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EFFECT OF EXTRACTION SOLVENTS ON POLYPHENOLS AND ANTIOXIDANT ACTIVITY OF MEDICINAL HALOPHYTES

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Abstract

This study was conducted to determine the most effective solvent for extraction of polyphenols and antioxidant activity of medicinally important coastal halophytes (*Thespesia populneoides, Salvadora persica, Ipomoea pes-caprae, Suaeda fruticosa* and *Pluchea lanceolata*) known for high antioxidant potential. Five different solvents (water, 80% methanol, 80% ethanol, acetone and chloroform) were used to quantify polyphenols including total phenolic (TPC), total flavonoid (TFC) and proanthocyanidin contents (PC) and antioxidant capacity using DPPH radical scavenging and Ferric reducing antioxidant power (FRAP) activities. Among solvents of different polarities 80% methanol appeared most effective for polyphenol extraction. *Thespesia populneoides* had the highest polyphenols (TPC, TFC and PC) followed by *Salvadora persica*. Highest antioxidant activity was also found in *T. populneoides* and *S. persica* using the same solvent (80% methanol) which appeared better than synthetic antioxidants (BHA and BHT). The correlation analyses of each solvent showed strong to weak relationships among all studied parameters with maximum values (r and R^2) in methanol followed by ethanol and water. Weaker correlation of acetone and chloroform indicates low capacity of these solvents both for polyphenol extraction and antioxidant activity. Our results reveal that aqueous methanol extracts of coastal halophytes had comparatively higher antioxidant activity than commercial antioxidants which indicate both their prospective efficacy and potential to replace synthetic derivatives from edible and medicinal products.

Key words: Arabian Sea, Coastal plants, DPPH, Economic potential, FRAP, Saline habitats, Salt tolerant plants, Secondary metabolites.

Introduction

Halophytes- plants adapted to grow in saline soils of arid/semi-arid regions could be a potential source of natural antioxidants (Meot-Duros *et al*., 2008) beside other economic uses (Gul *et al*., 2013; Koyro *et al*., 2013; Abideen *et al*., 2011; 2012). Extreme climatic conditions (e.g. salinity, drought, heat, irradiance) could reduce plant growth due to overproduction of reactive oxygen species (ROS) causing oxidative stress (Ksouri *et al*., 2007). Extremophile species like halophytes represent physiological plasticity which provides these plants a competitive advantage over other species in saline habitats (Falleh *et al*., 2011). They are able to withstand and quench toxic free radicals since they have efficient enzymatic and non-enzymatic antioxidant systems (Bose *et al*., 2013). The secondary compounds produced to detoxify ROS could be potentially used as medicines (Buhmann & Papenbrock, 2013; Oueslati *et al*., 2012; Trabelsi *et al*., 2012; Zrig *et al*., 2011). Recent reports suggest that halophytes may serve as potential sources of natural antioxidants (Jallali *et al*., 2014; Wang *et al*., 2013) and focus has been shifted towards the phyto-chemical screening of halophytes for their multiple health benefits (Joseph *et al*., 2013; Ksouri *et al*., 2012a & 2012b; Qasim *et al*., 2010).

Among phytochemicals, phenolic compounds (phenolic acids, flavonoids and proanthocyanidins) plays a significant role to protect plants from oxidative damage either individually or in various combinations (Choueiri *et al*., 2012; Lee *et al*., 2010). These compounds act as free radical scavengers, hydrogen donors as well as reducing agents (Razzaghi-Asl *et al*., 2013) with wide spectrum of biological activities and putative health effects (De-Pascual-Teresa *et al*., 2010). Increased dietary intake of these compounds is known to reduce the risk of coronary heart diseases (Kishimoto *et al*., 2013) and increases life expectancy (Halliwell, 2007) besides antiviral, anticancer and anti-inflammatory effects (Raghu *et al*., 2013). Halophytes are also rich in polyphenols and other bioactive substances which could be used for afore mentioned benefits (Buhmann & Papenbrock, 2013).

