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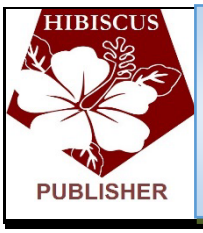

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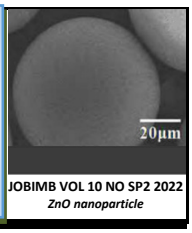

Antibacterial Activity of Alkaloid, Flavonoids and Lipids from Crude Extracts of *Azadirachta indica* on Some Selected Medically Important Bacteria

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ABSTRACT

Most tropical climates are home to the green perennial tree *Azadirachta indica*, which belongs to the Meliaceae family of Mahogany, and has long been known to have therapeutic effects. Secondary metabolites in plants cause biological activity in both humans and animals, which explains why they are used as herbs. For the investigation of the lipid, alkaloids, and flavonoids present in the *A. indica* extracts, thin-layer chromatography was carried out using several solvent systems. The thin layer chromatography-separated active components were tested for antibacterial efficacy against three multi-drug resistance pathogens namely: *Salmonella typhi, P. aeruginosa* and *S. aureus.* Alkaloids showed the highest antibacterial activity on *Salmonella* spp. (15 mm) and 12 mm *Staphylococcus aureus* isolates while lipids showed the least activity on the tested isolates. The minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) were calculated. The outcomes of the MIC and MBC revealed that the inhibitory concentrations of different plant extracts for certain bacteria varied. Values of MIC for *Salmonella typhi* were found in the range of 25 to 50 mg/mL, MBC 100 to 200 mg/mL and for *Staphylococcus aureus* MIC values ranged between 50 and 200 mg/mL, MBC 100 to 400 mg/mL and for *Pseudomonas aeruginosa* MIC values were found in the range of 100 to 200 mg/mL and MIC values ranged between 200 and 400 mg/mL using a different part of the plant extracted using three different solvents. The finding suggests that crude extract of *A. indica* might work well for the treatment of illnesses brought on by these microbes and that the activity of the crude extract is more than that of an individual component.

INTRODUCTION

Azadirachta indica, (neem) is a plant tree that belongs to the Meliaceae family, which includes mahogany, grows quickly and typically reaches heights of 15-20 meters [1]. It is a tropical green tree that is indigenous to the Indian continent and has been utilized for hundreds of years to address a range of human diseases in traditional medicine [2]. Many parts of the Neem plant, including the flowers, fruits, seeds, roots, leaves, gum, and stem have all been utilized as over-the-counter treatments for human ailments. Additionally, neem twigs are used by millions of people worldwide as chewing sticks for tooth hygiene. [2,3]. Bark, leaf, fruit, and root extracts have been utilized for an antacid, anti-parasitic, antibiotic, antivirus, contraceptives, anti-dermatitis, antitumor, anti-inflammatory, antioxidant, antifungal, and for dental treatments as well as treatment for respiratory

issues in children [4,5]. From various Neem plant components, more than 135 distinct chemicals have been discovered. There are two main categories of compounds: isoprenoids, which include diterpenoids and triterpenoids, and polysaccharides, proteins, and amino acids as examples of non-isoprenoids [6]. Tree bark polysaccharide reduces mice inflammation caused by carrageenin. Sulfur-containing substances, such as cyclic trisulphide and tetrasulphide, have antifungal action against Trichophyton mentagrophytes when separated from fresh, fully-grown neem leaves steam-distillate [7].

The main issue facing the entire world is the rise in antibiotic resistance in microorganisms that are crucial to medicine. Multiple drug resistance has been brought about by the careless usage of commercial antimicrobial medicines. Additionally, antibiotics may have negative side effects on the host, such as immunological suppression, allergies, and hypersensitivity. As a result, new antimicrobial medications are required for the treatment of infectious disorders. Plant-based antimicrobials have a huge medicinal promise. They effectively treat infectious infections while also reducing the number of the negative effects frequently seen with synthetic antimicrobials [8]. The majority of research on *A. indica* has been done on its extracts, but in this study, we want to extract some of its phytochemical components. We also want to find out what the Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of the crude extract of *A. indica* parts are, as well as what some of its secondary metabolites' antibacterial activity is on some medically important bacteria.

