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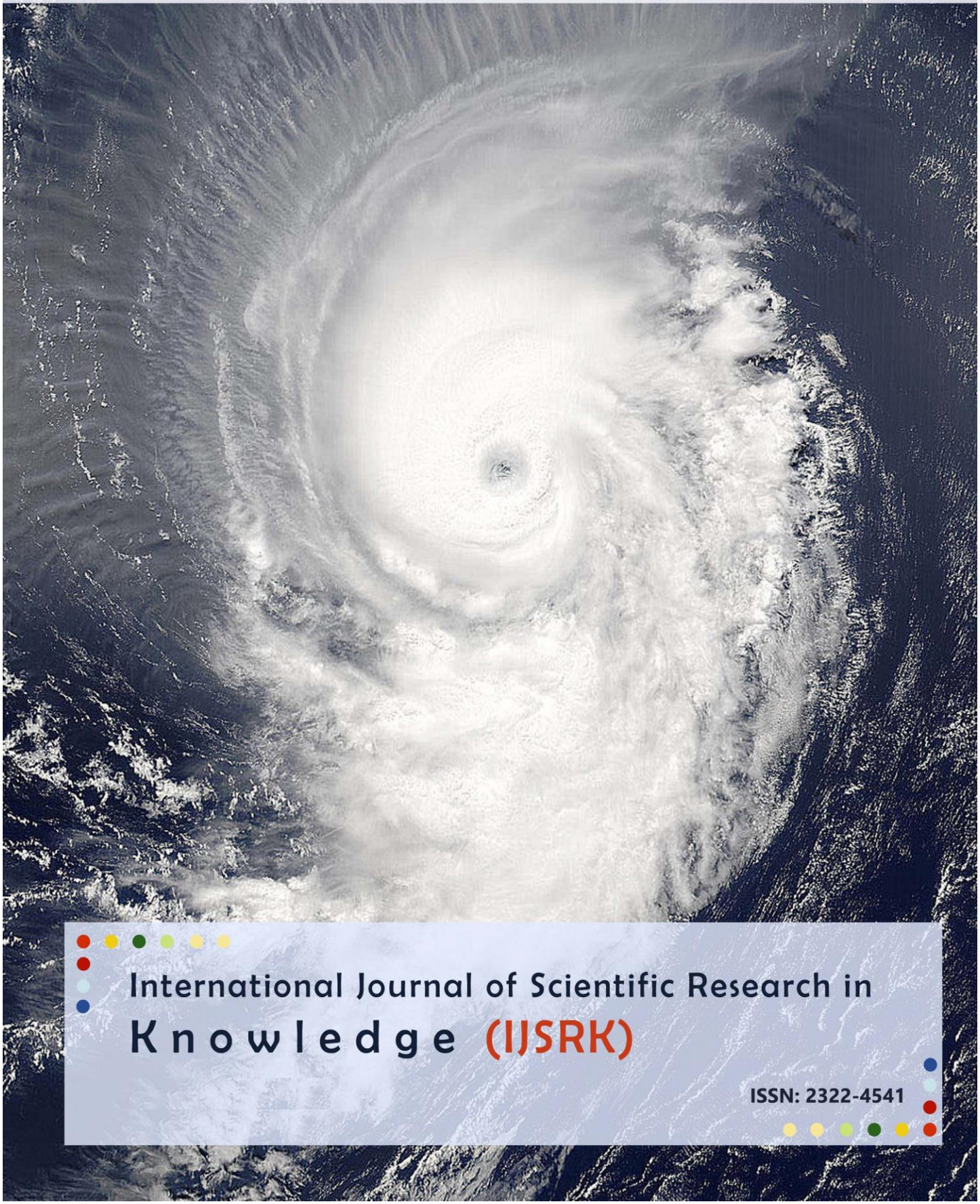
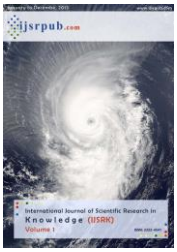


International Journal of Scientific Research in Knowledge (IJSRK)

