academicJournals

Vol. 13(3), pp. 486-491, 15 January, 2014
DOI: 10.5897/AJB2013.12833
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Antioxidant activity, phenolic and flavonoid content in leaves, flowers, stems and seeds of mallow (*Malva sylvestris* L.) from North Western of Algeria

Mohammed Choukri Beghdad[1]*, Chahid Benammar[1], Fatima Bensalah[1], Fatima-Zohra Sabri[1], Meriem Belarbi[1] and Farid Chemat[2]

[1]Université de Tlemcen, Département Biologie, Laboratoire Produits Naturels N° 14, 13000 Tlemcen, Algérie.
[2]Université d'Avignon et des Pays de Vaucluse, INRA, UMR408, Sécurité et Qualité des Produits d'Origine Végétale, F-84000 Avignon, France.

Accepted 8 January, 2013

The nutraceutical composition (phenolics and flavonoids) of all leaves, flowers, stems and seeds of mallow, *Malva sylvestris* L., as well as their antioxidant properties were studied using *in vitro* methods: ferric reducing antioxidant power (FRAP) assay, by scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and total antioxidant capacity (TAC) based on the reduction of molybdenum (VI) to molybdenum (V). Results show that all extracts possessed concentration-dependant antioxidant activity. Leaf extracts have a highest amount of total phenolics with 24.123 ± 0.718 mg GAE/g, and total flavonoids with 0.694 ± 0.017 mg RE/100 g. However, the seed extracts presented the lowest amount in the two assays used. In addition, the AcOEt (EC_{50} = 3.10 mg/ml) fraction showed the highest value of antioxidant activities for almost all parts of leaves.

Key words: *Malva sylvestris* L., antioxidant activity, ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), total antioxidant capacity (TAC).

INTRODUCTION

The originality of the Mediterranean diet in general, and that of North Africa in particular is often reduced to its richness of cereal and olive oil. In fact, vegetables are particularly important. Leafy vegetables such as mallows (*Malva sylvestris* L.) are omnipresent in the Algerian cuisine. Gastronomical traditions of *M. sylvestris* L. consider the usage of the leaves and stems in soups, stews and salad. Mallow is traditionally used to treat all kinds of inflammations, particularly used as antihemorrhodial agent, emollient and chest pain reliever for children (Gonda et al., 1990; Classen et al., 2001; Couplan, 2003).

Aqueous extracts of flowers of *M. sylvestris* L. are rich in anthocyanin (Mas et al., 1999) and are usually used to treat inflamed mucous membranes (Farina et al., 1995). Analytical data on the chemical and total phenolic contents of this species are very rare. However, previous chemical investigations have shown the presence of anthocyanins, flavones, flavonols, ferulic acid, hydroxycinnamic acids, sterols, sesquiterpenes, mono and diterpenes in leaves and stems of *M. sylvestris* L. (Nawwar et al., 1977; Nicoletti et al., 1989; Mas et al., 1999; Cutillo et al., 2006; Quave et al., 2008b). Therefore, it is important to study the antioxidant and antimicrobial activity of edible parts (leaves and stems) and non edible parts (flowers and seeds) from mallow (*M. sylvestris* L.), a green wild plant that is much consumed and appreciated for its culinary and medicinal virtues in

*Corresponding author. E-mail: cbeghdad@yahoo.fr. Tel: +213 662065448.

the Maghreb (Algeria, Morroco and Tunisia).

From the family *Malvaceae*, this specie is found on roadsides and forest edges. Thus, mallow (*M*. *sylvestris* L.), has been investigated in order to determine anti-oxidant molecules of its extracts from *M. sylvestris* L. stems, leaves, flowers and seeds as well as activity of some compounds previously isolated from mallow. Moreover, the relationships between total phenolic and total flavonoid content and antioxidant activity were also investigated. To the best of our knowledge, such investigation has never been achieved previously.

MATERIALS AND METHODS

Samples

2.0 kg of mallow (*M*. *sylvestris* L.) was purchased from local market in January (*M*. *sylvestris* L.) 2010. The green vegetables were washed, dried, with paper towels, cut into approximately 1 cm^2 squares, dried in an oven at 60°C for at least 24 h, and crushed before use. All analyses were conducted in triplicate and the results were based on the dry weight per 100 g of sample.

