Turkish Journal of Agriculture - Food Science and Technology, 10(4): 760-765, 2022
DOI: https://doi.org/10.24925/turjaf.v10i4.760-765.5060

Turkish Journal of Agriculture - Food Science and Technology

Available online, ISSN: 2148-127X | www.agrifoodscience.com | Turkish Science and Technology Publishing (TURSTEP)

Free Radical Scavenging, Reducing Power and Lipid Peroxidation Inhibition Activities of *M. communis* Berries Methanol Extract and its Fractions

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ARTICLE INFO

Research Article

Received : 22/01/2022
Accepted : 26/02/2022

Keywords:
Myrtus communis L.
Polyphenols
Free radical scavenging
Reducing Power
Lipid peroxidation inhibition

ABSTRACT

Free radicals or highly reactive oxygen species are capable of inducing oxidative damage to the human body Plants containing phenolic compounds have potent antioxidant capacity. The present study was undertaken to investigate the in vitro antioxidant activities of *Myrtus communis* L. (Myrtaceae), a plant widely used as natural remedy for digestive disorders in folk medicine. Total polyphenol contents were determined using Folin Ciocalteu's reagent; flavonoids were quantified employing the $AlCl_3$ Method. EAE extract showed the highest polyphenolic and flavonoids contents (358.37±2.28 GAE/g of dry extract and 105.44±3.48 QE/g of dry extract) respectively. The EAE had the highest antioxidant activity as measured by DPPH radical and hydroxyl radical scavenging activity. EAE and ME extracts exhibited the highest reducing power. EAE possess an IC_{50} close to BHT (0.074 mg/ml) as reference drug All extracts exhibited antioxidant activity in the linoleic acid emulsion system (76.81% -86.93 %). EAE showed an inhibition ratio of (86.93 %) close to that of BHT (94.9±1.52 %). These findings provide evidence that *Myrtus communis* L. *berries* are a potential source of antioxidant which have many benefits towards human health.

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Introduction

Free radicals are thought to contribute to several disorders in the body (Pisoschi et al., 2015; Sevindik et al., 2017). Antioxidants are a group of compounds that inhibit oxidation and reduce free radicals directly or indirectly (Kına et al., 2021). Oxidative stress may be alleviated in vivo by exogenous administration of antioxidants. Antioxidants can be natural or synthetic, but due to toxic and carcinogenic effects of some synthetic antioxidants, such as butylhydroxyanisole and butylhydroxytoluene, research attention is turning to exploration and discovery of effective, safe, and natural antioxidants to resist oxidative stress (Liu et al., 2014; Korkmaz et al., 2021; Mohammed et al., 2021; Pehlivan et al., 2021). For this, a great deal of attention has been focused in recent decades on natural antioxidants such as polyphenols present in medicinal and dietary plants, which might help in preventing oxidative damage (Aidi Wanness and Marzouk, 2016; Benchikh et al., 2018; Mohammed et al., 2020; Uysal et al., 2021).

Myrtus communis L. is one of the important aromatic and medicinal species belonging to the Myrtaceae family which includes approximately 100 genera and 3000 species growing in temperate, tropical and subtropical regions. It is native to Southern Europe, North Africa and Western Asia and also distributed in South America, North western Himalaya and Australia (Sumbul et al., 2011). In Algeria, *M. communis* is widespread especially in the Tell Atlas and in the coastal regions (Quezel and Santa, 1963). It is commonly known under the name of El-Reihan or Hlamouche.

The total phenolic content and flavonoids from methanolic, ethanolic and aqueous extracts of Myrtle leaves and berries were evaluated for their antioxidant activities (Amensour et al., 2009; Amira et al., 2012, Benchikh et al., 2018) and their hepatoprotective activities (Kumar et al., 2011; Hanaa A et al., 2020). Several studies have indicated the anti-inflammatory properties of the essential oil of *M. communis* in animal models (Hosseinzadeh et al., 2011; Amira et al., 2012, Soomro et al., 2019). Myrtus pulp and

seeds extracts exert a synergic effect with vitamin D in reducing inflammation and ROS production, protecting cells from oxidative stress damages (Cruciani et al., 2018).

