Optimization of extraction conditions of *Ganoderma applanatum* extract by ultrasound technique and evaluation of its antioxidant and antibacterial properties

Najmeh Khademipour[1], Sima Sharei [2*], Abdereza Aghajani

1- Ph.D. Student, Department of Food Biotechnology, Science and Research University, Tehran, Iran
2- M.S Student Department of Food science and technology, Science and Research University, Ahwaz, Iran
3- Assistant Professor, Department of Food science and technology, Science and research University, Qazvin, Iran

*Corresponding author (khademinajme@gmail.com)
Tel: +989139139388597
Tel.:02144866165
Fax: 02144866165
E-mail: khademinajme@gmail.com

Abstract

In the present study, the extract of *Ganoderma applanatum* was optimized using ultrasound and response surface method. For this purpose, the time range of 6 to 12 minutes, ultrasound power (150 to 250 W), and solvent types (ethanol, ether, and a mixture of ethanol and ether) were evaluated as independent parameters on total phenol total flavonoid and radical scavenging capacity. Analysis of variance showed that process time, ultrasound power, and solvent type significantly affected test responses ($p \leq 0.05$). Optimization results showed that if using ethanol and 250 W ultrasound power for 6 minutes, the highest phenolic and flavonoid amounts and the

lowest Ic50 can be achieved with a utility coefficient of 0.875. Examination of the optimal extract sample's antimicrobial properties also showed that MIC and MBC were 1250 and 2080 µg/mL for *Pseudomonas aeruginosa* and 2080 and 4150 µg/mL for *Staphylococcus aureus*, respectively. According to the HPLC results, chlorogenic acid and caffeic acid were the most phenolic compounds in extract. This study showed that using the ultrasound process with the specified conditions is a suitable tool for extracting *Ganoderma applanatum* extract with the highest bioactive compounds, which can be used due to its antioxidant activity food, pharmaceutical, and cosmetics industries.

Keywords: *Ganoderma applanatum*, Ultrasound, Optimization, Modeling, Response surface methodology.

1. Introduction

Natural biological products are secondary metabolites derived from microorganisms. These compounds can be complex mixtures extracted from raw materials or individual compounds(Lyu et al., 2020). Ganoderma is a well-known medicinal fungus that has been widely used in China, Japan, and Korea for the last two thousand years. Anti-tumor, anti-inflammatory, anti-viral, antibacterial, anti-parasitic, and immune-boosting properties have been attributed to this fungus (Mohammadifar et al., 2011).

There have also been many reports on this fungus's anti-obesity effects (Chang et al., 2015; Diling et al., 2020; Peng et al., 2019). Triterpenoids have been identified as bioactive components in Ganoderma (Zhu et al., 2018). *Ganoderma applanatum* belongs to the Polyporaceae family of basidiomycetes. This fungus is one of the oyster fungi with a diverse distribution (Mohammadifar et al., 2011). The history of *G. applanatum* therapeutic applications goes back thousands of years to the East Asian civilization. Studies have shown that an extract or drink prepared from *G.*

applanatum can cure rheumatism, and this fungus can also help relieve pain and prolong life (Peng et al., 2019; Zheng et al., 2020).

In recent years, extensive research has been done on extracting plant extracts, microorganisms, and algae. Ultrasound waves are effective methods in improving the extraction process of natural compounds such as antioxidant compounds (Haydari-Majd et al., 2012). Ultrasound waves lead to swelling, porosity in cell walls, better solvent uptake and excretion of compounds from the tissue into the solvent, and accelerate mass transfer (González-De-Peredo et al., 2020).

The extraction efficiency of phenolic compounds depends on some factors such as time, temperature, particle size, sample matrix porosity, solvent type, solvent concentration, pH, sample to solvent ratio, and solvent diffusion coefficient in the sample (Goltz et al., 2018; Irakli et al., 2018; Wang et al., 2008). Barranco et al. (2010) reported that *Ganoderma applanatum* is considered a potential source of non-toxic compounds with antioxidant, antimicrobial, and immune-modulating properties (Barranco et al., 2010). Nagaraj et al. (2013) also showed that this fungus is a rich source of chemical compounds such as saponins, phenols, steroids, glycosides, terpenoids, and flavonoids(Nagaraj et al., 2014). Oviasogie et al. (2015) investigated the antimicrobial properties of *Ganoderma applanatum* in Nigeria (Oviasogie et al., 2015). Mohammadifar et al. (2011) compared antioxidant activity and bioactive substances in *Ganoderma applanatum* and *Ganoderma lucidium* in Iran. Due to this fungus' importance, this study aimed to optimize the extraction conditions of *Ganoderma applanatum* extract by ultrasound technique and evaluate its antioxidant properties (Mohammadifar et al., 2011).

2. Materials and Methods

2-1. Preparation of *Ganoderma applanatum* extract

Ganoderma applanatum was obtained from a research farm in northern Iran (Gorgan: geographical location). They were dried in the open air and then pulverized and stored in the freezer at -20 ° C. The effect of three independent variables, solvent type (ethanol, ethyl ether, and ethanol and ethyl ether mixture), time (6 to 12 minutes), and ultrasonic power (150 to 250 Ws), were studied on yield and bioactive compounds contents.

Watman filtered the extracts No. 1 filter paper and separated them from *Ganoderma applantum* mas. The extracted filter was concentrated by rotary evaporator at 50 ° C to 60 ° C. Finally, it was kept in an oven at 40 ° C until completely dry (Mohammadifar et al., 2011).

2-2. Measurement of total phenol content

The amount of total phenol in the mushroom extract was measured by the colorimetric method based on Folin-Ciocalteu's reagent. In this method, about 0.5 ml of ethanolic extract was mixed with 2.5 ml of Folin-Ciocalteu's reagent (1: 10% diluted), and then 2 ml of sodium carbonate solution (7.5% v/v) was added. The samples' absorbance was measured after 30 minutes of incubation at 90 ° C with a spectrophotometer at 765 nm (Salamah et al., 2017).

