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EVALUATION OF ANTIMICROBIAL PROPERTIES OF N-HEXANE EXTRACT OF THE LEAVES OF *NAPOLEONEAE IMPERIALIS* FAMILY LECYTHIACEAE

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ABSTRACT

Napoleonaea imperialis is used to treat wounds in most parts of Nigeria. Against this background, N-hexane extract of the leaves were screened against some microorganisms to ascertain this claim and to recommend it for further investigation for possible inclusion into official compendium. The plant leaves were dried, powdered and extracted by cold maceration with N-hexane for 24hours. Phytochemical screening was done for alkaloids, saponin, steroidal nucleus, simple sugar, starch, glycoside, proteins and flavonoid using standard procedures. Antimicrobial screenings were carried out using agar diffusion technique. Antibacterial activity was conducted by screening against six pathogens comprising both Gram positive (*B. subtilis and S. aureus*) and Gram negative bacteria (*P. aeruginosa, Klebsiella, E. coli and S. typhi*) obtained from pharmaceutical Microbiology laboratory stock. The extract was screened against 24hour broth culture of bacteria seeded in the nutrient agar at concentrations 400, 200, 100, 50, 25, 12.5, 6.25and 3.125 mg/ml in DMSO and incubated at 37^0C, for 24 hours and measuring the inhibition zone diameter - IZD. The positive controls were ampicillin 20µg/ml for bacteria. The organisms were very sensitive to ampicillin. DMSO was used as negative control. The phytochemical screening showed the presence of alkaloids, saponins, tannins, glycosides and proteins whereas flavonoids, resins and steroids were absent. N-hexane extract exhibited activity against all the test bacteria at 400mg/ml and 200mg/ml. The N-hexane extract exhibited varying activity as the concentration is reduced, to *Staphylococcus aureus, E. coli, B. subtilis, P. saeruginosa, Kleb pneumonia, Salmonella typhi* with minimal inhibitory concentration of 50, 25, 200, 50, 400 and 200 mg/ml of N-hexane extract respectively. The extract demonstrated activities against certain bacteria confirming the use of the plant in ethno pharmacology and since the root extract are more often used, it is yet to be confirmed if it has more activity than the leaves against the test organisms. Taking the least inhibition zone diameter (IZD) of the standard (Ampicillin) as the breaking point, most of the extracts passed the breaking point.

INTRODUCTION: Medicinal plants are plants that can be put into culinary or medicinal use. In the past, substances with particular medicinal action are extracted from plants for use as drugs. Every culture relied on a vast variety of natural products in healing plants for their medicinal properties. The importance of plant in the present day method of treatment cannot be over emphasized. The use of herbs and medicinal plants as first medicines or remedies is assuming universal phenomenon. In developing countries, thousands of rural communities still depend mainly on folklore medicine to cure diseases.

Napoleonaea imperialis is a tree or shrub to 6 m high, low-branching, dense crown, in the understorey of the closed-forest in Nigeria and extending to Congo and Angola. The flowers are variable, usually cream at the circumference with red at the centre, but varying to apricot. In Nigeria, *Napoleonaea imperialis* have various vernacular names. It is called *ukpakonrisa* in Edo, *obu- anagbo* in Igala, *isi efe, (uburubu) akp'oiziga, akpodo* and *ukpodu* in various Igbo dialects [1, 2].

Napoleonaea imperialis is a Nigerian folklore medicinal plant. It has been reported to have wound healing properties [3, 4]. Today, there is need to study plants to properly establish those whose efficacy have been a claim. In this study, focus was on the anti-microbial activity of N-hexane extract of *Napoleonaea imperialis Ritchiea* leaves using Agar diffusion technique.

MATERIALS AND METHODS:

Chemicals and Solvent: The chemicals used for extraction processes include methanol, N-hexane, dimethyl sulphoxide (DMSO), Nutrient Agar and Sabourand dextrose agar. The reagents used were – concentrated sulfuric acid, naphthol solution in ethanol (Molisch reagents) picric acid, ammonium solution, nitric acid, Aluminum chloride solution, Fehling solution A and B, Wagner's reagents (iodine and potassium iodide), Hager's reagent (saturated solution of picric acid).

Sources of Microorganisms: The microorganisms used were bacteria obtained from laboratory stock of the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University Awka. The organisms include bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosae*, *Klebsiella* Species*, Escherichia coli, Bacillus subtilis* and *Salmonella typhi*

Equipment: Weighing Balance [Scout pro u401 made in China], Beakers, measuring cylinder, test tubes**,** incubators (Gent Lab UK), autoclave, test tubes, test tube racks, syringes and needle, Pasteur's pipette, conical flask, glass rod, inoculation loop, Tripod stand, filter paper (Whatman No. 1), Mortar and pestle , water bath, muslin- cloth, reagent bottles, Bunsen burner, and permanent marker.

