CODEN [USA]: IAJPBB ISSN : 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

SJIF Impact Factor: 7.187
https://doi.org/10.5281/zenodo.7497672

Available online at: http://www.iajps.com *Research Article*

ANTIBACTERIAL ACTIVITY OF ETHANOLIC EXTRACT OF *Hyophila Involuta* MOSS

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Article Received: October 2022 **Accepted:** November 2022 **Published:** December 2022

Abstract:

The frequency of life-threatening infections caused by pathogenic microorganisms has increased worldwide and is becoming an important cause of morbidity and mortality in immunocompromised patients in developing countries. These bacterial infections have a large impact on public health, however, bacterial resistance to antimicrobials is a rapidly growing problem with potentially devastating consequences. In the last decade, with more intensive studies for natural therapies, plants are used for treatment of various diseases especially skin problems and wounds. There has been continuous search for plants having antimicrobial activity, due to a growing concern about increased rate of infection caused by antibiotic resistant micro-organisms. Bryophytes chemical composition is increasingly growing, as high number of biologically-active compounds recently have been found in mosses and liverworts. Hyophila involuta possessed some active ingredients that can be of pharmaceutical importance, as well as exhibiting antimicrobial property on some pathogenic microorganism Many bryophytes have been assessed for secondary metabolites, antioxidant and antimicrobial potential. Many phytochemicals contribute towards the antibiotic properties of bryophytes against both gram-positive and gram-negative bacteria.

Key words: *Antibacterial activity, bryophytes, Hyophila involuta, zone of inhibition*

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Please cite this article in press Josna Priya Lewis et al, ***Antibacterial Activity Of Ethanolic Extract Of Hyophila Involuta Moss..,*** *Indo Am. J. P. Sci, 2022; 09(12).*

INTRODUCTION:

It is unbearable to conceive the existence of human survival, if the earth had no plants. The credibility of human on plants is from the very beginning. Plants are the accustomed authority for the medicines. The use of medicinal plants for the treatment and prevention of various diseases world widely, because of its efficiency and have low toxicity. According to the WHO more than 80% of the world's population relies on plant based herbal medicines for their primary healthcare needs.[1]

Infectious diseases are the leading cause of morbidity and mortality worldwide, especially in developing countries. Infectious diseases are caused by microorganisms, such as bacteria, viruses, parasites, or fungi; the diseases that can be spread, directly or indirectly, from one person to another. [2]

Bacteria are ubiquitous. They play an important role in maintaining the environment in which we live. Only a small percentage of the world's bacteria cause infection and disease. These bacterial infections have a large impact on public health. As a general rule, bacterial infections are easier to treat than viral infections, since the armamentarium of antimicrobial agents with activity against bacteria is more extensive. More so than with infectious diseases caused by viruses and parasites, however, bacterial resistance to antimicrobials is a rapidly growing problem with potentially devastating consequences.[3]

Today's, microbial infections, resistance to antibiotic drugs, have been the biggest challenges, which threaten the health of societies. Microbial infections are responsible for millions of deaths every year worldwide [4].

The long-term use of a single antibiotic (i.e., for more than 10 days) we select for bacteria, that are resistant not only to that antibiotic, but to several others. Under continued antimicrobial selection, the susceptible intestinal and/or skin flora may become colonized by organisms that are resistant not only to the ingested drug, but also to other, structurally unrelated drugs [5]. Hence with increase in resistance of microorganisms to antibiotics, there is a shift of choice from allopathic to ayurvedic and naturopathy, where herbs and spices are very common ingredients of medicine.[6] Herbal medicine is the fulcrum of complementary and alternative medicine, which in recent times is increasingly gaining widespread popularity all over the world and gradually streaming towards integration into the mainstream healthcare system.[7]

Man, since creation, has been dependent on plants for food, shelter, clothing and medicine. Medicinal plants are herbs that contain phytoactive components known to modern and ancient civilization for their healing properties. These medicinal plants used as disease remedies could be in any usual forms such as infusions, decoctions, tinctures, syrups, infused oils, essential oils, ointments and creams.[8]

