

# Isolation of Lichen Forming Fungus of *Everniastrum cirrhatum* and Evaluate its Antagonistic and Antimicrobial Activity

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## ABSTRACT

Mycobiont is a lichen forming fungi (LFF) symbiotically associated with phycobiont (algae) of lichens. Here LFF was isolated from *Everniastrum cirrhatum* lichen and checked its antagonist and antimicrobial properties against pathogens with minimum inhibitory concentration (MIC). The isolated LFF was inhibited the growth of several plant pathogens viz, *Fusarium moniliforme*, *F. oxysporum* and *F. udum* and Human pathogenic fungi viz, *Epidermophyton floccosum*, *Microsporium gypseum* and *Trichophyton rubrum* as well as pathogenic bacteria viz, *Streptococcus mutant*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Salmonella typhi*. The antagonistic activity of LFF was found most effective against *Fusarium udum* and caused 55.55% inhibition of mycelial growth and antimicrobial substances from LFF were also compared with natural thallus extract, maximum activity was observed by acetone extracts of LFF against *Microsporium gypseum* (21mm) and its MIC was found to be  $0.78125 \times 10^{-7}$   $\mu$ l against *Microsporium gypseum* and *Candida albicans*. Therefore acetone extracts of LFF found most effective inhibitor rather than other extracts. This is the 1<sup>st</sup> attempt to evaluate antagonistic and antimicrobial properties of LFF (*Everniastrum cirrhatum*) against pathogenic fungi and bacteria.

**Keywords-** Lichen forming fungus, *Everniastrum cirrhatum*, Antagonists, Antimicrobials.

## INTRODUCTION

Lichens are the small plants like organisms having a symbiotic association between fungus as mycobiont and algae as phycobiont<sup>1</sup>. The thallus of Lichen does not show any resemblance to either partner. Lichens are well known for having unusual secondary metabolites which have medicinal properties and been used in medicinal purposes from very long times. Some lichens such as *Cladonia islandica*, *Cladonia spereis* and *Lobaria pulmonaria* were found to be effective in the pulmonary tuberculosis treatment<sup>1</sup>.

Some secondary metabolites which not produced by higher plants or even in free living fungi are secreted by the mycobiont partner of Lichens Nobuo Hamada<sup>2-3</sup>. This is the main reason that's why the isolation and extraction of mycobiont which is also known as Lichen Forming Fungus (LFF) for secondary metabolites is necessary.

Antimicrobial secondary metabolites produced by some LFF have manifold more active biological properties like antibiotic, antiviral, allergenic, antitumor, plant growth inhibitory, ecological roles enzyme inhibitory and many more<sup>4-5</sup>.

The antibacterial and antifungal activities of lichens have studied by a number of investigators. The antibiotic property of lichen was firstly studied by Burkholder<sup>6</sup>. The antifungal and antibacterial activity of many lichens and their extracts was evaluated against different group of microorganisms like fungi, gram-positive and gram-negative bacteria<sup>7-9</sup>.

In this context the purpose of the present study was to standardize the methodology for isolation of Lichen Forming Fungus (LFF), study the antagonistic activity of this fungus and to screen their antimicrobial potential by solvent extracts of natural thallus and LFF of lichen *Everniastrum cirrhatum*.

## MATERIALS AND METHODS

### Collection and identification

The lichen was collected from Nainital (Utrakhand, India), identified by Dr. D. K Upreti, lichen laboratory, NBRI, Lucknow<sup>10</sup>. Then this lichen was dried at room temperature for 72 hours and preserved it in -20<sup>0</sup>C for further investigation<sup>10</sup>.

Some microorganisms which produced diseases viz. *Streptococcus mutans* (MTCC 890), *Staphylococcus aureus* (MTCC 7443), *Salmonella typhi* (MTCC 3216), *Salmonella typhimurium* (MTCC 3224), *Trichophyton rubrum* (MTCC 7859), *Fusarium oxysporum* (MTCC 6569), *Microsporium gypseum* (MTCC 6041), *Epidermophyton floccosum* (MTCC 7880), *Candida albicans* (MTCC 227), and *Fusarium moniliforme* (MTCC 6576), *Fusarium udum* (MTCC 4290) were obtained from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh. Colonies of bacterial cultures were preserved in NA (Nutrient agar) and fungi cultures were preserved in PDA (Potato dextrose agar) media at 4<sup>0</sup>C for further studies.

### Isolation of lichen forming fungus (LFF)

The separation and isolation of LFF give a better opportunity to gather the morphological and biochemical study of the mycobiont components of lichen. For isolation of LFF many types of media like Potato Dextrose Agar (PDA), 2% malt agar (MA), Sabordaud Dextrose Agar (SDA) and Malt Yeast Extract Agar (MYEA) were used<sup>11</sup>.

Lichen's apothecia were surface sterilized with 0.01% HgCl<sub>2</sub>, removed a drop of HgCl<sub>2</sub> from the surface of apothecia was triple time washed by distilled water. After it small pieces of apothecia were inoculated on a different medium. All inoculated plates were incubated at 24± 2<sup>0</sup>C

for 48 hrs. Plates were daily checked for contamination and immediate transfer the non-contaminated colony on to fresh culture plate<sup>11</sup>.

