Optimization of Extraction of Flavonoid, Total Phenolic, Antioxidant, and Antimicrobial Compounds from *Ganoderma Lucidum* by Maceration Method

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ABSTRACT: *Plants are a rich source of phenolic and flavonoid compounds which are among the most important natural antioxidants. The aim of this study was to optimize the extraction of flavonoid, total phenolic, antioxidant, and antimicrobial compounds from Ganoderma (G) Lucidum by the maceration method. To do so, independent variables including temperature, extraction time, and type of solvent along with Box-Behnken Response Surface Methodology (RSM) were used. The results of single optimization of the independent variables showed the highest flavonoid content (15.19 mg/g) with 100% desirability at an extraction time of 48 h, the temperature of 60 °C by using ethanol solvent. The highest total phenolic content (16.96 mg/g) with 99.96% desirability was observed at an extraction time of 26 h, a temperature of 60 °C by using ethanol solvent. The highest amount of antioxidant compounds (3.03 mg/g) or the lowest IC50 value (mg/mL) with 100% desirability was found at an extraction time of 48 h at a temperature of 60 °C by using ethanol solvent. The results of simultaneous optimization of the extraction conditions by maceration method showed the highest flavonoid content (15.20 mg/g), total phenolic content (16.01 mg/g), and the lowest IC50 (3.03 mg/mL) with 98.157% desirability at extraction time of 48 h, the temperature of 60 °C using ethanol solvent. The highest mean Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of G. lucidum extract obtained by the maceration method were 2500 and 5000 µg/mL, respectively against Clostridium perfringens and Escherichia E. coli. The predicted optimized treatment had superior antimicrobial activity against Staphylococcus aureus with a non-growth halo (Zone of inhibition) diameter of 10.60 mm as compared to C. perfringens and E. coli. The results revealed that the G. lucidum extract obtained by the maceration method could be introduced as an antioxidant and antimicrobial source in marketable food products.*

KEYWORDS: *Flavonoid; Total phenolics; Antioxidant; Ganoderma Lucidum; Maceration method.*

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INTRODUCTION

In recent years, the use of medicinal plants has been paid much attention worldwide due to the adverse effects of chemical medicines. Extraction is the first step in the study of medicinal plants and obtaining extracts from the plants is the starting point for the isolation and purification of chemical components of plants [1]. About 25% of new medicines are made directly or indirectly from plants and their other known components which exert biological activity especially antimicrobial, antifungal, antibacterial, and antioxidant effects [2, 3]. Plants contain a wide range of active compounds such as phenols, alkaloids, tannins, flavonoids, and antimicrobials as well as flavorings. Plant extracts are widely used in the pharmaceutical, food, and cosmetics industries. To produce such invaluable natural compounds and commercialize them, various extraction methods have been studied [4]. The selection of a proper method for extracting bioactive compounds from the plant matrix with minimum impurity as well as preservation of biological activity has been paid attention to in recent years. Traditional extraction with solvent is mainly based on the selection of appropriate solvents, temperature, and time which improve mass transfer. For traditional extraction of bioactive compounds, maceration and floatation methods can be used [5]. Due to the fact that the maceration method provides enough time for diffusion of the solvent into the cells and for antioxidants to leave their place, a higher number of bioactive compounds are extracted from the plant [6]. The maceration method is very simple and cheap and the extraction of phenolic compounds in this method is significant [7].

In the maceration method, the plant is placed in a wide-mouthed container to which the solvent is added and mixed uniformly. It is tightly closed and stored for several hours to several days. Its content is occasionally stirred and the pulp is removed through filtering or squeezing, fresh solvent is passed over the pulp and added to the previously prepared extract. The appropriate extraction temperature is 15-50ºC. The solution then is filtered with a decanter or sieve [8].

Other researchers including *Sardroodian et al*. (2016) [9] extracted the extract from Elaeagnus Angustifolia, *Izadi et al*. (2014) [10] from Echinacea purpurea L., *Dashtimakan et al*. (2018) [11] from Oliveria decumbens by maceration method.

Ganoderma (G) lucidum, belonging to the *Ganodemartaceae* family, is an annual herb known as the best medicinal mushroom because of its diverse therapeutic properties [12]. Numerous reports have been published on the bioactive compounds of this mushroom. The fruiting body, mycelium, and spores of *G. lucidum* contain 400 bioactive compounds with the most important ones being triterpenoids, polysaccharides, nucleotides, sterols, steroids, fatty acids, proteins, peptides, and many scarce substances [13]. Another component of *G. lucidum* is melanin. Melanin has antioxidant activity, boosts the immune system, and has a protective effect against radiation and anti-mutation properties [14]. Total polysaccharides contained in *G. lucidum* include D-B-glucans, heteropolysaccharides, and glycoproteins. The polysaccharides with β-1,3-glucan boost the immune system. They have antioxidant, antibacterial, antiviral, and radiation-protective properties. They stated that D-β- glucans prevented constipation, reduce the risk of colon cancer, produce short-chain fatty acids, lowered blood cholesterol and glucose, lowered insulin thereby controlling diabetes, and reduce the factors affecting the incidence of diseases such as obesity, hyperlipidemia, cardiovascular disease, cancer, and hypertension, boost the immune system, activate leukocytes, exert anti-tumor activity and prevent the spread of the activity of HIV. Chitin and chitosan polysaccharides, found in the cell wall of fungi, have therapeutic properties playing an important role in regulating hepatic, intestinal, and renal functions [15]. Therefore, the extraction efficiency and the antioxidant activity of the extracted bioactive compounds depend not only on the extraction methods but on the temperature, time, and type of solvent [16]. Considering the importance of the extraction method and conditions, the goal of this study was to optimize the extraction of flavonoid, total phenolic, antioxidant, and antimicrobial compounds from *G. lucidum* by maceration method.