Herbal extracts can be used directly or indirectly for the treatment of different ailments are termed medicinal plants. These herbs are considered to be a chemical factory of biopharmaceutical as they contain multitudes of naturally occurring chemical compounds [5]. These chemical compounds which may be primary, but often are secondary metabolites. Herbs contain secondary metabolites, which are organic compounds that are also capable of destroying or inhibiting the growth of microorganisms.[6]

Herbal extracts are effective because they interact with specific chemical receptors within the body and are in a pharmacodynamic sense. By using herbal medicines, patients have averted the many side effects that generally come with traditional medicines. Several popular conventional drugs on the market are derived from herbs. These include aspirin (from white willow bark), digitalis (from foxglove), and Sudafed (modelled after a component in the plant ephedra). Herbs may be good alternatives to current treatments for health problems. Herbal medicines had been considered in every culture; however, pharmaceutical companies overturned this type of thinking. [9]

Mosses belong to the simplest land plants. At the same time, they belong to the second largest taxonomic group in the plant kingdom: bryophytes. There are around 25,000 bryophyte species, which can be found in most ecosystems worldwide and include mosses (*Musci* ~ 8000 species), liverworts (*Hepaticae* ~ 6000 species) and hornworts (*Anthocerotae* ~ 1000 species). Due to their small size and identification problems, bryophytes have for a long time been neglected and considered almost useless as a source of biologically-active substances. Now, the situation has changed, and the interest in bryophyte chemical composition is increasingly growing, as a high number of biologically-active compounds recently have been found in mosses and liverworts. Many compounds that have been isolated from bryophytes have shown high biological activity. Therefore, extracts of bryophytes are prospects for the search of new pharmaceutically-active compounds. Well-expressed antibacterial, antifungal and antiviral activities have been

demonstrated in a number of bryophytes, and their cytotoxicity with respect to cancer cells, their antioxidant, antiplatelet, antithrombin, insecticidal and neuroprotective activity, as well as the ability to inhibit a number of biochemically important enzymes, as well as other kinds of activities, have been confirmed in several studies.[10]

Hyophila involuta (Hyophila Moss) is a species of plants in the family *Pottiaceae*. *H. involuta* possessed some active ingredients that can be of pharmaceutical importance, as well as exhibiting antimicrobial property on some pathogenic microorganism [11].

MATERIALS AND METHODS:

Plant Material:

The fresh *Hyophila involuta* moss was collected from local areas during monsoon. The moss was authenticated by Dr. Vijayalakshmi C. Bhat, Asst. Professor, HOD of Botany, Poornaprajna college, Udupi. The freshly collected moss was washed thoroughly to remove all the contaminants. The materials were shade dried and coarsely powdered using mechanical blender.

Preparation of the Extract:

The coarse powder was extracted by cold maceration by soaking with 500ml of ethanol for 1 week with occasional shaking. The resulting extract was filtered through Whatman No.1 filter paper and the filtrate was evaporated to dryness at 40°C on water bath. The extracts were then screened for the presence of secondary metabolites like alkaloids, anthraquinones, cardiac glycosides, flavonoids, steroids, tannins.

Phytochemical screening of the Extract:

A) Carbohydrates: 1ml extract + add 2-3 drops of alpha naphthol. Mix well. Add 2ml of conc. H_2SO_4 from sides of the test tube, reddish violet ring at the junction is formed. Indicates the presence of carbohydrates.

B) Alkaloids:

a) Wagner's Test: 2-3 ml extract + few drops Wagner's reagent, formation of reddish-brown precipitate.

b) Dragendorff's Test: 2-3 ml extract+ few drops. Dragendorff's reagent, formation of brown precipitate. Indicates the presence of alkaloids.

C) Flavonoids:

Lead acetate test: Extract + 10% lead acetate solution, formation of yellow color precipitate. Indicates the presence of flavonoids.

