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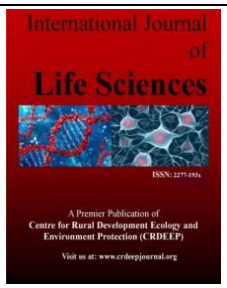

Full Length Research Paper

Secondary Metabolites and Bioactivities of two Species of *Nephrolepis* in Ekiti State, Nigeria

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ABSTRACT

The phytochemical screening of the fronds of two ferns: Nephrolepis cordifolia and Nephrolepis biserrata collected from Ekiti State, Nigeria was carried out to investigate the presence or absence of some secondary metabolites. Their antimicrobial potentials on selected organisms were also investigated. Crude aqueous and ethanolic extracts of the ferns were screened for the presence of secondary metabolites such as alkaloids, saponins, phenols, reducing sugar, cardiac glycosides, flavonoids, steroids, anthraquinones, terpenoids and tannins. Antimicrobial activity of the extracts was also carried out on Staphylococcus aureus, Escherichia coli, Baccillus subtilis, Enterobacter aerogenes and Candida albicans using agar diffusion method. The aqueous and ethanolic extracts of the ferns tested negative for steroids, anthraquinones and terpenoids while phenols, reducing sugar, saponins, cardiac glycosides, tannins and flavonoids were present in the extracts. Alkaloids were absent in N. cordifolia. The extracts of the two ferns showed significant activity on all the microorganisms. Enterobacter aerogenes was the most susceptible organism to the extracts of N. biserrata while the least was Escherichia coli and Baccillus subtilis. For N. cordifolia, the most susceptible organism was Staphylococcus aureus while the least susceptible was Enterobacter aerogenes. The results of the present study suggest that extracts of the fronds of the two ferns possess antimicrobial properties. This could be exploited further as sources of antimicrobials.

Introduction

Recently, the public demand for herbal medicine and the rise of antibiotic-resistant bacteria have motivated scientists to look for new natural sources with potential pharmaceutical capabilities. Besides, the indiscriminate use of antimicrobial drugs has created immense clinical problems in the treatment of infectious diseases (Davis *et al*., 1995). This has necessitated the development of alternative antimicrobial drugs from the medicinal herbs which are rich sources of novel antibacterial and antifungal chemotherapeutics (Jones and Firn, 1977). It has been reported that 80% of the world's population depend on plants to meet their primary health care needs (WHO, 2002). In order to solve issues related to antimicrobial resistance, drivers of resistance and possible solutions have been listed for future approaches. One of these could be the discovery and development of new antimicrobial agents that have clinical significant importance from natural sources. Obadoni and Ochuko (2001) reported that presently in industrialized nations, some 50% of all prescribed drugs are derived or synthesized from natural products and that their only available sources are animals, marine species, plants and microorganism. Among the estimated 250, 000 plant species existing worldwide, only a few percentages have been investigated phytochemically and the fraction submitted to biological and pharmacological screening is even smaller (Bindu *et al*., 2012). Though angiosperms are immense sources of therapeutics, lower plants are attracting more attention in recent times for the search for new and effective molecules. The medicinal values of pteridophytes have been known for several years. Hansel and Lagare (2005) reported that the antimicrobial properties of ferns are remarkable compared to the higher plants probably because of a large number of defensive biochemical compounds. Traditionally, people use pteridophytes as medicine and antibacterial agents. Generally, many species of pteridophytes are yet to be explored for their potential applications for future use and to isolate new active principles from them (Suvarnalatha *et al*., 2015).

N. biserrata and *N. cordifolia* belong to the family Nephrolepidaceae. Both have been reported to be of immense ethnobotanical importance. The ethnobotanical importance of *N. biserrata* in boils, abscesses and blisters have been documented (Baltrushes, 2006). Incidentally, boils and abscesses are due to bacterial infections (Lindenmayer *et al*., 1998; Smith *et al*, 2003) while blisters are caused by fungal infections (MERK, 2010). Hence, *N. biserrata* can be a potential plant to fight pathogenic microbes. *N. cordifolia* is being used in general disorders of renal and liver systems, skin diseases and as a contraceptive (Baltrushes, 2009).

