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Characterization of fruiting body exopolysaccharide from a milky mushroom *Calocybe* sp. TP and its antioxidant and antitumor activity

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Abstract

Calocybe is commonly known as milky mushroom and cultivated in India. Mushrooms have a good nutritional value and referred as healthy food. They are a good source of carbohydrate, proteins, vitamins and minerals while low in fat content. Mushrooms are well known as rich source of nutraceutical molecules too. They produce exopolysaccharides, having high antioxidant activity. Hence, the present study aims to characterize a mushroom exopolysaccharide for its antioxidant and antitumor activity. In present study, mushroom sample was collected from the Navsari Agricultural University, Navsari campus (20.9302° N, 72.9127° E). The sample was identified by morphological as well as 18S rRNA sequencing as *Calocybe* sp. TP (Accession No. MF737080). EPS was extracted from sample and characterized for its total carbohydrate 157.5±4.2 µg/ml. Chemical characterization of EPS by FTIR, TLC, HPLC and NMR analysis revealed that EPS contain glucose monomer in structure. Antioxidant activity measured by ABTS and DPPH showed 76.98% and 80.83% respectively. Antitumor activity was examined using MDA-MB-231 cell line and expressed 67.73% cell inhibition. Thus, EPS produced by *Calocybe* sp. TP can be used as antioxidant and antitumor molecule.

Keywords: antioxidant; antitumor; *Calocybe*; exopolysaccharide; mushroom

Introduction

Mushrooms are used since long for its nutraceutical properties. Reports revealed that it possesses various health benefits like, antimicrobial, antiviral, antioxidant, anticancer etc. In many Asian countries it is used for 3000 years for medicinal purpose to treat various diseases (Hobbs, 2002; Vyas *et al.*, 2021). Mushroom produced some bioactive molecules like terpenoids and polysaccharide which has an immno-modulatory effect. They can also suppress the growth of malignant tumours (Kheni and Vyas, 2017; Chaturvedi *et al.*, 2018).

Many mushrooms produce exopolysaccharide (EPS) during their growth cycle. These EPS are carbohydrates with high molecular weight and possess carbohydrate and protein conjugates. There is huge diversity in the EPS produced by different mushroom and thus in their medicinal properties. This uniqueness in the structure, bond, and chemical properties are responsible for their radical scavenging activity, antioxidant

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potential, inhibition of lipid peroxidation and also in lowering oxidative stress. Till date many mushrooms like *Agaricus, Calocybe, Ganoderma, Grifola, Inonotus, Lentinus, Phellinus, Pholiota, Pleurotus*, etc., have been reported for their antioxidant, antitumor and antiviral activities (Patel and Goyal, 2012). *Calocybe* also known as milky mushroom, produces EPS and has medicinal properties.

Today society is facing a major challenge in cancer treatment. Though chemotherapy is widely used it has many severe side effects also. Hence, in the current scenario, food with some nutraceutical value or food supplements having anticancer activity are in high demand. Milky mushrooms can be easily grown on a wide range of substrates containing cellulose, lignin, and hemicelluloses. Hence, the present study aims to characterize EPS produced by *Calocybe* and to evaluate its antioxidant and anticancer activity against the MDA-MB-231 cell line.

Materials and Methods

Collection and isolation of mushroom sample

The mushroom sample used in the present studies was collected from Navsari Agricultural University, Navsari campus (20.9302° N, 72.9127° E). The isolation of mycelium was carried out on potato dextrose agar (PDA) plates. For the isolation, a small piece of mushroom was inoculated in the PDA plates in aseptic condition, and the plates were incubated at 25 °C for 48-72 hrs. Subsequently, the culture was purified and stored on a PDA slant at 4 °C until the use.

Identification of mushroom

The morphological characterization was carried out macroscopically by observing the growth pattern of fungi and microscopically by the wet mounting of fungi in lactophenol. For molecular characterization, DNA from the mushroom sample was extracted according to the methods described by Graham *et al.* (1994). Amplification of the ITS region was done by ITS1 and ITS4 primers. PCR conditions were set as described by Kheni and Vyas (2017). Sequence was explored for homology and submitted to GenBank.

