

**RESEARCH ARTICLE**

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**OPTIMIZATION AND CHARACTERIZATION OF EXOPOLYSACCHARIDES FROM *PLEUROTUS SALMONEO-STRAMINEUS* AND ITS POSSIBLE APPLICATION****ABSTRACT:**

The present study revealed that the cultivation of *Pleurotus salmoneo-stramineus* on glucose yeast peptone medium at initial pH 6, sucrose and yeast peptone powder were used as sole carbon and nitrogen sources at 25°C under submerged incubation enhanced EPS production. Also, data indicated that the addition of palmitic acid at concentration 0.2% was the best stimulator for EPS production, where 1.278 mg/ml was obtained of EPS dry weight from experiments. HPLC analysis explained that exopolysaccharides is composing of glucose, galactose, fructose, mannose, arabinose and xylose at concentration 186.26, 4.765, 0.398, 154.78, 0.105, and 0.178 mg/gm, respectively. <sup>1</sup>HNMR confirmed presence of  $\alpha$  and  $\beta$  glycosidic linkages. EPS showed antioxidant activity 23.3% (25 mg/ml) and have antitumor activity against colon cancer cell DLD-1 where it inhibited tumour cell by 45%.

**KEY WORDS:**

Exopolysaccharides, structure, production, biological activity, *Pleurotus salmoneo-stramineus*

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<http://my.ejmanger.com/ejeb/>**INTRODUCTION:**

Many polysaccharides and polysaccharides-protein complexes have been isolated from fungi, algae, lichens and plant (Ooi and Liu, 2000). Many basidiomycete's mushrooms contain biologically active polysaccharides (Wasser *et al.*, 2000). The genus *Pleurotus* contain many biological compounds such as polysaccharides, protein, enzymes, dietary fibre and vitamins (Shen *et al.*, 2013). Xu *et al.* (2012) noticed that polysaccharide-protein complex isolated from *Pleurotus pulmonarius* had anticancer activity against liver cancer cells. Cao *et al.* (2015) reported that polysaccharide extracted from *Pleurotus ostreatus* mycelia markedly reduced both gastric cancer weight and volume.

The purpose of the present research was to evaluate the growth factors that might increase the amount of EPS. Also characterization of EPS using HPLC, IR and NMR were done. Finally, antioxidant and antitumor activity are analysed *in vitro*.

**MATERIAL AND METHODS:****Strain and Maintenance:**

*Pleurotus salmoneo-stramineus* was obtained from agricultural research centre (Central Laboratory for Agricultural Climate Giza, Egypt). The studied fungus was preserved on potato glucose agar (PGA) media which consisted of (gm/l): 20 glucose, 250 potato and 20 agar. The slants were incubated at 25°C for 7 days and then stored in refrigerator at 4°C.

**Determination of dry weight:**

The fungal biomass was separated by filtration and washed several times and dried at 80°C until constant weight (Nour EL-Dein *et al.*, 2004).

**Extraction of Exopolysaccharides (EPS):**

The culture filtrate was separated from the mycelia biomass followed by adding 5% Trichloroacetic acid (TCA) for removing

protein (Khalil, 2002) and stored at freezer overnight. The supernatant was mixed with two volumes of 95% of ethanol, stirred vigorously overnight at 4°C. The resultant precipitate was recovered by centrifugation at 3000 rpm for 20 minutes (Wu *et al.*, 2008) by centrifugation (Hettich, Germany).

#### **Measurement of carbohydrate content:**

The content of EPS was determined by phenol-sulfuric colorimetric method (Dubois *et al.*, 1956) using glucose as standard.

#### **Purification of polysaccharides:**

Crude EPS were partially purified by dialysis membrane (Berg *et al.*, 2007).

#### **Effect of different physiological parameters on exopolysaccharides production and mycelial growth:**

#### **Effect of different media on exopolysaccharides production and mycelial growth of *Pleurotus salmoneo-stramineus* under static and shaken conditions:**

*Pleurotus salmoneo-stramineus* mycelial growth and exopolysaccharides was studied by three different medium types, PG media, mushroom complete media (MCM) that consisting 20 g/l Glucose, 2 g/l Peptone, 2 g/l Yeast extract, 0.46 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{KHPO}_4$  and 0.5 g/l  $\text{MgSO}_4$  and glucose yeast peptone media (GYP) (40 g/l glucose, 10 g/l yeast extract and 5 g/l peptone). Inoculate each media with one fungal disc of *P. salmoneo-stramineus* with size 1cm in diameter. After incubation for 7 days under shaken and static culture at 25°C, the EPS was determined (Nour EL-Dein *et al.*, 2004).

