
	6.25	6.00	10.00	17.50	14.00	10.50	14.00
	3.13	-	-	-	11.00	16.50	No
	1.57	-	-	-	14.50	16.00	No
Ciprofloxacin	100	20	14.50	21.	-	-	-
Fluconazole	100	-	-	-	12.00	15.5.	13.40

Keys: No = no activity i.e. no zone of inhibition, - = this means not tested.

Table 9. *Antimicrobial Activity of Methanolic Extract of Neem Leaf and Standard Drugs Zones of Inhibition (mm).*

Type of Concentration		Zones of Inhibition by each Microbe (mm).					
Extract.	**mg/ml.**	***Staphyloccus aureus.***	***Pseudomonas aeruginosa.***	***Escherichia coli.***	***Aspergillus niger.***	***Aspergillus fumigatus.***	***Candida albicans***
Leaf	100	14.50	18.00	18.00	11.00	12.00	11.50
	50	11.00	16.00	10.00	9.00	15.00	7.50
	25	9.00	11.00	12.00	10.00	20.00	No
	12.50	9.40	10.50	9.50	No	10.00	13.00
	6.25	7.00	10.00	8.50	No	14.50	13.50
	3.13	-	-	-	10.00	13.00	11.00
	1.56	-	-	-	12.00	12.00	14.00
Ciprofloxacin	100	20	14.50	21.	-	-	-
Fluconazole	100	-	-	-	12.00	15.5.	13.40

Keys: No = no activity i.e. no zone of inhibition, **-** = this means not tested.

Table 10. *Antimicrobial Activity of Methanolic Extract of Neem Stem Bark and Standard Drugs Zones of Inhibition (mm).*

Type of Concentration		Zones of Inhibition by each Microbe (mm).					
Extract.	**mg/l**	***Staphyloccus aureus.***	***Pseudomona aeruginosa.***	***Escherichia coli.***	***Aspergillus niger.***	***Aspergillus fumigatus.***	***Candida albicans***
StemBark	100	17.50	17.00	15.00	22.50	No	16.00
	50	13.00	14.00	14.00	11.50	14.50	12.00
	25	11.50	13.00	9.00	15.50	No	No
	12.5	9.00	12.00	14.50	7.50	10.50	No
	6.25	10.50	No	8.50	19.50	10.00	14.00
	3.13	-	-	-	14.50	14.00	No
	1.56	-	-	-	14.00	20.00	No
Ciprofloxacin	100	20	14.5	21.	-	-	-
Fluconazole	100	-	-	-	12.00	15.5.	13.40

Keys: No = no activity i.e. no zone of inhibition, **-** = this means not tested.

Table 11. *Minimum Inhibitory Concentration Values of the Acetonic Neem Plant Parts Extracts in mg / ml.*

Name of Microbe.	Neem Plant Parts mg / ml.	
	Leaf Extract.	**Stem Bark Extract.**
Staphylococcus aureus	25	No
Pseudomonas aeruginosa	25	6.25
Escherichia coli	25	6.25
Aspergillus niger	25	12.50
Aspergillus fumigatus	25	12.50
Candida albicans	12.50	3.13

Key: No = value for MIC was obtained.

Table 12. *Minimum Inhibitory Concentration Values of the Ethanolic Neem Plant Parts Extracts in mg / ml.*

Name of Microbe.	Neem Plant Parts mg / ml.	
	Leaf Extract.	**Stem Bark Extract.**
Staphylococcus aureus	No	6.25
Pseudomonas aeruginosa	25	6.25
Escherichia coli	No	No
Aspergillus niger	12.50	6.25
Aspergillus fumigatus	12.50	6.25
Candida albicans	6.25	6.25

Key: No = value for MIC was obtained

Table 13. *Minimum Inhibitory Concentration Values of the Methanolic Neem Plant Parts Extracts in mg / ml.*

Name of Microbe.	Neem Plant Parts mg / ml.	
	Leaf Extract.	**Stem Bark Extract.**
Staphylococcus aureus	25	6.25
Pseudomonas aeruginosa	6.25	12.50
Escherichia coli	No	6.25
Aspergillus niger	25	25
Aspergillus fumigatus	12.50	6.25
Candida albicans	6.25	6.25

Key: No = value for MIC was obtained

Table 14. *MBC values of Acetone, Ethanol and Methanol Extracts of Neem Leaf.*

Type of Microbe.	Neem Leaf Extract.		
	Acetone.	**Ethanol.**	**Methanol.**
Staphylococcus aureus	25	No	25
Pseudomonas aeruginosa	25	25	25
Escherichia coli	25	No	No
Aspergillus niger	25	12.50	25
Aspergillus fumigatus	25	25	25
Candida albicans	No	25	25

Key: No = value for MBC was obtained.