ISSN: 2322-4541

International Journal of Scientific Research in Knowledge, 4(3), pp. 056-063, 2016
Available online at http://www.ijsrpub.com/ijsrk
ISSN: 2322-4541; ©2016; Author(s) retain the copyright of this article
http://dx.doi.org/10.12983/ijsrk-2016-p0056-0063



Full Length Research Paper

Evaluation of Phytochemicals, Antioxidant and Antibacterial Activity of *Hyophila involuta* (Hook.) Jaeg. and *Entodon plicatus* C.Muell. (Bryophyta) from Rajasthan, India

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Received 27 January 2016; Accepted 14 April 2016

Abstract. Plants are the ultimate source of drugs against many communicable diseases since time immemorial. Multitudinous drugs have been obtained from countless plant species. However, invariably angiosperms were the preferred choice of most of the workers and other plant groups remain somewhat unexplored in this direction. Hence, the aim of the present study was to examine the phytochemicals, antioxidant and antibacterial activity of two such neglected mosses (bryophyta), *Hyophila involuta* (Hook.) Jaeg. and *Entodon plicatus* C. Muell. (Bryopsida). In preluded phytochemical analysis cardiac glycosides, flavonoids, saponins, anthroquinone, terpenoids, tannins, phenolic, proteins, fats and fixed oils were observed by using standard tests. It was found that *E. plicatus* contains more amount of phytochemicals than *H. involuta*. The antioxidant activity of both the plants was also determined according to standard protocols and appealing results were found. Comparative analysis was done for the activity of Catalase, Peroxidase, Ascorbate peroxidase, Glutathione reductase and Superoxide dismutase and interesting observations were made. Antimicrobial activity of methanolic extracts of both the plants was evaluated against *Bacillus subtilis*, *B. cereus* and *Escherichia coli* by using the Agar well diffusion method. Extract of *H. involuta* showed a greater inhibitory activity than *Entodon plicatus*. *Bacillus* spp. (gram+ve) were found more affected than *E. coli* (gram-ve).

Keywords: Antibacterial, Antioxidant, Bacteria, Mosses, Phytochemicals

1. INTRODUCTION

Communicable bacterial diseases are the world's primary reason of untimely deaths, killing almost 50,000 people every day. In recent past, resistance against drug to many pathogenic bacteria has been frequently reported from all over the globe. The foul and arbitrary application of antimicrobial compounds is the main factor accountable for the manifestation of the incident of bacterial resistance to such compounds. With the augmented occurrence of resistance to common antibiotics, natural products from plants could be appealing choice. Numerous plant extracts and phytochemicals are now known to have remarakable antimicrobial properties, and can be of immense implication in remedial actions (Alam et al., 2015). Consequently, during last few years, a number of studies have been performed in different countries to exhibit such efficiency, including India (Vats and Alam, 2013; Bishnoi et al., 2016). Conversely, free radicals are recognized to be the major reason of a variety of persistent and degenerative diseases. Oxidative stress is linked with pathogenic mechanisms of many diseases, in addition to aging courses. It is defined as an unevenness between generation of free radicals and reactive metabolites (oxidants), and it also includes their removal by defensive mechanisms, known to as antioxidative mechanisms. This unevenness leads to smash up of essential biomolecules and organs with impending blow on the entire organism. Antioxidants can holdup, slow down or avert the oxidation of oxidisable suctances by hunting free radicals and thinning the impact of oxidative stress (Prassas and Diamandis, 2008). Natural antioxidants have been studied comprehensively for decades to find out those compounds which can provide safeguard against a many diseases associated to oxidative stress and damages induced by free radicals. So far, lots of plants have been documented to have valuable health belongings such as antioxidant assets. According to World Health Organization (WHO), something like 65 - 80% of the earth populations depend upon the conventional remedy to cure different diseases (Kaur and Arora, 2009).

