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Enhancing bioactive production in *Ganoderma* species: a statistical optimization strategy from submerged fermentation to fruiting bodies

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Abstract

Ganoderma mushrooms are renowned for their rich repertoire of bioactive compounds. To optimize the production of secondary metabolites with potent anticancer and antimicrobial properties, this study employed a two-stage strategy: statistical optimization of nanoparticle-mediated enhancement of secondary metabolites via submerged fermentation, followed by transfer of the optimal conditions to solid-state fermentation for fruit body production in *Ganoderma* species. Two fungal strains were isolated, identified via ITS sequencing, and phylogenetically analyzed, which confirmed the isolates as *Ganoderma lucidum* (*G. lucidum*) and *Ganoderma lingzhi* (*G. lingzhi*). One factor at time screening method revealed that iron and magnesium nanoparticles significantly stimulated ganoderic acid and phenolic production respectively. Subsequently, response surface methodology (RSM) optimized the key substrates to maximize secondary metabolite production, and these conditions were implemented in solid-state fermentation to stimulate bioactive components in *Ganoderma* fruiting bodies. Ganoderic acid extracts derived from the fruiting bodies demonstrated potent, broad-spectrum antibacterial activity, with minimum inhibitory concentrations (MICs) as low as 22.5 µg/mL against both *Escherichia coli* and *Staphylococcus aureus*. Furthermore, these extracts exhibited significant cytotoxicity against the MDA-MB-231 breast cancer cell line, with *G. lingzhi* showing superior potency (IC₅₀ = 299.78 µg/L) compared to *G. lucidum* (IC₅₀ = 581.05 µg/L). This statistical optimization approach resulted in a marked increase in the production of therapeutically valuable bioactive components from *Ganoderma*.

Introduction

Fungi are prolific producers of natural products; approximately 9,000 bioactive compounds have been obtained from fungi out of the 22,500 discovered from microorganisms [1]. Due to their reputation as natural sources of bioactive compounds with significant therapeutic attributes, among various species of fungi, medicinal mushrooms have become a primary focus in contemporary biotechnology and pharmacology research[2,3]. Given their prolonged use in traditional medicine and their extraordinary capability to biosynthesize high-value secondary metabolites, species of the genus *Ganoderma* have garnered significant scientific focus within this group of fungi [4,5]. These metabolites include polysaccharides—especially β -glucans—highly oxygenated triterpenoids such as ganoderic acids, and diverse phenolic compounds, which collectively exhibit potent anticancer, antimicrobial, antioxidant, immunomodulatory, and antitumor activities [6–8]. Over the past two decades, extensive studies have focused on the production and characterization of polysaccharides, ganoderic acids, and phenolic compounds from species of the genus *Ganoderma* cultivated on various formulated deciduous wood substrates [9,10]. Solid-state fermentation (SSF) which closely resembles the natural ecological habitat of *Ganoderma* species, has been widely employed for fruiting body and secondary metabolite productions [11,12]. SSF offers advantages such as the use of agricultural and industrial waste, reduced water usage, lower energy consumption, and simpler techniques, leading to concentrated products and waste minimization [13,14]. However, SSF presents challenges in controlling physical and chemical conditions of media, as well as prolonged cultivation periods, strong dependence on substrate composition, and difficulty in managing heterogeneity, quantifying biomass and metabolite production before fruiting body maturation [7,15]. In contrast, submerged fermentation (SmF) has emerged as an attractive alternative for the production of fungal biomass and secondary metabolites. SmF offers several advantages over SSF, including precise control of physical and chemical conditions of media, reduced contamination risk, shorter fermentation time, scalability, and cost-effectiveness, as well as compatibility with advanced statistical optimization tools [5,16]. Recent studies emphasize optimizing fermentation conditions to enhance the efficacy of bioactive components in curative and edible mushrooms for nutraceutical and pharmaceutical applications[7,11,16]. There is broad consensus that the quality and quantity of macroelements—particularly carbon, nitrogen, and mineral sources—strongly influence biomass proliferation and secondary metabolite synthesis in

Ganoderma species under submerged fermentation [5,17]. Beyond conventional substrates, colloidal solutions of nanoparticles (NPs) have recently been recognized as critical fermentation enhancers, capable of modulating fungal metabolism and markedly improving secondary metabolite biosynthesis [18,19][20,21]. However, there is limited research on the combined impact of colloidal nanoparticle solutions on the production of ganoderic acid and phenolic compounds in *Ganoderma* species under submerged fermentation conditions. To enhance metabolite productivity, a substantial body of literature has emphasized the importance of applying robust statistical approaches for fermentation optimization. Of these methods, RSM is extensively used to identify the best combinations of several key substrates which optimally affect metabolite yield, after initial screening method such as one-factor-at-a-time or Plackett–Burman design [5,7]. Despite numerous optimization studies on medicinal mushrooms, limited research has simultaneously addressed ganoderic acid and phenolic compound production in *Ganoderma* species using various nanoparticlen-supplemented in submerged fermentation. The current research was structured to boost the biosynthesis of ganoderic acid and phenolic compounds in *G. lucidum* and *G. lingzhi* which were identified using ITS region sequencing. Initially, a one-factor-at-a-time approach was used to identify the most impactful nanoparticles on metabolite production from the set of nanoparticles tested. Subsequently, RSM was employed to optimize the key substrates identified through the one-factor-at-a-time screening. Optimized fermentation condition was later employed in the solid-state cultivation to boost secondary metabolite production in fruit bodies. The ganoderic acids content in the fruiting bodies produced were also evaluated to determine the antimicrobial and anticancer bioactivities of their extracts, thus allowing for a comprehensive assessment of the connection between optimized fermentation conditions, metabolite accumulation, and functional biological efficacy.

Resultes and disscustion

Identification and Phylogenetic Analysis of Fungal isolates

Fungal isolates were identified through PCR amplification and sequencing of the internal transcribed spacer (ITS) region using primers ITS1 and ITS4. Amplification successfully yielded a fragment of approximately 500–700 base pairs, encompassing the ITS1, 5.8S, and ITS2 regions, for both isolates. Subsequent sequence analysis and BLAST searches against the GenBank database revealed significant matches for each isolate. Isolate SH showed high

sequence similarity to *G. lucidum* (98.75%; GenBank accession no. JQ520187.1) and *G. resinaceum* (98.28%; MH571692.1). In contrast, isolate GL exhibited a 99.84% match with *G. lucidum* (GU213487.1) and a 99.68% match with *G. lingzhi* (JQ781862.1).

