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Flavonoids from *Eupatorium illitum* and Their Antiproliferative Activities

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ABSTRACT

Objective: To isolate the chemical constituents of the aerial parts from *Eupatorium illitum*, elucidate their structures and evaluate their antiproliferative activity on human cancer cell lines. **Materials and Methods:** The ethanolic extract of *Eupatorium illitum* afforded five compounds, which were characterized using spectroscopic techniques and by comparison with data from the literature. Antiproliferative activities of selected isolates were evaluated. **Results:** The flavonoids Kumatakenin (**1**), Ermanin (**2**), 7-methoxy-aromadendrin (**3**) and Naringenin (**4**), together with 4-hydroxybenzoic acid (**5**) were isolated. Compounds **1**, **2**, **4** and **5** were evaluated for their antiproliferative activity on the human cancer cell lines A549 (lung), HBL-100 (breast), HeLa (cervix), SW1573 (lung), and T-47D (breast) presenting a wide range of bioactivities. In general, best results were observed for **5**. **Conclusion:** Compounds **1-5** are reported for first time from *Eupatorium illitum*. Isolated phytochemicals show moderate to low antiproliferative activities when evaluated on the aforementioned human cancer cell lines.

Key words: *Eupatorium illitum*, Ermanin, 4-Hydroxybenzoic acid, Kumatakenin, 7-Methoxy-aromadendrin, Naringenin.

INTRODUCTION

Eupatorium illitum Urb. (Asteraceae) is an endemic plant from Dominican Republic.[1] The genus Eupatorium comprises of nearly 1,200 species distributed mainly in the tropical regions of Americas, Europe, Africa and Asia[2]. Flavonoids isolated from species of this genus have been reported to show cytotoxic activity against cancer cell lines.[3-5] In the present study, we report compounds **1-5**, isolated for first time from *E. illitum*, and their antiproliferative activities on human cancer cell lines A549, HBL-100, SW1573, HeLa, and T-47D.

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MATERIALS AND METHODS

General

IR spectra were recorded using a Hyperion 3000 Fourier transform infrared microscope. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance III spectrometer with cryoprobe operating at 700 MHz in ^{1}H and 175 MHz in

^{13}C NMR respectively. DMSO–d_6 was used as solvent. HRESIMS was performed with a Bruker Maxis 4G QTOF mass spectrometer; presented results are for negative ion mode. Silica gel 60 (particle sizes 0.040-0.063 mm and 0.015-0.040 mm, Merck KGaA, Darmstadt, Germany) was used for column chromatography. Analytical and preparative TLC was developed on Silica gel 60 F_{254} plates (Merck KGaA, Darmstadt, Germany).

Plant Material

The aerial parts of *Eupatorium illitum* Urb. (Asteraceae) were collected on June 2010 at Sierra de Bahoruco, Provincia Pedernales, Dominican Republic. The plant material was identified by Teodoro Clase, botanist at Jardín Botánico Nacional "Dr. Rafael Ma. Moscoso", Santo Domingo, Dominican Republic, where a voucher specimen (JBSD 121457) has been deposited.

Extraction and Isolation

Aerial parts of *Eupatorium illitum* were shade, air dried, and ground to a fine powder. The ground material (617 g) was exhaustively extracted with 95% EtOH in a Soxhlet apparatus. The resulting crude extract (90.9 g) was dissolved in 95% EtOH (1.2 L) and treated with a 5% lead acetate solution (1L) to precipitate chlorophyll. After 24 hours, the mixture was filtered over paper, concentrated *in vacuo* to remove most of the EtOH, and extracted successively with hexane (6 x 750 mL) and ethyl acetate (6 x 1L). The ethyl acetate residue (10.7 g) was washed with distilled water (2 x 1L), dried over anh. Na_2SO_4, and subjected to CC (Si gel 0.040-0.063 mm) using hexane-acetone mixtures of increasing polarity to afford 22 fractions (F1-F22). Fraction 15 afforded **1** (190.6 mg) as a yellow precipitate; supernatant F15 was eluted over a column of Si gel (0.015-0.040 mm) with a gradient system of hexane-AcOEt (3:1 to pure AcOEt) affording 19 subfractions. Subfraction F15-13 gave **2** (9.2 mg) after PTLC (Si gel) using a mixture of hexane-AcOEt (7:3).

