International Journal of Ecology and Environmental Sciences 49: 375-379, 2023
ISSN: 2320-5199 (Online); https://doi.org/10.55863/ijees.2023.2700

Antifungal Activity of Leafy Liverworts Against Selected Plant Pathogenic Fungi

NEHA BINWAL[1], PRACHI JOSHI[2*], S.D. TEWARI[1], SARASWATI BISHT[1], MANJU KANDPAL[3], RUCHI JALAL[1] AND NEHA KOHLI[1]

[1]*Department of Botany, Indira Priyadarshini Govt. Girls P.G. College of Commerce, Haldwani, Nainital, Uttarakhand, India.*

[2]*Department of Botany, SSJDWSSS Government P.G. College Ranikhet, Almora, Uttarakhand, India*

[3]*Department of Chemistry, Government Degree College Daniya, Almora, Uttarakhand, India*

E-mail: nehabinwal171@gmail.com (NB); prachi.pragya21@gmail.com (PJ); shiv.tew@gmail.com (SDT);saraswatibisht.4321@gmail.com (SB); manjukandpal22@gmail.com (MK); ruchijalal13@gmail.com (RJ); nehakohli633@gmail.com (NK)

***Corresponding Author**

ABSTRACT

Bioactivity of bryophytes is one of the most required area of research nowadays due to their rich phytochemical potential. Antifungal activity of selected leafy liverworts was tested against the phytopathogenic fungi viz, *Fusarium oxysporum, F. solani* and *Rhizoctonia solani* by using the agar well diffusion method. The extract of *Plagiochila asplenioides* and *Heteroscyphys hyalinus* were prepared in different solvents (ethanol, methanol, acetone and chloroform). Interestingly, the ethanol and acetone extracts were found most effective followed by methanol and chloroform. The ethanolic extract of *H. hyalinus* possessed the highest antifungal activity against *R. solani* with percent inhibition (85±1.5%) and acetone extract of *P. asplenioides* showed the highest inhibitory activity against *F. oxysporum* (51±2.5%). The percent inhibition activity was also compared by using carbendazim. In future, these bryophytes may be utilised as potent bryo-fungicide against the plant pathogenic fungi. The antifungal activity of *P. asplenioides* and *H. hyalinus* against selected pathogenic fungi is being presented for the first time.

Key words: Antifungal activity, Liverworts, Phytopathogens, Solvents

INTRODUCTION

Bryophytes are naturally occurring surface dwelling small plants represented by approximately 34,556 species accepted worldwide (Plant List 2013), representing the second largest group in the plant kingdom after the Angiosperms (Mishler 2001). Generally, these plants are not known to be infected by either bacteria or fungi, nor do they get damaged by insects and other animals due to the presence of several complex organic compounds (McCleary and Walkington 1966). The secondary metabolites synthesised by the plants have been variously used as drugs, and there is an increasing demand for these natural products. In contrast to the extensive utilization of bioactive compounds obtained from higher plants, bryophytes have not been extensively studied, despite of their rich bioactive potential. The reason lying behind is, their very small size, intermingled population, difficulty in the collection, segregation and finally identification due to lack of bryologist with trained taxonomic eyes.

A great wealth of information is available today from widely scattered parts of the world on bryophytes for their biologically active compounds and antimicrobial activity, (Asakawa 2008, Veljic et al. 2010, Ulka et al. 2010, Azuelo et al. 2011, Asakawa et al. 2013, Yayintas and Irkin 2018, Ludwiczuk and Asakawa 2018, Commisso et al. 2021, Dziwak et al. 2022). However, in India in spite of the rich bryodiversity of both liverworts and mosses, very little information is available related to the phytochemical potential of these naturally occurring poikilohydrous group of plants (Banerjee and Sen 1979, Saxena and Harinder 2004, Alam et al. 2011, 2015, Bandyopadhyay and Dey 2022). From Kumaun region of Western Himalaya very scattered information is available regarding the screening of antimicrobial activity of bryophytes (Dubey et al. 2001, Mewari et al. 2007, Vashistha et al. 2007,

Dhondiyal et al. 2013, Negi and Chaturvedi 2016, Negi et al. 2018, 2020). Keeping in view, the limited information available on the antifungal potentiality of Kumaun Himalayan bryophytes, the present study was undertaken to investigate the antifungal activity of two selected leafy liverworts, viz., *Plagiochila asplenioides* (L.) Dumort. (Plagiochilaceae) and *Heteroscyphys hyalinus* (Stephani) A. Srivast. & S.C. Srivast. (Lophocoleaceae) against selected plant pathogenic fungi.

