J Plant Mol Breed (2021) 9(2): 24 - 34
DOI: 10.22058/JPMB.2022.563277.1265

RESEARCH ARTICLE

Establishment of adventitious root culture in *Echinacea purpurea* and enhanced accumulation of caffeic acid derivatives by biotic and abiotic elicitors

Mohammad Abdoli[1*], Nooshin Koolivand[1], Arash Babaei[2]

[1] Department of Plant Production and Genetics, Faculty of Agriculture, Malayer University, Malayer, Iran.
[2] Department of Biology, Faculty of Sciences, Malayer University, Malayer, Iran.

ABSTRACT: The present study aimed to develop a protocol for root induction and evaluate the effects of salicylic acid (SA) (0, 80 and 160 mg/l) and yeast extract (YE) (0, 0.75 and 1.5 g/l) on chlorogenic acid, caftaric acid, cichoric acid, cynarin and echinacoside production in *Echinacea purpurea* adventitious roots. Also, the effect of NH_4NO_3 (0, 0.25, 0.75, 1.0 X) concentration in MS medium supplemented with indole-3-acetic acid (IAA) (1 and 3 mg/l) on root induction was investigated. The results showed that adventitious root induction in coneflower was significantly influenced by NH_4NO_3 and IAA concentrations ($p\leq0.01$). The highest percentage of root induction (100%) and average number of roots formed on each explant (14.3 roots) was observed in 1 mg/l IAA×1/4NH_4NO_3 MS culture medium treatment. The main effect of SA and YE and their interaction effects with exposure time on the measured traits (except for echinacoside) was significant ($p\leq0.01$). The result showed that application of 1.5 g/l YE and 160 mg/l SA when harvested 96 hours post-elicitation are the most effective treatments to elicit caffeic acid derivatives (CADs) content. The highest chlorogenic acid, cichoric acid, caftaric acid, and cynarin production was obtained in 160 mg/l SA at 96 hours post-elicitation that was 2.13, 1.83, 2.39 and 2.97-fold higher compared to control respectively. The heatmap diagram showed that the CADs content in SA and YE treatments was clearly separated from each other and control treatment.

KEYWORDS: Cichoric acid, Coneflower, Elicitor, *In vitro*, Medicinal plant, Yeast extract.

ABBREVIATIONS: ANOVA, Analysis of variance; CADs, Caffeic acid derivatives; DW, Dry weight; HPLC, High-performance liquid chromatography; MS, Murashige and Skoog; SA, Salicylic acid; WPM, Woody plant medium; YE, Yeast extract.

INTRODUCTION

Plants are regarded as resources of food and valuable phytochemical compounds which have usefulness as agrochemical, pharmaceutical, food additives or aromatic products [13, 31]. Purple coneflower, *Echinacea purpurea* (L.) Moench is a perennial herb belonging to the *Asteraceae* family that is an important raw material for the pharmaceutical and ornamental industries [19]. High economic importance and extensive use of *E. purpurea* is related to the variety of bioactive compounds (216 different medicinally components) [26]. Several studies have revealed that extracts from *E. purpurea* has different pharmacological activities such as antioxidative, antibacterial, antifungal properties. It is commonly used to prevent and treat many diseases such as common colds, flu, respiratory, urinary diseases and skin inflammation. The therapeutic effects of *E. purpurea* could be due to its

*Corresponding author (✉):Abdoli_m@malayeru.ac.ir
Received: 10 October 2022/ Revised: 17 October 2022
Accepted: 17 October 2022

ingredients including alkamides, glycoproteins, caffeic acid derivatives (CADs), and polysaccharides [7]. Among these compounds, phenolic acids are an important group of *E. purpurea* metabolites with extensive pharmacological activity [5]. Prolonged growth period, diversity of components of active compounds, lack of pure plant material and contamination of plant materials by microorganisms have made it difficult to produce medicine from this medicinal plant commercially [1, 28]. For that reason, secondary metabolites could be produced under controlled conditions by using plant tissue culture technology [32]. The progress made in *in vitro* culture systems in aromatic and medicinal plants is essential for the sustainable production of high-value secondary compounds [15]. Adventitious roots are considered suitable biological materials and a good alternative for valuable bioactive compounds production in various plant species. Adventitious root culture has several advantages, as it is genetically stable, biosynthetic capabilities is stable [26, 27]. In addition, growth rate is higher, a little of inoculum is required in culture medium, and it helps to enhance important secondary compounds production [15]. The effects of some parameters on *E. purpurea* adventitious root culture have been discussed in several studies [14, 26, 35]. Elicitation is one of the strategies employed for increased production of high-value secondary metabolites from both *in vitro* and *ex vivo* grown medicinal plants [6, 26]. SA (2-hydroxybenzoic acid) is a phenolic compound and signaling molecule with important roles in stimulating secondary compounds biosynthesis [12, 22, 23]. Furthermore, the stimulating influence of biotic elicitor (YE) in enhancement of active compounds production was confirmed [12, 16, 22]. Elicitation strategies using biotic and abiotic factors were used to enhance the levels of caffeic acid derivatives in different *in vivo* and *in vitro* cultures of *E. purpurea* such as foliar application, adventitious and hairy roots, and cell suspensions. For instance, the effects of foliar application of different concentrations of SA and YE on *E. purpurea* aerial part has been evaluated that 160 mg/l SA and 1.5 g/l YE after 96 hours exposure time were appropriate for CADs production [22]. Abdoli et al. (2013) stated that content of chlorogenic and cichoric acid in hairy root cultures of *E. purpurea* was affected by KNO_3, $CaCl_2$ and $MgSO_4$ concentrations [3]. Also, nitric oxide was used as an elicitor for higher production of CADs in root cultures of coneflower [36]. The hypothesis of current study was that elicitation with abiotic (SA) and biotic (YE) elicitors would have stimulation effect on caffeic acid derivatives production in *E. purpurea*. The present study developed a protocol for induction of adventitious root in coneflower using the different levels of IAA or NH_4NO_3, and investigated the effects of SA and YE as elicitors on production of CADs in *E. purpurea* root cultures in Erlenmeyer flasks.