Chemicals

Folin Ciocalteu phenol reagent, gallic acid, ascorbic acid, DPPH (1,1 -diphenyl-2-picrylhydrazyl), trichloroacetic acid (TCA) and aluminium trichloride ($AlCl_3$) were obtained from Sigma Chemical Co. (St. Louis.MO, USA). Trolox was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other reagents and solvents used were of analytical grade.

Extraction

The dried powder of mallow (10.0 g) was extracted in triplicate with EtOH (96% v/v) at room temperature, under stirring. Aqueous suspension of the concentrated EtOH extract was evaporated to dryness and used for all investigations (Kukic et al., 2008).

Total phenol content analysis

The total phenolics content (TPC) was determined using Folin Ciocalteu reagent (Singleton et al., 1999). Briefly, an amount of 5 µl of the extract was added to 1.70 ml of distilled water and 300 µl of Folin Ciocalteu reagent (previously diluted 3-fold with distilled water). The mixture was allowed to stand for 3 min, then 0.5 ml of Na_2CO_3 (20%, w/v) was added to the mixture. After 1 h in dark at room temperature, the absorbance was measured at 760 nm. Results were expressed as gallic acid equivalents (mg of gallic acid/mg dry weight extract).

Total flavonoid content analysis

The concentration of total flavonoid (TFC) in each extract was determined with aluminium trichloride solution ($AlCl_3$) according to the colorimetric method adapted by Djeridane et al. (2006). An aliquot of the crude extract (500 µl) was mixed with 500 µl of aluminium trichloride solution ($AlCl_3$) at 2%. Then, the intensity of pink color was measured at 420 nm after 15 min. Rutin was used as standard. Results were expressed as mg rutin equivalents/100 g of dry weight.

Antioxidant activity

DPPH radical assay

Extracts were dissolved in appropriate solvents, mixed with 1 ml of 0.5 mM 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) in methanol (MeOH), and the final volume was adjusted to 5 ml. The mixtures were vigorously shaken and left for 30 min in the dark. Absorbance was measured at 517 nm using MeOH as blank. 1 ml of 0.5 mM DPPH diluted in 4 ml of MeOH was used as control. Neutralisation of DPPH radical was calculated using the equation: S (%) = 100 × (A_0 - As)/A_0, where A_0 is the absorbance of the control (containing all reagents except the test compound), and A_S is the absorbance of the tested sample. The EC_{50} value represents the concentration of the extract that caused 50% of neutralisation (Cuendet et al., 1997). These EC_{50} were changed to antiradical activity (A_{AR}) which is defined as $1/EC_{50}$, since this parameter increases with antioxidant activity. The results were compared with the activity of L-ascorbic acid.

FRAP assay

The total antioxidant activity (TAA) was investigated using the ferric reducing antioxidant power (FRAP) assay, which is based upon the reduction of Fe^{3+}-TPTZ (2,4,6-tripiridyl-2-triazine) complex under acidic conditions. The increase in the absorbance of the blue-coloured ferrous form (Fe^{2+}-TPTZ complex) is measured at 593 nm. The FRAP reagent was freshly prepared by mixing 25 ml of acetate buffer (300 mM, pH 3.6), 2.5 ml of TPTZ solution (10 ml TPTZ in 40 mM HCl) and 2.5 ml of $FeCl_3$ (20 mM) in water solution. An amount of 100 µl of each extract dissolved in appropriate solvent was added to 4.5 ml of FRAP reagent, stirred and incubated for 30 min. The absorbance was measured at 593 nm, using FRAP working solution as blank. A calibration curve of ferrous sulphate (100 to 1000 µM) was used, and the results were expressed in µmol Fe^{2+}/mg dry weight of extract. The relative activity of the sample was compared to L-ascorbic acid (Pellegrini et al., 2003).

Total antioxidant capacity assay

The assay was based on the reduction of molybdenum (VI) to molybdenum (V) by the sample and subsequent formation of a green phosphate/moolybdenum (V) complex at acidic pH (Prieto et al., 1999). An aliquot of 0.1 ml of the sample solution (100 µg/ml) was combined in an eppendorf tube with 1 mg of reagent solution (600 mM sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). Then, the samples were incubated in a thermal block at 95°C for 90 min and cooled to room temperature. The absorbance was measured at 695 nm against a blank (Ardestani and Yazdanparast, 2007). The antioxidant capacity of extracts was expressed as mg equivalent of ascorbic acid/g of dried weight (mg EAA/g).

