BETA GLUCAN SYNTHESIS AND MYCOCHEMICALS SCREENING OF LYCOPERDON PERLATUM COLLECTED FROM SAYALGUDI, SOUTHERN TAMILNADU, INDIA

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***Abstract:** This work targets on β-glucan synthesis and analysis of mycochemicals present in the mushroom, Lycoperdon perlatum. The samples were collected from coastal region of southern Tamilnadu. The sample, that the surface sterilized fruit body was dried and stored in powder form. The mycochemicals were examined from the mushroom fruit body dried powder. The mushroom mycelium was grown on PDA plate by the spore inoculation. The production of extracellular enzymes from mushroom mycelium was detected. Beta-glucan was isolated from mycelium using submerged fermentation technique and purified. It was acid hydrolyzed using tri-fluoro acetic acid (TFA) and analyzed by thin-layer chromatography. Total protein content present in the mushroom was measured by Lowry's method. As like total carbohydrate content was measured by phenol and sulfuric acid method that contain 56% crude β-glucan. The result obtained from this study has shown the potential of Lycoperdon perlatum mushroom extract as a potent therapeutic agent and a food supplement.*

***Index Terms** – Lycoperdon perlatum, Beta-glucan, Mycochemicals, Extra cellular enzymes*

1. INTRODUCTION

Mushroom is the fruiting body of macro fungi (*Basidiomycota*) and produces only a short reproductive stage in their lifecycle (Hankin and Anagnostakis ,1975). Mushroom can be epigeous, large enough to be seen with the naked eyes and can be picked by hand (Chang and Miles, 1989). Mostly *Basidiomycetes* and *Ascomycetes* are the fruiting body producing fungi. Total mushrooms on the earth are estimated to be 140,000 species in which 10% (14,000 approximately) are known. From the thousands of known species, 2000 species are safe for human consumption and about 650 species of these having medicinal importance (Persoon, 1796). *Lycoperdon perlatum*, commonly known as puffball mushroom, is a species of puffball fungus in the family *Agaricaceae*. Mushrooms are the effective functional food as well as sources for the production of drugs having antioxidant, antitumor and antimicrobial activities (Kanad Das, 2010). Some mushrooms are consumed as potential nutriceuticals (compounds that having medicinal and nutritional characteristics and are consumed as medicines in the form of tablets or capsules) (Rai et al, 2005). The secondary metabolites of mushrooms are chemically diverse and possess a wide spectrum of biological activities, which are explored in traditional medicines and in new targets of molecular biology (Persoon, 1796. In twentieth century, mushrooms are well known to people all over the Asian countries as an important bio-source of novel secondary metabolites.

The puffball mushrooms are the good sources of protein, carbohydrates, fats and several micronutrients (Miura, 2003). The predominant fatty acids in the puffball are linoleic acid (37% of the total fatty acids), oleic acid (24%), palmitic acid (14.5%) and stearic acid (6.4%) (Perdeck, 1950). One of the anti-oxidant is β-glucan. β- Glucan is a polysaccharide (glucose polymers) that is found in many foods such as oats, barley, mushrooms and yeasts. Also, it is lesser extent in rye and wheat. It is extremely difficult to extract and purify. However, Oat bran contains about 7 percent beta glucan and is inexpensive. It is not enough to use as a supplement food. Non-cellulosic β-glucans are now recognized as potential immunological inducers and some are used medically in some countries (Rohrmann and Molitoris, 1992).

These β-glucans consist of a backbone of glucose residues linked by β-(1→3)-glycosidic bonds **(**Lakhanpal and Rana, 2005) often with attached side-chain glucose residues joined by β-(1→6) linkages. Instead of other carbohydrates, the use of β-glucans reduces the cholesterol and triglycerides (Cisneros et al, 1996). The effects on your face are dramatic and it gives a good result in your skin care by the routine massage (Robeiro, 2007) (Satitmanwiwat, 2012). The regular usage of β-glucans used to control blood sugar level in diabetic patients. β-glucan gives protection from ionizing radio activity. The literature suggests β-glucans are effective in treating diseases like cancer **(**Cheung, 2002) a range of microbial infections and hyper cholesterolaemia **(**Chen and Seviour, 2007). *Lycoperdon perlatum* was first studied by mycologist Christian Hendrik Persoon in 1796 (Almendros et al, 1987). The main reason to take beta glucan from the other contents is to induce our immune system. β-glucans have several different structures and different affinities towards receptors. This nature produces different host immune responses.

