Assessment of some phenolic acids of artichoke callus under *in vitro* conditions

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Abstract

Medicinal plants are the most important sources of life saving drugs for the majority of world's population. Artichoke is one of the oldest medicinal plants from asteraceae family. In the present study, caffeic acid and chlorogenic acid accumulation of different organs of artichoke callus were studied under *in vitro* conditions in MS, SH, and B5 media. The experiment was performed based on a completely randomized design with 3 replications. The artichoke seeds were transferred to the sterile Petri dishes containing filter paper immediately after disinfecting under sterile conditions. The obtained seedlings were transferred to half-strength MS medium. Only non-contaminated seedlings were used for preparation of root, leaf, and petiole explants. The SH, MS, and B5 media containing 0.8% agar, 3% sucrose, and 0, 0.5, 0.75, and 1 (mg/l) 2,4-D were used for callus induction. According to the results, caffeic acid accumulation was not found to be statistically different ($\alpha<0.05$) among various media but maximum amount of chlorogenic acid accumulation between different media was recorded in SH medium containing 0.75 (mg/l) 2,4-D and with the leaf explant. In all culture media callus formation was related to the presence of 2.4,D. Otherwise, no callus formation was observed in hormone-free media.

Keywords: artichoke; callus; explant; 2,4-D; media culture; phenolic acids

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Introduction

Plants have been the important source of pharmacologically active compounds for thousands of years. Medicinal plants are the most important sources of life saving drugs for the majority of world's population (Shrivastava and Pratibha, 2011). Artichoke is one of the oldest medicinal plants and is a perennial plant of the asteraceae family. The food value of artichoke is related to the phenolic compounds, inolin, and salt minerals (Adzet et al., 1987). The most common hydroxycinnamic acid derivatives of artichoke are esters of caffeic acid (CFA) with quinic acid, predominantly chlorogenic acid (CGA, IUPAC name: 5-caffeoylquinic acid). Several studies have registered the presence of mono-dicaffeoylquinic acids, such as chlorogenic, neochlorogenic, and cryptochlorogenic acids and dicaffeoylquinic acids such as ascynarine in *C. scolymus* L. (Trajtemberg et al., 2006). About the effect of environmental conditions on phenolic compound accumulation of artichoke some studies were carried out (Abdin et al., 2003).

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Biotechnological tools are important for multiplication and genetic enhancement of the medicinal plants by adopting techniques such as *in vitro* regeneration and genetic transformations. *In vitro* propagation of plants holds tremendous potential for the production of high-quality plant-based medicines (Murch et al., 2000). Plant growth regulators and media culture are the most important factors affecting cell growth, differentiation, and metabolite formation (Lianget al., 1991).

The appropriate concentration of the medium is one of the critical determinants in controlling callus growth and metabolite production. Plant cell culture holds much promise as a method for producing complex secondary metabolites *in vitro*. There are a number of examples of cultured cells producing metabolites not observed in the plant, i.e., Lithospermum erythrorhizon cultures have been observed to synthesize rosmarinic acid (Fukui et al., 1984). It has become apparent that the choice of original plant material having high yields of the desired phytochemicals may be important in establishing high-yielding cultures (Deus and Zenk, 1982). Furthermore, the need to repeatedly screen plants for high-producing lines (due to inherent instability of cell lines) has been emphasized, although the nutritional composition of the medium is also important (Whitaker et al., 1986). Thus, a variety of approaches are being investigated in many research studies to increase productivity of useful plant metabolites in plant cell cultures. Tissue culturing of medicinal plants is widely used to produce active compounds for herbal and pharmaceutical industries (Sidhu, 2010). The aim of the present study was to determine the content of caffeic and chlorogenic acids of the artichoke callus and optimize a secondary metabolite producing culture.

Materials and Methods

Chemicals and standards

Chromatographic grade double distilled water, HPLC grade methanol (Merck-1, 06007), acetonitrile, and acetic acid were used for the HPLC analysis. CFA (SC0625) and CGA (SC3878), were purchased from Sigma Company. All other chemicals and reagents used were of the highest commercially available purity.

Surface sterilization

To obtain disinfected explant from newly germinated seeds, the viability of seeds of *Cynarascolymus* L. was performed via germination percentage test. After that, seeds were soaked in 200 (mg/l) GA_3 for 24 hours. Then seeds were washed by water. Treated seeds were surface sterilized with 70% methanol for 10 seconds, then they were washed with sterilized water. Next, the seeds were soaked in 20% sodium hypochlorite for 20 minutes and rinsed with autoclaved double distilled water 3 times in laminar flow hood (Michalak, 2006). Some seeds were cultured and the rest were stored in sterile glass bottle.