Extraction of EPS

The sample was dried at 60 °C in a hot air oven to remove water content for EPS extraction. The fine powder was prepared from dried samples using a blender for EPS extraction. Three different methods were used for eps extraction, i.e., hot water extraction (Zhang *et al.*, 2011), ethanol extraction, and methanol extraction (Xu *et al.*, 2009).

Quantification of carbohydrates

The carbohydrate from the crude sample and extracted EPS was measured using the method mentioned by Dubois *et al.* (1956).

FT-IR

For characterization of EPS, dried EPS powder was ground with KBr and pressed to obtain pellets. Infrared absorption spectra (Nicolet IR200 FT-IR Spectrometer) were recorded on an FT-IR in the 4000-400 cm^{-1} range. KBr pellet was used as the background reference.

Characterization of EPS

For chemical characterization sample was partially purified as described by Kheni and Vyas (2017). Briefly, sample was hydrolyzed using 5M trichloroacetic cacid (TCA) for 4 hrs in a boiling water bath.

Subsequently, dialysis was carried out in nitrocellulose beg at 4 °C for 2 days. After partial purification, characterization of EPS was carried out by TLC, HPLC, NMR, TGA and DSC analysis.

Thin Layer Chromatography (Stahl, 1969)

The sugars were detected by spotting the sample on silica gel 60 (F254 Merck, Germany) using ethyl acetate:acetic acid:methanol:distilled water (60:15:15:10) as solvent. The TLC plate was sprayed with an anisaldehyde-sulfuric acid reagent followed by heating at 100 °C for 5-10 minutes for the development of coloured spots. The type of sugar present in the sample was identified by comparing the Rf value of standard sugar run along with the sample.

HPLC (High-Performance Liquid Chromatography)

Hydrolyzed EPS sample was analysed using an analytical HPLC-ECD coupled with an RI detector. 0.1% H_2SO_4 in water was used as a mobile phase, and the flow rate was 0.5 ml/min. The monomers in EPS were detected by an RI detector.

NMR

Characterization of EPS was done by ^{1}H and ^{13}C NMR analysis. Water was used as a solvent during the analysis. Analysis was carried out at Central Salt & Marine Chemical Research (CSMCRI), Bhavnagar, India.

Antioxidant activity

To estimate the antioxidant activity, three different methods, Ferric Reducing Antioxidant Power (FRAP) assay, ABTS assay, and DPPH assay, were used. FRAP was measured as the method Benzie and Strain (1996) described where colorless Fe^{3+}-tripyridyltriazine complex was converted to a blue-coloured Fe^{2+}-tripyridyltriazine complex. The reduction of Fe^{3+} was monitored at 593 nm by a change in absorbance. The reducing efficiency was calculated using Fe^{2+} solution as a standard and expressed as Fe^{2+} µmol/gm.

In ABTS assay, radical scavenging ability of EPS was measured by inhibition of ABTS radical cation (ABTS+•) as described by Re *et al.* (1999) using ascorbic acid as standard. The Free radical scavenging ability of EPS was measured using the DPPH assay, as mentioned by Tailor and Goyal (2014). The nitrogen atom in DPPH is reduced by antioxidant to the corresponding hydrazine molecule. Thus, all the assay used to estimate their ability to scavenge free radical and subsequently antioxidant activity was calculated.

Antitumor activity

Antitumor activity was measured in vitro by cell growth inhibitory activity against the human breast cancer cell lines (MDA-MB-231) by MTT assay (Mossmann, 1983). For the assay, the cells were grown in tissue culture flasks in a Leibovitz medium at 37 °C. 10^5 cells/ml was transferred to each well in a 96-well tissue culture plate. The cells were allowed to grow for 24 hrs and then treated with the sample. 100 µl test sample (concentration range from 100 - 1000 µg/ml) was added to the wells, and cells were further incubated for another 24 hrs at 37 °C in an incubator. 20 µl MTT (5 mg/ml in phosphate-buffered saline) was added after incubation to each well, and cells were further allowed to grow for 4 hrs. Media was removed afterward, and100 µl dimethyl sulfoxide (DMSO) was added to each well. The absorbance was measured on a microplate reader (Multiskan, Thermo Scientific) at 570 nm. The experiment was done in triplicate. Control comprised cell growth without test sample. The inhibition percentage was calculated using the following formula:

Inhibition activity (%) = $[(A_{control}-A_{sample})/A_{control}] \times 100$

Where, $A_{control}$ was the absorbance of the control reaction and A_{sample} is the absorbance of the of the sample.

