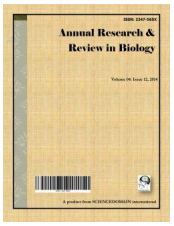

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The Evaluation of Antioxidant Capacity of Different Fractions of *Myrtus communis* L. Leaves

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Authors' contributions

This work was carried out in collaboration between all authors. Author SA designed the study, supervised the work and managed the literature searches. Author FB carried out the experimental work and wrote the first draft of the manuscript. Author HB performed the data analysis. All authors read and approved the final manuscript.

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ABSTRACT

Aims: *Myrtus communis* L. (Myrtle) is a plant widely used in traditional medicine in the Mediterranean Sea. The aim of this study was to determine the phenolic content of *Myrtus communis* L. leaves and to investigate the antioxydant activity of the methanol extract and its fractions.
Methodology: *Myrtus communis* L. leaves were extracted using organic solvents with different polarities to get the following fractions: Methanol extract (ME), chloroform extract (CE), ethyl acetate extract (EE) and aqueous extract (AqE). Total polyphenol, flavonoid and tannins contents were evaluated for all extracts. The antioxidant capacity of different extracts was assessed using nine *in vitro* tests.
Results: The results show that ME had the highest total phenolic content (149.25±3.11 mg GAE/g of dry extract), the aqueous extract had the highest tannins content (83.35±0.36 mg TAE/ g of dry extract), whereas, EE had the highest total flavonoid content (38.4±0.9 mg QE/ g of dry extract).The EE exhibited an interesting antioxidant activity using ABTS radical scavenging assay (IC_{50}=0.0015 mg/ml) and the DPPH test (IC_{50}=0.004 mg/ml), while EA showed an important activity

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in the hydroxyl radical scavenging test (IC_{50}=0.08 mg/ml), H_2O_2, (IC_{50}=0.015 mg/ml), iron chelating (IC_{50}=0.5 mg/ml) and reducing power (EC_{50}=0.03 mg/ml). The greatest activity in inhibiting the oxidation of β-carotene/ linoleic acid was induced by CE and EE extracts (93.95%, and 90.29%, respectively). All extracts showed a very strong antiperoxidant effect against FTC and MDA tests.
Conclusion: *Myrtus communis* L. leaves extracts have an important antioxidant activity which is most likely due to their polyphenolic content.

Keywords: Myrtus communis L.; polyphenols; free radicals; lipid perxidation; scavenging activity.

1. INTRODUCTION

Free radicals generated in aerobic metabolism are involved in a series of regulatory processes such as cell proliferation, apoptosis and gene expression. When generated in excess, free radicals can affect the defence capability of the antioxidant system, impairing the essential biomolecules in the cell by oxidizing membrane lipids, cell proteins, carbohydrates, DNA and enzymes. Oxidative stress results in cytotoxic compounds occurrence (malonyl dialdehyde, 4-hydroxynonenal) and alters the oxidant-antioxidant balance (redox homeostasis) that characterizes normal cell functioning [1].

Antioxidants can be natural or synthetic, but due to toxic and carcinogenic effects of the synthetic antioxidants, such as butylhydroxyanisole and butylhydroxytoluene), a great deal of attention has been focused in recent decades on natural antioxidants such as polyphenols derived from various natural sources.

Myrtus communis L. is a genus belonging to the Myrtaceae family which includes approximately 100 genera and 3000 species growing in temperate, tropical and subtropical regions [2]. In Algeria, the genus *Myrtus* L. (Myrtaceae) presently includes two species, *Myrtus communis,* known as common myrtle and *Myrtus nivellei* Batt. & Trab., known as Sahara myrtle [3]. Different parts of the plant find various uses in traditional and in the food, beverage and cosmetic industries [4-6]. Myrtle possess several biologic activities such as antibacterial, antiviral, antifungal, anti-inflammatory, analgesic, antioxidant, antimutagenic, anti-hemorrhagic, hepatoprotective, wound healing and anti-hyperglycaemic activities [7,8].

Several studies have pointed out to the richness of myrtle with phytochemical constituents including different classes of polyphenols [7,8,9,10]. The aim of this study, was thus to quantify the phenolic, flavonoid and tannins contents in the leaves of this plant as potential source of natural antioxidants and also to evaluate its antioxidant activity using different assays.

2. MATERIALS AND METHODS

2.1 Plant Material

The fresh leaves of *M. communis* L. were harvested in November, 2016 from Jijel (North-East of Algeria, 50 km to the south of the Mediterranean sea at an altitude of 300 meters). The taxonomic identity of the plant was done by Professor Gonzalez-Tejero and Casares-Porcel Department of Botany, University of Granada, Spain and a voucher number ML 11/16 was deposited at the Laboratory of Phytotherapy Applied to Chronic Diseases, University Setif 1. The collected plant was dried in shade at room temperature for 3 weeks. After drying, plant material was ground to a fine powder using an electric grinder (Moulinex, France).