D) Glycosides:

a) Modified Borntrager's test: Plant extract + ferric chloride solution + boil for 5min. + cooled + equal volume of benzene + benzene layer is separated + ammonia solution, formation rose-pink to blood red colored solution. Indicates the presence of anthraquinone glycosides.

b) Keller Killani test: Plant extract + glacial acetic acid + 1 drop 5% $FeCl_3$ + conc.H_2SO_4, formation of reddish-brown color appears at junction of the 2 liquid layers. Indicates the presence of cardiac glycosides.

E) Steroids:

Liebermann- Burchard's test 2mg extract + acetic anhydride, heated to boiling, cooled and then 1ml of concentrated sulphuric acid was added along the sides of the test tube, Formation of green color. Indicates the presence of steroids.

F) Tannins:

Ferric chloride test: Plant extract +5% of $FeCl_3$ solution, formation of deep blue- black color. Indicates the presence of tannins.[11]

Evaluation of Antimicrobial activity:

Test Organisms:

The organisms used to test their growth inhibition by the extracts were *Escherichia coli* and *Staphylococcus aureus* the strains were obtained from the Department of Microbiology, Srinivas college of Pharmacy, Mangalore.

Preparation of media:

The media was prepared by adding 2.8 g of nutrient agar to 100 ml of distilled water and boiled. The solution was sterilized by autoclaving at 121°C for 15 min and cooled. The test microorganisms were inoculated. The inoculated media was transferred into sterile plates. Allowed to cool and solidify under sterile conditions, and then incubated for 24 hours at 37°C to ensure that there was no bacterial contamination.[12]

Agar Well Diffusion Method:

Agar well diffusion method is widely used to evaluate antimicrobial activity of plant extracts. The prepared inoculated media was poured into the agar plate. A hole with the diameter of 6-8 mm is punched aseptically with a sterile borer, and a volume (20-100 µL) of the antimicrobial agent/ extract solution at desired concentration was introduced into the well. The agar plates were incubated at 370C for 24 hours. The antimicrobial agent diffuses in the agar medium and inhibits the growth of microbial strain tested.[12]

Preparation of ointment base:
Hard paraffin, Beeswax, Yellow soft paraffin, Cetostearyl alcohol was placed into an evaporating dish and melted in the decreasing order of their melting point over a water bath, Refer Table No.1. The mixture was continuously stirred to ensure homogeneity, at the same time avoid incorporation of excess air.[13]

Preparation of ointment:
0.6g of *Hyophila involuta* extract was mixed with 9.4 g of ointment base and stirred thoroughly till a homogeneous product is formed, Refer Table No.2. The prepared herbal ointment was placed in ointment tubes, labelled and were stored at room temperature.[13]

Quality Control of Ointment

- **Appearance**: The general appearance of the formulated ointment was observed and recorded. Qualities included colour granular/lumpy surface.
- **Consistency**: A small amount of ointment was slowly rubbed between the thumb and fore finger to gauge consistency of the ointment.
- **Spread ability:** The time taken by the upper slide to slip off was noted. 1g of the herbal ointment was sandwiched between two glass plates and 250g load was applied over the plates. The spread ability was evaluated by measuring diameter.
- **Washability**: A small amount of ointment was rubbed on the back of the hand after which it was washed off with warm water. The time taken was noted.
- **pH of the ointment**: The pH meter was calibrated using standard buffer solution. About 0.5g of the ointment was weighed and dissolved in 50 ml of distilled water and its pH was measured.
- **Viscosity**: Viscosity of ointment was done by using Brooke field viscometer at a temperature of 25 ℃ using spindle No. 64 at 0.3 RPM
- **Homogeneity**: The formulation was tested for homogeneity by visual appearance and touch.