Brophytes, the first land plants, also known as amphibians of plant kingdom, earlier used sporadically by the native to cure many diseases (Alam et al., 2015). Though, they are always known for their antimicrobial potential, up till now convincing proofs were somewhat missing. But now these plants are now emerging as novel source of natural remedies and researchers consider these plants as a reservoir of novel medication. Consequently, they have been extensively studied in respect to their phytochemistry. Accordingly, many bryophytes have been assessed for secondary metabolites, antioxidant and antimicrobial potential (Asakawa, 2007). The presence of remarkable phytochemicals confers that these plants have many biologically active compounds which are actively play a part in the defense mechanism including microbial infections (Beike et al., 2010). Although, many attempts have been made in this direction but lots of bryophytes (liverworts, hornworts and mosses) are still unexplored. Compared to angiosperms, only a handful of publications are available on bryophytes till date (Adebiyi et al., 2012). Hence there is a need to evaluate more and more bryophytes for the identification of biologically active compounds. Taking into consideration two moss species of Rajasthan (India) have been analyzed for their phytochemicals, antioxidant and antibacterial activity and obtained results are interesting. The findings of this study are useful in new drug development against many communicable pathogenic diseases. The study highlights the importance of these miniature sized plants as an exceptional reservoir of novel phytochemicals that can be assessed further particularly in the field of ethnomedicine and pharmacology.

2. MATERIALS AND METHODS

2.1.1. Plant material

Plant materials for this study were collected from the Mount Abu, Rajasthan, altitude 1400 m; 72.7083°E 24.5925°N, during July and august, 2014. Voucher specimens nos.: BURI-7860307 (*Entodon plicatus*); BURI-7860318 (*Hyophila involuta*); Legit.: A. Alam and S. C. Sharma; Det.: A. Alam) are deposited in the Banasthali University Rajasthan India (BURI) Herbarium, Banasthali Vidyapith, India.

2.1.2. Test Microorganisms

The pathogenic bacteria were obtained from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh. *Escherichia coli* (Migula) Castellani *et* Chalmers (MTCC 118); *Bacillus subtilis* (Ehrenberg) Cohn (MTCC 619) *Bacillus cereus* Frankland *et* Frankland (MTCC 430).

2.1.3. Preparation of extract

The collected plant samples were washed properly and stored at -80^{o} C. Prior to use, the samples were dried and grounded. The powdered material (10g) was extracted exhaustively using continuous extraction method with 200 mL methanol in a soxhlet extractor (Idris et al., 2009). The dried crude extracts were then dissolved in methanol and then tested for different phytoconstituents. The plant extracts were subjected to different tests using standard protocols.

2.2. Biochemical assays

2.2.1. Total chlorophyll content

The amount of chlorophyll was estimated using technique described by Arnon (1949) with some modifications. Absorbance was taken at 645nm, 652nm and 663nm.

2.2.2. Total carbohydrate content

For carbohydrate estimation fresh plant sample will be collected and processed by anthrone method (Dubois et al., 1951).

2.2.3. Total protein content

0.5g of the sample was ground well using mortar pestle in 5-10 mL of the buffer. The homogenate was centrifuged and the supernatant was used for protein estimation. Absorbance was measured at 660nm and amount of protein was calculated from the standard graph (Lowry et al., 1951).

2.2.4. Lipid peroxidation

0.3g of the sample was homogenized in 10 mL of 0.25% TBA which was prepared in 10% TCA. The extract was heated at 95^{o} C for 30m and them cooled rapidly. The content was centrifuged at 10000rpm for 10m and absorbance was taken at 600nm and 532nm.The level of lipid per oxidation is expressed in molar MDA/g fresh weight by using the extinction coefficient of 155mM^{-1}cm^{-1} (De Vos et al., 1989).

2.3. Enzyme extracts preparation

Plant materials were washed properly and then homogenized under ice cold conditions in 3 mL of extraction buffer. Extraction buffer contains 1mM EDTA, 0.05% Triton-X 100, 2% PVP and 1mM

ascorbate in 50mM phosphate buffer of pH 7. The obtained homogenate was centrifuged for 20m at 10000 rpm (4^o C). The resultant supernatant was used for enzyme assays.

2.3.1. Catalase activity

CAT activity was measured by the decrease of light absorption at 240nm, caused by the decomposing activity of catalase on hydrogen peroxide (Aebi, 1974). 0.1 mL of enzyme extract was made up to 3 mL with assay mixture containing 2.8 mL of 50mM of phosphate buffer and 0.1mL of 3.125mM H_2O_2. CAT activity was calculated by taking extinction coefficient 0.039mM^{-1}cm^{-1}.