2.2 Extraction and Fractionation

Phenolic compounds were extracted from Myrtus powder according to [11] with slight modification. One hundred grams of the plant powder was extracted with methanol (85%) at room temperature for 3 days. The resulting suspension was then filtered and concentrated by evaporation at 50 C° and splitted by successive washing with different solvents of increasing polarity (hexane, chloroform and ethyl acetate). Each fraction was evaporated to dryness to obtain the following fractions: methanol extract (ME), hexane extract (HE), chloroform extract (CE), ethyl acetate extract (EE) and the remaining aqueous extract (AE). The extracts were stored at 4°C until use.

2.3 Determination of Total Phenolic Content

Total phenolic content was assessed by Folin Ciocalteu reagent [12]. A volume of 100 µl of each extract was mixed with 500 µl of Folin

Ciocalteu reagent (diluted 10 times). After 4 min, 400 µl of 7.5% of Na_2CO_3 solution was added. The final mixture was shaken and incubated in dark at room temperature for 1 hour and the absorbance of the reaction mixture was measured at 760 nm. The amount of total polyphenols in different extracts was determined from a standard curve of gallic acid. The results were expressed as mg of gallic acid equivalent (GAE) per gram of dried plant extract.

2.4 Determination of Total Flavonoid Content

Total flavonoid content was determined using aluminum chloride assay [13]. Briefly, 1 ml of each tested extract or standard (quercetin) were mixed with 1 ml of $AlCl_3$ (2%). After 10 min of incubation, the absorbance against a prepared blank was measured at 430 nm. The results were expressed as quercetin equivalent per gram of dry plant extract weight (mg QE/g DW) using a calibration curve of quercetin.

2.5 Determination of Total Tannins Content

This was achieved by testing the capacity of the different extracts to precipitate haemoglobin from fresh bovine blood according to the method described by Bate-Smith [14]. Briefly, a volume of each plant extract was mixed with an equal volume of hemolized bovine blood (absorbance = 1.6). After 20 minutes of incubation at room temperature, the mixture was centrifuged at 4000 rpm, and the absorbance of the supernatant was measured at 576 nm and the results were expressed as mg equivalent tannic acid per gram of extract dry weight (mg TAE/g DW) using a calibration curve of tannic acid.

2.6 Determination of *in vitro* Antioxidant Activity

Since using a single assay would not give the correct result properties, and since the antioxidant activity of a plant extracts is influenced by many factors, such as the test system and the composition of extract. Therefore, it is important to carry out more than one type of antioxidants activity measurement to cover the various mechanisms of antioxidant activity.

2.6.1 ABTS radical cation decolorization assay

The radical scavenging assay against ABTS was measured [15]. 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:

> % inhibition = [(Absorbance of control – Absorbance of test sample)/Absorbance of control] x100.

To determine the IC_{50} values, a dose response curve was plotted. IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum scavenging capacity.

2.6.2 DPPH scavenging activity

The DPPH assay was based on the measurement of altering the purple colour to yellow of DPPH radical at 517 nm after reaction with antioxidant compound. The effect of antioxidants on DPPH radical was thought to be due to their hydrogen donating ability. DPPH· is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [16].

The 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity of the extracts was determined spectrophotometrically in an MRXe tc (DYNEX Technologies GmbH, Denkendorf, Germany), by monitoring the disappearance of DPPH at 515 nm [17]. Briefly, 20 µl of *M. communis* L. extracts or standard solution (ascorbic acid) in absolute methanol was added to 180 µl of DPPH reagent (0.004%) in 96 well plates. Absolute ethanol was used for reagent blank. All reagents were mixed and incubated for 30 minutes at room temperature and protected from light. Experiments were done in triplicates. The percentages of the DPPH free radical scavenging activity were calculated as follows:

> % inhibition = [(Absorbance of control – Absorbance of test sample)/Absorbance of control] x100.

To determine the IC_{50} values, a dose response curve was plotted. IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum scavenging capacity.

2.6.3 Hydroxy radical scavenging activity

The hydroxyl radical scavenging activity of *M. communis* extracts was measured by the salicylic method [18]. The reaction mixture 3.0 ml contained 1.0 ml of 1.5 mM FeSO4, 0.7 ml of 6 mM hydrogen peroxide, 0.3 ml of 20 mM sodium salicylate and varied concentrations of the extract. After incubation for 1 hour at 37°C in water bath, the absorbance of the hydroxylated salicylate complex was measured at 562 nm. The scavenging activity of hydroxyl radical effect was calculated according to the following equation:

$$[1- (A_1-A_2) / A_0] \times 100$$

Where A_0 is absorbance of the control (without extract) and A_1 is the absorbance in the presence of the extract, A_2 is the absorbance without sodium salicylate.

