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Synergistic Effect of Elicitors in Enhancement of Ganoderic Acid Production: Optimization and Gene Expression Studies

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Abstract

Ganoderma lucidum is one of the most well-known fungi, and has many applications in medicine. Ganoderic acid is among the valuable secondary metabolites of *Ganoderma lucidum*, and responsible for the inhibition of the tumor cell growth and cancer treatment. Application of ganoderic acid has been limited because of low yields of its production from *Ganoderma lucidum*. The present study aims to investigate the synergistic effect of elicitors including methyl jasmonate and aspirin on the production of ganoderic acid derived from *Ganoderma lucidum* mushroom in a shaken flasks using response surface methodology. The results showed that the optimal dose of methyl jasmonate and asprin significantly impacts on the amount of ganoderic acid production as a response ($p<0.05$). The proposed model predicted the maximum ganoderic acid production as 0.085 $mgml^{-1}$ in which the optimal concentrations obtained for methyl jasmonate and asprin were 250 mM and 4.4 mM, respectively. Also the influence of ganoderic acid production on the expression of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase and squalene synthase (two important metabolic pathway genes in ganoderic acid) was investigated, and the results showed that these genes' expression has increased by 10 and 11 folds, respectively.

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1.Introduction

The concept of "functional foods" started to form due to the increasing awareness of the relationship between diet and disease. According to the American Academy of Sciences, any food that is beneficial to the health is introduced as a functional food [1]. Consequently, the concept of "food as medicine" is proposed as functional foods. Nowadays, functional foods not only are able to cure the diseases, but based on a huge burden of evidence, they have key role in disease prevention as well.

At least 270 species of fungi have been identified to have various therapeutic properties, and in fact, the concept of "medicinal mushrooms" is under development [1]. *Ganoderma lucidum* is one of the popular medicinal mushrooms in the traditional Chinese and many Asian countries' medicine for more than 2000 years. These medicinal mushroom

species produce metabolites, including drug metabolites, called "triterpenes". Ganoderic acid (GA), which has been found in *Ganoderma lucidum*, is important due to its outstanding properties such as the ability to inhibit cholesterol synthesis [2], inhibition of tumor growth and cancer treatment [3-5], having anti-hepatitis B [6] and anti-human immunodeficiency virus (HIV) properties [7, 8]. Since *Ganoderma lucidum* is scarcely flourished in nature; hence, the commercial cultivation of industrial products and finding ways to increase production of its metabolites have been highlighted [9, 10]. Some of these methods including optimization of the environmental conditions, induction of elicitors, and genetic manipulation were applied for proliferation of their useful metabolites [11-15]. To increase GA production, elicitors were studied, and the results verified that various type of elicitors including phenobarbital, fungus extracts, and ether extract from the medicinal insects are effective [12-13, 16-17].

In 2010, increase in GA production under induction of methyl jasmonate (MeJA) was reported. Also, in other investigation, the use of aspirin (AS) in order to stimulate the production of GA was successfully proved [18, 19].

Literature survey on *Ganoderma lucidum* and GA production indicated that investigation on the interaction between these important parameters was ambiguous. In previous studies, one-factor-at-a-time methodology has been applied to optimize the abovementioned parameters [17-19]. Since more than one factor is considered, this method has not good efficacy, and also interactions between parameters are not analyzed. The only methodology capable of providing an answer to this question is factorial design of experiments (DOE), which through the use of techniques such as Response Surface Methodology (RSM), is able to simultaneously consider several factors at different levels, and give a suitable model for the relationship between the various factors and the response [20, 21].

Therefore, the objective goal of this study is to optimize the concentration of MeJA and AS, and investigate the synergistic effects of elicitors using RSM. Optimum concentrations to achieve maximum GA production were identified, and their effect on the expression levels of GA biosynthesis genes, including 3-hydroxy-3-methylglutaryl coenzyme A reductase (hmgr) and squalene synthase (sqs) was examined.

2. Materials and Methods

2.1. Fungus strain

Ganoderma lucidum fungus was purchased from the Cell Bank of Ferdowsi University of Mashhad (Iran) on solid medium, and cultured on potato dextrose agar (PDA).

2.2. Medium and culture conditions

500 ml flask containing 125 ml of liquid culture medium of yeast extract, peptone, glucose (YPG) was used to perform the experiments. Incubator temperature and shaking were set at around 28°C and 150 rpm, respectively. A solid punch with a diameter of 10 mm was added to 30 ml of the culture medium in each run [22].

