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Research Article

IN VITRO ANTIOXIDANT ACTIVITY OF ETHANOLIC AND AQUEOUS EXTRACTS FROM CRUDE *MALVA PARVIFLORA* L. GROWN IN LEBANON

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ABSTRACT

In the present work the total phenolic and total flavonoid contents from the extracts of crude leaves and stems of *Malva parviflora* grown in Lebanon have been determined using the Folin–Ciocalteu reagent and aluminum chloride methods. Also, different classic methods have been used for the determination of total saponins, alkaloids, lipids and ash content in these two parts of this plant. On the other hand the antioxidant ability of these extracts have been estimated using three different methods 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydrogen peroxide (H_2O_2) and chelating of ferrous ions tests. The obtained results demonstrated that ethanolic extracts of leaves of *M. parviflora* showed higher contents in both total phenolic and total flavonoid than found in the aqueous extract. The DPPH and H_2O_2 methods showed strong antioxidant ability of the different extracts from leaves of *M. parviflora*. Therefore, the chelating of ferrous ions test indicated strong antioxidant ability only for the aqueous extract. The extract of both crude leaves and stems of *M. parviflora* presents good antioxidant potential and it will be useful as good source of natural products.

Keywords: *Malva parviflora* L., Total phenolic, Total flavonoid, Antioxidant potential

INTRODUCTION

Oxidative stress is involved in the pathology of cancer, arteriosclerosis, malaria and rheumatoid arthritis, and could play a role in neurodegenerative diseases and ageing processes (Rammal et al., 2008a,b). It is well known that plants contain components of therapeutic value that may be used as alternative remedies for many diseases. About three quarter of the world's population relies on plants and plant extracts for their healthcare.

Malva parviflora L. belongs to the family Malvaceae. A hot poultice made from leaves is also used to treat wounds and swelling and is incorporated into a lotion to treat bruised and broken limbs (Shale et al., 1999). The leaves of *M. parviflora* have been used by the Xhosa people of South Africa for drawing swollen, inflamed purulent wounds (Watt and Breyer-Brandwijk, 1962). However, if ingested it could be toxic as *M. parviflora* is reported to cause mortality in foraging livestock, such as sheep, horses and cattle. Sheep are the most often affected and develop clinical signs including staggering, trembling arched back and labored breathing (Watt and Breyer-Brandwijk, 1962). In recent study, it was found that methanolic fraction of polyphenols from leaves and stems of this plant contain different amounts of phenols, flavonoid, saponin, alkaloid, resin and tannin (Farhan et al., 2012a). In this same study, this methanolic fraction showed high antioxidant potential (Farhan et al., 2012a).

The present study is aimed to quantify the total phenolic and total flavonoids compounds among others of the crude extracts from the stems and leaves of *M. parviflora*, an endemic Lebanese plant, and to evaluate its antioxidant capacity using three different tests, the DPPH, H_2O_2, and chelating of ferrous ions. Furthermore, spectrophotometric analyses were employed for the determination of total phenolic and total flavonoid concentrations of the crude extracts of this plant.

MATERIALS AND METHODS

Materials

Fresh plant was gathered from different regions in Lebanon on spring season between March and May in 2011 and the biological authentication was carried out by Professor George Tohme, president of C.N.R.S of Lebanon. Stems and leaves of *M. parviflora* were left on air at room temperature for two weeks to be very well dried. After that, they were crushed up and ground to get homogeneous fine powder by a grinder and then kept in a dark place at room temperature till their use in the different studies.

Chemicals

All the chemicals used were of analytical grade. Absolute ethanol and sodium hydroxide n-butanol, petroleum ether, ammonium hydroxide, acetic acid and sodium chloride were purchased from BDH England. Aluminum chloride and $FeSO4.7H_2O$ from were purchased Merck Germany. Sodium carbonate and hydrogen peroxide were purchased from Unichem India. Ascorbic acid, gallic acid, Folin-Ciocalteau reagent, Ferrozine and DPPH were purchased from sigma Aldrich, USA. Phosphate buffer solution (PBS) was purchased from Gibco, UK.