2-3. Measuring the content of total flavonoid compounds

The total amount of flavonoid compounds was determined according to Mohammad and Sanbol's method (2010). Initially, about 0.5 ml of ethanolic extract was mixed with 2 ml of distilled water. Then 0.15 ml of sodium nitrite solution (15%) was added to it. After 6 minutes, 0.15 ml of aluminum chlorate solution (10%) was added and allowed to stand for 6 minutes. Finally, 2 ml of sodium hydroxide solution (4%) was mixed with the resulting solution, and the final solution was reached to 5 ml using water. The resulting solution was then thoroughly mixed and allowed to stand for 15 minutes. Finally, the absorbance of the mixture was read by a spectrophotometer at

510 nm. Quercetin (manufactured by Merck) was used as the standard to draw the calibration curve, and the amounts of total flavonoids were calculated based on the amount of mg equivalent to quercetin (Esmaeili & Sonboli, 2010).

2-4. Measurement of free radical scavenging activity

The antioxidant activity of the extract obtained from *Ganoderma applanatum* by 1 and 2 diphenyl 2-picryl hydrazyl solution was measured according to Hayati et al., (2021) method. Absorption of the control sample was performed using a spectrophotometer at a wavelength of 517 nm. BHT was used as a positive control. 50 µl of the studied concentration of the extract in ethanol was added to 5 ml of 0.004% DPPH solution in ethanol (Hayati et al., 2021).

After 30 minutes of incubation at room temperature, the samples were read at 517 nm against the control sample, and the percentage of free radical scavenging was calculated using Equation 1.

$$\text{Inhibitory DPPH (\%)} = ((\text{A-B})/\text{A}) \times 100$$

The radical scavenging activity of *Ganoderma applanatum* extracts were interpreted via IC50 value. The IC50 is a concentration that can scavenge the 50% of DPPH free radical.

2-5. identification of phenolic acid compounds by high-performance liquid chromatography (HPLC)

Isolation and identification of phenolic compounds of *Ganoderma applanatum* extract according to Mello method and using high-performance liquid chromatography device, made by Agilent company, HPLC 1100 model from the USA. For this purpose, 300 µL of each solution was injected into the chromatogram. The mobile phase consisted of water: citric acid in a ratio of 1: 19 v/v as solvent A and methanol as solvent B at a constant flow rate of 1 mL/1. The gradient started with 30% solvent B and continued with 60% in 45 min, 75% in 85 min, 90% in 95 min, and a return to

30% in 105 min. The column's temperature was kept constant at 30 ° C, and the chromatogram was processed using Chemstate computer chromatography software from Agilent, USA (Mello et al., 2010).

3. Results

Table 1 lists the variables used in coded form, including time (hour), ultrasound power (power), and solvent type (ethanol, ether, and a mixture of ethanol and ether). The experiments determined using the response surface method and the D-Optimal design by the Design-Expert software version 12, presented in Table 2, also state the values obtained for each response.

Table1.

Table 2.

According to the results presented in Table 3, it was found that extraction time, ultrasound power, and solvent type had a significant effect on the total phenol content of the mushroom extract ($p \leq 0.05$).

Table 3.

3-1. Evaluation total phenol

As shown in Figure 1, with increasing extraction time, the amount of total phenol in the extract decreased for up to 9 minutes and then increased ($p \leq 0.05$). As the ultrasound's power increased from 150 W to 200 W, the phenolic compounds' content decreased and increased again to a power of 250 W ($p \leq 0.05$). The highest amount of extraction of total phenolic compounds was related to extraction with ethanol solvent, followed by ethanol and ether mixture and finally ether solvents.

The Lack of Fit calculated for the model predicted total phenolic compounds was non-significant, indicating the model's high efficiency.

FIG.1.

The presented equation for predicting total phenol is the quadratic equation expressed with the fitting coefficients in Table 4. Based on the results, R^2 and R^2 Adj's values were equal to 1, indicating the model's high adequacy. The interaction of extraction time variables and ultrasound power for each solvent is also shown in Figure 2.

FIG.2.

Figure 2 also shows that the highest extraction rate of phenolic compounds was related to the extracted samples with ethanol as a solvent and at the highest process power (250 W) and process time (12 minutes).

Table 4.

According to Figure 3, the proposed model was affected by three factors, process time, ultrasound power, and solvent type ($p \leq 0.05$).

3-2. Evaluation of flavonoid compounds

According to Figure 1 (b), the amounts of flavonoid compounds increased with increasing process time and ultrasound power ($p \leq 0.05$). The highest extraction of flavonoid compounds was related to ethanol solvent. The deliberate lack of fit was non-significant, which confirms the efficiency of the model. According to Figure 3, the highest extraction of flavonoid compounds was related to

the extracted samples with ethanol solvent, followed by ethanol and petroleum ether and petroleum ether solvent.

FIG.3.

The appropriate model for predicting the amounts of flavonoids in Ganoderma extract was linear. Furthermore, fitting coefficients showed that the expressed model was sufficient to predict flavonoid values.

3-3. Evaluation of IC50

According to Table 3 and Figure 1 (c), the Ic50 of Ganoderma extract samples decreased significantly with increasing extraction time ($p \leq 0.05$). With increasing ultrasound power, the value of Ic50 also decreased, which was significant at 95% confidence level ($p \leq 0.05$), Solvent type also had a significant effect on Ic50 ($p \leq 0.05$), According to Figure 1 (c), the lowest amount of Ic50 was in the sample extracted with ethanol solvent, and the highest amount of Ic50 was related to the extracts extracted with ether solvent.

FIG.4.

