COMPARATIVE STUDY ON THE PRODUCTION, PURIFICATION AND CHARACTERIZATION OF EXOPOLYSACCHARIDES FROM OYSTER MUSHROOMS, *PLEUROTUS FLORIDA* AND *HYPSIZYGUS ULMARIUS* AND THEIR APPLICATIONS

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ABSTRACT

Mushroom polysaccharides have attracted great deal of attention due to many health benefits such as immunomodulation, anticancer activity, prevention and treatment of cardiovascular diseases, antiviral and antimicrobial effects. The present work involved the production and purification of extracellular polysaccharides (EPS) from two oyster mushrooms, *Pleurotus florida (PF)* and *Hypsizygus ulmarius (HU)* and investigation of their effect in relation to antioxidant and anticancer activities. Several parameters such as pH, temperature and media for the growth of mycelial culture and for the production of EPS were optimized to be 4.5, 27 °C and GPKM media, respectively. Purification of exopolysaccharides was carried out by DEAE Sephacel (anion exchange chromatography) and high yield of exopolysaccharides were obtained as 0.726 mg/ml, and 0.665 mg/ml for PF-EPS and HU-EPS, respectively. The samples were further subjected to characterization, antioxidant and anticancer studies. Characterization by NMR showed relative peaks corresponding to polysaccharides. The results of antioxidant assay for the two polysaccharide samples (PF-EPS and HU-EPS) by phosphomolybdenum method was found to be 20.57 and 21.93 ìM AAE/g of tissue, respectively. Anticancer potential of purified polysaccharides were assessed by MTT assay on MCF breast cancer cell lines and two samples, PF-EPS and HU-EPS exhibited percentage of cell viability at 66.48% and 47.63%, respectively. On the whole comparatively, HU-EPS demonstrated encouraging results in terms of anticancer potential.

Keywords: *Pleurotus florida, Hypsizygus ulmarius,* exopolysaccharides, anticancer

INTRODUCTION

Polysaccharides derived from mushrooms have emerged as an important class of bio-active substances. Polysaccharides isolated from macrofungi had already been considered to have antitumour activity. Schizophyllan, Lentinan and Krestin (mushroom polysaccharides), which were in Japan and China, are used for antitumour activity since it stimulates T and B lymphocytes, monocytes and macrophages leading to secretion of TNF-α (Tumour Necrosis Factor) or interleukins in both cell culture and humans.

Cultivated mushrooms have been limited in application since it consumes more time for cultivation and they have the ability to accumulate many toxic metals such as cadmium, lead, arsenic, copper, nickel, silver, chromium and mercury from contaminated soil. Thus, polysaccharides were produced from submerged culture under controlled environment and has been a best alternative [1]. Exopolysaccharides have been found in many food and pharmaceutical applications. A wide variety of applications includes thickening and stabilizing agents in chemical industry, immuno stimulating and antitumour agents for clinical use. It has also the ability to heal and protect skin against infection. It is also used for enhancing collagen biosysnthesis and increasing cell proliferation [2].

Polysaccharides from mushrooms are reported to have free radical scavenging activity, superoxide radical scavenging, reducing properties, lipid peroxidation inhibition, suppression of proliferation and oxidative stress etc. Polysaccharides isolated from different mushrooms genera are capable of providing antitumor activity eg., *Agaricus, Calocybe, Ganoderma, Grifola, Inonotus, Lentinus, Phellinus, Pholiota, Pleurotus*,etc., These include the activation of macrophages, T lymphocytes and natural killer cells, which are able to secrete inflammatory mediators of cytokines such as the tumor

necrosis factor, α-interferon, α interleukin. Polysaccharides can depress the E-selectin protein and gene expression, which inhibit tumor cell to cell adhesion. Other mechanism include antiproliferative effects, apoptosis induction and differentiation of the tumor cells [3].

Pleurotus florida (PF) and *Hypsizygus ulmarius (HU)* are the two edible oyster mushrooms used in the present study. Many bio active compounds have been reported from the fruiting body of *PF* and *HU*. The most important materials among these were the polysaccharide fractions obtained mainly from the fruiting body and also partly from the mycelium of PF and HF.

Pleurotus species are commonly called as Oyster mushrooms. It is a lignocellulolytic fungus that grows naturally in the temperate and tropical forests on dead and decaying matter and it is in second grade among the important cultivated mushrooms in the world.There are about 40 species coming under *Pleurotus* mushroom, among the 25 species are commercially cultivated [4].

