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Norditerpene alkaloids from *Delphinium linearilobum* and antioxidant activity

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Abstract

From the roots of *Delphinium linearilobum* (Trautv.) N. Busch two new norditerpene alkaloids linearilobin and linearilin, and the known alkaloids lycoctonine, 14-acetyltalatizamine, browniine, cammaconine, talatizamine, and cochlearenine were isolated. Spectroscopic techniques were used for structure determination. Antioxidant activity was performed by DPPH and metal chelating activity assays.

Keywords: *Delphinium linearilobum*; Ranunculaceae; Alkaloids; Linearilobin; Linearilin; Antioxidant activity

1. Introduction

The name *Delphinium* is derived from the ''dolphine-delphine'' like shape of the flower buds of *Delphinium* species. Since Dioscorides they have been used against lice and scorpions (Gunther, 1968). In Turkish traditional medicine, *Delphinium* extracts were used against fits of epilepsy and tremors of tetanus, as well as against rabies, and as a vomiting agent (Baytop, 1994). Because the plants are very toxic, their extracts are used externally. Diterpene and norditerpene alkaloids obtained from these plants are neurotoxic, causing bradycardia, muscle system strokes, hypotension and cardiac arrest. Some of these alkaloids show insect repellent, antioxidant, antiinflammatory, and tyrosinase inhibition activities (Ulubelen et al., 2001; Shaheen et al., 2005).

In Turkey, 31 *Delphinium* species grow naturally, and 19 of them are endemic. Our research group has studied 16 Turkish *Delphinium* species, together with Pelletier's group (Meriçli et al., 2001; Ulubelen et al., 1996) and four species with a Pakistan group (Ulubelen et al., 1998) since 1990.

There are no phytochemical and biological studies previously reported with this species. Since the aerial parts yielded only a small amount of crude alkaloid, in the present study the roots of *D. linearilobum* (Trautv.) N. Busch (Ranunculaceae) were investigated and two new norditerpene alkaloids, linearilobin (**1**) and linearilin (**2**) isolated, in addition to the five known norditerpene alkaloids lycoctonine (**3**) (Pelletier et al., 1980), 14-acetyltalatizamine (**4**) (Konno et al., 1982), browniine (**5**) (Pelletier et al., 1980), cammaconine (**6**) (Yue et al., 1994), talatizamine (**7**) (Konno et al., 1982), and a known diterpene alkaloid cochlearenine (*N*-ethyl-1α-hydroxydictizine) (**8**) (Shaheen et al., submitted). The spectroscopic data of the known alkaloids were similar to those given in the literature. The antioxidant activity of the alkaloids **1** and **3**–**8** was performed using the DPPH and metal chelating activity assays.

2. Results and discussion

The roots of *D. linearilobum* (Trautv.) N. Busch were extracted with MeOH. The alkaloidal mixture was

