Article

J. Mex. Chem. Soc. **2007**, *51(4),* 193-197

ISSN 1870-249X

Coumarin Derivatives from *Loeselia mexicana*. Determination of the Anxiolytic Effect of Daphnoretin on Elevated Plus-maze.

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Recibido el 9 de agosto de 2007; aceptado el 20 de octubre de 2007

Abstract. The *bis*-coumarin daphnoretin (**1**) and its monomeric precursors scopoletin (**3**) and umbelliferone (**4**) were isolated for the first time from *L. mexicana*, a vegetal species used in Mexican traditional medicine. The unambiguous ^{13}C NMR chemical shifts assignments of daphnoretin (**1**) and its acetyl derivative **2** were achieved through one-bond (gHMQC) and long-distance (gHMBC) heteronuclear correlations. The crude hydroalcoholic extract and pure daphnoretin **1** obtained from this plant, showed anxiolytic effect in male ICR mice exposed to elevated plus-maze. Different doses of daphnoretin (**1**) were not able to modify the spontaneous locomotor activity measured in the open field test. This is the first report regarding to the anxiolytic effect of daphnoretin (**1**).

Keywords: *Loeselia mexicana* Brand, daphnoretin, 7-acetyldaphnoretin, ^{13}C NMR chemical shift, elevated plus maze, anxiety.

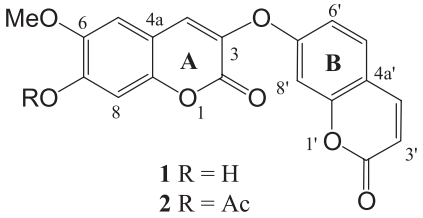
Resumen. La *bis*-coumarina dafnoretina (**1**) y sus precursores monoméricos escopoletina (**3**) y umbelliferona (**4**) se aislaron por vez primera de la parte aérea de *L. mexicana* Brand, una especie vegetal usada en la medicina tradicional mexicana. Los desplazamientos químicos de RMN ^{13}C de dafnoretina (**1**) y su derivado acetilado **2** se establecieron de manera inequívoca mediante experimentos de correlación heteronuclear (gHMQC y gHMBC). El extracto etanólico crudo y la dafnoretina (**1**) pura obtenidos de esta planta mostraron efecto ansiolítico en ratones machos ICR sometidos al experimento de laberinto elevado en forma de cruz. Los diferentes tramientos de dafnoretina (**1**) no modificaron la actividad motora espontánea medida con el método de campo abierto. Este es el primer estudio que describe el efecto ansiolítico de la dafnoretina (**1**).

Palabras clave: *Loeselia mexicana*; dafnoretina, 7-acetildafnoretina, RMN ^{13}C, laberinto elevado en forma de cruz, ansiedad.

Introduction

Loeselia mexicana Brand is a Polemoniaceae plant endemic of America growing from Texas to Central America. Its most common name in Mexico is *espinosilla*, where is largely appreciated due to its medicinal properties as anti-diarrhea [1], anti-pyretic, disinfectant, against dandruff, fall of hair and shock (*susto*: this meaning the people has a sensation of risk –real or imaginary– front to external stimuli) [2,3]. Such definition is similar to the medical anxiety disorder, which can be defined as "maladaptive, either because it is too intense or because it is inappropriately provoked by events that present no real danger" [4].

Previous phytochemical studies of *L. mexicana* have described the isolation of a pentacyclic triterpene [5] and other qualitative analyses have demonstrated the presence of flavonoid derivatives [6]. In this work the isolation of daphnoretin **1** from *L. mexicana* is described for the first time, which is a biscoumarin derivative widely distributed in the Thymelaeaceae, Leguminosae and Rutaceae. Its structure was established [7] since 1963 and corroborated by total synthesis three years later [8]. Since its isolation, daphnoretin **1** has been submitted to diverse antimicrobial [9] and antineoplastic studies [10], and more recently its ability to inhibit the lyase activity of DNA polymerase β has been described [11]. Further, a revision of the ^{13}C NMR chemical shifts (δ) assignments of daphnoretin **1**, as well as its acetylated derivative **2**, has been undertaken due to δ discrepancies observed in two previous works [12,13].