Hypsizygus ulmarius (elm oyster mushroom) is a high yielding mushroom for which commercial cultivation technology has been released and is gaining popularity. Previous reports suggests that this mushroom is rich in antioxidants and proved for its anti-diabetic activity [5].

The present study aims to compare the production, purification and characterization of exopolysaccharides of two edible oyster mushrooms, *Pleurotus florida (PF)* and *Hypsizygus ulmarius (HU)* and their application in relation to antioxidant and anticancer activities.

MATERIALS AND METHODS

Sample Collection and cultivation

Bed spawn of both PF and HU were collected from TNAU (Tamil Nadu Agricultural University, Coimbatore) and used for exopolysaccharide production. The collected spawn was grown over paddy straw at 25 °C for 25 days. Grown mushrooms were harvested after 25 days.

Isolation of exopolysaccharide from PF and HU mycelial broth

The exopolysaccharides present in the culture broth of PF and HU was extracted following the methodology of Xu *et al*. [6]. For the extracellular product isolation, the mycelial cells were removed from culture broth using a filter paper. The filtrate was concentrated to one-fifth of its original volume with a rotary evaporator under reduced pressure and mixed with 4 volumes of chilled 95% (v/v) ethanol, then stirred vigorously and left at 4 °C overnight. The precipitate was collected by centrifugation at 5000 rpm for 20 min and lyophilized (exoploysaccharide, EPS - I). The EPS-I was further treated with the Sevag reagent to remove the protein, yielding the EPS-II fraction.

Screening of media: Spawn was cultured in PDA plate and small piece was cut using knife and inoculated in respective broth.The mycelia of PF were grown in YMG liquid media prepared by dissolving 1 g of yeast extract, 3 g of malt extract, and 2 g of dextrose in 100 ml distilled water. Mycelial plugs of PF and HU were grown in YMG media for 20 days at 25 ºC and were seeded to liquid media under aseptic conditions. The flasks were placed on a rotary shaker at 120 rpm, 25 ºC. (Modified Yeon-Ran Kim, 2003) [7]. The seed medium was also used as the basal medium consisting of 30 g/l glucose, 5 g/l peptone, 5 g/l KH_2PO_4, 1 g/l $MgSO_4.7H_2O$, (modified Baojing Yuan *et al*. [8] for screening of media.

Optimization of pH and temperature: Screened media was taken for optimal pH and temperature. pH optimization was carried out at 3.5, 4, 4.5, 5, 5.5, respectively [9]. Temperature optimization was carried out at 25, 27, 30, 32 °C [10].

Mass production of exopolysaccharides: With the screened media and optimized pH and temperature, mycelial culture of both PF and HU was cultivated in large volume for extraction of exopolysaccharides [6] and assayed using phenol sulphuric acid method for total carbohydrates.

Purification of exopolysaccharides

Purification of exopolysaccharides was carried out using DEAE Sephacel anion exchange chromatography [11]. Fractions are gradient eluted with 0.5 M NaCl at the rate of 5ml/min. Collected fractions were assayed for total carbohydrates by phenol sulphuric acid method. Eluates showing highest concentration of exopolysaccharides were pooled and lyophilized.

Determination of nuclear magnetic resonance of exopolysaccharides

The ^1H nuclear magnetic resonance (NMR) spectra of exopolysaccharides in D_2O were obtained with 300MHz Bruker NMR Spectrometer [12].

Evaluation of total antioxidant capacity (TAC) by Phosphomolybdenum method

The total antioxidant capacity of the exopolysaccharides was evaluated by the phosphomolybdenum method according to the procedure described by Prieto *et al.* [13]. The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of green phosphate/Mo (V) complex at acidic pH. A 0.2 ml extract was combined with 2 ml of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate). The absorbance of the reaction mixture was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. The antioxidant activity was expressed as the number of gram equivalent of ascorbic acid. The calibration curve was drawn using ascorbic acid.