2.6.4 Hydrogen peroxide-scavenging activity

The ability of *M. communis* extracts to scavenge H_2O_2 was determined [19]. A solution of H_2O_2 (40 mM) was prepared in Na_2HPO_4–NaH_2PO_4 buffer solution (pH = 7.4, 0.1 mol/L). H_2O_2 concentration was determined spectrophotometrically from absorption at 230 nm. Different concentrations of samples in distilled water were added to a H_2O_2 solution (0.6 mL). Absorbance of H_2O_2 at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without H_2O_2. The activity of all samples to scavenge H_2O_2 was calculated using the following equation: Scavenging effect (%) = (1 − Abs of sample 230 nm/Abs of control 230 nm) × 100.

2.6.5 Ferrous ion chelating activity

The chelating effect of the extracts was determined according to the method of Decker and Welch [20] (which is based on the inhibition of the formation of Fe^{2+}-ferrozine complex after treatment of samples with Fe^{2+} ions. Briefly, 250 µl of test material or EDTA at different concentration were added to 50 µl of $FeCl_2$ (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^{2+}-ferrozine complex was measured at 562 nm. The chelating activity was expressed as a percentage using the following equation:

Chelating activity (%) = [(Abs of control – Abs of test sample)/Abs of control] x100.

To determine the IC_{50} values, a dose response curve was plotted. IC_{50} is defined as the effective concentration of the test material that is required to chelate 50% of iron ions.

2.6.6 Reducing power

The reducing powers of the extracts from *M. communis* L. and BHT were determined [21]. A volume of 0.1 ml of each extract BHT was mixed with an equal volume of 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide, and then incubated at 50°C for 20 min. 0.25 ml of 1% trichloroacetic acid was added to the mixture to stop the reaction, and then the mixture was centrifuged at 2790 g for 10 min. The supernatant (0.25 ml) was mixed with 0.25 ml distilled water and 0.1% FeCl3 (0.5 ml) and then the absorbance was measured at 700 nm. The reducing powers of the tested samples increased with the absorbance values.

2.6.7 Antioxidant activity determined by β-carotene bleaching method

This assay is based on the capacity of antioxidant molecules to inhibit β-carotene oxidative degradation that is caused by oxidative compounds of linoleic acid [22]. β-carotene/linoleic acid emulsion was prepared by mixing 0.5 mg of ß-carotene in 1 ml of chloroform, 25 µl of linoleic acid and 200 mg of Tween 40. Chloroform was was completly evaporated 40°C using a vacuum evaporator. Then 100 ml of oxygenated distilled water was added with vigorous shaking. To an aliquot of 2.5 ml of this emulsion, 350 µl of *M. communis* L. or the reference antioxidant (BHT) were added and well mixed. The absorbance was recorded after 0, 1, 2, 4, 6 and 24 hours at 490 nm. A negative control consisted of 2.5 ml distilled water or solvent instead of extract or reference antioxidant. All samples were assayed in triplicate. The antioxidant activity (AA) was measured in terms of successful bleaching of β-carotene by using the following equation:

$$AA=[1 - \frac{A_0 - A_t}{A_0^0 - A_t^0}] \times 100$$

Where, A_0 and A_0^0 were the absorbance values measured at zero time of the incubation for test sample and control, respectively. A_t and A_t^0 were

the absorbance values measured in the test sample and control, respectively after incubation for 24 hours.

2.6.8 Ferric thiocyanate (FTC) assay

The FTC method was used to determine the amount of peroxide at the initial stage of lipid peroxidation. [23]. 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. Exactly, 3 min after the addition of 0.1 ml of 0.02 M $FeCl_2$ 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:

> Inhibition% = (1 – Absorbance of sample at 500 nm/Absorbance of control at 500 nm) × 100.

2.6.9 Thiobarbituric acid (TBA) assay

The TBA test was conducted on the final day of FTC according to the method described by Kikuzaki and Nakatani [24] 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 recorded based on the absorbance of the final day of the FTC assay using the following equation:

> % inhibition = 100 – [(Abs sample /Abs control) × 100]

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

2.7 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 Graph Pad Prism Version 6.0 (GraphPad Software, Inc, La Jolla, CA, USA).

3. RESULTS

3.1 Total Phenolics, Flavonoids and Tannins Contents of *Myrtus communis* L. Leaves Extracts

The total phenolics, flavonoids and tannins contents among the different extracts of *Myrtus communis* L. leaves extracts are presented in Table 1. The total phenolic content in terms of mg GAE/g of dry weight of extract decreased in the following order: ME > AE > EE > CE, whereas the highest total flavonoids were found in EE (38.4 ± 0.9 mg QE/g DW) and tannins contents in AE (95.29 ± 0.68 mg TAE/g DW).