Table No 1: Formulation of ointment base

S. No	Components	Amount (g)
1.	Beeswax	0.5
2.	Hard paraffin	0.5
3.	Cetostearyl alcohol	0.5
4.	Yellow soft paraffin	8.5

Table No 2: Formulation of ointment

S. No	Components	Amount (g)
1.	Extract	0.6
2.	Ointment base	9.4

RESULTS:

Preliminary Phytochemical Screening:
Preliminary phytochemical analysis of the Moss extract revealed the presence of following phytochemicals as shown in Table No 3:

Table No 3: Results of Preliminary phytochemical screening

S. No.	Test	Results
1	Carbohydrate	+
2	Alkaloids	+
3	Anthraquinone glycosides	+
4	Cardiac glycosides	+
5	Flavonoids	+
6	Steroids	+
7	Tannins	-

Antibacterial activity of ethanolic extract of *Hyophila involuta* moss:

The moss extract shows significant antibacterial activity against both Gram-negative *Escherichia coli* and gram-positive *Staphylococcus aureus*. The zone of inhibition ranges from 1.2± 0.02 to 8.46±0.36 which is comparable to zone of inhibition of reference drug chloramphenicol. The zone of inhibition increased on increasing the concentration of the extract. The standard chloramphenicol showed significantly increased zone of inhibition against the tested organism when compared to the extract.

Zone of inhibition is summarized in Table No. 4

Table No. 4: Antibacterial activity of the *Hyophila involuta* moss

Concentration of extract (mg/ml)	**Gram negative *Escherichia coli***	**Gram positive *Staphylococcus aureus***
	Zone of Inhibition(mm) (Mean ± SD)	
20	1.8± 0.025	1.2± 0.02
40	5.2± 0.21*	4.9±0.2*
60	8.46±0.36**	7.9±0.3**
Positive control (Chloramphenicol 0.05%)	16.2±0.16***	16.0±0.16***
Negative control	Nil	Nil
Values are expressed as mean ± SEM. (n=3), $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ between negative control and treated.		

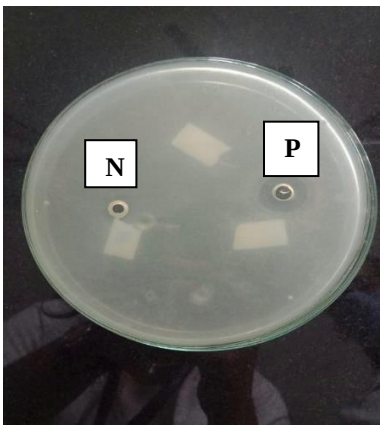

Fig1. Zone inhibition of Standard and control
N -Negative control
P -Positive control

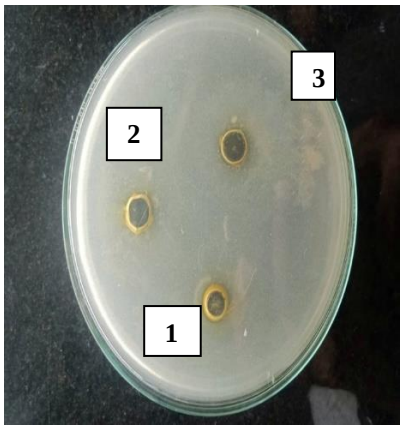

Fig2. Zone of inhibition of ethanolic extract of *Hyophila involuta* moss for *Escherichia coli.*
1 – 20 mg/ml of extract.
2 – 40 mg/ml of extract
3 – 60 mg/ml of extract

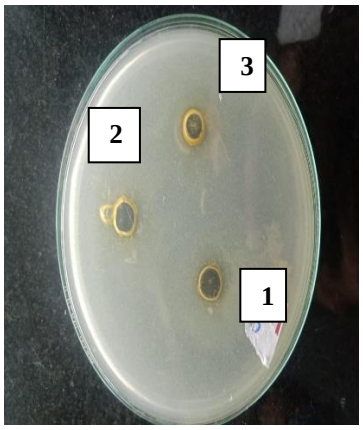

Fig3. Zone of inhibition of ethanolic extract of *Hyophila involuta* moss *Staphylococcus aureus.*
1– 20 mg/ml of extract.
2- 40mg/ml of extract
3- 60mg/ml of extract

Antibacterial Activity of Formulated ointment (*Hyophila involuta* moss):

The moss extract shows significant antibacterial activity against both Gram-negative *Escherichia coli* and gram-positive *Staphylococcus aureus*. It shows significant zone of inhibition against both the test organisms i.e., 4.1±0.3mm and 3.8±0.3mm. The standard chloramphenicol showed significantly increased zone of inhibition against the tested organism when compared to the formulated ointment. The zone of inhibition of the formulated ointment is summarized in Table No. 5.