The estimated model for predicting Ic50 is linear and is suit for usage, according to the fit coefficients stated in Table 4. *Ganoderma applanatum* extract's Extraction conditions were optimized to achieve the highest level of total phenol and total flavonoids and the lowest level of IC50 (Figure 5).

FIG.5.

3-4. Optimization

According to the optimization results, if 6 minutes of process time and 250 W of ultrasound power and ethanol solvent are used, the highest compounds of total phenol and flavonoids and the lowest IC50 in the amounts of 222.9 mg GAL/ml, 13.19 mg QE/ml and 2.58, respectively will be obtained.

In this case, the utility coefficient will be equal to 0.875. Validation of the model was performed by extracting *Ganoderma applanatum* under the proposed conditions in three replications, and the results of total phenol content, total flavonoids, and IC50 were evaluated. The results are shown in Table 5. According to table 5, the obtained values were close to the predicted values, *Ganoderma applanatum* extract's antimicrobial properties against two bacteria, *Staphylococcus aureus* and *Pseudomonas aerogenesis*, were investigated and the MIC and MBC values were reported in Table 5.

Table 5.

The minimum inhibitory concentration for *Pseudomonas aeruginosa* was 1250 µg/mL, and for S*taphylococcus aureus*, it was 2080 µg/mL, and the minimum lethal concentrations for *Pseudomonas aeruginosa* and *Staphylococcus aureus* were 2080 and 4150 µg/mL were reported, respectively. Due to the growth inhibitory and lethality of *Ganoderma applanatum* extract, it was found that this extract has antimicrobial activity against gram-positive and gram-negative bacteria.

3-5. Identification of phenolic acid compounds in *Ganoderma applanatum* extract

The type and amounts of phenolic compounds in *Ganoderma applanatum* extract are shown in Table 6. According to the observed results, chlorogenic acid and caffeic acid with 219.47 (g/mL) values and 170.02 (g/mL) were the most phenolic compounds in *Ganoderma applanatum* extract.

Table 6.

FIG.6

4. Discussion

4-1. Effect of ultrasound power, time, and solvent type on total phenol, total flavonoid, and IC50

Advanced extraction techniques, such as ultrasound extraction, have overcome conventional methods' limitations by increasing efficiency and selection. In this regard, using ultrasound waves to break cell membranes has reduced extraction time and increased efficiency. Sound waves with frequencies above 20 kHz cause mechanical oscillations in a material (González-De-Peredo et al., 2020). Unlike electromagnetic waves, sound waves must propagate in material and have cycles of expansion and contraction during propagation in the environment. In the expanding state, bubbles form in the liquid and produce negative pressure. The bubbles form, grow and eventually disintegrate. The two general modes of extraction are ultrasound baths and the ultrasound probe system. The mechanical effects of ultrasound cause more penetration of the solvent into the cellular material and improve mass transfer. Ultrasound during extraction can also destroy cell walls and facilitate the release of its contents; Thus, efficient cell destruction and effective mass transfer are the two main factors that increase ultrasound extraction (Martínez-Ramos et al., 2020). Ultrasound frequency has many effects on efficiency and extraction speed. Also, the effects of ultrasound on the efficiency and speed of ultrasound extraction vary depending on the extracted plant material's nature. For the ultrasonic process, the ionization power also affects the efficiency (González-De-Peredo et al., 2020).

The present study showed that with increasing power and using ethanol solvent, the highest amount of phenol and flavonoid compounds and the lowest IC50 was obtained, and with increasing the extraction process time, Ic50 decreased, and flavonoid content increased. However, in phenolic

compounds, a downward curve was observed up to 9 min and then increased. Consistent with this study's results, Saifullah et al. (2020) have shown that the extraction process's time and temperature positively affected the extraction efficiency of phenolic, flavonoid, and pro-anthocyanin compounds from lemons and the effect of ultrasound power on extraction were not significant (Saifullah et al., 2020). The extraction efficiency of total phenol compounds increased with increasing extraction time. Besides, the phenomenon of cavitation collapse due to high-pressure ultrasound can also lead to an increased outflow of phenolic compounds (Cheng et al., 2007). The ultrasound process may increase the hydroxyl groups attached to the aromatic rings and, as a result, increase the amounts of phenolic compounds (Aadil et al., 2013).

In the study of Espada-Bellido et al. (2017), the optimization of anthocyanins' extraction conditions and total phenolic compounds from white berries was investigated by ultrasound process. It had no phenolic compounds. As the processing time increased, the mass transfer time increased and increased the amounts of flavonoid compounds (Espada-Bellido et al., 2017).

Extraction time has an essential effect on the extraction rate of total phenolic compounds. Over time, the solvent can penetrate plant tissue, and phenolic compounds have ample opportunity to separate from their substrate and enter the surrounding solvent; this has been confirmed by the research of Spigno et al. (2007). The use of higher ultrasonic powers has led to increased extraction of phenolic compounds, which has resulted in more cellular destruction as a result of cavitation and extraction of compounds (Spigno et al., 2007).

Zheng et al. (2020) investigated the optimization of the extraction process of antioxidant, anti-diabetic, and anti-inflammatory compounds from *Ganoderma lucidium*. They showed that if used at 64.5 and 70 ° C for 1.2 Hours, the highest antioxidant activity will be observed (Zheng et al., 2020).

Alzorqi et al. (2017) optimized the extraction of polysaccharide compounds from Ganoderma lucidium and stated that the optimal extraction conditions are 590 W of ultrasound power, irradiation time, 58 minutes, and a temperature of 81 ° C. The extracted polysaccharide will be equal to 22.58 mg (Alzorqi et al., 2017). Pan et al. (2013) also optimized the extraction conditions of polysaccharides from *Ganoderma lucidium*. In this study, it has been reported that if the extraction time is equal to 230 minutes, the extraction temperature of 95 ° C, the extraction rate of polysaccharide compounds will be 1.45% (Pan et al., 2013).