RESULTS AND DISCUSSION

Yield and total phenolic content

The amount of phenolic and flavonoid of extracts obtained from *M*. *sylvestris* L. are reported in Table 1. Maximum yield of ethanolic extraction was obtained for

Table 1. Yield, total phenolics content and total flavonoids content of different extracts of *Malva sylvestris* L.

Parameter	Leaves	Stem	Flower	Seed
Yield	26.143±2.960	26.658±2.779	17.25±3.181	15.125±5.289
Total phenolics content (TPC)	24.123±0.718	2.173±0.038	6.978±0.602	3.714±0.096
Total flavonoids content (TFC)	5.694±0.017	0.018±0.001	0.170±0.033	0.031±0.002

Yield expressed as percentage (%); TPC, total phenolics content expressed as mg gallic acid equivalent (GAE)/g dried weight; TFC, total flavonoids content expressed as mg rutin equivalent (RE)/100 g dried weight; results are expressed in mean ± standard deviation, n = 3.

the extracts of stem samples. No correlation was found between the extraction yields of the total phenolics content (TPC) and total flavonoids content (TFC). Generally, the concentration of phenolic compounds was higher in the extracts isolated from leaves (24.123 ± 0.718 mg GAE/g), while in stem extract, this concentration was lower (2.173 ± 0.038 mg GAE/g). This variation can be explained by the presence of other compounds and/or different types of phenols. For example, contrary to the very visible flavonoids in flower petals, in leaves they are completely hidden by the ubiquitous green of the chlorophylls. Nevertheless, there is increasing evidence that these flavonoids, particularly when they are located at the upper surface of the leaf or in the epidermal cells, have a role to play in the physiological survival of plants (Harbone and Williams, 2000). The amount found in our sample of leaves was similar to the one found by Conforti et al. (2008) (28 ± 0.35 mg ACE/g). Other authors also found a higher phenolic content in leaves than in flowers (Barros et al., 2010).

Total flavonoid content

The TFC of our samples was calculated using quercetin standard. The TFC of leaves (5.694 ± 0.017 mg RE/100 g) was found slightly higher than the value found by Conforti et al. (2008) (4.77 ± 0.07 mg RE/100 g). This difference can be attributed to the different solvents used in the extraction process. In fact, for an efficient and most favourable extraction of polyphenol, some conditions were indispensable, that is, a) methanol is the best solvent for polyphenol extraction; b) the addition of water to the extraction system improves the yield; however, the higher the water concentration, the bigger the polyphenol content, which leads to the diminution of the polyphenol content; c) the solid/liquid ratio affects significantly polyphenol and flavonoid contents and the best ratio found is 1/6; d) it was noted that the higher the polyphenol and flavonoid contents, the longer the extraction time (Telli et al., 2010). Table 1 shows the amount of total flavonoids of the crude extracts from all parts of *M*. *sylvestris* L. increasing in the order of leaves > flowers > seeds > stems. The leaves are the richest in flavonoids and this explains their therapeutic properties in traditional medicine. In addition, it is not coincidence that leaves are consumed in Algeria.

DPPH free radical scavenging activity

The antioxidant potential was evaluated by measuring the decrease in DPPH radical absorption of extracts from four parts of *M*. *sylvestris* L. expressed as % inhibition, EC_{50} and A_{AR} (Table 2). All extracts were involved in the proton transfer with different degrees. This method evaluates the radical scavenging ability of a compound through its reaction with the stable DPPH radical. Concerning DPPH inhibition (%), all the samples proved to have high antioxidant activities (between 98.52 and 62.01%), with the exception of the *n*-Butanol (*n*-BuOH) fraction of flowers which was the lowest value (47.18%). So, the greatest EC_{50} obtained was that of extracts of flavonoids especially ethyl acetate (AcOEt) with 3.10 mg/ml. In the AcOEt fraction, the ranking of EC_{50} values is: leaves > stems > flowers > seeds. The results obtained from the evaluation of the total flavonoid content showed that leaves had the highest amount of flavonoids (flavonols) which have a high antioxidant activity in the AcOEt fraction (A_{AR} = 0.322) and in the *n*-BuOH fraction (A_{AR} = 0.118). This explains the nature of these molecules which are polar because the solvent used (AcOEt) is a polar solvent compared to *n*-BuOH which is a non polar solvent.

