Industrial Crops and Products 74 (2015) 858–866

Contents lists available at ScienceDirect

Industrial Crops and Products

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

HPLC-DAD profile of phenolic compounds and antioxidant activity of leaves extract of *Rhamnus alaternus* L.

Kamal Moussi[a,*], Balunkeswar Nayak[b], L. Brian Perkins[b], Farid Dahmoune[a], Khodir Madani[a], Mohamed Chibane[c]

[a] *Laboratoire de Biomathématiques, Biophysique, Biochimie, et Scientométrie, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, 06000 Bejaia, Algeria*

[b] *Food Science and Human Nutrition, School of Food and Agriculture, University of Maine, Orono, ME 04469, United States*

[c] *Laboratoire de Gestion et de Valorisation des Ressources Naturelles, Assurance Qualité, Faculté des Sciences de la Nature et de la Vie et des Sciences de la Terre", Université de Bouira, Algeria*

ARTICLE INFO

Article history:
Received 8 January 2015
Received in revised form 2 June 2015
Accepted 4 June 2015

Keywords:
Rhamnus alaternus L.
Phenolic compounds
Free radical scavenging activity
HPLC-DAD

ABSTRACT

This research investigated the polyphenols profiling and antioxidative properties of *Rhamnus alaternus* L. A comparative analysis of the defatted and eluted fractions of methanol extract from leaves of *R. alaternus* L. was performed on high-performance liquid chromatography coupled to diode array detection. The total phenolic, flavonoid, monomeric anthocyanin contents, and antioxidant activity of all the fractions of the leaves extract were quantified. All fractions showed the presence of phenolic compounds and exhibited different levels of free radical scavenging activity. Seven individual phenolic compounds and anthraquinones as a group of phenolic compounds in the samples were identified. Luteolin, quercetin-3-rhamnoside, *p*-coumaric acid, ferulic acid, gallic acid, and rutin were the newly identified compounds in the extract, confirmed by the presence of kaempferol and anthraquinones. Knowledge of these compounds in the leaves extract will help in formulating pharmaceutical products for various diseases.

1. Introduction

Ethnomedicine has been practiced since prehistory by virtually all human cultures around the globe, including Europe, Asia, Americas, and Africa (Slikkerveer, 2006). North Africa has one of the oldest and richest traditions associated with the use of medicinal plants (Batanouny et al., 2005), where Herodotus (430 B.C) wrote that medicine in Egypt is practiced among them on a plan of separation, by using an infinite number of drugs from medicinal plants (Rawlinson and Blakeney, 1910). Farther, North Africa is known for its illustrious savant king Juba II of Libya (Mauritania), who died about the year 20 AD, leaving at least a dozen scientific works. One of his most esteemed treaties is that which Pliny and Galen attributed the title "Deherba euphorbia.„ it is the variety of medicinal plant, to which he discovered the medicinal potential (Eugène-Humbert, 1952).

In North Africa, there are about 10,000 vascular plant species and about 70% found in the wild have medicinal, aromatic, and other uses. In Algeria, an estimated number of 3164 identified species are qualified as traditional medicinal plants (Vasisht and Kumar, 2004). Among the identified plant species on the earth, only a small percentage has been phytochemically investigated and the fraction submitted to biological or pharmacological screening is even smaller. Moreover, a plant extract may contain several thousand different natural products and any phytochemical investigation of a given plant will reveal only a narrow spectrum of its constituents (Hostettmann et al., 2000).

Plants represent a rich source of natural products, with almost infinite molecular diversity (Hostettmann and Marston, 2002), of which active ingredients of medicinal plants are mostly secondary metabolites (Zeng et al., 2013). The phenolic compounds are large and heterogeneous groups of these secondary metabolites, that are distributed throughout the plant kingdom (Maestri et al., 2006). Phenolic compounds are amongst the most desirable phytochemicals due to antimicrobial, antiviral, anti-inflammatory properties, and high antioxidant capacities (Ignat et al., 2011). Antioxidants are defined as compounds that can delay, inhibit, or prevent the

* Corresponding author at: Laboratoire de Biomathématique, Biophysique, Biochimie, et Scientométrie. Faculté des Sciences de la Nature et de la Vie, Université Abderrahmane Mira, Route Targa Ouzemour, 06000 Bejaia. Algerie. Fax: +213 34 21 47 62.

E-mail addresses: moussi.kamal2012@yahoo.fr (K. Moussi), balunkeswar.nayak@maine.edu (B. Nayak), BPerkins@Maine.edu (L.B. Perkins), farid.dahmoune@univ-bejaia.dz (F. Dahmoune), khodir.madani@univ-bejaia.dz (K. Madani), chibane18156@yahoo.fr (M. Chibane).

http://dx.doi.org/10.1016/j.indcrop.2015.06.015
0926-6690/

oxidation of oxidizable materials by scavenging free radicals. Phenolics have been considered powerful antioxidants in vitro (Dai and Mumper, 2010). Many efforts have been made to provide a highly sensitive and selective analytical method for the determination and characterization of phenolic compounds. It is very important to understand the profile of phenolic compounds in medicinal plants by using highly effective extractions and analytical methods (Ignat et al., 2011).

Rhamnus alaternus L. is a fleshy-fruited, shrub of the Mediterranean region (Ben Ammar et al., 2005). One of the species commonly used in reforestation programs, due to its fruit characteristics and ability to survive in xeric environments, which represents an important water and nutrient source for birds and small mammals (Gulias et al., 2004). It has traditionally been used as laxative; purgative; hypotensive; and it is also used for treatment of the dermatological, ocular, burning, odontological, hepatic (Ben Ammar et al., 2008; Ben Ammar et al., 2005), psychological, depression, and goiter problems (Mati and de Boer, 2011). The wood has been also used as cosmetic for split hair (Lardos, 2006) and as a dyeing agent for wool (Guarrera, 2006). In addition, it has been reported that the leaves extracts of *R. alaternus* showed the highest antimutagenic level in a bacterial assay system, high xanthine oxidase inhibition (Ben Ammar et al., 2008) and inhibition of both rat intestine and purified porcine liver carboxylesterase (Stocker et al., 2004).

As a prominent local medicinal plant, *R. alaternus* L. has been widely distributed in the Northern Algeria. The leaves are used locally to in Kabylia by decoction as purgative and laxative, and also used fresh for treatment of jaundice. The purpose of this study was (i) defatting and fractionation of methanol extract from leaves of *R. alaternus* L. (ii) to investigate profiling of the phenolic compounds including phenolic acids, flavonoids, and anthocyanins by HPLC-DAD, and (iii) assessing total phenolic compounds, flavonoids, monomeric anthocyanins using colorimetric methods, and antioxidant activity using a radical scavenging assay of all fractions of defatting and fractionation steps of methanol leaves extract.

2. Materials and methods

2.1. Chemicals

Acetonitrile, hexane, acetic acid, sodium carbonate, Folin–Ciocalteau reagents, aluminium trichloride ($AlCl_3$), potassium chloride, and sodium acetate trihydrate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ethyl acetate and DPPH (2,2-diphenyl-1-picrylhydrazyl) were obtained from Sigma–Aldrich (St. Louis, MO, USA). The phenolic standards, i.e., catechin, caffeic acid, quercetin-3-rhamnoside, *p*-coumaric acid, gallic acid, ferrulic acid, luteolin, apegenin, vanillic acid, and (−) epigallocatechin were purchased from Sigma–Aldrich (St, Louis, MO, USA). Chlorogenic acid and rutin were purchased from Acros organics (Thermo Fisher Scientific, Fair Lawn, NJ, USA) and naringenin and kaempferol from Sigma–Aldrich (Gillingham, Dorset, United Kingdom). All solvents used for this work were of HPLC grade.