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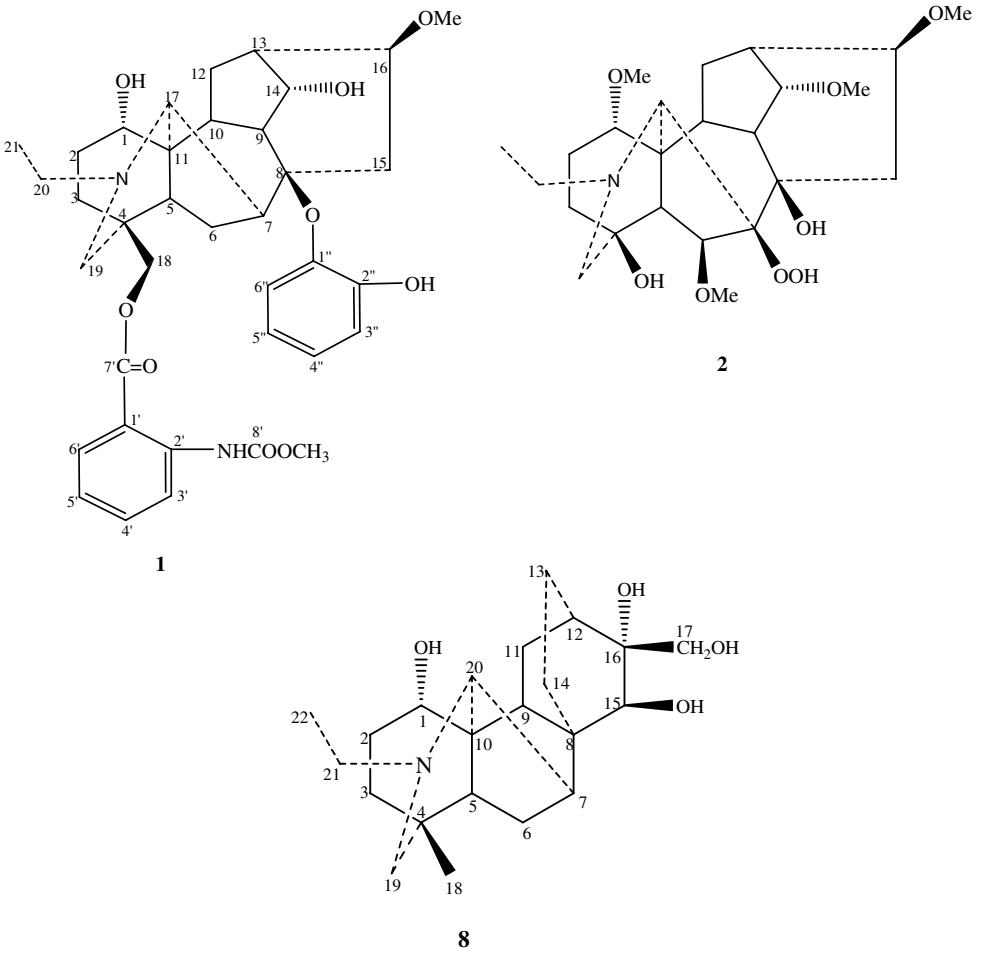

processed in two parts using a Chromatotron, yielding two new and six known alkaloids. The first new alkaloid was named linearilobin (**1**) and its CI–MS spectrum showed the molecular ion peak at *m/z* 662. The HRMS *m/z* 662.3200 (calcd. for $C_{37}H_{46}N_2O_9$, 662.3203) and ^{13}C NMR findings (one methyl, two methoxy, eight methylene, seventeen methine and nine quaternary C signals) indicated a molecular formula $C_{37}H_{46}N_2O_9$ for linearilobin (**1**) with 16 double bond equivalents of which six were accounted by a norditerpene skeleton, eight from two aromatic rings and two for the two carbonyl groups. The mass fragmentation of **1** showed the presence of a complex side chain, at *m/z* 603 $[M-COOCH_3]^+$ (a), at *m/z* 454 $[M-C_{10}H_{10}NO_4]^+$ (b), as well as a peak at *m/z* 569, indicating the presence of an another aromatic ring at *m/z* 569 $[M-C_6H_5O]^+$ (c), and *m/z* 553 $[M-C_6H_5O_2]^+$ (d) (see Fig. 1). ^1H NMR spectrum showed the presence of an aconitine-type norditerpene alkaloid (Table 1). The *N*-ethyl group was at δ_H 1.32 (*t*, $J = 4.5$ Hz, Me-21) and the methylene was found at δ_H 2.54 (*m*, H-20a) and 2.48 (*m*, H-20b). The two carboxyl car-

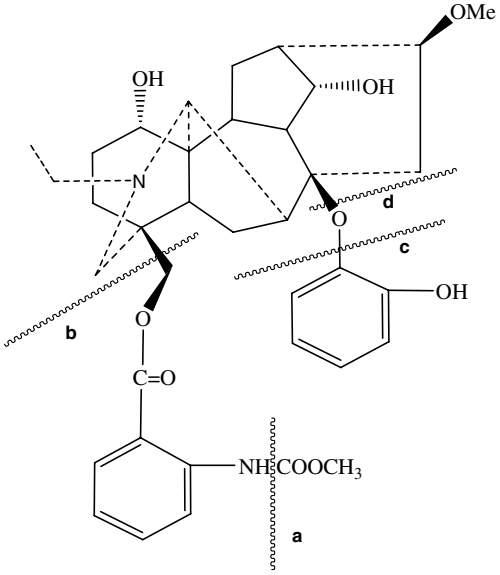

Fig. 1. Mass fragmentation of linearilobin (**1**).

