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Chromatographic fractionation, antioxidant and antibacterial activities of *Urginea maritima* methanolic extract

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Abstract: The present work concerns a phytochemical study of *Urginea maritima* L. from Algeria, and an evaluation of antioxidant activity of the methanolic extract (UMME) and its chromatographic fractions. UMME was fractionated using open glass chromatography on silica gel and antioxidant effects were evaluated using DPPH and β-carotene/linoleate assays. The phytochemical screening revealed that the bulb of plant contains flavonoids, glycosides, tannins, reducing compounds, anthraquinones combined, anthocyanins, mucilage, triterpenes and steroids. DPPH method showed that the UMME has a scavenger effect on radical DPPH with an IC_{50}=57.83±1.59µg/ml. The fractions isolated from *U. maritima* (L.) presented an IC_{50} ranging between 499.23 and 39.68µg/ml. In ~~of~~ β-carotene/linoleate test, UMME and fractions give an I% =69.56±0.08% and between 31.29±0.49% and 90.79±0.29%, respectively. UMME showed a high inhibitory effect on the xanthine oxidase (IC_{50}=0.67±0.01 mg/ml) and on the cytochrome c reduction (IC_{50}=0.68 mg/ml). Wide range of phytochemical constituents in *Urginea maritima* were detected in methanolic extract which exhibited antioxidant and antibacterial activity. This plant could serve as pilot for the development of novel agents for pathological disorders.

Keywords: *Urginea maritima*, phytochemical screening, column chromatography, Antioxidant, DPPH, secondary metabolites.

INTRODUCTION

Urginea maritima L. Baker is a perennial bulbous geophyte (a herbaceous plant with an underground storage organ) of the family Liliaceae (Bruneton 1996), native to the Mediterranean basin and well-adapted to its type of climate (Kopp *et al.*, 1996). It generally occurs in the slopes of hills, the sandy grounds near the Mediterranean Sea and in certain regions of Northern Africa (Bellakhdar, 1997), Middle East and Europe. *Urginea maritima* has two varieties: Red and white. The red variety (red squill) is predominant in Algeria (Sandberg and Corrigan, 2001) and Greece (Altardeh *et al.*, 2006). The white variety is predominant in Morocco (Bellakhdar, 1997). From the phytochemical point of view, it has been reported that the major constituents of *U. maritima* bulbs are glycosides (Kopp *et al.*, 1996; Krenn *et al*., 2000), Anthocyanins (Vega *et al.*, 1972), flavonoids (Fernandez *et al.*, 1972), fatty acids, polysaccharides (Spies *et al.*, 1992) and calcium oxalate (Cogne *et al.*, 2001). The cardiac glycosides (scillaren and scillarenin) are used in Europe as a cardiotonic diuretic for the treatment of cardiac marasmus and edema (Mitsuhashi *et al.*, 1994). It was then expected that this plant inhibited Na^+/K^+ adenosine triphosphatase (Schonfeld *et al.*, 1985). Furthermore, Bayazit and Konar (2010) showed that Squill bulb scillioside can reduce the musculoskeletal pains. It has been shown that extracts of bulbs have been active against stored product pests. This suggests that squill should be investigated for activity against other insects and pests (Pascual-Villalobos and Fernández, 1999). The objective of this research was to study the phytochemical and the fractionation of the methanolic extract of *U. maritima.* The evaluation of antioxidant activity and antibacterial effects of the methanolic extract and each chromatographic fraction was also carried out.

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MATERIALS AND METHODS

Reagents

All reagents used were of analytical grade. Tween-20, methanol, ethanol, benzene, acetone, β-carotene, chloroform, linoleic acid were purchased from E. Merck (Darmstadt, Germany). 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated-hydroxytoluene (BHT) were obtained from sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were analytical grade.