Source and Identification of Plant Materials: The fresh leaves of *Napoleonaea imperialis* were obtained from Ogidi in Idemili North local Government Area, Anambra state in July 2011. The plant was identified by Dr C.O. Ezugwu of the Department of Pharmacognosy and Traditional Medicine, Nnamdi Azikiwe University. The stalk and other impurities were removed from the leaves. The leaves were air dried in the Pharmacognosy Laboratory and then were pulverized to produce 450g of powdered plant leaf.

Extraction Process: Extraction was done by macerating the 450g of the powdered drug with 900ml of n-hexane solution for 48hrs. At the end it was strained using white muslin cloth and then filtered using Whatman No. 1 filter paper. The filtrate was concentrated using rotary evaporator.

Phytochemical Screening of the Plant: Standard screening tests were carried out on both powdered leave for various phytochemical constituents [5, 6, 7].

Test for Protein:

- **Xanthoproteic reaction test**: 5 ml volume of the filtrate obtained from boiling few grams of powdered plant is heated with few drops of concentrated nitric acid; yellow colour that changes to orange on addition of alkali indicates the presence of protein.

Test for Carbohydrates: 0.1g of the powdered leave was boiled with 2mL of distilled water and was filtered .To the filtrate, few drops of naphthol solution in ethanol (Molisch reagent) were added. Concentrated sulphuric acid was then poured gently down the test tube to form a lower layer. A purple interfacial ring indicates the presence of carbohydrate (starch).

Test for Alkaloids: About 5 g of powdered leave placed in the test tube and 20ml methanol added to the tube, the mixture was heated in water bath and allowed to boil for two minutes .It was cooled and filtered. 5ml of the filtrate was tested with two drops Wagner's reagent (solution of iodine and potassium iodide).

To another 5mL portion of the extract 2 drops of Hager's reagent (saturated picric acid solution) was added. The presence of precipitate indicates alkaloid.

Test for Steroids: About 9ml of ethanol was added to 1g of the extracts and refluxed for a few minutes and filtered. The filtrate was concentrated to 2.5ml on a boiling water bath. 5ml of hot distilled water was added to the concentrated solution. The mixture was allowed to stand for 1 hour and the waxy matter was filtered. The filtrate was extracted with 2.5ml of the chloroform using separating funnel. To 0.5ml of the chloroform extract in a test tube, 1ml of concentrated sulfuric acid was added to form a lower layer. A reddish brown interface shows the presence of steroids.

Tests for Saponins: About 20ml of water was added to 0.25g of crude extract and boiled gently in a hot water bath for 20 minutes. The mixture was filtered hot and allowed to cool and the filtrate was used for the following tests.

i. Frothing test: 5ml of filtrate was diluted with 20ml of water and vigorously shaken. The test tube was observed for the presence of stable foam upon standing.

ii. Emulsion test: To the frothing solution, 2 drops of olive oil was added and the content shaken vigorously and observed for the formation of emulsion.

Test for Flavonoids: About 10ml of ethyl acetate was added to 0.2g of the (crude extract) extract and heated on a water bath for 3 minutes. The mixture was cooled, filtered and used for the following test.

- **Ammonium test**: 4ml of filtrate was shaken with 1ml of dilute ammonium solution. The yellow colour in the ammoniacal layer indicates the presence of the flavonoids.

Antimicrobial Assay:

- **Microorganisms:** 24hour Cultures of seven human pathogenic bacteria made up of both gram positive (*S. aureus,* and *B.subtilis*) and gram negative (*P. aeruginosa, Klebsiella Spp, E. coli* and *S. typhi*) bacteria were used for the *in-vitro* antibacterial assay. All microorganisms were obtained from the laboratory stock of the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences Nnamdi Azikiwe University Awka.

- **Preparation of media:** Nutrient broth, nutrient agar, sabouraud dextrose agar (SDA) was used in the assays. Dimethylsulphoxide (DMSO) was used in solublising the extracts and drugs and as a negative control in the study. The media were prepared by dispersing the weighed amount in water and then were sterilizing them with autoclave. The plates of nutrient agar were poured and allowed to solidify after the appropriate organisms were seeded.

- **Antimicrobial agents:** Ampicillin, 20ug/ml (Mecure Industrial Ltd Lagos Nigeria.); was included in the study as standard reference drug.

