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Full Length Research Paper

Effect of growth stages on phenolics content and antioxidant activities of shoots in sweet marjoram (*Origanum majorana* L.) varieties under salt stress

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We investigated the contributions of salinity and development stage on total polyphenols, flavonoids, condensed tannins contents, phenolic content, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide scavenging activities at three phenolic stages: early vegetative stage (EVS), late or prefloral vegetative (LVS) stage and flowering stage (FS). The total phenolic, flavonoids and condensed tannins contents were more important in the presence and absence of salt at prefloral or building stage than at EVS and FS. In addition, salt stress increased total polyphenol and individual phenolic contents in shoots of *Origanum majorana*. This increase was more important in LVS than in EVS and FS. Moreover, the antiradical activity of the shoots (DPPH) decreased with the addition of NaCl. In fact, LVS extracts showed the highest antioxidant properties. Furthermore, at LVS, marjoram grown at 75 mM showed a higher antiradical ability against DPPH radical and antioxidant activity, compared to EVS and LVS. Data reported here revealed the variation of phenolic compound contents at different stages of growth of *O. majorana*, and the possible role of these changes in the response of the plant to salt was also discussed.

Key words: Antiradical activity, *Origanum majorana*, early vegetative stage (EVS), late or prefloral vegetative (LVS) stage and flowering stage (FS), phenolic content.

INTRODUCTION

Plants constitute a valuable source of natural antioxidants including phenolic compounds and flavonoids (El-Ghorab et al., 2007). Because of their potential carcinogenicity, the utilization of synthetic antioxidants is progressively restricted. This trend is concomitant with an increasing interest for the identification and valorization of natural antioxidants of plant's origin. Consumers give more attention to healthy and balanced diets, which greatly rely on the utilization of natural preservatives for a healthier lifestyle and natural ways of preserving nutrients (Raghavan, 2007).

In plants, polyphenol biosynthesis and accumulation are generally stimulated in response to biotic/abiotic constraints (Naczk and Shahidi, 2004). Thus, salt-stressed plants might represent potential sources of polyphenols, by increasing polyphenol concentration in the tissues and restricting biomass production. Consequently, one may hypothesize that optimal polyphenol yield would be obtained using stress-tolerant species (De Abreu and Mazzafera, 2005).

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Abbreviations: EVS, Early vegetative stage; **LVS,** late vegetative stage; **FS,** flowering stage.

Nowadays, phenolic compounds play an important role in human health because of their anti-inflammatory, antiallergic, antimicrobial, anticarcinogenic and antiviral activities (Medina et al., 2007). They prevent lipid peroxidation and oxidative modification of low density lipoprotein by means of their antioxidant activities (Servili et al., 2004). Phenolic composition of food materials has been the scope of many studies lately due to their antioxidant effects.

Additionally, it has been determined that the antioxidant effect of plant's products is mainly attributed to phenolic compounds, such as flavonoids, phenolic acids, tannins, and phenolic diterpenes (Pietta, 2000).

Lamiaceae plants are widely studied as natural antioxidant sources because of their high phenolic concentrations (Trouillas et al., 2003; Miura et al., 2002). Lamiaceae family includes a large number of volatile oil plants and the most important member is marjoram (*Origanum majorana*) (Paterson et al., 2006), a herb commonly found in Cyprus and the Eastern Mediterranean (Novak and Bitsch, 2002). This species has strong antioxidant activity, mainly because of its high phenolic acid and flavonoid contents, which are useful in health supplements and food preservation (Heo et al., 2002).

In a current data, it has been demonstrated that at prefloral stage, the richness of *O. majorana* in volatile and phenolic active compounds (known for their antioxidant, antimicrobial and insecticidal activities) could support the utilization of this plant in a large field of application including cosmetic, pharmaceutical, agro alimentary and biological defense.

This work was conducted to elucidate the effects of salinity, development stage and their interaction on the antioxidant properties of *O. majorana* shoot extracts through a quantification of their phenolic contents and an evaluation of 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide radical-scavenging capacities. The aim of this paper was to separate and quantify the effects of salinity and growth phase on antioxidant properties of *O. majorana*, which will be useful for crop breeders and growers attempting to produce it with high levels of natural antioxidants.

MATERIALS AND METHODS

Plant material

Marjoram seeds were taken from local plant nursery existing at Soliman in North east of Tunisia. Seeds of *O. majorana* varieties were germinated for 10 days in culture chamber with a 16 h photoperiod (150 $\mu mol.m^{-2}.s^{-1}$). Temperature and average relative moisture were respectively 22°C and 40% for the day and 18°C and 86% for the night. Eighty of each variety seedlings, having ten centimetre length stems and six leaves, were transferred into eight-strength Hoagland and Arnon (1950) nutrient solution (1.25 mM KNO_3, 1.25 mM $Ca(NO_3)_2.4H_2O$, 0.50 mM $MgSO_4.7H_2O$, 0.25 mM KH_2PO_4, 0.01 mM H_3BO_3, 0.001 mM MnSO4.$4H_2O$, 0.0005 mM $CuSO_4.5H_2O$, 0.0005 mM $ZnSO_4.6H_2O$, and 0.00005 mM $(NH_4)_6Mo_7O_{24}.4H_2O$) in a culture chamber with a 16 h photoperiod (150 $\mu mol.m^{-2}.s^{-1}$). After 20 days of acclimation, NaCl (75mM) was added to nutritive solution. The plant's aerial parts were harvested 17 days at the onset of stress symptoms.

