

ANTIOXIDANT ACTIVITY OF POLYPHENOL AND FLAVONOID RICH EXTRACTS FROM LEAVES OF MYRTLE (*MYRTUS COMMUNIS* L)

Eslami, Sh.; Mozdastan, Sh.; Ebrahimzadeh M.A.*

Pharmaceutical Research Center, School of Pharmacy, and Student Research Committee, Mazandaran University of Medical Sciences, Sari, Iran.

*zadeh20@gmail.com

Abstract

Antioxidant activities of polyphenol (PP) fraction and flavonoid rich (FR) fraction from *M. communis* L. leaf were evaluated with four different in vitro antioxidant tests. PP fraction exhibited very strong DPPH radical scavenging activities ($IC_{50} = 3.5 \pm 0.07 \mu\text{g ml}^{-1}$) than did controls ($p < 0.001$ and $p > 0.05$) and FR fraction ($10.8 \pm 0.1 \mu\text{g ml}^{-1}$, $p < 0.05$). PP fraction exhibited high reducing power at 5-80 $\mu\text{g ml}^{-1}$. It showed better reducing power than did FR fraction ($p < 0.05$), but vitamin C showed the highest activity ($p < 0.05$). PP fraction showed good Fe^{2+} chelating ability (70% at 400 $\mu\text{g ml}^{-1}$). Extracts also showed very strong nitric oxide scavenging activity between 5 and 160 $\mu\text{g ml}^{-1}$. The inhibition increased with increasing concentration of the extract. PP fraction showed better activity than FR fraction. The plants high phenolic and flavonoid contents could be responsible for its antioxidant activity and pharmacologic actions.

Key words: DPPH, *M. communis*, Polyphenol fraction, flavonoid rich fraction.

Introduction

Free radicals have been shown to be involved in many biological processes that cause damage to important cellular components and tend to a variety of diseases [1]. Antioxidants are molecules that inhibit the oxidation of other molecules. Antioxidants are of great importance in terms of oxidative stress prevention. Nowadays, research has focused on medicinal plants to extract new natural antioxidants. Intensive research is being carried out on utilization of natural antioxidants that may serve as potent antioxidant [2]. It has been shown that the antioxidant activities of plant products are mainly due to presence of phenolic acids and flavonoids [3,4]. *Myrtus communis* L. (Myrtle, Myrtaceae) is an example of functional fruit traditionally used. Myrtle is traditionally used as an antiseptic, antiinflammatory and hypoglycaemic agent [5]. Different parts of the plant find various uses in food and cosmetic industries [6]. Effect of increasing the polarity of solvent and impact of different extraction methods on total phenol and flavonoid contents and antioxidant activity of Myrtle have been reported recently [7,8]. Some studies on myrtle have focused on its phenolic compounds in leaves and berries [5]. Previous studies on *M. communis* L. Leaf have revealed the presence of several specific chemical compounds such as phenolic acids and flavonoids [6]. Polyphenols are an important class of secondary metabolites of plant possessing a variety of pharmacological activities. Plant phenolics are multifunctional and can act as reducing agents, metal chelators and singlet oxygen quenchers. Studying medicinal plants are of particular interest as they often contain high amounts of bioactive compounds [9]. In spite of some reports pertaining to antioxidant activity of Myrtle, very limited data was found regarding polyphenol and flavonoids rich fractions [10,11]. The aim of this work was to evaluate the antioxidant activities of polyphenol and flavonoids rich extracts from *M. communis* L. leaf to identify possible constituents responsible for antioxidant activity. Such information is valuable because it can prove beneficial for maintenance of optimal health and may increase the demand of these bioactive substances by food, cosmetic and pharmaceutical industries.

Methods

Plant material and preparation of extracts

Myrtle (*Myrtus communis* L) leaves were collected from Lorestan, Iran in the summer of 2013, and identified by Dr Bahman Eslami. Voucher specimen

was deposited in the Herbarium of Sari Faculty of Pharmacy (No. 1385). Leaves were dried at room temperature and coarsely ground (2-3 mm) before extraction.

