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THE ROLE OF EXOPOLYSACCHARIDE PRODUCED BY *Pleurotus Sapidus* AS MEDICAL AGENT

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activity, *Pleurotus
sapidus*

ABSTRACT

Mushrooms have been used since centuries for the treatment of different diseases and efforts were made to isolate different compounds from the mushrooms responsible for medicinal activities. Among the mushroom genus *Pleurotus* is worldwide in distribution due to their medicinal properties such interventions ameliorate hepatic lipid profiles, reduce inflammation and oxidative stress, noticeable antioxidant, anti-inflammatory, antimicrobial, antitumor and analgesic properties. These properties are attributed mainly to polysaccharides synthesized by *Pleurotus*. The aim of current study is to improve of Exopolysaccharide production by *Pleurotus sapidus* through manipulation of different culture and environmental conditions using response surface methodology (RSM). Plackett–Burman design indicated that; shaking, starch and incubation temperature are the most effective parameters. Box–Behnken RSM indicated that; the optimum concentration for each parameter was: 148.9 rpm for shaking, 30.1g/l starch and 25.2 °C for incubation temperature. After production, EPS was extracted, purified, identified using Fourier transform infrared (FTIR), Nuclear Magnetic Resonance (¹HNMR) and High Performance Liquid Chromatography (HPLC). The result indicated that; it consisting of β-(1→3) glucans, β-(1→6) glucans branched, Chitin, Mannans and Galactans with different concentrations in EPS. Exopolysaccharides extracted from *Pleurotus sapidus* showed antitumor, antiviral, antibacterial, anticoagulant and antioxidant activity.

1. Introduction

Microorganisms regularly preserve polysaccharides as a functional adaptation to prevent them from drying out and to help them adhere to surfaces (Hussien, 2019). Edible mushrooms have become attractive as “health foods” and as source materials for immunomodulators (Chen *et al.*, 2018). They have beneficial effects such as interventions ameliorate oxidative stress, hepatic lipid profiles, and reduce inflammation (Fontes *et al.*, 2019).

Several mushrooms belonging to *Pleurotus* species are confirmed to be producer of bioactive polysaccharide. It is one of most extensively studied white-rot fungi due to its exceptional ligninolytic properties (Hao *et al.*, 2017 & Bellettini *et al.*, 2016). *Pleurotus*, *Flammulina*, *Ganoderma*, *Grifola*, *Trametes*, *Lentinus*, *Auricularia*, *Hericium*, *Schizophyllum*, *Tremella* and *Phellinus* species are considered to be medically important mushrooms with essential bioactive properties (Deveci *et al.*, 2019).

The main polysaccharides present in mushrooms are β -glucans with potential biological activities. β -glucans are soluble fibers and have physiological functions. One of this functions is interference with absorption of sugars and reduction of serum lipid levels (Rahar *et al.*, 2011). Mushroom polysaccharides such as β -1, 3 glucan, β -1,6 glucan and lentinan have showed various health benefits i.e. anticancer, immunosuppressive hypertension, and diabetes (Bhakta and Kumar, 2013).

Mushrooms act as antibacterial and immune system enhancer; additionally, they are important sources of bioactive compounds. As a result of these properties, some mushroom extracts are used to promote human health (Valverde *et al.*, 2018).

The genus *Pleurotus* contain many biological compounds such as polysaccharides, enzymes, protein, dietary fiber and vitamins (Shen *et al.*, 2013). It has been recently reported that many potential natural antioxidants are derived from lots of sources such as oilseeds, cereal crops, vegetables, fruits, leaves, roots, spices, herbs and mushrooms (Lee and Yoon, 2009).

Mushroom cultivation is reported as an economically viable bio-technology process for conversion of various lignocellulosic wastes. The mycelium growth of mushroom species is mainly depends upon the substrate, nutrients and the growing conditions (Girmay *et al.*, 2016). Compared with conventional methods, RSM can be used to design experiments, build models, search optimum factors for desirable responses, and evaluate the relative significance of several influence factors even in the presence of complex interactions. (Khuri *et al.*, 2010). The purpose of the present research was to evaluate the growth factors that might increase the productivity of exopolysaccharides. Also, characterization of Exopolysaccharides using HPLC. Finally, antioxidant activity were analysed in vitro.

2. Materials and methods

2.1. Producing organism and preservation

Pleurotus sapidus species was obtained from mushroom and Mycology lab., department of plant pathology, Faculty of agriculture, Al-Azhar University, Cairo, Egypt. *Pleurotus sapidus* was preserved on sterilized slants containing Glucose yeast extract peptone media (GYP) which consisted of $g\ l^{-1}$: glucose 40, yeast extract 10, peptone 5, agar 15-20, chloroamphenicol 0.05 (as antibacterial agent), dissolved in 1L of distilled water and autoclaved it at 121 °C for 15 min. Prepared GYP slants were inoculated

by the pure fungal cultures and incubated at 28 °C. Slant cultures were transferred every month.

2.2. Determination of *Pleurotus sapidus* dry weight

Pleurotus sapidus was cultured in autoclaved 250 ml flasks each was containing 100 ml of Glucose yeast extract peptone media medium and incubated at 25°C for 7 days. After incubation the fungal biomass was separated by filtration and washed several times with sterile distilled water then dried at 80°C until a constant weight (Nour EL-Dein *et al.*, 2004).

2.3. Determination of EPS production

Fungal culture filtrates were treated with 5% Tri- chloroacetic acid (TCA) and left overnight to precipitate protein. The supernatant was mixed with two volumes of 95% (v/v) of ethanol, stirred vigorously overnight at 4°C. The resultant precipitate was recovered by centrifugation (Hettich EBA 12R, Germany) at 3000 rpm for 20 minutes (Wu *et al.*, 2008). This step was repeated twice to remove impurities of low molecular weight compounds. Exopolysaccharide and carbohydrate contents were dissolved in deionized water and quantified using phenol-sulfuric acid colorimetric method. All experiment was repeated in triplicate to calculate mean value of results (Dubios *et al.*, 1956). Crude exopolysaccharides were dialyzed with dialysis membrane to get ride of salts (Berg *et al.*, 2007).

2.4. Effect of different physiological parameters on mycelial growth and exopolysaccharides production of *Pleurotus sapidus*

Different experiments were made to select the most favorable conditions for high production of exopolysaccharides dry weight.

