Free Radical Scavenging, Metal chelating and Antiperoxidative Activities of *M. communis* Berries Methanol extract and its Fractions

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**ABSTRACT**

Oxidative stress resulted from free radicals and reactive oxygen species (ROS) are associated with many diseases. Phytotherapy has known a great evolution all the world and some medicinal plants are important remedies of some diseases. Myrtle (*Myrtus communis* L.) is a plant of Myrtaceae family which is common in the Mediterranean region, with flowering, always green leaves and fruit. This study aims to investigate *in vitro* antioxidant capacity of *M. communis* berries methanol extract and its three fractions using five assays: ABTS scavenging radicals, metal chelating, hydrogen peroxide and inhibition of lipid peroxidation assays. EAE extracts possessed the highest antioxidant activity in ABTS (EAE IC₅₀=2.5 µg/mL) and lipid peroxidation models (90.17%). Whereas, AqE is the most active extract in metal chelating activity (IC₅₀=0.73±0.03 mg/mL) and H₂O₂ assay. These results support the traditional use of this plant in healthcare and it could be a new source of antioxidant natural drugs.

**Keywords:**

*Myrtus communis* L.  
Polyphenols  
Antioxidant activity  
Metal chelating activity  
Antiperoxidative activity

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**Introduction**

Free radicals are part of normal metabolities for many organisms, and a complex system of endogenous and exogenous antioxidant sources in the body are employed to mitigate the potential damage from free radicals (Sevindik et al., 2017; Mohammed et al., 2021). When the body is in a state of aging or stress, these highly reactive chemical species are produced excessively, and structural abnormalities and dysfunction of the cell and mitochondrial membranes can arise (Shang et al., 2018; Korkmaz et al., 2021; Akgül et al., 2022).

The role of free radicals in many disease conditions has been well established. Several biochemical reactions in our body generate reactive oxygen species and these are capable of damaging crucial bio-molecules (Kina et al., 2021). If they are not effectively scavenged by cellular constituents, they lead to disease conditions (Halliwell et al. 1995). The harmful action of free radicals can be blocked by antioxidant substances, which scavenge the free radicals and detoxify the organism (Uysal et al., 2021).

The search for newer natural antioxidants, especially of plant origin, has been increasing ever since. Plants have been a constant source of drugs and recently, much emphasis has been placed on finding new therapeutic agents from medicinal plants. Today many people prefer to use medicinal plants rather than chemical drugs (Mohammed et al., 2020).

Myrtle (*Myrtus communis* L.) is a plant of Myrtaceae family which is common in the Mediterranean region, with flowering, always green leaves and fruit. MC leaves contain phenolic acids (gallic acid,ferulic acid, caffeic acid, syringic acid and vanillic acid), flavonoids, hydroxosable tannins (gallotannins), proanthocyanidins, essential oil (α-pinene, myrtyl acetate, 1,8-cineole, limonene and linalool) as the main compounds (Ozcan et al., 2019).

*M. communis*, or myrtle, is an evergreen shrub typical of the Mediterranean flora. Both the leaves and the flowers are delicately and pleasantly scented. It is a plant that has long been known for its medicinal properties. It is also used in the production of liqueurs, for which the berries or a...
mixture of leaves and berries is macerated in alcohol for a prescribed amount of time (Pereira et al., 2017). Fruit has carminative properties and have been used traditionally for the treatment of diarrhoea (Benchik et al., 2016 a,b), hemorrhoids (Mahboubi et al., 2017; Malekuti et al., 2019), inflammation (Amira et al., 2012; Soomro et al., 2019), hepatotoxicity (Kumar et al., 2011; Hanaa et al., 2020), burns (Ozcan et al., 2019), pancreatitis (Oz beyli et al., 2019) and skin diseases (Baharvand-Ahmadi et al., 2015).

Polyphenolic composition of the berries was characterized by high concentrations of flavonol glycosides, flavonols and flavanols. The major fatty acids of berries were reported as linoleic, palmitic, oleic and stearic acids (Aidi Wannes et al., 2010; Barboni et al., 2010).

Antioxidant activity of plant extracts cannot be evaluated by a single method. Therefore, commonly accepted assays were used to assess the antioxidative effect of M. communis different extracts. In a preliminary study, the antioxidant activity of the different extractives was evaluated employing ABTS scavenging radicals, metal chelating, hydrogen peroxide and inhibition of lipid peroxidation assays.