#### **Effect of different incubation periods:**

GYP media flasks were tested for different incubation periods of 5, 10, 12 and 14 days (Elshamy and Nehad, 2010). At the end of incubation periods the EPS, growth biomass were determined.

#### **Effect of different pH values:**

To determine the optimum pH for maximum production of EPS, the prepared liquid GYP media was adjusted at different values of pH (4.5, 5, 5.5, 6, 6.5, 7, 7.5, and 8) and incubated at 25°C at 150 rpm for 10 days (Elshamy and Nehad, 2010).

#### **Effect of different temperatures:**

The optimum temperature was determined by incubation of the tested organism at different degrees of temperature as 20, 25, 30 and 35°C for 10 days under shaken condition at 150 rpm.

#### **Effect of different carbon sources:**

The glucose of GYP media will be replaced with equimolecular weight concentrations of the following different carbon sources (maltose, fructose, starch and sucrose) which were tested for maximum production of EPS (Wu *et al.*, 2008).

Different nitrogen sources such as urea, glutamic acid, sodium nitrate, glycine and asparagine were added with equimolecular weight with initial nitrogen source (Wu *et al.*, 2008).

#### **Effect of different fatty acids at 0.2% (v/v):**

Different fatty acids (oleic, stearic and palmitic acids) at concentrations (0.2% v/v) were studied for giving the optimum mycelial growth and EPS production (Yun *et al.*, 2002).

#### **Characterization of the exopolysaccharides structure extracted from culture filtrate of *Pleurotus salmoneo-stramineus*:**

#### **Fourier Transform -Infrared Spectroscopy:**

Fourier transform-infrared (FT-IR) spectroscopy (FT/IR-4100, Japan) was employed using the KBr disc for the analysis and detecting of functional groups. (Shen *et al.*, 2013).

#### **Nuclear Magnetic Resonance:**

The  $^1\text{H}$  nuclear magnetic resonance (NMR) spectra of exopolysaccharides in  $\text{D}_2\text{O}$  were obtained with 300MHz Bruker NMR Spectrometer.

#### **High Performance Liquid Chromatography (HPLC):**

Exopolysaccharides were hydrolysed following the method of Chen *et al.* (2005). Analysis of the carbohydrate in the filtrate was performed by using HPLC, Shimadzu Class-VPV 5.03 (Kyoto, Japan) equipped with refractive index RID-10A Shimadzu detector.

#### **Biological activities:**

#### **2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity:**

Total antioxidant activity was estimated according to the method described by Thaipong *et al.* (2006) and Molyneux (2004) using different exopolysaccharides concentrations (5, 10, 15, 20, and 25 mg) dissolved in 1ml distilled  $\text{H}_2\text{O}$ .

#### **Antitumor activity:**

The human colon adenocarcinoma cancer (DLD-1) was kindly provided by the National Cancer Institute Cairo Egypt. They grow on RPMI-1640 medium supplemented with 5% heated Foetal Bovine Serum (FBS), 2mM glutamine and antibiotics (penicillin 100U/ml, streptomycin 100µg/ml), at 37°C in humidified atmosphere containing 5%  $\text{CO}_2$ . Exponentially growing cells were obtained by plating  $1.5 \times 10^5$  cells/ml for human colon cancer DLD-1  $10.75 \times 10^4$  cells/ml followed by 24h of incubation (Monks *et al.*, 1991). The protein-binding dye sulforhodamineB was used to estimated cell growth. The bound stain was solubilized and the absorbance was measured at 492 nm in plate reader. For each tested compound and cell lines, a dose response curve was obtained and the minimum inhibitory concentration (IC50) cell was calculated as described.

