

Development and Optimization of Culture Medium for the Production of Glabridin by *Aspergillus eucalypticola*: An Endophytic Fungus Isolated from *Glycyrrhiza glabra* L. (Fabaceae)

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ABSTRACT

Glabridin is a well-known active isoflavone found in the root of licorice (*Glycyrrhiza glabra* L.) that possess a wide range of biological activity. Plant cells, hairy roots, and fungal endophytes cultures are the most important alternative methods for plant resources conservation and sustainable production of natural compounds, which has received much attention in recent decades. In the present study, an efficient culture condition was optimized for the biomass accumulation and glabridin production from fungal endophyte *Aspergillus eucalypticola* SBU-11AE isolated from licorice root. Type of culture medium, range of pH, and licorice root extract (as an elicitor) were tested. The results showed that the highest and lowest biomass production was observed on PCB medium (6.43 ± 0.32 g/l) and peptone malt (5.85 ± 0.11 g/l), respectively. The medium culture PCB was produced the highest level of glabridin (7.26 ± 0.44 mg/l), while the lowest level (4.47 ± 0.02 mg/l) was obtained from the medium peptone malt. The highest biomass (8.51 ± 0.43 g/l) and glabridin (8.30 ± 0.51 mg/l) production were observed from the PCB medium adjusted with pH = 6, while the lowest value of both traits was obtained from the same medium with pH = 7. The highest production of total glabridin (10.85 ± 0.84 mg/l) was also obtained from the culture medium treated with 100 mg/l of the plant root extract. This information can be interestingly used for the commercialization of glabridin production for further industrial applications.

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1. Introduction

The plant taxa include a large number of medicinal and aromatic plants (MAPs) which contain various bioactive molecules [1,2]. Despite the progress in modern medicine, the use of MAPs has been an important part of the health ensuring of human societies for centuries. Currently, the active ingredients and phytochemical compounds of MAPs are widely used in the pharmaceutical, food, and cosmetic industries [3–5]. Therefore, these plants are still introduced as a source of new and valuable medicinal compounds.

Due to the increasing demand for plant-derived compounds, their low concentration in the plant materials, low regeneration rate of plants in nature, the risk of vulnerable plants extinction [6], looking for high-yielding alternative and sustainable sources of biologically active compounds is critically needed. The chemical synthesis of many phytochemical compounds is still complex and expensive. Therefore, cell and tissue culture, as well as isolation and culture development of plant endophytes are

the most important alternative methods for the production of specialized metabolites (SMs) [4,7].

Plant endophytes contain a variety of nonpathogenic and beneficial microorganisms including fungi and bacteria. These sources reside in the host cells without any symptoms and increase plant function [8]. Studies have shown that fungal endophytes are new and alternative sources of SMs [9]. These microorganisms are capable to produce many bioactive compounds similar to the host plants and a variety of new metabolites, as well. Fungal endophytes are a climate-change independent source of many SMs including alkaloids, flavonoids, phenolic acids, steroids, terpenoids that grow well at normal temperatures and neutral pH [10,11]. Many SMs-producing endophytic fungi such as *Fusarium mairei* [12], *Epicoccum nigrum* strain YEF2 [13], *Nigrospora* sp. LLGLM003 [14], *Aspergillus niger* 58 [15], *Alternaria brassicicola* [16], and *Penicillium canescens* [17] have been reported so far.

Optimization of culture medium is an efficient strategy for mass production of valuable bioactive compounds through fungal endophytes cultures.

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The effect of pH, temperature, level of carbon and amino acids, biotic and abiotic elicitors to increase biomass yield and the production of SMs in the fungal endophytes cultures has been widely studied [18–20]. The optimization of the culture medium has also a significant effect on the level of their antimicrobial activity [21].

Licorice (*Glycyrrhiza glabra* L.), belongs to the family Fabaceae, is one of the most important and widely used medicinal plant [22]. The plant is native to southern Europe and parts of Asia. It is widely cultivated in Belgium, England, France, Iran, Germany, and Italy [23,24]. The plant roots contain important flavonoids including glycyrrhizic acid, glabridin, isoliquiritigenin, and liquiritin [25], which have many commercial applications in the pharmaceutical, food, and tobacco industries as flavorings and sweeteners [26,27].