This study focuses on the synthesis of β-glucan and characterization of mycochemicals present in the mushroom, *Lycoperdon perlatum* was analyzed. This mushroom was collected from coastal regions of southern Tamilnadu, India. It was surface sterilized and the dried fruit body was stored in a powder form. The mushroom mycelium was isolated in PDA plate using spores. Mycochemical analysis was done and various fungal metabolites were examined.

2. METHODOLOGY

2.1. Sample collection

Fresh wild-growing fruiting bodies of mushroom and its spores from well grown mushroom *Lycoperdon perlatum* were collected from coastal resources of Sayalkudi in Ramanadhapuram district, Southern Tamil Nadu in India. Immediately after it was brought to laboratory it was washed and surface sterilized and dried at 40°C for 6 – 8 hrs. Dried mushroom were grinded using Mortar and Pistil and stored in dry container in dry place.

2.2. Processing of samples

Spores were inoculated in potato dextrose agar medium and incubated at room temperature. After mycelial growth it was stored at 4°C.

2.3. Extract preparation

10g of dried mushroom was used to prepare each 100 ml of ethanol, methanol and water extract using Soxhlet apparatus. Extraction was carried out for 6 hours and then cooled and stored in screw cap tubes.

2.4. Mycochemical screening

The mycochemical analysis of the mushroom extracts were carried out by the method of Adebayo and (Ishola Adebayo and Ishola, 2009) and the following mycochemicals were examined from the mushroom extracts.

2.4.1. Test for Alkaloids

About 0.2 g of extract was warmed with 1% of aqueous hydrochloric acid for two minutes. The mixture was filtered and few drops of Dragendorff's reagent were added. A reddish-brown color and the turbidity formation with the addition of reagent indicate the presence of alkaloids.

2.4.2. Test for Flavonoids

Small quantity (2g) of the extract was dissolved in 10% of sodium hydroxide (NaOH) and Hydrochloric acid (HCl). A yellow solution that turned to colorless solution with the addition of HCl indicates the presence of flavonoids.

2.4.3. Test for Anthraquinones

5g of extract was shaken with 10ml of benzene. The solution was filtered with the filter paper and 5ml of 10% NH_4OH solution was added. Formation of violet color in the phase indicates the presence of anthraquinones.

2.4.4. Test for Glycosides

A small quantity of the extract was dissolved in 2ml of acetic acid and cooled in ice. Concentrated H_2SO_4 was then carefully added. Colour change from violet to blue or green indicates the presence of glycoside.

2.4.5. Test for Steroids

A small amount of extract was treated with few drops of acetic acid anhydride. Concentrated H_2SO_4 was carefully added to the test tube by the wall. The presence of a brown ring at the boundary of the mixture was taken as positive result to presence of steroids.

2.4.6. Test for Saponins

0.1g of dried mushroom powder was used to make extraction. Mushroom powder was boiled in 10ml of distilled water for 5 minutes and cooled down. Then it was filtered and the filtrate was used for the following tests.

(i). Frothing test

1.0 ml of filtrate was diluted with 4.0 ml of distilled water and mixture was shaken vigorously and observed for persistent foam which lasted for at least 15minutes.

(ii). Emulsion test

This was performed by adding 2 drops of olive oil to the frothing solution and shaken vigorously. Formation of an emulsion indicates the positive test.

2.4.7. Test for Phlobatannin

A small amount of extract was boiled with 1% aqueous hydrochloric acid. Red color precipitate formation indicates the presence of phlobatannin.

2.4.8. Test of Coumarin

To 2.0 ml of the test solution, add a few drops of alcoholic sodium hydroxide. Appearance of yellow color indicates the presence of coumarin.

2.4.9. Test for Phenol

To the 2.0 ml of test alcoholic solution, add few drops of ferric chloride solution. Development of bluish green or red color indicates the presence of phenol.

2.4.10. Test for Quinone

The test solution was treated with few drops of concentrated sulphuric acid or aqueous sodium hydroxide solution. Color development indicates the presence of quinoid compounds.

2.4.11. Test for Tannins

A small amount of extract was mixed with basic lead acetate solution. White color precipitate formation indicates the presence of tannins.

2.4.12. Test for Sugar

The test solution was mixed with equal volume of Fehling's solution A and B and heated. Formation of red coloration indicates the presence of sugar.