MATERIAL AND METHODS

Collection of bryo samples

The collection of selected desired leafy liverworts was made from various areas of the Nainital district in the Kumaun region of Western Himalaya within an altitudinal range of 1000-2000 m. The collected samples were brought to the laboratory in sealed polyethylene bags. The pure leafy shoots were morphologically segregated and further examined under the microscope for proper identification. The voucher specimens are kept in the laboratory for future reference.

Micro-organisms selected

In the present study three plant pathogenic fungi namely, *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen, *Fusarium solani* (Mart.) Sacc., and *Rhizoctonia solani* J.G. Kuhn were selected for testing the antifungal potential of two leafy liverworts viz., *P. asplenioides* and *H. hyalinus*. Pure culture of plant pathogens was prepared from the infected part of the plant showing prominent disease symptoms. Symptomatic plant part was carefully brought to the laboratory and surface sterilized using 70% ethanol followed by sterile water. The plant parts showing symptoms were cut into square-shaped bits and transferred aseptically to the Potato Dextrose Agar (PDA) medium in a Petri plate. These plates were incubated at 25 ± 2°C for 3-4 days. The fungal mycelium grows out of the plant tissue and then the fungal mycelium was transferred to fresh medium in a Petri-plate for preparing the pure culture. The identification was done based on morphological characters and then by molecular analysis of all the fungi outsourced to Bio Edge Solutions, Bangalore, India, using 18s rRNA ITS sequencing.

Preparation of plant extracts

The segregated samples were washed thoroughly under the running tap water and shade dried at room temperature. The samples were crumbled into powder using an electric grinder. The powdered plant samples were extracted in different polar (ethanol, methanol, acetone) and non-polar solvents (chloroform) 10 gm/100 ml accordingly. The materials (plant powder + solvent) were shaken on a rotatory shaker at 280 rpm for 24 hrs and then the mixtures were filtered with Whatman filter paper no.1. Crude extracts were stored aseptically in airtight containers for future use.

Antifungal assay

The antifungal activity of selected leafy liverworts extract was tested against the plant pathogenic fungi using the "Agar Well" technique (Joshi et al. 2018, Grover and Moore 1962). Sterile PDA medium was poured into the Petri plates. After solidification of the medium, an appropriate well was made on an agar plate by using a cork borer of 9 mm, and 200 µl of the extract was added to each well. A mycelial disk of 9 mm was cut from the periphery of the actively growing colonies of the tested fungi and aseptically placed 2.5 cm apart from the well in the assay plates. Standard antifungal drug carbendazim 50WP was used as a positive control in the experiment and respective solvents as a negative control. Percent inhibition of fungal growth was analysed by measuring the radial growth of the test fungi after 4-5 days of incubation in 2 directions: R1 (radius in the opposite direction of the well) and R2 (radius in direction of the well filled with plant extract) as suggested by Joshi et al. (2018). Percent inhibition was calculated by using the formula:

R1-R2/R1 x 100.

All the experiments were performed in triplicates. Values were expressed as mean ± Standard Deviation. In all 24 sets of experiments were performed.

Determination of activity index (AI) and proportion index (PI)

The activity index (AI) and proportion index (PI) of the plant extract were calculated by using the following formula (Borkataky et al. 2013).

Table 1. Sensitivity results of *P. asplenioides* and *H. hyalinus* extracts

Solvents	Bryo extract of *H. hyalinus*			Bryo extract of *P. asplenioides*		
	F. oxysporum	***F. solani***	***R. solani***	***F. oxysporum***	***F. solani***	***R. solani***
Ethanol	+	+	+++	+	+	++
Methanol	-	+	+	+	+	+
Acetone	+	+	++	++	+	+
Chloroform	NT	NT	NT	+	+	-
Carbendazim	++	+	++	++	+	++

+ means Active**,** + means <50%**,** ++ means >50%**,** +++ means >80**,** - means No Inhibition**,** NT- Not Tested

Table 2. Antifungal activity of selected bryo-extracts representing percent zone of inhibition ± SD

S.No.	Botanical name	Extracts	***F. oxysporum***	***F. solani***	***R. solani***
1.	*Heteroscyphus hyalinus*	Ethanol	44±0.5	48.4±2.5	85±1.5
		Methanol	NA	48±0.5	36±0.9
		Acetone	46±1.1	31±0.5	51±1.5
		Chloroform	NT	NT	NT
2.	*Plagiochila asplenioides*	Ethanol	37±1	40±1.45	50.8±1.5
		Methanol	30±0.3	40±1.9	23±3.8
		Acetone	51±2.5	43±1.7	31.2±0.9
		Chloroform	24±2.5	20±1.73	NA
3.	Carbendazim (Bavistin)		52.4±1	47.3±2.5	54.3±0.6

NA= Not Active, NT= Not Tested

Activity index (AI) = Mean zone of inhibition of the extract / Mean zone of inhibition of Standard antibiotic drug
Proportion index (PI) = Number of positive results obtained for extract / total number of tests carried out for each extract.

