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Phytochemical Study and Antioxidant Activities of Leaves Extracts from *Rhamnus alaternus* L.

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ABSTRACT: This study was designed to examine the chemical composition and in vitro antioxidant activities of leaves extracts from *Rhamnus alaternus* L.; we submitted two extracts (methanolic and aqueous) of different polarity to a deep compositional analysis through the use of an advanced hyphenated technique like LC/Uv-vis-DAD/MS, to our knowledge no metabolic fingerprint studies have been done on this species so far. So, we report for the fist time the complete secondary metabolic fingerprint of *R. alaternus* polar extract, the chromatographic pattern from aqueous extract has several similarities with the methanolic one, two triglycoside flavonoids were identified in both extracts (kaempferol 3-*O*-[α-L-rhamnopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside, rhamnetin 3-*O*-[α-L-rhamnopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside), we note the presence of quercetin, kaempferol and rhamnetin derivatives. The samples were also subjected to a screening for their possible antioxidant activities by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and β-carotene-linoleic acid assays, in the first case the IC_{50} value was of 0.082 ± 0.0006 mg/ml for the methanolic extract and 0.398 ± 0.007 mg/ml for the aqueous one, in the β-carotene-linoleic acid system, the inhibition values of linoleic acid oxidation were estimated as 89.007 ± 1.914% and 59.639 ± 3.824% for the methanolic and aqueous extract respectively, in both tests the methanolic extract was the most active. On the other hand, total phenolics determination in the test solutions was carried out according to the spectrophotometric Prussian blue assay and the determination of flavonoids was performed using the method of aluminum trichloride, ($AlCl_3$) all the results indicated that the methanolic extract has higher total phenolics and flavonoids being of: 33.655 ± 2.503 mg GAE/g for the first method and 61.127 ± 1.217mg EQ/g; 129.681 ± 1.546 mg ER/g, for the second one. According to all these results, we conclude that there is a clear relationship between chemical composition of *R. alaternus* and its antioxidant activities.

KEYWORDS: β-carotene, Chemical structure, DPPH, Flavonoids

INTRODUCTION

Formation of free radicals may play an important role in the origin of life and biological evolution, implying their beneficial effects on organisms.[1] Radicals are known to take part in lipid peroxidation, which causes food deterioration, aging of organisms, and cancer promotion.[2] However, free radicals and other relative species can also cause the oxidation of biomolecules (e.g., protein, aminoacids, lipid, and DNA) which leads to cell injury and death.[3] Scientists, in many different disciplines, have become more interested in natural sources which could provide active components to prevent or reduce the impact of oxidative stress on cells.[4–5] Many plants contain natural antioxidants that act in metabolic response to the endogenous production of free radicals and other oxidant species.[6]

The selected plant in this study, *Rhamnus alaternus* (Rhamnaceae) is a perennial dioecious shrub distributed

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throughout the Mediterranean Basin, which flowers during late winter and early spring, with a peak in mid-February. It produces fleshy fruits that ripen in late spring and early summer, and thus represents an important water and nutrient source for birds and small mammals.[7] This medicinal plant has been used in North Africa as a laxative, purgative, diuretic, antihypertensive and depurative.[8] In Algeria this species is generally used for the treatment of hepatic and dermatological complications. Previous studies have shown potent antioxidant, free radical scavenging, antimutagenic and antigenotoxic activities of crude extracts from *R. alaternus*,[8–12] and others biological activities of *R. alaternus* extracts: antibacterial and antiproliferative was reported, in human cells, extracts of *R. alaternus* leaves modulate the expression levels of genes implicated in both DNA repair and oxidative defense systems.[8–13] The genus *Rhamnus* is characterized from a phytochemical point of view by the abundance of phenolic substances, especially flavonoids, anthraquinones and tannins,[14–10] which are described by numerous authors as antioxidant molecules.[15–16] These compounds, which are widely distributed across the plant kingdom, represent the most abundant antioxidants in the diet and they have gained tremendous interest as potential therapeutic agents against a wide variety of diseases, most of which involve oxidant damage.[17] On the other hand, numerous studies have shown structure–activity relationships governing antioxidant capacities of flavonoids.[18–20]

The aims of this work are to evaluate the chemical composition of the methanolic and aqueous extracts from the leaves of *Rhamnus alaternus* L. by using LC/Uv-vis-DAD/MS analyses, to identify the amount and the chemical structure of the compounds present in the extracts, to determine the amount of total phenolics and flavonoids according to Prussian blue assay and aluminum trichloride method respectively and to evaluated the *in vitro* antioxidant activities of the extracts by measuring their ability to scavenge the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and β-carotene-linoleic acid systems, showing that the activities have some relationships with the amount and the chemical structure of the phenolics present in the extracts.

MATERIALS AND METHODS

Plant material

The leaves of *Rhamnus alaternus* was collected in August 2009 from Teniet En Nasr in Bordj–Bou-Arriridj city located in the east of Algeria. Identification of the species was based on the work of Quezel and Santa.[21] and validated by botanists in the Department of Botany and Ecology at the University of Setif. After collection, the leaves were separated from the rest of the aerials, dried for 4 days in the shade (25–28°C), and then finely ground to obtain a brownish homogeneous powder, which was kept in a dry place in the dark until use.