Evaluation of anticancer activity by MTT assay

A colorimetric assay based on the MTT method by Sara silva *et al.* [14] was used to measure the growth inhibition of MCF breast cancer cell lines in microtitre plates of 96 wells containing DMEM medium. Appropriate concentrations of exopolysaccharides (1mg/ml) were added to each well (2 x 10^5 cells per well) and the cultures were incubated with CO_2 incubator with 5% CO_2 at 37 °C for 48h. Cell viability was determined by addition of 20µl of 3 mg/ml MTT to each microtitre well and after 4h of incubation, the supernatant was removed and 50 µl of dimethyl sulfoxide was added to each well to solubilize the precipitate. The survival rate of MCF breast cancer cell lines was assayed by measuring the optical density in a microtitre plate reader at 650 nm. Results were expressed as % of cell viability.

$$cell\ viability\ \% = \frac{test}{sample} * 100$$

All assays were carried out in triplicates and the results are expressed as mean ± standard of three replicates.

RESULTS AND DISCUSSION

Media screening

In order to produce exopolysaccharides (EPS) from PF and HU, culture media has to be optimized with certain parameters. Screening of media was carried out for culturing the mycelia in broth. From the Fig. 1, it was shown that YGM media produces polysaccharide (0.068 mg/ml) in mycelial powder, but at long incubation period (20 days). On the other hand, GPKM media produces the polysaccharide in mycelial broth (0.089 mg/ml) within 7 days of incubation. In order to carry out further study, GPKM was prefered, which gives high yielding exopolysaccharides. However, work on *Grifola frondosa* in YMG media displayed polysaccharide extraction at 7.73 mg/l [7]. Similar studies by Duan Yan-Quing *et al.* [15] in GPKM media on *A.aegirita* Mo-Aa exhibited the yield at 3.72g/l.

Optimization of pH and temperature

While working with mushroom mycelia, it became an important criteria to optimize the pH and temperature, since mushrooms belong to the family of fungi. Generally fungi prefer acidic pH and low temperature. Here, the study was carried out to find the pH and temperature for better yield of mycelial growth and exopolysaccharides production. From Fig. 2, it was inferred that with slight change in pH, growth of the mycelia was affected. At the pH 3, 5 and 4, the mycelial growth was

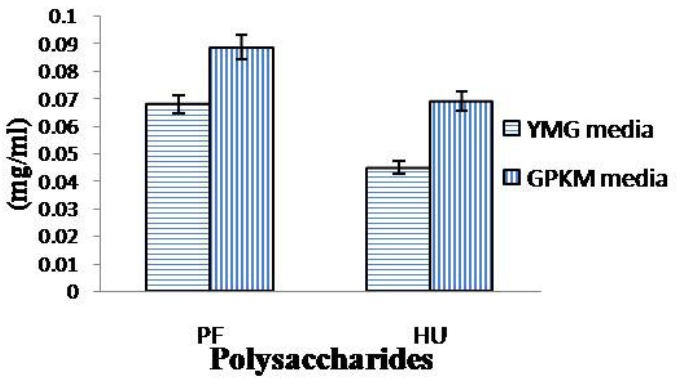

Figure 1. Screening of media for mass production of *Pleurotus florida* and *Hypsizygus ulmarius*

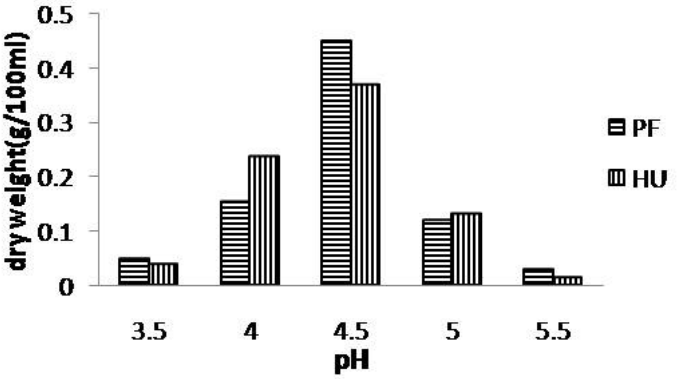

Figure 2. Optimization of pH for mycelia growth

limited, and in the pH 5 and 5.5 was tend to be negligible. However, at pH 4.5, there was reasonable quantity of mycelial growth. Since mycelial growth was directly proportional to the EPS, pH 4.5 is optimized for further studies. Other researchers reported optimum pH for the growth of *Alternania alternata* to be 3.0 [10] and study by Zhicai Zhang *et al*. [9] showed that there is no significant difference from pH 5 to 7.5 for the growth of mycelia *Tremella aurantialba*.