To maximize the polyphenol extraction several solvent systems and methods have been used (Buhmann & Papenbrock, 2013; Falleh *et al*., 2013). A good solvent system allows optimal extraction of desired compounds without modifying their chemical nature (Harborne, 1998). It is also reported that an optimum extraction of polyphenols is usually obtained in polar rather than non-polar solvents (Liu *et al*., 2007). Therefore, water and organic solvents (methanol, ethanol, acetone and chloroform) are widely used for extraction of plant materials (Dai & Mumper, 2010). Furthermore, these solvents are used individually or in combination (Boulekbache-Makhlouf *et al*., 2013; Hayouni *et al*., 2007) e.g. water and aqueous mixture of methanol and ethanol are used for higher yield of polyphenols (Rubio-Moraga *et al*., 2013; Stanojevic *et al*., 2009). Wang & Helliwell (2001) suggested aqueous ethanol as a superior solvent for polyphenols than methanol and acetone. Other researchers suggested that acetone is a better solvent for polyphenol extraction (Hayouni *et al*., 2007; Zhou & Yu, 2004) than water and chloroform. Above findings indicate that the yield of polyphenols depends upon type and polarity of

extracting solvents, beside the physical characteristic of plant samples (Buhmann & Papenbrock, 2013). To date, no particular or appropriate solvent is recommended for optimal yield of plant phenolics (Prior *et al*., 2005) particularly in halophytes. Owing to a greater variation in quality of plant extracts and unique chemistry of halophytes, identification of specific solvent is critical to optimize extraction procedure. Therefore, the objective of this study was to evaluate the efficiency of different solvents for polyphenol extraction and subsequent antioxidant activity from halophytes with known medicinal value.

Materials and Methods

Chemicals and reagents: Catechin, DPPH, folin–ciocalteu's phenol reagent, gallic acid, quercetin, sodium phosphate dibasic and vanillin were purchased from Sigma-Aldrich (GmbH, Sternheim, Germany). Iron (III) chloride 6-hydrate and iron (II) sulfate 7-hydrate were obtained from BDH (Poole, UK). Acetone, Butylated hydroxyanysol (BHA), chloroform, ethanol, hydrochloric acid, methanol and 2,4,6-Tri (2-pyridyl)-s-triazine (TPTZ) were obtained from Merck (Darmstadt, Germany).

Sample collection and preparation: In our pervious study we screened 100 medicinal plants (unpublished data) and top five species with highest antioxidant potential were selected for this study. These plants are of high medicinal importance and traditionally used in different areas of Pakistan and other parts of the world (Table 1). Plants were collected from natural populations distributed in coastal areas of Pakistani. Plant samples were dried under shade and leaves were ground using ball mill (Retsch MM-400). Five solvents including water, 80% methanol, 80% ethanol, acetone and chloroform were used for polyphenol extraction and antioxidant activity. One gram of powdered material was extracted with 20 ml of each solvent at 40°C for 3 hours using a shaking water bath (GFL-1092). Samples were then cooled and centrifuged (at 4500 rpm for 15 min) to recover supernatant for further analysis (Abideen *et al*., 2015).

Determination of total phenolic content (TPC): Folin-Ciocalteu colorimetric method was used to determine total phenolic content of plant extracts (Singleton & Rossi, 1965). Each extract was mixed with Folin-Ciocalteu reagent (0.2 N) and after 5 min aqueous sodium carbonate (75 g/L) was added and the mixture was incubated for 90 min at room temperature. Absorbance was measured using a UV/Vis spectrophotometer (Beckman Coulter DU530) at 760 nm. Results were expressed in milligram per g dry weight.

Determination of total flavonoids (TFC): Aluminum chloride colorimetric method was used to quantify total flavonoids (Chang *et al*., 2002). Leaf extracts were mixed with 1.5 ml of alcohol, 0.1 ml of aluminum chloride (10%), 0.1 ml of potassium acetate (1M) and 2.8 ml of de-ionized water. Absorbance of reaction mixture was recorded at 415 nm after incubation of 40 min at room temperature. Calibration curve was prepared using Quercetin and results were calculated as milligram per gram dry weight.

Determination of proanthocyanidin content (PC): Proanthocyanidin content was determined by the method of Sun *et al*. (1998). Briefly, 50 µL of diluted plant extracts were mixed with 3 mL of vanillin–methanol solution (4%) and 1.5 mL HCl (1.0 N). The mixture was left for 15 min and the absorbance was recorded at 500 nm. Catechin was used as standard compound and results were expressed as mg per gram dry weight (mg CE g^{-1} DW).