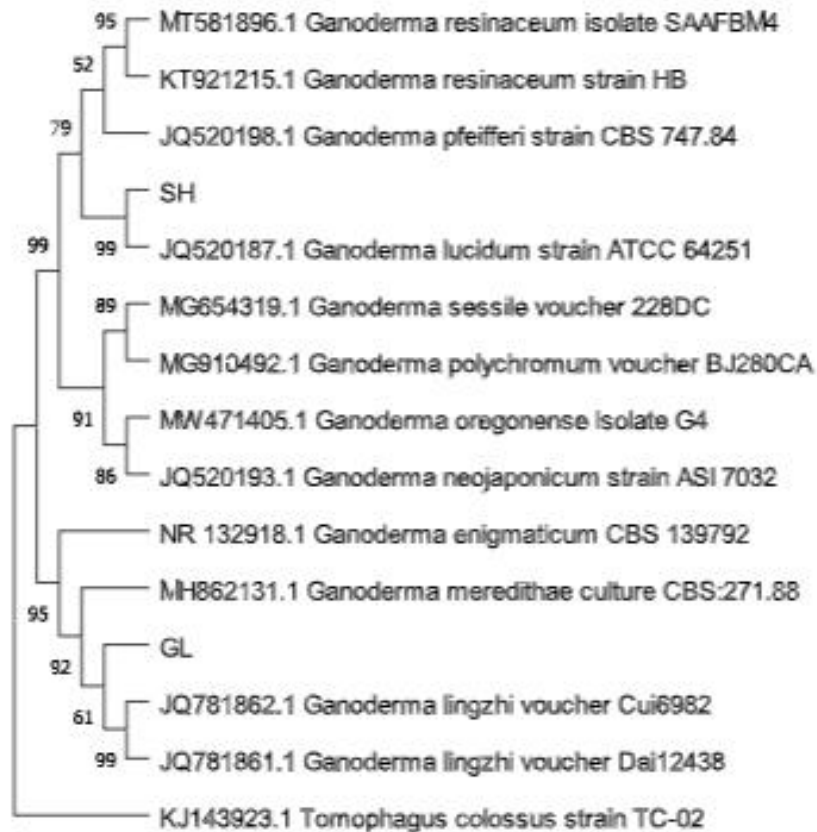


Figure-1 the gene tree of SH and GL isolates, constructed based on the ITS1-5.8s-ITS2 region. This tree was constructed using the Neighbor Joining method with a 1000-bootstrap analysis via MEGA11 software with *Tomophagus colossus* species serving as the outgroup.

The consensus phylogenetic tree, depicted in Figure 1, illustrates the evolutionary relationships of both isolates. This analysis is underpinned by bootstrap support values of 99 for isolate SH and 61 for isolate GL, indicating robust phylogenetic affinity with *G. lucidum* and *G. lingzhi*, thereby reinforcing their classification within this group. Additionally, the sequences for both isolates (SH and GL) are cataloged in the NCBI database and can be accessed through the accession number PP064858 and PP064859.

Investigation of the effect of nanoparticles on biomass production in G. lucidum and G. lingzhi

Figure 2 demonstrates that Fe₂O₃ NPs significantly enhanced biomass production in both *G. lucidum* and *G. lingzhi* compared to control samples. The recent findings indicated that low

levels of metal and metal-oxide NPs can stimulate fungal secondary metabolites [27]. In contrast, magnesium and zinc nanoparticles did not produce a statistically significant effect on biomass growth in either fungal species, which is consistent with literature indicating that not all metal oxide nanoparticles uniformly promote microbial biomass and that their effects are highly dependent on nanoparticle type, size, and biological context [28,29]. Furthermore, titanium and silicon nanoparticles exhibited a negative influence on biomass production for both fungal species, with the most substantial reduction observed in cultures containing silica nanoparticles. Additionally, the study revealed that increasing the pH from 5 to 6 led to decreased biomass accumulation, indicating a detrimental impact on fungal growth.

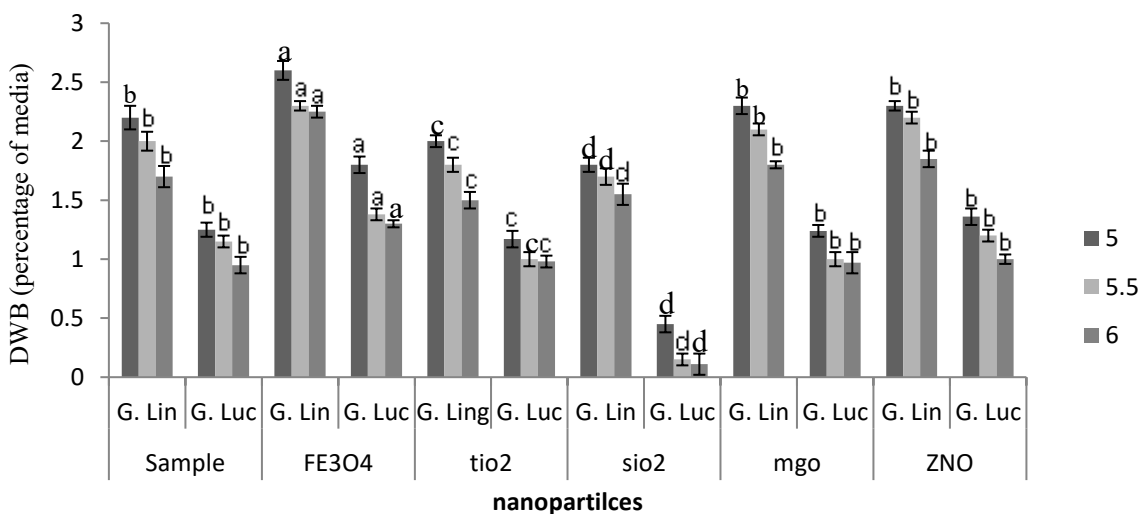


Figure-2 the effect of nanoparticles on DWB production in *G. lucidum* and *G. lingzhi*

Influence of Nanoparticles on Phenolic Compound Biosynthesis in *G. lucidum* and *G. lingzhi*

Figure 3 presented below elucidates that the application of magnesium nanoparticles resulted in a pronounced enhancement in the production of phenolic compounds across both fungal species when compared to control samples. Furthermore, the culture medium supplemented with silica nanoparticles exhibited a notable stimulatory effect on the biosynthesis of phenolic compounds. While titanium nanoparticles were similarly found to facilitate the production of phenolic compounds, their stimulating effect proved comparatively less substantial than that observed with silica nanoparticles. Conversely, the introduction of iron and zinc nanoparticles did not

yield a statistically significant increase in phenolic compound levels when assessed against the control group.