2.3. Elicitors induction

Since MeJA (Sigma, USA) cannot directly dissolve in the aqueous phase of medium, it was dissolved in ethanol. Also AS (Sigma, USA) was dissolved in ethanol, and both of them were sterilized by filtering. The same volumes of ethanol were added to the culture medium as a control sample**.**

2.4. GA measurement

After the cultivation process, GA was extracted according to Tang et al. (2002). Briefly, compounds from dried mycelia (100 mg) were dissolved in ethanol 50% (vv^{-1}) twice for one week. After centrifugation to remove mycelia, the supernatants were dried under vacuum and at 50°C. The residue was dissolved in water, and the precipitate was isolated by chloroform. The GA in chloroform was removed by 5% NaHCO3 (wv^{-1}), and then 2 N HCL was added to adjust the pH of the NaHCO3 phase below 3.0. Then GA was isolated by chloroform, and evaporated at 40°C. GA was dissolved in absolute ethanol, and the absorbency at 245 nm was detected [19].

2.5. Experimental design for RSM

A central composite design (CCD) was adopted to study two parameters at three levels According to the literature, a range was selected for two elicitors (Table 1). Therefore, to investigate the synergistic effect of MeJA and AS, 13 experiments were designed using MINITAB, release 15 (Table 2).

The analysis of variance (ANOVA) was used to evaluate the statistical significance of the full quadratic models predicted. The significance and the magnitude of the effect estimates for each variable and their possible linear and quadratic interactions effects were also determined. The significance level employed in the analysis was 5% ($p<0.05$).

Finally, the model was used to predict the optimum value of the factors, which gives maximum or fairly high GA. To achieve the greatest effect, the induction time of MeJA and AS was appointed in growth logarithm phase and stationary phase, respectively [19, 20]. To find the influence of each elicitor on GA production, the experiments were repeated by each elicitor separately.

Table 1. Variable levels for optimization of Ganoderic acid production

Variable	Level (-1)	Level (0)	Level (1)	Ref.
Methyl jasmonate (µM)	10	130	250	[18,19]
Asprin (mM)	0.5	4.25	8	[20]

Table 2. Design experiment of variables data based on RSM

	Factors		Response
Experiment number	MeJA (µM)	AS (mM)	GA ($mgml^{-1}$)
1	10	0.50	0.019
2	10	8.00	0.033
3	250	0.50	0.075
4	250	8.00	0.077
5	130	0.50	0.055
6	130	8.00	0.050
7	10	4.25	0.033
8	250	4.25	0.091
9	130	4.25	0.070
10	130	4.25	0.060
11	130	4.25	0.062
12	130	4.25	0.069
13	130	4.25	0.062

2.6. RNA isolation and quantitative real-time PCR

Aliquot of 0.1g of mycelium was separated from the culture medium and frozen by liquid nitrogen. Total RNA was extracted by RNX plus (Sinacolon, Iran). cDNA synthesis was performed by Synthesize Kit (Amplicon, Denmark). The random primer was used for cDNA synthesis. Then the transcript levels of hmgr and sqs were determined by quantitative real-time PCR using Cyber Green (Applied Biosystem, USA). A reliable source was adopted to design the primers (Table 3). 18srRNA gene was used as an internal control, and $2^{-\Delta\Delta CT}$ was applied for analysis.

2.7. Statistical analysis

The experimental results were analyzed statistically using Student's t-test (S) with SPSS (version 17). All experiments were carried out in triplicate. P-values <0.05 were defined as significant.

3. Results and Discussion

3.1. Model fitting

Table 2 lists the values of GA at each of the 13 combinations of factor levels with the values ranging from 0.019 to 0.091 $mgml^{-1}$. The values of the regression coefficients are presented in Table 4. The linear terms, as well as the second order terms of the independent parameters were significant. The statistical analysis of the interaction terms showed that, at 5% significance level, there were prominent interactions between MeJA and AS.

Table 3. Primer sets used for quantitative real-time PCR

Primer names	Sequences	Product length (bp)	Resource
Gl-hmgr	5-GTCATCCTCCTATGCCAAAC-3 5-GGGCGTAGTCGTAGTCCTTC-3	166	[19]
Gl-SQS	5-ACAGTTGTCAGCGAAGAGC-35-CGTAGTGGCAGTAGAGGTTG-3	170	[19]
18srRNA	5-GTCATCCTCCTATGCCAAAC-35-GGGCGTAGTCGTAGTCCTTC-3	200	[14]

Based on the calculated values of the regression coefficients (Table 4), a polynomial regression model equation that fitted 95.56% of the variation in the data is proposed as follows:

$$GA = 0.042 - 0.0057As + 0.0221Mj - 0.0236As.As + 0.0049Mj.Mj - 0.0035As.Mj$$

According to the results presented in Table 4, MeJA and AS are effective parameters for having higher regression coefficients. Figure 1 illustrates the contour plots of response versus the variation of the two significant parameters (MeJA and AS).
That's shown, for obtaining the highest value of response (GA), the level of MeJA should be around 250 µM (dark green regions in Figure 2). Furthermore, GA is in its maximum value when AS is in its lowest level; that is the minimum dose of AS correlates to the maximum production of GA. In fact, inoculation of the optimized value of elicitors (MeJA: 250 µM and AS: 4.40 mM) yielded 0.8 $mgml^{-1}$ GA, which was higher than the control sample (0.2 $mgml^{-1}$). In previous works, this increasing was reported almost 45% with the induction of MeJA [19].