Results

Collection and isolation of mushrooms

A mushroom sample was collected from Navsari Agricultural University, Navsari, Gujarat, India (Figure 1). A portion of the mushroom sample was taken for isolation and purification of mushroom. The rest of the biomass was dried at 60 °C. Isolation and subsequent purifications were done, and pure culture was stored on a PDA slant at 4 °C until use.

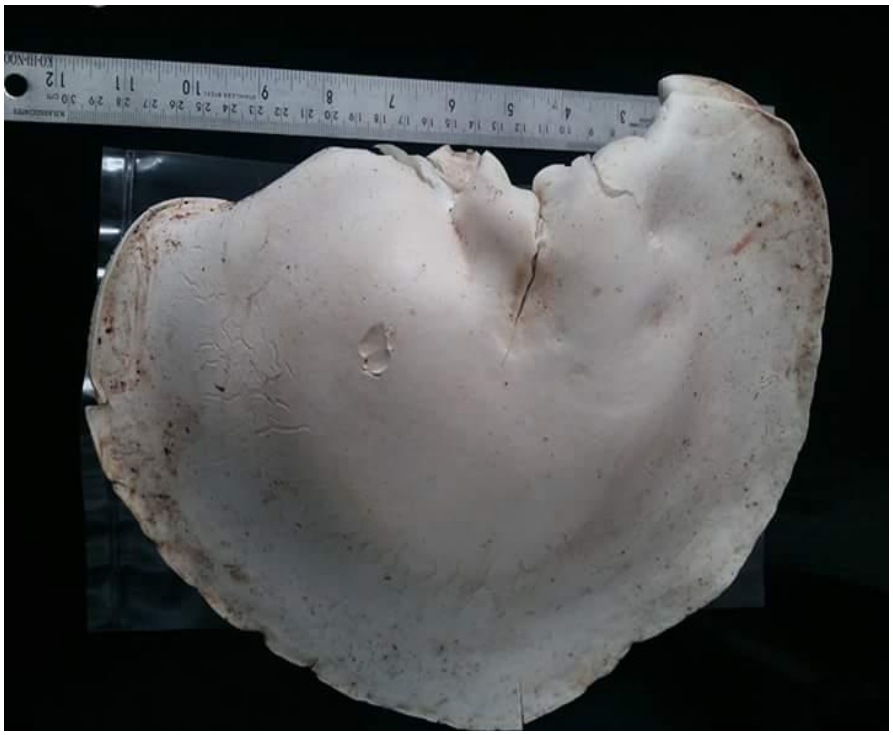

Figure 1. Mushroom sample collected from Navsari Agricultural University

Identification of mushroom

The pure culture was used to study the morphological characteristic of the isolate. The isolate showed secondary mycelia and clam connection when observed under a microscope with lactophenol cotton blue mounting stain. To identify the isolate, the ITS region is located between 18S rRNA and 5.8S rRNA gene, and hence, it was amplified with ITS 1 and ITS 4 primers. It produced a characteristic amplicon of approximately 650bp (Figure 2a). Amplified PCR product was sequenced at Saffron Life Sciences, India, and resulted in sequenced data being aligned using the NCBI blast tool. It showed homology with *Calocybe* sp., and hence it is named *Calocybe* sp TP (Figure 2b). The sequence data was submitted to GenBank with accession number MF737080.

Extraction of EPS

EPS from the mushroom sample was extracted by three different methods for higher EPS recovery. EPS was extracted by hot water, methanol, and ethanol extraction method. Maximum EPS was extracted using ethanol extraction (72.25 mg/g) followed by methanol (69.20 mg/g). The least EPS was extracted by hot water extraction (65.30 mg/g).