1 R = H
2 R = Ac

Results and discussion

Structural characterization and ^{13}C NMR chemical shift assignments of daphnoretin (1). HRFAB-MS analysis of daphnoretin (**1**) was consistent with a $C_{19}H_{12}O_7$ molecular formula. In addition, the ^{1}H NMR data match with those described by Cordell [13]. However, it was necessary to confirm the chemical shifts (δ) assignment for the singlets H-5 and H-8 by means of a *nOe* diff. experiment, since its correct assignment was critical to achieve a proper interpretation of the ^{13}C NMR data of **1**. Thus, singlet at δ 7.22 was enhanced (6%) upon irradiation of the OMe group at δ 3.83, meaning this signal belongs to H-5; therefore, signal for H-8 (δ 6.85) was implicitly assigned. The other single signal appearing at lower field (δ 7.82) belongs to H-4.

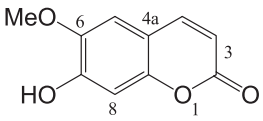
In relation to the ^{13}C NMR resonances assigned by Cordell [13] for daphnoretin (**1**), they showed strong inconsistencies with those described by Chakrabarti [12]. This encouraged us to carry out a revision of all ^{13}C chemical shifts of daphnore-

tin (**1**), since these two works are often taken as references to describe its presence in other plant species [9,11], or to assign the ^{13}C chemical shifts of structurally related compounds [14,15].

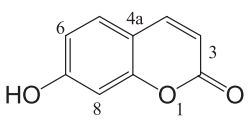
Thus, ^{13}C NMR chemical shifts assignments of all protonated carbons (C-4, C-5, C-8, and C-3'-C-7') were accomplished by means of a ^{1}H-^{13}C gHMQC experiment. While the δ values for these carbons agree well with those described by Cordell [13], several inconsistencies with those reported by Chakrabarti [12] remained. The 10 quaternary carbons were assigned by using a ^{1}H-^{13}C gHMBC experiment (Table 1). The results (Table 1) revealed that there are solely discrepancies in the assignment of C-2 (157.01) and C-7' (159.74) with those described by Cordell [13] (C-2, δ 159.75; C-7', δ 157.07), whereas the most of these δ values disagree with those described by Chakrabarti[12]. In order to clarify these discrepancies, signals of both H-4 and H-5' provided the key long-distance correlations to assign C-2 and C-7' unequivocally. Thus, H-4 (δ 7.82) clearly shows a three-bond (^{3}J) correlation with the signal assigned to C-2 at δ 157.01, whereas H-5' (δ 7.64) shows a similar correlation with the signal at δ 159.74.

According to the δ values discrepancies found for daphnoretin (**1**), it was also necessary to revise the ^{13}C NMR δ for 7-*O*-acetyldaphnoretin (**2**), which were described for the first time by Chakrabarti [12]. Firstly, daphnoretin (**1**) was converted to its acetylated derivative **2** by treatment with Ac_2O and Py for 16 h at room temperature. Once assigned the ^{1}H NMR spectrum of **2** (see experimental), it was taken as reference to achieve the assignments of its ^{13}C NMR spectrum. Again, the one-bond (^{1}H-^{13}C gHMQC) and long-distance (^{1}H-^{13}C gHMBC) heteronuclear correlations were determined to attain this purpose. Results shown in Table 1 revealed several δ inconsistencies between this work and those previously described [12].

Finally, compounds **3** and **4** were also isolated from the less polar fractions of the CH_2Cl_2 extract. According to their NMR data, the pattern of substitution of these compounds agree the distribution of substituents at C-6, C-7 (in **3**) and C-7' (in **4**) positions of daphnoretin (**1**). Accordingly, the linkage between A and B rings, to form daphnoretin (**1**), must be at C-3 and C-7' positions. Therefore, it can be assumed that such compounds correspond to the two coumarin units of daphnoretin (**1**).



scopoletine **3**



umbelliferone **4**

Anxiolytic Assays

Elevated plus-maze (EPM). Pure daphnoretin (**1**) isolated from the CH_2Cl_2 extract was submitted to this test. The data showed that the intraperitoneal administration of 1.8, 3.7, 7.5 and 15.0 mg/kg of daphnoretin (**1**) induced an increment of the time that mice spent in the open arms and the percentage of entries to them; such activities were doses-dependent with respect to the control group ($p < 0.001$) (Fig. **1**). When the anxiolytic drug diazepam (DZP) was administrated (1.0 mg/kg), also provoked an increase of these parameters ($p < 0.001$).

