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Cytotoxic Sesquiterpene Lactones from *Centaurothamnus maximus* and *Vicoa pentanema*

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The aerial parts of *Centaurothamnus maximus* yielded three cytotoxic guaianolides, chlorojanerin (1), cynaropicrin (2) and janerin (3). The structure elucidation of 1–3 was based on ^{1}H and ^{13}C NMR data, mainly 2D-NMR ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR experiments. Compounds 1–3 showed *in vitro* cytotoxic activity against human cancer cell lines of malignant melanoma (SK-MEL), epidermoid (KB), ductal (BT-549) and ovarian (SK-OV-3) carcinomas with IC_{50} values of 2–6 μg/mL. In addition, 12 sesquiterpene lactones (4–15), isolated previously from the aerial parts of *Vicoa pentanema*, were evaluated for cytotoxic and antimicrobial activities. 2α- Acetoxy-3β-hydroxyalantolactone (10) and 8β-hydroxyparthenolide (14) were found to be the main cytotoxic agents (IC_{50} values of 2–6 μg/mL against SK-MEL, BT-549 and SK-OV-3), while lactones 4, 5, 11 and 15 selectively inhibited the growth of human malignant melonoma (IC_{50} value of 3.6–7.3 μg/mL). Cell aggregation and cell adhesion assays, using HL-60 and HeLa cell lines, evaluated the effect of cytotoxic constituents 1–3, 10 and 14 on immune response and inflammation. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords: Centaurothamnus maximus; *Vicoa pentanema*; Compositae; sesquiterpene lactone; cytotoxic; cell aggregation; cell adhesion.

INTRODUCTION

Centaurothamnus maximus Wagentz & Dittri. (Fam. Compositae) is a leafy shrub with many branches and about 1.5 m tall (Collenette, 1999). The leaves have a silvery undersurface and the magenta flowers are about 4 cm wide with a faint sweet scent. The plant is found in many localities in the southern part of Saudi Arabia, on cliffs and steep hillsides. It has no known medicinal uses, perhaps because it is difficult to access. This plant has not previously been the subject of phytochemical analysis. Plants from Compositae, especially the genus *Centaurea*, have yielded a wide array of acetylenes (Bohlman *et al.*, 1973) and sesquiterpene lactones (Massiot *et al.*, 1986; Wang *et al.*, 1991), including germacranolides (Barrero *et al.*, 1989), guaianolides (Oksuz *et al.*, 1994; Youssef and Frahm, 1994) and elemanolides (Tortajada *et al.*, 1988) as the main secondary metabolites. Examination of the aerial parts of *C. maximus* has led to the isolation and characterization of three cytotoxic sesquiterpene lactones, namely the guainolides chlorojanerin (**1**), cynaropicrin (**2**) and janerin (**3**).

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Earlier investigation of the aerial parts of *Vicoa pentanema* Aitch. & Hemsl., another Compositae plant, yielded 12 sesquiterpene lactones (**4–15**), of which four were reported as new compounds (Mossa *et al.*, 1997). In this paper we wish to report the isolation and characterization of compounds **1–3** from *C. maximus*, and the cytotoxic and antimicrobial activities of compounds **1–15**, as well as cell aggregation and adhesion studies of the cytotoxic sesquiterpene lactones.

MATERIALS AND METHODS

General. The NMR spectra were taken on a Varian instrument at 300 MHz (^{1}H) and 75 MHz (^{13}C), using $CDCl_3$ as solvent and tetramethylsilane (TMS) as internal standard. Multiplicity determination (APT and DEPT) and 2D NMR spectra (COSY and HETCOR) were obtained using a standard Varian pulse-program. ESI-MS was run using a Bruker Bioapex instrument. Optical rotations were obtained at ambient temperature in $CHCl_3$, unless otherwise stated, using a Perkin-Elmer 241 MC polarimeter. TLC was performed on silica gel GF_{254}, using petroleum ether (40°–60°)–EtOAc (8:2) as solvent, with visualization using vanillin–H_2SO_4 spray reagent. Centrifugal preparative TLC (CPTLC, using a Chromatotron® instrument, Harrison Research Inc. Model 7924) was run with 4 mm silica gel PF_{254} disc, using a flow rate of 2 mL/min. The isolation and structure elucidation of sesquiterpene lactones (**4–15**) from the aerial parts of *V.*