The EC_{50} values of the phenolic and flavonoid compounds have revealed that the leaf sample have lower DPPH radical-scavenging activity (EC_{50} = 3.10 mg/ml) compared to leaves analysed by Conforti et al. (2008) (EC_{50} = 0.61 mg/ml) and higher than ethanolic extracts by Ferreira et al. (2006). Therefore, the ethanolic extracts and their fractions used in our study proved to be more effective. Other authors (DellaGreca et al., 2009) reported, in a study on the aerial parts of mallow, a DPPH scavenging activity equal to 24% at 20 µg/ml.

Ferric reducing antioxidant power assay

Different studies have indicated that the electron donation capacity (reflecting the reducing power) of bioactive compounds is associated with their antioxidant activity

Table 2. DPPH free radical scavenging activity of different extracts of *Malva sylvestris* L.

Extract	Total phenolics content (TPC)		
	% inhibition	EC_{50}	A_{AR}
Leaves	93.11	16.691	0.059
Stems	98.52	76.483	0.013
Flowers	93.70	13.92	0.071
Seeds	91.08	59.389	0.016

Total flavonoids content (TFC)						
	AcOEt	*n*-BuOH	AcOEt	*n*-BuOH	AcOEt	*n*-BuOH
Leaves	81.29	87.78	3.10	8.43	0.322	0.118
Stems	79.62	62.01	3.359	16.35	0.297	0.061
Flowers	92.467	47.18	3.486	17.774	0.286	0.056
Seeds	95.397	64.033	3.787	14.242	0.264	0.070
Ascorbic acid	93.62		0.065		15.38	
Quercetin	94.616		0.049		20.408	

DPPH, expressed as percentage of inhibition (% inhibition), expressed as EC_{50} [the plant extract concentration (mg/ml) to determine the concentration of extract necessary to decrease DPPH radical scavenging by 50%] and expressed as antiradical activity (A_{AR}) defined as $1/EC_{50}$.

Table 3. FRAP assay of different extracts of *Malva sylvestris* L.

Extract	FRAP assay at 750 µg/ml		% of Reduction	
Total phenolics content (TPC)				
Leaves	0.144±0.083		10.632	
Stems	0.152±0.087		11.184	
Flowers	0.040±0.023		2.980	
Seeds	0.091±0.053		6.732	
Total flavonoids content (TFC)				
	AcOEt	*n*-BuOH	AcOEt	*n*-BuOH
Leaves	0.586±0.393	0.444±0.252	43.119	32.671
Stems	0.324±0,187	0.066±0.038	23.877	4.856
Flowers	0.363±0,209	0.102±0.062	26.747	7.505
Seeds	0.05±0,028	0.066±0.038	3.679	4.893
Ascorbic acid	1.359±0.245		/	/
Quercetin	1.142±0.125		/	/

FRAP, Ferric reducing ability expressed as µmol Fe^{2+}/mg dry weight of extract; percentage (%) of reduction was compared to ascorbic acid; results are expressed in mean ± standard deviation, $n = 3$.

(Siddhuraju et al., 2002; Yen et al., 1993). In this assay, the ability of extracts to reduce iron (III) to iron (II) was determined and compared to that of ascorbic acid, which is known to be a strong reducing agent (Arabshahi-Delouee and Urooj, 2007) and quercetin (Table 3). The lowest value for the phenolic extracts was detected in the flowers matrixes (0.040 ± 0.023). However, the flavonoid extracts, (AcOEt = 0.05 ± 0.028 and *n*-BuOH = 0.066 ± 0.038) showed the lowest values for seeds. All the extracts exhibited some degree of electron-donating capacity in a concentration-dependant manner, but the capacities were inferior to that of ascorbic acid (1.359 ± 0.245) and quercetin (1.142 ± 0.125). At 750 µg/ml, the most potent reducing agents were the flavonoid extracts from AcOET (43.110%) and n-BUOH (32.671%) fractions of leaves; whereas, the phenolic extracts from stems (11.184%) had a reducing power greater followed by leaf extracts (10.632%). This was possibly due to the flavonoid content of leaves which had the highest values. Leaf extract containing the highest amount of total phenols is the most potent reducing agent (AcOEt = 43.110%), while seed extracts containing the least amount of phenolics, was the weakest in the activity.