2.5. Detection of extra cellular enzyme production

The methods described by Hankin and Anagnostakis (Galichet et al, 2001) and Rohrmann and Molitaris (Robeiro, 2007) were used to detect the production of extracellular enzymes by the mushrooms.

(i). Amylolytic activity

GYP medium (i.e., 1g glucose + 0.1g yeast extract + 0.5g pentone, 16g agar in 1000ml distilled water) plus 0.2% soluble starch at pH-6 was used. After 3 – 5 days, colonies were formed with clear zone otherwise blue medium indicated amylolytic activity.

(ii). Proteolytic activity

GYP medium amended with 0.4% gelatin (pH-6) was used. A solution of gelatin in water (8%) was sterilized separately and added to the GYP medium at the rate of 5 ml per 100 ml of medium constituting 0.4% gelatin. After incubation, degradation of the gelatin was seen as a clearing opaque and enhanced the clear zones around the fungal colony.

(iii). Cellulase activity

GYP medium containing Na-carboxy-methylcellulose (0.5%). After 3 – 5 days of colony growth, the plates were flooded with 0.2% aqueous Congo red solution and destained with 1M NaCl (15 minutes each). Appearance of yellow areas around the fungal colony due to cellulase production otherwise red medium indicates cellulase activity.

(iv). Tyrosinase activity

Mushrooms were grown in GYP medium. Formation of red brown color around the fungal colony after addition of p-cresol (1.08g / 1000ml) with 0.05% glycine to the surface of fungal colony indicates tyrosinase activity.

(v). Lipolytic activity

Tween 20 was sterilized by autoclaving for 15 minutes at 103 Kpa pressure and 1 ml was added to 100 ml of sterile, cooled agar medium (Peptone 10g, NaCl 5g, $CaCl_2.2H_2O$ 0.1g and agar 20g in 1000 ml of distilled water (pH 6). Clearing or precipitation around the fungal colony indicates lipolytic activity.

2.6. Submerged production of beta-glucan

Polysaccharide production − Seed culture medium for submerged fermentation consists of the following components:

Glucose	− 40g
Yeast extract	− 10g
KH_2PO_4	− 0.5g
K_2HPO_4	− 0.5g
$MgSO_4.7H_2O$	− 0.5g
dH_2O	− 1000ml

In a 250 ml shaker flask add 50ml liquid medium and incubated at 25°C on a rotary shaker at 110 rpm for 5 days. The broth medium was inoculated with 5mm square of well grown slant cultured mushroom. The contents of polysaccharides in the culture medium were from the total sugars using the phenol-sulfuric acid method.

2.7. Extraction of β-glucan

Mycelial growth of mushroom was cultivated in the following growth medium incubated at rotatory shaker.

Glucose	– 40g
Yeast extract	– 10g
KH_2PO_4	– 0.5g
K_2HPO_4	– 0.5g
$MgSO_4$	– 0.5g
dH_2O	– 1000ml

After 5 days of incubation the culture was filtered through cheese cloth. After filtration the mycelial mat was washed with 95% ethanol. Then the mycelial mat was centrifugation at 3000 rpm/minutes for 10minutes under cooling 4°C. Collect the pellet and boil for 10 minutes in hot water bath, then cool it.

Again the pellet was centrifuged at 6000 rpm/min for 15 minutes under 4°C. Add equal volume for ethanol (95%) and leave it for 18 hours at 4°C. Centrifuge at 6000 rpm/min for 15min under cooling 4°C. Take pellet and dissolved in PBS buffer and dialyzed against tap water for 3 days at 4°C with changed the distilled water every day. To remove protein from crude polysaccharide, (chloroform: butanol ratio of (4: 1) was employed. Briefly, the crude polysaccharide was dissolved with water to 10mg/ml.

The Sevag reagent was added to the sample with a ratio of 1:1 and subsequently mixed and vortex. The mixture was centrifuged at 10000 rpm for 20 minutes at room temperature. After centrifugation, the sample was separated into two layers. The top layer is the aqueous solution which contains the polysaccharide and the bottom layer is the Sevag reagent.

The top layer was carefully pipetted out without picking up the interface which contains some proteins and transferred to a clean tube. The solution was pooled and the extraction was repeated twice. Then, the aqueous layer was dialyzed (10KDa) with 2 litre distilled water. The water used for dialysis was changed every day until the volume of sample did not change. The components of the extract were analyzed with Fourier Transform Infra-Red spectroscopy (FT-IR).

