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Sulfated phenolic compounds from *Limonium caspium*: Isolation, structural elucidation, and biological evaluation

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ABSTRACT

Three new compounds, (2S,3S)-5-methyldihydromyricetin (**1**), (2S,3S)-5-methyldihydromyricetin-3'-O-sulfate (**2**) and β-D-glucopyranoside, 3-methyl, but-3-en-1-yl 4-O-α-L-rhamnopyranosyl (**3**) have been isolated from the *Limonium caspium*, together with dihydromyricetin (**4**), dihydromyricetin-3'-O-sulfate (**5**), myricetin-3'-O-sulfate (**6**), 5-methylmyricetin (**7**), myricetin (**8**), myricetin-3-O-β-glucoside (**9**), as well as phloridzin (**10**), and tyramine (**11**). Compounds **5** and **6** were isolated for the first time as acids. This is the first report of all these compounds from this plant. Their structures were established by extensive NMR studies (¹H NMR, ¹³C NMR, DEPT, ¹H-¹H COSY, HSQC, HMBC) as well as HRESIMS. All isolated compounds were evaluated for their antibacterial, antifungal, antimalarial and antileishmanial activities. Compounds **7**, **8** and **9** exhibited good antifungal activity against *Candida glabrata* with IC₅₀ values of 6.79, 15.37 and 8.53 μg/mL, respectively. Compound **8** displayed significant antimalarial activity against resistant and sensitive strains of *Plasmodium falciparum* with IC₅₀ values of 1.82 and 1.51 μg/mL, respectively. Compounds **1**, **4**, **6**, **8** and **9** showed excellent activity against *Trypanosoma brucei* with IC₅₀ values of 6.93, 9.65, 8.52, 7.67 and 6.31 μg/mL, respectively. To date, this is the first report on the phytochemical and biological activity of secondary metabolites from *L. caspium*.

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1. Introduction

Ethnopharmacology, the study of plants traditionally used for medical purposes with the aim of discovering their pharmacological properties, is a promising area for the discovery of novel medicinal compounds [1]. Salt tolerant plants, known as halophytes, for example, have traditionally been used to treat a wide variety of medical conditions, including viruses and other diseases and symptoms of aging, and continue to be used in rural areas for these therapeutic purposes [2]. Among the more than 2500 known salt-tolerant halophytes there are several that could be useful as cash crops. *Cakile maritima* has various potential medical uses, including antiscorbutic, diuretic, purgative and digestive properties [3]. The *Limonium* genus of salt-tolerant halophytes has a number of potentially useful species. As examples, *Limonium brasiliense* has been reported to possess anti-inflammatory and anti-bacterial qualities [4], *Limonium wrightii* is reported to treat arthritis and fever [5], *Limonium tetragonum* and *Limonium sinense* have been reported to

possess antiviral properties [6], and *Limonium axillare* and *Limonium californicum* have been shown to possess antibacterial and cytotoxic effects [7].

As a part of our search for novel, plant-derived biological agents, *Limonium caspium* was studied. Previous phytochemical studies of *Limonium* revealed the presence of several kinds of active chemical components such as polysaccharides, tannins, alkaloids, flavonoids, terpenes, aliphatic compounds, amino acids, and minerals [8]. The present study was aimed to investigate the constituent of *L. caspium* aerial parts. The fractionation, isolation and structural elucidation yielded a total of **11** compounds, which are shown in Fig. 1. All isolated compounds were evaluated for their antimalarial, antileishmanial, antifungal and antimicrobial activities.

2. Results and discussion

In this study the ethanolic extract derived from the aerial parts of *L. caspium* was subjected to multiple chromatographic fractionations over silica gel yielding a series of myricetin derivatives including two new dihydromyricetins: (2S,3S)-5-methyldihydromyricetin (**1**), and (2S,3S)-5-methyldihydromyricetin-3'-O-sulfate (**2**), six previously

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reported myricetins; dihydromyricetin (**4**) [9], dihydromyricetin-3'-O-sulfate (**5**) [11], myricetin-3'-O-sulfate (**6**) [11], 5-methylmyricetin (**7**), myricetin (**8**) [10], myricetin-3- β -glucoside (**9**) [12], and a new β -D-glucopyranoside, 3-methyl, but-3-en-1-yl 4-O- α -L-rhamnopyranosyl (**3**), phloridzin (**10**) [13], and tyramine (**11**) [14] (Fig. 1).