## RESULTS AND DISCUSSION:

### Effect of different media on exopolysaccharides production and mycelial growth of *Pleurotus salmoneo-stramineus* under static and shakedd conditions:

Figure 1 a&b showed that GYP medium was the best medium for high mycelial biomass and EPS production. *Pleurotus salmoneo-stramineus* mycelial dry weight ( $6.92 \pm 0.43$  mg/ml) and EPS dry weight ( $0.34 \pm 0.03$  mg/ml) was in case of shaking condition, however the biomass ( $6.86 \pm 1.14$  mg/ml) and EPS dry weight ( $0.23 \pm 0.06$  mg/ml) were decreased under static culture. From our results, shakedd culture approved its efficiency in maximum EPS production than static culture. These results was agree with finding of Anike *et al.* (2015) for *Lentinus squarrosulus* and also Osman *et al.* (2014) for *Flammulina velutipes* growing on mushroom fermentation medium. Nour EL-Dein *et al.* (2004) reported that malt medium was the preferred medium for EPS production from *Pleurotus pulmonarius*.

### Effect of different incubation periods:

Incubation period has an essential role for high mycelia growth and high production of EPS. In figure 1c, it was observed that EPS dry weight was  $0.39 \pm 0.02$  mg/ml after 10 days and after that it decreased. The optimum incubation time was 10 days and this is was consistent with Rosado *et al.* (2003) who found that it was 9 days for *Pleurotus ostratoroseus* sing and *Pleurotus ostreatus* "florida". The same conclusions were detected by Elshamy and Nehad (2010) for *Alterneria alternate*.

### Effect of different pH values:

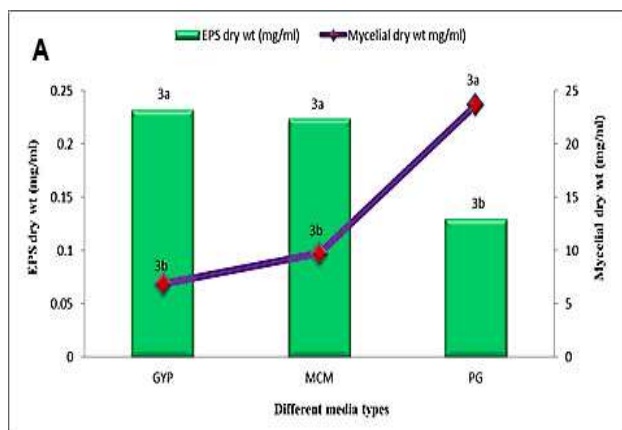
The medium pH was vital factor affecting cell membrane function, cell morphology and structure, the uptake of various nutrients, and product biosynthesis. Results in figure 1d, showed that pH 6 was the optimum pH value for high EPS production dry weight which gave  $0.39 \pm 0.01$  mg/ml and dry mycelia weight was  $7.32 \pm 0.77$  mg/ml. Lai *et al.* (2014) and Osman *et al.* (2014) reported that initial pH 6 achieved a maximum mycelial biomass and EPS obtained from *Lignosus rhinoceros* and *Flammulina velutipes* 6.

### Effect of different temperatures:

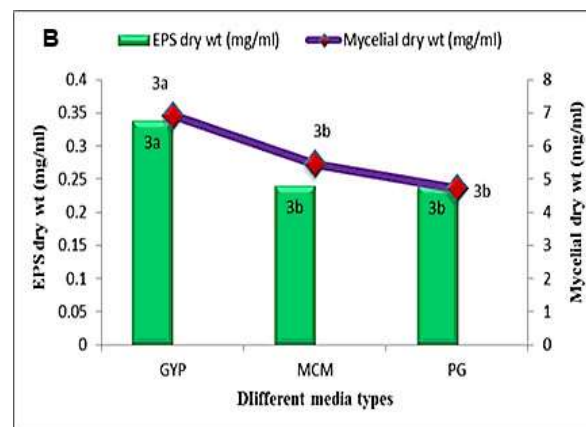
It was evident that temperature at 25°C was the most suitable degree for high EPS production and growth of *Pleurotus salmoneo-stramineus*. From figure 1e; the results showed a gradual increase of exopolysaccharides at 25°C and after which EPS concentration decreased but dry mycelial weight was optimum at 30°C. This was consistent with finding of Lee *et al.* (2008) for *Lentinus lepideus* and Choi *et al.* (2006) for *Pleurotus nebrodensis inzenga*.

