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Secondary Metabolites and Bioactivity of *Hyophila involuta* (Hook) Jaeg.

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Abstract

The phytochemical screening of *Hyophila involuta* collected from the Biological Garden of the Obafemi Awolowo University, Ile-Ife, Nigeria, was carried out to investigate the presence or absence of some secondary metabolites and its antibiotic potentials, using different extracts (with acetone and ethanol) on selected organisms. The extracts obtained were screened for the presence of secondary metabolites like alkaloids, anthraquinones, cardiac glycosides, flavonoids, phlobatanins, saponins, steroids, tannins, triterpenes and xanthoproteins. Antimicrobial activity of the extracts was carried out on *Escherichia coli*, *Staphylococcus aureus*, *Aspergillus flavus* and *Candida albicans*. Only acetone extract tested positive for the presence of flavonoids, while alkaloids and cardiac glycosides were detected present in both the acetone and ethanolic extracts. Flavonoids were detected present only in the acetone extract. Saponins were detected present only in the ethanolic extract. The extracts (acetone and ethanolic) showed significant activity on *Staphylococcus aureus* and *Aspergillus flavus*. The results obtained from this study indicated that *H. involuta* has medicinally important compounds, having therapeutic potential from which effective antimicrobial medicine can be sourced.

Keywords: antimicrobial, bryophytes, extract, medicinal, moss, pharmaceutical, phytochemical, screening

Introduction

Bryophytes have long been used for medicinal purposes; their use is well known in Asia (Ding, 1982; Wu, 1982) and Europe (Frahm, 2004). They produce a broad-range of antibiotics. These small, slow-growing groups of plants are often associated with disturbed habitat, barren rock surface and extreme climatic conditions. Bryophytes are abundant in many different types of plant communities and have a substantial and distinctive influence on the functioning of ecosystem where they occur, most especially in moist areas, as they possess adaptive mechanisms to survive water stress (Dilks and Proctor, 1974). Bryophytes assist in the stabilisation of soil crust by colonising bare ground and rocks, and are essential in nutrient recycling, biomass production as well as carbon fixing. Moreover, bryophytes are very efficient at regulating water flow by means of an effective water-retention mechanism (Hallingbäck and Hodgetts, 2000). They also have an economic value, being used as peat for fuel, in horticulture, oil absorption, or as sources for a wide variety of chemical compounds.

Bryophytes are known to contain numerous potentially useful compounds, including oligosaccharides, polysaccharides, sugar alcohols, amino acids, fatty acids, aliphatic compounds, phenylquinones, aromatic and phenolic substances (Pants and Tewari, 1990). However, the small size and inconspicuous distribution has made them apparently of no therapeutic use when compared to their tracheophyte counterparts (Glime, 2007; Harris, 2008). Nevertheless, mosses contain polyunsaturated fatty acids that are already known to have important potentials in human medicine, such as preventing atherosclerosis and cardiovascular disease, reducing collagen-induced thrombocyte aggregation and lowering triacylglycerols and cholesterol in plasma (Radwan, 1991). Scientists have found innumerable kinds of biological activity in compounds from bryophytes (Alam, 2012; Isa *et al*., 2014). Even in a single species, one might find multiple kinds of activity. For example, the liverworts *Plagiochasma japonica* and *Marchantia tosana* exhibit antitumor activity, antifungal and antimicrobial activity, inhibition of superoxide release, inhibition of thrombin activity and muscle relaxation (Lahlou *et al*., 2000). As it is often the case with herbal medicine, the effect of the total extract is more beneficial than that of the isolated compounds, perhaps due to a synergistic effect (Frahm, 2004). Earlier, Mc Cleary and Walkington (1966) reported that three species of mosses (*Anomodon rostratus*, *Plagiomnium cuspidatum*, *Orthotrichum rupestre*) produce substances that inhibit bacteria and fungi, but these inhibitors seem to be unstable products that vary considerably among species and even between seasons.