Free radical-scavenging ability using DPPH radical: Antioxidant activity was determined using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay according to Brand-Williams *et al*. (1995). Sample extract (1 ml) was added in the same proportion of DPPH reagent (100 µM in methanol) and kept in dark for 30 min and absorbance was recorded at 515 nm. The radical scavenging activity was calculated as percent inhibition (I %) of DPPH radical using equation:

$$I\ \% = [(A_{control} - A_{sample})/A_{control}] \times 100$$

A_{sample}: absorbance of sample, $A_{control}$: absorbance of control. Radical scavenging activity is presented as IC_{50} value (defined as the extract concentration in µg ml^{-1} which caused a 50% inhibition).

Ferric reducing antioxidant power (FRAP) assay: Ferric reducing antioxidant power activity was carried out using modified method of Benzie & Strain (1996). The method determines the ability of extract to reduce Iron (Fe^{3+} to Fe^{2+}). In the presence of TPTZ, the Fe^{+2}-TPTZ complex shows blue color which can be measured. FRAP reagent (2 ml) was added to the appropriate concentration of sample extract. After incubation for 5 min at room temperature, the absorbance was recorded at 593 nm against $FeSO_4$ standard and the values were expressed as millimole Fe^{+2} per gram dry weight (mMol Fe^{+2} g^{-1} DW).

Table 1. Description of medicinal halophytes used in present study.

Species (Family)	Common name	Habit	Plant type	Medicinal use	References
Ipomoea pes-caprae (Convolvulaceae)	Beach morning glory	Climber	Xero-halophyte	Diarrhea, vomiting, inflammation	Agoramoorthy, 2008
Pluchea lanceolata (Asteraceae)	Rasna	Shrub	Xero-halophyte	Antiinflammatory, analgesic, bronchitis	Arya *et al*., 2008
Salvadora persica (Salvadoraceae)	Toothbrush tree	Shrub	Psammo-halophyte	Toothache, Antiinflammatory	Qasim *et al*., 2014
Suaeda fruticosa (Amaranthaceae)	Shrubby sea-blite	Shrub	Xero-halophyte	Antibacterial	Qasim *et al*., 2011
Thespesia populneoides (Malvaceae)	Pacific rosewood	Tree	Hydro-halophyte	Hepatitis, Jaundice, Antimicrobial	Qasim *et al*., 2014

Statistical analysis: Values are presented as means (± S.E) of five replicates and post-hoc LSD test was used to compare individual means Correlation analyses ($p<0.05$) among different parameters were also performed using both correlation coefficient (r) and coefficient of determination (R^2). SPSS software (SPSS for windows ver. 11.0) was used to perform statistical analyses and graphs were plotted using Sigma plot.

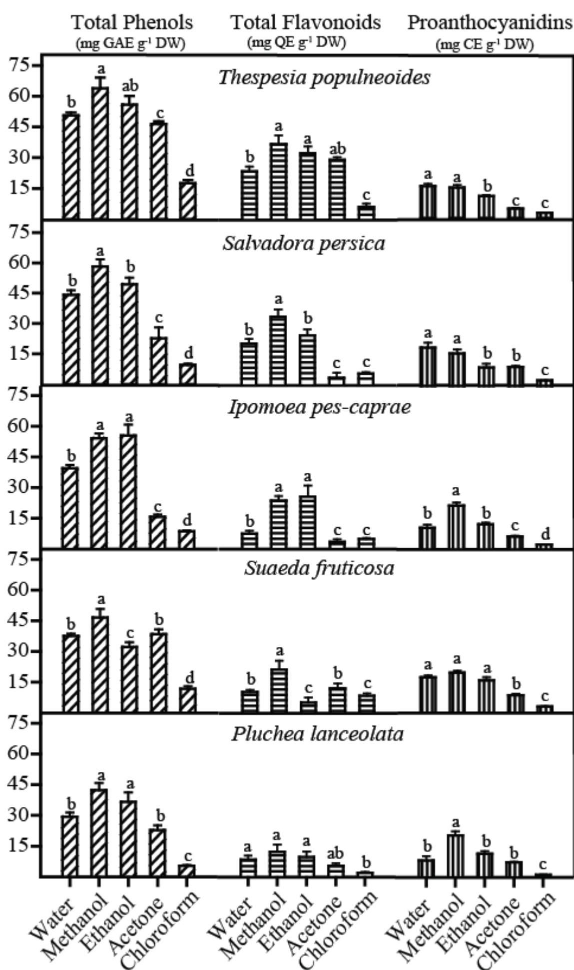

Fig. 1. Effect of different solvents on total phenolic, flavonoid and proanthocyanidin content of five medicinal halophytes. Bars represent means (± standard error) of 5 replicates. Similar letters over means are not significantly different at $p<0.05$.

