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Optimization of Production Conditions for Bioactive Polysaccharides from *Fomes fomentarius* and Investigation of Antibacterial and Antitumor Activities

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ABSTRACT

Background: One of the medicinal fungi that has been used in traditional medicine for a long time is the Basidiomycete fungus *Fomes fomentarius*, which is widely distributed in Iran. Polysaccharides as one of the metabolites of this fungus have anti-inflammatory, anti-diabetic, antibacterial, antioxidant, and anti-cancer properties.

Materials & Methods: Optimization of independent variables of $MgSO_4.7H_2O$ concentration, initial pH, yeast extract, and inoculum percentage to increase biomass and polysaccharide production of *F. fomentarius* was investigated using the Taguchi method. Then, the biological properties of the produced polysaccharide including antibacterial activity was investigated by bacterial colony counting method, antioxidant activity using DPPH free radical, and antiproliferative effect on 5 cancer cell lines MKN-45, AGS, A549, KYSE-30 and 5637 using MTS test.

Results: The concentration of $MgSO_4.7H_2O$ and initial pH had a significant effect ($P<0.05$) on the production of *F. fomentarius* polysaccharide and in optimal conditions polysaccharide production reaches 5.410 g/L. The polysaccharide of this fungus inhibits the growth of *Staphylococcus aureus* and *Escherichia coli* bacteria by 50% and 25%, respectively. The antioxidant activity of this polysaccharide in the DPPH test is 16.11%. The antiproliferative effect of this polysaccharide on cancer cells is different (KYSE-30> A549 ≥5637> AGS> MKN-45). This effect increases with increasing concentration. In KYSE-30 cell line treatment with 200 g/mL polysaccharide, cell viability reaches 40% after 72 hours.

Conclusion: Optimizing the culture medium of the medicinal fungus *Fomes fomentarius* increases the production of polysaccharides up to 5.410 g/L. Optimization increases the biological activity of polysaccharides. Antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* is 50% and 25%, respectively. The antioxidant activity of polysaccharides is 16.11% and the viability of KYSE-30 cancer cells reaches 40% after 72 hours.

Keywords: *Fomes fomentarius,* Optimization, Taguchi, Antibacterial, Antioxidant, Anticancer

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Introduction

The history of using medicinal fungi in East Asian countries dates back to thousands of years ago. Many of these fungi are still used to treat diseases **(1)**. The medicinal fungi properties include stimulating the proliferation of lymphocytes, reducing the proliferation of cancer cells, and anti-inflammation. The increasing importance of medicinal

fungi and their metabolites in the treatment of various diseases, the appropriate biodiversity of medicinal fungi in Iran, and the economic value of metabolites derived from these fungi indicate the need to pay attention to this branch of biological sciences **(2)**. Among the medicinal fungi, Basidiomycete *Fomes fomentarius* has long been used in the treatment of gastrointestinal diseases, liver cirrhosis, and various cancers. The distribution of this fungus is widespread in Iran and its presence has been reported in Mazandaran, Golestan, Gilan, Isfahan, Tehran, Kurdistan, Kermanshah, Khorasan and Azerbaijan provinces **(2, 3)**. This fungus has important properties in various aspects. This species is considered as one of the important causes of white heart rot on forest trees. Its extract has antioxidant and anti-cancer properties. *F. fomentarius* is also considered for the production of the laccase enzyme and use in processes such as decolorization and biodegradation **(3-5)**. One of the bioactive compounds of this fungus is polysaccharides that have anti-cancer, anti-inflammatory, anti-diabetic, and immune-enhancing activities. *F. fomentarius* β-glucan polysaccharide prevents tumor angiogenesis and metastasis **(6, 7)**. The anti-proliferative effect of this polysaccharide has been observed on SGC-7901, A549, MCF7, and MKN-45 cancer cell lines **(4, 5, 8)**. Studies show that the polysaccharides of *F. fomentarius* also have antibacterial and antiviral activity **(9, 10)**. Chen *et al.* (2008), examined the biomass and polysaccharide production of *F. fomentarius* in submerging culture conditions. A temperature of 25°C and an initial pH in the range of 5-6 are suitable for the growth of this fungus. Also, using glucose as carbon source, yeast extract as a nitrogen source, $CaCl_2$ and $MgSO_4.7H_2O$ improves mycelial growth and increases polysaccharide production **(11)**. Cultures of *F. fomentarius* in stirred bioreactor and solid bed bioreactor have also been studied **(4, 12)**. Many studies have been done on optimizing the culture medium for the growth of other fungi and their metabolite production. One of these statistical methods is the Taguchi array design, which allows the study and optimization of several variables simultaneously (13). Therefore, in this study, the production of biomass and polysaccharide of the Iranian medicinal fungus *F. fomentarius*, which has been isolated from the forests of Mazandaran, was investigated. Also, the composition of the culture medium of this fungus was optimized to increase the production of biomass and polysaccharide by the Taguchi method. Then the biological activities of polysaccharides including antibacterial, anti-oxidant, and cytotoxicity were investigated.

