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Polyphenol composition and antioxidant activity of *Searsia tripartita* and *Limoniastrum guyonianum* growing in Southeastern Algeria

Soumia Hadjadj^{a,*}, Marie-Andrée Esnault^b, Solenne Berardocco^c,
Sylvain Guyot^d, Alain Bouchereau^c, Fatima Ghouini^e, Razika Lamini^e,
Aminata Ould El Hadj-Khelil^a

^aLaboratoire de Protection des Ecosystèmes en Zones Arides et Semi-Arides. Université KASDI Merbah-Ouargla, BP 511, Ouargla 30000, Algérie

^bUnité Mixte de Recherche 6553, Ecosystèmes- Biodiversité- Evolution, Université de Rennes1, Campus de Beaulieu, 35042 Rennes Cedex, France

^cUMR 1349 Institut de Génétique, Environnement et Protection des Plantes (IGEPP), INRA - Agrocampus Ouest - Université de Rennes 1, France

^dINRA, UR117, Unité de Recherches Cidricoles, Biotransformation des Fruits et Légumes, Le Rheu F-35653, France

^eUniversité Kasdi Merbah-Ouargla, BP 511, Ouargla 30000 Algérie

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ABSTRACT

Due to the notable medicinal value of *Searsia tripartita* and *Limoniastrum guyonianum*, it was of great interest to conduct phytochemical and antioxidant investigations. Polyphenol extracts of the dried powder of plants were prepared by maceration in 70% of methanol. The concentrated extracts were successively fractionated with distilled water, ethyl acetate and *n*-butanol. Obtained extracts were analysed for their phenol compositions by colorimetric processes and were evaluated for their total antiradical capacity by ABTS, DPPH and ORAC assays. Additionally, the phytochemical components were characterized in the methanolic extracts by HPLC-DAD-ESI-MS/MS in the negative ionisation mode. *S. tripartita* exhibited the higher quantity of phenolic compounds. The ethyl acetate extract of *S. tripartita* had the greatest quantities of polyphenols (55.5 ± 4.9 mg gallic acid equivalent per gram of dry residue (GAE/g DR)), flavonoids (44.2 ± 0.8 mg rutin equivalent per gram of dry residue (RE/g DR)), and tannins (12.5 ± 2.5 mg GAE/g DR). In *L. guyonianum*, the aqueous fraction had the highest quantity of polyphenols and tannins (10.2 ± 1.4 and 3.2 ± 1.9 mg GAE/g DR, respectively), whereas flavonoids (1.8 ± 0.2 mg RE/g DR) were higher in the ethyl acetate portion. The antioxidant capacity of the hydromethanolic extract of *S. tripartita* was found to be 3–16 times more effective than that of *L. guyonianum* using ORAC, DPPH, ABTS tests. HPLC analysis of hydromethanolic extracts provided tentative identification of four flavonoid glycosides in *S. tripartita* (myricetin-3-O-glucoside, myricetin-3-O-rhamnoside, quercetin-3-O-glucoside, and quercetin-3-O-rhamnoside) and five phenolic metabolites of the flavonoid class in *L. guyonianum* (myricetin-3-O-rhamnosylglucoside, myricetin-3-O-glucuronide, myricetin-3-O-pentoside, quercetin-3-O-glucuronide, and eriodictyol-7-O-rutinoside). Our findings revealed that these plants could be used as a potent source of health molecules.

* Corresponding author.

E-mail address: hadjajsumiabo@gmail.com (S. Hadjadj).

Background

The potential detrimental effects of molecular oxygen are believed to be due to the synthesis of partially reduced and highly harmful species, called free radicals such as reactive oxygen and/ or nitrogen species (ROS/RNS) [1,2]. These species are generated from normal physiological or biochemical processes in all living organisms. The overproduction and disruption of homeostasis between these toxic species and the overcapacity of detoxification systems lead to the appearance of oxidative stress phenomenon [3,4]. This overconcentration of ROS/RNS is able to oxidize biomolecules like, unsaturated lipids in cellular membrane, structural and functional proteins, carbohydrates and deoxyribonucleic acids, causing tissue destruction and cell injury that may be responsible for various chronic and degenerative illnesses, such as tumour, cardiovascular disease, neural diseases, skin irritation and inflammation [5,6]. Fortunately, there are well-designed protective systems in place, with functions based on enzymes (mainly superoxide dismutase, glutathione and ascorbate peroxidase, and catalase), primary antioxidant compounds (e.g., ascorbic and caffeic acids, kaempferol, glutathione, anthocyanins and polyphenols), and certain macromolecules (such as albumin, ceruloplasmin, and ferritin) [3], although these systems may become overwhelmed.

Currently, there is an increase in the number of studies aiming to identify new sources of natural antioxidants that are important for health care benefits. Various classes of secondary antioxidants generally occur in plants. Among these phytochemical substances, polyphenols have been largely described for their ability to act as antioxidant agents. Polyphenols are of vast importance owing to their multiple biological activities, such as antimicrobial, anticoagulant, anticancer, hepatoprotector, cardioprotector and vasodilatory effects [7]. These actions have been attributed to their reducing power that relates to the mobility of phenolic hydrogen atoms, which allows human cells to defend against oxygen-induced injury [8].

Algeria, with its geographical position and extensive area, enjoys a very diverse range of ecosystems and soils. This promotes the development of a rich and diverse flora, represented by 3000 plant species, of which approximately 1000 are considered to be medicinally important [9,10]. These phytoresources constitutes an inestimable treasure that should be valorised and utilised as a potential source of natural biological compounds. Therefore, to appreciate the natural plant resources in Algeria, we undertook to study two local Saharan species, *Searsia tripartita* (Ucria) Moffett (synonym: *Rhus tripartita*) and *Limoniastrum guyonianum* Durieu ex Boiss., which grow especially well in North Africa. The leaves of both species are commonly used in Algeria for various medicinal purposes, such as for treating various digestive, skin, circulatory, metabolic, and respiratory diseases [11,12]. Leaves and galls of *L. guyonianum* are also traditionally used for their hypoglycaemic activity, although no study has been undertaken to explore this aspect [13].

Our study was focused to characterize and quantify the phenolics, flavonoids, and tannins in the aerial parts of *S. tripartita* and *L. guyonianum* and to evaluate whether these phytomolecules have free radical quenching potential. Furthermore, the main polyphenols were characterized by reverse-phase high-performance liquid chromatography coupled to UV-visible spectrometry and ion-trap mass spectrometry used in the MS and MS/MS modes, in order to examine their therapeutic potential, as suggested by their use in traditional medicine.