Extraction of phenolic compounds from artichoke waste by Rabelo et al. (2016) in their research showed that the most critical factor affecting the efficiency of the process based on ultrasound and membrane technology was the solvent composition (ethanol/water), and the use of ultrasound power above 240 W has a significant effect on the efficiency of the process (Rabelo et al., 2016).

In line with the present study, Kan et al. (2015) investigated and showed the antioxidant activity of *Ganoderma applanatum* and Lucidum polysaccharide extracts by the response surface method, using a time of 137 minutes, a temperature of 66 ° C and a solvent to substance ratio of mL/g, 35 had the best extraction conditions. The results also showed that increasing the concentration gradient by increasing the solvent to substance ratio and increasing the cell wall degradation due to increasing temperature and increasing the duration has led to an increase in the extracted extracts (Kan et al., 2015).

Ma et al. (2013) also investigated the conditions for extraction of polysaccharides from Ganoderma lucidem using ultrasound process. Ganoderma lucidum and iron reduction capacity was equal to 1956 mmol Trolox/g (Ma et al., 2013). Phenolic compounds are usually present in solution in plant tissues, and the high-pressure ultrasound process leads to the destruction of cell walls and vacuoles, which increase the amounts of phenolics (Costa et al., 2013). The ultrasound process may increase

the hydroxyl groups attached to the aromatic rings and increase the amounts of phenolic compounds (Aadil et al., 2013).

4-2. Antioxidant and Antibacterial effects

Mohammadifar et al. (2011) study antioxidant activity, and bioactive compounds in *Ganoderma lucidium* and *applanatum* showed that phenolic and flavonoid, and butalic acid compounds, as well as antioxidant activity *in Ganoderma applanatum* and polysanidarum Protein in *Ganoderma lucidium*, was higher than *Ganoderma applanatum*. Oviasogie et al. (2015), in a study, showed that *Ganoderma applanatum* extract has antimicrobial activity *against Staphylococcus aureus* and *Pseudomonas aeruginosa*, *Candida albicans*, and the ethanolic extract is more active than aqueous extract (Oviasogie et al., 2015).

4-3. phenolic acid compounds in *Ganoderma applanatum* extract

Phenolic acids are hydroxylated derivatives of benzoic acid and cinnamic acid. Hydroxycinnamic acids are found in abundance in most organisms, the most common of which are chlorogenic acid, caffeic acid, paracoumaric acid, and ferulic acid. Most studies have linked the health benefits of phenolic acids to these compounds' antioxidant activity (Mattila & Hellstrom, 2007).

Zengin et al. (2015) examined the phenolic compounds in Ganoderma applanatum and G. resinaceum and reported that methanolic extracts of both Ganoderma, protocatechuic acid, catechin, chlorogenic acid, and ferulic acid were observed. Heleno et al. (2012) also examined the phenolic acid composition of Ganoderma lucidum and showed that p-hydroxybenzoic acid, p-coumaric acid, and cinnamic acid were present in Ganoderma extract. Oludemi et al. (2018) in their study of the phenolic compounds of Ganoderma lucidum, stated that the major compounds identified were P-hydroxybenzoic acid and syringic acid. Veljović et al. (2017) also stated that the

most phenolic compounds identified in Ganoderma lucidum extract are gallic acid, trans-cinnamic acid, quercetin, camphor, hesperidin, and naringin. Dong et al. (2019) also examined Ganoderma lucidum extract's phenolic compounds and stated that the phenolic compounds identified included camferol, cinnamic acid, quercetin, coumaric acid, and rutin. Costa et al. (2013) also examined the phenolic compounds in Ganoderma lipsiense and stated that caffeic acid, syringic acid, vanillin, p-coumaric acid, salicylic acid, and ferulic acid were the most critical compounds identified. In similar studies, Si et al. (2019) stated that in addition to mannose, rhamnose, and glucose, Ganoderma lingzhi extract contains significant uric acid, which plays a role chelating metals and trapping free radicals and thus reducing oxidation activity.

Hye Ryu et al. (2020) also showed that Ganoderma lucidum contains ganoderic acid and lucidenic acid. Therefore, studying the amount of these compounds in *Ganoderma applanatum* extract was critical to emphasize its medicinal, nutritional, and antioxidant properties.

5. Conclusion

This study showed that the use of ultrasound in *Ganoderma applanatum* extract is an effective method. The duration of the process, ultrasound power, and solvent type significantly affected the extraction efficiency of phenolic compounds flavonoids and antioxidant activity. According to the findings of this study, with using ethanol, ultrasound power of 250 W, and for 6 minutes, the highest amount of phenol, flavonoids, and Ic50 equal to (222.9 mg GAL/mL), 13.19 (QE/ml), and 2.58 (%) can be expected. The results of model validation also showed that the phenol and flavonoids and IC50 levels were close to the software's values, and the minimum growth inhibitory concentration and the minimum bactericidal concentration of the extract on *Pseudomonas aeruginosa* and *Staphylococcus aureus* also indicated the antimicrobial activity of Ganoderma extract.