2.5. Effect of different media on mycelial growth and exopolysaccharides production of *Pleurotus sapidus*

Four different liquid media; Potato dextrose broth (PDB) (Modified) It composed of gl^{-1} ; suspension / solution of potato extract 20, dextrose 20, dissolved in 1L of distilled water and autoclaved it at 121 °C for 15 min., Glucose yeast extract peptone media (GYP) It composed of gl^{-1} ; glucose 40, yeast extract 10 , peptone 5, dissolved in 1L of distilled water and autoclaved it as described earlier., Malt extract Agar (MEA) It composed of gl^{-1} ; malt extract 20, dextrose 20 and dissolved in 1L of distilled water and mushroom complete media (MCM) It composed of gl^{-1} ; glucose 20, peptone 2, yeast extract 2, K_2HPO_4 1, MgSO_4 0.5 and dissolved in 1L of distilled water were used. Inoculate each medium with one fungal disc (with diameter size 0.7cm) of *Pleurotus sapidus*. After incubation for 7 days at 25°C, the mycelial dry weight and exopolysaccharides were determined (Nour EL-Dein *et al.*, 2004).

2.6. Optimization of EPS using Plakett–Burman (PB) design

The Plakett–Burman experimental design was applied to screen the significant variables that influence EPS production (Chen *et al.*, 2011). Fourteen variables of medium composition and culture conditions were tested at low (–1) and high (+1) levels as shown in Table1. Based on PB matrix design, two-level factorial design, it allows the investigation of 14 variables (n+1) (Plackett and Burman, 1946). On this design all trials were performed in triplicate and the averages of EPS production were treated as the responses. The main effect of each variable was determined with the following equation:

$$E_{xi} = (\sum M_{i+} - \sum M_{i-}) / N$$

Where E_{xi} is the variable main effect, M_{i+} and M_{i-} are EPS production in trials where the independent variable (x_i) was present in high and in low settings, respectively, and N is the number of trials divided by 2. For determination of variable significance, statistical t -values for equal unpaired samples were calculated with respect to observations. The variables significant at 95% level ($p < 0.05$) were considered to have significant effect on EPS production and thus used for further optimization.

2.7. Response surface methodology

Box–Behnken design (BBD) with three factors and three levels, including three replicates at the center point, were used for fitting a second-order response surface to provide a measure of process stability and inherent variability (Khani *et al.*, 2015). The center points and parameters were selected according to Plackett–Burman design. In this model, the most significant independent variables, named (X_1), (X_2) and (X_3) are included and each factor can be examined at the three different levels, low (-), high (+) and central or basal (0) as shown in Table 2. All experiments were performed in triplicates. Here, three different variables including medium consisted of shaking (X_1), starch (X_2) and incubation temperature (X_3) were treated as independent variables. Fifteen trials and their observations (shown in the results section) were fitted to the following second order polynomial mode:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + \dots \dots \dots (1)$$

Where, Y is the dependent variable (EPS production); X_1 , X_2 and X_3 are the independent variables; b_0 is the regression coefficient at center point; b_1 , b_2 and b_3 are linear coefficients; b_{12} , b_{13} and b_{23} are second-order interaction coefficients; and b_{11} and b_{33} are quadratic coefficients. The values of the coefficients were calculated and the optimum concentrations were predicted using Minitab

16.2.4 (ref USA) software. The quality of the fit of the polynomial model equation was expressed by R^2 , the coefficient of determination.

2.8. Purification of EPS by diethylaminoethyl-cellulose (DEAE-cellulose)

The previously obtained precipitate was dissolved in distilled water and then lyophilized in a vacuum freeze dryer to obtain the crude polysaccharide. A sample of the polysaccharide was redissolved in distilled water, and then purified with the DEAE-cellulose A52 column (2.6 cm × 60 cm), which was equilibrated with distilled water. The polysaccharide was fractionated by stepwise elution with distilled water (Gabriela, *et al.*, 2003; Liu, Lu, *et al.* 2015). Polysaccharide containing fractions were determined for the presence of the total sugar by phenol-sulfuric acid method (Dubois *et al.*, 1956). The fractions containing sugar were pooled, concentrated by ultra-filtration, and precipitated with 2 volumes of ethanol, respectively.

2.9. Characterization of exopolysaccharides produced by *Pleurotus sapidus*

1-High Performance Liquid Chromatography Analysis:

Analysis of the carbohydrate in the filtrate was performed by using High Performance Liquid Chromatography (HPLC). Exopolysaccharides were hydrolyzed following the method of (Chen *et al.*, 2005). Sample separation was carried on a Shimpack VP-ODS C18 column (250 mm × 4.6 mm, 5 μ m, Shimadzu, Kyoto, Japan) kept at 30 °C. The mobile phase was composed of 0.1 mol/l ammonium acetate (adjusted to pH 4.5 with acetic acid): acetonitrile (83:17, v/v). The flow rate was 1.0 ml/min. The injection volume was 20 μ l.

2-Fourier Transform-Infrared spectroscopy FT-IR of EPSs produced from *Pleurotus sapidus*

The structure of EPS was investigated by FT-IR spectroscopic analyses using Fourier Transform Infrared Spectrophotometer (FT/IR-4100, Japan). The purified EPS fractions (1 mg) were ground with 300 mg KBr powder and then pressed into pellets for transform IR spectral measurement on a Mattson Instrument (Mattson Instruments, Inc., Madison, WI, USA) and the absorbance of samples was scanned from 400-4000 cm^{-1} . The spectra were corrected for wavenumber-dependent signal-detection efficiency of the setup using a white light spectrum of a temperature calibrated tungsten band lamp (Shen *et al.*, 2013).

3- Nuclear Magnetic Resonance (^1H NMR) of exopolysaccharides produced from *Pleurotus sapidus*.

The exopolysaccharide was subjected to the proton (^1H) nuclear magnetic resonance (NMR) spectra and were recorded in deuterated dimethyl sulphoxide (DMSO- d_6) on a Burker High Performance Digital FT-NMR Spectrometer Avance 111 400 MHz (Central lab., Faculty of pharmacy, Egypt) (Kogan *et al.*, 2002).

2.10. Applications of EPSs produced by *P. sapidus*

Antioxidant activity:

Total antioxidant activity of purified exopolysaccharides was estimated according to the method described by (Thaipong *et al.*, 2006) using different exopolysaccharides concentrations (0.0, 0.25, 0.50, 1, 2 mg) each dissolved in 1ml distilled H_2O .

