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Original Article

Bioactive components of *Azadirachta indica* (neem) seed oil on some pathogenic bacteria isolates

O. Dogun[1], R. A. Ogenyi[1], E. Orewere[2]*, M. U. Henry[1], S. Z. Longchi[3]

[1]Department of Science Laboratory Technology, Federal College of Forestry, Jos, Plateau, Nigeria, [2]Department of Horticulture and Landscape Technology, Federal College of Forestry, Jos, Plateau, Nigeria, [3]Department of Plant Science and Biotechnology, Faculty of Natural Sciences, University of Jos, Jos, Plateau, Nigeria

ABSTRACT

The purpose of this study was to evaluate the antibacterial efficacy of *Azadirachta indica* oil against several selected pathogenic bacteria. The oil was extracted with n-hexane, petroleum ether, and methanol. The results of the phytochemical screening revealed the presence of secondary metabolites such as steroids, terpenoids, cardiac glycosides, anthraquinones, flavonoids, and alkaloids. At $P \leq 0.05$, there was a significant difference in antibacterial activity. The methanol extract showed the highest inhibitory effect against the test bacteria, followed by the n-hexane extract, while the petroleum ether oil had no effect against the test bacteria. *Salmonella* typhi was the most sensitive to the n-Hexane oil, with inhibition rates ranging from 6.83 ± 0.27 to 17.70 ± 0.17, followed by *Escherichia coli* at 2.17 ± 0.12–15.63 ± 0.32, *Staphylococcus aureus* at 1.50 ± 0.29–14.80 ± 0.20 and *Bacillus subtilis* was the least sensitive at 0.00 ± 0.00–9.97 ± 0.15. The *S.* typhi was the most inhibited by the methanolic oil extract, followed by *E. coli*, *B. subtilis*, and *S. aureus*. The results of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for n-Hexane oil showed that *E. coli* and *S. aureus* had MIC's of 25%, *B. subtilis* 50% and *S.* typhi 12.5% with MBC's of 50%, 100% and 25%, respectively. Similarly for the methanolic oil *E. coli* and *S.* typhi had MIC's of 6.25%, *S. aureus* 25%, and *B. subtilis* 12.5%. The results obtained support the use of oil for the treatment of diseases.

Keywords: *Azadirachta indica*, bacteria, bioactive, neem, phytochemical

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INTRODUCTION

Plants produce a wide range of phytochemical constituents, which are a rich source of medicinal products; thus, their use in herbal medicine continues to increase. The emergence of drug-resistant bacteria poses a serious global problem for clinicians and the pharmaceutical industry[1] and is of concern.[2] Antimicrobial resistance is responsible for hundreds of thousands of deaths annually and is projected to increase, and WHO has identified it as a major global health threat.[3] The use of herbal medicines in developed countries continues to increase as they are a rich source of novel medicines, and their bioactive principles form the basis of important compounds of pharmaceuticals, pharmaceutical intermediates, and synthetic medicines.[1,2] The global scenario is now shifting toward the use of medicinal and non-toxic botanical products. In recent years, many experiments have shown that plant-derived compounds have significant and possibly different antimicrobial effects compared to microbial-derived antibiotics so people are increasingly relying on plants in the discovery and development of new antibiotics.[4-7] Screening of medicinal plants for biologically active compounds provides clues for the development of new antibacterial agents. Neem (*Azadirachta indica*) is a versatile medicinal plant and a source of several compounds with different chemical structures and biological effects.[5] Extensive research has been done in the past to understand the chemical properties and medicinal uses of different parts of neem for therapeutic and industrial uses.[8] *A. indica* is now used in traditional medicine as a source of many therapeutic agents *A. indica* (seed) is known to have antiviral activity against vaccinia, chikungunya, measles, and coxsackie B viruses, as well as antibacterial and antifungal activity against various pathogenic microorganisms.[5] Neem seeds have been shown to have a wide range of pharmacological

Address for correspondence: E. Orewere, Department of Architecture, Faculty of Environmental Sciences, Bingham University Karu, Nasarawa, Nigeria. E-mail: emmamoge3603@gmail.com

activities, including anti-oxidant, anti-malarial, anti-mutagenic, anti-carcinogenic, and anti-inflammatory properties.[8] These bioactivities are believed to result from the presence of many bioactive compounds in different parts of the plant. Therefore, this study was performed to determine the antibacterial activity of *A. indica* seed oil against several selected pathogenic bacteria.