Table 1. Total phenolics, flavonoids and tannins contents of *Myrtus communis* L. leaves extracts

Extracts	Total phenolics (mg GAE/g Dw)	Total flavonoids (mg QE/g DW)	Total tannins (mg TAE/g DW)
ME	149.25 ± 3.11	26.38 ± 0.13	83.35 ± 0.36
CE	81.0 ± 1.53	28.05 ± 0.15	52.3 ± 0.25
EE	101.88 ± 1.73	38.4 ± 0.9	49.7 ± 0.98
AE	86.93±0.68	3.02±0.02	95.29±0.68

ME: Methanol extract, CE: Chloroform extract, EE: Ethyl acetate extract, AE: Aqueous extract, DW: Dry, GAE: Gallic acid equivalent, QE: Quercetin equivalent, TAE: Tannic acid equivalent

3.2 *In vitro* Antioxidant Activities of *Myrtus communis* L. Leaves Extracts

3.2.1 ABTS radical scavenging activity of *Myrtus communis* L. leaves extracts

The ability of *Myrtus communis* L. leaves extracts to scavenge the radical ABTS are shown in Fig. 1. All extracts exhibited high antioxidant activity and in the following order: EE (IC_{50} = 0.0015 mg/ml) > AE (IC_{50} = 0.004 mg/ml) > CE (IC_{50} = 0.0096 mg/ml) > ME (IC_{50} = 0.0098 mg/ml).

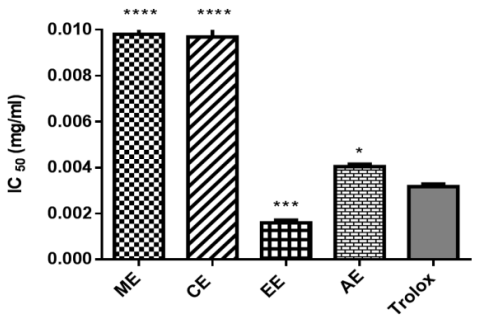

Fig. 1. ABTS radical scavenging activity of *Myrtus communis* L. leaves extracts

*ME: methanol extract, CE: chloroform extract, EE: ethyl acetate extract; AE: aqueous extract. Data were presented as IC_{50} means ± SD (n=3) (****P ≤ 0.0001; ***P ≤ 0.001, *P ≤ 0.01; ns: not significant) vs Trolox as standard*

3.2.2 DPPH radical scavenging activity of *Myrtus communis* L. leaves extracts

Among the chemical methods applied for determining the antioxidant capacity of a composite for capturing free radicals, the DPPH method (1.1,-Diphenil-2-picrilhydrazil) is one of the most used, for being considered practical, rapid and stable. The scavenging ability of the extracts was expressed as IC_{50} value (the concentration of substrate that causes 50% loss of DPPH activity). Low IC_{50} values indicate strong ability of the extracts to act as DPPH scavenger.

The results show that EE extracts exhibited the highest antioxidant activity (close to vit C as standard), followed by ME (IC_{50} = 0.009 mg/ml), AE (IC_{50} = 0.011 mg/ml) then CE (IC_{50} = 0.035 mg/ml). The IC_{50} for Vit C was 0.003 mg/ml) (Fig. 2).

3.2.3 Hydroxyl radical scavenging activity of *Myrtus communis* L. leaves extracts

The results showed that hydroxyl radical activity of AE showed better activity (IC_{50} = 0.08 ± 0.01 mg/ml) than the standard tocopherol (IC_{50} = 0.13 ± 0.02 mg/ml), followed by CE (IC_{50} = 0.12, $P \leq 0.01$ mg/ml) and ME (IC_{50} = 0.14 mg/ml, $P \leq 0.05$). However EE showed the lowest activity (IC_{50} = 0.18, P ≤ 0.0001) compared with tocopherol (Fig. 3).

3.2.4 Hydrogen peroxide scavenging activity of *Myrtus communis* L. leaves extracts

The scavenging effect of the extracts on hydrogen peroxide decreased in the following order: BHT > AE > EE > CE > ME and their IC_{50} values were found to be: 0.011; 0.015; 0.023; 0.037 and 0.109 mg/ml, respectively (Fig. 4).

3.2.5 Ferrous ion chelating activity of *Myrtus communis* L. leaves extracts

All the extracts demonstrated an ability to chelate ferric iron (II) ions. The chelating abilities on ferrous ions were in descending order: AE (IC_{50} = 0.5 ± 00 mg/ml) > ME (IC_{50} = 0.61 ± 0.25 mg/ml) >CE (IC_{50} = 2.56 ± 0.05 mg/ml) > EE (IC_{50} = 6.14 ± 0.058 mg/ml). None of the extracts appeared to be better chelators of ferric iron (II) ions than the positive control EDTA (IC_{50} = 0.02 ± 00 mg/ml) in this assay system (Fig. 5).