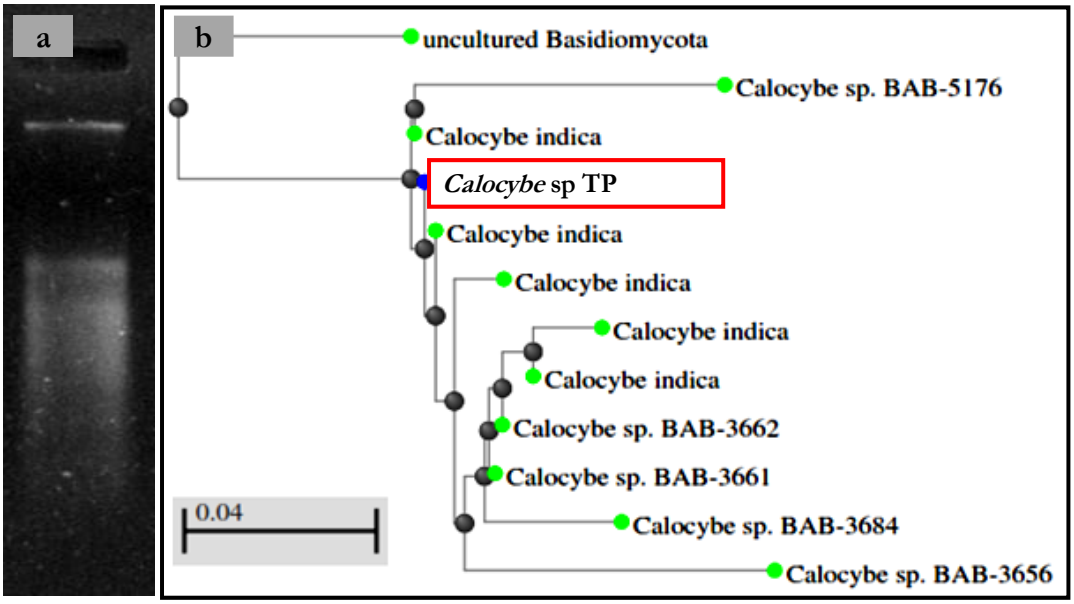

Figure 2. DNA of mushroom (a) phylogenetic tree of *Calocybe* sp TP (b)

Characterization of EPS

To find out the amount of carbohydrate present in the sample, 1 mg/ml of crude mushroom sample and extracted EPS were separately analyzed by phenol sulphuric acid method for carbohydrate content. Carbohydrate content was 157.5±4.2 (µg/ml) in extracted EPS sample.

FTIR analysis

The FTIR spectrum of an unhydrolyzed sample of *Calocybe* sp. TP EPS showed the characteristic absorbance of polysaccharides (Figure 3). The intensity of bands in the region 3,600-3,200 cm^{-1} was due to the hydroxyl stretching vibration of the polysaccharide. The absorption of a band at 2935 cm^{-1} indicates the C-H stretching vibration. The absorption between 1630-1402 cm^{-1} indicates a deprotonated carboxyl group. A broad stretch of C–O–C, C–O at 1,000^{-1}, 200 cm^{-1} corresponded to the presence of carbohydrates.

