Activity-Guided Isolation of Antioxidant Principles from *Limoniastrum feei* (Girard) Batt.

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Bioguided fractionation of a leaves extract from *Limoniastrum feei* (Girard) Batt. (Plumbaginaceae) led to the isolation of seven polyphenolic constituents: gallic acid (**1**), myrciaphenone A (**2**), myricetin-3-*O*-β-galactopyranoside (**3–1**), epigallocatechin gallate (**3–2**), myricetin 3-*O*-α-rhamnopyranoside (**4**), quercetin (**5**) and myricetin (**6**). Gallic acid was the most antioxidant compound in DPPH [(0.94 ± 0.68) μg/mL] and FRAP [(0.83 ± 0.15) μM Fe^{2+}/mL] tests, whereas myricetin was a more specific superoxide radical scavenger since it was the most active product in the superoxide nitroblue tetrazolium hypoxanthine/xanthine oxidase test [(1.86 ± 0.12) μg/mL].

Key words: *Limoniastrum feei*, Polyphenols, Oxidative Stress

Introduction

Plumbaginaceae is a cosmopolitan family which includes 836 species grouped in 27 genera. Only two of them, *Limonium* and *Limoniastrum,* are present in the Sahara (Ozenda, 2004). *Limoniastrum feei* (Girard) Batt. is a species endemic to Algeria and Morocco which can resist to extremely arid conditions (Quezel and Santa, 1963). A recent study revealed its good antibacterial activity (Belboukhari and Cheriti, 2005). However, to the best of our knowledge, no phytochemical studies have been conducted on the genus *Limoniastrum* until now.

Oxidative stress is caused by an imbalance in the oxidant/antioxidant equilibrium. Reactive oxygen species (ROS), generated either by endogenous or exogenous factors, are associated with the pathogenesis of various diseases such as inflammation, atherosclerosis, diabetes, cancer and arthritis (Halliwell and Gutteridge, 2000). During the past decade, interest in polyphenols, including flavonoids, has considerably increased due to the discovery of their various biological properties, principally their antioxidant effects and therefore their possible role in the prevention of several chronic diseases involving oxidative stress. Polyphenols inhibit cGMP and cAMP phosphodiesterases, xanthine oxidase and elastase, for example (Plessi *et al*., 1998).

The aim of the present study was to assess the antioxidant activity of the semidesertic species *L. feei*, and to isolate its active consituents by bioguided fractionation using the 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) assay, an electron transfer (ET)-based method. The results were compared with those of two other complementary assays: FRAP (ferric reducing ability of plasma), another ET-based method, as well as the superoxide anion scavenging assay, a hydrogen atom transfer (HAT)-based method.

Material and Methods

Reagents and drugs

All chemicals used were of at least analytical grade. 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) were obtained from Sigma Chemicals Co. (St. Louis, USA). Anhydrous sodium carbonate (Na_2CO_3), cupric sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), Folin-Ciocalteu phenol reagent, hydrochloric acid (HCl), glacial acetic acid, methanol, and sodium acetate trihydrate were obtained from Merck

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(Darmstadt, Germany) and Neu reagent from Roth (Karlsruhe, Germany).

Plant material

The whole plant *L. feei* (Plumbaginaceae) was collected in Bechar region, in the southwest of Algeria, during the flowering stage in April 2003. It was identified by Prof. Khalfallah, Institute of Biology of Constantine, Algeria. A voucher specimen (LF98B0501) was deposited in the herbarium of University Mentouri of Constantine.

The dried plant material [309 g of leaves (L), 950 g of roots (R), and 1174 g of flowers (FL)] was exhaustively extracted with 80% EtOH and then evaporated under reduced pressure to yield 25.5 g (8.2% w/w), 78.4 g (8.2% w/w) and 181.4 g (15.4% w/w) of extracts, respectively (Table I).

Table I. Yield (g) of successive liquid/liquid extractions with solvents of increasing polarity of leaves (L), flowers (FL), and roots (R).

