PARTS FROM LIFE-CYCLE OF *H.ERINACEUS*: RESPONSE SURFACE METHODOLOGY APPROACH TO OPTIMIZE EXTRACTION CONDITIONS AND DETERMINATION OF ITS ANTIOXIDANT, ANTIDIABETIC AND ANTIMICROBIAL EFFECT

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ABSTRACT

In this article, optimization of extraction conditions of different parts from the life cycle of *H.erinaceus* was investigated based on Response Surface Method. Furthermore, antioxidant, antidiabetic, and antimicrobial effects of optimized extracts were studied. Results: Temperature, liquid / solid ratio, and solvent concentration significantly affected the phenolic content and antioxidant capacities of all samples. The young fruit body of *H.erinaceus* had higher antioxidant (43.11±2.74, 51.39±2.34, 62.84±1.59 µmol TE/g for DPPH, FRAP and ABTS, respectively) and antidiabetic effects (93.54±2.3 and 132.25±1.38 µg/mL for IC50 values of α-glucosidase and α-amylase, respectively) than mycelium. However, these effects partially decrease as the fruit body ages. Besides, the optimized extracts exhibited more antimicrobial effects on Gram-positive bacteria than Gram-negative bacteria. Conclusion: Both the fruit body and the mycelium of *H.erinaceus* have many bio-functional properties. However, these properties of the fruit body decrease significantly with the delay of the harvest time. The extraction process conditions we optimize in this study can be adapted to large-scale separation processes in the industry.

Keywords: Antidiabetic, Antimicrobial, Antioxidant, *Hericium erinaceus*, Optimization

INTRODUCTION

Hericium erinaceus is an edible medicinal mushroom species in the Hericiaceae family. *H. erinaceus,* also known by names like lion's mane and monkey head, has a unique appearance with spines longer than 1 cm. Numerous bioactive components of this mushroom have many effects, such as antioxidant (**Ferreira *et al.*, 2009**), anti-aging (**Nakatsugawa *et al.*, 2003**), immunomodulatory (**Lee and Hong, 2010**), antitumor (**S. P. Kim *et al.*, 2011**), antimicrobial (**Wong *et al.*, 2009**), and antihyperglycemic (**Wang *et al.*, 2005**). These components in *H.erinaceus* are found both in the fruit body and in the mycelia. However, the amount of components in the fruit body of the mushroom varies according to the degree of fruit maturity (**Li *et al.*, 2015**). The production of medicinal mushrooms is a complex process due to parameters such as media content, hygienic requirements, and adequacy of artificial conditions. Also, even if previously supplied liquid or grain spawn is used in cultivation, the fruit body can usually be collected after a few months (**Stamets, 2011**). Mycelial biomass can be produced in a short time with high efficiency with the technique of submerge fermentation (**Fang and Zhong, 2002**). Whether it is mycelium or the fruit body, *H.erinaceus* has significant effects on human health, which has rapidly increased its use in food supplements in recent years.

Extraction conditions are vital for the recovery and subsequent purification of bioactive components from biomass. These conditions, such as temperature, liquid/solid ratio, and solvent concentration, may significantly change the nature and activity of the extract (**Chirinos *et al.*, 2007**). Therefore, extraction conditions need to be optimized for the desired objectives. The traditional optimization method, where only one variable is optimized at a time, can only be completed by performing a large number of experiments that cause excessive resource and time consumption. Moreover, interactions between variables may be ignored, and discrepancies may occur when individually optimized variables are applied together. In Response Surface Methodology (RSM), which was first introduced by **Box and Wilson (1992)**, unlike the traditional optimization method, the effect of multiple variables on responses can be evaluated as a whole. Thus, errors that may occur due to the interaction of variables are minimized, and the results can be better evaluated. Furthermore, RSM requires few experiments in the optimization process, which means time and resource

savings. Therefore, many researchers have successfully applied RSM to extract components from mushrooms and to optimize extraction process conditions (**Gogoi *et al.*, 2019; XuJie and Wei, 2008**). However, studies on the optimization of the extraction process of *H.erinaceus* are limited.

The objectives of this work were; to optimize extraction processes of mycelium and fruit body (with two different maturity stages) of *H.erinaceus* according to the total phenolic content (TPC) and antioxidant capacity based on RSM approach, and to determine and compare the antioxidant, antimicrobial and antidiabetic effects of optimized extracts.