Considering the diversity of medicinal plants, including pteridophytes, the screening of plant extracts for

phytochemical and antibacterial activity may be beneficial for human diseases. The synergistic interaction among crude extracts or the active compounds may be useful in the preparation of improved herbal or drug formulations.

The present study focussed on *N. biserrata* and *N. cordifolia* with a view to investigating the phytochemicals in their aqueous and ethanolic extracts as well as testing their antibiotic potentials on selected microorganisms.

Materials and Methods

The ferns were collected in Ado Ekiti, Ekiti State, Nigeria and were brought to the herbarium unit of the Department of Plant Science and Biotechnology, Ekiti State University, Ado Ekiti, Ekiti State, Nigeria for identification.

Preparation of Plant Extracts

Healthy disease free fronds of the ferns, *N. biserrata* and *N. cordifolia* were used for the preparation of the aqueous and ethanolic extracts. The fronds were thoroughly washed, shade dried for six weeks and pulverized using a blender. 5g of each plant was separately dispersed into 50ml of water and 70% ethanol. The solution was left at room temperature for 24 h and was filtered using Whatman No 1 filter paper. The filtrates were evaporated to dryness to obtain the extracts which were used for phytochemical screening.

Phytochemical screening

The extracts were screened for the presence of secondary metabolites like alkaloids, anthraquinones, cardiac glycosides, flavonoids, phenols, reducing sugars, saponins, steroids, tannins, terpenoids using the methods of Trease and Evans (2002), Sofowora (1984) and Isa *et al*. (2014).

Test for Alkaloids

0.5 g of the extract was stirred with 5 ml 1% aqueous hydrochloric acid on a steam bath and filtered. Then, 1 ml of the filtratewas treated with few drops of Mayer's reagent and another 1 ml portion of the substrate was treated with Dragendroff's reagent. Turbidity or precipitation with either of these reagents was taken as an evidence for the presence of alkaloids.

Test for Anthraquinones

0.5g of each extract was boiled in 2 ml of diluted sulphuric acid and then filtered while it was hot. 25 ml of benzene was added to the filtrate. It was shaken and the benzene layer was separated. Few drops of 10% (v/v) ammonia solution were added and the mixture was observed for colour change. Formation of a pink, red or violet colouration in the ammonia layer indicated the presence of anthraquinones in the extracts.

Test for Cardiac Glycosides

To 2 ml of the extracts, 3 ml of glacial acetic acid and 1 drop of 5% ferric chloride were added in a test tube. 0.5 ml of concentrated sulphuric acid was added by the sides of the test tube. Formation of blue colour in the acetic acid layer indicated the presence of cardiac glycosides.

Tests for Flavonoids

0.5 g of the extract was added to 1% Aluminium chloride and dissolved in methanol. Few drops of concentrated HCl, magnesium turnings and potassium hydroxide solutions were added. Appearance of orange or pink colour was taken as an evidence for the presence of flavonoids.

Test for Phenols

Ferric chloride test: A fraction of the extract was treated with 5% ferric chloride solution and observed for the formation of deep blue or black colour. To 1 ml of the extract, 2 ml of distilled water, 3 drops of 10% aqueous ferric chloride ($FeCl_3$) and 3 drops of potassium Ferro cyanide were added. Formation of blue or green colour showed the presence of polyphenols.

Test for Reducing Sugar

To 1 ml of aqueous extract, 1 ml of Fehling's A and 1 ml of Fehling's B solutions were added in a test tube and heated on a water bath for 10 min. Formation of red precipitate indicated the presence of reducing sugar.

Test for Saponins

This was determined by double extraction gravimetric method in which 0.5 g from each of the plant extracts was shaken with water in a test tube. Frothing which persisted on warming was taken as an evidence for the presence of saponins.