Solvent	L	FL	R
80% EtOH	25.5	181.4	78.4
C_6H_{12}	2.5	2.1	2.5
CH_2Cl_2	1.2	4.7	4.5
EtOAc	4.2	10.2	7.3
BuOH	8.6	45.7	20.4

Extraction and bioguided fractionation

Crude hydro-alcoholic extracts were extracted by liquid/liquid partition with solvents of increasing polarity: C_6H_{12}, CH_2Cl_2, EtOAc and finally *n*-BuOH (Fig. 1). The yield of each extract is reported in Table I. The comparison of their antiradical activity using the DPPH$^\bullet$ test showed that the EtOAc leaves extract was the most active (Table II); thus, fractionation of the EtOAc leaves extract was pursued on a silica gel open column (150 g, mesh 60 cm × 3 cm) using stepwise gradient elution (50 mL/fraction): C_6H_{12} (3 L); 1:1 (v/v) C_6H_{12}/$CHCl_3$ (1.5 L); $CHCl_3$ (2 L); 5% $CHCl_3$ in MeOH (400 mL); 10% $CHCl_3$ in MeOH (250 mL); 15% $CHCl_3$ in MeOH (200 mL); 20% $CHCl_3$ in MeOH (300 mL); 30% $CHCl_3$ in MeOH (300 mL); 40% $CHCl_3$ in MeOH (300 mL); and finally 50% $CHCl_3$ in MeOH (400 mL). Fractions were combined according to their TLC profiles (silica gel F_{254}; 5554, Merck) using EtOAc/MeOH/H_2O (100:17:13) (v/v/v) as eluent system. Plates were then revealed with NeuPEG (1% methanolic diphenylboric acid/β-ethanolamino ester/polyethylene glycol) reagent and detected at 254 and 366 nm to afford 7 major fractions (Fig. 1). According to the antiradical activity of the seven fractions tested by the DPPH assay, purification was continued on fractions 80–196 by means of semipreparative C18-HPLC, using 0.01 M H_3PO_4 (phase A) and acetonitrile (phase B) as eluents. The elution was carried out under the following conditions: in 20 min from 5% to 50% B, then in 5 min from 50% B to 100% B. The fractions were monitored at 280 nm (UV detector, Gilson) (volume of injection: 400 μL). The purification afforded 6 subfractions which were controlled by analytical C18-HPLC. Thus, pure compounds obtained were **1** (157 mg), **2** (5 mg), **4** (496.8 mg), **5** (4.7 mg) and **6** (20.4 mg). Subfraction 3 was further purified by Sephadex LH-20 column chromatography (10 g, 30 cm × 1.5 cm) using MeOH (200 mL) as eluent and afforded compounds **3–1** (30 mg) and **3–2** (50 mg) (Fig. 1).

Material

The NMR spectra were recorded on Bruker DRX NMR (200 MHz and 300 MHz) spectrometers. Samples were dissolved in deuterated methanol (CD_3OD). Mass spectroscopy was carried out on an Agilent LC/MSD instrument. Absorbances were measured in a Shimadzu UV 1205 spectrophotometer. Semipreparative C18-HPLC: pump, Gilson 305; UV detector, Gilson 115; column, Nucleodur®, Macherey-Nagel, 250 × 21 mm, 10 μm. Analytical C18-HPLC: pump, Varian 9010; DAD UV detector, Varian Prostar®, Les Ulis, France; column Nucleodur®, Macherey-Nagel, 250 × 4.6 mm, 5 μm. TLC: silica gel F_{254} (Merck), Sephadex LH-20 (Sigma-Aldrich).

Determination of total phenolic content (TPC)

An aliquot of 100 μL of extract was mixed with 2.5 mL of Folin-Ciocalteu phenol reagent and allowed to react for 5 min. Then, 2.5 mL of saturated Na_2CO_3 solution were added and allowed to stand for 1 h before the absorbance of the reaction mixture was read at 725 nm. The TPC of the extract was expressed as mg gallic acid equivalents per gram of plant material on dry weight basis (Parejo *et al.*, 2002).

