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Original Article

Qualitative and Quantitative Study of Quercetin and Glycyrrhizin in *In Vitro* Culture of Liquorice (*Glycyrrhiza glabra* L.) and Elicitation with $AgNO_3$

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Abstract

Liquorice (*Glycyrrhiza glabra* L.) is a plant that has been considered for a long time due to its valuable secondary metabolites. This study was conducted to obtain quercetin and glycyrrhizin under controlled conditions and the use of silver nitrate ($AgNO_3$) as an elicitor to increase their production. For this purpose, the seeds were cultured in MS media containing various concentrations of $AgNO_3$ (0, 2, 4, 8 and 10 mg L^{-1}). Quercetin in the aerial parts extract of three-month seedlings prepared with methanol solvent 95% and acetic acid (9:1), and glycyrrhizin in the root extract of four-month seedlings prepared with ethanolic extract (30%) were evaluated qualitatively and quantitatively using HPLC. The results obtained from three replications showed the presence of quercetin and glycyrrhizin in all samples. The amount of quercetin in all samples treated with $AgNO_3$ was significantly higher than control ($P \leq 0.05$) and this increase was higher at concentrations of 8 and 10 mg L^{-1} in comparison with other concentrations. Glycyrrhizin content increased under the influence of different concentrations of $AgNO_3$ as compared to the control; however, this increase was not significant. Our results clearly showed that this method is a practical method to produce and elicit more these compounds with medicinal value.

Keywords: anti-ethylene; biotechnology; elicitor; flavonoids; high performance liquid chromatography; non-biological stresses; terpenoids

Abbreviations: $AgNO_3$: Silver nitrate; ANOVA: Analysis of variance; HPLC: High performance liquid chromatography; L: Liter; µ: micro; m: milli 10^{-3}; mol: mole; MS: Murashige and Skoog nutrient medium; nm: nanometer; ROS: Reactive oxygen species; SE: Standard error; SOD: Superoxide dismutase; UV: Ultraviolet; v: volume; w: weight

Introduction

Plants are the source of a large group of organic compounds called secondary metabolites. The studies have shown that the market value of herbal medicines is always on the rise worldwide with substantial growth, and much of it is related to the production and supply of secondary metabolites derived from plants (Schäfer and Wink, 2009; Seca and Pinto, 2018; Yang *et al*., 2018). The complexity and costly chemical synthesis of the compounds (Smetanska, 2008; Kanwal and Sherazi, 2017) as well as their slow formation has led to an interest in using various methods of biotechnology including seeds, organs, tissues and cells culture for mass and fast production of these compounds under *in vitro* conditions (Naik and Al-Khayri, 2016). In this regard, manipulation of the medium and the use of elicitors (Tiwari and Rana, 2015) such as $AgNO_3$ with features including ease of access, solubility in water, and stability (Kumar *et al*., 2009) could be helpful for mass production of these metabolites (Bota and Deliu, 2011; Naik and Al-Khayri, 2016). There are several reports on increased production of secondary metabolites in plants by using $AgNO_3$ elicitor (Ramirez-Estrada *et al*., 2016; Shakeran *et al*., 2015; Patel and Krishnamurthy, 2013).

Liquorice *(Glycyrrhiza glabra* L.) is a perennial species belonging to the Fabaceae family. As an industrial plant, it is

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of interest to pharmaceutical and food industries. The plant height is up to 200 cm. Leaves are compound, flowers are purple and fruits are 2 cm long. It is a Mediterranean plant, growing in South Asia and from southern Europe to central Asia (Karaogul *et al*., 2016; Dastgir and Rizvi, 2016). So far, more than 300 types of flavonoids have been reported from different species of the genus *Glycyrrhiza*, of which 70 have been extracted from *Glycyrrhiza glabra* (Fukai *et al*., 1998; Ranganathan and Punniamurthy, 2013). Flavonoids are secondary metabolites that are physiologically important for plants. They increase plant resistance to stress (Treutter, 2006; Mierziak *et al*., 2014) and are involved in pollination, seed dispersal and thus in plant reproduction (Raja and Sreenivasulu, 2015). These compounds are also important in flower color determination (Panche *et al*., 2016), protect the plant cells against oxidative damage, and increase the efficiency of nutrient absorption at the time of aging (Feild *et al*., 2001). Protection of plants against pathogens and herbivores and involvement in plant competition and symbiosis of plants and microbes are other features of flavonoids (Wink, 2010; Mierziak *et al*., 2014). These compounds are also used in the treatment of many human diseases (Kumar and Pandey, 2013; Zeka *et al*., 2017).

Quercetin is a flavonoid. This compound is a strong dietary supplement and antioxidant, cleaning up the body from free radicals that cause cancer and diseases such as atherosclerosis. Furthermore, it is helpful to deal with viruses and lower the blood pressure (Edwards *et al*., 2007; Larson *et al*., 2010). In addition, this drug compound prevents the release of histamine, an inflammatory substance involved in allergic symptoms such as itching and sneezing (Maalik *et al*., 2014; Mlcek *et al*., 2016). Anti-aging property of quercetin is also reported by some researchers (Chondrogianni *et al*., 2010; Nagaich *et al*., 2016). In some studies, the presence of quercetin in liquorice has been reported (Singh *et al*., 2009; Khalaf *et al*., 2010).

