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Research Article

Production and Antiproliferative activity of Various Crude Extract from *Lentinus squarrosulus* mycelium

Rahayu Ahmad, Siti Mariatul Usyida Alias, Aidil Abdul Hamid, Wan Mohtar Wan Yusoff, Endom Ismail, Fauzi Daud*

School of Bioscience and Biotechnology, Faculty Science and Technology, National University of Malaysia, 43600 Bangi, Selangor, Malaysia.

***Corresponding author**
Fauzi Daud
Email: fauzid@ukm.my

Abstract: Production of extracellular and intracellular polysaccharides (EPS, IPS) from *Lentinus squarrosulus* mycelium was carried out through submerged fermentation. The one-factor-at-a-time method was adopted to investigate the effect of medium components (carbon, nitrogen), and environmental factor (initial pH) on dry weight of mycelium and EPS concentration. Sucrose and yeast extract gave the highest EPS concentration with 3.56 mg/mL and 1.23 mg/mL respectively when carbon and nitrogen sources were employed. Combination of ammonium chloride and yeast extract (30:70) ratio increased the mycelium dry weight up to 10.8 g/L and EPS concentration of 4.39 mg/mL. An initial pH of 7.5 showed maximum EPS concentration of 3.82 mg/mL with mycelium dry weight of 7 g/L. Crude extracts from hot water and cold water extraction of *Lentinus squarrosulus* mycelium (IPS HWE, IPS CWE) and water extract from supernatant (EPS WE) was proved to have antiproliferative activity against A549 lung carcinoma cell lines (ATCC) as compared to ethanol extract.
Keywords: polysaccharides, *Lentinus squarrosulus*, mycelium, antiproliferative activity

INTRODUCTION

The global awareness of cancer as the second largest cause of death in people of various ages and background has led to so much research effort and clinical studies in the fight against the disease [1]. Cancer is defined as a disease in which a group of abnormal cells grow uncontrollably by disregarding the normal rules of cell division[2]. There are several treatments for cancer which includes surgery, immunotherapy, radiotherapy and chemotherapy. Most of these techniques are useful in particular situations, but the combination of them offer a more efficient treatment for cancer. Most cytotoxic drugs used in cancer chemotherapy are also highly toxic to a wide spectrum of normal tissues, such as those found in gastrointestinal tract, bone marrow, heart, lungs, kidney and brain. Iatrogenic failure of these organs is a frequent cause of death from cancer[3]. The antitumor activities of mushrooms polysaccharides have drawn the most attention in recent years. The search for new antitumor and other medicinal substances from the higher Basidiomycetes and the study of medicinal value of these edible mushrooms have become matters of great interest.

Mushrooms are nutritionally functional food and a source of physiologically beneficial and non-toxic medicines[4]. A wide variety of mushrooms have been used traditionally for the maintenance of health and for prevention and treatment of diseases such as cancer, inflammation, viral diseases, hypercholesterolemia, blood platelet aggregation and hypertension[4-9]. The use of medicinal mushrooms in anticancer activity has been reported in China, Korea, Japan, Russia, United States and Canada and it was effective against cancers of the stomach, oesophagus, prostate and lung, belong to the family of Polyporaceae[10]. Some species of edible higher Basidiomycetes have been reported to exhibit antitumor activity and approximately 200 species of higher Basidiomycetes have been reported to exhibit antitumor activity[11-17].

Chihara and coworkers[18-20] were first to isolate a water-soluble anti-tumor polysaccharide from the fruit bodies of *Lentinusedodes*, which was name “Lentinan” [β(1-3), β(1-6) glucan] after the generic name of this mushroom. The molecular formula of Lentinan is $(C_6H_{10}O_5)n$, the mean molecular weight is about $1 \times 10^5 - 5 \times 10^5$ Da, [α]D + 20°-22° (NaOH). It was confirmed to be a β-D-glucan, as shown by electrophoresis and ultracentrifugation, as well as by various techniques and instrumental analysis[21]. The investigation of antitumor properties of *L. edodes* proved that lentinan was found to almost completely regressed the solid type of tumors in synergic host-tumor system A. The antitumor effect of Lentinan was originally confirmed

by using Sarcoma 180 transplanted in CD-1/ICS mice [18]. Antitumor activity of lentinan was found to be significantly higher than that of polysaccharides isolated from many other fungi and higher vascular plants[22].

To the best of our knowledge, there is no study reported of the antiproliferative activity of polysaccharides from *Lentinus squarrosulus* against lung cancer cell, A549 (ATCC). Thus in this study, various crude extracts from different extraction method from mycelium and culture broth of *Lentinus squarrosulus* was used to investigate their antiproliferative activity against lung carcinoma cell, A549 (ATCC cell line).