Preparation of the extracts

100 g of the powder was macerated in 1 liter of methanol to obtain the methanolic extract and another 100 g of the same powder was macerated is 1 liter of distillated water to obtain the aqueous extract, the resulting heterogeneous mixtures were kept stirring under reflux, then filtered. We add hexane (v/v) to the methanolic extract to separate two fractions (organic and methanolic phases) in our experiment we used the methanolic phase. All the solvents were recovered from the filtrates by evaporation under vacuum. The yields of this extraction are: 14.48%, 14.20% for the methanolic and aqueous extract respectively.

LC/Uv-vis-DAD/MS analyses of *Rhamnus alaternus* extracts

We submitted the two *R. alaternus* extracts (methanolic and aqueous) of different polarity to a deep compositional analysis through the use of an advanced hyphenated technique like LC/Uv-vis-DAD/MS. The combined use of DAD and MS detectors allowed us to obtain two independent sets of analytical data, namely, Uv-vis spectra and mass spectra. Particularly mass spectra are of pivotal importance, as they give us information about the chemical structure of the entire molecule, not only a part if it (as in energy-matter related techniques such as Uv-Vis spectroscopy or NMR). Considering also the interactions taking place in the chromatographic column between the stationary phase and the analytes, we obtain three independent set of analytical data. The study of these data allowed us to determine and identify the majority of the chromatographic signals (peaks) in the chromatograms.

Antioxidant activity

DPPH essay

The DPPH assay was carried out following the same method reported by Güllüce et al.[22] fifty microliters of various concentrations of *Rhamnus alaternus* extracts was added to 5 ml of a 0.004% methanol solution of DPPH (Sigma). After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition percent (I%) of DPPH. was calculated as follows:

$$I\% = (A_{blank} - A_{sample}/A_{blank}) \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. Inhibition is concentration dependent, and extract concentration providing 50% inhibition (IC_{50}) is calculated from the graphplotted inhibition percentage against extract concentration. The assay was carried out in triplicate.

β-carotene-linoleic acid assay

In this assay antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation, causing discoloration of β carotene.[23]

The antioxidant activity was evaluated according to the method described by Tepe et al.[24] A stock solution of β-carotene-linoleic acid mixture was prepared as follows: 0.5 mg β-carotene (Sigma) was dissolved in 1 ml of chloroform, 25 µl linoleic acid and 200 mg Tween 40 (Sigma) were added. Chloroform was completely evaporated using a vacuum evaporator, and then 100 ml of distilled water saturated with oxygen (30 min, 100 ml/min) was added with a vigorous shaking. Two thousand five hundred microliters of this reaction mixture was distributed to test tubes and 350 µl portions of the extract, prepared at 2 g/l concentrations were added and the emulsion system was incubated for up to 48 h at room temperature. The same procedure was repeated with the synthetic antioxidant, BHT (Sigma) as a positive control, and a blank. After this incubation period, absorbances of the mixtures were measured at 490 nm. Antioxidant capacities of the extracts were compared with those of BHT and blank.

Determination of the total phenolic contents

Total phenolics determination in the test solutions was carried out according to the spectrophotometric Prussian blue assay sited elsewhere with some modification.[25–27] Following this method 0.1 ml of each extract was added to 3 ml of distilled water, to this solution 1 ml of $K_3Fe(CN)_6$ (0.016M) dissolved in distilled water and 1 ml of $FeCl_3$ (0.02M) dissolved in HCL (0.1 N) were added. The mixture was incubated for 15 minutes at room temperature, and then 5 ml of stabilizing solution (comprising 30 ml of gum arabic "1%", 30 ml of phosphoric acid "85%" and 90 ml of distilled water) was added. The absorbance was measured at 700 nm and the amount of total phenols was calculated as mg/g gallic acid equivalents (GAE) from calibration curve of gallic acid standard solution. For the gallic acid, the curve absorbance (y) versus concentration (x) is described by the equation; $y=0.0023x + 0.0247$ ($R^2 = 0.9983$).

Assay for total flavonoids

The determination of flavonoids was performed using the method of aluminum trichloride, ($AlCl_3$) given elsewhere with slight modification.[28–29] To 1 ml of each extract 1 ml of $AlCl_3$ solution (2%) was added, then the absorbance readings at 430 nm were taken after 10 minutes of incubation at room temperature. The total flavonoids content was determined as milligrams of quercetin or rutin equivalent per gram of extract using a standard curve of quercetin and rutin at 0–40 µg/m.

Statistical analysis

All the samples were analyzed in triplicate and data were expressed as means ± SD using Graphpad. Analysis of variance was determined by one-way ANOVA. The Tukey test was used to determine the significance of differences between the mean values of the treatment groups at the level of $p < 0.1$.

