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Bioactive polyphenols from *Ranunculus macrophyllus* Desf. Roots: Quantification, identification and antioxidant *activity*

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ABSTRACT

Ranunculus macrophyllus Desf. is an Algerian medicinal plant whose roots are used in traditional medicine to cure feminine infertility and other diseases, however there are no studies regarding its phytochemistry and biological activities. The aim of this work is to study the phytochemical composition and antioxidant activity of different solvents fractions from the roots of *Ranunculus macrophyllus* Desf. Spectrophotometric and chromatographic methods were used to study the phytochemical composition; while antiradical, iron chelating ability, reducing power and lipid peroxidation were studied *in-vitro*. The ethyl acetate fraction showed the highest values of total phenolic compounds (271.0 ± 0.2 μg GAE/mg dry extract (d.e), flavonols (24 ± 5 μg RE/ mg d.e) and condensed tannins (129 ± 10 μg CE/mg d.e), while the hexane fraction contained the highest amount of triterpenoids (91 ± 7 μg UAE/mg d.e). The highest radical scavenging ability was recorded for the ethyl acetate fraction against DPPH (IC_{50} = 3.7 ± 0.1 μg/mL) and ABTS (IC_{50} = 81 ± 3 μg/mL) whereas the hexane fraction had the best hydrogen peroxide radical scavenging (IC_{50} = 380 ± 4 μg/mL). The ethyl acetate fraction had the best total antioxidant capacity (TAC = 361 ± 1 μAAE/ mg extract) and reducing power (310 ± 2 μAAE/mg extract). The β-carotene bleaching was inhibited at high rate even after 24 h by the ethyl acetate fraction (81.0 ± 0.5 %). All activities were correlated with the polyphenolic content of the fractions. Capillary LC-DAD and LC-MS/MS analysis of ethyl acetate fraction revealed high amounts of gallic acid (9.3 ± 0.6 mg/g d.e), dihydroxybenzoic acid (8.1 ± 0.2 mg/g d.e) and hesperidin (5.9 ± 0.6 mg/g d.e). With such high amounts of polyphenols and strong antioxidant activity *Ranunculus macrophyllus* Desf. roots could have a potential use in pharmaceutical and nutraceutical industries.

1. Introduction

Humans have always relied on plants to fulfil their basic needs, like food, and also to cure diseases (Huy et al., 2018; Du, 2018). The use of medicinal plants to cure diseases and relieve pain is still relevant, even with the great advancements in modern medicine, medicinal plants still largely contribute to general health care. In this context, 11% from the basic and essential drugs are exclusively from plant origin, additionally many synthetic drugs come from natural precursors (Pandey et al., 2019). The increasing interest in medicinal plants is mainly driven by the thoughts that natural products are more effective than synthetic ones, also having less side effect. They are also an economical alternative to conventional pharmacological treatments, especially in developing countries (Alamgir, 2018; Pandey et al., 2019). Crude drugs and their products are of economic importance and profitable commercial products (Shah and Seth, 2010). In addition to the therapeutic use of natural products deriving from medicinal plants, they are also used in other industries, beverages, condiments, spices, cosmetics, in confectioneries and as technical products (Shah and Seth, 2010; Du, 2018). The medicinal plants market is expected to reach the 5 trillion USD mark by 2050 (Alamgir, 2018). On the other hand, the efficiency of medicinal plants is due to their richness in bioactive compounds. Among these phytochemicals, polyphenols are the most distributed and structurally diverse group of bioactive compounds in the medicinal plants. Multiple biological activities have been ascribed to polyphenols, particularly well-known to be excellent antioxidants (Lingua et al., 2016; Alamgir, 2018). Their antioxidant ability enables them to scavenge the harmful free

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radicals, and to protect cells from the devastating effects of oxidative stress caused by high levels of free radicals (Belščak-Cvitanović et al., 2018). The latter was associated with the pathology of several diseases, including cancer, diabetes mellitus, atherosclerosis, neurodegenerative and inflammatory diseases, psychiatric disorders and/or aging process (Rodrigo, 2009; Zdeňka, 2014). Due to their several health promoting benefits, there is still an increasing demand on polyphenols in the market, which reached 1.28 billion USD in 2018 with a compound annual growth rate of 7.2% from 2019 to 2025 (Grand View Research, 2019).

On the other hand, members of the genus *Ranunculus* have been reported to have interesting biological activities, such as anti-cancer (*Ranunculus sieboldii*), anti-inflammatory (*Ranunculus pedatus*), antioxidant (*Ranunculus auricomus*), antibacterial (*Ranunculus muricatus*) and anti-hypertensive (*Ranunculus japonicus*) (Da-Cheng, 2018). The wide range of bioactivities exhibited by members of the genus *Ranunculus* is mainly attributed to their content of bioactive phytochemicals, specially polyphenols, flavonoids, triterpenoids and saponins, alkaloids and volatile compounds (Aslam et al., 2012).

Particularly, *Ranunculus macrophyllus* Desf. is one of the 50 *Ranunculus* species found in Algeria (Hachelaf et al., 2013). Its leaves are used for the treatment of certain skin diseases (Rai et al., 2011), while roots are traditionally used to treat feminine infertility and to gain weight (Rivera et al., 2006, Ouarghidi et al., 2013). Despite the frequent use of *Ranunculus macrophyllus* Desf. roots in traditional medicine, there is a dearth of scientific information on their chemical composition and bioactivity. Thus, the aim of this work is to elucidate for the first time the phytochemical composition of *Ranunculus macrophyllus* Desf. roots both quantitatively and qualitatively. In addition, the antioxidant activity of their different solvent extracts was estimated *in vitro*.

2. Materials and methods

2.1. Chemicals

All chemicals and solvents were of analytical grade, and purified water from a Milli-Q system (Millipore, Bedford, MA, USA) was used. For LC-MS/MS analysis, methanol, water and formic acid were of OptimaTM LC/MS grade (Fisher Scientific, Fair Lawn, NJ, USA). Acetonitrile (MeCN) and methanol (MeOH) of gradient HPLC quality were provided by Scharlab (Barcelona, Spain). Hexane, chloroform, ethyl acetate, butanol, folin-ciocalteu, sodium bicarbonate, aluminum chloride, bovine serum albumin, sodium dodecyl sulfate (SDS), triethanolamine (TEA), tannic acid, vanillin, acetic acid, perchloric acid, ursolic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, hydrogen peroxide, disodium hydrogen phosphate, sodium phosphate monobasic dihydrate, ferrous chloride, ferrozine, EDTA, trichloroacetic acid (TCA), ferric chloride, Potassium hexacyanoferrate(III), ammonium molybdate tetrahydrate, sulphuric acid, β-carotene, linoleic acid, tween 40, butylated hydroxyanisol (BHA), ascorbic acid (vitamin C) and trifluoroacetic acid (TFA, 99%), were supplied by Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plant material

The Roots of *Ranunculus macrophyllus* Desf. were gathered from Ras El Oued- Bordj Bou Arreridj province north-east Algeria in May 2018. The plant was identified by Dr. Bouadam from the department of physico-chemical biology of the university of Bejaia and a voucher specimen (AB-108) has been deposited at the herbarium of the life and natural science department, M'sila university. The roots were well washed with milli-Q water then air dried for one month and powdered with and an electric grinder.