Results

Total phenolic, flavonoid and proanthocyanidin content: Total phenolic (TPC), flavonoid (TFC) and proanthocyanidin contents (PC) are shown in Fig. 1. Significant differences ($p<0.05$) in phenolic (TPC), flavonoid (TFC) and proanthocyanidin (PC) contents were observed where leaf TPC (64 to 5 mg g^{-1}), TFC (37 to 2 mg g^{-1}) and PC (21 to 1 mg g^{-1}) varied considerably with the nature of solvent (Fig. 1). Leaf extracts of all halophytes generally showed higher quantities of polyphenols (TPC, TFC and PC) with significant variation ($p<0.05$) among solvent types. Aqueous methanol (80%) appeared most efficient solvent for TPC (64 to 42 mg g^{-1}) followed by 80% ethanol (56 to 37 mg g^{-1}) and water (51 to 31 mg g^{-1}) while acetone (46 to 23 mg g^{-1}) and chloroform (17 to 5 mg g^{-1}) were found less effective. Similarly, highest values of TFC (37 to 12 mg g^{-1}) and PC (21 to 15 mg g^{-1}) were also obtained in aqueous methanol followed by aqueous ethanol (TFC: 33 to 10 mg g^{-1}; PC: 16 to 9 mg g^{-1}) and water (TFC: 24 to 8 mg g^{-1}; PC: 19 to 8 mg g^{-1}). Acetone (29 to 5 mg g^{-1}; PC: 9 to 4 mg g^{-1}) and chloroform (6 to 2 mg g^{-1}; PC: 3 to 1 mg g^{-1}) showed lower extraction capacity.

All species showed considerably high antioxidant compounds (TPC, TFC and PC), of which *Thespesia populneoides* had the highest TPC (64 mg g^{-1}), TFC (37 mg g^{-1}) and PC (15 mg g^{-1}) followed by *Salvadora persica* (TPC 58 mg g^{-1}; TFC 34 mg g^{-1}; PC 18 mg g^{-1}) and *Ipomoea pes-caprae* (TPC 54 mg g^{-1}; TFC 24 mg g^{-1}; PC 21 mg g^{-1}). *Suaeda fruticosa* and *Pluchea lanceolata* had relatively lesser amount of TPC (46 mg g^{-1}; 42 mg g^{-1}), TFC (21 mg g^{-1}; 12 mg g^{-1}) and PC (20 mg g^{-1}; 20 mg g^{-1}).

Antioxidant activity: In general, leaf extracts of all species showed high antioxidant activity with significant variation ($p<0.05$) among solvent type. The IC_{50} values using DPPH radical scavenging test ranged from 885 to 17 µg ml^{-1} (Table 2) and in case of FRAP reducing power test the variation was from 6.54 to 0.85 mM Fe^{+2} g^{-1} (Table 3). Our data shows that aqueous mixtures of methanol (39 to 17 µg ml^{-1}) and ethanol (77 to 25 µg ml^{-1}) had higher DPPH activity than water extracts (106 to 40 µg ml^{-1}). In acetone, although the DPPH values were lower (111 to 55 µg ml^{-1}) than water and other alcoholic extracts however, they were significantly higher than chloroform (885 to 437 µg ml^{-1}). In case of FRAP, similar results were found where the highest activity was observed in aqueous methanol (6.54 to 4.81 mMol Fe^{+2} g^{-1}) and ethanol (6.23 to 4.43 mMol Fe^{+2} g^{-1}) followed by water (5.67 to 3.86 mMol Fe^{+2} g^{-1}) and acetone (4.22 to 2.11 mMol Fe^{+2} g^{-1}) while the lowest activity was recorded in chloroform (2.03 to 0.85 mMol Fe^{+2} g^{-1}). Hence, antioxidant activity in descending order was: 80% methanol > 80% ethanol > water > acetone > chloroform. This order is similar for total phenols except for acetone which showed lowest correlation.

Correlation between antioxidant activity and polyphenols: Simple linear regression was used to analyze the correlation coefficient (r) and coefficient of determination (R^2) between antioxidant activities (DPPH and FRAP) and polyphenols (TPC, TFC and PC) from different extracts of medicinal halophytes. When compared among solvents, highest correlation (r and R^2) between polyphenols (TPC, TFC and PC) and antioxidant activity (DPPH and FRAP) was found in 80% methanol followed by 80% ethanol and water whereas, a weaker correlation was observed in acetone and chloroform extracts (Tables 4 to 6).