### Effect of Different carbon sources:

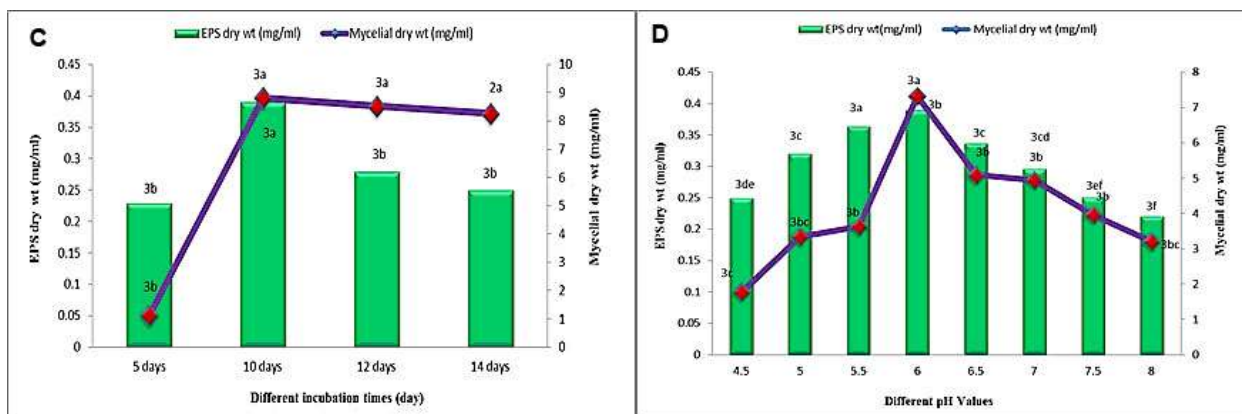
Figure 1f indicated that sucrose was the suitable carbon source for high production of EPS ( $0.603 \pm 0.01$  mg/ml) and mycelial growth ( $10.79 \pm 0.73$  gm/ml). Also starch increased mycelial growth ( $10.81 \pm 0.11$  mg/ml). This results were similar to Kim *et al.* (2002) for *Paecilomyces sinclairii*. In contrast, Shen *et al.* (2013) reported that xylose was the suitable carbon source for maximum production of EPS that obtained from *Pleurotus pulmonarius*.



Effect of different media on exopolysaccharides production and mycelial growth of *Pleurotus salmoneo-stramineus* under static condition.

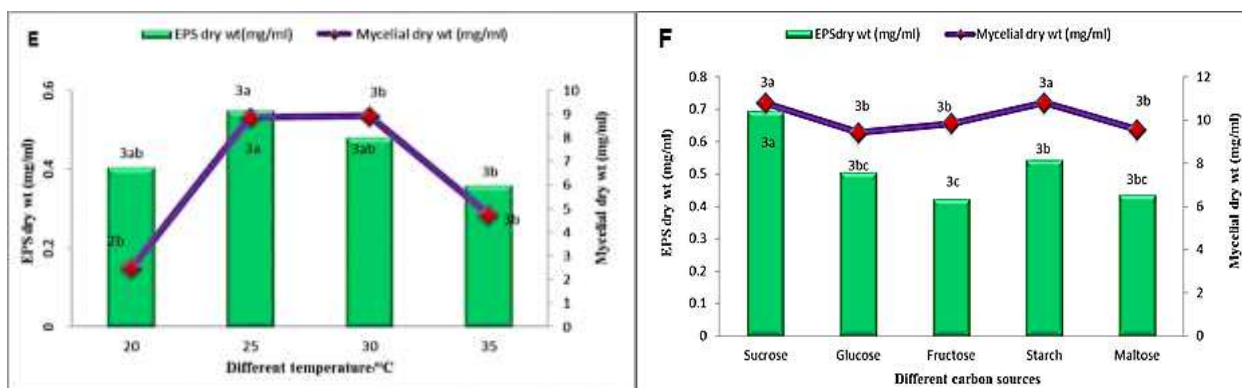


Effect of different media on exopolysaccharides production and mycelial growth of *Pleurotus salmoneo-stramineus* under shakedd condition.



Effect of different incubation periods on exopolysaccharides production and mycelial growth of *Pleurotus salmoneo-stramineus* under shaken condition.