2.8. Hydrolysis of β-glucan

The different β-glucans were acid hydrolysed with tri-fluoro acetic acid (TFA), hydrochloric acid (HCl) and sulphuric acid (H_2SO_4) to determine their monosaccharide content. The acid hydrolysis of β-glucan was done by heating in 3 M TFA at 120°C in an oven for one hour. The solutions were neutralised by concentrated ammonia. The non-cellulosic polysaccharides of the insoluble fibre fractions were determined by hydrolysis using 2 M TFA at 120°C for one hour.

2.9. Determination of carbohydrate

Total carbohydrate in the sample was determined using the phenol-sulfuric acid method (Dubois et al, 1956). Briefly, 500 µl 0.25 mg/ml sample was mixed with 500 µl 5% phenol. Then, 2 ml sulfuric acid was added to the mixture and incubated for 20 minutes at room temperature. The OD at 470 nm was measured and the total sugar was calculated by comparing with a standard curve from glucose.

2.10. Fourier Transform Infra-Red spectroscopy

The IR spectra of the compound were measured on Perkinelmer FTIR- Spectrum- RX I series instrument. Similarly the substance was grounded with IR grade potassium (KBr) (1: 10) pressed into discs under vacuum using spectra lab pelletizer. The IR spectrum was recorded in the region 4400 – 400 cm^{-1} and the typical stretching frequency of the bioactive substance was recorded for further characterization study.

2.11. HPLC analysis

The mushroom extract was subjected to HPLC separation, was performed using a SHIMADZU LC-10AT-VP instrument. Separation was performed on a C-18 column. For elution, the solvent gradient system was used (solvent A: H_2O; solvent B: methanol). The gradient was at a flow rate of 1ml/min, followed by an isocratic part of 5minutes. The column effluent was monitored with UV photo diode-array detector (DAD-UV) at 230 nm. Based on HPLC analysis the mushroom secondary metabolites were quantified by comparing the peak area of the samples with that of standard peak of compounds.

3. RESULTS AND DISCUSSION

3.1. The mycochemical analysis

The mycochemical analysis of *Lycoperdon perlatum* showed positive result for coumarin, phenol, quinine, saponins, tannins, sugar, alkaloids, flavonoids, steroids, anthraquinones, glycoside tannins and saponins by frothing test and emulsifications.

Table: 1 Myco-chemical analysis of wild edible mushroom, *Lycoperdon perlatum*

Mushroom Extract	AL	FL	AN	GL	ST	SA	PHL	CO	PH	QU	TA	SU
Ethanol Extract	+	+	+	++	++	+	++	+	++	++	++	++
Methanol Extract	++	+	+	++	++	+	+	+	++	++	++	++
Water Extract	+	–	–	–	+	–	–	–	+	+	+	+

++: Strongly present; +: Present; –: Absent
AL: Alkaloids; FL: Flavonoids; AN: Anthraquinones; GL: Glycosides;
ST: Steroids; SA: Saponins; PHL: Phlobatannins; CO: Coumarin;
PH: Phenol; QU: Quinone; TA: Tannins; SU: Sugar

The mycochemical analysis of *Lycoperdon perlatum* revealed that contain coumarin, phenol, quinine, saponins, tannins, sugar, alkaloids, flavonoids, steroids, anthraquinones, glycoside tannins and saponins by frothing test and emulsifications. The mycochemical analysis of edible mushrooms *Lycoperdon perlatum* disclosed the presence of major myco-constituents visualized. Among this, three solvents were used for extraction inwhich hot water extract showed less number of myco-constituents as compared to ethanol and methanol extracts. Ethanol and methanol solvents show more or less same to each other.

3.2. Detection of extra cellular enzyme production

In the assay of extra cellular enzyme production, amylolytic activity, proteolytic activity and cellulase activity showing positive results while tyrosinase and lipolytic activity shows negative results.

