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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Determination of antioxidant capacity, phenolic acid composition and antiproliferative effect associated with phenylalanine ammonia lyase (PAL) activity in some plants naturally growing under salt stress

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Abstract

Being a considerable abiotic stress factor particularly for arid and semi-arid regions, salt stress may significantly limit the plant growth and yield. Plant's response to salt stress involves secondary metabolites and especially phenylpropanoids that significantly contribute to the antioxidant activity of plant tissues. In addition to their important role in the control of cancer, phenylpropanoid compounds act as quenchers of singlet oxygen formation, free radical scavengers and reducing agents. One of the important gateway enzymes in the secondary metabolic pathway leading to the synthesis of phenylpropanoids is phenylalanine ammonia lyase (PAL). The aim of this study is to determine the phenolic acid composition, antioxidant capacity and antiproliferative effect associated with PAL activity in some plants that grow naturally under salt stress. The PAL activities of *Salsola nitraria, Salvia halophil*a and *Cyathobasis fruticulosa* were evaluated. The antioxidant content of the extracts was studied and they were evaluated for their antioxidant activity. MTT assay was used to determine the antiproliferative effects of the extracts on HT-29 cells. Also, phenolic acids in extracts, namely *p*-coumaric acid, vanillic acid, gallic acid, caffeic acid, chlorogenic acid and syringic acid were screened using LC-MS/MS. Considering all results, *C. fruticulosa* with its highest PAL activity (62.85 μmol.min^{-1}.mg^{-1} protein) has become prominent among the three plants. *C. fruticulosa* extract exhibited the highest antioxidant content with total phenolic content (120.36 mg/g) as the major antioxidant component. It was also found to be the plant extract richest in *p*-coumaric acid, vanillic acid, gallic acid, caffeic acid, chlorogenic acid and syringic acid as phenolic acids. Besides its marked antiproliferative activity against HT-29 cells, *C. fruticulosa* extract had the highest antioxidant activity compared with other extracts. In conclusion, the antioxidant and anticancer properties of plants naturally growing under salt stress may partly arise from their high PAL activities. Therefore, compounds obtained through plants exhibiting high levels of PAL activities could be used in the development of new pharmaceuticals as an antioxidant and anticancer agent.

Keywords Antioxidant · Antiproliferative · Phenylalanine ammonia lyase (PAL) · Phenolic acid · Salt stress

Introduction

Being a considerable abiotic stress factor particularly for arid and semi-arid regions, salt stress may significantly limit the plant growth and yield. Salt stress may result in the closure of stomata, thus reducing the availability of carbon dioxide (CO_2) in the leaves and inhibiting carbon fixation, exposing chloroplasts to excessive excitation energy, which in turn could increase the generation of reactive oxygen species (ROS) and induce oxidative stres (Gao et al. 2008).

ROS overproduction in plants may ultimately cause membrane damage and multiple cellular damages resulting in alterations in transcription, translation, protein activity, metabolic changes and thus in programmed cell death. As a safeguard against this ROS burst, plants have evolved adaptive antioxidative strategies to reduce oxidative damage due to salt stress (Gholizadeh and Kohnehrouz 2010). Plants have developed antioxidant enzymes like glutathione reductase (GR), catalase (CAT), peroxidase (POD),

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superoxide dismutase (SOD), ascorbate peroxidase (APX) and non-enzymatic scavengers, such as glutathione, flavonoids, tocopherols, phenolics, carotenoids, ascorbic acid, lycopenes and alkaloids among others (Caverzan et al. 2012).

An important pathway of the plant secondary metabolism is the phenylpropanoid pathway, which yields a range of phenolic compounds having structural and defence-related functions. These phenolic compounds include lignins, phenolic acids (hydroxycinnamic acid (chlorogenic acid, caffeic acid and *p*-coumaric acid) and hydroxybenzoic acid (gallic acid, vanillic acid and syringic acid)), flavonoids and stilbenes (Vogt 2010). The non-oxidative deamination of phenylalanine (Phe) to trans-cinnamic acid (TCA) is catalysed by phenylalanine ammonia lyase (PAL, EC 4.3.1.5) (Hemmati 2015). Induction of this enzyme by various biotic (infection by viruses, bacteria, fungi, etc.) and abiotic (UV-B light, high and low temperatures, wounding and salt, etc.) stresses resulted such phenolic compounds to accumulate as flavonoids and phenolic acids (Tovar et al. 2002).

