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CHEMICAL CONSTITUENTS AND ANTIPROLIFERATIVE ACTIVITY OF *Euphorbia bivonae*

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Euphorbia is a genus of plants belonging to the family Euphorbiaceae, consisting of about 2160 species. This family occurs mainly in the Indo-Malayan region and tropical America. *Euphorbia* also has many species in non-tropical areas such as the Mediterranean area, the Middle East, South Africa, and southern USA.

Phytochemical studies of species from the genus *Euphorbia* report the presence of skin-irritating and tumor-promoting diterpenoids [1], several macrocyclic diterpenoids with antibacterial, anticancer, PGE2-inhibitory, antifeedant, anti-HIV, and analgesic activities [2–4]. Some of the Euphorbiaceae species are used in folk medicine for the treatment of skin diseases, gonorrhea, migraines, intestinal parasites, and as a purgative [5].

Euphorbia bivonae Steudel is distributed in Southern Italy, Morocco, Algeria, Tunisia, Lebanon, and Palestine. There are no previous phytochemical studies reported in the literature on the chemical composition of the aerial parts.

This paper concerns the determination of fatty acid components by GC-MS, the isolation of triterpene and coumarin constituents by chromatographic purification, and the antimicrobial and antiproliferative activities (by microdilution assays) of the acetonic extracts of *Euphorbia bivonae* stem and leaf. Leaf and stem fatty acid (%) contents of *E. bivonae* are reported in Table 1. Stearic, linoleic, and palmitic acids, detected as methyl esters by GC-MS, are the main constituents in leaves; palmitic, lauric, decadioic, and oleic acids are the main constituents in stems.

From the acetonic extract of the leaves were isolated eight triterpene compounds: lupeol (**1**), β-amyrin (**2**), α-amyrin (**3**), β-amyrin acetate (**4**), olean-12-en-3-one (**5**), campesterol (**6**), stigmasterol (**7**), and β-sitosterol (**8**) together with five known coumarins: osthol (**9**), bergapten (**10**), xanthoxin (**11**), isopimpinellin (**12**), and imperatorin (**13**), and from the acetonic extract of the stems were isolated compounds **1**, **3**, **6**–**9**, **11**, and **12**.

All compounds were identified by their physical (mp, $[\alpha]_D^{20}$) and spectroscopic data (^{1}H NMR, ^{13}C NMR, MS) and by direct comparison with original samples. The obtained spectra of the compounds were in agreement with data reported in the literature.

Plant Material. Plant samples (leaves and stems) were collected in Monte Inici near Castellammare del Golfo (Trapani) during the spring and identified by members of the Department of Botanical Science, University of Palermo. A voucher specimen is deposited in the Herbarium of the Botanical Garden of Palermo (Italy).

Extraction, Isolation, and GC-MS Analyses. Leaves (140 g) and stems (300 g) of the plant were air dried, pulverized, and individually extracted with acetone at room temperature for 7 days to give, after filtration and *in vacuo* evaporation, two brown residues: 21 and 10.90 g, respectively.

The acetonic residue of the leaves (10 g) was suspended in 85% aq. MeOH and treated with *n*-hexane (3 × 100 mL). The obtained residue (3.5 g) after evaporation *in vacuo* of the *n*-hexane phase was suspended in 10% KOH in MeOH (100 mL) and kept at reflux for 4 h until complete dissolution. After evaporating the MeOH, the remaining aqueous phase was extracted with Et_2O (3 × 50 mL). The collected Et_2O layers containing the nonsaponifiable fraction were dried with Na_2SO_4 and, after Et_2O phase evaporation, yielded 1.80 g of residue.

The remaining aqueous phase was acidified with HCl, diluted, and extracted with Et_2O (3 × 50 mL).

The collected Et_2O layers containing the free fatty acid fraction were dried with Na_2SO_4, and after evaporation of the Et_2O phase, yielded a residue (0.18 g), which was methylated with diazomethane to obtain the methyl esters of the fatty acids.

