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Review Article

"A Study On Lichen Forming Fungi And Its Secondary Metabolites"

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ABSTRACT

Lichens are the slow-growing symbiotic association between a fungus (ascomycetes and rarely basidomycetes) and algae (cynobacteria). Mycobionts grown without their photobionts produce secondary lichen compounds under certain conditions and can also produce substances that are different from the metabolites found in symbiosis. The secondary metabolites are extracellular produced by fungal partner and are deposited in the fungal hyphae surface. These metabolites form crystals and are extracted by organic solvents. The lichen metabolites were extracted using acetone and the secondary metabolites were identified. The extract was also quantitatively analyzed for total polyphenols and flavonoids. They were also tested for antioxidant activity by DPPH assay and FRAP method. The culture conditions were optimized for maximum secondary metabolite production. The lichen extracts show great Antilarval activity on house mosquito. They are also active against MCF-7 cancer cell lines. The culturing of lichens and its mycobionts are difficult and it requires axenic condition. The lichenous fungi produce unique metabolites that are not produced by free fungi. The metabolites have unique biological activities and cytotoxic effects. They play an important role in the field of pharmaceutical biology especially in the cancer study.

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INTRODUCTION:

Lichens are formed from a combination of mycobiont and a phycobiont. Majority of the lichen physical structure and shape is provided by the fungal element which surrounds and grows around the algal cells. Lichens are terrestrial and they generally grow on rocks, tree trunks, leaves and exist as epiphytes. Lichens are also capable of growing at extreme conditions. The algal partner of lichens can be either green algae or cyanobacteria, Lichens are also natural pollution indicators and are involved in monitoring industrial pollution. They produce organic compounds known as lichen substances. Lichens have been used as medicines from the times of world wars. Lichens have been used in the medicines, food industry, perfumery and cosmetic industries for many years. The secondary metabolites of lichens show diverse biological activities such as antiproliferative, antioxidant, antitumor, antipyretic, analgesic, anti-inflammatory and photoprotective.More than 1000 lichen metabolites are known and nearly 550 of them are unique to lichens. The mycobionts of lichens produce unique secondary metabolites that are not even found in higher plants and free-living fungi. The classes of unique metabolites include depsides, depsidones, dibenzofurans, xanthones and pulvinic acid derivatives. These metabolites are produced by specialized pathways known as shikimic acid pathway, polyketide pathway and mevalonic acid pathway. Polyketide synthases (PKS) catalyses the biosynthesis of polyketides. They are the large multi-functional enzyme which catalyses the repetitive condensation of acetyl coenzyme A (CoA) starting with malonyl-CoA elongation units. Lichens are able scavenge free radicals due to the presence redox system in their metabolic process. Lichen substances have also been used in cosmetic and nutraceutical industry.

MATERIALS AND METHODS

Sample collection

The lichens were collected from the trees of Pichavaram Mangrove forest. They were attached to the barks of trees and rocks. They were carefully collected from bark and wood using a knife and transferred into breathable bags.

In vitro lichen culture

The work on the cultures of lichens was started within 7 days after the collection from localities following the methodology established for lichen culture by Yamamoto & all (1985, 1987). Now it was transferred to SDA medium to isolate LFF (lichen forming fungi) from the culture. To extract secondary metabolites, LFF were cultured in MYE liquid medium. The culture media used for lichen culture are Malt-Yeast Extract (MYE) (Ahmadjian, 1993); Bischoff and Bold's (Bischoff & Bold, 1963) and Modified Bold's Basal medium (MBB) (Behera & all, 2000), were used.

Extraction of lichen secondary metabolites

Cold extraction

The ground lichen was extracted sequentially with 50 ml of acetone at room temperature for a period of 6 hours. The extracts were filtered and then concentrated to dryness.

Soxhlet extraction

The extraction was done using a soxhlet apparatus and acetone as a solvent for 6 hours.

Qualitative Analysis of lichen extract

Phytochemical tests

Preliminary phytochemical analysis was done for the extract as per standard methods described by Brain and Turner (1975) and Evans (1996).

Thin layer Chromatography

Using capillary tube load few drops of extract in acetone, and let the solvent of the extract evaporated (mark a loading line on the plate, 2 cm from one edge of the plate). Run the chromatogram in a suitable solvent system in a TLC tank until the solvent covers 90% of the plate. Take the chromatogram out of the tank and

evaporate the solvent. View the chromatogram and mark any coloured spots. Note down the colour and Rf value of the spot. Compare with the standard and identify the compound.