Glabridin (C₂₀H₂₀O₄) is a well-known prenylated isoflavones (4-[(3*R*)-8,8-dimethyl3,4-dihydro-2*H*-pyrano[2,3-*f*]chromen-3-yl]benzene-1,3-diol) (Figure 1) extracted from the licorice root that possessive many biological activities such as anti-inflammatory, anti-bacterial, anti-tumor, anti-osteoporosis, and diabetes treatment properties [25,28,29]. It also has tyrosinase and melanogenesis activities, inhibits skin inflammation, and can act as a skin brightener [30]. Due to the low concentration of glabridin in licorice materials [31], looking for other sources or alternative production methods such as biotechnological techniques is a great interest. To date, several attempts have been performed to produce glycyrrhizic acid through tissue, cell, and hairy root cultures of licorice [32,33], but our literature survey revealed that the production of glabridin through biotechnological methods has not been reported yet.

Recently, *Aspergillus eucalypticola* SBU-11AE was isolated from *G. glabra* root (Figure 2(A,B)) as a

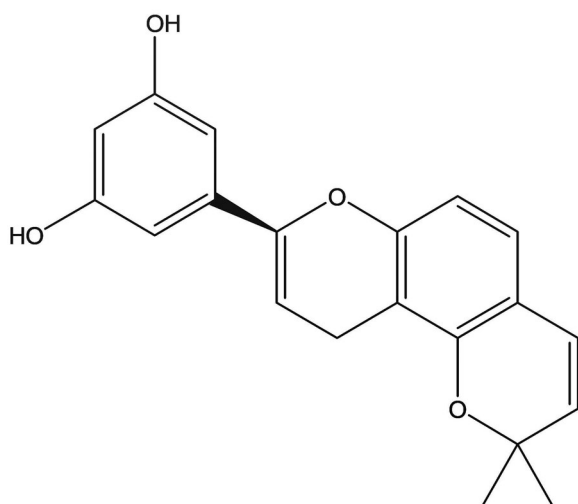


Figure 1. Chemical structure of glabridin.

glabridin-producing endophytic fungus (Data not shown). Due to the commercial and medicinal importance of glabridin, this study was aimed to increase fungal biomass and glabridin production of *A. eucalypticola* SBU-11AE culture by screening of the fungal culture media constitution, optimizing the pH level in promising medium as well as adding of licorice root extract to promise medium under optimized pH condition. The results obtained can be interestingly used for further commercial production of glabridin in fermentation systems.

2. Materials and methods

2.1. Fungal endophyte

Licorice endophyte fungus, *A. eucalypticola* SBU-11AE (stored on the sand at 4 °C) that produce glabridin (data not published), obtained from Beneficial Microorganisms Lab, Department of Agriculture, Medicinal Plant and Drugs Research Institute (MPDRI), Shahid Beheshti University, Tehran, Iran. To prepare the working culture of fungus, 100–200 mg of inoculated sand was cultured on 8 cm Petri dish containing PDA (Potato Dextrose Agar) medium and incubated at 28 °C for seven days (Figure 2(C)) [34,35].

2.2. Media culture and treatments

Five media culture including of potato dextrose broth (PDB: 200 g potato + 20 g dextrose per/l) (Figure 2(D)), potato carrot broth (PCB: 100 g carrot, 100 g potato + 20 g dextrose per/l) (Figure 2(E)), yeast extract peptone dextrose (YEPD: 10 g of yeast extract, 10 g peptone + 20 g dextrose per/l) (Figure 2(F)), malt (30 g/l + 20 g dextrose per/l) (Figure 2(G)), and malt + peptone (30 g malt + 5 g peptone per/l) (Figure 2(H)), were selected according to previous studies described [36–39]. Briefly, *A. eucalypticola* SBU-11AE spore suspension (10⁵ spore per mL) added to 500 mL Erlenmeyer flask containing 150 mL medium culture and then incubated at 28 °C under darkness condition for two weeks.