### Antagonistic effect of LFF

The antagonistic effect of LFF of lichen *Everniastrum cirrhatum* was evaluated against different pathogenic fungi viz., *Fusarium moniliforme*, *F. oxysporum*, *F. udum*, *Trichophyton rubrum*, *Microsporium gypseum* and *Epidermophyton floccosum* by dual culture technique<sup>12</sup> in triple replications. 5 mm diameter discs of LFF were placed on one side of poured PDA plates aseptically while, the discs of fungus pathogen were placed on opposite sides of LFF in the same Petri plates here control were also maintained with both LFF as well as pathogen separately. These Petri plates were incubated at  $20 \pm 2^{\circ}\text{C}$  for 12 days. After each 24 hours, the mechanism of interaction was observed and the data were recorded as per cent inhibition by the formula given here:

Percent growth inhibition over control =  $\frac{dc - dt}{dc} \times 100$ .

where,

dc = colony diameter in control

dt = colony diameter in treatment

### Preparation of extracts

4 g air dried natural thallus (NT) of lichen powder and same amount of air dried LFF was dipped in 8 ml of each solvent (i.e. ethanol and acetone) separately for 48 hours at room temperature<sup>10</sup>. By using filter paper (Whatman No.1) all extracts were filtered and under reduced pressure they evaporated till dryness. The obtained residues, 8 ml of Dimethyl sulfoxide (DMSO) were added in each filtrate for final concentration<sup>10</sup>. The obtained solutions were kept at  $4^{\circ}\text{C}$  for further study<sup>10</sup>.

### Antimicrobial screening of LFF and NT of lichen extracts against pathogens

For antimicrobial screening disc-diffusion method was used<sup>12,13</sup>. A sterile disc of 6 mm (Hi Media) was impregnated with 20 $\mu$ l of the extracts and placed in a Luria Bertani agar plate which is inoculated with the pathogen. All plates were incubated at  $30 \pm 2^{\circ}\text{C}$  for 24 hours and with DMSO only the control was also maintained.

### MIC of acetone (LFF) extract of lichen against pathogens

Minimum Inhibitory Concentration (MIC) is the lowest concentration which inhibited the growth of microorganisms and judged by the lack of turbidity in a tube. The MIC of acetone extract of LFF was checked against pathogens by microtiter plate assay used by Sarker *et. al.*, 2007<sup>14</sup>.

## RESULTS AND DISCUSSION

### Isolation of LFF

From different media tried, Malt, Yeast Extract Agar medium was supported best for the isolation and good growth of LFF. The pure culture of LFF, colony texture was white smooth with wavy margin (figure 1). Similar results were found by Dharmadhikari *et al.*, 2010 but they found MGYB medium best for the growth of the mycobiont of lichen *Parmelinella simplicior*<sup>11</sup>. For the analysis of secondary metabolites and their mass production, liquid media were found to be more convenient so that LFF can easily separate from liquid medium as compare to solid medium. The growth rate of the cultured LFF in the laboratory could be improved to harvest large quantities of these novel secondary metabolites. Thus, to identify the chemical constituents of the extracts of LFF more study is necessary. In addition, the data may also suggest that the extracts of LFF could be used as an easily accessible source of secondary metabolites for the antimicrobial properties in the pharmaceutical purpose.

### Antagonistic effect of LFF

The antagonist effect of LFF of lichen *Everniastrum cirrhatum* was evaluated against different pathogenic fungi viz., *Fusarium oxysporum*, *F. moniliforme*, *F. udum*, *Trichophyton rubrum*, *Microsporium gypseum* and *Epidermophyton floccosum* were estimated on the basis of the percentage of inhibition of pathogenic strains, the results were presented in table 1.

The data showed that the LFF of lichen was found a most effective antagonist against *Fusarium udum* and caused 55.55% inhibition of mycelial growth and against *Trichophyton rubrum* showed minimum antagonist i.e. 16.66%.

### Antimicrobial screening of extracts of LFF and NT against test pathogens

The antibacterial activity of acetone and ethanol extracts of dried lichen and dried LFF against the different pathogenic microorganisms was estimated on the basis clear zone inhibition of pathogenic strains, the results were presented in table 2.

The aqueous extract (NT) showed no activity *Staphylococcus aureus*, *Salmonella typhi*, *Salmonella typhimurium*, *Trichophyton rubrum* and *Microsporium gypseum*. The minimum activity was recorded against *Streptococcus mutant* (05mm), maximum activity was found against *Candida albicans* (06mm). The acetone extract of natural thallus showed minimum activity against *Streptococcus mutant* (12mm), maximum activity was observed against *Microsporium gypseum* (16mm). The acetone extract of LFF showed minimum activity against *Salmonella typhi* (14mm), maximum activity was observed against *Microsporium gypseum* (21mm). The ethanol extract of natural thallus showed minimum activity against *Salmonella typhi* (05mm), maximum activity was observed against *Salmonella typhimurium* (13mm).