The phytochemical study of bryophytes for pharmaceutical "lead" compounds has been overlooked because of their minuscule nature which makes it difficult to identify and to collect large quantity of pure samples for producing drugs. However, recent studies of some *in vitro* cultures of bryophytes have shown that they synthesize distinct antibiotically active substances (Ilhan *et al*., 2006; Isa *et al*., 2014). The various secondary metabolites present in bryophytes are responsible for their bioactivity. Such metabolites are also effective as antitumor, antibiotics, anti-fungal, anti-feedants and repellents (Huneck, 1983). In recent years, many possible sources of natural antibiotics have been in use for several infectious diseases, being represented by many bacteria and fungi. In this respect, the most investigated taxa were from the angiosperms, whereas very little data are available on other groups of plants, including bryophytes (Subhisha and Subramonia, 2005).

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In this study, the research focused on *Hyophila involuta* with a view to investigating the phytochemicals in its acetone and ethanolic extracts, as well as testing its antibiotic potentials on selected microorganisms.

Materials and Methods

Biological material

The moss plant studied hereby was *Hyophila involuta* (Hook) Jaeg. and the samples were collected from the top of some concrete walls in the Biological Garden, Obafemi Awolowo University, Ile-Ife, Nigeria (7°3' and 7° 34' N & 4°30' and 4°32' E). An initial collection was made, when few samples of the mosses were collected, kept in Herbarium packets and brought to the Herbarium Unit, Department of Botany of the same university where they were properly observed and identified. Thereafter, the moss samples were collected fresh from the field, air dried, grounded and used for the experiments.

Extraction and screening for phytochemical substances

The extracts were procured by separately soaking the moss plants in acetone and ethanol for seventy-two (72) hours. The resulting solutions were filtered and the filtrates evaporated to dryness to obtain the extracts. The extracts were then screened for the presence of secondary metabolites like alkaloids, anthraquinones, cardiac glycosides, flavonoids, phlobatanins, saponins, steroids, tannins, triterpenes and xanthoproteins using the methods of Sofowora (1984), Oyedapo *et al*. (1999) and Oyesiku (2005) as reported by Isa *et al*. (2014).

Alkaloids: Acidic extracts were prepared separately by mixing 50 mg of each extract (methanol and chloroform) with 10 ml of 10 % (v/v) HCl. These were heated and thereafter filtered. To 1 ml of the filtrate, few drops of Picric acid and Wagner's reagent were added separately. The mixtures were then examined for colour change, turbidity or formation of precipitate. The formation of precipitate indicated the presence of alkaloids.

Anthraquinones: Each extract (0.5 g) was boiled in 2 ml of diluted sulphuric acid and then filtered while it was hot. To the filtrate, about 2.5 ml of benzene were added, shaken and the benzene layer was separated. Few drops of 10% (v/v) ammonia solution were then 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 extract.

Cardiac glycosides: Each extract (0.5 g) was dissolved with 2 ml of chloroform, filtered and the concentrated sulphuric acid was carefully layered at the bottom of the tube to form a lower layer. The chloroform/sulphuric acid interphase was then observed for the formation of a reddish brown colour ring, indicating the presence of cardiac glycoside in the extract.

Flavonoids: About 5 ml of ethanol were added to 5 mg of each of the extracts, shaken and then filtered. To 1 ml of the filtrate, few drops of 0.5 N ethanolic potassium hydroxide solutions were added. Formation of suspension, cloudiness or precipitate indicated flavonoids' presence in the extracts.

Phlobatanins: About 0.5 g of the extracts were heated with 10% (v/v) HCl in boiling water. The solutions were then observed for formation of red precipitate, which indicated the presence of phlobatanins.

Saponins: Each extract (0.1 g) was suspended in water in a test tube, shaken vigorously and noting the frothing. The solutions were warmed at 70 °C for about 15 min in water bath. The mixtures were shaken vigorously after warming. Persistence of frothing after warming indicated the presence of saponins.

Steroids: To 1 ml of concentrated sulphuric acid was added 1 ml of aqueous extract. It was allowed to stand for 5 min and then examined for the formation of reddish brown precipitate, which was an indication of the presence of steroids.

Tannins: The extract (10 mg) was dissolved in 10 ml distilled water and then filtered. To 1.0 ml of the filtrate were added few drops of 0.5 M ferric chloride in glacial acetic acid. The mixture was examined for the formation of blue, blue-black or greenish precipitate, which indicated the presence of tannins.