Other pharmacological studies on *M. communis* have been focussing on its antibacterial activity (Mansouri et al., 2001), its anti-ulcerogenic effects in rats (Benchikh, 2017) and anti-alzheimer activity (Aykac et al., 2018). The antidiarrhoeal and antisecretory effects of both essential oil (Benchikh et al., 2016 a). and different extracts (Benchikh et al., 2016 b) from the leaves were also assessed in mice using different doses and different mechanisms. The authors found that these elements had effective anti-diarrhoeal actions. The aim of the present study, was thus to quantify the phenolic and flavonoid contents in the berries of this plant as potential source of natural antioxidants and also to evaluate its antioxidant activity using different methods.

Materials and Methods

Plant Material

The fresh berries of *M. communis* were collected from Jijel (North-East of Algeria) in November, 2018. The taxonomic identification of the plant was carried by Professor Hocine Laouer, Department of Plant and Ecology, University Setif 1, Algeria. A voucher number 52 MB 07/12/18 JiJ/SA/ was deposed at the laboratory of Phytotherapy Applied to Chronic Diseases, Faculty of Nature and Life Sciences, University of Setif 1, Algeria. The collected plant was dried under shade and grounded into a fine powder using an electric mill.

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 (MBE) were 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 tetraglycosides 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.

Determination of Total Phenolic Content

Total phenolic content was assessed by Folin Ciocalteu reagent as described by Mamache et al. (2020). 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.

Determination of Total Flavonoid Content

Total flavonoid content was determined using aluminum chloride assay (Bahorun et al., 1996). One 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.

Determination of In vitro Antioxidant Activities

DPPH scavenging capacity

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, according to the method described by Amira et al. (2020). Briefly, 20 µL of plant 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:

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

AC : Absorbance of control
ATS : Absorbance of test sample

Reducing power

The reducing powers of MBE extracts were determined according to the method described by Mamache et al. (2020). A 0.1 mL aliquot of each extract BHT were 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% $FeCl_3$ (0.5 mL) and then the absorbance was measured at 700 nm. The reducing powers of the tested samples increased with the absorbance values.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of the plant extracts was measured by the salicylic method described by Benchikh (2017). 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.

β-carotene/linoleic acid 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 according to the method of Kartal et al. (2007). 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 completely 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 the plant tested extract 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 calculated in terms of powerful bleaching of β-carotene according to 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.

Statistical Data Analysis

Results were expressed as means ± standard deviation (SD) and were analysed 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).

Results and Discussion

Total Phenolics and Flavonoids Contents

The total phenolic content in terms of mg GAE/g of dry weight of extracts decreased in the following order: EAE>ME >AqE>CHE (Table 1), whereas, the total flavonoid content in terms of mg QE/g of dry weight of extract decreased in the following order EAE>CHE>ME>AqE. EAE extract contain the highest amounts both for phenolics (358.37±2.28GAE/g DW) and flavonoids compounds (105.44±3.48 QE/g DW), indicating that ethyl acetate could be the most powerful solvent to extract phenolic compounds.

In vitro Antioxidant Activities of MBE Extracts

DPPH radical scavenging activity

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 EAE extract exhibited the highest antioxidant activity (IC_{50}=0.0057 mg/mL), followed by ME (IC_{50}=0.016 mg/mL), CHE (IC_{50}=0.02 mg/mL) then AqE (IC_{50}=0.048 mg/mL) (Figure 1).

Table 1. Total phenolics and flavonoids contents of MBE extracts

Extract	Total phenolics (mg GAE/g Dw)	Total flavonoids (mg QE/g DW)
ME	147.85±2.52	30.13±1.41
CHE	72.70±0.83	46.11±1.65
EAE	358.37±2.28	105.44±3.48
AqE	73.96±0.50	2.38±0.24

ME: methanol extract, CHE: chloroform extract, EAE: ethyl acetate extract, AqE: Aqueous extract, DW: Dry weight, GAE: Gallic acid equivalent, QE: Quercetin equivalent. Results are expressed as means ± SD (n=3).

