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Original Research Article

Phytochemical Constituents and Comparative *in vitro* Antibacterial Activity of Leaf and Stem Extracts of *Pneumatopteris afra* (Christ) Holttum

Adebiyi, Adedeji Olayinka[1]* and David, Oluwole Moses[2]

[1]Department of Plant Science and Biotechnology, Ekiti State University, Ado-Ekiti, Nigeria.
[2]Department of Microbiology, Ekiti State University, Ado-Ekiti, Nigeria.

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Ferns (pteridophytes) have been reported to have different phytochemicals. They have been used in the treatment of many infections. The phytochemical analyses and antibacterial activities of *Pneumatopteris afra* were investigated in this study. Standard physical-chemical methods were used to determine the phytochemical constituents of the leaf and the stem of the *P. afra*. Disc diffusion dilution method was used to determine the antibacterial activities of the ethanolic, ethyl acetate and n-hexane extracts of the leaf and stem of the plant. *Escherichia coli* ATCC 8739 did not produce the zone of inhibition when exposed to the n-hexane extracts of the plant. Unlike ethanolic extract, the n-hexane extract of the leaf of *P. afra* performed better than the stem extract. Ethyl acetate extract of the stem performed better than the leaf extract. Except for organisms, *Serratia marcescens* ATCC 9986 and *Shigella sonnei* ATCC 29930 all the test organisms showed more susceptibility to ethyl acetate extract of the stem. *In vivo* evaluation of the extract and possible mechanism(s) of action of the extracts of the plant is still open to investigation.

Keywords: *Pneumatopteris afra*, Ferns, Antibacterial, Phytochemical.

INTRODUCTION

In recent years, antimicrobial resistance has become a major public health concern globally. It has been reported that over 70% of pathogens found in US hospitals acquired resistance for at least one antibiotic, resulting in mortality of more than 14,000 patients annually from nosocomial infections (Rezai and Weinstein, 2010). The continued emergence or persistence of drug resistant organisms and the increasing evolutionary adaptations of pathogenic organisms to commonly used antibacterial have reduced the efficacy of antibacterial agents currently in use. In addition to this, antibiotics are associated with adverse effects; therefore, the search for new drugs from novel sources such as plants is necessary.

It has been pointed out that more than 80% of the world's population depend on plants to meet their primary health care needs (WHO, 2002). In order to solve antimicrobial resistance issue, drivers of resistance and possible solutions have been listed for future approaches. One of the effective approaches could be the discovery and the development of new antimicrobial agents that have clinical significant importance from natural sources. In industrialized nations at present, some 50% of all prescribed drugs are derived or synthesized from natural products, the only available sources for which are animals, marine species, plants and microorganisms (Obadoni and Ochuko, 2001). Among the estimated 250,000 plant species existing worldwide, only a small percentage have been investigated phytochemically and the fraction submitted to biological and pharmacological screening is even smaller (Bindu *et al.,* 2012). Nowadays, more and more angiospermic plants are being used as medicine for many diseases. However, the lower group of plants like pteridophytes is largely neglected and has not been well documented.

Notwithstanding, recent ethnobotanical, pharmacological and biological searches have revealed medicinal, pharmaceutical and phytochemical attributes of pteridophytes, which have valuable potential applications for health and industry. These plants are not infected by microbial pathogens which may be one of the important factors for their evolutionary success and the fact that they survived for more than 350 million years (Sharma and Vyas, 1985). These plants have been successfully used in different systems of medicine like Ayurvedic, Unani, Homeopathic and other systems of medicine (Suvarnalatha *et al*., 2015).