Compound **1** was isolated as a yellowish amorphous solid. The ^1H NMR showed a three proton singlet signal at δ_{H} 3.75, suggesting one methoxy group, and two protons resonating at δ_{H} 6.38 (2H, d, $J = 2.16$ Hz) related to the B-ring (H-2' and H-6'). A dihydromyricetin skeleton was evident from the coupling pattern of C-3 and C-2, which

resonate at δ_{H} 4.16 (1H, d, $J = 10.6$), and δ_{H} 4.81 (1H, d, $J = 10.45$). Analysis of the ^{13}C NMR spectrum and DEPT experiments, confirmed a dihydromyricetin moiety with the presence of a ketone carbonyl signal at δ_{C} 189.8, and a typical non-substituted A-ring with signals at δ_{C} 93.3 and 95.5 for C-8 and C-6, respectively. The ^1H NMR and ^{13}C NMR spectroscopic data of **1** were found to be similar to those of dihydromyricetin (**4**) [9] except for the presence of a methoxy group (Table 1). The nature and identity of this dihydromyricetin were deduced from the NMR experiments (COSY, NOESY, TOCSY, and HSQC). HMBC correlations allowed the complete assignments: the proton at δ_{H} 4.81 (1H, d, $J =$

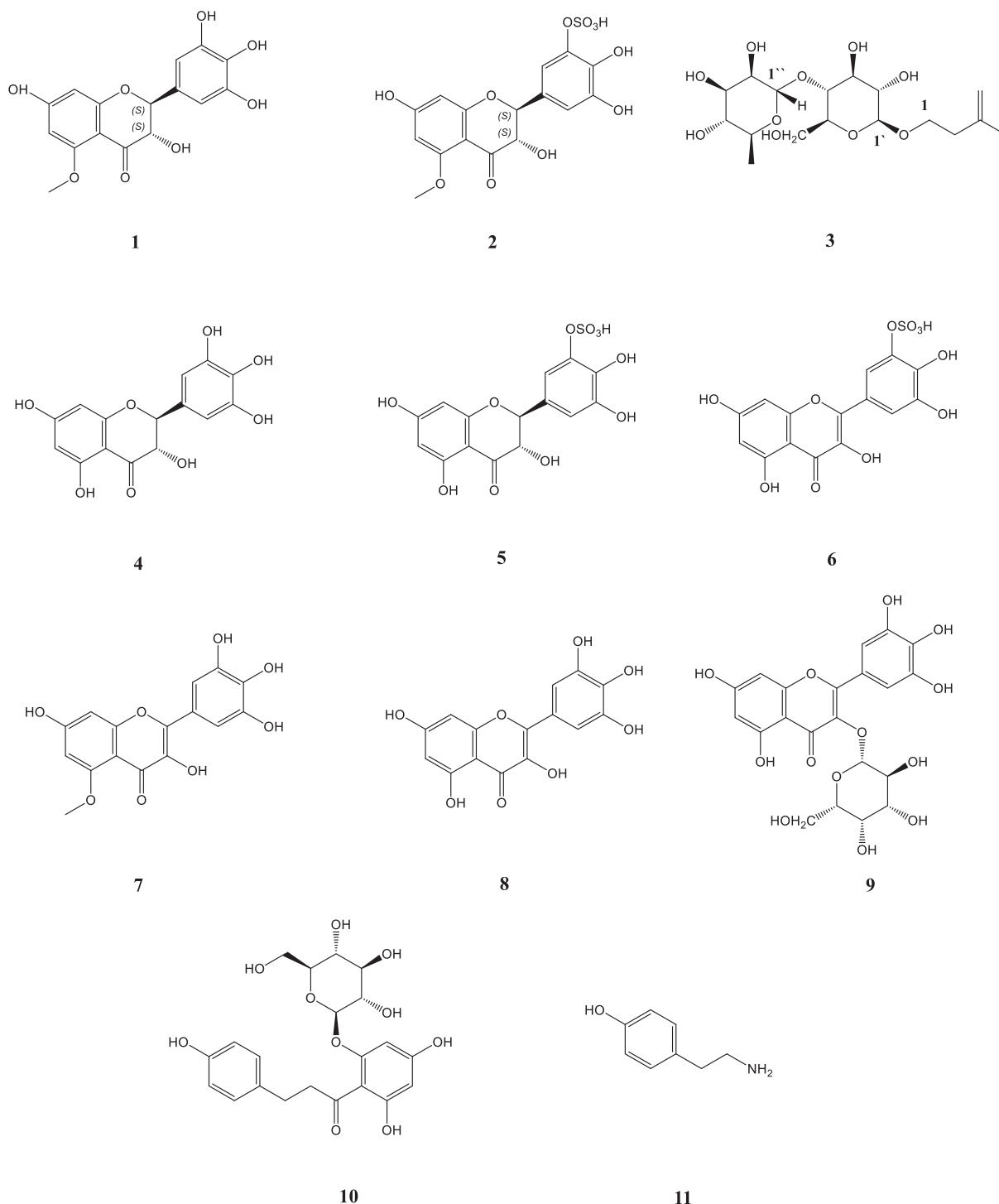


Fig. 1. Compounds isolated from *Limonium caspium*.

Table 1
¹H and ¹³C NMR data for **1** and **4** (in DMSO).

No.	1		4	
	δ_{H} (mult; J in Hz) ^a	δ_{C} ^b	δ_{H} (mult; J in Hz) ^a	δ_{C} ^b
2	4.81 (d, 10.45)	82.7	4.91 (d, 10.8)	83.7
3	4.16 (d, 10.6)	72.9	4.42 (d, 10.72)	72.1
4		189.8		198.0
5		162.2		162.9
6	5.93 (s)	95.5	5.91 (d, 1.8)	96.4
7		164.8		167.2
8	6.08 (s)	93.3	5.86 (d, 1.76)	95.4
9		163.7		163.8
10		102.6		100.9
1'		127.5		127.7
2'	6.38 (s)	106.9	6.40 (s)	107.2
3'		145.8		146.1
4'		133.4		133.8
5'		145.8		146.1
6'	6.38 (s)	106.9	6.38 (s)	107.2
—CH ₃	3.75 (s)	55.8		

^a ¹H NMR measured at 400 MHz.

^b ¹³C NMR measured at 100 MHz [9].