Materials and Methods

Collection of Mushroom Samples

The *F. fomentarius* was isolated from Mazandaran forests in 2017 with the cooperation of Sari Agricultural Sciences and Natural Resources University. In each case, the fungal specimen was harvested in a healthy, complete and appropriate manner based on the color, shape, size and surface decoration of the cap, base, blades and many other characteristics. After morphological confirmation by mycologists, it is kept at 4°C.

Cultivation of *F. fomentarius* and Polysaccharides Extraction

The *F. fomentarius* mycelium was cultured in potato dextrose agar (PDA) (manufactured by Merck, Germany) in a petri dish and incubated for 5 days at 28°C (BINDER, USA). For seed culture, 10 mm^2 pieces of fungus grown in PDA medium were transferred to 100 mL of Potato dextrose broth (PDB) medium (manufactured by Merck, Germany) for 7 days at 28°C and 150 rpm in shaker incubator (JAL TAJHIZ, Iran). Suitable culture medium of this fungus includes glucose (5%), peptone (0.2%), malt extract (1%), yeast extract (0.2%), $MgSO_4.7H_2O$ (0.25%) and KH_2PO_4 (0.5%). The initial pH of this medium was adjusted to 6 by adding NaOH (1M) and HCl (1M). After autoclaving, 5% v/v inoculum was added and incubated at 28°C and 150 rpm for 4 days **(11)**. To extract the *F. fomentarius* polysaccharide, after separating the biomass using Whatman paper, absolute ethanol with a ratio of 4:1 v/v was added to the supernatant. After overnight refrigeration, the polysaccharide precipitates were centrifuged for 10 min and 10,000 rpm (AWEL/MF20-R/France). The biomass and polysaccharide were lyophilized (OPERON) and the final product was stored at room temperature.

Optimization of Culture Medium Compositions by Taguchi Method

Taguchi method was used to investigate the effect of independent variables of $MgSO_4.7H_2O$, pH, yeast extract, and inoculum percentage on biomass and polysaccharide production. The Taguchi L9 array was used to examine four variables at three levels (Table 1), the experiments were performed according to this array, and the results were analyzed using Design Expert 11 software (State-Ease, USA).

Biological Assessments

Antibacterial Activity

The colony-forming unit (CFU) method was used to evaluate the antibacterial activity of polysaccharides. *Staphylococcus aureus* (UTMC 1429) and *Escherichia coli* (PTCC 1269) were obtained from the the Research Center for Technology and Microbial Products of the University of Tehran and Persian Type Culture Collection. Bacteria were cultured in Müller-Hinton Broth (manufactured by Merck, Germany) and after 24 hours of incubation (BINDER, USA), 0.5 McFarland solution was prepared from them. A 5% solution of each polysaccharide with 0.5 McFarland solution was prepared from each bacterium in a volume of 500 µL and kept for 24 hours in a shaker incubator (JAL TAJHIZ,

Iran) at 37°C and 140 rpm. The mixture was diluted with phosphate buffer saline (1:10) and 10 µL of it cultured in nutrient agar medium (manufactured by Merck, Germany). In the control state, 10 µL of McFarland 0.5 solution of each bacteria was cultured. Then, all culture media were incubated and after 24 hours, bacterial colony count was performed with a gel dock (Quantum, France) **(14)**.