Effect of different pH values on exopolysaccharides production and mycelial growth of *Pleurotus salmoneo-stramineus* under shaken condition



Effect of different temperature degrees on exopolysaccharides production and mycelial growth of *Pleurotus salmoneo-stramineus* under shaken condition

Effect of different carbon sources on exopolysaccharides production and mycelial growth of *Pleurotus salmoneo-stramineus* under shaken condition

Fig. 1. a-f: Different physiological parameters were studied to optimize the exopolysaccharides production and its effect on mycelial growth of *Pleurotus salmoneo-stramineus*

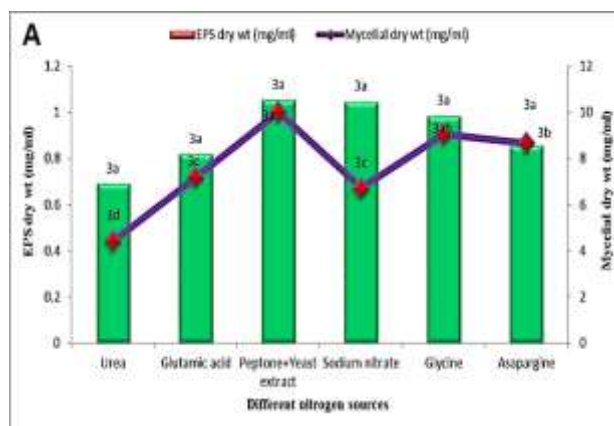
#### Effect of different nitrogen sources:

Different nitrogen sources have performed effect on EPS and biomass production. Results in figure 2a showed that yeast extract and peptone achieved high production of EPS ( $1.053 \pm 0.11$  mg/ml) and gave the optimum mycelial dry weight ( $10.02 \pm 1.34$  mg/ml). These results were consistent well with finding of Osman *et al.* (2014) for *Flammulina velutipes* on intracellular polysaccharides production. Elshamy and Nehad (2010) recorded for *Alternaria alternata* that yeast extract followed by sodium nitrate were the best source of nitrogen for EPS production. The stimulatory effect of yeast extract is due to its protein, amino acid and vitamin content (Botton and Blair, 1982).

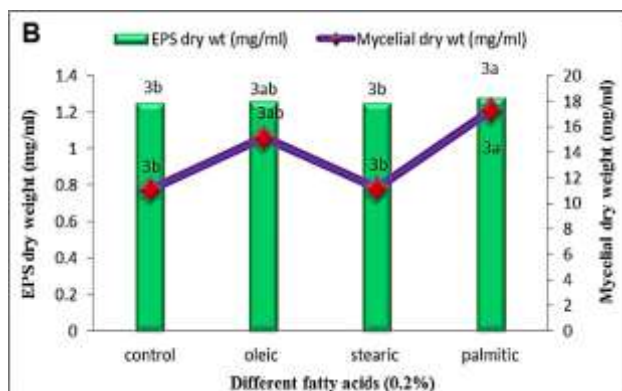
#### Effect of different fatty acid at concentration 0.2% (v/v):

It was noticed that maximum exopolysaccharides was achieved by using palmitic acid at concentration 0.2% (v/v) ( $1.278 \pm 0.01$  mg/ml) as shown in figure 2b. The present results agreed with Yang *et al.* (2000) and Abd El-Zaher *et al.* (2005) for *Ganderma lucidum*. Wang *et al.* (2011) in his

research on *Cordyceps sinensis* CS001 found that at 55 mg of palmitic acid showed stimulatory effects on EPS production, the EPS yield significantly increased from 353.1 to 401.7 mg/l.



Effect of different nitrogen sources on exopolysaccharides production and mycelial growth of *Pleurotus salmoneo-stramineus* under shaken condition



Effect of different fatty acids (0.2% v/v) on exopolysaccharides production and mycelial growth of *Pleurotus salmoneo-stramineus* under shakedd condition

Fig. 2. A&b. Different physiological parameters were studied to optimize the exopolysaccharides production and its effect on mycelial growth of *Pleurotus salmoneo-stramineus*

### Characterization of the exopolysaccharides structure extracted from culture filtrate *Pleurotus salmoneo-stramineus*:

#### Fourier Transform -Infrared Spectroscopy of polysaccharides (FT-IR):

From figure 3a, EPS showed peaks number at  $3426\text{ cm}^{-1}$  (no.1) which means presence of OH,  $1652\text{ cm}^{-1}$  (no.5) means presence of C=O,  $1162\text{ cm}^{-1}$  (no.11) indicated for the presence of COC stretching of glycosidic bonds. The bands at  $1162$  (no.11),  $1079$  (no.12) and  $1037\text{ cm}^{-1}$  (no.13) were characteristic to presence of  $\beta$  glucan.  $\alpha$ 1, 3 glucan was appeared at the bands  $551$  (no.15) and  $469$  (no.16)  $\text{cm}^{-1}$ .  $2933$  (no.2)  $\text{cm}^{-1}$  explained the presence of carbohydrate rings. Jantaramanant *et al.* (2014) showed that a peak at  $1,078\text{ cm}^{-1}$ , which represents the  $\beta$  (1 $\rightarrow$ 3)-glucans for *Pleurotus sajor-caju*. By analyzing *Pleurotus sajor-caju* exopolysaccharides under IR spectroscopy,