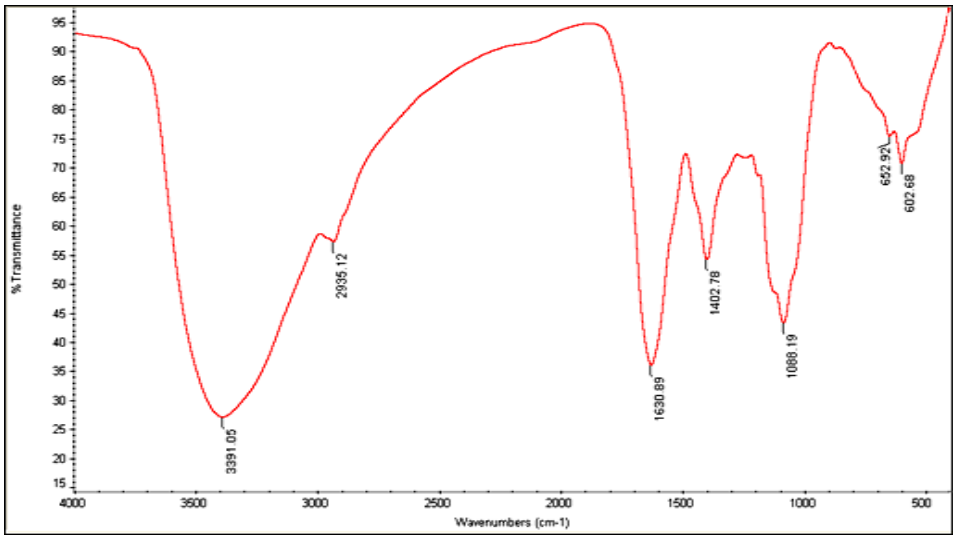

Figure 3. FTIR spectrum of EPS from *Calocybe* sp TP

TLC analysis

To identify the monomer of the EPS sample, partial purification of EPS was done using acid hydrolysis. Hydrolysis was monitored using TLC analysis at 1 hr intervals. The product was completely hydrolysed after 4 hrs of incubation in a boiling water bath. Hydrolysed product was neutralized with sodium bicarbonate and used further for TLC analysis and HPLC analysis. On a thin layer chromatogram, single spot was detected and had a similar Rf value to standard glucose.

HPLC analysis

A partially purified sample was analyzed for presence of monomers in the EPS structure. A single peak was matched with the glucose standard. TLC results also corroborate with HPLC analysis.

NMR

EPS sample was characterized by ^{1}H NMR and ^{13}C NMR analysis. In ^{1}H NMR peak between 3.03 – 3.64 indicates the sugar proton, whereas δ 4.83 indicates the anomeric carbon (Figure 4a). The peak at 4.207 showed H^{-1} with β (1→6) linkage. In ^{13}C, NMR peaks at 63.655, 65.399, 66.123, and 66.783 indicated the presence of C-6 (Figure 4b). Moreover, the peak at 69.047 suggested a large amount of terminal anhyro glucose units. Peaks at 72.155 and 72.545 revealed the presence of β (1→6) linkage, whereas the peak at 102.98 suggested the presence of β (1→3) in linkage in the backbone.

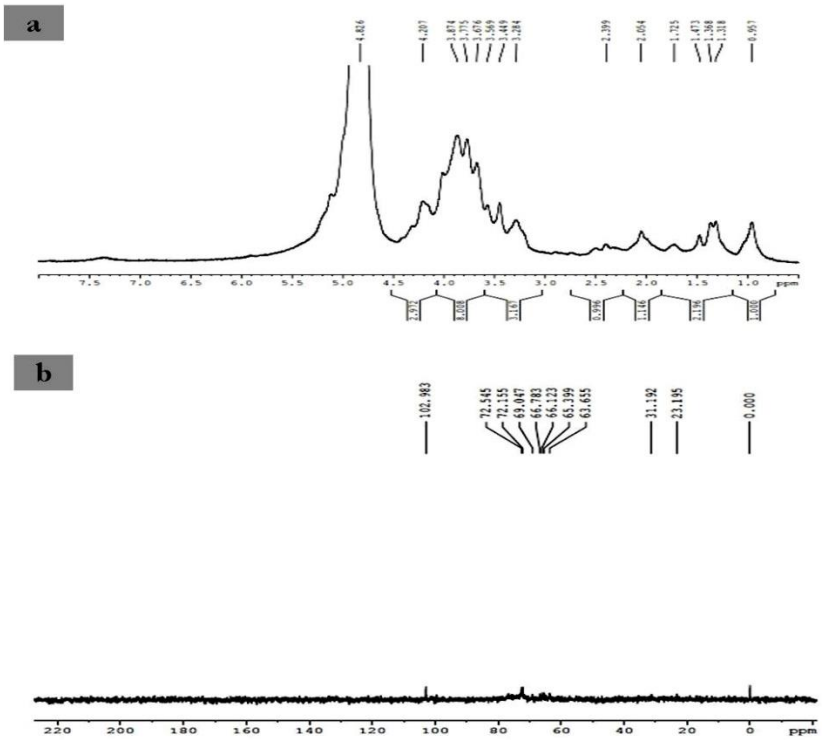

Figure 4. ^{1}H NMR (a) and ^{13}C NMR (b) spectrum of EPS produced by *Calocybe* sp TP

Antioxidant activity

Antioxidant activity of EPS was measured using FRAP assay. If molecule have antioxidant ability it converts the FeIII molecule to FeII. Results revealed that EPS has 10.5 mM/gm antioxidant activities. The second assay used for antioxidant activity was the ABTS assay. EPS showed 76.98% inhibition of radical ABTS molecule. The third assay used for measuring antioxidant activity was the DPPH assay. In this assay, EPS showed 80.83% antioxidant activity.