Materials and methods

Plant materials collection

The plant material consisting of the aerial part of *S. tripartita* (Anacardiaceae) and *L. guyonianum* (Plumbaginaceae), were harvested during the autumn season from their natural biotopes, Ghardaïa (32° 13' 09.1" N, 003° 30' 00.1" E, altitude 519 m) and Ouargla (33° 12' 30.9" N, 006° 07' 59.6" E, altitude 299 m), that are regions located in the north-eastern Algerian Sahara. The botanical identification of species was carried out according to the Flora of Sahara [14].

To dehydrate the plant material by a similar method to that employed by traditional medicine, the fresh aerial parts were left to dry in air and in darkness at 25–30 °C room temperature for two weeks. The dried samples were powdered into a fine powder using an electric mortar and were put in glass vials maintained at a low temperature (–20 °C) until phytochemical and biological analysis were begun.

Extraction of phenolic metabolites

A sample of 100 g of the dried and powdered aerial parts of each plant was extracted by immersion in 500 mL of hydroalcoholic mixtures (methanol/ water, 70/30: V/ V) under magnetic stirring for 24 h at laboratory temperature and then

filtered through filter paper (Whatman No. 1). Then, the residue was exhausted successively by maceration twice using the same volume of the initial solvent.

The filtrates were combined and evaporated to a dry residue under reduced pressure at 40 °C using a rotary evaporator until dryness was achieved. The obtained dry residue constituted the “crude extract”. A part of the dried crude extract was solubilized in absolute methanol (1 mg/mL) to measure their ability to scavenge free radicals and for quantifying the total extractable polyphenols. The remaining crude extract was suspended in 100 mL distilled water. Next, the extract was extracted three times with ethyl acetate (3 × 100 mL) and then with *n*-butanol (3 × 100 mL). The aqueous, ethyl acetate, and butanolic phases were evaporated at reduced pressure at 40 °C using a rotary evaporator until dryness was achieved [15]. Residues corresponding to the ethyl acetate, butanolic, and aqueous fractions were solubilized in absolute methanol to make a final concentration of 1 mg/mL for subsequent analysis.

Total phenolic content

The concentration of total phenols was estimated by the Folin–Ciocalteu method [16]. A volume of 100 µL of the sample was combined with 500 µL of the Folin–Ciocalteu reagent (diluted 10-fold) and 1000 µL of distilled water. After vigorous shaking and incubation for 5 min in the dark, 1500 µL of an aqueous solution of sodium carbonate (200 g/L) were added. The reaction mixture was left for 2 h in the dark to develop blue colour. Finally, the optical density was measured at 765 nm using a SHIMADZU UV mini-1240 UV–Vis spectrophotometer (Shimadzu, Japan). The total phenolic amount was estimated by referring to a standard curve made with gallic acid and was expressed as mg gallic acid equivalent per gram of dry residue (mg GAE/g DR).

Total flavonoid content

The flavonoid content was determined according to the colorimetric method of Lamaison and Carnatusung [17], based on the formation of a yellow complex, due to the interaction between the flavonoid and aluminium and which absorbent at 430 nm [18]. An aliquot of 1 mL of each extract was mixed with 1 mL of a 2% aluminium chloride solution (2% in methanol). After incubation at room temperature for 10 min, the optical density was measured with a SHIMADZU UV mini-1240 UV–Vis spectrophotometer. The flavonoid content was determined by using a standard curve made with rutin and was expressed as mg rutin equivalent per gram of dry residue (mg RE/g DR).

Total tannin content

The total tannin level was measured according to the protocol developed by Makkar et al. [19] with some modifications. 100 mg of gelatin protein was mixed with 1 mL of distilled water and 1 mL of each sample. After incubation for 15 min at 4 °C, the mixture was centrifuged for 10 min at a centrifugal force of 3000 g. 200 µL of supernatant were used to measure the non-tannin phenolics using the Folin–Ciocalteu method. Total tannin amount was calculated by subtracting non-tannin phenolic from total phenolic amount.

Measurements of total antioxidant capacity (TAC)

Antioxidant activities were evaluated by three complementary radical scavenging assays involving different mechanisms because multiple reactions are involved in natural extracts [20]. The antioxidant activity measurements were performed with respect to the regression equation of the calibration curve of trolox and expressed as trolox equivalent in micromoles per gram of dry residue (µmol TE/g DR).

ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assay

The antiradical activity against the ABTS radical of each sample was made following the procedure reported by Re et al. [21]. The stock solutions contain 7 mM ABTS and 2.45 mM potassium persulfate. The ABTS radical cation (ABTS⁺) was prepared by mixing 5 mL of the ABTS solution with 88 µL of potassium persulfate, the mixture left in the dark at room temperature for 12–16 h before use. The solution was diluted daily with distilled water to obtain an absorbance of 0.7 ± 0.02 at 734 nm. The assay was adapted to 96-well Microplates, and 10 µL of extracts or standard was added to 290 µL of freshly diluted ABTS⁺ solution and incubated for 5 min in the dark. The absorbance was measured at 734 nm using a VERSAmax Microplate Reader (Molecular Devices, CA, USA) with Softmax Pro-software

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The DPPH assay is based on measurement of the loss of the DPPH[°] purple colour to yellow at 515 nm after reaction with antioxidants and carried out according to the procedure described by Brand-Williams et al. [22]. A total of 10 mL of each extract or standard was mixed with 290 µL of DPPH[°] methanolic solution (60 µM) in a 96-well Microplate. The decrease in absorbance was monitored at 515 nm every minute on the VERSAmax Microplate Reader until the reaction reached a plateau.

ORAC (oxygen radical absorbance capacity) assay

The ORAC test evaluates the antiradical ability against peroxy radicals. This test was performed according to the method described by Ou et al. [23]. Analyses were conducted in a 75 mM phosphate buffer solution with pH 7.4 at 37 °C. The peroxy radical was generated using AAPH that was freshly prepared for each run. Fluorescein was used as the substrate. Fluorescence was recorded using a SAFAS (Monaco) FLX-Xenius spectrofluorometer. ORAC analyses were performed in 96-well black opaque Microplates. The excitation wavelength was 485 nm and emission wavelength was 520 nm. 20 µL of antioxidant solution was mixed with 180 µL of 120 nM fluorescein and incubated for 15 min at 37 °C. The reaction was initiated with 100 µL of 12 mM AAPH solution. The fluorescence was monitored every minute for 2 h. Fluorescence data were plotted and the area under the curve (AUC) was calculated. The net AUC was calculated by subtracting the AUC of the blank from the AUC obtained in the presence of an antioxidant (AUC extracts - AUC blank).