Table 1 Solvent systems used in the TLC of lichen substances

Solvent system	Suitable for the separation of
Toluene: acetic acid = 85: 15	All lichen substances
Chloroform: acetone = 4: 1	Pulvinic acids and usnic acid

Table 2 Reagents for the visualization of TLC spots of lichen substances

Reagent	Suitable for the identification
20% Sulphuric acid, 15min at 150°C	All lichen substances, grey, brown, blue spots
0.5 ml Anisaldehyde + 1.0 ml sulphuric acid + 8.5 ml methanol, 10min 100-110°C	Depsides and usnic acid, red to blue-violet

Determination of cell biomass concentration by wet/ dry weight method

Stir the flask to suspend the culture evenly. Pour out 100 ml of the culture into a graduated cylinder. Separate the cells from the broth either by centrifugation at 10,000 g for 5 minutes or by filtration. In the case of centrifugation, carefully discard the clear broth and scrape the cell paste from the centrifuge tube completely into the Petri plate and note the weight. In case of dry weight method, dry the paste in an oven set at 100ºC. The mycelium will be charred and the filter membrane will be burned if the temperature of the oven is set too high. Measure the weight of the pan/filter plus the cell paste periodically until there is of Gallic acid equivalent.

$$\text{Inhibition\%} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

FRAP Assay

Different concentrations of lichen extracts were mixed with 2.5 ml phosphate buffer (200 mM, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was placed in a water bath for 20 min at 50°C, cooled rapidly and mixed with 2.5 ml of 10% trichloroacetic acid. Then, the supernatant was added with 0.5 ml of 0.1% ferric chloride. The amount of iron (II)–ferricyanide complex formed was determined by measuring the formation of Perls' Prussian blue at 700 nm. The higher absorbance of the reaction mixture indicates greater reducing power.

Standardization of Lichen forming fungal Culture

The standardization of fungal culture was done by altering basic growth conditions such as temperature, pH, light intensity and shaking to get maximum growth and maximum production. MYE liquid medium was used with different pH conditions (pH- 2, 4, 6 and 8), temperature

change (15ºC, 37ºC and 50ºC) and shaking, non-shaking conditions and culturing in the dark.

Estimation of Ester groups for Depsidone quantification

Procedure

Samples containing ester bonds were dissolved in 1 ml of ethanol was added to a 5 ml volumetric flask. KOH and hydroxylamine hydrochloride solution were mixed and 2 ml of that solution was added to the flask followed by 0.6 ml of water to dissolve the precipitate that was formed. The solution was kept at room temperature for 1 hour. Successively, 0.5 ml each of HCl solution and FeCl3 were added and followed by water upto 5 ml mark.

Estimation of Usnic Acid content

A colorimetric method to estimate usnic acid was designed by N. P. Jayasankar et al (1968). Ehrlich reagent or PDAB (p-dimethylaminobenzaldehyde) is used for the chromatographic detection of phenol compounds).

Anti-larval Activity of Lichen extract against the larvae of house mosquito

Mosquito larval culture used in the assays was collected from an open tank with stagnant water. The protocol involved direct and diluted method. The acetone extracts of lichen forming fungi were directly applied on mosquito larva and alternatively in diluted method, the extracts were diluted by 1:10 (extract/water). The timely observations were noted.

Anticancer Activity against MCF-7 Cell lines

MTT Assay

The tetrazolium ring of MTT is cleaved by dehydrogenases in mitochondria of living cells to produce a purple formazan. The MTT test was carried out according to the protocol described by Berridge et al. (2005). Briefly, after 72 hours of incubation with the tested substances, the medium was aspirated, and 170 µl of the mixture of the medium and the MTT solution (7.5:1) was added to the wells. The cells were incubated for 4 hours. Excessive MTT was then aspirated, and the formazan that formed was solubilized by the addition of 150 µl SDS.