MATERIALS AND METHOD

Optimization of fermentation medium using one-factor-at-a-time method

Mushroom mycelium of *Lentinus squarrosulus* were maintained on potato dextrose agar (PDA), 30 g/L added with 2% yeast extract, stored at 4°C for a long term use. The mycelium from stock culture was placed in the centre on PDA plate and incubated for 7 days prior to submerged liquid fermentation. Glucose yeast extract (GYE) medium consists of (per liter) glucose 30 g, yeast extract 3 g, Mg.SO4.7H_2O 0.5 g, KH_2PO4 1 g with initial pH of 5.5 [23] was used in this study. 10 plugs of *Lentinus squarrosulus* mycelium were inoculated in each flask and fermentation was carried out at 28°C, 150 rpm for 7 days. The effect of different carbon sources, nitrogen sources, manipulation of their concentrations and various initial pH were investigated. For carbon sources, glucose was substituted with different carbon sources such as sucrose, fructose, maltose, lactose, arabinose, sucrose and xylose. Initially all carbon sources were screened at 30 g/L and after that carbon source concentration were varied from 25 to 200 g/L. The effect of nitrogen sources was carried out by replaced yeast extract with other organic nitrogen sources such as peptone, beef extract, and inorganic nitrogen sources like ammonium chloride, ammonium nitrate, ammonium sulphate, sodium nitrate and urea. Keeping total nitrogen source at 3 g/L, the ratio of organic to inorganic nitrogen source was varied from 0:100 to 100:0. The effect of initial pH on growth of mycelium and EPS production was carried out with different pH value. pH values were varied from 4.5 to 8.0.

Analytical Methods

Samples collected were centrifuged at 10 000 rpm for 20 min, and the resulting supernatant (1 mL) were subjected to the phenol-sulfuric acid assay. The precipitation of EPS was conducted as followed the methodology of [24]. The supernatant was mixed with 4 times (4:1 v/v) the volume of absolute ethanol, stirred vigorously and kept overnight at 4°C. The precipitated extracts were centrifuged at 10 000 rpm for 20 min discarding the supernatants. The insoluble component was suspended in 1 mL distilled water and was assayed by phenol-sulfuric acid method [25] using D-glucose as a standard. The dry weight of mycelium was measured after repeated washing of the mycelial pellet with distilled water and drying at 70°C to a constant weight.

Production of polysaccharides

Submerged liquid fermentation was carried out for production of mycelium using optimized culture conditions (sucrose concentration 114.61 g/L, yeast ectract 1.62 g/L, Mg.SO4.7H_2O 0.5 g/L, KH_2PO4 1 g/L and initial pH 5.81) as described by[26]. A 5 mm plug of *Lentinus squarrosulus* mycelia was removed with a cork borer from 7 day-old-culture. The mycelia cut (10 plugs) was transferred into liquid medium, incubated in orbital shaker for 7 days at 28°C, 150 rpm for mycelia growth. Cultured mycelia were then dried using oven until constant weight. Dried mycelia were used for intracellular polysaccharide (IPS) extraction using ethanol, cold water and hot water method. Culture filtrates were used for extracellular polysaccharide (EPS) extraction using ethanol and water extraction method. EPS water extract were prepared by directly subjecting the culture filtrate to freeze-dryer yielding the exopolysaccharide water extract (EPS WE).

Extraction of Polysaccharides

Cold Water Extraction

Approximately, 5 g of mycelia powder were mixed with 500 mL of distilled water and stirred vigorously for 3 h at room temperature. The extracts were filtered using Whatman No 1 and the culture filtrates were freeze dry for intracellular polysaccharides cold water extracts (IPS CWE). Exopolysaccharide cold water extract were prepared by directly subjected the culture filtrate (after separated from mycelium) to freeze-dryer yielding EPS WE.

Hot Water Extraction

Intracellular polysaccharide hot water extracts (IPS HWE) were extracted using the method as described by [27]. Briefly, 40 g of dried mycelia powder was extracted twice with distilled water (600 mL) at 100°C for 3 h in water bath. The extracts were cooled, filtered and the filtrates were dried using freeze dryer.

Ethanol Extraction

Ethanol extraction was conducted according to the method used by[24]. Culture filtrates were mixed with 4 times of absolute ethanol, stirred vigorously and kept overnight at 4°C. Ethanol was removed using rotary evaporator and crude extracts were dried using freeze dryer yielding the extracellular polysaccharide ethanol extracts (EPS EE). For intracellular polysaccharide ethanol extracts (IPS EE), a modified hot water extraction as described by[27] was used. Briefly, the powders of dried mycelia (40 g) were extracted twice with distilled water (600 mL) at 100°C for 3 h in a water bath. The extracts were cooled,

filtered using filter paper (Whatmann No 1) and precipitated using ethanol before dry using freeze dryer.