Pneumatopteris afra belongs to the family Thelypteridaceae and has been used from time immemorial in traditional systems of medicine for relieving ascaris disease, cold, diarrhea, burn, trauma, bleeding and much more in many

*Corresponding Author: Adebiyi, Adedeji Olayinka. Department of Plant Science and Biotechnology, Ekiti State University, Ado-Ekiti, Nigeria.
Email: djadebiyi@yahoo.com

countries (Yapar *et al*., 2006). 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). More so, the medicinal importance of a plant is due to the presence of some special compounds like alkaloids, flavonoids, phenols tannins and saponins which are usually concentrated in the storage organs such as roots, stems and leaves. In view of these, the present investigation is designed to find out the phytochemical analysis and antibacterial activities of *P. afra*.

MATERIALS AND METHODS

Plant Collection, Identification and Processing

Different parts of *P. afra* (leaf and stem) were collected in growing area of a river bank in Ado-Ekiti, Nigeria. The species was authenticated at the Herbarium section of the Department of Plant Science, Ekiti State University, Ado-Ekiti, Nigeria. The plant was washed with distilled water to remove any adhering soil or extraneous materials and was then air dried. Matured leaves were detached from the stems and ground to fine powder.

A 50 g of ground plant sample was soaked in 500 ml of extracting solvents (ethanol, ethyl acetate and n-hexane) for 48 h and shook periodically. The sample was then filtered through Whatman Number 1 filter paper and washed with another 200 ml solvent. The filtrate was concentrated. The dried extract was dissolved in 4% dimethyl sulfoxide (DMSO) in their extracting solvent and topped up with water to make the required concentrations. The reconstituted extracts were filtered by 0.45 µl pore size membrane filter for sterility.

Qualitative Determination of Phytochemicals

The phytochemical components of the plant were determined using standard procedures.

- ***Test for Saponin***

  Two grams of sample were weighed in a beaker; 5ml of distilled water was added and heated to boil. Persisted foaming on warming was taken as an evidence for the presence of saponin (Sofowora, 1982).

- ***Test for Tannin***

  Two grams of sample were weighed and mixed with 10 ml of distilled water. The mixture was filtered and two drops of 5% ferric chloride ($FeCl_3$) was added to the filtrate. Blue-black colouration was taken as an indication of the presence of tannin (Trease and Evans, 1983).

- ***Test for Alkaloid***

  Two grams of sample were weighed in a beaker and it was extracted with 10 ml 2% hydrochloric acid by heating gently for about 5 minutes. The HCL extract was filtered with Whatman No 1 filter paper to have a clear solution and prevent false result. 2.5 ml of the filtrate was treated with few drops of Dragendoff's reagent. Appearance of precipitate indicated the presence of alkaloid in the extract (Trease and Evans, 1983).

- ***Test for Cardiac Glycosides***

  Two grams of the sample was dissolved in 2 ml glacial acetic acid containing one drop of ferric chloride ($FeCl_3$). The solution was underplayed with 1.0 ml of concentrated sulphuric acid (H_2SO_4). A reddish brown colour at the interface indicates the presence of a steroidal ring, that is, a glycine portion of the cardiac glycosides (Keller-Killiani's test).

- ***Test for Flavonoid***

  Five mililitres of the diluted ammonia solution were added to a portion of aqueous filtrate of sample extract followed by the addition of concentrated sulphuric acid. Formation of yellow colour indicated the presence of flavonoid (Sofowora, 1982).

- ***Test for Phenol***

  Five grams of the powdered sample was mixed with 20 ml of H_2SO_4 in ethanol and heated for five minutes. 1 ml of the filtrate of the heated mixture and two drops of ferric chloride were mixed to observe green, blue or black coloration (Sofowora, 1982).

- ***Test for Phlobatannins***

  Deposition of a red precipitate when aqueous extract of the mushroom was boiled with 1% aqueous HCl acid was an evidence for the presence of phlobatannins (Sofowora, 1982).

- ***Quantitative Analysis***

  The quantitative amount of phytochemicals which were found in the fern extract were determined using standard procedure as described by Obadoni and Ochuko (2001), Trease and Evans (2002) and Amakura *et al.* (2009).