Triterpenes: Extracts (20 mg) were suspended in 10 ml of chloroform, warmed slightly in water bath and then filtered. About 5 ml of concentrated sulphuric acid were then added to the chloroform filtrate and it was properly mixed. The mixtures were examined for the formation of red colour, which indicated the presence of triterpenes in the extract.

Xanthoproteins: Few drops of nitric acid were added to 1 ml of aqueous extract followed by the addition of few drops of ammonia solution. Formation of reddish or slightly brown precipitate indicated the presence of xanthoproteins.

Investigation of antimicrobial potentials

Test organisms

The organisms used to test their growth inhibition by the extracts were *Escherichia coli* (NCIB 86), *Staphylococcus aureus* (NCIB 8588), *Aspergillus flavus* and *Candida albicans*; the strains were obtained from the Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria.

Preparation of media

The appropriate quantity of each medium used was accurately weighed and dissolved in the appropriate amount of distilled water according to the recipe for use; the solutions were heated to boil when necessary, dispensed into McCartney bottles and then autoclaved. After autoclaving, the nutrient agar (for the bacteria) was kept in molten form for subsequent use, while the broth was allowed to cool before sub-culturing. The eighteen hour peptone broth cultures were used. With the aid of a 1 ml sterile pipette, 0.1 ml of the broth culture of each test organism (bacteria) was added to the 20 ml sterile molten nutrient agar (sabourand dextrose agar in the case of *A. flavus* and *C. albicans*), which had already cooled to 44 °C. Each bottle was gently rotated to mix the inoculums with the medium and then poured into a properly labelled sterile Petri-dish and allowed to set.

Determination of antibiotic activity of the extracts

The agar diffusion method as described by Kudi *et al*. (1999), Ogundipe *et al*. (2000) and Isa *et al*. (2014) was used to determine the growth inhibition of the test organisms by the plant extracts. All bacteria were maintained at 40 °C on nutrient agar plates before use. The tests were carried out using crude extract stock solutions of the plant, prepared by dissolving the acetone extract into acetone and the ethanolic extract into ethanol in different Bijou bottles as described in Table 1.

Using a sterile cock-borer of 0.5 mm diameter, six equidistant holes per plate were made in the set agar with the control at the centre. Thereafter, the wells and the ditches were filled with the prepared different concentrations of the extract solutions, using

sterile Pasteur pipettes. These were done in duplicates. The culture plates were incubated at 35 °C for 24 hours and the relative susceptibility of each organism to the extracts, as indicated by clear zones of growth inhibition around the wells, were examined and recorded.

Results

Extraction and screening for phytochemical substances

The results of the phytochemical screening are presented in Table 2. Only acetone extract tested positive for the presence of flavonoids, while alkaloids and cardiac glycosides were tested positive in both the acetone and ethanolic extracts; saponins was tested present only in the ethanolic extract. None of anthraquinones, phlobatanins, steroids, tannins, triterpenes and xanthoproteins were detected present in both extracts throughout the experiment.

Antibiotic activity

The summary of the results of antimicrobial test of the extracts are presented in Table 3. The study showed different reactions to the extracts by each of the test organisms. *Escherichia coli* was totally resistant to ethanolic extract at the concentration used, while it showed only a slight resistance to the acetone extract. The growth of *Staphylococcus aureus* was inhibited by acetone extract, but that of *Aspergillus flavus* and *Candida albicans* were not. *Staphylococcus aureus* was also observed to be totally resistant to the ethanolic extract, whereas *Aspergillus flavus* was not (e.g. inhibited), but *Candida albicans* was observed to be slightly resistant to the acetone extract.

Discussion

The search for effective antimicrobial agents had developed recently. One of the major reasons for this is the increasing resistance posed by microorganisms to commercial antibiotics (Olofin *et al*., 2013). The results obtained from the current study showed that the bryophyte *H. involuta* possessed some active ingredients that can be of pharmaceutical importance, as well as exhibiting antimicrobial property on some pathogenic microorganisms. Plants produce secondary metabolites in order to protect themselves from microorganisms, herbivores and insects, thus antimicrobial effect is somehow expected (Vats and Tiwari, 2014).