Chemicals and standards

All solvents used in the experiments (methanol) were purchased from Merck (Darmstadt, Germany). Folin-Ciocalteu reagent sodium carbonate (Na_2CO_3), aluminium chloride ($AlCl_3$), sulphuric acid (H_2SO_4), sodium hydroxide (NaOH), sodium nitrite ($NaNO_2$), hydrochloric acid (HCl), and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Steinheim, Germany). Authentic standards of phenolic compounds were purchased from Sigma and Fluka. Stock solutions of these compounds were prepared in high performance liquid chromatography (HPLC)-grade methanol. These solutions were wrapped in aluminum foil and stored at 4°C. All other chemicals used were of analytical grade.

Phenolic extraction

Marjoram aerial parts were dried at room temperature for two weeks. Samples of dried aerial parts were finely ground with a blade-carbide grinding (IKA-WERK Type: A: 10). Triplicate sub-samples of 1 g of each ground aerial parts were separately extracted by stirring with 10 ml of pure methanol for 30 min. The extracts were then kept for 24 h at 4°C, filtered through a Whatman No. 4 filter paper, evaporated under vacuum to dryness and stored at 4°C until analysis (De Abreu and Mazzafera, 2005).

Total polyphenol quantification

The total phenolic content was assayed using the Folin–Ciocalteu (F-C) reagent, according to the method described by Mau et al. (2001). An aliquot of 125 µL of diluted extract (20% (v/v)) was added to 500 µL of deionized water and 125 µL of F-C reagent. After shaking, the mixture was incubated for 3 min at room temperature. Then, 1250 µL of 7% Na_2CO_3 solution was added. The volume obtained was adjusted to 3 ml using distilled water, mixed vigorously, and held for 90 min at ambient temperature. The absorbance of the solution was then measured at 760 nm against a blank. The sample was analysed in triplicate and the total phenolic content was expressed as mg of gallic acid equivalents (GAE) per gram of dry weight through a calibration curve range of 50 to 400 $\mu g\ ml^{-1}$ (R2 =0.99).

Flavonoid quantification

The flavonoid content was measured (Dewanto et al., 2002). 1 ml of appropriately diluted marjoram samples (5% (v/v)) was added to a 10 ml volumetric flask containing 4 ml of distilled water, followed by immediate addition of 0.3 ml of 5% (w/v) $NaNO_2$, 0.6 ml of 10% (w/v) $AlCl_3$ after 5 min and 2 ml of 1 M NaOH after 6 min; contents of each reaction flask were diluted with 2.4 ml of distilled water and mixed immediately. Absorbance of resulting pink-coloured solution was read at 510 nm against the blank (distilled water). Samples were analysed in triplicate. Flavonoid content was expressed as mg

catechin equivalents (CE) per g of dry herb. The calibration curve range was 50 to 500 mg/ml. Triplicate measurements were taken for all samples.

Tannin quantification

0.05 ml of *O. majorana* samples extracted in non diluted methanol was added to 3 ml of vanilline (4%), and 1.5 ml of concentrated H_2SO_4. Absorbance of resulting pink-coloured solution was read at 500 nm against extract solvent as a blank (Zhishen et al., 1999). The amount of total condensed tannins is expressed as mg (+)-catechin /g DW. The calibration curve range was 50 to 400 µg/ml (R2=0.99). All samples were analysed in three replications.

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) and superoxide anion radical-scavenging activity

The electron donation ability of the obtained methanol extracts was measured by bleaching of the purple coloured solution of 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) according to the method of Sun et al. (1988). Methanolic extracts (2 ml, 10 to 1000 µg ml^{-1}) were added to 0.5 ml of 0.2 mM DPPH. After an incubation period of 30 min at room temperature, the absorbance was measured against a blank at 517 nm. The antiradical activity was expressed as IC_{50} (µg/ml), the concentration required to cause 50% DPPH inhibition. The ability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging effect (%) = [(A blank – A sample)/A blank] × 100 (1)

Where, A blank is the absorbance of the control reaction and A sample is the absorbance in the presence of plant extract. Samples were analyzed in triplicate

Superoxide anion scavenging activity

Superoxide anion scavenging activity was assessed using the method described by Duh et al. (1999). The IC_{50} index value was defined as the amount of antioxidant necessary to reduce the generation of superoxide radical anions by 50%. The IC_{50} values (three replicates per treatment) were expressed as µg ml^{-1}. As for DPPH*, a lower IC_{50} value corresponds to a higher antioxidant activity of plant's extract (Patro et al., 2005). The inhibition percentage of superoxide anion generation was calculated using the following formula:

Superoxide quenching (%) = [(A blank – A sample)/A blank] × 100

Where, A blank and A sample have the same meaning as in Equation (1)

Extraction and RP–HPLC analysis of phenolic compounds

Polyphenol extraction

Free polyphenols were extracted by maceration into methanol according to the method of Mau et al. (2001). 1 g of dried and powdered sample was immersed into 10ml of methanol and the suspension was stirred for 30 min; then the mixture was kept at rest and obscurity at 4°C for 24 h. The mixture was then filtered through a Whatman filter paper (No. 4), then 3, 4-dihydroxybenzoic acid was added as an internal standard and the methanol macerate was evaporated to dryness under vacuum and stored at 4°C for further analysis.