MATERIAL AND METHOD

Preparation of plant matter
The leaves, roots, and bark of *Azadirachta indica* were harvested, washed under running water to eliminate any dirt or foreign items, and then allowed to dry for Six (6) weeks in the shade in a postgraduate lab at Abubakar Tafawa Balewa University. Before being used, the dry ingredients were packaged and sealed in plastic bags after being pounded in a mortar [9].

Extraction of plant material
A continuous orbital shaker was used to mix 400 grams of dried, pulverized *Azadirachta indica* leaf, bark, and root for 72 hours in 2.5 litres of acetone, followed by ethanol, methanol, and then water. The extracts were concentrated using a rotary vacuum evaporator at a temperature not to exceed 40°C after being passed twice via cotton wool and once with Whatman No. 1 filter paper in an aerated oven set at 40°C, the condensed extracts were completely dried and stored inside a refrigerator at 4°C according to Supelco Bulletin [10].

Phyto-chemical Analysis
The protocols outlined by Talukdar and Choudhary [11] were used to evaluate the extracted materials. To conduct the following tests to determine whether there are alkaloids, saponins, tannins, Terpenoids, flavonoids, glycosides, volatile oils, and reducing sugars present

Saponins
Using the froth test, Saponins were identified. The sample was weighed at 1g and placed in a conical flask with 10mL of sterilized distilled water, which was then brought to a boil for 5 minutes. After filtering the mixture, 2.5 milliliters of the filtrate were mixed with 10 milliliters of sterile, distilled water in a test tube. For around 30 seconds, the test tube was rotated and forcefully shook. After then, it was permitted to stand for 30 minutes. The appearance of saponins was suggested by honeycomb froth.

Tannins
Three to four drops of a 10percent ferric chloride solution were added to an extract that had been diluted with water. Gallic tannins are seen to be blue and catecholatic tannins to be green.

Reducing Sugars
A water bath was used to heat 0.5mL of plant extracts, 1mL of water, and 5-8 drops of Fehling's solution. Precipitation that is brick red suggests that there are reducing sugars present.

Glycosides
In a test tube, 5mL of extract was combined with 25mL of diluted Sulphuric acid, which was then heated for 15 minutes, cooled, and neutralize with 10%NaOH before 5mL of Fehling solution was added. A brick-red precipitate is a sign of glycosides.

Alkaloids
In a test tube, two (2mL) of the extract were measured before the picric acid solution had been added. The appearance of alkaloids was indicated by an orange color.

Flavonoids
1.5mL of a 50% methanol solution was added to 4mL of extract solution for the treatment. The solution is warmed, before adding magnesium metal. A red color was seen for flavonoids and an orange color for flavones after 5–6 drops of strong hydrochloric acid were added to this solution.

Volatile oils
A small amount of diluted HCl and diluted 0.1mL NaOH were added to two 2mL of the extracts and were mixed. In the presence of volatile oils, a white precipitate forms.

Terpenoids
A combination of 0.5 mL acetic anhydride and 0.5 mL chloroform were used to process four milligrams of extract. After that, gradually strong sulphuric acid solution was added to it, terpenoid was visible as a red violet tint.

Collection and Culture of Organisms
Isolates of *P. aeroginosa, S. aureus and S. typhii* were acquired from Abubakar Tafawa Balewa University Teaching Hospital. To validate the precise species of these clinical isolates, additional identification was made using biochemical assays (Urease, Catalase, Coagulase, Motility, sugar fermentation, Indole, citrate utilization test), and tested for multi-drug resistance activity. Before use, the clinical isolates were inoculated into nutrient agar slants and then kept in the refrigerator at 4°C.