Test for Steroids

The extract was treated with chloroform and filtered. Few drops of concentrated sulphuric acid were added, shaken and allowed to stand. It was examined for the formation of reddish precipitate which indicated the presence of steroids.

Tests for Tannins

0.5 g of the extract was stirred with 10 ml of distilled water, filtered and ferric chloride salt was added. A blue-black, green or blue-green precipitate was taken as an evidence for the presence of tannins.

Test for Terpenoids

The extract was treated with chloroform and filtered. Few drops of concentrated sulphuric acid were added, shaken and allowed to stand. It was examined for the formation of reddish precipitate which indicated the presence of steroids. Presence of golden yellow precipitate indicated the presence of terpenoids.

Preparation of crude extracts

50 g of the ground plant material was soaked in 250 ml of distilled water and 70% ethanol separately for 24 h. The extracts were filtered through a sieve to remove debris. The filtrate was then filtered again using Whatman No 1 filter paper. The final filtrate was then evaporated in a water bath at 40^0C to get the crude extract (Idris *et al*., 2009). All the extracts were preserved in airtight bottles until further use.

Preparation of concentrations of plant extracts

1 g each of both ethanolic and aqueous extracts was added to 5 ml of 70% ethanol and distilled water respectively to give a concentration of 200mg/ml. Other concentrations of 150, 100, 50 and 25 mg/ml were prepared by double dilution method as described by Iqbal and Arina (2001).

Test organisms

The organisms used were obtained from the Department of Microbiology, Afe Babalola University, Ado Ekiti, Ekiti State, Nigeria. They included *Staphylococcus aureus, Escherichia coli, Baccillus subtilis, Enterobacter aerogenes and Candida albicans*.

Antibiotic Activity of the Extracts

The antibiotic activity of the extracts was tested using agar diffusion method. Sterile nutrient agar plates were prepared and allowed to solidify. Standardized organisms of 0.1 ml of a

day old were introduced into the plates and sterile cotton swab was used to spread the inocula evenly on the surface of the agar and the excess drained off. The plates were left on the bench for 1 h so that the inocula will diffuse into the agar. A sterile cork borer of 4mm diameter was used to make ditches on the plates. Varying concentrations of extracts i.e. 200 mg/ml, 150 mg/ml, 100 mg/ml, 50 mg/ml and 25 mg/ml were made and 0.5 ml of the extract was dropped in each of the appropriately labelled plate. Control was set up by adding 0.5 ml of the appropriate solvent into the ditch. Experiments were done in triplicates and left on the bench for few minutes for the extracts to diffuse into the agar. The plates were then incubated at 37^0C for 24 h to allow for maximum growth of the microorganisms. Then antibacterial activity of the extracts was determined by measuring and recording the zone of inhibition.

Results

Phytochemical screening of the extracts of the ferns revealed the presence of various secondary metabolites and the results are summarized in Table 1. The phytochemical constituents of the two ferns were very similar. Ethanolic and aqueous extracts of the ferns had phenols, reducing sugar, tannins, cardiac glycosides, saponins and flavonoids but tested negative to steroids, anthraquinones and terpenoids. Alkaloids were absent in *N. cordifolia*.

The antimicrobial activity of *N. biserrata* and *N. cordifolia* are shown in Tables 2 and 3 respectively. The zone of inhibition in aqueous extracts of *N. biserrata* fronds ranged between 6.5 mm and 12.0 mm while it ranged between 6.5mm and 14.8 mm in ethanolic extracts at 200 mg/ml concentration. For *N. cordifolia*, the zone of inhibition ranged between 8.2 mm and 22.0 mm in aqueous extracts while it ranged between 9.5 mm and 16.4 mm in ethanolic extracts at 200 mg/ml concentration. Results revealed that *Enterobacter aerogenes* was the most susceptible organism to the extracts of *N. biserrata* while the least was *Escherichia coli* (for aqueous extract) and *Baccillus subtilis* (for ethanolic extracts). *Staphylococcus aureus* was the most susceptible organism to the extracts of *N. cordifolia* while the least was *Enterobacter aerogenes*. Aqueous extract of the two ferns did not inhibit the growth of *Candida albicans* at 25 mg/ml concentration. Also, aqueous extract of *N. biserrata* did not inhibit the growth of *Escherichia coli* and *Baccillus subtilis* at 25 mg/ml concentration.