Fraction 16 was chromatographed over a column of Si gel (0.015-0.040 mm) with a gradient system of hexane-AcOEt (7:3 to pure AcOEt), affording 16 subfractions. Sub fraction F16-15 was recromatographed over Si gel (0.015-0.040 mm) using a gradient system of hexane-AcOEt (7:3 to pure AcOEt) to yield 21 subfractions. PTLC (Si gel) of subfraction F16-15-15 using a mixture of hexane-AcOEt (6.5:3.5, twice) afforded **3** (5.8 mg) and **4** (5.6 mg).

Fraction 21 was chromatographed over a Si gel (0.015-0.040 mm) column with a gradient system of hexane-acetone (6.5:3.5 to pure acetone) to yield **5** (13.9 mg).

Antiproliferative assays

Biology

All starting materials were commercially available research-grade chemicals and used without further purification. Fetal calf serum (FCS) was purchased from Gibco (Grand Island, NY), trichloroacetic acid (TCA) and glutamine were from Merck (Darmstadt, Germany), and RPMI 1640 medium penicillin G, streptomycin, DMSO and sulforhodamine B (SRB) were from Sigma (St Louis, MO).

Cell lines and culture

The human solid tumor cell lines A549, HBL-100, HeLa, SW1573, and T-47D were used in this study. These cell lines were a kind gift from Prof. G. J. Peters (VU Medical Center, Amsterdam, The Netherlands). Cells were maintained in 25 cm^2 culture flasks in RPMI 1640 supplemented with 5% heat inactivated fetal calf serum and 2 mM L-glutamine in a 37ºC, 5% CO_2, 95% humidified air incubator. Exponentially growing cells were trypsinized and re-suspended in antibiotic containing medium (100 units penicillin G and 0.1 mg of streptomycin per mL). Single cell suspensions were counted using Orflow's Moxi^Z automated cell counter (Ketchum, ID) and dilutions were made to give the appropriate cell densities for inoculation onto 96-well microtiter plates. Cells were inoculated in a volume of 100 µL per well at densities of 10 000 (A549, HBL-100, HeLa and SW1573), and 15 000 (T-47D) cells per well, based on their doubling times.

Chemosensitivity testing

Compounds were initially dissolved in DMSO at 400 times the desired final maximum test concentration. Control cells were exposed to an equivalent concentration of DMSO (0.25% v/v, negative control). Each agent was tested in triplicate at different dilutions in the range of 1–100 µM. The drug treatment was started on day 1 after plating. Drug incubation times were 48 h, after which time cells were precipitated with 25 µL ice-cold TCA (50% w/v) and fixed for 60 min at 4ºC. Then the SRB assay was performed.[6] The optical density (OD) of each well was measured at 492 nm, using BioTek's PowerWave XS Absorbance Microplate Reader (Winooski, VT). Values were corrected for background OD from wells only containing medium.

RESULTS AND DISCUSSION

The ethyl acetate residue (10.7 g) of the ethanolic extract from *E. illitum*, afforded, after chromatographic

procedures, compounds **1-5** (Figure 1). Their chemical structures were identified using spectroscopic techniques (FTIR, HRESIMS, and NMR) and by comparison with data reported in literature. Their found spectral data are shown below.

Kumatakenin (1)

Yellow powder; IR ν_{max}: 3236, 2948, 2362, 2337, 1656, 1600, 1583, 1497 cm^{-1}; HRESIMS obsd. m/z 313.0663 [M – H]$^-$ calcd. for $C_{17}H_{14}O_6$, 313.0790; ^{1}H NMR (700MHz, DMSO-d_6) δ = 12.69 (1H, s, 5-OH), 10.31 (1H, br s, 4'-OH), 7.99 (2H, d, *J* = 8.9 Hz, H-2', H-6'), 6.97 (2H, d, *J* = 8.6 Hz, H-3', H-5'), 6.76 (1H, d, *J* = 1.6 Hz, H-8), 6.39 (1H, d, *J* = 1.6 Hz, H-6), 3.87 (3H, s, 7-OMe), 3.81 (3H, s, 3-OMe). ^{13}C NMR (176MHz, DMSO-d_6) δ = 178.5 (C-4), 165.6 (C-7), 161.4 (C-5), 160.8 (C-4'), 156.8 (C-9), 156.4 (C-2), 138.3 (C-3), 130.7 (C-2', C-6'), 120.9 (C-1'), 116.1 (C-3', C-5'), 105.7 (C-10), 98.2 (C-6), 92.8 (C-8), 60.2 (3-OMe), 56.6 (7-OMe). The spectroscopic data (^{1}H and ^{13}C NMR) were comparable with published values.[7,8] Assignments were confirmed by HSQC and HMBC experiments.