The number of reports about the therapeutic advantages of consumption of phenolics derived from the phenylpropanoid pathway increased substantially (Tohge and Fernie 2017). In addition to their important role in the control of cancer, phenylpropanoid compounds act as quenchers of singlet oxygen formation, free radical scavengers and reducing agents (Ghasemzadeh and Ghasemzadeh, 2011). As revealed by studies conducted so far, phenolic compounds can have overlapping and complementary mechanisms of action, such as gene expression regulation in cell differentiation, cell proliferation, tumour suppressor genes and oncogenes; antioxidant activity and scavenging-free radicals; modulation of enzyme activities in oxidation, detoxification and reduction; immune system stimulation; induction of cell-cycle arrest and apoptosis; hormone metabolism regulation (Liu, 2004).

Cyathobasis fruticulosa (BUNGE) AELLEN is a monotypic single species/single genus plant belonging to the genus *Cyathobasis*. It is endemic to saline soils of Central Anatolia in Turkey. It is a member of the tribe Salsoleae of the family Chenopodiaceae that comprises a large variety of plants containing alkaloids (Şirin et al. 2016).

Salsola belongs to the family Chenopodiaceae and is dispersed in the arid desert. Worldwide there are about 130 species (Ning et al. 2015), 17 of which are in Turkey. *Salsola nitraria* PALL is not endemic to Turkish flora.

Widespread throughout the world in the Lamiaceae family, *Salvia* is the largest genus consisting of about 900 species. This genus is represented in Turkish flora by 88 species and 93 taxa, 45 of which are endemic. *Salvia halophila* HEDGE is endemic to Turkish flora (Albayrak et al. 2008).

The main objectives of this study were (i) to determine the PAL activity of *C. fruticulosa*, *S. nitraria* and *S. halophila* naturally growing under salt stress, (ii) to investigate the antioxidant content of the extracts by determination of phenolic, flavonoid, ascorbic acid, β-carotene, lycopene and total alkaloid contents, (iii) to characterise the phenolic acid composition of the extracts by LC-MS/MS, (iv) to evaluate the antioxidant activity of the extracts by metal chelating ability on ferrous ions, plasma lipid peroxidation inhibitory and scavenging ability on DPPH radicals, (v) to investigate the antiproliferative effect of the extracts on HT-29 cells by MTT assay, (vi) it is first time to determine the relationship between PAL activity, antioxidant capacity, phenolic acid composition, antiproliferative effect of plants naturally growing under salt stress.

Materials and methods

Plant materials

Cyathobasis fruticulosa, *Salsola nitraria* and *Salvia halophila* naturally growing in field rich with saline salts in Beypazarı, Ankara, Turkey and were collected during the flowering stage in August 2011. The identification of plant materials were confirmed by plant taxonomist, Prof. Dr. Zeki AYTAÇ, in the Department of Biology, Gazi University, Ankara, Turkey. A voucher specimens were deposited at the Herbarium of Gazi University, Ankara, Turkey (voucher ID: ZA-10439, 36, 43).

Determination of phenylalanine ammonia lyase (PAL) activity

The leaves were weighed, frozen in liquid nitrogen and ground in a mortar. The final pulverized powder was extracted in 50 mM Tris-hydrochloric acid (HCl) buffer (pH 8.8) containing 10 mM 2-ß-mercaptoethanol, 1 mM ethylenediaminetetraacetic acid (EDTA) and 2.5% polyvinylpyrrolidone-40 (PVP-40). The mixture was centrifuged at 21180*xg* for 20 min, and the clear supernatant was desalted in aliquots using an Amicon 50 K column (Millipore) and assayed for PAL activity. The aliquots were stored at −20 °C until use (Goldson et al. 2008). Protein concentrations were determined by a dye-binding method using bovine serum albumin (BSA) as the protein standard (Bradford 1976). PAL activity was assayed by measuring the trans-cinnamic acid (TCA) formation at 290 nm and calculated using a standard curve. The enzyme reaction mixture contained 100 mM Tris–HCl, 40 mM L-phenylalanine (Phe) and an aliquot of the enzyme in a total volume of 1.0 mL at pH 8.8. The reaction was carried out at 37 °C for 30 min and terminated by the addition of 50 µL of 4 M HCl. PAL activity was expressed in μmol.min^{-1}.mg^{-1} (Goldson et al. 2008).

Preparation of the extracts

Thirty grams of the dried and powdered plant materials were extracted with methanol (HPLC grade) by using Soxhlet apparatus at 60 °C for 4 h. The extracts were filtered and concentrated under vacuum at 80 °C by using a rotary evaporator (Heidolph, Laborota 4000, Schwabach, Germany), freeze dried and stored at 4 °C until usage with in a maximum period of 1 week.

Determination of antioxidant content

Total phenolic

In tota, 0.1 mL of the extracts (1 mg extract/mL methanol) were mixed with 0.2 mL of 50% Folin–Ciocalteu reagent. The mixture was allowed to react for 3 min, and 1-mL aqueous solution of 2% sodium carbonate (Na_2CO_3) was added. Then, the mixture was vortexed vigorously. At the end of incubation for 45 min at room temperature, absorbance of each mixture was measured at 760 nm. The results were expressed as mg of gallic acid/g of the extracts (Yaltirak et al. 2009, Singleton and Rossi, 1965).

