HOSTED BY
ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Document heading doi: 10.1016/S2221-1691(15)30165-9

Phytochemical analysis, hypotensive effect and antioxidant properties of *Myrtus communis* L. growing in Algeria

Amel Bouaziz[1], Seddik khennouf[1*], Mussa Abu zarga[2], Shtaywy Abdalla[3], Abderahmane Baghiani[4], Noureddine Charef[4]

[1]*Laboratory of Phytotherapy Applied to Chronic Diseases, Department of Biology and Animal Physiology, Faculty of Nature and Life Sciences, University Setif 1, Setif, 19000, Algeria*

[2]*Natural Products Laboratory, Department of Chemistry, Faculty of Science, the University of Jordan, Amman, Jordan*

[3]*Animal & Human Physiology Laboratory, Department of Biology, Faculty of Science, the University of Jordan, Amman, Jordan*

[4]*Laboratory of Applied Biochemistry, Department of Biochemistry, Faculty of Nature and Life Sciences, University Setif 1, Setif, 19000, Algeria*

ARTICLE INFO

Article history:
Received 28 Sep 2014
Received in revised form 13 Oct 2014
Accepted 20 Oct 2014
Available online 29 Oct 2014

Keywords:
Myrtus communis
Phytochemistry
Phenolic content
Antioxidant
Hypertension

ABSTRACT

Objective: To analyze *Myrtus communis* chemically and evaluate the hypotensive effects and antioxidant properties of methanol, chloroform, ethyl acetate and aqueous extracts from the leaves of this plant.

Methods: Total phenolic and flavonoid contents as well as the antioxidant potential of methanol, chloroform, ethyl acetate and aqueous extracts have been investigated by using different *in vitro* methods. The hypotensive effects of methanol and ethyl acetate extracts were evaluated in anaesthetized rats by using the method of invasive blood pressure recording. Moreover, ethyl acetate extract was subjected to analysis by different chromatographic methods in order to identify new compounds.

Results: Chemical analysis of ethyl acetate extract revealed the presence of myrecitin-3-O-α-rhamnoside. Ethyl acetate extract was found to have the highest total phenolic and total flavonoid contents with the values of 435.37 mg gallic acid equivalents/g dried weight and 130.75 mg quercetin equivalent/g dried weight, respectively. Ethyl acetate extract also exhibited the highest activity in scavenging 2,2-diphenyl-1-picrylhydrazyl, 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid), hydroxyl radical and reducing power; whereas, methanol extract exhibited higher chelating activity than ethyl acetate extract did. Chloroform was found to be strong inhibitor of lipid peroxidation in β-carotene bleaching assay (91.19%), ferric thiocyanate method (87.55%), and thiobarbituric acid method (82.59%) as compared to butylated hydroxytoluene. Intravenous administration of methanol and ethyl acetate extract (0.04 to 12 mg/kg body weight) decreased the maximum mean arterial blood pressure with values of 20.6% and 32.49% at 12 mg/kg body weight, respectively in anesthetized rats.

Conclusions: This study provides a scientific basis for the use of *Myrtus communis* in traditional medicine as hypertensive agent as well as additional resources for natural antioxidants.

1. Introduction

The excessive production of reactive oxygen species (ROS) overwhelming the antioxidant defense mechanisms of the cells has been shown to oxidize biological molecules and induce damage to the cell membrane, proteins, carbohydrates, and DNA. This oxidative stress is involved in several pathological situations including hypertension, hypercholesterolemia, diabetes, and heart failure[1]. In pathophysiology of hypertension, ROS especially superoxide radical (O_2^-) accelerate the breakdown of nitric oxide (NO) by binding it to form peroxynitrite ($ONOO^-$). NO is the main vasodilator factor that maintains vascular homeostasis and normal vasomotor tone, and inhibits renal tubular sodium reabsorption. Thus, reduction in bioavailability of NO leads to hypertension through increasing

*Corresponding author: Prof. Seddik khennouf, Department of Biology and Animal Physiology, Faculty of Nature and Life Sciences, University Setif 1, Setif, 19000, Algeria.
Tel: +213552 747103
E-mail: khennouf_sed@yahoo.fr
Foundation Project: Supported by Thematic Agency for Research in Health Sciences (Grant No. 361/25/09/2011).

peripheral vasoconstriction and sodium retention[2,3].

Plants synthesize a diverse spectrum of antioxidant phenolic compounds as secondary products, which not only prevent oxidative damage in planta but also protect human from diseases when the plants are consumed as food[4]. Use of synthetic antioxidant in the prevention of the free radical damage had many toxicological side effects including carcinogenicity. For this reason, there has been a particular interest in the potential health benefits of natural antioxidants with the strong capacity to scavenge ROS and inhibit lipid peroxidation[5].

Myrtle [*Myrtus communis* L. (*M. communis*)] is an evergreen shrub belonging to the Myrtaceae family. It is widespread spontaneously throughout the Mediterranean area and has been used for medicine, food and spice purposes. In folk medicine, the leaves and fruits are used as antiseptic, antibacterial, antihyperglycemic, analgesic, antiinflammatory agents[6,7]. Also, in Algeria, the decoction of aerial parts of myrtle is recommended as antihypertensive drug[8,9].

Many reports have described antioxidant activities of different extracts of *M. communis*, and certain ingredients implying good benefit for the treatment of diseases related to oxidative stress and various known antioxidants including flavonoids, phenolic acids, tannins and α-tocopherol have been previously isolated from this plant[10-12].

Many investigations on myrtle have focused on its volatile fraction and on phenolic compounds in berries[11,13-16]. However, few studies have investigated the antioxidant activity of myrtle leaf extracts. In addition, there is no pharmacological study on the effects of *M. communis* extract on hypertension. Thus, the present study was undertaken to evaluate the hypotensive effect on *in vivo* and the *in vitro* antioxidant properties of various extracts from *M. communis* leaves and to find any correlation between the antioxidant activity and total phenolic, total flavonoid contents of the extracts as well to identify possible constituents responsible for this activity.

2. Materials and methods

2.1. Chemicals

Linoleic acid, ammonium thiocyanate, β-carotene, butylated hydroxytoluene (BHT) were purchased from Fluka Chemical Co. (Buchs, Switzerland). The chemicals such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethylenediamine tetraacetic acid (EDTA), gallic acid, 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid (trolox), 2.2´-Azino-bis(3-ethylbenzenothiazoline-6-sulfonic acid) (ABTS), Folin-Ciocalteu reagent were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Potassium persulphate, potassium ferricyanide, trichloroacetic acid, thiobarbituric acid (TBA), ferrozine, ferrous and ferric chloride were obtained from Merck. All other reagents were of analytical grade.

2.2. Animals

Healthy male and female adult albino rats weighing 200–300 g were used. Animals were housed in an air-conditioned animal room, with 12 h/12 h light/dark photoperiod, and maintained with free access to water and feeding *ad libitum*. The hypotensive experiments in anesthetized rats were conducted after the experimental procedures were revised and approved by the Animal Ethics Committee of the University of Jordan, Jordan.