RESULTS

LC/Uv-vis-DAD/MS analyses

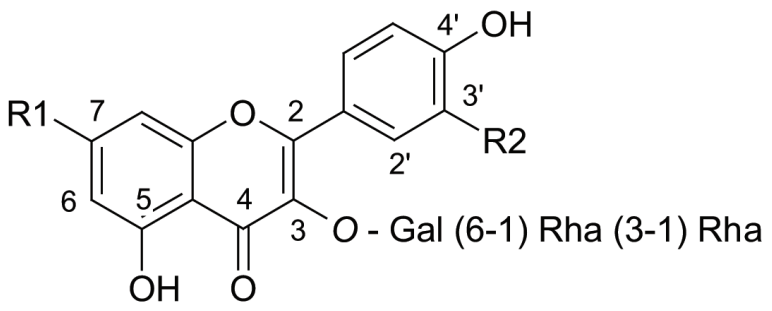
Recent papers reported the presence of highly hydroxylated flavonoids (kaempferol, quercetin and isorhamnetin) in the polar extracts from Rhamnus leaves,[30] to our knowledge no metabolic fingerprint studies have been done on this species so far. So, we here report for the fist time the complete secondary metabolic fingerprint of *Rhamnus alaternus* polar extract (Figure 1).

The chromatographic pattern from aqueous extract in Figure 3 has several similarities with the previous one (Figure 2), including the presence of peaks 2 and 4 and here numbered as 2 and 7 identified according to the literature.[30] Flavones (quercetin, kaempferol and rhamnetin derivatives) kept dominating this extract, which is also characterized by the presence of their corresponding aglycones (peak 10, 11 and 12, respectively).

Antioxidant activity

The DPPH scavenging activity is usually presented by IC_{50} value, defined as the concentration of the antioxidant needed to scavenge 50% of DPPH present in the test solution, it should be noted that lower IC_{50} value reflects better DPPH radical-scavenging activity.[31] As can be seen from the Table 1, Antioxidant activity of the methanolic extract was superior to that of the aqueous extract with an IC_{50} value of 0.082 ± 0.0006 mg/ml. On the other hand, none of the samples showed activity as strong as the positive control BHT (0.032 ± 0.018 mg/ml).

In β-carotene/linoleic acid model system, the relative antioxidative activities (RAAs) of the extracts were



Peak number	R1	R2
1	OH	OH
2	OH	H
4 (7)*	OCH_3	OCH_3
5	OCH_3	H

*aqueous extract

Figure 1: Chemical structures of compounds discussed in the text. Peak numbers refer to Figure 2 and 3. Among the *ca* 15 peaks (including minor components) appearing in the chromatogram of *R. alaternus* methanolic extract in Figure 2, 8 peaks were tentatively identified by means of their Uv-vis and mass spectra. Speculations have also been done on the basis of their relative retention times. These data allowed us to identify peaks 2, 4 and 5 as kaempferol 3-*O*-[α-L-rhamnopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside, rhamnetin 3-*O*-[α-L-rhamnopyranosyl(1→3)-*O*- α -L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside that represent the main peak (29,63%) and rhamnocitrin 3-*O*-[α-L-rhamnopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside, respectively in agreement with the work of Ben Ammar et al.,[30] we also identify peak 1 as quercetin 3-*O*-[α-L-rhamnopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside specially on the basis of its mass spectrum and diagnostic Uv-Vis spectrum. All the other peaks appear to be kaempferol, quercetin and rhamnetin sugar derivatives (Figure 2). The most recurrent sugar is definitely rhamnose. Interestingly, no organic nor cinnamic acids were detected in both extracts.

Table I: Antioxidant activity of the aqueous and methanolic extract of *Rhamnus alaternus*[a]

Samples	DPPH[b]	β-carotene/linoleic acid[c]
Aqueous extract	0.398±0.007***	59.639±3.824***
Methanolic extract	0.082±0.0006***	89.007±1.914***
BHT (Positive control)	0.032±0.018	99.256±0.663

The data are expressed as means ± SD, (n=3);
[a]Results are means of two different experiments.
[b]IC_{50} values of DPPH assay (mg/ml).
[c]Given as percentage "%" of inhibition of the linoleic acid.

calculated from the equation; $RAA = A_{sample}/A_{BHT}$, where A_{BHT} is the absorbance of the control (BHT) and A_{sample} is the absorbance of the extracts. The inhibition values of linoleic acid oxidation were estimated as 89.007 ± 1.914% and 59.639 ± 3.824% in the presence of the methanolic and aqueous extract respectively. The results are resumed in Table 1, as we see also in this essay the methanolic extract exhibited the best results.

Table 2: Amount of total phenolics and flavonoids in the extracts of *Rhamnus alaternus*[a]

Samples	Phenolics content (mg GAE /g extract)[b]	Teneur en flavonoids content	
		Quercetine (mg EQ/g)[c]	Rutin (mg ER/g)[d]
Methanolic extract	33.655 ± 2.503	61.127 ± 1.217	129.681 ± 1.546
Aqueous extract	8.549 ± 0.553	1.950 ± 0.063	1.710 ± 0.013

The data are expressed as means ± SD, (n=3);
[a]Results are means of two different experiments.
[b]GAE Gallic acid equivalents.
[c]EQ quercetin equivalents.
[d]ER rutin equivalents.