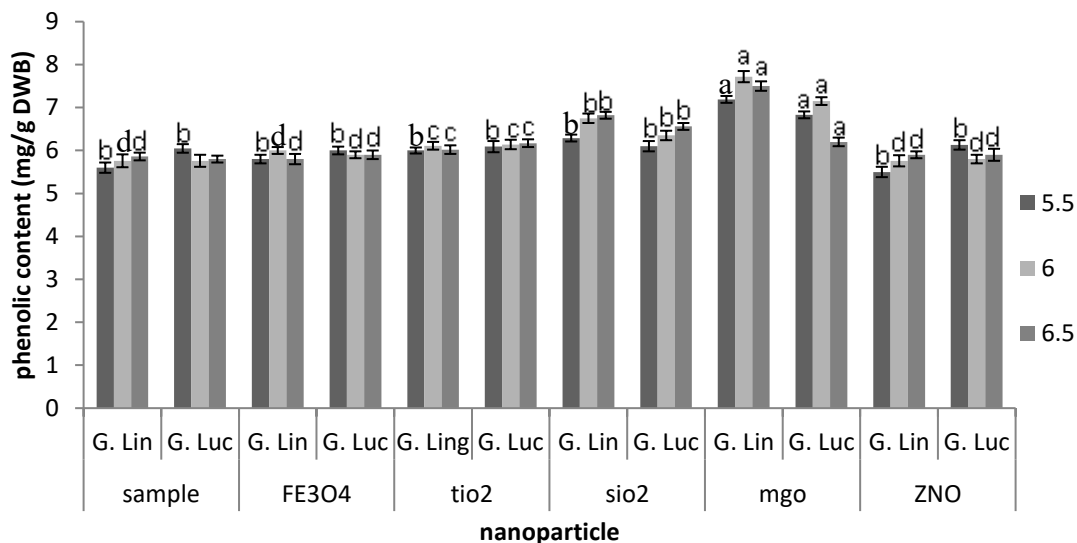


Figure-3 the effect of nanoparticles on phenolic production in *G. lucidum* and *G. lingzhi*

Investigation of the effect of nanoparticles on ganoderic acid production in *G. lucidum* and *G. lingzhi*

G. lucidum exhibited a significantly higher concentration of ganoderic acid than *G. lingzhi* under the experimental conditions. This species-dependent variation in secondary metabolite production is consistent with previous studies demonstrating that genetic regulation and enzymatic pathways involved in triterpenoid biosynthesis differ among closely related *Ganoderma* species, thereby influencing ganoderic acid accumulation [4,30]. With the exception of iron and zinc nanoparticles, all other nanoparticle treatments adversely affected ganoderic acid synthesis in *G. lucidum*. Metal-based nanoparticles are known to induce oxidative stress, membrane damage, and metabolic imbalance in fungal systems, which may disrupt microbial growth when exposure exceeds tolerance levels [31,32]. Elevated reactive oxygen species (ROS) generation under nanoparticle stress has been shown to downregulate biosynthetic gene clusters responsible for secondary metabolite production[33]. Among the nanoparticles evaluated, iron nanoparticles induced the highest ganoderic acid concentration and simultaneously enhanced biomass production in both fungal species. Metal nanoparticles have been reported to stimulate microbial growth and metabolic activity by enhancing nutrient uptake, facilitating electron transport processes, and acting as catalytic cofactors in enzymatic systems

[34–36] .In contrast, in *G. lingzhi*, only magnesium and iron nanoparticles significantly stimulated ganoderic acid production, whereas the remaining treatments did not differ statistically from the control (Figure 4).

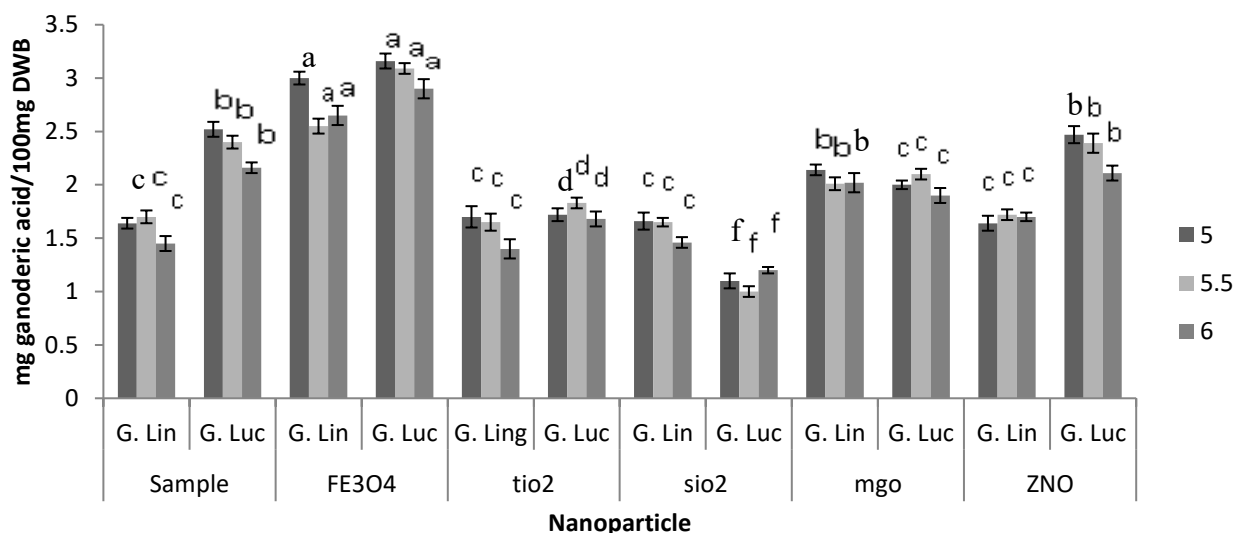


Figure-4 the effect of nanoparticles on ganoderic acid production in *G. lucidum* and *G. lingzhi*

In light of both biomass accumulation and secondary metabolite production, a clear positive correlation was observed between maximal biomass yield and elevated concentrations of ganoderic acid across experimental treatments. Specifically, culture media enriched with iron nanoparticles exhibited the highest ganoderic acid content, concomitant with the most substantial biomass production in both *G. lucidum* and *G. lingzhi*. In contrast, the most adverse effects on ganoderic acid biosynthesis were noted in medium that negatively influenced biomass accumulation, with silica nanoparticle supplementation yielding the lowest ganoderic acid levels. Interestingly, although silica nanoparticles exerted the most pronounced detrimental impact on overall biomass content, they simultaneously stimulated the production of phenolic compounds; a similar outcome was reported by [37]. Both iron and magnesium nanoparticles were found to effectively induce the production of ganoderic acid as well as phenolic compounds in both fungal species.