Antioxidant Activity

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) test was used to evaluate the antioxidant activity of polysaccharides. After preparing 2 mg/mL polysaccharide solution in water, the polysaccharide solution and DPPH solution were mixed in a ratio of 1:4 v/v and placed in a shaker incubator for 30 minutes **(10)**. Then the absorbance of the samples was measured at 517 nm with ELISA reader (Carry 100 Bio, Australia) and antioxidant activity was calculated according to the following formula:

:(eq.1)

DPPH• scavenging effect (%) = [(A0 – sample A)/A0] × 100

A0: Adsorption of DPPH solution at 517 nm

sample A: Absorption of the test sample at 517 nm

Cytotoxicity Assay

MKN45, AGS, A549, KYSE-30, and 5637 cancer cell lines were purchased from the Iranian Genetic Resources Center. Cancer cells were cultured in RPMI 1640 medium with 2 mM L-glutamine and 15% FBS and stored in a CO_2 incubator at 37°C. To evaluate the effect of polysaccharide, its solution was prepared in sterile water (1 mg/mL) and used at specific concentrations (50, 100, 200 µg/mL) to treat cells for 24 and 72 hours. In this experiment, positive control included culture medium and negative control included untreated cell culture. MTS test was used to evaluate cell viability. A total of 2000 cancer cells were cultured in each well of 96 well plates (24 hours). After treatment with specified concentrations of polysaccharide for 24 and 72 hours, 10 µM MTS solution (1:10 diluted in RPMI 1640) was added to each well and incubated for 3 hours (15). Then, adsorption of each sample was read at 495 nm using ELISA reader (Carry 100 Bio, Australia) and the cell viability percentage was calculated using the following formula:

:(eq.2)

Cell viability: Sample absorbance/ Control absorbance × 100

Statistical Analysis

Data were analyzed statistically by ANOVA method using Design Expert, version 11 (State Ease, USA). P-values below 0.05 (P<0.05) were considered to be statistically significant.

Results

The results of initial experiments on the biomass and polysaccharide production of the *F. fomentarius* showed that four days after the cultivation of the fungus in the desired culture medium, biomass production reaches 15 g/L and polysaccharide production reaches 4.06 g/L. Therefore, the culture medium that includes glucose, peptone, malt extract, yeast extract, $MgSO_4.7H_2O$, and KH_2PO_4 is a suitable culture medium for the growth and production of polysaccharides of this fungus.

Optimization of Biomass and Polysaccharide Production

According to the preliminary results, the optimization of the culture medium was designed and performed using the Taguchi method with four factors at three levels (Table 1).

Table 1. Taguchi L9 array test design layout and results obtained for the production of biomass and polysaccharide of *F. fomentarius*

Parameters					Response	
Runs	A:$MgSO_4.7H_2O$ (g/L)	B:pH	C:Yeast extract (g/L)	D:Inoculum (% v/v)	Biomass (g/L)	Polysaccharide (g/L)
1	2.5	8	2	5	9.890	4.498
2	2.5	4	4	10	10.560	2.337
3	1	4	2	3	11.840	1.274
4	1	6	4	5	14.522	2.785
5	4	4	6	5	11.626	4.201
6	1	8	6	10	9.920	2.878
7	2.5	6	6	3	14.690	3.573
8	4	8	4	3	12.150	5.410
9	4	6	2	10	7.600	3.110

Figure 1 (A) shows a comparison chart of the amount of biomass produced in each experiment and the amount predicted by the software. As can be seen, these results are very close to the data predicted by the software. R^2 for biomass production is 90.54%. Analysis of the results with ANOVA using Design Expert showed that inoculum have a significant effect (P<0.05) on the biomass production of *F. fomentarius*. The linear model for biomass production based on each of the variables is as follows:

:(eq.3)

Biomass: 10.44 - 1.44A – 0.4200B + 1.55C - 3.18D

In this equation (A) is the concentration of $MgSO_4.7H_2O$, (B) pH, (C) yeast extract, and (D) the percentage of inoculum. The two-dimensional and three-dimensional diagrams of the effect of variables on biomass production are shown in Figure 1.

Table 1. Analysis of variance and regression coefficients for biomass production of *F. fomentarius*.

