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Study the Effect of Salt Stress on Antioxidant Compounds of Artichoke under Invitro Conditions

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Abstract

Artichoke (*Cynara scolymus*) is one of the oldest herbs and nowadays because of its ingredient is concerned by pharmaceutical industry. Abiotic stresses including salinity, changes the metabolic pathways of cultured cells and leads to the production of secondary metabolites. This study was aimed to determine the effect of salt stress on antioxidant compounds of callus focusing on the phenol, flavonoid, and chlorogenic acid and caffeic acid production under in vitro conditions. The present study was carried out as two separate experiments as direct culture (cultured explant) on MS solid medium with different salinity concentrations (0, 50, 150, 300, 600, 1200 µM NaCl) and indirect culture (callus culture) in different salin concentrations of 0, 50, 150, 300, 600, 1200, 2000, 4000, 6000 and 75000 µM NaCl. Four weeks after culture the mentioned compounds were measured via distinctive methods. Results showed that, versus to indirect culture radical scavenging percentage, the content of chlorogenic acid and caffeic acid were significantly influenced by salinity and the highest amount of them were recorded in the samples in which treated with 300 µM NaCl, control and 600 µM NaCl, respectively. In indirect culture no significant difference was observed on other phenolic compounds and antioxidant activity except caffeic acid.

1. Introduction

Salinity is one of the most important environmental factors that negatively influence plant growth, development and productivity. Salt stress changes the morphological, physiological and biochemical responses of plants (Sevengor *et al*., 2011). There is evidence that high salt concentrations cause an imbalance in cellular ions, resulting in ion toxicity and osmotic stress, leading to the generation of reactive oxygen species (ROS) which cause damage to DNA, lipids and proteins. (Aazami *et al*., 2010; Sabir *et al*., 2012). to mitigate and repair the damage initiated by various ROS, plants have evolved specific protective mechanisms. Antioxidants can be divided into two classes: the low-molecular-mass non-enzymatic free radical scavengers and enzymes. These include non-enzymatic antioxidants such as ascorbic acid, glutathione and flavonoids. (Ahmadkhan. 2010, Sevengor *et al*., 2011).
Cynara scolymus L. is a perennial herbaceous plant fszof the Asteraceae family and native to southern Europe, the North Africa and canary island (Zeyaei et al., 2005). This plant has moderate resistance to salinity (Francois,1995). Tissue culture technique provides a unique chance for studying many aspects of plant growth and development. Furthermore, tissue culture gives a good tool for studying the physiological effect of salt stress at the cellular level under invitro conditions (Nashermohamad *et al*., 2011). It also has the potential for the selection of stress-tolerant variants using a low cost of laboratory set up. The effect of salt stress on secondary plant products have been reported for in vitro culture of Helianthus annus (Santos, *et al*., 2000), Catharanthus roseus (Garj, 2010), Trigonella (Sarahinobar *et al*., 2011), Phaseolus vulgaris (Babu *et al*, 2008), and Cotton ovules (Rajguru, 1999).The aim of this study was to determine the effect of salt stress on the content of antioxidant compounds of callus of cynara scolymus under in vitro conditions.

2. Material and Methods

2.1. Plant Material and Callus Induction

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Before sowing, the seeds of artichoke were kept at 40C for three days. Treated seeds were cultured in culture media containing coco peat, perlite and sand. Seedlings were surface sterilized first by washing with 70% ethanol for 10 seconds followed by 35% hypochlorite sodium for 8 minutes. Samples were then washed with double distilled sterilized water 6 times and were cultured on MS medium containing (NAA (5 mg/l) + BA (2 mg/l). Cultures were maintained 30 days in dark conditions at 25 ± 2 °C.

2.2. Salt Stress Treatments

*Experiment 1***:** In this experiment for callus induction aseptic cotyledons were transferred directly to MS medium containing different concentrations of NaCl (0, 50,150,300,600.1200 µm/L). Then, explant culture was maintained at 25 ± 2 °C in the dark conditions. After 4-5 weeks the callus formation completed and the measurements were done.

*Experiment 2***:** In this experiment the calli were induced from cotyledons of seedling on non-saline MS medium. Then the callus was transferred to the same medium containing different salt level (0, 50, 150, 300, 600, 1200, 2000, 4000, 6000 and 75000 µm/L). After 4-5 weeks the measurements were done.