Terpenoids are the largest class of natural compounds that are made of mevalonic acid pathway and plants use these compounds in growth, but their main role is to preserve the plant against a variety of biological and non-biological stresses (Tholl, 2015). Glycyrrhizin or glycyrrhizic acid is a terpenoid compound with the molecular formula of $C_{42}H_{62}O_{16}$ and the most important ingredient in the root of *Glycyrrhiza glabra*, making it sweet. Glycyrrhizin has medicinal properties in humans including antioxidant and anti-cancer, anti-viral, anti-hepatitis, anti-skin allergies, anti-inflammatory, anti-wound, and anti-peptic ulcers. Glycyrrhizin is also used as a food sweetener (Tian *et al*., 2008; Shabani *et al*., 2009; Liao *et al*., 2016).

Regarding the importance of liquorice as a medicinal plant, this research was aimed to investigate the quercetin and glycyrrhizin quantitatively and qualitatively under *in vitro* condition and also study the effects of $AgNO_3$ elicitor at different concentrations (0, 2, 4, 8, 10 mg L^{-1}) on increased production of these secondary metabolites in this species.

Materials and Methods

Preparation of seeds and cultivation conditions

The seeds of *Glycyrrhiza glabra* were procured from Isfahan PakanBazr Company (Isfahan, Iran) and surface-sterilized with sodium hypochlorite (3%) for three minutes. Then, the seeds were rinsed with distilled water several times and cultured in plates with a diameter of 8 cm, containing MS medium (Murashige and Skoog, 1962) and various concentrations of $AgNO_3$ (0, 2, 4, 8, 10 mg L^{-1}). All culture media contained 3% sucrose and 0.8% agar (w / v). The pH was adjusted to 5.8. The media were autoclaved at 1 atmosphere and 121 °C for 20 minutes. The plates containing culture media and seeds were placed under a photoperiod of 16 hours light (using 36-watt fluorescent lamps at a distance of 30 cm from the samples) and 8 hours darkness at 28 ± 2 °C.

Extracts preparation

To evaluate the quercetin qualitatively and quantitatively, after 3 months of seed culture, 0.1 g of dried powder of aerial parts (leaves and stems) were poured into lidded test tubes and 1.5 mL of methanol-acetic acid at a ratio of 9:1 (v / v) was added to each sample. The test tubes containing dried powder of aerial parts and solvent were placed in water bath at 50 °C for 20 minutes (Dmitriensko *et al*., 2012; Jaimand *et al*., 2013).

In order to qualitative and quantitative studies on glycyrrhizin, dried and powdered roots of four-month seedlings were used. For this purpose, 0.04 g dry powder of roots and 2 ml of ethanol solution (30%) were poured into the test tubes and mixed with a shaker several times and placed at 50 °C for one hour (Tian *et al*., 2008). The extracts, obtained by BioFIL filters with a diameter of 0.45 mμ, were kept in the refrigerator until injection into the HPLC.

HPLC conditions

Isolation of quercetin and glycyrrhizin was performed by high-performance liquid chromatography (HPLC) from Knauer Company (Berlin, Germany). Detection was made by a UV / V is detector set to 290 nm for quercetin and 254 nm for glycyrrhizin. The column was C18 (250 × 4.6 mm, 30 °C) with a pump (model K-1001). Methanol-water-acetic acid at a ratio of 50:45:5 (v / v / v) was used as the mobile phase for quercetin and 20 μl was injected. For glycyrrhizin, the mobile phase included methanol-water (30:70, v/v) and 1% acetic acid that 50 μl was injected. For both compounds, the flow rate was 1 mL/sec and the experiments lasted 30 minutes.

Preparation of standards and calibration curves

Quercetin with molecular formula $C_{15}H_{10}O_7$ and molecular weight of 302.24 g/mol was used as quercetin standard. It was purchased at 10 grams from Sigma-Aldrich. Concentrations of 5, 10, 20, 40, 80 mg of standard sample were prepared in one liter of methanol-acetic acid (9:1, v / v) and injected to the HPLC. The quercetin content was calculated based on the equation of the standard curve and the area under spectrums.

Ammonium salt of glycyrrhizic acid ($C_{42}H_{65}NO_{16}$) with molecular weight of 839.96 g/mol at 25 g with brand Sigma-Aldrich was used as glycyrrhizin standard. This compound was used at concentrations of 10, 20, 30, 40, and 50 mg, solved in one liter of ethanol solvent (30%) and injected to the device to draw the calibration line curve

of glycyrrhizin. Then, the glycyrrhizin content in samples was calculated based on the equation of the standard curve and the area under spectrum.