Ermanin (2)

Yellow solid; IR ν_{max}: 3130, 2936, 2840, 2361, 2050, 1650, 1604, 1498 cm^{-1}; HRESIMS obsd. m/z 313.0706 [M – H]$^-$ calcd. for $C_{17}H_{14}O_6$, 313.0790; ^{1}H NMR (700MHz, DMSO-d_6) δ = 12.65 (1H, s, 5-OH), 8.03 (2H, d, *J* = 8.8 Hz, H-2', H-6'), 7.14 (2H, d, *J* = 9.1 Hz, H-3', H-5'), 6.46 (1H, d, *J* = 1.7, H-8), 6.21 (1H, d, *J* = 1.9 H-6), 3.87 (3H, s, 4'-OMe), 3.80 (3H, s, 3-OMe). ^{13}C NMR (176MHz, DMSO-d_6) δ = 178.0 (C-4), 164.4 (C-7), 161.4 (C-4'), 161.3 (C-5), 156.5 (C-9), 155.2 (C-2), 138.0 (C-3), 130.0 (C-2', C-6'), 122.2 (C-1'), 114.3 (C-3', C-5'), 104.2 (C-10), 98.7 (C-6), 93.9 (C-8), 59.8 (3-OMe), 55.5 (4'-OMe). The spectroscopic data (^{1}H and ^{13}C NMR) were comparable with published values.[9,10] Assignments were confirmed by HSQC and HMBC experiments.

7-Methoxy-aromadendrin (3)

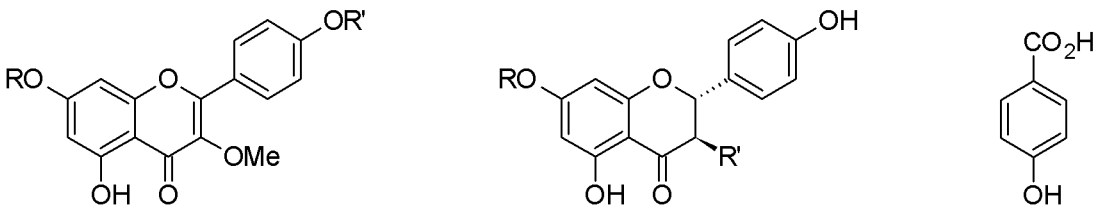
Yellow solid; IR ν_{max}: 3144, 2939, 2258, 1716, 1636, 1616, 1595, 1573, 1501, 1443 cm^{-1}; HRESIMS obsd. m/z 301.0717 [M – H]$^-$ calcd. for $C_{16}H_{14}O_6$, 301.0790; ^{1}H NMR (700MHz, DMSO-d_6) δ = 11.88 (1H, s, 5-OH), 9.57 (1H, s, 4'-OH), 7.33 (2H, d, *J* = 8.2 Hz, H-2', H-6'), 6.80 (2H, d, *J* = 8.5 Hz, H-3', H-5'), 6.12 (1H, d, *J* = 2.1 Hz, H-6), 6.10 (1H, d, *J* = 1.9 Hz, H-8), 5.82 (1H, d, *J* = 6.4 Hz, 3-OH), 5.11 (1H, d, *J* = 11.3, H-2), 4.66 (1H, dd, *J* = 11.5, 6.3 Hz, H-3), 3.79 (3H, s, 7-OMe); ^{13}C NMR (176MHz, DMSO-d_6) δ = 199.0 (C-4), 168.0 (C-7), 163.4 (C-5), 163.0 (C-9), 158.2 (C-4'), 130.0 (C-2', C-6'), 127.9 (C-1'), 115.4 (C-3', C-5'), 101.8 (C-10), 95.4 (C-6), 94.3 (C-8), 83.5 (C-2), 72.0 (C-3), 56.4 (7-OMe). The spectroscopic data (^{1}H and ^{13}C NMR) were comparable with published values.[11] Assignments were confirmed by HSQC and HMBC experiments.