Temperature was also an important factor to be considered, while working on mushrooms, since it prefers comparatively low temperature than bacteria. From figure 3, it was clearly noticed that at temperature 25 °C, there was limited growth and at 32 °C, lot of bacterial growth was noted during the growth of mycelia. Thus optimum temperature for the growth of mycelial culture was found to be 27 °C. Our results are in concurrence with studies by Shamy and Nehad [10] on *Alternaria alternata* who showed that optimal temperature to be 30 °C.

Mass production of exopolysaccharides

In order to get bulk product, mass production step was necessarily carried out with the screened media, optimized pH and temperature. Production of exopolysaccharide (EPS) was carried out by method of Xu *et al*. [6] for both PF and HU.

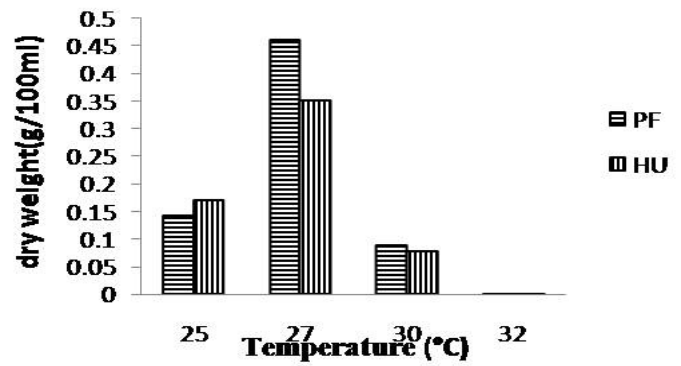

Figure 3. Optimization of temperature for mycelial growth

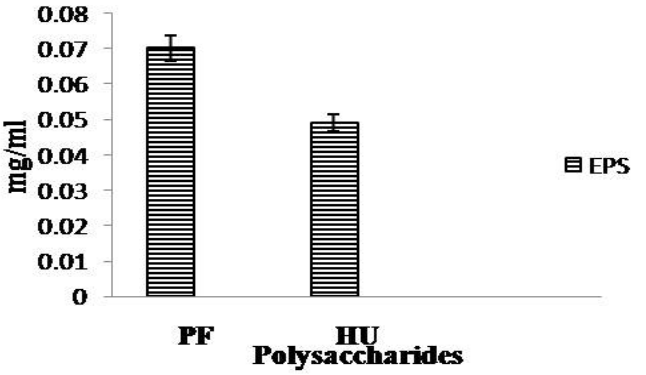

Figure 4. Mass production of exopolysaccharides

Figure 4. depicts the mass production of exopolysacharides. The mass production was carried out for samples namely *P. florida* (PF) and *H. ulmarius* (HU), exopolysaccharides (EPS), respectively. These obtained exopolysaccharides were used for further purification.

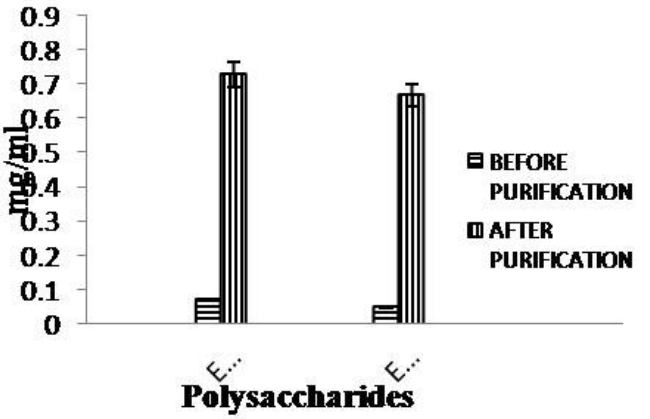

Figure 5. Comparison of Exopolysaccharides yield

Purification of exopolysaccharides

Purification by DEAE Sephacel anion exchange chromatography was carried out to remove the residual proteins. The unbound fractions containing exopolysaccharides was collected by eluting the column with sodium phosphate buffer of pH 8. Collected fractions were checked for total carbohydrates and high polysaccharide fractions were pooled, dialyzed and lyophilized [11].

After purification, exopolysaccharide production of both PF and HU was increased by 10 and 13 fold, respectively compared to the crude exopolysaccharides (Fig.5). Thus, these polysaccharides namely PF-EPS and HU-EPS were used for further studies such as characterization and application studies based on the yield. Similar studies by Sara Silva *et al*. [14] from *Letinula edodes* showed 0.037 g/l of exopolysaccharide after purification.

