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EVALUATION OF THE ANTIMICROBIAL ACTIVITIES OF METHANOL AND AQUEOUS EXTRACT OF *LEPTADENIA HASTATA* ROOT ON CLINICAL ISOLATES

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Keywords:

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ABSTRACT: Using the plant as a source of medicine is one of the major campaigns in the field of alternative therapy. This study was conducted to investigate the antimicrobial activity of *Leptadenia hastate* root. The root of this plant was collected, dried and ground. The powder was subjected to extraction by cold maceration using water and methanol. The *in-vitro* antimicrobial activity of the extract was tested against four strains of bacteria: Staphylococcus aureus, *Pseudomonas aeruginosa*, *E. coli*, *Bacillus cereus* and one fungus *Candida albicans* using the agar diffusion method. DMSO was used as a negative control; Ciprofloxacin was used as a positive control for bacteria and fluconazole as a positive control for fungi. The agar dilution method determined the minimum inhibitory concentration (MIC) of crude extract. The phytochemical analysis was conducted to determine the secondary metabolites. The antimicrobial activities show that methanol extract of *Leptadenia hastate* was only sensitive against *Bacillus cereus* and *Candida albicans* out of five organisms tested, while aqueous extract does not show activity against any organism tested. MIC of methanol extract of *Leptadenia hastate* against *Candida albicans* and *Bacillus cereus* was 125 mg/ml. The phytochemical analysis revealed the presence of alkaloids, tannins, flavonoids, saponins, terpenoids and other bioactive components. The result suggested that the methanol and aqueous extract of *Leptadenia hastate* contains various pharmacology active compounds. There is a need for further study on the crude extract of this plant in order to identify and purify the active ingredients.

INTRODUCTION: The ancient scholars only believed that herbs were the only solutions to cure many health-related problems and diseases [1]. From the experimental studies conducted, they arrived at accurate conclusions about the efficacy of different herbs that have medicinal value.

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Thus formulated, most drugs are free of side effects or reactions. This is the reason why herbal treatment is growing in popularity across the globe. These herbs that have medicinal quality provide rational means for the treatment of many internal diseases, which are otherwise considered difficult to cure [2].

Medicinal plants are considered rich ingredients resources that can be used in drug development pharmacopoeial, non- pharmacopoeial or synthetic drugs [3]. Medicinal plants such as *Aloe, Tulsi, Neem, Turmeric, Ginger and Leptadenia hastata* cure

several common ailments. These are considered as home remedies in many parts of the country. *Leptadenia hastata* is a common edible, non-domesticated wild plant found in Saharan and sub – Saharan African [4]. The leaves, the most widely used part of the plant, are more abundant and fresh during the rainy season [5].

A proper study of African plants' antimicrobial properties, constituents, bactericidal and bacteriostatic activities would help and facilitate the development of the desired effect of a given plant.

In this work, screening was conducted using *Leptadenia hastata* roots for antibacterial activity against four strains of bacteria: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *E. coli*, *Bacillus cereus,* and one fungus, *Candida albicans*.

MATERIALS AND METHODS:

1. Bacterial and Fungal Strains Used in Preliminary Studies: Preliminary screening of the crude extract was carried out using clinical isolates of *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Bacillus subtilis, Salmonella typhi, Candida albican,* and *Enterococcus* collected from the department of Pharmaceutical Microbiology and Biotechnology laboratory, University of Nigeria, Nsukka.

1.1 Nutrient Media: Nutrient agar (Fluka limited) for bacteria was used for inoculation on plates, slants and tubes. Sabouraud Dextrose agar (FLUKA Limited) for fungi was also used for inoculation of plates, slants, and tubes.

1.2 Antibiotic: Ciprofloxacin.

1.3 Solvents: Methanol (Merck, Germany), Dimethyl sulphoxide (DMSO) (May & baker, England), Distilled Water (Laboratory grade).