Preparation of polyphenol fraction

Polyphenols were extracted according to our recently published paper [12]. The extraction was performed twice at 20°C with a shaking incubator. Extracting time was 30 min, and extracting solvent was methanol/acetone/water (3.5/ 3.5/ 3) containing 1% formic acid. All extracts were collected and evaporated under vacuum at 35-40°C to remove methanol and acetone. Lipophilic pigments were then eliminated from the aqueous phase by extraction with petroleum ether. The aqueous phase was collected and further extracted three times by ethyl acetate. Organic phases were collected and concentrated over a rotary vacuum until a crude solid extract was obtained, which was then freeze-dried for complete solvent removal and used as polyphenol (PP) fraction (yield: 3.6%).

Preparation of flavonoid rich fraction

For preparation of flavonoid rich fraction, plant leaf powder was defatted twice with CHCl₃ and extracted twice with 60% acetone for 12 hours at room temperature. The solvent was removed, leaving the crude acetone extract. After preparation of 10% methanol slurry, the crude acetone extract was fractionated sequentially with n-hexane and ethyl acetate. The ethyl acetate was used as flavonoid rich fraction (yield: 1.8%) [13].

Determination of total phenolic content

Total phenolic content was measured by colorimetric method using the Folin-Ciocalteu [14,15]. The extract samples (0.5 ml) were mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) for 5 min and aqueous Na₂CO₃ (4 ml, 1 M) were then added. The absorbance of the reaction mixture was measured at 760 nm after incubation for 2 h at room temperature. Gallic acid was used as a reference standard. The standard curve was prepared by 0, 50, 100, 150, 200, and 250 mg ml⁻¹ solutions of gallic acid in aqueous methanol (50%). The results were expressed as milligram gallic acid equivalent (mg GAE) /g of extract.

Determination of total phenolic compounds and flavonoid content

The total flavonoid content was determined by our recently published paper [14,15]. 0.5 ml solution of each extracts (in methanol) were separately mixed

with 1.5 ml of methanol, 100 μ L of 10% AlCl_3 , 100 μ l of 1 M potassium acetate, and 2.8 ml of distilled water, and left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm with a double beam UV/Visible spectrophotometer. Total flavonoid contents were calculated as quercetin equivalent from a calibration curve. The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100 mg ml⁻¹ in methanol.

DPPH radical-scavenging activity

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extracts [16]. 2 ml of each extracts (with different concentrations) were added to 2 ml of methanolic solution of DPPH (100 μ M). After 15 min at room temperature in the dark, the absorbance was read at 517 nm. The experiment was repeated for three times. Percentage of inhibition was calculated as $[(A_0 - A_s)/A_s] \times 100$, where A_0 was the absorbance of the control, and A_s was the absorbance of the extract/ standard. Vitamin C and BHA were used as control for comparison. IC₅₀ values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

Determination of reducing power

Determination of reducing power was determined according to our recently published paper [2]. Different amounts of each extracts in water (1 ml) were mixed with phosphate buffer (1 ml, 0.2 M, pH 6.6) and potassium ferricyanide (1 ml, 1%). After incubation of the mixture for 20 min at 50 $^{\circ}\text{C}$, 1 ml of trichloroacetic acid (10%) was added to stop the reaction. The mixture was centrifuged at 3000 rpm for 10 min. 1 ml of the resulting supernatant was mixed with 1 ml of distilled water, and FeCl_3 (100 μ l, 0.1% in water). The absorbance was measured at 700 nm. Vitamin C was used as control for comparison.

Fe²⁺ chelating activity

The chelating of ferrous ions by extracts was estimated by our recently published paper [3,4]. 1 ml of different concentrations of extracts were added 2.8 ml of distilled water and then mixed with 50 μ l of 2 mM FeCl_2 and 150 μ l of ferrozine (5 mM). The mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine- Fe^{2+} complex formation was calculated as

$[(A_0 - A_s)/A_s] \times 100$, where A_0 was the absorbance of the control, and A_s was the absorbance of the extract/ standard. Na₂EDTA was used as positive control.

