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Original Research Paper

ANTIMICROBIAL ACTIVITY OF METHYLENE CHLORIDE/METHANOLIC EXTRACT (50:50) OF THE LEAVES OF *RITCHIEA LONGIPEDICELLATA* FAMILY CAPPARIDACEAE

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ABSTRACT

Ritchiea longipedicellata had been reported to exhibit antimicrobial properties. This study is to determine the antimicrobial activities of Ritchiea longipedicellata leaves against microorganisms and to serve as criteria to recommend the ethno pharmacological uses of the plant. The plant leaves were dried, powdered and extracted by cold maceration with 1:1 mixture of methylene chloride and methanol for 24hours. Phytochemical screening was done for alkaloids, saponin, essential oil, phenolic group, steroidal nucleus, simple sugar, starch, cyanogenic glycoside, proteins and flavonoid using standard procedures. Antimicrobial screenings were done using agar diffusion technique. Antibacterial activity test was conducted by screening against seven pathogens comprising both Gram positive and Gram negative bacteria obtained from pharmaceutical Microbiology laboratory stock. The extracts were screened against 24hour broth culture of bacteria seeded in the nutrient agar at concentrations 200, 100, 50, 25, 12.5 and 6.25 mg/ml in DMSO and incubated at 37ċ, for 24 hours and measuring the inhibition zone diameter - IZD. The same was done for antifungal; however, fungi were seeded into a sabouraud dextrose agar and incubated for 72 hours at 25ċ. *Aspergillus niger* and *Candida albican* were used. The positive controls were ampicillin 20µg/ml and clotrimazole cream 1mg/ml for bacteria and fungi respectively. DMSO was used as negative control. The results of phytochemical screening showed moderate availability of alkaloid, simple sugar and abundance of flavonoid, steroidal nucleus, essential oil, phenolic group, cyanogenic glycoside; absence of starch and protein and doubtful quantity of saponin. The extracts displayed various activities against bacteria inhibiting it at various concentrations ranging from 200 to 6.25 mg/ml. The Crude extract (200 mg/ml) had IZD of 5.0, 12.0, 4.0, 4.0, 7.0, and 5.0 mm against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis, Sarcina lutea, and Salmonella typhi* respectively. The Crude inhibited most appreciably *Pseudomonas aeruginosa* at all the

concentration with IZD ≥6mm, however no activity against *Klebsiella* was recorded. The extract demonstrated activities against certain bacteria and fungi confirming the use of the plant in ethno pharmacology. Taking the least IZD of the standard (Ampicillin) as the breaking point, the extracts passed the breaking point.

Keywords: *Ritchiea longipedicellata*, Antimicrobial screening, Breaking point and activity.

INTRODUCTION

Over the past decade herbal medicine has become a topic of global importance, making an impact on both world health and international trade. Medicinal plants continue to play central roles in the healthcare system of large proportion of the world's population. This is particularly true in the developing countries, where herbal medicine has a long and uninterrupted history of use. Recognition and development of medicinal and economic benefits of these plants are on the increase in both developing and industrialized nations (Srinivas *et al.,* 2007). Continuous usage of herbal medicine by a large proportion of the population in the developing countries is largely due to the high cost of western pharmaceuticals, health care, adverse effects that follow their use (in some case) and the cultural and spiritual point of view of the people of the countries (Srinivas *et al.)*.

In Western developed countries however, after a downturn in the pace herbal use in recent decades, the pace is again quickening as scientists realize that the effective life span of any antibiotic is limited (Satyejji *et al., 2007)*. Worldwide spending on finding new anti-infective agents (including vaccines) was expected to increase 60% from the spending levels in 1993. New sources, especially plant sources, are also being investigated. Secondly, the public is becoming increasingly aware of problems with the over - prescription and misuse of traditional antibiotics. In addition, many people are interested in having more autonomy over their medical care. All these makes the knowledge of chemical, biological and therapeutic activities of medicinal plants used as folklore medicine become necessary. (Fagbohun *et al.,* 2010).

Traditional medicine use in Nigeria is as old as the people; and has remained relevant among every other types of therapy. Presently, WHO has defined traditional medicine as comprising therapeutic practices that have been in existence often for hundreds of years before the development of and spread of modern scientific medicine and are still in use today (Sofowora in Evans). The practice of traditional medicine has been noted by WHO in 1991 to vary widely in keeping with the social and cultural heritage of different countries in Africa .The variation is extended to the various regions and group in the countries.