MATERIAL AND METHODS

Materials

Pure culture of *H.erinaceus* obtained from Culture Collection at the Ahi Evran University (Kırşehir, Turkey) were inoculated on Malt Extract Agar (MEA) plates. After incubation at 25°C for ten days, it was used for both mycelial biomass production and mushroom cultivation. Wheat bran and poplar sawdust used for the growing medium of mushrooms were provided from local suppliers in Yozgat province. All chemicals used in this study were purchased from Merck KGaA unless otherwise stated.

Mycelial biomass production

The fully colonized media was divided into two equal parts with a scalpel. Then one of them was transferred in a sterile stainless steel blender container (Waring 8011 ES blender, NJ, USA) and homogenized with 100 mL of distilled water. After homogenization, 10 mL of mycelium-enriched liquid was poured into a 250 mL Erlenmeyer flask containing 90 mL of broth prepared with 20 g/L light malt extract, 5 g/L dry yeast extract, 1 g/L K_2PO_4 and 0.5 g/L $MgSO_4$. After the Erlenmeyer flask was closed with non-absorbent cotton, mixture, which was formulated based on **Lee *et al.* (2009)** with some modifications, was incubated in a shaking water bath (Daihan MaXturdy-45, Korea) at 25 °C. At the end of the 14 days incubation, the liquid was centrifuged at 4000 rpm for 5 min (Nüve, Turkey), and the precipitate was named as mycelial biomass (MB).

Preparation and inoculation of substrate

The method used in this section was performed based on **Ko *et al.* (2005)** with some modifications. Poplar sawdust was mixed with wheat bran in a ratio of 4:1, and its moisture content was adjusted to 65% with distilled water. In order to adjust the pH, 1% $CaCO_3$ was added to the substrate. 1 kg of the substrate was filled into polypropylene bags with 5 µ pore size filter (Type 14A, Unicorn Bags, TX, USA), autoclaved at 121 °C for 90 minutes, and then cooled to 25 °C. The sterile substrate was inoculated with five mycelial discs taken from fully colonized plates using a 7mm diameter cork borer. After bags were laminated with the heat sealer, they were shaken by hand to distribute the discs into the substrate.

Mushroom cultivation

Substrate bags were transferred to the climate-controlled room and incubated at 25 °C with 85% RH for the vegetative phase. At this stage, the room was dark, and CO_2 was uncontrolled. After the hyphae of *Hericium* completely covered the bags, six different points on the body of the bags were cut in 3 cm lengths shaped of the cross for the development of basidiomata. Then generative phase was conducted under the following conditions: at 17°C, 95% RH, below 800 ppm CO_2 concentration, and under artificial lighting for 12 hours a day. Half of the mushrooms, whose fruit body spines were reaching 2 cm, were immediately harvested and named as young fruit body (YFB). The other half of the mushrooms were collected after waiting for five more days and named as over-ripe fruit body (OFB).

Sample preparation for extractions

After the mushrooms were cut in 0.5 cm thickness, they were dried with MB samples at 40 °C for 24h. Dried samples were then powdered using a blade mill (Bosch MKM6000, Germany). After methanol was chosen as the best solvent in preliminary experiments, powdered samples (1 g) was placed in screw-capped tubes with aqueous methanol at various concentrations. Extraction was performed in a shaking water bath (Wisd, Korea) at the temperature and solvent/solid ratio specified at the experimental design for 60 minutes.

Experimental design and statistical analysis

The Box-Behnken Design (BBD) of Response Surface Method (RSM) was used to determine the effect of extraction conditions on TPC and 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant capacity of samples, as well as to determine the optimized extraction method according to maximized TPC and DPPH. Extraction conditions such as temperature, liquid/solid ratio, and methanol concentration were determined as independent variables and examined at three levels, while TPC and DPPH were selected as responses (Table 1).

Table 1 Levels of independent variables

Independent variables	Units	Symbol	Coded levels		
			-1	0	1
Temperature	°C	X_1	30	45	60
Liquid/solid ratio	mL g^{-1}	X_2	10	20	30
Methanol concentration	%	X_3	30	55	80

Experimental designs consisting of 15 experimental points were created for each of the three samples (MB, YFB, and OFB), as shown in Table 2.