Table 2. Effect of different solvents on DPPH radical scavenging activity (IC_{50} µg ml^{-1}) of medicinal halophytes. Values are means (± standard error) of 5 replicates. Similar letters over means are not significantly different at $p<0.05$.

	Thespesia populneoides	*Salvadora persica*	*Ipomoea pes-caprae*	*Suaeda fruticosa*	*Pluchea lanceolata*
Water	40.04 (0.71) b	30.11 (1.40) a	71.56 (5.03) b	62.56 (5.03) b	105.87 (7.18) c
Methanol (80%)	16.64 (1.24) a	20.92 (1.52) a	32.11 (3.25) a	33.31 (3.54) a	38.76 (6.11) a
Ethanol (80%)	25.45 (1.24) a	22.35 (1.76) a	45.21 (3.89) a	92.67 (8.76) c	76.56 (8.72) b
Acetone	55.43 (8.76) c	187.65 (4.32) b	135.87 (12.43) c	98.54 (9.56) c	411.32 (21.43) d
Chloroform	637.65 (25.67) d	754.32 (31.23) c	885.15 (17.54) d	478.12 (9.51) d	789.02 (11.49) e

BHA: 42.15 (2.51); BHT: 35.24 (1.65)

Table 3. Effect of different solvents on FRAP reducing power activity (mMol Fe^{+2} g^{-1}) of medicinal halophytes. Values are means (± standard error) of 5 replicates. Similar letters over means are not significantly different at $p<0.05$.

	Thespesia populneoides	*Salvadora persica*	*Ipomoea pes-caprae*	*Suaeda fruticosa*	*Pluchea lanceolata*
Water	5.32 (0.33) b	5.67 (0.55) a	3.87 (0.42) b	4.49 (0.21) b	3.86 (0.84) b
Methanol (80%)	6.54 (0.56) a	5.96 (0.29) a	5.46 (0.15) a	5.32 (0.56) a	4.81 (0.34) a
Ethanol (80%)	6.01 (0.86) a	6.23 (0.54) a	5.33 (0.31) a	4.43 (0.11) b	4.76 (0.21) a
Acetone	2.84 (0.22) c	4.22 (0.43) b	2.11 (0.28) c	3.76 (0.15) c	3.22 (0.21) b
Chloroform	1.15 (0.11) d	1.66 (0.25) c	2.03 (0.08) c	0.85 (0.04) d	1.32 (0.09) c

BHA: 5.32 (0.42); BHT: 7.13 (0.54)

Table 4. Correlation between total phenolic content (TPC) and each of DPPH radical scavenging and FRAP reducing power activities using different solvent extracts of medicinal halophytes.

	TPC x DPPH			TPC x FRAP		
	r	R^2	Equation	r	R^2	Equation
Water	-0.883	0.780	$y = -3.493x + 203.2$	0.786	0.617	$y = 0.0824x + 1.321$
Methanol	-0.951	0.904	$y = -1.003x + 81.5$	0.968	0.937	$y = 0.0731x + 1.743$
Ethanol	-0.896	0.802	$y = -2.546x + 169.3$	0.814	0.663	$y = 0.0576x + 2.709$
Acetone	-0.771	0.594	$y = -2.949x + 203.9$	0.161	0.026	$y = 0.0104x + 2.926$
Chloroform	-0.827	0.684	$y = -36.48x + 1054.1$	0.424	0.180	$y = -0.043x + 1.8556$

Table 5. Correlation between total flavonoid content (TFC) and each of DPPH radical scavenging and FRAP reducing power activities using different solvent extracts of medicinal halophytes.

	TFC x DPPH			TFC x FRAP		
	r	R^2	Equation	r	R^2	Equation
Water	-0.821	0.674	$y = -3.271x + 108.2$	0.939	0.881	$y = 0.1045x + 3.1668$
Methanol	-0.986	0.971	$y = -0.914x + 51.7$	0.980	0.961	$y = 0.065x + 3.9521$
Ethanol	-0.942	0.887	$y = -2.549x + 102.3$	0.887	0.786	$y = 0.0597x + 4.1844$
Acetone	-0.833	0.693	$y = -3.707x + 158.2$	0.148	0.022	$y = -0.011x + 3.3502$
Chloroform	-0.705	0.496	$y = -59.97x + 1001.1$	0.458	0.210	$y = -0.0897x + 1.8999$

Table 6. Correlation between proanthocyanidin content (PC) and each of DPPH radical scavenging and FRAP reducing power activities using different solvent extracts of medicinal halophytes.