Table 1. ^{13}C NMR chemical shifts assignments for daphnoretin (**1**) and its acetyl derivative **2**.

# C	δ,[a] Daphnoretin (1)	δ,[b] 7-O-Acetyldaphnoretin (2)
2	157.01	157.40
3	135.67	139.99
4	131.05	126.74
4a	110.18	116.62
5	109.32	111.93
6	145.71	148.96
7	150.43	142.03
8	102.74	108.90
8a	147.48	145.59
2'	160.03	160.43
3'	113.87	115.14
4'	144.08	142.91
4a'	114.39	115.29
5'	129.92	129.37
6'	113.41	114.26
7'	159.74	158.66
8'	103.92	105.72
8a'	155.04	155.36
OMe	55.97	56.39
O<u>C</u>OMe		168.41
OCO<u>Me</u>		20.58

[a] Referenced to 39.43 ppm. ^{13}C δ for the methyl groups of DMSO-d_6.
[b] Referenced to 77.00 ppm, ^{13}C δ for $CDCl_3$.

Open Field Test (OFT). The OFT paradigm was done in order to determine the effect of the administration of the daphnoretin **1** on spontaneous motor activity. The different doses of this compound (1.8, 3.7, 7.5 and 15.0 mg/kg) and the DZP group, did not induce changes statistically significant with respect to control group ($p > 0.05$) on the total number of crossing and rearing in the open field test (Fig. **2**).

The World Health Organization reports that approximately 450 million of people suffer from mental disorder (WHO, 2001) [16]; from which anxiety and depression are the most frequent [17]. The search for new therapeutic products for the treatment of anxiety from medicinal plants has increased constantly and its effectiveness has been showed in a variety of models [18]. In this work daphnoretin (**1**) was able to reduce

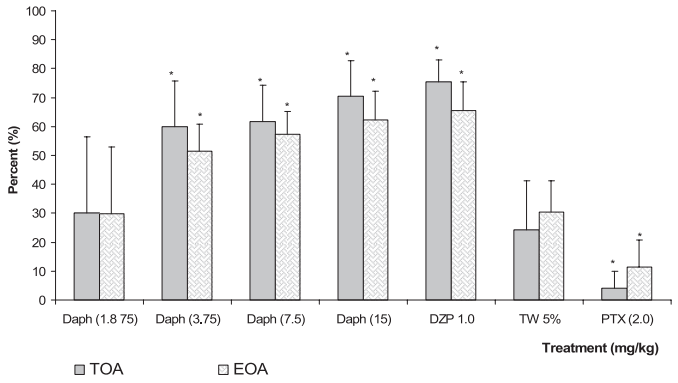


Fig. 1. Effect produced by daphnoretin (**1**) (1.87, 3.75, 7.5 and 15 mg/kg) from *Loeselia mexicana* upon percentage of entries and percentage of time spent by mice in open arms on the elevated plus maze test.
TW 5% = negative control (Tween 20, 5% solution); DZP= diazepam; Daph. = daphnoretin (**1**). TOA= Percentage of time spending to open arms; EOA= Percentage of entries to open arms. ANOVA post-hoc Dunnet test * = $p<0.05$. All groups were administrated intraperitoneal pathway and compared to the control group (TW 5%).

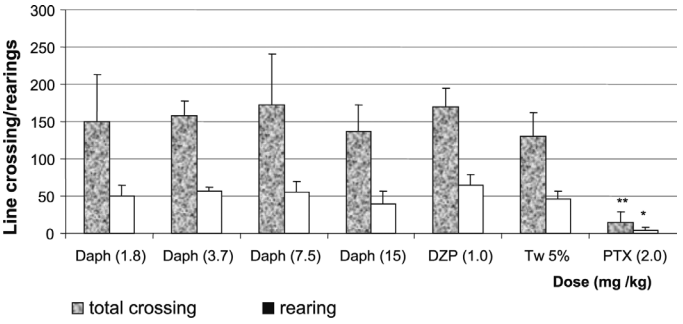


Fig. 2. Effect produced by different doses of daphnoretin (**1**) from *Loeselia mexicana* upon the total number of crossings and rearings showed by ICR mice exposed to the open field paradigm.
TW 5% = negative control (Tween 20, 5% solution); DZP= diazepam; Daph= daphnoretin (**1**). ANOVA post-hoc Dunnet test * = $p<0.05$, ** = $p<0.001$. All groups were compared to the control group (TW 5%).