In vitro seed germination and callus production

For germination, surface sterilized seeds were placed in the Petri dishes containing filter paper, and were incubated in culture room at 23±2 °C with 16 h photoperiod. After peeling off sprout coats, the obtained sprouts were placed individually in 10x15 cm culture tubes containing 2 cm of solidified 0.8% agar of half-strength MS medium culture (1/2 MS). The culture tubes were transferred to the culture room at 23±2 °C for four weeks.

The disease-free seedlings were used as the initial material for preparation of root, leaf, and petiole explants (1x1.5 cm). Prepared explants were cultured in culture tubes (10x15 cm) containing 30 ml of each of the B5 (Schenk and Hildebrandt, 1972), SH (Gamborg et al., 1968), and MS (Murashige and Skoog, 1962) media cultures containing 8 g/l agar, 3% sucrose, and different 2,4-D concentrations. All of the calluses were transferred to dark culture room at 23 ± 2 °C.

Secondary metabolite extraction

Different extraction procedures of the polyphenols have been reported, mainly as hot water, methanol, ethanol, acetone, and ethyl acetate. Among them methanol and ethyl acetate

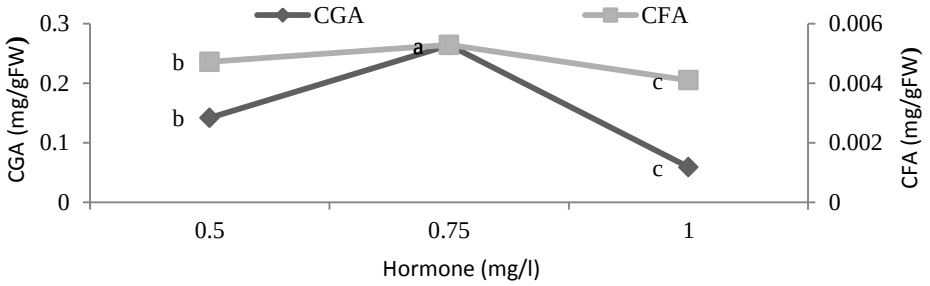

Fig. I. Effect of different levels of 2, 4-D on phenolic acids accumulation; * Each value in the table is the average of three replicates. Values sharing the same letter in each column are not significantly different from each other by protected LSD analysis (α<0.05).

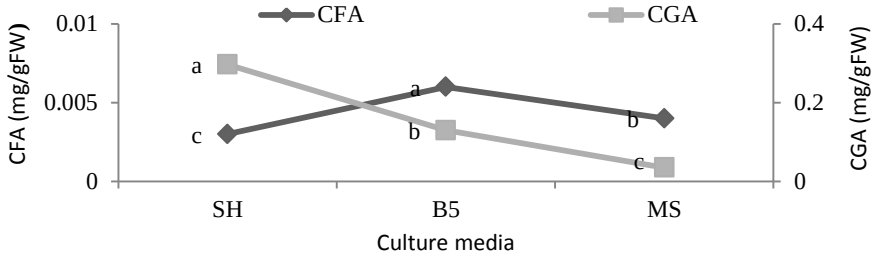

Fig. II. The variation of polyphenolic compound in different culture media; * Values sharing the same letter in each column are not significantly different from each other by protected LSD analysis (α<0.05).

are the most appropriate solvents (Robbins, 2003). Thus methanol was used as the extraction solvent in the present work. For extracting, the fresh callus (2 g) and methanol (10 ml) were blended with a cooled manual blender and the obtained suspensions were placed in a dark tube. Then the mixture was subjected to ultrasound for 10 min and shaken in next 12 hours. Finally, suspensions were centrifuged at 3500 rpm for 10 min and the obtained supernatant was used in HPLC analyzing. All of the samples and solvent were filtered through Whattman paper.

The used HPLC apparatus for caffeic acid and chlorogenic acid determination was equipped with a UV diode array detector in RP-C18 column (250 x 4.6 mm, 5 µm). The flow rate was 1 ml/min and column temperature was maintained at 40 °C. The mobile phase was consisted of a mixture of H_2O, acetonitrile and acetic acid (89:10:1, V/V). The detecting wavelengths were 270, 280, and 330 nm.

Statistical analysis

Multi-factorial experiment was conducted in a completely randomized design with three replications for each of the 24 treatments. The results were subjected to analysis of variance in SPSS 17.0 and Excel was used for figure preparation.

Results

Effect of different levels of 2,4-D on the total phenolic compounds accumulation

Result indicates that the amount of produced caffeic acid and chlorogenic acid was increased when the concentration of 2,4-D increased to 0.75 mg/l (Fig. I).