2.3. Extraction, fractionation and purification

The extraction procedure for phenolic compounds was conducted as the method described by Markham[17]. The leaves of *M. communis* were collected from Jijel Region in North Eastern Algeria during September, 2012. The plant was identified by Prof. Hocine Louar from the Laboratory of Botany, Faculty of Natural and Life Sciences, University, Setif, Algeria. The leaves were separated, dried under shadow and powdered. Then, the grounded plant material (1 kg) was extracted by maceration with 5 L methanol (85%) at room temperature for 5 d. The filtered solvent was evaporated under vacuum and lyophilized to obtain a crude methanolic extract (190.8 g). The residue (100 g) was dissolved in 1 L H_2O–methanol mixture (9:1) and was then partitioned by successive extractions with hexane, chloroform and ethyl acetate. Each fraction was evaporated to dryness under reduced pressure to give hexane, chloroform, ethyl acetate, and the remaining aqueous extract. After that, the ethyl acetate extract (23 g) was chromatographed over silica gel column (0.063-0.200 mm, Riedel-Dehaen, 120 g, 3 cm i.d.) and eluted with chloroform, and then with chloroform-methanol mixtures of increasing polarity (100; 90:5-50:50) in a gradient system. On the bases of thin layer chromatography behavior, 48 fractions (250 mL for each) obtained were combined together into 14 fractions. Then, the fractions were purified by preparative thin layer chromatography and the structures of the isolated compounds were determined by mass spectrometry, ^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR spectra.

2.4. Determination of total polyphenols content

The total polyphenols content was determined by the Folin-Ciocalteu method as described by Li *et al.* with slight modification[18]. In brief, 0.1 mL of *M. communis* extracts were mixed with 0.5 mL of Folin-Ciocalteu reagent (diluted 10 times). After 4 min, 0.4 mL of 7.5% sodium carbonate solution was added. The final mixture was shaken and then incubated for 90 min in dark at room temperature. The absorbance of all samples was measured at 760 nm and the results were expressed in milligrams of gallic acid equivalents per gram dried weight (mg GAE/g DW).

2.5. Determination of total flavonoids content

The total flavonoids content of each extract was determined by a colorimetric method as described by Bahorun *et al*[19]. Each sample (1 mL) was mixed with 1 mL of aluminium chloride solution (2%) and allowed to stand for 10 min. Absorbance of the

mixture was then determined at 430 nm versus prepared methanol blank. Results were expressed as quercetin equivalent per gram dried weight (mg QE/g DW).

2.6. In vitro antioxidant activity

2.6.1. DPPH radical scavenging assay

Free radical scavenging activity against DPPH radical was measured by using the method described by Burits and Bucar[20]. About 50 μL of extract dilutions ranged from 0.1 to 1.5 mg/mL were added to 5 mL of DPPH solution (0.004%) dissolved in methanol. After 30 min at room temperature, the absorbance was measured at 517 nm. BHT was used as standard. Inhibition of free radical DPPH in percent (I%) was calculated in following way:

$$I\% = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where, $A_{control}$ is the absorbance of the blank solution (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the plot of inhibition percentage against extract concentration.

2.6.2. ABTS radical scavenging activity assay

The spectrophotometric analysis of $ABTS^+$ radical scavenging activity was determined according to method of Re *et al.* with some modifications[21]. The $ABTS^+$ solution was produced by the reaction of 7 mmol/L of ABTS solution in 2.45 mmol/L potassium persulfate (final concentration). The mixture was kept in the dark at room temperature for 24 h before use. The solution was diluted with methanol and equilibrated at room temperature to give an absorbance of 0.70±0.02 at 734 nm in a 1 cm cuvette. Then, 50 μL of the extract dilutions (0.012 5, 0.025, 0.05, 0.1, 0.2, 0.3, 0.5 mg/mL) was mixed with 1 mL $ABTS^+$ solution and kept for 30 min at room temperature; the absorbance of reaction mixture was measured at 734 nm. Trolox was used as positive control. The $ABTS^+$ radical scavenging ability was calculated according to the same equation in the DPPH assay.

2.6.3. Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity was assayed according to the method of Li *et al.* with slight modification[22]. Briefly, A mixture of 100 μL 1,10-phenanthroline (5 mmol/L), 100 μL $FeSO_4$ (5 mmol/L) and 100 μL EDTA (15 mmol/L) was mixed with 70 μL of sodium phosphate buffer (0.2 mol/L, pH 7.4). Then, 100 μL sample (1 mg/mL) and 140 μL H_2O_2 (0.01%) were added. The mixture was incubated at 37 °C for 60 min, and the absorbance was measured at 536 nm. Vitamin C was used as a positive control. To calculate hydroxyl radical scavenging, the following equation was used:

$$\text{Hydroxyl radical scavenging activity } (\%) = \frac{(A_s - A_0) \times 100}{(A_c - A_0)}$$

Where, A_s is the absorbance of the sample, A_0 is the absorbance of the blank solution (distilled water); and A_c is the absorbance of a control solution in the absence of H_2O_2.

2.6.4. Ferrous ion chelating activity

Ferrous ion chelating activity was measured following the method of Decker and Welch[23]. The reaction mixture (1.5 mL) contained 500 μL of extract dilutions ranged from 0.1 to 30 mg/mL or EDTA, 100 μL $FeCl_2$ (0.6 mmol/L in water) and 900 μL methanol. The control contained all the reaction reagents except the extract and EDTA. The mixture was shaken well and allowed to react at room temperature for 5 min and 100 μL ferrozine (5 mmol/L in methanol) was then added. The absorbance of the Fe^{2+}-ferrozine complex was measured at 562 nm. The chelating effect was calculated as a percentage by using the same equation in the DPPH assay. IC_{50} value was defined as the effective concentration of test material that is required to chelate 50% of iron ions.

2.6.5. Reducing power

The reducing power of the *M. communis* extracts was determined according to the method of Chung *et al*[24]. A 0.1 mL aliquot of different concentrations of extracts ranged from 0.001 to 0.25 mg/mL or BHT was mixed with an equal volume of 0.2 mol/L phosphate buffer (pH 6.6) and 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min to reduce ferricyanide into ferrocyanide. After that, 0.25 mL trichloroacetic acid was added into the mixture to stop the reaction, and the mixture was centrifuged at 3 000 r/min for 10 min. The supernatant (0.25 mL) was added into distilled water (0.25 mL) and 0.1% ferric chloride (0.5 mL), and then the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates increase in reducing power.

2.6.6. β-carotene bleaching assay

In this assay, antioxidant capacity was determined by measuring the inhibition of discoloration of β-carotene according to the method of Dapkevicius *et al*[25]. A stock solution of β-carotene-linoleic acid mixture was prepared as follows: 0.5 mg β-carotene was dissolved in 1 mL of chloroform, 25 μL linoleic acid and 200 mg of Tween 40 (Merck) were added. Chloroform was completely evaporated, and then, 100 mL distilled water saturated with oxygen (30 min, 100 mL/min) was added with vigorous shaking. About 2 500 μL of this reaction mixture was dispensed into test tubes and 350 μL of the various extracts, BHT and blanks (H_2O and methanol), prepared at concentration of 2 mg/mL were added and the emulsion system was incubated for 24 h at room temperature. The absorbances of the mixtures were measured at 490 nm. The antioxidant activity was measured in terms of successful bleaching of β-carotene by using the following equation:

$$\text{Antioxidant activity}\% = \frac{A_{sample}}{A_{BHT}} \times 100$$

Where, A_{sample} is the absorbance of the extract; A_{BHT} is the absorbance of positive control BHT.