MATERIALS AND METHODS

Experimental set up and growth conditions

Echinacea purpurea (L.) Moench seeds were purchased from Pakanbazr Company (Isfahan, Iran). The seeds were washed under running tap water for 5-10 min and surface sterilized with 70% (v/v) ethanol for 1 min, followed by soaking in 5% (w/v) sodium hypochlorite solution for 20 min. Seeds were rinsed three times (5 min) in sterile distilled water. For germination, sterilized seeds were placed into glass jar (8 cm in height and 5.5 cm in diameter) containing 50 ml MS [24] basal medium supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar (Merck). The pH of medium was adjusted to 5.8 and sterilized by autoclaving (20 min at 121 ºC). All the cultures were maintained at 25 ± 2 ºC in a growth chamber with a 16-h photoperiod (60 µmol m^{-2} s^{-1}) provided by cool white fluorescent lamps to develop plantlets. The 45-days-old seedlings were used as the source of leaf explants for the subsequent experiment.

Adventitious root induction

Leaf explants from 45-day-old seedlings were excised and placed on various concentrations of ammonium nitrate (NH_4NO_3) i.e. 0, 0.25, 0.75 and 1.0X of MS basal media supplemented with indole-3-acetic acid (IAA) (1 and 3 mg/l) and 3% (w/v) sucrose. The pH of all media tested in this experiment was adjusted to 5.8, before autoclaving (20 min at 121 ºC). The selected leaf explants were placed on 25 ml of the media in a 10 cm Petri dish (5 explants/dish, 3 dishes/treatment) for *E. purpurea* root induction and sealed using Parafilm. The cultures were kept at 25 ± 2 ºC in a growth chamber with a 16-h photoperiod for 4 weeks. Each treatment consisted of three plates containing five explants each. After 4 weeks of culture (Fig. 1a), data were recorded and percentage of root induction, average number of roots formed on each explant and average roots length was quantified compared to the control. The roots produced from these cultures (after 4 weeks) were used for establishing root suspension culture. To evaluate caffeic acid derivatives accumulation, about 1 g of direct adventitious roots

emerging from leaf explants were grown in 100 ml Erlenmeyer flasks containing 50 ml of liquid WPM basal medium, wrapped with aluminum foil, and incubated in the dark (25 ± 2 ºC) on an orbital shaker at 110 rpm.

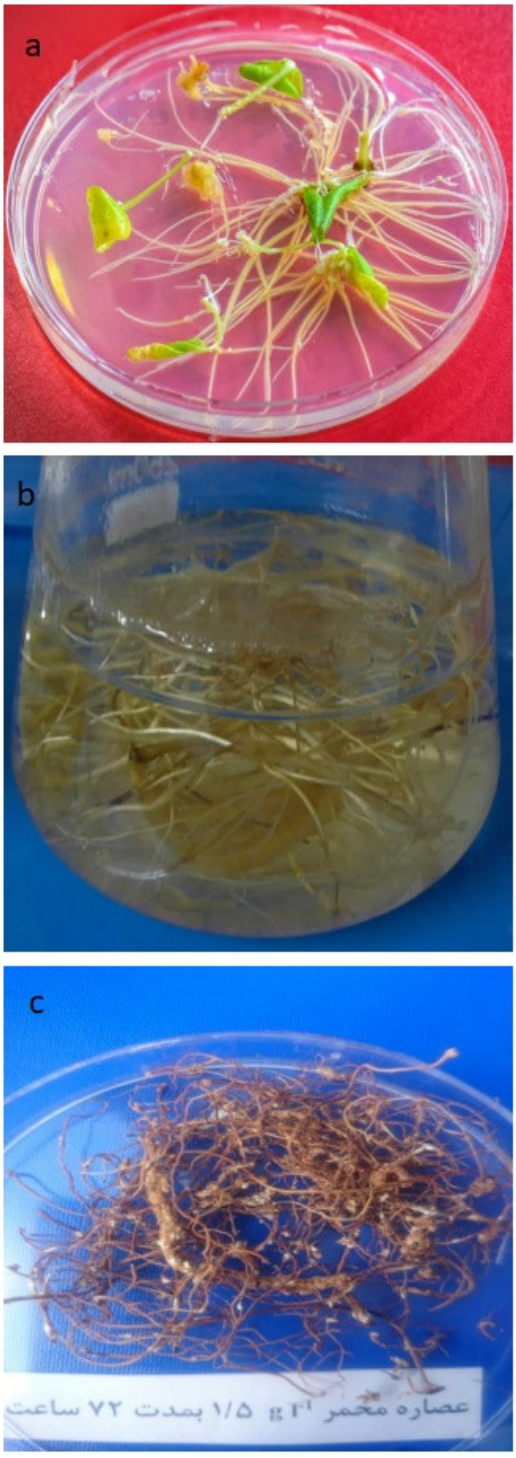

Figure 1. Adventitious roots formation of *E. purpurea* leaf segment after 28 days in solid ¼ NH_4NO_3MS basal medium supplemented with 1 mg/l IAA (a), roots of grown in WPM liquid medium for 28 days (b), Harvested dry roots after elicitation (c).

Elicitation process

In this study, two different factorial experiments based on completely randomized design were performed on CADs production in *E. purpurea* adventitious roots. In the first experiment, woody plant medium (WPM) was supplemented with salicylic acid (Merck) at concentrations of 0 (distilled water + 1% ethanol as a solvent), 80 and 160 mg/l and each at two elicitation times (72 and 96 h) and in the second experiment, the medium was supplemented with YE (Merck) at concentrations of 0 (distilled water), 0.75 and 1.5 g/l and each at two elicitation times (72 and 96 h) [22]. Each treatment consisted of three replicates. After sterilization, SA and YE were added to 28-days-old roots (Fig. 1b). Three and four days after elicitation, all adventitious roots cultured in the Erlenmeyer flasks were harvested (Fig. 1c) and analyzed for CADs production. The samples were collected from the media and then they were shade dried for two days.

