JadounScience
http://www.jadounscience.com
International Journal of Drug Research and Technology
Scientific Publication of JadounScience
ISSN 2277-1506

Int. J. Drug Res. Tech. 2011, Vol. 1 (1), 45-51

International Journal of Drug Research and Technology

Available online at http://www.ijdrt.com/

Original Research Paper

EVALUATION OF ANTIMICROBIAL PROPERTIES OF ETHYL ACETATE EXTRACT OF THE LEAVES OF *NAPOLEONEAE IMPERIALIS* FAMILY LECYTHIACEAE

A.F. Onyegbule[1]*, C.F. Anowi[2], T.H. Gugu[3] and A.U. Uto-Nedosa[4]

[1]* Dept of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria

[2]Dept of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical sciences, Nnamdi Azikiwe University, Awka, Nigeria

[3] Dept of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria

[4]Dept of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria

ABSTRACT

Napoleonaea imperialis is used to treat wounds in Anambra State, Nigeria. Against this background, ethyl acetate extract of the leaves were screened against some microorganisms so as to ascertain this claim and to recommend it for further investigation for possible inclusion into official compendium. The plant leaves were dried, powdered subjected to cold maceration with ethyl acetate for 24 hours. 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 six pathogens comprising both Gram-positive and Gram-negative bacteria obtained from pharmaceutical microbiology laboratory stock. The extract was screened against 24 hour broth culture of bacteria seeded in the nutrient agar at concentrations 400, 200, 100, 50, 25, 12.5, 6.25 and 3.125 mg/ml in DMSO and incubated at 37^{o}C, for 24 hours and measuring the inhibition zone diameter - IZD. The phytochemical screening showed availability of alkaloid, saponins, tannins, glycosides and proteins. Ethyl acetate extract exhibited activity against *E. coli*, *B.subtilis* and *Pseudomonas aeruginosa* with minimal inhibitory concentration of about 50 mg/ml for *E. coli*, 100 mg/ml for *Pseudomonas aeruginosa* and 200 mg/ml for *B. subtilis*. 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 IZD of the standard (Ampicillin) as the breaking point, most of the extracts passed the breaking point.

Keywords: *Napoleonaea imperialis*, 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 Evans*). 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 and Lutfun, 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 60 million people are estimated to use herbal remedies each year affording cost of about 3.2billion Dollars in USA, $6billion 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 have been a claim (Evans).

In this study, focus was on the anti-microbial activity of Ethyl acetate extract of *Napoleonaea imperialis Ritchiea* leaves using Agar diffusion techniques.

MATERIALS AND METHODS

Chemicals and Solvent

The chemicals used for extraction processes include, ethyl acetate, dimethyl sulfoxide (DMSO), nutrient agar and sabouraud dextrose agar. The reagents used, 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 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 - *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella* Species, *Escherichia coli, Bacillus subtilis, and*, *Salmonella typhi.*

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.

Methods

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 1000ml of ethyl acetate 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 and crude extract for various phytochemical constituents. The procedure used was obtained from Trease and 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.

Test for carbohydrates

0.1g of the powdered leave was boiled with 2 ml 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 20 ml 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 5 ml 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 2.5 ml on a boiling water bath. 5 ml 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 2.5 ml of the chloroform using separating funnel. To 0.5 ml 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.

Test for 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.

Antimicrobial Assay

Microorganisms

24 hour Cultures of six 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, was used for the assays. Dimethyl sulfoxide (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 McFarland 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; 400mg/ml, 200mg/ml,100mg/ml,50mg/ml,25mg/ml12.5mg/ ml 6.25mg/ml and 3.125mg/ml of extract were prepared in dimethyl sulfoxide (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. This test was conducted on the crude and the solvent dimethyl sulfoxide was used as negative control while ampicillin and was used as positive control.

RESULTS

Table 1: Phytochemical Screening of *Napoleonaea imperialis*

2° Metabolites (Plant Leaves)	Tests/Observations	Inference
Proteins	Xanthoproteic reaction test (no orange coloration)	+
Alkaloids	Wagner and Hager 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)	---
Saponins	Frothing and Emulsion tests (formation of froth & emulsion)	++
Resins	Precipitation and Colour Test (negative)	--

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

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	+	+	+	+	+	+	+	+	6.0
P. aeruginosa	6	4	2	+	+	+	+	+	6.0
Klebsiella	+	+	+	+	+	+	+	+	16.0
E. coli	6	4	3	2	+	+	+	+	9.0
B. subtilis	4	2	+	+	+	+	+	+	5.0
S. typhi	+	+	+	+	+	+	+	+	6.0

Key: + Means presence of antimicrobial activity; - Means absence of antimicrobial activity.

DISCUSSION, CONCLUSION AND RECOMMENDATION

The results of 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 400 mg/ml concentration, the extract is effective against *E. coli*, *B.subtilis*, and *Pseudomonas aeruginosa* with inhibition zone diameter IZD of 6 mm, 4 mm and 6 mm respectively. At 200 mg/ml, the extract is effective on *E. coli*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* with IZD of 4 mm, 2 mm, and 4 mm respectively. At 100 mg/ml, *E. coli* and *Pseudomonas aeruginosa* had IZD of 3 mm and 2 mm. Also at 50 mg/ml the extract showed activity against *E. coli* with inhibition zone diameter of 2 mm. The DMSO used did not show any activity against the bacteria used. When compared with a standard antibiotic ampicillin at 20µg/ml the crude 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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