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452

453

Figure caption

FIG.1 The effect of independent factors on total phenol(a), total flavonoid(b), and IC50(c)

FIG.2 Effect of interaction between time and ultrasonic power on total phenol with difference solvents ethyl ether(a), ethanol (b), ethyl ether and ethanol (c)

FIG.3 Effect of interaction between time and ultrasonic power on total flavonoid with difference solvents ethyl ether(a), ethanol (b), ethyl ether and ethanol (c)

FIG.4 Effect of interaction between time and ultrasonic power on IC50 with difference solvents ethyl ether(a), ethanol (b), ethyl ether and ethanol (c)

FIG.5 Optimization condition for extracting *Ganoderma applanatum* extract by ultrasound technique

FIG.6 Chromatogram of identified phenolic acid compounds

Table 1. Independent factors and their investigated coded level for D-optimal design

Factor	Name	Type	Minimum	Maximum	Coded Low	Coded High	Mean	Std. Dev.
A	Time	Numeric	6.00	12.00	-1 ↔ 6.00	+1 ↔ 12.00	9.00	2.27
B	Power	Numeric	150.00	250.00	-1 ↔ 150.00	+1 ↔ 250.00	200.00	37.80
C	Solvent	Categoric	Level 1 of C	Level 3 of C			Levels:	3

468 Table 2. Levels of independent factors and actual values of dependent for D-optimal design

	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3
Run	A: Time	B: Power	C: Solvent	IC50	Phenol	Flavonoid
1	6	150	Ethanol	3.48	22.58	12.45
2	12	150	Ethanol	1.93	24.25	13.67
3	6	250	Ethanol	2.71	222.9	13.26
4	12	250	Ethanol	1.74	25.25	14.7
5	6	200	Ethyl ether	4.66	4.929	7.62
6	12	200	Ethyl ether	4.04	6.9	10.36
7	6	200	Ethyl ether and ethanol	3.52	21.929	11.79
8	12	200	Ethyl ether and ethanol	2.69	23.076	13.29
9	9	150	Ethyl ether	6.71	3.753	6.89
10	9	250	Ethyl ether	4.212	6.047	9.16
11	9	150	Ethyl ether and ethanol	3.61	20.16	11.71
12	9	250	Ethyl ether and ethanol	3.45	22.63	12.93
13	9	200	Ethanol	2.45	23.07	13.29
14	9	200	Ethanol	3.08	22.69	13.15
15	9	200	Ethanol	2.43	23.13	13.24

469

470

471 Table 3. ANOVA for response surface of the quadratic polynomial (total phenol) and linear model (Flavonoid and

472 IC50)

Total Phenol (mg GAL/ml)	Source	Sum of Squares	df	Mean Square	F-value	p-value	
	Model	40122.50	11	3647.50	92583.06	< 0.0001	Significant
	A-TIME	4649.47	1	4649.47	1.180E+05	< 0.0001	
	B-POWER	5308.83	1	5308.83	1.348E+05	< 0.0001	
	C-SOLVENT	4987.01	2	2493.50	63291.63	< 0.0001	
	AB	9932.12	1	9932.12	2.521E+05	< 0.0001	
	AC	4955.17	2	2477.59	62887.59	< 0.0001	
	BC	4829.29	2	2414.65	61289.99	< 0.0001	
	A^2	2480.92	1	2480.92	62972.19	< 0.0001	
	B^2	2281.98	1	2281.98	57922.60	< 0.0001	
	Residual	0.1182	3	0.0394			
	Lack of Fit	0.0043	1	0.0043	0.0760	0.8087	not significant
	Pure Error	0.1139	2	0.0569			
Flavonoid (mg QE/ml)							
	Model	70.14	4	17.53	64.84	< 0.0001	Significant
	A-TIME	5.95	1	5.95	22.01	0.0011	
	B-POWER	3.55	1	3.55	13.13	0.0055	
	C-SOLVENT	60.63	2	30.32	112.11	< 0.0001	
	Residual	2.43	9	0.2704			
	Lack of Fit	2.42	8	0.3030	30.92	0.1382	not significant
	Pure Error	0.0098	1	0.0098			
	Cor Total	72.57	13				
IC50(%)	Source						
	Model	17.80	4	4.45	11.98	0.0008	Significant
	A-TIME	1.97	1	1.97	5.30	0.0440	
	B-POWER	1.64	1	1.64	4.41	0.0622	
	C-SOLVENT	14.20	2	7.10	19.11	0.0004	
	Residual	3.71	10	0.3714			
	Lack of Fit	3.44	8	0.4301	3.15	0.2634	not significant
	Pure Error	0.2733	2	0.1366			
	Cor Total	21.52	14				

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474

475 Table 4. Fit statistics of proposed models for dependent factors

R^2 adj	R^2	CV	std	Model		Source
1	1	0.64	0.198	4.76+14.9A-0.96B-0.33AB+2.88A2+0.009B2	Ethyl ether	total phenol (mg GAL/ml)
				22.19+14.78A-0.96B-0.33AB+2.88A2+0.009B2	Ethyl ether and ethanol	
				0.92-1.73A+0.018B-0.33AB+2.88A2+0.0009B2	Ethanol	
0.95	0.96	4.67	0.5	2.65+2.28A+0.013B	Ethyl ether	total flavonoid mg QE/ml)((
				6.57+0.28A+0.01B	Ethyl ether and ethanol	
				7.56+0.28A+0.01B	Ethanol	
0.758	0.827	14.58	0.609	9.003-0.16A-0.009B	Ethyl ether	(%) IC50
				7.41-0.16A-0.009B	Ethyl ether and ethanol	
				6.64-0.16A-0.009B	Ethanol	

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478 Table 5. MIC and MBC for *Pseudomonas aeruginosa* and *Staphylococcus aureus*

total phenol mg GAL/ml)(	total flavonoid mg QE/ml)(	IC50(%)	MIC(µg/mL)		MBC(µg/mL)	
			Pseudomonas aeruginosa	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
219.3	11.5	2.88	1250	2080	2080	4150