2.3. Extraction and fractionation

The extraction of the plant powder was conducted according to Prieto et al. (2003). The first step consisted in a solid-liquid extraction, in which an amount of 100 g of root powder was macerated at room temperature, three times, with 500 mL of pure methanol. The combined filtrates were evaporated under low pressure using a rotary evaporator (40° C), yielding the crude methanolic extract. The latter was dissolved in 100 mL of an aqueous methanol solution (10%, v) and subjected to a liquid-liquid extraction in a separating funnel as a second step. The crude extract was sequentially extracted using solvents of increasing polarity; hexane (HR), chloroform (CR), ethyl acetate (EAR) and *n*-butanol (BR) to provide 4 fractions. The remaining aqueous phase was designed as the fifth fraction (AR).

2.4. Total polyphenols content (TPC)

The total phenolic content of the plant fractions was estimated by the method of Singleton and Rossi (1965). A volume of 200 μL of the sample was mixed with 1 mL of 10% (v) Folin-Ciocalteu reagent and incubated for 4 min. Then 800 μL of 7.5% (w) sodium carbonate was added. The resulting mixture was finally incubated for 2 h and the absorbance was measured at 765 nm against the corresponding blank. Gallic acid was used as standard and a calibration curve was prepared in the same conditions of samples. The results were expressed as μg of gallic acid per mg of dried extract (μg GAE/mg d.e).

2.5. Total flavonoids content (TFC)

The total flavonoids content was evaluated following the aluminum trichloride method. A volume of 0.50 mL of each extract was mixed with the same volume of aluminum trichloride (2%, w) and then incubated during 15 min. The absorbance was measured at 430 nm. Quercetin was used to obtain a calibration curve under same conditions. The results were expressed as μg of quercetin per mg of dried extract (μg QE/mg d.e) (Djeridane et al., 2006).

2.6. Total tannins content (TTC)

The total tannins content of all sample fractions was estimated according to the method based on their ability to precipitate proteins. One volume of sample was mixed with two volumes of BSA (1mg/mL), and then incubated during 15 min at room temperature. The mixture was centrifuged for 15 min at 3000*g* and the supernatant was discarded. In a second step the platelets remaining in the test tube were dissolved in 4 mL SDS/TEA solution (1% SDS, 5% v/v TEA in distilled water), and 1 mL of $FeCl_3$ was added. The mixture was then incubated again for 15 min and the absorbance was measured at 510 nm. A blank solution was prepared by mixing 1 mL of $FeCl_3$ and 4 mL of the SDS/TEA solution. Tannic acid was used as reference compound and the results were expressed as μg of TEA per mg of dried extract (μg TEA/mg d.e) (Hagerman and Butler, 1978).

2.7. Flavonols content (FOL)

To evaluate flavonols content of plant fractions, 1 mL of sample extract and 1 mL of aluminum trichloride (2%, w) were mixed with 1.5 mL of 5% aqueous sodium acetate (w) and incubated at 25°C for 2.5 h. The absorbance was then registered at 440 nm and the results were expressed as μg of rutin per mg of dried extract (μg RE/mg d.e) using the calibration curve prepared with rutin standard (Kumaran and Karunakaran, 2007).

2.8. Condensed tannins content (CTC)

The estimation of condensed tannins was performed according to the optimizations of Sun et al. (1998). A volume of 250 μL of each extract (1mg/mL in methanol) was mixed with 625 μL of 1% (w) vanillin (prepared in methanol) and 625 μL of 10% (v) sulphuric acid (in methanol). The mixture was incubated at room temperature for 15 min and the absorbance was measured at 500 nm. A calibration curve was prepared using catechin as standard, and the results were expressed as μg of catechin per mg of dried extract (μg E/mg d.e).

2.9. Triterpenoid content (TC)

For the triterpenoid estimation, 30 μL of the plant extracts, 50 μL of 5% (w) of vanillin prepared in acetic acid, and 100 μL of perchloric acid were mixed. The resulting mixture was incubated at 60°C for 45 min and cooled in an ice-water bath. Then, 450 μL of acetic acid was added. The absorbance was measured at a wavelength of 548 nm. Ursolic acid was used as standard to prepare the calibration curve and the results were expressed as μg of ursolic acid per mg of dried extract (μg UAE/mg d.e) (Chang et al., 2012).

2.10. Chromatographic analysis of phenolic compounds

2.10.1. Capillary LC-DAD analysis

The method described by León-González et al. (2018) was adopted to analyze the phenolic content of the plant ethyl acetate fraction using an Agilent cLC instrument Mod. 1100 Series (Agilent Technologies, Madrid, Spain), equipped with a G1376A binary capillary pump, a G1379A degasser and a G1315B diode array detector (500 nL, 10 mm pathlength). Reversed-phase high-performance liquid chromatography was performed on a Synergi™ Fusion 4 μm C18 (150 mm × 0.3 mm I.D.) capillary analytical column supplied by Phenomenex (Torrance, CA, USA), which was maintained at room temperature. An external stainless-steel loop of a volume of 10 μL was placed into a Rheodyne® injection valve. Detection was made using a diode array HP8543 UV/Vis detector (Agilent Technologies), connected to HP Chemstation software. The Agilent Chemstation software package for Microsoft Windows was used to acquire and analyze data.

The composition of the mobile phase consisted in a mixture of two solvents; acetonitrile (A) and TFA aqueous solution 0.1% (v) at pH 3.2 (B). The gradient composition was 8% (v) A for 3 min, followed by a linear increase to 34% (v) A during 11 min, an isocratic step at 34% (v) A for 7 min, and then a linear decrease to 8% (v) A, which was maintained until the end of the analysis. The mobile phase flow rate was set at 10 μL/min.

Polyphenols were detected at 220, 260, 292, 310 and 365 nm and identified on the basis of their retention times and UV absorption in comparison to that of pure standards. Compounds were quantified at their maximum absorption wavelength; 260 nm for ,4-dihydroxybenzoic acid, 292 nm for gallic acid, naringin and hesperidin, 365 nm for rutin, quercetin, myricetin and kaempferol, and 310 nm for chlorogenic acid, *trans*-ferulic, *p*-coumaric acid, caffeic acid and resveratrol, by using their respective external calibration curves. Linear ranges were within10–310 μg/L for gallic acid, 10–75 μg/L for 3,4-dihydroxybenzoic acid, 40–400 μg/L for chlorogenic acid, 20–400 μg/L for *p*-coumaric and 15–200 μg/L for *trans*-ferulic acids, 30–200 μg/L for rutin, 15–60 μg/L for naringin, 10–60 μg/L for resveratrol, 5–60 μg/L for quercetin, 12–60 μg/L for kaempferol and 100–500 μg/L for hesperidin. Linearity was evaluated in terms of the squared correlation coefficients (r^2). All r^2 values were higher than 0.95 for the polyphenol calibration curves. For on-column focusing purposes, all injection solutions were prepared in 0.1% (v) TFA aqueous solution adjusted at pH 3.2, containing 1% (v) of pure acetonitrile.

The quality parameters of the capillary LC-DAD method, including detection and quantitation limits, linearity range, run-to-run precision and day-to-day precision can be found in the studies performed by León-González et al., (2018), Gómez-Mejía et al., (2019) and Ramón-Gonçalves et al., (2019).

2.10.2. LC-MS/MS analysis

The identities of compounds were corroborated by LC-MS/MS in negative electrospray ionization (ESI) mode, using a Shimadzu LC-MS-8030 triple quadrupole system (Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a Nexera LC-30AD solvent delivery unit, a Nexera SIL-30AC autosampler with temperature-controlled tray, and a CTO-20AC column oven. A SynergiTM C18 Fusion-RP 80 Å analytical column from Phenomenex (150 × 3 mm I.D., 4 μm) at room temperature was used as stationary phase. Data were acquired and processed with the LabSolutions LC-MS software provided by Shimadzu. The MS/MS equipment used nitrogen as both nebulizing (1.5 L/min) and drying (15.0 L/min) gas. Fragmentation was performed using argon as the collision gas at a pressure of 230 kPa in the collision cell, and the collision energy voltages applied were in the range 10–55 eV. ESI for ionization voltage was set at −4.5 kV. The interface current was fixed at 6.2 μA, and the detector voltage at 1.84 kV.