RP-HPLC analysis of phenolic compounds

Phenolic compound analysis was carried out using an Agilent Technologies 1100 series liquid chromatograph (RP–HPLC) coupled with a UV– vis multiwavelength detector. The separation was carried out on a 250 × 4.6 mm, 4-µm Hypersil ODS C18 reversed phase column at ambient temperature.

The mobile phase consisted of acetonitrile (solvent A) and water with 0.2% sulphuric acid (solvent B). The flow rate was kept at 0.5 ml/min. The gradient programme was as follows: 15%A/85%B, 0–12 min; 40%A/60%B; 12 to 14 min; 60%A/40%B, 14 to 18 min; 80%A/20%B, 18 to 20 min; 90%A/10%B, 20 to 24 min; 100%A, 24 to 28 min (Nuutila et al., 2002). The injection volume was 20 µl, and peaks were monitored at 280 nm. Samples were filtered through a 0.45 µm membrane filter before injection. Phenolic compounds were identified according to their retention times and spectral characteristics of their peaks against those of standards. Analyses were performed in triplicate.

Statistical analysis

All extractions and determinations were conducted in triplicate. Data were expressed as mean ± S.D. Means were statistically compared using the STATISTICA (v 5.1) program with Student's t-test at the $p < 0.05$ significance level.

RESULTS

Total polyphenol contents

Salinity influenced significantly the total phenol contents of *O. majoran* at different growth stages. In fact, the content increased in the shoots by 2.5, 1.3 and 1.5 folds at early vegetative stage (EVS), late vegetative stage (LVS) and flowering stage (FS), respectively (Figure 1).

At LVS, phenolics of *O. majorana* showed higher content of 7.24 to 8.86 mg GAE/g DMW respectively at 0 mM and 75 mM NaCl , compared to flowering stage and EVS (Figure 1). These results indicated that salinity influences significantly the secondary metabolism of *O. majorana*, which could be a defense mechanism and a biochemical adaptation to environmental stress (Dixon and Paiva, 1995). Our findings suggest that the late vegetative stage could be characterized by the maximum phenolic content (Figure1). After this stage, the plant must reduce its growth and prepare itself for the budding stage. In fact, it could be postulated that during the late vegetative stage, *O. majorana* plant accumulates phenolics to prepare itself for the lignification process in order to slow down its growth. Indeed, many studies reported

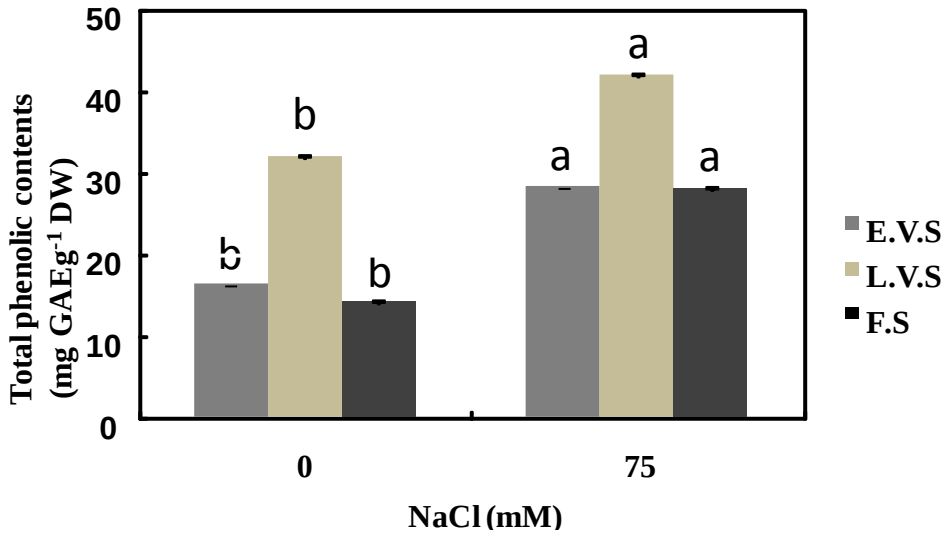

Figure 1. Total phenolic contents (mg GAEg-1 DW) in *Origanum majorana* L at different growth stages, plants grown over 17 days in the presence of 0 and 75mM NaCl. Means of 3 replicates. Means followed by different letters are significantly different at P≤0.005. Level as determined by analysis of variance (ANOVA).