2.3. Sample Preparation for Phenol, Flavonoid and Antioxidant Measurements

Samples (0.5 gr) were homogenized in 5 ml of 80% methanol (1:10) and shacked for 24 hours. Then the samples were centrifuged at 3,000 rpm for 5 min and the Supernatant was used sample analysis.

2.4. Total Phenolic Content

Total phenolics were estimated according to Ebrahimzadeh (2008). For that 20 µL of the methanolic crude extract was mixed with 1.16 ml distill water and 100 µL foline ciocalteau reagent. After 8min, 300 µL of sodium carbonate (1M) was added to the mixture. Samples were left for 30 min at room conditions and the reading was done against a blank at 750 nm. A calibration curve was obtained in parallel under the same operating conditions using Gallic acid as a positive control. The results were expressed as mg Gallic acid equivalent per gram (mg/GA/g) of callus extract.

2.5. Total Flavonoid Content

Total flavonoid was estimated according to Ebrahimzadeh (2008). For that 0.5 ml of the methanolic crude extract was mixed with 2.8 ml distilled water and subsequently with 1.5 ml methanol, 0.1ml of aluminum chloride (10%) After acetate potassium (1M) solution addition, it was allowed mixture to stand for 30 min in the dark. Absorbance of the mixture was then determined at 415 nm versus prepared pure methanol blank. Results were expressed as mg quercetin/g callus sample.

2.6. Determination of the Radical Scavenging Effect of Extract

A methanolic solution (1ml) of each crude extracts at different concentrations was added to 1mL of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) solution 0.1mM (4mg DPPH 100ml in methanol). The studied compounds were tested with methanol as control and absorbance at 517 nm was determined after 30 min in the dark conditions. The absorbance (A) of the control and samples was measured, and the DPPH scavenging activity (SA) in percentage was determined as follow: SA % = [(A control – A sample) / A control] ×100 (Ebrahimzadeh *et al*., 2008). The antioxidant activity was calculated by the amount of DPPH that was scavenged by using a calibration curve of DPPH in methanol. It was expressed as number of reduced DPPH molecules per gram fresh weight callus.

2.7. Preparation of Crude Methanol Extracts for Chlorogenic Acid, Caffeic Acid Measurement

About 0.5 g of the fresh callus was weighed accurately and dissolved in 5 ml HPLC grade methanol (1:5). After 10 min of ultrasonication, extract was shacked for 12 hours. Then the extract was centrifuged for 10 min at 3500 rpm. The supernatant was transferred into a 2ml flask. The combined supernatants of the extracted sample were filled up to 2 ml and filtered through a folded filter (Santos *et al*., 2003; Trajtemberg *et al*., 2006).

The chlorogenic and caffeic acid content of extract was measured by HPLC apparatus (model Merck –Hitachi El-7100) equipped with diode RE-Hitachi L2450 detector and Hitachi L-2300 oven. Sample constituent was separated through RP-18 type column (250×4/6 mm) with particles size 5 µm. The injection volume was 20µl and solvent flow rate was set at 1ml/min. In this case a mixture of acetonitril, acetic acid and deionized water in a rate of 10:1:89 was used as mobile phase. Chromatograms were detected at a wavelength of 330 nm. Quantification was accomplished using a calibration curve of pure chlorogenic acid and caffeic acid as standard.

2.8. Statistical Analysis

Analysis of data was performed based on the completely randomized design (CRD). The test was performed under controlled conditions in a culture chamber with 10 salt treatments with 3 replications. Data analysis of

variance was done using software SPSS. And the mean value was compared based on the least significant difference (LSD) test.

3. Results

Table 1 shows the variance analysis of data. According to the table 1, chlorogenic acid as an important precursor of cynarin and Cynaroside was significantly influenced by both culture method and salinity

3.1 Total Phenolic Content

According to the results, in indirect culture, salinity showed significant effect on the total phenolic content. The highest phenolic content was observed at control samples, while the lowest phenolic accumulation was observed in the samples in which treated with high levels of salinity (Fig. 1). In direct culture there was no significant difference in total phenolic content. Where the highest content was observed in 600 mM NaCl and the lowest was related to the control (Fig. 2). Data analysis expressed that, between salinity and culture methods no significant difference is presented.