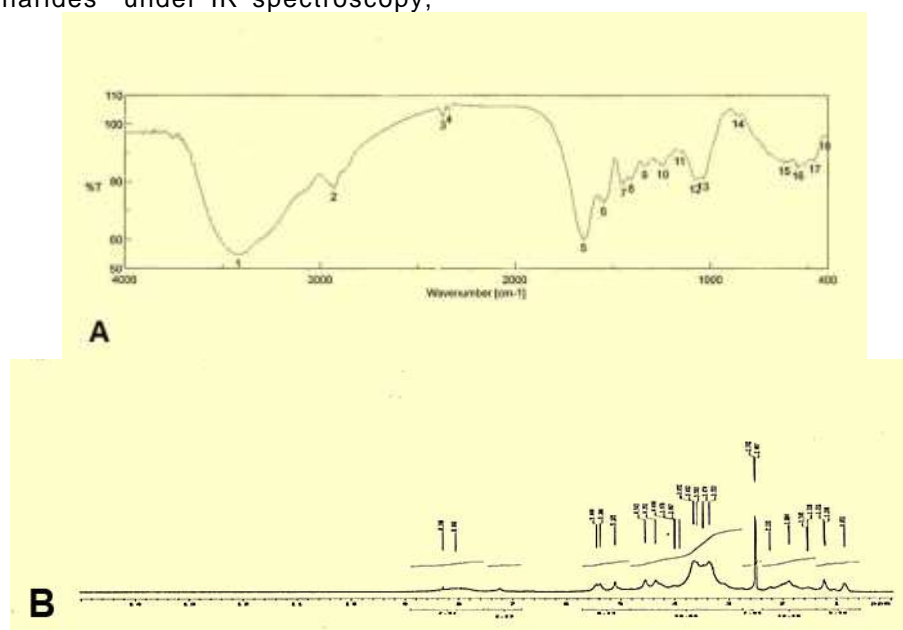
peaks at  $2,920$ ,  $3,394\text{ cm}^{-1}$  are C-H stretching vibration and hydroxyl stretching vibration, respectively (Satitmanwiwat *et al.*, 2012). Shen *et al.* (2013) noticed that exopolysaccharides from *Pleurotus pulmonarius* gave continuous absorption beginning at the region.  $3278.4\text{ cm}^{-1}$  which is characteristic of a carbohydrate ring.

#### Determination of Nuclear Magnetic Resonance of exopolysaccharides:

By studying structure of EPS by  $^1\text{H}$  NMR spectrum, the peaks at 5.10 and 4.5 ppm, is characteristic of the  $\alpha$  and  $\beta$  linkages, respectively. The ring proton regions 3.0-4.2 showed overlapping peaks that were assigned to protons of carbons C2 to C5 (or C6) of the glycosidic ring as shown in figure 3b. This finding was with agreement of Latha and Baskar (2014) and Li *et al.* (2012) for exopolysaccharides from *Pleurotus florida*, *hypsizygus ulmarius* and *Pleurotus abalonus*, respectively

#### High Performance Liquid Chromatography Analysis:

The HPLC chromatography (Fig. 3c) showed that EPS is consisting of six peaks refer to the presence of glucose, galactose, fructose, mannose, arabinose and xylose at concentration 186.26, 4.765, 0.398, 154.78, 0.105, and 0.178 mg/g. Ren *et al.* (2015) showed that polysaccharides from *Pleurotus abalonus* fruiting bodies consisted of D-mannose, D-ribose, l-rhamnose, D-glucuronic acid, D-glucose and D-galactose, and their corresponding mole percentages were 3.4%, 1.1%, 1.9%, 1.4%, 87.9%, and 4.4%, respectively. HPLC analysis for *Pleurotus ostreatus* and *Pleurotus sajor-caju*. Polysaccharides were consisting of maltose, glucose, mannose and fructose (Ullah *et al.* 2014).