Analysis of individual phenolic compounds by analytical RP-HPLC-IT-MS

The detection and identification of phenolic compounds were performed on the aerial part of the plants. To that end, 1 g of dried material was prepared by dissolving in 5 mL of 70% (v/v) aqueous methanol containing 1% v/v formic acid for 30 min in an ultrasonic bath (Brasson 2200, USA). The procedure was repeated three times and the combined extracts were filtered through a polytetrafluoroethylene (PTFE) membrane (0.45 µm, Uptidisc Interchim, France) and injected into an RP-HPLC-UV-visible-MS system. Analyses were performed using an LC-DAD-ESI-MS (liquid chromatography coupled to diode array detection and electrospray ionization mass spectrometry) system composed of an SCMA1000 degasification system (ThermoQuest, San Jose, CA, USA), an automatic injection system (ThermoFinnigan, San Jose, CA, USA), an 1100 series binary pump (Agilent Technologies, Palo Alto, CA, USA), and a Spectra photodiode array detector system UV6000LP (ThermoFinnigan, San Jose, CA, USA). The mass spectrometer was an ion trap LCQ Deca (ThermoFinnigan, San Jose, CA, USA) equipped with an electrospray ionization source. A sample volume of 2 µL was injected onto a Zorbax Eclipse XDB-C18 column (2.1 mm × 150 mm, 3.5 µm; Agilent Technologies) and maintained at 30 °C. The mobile phase consisted of solvent A (aqueous formic acid, 0.1% v/v) and solvent B (acetonitrile containing 0.1% formic acid v/v). The solvents were filtered through a 0.45 µm PTFE membrane. The following linear gradient elution was applied at a constant flow rate of 0.2 mL/min: 97%–91% A, 0–5 min; 91%–84% A, 5–15 min; 84%–50% A, 15–45 min; 50%–10% A, 45–48 min; and 48–51 min isocratic and reconditioning the column for 15 min.

Statistical analysis

The data were expressed as means ± standard deviation (SD) and statistically analysed using a Tukey's HSD test, differences were considered statistically significant at the 5% level. Linear regressions were used to examine the relationships between antioxidant activity and phenolic component content according to Bravais-Person correlation test. All statistical analyses were performed using XLSTAT statistical software version 2009.6.01. All experiments were executed in triplicate.

Results

Phenolic fractionation in the plant species

Solvent-directed extraction is an essential step for the elucidation of bioactive components from medicinal plants. However, the nature of the extracting solvent is the most controversial parameter that can influence the quantity and the quality of molecules isolated from the plant extracts, namely, phenolics [24]. In our work, four solvents with increasing polarity were used to evaluate the polyphenol content in the extracts. *S. tripartita* possesses significantly higher content ($p < 0.001$) of total phenolic compounds, flavonoids, and tannins than that of *L. guyonianum* except for the content of total phenolics ($p = 0.102$) and tannins ($p = 0.248$) in the aqueous fraction (Table 1). Together, these compounds represent 16.3% of the dried *S. tripartita* plant versus 3.4% in *L. guyonianum*. For both species, phenolics were quantitatively the most abundant. Based on the solvent fraction, the flavonoid content ranged from 0.3 ± 0.1 to 53.6 ± 1.8 mg RE/g DR for *S. tripartita* and from 0.2 ± 0.0 to 5.7 ± 0.1 mg RE/g DR for *L. guyonianum*. The tannin content ranged from 7.9 ± 1.9 to 28.5 ± 5.2 mg GAE/g DR in *S. tripartita* and from 0.3 ± 0.1 to 5.4 ± 1.7 mg GAE/g DR in *L. guyonianum*. The ethyl acetate fraction was the richest in *S. tripartita* for the three classes of compounds (57.1% for phenolics, 75.8% for flavonoids, and 40.6% for tannins). The residual aqueous fraction had the lowest content, whereas *L. guyonianum* exhibited higher content of polyphenols (70.3%) and tannins (80%). The opposite was true for flavonoids, as the ethyl acetate extract was the richest in flavonoids (51.4%).

Total antioxidant capacity

Due to the complexity of the oxidation processes and the chemical diversity of antioxidants, with both hydrophilic and hydrophobic components, the total antioxidative ability cannot be fully evaluated by one single chemical reaction. Therefore, more than one type of radical system is required to investigate the total antioxidant activity of natural extracts and pure isolated substances [25]. In the current study, total antiradical capacity was measured using the ABTS, DPPH and ORAC

Table 1Total phenolic, flavonoid and tannin contents of crude extract and fractions of *S. tripartita* and *L. guyonianum*.

Extract and fractions	Amount (mg/ g) \pm standard deviation					
	Total phenolic		Flavonoids		Tannins	
	<i>S. tripartita</i>	<i>L. guyonianum</i>	<i>S. tripartita</i>	<i>L. guyonianum</i>	<i>S. tripartita</i>	<i>L. guyonianum</i>
Crude extract	80.7 \pm 2.8 ^a	23.2 \pm 0.6 b	53.6 \pm 1.8 a	5.7 \pm 0.1 b	28.4 \pm 5.2 a	5.4 \pm 1.7 b
Ethyl acetate fraction	55.5 \pm 4.9 ^a	3.1 \pm 0.3 b	44.2 \pm 0.8 a	1.8 \pm 0.2 b	12.5 \pm 2.5 a	0.5 \pm 0.2 b
Butanolic fraction	26.8 \pm 0.8 ^a	1.2 \pm 0.2 b	13.8 \pm 1.3 a	0.2 \pm 0.0 b	10.4 \pm 1.9 a	0.3 \pm 0.1 b
Aqueous fraction	14.9 \pm 3.6 ^a	10.2 \pm 1.4 a	0.3 \pm 0.1 b	1.5 \pm 0.1 a	7.9 \pm 1.9 a	3.2 \pm 1.9 a

The total phenolic and tannins content values were expressed as mg gallic acid equivalent/ g of dry residue. The flavonoids content was expressed as mg rutin equivalent /g of dry residue.

Values with different letters in the same row represent significant differences between the similar fractions of the two species ($p < 0.05$). The differences were tested using Tukey's test.

Table 2Total antioxidant capacity of crude extract and fractions of *S. tripartita* and *L. guyonianum*.

Extract and fractions	TAC (μ mol/ g) \pm standard deviation					
	ABTS assay		DPPH assay		ORAC assay	
	<i>S. tripartita</i>	<i>L. guyonianum</i>	<i>S. tripartita</i>	<i>L. guyonianum</i>	<i>S. tripartita</i>	<i>L. guyonianum</i>
Crude extract	214.7 \pm 5.2 a	14.2 \pm 1.0 b	551.9 \pm 3.9 a	33.0 \pm 0.9 b	1243.1 \pm 46.4 a	369.5 \pm 85.7 b
Ethyl acetate fraction	158.6 \pm 3.2 a	1.5 \pm 0.1 b	234.3 \pm 7.8 a	5.1 \pm 0.1 b	786.5 \pm 14.3 a	43.8 \pm 2.1 b
Butanolic fraction	64.8 \pm 1.2 a	1.4 \pm 0.1 b	163.1 \pm 2.3 a	0.2 \pm 0.0 b	283.8 \pm 1.0 a	21.2 \pm 0.8 b
Aqueous fraction	18.2 \pm 1.0 a	5.9 \pm 0.5 b	57.6 \pm 0.6 a	9.7 \pm 0.5 b	48.1 \pm 2.2 a	52.3 \pm 2.2 a

TAC: The total antioxidant capacity values were expressed as μ mol trolox equivalent/g of dry residue.