1.4 Materials Used: Test tubes, petri -dishes, pipette, measuring cylinder, flatbottom flask, burnsen burner, autoclave, refrigerator, cotton wool, Weighing balance, foil, wire loop, masking tape.

1.5 Collection and Authentication of Plant Material: The root of *Leptadenia hashata* were collected from Orba in Udenu Local Government Area of Enugu state on the 13th August 2019; Mr. Alfred Ozoiko did authentication, a taxonomist with the International Center for Ethnomedicine and Drug Development (InterCEDD) Nsukka with Voucher no inter CEED 042.

1.6 Preparation and Extraction of Plant Material: The harvested roots were air dried for up to one month, after which the roots were ground into powdered particles, and poured into the clean container until needed. The ground powder was subjected to extraction by cold maceration. A 500 ml volume of methanol and aqueous was poured into two containers containing the powdered root and left for 24 h. This was filtered with gauze and a funnel into a vessel. The filtrate in a closed container was poured into an evaporating dish and left for days to dry. The dried extract was collected into clean containers and placed in the refrigerator at 4°C until needed.

1.7 Preparation of Extract: One kilogram each (1 kg) of the powdered ground root was weighed and subjected to extraction with 80% methanol and water by cold maceration. A 500 ml volume of methanol and water was poured into two different containers containing the powdered root and left for 24 h. It was filtered with gauze and a funnel into a vessel. The filtrate in a closed container was poured into evaporating dishes and left to dry at 30°C. After extraction, 4 g and 3 g of methanol and water of dried extract were gotten and poured into clean containers and placed in the refrigerator at 4°C.

1.8 Antimicrobial Screening: The antimicrobial activity of methanol and aqueous extracts of *Leptadenia hastata* root was determined by agar well diffusion and agar disc diffusion methods for standard antibiotics. The test organisms were sub-cultured into a fresh nutrient agar, and the concentration of working stock culture was assessed a 10^6 CFU/ml. For the susceptibility test, 100µl of inoculums; was mixed with 19 ml of sterilized Mueller Hinton agar and poured immediately into the sterile petri dishes. The petri dishes were left to solidify for 10 minutes. A sterilized 6 mm cork borer was used to make wells in the center of the divided areas. Few drops of each extract were pipetted into the wells. The petri dishes were incubated at 37ºC for 24 h. The experiment was done three times to minimize error.

After the incubation period, the antimicrobial activity was evaluated by measuring the inhibition zone. For *candida albicans*, Sabouraud agar was included. The inoculated Petri dishes were incubated at 25ºC for 48 h. The bacterial inhibitions were compared with ciproflaxacin disc (5µg). As negative control, few drops of DMSO were pipetted into each well for bacteria and fungi.

The extracts that exhibited inhibition zones were subjected to a minimum inhibitory concentration (MIC) assay using the diffusion method. A quantity of 1 g of the extract was dissolved in 4ml of DMSO, which yielded an initial concentration of 250mg/ml. Subsequently, two –folds serial dilution was made from the stock to obtain 125 mg/ml, 62.5 mg/ml, 31.5 mg/ml and 15.625 mg/ml concentrations. The agar plates were prepared and allowed to solidify. A 0.1 ml of the test organisms was smeared on the surface of the mueller Hinton agar in the petri dishes using a sterile glass rod and allowed to stand for five minutes in aseptic condition. Five wells were made on the agar using a cork borer of 6 mm [6, 7]. Six drops of each concentration was transferred into each and labeled. The positive control, standard drug (ciprofloxacin), and negative control DMSO, which was used to fill one of the wells. A prediffusion time of 30 mins was allowed before incubation. Bacteria were incubated at 37ºC for 24 h and fungus at 24^{o}C for 6 days. Zone of inhibition was determined using measuring ruler. The zones of inhibition were determined using a meter rule. The MIC was estimated as follows;

The MIC was estimated as follows;

- Obtaining the means of the inhibitory zones.
- Diameter (IZD) of each well of the organisms.
- Calculating logarithm of the concentrations.
- Plotting the graph of mean IZD against Log concentration.
- Finding the intercept and calculating antilog of the intercept, which is the MIC.