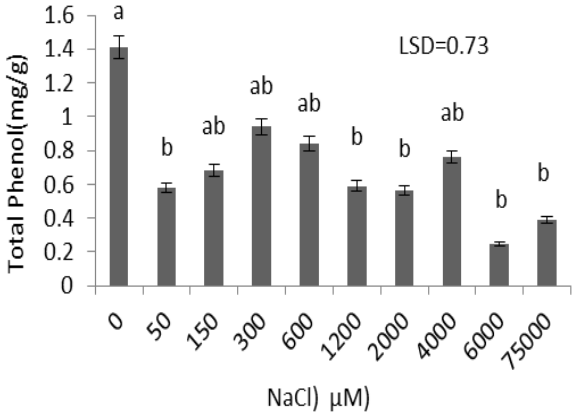

Fig. 1 Effect of salinity on the total phenolic content under indirect culture conditions

Different letters indicate significant differences, ($p < 0.05$).

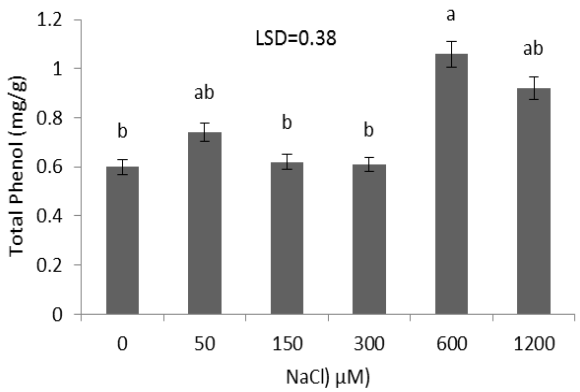

Fig. 2 Effect of salinity on total phenolic content in direct culture

Different letters indicate significant differences ($p < 0.05$).

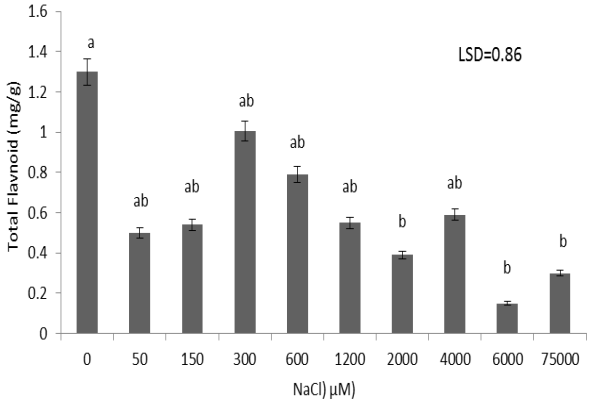

Fig. 3 Effect of salinity on total Flavonoid content in indirect culture

Different letters indicate significant differences ($p < 0.05$).

In both salinity and the methods of culture was observed no significant difference on total phenol content was observed (Table 1).

3.1. Total Flavonoid Content

According to the obtained data of indirect culture, salinity showed no significant effect on total phenolic content. The highest amount of phenolic content was observed at control callus samples.

Table 1 Variation of antioxidant agents of artichoke callus affected by salinity and the methods of culture

Parameter	Total Phenol (mg/g)	Total Flavonoid (mg/g)	Antioxidant (DPPH)	Acid Chlorogenic (mg/g)	Acid Caffeic (mg/g)
Culture method	0.054^{ns}	0.095^{ns}	679.03^{***}	1.11^{*}	0.563^{***}
Salinity	0.196^{ns}	0.178^{ns}	45.36^{ns}	0.566^{*}	0.003^{ns}
Culture method× salinity	0.175^{ns}	0.571^{ns}	92.56^{ns}	0.555^{*}	0.002^{ns}

***Significant at $P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ levels, ^{ns}: not significant

While the lowest amount of flavonoid content was observed in the samples cultured under high salinity conditions (Fig. 3). Similar to the direct culture, in indirect culture and among different levels of salinity no significant difference was observed in total flavonoid content. Despite to that, mean value comparison showed that there is significant difference among different levels of salinity. In which the highest flavonoid content was observed in the calluses treated with150 Mm NaCl and the lowest was recorded in control samples (Fig. 4).