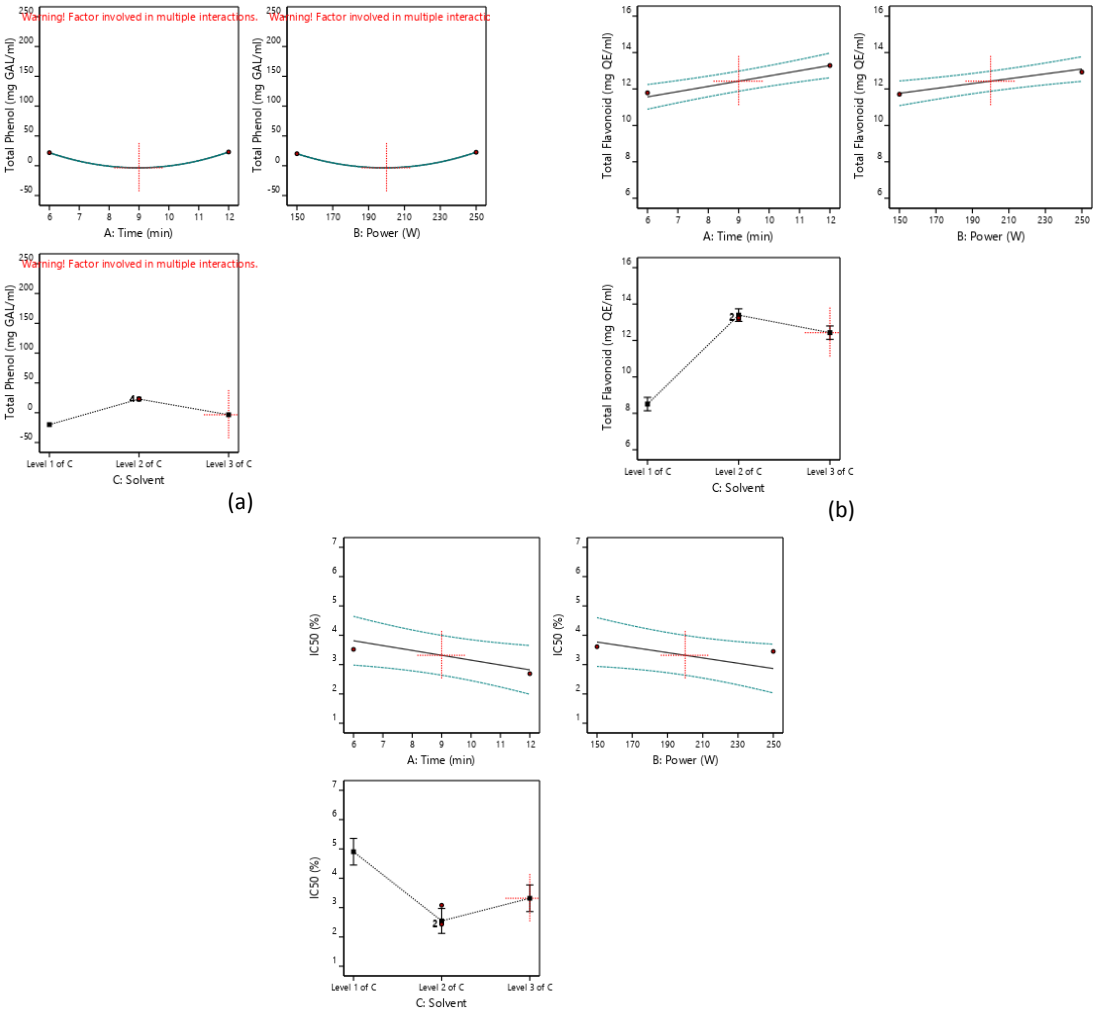
479

480

481 Table 6. Phenolic compounds in *Ganoderma applanatum* extract

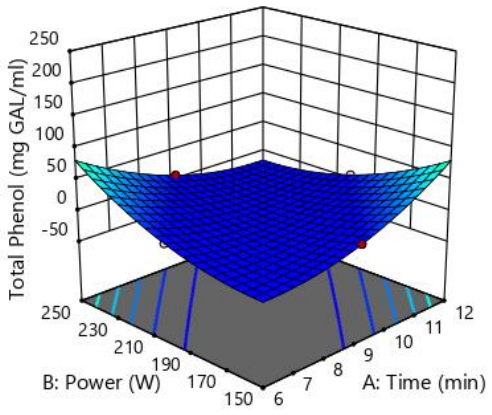
Peak number	Phenolic compound	Retention time (minutes)	Peak Area (percentage)	Recycling percentage	Concentration (µ g / mL)
1	Quercetin	3.51	4.68	98	30.21
2	Cinnarine	6.43	4.03	98	37.1
3	Benzoic acid	6.81	6.92	99	48.22
4	Quercetin	7.59	14.12	99	108.31
5	Caffeic acid	7.81	25.84	98	170/02
6	Luteolin	7.91	4.11	95	41.58
7	Chlorogenic acid	8.11	30.99	100	219.47
8	p-coumaric acid	10.55	5/01	98	41.42
9	routine	10.59	3.81	99	30.21

482



483 *Level 1 0f C is Ethyl ether, Level 2 0f C is Ethanol and Level 3 0f C is Ethyl ether and ethanol

484 FIG.1.



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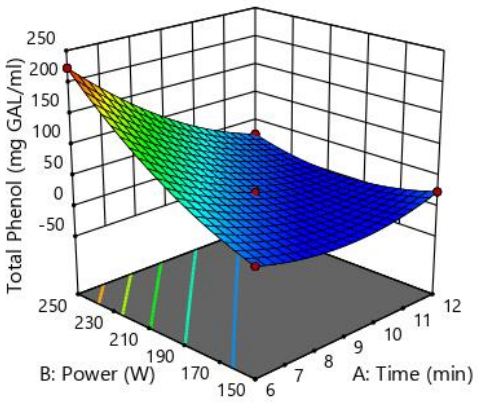

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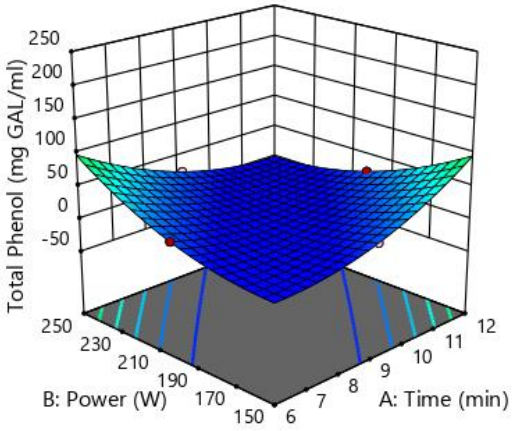