EXPERIMENTAL SECTION

Materials

G. lucidum mushroom was purchased from a grocery in Qom, Iran. Then its scientific name was confirmed by the medicinal herb's herbarium of the faculty of pharmacy, at Tehran University. *G. lucidum* belongs to the Ganodermatacen family. Other chemicals including Müller-Hinton broth, ethyl acetate, ethanol, methanol, 0.2 normal Follin-Ciocaltea reagent, sodium carbonate, gallic acid, aluminum chloride, 1 M potassium acetate, quercetin,

2,2 –diphenyl-1-picrylhydrazyl were prepared from Merck company (Germany).

G. lucidum preparation

First, the mushrooms were rinsed and cut into small pieces. Then they were dried in the oven (Model UF55/UN, Mommert company, Germany) at 45 °C for 24 h to reach a constant weight. Next, they were shaped into floury granules by a mill (Model ML- 32o f, Pars khazar company, Iran) and then sieved with mesh 16. The powder was kept in polyethylene containers in a dry place at 25 °C.

Extraction of *G. lucidum* extract by maceration method

To prepare the *G. lucidum* extract, the method of *Ebrahimzadeh et al*. (2008) [17] with some modifications was used. Thus the rates of independent variables were selected based on preliminary studies. The first 20 g of dried *G. lucidum* powder was mixed with each of the solvents presented in Table 1 in a ratio of 1:5 and the extract were obtained under the mentioned extraction conditions. It should be noted that to prevent solvent evaporation, the Erlenmeyer was closed tightly with a polyethylene lid and kept in an orbital shaker (Model WB22-MEMMERT, Germany) at the appropriate extraction temperature and time. The obtained extract then was separated from *G. lucidum* by using Whatman filter paper No.1 and concentrated by a rotary evaporator (Model EKA over 16) at 40°C to reach a Brix value of 60 [17].

Total flavonoid content measurement

The total flavonoid content of *G. lucidum* extract was measured by the colorimetry method. 0.5 mL of the extract was dissolved in 1.5 mL of methanol in a test tube to which 0.1 mL 10% aluminum chloride and 0.1 mL of 1 M potassium acetate were added. Finally, 2.8 mL of distilled water was added and kept at room temperature for 30 min and then the absorbance was read at 415 nm by spectrophotometer (Model L800 Aquatic, Germany). Quercetin (Merck company, Germany) was used as the standard for drawing the calibration curve. The flavonoid content was expressed in mg quercetin equivalent per g of dry sample [18].

Total phenolic content measurement

Total phenolic content was measured by the Follin-Ciocaltue method. 1 mL/mg of each extract was prepared. 0.5 mL of each extract was mixed with 2.5 mL of 0.2 normal Follin-Ciocaltue reagents and stirred for 5 min. Next 2 mL of sodium carbonate at a concentration of 7.5 % w/v were added. The absorbance of samples was read at 760 nm by UV-spectrophotometer (UV2100, US). The results were expressed in gallic acid equivalent values. To do this, the average resulting absorbance was put in the equation of line obtained from the drawn standard gallic acid curve and the result was reported as total phenolic content in mg gallic acid equivalent per gram of the extract. The experiments were conducted in triplicate for each extract and standard [17].

Table 1: Extraction conditions for G. lucidum extract by Box-Behnken RSM.

Treatment	Solvent type*	Time (h)	Temperature (ºC)
1	2	12	20
2	2	12	60
3	2	48	20
4	2	48	60
5	1	30	20
6	1	30	60
7	3	30	20
8	3	30	60
9	1	12	40
10	1	48	40
11	3	12	40
12	3	48	40
13	2	30	40
14	2	30	40
15	2	30	40

1: Ethyl acetate, 2: Ethanol, 3: 50% Ethanol+ 50% Ethyl acetate

DPPH free radical scavenging measurement

The ability of Ganoderma to transfer hydrogen or electron atoms was measured by DPPH ethanolic solution bleaching assay. 2,2-diphenyl-1-picrylhydrazyl is a purple-colored stable free radical which is turned into yellow diphenylpicrylhydrazyl when it is reduced by electron or hydrogen donor elements (antioxidants). To do so, 5 μL of the prepared extract were mixed with 50% ethanol (1.4 mL) and then added to 0.004% DPPH (1 mL)

in ethanol. Next, the samples were shaken for 1 min, kept in the dark for 30 min and their absorbance was read at 517 nm by spectrophotometer (Model L800 Aqualytic, Germany), Distilled water was used as a control. The percentage of free radical scavenging was calculated by using Equation (1) [19].

$$\text{DPPH} = \frac{\text{control absorbance}(\%) - \text{sample absorbance }(\%)}{\text{sample absorbance }(\%)} . 100 \quad (1)$$

To better evaluate the antioxidant activity, IC50 indicates the percentage of the extract which can neutralize 50% of DPPH free radicals was used.

Microbial tests

The predicted optimal treatment was prepared by the maceration method with the highest antioxidant activity and after their antioxidant property was confirmed, their antimicrobial effect was measure by well and disc diffusion methods against *Escherichia coli, Staphylococcus aureus*, and *Clostridium perfringens*. The antimicrobial properties of the extracts were evaluated by 96-well microplates.

First, 95 µL of Müller-Hinton broth and 5 µL of the bacterial suspension corresponding to 0.5 McFarland standard were added to each well of ELISA plate to which 100µL of serial dilutions of the extract was added. The samples were mixed by a shaker (300 rpm, 20 S) and then incubated at 37°C for 24 h. Then the turbidity was read at 540 nm by ELISA reader (Model LX800 Biotech company, USA).