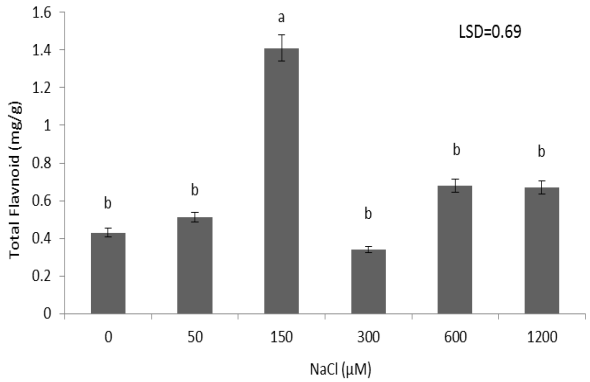

Fig. 4 Effect of salinity on the total flavonoid content of callus under in direct culture
Different letters indicate significant differences (p <0.05)

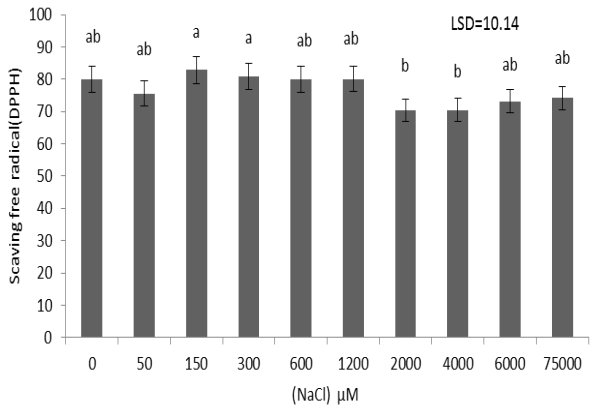

Fig. 5 Effect of salinity on the percentage of free radical scavenging activity in indirect culture
Different letters indicate significant differences (p <0.05.)

3.2. Free Radical Scavenging Activity

The results showed that, the percentage of free radical scavenging activity of callus extract was significantly influenced by salinity (P<0.01). In, in direct culture conditions when callus treated with moderate concentrations of NaCl (150 and 300 µM), free radical scavenging activity of extract was at its highest amount (Fig. 5). Opposite to that, in direct culture, salinity had no significant effect on free radical scavenging activity. Data showed that the highest free radical scavenging activity of callus extract was observed in samples which were treated with 150 and 300µM NaCl and the samples which were treated with 2000 and 4000 µM NaCl showed the lowest radical scavenging activity (Fig. 6)

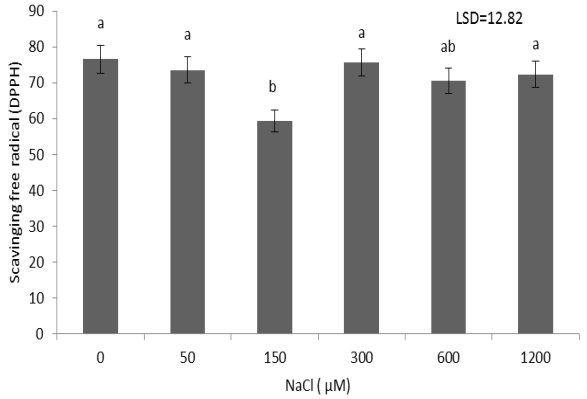

Fig. 6 Effect of salinity on the percentage of free radical scavenging in direct culture
Different letters indicate significant differences (p <0.05).

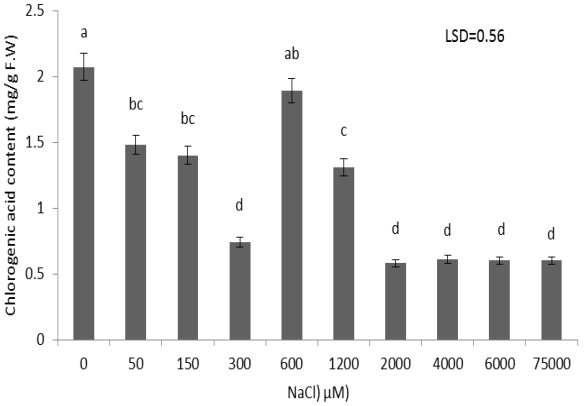

Fig. 7 Effect of salinity on chlorogenic acid content in indirect culture
Different letters indicate significant differences (p <0.05).

3.3. Measurement of Chlorogenic Acid

In, in direct culture, salt stress had significant influence on the chlorogenic acid accumulation (P<0.001). Among different levels of the salinity the highest chlorogenic acid content was observed in the samples in which treated with 600 µM NaCl by salinity increasing up to 600 µM, the chlorogenic acid content was decreased (Fig. 7). Although in direct culture salinity did not have significant influence on chlorogenic acid content of samples, but the comparison of mean value showed that the samples in which treated with 600 µM NaCl had the highest chlorogenic acid accumulation

(Fig. 8). Results showed that the chlorogenic acid accumulation of samples was significantly influenced by the interaction effect of culture method and salinity (p=0.05) (Table1).