10.45 Hz, H-2) showed strong correlation with the carbon at δ_{C} 106.9 (C-2') and a weak one with δ_{C} 127.5 (C-1'), as well as, the correlation between the signal at δ_{H} 6.38 (2H, s) and the signals at δ_{C} 82.7 (C-2), δ_{C} 145.8 (C-3'), and δ_{C} 133.4 (C-4') (Table 1). The position of the methoxy group assigned through the strong correlation of the signal at δ_{H} 3.75 with the carbon C-5 resonating at δ_{C} 162.2, which together with published data [9], identified this compound (**1**) as 4*H*-1-benzopyran-4-one, 2,3-dihydro-3,5-dihydroxy-5-methoxy-2-(3,4,5-trihydroxyphenyl). Compound **1** had $[M + H]^+$ and $[M - H]^-$ ions in the HRESIMS at m/z 335.0750 and 333.0393 respectively, which is consistent with the molecular formula C₁₆H₁₄O₈. To determine the absolute configuration of the stereogenic center at C-2 and C-3, its ECD spectrum was measured and compared to calculated values for the 2*R*,3*R* and 2*S*,3*S* enantiomers. While both 2*R*,3*S* and 2*S*,3*R* enantiomers were eliminated based on the coupling constant of the signals for H-2 and H-3. The two proton signals of **1** were double and double peaks: 4.81 (1H,

$J = 10.45$ Hz, 2-H), 4.16 (1H, $J = 10.6$ Hz, 3-H), whereas in its 2*R*,3*S* isomer they were both single peaks, which indicated that 2-H and 3-H are either 2*R*,3*R* or 2*S*,3*S* enantiomers [27]. The experimental ECD spectrum showed a positive Cotton effect at 197 nm and a negative Cotton effect at 217 nm (Fig. 2). The B3LYP/6-31G simulated ECD spectrum [28] for 2*S*,3*S* enantiomer, generated from 40 excited states using Gaussian band shapes for the peaks, had peaks at 197 and 217 nm. The calculated ECD of the 2*S*,3*S* enantiomer showed excellent agreement with the experimental data. The compound **1** is a newly reported dihydromyricetin-5-methyl ether, and is named (2*S*,3*S*)-5-methylampelopsin.