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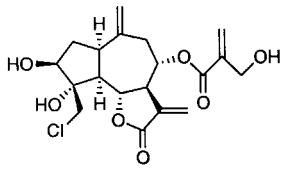
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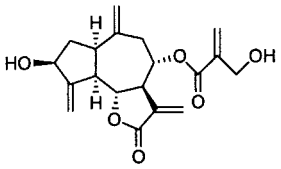
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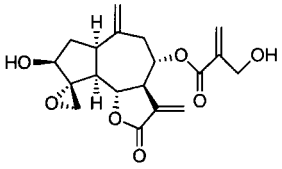
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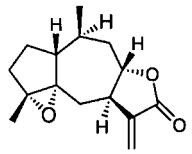
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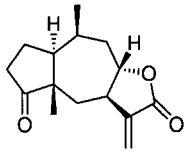
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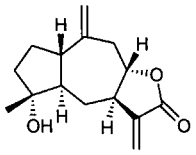
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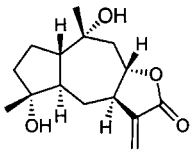
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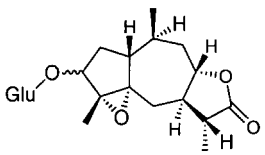
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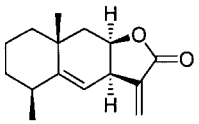
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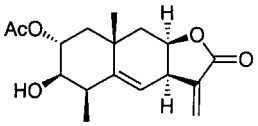
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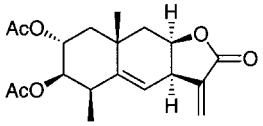
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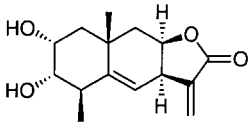
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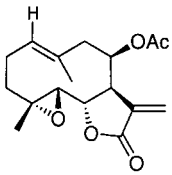
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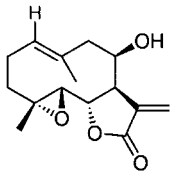
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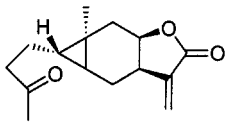
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pentanema was described previously (Mossa *et al.*, 1997).

Plant material. The aerial parts of *C. maximus* were collected in Abha, Saudi Arabia on 16 May 1995. A voucher specimen (no. 13317) was deposited at the herbarium of the MAPPRC, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. The aerial parts of *V. pentanema* were collected in Abha on 13 May 1993 (voucher no. 13063).

Extraction and isolation of sesquiterpene lactones from *C. maximus*. The dried ground leaves of *C. maximus* (800 g) were percolated at room temperature with 95% EtOH and the extract was evaporated *in vacuo* to leave 39 g of gummy residue. The active EtOH extract (15 g) was flash chromatographed on silica gel (450 g), using initially CH_2Cl_2 (1 L) followed by 1%–2% MeCN–CH_2Cl_2 as solvent, to afford chlorojanerin as needles [**1**; 50 mg; mp 155°–156°C (crystallized from CH_2Cl_2/*n*-hexane), $[\alpha]_D$ +69° (*c* 5.0, MeOH); Lit.† mp 151°–153°C, $[\alpha]_D$ +73°], followed by mixtures of compounds **2** and **3** (2 g). The mixture (1 g) was subsequently separated by CPTLC (4 mm silica gel P_{254} disc; solvent: 15% EtOAc in *n*-hexane) which afforded cynaropicrin [**2**, 300 mg, $[\alpha]_D$ +105° (*c* 3.0, MeOH); Lit.† $[\alpha]_D$ +108.6°), followed by janerin (**3**, 350 mg, $[\alpha]_D$ +75° (*c* 5.0, $CHCl_3$); Lit.† $[\alpha]_D$ +69.5°) as transparent gums.

Cytotoxicity assay. The *in vitro* cytotoxic activity was determined against four human cancer cell lines, SK-MEL, KB, BT-549 and SK-OV-3 (Table 2), obtained from the American Type Culture Collection (ATCC, Rockville, MD). A primary assay for initial extracts/fractions used a single concentration (100 μg/mL). In a secondary assay active extracts/pure compounds were tested at three concentrations (10, 3.3 and 1.1 μg/mL),

† Dictionary of Natural Products on CD ROM (1999). Chapman and Hall, CR Cnet Base, Version 8:1, Chapman and Hall #"s MTS50-D, JZL71-K JZL62-I.

using a culture-treated 96-well microplate (Dou *et al.*, 1996). The level of general toxicity of each sample was also determined by measuring their effect on a fibroblast cell line from African green monkey kidney (VERO; non-transformed). The assay is based on the accumulation of neutral red dye in the lysosomes of viable cells (Borenfreund *et al.*, 1990). A subsequent addition of 2-propanol will lyse the cells, releasing the dye into solution and the absorbance is measured at 490 nm and 630 nm. Corresponding growth inhibition was calculated and graphed. For secondary assays, IC_{50} values were determined from logarithmic graphs of growth inhibition values. The cytotoxic agents doxorubicin and 5-fluorouracil were used as positive controls, while the DMSO vehicle was used as a negative control.