Total flavonoid

In total, 2 mL of the extracts (0.3 mg extract/mL methanol) were mixed with 0.1 mL of 10% aluminium chloride hexahydrate ($AlCl_3{\cdot}6H_2O$), 0.1 mL of 1 M potassium acetate (KCH_3COO) and 2.8 mL of deionized water. After the 40 min incubation at the room temperature, the absorbance of the reaction mixture was determined at 415 nm. The results were expressed as mg of rutin/g of the extracts (Stanojević et al. 2009).

Ascorbic acid

The extracts (100 mg) were extracted with 10 mL of 1% metaphosphoric acid for 45 min at the room temperature and filtered. In total, 1 mL of the filtrates were mixed with 9 mL of 2,6-dichlorophenolindophenol (DCPIP, DCIP or DPIP), and the absorbance was measured at 515 nm against a blank. Content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid. The results were expressed as mg of ascorbic acid/g of the extracts (Klein and Perry, 1982).

β-carotene and lycopene

The extracts (100 mg) were vigorously shaken with 10 mL of acetone–hexane mixture (4:6) and filtered. The absorbance of the filtrates were measured at 453, 505 and 663 nm. The results were expressed as mg of carotenoid/g of the extracts (Nagata and Yamashita, 1992).

Total alkaloid

The extracts (1 mg) were dissolved in 2 M HCl and then filtered. Then 5 mL of bromocresol green solution and 5 mL of phosphate buffer were added to this solution. The mixture was shaken and the complex formed was extracted with chloroform by vigorous shaking. The absorbance of the complex in chloroform was measured at 470 nm. The results were expressed as mg of boldine/g of the extracts (Ajanal et al. 2012; Fazel et al. 2010).

Determination of phenolic acid composition

Analysis was performed by METU Central Laboratory, Molecular Biology-Biotechnology Research and Development Center, Mass Spectroscopy Laboratory, Ankara, Turkey, with AGILENT 6460 Triple Quadrupole System (ESI + Agilent Jet Stream) coupled with AGILENT 1200 Series HPLC. The quantitative data of phenolic acids in the extracts were calculated using their respective concentration vs. peak area calibration curves.

Liquid chromatography	
Equipment	AGILENT 1200 HPLC Series
Column	Zorbax SB-C18 (2.1 × 50 mmx1.8 μ)
Mobile phase	Solvent A: 0.05% formic acid (Merck) + 5 mM ammonium formate (Merck) Solvent B: Methanol (MS grade, Merck)
Column temperature	35 °C
Flow	0.5 mL/min
Run time	13 min
Flow mode	Gradient
Enjection volume	5 μL
Standart curve range	0.019; 0.039; 0.078; 0.156; 0.31; 0.625; 1.25 ppm
Mass spectroscopy	
Equipment	AGILENT 6460 LCMSMS
Ionosation source	ESI + Agilent Jet Stream
Pump	AGILENT BinPump-SL (G1312B9)
Autosampler	AGILENT h-ALS-SL + (G1367D)
Column compartment	AGILENT G1316B 1200 Series Thermost. Col. Compart SL

Table (continued)

Liquid chromatography	
Microdagasser	AGILENT G1379B 1200 Series Micro Degasser
Software	AGILENT G3793AA Mass Hunter Optimizer Software
Nitrogen generator	Peak Scientific
Scan mode	MRM
Gas temperature	300 °C
Gas flow	9 mL/min
Nebulizer	45 psi
Sheath gas Temperature	300 °C
Sheath gas flow	9 mL/min
Capillary	4000 V
Nozzle voltage	500 V

Determination of antioxidant activity

Metal chelating ability on ferrous ions

In total, 1 mL of the extracts (5 mg extract/mL methanol) were mixed with 3.7 mL of methanol and 0.1 mL of 2 mmol/L ferrous chloride ($FeCl_2$). The reaction was initiated by the addition of 0.2 mL of 5 mmol/L ferrozine. Then, the mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was then measured at 562 nm. IC_{50} values denote the concentration of sample, which is required to chelate 50% of the ferrozine–Fe^{2+} complex formation and calculated both for the extracts and the synthetic chelator EDTA (Danis et al. 1994, Decker and Welch, 1990).