2.2. Preparation of methanol extract

R. alaternus L. leaves were collected in March 2013, from Hengued, Adekar, North-West part of Bejaia in Kabylia (Algeria) (latitude: 36°43′15.46″N and longitude: 4°34′54.15″E) and kept for drying under a forced air oven at 60 °C up to moisture content of about 3.25% (determined by dry weight in oven at 105 °C until constant weight) (Spigno et al., 2007). The dried material was crushed to prepare powder, which was milled through a 1 mm sieve (final powder size <1 mm). Fifty grams of powder were macerated with 500 mL of methanol for 72 h at room temperature. After filtration through a filter paper, methanol was entirely removed using a rotary evaporator (Buchi, Flawii, Switzerland) at 45 °C (at 337 mbar). The dry extract was weighed and then re-dissolved in methanol to obtain a solution, with known concentration which was designated as crude extract (CE). The extractive value was calculated on dry weight basis from the formula given below:

$$\%\text{extractivevalue(yield\%)} = \frac{\text{Weightofdryextract}}{\text{Weighttakenforextraction}} \times 100$$

2.3. Defatting and fractionation of extract

All the steps of extraction, defatting, and fractionation are summarized in Fig. 1. Fifty milliliters of crude extract were subjected to entire evaporation of the methanol on a rotary evaporator (Buchi, Flawii, Switzerland) at 45 °C (at 337 mbar). The resulting dry extract was dissolved in 90 mL of Ultrapure water (Milli-Q) to obtain aqueous solution and was defatted several times, with the same volume of hexane, until a clear solution of hexane resulted. The hexane in the solution was evaporated using a rotary evaporator to get dry extract at 45 °C (at 335 mbar). The dry extract was re-dissolved in 40 mL of methanol and designated as hexane fraction (HF). The insoluble fraction formed between hexane and aqueous solution was washed several time with ultrapure water (Milli-Q), dried, dissolved in 25 mL of methanol and is designated as intermediate fraction (INT).

C-18 Sep-Pak Vac 35-cc (Waters, Milford, MA, USA) column was sequentially rinsed with 50 mL of ethyl acetate, acidified methanol (0.01% v/v HCl), and acidified water (0.01% v/v HCl), and charged separately with 20 mL of defatted aqueous solution of extract. The column was then washed with 60 mL of acidified Ultrapure water (Milli-Q) (AW), to remove any organic sugars and acids remaining in the solution (fraction AW), followed by elution with 60 mL of ethyl acetate and was designated as fraction EA. The final elution was made with 60 mL of acidified methanol and was designated as fraction AM (Lacombe et al., 2012). The ethyl acetate fraction EA contained a small amount of water, resulting from the previous elution with acidified water, which led to the separation of ethyl acetate solution designated as EA1 and water solution designed as EA2.

The solvents of the fractions HF, EA1, AM were removed completely on a rotary evaporator (Buchi, Flawii, Switzerland) at 45 °C (at 240 mbar). For the aqueous solutions AW and EA2, the solvents were removed by lyophilization (VirTis, New York, USA). All dry extracts HF, EA1, AM, AW, EA2, and INT fractions were dissolved in HPLC grade methanol and were subjected to HPLC analysis, phytochemical dosages, and antioxidant activity.

2.4. Determination of total phenolic compounds

The total phenolic content of the standards, crude extract (2 mg/mL) and all fractions (INT, HF, AW, EA1, EA2, and AM) without dilution were determined by Folin–Ciocalteu's phenol method following the procedures of Velioglu et al. (1998). Briefly, 20 μL of standard or samples were mixed separately with 150 μL of Folin–Ciocalteu reagents (previously diluted 1:10 with distilled water) and allowed to stand at 22 °C for 5 min. After incubation, 150 μL of sodium bicarbonate (6 g/100 mL) were added and after 90 min at 22 °C, the absorbance was read on the BMG LABTECH FLUOstar Omega plate reader (Germany) at 725 nm against a blank. The experiment was carried out in triplicate and the concentration of total phenolic compounds in the extract were expressed in mg gallic acid equivalent (GAE) per gram plant powder (PPW) i.e., (mg GAE/g PPW).

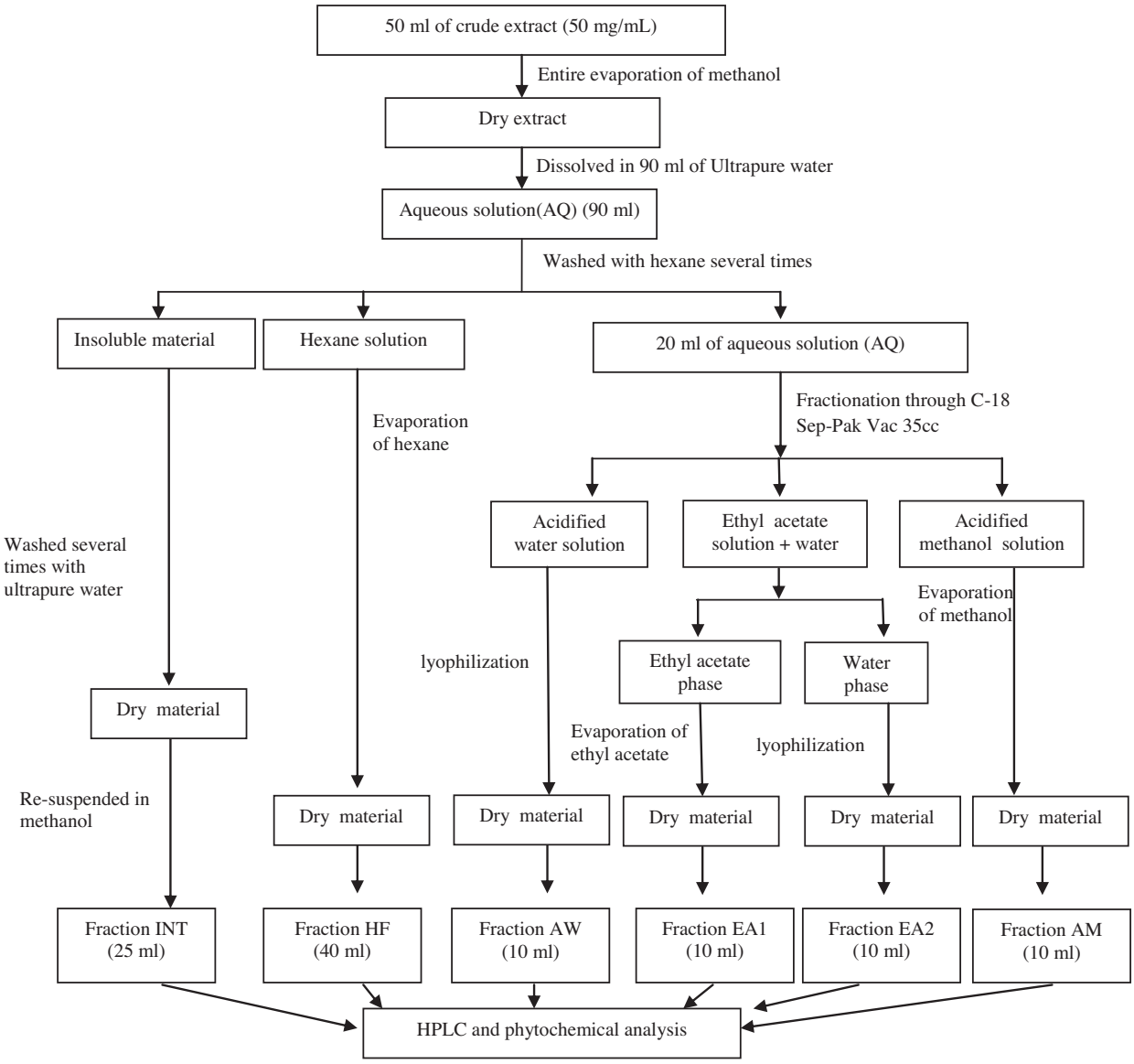

Fig. 1. Schematic diagram of fractionation of extract of *Rhamnus alaternus* L. leaves.