Preparation of crude extracts

10 grams of powdered leaves and stems of *M. parviflora* were putted into a flask with 500 mL of ethanol, and the mixture was then extracted by agitation for five hours at 25 °C. Then, a maceration of the extracts was done overnight for 24 h. After, the ethanolic layer containing the extract was taken. The extraction was repeated on the remaining amount of the precipitate using 150 mL of ethanol and all extracts were filtered by using a 0.45 millipore filter paper. After that, the two fractions of extracts were mixed together and then concentrated using a rotary evaporator at 40 °C under reduced pressure (227 m/bar for ethanol). After that, the extracts were stored at -20 °C till their usage in the different tests. The extracts resolved in ethanol and distilled water.

The aqueous extract has been prepared using the same steps of ethanolic extraction except the temperature of the extraction that should be 60 °C (Harborne, 1973).

Total phenolic content (TPC)

The Folin–Ciocalteau reagent method has been used for the estimation of total phenolic extracts quantities according to Lister and Wilson (2001) with slight modification. Five concentrations of all crude extracts of the used plant have been prepared and then 100 µL have been taken from each concentration and mixed with 0.5 mL of Folin–Ciocalteau reagent (1/10 dilution) and 1.5 mL of Na_2CO_3 2% (w/v). The blend was incubated in the dark at room temperature for 15 min. The absorbance of blue-colored solution of all samples was measured at 765 nm using a Gene Quant 1300 UV-Vis spectrophotometers. The results were expressed in mg of gallic acid equivalent (GAE) per g of dry weight of plant powders.

Estimation total flavonoids content

The aluminum chloride method was used according to Quettier-deleu et al. (2000) for the determination of total flavonoids content of all crude extracts of the studied plant. 1 mL of various concentrations of all crude extracts was mixed with 1 ml of 2%

methanolic aluminum chloride solution. After an incubation period at room temperature in the dark for 15 min, the absorbance of all samples was determined at 430 nm using a Gene Quant 1300 UV-Vis spectrophotometers. The results were expressed in mg per g of rutin equivalent (RE) and methanol was used as blank.

Saponins determinations

The determination of total saponin was done according to the method used by Obadoni and Ochuko (2001) with minor modifications. 1 g of powdered plant has been added to 100 ml of 20% aqueous ethanol and kept in a flask on stirrer for half hour and then heated over a for 4 h at 45 °C with mixing. The mixture was filtered by using filter paper whatman and the residue again extracted with another 100 ml of 25% aqueous ethanol. The combined extracts were concentrated by using rotary evaporator in 40 °C to gets 40 ml approximately. The concentrate was transferred into separator funnel and extracted twice with 20 ml diethyl ether. The ether layer was discarded while the aqueous layer was kept and then re- extracted with 30 ml n-butanol was added. The n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was evaporated. After evaporation, the samples were dried in the oven at 40°C to a constant weight. The saponin content was calculated using the following formula:

% saponin = [final weight of sample/initial weight of extracts] × 100

Total alkaloids

The quantification method for alkaloids determinations has been used according to Harborne (2005) with some modifications. 100 ml of 10% acetic acid in ethanol was added to 1 gram of dry powdered plant and then the extracts were covered and allowed to stand for 4 h. After that, the extracts have been filtrated and concentrated on a water bath to 25 ml of its original volume. The droplets of concentrated ammonium hydroxide were added to the extract until the precipitation the whole solution was allowed to settle, and then the precipitates were washed with dilute ammonium hydroxide and then filtered using filter paper whatman. The residue was dried in the oven at 40 °C and weighed. The alkaloid content was determined using the following formula:

% alkaloid = [final weight of the sample / initial weight of the extract] × 100.