Lack of turbidity determined the Minimum Inhibitory Concentration (MIC). Next, the samples from wells showing no turbidity were sub-cultured on Müller-Hinton agar, and the colony count was performed by serial dilutions. The first tube with a reduction in a bacterial number greater than 0.001 compared to zero time for the control tube was determined as the Minimum Bacterial Concentration (MBC) [20].

Identification of phenolic compounds by High-Performance Liquid Chromatography (HPLC)

Phenolic compounds were isolated and identified using the Mello method [21] by HPLC (Model HPLC 1100, Agilent Company) equipped with a diode array detector at 260 nm. To do this, 100 µL of each solution was injected into the chromatogram device. The chromatographic assay was performed on a Eurospher C18 column (150×4.6mm i.d., 3.5 µm, Waters). The mobile phase consisted of water: citric acid (1:19 V/V) as solvent A and Methanol as solvent B, at a constant flow rate of 1 mL/min. Chromatographic The gradient was started with 30% solvent B and continued with 60% within 45 min, 75% within 85 min, 90% within 95 min, and again 30% within 105 min. The column temperature was kept constant at 30 °C and the chromatogram was processed by Chemstate chromatography software (Agilent company, USA).

Statistical analysis

15 treatments were designed by Box-Behnken Response Surface Methodology (RSM). In order to analyze the data and optimize the extraction conditions for *G. lucidum* extract, Box-Behnken RSM and Minitab 16 software at 95% confidence interval were used.

RESULTS AND DISCUSSION

Analysis of variance for G. lucidum extracted under different conditions

As shown in table 2, the linear and quadratic effects of the temperature, time, and type of solvent as well as the interaction effect of type of solvent × extraction time on the flavonoid content of *G. lucidum* extract were significant (P≤0.05). However, the interaction effect of temperature × time and type of solvent × temperature on the flavonoid content were not significant (P>0.05). Considering the F value, the quadratic effect of the type of solvent had the greatest effect on the flavonoid content followed by the linear effect of the type of solvent.

As shown in Table 2, the linear effects of temperature, time, and type of solvent and the quadratic effect of temperature and type of solvent and the interaction effect of temperature × time on total phenolic content of *G. lucidum* extract were significant (p≤0.05) while the quadratic effect of time and the interaction effects of type of solvent × time and type of solvent × temperature on total phenolic content were not significant (p>0.05). Considering the F value, the quadratic effect of the type of solvent had the greatest effect on total phenolic content followed by the linear effect of the type of solvent.

As shown in Table 2, the linear effect of temperature, time, and type of solvent and the quadratic effect of time and type of solvent as well as the interaction effect of temperature × time on IC50 were significant (p≤0.05). however the quadratic effect of temperature and the interaction effect of type of solvent × time and type

Table 2: Variance analysis of response surface for extraction of G. lucidum extract by maceration method.

Source	Flavnoids (mg/g)		Total phenol compounds (mg/g)		IC50 (mg/mL)	
	P-value	F-value	P-value	F-value	P-value	F-value
Constant	*0.000	1008.74	*0.000	179.22	*0.000	432.56
Linear effects	*0.000	986.41	*0.000	282.55	*0.000	417.49
Temperature (A)	*0.000	398.12	*0.000	243.00	*0.000	119.51
Time (B)	*0.000	251.03	*0.001	58.13	*0.002	37.46
Solvent Type (C)	*0.000	2310.07	*0.000	546.53	*0.000	1095.51
Square effects	*0.000	2030.12	*0.000	238.04	*0.000	875.51
Temperature× Temperature (A^2)	0.007*	19.54	*0.003	30.72	0.322	1.21
Time × Time (B^2)	*0.000	166.93	0.141	3.06	0.001*	54.12
Solvent × Solvent (C^2)	*0.000	5736.84	*0.000	701.48	*0.000	2483.30
interaction	0.016*	9.68	0.005*	17.06	0.065	4.67
Temperature × Time (A×B)	0.877	0.03	*0.001	45.02	0.020*	11.33
Temperature× Solvent (A × C)	0.467	0.62	0.206	2.11	0.598	0.32
Time× Solvent (B × C)	0.003*	28.40	0.101	4.04	0.185	2.36
Lack of fit	0.086	10.84	0.058	25.26	0.059	51.89
Total	-	-	-	-	-	-

of solvent × temperature on IC50 was not significant ($p>0.05$). Considering the F value, the quadratic effect of the type of solvent had the greatest effect on IC50 (mg/mL) followed by the linear effect of the type of solvent.

Polynomial model for G. lucidum extract obtained under different conditions

The polynomial model for predicted flavonoid content is presented in Table 3. The coefficient of determination (R^2) and the adjusted coefficient of determination (R^2-adj) was 99.94% and 99.85%, respectively indicating the good fit of the model to the experimental data.

The polynomial model for predicted total phenolic content is presented in Table 3. R^2 and R^2-adj values were 99.69% and 99.13%, respectively showing the good fit of the model to the experimental data.

The polynomial model for the predicted IC50 value is presented in Table 3. As shown in Table, R^2, and R^2 -adj values were 99.87% and 99.64%, respectively showing the good fit of the model to the experimental data.

Flavonoid content of G. lucidum extract obtained by maceration method

Flavonoids are a group of compounds found abundantly in nature. So far more than 9000 flavonoids have been reported. Flavonoids are a class of low-molecular-weight compounds based on a 2-phenyl-chromone nucleus. Most importantly flavonoids are characterized by a C2=C3 bond which affects their functional properties and degree of oxidation [22].