To examine the effect of pH on fungus mycelia biomass and glabridin production in promising medium, the fungal spore suspension (10⁵ spore per mL) was added to PCB medium with different pH levels including 5 (Figure 2(I)), 6 (Figure 2(J)), and 7 (Figure 2(K)). The pH level of the PCB culture medium was adjusted by adding hydrochloric acid (HCl 1N) and sodium hydroxide (NaOH 1N) as described previously [40]. The PCB medium with initial pH = 5.5 ± 2 was considered as the control. The flasks were then incubated under the same

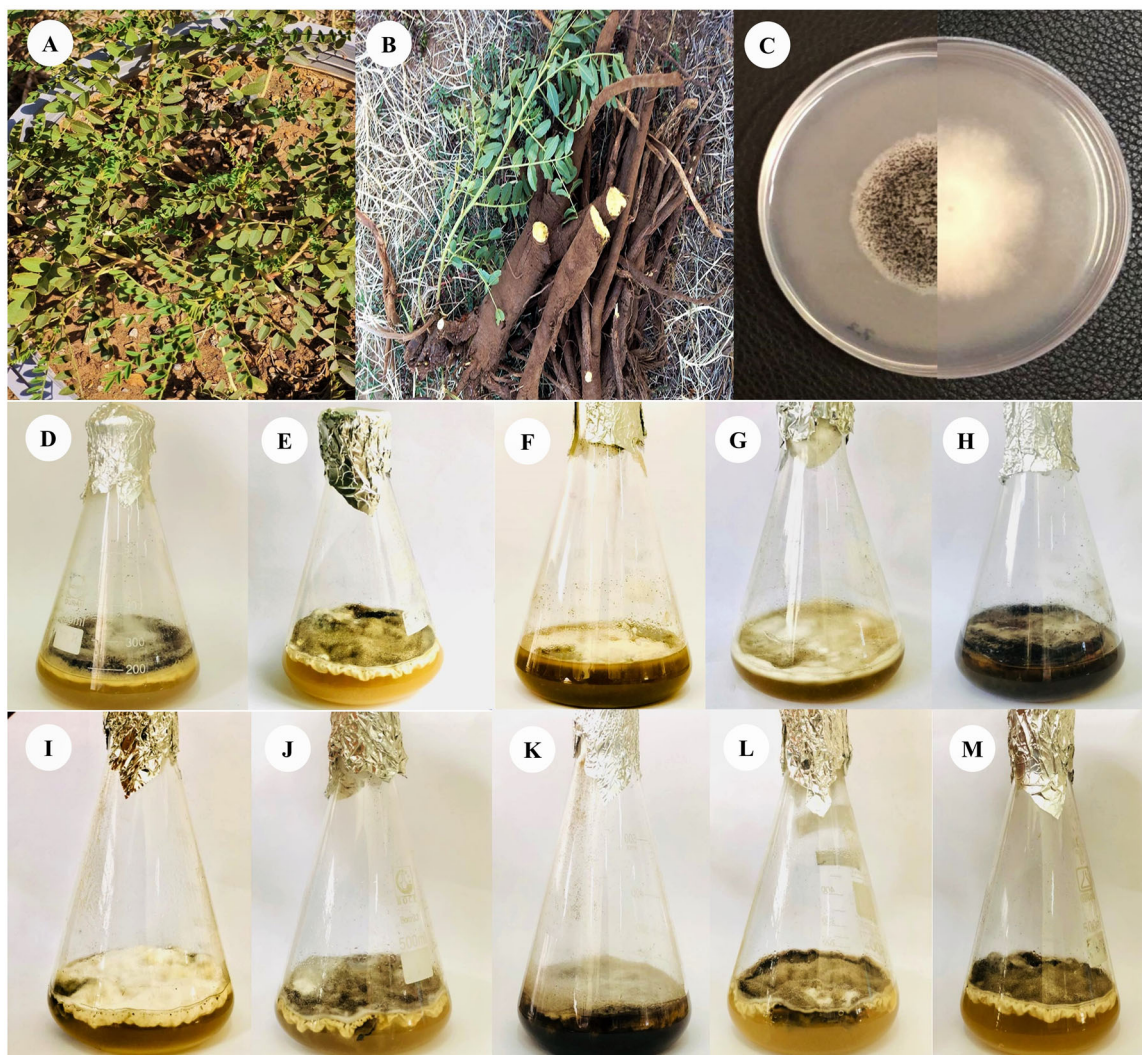


Figure 2. Representative information of *Glycyrrhiza glabra* used in this study. Wild plant that served as root source (A); collected of the plant roots used for the isolation of fungal endophyte (B); forward (left) and backward surface of isolate on PDA medium (C); fungal endophyte grown in PDA (D), PCB (E), YEPD (F), malt (G), and malt + peptone (H) media. Fungal endophyte cultured in PCB medium with pH of 5 (I), 6 (J), and 7 (K). Fungal endophyte elicited by 50 mg/l (L) and 100 mg/l (M) of the plant root (*Glycyrrhiza glabra*) methanolic extract.

conditions at 28 °C for two weeks. Fungal mycelia and culture medium were collected for further phytochemical analysis.