Table 1
^1H (500 MHz) and ^{13}C (125 MHz) NMR data of alkaloids **1** and **2** in $CDCl_3$

	1		**2**	
	δ_H (*m*, *J* Hz)	δ_C	δ_H (*m*, *J* Hz)	δ_C
1	3.76 (*t*, 3)	72.52 (*d*)	3.24 (*dd*, 10.5;6.8)	84.74 (*d*)
2	1.60 (*m*) 1.58 (*m*)	29.25 (*t*)	2.34 (*m*) 1.88 (*m*)	29.94 (*t*)
3	1.85 (*m*) 1.64 (*m*)	31.43 (*t*)	1.92 (*m*) 1.62 (*m*)	37.74 (*t*)
4	–	37.60 (*s*)	–	70.81 (*s*)
5	1.75 (*d*, 8)	43.22 (*d*)	2.40 (*s*)	44.17 (*d*)
6	1.79 (*dd*, 14;8) 1.56 (*m*)	25.43 (*t*)	3.97 (*s*)	90.63 (*d*)
7	2.42 (*d*, 8)	48.16 (*d*)	–	109.99 (*s*)
8	–	85.90 (*s*)	–	78.79 (*s*)
9	2.10 (*dd*, 10.4;4.7)	46.43 (*d*)	2.20 (*brt*, 6.3)	45.27 (*d*)
10	1.94 (*m*)	44.28 (*d*)	1.85 (*m*)	37.93 (*d*)
11	–	49.17 (*s*)	–	49.43 (*s*)
12	2.05 (*m*) 1.62 (*m*)	27.76 (*t*)	1.95 (*m*) 1.65 (*m*)	30.77 (*t*)
13	2.34 (*m*)	42.68 (*d*)	2.28 (*m*)	43.63 (*d*)
14	4.14 (*t*, 5)	76.32 (*d*)	3.56 (*t*,4.8)	84.74 (*d*)
15	2.15 (*dd*, 15;5) 3.30 (*dd*, 15;8.5)	43.24 (*t*)	2.54 (*m*)	33.76 (*t*)
16	3.25 (*brd*, 9)	82.63 (*d*)	3.35 (*dd*, 10;4.6)	83.08 (*d*)
17	2.71 (*brs*)	65.46 (*d*)	2.96 (*s*)	66.35 (*d*)
18	4.15 (*d*, 11) 4.22 (*d*, 11)	68.74 (*t*)	–	–
19	2.70 (*d*, 13) 2.80 (*d*, 13)	57.66 (*t*)	2.80 (*d*, 12) 3.50 (*d*, 12)	57.53 (*t*)
20	2.54 (*m*) 2.48 (*m*)	48.61 (*t*)	2.88 (*m*) 2.60 (*m*)	49.62 (*t*)
21	1.32 (*t*, 4.5)	13.22 (*q*)	1.05 (*t*, 4.5)	14.22 (*q*)
1-OMe	–	–	3.27 (s)	56.56 (*s*)
6-OMe	–	–	3.29 (*s*)	59.33 (*s*)
14-OMe	–	–	3.29 (*s*)	57.94 (*s*)
16-OMe	3.56 (*s*)	57.40 (*d*)	3.35 (*s*)	57.59 (*s*)
Ar—*C*=O	–	167.35 (*s*)		
Ar–N*H*	11.4 (*s*)	–		
Ar–NH*C*=O	–	169.00 (*s*)		
CO*OCH*$_3$	3.60 (*s*)	51.0 (*s*)		
1′	–	114.72 (*s*)		
2′	–	151.87 (*s*)		
3′	7.96 (*dd*, 8.5;1.5)	116.35 (*d*)		
4′	7.48 (*ddd*, 8.5;7.5;1.5)	135.83 (*d*)		
5′	7.04 (*ddd*, 8.5;7.5;1.5)	116.88 (*d*)		
6′	8.62 (*dd*, 7.5;1.5)	130.62 (*d*)		
1″	–	148.06 (*s*)		
2″	–	151.02 (*s*)		
3″	7.17 (*bd*, 8.3)	114.21 (*d*)		
4″	7.45 (*td*, 8.3;1.2)	121.30 (*d*)		
5″	6.75 (*td*, 8.3;1.2)	118.63 (*d*)		
6″	7.90 (*dd*, 8.3;1.5)	114.26 (*d*)		
OH	–	–	3.57 (*brs*)	–
OH	–	–	3.94 (*brs*)	–

