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Full Length Research Paper

Effects of physiological stage and solvent on polyphenol composition, antioxidant and antimicrobial activities of *Limonium densiflorum*

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Halophyte ability to withstand salt-triggered oxidative stress is governed by the synthesis of power antioxidant enzymes and substances. This might explain the utilization of some halophytes as traditional medicinal plants such as *Limonium* genus. In the present study ten extracts were used to examine the effects of solvent and physiological development stage on tissue phenolic contents, antioxidant and antimicrobial activities of *Limonium densiflorum*. Based on analysis of variance (ANOVA) test, results showed that solvent, physiological development stage and their interaction affected significantly phenolic composition and antioxidant activity. Moreover, solvent effect was much higher for flavonoid content, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene and Fe-reducing tests. While physiological stage effect was considerably larger than solvent effect for total antioxidant activity and tannin contents. Besides, shoot extracts exhibited high antiradical activity as compared to butylated hydroxytoluene (BHT). Furthermore, ethanol 95% extract showed the highest antibacterial activities mainly against gram positive germs. High-performance liquid chromatography (HPLC) analysis revealed several phenolic compounds including rutin hydrate and trihydrate as major phenolics. In conclusion, our findings identified the appropriate solvent and development stage for extracting phenolics which might provide a rich and novel source of natural antioxidants as additives replacing synthetic ones in industry.

Key words: *Limonium densiflorum,* extracting solvent, physiological development stage, phenolics, antioxidant activity, antimicrobial capacity.

INTRODUCTION

In recent years, natural antioxidants have gained a lot of importance, thanks to their potential as prophylactic and therapeutic agents in many diseases. The discovery of the role of free radicals in cancer, diabetes, cardiovascular diseases, autoimmune diseases, neurodegenerative disorders, aging and other diseases has led to a medical revolution that is promising a new paradigm of healthcare (Ratnam et al., 2006). In fact, these radicals are highly reactive molecules or chemical species containing unpaired electrons that cause oxidative stress, which is defined as “an imbalance” between oxidants and antioxidants in favor of the oxidants, potentially leading to damage (Jaleel et al., 2009). Oxidative stress can damage lipids, proteins, enzymes, carbohydrates and deoxyribonucleic acid (DNA) in cells and tissues, resulting in membrane damage, fragmentation or random cross linking of molecules like DNA, and even lead to cell death induced by DNA fragmentation and lipid peroxidation, therefore the challenge of diseases (Migliore and Coppedè, 2009). Although not many powerful antioxidants are listed in pharmacopoeias, extensive research is being carried out globally on these agents, especially when several of them have been proven pharmacologically active (Ratnam et al., 2006). Plants are potential sources of natural antioxidants; among them halophytes are salt-tolerant

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plants able to withstand hard climatic conditions which generate oxidative stresses (Ksouri et al., 2008). This particularity to overcome harmful environment is attribute to the powerful antioxidant system including enzymatic and non enzymatic molecules mainly phenolics (Ksouri et al., 2010).

The antioxidant activity of phenolic compounds is due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Ramarathnam et al., 1997). Recent studies have shown that many dietary polyphenolic constituents derived from plants are more effective antioxidants in *vitro* than vitamin E or C, and thus might contribute significantly to the protective effects in *vivo* (Auberval et al., 2010; Rene et al., 2001). Additionally to their role as antioxidant, these compounds exhibit a wide spectrum of medicinal properties, such as antimicrobials, anti-allergic, anti-inflammatory and cardio-protective effects (Balasundram et al., 2006). Previous studies have shown that phenolic content and antioxidant activities in plants depend on biological factors (genotype, organ, and physiological development stage) and technical factors mainly extracting solvent and methods of extraction (Bâno et al., 2003).

Furthermore, the content of phenolics present in plants depends on the interaction between all these factors separately (Moore et al., 2006). According to Lisiewska et al. (2006), the evolution of phenolic content in higher plants may reflect their physiological status and developmental stages.

In fact, Martin-Tanguy (1997) mentioned that some phenols (phenolamids) could be considered as markers of flowering induction. In this context, some phytoestrogens (isoflavonoids) of Soy stimulate the development of flowers. Further, 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*. Moreover, Verma and Kasera (2007) indicated that peak concentration of phenols was observed at flowering stage in *Boerhavia diffusa* and *Sida cordifolia.* On the other hand, different solvent systems have been used for polyphenols extraction from plant material (Pinelo et al., 2004) because the extraction yield is dependent on the polarity and the nature of solvents (Goli et al., 2004). Water and aqueous mixtures of ethanol, methanol and acetone are commonly used for phenolics extraction from plants (Sun and Ho, 2005). Trabelsi et al. (2010) showed that methanol is the best solvent to extract total phenolics and 80% acetone to remove tannins from the halophyte *Limonastrium monopetalum.*

However, in another work, water was found to be a better solvent, for extracting tea catechins, than 80% methanol or 70% ethanol (Khokhar and Magnusdottı´, 2002). Nevertheless, Bushra et al. (2009) reported that methanol 80% is the most appropriate solvent for phenolic compound extraction. Besides, it was showed that ethanol was the best solvent for extracting phenolic compounds from tassel, followed by methanol and then water (Sobhi et al., 2009). As there had not a suitable solvent for extraction of phenolic compounds in all species, it is interesting to establish the effect of solvent with different polarities phenolic content in *Limonium densiflorum.* In Tunisia, a considerable diversity of halophytic species with multiple interests including therapeutic practices occurs. For example, *L. densiflorum*, is a rosette plant from coastal regions and salt flat. It can tolerate a wide range of environmental conditions and resist to abiotic stresses such as salt, high temperature, and water deficit stress (Boushereau et al., 1999).

Moreover, *Limonium* spp. is known in a folk medicine, for example, *Limonium Wrightii* is used for the treatment of fever or arthritis (Aniya et al., 2002). However, biological and pharmacological proprieties of this species (*L. densiflorum*) have not been studied. For this reason, the objectives of this study were; (i) to investigate antioxidant activities using five solvent kinds with different polarity (hexane, acetone/water, ethanol/water, methanol/water and water) for extraction and two physiological stages (vegetative and flowering), (ii) to estimate the antimicrobial capacities against human pathogen strains, and (iii) to quantify and identify the main phenolic compounds present in *L. densiflorum*.

MATERIALS AND METHODS

Chemical and reagents

Folin–Ciocalteu reagent, sodium carbonate anhydrous (Na_2CO_3), gallic acid, sodium nitrite solution ($NaNO_2$), aluminum chloride hexahydrate solution ($AlCl_3$, $6H_2O$), vanillin, 2,2 Diphenyl-1-picrylhydrazyl (DPPH), trichloroacetic acid, iron(III)chloride anhydrous ($FeCl_3$), ascorbic acid and dimethyl sulfoxide (DMSO) were purchased from Fluka (Buchs, Switzerland). β-carotene, tween 80, linoleic acid, and butylated hydroxytoluene (BHT) were purchased from Sigma- Aldrich (GmbH, Sternheim, Germany). Sulfuric acid (H_2SO_4) and Kalium-hexacyanoferrat (III); $K_3Fe\ (CN)_6$ were obtained from Merck (Darmstadt, Germany).