Optimization of culture conditions for the production of ganoderic acid and phenolic components in the *G. lingzhi*

Optimization experiments were conducted to develop a mathematical model for predicting process behavior. Unlike conventional designs, optimization approaches consider fewer factors, which, despite their limited number, exert strong influences on the outcome. The primary objective of this experiment was to optimize the production of ganoderic acid and phenolic compounds in *G. lingzhi* under submerged fermentation conditions utilizing response surface methodology (RSM). A targeted concentration range of two critical nanoparticles—MgO and Fe₃O₄ NPs—was established, as delineated in table 1, and subsequently optimized through RSM techniques.

Table-1-Different levels of magnesium and iron used (g/l)

factor	-1	1	-1.414	+1.41
Mgo	0.1	0.5	0.017157	0.582843
Fe ₃ O ₄	0.1	0.5	0.017157	0.582843

Table 2 presents the regression coefficients and P values for the fitted models. The model and linear effects of magnesium oxide nanoparticles (A) and iron oxide nanoparticles (B) on ganoderic acid and phenolic acid formation in *G. lingzhi* were significant (*P* < 0.05). Both the quadratic term and the interaction effect were statistically significant on phenolic acid production.

Table-2 ANOVA for response surface quadratic model analysis of variance GA (Ganoderic acid).

Source	Sum of Squares		df		Mean Square		F Value		p-value Prob > F	
	Phenolic	GA	Phen	GA	Phenolic	GA	Phenolic	GA	Phenol	GA
Block	4.51584	0.578884	1	1	4.51584	0.578884				
Model	87.8736	5.629749	5	2	17.57472	2.814875	372.0189	126.8311	0.0002	< 0.0001
A	30.58946	1.193568	1	1	30.58946	1.193568	647.5129	53.77916	0.0001	0.0003
B	5.377089	4.227404	1	1	5.377089	4.227404	113.8214	190.4762	0.0018	< 0.0001
AB	12.14523	-	1	-	12.14523	-	257.0882		0.0005	
A^2	6.351779	-	1	-	6.351779	-	134.4534		0.0014	
B^2	40.76658	-	1	-	40.76658	-	862.9405		< 0.0001	
Residual	0.141724	0.133163	3	6	0.047241	0.022				
Cor	92.53116	6.341796	9	9						
Total										

Equation presents the regression models for ganoderic acid and phenolic acid production in submerge fermentation, derived from the analysis performed using Design-Expert software. The coefficient of determination (R²) values were 0.907 for ganoderic acid and 0.973 for phenolic acid, indicating strong agreement between the experimental data and the regression models.

These results suggest that the models effectively describe and predict the relationship between culture conditions (MgO and Fe₃O₄ NPs) and the formation of ganoderic and phenolic acids in *G. lingzhi*

$$\text{Ganoderic acid} = +1.08600 + 1.94997 \times \text{Mgo} + 3.83497 \times \text{Fe}_3\text{O}_4$$

$$\text{Phenolic} = +16.52791 - 20.87833 \times \text{Mgo} - 62.18763 \times \text{Fe}_3\text{O}_4 + 43.56250 \times \text{Mgo} \times \text{Fe}_3\text{O}_4 + 29.46875 \times \text{Mgo}^2 + 74.65625 \times \text{Fe}_3\text{O}_4^2$$

As illustrated in table 3, maintaining a medium and constant concentration of magnesium oxide nanoparticles at 0.3 g/L while progressively increasing the concentration of iron oxide nanoparticles from 0.01 g/L to 0.5 g/L resulted in a substantial enhancement of ganoderic acid production. Specifically, ganoderic acid concentrations increased from 1.5 mg/100 mg DWB to 3.9 mg/100 mg DWB, reflecting more than a twofold increase across samples 1, 2, and 8. Similarly, when iron oxide concentration was held constant at 0.3 g/L, elevating magnesium oxide levels from 0.017 g/L to 0.58 g/L also facilitated an increase in ganoderic acid synthesis, with concentrations rising from 2 mg/100 mg DWB to 3.4 mg/100 mg DWB—observed in samples 1, 2, and 6. However, this increase was notably less pronounced than that observed with iron oxide. Ultimately, the highest concentrations of both nanoparticles led to the most significant yields of ganoderic acid, achieving a maximum of 3.9 mg/100 mg DWB, particularly in sample 4. These results underscore the notion that elevated concentrations of nanoparticles exert a potent stimulatory effect on ganoderic acid production. In prior investigations employing a one-factor-at-a-time approach, magnesium oxide (MgO) exhibited a markedly greater influence on phenolic compound production compared to iron oxide (Fe₂O₃). This trend persisted in the current study; specifically, increasing the concentration of iron oxide, while maintaining a constant and moderate level of magnesium oxide, resulted in diminished phenolic acid formation, as evidenced by the results from samples 3 and 8. Conversely, when iron oxide concentration was held constant, elevating the levels of magnesium oxide led to a corresponding increase in phenolic acid synthesis, as indicated by samples 5 and 6. Notably, the highest concentrations of phenolic acid, quantified at 12.63 mg gallic acid/g DWB, were recorded in cultures supplemented with the lowest concentrations of iron oxide (sample 2).

Table-3 Results of FCCCD using two variables indicating observed results.

sample	Mgo	Fe ₂ O ₃	GA mg ganoderic /100mg DWB	Phenolic acid
1	0.3	0.3	3	5.3
2	0.3	0.017157	1.5	12.36
3	0.5	0.1	2.4	10.23
4	0.5	0.5	3.9052	12
5	0.017157	0.3	2.0992	4.63
6	0.582843	0.3	3.4432	10
7	0.1	0.5	3.3	4.3
8	0.3	0.582843	3.9	9.5
9	0.1	0.1	1.8672	9.5
10	0.3	0.3	2.8	4.5

As shown in figure 5, The simultaneous increase of these two nanoparticles resulted in a significant enhancement in the concentration of ganoderic acid