bonyl groups were observed at δ_C 167.35 *s* (C-7′) and 169.0 *s* (C-8′). One methoxy group was observed at δ_H 3.56 (3H, *s*), another one was at δ_H 3.60 (3H, *s*). The absence of methoxy groups at C-1 and C-14 was decided by the ^1H and ^{13}C NMR peaks δ_H 3.76 (*t*, $J = 3$ Hz) and δ_C 72.52 *d* versus δ_H 3.24 *dd* and δ_C 84.0 *d* and δ_H 4.14 (*t*, $J = 5$ Hz) and δ_C 76.32 *d* versus δ_H 3.56 *t* and δ_C 84.0 *d*, respectively. Biogenetically, one of the methoxy was placed at C-16 and the other at the side chain $C_{10}H_{10}NO_4$ due to the mass fragmentation. The absence of a Me-18 at around δ_H 0.7–0.9, and the presence of a C-18 methylene group at δ_H 4.15 (*d*, $J = 11$ Hz) and 4.22 (*d*, $J = 11$ Hz), δ_C 68.74 *t* and the mass fragmentation showed that the side chain $C_{10}H_{10}NO_4$ was present at C-4 (Zhou et al., 2004). Since the splitting pattern indicated the three hydrogen next to each other in the ^1H NMR spectrum (H-5, H-6 and H-7). There was only one site remaining for the placement of a cathecol group, namely C-8. Thus linearilobin was assigned structure **1**.

The second new norditerpene alkaloid linearilin (**2**) showed a molecular formula $C_{24}H_{39}NO_8$ from its HRMS and ^{13}C NMR spectra (one methyl, four methoxy, six methylene, nine methine, and four quaternary carbons signals). The alkaloid has a lycoctonine-type structure (Atta-ur-Rahman, 1990). It has six degrees of unsaturation accounted for in its skeleton. The alkaloid has four methoxy groups at δ_H 3.27 (3H, *s*), 3.29 (6H, *s*), and 3.35 (3H, *s*), two hydroxyl groups at δ_H 3.57 (*brs*) and 3.94 (*brs*), and a peroxyl group was established by an iodine test (see Section 4). From the chemical shifts (^1H and ^{13}C NMR, Table 1), the methoxy groups were placed at C-1 (δ_H 3.24 *dd*, δ_C 84.74 *d*), C-6 (δ_H 3.97 *s*, δ_C 90.63 *d*), C-14 (δ_H 3.56 *t*, δ_C 84.74 *d*), and C-16 (δ_H 3.35 *dd*, δ_C 83.08 *d*). Usually in norditerpene alkaloids, there are two methyl groups present, one appears at C-4 and the other at N–CH_2–CH_3 or N–CH_3. In the ^1H NMR spectrum of linearilin (**2**), there was only one methyl signal as a triplet at δ 1.05 *t* which is an *N*-ethyl group. Because of the absence of the second methyl and the signal at δ_C 70.81 *s* as in delbine (Jiang and Sung, 1985), one of the hydroxyl groups was placed at C-4. The second hydroxyl and the peroxyl group could be found at C-7 or C-8. When there is a hydro-