Source	Sum of squares	df	Mean square	F-value	P-value
Model	41.69	4	10.42	9.57	0.0251
$MgSO_4.7H_2O$ (A)	5.55	1	5.55	5.55	0.0868
pH (B)	1.06	1	1.06	0.9722	0.3800
Yeast extract (C)	6.41	1	6.41	5.88	0.0723
Inoculum (D)	28.67	1	28.67	26.34	0.0068
Residual	4.35	4	1.09		
Cor Total	46.05	8			

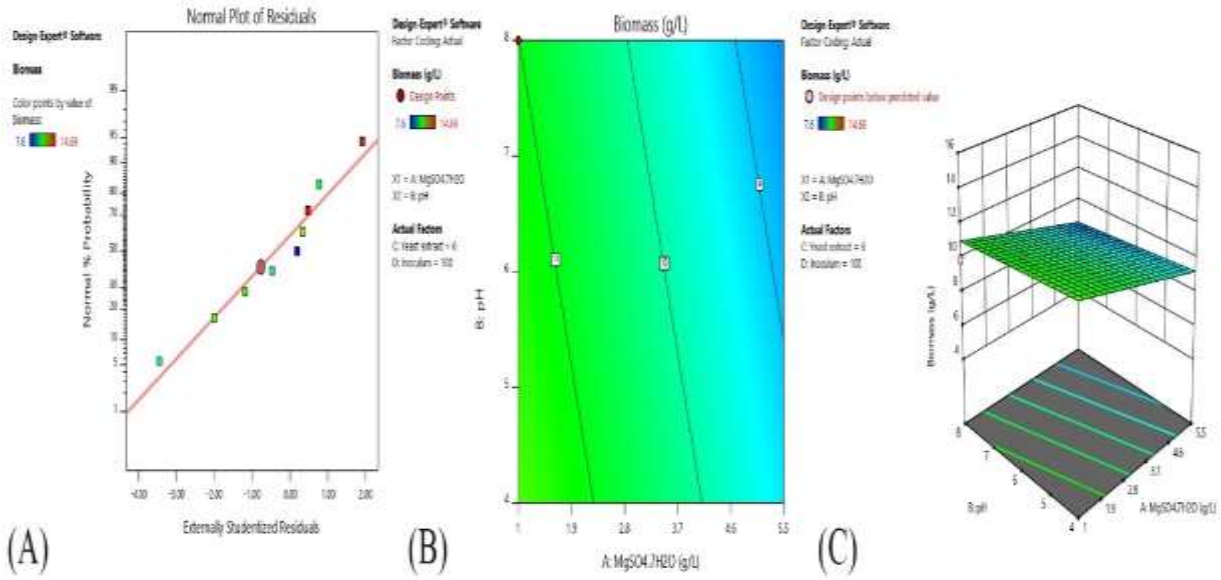

Figure 1. Comparison diagram of produced and predicted biomass (A), two-dimensional diagram (B) and three-dimensional (C) effect of $MgSO_4.7H_2O$ and initial pH on *F. fomentarius* biomass production.

A comparison of the amount of polysaccharide produced in each experiment and the amount predicted by the software showed that these results are very close. R^2 for biomass production is 91.68%. Analysis of the results with ANOVA using Design Expert showed that concentrations of $MgSO_4.7H_2O$ and initial pH have a significant effect (P<0.05) on the production of polysaccharides of *F. fomentarius*. The linear model for polysaccharide production based on each of the variables is as follows:

:(eq.4)

Polysaccharide=3.71+1.45A+0.8292B+0.4427C-0.6025D

In this equation (A) is the concentration of $MgSO_4.7H_2O$, (B) pH, (C) yeast extract and (D) the

percentage of inoculum. The two-dimensional and three-dimensional diagrams of the effect of variables on polysaccharide production are shown in Figure 2.

Table 2 . Analysis of variance and regression coefficients for polysaccharide production of *F. fomentarius*.

Source	Sum of squares	df	Mean square	F-value	P-value
Model	11.25	4	2.81	11.02	0.0196
$MgSO_4.7H_2O$ (A)	5.58	1	5.58	21.84	0.0095
pH (B)	4.13	1	4.13	16.16	0.0159
Yeast extract (C)	0.5227	1	0.5227	2.05	0.2257
Inoculum (D)	1.03	1	1.03	4.02	0.1153
Residual	1.02	4	0.2553		
Cor Total	12.27	8			