RESULTS AND DISCUSSION

The organic extracts obtained from the two leafy liverworts *P. asplenioides* and *H. hyalinus* were tested by using Agar Well Method against three plant pathogenic fungi namely- *F. oxysporum, F. solani,* and *R. solani.* The results obtained are given in Tables 1 and 2. It was observed that all the organic extracts of *P. asplenioides* and *H. hyalinus* exhibited significant antifungal activity against almost all the tested fungi. The highest antifungal activity was reported in the ethanolic extract of *H. hyalinus* against *R. solani* (85±1.5%) (Table 2). Interestingly, the percent inhibition was found to be significantly higher than that of the standard antifungal drug Carbendazim (54.3±0.6%) used as a positive control. The acetone extract of *P. asplenioides* against *F. oxysporum* (51±2.5%) and *H. hyalinus* against *R. solani* (51±1.5%), respectively, showed the second highest antifungal inhibitory activity (Table 2). The antifungal potential of the extracts was also compared to the standard drug by the activity index (AI) the highest activity index was observed in the acetone extract of *P. asplenioides* against *F. oxysporum* (AI=0.97), followed by ethanolic extract of the same plant against *R. solani* (AI=0.93) (Table 4). The highest activity index of *H. hyalinus* was found in ethanolic extract against *R. solani* (AI=1.5) followed by the methanolic extract against *F. solani* (AI=1.01) (Table 3). The antifungal potential of both the leafy liverworts was also compared in terms of the Potential Index (PI). Both *P. asplenioides* (PI = 0.88) and *H. hyalinus* (PI = 0.91) exhibited approximately equal potency in inhibiting the tested fungi. In the present investigation the activity order of selected plants was observed to be as *H. hyalinus* >*P.*

asplenioides.

The results obtained from the experiments showed that the selected leafy liverworts viz., *H. hyalinus* and *P. asplenioides* extracts exhibited antifungal properties against the selected plant pathogenic fungi nearly equal to that of the commercial fungicide (carbendazim). Further, more future studies are needed to determine the chemical composition of the bioactive compounds present in the plants which are responsible for the observed antifungal activity. The bioactivity of the plant extract may not be due to the action of a single active compound, but may be due to the mutualistic effect of several compounds that are present in minor proportion in a plant (Davicino et al. 2007). The choice of solvents used for the extraction procedure and the method used for preparing the extract of the plant affects the type of compounds extracted from the plant's material and hence, affects the zone of inhibition. Literature survey indicates that from Kumaun region of Western Himalaya the antifungal activity of these two leafy liverworts is being presented for the first time.

CONCLUSIONS

The results obtained from this study on the evaluation of the antifungal potential of these leafy liverworts will draw a line for future investigation on how to use bryophytes in the protection of crops against fungal diseases.

ACKNOWLEDGEMENTS

The authors are grateful to the Principal, Indira Priyadarshini Government Girls Post Graduate College of Commerce Haldwani for providing the necessary laboratory facilities. One of the authors NB is thankful to her research colleagues for their help in various ways.

Authors' contribution: NB: Experiments, Data analysis and Writing. PJ: Supervision and Resources. SDT: Conceptualization, Identification of Bryophytes and Finalising the Manuscript. SB: Experimental tips. MK: Inputs for Extract Preparation RJ: Lab Inoculation Techniques. NK: Collection and Photography.

Table 3. Activity Index of the crude extract of *H. hyalinus*

Solvents	*F. oxysporum*	*F. solani*	*R. solani*
Ethanol	0.83	1.02	1.50
Methanol	-	1.01	0.66
Acetone	0.87	0.65	0.93
Chloroform	-	-	-

Table 4. Activity Index of the crude extract of *P. asplenioides*

Solvents	*F. oxysporum*	*F. solani*	*R. solani*
Ethanol	0.70	0.84	0.93
Methanol	0.57	0.84	0.42
Acetone	0.97	0.90	0.57
Chloroform	0.45	0.42	0.00

Conflict of interest: Authors declare no conflicts of interest.

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Received:9th September 2022
Accepted: 28th November 2022