MATERIALS AND METHODS

Sample Collection and Identification

A. indica seeds were collected from Langtang North Local Government Area of Plateau state, the plant was identified and voucher specimens were prepared and stored at the herbarium unit at the Federal College of Forestry Jos, Plateau state.

Sample Preparation

A. indica seeds were air-dried in the shade and ground to powder with a mortar and pestle. The powder was sieved and stored in an airtight bottle until needed.

Extraction of Oil

Extraction was performed by cold maceration, taking 100 g and maceration in 500 mL of n-hexane, petroleum ether, and methanol, respectively, for 72 h. Each oil sample was filtered through filter paper (FHJ 368) and then dried in a water bath.[9] Extraction was performed based on solvent polarity.

Phytochemical Determination

Test for alkaloids

Approximately 0.5 g of each extract was stirred on a steam bath with 3 mL of 1% aqueous hydrochloric acid and each 1 mL of filtrate was treated with a few drops of Meyer's reagent, Dragendorff's reagent and picric solution. Precipitation with either of these reagents was utilized as preliminary evidence for the presence of alkaloids in the extract.[10]

Test for saponins

About 0.5 g of each plant extract was shaken with water in a test tube. Frothing which persisted on warming was taken as preliminary evidence for the presence of saponins.

Test for tannins

About 0.5 g of the extract was stirred with 1 mL of distilled water and filtered; ferric chloride was added to the filtrate. A blue-black, green, or blue-green precipitate indicated the presence of tannins.

Test for anthraquinones

Borntrager's test was used for the detection of anthraquinones, 0.5 g of each extract was put into a dry test tube and 5 mL of chloroform was added and shaken for 5 min. The extract was filtered, and the filtrate was shaken with an equal volume of 100% ammonia solution. A pink-violet or red color in the ammoniacal layer (lower layer) indicated the presence of free anthraquinones.

Test for cardiac glycoside

A total of 100 mg of the extract was dissolved in 70% alcohol and filtered. About 3 drops of lead sub-acetate were introduced into the filtrate and filtered. The filtrate was extracted with 10 mL of chloroform in a separating funnel and concentrated to dryness. The resulting residue was dissolved in 1 mL of glacial acetic acid containing one drop of ferric chloride solution. This was underplayed with 1 mL of concentrated sulphuric acid. A brown ring obtained at the interphase indicated the presence of a deoxysugar characteristic of cardenolides.

Test for steroid and terpenes

A little quantity of each extract was dissolved in chloroform, and 1 mL of acetic anhydride was added, then two drops of concentrated sulphuric acid were added. A pink color which changed to bluish-green on standing, indicated the presence of steroid and terpenes.

Test for flavonoids

5 mL dilute ammonia was added to 5 mL of the extract and then 5 mL concentrated sulfuric acid was added. The formation of a yellow color showed the presence of flavonoids.

Test for carbohydrates

100 mg of each extract was dissolved in 3 mL of distilled water and mixed with a few drops of molisch reagent (10% solution of napthol in alcohol) then 1 mL of concentrated sulphuric acid was carefully added down the side of the inclined tube so that the acid formed a layer beneath the solution. A white color at the base indicated the presence of carbohydrates.

Source of microorganisms

Standard isolates of the bacteria *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Salmonella* typhi were obtained from the veterinary research institute vom. The organisms were collected in a suspension of nutrient broth.

