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Original Article

Production of chlorogenic acid in *Varthemia persica* DC (var. *persica*) callus cultures

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Abstract

Chlorogenic acid, a pharmacologically important compound, is a phenolic compound that occurs in certain commonly used medicinal herbs. We looked for the presence of this compound in the callus cultures of *Varthemia persica* DC (var. *persica*). We have evaluated the conditions for establishment of callus cultures of *V. persica* and the *in vitro* production of chlorogenic acid. Callus was initiated by culturing seedling of *V. persica* on MS basal medium supplemented with different concentrations of kinetin, naphthalene acetic acid and 2,4-diphenoxy acetic acid. Also, the influence of light, and phytohormones on the production of chlorogenic acid was examined. Kinetin stimulated the production of chlorogenic acid. Replacement of 2,4-diphenoxy acetic acid with naphthalene acetic acid did not alter the chlorogenic acid production. The ability to induce the accumulation of chlorogenic acid in the *V. persica* callus cultures offers an opportunity to produce a phenolic compound with therapeutic value.

Keywords: Chlorogenic acid; *Varthemia persica*; Callus culture

INTRODUCTION

Chlorogenic acid (5-*o*-caffeoylquinic acid) is a phenolic compound widely distributed in plants including coffee (1,2). A literature survey revealed that chlorogenic acid possesses a wide range of biological activities such as anti-oxidant (3), anti-mutagenic (4), immunomodulatory properties (5), reduction of adrenocorticotropic hormone levels (6) and antiviral activities (7). Antioxidant effects of chlorogenic acid have been associated with a lower risk of a variety of liver diseases including liver cirrhosis and liver cancer (8,9). Chlorogenic acid constitutes a significant component of certain commonly used medicinal herbs. The herbs known for their chlorogenic acid content include *Chrysanthemum* flower, *Crataegus* fruit, *Artemisia* leaves, and *Epimedium* leaves (10,11). The amount of the compound which is present in most plants is miniscule. Thus, they are not an important part of medicinal herbs. Nevertheless, there is relatively little information available on the *in vitro* production of chlorogenic acid (12-14). In response to the growing evidence of the value of chlorogenic acid, we have investigated the production of this compound in cultured tissues of *Varthemia persica* (Asteraceae). The aromatic genus of *Varthemia* has only one species, *V. persica* DC, in Iran. There is no report on the pharmacological activity of this species. To the best of our knowledge, no *in vitro* culture of *V. persica* has previously been reported. However, the volatile constituents and flavonoid compositions of the plant have been studied (15,16).

MATERIALS AND METHODS

Reagents and solutions

Chlorogenic acid was supplied by Roth (Germany). HPLC grade acetonitrile was purchased from Caledon (Canada). Butylhydroxytoluene, KH_2PO_4, methanol, hydrochloric

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acid, acetic acid were from Merck (Germany). Naphthaleneacetic acid, kinetin, and 2,4-dichlorophenoxyacetic acid (2,4-D) were purchased from Sigma-Aldrich (UK). All reagents and solutions were either HPLC or analytical grades. Water was obtained by double distillation and purified additionally with a Milli-Q system.

Plant materials

The seeds of *V. persica* DC (var. *persica*) (Asteraceae) were collected from the northern slopes of Karkas mountains, at altitudes of 2200-2400 m in Isfahan province, Iran in the summer of the year 2005. The plant was identified, and a voucher specimen (No. 1278) has been deposited in the herbarium of the School of Pharmacy and Pharmaceutical Sciences of Isfahan University of Medical Sciences (15).

Sterilization and explants procedures

In the first stage of the sterilization procedure, seeds were soaked in sterile water containing 0.05% Tween 80 for 60 min. In a laminar flow hood, the water was poured off and the seeds were rinsed with more sterile water. This first step of sterilization was followed by a 3-min soaking in 80% ethanol with further rinsing with sterile water. The seeds were then germinated on a wetted filter paper in Petri dishes in darkness at 25 °C.