Plant material

Urginea maritima bulbs were collected at Bordj Bou Arreridj, Algeria, from September to October 2010 and were identified by Prof H. Laouer, Department of Ecology and Vegetal Biology, Faculty of Nature and Life Sciences, University Setif. Bulbs parts of the plant were air dried for several days. The dried plant material was ground to a coarse powder using a dry mill. For the extraction procedure, 1kg of powdered plant material was soaked in methanol for 7 than 5 days at room temperature with renewal of the solvent (Markham, 1982). The solvent

was then removed by rotary evaporation to obtain a dried extract called *Urginea maritima* methanolic extract (UMME).

Phytochemical screening

The screening is important to the next steps like extraction and/or fractionation of extracts to isolate compounds of interest. The phytochemical tests enable to detect the presence different groups of biochemical substances. Reducing compounds and alkaloids were performed according to the method described by Sofowora (1985) and Bruneton (2009). The tests were based on the extraction with suitable solvents of increasing polarity and the visual observation of color change or formation of a precipitate after the addition of specific reagents. These reactions are selective to types or groups of compounds, simple, rapid, sensitive and require a minimum of laboratory equipment.

Fractionation of methanolic extract

Open glass column chromatography, 100cm high and 4 cm of diameter, of UMME was carried out on silica gel 70-230 mesh, packed in dichloromethane. Elution was carried out using dichloromethane-methanol of increasing polarity. After TLC analysis, similar fractions were combined into pools called here fractions. The pool (fraction) 26 was subjected to further purification on a little column of 30cm high and 2cm of diameter using solvent mixtures of chloroform-methanol-acetic acid-H_2O of increasing polarity (Abbot and Andrews, 1970).

HPLC analysis

HPLC analysis was performed using a HPLC Model 9100 apparatus equipped with a ternary pump Model 9100 and UV–visible detector Model 9100. A reverse phase column C18 (250 x 4.6mm, 5 µm particles) was used. The mobile phase consisted of a binary mixture of methanol and water (60:40 v/v) at isocratic flow rate of 1ml. min^{-1}. The absorbance was monitored at λ=254 (Kuntić *et al*., 2007).

Determination of total Polyphenol and flavonoids Contents

The total polyphenols in *Urginea maritima* extracts were determined by the Folin–Ciocalteu method according to Cliffe *et al.* (1994) with slight modifications as we have previously described (Baghiani *et al*., 2012). Flavonoids were quantified using aluminium chloride reagent ($AlCl_3$), (Bahorun *et al.*, 1996). They were measured as quercetin and rutin equivalents. One ml of *Urginea maritima* extracts was dissolved in methanol, 1ml of $AlCl_3$ (2 %) in methanol was added, after incubation for 10 min, the absorbance was measured at 430 nm.

Free radical scavenging activity using DPPH

The free radical scavenging properties of *Urginea maritima* extracts were measured by decrease in the absorbance of methanol solution of DPPH (Burits and Bucar, 2000; Baghiani *et al*., 2012).

Antioxidant assay using ß-carotene-linoleate test

ß-carotene bleaching assay was carried out according to the method described by Dapkevicius *et al.* (1998) with modifications (Baghiani *et al.*, 2012). In this test, BHT was used as positive control and MeOH and H2O as blanks.

Effects of methanolic extract of Urginea maritima on xanthine oxidase activity

The effect of extract methanolic of *Urginea maritima* on xanthine oxidase (XO) activity was determined by measuring the absorbance 295 nm (Boumerfeg *et al.*, 2009). The enzymatic reaction is initiated by the addition of bovine XO, which was prepared in our laboratory with a specific activity of 1176 ηmole/min/mg of enzyme, to the xanthine prepared in phosphate buffer containing EDTA as we have previously described (Boumerfeg *et al.*, 2009). Allopurinol was used as a positive standard. The effects on the superoxide anions generation by XO was measured by following the cytochrome c (25 mM) reduction at 550 nm (Robak and Gryglewski, 1988).

