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Research Article

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In vitro estimation of antioxidant compounds of artichoke (*Cynara scolymus* L.) as affected by methyl jasmonate and salicylic acid

Atana Tanoori, Azim Ghasemnezhad and Mahdi Alizadeh*

Gorgan University of Agricultural Sciences and Natural Resources, Gorgan- Iran

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ABSTRACT

Nowadays, poor diet and stress of urban life cause many diseases and health disorders. Free radicals accumulation inside the body may be increased due to unsuitable diet and stresses in which most biochemical process is negatively affected. Due to the presence of a lot of antioxidant and radical scavenging compounds, the role of plant as a guarantor of human health is highlighted. Due to the biochemical properties of artichoke as a rich source of antioxidant compounds, the in vitro changes in antioxidant compounds namely phenols, flavonoids and radical scavenging properties of extract using methyl jasmonate and salicylic acid as stimulator were studied. The artichoke callus samples were transferred to a solid MS medium containing different concentrations of methyl Jasmonate and salicylic acid (0, 25, 50, 100, 200 µM). 28 days following subculture the biochemical compounds were investigated. Results showed that salicylic acid had significant effect on all measured parameters. The highest content of phenol and flavonoids of callus were observed in the samples in which treated with 200 µM salicylic acid. The highest radical scavenging percentage was obtained from the samples which were treated with 100 µM salicylic acid. Results showed that by increasing the amount of methyl jasmonate a reduction in the phenol compounds was observed. Contrary to that no clear trend was observed in flavonoid content. Based on the obtained results application of elicitors like Jasmonic and salicylic acids in artichoke media in certain amount stimulates may enhance the production of antioxidant compounds of callus extract.

Key words: antioxidant, callus, methyl jasmonate, salicylic acid

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INTRODUCTION

The mechanism of action of antioxidants are the most important defense strategies of living organism, that today due to intensification environmental stresses and depletion of immune system natural antioxidants as radical scavenger are necessary. Especially with evidence of adverse effects of synthetic antioxidants the necessity of adding compounds of antioxidant and anti radical with natural origin in diet is most recommended [1]. Phenolic and flavonoid compounds are large numbers of chemical compounds in plants, which play physiological roles such as antibacterial, antiviral, anticancer agent and scavengers of most types of oxidizing molecules [2,3].

Artichoke (*Cynara scolymus*) from Asteraceae family, with the main ingredient of polyphenols such as caffeic acid (CA), monocaffeolonic acid derivatives and other natural antioxidant is recognized as high value medicinal plant [4]. With increasing demand and decreasing arable land, the biotechnological production of valuable secondary metabolites using tissue culture techniques is an appropriate alternative method. Since the complex structures do not produce in their sufficient amounts, the production of these compounds can be enhanced by the treatment of the undifferentiated cells with elicitors such as methyl jasmonate (MJ) and salicylic acid (SA) [5,6].

Methyl jasmonate and salicylic acid are considered to be plant signaling molecules that play a key role in plant growth, development and defense responses[7,8]. The signal molecules are involved in some signal transduction systems, which induce particular enzymes of the secondary metabolite pathway to from defense compounds such as

polyphenolic compounds [9]. Recently there has been an upsurge of interest in therapeutic potentials of artichoke antioxidant compounds such as chlorogenic acid (CGA) and caffeic acid, therefore in this research, the effect of two commonly used phytochemical elicitors, SA and MJ on the production of antioxidant compound in artichoke callus cultures was studied.

EXPERIMENTAL SECTION

Seed germination and callus initiation

Initially, the seeds were washed thoroughly with normal tap water (30 min) and surface sterilized with ethanol (70%) for 10 seconds, followed by 20 min immersion in sodium hypochlorite (20%) and washed thoroughly with sterile distilled water (three times). Seeds were then germinated on sterile filter paper moistened with sterile distilled water in Petri dishes. Following germination, for better growth seedlings were transferred to half strength MS medium, 3% sucrose and 8% agar. Plantlets in six leaves stage were used as explant source. One or two centimeters of petioles were cultured in induction phase of MS medium containing BA (2 mg/l) + NAA (5 mg/l). After 28 days in two separate experiments the obtained callus, was treated with different levels of MJ and SA (0, 25, 50,100,200 µM) in solid MS medium containing BA and NAA. Four weeks after subculture, biochemical compounds of callus were studied.

Preparation of callus Extract

Fresh callus samples were ground and homogenized in cool mortar. Each sample was extracted with 1ml of methanol (80%). Homogeneous materials were placed in the dark for 24 hours on Shaker and then were centrifuged at 3000 rpm for 5 min and the supernatant was subjected to biochemical analysis.

