

Comparative studies on antioxidant activities of extracts from the leaf, stem and berry of *Myrtus communis* L.

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Abstract

Myrtle (*Myrtus communis* L.) leaf, stem and berry extracts were prepared to examine the total phenolics, flavonoids and condensed tannins contents. The antioxidant activities of crude extracts, ethyl acetate fraction, butanolic fraction, tannins and anthocyanins from these parts of plant were evaluated in vitro using reducing power and DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging tests. The total phenol contents varied between different myrtle parts: leaf extract had higher total phenol content expressed as gallic acid equivalents (GAE)/g dry matter (119.23 ± 0.77 mg/g) than stem (112.96 ± 0.53 mg/g) and berry (70.26 ± 0.71 mg/g) extracts. The highest contents of total flavonoids and condensed tannins expressed as catechin equivalents (CE)/g dry matter were observed in leaf (6.56 ± 0.57 mg/g) and berry extracts (27.20 ± 0.25 mg/g), respectively. The ethyl acetate fraction of leaf exerted greater antioxidant activity followed by leaf butanolic fraction and berry anthocyanins extracts. The efficient concentrations (EC_{50}) of this fraction were 0.09 ± 0.002 mg/mL for reducing power and 0.26 ± 0.004 mg/mL for scavenging DPPH radical. This better activity was confirmed by thin layer chromatography (TLC), where we identified the presence of gallic acid in all parts of plant, catechin in leaf and quercetin in berry.

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Introduction

Antioxidants are of great importance in terms of oxidative stress prevention, which may result from several degenerative diseases (Helen *et al.*, 2000). Nowadays, research has focused on medicinal plants to extract new natural antioxidants that can replace synthetic additives (Dastmalchi *et al.*, 2007). Polyphenols are an important class of secondary metabolites of plant possessing an impressive array of pharmacological activity (Djeridane *et al.*, 2007). They possess a wide range of biological effects, including antioxidant, antimicrobial, anti-inflammatory and vasodilatory actions (Wang *et al.*, 2009). They can act as antioxidants by donating hydrogen to highly reactive radicals, thereby preventing further radical formation (Lapornik *et al.*, 2005). The antioxidant effect of naturally occurring phenolic components has previously been studied by several authors.

Myrtus communis L. is one of the important aromatic and medicinal species from the Myrtaceae family. It is naturally widely distributed in the mediterranean regions (Pottier-Alapetite, 1979). In Algeria, it is present in Tell on the slopes of hills and on coastal areas from east to west, sometimes in remote areas (Quezel and Santa, 1963). Different parts of the plant find various uses in food and

cosmetic industries (Chalchat *et al.*, 1998). Myrtle is better known as a medicinal plant for its anti-hyperglycemic (Elfellah *et al.*, 1984), antiseptic and anti-inflammatory activities (Diaz and Abeger, 1987; Al-Hindawi *et al.*, 1989).

Myrtus species have been reported as very rich in volatile oils (Shikhiev *et al.*, 1978; Satrani *et al.*, 2006), phenolic acids, flavonoids (Joseph *et al.*, 1987; Romani *et al.*, 1999), tannins (Diaz and Abeger, 1986), anthocyanin pigments (Martin *et al.*, 1990) and fatty acids (Cakir, 2004). Previous studies on *M. communis* L. aerial parts have also revealed the presence of several specific chemical compounds, for example, the essential oils, phenolic acids, flavonoids and tannins in leaf and flowers (Messaoud *et al.*, 2005; Aidi Wannes *et al.*, 2010) and anthocyanin, fatty and organic acids in berries (Martin *et al.*, 1990; Tuberoso *et al.*, 2010; Messaoud *et al.*, 2012). However, little researches have undertaken the antioxidant activity of myrtle leaf, stem and flower essential oil (Yadegarinia *et al.*, 2006; Aidi Wannes *et al.*, 2010) and extract (Hayder *et al.*, 2004; Sacchetti *et al.*, 2007; Gardeli *et al.*, 2008; Amensour *et al.*, 2009), but there is no information regarding principal secondary metabolites of stem, leaf and berry. The aim of this work was to evaluate the antioxidant activity of methanolic crude extract, ethyl acetate fraction, butanolic fraction, tannins and anthocyanins

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extracts from *M. communis* L. leaf, stem and berry, and to identify possible constituents responsible for this activity by TLC.