ANTIMICROBIAL SUSCEPTIBILITY TESTING

Disk Diffusion Technique

Antimicrobial susceptibility testing was performed on clinical isolates using the disk diffusion technique described by[11] Bacterial inoculum was prepared from subcultures as follows. Day old bacterial colonies were suspended in broth and the turbidity was adjusted to the McFarland standard of 0.5. Bacteria were inoculated onto solidified nutrient agar plates using a sterile cotton swab method. Disks impregnated with 100, 50, 25, 12.5, and 6.25% oil were placed on the inoculation plate, incubated at 37°C for 24 h, and the zone of inhibition was measured to the nearest millimeter (mm).

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

MICs were determined using the broth dilution method, a standardized inoculum of 1 mL of broth containing the organism was introduced into a test tube containing 5 mL of sterile broth, and oil at various concentrations of oil was added to the test tube, and incubated at 37°C for 24 h and observed for growth in the form of turbidity.

Data Collection and Statistical Analysis

In vitro antibacterial assay

Experiments were performed in triplicate and the resulting data were obtained were subjected to analysis of variance and used to determine the significance of the inhibition regions, the difference between the antimicrobial activities of the neem oil extracts, and the susceptibility of the test organism. Significance was accepted at $P \leq 0.05$ using GraphPad Prism version 8.

RESULTS

The phytochemical investigation of the oil extracts of *A. indica* indicated the presence of secondary metabolites such as steriods, terpenoids, cardiac glycosides, and anthraquinones. Flavonoids were highly present in the methanolic oil extract of *A. indica,* whereas alkaloids were present in the n-Hexane and petroleum ether oil extracts. Saponins, tannins, and carbohydrates were absent in all the oil extracts. The phytochemical results of the oil extracts of *A. indica* are summarized in Table 1.

Results revealed in Table 2 shows at $P \leq 0.05$ there was a significant difference in the antibacterial activity of the oils from *A. indica* on the selected bacteria isolates activity in a concentration-dependent manner. Values are presented as mean ± standard error of means. The ranking was done across the neem oil extracts and values with the same superscript are not significant. The methanolic extract had the highest inhibitory activity on all the test bacteria, followed by the n-Hexane extract, but the petroleum ether oil had no activity on the test organism. *S.* typhi was the most susceptible to the n-Hexane oil with inhibition of 6.83 ± 0.27–17.70 ± 0.17, followed by *E. coli* at 2.17 ± 0.12–15.63 ± 0.32. *S. aureus* at 1.50 ± 0.29–14.80 ± 0.20 and *B. subtilis* had the least susceptibility at 0.00 ± 0.00–9.97 ± 0.15. Similarly, *S.* typhi had the highest inhibition with the methanolic oil extract followed by *E. coli*, *B. subtilis,* and *S. aureus*.

Table 1: Phytochemical screening of different oils of *Azadirachta indica* seeds

Constituents	n-hexane	Petroleum ether	Methanolic
Alkaloids	++	+++	-
Saponins	-	-	-
Tannins	-	-	-
Flavonoids	-	-	+++
Carbohydrates	-	-	-
Steroids	+	+	+++
Anthraquinones	+	+++	++
Cardiac glycosides	+++	+	++
Terpenoids	+	+	+

Key: +=presence, ++=more present, +++=Highly present, --=Absence

The results in Tables 3 and 4 reveal the MIC and minimum bacteriocidal concentration for n-Hexane oil showed that *E. coli* and *S. aureus* had MICs of 25%, *B. subtilis* 50%, and *S.* typhi 12.5% with MBC of 50%, 100% and 25%, respectively. For the methanolic oil *E. coli* and *S.* typhi had MIC's of 6.25%, *S. aureus* 25% and *B. subtilis* 12.5%.

Figures 1 and 2 summarize the antibacterial activity of n-Hexane and methanolic *A. indica* oil extracts on some bacteria isolates against a positive control (Gentamycin 50 mg/mL).