Materials and Methods

Plant Material

The fresh berries of M. communis were collected from Jijel (North-East of Algeria) in November, 2018. The taxonomic identity of the plant was done by Professor Hocine Laouer, Department of Plant Biology and Ecology, University Setif 1, Algeria. A voucher number 52 MB 07/12/18 JijjSA/ was deposited at the laboratory of Phytotherapy Applied to Chronic Diseases.

Extraction and Fractionation

The extraction procedure was conducted as described in our previous study (Benabdallah et al. 2014) with slight modification. This method has two major steps: the first is with methanol to dissolve the flavonoids and the second is with chloroform and ethyl acetate to separate aglycones and glycosylated fractions of flavonoids. The dried powder of M. communis berries was extracted with methanol (85%) at room temperature for 3 days. The resulting suspension was then filtered and concentrated by evaporation at low pressure at 40°C. The filtrate was freed of waxes, fats and chlorophyll by successive washings with n-hexane to give an aqueous phase. To separate aglycones flavonoids and glycosylated flavonoids, the aqueous phase was mixed with chloroform to obtain an organic phase containing the aglycones flavonoid and methoxylated aglycones. The remaining aqueous phase underwent a series of extractions with ethyl acetate to recover the organic phase which contained some aglycones flavonoid, but especially mono- and diglycosides flavonoids. The remaining aqueous phase contained more polar glycosylated flavonoids such as di-, tri- and tetracyglcosides flavonoids. In this study, four extracts were used: methanol (ME), chloroform (CHE), ethyl acetate (EAE) and aqueous (AqE) extracts. The collected fractions were submitted to a concentration at low pressure at 40°C and then dried and stored at 4°C until use.

Determination of in vitro Antioxidant Activity

\textbf{ABTS radical cation decolorization assay}

The radical scavenging assay against ABTS was measured using the method of Re et al. (1999) with slight modification. The ABTS radical stock solution (7 mM in water) was mixed with 2.45 mM potassium persulfate and kept for 12-16 h in the dark at room temperature. The solution was then diluted with methanol to give an absorbance of ~0.7 at 734 nm. Then 50 µL of sample was mixed with 1 mL of ABTS mixture and kept for 30 min at room temperature in the dark. The absorbance of reaction mixture was measured at 734 nm. Trolox was used as positive control. All determinations were performed in replicates. Scavenging capability of test compounds was calculated from the following equation:

\[
\% \text{ inhibition} = \frac{[(AC–ATS)/AC] \times 100.}
\]

\textbf{AC} : Absorbance of control

\textbf{ATS} : Absorbance of test sample

The antioxidant activity of plant extracts was expressed as IC\textsubscript{50}, which is defined as the concentration of extracts (in µg/mL) required to scavenge 50% of ABTS radicals. IC\textsubscript{50} values were estimated by a nonlinear regression. A lower IC\textsubscript{50} value indicates higher antioxidant activity.

\textbf{Hydrogen peroxide-scavenging activity}

The ability of M. communis extracts to scavenge hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) was determined according to the method of Ruch et al. 1(989). A solution of H\textsubscript{2}O\textsubscript{2} (40 mM) was prepared in Na\textsubscript{2}HPO\textsubscript{4} - Na\textsubscript{2}H\textsubscript{2}PO\textsubscript{4} buffer solution (pH = 7.4, 0.1 mol/L). H\textsubscript{2}O\textsubscript{2} concentration was determined spectrophotometrically from absorption at 230 nm. Different concentrations of samples in distilled water were added to a H\textsubscript{2}O\textsubscript{2} solution (0.6 mL). Absorbance of H\textsubscript{2}O\textsubscript{2} at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without H\textsubscript{2}O\textsubscript{2}. The activity of all samples to scavenge H\textsubscript{2}O\textsubscript{2} was calculated using the following equation:

\[
\text{SE (\%)} = \frac{(1–\text{AS 230 nm/ AC 230 nm}) \times 100}{\text{AS}}
\]

\textbf{SE} : scavenging effect

\textbf{AS} : Abs of sample

\textbf{AC} : Abs of control

\textbf{Ferrous ion chelating activity}

The chelating effect of the extracts was determined according to the method of Decker and Welch (1990) (which is based on the inhibition of the formation of Fe\textsuperscript{2+}-ferrozine complex after treatment of samples with Fe\textsuperscript{2+} ions. Briefly, 250 µL of test material or EDTA at different concentration were added to 50 µL of FeCl\textsubscript{2} (0.6 mM in distilled water) and 450 µL of methanol. After 5 min of incubation, the reaction was initiated by the addition of 5 mM ferrozine (50 µL), the mixture was stirred and allowed to react at room temperature for 10 min. The control contained all the reaction reagents except the extract and EDTA. The absorbance of the Fe\textsuperscript{2+}-ferrozine complex was measured at 562 nm. The chelating activity was expressed as a percentage using the following equation:

\[
\text{CA (\%)} = \frac{[(AC–ATS)/AC] \times 100}{\text{CA}}
\]