Antimicrobial assay. The preliminary antimicrobial activities of the crude extracts/fractions and the IC_{50} / MIC values of compounds **1–5** (Table 3) were determined using a modified microplate assay protocol with a 96-well format, as recommended by the National Committee for Clinical Laboratory Standards (1997). The test organisms used were ATCC strains of *Candida albicans* B311 (90028), *Cryptococcus neoformans* (90113), *Staphylococcus aureus* (6535), methicillin-resistant *S. aureus* (33591). Amphotericin B and rifampicin were used as positive controls, with DMSO as a negative control.

Cell aggregation assay. Cell aggregation was measured as previously described (Katagiri *et al.*, 1999). HL-60, the myelomonocytic cell line, was suspended at a density of 1×10^6 cells/mL. Then 150 μL of the cell suspension was added to each well of a 96-well plate. After incubation with sample for 10 min, phorbol myristate acetate (PMA, Sigma) (10 ng/mL, final) was added. Plates were placed in a CO_2 incubator and aggregation of the cells was observed microscopically 16 h after the PMA addition. Cytochalasin B, anti-LFA-1 and anti-ICAM-1 monoclonal antibodies were used as positive control cell aggregation inhibitors.

XTT assay for cytotoxicity. Following the cell aggregation assay, the XTT (3′-1[(phenylamono)-carbonyl]-3,4-tetrazolium-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate) assay was performed using the methods described by Scudiero *et al.* (1998). Briefly, 25 μL of XTT-phenazine methosulfate (PMS) solution (1 mg/mL XTT solution supplemented by 25 μM of PMS) was added to the cells in each well on the microplates. After incubating for 4 h at 37 °C, absorbance at 450 nm was measured by a microplate reader (reference absorbance at 630 nm).

Cell adhesion assay. (Musza *et al.*, 1994) HL-60 cells which express LFA-1, were stained with a CFSE (carboxyfluorescein diacetate succinimyl ester, Molecular Probes) (Bronner-Fraser, 1985). CFSE labelled HL-60 cells and potential inhibitors were added to the wells of 96 well microtitre plates which contained confluent monolayers of HeLa cells, a carcinoma cell line which expresses ICAM-1. Then, 50 ng/mL PMA was added to stimulate the HL-60 cells to convert LFA-1 to its high avidity binding state (Martin and Springer, 1987). The cultures were incubated for 45 min at 37 °C. Nonadherent HL-60 cells were washed away, the remaining cells were solubilized with 1% Triton X-100 (Sigma) and the fluorescence quantitated using a CytoFluor 2350, Fluorescence Measurement System (Millipore) with an excitation wavelength of 496 nm and emission at 519 nm. Anti-ICAM-1 monoclonal antibody and cytochalasin B were used as positive controls.

Table 1. ^{13}C NMR chemical shift values[a] for compounds 2 and 3

Carbon	2[b]	3[c]
1	45.3 s[d]	45.6 s[d]
2	39.0 t	36.4 t
3	73.7 d	76.0 d
4	152.2 s	68.2 s
5	51.4 d	53.0 d
6	78.5 d	76.6 d
7	47.5 d	47.9 d
8	74.3 d	74.2 d
9	37.0 t	37.6 t
10	137.3 s	137.0 s
11	139.3 s	139.2 s
12	169.1 s	169.0 s
13	122.7 t	122.7 t
14	118.2 t	118.6 t
15	113.5 t	48.4 t
16	165.3 s	165.3 s
17	141.7 s	141.4 s
18	62.1 t	62.1 t
19	126.7 t	126.7 t

[a] Spectra recorded at 75 MHz in $CDCl_3$.
[b] Assignments for carbon 8, 10, 13, 14, 15 and 18 of **2** were incorrectly reported by Wang *et al.* (1991).
[c] Data assigned for comparison; assignment for carbon 10 of **3** was incorrectly reported by Massiot *et al* (1986).
[d] Multiplicities of the carbon signals are determined by APT and / DEPT experiments. Assignments were aided 2D NMR COSY and HETCOR experiments.