In the practice of traditional medicine in Africa much emphasis is placed on supernatural forces so that practitioners are consulted not only for sicknesses but also when any misfortune occur in the families since many of the evil omen are ascribed to supernatural forces (Sofowora in Trease and Evans text). The medications are intended for both internal and external use but none for intravenous administration (besides those applied with scarification). This practice involves several techniques which are mainly diagnosis and treatment. Clearly, it is evident that almost all traditional practice all over the globe indicated herb as an important aspect in the treatment of disease. The importance of plant in the present day method of treatment cannot be over emphasized In developing countries; thousands of rural communities still depend mainly on folklore medicine to cure diseases (Fagbohun *et al.,* 2010). No surprise that as at today plant still forms one of the major sources of medicines used in clinics, generating about 50% medicinal compounds used by pharmaceutical industry, 25% of prescription drugs are derived from tropical plants three quarter of which from folkloric medicines (Inamul, 2004). Such drugs are *Digitalis* used as important drugs for the management of heart failure from *Digitalis purpurea,* Quinine used for treatment of cerebral malaria from *Cinchona* bark

etc. Undoubtedly, a lot of medicine have been isolated from plant that are employed in the health sector today even in the possibility of synthetic chemicals serving as drug, plants still hold many specie (Evans).

Today focus is changing and people are drifting from the use of conventional therapy to the use of natural product. Based on world Health Organization (WHO) report, some 3.4 billion people in the developing world depend on the plant based traditional medicines (Satyajii et al., 2007). So also according to WHO, 80% of the world populations rely chiefly on plant based traditional medicines especially for their primary health care needs. About 60million people are estimated to use herbal remedies each year affording cost of about 3.2 billion Dollars in USA, $6 billion in Europe, more than $2 billion in Germany, over 2.3 billion Dollars in china, $2.1 billion in Japan, and $1-2 billion in Malaysia etc. (Inamul, 2004).Though Nigeria Statistics is not documented, it is clear that huge amount of money is being spent on traditional medicine evidenced by ever increasing number of such products and their demands. Among the uses of herbal therapy is in the treatment of infective diseases which form a high percentage of the diseases affecting man all over the world today. The results presently arising from the use of available chemotherapeutic agents are even encouraging factors to the use of herbs. This becomes more serious especially with the claim of benefits of herbal medicines over synthetic counterpart. People seem to have understood and chose to avoid the debilitating side effects that come along with some synthetic chemicals.This coupled with the incidence of resistance to most of the existing chemotherapies by microorganisms, re-establish the strong need for antibiotic from natural sources. Antibacterial resistance among bacterial pathogens in recent time is a critical area of public health concern (Fagbohun *et al.,* 2010). There is need for the development of new antibiotics due to acquired resistance more importantly, from natural sources as this delays resistance (Ajaiyeoba, 2000). According to Denver Russell plant might prove to be a potentially fruitful source of new antimicrobial agent. Though he indicated toxicity as problem in the use of high plants, all plants might not be toxic plus optimization normally used for every drug developments. Today, there is need to study plants to properly establish those whose efficacy has been a claim (Evans).

In this study, focus was on the anti-microbial activity of dichloromethane : methanol (1:1) crude extracts, of *Ritchiea Longipedicellata Gilg* leaves using agar diffusion techniques.

Taxonomy and Plant Description

The has the Kingdom (Plantae), Division (Angiospermae), Class (Dicotyledonae), Subclass (Archichlamydae),Order (Papaverale/Brassicales), Suborder (Capparineae), Family (Capparidaceae), Genus (*Ritchiea)* and Species (*Ritchiea longipedicellata Gilg).*

The plant is an evergreen climber but when alone it is a self-supporting shrub with compound palmate leaves. The leaves can be collected all - year round as the plant can stand dry season. The roots are tuberous and with strong pungent odours when perceived. Like other capparidaceae, this herb is indigenous to the tropics, found mostly in the lowland area of rain forest, especially beside water body and virgin up-lands. As a shrub it grows to a height of few meter(s) and as climber can grow a considerable length of about 5 meters with several branches (Local source).

Distribution of *Ritchiea longipedicellata*

The plant *Ritchiea longipedicellata G*. is virtually all over the tropical land of Africa and particularly West Africa. In Nigeria, the plant is found in the south east where the plant is used locally for various indications. The local name springs from the number of leaves (three) present in a leaflet hence it is called Nchi-ato [3-ears] by the Ibo people (Ikwo)

Ethnobotanical Uses of *Ritchiea Longipedicellata*

The plant is used in Nigerian local villages (Particularly in Ikwo L. G. A. in Ebonyi State)

where the root and the leaves are used for treatment of various illness.–small quantity of the root can be chewed (with closed mouth) to relieve pain in the head, cold, upper respiratory tract infections. Local palm wine extract of the plant is used for the treatment typhoid fever and malaria and general illness that prove resistance to modern therapies (Local users and traditionalist).