2.6.7. Ferric thiocyanate (FTC) test

The antioxidant activity of plant extracts on the inhibition of linoleic acid peroxidation was assayed with a modified thiocyanate method described by Yen *et al*[26]. A total of 0.5 mL sample solution of 2 mg/mL of plant fractions was mixed with 2.5 mL of 0.02 mol/L linoleic acid emulsion at pH 7 and 2 mL of 0.2 mol/L phosphate buffer at pH 7. The linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid and 0.2804 g of Tween-20 as emulsifier in 50 mL phosphate buffer. The reaction mixture was incubated at 37 ˚C for 5 d. About 0.1 mL of the reaction mixture at 24 h intervals was added with 75% ethyl alcohol (4.7 mL), 30% ammonium thiocyanate (0.1 mL), 0.02 mol/L ferrous chloride and 3.5% HCl (0.1 mL). Three minutes after the addition of ferrous chloride to the reaction mixture, the absorbance was measured at 500 nm. The measurement was performed every 24 h until the absorbance of the control reached its maximum value. BHT and vitamin C was used as positive controls.

2.6.8. TBA test

The TBA test was conducted on the final day of FTC test according to the method described by Kikuzaki and Nakatani to determine the malonaldehyde formation from linoleic acid peroxidation[27]. The same sample preparation method as described in the FTC test 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 min. After cooling, it was then centrifuged at 3 000 r/min for 20 min. 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. Both methods (FTC and TBA) described antioxidant activity by percent inhibition:

$$\% \text{ Inhibition} = 100 - [(A_{sample}/A_{control}) \times 100]$$

Where, $A_{control}$ and A_{sample} are the absorbances of the control (without sample) and the experimental (with sample) reactions, respectively.

2.7. Blood pressure measurements in anesthetized rats

The blood pressure of the anesthetized rats was recorded by method described by Abdalla *et al*[28]. Male albino rats were anesthetized with sodium thiopental (50 mg/kg body weight; *i.p.*). The right common carotid artery was exposed and a catheter was introduced for the recording of blood pressure using P23AA Statham pressure transducer situated at the level of the heart and connected to a Gilson polygraph. The right femoral vein was also catheterized for the intravenous injection of methanol and ethyl acetate extracts. After a steady baseline of blood pressure was obtained (about 15 min), methanol and ethyl acetate extracts were injected in doses of 0.04, 0.12, 0.4, 1.2, 4, and 12 mg/kg body weight. Blood pressure was allowed to return to the resting level before every next dosing. The changes in systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial blood pressure (MABP) were recorded and expressed as percent of their respective control values obtained before extracts administration.

2.8. Statistical analysis

Statistical analysis was performed by using the Graph Pad Prism (version 5.01 for Windows). All *in vitro* results were calculated as mean±SD and were analyzed by One-way analysis of ANOVA followed by Dunnet's test. The pharmacological results were presented as mean±SEM of six experiments. Values were compared by using paired Student's *t*-test. In all cases, The *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Phytochemical analysis

The mother liquor of fraction from ethyl acetate extract gave one major spot which was purified by using preparative thin layer chromatography eluting with chloroform: methanol (80:20) to give a yellow powder identified as myricetin-3-O-α-rhamnoside and also known as Myricetrin (Figure 1)[18-28].

Its NMR spectroscopic data was as the following: ^{1}H-NMR [500 MHz, dimethylsulfoxide-d_6 δ/ppm]; 0.85 (3H, d, J=6.1 Hz, H-6"), 3.00-3.70 (4H, m, H-2", H-3", H-4", H-5"), 3.99 (1H, br s, H-1"), 6.44 (1H, br s, H-8), 6.47 (1H, br s, H-6), 6.91 (2H, s, H-2', H-6'), 12.66 (1H, br s, 5-OH).

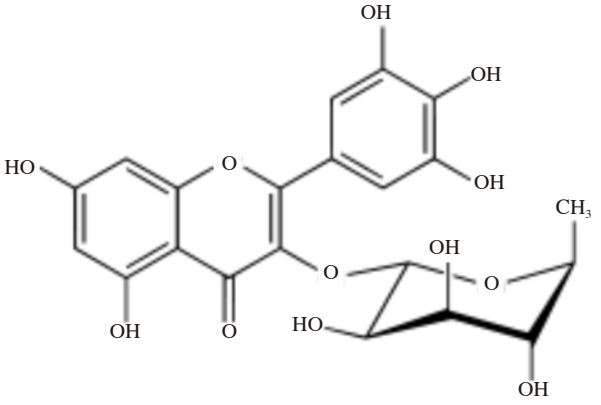

Figure 1. Myricetin–3-O-α-L-rhamnoside.

^{13}C-NMR (500 MHz, dimethylsulfoxide-d_6 δ/ppm); 18.0 (C-6"), 70.5 (C-2", C-5"), 70.9 (C-3"), 71.8 (C-4"), 102.4 (C-1"), 94.0 (C-8), 99.2 (C-6), 104.4 (C-10), 108.4 (C-2' and C- 6'), 120.1 (C-1'), 134.8(C-4'), 136.9 (C-3), 146.2 (C-3' and C-5'), 156.8 (C-2), 157.9 (C-9), 161.7 (C-5), 164.8 (C-7), 178.2 (C-4).

The mass spectrum of myricetin–3-O-α-rhamnoside gave a molecular ion peak at m/z=436.088 20 calculated for the molecular

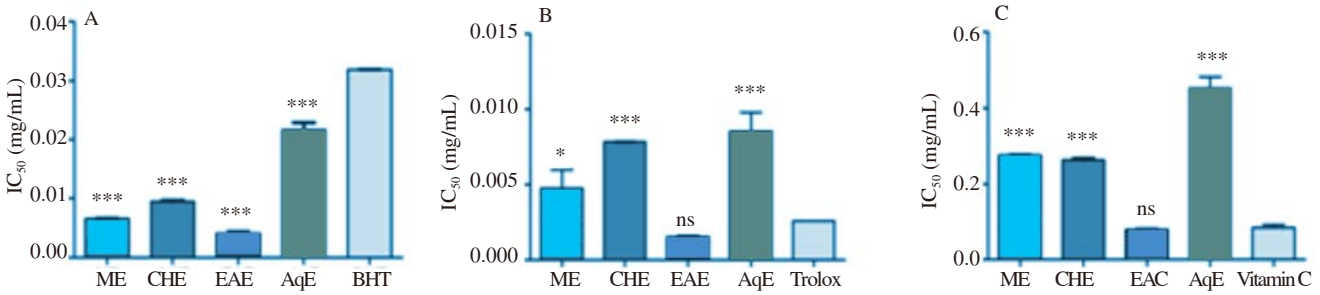

Figure 2. Free radical scavenging activity of different *M. communis* L. extracts.
A: DPPH assay; B: ABTS assay; C: Hydroxyl radical assay. Data were presented as means±SD (n=3). ns: No significant difference. *: P<0.01, ***: P<0.001 compared to standards; ME: Methanol extract; CHE: Chloroform extract; EAE: Ethyl acetate extract; AqE: Aqueous extract.

formula ($C_{21}H_{18}O_{12}$+H^+).