Table 2
Free radical scavenging activity (%) inhibition of the isolated alkaloids (**1**, **3**–**8**), TOC and BHT in methanol

Alkaloids	25 μg	50 μg	100 μg
1	2.32 ± 0.01	3.59 ± 0.04	7.75 ± 0.02
2	NT	NT	NT
3	3.04 ± 0.03	5.38 ± 0.02	10.77 ± 0.02
4	0.84 ± 0.01	0.86 ± 0.03	0.90 ± 0.04
5	5.18 ± 0.03	7.15 ± 0.01	15.11 ± 0.04
6	1.12 ± 0.01	1.45 ± 0.04	2.13 ± 0.02
7	1.19 ± 0.01	1.82 ± 0.04	1.91 ± 0.03
8	32.02 ± 0.02	49.61 ± 0.03	75.52 ± 0.04
TOC[a]	96.40 ± 0.02	96.67 ± 0.01	96.69 ± 0.00
BHT[a]	55.38 ± 0.02	71.79 ± 0.02	94.01 ± 0.01

[a] Reference compounds. NT, not tested.

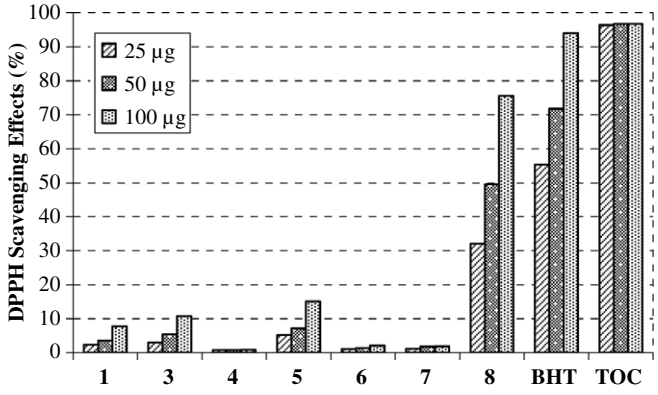

Fig. 2. Free radical scavenging activity of alkaloids (**1**, **3**–**8**), BHT and TOC by DPPH radical (TOC, α-tocopherol; BHT, butylated hydroxytoluene).

xyl either at C-7 or C-8, the chemical shifts should be around 88–89 ppm (C-7) and 77-79 ppm (C-8), respectively, in the ^{13}C NMR. In the present study, the signal at δ_C 78.79 showed that the hydroxy could be placed at C-8 and the peroxy at C-7 (δ_C 109.99). All of the integrated spectral data indicated this structure for linearilin (**2**).

Table 2 and Fig. 2 show the DPPH radical scavenging activity of alkaloids **1** and **3**–**8**. Radical scavenging activity of these alkaloids increases with increasing dose. The difference between the tested alkaloids and control was statistically significant ($p < 0.05$). The scavenging effect of the alkaloids and standards on the DPPH radical decreased in the order α-tocopherol > BHT > **8** > **5** > **3** > **1** > **6** > **7** > **4** at all concentrations (25 µg/mL, 50 µg/mL, and 100 µg/mL), demonstrating a linearity with increasing concentration. In addition, Shaheen et al. determined that cochlearenine (**8**), obtained from *Aconitum cochleare* Woroschin, caused a mixture of cardiac stimulation and depressant activities (Shaheen et al., submitted).

Ferrous ion chelating activities of alkaloids (**1**, **3**–**8**) and quercetin are shown in Table 3 and Fig. 3. As can be seen in Table 3, the alkaloids may chelate the ferrous ions with –NR_2 (Lindsay, 1996; Yuan et al., 2005). It was reported that compounds with structures containing two or more of the following functional groups: –OH, –SH, –COOH, –PO_3H_2, ─C═O, –NR_2, –S– and –O– in a favorable structure–function configuration can show metal chelating activity (Lindsay, 1996; Yuan et al., 2005). The difference between the tested alkaloids and control was statistically significant ($p < 0.01$). The chelating effect of alkaloids and standards on ferrous ions decreased in order **3** > **8** > **5** > quercetin > **6** > **4** > **7** > **1** at the 100 µg/mL concentration, and demonstrated a linearity with increasing concentration.