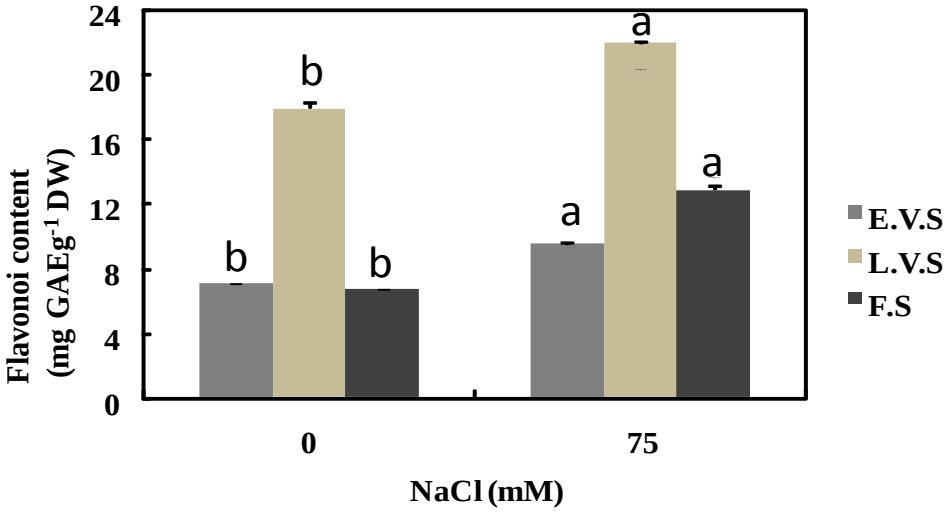

Figure 2. Flavonoid contents (mg GAEg-1 dw) in *Origanum majorana* L at different growth stages, plants grown over 17 days in the presence of 0 and 75 mM NaCl. Means of 3 replicates. Means followed by different letters are significantly different at P≤0.005. Level as determined by analysis of variance (ANOVA).

that lignin is a polymer synthesized from phenolic compounds of phenylpropane type and occurs under well-defined molecular associations (Boudet, 1998; Brunow, 1998). Variation of phenolic concentration during the growth of marjoram affirms the influence of both phonological stages and salt stress on production and release of these metabolites.

Flavonoid and condensed tannin contents

In the presence of salt addition, the evolution of flavonoid and tannin contents was similar to polyphenols at different growth stages. As shown in Figures 2 and 3, the flavonoid and tannin contents increased under saline addition. This change led to significant ($p \leq 0.05$)

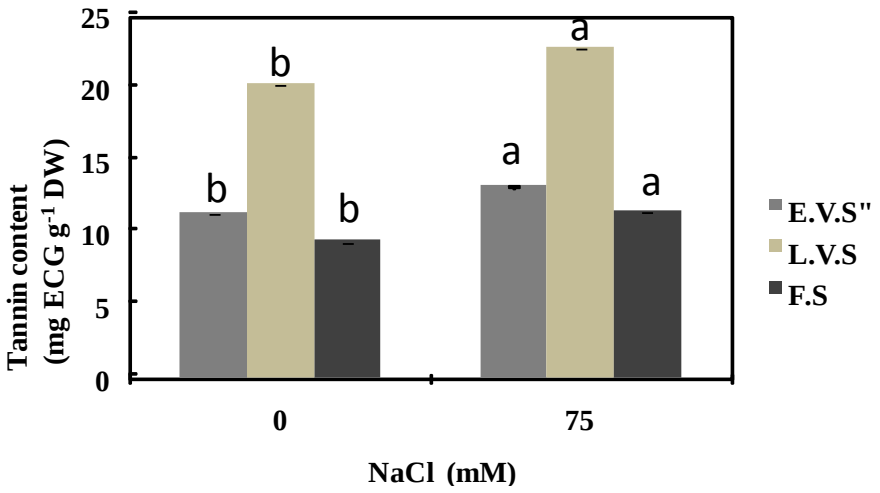

Figure 3. Tannin contents (mg GAEg-1 dw) in *Origanum majorana* L at different growth stages, plants grown over 17 days in the presence of 0 and 75mM NaCl. Means of 3 replicates. Level as determined by analysis of variance (ANOVA).

differences among treatments and growth stages. However, late vegetative stage had higher content of flavonoids and tannins than flowering and early vegetative stages, respectively at 0 and 75 mM NaCl. The effect of salt on the phenolic, flavonoid and tannin contents in the plant's extracts can be observed in more detail through HPLC analysis.

Individual phenolic acid contents

Our previous study demonstrated that phenolic composition of *O. majorana* shoots was characterized by the predominance of flavonoids at early vegetative stage, especially amontoflavone at 0 mM NaCl, and quercetin-3 –galactoside at 75 mM (Baatour et al., 2012).

Thus, the influence of growth stages on flavonoids and phenolic acid production was examined (Table 1). These two classes were stimulated by salt at different growth stages. Results showed that during the early vegetative stage and in absence of salt, flavonoids were predominant ($p < 0.05$) with 169.3 mg/100 g of DW against 131.1 mg/g DW for phenolic acids. However, during the late vegetative stage, phenolic acids became dominant ($p < 0.05$). But, at flowering vegetative stage, flavonoids became dominant with 135 and 191 mg/g DW, respectively at 0 and 75 mM NaCl.

