Phenolic Compounds from *Limonium pruinosum*

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A new phenolic compound, 6'-*O*-sulfonyl-salidroside (**1**), together with fifteen known compounds, were isolated from the all parts of *Limonium pruinosum*. Their structures were elucidated by 1D and 2D NMR experiments including 1D TOCSY, DQF-COSY, HSQC and HMBC spectroscopy, as well as ESIMS analysis. The antioxidant activity of the extracts and all isolated compounds was evaluated by DPPH test.

**Keywords:** *Limonium pruinosum*, Plumbaginaceae, Phenolic compounds, NMR.

The genus *Limonium* (Plumbaginaceae), also known as "Sea Lavender", "Statice", or "Marsh-rosemary", comprises about 300 species with a worldwide distribution. Some species are endemics in the Mediterranean region [1]. All species are herbaceous perennial plants with varying degree of colors from white to pink, produced on a branched panicle or corymb. *Limonium* species are traditionally used for the treatment of infections, fever, hemorrhage and other disorders [2]. Previous phytochemical studies of this genus led to the isolation and characterization of flavonoids, carbonyl compounds, hydrocarbons, fatty acids, naphthoquinone, tannins, alkaloids and amino acids [3]. Different pharmacological activities have been reported such as: antiviral, antitumor, antipyretic, hemostatic, depurative, antifungal and antimicrobial [4]. *L. pruinosum* Kuntze is among the plants that feed camels in the arid region of southern Tunisia. The antioxidant activity of *L. pruinosum* crude extract was previously reported, but phytochemical investigations have never been reported [5]. In this paper, we describe the isolation and structural identification by spectroscopic and spectrometric techniques of a new compound, together with known compounds, and the antioxidant activity of the extracts and pure compounds [6].

The ethanolic-aqueous extract of *L. pruinosum* whole plant was successively partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol, in the amounts 0.3, 0.8, 6.0 and 12.0 g, respectively. The free-radical scavenging activity of *Limonium* extracts was evaluated by DPPH test [6,7]. The polar ethyl acetate and *n*-butanol extracts showed a high potency in scavenging DPPH (EC_{50} 5.33 and 11.64 µg/mL, respectively) (Table 1), which correlated with their total phenolic content (251.7 and 158.8 µg/mg, respectively, and expressed as gallic acid equivalents). The other extracts did not exhibit activity in this assay up to 400 µg/mL. Fractionation and purification of ethyl acetate and *n*-butanol extracts by a combination of chromatographic techniques led to the isolation of one new compound **1** and fifteen known compounds (**2-16**).

Table 1: Total phenol content, and free-radical scavenging activity (DPPH test) of *L. pruinosum* extracts and compounds.

Extract or Compound	Phenolic content (µg/mg extract)[a]	DPPH test [IC_{50} (µg/mL)][b]
ext- Ethyl acetate	251.7 ± 3.7	5.3 ± 0.2
ext- *n***-BuOH**	158.8 ± 2.6	11.6 ± 0.6
1		10.6 ± 1.5
2		10.8 ± 1.7
3		13.3 ± 2.0
4		7.2 ± 0.5
5		1.5 ± 0.2
6		17.0 ± 1.5
7		156.2 ± 5.4
8		1.2 ± 0.09
9		1.9 ± 0.1
10		7.4 ± 0.6
11		35.7 ± 2.9
12		227.8 ± 6.7
13		7.0 ± 0.4
14		17.0 ± 1.8
15		16.0 ± 1.0
16		103.3 ± 4.4
α-tocopherol[c]		10.1± 1.3
L-ascorbic Acid[c]		5.8± 0.9

[a]gallic acid equivalents. [b]Mean ± S.D. of three determinations. [c]Positive control.
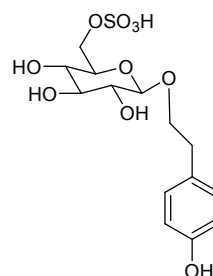
Figure 1: Compound **1**.