- **Antimicrobial activity determination:** An overnight broth culture used to obtain 0.5 Marcfarland standard of bacterium was used to seed sterile molten nutrient agar medium maintained at 45°C. Six holes (6mm) respectively, were bored in each of the plates (9cm, diameter) with an aseptic cork borer, when seeded plates had solidified; 200mg/ml,100mg/ml,50mg/ml,25mg/ml12.5mg/ml and 6.25mg/ml of extract were prepared in dimethylsulphoxide (DMSO) by preparing a stock solution and carrying out double fold dilutions on it and with the aid of a Syringe, the wells were filled with 0.25 ml (5drops) of different dilutions of the extract while the centre wells were filled with 20µg/ml and 1 mg/ml of ampicillin for bacteria. (Also dissolved in DMSO). Diameters of zones of inhibition were determined after incubating plates at 37°C for 24h for the bacteria. This test was conducted on the extract and the solvent dimethylsulphoxide was used as negative control while ampicillin was used as positive control.

RESULTS:

TABLE 1: PHYTOCHEMICAL SCREENING

2° Metabolites (Plant Leaves)	Tests/ Observations	Inference
Proteins	Xanthoproteic reaction test (no orange coloration)	+
Alkaloids	Wagner and Hagger test (precipitate formation)	++
Flavonoids	Ammonium test (formation of yellow coloration)	---
Glycosides	Picric acid test (brick-red coloration)	+
Steroids	Sulfuric acid test (reddish brown interface formation)	---
Resins	Precipitation and Colour Test (negative)	--
Saponins	Frothing and Emulsion tests (formation of froth & emulsion)	++

- = not detectable; + = low concentration; ++= medium concentration; +++ = High concentration

The Results of Antimicrobial Screening: Antibacterial Activity of Ethyl acetate extract.

TABLE 2: ANTIBACTERIAL ACTIVITY OF EXTRACT

N- hexane Fraction	Inhibition Zone Diameter For Bacteria in Different Concentrations of Extracts (mm)								
Bacteria Used	400	200	100	50	25	12.5	6.25	3.125 [mg/ml]	Am (20µg)
S. aureus	9	6	4	2	+	+	+	+	6.0
P. aeruginosa	8	6	4	2	+	+	+	+	9.0
Klebsiella	3	+	+	+	+	+	+	+	16.0
E. coli	11	8	6	4	2	+	+	+	6.0
B. subtilis	4	2	+	+	+	+	+	+	5.0
S. typhi	4	2	+	+	+	+	+	+	6.0

Key: + means presence of antimicrobial activity, - means absence of antimicrobial activity

DISCUSSION, CONCLUSION AND RECOMMENDATION: The phytochemical screening showed presence of alkaloid, saponins, tannins, glycosides and proteins. Flavonoids, resins and steroid were absent. The extracts displayed various activities against bacteria inhibiting it at various concentrations ranging from 400 to 50 mg/ml. At 400mg/ml concentration, the extract is effective against *Staphylococcus aureus*, *E. coli, B. subtilis, Pseudomonas aeruginosa, Klebsiella pneumonia* and *Salmonella typhi* with inhibition zone diameter IZD of 9mm, 11mm, 3mm, 8mm, 3mm, and 4mm respectively.

At 200mg/ml, the extract is effective against *S. aureus, E. coli, Bacillus subtilis, Pseudomonas aeruginosa and Salmonella typhi* with IZD of 6mm, 8mm, 2mm, 6mm, and 2mm respectively. At 100mg/ml, *S. aureus, E. coli, Pseudomonas aeruginosa,* were effective with IZD of 4mm, 6mm and 4mm. Also at 50mg/ml the extract showed activity against *S. aureus, E. coli and Pseudomonas aeruginosa* with IZD of 2mm, 4mm and 2mm at 25mg/ml the extract is effective against *E. coli*. The DMSO used did not show any activity against the bacteria used.

The extract is effective against *S. aureus, E. coli, B. subtilis, Pseudomonas aeruginosa, K. pneumonia* and *S. typhi* with minimal inhibitory concentration of about 50mg/ml for *S. aureus* and *P. aeruginosa*, 25mg/ml for *E. coli*; 200mg/ml for *B. subtilis* and *S. typhi* and 400mg/ml for *K. pneumonia*. When compared with a standard antibiotic – ampicillin at 20microgram/ml the extract is effective. The extract demonstrated activities against certain bacteria confirming the use of the plant in ethno pharmacology. More work should be done to ascertain the active principles responsible for this action.

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