Table 2 Box Behnken design used for Response Surface Methodology with experimental and predicted values for the independent variables

Standard order	Coded variables			Mycelial biomass				Young fruit body				Over-ripe fruit body			
	X_1 (°C)	X_2 (%)	X_3 (mL g^{-1})	TPC		DPPH		TPC		DPPH		TPC		DPPH	
				Pre.	Exp.	Pre.	Exp.	Pre.	Exp.	Pre.	Exp.	Pre.	Exp.	Pre.	Exp.
1	-1	-1	0	2.18	2.18	20.67	20.97	4.58	4.69	39.00	39.11	3.13	3.17	25.45	25.22
2	1	-1	0	2.43	2.37	22.64	22.55	4.99	4.97	40.04	39.97	4.06	4.10	30.03	30.23
3	-1	1	0	2.22	2.28	22.05	22.14	4.91	4.93	40.27	40.34	4.01	3.97	27.97	27.77
4	1	1	0	3.14	3.14	24.79	24.49	5.27	5.16	39.70	39.59	4.17	4.12	31.36	31.59
5	-1	0	-1	1.52	1.49	18.47	18.25	5.02	4.94	40.34	40.25	3.74	3.79	28.39	28.99
6	1	0	-1	2.31	2.35	23.47	23.64	5.29	5.34	40.24	40.33	4.20	4.25	33.08	33.24
7	-1	0	1	2.59	2.55	24.30	24.13	5.18	5.13	41.85	41.76	4.10	4.05	30.33	30.17
8	1	0	1	2.97	2.99	24.00	24.22	5.68	5.76	42.42	42.51	4.74	4.69	33.60	33.00
9	0	-1	-1	1.76	1.79	19.20	19.12	4.29	4.27	40.14	40.12	3.83	3.74	27.94	27.58
10	0	1	-1	1.98	1.94	21.23	21.36	4.53	4.59	40.53	40.55	3.99	3.98	28.71	28.32
11	0	-1	1	2.47	2.51	22.64	22.51	4.51	4.45	41.91	41.89	3.94	3.95	28.02	28.41
12	0	1	1	2.99	2.97	24.15	24.23	4.87	4.89	42.45	42.47	4.78	4.87	31.10	31.46
13	0	0	0	3.67	3.64	26.82	25.70	6.09	5.98	41.58	42.22	4.84	4.91	34.15	33.10
14	0	0	0	3.67	3.66	26.82	26.85	6.09	6.10	41.58	41.13	4.84	4.83	34.15	34.89
15	0	0	0	3.67	3.72	26.82	27.90	6.09	6.20	41.58	41.39	4.84	4.79	34.15	34.47

Legend: Exp.: Experimental values, Pre.; Predicted data

The relationship between factors and variables was expressed using the following equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

where; Y: predicted response, β_0: intercept, β_1, β_2, β_3: linear coefficients, β_{11}, β_{22}, β_{33}: interaction coefficients, β_{12}, β_{13}, β_{23}: quadratic coefficients, X_1, X_2, X_3: independent variables.

RSM was performed by using Design Expert 11.0.0 software (Stat-Ease Inc., Minneapolis, MN). Superimposed 3D surface plots were created to compare samples with each other. For this purpose, Design Expert software outputs were processed using Mathematica software (version 7; Wolfram Research, Champaign, IL). The significance of differences between data was determined using the SPSS 22.0 statistics package (SPSS Inc., Chicago, IL).

Total phenolic content

The TPC of the samples were determined by the method of **Singleton *et al.* (1999)** with minor modifications, as detailed in our previous study **(Doğan *et al.*, 2020).**

Antioxidant activity

DPPH, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and ferric reducing ability of plasma (FRAP) were used to determine the antioxidant capacity of the samples. The results of the analysis were expressed as Trolox equivalent (µM TE/g sample). For DPPH analysis, 0.1 mL sample was placed in the test tube containing 3.9 mL of 25 mg/L concentration of methanolic DPPH solution. After 30 min of incubation at room temperature in a dark place, the absorbance was read at 515 nm **(Brand-Williams *et al.*, 1995)**. In ABTS analysis, the ABTS solution, consisting of 30 mg ABTS and 7.8 mL of 2.46 mM potassium peroxodisulfate, was incubated for 16 hours. This solution was then diluted with phosphate-buffered saline (PBS) to achieve an absorbance of 0.70 ± 0.05 at 734 nm. After that, 1950 µL of diluted ABTS solution and 50 µL of the sample was vortexed for 30 s and absorbance was read after 6 min of incubation **(Cam *et al.*, 2009)**. FRAP analysis was performed according to **(Benzie and Strain, 1996)**. 10 µL of sample and FRAP reagent, consisting of 20 mM ferric chloride, 30 mM sodium acetate and 10 mM TPTZ, were mixed in a test tube. After 30 min incubation, the absorbance was read at 593 nm.