Materials and Methods

Plant material

The plant samples *M. communis* L. were collected during December 2009 and February 2010 from Honaine locality, 69 km north Tlemcen (Algeria). The plant material identification was carried out at the Laboratory of Botany (University of Tlemcen), according to the new flora of Algeria (Quezel and Santa, 1963). The three parts (leaves, stems, berries) were separated and dried at room temperature. After, each part was manually ground to a fine powder.

Extractions of chemical compounds from the leaves, the stems and the berries

Methanolic extract

One gram of each part (leaf, stem and berry) was extracted in 20 mL of methanol 96.6° for 24 h at room temperature, followed by rapid paper filtration through Whatman No 0.45 µm filter paper. The resulting solutions were evaporated under vacuum at 60°C by Buchi Rotavapor R-200 to dryness. The residues were then dissolved in 3 mL of methanol.

Ethyl acetate and butanolic fractions

The leaf, stem and berry dry residues obtained by the same procedure for methanolic extracts extraction were treated with 10 mL of boiling water to dissolve the flavonoids. Further filtration through filter paper No 0.45 µm, afforded the aqueous solution that was firstly extracted with 10 mL of ethyl acetate, then with 10 mL of n-butanol. The extracts were evaporated and weighed, then dissolved in 3 mL of methanol (Bekkara et al., 1998).

Tannins

The extraction of tannins from *M. communis* L. was obtained according to the method of Zhang et al. (2008). The powder (5 g) of each part (leaf, stem, berry) was extracted with 50 mL acetone-water (35/15, v/v) and stirred continuously for 72 h at room temperature. Then, the mixture was filtrated and evaporated under vacuum at 40°C to remove acetone. The remaining solution was washed with 15 mL dichloromethane to remove lipid soluble substances. After that, the solution was further extracted with ethyl acetate at a ratio of 15/15 (v/v). The water layer was separated and extracted twice more similarly. Then the resulting water layer was evaporated to dryness, and the resulting substance was weighed

and dissolved in methanol.

Anthocyanins

The extraction of anthocyanins was as described by Longo et al. (2007). 2.5 g of berry powder was extracted twice with 12.5 mL of HCl/methanol solution (v/v, 0.1 %) for 20 h at room temperature. After filtration and evaporation, the dry residue was dissolved in methanol.

Determination of total phenolic content

The total phenolic in leaf, stem and berry methanolic extracts was determined by spectrometry using Folin-Ciocalteu reagent assay (Singleton and Rossi, 1965). A volume of 200 µL of the extract was mixed with 1 mL of Folin-Ciocalteu reagent diluted 10 times with water and 0.8 mL of a 7.5% sodium carbonate solution in a test tube. After stirring and 30 min later, the absorbance was measured at 765 nm by using a Jenway 6405 UV-Vis spectrophotometer. Gallic acid was used as a standard for the calibration curve. The total phenolic content (three replicates per sample) was expressed as milligrams of gallic acid equivalents per gram of dry matter (mg GAE/g DM). The data were presented as the average of triplicate analyses.

Determination of flavonoids

The quantification of flavonoids was determined by a colorimetric assay using a method described by Zhishen et al. (1999). 500 µL of methanolic extract of each part from the plant (leaf, stem and berry) was mixed with 1500 µL of distilled water, follow-ups of 150 µL of sodium nitrite (5%). After 5 min, 150 µL of aluminum trichloride (10%, m/v) was added to mixture. After 6 min of incubation at room temperature, 500 µL of sodium hydroxide (4%) was added. Immediately, the mixture was completely agitated in order to homogenize the contents. The absorbance of the solution was measured at 510 nm against a blank. The mean (±SD) results of triplicate analyses were expressed as mg of catechin equivalents of total extractable compounds (mg CE /g DM).

Determination of condensed tannins

Proanthocyanidins were measured using the vanillin assay described by Julkunen-Titto (1985). A volume of 50 µL of the methanolic extracts was added to 1500 µL of vanillin/methanol solution (4%, m/v) then mixed using a vortex. Then, 750 µL of concentrated hydrochloric acid (HCl) was added. After incubation for 20 min at room temperature, the absorbance was measured at 550 nm against a blank using a spectrophotometer Jenway 6504 UV/Vis. The amount of total condensed tannins (three replicates

per sample) was expressed as milligrams of catechin equivalent per gram of dry matter (mg CE/ g DM) from the calibration curve.