Effect of media on polyphenol accumulation

Based on the obtained results, the B5 medium produced the highest amount of caffeic acid while culture in the SH medium produced the highest amount of chlorogenic acid (Fig. II).

Polyphenolic compound accumulation and antioxidant activity of callus based on the kind of explant

Metabolism of secondary products seemed to correlate with organized cell

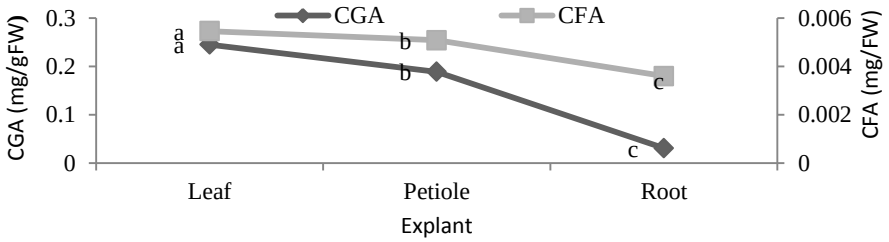

Fig. III. Effect of different explants on polyphenolic compound production; * Values sharing the same letter in each column are not significantly different from each other by protected LSD analysis (α<0.05).

structures. The amount of caffeic acid and chlorogenic acid was slightly similar among the explants. The highest amount of caffeic acid was produced in calluses originated from leaves and petiole explants. On the other hand, only the calluses originated from leaves had the highest amount of chlorogenic acid (Fig. III).

Effect of culture media and explant on the phenolic acids

Considering the interaction of effect of culture media and explant irrespective of hormone levels, it was found that the leaf explant in B5 medium produced maximum amount of caffeic acid (Fig. IV: A). On the other hand, the maximum amount of chlorogenic acid was accumulated in the SH medium with the leaf explant (Fig. IV: B).

Effect of different concentrations of 2,4-D and the kind of explant on the phenolic acids accumulation

The effect of hormone and explant on measured parameters is shown in Figure (V). The highest amount of caffeic acid was recorded in the petiole and leaf explants treated with 0.75 (mg/l) 2,4-D. The leaf explants with the concentration of 0.75 (mg/l) hormone contained the highest amount of chlorogenic acid. The lowest amounts of caffeic and chlorogenic acids were observed under the interaction of root explant and all levels of 2.4-D.

Interaction effect of the culture media and different levels of the hormone on total phenolic acids accumulation

While no significant difference (α<0.05) was observed on caffeic acid accumulation (Fig. 6:

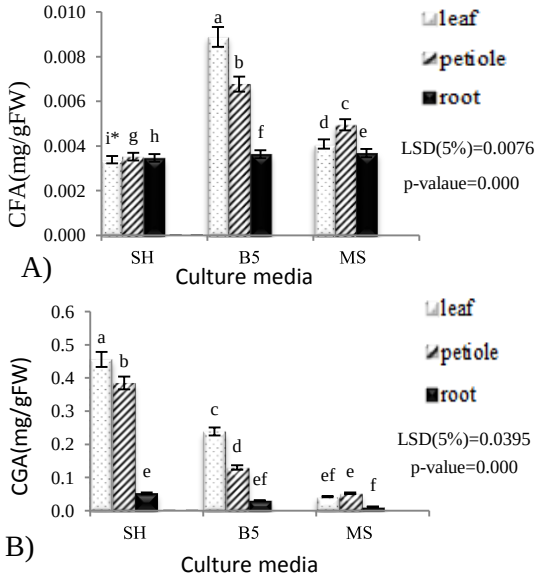

Fig. IV. Interaction of effects of culture media and explant on measures of polyphenolic compounds; * Values with the same letter in each column are not significantly different from each other by protected LSD analysis (α<0.05).

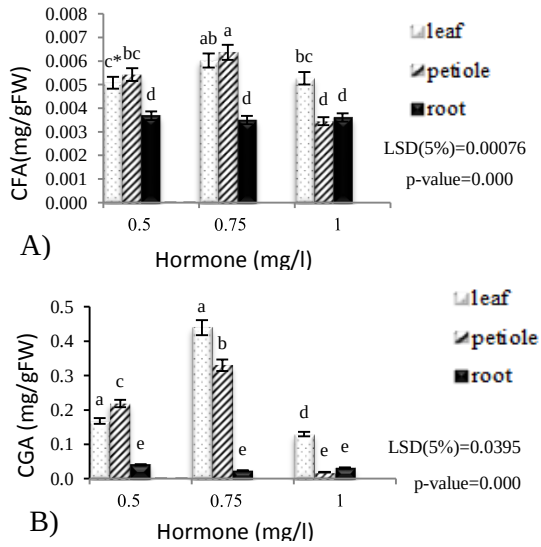

Fig. V. Interaction of effects of hormone and explant on measures of polyphenolic compounds; * Values with the same letter in each column are not significantly different from each other by protected LSD analysis (α<0.05).