Similar relations between iron (III) reducing activity and total phenol content have been reported in the literature (Benzie and Strain, 1999; Gao et al., 2000; Zhu et al., 2002); however the correlation may not always be linear (Yildirim et al., 2000).

Table 4. Total antioxidant capacity (TAC) of different extracts of *Malva sylvestris* L.

Extract	Total phenolics content (TPC)	Total flavonoids content (TFC)	
		AcOEt	*n*-BuOH
Leaves	0.051±0.002	0.138±0.006	0.092±0.012
Stems	0.052±0.002	0.087±0.004	0.091±0.001
Flowers	0.060±0.002	0.114±0.009	0.118±0.018
Seeds	0.100±0.008	0.155±0.036	0.085±0.007

TAC expressed as mg equivalent of ascorbic acid/g of dried weight (mg EAA/g), results are expressed in mean ± standard deviation, $n = 3$.

Total antioxidant capacity (TAC)

In the phosphor molybdenum assay, which is a quantitative method to evaluate water-soluble and fat-soluble antioxidant capacity (total antioxidant capacity), the extracts exhibited some degree of activity in a dose dependent manner (Table 4). The extracts showed electron-donating capacity and thus they may have acted as radical chain terminators, transforming reactive free radical species into more stable non-reactive products (Dorman et al., 2003). This is the first study on TAC of the four parts of *M*. *sylvestris* L. Comparison between values of phosphomolibdic assay showed a higher antioxidant capacity to reduce molybdenum (VI) to molybdenum (V) reported for AcOEt fraction of seeds (0.155 ± 0.036 mg EAA/g) followed by AcOEt fraction of leaves (0.138 ± 0.006 mg EAA/g) and *n*-BuOH fraction of flower (0.118 ± 0.018 mg EAA/g) and AcOEt fraction of flowers (0.114 ± 0.009 mg EAA/g). From the results obtained, it was generally observed that AcOEt fraction was more active than the *n*-BuOH fraction with DPPH and FRAP method and this has also been obtained in several studies, so they were more active compared to other extracts. Apparently, the AcOEt fraction has a better capacity compared to other fractions probably due to higher hydrogen-donating components extracted by the solvent (Jung et al., 2008). The increase in antioxidant activity may be explained by the increase of solvent polarity. These results have been confirmed by other studies using the FRAP (Ardestani and Yazdanparast, 2007; Atmani, 2009; Li et al., 2009) and DPPH (Tian et al., 2009; Fabri et al., 2009) assays.

Antioxidant and radical scavenging properties of plant extracts is associated with the presence of phenolic compounds which are able to donate hydrogen to the radical. Numerous reports have indicated good correlation between the RSA and the concentration of phenolic compounds measured by Folin-Ciocalteu method (LoŽienė et al., 2007). It is worth noting that ethanol has proven to be more efficient compared to the acetone solvent for extraction of antioxidants from leaf parts of *M*. *sylvestris* L. Other authors also found a higher phenolic and flavonoid contents and antioxidant activity in leaves than in leafy flower stems and flowers, but the differences were not as remarkable as those found in our case (Barros et al., 2010). Flavonoids, among the most diverse and widespread groups of natural compounds, are probably the most natural phenolics (Shimoi et al., 1998). The highest activity was obtained from the 3',4' dihydroxy functional group in the B ring and the 4-carbonyl functional group in the ring C, and this provides the stability to flavonoxy radical (A°) which reacts with another radical obtained as a stable structure (quinone for flavonoids) (Harbone and Wiliams, 2000; Sharififar et al., 2009; Garrido et al., 2013).

Conclusion

The results of the present investigation indicate that the evaluation of the antioxidant activity by three methods, including DPPH free radical scavenging activity, ferric reducing antioxidant power (FRAP) and total antioxidant capacity (TAC) showed that AcOEt and BuOH fractions in leaves have a highest values of antioxidant activity. Flavonoid extracts possess a higher antioxidant activity compared to other extracts. Finally, the kind of mallow analyzed here can be considered as good sources of some phenolic and antioxidant compounds.

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