The flavonoid content of *G. lucidum* extract obtained by the maceration method under different conditions by the test method and the predicted values are reported in Table 4. Different extraction conditions (temperature, time, and type of solvent) had a great effect on the flavonoid content of *G. lucidum* extract obtained by maceration as the flavonoid content ranged from 5.085 mg/100g to 15.127 mg/100g. The highest flavonoid content (15.127 mg/100g) was obtained at 60 °C, extraction time of 48 h using ethanol solvent, and the lowest content was observed at 20 °C, extraction time of 30 h using ethyl acetate solvent. As the extraction time increased from 12 h to 48 h and the temperature increased

Table 3: Polynomial model for predicting flavonoid content, total phenolic content, and IC50 of G. lucidum extract obtained by maceration method.

Source	Model	R^2	R^2-adj
Flavonoids (mg/g)	Y= 13.1159 + 0.8620 A + 0.6845 B + 2.0764C – 0.2811 A^2 +0.8216 B^2 -4.8164 C^2 - 0.0100 AB -0.0481AC+ 0.3256BC	99.94	99.85
Fenolic compounds (mg/g)	Y== 15.5878 + 2.2309 A + 1.0911 B + 3.3456 C -1.1675A^2 -0.3685B^2 -5.5792C^2 - 1.3579AB + 0.2941AC+ 0.4069BC	99.69	99.13
IC50 (mg/mL)	Y= 4.59030 - 0.71009 A -0.39755 B – 2.14990 C- 0.10502 A^2- 0.70334 B^2+ 4.76455C^2 + 0.30918 AB + 0.05165 AC+ 0.14125BC	99.87	99.64

from 20°C to 60°C by using ethanol solvent the flavonoid content increased. Given the polarity of flavonoids, using polar solvents increased their solubility and the rate of extraction. *Liazid et al*. (2011) [23] showed that using 50% ethanol and 40% methanol had the greatest effect on the extraction of phenolic compounds from the grape skin. The phenolic content in the solvent increased with increasing the concentration gradient between the solid matter and solvent. It was demonstrated that the extraction temperature had the greatest effect on the changes in flavonoids followed by the extraction time; As they increased the flavonoid content showed an increase. It should be noted that factors such as particle size, physical properties of the materials, time of maceration insolvent, environment and solvent temperature, the ratio of solvent to the materials as well as the extraction method affect the phenolic content [24]. Different amounts of phenolic compounds are extracted with different solvents because they may be soluble or insoluble due to their polarity or non-polarity in different solvents [25]. *Satarelli et al*. (2018) [26] investigated the phytochemical compounds and antioxidant properties of *G. lucidum.* To do so, they extracted Ganoderma mycelium extract with 80% ethanol at 4°C. The results showed that tetracycline and terpenoid structures were observed more than other structures and about 17.17 mg/g of phenolic compounds including flavonoid and tannins were observed which indicated a great antioxidant activity. They also used ethanol solvent which is consistent with the results of the present study. *Toprakçı et al.,* 2021 [27] Extracted carotenoids from orange peel and reported particle size, extraction time, and solvent type were effective factors for extracting carotenoid compounds, respectively.

Phenolic content of G. lucidum extract obtained by maceration method

Phenols are compounds characterized by one or more hydroxyl groups (-OH) bonded to an aromatic (nonpolar) ring and are distinguished by their polarity. So, the solubility of phenols in the solvent can be explained by their spatial structure (polar or nonpolar) and the intermolecular force (hydrogen bond) between them and the solvent [28]. The total phenolic content of *G. lucidum* extract obtained by the maceration method under different conditions by the test method as well as the predicted values are shown in Table 4.

As shown in the Table, different extraction conditions (temperature, time, and type of solvent) had a significant effect on total phenolic content as it ranged from 3.518 mg/100g to 16.733 mg/100g. The highest phenolic content (16.733 mg/100g) was observed at 60°C, extraction time of 12 h by using ethanol solvent, and the lowest phenolic content (3.518 mg/100g) was found at 20 °C, extraction time of 30 h by use of ethyl acetate solvent. The type of solvent had the greatest effect on the extraction of phenols from *G. lucidum* followed by the extraction temperature. The increasing trend in the early steps of extraction was due to the high gradient of antioxidants between the solvent and the cell wall which facilitates the extraction. At longer extraction times, the rate of extraction decreases due to the presence of bioactive compounds in the less accessible parts of the plant cells and decreased concentration gradient of such matters. Some previous studies have reported this initial increasing trend followed by a decrease in the antioxidant activity of the plant extracts [29]. Increased temperature caused by physical and chemical stresses led to an increase in the dissolution coefficient of phenolic compounds [30]. A higher amount of phenolic compounds are extracted by ethanol due to its polarity compared to ethyl acetate which is a nonpolar solvent. *Veljovic et al*. (2017) [31] extracted the Ganoderma purpurea extract by maceration method with ethanol, identified its phenolic compounds, and investigated its anti-inflammatory properties. The results of chromatography revealed that farnesyl, isolated from the extract, could reduce inflammation. *Akinyele et al*. (2011) [8]

Table 4: Flavonoid content, total phenolic content, and IC50 of G. lucidum extract obtained by maceration method under different conditions by test method and predicted values.