(c)

485 FIG.2.

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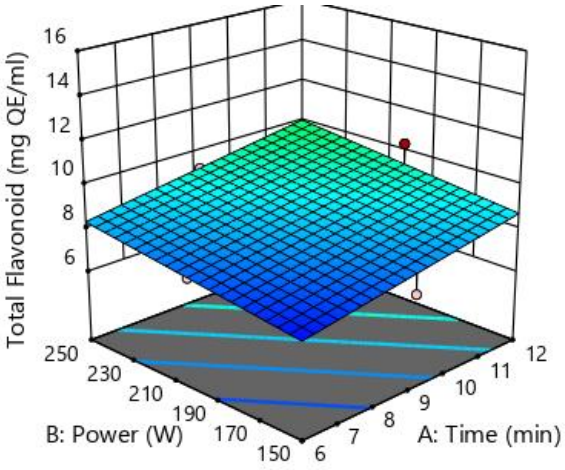

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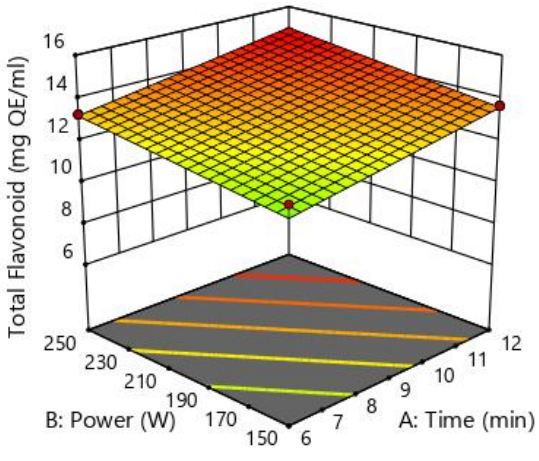

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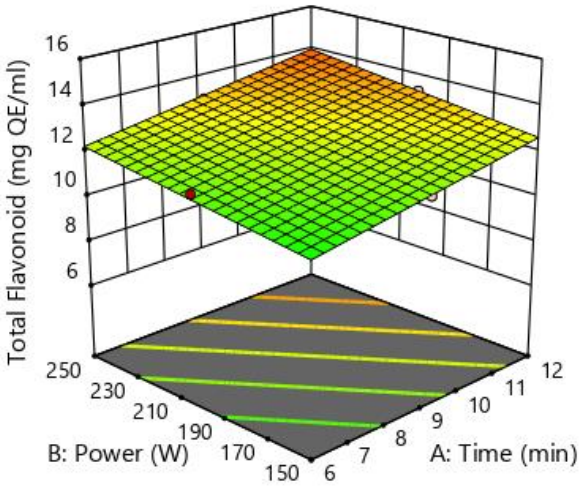

(c)

487 FIG.3.

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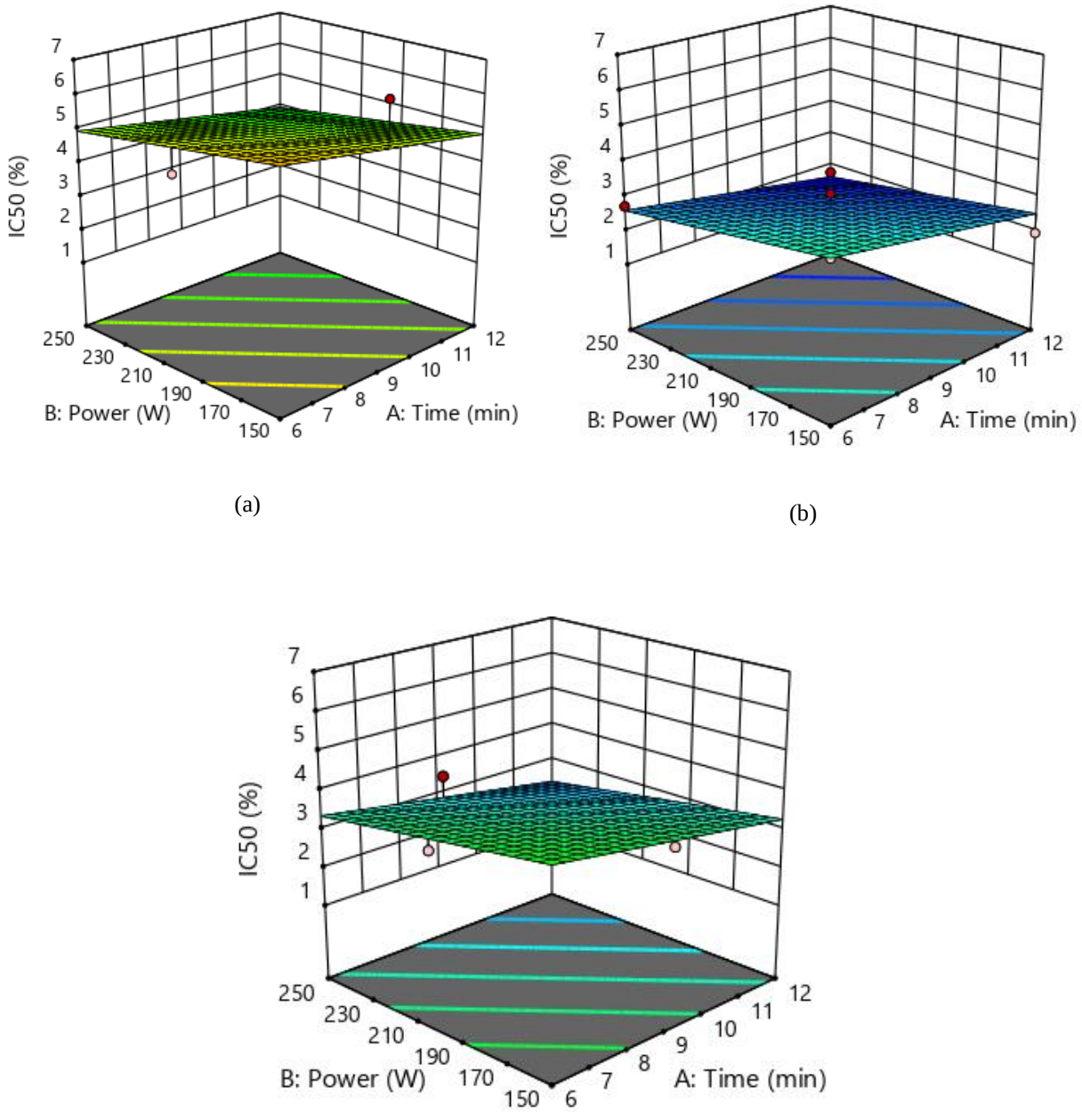