Antibacterial Activity

- ***Source and standardization of test bacteria***

  The bacterial (both local and typed strains) used in this study were collected from the Department of Microbiology, Ekiti State University, Ado-Ekiti, Nigeria. The bacterial isolates used include: *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 8739, *Klebsiella pneumoniae* ATCC 10031, *Klebsiella pneumoniae* ATCC 4532, *Proteus vulgaris* ATCC 6830, *Pseudomonas aeruginosa* ATCC 19582 and *Staphylococcus aureus* ATCC 6538. The test isolate multiple-antibiotic resistant *S. aureus* used in this study was resistant to ceftriaxone, cefuroxime, ceftazidime, cloxicilin, gentamicin, erythromycin, augmentin, vancomycin and ofloxacin. The bacterial isolate was cultured at 37 °C in the Nutrient broth (Oxoid) for 18 h and diluted to an optical density of 0.1 (0.5 McFarland Standard) at a wavelength of 625nm.

- ***Determination of minimum inhibitory concentration (MIC)***

  Macrobroth dilution method was used for the determination of minimal inhibitory concentration (MIC) of the extracts on vancomycin-resistant *Staphylococcus aureus* as described by CLSI (2012). Mueller Hinton broth was used to prepare different concentrations (mg/ml) of the extracts in serial dilutions. Each prepared concentration in tubes was inoculated with 100 µl of each of the standardized culture of the test bacterium. Tube without extract was used as negative control. All the tubes were incubated aerobically at 37 °C for 18 h. The first tube in the series with no sign of visible growth was taken as the MIC.

- ***Determination of minimum bactericidal concentration (MBC)***

  A loopful of culture from the broth that showed no growth in the MIC test were inoculated on sterile nutrient Agar plates and observed for growth after incubation at 37 ^{o}C for 24 h. The MBC was taking as the least concentration of the extracts that showed no growth. The minimum inhibitory concentration index (MICI) was calculated as follows:

$$MICI = (MBC/MIC)$$

- ***Determination of the Susceptibility of the Isolates***

  The agar well diffusion was used to determine the susceptibility of the isolates to the median activity concentrations (MAC) of the extracts against the multiple antibiotic resistant *Pseudomonas aeruginosa* ATCC 19582. The MAC was determined as follows:

$$MAC = [(MIC + MBC)/2]$$

The standardised test organisms [i.e. with optical density of 0.1 (0.5 McFarland Standard) at a wavelength of 625nm] was seeded on the agar and well were made in the agar medium and the MAC of the extracts were gently dispensed into the wells. The plates were incubated at 37 ºC for 18 h and observed for zone of clearance. An average of many radii of the inhibition zone was taken to the nearest millimetre. The clear area of inhibition was estimated as described by Aderiye and David (2014).

RESULTS AND DISCUSSION

The phytochemical constituents of the leaf and the stem of the plant were very similar. The two parts of the plant had alkaloid, phlobatamin, saponin and flavonoid while cardioglycosides was only present in the leaf (Table 1). The quantitative phytochemical composition of leaf and stem of *P. afra* is shown in Table 2. All the phytochemical detected were in abundance in the leaf than the stem except phlobatamin. Cardioglycosides had the highest values in the leaf (but not detected in the stem). Tanin recorded the least amount in leaf and the stem as shown in Table 2.

The MICs of the n-hexane and ethyl acetate extracts of the leaf were lower than that of the stem. While the n-hexane and ethyl acetate extracts of the leaf were the same (100 mg/ml) when tested on the indicator organism (VRSA). The MBC of the ethanolic extract of the stem was 100% higher than that of the leaf of the plant on the isolate. The MICI of the ethyl acetate extracts on VRSA were lower than the other extracts. This is an indication of bactericidal activity of the ethyl acetates while the activities of the ethanolic and n-hexane extracts were bacteriostatic on the test organism as explained by Shanmughapriya *et al.* (2008).