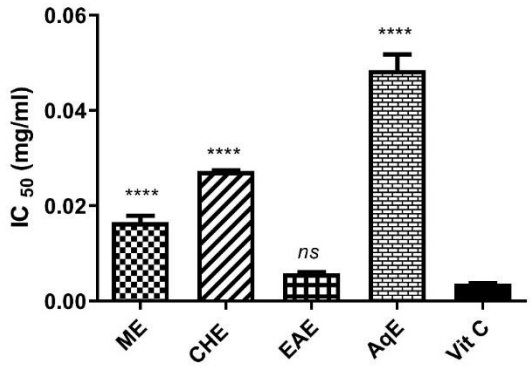

Figure 1. DPPH radical scavenging activity of M. communis L. berries extracts (MBE).

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; ns: not significant) vs vitamin C as standard.

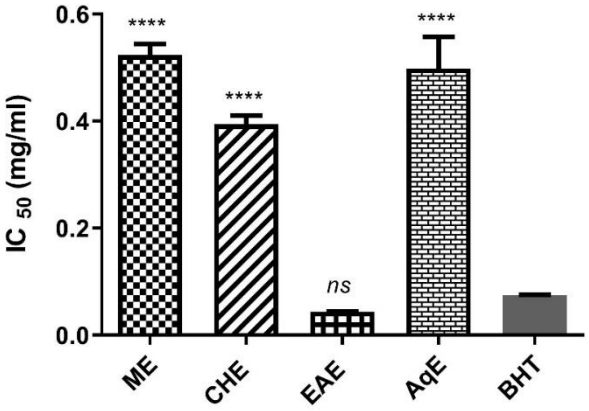

Figure 2. Reducing power activity of *M. communis* L. berries extracts (MBE).

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

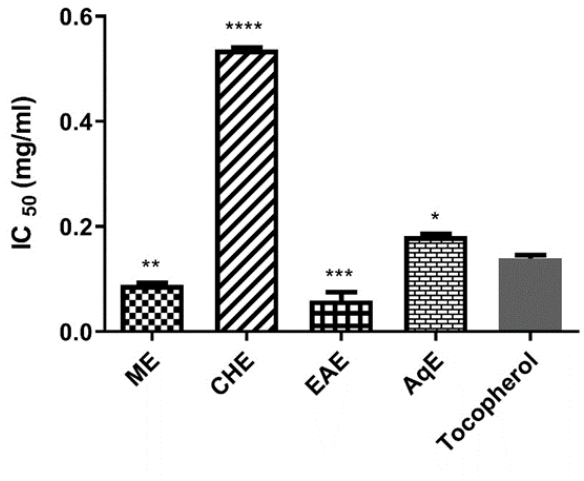

Figure 3. Hydroxyl radical scavenging activity of M. communis L. berries extracts (MBE).
ME: M methanol extract, CHE: chloroform extract, EAE: ethyl acetate extract; AqE: aqueous extract. Data were presented as IC50 means±SD (n=3) (****P≤0.0001; **P≤0.01; *P≤0.05; ns: not significant) vs Tocopherol as standard.