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TABLE 1. Leaf and Stem Fatty Acid % Contents of *E. bivonae* Detected as Methyl Esters by GC-MS, %

Fatty acid	Leaf	Stem	Fatty acid	Leaf	Stem
9:0	–	0.8	18:0	51.5	1.7
10:0	–	0.6	18:2	21.6	5.7
12:0	–	20.1	18:3	Tr.	Tr.
14:0	4.0	3.1	20:4	9.5	–
15:0	Tr.	–	20:1	0.5	–
16:0	10.1	33.6	22:0	0.7	–
10:1	–	15.1	23:0	0.2	0.8
16:1	0.2	1.5	26:0	–	0.7
18:1	0.4	13.1			

Tr.: trace (≤ 0.05%).

The residue containing the methyl esters of the fatty acids, diluted in dichloromethane to obtain samples of 100 ppb, was injected in the split mode (1:100) and analyzed by GC-MS using a Hewlett-Packard gas-chromatograph, model 6890. It was equipped with a DB-5MS column (30 m × 0.25 mm × 0.25 μm, Agilent, USA). The working conditions were as follows: injector temperature 250°C; programmed heating from 100 to 280°C at 5°C/min, followed by 14 min under isothermal conditions; helium carrier gas at 1.0 mL/min.

The GC was interfaced with a Micromass AutoSpec Ultima-OToF double-focusing magnetic sector mass spectrometer. Identification of the individual components was based on matching with the NIST 2005 mass spectra library and comparison with spectra of authentic samples and literature data. Total ion current peak areas gave good approximations to relative concentrations based on GC-MS peak areas.

The acetonic residue of the stems (10 g) treated using the same procedure as for the acetonic residae of the leaves to give nonsaponifiable (0.65 g) and fatty acid methyl ester fractions (0.07 g).

The nonsaponifiable leave fraction (1.80 g) was applied to a silica gel column using a gradient of petroleum ether–EtOAc. Seven fractions were collected and monitored by TLC. Fractions 1–3 were combined, and after further purification by preparative TLC using CH_2Cl_2–MeOH (99:1), CH_2Cl_2–EtOAc (25:1), and $CHCl_3$–EtO_2 (19:1), we isolated eight triterpene compounds: lupeol (**1**, 30 mg), *β*-amyrin (**2**, 12.5 mg), *α*-amyrin (**3**, 15.2 mg), *β*-amyrin acetate (**4**, 22.2 mg), olean-12-en-3-one (**5**, 5.2 mg), campesterol (**6**, 6.5 mg), stigmasterol (**7**, 5.9 mg), and *β*-sitosterol (**8**, 9.2 mg).

Lupeol (1). $C_{30}H_{50}O$, colorless needles, mp 177°C. EI-MS *m/z*: 426 $[M]^{\cdot+}$ (25), 411 (10), 393 (3), 218 (45), 207 (55), 203 (40), 189 (50) [6, 7].

***β*-Amyrin (2)**. $C_{30}H_{50}O$, colorless needlelike crystals, mp 196–198°C. EI-MS *m/z*: 426 $[M]^{\cdot+}$ (10), 411 (2), 218 (100), 203 (30), 189 (10) [6–8].

***α*-Amyrin (3)**. $C_{30}H_{50}O$, white needle, mp 182–184°C. EI-MS *m/z:* 426 $[M]^{\cdot+}$ (20), 411 (3), 218 (100), 203 (14), 189 (12) [6].

***β*-Amyrin Acetate (4)**. $C_{32}H_{52}O_2$, colorless crystals, mp 193°C. EI-MS *m/z:* 468 $[M]^{\cdot+}$ (20), 453 (5), 408 (3), 393 (3), 272 (10), 249 (8), 218 (100), 189 (30) [8].

Olean-12-en-3-one (5). $C_{30}H_{48}O$, white needle, mp 177–179°C. EI-MS *m/z*: 424 $[M]^{\cdot+}$ (8), 409 (5), 218 (100), 203 (50), 189 (15) [8].