Table: 2 Analysis of extracellular enzyme production by *Lycoperdon perlatum*
(+: Presence; –: Absence)

Amylolytic Activity	Proteolytic activity	Cellulase activity	Tyrosinase activity	Lipolytic activity
+	+	+	–	–

In amylolytic activity after 3 - 5 days of incubation, mushroom showed amylolytic activity by production of yellow zone around the colony when the plates were flooded with iodine solution. In proteolytic activity after incubation, degradation of the gelatin was seen as a clearing in the somewhat opaque and the clear zones around the fungal colony were enhanced when the plates were flooded with saturated aqueous solution of ammonium sulphate which result in formation of a precipitate and give a positive result. In cellulase activity, when Congo red solution was added and destained with 1M NaCl and kept for 15 minutes yellow areas around the fungal colony appeared which indicated cellulase activity positive. In this mushroom, tyrosinase activity was absent as no red brown color formation around the fungal colony after addition of p-cresol to the surface of fungal colony which indicates tyrosinase activity. In this mushroom, lipolytic activity was also absent as clearing or precipitation around the fungal colony was not produced.

3.3. Polysaccharide extraction and purification

Purified mushroom polysaccharide by submerged production which is white, sponge like powders of 2g yield was produced. The thin layer chromatography showed the presence of glucose confirmed in all the hydrolysates by calculation of retention factor to be 50.

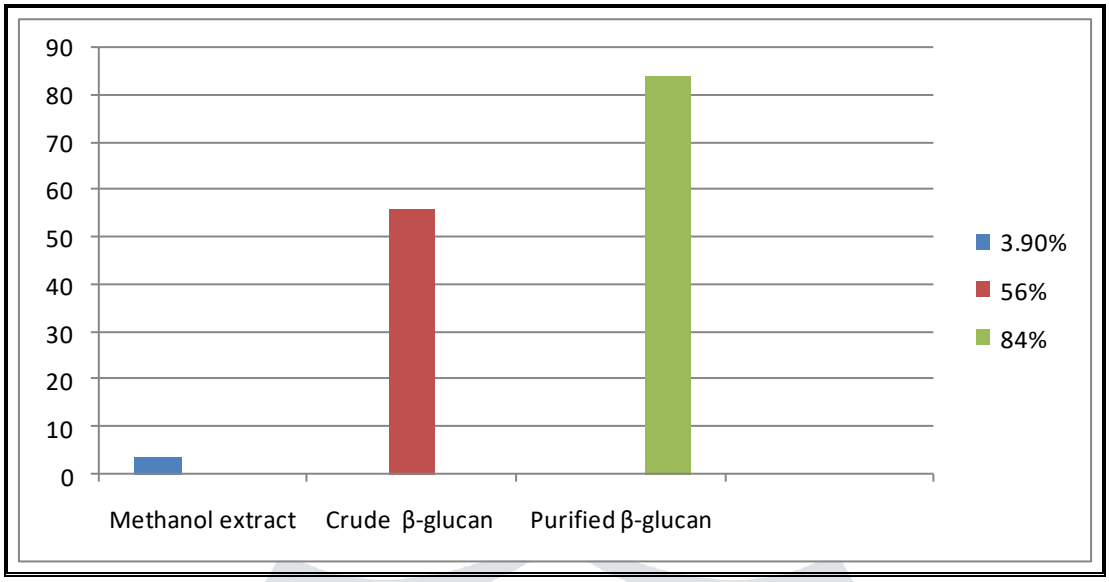


Figure: 1 Phenol Sulphuric acid Carbohydrate assay

3.4. Determination of the total carbohydrates by Phenol-Sulphuric acid method

The total carbohydrates present in the sample were analyzed by the phenol-sulfuric acid method. 1g of mushroom powder contains 3.9 mg of carbohydrate, and 100 mg of β-glucan contains 84mg carbohydrate. Phenol sulfuric acid method is a colorimetric method widely used in carbohydrate analysis. The crude polysaccharide was extracted using hot water. Proteins in the extract were removed by using the Sevag reagent. The total carbohydrates were estimated by the phenol-sulfuric modified method. We find the total carbohydrate content before and after the Sevag reagent was increased from 56% to 84%, respectively. Using ethanol extract of mushroom it was calculated that 1g of mushroom powder contain 3.9 mg of carbohydrate, and 100mg of beta-glucan contains 84mg of carbohydrate.

3.5. FT-IR analysis

The dialysed and powered beta-glucan samples were used for FTIR analysis. The FTIR spectra of polysaccharide extracts from *Lycoperdon perlatum* looked very similar to the standard FT-IR spectra of partially purified polysaccharide extracted from the dried grey oyster mushroom. The carbohydrate absorption peak may vary due to the fact that alkaline extraction of carbohydrates produces degradation especially at the basic chain.