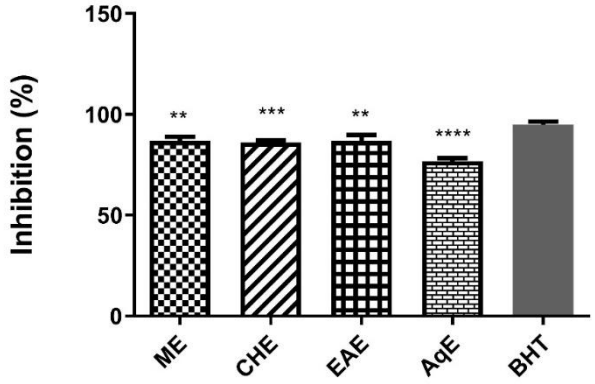

Figure 4. Antioxidant activity of M. communis L. berries extracts (MBE). (2 mg/mL) using β-carotene /linoleic acid bleaching assay after 24 h.
ME: methanol extract, CHE: chloroform extract, EAE: ethyl acetate extract; AqE: aqueous extract. Data were presented as IC50 means±SD (n=3). (****P ≤ 0.0001; ***P ≤ 0.001; **P ≤ 0.01) vs BHT as standard.

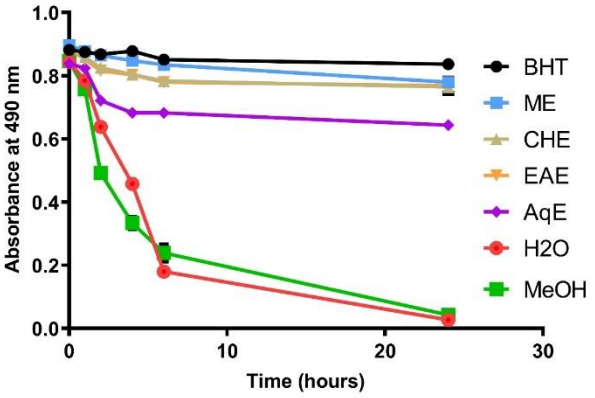

Figure 5. shows the decrease in absorbance of β-carotene in the presence of 2 mg/mL of different MBE extracts or reference antioxidant (BHT) compared with MeOH and H_2O as negative controls.

Reducing power capacity

From the results (Figure 2), we can see that the best reducing power (the effective concentration at which the absorbance was 0.5) was for EAE (EC_{50}=0.043 mg/mL). CHE, AqE and ME showed close reducing power values (EC_{50}=0.39, 0.49 and 0.52 mg/mL, respectively). BHT presented strong antioxydant activity (EC_{50}=0.074 mg/mL). This value is similar to that of the ethyl acetate fraction (AE), which means that use of synthetic antioxidants such as BHA or BHT could be avoided by replacing them with the natural ones.

Hydroxyl radical scavenging activity

The results showed that hydroxyl radical activity of EAE showed better activity (IC_{50}=0.059 mg/mL) than the standard tocopherol (IC_{50}=0.13±0.02 mg/mL), followed by ME (IC_{50}=0.08 mg/mL) and AQE (IC_{50}=0.18 mg/mL). However CHE showed the lowest activity (IC_{50}=0.53) compared with tocopherol (Figure 3).

Antioxidant activity of MBE extracts determined by β-carotene /linoleic acid bleaching assay

Figure 4 shows the effect of MBE 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. ME showed the highest antioxidant activity (86.93 %) and the AqE exhibited the lowest antioxidant activity (76.81%) compared to BHT (94.9±1.52%). Figure 5 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).

Discussion

In the present study, the polyphenols contents and the antioxidant activities of *M. communis* berries extracts were investigated. Antioxidant activity of plant extracts cannot be evaluated by a single method, therefore, commonly accepted assays were used to assess the antioxidative effect of the different extracts of MBE. Four different assays were used employing DPPH radical scavenging activity, hydroxyl radical scavenging activity, reducing power and β-carotene/linoleic acid bleaching assay.

In this study, it has been demonstrated that all MBE extracts contained phenolic compountds. EAE fraction showed the highest content (358.37±2.28). AqE and CHE showed the lowest content. This could be due to different degree of polarity of the solvents used for the extraction of polyphenolic compounds. It would be concluded that ethyle acetate is a good solvent to concentrate phenolic substances of intermediate polarity.

