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Assesment Of Antibacterial Activities Crude Leaf Extracts Of Selected Medicinal Plants From Ezza North Ebonyi State Nigeria Against Staphylococcus Aureus, Klebsiella Pneumoniae, Pseudomonas Aeruginosa. Escherichia Coli, And Streptococcus Mutans.

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Abstract
***Aim** :Medicinal plants have been used for ages as remedies for human diseases because they contain bioactive components of therapeutic value .This work was undertaken to investigate the in vitro antibacterial efficacy of six herbal extracts namely : Alstonia boonie,Morinda lucida, Parkia biglobosa,Olax subscopedia ,Anthocleista djolensis and Cusoniaspicataon six medicinally important bacterial strains (Klesbsiella spp, ,Staphylococcus, Streptococcus mutants, Pseudomonas aeruginosa,Salmonella spp, and E.coli) .*
***Method**: Agar well diffusion method, with varying concentrations of each extract (100,50,25, and 12.5 mg/ml) were used.*
***Results** : Alstonia boonei,showed no inhibition,Morindalucida showed maximum zone of inhibition against Klebsiella at 100mg/ml and 50mg/ml only, Parkia biglobosa exhibited inhibition from 25 mg/ml and maximum inhibitionwas recorded at 100mg/ml against E. coli,50 mg/ml against Staphylococcus whereas Cussoniaspicata showed maximum inhibition against E. coli and Pseudomonas at 100mg/ml only and no inhibition was observed against other isolates.Based on the present evaluationthis work suggest thatMorinda lucida,Parkia biglobosa,,Anthocleista adjolensis,and Cusonia spicata has inhibitory effects against some of the tested organisms and can be used in management of diseases caused by them while Alstonia boonie, and Olax may not be used.*
***Conclusion**: However, it may be inferred that these plants can be used as therapeutic natural agents that may serve as lead for development of new pharmaceuticals addressing the major therapeutic needs.*
***Keywords**: Pharmaceuticals, medicinal, therapeutic, plants, microorganisms, anti-biotics, agar well diffusion*

I. INTRODUCTION

Plants areused by the indigenous people in differentparts of Nigeria for treatment ofinfectious diseases such as cholera,diarrhoea, dysentery and othergastrointestinal disorders [1]. The use of plant extracts and phyto-chemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments [2]. In the last few years, a number of studies have been conducted in different countries to prove such efficacy. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of plants [3].Among the families of onpthalmological preparation for the treatment of human illnesses such as malaria, gatro-intestinal disorders, fever, dizziness, secondary sterility, diarrhoea, jaundice, nose bleeding and pain [4].

Alstonia boonei is a very large, deciduous, tropical-forest tree belonging to the dogbane family, Apocynaceae. It is native to tropical West Africa, with a range extending into Ethiopia and Tanzania[5]. Its common name in the English timber trade is *cheese wood, pattern wood* or *stool wood* while its common name in the timber trade is "emien" [6].

Morinda lucida is a very good plant in traditional medicine in west Africa and various studies have confirmed it therapeutic effectiveness of several uses[7]. In addition to anthraquinones, tannins, and flavonoids have been isolated from it[ ].Extracts of the plant have shown anti-inflammatory, febrifugal and pain-reducing activity, as well as promoting gastric emptying and intestinal motility**[8**]. Leaf extracts have shown in vitro antimalarial activity against *Plasmodiumfalciparum,* while in several other tests antidiabetic properties have been reported[9].*Olax subscorpioidea* is a shrub or tree that belongs to the family of Olacaceae widely used as a medicinal plant inWest African countries [10]. It is a highly valuedmedicinal plant in the traditional management of cancer, sexually transmitted diseases, pain-killers, asthma, mental illnesses [11]. The antimicrobial activities of ethanol extract of the roots of *O.subscorpioidea* has been reported on some selected microorganisms[12].

Anthocleista djalonensis is a tree growing up to 15 metres tall. The cylindrical bole is unbuttressed, it can be up to 40cm in diameter. The twigs sometimes have 2 erect spines or small cushions above the leaf axils[13]. This tree is one of several species in the genus that are much used in traditional medicine and for similar medicinal purposes [14]. They may all be used as substitutes for each other. The tree is commonly harvested from the wild[15]. *Anthocleista djalonensis* is widely used throughout its distribution area as a strong purgative and diuretic [16].The root is commonly taken to treat intestinal problems, including constipation, to regulate menstruation and as an abortifacient[17].

Cussonia spicata are traditionally used for treatment of indigestion [18]. The mashed roots, which are succulent and edible, have been used for the treatment of Malaria and as a food source in times of need [19]. A root decoction is used to treat fever, venereal disease, as a diuretic and laxative and to treat mental illness [20].