Antimicrobial activity

The antibacterial activity of UMME was tested against 10 bacteria strains: *Salmonella typhimurium* ATCC 13311, *Acinetobacter baumanii* ATCC 19606, *Klebsiella pneumoniae* ATCC 700603, *Bacillus cereus* ATCC 10876, *Enterobacter cloaceae*, *Listeria monocytogenes* ATCC 15313, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus,* ATCC 25923 and *Proteus vulgaris*. The disc difusion technique was used to evaluate the antibacterial activity of methanolic extracts. Ten µl of powder resin by disc of Whatman paper N°1 was applied into Muller Hinten medium inoculated by a bacterial suspension. The bacterial solution was diluted in order to obtain a concentration equivalent to 0.5Mc Farland (NCCLS, 2001). The dried material of plant extracts was dissolved at different concentrations in one ml of DMSO. Bacterial suspensions (100µl) were spread on Muller Hinton Agar medium. The extracts (10µl) were applied to discs of Whatman paper N°1 and applied into the medium inoculated by a bacterial suspension. The plates were incubated at 37°C for 48h.

RESULTS

Phytochemical screening

Urginea maritima powder was subjected to various phytochemical tests to identify the present chemical constituents. The results showed the presence of flavonoids, glycosides, tannins, reducing compounds, anthraquinones combined, anthocyanins, mucilage, triterpenes and steroids (table 1).

Table 1: Result of phytochemical screening of *Urginea maritima*

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Test			Results
Alkaloids			-
Tannins	Catechols		+ + +
	Gallic		+
Anthocyanins			+ +
Flavonoids			+ +
Leucoanthocyans			+ + +
Anthraquinones	Free		-
	combined	*o*-heterosides	+
		c-heterosides	+
triterpenes and steroids			+ + +
Saponins			-
Reducing compounds			+
Glycosides			+ + +
Coumarins			-
Mucilage			+ +

+ : present, - : absent

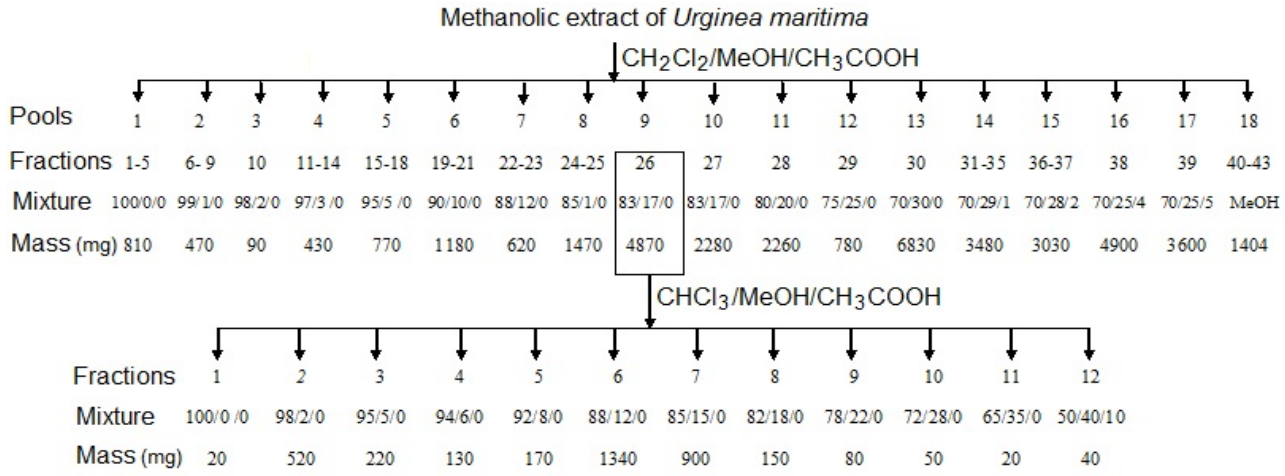

Fig. 1: The fractionation procedure of *Urginea maritima* methanolic extract by column chromatography in two steps.