The lowest level of phenols was detected in the flowering stage, whereas the highest content was detected during the late vegetative stage

Comparing the results of this study to bibliographic data, it seems that physiological stage could affect significantly the phenolics classes. In fact, Hamrouni et al. (2009) and Papageorgiou et al. (2008) indicated the predominance of phenolic acids during the early vegetative stage, whereas flavonoids predominated during the other stages of growth of *O. majorana.* In another study, Ayan et al. (2007) reported that total phenol content reached the highest level at floral budding in *Hypericum hyssopifolium* and *Hypericum scabrum* and at full-flowering in *Hypericum pruinatum*. In another study, Verma and Kasera (2007) indicated that peak concentration of phenols was observed in flowering stage in *Boerhavia diffusa* and *Sida cordifolia* except in *Asparagus racemosus* that showed maximal accumulation of phenols in the vegetative stage.

In fact, it could be postulated that during the late vegetative stage, *O. majorana* plant accumulates phenolics to prepare itself for the lignification process in order to slow down its growth. The biosynthesis of the different phenolic acids in the shoots was stimulated by the salt treatments. Baatour et al. (2012) reported that during the early vegetative stage, rosmarinic acid also trans-2-hydroxycinnamic acid was known as the major compound, followed by amentoflavone in the control. In addition, we noted a significant stimulation of gallic and trans-2 hydrocinnamic acids by 3 folds respectively and quercetin- 3- galactoside by 10.5 folds. In addition, we showed an important modification in phenolic acids class in the presence of salt addition. Moreover, an appearance of chlorogenic, syringic, vanillic and ferulic acids was observed.

The appearance of ferulic acid under osmotic stress could be correlated to the reinforcement of the plant wall, the decrease of cell wall plasticity and to the overall cell elongation (Wakabayashi et al., 1997). However,

Table 1. Phenolic contents (mg/ 100 g DW) of *Origanum majorana* shoots cultured under NaCl 75 mM at the early vegetative stage (E.V.S) and the late vegetative stage (L.V.S)

Phenolic compounds	Content (mg/100 g DW of dry sample)					
	E.V.S		L.V.S		F.S	
	0 mM	75 mM	0 mM	75 mM	0 mM	75 mM
Phenolic acids	**131.11±0.15^{b}**	**305.83±1.52^{a}**	**219.38±0.87^{b}**	**241.74±0.98^{a}**	**93.286±0.87^{b}**	**163.14±1.56^{a}**
Gallic acid	26.63±1.37^{b}	76.33±1.27^{a}	56.03±1.00 ^{b}	71.85±0.25^{a}	26.86±1.07^{b}	33.47±0.09^{a}
Caffeic acid	6.38±0.32^{b}	10.92±0.24 ^{a}	45.83±1.21^{b}	54.32±0.21^{a}	17.55±1.01^{b}	34.47±0.57^{a}
Dihydroxyphenolic acid	13.44±0.65^{a}	11.35±0.47 ^{b}	10.24±0.24^{a}	1.37±0.01^{b}	6.99±0.04^{b}	15.58±1.20^{a}
Chlorogenic acid	nd	17.71±0.57^{a}	0.85±0.09^{b}	25.78±1.20^{a}	6.70±1.13^{b}	11.41±0.57^{a}
Syringic acid	nd	20.26±0.17^{a}	2.69±0.07^{b}	2.45±0.08 ^{b}	2.36±0.07^{a}	6.70±0.09^{a}
Vanillic acid	nd	1.72±0.06^{a}	6.46±0.27^{a}	1.40±0.17^{b}	1.86±0.02^{a}	1.07±0.02^{b}
Rosmarinic acid	30.39±0.87^{a}	29.5±0.69 ^{a}	32.52±0.77^{a}	4.79±0.42^{b}	1.18±0.02^{b}	2.56±0.02^{b}
Cinnamic acid	18.54±0.67^{a}	9.46±0.97^{b}	21.73±0.23^{b}	34.35±0.16 ^{a}	6.58±1.01^{b}	21.22±0.9^{a}
Trans- 2 hydrocinnamic acid	28.76±1.03^{b}	88.50±1.37^{a}	33.49±1.47^{a}	26.42±1.13 ^{b}	2.10±0.09^{b}	6.16±0.03^{a}
Coumaric acid	6.97±0.09^{a}	2.51±0.02^{b}	6.14±0.28^{a}	5.46±0.25^{a}	20.01±1.35^{b}	28.69±0.18^{a}
Ferulic acid	nd	37.57±1.37^{a}	3.40 ±0.46^{b}	4.92±0.21 ^{a}	1.096±0.05^{b}	1.81±0.21^{a}
Flavonoids	**169.26±1.32^{b}**	**304.34±2.11^{a}**	**194.8±0.68^{b}**	**319.95±0.97 ^{a}**	**135±1.45^{b}**	**191.17±1.87^{a}**
Epigallocatechin	16.95±0.97^{b}	31.52±1.01^{a}	nd	3.11±0.02^{a}	1.53±0.02^{b}	1.82±0.12^{a}
Epicatechin	5.1±0.54^{b}	7.82±0.95^{a}	nd	7.46±0.01^{a}	6.61±0.25^{b}	13.27±0.09^{a}
Resorcinol	5.09±0.17^{b}	17.64±0.48^{a}	5.07±0.09 ^{b}	7.83±0.07^{a}	2.57±0.11^{a}	1.56±0.0^{b}
Rutin	22.64±0.99^{a}	3.17±0.85^{b}	20.51±0.67^{a}	14.28±0.36^{b}	1.89±0.57^{b}	6.51±1.27^{a}
Luteolin	13.96±1.01^{a}	3.17±0.01^{b}	17.03±0.69^{b}	23.94±1.05^{a}	7.62±0.37^{b}	9.98±0.47^{a}
Coumarin	nd	8.61±0.25 ^{a}	10.11±0.97^{b}	27.32±0.37^{a}	20.01±0.57^{b}	28.69±0.57^{a}
Quercetin	14.47±0.44^{b}	29.09±1.35 ^{a}	20.06±1.37^{a}	7.24±0.57^{b}	4.35±0.57^{b}	21.57±1.87^{a}
catechin	0.72±0.02^{b}	17.71±0.67 ^{a}	5.43±0.03 ^{b}	43.56±1.39^{a}	3.24±0.97^{b}	3.98±0.97^{a}
Quercetin-3 –galactoside	9.85±0.57^{b}	102.65±1.65^{a}	9.54±0.37^{b}	12.20±0.99^{a}	12.99±1.01^{b}	16.26±1.98^{a}
Quercitin-3-ramnoside	nd	8.72±1.27 ^{a}	1.54±0.01^{b}	3.80±0.47^{a}	1.49±0.15^{a}	2.86±0.27^{a}
Apigenin	15.48±0.97^{b}	21.40±1.23^{a}	29.10±1.37^{b}	43.36±1.57^{a}	12.89±1.98^{b}	17.50±1.47^{a}
Amentoflavone	65.72±0.21^{a}	65.99±0.30^{a}	76.41±1.65^{b}	125.85±1.7 ^{a}	60.65±0.07^{b}	67.17±0.07^{a}