the anxiety of mice exposed to elevated plus maze, this effect was similar to that showed by DZP (Fig. **1**). Such activity was dose-depend and did not induce changes of spontaneous motor activity (showed in the open field test, Fig. **2**). With these animal models it is not possible to establish the action mechanism of daphnoretin (**1**); however, the anxiolytic effect shown by daphnoretin (**1**) could be due to, at least in part, the activation of protein kinase C (PKC) on $GABA_A$ receptor. This compound is able to activate PKC in different biological models [19,20]. The PKC family of serine-threonine kinases regulates a variety of cell functions [21] and neural tissues display high PKC activity and isoform expression, which are important in the control of different neuronal functions associated with diverse brain pathologies [22]. The $GABA_A$ receptor is susceptible to regulation by PKCs isoforms, this modulation might produce different actions over the allosteric modulator drugs of $GABA_A$ [23]. PKC activation enhances the activity of $GABA_A$ by benzodiazepines and barbiturates [24] which are anxiolytic and sedative substances. Coumarin-type substances have sedative effects or are able to bind with benzodiazepine sites on $GABA_A$ receptors. For instance, 3-arylsulfonyl-4-hydroxycoumarin showed low sedative effects [25]. Furanocoumarins isolated from *Angelica dahurica* strongly inhibit the binding of [^{3}H] diazepam to the CNS [26]. Daphnoretin (**1**) is a good candidate to be used as an anxiolytic substance; however, further studies are necessary to measure its pharmacological interaction with GABAergic system and anxiolytic effect.

Experimental

General. Melting points were determined on an Electrothermal capillary melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 599B spectrophotometer. 1D and 2D ^{1}H and ^{13}C NMR spectra were obtained on a Varian Mercury-300 spectrometer at 300 and 75.4 MHz, respectively, using DMSO-d_6 or $CDCl_3$ as solvent and TMS as internal standard. Low- and high-resolution FABMS and CIMS (70 eV) data were collected on a JEOL JMS-AX 505 HA and JMS-SX 102 mass spectrometers, respectively. Thin-layer chromatograms were done on precoated TLC sheets of silica gel 60 F_{254} (Merck) and visualized by spraying a ceric sulfate/H_2SO_4 solution and heating.

Plant material. *L. mexicana* was collected in July, 2004, in Hueyapan, Morelos, Mexico. A voucher specimen (INAHM-2017) is preserved at the Cuernavaca's Botanic Garden, Cuernavaca, Morelos, Mexico.

Extraction and isolation. The whole plant of *L. mexicana* was dried during 4 weeks and finely powdered. The resulting powder (500 g) was extracted in 60% ethanol solution at 50 ºC for 2 h, and concentrated to afford the crude extract as brownish syrup (80 g), which was partitioned between H_2O (500 mL) and hexane (2 × 600 mL) to obtain 11 g hexane extract. The aqueous layer was extracted with CH_2Cl_2 (2 × 600 mL), the organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness, affording 20 g of crude extract as yellowish syrup, which was submitted to open column chromatography over Si gel using *n*-hexane-acetone (2:1) as mobile phase. Fractions 42 to 50 were combined based on their similar TLC patterns; the same criterion was applied to fractions 30 and 31. Both waxy residues were individually re-chromatographed as described above, yielding 160 mg of daphnoretin (**1**, mp 244-245 ºC), 40 mg of scopoletin (**3**) and 20 mg of the less polar umbelliferone (**4**).

Daphnoretin (1). Yellow crystals mp 244-245 ºC. ^{1}H NMR ($CDCl_3$): δ 8.03 (1H, d, J = 9.6 Hz, H-4'), 7.82 (1H, s, H-4), 7.70 (1H, d, J = 7.8 Hz, H-5'), 7.22 (1H, s, H-5), 7.17 (1H, d, J

= 2.3 Hz, H-8'), 7.07 (1H, dd, *J* = 7.8, 2.3 Hz, H-6'), 6.85 (1H, s, H-8), 6.36 (1H, d, *J* = 9.6 Hz, H-3'); ^{13}C NMR ($CDCl_3$): See Table 1. HRFABMS m/z calcd. for $C_{19}H_{12}O_7 + H^+$ 353.0661; Found 353.0659.