Plant sampling and preparation for extract

Seasonal sampling of *L. densiflorum* were performed in August 2008 (flowering stage) and December 2009 (vegetative stage) from salt flat in Sidi El Hani (region of Kairoun, semi-arid bioclimatic) located in centre of Tunisia. The harvested plants were identified at the Biotechnology Center at the Technopark of Borj-Cedria, and a voucher specimen (PLM30) was deposited at the Herbarium of the Laboratory of Extremophile Plants at the Biotechnology Center. Shoots were rinsed with distilled water, left at room temperature for 7 days in the dark, and then oven dried for 24 h at 60°C. Extracts were obtained by magnetic stirring of 2.5 g dry powder with 25 ml (hexane, ethanol/water (9,5/0.5), methanol/water (8/2), acetone / water (8/2) and water) for 30 min. Extracts were kept for 24 h at 4°C, filtered through a Whatman No. 4 filter paper, and evaporated under vacuum to dryness. Then, they were stored at 4°C until

analysis.

Phenolic quantification

Estimation of total phenolic content

Total phenolics were assayed using the Folin–Ciocalteu reagent, following Singleton and Rosi (1965) method. This method was based on the reduction of a phosphowolframate-phosphomolybdate complex by phenolics to blue reaction products and slightly modified by (Dewanto et al., 2002). An aliquot of diluted sample extract was added to 0.5 ml of distilled water and 0.125 ml of the Folin–Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 min, before addition of 1.25 ml of 7% Na_2CO_3. The solution was then adjusted with distilled water to a final volume of 3 ml and mixed thoroughly. After incubation in dark, the absorbance at 760 nm, was read versus the prepared blank. Total phenolic content of plant was expressed as mg gallic acid equivalents (GAE)/g DW through the calibration curve with gallic acid. The calibration curve range was 50-400 µg/ml (R^2=0.99). All samples were analyzed in triplicate.

Estimation of total flavonoid contents

Total flavonoids were measured by a colorimetric assay according to (Dewanto et al., 2002). An aliquot of diluted sample or standard solution of (+)-catechin was added to a 75 ml of $NaNO_2$ solution (5%), and mixed for 6 min before adding 0.15 ml $AlCl_3$ (10%). After 5 min, 0.5 ml of sodium hydroxide (NaOH) was added. The final volume was adjusted to 2.5 ml with distilled water and thoroughly mixed. Absorbance of the mixture was determined at 510 nm against the blank where the sample was omitted. Total flavonoid content was expressed as mg catechin per g of DW (mg CE/g DW), through the calibration curve of (+)-catechin, ranging from 0 to 400 mg/ml. All samples were analyzed in triplicate.

Quantification of total condensed tannins

Proanthocyanidins were measured using the modified vanillin assay described by Sun et al. (1998). To 50 µL of properly diluted sample, 3 ml of methanol vanillin solution and 2.5 ml of H_2SO_4 were added. The absorption was measured at 500 nm against extract solvent as a blank. The content of total condensed tannins is expressed as mg (+)catechin/g DW. The calibration curve range was 0–400 µg/ml. All samples were analyzed in three replications.

Antioxidant activities of shoot extracts

Total antioxidant activity

The total antioxidant capacity (TAC) of the plant extracts was evaluated by the phosphomolybdenum method of Prieto et al. (1999). A 0.1 ml aliquot of the plant extract was mixed with 1 ml of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The absorbance of the mixture was measured at 695 nm and standard curve was performed with ascorbic acid solution. The antioxidant capacity was expressed as mg gallic acid equivalent per gram dry weight (mg GAE/g DW). The calibration curve range was 0 – 500 µg/ml. All samples were analyzed in triplicate.

DPPH· radical scavenging activity

The DPPH scavenging activity was estimated according to Hanato et al. (1998). The dried plant extract was diluted in pure methanol at different concentrations ranging from 1 to 100 µg/ml, and then 2 ml were added to 0.5 ml of 0.2 mmol DPPH methanolic solution. The mixture was shaken vigorously and left standing at room temperature for 30 min in the dark, and then the absorbance was measured at 517 nm. For each dilution of the extract, the DPPH scavenging activity was calculated as $100 \times (A0-A1)/A0$, where $A0$ is the absorbance of the control at 30 min, and $A1$ is the absorbance of the sample at 30 min. The antiradical activity was finally expressed as IC_{50} (µg/ml), the extract concentration required to cause a 50% inhibition. All samples were analyzed in three replications.

Determination of reducing power

The ability of the extracts to reduce Fe^{3+} was assayed by the method of Oyaizu (1986). Briefly, 1 ml of shoot extract was mixed with 2.5 ml of phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 ml of $K_3Fe(CN)_6$ (1 g/100 ml). After incubation at 50°C for 25 min, 2.5 ml of trichloroacetic acid (10 g/100 ml) were added and the mixture was centrifuged at 650 × g for 10 min. Finally, 2.5 ml of the upper layer was mixed with 2.5 ml of distilled water and 0.5 ml of aqueous $FeCl_3$ (0.1 g/100 ml).

β-Carotene bleaching test (BCBT)

A slightly modified of Koleva et al. (2002) method was employed to estimate *L. densiflorum* shoots capacity to inhibit the β-carotene bleaching. β-carotene (2 mg) was dissolved in 20 ml chloroform and to 4 ml of this solution, linoleic acid (40 mg) and Tween 40 (400 mg) were added. Under vacuum at 40°C, the chloroform was evaporated and 100 ml of oxygenated water was added, then the emulsion was vigorously shaken. Sample extract and reference compounds (BHT and BHA) were prepared in ethanol. An aliquot (1500 µl) of the β-carotene/linoleic acid emulsion was distributed in each of the tubes and 100 µl of the test samples were added. Three replicates were prepared for each sample concentration. The tubes were incubated at 50°C for 120 min, and the absorbance was measured at 470 nm. Readings of all samples were performed immediately (t = 0 min) and after 120 min of incubation. The antioxidant activity (AA) of the extracts was evaluated in term of β-carotene bleaching inhibition using this formula: AA=100 x $((A0-A1)/(A1-A2))$ where $A0$ is the absorbance of the sample at 120 min, $A1$ is the absorbance of the control at 0 min, and $A2$ is the absorbance of the control at 120 min. The results were expressed as IC_{50} values (µg/ml).

Antimicrobial activity

Microorganisms

The antimicrobial activity was assessed against six human pathogenic bacteria, including Gram-positive, (*Staphylococcus aureus* ATCC 25923, *Micrococcus luteus* NCIMB 8166, *Enterococcus feacalis* ATCC 29212, *Listeria monocytogenes* ATCC 19115) and Gram-negative bacteria (*Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella thyphymurium* LT2) and four yeast (*Candida albicans (*ATCC 90028), *Candida parapsilosis* (ATCC 22019), *Candida glabatra* (ATCC 9030) and *Candida Krusei* (ATCC 6250).