Table 15. *MBC values of Acetone, Ethanol and Methanol Extracts of Neem Stem Bark.*

Type of Microbe.	Neem Leaf Extract.		
	Acetone.	**Ethanol.**	**Methanol.**
Staphylococcus aureus	No	25	12.50
Pseudomonas aeruginosa	12.50	25	25
Escherichia coli	25	No	12.50
Aspergillus niger	25	25	25
Aspergillus fumigatus	25	25	25
Candida albicans	No	No	No

Key: No = value for MBC was obtained.

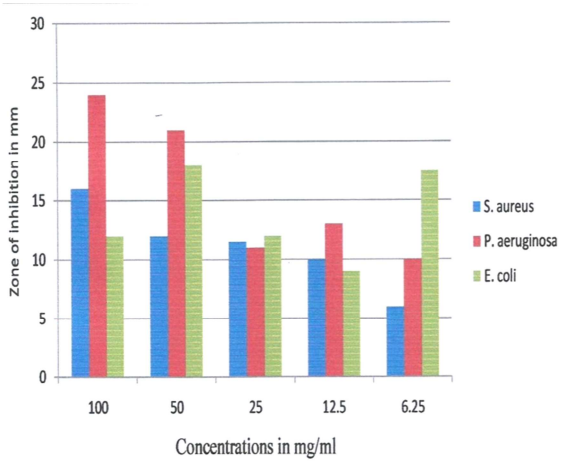

Figure 1. *Showing the Antibacterial Activity of Stem Bark Acetone Extract of Neem Plant.*

From the graph above, the most susceptible was *P. aeruginosa*, followed by *E. coli* and *Staphylococcus aureus* was the least susceptible to the acetone extract of the stem bark extract.

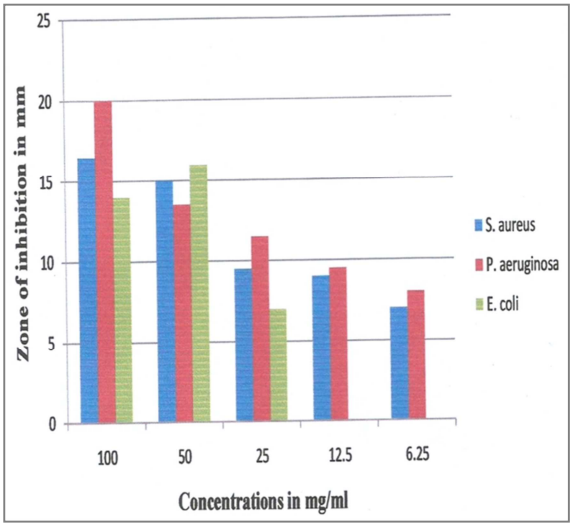

Figure 2. *Showing the Antibacterial Activities of Ethanol Leaf Extract of Neem.*

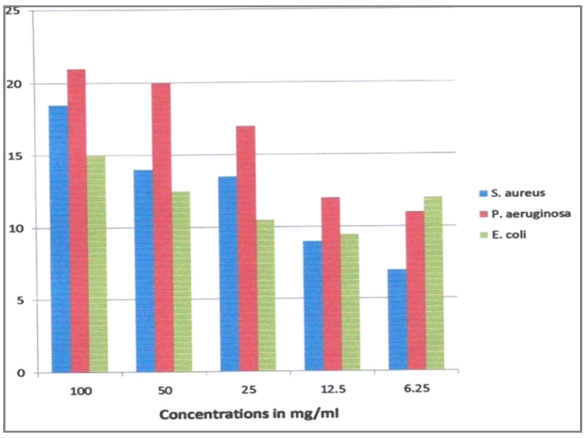

Figure 3. *showing the Antibacterial Activities of Ethanol Stem Bark Extract of Neem.*

The ethanol extract of stem bark from fig. 3 had more activity than that of the leaf extract showed in fig. 2 above. The ethanol leaf extract had no activity against *E. coli* at 12.5 mg / ml and 6.25 mg / ml but that of the stem bark extract had activity against *E. coli* at these concentrations.