In Vitro Cytotoxicity Assay

The A549 lung carcinoma cell lines and Chang cell from ATCC were used for the cytotoxicity test. The cells, A549 were grown in RPMI medium supplemented with 20% fetal bovine serum (FBS) and 1% Penicillin-streptomycin. All cultures were maintained at 37°C in humidified atmosphere of 5% CO_2. Cytotoxicity assay of five different crude extracts particularly from mycelium (IPS CWE, IPS HWE, IPS EE) and culture broth (EPS EE, EPS WE) of *Lentinus squarrosulus* on A549 cell lines were evaluated in vitro using the colorimetric 3-(4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide, MTT assay. Briefly, cell suspension in culture medium were seeded in 96-well plates with the concentration of 1 x 10^5 cells/well, and incubated at 37°C in humidified atmosphere of 5% CO_2. After 24 h, cells were treated with different concentration of crude extracts followed by incubation until 72 h. The 100 µL of MTT (5 mg/mL) was added to each well and incubated for 4 h. Finally the media were removed and DMSO was added to the wells (150 µL/well) and absorbance was measured at 570 nm in a micro plate reader. Tamoxifen drug (0.2 mg/mL) was used as positive control. Tamoxifen was dissolved in 0.1 ml DMSO and added with 10 mL of complete medium before diluted in different concentration range from 10 to 100 ug/mL. Each measurement was performed in triplicate. The half-maximal inhibitory concentration (IC_{50}) value was determined from the percentage of the cell viability versus final concentration of the extract curve.

RESULT AND DISCUSSION

Optimization Using One-Factor-at-a-time

In this study, we demonstrated the effect of medium components on the mycelia growth and production of EPS through submerged fermentation. The medium was supplemented with different carbon sources such as glucose, sucrose, fructose, lactose, maltose and xylose at a concentration of 30 g/L. Figure 1 shows the effect of different carbon sources on mycelial growth and EPS production. Among the carbon sources tested, glucose supported the mycelial growth with the highest dry weight of 7.92 g/L followed by maltose and fructose with 6.01 and 5.87 g/L respectively. However glucose exhibited the lowest EPS with 1.32 mg/ml. The production of EPS was high when sucrose was used as carbon source with 3.56 mg/mL and 3.47 mg/mL when fructose was supplied in the medium. Xylose showed lowest dry weight of mycelium with 3.90 g/L. The lowest concentration of EPS from glucose is contrast to some of previous research, which reported that glucose supported the mycelia growth as well as the production of EPS by *Paecilomyces tenuipes* C240 [28]. The highest EPS production obtained when sucrose was used as carbon sources and this result was similar to[29] which demonstrated that sucrose showed highest schizophyllan production (1.62 g/L) from *Schyzophyllum commune* NRCM after 168 h of fermentation. Previous research[30] also reported that sucrose was the most suitable carbon source for both cell growth (biomass concentration) and polysaccharide production. During the microbial fermentations, carbon source play a major role in building of cellular material and also used in the synthesis of polysaccharide as energy source[31-32].

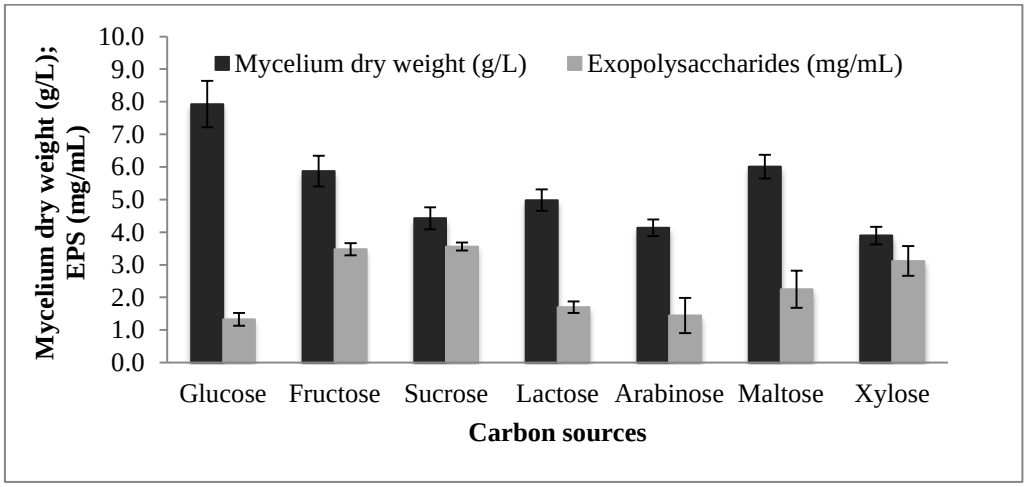


Fig-1: Effect of carbon sources on the mycelium dry weight and EPS concentration

In order to determine the suitable concentration of carbon source for mycelia growth and EPS production, sucrose concentration were varied from 25 to 200 g/L. It was observed that increasing concentrations of sucrose resulted in the increasing of mycelial growth up to 33 g/L. The EPS production was increased from 1.36 mg/mL to 4.69 mg/mL when the concentration of sucrose increased from 25 to 100 g/L. After that, the EPS concentrations were decreased to 3.63 g/L when the concentrations was further increased up to 200 g/L