Antitumor activity

Antitumor activity was measured by cell viability using an MTT assay. The assay is based on the cleavage of the tetrazolium salt MTT in the presence of an electron-coupling reagent. To examine antitumor activity MDA-MB-231 cells were grown in Leibovitz media (Figure 5a). For MTT assay, cells were grown in 96 well ELISA plate for 24 hrs. After 24 hrs of incubation, different concentrations of EPS (0.1 – 1 mg/ml) were added and cells were further incubated for 24 hrs (Figure 5b). MTT solution was added to a 96-well ELISA plate containing pre-grown cells with different EPS concentrations and incubated for 4 hrs. Formazan produced after this incubation period was quantified using a scanning multi-well spectrophotometer ELISA reader. The measured absorbance directly correlates to the number of viable cells. Cells were lysed after 24 hrs of incubation, as shown in Figure 5c. Clumps of EPS were also observed after 24 hrs when most of the cells lysed. In control, all wells showed higher formazan production, whereas, in test samples, formazan colour production decreased gradually as EPS concentration increased. Cell inhibition was started at a minimum concentration of 0.1 mg/ml of EPS concentration, and maximum cell inhibition of 67.73 % was observed at a 1mg/ml concentration.

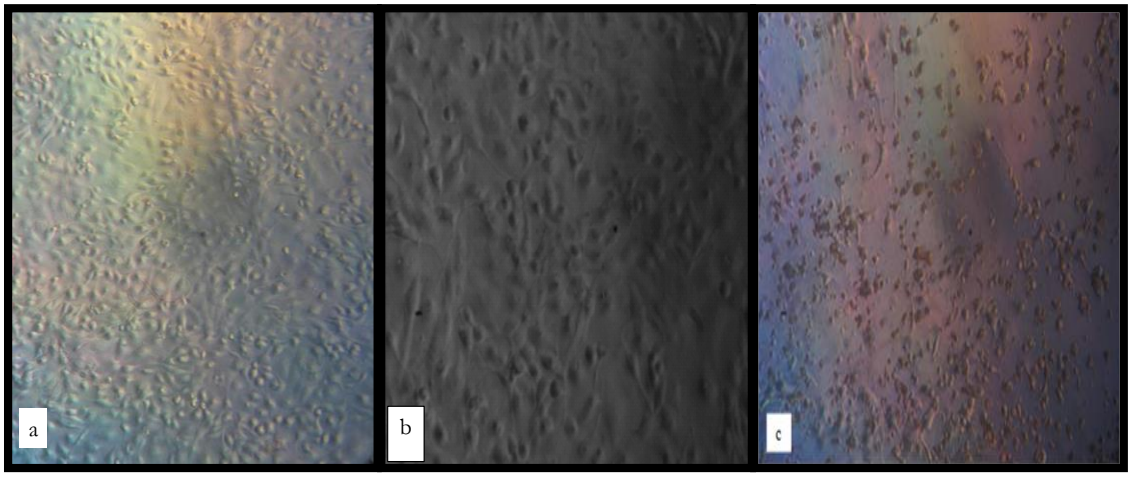

Figure 5. Antitumor activity against MDA-MB 231 cell line;(a) Normal growth of MDA-MB 231 breast cancer cell line; (b) cell line with addition of EPS; (c) Cell lysis in presence of EPS after 24-hour incubation

Discussion

In the present COVID 19 pandemic situation, everyone needs food with antioxidant activity. During metabolism, many free radicals are generated, which are harmful to DNA, protein, and other biomolecules. Long-term exposure to such free radicals may work as mutagens or carcinogens. Hence, food rich in antioxidants are advised by dieticians to fight against harmful caused by free radicals.