nd, Non detective; -Values (means of three replicates± SD) with different superscripts (a,b) are significantly different at $P < 0.05$.

Sampietro et al. (2006) indicated that syringic acid was reported to promote cell division and has synergic effect with vanillic acid. Thus, in view of the accumulation of syringic acid, in the shoots at 75 mM NaCl (Table 1), we can suppose that it mitigates vanillic and ferulic acid effects in order to support *O. majorana* shoots growth under salt addition. Baatour et al. (2012) showed that at early vegetative stage, the stimulation of flavonoids content biosynthesis in the presence of salt could be related to the improvement of quercetin biosynthesis. We also noted an increase of quercetin-3-galactoside and the appearance of quercetin-3-rhamnoside. This result suggested that *O. majorana* may be responding as an adaptive mechanism by activating enzymes involved in the galactosylation of quercetin into quercetin-3-galactoside and quercetin-3-rhamnoside, which could lead to avoiding of salt damage.

As revealed in Table 1, in late vegetative stage, gallic and caffeic acids reached the highest content (56.03 mg/100 g of DW and 45.83 mg/100 g of DW; respectively), followed by amentoflavone (76.41 mg/100 g of DW) at 0 mM NaCl. Salt induced a stimulation of gallic, caffeic acids and amentoflavone to richness (71.85 mg/100 g of DW; 54.32 mg/100 g of DW and 125.85 mg/100 g of DW, respectively).

Independently of the applied treatment, amentoflavone was detected as the major phenolic compound, at the flowering vegetative stage, with a fraction of 60.65 mg/100 g of DW and 67.17 mg/100 g of DW, followed by gallilic acid (33.47 mg/100 g of DW), respectively at 0 and 75 mM NaCl (Table 1).

NaCl provoked a stimulation of phenolic acids due to

Table 2. Amounts of free radical scavenging activity and antiradical activity of *Origanum majorana* aerial part cultured under NaCl 75 mM.

Extract	DPPH scavenging activity (µg/ml)	IC_{50} Antiradical activity (µg/ml)
EVS (0mM)	430±1.13[a]	53±0.24 [a]
LVS (0mM)	203±0.68[a]	50±0.01[a]
FS (0mM)	207±0.97 [a]	51±0.07[a]
EVS (75mM)	290±0.15[b]	44±0.16 [b]
LVS (75mM)	199±0.09 [b]	40±0.25[b]
FS (75mM)	208±0.04 [a]	41±0.05[b]

Values (means of three replicates) with different superscripts (a–b) are significantly different at $P < 0.05$.

significant increase of caffeic, dihydroxyphenolic, clorogenic, syringic, cinnamic and trans-2-hydrocinnamic acids by 1.96, 2.22, 1.7, 2.83, 3.22 and 2.93 folds, at flowering stage. The increase of flavonoids is the consequence of stimulation of epicatechin, rutin and quercetion by 2, 3.44 and 4.95 folds, at F.V.S (Table 1).