The NMR and MS data of compound **1** demonstrated the molecular formula $C_{14}H_{20}O_{10}S$ (HRESIMS at *m/z* 379.3597 [M-H]⁻), which suggested the presence of a sulfate group in the molecule. Acid hydrolysis of **1** followed by treatment with $BaCl_2$, confirmed the

Table 2: NMR data of compound **1** (CD$_3$OD, 600 MHz)[a].

position	δ$_H$	δ$_C$
	1	
1		155.2
2	6.72 d (8.0)	115.0
3	7.29 d (8.0)	130.1
4		129.6
5	7.29 d (8.0)	130.1
6	6.72 d (8.0)	115.0
7	2.86 t (6.7)	35.9
8	4.02, m	70.0
	3.73, m	
1'	4.32	102.9
2'	3.23 dd (8.5, 9.0)	74.1
3'	3.38 t (9.5)	76.7
4'	3.36 t (9.5)	70.4
5'	3.50 m	74.9
6'	4.35 dd (12.0, 2.5)	67.0
	4.16 dd (12.0, 5.0)	

[a]J values are in parentheses and reported in Hz; chemical shifts are given in ppm; assignments were confirmed by COSY, HSQC and HMBC experiments.

presence of a sulfate group. The ESI-MS in negative ion mode showed the [M-H]$^-$ ion peak at m/z 379, and fragments in the MS/MS analysis at m/z 241 [M-H-C$_8$H$_{10}$O$_2$]$^-$), and at m/z 97 corresponding to the sulfate group for the loss of a sugar moiety. The ^{1}H NMR spectrum (Table 2) showed signals of four aromatic protons [δ$_H$ 7.29 (2H, d, J = 8.0 Hz, H-2/6), 6.72 (2H, d, J = 8.0, H-3/5)], and of four methylene protons at [δ$_H$ 2.86 2H, br t, J = 6.7 Hz), 4.02 (1H, m), 3.73 (1H, m)]. The ^{1}H NMR spectrum also showed the presence of a β-glucopyranosyl unit (δ$_H$ 4.32, 1H, d, J = 7.8 Hz). A combination of 1D-TOCSY and COSY experiments provided evidence for the presence in the molecule of the segments H-1'–H$_2$-6', H-7–H-8, and H-2/5–H-3/6. The elucidation of the whole basic carbon skeleton from the above subunits was achieved on the basis of a series of ^1J (HSQC) and ^3J (HMBC) correlations. The HMBC spectrum exhibited correlations between the proton signal at δ$_C$ 2.86 and the carbons at δ$_C$ 129.6 (C-1) and 130.2 (C-2 and C-5) confirming the position of the 4-hydroxyphenylethanol structure. The location of glucopyranose was obtained on the basis of the HMBC correlations between the proton signal at δ$_H$ 4.32 (H-1') and the carbon signal at 70.0 (C-1') ppm, and between the proton signals at δ 4.02 and 3.73 (H$_2$-8) and the carbon signal at 102.9 ppm [8]. The position of the sulfate group was established at C-6 of the glucose unit on the basis of the downfield shifts of H$_2$-6' (δ$_H$ 4.35, dd, J = 12.0, 2.5 Hz; and 4.16, dd, J = 12.0, 5.0 Hz) and of C-6' δ$_c$ 67.0, consistent with the presence of an ester moiety [9]. Thus compound **1** was established as the new 6'-*O*-sulfonyl-salidroside.

The fifteen known compounds were identified as 1,6-*O*-digalloyl-β-D-glucopyranose (**2**) [10], myricetin-3-*O*-galactopyranoside (**3**), myricetin-3-*O*-(6''-galloylglucoside) (**4**) [11], *trans*-N-caffeoyl-tyramine (**5**) [1], myricetin 3-*O*-glucopyranoside (**6**) [11], kaempferol 3-*O*-rhamnopyranoside (**7**), gallic acid (**8**), methyl gallate (**9**), limoniastramide (**10**) [1], avicularoside (**11**) [2], kaempferol (**12**) [12], myricetine-3-*O*-arabinofuranoside (**13**) [4], quercitin-3-*O*-galactopyranoside (**14**), quercitin-3-*O*-rhamno-pyranoside (**15**) [11], and astragalin 6''-*O*-gallate (**16**) [12] by detailed NMR and MS analyses and comparison with literature data.