2. Phytochemical Analysis of the Extract:

2.1 Qualitative Tests: The phytochemical analysis was conducted at the department of Pharmacognosy, University of Nigeria Nsukka. The methanol and water extracts were both subjected to the following phytochemical test:

Test for Alkaloids: The dried extract was treated with a few drops of dilute hydrochloric acid and then filtered. The filtrate was subjected to the following test:

Dragendorff's Test: A few drops of Dragendorf's reagent were added to 3ml of the filtrate. The appearance of orange-brown precipitates confirmed the presence of alkaloids.

Mayer's test: A few drops of Mayer's reagent were added to 3 ml of the filtrate. The appearance of cream-colored precipitates showed the presence of alkaloids.

Wagner's Test: Wagner's reagent was added to 3 ml of the filtrate in little drops. The appearance of reddish brown precipitates detected alkaloids.

Test for Phenolic Compounds: The extract was dissolved in water, warmed over a bath, and filtered. The following tests were then carried out on the filtrate.

Lead Acetate Test: The test filtrate was treated with a 10% w/v basic lead acetate solution in distilled water. A white precipitate was observed, which indicates the presence of phenolics.

Potassium Dichromate Test: On adding potassium dichromate solution to the test filtrate, a black coloration was observed, indicating the presence of phenolic compounds.

Test for Flavonoids:

Lead Acetate Test: A little amount of lead acetate solution was added to the extract. The appearance of yellow-colored precipitates shows the presence of flavonoids.

Shinoda's Test: A little amount of test extract was dissolved in 5 ml ethanol (95% w/v) and then treated with a few drops of strong hydrochloric acid and 0.5 g of magnesium metal. The appearance of pink or crimson color indicates the presence of flavonoids.

Test for Proteins:

Test with Million's Reagent: A 5 ml of Millon's reagent was added to 3 ml of the extract. The

presence of proteins was shown by the appearance of white precipitates that turned red when heated.

Biuret Test: A 4 percent NaOH and a few drops of 1 percent $CuSO_4$ solution were added to 3 ml of aqueous extract. The presence of proteins was shown by the appearance of violet or pink color [27].

Tests for Glycosides:

Salkowski's Test: A 2 ml of concentrated H_2SO_4 was added to the Plant crude extract. A reddish brown color was formed, which indicated the presence of the steroidal aglycone part of the glycoside.

Test for Terpenoids: A 2.0 ml of chloroform was added to the 5ml extract filtrate. It was evaporated in a water bath and then boiled with 3 ml of concentrated H_2SO_4. A grey color formed, which showed the entity of terpenoids.

Test for Steroids: To 5 ml of extract filtrate, 2 ml chloroform, and concentrated H_2SO_4 were added. A red coloration appeared in the lower chloroform layer, indicating the presence of steroids.

Test for Tannin: A 0.5 g aqueous extract was mixed with 10 ml bromine water. The decolorization of bromine water detected the presence of tannins.

Test for Saponin:

The Frothing Test: A 0.5mg test extract was placed in a test tube, and a small amount of sodium bicarbonate and water was added. The solution was agitated vigorously. The presence of saponins was indicated by the appearance of characteristic honeycomb-like froth which does not disappear on standing.

Test for Reducing Sugars:

Benedict's Test: A 1ml of the extract filtrate was transferred into a clean test tube. A 2 ml of Benedict's reagent was added to the test and thoroughly mixed. The solution was heated over water bath for 5 min. A red brick precipitate was observed, which indicated the presence or reducing sugars.