Compound **2** was obtained as a pale yellow, amorphous powder. Treating compound **2** with 10% H₂SO₄ and heating on TLC gave a yellowish pink color. The ¹H NMR spectrum (Table 2) of **2** showed a meta-coupled two doublet proton at δ_{H} 6.10 ($J = 1.96$ Hz) and 5.98 ($J = 1.64$ Hz) corresponding to the C-6 and C-8 protons of a flavonoid skeleton. Protons at δ_{H} 7.05 (1H, d, $J = 1.8$ Hz, H-1') and 6.87 (1H, d, $J = 1.8$ Hz, H-6') showed meta-coupled doublets related to a flavonoid B-ring, which suggests that this ring possesses an unsymmetrical 3',4',5'-trisubstituted pattern. The ¹³C NMR spectrum of **2** revealed 15 carbon signals, including δ_{C} 82.9 (C-2), 72.9 (C-3), and 191.4 (C-4), which are characteristic features of a flavanone moiety. Dihydromyricetin (**4**) showed good agreement with the carbon chemical shifts of **2** (Table 2), except those of the B-ring [δ_{C} 128.0 (C-1'), 113.3 (C-2'), 146.3 (C-3'), 138.0 (C-4'), 140.2 (C-5'), and 112.0 (C-6')] and the C-5 of the A-ring. Compound **2** has $[M + H]^+$ ion in the HRESIMS at m/z 414.02053 consistent with the molecular formula C₁₆H₁₄O₁₁S. The structure of **2** was suggested to be 3'-*O*-sulfate-methyldihydromyricetin. The effect of *O*-sulfate as an electron-withdrawing group was observed in the ¹³C NMR spectrum [15]. The carbon atoms ortho to the sulfate group show a down-field shift of 5.1–6.9 ppm, which is the result of decreased electron density. This contrasts with the up-field shift of 3.7 ppm shown by the carbon atom bound to the sulfate group, which results from an increased electron density. These are in line with the chemical shifts calculated in the past for quercetin-3'-sulfate and phloroglucinol sulfates [16]. The