Plasma lipid peroxidation inhibitory assay

In total, 0.4 mL of plasma (Bloodcenter, Gazi University Hospital, Ankara, Turkey), 0.1 mL of 0.5 mM iron (II) sulphate solution ($FeSO_4$), 0.1 mL of 0.5 mM hydrogen peroxide (H_2O_2) and 0.2 mL of the extracts (2 mg extract/mL methanol) were mixed and incubated at 37 °C. After 12 h of incubation, the reaction solution was mixed with 375 µL of 4% tricarboxylic acid (TCA) and 75 µL of 0.5 mM butylhydroxytoluene (BHT) and held in an ice bath for 5 min. Supernatant was obtained by centrifugation at 5000*xg* for 15 min. In total, 0.2 mL of 0.6% thiobarbituric acid (TBA) was then added. The mixture was incubated at 95 °C for 30 min and allowed to cool. Supernatant was obtained by centrifugation at 5000*xg* for 15 min. The absorbance was then measured at 532 nm. Lipid peroxidation was expressed in nmoles of TBARS (TBA reacting substances) formed per mL of plasma sample (Rodriguez-Martinez and Ruiz-Torres, 1992).

Scavenging ability on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals

DPPH percent radical scavenging activities of the extracts and the synthetic antioxidants α-tocopherol, butylhydroxyanisole (BHA) and BHT as positive controls were determined by a spectrophotometric method based on the reduction of the methanol solution of DPPH. In total, 1 mL of various concentrations of the extract in methanol was added to 1 mL of a 0.004% methanol solution of DPPH. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank. Inhibition of a free radical, DPPH, in percent was calculated. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH-free radicals and calculated both for the extracts and synthetic antioxidants (Blois 1958).

Determination of antiproliferative effect

Human colon carsinoma HT-29 cells were obtained from Sap Institute (Ankara, Turkey). The cells were grown at 37 °C in humidified 5% CO_2, 95% air mixture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin, 1% L-glutamine and 40% MCDB-201. The medium was replaced three times in a week until the cells were near confluency.

The extracts were evaluated on HT-29 in order to examine their antiproliferative effect on cancer cells. HT-29 cells were seeded in a 96-well plates (10^4 cells/well) and incubated at 37 °C with 5% carbon dioxide (CO_2). After 24, 48 and 72 h incubation, the cells were treated with the extracts (100, 250, 500 and 1000 µg/mL). Following the removal of the extracts from the wells, the cells were washed with PBS and fresh medium were added to the wells. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

The MTT assay was used to assess the antiproliferative effect of the extracts. It is a colorimetric assay relying on the conversion of yellow tetrazolium bromide (MTT) to the purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (Mosmann 1983). In total, 20 µL of 0.5% MTT in phosphate buffered saline (PBS) was added to each microwell. Following 4 h incubation, the medium was removed and 200 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Absorbance was measured in an ELISA microplate reader (ELx800, BioTek, USA) at 570 nm. Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells that served as a control.

Data analysis

All experiments were conducted in triplicate, and mean values presented. The results were expressed as the means ± standard deviations (SD). Statistical analyses were performed using SPSS version 11.0 (SPSS, Chicago, IL, USA). One-way ANOVA analysis was used to determine the statistical significance of differences between the values.

Results and discussion

PAL activity of the plants

PAL plays a crucial role at the interface between plant primary and secondary metabolism by catalysing the deamination of L-phenylalanine (L-phe) to form trans-cinnamic acid (TCA), the first step in the general phenylpropanoid way (Reichert et al. 2009). Thousands of secondary metabolic products in plants, such as phenolic acids, flavonoids, anthocyanins, lignins and phytoalexins, are derived from phenylpropanoid (Hsieh et al. 2010).

In some studies, PAL activity was induced by several biotic and abiotic stresses, including salt stress, chilling, wounding, heavy metal and infection by viruses, bacteria or fungi. The defence response of plant cells involves PAL. Therefore, in different plant species, PAL has been generally considered as an environmental stress marker (MacDonald and D'Cunha 2007).

The present study described PALs from *S. nitraria* and *S. halophila* first time. The PAL activity of *C. fruticulosa* was established in our previous study (Şirin et al. 2016). *C. fruticulosa* (62.85 μmol.min^{-1}.mg^{-1} protein) exhibited the highest PAL activity among the others (Table 1). Considering PALs obtained from plants in previous literature (0 and 100 μmol.min^{-1}.mg^{-1} protein), these figures are notable (Goldson et al. 2008).

An increase in PAL activity may occur as a response to the cellular damage stimulated by higher sodium chloride (NaCl) concentrations. Apparently, in *C. fruticulosa*, enhanced PAL activity may be associated with the implication of this enzyme in the plant response to salt stress in soil. Controlling PAL activity seems to be an important element in the regulation of this pathway. A high PAL activity is associated with the accumulation of phenylpropanoid compounds in tissues of several plant species. Thus, the antioxidant and anticancer effects of organisms with a high PAL activity are also expected to be higher.