3.2.6 Reducing power capacity of *Myrtus communis* L. leaves extracts

From the results (Fig. 6), we can see that the best reducing power (the effective concentration at which the absorbance was 0.5) was for AE (EC_{50} = 0.033 ± 00 mg/ml) and ME (EC_{50} = 0.047 ± 00 mg/ml) which were stronger than BHT (EC_{50} = 0.074 ± 00 mg/ml) as positive standard. EE showed comparable effect (EC_{50} = 0.065 ± 00 mg/ml) as BHT, whereas, the reducing power of CE was significantly ($P \leq 0.0001$) lower (EC_{50} = 0.226 ± 0, 01 mg/ml) than BHT.

3.2.7 Antioxidant activity of *Myrtus communis* L. leaves extracts determined by β-carotene /linoleic acid bleaching assay

Fig. 7 shows the effect of *Myrtus communis* L. leaves extracts on the changes in the percentage of the inhibition ratio of linoleic acid oxidation compared to BHT as positive control during 24 h. The addition of the plant extracts and BHT at 2 mg/ml was markedly effective in inhibiting the oxidation of linoleic acid and subsequent bleaching of β-carotene, in comparison with the negative control which contained no antioxidant

component. CE showed the highest antioxidant activity (93.95 ± 0.53%) compared to BHT (94.9 ± 1.52%) followed by EE (90.29 ± 0.42%), AE (79.58±2.67%) and ME (73.24 ±1.52%). Fig. 8 shows the decrease in absorbance of β-carotene in the presence of 2 mg/ml extract or reference antioxidant (BHT) compared with the negative controls (MeOH and H_2O).

3.2.8 Antioxidant activity of *Myrtus communis* L. leaves extracts determined by FTC assay

As shown in Fig. 9, the different extracts showed good antioxidant potential with percent inhibition ranging from (67.65 ± 2.59%) to (92.77 ± 1.44%) as compared with BHT as positive control. The results indicated that both EE and CE exerted marked effects on inhibition of linoleic acid oxidation, which were as strong (92.77 ± 1.77 and 92.22 ± 1.42, respectively) as BHT (90.56 ± 1.7%) ($P \geq 0.05$).

3.2.9 Antioxidant activity of *Myrtus communis* L. leaves extracts determined by thiobarbutiric acid assay (TBA)

As shown in Fig. 10, the plant extracts inhibited MDA formation in the following order: BHT (96.51 ± 1.7%) > EE (94.77 ± 3.52%) > CE (94.31 ± 2.73%) > ME (88.18 ± 1.86%) > AE (81.81 ± 5.08%). The percentage of inhibition exhibited by MLEE and MLCE was comparable to the positive control (BHT).

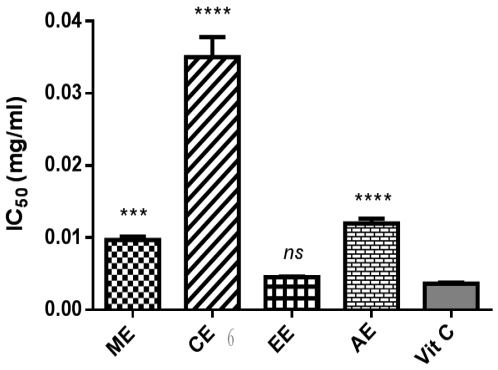

Fig. 2. DPPH radical scavenging activity of *Myrtus communis* L. leaves extracts

*ME: methanol extract, CE: chloroform extract, EE: ethyl acetate extract; AE: aqueous extract. Data were presented as IC 50 means ± SD (n=3). (****P ≤ 0.0001; ***P ≤ 0.001; ns: not significant) vs vitamin C as standard*

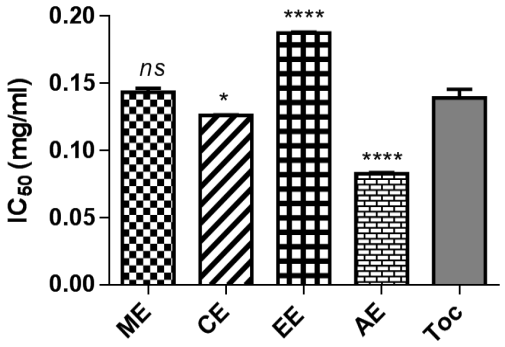

Fig. 3. Hydroxyl radical scavenging activity of *Myrtus communis* L. leaves extracts

*ME: methanol extract, CE: chloroform extract, EE: ethyl acetate extract, AE: aqueous extract, Toc: tocopherol. Data were presented as IC_{50} means ± SD (n=3). (****P ≤ 0.0001; (*P ≤ 0.01; ns: not significant) vs Tocopherol as standard*

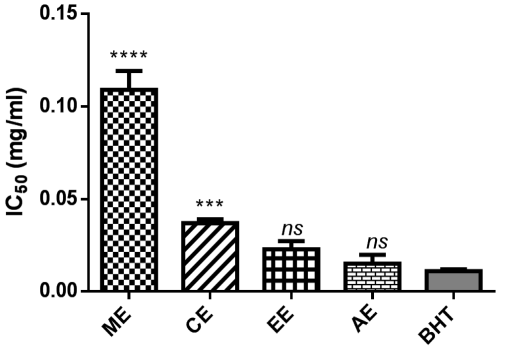

Fig. 4. Hydrogen peroxide scavenging activity of of *Myrtus communis* L. leaves extracts