Experimental design and statistical analysis

Preparation of culture media containing various concentrations of $AgNO_3$ (0, 2, 4, 8, 10 mg L^{-1}) with 6 seeds in each plate were performed three times with three replications for each group and each time, a culture medium containing different concentrations of $AgNO_3$ and seeds was considered as a replicate. The data were analyzed by analysis of variance (ANOVA) and the means were compared with Duncan's Multiple Range Test ($P \leq 0.05$) using SPSS software V.16. Charts were drawn in the EXCEL 2007 software.

Results

HPLC chromatograms for quercetin and glycyrrhizin standards

According to the HPLC chromatograms for quercetin standard solutions with different concentrations, maximum absorption was detected approximately 13 minutes after injection (Fig. 1A). The calibration curve, curve line equation ($Y = 7811.5x + 4970.9$), and regression coefficient ($R^2 = 96.34$) for quercetin standard solutions are shown in Fig. 2A.

According to the HPLC chromatograms for glycyrrhizin standard solutions at different concentrations, maximum absorption was detected approximately 24 minutes after injection (Fig. 1B). The calibration curve, curve line equation ($Y = 18401x - 21698$), and regression coefficient ($R^2 = 0.97$) for glycyrrhizin standard solutions are shown in Fig. 2B. These curves of two compounds show good linearity and regression coefficients.

HPLC chromatograms for quercetin and glycyrrhizin

Figs. 3 and 4 show the three-month and four-month seedlings derived from the growth of seeds at different concentrations of $AgNO_3$ in which quercetin and glycyrrhizin were investigated qualitatively and quantitatively in the aerial parts (leaves and stems) and root extracts, respectively. Chromatograms of quercetin spectra, at various concentrations of $AgNO_3$ (0, 2, 4, 8, 10 mg L^{-1}), indicated the presence of this compound in the extracts of aerial parts of seedlings with an absorption peak in approximately the thirteenth minute (Fig. 5A-E).

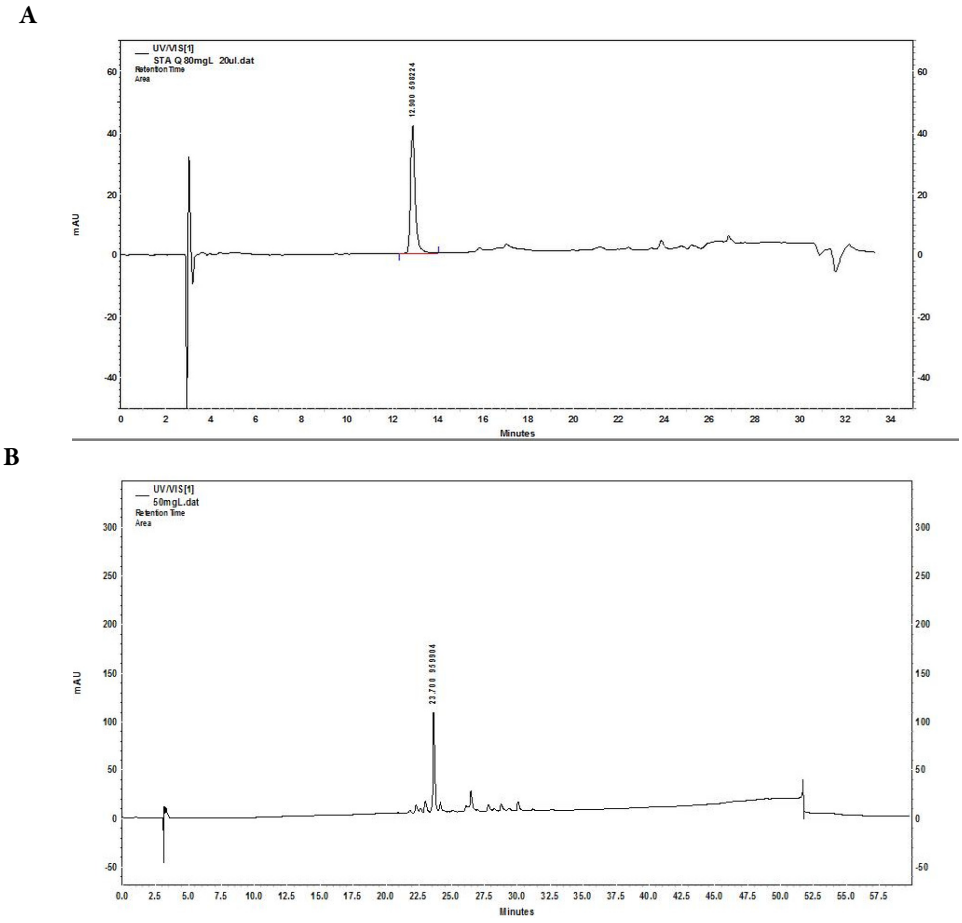

Fig. 1. Spectrum chromatograms of quercetin (A) and glycyrrhizin (B) standards. The y-axis of the chromatogram is the intensity of quercetin and glycyrrhizin absorptions (Area) in unit of milli-Absorbance Unit (mAU). The x-axis determines the Retention Time (RT) in unit of minute. The amount of injected sample for quercetin is 20 µL and for glycyrrhizin is 50 µL. For quercetin standard, maximum absorption is approximately 13 minutes after injection and for glycyrrhizin standard, it is approximately 24 minutes after injection