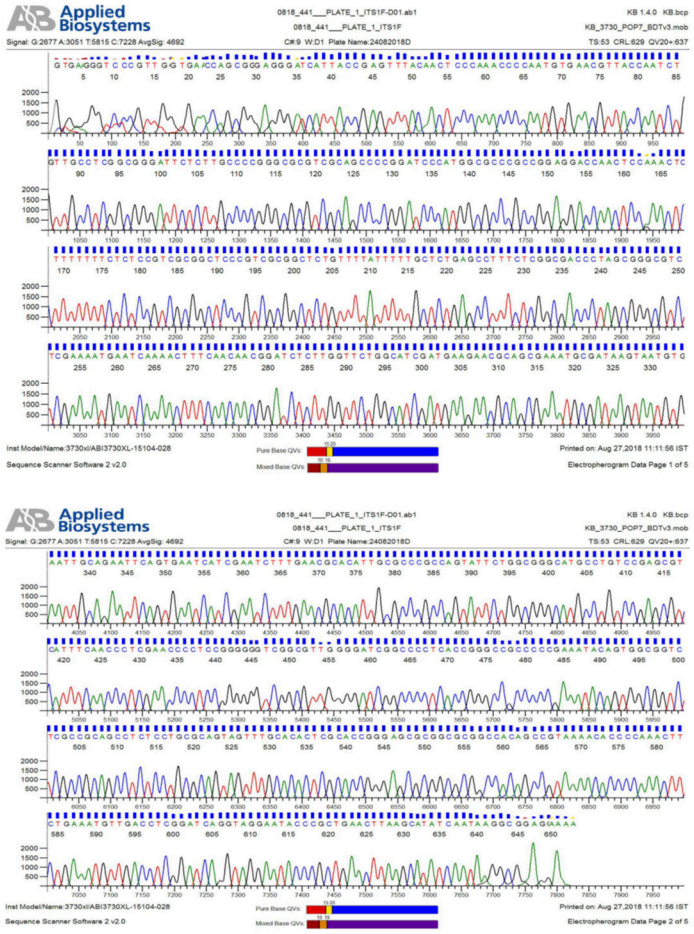
The plates were shaken (3 min, 650 rpm), and the formazan that formed was solubilized. The mixture of the medium and the MTT solution (7.5:1) was used as a blank. The absorbance was read at 570nm using a microplate reader. IC50 values were calculated from curves constructed by plotting the cell survival versus the drug concentration.

Microbial Identification by 18S RNA sequencing

DNA was isolated from the culture provided by the scientist. Its quality was evaluated on 1.0% Agarose Gel, a single band of high-molecular weight DNA has been observed. Fragment of ITS region was amplified by PCR. A single discrete PCR amplicon band of ~700 bp was observed when resolved on agarose. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with ITS1 and ITS4 primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. A consensus sequence of the PCR amplicon was generated from forward and reverse sequence data using aligner software. The ITS region sequence was used to carry out BLAST with the database of NCBI Genbank. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7.

Results and Discussion

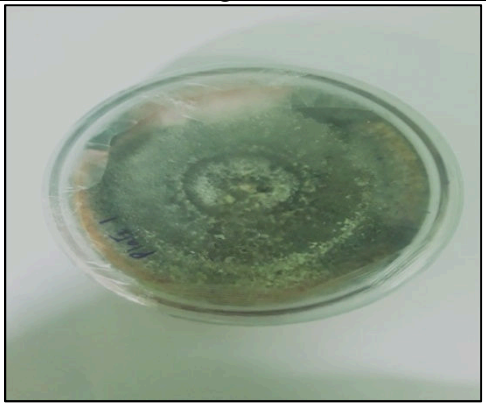
Identification of lichen forming fungi by 18S RNA Sequencing



From 18S RNA sequencing, the organism was found to be *Trichoderma longibrachiatim.* They are soil bacteria found in warmer countries. They are distinct organisms that produce various proteins and metabolites. They are also parasitic and saprophytic in nature.

Lichen forming fungal isolation

Fig. 1.1 Trichoderma longibrachiatum in SDA medium



Standard graph for Polyphenols

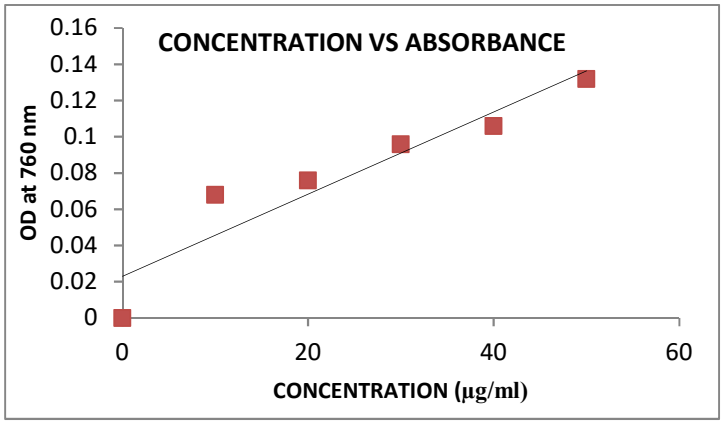
Thin layer Chromatography

The Rf class is Single lichen forming fungus was capable of producing multiple lichen metabolites