*ME: methanol extract, CE: chloroform extract, EE: ethyl acetate extract, AE: aqueous extract. Data were presented as IC_{50} means ± SD (n=3). (****P ≤ 0.0001; ***P ≤ 0.001; ns: not significant) vs BHT as standard*

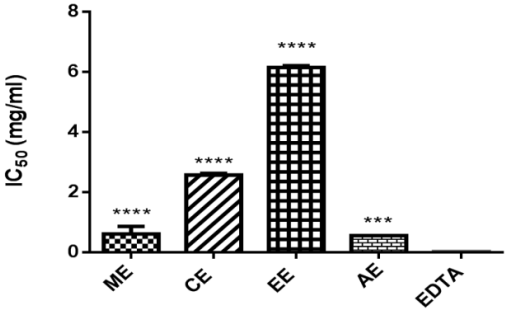

Fig. 5. Ferrous ion chelating activity of *Myrtus communis* L. leaves extracts

*ME: methanol extract, CE: chloroform extract, EE: ethyl acetate extract, AE: aqueous extract. Data were presented as IC_{50} means ± SD (n=3) (****P ≤ 0.0001; (***P ≤ 0.001; ns: not significant) vs EDTA as standard*

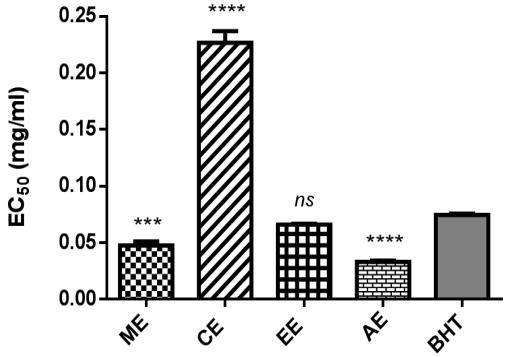

Fig. 6. Reducing power activity of *Myrtus communis* L. leaves extracts

*ME: methanol extract, CE: chloroform extract, EE: ethyl acetate extract, AE: aqueous extract. Data were presented as IC_{50} means ± SD (n=3) (****P ≤ 0.0001; ns: not significant) vs BHT as standard*

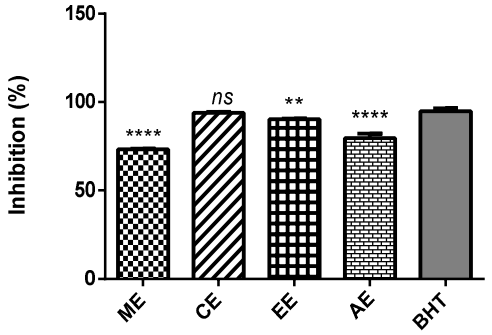

Fig. 7. Antioxidant activity of *Myrtus communis* L. leaves extracts (2 mg/ml) using β-carotene /linoleic acid bleaching assay after 24 h

*ME: methanol extract, CE: chloroform extract, EE: ethyl acetate extract, AE: aqueous extract. Data were presented as means ± SD (n=3) (****P ≤ 0.0001; **P ≤ 0.01; ns: not significant) vs BHT as standard*

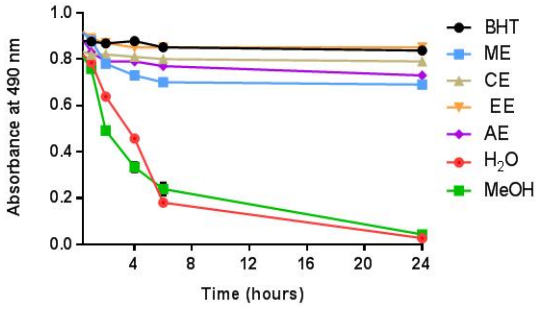

Fig. 8. Decrease in absorbance of β-carotene in the presence of 2 mg/ml of different of *Myrtus communis* L. leaves extracts or reference antioxidant (BHT) compared with MeOH and H_2O as negative controls

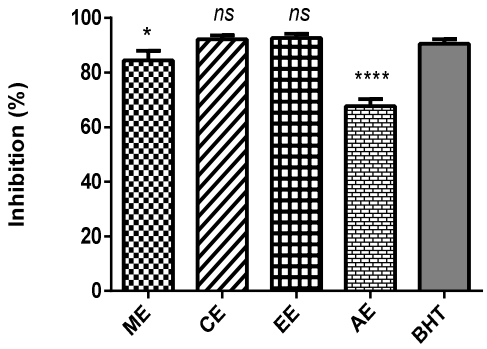

Fig. 9. Antioxidant activity of *M. communis* L. leaves extracts (2 mg/ml at 96 h of incubation) measured by FTC method