2.3. Elicitation

The oven-dried and powdered roots (10 g) of the plant host (*G. glabra*) were extracted with methanol 70% (100 ml) by sonication for 30 min. The extract was then centrifuged at 4400 rpm for 10 min and the organic phase was concentrated using a rotary evaporator (Laborota 4000 efficient, Heidolph, Germany) at 40 °C. Finally, the concentrated solution was poured into a plate and dried in an oven at 40 °C. Dried methanolic extract 50 (Figure 2(L)) and 100 (Figure 2(M)) mg/l was then added to the selected medium culture (PCB with pH = 6) as an elicitor. Each conical flask was incubated under the

same conditions at 28 °C for two weeks. The experiment was repeated three times. at the end of the growth period, extraction of the mycelium and culture medium was considered for the extraction and phytochemical analysis.

2.4. Extraction and HPLC-PDA analysis

The glabridin content of mycelia (intracellular) and supernatant fluid (extracellular) of fungus were determined separately. Briefly, the mycelia were separated and dried at 40 °C for 48 h. The dried and pulverized mycelia (1 g) were extracted with 70% methanol (10 ml) by sonication for 30 min as described previously [41]. The extracts were then centrifuged (4400 rpm) for 10 min and filtered by Whatman No 1 filter paper. The organic phase was evaporated with a rotary evaporator at 40 °C. The

extracts were dissolved in 2 ml of high-performance liquid chromatography (HPLC) grade methanol and used for the analysis.

The glabridin extraction from the supernatant fluid was carried out according to El-Sayed, et al. [42] method. For instance, 150 ml of the filtered culture media was shaken in a separating funnel with 50 ml of ethyl acetate. The organic phase was then evaporated using a rotary evaporator at 40 °C. The residue was dissolved in 2.0 ml of HPLC methanol and used for further analysis.

The Waters HPLC device equipped with a PDA 996 detector and C18 column (25 cm × 4.6 mm Eurospher 100-5) was used for quantification analysis of the glabridin. The mobile phase was acetonitrile (A), and water containing 0.3% phosphoric acid (B) with a flow-rate of 1 ml min⁻¹. The glabridin was measured at 230 nm. Different concentrations of glabridin (500, 250, 100, 50, 25, 10, 5 ppm) were used to draw the calibration curve. the correlation coefficient (*R*₂) was equal to 0.998.

2.5. Statistical analysis

This study was conducted in a completely randomized design (CRD) with three replications. The data were analyzed by Excel 2016 and SPSS 26 software. The mean values were compared using one-way ANOVA with Duncan's test.

3. Result and discussion

The analysis of variance (ANOVA) showed that the type of culture medium had a significant effect on the biomass and glabridin level produced by the fungal endophyte *A. eucalypticola*. The highest and lowest biomass production was observed on PCB medium (6.43 ± 0.32 g/l) and peptone malt (5.85 ± 0.11 g/l), respectively. The medium culture PCB was produced the highest level of glabridin (7.26 ± 0.44 mg/l), while the lowest level (4.47 ± 0.02 mg/l) was obtained from the medium peptone malt (Table 1). It seems that PCB medium had a better effect on the growth and development of metabolic susceptibility of endophytic fungus than other culture media due to the presence of starch as a carbon source. The superiority of the PCB culture medium may be also due to the presence of vitamin-rich (A, K, C, B, and E) of carrot roots, which favor the growth of the fungi. Previous studies have also shown that fungal growth and reproduction depend on a medium rich in minerals, carbohydrates, water, and nitrogen [43].