Disc-diffusion assay

Antibacterial activity of different shoot extracts was assessed using

Table 1. Total polyphenol, flavonoid, and condensed tannin contents in *Limonium densiflorum* collected in August and December.

	Polyphenols (mgEGA/gDW)		Flavonoids (mgEC/gDW)		Tannins (mgEC/gDW)	
	August	December	August	December	August	December
Hexane	0.18±0.02^{g}	0.18±0.01^{g}	0.11±0.03^{g}	0.07±0.01^{g}	1.118±0.04^{h}	2.43±0.43^{h}
Ethanol 95%	48.04±0.64^{b}	8.79±0.18^{f}	8.34±2.65^{c}	3.01±0.21^{e}	52.2±0.01^{a}	5.35±1.09^{g}
Acetone 80%	109.14±21.18^{a}	41.41±2.94^{c}	15.93±0.26^{a}	7±0.26^{d}	47.7±3.28^{b}	26.73±2.64^{d}
Methanol 80%	49.93±0.68^{b}	34.73±1.12^{d}	10.49±1.28^{b}	8.73±0.88^{c}	33.1±1.31^{c}	22.02±2.83^{e}
Water	16.65±0.77^{e}	5.40±0.77^{f}	3.04±0.21^{e}	1.48±0.19^{f}	10.877±0.06^{f}	0.768±0.06^{h}

For each column or line, values followed by one or more of the same letters were not significantly different at $P < 0.05$ according to the Newman-Keuls post-hoc test.

the paper disk agar diffusion method according to (Freney et al., 2002). Extracts were prepared at a concentration of 50 mg/ml and 12.5 mg/ml in DMSO. Inoculates grown in Muller Hinton Agar at 37°C for 24 h were diluted to approximately 2 × 10^6 CFU/ml in saline solution (9 g/L). The concentration of the suspension used for inoculation was standardized by adjusting the optical density to 0.5 at 570 nm. Absorbent disks (Whatman disk No 3 of 6 mm diameter) were impregnated with 20 µl of different extracts and then placed on the surface of inoculated plates (90 mm) and incubated at 37°C for 24 h. Negative controls were prepared using a disk impregnated with the same solvent as that used to dissolve the plant extracts (DMSO). Antimicrobial activity was assessed by measuring the inhibition zone. This was the diameter of the zone visibly showing the absence of growth, including the 6 mm disk. Standard discs of Gentamycin (10 UI) served as positive antibiotic controls. All the tests were performed in triplicate.

For the antifungal activity of the same extracts, the agar-disc diffusion method was used as previously described (Cox et al., 2000). Candida strains were first grown on Sabouraud chloramphenicol agar plate at 30°C for 18 – 24 h. Several colonies were transferred into saline solution (9 g/L) and adjusted to 0.38 at 520 nm. The inocula of the respective yeasts were streaked onto Sabouraud chloramphenicol agar plates at 30°C using a sterile swab and then dried. A sterile filter disc, diameter 6 mm (Whatman paper No 3) was placed in the plate. 20 µl of different extract concentrations (representing 500 and 1000 µg per disk) were dropped on each paper disc. The treated Petri dishes were incubated at 30°C for 18–24 h. As for the antibacterial activity, the antifungal one was evaluated by measuring the diameter of the growth inhibition zone around the discs. The susceptibility of the standard was determined using a disc paper containing 20 µg of amphoterecin B. Each experiment was carried out in triplicate and the mean diameter of the inhibition zone was recorded.

Analysis of individual phenolic compounds by analytical RP-HPLC

HPLC analysis was performed on AGILANT apparatus equipped with an autosampler model 1100, a Prostar Pump model 1100, and a Prostar diode array detector model 1100. A RPC18 column (Prontosil, 250 × 4.0 mm, 5 µm, Bischoff) was used for analysis. The mobile phase was composed of two solvents: A (0.025% trifluoroacetic acid, TFA in H_2O and B acetonetrile MeCN). The sample was dissolved in methanol/water (1/1) and filtered through a 0.45 µm Millipore filter. Total run time was 60 min. The elution program at 1ml/min was as follows 90 A/10 B (0 - 40 min); 50 A/50 B (40 - 41 min); 100% B (41 - 50 min); 90 A/10 B (50 - 59 min). Each sample was directly injected and chromatograms were monitored at 280 nm. The sample injection volume was 20 µl. Identification of compounds was achieved by comparing their retention time values with those of standards.

Statistical analysis

Means were statistically compared using the STATISTICA program. A two-way analysis of variance (ANOVA) and Duncan's multiple range test were carried out to test any significant differences between parameters at $P < 0.05$.

RESULTS

Assessment of polyphenol, flavonoid and tannin contents

Polyphenol content varied significantly mainly as function of solvent (S) and physiological development stage (D) and followed by their interaction (S x D). Considering the physiological development stage, polyphenol content, in all solvent extracts, was lower at vegetative stage as compared to flowering one, except for hexane shoot extracts (Table1). For example, 80% acetonic extract at vegetative stage (*ca.* 41.4 mg GAE/g DW) were much lower than at flowering one (*ca.* 109.14 mg GAE/g DW) (Table 1). We note also significant difference between *L. densiflorum* extracts depending of solvent nature. For instance at flowering stage, the highest level of polyphenols was achieved using 80% acetone (109 mg EAG/g DW), followed by 80% methanol (49.93 mg EGA/g DW), 95% ethanol (48.06 mg EGA/g DW), water (16.65 EGA/g DW) and at last hexane (0.186 mg EAG/g DW). The same order of polyphenol contents in shoot extracts was observed at the vegetative phase.

As with the previous parameter, solvent extracting and physiological stage, as well as their interaction showed an important effect on flavonoid contents (Table 1). In fact, as for polyphenols, the same tendency was observed, the content of these pigments (flavonoids) in sample extracts at the flowering period were higher as compared to those from vegetative stage (Table 2). For example, in summer period (flowering shoots), acetonic extracts displayed 2-folds higher than winter season. In

Table 2. Total antioxidant capacity of shoot plant expressed as mg EGA/g DW.

	Hexane	Ethanol 95%	Acetone 80%	Methanol 80%	Water
August	61.33±3.5^{d}	187.13±3.5^{b}	189.67±2.14^{a}	148.86±6^{c}	53.16±6.98^{d}
December	13.51±4^{f}	19.02±1.48^{f}	52.57±0.63^{e}	46.6±6.1^{g}	17.77±2.43^{f}

For each column or line, values followed by one or more of the same letters were not significantly different at $P < 0.05$ according to the Newman-Keuls post-hoc test.

Table 3. Scavenging activity, expressed as IC_{50} values (µg/ml), on DPPH test of *Limonium densiflorum* extracts and BHT. Means of three replicates followed by at least one same letter are not significantly different at P <0.05.