DPPH assay has become quite popular in natural antioxidant studies. One of the reasons is that this method is simple and highly sensitive. This assay is based on the theory that a hydrogen donor is an antioxidant. DPPH• is one of the few stable and commercially available organic nitrogen radicals (Moon et al., 2009). 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 (Ozsoy et al., 2008). 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 (Sahoo et al., 2013). Those results were comparable for the methanol extract (Aidi Wanness and Marzouk B, 2016). The same authors reported that myrtle seed, flower and leaf extracts showed stronger scavenging ability on DPPH and that they were rich in hydrolysable tannins.

The reducing power assay is often used to evaluate the ability of the natural antioxidant to donate an electron or hydrogen In this assay, the presence of reductants in the antioxidant sample causes the reduction of the Fe^{3+}/ferricyanide complex to the Fe^{2+} /ferrous form. It has been widely accepted that the higher absorbance at 700 nm, the greater is the reducing power (Gülçin, 2006). Results of the present study showed that the reducing capacity of the berries different extracts revealed strong activity in a dose dependant manner. It is believed that contents of polyphenols and flavonoids are related to reducing power (Duh et al., 1999). The fact that EAE fraction exhibited the strongest scavenger activity could be explained by its richness in both phenolic acids (358.37 mg GAE/kg DW) and flavonoids (105.44 mg QE/kg DW) compared to the other fractions.

Hydroxyl is very toxic free radical, causing damage to DNA, lipids and proteins. 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 (Liu et al., 2014). Removal of hydroxyl radicals can protect humans against some diseases (Shah et al., 2013). The present results reveal that EAE extract exhibited the most powerful scavenging effect. This strong activity could be attributed to the richness of this fraction in polyphenols and flavonoids, as mentioned above. In fact, polyphenols possess hydroxyl groups that confer scavenging ability against free radicals and reactive oxygen species (Halliwell et al., 1995).

It has long been known that β-carotene reacts with the peroxyl radical to produce β-carotene epoxides. Therefore, β-carotene has received attention as a radical scavenger or antioxidant. Later, an antioxidant assay using β-carotene combined with lipids, such as linoleic acid, was established. Lipids, such as linoleic acid, form a peroxyl radical (LOO•) in the presence of ROS and O_2. This peroxyl radical reacts with β-carotene to form a stable β-carotene radical. Subsequently, the amount of β-carotene reduces in a testing solution (Tsuchihashi et al., 1995). B-carotene in the absence of the antioxidant undergoes a rapid decolourization since the free linoleic acid radical attacks the β-carotene, which loses the double bonds and, consequently, its orange colour (Miguel et al., 2010). The degradation rate of β-carotene-linoleate depends on the antioxidant activity of the extracts. As for antiradical scavenging, all MBE extracts showed a higher ability to prevent the bleaching of β-carotene. If an antioxidant is present in a testing solution, it reacts competitively with the peroxyl radical, therefore, antioxidant effects are easily monitored by bleaching the colour of a test solution with a spectrophotometer at 470 nm, which is the typical absorbance by β-carotene. (Moon and Shibamoto., 2009).

In this study, MBE extracts significantly inhibited the degree of lipid peroxidation in the ß-carotene-linoleic bleaching inhibition assay which could be attributed to their phenolic contents, indicating that MBE extracts act as antioxidants by their ability to quench and neutralize free radicals or decompose peroxides. These results are in agreement with those of Kumar et al. (2011) and Gonçalves et al. (2013) who demonstrated that myrtle leaves extracts are effective inhibitors of lipid peroxidation.)

Conclusion

According to our results, in most cases, the highest radical scavenger capacity was detected in ethyl acetate and methanol extracts, which indicates that polyphenols may be responsible. The most interesting antioxidant activity, in order of effectiveness, was observed in DPPH and hydroxyl scavenging assays. Our finding increases the interest in the use of *M. communis berries* as a source of pharmacological agents. Further determination of compounds from this plant and the study of other biological effects may provide more information on their medicinal value.

Acknowledgements

This work was supported by the Algerian Ministry of Higher Education and Scientific Research (MESRS). We express our gratitude to these organizations.

Competing Interests

Authors have declared that no competing interests exist

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