Treatment	Flavonoids (mg/g)		Total phenol (mg/g)		IC50 (mg/mL)	
	Tested	Predicted	Tested	Predicted	Tested	Predicted
1	12.66	12.100	9.153	9.372	5.031	5.199
2	13.949	13.844	16.733	16.550	3.052	3.160
3	13.383	13.844	14.086	14.270	3.893	3.785
4	15.127	15.193	16.235	16.016	3.151	2.983
5	5.085	5.032	3.518	3.559	12.221	12.161
6	6.865	6.852	6.988	7.432	10.638	10.638
7	9.268	9.281	10.106	9.662	7.758	7.758
8	10.865	10.909	14.753	14.712	11.448	6.441
9	6.567	6.686	5.871	5.610	10.095	11.340
10	7.456	7.404	7.204	6.979	6.926	10.263
11	10.134	10.187	11.263	11.488	6.138	6.758
12	12.326	12.207	14.224	14.484	4.556	6.245
13	13.079	13.116	15.675	15.588	4.590	4.590
14	13.168	13.116	15.675	15.588	4.590	4.590
15	13.101	13.116	15.675`	15.588	4.590	4.590

extracted the *G. lucidum* extract by screening method. They studied the effect of different solvents [ethanol, methanol, methanol- ethanol (50:50), water, ethyl acetate, and n- hexane] on the extraction yield and showed that the extraction yield of active phytochemical compounds ranged from 0.90% to 8.010%. Methanol-ethanol mix resulted in the highest extraction yield. *Muniz marquez et al*., 2013 [21] Effects of several experimental factors, such as sonication time, solid/liquid ratio and concentration of solvent on the extraction of phenolic compounds were evaluated through a randomized complete block design with factorial treatment arrangement. The best extraction conditions were: 1 g plant sample with 12 mL of 35% ethanol, for 40 min, obtaining a yield of phenolic compounds of 17.32 ± 1.52 mg/g of plant. The extract of phenolic compounds from grapefruit leaves was assisted by ultrasound-assisted and they reported total phenolics content was found to be 19.04 mg gallic acid equivalents/ g dried leaf [32].

IC50 of G. lucidum extract obtained by maceration method

The process of extracting phenolic compounds is an important factor in determining the antioxidant properties of the extract. IC50 value in DPPH scavenging assay refers to the concentration of antioxidant extract which can scavenge 50% of the primary free radicals. IC50 of the *G. lucidum* extract obtained by maceration method under different conditions by the test method and the predicted values are presented in table 4. Different extraction conditions (temperature, time, and type of solvent) had a significant effect on IC50 of the *G. lucidum* extract obtained by the maceration method as the IC50 value ranged from 3.052 mg/mL to 12.221 mg/mL. The lowest IC50 (3.052 mg/mL) or the highest rate of free radical scavenging was found at 60°C, extraction time of 12 h by using ethanol solvent, and the highest IC50 (12.221 mg/mL) or the lowest rate of free radical scavenging was observed at 20 °C, extraction time of 30 h by using ethyl acetate. As shown in Table 4, the type of solvent had the greatest effect on the antioxidant activity followed by temperature and time. When using the maceration method, polar ethanol solvent has the greatest effect due to its high efficiency in extracting flavonoid and phenolic compounds. In some previous studies a good linear relationship between antioxidant activity and total

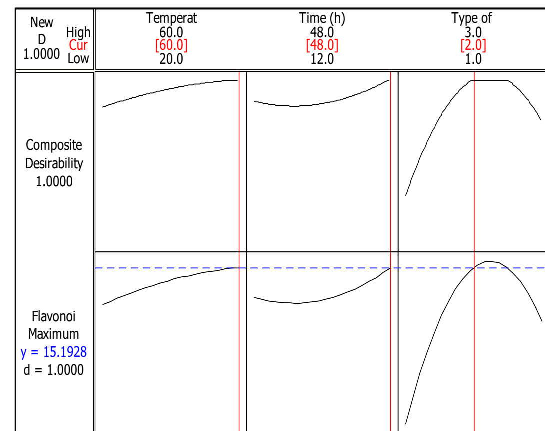
phenolic content was reported [33]. *Saltarelli et al.* (2009) [26] reported that the antioxidant compounds contained in *G. lucidum* were effective in preventing and controlling many diseases due to the presence of polyphenols and their antioxidant property was caused by the reducing activity and scavenging of oxygen radicals. They investigated the ethanolic extract of *G. lucidum* and showed that it had low-molecular-weight compounds with significant antioxidant activity which is in agreement with the results obtained in the present study. *Ozbek et al.,* 2018 [34] studied the phenolic and antioxidant compounds of Pistachio (Pistacia vera L.) and The obtained results indicated relationships between the tested parameters, i.e. ethanol concentration and extraction yield. The maximum yield was obtained with 50% ethanol (32.9 g dry extract/100 g dry matter). The total phenolic content of the extracts was found in the range of 21.3–39.3 mg/g extract as gallic acid equivalent. Antioxidant activity was determined by using three different tests. Using the DPPH test, the best antioxidant activity with the lowest IC_{50} value (0.70 mg/mL) was obtained for 40% ethanol, and the least antioxidant activity was obtained for 100% ethanol with the highest IC50 value of 2.73 mg/mL.