DISCUSSION

Phytochemical results of *A. indica* oil extract indicate the presence of secondary metabolites known to have both medicinal and physiological properties such as: steroids, terpenoids, cardiac glycosides, and anthraquinones. Flavonoids were highly present in the methanolic oil whereas alkaloids were present in the n-Hexane and petroleum ether oil. Saponins, tannins, and carbohydrates were absent in all the oil extracts. The results of this finding are consistent with other researchers who reported bioactive components of *A. indica* oil.[12-14] Ahmed *et al*., 2020 reported that phytochemical screening of neem oil extracts revealed high levels of cardiac glycosides and anthraquinones, as well as significant amounts of monosaccharides, tannins, and phenolic acids. Only small amounts of flavonoids were detected in neem seed oil and no saponins, tannins, or carbohydrates were detected.

At $P \leq 0.05$, the antibacterial activity results of different oil extracts showed significant differences in antibacterial activity in a concentration-dependent manner. That is, the higher the concentration of the oil, the higher the antibacterial activity of the oil. Methanol oil extract showed the highest inhibitory effect against all test bacteria, followed by n-hexane extract, whereas petroleum ether oil had no effect against the test microorganisms. *S.* typhi was the most sensitive to n-hexane oil with inhibitions of 6.83 ± 0.27 to 17.70 ± 0.17, followed by *E. coli* with 2.17 ± 0.1215.63 ± 0.32. *S. aureus* had the lowest susceptibility, ranging from 1.50 ± 0.29 to 14.80 ± 0.20, and

Table 2: The antibacterial activity of *Azadirachta indica* oil on some selected bacteria isolates

ORG.	Oil	6.25%	12.5%	25%	50%	100%
Escherichia coli	n-Hexane	2.17±0.12^{b}	5.80±0.20^{b}	8.37±0.35^{b}	11.97±0.6^{b}	15.63±0.32^{b}
	Methanolic	8.00±0.58^{a}	11.33±0.67^{a}	15.13±0.13^{a}	17.30±0.44^{a}	20.20±0.49^{a}
	Pet ether	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}
Staphylococcus aureus	n-Hexane	1.50±0.29^{b}	3.93±0.18^{b}	6.63±0.47^{b}	10.60±0.21^{b}	14.80±0.20^{b}
	Methanolic	3.67±0.20^{a}	6.57±0.35^{a}	10.13±0.47^{a}	12.83±0.27^{a}	16.87±0.24^{a}
	Pet ether	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}
Bacillus subtilis	n-Hexane	0.00±0.00^{a}	1.80±0.25^{b}	3.63±0.20^{b}	6.93±0.18^{b}	9.97±0.15^{b}
	Methanolic	4.77±0.62^{a}	8.80±0.42^{a}	12.50±0.29^{a}	15.50±0.25^{a}	18.17±0.44^{a}
	Pet ether	0.00±0.00^{b}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}
Salmonella typhi	n-Hexane	6.83±0.27^{b}	9.87±0.24^{b}	12.40±0.35^{b}	14.97±0.20^{b}	17.70±0.17^{b}
	Methanolic	12.17±0.44^{a}	18.00±0.58^{a}	21.13±0.47^{a}	23.93±0.23^{a}	28.30±0.36^{a}
	Pet ether	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}

L.S.D=1.02, P<0.0001 ****Level of Significance, ^{a,b,c}: Ranking,it is done to show where the significance is coming from.

Table 3: The MIC and MBC for the antibacterial activity of n-hexane oil on some bacteria isolates

Organism	6.25%	12.5%	25%	50%	100%	MIC	MBC
Escherichia coli	+	+	+	-	-	25	50
Staphylococcus aureus	+	+	+	-	-	25	50
Bacillus subtilis	+	+	+	+	-	50	100
Salmonella typhi	+	+	-	-	-	12.5	25

Key: -=No Growth, +=Growth

Table 4: The MIC and MBC for the antibacterial activity of methanolic oil on some bacteria isolates

Organism	6.25%	12.5%	25%	50%	100%	MIC	MBC
Escherichia coli	+	-	-	-	-	6.25	12.5
S. aureus	+	+	+	-	-	25	50
Bacillus subtilis	+	+	-	-	-	12.5	25
Salmonella typhi	+	-	-	-	-	6.25	12.5