A mixture of methanol (solvent A) and 0.2% (v) formic acid aqueous solution (solvent B) was used as mobile phase: 5% (v) A was maintained for 0.1 min, then a linear increase to 40% A (v) within 25 min, and finally a linear increase to 70% (v) A within another 10 min. This condition was held for 2 min, then changed to the initial conditions (5% A) within 1 min and equilibrated for 2 min. The flow rate was 0.50 mL/min. The injection solution was prepared as follows; 40 μL of plant extracts were added to 2.5 mL of methanol containing 0.2% (v) formic acid then diluted to 5 mL with LC/MS grade water. A volume of 20 μL of this mixture was injected into the chromatograph. For quantification purposes, calibration curves were performed using external calibrations. Linearity ranges were within 30–130 μg/L for gallic acid, 10–80 μg/L for dihydroxybenzoic acid, *p*-coumaric acid, hesperidin, caffeic acid and resveratrol, 10–100 μg/L for chlorogenic acid, 20–80 μg/L for *trans*-ferulic acid and kaempferol, 20–100 μg/L for naringin and rutin, 5–50 μg/L for quercetin and myricetin, and 15–130 μg/L for catechin (Gómez-Mejía et al., 2019).

3. In-vitro antioxidant activity

3.1. DPPH scavenging assay

A mixture of 0.5 mL of 0.1 mM methanolic DPPH solution and 1.5 mL of sample extracts was incubated in the dark for 30 min, and the absorbance was measured at 517 nm (Shen et al., 2010). BHT, BHA (synthetic antioxidant), and gallic acid and quercetin (pure compounds) were used as reference compounds. The inhibition of the DPPH radical was calculated by using the following equation:

% Inhibition = (Ac–At/Ac) * 100

Where Ac is the absorbance of the control and At is the absorbance of the test.

The calculated inhibition percentages where plotted against sample concentrations and the resulting plots were used to calculate the IC_{50} (concentration of the sample that reduce 50% of DPPH free radical initial concentration).

3.2. ABTS scavenging assay

The radical scavenging activity was also evaluated using the stable cation radical ABTS, as described by Re et al. (1999). ABTS radical was generated by mixing the ABTS solution (7mM) with 13.24 mg of potassium persulfate for 16 h. The resulting solution was refrigerated,

then diluted to reach an absorbance of 0.70 ± 0.02 at 734 nm. A volume of 100 μL of sample was mixed with 1.9 mL of $ABTS^{\bullet+}$ solution, and then incubated. After 7 min, the absorbance was measured at 734 nm. BHA, BHT, gallic acid and quercetin were used as standard compounds. The ABTS inhibition was calculated using the following formula:

% Inhibition = (Ac−At/Ac) * 100

Where Ac is the absorbance of the control and At is the absorbance of the test.

The estimated inhibition percentages where plotted against sample concentrations and the resulting plots were used to calculate the IC_{50} (concentration of the sample that reduce 50% of the ABTS initial concentration).

3.3. Hydrogen peroxide scavenging assay

Hydrogen peroxide scavenging activity of the plant fractions was assessed based on the method proposed by Ruch et al. (1989). A volume of 0.50 mL of the sample was dissolved in PBS (pH 7.4) and mixed with 1 mL of freshly prepared 20 μmol H_2O_2 solution. The mixture was incubated in the dark for 10 min, and then the absorbance was measured at 240 nm. A blank control was prepared for each concentration, by adding PBS instead of the sample extract. Gallic acid (GA) and BHA were used as reference compounds. The percentage inhibition of hydrogen peroxide was calculated according to the following equation:

% Inhibition = (Ac−At/Ac) * 100

Where Ac is the absorbance of the control and At is the absorbance of the test.

The inhibition percentages where plotted against sample concentrations and the resulting plots were used to calculate the IC_{50} (concentration of the sample that reduce 50% of the H_2O_2 initial concentration)

3.4. Reducing power assay

The reducing power of the extracts was evaluated according to Oyaizu (1986). Briefly, equal volumes of the sample extract, potassium hexacyanoferrate (1%, w) and phosphate buffer (0.2 M at pH 6.6) were mixed and incubated at 50°C for 20 min. The same volume of TCA (10%, w) was immediately added and the mixture was centrifuged at 700 rpm for 10 min. A supernatant volume of 1.25 mL was then mixed with 1.25 mL of milli-Q water and 0.5 mL of $FeCl_3$ (0.1%, w). The mixture was vortexed and the absorbance was registered at 700 nm. BHA, BHT, gallic acid and quercetin were used as standard compounds. A calibration curve using ascorbic acid was prepared in the same conditions that samples and the results were expressed as ascorbic acid equivalents per mg of dried extract (μg AAE/mg d.e).

3.5. Iron chelating capacity

To estimate the iron chelation ability of plant samples, 500 μL of the extract was mixed with 100 μL of $FeCl_2$ (0.6 mM) and 900 μL methanol. After 5 min, a volume of 100 μL of ferrozine (5mM) was added to the mixture. The reaction mixture was stirred and incubated for 10 min, and the absorbance was measured at 562 nm (Le et al., 2007). A blank solution was prepared by following the same procedure, except for the addition of the sample extract. EDTA was used as reference compound and the percent of chelation was calculated using the formula:

% Chelation = (Ac−At/Ac)*100

Where Ac is the absorbance of the control and At is the absorbance of the sample.

The calculated chelation percentages where plotted against sample concentrations and the resulting plots were used to calculate the IC_{50} (concentration of the sample that chelate the 50% of the initial iron concentration).

3.6. Total antioxidant capacity

The total antioxidant activity of plant fractions was determined using the phosphomolybdenum method. A sample volume of 0.1 mL was mixed with 1 mL of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate), and then incubated at 95°C for 90 min. The absorbance was measured at 695 nm against a blank containing methanol instead of the sample extract (Prieto et al., 1999). Standard compounds such as BHA, BHT, gallic acid and quercetin were used for comparison purposes. The results were expressed as ascorbic acid equivalents per mg of dried extract (μg AAE/mg d.e), using the ascorbic acid calibration curve elaborated in the same conditions.

3.7. β-Carotene bleaching assay

β-Carotene bleaching assay was used to establish the potential anti-lipid peroxidation effect of the sample extracts. As stated by Tepe et al. (2005), the β-carotene emulsion was prepared by mixing 0.5 mg of β-carotene, 1 mL of chloroform and 25 μL of linoleic acid. Then, an amount of 200 mg of tween 20 were added to the mixture. The solvent chloroform was evaporated under reduced pressure, and 100 mL of oxygen saturated milli-Q water was added and vigorously shacked. A volume of 2.5 mL of the previously prepared β-carotene solution was added in test tubes and mixed with 350 μL of sample extract. The same procedure was repeated with BHA, BHT and gallic acid as positive controls (2 mg/mL), while methanol and water were used as negative controls. The absorbance was measured at 490 nm after 1 h, 2 h, 3 h, 4 h, 6 h, 24 h and 48 h of incubation under the dark at room temperature. The inhibition of the β-carotene bleaching after 24 h was calculated according to the following equation:

$\%\ \text{Inhibition}_{(24\ h)} = (AE/AB) * 100$

Where AE is the absorbance in presence of the extract or standards at 0 h and AB is the absorbance in presence of the sample extracts or standards after 24 h.