ABTS and DPPH radical scavenging activity; ORAC: Oxygen radical absorbance capacity.

Values with different letters in the same row represent significant differences between the similar fractions of the two species ($p < 0.05$) by means with Tukey's test.

Table 3

Correlation analysis between the different antioxidant assays and phenolic compound content in crude extract and fractions.

Phenolic compounds	Total phenolic		Flavonoids		Tannins	
	R ²	P	R ²	P	R ²	P
ABTS assay	0.98	<0.0001	0.99	<0.0001	0.88	<0.0001
DPPH assay	0.96	<0.0001	0.93	<0.0001	0.93	<0.0001
ORAC assay	0.98	<0.0001	0.97	<0.0001	0.87	<0.0001

R²: Pearson correlation coefficient.

P: Probability level.

assays (Table 2). The ORAC technique measures the ability of an antioxidant to quenching free radicals (peroxyl radicals) by hydrogen atom transfer [26], whereas the other two radicals may be neutralized either *via* electron transfer or *via* hydrogen donation [27]. Overall, the total antioxidant properties of the two studied species were strongly different. The effectiveness differed between and within the two species, depending on the scavenging assay used and on the tested fraction. *S. tripartita* with high levels of phenolic compounds displayed very high significant antioxidant capacity ($p < 0.001$) in all assays (except for the aqueous fraction in the ORAC assay, $p = 0.080$). The crude extract possessed the highest antioxidant activity as determined by the ORAC (1243.1 \pm 46.4 μ mol TE/g DR), ABTS (214.7 \pm 5.2 μ mol TE/g DR) and DPPH (551.9 \pm 3.9 μ mol TE/g DR) assays. The results were 3 to 16-fold higher than those in *L. guyonianum* (369.5 \pm 85.7, 14.2 \pm 1.0 and 33.0 \pm 0.9 μ mol TE/g DR, respectively). Antioxidant capacity in *S. tripartita* was highest in the ethyl acetate extract, followed by the butanolic and aqueous portions, whereas in *L. guyonianum* the organic fractions exhibited the lowest antioxidant capacity, with the butanolic fraction always being the lowest.

The antiradical activity of plant extracts increased proportional to the phenolic content. The total phenolic, flavonoid, and tannin content all correlated strongly and positively with antioxidative potential (Table 3). The highly significant correlation coefficients were $0.96 \leq R^2 \leq 0.98$ ($p < 0.0001$) $0.93 \leq R^2 \leq 0.99$ ($p < 0.0001$), and $0.87 \leq R^2 \leq 0.93$ ($p < 0.0001$), respectively.

Phenolic structure elucidation by LC-ESI-MS/MS

A qualitative composition of phenolic compounds in the aqueous-methanol fractions was carried out by HPLC - DAD - ESI-MS/MS in negative ionization mode. This mode was selected owing to the greater sensitivity and selectivity for phenolics in comparison with the positive mode. Polyphenols are weakly acidic compounds, indicating that dissociation is easier

Table 4
Phenolic compounds identified in aerial part extracts of *S. tripartita* and *L. guyonianum* by HPLC-DAD-ESI-MS/MS.

Peak n°	Proposed compound	Rt (min)	λ max (nm)	[M - H] ⁻ (m/z)	Major MS/ MS ion fragments m/z (intensity %)	References
<i>S. tripartita</i>	1A Myricetin-3-O- glucoside	18.02	256, 357	479	317 (4), 316 (21)	[30]
	2A Myricetin-3-O- rhamnoside	20.76	255, 350	463	316 (24)	[31]
	3A Quercetin-3-O-glucoside	21.23	254, 355	463	300 (24)	[32]
	4A Quercetin-3-O-rhamnoside	23.95	263, 349	447	300 (34)	[33]
<i>L. guyonianum</i>	1B Myricetin-3-O-rhamnosylglucoside	18.09	263, 354	625	479 (100), 316 (38), 317 (13)	[30]
	2B Myricetin-3-O-glucuronide	18.36	263, 356	493	317 (29), 316 (7)	[30]
	3B Myricetin-3-O- pentoside	20.68	264, 350	449	316 (84), 317 (25)	/
	4B Quercetin-3-O-glucuronide	21.62	263, 354	477	301 (34)	[30]
	5B Eriodictyol-7-O- rutinoside	31.49	283	595	287 (6)	/

Rt: Retention time.

λ max: Wavelengths of maximum absorption.

[M - H]⁻: Deprotonated molecule.

than protonation [28,29]. Data acquisition and analysis were performed using the Xcalibur software version 2.1 (Table 4, Fig. 1). The main peaks on the base-peak chromatograms were identified based on the interpretation of their UV-visible spectrometry, full MS and MS/MS spectra and also in comparison with those reported in the literature.

Four flavonoid glycosides were found as the most predominant compounds in *S. tripartita*, i.e., myricetin-3-O-glucoside, myricetin-3-O-rhamnoside, quercetin-3-O-glucoside, and quercetin-3-O-rhamnoside.

Five phenolic-related phytochemicals represented the most abundant compounds in *L. guyonianum* extracts, i.e., myricetin-3-O-rhamnosylglucoside, myricetin-3-O-glucuronide, myricetin-3-O-rhamnoside, quercetin-3-O-glucuronide, and eriodictyol-7-O-rutinoside.

Compounds (peak 1A and 2A in *S. tripartita* and peak 2B and 3B in *L. guyonianum*) showed an m/z of 479, 463, 493, and 449 [M-H]⁻, which correspond to myricetin-3-O-glucoside (the fragment ions at m/z 317 and 316 correspond to the loss of a hexose moiety (-162 amu) and hexose+ H loss), myricetin-3-O-rhamnoside (the fragment ion at m/z 316 corresponds to deoxyhexose+ H), myricetin-3-O-glucuronide (the product ions at m/z 317 and 316 correspond to glucuronide (-176 amu) and glucuronide+ H loss), and myricetin-3-O-pentoside (the product ions at m/z 317 and 316 correspond to pentose (-132 amu) and pentose+ H loss), respectively (Fig. 2A and B) (Fig. 3A and B).