The production rate of SMs from cultured fungal cells depends largely on the carbon source and its concentration, and the biosynthetic pathway, as well

Table 1. The effect of culture medium studied on the biomass and glabridin production of *Aspergillus eucalypticola* SBU-11AE as an endophytic fungus isolated from *Glycyrrhiza glabra*.

| Treatment | Biomass (g/l) | Content of glabridin (mg/l) | | |
|----------------|--------------------------|-----------------------------|--------------------------|--------------------------|
| | | Extracellular | Intracellular | Total |
| Medium culture | | | | |
| PCB | 6.43 ± 0.32 ^a | 1.72 ± 0.03 ^a | 5.57 ± 0.41 ^a | 7.26 ± 0.44 ^a |
| PDB | 6.33 ± 0.39 ^b | 1.37 ± 0.03 ^b | 4.47 ± 0.29 ^b | 5.85 ± 0.26 ^b |
| YEPD | 6.08 ± 0.51 ^c | 1.20 ± 0.01 ^c | 3.29 ± 0.10 ^d | 4.50 ± 0.11 ^d |
| Malt | 5.97 ± 0.52 ^d | 1.23 ± 0.01 ^c | 3.75 ± 0.11 ^c | 4.96 ± 0.11 ^c |
| Malt + Peptone | 5.85 ± 0.11 ^e | 1.24 ± 0.00 ^c | 3.23 ± 0.02 ^d | 4.47 ± 0.02 ^d |

PCB: potato carrot broth; PDB: potato dextrose broth; YEPD: Yeast extract peptone dextrose.

Different superscript lower case letters indicate a significant difference at *p* = 0.05.

[44]. Optimization of fungal culture conditions with biotic elicitors, vitamins, and nutritional supplements can be satisfactorily increased fungal growth [43]. It has previously been shown that vitamin E may be effective on cell membrane stability, modulation of cellular signaling, regulation of gene expression, and control of cell proliferation [45]. FS Pradeep and B Pradeep [21] showed that the peptone culture medium produced the highest amount of biomass in *Fusarium moniliform* due to the presence of glucose as a carbon source.

The pH of the culture medium had also a significant effect on the biomass production and accumulation of glabridin. The highest biomass (8.51 ± 0.43 g/l) (Figure 3(A)) and glabridin (8.30 ± 0.51 mg/l) (Figure 3(B)) production were observed from the PCB medium adjusted with pH = 6, while the lowest values of both traits were obtained from the same medium with pH = 7. The biomass and glabridin production at pH = 6 was 1.17 and 1.13-fold more than the control. As it could be ascertained, *A. niger* is growing at the range of pH = 5–6 (Table 2). The pH of the medium culture is another factor affecting the metabolic activity and growth of the fungus [40]. The mycelium of various fungal species grows in a wide range of pH levels. However, the optimal pH for the most microorganisms is ranging from 5 to 7. It has been reported that the most filamentous fungi require an acidic pH (5–6) for growth and sporulation, including the *Fusarium* sp., which grows well at a pH of 5.5 [21]. In the study by F-C Yang and C-B Liao [46], the optimum initial pH for the growth of *Ganoderma lucidum* was reported to be about 5. In the study by H Zhao, L Huang, C Xiao, et al. [38], it was found that the maximum metabolites were obtained in media with an initial pH of 6, while the maximum biomass volume was obtained at an initial pH of 4.

The results from ANOVA also showed that the plant root extracts added to the fungal endophyte culture had a significant effect (*p* < 0.05) on the production of biomass and glabridin. The highest and lowest biomass was observed at a concentration of 100 mg/l