Extraction and determination of CADs in adventitious roots

Extraction and quantification of CADs (caftaric acid, chlorogenic acid, cynarin, echinacoside, and cichoric acid) (Fig. 2) from *E. purpurea* adventitious roots were performed and calculated on the basis of the High-performance liquid chromatography (HPLC) assay described by Brown, (2011) [8]. The elicitor treated adventitious roots were dried and powdered to a fine

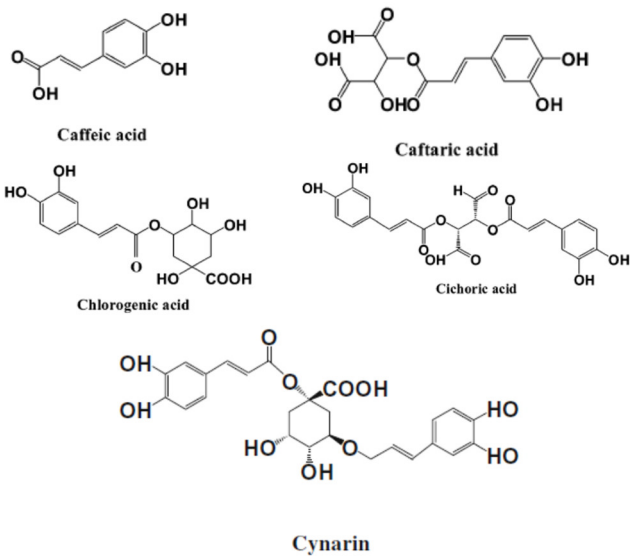

Figure 2. Chemical structures of biologically active phyto-chemicals extracted from *E. purpurea* root samples by HPLC [1].

powder using a mortar and pestle, homogenized in 50 ml conical tubes with 25 ml 60% methanol solution at room temperature for 30 min. The extract was centrifuged for 5 min at 5000 rpm. The supernatant was collected and filtered through 0.2 µm membrane filters and transferred to new vials. The identification and quantification of these 5 compounds were analyzed using an HPLC system (Unicam-Crystal 200 HPLC system). Analytical column was Cosmosil 5C18-AR-II, 150×4.6 mm id. Column temperature adjusted at 25 °C. 0.1 % (v/v) phosphoric acid (A) and acetonitrile (B) were used as the mobile phase. The flow rate of the mobile phase was 1.5 ml/min, injection volume was 20 µl and monitoring of CADs was 330 nm. Retention time of the reference standards including caftaric acid, chlorogenic acid, cynarin, echinacoside, and cichoric acid were 4.15, 4.65, 7.50, 7.92 and 13.00 min, respectively. The content of CADs in the dried adventitious roots on a dry weight basis (mg/g) was calculated [22].

Statistical analysis

All statistical analyses were conducted using the SPSS software version 18.0 (SPSS, Inc., Chicago, IL, USA). The Duncan's multiple range test was used for calculation of significant differences and $p \leq 0.05$ was considered as significant. The results represented as mean of three replications. Also, the data were analyzed by heatmap using the software ClustVis.

RESULTS

Effects of IAA and NH4NO3 on adventitious root induction

After four weeks, adventitious root induction (%), average number of adventitious roots, and average length of roots were recorded. The results of analysis of variance showed that adventitious root induction in *E. purpurea* was significantly influenced by NH_4NO_3, and IAA concentrations. The simple effects of NH_4NO_3, and IAA on root induction (%), average number of roots formed on each explant, and mean length of a roots were significant ($p \leq 0.01$). Also, the interaction effect of NH_4NO_3×IAA on the studied traits were significant ($p \leq 0.01$) (data not shown). Mean comparisons (Table 1) showed that there were high differences among NH_4NO_3 concentrations for ability of adventitious root induction. Adventitious root induction (%) of the leaf explants at 1 mg l^{-1} IAA×1/4NH_4NO_3 MS culture medium reaching up to 100% was the highest and each explant could generate 14.33 roots (Fig. 1a). The results showed that both 1 and 3 mg/l IAA were proven suitable for root induction; however, the highest percentage of root induction (100%) was related to 1 mg l^{-1} IAA in 1/4NH_4NO_3 MS culture medium and the lowest percentage of root induction was related to MS treatment with 3/4NH_4NO_3×3 mg l^{-1} IAA (33%). The highest average roots length was related to MS without NH_4NO_3 × IAA_3 (6.33 cm) and the lowest

Table 1. Effects of various concentration of NH_4NO_3 MS medium, and IAA on adventitious root induction in *E. purpurea*

Factor	Mean		
	Root induction percentage	Average number of roots per explant	Average root length (cm)
Concentration of NH_4NO_3 MS medium (X)			
Without NH_4NO_3	67	4.97	5.67
1/4 NH_4NO_3	78	8.38	3.50
3/4 NH_4NO_3	39	2.35	2.50
1.0 NH_4NO_3	67	3.93	3.50
Concentration of IAA (mg/l)			
IAA_1 -1	75	6.17	3.75
IAA_3 -3	66	3.65	3.83
NH_4NO_3 × IAA			
Without NH_4NO_3 × IAA_1	55 ^{bcd}	3.50 ^{c}	5.00 ^{b}
Without NH_4NO_3 × IAA_3	77 ^{ab}	6.43 ^{b}	6.33 ^{a}
1/4 NH_4NO_3 × IAA_1	100 ^{a}	14.33 ^{a}	4.33 ^{bc}
1/4 NH_4NO_3 × IAA_3	55 ^{bcd}	2.43 ^{c}	2.67 ^{d}
3/4 NH_4NO_3 × IAA_1	44 ^{cd}	2.73 ^{c}	2.33 ^{d}
3/4 NH_4NO_3 × IAA_3	33 ^{d}	1.97 ^{c}	3.67 ^{d}
1.0 NH_4NO_3 × IAA_1	66 ^{bc}	4.13 ^{bc}	3.33 ^{cd}
1.0 NH_4NO_3 × IAA_3	66 ^{bc}	3.73 ^{bc}	3.67 ^{bcd}

Means followed by the same letter in each column are not significantly different at $p \leq 0.05$ according to the Duncan's multiple range test. Data are presented as mean.

Table 2. Significant levels in ANOVA of the effect of salicylic acid (Experiment 1) and yeast extract (Experiment 2) and exposure times on caffeic acid derivatives production in *E. purpurea* adventitious root cultures.

Items	Experiment 1			%C.V.	Experiment 2			%C.V.
	Salicylic acid (SA)	Exposure times (T)	SA×T		Yeast extract (YE)	Exposure times (T)	YE×T	
Cichoric acid	**	**	**	3.80	**	**	**	3.31
Chlorogenic acid	**	**	*	5.74	**	**	**	3.85
Caftaric acid	**	**	**	2.32	**	**	*	2.19
Echinacoside	*	**	ns	23	**	**	ns	8.29
Cynarin	**	**	**	8.69	ns	**	**	12.73

*, **: significantly different at the 5 and 1% probability level, respectively. ns: not significant.

mean length of roots was related to 1 and 3 mg l^{-1} IAA in 1/4NH_4NO_3 MS culture medium. The results showed that with the decreasing NH_4NO_3 strength (0 and 0.25X MS), the measured traits increased significantly, in compared to standard MS (1X NH_4NO_3 MS) medium and MS treatment with 3/4NH_4NO_3×3 mg l^{-1} IAA (Table 2).