Tissue culture conditions

For all the experiments, the basic medium contained 4.2 g/l MS (Murashige and Skoog) minimal organic media (Sigma); 3% (w/v) sucrose, 1.2% agar (Sigma) and pH adjusted to 5.7. Approximately, 50 ml of medium was poured into 250-ml flasks. The flasks were sterilized at 120 °C for 15 min before the addition of seedlings. Three samples of seedlings were prepared on each medium. Callus was successfully induced. Most calluses were grown in 16/8 h light-dark cycles (Shahab white fluorescent lamp, 58w) at 27 °C. For comparison, some cultures were also grown in continuous darkness. Callus tissue was transferred every three weeks to fresh solid medium. The media was supplemented with hormones including naphthaleneacetic acid (NAA); kinetin; and 2,4-D. All hormones were added prior to autoclaving.

Preparation of the extracts

Extracts of the callus cultures were prepared after they had carefully been separated from adhering medium. Callus tissues were freeze dried for 24 h and then extracted. The dried materials were ground to a fine powder using a pestle and mortar. A known amount of the powder was quantitatively extracted with redistilled methanol as follows. The powdered callus (1 g) was mixed with 40 ml of 62.5% aqueous methanolic solution containing 1% (w/v) butyl hydroxytoluene (as internal standard) and 10 ml hydrochloric acid 6 M and then refluxed for 2 h. The mixture was cooled to room temperature and brought to 100 ml volume with methanol and a portion was filtered through a filter paper (17). Aliquots of 20 µl were injected into the HPLC column.

Chromatographic conditions

Chromatographic separation was performed using a Nova-Pack C_{18} column (150 × 3.9 mm) and a Nova-Pack C_{18} pre-cloumn (Waters Association, Milford, MA). The mobile phase consisted of 25% acetonitrile in 25 mM KH_2PO_4 buffer (pH 6.5) at a flow rate of 1 ml/min. The column effluent was monitored at 325 nm wavelength using a diode array detector at (PDA 2996, Water Millennium). UV spectra were obtained at wavelengths between 210-500 nm. Quantitation was achieved by comparison of the peak area ratios of the drug to the internal standard and reference to a standard curve. The mobile phase was prepared daily, filtered and degassed by ultrasonication before use. The mobile phase was not allowed to recirculate during the analysis.

Standard solutions of chlorogenic acid

A stock solution of chlorogenic acid was prepared by dissolving 5 mg of chlorogenic acid in 10 ml of methanol in a 10-ml volumetric flask to obtain a concentration of 500 µg/ml. Aliquots of 0.5, 1, 1.5, and 2 ml of this stock solution were transferred into 50-ml volumetric flasks, 20 ml of 62.5% aqueous