2.11. STATISTICAL ANALYSIS

Data were expressed as mean \pm S.D. (n = 3). Experimental data were statistically subjected to analysis of variance (ANOVA) using

Minitab 16.2.4 (ref USA) software to evaluate significant differences at probability level of $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Effect of different media on exopolysaccharides production and mycelial dry weight of *Pleurotus sapidus*

Figure 1 showed that Glucose Yeast peptone liquid medium (GYP) was the best medium for exopolysaccharide extract and high mycelial biomass of *Pleurotus sapidus* production which gave 1.138 ± 0.012 and 0.286 ± 0.010 mg/ml respectively. Among different growing agar media used in this investigation are GYP, PDA, MEA, and WEA; Glucose Yeast Pepton media has proved to be the best media for the mycelial growth and EPS production of *Pleurotus sapidus* followed by PDA with a slight difference. These results were agreed with Latha and baskar, (2014) who studied the effect of different media on production of polysaccharides from oyster mushroom *Pleurotus florida* and *Hypsizygu ulmarius* and found that that glucose yeast peptone (GYP) media was the best media. This difference of EPS production and mycelial growth on different media may be due to availability of different carbon sources and availability of other required nutrients for mushroom *Pleurotus sapidus* in GYP medium.

3.2. Effect of Static and Shaking condition on both EPS, IPS production and mycelial dry weight of *Pleurotus sapidus*

It was evident that Exopolysaccharides and mycelial dry weight in case of shaking is higher than that of static condition which were 1.795 ± 0.003 and 1.183 ± 0.066 mg/ml respectively (Fig. 2). This was consistent with finding of Anike *et al.*, (2015) who explained that shaken fermentation was higher than static in production of exopolysaccharides from *Lentinus squarrosulus*. This indicates that the

higher aeration (agitation) was an efficient strategy for enhanced EPS production (Chiu *et al.*, 2008). Agitation enhances mixing in the culturing broth, which is necessary to provide the nutrient and oxygen supply required for the growth of fungi and subsequently increase the production of biomass and EPS (Xu *et al.*, 2006).

3.3. Optimization of EPSs production using Plackett-Burman experimental design

The Plackett-Burman design is an important tool in screening the effects of variables process on yield; It can significantly reduce the number of repetitive experiments to be conducted in a further optimization study, using a response surface methodology (Ekpenyong *et al.*, 2017). The effect of the variables on EPS production was determined by conducting twenty experiments given by the model (Table 1). All experiments were performed in three replica and the average results as percentage of EPS production are presented in (Table 1) that show the maximum production of EPS from *Pleurotus sapidus* was obtained on trial 15 (+ve concentration of glucose, starch, peptone, yeast extract, KH_2PO_4 , stearic acid and shaking, while -ve concentration of pH, incubation time, temperature, sucrose, omega 3 and inoculum size) (Table 1). The main effect of each variable was calculated as the difference between both averages of measurements made at the high level (+) and at the low level (-) of that factor. The results of main effect are presented in Fig. (3). The results indicated that the presence of high levels of shaking, starch, glucose, KH_2PO_4 , stearic acid, sucrose and maltose in the basal medium positively affected EPS production. These results are confirmed from the Pareto chart as shown in Fig. (4), which indicates higher effects presented in the upper portion and then progress down to the lower effects.

It was deduced from Fig. (4) that the shaking condition was the most significant variable that affected EPS production, followed by starch and temperature, respectively. These variables were examined more thoroughly by applying the Box–Behnken design.

3.4. Optimization of EPS production by Box-Behnken design

In order to approach the optimum response region of EPS production, the effective independent variables including shaking (X_1), starch concentration (X_2) and temperature (X_3) suggested by the Plackett-Burman design were further investigated, each at three levels (-, 0 and +) according to the Box and Behnken design (Box and Behnken, 1960), (Table 2). However, glucose (40) g l^{-1} , sucrose (30) g l^{-1} , maltose (30) g l^{-1} , peptone (7) g l^{-1} , yeast extract (5) g l^{-1} , KH_2PO_4 (1) g l^{-1} , stearic acid (2) g l^{-1} , initial pH (4.5), incubation period (10 days) and inoculum size (1 disc) were treated as constant factors. (Table 2) represents the design matrix of the coded variables together with the experimental results of EPS production. All cultures were performed in triplicate and the average of the observations was used.

As shown in Table (2), the highest EPS production records (8.792, 8.827 and 8.779) mg/l which were achieved by the trials number 13, 14 and 15, respectively, 25 °C. On the other hand, it is clear that the lowest EPS production was (7.193, 7.200 and 7.207) mg/l which were achieved by the trials number 6, 4 and 1 respectively, 30 °C.

For a precise prediction of the optimal point, a second order polynomial function was fitted to the EPS production results of the applied Box-Behnken experiment. Using Minitab 16.2.4 (ref USA) software it has obtained a value for coefficient of determination R^2 of 0.99.75, which indicates that 99.75% of the variability of the response

could be explained by the model and about 0.25% of the total variation cannot be explained by this model.

According to the obtained statistical analysis results, the correlation between the response and the three independent variables can be described by the following equation (2): equation (2)

$$Y = -46.1934 + 0.4589X_1 + 0.5761X_2 + 0.9578X_3 - 0.0007X_1X_2 + 0.0020X_1X_3 + 0.0006X_2X_3 - 0.0016X_1^2 - 0.0079X_2^2 - 0.0252X_3^2$$

Where Y is the product of EPS (dependent variable); X₁, X₂ and X₃ are levels of shaking, starch and temperature, respectively. The degree of fitting of the model is relatively high as the calculated coefficient of determination (R²) was 0.9975. Also, the Predictive R-Square was 96.30%, which concluded that these values are in reasonable agreement with the Adjustive R-Squared of 99.29%, indicating that a good agreement between the experimental and predicted values on EPS production. The closer the value of R is to 1, the better is the correlation between the measured and the predicted values. This value indicates a high degree of correlation between the experimental and the predicted values.