Table 1: Phytochemical constituents of the ferns

Phytochemical	***N. biserrata***		***N. cordifolia***	
	Aqueous	**Ethanol**	**Aqueous**	**Ethanol**
Alkaloids	+	+	-	-
Anthaquinones	-	-	-	-
Cardiac glycosides	+	+	+	+
Flavonoids	+	+	+	+
Phenols	+	+	+	+
Reducing sugar	+	+	+	+
Saponins	+	+	+	+
Steroids	-	-	-	-
Tannins	+	+	+	+
Terpenoids	-	-	-	-

+: Present, - : Absent

Table 2: Antimicrobial activity of aqueous and ethanolic extracts of the fronds of *N. biserrata* (zone of inhibition in mm)

Organism	**Aqueous**					**Ethanol**				
	25	50	100	150	200	25	50	100	150	200
Staphylococcus aureus	2.0	3.5	4.0	10.2	12.0	3.8	5.0	7.3	10.8	11.2
Escherichia coli	-	0.8	2.5	4.0	5.5	0.5	2.5	4.8	7.4	8.0
Baccillus subtilis	-	2.0	4.0	4.5	5.8	-	1.5	3.5	5.8	6.5
Enterobacter aerogenes	2.5	6.0	7.5	13.5	18.0	1.5	2.6	3.8	6.5	14.8
Candida albicans	-	0.5	2.5	4.8	6.5	0.5	2.2	3.0	4.7	7.0

Table 3: Antimicrobial activity of aqueous and ethanolic extracts of the fronds of *N. cordifolia* (zone of inhibition in mm)

Organism	**Aqueous**					**Ethanol**				
	25	50	100	150	200	25	50	100	150	200
Staphylococcus aureus	4.0	7.0	12.5	15.0	22.0	2.5	6.3	10.5	12.0	16.4
Escherichia coli	2.5	3.3	7.0	8.5	8.8	3.0	3.5	8.2	9.5	11.5
Baccillus subtilis	1.0	2.5	6.0	8.0	9.6	2.2	2.5	5.8	7.5	12.0
Enterobacter aerogenes	0.5	1.7	3.2	5.0	7.5	2.0	4.0	5.6	7.0	9.5
Candida albicans	-	1.0	2.8	6.5	8.2	0.5	3.7	6.0	8.5	10.0

Discussion

In the last five decades, several studies focussing on the phytochemical constituents and antimicrobial activities of plants and their derivatives have been conducted. This has led to the manufacture of several drugs such as quinine, emetin, belladonna amongst others (El-Mahmood, 2010). It has been suggested that there is a direct relationship between the chemical structure of a bioactive compound in plant extracts and their antimicrobial activity (El-Mahmood and Ameh, 2007; El-Mahmood, 2010).Rani *et al.* (2010) reported that *N. biserrata* and *N. cordifolia* are good sources of antimicrobial compounds. The present study revealed the presence of some secondary metabolites such as alkaloids, flavonoids, cardiac glycosides, tannins, reducing sugar, saponins and phenols in