as shown in Table 1. The decreased of EPS concentration after further increased of the sucrose concentration (100-200 g/L) might be due to the substrate inhibition occurred when excess carbon source was supplied to the culture medium. The result was similar to the one reported by[33]. It was described that the conversion rates of sucrose at high concentration were found to be low and high carbon source (sucrose) might have increased the osmotic pressure of the medium and thus influenced exopolysaccharide production.

Table- 1: Effect of different sucrose concentrations on the mycelium dry weight and EPS concentration.

Sucrose concentrations (g/L)	Mycelium dry weight (g/L)	Final pH	Exopolysaccharides (mg/mL)
25	4.21 ± 1.71	4.9 ± 0.4	1.36 ± 0.16
30	4.45 ± 0.72	4.7 ± 0.1	3.52 ± 0.20
40	8.86 ± 0.68	4.8 ± 0.6	3.68 ± 1.11
60	13.47 ± 4.20	4.4 ± 0.3	3.75 ± 0.70
80	17.74 ± 4.27	4.3 ± 0.3	4.31 ± 0.43
100	20.41 ± 2.29	4.3 ± 0.3	4.69 ± 0.10
150	32.58 ± 3.61	4.4 ± 0.1	3.65 ± 0.03
200	33.0 ± 2.24	4.5 ± 0.4	3.63 ± 0.41

Fermentation was carried out for 7 days at 28°C with initial pH 5.5.
Values are mean ± S.D. of triplicate.

Various inorganic and organic nitrogen sources were tested out to enhance the mycelial growth and EPS production. It was found that when organic nitrogen sources were used, mycelial growth and EPS production were high compared to inorganic nitrogen sources. Figure 2 shows the effect of different organic and inorganic nitrogen sources on EPS production. Amongst eight kinds of nitrogen sources, yeast extract as organic and ammonium chloride as inorganic nitrogen source were favourable for the mycelial growth and EPS production. Yeast extract gave highest mycelium dry weight of 8.06 g/L with 1.23 mg/mL of EPS whereas ammonium chloride showed 3.58 g/L mycelium dry weight and 0.63 mg/L EPS. This suggests that yeast extract and ammonium chloride might contain the components necessary for mycelial growth and EPS production. Comparing the influence of different ammonium salts, the NH_4^+ ion played a central role in nitrogen metabolism as the form in which nitrogen incorporated into organic cell components (biomass)[34]. Amongst all inorganic nitrogen sources tested, ammonium nitrate gave highest mycelium biomass and EPS concentration. It was reported previously by[34] that solely supplied of inorganic nitrogen sources enhanced poor growth of mycelia.

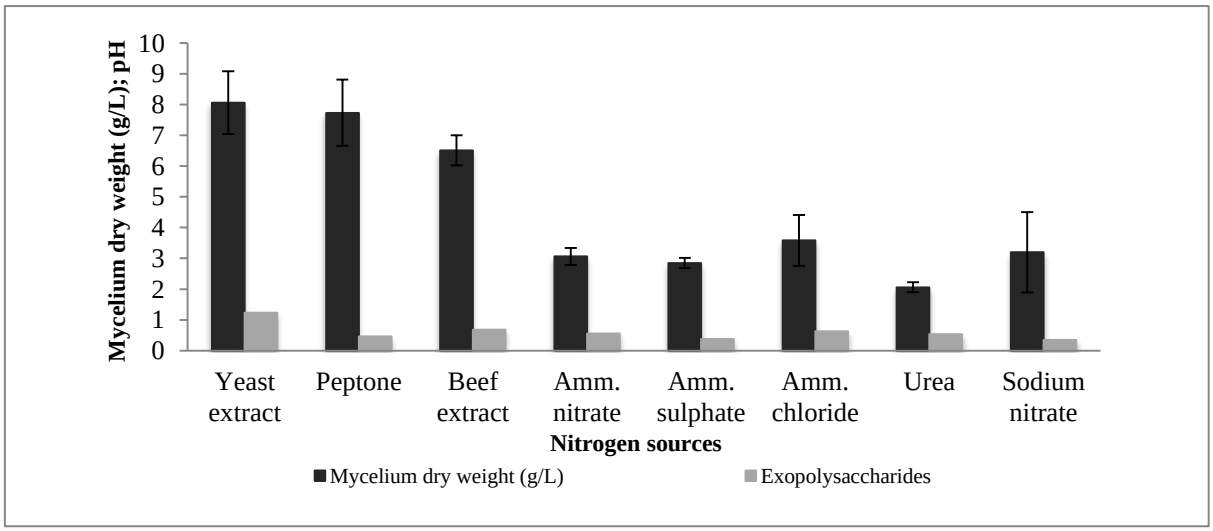


Fig-2: Effect of different nitrogen sources on the mycelium dry weight and EPS concentration.