Fractionation of methanolic extract

A total of 43 fractions were collected. These were pooled, based on similar thin-layer chromatograms into 18 pools. Results of column chromatography of the methanolic extract are shown in fig. 1. The bioactive fraction F26 (4.87g) was subjected for further fractionation (purification) on column chromatography. Elution was done with chloroform and chloroform-methanol to get 12 pooled sub fractions.

The nature of the sample source determines the isolation techniques, which vary among solid and liquid samples. Polyphenols and flavonoids in plant were isolated by extraction with chloroform-methanol, followed by separation on column chromatography. Elution was done with chloroform and chloroform-methanol, for obtaining enriched total flavonoids and polyphenols.

Total polyphenols and flavonoids contents

Table 2 presents the amount of phenolic and flavonoïds compounds in methanolic extracts of *U. maritima* and its chromatographic fractions. Total phenolic contents were expressed as mg Gallic acid equivalents per gram of dry weight (mg GA Eq/g extract) and total flavonoids contents as mg Quercetin and Rutin equivalents per gram of dry weight (mg Q Eq and R Eq/g extract).

In UMME fractions, the fraction 25 presents the highest amount of phenolic compounds (404.19±1.52 mg GA Eq/g) followed by crud UMME (table 2). Fractions 1 to 19 contained very low amount of polyphenols and flavonoids. In the fractions of F26, the fraction F11 contained the highest concentration of polyphenols (318.09±0.44 mg GA Eq/g) and total flavonoids contents (32.12±0.4mg Q Eq/g), (fig. 2).

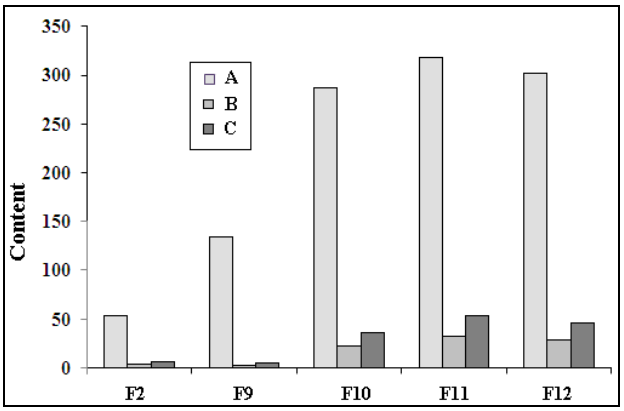

Fig. 2: Total phenolic and flavonoid contents of fraction 26 and its major chromatographic fractions. Polyphenols as mg GA-Eq/g. (A), Flavonoids as mg Q-Eq/g (B) and as mg R-Eq/g.

Free radical scavenging activity using DPPH method

Natural products isolated from medicinal plants have attracted considerable attention in recent years due to their various pharmacological properties. In the present study, DPPH radicals were used in the test to investigate the scavenging effects of methanolic extract and chromatographic fractions. The results showed that UMME possesses a scavenging effect on DPPH radical (EC_{50}=57.83±1.59µg/ml). The presence of compounds such as tannins, flavonoids, Anthocyanins and phenols in *Urginea maritima* extract may be the cause of this effect. The chromatographic fractions investigated showed different levels of DPPH free radical scavenging activity. In fractions of UMME, IC_{50} values ranged from 39.68±0.69µg/ml to 499.23±1.25µg/ml. The IC_{50} values of fractions of F26 were ranged between 39.91±0.08µg / ml and 619.76±4.85µg/ml (fig. 3).