A), the highest amount of chlorogenic acid was accumulated in the samples cultivated in SH medium containing 0.75 (mg/l) 2.4-D. Interestingly, under this condition the amount of chlorogenic acid was nearly 3-6 times higher than that of other treatments (Fig. 6: B).

Interaction effect of medium, hormone and explant on phenolic acids compound accumulation

Statistical analysis showed that there was no significant difference (α<0.05) between none of the treatments on caffeic acid, but in the case of chlorogenic acid situation was quite different. By comparing all of the treatments, it was identified that the highest amount of chlorogenic acid was in the SH media containing 0.75 (mg/l) 2.4-D in leaf explant. As caffeic acid is a precursor of chlorogenic acid, its low level could be explained by the conversion of caffeic to chlorogenic acid under hormonal stress (Fig. VII).

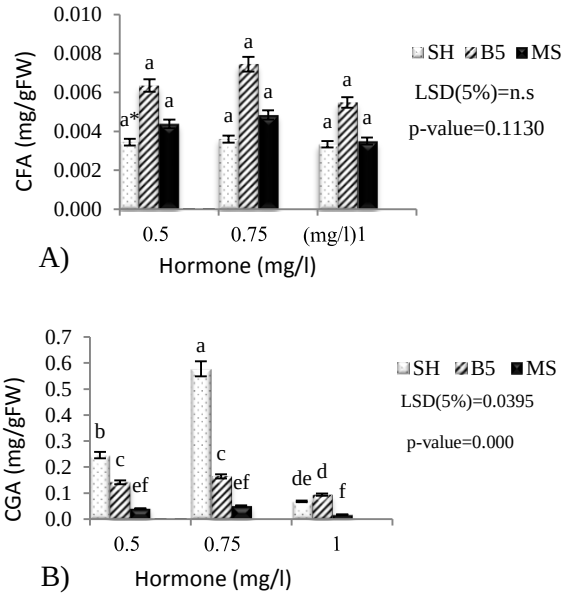

Fig. VI. Interaction of effects of culture media and hormone on measures of polyphenolic compounds; * Values sharing the same letter in each column are not significantly different from each other by protected LSD analysis (α<0.05).

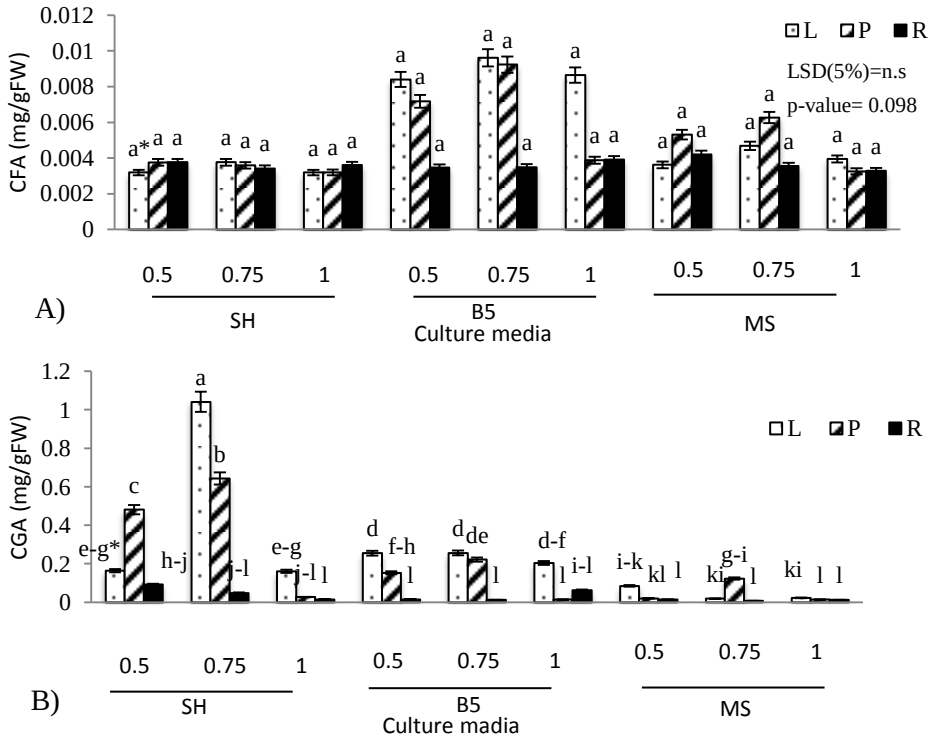

Fig. VII. Interaction of effects of media, hormone, and explants on amount of polyphenol acids compounds and antioxidant activity; * Values sharing the same letter in each column are not significantly different from each other by protected LSD analysis (α<0.05).