Combination of yeast extract and ammonium nitrate was carried out in order to enhance the mycelia growth and EPS production. The best combination obtained was 30:70 (inorganic to organic nitrogen source), which resulted in the increased of mycelium dry weight up to 10.8 g/L and EPS concentration of 4.39 mg/mL as showed in Figure 3. Interestingly, result obtained was contrast to the one reported by[29] where

combinations of yeast extract and ammonium nitrate at various ratios have decreased schizophyllan and mycelium dry weight. Taking into account that higher fungi usually require long cultivation period for successful submerged culture, introducing inorganic nitrogen source to the medium might possibly reduce the risk of culture contamination[28]. The influence of initial pH on mycelial growth and EPS production was examined in the range of 4.5 to 8.0. The optimal pH for mycelial growth and EPS production was 7.5 as shown in Figure 4. Initial pH of 7.5 supported the mycelial growth of 7.0 g/L and maximum production of 3.82 mg/mL EPS. It was reported that a pH of 6.0 to be optimum for schizophyllan production with 1.65 g/L[29] whereas [23] reported that pH 5.3 was optimum for schizophyllan production. The end of pH values was found in the range of 4.4 to 5.09 although different pH value was applied in the initial of experiment. It has been reported that many kinds of ascomycetes and basidiomycetes have more acidic pH during submerged culture[35]. It was also reported on previous study that the effect of carbon, nitrogen, C:N ratio and initial pH are amongst crucial factor on the production of polysaccharide and lectin production in both mycelium and culture medium[36] through submerged fermentation. Submerged fermentation of mushroom mycelium culture clearly provides a consistent mycelia formed composition under controlled conditions with a low risk of contamination[37-38].

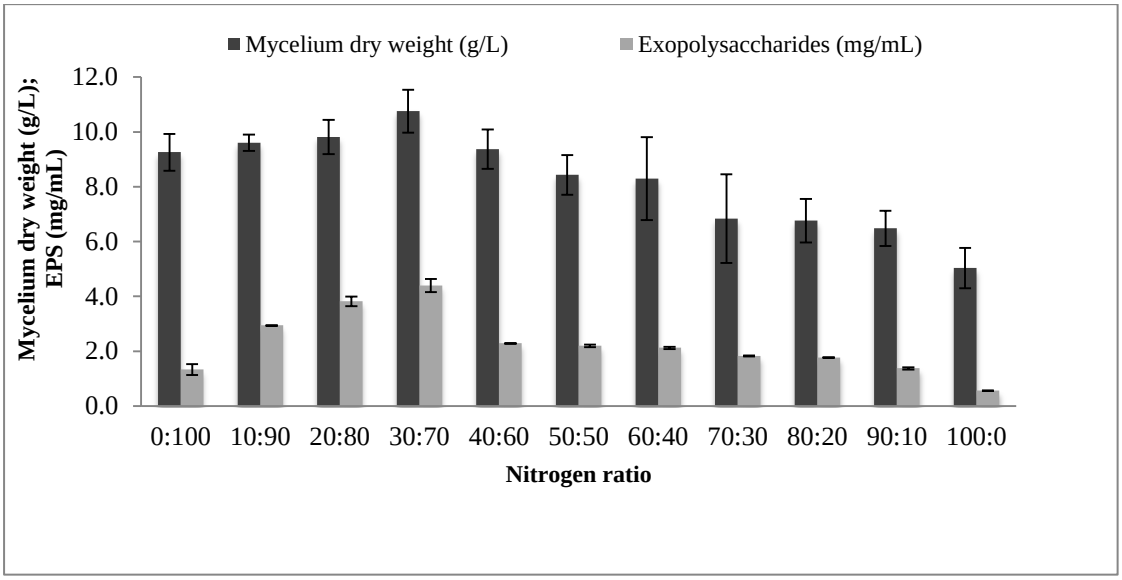


Fig-3: Effect of inorganic (ammonium chloride) to organic nitrogen sources (yeast extract) on mycelium dry weight and EPS concentration.