	Hexane	Ethanol95%	Acetone80%	Methanol80%	Water	BHT
August	>1000^{a}	6.03±0.94^{fe}	4±0.01^{f}	4.8±0.86^{f}	410±24^{b}	11.5±0.01^{e}
December	>1000^{a}	20±4.16^{d}	5.8±1.01^{fe}	6.06±0.94^{fe}	80±11^{c}	

For each column or line, values followed by one or more of the same letters were not significantly different at $P < 0.05$ according to the Newman-Keuls post-hoc test.

addition, and independently of development stage, solvent contributed to the highest proportion of total variance. In fact, among the solvent used for the extraction, the mixture acetone/water (8:2) displayed the highest capacity to extract flavonoids (15,093 mg EC/g DW) from *L. densiflorum* shoot followed by methanol 80%, ethanol 95%, water and eventually hexane that exhibited the lowest extracting power.

Results showed that *L. densiflorum* is rich in proanthocyanidin (condensed tannins) than flavonoid fraction (Table 1). The content of condensed tannins of the aerial parts exhibited also significant variation depending mostly on physiological stage (D) than solvent (S) and their interaction (Table 5). In fact, *L. densiflorum* shoots collected at flowering stage showed a content of condensed tannins ranging from 10 to 14-folds higher in 95% ethanol and water extracts respectively, than those collected at vegetative stage. Moreover, and independently of development stage, 95% ethanol exhibited the better extracting power for condensed tannins from plant extracts at flowering stage in contrast to the total phenolic and flavonoids. While, 80% acetone was more appropriate for proanthocyanidin extracting at vegetative stage.

Determination of antioxidant activities

Considering the multifaceted aspects of antioxidants and their reactivity, several antioxidant assays were applied. In fact, depending on the reaction involved, these assays can roughly be classified into two types: assays based on hydrogen atom transfer reactions and assays based on electron transfer (Huang et al., 2005). Accordingly, we combined numerous complementary analyses. Of these, Total antioxidant activity, DPPH or ABTS assays, ROS quenching assay, metal chelating, reductive potential, β-carotene linoleate system and linoleic acid method are the most commonly used for the determination of antioxidant activities of plant extracts (Ksouri et al., 2010).

Total antioxidant capacity

Excluding the solvent effect, the study reveals that the antioxidant activity of *L. densiflorum* extracts from reproductive stage (between 54 and 190 mg EGA) was higher than that of vegetative one (between 13.5 and 52 mg EGA) (Table 2). Moreover, acetone and ethanol (about 188 mg EGA), followed by methanol extracts (149 mg EGA) showed a strong antioxidant activity as compared to water and hexane (less than 62 mg EGA) at flowering period. These results were corroborated by statistical analysis which showed that (D) effects were considerably larger than (S) effect on total antioxidant activity.

Radical scavenging activity (DPPH test)

Data in Table 3 showed the antioxidant activity of the different extracts of *L. densiflorum* at two development stages. The DPPH quenching capacity varied significantly between solvents and physiological stage as depicted by ANOVA, while (S x D) and (D) effects were comparable and greatly lower than (S) effect (Table 5). In fact, antiradical activity varied considerably as function of extracting solvent and was ranged from 4 to over than 1000 µg/ml (Table 4). Moreover, independently of the harvesting period, three solvent classes were revealed. The first one, showing the lower antiradical activity

Table 4. Reducing power (EC_{50}, µg/ml) and inhibition of β-carotene bleaching activity (IC_{50}, µg/ml) of *L. densiflorum* aerial part extracts, BHT, and ascorbic acid. Means of three replicates followed by at least one same letter are not significantly different at P < 0.05.

	Fe-reducing power		Inhibition of β-carotene bleaching activity	
	August	December	August	December
Hexane	>1000[a]	>1000[a]	>1000[a]	>1000[a]
95% Ethanol	85±3.28[e]	690±49[b]	600±14.3[c]	>1000[a]
80% Acetone	99.67±1.43[e]	206±13.3[d]	760±7.1[b]	590±14.33[dc]
80% Methanol	58±5[f]	210±13.3[d]	340±7.14[e]	570±65[d]
water	580±20[c]	>1000[a]	>1000[a]	>1000[a]
ascorbic acid	40±0.5[g]			
BHT			75±0.2f	

For each column or line, values followed by one or more of the same letters were not significantly different at *P* < 0.05 according to the Newman-Keuls post-hoc test.

Table 5. Comparison of the effect of physiological stage (P) and extracting solvent (S) on antioxidant compounds and activities.

	S	P	(S*P)
Polyphenol	44.51	46.03	17.04
Flavonoids	57.85	33.66	8.49
Condensed tannins	33.96	54.38	11.66
Total antioxidant activity	10.87	83.6	5.53
DPPH	94.17	3.1	2.73
Reducing power	59.33	34.19	6.48
β-carotene	69.6	11.64	18.76

F values for the proportion of total variance attributed to physiological development stage, Solvents (S) and their interaction(S*P). All values correspond to *P* < 0.05.

includes pure hexane (IC_{50} > 1000 µg/ml). In the second class, the most polar solvent (water) depicted moderated activity (IC_{50}) values between 80 and 410 µg/ml. The third group includes the mixture of acetone or methanol or ethanol and water respectively exhibit the best antiradical activity against DPPH (IC_{50}) values ranged from 4 to 20 µg/ml. From the aforementioned results, the phenolic compounds from, acetonic, ethanolic and methanolic extracts of *L. densiflorum* proved their efficiency as antioxidants when compared to BHT.

Iron reducing power

Results showed that *L. densiflorum* exhibit a significant reducing power that depends on physiological stage and solvents (Tables 4 and 5). For instance, (ethanolic, methanolic and acetonic) extracts were more efficient than (hexanic and water) whatever the development stage. At the flowering one, (IC_{50}) values of ethanolic, methanolic and acetonic extracts were respectively 7, 2 and 5 times larger than in the vegetative stage. In addition, methanolic extract (IC_{50} = 58 µg/ml) have an activity comparable to the standard ascorbic acid (IC_{50} = 40 µg/ml).

Antioxidant assay using β-carotene linoleate system

Results showed (Table 4) that independently of development stage, 80% methanolic extract displayed the highest β-carotene bleaching activity as compared to other extracts. In addition, at the flowering stage, 80% methanolic extract exhibit the best activity to prevent the decouloration of β-carotene with IC_{50} value equal to 340 µg/ml.

Evaluation of antimicrobial activities

Previous results showed the superiority of flowering stage of *L. densiflorum* for phenolics extracting and the powerful antioxidant activity of this extract. For that, the antibacterial activity of this plant was only evaluated for the flowering stage (using different solvents) and was determined against 7 bacterial strains and 4 yeasts considered as among the common food-borne bacteria. All extracts were used at 500 and 1000 µg/disc. Results showed that ethanolic extracts has the best broad-spectrum action, (diameter of inhibition ranged from8 to 16 mm), followed by 80% acetone and 80% methanol extracts, while hexanic or water extracts have no

Table 6. Antibacterial activity of *Limonium densiflorum* extracts at different concentrations (µg/disc). Inhibition zone calculated in diameter around the disc (mm).