Determination of total lipids

The estimation of the percentage of total fat (lipids) was done according to the method AACC (1984). 10 g of powders from leaves and stems of *M. parviflora* were added in Soxhlet apparatus with 200 ml of petroleum ether (40-60 °C) and extracted during 8 hours. After that, the solvent was filtered using Büchner funnel under reduced pressure, and then an evaporation using a rotary evaporator at 40 °C has been done. Finally, the weight of lipids has been calculated.

Estimate the proportion of ash

1 gram of powders of leaves and stems of *M. parviflora* has been tested to estimate the proportion of ash using the standard methods of AOAC (1980). 1 g was putted in and burned in a furnace burning (muffle furnace) at 550 °C for five hours till the obtaining of an ovary gray color of the powders. Then, we weighted the residues and the percentage of ash has been estimated according the essential dry weight of plant powder.

Determination of humidity content

The method mentioned in AACC (1984) was done to determine the moisture content of *M. parviflora*. 1 g of fresh leaves and 1 g of fresh stems of *M. parviflora* have been taken and placed in an oven at 105 °C for one hour. Then, they were putted in a desiccator for half hour. After that, the mass of each content has been noticed. Then, the two was returned again to the oven for another one hour. After heating, they were putted again in the desiccator for half hour. These steps have led to dry leaves and stems and their mass has been noticed again in order to calculate the percentage of humidity in these samples.

Evaluation of the antioxidant activity

DPPH radical scavenging activity

The method of Chew et al. (2009) has been used for the scavenging ability of DPPH antioxidant test with slight modification. 1 ml of different concentrations of diluted extracts of the plant parts in ethanol was added to 1mL of DPPH (0.15 mM in ethanol) and at the same time, a control consisting on 1ml DPPH with 1 ml ethanol was prepared. The reaction mixtures were mixed very well by hand and then incubated in the dark at room temperature for 30 min and the absorbance was measured at 517 nm by a Gene Quant 1300 UV-Vis spectrophotometer. The ascorbic acid was used as a positive control and the ethanol was used as blank. The DPPH scavenging ability of plant extracts was calculated using the following equation:

% scavenging activity = [(Abs control − Abs sample)]/ (Abs control)] ×100

The Abs control is the absorbance of DPPH + ethanol; Abs sample is the absorbance of DPPH radical + sample. Also, three controls have been prepared.

Scavenging activity of H_2O_2 radical

The H_2O_2 scavenging of the crude extracts of *M. parviflora* was determined according to the method of Ruch et al. (1989). A solution of H_2O_2 (40 mM) was prepared in PBS (pH 7.4) and concentration was determined spectrophotometrically (Gene Quant 1300 UV-Vis) at 230 nm. Different concentrations of extracts from stems and leaves of this plant in distilled water were added to a H_2O_2 solution (0.6 ml, 40 mM) and the absorbance of H_2O_2 at 230 nm was determined after 10 min against a blank solution containing the plants extracts without H_2O_2. Ascorbic acid was used as stander reference.

The percentage scavenging of H_2O_2 was calculated using the following equation:

% scavenged [H_2O_2] = [(Abs control – Abs sample) / Abs control] × 100.

Chelating effects on ferrous ions

The method of Dinis et al. (1994) has been used to estimate the chelating effect on ferrous ions with some modifications. 0.5 ml of various concentrations of all extracts was mixed with 0.5 ml of $FeSO_4$ (0.12 mM), and with 0.5 ml of Ferrozine (0.6 mM). The mixtures were allowed to stand for 10 min at room temperature. After incubation, the absorbance was measured by Gene Quant 1300 UV- Vis spectrophotometrically at 562 nm. Ultra-pure water of sample solution was used as a control without extracts, ultra-pure water instead of Ferrozine solution was used as a blank. EDTA-Na_2 was used as reference standard. All measurements were performed in Triplicate. The Ferrozine solution (3-[2-Pyridyl]-5,6-diphenyl-1,2,4-triazine-4,4′-disulfonic acid Na-salt) (0.6 mM) was prepared in Ultra-pure water and stored in the dark at room temperature. The ability of the sample to chelate ferrous ion was calculated relative to the control (consisting of iron and Ferrozine only) using the formula:

Ferrous ion - chelating ability (%) = [(*Abs control − Abs sample) / Abs control]* ×100

STATISTICAL ANALYSIS

All analyses were carried out in triplicates. The results of scavenger activity and total phenolic and total flavonoids contents were performed from the averages of all samples reading Mean ± SD used Excel 2003.