Table 3
The metal chelating effect of the isolated alkaloids (**1**, **3**–**8**) and quercetin on ferrous ions

Alkaloids	25 µg	50 µg	100 µg
1	1.62 ± 0.15	2.32 ± 0.04	6.01 ± 0.05
2	NT	NT	NT
3	27.14 ± 0.03	61.28 ± 0.05	92.60 ± 0.05
4	3.66 ± 0.04	10.06 ± 0.04	16.59 ± 0.09
5	9.65 ± 0.02	22.71 ± 0.02	42.55 ± 0.03
6	8.81 ± 0.04	9.62 ± 0.04	16.64 ± 0.04
7	3.32 ± 0.03	5.35 ± 0.02	9.00 ± 0.04
8	10.95 ± 0.03	25.40 ± 0.03	45.01 ± 0.01
Quercetin[a]	5.65 ± 0.05	9.18 ± 0.01	19.27 ± 0.01

[a] Reference compound. NT, not tested.

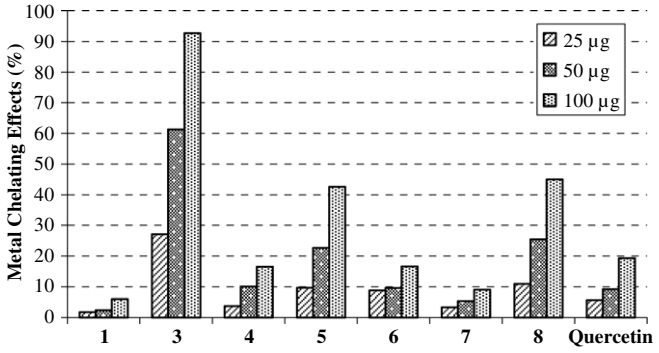

Fig. 3. Metal chelating effect of alkaloids (**1**, **3**–**8**), and quercetin by the Fe^{+2}-ferrozine method on ferrous ions.

3. Conclusions

In this study, the roots of *D. linearilobum* (Trautv.) N. Busch were investigated chemically for the first time, and two new and six known alkaloids were isolated. Among the new alkaloids, linearilobin (**1**), together with the known alkaloids lycoctonine (**3**), 14-acetyltalatizamine (**4**), browniine (**5**), cammaconine (**6**), talatizamine (**7**), and cochlearenine (**8**), cochlearenine showed the highest DPPH radical scavenging activity, while lycoctonine exhibited the highest metal chelating activity.

4. Experimental

4.1. General

UV spectra were recorded on a Shimadzu UV-1601 and IR spectra on a Perkin–Elmer Model 983 spectrometer. Optical rotations were determined in an Opt. Act. Ltd. AA-5 polarimeter. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded on a Varian Unity Inova instrument. EIMS were recorded on a Thermo Electron DSQ quadrupol and HRMS on a Zap Spec spectrometer.

4.2. Plant material

The roots of *D. linearilobum* (Trautv.) N. Busch were collected and identified by F. Özgökçe from Eastern Turkey (Van, Güzeldere passage, 2827 m) in August 2004. A voucher specimen is deposited in the Herbarium of Yüzüncü Yil University VANF 10948.