(a)

(b)

(c)

489 FIG.4.

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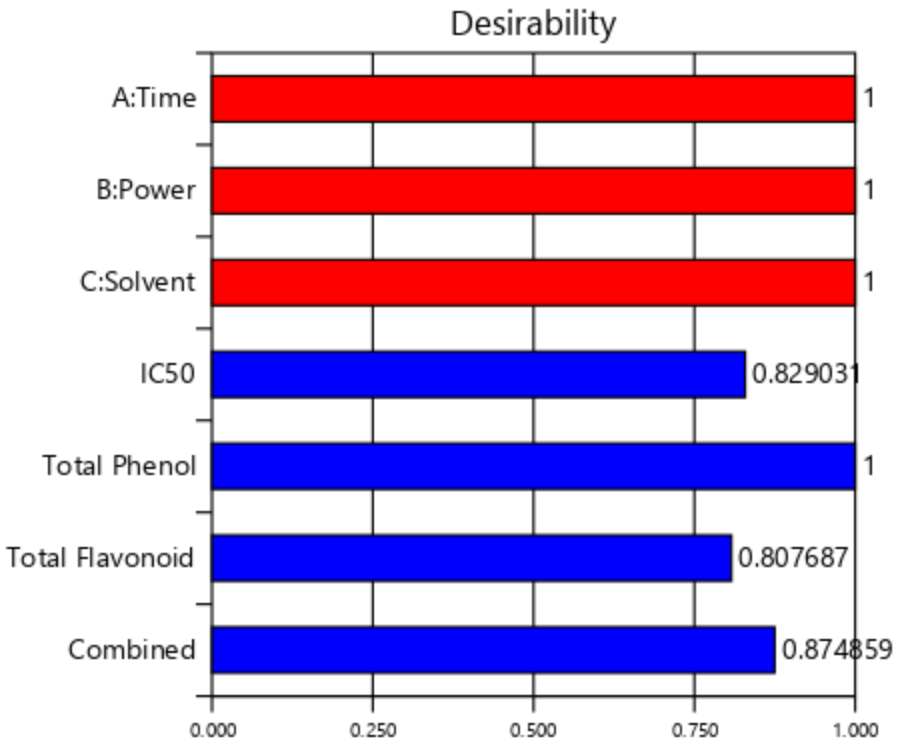

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492 FIG.5.

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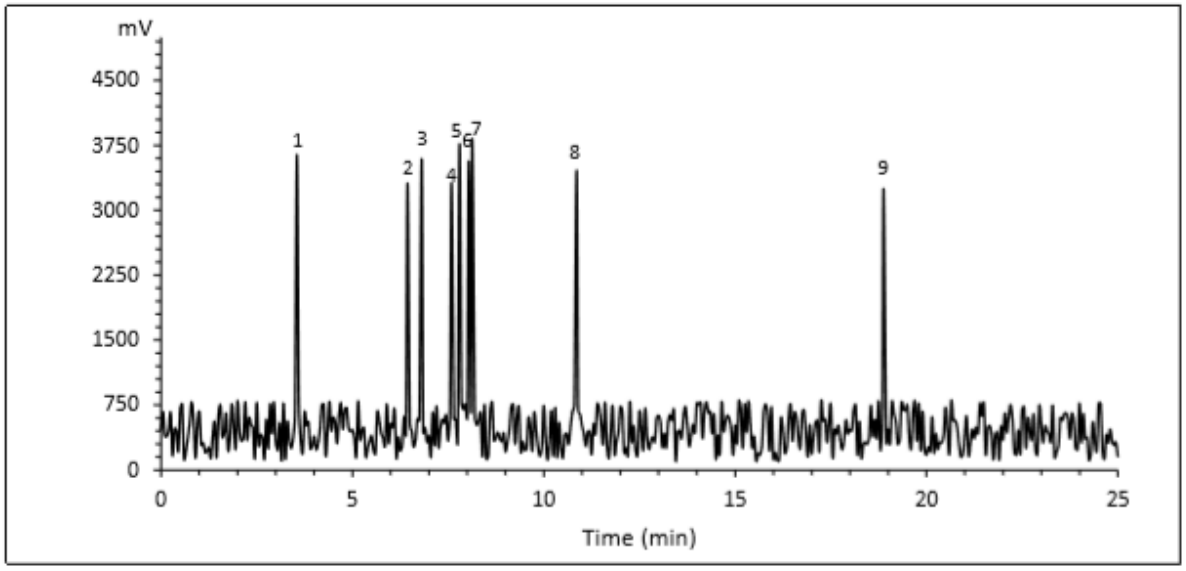

495

496 FIG.6.

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