3.2. Total polyphenol and flavonoid contents

The total phenolic contents in *M. communis* extracts measured by Folin-Ciocalteu reagent in terms of mg GAE/g DW decreased in the following order: ethyl acetate extract>methanol extract>chloroform extract>aqueous extract; whereas the flavonoid contents calculated as mg QE/g DW by using aluminum chloride methods were in this order: ethyl acetate extract>chloroform extract>methanol extract>aqueous extract (Table 1).

Table 1
Total polyphenol and flavonoid contents in *M. communis* extracts.

Extracts	Total phenolic content (mg GAE/g DW)	Total flavonoids content (mg QE/g DW)
Methanol	260.44±2.52	26.77±0.46
Chloroform	186.96±1.69	50.81±1.20
Ethyl acetate	435.37±3.15	130.75±2.86
Aqueous	157.70±2.65	2.64±0.22

Results are expressed as means±SD (n=3).

From this results, it is easily to conclude that the highest total polyphenol and flavonoid contents were found in ethyl acetate (435.37 mg GAE/g DW and 130.75 mg QE/g DW, respectively).

3.3. Antioxidant activity evaluation

3.3.1. DPPH radical scavenging activity

In this assay, all extracts scavenged significantly the DPPH radical (P<0.001) in concentration dependent manner with higher activity than BHT as standard control (IC_{50}=0.031 00±0.000 05 mg/mL) (Figure 2A). The IC_{50} of myrtle extracts increased in the order of ethyl acetate extract (0.004 0±0.000 3 mg/mL), methanol exract (0.006 00±0.000 05 mg/mL), chloroform extract (0.009 0±0.000 2 mg/mL) and aqueous extract (0.021±0.001 mg/mL), respectively.

3.3.2. ABTS radical scavenging activity

The results showed that all extracts effectively scavenged the ABTS cations with IC_{50} values ranging from 0.001 5 mg/mL to 0.008 mg/mL (Figure 2B). Such values are close to that of standard trolox [(0.002 500±0.000 005) mg/mL]. As seen in Figure 2B, the ethyl acetate extract and methanol extract had strong activity to scavenge ABTS radicals with IC_{50} values of (0.001 50±0.000 09) mg/mL and (0.004 80±0.000 08) mg/mL, respectively.

3.3.3. Hydroxyl radical scavenging activity

In this assay, hydroxyl radicals generated by the Fenton reaction could oxidize Fe^{2+} into Fe^{3+} which is reflected by the degree of decolorization of the reaction solution. Thus, all extracts of *M. communis* considerably decreased the degradation of Fe^{2+}-(1,10-phenanthroline/EDTA) by H_2O_2 compared to vitamin C [IC_{50} was (0.084±0.007) mg/mL] (Figure 2C). The level of antioxidant activity of *M. communis* extract decreased in the following order: ethyl acetate extract>chloroform extract>methanol extract>aqueous extract, and the IC_{50} values of these extracts were found to be (0.080±0.007) mg/mL, (0.262 8±0.005 0) mg/mL, (0.275 0±0.000 8) mg/mL and (0.454 0±0.200 0) mg/mL, respectively.

3.3.4. Metal chelating activity

As seen in Figure 3, all extracts interfered with the formation of ferrous and ferrozine complex, suggesting that the extracts exhibited appreciable chelating activity.

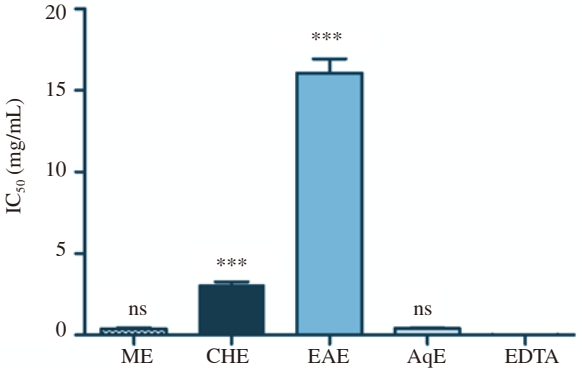

Figure 3. Metal chelating activity of different *M. communis* extracts.
Data were presented as means±SD (n=3). ns: No significant difference; ***: P<0.001 compared to EDTA as standard; ME: Methanol extract; CHE: Chloroform extract; EAE: Ethyl acetate extract; AqE: Aqueous extract.

By comparing myrtle extracts, the chelating activities decreased in the order of methanol extract [(0.39±0.06) mg/mL]>aqueous extract [(0.403±0.050) mg/mL]>chloroform extract [(3.05±0.21) mg/mL]>ethyl acetate extract [(16.05±0.88) mg/mL]. Methanol and

aqueous extracts were found to be as strong as positive control, EDTA [IC_{50}=(0.018 6±0.003) mg/mL] (no significant difference, P>0.05).

3.3.5. Reducing power

Figure 4 shows the dose-response curves for the reducing powers of the extracts from *M. communis*. In this assay, the extracts of *M. communis* showed a promising result. Reducing power of extracts and standard compound (BHT) decreased in the following order: ethyl acetate extract>BHT>methanol extract>aqueous extract>chloroform extract, with absorbance of 1.51±0.01, 0.99±0.007, 0.65±0.07, 0.60±0.02 and 0.55±0.01, respectively at 0.1 mg/mL.

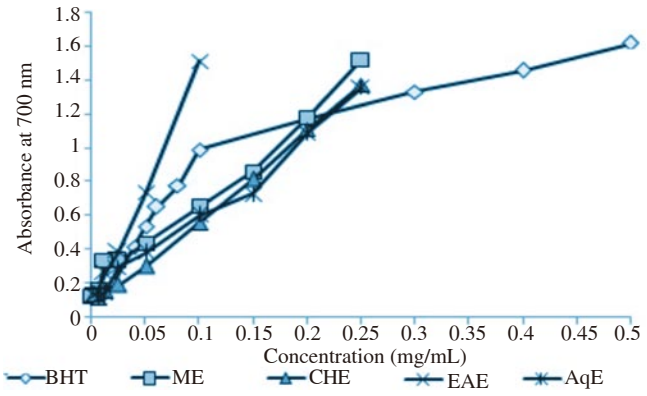

Figure 4. Reducing power of different *M. communis* extracts.
Values are expressed as means±SD (n=3). ME: Methanol extract; CHE: Chloroform extract; EAE: Ethyl acetate extract; AqE: Aqueous extract.

3.3.6. β-carotene/linoleic acid bleaching assay

In this assay, all extracts showed higher ability to prevent the bleaching of β-carotene compared to BHT as positive control (Figure 5).