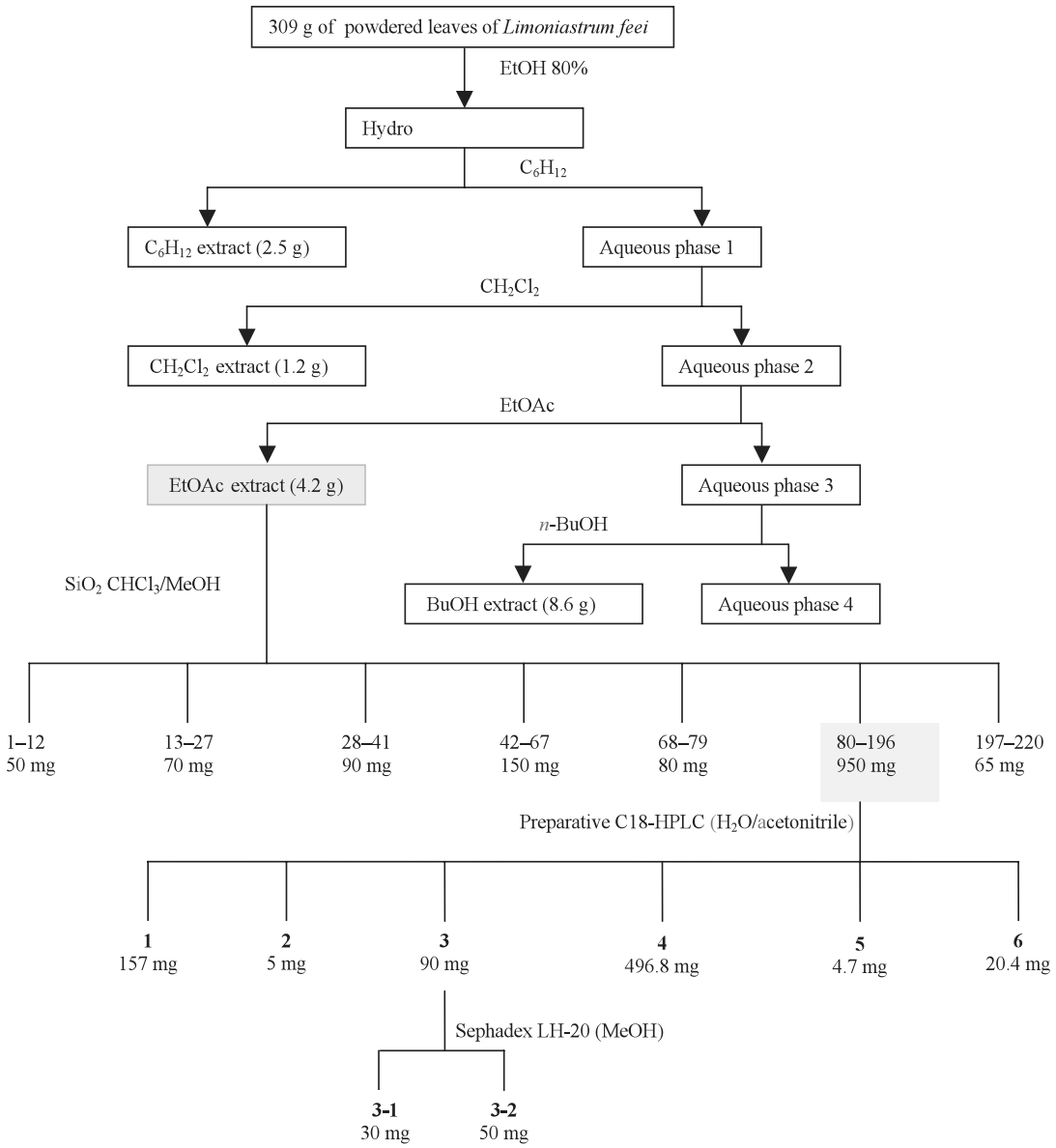


Fig. 1. Scheme of bioguided extraction and purification of *L. feei* EtOAc leaves extract. The same steps were followed to obtain different extracts of roots and flowers (C_6H_{12}, CH_2Cl_2, EtOAc and BuOH).