Antidiabetic activity

Inhibitions of α-amylase and α-glucosidase analyzes were used to determine the antidiabetic activities of the samples. In α-glucosidase analyzes, 50 µL sample, 1250 µL 67 mM monopotassium phosphate and 50 µL α-glucosidase were mixed in a test tube and incubated at 37 °C for 5 min. 125µL 10 mM 4-nitrophenyl-D-glucopyranoside solution was added to the tube to start the enzymatic reaction. After 20 min, 2 mL of 0.1 M sodium carbonate was added to the tube to stop the reaction, and the absorbance was read at 400 nm. In α-amylase analyzes, 1 mL sample, 1 mL potato starch solution, and 1 mL 20 mM monosodium phosphate were mixed in a test tube and incubated at 37 °C for 5 min. 1 mL α-amylase solution was added to the tube to start the enzymatic reaction. After 30 min, 0.5 mL of 5.31M sodium potassium tartrate (prepared with 2 M sodium hydroxide)

and 0.5 mL of 96 mM 3,5-dinitrosalicylic acid solution was added to the tube. The mixture was kept in boiling water for 5 minutes to stop the reaction, and then the absorbance was read at 540 nm (**Cam *et al.*, 2020; McDougall *et al.*, 2005**). Antidiabetic activity for the two methods described above was calculated using the following equation.

$$Antidiabetic\ activity\ (\%) = \frac{ABS_{control} - ABS_{sample}}{ABS_{control}} x100$$

where $ABS_{control}$ and ABS_{sample} express absorbance of control and sample respectively.

Phenolic concentrations of samples that inhibit 50% of α-amylase and α-glucosidase were expressed as IC_{50}.

Antimicrobial activity

The disk diffusion method used to determine antimicrobial activity was performed based on **Bauer (1966)**. The cultures incubated for 16-24 hours were suspended in 0.85% saline solution to obtain turbidity of 0.5 McFarland standard. Then, suspensions of *Bacillus cereus* (NRRL-B 37), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 25955) and *Candida albicans* (ATCC 90028) were inoculated onto Mueller-Hinton agar (MHA) plates. After 10 µL of the sample was impregnated to 6 mm diameter discs, they were placed on plates and incubated 37°C for 36h. Antimicrobial activity was determined by measuring the zone around each disc with a digital caliper.

RESULTS AND DISCUSSION

Preliminary experiments for solvent selection

Preliminary experiments based on the highest DPPH and TPC were performed to determine the best solvent among ethanol, methanol, acetone, and distilled water for YFB. 1 gram of dry sample obtained as described in the previous sections was mixed with 20 mL of solvent having 80% concentration and incubated at 45 °C for 60 min. The supernatant was analyzed after separate from the precipitate. The results showed that the highest TPC (5.06±0.21 mg GAE/g) and antioxidant capacity (43.11±2.74 µmol TE/g) belonged to methanolic extract as compared to other solvent extracts (Figure 1). In addition to the polarity of solvent and component, the water solubility of components plays a critical role in the separation of bioactive components from the organic material (**Barwick, 1997**). Snyder's solvent polarity index of ethanol, methanol, acetone, and distilled water is 5.2, 6.6, 5.4, and 9, respectively (**Snyder, 1978**). Except for hydrosol, a positive correlation was found between TPC, antioxidant capacity, and solvent polarity index (data not shown). The reason for the low extraction performance despite the high polarity index of distilled water may be explained by the fact that some of the bioactive components of *Hericium* are insoluble in water. On the

other hand, methanol is the alcohol with the best ionization potential. This may have caused the anions in *H.erinaceus* to dissolve more in methanol than other solvents through facilitated electron transfer (**Litwinienko and Ingold, 2003**). However, the entire extraction mechanism of the fungal material consisting of many different components with complex interactions may not be explained with a few parameters.
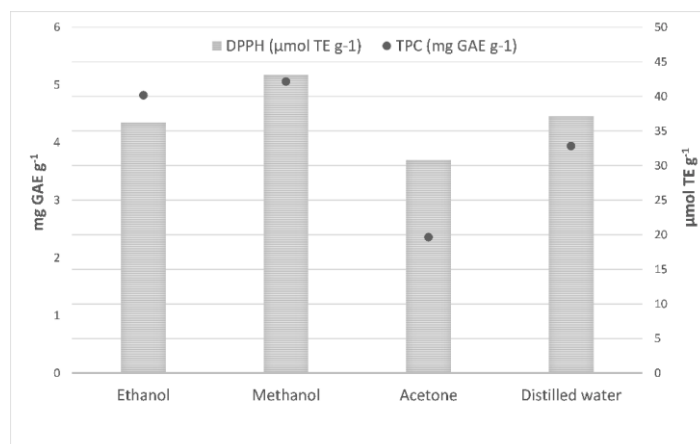