Furthermore, solving the model according to the data obtained from (Table 2) revealed an optimum response at X₁ = 150, X₂ = 30 and X₃ = 25 with a predicted Y (response) of 8.8026 mg/l. Thus, according to the results of the two optimization experiments, an optimum response (EPS production) is predicted under the following fermentation condition: glucose 40 g l⁻¹; sucrose 30 g l⁻¹; starch 30 g l⁻¹; maltose 30 g l⁻¹; peptone, 7 g l⁻¹; KH₂PO₄ 1 g l⁻¹; stearic acid 2 g l⁻¹; yeast extract 5 g l⁻¹; temperature 25 °C; shaking 150 rpm; initial pH 4.5; inoculum size 1 disc; and an incubation period of 10 days.

In addition, the simultaneous effects of the three independent factors on each response were revealed by three-dimensional graphs generated by Minitab 16.2.4 (ref USA) software. As shown in the surface plots and their corresponding contour plots of the Box-Behnken design variations in shaking (X₁), starch (X₂) and temperature (X₃) within the examined concentration ranges and under the present experimental conditions, were clearly effective. Figs. (5, 6 and 7) showed the influences of variations in shaking, starch and temperature on the experimental fungus with respect to EPS production. From this figures, it can be suggested that, up to approximately 25 °C, the more the temperature lead to decrease in the EPS production in the medium. It seems likely that increasing the speed of shaker to about 150 rpm would allow more EPS production. However, an optimum level of starch appears to be 30 g l⁻¹.

Thus, according to the results of the two optimization experiments, an optimum response (EPS production) is predicted under the following fermentation condition g l⁻¹: glucose 40; sucrose 30; starch 30.1; maltose 30; peptone 7; KH₂PO₄ 1; stearic acid 2; yeast extract 5; temperature 25.2 °C; shaking 148.9 rpm; initial pH 4.5; inoculum size 1 disc; and an incubation period of 10 days.

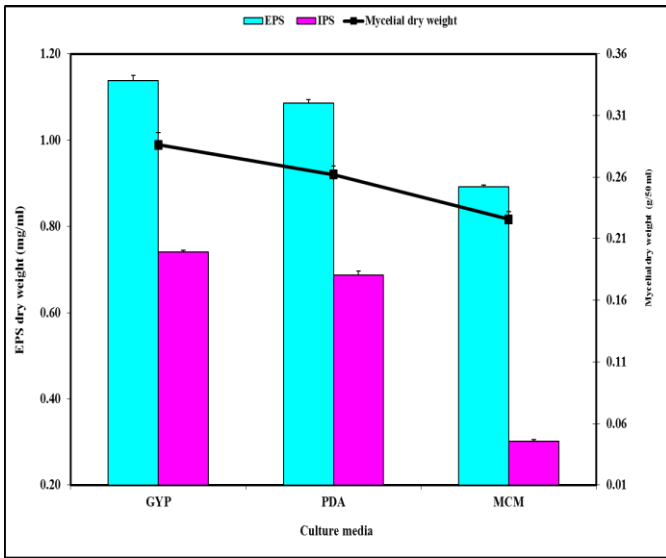


Fig. (1): Effect of different media on exopolysaccharides, endopolysaccharide production and mycelial dry weight of *Pleurotus sapidus*.

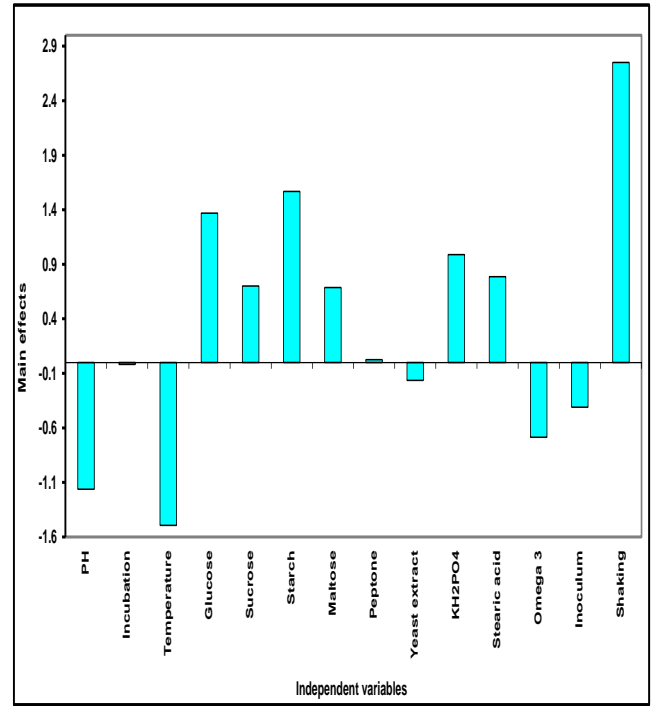


Fig. (3): Main effects of independent variables examined in Plackett-Burman design.

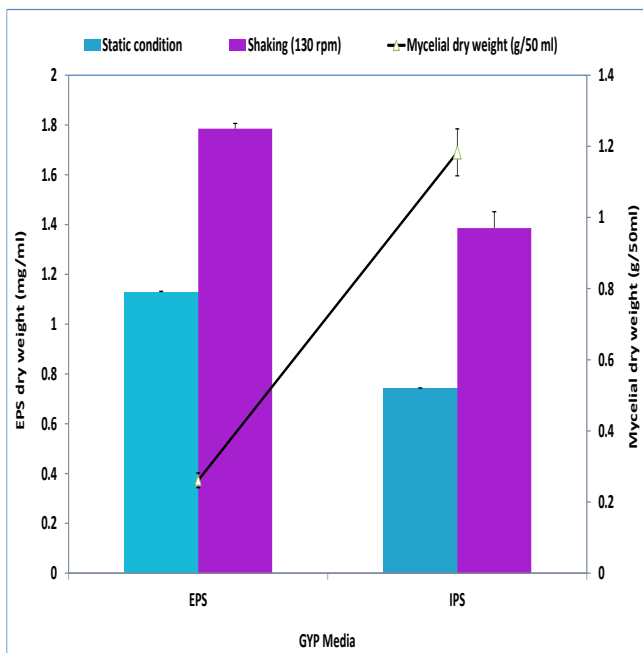


Fig. (2): Effect of Static and Shaking condition on both EPS, IPS production and mycelial dry weight of *Pleurotus sapidus*:

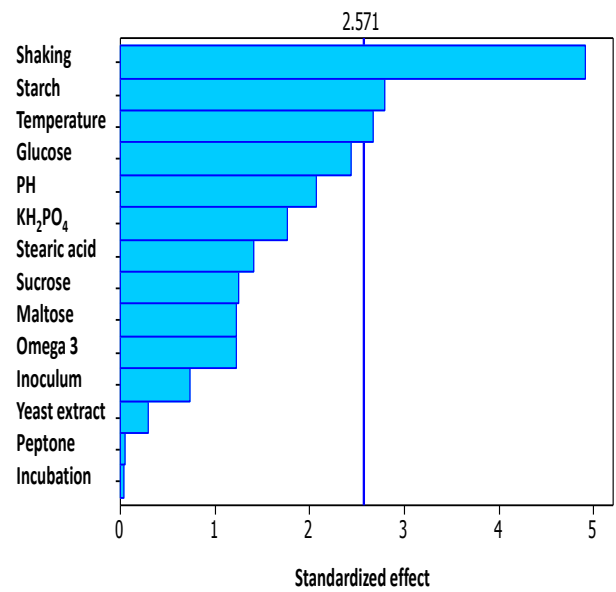


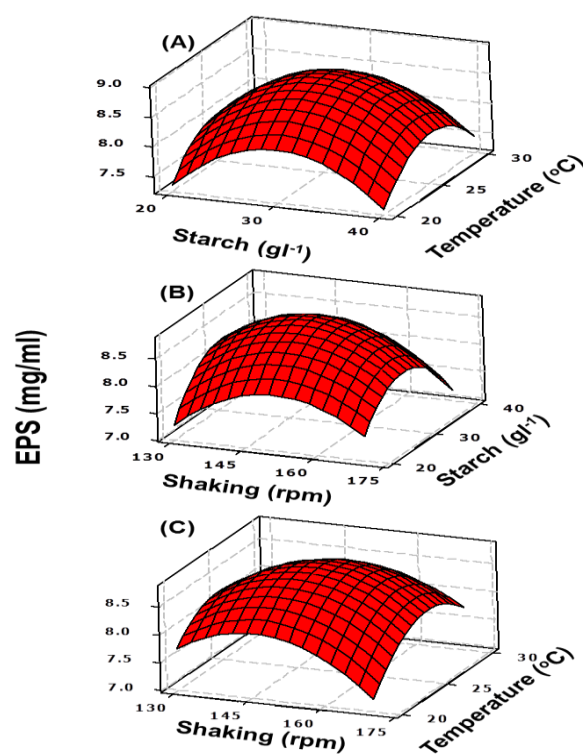
Fig. (4): Pareto chart of the standardized effects of EPS dry weight (mg/ml) examined in Plackett-Burmann design (Alpha = 0.05).

Table (1): Response to Plackett Burman design of 14 independent variables in 20 trials (data were average of three replica \pm SD)

Trials	PH	Incubation (day)	Temperature (°C)	Glucose (gl ⁻¹)	Sucrose (gl ⁻¹)	Starch (gl ⁻¹)	Maltose (gl ⁻¹)	Peptone (gl ⁻¹)	Yeast extract (gl ⁻¹)	KH ₂ PO ₄ (gl ⁻¹)	Stearic acid (gl ⁻¹)	Omega 3 (gl ⁻¹)	Inoculum (disc)	Shaking (rpm)	Mycelial dry weight (g/50 ml) \pm SD	EPS dry weight (mg/ml) \pm SD
1	+	-	+	+	-	-	-	-	+	-	+	-	+	+	1.439 \pm 0.013	0.501 \pm 0.005
2	+	+	-	+	+	-	-	-	-	+	-	+	-	+	0.698 \pm 0.006	3.966 \pm 0.009
3	-	+	+	-	+	+	-	-	-	-	+	-	+	-	2.190 \pm 0.245	0.908 \pm 0.286
4	-	-	+	+	-	+	+	-	-	-	-	+	-	+	1.125 \pm 0.192	3.143 \pm 0.407
5	+	-	-	+	+	-	+	+	-	-	-	-	+	-	1.270 \pm 0.036	1.200 \pm 0.016
6	+	+	-	-	+	+	-	+	+	-	-	-	-	+	0.170 \pm 0.016	2.103 \pm 0.002
7	+	+	+	-	-	+	+	-	+	+	-	-	-	-	1.570 \pm 0.073	0.644 \pm 0.056
8	+	+	+	+	-	-	+	+	-	+	+	-	-	-	0.947 \pm 0.095	0.239 \pm 0.003
9	-	+	+	+	+	-	-	+	+	-	+	+	-	-	2.323 \pm 0.306	0.586 \pm 0.045
10	+	-	+	+	+	+	-	-	+	+	-	+	+	-	2.130 \pm 0.191	0.632 \pm 0.091
11	-	+	-	+	+	+	+	-	-	+	+	-	+	+	1.231 \pm 0.077	7.944 \pm 0.026
12	+	-	+	-	+	+	+	+	-	-	+	+	-	+	1.530 \pm 0.180	3.523 \pm 0.12
13	-	+	-	+	-	+	+	+	+	-	-	+	+	-	1.083 \pm 0.078	1.848 \pm 0.751
14	-	-	+	-	+	-	+	+	+	+	-	-	+	+	1.543 \pm 0.039	2.936 \pm 0.421
15	-	-	-	+	-	+	-	+	+	+	+	-	-	+	1.220 \pm 0.269	7.966 \pm 0.005
16	-	-	-	-	+	-	+	-	+	+	+	+	-	-	1.653 \pm 0.241	0.888 \pm 0.111
17	+	-	-	-	-	+	-	+	-	+	+	+	+	-	0.970 \pm 0.016	0.307 \pm 0.034
18	+	+	-	-	-	-	+	-	+	-	+	+	+	+	1.580 \pm 0.289	2.253 \pm 0.165
19	-	+	+	-	-	-	-	+	-	+	-	+	+	+	0.930 \pm 0.033	0.603 \pm 0.005
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.263 \pm 0.029	0.17 \pm 0.008

Table (2): Independent variables studied by Box-Behnken design using 3 factors in 15 trials