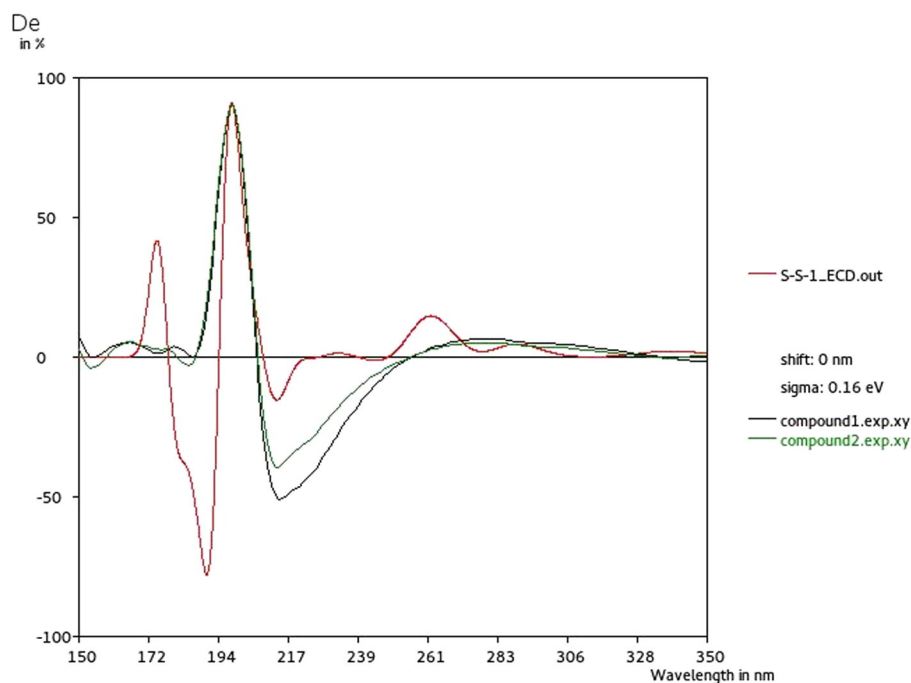


Fig. 2. Experimental (black for **1** and green for **2**) spectrum compared to the calculated (red) ECD spectrum of 2*S*,3*S* enantiomer (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Table 2
¹H and ¹³C NMR data for **2**, **5**, and **6** (in CD₃OD).

No.	6	5	2
	δ_{H} (mult; J in Hz) ^a	δ_{H} (mult; J in Hz) ^a	δ_{H} (mult; J in Hz) ^a
2		147.1	4.91 (d, 11.56)
3		137.6	4.53 (d, 11.64)
4		177.3	196.9
5		162.3	163.9
6	6.19 (s)	99.3	5.93 (d, 2.12)
7		165.6	167.3
8	6.42 (s)	94.5	5.90 (d, 2.2)
9		158.1	96.0
10		104.5	100.5
1'		123.3	127.8
2'	7.80 (s)	115.3	7.04 (d, 2.16)
3'		147.6	113.3
4'		141.1	146.3
5'		141.6	138.0
6'	7.68 (s)	113.5	140.2
—CH ₃		6.86 (d, 2.16)	112.1
			3.83 (s)
			55.0

^a ¹H NMR measured at 400 MHz.^b ¹³C NMR measured at 100 MHz [9].

experimental ECD spectrum showed a negative Cotton effect peak at 217 nm and a positive Cotton effect peak at 197 nm (Fig. 2) which is similar to that of **1**. Based on these results, (2S,3S)-3'-O-sulfate-5-O-methyldihydro-romyricetin was assigned as the structure of **2**. Two other sulfate flavonoids, **5** and **6**, were isolated in acidic form for the first time and were identified by spectroscopic analysis and comparison of the resulting data with the literature [11]. Although sulfated compounds occur ubiquitously in higher plants, it is unclear what physiological significance these compounds have in plants. It is possible that they play a role in neutralizing reactive hydroxyl groups. In addition, because these compounds accumulate in plants which grow in saline conditions, it is possible that they play a role in sequestering sulfate ions [17]. The presence of many sulfated-form flavonoids is well-known in plants [15]. Lignan, (+)-isolarisiresinol-3 α -O-sulfate [18], and the coumarin sulfates [19], both containing sulfated alcohol groups, are examples of other types of phenolic sulfates.

Compound **3** gave [M + Na]⁺ and [M + Cl][−] ions in the HRESIMS at m/z 417.1737 and 429.1544 respectively, consistent with the molecular formula C₁₇H₃₀O₁₀. The ¹H NMR spectrum displayed two anomeric proton signals at δ_{H} 5.19 (1H, s br) and 4.36 (1H, d, J = 7.6 Hz), corresponding to rhamnose and glucose anomeric protons, respectively.

Table 3
¹H and ¹³C NMR data for **3** (in CD₃OD).

No.	3
	δ_{H} (mult; J in Hz) ^a
1	4.0 (m)
2	3.6 (m)
3	2.36 (t, 7.6)
4	1.75 (d, 6.9)
5	1.75 (s)
1'	4.36 (d, 7.6)
2'	3.93
3'	3.40
4'	3.49
5'	3.29
6'	3.87
1''	5.19 (s br)
2''	3.97
3''	3.27
4''	3.41
5''	3.67
6''	1.23 (d, 6.1)

^a ¹H NMR measured at 400 MHz.^b ¹³C NMR measured at 100 MHz.