Figure 1 Effects of extraction solvents (ethanol, methanol, acetone and water) on the total phenolic content and antioxidant activity of *H.erinaceus*

Determination of model accuracy

The model equations, and statistical parameters such as regression (p-value), coefficient of determination (R^2), adjusted R^2 (R^2_{adj}), predicted R^2 (R^2_{pred}) and lack of fit were given in Table 3. The p-value of the analysis of variance (ANOVA) of all responses was found to be less than 0.05, indicating that the selected model was significant. The experimental and predicted values of the responses in the 15 experimental points were close to each other, which is an indicator of a reliable model (Table 2). The lack of fit determined for the TPC and DPPH of the samples was found insignificant. The R^2 values of the responses ranged from 0.956 to 0.997, which are entirely satisfactory for the quadratic model's ability to predict new values. Furthermore, R^2_{pred} of responses were in reasonable agreement with their R^2_{adj}. All statistical evaluations showed that the 2nd order quadratic model obtained from the software could be used to determine the effect of the extraction conditions on the responses, and in the process optimization.

Table 3 Polynomial equations and statistical parameters of models

Sample	Responses	2nd order polynomial equations	Regression (p-value)	R^2	R^2_{adj}	R^2_{pred}	Lack of fit
Mycelial biomass	TPC	$3.67+0.294X_1+0.185X_2+0.431X_3+0.168X_1X_2-0.105X_1X_3+0.078X_2X_3-0.569X_1^2-0.612X_2^2-0.759X_3^2$	0.0001	0.997	0.992	0.960	0.248
	DPPH	$26.82+1.18X_1+0.884X_2+1.59X_3+0.192X_1X_2-1.33X_1X_3-0.13X_2X_3-1.76\ X_1^2-2.52X_2^2-2.49X_3^2$	0.0024	0.971	0.920	0.880	0.947
Young fruit body	TPC	$6.09+0.193X_1+0.149X_2+0.136X_3-0.013X_1X_2+0.058X_1X_3+0.030X_2X_3-0.207X_1^2-0.949X_2^2-0.594X_3^2$	0.0005	0.986	0.959	0.833	0.450
	DPPH	$41.58+0.118X_1+0.232X_2+0.922X_3-0.403X_1X_2+0.168X_1X_3+0.038X_2X_3-0.936X_1^2-0.891X_2^2+0.569X_3^2$	0.0067	0.956	0.878	0.845	0.970
Over-ripe fruit body	TPC	$4.84+0.273X_1+0.248X_2+0.225X_3-0.195X_1X_2+0.045X_1X_3+0.170X_2X_3-0.472X_1^2-0.532X_2^2-0.177X_3^2$	0.0003	0.988	0.967	0.841	0.255
	DPPH	$34.15+1.99X_1+0.963X_2+0.614X_3-0.298X_1X_2-0.355X_1X_3+0.578X_2X_3-1.52X_1^2-3.93X_2^2-1.28X_3^2$	0.0025	0.971	0.919	0.748	0.681

Effect of the extraction conditions on the TPC

The effect of extraction process conditions on the TPC value of MB, YFB, and OFB were given in Figure 2.
The level of independent variables applied to all three samples significantly affected the extractable TPC value. Consistent with the mass transfer principle,

TPC increased significantly as the liquid / solid ratio increased from 10 to 20. However, it subsequently decreased. This may be explained by extracting the maximum amount of phenolic when the liquid / solid ratio is at a certain level and decreasing the phenolic concentration of the increasing liquid / solid ratio after this level.
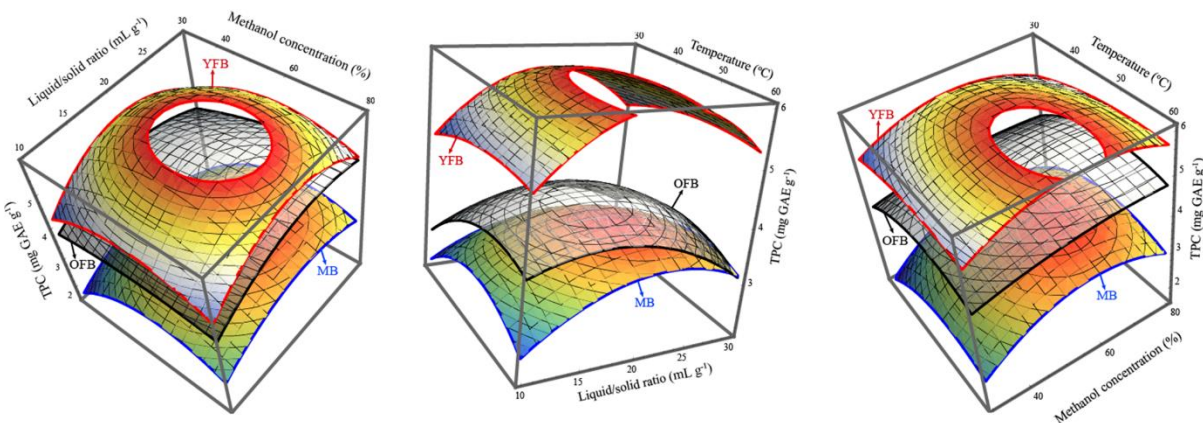
Figure 2 Superimposed 3-D surface plots for effect of the extraction conditions on the total phenolic content of mycelial biomass (MB), young fruit body (YFB) and, over-ripe fruit body (OFB)