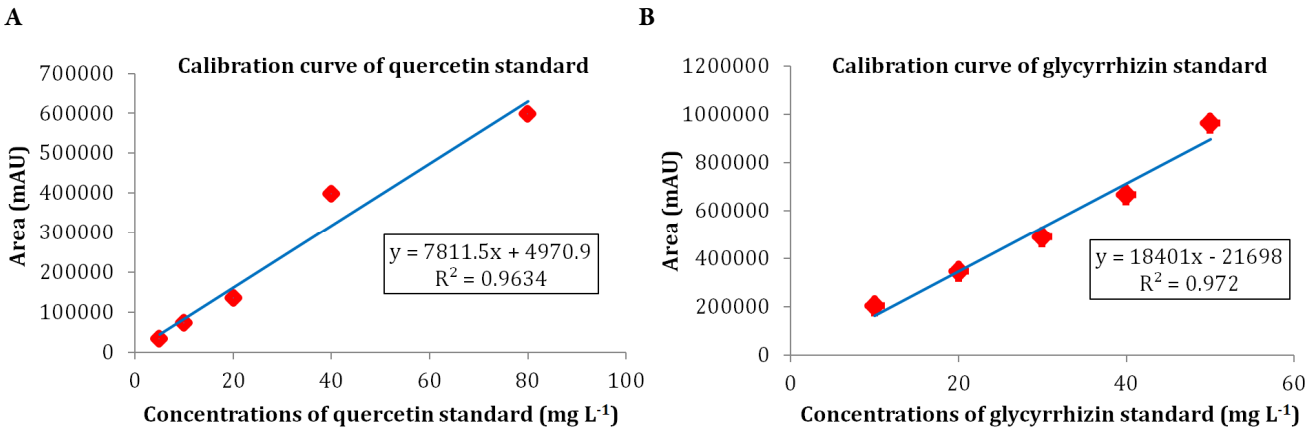

Fig. 2. A: Calibration curve of quercetin standard in different concentrations (5, 10, 20, 40 and 80 mg L^{-1}) and the regression equation (y = 7811.5x + 4970.9) with regression coefficient (R^2 = 0.9634). B: Calibration curve of glycyrrhizin standard in different concentrations (10, 20, 30, 40 and 50 mg L^{-1}) and the regression equation (y=18401-21698) with regression coefficient (R^2 = 0.972). These curves of two compounds show good linearity and regression coefficients

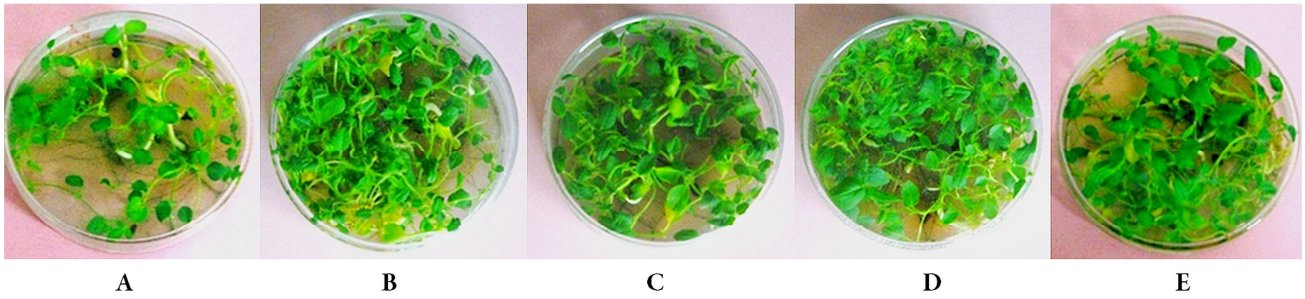

Fig. 3. Three-month-old seedlings of *Glycyrrhiza glabra* in MS medium containing different concentrations of $AgNO_3$ for qualitative and quantitative studies on quercetin. 0 mg L^{-1} (A), 2 mg L^{-1} (B), 4 mg L^{-1} (C), 8 mg L^{-1} (D), 10 mg L^{-1} (E)