\textbf{CA} : Chelating activity

\textbf{AC} : Abs of control

\textbf{ATS} : Abs of test sample

To determine the IC\textsubscript{50} values, a dose response curve was plotted. IC\textsubscript{50} is defined as the effective concentration of the test material that is required to chelate 50% of iron ions.
**Ferric thiocyanate (FTC) assay**

The FTC method was used to determine the amount of peroxide at the initial stage of lipid peroxidation using the method by Yen et al. (2003) with slight modifications. Linoleic acid emulsion (0.02 M) was prepared with linoleic acid (155 μL) and Tween 20 (155 μL) in phosphate buffer (50 mL, 0.02 M, pH 7.4). A reaction solution, containing extracts with different concentrations (0.5 mL), linoleic acid emulsion (2.5 mL), and phosphate buffer (2 mL, 0.02 M, pH 7.0) was placed in a glass vial with a screw cap and mixed with a vortex mixer. The mixture was incubated at 40°C in the dark. To 0.1 mL of reaction mixture, 4.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate were added. After 3 min of the addition of 0.1 mL of 0.02 M FeCl₂ in 3.5% HCl, the peroxide value was determined by recording the absorbance at 500 nm every 24 hours until the absorbance of the control reached a maximum. The positive and negative controls were subjected to the same procedures as the sample, except for the negative control, in which only the solvent was added, and for the positive control in which the sample was replaced with BHT and Vitamin C. The inhibition percentage of linoleic acid peroxidation was calculated as:

\[
\text{Inhibition\%} = (1 - \frac{\text{AS at 500 nm}}{\text{AC at 500 nm}}) \times 100
\]

where

- AS : Abs of sample
- AC : Abs of control

**Thiobarbituric acid (TBA) assay**

The TBA test was conducted on the final day of FTC according to the method described by Kikuzaki and Nakatani (1993) to determine the malonaldehyde (MDA) formation from linoleic acid peroxidation. The same sample preparation method as described in the FTC method was used. To 1 mL of sample solution, 20% trichloroacetic acid (2 mL) and thiobarbituric acid solution (2 mL) were added. The mixture was placed in a boiling water bath for 10 minutes. After cooling, it was then centrifuged at 3000 rpm for 20 minutes. Absorbance of the supernatant was measured at 532 nm. Antioxidant activity was determined based on the absorbance of the final day of the FTC assay using the following equation:

\[
\% \text{ inhibition} = 100 - \left[\frac{S}{C}\right] \times 100
\]

where

- S : Abs sample
- C : Abs control

Where Abs control and Abs sample are the absorbances of the control (without sample) and the experimental (with sample) reactions, respectively.

**Statistical Data Analysis**

Results were expressed as means ± standard deviation (SD) and were analyzed by one way analysis of variance (ANOVA) followed by Dunnet’s test. The P Values of P<0.05 were considered significantly different using GraphPad Prism Version 6.0 (GraphPad Software, Inc, La Jolla, CA, USA).

**Results**

**In vitro Antioxidant Activities of M. communis Leaves Extracts**

**ABTS radical scavenging activity of M. communis leaves extracts**

The ability of M. communis berries extracts to scavenge the radical ABTS are shown in Figure 1. All extracts exhibited high antioxidant activity and in the following order: EAE (IC₅₀=2.5 μg/mL)>ME (IC₅₀=13.6 μg/mL)>CHE (IC₅₀=16 μg/mL)>AqE (IC₅₀=25 μg/mL). EAE presented stronger antioxidant activity than Trolox (3 μg/mL), the reference drug used in this assay.

**Ferric ion chelating activity of M. communis berries (MBE) extracts**

A decrease in absorbance indicates a higher chelating power of the extract. All the extracts demonstrated an ability to chelate ferric iron (II) ions. The chelating abilities on ferrous ions were in descending order: AqE (IC₅₀=0.73±0.03 mg/mL)>ME (IC₅₀=0.82±0.03 mg/mL)>EAE (IC₅₀=1.14±0.07 mg/mL)>CHE (IC₅₀=1.43±0.12 mg/mL). None of the extracts appeared to be better chelators of ferric iron (II) ions than the positive control EDTA (IC₅₀=0.02±0.00 mg/mL) in this assay system (Figure 2). In this assay, all extracts and EDTA interfered with the formation of ferrous and ferrozone complex, suggesting that it has chelating activity and captures ferrous ion before ferrozone.