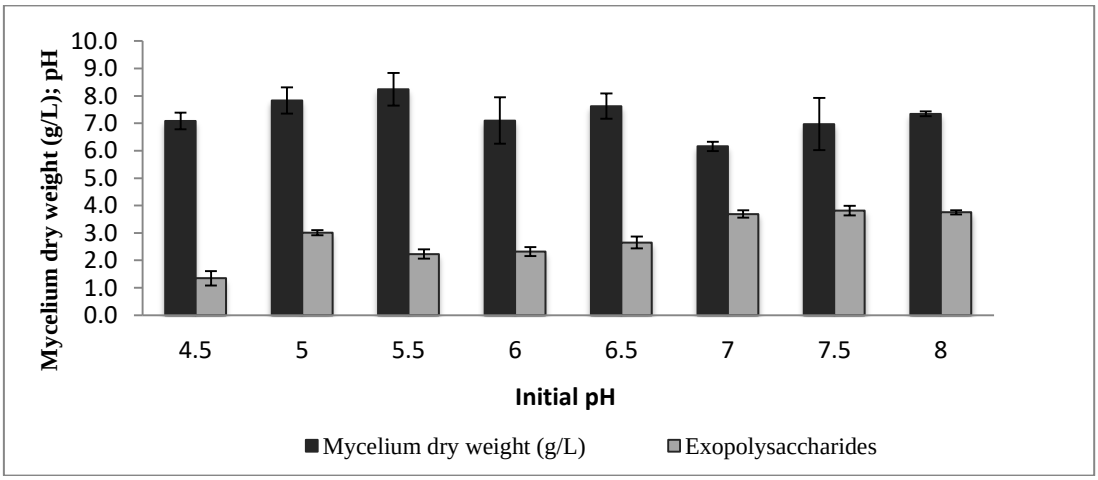


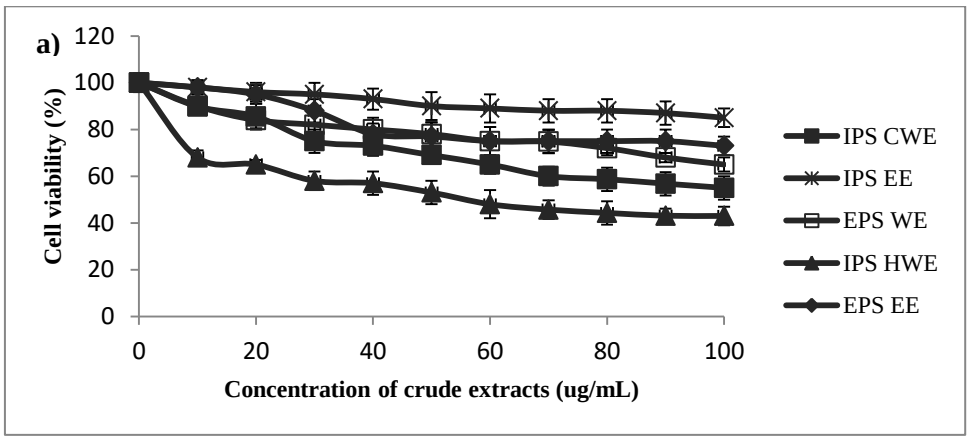
Fig-4: **Effect of different initial pH value on the mycelium dry weight and EPS concentration.**

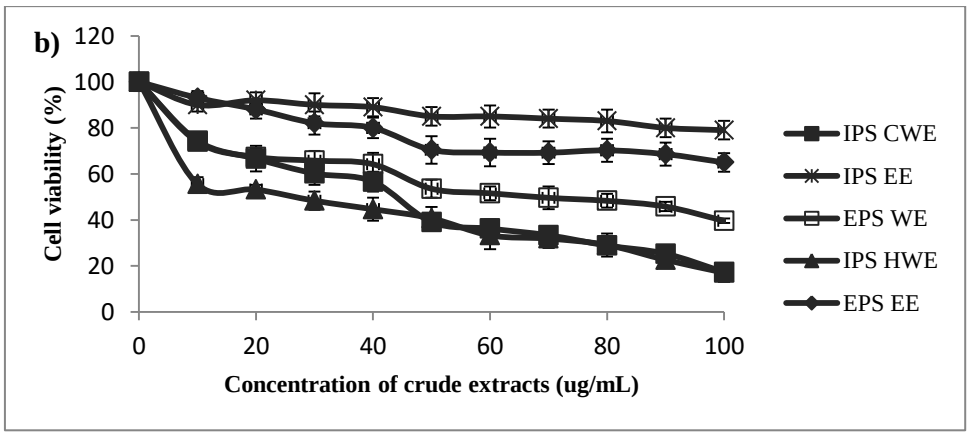
Cytotoxicity assay

According to our previous study conducted by[39], various crude extract from mycelium and culture broth were determined their carbohydrate concentration, total glucan, α-glucan and β-glucan content. It was reported that IPS HWE and IPS CWE were high in carbohydrate concentration, and this amount was correlated with the high content of β-glucan in these crude extract. In the present study, various crude extract namely EPS EE, EPS WE, IPS

CWE, IPS HWE and IPS EE were further tested their cytotoxicity effect towards lung carcinoma cell lines (A549) and Chang cells as the normal cell lines. The effect of different concentrations for 24, 48 and 72 hours incubation on the cell viability of lung carcinoma cell lines (A549) were showed in Figure 5a,b and c respectively.