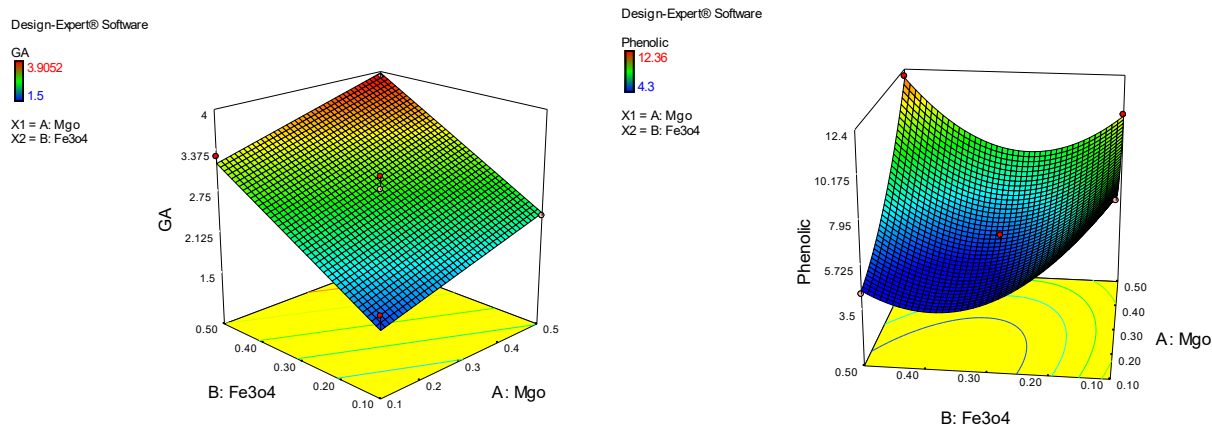


Figure-5 RSM curve for the production of ganoderic acid (mg/(100 g DWB) and phenolic content (mg gallic acid/g DWB) from the fungus *G. lingzhi* with the variables of iron oxide nanoparticles (B) and magnesium oxide nanoparticles (A)

The response surface analysis indicated that the lowest concentration of Fe₃O₄ and the highest

concentration of MgO provided the optimal conditions for the highest production of phenolic acids. Conversely, a reduction in MgO concentration had a significant inhibitory effect on phenolic acid production.

Validation of the optimized culture conditions

Under the optimal conditions, which involved the utilization of 0.5 g/L of mgo and 0.5 g/L of fe3o4, the models predicted that the ganoderic acid and phenolic content would peak at 3.93 mg ganoderic /100mg DWB and 11.92 mg gallic acid/g DWB respectively. In order to assess the accuracy of the model equation, the predicted optimal conditions were implemented in an experimental setting. Under these experimental conditions, the measured the phenolic and ganoderic acid production was determined to be 11.25 mg gallic acid/g DWB and 3.53 mg ganoderic /100mg DWB respectively.

Optimization of Ganoderic Acid and phenolic acid Production from *G. lucidium* Mushrooms Using Response Surface Methodology

Table 4 presents the regression coefficients and *P*-values for the fitted models. The model and linear effects of magnesium oxide nanoparticles (A) and iron oxide nanoparticles (B) on phenolic acid and ganoderic acid formation in *G. lucidium* were statistically significant (*P* < 0.05). The final model showed a non-significant lack of fit (*P* > 0.05), confirming the adequacy and reliability of the model.

Table 4- ANOVA for response surface quadratic model analysis of variance.

	Sum of Squares		df		Mean Square		F Value		p-value Prob > F	
Source	Phenolic	GA	Phen	GA	Phenolic	GA	Phenolic	GA	Phenol	GA
Model	86.52312	4.093858	4	2	21.63078	2.046929	71.77132	69.64321	0.0001	< 0.0001
A	5.722175	0.203422	1	1	5.722175	0.203422	18.98628	6.921069	0.007	0.0339
B	42.77891	3.890437	1	1	42.77891	3.890437	141.9412	132.3654	< 0.0001	< 0.0001
A ²	20.70362	-	1	-	20.70362	-	68.69498		0.0004	
B ²	32.60452	-	1	-	32.60452	-	108.1824		0.0001	
Residual	1.506924	0.205742	5	7	0.301385	0.029392				
Lack of Fit	1.344474	0.192942	4	6	0.336118	0.032157	2.069057	2.51	0.4748	0.448
Pure Error	0.16245	0.0128	1	1	0.16245	0.0128				
Cor Total	88.03004	4.2996	9	9						

Equation 4 presents the regression model for phenolic acid and ganoderic acid production, obtained through analysis with Design-Expert software. The coefficients of the second-order polynomial model were generated after eliminating non-significant terms using the Forward

selection method. The coefficient of determination (R^2) for the phenolic acid and ganoderic acid model was 0.984 and 0.93 indicating an excellent fit between the experimental data and the regression model. This result demonstrates that the model effectively describes and predicts the relationship between culture conditions (MgO and Fe₃O₄ nanoparticles) and response productions.

$$\text{Ganoderic acid} = +3.18316 - 0.79730 \times \text{Mgo} + 3.48678 \times \text{Fe}_3\text{o}_4$$

$$\text{Phenolic} = -0.20224 + 27.69319 \times \text{Mgo} + 51.62156 \times \text{Fe}_3\text{o}_4 - 53.20313 \times \text{Mgo}^2 - 66.76563 \times \text{Fe}_3\text{o}_4^2$$

The results indicate that increasing the concentration of iron oxide nanoparticles, while maintaining a constant level of magnesium oxide, significantly enhanced phenolic acid production in *G. lucidum*, as demonstrated by samples 2 and 4. Similarly, when the concentration of iron oxide was fixed at 0.3 g/L, moderate concentrations of magnesium oxide (0.3 g/L) also facilitated an increase in phenolic acid synthesis, as evidenced by samples 7 and 8. These findings imply that moderate concentrations of both nanoparticles, specifically around 0.30 g/L, exert the most pronounced influence on phenolic acid accumulation in *G. lucidum* under submerged conditions, particularly highlighted by the results from sample 7 (table-5).

Table-5 Results of FCCCD using two variables indicating observed results.

sample	Mgo	Fe ₂ O ₃	GA	Phenolic acid
1	0.5	0.1	3.19	5.1
2	0.3	0.582843	5.17	11
3	0.582843	0.3	3.6	6.9
4	0.3	0.017157	3	4
5	0.1	0.1	3.7	6.5
6	0.1	0.5	4.8	10.4
7	0.3	0.3	4	13.08
8	0.017157	0.3	4	10.27
9	0.5	0.5	4.6	9.8
10	0.3	0.3	3.84	12.51

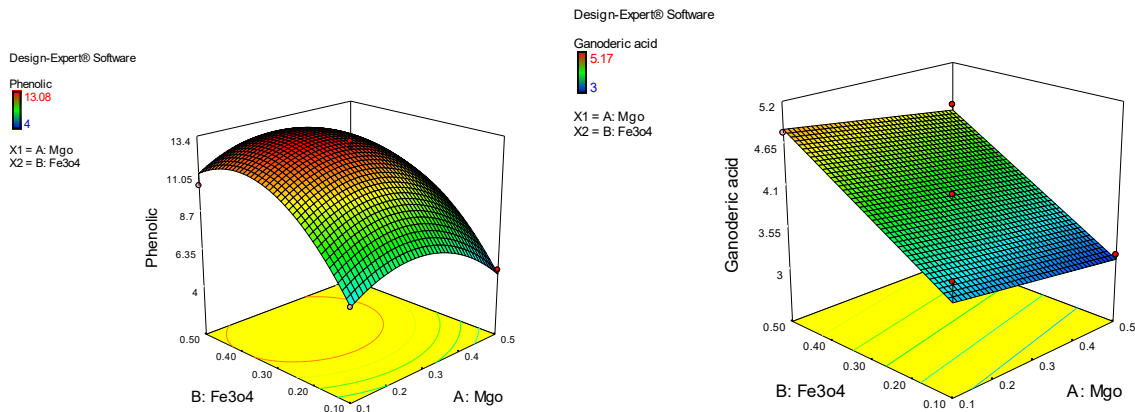


Figure-6 RSM curve for the production of ganoderic acid (mg/(100 g DWB) and phenolic content (mg gallic acid/g DWB) from the fungus *G. lucidium* with the variables of iron oxide nanoparticles (B) and magnesium oxide nanoparticles (A)