Reducing power assay

The reducing power of the extracts was determined according to the method of Oyaizu (1986). Various concentrations of the extracts (mg/mL) in distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and 1% of potassium ferricyanide water solution (2.5 mL, $K_3[Fe(CN)_6]$). The mixture was incubated at 50°C for 20 min. Aliquots of trichloroacetic acid (2.5 mL, 10%) were added to the mixture which was then centrifuged at 3000 rpm for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and a freshly prepared $FeCl_3$ solution (0.5 mL, 0.1%). The absorbance was measured at 700 nm. The reducing powers of the tested samples increased with the absorbance values. Ascorbic acid was used as a positive control.

DPPH scavenging assay

The DPPH radical scavenging ability was evaluated according to a method described by Sanchez-Moreno *et al.* (1998). Fifty microliters of various concentrations of methanolic extracts were added to 1950 μ L of DPPH[•] methanolic solution (0.025 g/L). After a 30 min incubation period at room temperature, the absorbance was read against a blank at 515 nm. DPPH free radical scavenging activity in percentage (%) was calculated using the following formula:

$$\text{DPPH scavenging effect (\%)} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100$$

Where A_{control} : absorbance of the control reaction;
 A_{sample} : absorbance of the test compound.

The antioxidant activity of extracts was expressed in terms of concentration required to inhibit 50 % DPPH radical formation (EC_{50} mg/mL). The ascorbic acid was used as positive control.

Thin-layer chromatography (TLC) of plant extract

The both fractions from ethyl acetate and n-butanol were submitted to TLC analysis (Silica gel GF254, Merck) using the following mobile phase system: ethyl acetate/formic acid/distilled water (65: 15: 20) (Males and Medic-Saric, 2001). The compounds of reference used made it possible to identify among the flavonoids, mainly Rutin, naringenin, quercetagenin, dihydroquinone, catechin, gallic acid, ρ -coumaric acid, ferrulic acid, syringic acid, resorcinol, phloroglucinol, hydroquinone and pyrocatechol.

Statistical analysis

Data reported were mean \pm SD from triplicate measurements. Correlation analyses of antioxidant activity were performed by using the Microcal Origin 6 and Tcwin 2 software.

Results and Discussion

Total phenolics, flavonoids and condensed tannins contents

The quantitative determination of phenolic compounds in methanolic extracts of three parts of *M. communis* was presented in Table 1. Total phenolic content was determined as gallic acid equivalents in milligrams per gram (mg GAE/g) while total flavonoid and condensed tannin contents were calculated as catechin equivalents in milligrams per gram (mg CE/g). The leaf (119.23 mg GAE/g) and stem (112.96 mg GAE/g) had higher total phenolic content than berry (70.26 mg GAE/g). These amounts were higher than those described by Aidi Wannes *et al.* (2010) in leaf (33.67 mg GAE/g), flower (15.70 mg GAE/g) and stem (11.11 mg GAE/g) of *M. communis*. Amensour *et al.* (2009) also found that the leaf of Portuguese Myrtle contained the lowest phenolic content with 31.2 mg GAE/g compared with our result but highest value in Greece leaf (373 mg GAE/g) reported by Gardeli *et al.* (2008). The leaf (6.56 ± 0.57 mg CE/g DM) and stem extracts (6.11 ± 0.30 mg/g) exhibited similar concentrations of total flavonoids, whereas the berry extract had more condensed tannins (27.20 ± 1.09 mg/g) than other extracts. This result showed higher compared to work of Aidi Wannes *et al.* (2010).

Antioxidant activity

The reductive capabilities of methanolic extracts from the three part of *M. communis* were shown in Table 2 and compared with ascorbic acid (positive control). The reducing power of all secondary metabolite in leaf, expressed as EC_{50} showed higher activities than that of others extracts. Also, the anthocyanins of berry exhibited stronger reducing power (0.21 mg/mL) followed by leaf butanolic and ethyl acetate fractions (0.24 and 0.27 mg/mL, respectively). However, the ascorbic acid concentration required to reduce the ferric iron was higher (0.06 mg/mL), indicating a better activity than all the extracts.