Trials	Shaking (rpm)	Starch (gl ⁻¹)	Temperature (°C)	EPS (mg/ml) \pm SD
1	(-) 130	(-) 20	(+) 30	7.207 \pm 0.016
2	(+) 170	(-) 20	(0) 25	7.399 \pm 0.005
3	(-) 130	(+) 40	(0) 25	7.607 \pm 0.005
4	(+) 170	(+) 40	(+) 30	7.200 \pm 0.016
5	(-) 130	(0) 30	(-) 20	7.743 \pm 0.022
6	(+) 170	(0) 30	(+) 30	7.193 \pm 0.001
7	(-) 130	(0) 30	(0) 25	7.439 \pm 0.002
8	(+) 170	(0) 30	(-) 20	7.689 \pm 0.008
9	(0) 150	(-) 20	(-) 20	7.324 \pm 0.012
10	(0) 150	(+) 40	(+) 30	7.248 \pm 0.003
11	(0) 150	(-) 20	(0) 25	7.445 \pm 0.014
12	(0) 150	(+) 40	(-) 20	7.484 \pm 0.003
13	(0) 150	(0) 30	(0) 25	8.792 \pm 0.031
14	(0) 150	(0) 30	(0) 25	8.827 \pm 0.032
15	(0) 150	(0) 30	(0) 25	8.779 \pm 0.017

**Fig. (5):** Response surface plots of EPS production by *Pleurotus Sapidus* using independent variables; (A) temperature and starch, (B) starch and shaking and (C) temperature and shaking.

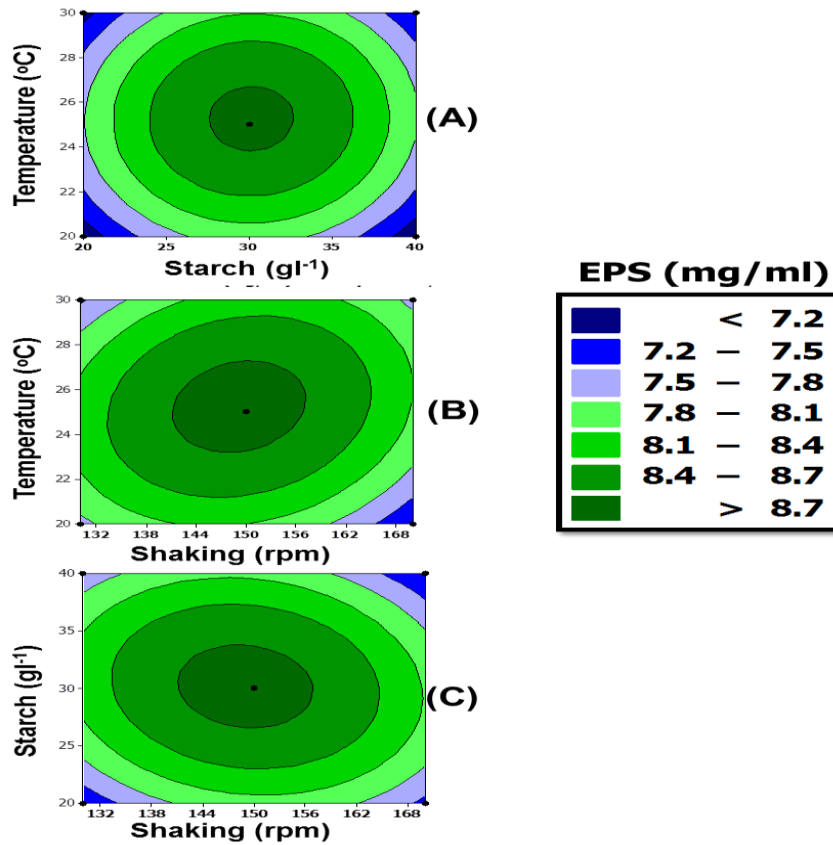


Fig. (6): Contour Plot of EPS (mg/ml) production against; (A) temperature and starch, (B) temperature and shaking, and (C) starch and shaking

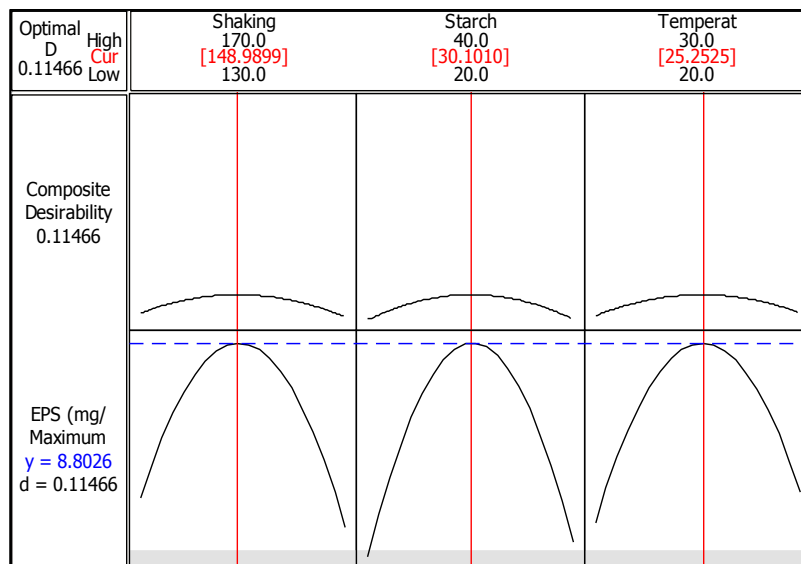


Fig. (7): Optimization plot of EPS production by *Pleurotus Sapidus* using Box-Behnken design.

Table (3): ANOVA for process variables.

Source	DF	Seq SS	Adj SS	Adj MS	F-value	P-value
Regression	9	4.99094	4.99094	0.55455	219.07	0.000
Linear	3	0.07431	0.07431	0.02477	9.78	0.016
Shaking	1	0.03308	0.03308	0.03308	13.07	0.015
Starch	1	0.00336	0.00336	0.00336	1.33	0.301
Temperature	1	0.03787	0.03787	0.03787	14.96	0.012
Square	3	4.66396	4.66396	1.55465	614.16	0.000
Shaking*Shaking	1	1.13389	1.57363	1.57363	621.65	0.000
Starch*Starch	1	2.06226	2.32561	2.32561	918.72	0.000
Temperature*Temperature	1	1.46780	1.46780	1.46780	579.85	0.000
Interaction	3	0.25267	0.25267	0.08422	33.27	0.001
Shaking*Starch	1	0.08960	0.08960	0.08960	35.40	0.002
Shaking*Temperature	1	0.15979	0.15979	0.15979	63.12	0.001
Starch*Temperature	1	0.00329	0.00329	0.00329	1.30	0.306
Residual Error	5	0.01266	0.01266	0.00253		
Lack-of-Fit	3	0.01140	0.01140	0.00380	6.03	0.145
Pure Error	2	0.00126	0.00126	0.00063		
Total	14	5.00359				

3.4. Characterization of exopolysaccharides produced by *Pleurotus sapidus*:

Exopolysaccharides were subjected to High Performance Liquid Chromatography analysis (HPLC). The HPLC chromatograph (Figure 8) showed that they consist of five peaks that referred to presence of β -(1 \rightarrow 3) glucans, β -(1 \rightarrow 6) glucans branched, Chitin, Mannans and Galactans with different concentrations in EPS. For 1 mg of EPS structure analysis, the concentrations of β -(1 \rightarrow 3) glucans, β -(1 \rightarrow 6) glucans branched, Chitin, Mannans and Galactans were 25.40%, 15.60%, 19.20%, 22.20%, 17.60% respectively.