4. Statistical analysis

All experiments were performed in triplicate and data were analysed using the software GraphPad prism 7.00. The results were expressed as mean values and standard deviation (SD). Mean values were compared statistically by using one way-ANOVA and significant differences (p-values < 0.05) were found. In order to analyse the difference pattern among means, Tukey test was applied ($\alpha = 0.05$). Pearson linear correlation was calculated to evaluate the correlation between total bioactive components and antioxidant activity results.

5. Results and discussions

5.1. Extraction yield

The methanolic extraction of *Ranunculus macrophyllus* Desf. roots provided a gummy yellowish extract that represented (15.3 ± 1.0) % (w/w) of the initial root powder. The liquid-liquid fractionation of the methanolic crude extract with solvents of increasing polarity afforded 5 fractions. The yield of the obtained fractions is presented in Fig. 1.

The aqueous fraction (AR) is the most important part of the methanolic crude extract, which represents (58 ± 10) % (w/w), followed by the butanolic fraction (BR) with (12 ± 1) % (w/w) and the hexane fraction (HR) with (5.5 ± 0.6) % (w/w), while chloroform fraction (CR) and ethyl acetate fraction (EAR) represent only (1.6 ± 0.4) % (w/w) and (1.3 ± 0.5) % (w/w), respectively. Khan et al. (2006) reported a

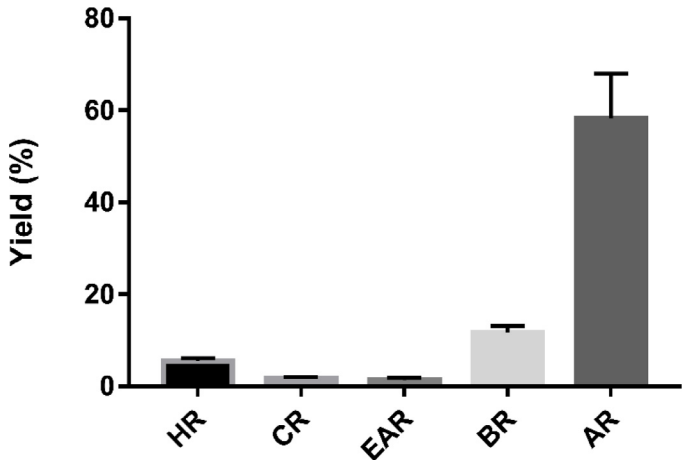

Fig. 1. Yields obtained for the different solvents fractions from the methanolic extract of *Ranunculus macrophyllus* Desf. HR (hexane fraction), CR (chloroform fraction), EAR (ethyl acetate fraction), BR (butanolic fraction) and AR (aqueous fraction).

lower yield for the methanolic crude extract of *Ranunculus repens*. In the same study, the chloroform fraction presented 13% (w/w) of the crude methanolic extract, being this result higher than the obtained one in the present study. The yield of the hydro-ethanolic extract from roots of *Ranunculus ternatus* as found by Deng et al. (2013) was similar to that reported in this work (16%, w/w). The same research also reported a comparable yield for the aqueous fraction (64.5%, w/w).

The extraction yield is affected by several factors. In fact, in addition to the inter-species variation, there is also intra-species variation. The first one could be explained by genetic and environmental factors (Siddhuraju and Becker, 2003), while the latter could be explained by the extraction method and the conditions in which the procedure is performed (temperature, pH, extraction time) (Do et al., 2013).

5.2. Spectrophotometric studies of bioactive compounds

Polyphenols and triterpenoids were characterized and quantified using spectrophotometric methods as described in the experimental Section. The obtained results are shown in Table 1.

The EAR fraction contains the highest amount of total polyphenols (TPC), total flavonoids (TFC) and flavonols (FOL). Total tannins (TTC) were distributed equally between EAR and CR, while the other fractions contained only small tannins amounts. The condensed tannins (CTC) are present in all fractions at similar amounts, except for the EAR fraction, which contains a significantly greater amount than the other fractions analysed. Regarding triterpenoids, the estimated amounts decrease with the increase of the solvent polarity. Thus, the highest amount is found in the HR fraction, while the lowest amount is observed in both AR and BR fractions. Total polyphenol content of different fractions from aerial parts of *Ranunculus sceleratus*, studied by Shahid et al. (2015), showed the same general distribution of polyphenols between the solvents, though different amounts. The polyphenol content in chloroform, ethyl acetate and butanol reported in this study were lower than our estimated polyphenol content for the same fractions, while hexane and aqueous fractions showed higher TPC values than our corresponding fractions. The presence of flavonoids, tannins and terpenoids were reported for *Ranunculus arvensis* as well as other species, although they were not quantified (Hachelaf et al., 2013; Khan et al., 2017).

For the same extraction conditions (temperature, extraction time, pH and others), the extraction yield (whether expressed as w/w or as amount of metabolite extracted) is mainly affected by two important factors: the solvent nature and the chemical properties (polarity) of the molecules to be extracted (Do et al., 2013). Polar molecules will be more soluble in a polar solvent, whilst a non-polar solvent will more likely extract non-polar compounds (Ramawat and Mérillon, 2013). Methanol is an appropriate solvent for the quantitative extraction of polyphenolic compounds, allowing the extraction of different groups of polyphenols and terpenoids (Oreopoulou et al., 2019). Solvent fractionation of increasing polarities may allow the separation of polyphenol compounds into separate groups (Rafińska et al., 2019). It has been reported that ethyl acetate fraction is able to dissolve phenolic diterpenes and flavonoids aglycones (Oreopoulou et al., 2019), while triterpenoids are often extracted with non-polar solvents (Mutai et al., 2007).

5.3. In vitro *antioxidant activity*

5.3.1. *Radical scavenging activity*

The radical scavenging activity of the different fractions was evaluated against, DPPH, ABTS and hydrogen peroxide. The obtained results are presented in Table 2.

The ethyl acetate fraction exhibited a strong radical scavenging activity, with very low IC_{50} value for both, DPPH (3.7 ± 0.1) μg/mL and ABTS (80.7 ± 3) μg/mL. This activity was similar to that of synthetic antioxidants (BHA and BHT) as well as to pure standard compounds (quercetin and gallic acid), used as reference drugs. The

Table 2
Radical scavenging and iron chelation activities of different fractions from *Ranunculus macrophyllus* Desf. roots.

	DPPH	ABTS	H_2O_2	Iron chelation
IC_{50} (μg/mL)				
HR	$81^{d} \pm 5$	$9081^{e} \pm 112$	$380^{c} \pm 4$	2366 ± 84
CR	$19^{b} \pm 1$	$369^{b} \pm 19$	$709^{f} \pm 6$	4978 ± 54
EAR	$3.7^{a} \pm 0.1$	$81^{a} \pm 3$	$645^{e} \pm 5$	> 5000
BR	$58^{c} \pm 2$	$1228^{c} \pm 33$	$4027^{g} \pm 34$	2572 ± 51
AR	$319^{e} \pm 4$	$6271^{d} \pm 49$	>10000	1012 ± 30
BHA	$1.9^{a} \pm 0.2$	$41.1^{a} \pm 0.8$	$81^{a} \pm 2$	–
BHT	$4.1^{a} \pm 0.2$	$38.5^{a} \pm 0.9$	–	–
Quercetin	$1.3^{a} \pm 0.1$	$19.4^{a} \pm 0.1$	–	–
Gallic Acid	$0.5^{a} \pm 0.0$	$59^{a} \pm 4$	$278^{b} \pm 7$	–
EDTA	–	–	–	22 ± 1

[b] Results are expressed as mean ± SD (n=3). Values on the same column with different superscript letters indicate significant differences (p-value < 0.05). CR (chloroform fraction), EAR (ethyl acetate fraction), BR (butanolic fraction) and AR (aqueous fraction).