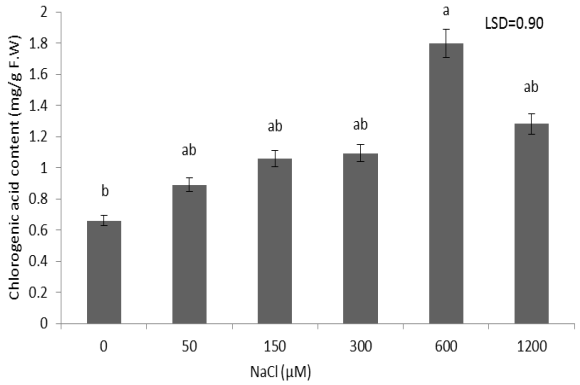

Fig. 8 Effect of salinity on chlorogenic acid accumulation in direct culture conditions
Different letters indicate significant differences (p <0.05)

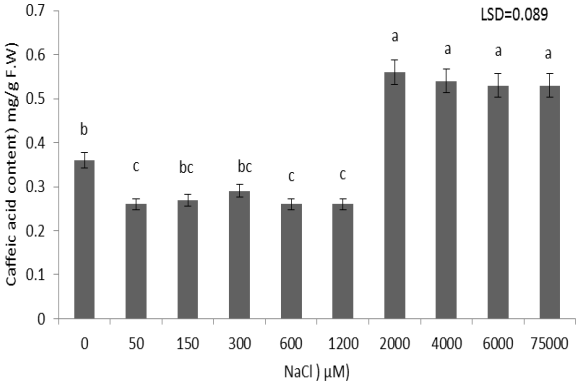

Fig. 9 Effect of salinity on the caffeic acid accumulation in indirect culture
Different letters indicate significant differences (p <0.05).

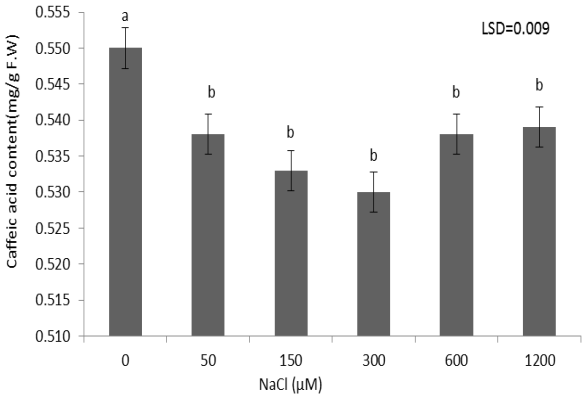

Fig. 10 Effect of salinity on the concentration of caffeic acid in direct culture
Different letters indicate significant differences (p <0.05).

3.4. Caffeic Acid Measurement

The effect of in indirect culture and salt stress on the caffeic acid accumulation showed that, this compound was significantly influenced by treatments. The highest amount of caffeic acid content was observed at the highest salinity levels (Fig. 9). In direct culture, salinity had significant effect on caffeic acid content (P <0.01). Versus to the indirect culture, the direction of effect was quite different and in this case the caffeic acid accumulation decreased as salinity increased (Fig. 10).

4. Discussion

According to the results of the present study, salinity showed significant changes on radical scavenging activity its agents like chlorogenic acid and caffeic acid contents in indirect (culture callus). But salinity showed no significant effect on total phenolics and flavonoid content in direct culture. The measured parameters except caffeic acid of direct culture were relatively higher than that of indirect culture.

It can be suggested that due to greater sensitivity of callus tissue to explants, salt stress and the salinity may increase cell heterogeneity in calli (Santos., 2000) and leading to significant different among compounds in indirect culture than direct culture. The antioxidant activity in artichoke tissue mostly comes from poly phenolic compounds including flavonoids. The released secondary metabolites from plants may vary from plant to plant and species to species. It also can be varied in different environmental conditions (Selvam. 2013). It has been reported that in some species callus are more salt tolerant than plants (Smith and McComb., 1981; Tal., 1990, Santos., 1998; Santos and Caldeira, 1999). In our results, the amount of measured secondary metabolite of direct culture was higher than that of callus refers to the less sensitivity of callus cells to salt stress than explant cell. Indeed, the level of stress in which encourages the response to the salinity in callus cells which appears by secondary metabolite accumulation might be higher.

