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Identification of the Main Specialized Metabolites of *Ceanothus caeruleus* and Cytotoxic Effects of a-*nor*-Lupane Derivatives†

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Abstract: The dichloromethane (DCM) and ethyl acetate (EtOAc) extracts of *C. caeruleus* yielded nine known compounds, including an A-*nor*-lupane triterpenoid identified as gouanic acid B (**1**). Additionally, its acetyl derivative, acetylgouanic acid B (**2**), is reported here for the first time as a natural product. Furthermore, we tested the bioactivity of natural products **1** and **2** and their dimethyl ester derivatives **3** and **4** against cancer cell lines MCF-7, A549, HeLa, and K562. Among these, compounds **1** (IC_{50} = 36.4 ± 4.0 µM), **2** (IC_{50} = 21.6 ± 4.3 µM), and **4** (IC_{50} = 33.0 ± 2.0 µM) demonstrated moderate to good activity against the K562 cell line while maintaining a satisfactory survival rate in non-cancerous bMEC cells. Notably, the natural triterpenes **1** and **2** and derivative **4** showed remarkable outcomes in cytotoxicity tests due to their specificity against K562 leukemia cells.

Keywords: *Ceanothus caeruleus*; triterpenoid; bioactive compounds; cytotoxicity activity; non-cancerous cell line.

1. Plant Source

Certain species of *Ceanothus* play a significant role in traditional medicine. For example, *Ceanothus caeruleus*, native to the State of Michoacán in Mexico and commonly referred to as

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"chaquira's flower" or "leather rod," is used to treat various ailments, including wounds, pimples, insect bites, foot inflammation, stomach issues, and diarrhea [1, 2].

Flowers, leaves, shrubs, and stems of *Ceanothus caeruleus* Lag. were collected on February 2023 near the Estribo Grande Panoramic Viewpoint (19°30′52.5′ffN, 101°38′36.8′′W, 2348 masl) at Pátzcuaro, Michoacán, Mexico. A voucher specimen (EBUM 3658) was identified by M.Sc. Patricia Silva-Sáenz and deposited at EBUM Herbarium of the Facultad de Biología of the Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, Mexico.

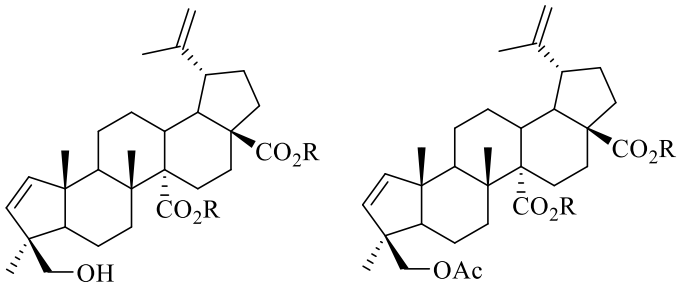
2. Previous Studies

There are no previous reports about the chemical composition of this plant.

3. Present Study

Organic extracts from *Ceanothus caeruleus* were prepared using maceration and reflux extraction with dichloromethane (DCM) and ethyl acetate (EtOAc) (see Supplementary Information). DCM maceration of the flowers produced a mixture of β-sitosterol and stigmasterol (0.8%), ceanothenic acid (3.7%), alphitolic acid (5.2%), veratric acid (4.5%), and β-sitosterol β-D-glucopyranoside (2.2%), with structures confirmed by ^{1}H NMR data comparison [3, 4-14]. Notably, this is the first report of alphitolic acid in the *Ceanothus* genus. Subsequent EtOAc extraction yielded lower amounts of ceanothenic acid (0.5%), β-sitosterol β-D-glucopyranoside (0.8%), and kaempferol (0.9%) [15]. For the leaves, DCM extraction isolated ceanothenic acid (2.7%), alphitolic acid (5.5%), β-sitosterol β-D-glucopyranoside (2.0%), and gouanic acid B (**1**) (2.0%) similar to flower yields, along with betulinic acid as a minor product (0.7%) [16]. EtOAc extraction gave β-sitosterol β-D-glucopyranoside (0.8%) and acetylgouanic acid B (**2**) (1.4%), whose structure was elucidated through 1D and 2D NMR, as it has not been previously reported as a natural product (see Supplementary Information). From the stems, DCM extraction yielded ceanothenic acid (2.1%) and β-sitosterol β-D-glucopyranoside (4.2%). DCM extraction of the roots provided high yields of dehydroabietic acid (22.8%) [17], while EtOAc extraction identified A-*nor*-lupane ceanothic acid (21%) [18-21] (see Tables S1 and S2). The presence of component **2** in the plant has been confirmed through methanolic extraction by macerating the leaves without using any other solvent beforehand. The ^{1}H NMR spectrum of the methanol extract showed a distinctive singlet signal attributed to the acetyl group, which verifies the presence of acetyl A-*nor*-lupane (See Figure S21 and S22). Additionally, the same methanolic extract of the leaves underwent HPLC analysis, using compound **2** as a standard. Data were recorded at a wavelength of 220 nm, with characteristic peaks for compound **2** observed at a retention time of 3.298 minutes for the pure compound and 3.402 minutes for the crude leaves extract, falling within a normal range variation [22], providing compelling evidence of its presence in *C. caeruleus* (see Figures S24-S28).