II. MATERIALS AND METHODS

Materials:

Media - Media used include: Nutrient agar (Fluka, Chemie India), Mueller Hinton agar and Nutrient agar (Oxoid, Uk), MacConkey agar (Fluka, Chemie, India), Simmons Citrate agar (BIOTECH Laboratories Ltd, Uk). Broth media used include peptone water, nutrient broth and Mueller – Hinton broth, Tryptone soya broth (Oxoid, Uk). all culture media broth media were prepared in line with the manufacturers' instructions.

Equipment

Equipment used includes hot air oven (Genlab, UK, model No: MIN0/50), refrigerator (samsung, China, model No: GC-051SA), incubator (Merck, Germany, MINI/50), Bunsen burner (SEDI, Akuke, Nigeria), microscope (Olympus, Germany), autoclave (Medica Instrument MFG. Co).

Instruments

Instrument used include: wire gauze, tripod stand, inoculating loop, test tube rack, spatula, laboratory forceps, meter rule, electronic weighing balance (Scout Pro, China, model No: SPU401), conical flask, beaker, Petri dish, stirring rods, filter papers, glass slides, aluminum foil, injection syringes, micropipette tips, INTEC micropipette (10-100µl), cork borer (6mm), sucker, mortar and pestle, Whattmann Number (1) filter paper.

Chemicals Used

Chemicals used include: methanol, distilled water, normal saline, immersion oil, Lugol's iodine, ethanol, safranin, crystal violet, Hydrogen peroxide, peptone pellets, tetramethyl-p-phenylenediamine, sodium chloride, dimethylsulphoxide (DMSO), kovac's reagent (Fisher Scientific Company, USA).

Plants Materials

The plant materials used in the present study are*Alstonia boonie,Morinda lucida*, *Parkia biglobosa,Olax subscopedia* ,*Anthocleista adjolensis* and *Cusoniaspicata***.**The leaves of selected plants were collected from Ezza North in Ebonyi State Nigeria .It was identified by a taxonomist Professor Okafor J. of the department of Biology Ebonyi State University Abakaliki Nigeria where specimen has been deposited at the herbarium for future reference.The sample were cleaned, air driedfor two weeks, and ground to powder using mechanical grinder (corona machine model E 46) and sieved with 2mm size sieve .

Methods

Extraction of the plant material

The powdered plant (150 g) was macerated with 750 ml 70 % methanol for four days after which , it was filtered and evaporated to dryness on water bath to a reddish –brown residue, the residue was stored in an air tight container until required for further use.

Test Organisms

Five antibiotic – resistant bacteria organisms used are *Staphylococcus aureus*, *Klebsiella pneumoniae, Pseudomonas aeruginosa*, *Escherichia coli*, and *Streptococcus mutans.* These test organisms were collected from microbiology laboratory unit of Federal Teaching Hospital Abakaliki (FETHA) Nigeria.

Purification and Re-Identification of Bacterial Isolates

All the bacterial isolates (*E. coli*, *Klebsiella pneumonia,Staphylococcus aureus,Streptococcus mutans Salmonella* spp and *Pseudomonas aeruginosa*.) were purified on nutrient agar and MacConkey's agar plates. Gram staining and conventional biochemical test such as Indole test, Oxidase test, Coagulase test, Catalase test, Voges Proskauer (VP) test and sugar fermentation test. Identified and purified test organisms were stored in the refrigerator in a nutrient agar slant [20].

Preparation of 0.5 McFarland Turbidity Standard

Turbidity standard equivalent of 0.5 McFarland Standard was prepared by adding 1 ml of concentrated H_2SO_4 to 99 ml of distilled water, and dissolving 0.5 g of dehydrated barium chloride ($BaCl_2.2H_20$) in 50 ml of distilled water in separate reaction flasks respectively. Barium chloride solution (0.6 ml) was added to 99.4 ml of the H_2SO_4 solution in a separate test tube, and was mixed well to obtain 0.5 McFarland turbidity standard. Small portion of the turbid solution was transferred to a capped test tube stored at room temperature (28^{o}C). This was used to adjust and to compare the turbidity of the test bacteria in order to get a confluent growth on a growth or culture plate [21]when performing Antimicrobial Susceptibility Testing (AST).

Preparation of Nutrient Broth

Nutrient broth was prepared by dissolving 1.3 g of nutrient broth powder (Oxoid, Uk) in 100 ml of distilled water according to the manufacturer's instructions. Then, 5 ml each of the nutrient broth was dispensed aseptically into capped test tubes which were sterilized by autoclaving at 121^{o}C for 15mins at 15psi. [22].