Concerning methanol concentration, the TPC of MB and YFB increased with the increase of methanol concentration up to 65% and 55%, respectively, then slightly decreased. TPC of the OFB increased until the methanol concentration was 70% and remained constant at its maximum level. The transfer of bioactive components such as phenolics from biomass to solvent largely depends on the difference in polarity between solvent and solute. Therefore, solvents consisting of a combination of alcohol and water can provide more efficient extraction performance than solvents using only one type of alcohol (**Markom *et al.*, 2007).** Two different situations can be mentioned about the effect of temperature on the recovery of phenolics during the extraction process. The first is that the temperature softens the tissues, increases the solubility of the phenolics, and, accordingly, increases the diffusion ability of the amount of phenolic substance to a certain level. Moreover, high temperature increases the efficiency of phenolic

transfer by reducing solvent viscosity and surface tension. The second is that excessive temperature causes the amount of phenolics extracted to decrease as a result of chemical and thermal degradation (**Dent *et al.*, 2013)**. Hence, temperature is one of the most crucial parameters for extraction processes. Herein, considering only the temperature, 48℃ stood out as the breaking point for TPC of all three samples.

Effect of the extraction conditions on the DPPH

The effect of variables on the antioxidant capacity of the samples was significant, as shown in Figure 3.
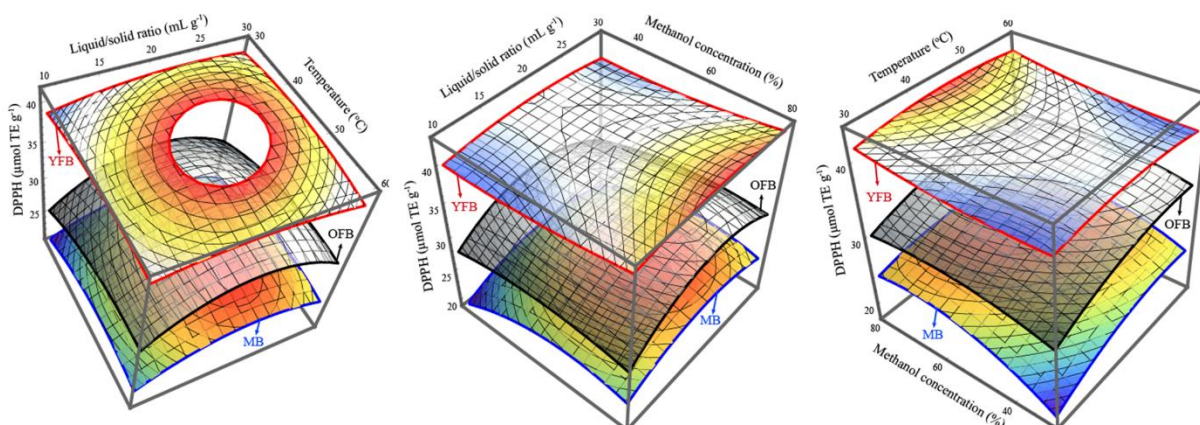

Figure 3 Superimposed 3-D surface plots for effect of the extraction conditions on the antioxidant capacity (DPPH) of mycelial biomass (MB), young fruit body (YFB) and, over-ripe fruit body (OFB)

The effect of the independent variables and the quadratic model was statistically significant for all three samples. When the temperature and liquid/solid ratio were kept constant at a medium level, the curved effect of methanol concentration on antioxidant capacity in MB and OFB samples, as in TPC, was determined. This type of effect is also a sign that there is a positive correlation between TPC and antioxidant capacity since some of the phenolic components have antioxidant effects. In accordance with our study, **Liyana-Pathirana and Shahidi (2005)** have stated that water-alcohol composite (%50) solvents showed the highest performance in extracting antioxidant components. However, in the YFB sample, the methanol concentration affected the antioxidant capacity linearly. This result indicated that antioxidant components in YFB that are degraded as the fruit body ages, are probably better soluble in lower polar solvents.