The ¹³C NMR displayed signals at 16.6 (C-6''; q), 68.3 (C-5''; d), 72.6 (C-4''; d) 70.4 (C-3''; d), 70.9 (C-2''; d) and 100.8 (C-1''; d) attributable to a terminal α -L-rhamnose, while the δ_{C} 61.3 (C-6'; t), 76.3 (C-5'; d), 77.9 (C-4'; d), 77.7 (C-3'; d), 70.8 (C-2'; d) and 101.6 (C-1'; d) illustrated the inner β -D-glucose (Table 3). The glycosylation shift of the C-4' [20,21] suggested that the terminal rhamnose unit is connected to the C-4' of the glucose moiety. In addition to the signals for the sugars, the ¹H NMR showed signals representative of one methyl at δ_{H} 1.75 (3H, s), one aliphatic methylene at δ_{H} 2.36 (2H, t, J = 7.6 Hz), one hydroxyl methylene at δ_{H} 4.0 (1H, m) and 3.6 (1H, m) and an exomethylene at δ_{H} 1.75 (2H, d, J = 6.9 Hz). Their ¹³C NMR signals appeared at δ_{C} 21.5 (C-5; q), 37.3 (C-2; t) 67.8 (C-1; t), 110.7 (C-4; t) and 142.3 (C-3; s), respectively, suggesting the presence of CH₂=C(CH₃)CH₂CH₂O-. In the HMBC experiment H-1' showed a clear correlation with C-1, and H-1'' with C-3' and C-2'. In the HMBC experiment the proton at δ_{H} 1.75 (3H, s) showed strong correlations with the carbon at δ_{C} 142.3 (C-3), 110.7 (C-4) and 37.3 (C-2), as well as, the correlations between the signal at δ_{H} 2.36 (1H, t, J = 7.6) and the signals at δ_{C} 142.3 (C-3), 110.7 (C-4) and 67.8 (C-1). The assignments were based on COSY, HMBC and HMQC correlations. The above evidence was used to propose the structure of compound **3** which is β -D-glucopyranoside, 3-methyl, but-3-en-1-yl 4-O- α -L-rhamnopyranosyl (Fig. 1).

The structures of the known compounds were confirmed by comparison of their spectroscopic properties with published data. To date, this is the first phytochemical and biological evaluation of *L. caspium*. The isolated compounds **1–11** (Fig. 1) were evaluated for their antibacterial, antifungal, antimalarial and antileishmanial activities. The antibacterial activities were evaluated using *Staphylococcus aureus*, *Methicillin-resistant S. aureus* (MRSA), *Escherichia coli*, *Pseudomonas aeruginosa*, and *Mycobacterium intracellulare*. None of these compounds showed in vitro antibacterial activity. The antifungal activities were evaluated against a panel of pathogenic fungi (*Candida albicans*, *Candida glabrata*, *Candida krusei*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*) associated with opportunistic infections. Compounds **7**, **8** and **9** exhibited good antifungal activity against *C. glabrata* with an IC₅₀ values of 6.79, 15.37 and 8.53 μ g/mL, respectively. Compound **8** showed potent antimalarial activity against both resistant and sensitive strains of *Plasmodium falciparum* with IC₅₀ values of 1.82 and 1.51 μ g/mL, respectively. Compounds **1**, **4**, **6**, **8** and **9** showed significant activity against *Trypanosoma brucei* with IC₅₀ values of 6.93, 9.65, 8.52, 7.67 and 6.31 μ g/mL, respectively. Compounds **1–11** were evaluated at a concentration of 10 μ M for their affinity to bind with cannabinoid (CB1 and CB2) and opioid (μ , κ and δ) receptors. None of these compounds showed affinity for these receptors.

3. Experimental

3.1. Plant material and extraction

The plant, *L. caspium* (Willd), was collected during its flowering stage on July, 29th 2004 in the piedmont steppe of the Ulytau Mountains and identified by Botanical Garden, Institute of Botany and Phytointroduction, Almaty, Kazakhstan.

The aerial parts (0.24 kg) were air-dried followed by grinding in a Willey-Mill plant grinder. Ground plant material was extracted at room temperature using two different solvents, methylene chloride (1.7 L) and ethanol (2.2 L) provided 7.4 g and 11.3 g respectively of the crude extracts after evaporation of the solvents. Lastly, extraction with water (2.5 L) provided 17.8 g of extract after lyophilization to remove the water.