**Hydrogen peroxide scavenging activity of M. communis berries (MBE) extracts**

The scavenging effect of the extracts on hydrogen peroxide decreased in the following order: AqE>EA>ME>CHE (Figure 3). Aqueous extract displayed very strong H₂O₂ scavenging activity (IC₅₀=2.6 μg/mL), whereas chloroform extract exhibited an IC₅₀ of 14 μg/mL. **Antioxidant activity of M. communis berries (MBE) extracts determined by FTC assay**

As shown in Figure 4, all plant extracts showed good antioxidant potential with percent inhibition ranging from (82.76±0.48%) to (90.17±1.21%) as compared with BHT as positive control. The results indicated that EAE exerted marked effects on inhibition of linoleic acid oxidation, which was as strong (90.17±1.21) as BHT (90.82±1.05%).

![Figure 1. ABTS radical scavenging activity of M. communis berries extracts (MBE).](image-url)

ME: M methanol extract, CHE: chloroform extract, EAE: ethyl acetate extract; AqE: aqueous extract. Data were presented as IC₅₀ means±SD (n=3) (**P<0.0001; ***P<0.001; ns: not significant) vs Trolox as standard.
Antioxidant activity of M. communis berries extracts (MBE) determined by thiobarbituric acid assay (TBA)

As shown in figure 5, all plant extracts strongly inhibited MDA formation in the following order: EAE > (94.61±0.4 %) > ME (94.28±0.76 %) > CHE (92.26±0.55%) > AqE (91.99±0.51%). The percentage of inhibition exhibited by EAE and CHE was comparable to BHT as positive control (98.47±0.55).

Discussion

The antioxidant properties of polyphenols are due to their redox properties, which allow them to act as reducing agents, hydrogen donors, metal chelators and single oxygen quenchers. Polyphenolics exhibit a wide range of biological effects including antibacterial, anti-inflammatory, antiallergic, hepato-protective antithrombotic, antiviral, anticarcinogenic and vasodilatory actions; many of these biological functions have been attributed to their free radical scavenging and antioxidant activity (Piluzza and Bullitta, 2011).

The ABTS free radical is formed by oxidation of ABTS with potassium persulfate which is reduced by hydrogen donating ability of different extracts of M. communis was evaluated by measuring the decrease in the absorbance spectrum in the blue-green ABTS radical reaction.

Extracts examined in this study (Figure 1) efficiently scavenged ABTS radicals generated by the reaction between 2,2'-azinobis (3-ethylbenzothiazolin-6-sulphonic acid) (ABTS) and ammonium persulfate, and showed a very good ABTS scavenging activity ranging from 2.5 to 25 µg/mL in the order of EAE>ME>CHE>AqE. EAE extract presented stronger antioxidant activity (IC50=2.5 µg/mL). This value is close to that of the ethyl acetate fraction (Trolox), which means that use of synthetic antioxidants such as Trolox could be avoided by replacing them with the natural ones. These results were in

Figure 2. Ferrous ion chelating activity of M. communis berries extracts (MBE).
ME: methanol extract, CHE: chloroform extract, EAE: ethyl acetate extract; AqE: aqueous extract. Data were presented as IC50 means ± SD (n=3). Data were presented as IC50 means ± SD (n=3) (****P≤0.0001) vs EDTA as standard.

Figure 3. Hydrogen peroxide scavenging activity of M. communis berries extracts (MBE).
ME: M methanol extract, CHE: chloroform extract, EAE: ethyl acetate extract; AqE: aqueous extract. Data were presented as IC50 means ± SD (n=3) (****P≤0.0001; **P≤0.01; ns: not significant) vs BHT as standard.

Figure 1. Antioxidant activity of M. communis berries extracts (MBE). (2 mg/mL at 96 h of incubation) measured by FTC method.
ME: M methanol extract, CHE: chloroform extract, EAE: ethyl acetate extract; AqE: aqueous extract. Data were presented as IC50 means±SD (n=3) (****P≤0.0001; ***P≤0.001; ns: not significant) vs BHT as standard.