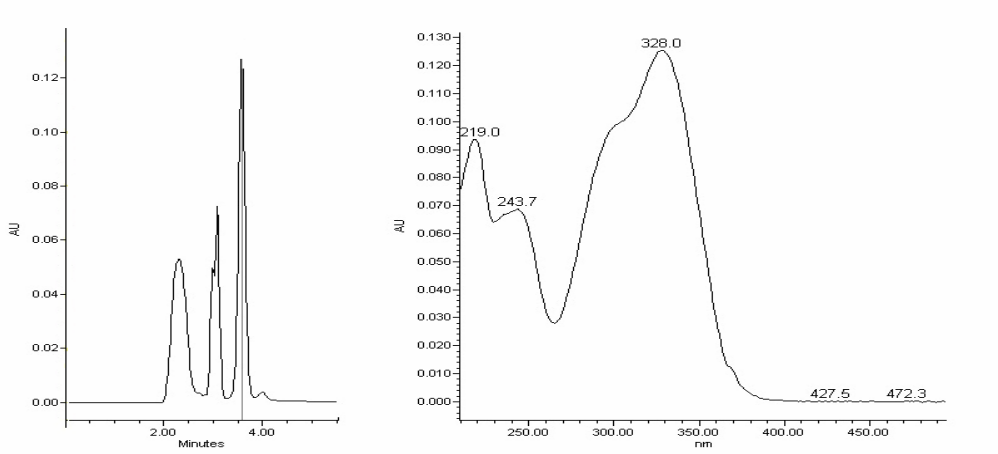

Fig. 1. HPLC-PDA chromatogram and UV spectrum of chlorogenic acids from callus of *Varthemia persica*. Peak (Rt: 2.25) Internal standard; Peak (Rt: 3.08): 5-chlorogenic acid and 4-chlorogenic acid; Peak (Rt: 3.57): 3-chlorogenic acid

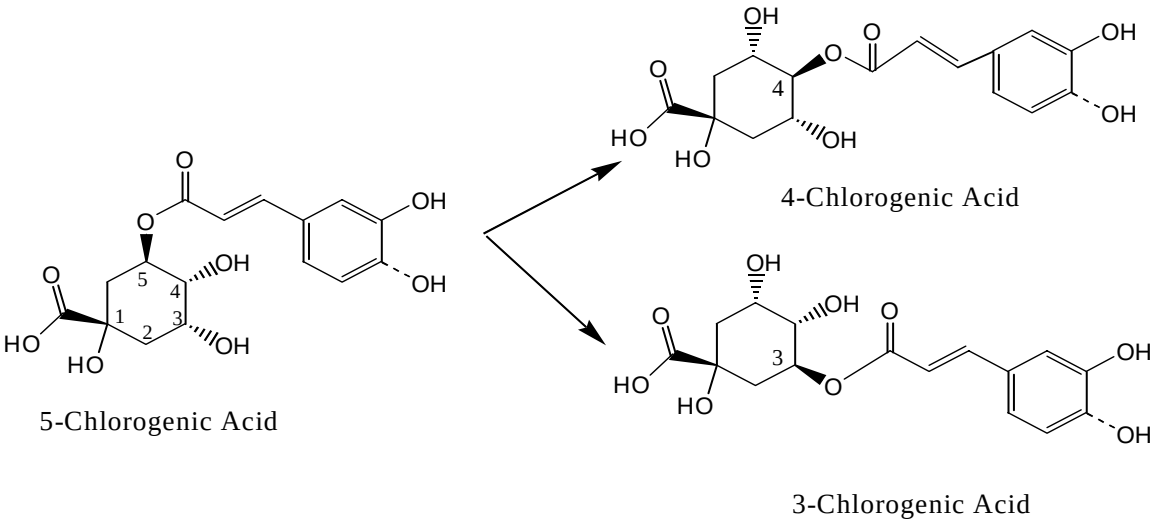

Fig. 2. Isomers of chlorogenic acid

methanolic solution containing 1% w/v butyl hydroxytoluene and 5 ml of 6 M HCl acid was added and brought to volume with methanol. This resulted in a series of standard solutions at concentrations of 5, 10, 15, 20 µg/ml and 20 µl aliquotes were injected into the HPLC column.

Chlorogenic acid identification and quantification

The spectra of compounds were recorded at wavelengths between 210-500 nm for the identification of the chlorogenic acid and for the test of peak purity. Identification of chlorogenic acid was achieved by comparison of its retention time and UV spectra with that of standard compound (Fig. 1). Linear regression was used to quantitate chlorogenic acid concentrations in samples. Chlorogenic acid content was calculated as the sum of the three isomers of 3-chlorogenic acid, 4-chlorogenic acid and 5-chlorogenic acid in callus extract (Fig. 2**).** Calibration curves were constructed by plotting peak area ratio (y) of chlorogenic acid to the internal standard versus chlorogenic acid concentrations (x).

All experiments were performed in triplicate. The analysis of data was performed by ANOVA and Hierarchical cluster analysis using the SPSS program. The differences between treatments were estimated using the Duncan test at $P<0.05$.