Multi-drug resistance screening of the isolates
Utilizing Mueller-Hinton agar plates and commercially available antibiotic discs, antimicrobial susceptibility testing was done using Kirby-Bauer disc diffusion technique based entirely on the Clinical and Laboratory Standards Institute guidelines (CLSI) (Hardy Disc-HDx) [12]. To produce confluent growth for the sensitivity test, the confirmed isolates were cultured on pre-made Brain Heart Infusion (BHI) agar plates and incubated at 37 °C for a period of 24 hours for the sensitivity tests, as detailed elsewhere [13], a loop-full of isolates from BHI plates were dispensed in sterile normal saline to match the 0.5 McFarland Turbidity Standard (1.0 x 108 CFU/L). The iso-sensitivity test agar plates were inoculated with about 100 µL of the bacterial suspension, and the extra was removed using sterile Pasteur pipettes. The

plates were dried in a laminar flow at room temperature. Using a sterile set of forceps, the antibiotic disks also were placed into the plates inoculated with the isolates and carefully pressed to achieve full contact with the agar. The discs were spaced 25 mm apart from one another and 15 mm from the plate's edge. The plates were incubated aerobically at 35–37°C for 18–24 hours. Standards authorized by the Clinical and Laboratory Standard Institute were used to measure and evaluate the sizes of the zones of inhibition [14].

The antibiotic discs used include: Ampicillin (10 µg), Amoxicillin/clavulanic acid (25/5 µg), Amikacin (30 µg), Ciprofloxacin (5 µg), Chloramphenicol (30 µg), Erythromycin (15 µg)Gentamycin (10 µg), Kanamycin (30 µg), Sulphate Colistin (10 µg), Streptomycin (300 µg), Penicillin (30 µg), Tetracycline (30 µg), Vancomycin (30 µg),Cotrimoxazole (30 µg), Sparfloxacin (5 µg), and levofloxacin (10 µg) (Oxoid). As a control, *E. coli* American Type Culture Collection (ATCC) 25922 was employed. Resistance to three or more types of antibiotics was used to characterize the multidrug resistance phenotype [15].

Growth Media
In this investigation, the nutrient broth was used. The media was made in accordance with the manufacturer's manual. [16].

Standardization of the Inoculum
A tube holding around 2.0ml of normal saline was filled with enough material from the overnight culture of the test organisms using an inoculum loop until the turbidity met the turbidity standard of 1% BaSO4. (1.0×10^8 CFU/µL) [16].

Determination of Minimum Inhibitory Concentration (MIC)
The tube dilution method was used to calculate the extracts' minimum inhibitory concentration (MIC) ([17]. Plant extracts were diluted and added to the nutritional broth at a 1:1 ratio. To determine the range of MIC values, estimates of the MIC values of the plant extracts against the test organisms were made. Using the dilution formula, the following concentrations were created for each extract: 400 mg/mL, 200 mg/mL, 100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL and 6.25 mg/mL. Each tube also received 0.1 mL of the test organisms' standard suspension. After that, the tubes were incubated for 24 hours at 37 °C. As a control, a tube containing nutrient broth with extract without inoculum was added. At the conclusion of the incubation time, the presence of growth (turbid solution) or absence of growth (clear solution) was noted. The Minimum inhibitory concentration was defined as the extract's lowest concentration at which no growth was observed (MIC).

Determination of Minimum Bactericidal Concentration (MBC)
The last test dilution that displayed visible growth (turbidity) and all others that did not show any growth were sub-cultured on a fresh medium and incubated for an additional 24 hours to determine the minimum bactericidal concentration (MBC) According to a report by [13,17], the minimum bactericidal concentration (MBC) was determined to be the maximum dilution that revealed no single bacterial colony.

Active Compound Separation from Neem Extracts Suspension by TLC

Chromaplate preparation
In a hot air oven, different glass slides were wiped and dried. In a clean beaker, a slurry was made by combining silica gel with twice as much distilled water. Using the other slide edge, one (1) drop of the slurry was applied to the slide and spread out to create a thin coating. The slides were left in this state for a short while. The chromoplates were then heated in a hot air oven for 30 minutes at 120 ºC to activate them.

Sample Loading
The origin was noted on the slides around Two (2 cm) from the bottom after allowing them to cool at ambient temperature. At each slide's center, up from the edge, the working suspensions are being loaded.

Chromatogram Development
The tank was developed using the Eskil Hultin [18] method. The tank was filled to capacity with the following solvent systems. alkaloids: benzene and methanol-80:20, flavonoids: chloroform and ethanol-70:30 and lipid: chloroform:methanol and water-10:10:3. Without touching the baseline, the slides were held in the tank by solvent. The slides were dried after marking the final solvent front.