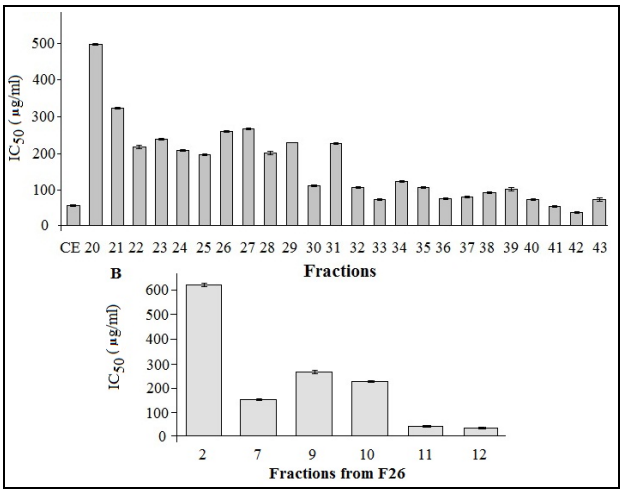

Fig. 3: IC_{50} values of plant extracts for free radical scavenging activity by DPPH method. Lower IC_{50} value indicates higher antioxidant activity. (A): Fractions of crud extract, (B): Fractions from faction 26. Each value is represented as mean of three determinations ± S.D.

Antioxidant assay using ß-carotene-linoleate

The antioxidant activities of UMME and its fractions compared to butylated hydroxytoluene (BHT) are presented in fig. 4. The I% value of UMME was 69.65%. The best activity was shown by fraction 38 of methanolic extract with an I % value of 87%. The lowest inhibition was 51.19% shown by the fraction 23. In the fractions of F26, the fraction 12 gave a highest inhibition compared to BHT (fig. 4) with an I% =90.79%.

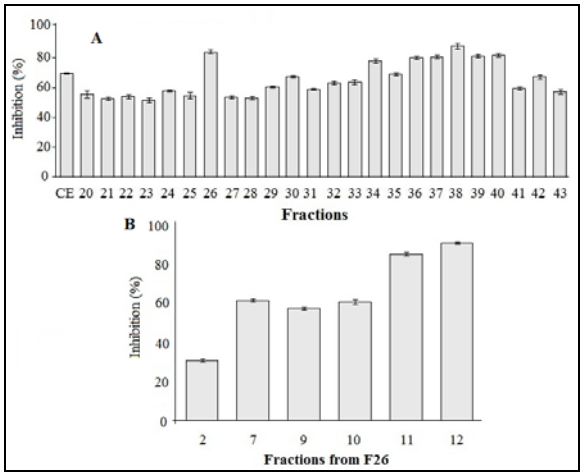

Fig. 4: Antioxidant activity of the methanolic extract and its chromatographic fractions, compared to BHT (positive blank) and, H_2O and Methanol (negative blanks) using *ß*-carotene-linoleate assay after 24 hours of incubation. Results are means of three different experiments ± S.D.

Qualitative HPLC analysis

Due to the complexity of the natural mixtures of phenolic compounds of various plants, it is rather difficult to elucidate their structure and assess the antioxidant and biological potentials. Hence, it was aimed in this work to isolate and identify some of the phenolic compounds present in the methanolic extract and major fractions using HPLC which is a high-resolution chromatographic technique probably the most widely used analytical technique for characterizing the polyphenolic compound (Gomez-Caravaca *et al*., 2006). Crude extract and four more active fractions of *Urginea maritima* bulb (F26, F30, F36 and F42) were selected in this analysis. The preliminary results presented in table 3 showed the presence of at ten major compounds in the crude methanolic extract and two to four compounds in the chromatographic fractions.

Effects of Urginea maritima methanolic extract on XO activity

The extract inhibited the activity of xanthine oxidase in a concentration dependent manner (fig. 5) with an IC_{50} (mg/ml) value of 0.67±0.01. Additionally, UMME was able to reduce the cytochrome c^{+3} in concentration-dependent manner. It has a potent scavenging activity of superoxide anion radical with an IC_{50} value of 0.68± 0.001mg/ml (fig. 5).

Table 2: Total phenolic and flavonoid contents of *U. maritima* methanolic extract and its major chromatographic fractions.