RESULTS AND DISCUSSION

Antioxidants are substances that delay the oxidation process, inhibiting the polymerization chain initiated by free radicals and other subsequent oxidizing reactions (Halliwell and Aruoma, 1991). It has been proposed that the health beneficial effects of polyphenols could result from either their antioxidant functions and/or independently from these properties e.g. by acting as modulators of cellular signaling processes (Bouayed, 2010; Rammal et al., 2012).

Our obtained results showed that both leaves and stems of *M. parviflora* have exerted high antioxidant power at different concentrations. The DPPH test demonstrated that 4 mg/ml of the aqueous and ethanolic extracts from leaves of *M. parviflora* have significantly increased the % of scavenger activity by 59 % and 70 % respectively as shown in Fig 1.

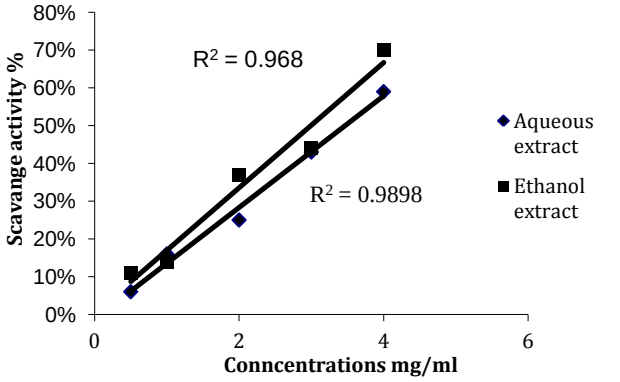

Fig 1:Positive correlation between the concentration of leaves of *M. parviflora* and the DPPH test.

On the other side, 4 mg/ml of the aqueous and ethanolic extracts from stems of *M. parviflora* have significantly increased the % of scavenger activity by 51 % and 56 % respectively as shown in Fig 2.

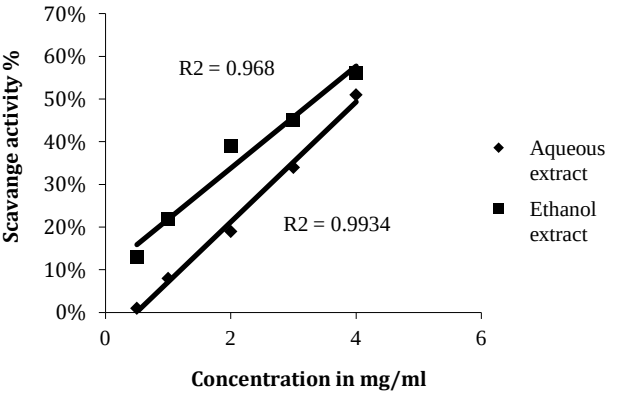

Fig 2:Positive correlation between the concentration of stems of *M. parviflora* and the DPPH test.

On the other hand and in order to confirm the antioxidant power of this plant another known test has been used in our study, the H2O2 test. Hydrogen peroxide is an important reactive oxygen species because of its ability to penetrate biological membranes. However, it may be toxic if converted to hydroxyl radical in the cell (Gulcin et al., 2003). Scavenging of H2O2 by the plant extracts may be attributed to their phenolics, which donate electron to H2O2, thus reducing it to water.

Our results demonstrated that both extracts from leaves of *M. parviflora* were capable of scavenging hydrogen peroxide in a concentration dependent manner. The % of scavenger activity of 3 mg/ml of leaves of *M. parviflora* was 83 % and 73 % for the ethanolic and aqueous extracts respectively as shown in Fig 3 and Fig 4. Therefore, the % of scavenger activity of 3 mg/ml of stems of *M. parviflora* was 58 % and 64 % for the ethanolic and aqueous extracts respectively as shown in Fig 3 and Fig 4.