Determination of total flavonoid content

Aluminum chloride colorimetric method was used for flavonoids determination [10]. Each extract (0.5 ml of 1:10 g ml^{-1}) was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water and kept at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm using spectrophotometer. The calibration curve was prepared by preparing quercetin solutions at different concentrations dissolved in methanol.

Determination of total phenolic compounds

Total phenols were measured via Folin Ciocalteau reagent method [11]. Each sample (40 µl of 1:10 g ml^{-1}) was mixed with 200µl of Folin Ciocalteau, 2.32 ml of distilled water and 600 µl of sodium carbonate (Na_2CO_3). The mixtures were allowed to stand for 15 min and the absorbance reactions of total phenols were determined by spectrophotometer at 765 nm. The standard curve was prepared using 0, 100, 200, 600, 700 mg L^{-1} solutions of gallic acid in methanol: water solution (50:50, v/v).

Free radical scavenging activity determination

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extracts [11]. Each methanolic extract was added at an equal volume to methanolic solution of DPPH (0.1 mM). After 15 min incubation at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated for three times.

HPLC analysis

Methanol extract were placed on ultrasound for 30 min and then was centrifuged at 5000 rpm for 15 min at room temperature. The supernatants were transferred to a new tube and filtered through 0.4 µm filter. The resultant extracts were then used for subsequent HPLC analyses. Chlorogenic acid standard was HPLC-grade purity procured from (Sigma, USA). HPLC was performed with a Merck Hitachi (MH) system (Merck Hitachi, Japan) comprising a quaternary pump (MH, L-7100), a vacuum degasser (Merck L-7614), a UV detector (Merck L-7400), and a 20-µL sample injector (injector 2041 series, SA). Compounds were separated on a 250 mm × 4.6 mm, C-18 column (Merck, Germany). The mobile phase comprised a mixture of acetonitril (10 ml), acetic acid (1 ml) and deionized water (98 ml) and the flow rate and injection volume were 1 ml/min and 20 µl [12, 13]. UV absorbance was measured at 330 nm. The operating temperature was maintained 40 $^{\circ C}$. Acetonitril, acetic acid and water were of HPLC-grade purity. A calibration curve also was constructed using the integrator values obtained from the quantification of standard solutions.

RESULTS

The results were analyzed using SAS software and the mean values were compared by LSD test in $p < 0.01$ probability. The results showed that, accumulation of phenolic and flavonoid compounds of callus were enhanced 28

days following callus culture. Different concentrations of SA particularly the highest and lowest concentrations ones had significant influence on callus (Table 1). Especially, SA at the rate of 200 µM induced the accumulation of phenolic compounds by approximately 5.5- fold as compared to the control. When SA was applied as 100 µM the flavonoids content was accumulated to a maximum of 2.19 (mg/g F.W) as compared to control. Results showed that SA significantly increased radical scavenging percentage of extract and the highest activity was observed in samples in which treated with 100 µM SA (Table 1). The content of CGA (chlorogenic acid) and CA in time course samples after addition of SA of different concentration was detected (Fig. 1) which shows that SA at concentration ranging from 0-200 µM induced this metabolite content. The maximum of these two metabolite content accumulations was observed in the treatment with 100 µM. But with the increasing concentration of SA from 100 to 200 µM, the content of chlorogenic acid and caffeic acid were dramatically reduced.

Table 1. The effect of SA on biochemical compounds content on fresh callus of *C. scolymus*

Treatment (SA)	Antioxidant compounds		RSP* (%)
	Total phenols (mg/l F.W)	Flavonoid (mg/l F.W)	
control	0.47[e]	0.66[e]	9.04[e]
25 µM	1.07[d]	0.85[d]	46.68[d]
50 µM	1.26[c]	1.15[c]	76.79[c]
100 µM	2.33[b]	3.06[a]	87.78[a]
200 µM	2.56[a]	2.19[b]	81.67[b]
LSD	0.08	0.13	4.16

**RSP: radical scavenging percentage*

Table 2. The effect of MJ on biochemical compound content on fresh callus *C. scolymus*

Treatment (MJ)	Antioxidant compounds		RSP (%)
	Total phenols (mg/l F.W)	Flavonoid (mg/l F.W)	
control	0.47[e]	0.66[e]	9.04[d]
25 µM	0.62[d]	0.41[d]	84.92[a]
50 µM	0.87[c]	0.45[c]	78.67[b]
100 µM	1.04[b]	0.7[a]	64.72[c]
200 µM	1.86[a]	0.65[b]	86.71[a]
$LSD_{5\%}$	0.06	0.42	2.97