Discussion

Regarding the effect of different levels of 2,4-D on the total phenolic compounds accumulation it was found that lower concentrations (0.5 and 0.75 (mg/l)) of hormone was more suitable for phenolic acids compound production. Higher concentration of 2,4-D on the other hand causes salient decrease in vital performances of plant and also in secondary metabolite production. These results can be explained by the chemical behavior of the hormone. At high concentrations, 2,4-D acts as a selective herbicide which is used for killing broadleaf weeds (Howard, 1991). This result is in agreement with the finding of Stefancic et al. (2007) who showed that the chlorogenic acid content of cherry rootstock did not decrease when the 2.4-D concentration increased. It has been shown that 2,4-D stimulates dedifferentiation of the cells and consequently, diminishes the level of secondary metabolites, and such phenomena have been observed in chlorogenic acid production in Echinaceae angustifolia *in vitro* culture (Lucchesini et al., 2009). Enhanced synthesis of determined secondary metabolites in response to the stressful conditions is believed to protect the cellular structures from oxidation (Chanwitheesuk et al., 2005).

Considering the effect of media on polyphenol accumulation, the observed variation in the measured parameters is explainable with the composition of media. In a study by Kim et al. (2013), suitable culture medium for secondary metabolite production in Tea tree (*Camellia sinensis* L.) was determined. Among the treatment media the highest amounts of caffeine and epicatechin-gallate (ECG) were observed in B5 liquid medium. Interestingly, in comparison with caffeine, catechin production in adventitious root cultures was higher than that of intake leaf. The role of medium on secondary metabolite production was intensively investigated by other researchers. In a study of optimization of flavone production in *Scutellaria baicalensis*, Kim et al. (2012) compared the effects of media with full- and half strength of B5, MS, and SH showing that the type of media had a significant influence on flavone production in *S. baicalensis*. The levels of the flavones baicalin, baicalein, and wogonin were higher in the hairy root cultures using half-strength B5 compared with those of the other media used in their study. Similar results were reported for different plants (Yonemitsu et al., 1990; Sauerwein et al., 1991; Granicheret al., 1995; Bálványos et al., 2001; Washida et al., 2004; Lee et al., 2007; Murthy et al., 2008).

Results showed that the type of explant and its anatomical structure played an important role in phenolic compounds production. Such variations could be pointed to the physiological conditions of the explant, which are determined by genetic factors (Nagarathna et al., 1991). Variation in phenolic compounds formation ability of different explant types also has been reported in many other species that reflect the existence of a large inter-explant variability (Trajtemberg et al., 2006; Sgherri et al, .2004). As it is known, phenolic compounds are synthesized in leaves and then moved to other tissues and organs. Therefore, amounts of total phenolic compounds in leaves are more than the other tissues and organs of the plants (Ozyigit, 2008).

Phenolic acids are synthesized in plant in response to physical injuries, illness or other stresses (Beckman, 2000). In explaining patterns of environmental effects on plant secondary metabolites, there is the energy-nutrients balance hypothesis. According to this hypothesis, when nutrients are available, plant allocates carbon to growth preferably. Considering the fact that the stress limits growth more than photosynthesis, thereby photosynthesis leads to the formation of carbohydrates and the energy and nutrients which are directly involved in defense synthesis are not available for growth and production of secondary metabolites carbonaceous (Coley, 1986).

Conclusion

The findings of this study showed that the phenolic acids synthesis is affected by 2.4-D in *in vitro* culture conditions. Accumulation rate of polyphenols was dependent on medium formulation, growth regulator concentrations and explant type. Leaf explant in SH medium containing 0.75 (mg/l) 2,4-D was optimal for producing chlorogenic acid, but for producing

caffeic acid, leaf explant in B5 medium containing 0.75 (mg/l) 2,4-D was preferred. Therefore, taking advantage of the callus of this plant to obtain valuable secondary metabolites is recommended for commercial proposes. Our findings indicate that *C. scolymus* L. culture can be a valuable alternative approach for the production of polyphenolic compounds. By using a selective culture and exogenous auxin treatments, a relatively high antioxidant activity and polyphenol production can be achieved.

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