*ME: methanol extract, CE: chloroform extract, EE: ethyl acetate extract, AE: aqueous extract. Data were presented as means ± SD (n=3). (****P ≤ 0.0001; *P ≤ 0.05; ns: not significant) vs BHT as standard*

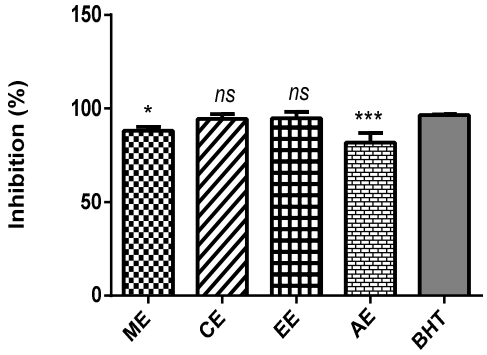

Fig. 10. Antioxidant activity of *M. communis* L. leaves extracts (2 mg/ml at 96 h of incubation) measured by TBA assay

*ME: methanol extract, CE: chloroform extract, EE: ethyl acetate extract, AE: aqueous extract. Data were presented as means ± SD (n=3). (***P ≤ 0.001; *P ≤ 0.05; ns: not significant) vs BHT as standard*

4. DISCUSSION

Several reports have described *M. communis* L. as being rich in phenolic acids, flavonoids, tannins, essential oils and fatty acids [7,8,10]. The results of the present study revealed that *M. communis* extracts are rich in polyphenols. The highest levels of polyphenols were identified in methanol extract. These results were quite close to that found by the following authors [25,26] but lower than that found by others [10,27]. The flavonoids content of this study were in line with those of [28], but lower than that of [10,29,30] and higher than those of other authors [25,26,31]. The highest levels of tannins were

detected in the methanol extract. These values were comparable to those of [32] and higher than [26] and lower than [30], although the last three studies were from the same region and represented the closest area to the location of our study, this may be explained by the composition of the soil. These discrepancies are probably due to different degrees of polarity of the solvents used for the extraction procedure, methods of quantification, geographic region, and the season of harvest [27].

Free radicals are thought to contribute to several disorders in the body [1,33]. Antioxidants are a group of compounds that inhibit oxidation and reduce free radicals directly or indirectly. Oxidative stress may be alleviated in vivo by exogenous administration of antioxidants. Some synthetic antioxidants showed potential adverse effects on the body. Thus, research attention is turning to exploration and discovery of effective, safe, and natural antioxidants to resist oxidative stress [34]. There is an increasing interest in natural antioxidants, e.g., polyphenols, present in medicinal and dietary plants, which might help in preventing oxidative damage.

In the present study, *M. communis* leave extracts exhibited a strong scavenging effect on ABTS, DPPH, Hydroxyl and H_2O_2. The most probable cause by which the extracts induce their antioxidant activity is due to the wide range of their constituents. The estimation of some classes of these constituents in this study and others revealed the presence of several classes of phenolic compounds such as, flavonoids and tannins at high concentration. Some of these chemicals have been already implicated in different antioxidant systems [35-40]. These phenolics either as single compounds or in many plant extracts have also been suggested to act as antioxidants [41-43].

Polyphenols possess ideal structural chemistry for free radical scavenging activity, and they have been shown to be more effective antioxidants *in vitro* than tocopherols and ascorbate. Antioxidant properties of polyphenols arise from their high reactivity as hydrogen or electron donors, and from the ability of the polyphenol derived radical to stabilise and delocalise the unpaired electron (chain breaking function), and from their ability to chelate transition metal ions [44]. Flavonoids and tannins are also important secondary metabolites present in plants, and studying variation in their contents is important because the biological effects of many plant materials relay on these compounds [45].

The effect of antioxidants on DPPH radical scavenging was conceived to be due to their proton-donating ability. In DPPH test, the antioxidants were able to reduce the stable radical DPPH to the yellow-coloured diphenyl picrylhydrazine [46]. In the present study, DPPH scavenging activity ranged from 0.009 to 0.035 mg/ml among the four plant fractions, with the highest scavenging capacity exhibited by ethyl acetate extract, followed by methanolic extract. These results were comparable to those found by [25,30] for the methanol and ethyl acetate extracts and higher than those found by Amensour [47]. Aidi Wannes [48] reported that myrtle seed, flower and leaf extracts showed stronger scavenging ability on DPPH and that they were rich in hydrolysable tannins. The observed antioxidant activity of the extracts may be due to the neutralization of free radicals (DPPH), either by transfer of hydrogen atom or by transfer of an electron [49].