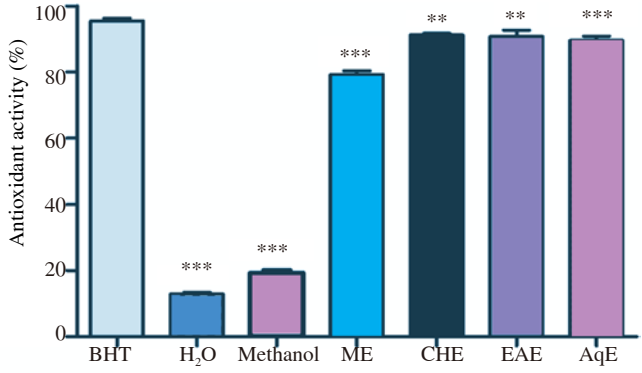

Figure 5. Antioxidant activities of *M. communis* extracts (2 mg/mL at 24 h of incubation) measured by β-carotene bleaching method.
Values are expressed as means±SD (n=3). **: P<0.01, ***: P<0.001 compared to BHT as standard. ME: Methanol extract; CHE: Chloroform extract; EAE: Ethyl acetate extract; AqE: Aqueous extract.

The highest relative antioxidant activity was found in chloroform extract (91.19%±0.51%) followed by ethyl acetate extract (90.73%±1.90%), aqueous extract (89.54%±1.20%) and methanol extract (79.13%±1.20%). There was a significant difference (P<0.05) between the means of total antioxidant activity among the myrtle extracts and BHT as standard (95.40%±0.88%).

3.3.7. Ferric thiocyanate test

As shown in Figure 6, all extracts significantly slackened the peroxidation in the linoleic acid emulsion system throughout the incubation period when compared to standard control BHT and vitamin C. The best activity was observed in chloroform extract (87.55%±1.40%), which was as strong as that of the positive control, BHT (no significant difference, P>0.05) followed by methanol extract (84.59%±1.8%), ethyl acetate extract (81.05%±0.87%) and aqueous extract (46.95%±0.49%), respectively.

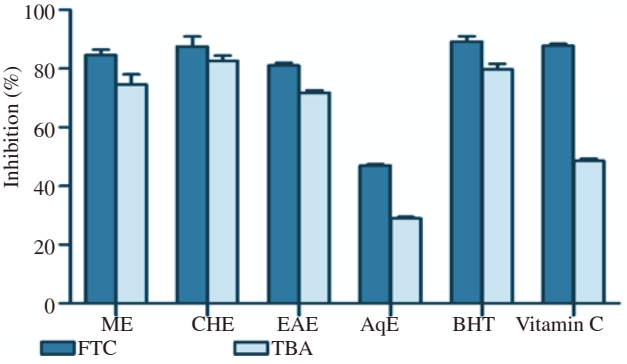

Figure 6. A comparison of total antioxidant activity of *M. communis* extracts by using the FTC and TBA methods.
Values are expressed as means±SD (n=3). ME: Methanol extract; CHE: Chloroform extract; EAE: Ethyl acetate extract; AqE: Aqueous extract.

3.3.8. TBA test

The results from TBA test (Figure 6) strongly correlated with the FTC data, and all fractions of *M. communis* exhibited a good antioxidant potential when compared to BHT and vitamin C. The percentage inhibition of peroxidation in a linoleic acid system by plant fractions and standard compound (BHT) decreased in the following order: chloroform extract (82.59%±1.90%)>methanol extract (74.64%±2.47%)>ethyl acetate extract (71.78%±0.61%)>aqueous extract (28.96%±0.49%).

Therefore, from the results, the apolar chloroform extract was the most effective in inhibiting the lipid peroxidation than the other extracted fractions, and the activity of this extract was superior to vitamin C (48.60%±1.5%) and BHT (79.75%±0.61%).

3.4. Hypotensive effect in anesthetized rats

The results showed that intravenous injection of methanol and ethyl acetate extract at 0.04 to 12 mg/kg induced dose-dependent and transitory decrease in SBP, DBP and MABP of the anesthetized rats (Figures 7 and 8). As shown in Figure 7, the maximum decrease in SBP and DBP was 18.67%±2%; 21.57%±3.17% and 30.46%±2.88%; 33.51%±1.07% for methanol and ethyl acetate extract respectively at the dose of 12 mg/kg. Also, the maximum fall in MABP of 20.60%±2.78% and 32.49%±2.88% was recorded for methanol and ethyl acetate extract respectively at the same dose (Figure 8).

From the above results, it was found that both extracts decreased significantly (P<0.001) the MABP at the dose of 0.4 to 12 mg/mL.

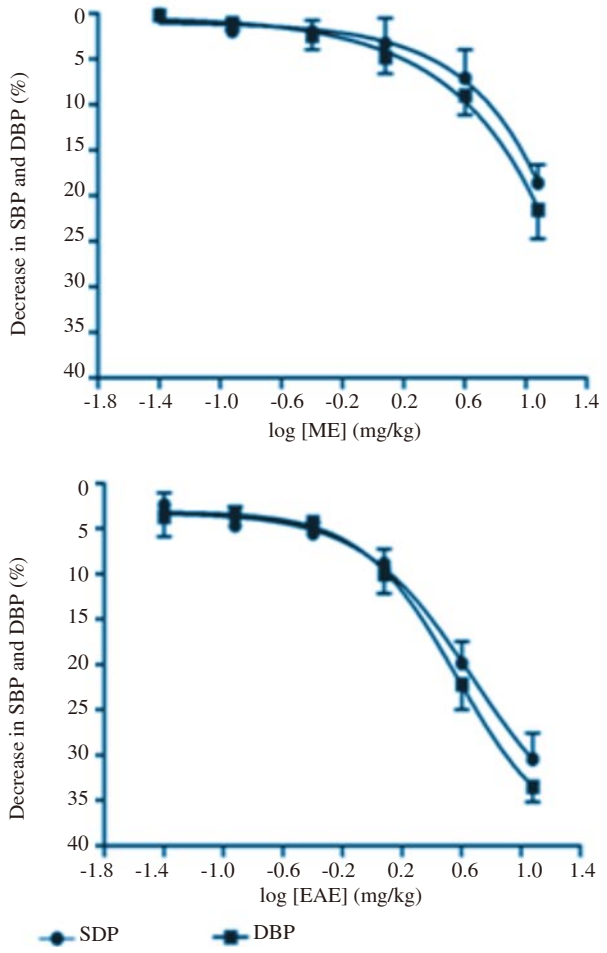

Figure 7. Concentration-response curves of methanol and ethyl acetate extract of *M. communis* on SBP and DBP of anesthetized rats.
Values are expressed as means±SEM (n=6). ME: Methanol extract; EAE: Ethyl acetate extract.