In order to identify the compounds responsible for the observed activity of *Limonium* crude extracts, the free radical scavenging activity of the new compound **1** and the known compounds (**2-16**) was evaluated. The new sulfate phenylpropanoid glycoside (**1**) showed a DPPH scavenging activity superimposable with that of the positive control α-tocopherol (EC$_{50}$ 10.6 and 10.1 μg/mL, respectively,) and about two fold lower than that of L-ascorbic acid (EC$_{50}$ 5.85 μg/mL). Gallic acid (**8**), methyl gallate

(**9**), and *trans*-N-caffeoyltyramine (**5**) were the most active compounds (EC$_{50}$ 1.20, 1.92, and 1.50 μg/mL, respectively). Also the dimer of the phenolic acid amide, limoniastramide (**10**) displayed a strong capacity to scavenge the free radical DPPH (EC$_{50}$ 7.4 μg/mL). Moreover, myricetin and quercetin derivatives (**3**, **4**, **6**, **11**, **13-15**) exhibited considerable antioxidant activities with EC$_{50}$ values ranging from 7.0 to 35.7 μg/mL. On the other hand, kaempferol (**12**) and its derivatives (**7**, **16**) had a weak free radical scavenging activity.

Experimental

General experimental procedures: Optical rotations were measured on a Perkin-Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 1 dm microcell. UV spectra were recorded on a Perkin-Elmer-Lambda spectrophotometer. NMR experiments were performed on a Bruker DRX-600 spectrometer at 300 K. All the 2D NMR spectra were acquired in CD$_3$OD. Standard pulse sequences and phase cycling were used for DQF-COSY, TOCSY, HSQC, and HMBC experiments. HRESIMS were acquired in positive ion mode on a Q-TOF premier spectrometer equipped with a nanoelectrospray ion source (Waters-Milford, MA, USA). Column chromatography was performed over Sephadex LH-20. HPLC separations were conducted on a Shimadzu LC-8A series pumping system equipped with a Shimadzu RID-10A refractive index detector and Shimadzu injector on a C$_{18}$μ-Bondapak column (30 cm x 7.8 mm, 10 μm Waters, flow rate 2.0 mL min^{-1}) [13].

Plant material: The whole plant of *L. pruinosum* was collected during the flowering period, in April 2012, in the region of Bechar, southwest Algeria. The plant was identified by Prof. Mouhammed Kaabache of the University of Setif, Algeria. A voucher specimen, number 116LP/VAREBIOL/412, was deposited in the Herbarium of the Chemistry Department at the University of Constantine 1, Algeria.

Extraction and isolation: The whole plant of *L. pruinosum* (1.8 Kg) was powdered and successively extracted by exhaustive maceration in ethanol (80%), filtered and the solvent removed under vacuum at 40°C in a rotary evaporator obtaining 27.3 g of dried extract. Part of this (25.0 g) was partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol to yield 0.3, 0.8, 6.0 and 12 g of the respective residues. The BuOH extract was successively partitioned between *n*-BuOH and H$_2$O. A portion (2.5 g) of the *n*-BuOH-soluble fraction was separated by Sephadex LH-20 with methanol as eluent (flow rate 1.1 mL/min). Fractions of 10 mL were collected, analyzed by TLC on silica 60 F254 gel-coated glass sheets with CHCl$_3$:MeOH:H$_2$O (40:9:1 v/v/v) and *n*-BuOH–AcOH–H$_2$O (60:15:25 v/v/v) and grouped to obtain 8 fractions (A–H). Fraction D (432 mg) was purified by RP-HPLC using MeOH–H$_2$O (15:85) to give compound **1** (3 mg, t_R 10 min). Fraction E (226 mg) was purified by RP-HPLC using MeOH–H$_2$O (1:4) to give 1,6-*O*-digalloyl-β-D-glucopyranose (**2**) (1.4 mg, t_R 22 min) and myricetin-3-*O*-galactopyranoside (**3**) (1.4 mg, t_R 37 min). Fraction G (70 mg) was a pure compound myricetin 3-*O*-(6''-galloylglucoside) (**4**). All the ethyl acetate extract (6.0 g) was subjected to CC using silica gel and eluting with CH$_2$Cl$_2$, followed by increasing concentrations of MeOH (between 1% and 100%) Fractions of 50 mL were collected, analyzed by TLC (silica gel plates, in CH$_2$Cl$_2$ and mixtures CH$_2$Cl$_2$−MeOH) and grouped into 7 fractions (A−G). Fraction B (82 mg) was purified by RP-HPLC using MeOH–H$_2$O (3:7) to give *trans*-N-caffeoyltyramine (**5**) (1.4 mg, t_R 90 min). Fraction C (92 mg) was purified by RP-HPLC using MeOH–H$_2$O (45:55) to give myricetin 3-*O*-glucopyranoside (**6**) (1.1 mg, t_R 12 min) and kaempferol 3-*O*-rhamnopyranoside (**7**) (2.1 mg, t_R 60 min). Fraction