RESULTS AND DISCUSSION

A preliminary cytotoxicity screening showed strong activity of the *C. maximus* crude extract. A similar cytotoxicity assay was performed on the EtOH extract of *V. pentanema*, which was also found to be active against human cancer cell lines. The active EtOH extract of *C. maximus* was flash chromatographed over silica gel to give the sesquiterpene lactones **1–3** in 0.008%, 0.02% and 0.025% yields with respect to the dried plant material, respectively. These compounds were identified as chlorojanerin (**1**) (Gonzalez *et al.*, 1977; Youssef and Frahm, 1994), cynaropicrin (**2**) (Corbella *et al.*, 1972) and janerin (**3**) (Gonzalez *et al.*, 1977) by comparison of their physical and spectroscopic data with those reported previously. The ^{13}C-NMR data for **1** and **3** were generally in agreement with those reported by Youssef and Frahm, (1994) and Massiot *et al.* (1986), respectively. In addition, a revised ^{13}C-NMR data for cynaropicrin (**2**) (Wang *et al.*, 1991) is assigned in Table 1, using 2D NMR COSY and HETCOR experiments, as well as by comparison with those of **1** and **3**. In addition, a total of 12 sesquiterpene lactones were isolated, including five guaianolides (**4–8**), four eudesmanolides (**9–12**), two

Table 2. Cytotoxic activities of sesquiterpene lactones

Compound	IC_{50} (μg/mL) SK-MEL	KB	BT-549	SK-OV-3	VERO[a]
Chlorojanerin (**1**)	2.3	6.0	5.9	6.3	5.9
Cynaropicrin (**2**)	2.0	5.5	5.7	6.3	6.0
Janerin (**3**)	2.7	6.0	5.8	6.2	6.7
4α,5α-Epoxy-10α,14H-1-*epi*-inuviscolide (**4**)	4.1	–	>10[b]	>10[b]	3.7
8-*Epi*confertin (**5**)	3.6	–	>10[b]	>10[b]	5.0
Inuviscolide (**6**)	5.3	9.0	5.0	7.3	5.4
10α-Hydroxy-14H-inuviscolide (**7**)	>10	–	5.0	8.1	>10
4α,5α-Epoxy-10α,11β,12H, 14H-1-*epi*-inuviscolide-3β-glucoside (**8**)	–	–	>10[b]	>10[b]	>10[b]
2α-Acetoxy-3β-hydroxyalantolactone (**10**)	2.5	–	5.9	6.3	4.9
2α,3β-Diacetoxyalantolactone (**11**)	7.3	–	–	–	>10[b]
2α,3α-Dihydroxyalloalantolactone (**12**)	5.5	–	>10[b]	>10[b]	7.4
8β-Hydroxyparthenolide (**14**)	1.8	>10	5.0	6.0	5.2
Carabrone (**15**)	5.1	–	>10[b]	>10[b]	5.0
Doxorubicin	<1.1	<1.1	<1.1	<1.1	6.9
5-fluorouracil	6.3	>10[b]	–	>10[b]	>10[b]

SK-MEL, human malignant melanoma; KB, human epidermoid carcinoma; BT-549, human ductal carcinoma; SK-OV-3, human ovary carcinoma.
[a] VERO (kidney, African green monkey) used as normal cell line; –, inactive at 20 μg/mL
[b] weak activity.

germacranolides (**13, 14**) and one elemenolide (**15**), from *V. pentanema* (Mossa *et al.*, 1997).

Most of the sesquiterpene lactones tested for *in vitro* cytotoxic activity against human cancer cell lines of SK-MEL, KB, BT-549 and SK-OV-3 were found to be active (Table 2). Among the guaianolides isolated from *C. maximus* and *V. pentanema*, compounds **1–3** and inuviscolide (**6**) demonstrated the most potent activities (IC_{50} values between 2 and 9 μg/mL) against these cell lines. The eudesmanolide **10** and germacranolide **14** showed consistent cytotoxicities against SK-MEL, BT-549 and SK-OV-3 with IC_{50} values between 1.8 and 6.3 μg/mL, while the compounds **4, 5, 11** and **15** showed selective activities against human malignant melanoma (SK-MEL), with IC_{50} values of 4.1, 3.6, 7.3 and 5.1 μg/mL, respectively. When tested against opportunistic infectious pathogens (*Candida albicans* and *C. neoformans*) guaianolides **4** and **5** were found to be weakly active only against *C. albicans*, with IC_{50} values at 15 and 20 μg/mL, respectively (Table 3). Interestingly, the main cytotoxic constituents **1–3** from *C. maximus*, as well as **10** and **14** from *V. pentanema* were found to be devoid of antimicrobial activity at 50 μg/mL, while most of the sesquiterpene lactones, except **7, 8** and **11** were found to be toxic to the VERO cell line (IC_{50} of 3.7–7.4 μg/mL). The guaianolide glucoside **8**, which is devoid of the cytotoxic α-methylene-γ-lactone chromophore (Kupchan *et al.*, 1971), was found to be inactive against these cell lines. Cynaropicrin had previously been reported for its cytotoxic activity against solid and ascites tumours (S-180 sarcoma and Ehrlich carcinoma) (Zong *et al.*, 1994) and was also reported to be one of the neurotoxic agents of yellow star thistle that cause nigro-pallidal encephalomalacia in horses upon oral ingestion (Wang *et al.*, 1991). The cytotoxic activity of alantolactone (**9**) and its ability to induce apoptosis in human T-cell leukaemia cells have recently been reported (Dirsch *et al.*, 2000.), while lipiferolide (**13**) previously demonstrated activities against KB cell lines (Doskotch *et al.*, 1975).