Effect of elicitors on CADs production

The effect of YE and SA on CADs (caftaric acid, chlorogenic acid, cynarin, echinacoside, and cichoric acid) production from E. purpurea adventitious roots was shown in Tables 2-4.

Effect of salicylic acid

The results of variance analysis (ANOVA) showed that there were highly significant differences among various concentrations of SA for amount of four phenolic compounds of roots including caftaric acid, cichoric acid, cynarin, and chlorogenic acid production at 1% probability level and echinacoside at 5% probability level (Table 2). The simple effect of exposure times on CADs production were significant ($p \leq 0.01$). Also, the interaction effect of SA×exposure time on the studied traits was significant ($p \leq 0.01$). The results of mean comparison indicated that SA significantly altered the amount of CADs content (mg/g DW) in *E. purpurea* (Table 3). The results showed that the amount of caftaric acid varied from 2.03 to 4.69 mg/g DW. As shown, the highest caftaric acid production (4.69 mg/g DW) was obtained in 160 mg/l SA × 96 hours treatment, which was 2.13-fold higher than control. This was followed by 160 mg/l SA × 72 hours. Also, lowest level of caftaric acid content was observed in control (2.03 mg/g DW). With increasing the concentration of SA and exposure time, the amount of caftaric acid increased. The highest cichoric acid contents (2.18 mg/g DW) were approximately 1.82 times more than the control, and were observed in the roots treated with 160 mg/l SA × 96 hours exposure time. The lowest level of cichoric acid was observed in the control. The highest chlorogenic acid accumulation (1.67 mg/g DW) was produced by the interaction of 160 mg/l SA × exposure time of 96 hours. It showed that elicitation by 160 mg/l SA × 96 hours exposure time increased the accumulation of chlorogenic acid (2.38-fold) higher than the control roots. As compared with the control group, SA could stimulate accumulation of echinacoside. According to the results stated in the Table 3, with increasing the doses of SA from 80 to 160 g/l, the echinacoside content from 0.44 to 0.67 mg/g DW increased. Also, the results of mean comparison showed that with increasing the elicitation time from 72 to 96 hours, the amount of echinacoside has increased. The results indicated that the content of cynarin in coneflower roots increased gradually with increasing SA concentration and exposure time (Table 3). The amount of cynarin varied from 0.35 to 1.04% mg/g DW. The highest cynarin production (1.04% mg/g DW) was obtained at 160 mg/l SA treated roots for 96 hours that was higher (2.97-fold) compared to the control. The lowest cynarin production was observed in the control roots.

Effect of yeast extract

In the second experiment, statistical analysis indicated that the effects of six treatments consisting various concentrations of YE and exposure time on content of caftaric acid, echinacoside, chlorogenic acid, and cichoric acid content in *E. purpurea* adventitious roots was significant ($p \leq 0.01$) (Table 2). The effect of YE treatment on cynarin production was not significant ($p > 0.05$). In the present study, considering the significant interaction effect of YE× time after elicitation (except for echinacoside), we have focused on the interaction of the two studied factors (Table 4) [34]. The results indicated that the amount of cichoric acid varied from 1.20 to 2.25 mg/g DW. As shown, the highest cichoric acid content (2.25 mg/g DW) was obtained by 1.5 g/l YE × 96 hours

Table 3. Effects of salicylic acid and exposure time on caffeic acid derivatives production in *E. purpurea* adventitious root cultures.

Factor	Bioactive compound contents (mg/g DW)				
	Cichoric acid	Chlorogenic acid	Caftaric acid	Echinacoside	Cynarin
Salicylic acid (SA) (mg/l)					
SA_0-0	1.26	0.66	2.12	0.44 ^{b}	0.37
SA_{80}-80	1.66	1.09	3.46	0.58 ^{a}	0.61
SA_{160}-160	2.04	1.73	4.44	0.67 ^{a}	0.95
Exposure times (T) (hours)					
T_{72}-72	1.59	0.99	3.11	0.44 ^{b}	0.58
T_{96}-96	1.73	1.21	3.56	0.68 ^{a}	0.70
SA × T					
$SA_0 \times T_{72}$	1.30 ^{e}	0.60 ^{e}	2.03 ^{e}	0.40	0.39 ^{ed}
$SA_0 \times T_{96}$	1.20 ^{e}	0.70 ^{e}	2.20 ^{e}	0.48	0.35 ^{e}
$SA_{80} \times T_{72}$	1.51 ^{d}	0.93 ^{d}	3.10 ^{d}	0.47	0.50 ^{d}
$SA_{80} \times T_{96}$	1.80 ^{c}	1.25 ^{c}	3.80 ^{c}	0.70	0.72 ^{c}
$SA_{160} \times T_{72}$	1.98 ^{b}	1.49 ^{b}	4.18 ^{b}	0.55	0.86 ^{b}
$SA_{160} \times T_{96}$	2.18 ^{a}	1.67 ^{a}	4.69 ^{a}	0.81	1.04 ^{a}

Means followed by the same letter in each column are not significantly different at $p \leq 0.05$ as determined by the Duncan's multiple range test. Data are presented as mean (n = 3). SA: Salicylic acid (mg/l). T: Exposure time (hours).

Table 4. Effects of yeast extract and exposure time on caffeic acid derivatives production in *E. purpurea* adventitious root cultures.