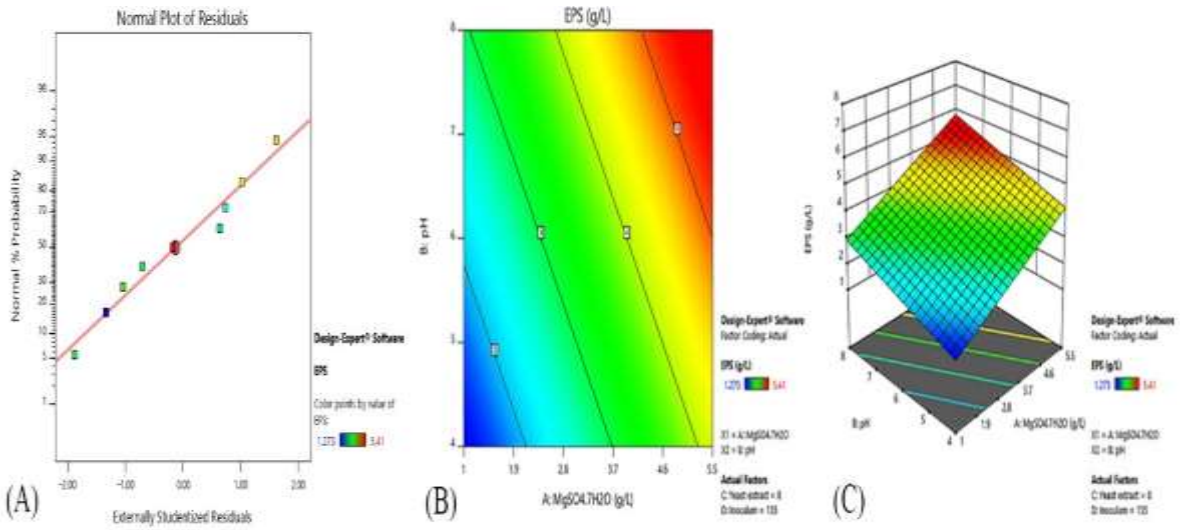

Figure 2. Comparison diagram of produced and predicted polysaccharide (A), two-dimensional diagram (B) and three-dimensional (C) effect of $MgSO_4.7H_2O$ and initial pH on *F. fomentarius* polysaccharide production.

Biological Assessments

Antibacterial Activity

The antibacterial activity of polysaccharide produced by *F. fomentarius* in Taguchi experiments was tested against Gram-positive bacteria *S. aureus* and Gram-negative bacteria *E. coli*. Bacterial colony count using a gel dock device showed that in the control sample of both strains, bacterial growth is 100%. Colony counts showed that the polysaccharide of this fungus has an inhibitory effect on bacterial growth. This effect is greater on the gram-positive bacteria *S. aureus*. Sample 7 with 50% inhibition of *S. aureus* had the highest inhibitory effect ($P<0.05$). It can also inhibit the growth of *E. coli* by up to 24%. The highest inhibitory effect on *E. coli* was observed in sample 3 (25%). There was no significant difference between the antibacterial activities of polysaccharides on *E. coli*. The Figure 3 (A) shows the antibacterial activity of the polysaccharide of the fungus *F. fomentarius*.

Antioxidant Activity

The antioxidant activity of polysaccharides produced by *F. fomentarius* in Taguchi experiments was investigated using DPPH free radicals. When the antioxidant substance donates protons to this free radical, its absorption in 517 nm decreases; this decrease indicates the level of antioxidant activity. The antioxidant activity of polysaccharides produced by this fungus varies in different environments (Figure 3, B). Analysis of DPPH results showed that the antioxidant activity in polysaccharide produced in environment 7 shows the highest antioxidant activity (16.11%). The lowest antioxidant activity is related to sample 2.

Cytotoxicity Assay

Biological assessments in previous stages showed that sample 7 has the most biological activity; therefore, this sample was used to evaluate cytotoxicity. In this study, the antiproliferative effect of polysaccharide of this

fungus in different concentrations on 5 cancer cell lines MKN-45, AGS, A549, KYSE-30 and 5637 was investigated using MTS test (Figure 4). In general, *F. fomentarius* polysaccharide inhibits the growth of all studied cancer cells significantly (P<0.05). After 24 hours, the antiproliferative effect of polysaccharide at a concentration of 50 µg/mL on AGS was significantly (P<0.05) greater than that KYSE-30 and 5637, but no difference was observed between the antiproliferative effect of polysaccharide in other cancer cell lines. At a concentration of 100 µg/mL polysaccharide had the highest antiproliferative effect on the AGS cell line and it was significantly higher than other cell lines (P<0.05). At a concentration of 200 µg/mL, the polysaccharide has a significant antiproliferative effect on A549 cancer cells compared to other cell lines, and it inhibits the growth of this cell by more than 50%. After 72 hours, there was no significant difference between the antiproliferative effects of polysaccharide on the cancer cell lines. At a concentration of 100 µg/mL polysaccharide of *F. fomentarius* had the most antiproliferative effect on 5637 cells and it was significantly different from other cell lines (P<0.05). At a concentration of 200 µg/mL, polysaccharide inhibits the growth of A549, KYSE-30 and 5637 cells by up to 40% and has a significant difference compared to this effect on MKN-45 and AGS cells (P<0.05).