Key: -=No Growth, +=Growth

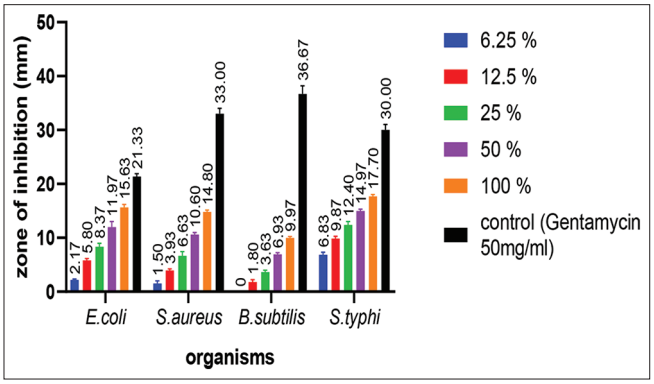

Figure 1: The antibacterial activity of the n-Hexane oil of *Azadirachta indica* on some bacteria isolates

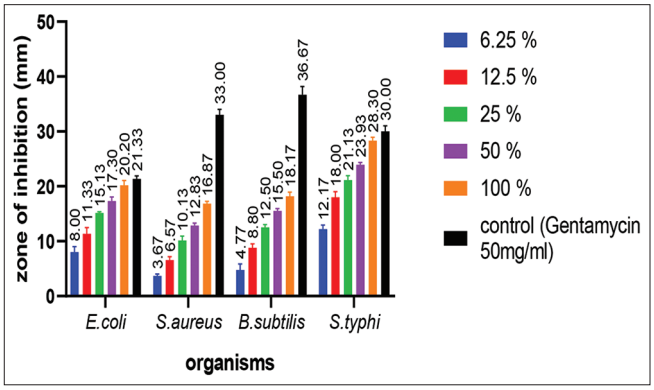

Figure 2: The antibacterial activity of the methanolic oil extract of *Azadirachta indica* on some bacteria isolates

B. subtilis susceptibility ranged from 0.00 ± 0.00 to 9.97 ± 0.15. Similarly, the highest rate of inhibition by methanol oil was for *S.* typhi, followed by *E. coli*, *B. subtilis*, and *S. aureus*. The results of the MIC and MBC for n-Hexane oil showed that *E. coli* and *S. aureus* had MIC's of 25%, *B. subtilis* 50%, and *S.* typhi 12.5% with MBC of 50%, 100% and 25%, respectively.

For the methanolic oil *E. coli* and *S.* typhi had MIC's of 6.25%, *S. aureus* 25% and *B. subtilis* 12.5%. The results of this finding are in line with the work of other researchers[15-18] who reported the antibacterial activity of neem oil extracts. Neem seed oil extract has a broad spectrum of activity as it is effective against both Gram-positive and Gram-negative bacteria. Gram-positive bacteria are generally more sensitive to antimicrobial agents than Gram-negative bacteria, mainly due to differences in genetic makeup.[19] Gram-positive bacteria have a thin peptidoglycan layer making them more susceptible, while Gram-negative bacteria have a phospholipid bilayer that increases resistance to antimicrobial agents.[20] The results of this study are consistent with another finding that neem seed oil extract is more effective against *E. coli*, which has a 19.5 mm zone of inhibition, than *S. aureus*, which has a 19 mm zone of inhibition.[17] The antibacterial effect of neem seed oil is likely due to the presence of chemical substances such as flavonoids, tannins, and phenols that are known to inhibit bacterial growth.[15] It is also brought about by inhibiting DNA synthesis and cytoplasmic membrane function as well as inhibition of energy metabolism in bacteria.[21]

CONCLUSION

This study confirmed that *A. indica* (Neem) has some antibacterial activity and these activities are due to the presence of certain bioactive compounds. These findings support the use of neem oil in traditional medicine.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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