MATERIALS AND METHODS

Chemicals and Solvents

The chemicals used for extraction processes include methanol, n-hexane methylene dichloride (Qualichem Pvt ltd), dimethyl sulfoxide (DMSO), Nutrient Agar and Sabouraud dextrose agar. The reagents used were – concentrated sulfuric acid, naphtha solution in ethanol (Mulish 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 both bacteria and fungi 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 aeruginosa*, *Klebsiella* species, *Escherichia coli, Bacillus subtilis, Sarcina lutea*, *Salmonella typhi*) and *Aspergillus niger Candida albican* were the two fungi used.

Equipment

Weighing Balance[Scout pro u401 made in China], Beakers, measuring cylinder, test tubes, incubators (GentLab 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 *Ritchiea Longipedicellata* were obtained from Echialike in Ikwo local Government Area, Ebonyi state in November 2010. The plant was identified by Dr C. O. Ezugwu of the Department of Pharmacognosy and traditional medicine Nnamdi Azikiwe University Agulu. 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 250g of powdered plant leaf.

Extraction Process

Extraction was done with ratio 1:1 combination of Dichloromethane and methanol. The 250g of powdered dried leaves was macerated with 500ml of the mixed solvent in a container for six days with occasional agitations. 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 and crude extract for various phytochemical constituents. The procedure used was obtained from Evans (2002).

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 (Roof, 1921).

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.

Fixed oil

Whole extract solution (0.5ml) with two drops of 1M alcoholic $K_2Cr_2O_7$ and 3 drops of phenolphthalein were added in a clean test tube. Soap formation shown by frothing indicated the presence of fixed oil.

Phenolic group

Alcoholic plant extract (0.5ml) was taken in a test tube. Two drops of 1M ferric chloride was added. Appearance of intense color indicated the presence of phenolic groups.

Cyanogenetic glycosides

About 1 g of powdered sample was boiled with distilled water and moist sodium picrate paper held inside the tube with a cork. A colour change from yellow to Brick-red of the picrate paper is positive for cyanogenetic glycosides.

Antimicrobial Assay Microorganisms

24hour Cultures of seven human pathogenic bacteria made up of both gram positive (*S. aureus, S. lutea* and *B.subtilis*) and gram negative (*P. aeruginosa, Klebsiella spp, E. coli* and *S. typhi*) bacteria were used for the *in-vitro* antibacterial assay. For the antifungal assay, two fungi were utilized for the studies and these were made up of *Aspergillus niger* and *Candida albican*. 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.); Clotrimazole cream, 1mg/ml (Drug field, Nigeria) were included in the study as standard reference drugs.

Antimicrobial Activity Determination

An overnight broth culture used to obtain 0.5 McFarland standard of bacterium was used to seed sterile molten nutrient agar medium maintained at 45°C. Sabouraud dextrose agar plate was similarly seeded with fungi. Seven 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 and clotrimazole cream for bacteria and fungi respectively (also dissolved in DMSO). Diameters of zones of inhibition were determined after incubating plates at 37°C for 24h for the bacteria and at 25°C for 72 hours for fungi respectively. This test was conducted first on the crude extract and then on each of the different fractions and the solvent dimethylsulphoxide was used as negative control while ampicillin and clotrimazole cream were used as positive control.

RESULTS AND ANALYSIS

Table 1: Phytochemical Screening of *Ritchiea longipedicellata Gilg*

2° Metabolites (Plant Leaves)	Tests/ Observations	Inference
Proteins	Xanthoproteic reaction test (no orange coloration)	-
Alkaloids	Wagner and Hager test (precipitate formation)	++
Cyanidins	Picrate paper (from yellow to brick- red coloration)	+++
Flavonoids	Ammonium test (formation of yellow coloration)	+++
Glycosides	Picric acid test (brick-red coloration)	++
Steroids	Sulfuric acid test (reddish brown interface formation)	+++
Phenolic group	Ferric chloride test (intense coloration)	+++
Starch	Molisch test (no purple interfacial ring formed)	-
Reducing sugar	Benedict's test (rusty brown coloration)	+++
Essential oil	Potassium chromate test (soap formation via frothing)	+++
Saponins	Frothing and Emulsion tests	+