*Acetylgouanic acid B (**2**):* colorless amorphous solid (decomposes above 100 °C), $[\alpha]_{589}$ = +60, $[\alpha]_{578}$ = +62, $[\alpha]_{546}$ = +72, $[\alpha]_{436}$ = +129, $[\alpha]_{365}$ = +220 (*c* 0.26, acetone at 25 °C, see supporting information for details of measurement); IR (KBr) ν_{max} 3450-2650 (-COOH), 2942 and 2869 (C-H), 1739 and 1687 cm^{-1} (C=O). ^{1}H NMR (300 MHz, pyridine-d_5) δ 6.19 (1H, d, *J* = 5.9 Hz, H-2), 5.48 (1H, d, *J* = 5.9 Hz, H-3), 5.08 (1H, brs, H-29), 4.85 (1H, brs, H-29′), 4.23 (1H, d, *J* = 11.1 Hz, H-24), 4.11 (1H, d, *J* = 11.1 Hz, H-24′), 3.68 (1H, m, H-19), 2.04 (3H, s, AcO), 1.94 (3H, s, CH_3-30), 1.18 (3H, s, CH_3-26), 1.04 (3H, s, CH_3-25), 1.02 (3H, s, CH_3-23); ^{13}C NMR (75.4 MHz, pyridine-d_5) δ 179.6 (C-28), 178.9 (C-27), 171.2 (AcO), 151.6 (C-20), 144.3 (C-2), 135.1 (C-3), 110.7 (C-29), 68.8 (C-24), 63.2 (C-5), 60.7 (C-14), 56.9 (C-17), 52.6 (C-18), 51.2 (C-10), 49.2 (C-4), 48.9 (C-9), 48.3 (C-19), 42.0 (C-8), 40.5 (C-13), 39.0 (C-7), 38.1 (C-22), 35.7 (C-16), 31.5 (C-21), 29.2 (C-15), 26.9 (C-12), 24.6 (C-23), 24.0 (C-11), 21.1 (AcO), 20.8 (C-25), 19.6 (C-30), 18.7 (C-26), 18.5 (C-6); EIMS *m/z* (rel. int.): 512 $[M]^+$ (0.4), 452 (4), 439 (17), 393 (16), 371 (54), 325 (12), 173 (100), 119 (82), 107 (84), 105 (81), 91(59). HRESIMS *m/z* 513.3213 (calcd. for $C_{31}H_{45}O_6$ + H, 513.3211).



1 R = H
3 R = Me

2 R = H
4 R = Me

Figure 1. A-*nor*-lupane-type triterpenes isolated from *C. caeruleus* (**1** and **2**) and their dimethyl ester derivatives (**3** and **4**).

Triterpene compounds with a carboxylic group at position C-28 are challenging to modify due to steric hindrance. A carbonyl group moiety is reportedly essential for better antitumor activity, and small-chain esters have been shown to enhance cytotoxicity. Consequently, even "simple" modifications, such as methylation and acetylation, can significantly improve the biological activity of natural products [23]. Thus, the dimethyl ester derivative **3** was prepared from **1** through methylation with diazomethane (see Supplementary Information), and the compound **4** was obtained by acetylation of **3** (see Supplementary Information).