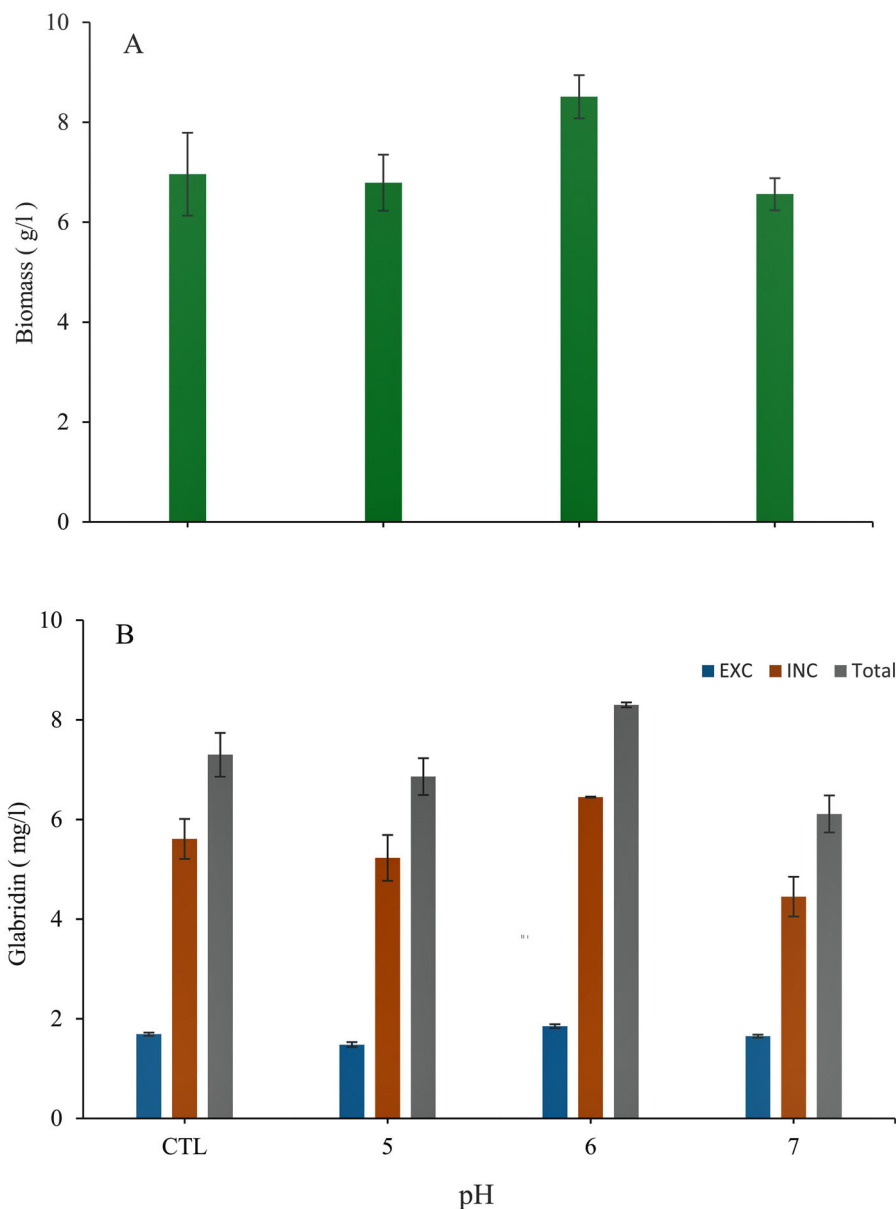


Figure 3. The effect of pH studied on the biomass (A) and glabridin (B) production of *Aspergillus eucalypticola* SBU-11AE as an endophytic fungus isolated from *Glycyrrhiza glabra*. EXC: extracellular content; INC: intercellular content; CTL: control.

Table 2. The effect of pH level of the culture medium on the biomass and glabridin production of *Aspergillus eucalypticola* SBU-11AE as an endophytic fungus isolated from *Glycyrrhiza glabra*.

| pH level | Biomass (g/l) | Glabridin (mg/l) |
|---------------|--------------------------|--------------------------|
| 5.5 (Control) | 6.96 ± 0.83 ^b | 7.3 ± 0.44 ^b |
| 5 | 6.79 ± 0.56 ^c | 6.86 ± 0.37 ^c |
| 6 | 8.51 ± 0.43 ^a | 8.3 ± 0.05 ^a |
| 7 | 6.56 ± 0.32 ^d | 6.11 ± 0.37 ^d |

Different superscript lower case letters indicate a significant difference at $p = 0.05$.

(10.75 ± 1.0 g/l) and 50 mg/l (8.12 ± 0.76 g/l) (Figure 4(A)), respectively. The highest production of total glabridin (10.85 ± 0.84 mg/l) was also obtained from the culture treated with 100 mg/l of the plant root extract (Figure 4(B)). HPLC-PDA chromatogram of the methanolic extract of fungal mycelia cultured in PCB medium (pH = 6) and treated with 100 mg/l of the plant root extract is shown in (Figure 5).