Characterization of exopolysaccharides by NMR

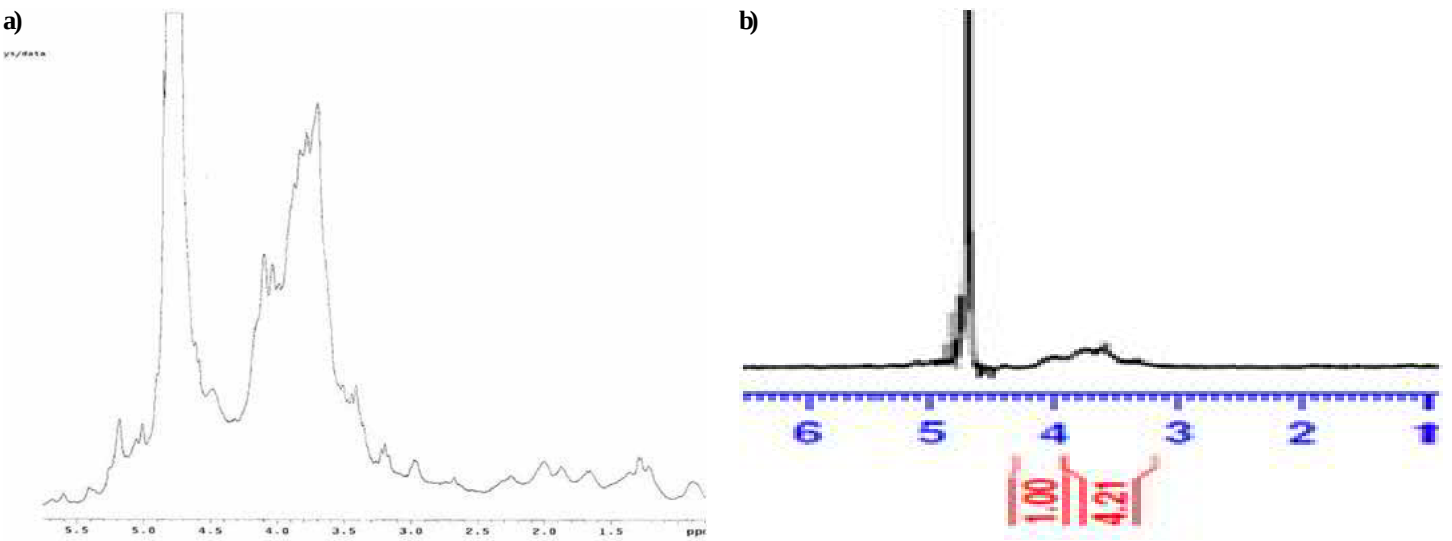

Figure 6. NMR spectrum of a) PF exopolysaccharides b) HU exopolysaccharides

H^1 NMR spectra for the two polysaccharide samples were analyzed by comparing with previous similar works. As per spectral Fig. 6 and 7, the peaks at 5.10 and 4.51 ppm, is characteristic of the α and β linkages respectively [12]. The anomeric region 4.3- 4.9 ppm were found to be characteristic of β anomers.

The ring proton regions 3.0-4.2 ppm showed overlapping peaks and were assigned to protons of carbons C2 to C5 (or C6) of the glycosidic ring [16], such that axial, equatorial protons and CH_2OH protons are appearing as multiplet.

Evaluation of total antioxidant capacity (TAC) by phosphomolybdnum method

Antioxidant compounds are present in many of fruits and vegetables naturally. Nowadays in the emerging field, antioxidant compound is necessarily produced in excess separately in order to act against free radicals, since free radicals destroy the