Quercetin-3-O-glucoside and quercetin-3-O-rhamnoside (peak 3A and 4A) in *S. tripartita* and quercetin-3-O-glucuronide (peak 4B) in *L. guyonianum* produced deprotonated molecules at m/z 463, 447, and 477, respectively. The MS/MS fragmentation pattern obtained from these compounds revealed a characteristic m/z at 301 or 300 (m/z) corresponding to quercetin aglycone, suggesting neutral losses of the sugar moiety hexose (loss of 162 amu from [M-H]⁻ for peak 3A), deoxyhexose (loss of 146 amu from [M-H]⁻ for peak 4A), and glucuronide (loss of 176 amu from [M-H]⁻ in peaks 4B) (Fig. 2C and D) (Fig. 3C).

Peak 1B in *L. guyonianum* was tentatively identified as myricetin-3-O-rhamnosylglucoside because the deprotonated molecule showed a [M - H]⁻ at m/z 625 and MS/MS fragments at m/z 479, m/z 317, and m/z 316, which correspond to deoxyhexose (-146 amu), diglycoside (-308 amu), and diglycoside molecule + H loss, respectively (Fig. 3D).

Compound 5 exhibited a deprotonated molecule at m/z 595 (aglycone + disaccharides) and a product ion at m/z 287 characteristic of the aglycone eriodictyol, which may be attributed to the loss of a rutinoside moiety. This compound has been assigned as eriodictyol -7-O-rutinoside (Fig. 3E).

Discussion

Polyphenols are among the important group of secondary metabolites in plants and they are detected at high concentrations in many medicinal plants [34,35]. Undoubtedly, structure-related and dose-related mechanistic investigations are required for understanding the basis of phenolic compound pharmaceutical activity. *S. tripartita* and *L. guyonianum* are two halophyte species widely used as medicinal plants that possess several pharmacological effects. The root cortex extract of *R. oxyacantha* has been indicated to contain proanthocyanidic oligomers and polymers with high antioxidant property and the ability to prevent thymocyte death in rats [36]. Stems of *R. tripartita* are essentially rich in phenolic compounds and show great cardioprotective potential in human cells and rats [37]. Leaf and gall infusions of *L. guyonianum* have been utilized by the Tunisian traditional healers for the treatment of diarrhoea and dysentery [38]. Recently, Krifa et al. [39] stated that an aqueous gall extract of this plant exhibited antioxidant activities, as well as immunomodulation actions on splenocytes, natural killer cells, and macrophages.

Since the two species in the present study may be characterized by diversified classes of phenolic compounds, we first explored the qualitative and quantitative extraction performance of different solvent systems. The present study showed a great variation of phenolic content as a function of species and solvent polarity. *S. tripartita* exhibited higher quantities of phenolic compounds than that of *L. guyonianum*. Together, these compounds represented 16.3% of the dried extract for the

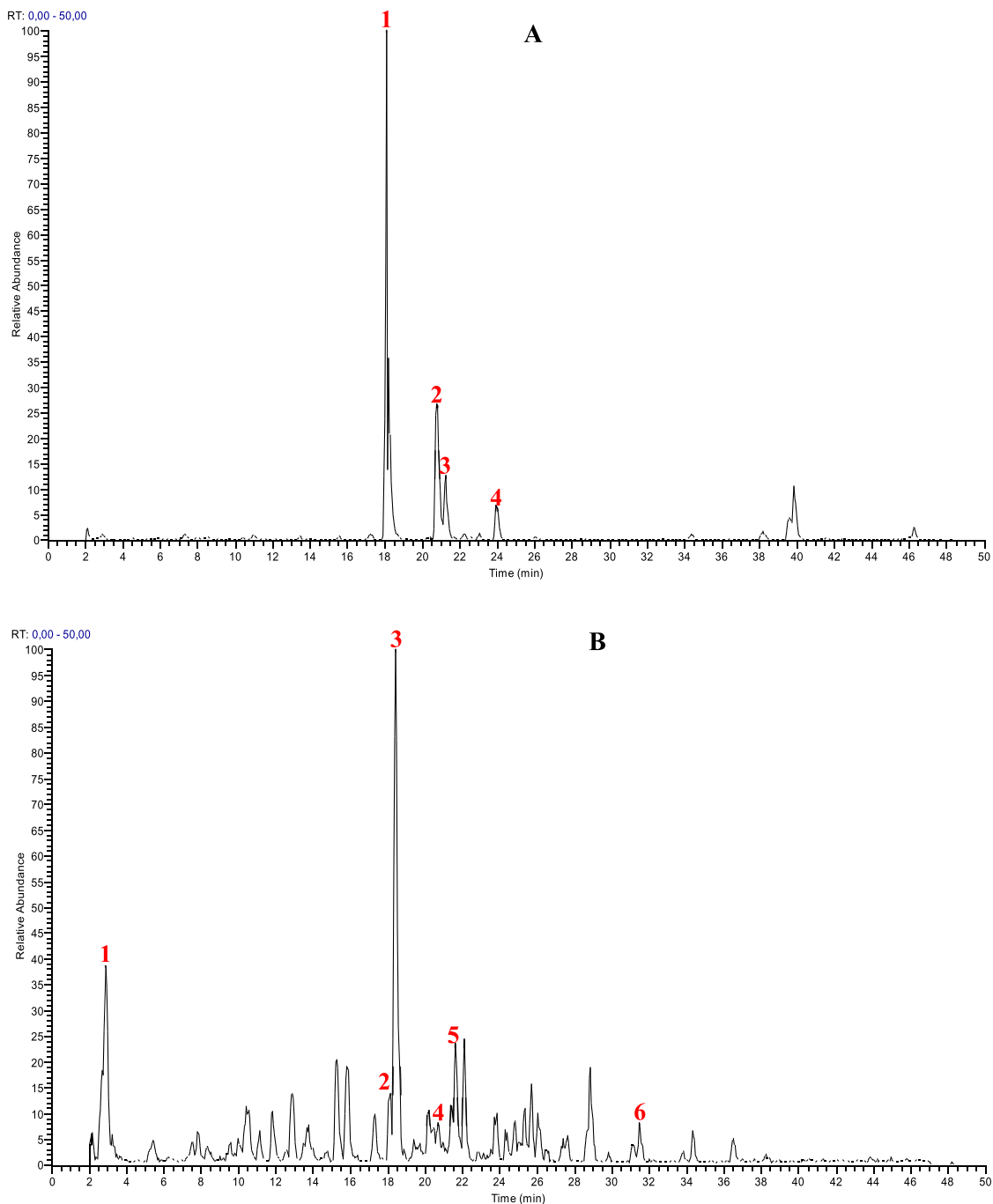


Fig. 1. RP-HPLC chromatograms of methanolic extracts of *S. tripartita* (A) and *L. guyonianum* (B). Peak numbers correspond to **1A**, myricetin-3-O-glucoside, **2A**, myricetin-3-O-rhamnoside, **3A**, quercetin-3-O-glucoside and **4A**, quercetin-3-O-rhamnoside for *S. tripartita* and **1B**, myricetin-3-O-rhamnosylglucoside, **2B**, myricetin-3-O-glucuronide, **3B**, myricetin-3-O-pentoside, **4B**, quercetin-3-O-glucuronide and **5B**, eriodictyol -7-O-rutinoside for *L. guyonianum*.