Free radical scavenging activity

The samples were measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH•. The percentage of DPPH• decolouration of the sample was calculated according to the equation: % decolourization = $[1 - (Abs_{sample}/Abs_{control})] \cdot 100$, where Abs_{sample} is the absorption of the sample and $Abs_{control}$ is the absorption of the blank without sample. Decolouration was plotted against the sample extract concentration, and a logarithmic regression curve was established in order to calculate the IC_{50} value, which is the amount of sample necessary to decrease the absorbance of DPPH• by 50% (Parejo *et al.*, 2002).

Superoxide anion scavenging activity

The superoxide radicals were generated *in vitro* by the hypoxanthine/xanthine oxidase system. The scavenging activity of the extract was determined by the NBT reduction method. In this method, $O_2^{\bullet -}$ reduces the yellow dye NBT^{2+} to produce the blue formazan, whose absorbance was measured spectrophotometrically at 560 nm. Antioxidants are able to inhibit the formation of purple NBT. The results are expressed as the percentage inhibition of the NBT reduction with respect to the reaction mixture without a sample (buffer only) and were calculated according to the equation: % inhibition = $\{[(C_{abs} - CB_{abs}) - (S_{abs} - SB_{abs})]/(C_{abs} - CB_{abs})\} \cdot 100$, where S_{abs}, SB_{abs}, C_{abs}, and CB_{abs} are the absorbance of the sample, the blank sample, the control, and the blank control, respectively (Parejo *et al*., 2002).

Ferric reducing ability of plasma (FRAP)

The ability to reduce ferric ions was measured using a modified version of a previously described method (Benzie and Strain, 1996). An aliquot (200 μL) of an extract (with appropriate dilution, if necessary) was added to 3 mL of FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10 mM TPTZ solution and 1 part of 20 mM $FeCl_3 \cdot 6H_2O$ solution), and the reaction mixture was incubated in a water bath at 37 °C. The increase in absorbance at 593 nm was measured after 30 min. The antioxidant capacity based on the ability to reduce ferric ions of the extract was expressed as μM equivalent to Fe^{2+}/mL.

Results and Discussion

The comparison of the antiradical activities of crude hydro-alcoholic extracts of *L. feei* showed that the leaves (L) extract was 5- and 8-fold more active than the flower (FL) and root (R) extracts, respectively (Table II). In general, EtOAc extracts

Table II. Radical scavenging activities (DPPH test) of crude 80% EtOH extract along with CH_2Cl_2, EtOAc, BuOH extracts from roots (R), flowers (FL) and leaves (L) of *Limoniastrum feei*. Results are expressed as IC_{50} (μg/mL) values, mean ± SD (standard deviation), of triplicate determinations.

Extract	IC_{50} (R)	IC_{50} (FL)	IC_{50} (L)
80% EtOH	3.3 ± 1.43	1.75 ± 0.74	0.38 ± 0.06
CH_2Cl_2	44.04 ± 12.37	5.75 ± 0.74	8.67 ± 4.49
EtOAc	1.44 ± 0.08	1.63 ± 0.32	1.21 ± 0.19
BuOH	3.93 ± 0.89	2.82 ± 0.24	1.30 ± 0.24