ABTS assay is an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants and of chain-breaking antioxidants. The ABTS•+ radicals are more reactive than DPPH radicals and, unlike the reactions with DPPH radicals, which involve H atom transfer, the reactions with ABTS•+ radicals involve electron-transfer [50]. Extracts examined in this study 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 0.0015 to 0.0095 mg/ml in the order of ethyl acetate>aqueous extract>chloroform extract >methanolic extract. The order of ABTS radical scavenging activity of the different extracts in the present study was almost similar to that observed for DPPH, where in both tests; Ethyl acetate exhibited the strongest scavenging activity (lowest IC_{50}). From the above extracts, ethyl acetate extract was shown to possess the highest radical scavenging activity against ABTS and DPPH, which could be explained by its ability of having extracted a considerable amount of polyphenols and flavonoids with specific structure containing many hydroxyl groups.

Hydroxyl radical ($OH^{\bullet}$) is extremely reactive, more toxic than other radical species and can attack biologic molecules such as DNA, proteins and lipids. The radical $OH^{\bullet}$ is widely believed to

be generated from the Fe^{2+} (or Cu^{+})/H_2O_2 Fenton reaction system, by simply incubating $FeSO_4$ and H_2O_2 in aqueous solution. Thus, scavenging ability of hydroxyl radical is widely accepted as a tool to evaluate the potential of antioxidants and can be accomplished through direct scavenging or preventing of OH formation through the chelation of free metal ions or converting H_2O_2 to other harmless compounds [33]. In the present study, the ability of *M. communis* extracts to quench hydroxyl radicals seems to be directly related to the prevention of propagation of the process of lipid peroxidation and it seems to be a good scavenger of reactive oxygen species [51]. The present results reveal that aqueous extract exhibited the most powerful scavenging effect. This strong activity could be attributed to the richness of this fraction in tannins (95 mg/kg) compared to the other fractions.

Hydrogen peroxide is capable of reacting with major cell components and participates in lipid peroxidation and also causing DNA damage [52,53]. It plays an important role in the formation of other ROS molecules such as superoxide anion radicals or hydroxyl radicals upon reaction with Fe^{2+} in the cell. In the present study, the capacity for inhibiting the oxidative effect of the H_2O_2 increased with increasing the concentration of *M. communis* tested extracts. This observed H_2O_2 scavenging activity may be attributed to the presence of phenolic components which can easily donate electrons to hydroxyl radicals.

Fe (III) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action, and can be strongly correlated with other antioxidant properties [54]. In reducing power test, the change of colour from yellow to green depends on the amount of reductants in test sample. The presence of reductants in test specimen causes the reduction of ferricyanide complex to ferrous form by donating an electron. The present results revealed that the reducing power of the extracts were in the order of Aqueous extract followed by methanolic extract, then ethyl acetate extract and finally, the chloroform extract. Their EC_{50} ranging from 0.033 to 0.226 mg/ml. These findings are comparable to those found by Kanoun [25], higher than those found by Amensour [47] and lower than those found by Gardelli [27].

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. In the present study, extracts showed moderate chelating activity compared to other tests and none of the extracts appeared to be better chelators of iron (II) ions than the positive control EDTA in this assay system. Although flavonoid contents in methanolic and aqueous extracts are lower than that in ethyl acetate extract, they showed better chelating activity than that of ethyl acetate extract. This activity could be related to the richness of these extracts in tannins.

Three tests were used to study the effects of myrtle extracts on lipid peroxidation. The *β*-carotene bleaching method (coupled oxidation of *β*-carotene and linoleic acid) estimates the relative ability of antioxidant compounds in plant extracts to scavenge the radical of linoleic acid peroxide that oxidizes *β*-carotene in the emulsion phase. *β*-carotene in the absence of the antixodant undergoes a rapid decolourization since the free linoleic acid radical attacks the *β*-carotene, which loses the double bonds and, consequently, its orange colour. In the ferric thiocyanate (FTC) method; during linoleic acid oxidation, peroxides are formed, which oxidise Fe^{2+} to Fe^{3+}. The latter ions form a complex with thiocyanate, and this complex has a maximum absorbance at 500 nm. Therefore, high absorbance indicates high linoleic-acid oxidation [55]. The TBA test measures the malondialdehyde (MDA) formed after lipid hydroperoxide decomposition, which forms a pink chromophore with thiobarbituric acid (TBA). This coloured complex absorbs at 532 nm and results in the condensation of TBA and malondialdehyde in an acidic environment [56].

Myrtle extracts inhibited lipid peroxidation in all three tests. The highest activity was exerted by methanol and aqueous extracts. These results are in agreement with those of Kumar and co-workers [57] and Gonçalves [58] who demonstrated that myrtle extracts are effective inhibitors of lipid peroxidation.

5. CONCLUSION

M. communis L. fractions are rich in polyphenols, flavonoids and tannins. They have significant antioxidant and free radical scavenging activities in different in *vitro* assay systems. However, whether these compounds work singularly or in

synergy with other constituents deserve further investigations. These findings give strong support for expanding the investigations of myrtle for utilisation in food and cosmetic industries as well as in traditional folk medicine.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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