3.2. Isolation

The ethanol extract of *L. caspium* (11.3 g) was fractionated using column chromatography with reversed phase C18 silica gel stepwise from

water to methanol to yield seven fractions (water; 4:1 H₂O–MeOH; 3:2 H₂O–MeOH; 1:1 H₂O–MeOH; 2:3 H₂O–MeOH; 1:4 H₂O–MeOH and MeOH). The fraction eluted with water was subsequently chromatographed on an open column using reversed phase C18 silica gel starting with water and followed with a stepwise gradient elution to MeOH, yielding nine subfractions (water (**1**); 95:5 H₂O–MeOH (**2**); 90:10 H₂O–MeOH (**3**); 80:20 H₂O–MeOH (**4**); 70:30 H₂O–MeOH (**5**); 60:40 H₂O–MeOH (**6**); 40:60 H₂O–MeOH (**7**); 20:80 H₂O–MeOH (**8**) and MeOH (**9**)). The subfraction (**2**), eluted with 5% MeOH in water, was rechromatographed using Sephadex LH-20 eluted with methanol to afford compounds **2** (22 mg) and **5** (12 mg). Subfraction (**3**), eluted with 10% MeOH in water, was rechromatographed using Sephadex LH-20 eluted with methanol to yield compounds **1** (34 mg), **7** (33 mg) and **4** (15 mg). Subfraction (**4**), eluted with 20% MeOH in water, was rechromatographed using Sephadex LH-20 eluted with MeOH yielding eighteen subfractions (**A–R**) (50 mL each). Subfraction **P** and **Q** afforded as compound **8** (50 mg). Subfraction **E** was purified on Sephadex LH-20 to furnish compound **11** (6 mg). Compound **10** (25 mg) was purified with Sephadex LH-20 eluted with MeOH from subfraction (**7**), eluted with 60% MeOH in water. The subfraction (**5**), eluted with 30% MeOH in water, was rechromatographed on Sephadex LH-20 eluted with methanol to yield compounds **3** (15 mg), **6** (12 mg), and **9** (21 mg).

3.3. General experimental procedures

¹H and ¹³C NMR spectra were obtained on a Bruker model AMX 500 NMR spectrometer with standard pulse sequences, operating at 500 MHz in ¹H and 125 MHz in ¹³C. The chemical shift values were reported in parts per million units (ppm) and trimethylsilane (TMS) or known solvent shifts, used as internal chemical shift references. Coupling constants were recorded in Hertz (Hz). Standard pulse sequences were used for COSY, HMQC, HMBC, TOCSY, NOESY and DEPT. High-resolution mass spectra (HRMS) were measured on a Micromass Q-ToF Micro mass spectrometer with a lock spray source. Column chromatography was carried out on silica gel (70–230 mesh, Merck) and Sephadex LH-20 (Mitsubishi Kagaku, Tokyo, Japan). TLC (silica gel 60 F254) was used to monitor fractions from column chromatography. Visualization of the TLC plates was achieved with a UV lamp (λ = 254 and 365 nm) and anisaldehyde/acid spray reagent (MeOH:acetic acid:anisaldehyde:sulfuric acid, 85:9:1:5). All chemicals used were purchased from Sigma-Aldrich (St. Louis, MO) with the following exceptions: for the binding experiments [³H]-CP-55,940 (174.8 Ci/mmol), [³H]-DAMGO (53.4 Ci/mmol), [³H]-U-69,593 (42.7 Ci/mmol), and [³H]-Enkephalin (45 Ci/mmol) were purchased from Perkin-Elmer Life Sciences Inc. (Boston, MA, U.S.A.). CP-55,940, DAMGO, DPDPE, nor-Binaltorphimine and Win 55,212-2 were purchased from Tocris Bioscience (Ellisville, MO, U.S.A.). CD spectra were measured on a JASCO J-715 spectrometer.

3.4. In vitro antimicrobial assay

Compounds **1–11** were tested for antimicrobial activity against a panel of microorganism obtained from the American Type Culture Collection (Manassas, VA) and included *C. albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, *C. neoformans* ATCC 90113, and *A. fumigatus* ATCC 204305, *S. aureus* ATCC 29213, methicillin-resistant *S. aureus* ATCC 33591 (MRSA), *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068. The bioassays were performed as previously described [22,24].