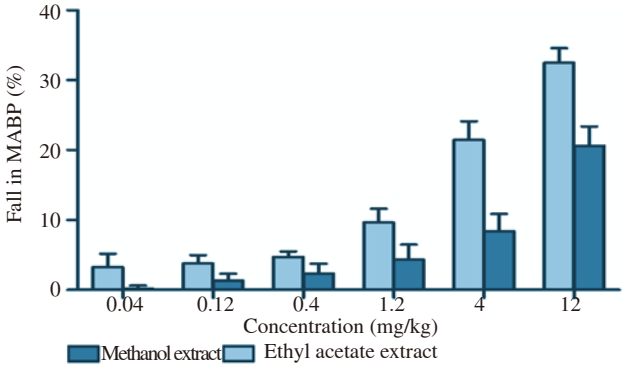

Figure 8. Dose-dependent hypotensive effect of methanol and ethyl acetate extract of *M. communis* on MABP in anaesthetized rats.
Values are expressed as mean±SEM (n=6).

4. Discussion

In the present study, the hypotensive effect, the radical scavenging activity, reducing power, iron chelating activity and inhibition of lipid peroxidation of *M. communis* extracts were investigated. The antioxidant activities of *M. communis* extracts were also compared with that of reference synthetic antioxidant. In addition, the phytochemical analysis of ethyl acetate extract was carried out.

Many reports had described the chemical constituents of different extracts of myrtle including polyphenols and flavonoids[10,12,15]. In the present study, phytochemical analysis of ethyl acetate extract from *M. communis* revealed the isolation of myricetin-3-O-rhamnoside, a major flavonol in this plant, which was isolated previously from myrtle liqueur, and myrtle leaves[11,16,29]. The proposed structure of this flavonol was confirmed by comparison of NMR and mass data with those reported in the literature[30].

Phenolic compounds such as flavonoids, phenolic acids and tannins are widely distributed in plants and have gained much attention due to their antioxidant activities and free radical scavenging abilities, which potentially have benefit for human health[14].

Results obtained in the present study revealed that the level of these phenolic compounds in the various extracts of the myrtle leaves were considerably higher in ethyl acetate extract than that in other extracts, and this could be due to different degree of polarity of the solvents used for the extraction of polyphenolic compounds. Moreover, the phenolic content of *M. communis* leaves observed in this study corroborated with the findings of Gardeli *et al*. and Nassar *et al*. on different fractions of this plant from different agro-climatic regions[10,31].

ROS play a role in signal transduction; whereas excessive ROS production leads to oxidative stress which has been involved in the pathophysiology of many cardiovascular diseases such as endothelial dysfunction, atherosclerosis and hypertension[2,32].

From the above results, it can be noted that *M. communis* extract exhibited different but effective degrees of *in vitro* antioxidant activity with all the methods and lipid systems used.

Among all the extracts, ethyl acetate extract was shown to possess significant radical scavenging activity against DPPH, ABTS, hydroxyl radicals and reducing power, evidencing its ability of having extracted a considerable amount of polyphenols and flavonoids with specific structure with many hydroxyl groups. Remaining extracts can't be considered to be inferior because satisfactory phenolic contents were obtained in this study and enough to show their antioxidant activity. According to many reports, there is a highly positive correlation between polyphenols, flavonoids, and antioxidant activities in many plant species, and this is mainly due to their redox properties, which allows them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers[33-35]. Furthermore, they scavenge free radicals and have a metal chelating potential[11].

DPPH and ABTS radical scavenging methods are common spectrophotometric procedures for determining antioxidant capacities of components, and they are based on the ability of ABTS and DPPH radical to decolorize in the presence of antioxidants by accepting an electron or hydrogen donated by an antioxidant compound[36]. Thus, the strong scavenging capacity of *M. communis* extracts

was possibly due to the hydrogen/electron donating ability of the polyphenolic compounds present in these extracts, which made them good antioxididants acting as free radical inhibitors or scavengers. Our results is in agreement with that obtained by Hayder *et al*., who studied the effect of extraction solvent on DPPH scavenging activity of myrtle leaf extracts from Tunisia[37].

Hydroxyl radical is the most ROS and it can cause lipid peroxidation and enormous biological damage. It is a potent cytotoxic agent and able to attack and damage almost every molecule found in living tissues. Therefore, the scavenging of hydroxyl radical by extracts may provide a significant protection to biomolecules against free radicals[38].

Myrtle extracts showed potent antioxidant activity mainly due to their richness in phenolic compounds. Therefore, these polyphenols should be considered to contain conjugated ring structures and hydroxyl groups that have the potential to function as antioxidant *in vitro* by scavenging free radicals, lipid peroxy radicals and stabilizing free radicals involved in this oxidative processes[39].

The reducing power of the extracts may provide a significant indication about the potential antioxidant capacity of the plant. The presence of antioxidants in the sample would result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. The amount of Fe^{2+} complex can then be monitored by measuring the absorbance at 700 nm[40]. In this assay, *M. communis* extracts showed good reducing power in a concentration-dependent manner, indicating the compounds in this plant, performing as good electron donors and therefore should be able to terminate radical chain reaction by converting free radicals and ROS into more stable products.

The metal chelating assay is based on the ability of extract to chelate transition metals by binding them to ferrous (Fe^{2+}) ion catalyzing oxidation and disrupting the formation of Fe^{2+}- ferrozine complex (intense red purplish in color). This chelating capacity is important, since it reduces concentration of the catalyzing transition metal in lipid peroxidation through the inhibion of lipid peroxides to peroxyl and alkoxyl radicals via the Fenton reaction[40,41]. The observed results demonstrate that all extracts interfered with the formation of ferrous and ferrozine complex, suggesting that the extracts exhibited appreciable chelating activity.

Although phenolic contents in methanol and aqueous extracts are lower than that in ethyl acetate extract, they showed the better chelating activity than that of ethyl acetate extract, and this finding was supported by the correlation analysis, which showed that there is a negative correlation of phenolic compounds with chelating activity (R^2=0.82). In fact, although phenolic compounds are the most abundant and potent antioxidants in plants, there is also a number of non-phenolic compounds that contribute to the overall antioxidant activity of plant extracts[5]. The antioxidant activity of phenolic compounds critically depends on the number and position of phenolic hydroxyls in the aromatic ring moieties. Generally, monophenols are less effective than catecholic phenols, and phenolic aglicons have higher antioxidant activity than their respective glycosides[11].

Lipid peroxidation is a marker of oxidative damage to membranes, producing derivatives such as hydroperoxides and malonaldehyde that can be used to quantify the amount of damage in the cell, and this later plays a crucial role in the pathogenesis of many diseases[5]. We therefore tested the ability of *M. communis* to inhibit lipid peroxidation by three methods (β-carotene bleaching test, FTC method and TBA method).

β-carotene bleaching test measures the capacity of antioxidant to retard β-carotene discoloration induced by the conjugated diene hydroperoxides arising from linoleic acid oxidative degradation[27]. However, the FTC method measures the amount of peroxide at the primary stage of linoleic acid peroxidation, and the TBA method test measures the amount of the thiobarbituric acid reactive substances content produced at the secondary stage after the decomposition of the lipid peroxide during the oxidation process. Malonaldehyde was one of the low molecular weight compounds, which is a very unstable compound causing DNA damage, generation of cancer and aging related diseases[42]. In our study, *M. communis* extracts significantly inhibited the degree of lipid peroxidation in the three methods tested which could be attributed to their phenolic contents, indicating that *M. communis* extracts act as antioxidant at an early and later stages of lipid peroxidation by their ability to quench and neutralize free radicals or decompose peroxides[43].