*Dimethyl gouanate B (**3**):* colorless amorphous solid (decomposes above 100 °C), $[\alpha]_{589}$ = +38, $[\alpha]_{578}$ = +40, $[\alpha]_{546}$ = +46, $[\alpha]_{436}$ = +83, $[\alpha]_{365}$ = +142 (*c* 0.82, chloroform at 25 °C, see supporting information for details of measurement); IR ($CHCl_3$) ν_{max} 3612 (O-H), 2946, and 2864 (C-H), 1709 cm^{-1} (C=O). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.08 (1H, d, *J* = 5.9 Hz, H-2), 5.44 (1H, d, *J* = 5.9 Hz, H-3), 4.75 (1H, brs, H-29), 4.64 (1H, brs, H-29′), 3.69 (3H, s, OMe), 3.67 (3H, s, OMe), 3.62 (1H, d, *J* = 10.8 Hz, H-24), 3.53 (1H, d, *J* = 10.8 Hz, H-24′), 3.03 (1H, m, H-19), 2.33 (1H, m, H-15), 2.32 (1H, m, H-13), 2.07 (1H, m, H-16), 2.05 (1H, m, H-12), 1.89 (1H, m, H-22), 1.89 (1H, m, H-21), 1.72 (1H, m, H-16′), 1.69 (3H, s, CH_3-30), 1.67 (1H, m, H-9), 1.60 (1H, m, H-7), 1.58 (1H, m, H-6), 1.56 (1H, m, H-18), 1.52 (2H, m, CH_2-11), 1.43 (1H, m, H-7′), 1.40 (1H, m, H-6′), 1.35 (1H, m, H-21′), 1.34 (1H, m, H-22′), 1.27 (1H, m, H-12′), 1.21 (1H, m, H-5), 1.19 (1H, m, H-15′), 1.11 (3H, s, CH_3-23), 1.01 (3H, s, CH_3-26), 0.99 (3H, s, CH_3-25). ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 176.6 (C-28), 176.0 (C-27), 149.9 (C-20), 143.5 (C-2), 134.6 (C-3), 110.0 (C-29), 67.3 (C-24), 62.1 (C-5), 60.2 (C-14), 56.2 (C-17), 51.7 (C-18), 51.4 (OMe), 50.6 (OMe), 50.6 (C-4), 50.4 (C-10), 48.3 (C-9), 46.9 (C-19), 41.4 (C-8), 39.5 (C-13), 38.1 (C-7), 36.8 (C-22), 34.1 (C-15), 30.3 (C-21), 27.9 (C-12), 25.6 (C-16), 23.8 (C-23), 23.0 (C-11), 20.6 (C-25), 18.9 (C-30), 18.0 (C-26), 17.9 (C-6). EIMS *m/z* (rel. int.): 498 $[M]^+$ (0.2), 483 (0.2), 467 (7), 407 (10), 399 (24), 279 (10), 247 (8), 173 (38), 79 (100), 77 (52). HRESIMS *m/z* 499.3427 (calcd. for $C_{31}H_{46}O_5$ + H, 499.3418).

*Acetylgouanic acid B dimethyl ester (**4**):* colorless amorphous solid (decomposes above 100 °C). $[\alpha]_{589}$ = +40, $[\alpha]_{578}$ = +41, $[\alpha]_{546}$ = +47, $[\alpha]_{436}$ = +86, $[\alpha]_{365}$ = +145 (*c* 0.17, chloroform at 25 °C, see supporting information for details of measurement); IR ($CHCl_3$) ν_{max} 2944 and 2864 (C-H), 1717 cm^{-1} (C=O). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.08 (1H, d, *J* = 5.9 Hz, H-2), 5.40 (1H, d, *J* = 5.9 Hz, H-3), 4.75 (1H, brs, H-29), 4.64 (1H, brs, H-29′), 4.03 (1H, d, *J* = 10.8 Hz, H-24), 3.97 (1H, d, *J* = 10.8 Hz, H-24′), 3.69 (3H, s, OMe), 3.67 (3H, s, OMe), 3.05 (1H, m, H-19), 2.36 (1H, m, H-16), 2.31 (1H, m, H-16′), 2.08 (1H, m, H-15), 2.04 (2H, m, CH_2-12), 2.04 (3H, s, OAc), 2.02 (1H, m, H-15'), 1.90 (1H, m, H-22), 1.88 (1H, m, H-21), 1.69 (3H, s, CH_3-30), 1.68 (1H, m, H-9), 1.63 (1H, m, H-13), 1.62 (1H, m, H-7), 1.60 (1H, m, H-6), 1.57 (1H, m, H-18), 1.51 (2H, m, CH_2-11), 1.43 (1H, m, H-6′), 1.40 (1H, m, H-7′), 1.37 (1H, m, H-21′), 1.35 (1H, m, H-22'), 1.25 (1H, m, H-5), 1.08 (3H, s, CH_3-23), 1.01 (3H, s, CH_3-26), 1.00 (3H, s, CH_3-25). ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 176.7 (C-28), 176.1 (C-27), 171.3 (OAc), 150.0 (C-20), 143.7 (C-2), 134.2 (C-3), 110.1 (C-29), 68.4 (C-24), 62.3 (C-5), 60.2 (C-14), 56.2 (C-17), 51.8