Table 1
Spectrophotometric quantification of phenolic compounds and triterpenoids in the different fractions from *Ranunculus macrophyllus* Desf. roots.

	TPCμg GAE/mg (d.e)	TFCμg QE/mg (d.e)	FOLμg RE/mg (d.e)	TTCμg TAE/mg (d.e)	CTCμg CE/mg(d.e)	TCμg UAE/ mg(d.e)
HR	$10^{d[a]} \pm 2$	$0.70^{c} \pm 0.01$	$2.5^{b} \pm 0.2$	$0.5^{b} \pm 0.2$	$86.5^{b} \pm 0.2$	$91^{a} \pm 7$
CR	$111^{b} \pm 4$	$1.2^{b} \pm 0.1$	$3.8^{b} \pm 0.2$	$33^{a} \pm 3$	$94.1^{b} \pm 0.4$	$64.6^{b} \pm 0.8$
EAR	$271.0^{a} \pm 0.2$	$3.5^{a} \pm 0.1$	$24^{a} \pm 5$	$31^{a} \pm 1$	$129^{a} \pm 10$	$38^{c} \pm 5$
BR	$46.1^{c} \pm 0.9$	$1.1^{b} \pm 0.0$	$4.6^{b} \pm 0.6$	$1.0^{b} \pm 0.1$	$102^{b} \pm 11$	$20^{d} \pm 2$
AR	$6.0^{d} \pm 1.1$	$0.5^{d} \pm 0.0$	$3.5^{b} \pm 0.4$	$1.4^{b} \pm 0.2$	$107^{b} \pm 8$	$18^{d} \pm 7$

[a] Results are expressed as mean ± SD (n=3). Values on the same column with different superscript letters indicate significant differences (p-value < 0.05). CR (chloroform fraction), EAR (ethyl acetate fraction), BR (butanolic fraction) and AR (aqueous fraction). TPC (total polyphenols content), TFC (total flavonoids contents), FOL (flavonols content), TTC (total tannins content), CTC (condensed tannins content) and TC (triterpenoid content).

chloroform fraction also showed a considerable antiradical activity with IC_{50} (19 ± 1) and (369 ± 19) μg/mL for DPPH and ABTS, respectively. Hexane and aqueous fractions exhibited the weakest antiradical activity compared to the one showed by the other fractions and the standards drugs analysed. As can be also observed in Table 2, synthetic antioxidants and natural pure compounds showed similar radical scavenging ability against both DPPH and ABTS.

Radical scavenging is one of the main mechanisms by which polyphenols exert their antioxidant activity (Belščak-Cvitanović et al., 2018); it can be accomplished by donating hydrogen atom or singlet electron transfer. Moreover, this activity depends on the number and position of hydroxyl groups in the compound (Shahidi and Ambigaipalan, 2015).

Although hydrogen peroxide has a low reactivity, it can still damage cells through its ability to penetrate the membrane of the cells and oxidize a series of molecules inside. It can also react with transition metals, such as iron, to generate hydroxyl radical, known to be very reactive and destructive (Halliwell et al., 2000). In addition to being produced physiologically in our organism, hydrogen peroxide also occurs naturally in food, water and air, which increases our exposure to this reactive species (Bhatti et al., 2015). These statements underscores the importance of the hydrogen peroxide scavenging activity.

The anti-hydrogen peroxide activity revealed that HR had an excellent scavenging capacity (IC_{50} = 380 ± 4 μg/mL). Together with EAR (IC_{50} = 645 ± 5 μg/mL), they exhibited better hydrogen peroxide scavenging activity than the other studied fractions; at the same time their activity remain significantly lower than the ones of the standard drugs. Moderate activity was observed for CR with an IC_{50} value equal to 4027 ± 34 μg/mL. BR presented the lowest hydrogen peroxide scavenging activity. It is also worth mentioning that the synthetic antioxidant tested (BHA) had better hydrogen peroxide scavenging effect than gallic acid (IC_{50} = 278 ± 7 μg/mL). Other species such as R*anunculus arvensis* and *Ranunculus sceleratus*, showed moderate antiradical activities (Khan et al., 2017; Shahid et al., 2015) meanwhile *Ranunculus reptans* exhibited strong antiradical activity (Paudel et al., 2014).

In order to understand the relationship between the secondary metabolites and the antioxidant activities studied, a correlation analysis was performed (Table 3). According to it, DPPH and ABTS were significantly correlated with total flavonoid content (r = 0.99), total polyphenols (r = 0.97) and total flavonols (r = 0.96), suggesting that polyphenols are the main contributors to the DPPH and ABTS scavenging effects. These results are in agreement with those reported by Kiselova et al. (2006), Li et al. (2009) and Kumar et al. (2014).

In contrast to DPPH and ABTS, the hydrogen peroxide scavenging ability was not significantly correlated with the phenolic content of the samples; it was even negatively correlated with condensed tannins content (CTC). Thus, as has been formerly reported, the hydrogen peroxide scavenging ability of many plants extracts is not always linked to their polyphenol contents (Bayliak et al., 2016). In fact, a previous study of anti-H_2O_2 activity of several polyphenols revealed that diosmin and narirutin do not have such activity, while others like luteolin-7-*O*-rutinoside shows only a weak anti-H_2O_2 activity (Sroka et al., 2005). It seems that this activity has some structure requirement, specially the position of hydroxyl groups on the aromatic ring (Sroka et al., 2005).

The ability of the samples to scavenge hydrogen peroxide was significantly correlated to triterpenoids content (r= 0.93), indicating that this ability could be mainly attributed to the content of bioactive triterpenoids. Many terpenoids have been proven to be excellent antioxidants, whether in *in vitro* or *in vivo* studies (González-Burgos and Gómez-Serranillos, 2012). Moreover, plant bioactive compounds are not limited to polyphenols and thus, this should be taken into consideration when studying the antioxidant activity of plant extracts.

Table 3
Pearson correlation coefficient between antioxidant activities and bioactive compounds.

	DPPH	ABTS	H_2O_2	Iron chelation	FRAP	TAC	β-carotene
TPC	0.97*	0.98*	0.19	0.24	0.96*	0.95*	0.71
TFC	0.99*	0.99*	0.19	0.33	0.98*	0.92*	0.62
FOL	0.96*	0.96*	0.06	0.55	0.96*	0.84	0.43
TTC	0.70	0.73	0.23	−0.10	0.69	0.83	0.83
CTC	0.84	0.85	−0.28	0.72	0.83	0.64	0.14
TC	−0.10	−0.12	0.93*	−0.54	−0.06	0.20	0.61

* significant correlation (p-value < 0.05). TPC (total polyphenols content), TFC (total flavonoids contents), FOL (flavonols content), TTC (total tannins content), CTC (condensed tannins content) and TC (triterpenoid content).

5.3.2. Iron chelating capacity

The different fractions were tested for their iron chelation ability and the results are presented in Table 2. All the fractions showed different and dose-dependent iron chelation capacities. Consequently, the fractions can be ordered from the highest to the lowest capacity as follows: AR > HR > BR > CR > EAR. Compared to the EDTA standard iron chelating agent (IC_{50} equal to 22 ± 1 μg/mL), all the fractions showed, in general, poor iron chelating ability. Even with the highest polyphenol amounts, EAR fraction showed the weakest iron chelation ability. When studying the correlation between bioactive compounds and iron chelation (Table 3), significant correlations between the iron chelation and the quantified bioactive compounds was not found. For CTC this correlation was notable (r = 0.72), but not significant. For polyphenols this result is in accordance with the studies of Chai et al. (2014) and Ebrahimzadeh et al. (2008). In fact, not all phenolic compounds are good iron chelating agents. Again, this ability is strongly related to the structural features of the compounds, and the presence of some functional groups could hinder or strengthen this ability (Andjelković et al., 2006; Zhang and Tsao, 2016).