The positive effect of simultaneous culture of endophytic fungi and host plant (licorice) on synthesis and accumulation of phenolic and flavonoid compounds in three endophytes *Stagonosporopsis cucurbitacearum*, *Bionectria* sp., and *Aspergillus terreus* has also been reported [47]. It has been previously shown that plant fungal endophytes secrete indole-3-acetic acid (IAA) and abscisic acid in the interaction with the host plant [48,49]. The active ingredients of an endophytic fungus stimulant sometimes include polysaccharides or components of the fungal cell wall; which may be absorbed or identified by the plant and its stimulus receptors [50]. All of these activities can effectively contribute to the synthesis of SMs. The ability of endophytes to produce active substances may be due to their acquired ability to biosynthesize compounds similar to the host plant. It seems that the symbiotic

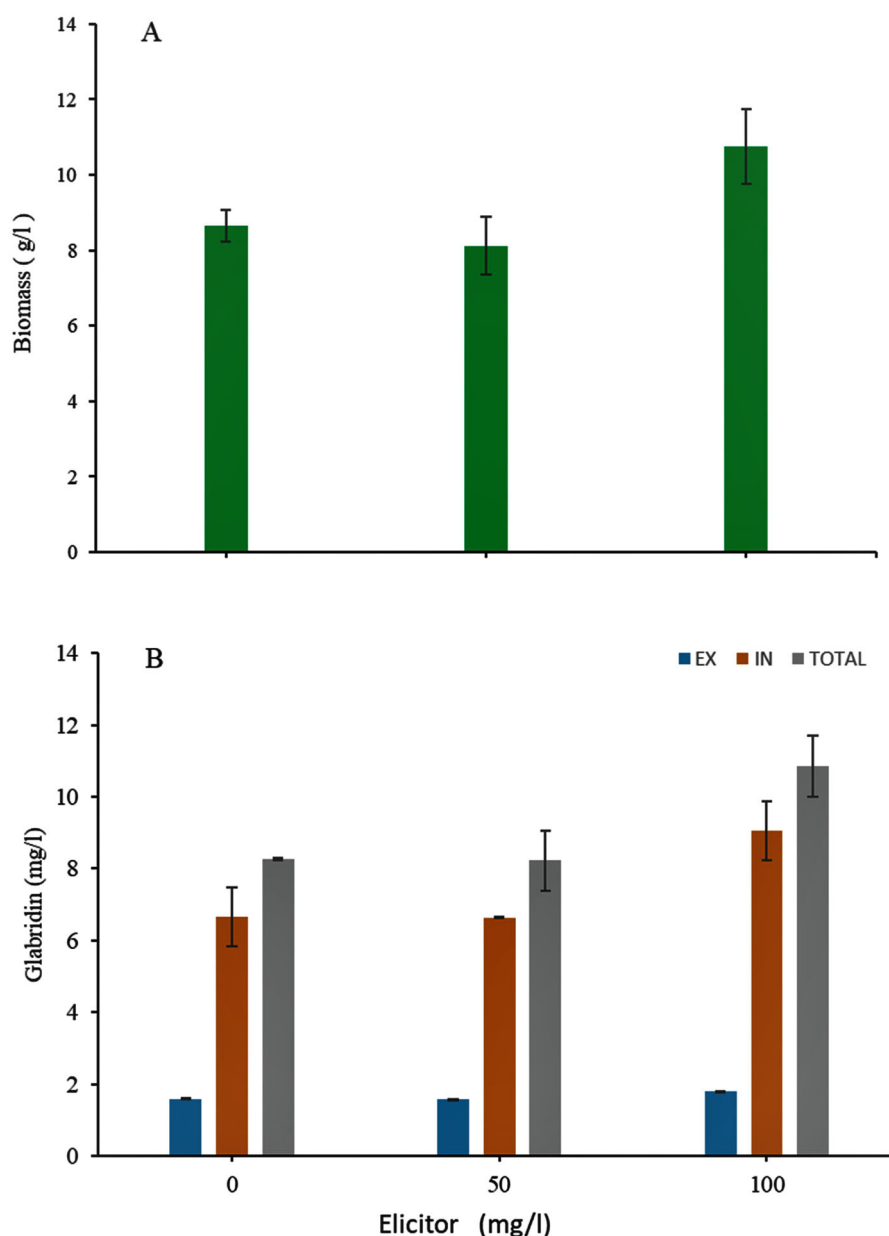


Figure 4. The effect of elicitors studied on the biomass (A) and glabridin (B) production of *Aspergillus eucalypticola* SBU-11AE as an endophytic fungus isolated from *Glycyrrhiza glabra*. EXC: extracellular content; INC: intercellular content.

relationship of endophytic fungi can be very effective in the path of metabolic processes of the host plant including biosynthesis and breakdown of hormones, amino acids, various SMs i.e. flavonoids, polysaccharides, and terpenes, lipids, sugars, vitamins and proteins [49,51]. In another study, it was also stated that the simultaneous culture system of *Rumex gmelinii* Turcz. ex Ledeb. endophytes with the plant *in vitro* seedlings had a positive effect on the production of SMs compared to the control [52].