Figure 6 illustrates the effects of varying concentrations of MgO and Fe₃O₄ NPs nanoparticles on the synthesis of phenolic acid and ganoderic acid. The response surface analysis indicates that optimal nanoparticle concentrations must be maintained within a specific range to maximize phenolic acid production. Notably, the highest yield of phenolic acid was achieved at moderate concentrations of MgO and Fe₃O₄ NPs. Conversely, elevated concentrations of Fe₃O₄ NPs in conjunction with the lowest concentrations of MgO NPs were found to effectively stimulate ganoderic acid production.

Validation of the optimized culture conditions

Under the optimal conditions, which involved the utilization of 0.27 g/L of Mgo and 0.34 g/L of Fe3o4 and 0.1 g/L of Mgo and 0.5 g/L of Fe₃O₄ the models predicted that the phenolic and ganoderic acid production content would peak at 13.22 mg gallic acid/g DWB and 4.85 mg ganoderic /100mg DWB respectively. In order to assess the accuracy of the model equation, the predicted optimal conditions were implemented in an experimental setting. Under these experimental conditions, the measured the phenolic and ganoderic acid production was determined to be 13.5 mg gallic acid/g DWB and 4.65 mg ganoderic /100mg DWB respectively. The achieved value of 46.5 mg/g DWB surpasses all reported submerged fermentation studies: it is 1.3× higher than Xu et al. (2008) (35.3 mg/g) [38], 3.7× higher than Fang & Zhong (2002) (12.7 mg/g) [24], 4.2× higher than Tang & Zhong (2002) (11.2 mg/g) [39]. It also exceeds triterpenoid levels in antler-type *G. sichuanense* (6.37-11.28 mg/g) reported by Apiwatanapiwat et al. (2025)

[40] and falls within the upper range of values (0.38-35.43 $\mu\text{g}/\text{mg}$ extract) reported by Tokul-Ölmez et al. (2025) [41]. The phenolic content of 13.5 mg GAE/g DWB is competitive with literature values: it falls at the upper end of Masjedi et al. (2022) (6.71-14.53 mg/g) [42], exceeds Peng et al. (2024) (11.27 mg/g) [43], and is comparable to Apiwatanapiwat et al. (2025) (12.77-18.31 mg/g) [40]. Nanoparticle-supplemented submerge fermentation produced ganoderic acid levels exceeding all referenced studies and phenolic content comparable to or higher than recent reports, demonstrating this strategy's effectiveness for enhancing bioactive compounds in *G. lucidum*.

Solid state formulation toward ganoderma fruiting

The solid-state fermentation media, formulated from equal parts beech wood sawdust and chips and supplemented with 50 g/L glucose and 30 g/L corn steep liquor (CSL) [5], were enriched with nanoparticles. For *G. lucidum*, the media was supplemented with 0.1 g/L of MgO NPs and 0.5 g/L of Fe_3O_4 NPs, while for *G. lingzhi*, 0.5 g/L of both MgO and Fe_3O_4 NPs were used. All bags were then incubated following established protocols [44] to stimulate ganoderic acid production. After one month, all fruit bodies were harvested (figure 7). The highest ganoderic acid content recorded was 6 ± 0.8 of mg ganoderic /100mg DWB for *G. lucidum* and 5 ± 0.6 mg ganoderic /100mg DWB for *G. lingzhi*.



Figure-7 Fruiting bodies of *G. lucidum* (yellow) and *G. lingzhi* (red) cultivated under the optimal conditions predicted by RSM.

The study findings indicated a substantial enhancement in ganoderic acid through solid-state fermentation in comparison to submerged fermentation. Compared to submerged fermentation,

solid-state fermentation has different records of successful applications in the production of secondary metabolites [45].

The results of the MIC/MBC microdilution for both *G. Lucidum* and *G. Lingzhi*

Based on the initial experimental data, ganoderic acid extracted from *G. lucidum* demonstrated potent antibacterial activity against both Gram-positive and Gram-negative bacteria [46,47]. Complete inhibition of *Bacillus subtilis* (PTCC1715), *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC25923), *Micrococcus luteus* (ATCC9341), and *Pseudomonas aeruginosa* (ATCC27853) was observed at concentrations of 46, 30, 30, 46, and 46 µg/mL, respectively, with no detectable colony formation after 48 h of incubation. Lower concentrations (38, 23, 23, 38, and 38 µg/mL) resulted in minimal residual growth. These findings are consistent with previous reports documenting the antibacterial properties of *Ganoderma* extracts against pathogenic bacteria [48,49], with methanolic and other solvent extracts showing particular efficacy against *E. coli* and *Pseudomonas aeruginosa* [49,50]. Further optimization of concentration parameters revealed complete eradication of *B. subtilis*, *E. coli*, *S. aureus*, and *M. luteus* at 45, 26, 26, and 45 µg/mL, respectively, while concentrations of 34, 22.5, 22.5, and 34 µg/mL corresponded to the minimum inhibitory concentrations (MICs). The determination of MIC values followed established methodologies for assessing antimicrobial activity [51–53], providing quantitative measures of antibacterial potency comparable to those reported for other natural products and antimicrobial agents [53,54]. Similarly, ganoderic acid derived from *G. lingzhi* demonstrated complete inhibition of the tested bacterial strains at concentrations of 50, 32, 32, 50, and 50 µg/mL, while MIC values were recorded at 35, 23, 23, 35, and 35 µg/mL (table 6). The observed antibacterial activity against both Gram-positive and Gram-negative bacteria aligns with studies demonstrating the broad-spectrum potential of *Ganoderma* extracts [46,55]. Specifically, previous investigations have shown that *Ganoderma* extracts are particularly effective against *S. aureus* [56] and other Gram-positive bacteria [57], with bioactive compounds such as triterpenoids [55] implicated in mechanisms involving disruption of cell membrane permeability and integrity [55]. Notably, the relatively lower MIC values observed in the current investigation reflect the effectiveness of the statistical method used to promote bioactive components, resulting in an enhanced antimicrobial effect compared to extracts described in previous studies [48–50].