The temperature was another major factor affecting antioxidant capacity, and the antioxidant capacity of all samples continued to increase up to ~48℃. After this temperature, the antioxidant capacities of MB and YFB partially decreased, while the antioxidant capacity of OFB remained almost constant. Fungi have cell walls formed by crosslinking chitin, glucan, and glycoproteins (**Bowman and Free, 2006).** The reason for OFB's antioxidant capacity to be positively diverged from other samples after ~48℃ may be that OFB's thickened cell wall needs higher temperature to release antioxidant compounds, as well as the cell wall provides some protection to compounds that are degraded due to excessive temperature.

For all samples, a curved effect of liquid/solid ratio on the antioxidant capacity with a peak of 20 mL/g, similar to TPC, was determined. This may be explained by the increase in antioxidant components that can contact the solvent as the liquid/solid ratio increases to 20 mL/g. However, as this rate increased further,

the components in the solution may be diluted, and the antioxidant capacity measured may decrease.

Optimization and validation of models

Numerical optimization was used to determine the most suitable extraction conditions for each fungal sample. Hence, the criteria of dependent variables were set as a maximum for TPC and DPPH. Factor combinations with the highest desirability value were selected as optimum. The desirability approach is a method that assigns "score" to a series of responses and selects factor settings that maximize that score (**Natrella, 2010)**. The optimal levels of independent variables and desirability values for samples are shown in Table 4.

In order to determine the accuracy of the model, the predicted data were compared with the experimental values. Although differences of less than 5% were detected between experimental and predicted data, they were compatible (Table 4). Hence, it was concluded that the data predicted by the model were dependable.

Table 4 Optimal extraction conditions with experimental and predicted values at this conditions

Samples	Optimal conditions				Response	Predicted value	Experimental value	Differences (%)
	Temperature (°C)	Liquid/solid ratio(mL g⁻¹)	Methanol conc. (%)	Desirability score				
Mycelial biomass	48.7	21.8	61.2	0.96	TPC	3.79	3.82±0.32	0.79
					DPPH	27.24	26.96±0.85	1.03
Young fruit body	49.1	20.9	72.6	0.93	TPC	5.95	5.87±0.41	1.35
					DPPH	42.51	43.11±2.74	1.41
Over-ripe fruit body	53.4	21.2	60.8	0.98	TPC	4.91	4.89±0.63	0.41
					DPPH	34.87	33.56±1.31	3.76

Legend: conc.: concentration

Antioxidant and antidiabetic capacity

The antioxidant and antidiabetic effects of optimized extracts are given in Table 5. The hierarchy of antioxidant and antidiabetic effects of samples in all analysis methods was following: YFB>OFB>MB. Oxygen radicals are cancer-related molecules that can cause DNA, RNA, and protein damage within the cell. Antioxidants can be protective against cancer because they inhibit these radicals (**Lambert and Elias, 2010**). In this study, DPPH, FRAP, and ABTS were used to evaluate antioxidant capacity because the mechanisms of antioxidants to inhibit radicals are different. DPPH, FRAP and ABTS values of young fruit were 59.9%, 30.9%, and 39.2% higher than mycelia, respectively. But when the fruit body aged, it lost its antioxidant effect by 22.2%, 17.2%, and 10.6%, respectively. Probably the aging process increases oxidative reactions, which speeds up the breakdown of antioxidant components. The results were higher than in the previous study (**Atila et al., 2018**), where the FRAP value ranged from 1.76 to 4.92 µmol TE/g. Moreover, unlike our result, **Wong et al. (2009)** reported that *H.erinaceus* mycelium extract had a higher antioxidant effect than the fruit body. These differences may have resulted from the uniqueness of the extraction process conditions obtained as a result of optimization in our study.