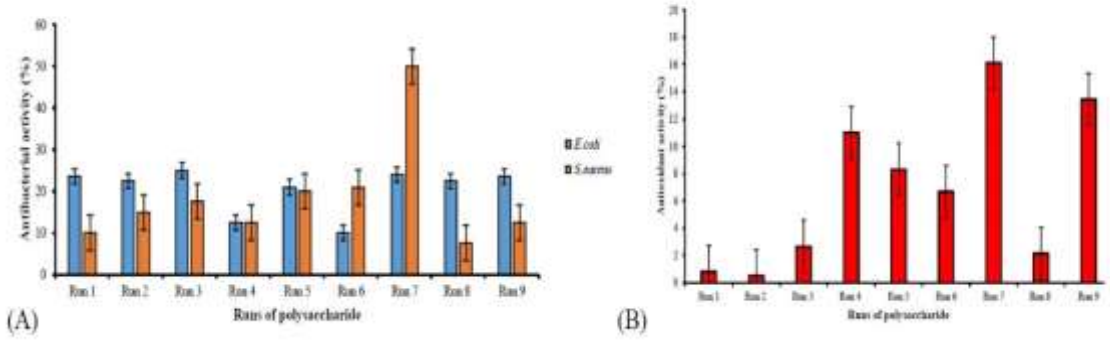

Figure 3. Antibacterial (A) and antioxidant (B) activity of polysaccharide obtained from the Taguchi L9 array.

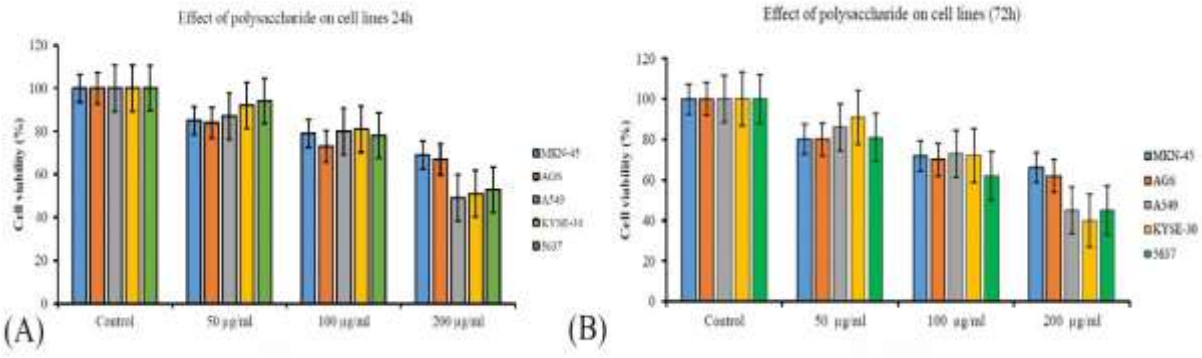

Figure 4. Cell viability diagram of 5 cancer cell lines treated with F. *fomentarius* polysaccharide after 24 (A) and 72 hours (B

Discussion

In this study, the optimization of biomass and polysaccharide production of the medicinal fungus *F. fomentarius* and its polysaccharide biological properties were investigated. As observed in previous studies **(4)** and this study, the suitable culture medium for this fungus includes glucose, peptone, malt extract, yeast extract, $MgSO_4.7H_2O$, and KH_2PO_4. Studies show that peptone in this environment plays an important role in increasing the growth and production of fungal enzymes **(16)**. Optimization of culture medium using the Taguchi method showed that $MgSO_4.7H_2O$ concentration and initial pH have a significant effect (P<0.05) on polysaccharide production of this fungus. With the increase of $MgSO_4.7H_2O$ and the initial pH, the production of polysaccharide of *F. fomentarius* increases. pH is involved in permeability, cell membrane function, and production of secondary metabolites **(11)**. Magnesium is an important element in the metabolism of fungi and the stability of cell membranes. It is also involved in DNA replication, cell division, and many enzymatic reactions **(11, 17, 18)**. Chen *et al.* (2008), increased the polysaccharide production of this fungus (3.64 g/L) by optimizing the culture medium. In this study, under optimal conditions (4 g/L $MgSO_4.7H_2O$, initial pH=8),

the production of polysaccharide of the *F. fomentarius* reached 5.410 g/L. This amount is 1.5 times the production of polysaccharides in common fungal culture medium (PDB) **(11)**.