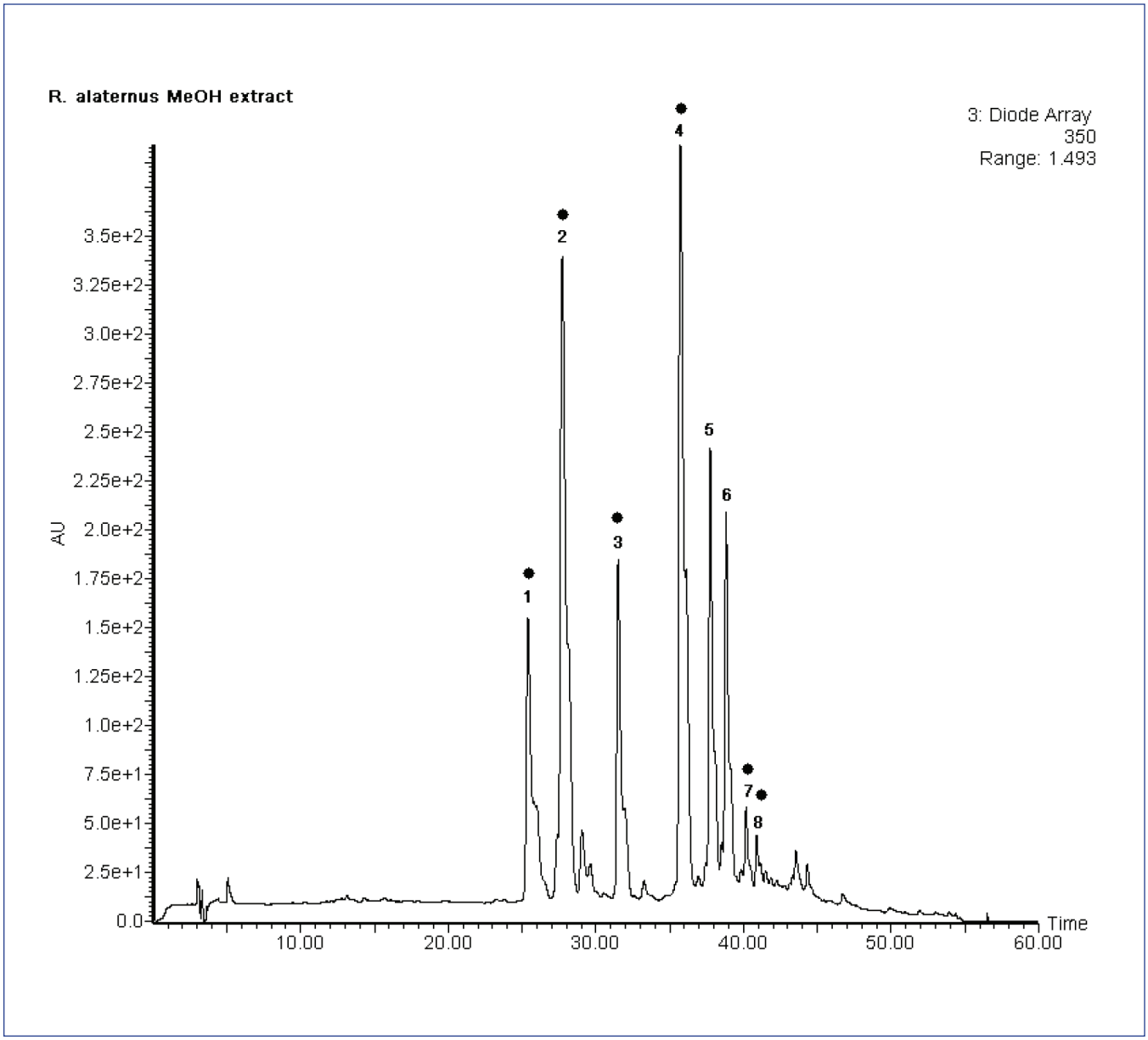


Figure 2: *Rhamnus alaternus* methanolic extract (visualised at 350 nm).Tentative identification of peaks present on the chromatogram (peaks found also in the aqueous extract highlighted): **1.** quercetin 3-*O*-[α-L-rhamnopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside (11.04%); **2.** kaempferol 3-*O*-[α-L-rhamnopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside (27.60%); **3.** kaempferol derivative (9.67%); **4.** rhamnetin 3-*O*-[α-L-rhamnopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside (29.63%, main peak); **5.** rhamnocitrin 3-*O*-[α-L-rhamnopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside (10.29%);[30] **6.** quercetin derivative (9.38%); **7.** kaempferol derivative (1.32%); **8.** rhamnetin derivative (1.06%).

Assays for total phenolics and flavonoids

The amounts of total phenolics in the extracts were determined spectrometrically according to Prussian blue assay[25–27] and calculated as gallic acid equivalents; gallic acid is a water soluble polyhydroxyphenolic compound which can be found in various natural plants.[32] Results of the analysis of total phenolics and flavonoids are given in Table 2. As can be seen from the table the total phenolics contents of the methanolic and aqueous extracts of *Rhamnus alaternus* are: 33.655 ± 2.503 mg/g and 8.549 ± 0.553 mg/g, respectively. The results indicated that the methanolic extract has higher total phenolics and flavonoids content than the aqueous extract with the two standards quercetine and rutin.