**RSP: radical scavenging percentage*

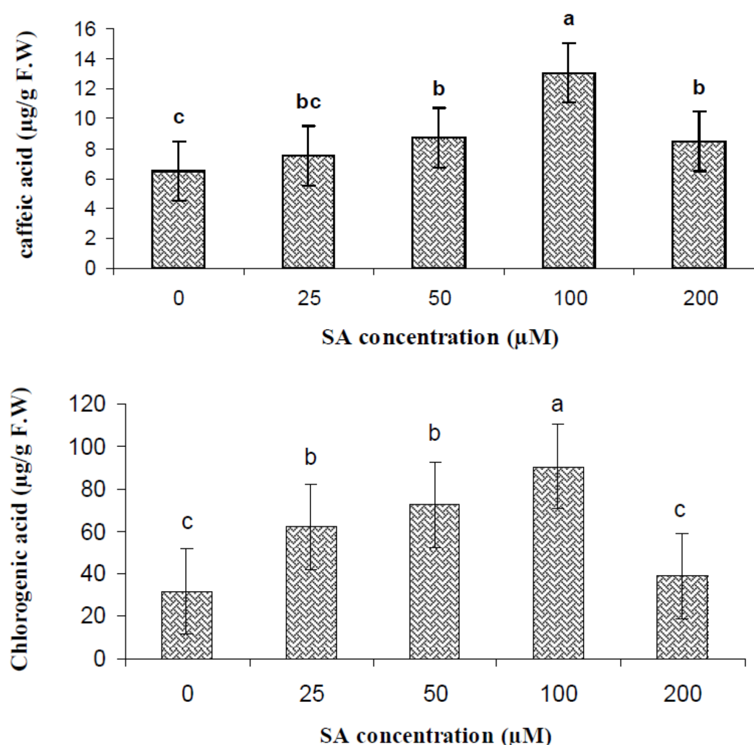

Fig 1. Effects of salicylic acid on CA (caffeic acid) and CGA (chlorogenic acid) in callus culture of *Cynara scolymus*

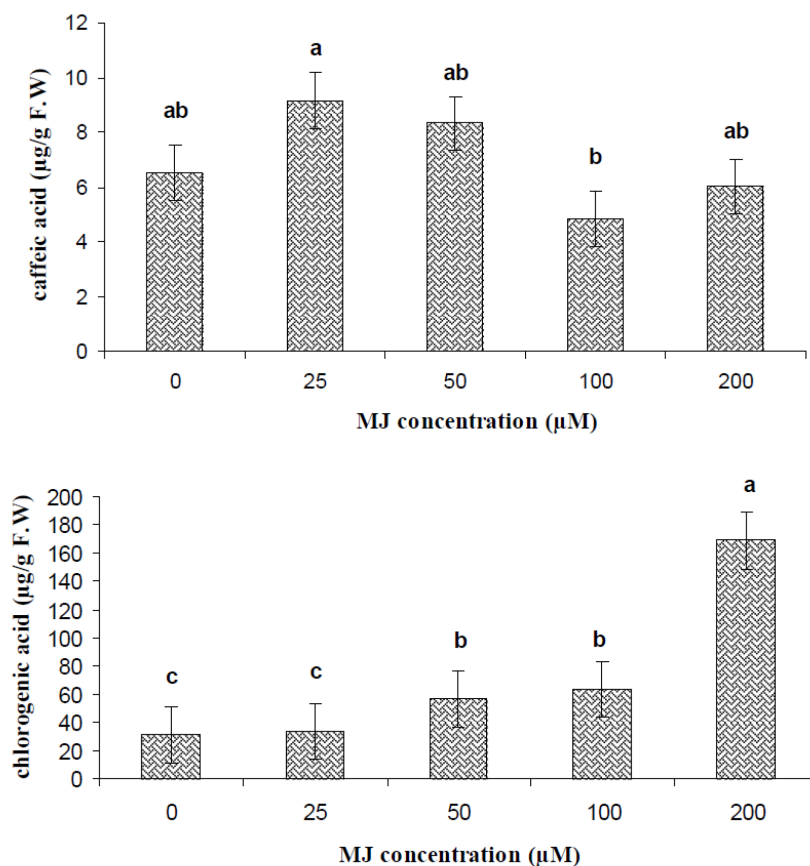

Figure 2. Effect of Methyl jasmonate on CA and CGA in callus culture of *Cynara scolymus*

MJ (methyl Jasmonate) had also significant effect ($p < 0.001$) on antioxidant compounds (Table, 2). The results suggested that callus produced the maximum total phenols when cultured in MS medium supplemented with 200 µM MJ. It was increased 3.96 fold over than control. The maximum flavonoid production (0.7 mg/g F.W) was observed at a MJ concentration of 100 µM. Results showed that different concentration of MJ also had significant effect on radical scavenging percentage. The effect of MJ on radical scavenging percentage was at the highest amount when treated with both 25 and 200 µM.