Table 1 PAL activity of the plants[a]

	PAL activiy (μmol.min^{-1}.mg^{-1} protein)
CF	62.85 ± 0.11^{a}
SN	53.56 ± 0.09^{b}
SH	45.74 ± 0.07^{c}

CF Cyathobasis fruticulosa, *SN* Salsola nitraria, *SH* Salsola halophila

[a]Values represent averages ± SD for triplicate experiments. Values in the same column with different superscript upper case letters are significantly ($p < 0.05$) different.

Antioxidant content of the extracts

Total phenolic, total flavonoid, ascorbic acid, β-carotene, lycopene and total alkaloid contents in the extracts were determined. Futhermore, plants' antioxidant contents, especially of *C. fruticulosa* and *S. nitraria* were described for the first time.

Being biologically active components, phenolics are the main compounds that are able to donate hydrogen to free radicals, thus breaking the lipid oxidation chain reaction at the first initiation step. This high radical scavenging potential of phenolics may be attributed to their phenolic hydroxyl groups (Sawa et al. 1999).

Total phenolic content was shown to be solvent-dependent and was the major antioxidant component found in the extracts. Various studies confirmed methanol as a better extraction solvent for phenolics from plant materials as compared with less polar solvents, such as acetone (Mohsen and Ammar 2009; Stankovic et al. 2012). The highest total phenolic content (120.36 mg/g) was determined in *C. fruticulosa* (Table 2). Its total phenolic content in the present study was higher than the values reported by Shehab and Abu-Gharbieh 2014 and Küçükboyacı et al. 2016. According to Shehab and Abu-Gharbieh (2014), for different extracts, the total phenolic content of *Salsola stenoptera* was about 0.64–4.00 mg/g. Küçükboyacı et al. (2016) reported that the total phenolic content of *Salsola grandis* was in the range of 18.60–56.10 mg/g for different extracts. *C. fruticulosa*, *S. stenoptera* and *S. grandis* are members of the same family (Chenopodiaceae) and different localities or extracts belonging to the same family may exhibit varying antioxidant contents.

The highest total flavonoid content (119.56 mg/g) was determined in *C. fruticulosa* (Table 2). Its total flavonoid content in the present study was higher than the values reported by Asan-Ozusaglam et al. (2015) and Shehab and Abu-Gharbieh (2014). Asan-Ozusaglam et al. (2015)

Table 2 Antioxidant content of the extracts[a]

	Total phenolic (mg/g)	Total flavonoid (mg/g)	Ascorbic acid (mg/g)	*β*-carotene (mg/g)	Lycopene (mg/g)	Total alkaloid (mg/g)
CF	120.36 ± 0.25[a]	119.56 ± 0.23[a]	11.85 ± 0.02[a]	0.70 ± 0.00[a]	0.4 ± 0.00[a]	0.40 ± 0.00[a]
SN	105.58 ± 0.20[b]	42.35 ± 0.08[b]	5.87 ± 0.01[b]	0.70 ± 0.00[a]	0.3 ± 0.00[a]	0.19 ± 0.00[b]
SH	32.67 ± 0.14[c]	16.58 ± 0.03[c]	4.53 ± 0.01[b]	0.30 ± 0.00[b]	0.03 ± 0.00[b]	0.07 ± 0.00[c]

CF Cyathobasis fruticulosa, *SN* Salsola nitraria, *SH* Salsola halophila

[a]Values represent averages ± SD for triplicate experiments. Values in the same column with different superscript lower case letters are significantly ($p < 0.05$) different

Table 3 Phenolic acid composition of the extracts[a]

	CF	SN	SH
Vanillic acid (μg/g)	0.4364 ± 0.0030[a]	0.3419 ± 0.0119[a]	0.2225 ± 0.0019[a]
Caffeic acid (μg/g)	0.3205 ± 0.0012[b]	0.1154 ± 0.0013[b]	0.0201 ± 0.0000[b]
Syringic acid (μg/g)	0.2637 ± 0.0043[c]	0.2058 ± 0.0046[c]	0.0895 ± 0.0001[c]
p-coumaric acid (μg/g)	0.1863 ± 0.0003[d]	0.0993 ± 0.0011[b]	0.0355 ± 0.0005[b]
Chlorogenic acid (μg/g)	0.1439 ± 0.0014[e]	0.0711 ± 0.0001[b]	0.0324 ± 0.0001[b]
Gallic acid (μg/g)	≤0.019[f]	≤0.019[d]	≤0.019[d]

CF Cyathobasis fruticulosa, *SN* Salsola nitraria, *SH* Salsola halophila

[a]Values represent averages ± SD for triplicate experiments. Values in the same column with different superscript lower case letters are significantly ($p < 0.05$) different

reported that the total flavonoid content of *S. stenoptera* was in the range of 5.76–61.51 μg/mg for different extracts. According to Shehab and Abu-Gharbieh (2014), for different extracts, the total flavonoid content of *Salsola imbricata* was about 0.11–0.571 g/100 g. *C. fruticulosa* and *S. imbricata* are members of same family (Chenopodiaceae). These differences in the results may be attributed to stress conditions, differences in the plant collection site, time and the method used.