Assay of nitric oxide-scavenging activity

Nitric oxide scavenging activity was measured spectrophotometrically. For this experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline (pH 7.4) was mixed with different concentrations of each samples dissolved in water (and methanol as a co-solvent) and left at 25 $^{\circ}\text{C}$ for 150 min. The same reaction mixture, without extract or essential oil but with an equivalent amount of water, served as control. After the incubation period, 0.5 ml of Griess reagent (sulphanilamide (1%) and N-1-naphthylethylenediamine dihydrochloride (0.1%) in phosphoric acid (2%)) was added. The absorbance of the pink color formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with N-1-naphthylethylene diamine dihydrochloride was measured at 546 nm and the percentage of scavenging activity was measured with reference to standard (quercetin) [16]. The IC₅₀ value is the inhibitory concentration at which nitric radicals were scavenged by 50%.

Results and Discussion

Polyphenols are an important class of secondary metabolites of plant possessing various pharmacological activities such as vasodilatory, antimicrobial, antioxidant and anti-inflammatory actions. They can act as antioxidants by donating hydrogen to highly reactive radicals, thereby preventing further radical formation [17]. The antioxidant effect of naturally occurring phenolic components has previously been studied by several authors. Myrtle leaf had high total phenolic content (3.6%, 1218.3 \pm 26.3 mg ml⁻¹ GAE). Highly positive linear relationship exists between antioxidant activity and total phenolic contents in many spices and herbs [3,4]. Consistent with this, polyphenol fraction may be a resource of efficient natural antioxidant and could be useful in relation to diseases involving free radical reactions. Flavonoids are of interest in food and cosmetic industries because of their antioxidant activity [3,4]. Myrtle leaf had high flavonoids contents (1.8%, 596.7 \pm 18.5 mg of QE ml⁻¹). Flavonoids may slow the pathogenesis of cardiovascular disease and atherosclerosis and by their free radical scavenging activities. A good relationship between intake of dietary flavonoids and decrease in the risk of cardiovascular disease has been reported [18].

DPPH radical-scavenging activity has been extensively used for screening antioxidants. Polyphenols donate hydrogen, forming the reduced DPPH. This method has been used widely to evaluate the radical scavenging ability of antioxidants from different plants due to its advantage of short time and sensibility [12]. PP fraction exhibited very strong DPPH radical scavenging activities ($IC_{50} = 3.5 \pm 0.07 \mu\text{g ml}^{-1}$) than did controls (IC_{50} of BHA was $53.8 \pm 1.4 \mu\text{g ml}^{-1}$, $p < 0.001$, and vitamin C, $5.1 \pm 0.02 \mu\text{g ml}^{-1}$, $p > 0.05$) and FR fraction ($10.8 \pm 0.1 \mu\text{g ml}^{-1}$, $p < 0.05$) (Table 1). In the reducing power assay, antioxidants reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) by donating an electron. Amount of Fe^{2+} complex can be detected by measuring the formation of Prussian blue at 700 nm [9]. A dose-response relationship was also found in the reducing power assay (Figure 1). PP fraction showed better reducing power than did FR fraction ($p < 0.05$), but vitamin C showed better activity ($p < 0.05$). It seems PP fraction has reductive potential and can serve as electron donors, terminating the radical chain reaction. Chelation therapy reduces iron-related complications in human and improves quality of life in some diseases such as Thalassemia pathology and Alzheimer's disease [2]. Iron ions generate free radicals from peroxides via Fenton reactions. Ferrozine can form complex with ferrous ion. In the presence of other chelating agents, this complex is less formed and the red colour of complex decreases. Extracts interfered with the formation of this complex, i.e. they have chelating activity and can capture Fe^{2+} before ferrozine. PP fraction showed good Fe^{2+} chelating ability (70% at $400 \mu\text{g ml}^{-1}$) (Table 1).