Studies show that physicochemical conditions of the culture medium such as carbon source, nitrogen source, mineral sources, pH and culture temperature play an important role in the biological activity of fungal metabolites **(19, 20)**. *F. fomentarius* polysaccharide inhibits the growth of *S. aureus* and *E. coli* by 50% and 25%, respectively. Polysaccharide of this fungus contains terpenoids and polyphenols with antibacterial activity and with their increase this activity increases **(21)**. Studies show that the type and volume of solvent used to extract polysaccharides play a role in the content of polyphenols. The use of polar solvents such as ethanol increases polyphenols **(10, 22).** Kalyoncu *et al.* (2010), found that the antioxidant activity of the polysaccharide of the *F. fomentarius* was 5.97%. By optimizing the culture conditions in this study, the antioxidant activity of polysaccharide increased to 16.11%. Most biological properties such as antioxidant, antibacterial, and antiproliferative activity are observed when the initial pH of the fungal culture medium is in the range of 5-7 **(23)**. The difference between the initial pH and the composition of the culture medium causes a difference in the phenolic content of the polysaccharide. These compounds are associated with antioxidant activity and reduction of $OH^{\bullet}$, $O_2^{\bullet}$, and $NO^{\bullet}$ radicals **(24)**. Investigations in this study and previous studies **(4, 5, 11)** show that this polysaccharide has an antiproliferative effect on cancer cells, but this effect varies in cell lines and increases with increasing of the concentration. The antiproliferative effect of *F. fomentarius* polysaccharide was observed (KYSE-30> A549 ≥563749> AGS> MKN-45) after 72 hours. In the treatment of KYSE-30 cells with 200 µg/mL polysaccharide, cell viability reaches 40% after 72 hours. Also, after 72 hours, the life expectancy of A549 and 5637 cancer cells was 45% and that of MKN-45 and AGS cancer cells was 66% and 62%, respectively. Today, the polysaccharides of the fungi like *Ganoderma lucidum* and *Tinea versicolor* are used as supplements in the treatment of various cancers. The anti-cancer activity of these polysaccharides includes inhibiting the growth of cancer cells and stimulating the immune system **(25)**. The antiproliferative activity of fungal polysaccharides is related to the chemical structure and composition of their monosaccharides. The polysaccharide of *F. fomentarius* is rich in beta-glucan, which has anti-cancer properties. This compound destroys transcription proteins in cancer cells and arrests the cell cycle in the G1 phase. It also induces apoptosis in cells by damaging organelles, the cell nucleus, and fragmenting DNA **(10, 26, 27, 28)**. The results of this study showed that the polysaccharide of the medicinal *F. fomentarius*, due to its antibacterial, antioxidant and antiproliferative properties, can be a suitable option for the treatment of many diseases and can be used as a dietary supplement.

Conclusion

In this study, the production of biomass and polysaccharide of the Iranian medicinal *F. fomentarius* was optimized using the Taguchi method. Inoculum percentage had significant effects on biomass production and $MgSO_4.7H_2O$ concentration and initial pH had significant effects (P<0.05) on polysaccharide production of this fungus and under optimal conditions (4 g/L $MgSO_4.7H_2O$, initial pH=8) polysaccharide production increases 1.5 times and reaches 5.410 g/L. Biological assessments showed that this polysaccharide inhibits the growth of *S. aureus* and *E. coli* 50% and 25%, respectively (P<0.05). Also, its antioxidant activity increases 2 times and reaches 16.11%. The antiproliferative effect of *F. fomentarius* polysaccharide is different on different cancer cells and increases with increasing concentration. The cell viability of KYSE-30 treatment with 200 µg/mL polysaccharide, reaches 40% after 72 hours. Also, the cell viability of A549 and 5637 cancer cells is 45% and the cell viability of MKN-45 and AGS cancer cells is 66% and 62%, respectively.

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Conflict of Interest

Authors declared no conflict of interests.