Bacteria	µg/disc	Hexane	80% acetone	95% ethanol	80% methanol	Water	Gentamycin (10UI)
E. coli	1000	_	9^{e}	13.33^{b}	7.66^{f}	_	23^{a}
	500	_	10^{d}	11.33^{c}	8^{f}	_	
P. aeruginosa	1000	_	7.66^{c}	14^{b}	7^{cd}	_	16^{a}
	500	_	$_{}^{d}$	12.66^{c}	$_{}^{d}$	_	
E. faecium	1000	_	7.66^{c}	13.33^{b}	8^{c}	_	20^{a}
	500	_	8^{c}	13^{b}	$_{}^{d}$	_	
Salmonelle typhi	1000	_	8^{d}	12.33^{b}	7.66^{d}	_	24^{a}
	500	_	8^{d}	11^{c}	8^{d}	_	
S. aureus	1000	_	10^{d}	16.66^{b}	8^{f}	_	19^{a}
	500	_	9.66^{e}	14^{c}	7.66^{f}	_	
L. monocytogenes	1000	_	10^{c}	15.66^{a}	8^{d}	_	0^{e}
	500	_	10^{c}	14.66^{b}	7.66^{d}	_	
M. luteus	1000	_	13^{b}	8^{c}	7.66^{c}	_	26^{a}
	500	_	13^{b}	8.33^{c}	8^{c}	_	

No antimicrobial activity (-). For all bacteria, the inhibition zone of the control (+) gentamycin (10 UI) was higher than 15 mm (++++). The diameter of disc was 6 mm. For each column or line, values followed by one or more of the same letters were not significantly different at $P< 0.05$ according to the Newman-Keuls post-hoc test.

Table 7. Anticandidal activity of *L. densiflorum* extracts at different concentrations (µg/disc). Inhibition zone calculated in diameter around the disc (mm).

	µg/disc	Hexane	Acétone 80%	Ethanol 95%	Methanol 80%	Water	AmphotericinB 20 µg
C. krusei	1000	_	_	12^{b}	7.66^{c}	_	29^{a}
	500	_	_	12^{b}	8^{c}	_	
C. parapsilosis	1000	_	8^{d}	14^{b}	$_{}^{e}$	_	28^{a}
	500	_	7.66^{d}	12.66^{c}	$_{}^{e}$	_	
C. glabatra	1000	_	7.33^{d}	13.33^{c}	8^{d}	_	22^{a}
	500	_	8^{d}	14^{b}	7.66^{d}	_	
C. albicans	1000	_	8^{c}	12.33^{b}	12^{b}	_	27^{a}
	500	_	8^{c}	12^{b}	12.33^{b}	_	

No antimicrobial activity (-). For all Candida, the inhibition zone of the control (+) Amphoterecin B (20 µg) was higher than 9 mm (++++). The diameter of disc was 6 mm. SD: standard deviation. For each column or line, values followed by one or more of the same letters were not significantly different at $P< 0.05$ according to the Newman-Keuls post-hoc test.

antimicrobial activities (Tables 6 and 7). Moreover, ethanolic extract showed an important antifungal activity (inhibition diameter was ranged from 12 to 14 mm). Furthermore, aqueous acetonic and methanolic extracts showed similar behavior against 3 strains (*P. aeruginosa, E. faecuim* and *Salmonelle thyphi*) since the inhibition diameters is variable for the other strains (*E. coli, Staphylococcus aureus, L isteria monocytogenes and M. luteus*). In contrast, methanolic extract showed an antifungal activity more important than acetonic one with an inhibition diameters ranged from 7 to 12 mm. Indeed, the different extracts evaluated for their effect on microbes growth, the inhibition zones increased generally with the extract concentration. Besides, gram-positive

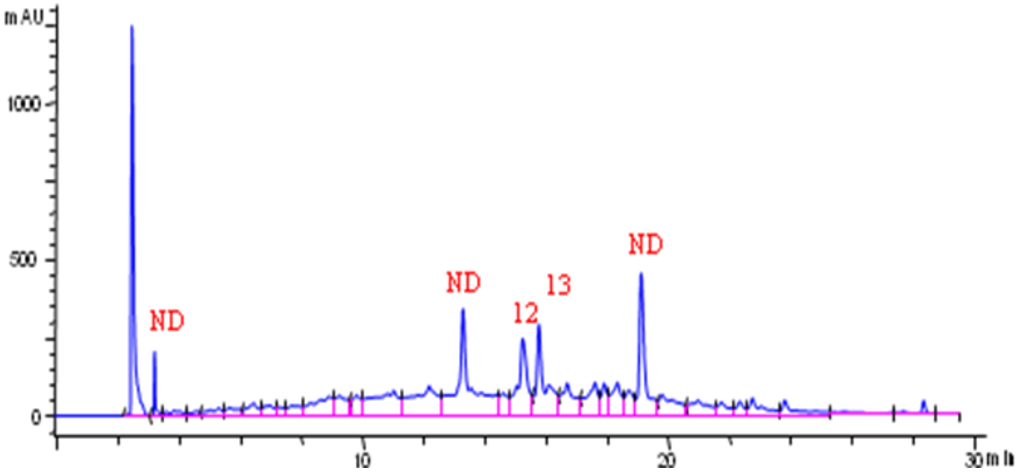

Figure 1. RP-HPLC chromatogram of *L. densiflorum* extract (winter period). Signal was recorded at 280 nm. Numbering of peaks refers to their identification as shown in Table 8 and corresponding to 12, Rutin hydrate and 13, Rutin trihydrate.

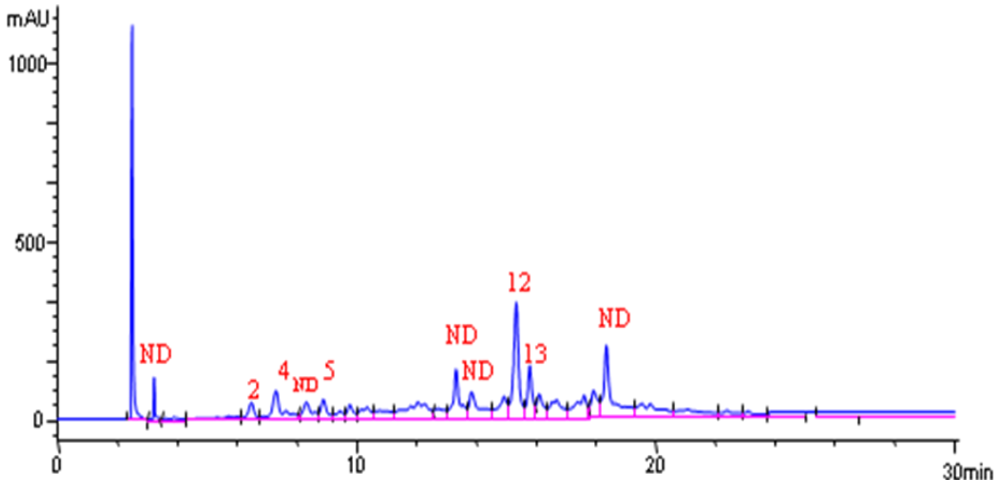

Figure 2. RP-HPLC chromatograms for *L. densiflorum* extract (summer period). Signal was collected at 280 nm. Peaks numbers corresponding to: 2, Protocatechiuc acid, 4, Epigallocatechin, 5, Chlorogenic acid, 12, Rutin hydrate and 13, Rutin trihydrate.