Factor	Bioactive compound contents (mg/g DW)				
	Cichoric acid	Chlorogenic acid	Caftaric acid	Echinacoside	Cynarin
Yeast extract (YE) (g/l)					
YE_0-0	1.25	0.63	2.12	0.44 ^{c}	0.37
$YE_{0.75}$-0.75	1.62	1.52	2.38	0.67 ^{b}	0.39
$YE_{1.5}$-1.50	2.10	2.06	3.02	0.91 ^{a}	0.55
Exposure times (T) (hours)					
T_{72}-72	1.57	1.27	2.36	0.62 ^{b}	0.43
T_{96}-96	1.74	1.53	2.65	0.73 ^{a}	0.45
YE × T					
$YE_0 \times T_{72}$	1.30 ^{e}	0.55 ^{f}	2.04 ^{e}	0.39	0.39 ^{b}
$YE_0 \times T_{96}$	1.20 ^{e}	0.70 ^{e}	2.21 ^{d}	0.60	0.34 ^{b}
$YE_{0.75} \times T_{72}$	1.49 ^{d}	1.33 ^{d}	2.22 ^{d}	0.85	0.40 ^{b}
$YE_{0.75} \times T_{96}$	1.75 ^{c}	1.71 ^{c}	2.53 ^{c}	0.48	0.36 ^{b}
$YE_{1.5} \times T_{72}$	1.93 ^{b}	1.93 ^{b}	2.80 ^{b}	0.73	0.47 ^{b}
$YE_{1.5} \times T_{96}$	2.25 ^{a}	2.17 ^{a}	3.21 ^{a}	0.96	0.61 ^{a}

Means followed by the same letter in each column are not significantly different at $p \leq 0.05$ as determined by the Duncan's multiple range test. Data are presented as mean (n = 3). YE: Yeast extract (g/l). T: Exposure time (hours).

treated roots, that was higher compared to the control (1.87-fold). The lowest content of cichoric acid was observed in the control (distilled water) after 96 hours of treatment (1.20 mg/g DW). There were notable differences among interaction of YE × exposure times on chlorogenic acid production in adventitious roots of *E. purpurea*. The highest chlorogenic acid content (2.17 mg/g DW) were 3.94 times higher than the control level at 72 hours after elicitor treatment, and were observed in the roots treated with 1.5 g/l YE × 96 hours after elicitation. According to the values stated in the Table 4, the content of caftaric acid varied from 2.04 to 3.21 mg/g DW. The highest caftaric acid accumulation (3.21 mg/g DW) was obtained at 160 mg/l YE treated roots for 96 hours that was higher (1.57-fold) compared to the control. The lowest level of caftaric acid content was observed in the control roots. It is observed that with increasing the concentration of YE from 0 to 1.5 g/l, the echinacoside content from 0.44 to 0.91 mg/g DW increased. Also, the results indicated that with increasing the exposure time, the amount of echinacoside increased. The interaction effect of YE × exposure time on echinacoside was not significant at 5% probability level.

The heatmap diagram based on the concentration of five CADs (caftaric acid, chlorogenic acid, cynarin, echinacoside, and cichoric acid) of *E. purpurea* adventitious root treated with various concentrations of elicitors (YE and SA each in two exposure time) were visualized (Fig. 3). Among the treatments (YE and SA each in two exposure times), the *E. purpurea* adventitious

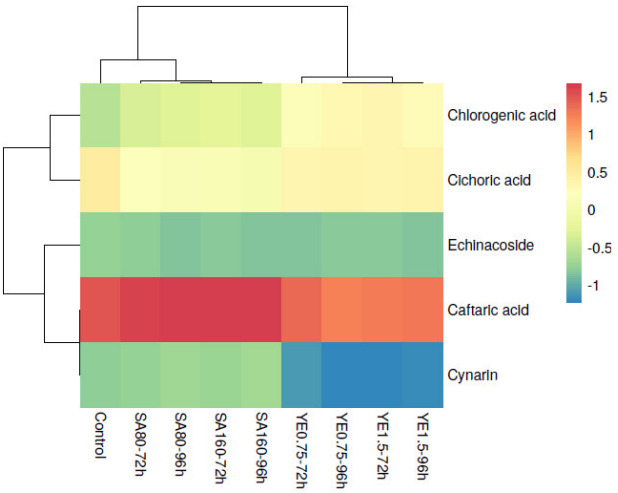

Figure 3. Heatmap diagram based on the relative concentration of 5 caffeic acid derivatives of *E. purpurea* adventitious root treated with yeast extract and salicylic acid. Each column and each row represent various concentrations of elicitors and individual compounds, respectively. Compounds that are classified in the same group responded similarly to the experimental treatments. Color scale of heatmap represents relative intensity values. The colors (blue to red) of the tiles in the heatmap represent the values varying from low to high

roots elicited with SA (80 and 160 mg/l) each in 72 and 96 exposure times grouped in one cluster; YE (0.75 and 1.5 g/l) each in 72 and 96 exposure times treatments also grouped together; while other treatment (control) formed another cluster. The higher caftaric acid content was achieved by SA treatments, while YE treatments yielded the lower level of caftaric acid content in our experimental condition. The lowest level of cynarin content was achieved by YE treatment. In our experimental condition, the highest concentration of five CADs of *E. purpurea* adventitious root was subjected to caftaric acid.

DISCUSSION

Adventitious root culture which develop from the differentiated cells provides biomass as well as valuable bioactive compounds with great potential for large-scale production [33]. Adventitious root formation is influenced by several internal and environmental factors [18]. It is well known that optimization of the root culture condition of target plant tissues is important factor for *in vitro* bioactive compounds studies. The concentration of NH_4NO_3 and IAA were optimized for better responses of rooting to achieve higher secondary metabolites under *in vitro* condition. In our study, leaf explant of *E. purpurea* was suitable for direct root induction. The results showed that both 1 and 3 mg/l IAA were proven suitable for root induction; however, the highest percentage of adventitious root induction (100%) was related to 1 mg l^{-1} IAA in 1/4NH_4NO_3 MS culture medium. It is commonly accepted that auxins have a positive role in initiation of roots. According to the results stated in the Table 1, the root induction was significantly affected by the concentration of IAA. The most abundant natural auxin (IAA) derived from L-tryptophan, controls overabundance of growing programs in plants, including adventitious root formation [18]. In many plants, adventitious root formation is correlated with expression levels of genes involved in auxin pathway [20]. The results of present study suggested that the root induction was significantly affected by the concentration of NH_4NO_3. In our finding, 1 mg l^{-1} IAA in 1/4NH_4NO_3 MS culture medium was found as best treatment, which gave higher number of root (14/33). With the decreasing NH_4NO_3 strength (without and 0.25X MS), the measured traits increased significantly, in compared to standard MS. Nitrogen is an essential mineral element required in the greatest quantity in plants. Roots can utilize NO_3^- and NH_4^+; however, for root development, nitrate (NO_3^-) is the most important source of nitrogen [30]. In accordance with our results, it reported that media salt strength significantly influenced root culture of *P. sepium*. Their findings showed that lower (1/4 -1/2) MS salt strength could induce more roots than higher (1- 2) MS salt strength treated cultures. In other words, it reported that the increase of media salt strength was in inverse proportion with adventitious roots growth [38]. Sivakumar et al. (2005) reported that nutrient medium treatment could be effective for enhancing growth and bioactive compound synthesis in hairy root cultures [30]. According to these findings, SA as an abiotic elicitor and YE as a biotic elicitor at various concentrations can stimulate an increased accumulation of CADs compounds in the root cultures of *E. purpurea*. All of the used concentrations and exposure times had a stimulatory effect on the production of examined CADs. In accordance with our results, it is reported that dosage and type of the elicitors, and time after elicitor treatments were the factors that affected secondary metabolite production in *in vitro* cultures [4]. Abiotic elicitor (SA) that widely used is presented as an eco-friendly, highly potent, cost effective, and quick strategy for metabolite accumulation in medicinal plants [4]. In the present investigation, the results indicated that SA at all doses and exposure times significantly increased the CADs contents