Table No. 5: Antibacterial activity of formulated ointment

Concentration	**Gram negative** ***Escherichia coli***	**Gram positive** ***Staphylococcus aureus***
	Zone of Inhibition(mm) (Mean ± SD)	
0.6g	4.1±0.3*	3.8±0.3*
Positive control (Chloramphenicol 0.05%)	16.2±0.16***	16.0±0.16***
Negative control	Nil	Nil
*Values are expressed as mean ± SEM. (n=3), *P<0.05, **P<0.01, ***P<0.001 between negative control and treated.*		

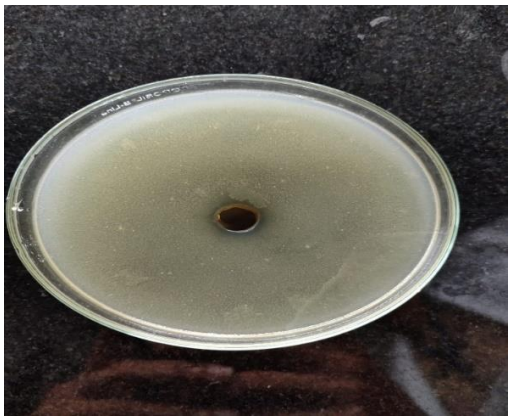

Fig4. Zone of inhibition of formulated ointment against *Escherichia coli*

Fig5. Zone of inhibition of formulated ointment against *Staphylococcus aureus*

Physiochemical Evaluation of Ointment:
The formulated ointment was evaluated for physicochemical and tests and the results were shown in Table 5. The ointment was coloured, having good appearance, and better spreadability. The ointment was greasy and had thicker consistency. The pH of the formulated ointment was found to be 6.7 which was found to be ideal for the skin. The ointment was homogeneous with no lumps as shown in Table No 6.

Table No. 6: Results of Physiochemical evaluation of Ointment

Evaluation test	Results
Appearance	Good
pH	6.7
Color	Moss Green
Spread ability	Good
Viscosity	33000cp
Washability	15 seconds
Homogeneity	No lumps
Consistency	Greasy and Thicker

DISCUSSION:

According to WHO, 80% of the population of developed countries depend on plants or plant products as conventional source of medicine. Thus, there is need for investigation of plants for their properties, safety and efficacy with experimental validation.[14]

Use of medicinal plants has been appreciated due to low cost and lesser side effects. Herbal drugs have been used successfully in the treatment of various ailments over the last few decades. Development of drug resistance in pathogens is one of the major problems in medicine. Natural products derived from the botanicals can be used as a substitute to solve the problem. A number of herbal compounds have been discovered with immense therapeutic potential. Therefore, to meet the potential future demand for various bioactive compounds used as drugs, a new production system is required significantly.[15]

Bryophytes are known to be Lilliputians of plant kingdom. These are mainly found in humid climatic conditions. Bryophytes have been used traditionally in Indian culture and as natural diapers owing to their antimicrobial activity. Bryophytes used by traditional healers for the treatment of boils, burns, eczema, skin disorders, and wounds and are also reported as an antimicrobial, antioxidant, antipyretic, and antidote agent. The use of bryophytes in herbal medicines has been common in China, India, and among Native Americans since ancient times. Numerous

compounds, including oligosaccharides, polysaccharides, sugar alcohols, amino acids, fatty acids, aliphatic compounds, phenyl quinines, aromatic and phenolic compounds occur in bryophytes.[16]
The ethanolic extract of *Hyophila involuta* inhibited the growth of Gram-positive as well as Gram-negative bacteria.

The aim of the study was to determine the antibacterial activity of ethanolic extract of *Hyophila involuta* Moss. The safety profile of antibacterial drugs more closely resembles that of today's cytotoxic, chemotherapeutic agents used in oncology with narrow therapeutic windows and considerable side effects.