2.5. Determination of total flavonoid

The total flavonoid content was determined using the aluminium trichloride method (Adedapo et al., 2008). Briefly, 150 μL of 2% aluminium trichloride ($AlCl_3$) in methanol were mixed with the same volume of a standard or crude extract (2 mg/mL) or fractions (INT, HF, AW, EA1, EA2, and AM). Absorption readings at 430 nm were taken after 10 min against a blank using a BMG LABTECH FLUOstar Omega plate reader. The experiment was carried out in triplicate and the total flavonoid contents were expressed as milligram quercetin-3-rhamnoside equivalent (QE) per gram plant powder (PPW) i.e., (mg QE/g PPW).

2.6. Determination of total monomeric anthocyanin

The total monomeric anthoyanin content was determined by the pH differential method with pH 1.0 buffer (potassium chloride, 0.025 M) and pH 4.5 buffer (sodium acetate, 0.4 M) (Lee, 2005). The crude extract and all fractions (INT, HF, AW, EA1, EA2, and AM) were diluted with pH 1.0 and pH 4.5 buffers. 350 μL of buffered samples at pH1 and pH 4.5 were added separately to appropriate microplate wells and readings were recorded, using a BMG LABTECH FLUOstar Omega plate reader (Germany). The absorbance was then measured at 520 and 700 nm. The experiment was carried out in triplicate and the concentration of anthocyanin was calculated as follows:

$$\text{Totalmonomericanthocyanin(cyd} - 3 - \text{glu, mg/L)}$$

$$= x = \frac{\text{A} \times \text{MW} \times \text{DF} \times 10^3}{\epsilon \times \text{l}}$$

where $A = (A_{520nm} - A_{700nm})$ pH 1.0 $- (A_{520nm} - A_{700nm})$ pH 4.5; MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu); DF = dilution factor established in D; l = path length in cm; ϵ = 26 900 molar extinction coefficient ($L \times mol^{-1} \times cm^{-1}$) for cyd-3-glu; and 10^3 = factor for conversion from g to mg.

2.7. Determination of antioxidant activity

DPPH is a stable radical with a deep purple color. Reaction with other radicals, electrons, or hydrogen atoms leads to loss of color at 515 nm and loss of the EPR free radical signal (Schaich et al., 2015). The antioxidant activity of crude extract and fractions of

Table 1
Total phenolics, total flavonoids, total monomeric anthocyanins contents, and antioxidant activity of *Rhamnus laternus* L. leaves extract and fractions.

	Fraction	Total phenolic contents (μg GAE/mL)	Total flavonoids (μg QE/mL)	Total monomeric anthocyanins (mg c3g/L)	Antioxidant activity (%) DPPH inhibition)
Crude extract	CE	155.60 ± 0.77 ^{a2}	60.22 ± 5.76 ^{a3}	0.0 ± 0.0	66.83 ± 3.88 ^{a3}
Fractions after defatting	INT	165.51 ± 7.87 ^{a1}	156.39 ± 9.02 ^{a1}	0.0 ± 0.0	90.36 ± 0.45 ^{a2}
	HF	245.53 ± 11.58 ^{a2}	163.29 ± 12.55 ^{a1}	0.0 ± 0.0	80.14 ± 0.52 ^{a1}
Fractions through C–18	EA1	317.47 ± 0.65 ^{b2}	103.18 ± 5.96 ^{b2}	0.0 ± 0.0	90.81 ± 2.46 ^{b1}
	EA2	78.91 ± 7.72 ^{b4}	13.53 ± 0.57 ^{b3}	0.0 ± 0.0	25.93 ± 5.41 ^{b2}
	AM	712.58 ± 4.35 ^{b1}	705.30 ± 2.04 ^{b1}	0.0 ± 0.0	86.04 ± 0.09 ^{b1}
	AW	204.88 ± 4.14 ^{b3}	0.00 ± 0.00 ^{b4}	0.0 ± 0.0	12.60 ± 1.24 ^{b3}

CE: crude extract; INT: intermediate fraction; HF: hexane fraction; EA1: ethyl acetate fraction 1; EA2: ethyl acetate fraction 2 (containing water); AW: aqueous fraction (containing organic sugars); AM: acidified methanol fraction (see Fig. 1 for detailed separation method); GAE: gallic acid equivalent; QE: quercitin equivalent; C3G: cyanindin-3-glucoside. a, b are notations for comparison between samples and 1-4 are notations for the level of statistical differences.

the extract was determined by measuring the DPPH free radical scavenging activity (Dudonne et al., 2009). The DPPH• solution in methanol (6×10^{-5} M) was prepared, and 300 μL of this solution was mixed separately with 10 μL of crude extract and all fractions (INT, HF, AW, EA1, EA2, and AM) in flat bottom wells, by using BMG LABTECH FLUOstar Omega plate reader (Germany) set at 37 °C. After 20 min of incubation, a decrease in the absorbance at 517 nm was measured (A_{sample}). A control containing 10 μL of methanol in the DPPH• solution was prepared and its absorbance was measured ($A_{control}$). DPPH is a free radical that produces a blue–violet solution in methanol. In the presence of antioxidant compounds, DPPH radical is reduced, producing a non-color methanol solution. The experiment was carried out in triplicate. Radical scavenging activity was calculated using the following formula:

$$\%\text{inhibition} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

where $\text{Abs}_{\text{control}}$ was the absorbance of control and $\text{Abs}_{\text{sample}}$ was the absorbance of sample.

2.8. HPLC-DAD analysis

The analysis for phenolic compounds was performed on the Agilent 1200 series rapid resolution liquid chromatograph (Agilent Technologies, Santa Clara, CA) consisting of a vacuum degasser, an auto-sampler, a binary pump and diode-array detection (DAD) system. Data analysis was performed with Agilent HPLC Chem-Station software. This instrument was equipped with a Phenomenex Prodigy 5 ODS-2 column (4.60 mm × 250 mm, 5 micron, CA, USA). Samples were centrifuged at 2655 × *g* for 10 min prior to HPLC injection. Simultaneous monitoring was performed for determination at 254, 280, 300, 520, and 640 nm; and spectral data were collected from 200 to 700 nm. The column temperature was set at 30 °C, and the injection volume was 20 μL, with a flow rate of 0.5 mL/min. Acidified water (6% acetic acid, v/v) and acetonitrile were used as mobile phases A and B, respectively (Chirinos et al., 2008). The gradient was programmed as follows: 0–40 min, 0–25% B; 40–80 min, 25–85% B; 80–90 min, 85–100% B; 90–95 min, 100% B. All identified phenolic compounds (phenolic acids and flavonoids) were quantified with external standards, using calibration curves. The standard response curve was a linear regression fitted to values obtained at each concentration within the range of 12.5–200 μg/mL for phenolic acids and 20–350 μg/mL for flavonoids.