Table-6 the antibacterial properties of ganoderic acid, extracted from both *G. Lucidum* and *G. Lingzhi*, using the microdilution approach

Bacteria	MIC/MBC (µg/ml)	
	<i>G. Lucidum</i>	<i>G. Lingzhi</i>
Bacillus cereus	34/45	35/46
Escherichia coli	22.5/26	23/29
Staphylococcus aureus	22.5/26	23/29
Micrococcus luteus	34/45	35/46
Pseudomonas aeruginosa	34 /45	34/46

Cytotoxicity of shiitake against human *breast cancer cell line MDA-MB-231*

An investigation into the cytotoxic effects of extracts derived from *G. lucidum* and *G. lingzhi* on the human triple-negative breast cancer (TNBC) cell line MDA-MB-231 demonstrated significant reduction in cell viability following 48 h of incubation. These findings align with previous studies reporting dose- and time-dependent cytotoxicity of *Ganoderma* extracts in TNBC models [58,59]. At a concentration of 500 µg/L, both extracts induced marked cytotoxicity, consistent with the anti-proliferative properties documented across multiple *Ganoderma* species [60–62]. The half-maximal inhibitory concentration (IC₅₀) values were determined to quantify antiproliferative potency, yielding IC₅₀ values of 581.05 µg/L for *G. lucidum* and 299.78 µg/L for *G. lingzhi* (figure8-9)

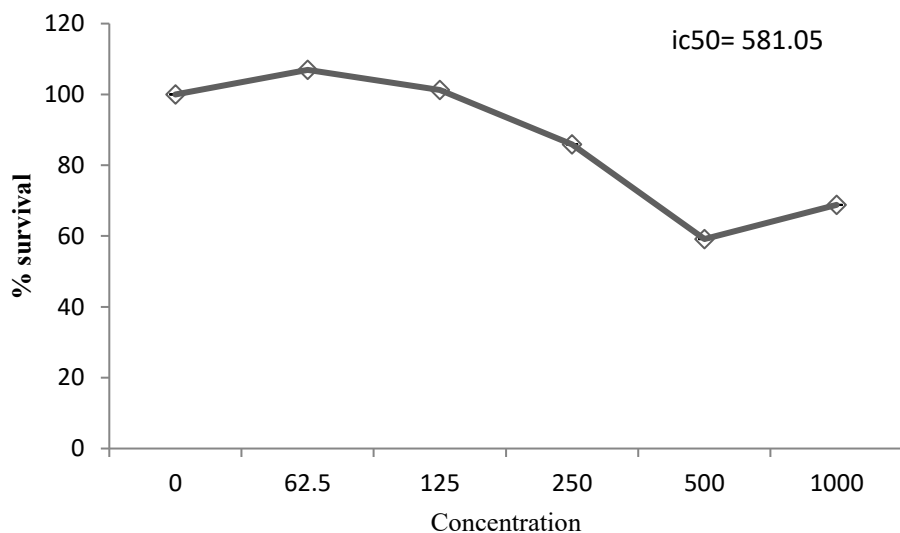


Figure-8 Cytotoxic impact of *G. lucidum* on MDA-MB-231 cells. Each bar represented the mean $\pm$ SD of three experiments.

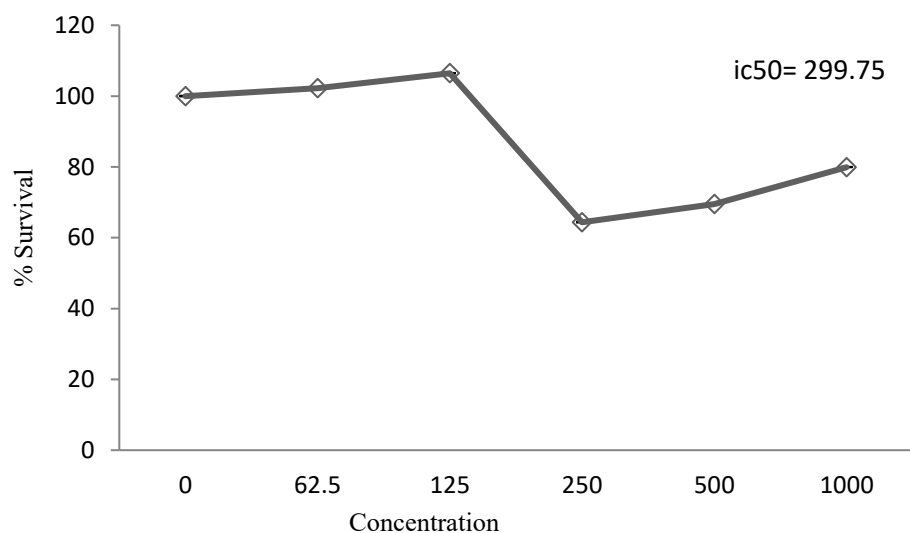


Figure-9 Cytotoxic impact of *G. lingzhi* on MDA-MB-231 cells. Each bar represented the mean $\pm$ SD of three experiments.

These values fall within the range of active concentrations reported in the literature, where crude extracts typically exhibit IC_{50} values of approximately 1.4–1.5 mg/L (1400–1500 μ g/L) [59] and fractionated preparations show variable potency depending on bioactive compound composition

[61]. The lower IC₅₀ for *G. lingzhi* indicates greater antiproliferative potency than *G. lucidum*, likely due to higher concentrations of bioactive constituents achieved through our innovative statistical approach

Conclusion

Two fungal isolates were conclusively identified through ITS sequencing and phylogenetic analysis, confirming their classification as *G. lucidum* and *G. lingzhi*. The investigation revealed that metal oxide nanoparticles exert diverse and species-specific effects on fungal metabolism. The optimized fermentation process not only significantly increased the yields of target metabolites but also produced extracts with potent biological activities. The ganoderic acid obtained exhibited strong, broad-spectrum antibacterial effects against clinically relevant Gram-positive and Gram-negative bacteria, with minimum inhibitory concentration (MIC) values comparable or superior to those reported in previous studies. Furthermore, both *Ganoderma* extracts demonstrated significant cytotoxicity against the aggressive, triple-negative breast cancer cell line MDA-MB-231. The superior potency observed for the *G. lingzhi* extract highlights its potential as a rich source of anticancer agents. Collectively, these findings establish a solid and efficient statistical framework for optimizing the synthesis of bioactive metabolites with significant therapeutic properties in *G. lucidum* and *G. lingzhi*.