One of the primary treatment approaches for type 2 diabetes is based on the inhibition of enzymes such as α-glucosidase and α-amylase that break down carbohydrates. In this study, the effectiveness of optimized extracts in inhibiting these enzymes was tested. D-threitol, D-arabinitol, palmitic acid, and α-D-glucan are important components that show antidiabetic effects in *H.erinaceus* (**Wang et al., 2005**). The antidiabetic effect of YFB was more than MB, and this effect was found to be low in OFB, probably because the components as mentioned earlier were degraded by aging of the fruit. In a study, the antidiabetic effect of different mushroom varieties was investigated (**Stojkovic et al., 2019**). Accordingly, YFB showed lower α-amylase inhibition than *A.blazei* and *P.linteus*, whereas it showed higher alpha α-glucosidase inhibition than the same mushrooms. In another study, α-amylase and α-glucosidase IC_{50} value of *P.florida* was determined as 35.96±0.35µg/mL and 202.02±3.84µg/mL, respectively (**Prabu and Kumuthakalavalli, 2017**). People with diabetes may take acarbose, an antidiabetic drug, at an initial dose of 3x25mg daily (**Lebovitz, 1995**). With the calculation made according to the inhibition of α-glucosidase by acarbose, it was concluded that YFB powder might be taken at a dose of 3x66.36 g/day (Table 5).

Table 5 Antioxidant and antidiabetic activity of samples

Sample	TPC (mg GAE g⁻¹)	Antioxidant capacity			Antidiabetic activity		
		DPPH (µmol TE g⁻¹)	FRAP (µmol TE g⁻¹)	ABTS (µmol TE g⁻¹)	α-glucosidase IC_{50} (µg mL⁻¹)	α-amylase IC_{50} (µg mL⁻¹)	DDR
Mycelial biomass	3.82±0.32[c]	26.96±0.85[c]	39.27±1.12[c]	45.14±0.64[c]	186.77±1.61[a]	226.61±0.44[a]	3x132.5 g
Young fruit body	5.87±0.41[a]	43.11±2.74[a]	51.39±2.34[a]	62.84±1.59[a]	93.54±2.3[c]	132.25±1.38[c]	3x66.36 g
Over-ripe fruit body	4.89±0.63[b]	33.56±1.31[b]	42.53±0.93[b]	56.17±2.97[b]	137.21±2.15[b]	191.72±0.56[b]	3x97.34 g
Acarbose	-	-	-	-	35.24±1.73[d]	72.96±3.18[d]	3x25 mg

Legend: DDR: Drug Dose Regimen; it expresses mg of *H.erinaceus* powder equivalent to acarbose with 3×25 mg tablets given to a diabetic patient daily and calculated on the basis of α-glucosidase inhibition. Means with different letters in the same column are significantly different ($p<0.05$)

Antimicrobial capacity

Antimicrobial activities of the optimized extracts were evaluated according to the diameter of the inhibition zone, as in Table 6. While MB showed moderate level inhibition against Gram-positive bacteria B. cereus and *S.aureus*, YFB showed moderate level inhibition against *B.cereus* and high-level inhibition against *S.aureus*. All samples showed a low level of inhibition against Gram-negative bacteria *E.coli* and *K.pneumoniae*. Against to *C.albicans*, a pathogenic fungus,

only YFB had an antimicrobial effect, but it was low level. Antimicrobial capacity tests clearly demonstrated that *H.erinaceus* had more inhibitory effects against Gram-positive bacteria than Gram-negative bacteria. This can be explained by the fact that Gram-negative bacteria have an outer membrane that makes them resistant to many antibiotics (**Nikaido and Vaara, 1985**). The results were in agreement with **Wong et al. (2009)** and **D.-M. Kim et al. (2000)**.

Table 6 Antimicrobial activity of samples

Sample	B. cereus	S. aureus	E. coli	K. pneumoniae	C. albicans
Mycelial biomass	++	++	+	+	ND
Young fruit body	++	+++	+	+	+
Over-ripe fruit body	+	+	+	+	ND

Legend: Diameter of inhibition zone: + low level of activity (8 to 15 mm), ++ moderate level of activity (15 to 21 mm), +++ high level of activity (>21 mm), ND not determined

CONCLUSION

RSM was successfully applied in the optimization of extraction conditions that maximize antioxidant capacity and phenolic content in this study. Optimization results showed that the best conditions for the extraction process were at an average of 50.4 °C, 21.3 mL/g liquid to solid ratio, and 64.87% methanol concentration. Results indicated that the optimized extracts also had high levels of antioxidant, antimicrobial, and antidiabetic effects. Also, the harvest time of *H.erinaceus* played an essential role in these effects. Although mycelial biomass bioactivity was less than the fruit body, it cannot be ignored due to its short production time and relatively easy production process.

Optimization of the green-novel extraction processes should be investigated in more detail based on the biofunctional properties of *H.erinaceus* that are very important for human health.

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