bacteria (*S. aureus,* and *L. monocytogenes*) were more sensitive than the gram-negative ones (*E. coli* and *S. typhi*). Concerning antifungal activity, *C. glabatra* was the most sensible to ethanolic and aqueous acetonic extract with an inhibition diameter 14 and 8 mm respectively. Whereas, *C. albicans* was more sensible to methanolic extract (inhibition diameter 12 mm).

Phenolics identification in L. densiflorum by RP-HPLC

The characterization of polyphenolic compounds of *L. densiflorum* was carried out using HPLC–DAD technique. Chromatograms of the phenolic extracted from this halophyte are presented in Figures 1 and 2. The identified compounds were confirmed by comparison of the data with those obtained for standard compounds listed in the experimental part (Table 8). In fact, chromatogram analysis depicts differences between two periods. The results revealed a phenolic fingerprint composed by two phenolic acids (chlorogenic and Protocatechuic acid), and two flavonoids (epigallocatechin and rutin) in *L. densiflorum* collected in the summer period and only one flavonol (rutin) in the winter period. In fact, phenolics were more largely represented in *Limonium* from the summer than in the winter.

Table 8. Phenolic acid and flavonoid standard retention times determine by RP-HPLC. RT: retention time.

S/N	Standard	Retention times
1	Gallic acid	3.993
2	Protocatechuic acid	6.407
3	3,4-dihydroxyphenol acetic acid	6.835
4	Epigallocatechin	7.124
5	Chloroginic acid	8.873
6	4-hydroxybenzoic acid	9.293
7	2,5-dihydroxybenzoic acid	9.701
8	Vanillic acid	10.441
9	Caffeic acid	10.839
10	3,5 dimethoxy-4-hydroxybenzoic acid	10.919
11	P-coumaric acid	14.771
12	Rutin hydrate	15.679
13	Rutin trihydrate	15.7
14	Trans-4-hydroxy-3-methoxycinnamic acid	16
15	Sinapic acid	16.026
16	3,4-Dimethoxybenzoic acid	16.234
17	O-coumaric acid	19.325
18	Trans-2-hydroxycinnamic acid	19.384
19	Rosmarinic acid	20.231
20	Salicylic acid	20.422
21	Naphtoresorcinol	24.039
22	Quercitin dihydrate	25.988
23	Trans-cinnamic acid	25.999
24	4-methoxycinnamic acid	26.812
25	4',5,7-trihydroxyflavone	29.368
26	Kaempferol	30.963

DISCUSSION

In this study, *L. densiflorum*, halophyte belonging to the *Plumbaginacea* family was harvested at two physiological development stages (vegetative and flowering) and the extraction was performed using five solvents of increasing polarity. The ANOVA for all parameters assessed a significant influence on phenolics contents, antioxidant and antimicrobial activities of plant extracts as function of (S) and (D), and their interaction (S × D). Moreover, our results indicated that solvent effect was the main contributor for flavonoids, DPPH, reducing power and β-carotene. Whereas (D) was the main contributor for total phenolic, tannin content and total antioxidant activity. Besides, (S) followed by (D) were the predominant contributors to phenolic content, antioxidant and antimicrobial properties than their interaction (E × D). The highest phenolic contents were obtained at the flowering stage (summer period). These results could be mainly attributed to environmental parameters, for example an increase of the content of ultraviolet (UV) radiation associated with an increase in solar radiation received by the plants. The increased temperature and sunshine of plant may have also caused an accumulation of phenolics to protect plants (Too et al., 2004). Indeed, it is well known that flavonoids and phenolic acids have an important role in plant defense mechanisms (Dixon and Paiva, 1995). It has been reported by Hunt and Baker (1980) that light increases increased the rate of phenolic biosynthesis in plants by stimulating of enzyme activities, especially phenylalanine ammonia-lyase (PAL) which plays an important role in converting phenylalanine (from shikimic acid pathway) into coumaric acid, which is the initial precursor molecules involved in the synthesis of phenolic components (flavonoids and hydroxycinnamtes) in plants (Smith, 1973). Therefore, at flowering stage, *L. densiflorum* received higher content of light and UV radiations and showed high content of phenolics than at vegetative one (winter season). Studying seasonal changes of phenolic contents in *Boerhavia diffusa* and *Sida cordifolia* (Verma and Kasera, 2007) revealed that the total phenol content increased at the flowering stage as compared to vegetative one. Nevertheless, Males et al. (2003) indicated that the aerial parts of *Crithmum maritimum* harvested before flowering and at the beginning of flowering stage showed the highest total polyphenol contents. Besides, phenolic extrability depend on solvent nature. For instance, Trabbelsi et al. (2010)

reported that methanol extract of *limoniastrum spp* exhibited the highest level of total polyphenols (15.85 mg EGA/g DW) for the plant harvested in summer period. Sobhi et al. (2009) found that ethanol is the best solvent for the extraction of total polyphenols from the corn tassel.

On the other hand, the extracts (acetonic, ethanolic and methanolic) which showed relatively high antioxidant activity exihibited the highest content of total phenolics. It has been reported that antioxidant activity of plant extracts is mainly ascribed to the concentration of the phenolic compounds present in the plants (Heim et al., 2002). In this context, several studies showed good correlation between total phenols and antioxidant activity (Bourgou et al., 2008; Huang et al., 2005; Falleh et al, 2008).

For the DPPH test, IC_{50} value of *L. densiflorum* was more interesting than that observed in other halophytic plant such as *Cakile maritima* (Ksouri et al., 2007) *Mesembryanthemum edule* (Falleh et al., 2009) and *Limoniastrum monopetalum* (Ksouri et al., 2008). Indeed, the phenolic compounds in acetonic, ethanolic and methanolic extracts of *L. densiflorum* proved their efficiency as antioxidants when compared to BHT. Therefore, these data suggested that natural antioxidants could replace synthetic antioxidant in food products.