Table: 3 FTIR analysis of β-glucan sample

Sl. No.	Peak (cm)	Functional Group	Bonding
1.	3000 – 3500 cm	–OH	Hydrogen bonding
2.	1147 cm	–C–O–C	Glycosidic linkage
3.	1040 cm	–C–O	Glycosidic linkage
4.	890 – 850 cm	–O–	α-and β-Glycosidic linkage
5.	1080 cm	–O–	β-Glycosidic linkage
6.	1647 cm	–	Protein
7.	1249 cm	–	Protein

To further confirm the presence of β (1→3)-glucan in our partially purified extract from the dried mushroom polysaccharide, the FT-IR spectroscopy technique was employed. Standard FT-IR analysis of mushroom polysaccharide and purified β-glucan was used as references (Galichet et al, 2001) (Satitmanwiwat et al, 2012). For example, the peaks in the range of 950 – 1,200 cm^{-1} indicate the presence of polysaccharides and anomeric region in the range 950 – 750 cm^{-1}. The peaks at 1,150 - 1,160 cm^{-1} define the stretching of glycosidic bonds. Other peaks and shoulders for β-glucan can be found at 1,376, 1,160, 1,100, 1,080, 1, 078, 1044 and 890 cm^{-1} (Synytsya et al, 2009). Additional peaks at 1,153 and 1,025 cm-1 correspond to the pyranose ring and β(1→4) glucans, respectively (Galichet et al, 2001) (Satitmanwiwat et al, 2012), suggesting cellulose contamination in our sample in the range 1155,1023,930,850 and 765 cm^{-1}. No evidence of other sugars was found in the FT-IR analysis.

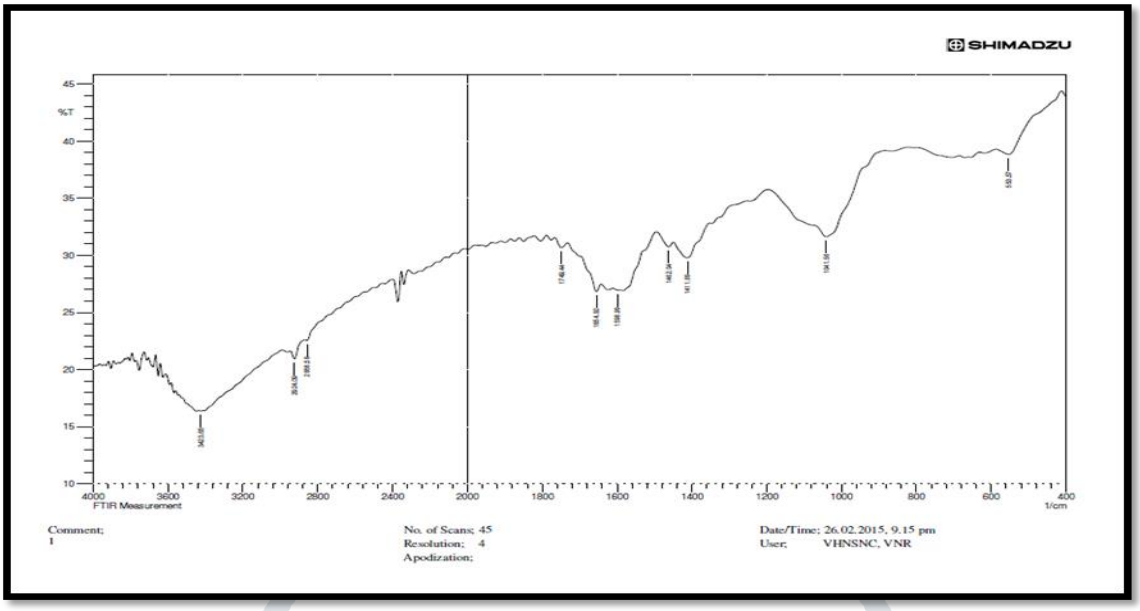
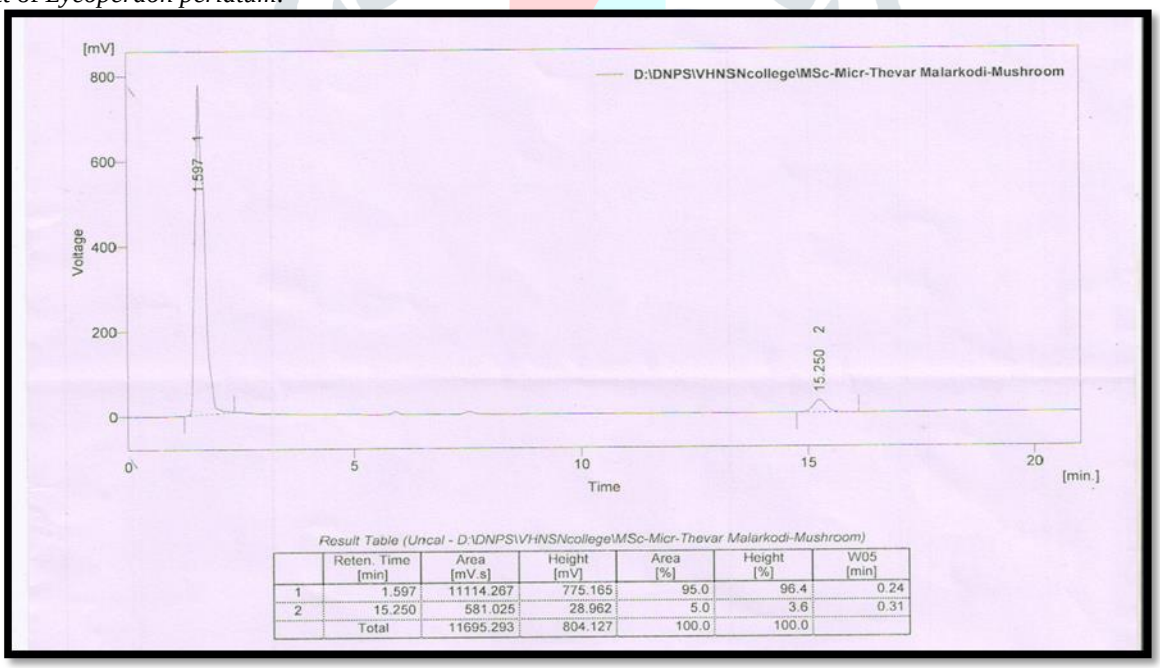