4.3. Extraction and isolation

Dried and powdered roots (493 g) were macerated with MeOH and the solvent was evaporated in vacuo. The MeOH extract (8 g) was dissolved in MeOH and acidified with 5% HCl to pH 2.8 and extracted with CH_2Cl_2 (10×150 mL). The remaining aq. soln. was basified with 10% NaOH to pH 8–10 and extracted with CH_2Cl_2 (15×150 mL) to yield of a crude alkaloidal mixture (1.59 g). Half of the crude extract was directly separated on Al_2O_3 60 GF_{254} neutral (Typ E) (Merck Art. 1092) rotor 1 mm thick on a Chromatotron eluting with a gradient of petroleum ether, CH_2Cl_2 and methanol. Similar fractions were combined by silica gel TLC plates (Merck Art. 5554) (Fractions A–F). Eight alkaloids were purified by preparative TLC plates (Merck Art. 5554) using following solvent systems: Fraction A afforded 14-acetyltalatizamine (**4**) (20 mg) (Konno et al., 1982) (PE:T:CH_2Cl_2:DEA – 3:1:0.5:0.1) and talatizamine (**7**) (15 mg) (Konno et al., 1982) (T:CH_2Cl_2:DEA – 3:1:0.1), fraction B gave linearilin (**2**) (8 mg), browniine (**5**) (6 mg) (Pelletier et al., 1980) and cammaconine (**6**) (12 mg) (Yue et al., 1994) (T:CH_2Cl_2:DEA – 3:1:0.1), from fraction E, linearilobin (**1**) (7 mg) and lycoctonine (**3**) (50 mg) (Pelletier et al., 1980) were obtained (T:CH_2Cl_2:MeOH:DEA – 2:1:0.5:0.1), and from fraction F cochlearenine (**8**) (10 mg) (Shaheen et al., submitted) (T:CH_2Cl_2:MeOH:-DEA – 1:2.5:0.5:0.1).

4.4. Peroxide test

Alkaloid **2** (5 mg) was dissolved in EtOH, a few drops of 0.1 N HCl (Merck) and a few drops of 1% KI (Merck) aq. soln. were added. A yellow colour was formed which turned blue on the addition of starch solution.

4.5. Linearilobin (*1*)

Amorphous alkaloid, $[\alpha]_D^{20}$ 0° ($CHCl_3$; c 1); UV λ_{max}^{MeOH} nm ($\log\varepsilon$): 222 (4.5), 252 (3.6), 308 (2.0); IR $\nu_{max\ 3}^{CHCl}$ cm^{-1}: 3445, 2924, 1847, 1830, 1733, 1686, 1654, 1626, 1606, 1540, 1458, 1091; EIMS 70 eV, *m/z* (rel. int.): 662 $[M]^+$ (3), 603 $[M-COOCH_3]^+$ (3), 587 $[603\text{-}NH_2]^+$ (6), 569 $[M-C_6H_5O]^+$ (12), 553 $[M-C_6H_5O_2]^+$ (15), 454 $[M-C_{10}H_{10}NO_4]^+$ (15), 436 (10), 390 (5), 267 (12), 240 (9), 183 (32), 151 (28), 119 (35), 84 (45), 58 (100). ^1H (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data: see Table 3. HRMS: *m/z* 662.3200 (calcd. for $C_{37}H_{46}N_2O_9$, 662.3203).

4.6. Linearilin (*2*)

Amorphous alkaloid, $[\alpha]_D^{20}$ + 18.4° ($CHCl_3$; *c* 0.5); UV λ_{max}^{MeOH} nm ($\log\varepsilon$): 201 (4.0), 226 (sh) (3.1), 257 (2.5); IR $\nu_{max\ 3}^{CHCl}$ cm^{-1}: 3426, 2926, 1734, 1652, 1560, 1458, 1260, 1089; EIMS 70 eV, *m/z* (rel. int.): 469 $[M]^+$ (3), 452 $[M-OH]^+$ (35), 436 $[M-OOH]^+$ (20), 418 $[436\text{-}H_2O]^+$ (5), 344 (4), 282 (3), 238 (5), 202 (7), 192 (11), 178 (14), 128 (17), 114 (20), 108 (26), 91 (32), 85 (58), 71 (68), 58 (100). ^1H (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data: see Table 3. HRMS: *m/z* 469.2669 (calcd. for $C_{24}H_{39}NO_8$, 469.2675).