Figure 5. Antioxidant activity of M. communis berries extracts (MBE). (2 mg/mL at 96 h of incubation) measured by TBA assay.
ME: M methanol extract, CHE: chloroform extract, EAE: ethyl acetate extract; AqE: aqueous extract. Data were presented as IC50 means ± SD (n=3) (****P≤0.0001) vs BHT as standard.

Figure 4. Hydrogen peroxide scavenging activity of M. communis berries extracts (MBE).
ME: M methanol extract, CHE: chloroform extract, EAE: ethyl acetate extract; AqE: aqueous extract. Data were presented as IC50 means ± SD (n=3) (****P≤0.0001; **P≤0.01; ns: not significant) vs BHT as standard.
accordance with those of Aidi Wannes et al. (2016) who reported that myrtle seed, flower and leaf extracts showed stronger scavenging ability and that they were rich in hydrolysable tannins.

Metal ion chelating activity of an antioxidant molecule prevents oxyradical generation and the consequent oxidative damage. Metal ion chelating capacity plays a significant role in antioxidant mechanisms, since it reduces the concentration of the catalysing transition metal in lipid peroxidation (Jomova and Valk, 2011). In this assay, both extracts and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine.

As in the case of ABTS radical scavenging, AqE and ME extracts exhibited the highest metal chelating capacity. (IC_{50}= 0.73 and 0.82 mg/mL, respectively). This activity could be related to the richness of these extracts in tannins as proved in our previous study (Benchikh et al., 2018). EAE and CHE extracts showed weak chelating activity (IC_{50}=1.14 and 1.43 mg/mL, respectively). Although flavonoids contents in EAE and ME extracts are higher than that in AqE and CHE extract, they showed lower chelating activity. This could be explained that flavonoids are not good metal chelators. None of the extracts appeared to be better chelators of iron (II) ions than the positive control EDTA in this assay system (IC_{50}= 0.02 mg/mL).

Hydrogen peroxide is a weak oxidizing agent which inactivates enzymes by oxidation of the essential thiol (SH-) groups. It rapidly transverses cell membranes and once inside the cell interior, interacts with Fe_{2+} and Cu_{2+} to form hydroxyl radicals, which is harmful to the cell. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate (Peng et al., 2011).

All extracts were capable of scavenging hydrogen peroxide in a concentration-dependent manner. AqE exhibited the highest scavenging capacity (2.6 μg/mL). This observed H_{2}O_{2} scavenging activity may be attributed to the presence of phenolic components which can easily donate electrons to hydroxyl radicals.

Lipid peroxidation is the mechanism by which lipids are attacked by ROS to form a carbon radical that reacts with oxygen, resulting in a peroxy radical and thus generating lipid peroxides (Audin et al., 2014). Lipid peroxidation leads to oxidative degradation of unsaturated fatty acids and leads to the alteration of the structural integrity of membranes and their permeability. However, the conversion of Thiobarbituric Acid Reactive Substances (TBARS) equivalent of MDA is widely used to assess the importance of lipid peroxidation (Benabdallah et al., 2014). Flavonoids, phenolic acids and tannins inhibit mechanisms of enzymatic and non-enzymatic initiation of lipid peroxidation (Morton et al., 2000).

Two tests were used to study the effects of myrtle berries extracts on lipid peroxidation. The ferric thiocyanate method (FTC) measures the ability of antioxidants to scavenge peroxyl radicals produced during the initial stages of oxidation, which react with polyunsaturated fatty acids, through hydrogen donation (Huang et al., 2005).

In this study, after 48h of testing, the percentage inhibition of peroxidation in linoleic acid system by all MBE extracts was high. EAE showed the strongest activity (90.17±1.21 %). This value is similar to BHT as drug reference (90.82±1.05%). These results are in agreement with those of Kumar et al. (2011) who demonstrated that myrtle extracts are effective inhibitors of lipid peroxidation. Phenolic compounds and other chemical components present in the extract may suppress lipid peroxidation through different chemical mechanisms, including free radical quenching, electron transfer, radical addition or radical recombination (Galvez et al., 1995).

Conflict of Interest Statement

We declare that we have no conflict of interest.

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Conclusion

Extracts of M. communis berries contain high levels of total phenolic compounds, and they were able to scavenge free radicals and thereby terminate the radical chain reactions. This study indicates that MBE contains relevant antioxidant compounds responsible, at least in part, for its antioxidant and radicals scavenging activity. Further work is required to isolate and characterize the bioactive compounds that are responsible for this antioxidant activity.

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