Our findings are in agreement with the work of Gonçalves *et al.* and Kumar *et al.* who demonstrated that extracts of myrtle prepared using different organic solvents were effective inhibitors of lipid peroxidation[5,44].

Despite the highest polyphenolic content of ethyl acetate extract, the non-polar chloroform extract is more active than the polar ethyl acetate extract in three anti-lipid peroxidation methods tested. Frankel and Meyer have suggested that the polarity of an extract is important in water: lipid emulsions[45]. The non-polar extracts exhibit most important antioxidant properties as they are concentrated within lipid-water interface, thus they can help to prevent radical formation of β-carotene and lipid oxidation. While, polar extracts are diluted in the aqueous phase and are thus less effective in inhibiting lipids peroxidation.

Although the leaves of *M. communis* are commonly used in traditional Algerian medicine as antihypertensive therapy, the pharmacological evidences of their activity are lacking. Therefore, in this study, the hypotensive effect of methanol and ethyl acetate extracts from *M. communis* leaves was reported for the first time. Our data reveal that ethyl acetate extract showed a significant hypotensive effect than methanol extract, and this is most possibly due to its high total contents of phenolic compounds and flavonoids such as myricetin-3-O-rhamnoside isolated in this study.

Previous studies reported that these secondary metabolites are capable of exerting antihypertensive effects by different pathways, such as endothelium-dependent vasodilation involving NO production[46], angiotensin converting enzyme inhibition and a reduced oxidative status caused by the antioxidant capacity of these compounds. Nevertheless, the exact mechanisms involved in the antihypertensive effect of polyphenols have not been clarified yet. Thus, a good understanding of these mechanisms will allow the development of natural treatments with polyphenols for blood pressure lowering effect[47].

In the present study, our data highlight the good antioxidant proprieties of different extracts from myrtle leaves. This antioxidant potential is probably attributed to the presence of polyphenolic compounds which may have many benefits in treating oxidative stress related diseases. Furthermore, this study shows for the first time that methanol and ethyl acetate extract of *M. communis* have

dose-dependent blood pressure lowering effect in rats, providing a rational basis to understand the use of this plant in hypotensive treatment in folk medicine. These results lay the ground work for further studies on the molecular mechanisms underlying the biological profile of the extracts and isolation and purification of more active principles in each extract as well as clarification of their mode of action.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This work was supported by the Algerian Ministry of Higher Education and Scientific Research and the Thematic Agency for Research in Health Sciences with grant number (361/25/09/2011). We express our gratitude to these organisations.

References

[1] Gönenç A, Hacışevki A, Tavil Y, Çengel A, Torun M. Oxidative stress in patients with essential hypertension: a comparison of dippers and non-dippers. *Eur J Intern Med* 2013; **24**: 139-144.

[2] Frombaum M, Le Clanche S, Bonnefont-Rousselot D, Borderie D. Antioxidant effects of resveratrol and other stilbene derivatives on oxidative stress and *NO bioavailability: potential benefits to cardiovascular diseases. *Biochimie* 2012; **94**: 269-276.

[3] Pendyala LK, Li J, Shinke T, Geva S, Yin X, Chen JP, et al. Endothelium-dependent vasomotor dysfunction in pig coronary arteries with paclitaxel-eluting stents is associated with inflammation and oxidative stress. *JACC Cardiovasc Interv* 2009; **2**(3): 253-262.

[4] Nićiforović N, Mihailović V, Masković P, Solujić S, Stojković A, Pavlović Muratspahić D. Antioxidant activity of selected plant species; potential new sources of natural antioxidants. *Food Chem Toxicol* 2010; **48**: 3125-3130.

[5] Gonçalves S, Gomes D, Costa P, Romano A. The phenolic content and antioxidant activity of infusions from Mediterranean medicinal plants. *Ind Crops Prod* 2013; **43**: 465-471.

[6] Mimica-Dukić N, Bugarin D, Grbović S, Mitić-Ćulafić D, Vuković-Gačić B, Orčić D, et al. Essential oil of *Myrtus communis* L. as a potential antioxidant and antimutagenic agents. *Molecules* 2010; **15**: 2759-2770.

[7] Chidouh A, Aouadi S, Heyraud A. Extraction, fractionation and characterization of water-soluble polysaccharide fractions from myrtle (*Myrtus communis* L.) fruit. *Food Hydrocoll* 2014; **35**: 733-739.

[8] Boudjelal A, Henchiri C, Sari M, Sarri D, Hendel N, Benkhaled A, et al. Herbalists and wild medicinal plants in M'Sila (North Algeria): an ethnopharmacology survey. *J Ethnopharmacol* 2013; **148**: 395-402.

[9] Amel B. Traditional treatment of high blood pressure and diabetes in Souk Ahras District. *J Pharmacognsy Phytother* 2013; **5**(1): 12-20.

[10] Nassar MI, Aboutabl El-SA, Ahmed RF, El-Khrisy ED, Ibrahim KM, Sleem AA. Secondary metabolites and bioactivities of *Myrtus communis*. *Pharmacognosy Res* 2010; **2**: 325-329.

[11] Tuberoso CIG, Rosa A, Bifulco E, Melis MP, Atzeri A, Pirisi FM, et al. Chemical composition and antioxidant activities of *Myrtus communis* L. berries extracts. *Food Chem* 2010; **123**: 1242-1251.

[12] Pereira P, Cebola MJ, Bernardo-Gil MJ. Comparison of antioxidant activity in extracts of *Myrtus communis* L. obtained by SFE vs. solvent extraction. *J Environ Sci Eng A* 2012; **1**: 115-120.

[13] Bouzabata A, Castola V, Bighelli A, Abed L, Casanova J, Tomi F. Chemical variability of Algerian *Myrtus communis* L. *Chem Biodivers* 2013; **10**: 129-137.

[14] Aidi Wannes W, Mhamdi B, Sriti J, Ben Jemia M, Ouchikh O, Hamdaoui G, et al. Antioxidant activities of the essential oils and methanol extracts from myrtle (*Myrtus communis* var. *italica* L.) leaf, stem and flower. *Food Chem Toxicol* 2010; **48**(5): 1362-1370.

[15] Tuberoso CI, Boban M, Bifulco E, Budimir D, Pirisi FM. Antioxidant capacity and vasodilatory properties o Mediterranean food: the case of Cannonau wine, myrtle berries liqueur and strawberry-tree honey. *Food Chem* 2013; **140**: 686-691.

[16] Montoro P, Tuberoso CI, Piacente S, Perrone A, De Feo V, Cabras P, et al. Stability and antioxidant activity of polyphenols in extracts of *Myrtus communis* L. berries used for the preparation of myrtle liqueur. *J Pharm Biomed Anal* 2006; **41**: 1614-1619.

[17] Markham KR. *Techniques of flavonoid identification*. Waltham: Academic Press; 1982, p. 113.