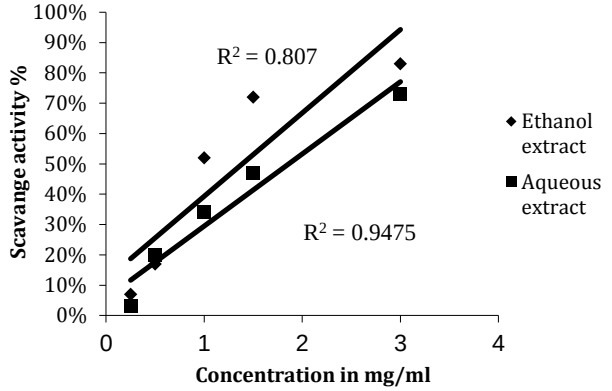

Fig 3:Positive correlation between the concentration of leaves of *M. parviflora* and the H_2O_2 test.

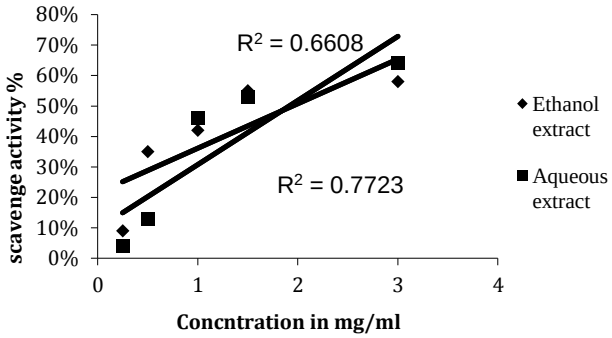

Fig 4:Positive correlation between the concentration of stems of *M. parviflora* and the H_2O_2 test.

Antioxidants inhibit interaction between metal and lipid through formation of insoluble metal complexes with ferrous ion (Hsu et al., 2003). The iron-chelating capacity test measures the ability of antioxidants to compete with ferrozine in chelating ferrous ion (Elmastas et al., 2006).

Fig 5 and Fig 6 show the metal chelating effect of two extracts from *M. parviflora* leaf and stem. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of samples possessing chelating activity, the formation of complexes is decreased. Therefore, measurement of the rate of color reduction helps to estimate the chelating activity of the samples. As shown in fig 5, chelating capacity of the extracts increased with increase in concentration. The order of metal chelating effect of extracts of 5 mg/ml of leaves is: Aqueous extract (72 %) > Ethanolic extract (65 %). Chelating effect of extracts of 5 mg/ml of stems is: Aqueous extract (67 %) > Ethanolic extract (58 %). Among the two extracts of leaves and stems, aqueous extract show higher chelating activity compared to the ethanolic extracts of both leaves and stems of this plant.

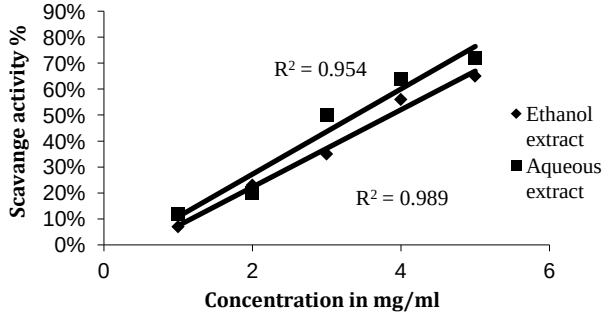

Fig 5:Scavenging activity of the iron chelating radical potential of leaves of *M. parviflora.*