5.3.3. Reducing power

The reducing power assay can be described as an electron transfer based assay (Huang et al., 2005) that measures the reducers (antioxidants) present in a sample on the basis of a spectrophotometric redox reaction (Hajimahmoodi et al., 2008). The estimated reducing power (FRAP) of the target plant fractions is represented in Fig. 2.

Among all the studied fractions, EAR showed the best reducing power with a FRAP value of (310 ± 2 μg) AAE/mg (d.e), comparable to the reducing power value of BHT and significantly higher than the ones determined for the other analysed fractions. CR fraction exhibited moderate reducing power (53.2 ± 0.5) μg AAE/mg (d.e), while the reducing power of the remaining fractions did not exceed (21.7 ± 0.1) μg AAE/mg (d.e).

The reference standards displayed higher activity than the plant fractions, with a registered maximum value for gallic acid (1129 ± 5), and a minimum value for BHA (435 ± 4) μg AAE/mg (d.e). Moreover, standard pure compounds (quercetin and gallic acid) showed superior reducing power than synthetic compounds (BHA and BHT). The correlation study (Table 3) revealed that there was no relationship between triterpenoid content of the fractions and their reducing power (r = −0.06). Nevertheless, a strong significant relation among flavonoids (r = 0.98), flavonols (r = 0.96) and total polyphenols content of the fractions (r = 0.96) was found. The correlation with total tannins (r = 0.69) and condensed tannin contents (r = 0.83) was substantial, but not significant. Therefore, it could be assumed that polyphenols are the main compounds responsible for the reducing power of the plant fractions, which is in complete agreement with the results reported by other studies (Balík et al., 2008; Zhang et al., 2013; Aklima et al., 2014). In contrast to the results obtained by Amessis-Ouchemoukh et al. (2014), which did not find a significant correlation between total polyphenol content and the FRAP reducing

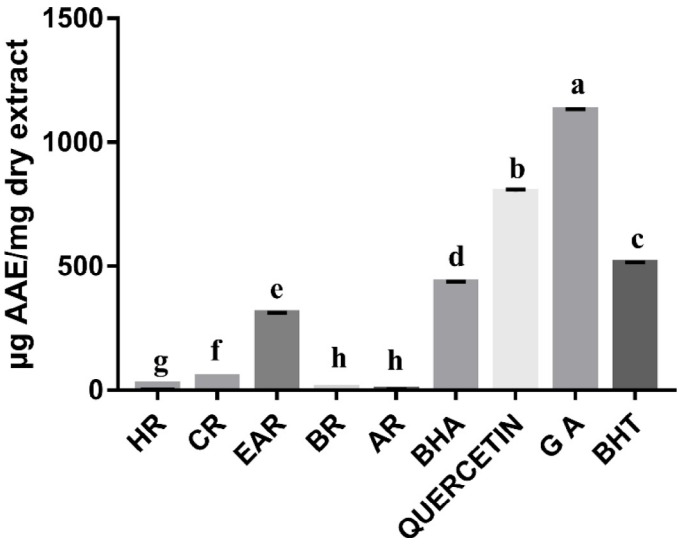

Fig. 2. Reducing power of different fractions from roots of *Ranunculus macrophyllus* Desf., BHA, quercetin, gallic acid (GA) and BHT. Results are expressed as mean ± SD of triplicates. Bars with different letters indicate significant differences (p-value < 0.05). CR (chloroform fraction), EAR (ethyl acetate fraction), BR (butanolic fraction) and AR (aqueous fraction).

power. The reducing capacity of the samples studied in this work could be due to the presence of reductants acting as electron donors. In this regard, polyphenols are known to be excellent electron donors (Bendary et al., 2013), allowing them to scavenge free radicals and act as chain breaking antioxidants. They may also react with some peroxide precursors, inhibiting the peroxide formation (Loganayaki et al., 2013). In addition, this electron donating ability may allow polyphenols to regenerate or to repair other oxidized antioxidants, such as vitamin E (Shahidi et al., 1992).

5.3.4. Total antioxidant capacity

The total antioxidant capacity (TAC) of the different fractions and the evaluated standards compounds are shown in Fig. 3. EAR exhibited the strongest total antioxidant capacity (361 ± 1) μg AAE/mg (d.e) compared to the other fractions. Both CR and HR fractions showed moderate TAC with (210 ± 9) and (154 ± 6) μg AAE/mg (d.e), respectively; while BR (93 ± 2) μg AAE/mg (d.e) and AR (84 ± 4) μg AAE/mg (d.e) showed the lowest activity. The activity of EAR was significantly higher than the one of the standards compounds BHA (284 ± 2) μg AAE/mg (d.e) and quercetin (243 ± 8) μg AAE/mg (d.e). Similar to BHT (353 ± 6) μg AAE/mg (d.e), but still lower than gallic acid activity (737 ± 2) μg AAE/mg (d.e). Additionally, in this test, gallic acid had higher total antioxidant capacity than synthetic antioxidants, while quercetin presented lower total antioxidant capacity than BHA and BHT.

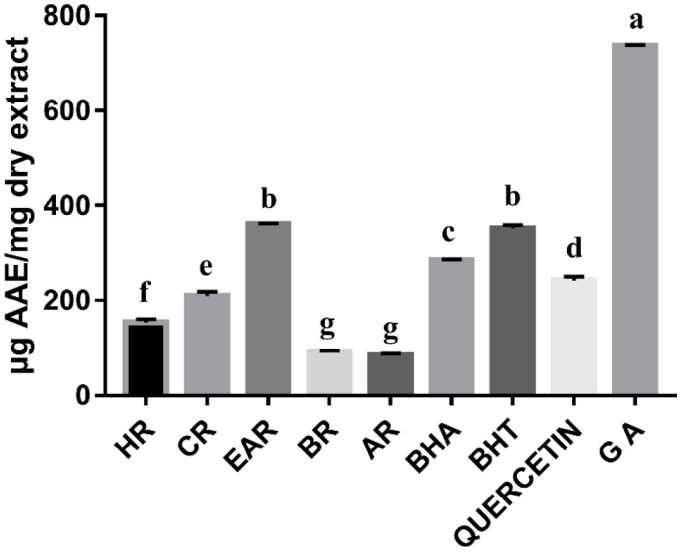

Fig. 3. Total antioxidant capacity of different fractions from roots of *Ranunculus macrophyllus* Desf., BHA, quercetin, gallic acid and BHT. Results are expressed as mean ± SD of triplicates. Bars with different letter indicates significant differences (p-value < 0.05). CR (chloroform fraction), EAR (ethyl acetate fraction), BR (butanolic fraction) and AR (aqueous fraction).

The TAC of the EAR fraction suggests the presence of effective antioxidants in this fraction. The correlation study (Table 3) revealed a significant correlation with total polyphenol contents (r = 0.95) and total flavonoid contents (r = 0.92). The correlation with total tannin content (r = 0.83) and flavonols (r = 0.84) was also high, but not significant. This fact suggests that polyphenols are the main contributors to the antioxidant activity. Jan et al. (2013) and Zhao et al. (2014) also reported this observation, while Sarikurkcu et al. (2017) found a significant correlation only with flavonoids and flavonols, although not with total polyphenol content. The fact that HR fraction showed higher activity than BR, despite the lower phenolic content, may suggest the involvement of other bioactive compounds in the total antioxidant activity exhibited by HR. For example, carotenoids and α-tocopherol, are common non-phenolic compounds involved in the total antioxidant capacity of non-polar extracts (Prieto et al., 1999). Triterpenoids, may at least partially be responsible for the activity of the HR fraction. Sarikurkcu et al. (2017) found that triterpenoids were significantly correlated with TAC of several plant extracts.