2.3.2. Ascorbate peroxidase activity

CAT activity was assayed by the method described by Chen and Asada (1990). Activity of APX was determined by the decrease in absorbance at 290nm, caused by H_2O_2 dependent oxidation of ascorbic acid. 0.1 mL of enzyme extract was added to the reaction mixture containing 0.5mM ascorbic acid in 2.8 mL phosphate buffer (pH 7.2) and 0.1mL H_2O_2.

2.3.3. Superoxide dismutase activity

SOD activity was determined by measuring its ability to inhibit the reduction of nitro blue tetrazolium (NBT) (Beauchamp and Fridovich, 1971). The absorbance was read at 560nm.

2.3.4. Glutathione reductase activity

GR activity was measured according to Kocsy et al. (2000) with slight modifications. Absorbance was taken at 412nm for 3m (extinction coefficient 6.2mM^{-1}cm^{-1}).

2.3.5. Peroxidase activity

POD activity was determined by the method used by Ghamsari et al. (2007) with slight modifications. 20µl of enzyme extract was added to the reaction mixture containing 2.5 mL of 50mM potassium phosphate buffer (pH 6.1), 1 mL of 1% H_2O_2 and 1 mL of 1% guaiacol. The absorbance was recorded at 420nm using extinction coefficient of 26.6mM^{-1}cm^{-1}.

2.4. Quantitative analysis of various bioactive compounds

2.4.1. Total flavonoid content

Total flavonoid content was determined by using the method described by (Sakanaka et al., 2005) with some modifications (Srinivasan et al., 2014). The flavanoid content was determined by aluminum chloride method using quercetin as standard. Extracts and quercetin were prepared in methanol (1mg/mL). The absorbance was measured at 425nm using a spectrophotometer. Results were expressed as mg of quercetin equivalent per gram of dry weight (mg QE/g) of extracts.

2.4.2. Total phenolic content

Total phenolic content determined using the method of Singleton and Rossi (1965). Methanolic solution of the samples and gallic acid were produced in a concentration of 1mg/mL. The reaction mixture was prepared by mixing 0.5 mL methanolic solution of extract, 2.5 mL of 10% Folin-Ciocalteu reagent dissolved in water and 2.5 mL of 7.5% $NaHCO_3$. For Blank, 0.5 mL methanol was used instead of the plant samples. The samples were incubated in a thermostat at 45^o C for 45m. The absorbance was measured at 765nm. Results were expressed as mg of gallic acid equivalent/g of dry weight (mg GA/g) of extracts.

2.5. Antibacterial assays

The extracts so obtained were subjected to solvent evaporation and then dried in desiccator. Three different concentrations (10mg/mL, 20mg/mL, 30mg/mL) of the plant extract were prepared using methanol. Cultures of *Bacillus subtilis, Bacillus cereus, Escherichia coli* were revived by inoculating culture vials containing 10 mL of autoclaved Nutrient Media (for *Bacillus subtilis* and *Bacillus cereus*) and Luria broth (for *Escherichia coli*). This was done in aseptic conditions under Laminar Air Flow. The culture vials then incubated in an incubatory shaker at 37^o C for 24h. Turbidity was observed in the broths that indicates proper growth of the bacterial strains. Revived culture strains were then stored in cold room to inhibit their over growth. Before the screening process, revived culture were taken out of the cold room and then kept in an incubatory shaker for 30m for the activation of the strains. The activated strains then used for inoculums. After solidification of the media, 100µl of bacterial suspension was uniformly spread on the agar plate using a sterile glass spreader. 3-4 mm deep wells were punched with the help of a well puncher on the freshly seeded agar plates. 20µl of the plant extract was put into the well using micropipette. For positive control sterile discs of Streptomycin were used. For negative control, solvent was put into the wells. The plates were incubated at 37^o C for 24h. Finally, antibacterial activity of the

plant extracts was observed by the observation and measurement of the zone of inhibition around the wells.