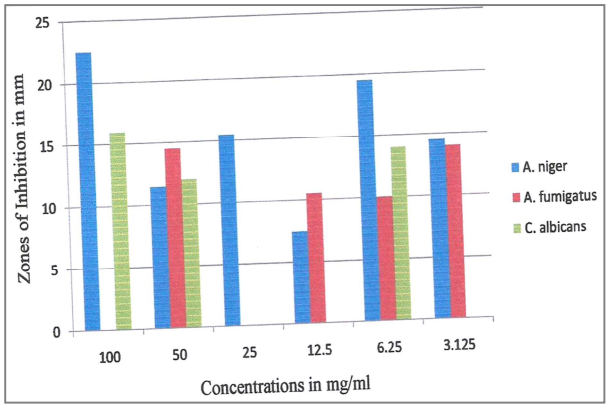

Figure 4. *Showing Antifungal Activities of Stem Bark Methanol Extract of Neem.*

From the figure above, *A. niger* was the most susceptible against the methanol extract of stem bark followed by *A. fumigatus* while *C. albicans* showed the least activity. *C. albicans* was resistance against this extract at 25 g / ml, 12.5 mg / ml, 3.13 mg / ml and 1.56 mg / ml.

4. Discussion

Plant metabolites (phytochemical), with unknown pharmacological activities have been extensively investigated as a source of medicinal agents. The findings of the preliminary phytochemicals investigations and the results of antimicrobial activity were depicted in the respective tables and figures. The results of phytochemicals in the present investigation showed that the Neem plant leaf contain eight phytochemicals components which agree with the works of some researchers as Mohapatra *et al.,* 2014; Abdullah *et al.,* 2011; Chattopadhyay, 1993; such as alkaloids, tannin, glycoside, anthraquinones, reducing sugar, polyphenol, terpenoid and steroid. The presence of these phytochemicals constituents are the reasons leaf and bark acetone, ethanol and methanol extracts have antimicrobial activity.

The difference in the antimicrobial efficacy could be due to variable distribution of phytochemicals compounds in different parts, also the Neem plant stem bark extracts contain the following components like alkaloids, tannin, reducing sugar, polyphenol, and flavonoids. From the results of the phytochemical analysis, it was observed that the stem bark methanol extract contains alkaloid which is absent in the leaf extract. The reducing sugar content is strongly present in the leaf extract while it is present in the bark. Polyphenol is present in the bark's methanolic extract but it is absent in the leaf methanolic extract. The leaf methanolic extract contains tannin but this is absent in the bark extract. Both methanolic extract of leaf and bark do not contain anthraquinones, terpenoid, steroids, glycosides and flavonoid, this in line with

the work of Asif (2012).

The extracts that showed high antibacterial activity is acetonic stem bark extract against *Pseudomonas aeruginosa* (22 mm) followed closely by ethanolic stem bark extract against the same organism (21 mm). For the leaf extracts, the ethanolic leaf extract had the highest antibacterial activity against *Pseudomonas aeruginosa* (20 mm), while the least antibacterial activity was against *Escherichia coli* in acetonic and ethanolic extracts. More so ever, it was observed that the methanolic stem bark extract of Neem plant had the highest antifungal activity against *Aspergillus niger* (22.50 mm). This is followed by ethanolic stem bark extract against *Aspergillus fumigatus.* Stem bark extracts had low antifungal activity especially against *Candida albicans,* with these results obtained; it revealed that the studied plant parts (stem bark and leaves) contains both antifungal and antibacterial agents and which in conformity with the works of Asif (2012) and Jayasree *et al.*, (2014).

Overall, the extract of the neem plant part with no antimicrobial activity was that of the stem bark methanolic extract against *C. albicans.* In addition, stem bark extracts, with maximum zone of inhibition as 22.50 mm, had significant antimicrobial activity than the leaf extracts. The highest and lowest zones of inhibitions were 22.5 mm and 6 mm respectively. From the experiment done, about one quarter of the results showed that as the concentrations of the plant extracts increases, the zone of inhibitions also increases. But majority of the results obtained do not agree with this relationship. This means that in some situations as the concentrations of the extracts increase, there is a decrease and later a sharp increase in the zones of inhibition.

From the results of the colonial characteristics, the morphological characteristics and the biochemical characteristics of the isolates used for this experiment showed that they were *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus.* Also, the morphological characteristics and the slide culture results showed that the moulds used were that of *Aspergillus niger* and *Aspergillus fumigatus.* The germ tube test showed that the isolate was *C. albicans* as were described by Joanne *et al.*, (2011).