Natural products may constitute an appropriate source of anti-infective agents. For example, flavonoids showed antimicrobial activity, and quercetin and other related phenolic compounds act essentially through enzyme inhibition of DNA gyrase (Cushnie and Lamb, 2005). Our results showed that *L. densiflorum* extracts are more active against Gram-positive bacteria than Gram-negative bacteria. This is in agreement with previous study (Kabuki et al., 2000) which reported that the antimicrobial spectrums of the catechins. The aflavins, and mango seed kernel ethanol extract were more effective against gram-positive bacteria than Gram-negative bacteria. Besides, Rios and Recio (2005) showed that phenolics are the predominant active chemicals in medicinal plants, with Gram positive bacteria being the most sensible germs. This result could be explained by the structures of cell envelope, including cytoplasmic membrane and cell wall component are different between Gram-positive and Gram-negative bacteria. Gram-negative bacteria possess an outer membrane surrounding the cell wall, which restricts diffusion of hydrophobic compounds through its lipo-polysaccharide covering. Without outer membrane, the cell wall of Gram-positive ones can be permeated more easily and extracts can disturb the cytoplasmic membrane, disrupt the proton motive force (PMF), electron flow, active transport and coagulation of cell contents (Burt, 2004). Therefore, the structural difference of bacteria plays an important role in their susceptibility. In addition, antimicrobial activity is particularly important with the concentrations of 1000 µg/ml than 500 µg/ml. These results suggest that the antibacterial capacity need more concentration and even purification of phenolic compounds.

HPLC analysis showed that chlorogenic acid, epigallochatechin and protocathechuic acid appearing in *L. densiflorum* harvested in August (flowering stage) are absent at the winter period (vegetative stage). In addition, rutin hydrate is the major compound mainly in the flowering stage. These data established that the antioxidant and antimicrobial activities of *L. densiflorum* shoot could be attributed to their polyphenol compounds. In fact, rutin displayed various biological activities that are beneficial to human health such as antioxidant anti-inflammatory capacities and protective effect against hepatotoxicity (Koda et al., 2008). Studies showed that rutin give high positive correlation with DPPH, superoxide anion scavenging, lipid peroxydation assay and reducing power. Moreover, protocheuic and chlorogenic acid have a potent ability as iron chelating agents. Besides, these phenolic acids were reported for their antimicrobial activity against bacteria and yeasts (Han et al., 2009, Tsutomu et al., 2008; Zhao et al., 2010). In this way, *L. densiflorum* superiority observed in summer period at the level of antioxidant and antimicrobial activities may be explained by the content and nature of individual phenolic compounds. Previous studies indicated that benzoates compounds were a potent antioxidant agent, quenching radicals, singlet oxygen and hydrogen peroxide (Bourgou et al., 2008). For that, natural antioxidants such as polyphenols are often added to foods to stabilize them and prevent off-flavor development and have considerable interest for their potential role as functional foods or nutraceuticals (Espin et al., 2007). For instance, phenolic acids such as gallic and vanillic acids account for almost one-third of dietary phenols and are associated with organoleptic, nutritional and antioxidant properties of foods (Rodríguez et al., 2008). In fact, phenolics may contribute to the bitterness, astringency, color, flavor, odor and oxidative stability of food (Naczk and Shahidi, 2006). The mechanism by which antioxidants protect food from oxidation is by scavenging of free radicals via donation of an electron or a hydrogen atom, or by deactivation of metal ions and singlet oxygen (Soong and Barlow, 2006).

Abbreviations: BHT, Butylated hydroxytoluene; **DPPH,** 2,2-diphenyl-1-picrylhydrazyl; **HPLC,** high-performance liquid chromatography; **DNA,** deoxyribonucleic acid; **DMSO,** dimethyl sulfoxide; **$FeCl_3$,** iron(III)chloride anhydrous; **H_2SO_4,** Sulfuric acid; **$K_3Fe(CN)_6$,** Kalium-hexacyanoferrat (III); **Na_2CO_3,** sodium carbonate anhydrous; **$NaNO_2$,** sodium nitrite solution; **$AlCl_3.6H_2O$,** aluminum chloride hexahydrate solution; **NaOH,** sodium hydroxide; **TAC,** total antioxidant capacity; **BCBT,** ß-Carotene bleaching test; **AA,** antioxidant activity; **TFA,** trifluoroacetic acid; **ANOVA,** analysis of variance; **UV,** ultraviolet; **PAL,** phenylalanine ammonia-lyase; **PMF,** proton motive force.

Conclusion

This work demonstrated the involvement of extracting solvents and developmental stage on the variation of antioxidant molecules and their activities in the halophyte *L. densiflorum*. It can be stated that some exogenous factors (solvents of extraction) are highly responsible of the polyphenolic level variability. In this context, organic extract (acetonic, methanolic and ethanolic) at the flowering period exhibited the highest antioxidant and antimicrobial capacities. As a whole, we can recommend *L. densiflorum* as a potent source of antioxidant and antimicrobial suggesting it use in nutraceuticals and food industries.

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REFERENCES

Aniya Y, Miyagi C, Nakandakari A, Kamiya S, Imaizumi N, Ichiba T (2002). Free radical scavenging action of the medicinal herb *Limonium wrightii* from the Okinawa islands. Phytomed., 9: 239-244.

Auberval N, Dal-Ros S, Schini-Kerth V, Pinget M, Sigrist S (2010). Effet antioxydant des extraits de polyphénols de vin rouge et de thé vert sur des cellules RINm5F après induction d’un stress oxidant. Diab.Metab., 36: A79.

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, Bangla. J. Bot., 36: 39-46.

Balasundram N, Sundram K, Sammar S (2006). Phenolic compounds in plants and agri-industrial by-products, Antioxidant activity, occurrence, and potential uses. Food. Chem., 1: 191-203.

Bourgou S, Ksouri R, Bellila A, Skandrani I, Falleh H, Marzouk B (2008). Phenolic composition and biological activities of Tunisian *Nigella sativa* L. shoots and roots. C. R. Biol., 331: 48–55.

Burt S (2004). Essential oils: their antibacterial properties and potential applications in foods-a review. Int. J. Food Microbiol., 94: 223-253.

Bushra S, Farooq A, Ashraf M (2009). Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. Molecules, 14: 2167-2180.

Cox S, Mann C, Markham J, Bell H, Gustafson J, Warmington J, Wyllie K (2000). The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil). J. Applied Microbiol., 88: 170-175.

Cushnie T, Lamb A (2005). Antimicrobial activity of flavonoids. Int. J. Antimicrob. Agents, 26(5): 343-356.

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

Espin JC, Garcia-Conesa MT, Tomas-Barberan FA (2007). Nutraceuricals facts and fiction. Phytochem., 68: 2986-3008.

Falleh H, Ksouri R, Chaieb K, Karray-Bouraoui N, Trabelsi N, Boulaaba M, Abdelly C (2008). Phenolic composition of *Cynara cardunculus* L. organs, and their biological activities. C. R. Biol.,. 331: 372-379.

Freney J, Renaud F, Hansen W, Bollet C (2002). Précis de bactériologie clinique, ESKA, Paris, p. 565.

Goli A, Barzegar M, Sahari MA (2004). Antioxidant activity and total phenolic compounds of pistachio (*Pistachia vera)* hull extracts. Food Chem., 92: 521-525.