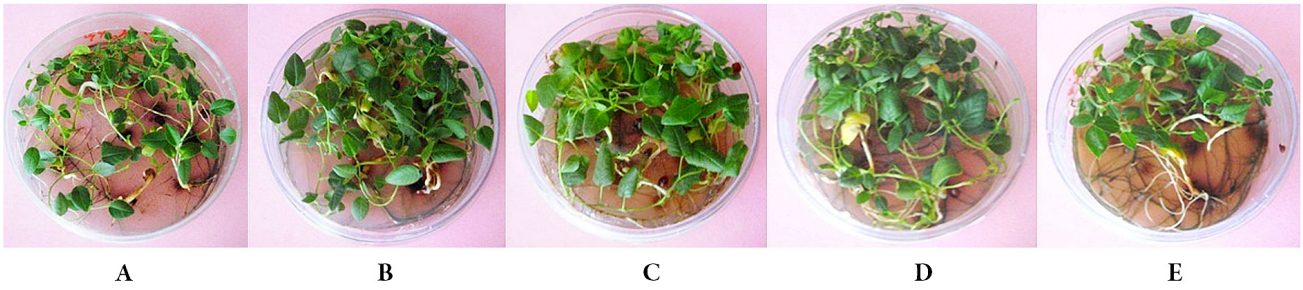

Fig. 4. Four-month-old seedlings of *Glycyrrhiza glabra* in MS medium containing different concentrations of $AgNO_3$ for qualitative and quantitative studies on glycyrrhizin. 0 mg L^{-1} (A), 2 mg L^{-1} (B), 4 mg L^{-1} (C), 8 mg L^{-1} (D), 10 mg L^{-1} (E)

According to the results, the mean maximum absorption at different concentrations of $AgNO_3$ was higher as compared with control samples. In addition, the content of quercetin significantly increased at concentrations of 2 (5.94 ± 0.8), 4 (5.20 ± 0.3), 8 (10.74 ± 0.8), and 10 (10.95 ± 1.1) mg L^{-1} to compared with control group (2.45 ± 0.5) ($P \leq 0.05$). Furthermore, the difference between concentrations of 2 and 4 mg L^{-1} with 8 and 10 mg L^{-1} was significant. No significant difference was found between concentrations of 2 and 4 mg L^{-1} as well as between concentrations of 8 and 10 mg L^{-1} (Fig. 7A).

Chromatograms of the glycyrrhizin spectrum at various concentrations of $AgNO_3$ (0, 2, 4, 8, 10 mg L^{-1}), showed the presence of this compound in the root extract of seedlings with the appearance of absorption peaks in approximately the twenty-fourth minute (Fig. 6A-E). According to the results, the content of glycyrrhizin insignificantly increased at concentrations of 2 (8.31 ± 1.1), 4 (9.59 ± 1.5), 8 (9.08 ± 1.2), and 10 (8.13 ± 0.3) mg L^{-1} to compared with control group (8.05 ± 1.3) (Fig. 7B).

Discussion

The results of this study clearly showed the presence of quercetin and glycyrrhizin in control samples and those treated with $AgNO_3$. In addition, increased content of these secondary metabolites in the treated samples was observed compared to controls. In some reports it has been shown that the production of secondary metabolites in plants is a sign of cell differentiation (Bourgaud *et al*., 2001). Due to the slow formation of these compounds in nature, the use of biotechnology including seed, tissue, and cell culture under *in vitro* conditions could result in faster production of these compounds and increase the possibility of access to natural medicines with no side effects (Rates, 2001; Zhao *et al*., 2005).Thus, the presence of quercetin and glycyrrhizin in all samples could be attributed to the *in vitro* culture and accelerated growth of plants. Furthermore, it is proven that the use of elicitors such as salts of heavy metals including $AgNO_3$ can cause messages to induce the production of secondary metabolites (Bota and Deliu 2011; Vildova *et al*., 2016).

Silver is a heavy metal with a molecular weight of 107.868 $gmol^{-1}$ and a density of 10.5 gcm^{-3} and the most important form in nature is nitrate (Bais *et al*., 2000). Studies have shown that the ions of heavy metals such as silver can cause oxidative stress induction (Fuente *et al*., 2014). Under oxidative stress conditions, the production of reactive oxygen species (ROS) is the first process occurring within cells based on stress intensity and physicochemical conditions (Zhao *et al*., 2005; Tripathy and Oelmüller, 2012; Sewelam *et al*., 2016), causing damage to DNA, structural proteins and lipids and induction of out-of-control chain reactions such as oxidation and peroxidation (Sharma *et al*., 2012; Tripathy and Oelmüller, 2012;