5.3.5. β-Carotene bleaching assay

β-Carotene is a red-orange natural pigment and a strong antioxidant, when oxidized by a radical or an oxidizing agent, β-carotene quickly decolorizes. In β-carotene assay the radicals are generated from autoxidation of linoleic acid in presence of oxygen, and the addition of another antioxidant to the β-carotene will prevent or delay its decolorization. The stronger is the antioxidant, the longer is the delay of decolorization. This effect is due to the competition between β-carotene and the other antioxidant in the system.

The capacity of a target plant fractions and standards to inhibit the β-carotene bleaching is summarized in Fig. 4. All samples were able to delay the decolorization of β-carotene solution at different rates, as shown by the kinetics of β-carotene bleaching included in Fig. 4 (a). EAR and CR were the most efficient fractions inhibiting the β-carotene bleaching by (81.0 ± 0.5) % and (80.2 ± 0.8) % respectively, after 24 h. At the same time, HR inhibition rate was (59.0 ± 0.2) %, while the inhibition rate of BR was just (27 ± 5) %. Therefore, it could be concluded that after 24h, the effect of AR was quite weak (13 ± 2) %, and even close to the one of methanol, used as a negative control. Both EAR and CR fractions are more effective than gallic acid (72 ± 5) %, and synthetic antioxidants presented the best inhibition of β-carotene bleaching; after 24 h the inhibition of BHA was (97 ± 2) % and that of BHT was (94.4 ± 0.5) %.

β-Carotene bleaching was correlated (Table 3) with the different bioactive compounds in the following order; TTC (r = 0.83) > TPC (r = 0.7) > TFC (r = 0.62) > TC (r = 0.6) > FOL (r = 0.42) > CTC (r = 0.13), but none of them was significant. This fact could suggest that β-carotene bleaching is a synergic result between the different sample components. Sarikurkcu et al. (2017) reported a significant correlation of β-carotene bleaching only with FOL, nonetheless no correlation was described with TPC, TFC and TC contents.

While other assays as FRAP are based on the capacity of reductants for transferring electron within a sample, β-carotene bleaching is a hydrogen transfer-based test (Nićiforović et al., 2017). In fact, polyphenols are known to react with lipid radicals by hydrogen atom transfer (Prior et al., 2005). Therefore, the great β-carotene bleaching

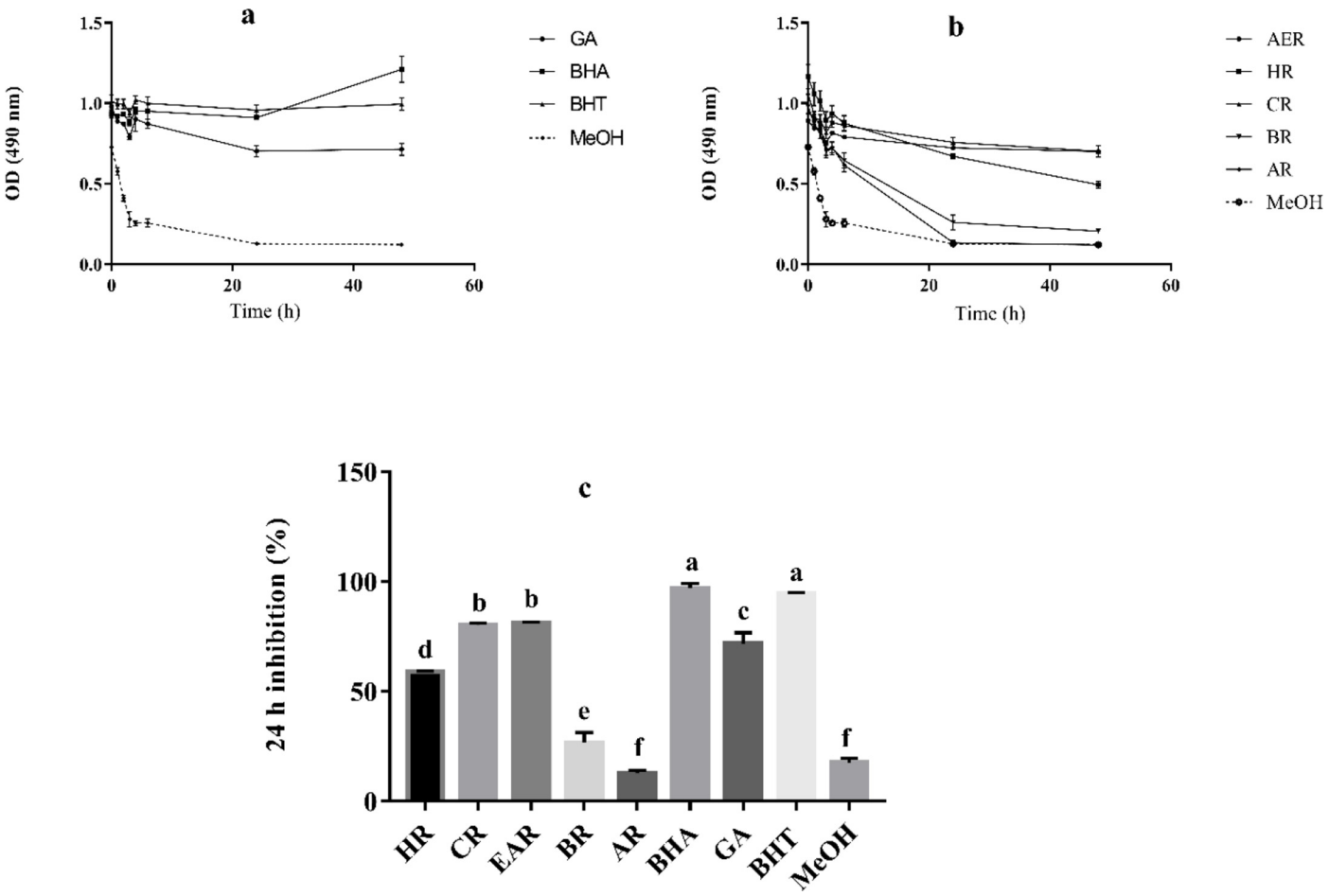

Fig. 4. Results obtained from the β-carotene bleaching assay: (a) kinetics of β-carotene bleaching for BHA, BHT, gallic acid (GA) and methanol (MeOH), (b) kinetics of β-carotene bleaching for different fractions and methanol, (c) inhibition of β-carotene bleaching after 24 h for fractions, standards and MeOH. Results are expressed as mean ± SD of triplicates. Bars with different letters indicate significant differences (p-value < 0.05). CR (chloroform fraction), EAR (ethyl acetate fraction), BR (butanolic fraction) and AR (aqueous fraction).

activity showed by the studied fractions reflects their excellent hydrogen donating ability.

As it can be seen from the results included in Fig. 4(b), non-polar fractions (HR, CR) presented better activity than polar fractions (BR and AR), which could be attributed to the polarity of the reductants in the non-polar fractions. Actually, β-carotene reaction medium is an emulsion of linoleic acid, consequently non-polar molecules will have a better access to the lipid radicals than the polar ones (Nićiforović et al., 2017). In this regard, the richness of HR and CR fractions with liposoluble triterpenoids must be pointed as they may be partially responsible for their activity.