Single optimization of extraction conditions for G. lucidum extract by maceration method

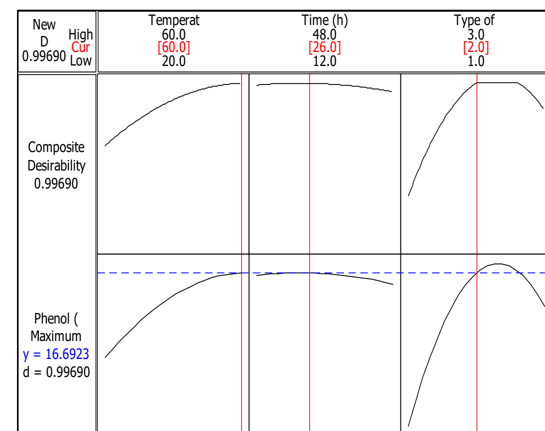
Fig. 1 (a) shows the optimal conditions for containing the maximum amount of flavonoid from *G. lucidum* extract by the maceration method. The maximum amount of flavonoid was predicted to be 15.1928 mg/g with 100% desirability at 60 °C, an extraction time of 48 h by using ethanol solvent. A flavonoid content 15.127 mg/100g was obtained under optimal laboratory conditions which showed no significant (P>0.05) difference from the flavonoid content predicted by RSM.

Fig. 1 (b) shows the optimal conditions for obtaining the maximum total phenolic content from *G. lucidum* extract by the maceration method. The maximum phenolic content was predicted to be 16.6923 mg/g with 99.69% at 60 °C, extraction time of 26 h by using ethanol solvent. Total phenolic content of 16.597 mg/100g was obtained under optimal laboratory conditions which showed no significant (P>0.05) difference from the total phenolic content predicted by RSM.

Fig. 1 (c) shows the optimal extraction conditions for obtaining the maximum amount of antioxidant compounds



(a)



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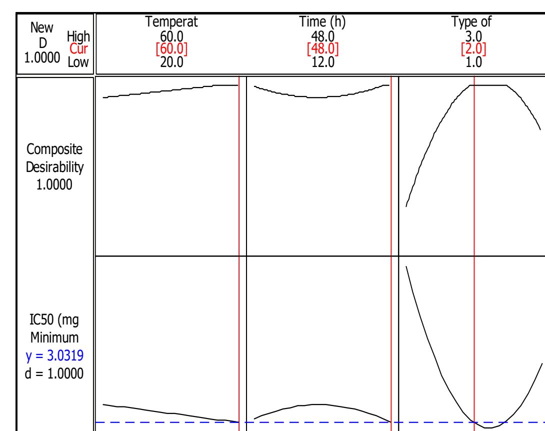

(c)

Fig. 1: Diagram of single optimization of (a) flavonoids, (b) total phenolic content, (c) IC50 of G. lucidum extract by maceration method under different conditions.

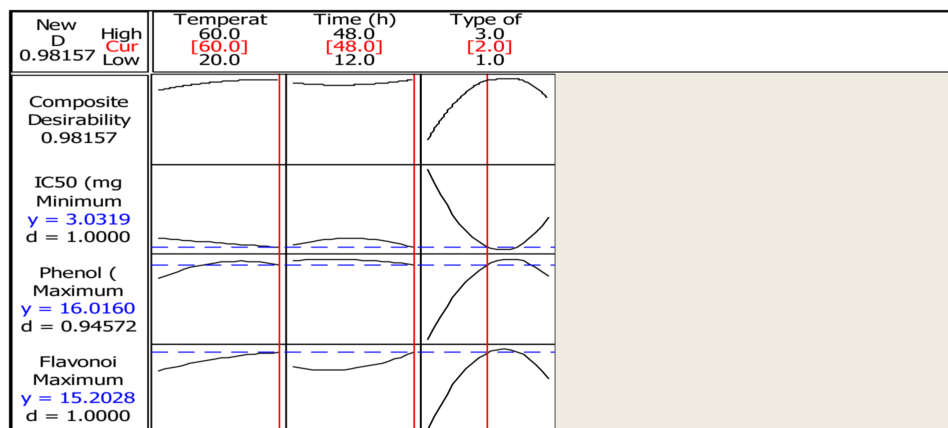

Fig. 2: Simultaneous optimization of flavonoids, total phenolic content, and IC50 of G. lucidum extract by maceration method under different conditions.

(the lowest IC50) from *G. lucidum* extract by the maceration method. The lowest IC50 with 100% desirability was predicted to be 3.0319mg/mL at 60 °C, extraction time of 48 h by using ethanol solvent. The IC50 value of 3.027 mg/mL was obtained under optimal laboratory conditions which showed no significant (P>0.05) difference from IC50 predicted by RMS.

Simultaneous optimization of extraction conditions for G. lucidum extract by maceration method

The simultaneous optimization of extraction conditions for *G. lucidum* extract by maceration method in order to obtain the maximum amount of flavonoid and total phenols and the lowest IC50 with 98.157% desirability at 60 °C, extraction time of 48 h by using ethanol solvent was predicted (Fig. 2). The predicted values of flavonoid, total phenols and IC50 were 15-2028 mg/g, 16.0160mg/g and 3.0319 mg/mL, respectively. The IC50 value of 3.305mg/mL, total phenolic content of 16.6923 mg/g, and flavonoid content of 15.1928 mg/g were obtained under optimal laboratory conditions which showed no significant (P>0.05) difference from the values predicted by RSM.

Antimicrobial effects of G. lucidum extract obtained by maceration method

Simultaneously optimized conditions for obtaining the maximum amount of flavonoid and antioxidant compounds from *G. lucidum* extract were selected and the antimicrobial effects of the optimized treatment were examined by disc and well diffusion methods against *S. aureus*, *E. coli*, and *C. perfringens* (Fig. 3). Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for the predicted optimal treatment against *S. aureus*, *E. coli*, and *C. perfringens* are presented in Table 5. The results showed that there was no significant (P>0.05) difference between MIC and MBC of the *G. lucidum* extract obtained by maceration method against *C. perfringens* and *E. coli,* however, a significant (P≤0.05) difference was found between MCB of the *G. lucidum* extract against *C. perfringens*, *E. coli,* and *S. aureus*. The lowest mean MIC and MBC were 1666 and 2500 µg/mL, respectively against *S. aureus* and the highest mean MIC and MBC were 2500 and 5000 µg/mL, respectively against *C. perfringens* and *E. coli*. Finally, the predicted optimal treatment had a superior antimicrobial effect on *S. aureus* with the largest non-growth halo (zone of inhibition) (10.66 mm) compared to *C. perfringens* and *E. coli*.