According to **Jahan and Singh (2019)** mushrooms are a good source of insoluble fiber in the form of chitin (polymer of N-acetylglucosamine) and non-starch polysaccharides like β -glucans. Also, According to **Khan and Tania (2012)** the carbohydrates in *Pleurotus* spp. are mainly in the form of polysaccharides or glycoproteins. The most abundant polysaccharides are chitin, α - and β -glucans, and other hemicelluloses (e.g., mannans, xylans, and galactans).

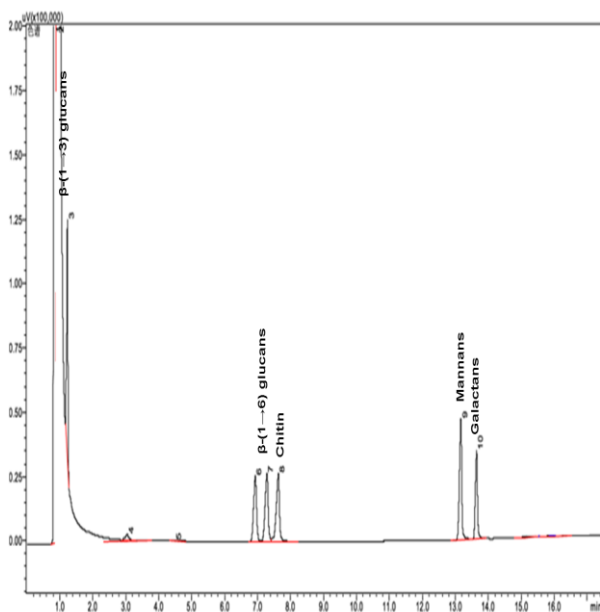


Fig. (8): HPLC of monomer of exopolysaccharides in culture filtrate produced by *pleurotus sapidus*.

3.5. DPPH scavenging activity of exopolysacch-arides

DPPH method is usually to evaluate antioxidant activity of various natural compounds by reducing stable DPPH radicals to yellow-colored diphenylpicryl-hydrazine. DPPH radical scavenging ability is responsible for hydrogen donating efficiency of antioxidants. As shown in **(Fig. 9)** DPPH radical scavenging activity of endopolysaccharides increased gradually with increasing concentration. At 2 mg/ml scavenging effects of EPS were 94%. These findings also agree with findings by **Jeena *et al.* (2016)**.

The present results also agree with the results of **Li *et al.* (2012)** who suggested that all the polysaccharides from *Hypsizygus marmoreus*, *Lentinus edodes*, *Russula vinosa* Lindblad, *Hohenbuehelia serotina*, *Auricularia auricula* and *Hericium erinaceus* had significant antioxidant capacities (ranged from 18.54% to 100%) at concentration of 20 mg/ml. Also, **Zhao *et al.* (2005)** reported that radical scavenging ability is responsible for hydrogen donating efficiency of antioxidants. Therefore,

the antioxidant activity may be attributed to the proton donating ability, indicating that it may contain lots of reductones to react with radicals to stabilize and terminate radical chain reactions.

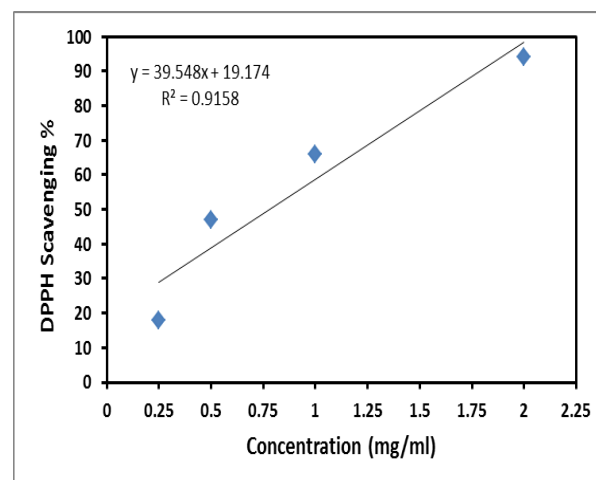


Fig. (9): Antioxidant activity of exopolysaccharides production from *Pleurotus sapidus*.

3.6. Anti-tumour activity of *Pleurotus sapidus* EPS

In the present study, the growth inhibitory effects of the EPS obtained from *P. sapidus* against human colon cancer cell (Caco-2) were examined. The control (Caco-2) cells viability was assessed as 100%. The inhibitory effect was estimated using different concentration (10000, 5000, 2500, 1250, 625, 312.5, 156.25 and 78.125 mg/ml) of EPS on both cell line included. Current results showed that the produced EPS exhibited various degree of antitumor effect toward the tested cell lines with increasing concentrations of EPS resulted in increased rates of tumour inhibition. EPS presented anti-proliferative activity at dosage 10000 mg/ml where inhibit 91% of tumour viability cells of colon as shown in **(Fig. 10)**. The IC_{50} for colon was 185.021 mg/ml. The effects of EPS on cell line were exhibited in **(Fig.11)** in which at concentration 78.125 mg/ml there is slightly disintegration of cells, at 156.25 mg/ml disintegration of cells increased, at

312.5 mg/ml partial disintegration and at 10000 mg/ml increased lysis percentage, subsequently the number of individual tumour cell decreased. In what refers to the polysaccharides from *Pleurotus spp.*, **Facchini et al. (2014)** reported the efficacy of a polysaccharide fraction obtained from the mycelium of *P. ostreatus* with NH₄-oxalate at 100°C inhibiting the development of Ehrlich Tumour (ET) and Sarcoma 180 (S-180).