DISCUSSION

The technique LC/Uv-vis-DAD/MS analyses allow us to obtain the complete secondary metabolic fingerprint of *R. alaternus* polar extract, the chemical structures

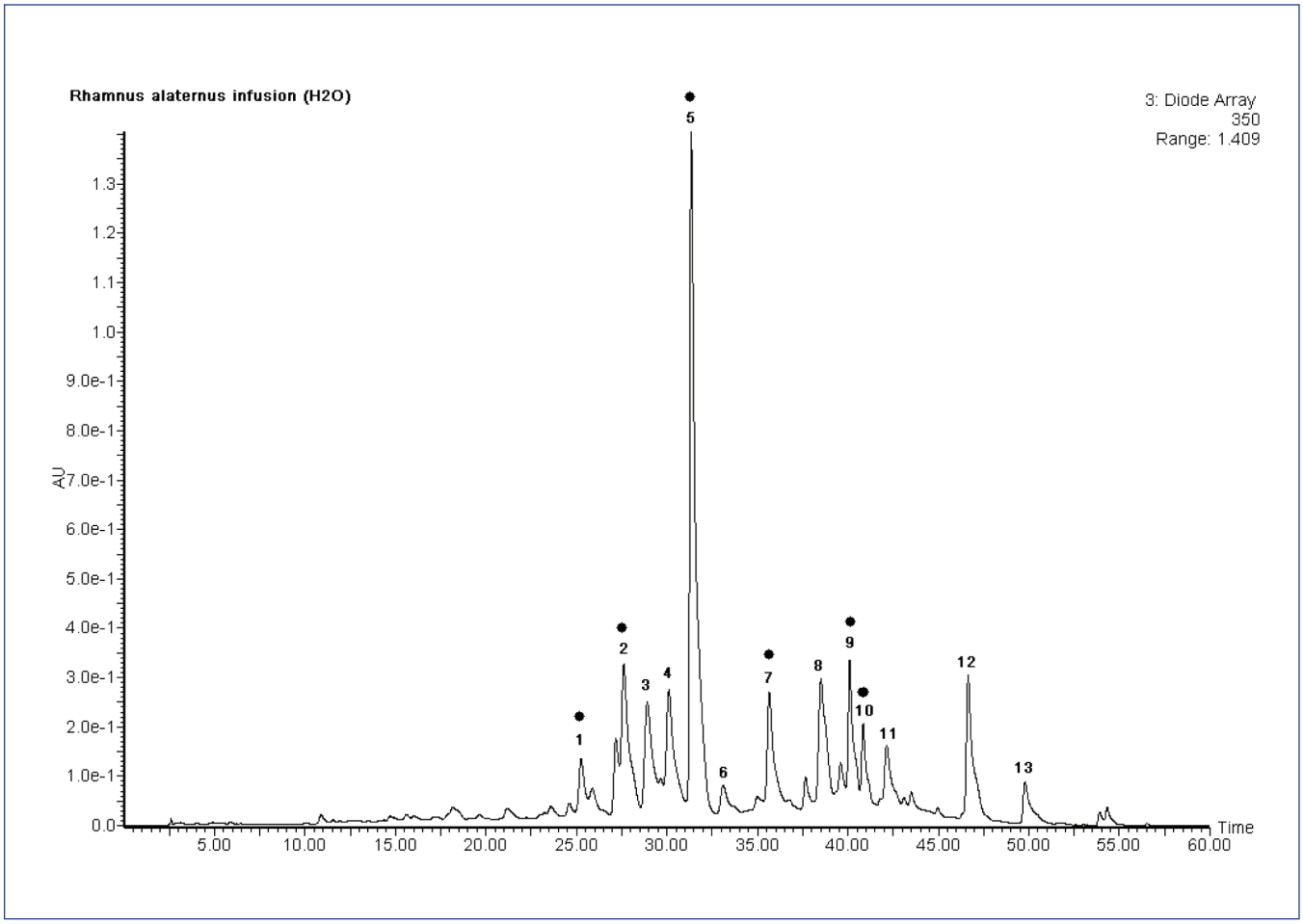


Figure 3: *Rhamnus alaternus* aqueous extract (visualised at 350 nm). Tentative identification of peaks present on the chromatogram: **1.** quercetin 3-*O*-[α-L-rhamnopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside (2.74%); **2.** kaempferol 3-*O*-[α-L-rhamnopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside (13.38%); **3.** quercetin derivative (5.43%); **4.** kaempferol derivative (5,10%); **5.** kaempferol derivative (31,41%, main peak); **6.** rhamnocitrin derivative (1.83%); **7.** rhamnetin 3-*O*-[α-L-rhamnopyranosyl (1→3)-*O*-α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside (9.78%); **8.** rhamnetin derivative (7.21%); **9.** kaempferol derivative (4.84%); **10.** rhamnetin derivative (2.28%); **11.** quercetin (3.27%); **12.** kaempferol (9.55%); **13.** rhamnetin (3.19%).