Phenolics have been regarded as secondary metabolites, believed to function in plants to regulate responses to stress (Lattanzio *et al*., 2009). Based on the obtained results, the highest phenolic accumulation in the samples of media containing 300µM indicates that the phenolic compounds as defence agents are the most popular in plant cells against stresses. Till 300µM cells try to overcome the stresses using phenolic compounds. No observed direct relation between stress levels and phenolic compounds accumulation in salinity higher than 300µM, means that the role of phenolic compounds in this cases has been substituted by other

stronger anti-stresses compounds, the finding in which reported by some other researchers (Santos *et al*, 2000, Sarahinobar *et al*.,2011; Sbir *et al*., 2012,).

Plants widely vary in their phenolic composition and the type and level of phenolic compounds affected by both genetic and environmental parameters (DeAbreu and Mazzafera. 2005). Increase total phenolics content was observed with moderate salin levels in red peppers (Navarro *et al*., 2006). Phenolic compounds exhibit antioxidant activity by inactivating lipid free radicals or protect hydro peroxides against decomposition of into free radicals (Balasundram *et al*., 2006). Phenolics have been regarded as secondary metabolites, believed to function in plants to regulate responses to stress (Lattanzio *et al*., 2009).

Salinity stress caused generation of excessive reactive oxygen species (ROS), which leads to cell toxicity membrane disfunction and cell death (Sevengor, 2011). Production of reactive oxygen species (ROS) is an unavoidable process in photosynthetic tissues, but ROS are also produced in mitochondria and cytosol. ROS including singlet oxygen, superoxide, hydrogen peroxide, and hydroxyl radicals and can damage proteins, membrane lipids, and other cellular components. Some ROS also serve as signaling molecules. These Reactive oxygen species are scavenged by resident enzyme systems and non-enzymatic antioxidants. Plants have several mechanisms for adaptation to osmotic stress caused by high salinity, which includes Enzymatic and non-enzymatic systems (Bohnert *et.al*., 1999). Systems consisting of non-enzymatic antioxidant compounds, (glutathione, flavonoids, alkaloids, carotenoid, vitamin E (Tocopherol), Vitamin C (ascorbate) and osmotic materials (proline, betaine and soluble sugars solution) cannot play alone important roles in plant adaptation to stress. So it is necessary for enzyme protection systems supplement act in cases failure of non-enzymatic systems (Bohnert *et.al*., 1999). When salt was tolerably for plant, plant induced mechanism else to deal with oxidative compound that acts in accordance with phenolic compounds. In stressed plants, increase antioxidant enzyme activities in addition to osmotic adjustment leading to increase resistance. Despite to that, osmotic regulation and ionic changes, change in antioxidant enzyme activity can be considered as one of influence of salinity on plants. Activity of these enzymes will change depending on the sensitivity of plant species, strange of growth, intensity and duration of stress concentrations (Heidary *et al*., 2011).

Caffeic acid and its derivatives as chlorogenic acid are antioxidants, whose activity may be accomplished by two mechanisms: by free-radical scavenging or by inhibiting the action of enzymes involved in oxidative reactions (Trajtemberg *et al*., 2006). Caffeic acid is considered as a phenolic compound and precursor of other phenolic compounds such as chlorogenic acid cynarin and cynaroside and always available in small amount in plant. In most cases high amount of this compound in tissue refers to the high level of salinity in which stopped the conversion of caffeic acid to its derivatives such as chlorogenic acid and cynarin (Trajtemberg, 2006).

5. Conclusins

The results of the present study showed that, salinity did not have strong influence on the phenol and flavonoid contents both in direct and indirect culture conditions, but the free radical scavenging ability and the content of chlorogenic acid were significantly different in the indirect culture. Increase in phenolic, flavonoids and antioxidant compounds increase with rising salinity level to a certain extent reflects the relative resistance of plants to oxidative compounds is caused by salinity. When plants reach to the level of salinity in which cannot protect them, another tolerance system will activate. The ability of plants to this conversion defined their tolerance. Secondary metabolites plays important role in this case. Managing secondary metabolite production via manipulating environmental stresses especially in invitro conditions help us to increase the production of these compounds. Thus, further investigation in this area help us to have a good understanding about the response of cynara scolymus tissue to saline stress.

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