2.2 Quantitative Test:

Determination of Total Alkaloids: A 1 g of the sample was weighed into a 250 ml beaker and 50 ml of 10 % acetic acid in ethanol was added. The solution was covered and allowed to stand for 4 h. It was filtered, and the filtrate evaporated to a quarter of its original volume in a water bath. Concentrated ammonium hydroxide was added in drops to the extract until the precipitation was complete. The solution was allowed to settle, after which the precipitated was collected and rinsed with dilute ammonium hydroxide before being filtered. The resulting residue is the alkaloid, which was dried and weighed.

Determination of Saponin: 1g of each extract was put into a conical flask, and $5cm^3$ of 20 % aqueous ethanol was added. The sample was heated over a hot water bath for 4 hours with continuous stirring at about 55°C. The mixture was filtered, and the residue was re-extracted with another 10 ml 20 % ethanol. The combined extracts were reduced to 5 ml over a water bath at about 90°C. The concentrate was poured into a separator funnel, along with 3 ml of diethyl ether, and rapidly agitated. The aqueous layer was recovered while the ether layer was discarded. It was evaporated and dried in a hot air oven, after which the saponin content was calculated.

Determination of Tannin: A 500 mg sample was weighed out and transferred into a 50 ml bottle. A 50 ml of distilled water was added, and the solution was agitated for about an hour in a mechanical shaker.

It was then filtered into a 50 ml volumetric flask and made up to the mark. 5 ml of the filtrate was transferred into a test tube and 2 ml of 0.1 M $FeCl_3$ in 0.I N HCl and 0.008 M potassium ferrocyanide were added. The solution was properly mixed, and the absorbance was taken.

Determination of Phenolic Compounds: A 100 mg of sample extract was carefully weighed and diluted in 100 ml of triple distilled water. 1 ml of this solution was transferred to a test tube, followed by the addition of 1.5 ml 20 percent Na_2CO_3 solution and 0.5 ml 2 N Folin-Ciocalteu reagent. TDW was used to make up the volume to 8ml, followed by vigorous agitation.

It was allowed to stand for two hours, and the absorbance was taken at 765nm afterward. The phenolic compound was then estimated from the calibration curve obtained.

Determination of Total Flavonoids: The approach relies on the production of a flavonoids-aluminum complex with a maximum absorptivity of 415 nm.

A 20 % aluminium trichloride in methanol was added to 100 µl of the sample extract in methanol. The solution was allowed to stand for 40 minutes and the absorbance was read at 415 nm. The concentration of flavonoids was then determined from the absorbance obtained [28].

Determination of Steroids: Known volumes (0.1 ml and 0.2 ml) of triple acid extract and a set of standards (0.5 to 2.5 ml) were taken and made up to 5 ml with ferric chloride diluting reagent. A blank was prepared simultaneously by taking 5.0 ml diluting reagent.

Then 4.0 ml of concentrated sulphuric acid was added to each tube. After 30 minutes of incubation, the intensity of the color developed was read at 540 nm.

Determination of Terpenoids: To 1 ml of the plant extract, 3 ml of chloroform was added. The sample mixture was thoroughly agitated and left for 3 min, and then 200 µl of concentrated sulfuric acid (H_2SO_4) was added. Then it was incubated at room temperature for 2 h in dark conditions and a reddish brown precipitate was formed during incubation. Then carefully and gently, all supernatant of the reaction mixture was decanted without disturbing the precipitation. A 3 ml of 95 % (v/v) methanol was added and thoroughly agitated until all the precipitates were completely dissolved in the methanol. The absorbance was read at 538 nm using UV/visible spectrophotometer. The total terpenoid content was calculated by calibration curve of Linalool and the results were expressed as Linalool equivalent (mg/g).

Determination of Reducing Sugar: Reducing sugars present were estimated using the Dinitrosalicylic Acid (DNSA) method to 3 ml of the extract in test tubes 3 ml with water was added and this was followed by the addition of 3 ml of DNS reagent. The mixture was heated in a water bath for 5 min. 1 ml of 40 % Rochelle salt solution was added while the tubes' contents were warm. The test tube containing the test and blank was cooled, and the absorbance read at 510 nm. The concentration was calculated from a glucose standard curve.