While the amount of ascorbic acid found in the extracts was moderate, lycopene and β-carotene were only found in residual amounts. They were found owing to their lipophilic character. Highest β-carotene (0.70 mg/g), lycopene (0.40 mg/g), ascorbic acid (11.85 mg/g) and total alkaloid (0.040 mg/g) contents were determined in *C. fruticulosa* (Table 2).

PAL is an important intermediate where the synthetic pathways of phenolics and flavanoids meet. PAL, catalysing the deamination of Phe to TCA, was shown to be regulated upon stress exposure. In this plant, the overall activity pattern of the PAL enzyme was found to be similar to the total antioxidation pattern. This suggests that PAL could be a crucial component of this salt-induced antioxidative system.

Phenolic acid composition of the extracts

In this study, total phenolic content was the highest antioxidant content. Therefore, the phenolic acid compositions of the plants were determined. In *C. fruticulosa* and *S. nitraria* extracts, three cinnamic acid derivatives (chlorogenic, *p*-coumaric and caffeic acids), two benzoic acid derivatives (vanillic and syringic acids) and one shikimic acid derivative (gallic acid) as phenolic acids were reported first time. *C. fruticulosa* was found to be the richest plant extract in phenolic acids (Table 3).

Vanillic acid is a widely used compound in foods, beverages, drugs and cosmetics and it has been reported to have multiple functions by its antimutagenic and anti-angiogenetic effects. Vanillic acid is a stronger antioxidant regarding the reducing power, lipid peroxidation inhibition and protein oxidation inhibition (Chou et al. 2010). It exhibited metal chelating activity (Acton, 2011) and showed radical scavenging activity (Tai et al. 2012). It can treat critical diseases like cancer among others (Kim et al. 2010).

In the present study, the analysis revealed vanillic acid as the major phenolic component in *C. fruticulosa*. It may constitute an essential ingredient in the antioxidant content and antioxidant activity of *C. fruticulosa*.

Caffeic acid shows anti-inflammatory and anticancer activity (Magnani et al. 2014; Murtaza et al. 2015). Its antioxidant effects cover four aspects: (i) anti-lipid peroxidation; (ii) radical scavenging; and (iii) antioxidation of low density lipoprotein; (iv) chelating ability (Decker et al. 2010). Its therapeutic potential for various pathologies including inflammation and cancer are promising (Murtaza et al. 2015).

Syringic acid has shown antioxidant, antiproliferative, chemoprotective and anticancer activity. It also strongly inhibited low density lipoprotein oxidation, supported the

Table 4 Antioxidant activity of the extracts[a]

	Metal chelating IC_{50} (mg/mL)	DPPH IC_{50} (mg/mL)
CF	2.12 ± 0.00^a	0.053 ± 0.00^a
SN	4.45 ± 0.00^b	0.065 ± 0.00^b
SH	6.23 ± 0.01^c	0.089 ± 0.00^c
Plasma lipid peroxidation TBARS (nmol/mL)		
Control	3.5 ± 0.00	
CF	2.52 ± 0.00^a	
SN	2.70 ± 0.00^b	
SH	2.84 ± 0.00^c	

CF Cyathobasis fruticulosa, *SN* Salsola nitraria, *SH* Salsola halophila

[a]Values represent averages ± SD for triplicate experiments. Values in the same column with different superscript upper case letters are significantly ($p < 0.05$) different

scavenging of free radicals, reduced the production of malondialdehyde (MDA) (Güven et al. 2015) and displayed metal chelating activity (Acton, 2011).

p-coumaric acid exhibits antioxidant, anti-inflammatory and anticancer activities (Pei et al. 2016). It has been described as a chain-breaking antioxidant acting through radical scavenging activity related to its electron- or hydrogen-donating capacity and to the ability to delocalize/stabilize the resulting phenoxyl radical within its structure. The free radical scavenging ability of antioxidants can be predicted by standard one-electron potentials (Teixeira et al. 2013). It decreases low density lipoprotein peroxidation (Boz, 2015) and has metal chelating ability (Rivas et al. 2001).

Chlorogenic acid possesses attributed antioxidant (acting as scavengers of reactive oxygen species, inhibiting in vitro lipid peroxidation (Ohnishi et al. 1994), metal chelating ability (Andjelković et al. 2006)), antimutagenic and anti-inflammatory effects (Xiang and Ning, 2008).