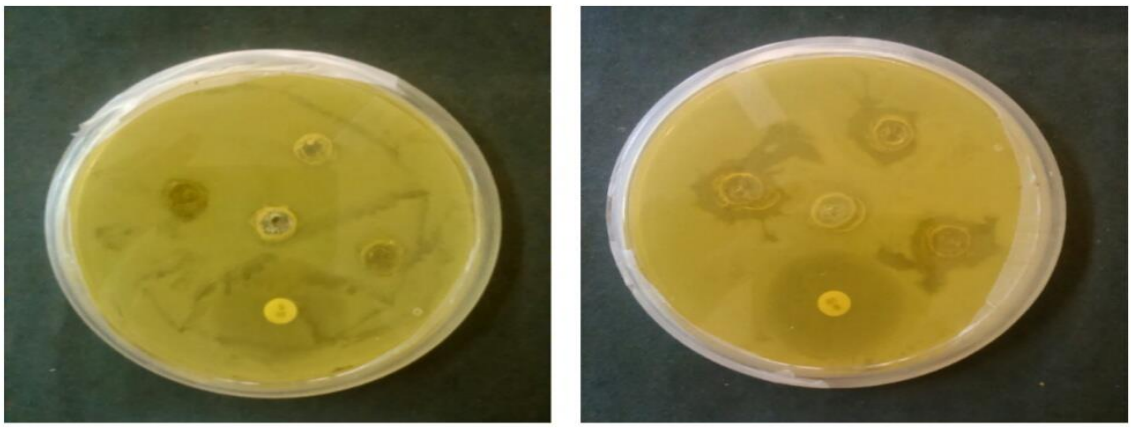


Plate 1: Comparative study of antibacterial activity of methanolic extracts of *Entodon plicatus* (left) and *Hypohila involuta*(right)

Table 1: Comparative analysis of phytochemical screening

		Presence or Absence	
Phytochemical	**Test**	***Hyophila involuta***	***Entodon plicatus***
Amino Acids	Millon's test	+	+
Carbohydrate	Molisch's test	+	+
Fats	Saponification test	+	+
Flavanoids	Shinoda test	+	+
Anthraquinone	Borntrager's test	+	+
Cardiac Glycosides	Kellar- Killani test	+	+
Saponin glycosides	Froth formation test	-	-
Tannins	Ferric Chloride test	+	+
Proteins	Xanthoprotein test	+	+
Steroids	Salkowski test	+	-
Terpenoids	Salkowski test	-	+
Alkaloids	Dragendorff's reagent test	+	+

Table 2: Quantitative analysis of flavonoids and phenolics

	Hyophila involuta	*Entodon plicatus*
Flavanoid content (in mg/g) (Sakanaka et al., 2005)	198.6	146
Phenolic content (in mg/g) (Singleton et al., 1965)	161	218

Table 3: Antibacterial activity of various plant extracts against the bacterial species

Sr. No.	Plant	Concentration of the extract	*B. subtilis*	*B. cereus*	*E.coli*
1	*H. involuta*	10mg/mL	+	+	+
		20mg/mL	+	-	+
		30mg/mL	+	+	+
2	*E. plicatus*	10mg/mL	-	+	-
		20mg/mL	-	+	-
		30mg/mL	+	-	-

3. RESULTS AND DISCUSSIONS

Enzyme extracts of both mosses were used to perform various tests to determine the presence of antioxidant enzymes in it. The analysis showed the presence of various antioxidants' activity in these plants via. CAT, APX, SOD, GR and POD. The activity of CAT, SOD and GR was reported higher in *E. plicatus* (Plate 2; Figs. 1, 3, 4), while activity of POD and SOD was found more in case of *H. involuta* (Plate 2; Figs. 2, 5). The occurrence of these enzymes confirms the free radical scavenging potential of bryophytes that is comparable to Vitamin C, which aggressively reacts with free radicals and retard the formation of hydro peroxides (Manoj and Murugan, 2012; Mukhopadhyay et al., 2013).

Protein content was reported more in *Hyophila involuta* as compared to *Entodon plicatus* (Plate 3; Fig. 6). Lipid peroxidation was found higher in *Entodon plicatus* compared to *H. involuta* (Plate 3; Figs. 7). Total chlorophyll was reported higher in *Entodon plicatus*, consequently, the amount of carbohydrate is also higher in *Entodon plicatus* (Plate 3; Figs. 8, 9).