Phytochemical analysis of lichen forming fungal extract

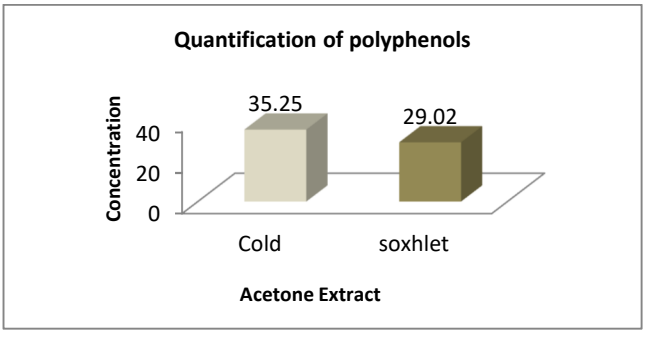
Phytochemical analysis	***Trichoderma longibrachiatum***
Dragendoff's test for Alkaloids.	Positive
Ferric chloride test for flavonoids.	Positive
Ferric Chloride test (5%) for tannins and phenols.	Positive
Libermann-Buchard test for Sterols.	Positive
Salkouski Test for Triterpenoids	Positive

Graph 1 Gallic acid standard graph



Standard graph was obtained using Gallic acid for quantification of polyphenols using Follin-Ciocalteau method. Standard graph helps to obtain the concentration of polyphenols in unknown extracts from the O.D obtained.

Polyphenol Quantification



Graph 2 Total polyphenols concentration

sample extract	O.D at 415nm cold	O.D at 415nm soxhlet
Trichoderma longibrachiatum	0.025	0.057

Total flavonoids quantification

The presence of flavonoids also leads to high antioxidant activity which prevents the occurrence of non- communicable diseases like cancer, diabetes, dementia and myocardial infarction for which free radicals are considered one of the major contributing factors.

Antioxidant Activity by DPPH Assay

Trichoderma longibrachiatum shows the high antioxidant activity. This shows that lichens may be used as possible natural antioxidant, antimicrobial and anticancer agents to control various human, animal and plant diseases (Branislav R Ranković *et al*, 2011).

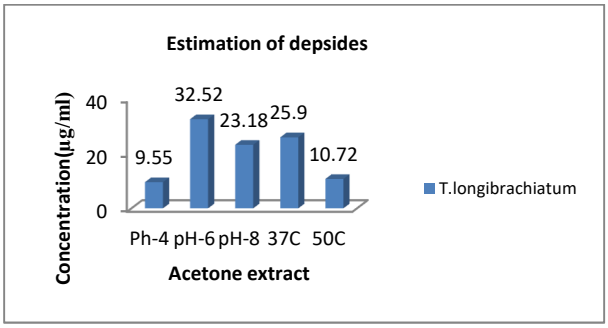
Sample Extract	O.D at 517nm	Inhibition % Cold	Inhibition % Soxhlet
T. longibrachiatum	0.103	14.16	21.83

Antioxidant Activity By FRAP Assay

Sample Extract	O.D AT 700nm
T. longibrachiatum	0.060

Since the Optical density is directly proportional to its ferric reducing capacity, It can be concluded that *Trichoderma longibrachiatum* has a high reducing capacity. FRAP assay measures the reducing power of samples via direct electron donation and the reduction of ferric tripyridyltriazine [Fe^{3+}-TPTZ] complex to ferrous tripyridyltriazine [Fe^{2+}-TPTZ].