RESULTS

Fig. 1 shows a typical chromatogram of chlorogenic acid isomers as well as the internal standard extracted from callus culture of *V. persica*. The flow rate of the mobile phase seemed to optimize at 1 ml/min. This flow rate resulted in the retention times of about 3.08 for 5-chlorogenic acid and 4-chlorogenic acid which were not resolved completely. 3-chlorogenic acid appeared at 3.57 min and was completely resolved from the other two isomers of chlorogenic acid. Under the chromatographic conditions described, chlorogenic acid isomers and the internal standard peaks were well resolved and no peak tailing was noticed. No interfering peaks were observed in the chromatogram. Quantification was not hampered by potential interferences from the internal standard (Fig. 1). The standard curves were linear over the concentration ranges of 5-20 µg/ml using line-fit plot in regression analysis with a coefficient of 0.998 (Fig. 3). In these studies, different concentrations and combinations of NAA, kinetin and 2,4-D in addition to the effect of light/darkness treatment were studied in order to establish a basic formula which could be employed to evaluate chlorogenic acid production (Table 1).The chlorogenic acid yields on different hormone and light treatments are presented in Table 1. The highest chlorogenic acid content was observed when kinetin was used at the concentration of 0.1 g/l alone with no auxin hormone. The chlorogenic acid content was increased almost three folds when 2,4-D was replaced with NAA in the presence of kinetin. However, no significant difference was recorded by variation of light treatments in cultures carried out in the presence of kinetin and 2,4-D. At least two of the three isomers were detected in

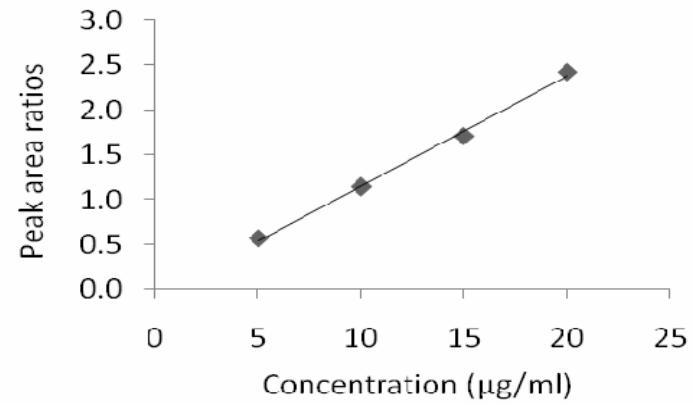

Fig. 3. Standard curve of chlorogenic acid

Table 1. Effects of phytohormones and light cycle on chlorogenic acid production in callus cultures of *V. persica*

Treat	K (mg/l)	2,4-D (mg/l)	NAA (mg/l)	Light/Dark cycle (h)	Chlorogenic acid (µg/g dw)
A	0.2	0.1	0	16/8	[a]39 ± 2
B	0.2	0	0.1	16/8	[b] 93 ± 4
C	0.2	0	0	16/8	[b] 98 ± 4
D	0.1	0	0	16/8	[c] 182 ± 8
E	0.2	0.2	0	0/24	[a] 37 ± 1
F	0.2	0.2	0	16/8	[a] 36 ± 2

Chlorogenic acid contents are expressed as sum of the three chlorgenic acid isomers and µg/g of dry weight of the plant tissue. K: kinetin; NAA: naphthalene acetic acid; 2,4-D: 2,4-dichlorophenoxyacetic acid. Duncan and cluster test results are indicated as superscript letters (a, b, c).

all samples tested (results not shown). None of the culture conditions investigated resulted in the production of only one isomer.