3.2 Interaction amongst the factors involved in GA production

In the cases where interaction between the factors is statistically significant, surface plots give more complete information regarding the effect of a factor on the response. Examination of the surface plot presented in Figure 2 shows that the effect of MeJA on GA production as pharmaceutical metabolite is higher than that of AS.

Amounts of GA in various conditions and the effect of inoculation of AS (as the induction of apoptosis) and MeJA (as an inducer) on the production of GA are illustrated in Figure 3. Also the optimal synergy of the two elicitors comparing to the control is shown in the figure.

Table 4. Regression coefficients for determination of variable significance on Ganoderic acid and their significance for the response surface model

Independent factor	Regression coefficient	P-value
Constant	0.042	< 0.001
AS	-0.0057	< 0.001
MeJA	0.0221	< 0.001
AS.AS	-0.0236	0.004
MeJA. MeJA	0.0049	0.010
AS. MeJA	-0.0035	0.019
R^2	95.56	

The model showed that the synergistic effect of these two elicitors on GA production is far greater than the effect of each of them. To date, the interaction of these two elicitors has not been reported, but several studies have been performed using these elicitors to investigate the effect of AS and MeJA separately on GA production. They reported that influence of MeJA was 45.3% higher than the untreated control sample [19, 20].

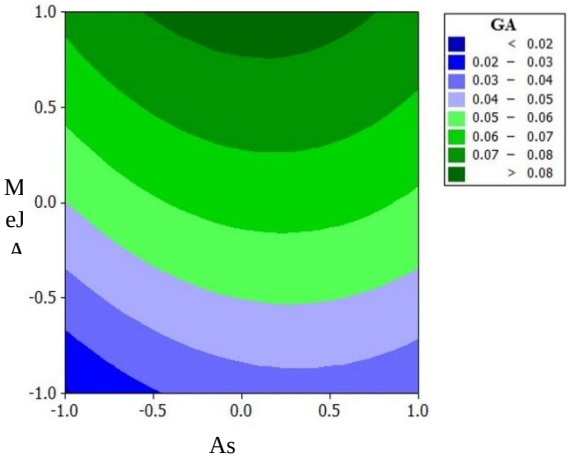

Figure 1. Contour plots for response (GA production) with respect to MeJA and AS (legend; GA: Ganoderic acid, AS: Asprin, MJ: Methyl jasmonate)

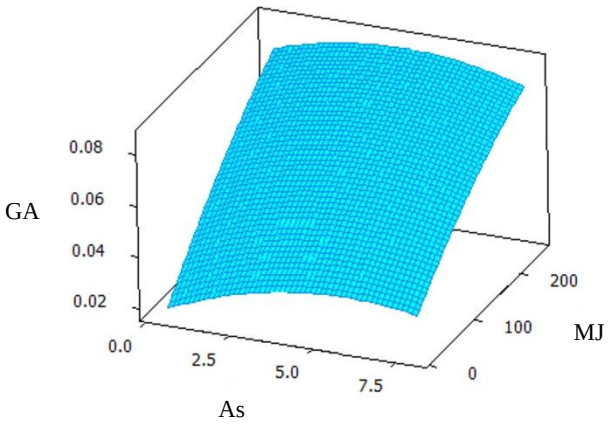

Figure 2. Surface plots for response (GA production) with respect to MeJA and AS (legend; GA: Ganoderic acid, AS: Asprin, MJ: Methyl jasmonate)

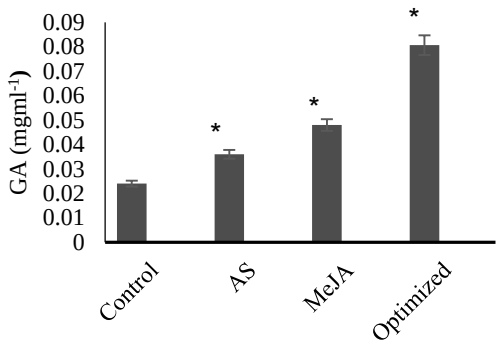

Figure 3. GA production ($mgml^{-1}$) in the optimal dose of aspirin (alone), MeJA (alone), and the synergistic effects of elicitors (optimized) (*p <0.05, n=3)

3.3. Transcriptional responses to stimulation of elicitors

GA is one of the important active triterpenoid components, and is synthesized via mevalonate pathway (Figure 4), and hmgr and sqs are considered as critical enzymes in the terpenoeid synthesize pathway.