The presence of alkaloids and cardiac glycosides in *H. involuta* is an indication that this particular plant has medicinal properties. Alkaloids were reported to be also poisonous, but in specific quantity they can be used medicinally (Oyesiku, 2005). Plants that possess alkaloids are pharmacologically active as they have physiological effects and serve as therapeutic and antimalarial drugs (Murugan *et al*., 2012). Cardiac glycosides also have therapeutic applications, being used for treating heart related problems (Seigler, 1998). Saponins were reported to possess expectorant action, which is very useful in the management of upper respiratory tract inflammation; saponin present in plants is cardiotonic in nature (Finar, 1989; Trease and Evans, 1989). Saponin is also reported to have antidiabetic properties (Kamel, 1991). Blazovics *et al*. (1993) reported that some flavonoids have been shown to prevent liver cancer (hepatoma) and to prevent the liver from lipid peroxidative effects in experimental hyperlipidaemia. Flavonoids are used as natural anti-oxidants in foods, medicinal and non-nutritive plant materials due to their ability to inhibit and scavenge reactive oxygen species (Larson, 1988; Kim *et al*., 1990).

Microbial infections pose a health problem throughout the world, whereas plants are a possible source of antimicrobial agents (Burapadaja and Bunchoo, 1995; Adenisa *et al*., 2000). Medicinal plants contain active principles which can be used as an alternative, cheap and effective source of drugs against common bacterial and fungal infections. A number of bryophytes have been identified, classified and reported to express interesting bioactivities (Dulger *et al*., 2009; Sabovljevic, 2011). Thus many researchers have found innumerable kinds of biological activity in compounds from bryophytes (Isa *et al*., 2014).

The antimicrobial efficacy of *H. involuta* extracts tested in the current study in terms of the highest activity for acetone extract was found to be against *Staphylococcus aureus,* while that of the ethanolic extract was found to be against *Aspergillus flavus. Aspergillus flavus* and *Candida albicans* were noted resistant to acetone extract. In the same vein, *Escherichia coli* and *Staphylococcus aureus* were also resistant to ethanolic extract.

In the past, bryophytes have been regarded as having little economic importance and of no pharmaceutical values (Oyesiku, 2005), but, recently, bryophytes have been recognised as a possible source of pharmaceutical activities (Zinsmeister, 1991). Earlier in his work, Flowers (1957) reported the use of mosses in the treatment of burns and open wounds.

In the light of these aspects and the results obtained from the hereby work, *H. involuta* can be recommended as a source of antimicrobial agents in the treatment of diseases caused by *Staphylococcus aureus* and *Aspergillus flavus.*

Table 1. The concentrations of crude acetone and ethanolic extracts

Bottle number	1	2	3	4	5	6
Concentration of crude acetone extract (ppm)	3.330	1.110	0.370	0.123	0.041	0.014
Concentration of crude ethanolic extract (ppm)	1.670	0.557	0.186	0.061	0.020	0.009

Table 2. The results of phytochemical screening of *H. involuta*

Phytochemicals present	Acetone extract	Ethanol extract
Alkaloids	+	+
Anthraquinones	-	-
Cardiac glycosides	+	+
Flavonoids	+	-
Phlobatanins	-	-
Saponins	-	+
Steroids	-	-
Tannins	-	-
Triterpenes	-	-
Xanthoproteins	-	-

Table 3. Antibiotic activity results of *H. involuta* extracts

Tested organism	Resistance to crude extract	
	Acetone extract	Ethanol extract
Escherichia coli	SR	R
Staphylococcus aureus	S	R
Aspergillus flavus	R	S
Candida albicans	R	SR

Key: R = Resistant; S = Sensitive; SR = slightly resistant

Conclusions

The study revealed that *H. involuta* can serve as a potent source of alternative medicine. The range of antimicrobial activity showed by the acetone and ethanol extracts bring evidence that the studied bryophyte might be considered as a potential antimicrobial agent, having a broad spectrum activity. Moreover, extensive studies can provide clues about its possible pharmaceutical exploration.

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