4.7. Cochlearenine (*N-ethyl-1-α-hydroxydictizine*) (*8*)

Amorphous alkaloid, $[\alpha]_D^{20}$ − 35.5° ($CHCl_3$; *c* 0.5); UV λ_{max}^{MeOH} nm ($\log\varepsilon$): 203 (4.2), 226 (sh) (3.0), 284 (2.5); IR $\nu_{max\ 3}^{CHCl}$ cm^{-1}: 3408, 2923, 2853, 1701, 1653, 1627, 1560, 1543, 1458, 1375, 1215, 1066, 951, 758; EIMS 70 eV, *m/z* (rel. int.): 377 $[M]^+$ (12), 346 $[M-CH_2OH]^+$ (11), 318 (10), 286 (15), 258 (5), 198 (15), 187 (10), 186 (65), 127 (10), 99 (20), 97 (33), 85 (50), 71 (66), 57 (100). ^1H (500 MHz, $CDCl_3$): δ 0.64 (3H, *s*, H-18), 0.98 (3H, *t*, $J = 7$ Hz, H-22), 3.43 (1H, *d*, $J = 11.2$ Hz, H-17a), 3.57 (1H, *s*, H-20), 3.77 (1H, *dd*, $J = 6$; 10 Hz, H-1β), 3.96 (1H, *s*, H-15α), 4.14 (1H, *d*, $J = 11.2$ Hz, H-17b); ^{13}C NMR (125 MHz, $CDCl_3$): δ 70.9 (*d*,C-1), 29.9 (*t*, C-2), 27.1 (*t*, C-3), 33.8 (*s*, C-4), 48.3 (*d*, C-5), 29.9 (*t*, C-6), 42.6 (*d*, C-7), 42.7 (*s*, C-8), 40.5 (*d*, C-9), 51.4 (*s*, C-10), 21.6 (*t*, C-11), 37.0 (*d*, C-12), 23.8 (*t*, C-13), 24.0 (*t*, C-14), 87.7 (*d*, C-15), 79.0 (*s*, C-16), 68.1 (*t*, C-17), 26.2 (*q*, C-18), 57.2 (*t*, C-19), 67.4 (*d*, C-20), 50.3 (*t*, C-21), 14.4 (*q*, C-22); HRMS: *m/z* 377.2558 (calcd. for $C_{22}H_{35}NO_4$, 377.2566).

4.8. DPPH (*1,1-diphenyl-2-picrylhydrazyl*) free radical scavenging activity assay

Radical scavenging activity of the alkaloids (**1**, **3**–**8**) was determined using DPPH as a reagent. The reaction mixture containing test sample (1 mL) in different concentrations (500 ppm in MeOH) and DPPH (4 mL) (Sigma, 100 μM) in methanol was taken and incubated in the dark at 37 °C for 30 min. Scavenging capacity was read spectrophotometrically by monitoring the decrease in absorbance at 517 nm. A lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Percent radical scavenging activity was determined by comparison with a MeOH containing control. BHT (2,6-di-*t*-butyl-1-hydroxytoluene) and α-tocopherol were used as positive controls (Shaheen et al., 2005). All the chemicals used were of analytical grade (Sigma, USA). The percent DPPH scavenging effect was calculated using the following equation:

$$\text{DPPH scavenging effect } (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of the control, and A_{sample} is the absorbance of the sample.

4.9. Metal chelating activity assay

The chelating activity of alkaloids (**1**, **3**–**8**) on Fe^{2+} was measured as reported by Decker and Welch (1990). Samples (**1**, **3**–**8**) were mixed with 3.7 mL of methanol, and then

the mixture was reacted with $FeCl_2$ (2 mM, 0.1 mL) and ferrozine (5 mM, 0.2 mL) for 10 min, and the absorbance at 562 nm determined spectrophotometrically (Gülçin et al., 2003). Quercetin was used as positive control. All the chemicals used were of analytical grade (Sigma, USA). The percent chelating activity of samples on Fe^{2+} was calculated as follows:

Metal chelating activity (%)
$= [1 - (\text{absorbance of sample})/(\text{absorbance of control})] \times 100$

4.10. Statistical analysis

Experimental results were mean ± SD of three parallel measurements. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple range tests, p values <0.05 were regarded as significant, p values <0.01 were regarded as very significant.

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