Extracts	Total polyphenols (mg GA-Eq/g)	Total flavonoids	
		(mg Q-Eq/g)	(mg R-Eq/g)
L'extrait brut	234.25±0.35	11.42±0.35	18.69±0.57
Fraction 20	32.38±0.05	1.84±0.02	2.88±0.11
Fraction 21	101.07±0.3	5.14±0.05	8.37±0.15
Fraction 22	178.3±0.3	7.09±0.43	11.49±0.74
Fraction 23	88.23±0.23	6.48±0.53	10.58±0.89
Fraction 24	235.23±0.26	17.92±0.62	29.48±1.08
Fraction 25	404.19±1,52	25.73±1.88	42.35±3.8
Fraction 26	124.52±0.33	6.6±0.11	10.45±0.42
Fraction 27	103.85±2.62	7.41±0.41	12.18±0.72
Fraction 28	118.33±1.14	8.89±0.38	14.58±0.77
Fraction 29	76.71±1.95	7.29±0.31	11.83±0.45
Fraction 30	196.14±0.2	14.97±0.99	24.77±0.48
Fraction 31	126.14±1.31	15.05±0.33	24.65±0.48
Fraction 32	182.42±1.71	4.91±0.33	7.75±0.55
Fraction 33	181.96±0.45	7.53±1.32	12.36±2.1
Fraction 34	100±0.2	16.58±0.67	27.31±1.05
Fraction 35	138.11±0.84	9.41±0.13	15.45±0.14
Fraction 36	178.42±0.8	15.09±0.89	20.82±1.02
Fraction 37	177.02±0.03	9.35±0.12	15.71±0.15
Fraction 38	126.61±0.26	20.52±1.78	34.83±2.89
Fraction 39	83.61±0.13	13.14±0.26	23.27±0.5
Fraction 40	123.96±0.55	19.89±2.25	32.78±3.67
Fraction 41	221,07±1,56	13.60±0.24	22.37±0.34
Fraction 42	301.71±0.4	19.41±1.79	31.65±2.89
Fraction 43	92.78±0.3	3.48±0.05	5.66±0.12

Table 3: Major compounds identified by HPLC analysis of crude extract (CE) and chromatographic more active fractions of *Urginea maritima* bulb.

Major Compounds	CE	F26	F30	F36	F42
Tannic acid	+/-			+	+
Quercetin	+		+		
Gallic acid	+	+			
Reserpin	+/-			+	
Catechin	+				
Caffeic acid	+	+			+/-
Rutin	+/-	+	+		
Vanilin	+				
Kaemferol	+				
Naringin	+	+	+		

Xanthine oxidase mechanism leads to the production of ROS (Harrison, 2002). These ROS have great effects on different biomolecules (Castro and Freeman, 2001).

Antimicrobial activity

Preliminary screening of the *in vitro* antibacterial activity of methanolic extract was studied against various pathogens. The results showed different antimicrobial properties of plant extracts (table 4). *Bacillus cereus* ATCC10876 was found to be the most inhibited pathogen by the UMME with a diameter of inhibition zone of 11 mm; followed by *Acinetobacter baumanii* (10mm) and *Salmonella typhimurium* (9mm). Ivancheva *et al*. (2006) attributed this activity to the presence of, secoiridoid glucosides, phenylethanoids and flavonoids contained in the extract.

DISCUSSION

The medicinal plants are rich in secondary metabolites including polyphenols, alkaloids, glycosides and flavonoids. They are of great clinical importance and are

widely used in pharmaceutical industry. Polyphenols are known as antioxidant and scavenging compounds against free radicals associated with oxidative stress (Fergusion *et al*., 2006).