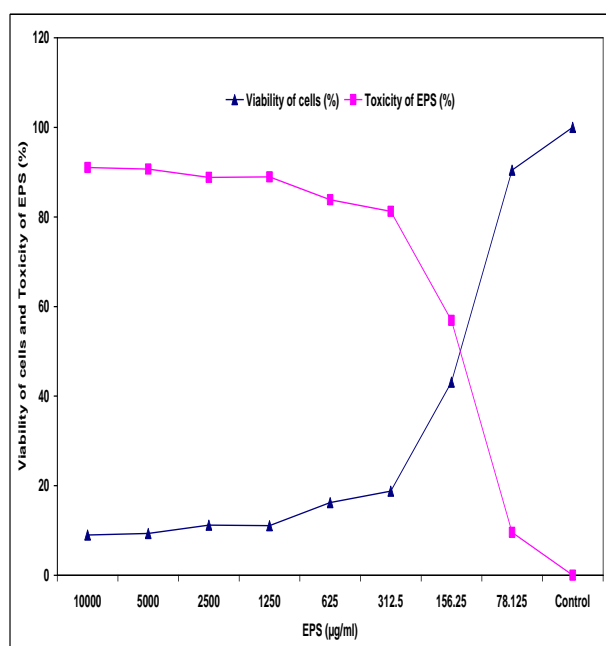


Fig. (10): Effect of EPS from *Pleurotus sapidus* (toxicity) on survival Caco-2 at different concentration in relation to cell viability of tumor.

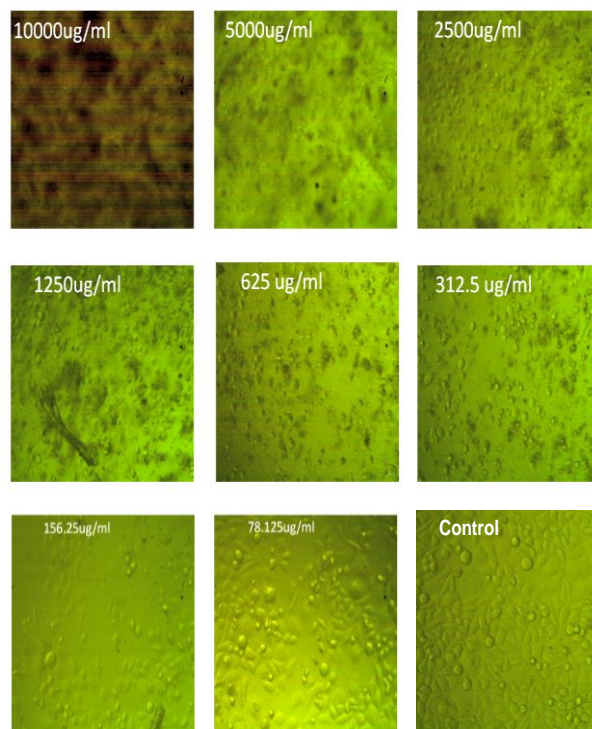


Fig. (11): Effect of EPS produced by *Pleurotus sapidus* on Caco-2 cells at different concentrations.

3.7. Antiviral activity of *Pleurotus sapidus*

EPS

In the present study, the growth inhibitory effects of the EPS obtained from *P. sapidus* against HAV (Hepatitis A Virus) was examined. The HAV was injected to Vero cell causing 53.8% toxicity of Vero cell which represents 100% of its actual virulent power. By application of EPS crude extract, the toxicity of virus to Vero cell became 48.3% which represent 83% of viral activity. So, the EPS of *P. sapidus* exhibited antiviral activity in percentage of 16.8%. These data are shown in (Fig.12).

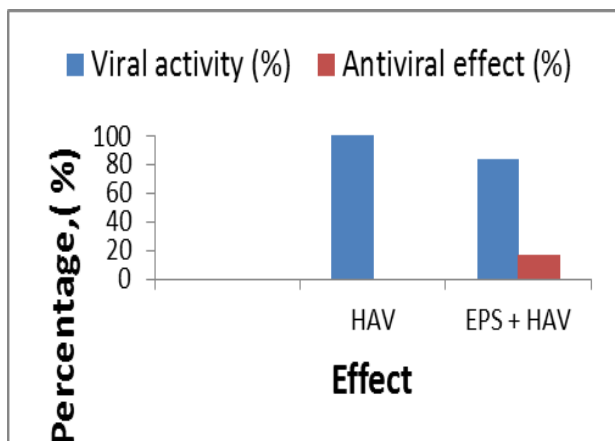


Fig. (12): Effect of different concentrations of EPS obtained from *P. Sapidus* on survival HAV (Dilution used = 78.125 mg/ml).

4. Conclusion and Recommendation:

The recorded results support the efficiency of multi-factorial experimental designs in elucidating the significant factors and predicting their optimum settings for achieving EPS maximum productivity. The current study indicates that exopolysaccharides extracted from *P.sapidus* has efficient scavenging activity.

It can be recommended that supplementation of natural products as *P. sapidus* mushroom is an important powerful anti tumor, antioxidant, antiviral and can be used for medical purposes. Future studies are needed to evaluate the feasibility of *P. sapidus* extract for developing potent antitumor, antioxidant drugs and detect their biological activities.

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دور السكريات عديدة التسكر الخارجيه المنتجه من بلوروتس سايبديس كعامل طبي

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أظهرت الدراسة ان زراعة فطر البلوروتس سايبديس على وسط غذائى يحتوى على النشا والجلوكوز لمدة (١٠) يوم كفترة تحضين عند درجة (٢٥,٢) درجة سليوزية أعطت أعلى إنتاجية من السكريات عديدة التسكر المستخرجة خارجيا. وأوضحت النتائج بعد التحليل باستخدام جهاز HPLC أن عديدة التسكر الخارجيه المستخلصة من فطر البلوروتس سايبديس يتكون من السكريات بيتا-جلوكان ، كيتين ، جالاكتان ، المانان . أثبتت الدراسة ان السكريات عديدة التسكر الخارجيه المستخلصة من فطر البلوروتس سايبديس لها نشاط مضاد للسرطانات بنسبه (٩١%) ومضاد للأكسدة بنسبة ٩٤ % عند ٢ ملليجرام/مل .