HPLC analysis of CGA and CA content of the callus during 28 days after elicitation with MJ (Fig.2) showed that chlorogenic acid content increased ($p<0.001$) and reached to maximum value (168.93 µg/g F.w) at MJ concentration of 200 µM. The Fig 2 shows that MJ at concentrations ranging 0, 25, 50, and 200 did not have significant difference and the minimum CA content was observed in samples treated with 100 µM. Results showed a negative correlation between chlorogenic acid and caffeic acid content under MJ treatment (table 2).

DISCUSSION

Artichoke produces wide range of phenolic compounds and many efforts have been performed to increase its production rate. However there are few reports on in vitro changes of secondary compounds with the help of elicitors. Tissue culture is an efficient system for the study of biochemical changes occurred in cultured plant cell, tissue and organ cultures. Therefore, evaluation of biochemical changes in the plant influenced of defense system stimulants by this method is relatively easy. Matkowasky [14] reported that secondary metabolite production in cell with addition of elicitors such as methyl jasmonate and salicylic acid to the nutrient medium under *in vitro* condition was induced and increased. In fact, the plant due to their plant cell membranes structure, when encounter to stressors, active a range of defense reaction which followed of defense mechanisms in plant expands. As soon as inoculation with stressors the cells of host plant show hypersensitive response dependent to reactive oxygen species (ROS). The mechanism of the HR is very complicate and it is occurrence coincides with a myriad of physiological, molecular, and biochemical events such as, the production of low molecular weight antimicrobial compounds termed phytoalexins, the increased expression of genes encoding enzymes in the phenylpropanoid pathway, which eads to the production of phytoalexins and other phenolic compounds, increased peroxidase activity, the expression of genes encoding proteinase inhibitors that can inhibit insect and microbial proteinases; the expression of genes

encoding the pathogenesis-related (PR) proteins. Activation of HR and other defense pathways causes increase ability of an entire plant resistance and systemic acquired resistance (SAR) [15, 16].

Salicylic acid is an important regulatory molecule in plant defense that independent pathways of NPR_1 cause stimulate sensitive pathway to salicyhydroxamic acid and resulting induction PR protein and SAR [17, 18]. Also SA with binding to catalase and proxidas increased H_2O_2 and other ROS, which could activate PR protein and then serve second messengers in the defense signaling pathway [19, 20]. Salicylic acid as a natural phenolic compound has a similar pathway with other phenolic compound. Thus, its increase in medium culture could of the primary reasons for the synthesis and increase other phenolic compounds. Phenylalanine ammonia lyase (PAL) as an intermediate of pathway SA biosynthetic has the potential to become the most phenolic compound [21]. Chlorogenic and (CGA) cafeic acids (CA) are phenolic compounds with strong antioxidant capacity in time of increase stress. Therefore, creation pseudo- stresses condition with using elicitor compounds cause increase effective compound in the antioxidant activity. It seems that CA as a phenolic compound and precursor of CGA is there always a few amounts in the plant and in stress condition changes to CGA and other compounds.

Baber Ali and et al [22] showed that MJ with similar mechanism of SA plays role in the enzymes activity involved in the synthesis of secondary metabolite. They stated that MJ with induction of proteinase inhibitors of enzymes cause reduce primary metabolite activity and in contrast with the induction of oxidative enzymes, ROS, gene expression and induced the secondary metabolite. Indeed, MJ can be act as a stress factor and signaling molecules by induction NADH oxidase and increase content of H_2O_2. Rapid production of H_2O_2 in tomato plant at 3min after treatment with MJ has been observed [23].

Jasmonates are known to enhance phenolic compounds accumulation in plant cell. Thus, exposure of cell culture to MJ led to an increase in PAL activity [24, 25].

Increased of Phenolic and Flavonoid compounds in this study was similar with Rudell and Mattheis [26]. Kim and et al [27] explained that treatment of MJ with increased H_2O_2 content and with changes in enzyme involved in the synthesis of metabolites such PAL was increased Phenolic and flavonoid compounds.

Baber Ali and et al [22] reported that high concentration of MJ and SA decreased ratio of fresh to dry weight but at concentration of 200 M increased CGA and CA content. In this research MJ and SA significantly increased total phenols (CGA & CA) and total flavonoids in artichoke callus. Increase these compounds as a secondary metabolite with antioxidant activity, followed increases MJ and SA, came from the effect of stimulus on radical scavenging and antioxidant ability in artichoke callus.

CONCLUSION

The MJ and SA as important phyto-hormones play noticeable role in most physiological and biochemical processes in artichoke callus. The results of the present study showed that the produced secondary metabolites in artichoke callus can be balanced between the different products of polyphenols and interaction with other hormones to protect itself from the risk of oxidative destruction.

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