the fronds of *N. biserrata* and *N. cordifolia* which may be correlated to their antimicrobial activity. Alkaloids have a wide range of pharmacological activities which include their actions on the autonomic nervous system, blood vessels, promotion of dieresis, respiratory system, gastrointestinal tract, malignant infection and malaria diseases (Omotayo and Omoyeni, 2009). Flavonoids are polyphenols commonly found in plants. They contribute significantly as natural antioxidants. Flavonoids have been shown to contain anti-mutagenic and anti-malignant effect along with its role in preventing diseases like cancer and inhibiting low density lipoproteins (LDL) oxidation induced by free radicals. Flavonoids have also shown antibacterial, antiviral, antitumor and anti-inflammatory activities (Sellappan and Caimir, 2002; Islam *et al.,* 2013). Cardiac glycosides also have therapeutic applications, being used for treating heart related problems (Seigler, 1998). Saponin has been reported to have anti-diabetic properties (Kamel, 1991). Saponin also lowers the cholesterol level; possess anti-carcinogenic activities as well as stimulating immune response (Edward, 2011). Tannin is one of the groups of phenolic compounds that act as primary antioxidants and possess antimicrobial, anti-inflammatory, anti-allergic, anticancer activity and for the treatment of intestinal disorders (Rievere *et al.,* 2009). Reducing sugars act as antioxidants (Richard, 2006). Phenolics are important components of plants and many of the pharmacological effects exerted by plants could be attributed to the presence of valuable constituents contained in plants (Yang *et al.,* 2002). The antibacterial activity of plants is believed to be due to the presence of some bioactive compounds like alkaloids, tannins, saponins, terpenes and flavonoids (Dash *et al*., 2013).

In developing countries, infectious diseases impose a big threat and this may be due to unavailability and high cost of medicines (Ayoola *et al*., 2008). Recently, the interest in plants as sources of antimicrobial agents has increased worldwide probably due to their use from ancient time particularly in developing countries and a good proportion of the world's population depends on plants for the treatment of infectious and non-infectious diseases (McLauglin *et al*., 1993). Despite the fact that the ethnobotanical importance of pteridophytes have been investigated and studied, very less work has been done on their antimicrobial activity (Cracelin *et al.,* 2012). The results of the present investigation revealed that the ferns possess antimicrobial properties. Bhabbie *et al.* (1972) studied the phytochemical composition of *Adiantum radiata* and found out that the isolated phytochemicals were effective against the growth of microorganisms. Bernejee and Sen (1980) studied the antibiotic activity of pteridophytes while Pandey and Bhargava (1972) also analysed the antiviral activity of crude extracts of some pteridophytes and reported significant antimicrobial activity. Guha *et al.* (2004) studied the antibacterial activity of *Adiantum capilllus-veneris* and found out that nearly all the extracts were effective against the selected microorganisms. Rani *et al.* (2010) also studied the antimicrobial activity of three pteridophytes and reported their various antimicrobial properties. Results of the present study tend to agree with these findings.

In an earlier exhaustive study of some pteridophytes, 73 displayed antimicrobial properties but *N. biserrata* was not part of it (Bernejee and Sen, 1980). Result of the present study does not agree with this. This may be due to potential differences in seasonal collection, locality and extraction/experimental procedure (Russell, 2010). Therefore further studies may be needed to explore the factors affecting pteridophyte activity.

The extracts of the two ferns showed almost similar pattern of inhibition zones against the organisms tested. However, the observed variations in the dimensions of the inhibition zone in the present study may be due to various strains and species of the microorganisms (Kaushik *et al.,* 2012).

In the present study, the bioactivities of the aqueous and ethanolic extracts were similar. This does not agree with the previous assertion that the aqueous extracts of pteridophytes are more effective in inhibiting microbial growth (Bernejee and Sen, 1980).

The present study revealed that the susceptibility of the Gram-negative bacteria and Gram-positive bacteria were similar as against the general assertion that Gram-negative bacteria are more resistant to antibiotics than Gram-positive bacteria (Chowdhury and Islam, 2004). This may be due to the presence of broad spectrum of antibiotic compounds present in the two ferns (Gracelin *et al.,* 2012)

The activity of the extracts in the present study was concentration dependent. This agrees with the work of El-Mahmood *et al.* (2008).

Conclusion

The findings of this study revealed that *N. biserrata* and *N. cordifolia* can serve as a potent source of alternative medicine. The range of antimicrobial activity showed by the aqueous and ethanolic extracts of their fronds indicated that they might be considered as antimicrobial agents. However, further investigation is required to isolate and characterize the active compounds responsible for the bioactivity.

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