3.5. In vitro antimalarial and antileishmanial assays

Antimalarial activity was determined in vitro against chloroquine sensitive (D6, Sierra Leone) and resistant (W2, Indo China) strains of *P. falciparum* by measuring plasmodial LDH activity, as described earlier

[24]. The antileishmanial activity of the compounds was tested in vitro against a culture of *Leishmania donovani* promastigotes. Pentamidine and Amphotericin B were used as positive controls [22,23].

3.6. Radioligand displacement for cannabinoid receptor subtypes

Compounds evaluated in the assay were run in competition experiments using both cannabinoid receptor subtypes, CB1 and CB2 [25,26]. Cannabinoid receptor binding assays were performed under the following conditions: 10 μM of each compound was incubated with 0.5 nM [³H]-CP 55,940, a potent cannabinoid agonist with affinity to both receptor subtypes, and 10 μg CB1 or CB2 membrane [26] for 90 min in a 96-well plate. The reaction was terminated via rapid vacuum filtration through GF/C filters presoaked with 0.3% BSA using a Perkin Elmer 96-well Unifilter (Perkin Elmer Life Sciences Inc., Boston, Mass. U.S.A.) followed by 10 washes with 50 mM Tris–EDTA buffer containing 0.2% BSA. Plates were read using a Perkin Elmer Topcount (Perkin Elmer Life Sciences Inc., Boston, Mass. U.S.A.). Total binding was defined as binding in the presence of 1.0% DMSO. Nonspecific binding was the binding observed in the presence of 1.0 μM CP-55,940. Specific binding was defined as the difference between total and nonspecific binding. Percent binding was calculated using the following formula: 100 – (binding of compound – nonspecific binding) * 100 / specific binding.

K_i and IC₅₀ values were calculated using Graph-Pad Prism 5.

3.7. Radioligand displacement for opioid receptor subtypes

All compounds evaluated in the assay were run in competition binding against the opioid receptor subtypes (δ, κ, μ), as well as the cannabinoid receptor subtypes mentioned above [26]. Saturation experiments were performed after each batch of membrane was scraped for each of the three opioid cell lines (δ, κ, μ) to determine the optimal tritium and membrane concentration to be used in the assay. Saturation experiments determine the receptor number and radioligand affinity for the membrane. Opioid binding assays were performed under the following conditions: 10 μM of each compound was incubated with [³H]-DAMGO (μ), [³H]-U-69,593 (κ), or [³H]-enkephalin (δ) for 60 min in a 96-well plate. The reaction was terminated via rapid vacuum filtration through GF/B filters presoaked with 0.3% BSA using a Perkin Elmer 96-well Unifilter followed by 10 washes with 50 mM Tris–HCl. Plates were read using a Perkin Elmer Topcount. Total binding was defined as binding in the presence of 1.0% DMSO. Nonspecific binding was the binding observed in the presence of 10 μM DAMGO (μ), nor-binaltorphimine (κ), or DPDPE (δ). Specific binding was defined as the difference between total and nonspecific binding. Percent binding was calculated using the following formula: 100 – (binding of compound – nonspecific binding) * 100 / specific binding.

K_i and IC₅₀ values were calculated using Graph-Pad Prism 5.

3.8. Computational

Conformational analysis of compounds **1** and **2** were performed with Schrödinger Macromodel 9.9 (Schrödinger, LLC, New York) employing the OPLS2005 (optimized potential for liquid simulations) force field in MeOH [29]. Four conformers within a 2 kcal/mol energy window from the global minimum were selected, based on Boltzmann distribution calculated from Schrödinger software [29]. The output files for these conformers were prepared by Avogadro software for Gaussian 09 calculation, geometrical optimization and energy calculation applied on these conformers at B3LYP/6-31G [30]. Vibrational evaluation was done at the same level to confirm minimal excitation energy (denoted by wavelength in nm), rotatory strength dipole, and dipole length that were calculated in MeOH by TD-DFT/B3LYP/6-31G performed by the Gaussian 09 software package [30,31]. ECD curves were obtained in the SpecDis 1.62 program [32].

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