The comparison between salt constraints revealed an inhibition of phenolics acids contents attested by an important decrease of syringic, rosmarinic, trans-2 hydrocinnamic and ferulic acids contents by 8, 6, 3 and 7 folds (Table 1). Indeed, it is well known that an important function of flavonoids and phenolic acids is their action in plant's defense mechanisms (Dixon and Paiva, 1995). Regarding theses variations in the accumulation of secondary metabolites in marjoram plants, it could be concluded that the physiological stage of the plant affects the choice of best harvesting time. In fact, when phenolics and flavonoids are desired, the late vegetative stage and early vegetative stage could be favourable to harvesting than flowering stage.

Antiradical and antioxidant activities

In the present study, the free radical scavenging activity of *O. majorana* extracts at different growth stages is as shown in Table 2. The stable DPPH radical was widely used to evaluate this activity in many plants' extracts.

The assessment of antioxidant activity showed that *O. majorana* shoots were able to scavenge this radical at three growth stages (Table 2). However, LVS displayed a higher activity based on IC_{50} = 50 and 40 µg ml^{-1}, respectively at 0 and 75 mM, than EVS and FS. Antioxidant activity increased under salt treatment at all growth stages. These findings showed that marjoram had a great antioxidant capacity at LVS as compared to FS and EVS with salt addition. *O majorana* shoots exhibited significant superoxide anion scavenging capacity (Table 2). As for DPPH, at NaCl 75 mM, shoot extracts were more effective O-2 scavengers than control ones with 44, 40 and 41 µg ml^{-1} and 53, 50 and 51 µg ml^{-1}, respectively in EVS, L.S and FS. These results revealed that methanolic extracts of *O. majorana* varieties were free radical scavengers, acting as primary antioxidants.

These three extracts exhibited higher activities at 75 mM NaCl. In control, there were no differences between LVS and FS; meanwhile, LVS extracts showed the highest antioxidant properties. Furthermore, at LVS, marjoram grown at 75 mM showed a higher antiradical ability against both DPPH radical and antioxidant activity, as compared to E.V.S and L.V.S. In fact, the lowest IC_{50} value corresponds to the highest antioxidant activity.

ROS are mostly generated in the chloroplasts during the electron transfer along the electron chain (Foyer et al., 1994) and an overproduction of ROS leading to an oxidative stress is known to be elicited by salinity (Hasegawa et al., 2000). So, to counteract NaCl-induced oxidative stress, plants are endowed with antioxidant defense system including enzymatic and non enzymatic ones, and it has been demonstrated recently that the improvement of DPPH radical scavenging property is involved in salt tolerance (Xie et al., 2008). The antioxidant capacity estimated by DPPH assay could be thus contributed by phenolic compounds.

Conclusion

This study showed that salinity induced biochemical changes in the *O. majorana* tissues, which could reflect an adaptation response to the stress. Moreover, our results demonstrated that cultivation of medicinal plants like *O. majorana* under salt conditions could increase its secondary metabolism as shown by the enhancement antioxidant activities and phenolic content.

In fact, the differences in phenolic compound contents between growth stages could be attributed to phenolic composition. L.V.S had an important activity in real food systems relative to commercially used, antioxidant extracts and economic feasibility of practical applications

due to high phenolic contents and antioxidant activities as compared to E.V.S and F.S.

Therefore, the favored harvest of marjoram could be in L.V.S and E.V.S than F.S., which supports the utilization of this plant in a large field of application including cosmetic, pharmaceutical, agro alimentary and biological defense.

REFERENCES

Ayan AK, Yanar P, Cirak C, Bilgener M (2007). Morphogenetic and diurnal variation of total phenols in some Hypericum species from Turkey during their phonological cycles. Banglad. J. Bot. 36:39-46.

Bâatour O, Mahmoudi H, Tarchoun I, Nasri N, Kaddour R, M Zaghdoudi, Wissal A, Hamdaoui G, Lachaâl M, Marzouk B (2012). Salt effect on phenolics and antioxidant activities of Tunisian and Canadian sweet marjoram (*Origanum majorana* L.) shoots. J. Sci. Food. Agric. DOI 10.1002/jsfa.5740.

Boutet AM (1998). A new view of lignification. Trends Plant Sci. 3:67-71.

Brunow G (1998) In: Lewis NG, Sarkanen S (Eds.), Oxidative Coupling of Phenols and the Biosynthesis of Lignin and Lignan Biosynthesis. Am. Chem. Soc. Washington. DC. pp. 131-147.

De Abreu IN, Mazzafera P (2005). Effect of water and temperature stress on the content of active constituents of *Hypericum brasilienne* Choisy. Plant Physiol. Biochem. 43:241-248.

Dewanto V, Wu X, Adom KK, Liu RH (2002). Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J. Agric. Food Chem. 50:3010-3014.

Dixon RA, Paiva NL (1995). Stress-induced phenylpropanoid metabolism. Plant Cell 7:1085-1097.