Key: - = Not detectable; ± = Doubtful; + = Low concentration; ++= Medium concentration; +++= High concentration

Antimicrobial Screening

Table 2: Antibacterial activity of dichloromethane/methanol extract

Crude Extracts	Inhibition Zone Diameter For Bacteria in Different concentrations of Extracts and Standard (mm)						
Bacteria	200	100	50	25	12.5	6.25 (mg/ml	Am(20ug)
S. aureus	5.0	4.0	3.0	-	-	-	6.0
P. aeruginosa	12.0	9.0	8.0	7.0	7.0	6.0	6.0
Klebsiella	-	-	-	-	-	-	16.0
E. coli	4.0	4.0	3.0	3.0	-	-	9.0
B. subtilis	4.0	3.0	-	-	-	-	5.0
Sarcina lutea	7.0	5.0	-	-	-	-	39.0
S. typhi	5.0	-	-	-	-	-	6.0

Table 2: Antifungal activity

Fungi	200	100	50	25	12.5	6.25(mg/	CLT (1mg/ml)
Aspergillus	8.0	-	-	-	-	-	-
Candida	-	-	-	-	-	-	14

Key: - = Means absence of antimicrobial activity

DISCUSSION, CONCLUSION AND RECOMMENDATION

Result of Phytochemical Screening of *Ritchiea longipedicellata Gilg* and yields of extracts. The percentage yield of powdered plant was 8.4g% with methanol/dichloromethane extract. The results of phytochemical screening showed moderate presence of alkaloid, simple sugar and abundance of flavonoid, steroidal nucleus, essential oil, phenolic group, cyanogenic glycoside; starch and protein were absent and doubtful quantity of saponin. The extracts displayed various activities against bacteria inhibiting it at various concentrations ranging from 200 to 6.25 mg/ml. Crude extract (200 mg/ml) had IZD of 5.0, 12.0, 4.0, 4.0, 7.0, and 5.0 mm against *S. aureus*, *P. aeruginosa*, *E. coli*, *B. subtilis*, *Sarcina lutea*, *S. typhi* respectively. Crude extract inhibited most appreciably *Pseudomonas aeruginosa* at all the concentration with IZD ≥6mm, however no activity against *Klebsiella* was recorded. The DMSO used did not show any activity against the bacteria used.

The results of phytochemical screening showed moderate presence of alkaloid, simple sugar and abundance of flavonoid, steroidal nucleus, essential oil, phenolic group, cyanogenic glycoside; absence of starch and protein but doubtful quantities of saponin in the leaves crude extract and powder screened for secondary metabolites. Some of these active principles (secondary metabolites) have been reported to have activity against micro-organisms. Flavonoid, phenolic, Alkaloids, triterpenes and essential oils have been shown to have activities (Majorie, 1999). The Presence of alkaloids, cyanogenetic glycosides, steroidal nucleus and reducing sugars, phenolic group and essential oil are normal with the plants of this family capparidaceae (Lather *et*

al, 2010; Ajaiyeoba E. O., 2000).The crude extract yielded enormous quantity of fixed oil.

The activity of the crude extracts was well demonstrated against *pseudomonas aeruginosa* were all the dilutions exerted some effect ranging from 12 to 6 mm for concentration 200 to 6.25mg/ml. It also exerted some effect against certain other organisms like *Sarcina lutea* with 7 and 5 mm for 200 and 100mg/ml. It also inhibited *S. aureus* and *S. typhi* to some extents but displayed very discouraging effects against *E. coli* and *B. subtilis* (4mm each). It however had no effect against *Klebsiella*. The activity against fungi was quite discouraging with no activity against *Candida* and of 8mm at 200mg/ml against *Aspergillus niger*.

The fact that the extract and demonstrated activities against certain bacteria and fungi confirming the use of the plant in ethno pharmacology. Since the root extract is more often used locally, it is yet to be confirmed if it has more activity than the leaves against the tested organisms. Taking the least IZD of the standard (Ampicillin) as the breaking point inhibition, the extract passed the breaking point as. It is recommended that further test be conducted to determine the activity of the root against bacteria and fungi since active principle in the plant is very suggestive of a good antibacterial and antifungal activity. The toxicity of the extracts should be tested in animals to rule out possibilities of poisoning since cyanogenic glycosides and heavy metal accumulation of some plants of the species are suggestive of toxicity.

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