D (217 mg) was purified by RP-HPLC using MeOH–H$_2$O (2:3) to give gallic acid (**8**) (2.7 mg, t_R 4 min), methyl gallate (**9**) (1.7 mg, t_R 6 min), limoniastramide (**10**) (1.6 mg, t_R 30 min), avicularoside (**11**) (1.8 mg, t_R 55 min) and kaempferol (**12**) (22.0 mg, t_R 55 min). Fraction E (422 mg) and F (430 mg) were separately purified by RP-HPLC using MeOH–H$_2$O (35:65) to give methyl gallate (**9**) (2.2 mg, t_R 13 min), myricetin-3-*O*-arabinofuranoside (**13**) (8.2 mg, t_R 47 min), quercetin-3-*O*-galactopyranoside (**14**) (2.2 mg, t_R 52 min) and quercetin-3-*O*-rhamnopyranoside (**15**) (2.8 mg, t_R 13 min) from fraction E, and myricetin-3-*O*-galactopyranoside (**3**) (1.5 mg, t_R 40 min), quercetin-3-*O*-galactopyranoside (**14**) (3.4 mg, t_R 52 min), and astragalin 6”-*O*-gallate (**16**) (1.3 mg, t_R 64 min) from fraction F. The purity of each compound was determined by NMR and HPLC.

Compound 1
[α]$_D$:-20.2.4 (*c* 0.11, MeOH).
^{1}H and ^{13}C NMR: Table 2.
ESIMS *m/z*: 379 [M + H]$^-$, 241 [(M – H) –C$_8$H$_{10}$O$_2$]$^-$,
HRESIMS *m/z*: 379,3597 [M-H]$^-$ (calcd for C$_{14}$H$_{20}$O$_{10}$S, 379.3577).

Quantitative determination of total phenols: Ethyl acetate and *n*-butanol extracts of *L. pruinosum*, dissolved in MeOH, were analyzed for their total phenolic content according to the Folin-Ciocalteau colorimetric method. Total phenols were expressed as gallic acid equivalents (μg/mg extract) [14].

Bleaching of the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH• test): The antiradical activities of *L. pruinosum* extracts, pure compounds (**1-16**), α-tocopherol, and L-ascorbic acid were determined using the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH•) and the procedures described by Mencherini *et al.* [15]. In its radical form, DPPH• has an absorption band at 517 nm, which disappears upon reduction by an antiradical compound. An aliquot (37.5 μL) of the MeOH solution containing different amounts of the extract or compounds from *L. pruinosum* was added to 1.5 mL of daily prepared DPPH• solution (0.025 g/L in MeOH); the maximum concentration employed was 400 μg/mL. An equal volume (37.5 μL) of the vehicle alone was added to the control tubes. Absorbance at 517 nm was measured on a Shimadzu UV-1601UV–visible spectrophotometer 10 min after starting the reaction. The DPPH• concentration in the reaction medium was calculated from a calibration curve analyzed by linear regression. The percentage of remaining DPPH• (%DPPH• REM) was calculated as follows:

$$\% \text{ DPPH• REM) } [\text{DPPH•}]T/[\text{ DPPH•}]0 \times 100,$$ where T is the experimental duration time (10 min).

α-Tocopherol and L-ascorbic acid were used as positive controls in the test. All experiments were carried out in triplicate, and the mean effective scavenging concentrations (EC$_{50}$) were calculated by using the Litchfield and Wilcoxon test. Results are reported in Table 1.

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