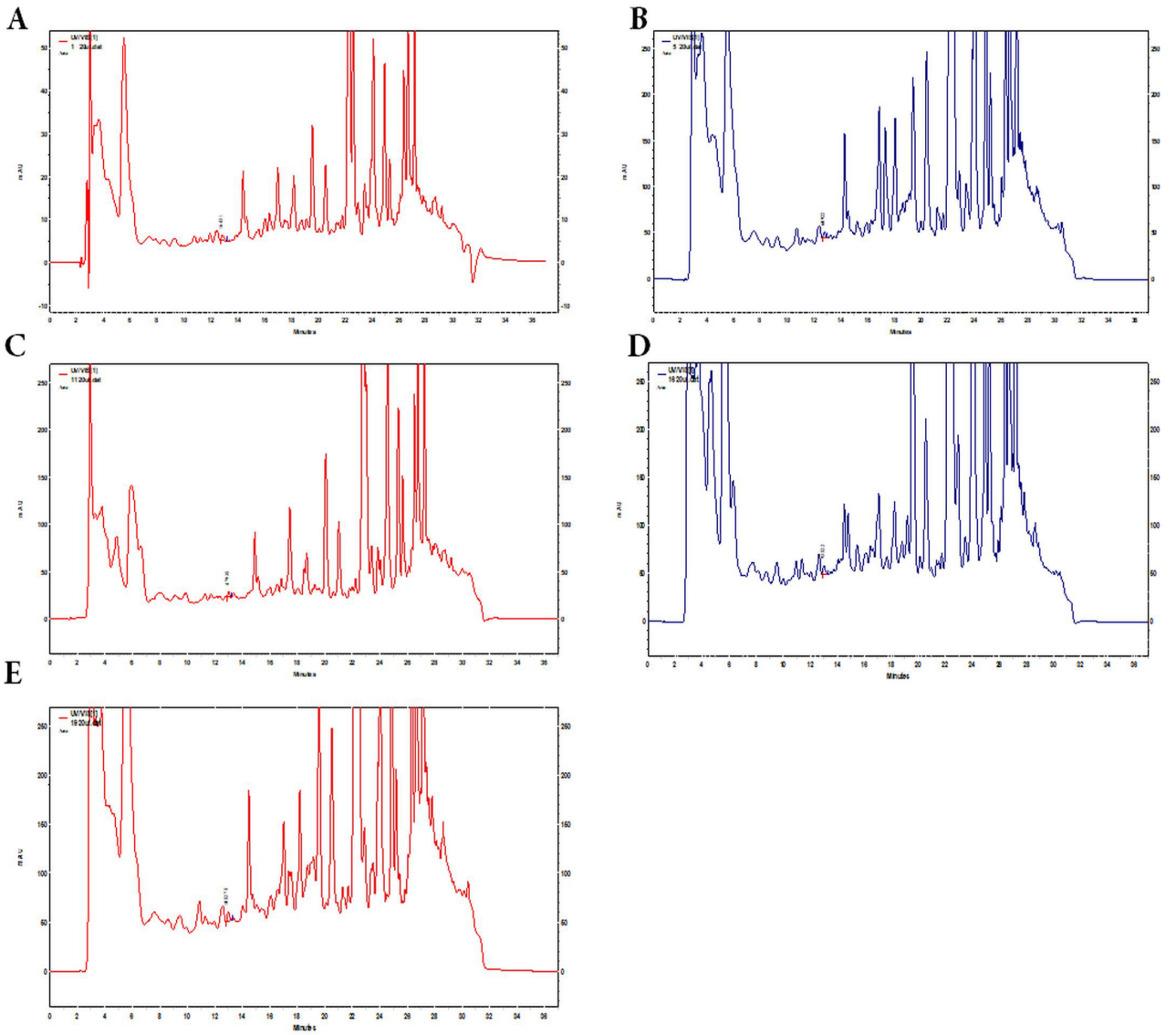

Fig. 5. Spectrum chromatograms of quercetin available in the aerial parts (stems and leaves) of three-month-old seedlings of *Glycyrrhiza glabra* treated with $AgNO_3$ at different concentration. 0 mg L^{-1} (A), 2 mg L^{-1} (B), 4 mg L^{-1} (C), 8 mg L^{-1} (D), 10 mg L^{-1} (E). Chromatograms of quercetin spectra at various concentrations of $AgNO_3$, show the presence of this compound with an absorption peak in approximately the thirteenth minute. Y-axis: Intensity of quercetin absorption in unit of mAU. X-axis: Retention Time (RT) in unit of minute