Morbidity and mortality due to infectious diseases in the resource-poor nations of the world account for about 50% of all deaths (Khosravi and Behzadi, 2006; Ahmad and Aqil, 2009). This largely due to the increase in resistant of pathogenic microbes to the antimicrobials which has been of a global concern (Al-Bari *et al*., 2006; Simoes *et al*., 2007). The ethanolic extract of stem of the plant had better antibacterial activity than the ethanolic extract of the leaf. *Bacillus cereus* ATCC 10702 and *S. aureus* ATCC 6538 did not produce zone of inhibition when exposed to the ethanolic extracts of the plant. *Pseudomonas aeruginosa* ATCC 19582 and *Se. marcescens* ATCC 9986 had the highest zone of inhibition (95.07 mm^2) when exposed to the extracts.

The zone of inhibition in both extracts ranged between 0 and 95.07 mm^2 and 0 and 86.63 mm^2 in stem and leaf extracts of *P. afra* respectively as shown in Table 4. The n-hexane extracts performed better than the ethanolic extract. *E. coli* ATCC 8739 did not produce the zone of inhibition when exposed to the n-hexane extracts of the plant. Unlike ethanolic extract, the n-hexane extract of the leaf of *P. afra* performed better than the stem extract as shown in (Tables 4 and 5). *Serratia marcescens* ATCC 9986 showed the highest sensitivity to the n-hexane extract of the plant with zone of inhibition of 113.14 mm^2 while *S. aureus* ATCC 6538 had the highest zone of inhibition to n-hexane extract of the leaf.

Medicinal plants have been a source of therapeutic agents playing active roles in the management of diseases caused by microbes (Ichor and Ekoja, 2011). Phytomedicine has been an alternative to synthetic antimicrobial agents due to their easy accessibility and less toxicity compared to the orthodox medicine (Satish *et al*., 2009; Kumar *et al.*, 2010). Secondary metabolites such as saponins, tannins, alkaloids, phenols, glycoalkaloids, flavonoids, sesquiterpenes, lactones and terpenoids have been reported to be responsible for the antimicrobial and pharmacological actions of some of the medicinal plants (Nawrot et al., 2007; Srivastava *et al*., 1996). Some of the plants have dual roles; they serve as a source of food and also serve medicinal purposes (McChesney *et al*., 2007; Mohanasundari *et al*., 2007).

In Table 6, it is evident that the ethyl acetate extract of the stem performed better than the leaf extract. *E. coli* ATCC 8739, *K. pneumoniae* ATCC 10031 and *P. aeruginosa* ATCC 19582 showed complete susceptibility to the ehylaceteate extract of leaf of *P. afra. Escherichia coli* ATCC 8739 was most resistant to stem extract while *S. aureus* ATCC 6538 was the most susceptible. Except for *Se. marcescens* ATCC 9986 and *Sh. sonnei* ATCC 29930 all the test organisms showed more susceptibility to ethyl acetate extract of the stem. The extracts of the stem of *P. afra* had better antibacterial properties compared with the leaf extract.

Out of the three extracts, ethyl acetate had the best antibacterial activity followed by n-hexane extract while the least activity was observed in ethanolic extract. The n-hexane of the plant had the highest antibacterial property while the ethanolic extract had the least. The activity of this plant is due to the presence of the phytochemicals present in it. These metabolites have various target sites in the microbes.

Table 1: Qualitative phytochemical composition of leaf and stem of *P. afra*

Phytochemicals	Samples	
	Leaf	Stem
Alkaloid	+	+
Saponins	+	+
Steroids	-	-
Tanin	+	+
Flavonoid	+	+
Terpenoid	-	-
Cardic glycosides	+	-
Phlobatamin	+	+

+ = presence, - = absent

Table 2: Quantitative phytochemical composition (%) of leaf and stem of *P. afra*

Phytochemicals	Samples	
	Leaf	Stem
Alkaloid	0.74±0.05	0.71±0.06
Saponins	0.82±0.10	0.78±0.06
Steroids	0	0
Tanin	0.38±0.04	0.35±0.02
Flavonoid	0.58±0.06	0.43±0.03
Terpenoid	0	0
Cardic glycosides	0.92±0.10	0
Phlobatamin	0.32±0.04	0.33±0.02