Campesterol (6). $C_{28}H_{48}O$, EI-MS *m/z*: 400 $[M]^{\cdot+}$ (30), 382 (26), 367 (17), 289 (31), 213 (26), 161 (13), 145 (21).

Stigmasterol (7). $C_{29}H_{48}O$, EI-MS *m/z*: 412 $[M]^{\cdot+}$ (20), 397 (6), 369 (7), 300 (21), 271 (6), 255 (13), 159 (11), 133 (22).

***β*-Sitosterol (8)**. $C_{29}H_{50}O$, colorless crystals. EI-MS *m/z*: 414 $[M]^{\cdot+}$ (32), 396 (10), 381 (5), 329 (11), 303 (6), 273 (12), 255 (15), 213 (20) [9].

Fractions 4–7 were combined, and after further purification we isolated five coumarin compounds: osthol (**9**, 19.5 mg), bergapten (**10**, 17.2 mg), xanthoxin (**11**, 9.8 mg), isopimpinellin (**12**, 10.6 mg), imperatorin (**13**, 11.7 mg).

Osthol (9). $C_{15}H_{16}O_3$, amorphous solid, mp 82–84°C. EI-MS *m/z*: 244 $[M]^{\cdot+}$ (100), 229 (80), 213 (40), 201 (60), 189 (55), 175 (20), 159 (15), 131 (30), 115 (25), 103 (10). ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm, J/Hz): 1.67 (3H, s, H-5′), 1.84 (3H, s, H-4′), 3.49 (2H, d, J = 7.2, H-1′), 3.92 (3H, s, OCH_3-7), 5.22 (1H, t, J = 7.2, H-2′), 6.23 (1H, d, J = 9.6, H-3), 6.84 (1H, d, J = 8.4, H-6), 7.29 (1H, d, J = 8.4, H-5), 7.62 (1H, d, J = 9.6, H-4). ^{13}C NMR (75 MHz, $CDCl_3$, δ, ppm): 160.15 (C-2), 112.71 (C-3), 143.79 (C-4), 126.25 (C-5), 117.70 (C-6), 161.32 (C-7), 107.33 (C-8), 152.68 (C-9), 112.86 (C-10), 21.83 (C-1′), 121.12 (C-2′), 132.44 (C-3′), 17.83 (C-4′), 25.69 (C-5′), 55.96 (OCH_3) [10].

Bergapten (10). $C_{12}H_8O_4$, amorphous solid, mp 190–191°C, EI-MS *m/z*: 216 $[M]^{\cdot+}$ (100), 201 (40), 188 (29), 173 (64), 145 (35). ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm, J/Hz): 4.27 (3H, s, OCH_3), 6.27 (1H, d, J = 9.8, H-3), 7.02 (1H, d, J = 2.5, H-9), 7.13 (1H, s, H-8), 7.59 (1H, d, J = 2.5, H-10), 8.15 (1H, d, J = 9.8, H-4). ^{13}C NMR (75 MHz, $CDCl_3$, δ): 160.3 (C-2), 112.5 (C-3), 139.8 (C-4), 149.7 (C-5), 114.0 (C-6), 158.3 (C-7), 94.0 (C-8), 105.9 (C-9), 152.7 (C-10), 105.2 (C-4a), 144.9 (C-8a), 60.16 (OCH_3) [11, 12].

Xanthoxin (11). $C_{12}H_8O_4$, white powder, mp 147°C. EI-MS *m/z*: 216 $[M]^{\cdot+}$ (100), 201 (38), 173 (58), 145 (23), 89 (18). ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm, J/Hz): 4.30 (3H, s, OCH_3), 6.38 (1H, d, J = 9.6, H-3), 6.82 (1H, d, J = 2.4, H-9), 7.36 (1H, s, H-5), 7.69 (1H, d, J = 2.4, H-10), 7.77 (1H, d, J = 9.6, H-4). ^{13}C NMR (75 MHz, $CDCl_3$, δ): 160.7 (C-2), 114.8 (C-3), 144.4 (C-4), 112.9 (C-5), 126.3 (C-6), 147.7 (C-7), 132.6 (C-8), 106.9 (C-9), 146.7 (C-10), 106.4 (C-4a), 152.7 (C-8a), 61.2 (OCH_3) [13, 14].