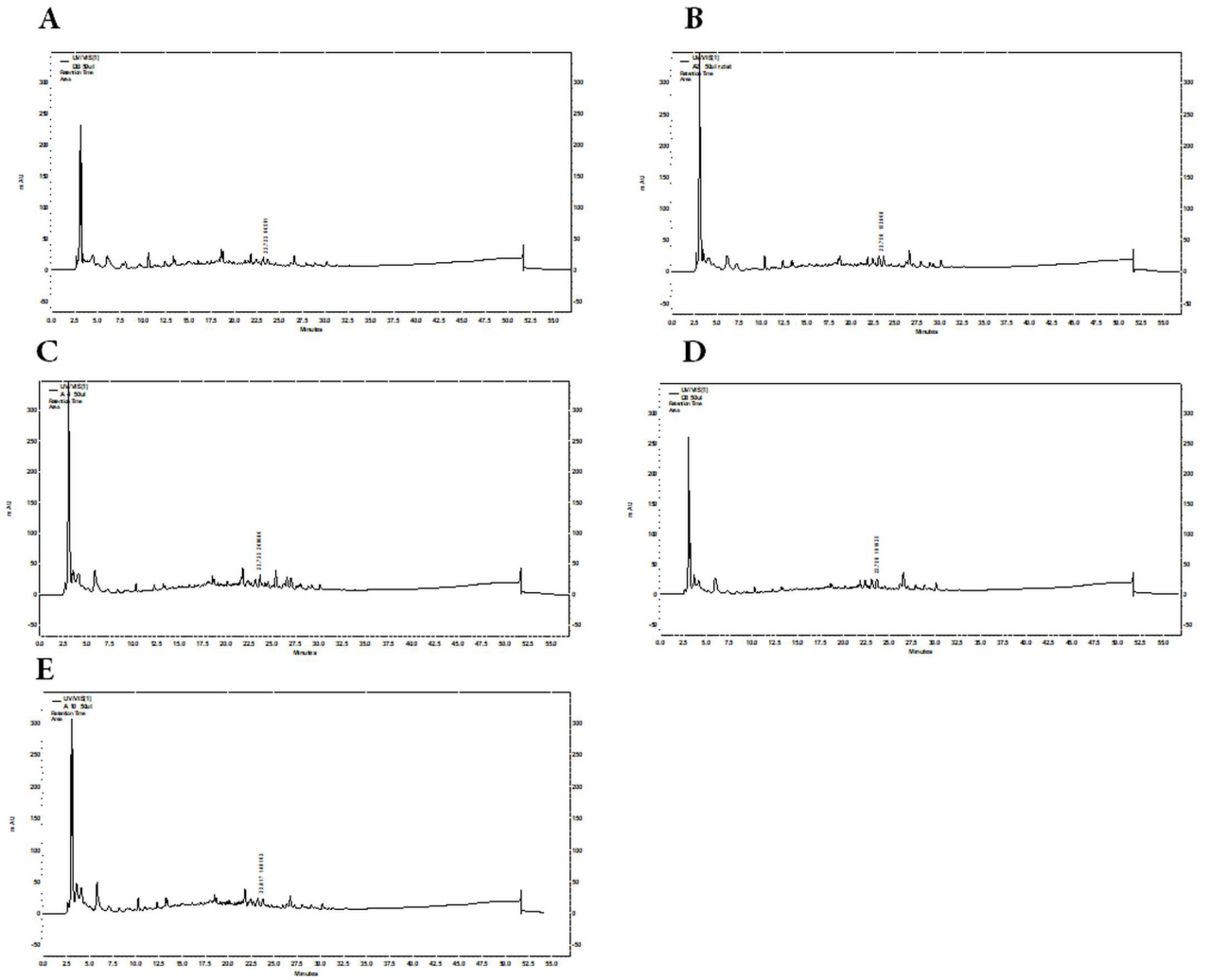

Fig. 6. Spectrum chromatograms of glycyrrhizin available in the roots of *Glycyrrhiza glabra* seedlings treated with $AgNO_3$ at different concentration. 0 mg L^{-1} (A), 2 mg L^{-1} (B), 4 mg L^{-1} (C), 8 mg L^{-1} (D), 10 mg L^{-1} (E). Chromatograms of glycyrrhizin spectra at various concentrations of $AgNO_3$, show the presence of this compound with the appearance of absorption peaks in approximately the twenty-fourth minute. Y-axis: Intensity of glycyrrhizin absorption in unit of mAU. X-axis: Retention Time (RT) in unit of minute