in the adventitious roots. The positive effect of SA on production of CADs has also been reported in *E. purpurea* aerial parts in greenhouse condition [22]. SA foliar application on field-grown *E. purpurea* plants elicits a twofold increase in two bioactive compound (cichoric and caftaric acid) in flower heads, and an almost fourfold increase of CADs in the roots [17]. There are already other investigations showing that applications of SA, stimulate the production of *in vitro* secondary compounds in different plants [9, 21]. Also, similar results of increasing in secondary metabolites in response to elicitation with SA have been reported in other medicinal plant species such as *M. piperita* [23], *M. officinalis* [12], *A. millefolium* [11]. As inferred, the enhanced CADs production in the elicited coneflower might be ascribed to the activation of genes involved CADs pathway. Abbasi et al. (2012) reported that the increase in CADs accumulation is related to PAL enzyme that catalyzes the first metabolic step in phenylpropanoid metabolism and bioactive compounds production in hairy roots of *E. purpurea* [2]. Ghasemzadeh et al. (2012) reported that as a result of SA application is increase in activity of phenylalanine ammonia lyase and chalcone synthase [10]. According to these findings, the content of CADs except for echinacoside reached the highest levels in the roots 96 hours after 1.5 g/l YE treatment. The result of present study is in agreement with the results of our previous study on CADs production in *E. purpurea* plants under greenhouse conditions. The results of the study indicated that highest cichoric acid content was obtained in plants treated with 1.5 g/l YE for 96 hours, that was higher compared to respective control (3.58-fold). Also, for accumulation of CADs in *E. purpurea* aerial parts, longer time (72 and 96 hours) of elicitation was required [22]. The positive effects of this biotic elicitor on accumulation of secondary metabolites in *M. officinalis* [12], *M. piperita* [23], American ginseng hairy root cultures [16] have been previously reported. Different constituents of YE are minerals, amino acids and vitamins. The positive and stimulating effects of YE on secondary compounds production can be related with the concentrations of Ca^{2+}, Co^{2+} and Zn^{2+} cations [29]. Yan et al. (2006) reported that YE increased phenolic acids that may be for its effects on phenylpropanoid pathway and mainly through a tyrosine-derived pathway [37]. The heatmap diagram (Fig. 4) showed that YE and SA clusters are completely separated from each other. Therefore, elicitor type (SA or YE) was a key factor for the differences observed within treatments. Also, the results indicated that the content of compounds in control treatment was clearly separated in the heatmap diagram from the elicited with SA and YE treatments that was in agreement with results has been reported in other plant species [12]. Compounds that are classified in the same group responded similarly to the experimental treatments. This study highlights the differences in quantity of CADs content among concentrations and type of elicitors. Also, it was indicated that SA and YE elicitors had positive effects on CADs production in the induced roots of *E. purpurea*.

CONCLUSION

In conclusion, for root induction in *E. purpurea*, it is appropriate to use 1 mg/l IAA×1/4NH_4NO_3 MS culture medium. The present study clearly indicates that abiotic (SA) and biotic (YE) elicitors at different concentrations and exposure time were acted as effective elicitors for stimulating the enhanced production of CADs contents in *E. purpurea* adventitious root cultures. The findings of the present study showed that elicitation for 96 hours promoted CADs synthesis more than 72 hours in root cultures. The yeast extract at a concentration of 1.5 g/l × 96 hours harvesting time proved to be optimal for efficient biosynthesis of CADs. The lowest level of bioactive compound contents was observed in the control roots. Valuable bioactive compounds in *E. purpurea* can be produced by these efficient *in vitro* systems.

AUTHOR CONTRIBUTION STATEMENT

N. K. performed the experiments and data gathering. M. A. designed/supervised the research, analyzed the data and wrote the manuscript. A. B. was advisor in the present research.

DECLARATION OF COMPETING INTEREST

The authors declare no competing financial interest.

ACKNOWLEDGEMENTS

This study was supported by the University of Malayer, Iran.

REFERENCES

[1] Abbasi, B.H., Saxena, P.K., Murch, SJ. and Liu, C.Z. 2007. Echinacea biotechnology: challenges and opportunities. In Vitro Cellular & Developmental Biology-Plant 43: 481-492. doi: 10.1007/s11627-007-9057-2.

[2] Abbasi, B.H., Stiles, A.R., Saxena, P.K. and Liu, C.Z. 2012. Gibberellic acid increases secondary metabolite production in *Echinacea purpurea* hairy roots. Applied Biochemistry and Biotechnology 168:2057–2066. doi: 10.1007/s12010-012-9917-z.

[3] Abdoli, M., Moieni, A. and Naghdi Badi, H. 2013. Influence of KNO3, CaCl2 and MgSO4 concentrations on growth and cichoric acid accumulation in hairy root culture of purple coneflower (*Echinacea purpurea* L.). Journal of Medicinal Plants 12: 75-84.

[4] Ali B. 2020. Salicylic acid: An efficient elicitor of secondary metabolite production in plants. Biocatalysis and Agricultural Biotechnology 31: 101884. doi: 10.1016/j.bcab.2020.101884.

[5] Arceusz, A., Wesolowski, M. and Konieczynski, P. 2013. Methods for extraction and determination of phenolic acids in medicinal plants: a review. Natural Product Communications 8 (12): 1821–1829.

[6] Baenas, N., Garcia-Viguera, C. and Moreno, D.A. 2014. Elicitation: A tool for enriching the bioactive composition of foods. Molecules 19: 13541-13563. doi: 10.3390 / molecules190913541.

[7] Barrett, B. 2003. Medicinal properties of Echinacea: a critical review. Phytomedicine 10 (1): 66–86. doi: 10.1078/094471103321648692.