(C-18), 51.4 (OMe), 50.7 (OMe), 50.4 (C-10), 48.4 (C-4), 48.2 (C-9), 47.0 (C-19), 41.4 (C-8), 39.6 (C-13), 38.1 (C-7), 36.9 (C-22), 34.1 (C-16), 30.4 (C-21), 27.9 (C-15), 25.7 (C-12), 24.3 (C-23), 23.0 (C-11), 20.9 (OAc), 20.4 (C-25), 18.9 (C-30), 18.0 (C-26), 17.8 (C-6). EIMS *m/z* (rel. int.): 540 $[M]^+$ (0.17), 525 (0.24), 467 (4), 399 (35), 339 (15), 279 (26), 247 (11), 173 (75), 105 (100), 91 (89), 79 (58). HRESIMS *m/z* 541.3527 (calcd for $C_{33}H_{48}O_6$ + H, 541.3524).

Cytotoxic Activity Tes:. The most common types of cancer are breast cancer (MCF-7), lung cancer (A549), cervical cancer (HeLa), and leukemia (K562). The two natural products, **1** and **2**, and their respective dimethyl ester derivatives, **3** and **4**, were tested against the four cancer cell lines (Table 1). These results showed that the four compounds have IC_{50} values greater than 50 μM for the MCF-7 and A549 cell lines. In the HeLa cell line, the same trend was observed for three compounds, except for the dimethyl ester of gouanic acid B (**3**) with an IC_{50} value of 18.3 μM. Regarding the K562 cell line, gouanic acid B (**1**) and the acetyl dimethyl ester derivative **4** showed an IC_{50} with 36.4 and 33.0 μM values, respectively. In this cell line, the natural product acetylgouanic acid B (**2**) showed a good IC_{50} value of 21.6 μM, while gouanic acid dimethyl ester **3** showed a better IC_{50} value of 7.1 μM. The survival rate in the percentage of bovine mammary epithelial cells (bMECs) treated with **3** was low (35%), but cells treated with **1** showed an acceptable survival rate (89%) (Table 1).

Table 1. IC_{50} of compounds (μM) **1**, **2**, **3**, and **4** against MCF-7, A549, HeLa, and K562 cancer cell lines and survival percentage of bMEC non-cancerous cells .

Compound	MCF-7[a]	A549[a]	HeLa[a]	K562[a]	bMEC[a]	
					CT[b]	survival (%)
1	>50	>50	>50	36.4 ± 4.0	40	88
2	>50	>50	>50	21.6 ± 4.3	26	89
3	>50	>50	18.3 ± 2.4	7.1 ± 1.8	21	35
4	>50	>50	>50	33.0 ± 2.0	35	92
Actinomycin D	63[c]	73[c]	9[c]	71[c]	—	73[c]

[a] Experiments were performed in triplicate.
[b] Concentrations assessed (CT) for survival experiment on bMEC were obtained by adding IC_{50} + SE for each cell line, CT = IC_{50} + SE.
[c] Survival (%) of each cell line at 10 μM of actinomycin D as control.

It is worth mentioning that in all assays, the concentration tested (CT) in normal cells had higher concentrations than the corresponding IC_{50}. CT values were obtained by adding the corresponding maximum standard error (SE) value to each IC_{50} value. Thus, for compounds **1**, **2**, **3**, and **4**, the CT was 40, 26, 21, and 35 μM, respectively. Noteworthy, the survival percentage of non-cancerous cells (bMEC) treated with **1**, **2**, and **4** was 88, 89 and 92%, respectively. Also, these three triterpenes showed selective cytotoxicity on K562 myelogenous leukemia cells since they showed no activity against breast (MCF-7), lung (A549), and cervical carcinoma (HeLa) cancer cells.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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