Methanolic extracts of the both the studied bryophytes were screened for antibacterial activity against both Gram positive (*B. subtilis and B. cereus*) and Gram negative (*E. coli*) bacterial strains. Both the plants show substantial antimicrobial activity and clear zone of inhibition were seen (Plate 1). However, the comparatice results showed that *H. involuta* was found more active against all the three bacterial strains even at lower concentration (10mg/mL), indicating that more quantity of secondary metabolites and elevated antioxidant enzymatic activity are the possible reasons for this enhanced inhibitory response (Xie and Lou, 2009).

4. CONCLUSION

The present study on the selected mosses gives the valuable information about these plants with respect to the phytochemicals, antioxidant and antibacterial activity. The phytochemical screening reveals the presence of alkaloids, flavanoids, phenols etc., that can play an important role in pharmaceutical preparations where these can be exploited for the manufacturing of novel drugs related to antioxidant and antibiotic production. Biologically active constituents like alkaloids, flavonoids, phenols, antraquinone glycosides and steroids were present in both the plants while saponin glycosides was absent in both the plants and terpenoid was present only in *Entodon plicatus* (Table 1).

Further, quantitative estimation of both plants has indicated comapartively higher content of phenols and flavanoids; interestingly, flavonoid content was higher in *Hyophila involuta* while Phenolic content was greater in *Entodon plicatus* (Table 2).