The Hydroxyl group in phenolic compounds binds to the active part of enzymes and prevents their metabolism. As the first para-cement swells the cell membrane of the microorganism which facilitates the entry of more carvacrol into the cell and finally, carvacrol exerts its bactericidal effect on the microorganisms and kills them [35]. By another mechanism, carvacrol binds to the cell membrane which reduces the selective permeability and increases the membrane permeability and causes the destruction of microbial cells [35].

Mohammadi et al. (2015) [36] investigated the antimicrobial effects of phenolic compounds of Scutellaria pinnatifida extract and showed that the extract could inhibit the important pathogenic bacteria as *S. aureus* with the largest non-growth halo (zone of inhibition) showed to be the most sensitive bacteria when assessed by disc and

Table 5: Mean MBC and MIC of G. lucidum extract obtained by maceration method against S. aureus, E. coli, and C. perfringens Similar small letters in each column represent no significant difference (P>0.05).

Microorganism	Well (mm)	Disk (mm)	MIC µg/mL	MBC µg/ml
Staphylococcus aureus	15.66±0.577^{c}	10.66±0.577c	1666±721.68^{b}	2500±0.00b
E. coli	13.66±0.577^{b}	5.33±0.577^{b}	2500±0.00^{a}	5000±0.00^{a}
Clostridium perfringens	12.66±0.577^{a}	5.00±0.577^{a}	2500±0.00^{a}	5000±0.00^{a}

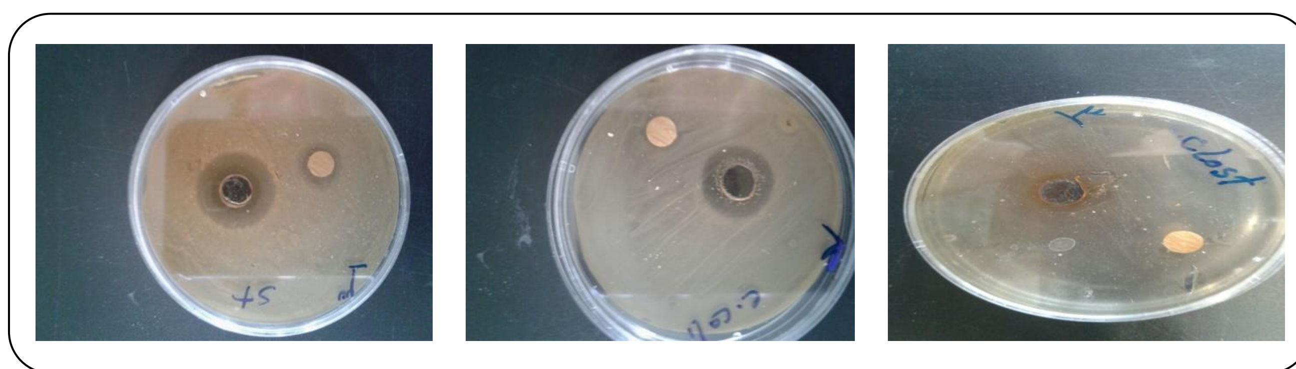

Fig. 3: Non-growth halo diameter cleated by G. lucidum extract against (a) S. aureus, (b) E.coli, (c) C.perfringens.

well diffusion methods, whereas it had no significant effect on gram-negative bacteria and fungi. *Shanmugapriya et al*. (2012) [37] studied the antioxidant and antimicrobial effect and chemical compounds of black pepper and showed that the phenolic compounds in black pepper could exert inhibitory effects on important pathogenic bacteria, yeasts, and some fungi strains. In research, the maceration extraction method with ethanol and water was used to obtain S. schtschegleevii extract, and extraction yield, total phenol content, and antioxidant and antimicrobial activity of the obtained extracts were evaluated. The extraction yield of S. schtschegleevii ethanolic extract was higher than that of aqueous extract (8. 8± 0. 27 vs. 6. 9± 0. 33%), and its total phenol content was also higher compared to the aqueous one (55. 35± 0. 28 vs. 49. 4± 0. 62 mg gallic acid/g dried extract). Antioxidant activity based on IC50 showed that the ethanolic extract, due to its higher total phenol content, has the ability to deactivate and neutralize free radicals more efficiently in comparison to the aqueous extract. Antimicrobial results (disk diffusion agar, minimum inhibitory concentration, and minimum bactericidal concentration) indicated that bacteria Staphylococcus aureus, Listeria innocua, Escherichia coliand Pseudomonas aeruginosa were more sensitive to the ethanolic extract, and at the same concentration of ethanolic and aqueous extracts, gram-positive bacteria (Staphylococcus aureus and Listeria innocua) had higher sensitivity than the gram-negative ones (Escherichia coli and Pseudomonas aeruginosa) [38].

Identification of phenolic acids of G. lucidum extract

The type and number of phenolic compounds of the *G. lucidum* extract are presented in Table 6 and Fig. 4. Gallic acid (189.45 µg/mL) and chlorogenic acid (158.1 µg/mL) were the most abundant phenols in the *G. lucidum* extract.