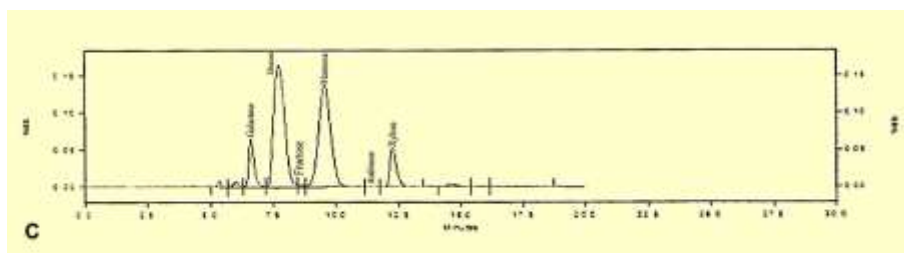


Fig. 3a-c. Characterization the exopolysaccharides structure extracted from culture filtrate of *Pleurotus salmoneo-stramineus*. a) FT-IR analysis b) <sup>1</sup>H NMR spectrum c) HPLC analysis

### Biological activities:

#### Antioxidant activity:

DPPH method is usually to evaluate antioxidant activity of various natural compounds by reducing stable DPPH radicals to yellow-colored diphenylpicrylhydrazine. DPPH radical scavenging ability is responsible for hydrogen donating efficiency of antioxidants. As shown in figure 4, DPPH radical scavenging activity of EPS increase gradually with concentration increase. At 25 mg/ml, scavenging effects of EPS were 23.3%, respectively. Li *et al.* (2012) suggested that all the polysaccharides from

*Dictyophora indusiata*, *Hypsizygus marmoreus*, *Lentinus edodes*, *Russula vinosa* Lindblad, *Hohenbuehelia serotina*, *Pleurotus eryngii* Quel, *Hericium erinaceus* and *Auricularia auricula* had significant antioxidant capacities (ranged from 18.54% to 100%) at concentration of 20 mg/ml. On contrast of Sharma *et al.* (2015) for *Cordyceps gracilis*, the intracellular polysaccharides of both strains *P. ostreatus* PBS281009 and M2191 were found to show a higher DPPH scavenging activity than those of the EPS synthesized.

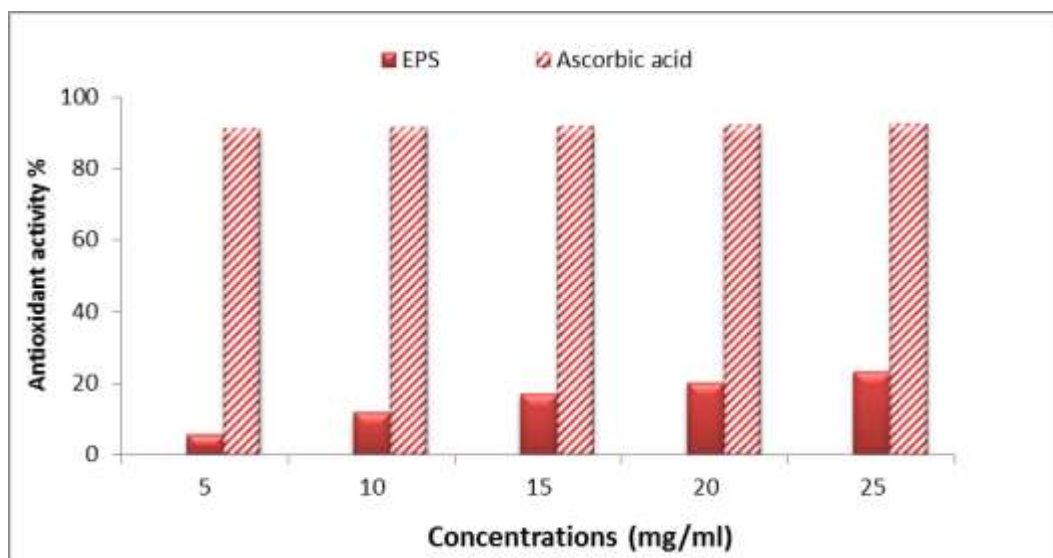
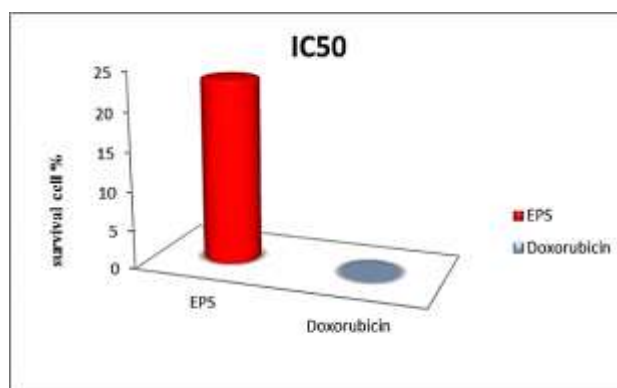