[18] Li HB, Cheng KW, Wong CC, Fan KW, Chen F, Jiang Y. Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae. *Food chem* 2007; **102**: 771-776.

[19] Bahorun T, Gressier B, Trotin F, Brunet C, Dine T, Luyckx M, et al. Oxygen species scavenging activity of phenolic extracts from hawthorn fresh plant organs and pharmaceutical preparations. *Arzneimittelforschung* 1996; **46**: 1086-1089.

[20] Burits M, Bucar F. Antioxidant activity of *Nigella sativa* essential oil. *Phytother Res* 2000; **14**: 323-328.

[21] Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med* 1999; **26**: 1231-1237.

[22] Li YH, Jiang B, Zhang T, Mu WM, Liu J. Antioxidant and free radical-scavenging activities of chickpea protein hydrolysate (CPH). *Food Chem* 2008; **106**: 444-450.

[23] Decker EA, Welch B. Role of ferritin as a lipid oxidation catalyst in muscle food. *J Agric Food Chem* 1990; **38**: 674-677.

[24] Chung YC, Chen SJ, Hsu CK, Chang CT, Chou ST. Studies on the antioxidative activity of *Graptopetalum paraguayense* E. Walther. *Food Chem* 2005; **91**: 419-424.

[25] Dapkevicius A, Venskutonis R, Van Beek TA, Linssen JPH. Antioxidant activity of extracts obtained by different isolation procedures from some aromatic herbs grown in Lithuania. *J Sci Food Agric* 1998; **77**: 140-146.

[26] Yen GC, Chang YC, Su SW. Antioxidant activity and active compounds of rice koji fermented with *Aspergillus candidus*. *Food Chem* 2003; **83**: 49-54.

[27] Kikuzaki H, Nakatani N. Antioxidant effects of some ginger constituents. *J Food Sci* 1993; **58**(6): 1407-1410.

[28] Abdalla S, Abu Zarga M, Sabri S. Effects of the flavone luteolin, isolated from *Colchicum ritchii*, on guinea-pig isolated smooth muscle and heart and on blood pressure and blood flow. *Phytother Res* 1994; **8**: 265-270.

[29] Hayder N, Bouhlell I, Skandrani I, Kadri M, Steiman R, Guiraud P, et al. *In vitro* antioxidant and antigenotoxic potentials of myricetin-3-o-galactoside and myricetin-3-o-rhamnoside from *Myrtus communis*: modulation of expression of genes involved in cell defence system using cDNA microarray. *Toxicol In Vitro* 2008; **22**: 567-581.

[30] Shen CJ, Chen CK, Lee SS. Polar constituents from *Sageretia thea* leaf characterized by HPLC–SPE–NMR assisted approaches. *J Chin Chem Soc* 2009; **56**: 1002-1009.

[31] Gardeli C, Vassiliki P, Athanasios M, Kibouris T, Komaitis M. Essential oil composition of *Pistacia lentiscus* L. and *Myrtus communis* L.: evaluation of antioxidant capacity of methanolic extracts. *Food Chem* 2008; **107**: 1120-1130.

[32] Kvietys PR, Granger DN. Role of reactive oxygen and nitrogen species in the vascular responses to inflammation. *Free Radic Biol Med* 2012; **52**: 556-592.

[33] Govindan P, Muthukrishnan S. Evaluation of total phenolic content and free radical scavenging activity of *Boerhavia erecta*. *J Acute Med* 2013; **3**(3): 103-109.

[34] Farhat MB, Landoulsi A, Chaouch-Hamada R, Sotomayor JA, Jordán MJ. Phytochemical composition and *in vitro* antioxidant activity of by-products of *Salvia verbenaca* L. growing wild in different habitats. *Ind Crops Prod* 2013; **49**: 373-379.

[35] Bouaziz A, Khennouf S, Abdalla S, Djidel S, Abu Zarga M, Bentahar A, et al. Phytochemical analysis, antioxidant activity and hypotensive effect of Algerian azarole (*Crataegus azarolus* L.) leaves extracts. *Res J Pharm Biol Chem Sci* 2014; **5**(2): 286-305.

[36] Apostolou A, Stagos D, Galitsiou E, Spyrou A, Haroutounian S, Portesis N, et al. Assessment of polyphenolic content, antioxidant activity, protection against ROS-induced DNA damage and anticancer activity of *Vitis vinifera* stem extracts. *Food Chem Toxicol* 2013; **61**: 60-68.

[37] Hayder N, Abdelwahed A, Kilani S, Ammar RB, Mahmoud A, Ghedira K, et al. Anti-genotoxic and free-radical scavenging activities of extracts from (Tunisian) *Myrtus communis*. *Mutat Res* 2004; **564**: 89-95.

[38] Tanzadehpanah H, Asoodeh A, Chamani J. An antioxidant peptide derived from ostrich (*Struthio camelus*) egg white protein hydrolysates. *Food Res Int* 2012; **49**: 105-111.

[39] Priyadharshini SD, Sujatha V. Antioxidant assessment for various solvent fractions of *Cassia fistula* Linn. flowers. *Int J PharmTech Res* 2012; **4**(1): 510-517.

[40] Sen S, De B, Devanna N, Chakraborty R. Total phenolic, total flavonoid content, and antioxidant capacity of the leaves of *Meyna spinosa* Roxb., an Indian medicinal plant. *Chin J Nat Med* 2013; **11**(2): 149-157.

[41] Patel BD, Kamariya YH, Patel MB. Free radical scavenging potential of ethanolic extract of *Uraria picta* Linn. *Pharmacolonline* 2011; **2**: 134-145.

[42] Norhaizan ME, Ng SK, Norashareena MS, Abdah MA. Antioxidant and cytotoxicity effect of rice bran phytic acid as an anticancer agent on ovarian, breast and liver cancer cell lines. *Malays J Nutr* 2011; **17**(3): 367-375.

[43] Moyo B, Oyedemi S, Masika PJ, Muchenje V. Polyphenolic content and antioxidant properties of *Moringa oleifera* leaf extracts and enzymatic activity of liver from goats supplemented with *Moringa oleifera* leaves/ sunflower seed cake. *Meat Sci* 2012; **91**: 441-447.

[44] Kumar MR, Phaneendra P, Bodhanapu S, Rahiman OMF, Niyas KM, Tamizmani T. Antioxidant and hepatoprotective activity of the aqueous extract of *Myrtus Communis* (Myrtle) Linn. leaves. *Pharmacologyonline* 2011; **1**: 1083-1090.

[45] Frankel EN, Meyer AS. The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *J Sci Food Agric* 2000; **80**(13): 1925-1941.

[46] Nugroho AE, Malik A, Pramono S. Total phenolic and flavonoid contents, and *in vitro* antihypertension activity of purified extract of Indonesian cashew leaves (*Anacardium occidentale* L.) *Int Food Res J* 2013; **20**(1): 299-305.

[47] Quiñones M, Muguerza B, Miguel M. Aleixandre A. Evidence that nitric oxide mediates the blood pressure lowering effect of a polyphenol-rich cocoa powder in spontaneously hypertensive rats. *Pharmacol Res* 2011; **64**: 478-481.