The key cytotoxic sesquiterpene lactones from both plants were subjected to lymphocyte-associated antigen-1 (LFA-1: CD 11a/CD 18)/intercellular adhesion molecule-1 (ICAM-1: CD 54) mediated aggregation and adhesion assays (Table 4), using HL-60 and HeLa cell lines, in order to determine their effects on immune response and inflammation (Carlos and Harlan, 1994; Hynes, 1992; Springer, 1990). For example, leukocyte adherence to the endothelial cell is an essential event in the process of inflammation and immune recognition (Picker and Butcher, 1992; Osborn, 1990), and the extracellular interactions between specific CAMs expressed on the endothelium and leukocytes will mediate leukocyte entry into tissues, T-cell proliferation and antigen presentation. Therefore, an agent that could inhibit leukocyte adhesion and transmigration would represent a novel mechanism of action as an immunosuppressive and antiinflammatory drug. 8β-Hydroxyparthenolide (**14**) potently inhibited cell aggregation with a MIC value of <0.15 μg/mL (vs 0.39 μg/mL for cytochalasin B), but was found to be less active in the

Table 3. Antimicrobial activity of compounds 4–6, 10, 11 and 14

Compound	IC_{50}/MIC (μg/mL)[a] *C. albicans*	*C. neoformans*	*MR S. aureus*
4	15/+[b]	–	–
5	20/+	–	–
6	50/+	–	–
10	–	–	50/+
11	35/+	–	–
15	25/+	20/+	–
AMB	0.015/0.039	0.045/0.156	–
RIF	NT	NT	0.0025/0.3125

[a] IC_{50}/MIC values after 48 h of incubation at 37 °C;
[b] +, MIC not determined; –, inactive at 50 μg/mL; NT, not tested; AMB, amphotericin B; RIF, rifampicin.

Table 4. Cell proliferation, cell aggregation and cell adhesion activity[a] of compounds 1–3, 10 and 14

Compound	Cell agg MIC (μg/mL) (A)	Cell prolif (HL-60) XTT IC_{50} (μg/mL) (B)	Specific index (B)/(A)	Cell adh IC_{50} (μg/mL) (C)	Specific index (B)/(C)
1	0.5	0.8	1.6	–	–
2	1.4	1.1	0.8	–	–
3	1.4	1.1	0.8	–	–
10	1.4	4.0	2.8	7.0	0.6
14	<0.15	1.2	>8.0	5.0	0.2
Cytochn[b]	0.39	>30	76.9	0.25	>120

[a] Assay system for inhibitors of LFA-1/ICAM-1-mediated aggregation combined with XTT assay as a primary assay. Following LFA-1/ICAM-1-mediated adhesion assay was performed with HL-60 cells and HeLa cells as a secondary assay. –, inactive at 12.5 μg/mL.
[b] Cytochalasin B.

secondary cell adhesion assay (IC_{50} 5 μg/mL vs 0.25 μg/mL for cytochalasin B). On the other hand, guaianolides **1–3** from *C. maximus* demonstrated potent cytotoxic activity against human leukaemia cell lines in the primary cell proliferation assay (MIC 0.8–1.1 μg/mL), while 8β-hydroxyparthenolide (**14**) was >25 fold more cytotoxic than cytochalasin B as determined by XTT assay. The inhibitory effects on cell aggregation of compounds **1–3** and **10** were probably caused by the toxic effects of these sesquiterpene lactones, while **14** showed a more selective effect on cell aggregation that appears to be irrespective of its cytotoxicity. Thus, 8β-hydroxyparthenolide (**14**) decreased the expression of ICAM-1 induced by PMA on HL-60 cells. In the cell adhesion assay, between HL-60 cells and HeLa cells, **14** had negligible specificity compared with that of cytochalasin B. Therefore, **14** appeared to inhibit ICAM-1 but had no direct effect on protein-protein interaction between LFA-1 and ICAM-1.

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