Our results exhibited that out of five crude extracts; only three crude extracts from water extraction namely IPS HWE, IPS CWE and EPS WE showed antiproliferative activity against lung carcinoma cell lines (A549) as compared to ethanol extraction. For 24 hours incubation time, only IPS HWE inhibits the growth of A549 lung carcinoma cells with 43% of cell viability and $IC_{50}$of 56 µg/mL. As the incubation time prolonged to 48 hours, crude water extract from mycelium (IPS HWE, IPS CWE) and from culture broth (EPS WE) both were significantly decreased the cell viability. IPS HWE showed the lowest IC_{50} with 26 µg/mL followed by IPS CWE and EPS WE with 43.5 µg/mL and 65 µg/mL respectively. The IC_{50} of all these three crude extracts were further decreased as the incubation time increased to 72 hours with 20.8 µg/mL for IPS HWE, IPS CWE (26.3 µg/mL) and EPS WE (55 µg/mL). It is suggested that the antiproliferative activity of IPS HWE, IPS CWE and EPS WE were time dependent because of the significant decreased of IC_{50} as the incubation time increased to 72 hours and for IPS CWE and EPS WE, the cell viability only starts to decrease at 48 hours of incubation. It is also were pronounced as a dosage-dependent due to the decreased of cell viability with the increased of crude extract concentration. In this study, we found out that crude extract from hot water extraction (IPS HWE) exhibited higher antiproliferative activities as compared to the other crude extract. This might be due to the mushroom extracted with hot water usually consists a polysaccharide-protein complex which has been proved to possess a strong antitumor effect[40]. It was also suggested that the higher temperature applied in the hot water extraction process managed to solubilize large amount of cellular structural/storage proteins[41]. Previous studies also reported that hot water extract and cold water extract from *Lignosus rhinoceros* exhibited significant antipoliferative activity against different kinds of leukemic cells, breast cancer cell (MCF-7) and lung cancer cell (A549) respectively[41-42]. Our result also showed that IPS HWE, IPS CWE and EPS WE were essentially not cytotoxic against normal cell lines (Chang). Tamoxifen drug (positive control) range from 10 to 100 ug/ml clearly inhibited the growth of normal cell line. In contrast our water extract (IPS CWE and IPS HWE) could support the growth of normal cell lines (Chang) above 60% of cell viability. As far as it is concern, this is the first report to demonstrate that *Lentinus squarrosulus* water extract is not toxic to normal cells and suggested as potentially bioactive compound in anticancer treatment.





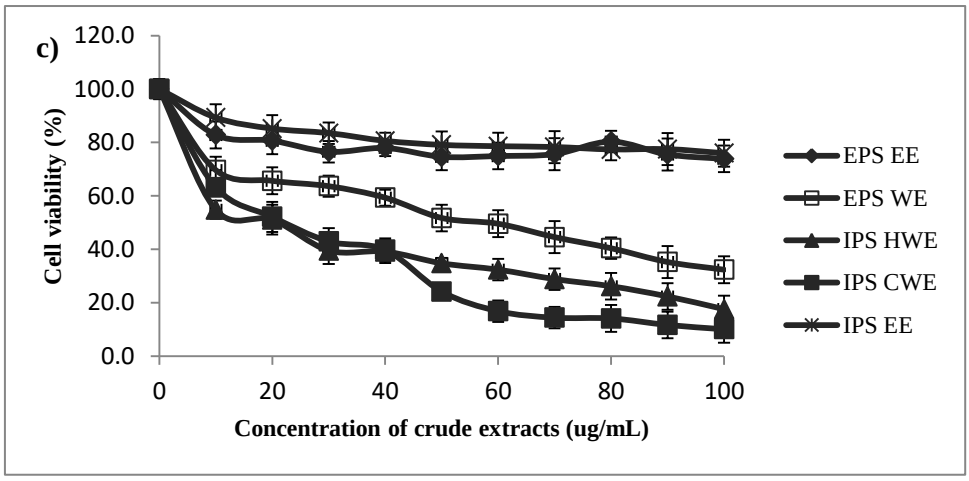


Fig- 5: Antiproliferative activities of various crude extract of *Lentinus squarrosulus* mycelial and culture broth. Lung carcinoma cells, A549 (ATCC) were treated with different concentrations of crude extract ranging from 10 µg/mL to 100 µg/mL at 24 h (A), 48 h (B) and 72 h (C). Values are mean ± S.D. of triplicate.