Materials and Methods

All analytical-grade chemicals and reagents were purchased from Merck (Darmstadt, Germany). Potato dextrose agar (PDA) was obtained from QUELAB (England). Titanium dioxide nanoparticles (TiO₂ NPs; ~15 nm), magnesium oxide nanoparticles (MgO NPs; ~20 nm), Iron Oxide Nanopowder (Fe₃O₄ NPs; ~15nm), Silicon Oxide Nanoparticles (SiO₂ NPs; ~20nm), Zinc Oxide Nanoparticles (ZnO NPs; ~40 nm) with ≥99% purity were purchased from US Research Nanomaterials Inc. Concentrations of extracts were obtained under reduced pressure using a rotary evaporator (Heidolph Laborota 4000, Germany), and dried using a vacuum freeze-dryer (Christ Alpha 1–2, Germany).

Fungal Isolation and Molecular Identification

Ganoderma spp. were purchased from local market and transferred to the Faculty of Agriculture, Shahrood University of Technology, Iran. Samples were cultured on PDA plates and incubated

at 25 ± 2 °C for 5 days to obtain pure isolates. Genomic DNA was extracted from actively growing mycelia following the protocol described by [22]. DNA quality and concentration were assessed using 1% (w/v) agarose gel electrophoresis and a NanoDrop spectrophotometer (Thermo Scientific, USA). Molecular identification was performed by amplifying the ITS1–5.8S–ITS2 rDNA region using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers (White et al., 1990). Additionally, the calmodulin (CaM) gene was amplified using CMD5 and CMD6 primers to ensure accurate species-level identification (Hong et al., 2005). PCR conditions included initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation (94 °C, 1 min), annealing (57 °C, 30 s), extension (72 °C, 1 min), and a final extension at 72 °C for 8 min. PCR products were visualized on 1.2% agarose gels under UV illumination and sequenced commercially (Bio Magic Gene, Iran). Sequence quality was analyzed using Chromas Lite v2.1 and compared with reference sequences in GenBank using BLAST. Phylogenetic analysis was conducted using MEGA X software, and a maximum parsimony tree was constructed with 1,000 bootstrap replicates.

Microorganisms, Inoculum preparation, and fermentation conditions

The identified *Ganoderma* strain was maintained on PDA slants at 4 °C for long-term storage. For inoculum preparation, mycelial plugs were transferred into 250 mL Erlenmeyer flasks containing 50 mL of seed medium composed of glucose (30 g/L), yeast extract (10 g/L), and macro-mineral elements. Cultures were incubated at 28 °C and 185 rpm for 5 days to obtain actively growing inoculum. Subsequently, 5% (v/v) fresh seed culture was inoculated into fermentation media to enhance biomass formation and secondary metabolite production [23]

Fermentation Media and Experimental Design

Submerged fermentation experiments were performed to evaluate the effects of nanoparticles supplementation on ganoderic acid and polysaccharide production. The basal medium consisted of 50 g/L glucose, 30 g/L CSL syrups, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1 g/L), CaCl_2 (0.2 g/L), KH_2PO_4 (1.5 g/L), and CaCO_3 (1.5 g/L), with incubation at 28 °C and 185 rpm as constant parameters [5]. TiO_2 , Mgo, Fe_2O_3 , ZnO, SiO_2 NPs were added separately at concentrations of 0.01, 0.05, and 0.1 g/L to investigate their effects on secondary metabolite biosynthesis.

Analytical Methods

Determination of Ganoderic Acid (GA)

Ganoderic acid was extracted from dry weight biomass (DWB) using 70% (v/v) ethanol following the method of [24]. Briefly, 0.1 g of powdered biomass was extracted with 10 mL ethanol, centrifuged, and evaporated. Liquid–liquid extraction was performed using chloroform and NaHCO₃ (5%, pH 3). The final GA extract was dissolved in ethanol and quantified spectrophotometrically at 245 nm. Thymol (Merck, 108,167, Crystal) and absolute ethanol were used as standard solution and control solution, respectively. The concentration of thymol calibration solutions ranged from 0.1 to 1 mg/mL.

Solid-State Fermentation for Fruiting Body Production

The optimized submerged fermentation medium was adapted for solid-state cultivation. Beech wood sawdust and chips (1:1, w/w) were detoxified using alkaline treatment and soaked in 50 ppm NaOCl for 6 h, then supplemented with optimal key substrates. All bages were sterilized at 121 °C for 2.5 h, inoculated with spawn, and incubated under controlled conditions to induce fruiting body formation [5].

Extraction of Phenolic Compounds

Dried tissues of mushroom were ground and extracted with 90% ethanol at 25 °C for 72 h, then centrifuged, the residue was concentrated by rotary evaporator under vacuum (Heidolph Laborota, Germany; Nickavar and Esbati, 2012). Extracts were analyzed for total phenolic content using the Folin–Ciocalteu method, expressed as mg gallic acid equivalents (GAE)/g dry weight [25].

Antimicrobial Activity

Antibacterial activity was assessed against *Bacillus subtilis* (PTCC1715), *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC25923), *Micrococcus luteus* (ATCC9341), and *Pseudomonas aeruginosa* (ATCC27853) using the broth microdilution method. MIC and MBC values were determined using TTC dye and BHI agar confirmation, respectively [26].

Cell Culture and Cytotoxicity Assay

MDA-MB-231 breast cancer cells and MCF-10A normal epithelial cells were cultured in DMEM supplemented with 10% FBS and antibiotics. Cytotoxic effects of *Ganoderma* extracts were evaluated using the MTT assay after 48 h exposure at concentrations ranging from 62.5–1000 µg/mL [7].

Statistical Analysis

One-factor-at-a-time (OFAT) experiments were conducted to identify key substrates. Central composite design (CCD) under response surface methodology (RSM) was applied using Design-Expert v7.0.0. ANOVA evaluated model significance, and Duncan's multiple range test ($p < 0.05$) was used for mean comparisons. All experiments were performed in triplicate.

Author contributions

Ahmad Najafi Moghadam: Data curation, Formal analysis, Investigation, Resources, Software, editing. Hamid Reza Samadlouie: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing – review & editing. Amir Salek Farrokhi: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing – review & editing. Shideh Mojerlou: Data curation, Investigation, Project administration, Resources, Writing – review & editing. Mohamad Reza Najmedi: Data curation, Formal analysis, Investigation, Resources, Software

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Declarations

Competing interests

The authors declare no competing interests.

Research involving plants

All authors comply with the IUCN Policy Statement on Research Involving Species at Risk of Extinction.

Data availability

Sequence data that support the findings of the isolated species in this study have been deposited in The National Center for Biotechnology Information with the accession number PP064858 and PP064859. All data generated or analyzed during this study are included in this published article.

Additional information

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