of the three glycoside flavonoids identified as: kaempferol 3-*O*-[α-L-rhamnopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside, rhamnocitrin 3-*O*-[α-L-rhamnopyranosyl(1→3)-*O*-α-L rhamnopyranosyl (1→6)]-β-D-galactopyranoside and rhamnetin 3-*O*-[α-L-rhamnopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl (1→6)]-β-D-galactopyranoside, were in agreement with the work of Ben Ammar, et al.[30] These compounds were also previously isolated from other species of the genus *Rhamnus*.[33–37] Our results confirm their presence in the methanoilc extract and only two of them are present in the aqueous extract, this second extract was mainly constituted of rhamnetin, kaempferol, quercetin, recent papers reported the presence of such compounds in *Rhamnus* leaves.[30]

Antioxidant activity is a complex process that can occur through several mechanisms, due to its complexity more than one test must be carried out when evaluating the antioxidant activity of pure compounds or extracts.[38] In this work, two classical antioxidant tests were carried out, the DPPH and β-carotene/linoleic acid tests. The first gives information about the ability of the tested compounds to scavenge free radicals and the second about the ability of tested extracts to delay lipid peroxidation by reacting with chain-propagating peroxyl radicals faster than these radicals can react with proteins or fatty acid side-chains.[39]

In DPPH essays; when a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form 1,1-diphenyl-2-picryl hydrazine (non radical) with the loss of this violet color.[40] The antioxidants reduce the DPPH radical to a yellow-colored compound, diphenylpicrylhydrazine, and the extent of the reaction will depend on the

hydrogen-donating ability of the antioxidants.[41] In this assay, the two extracts reduced the stable radical DPPH to the yellow-colored diphenylpicrylhydrazine, it was fond that the methanolic extract was the most potent DPPH radical-scavenging activity.

In the evaluation of the antioxidant activities by the spectrophotometric β-carotene bleaching test the β-carotene undergoes rapid discoloration in the absence of an antioxidant. β-Carotene bleaching method is based on the loss of the yellow color of β-carotene due to its reaction with radicals formed by linoleic acid oxidation in an emulsion. The rate of β-carotene bleaching can be slowed down in the presence of antioxidants.[42] From our results, it also appeared that the methanolic extract present the best results in this essay, a relationship between the DPPH scavenging ability and β-carotene bleaching extent was found, it may concern the chemical components of the extracts that reflect the activities. Many natural molecules, especially those produced in the plant kingdom, have at least one benzene ring with a hydroxyl functional group in their skeleton, such compounds are collectively known as phenolic compounds and due to their hydrogen or single electron donating potentials, usually play important rules in the antioxidant activity of the plant extracts.[43] In fact our results in essays of total phenolics and flavonoids content confirm the presence of natural antioxidant phenolic compounds in the methanolic and aqueous extracts and demonstrate that the methanolic extract has also higher total phenolic and flavonoids compounds than the aqueous extract, according to these results, it is clear that there is a relationship between phenolic contents and antioxidant activities, significantly high total polyphenols and flavonoids content of the *R. alaternus* methanolic extract may be corroborated with the observed antioxidant activities. Such compounds were previously described to possess antioxidant activities;[44–45] also previous studies on other extracts from *R. alaternus* evaluated antioxidant activity in other models and showed good properties.[8–11]

The differences between the extracts in the results are probably due to the differences in their chemical composition. The mechanism of reaction between antioxidant and DPPH depends on the structural conformation of the antioxidant. Some compounds react very quickly with DPPH, reducing a number of DPPH molecules equal to the number of the hydroxyl groups.[41] Ben Ammar, et al.[30] found that the antioxidant activity of the 3 flavonoids isolated from leaves of *Rhamnus alaternus* showed different potency in DPPH radical-scavenging essay, they revealed that the radical scavenging activities of the tested flavonoids were correlated with the number and position of phenolic hydroxyl groups[30] their results tended to follow the results of Bors et al. and Cai et al.[18,19] revealing that the required structural criteria for high radical-scavenging and antioxidant activities of flavonoids include the ortho-dihydroxyl moiety (catechol substructure) in the B-ring or the A-ring, the 3-hydroxyl group in the C-ring, and the 2,3-double bond in conjugation with 4-oxo function (carbonyl group) in the C-ring; and finally the additional presence of both 3-, 5- and 7-hydroxyl groups; Ben Ammar, et al.[30] showed that the compound: rhamnetin 3-*O*-[α-L-rhamnopyranosyl(1→3)-*O*-α-L-rhamnopyranosyl(1→6)]-β-D-galactopyranoside presents the best potent activity in DPPH essays; this compound not only possesses the 2,3-double bond in conjugation with 4-oxo function in the C-ring, but also possesses 3,4-dihydroxy groups in the B-ring and another free 5-OH in the A-ring, which are amongst the essential structural elements for potent radical-scavenging activities of the flavonoids.[30] If we relate their results to our results we can say that since this same compound present the main peak in our methanolic extract but not the main in the aqueous one, so we can attribute the high activity of methanolic extract to the large presence of this flavonoid.