Reducing power of extracts and ascorbic acid followed the order: ascorbic acid > berry anthocyanins > leaf butanolic fraction > leaf ethyl acetate fraction > leaf tannins > leaf methanolic extract > stem methanolic extract > berry tannins > stem tannins > stem butanolic fraction > berry butanolic fraction >

Table 1. Total phenolics, flavonoids condensed tannins contents

	Leaf	Stem	berry
Total phenolics (mg GAE/g DM)	119.23±0.77	112.96±0.53	70.26±0.71
Total flavonoids (mg CE/g DM)	6.56±0.57	6.11±0.30	3.87±0.25
Condensed tannins (mg CE/g DM)	13.78±0.89	22.47±0.70	27.20±1.09

Table 2. EC₅₀ concentrations of antioxidant activity from bioactive compounds

Extracts	Reducing power (EC ₅₀ mg/mL)			DPPH scavenging assay (EC ₅₀ mg/mL)		
	Leaf	Stem	berry	Leaf	Stem	berry
Methanolic extract	0.45±0.007	0.49±0.013	3.41±0.247	0.47±0.021	0.25±0.005	1.09±0.02
Ethyl acetate fraction	0.26±0.004	9.87±2.213	12.63±1.312	0.09±0.002	0.87±0.015	0.60±0.054
Butanolic fraction	0.23±0.007	2.05±0.100	2.16±0.297	0.21±0.007	0.58±0.018	0.51±0.037
Tannins	0.41±0.016	1.64±0.026	0.89±0.046	0.22±0.004	0.32±0.011	0.35±0.006
Anthocyanins			0.21±0.000			0.20±0.008
Ascorbic acid		0.06±0.00			0.12±0.000	

berry methanolic extract > stem ethyl acetate fraction > berry ethyl acetate fraction.

DPPH radical scavenging based antioxidant potential of the extracts was evaluated using the parameter EC₅₀ (Table 2). It was seen that the leaf ethyl acetate fraction had a stronger effect of DPPH scavenging free radical with an average EC₅₀ value of 0.09 mg/mL compared to ascorbic acid (0.12 mg/mL). The leaf butanolic fraction (0.21 mg/mL), berry anthocyanins (0.20 mg/mL), leaf tannins (0.22 mg/mL) and stem methanolic extract (0.25 mg/mL) present an interesting antioxidant activity on the DPPH radical, while the others extracts showed a considerably less hydrogen donating ability. The EC₅₀ ranged between 0.32 to 1.09 mg/mL.

Scavenging abilities on DPPH radicals were in descending order: Leaf ethyl acetate fraction > ascorbic acid > berry anthocyanins > leaf butanolic fraction > leaf tannins > stem methanolic extract > stem tannins > berry tannins > leaf methanolic extract > berry butanolic fraction > stem butanolic fraction > berry ethyl acetate fraction > stem ethyl acetate fraction > berry methanolic extract.

It has been reported that the antioxidant activity of *M. communis* was attributed to phenolic compounds like flavonoids, to its redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers and have also metal chelating properties (Umadevi *et al.*, 1988; Rice-Evans *et al.*, 1995; Romani *et al.*, 2002; Gardeli *et al.*, 2008). Among these compounds, we could identify by TLC the gallic acid and catechin in leaf and stem of plant. Moreover, the berry revealed the presence of quercetin and gallic acid. This result is in agreement with the work of Aidi Wannes *et al.* (2010). These authors identified in the different myrtle organs, five phenolic acids (gallic acid, caffeic acid, syringic acid, vanillic acid and ferulic acid), eight flavonoids (quercetin-3-rutinoside, myricetin-3-D-galactoside, quercetin-3-D-galactoside, myricetin-3-D-rahmnoside, quercetin-3-D-rahmnoside, myricetin,

quercetin and catechin) and hydrolysable tannins (gallotannins). Schlesier *et al.* (2002) also showed that the gallic acid is the most active compound in tests DPPH and FRAP by comparison with other antioxidants. Other studies showed that the phenolic substances, like the phenolic acids and the flavonoids, are considerably more antioxidant than the vitamin C and the vitamin E (Cad *et al.*, 1997; Vinson *et al.*, 1995). Others authors were suggested that the antioxidant activity of the plant extracts is in correlation with their composition of polyphenols (Montoro *et al.*, 2006).

Conclusion

The medicinal plants are the source of the majority of natural antioxidants and they remain still under exploited in the field medical. In the drug company, knowing that the antioxidants would seem significantly with the prevention of the diseases, the development of new drugs containing antioxidants of natural origin must be with the order of day. According to the results obtained in this study, the extracts of *M. communis* showed a good antioxidant activity, mainly leaf ethyl acetate fraction, to iron reducing power and to scavenge free radicals. These findings may confirm the interesting potential of this plant as a valuable source of natural bioactive molecules in food and medical industry. Further scientific work is in progress to ensure the medicinal properties of the plant *in vivo* correlate with its antioxidant activity.

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