Preparation of Nutrient Agar, Mueller-Hinton And Macconkey Agar Plates

Agar plates were prepared by dissolving 2.8g, 3.8g and 5.2g respectively of nutrient agar, Mueller-Hinton and MacConkey agar in 100 ml of distilled water each according to the manufacturer's instructions. This was heated over a Bunsen burner flame to dissolve and twenty milliliters each was dispensed aseptically into McCartney bottles and was sterilized by autoclaving at 121^{o}C for 15mins at 15psi.

Standardization of Test Bacteria

All test bacteria were standardized individually before use by inoculating a 5 ml normal saline in sterile test tubes with loopful of a 24hr young culture of the test organism from a nutrient agar slant. Afterwards, dilutions using loopful of the test bacterium and sterile water were carried out in order to get microbial population of 10^5 colony forming unit per milliliter (CFU/ml) by comparing it with 0.5 McFarland turbidity standards [23].

Screening for Antimicrobial Activity herbal Extracts Using Agar Well Diffusion Method

Twenty milliliter each of sterilized molten Muller Hinton agar was poured aseptically into sterile Petri dishes of equal sizes (20ml) and then allowed to solidify (gel). The surface of the Mueller Hinton agar plates were then streaked with standardized inoculums of the test bacteria that was adjusted to 0.5 McFarland turbidity standards. Thereafter, a sterilized 6 mm cork borer was used to bore 5 holes on the Mueller Hinton agar plate(s), and 4 of the holes were filled with equal volumes of the respective plant extracts that was diluted with 90 % DMSO [24]. Sterilized distilled water was used as the negative control. The plates were allowed for about 30mins for pre-diffusion of the plant extracts, and these were incubated at 37^{o}C for 24hrs. After incubation, the inhibition zone diameters were measured in millimeter using a meter. The inhibition zone diameter (IZD) of each plant extracts were evaluated by subtracting the size of the cork borer from the IZD measured[25].

Determination of Minimum Inhibitory Concentrations (Mic) of the Plant Extracts Against Test Organisms Using Agar Well Diffusion Metheod

Varying concentration of each extract (100 mg/ml, 50 mg/ml, 25mg/ml, 12.5mg/ml), were prepared. The surface of the Mueller Hinton agar plates were then streaked with standardized inoculums of the test bacteria that was adjusted to 0.5 McFarland turbidity standards. Thereafter, a sterilized 6 mm cork borer was used to bore 5 holes on the Mueller Hinton agar plate(s), and 4 of the holes were filled with equal volumes of the respective plant extracts and a positive control (Ciprofloxacin 5µg) was placed on the surface of the Mueller Hinton Agar and a hole was bored and was filled with sterile water as a negative control. The inoculated plates were incubated for 18 – 24 hr using incubator. After 18 – 24 hr incubation, the plates with clear inhibition around a bored hole with the lowest concentration was considered to be the minimum inhibitory concentration [26].

III. RESULTS

Table 1 : Antimicrobial activity of methanol extract of leaves of *Morinda lucida*

Microorganisms	100Mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml
Escherichia coli	No inhibition	No inhibition	No inhibition	No inhibition
Pseudomonas aeruginosa.	11 mm	No inhibition	No inhibition	No inhibition
Staphylococcus aureus	No inhibition	No inhibition	No inhibition	No inhibition
Klebsiella pneumoniae	23mm	16 mm	No inhibition	No inhibition
Salmonella	No inhibition	No inhibition	No inhibition	No inhibition

Table 2 : Antimicrobial activity of methanol extract of leaves of *Parkia biglobosa*

Microorganisms	100Mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml
Escherichia coli	16mm	**13 mm**	**14 mm**	No inhibition
Pseudomonas aeruginosa.	13 mm	10 mm	13 mm	No inhibition
Staphylococcus aureus	19 mm	14 mm	13 mm	No inhibition
Klebsiella pneumoniae	18 mm	14 mm	12 mm	14 mm
Salmonella typhi	15 mm	15 mm	13 mm	No inhibition

Table 3: Antimicrobial activity of methanol extract of leaves of *Annthocleista adjolensis*

Microorganisms	100Mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml
Escherichia coli	14 mm	No inhibition	No inhibition	No inhibition
Pseudomonas aeruginosa.	No inhibition	No inhibition	No inhibition	No inhibition
Staphylococcus aureus	No inhibition	15 mm	No inhibition	No inhibition
Klebsiella pneumonia	No inhibition	No inhibition	No inhibition	No inhibition
Salmonella typhi	No inhibition	No inhibition	No inhibition	No inhibition

Table 4: Antimicrobial activity of methanol extract of leaves of *Cussonia spicata*