[8] Brown, P.N. 2011. Determination of major phenolic compounds in Echinacea spp. raw materials and finished products by high-performance liquid chromatography with ultraviolet detection: single-laboratory validation matrix extension. Journal of AOAC International 94 (5): 1400-1410. doi:10.5740/jaoacint.11-142.

[9] Demirci, T., Aras Ascı, . And Gktrk Baydar, N. 2021. Influence of salicylic acid and L-phenylalanine on the accumulation of anthraquinone and phenolic compounds in adventitious root cultures of madder (*Rubia tinctorum* L.). Plant Cell, Tissue and Organ Culture 144: 313–324. https://doi.org/10.1007/s11240-020-01952-w.

[10] Ghasemzadeh, A., Jaafar, H.Z., Karimi, E. and Ibrahim, M.H. 2012. Combined effect of CO_2 enrichment and foliar application of salicylic acid on the production and antioxidant activities of anthocyanin, flavonoids and isoflavonoids from ginger. BMC Complementary Medicine and Therapies 12: 229. doi: 10.1186/1472-6882-12-229.

[11] Gorni, P.H., Pacheco, A.C., Moro, A.L., Silva, J.F.A., Moreli, R.R., de Miranda, G.R., Pelegrini, J.M., Spera, K.D., Junior, J.L.B. and da Silva R.M.G. 2020. Salicylic acid foliar application increases biomass, nutrient assimilation, primary metabolites and essential oil content in *Achillea millefolium* L. Scientia Horticulturae 270: 109436. doi:10.1016/j.scienta.2020.109436.

[12] Hedayati, A. and Abdoli M. 2021. Foliar application of yeast extract and salicylic acid affect chemical composition and content of lemon balm (*Melissa officinalis* L.) essential oil. Journal of Plant Molecular Breeding 9 (1): 12 – 24. doi: 10.22058/JPMB.2022.544981.1248.

[13] Ho, T.T., Murthy, H.N. and Park, S.Y. 2020. Methyl Jasmonate induced oxidative stress and accumulation of secondary metabolites in plant cell and organ cultures. International Journal of Molecular Sciences 21(3): 716. https://doi.org/10.3390/ijms21030716.

[14] Jeong, J.A., Wu, C.H., Murthy, H.N., Hahn, E.J. and Paek, K.Y. 2009. Application of an airlift bioreactor system for the production of adventitious root biomass and caffeic acid derivatives of *Echinacea purpurea*. Biotechnology and Bioprocess Engineering 14:91–98.

[15] Khanam, M.N., Anis, M., Javed, S.B., Mottaghipisheh, J. and Csupor, D. 2022. Adventitious Root Culture-An Alternative Strategy for Secondary Metabolite Production: A Review. Agronomy 12: 1178. https://doi.org/10.3390/agronomy12051178.

[16] Kochan, E., Szymczyk, P., Kuźma, Ł., Lipert, A. and Szymańska, G. 2017. Yeast extract stimulates ginsenoside production in hairy root cultures of American ginseng cultivated in shake flasks and nutrient sprinkle bioreactors. Molecules 22 (6): 880. doi: 10.3390/molecules22060880.

[17] Kuzel, S., Vydra, J., Triska, J., Vrchotova, N., Hruby, M. and Cigler, P. 2009. Elicitation of pharmacologically active substances in an intact medical plant. Journal of Agricultural and Food Chemistry 57: 7907-7911. doi: 10.1021/jf9011246.

[18] Lakehal, A. and Bellini C. 2019. Control of adventitious root formation: Insights into synergistic and antagonistic hormonal interactions. Physiologia Plantarum, Wiley 165 (1): 90-100 pp.

[19] Lema-Rumińska, J. Kulus, D., Tymoszuk, A., Varejo, J.M.T.B. and Bahcevandziev, K. 2019. Profile of secondary metabolites and genetic stability analysis in new lines of *Echinacea purpurea* (L.) Moench micropropagated via somatic embryogenesis. Industrial Crops & Products 142: 111851. https://doi.org/10.1016/j.indcrop.2019.111851.

[20] Liu, M., Zhang, H., Fang, X., Zhang, Y. and Jin, C. 2018. Auxin acts downstream of ethylene and nitric oxide to regulate magnesium deficiency-induced root hair development in Arabidopsis thaliana. Plant and Cell Physiology 59: 1452-1465. https://doi.org/10.1093/pcp/pcy078.

[21] Mahalakshmi, R., Eganathan, P. and Parida, A.K. 2013. Salicylic acid elicitation on production of secondary

metabolite by cell cultures of *Jatropha curcas* L. International Journal of Pharmacy and Pharmaceutical Sciences 5: 655–659.

[22] Mehrpooya, Zh., Abdoli, M. and Talebian, M.A. 2021. Effect of salicylic acid and yeast extract on caffeic acid derivatives production in *Echinacea purpurea* L. Journal of Medicinal Plants 20 (78): 36-47. doi: 10.52547/jmp.20.78.36.

[23] Motiee, M. and Abdoli, M. 2021. Changes in essential oil composition of peppermint (*Mentha x piperita* L.) affected by yeast extract and salicylic acid foliar application. Journal of Medicinal Plants 20 (79): 47-58. doi: 10.52547/jmp.20.79.47.

[24] Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15: 473–497. https://doi.org/10.1111/j.1399.

[25] Murch, S.J., Peiris, S.E., Shi, W.L., Zobayed, S.M.A. and Saxena P.K. 2006. Genetic diversity in seed populations of *Echinacea purpurea* controls the capacity for regeneration, route of morphogenesis and phytochemical composition. Plant Cell Report 25:522–532. doi:10.1007/s00299-006-0118-5.

[26] Murthy, H., Lee, E.J. and Paek, K.Y. 2014. Production of secondary metabolites from cell and organ cultures: strategies and approaches for biomass improvement and metabolite accumulation. Plant Cell, Tissue and Organ Culture 118: 1-16. doi: 10.1007/s11240-014-0467-7.

[27] Paek, K.Y., Murthy, H.N., Hahn, E.J. and Zhong, J.J. 2009. Large scale culture of ginseng adventitious roots for production of ginsenosides. Advances in Biochemical Engineering/Biotechnology113: 151–176. doi: 10.1007/10_2008_31.