Han Y (2009). Rutin has therapeutic effect on septic arthritis caused by *Candida albicans*. Intern J. Immuno-pharmacol., 9: 207-211.

Hanato T, Kagawa H, Yasuhara T, Okuda T (1988). Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects. Chem. Pharmacol. Bull., 36: 2090-2097.

Heim K, Taigliaferro A, Bobilya D (2002). Flavonoid antioxidants: Chemistry, metabolism and structure-activity relationships. J. Nutr. Biochem., 13: 572-584.

Huang D, Ou B, Prior L (2005). The chemistry behind antioxidant capacity assays. J. Agri. Food. Chem., 51: 1841-1856.

Hunt G, Baker E (1980). Phenolic constituents of tomato fruit cuticles. Phytochem., 19: 1415-1419.

Martin-Tanguy J (1997). Conjugated polyamines and reproductive development: Biochemical, molecular and physiological approaches. Physiol. Plant, 100: 675-688.

Jaleel A, Ksouri R, Gopi R, Manivannan P, Jallali I, Chang-Xing Z, Hong-Bo R, Panneerselvam S (2009). Antioxidant defense responses: Physiological plasticity in higher plants under abiotic constraints. Acta Physiol. Planta., 31(3): 427-436.

Kabuki T, Nakajima H, Arai M, Ueda S, Kuwabara Y, Dosako S (2000). Characterization of novel antimicrobial compounds from mango (*Mangifera indica* L.) kernel seeds. Food Chem., 71: 61-66.

Khokhar S, Magnusdottır SGM (2002). Total phenol, catechin, and caffeine contents of teas commonly consumed in the United Kingdom. J. Agric. Food Chem., 50: 565-570.

Koda T, Kuroda Y, Imai H (2008). Protective effect of rutin against spatial memory impairment induced by trimethyltin in rats. Nutr. Res., 28: 629-634.

Koleva I, Teris A, Jozef P, Linssen A, Lyuba N (2002). Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochem. Anal., 13: 8-17.

Ksouri R, Falleh H, Megdiche W, Trabelsi N, Mhamdi B, Chaieb K, Bakrouf A, Magné C, Abdelly C (2010). Antioxidant and antimicrobial activities of the edible medicinal halophyte *Tamarix gallica* and related polyphenolic constituents. Food Chem. Toxico., 47: 2083-2091.

Ksouri R, Megdiche W, Debez A, Falleh H, Grignon C, Abdelly C (2007). Salinity effects on polyphenol content and antioxidant activities in leaves of the halophyte *Cakile maritima*. Plant Physiol. Biochem., 45: 244-249.

Ksouri R, Megdiche W, Falleh H, Trabelsi N, Boulaaba M, Smaoui A, Abdelly C (2008). Influence of biological, environmental and technical factors on phenolic content and antioxidant activities of Tunisian halophytes. C. R. Biol., 331: 865-873.

Lisiewska Z, Kmiecik W, Korus A (2006). Content of vitamin C, carotenoids, chlorophylls and polyphenols in green parts of dill (*Anethum graveolens* L.) depending on plant height. J. Food. Comp. Analy. 19: 134-140.

Males Z, Zuntar I, Nigovic B, Plazibat M, Vundac V (2003). Quantitative analysis of the polyphenols of the aerial parts of rock samphire (*Crithmum maritimum L.*). Acta Pharmacol., 53: 139-144.

Migliore L, Coppedèb F (2009). Environmental-induced oxidative stress in neurodegenerative disorders and aging. Mut. Res., 674: 73-84.

Moore J, Liu J, Zhou K, Liangli Y (2006). Effects of genotype and environment on the antioxidant properties of hard wheat bran. J. Agric Food Chem., 54: 5313-5322.

Naczk M, Shahidi F (2006). Phenolics in cereals, fruits and vegetables: occurrence, extraction and analysis. J. Pharma Biomed. Analy., 41: 1523-1542.

Oyaizu M (1986). Studies on products of the browning reaction: antioxidative activities of browning reaction. Jpn. J. Nutr., 44(66): 307-315.

Pinelo M, Rubilar M, Sineiro J, Nunez MJ (2004). Extraction of antioxidant phenolics from almond hulls (*Prunus amygdalus*) and pine sawdust (*Pinus pinaster*). Food Chem., 85: 267-273.

Prieto P, Pineda M, Aguilar M (1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. Anal. Biochem., 269: 337-341.

Ramarathnam N, Ochi H, Takeuchi M (1997). Antioxidant defense system in vegetable extracts, in: F. Shahidi (Ed.). Natural antioxidant chemistry, health effects and applications, 1: 76-87.
Ratnam D, Ankola D, Bhardwaj V, Sahana D, Ravi Kumar M (2006). Role of antioxidants in prophylaxis and therapy: A pharmaceutical perspective. J. Cont. Rel., 113: 189-207.
Rene DT, Yolanda V, Zeno A (2001). Comparison of the antioxidant content of fruits, vegetables and tea measured as vitamin C equivalents. Toxicol., 166: 63-69.
Rodríguez H, Landete JM, De las Rivas B, Munõz R (2008). Metabolism of food phenolic acids by *Lactobacillus plantarum* CECT 748T. Food. Chem., 107: 1393-1398.
Rios JL, Recio M C (2005). Medicinal plants and antimicrobial activity. J. Ethnopharmacol., 100: 80-84.
Singleton V, Rosi J (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Oenol. Vitic., 16: 144-158.
Smith H (1973). Regulatory mechanisms in the photocontrol of flavonoid biosynthesis. In: Milborrow, B.V. (Ed.), Biosynthesis and Its Control in Plants. Academic Press, New York, pp. 303-320.
Sobhy M, Abdalla M, Ammar S (2009). Total phenolic contents and antioxidant activity of corn tassel extracts. Food Chem., 112: 595-598.
Soong Y, Barlow P (2006). Quantification of gallic acid and ellagic acid from longan (*Dimocarpus longan Lour*.) seed and mango (*Mangifera indica* L.) kernel and their effects on antioxidant activity. Food Chem., 97: 524-530.
Sun B, Richardo-da-Silvia J, Spranger I (1998). Critical factors of vanillin assay for catechins and proanthocyanidins. J. Agric. Food. Chem., 46: 4267-4274.
Sun T, Ho C (2005). Antioxidant activities of buckwheat extracts. Food Chem., 90: 743-749.
Trabelsi N, Megdiche W, Ksouri R, Falleh H, Oueslati S, Soumaya B, Hajlaoui H, Abdelly C (2010). Solvent effects on phenolic contents and biological activities of the halophyte *Limoniastrum monopetalum* leaves. LWT, pp. 1-8.
Verma V, Kasera K (2007). Variations in secondary metabolites in some arid zone medicinal plants in relation to season and plant growth, Indian. J. Plant. Physio., 12: 203-206.
Zhao M, Wang H, Yang B, Tao H (2010). Identification of cyclodextrin inclusion complex of chlorogenic acid and its antimicrobial activity. J. Food Chem., 120: 1138-1142.