2.9. Statistical analysis

The experimental results were expressed as means ± standard deviation for triplicate measurements. Correlation analysis of antioxidant activity (Y) versus the total phenolic content (X) and flavonoid content (X) was carried out using the correlation program in Microsoft Excel software. Differences between fractions were determined by ANOVA procedures, followed by the Tukey's honestly significant difference (HSD) at $P < 0.05$ using statistical software (SAS Version 7.0, Trial version 8.0.7.1).

3. Results and discussion

3.1. Quantification of phenolic compounds

Soluble phenolic compounds can be isolated easily from plant tissue by extraction into methanol (Vermerris and Nicholson, 2007), which is the most suitable solvent due to its ability to inhibit the action of polyphenol oxidase, that causes the oxidation of polyphenols (Lim and Quah, 2007). The methanol extractive value (yield) from leaves of *R. alaternus* L. was 22% of plant powder (PPW). The total phenolic and flavonoid contents of crude extract (CE) were 155.60 ± 0.77 μg GAE/mL and 60.22 ± 5.76 μg QE/mL, respectively (Table 1). Considering the extraction yield, the results represent 17.13 ± 0.09 mg GAE/g PPW and 6.63 ± 0.63 mg QE/g PPW, respectively.

Determination of the total monomeric anthocyanin contents by the pH differential method was based on the anthocyanin structural transformation, that occurs with a change in pH (colored at pH 1.0 and colorless at pH 4.5) (Lee, 2005). The analysis of monomeric anthocyanin showed no change in color or absorbance between pH 1.0 and pH 4.5, which indicates the absence of monomeric anthocyanin in crude extract (CE) of *R. alaternus* L.

Plant crude extracts usually contain large amounts of carbohydrates and/or lipoidal material. The concentration of phenolics in the crude extract may be low. To concentrate and obtain polyphenol-rich fractions before analysis, strategies including sequential extraction or liquid–liquid partitioning and/or solid phase extraction, based on polarity and acidity have been commonly used. In general, elimination of lipoidal material can be achieved by washing the crude extract with non-polar solvents, such as hexane, dichloromethane, or chloroform (Dai and Mumper, 2010). However, in the present study, defatting step gave rise to three phases, HF, INT, and AQ (aqueous solution). The non-phenolic compounds were eliminated, but also the phenolic compounds were found in HF and INT fractions, with the quantities of 245.53 ± 11.58 and 165.51 ± 7.87 GAE μg/mL, respectively (Table 1). Considering the dilution factor, these amounts represent 3.44 ± 0.16 and 1.45 ± 0.07% of total phenolic content in crude extract (CE), respectively. The flavonoid content was 66.51 ± 5.11 and 94.49 ± 5.45% of total phenolic content in HF and INT, respectively.

Reverse phase C18 cartridges have been most widely used in phenolic compounds separation (Nayak et al., 2011). After the aqueous sample was passed through preconditioned C18 cartridges, the cartridges were washed with acidified water to remove

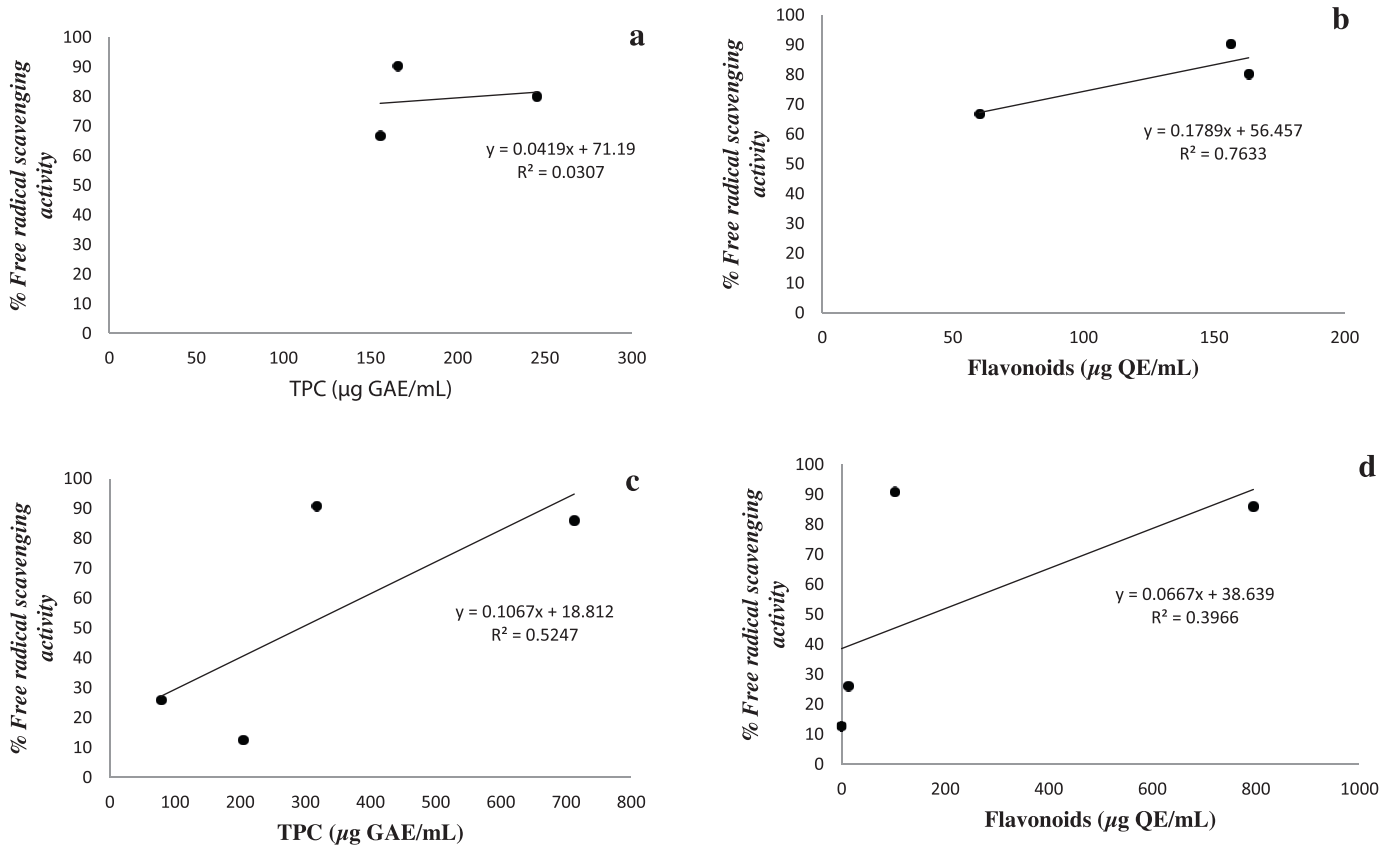

Fig. 2. Linear correlation of free radical scavenging activity (Y) in terms of % DPPH inhibition versus the total phenolic compounds (TPC) (X) and versus flavonoid content (X) in ascending order concentrations (a) and (b) for crude extract (CE), intermediate fraction (INT) and hexane fraction (HF), (c) and (d) for eluted fractions through reverse phase C-18 Sep-Pak column.

sugar, organic acids, and other water-soluble constituents (Dai and Mumper, 2010; Lacombe et al., 2012). The polyphenols were then eluted with absolute methanol or aqueous acetone (Dai and Mumper, 2010). Monomeric phenolic acids were then eluted with ethyl acetate followed by elution of anthocyanins and proanthocyanidins with acidified methanol (Lacombe et al., 2012).