The analysis in the present study revealed that caffeic acid, *p*-coumaric acid, chlorogenic acid and syringic acid were the moderate phenolic acid component in *C. fruticulosa*. Their content seems to be relevant to the antioxidant content and antioxidant activity of *C. fruticulosa*.

Gallic acid is a potent antioxidant. It acts by inhibiting in vitro lipid peroxidation (Watson and Preedy 2013) and as a scavenger of reactive oxygen species (Locatelli et al. 2013). Gallic acid possesses a weak chelating and a strong reducing property (Barcelo et al. 2014). In addition to its use in treating critical diseases like cancer, previous reports indicate it as an anticarcinogenic, antimutagenic, anti-angiogenic and anti-inflammatory agent (Choubey et al. 2015).

The analysis revealed gallic acid as the minor phenolic acid component in *C. fruticulosa*. Gallic acid content seems to be not relevant to the antioxidant content and antioxidant activity of *C. fruticulosa*.

An increase in the PAL activity may also increase the accumulation of phenolic acids, which in turn may be related to the antioxidant content and antioxidant activity. Futhermore, an increase in phenolic acids in response to salinity has also been reported in extracts of different tissues of other plants (Colla et al. 2013; Lim et al. 2012; Ksouri et al. 2007). This increase supports the theory that phenolic acids protect the plants against the oxidative stress due to salinity, and secondary metabolites may play a role in the plant's tolerance to salinity.

Antioxidant activity of the extracts

Metal chelating ability, plasma lipid peroxidation inhibitory and scavenging ability in the extracts were determined. Antioxidant activity of the plants, particularly of *C. fruticulosa* and *S. nitraria*, was described for the first time.

Due to its high reactivity, iron is regarded as the most important lipid oxidation pro-oxidant among transition metals. Ferrous iron accelerates lipid oxidation by breaking down hydrogen peroxide and lipid peroxides to reactive free radicals via the Fenton reaction ($Fe^{2+} + H_2O_2\ Fe^{3+} + .OH + OH^-$). While Fe^{3+} ions also produce radicals from peroxides, the rate is 10-fold less than that with Fe^{2+} ions (Miller et al. 1996). The production of these radicals may result in protein modification, lipid peroxidation and DNA damage. With their ability to inactivate metal ions, chelating agents may potentially inhibit the processes dependent on metals (Finefrock et al. 2003). *C. fruticulosa* demonstrated the highest ferrous iron chelating ability (IC50 = 2.12 mg/ml) (Table 4). The synthetic chelating agent EDTA had potent excellent chelating ability at the 5 mg/mL (~95%).

Lipid peroxidation and oxidative stress are indicated by the plasma concentrations of TBARS. Especially the polyunsaturated fatty acids in the cells and blood are quite open to attacks, resulting the generation of lipid peroxides (Halliwell and Gutteridge 2015). During lipid peroxidation, low molecular-weight end products, probably malonaldehyde, are formed by oxidation of polyunsaturated fatty acids and they can be reacted with two molecules of TBA. The generation of MDA was substantially controlled by the extracts where *C. fruticulosa* exhibited the highest inhibitory activity (2.52 nmol/mL) (Table 4).

Their hydrogen donating ability or radical scavenging activity was believed to cause the effect of antioxidants on DPPH radical scavenging. IC_{50}, the antioxidant concentration required to decrease (by 50%) the initial substrate concentration, is a parameter widely used to measure the antiradical efficiency where a lower IC_{50} value indicates a higher free radical scavenging activity. *C. fruticulosa* extract ($IC_{50} = 0.053$ mg/mL) exhibited a higher scavenging ability on DPPH radicals (Table 4). Furthermore, DPPH scavenging abilities of the extracts were lower than that of synthetic antioxidants butylhydroxyanisole ($IC_{50} = 0.00$ mg/mL), butylhydroxytoluene (BHT) ($IC_{50} = 0.02$ mg/mL) and α-tocopherol ($IC_{50} = 0.01$ mg/mL).

Antioxidant activity may protect plants against oxidative damage under environmental stress. In plants exposed to salt stress, an increase in the antioxidant content and phenolic acids concentration along with enhanced antioxidant ability is often observed indicating a potential correlation between these variables. Increased antioxidant activity response to salinity has also been reported in extracts of different tissues of some other plants (Corrêa et al. 2013; Sharma et al. 2013; Farhoudi et al. 2012; Yuan et al. 2010; Sairam et al. 2005).