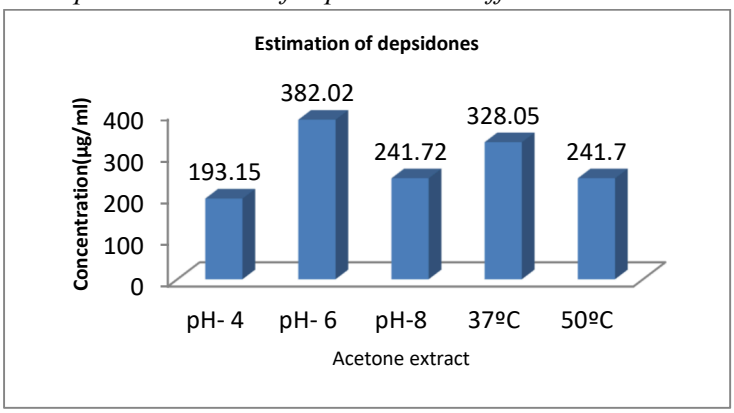
Quantification of Depsides by Polyphenol Estimation



Graph 3 Estimation of depsides at different conditions.

A depside is a type of polyphenolic compound composed of two or more monocyclic aromatic units linked by an ester bond. Depsides are most often found in lichens, but have also been isolated from higher plants. Certain depsides have antibiotic, anti-HIV, antioxidant, and anti-proliferative activity *in vitro*.

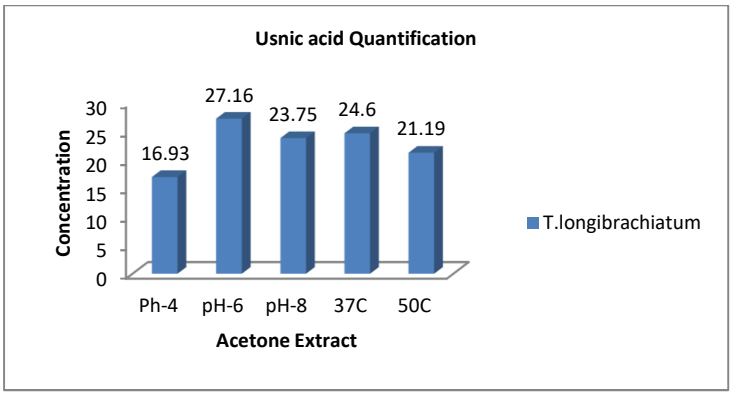
Graph 4 Estimation of depsidones at different conditions.



Quantification of depsidones by ester estimation

Depsidones are chemical compounds that are sometimes found as secondary metabolites in lichens. They are esters that are both depsides and cyclic ethers.

Graph 5 Estimation of depsidones at different conditions.



Usnic acid quantification based on pH and Temperature

The lichen compound usnic acid is used for its antimicrobial activities in cosmetic products and is also a component of slimming agents.. Its effect against cancer cells was first noted over 30 years ago. Usnic acid, a lichen acid, is a compound found in crude medicines and dietary supplements, including Lipokinetix1, a supplement marketed as a weight loss agent that caused hepatotoxicity and acute liver failure in patients.

Acetone extract	pH/temperature	Wet weight	Dry weight
Trichoderma longibrachiatum	2	No growth	No growth
	4	4.88 g	0.98 g
	6	7.02 g	1.28 g
	8	3.25 g	0.48g
	15ºC	No growth	No growth
	37ºC	5.02 g	1.05 g
	50ºC	4.34 g	0.90 g

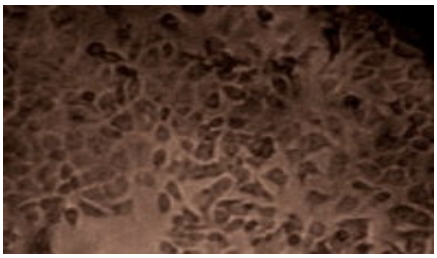
Cellular biomass determination by wet/dry weight method

The growth rate of the mycobiont was very slow at low temperatures, and the colonies of the mycobiont could not be seen by the naked eye until about 110 days after cultivation at 5ºC and about 50 days at 10ºC. The average weight of the mycelia was 0.32 mg at 50C, and increased greatly to 31.3 mg at 25ºC.

The weight of the mycelia increased about 100-fold between 5 and 25ºC, and increased linearly with temperature on a logarithmic scale, especially until 15ºC. On the other hand, the content of the depside increased approximately 80-fold from 0.013 to 1.02% with the elevation of temperature from 5 to 15ºC, and then decreased from 1.02 to 0.009% between 15 and 25ºC(Nobuo Hamada 1989).