7-*O*-Acetyldaphnoretin (2). To a solution of 50 mg (0.014 mmol) of daphnoretin (**1**) in 1 mL of Py were added 2 mL of Ac_2O and the mixture was stirred at room temperature for 12 h. The reaction mixture was poured into ice-water, extracted with EtOAc, washed successively with water, an aqueous 5% HCl solution and water. The organic layer was dried with anhydrous Na_2SO_4, evaporated to dryness and purified by column chromatography (Silica gel 60, .040-.063 mm) using a mixture of hexane/EtOAc (6:4) as mobile phase, affording 40 mg (80%) of 7-*O*-acetyldaphnoretin **2** as a colorless solid, mp 231-232 °C. ^{1}H NMR ($CDCl_3$): δ 7.68 (1H, d, *J* = 9.6 Hz, H-4'), 7.50 (1H, d, *J* = 7.8 Hz, H-5'), 7.37 (1H, s, H-4), 7.16 (1H, s, H-5), 7.19 (1H, d, *J* = 2.3 H_2, H-8), 6.95 (1H, dd, *J* = 7.8, 2.3 Hz, H-6'), 6.94 (1H, s, H-8), 6.36 (1H, d, *J* = 9.6 Hz, H-3'). ^{13}C NMR ($CDCl_3$): See Table 1.

Drugs. Diazepam (DZP, 1.0 mg/kg, Sigma) was used as the standard anxiolytic (elevated plus maze and open field test), Picrotoxin (PTX, 2 mg/Kg, Sigma) was used as anxiogenic drug. A Tween 20 solution (TW, 5%, Merck) was used to treat control group, and hydroalcoholic extract from *L. mexicana* (Lm, 100 mg/kg) and pure daphnoretin (**1**, Daph, 1.8, 3.7, 7.5 and 15 mg/kg), isolated from the above extract, were used for the treated groups.

Animals and treatments. Male ICR mice (32-38 g) purchased at Harlan Mexico were used. All animals were housed 8 per cage at room temperature (23-25 °C) under a 12 h light-dark cycle (lights on 07:00 h) for at least 3 weeks prior to testing, with free access to water and food (Harlan rodent lab diet). All assays were conducted from 8 to 13 h, in an especial noise-free room with controlled illumination. The animals were treated with different doses of daphnoretin **1** for elevated plus-maze and open field tests, for anxiolytic and spontaneous motor activity tests (1.8, 3.7, 7.5, 15.0 mg/kg). The negative control group was treated with 5% Tween 20 solution (intraperitoneal), the anxiolytic drug was DZP (1.0 mg/kg) and the anxiogenic drug was PTX (2.0 mg/kg). Eight animals for all treatment were used, which were administered by intraperitoneal pathway, 30 min before each test. All experiments were conducted in accordance with the Mexican Official Norm [27].

Elevated plus-maze (*EPM*). This test has widely been validated for measuring anxiolytic and anxiogenic-like activities in rodents [28]. Each animal was placed at the center of the maze, facing one of the enclosed arms. The number of entries and the time spent in enclosed and open arms were recorded for 5 minutes. Entry into an arm was defined as the animal placing all four paws onto the arm. Total exploratory activity (number of entries) and other ethologically derived measures (grooming, rearing, stretched attend postures and head dipping) were also registered.

Open field test (*OFT*). The open-field area was made of acrylic transparent walls and black floor (30 cm X 30 cm X 15 cm) divided into nine squares of equal area. The open field was used to evaluate the exploratory activity of the animal [29]. The observed parameters were the number of squares crossed (with the four paws) and number of rearings.

Statistics Analysis. Data were analyzed by ANOVA for one-way and post-hoc tests were then performed using the Dunnet, the level of significance was set at $p<0.05$.

Acknowledgements

LGZ acknowledges CGPI/IPN (grants 20020683, 20030702 and 20040199) for financial support. VMNG thanks CONACyT-Mexico (52646) and IMSS-FOFOI (2005/1/I/066) for financial support.

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