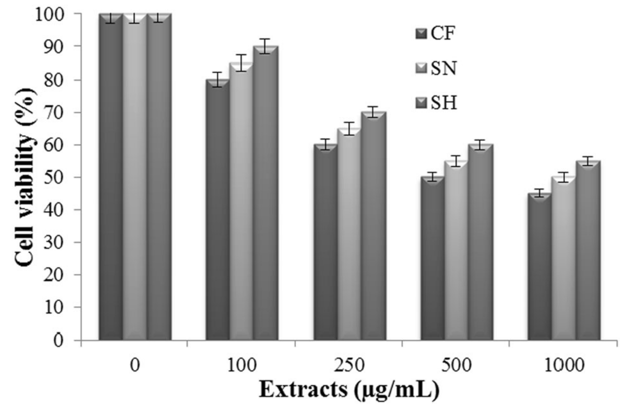

Fig. 1 Antiproliferative effect of the extracts on HT-29 cells for 24 h

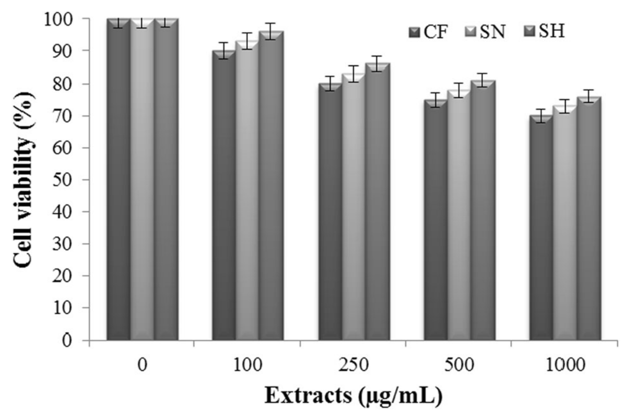

Fig. 2 Antiproliferative effect of the extracts on HT-29 cells for 48 h

Antiproliferative effect of the extracts

In order to examine their antiproliferative effects, *C. fruticulosa*, *S. nitraria* and *S. halophila* extracts (100, 250, 500 and 1000 µg/mL) were evaluated on HT-29 cells. Furthermore, antiproliferative effects of the plants, particularly of *C. fruticulosa* and *S. nitraria* on cancer cells were described for the first time. The antiproliferative effect of the *C. fruticulosa* extract on HT-29 cells was found to be superior to other plant extracts following 24-h (Fig. 1), 48-h (Fig. 2) and 72-h incubations. Of the plant extracts, *C. fruticulosa* extract exhibited the highest antiproliferative effect at all concentrations after 48 h incubation: from 44 to 79% cell death. Since the cell death rate over 72 h of treatment was 64–99%, results were not provided. The data obtained indicate that various species may differ greatly, and the results strengthen the evidence that *C. fruticulosa* might be considered a natural resource of anticancer agent.

In the literature, following a 48 h incubation, crude and fine polysaccharides of *Salicornia herbecea* exerted antiproliferative effects on HT-29 cells at all concentrations (0.5, 1, 2 and 4 mg/mL): from 30 to 80% cell death. In the present study, the antiproliferative effect of *C. fruticulosa* was higher compared to the values reported by Ryu et al. 2009. *S. herbecea* and *C. fruticulosa* are belong to the same family (Chenopodiaceae).

These results suggest a possible direct correlation between antioxidant capacity and antiproliferative effect. A large increase in level for PAL, a key enzyme in the phenylpropanoid pathway leading to the biosynthesis of a wide array of phenylpropanoids. Phenylpropanoid components play important roles in the control of cancer. Phenylpropanoid compounds may exert their chemopreventive activity through various possible mechanisms, such as enhancing the level or fidelity of DNA repair, preventing the carcinogen from binding to DNA, deactivating or detoxifying the carcinogen, inhibiting the formation or activation of the carcinogen, and inhibiting the uptake of the carcinogen. Antioxidant properties further include the scavenging of oxygen radicals and reactive electrophiles as well as the inhibition of arachidonic acid metabolism (Nichenametla et al. 2006). Instead of a single compound, combinations of phenylpropanoid compounds increase the potency and efficacy of this chemopreventive effect by targeting the overlapping and complementary phases in the carcinogenic process (Araújo et al. 2011). It is necessary to conduct further studies with other cell lines and in vivo animal models to discover the molecular mechanism of the extracts on colon cancer.

Conclusions

Cyathobasis fruticulosa, *Salsola nitraria* and *Salvia halophila* naturally growing under salt stress were reported for the first time together for the relationship of their PAL activity, antioxidant content, antioxidant activity, phenolic acid composition and antiproliferative effect. High PAL activity in plants naturally growing under salt stress was associated with high antioxidant and anticancer effects. According to the results of this study, plants naturally growing under salt stress may be suggested as a new potential source of natural antioxidant and novel anticancer agents for the development of pharmaceuticals.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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