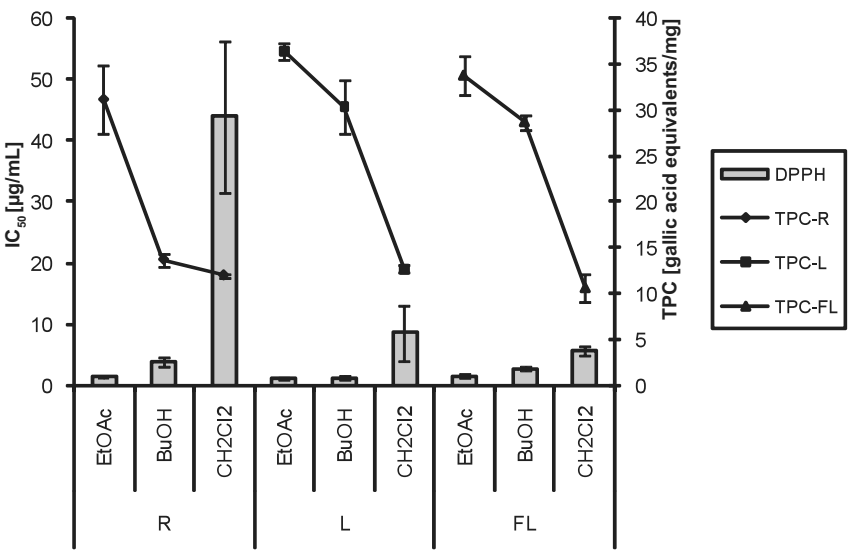


Fig. 2. Correlations between total phenolic content and antiradical activity of different extracts (CH_2Cl_2, EtOAc, BuOH) obtained from roots, leaves and flowers of *L. feei*. TPC, total phenolic content (gallic acid equivalents/mg dry weight); DPPH assay expressed in IC_{50} (μg/mL); R, root extract (R = 0.6); L, leaves extract (R = 0.97); FL, flower extract (R = 0.99).

were more active than CH_2Cl_2 and BuOH extracts (Table II), suggesting that they were more enriched in antiradical compounds. Indeed, TLC and HPLC profiles of EtOAc extracts of L and FL were quite similar and showed that they contain mainly flavonoids and phenolic acids (data not shown). These observations were strengthened by linear regression analysis of the antiradical activity and total phenolic content (Fig. 2). A good correlation between the antiradical effect and the total phenolic content of extracts (CH_2Cl_2, EtOAc and BuOH) was observed with $R = 0.97$ and 0.99 for L and FL extracts, respectively, which suggests that polyphenols present in these extracts are responsible for the scavenging activity. However, there is a mild correlation between the phenolic content and the antioxidant activity in R extracts ($R = 0.6$). Moreover, the excellent linear correlations between the "total phenolic profiles" and "the antioxidant activity" is not surprising if one considers similar chemistry of the two assays which are based on the electron transfer mechanism (Huang *et al*., 2005; Prior *et al*., 2005). Other studies have shown a high correlation between the total antioxidant activity and phenolic contents (Parejo *et al*., 2002; Guo *et al*., 1997).

Flavonoids are potent antioxidants *in vitro*, but antioxidation is, however, only one of the many mechanisms through which flavonoids can exert their actions (Erlund, 2004). Indeed, the actions ascribed to polyphenols are almost certainly mediated partly by their free radical scavenging, antioxidant and metal-complexing actions (Bahorun *et al*., 2004; Rice-Evans *et al*., 1996).

The anti-DPPH activity of the *L. feei* EtOAc leaves extract [$IC_{50} = (1.21 \pm 0.19)\,\mu g/mL$] was compared with that of corresponding extracts prepared from some medicinal plants: *Achillea millefolium*, *Artemisia dracunculus*, *Foeniculum vulgare*, *Lavandula latifolia*, *Melilotus officinalis* [$IC_{50} = (9.29 \pm 0.2)\,\mu g/mL$; $(10.87 \pm 0.3)\,\mu g/mL$; $(12.06 \pm 1.66)\,\mu g/mL$; $(24.26 \pm 1.68)\,\mu g/mL$; $(101.73 \pm 14.39)\,\mu g/mL$, respectively] (Parejo *et al*., 2002), and highlighted that *L. feei* was far more active. Thus, activity-guided fractionation using the DPPH assay was continued on the EtOAc leaves extract, which gave 7 fractions, which were combined according to their TLC profiles (Fig. 1). These fractions were again tested for their antiradical power. Fractions 80–196 were as active as fractions 197–220 (Table III) and possessed all the compounds of the latter fraction (data not shown). Furthermore, fractions 80–196 were 14-times in weight more than fractions 197–220 (Fig. 1); thus, purification was pursued on fractions 80–196. The purification of the 6 major peaks of this fraction was carried out by semipreparative HPLC and Sephadex LH-20 chromatography and afforded 7 pure compounds (Fig. 1) identified by combined spectroscopic techniques (1D and 2D NMR, ESI-MS, UV and co-injection with authentic standards) (copies of the original spectra are obtainable from the author of correspondence). These are gallic acid (**1**), myrciaphenone A (**2**), myricetin-3-*O*-β-galactopyranoside (**3–1**), epigallocatechin gallate (EGCG) (**3–2**), myricetin 3-*O*-α-rhamnopyranoside (**4**), quercetin (**5**) and myricetin (**6**) (Fig. 3). These polyphenolic compounds are encountered in different *Limonium* species like *L. axillare* (Ahmed *et al*., 1999; Bashir *et al*., 1994), *L. brasiliense* (Murray *et al*., 2004), *L. wrightii* (Aniya *et al*., 2002), and *L. sinense* (Lin and Chou, 2000). Thus, our results show a close chemotaxonomic relationship between *Limonium* and *Limoniastrum*.