Fig. 4. Scavenging effect of exopolysaccharides from *Pleurotus salmoneo-stramineus* on DPPH radical. Each value is expressed as mean  $\pm$  standard deviation (n=3).

#### Antiproliferative activity:

EPS presented antiproliferative activity at dosage of 10 mg/ml which inhibited 45% of tumour as shown in figure 5. The IC<sub>50</sub> (mg/ml) for EPS and doxorubicin (as standard) were  $23.62 \pm 2.34$  and  $0.06 \pm 0.008$  mg/ml, respectively. Polysaccharide-Kureha known as krestin (PS-K) has been demonstrated to increase NK/LAK activation (Fisher and Yang 2002). Latha and Baskar (2014) approved that polysaccharides of *Pleurotus florida*-EPS and *Hypsizygus ulmarius*-EPS had effect against breast cancer cell lines, where it exhibited percentage of cell viability at 66.48% and 47.63%, respectively. Tong *et al.* (2009) showed that polysaccharides extracted from

fruiting bodies of *Pleurotus ostreatus* presented significantly higher anti-tumour activity against Hela tumour cell *in vitro*.



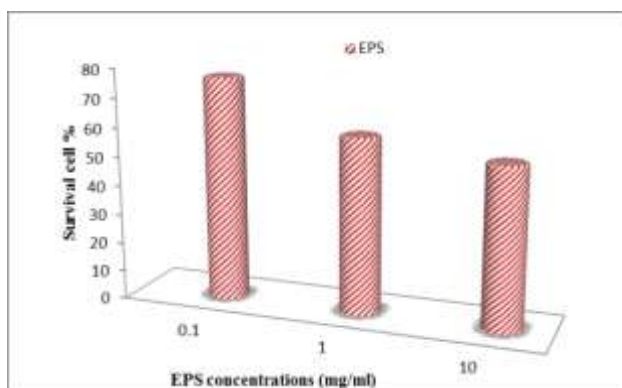


Fig. 5. Effect of different concentrations of exopolysaccharides from *Pleurotus salmoneo-stramineus* on human colon cancer DLD-1 survivability.

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## الظروف المثلى وتوصيف السكريات عديدة التسكر الخارجية من سلمونو استرامينيس وتطبيقاتها الممكنة

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فطر البلوروتس سلمونو استرامينيس يتكون من السكريات الجلوكوز، الجالاكتوز، الفركتوز، المانوز، أرابينوز وزيلوز بتركيزات 186.26، 4.765، 0.398، 154.78، 0.105 و 0.178 ملليجرام/جم. أيضا تحليل HNMR أكد وجود رابطة ألفا وبيتا الجليكوزيدية. أثبتت السكريات عديدة التسكر الخارجية المستخلصة من فطر البلوروتس سلمونو استرامينيس أن لها نشاط مضاد للأكسدة بنسبة 23.3% عند 25 ملليجرام/مل وأيضا لها نشاط مضاد للأورام ضد خلية سرطان القولون DLD-1 حيث تثبط الخلايا السرطانية بنسبة 45%.

أظهرت الدراسة أن زراعة فطر البلوروتس سلمونو استرامينيس على وسط غذائي يحتوى على السكر كـ مصدر كربونى ومستخلص الخميرة والبيتون كمصدر نيتروجينى عند رقم هيدروجينى 6 ومضاف اليه حمض الباليك بتركيز 0.2% عند درجة 25 درجة سليوزية لمدة 10 أيام كفترة تحضين أنتجت 1.278 ملليجرام/مل من السكريات عديدة التسكر المفزة خارجيا فى الوسط الغذائى. وأوضحت النتائج بعد التحليل باستخدام جهاز HPLC أن عديدة التسكر الخارجية المستخلصة من