The elution of defatted solution (aqueous solution (AQ)) by C-18 Sep-Pak showed the presence of phenolic compounds in all fractions of EA1, EA2, AM, and AW. The total phenolic contents of 24.16 ± 0.04, 6.01 ± 0.59, 54.24 ± 0.33 and 15.59 ± 0.22% were found in EA1, EA2, AM, and AW fractions, respectively, with a high amount of elution in AM fraction, which is constituted with 98.98 ± 0.29% of flavonoid of total phenolic content in AM fraction. It was observed that a significant amount of phenolic compounds were lost during washing of the C18 column with acidified water. The flavonoids were present in all eluted fractions of extract except in AW. The results confirmed the absence of anthocyanin in all fractions, even in the fraction that eluted with acidified methanol (AM) (Table 1).

3.2. Antioxidant activity

The antioxidant activity using scavenging of DPPH free radicals was correlated with the total phenolic, flavonoid contents of crude extract and all fractions (INT, HF, AW, EA1, EA2, and AM). The free radical scavenging activity showed that the INT fraction had the higher activity, with a rate of 90.36 ± 0.45%, followed by HF with 80.14 ± 0.52% and CE with 66.83 ± 3.88%. For the eluted fractions derived from the aqueous solution (AQ), the higher activity of free radical scavenging was observed in EA1 fraction (90.81 ± 2.46%) followed by AM (86.04 ± 0.09%), EA2 (25.93 ± 5.41%) and AW (12.60 ± 1.24%) fraction despite the higher quantities of total phenolic and flavonoid contents in AM fraction compared with EA1 (Table 1). Correlation analysis (Fig. 2) of free radical scavenging activity showed different levels of linear positive correlation with total phenolic and flavonoid contents in all fractions. Furthermore, the correlation results of the antioxidant activity (Y) versus total phenolic (X) and flavonoid (X) in crude extract (CE), INT and HF fractions (Fig. 2a and b) suggest that only 17.52% of the antioxidant capacity depend on the total phenolic contents. However, for flavonoid, the antioxidant activity dependence was 87.36%, with partial accordance to the statistically significant difference level (Table 1). The antioxidant activity of the eluted fractions derived from the aqueous solution (AQ) showed a dependence on the total phenolic content (Fig. 2c) and flavonoid (Fig. 2d), with a rate of 72.43% and 62.97%, respectively, which was in partial accordance with the statistically significant difference level of total phenolic content and with no accordance for flavonoid content.

In previous studies, it has been reported that the antioxidant activities of many plant extracts were proportional to their phenolic content, suggesting a causative relationship between them (Nayak et al., 2013), which is mainly due to the redox properties of phenolic compounds, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. They may also have a metal chelating potential (Javanmardi et al., 2003). The antioxidant activity is strongly dependent on the model system in which it is evaluated, and a single analytical assay to measure the antioxidant activity may be inadequate. The polarity of both the extracting and the reacting medium during the assays is usually determined for the protection against oxidation (Moure et al., 2001). For this, the evaluation of antioxidants must be followed by assays with other systems, as lipids in micellar systems, bulk

Table 2
Identification of individual phenolic compounds and anthraquinones as a group of phenolic compounds at 280 nm using HPLC-DAD system.

Name of phenolics	Compound notations	Retention times	Maximum absorbance (200–700 nm)
Rutin	1	38.03	256, 355
Antraquinones	2	[a]	[a]
Quercetin-3-rhamnoside	3	54.55	231, 255, 371
Kaempferol	4	59.34	232, 265, 336
Gallic acid	5	8.73	232, 270
p-Coumaric acid	6	36.20	214, 234, 312
Ferulic acid	7	39.52	220, 226, 238, 323
Luteolin	8	54.34	230, 253, 347

[a] 17 peaks of anthraquinones with a different retention times and absorbance. Identification of spectra absorbance of anthraquinones by comparing with bibliography (Marković et al., 2008).

oils, foods, etc., because the more potent proton donor is not necessarily the best antioxidant in different systems (Franco et al., 2008). However, phenolic compounds do not contribute to 100% of antioxidant activity in plant extracts. Some contribution may also come from the presence of other antioxidant secondary metabolites, such as volatile oils, carotenoids, and vitamins (Javanmardi et al., 2003). Moreover, it has been reported that the content of chlorophylls contributed to the antioxidant capacity and studies have shown that chlorophylls act as electron donors, reducing free radicals such as DPPH (Fernandez-Orozco et al., 2011). The study concluded that the solubility of phenolic compounds derived from defatting and fractionation in the evaluated antioxidant system, qualitative effect of phenolic compounds in different fractions, the presence of chlorophyll in CE, INT, and HF and the eventual presence of other metabolites may be responsible for the antioxidant capacity.

3.3. Determination of phenolic compounds HPLC analysis

The HPLC-DAD method developed for this study achieved an appropriate separation among the fourteen standards and the method validation data obtained for the seven individual identified phenolic compounds and anthraquinones as a group of phenolic compounds at 280 nm is shown in Table 2. HPLC profile of all fractions is summarized in Table 3 and chromatograms recorded at 280 nm for all fractions are shown in Fig. 3. The peaks of interest were identified by their retention time and UV–vis spectra in samples and standards. The HPLC profile of all fractions of the resulting solutions of the defatting (INT and HF) and fractionation of the retained compounds of aqueous solution (AQ) by the C-18 Sep-Pak (EA1, EA2, AW, AM) showed several peaks corresponding to different phenolic compounds with quantitative and qualitative difference of identified compounds. Presence of high amounts of total phenolic and flavonoid contents in AM fraction (Table 1) were confirmed by HPLC analysis with reference to the peaks recorded (36–53 min), with very high absorbance (Fig. 3F), with characteristic spectra absorbance of flavonoids, with clear spectra for peaks with retention times of 36.885, 43.458, and 52.638 min (Supplement Fig. 1a). For the qualitative, the identified phenolic compounds were rutin (HF and EA2), quercetin-3-rhamnoside (HF, INT, and EA1,) kaempferol (HF, INT, EA1, EA2, and AM), *p*-coumaric acid (EA1), ferulic acid (EA1), gallic acid (AW), luteolin (AM), antraquinones (HF, INT, EA1, EA2, and AM) and in the end chlorophyll (HF and INT) as no phenolic compounds.

Anthocyanins are responsible for the red, purple, and blue hues present in fruit, vegetables, and grains; have typical absorbance in the visible band 400 and 600, with a maximum around 520 nm (Lee, 2005). In the present study, the results of colorimetric method for total monomeric anthocyanin were confirmed by recording the chromatograms of all fractions at 520 nm, which are characterized by the absence of peaks.