4. Conclusion

The present study showed that optimizing the culture medium of endophytes is an effective strategy to increase the production of bioactive compounds. Environmental factors such as culture medium,

nutrients, temperature, pH, culture period and stimuli affect biomass and SMs production. Fungal endophyte, *A. eucalypticola*, isolated from licorice root is a promising source for producing of glabridin. In the present study, *A. eucalypticola* SBU-11AE cultured in the PCB culture medium (pH = 5–6) and elicited with dry extract of host plant root (100 mg/l) during the 14-day culture period was produced the highest level of glabridin. The results obtained can be interestingly exploited in the development of mass culture systems like fermentors for the commercial production of glabridin as valuable pharmaceutical compounds.

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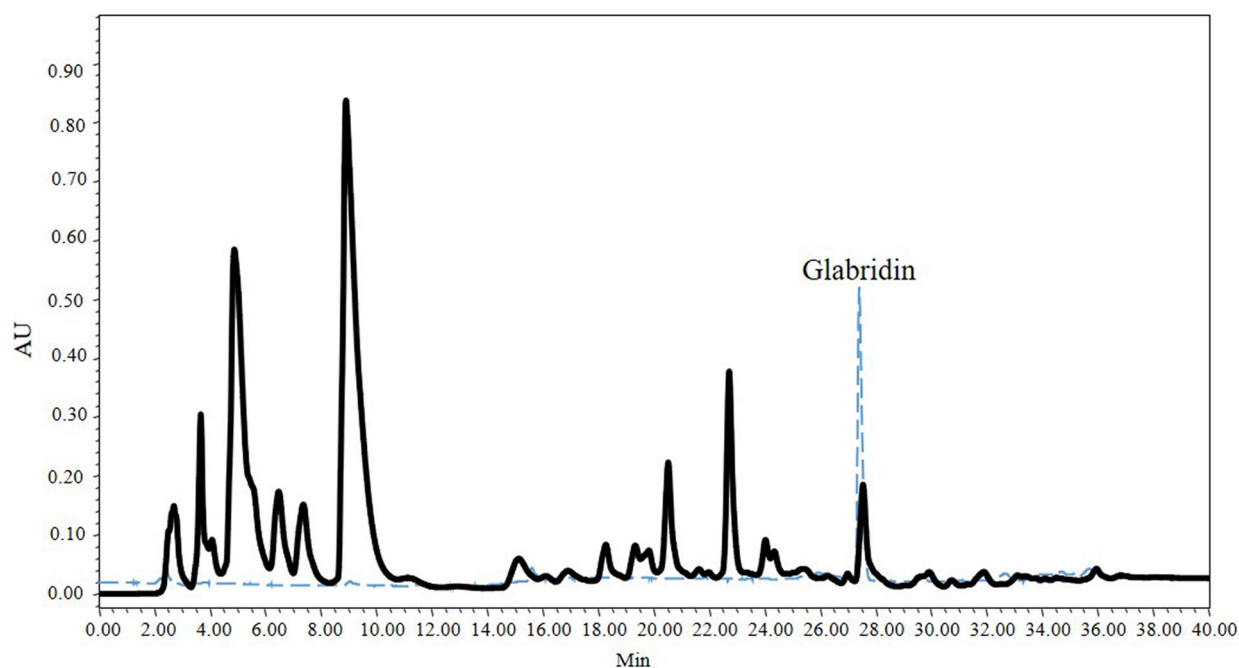



Figure 5. The HPLC-PDA chromatogram of the methanolic extract of *Aspergillus eucalypticola* SBU-11AE mycelia (bold line) and standard of glabridin (dotted line). The condition is explained in the experimental section.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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