Figure 1

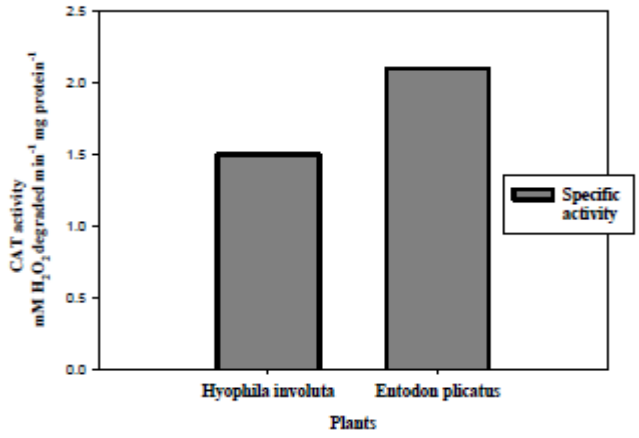


Figure 2

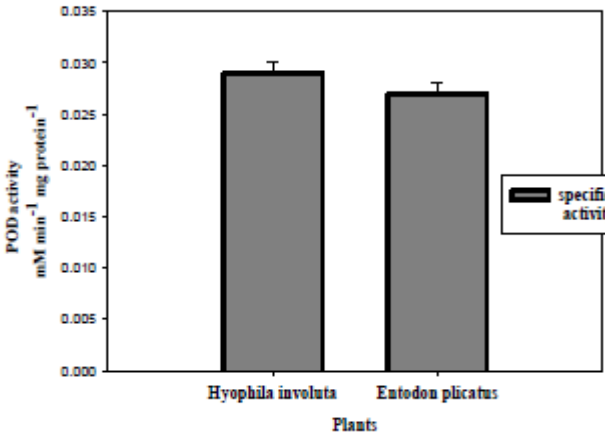


Figure 3

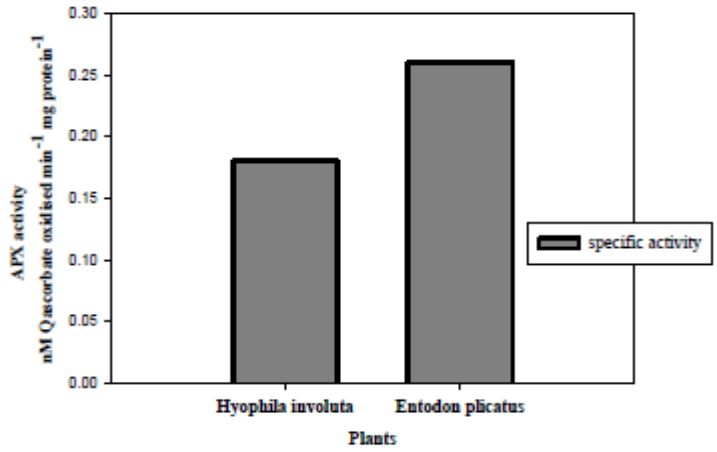


Figure 4

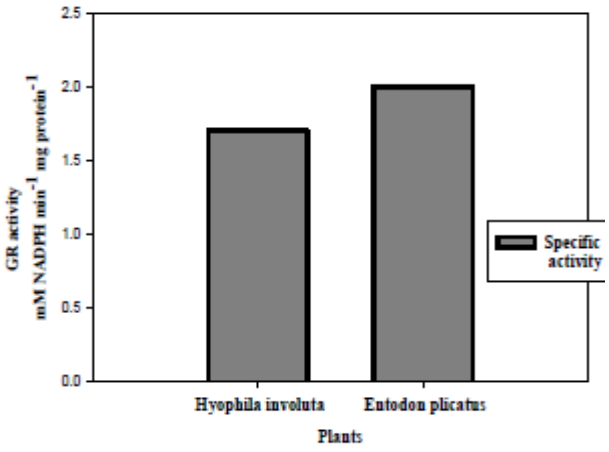
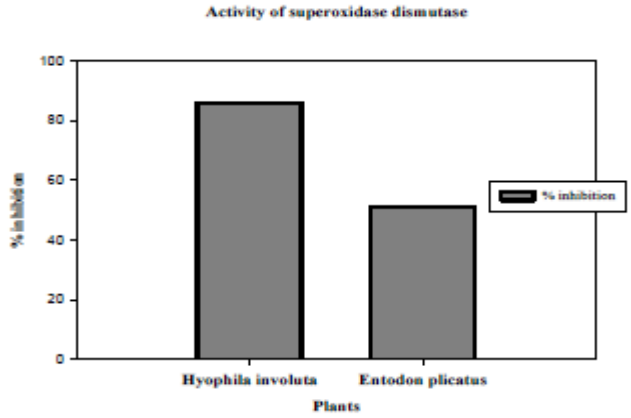
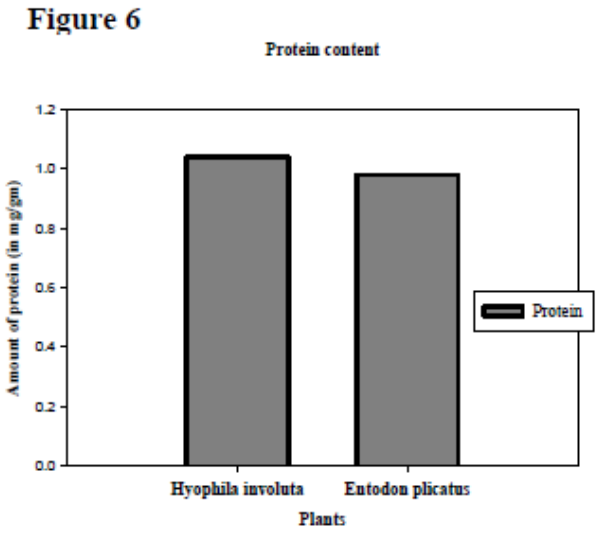