S. tripartita plant versus 3.4% for *L. guyonianum*. The high level of phenolic compounds from *S. tripartita* could be related to intrinsic (genetic) factors also extrinsic (environmental and extraction conditions) factors [40]. It has been demonstrated that their concentrations are higher when the living environment of the plant is not adequate, whereby the plant promotes the synthesis of secondary metabolites to adapt and survive [41]. In the region of Ouargla and Ghardaïa, the environmental conditions are drastic. For example, the average temperature reached respectively 36.7 °C and 35.25 °C in summer (2010–

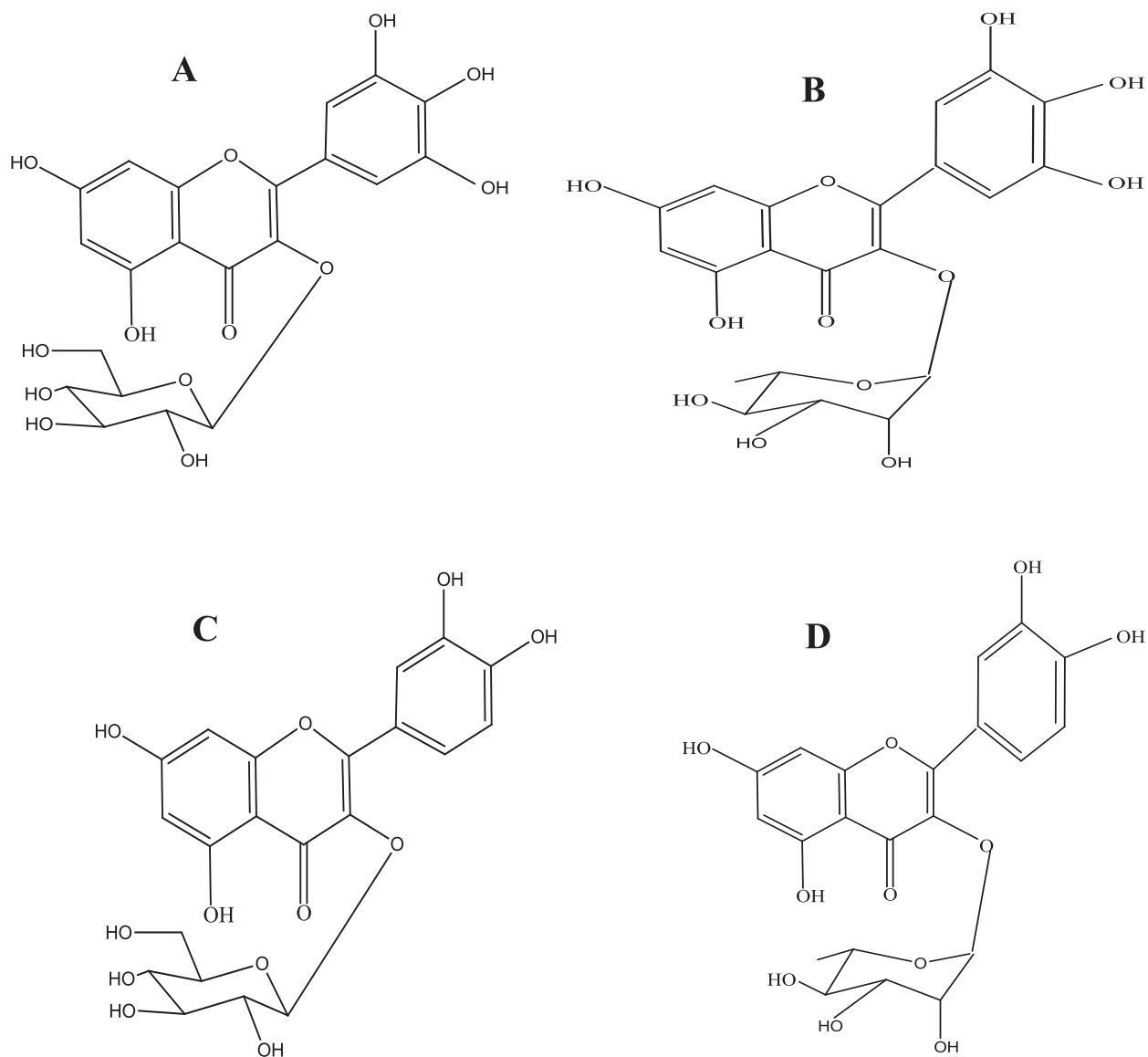


Fig. 2. Chemical structures of main flavonoids identified in *S. tripartita*. Myricetin-3-O- glucoside (A) and myricetin-3-O- rhamnoside (B), quercetin-3-O-glucoside (C) and quercetin-3-O-rhamnoside (D).

2015), and the pluviometry was at the most 7 mm and 11.6 mm a month. These conditions probably contributed to favour metabolic pathways producing metabolites that allow plants to survive.

For both species, high variation in polyphenol content was found between the different fractions depending on the solvent polarity. The content of all phenolic classes was in the following order from high to low: ethyl acetate fraction > butanolic fraction > aqueous fraction in *S. tripartita* and aqueous fraction > ethyl acetate fraction > butanolic fraction in *L. guyonianum* (except flavonoids). The richness of the ethyl acetate portion of the crude extract of *S. tripartita* in phenolic compounds might be linked to the richness of this species in moderately polar compounds (e.g., aglycones and monoglycosylates). However, the presence of higher amounts of polyphenols and tannins in the aqueous fraction of the crude extract of *L. guyonianum* than that in the organic fractions suggests the richness of this species in hydrolysable glycosylated polyphenols. The solubility of phenolic compounds is particularly dependent to the polarity of solvent used, the molecular weight and length of constituent hydrocarbon chains, the presence and position of hydroxyl groups, and the degree of polymerization of phenolics [42]. Many previous studies support the observation that phenol content can vary widely as a function of the polarity of the solvent in liquid-liquid extraction and fractionation systems. Kosar et al. [43] proved that the ethyl acetate fraction had the greatest phenolic content in *Rhus coriaria* L. (540 mg GAE/g extract), which is an Anacardiaceae species as well as *S. tripartita*, followed by the water fraction (5.1 mg GAE/g extract). Kim et al. [44] reported in *R. vernici-*