Determination of Glycosides: A known volume (2.5) ml of 15 % lead acetate was added to 1 ml of the extract, and the mixture was shaken and filtered. 2.5 ml of chloroform was added to the filtrate and shaken vigorously. The lower layer was collected and evaporated to dryness. The residue was dissolved with 3 ml of glacial acetic acid and 0.1 ml of 5 % ferric chloride. 0 .25 ml of Concentrated H_2S0_4 was added, and the container was kept in the dark for 2 hours. Absorbance was measured at 530 nm.

Haemolysis Test: A 0.5 g extract was dissolved in 3 ml of distilled water and filtered.0.5 ml of animal blood was added to the filtrate in the test tube. Another 0.5ml of animal blood was added to 0.5 ml of normal sodium chloride Solution (0.9%) for control, allowing standing for10 min. The solution in the test tube containing extract and blood turned dull- red with no precipitate. At the same time, the tube containing normal sodium chloride and blood maintained the initial red color.

Test for Reducing Sugar: A quantity 0.2 g weight of the extract was dissolved in 5 ml distilled water, 5 ml of equal mixture of Fehling solutions A & B was added and boiled. A precipitate of brick-red color was formed.

Test for Carbohydrate:

Molish Test: A quantity of 0.5 g of the extract was dissolved in 3 ml of water and heated. Three drops of molish reagent was added, and small amount of concentrated sulphuric acid was carefully added from the side of the test tube to form a lower layer. A reddish-colored ring at the interfacial ring was observed.

Test for Sterols and Triterpenes: A 2 ml extract was taken in a test tube, few drops of acidic anhydride and concentrated sulphuric acid were added to the test tube slowly. The formation of a reddish brown ring was observed.

Test for Anthraquinone Glycoside: A 2 ml extract was taken in a test tube; 1 ml of ammonia was added and stirred. The appearance of reddish brown color in the aqueous layer and green color in the bottom was observed.

RESULTS AND DISCUSSION:

3.1 Phytochemical tests: Table 1 below shows the qualitative phytochemical test. **Table 2** below shows the quantitative phytochemical test.

TABLE 1: PHYTOCHEMICAL COMPOSITION OF *LEPTADENIA HASTATA* LEAVES

Phytochemicals	Methanol extract	Aqueous extract
Tannins	++	+++
Glycosides	++	++
Alkaloids	++	++
Flavonoids	+++	+++
Phenolic compounds	+++	+++
Terpenoids	++	++
HCN	+	+

Key: Percentage yield (%) Aqueous =14.570, Methanol = 7.510,'-' = Not present, '+' = Present in small concentration, '++'=Present in moderately high concentration,'+++' = Present in very high concentration.

TABLE 2: PHYTOCHEMICAL COMPOSITION OF *LEPTADENIA HASTATE* ROOTS

Phytochemicals	Methanol extract mg/g	Aqueous extract mg/g
Phenols	2120.43	1653.763
Tannins	122.6550	244.4196
Flavonoids	1558.025	1312.757
Steroids	0.37095	1.195496
Terpenoids	130.1412	121.4242
Saponins	0.068373	0.093089
Alkaloids	366.3289	139.8632
Glycosides	0.268563	0.231181

3.2 Preliminary Sensitivity Test Result: The preliminary sensitivity test result shows that the methanol extract of *Leptadenia hastate* leaves has activity against *E. coli, Pseudomonas aeruginosa, Staphylococcus aureus and Candida albicans* **Table 2.**

TABLE 3: PRELIMINARY SUSCEPTIBILITY OF THE ORGANISMS TO METHANOL AND AQUEOUS EXTRACTS OF *LEPTADENIA HASTATE* ROOTS

Organism	Methanol	Aqueous
E. coli	-	-
Pseudomonas aeruginosa		
Bacillus cereus	+	+
Staphylococcus aureus		
Candida albicans	-	+