The mushroom sample collected was identified as *Calocybe* sp. TP by ITS region sequencing was submitted to Genbank. There were three different methods used for EPS extraction. Out of these methods used, the highest EPS was recovered using the ethanol extraction method, followed by methanol and hot water extraction. EPS extracted using ethanol extraction showed a higher extractive index, and the results are in accordance with many published data (Bae *et al.*, 2005; Zong *et al.*, 2012; Su *et al.*, 2013). In the ethanol extraction method, 72.25 mg/g EPS was extracted from *Calocybe* sp. TP, which contained 157.5±4.2 µg/mg of carbohydrate. Vishwakarma *et al.* (2016) estimated carbohydrate content from *Calocybe gambosa* and *Calocybe indica*. Carbohydrate content was 55.12±0.66% and 55.12±0.66%, whereas protein content was 31.44±0.22% and 52.13±0.54% in *Calocybe gambosa* and *Calocybe indica,* respectively.

Being a carbohydrate in nature, based on monomer, EPS can be homopolymer or heteropolymer. Moreover, it can be either linear or branched and contain β- (1→3) and β- (1→6) glycosidic bonds (Zong *et al.*, 2012). EPS is responsible for biofilm formation and cell to cell communication. Structural characterization using FTIR, TLC, and HPLC analysis was used to identify structural monomers of *Calocybe* sp. TP EPS. Among this analysis, characteristics of bonds present in carbohydrate structure can be easily identified using FTIR analysis. TLC and HPLC analysis of the exopolysaccharide extracted from *Calocybe* sp. TP showed the presence of a glucose monomer. However, Tejasvini *et al.* (2013) reported the presence of rhamnose, arabinose, xylose, galactose, and glucose in the EPS extracted from *Calocybe* indica.

Antioxidant activity is important as part of nutraceutical properties. Hence, in the present study, three different methods were used to estimate the antioxidant activity of the same. The result of antioxidant activity by EPS suggests that it has almost similar activity by ABTS and DPPH assay. Prabhu and Kumuthakalavalli (2016) worked on *Calocybe indica* and found 57.38±0.37mM/g Fe^{+2}, which is higher (10.5 mM/g Fe^{+2}) than the reported in present study. Mirunali *et al.* (2012) have also worked on *Calocybe indica* and reported 63% inhibition in ABTS assay, which is lower than % inhibition of the antioxidant activity of EPS from *Calocybe* sp. TP (76.98%). Prabhu and Kumuthakalavalli (2016) reported 28.04±0.41% antioxidant activity of DPPH assay in *Calocybe indica,* which was also was found lower than *Calocybe* sp. TP (80.83%) found in the present study.

Here, EPS extracted from *Calocybe* sp. TP was evaluated on the MDA-MB-231 breast cancer cell line by MTT assay showed 67.73 % inhibition of the tumour cell line at 1mg/ml EPS concentration. Selvi *et al.* (2011) reported 68±2.80% inhibition of urinary bladder carcinoma T24 cell line at 100 µg/ml EPS concentration of *C. indica,* which was found to be higher than the result of the present study. Ghosh (2015) extracted EPS by water and methanol extraction method from *Calocybe indica.* They have examined antitumor activity on the MHH-ES-1 cell line and reported 55.25±1.201 mg/ml and 46.56±0.134mg/ml IC50 values of water and methanol extract, respectively. While on MCF-7 cell lines, they have found 52.12±0.15mg/ml and 47.94±0.09 mg/ml IC50 values of water extract and methanol extract, respectively.

Conclusions

Calocybe sp TP (Accession No. MF737080) contains 104.25±0.15 µg/ml of total and 140±10 µg/ml protein in its exopolysaccharide molecule. Chemical characterization of EPS by various analyses revealed that *Calocybe* sp TP contains a single monomer Glucose in structure. TGA analysis revealed 86.34 % weight loss after the second stage. EPS possesses higher antioxidant activity measured by ABTS and DPPH suggests that it can scavenge free radicals. EPS can suppress tumor cell line, as evident by 67.73 % cell inhibition of the MDA-MB-231 cell line. Hence, EPS produced by *Calocybe* sp TP can be used as an antioxidant and antitumor molecule.

Authors' Contributions

Conceptualization and design of experiments (TKV); Methodology (PSV); data collection, analysis and interpretation (PSV); MTT assay (KKS); Supervision (TKV and KKS); Writing - original draft (TKV); Writing - review and editing (TKV). All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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