CONCLUSIONS

When Comparing all the results obtained in this work we can conclude that the antioxidant activities of the tested extracts could be explained by the large presence of phenolic compounds as it was enriched in this compounds, the flavonoids were the major components of the methanolic extract witch was the best potent in all the results, the activities obtained with this extracts, can be obtained by isolation of this compounds therefore testing of their anti radical properties is of interest, primarily in order to find new promising sources for natural antioxidants; the isolation and preparation of bioactive compounds from *Rhamnus alaternus* leaves could be used to produce potent natural antioxidants or functional ingredients with commercial applications.

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REFERENCES

1 McCord JM. The evolution of free radicals and oxidative stress. Am J Med. 2000; 108:652–659.

2 Ashok BT, Ali R. The aging paradox: free radical theory of aging. Exp Gerontol. 1999; 34:293–303.

3 Ignarro LJ, Cirino G, Casini A, Napoli C. Nitric oxide as a signaling molecule in the vascular system: an overview. J Card Pharm. 1999; 34: 879–886.

4 Ulubelen A, Topcu G, Tan N, Olcal S, Johansson C, Ucer M. Biological activities of Turkish medicinal plant, Prangos platychlaena. J Ethnopharm. 1995; 45:193–197.

5 Yan X, Murphy BT, Hammond GB, Vinson JA, Neto CC. Antioxidant activities and antitumor screening of extracts from cranberry fruit (*Vaccinium macrocarpon*). J Agric Food Chem. 2002; 50:5844–5849.

6 Mata AT, Proenc C, Ferreira AR, Serralheiro mIM, Nogueira JMF, Araújo MEM, Antioxidant and antiacetylcholinesterase activities of five plants used as Portuguese food spices. Food Chem. 2007; 103:778–786.

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

8 Ben Ammar R, Bouhlel I, Valenti K, Ben Sghaier M, Kilani S, Mariotte AM, et al. Transcriptional response of genes involved in cell defense system in human cells stressed by H_2O_2 and pre-treated with (Tunisian) *Rhamnus alaternus* extracts: Combination with polyphenolic compounds and classic *in vitro* assays. Chem-Biol Interac. 2007; 168: 171–183.

9 Chevolleau S, Debal A, Ucciani E. Détermination de l'activité antioxydante d'extraits végétaux. Rev Fr Corps Gras. 1992; 39:3–8.

10 Ben Ammar R, Kilani S, Abdelwahed A, Hayder N, Mahmoud A, Chibani J, et al. In vitro mutagenicity, antimutagenicity and free radical scavenging activities of *Rhamnus alaternus* L. (Rhamnaceae) extracts. Pak J Biol Sci. 2005;8(3):439–445.

11 Ben Ammar R, Kilani S, Bouhlel I, Ezzi L, Skandrani I, Boubaker J, et al. Antiproliferative, antioxidant and antimutagenic activities of flavonoids enriched extracts from (Tunisian) *Rhamnus alaternus* L. combination with the phytochemical composition. Drug Chem. Toxicol, 2008; 31: 61–80.

12 Ben Ammar R, Ben Sghaier M, Boubaker J, Bhouri W, Naffeti A, Skandrania I, et al. Antioxidant activity and inhibition of aflatoxin B1-, nifuroxazide-, and sodium azide-induced mutagenicity by extracts from *Rhamnus alaternus* L. Chem-Bio Interac. 2008; 174:1–10.

13 Ben Ammar R, Kilani S, Bouhlel I, Skandrani I, Naffeti A, Boubaker J, et al. Antibacterial and cytotoxic activities of extracts from (Tunisian) *Rhamnus alaternus* (Rhamnaceae). Ann Microbiol. 2007; 57:453–460.

14 Coskun M. HPLC Analysis of the anthroquinones from *Rhamnus* species growing in Turkey. Int J Pharmacog. 1992; 30:151–156.

15 Yokozozawa T, Chen CP, Dong E, Tanaka T, Nishioka GI. Study on the inhibitory effect of tannins and flavonoids against DPPH radical. Biochem Pharmacol. 1998; 56:213–222.

16 Park KY, Jung GO, Lee KT, Choi J, Choi MY, Kim GT, et al. Antimutagenic activity of flavonoids from the heartwood of *Rhus verniciflua*. J Ethnopharmacol. 2004; 90:73–79.

17 Ross JA, Kasum CM. Dietary flavonoids: Bioavailability, metabolic effects and safety. Annu Rev Nutr. 2002; 22:19–34.

18 Bors W, Michel C, Stettmaier K. Structure–activity relationships governing antioxidant capacities of plant polyphenols. Method Enzimol. 2001; 335:166–180.

19 Cai YZ, Sun M, Xing J, Luo Q, Corke H. Structure–radical scavenging activity relationships of phenolic compounds from traditional Chinese medicinal plants. Life Sci., 2006; 78:2872–2888.

20 Cotelle N, Bernier J, Catteau J, Pommery J, Wallet J, Gaydou EM. Antioxidant properties of hydroxy-flavones. Free Radical Bio Med. 1996; 20:35–43.