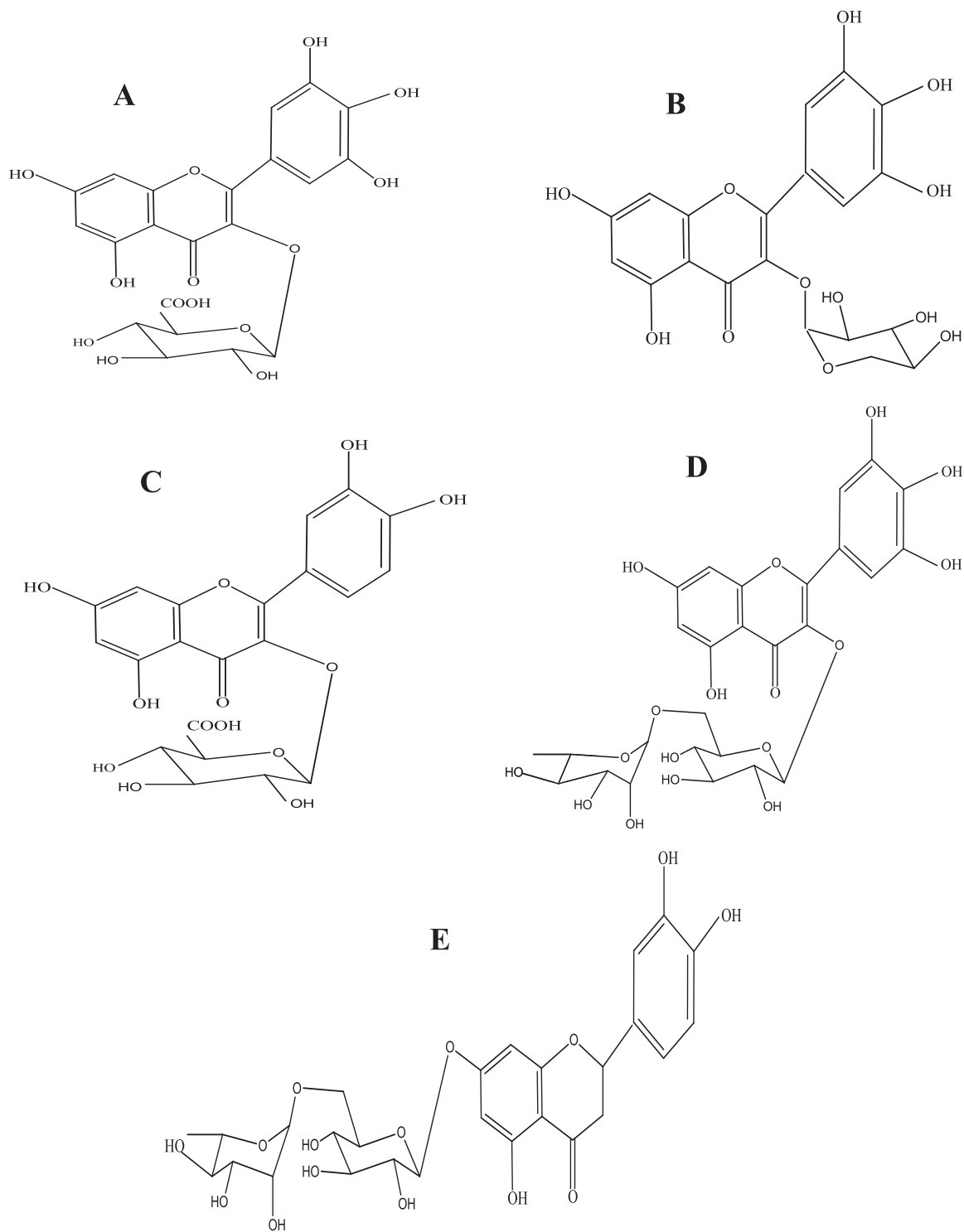


Fig. 3. Chemical structures of main flavonoids identified in *L. guyonianum*.

Myricetin-3-O- glucuronide (A), myricetin-3-O- pentoside (B), quercetin-3-O-glucuronide (C), myricetin-3-O-rhamnosylglucoside (D) and eriodictyol –7-O- rutinoside (3E).

flua, another Anacardiaceae species, that the total phenolic content in the ethyl acetate fraction (723 mg GAE/g) and in 80% ethanolic extract (597 mg GAE/g) was higher than that in other fractions, whereas the 80% ethanolic extract was the highest in flavonoids, equalling 201 mg QE/g (quercetin equivalent).

In a study dedicated to *L. guyonianum*, Belfar et al. [45] first extracted the polyphenols with 60% ethanol before partitioning. These authors found high quantities of both total phenolic and total flavonoid content, 75.81 mg GAE/g and 13.44 mg QE/g (quercetin equivalent) in a butanolic fraction, and 15.51 mg GAE/g and 3.56 mg QE/g in an ethyl acetate fraction, respectively. In comparison, our results in *L. guyonianum* for total phenolic and total flavonoid content were 1.2 mg GAE/g DR and 0.2 mg RE/g DR in the butanolic fraction, and 3.1 mg GAE/g DR and 1.8 mg RE/g DR in the ethyl acetate fraction, respectively. These differences might be related to the fact that in the present study the reference was rutin, whereas it was probably (although not indicated) quercetin for the study by Belfar et al. [45]. Furthermore, they did not analyse the aqueous fraction, which was demonstrated to be important for the total phenolic content (10.2 mg GAE/g DR in our analysis). For *S. tripartita*, the total phenolic content in the aerial parts was 80.7 mg GAE/g DR in our study. In Tunisia, Itidel et al. [46] found levels of phenolics of 45–102.1 mg GAE/g in leaves, 77–219 mg in the stem cortex, and 39–121 mg in fruits of the same species (under the name *R. tripartita* (Ucra) Grande). For flavonoids, we found 53.6 mg RE/g DR and Itidel et al. [46] reported quantities of 50.7–74 mg RE/g in leaves, 5.6–12.7 in the stem cortex, and 5–11.9 in fruits.

Currently, great importance is given to the indication of total antioxidant capacity as a tool for identifying natural products that may have beneficial effects against degenerative diseases [47]. TAC values of plant extracts were largely different depending on the polarity of the solvent. Thus, effective antioxidant activity was found in *S. tripartita*. Our findings are in concord with those from a previous study by Mahjoub et al. [48] in Tunisian *R. tripartitum* (= *R. tripartita*) aerial parts after methanolic extraction. The ABTS assay showed TAC values of 1.20 mM TE/mg extract. Our results were 214.7 $\mu\text{mol TE/g DR}$. Ben Miled et al. [49] reported that polar extracts of *R. tripartitum* root (water and methanol) have a same and better ORAC activity with respective values of 8.95 and 8.55 $\mu\text{mol TE/ mg}$ than non-polar ones (hexane and dichloromethane). On the other hand, Wu et al. [50] indicated effective antioxidant activity of 1544 $\mu\text{mol TE/g}$ in the ORAC assay for an ethanolic extract of *R. hirta* L. fruits that was higher than that of our methanolic extract (1243.1 $\mu\text{mol TE/g DR}$). For *L. guyonianum* extract, Trabelsi et al. [51] found a powerful ability to quench DPPH radical with an IC50 of 120 $\mu\text{g/mL}$, which is difficult to compare to our extract with a TAC in the DPPH assay of 33 $\mu\text{mol TE/g DR}$. Furthermore, Souid et al. [52] demonstrated that the hydromethanolic extracts obtained from leaves of eight *Limonium* species have a highly ORAC levels, varied between 2631.50 $\mu\text{M TE/G DW}$ for *L. spathulatum* and 5108 for *L. virgatum*.