Anthraquinone derivatives are a group of phenolic compounds, present in plants, usually in the form of oxidised 1,8-dihydroxyanthrone with a specific substitution pattern (Kosalec et al., 2013). Emodin is a biologically active, naturally-occurring anthraquinone derivative from plant (Sucheta et al., 2011), presents spectra absorbance in methanol between 200 and 580 nm, with absorption maxima (A_{max}) located at 203.10, 221.72, 253.59, 263.85, 289.43, and 436.83 nm (Marković et al., 2008). In the present study, 17 peaks with different retention times and absorption maxima were detected (Table 3) and constitute 6.18% of the area of total fractions, with typical spectra of anthraquinones, for example, the peak with retention times at 75.88 min, which represents 20.98% of the area of HF fraction, presents spectra absorbance between 200 and 510 nm, with absorption maxima (A_{max}) located at 204, 230, 253, 266, 288, and 440 nm (Supplement Fig.1.b).

Chloroplastic pigments are lipophilic compounds (Fernandez-Orozco et al., 2011) and chlorophylls as no phenolic compound (in all known forms) have two major absorption bands in the visible range, “red” and “blue” and absorption maxima (A_{max}) for Chlorophyll “a” and “b” in acetone are located at 662.10 and 645.50 nm, respectively (Zvezdanović et al., 2009). In this context, the INT and lipophilic HF fractions present the peaks at different retention times (between 88 and 92 min) with typical spectra of chlorophyll (Supplement Fig.1.c).

Polyphenols are hydrophilic (Fernandez-Orozco et al., 2011) and in order to eliminate the lipophilic compounds such as lipids, carotenoids, and chlorophyll from the plant extract, hexane was used (Dai and Mumper, 2010). In contrast, the results of colorimetric method for total phenolic and flavonoid contents were confirmed in HPLC profile of INT and HF fractions, by the presence of peaks with spectra characteristic of phenolic compounds. Indeed, substantial quantitative losses were recorded upon defatting. 60.88% of anthraquinones, 53.47% of kaempferol and 49.43% of quercetin-3-rhamnoside were lost, which represents a distribution respectively in INT and HF with 46.93 and 13.96%; 21.46 and 32.01%; 12.43 and 37.00%. For fractions derived from the aqueous solution (AQ) (C-18 Sep-Pak eluted fractions), these compounds are present respectively in EA1, EA2, and AM with 32.94, 4.43, and 1.75% for anthraquinones, 33.13, 7.62, and 5.79% for kaempferol and 50.57% only in EA1 for quercetin-3-rhamnoside. Unlike these results, the fractions derived from the aqueous solution (AQ), 100% of rutin (with traces in HF) was found in EA2; 100% of luteolin in AM; 100% of *p*-coumaric acid and ferulic acid present in EA1 and 100% of qualitative losses of gallic acid was recorded upon washing (AW).

Around 12.85% of the total area of total peaks detected by HPLC-DAD at 280 nm of identified compounds for all fractions is constituted by 48.06% of anthraquinones, 26.12% of kaempferol, 9.46% of luteolin, 7.97% of quercetin-3-rhamnoside, 5.53% of gallic acid, 1.26% of *p*-coumaric acid, 0.99% of ferulic acid, 0.34% of rutin and 0.27% of chlorophyll.

Table 3
Quantification of identified individual phenolic compounds and anthraquinones as a group of phenolic compounds at 280 nm using HPLC-DAD system.

	Fractions 280 nm	Total area mAU[a]s	Identified compounds	Time (min)	Area (mAU[a]s)	Area(%) in fraction	Quantity (μg/g PPW)
	HF	59858.500	Rutin	38.80	90.30	0.15	UQ[a]
			Antraquinones[a]	53.75 55.51 58.00	68.80	0.12	–
				58.60 65.38 66.60	557.90 104.80	0.93	
				75.88 86.72 93.33	574.90 561.90	0.18	
					879.40 12,559.90	0.96	
					3539.50 207.40	0.94	
						1.47 20.98 5.91	
						0.35	
			Quercetin-3-rhamnoside	54.41	780.00	1.30	43.35
			Kaempferol[a]	59.45	3490.20	5.83	–
	INT	103010.000	Quercetin-3-rhamnoside	54.55	3993.30	3.88	272.63
			Antraquinones[a]	58.72	3152.10	3.06	–
				75.66	6598.30	6.41	
			Kaempferol[a]	59.45	8960.60	8.70	–
Obtained fractions by C-18 Sep-Pak Vac fractionation	AW	13594.100	Gallic acid	8.66	6973.10	51.30	470.50
			p-coumaric acid	36.48	1391.80	1.82	19.79
			Ferulic acid	39.78	1097.80	1.44	28.45
			Quercetin-3-rhamnoside	54.49	4052.20	5.30	276.84
	EA1	7,650,000.000	Antraquinones[a]	55.54	3659.1	4.79	–
				58.72	9404.60	12.30	
				61.74	1510.80	1.98	
				75.85	2510.00	3.28	
			Kaempferol[a]	59.46	6883.60	9.00	
			Rutin	38.83	283.30	4.71	24.06
	AE2	6016.595	Antraquinone[a]	58.59	181.00	3.01	–
			Kaempferol[a]	59.40	124.70	2.07	–
	AM	602701.000	Antraquinone[a]	53.82	7157.00	1.19	–
			Luteolin[a]	54.29	10480.10	1.74	–

[a] UQ: detected but unquantifiable. Luteolin and Kaempferol were only identified but not quantified. Anthraquinones identified by comparing the spectra absorbance with bibliography (Marković et al., 2008).