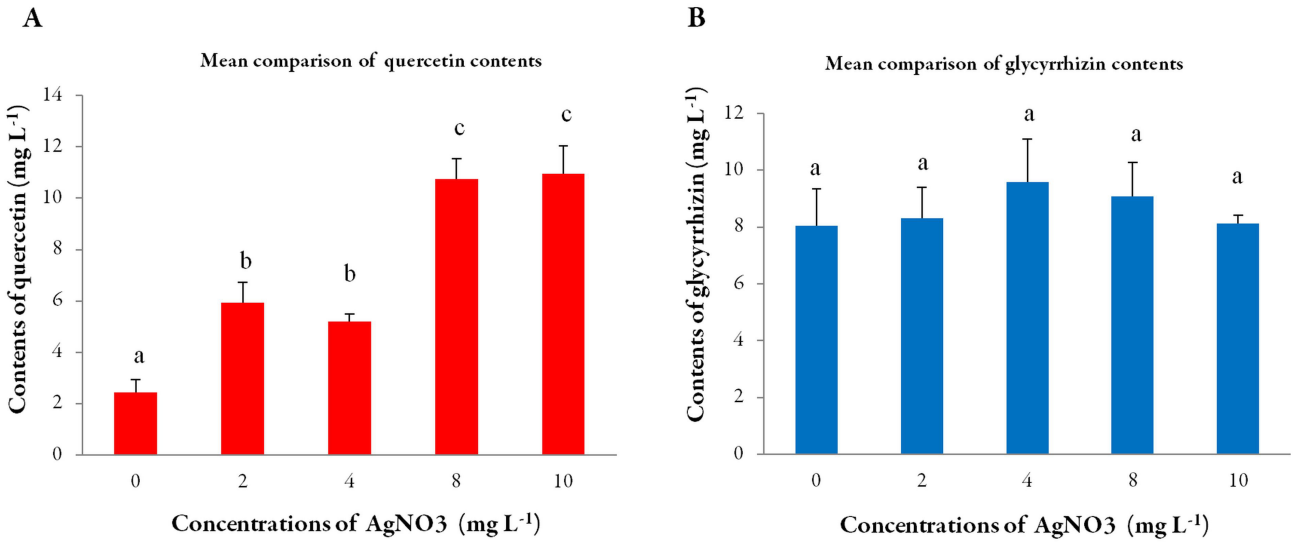

Fig. 7. Mean comparison of quercetin (A) and glycyrrhizin (B) contents at different concentrations of $AgNO_3$. Values are the mean ± standard error and the different letters are significantly different at $P \leq 0.05$ using Duncan's multiple range. The results show the increase of quercetin and glycyrrhizin at different concentrations of $AgNO_3$ as compared with control (0 mg L^{-1})

Fryzova *et al*., 2018). Evidence has shown that plants reduce the toxicity of these radicals through enzymatic and nonenzymatic antioxidant activity (Racchi, 2013). Superoxide dismutase (SOD), one of plants defense enzymes, is increased under stress conditions caused by heavy metals (Sharma and Dubey, 2005; Groppa *et al*., 2007). This enzyme plays a key role in the conversion of superoxide radical (O_2^-) into hydrogen peroxide (Perry *et al*., 2010). Hydrogen peroxide as a signaling molecule induces the production of antioxidants such as flavonoids (Agati *et al*., 2012). Under stress conditions such as exposure to heavy metals, plant non-enzymatic defense includes the production of secondary metabolites and antioxidant compounds such as flavonoids that causes preservation and survival of plants (Racchi, 2013; Sytar *et al*., 2013; Kubalt, 2016). Studies have shown that due to the reducing property, flavonoids increase the stability of the cell wall and create a physical barrier to protect the cells against the heavy metals (Díaz *et al*., 2001). It is reported that, under oxidative stress, secondary metabolites are increased to maintain metabolic activity and help to increase the tolerance of cells (Nammi *et al*., 2003).

According to the contents, it seems that in this research, the stress caused by the application of $AgNO_3$ induced enzymatic and non-enzymatic defense systems and thus the biosynthesis of quercetin and glycyrrhizin was increased to maintain the survival of the plant. In some experiments, it is observed that the activity of defense mechanisms increases as oxidative stress becomes more sever (Wong *et al*., 2006).

This may be due to the further increase of quercetin content at higher concentrations of $AgNO_3$ (8 and 10 mg L^{-1}), compared with lower concentrations (2 and 4 mg L^{-1}). In addition, there are several reports on anti-ethylene effect of $AgNO_3$ on *in vitro* culture conditions (Curtis, 1981; Turhan, 2004; Kumar *et al*., 2009; Harwansh *et al*., 2011; Tamimi, 2015). Since the high concentrations of ethylene inhibit the production of secondary metabolites (Zhang, 2004), it seems that the positive effect of silver ions (Ag^+) on production and increase of secondary metabolites, including flavonoids and terpenoids could be related to its inhibitory effect on the activity or synthesis of ethylene.

In studying the effect of abiotic elicitors on *Salvia miltiorrhiza*, it was found that the Ag^+ ions had the greatest effect on production of phenolic compounds as compared with other ions and also it has shown that these elicitors like $AgNO_3$ induces the activation of gene expression responsible for biosynthesis of plant metabolites in *in vitro* conditions (Naik and Al-Khayri, 2016). As a result, it seems that this can lead to the biosynthesis and accumulation of secondary plant compounds.

The use of $AgNO_3$ at a concentration of 5.887×10^{-4} mol/L in the culture medium of *Silybum marianum* L. and after 72h, led to the increased production of flavonoid compounds (Vildova *et al*., 2016). In a study on *Ononis arvensis* L., it was reported that 0.5 mg of $AgNO_3$ in the culture medium could increase the flavonoid content (Tumova and Polivkova, 2006). The results are consistent with the results of this study. It should be noted that the response of plants to heavy metals could be different depending on the metal concentration and time of stress.

Conclusions

Our results clearly showed that this method and the use of $AgNO_3$ elicitor with different concentrations (0, 2, 4, 8, 10 mg L^{-1}) in the culture medium of *Glycyrrhiza glabra* could be a practical method to produce more quercetin and glycyrrhizin with medicinal value. It should be noted that the use of higher concentrations of $AgNO_3$ is recommended for a significant increase in glycyrrhizin content. Further pharmacological studies on this compound are recommended to optimize the use and consumption of quercetin and glycyrrhizin based on the method used in this research.

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