Figure: 2 FT-IR spectra of purified β-glucan extracted from *Lycoperdon perlatum*

Our FT-IR result (figure: 2) shows that the sample still contains some proteins, which might not completely be removed by the Sevag reagent (Synytsya et al, 2009). We also found a significant loss in the total carbohydrate after the Sevag extraction. Therefore, this extraction method might not be the best technique for eliminating proteins from the polysaccharide enzymatic purification must be employed.

3.6. HPLC Analysis

The mushroom cultured broth was separated by filtration followed by extraction with methanol. The compounds present in the methanol extract were detected on the TLC plates by UV - absorption under UV - lamp. The production of metabolites was further demonstrated by means of HPLC analysis. Figure (3) shows the HPLC chromatogram of the crude extract of *Lycoperdon perlatum*. Separation of the mixture of compounds in the extract was done with methanol: water (70: 30) over a period of 10 minutes. The presence of several metabolites was detected in the crude extract of *Lycoperdon perlatum*.



	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	1.597	11114.267	775.165	95.0	96.4	0.24
2	15.250	581.025	28.962	5.0	3.6	0.31
	Total	11695.293	804.127	100.0	100.0	

Figure: 3 HPLC analysis of methanol crude extract from *Lycoperdon perlatum*

The method of extracting Beta-glucan used in this work has proven to be efficient for *Lycoperdon perlatum* mushrooms. The quantification method HPLC was found to be efficient for measuring the presence of Beta-glucan. The first peak in HPLC is the solvent which is methanol and second peak is Beta-glucan in the range 15.250 cm^{-1}.

CONCLUSION

The present study focuses on the edible mushroom, *Lycoperdon perlatum*. The sample was collected from coastal regions. The mushroom mycelium was grew and isolated on potato dextrose agar plates using spores. Mycochemical screening was done and various fungal metabolites were examined in which methanol and ethanol solvents gave the better results than the hot water. The productions of extracellular enzymes by mushroom mycelium were detected. Beta-glucan was isolated from mycelium using submerged fermentation technique and purified. It was acid hydrolyzed using TFA and analyzed using thin layer chromatography. And the presence protein content should be measured using Lowry's method. The carbohydrate content was measured using phenol and sulfuric acid method that contain 56% crude β-glucan and 84%

purified β-glucan. The results obtained from this study has shown the potential of *Lycoperdon perlatum* mushroom extract as a potent therapeutic agent and a food supplement.

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