Duh PD, Tu YY, Yen GC (1999). Antioxidant activity of water extract of harng jyur (*Chrysanthemum morifolium*, Ramat). Lebensm. Wiss. Technol. 32:269-277.

El-Ghorab AH, Shibamoto T, Ozcan M (2007). Chemical composition and antioxidant activities of buds and leaves of capers (*Capparis ovata* Desf. Var. Canesencene) cultivated in Turkey. J. Essent. Oil Res. 19:72-77.

Foyer H, Descourvierse P, Kunert KJ (1994). Protection against oxygen radicals: an important defense mechanism studied in transgenic plants. Plant Cell. Environ. 17:507-523.

Hamrouni SI, Maamoui E, Chahed T, Aidi WW, Kchouk ME, and Marzouk B (2009). Effect of growth stage on the content and composition of the essential oil and phenolic fraction of sweet marjoram (*Origanum majorana* L.). Ind. Crops. Prod. 30:395-402.

Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ (2000). Plant cellular and molecular responses to high salinity. Annu. Rev. Plant. Physiol. Plant Mol. Biol. 51:463-499.

Heo HJ, Cho HY, Hong B, Kim HK, Heo TR, Kim EK, Kim SK, Kim CJ, Shin DH (2002). Ursolic acid of *Origanum majorana* L. reduces Abeta-induced oxidative injury. Mol. Cells 13:5-11.

Hoagland DR, Arnon DI (1950). The water culture method for growing plants without soil. Calif Agric Exp Sta Berkley, Circ 347, CA. p. 32.

Mau JL, Chao GR, Wu KT (2001). Antioxidant properties of methanolic extracts from several ear mushrooms. J. Agric. Food Chem. 49:5461-5467.

Medina E, Brenes M, Romero C, Garcia A, De Castro A (2007). Main antimicrobial compounds in table olives. J. Agric. Food. Chem. 55:9817-9823.

Miura K, Kikuzaki H, Nakatani N (2002). Antioxidative activity of chemical components from sage (*Salvia officinalis* L.) and thyme (*Thymus vulgaris* L.). J. Agric. Food Chem. 50:1845-1851.

Naczk M, Shahidi F (2004). Extraction and analysis of phenolics in food. J. Chromatogr. A 105:95-111.

Novak JC, Bitsch J, Langbehn F, Pank M, Skoula GY (2000). Ratios of cis and trans-sabinene hydrate in *Origanum majorana* L. and *Origanum microphyllum* (Bentham) Vogel. Biochem. Syst. Ecol. 28:697-704.

Nuutila AM, Kammiovirta K, Oksman-Caldentey KM (2002). Comparison of methods for the hydrolysis of flavonoids and phenolic acids from onion and spinach for HPLC. Anal. Food Chem. 76:519-525.

Paterson JR, Srivastava R, Baxter GJ, Graham AB, Lawrence JR (2006). Salicylic acid content of spices and its implications. J. Agric. Food Chem. 54:2891-2896.

Patro BS, Bauri AK, Mishra S, Chattopadhyay S (2005). Antioxidant activity of Myristica malabarica extracts and their constituents. J. Agric. Food Chem. 53:6912-6918.

Pietta PG (2000). Flavonoids as antioxidants. J. Nat. Prod. 63:1035-1042.

Raghavan S (2007). Handbook of spices, seasonings, and flavorings (2nd ed.). Boca Raton: CRC, Taylor and Francis Group.

Sampietro DA, Vattuone, MA, Isla MI (2006). Plant growth inhibitors isolated from sugarcane (*Saccharum officinarum*) straw. J. Plant Physiol. 163:837-846.

Servili M, Selvaggini R, Esposto S, Taticchi A, MontedoroG, Morozzi G (2004). Health and sensory properties of virgin olive oil hydrophilic phenols: Agronomic and technological aspects of production that affect their occurrence in the oil. J. Chromatogr. A 1054:113-127.

Sun Y, Oberley LW, Li Y (1988). A simple method for clinical assay of superoxide dismutase. Clin. Chem. 34:497-500.

Trouillas P, Calliste CA, Allais DP, Simon A, Marfak A, Delage C, Duroux JL (2003). Antioxidant, anti-inflammatory and antioxidant antiproliferative properties of sixteen water plant extracts used in the Limousin countryside as herbal teas. Food Chem. 80:399-407.

Verma V, Kasera PK (2007). Variations in secondary metabolites in some arid zone medicinal plants in relation to season and plant growth. Indian J. Plant Physiol. 12:203-206.

Wakabayashi K, Hoson T, Kamisaka S (1997). Osmotic stress suppresses cell wall stiffening and the increase in cell wallbound ferulic and diferulic acids in wheat coleoptiles. Plant. Physiol. 113:967-973.

Xie Z, Duan L, Tian X, Wang B, Eneji AE, Li Z (2008). Coronatine alleviates salinity stress in cotton by improving the antioxidative defense system and radical-scavenging activity. Plant Physiol. 165:375-384.

Zhishen J, Mengcheng T, Jianming W (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem. 64:555-559.