5.4. Capillary LC-DAD and LC-MS/MS analysis of phenolic compounds

Spectrophotometric estimations of bioactive compounds and correlation studies revealed that EAR fraction had the highest polyphenol content and the best antioxidant activities. These activities were highly correlated with total polyphenols content. Therefore, the individual polyphenol profile of EAR was investigated by using capillary LC-DAD and LC-MS/MS, according to the procedures described in Section 2.10.1 and 2.10.2.

The individual polyphenolic profile of the EAR fraction was corroborated by using the LC-MS/MS method. Three different transitions were employed to unequivocally verify the identity of the polyphenols determined, the most abundant was used for quantification, whereas the others were for identification purposes (Table 4). Although all studied compounds were detected by LC-MS/MS, quantification was not possible as they were at concentration levels below the quantification limits of the method. Thus, quantitative analyses were performed by capillary LC-DAD. Therefore, combination of DAD and MS/MS detection systems provided both, an identification and compositional information of polyphenols presented in *Ranunculus macrophyllus* Desf. roots.

Table 4
Analysis of polyphenols by capillary LC-DAD and LC-MS/MS: identification and quantification results.

Compound	Capillary LC-DAD			LC-MS/MS					
	Rt, min	UV [a], nm	Extracted amount, mg/g (d.e)	Rt, min	PrecursorIon, m/z[M−H]⁻	Quantification Transition	Confirmation Transitions		Extracted amount, mg/g (d.e)
						Product ion I, *m/z* (Ce, eV)[b]	Product ion II, m/z (Ce, eV)[b]	Product ion III, m/z (Ce, eV)[b]	
Gallic A.	3.70	292	9.3 ± 0.6	6.25	169.0	125.1 (19)	79.0 (23)	53.1 (20)	LOD[c]
Dihydroxybenzoic A.	5.09	260	8.1 ± 0.2	10.22	153.0	109.0 (16)	108.1 (25)	53.0 (25)	LOD
Chlorogenic A.	8.29	260	3.5 ± 0.2	16.76	353.1	191.0 (16)	85.1 (44)	93.1 (42)	LOD
Caffeic A.	10.44	310	6.7 ± 0.4	18.07	179.0	135.0 (18)	134.0 (30)	89.0 (34)	LOD
p-Coumaric A.	12.95	310	3.0 ± 0.1	23.00	163.0	119.0 (17)	93.0 (31)	117.1 (34)	LOD
trans-Ferulic A.	13.74	310	2.1 ± 0.1	23.98	193.2	134.0 (18)	178.0 (17)	149.1 (15)	LOD
Hesperidin	15.35	292	5.9 ± 0.6	27.56	609.1	301.1 (23)	286.1 (48)	164.0 (55)	LOD
Rutin	–	–	nd	28.47	609.0	300.1 (42)	300.9 (33)	271.0 (45)	LOD
Resveratrol	17.18	310	3.3 ± 0.2	30.71	227.1	143.1 (25)	113.0 (10)	69.1 (21)	n.d.

[a] wavelength used for quantification.
[b] Collision energy, electron Volt.
[c] LOD: determined at the levels of the method detection limit; n.d: not detected; Rt: retention time; (d.e): dry weight.

Table 4 shows the compounds identified and/or quantified in the ethyl acetate fraction from *Ranunculus macrophyllus* Desf. This fraction contains high amounts of phenolic acids and flavonoids. Two categories of phenolic acids were present; cinnamic acid derivatives such as chlorogenic acid (3.5 ± 0.2) mg/g (d.e), caffeic acid (6.7 ± 0.4) mg/g (d.e), *p*-coumaric acid (3.0 ± 0.1) mg/d (d.e) and *trans*-ferulic acid (2.1 ± 0.1) mg/g (d.e); and benzoic acid derivatives like gallic acid (9.2 ± 0.6) mg/g (d.e) and dihydroxybenzoic acid (8.1 ± 0.2) mg/g (d.e). Flavonoids identified were hesperidin (5.9 ± 0.6) mg/g (d.e), and rutin. The latter one was only detected using LC-MS/MS, which could be explained by the highest sensitivity of the method for this particular compound. The stilbene resveratrol (3.3 ± 0.2) mg/g (d.e) was also identified by both chromatographic methods. The individual polyphenol profile of the EAR fractions, as well as the extracted amounts, has been established properly according to the criteria mentioned before.

As far as the author's knowledge, this is the first time that all the mentioned compounds have been identified and quantified in *Ranunculus macrophyllus* Desf. roots. According to the literature, especially reviews on the genus *Ranunculus* (Aslam et al., 2012; Da-Cheng, 2018), the polyphenol *trans*-ferulic acid was earlier detected in *Ranunculus muricatus* (Da-Cheng, 2018), *p*-coumaric acid and gallic acid were also detected in the roots of *Ranunculus ternatus* (Deng et al., 2013; Da-Cheng, 2018). Likewise, caffeic acid and rutin were reported in *Ranunculus arvensis* (Bhatti et al., 2015). However, to the best of the author's knowledge, this is the first time that hesperidin, chlorogenic acid and resveratrol are identified in the genus *Ranunculus*.

It is worth mentioning the importance of the compounds identified, as they are widely spread in fruits and vegetables. Thus, they constitute one of the most common polyphenol groups in human food. In fact, the quantities found in our study are similar or higher than those found in common food sources. The amount of resveratrol found is similar to the levels reported in red and white grape seeds (Counet et al., 2006). From the same study it can be seen that the amount of *trans*-ferulic acid in chocolate extract (2.4 ± 0.1) mg/g (d.e) is close to the one observed in the present study (2.1 ± 0.1) mg/g (d.e). The amount of hesperidin, also known as orange peel glucoside, quantified in this study was higher than the one reported in *Citrus mitis* Blanco (1.5 ± 0.2) mg/g (d.e) (Lou et al., 2014). Both gallic acid and ferulic acid quantities in pineapple peels are lower than in the roots of *Ranunculus macrophyllus* Desf. (Li et al., 2014). Caffeic acid content is almost 30 times higher than it is in the roots of red beet (*Beta vulgaris*), while chlorogenic acid content is 17 times higher than that found on the stems of red beet (Koubaier et al., 2014). In addition, *Ranunculus macrophyllus* Desf. roots contained considerable amounts of hydroxybenzoic acid derivatives. This is very important because it is known that the levels of these compounds are very low in edible plants, except in some red fruits (Gómez-Maqueo et al., 2018). This fact makes *Ranunculus macrophyllus* Desf. roots a valuable source of hydroxybenzoic acid derivatives. Therefore, the roots of *Ranunculus macrophyllus* Desf. can be seen as an appreciated source of high added-value polyphenols, like ferulic acid, hesperidin and resveratrol, with potential interest in cosmetic, pharmaceutical, and agri-food industries.

Conclusions

The liquid-liquid separation showed that ethyl acetate fraction contains most of the phenolic compounds of *Ranunculus macrophyllus* Desf. roots, while the chromatographic analysis revealed for the first time the presence of gallic acid, dihydroxybenzoic acid, caffeic acid and hesperidin as major compounds, some of them are described for the first time in the genus ranunculus. Thus, the EAR fraction could provide a total amount of phenolic compounds of 41.9 mg/g (d.e). Ethyl acetate fraction exhibited strong radical scavenging ability, high metal ions reducing power and potent anti-lipid peroxidation activity and these activities were strongly correlated with the phenolic content. Consequently, *Ranunculus macrophyllus* Desf. roots may be considered as a valuable source of strong polyphenolic antioxidants with many therapeutic and industrial applications, and as an ingredient for preparation of nutraceutical and functional food.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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