[28] Raman, P., Patino, L.C. and Nair, M.G. 2004. Evaluation of metal and microbial contamination in botanical supplements. Journal of Agricultural and Food Chemistry 52: 7822–7827. https://doi.org/10.1021/jf049150.

[29] Skandari, S.A., Piri, K.H., Kayhanfar, M. and Hasanloo, T. 2012. Influence of jasmonic acids, yeast extract and salicylic acid on growth and accumulation of hyosciamine and scopolamine in hairy root cultures of *Atropa belladonna* L. International Journal of Agriculture: Research and Review 2:403-409.

[30] Sivakumar, G, Yu, K.W., Hahn, E.J. and Paek, K.Y. 2005. Optimization of organic nutrients for ginseng hairy roots production in large-scale bioreactors. Current Science 89: 641-649.

[31] Thakur, M., Bhattacharya, S., Khosla, P.K. and Puri, S. 2019. Improving production of plant secondary metabolites through biotic and abiotic elicitation. Journal of Applied Research on Medicinal and Aromatic Plants 12:1–12. doi:10.1016/j.jarmap.2018.11.004.

[32] Udomsuk, L., Jarukamjorn, K., Tanaka, H. and Putalun, W. 2009. Isoflavonoid production in hairy roots culture of *Pueraria candollei*. Journal of biosciences 64: 687-91. doi: 10.1515/znc-2009-9-1013.

[33] Wang, J., Man, S., Gao, W., Zhang, L. and Huang, L. 2013. Cluster analysis of ginseng tissue cultures, dynamic change of growth, total saponins, and specific oxygen uptake rate in bioreactor and immunoregulative effect of ginseng adventitious root. Industrial Crops & Products 41:57–63. https://doi.org/10.1016/j.indcrop.2012.04.005.

[34] Wickens, T.D. and Keppel, G. 2004. Design and analysis: A researcher's handbook (4th ed.). Englewood Cliffs, NJ: Prentice Hall.

[35] Wu, C.H., Murthy, H.N., Hahn, E.J. and Paek, K.Y. 2007a. Improved production of caffeic acid derivatives in suspension cultures of *Echinacea purpurea* by medium replenishment strategy. Archives of Pharmacal Research 30: 945–949. doi: 10.1007/BF02993961.

[36] Wu, C.H., Tewari, R.K., Hahn, E.J. and Paek, K.Y. 2007b. Nitric oxide elicitation induces the accumulation of secondary metabolites and antioxidant defense in adventitious roots of *Echinacea purpurea*. Journal of Plant Biology 50: 636–643.

[37] Yan, Q., Shi, M., Ng, J. and Wu, J.Y. 2006. Elicitor-induced rosmarinic acid accumulation and secondary metabolism enzyme activities in *Salvia miltiorrhiza* hairy roots. Plant Science 170: 853-858. https://doi.org/10.1016/j.plantsci.2005.12.004.

[38] Zhang, J., Gao, W.Y., Wang, J. and Li, X.L. 2011. Effects of explant types and media salt strength on growth and secondary metabolite accumulation in adventitious roots of *Periploca sepium* Bunge. Acta Physiologiae Plantarum. 33: 2447-2452. doi:10.1007/s11738-011-0785-x.

کشت ریشه نابجا در سرخارگل (*Echinacea purpurea* L.) و افزایش تجمع مشتقات اسید کافئیک از طریق محرک‌های زنده و غیر زنده

محمد عبدلی[1*]، نوشین کولیوند[1] ، آرش بابایی[2]

[1] گروه تولید و ژنتیک گیاهی دانشکده کشاورزی دانشگاه ملایر ملایر ایران.

[2] گروه زیست شناسی، دانشکده علوم، دانشگاه ملایر، ملایر، ایران.

*نویسنده مسئول: Abdoli_m@malayeru.ac.ir

چکیده

این تحقیق با هدف ایجاد دستورالعملی برای القای ریشه نابجا و بررسی اثرات اسید سالیسیلیک (۰، ۱۶۰ و ۳۲۰ میلی‌گرم در لیتر) و عصاره مخمر (۰، ۰/۷۵ و ۱/۵ گرم در لیتر) بر تولید مشتقات کافئیک اسید (اسید شیکوریک، اسید کلروژنیک، اسید کافتاریک، اکیناکوزید و سینارین) در ریشه‌های نابجای سرخارگل انجام شد. همچنین اثر نیترات آمونیم ۰، ۰/۲۵، ۰/۷۵ و ۱ برابر غلظت محیط MS همراه با دو غلظت ایندول استیک اسید (۱ و ۳ میلی‌گرم در لیتر) بر القای ریشه مورد بررسی قرار گرفت. نتایج نشان داد که القای ریشه نابجا در سرخارگل به طور معنی داری تحت تأثیر میزان نیترات آمونیم و غلظت IAA قرار گرفت ($p\leq$۰/۰۱). بیشترین القای ریشه (۱۰۰٪) و میانگین تعداد ریشه های نابجا در ریزنمونه‌های کشت شده (۱۴/۳ ریشه) در تیمار ۱ میلی‌گرم در لیتر IAA در محیط کشت MS با یک‌چهارم نیترات آمونیم مشاهده شد. اثرات اصلی و متقابل اسید سالیسیلیک و عصاره مخمر با مدت زمان بر صفات اندازه گیری شده (به استثناء اکیناکوزید) معنی دار بود ($p\leq$۰/۰۱). نتایج نشان داد کاربرد ۱/۵ گرم در لیتر عصاره مخمر و ۱۶۰ میلی گرم در لیتر سالیسیلیک اسید به مدت زمان ۹۶ ساعت بهترین تیمارها برای تحریک میزان مشتقات کافئیک اسید می باشد. بیشترین میزان تولید اسید کافتاریک، اسید شیکوریک، اسید کلروژنیک و سینارین در ریشه‌های نابجا، پس از ۹۶ ساعت تیمار با غلظت ۱۶۰ میلی‌گرم در لیتر سالیسیلیک اسید بدست آمد که در مقایسه با شاهد ۲/۱۳، ۱/۸۳، ۲/۳۹ و ۲/۹۷ برابر بیشتر بود. نمودار حرارتی (هیت مپ) نشان داد که میزان مشتقات کافئیک اسید در تیمارهای عصاره مخمر و سالیسیلیک اسید در گروههای مجزایی از هم و شاهد قرار گرفتند.

کلمات کلیدی: اسید شیکوریک، سرخارگل، محرک، درون شیشه‌ای، گیاه دارویی، عصاره مخمر