Table III. Radical scavenging activities (DPPH test) of 7 major fractions obtained after silica gel fractionation of an EtOAc leaves extract. Results are expressed as IC_{50} ($\mu g/mL$) values, mean ± SD (standard deviation), of triplicate determinations.

Fractions	IC_{50} [$\mu g/mL$]
1– 12	NS
13– 27	NS
28– 41	30.93 ± 17.84
42– 67	9.07 ± 2.47
68– 79	18.35 ± 4.42
80–196	1.74 ± 0.13
197–220	1.44 ± 0.09

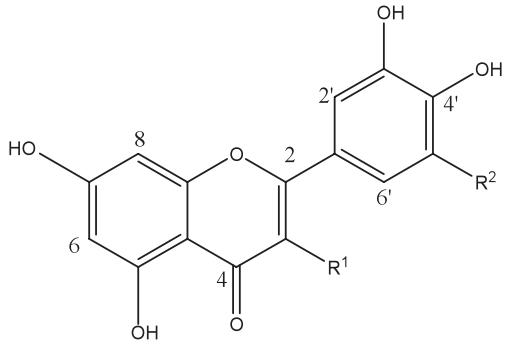
NS, not significant ($IC_{50} > 100\,\mu g/mL$).

The antioxidant activities of pure compounds were compared with the activities of the EtOAc extract and ascorbic acid taken as the reference antioxidant. Therefore, in the DPPH assay (Table IV), all compounds isolated, with the exception of **2**, showed a potent antioxidant effect, where gallic acid was as active as the reference antioxidant ($IC_{50} = 0.94\,\mu g/mL$). In fact, compounds **1**, **3–2** and **5** are routinely used as reference drugs in antioxidant tests (Erlund, 2004). In the same way, gallic acid was also the most active compound in the FRAP assay (Table IV); moreover, it was 2-fold more active than the reference compound ascorbic acid, but the other compounds **4–6** also had strong

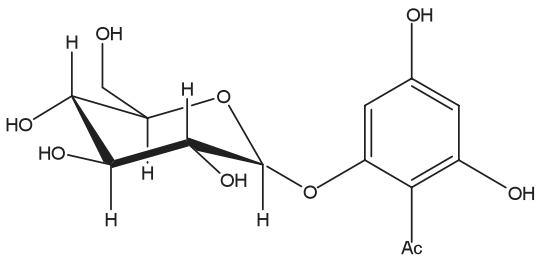
reducible power ($IC_{50} < 2\,\mu g/mL$). Another research team has found that gallic acid was the most active compound in DPPH and FRAP assays in comparison with other antioxidants (Schlesier *et al*., 2002). Some studies have indicated that phenolic substances, such as flavonoids and phenolic acids, are considerably more potent antioxidants than vitamins C and E (Cao *et al*., 1997; Vinson *et al*., 1995). Gallic acid (**1**) has been identified as the active compound of the water extract of *Limonium wrightii* with a strong free radical scavenging action (Bashir *et al*., 1994). It is noteworthy that gallic acid is the major compound in black tea (Hodgson *et al*., 2000). Black teas are also rich in EGCG, quercetin and myricetin (Ahmed *et al*., 2002; Luximon-Ramma *et al*., 2005). These compounds are well recognized as being responsible for the antioxidant properties of tea (Murray *et al*., 2004). In the superoxide scavenging activity assay (Table IV), unlike the two last tests, myricetin was the most antioxidant compound (IC_{50} = 1.86 μg/mL); it was 3-fold more active than ascorbic acid. Compounds **1**, **3–5** were also a little or a lot more active than the reference vitamin C.