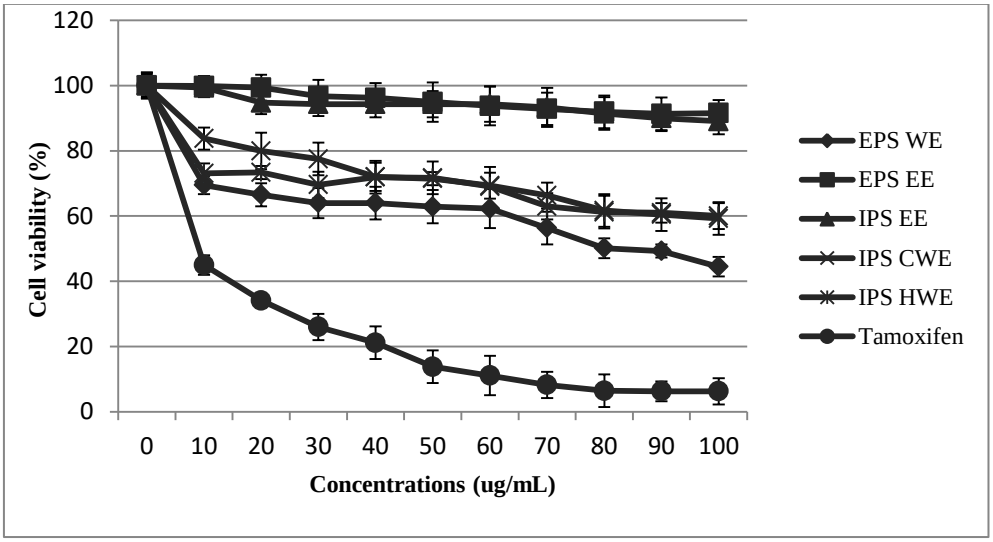


Fig-6: **Antiproliferative activities of various crude extract of *Lentinus squarrosulus* mycelial and culture broth. Normal cells (Chang), were treated with different concentrations of crude extract ranging from 10 µg/mL to 100 µg/mL at 72 h. Values are mean ± S.D. of triplicate.**

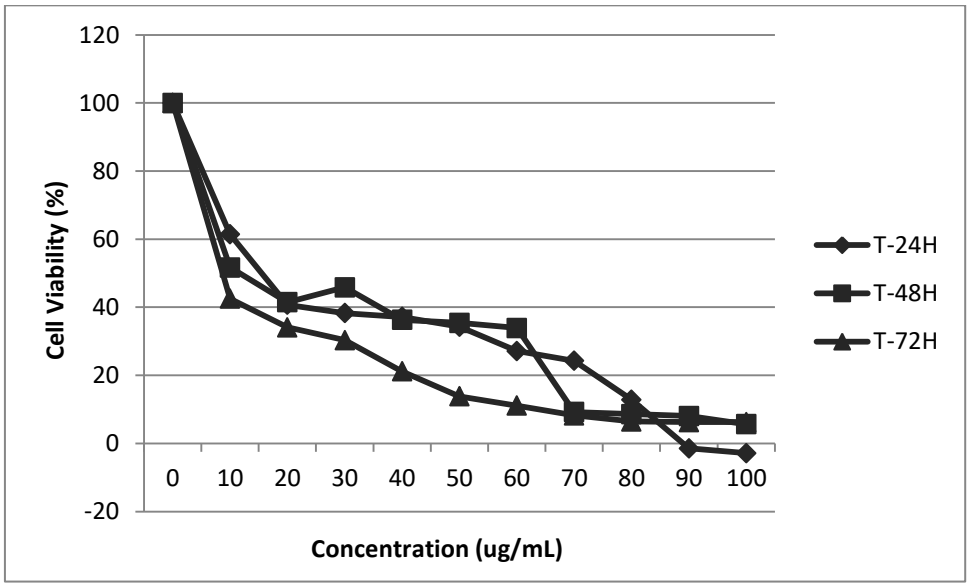


Fig-7: **Antiproliferative activities of various crude extract of *Lentinus squarrosulus* mycelial and culture broth. Lung cancer cells (A549) were treated with different concentrations of Tamoxifen ranging from 10 µg/mL to 100 µg/mL. Values are mean± S.D. of triplicate.**

CONCLUSION

Mycelium dry weight and EPS concentration had increased when sucrose and yeast extract were utilized as carbon and nitrogen sources respectively. Combination of ammonium chloride and yeast extract enhanced the mycelium dry weight up to 10.8 g/L with 4.4 mg/mL of EPS concentration. Crude water extract from mycelium of *Lentinus squarrosulus* possess a strong antiproliferative activity against A549 lung carcinoma cell lines.

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