Naringenin (4)

Yellow solid; IR ν_{max}: 3115, 3055, 2019, 2819, 2831, 2700, 2349, 2286, 2051, 1898, 1627, 1600, 1586, 1519, 1496 cm^{-1}; HRESIMS obsd. m/z 271.0603 [M – H]$^-$ calcd. for $C_{15}H_{12}O_5$, 271.0685; ^{1}H NMR (700MHz, DMSO-d_6) δ = 12.16 (1H, s, 5-OH), 10.80 (1H, br s, 7-OH), 9.59 (1H, br s, 4'-OH), 7.32 (2H, d, *J* = 8.5 Hz, H-2', H-6'), 6.80 (2H, d, *J* = 8.3 Hz, H-3', H-5'), 5.88 (2H, s, H-6, H-8), 5.45 (1H, dd, *J* = 12.8, 2.8 Hz, H-2), 3.28 (1H, dd, *J* = 17.0, 12.9, H-3 trans.), 2.69 (1H, dd, *J* = 17.1, 2.9 Hz, H-3 cis.); ^{13}C NMR (176MHz, DMSO-d_6) δ = 196.8 (C-4), 167.2 (C-7), 163.9 (C-5), 163.4 (C-9), 158.2 (C-4'), 129.3 (C-1'), 128.8 (C-2', C-6'), 115.6 (C-3', C-5'), 102.2 (C-10), 96.3 (C-6), 95.5 (C-8), 78.9 (C-2), 42.4 (C-3). The spectroscopic data (^{1}H and ^{13}C NMR) were comparable with published values.[12,13]

4-Hydroxybenzoic acid (5)

White solid; IR ν_{max}: 3400, 2255, 2128, 1654, 1049, 1023, 999, 824, 762, 625, 614 cm^{-1}; HRESIMS obsd. m/z 137.0242 [M – H]$^-$ calcd. for $C_7H_6O_3$, 137.0317; ^{1}H NMR (700MHz,



1 R = CH_3, R' = H

2 R = H, R' = CH_3

3 R = CH_3, R' = OH

4 R = H, R' = H

5

Figure 1: Structures of compounds 1-5 isolated from the aerial parts of *Eupatorium illitum*

Table 1: Values of GI_{50} (μg.ml^{-1}) for selected compounds from *Eupatorium illitum* on human cancer cell lines

Compound	A549	HBL-100	SW1573	HeLa	T-47D
1	163	12	5	10	250
2	16	11	7	8	58
4	40	22	10	7	49
5	12	10	8	7	13

DMSO-d_6) δ = 7.79 (2H, d, *J* = 8.5 Hz, H-2, H-6), 6.82 (2H, d, *J* = 8.7 Hz, H-3, H-5); ^{13}C NMR (176MHz, DMSO-d_6) δ = 167.6 (COOH), 162.0 (C-4), 132.0 (C-2, C-6), 121.8 (C-1), 115.6 (C-3, C-5). The spectroscopic data (^{1}H and ^{13}C NMR) were comparable with published values.[14]

Antiproliferative activity

Selected isolates were evaluated for their antiproliferative activity against the human solid tumor cell lines A549 (lung), HBL-100 (breast), HeLa (cervix), SW1573 (lung), and T-47D (breast). The effect of these compounds on the aforementioned cell lines, expressed as the value GI_{50} (concentration causing 50% of growth inhibition), is presented in Table 1.

CONCLUSION

In summary, we have reported the isolation of four flavonoids from Eupatorium illitum in addition to 4-Hydroxybenzoic acid. All compounds are reported for first time in this plant. The study of the antiproliferative activity against human solid tumor cell lines showed that the compounds are able to inhibit cell growth in all cell lines tested.

CONFLICT OF INTEREST

Authors do not have any conflict of interest.

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