Visualization of Spot
1% ethanolic aluminium chloride solution was utilized to visualize flavonoids, which were then seen under 560 nm UV light. Alkaloids were seen when exposed to UV radiation. In order to fill the tank with iodine vapor and detect lipids, a few pieces of iodine crystals were placed inside and covered with a glass plate. The plate was left in an iodine-vapor saturated tank for a few hours while being visually examined.

Recovery of the Active Compound
Scratches were made on the preparatory silica gel slides using a clean, dry spatula. The spots were then collected in a beaker with the right solvents and left overnight. The beaker's contents were mixed before being filtered using Whatman No. 1 filter paper. A clean, dry beaker was used to collect the filtrate. By using the cup diffusion method, the filtrate containing the active ingredient was used to assess the antibacterial action [19].

$$Rf = \frac{\text{Distance travelled by the substance being considered}}{\text{Total distance travelled by the mobile phase}}$$

RESULTS

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)
Fig. 1 shows the results for the MIC and that of MBC of the leaf extracts of *A. indica* on the tested medically important bacteria. MIC values obtained were in the range of 50 mg/mL-100 mg/mL across the tested organisms. However, MBC values were in the range of 100 mg/mL-400 mg/mL. The result for MIC and MBC of the bark extracts of *A. indica* is presented in **Fig. 2**. MIC values for the tested organism using the bark of *A. indica* extracted using different were found to be in the range of 50 mg/mL-200 mg/mL whereas MBC values 200 mg/mL-400 mg/mL.

Fig. 3 showed that MIC values using methanol extract of root for *S. typhi, P. aeruginosa* and *S. aureus* were 25 mg/mL, 50 mg/mL and 50 mg/mL respectively and MBC values were 100 mg/mL, 200 mg/mL and 400 mg/mL for *Salmonella typhi*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* respectively

Active ingredients in A. indica's root, bark, and leaf extract antimicrobial activity

The results for some of the active components that were extracted from Azadirachta indica's leaves, stem, and root are shown in **Table 1**. These components are lipids, alkaloids, and flavanoids. The highest activity was found in alkaloids, which were followed by flavanoids and lipids. Alkaloid had the highest activity (15 mm zone of inhibition) and lipids had the lowest activity against *Salmonella typhi.* **Table 2** shows the retention Factor (RF) values of some of the extracted active ingredients from the stem, root and leaves of *Azadirachta indica* using three different solvents namely methanol ethanol and acetone.

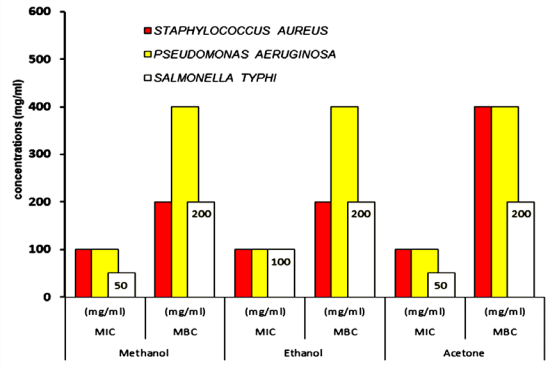

Fig. 1. Minimum Bactericidal concentration (MBC) amd Minimum inhibitory concentration (MIC) of the leaves extracts of *Azadirachta indica.*

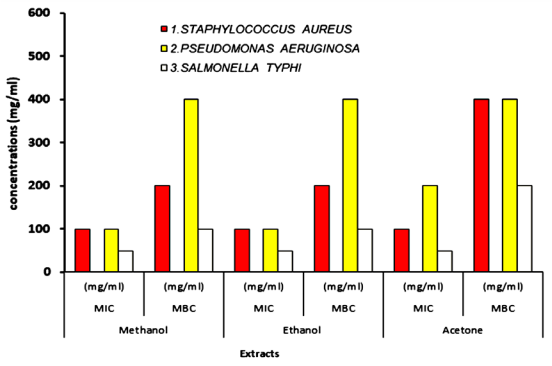

Fig. 2. Minimum Bactericidal concentration (MBC) and Minimum inhibitory concentration (MIC) of the bark extract of *Azadirachta indica.*