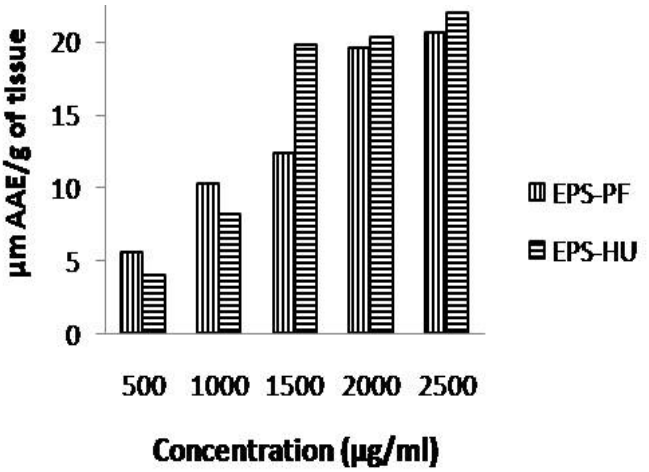

Figure 7. Total Antioxidant Capacity (TAC) of mushroom exopolysaccharides

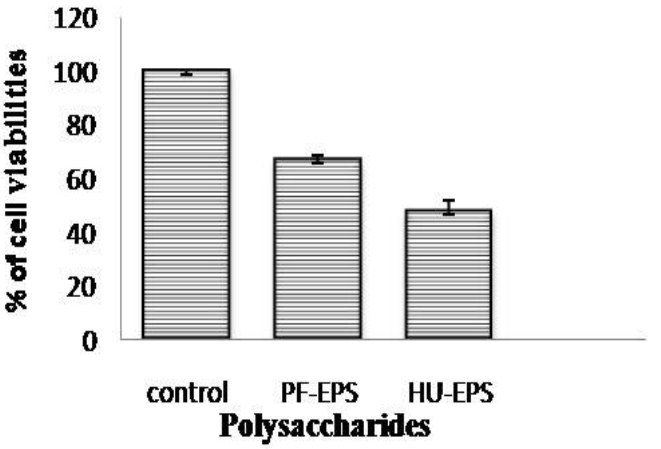

Figure 8. Cytotoxic effect of the polysaccharide samples on MCF-7 cancer cells

body's macromolecules such as protein, DNA, RNA, etc. In the present study, antioxidant assay was carried out in order to evaluate the antioxidant capacity of these two purified polysaccharide samples. The results of the assay were expressed as micromolar of ascorbic acid equivalents, which was then tabulated (Fig. 8).

At 2500 µg/mL, PF fruiting body polysaccharide showed higher ascorbic acid equivalents. Out of these two exopolysaccharides, the antioxidant activity was more or less similar as 20.57, 21.93 µM AAE/g of tissue for PF-EPS and HU-EPS respectively. Previous work by Adebayo *et al*., (2012) on *Pleurotus pulmonarius* showed 25.6 µM AAE/ g for 1mg/ml of fruiting body polysaccharides.

Evaluation of Anticancer activity by MTT assay

In recent years, occurrence of breast cancer is gradually increasing. It is a type of cancer originating from breast tissue,most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk. Emerging trend was to find the alternative medicine to cure these types of cancer in early stages, since breast cancer had caused 4,58,503 deaths worldwide in 2008. Thus, the present work has been extended to MCF breast cancer cell lines by observing cell viability on treatment with purified exopolysaccharides. Anticancer activity was done by MTT assay for purified polysaccharide samples at concentration of about 1mg/ml.

Results showed that HU-EPS has low cell viability towards the MCF breast cancer cell lines at about 47% compared to other polysaccharides. Studies by Thetsrimuang *et al.* [17] on MCF breast cancer cell lines by crude polysaccharides from *Lentinus polychorus* at 1mg/ml concentration have shown cell viability of 55%. Similar work by Larissa *et al*. [18] in *Hypsizygus marmoreus* showed inhibition of tumor growth upto 59%.

CONCLUSION

The present study aimed at the exopolysaccharides(EPS) production from PF and HU with screened media (GPKM), optimized pH (4.5) and temperature (27 °C) respectively. Purification of exopolysaccharides was carried out by DEAE Sephacyl anion exchange chromatography and has a yield upto 0.726 mg/ml, and 0.665 mg/ml, respectively. These exopolysaccharide samples were subjected to further analysis such as characterization by NMR, antioxidant and anticancer activity. Characterization by NMR showed relative peaks corresponding to polysaccharides such as OH and CH stretch, glycosidic linkages etc., respectively. Antioxidant assay was carried out for exopolysaccharide samples (PF-EPS and HU-EPS) and results obtained as 20.57, 21.93 µM AAE/g of tissue, respectively and the dosage for anticancer studies were fixed at 1mg/ml. MTT assay was carried out on MCF breast cancer cell lines to assess the anticancer activity of the purified exopolysaccharides. The anticancer activity of two samples PF-EPS and HU-EPS was denoted as percent viability of

66.48% and 47.63%, respectively. Hence, the present study showed that HU-EPS displayed high anticancer effect comparatively. Mushroom polysaccharides are yet to be explored for a lot of various pharmaceuticals for applications in near future.

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