From the results obtained, it revealed that, the most susceptible bacterium was *P. aeruginosa*, followed by *E. coli* and *Staphylococcus aureus* was the least susceptible to the acetone extract of the stem bar extract. The ethanol extract of stem bark from fig. 3 had more activity than that of the leaf extract showed in fig. 2 above. The ethanol leaf extract had no activity against *E. coli* at 12.5 mg / ml and 6.25 mg / ml but that of the stem bark extract had activity against *E. coli* at these concentrations. *A. niger* was the most susceptible against the methanol extract of stem bark followed by *A. fumigatus* while *C. albicans* showed the least activity. *C. albicans* was resistance against this extract at 25 g / ml, 12.5 mg / ml, 3.13 and 1.56 mg / ml. That means, the acetonic stem bark extract contains more antimicrobial agent than the leaves extract which in conformity with the work of Asif (2012).

All test strains of fungi were found to be sensitive to the standard drugs (antimicrobial agents) used in this work; Fluconazole and bacteria strain were also sensitive to ciprofloxacin. Results of the agar well diffusion method are shown above, that the leaf extract exhibited antimicrobial activity against all the tested organisms at all concentrations. The bark acetonic extract exhibited significant antimicrobial activity on *Pseudomonas aeruginosa, E. coil, Staphylococcus aureus* and *Aspergillus fumigatus.* The bark ethanolic extract exhibited significant antimicrobial activity on *P. aeruginosa, Staphylococcus aureus* and its fungi activity was highest at high concentration against *Aspergillus fumigatus.* The bark methanolic extract exhibited significant antimicrobial activity on *Aspergillus niger, Candida albicans* and unusually significant activity against *A. fumigatus* at low concentration its bacterial activity was highest at high concentration on *Staphylococcus aureus, P. aeruginosa* and *E. coli.* The leaf acetonic extract exhibited significant antifungal activity on *A. fumigatus* and *C. albicans* at lower concentrations. The leaf ethanolic extract exhibited significant antimicrobial activity on *P. aeruginosa, S. aureus* and *E. coli* and it fungi activity was highest on *A. fumigatus* followed by *C. albicans.* The leaf methanolic extract exhibited significant antimicrobial activity on *P. aeruginosa, S. aureus* and its fungi activity was highest on *A. fumigatus.* This showed and proved that the plant parts of *A. indica* contains some antimicrobials agents responsible these activities.

Minimum inhibitory concentration (MIC) values of the *A. indica* acetonic, methanolic and ethanol extracts of leaf and bark and Minimum inhibitory concentration was tested for the acetonic, ethanol and methanol extracts of leaves and bark were ascertained. The results of this study revealed that MIC for leaf acetonic extract was 25 mg / ml for all organisms except C. albicans which was 12.5 mg / ml. MIC values for the ethanol extract of leaf and bark for fungi were 12.5 mg / ml and 6.25 mg / ml respectively. No MIC values were obtained for acetone bark extract for *Staphylococcus* aureus and ethanol extract also for *S. aureus.*

The same result was obtained for the *E. coli* in ethanol and methanol leaf extracts. Minimum bactericidal concentration (MBC) values of the Neem acetone, ethanol and methanol extracts of leaf and bark were also obtained. Minimum bactericidal concentration was tested for the acetone, ethanol and methanol extracts of leaves and bark. Results revealed that the MBC for leaf acetone extract was 25 mg / ml except for *C. albicans* whose MBC was not obtained. The MBC value for the methanol extract of leaf was 25 mg / ml for all organisms except for *E. coli* whose MBC value was not obtained. No MBC values were obtained for ethanol and methanol leaf extracts and ethanol extract of bark for *E. coli.* Also, no MBC values were obtained for acetone extract of leaf and acetone, ethanol and methanol extracts of bark for *C. albicans.* The standard antibacterial drug used as control (ciprofloxacin) indicated that *E. coli* was the most susceptible to the drug followed by *Staphylococcus aureus* and *Pseudomonas aeruginosa.* These present study revealed that, there are some phytochemicals with the potency and efficacy for antimicrobial activities and support the works of most researchers on the medicinal plants, especially the Neem plant in concerned.

5. Conclusion

This investigation was done to find out the antimicrobial activity of leaf and bark extracts of Neem plants against selected pathogens. From the above study, *A. indica* which is used in folkloric traditional medicine showed that it is active against bacterial and fungal strains but there were some degree of variation in their antimicrobial activities. Thus, it may be concluded that *A. indica* leaf and bark extracts have antimicrobial activity against these pathogens. This plant could be utilized as an alternative source of useful antimicrobial drugs. However, extensive research still needs to be done on phytochemicals of this plant for the development of cost effective drugs for the future. More so, since many of the existing synthetic drugs cause various side effects, drug development using plant based compounds could be useful in meeting this demand for newer drugs with minimal side effects.

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