Antilarval Activity Of Lichen Extract Against The Larvae Of House Mosquito

Acetone extracts	Larvicidal activity	Time
Trichoderma longibrachiatum	100 % Mortality rate	42 mins 32 sec

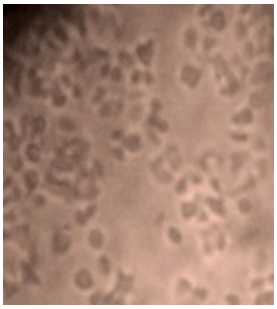


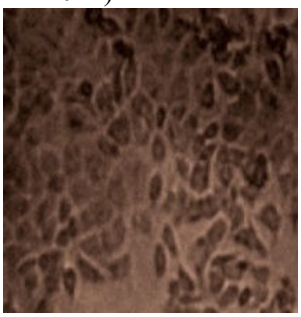
The acetone extract of Trichoderma longibrachiatum, showed 100% mortality of mosquito larva. They can also be studied for their anti-feedant capacity. The larvicidal activity of the methanol extract, fractions and compounds 2-hydroxy-4-methoxy-6-propyl-methyl benzoate and usnic acid identified from the lichen *Ramalina usnea* (L.). Ramalinaceae, was tested against the third instar larvae of the *Aedes aegypti* mosquito. The methanol extract and three fractions showed activity, killing 100% and 96.6% of the larvae at a concentration of 150 µg/ml in 24 hours. This was the first study of its kind reporting the larvicidal activity against the *A. aegypti* mosquito with compound (Antônio S.N.Moreira *et el* 2016).

Anticancer effect of *Trichoderma longibrachiatum* on MCF-7 cell line

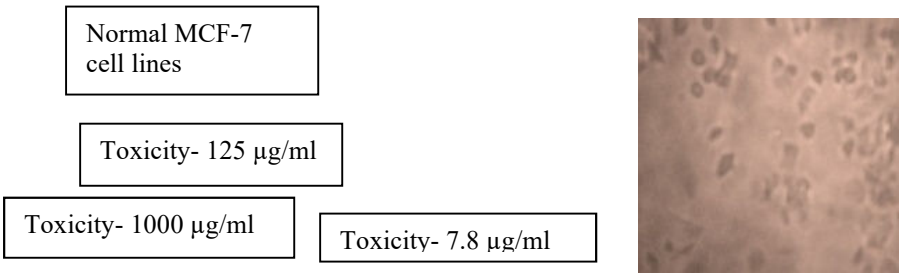
Trichoderma longibrachiatum acetone extract was tested against MCF-7 cell lines (Breast cancer cell lines) after optimizing the conditions. The extract responded positively against MCF-7 cell lines. However, the extract from *Trichoderma longibrachiatum* showed 50.32% viability at a concentration of 125µg/ml whereas the unknown sample extract showed slightly higher viability of 54% yet at a lower concentration of just 62.5µg/ml.

Three lichen secondary metabolites; atranorin, gyrophoric acid and physodic acid on A375 melanoma cancer cell line by activating the apoptotic process (Cardile V *et al 2017)*. Physodic acid showed anticancer activity was tested against FemX (human melanoma) and LS174 (human colon carcinoma) cell lines using the MTT method (MarijanaKosanićet al 2012).





Anticancer effect of *Trichoderma longibrachiatum* on MCF-7 cell line



Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1000	Neat	0.412	24.42
500	1:1	0.552	32.72
250	1:2	0.696	41.25
125	1:4	0.849	50.32
62.5	1:8	1.011	59.92
31.2	1:16	1.112	65.91
15.6	1:32	1.213	71.90
7.8	1:64	1.305	77.35
Cell control	-	1.687	100

CONCLUSION

The isolation of the mycobiont was successfully done followed by its secondary metabolite expression study. Since the mycobiont was cultured separately without the influence of photobiont, the expression of secondary metabolites was considerably less compared to natural lichen. It is also difficult to culture lichens *in vitro* as it requires axenic culture condition. The work on lichen mycobionts and its secondary metabolites are quite valuable. Lichens are complex organisms and there are wide range secondary metabolites produced by them. The depsides and depsidones produced by lichen or its fungal partner are valuable in the pharmaceutical industry. Although many natural and cultured lichens have been studied for their biological properties and several compounds

have been purified and identified, their therapeutic potential has not yet been fully explored. Particularly, Limited information is available on the anticancer effects of pure compounds obtained from lichens.

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