Microorganisms	100Mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml
Escherichia coli	12 mm	No inhibition	No inhibition	No inhibition
Pseudomonas aeruginosa.	12 mm	No inhibition	No inhibition	No inhibition
Staphylococcus aureus	No inhibition	No inhibition	No inhibition	No inhibition
Klebsiella pneumonia	No inhibition	No inhibition	No inhibition	No inhibition
Salmonella typhi	No inhibition	No inhibition	No inhibition	No inhibition

Table 5: Antimicrobial activity of methanol extract of leaves of *Alstonia boonie*

Microorganisms	100mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml
Escherichia coli	No inhibition	No inhibition	No inhibition	No inhibition
Pseudomonas aeruginosa.	No inhibition	No inhibition	No inhibition	No inhibition
Staphylococcus aureus	No inhibition	No inhibition	No inhibition	No inhibition
Klebsiella pneumonia	No inhibition	No inhibition	No inhibition	No inhibition
Salmonella typhi	No inhibition	No inhibition	No inhibition	No inhibition

Table 6: Antimicrobial activity of methanol extract of leaves of *olax subscopedia*

Microorganisms	100 Mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml
Escherichia coli	No inhibition	No inhibition	No inhibition	No inhibition
Pseudomonas aeruginosa.	No inhibition	No inhibition	No inhibition	No inhibition
Staphylococcus aureus	No inhibition	No inhibition	No inhibition	No inhibition
Klebsiella pneumonia	No inhibition	No inhibition	No inhibition	No inhibition
Salmonella typhi	No inhibition	No inhibition	No inhibition	No inhibition

IV. DISCUSSION

The antimicrobial activity of plants can be detected observing the growth response of various microorganisms to the plant tissues and extracts that areplaced incontact with them[27]. The choice of organisms to be used would depend on the purpose of the investigation.*Morinda lucida* extract was active against *Pseudomonasaeruginosa* as revealed by the zones of inhibition 11mm,23mm and 16 mmagainst *Klebsiella mutant*, respectively[Table 1].This can be explained by the differences in the cell wall permeability of the organisms to antimicrobial agents.This is in line with the work of [28],which reported that the extract of *piperguineense* had significantly inhibited the growth of bacteria*Salmonella typhi*,*Staphylococcusaureus*.Report have shown that a similar plant species *Gongronemalatifolium* containing saponins,flavonoids,tannins and anthraquinones was found to have very potent antibacterial activities of different plant species [29].Flavonoids have been reported to be synthesized by plants in response to microbial infections and are good antibacterial agents,tannins have been demonstrated to have antibacterial activities .However,*E.coli*,*Pseudomonas* ,*Staphylococcus*,*Klebsiella* and *Salmonella* showed susceptibility to the extract of *Parkiabiglobosa*[Table 2] .Maximum inhibition (19 mm) against *staphylococcus* while low inhibition 12 mm against *Klebsiella* at 25 mg/ml were recorded.The cell wall of gram negative organisms makes themless permeable to antimicrobials, because of its high lipid content and that the extract was inactive on the other bacterial species probably because of innate resistance [30].

Furthermore, *Anthocleistaadjalonensis* showed positive activity (14 mm) against *E.coli* at 100 mg/ml,*staphylococcus* 15 mm at 50 mg/ml [Table 3],while *Cussoniaspicata* 12 mmagainst *E.coli* at 100 mg/ml ,12 mm against *Pseudomonasaeruginosa*[Table 4].This is in line with the work of [31], that showed antibacterial efficacies of herbal extracts concentrations under study .*Olaxsubscorpedia* and *Alstoniaboonie* were inactive against the organisms under study at all concentrations and showed negative effects on anti-bacterial activity in all the microorganisms tested [Table 5 and 6].This investigation indicated that*Olax subscorpedia* and *Alstonia boonie*extracts did not show antibacterial activity which similar to the recent study of [32] ,which suggested that the methanolic *Bliagh euginata*extract has not shown antifungal activity. However, negative results do not mean absence of bioactive constituents nor is the plant inactive. Crude plant extracts are generally a mixture of active and non active compounds .Same observations have been reported earlier by various authors [33]. Active compounds present in insufficient quantities in the crude extracts to show activity with the dose levels employed . Lack of activities can thus be proven by using large doses [34]. With no antibacterial activities extract may be active against other bacteria species which are not tested .Our previous study had shownthat *Anthocleista adjalonensis*extract contain medicallyuseful phytochemicals such as alkaloids saponins and steroids[34]. These substances could be extracted for food industries or health products as medicinal food,pharmaceutical exploits, biotechnology and general medicine.

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