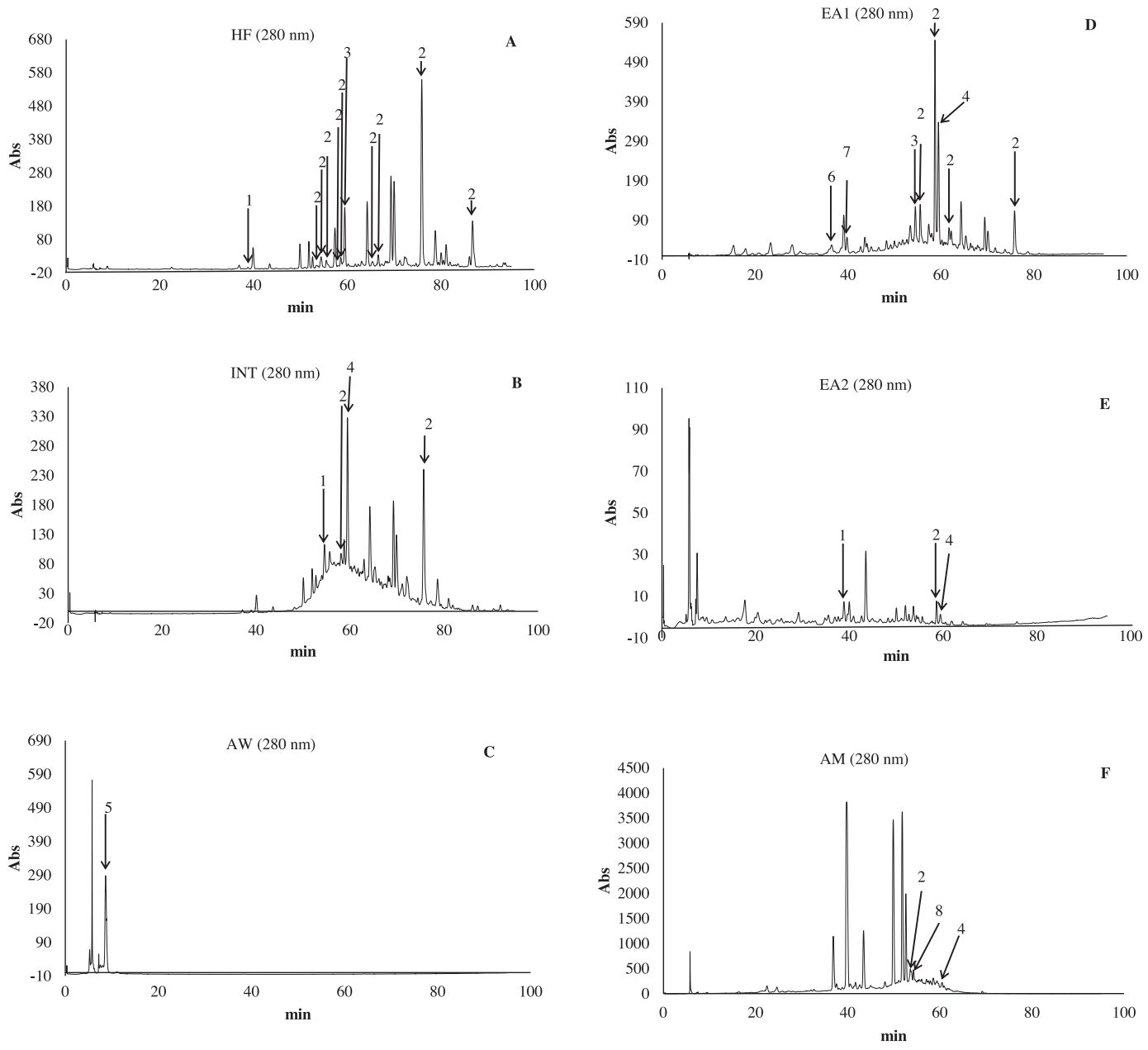

Fig. 3. Identification of selected individual phenolic compounds and anthraquinones as a group of phenolic compounds in different fractions of extract i.e. (A) HF, (B) INT, (D) EA1, (E) EA2, and (F) AM from leaves of *R. alaternus* L. The phenolic compounds were identified at 280 nm using a HPLC-DAD system. Phenolic compounds identified were Rutin (1), antraquinone (2), quercetin-3-rhamnoside (3), kaempferol (4), gallic acid (5), *p*-coumaric acid (6). ferulic acid (7), and luteolin (8). Phenolic compounds of other peaks were either unknown.

At present, the composition of phenolic compounds in *R. alaternus* L. is still limited. From the previous studies, it is known that the extracts from *R. alaternus* L. are rich in compounds as anthraquinones and flavone heterosides (Stocker et al., 2004). In addition, four anthraquinine aglycones (emodin, chrysophanol, alaternin, and physcion) were isolated (Ben Ammar et al., 2008; Izhaki et al., 2002) from the above-ground parts of the plant, with emodin being the most abundant of these (Izhaki et al., 2002). Moreover, the leaves extracts showed the presence of various quantities of anthraquinones; coumarins; tannins; and flavonoids (Ben Ammar et al., 2005), as kaempferol 3-*O*-isorhamninoside, rhamnocitrin-3-*O*-isorhamninoside, rhamnetin-3-*O*-isorhamninoside, apigenin (Ben Ammar et al., 2009), kaempferol and quercetin (Ben Ammar et al., 2008, 2009). In comparison, the presence of anthraquinones and kaempferol was confirmed; apigenin was absent; and luteolin, quercetin-3-rhamnoside, *p*-coumaric acid, ferulic acid, gallic acid, and rutin are the newly identified compounds in *R. alaternus* L. The previous studies shows, that the anthraquinone derivatives present in plants exhibit laxative effects (Kosalec et al., 2013). In fact, the use of *R. alaternus* L. in traditional medicine as laxative may be related principally to the presence of anthraquinones.

4. Conclusion

In this study, HPLC-DAD analysis of methanol extract of leaves shows the characterization of six new phenolic compounds. All fractions obtained in defatting (INT and HF) and fractionation by C-18 Sep-Pak cartridges (EA1, EA2, AW, AM) of methanol extract of *R. alaternus* L. showed the presence of phenolic compounds with

qualitative and quantitative differences. In order to eliminate the lipoidal material using hexane, considerable qualitative, and quantitative losses of phenolic compounds were observed in the INT and HF fractions. In the washing step of C-18 Sep-Pak cartridges contained aqueous solution (AQ), in order to remove sugar and organic acids; the resulting acidified water shows the elimination of phenolic compounds. For antioxidants activity, all obtained fractions exhibited free radical scavenging activity. Anthraquinones can be considered as multipotent antioxidants. In addition to their antioxidant activity, they are shown to possess other biological activities, including antibacterial, antiviral, antifungal, anticancer (Kosalec et al., 2013), and possess potent antimicrobial activity against skin infecting pathogenic organisms (Sucheta et al., 2011). *R. alaternus* L., by its richness of anthraquinones, can be a potential candidate for investigation and therapy as antimicrobial and anticancer.

Acknowledgements

We acknowledge Katherine Davis-Dentici and Mike Dougherty for their technical support in analyzing some of the phenolic compounds. The authors also thank Kaitlyn E. Feeney for her help in conducting HPLC analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2015.06.015

References

Adedapo, A.A., Jimoh, F.O., Koduru, S., Afolayan, A.J., Masika, P.J., 2008. Antibacterial and antioxidant properties of the methanol extracts of the leaves and stems of *Calpurnia aurea*. BMC Complement. Altern. Med. 8, 1–8.

Batanouny, K., abdul Rahman, F., Benhouhou, S., Chemli, R., Ghrabi, Z., Hammouda, F., Ismail, S.I., Abdel-azim, N.S., Shams, K.A., Rais, C., Lamnaouer, D., 2005. A guide to medicinal plants in north Africa. IUCN centre for Mediterranean Cooperation, Malaga (Spain).

Ben Ammar, R., Ben Sghaier, M., Boubaker, J., Bhouri, W., Naffeti, A., Skandrani, I., Bouhlel, I., Kilani, S., Ghedira, K., Chekir-Ghedira, L., 2008. Antioxidant activity and inhibition of aflatoxin B(1)-, nifuroxazide-, and sodium azide-induced mutagenicity by extracts from *Rhamnus alaternus* L. Chem. Biol. Interact. 174, 1–10.

Ben Ammar, R., Bhouri, W., Ben Sghaier, M., Boubaker, J., Skandrani, I., Neffati, A., Bouhlel, I., Kilani, S., Mariotte, A.M., Chekir-Ghedira, L., Dijoux-Franca, M.G., Ghedira, K., 2009. Antioxidant and free radical-scavenging properties of three flavonoids isolated from the leaves of *Rhamnus alaternus* L. (Rhamnaceae): a structure-activity relationship study. Food Chem. 116, 258–264.

Ben Ammar, R., Kilani, S., Abdewahed, A., Hayder, N., Mahmoud, A., Chibani, J., Chekir-Ghedira, L., Ghedira, K., 2005. In vitro mutagenicity, antimutagenicity, and free radical scavenging activities of *Rhamnus alaternus* L. (Rhamnaceae) extracts. Pak. J. Biol. Sci. 8, 439–445.

Chirinos, R., Campos, D., Costa, N., Arbizu, C., Pedreschi, R., Larondelle, Y., 2008. Phenolic profiles of andean mashua (*Tropaeolum tuberosum* Ruíz & Pavón) tubers: identification by HPLC-DAD and evaluation of their antioxidant activity. Food Chem. 106, 1285–1298.