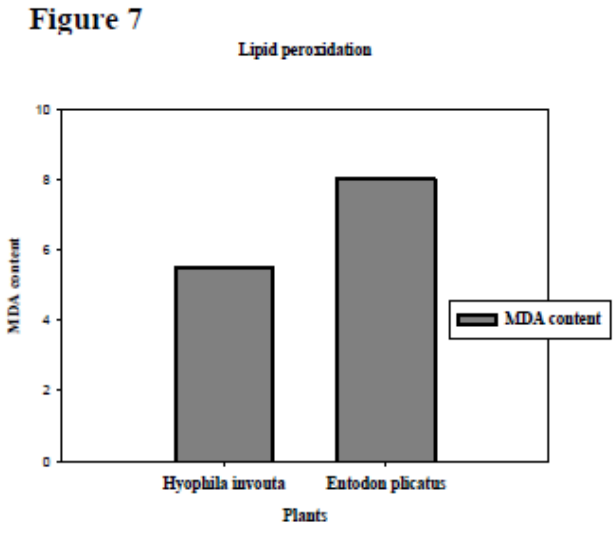
Figure 5

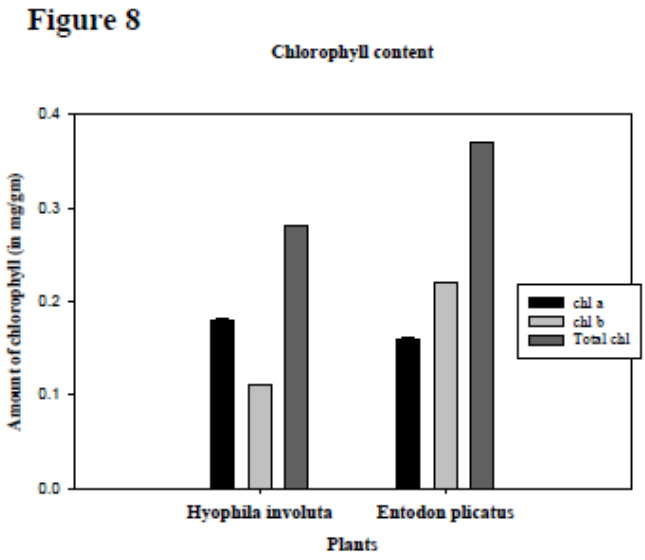


Biochemical analysis of primary metabolites

Plate 2: Fig 1- Catalase activity in *H .involuta* and *E. plicatus*, Fig 2- Activity of peroxidase enzyme in *H. involuta* and *E. plicatus*, Fig 3- Activity of ascorbate peroxidase in *H. involuta* and *E. plicatus*, Fig 4- Activity of glutathione reductase in *H. involuta* and *E. plicatus*, Fig 5- Activity of superoxide dismutase in *H. involuta* and *E. plicatus.*







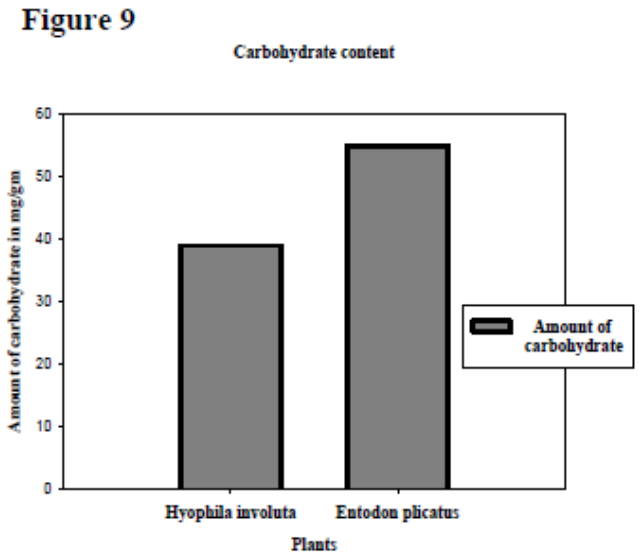


Plate 3: Fig 6- Amount of total protein in *H. involuta* and *E. plicatus,* Fig 7- Amount lipid peroxidation in *H. involuta* and *E. plicatus,* Fig 8- Amount of total chlorophyll content in *H. involuta* and *E.plicatus,* Fig 9- Amount of total carbohydrate in *H. involuta* and *E. plicatus.*

Presence of steroids is an asset due to its importance in few compounds that are used as sex hormones. Hence, the moss species can be seen as a probable source of valuable medicinal preparations and drug additives equally in traditional medicine system and pharmaceuticals production.

ACKNOWLEDGMENTS

The authors are thankful to Prof. Aditya Shastri, Vice Chancellor, Banasthali University, Rajasthan and Prof. Vinay Sharma, Dean & Head, Faculty of Science and Technology, Banasthali University for providing necessary facilities for this work

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