Extracts also showed very good nitric oxide scavenging activity between 5 and $160 \mu\text{g ml}^{-1}$. The inhibition increased with increasing concentration of the extract. PP fraction showed better than FR fraction. Quercetin, used as control, showed higher scavenging activity with $IC_{50} = 17.01 \pm 0.11 \mu\text{g ml}^{-1}$. In addition to reactive oxygen species, nitric oxide is also implicated for other pathological conditions such as cancer, inflammation and central nervous system problems. Scavenging activity of nitric oxide may help to stop the chain of reactions initiated by the detrimental excess generation of NO [16]. In conclusions, polyphenol and flavonoid rich fractions had significant antioxidant and free radical scavenging activities in different test. This gives strong support for expanding the investigations of Myrtle for use in food and cosmetic industries and in traditional folk remedy.

Acknowledgments

This research was supported by a grant from the research council of Mazandaran University of Medical Sciences, Iran.

References

1. Nabavi, S.F., Nabavi, S. M., Ebrahimzadeh, M. A., Eslami, B., Jafari, N., In vitro antioxidant and antihemolytic activities of hydroalcoholic extracts of *Allium scabriscapum* Boiss. & Ky. aerial parts and bulbs, *Int J Food Prop* 2013; 16:4, 713-722
2. Ebrahimzadeh, M.A., Nabavi, S.M., Nabavi, S.F., Eslami, Sh., Antioxidant and free radical scavenging activities of culinary-medicinal mushrooms, golden chanterelle *Cantharellus cibarius* and angel's wings *Pleurotus porrigens*. *Int J Med Mushrooms* 2010; 12: 265-272.
3. Ebrahimzadeh, M.A., Nabavi, S.M., Nabavi, S.F., Correlation between the invitro iron chelating activity and polyphenol and flavonoid contents of some medicinal plants. *Pak J Biol Sci* 2009; 12, 934-938.
4. Ebrahimzadeh, M.A., Pourmorad, F., Bekhradnia, A.R., Iron chelating activity screening, phenol and flavonoid content of some medicinal plants from Iran. *Afr J Biotech* 2009; 7: 3188-3192.
5. Wannes, W.A., Mhamdi, B., Mariem Ben Jemia, J.S., Ouchikh, O., Hamdaoui, G., Elyes Kchouk, M., Marzouk, B., Antioxidant activities of the essential oils and methanol extracts from myrtle (*Myrtus communis* var. *italica* L.) leaf, stem and flower. *Food Chem Toxicol* 2010; 48: 1362-1370.
6. Kanoun, K., Belyagoubi-Benhammou, N., Ghembaza, N., Atik Bekkara, F., Comparative studies on antioxidant activities of extracts from the leaf, stem and berry of *Myrtus communis* L. *Int Food Res J* 2014; 21: 1957-1962.
7. Mozdastan, Sh., Ebrahimzadeh, M.A., Khalili, M., Comparing the impact of different extraction methods on antioxidant activities of myrtle (*Myrtus communis* L.). *J Mazandaran Univ Med Sci* 2015; 25(127): 10-24
8. Mozdastan, Sh., Ebrahimzadeh, M. A., Eslami Sh., Effect of increasing the polarity of solvent on total phenol and flavonoid contents and antioxidant activity of Myrtle (*Myrtus communis* L.). *J Mazandaran Univ Med Sci* 2015; 25(126): 68-81
9. Ebrahimzadeh, M.A., Nabavi, S.F., Nabavi, S.M., Eslami, B., Antihemolytic and antioxidant activities of *Allium paradoxum*. *Cen Eur J Biol* 2010; 5: 338-345.
10. Montoro, P., Tuberose, C.L., Piacente, S., Perrone, A., Feo, V., De Cabras, P., Pizza, C., Stability and antioxidant activity of polyphenols in extracts of *Myrtus communis* L. *J Pharm Biomed Anal* 2006; 41: 1614-1619.
11. Amensour, M., Sendra, E., Abrini, J., Perez-Alvarez, J. A., Fernandez-Lopez, J., Antioxidant activity and total phenolic compounds of Myrtle extracts. *CyTA- J Food* 2010; 8: 95-101.
12. Rabiei, Kh., Bekhradnia, S., Nabavi, S.M., Nabavi, S.F., Ebrahimzadeh, M.A., Antioxidant activity of polyphenol and ultrasonic extracts from fruits of *Crataegus pentagyna* subsp. *elburensis*. *Nat Prod Res* 2012; 26: 2353-2357.
13. Naqinezhad, A., Nabavi, S.M., Nabavi, S.F., Ebrahimzadeh, M.A., Antioxidant and antihemolytic activities of flavonoid rich fractions of *Artemisia tschernieviana* Besser. *Eur Rev Med Pharmacol Sci* 2012; 16: 88-94.
14. Ebrahimzadeh, M.A., Nabavi, S.M., Nabavi, S.F., Eslami, B., Ehsanifar, S., Antioxidant activity of *Hyoscyamus squarrosus* fruits. *Pharmacologyonline* 2009; 2: 644-650.
15. Ebrahimzadeh, M.A., Nabavi, S.M., Nabavi, S.F., Dehpour, A.A., Antioxidant activity of hydroalcoholic extract of *Ferula*