	PC x DPPH			PC x FRAP		
	r	R^2	Equation	r	R^2	Equation
Water	-0.875	0.765	$y = -5.7858x + 143.25$	0.837	0.701	$y = 4.5251x - 6.9677$
Methanol	-0.928	0.862	$y = 2.8653x - 24.465$	0.869	0.756	$y = -3.9409x + 40.572$
Ethanol	-0.834	0.696	$y = 10.046x - 66.823$	0.851	0.724	$y = -2.8407x + 27.076$
Acetone	-0.720	0.518	$y = 18.273x - 13.911$	0.778	0.605	$y = 1.828x + 1.3015$
Chloroform	-0.496	0.246	$y = -141.3x + 1008.3$	0.187	0.035	$y = -0.2861x + 2.8031$

Discussion

Antioxidant properties of medicinal plants cannot be evaluated by single method due to variety of complex phytochemicals (Li *et al*., 2008). Thus, two widely used methods DPPH and FRAP, which are based on free radical scavenging and reducing power abilities of plant extracts (Sreeramulu *et al*., 2013), have been employed in this study. In general, leaf extracts of studied halophytes displayed strong antioxidant activity which was higher than commercial antioxidants. Aqueous methanol and ethanol extracts showed significantly higher antioxidant activity in these plants which is in agreement with previous reports (Sahreen *et al*., 2010; Zhou & Yu, 2004). There is growing evidence that aqueous methanol extract exhibit higher antioxidant activity when compared with other solvents (Sen *et al*., 2013; Khan *et al*., 2012). Cai *et al*. (2004) reported similar results for Chinese medicinal plants while Li *et al*. (2008) and Boulekbache-Makhlouf *et al*. (2013) reported that aqueous alcoholic extracts are usually strong free radical inhibitors and reducing agents. Hence, it can be concluded that aqueous alcoholic extract (particularly 80% methanol) is a better solvent for polyphenol extraction of halophytes.

Change in solvent polarity can affect the extraction of selected group(s) of antioxidant compounds and subsequent antioxidant activity (Razali *et al*., 2012; Roby *et al*., 2013). Tables 4-6 indicates that TPC, TFC and PC of methanol extracts were highly correlated with antioxidant activity tests followed by ethanol and water extracts. These solvents yielded higher polyphenols (TPC, TFC and PC) which are the major contributors of overall antioxidant activity (Quideau *et al*., 2011). These results are in agreement with previous studies where high correlation between polyphenols and antioxidant activity was reported (Fredes *et al*., 2014; Katalinic *et al*., 2013; Goulas & Manganaris, 2012; Ramful *et al*., 2011). However, acetone and chloroform had relatively weak to poor correlation which is in line with their polyphenolic yields.

All test species showed considerably higher amount of polyphenols (TPC, TFC and PC) and antioxidant activity. *Thespesia populneoides* was found as antioxidant rich plant followed by *Salvadora persica* and *Ipomoea pes-caprae*. Although, *Suaeda fruticosa* and *Pluchea lanceolata* had lower amount of polyphenols and antioxidant activity, their values were even higher than most of the polyphenol rich vegetables (Kim *et al*., 2013; Sreeramulu *et al*., 2013), fruits (Reddy *et al*., 2010) and other medicinal plants (Raja & Pugalendi, 2010; Lin *et al*., 2013; Proestos *et al*., 2013). Our results also indicate that these halophytes are rich in natural antioxidants with higher activity than commercial ones (BHA and BHT) and displayed immense potential as natural alternative to harmful synthetic chemicals for food, pharmaceutical and cosmetic industry.

Conclusion

Alcoholic extracts (80% methanol and ethanol) were found most effective solvents for recovering antioxidant compounds from halophytes while chloroform appeared least affective. A higher degree of relationship between antioxidant activity and polyphenols indicate that antioxidant activity is a function of polyphenolic pool. In addition, the antioxidant activity of halophytes was better than those of commercial antioxidants which could be related to their adaptation in extreme climatic conditions. Cultivation of halophytes on salt affected barren lands with saline/brackish water would provide better alternative to obtain antioxidant rich raw material. Therefore, characterization of phenolic metabolites is recommended which would be helpful in developing natural alternatives to replace synthetic antioxidants from edible and medicinal products.

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