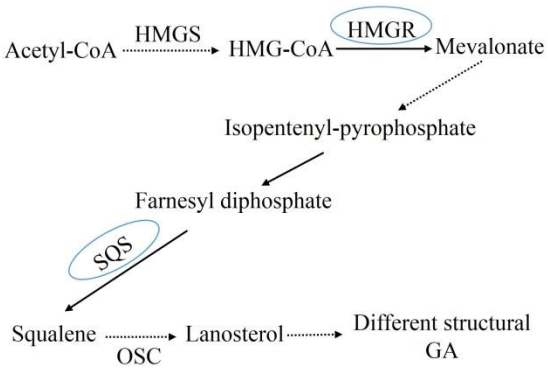

Figure 4. Mevalonate pathway of GA biosynthesis in G. lucidum
HMG-CoA: hydroxy-3-methylglutaryl-Coenzyme A; HMGS: HMG-CoA synthase; HMGR: HMG-CoA reductase; MVD: mevalonate-5-pyrophosphate decarboxylase; IPP: isopentenyl-pyrophosphate; FPS: farnesyl pyrophosphatesynthase; FPP: farnesyl diphosphate; SQS: squalene synthase; OSC: oxidosqualene cyclase [12].

By comparing the expression level of hmgr and sqs in the optimal doses of MeJA and AS induction with the control, it was revealed that the level of gene expression of hmgr and sqs has been increased by 10 and 11 folds, respectively (Figure 5). The results indicated that hmgr and sqs are prominent genes in the GA biosynthesis pathway. Prior investigation on increasing GA production has indicated that the transcript levels of hmgr and sqs were 10-fold higher than that of the control [19].

4. Conclusion

The yield of GA production as secondary metabolite, naturally derived from *Ganoderma*

lucidum mushroom, is extremely low, and consequently, the application of this worthwhile metabolite as an anticancer is limited. MeJA as a factor that establishes signaling pathway, causing to enhance triterpene production, leads to increased production of GA. In addition, GA production can be stimulated by adding AS to the medium of *Ganoderma lucidum* during the culturing where it causes to apoptosis.

In this study, for the first time, the combinatory effect of induction of MeJA and AS on GA production was examined. The results showed that the highest amount of GA (0.085 $mgml^{-1}$) occurred when the concentration of MeJA and AS was 250 µM and 4.4 mM, respectively. The optimum concentrations were performed in triplicate, and the amount of average GA was found to be 0.081 mg per ml, showing 95% confidence interval for GA production under optimized conditions. Furthermore, the gene expression analysis of GA biosynthesis pathway confirmed these results.

The synergistic effect of elicitors on GA production was also investigated. Induction of MeJA in the growth logarithm phase caused to the maximum rate of GA production by *Ganoderma lucidum*. Then by adding AS to the medium in the stationary phase, crisis in the growth of *Ganoderma lucidum* was generated, which was highly effective on GA production. In fact, after initial growth of *Ganoderma lucidum*, by induction of apoptosis and establishment of its signaling pathway, *Ganoderma lucidum* was forced to produce greater amounts of GA. Statistical analysis of the interactions between MeJA and AS showed a significant relationship between the parameters at 5% level of confidence. MeJA (as inducer of GA production) and AS (causing to apoptosis) were independent to each other, and had their own effect on metabolite production. Finally, the optimization results were confirmed by increasing in the gene expression levels of two important genes involved in the mevalonate pathway.

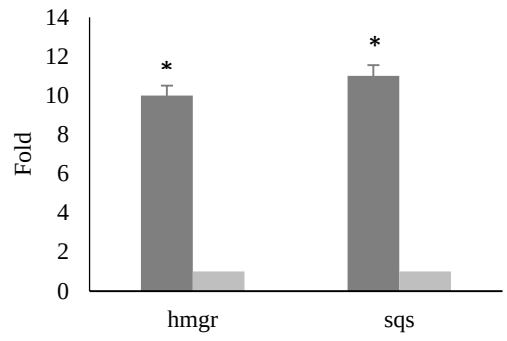

Figure 5. Analysis of gene expression of hmgr and sqs in optimal conditions comparing to the control (*p <0.05).

This present research is the first report on the synergistic effect of two elicitors, and provides useful information for future investigations on GA production from *Ganoderma lucidum.* This new approach could be developed by examining the influence of new elicitors on GA production.

5. Acknowledgment

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6. Conflict of interest

The authors have declared that there is no conflict of interest.

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