DISCUSSION

As shown in Table 1 the conditions of callus growth, including hormone supplementation and light darkness treatment, had a significant effect on the production level of chlorogenic acid in cultures of *V. persica*. Although chlorogenic acid production was observes on all media tested, medium containing 0.1 mg/l kinetin produced the best results for the chlorogenic acid production. It has been reported, for several plant cell lines, that manipulation of the hormone concentration in the medium can produce secondary metabolites. Sometimes very little changes in the hormone level are needed to produce specific metabolite (18). It is interesting to see that increasing kinetin concentration to 0.2 mg/l did not result in the production of more chlorogenic acid. Variation of other hormones concentration also influenced chlorogenic acid production. The interesting point to note is that transfer of the *V. persica* callus from the medium containing 0.1 mg/l 2, 4-D and 0.2 mg/l kinetin to medium containing only 0.1 mg/l kinetin caused two-fold increase of chlorogenic acid level. On the contrary, it has been reported that, differences in the auxin levels caused no changes in the chlorogenic acid production in cherry rootstock (19). This suggests that, kinetin has shown the most remarkable effects on production of chlorogenic acid in *V. persica* callus cultures. In general, an increase in auxin levels, such as 2,4-D, in the medium stimulates dedifferentiation of the cells and consequently diminishes the level of secondary metabolites, such phenomena has been observed on chlorogenic acid production in *Echinaceae angustifolia in vitro* tissue culture (20). Similarly, it has been reported that cytokinins stimulates alkaloid synthesis which is induced by removing auxin from the medium of a cell line of *N. tabacum* (21). However, cytokinins have different effects depending on the type of metabolite and species concerned. Thus, kinetin stimulated the production of anthocyanins in *Haplopappus gracilus* but inhibited the formation of anthocyanin in *Populus* cell cultures (22). As shown in Table 1, a significant difference in chlorogenic acid production was observed when NAA was applied into the medium instead of 2,4-D (treatment B). Moreover, the use of kinetin alone at low concentrations resulted in the highest production of chlorogenic acid (treatment D). Elimination of 2,4-D or replacement of 2,4-D by NAA or indole acetic acid has been shown to enhance the production of shikonin in suspensions of *L. erythrorhizon* (23). Also, it has been shown that in the cultured cells of *Camptotheca acuminata*, anthocyanin production is significantly greater in the presence of kinetin, compared to benzyladenine (24).

No significant changes were observed when the light effect on chlorogenic acid production in *V. persica* cultured cells was evaluated. On the contrary, the amount of chlorogenic acid production has been reported to increase in cultured callus tissues of *Haplopappus gracilis* cultured in blu light comparing with red light and cultured somatic embryos of *Eleutherococcus senticosus* treated in light (25,26). Also, it has been reported that chlorogenic acid and cichoric acid production is optimum in *Echinacea purpurea* cultures incubated in the light conditions specially 3/21 h light and dark photoperiod and in *Eucommia ulmoides* callus culture incubated on 12 h l/d illumination (27,28). Similar results have been reported for the chlorogenic acid accumulation in *Fabiana imbricata* callus cultures incubated under dark illumination (29).

However, there are several reasons for the secondary metabolites production under light-stimulated conditions. It has been reported that, the activity of the enzymes involved in the biosynthesis of cinnamic acids, coumarins, lignins, flavones, flavonols, chalcones, and anthocyanins are influenced significantly by light (30). It has been shown that the accumulation of anthocyanin is strongly stimulated by light in cell cultures of *D. carota* and *Vitis hybrids* (22). Also, exclusion of light in callus cultures of *Citrus limon* prompted the accumulation of monoterpenes (31). It has been suggested that the light effects on

accumulation of secondary compounds may occur via direct control of product concentration or via influences on enzymatic reactions (27,32).

The ability to produce chlorogenic acid biotechnologically in the *V. persica* callus culture offers an opportunity to produce a phenolic compound with therapeutic value.

CONCLUSION

This is the first report on production of chlorogenic acid *in vitro* using cultures of *V. persica*. It was underlined how *in vitro* production of *V. persica* callus from plant seedlings and the development of the proper cells treatments allowed obtaining biomass capable of production of active compound with therapeutic qualities. The results indicated that if cell medium and/or callus environment improves, the content of chlorogenic acid would be increased.

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