Isopimpinellin (12). $C_{13}H_{10}O_5$, white powder, mp 145–146°C. EI-MS *m/z:* 246 $[M]^{\cdot+}$ (94), 231 (100), 203 (20), 175 (14), 160 (24), 147 (17), 104 (10). ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm, J/Hz): 4.07 (3H, s, OCH_3-8), 4.09 (3H, s, OCH_3-5), 6.20 (1H, d, J = 9.8, H-3), 7.0 (1H, d, J = 2.2, H-9), 7.63 (1H, d, J = 2.2, H-10), 8.07 (1H, d, J = 9.8, H-4). ^{13}C NMR (75 MHz, $CDCl_3$, δ): 160.9 (C-2), 113.25 (C-3), 139.82 (C-4), 144.08 (C-5), 115.18 (C-6), 150.41 (C-7), 128.50 (C-8), 105.20 (C-9), 145.53 (C-10), 61.22 (OMe-5), 62.13 (OMe-8), 108.01 (C-4a), 144.69 (C-8a) [13–15].

Imperatorin (13). $C_{16}H_{14}O$, white powder, mp 103–101°C. EI-MS *m/z:* 270 $[M]^{\cdot+}$ (20), 202 (100), 174 (20), 145 (10), 118 (8), 89 (15), 69 (18), 53 (3). ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm, J/Hz): 1.72 (3H, s, H-5′), 1.74 (3H, s, H-4′), 5.00 (2H, d, J = 7.2, H-1′), 5.56 (1H, m, H-2′), 6.37 (1H, d, J = 10, H-3), 6.82 (1H, d, J = 2.0, H-9), 7.36 (1H, s, H-5), 7.69 (1H, d, J = 2.0, H-10), 7.77 (1H, d, J = 9.6, H-4). ^{13}C NMR (75 MHz, $CDCl_3$, δ): 159.8 (C-2), 114.2 (C-3), 145.3 (C-4), 114.1 (C-5), 125.7 (C-6), 147.8 (C-7), 130.5 (C-8), 107.1 (C-9), 146.4 (C-10), 116.3 (C-4a), 143.2 (C-8a), 69.3 (C-1′), 119.6 (C-2′), 139.1 (C-3′), 17.8 (C-4′), 25.4 (C-5′) [11–15].

The nonsaponifiable stem fraction (0.650 g) was treated with the above-mentioned procedure to isolate compounds **1**, **3**, **6–9**, **11**, and **12**, which were present in the nonsaponifiable leaf fraction too.

Antimicrobial and Antiproliferative Assays. Assessment of antimicrobial activity of acetone extracts was made on *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 10231, and *Candida tropicalis* ATCC 13803 microbial strains.

The acetonic extracts showed inactive antimicrobial activity at the maximum tested concentration of 200 μg/mL against the tested microorganisms.

The acetone extracts were tested *in vitro* for their antiproliferative activity against K562 (human chronic myelogenous leukemia), MCF-7 (breast tumor), and NCI-H460 (human lung carcinoma) cell lines.

The antiproliferative effect of the extracts was estimated as percentage of growth inhibition using the methods described in [16].

The acetone extracts showed good activity against K562, with IC_{50} values of 65.7 μg/mL for leaves and 1.7 μg/mL for stems, and only moderate activity on MCF7 and NCI-H460.

In fact, growth inhibition percentages recorded on NCI-H460 cell lines at a screening concentration of 100 μg/mL were 95.5% for stem and 83.1% for leaf extracts.

The cytotoxicity of the extracts evaluated as growth inhibition percentages on MCF7 at a single dose of 100 μg/mL was 22.0% for leaves and 56.2% for stems.

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