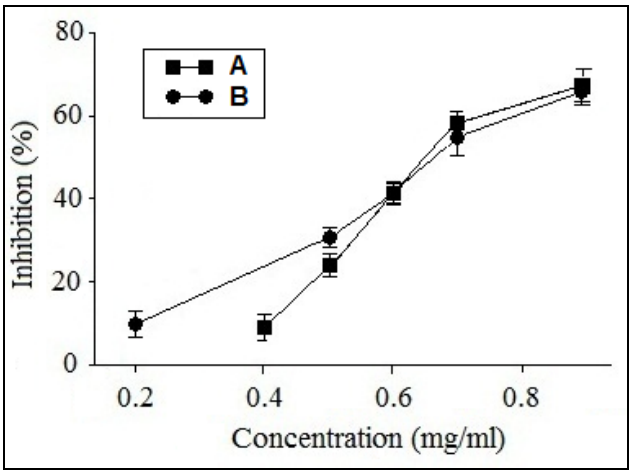

Fig. 5: Inhibition of bovine xanthine oxidase activity (A) and super oxide anion radical generation from xanthine/xanthine oxidase system (B) by the methanolic extract of *Urginea maritima*. Results are expressed as percentage of control where no inhibitor was added. Each value is represented as mean ± S.D (n=3).

The presence of these compounds such as tannins, flavonoids, proanthocyanidins and phenols in *Urginea maritima* extract may give credibility to its local use for the management of oxidant related pains. Tannins have been found to have antiviral, antibacterial, anti-parasitic effects, anti-inflammatory, antiulcer and antioxidant property for possible therapeutic applications (Ly *et al.*, 2004; Akiyama *et al.*, 2001). The composition of tannins as observed in this study may justify its traditional usage come anti-inflammatory. Flavonoids, the major group of phenolic compounds are reported for their antimicrobial, antiviral and spasmolytic activity. Flavonoids are able to scavenge hydroxyl radicals, super oxide anion radicals and lipid peroxy radicals, it has been confirmed that pharmacological effect of flavonoids is correlating with their antioxidant activities (Boumerfeg *et al.*, 2009). In the present study we observed the presence of glycosides in the methanolic extract of *Urginea maritime.* Glycosides have a history of pharmacological effects for their cardiotonic and diuretic effects. Therefore, the concentration of these compounds could contribute synergistically to the significant cardiotonic potency of this plant and thus may support the local usage for the treatment of cardiac marasmus and edema (Mitsuhashi *et al.*, 1994). These results reveal that the plant has quite a number of chemical constituents, which may be responsible of the many pharmacological actions.

Several techniques were used in this study to assess the free radical scavenging and reducing properties of the methanolic extracts of *Urginea maritima*, along with evaluation of the total flavonoids and polyphenols contents. *Urginea maritima* extract acts as reducing agent, exhibiting antioxidant role in inhibiting and scavenging free radicals and providing protection to humans against infections and degenerative diseases (Nagler *et al.*, 2006). The present study showed the ability of the methanolic extract and its fractions to inhibit hydroxyl radical, with a concentration dependent manner.

Flavonoids and other phenolic compounds of plant origin have been reported as scavengers and inhibitors of lipid peroxidation (Adithya *et al.*, 2013). In fact, the antioxidant activity was dependent upon the presence and quantity of polyphenols and/or flavonoids (Ibraheem *et al*., 2014). Thus, the degradation rate of β-carotene-linoleate depends on the antioxidant activity of the extracts (Maisarah *et al*., 2013).

CONCLUSION

The main classes of secondary metabolites such as alkaloids, flavonoids, tannins, sterols and terpenoids, were found in *U. maritima*. The phytochemical analyses showed that the methanolic extract and chromatographic fractions were rich in polyphenols and flavonoids. Additionally, UMME and some of its fractions exhibited substantial potency in scavenging DPPH radical, inhibiting lipid peroxidation, XO activity and cytochrome c reduction. Finally, methanolic extract has an antimicrobial activity against seven species of bacteria. In conclusion, *Urginea maritima* methanolic extract contains several constituents which have antioxidant and antibacterial activities. Further studies regarding isolation and purification of active phyto-constituents with broad spectrum of antioxidant and antibacterial activities are under investigation.

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