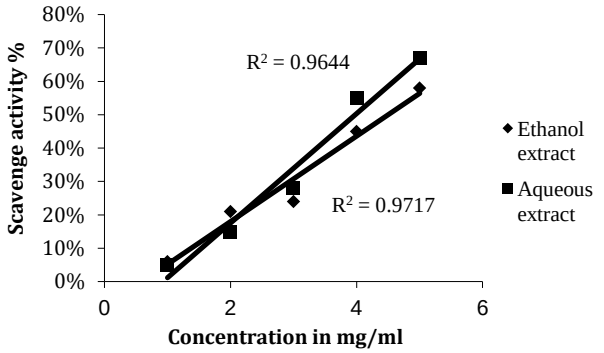

Fig 6:Scavenging activity of the iron chelating radical potential of stems of *M. parviflora.*

In order to determine the concentration of the extracts at which 50 % of the initial DPPH were decreased, the IC_{50} was studied. Our results showed that the IC_{50} of DPPH, H_2O_2 and Iron chelating tests was different between leaves and stems of the studied plant as shown in Table 1, Table 2 and Table 3 respectively.

Table 1:IC_{50} values of stems and leaves of the plant for the DPPH test

Scavenge activity	Ic_{50}
Ascorbic acid	2.5 µg/mL
Aqueous extract of stems	4 mg/ml
Ethanol extract of stems	3.3 mg/ml
Aqueous extract of leaves	3.4 mg/ml
Ethanol extract of leaves	2.9 mg/mL

Table 2: IC_{50} values of stems and leaves of the plant for the H_2O_2 test

Scavenge activity	Ic_{50}
Ascorbic acid	10 µg/mL
Aqueous extract of stems	1.5 mg/ml
Ethanol extract of stems	1.9 mg/ml
Aqueous extract of leaves	1.8 mg/ml
Ethanol extract of leaves	1 mg/ml

Table 3. IC_{50} values of stems and leaves of the plant for the Iron chelating test

Scavenge activity	Ic_{50}
EDTA	5 µg/mL
Aqueous extract of stems	3.9 mg/ml
Ethanol extract of stems	4.5 mg/ml
Aqueous extract of leaves	3 mg/ml
Ethanol extract of leaves	3.8 mg/ml

A number of reports on flavonoids, triterpenoids, and polyphenols indicated that they possess antioxidant and free radical scavenging activity (Farhan et al., 2012a,b), 1995). These phytoconstituents may exert multiple biological effects against tumors, heart disease, AIDS, and different pathologies due to their free radical scavenging activities. The total phenolic content (TPC) and total flavonoid content (TFC) in leaves and stems of the studied plant have been evaluated. As shown in Table 4, both leaves and stems contain high amounts of TPC and TFC. This amount has increased with the concentration of the extracts.

Table 4:The amount of TPC and TFC in the leaves and stems of *M. parviflora* (mg CAE/g dry weight).

Extracts	Leaves		Stems	
	Aqueous	Ethanol	Aqueous	Ethanol
Total phenol	1.99 ± 0.070	2.24 ± 0.031	1.47 ± 0.028	1.61 ± 0.043
Total flavonoid	0.83 ± 0.063	1.07 ± 0.031	0 .91 ± 0.023	0. 98 ± 0.041

Values are the average of triplicate experiments and values expressed as mean ± SD

Both studied parts of *M. parviflora* contain different amounts of active compounds as shown in Table 5. These amounts were different between leaves and stems.

Table 5: Percentage of active contents of both parts of *M. parviflora*

Parts	Leaves	Stems
Total saponin	0.0184 ± 0.016	0.0313 ± 0.028
Total alkaloid	2.2 ± 0.011	1.46 ± 0.07
Total ash	0.0871 ± 0.010	0.0551 ± 0.018
Total lipids	8.66 ± 0.013	6.49 ± 0.04
% Humidity	79.85 ± 0.011	76.10 ± 0.005

Values are the average of triplicate experiments and values expressed as mean ± SD

CONCLUSION

In the light of all these results we can suggest that the studied plant possesses higher antioxidant activity and by consequence, it can be considered as good sources of natural products that may be employed in the treatment of the different diseases associated to the oxidative stress.

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