Values are means of three determinations

Table 3: The minimum inhibitory concentrations and minimum bactericidal concentrations of the extracts of both stem and leaf of *P. afra* against vancomycin-resistant *S. aureus* (mg/ml)

Extracts	Leaf			Stem		
	MIC	MBC	MICI	MIC	MBC	MICI
Ethanolic	50	150	3	12.5	75	6
n-Hexane	12.5	100	8	50	100	2
Ethylacetate	75	100	1.3	100	150	1.5

Table 4: shows the antibacterial activity of ethanolic extracts of *P. afra* (zone of inhibition in mm^2)

Organisms	Stem	Leaf
Bacillus cereus ATCC 10702	0.00	0.00
Bacillus pumilis ATCC 14884	50.29	63.64
Enterococcus faecalis ATCC 29212	56.77	50.29
Staphylococcus aureus ATCC 6538	0.00	0.00
Escherichia coli ATCC 8739	63.64	50.29
Enterobacter cloaca ATCC 13047	44.20	38.50
Klebsiella pneumonia ATCC 10031	50.29	86.63
Pseudomonas aeruginosa ATCC 19582	95.07	44.20
Serratia mercescens ATCC 9986	95.07	0.00
Shigella sonnei ATCC 29930	0.00	56.77

Table 5: shows the antibacterial activity of n-hexane extracts of *P. afra* (zone of inhibition in mm^2)

Organisms	Stem	Leaf
Bacillus cereus ATCC 10702	44.20	28.29
Bacillus pumilis ATCC 14884	56.77	50.29
Enterococcus faecalis ATCC 29212	86.63	78.57
Staphylococcus aureus ATCC 6538	44.20	103.91
Escherichia coli ATCC 8739	0.00	0.00
Enterobacter cloaca ATCC 13047	50.29	50.29
Klebsiella pneumonia ATCC 10031	63.64	95.07
Pseudomonas aeruginosa ATCC 19582	63.64	95.07
Serratia mercescens ATCC 9986	113.14	50.29
Shigella sonnei ATCC 29930	63.64	56.77

Table 6: shows the antibacterial activity of ethyl acetate extracts of *P. afra* (zone of inhibition in mm^2)

Organisms	Stem	Leaf
Bacillus cereus ATCC 10702	63.64	50.29
Bacillus pumilis ATCC 14884	56.77	56.77
Enterococcus faecalis ATCC 29212	70.91	50.29
Staphylococcus aureus ATCC 6538	113.14	70.91
Escherichia coli ATCC 8739	0.00	0.00
Enterobacter cloaca ATCC 13047	78.57	70.91
Klebsiella pneumoniae ATCC 10031	50.29	0.00
Pseudomonas aeruginosa ATCC 19582	50.29	0.00
Serratia mercescens ATCC 9986	50.29	113.14
Shigella sonnei ATCC 29930	56.77	63.64

Terpenes involved in membrane disruption by lipophilic compounds and also promoted membrane disruption (Ahmed *et al.* 1993; Chung *et al.,* 1998). Tannins acted on microorganism membranes and polysaccharides; it also involves in the enzymes promoting inactivation. Flavonoids inhibit the cytoplasmic membrane function and DNA gyrase in the cell of bacteria.

The activities of the extracts of the plant authenticate its folkloric claims, to cure bacterial infectious diseases, by the trado-medical practitioners'. The extracts tested in this work showed *in vitro* activity against both gram positive and Gram negative bacteria. The extracts of the plant still have to be partitioned and fractions activity should be investigated. This activity guided procedure may assist in discovering another biological active phytochemical candidate(s) in the fight against drug-resistant bacteria. Also *in vivo* evaluation of the extract and possible mechanism(s) of action of the extracts of the plant is still open to investigation.

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