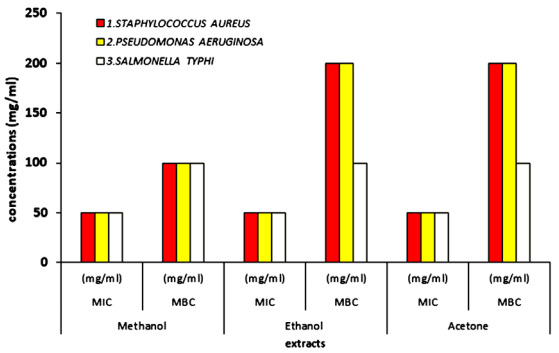

Fig. 3. Minimum Bactericidal concentration (MBC) Minimum inhibitory concentration (MIC) of the Root extract of *A. indica.*

Table 1. Antibacterial activity of the active compounds from TLC for the leaves,` root and bark extract of *A. indica.*

Mean values for the Diameter of Zones of inhibitions (mm) on Test organisms

Active compounds	Plant part	*Salmonella tyhi*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
	Root	15.0	10.0	12.0
Alkaloids	Stem	9.0	3.0	7.0
	leaves	5.0	5.0	5.0
flavanoids	Root	6.0	4.0	9.0
	Stem	7.0	nil	7.0
	Leaves	5.0	2.0	5.0
Lipids	Root	4.0	11.0	5.0
	Stem	2.0	1.0	6.0
	Leaves	Nil	nil	Nil

Table 2. RF values obtained in TLC (cm) for the root, bark and leaves of *Azadirachta indica* extracts.

Solvents	Plant parts	Components		
		Alkaloid	flavonoid	Lipid
Methanol	Root	0.40	0.30	0.30
	Stem	0.52	0.43	0.51
	Leaves	0.90	0.50	0.72
Ethanol	Root	0.91	0.56	0.62
	Stem	0.92	0.41	0.54
	Leaves	0.31	0.91	0.81
Acetone	Root	0.70	0.46	0.50
	Stem	0.62	0.75	0.81
	Leaves	0.95	0.85	0.98

DISCUSSION

The antimicrobial activities of *A. indica* crude extract and several of its secondary metabolites, notably alkaloids, flavonoids, and lipids, were investigated on Gram-negative and Gram-positive bacteria (*S. typhi and P. aeruginosa*) (*S. aureus*). The MIC and MBC values for *A. indica* leaves, root, and stem extracts extracted using different solvents show that the root extracts had higher activity against the studied organism. The extract demonstrated the greatest effectiveness against *Salmonella* species, followed by *S. aureus* and *P. aeruginosa*. The efficiency of different extracts varies depending on extrinsic and intrinsic elements such as temperature and PH levels [20].

Secondary metabolites of plants such as alkaloids, glycosides, flavonoids, tannins, steroids, terpenoids, and several other chemicals act as a defensive mechanism against many microbes, insects, and other herbivores [1,3]. *S. typhi* was discovered to be the most sensitive to different plant parts, while *P. aeruginosa* was shown to be the least susceptible. Because antibacterial agents have different modes of action, The disparity in the effect of these plant extracts on the organisms implied that there were various antimicrobial components inside the plant parts extract and, therefore the component that acted on one might not be the same as the component that acted on the others because antibacterial agents have various mechanisms of action [3].

Observations of these phenomena of variable susceptibility were made by [11] and [10]. The findings of [9] are similar to the results of this study, which demonstrate that both Gram-positive and Gram-negative bacteria have been sensitive to the extracts of *A. indica*. Because *A. indica* contains so many different active ingredients, its extract exhibits a wide range of action against both Gram-negative and Gram-positive bacteria. In conclusion, Alkaloids, flavonoids, and lipids were some secondary metabolites that were isolated from the crude extract of *A. indica* using TLC, but the findings from this work showed that a combination of active components is needed to treat the disease of bacterial aetiology. A preliminary scientific corroboration of the use of these plant components for the treatment of microbiological diseases, particularly those brought on by multidrug-resistant organisms, is provided by the findings, which also validate the traditional understanding of the local users. Since the plant is widely available and the extract is easily prepared using a straightforward maceration or infusion procedure, it may be a genuine and more affordable alternative to traditional medications. The study shows the value of plant components in creating effective antibacterial agents.

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