Zones of inhibition for *P. aeruginosa* and *S. aureus* for both extracts are: negative. Kindly insert the negative sign (-),

TABLE 4: SUSCEPTIBILITY OF THE ORGANISMS TO THE AQUEOUS EXTRACTS OF LEPTADENIA HASTATE ROOTS

Test organisms	Zones of inhibition (mm) Concentrations of the extract (mg/ml)					Control	
	250	125	62.5	31.25	15.625	Positive control Ciprofloxacin	Negative control DMSO
Pseudomonas aerugenosa	0	0	0	0	0	30	0
Staphylococcus aureus	0	0	0	0	0	21	0
Escherichia coli	0	0	0	0	0	10	0
Bacillus cereus	22	0	0	0	0	31	0

TABLE 5: SUSCEPTIBILITY OF THE ORGANISMS TO THE METHANOL EXTRACTS OF *LEPTADENIA HASTATE* ROOTS

Test organisms	Zones of inhibition (mm) Concentrations of the extract (mg/ml)					Control	
	250	125	62.5	31.25	15.625	Positive control Ciprofloxacin	Negative control DMSO
Pseudomonas aerugenosa	0	0	0	0	0	30	0
Staphylococcus aureus	0	0	0	0	0	21	0
Escherichia coli	0	0	0	0	0	10	0
Bacillus cereus	23	22	19	18	19	30	0

TABLE 6: SUSCEPTIBILITY OF FUNGI TO THE METHANOL EXTRACTS OF *LEPTADENIA HASTATE* ROOTS

Test organisms	Zones of inhibition (mm) Concentrations of the extract (mg/ml)					Control	
	250	125	62.5	31.25	15.625	Positive control Ciprofloxacin	Negative control DMSO
Candida albicans	35	35	32	28	28	35	0

TABLE 7: SUSCEPTIBILITY OF FUNGI TO THE AQUEOUS EXTRACTS OF *LEPTADENIA HASTATE* ROOTS

Test organisms	Zones of inhibition (mm) Concentrations of the extract (mg/ml)					Control	
	250	125	62.5	31.25	15.625	Positive control Ciprofloxacin	Negative control DMSO
Candida albicans	0	0	0	0	0	35	0

3.3 Minimum Inhibitory Concentration: The minimum inhibitory concentrations (MIC) of methanol extract of *Leptadenia hastate* leaves against the test organisms is shown in the table below.

TABLE 8: MINIMUM INHIBITORY CONCENTRATIONS OF THE METHANOL EXTRACT

Test organisms	Minimum inhibitory concentration (mg/ml)
Candida albicans	125
Bacillus cereus	125

DISCUSSION: The phytochemical analysis demonstrated the presence of important photochemical and also revealed that the methanol and aqueous contain a variety of constituents ranging from glycoside to saponins, carbohydrates, reducing sugar, alkaloids, and flavonoids. These are well known to have antimicrobial activity, which will help explain why the extract had antimicrobial activity against some of the isolates tested.

However, the quantitative assay revealed that methanol extract has a high content of phenol, glycosides, alkaloids, flavonoids, and terpenoids, and this may be responsible for the high activity observed with methanol extract when compared to aqueous extract.

This also implies that phytochemicals are more soluble in organic solvents than in aqueous. The susceptibility test shows that the methanol extract at various concentrations had inhibitory effect against *Bacillus cereus* and *Candida albicans.* The inability of aqueous extract to inhibit most of the organisms tested may be due to environmental factors or the inability of the aqueous solvent to extract enough active constituents to inhibit microbial growth. Methanol extract of *Leptadenia hastate* was preferred as it has both antibacterial and antifungal activity. This knowledge can be exploited in the development of a novel drug with antibacterial and antifungal activity upon isolation and purification of active compounds.

The minimum inhibitory concentrations of methanol extract of *Leptadenia hastate* against *Bacillus cereus* and *Candida albicans* are 125 mg/ml

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