21 Quezel P, Santa S. Nouvelle flore de l'Algérie et des régions désertiques méridionales. Paris: Editions du Centre National de la Recherche Scientifique, p. 475–476; 1962–1963.

22 Güllüce M, Somken M, Daferera D, Agar G, Ozkan H, Kartal N, et al. *In vitro* antibacterial, antifungal, and antioxidant activities of the essential oil and methanol extracts of herbal parts and callus cultures of *Satureja hortensis* L. J Agric Food Chem. 2003; 51:3958–3965.

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

24 Tepe B, Daferera D, Tepe AS, Polissiou M, Sokmen A. Antioxidant activity of the essential oil and various extracts of *Nepeta flavida* Hub-Mor. from Turkey. Food Chem. 2007;103:1358–1364.

25 Price MP, Butler LG. Rapid visual estimation and spectrophotometric determination of tannin content of *sorghum* grain. J Agric Food Chem. 1977; 25:1268–1273.

26 Graham HD. Modified Prussian Blue assay for total phenols. J Agric Food Chem. 1992; 40:801–805.

27 Nakiboglu M, Urek RO, Kayali HA, Tarhan L. Antioxidant capacities of endemic *Sideritis sipylea* and *Origanum sipyleum* from Turkey. Food Chem. 2007; 104:630–635.

28 Sarikurkcu C, Tepe B, Yamac M. Evaluation of the antioxidant activity of four edible mushrooms from the Central Anatolia, Eskisehir – Turkey: *Lactarius deterrimus, Suillus collitinus, Boletus edulis, Xerocomus chrysenteron*. Bioresource Technol. 2008; 99:6651–6655.

29 Bahorum T, Gressier B, Trotin F, Brunete C, Dine T, Vasseur J, et al. Oxygen species scavenging activity of phenolic extracts from hawthorn fresh plant organs and pharmaceutical preparation. Arzneimittel-Forsch. 1996; 46:1086–1089.

30 Ben Ammar R, Bhouri W, Ben Sghaier M, Boubaker J, Skandrani I, Neffati A, et al. Antioxidant and free-radical-scavening properties of three flavonoids isolated from the leaves of *Rhamnus alaternus* L. (Rhamnaceae): a structure-activity relationship study. Food Chem. 2009; 116:258–264.

31 Gholivand MB, Rahimi-Nasrabadi M, Hossein Batooli H, Ebrahimabadi AH. Chemical composition and antioxidant activities of the essential oil and methanol extracts of *Psammogeton canescens*. Food Chem Toxicol. 2010; 48:24–28.

32 Sun J, Chu YF, Wu X, Liu RH. Antioxidant and antiproliferative activities of common fruits. J Agric Food Chem. 2002; 50:449–454.

33 Satake T, Hori K, Kamiya K, Saiki Y, Fujimoto Y, Kimura Y, et al. Studies on the constituents of Turkish Plants. I. Flavonol Triglycosides from the fruit of *Rhamnus thymifolius*. Chem Pharm Bull. 1993; 41:1743–1745.

34 Marzouk MS, El-Toumy SAA, Merfort I, Nawwar MAM. Polyphenolic metabolites of *Rhamnus disperma*. Phytochemistry. 1999; 52:943–946.

35 Lin, CN, Wei BL. Flavonol and naphthalene glycosides from *Rhamnus nakaharai*. J Nat Prod. 1994; 57:294–297.

36 Özipek M, Calis I, Ertant M, Rüedi P. Rhamnetin 3-p-Coumaroylrhamninoside from *Rhamnus petiolaris*. Phytochemistry. 1994; 37:249–253.

37 Lin CN, Chung MI, Can KH, Lu ChM. Flavonol and anthraquinone glycosides from *Rhamnus formosana*. Phytochemistry. 1991; 30: 3103–3106.

38 Aruoma OI. Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. Mutat Res. 2003; (523–524):9–20.

39 Mata AT, Proença C, Ferreira AR, Serralheiro mIM, Nogueira JMF, Araújo MEM. Antioxidant and antiacetylcholinesterase activities of five plants used as Portuguese food spices. Food Chem. 2007; 103:778–786.

40 Molyneux P. The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin J Sci Technol. 2004; 26:211–219.

41 Bondent V, Brand-Williams W, Bereset C. Kinetic and mechanism of antioxidant activity using the DPPH free radical methods. Lebensm Wiss Technol. 1997; 30:609–615.

42 Kulisic T, Radonic A, Katalinic V, Milos M. Use of different methods for testing antioxidative activity of oregano essential oil. Food Chem. 2004; 85: 633–640.

43 Ebrahimabadi AH, Ebrahimabadi EH, Djafari-Bidgoli Z, Kashi FJ, Mazoochi A, Batooli H. Composition and antioxidant and antimicrobial activity of the essential oil and extracts of *Stachys inflata* Benth from Iran. Food Chem. 2010; 119:452–458.

44 Urquiaga I, Leighton F. Plant polyphenol antioxidant and oxidative stress. Biol Res. 2000; 33:55–64.

45 Dasgupta N, De B. Antioxidant activity of some leafy vegetables of India. A comparative study. Food Chem. 2007; 101:471–474.