Owing to the complexity of the oxidation/antioxidation processes, it is obvious that no single testing method is capable of providing a comprehensive picture of the antioxidant profile of a studied sample (Parejo *et al*., 2002; Prior *et al*., 2005). In addition, the total antioxidant capacity needs to reflect both lipophilic and hydrophilic capacity, and at least for physiological activity it needs to reflect and differentiate both the hydrogen atom transfer (radical quenching) and the electron transfer (radical reduction). Moreover, to fully elucidate its antioxidant capacity, tests evaluating the effectiveness against various reactive oxygen species (ROS)/reactive nitrogen species (RNS), such as $HO^{\bullet}$ and $ONOO^{\bullet -}$ are needed, and this may require future development of additional methods specific to each radical source (Prior *et al*., 2005).

In conclusion, *Limoniastrum feei* is a natural source rich in polyphenols (gallic acid, quercetin, myricetin and EGCG) possessing strong antioxidant activity, which could have a direct action on different diseases in relation with ROS/RNS.



	R^1	R^2
3–1	O–Glu	OH
4	O–Rha	OH
5	OH	H
6	OH	OH



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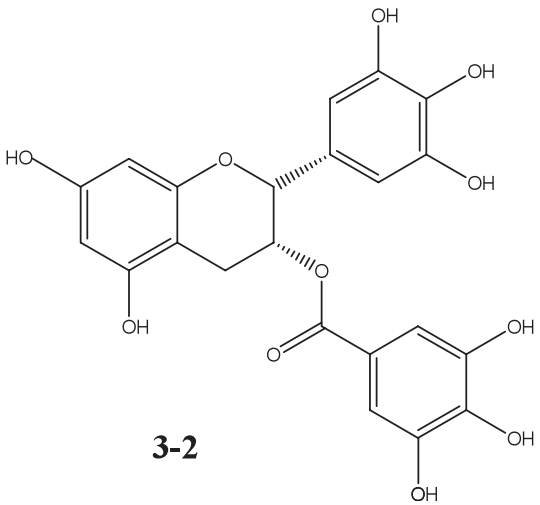

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Fig. 3. Chemical structure of compounds **2**–**6**.

Sample	DPPH[a]	FRAP[b]	Superoxide[a]
EtOAc extract	1.21 ± 0.19	2.25 ± 0.12	3.61 ± 0.56
Gallic acid (**1**)	0.94 ± 0.68	0.83 ± 0.15	3.49 ± 0.99
Myrciaphenone A (**2**)	60.92 ± 3.34	7.61 ± 1.68	NS >>10
Myricetin-3-*O*-*β*-galactopyranoside (**3–1**)	2.84 ± 0.69	2.89 ± 0.02	2.85 ± 0.2
Epigallocatechin gallate (**3–2**)	2.54 ± 0.45	2.2 ± 0.06	2.1 ± 0.08
Myricetin 3-*O*-*α*-rhamnopyranoside (**4**)	1.82 ± 0.26	1.38 ± 0.39	3.83 ± 0.82
Quercetin (**5**)	2.23 ± 0.13	1.48 ± 0.19	5.79 ± 0.49
Myricetin (**6**)	3.27 ± 0.55	1.60 ± 0.14	1.86 ± 0.12
Ascorbic acid	0.59 ± 0.12	1.62 ± 0.62	5.8 ± 0.79

Table IV. Radical scavenging activities (DPPH, FRAP and superoxide) of 7 pure polyphenols purified by semipreparative C18-HPLC in comparison with their respective EtOAc extract. Results are expressed as mean ± SD (standard deviation) of triplicate determinations.

[a] Values expressed as IC_{50} [μg/mL].
[b] Values expressed as EC1 in μM equivalent to Fe^{2+}/mL.

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