Phenolic acids are hydroxylated derivatives of benzoic acid and cynarine acid. Hydroxycinnamic acid is abundantly found in most living organisms. The most common phenolic acids are chlorogenic, caffeic, paracoumararic, and ferulic acids. Many studies have demonstrated the link between the healthful properties of phenolic acids and their antioxidant activity [39]. Hydroxycinnamic acids have greater antioxidant activity than hydroxyl benzoic acid due to their propanoic side chain instead of a carboxyl group [40]. Researchers have shown that derivatives of hydroxycinnamic acids such as caffeic and ferulic acids had effective radical scavenging activity [41, 16]. Flavonoids are the most effective antioxidant among the phenolic compounds. They are potent scavengers of hydroxyl and peroxide radicals and their effect on superoxide radicals has not yet been determined [42]. Quercetin is a flavonol and is among

Table 6: Phenolic compounds of G. lucidum extract.

Peak No.	Identified phenolic compound	Inhibition time (min)	Area peak (%)	Recovery percentage	Concentration (µg/mL)
1	Quercetin	3.55	7.75	99	29.52
2	Cynarine	6.44	6.22	99	28.10
3	Benzoic acid	6.81	8.151	96	44.63
4	Quercetin-3-methyl-ether	7.59	17.71	98	98.27
5	Chlorogenic acid	7.8	27.73	99	158.1
6	Luteolin	8.04	5.77	94	26.67
7	Gallic acid	8.13	41.24	101	189.45
8	Caffeic acid	10.86	9.44	96	33.24
9	Rutin	10.88	6.89	98	27.23

the most powerful natural antioxidants. There is a direct relationship between the increased number of hydroxyl groups and the antioxidant power of flavonoid compounds [43]. Rutin, quercetin, and luteolin have therapeutic, antibacterial, and antioxidant properties as luteolin can reduce cholesterol synthesis by indirectly interfering with cholesterol synthesis and inhibiting hepatic enzyme hydroxyl methylglutrayl CoA reductase. Cynarine is also a derivative of dicaffeil quinic with a high level of antioxidant and anti-cholesterol activity [37]. Antioxidant effects of quercetin are the result of chelating metals, scavenging radicals, and stimulating the expression of protective enzymes [41, 44]. Quercetin is a plant pigment (flavonoid) with potent antioxidant properties. Quercetin-3-methyl ether is one of those flavonoids and shares a similar structure with quercetin.

Rutin is a natural pigment and a food stabilizer and preservative used in different industries. It has various biological activities including antioxidant, anti-inflammatory, and hepatic protective effects [43]. Therefore, in order to demonstrate its medicinal, nutritional, and antioxidant properties, it is very important to measure these compounds in *G. lucidum* extract. The chromatogram of analyzed phenolic compounds is presented in Fig. 4.

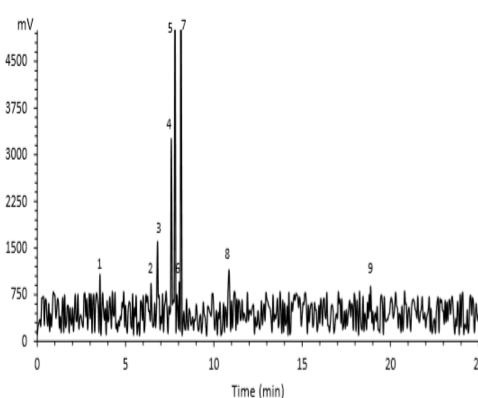

Fig. 4: Chromatogram of identified phenolic compounds.

Peaks 1-9 are presented in Table 6.

Zengin et al. (2015) [45] investigated the phenolic compounds in G. applanatum and *G. resinceua* and found protocatechuic acid, catechin, chlorogenic acid, and ferulic acid in the methanolic extract of both Ganoderma species. *Heleno et al*. (2012) [46] studied the phenolic compounds in *G. lucidum* and showed that the Ganoderma extract contained P-hydroxybenzoic acid and syringic acid. *Veljovic et al*., (2017) [31] reported that the most predominant phenolic compounds in *G. lucidum* were gallic acid, trans-cinnamic acid, quercetin, kaemferol, hesperidin, and naringin. *Dong et al.,* (2019) [47] also examined the phenolic compounds of G.lucidum and identified kaempferol, cinnamic acid, quercetin, coumaric acid, and rutin.

Costa et al., (2018) [48] identified the main phenolic compounds caffeic acid, syringic acid, vanillin, p-coumaric acid, salicylic acid, and ferulic acid. Similarly, *Si et al*., (2018) [49] found a considerable amount of uric acid in the *G. lucidum* extract in addition to mannose, rhamnose, and glucose which plays a role in chelating metals and scavenging free radicals thereby reducing the oxidation.

CONCLUSIONS

The objective of this study was to optimize the extraction conditions for *Ganoderma lucidum* extract

by maceration method at different extraction temperatures, times, and by using different solvents and to evaluate flavonoid and total phenolic compounds as well as their antimicrobial properties. The results showed that increasing the extraction time and temperature and using ethanol solvent had a significant effect on increased flavonoid and total phenolic compounds and reduced IC50 value (mg/mL) as the highest flavonoid content (15.2028mg/g), the highest total phenolic content (16.6743mg/g) and lowest IC50 value (2.9835) were found at 60ºc, extraction time of 48 h and by using ethanol solvent. Optimization of the extraction conditions for *G. lucidum* extract by Box-Behnken response surface methodology could increase the extraction yield of bioactive compounds such as flavonoids, phenols, and antioxidants. So maceration can be a suitable method for extracting phenolic compounds from *G. lucidum* mushroom. By optimizing the extraction conditions, more antioxidant and antimicrobial compounds can be extracted from *Ganoderma lucidum*.

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