- gummosa Boiss roots. *Eur Rev Med Pharmacol Sci* 2011; 15(6): 658-664.
16. Mahmoudi, M., Ebrahimzadeh, M.A., Nabavi, S.F., Hafezi, S., Nabavi, S.M., Eslami, Sh., Antiinflammatory and antioxidant activities of gum mastic. *Eur Rev Med Pharmacol Sci* 2010; 14: 765-769.
17. Cai, Y.Z., Luo, Q., Sun, M., Corke, H., Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sciences* 2004; 74: 2157-2184.
18. Hertog, M.G., Feskens, E.J., Hollman, P.C., Katan, M.B., Kromhout, D., Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. *Lancet* 1993; 342: 1007-1011.

Table 1. Phenolic contents and antioxidant activities of *M. communis* leaf polyphenol (PP) and flavonoid rich (FR) fractions.

Extracts	Total phenolic contents (mg ml ⁻¹)	Flavonoid contents (mg ml ⁻¹)	DPPH radical scavenging, IC ₅₀ (µg ml ⁻¹) ^a	Nitric oxide scavenging IC ₅₀ (µg ml ⁻¹) ^b	Fe ²⁺ chelating (%) ^c
PP fraction	1218.3 ± 26.3	510.2 ± 16.4	3.5 ± 0.07	57.9 ± 2.3	70.2 ± 1.4
FR fraction	596.7 ± 18.5	153.3 ± 9.1	10.8 ± 0.1	151.1 ± 6.7	63.2 ± 0.9

^a IC₅₀ of BHA was 53.8 ± 1.4, vitamin C, 5.1 ± 0.02 µg ml⁻¹, respectively.

^b IC₅₀ for quercetin was 17.01 ± 0.11 µg ml⁻¹.

^c At 400 µg ml⁻¹, EDTA used as control (IC₅₀ = 4.2 ± 0.2 µg ml⁻¹).

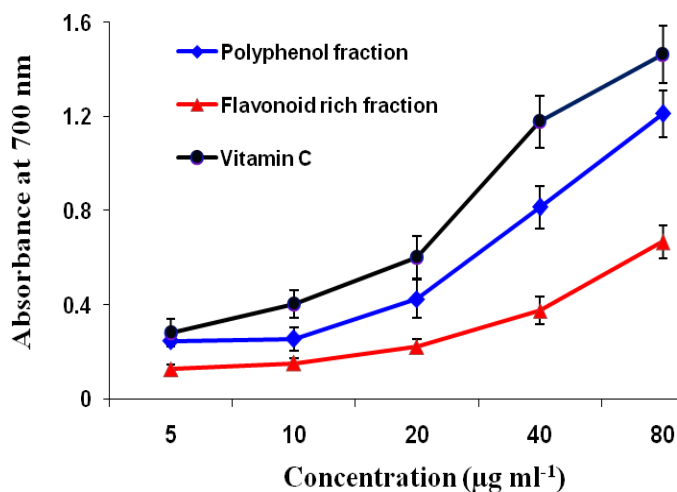


Figure 1. Reducing power of *M. communis* leaf polyphenol (PP) and flavonoid rich (FR) fractions. Vitamin C used as control.