Antibiotic resistance is a serious and growing phenomenon in contemporary medicine and has emerged as one of the pre-eminent public health concerns. Research in alternative medicine has begun and one such alternative is use of herbal drugs to treat infections.

Bryophytes are the small group of plants placed between algae and the vascular plant. *Hyophila involuta* Moss belongs to the family *Pottiaceae*. The *Hyophila involuta* Moss was collected during monsoon and dried, extract was prepared using maceration process with 70% ethanol. The extract was then subjected to preliminary phytochemical screening. It revealed the presence of carbohydrates, alkaloids, glycosides, flavonoids and steroids and this implied the antibacterial activity of the extract. The antibacterial activity of the extract was evaluated using agar well diffusion method. Our study showed that the ethanolic extract of *Hyophila involuta* inhibited the growth of both Gram-negative as well as Gram-positive bacteria. The extract was formulated into simple ointment using yellow soft paraffin base, Ointment of *Hyophila involuta* extract was formulated. The antibacterial activity was measured using agar well diffusion method, the antibacterial activity was well maintained when it was converted into ointment formulation. Quality control test of formulated ointment were performed, test for the appearance, spreadability, washability, consistency, Viscosity, pH was performed. Evaluation of antimicrobial activity of the formulated ointment was performed and showed acceptable zone of inhibition. All the extract showed good antibacterial activity, though the antibacterial activity of standard showed comparatively increased effect than the extract. The formulated ointment also showed good antibacterial activity, *Hyophila involuta* Moss is found to be rich in phytoconstituents like alkaloids, flavonoids. The presence of alkaloids, glycosides, flavonoids implied that they may be the active compound which may be responsible for the antibacterial activity in this study. Antibacterial mechanism of flavonoid is due to inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function and inhibition of energy metabolism in bacteria. The antibacterial activity of alkaloids mechanism involves inhibition of bacterial nucleic acid and protein synthesis, modification of bacterial cell membrane permeability, damage of cell membrane and cell wall, inhibition of bacterial metabolism and inhibition of efflux pump. Moreover, the complexation of metal ions from the bacterial growth environment could also be a possible mechanism for their antimicrobial properties.[29]

The study shows that ethanolic extract of *Hyophila involuta* is effective against Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli.* The formulated ointment showed effective antibacterial property. The standard chloramphenicol showed significantly increased zone of inhibition against the tested organism when compared to extract and formulated ointment.

In the past, bryophytes have been regarded as having little economic importance and of no pharmaceutical values, Bacterial infections increase the morbidity and mortality, increase the cost of treatment, and prolong hospital stay adding economic burden on the nation. In recent time, bryophytes have been recognised as a possible source of pharmaceutical activities and moss is reported to be used in the treatments.[26]

CONCLUSION:

The results showed that *E. coli* and *S. aureus* were found to be very sensitive test microorganisms to the test extract. The study showed that ethanolic extract of *Hyophila involuta* Moss showed potential antibacterial activity against both Gram-positive and Gram-negative bacteria. The observed antibacterial activity of the formulations is due to the presence of active constituents in the ethanolic extract of *Hyophila involuta*. The phytochemical screening showed the presence of alkaloids, glycoside, flavonoids and steroids the presence of these phytochemicals in the extract *Hyophila involuta* implied that they are the active compound which may be responsible for the antibacterial activity in this study. The antibacterial activity was maintained even on formulating into ointment. Unfortunately, very few biologically active substances so far obtained from bryophytes have been proved for its economical use, at least in part due to the slow-growing nature and difficulty of culturing bryophytes. And, while their pharmaceutical use

seems promising. Several secondary metabolites have been isolated so far from different species but the mechanisms behind their activity are still widely unexplored. Creation and development of production system by using bryophyte cells could solve the future demand of novel plant-based production system.

ACKNOWLEDGEMENT:

We express our sincere thanks to the Management of Srinivas College of Pharmacy, for providing the necessary facilities to carry out research work. We thank Rajiv Gandhi University of Health Sciences, Bangalore and Vision Group of Science and Technology, Bangalore for providing financial assistance.

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