The difference in the activity between the different fractions can be connected to the nature of the phenolic compounds in each fraction. Based on a study by Cowan [53], during liquid-liquid extraction, the phytomolecules are distributed between the solvents based on their polarity and solubility. Consequently, a change in solvent nature alters its efficacy to extract a specific group of antioxidant compounds and influence the antioxidant properties of the extract [54]. Kratchanova et al. [55] mentioned the significant effect of solvent extraction on the assessment of antioxidant activity and polyphenol extraction. It is consequently advocated to utilize more than one extraction system to improve the investigation of the antioxidant activity of natural products.

The values of TAC estimated by the ORAC assay were notably higher than those obtained by the ABTS and DPPH assay methods. The diversity in radical scavenging between the diverse systems shown in these experiments may be ascribed to the difference in the reaction stoichiometry between the natural antioxidants in the extracts and various radicals. Free radical scavenging by antioxidants is dependent upon two types of mechanisms as follows: *via* release of a hydrogen atom from hydroxyl groups (i.e., fast kinetics of some phenolic acids and derivatives) or *via* the release of an electron (i.e., slow kinetics derived glycosylated and anthocyanins) [20].

Similar to other studies, we established a positive linear relationship between the content of the phenolic compounds and the antioxidant power, suggesting that phenolic components present in these extracts are the principal contributors to the antioxidant action of the selected plants [43,50,56,57]. Indeed, phenolic compounds encompass a range of structures that are widely distributed in plants and they have been informed to have various biological effects, including antiradical capacity. The antioxidant activities of the compound are primarily due to their redox properties, hydrogen donors, singlet oxygen quenchers, and metal chelators, which can act essential roles in trapping free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [58].

Considerable effort has been dedicated to the elucidation of the structure of phenolic compounds. In recent decades, high-performance liquid chromatography (HPLC) coupled to mass spectrometry (LC-MS) has been considered the platform of choice for such work [59]. From these studies, chromatographic resolution and spectrometric parameters allowed identification of four flavonoids in *S. tripartita*, i.e., myricetin-3-O-glucoside, myricetin-3-O-rhamnoside, quercetin-3-O-glucoside, and quercetin-3-O-rhamnoside. Five probable phenolic compounds, i.e., myricetin-3-O-rhamnosylglucoside, myricetin-3-O-glucuronide, myricetin-3-O-pentoside, quercetin-3-O-glucuronide, and eriodictyol-7-O-rutinoside, have been proposed to occur abundantly in *L. guyonianum*. To our knowledge, these compounds are stated for the first time in these species, and they can contribute to the antioxidant property displayed by the corresponding extracts, although direct evidence is yet to be proposed. Our results are in agreement with those from phytochemical investigations performed on *R. tripartita* by Mahjoub et al. [48], another beneficial natural resource with antioxidant properties. Four compounds were isolated including a new biflavonoid, masazinoflavanone (S1) in the chloroformic extract and three other natural substances from the ethyl acetate/methanol fraction (myricetin (S1), (-)-lyoniresinol 3a-O-b-d-glucopyranoside (S3), and (-)-methyl shikimate (S4)). It has been recorded that the corresponding pure substances exhibited antioxidant activity towards the ABTS radical with 1.20,

2.40, 1.58, and 0.28 mM TE/mg sample, respectively. Recently, Abd El-Salam [60] studied *R. tripartita* plants of Egyptian origin and isolated six bioactive constituents from the alcoholic extract of leaves, including gallic catechin, quercetin, myricetin, kampferol-3-O- α -L-rhamnopyranoside, kampferol-7-O- α -L-rhamnopyranoside, and β -sitosteryl-3-O- β -glucopyranoside.

Earlier studies showed that related polyphenolic compounds were encountered in different *Limonium* species [52,61]. Chaabi et al. [56] isolated four flavonoids from the leaf extract of *L. feei*, i.e., myricetin-3-O- β -galactopyranoside, myricetin 3-O- α -rhamnopyranoside, quercetin, and myricetin where myricetin was shown to be the most active product in the superoxide nitroblue tetrazolium hypoxanthine/xanthine oxidase test.

In contrast to our results, Trabelsi et al. [62], demonstrated that different fractions from an ethyl acetate extract of *L. guyonianum* leaves contained variable and powerful antioxidants, such as a phenolic acid (*p*-coumaric acid) and two flavonoids (catechin and epigallocatechin-3-O-gallate) in their fraction 3, four phenolic compounds (gallo-catechin, sinapic acid) and two amides of phenolic acids (N-E-caffeoyl tyramine (1) and N-E-feruloyl tyramine) in fraction 4 and a new dimer of phenolic acid amide (Limoniastramide) in fraction 5. In addition, Trabelsi et al. [51] demonstrated that phenolic compound composition can vary, both qualitatively and quantitatively, within the same species depending on the provenance. Thus, six phenolic compounds were identified from the *L. guyonianum* collected in El Akarit (Tunisia) with three phenolic acids (gallic, 4-hydroxybenzoic and 3,4-dimethoxybenzoic acids) and three flavonoids (gallic catechin, catechin and epigallocatechin-3-O-gallate). However, only four compounds were identified from this medicinal halophyte native to the Oued Ran station, namely, gallic catechin, catechin 3,4-dimethoxybenzoic acid and vanillic acid.

Conclusions

Based on the present results, extracts and fractions of the two medicinal plants *L. guyonianum* and *S. tripartita* exhibited high quantities of bioactive substances and powerful antioxidant activities. The HPLC–DAD–ESI–MS/MS analysis of the crude extracts provided a tentative identification of major phytochemicals. The identified compounds belong to the flavonoid glycoside class, particularly the flavonol and flavanone classes (derived from aglycones; quercetin, myricetin, and eriodictyol). The sugar moieties consist of hexosides, disaccharides, deoxyhexosides, and pentosides. The presence of this group of secondary metabolites is probably the main contributor to the antiradical capacity of these plants, as well as supporting their uses in folk medicine to treat certain illness in relation to oxidative stress. Consequently, we propose a detailed study of these species to isolate the individual phenolic compounds to extensively elucidate their antioxidant capacity, which may be further exploited in herbal formulations.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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