The ethanol extract of LFF showed minimum activity against *Microsporium gypseum* (13mm), maximum activity was observed against *Streptococcus mutant* and *Salmonella typhimurium* (16mm). The differences of antibacterial activity between lichen extracts were dependent upon the solvent used for extraction. Rankovic *et. al.*, 2007 found that the extracts of lichens *Lasallia pustulata*, *Umbilicaria crustulosa*, *Parmelia sulcata* and extracts of *Umbilicaria cylindrical*, with solvents used were acetone, methanol and aqueous have active antimicrobial properties against bacteria<sup>15</sup>. Rowe *et. al.*, 1989 were also reported that many different extracts of lichens viz. *Evernia prunastri*, *Pseudovernia furfuracea* and *Alectoria capillaries* were active against gram-positive bacteria and *Candida albicans*<sup>16</sup>. All these studies indicated that the lichens inhibited the growth of mostly gram-positive bacteria. However, this may be due to the biochemical variations between gram-positive and gram-negative bacteria. If so, it is great to note that *Everniastrum cirrhatum* inhibited the growth of both gram positive and gram negative bacteria as well as the growth of fungi also.

### MIC of acetone (LFF) extract against pathogens

The MIC is the lowest concentration at which the bacterial growth is inhibited by the lichen extract and could be detected by pink well showing reduction of resazurin in the dilution series. The results obtained after microtiter plate assay are shown in table 3.

The MIC of acetone (LFF) extract showed  $0.78125 \times 10^{-7}$   $\mu$ l against *Microsporium gypseum* and *Candida albicans*, while  $3.125 \times 10^{-5}$   $\mu$ l against *Staphylococcus aureus*, *Salmonella typhimurium* and *Trichophyton rubrum*. The MIC of *Streptococcus mutant* and *Epidermophyton floccosum* was found to be  $6.25 \times 10^{-4}$   $\mu$ l and the MIC of *Salmonella*

*typhi* was found to be  $12.5 \times 10^{-3}$  µl where as Santiago *et. al.*, 2010 were shown the Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of the crude extracts of lichen *Ramalina dendriscoides* (Rd), against *S. aureus*, which gave MIC/MBC of 156 µg/ml and 2500 µg/ml, respectively<sup>17</sup>.

## CONCLUSIONS

Malt, Yeast Extract Agar medium was the best medium for the isolation of mycobiont, antagonistic activity of mycobiont was observed maximum against *Fusarium udum* (55.55%) while, out of the extracts, acetone extract of LFF (*Everniastrum cirrhatum*) showed maximum antimicrobial activity against *Microsporium gypseum* (21mm), *Salmonella typhimurium* (19mm) and *Candida albicans* (19mm).

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**Table 1.** Antagonistic effect of lichen against different pathogenic fungi

S. No	Name of pathogen	Percent of inhibition after 14 days
1.	<i>Fusarium moniliforme</i> ,	38.88
2.	<i>Fusarium oxysporum</i>	36.11
3.	<i>Fusarium udum</i>	55.55
4.	<i>Trichophyton rubrum</i>	25.27
5.	<i>Microsporium gypseum</i>	30.55
6.	<i>Trichophyton rubrum</i>	16.66

**Table 2.** Inhibition of pathogenic bacteria and fungi by natural extract and LFF

S. No.	Extracts	Zone of inhibition against pathogens (mm)							
		Sm	Sa	St	Sty	Tr	Mg	Ef	Ca
1.	Acetone (NT)	12	15	13	15	14	16	15	14
2.	Ethanol (NT)	09	10	05	13	10	09	12	12
3.	Acetone (LFF)	17	18	14	19	18	21	17	19
4.	Ethanol (LFF)	16	15	15	16	15	13	14	15
5.	Aqueous extract (NT)	-	05	-	-	-	-	05	06

*Streptococcus mutant* (SM), *Staphylococcus aureus* (SA), *Salmonella typhi* (ST), *Salmonella typhimurium* (Sty), *Trichophyton rubrum* (Tr), *Microsporium gypseum* (Mg), *Epidermophyton floccosum* (Ef), *Candida albicans* (Ca), Cultured lichen (LFF) and Natural thallus (NT).

**Table 3.** MIC of acetone extract (LFF) against pathogens

S. No.	Pathogenic culture strains	Minimal inhibitory concentration ( $\mu$ l/ml)
1.	<i>Streptococcus mutant</i>	$6.25 \times 10^{-4}$
2.	<i>Salmonella typhimurium</i>	$3.125 \times 10^{-5}$
3.	<i>Salmonella typhi</i>	$12.5 \times 10^{-3}$
4.	<i>Staphylococcus aureus</i>	$3.125 \times 10^{-5}$
5.	<i>Trichophyton rubrum</i>	$3.125 \times 10^{-5}$
6.	<i>Microsporium gypseum</i>	$0.78125 \times 10^{-7}$
7.	<i>Epidermophyton floccosum</i>	$6.25 \times 10^{-4}$
8.	<i>Candida albicans</i>	$0.78125 \times 10^{-7}$



**Figure 1.** Mycobiont of Lichen *Everniastrum cirrhatum*