Dai, J., Mumper, R.J., 2010. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. Molecules 15, 7313–7352.

Dudonne, S., Vitrac, X., Coutiere, P., Woillez, M., Merillon, J.-M., 2009. Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. J. Agric. Food Chem. 57, 1768–1774.

Eugène-Humbert, G., 1952. Un roi qui, selon Plutarque, fut le plus savant des èrcivains grecs. Revue d'histoire Pharm. 40, 353.

Fernandez-Orozco, R., Roca, M., Gandul-Rojas, B., Gallardo-Guerrero, L., 2011. DPPH-scavenging capacity of chloroplastic pigments and phenolic compounds of olive fruits (cv. Arbequina) during ripening. J. Food Compos. Anal. 24, 858–864.

Franco, D., Sineiro, J., Rubilar, M., Sánchez, M., Jerez, M., Pinelo, M., Costoya, N., Núñez, M.J., 2008. Polyphenols from plant materials: extraction and antioxidant power. EJEAFChe 7, 3210–3216.

Guarrera, P.M., 2006. Household dyeing plants and traditional uses in some areas of Italy. J. Ethnobiol. Ethnomed. 2, 1–7.

Gulias, J., Traveset, A., Riera, N., Mus, M., 2004. Critical stages in the recruitment process of *Rhamnus alaternus* L. Ann. Bot. (Lond.) 93, 723–731.

Hostettmann, K., Marston, A., 2002. Twenty years of research into medicinal plants: results and perspectives. Phytochem. Rev. 1, 275–285.

Hostettmann, K.M., Marston, A., Ndjoko, K., Wolfender, J.L., 2000. The potential of African plants as a source of drugs. Curr. Org. Chem. 4, 973–1010.

Ignat, I., Volf, I., Popa, V.I., 2011. A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. Food Chem. 126, 1821–1835.

Izhaki, I., Tsahar, E., Paluy, I., Friedman, J., 2002. Within population variation and interrelationships between morphology, nutritional content, and secondary compounds of *Rhamnus alaternus* fruits. New Phytol. 156, 217–223.

Javanmardi, J., Stushno, C., Lockeb, E., Vivanco, J.M., 2003. Antioxidant activity and total phenolic content of Iranian Ocimum accessions. Food Chem. 83, 547–550.

Kosalec, I., Kremer, D., Locatelli, M., Epifano, F., Genovese, S., Carlucci, G., Randic, M., Koncic, M.Z., 2013. Anthraquinone profile, antioxidant, and antimicrobial activity of bark extracts of *Rhamnus alaternus*, R., fallax, R. intermedia and R. pumila. Food Chem. 136, 335–341.

Lacombe, A., Wu, V.C.H., White, J., Tadepalli, S., Andre, E.E., 2012. The antimicrobial properties of the lowbush blueberry (*Vaccinium angustifolium*) fractional components against foodborne pathogens and the conservation of probiotic *Lactobacillus rhamnosus*. Food Microbiol. 30, 124–131.

Lardos, A., 2006. The botanical materia medica of the Iatrosophikon—a collection of prescriptions from a monastery in Cyprus. J. Ethnopharmacol. 104, 387–406.

Lee, J., 2005. Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study. J. AOAC Int. 88, 1269–1278.

Lim, Y.Y., Quah, E.P.L., 2007. Antioxidant properties of different cultivars of Portulaca oleracea. Food Chem. 103, 734–740.

Maestri, D.M., Nepote, V., Lamarque, A.L., Zygadlo, J.A., 2006. Natural products as antioxidants. In: Imperato, F. (Ed.), Phytochemistry: Advances in Research. Research Signpot, Trivandrum, India, pp. 105–135.

Marković, Z., Manojlović, N., Zlatanović, S., 2008. Electronic absorption spectra of substituted anthraquinones and their simulation using ZINDO/S method. J. Serb. Soc. Comput. Mech. 2, 73–79.

Mati, E., de Boer, H., 2011. Ethnobotany and trade of medicinal plants in the Qaysari Market, Kurdish Autonomous Region, Iraq. J. Ethnopharmacol. 133, 490–510.

Moure, A., Franco, D., Sineiro, J., Domıínguez, H., Núñez, M.a.J., Lema, J.M., 2001. Antioxidant activity of extracts from *Gevuina avellana* and *Rosa rubiginosa* defatted seeds. Food Res. Int. 34, 103–109.

Nayak, B., Berrios, J.D.J., Powers, J.R., Tang, J., 2011. Thermal degradation of anthocyanins from purple potato (Cv. Purple Majesty) and impact on antioxidant capacity. J. Agric. Food Chem. 59, 11040–11049.

Nayak, B., Liu, R.H., Tang, J., 2013. Effect of processing on phenolic antioxidants of fruits, vegetables and grains a review. Crit. Rev. Food Sci. Nutr., http://dx.doi.org/10.1080/10408398.2011.654142

Rawlinson, G., Blakeney, E.H., 1910. The History of Herodotus. J. M. Dent & Sons Ltd, London.

Schaich, K.M., Tian, X., Xie, J., 2015. Hurdles and pitfalls in measuring antioxidant efficacy: a critical evaluation of ABTS, DPPH, and ORAC assays. J. Funct. Foods 14, 111–125.

Slikkerveer, L., 2006. The challenge of non-experimental validation of mac plants, towards a multivariate model of transcultural utilization of medicinal, aromatic and cosmetic plants. In: Bogers, R.J., Craker, L.E., Lange, D. (Eds.), Medicinal and Aromatic Plants: Agricultural, Commercial, Ecological, Legal, Pharmacological, and Social Aspects. Springer, pp. 1–28.

Spigno, G., Tramelli, L., De Faveri, D.M., 2007. Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics. J. Food Eng. 81, 200–208.

Stocker, P., Yousfi, M., Djerridane, O., Perrier, J., Amziani, R., El Boustani, S., Moulin, A., 2004. Effect of flavonoids from various Mediterranean plants on enzymatic activity of intestinal carboxylesterase. Biochimie 86, 919–925.

Sucheta, G.A., Asha, K.A., Tushar, G.V., Nirmala, D.R., Jyoti, S.P., 2011. Standardization of emodin-an bioactive molecule, using spectral methods. Int. J. Drug Dev. Res. 3, 259–265.

Vasisht, K., Kumar, V., 2004. Compendium of Medicinal and Aromatic Plants AFRICA. United Nations Industrial Development Organization and the International Centre for Science and High Technology.

Velioglu, Y., Mazza, G., Gao, L., Oomah, B., 1998. Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. J. Agric. Food Chem. 46, 4113–4117.

Vermerris, W., Nicholson, R.L., 2007. Isolation and identification of phenolic compounds. In: Phenolic Compound Biochemistry. Springer, pp. 151–190.

Zeng, Y., Guo, L.-P., Chen, B.-D., Hao, Z.-P., Wang, J.-Y., Huang, L.-Q., Yang, G., Cui, X.-M., Yang, L., Wu, Z.-X., Chen, M.-L., Zhang, Y., 2013. Arbuscular mycorrhizal symbiosis and active ingredients of medicinal plants: current research status and prospectives. Mycorrhiza 23, 253–265.

Zvezdanović, J., Cvetić, T., Veljović-Jovanović, S., Marković, D., 2009. Chlorophyll bleaching by UV-irradiation in vitro and in situ: absorption and fluorescence studies. Radiat. Phys. Chem. 78, 25–32.