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A new γ-pyrone, sterols and triterpenes from *Helichrysum bracteatum, Gazania nivea and Dimorphotheca ecklonis*

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ABSTRACT

A new γ-pyrone, 3-ethyl-6-(7'-hydroxy-7'-methyl-nonyl)-2-methoxy-5-methyl-pyran-4-one (helichrysone) (**3**) was isolated from the aerial parts of *Helichrysum bracteatum*. In addition, twelve known compounds; six triterpenes *viz*. α-amyrin (**1**), oleanolic acid (**6**), lupeol acetate (**8**), ß-amyrin (**9**), ursolic acid (**11**) and lupeol (**13**), three steroidal compounds *viz*. spinasterol (**2**), stigmasterol (**5**) and ß-sitosterol (**10**) and their glycosides **4**, **7** and **12**, respectively, were isolated from *H. bracteatum*, *Gazania nivea* and *Dimorphotheca ecklonis*. This is the first report for the isolation of compounds **1, 5, 6**, and **7** from *H. bracteatum*, compounds **2** and **4** from genus *Helichrysum*, compounds **1, 8** and **10** from *G. nivea*, compounds **5, 7, 9, 11** and **12** from genus *Gazania* and compounds **1, 5, 7, 9, 10, 12** and **13** from genus *Dimorphotheca*. Compound **3** showed only a mild activity against cervix and breast cancer cell lines *in-vitro* as well as a mild anti-HIV activity.

KEYWORDS: γ-pyrone, sterols, triterpenes, *Helichrysum bracteatum, Gazania nivea, Dimorphotheca ecklonis*.

INTRODUCTION

The medicinal potentials of sterols and triterpenes have been considered a long time ago. Spinasterol, ß-sitosterol and α-amyrin were reported to have antitumour effect [1-3]. In addition, both spinasterol and ß-sitosterol were found to increase the cholesterol excretion and decrease the plasma and liver cholesterol levels [4]. Many pharmacological properties for oleanolic acid have been demonstrated, such as anti-inflammatory, antitumour, hepatoprotective, antibacterial and anti-HIV [5]. Moreover, oleanolic and ursolic acids were found to prevent the development of hypertension and have a potent diuretic activity [5]. For these reasons and because no previous work was traced concerning the triterpene and steroidal contents of *Helichrysum bracteaum* (Vent.) Andrews, *Gazania nivea* DC. and *Dimorphotheca ecklonis* DC., Family Asteraceae (Compositae), it was of interest to study in details the petroleum ether extracts of these plants.

MATERIALS AND METHODS

General experimental procedures

Melting points were determined on electrothermal 9100 (UK). ^{1}H- (300 MHz) and ^{13}C- (75 MHz) NMR spectra were recorded on Varian Mercury instrument, while ^{1}H- (400 MHz), ^{13}C- (100 MHz), ^{1}H-^{1}H-COSY, HMQC, HMBC were measured on Jeol JHA-LAA 400 WB-FT instrument, spectrometer (Japan) using TMS as internal standard. Mass spectra were recorded on Jeol GC mat instrument, 70 ev (Japan). IR was measured on a Shimadzu-IR-435 Infrared Spectrophotometer (Microanalytical center, Cairo University, Cairo, Egypt). UV measurement was done on Shimadzu UV-1650 PC Spectrophotometer (Faculty of Pharmacy, University of Cairo, Cairo, Egypt). Thin-layer chromatography (TLC) was performed on silica gel GF_{254} precoated plates (Machery Nagel, Germany) and silica gel G60 (E-Merck) for preparative TLC. Silica gel 60 (Machery Nagel 230-400 mesh ASTM) and silica gel H (E-Merck) for VLC were used for column chromatography.

Plant material

The flowers and the remaining aerial parts (after separation of the flowers) of *H. bracteatum* (Vent.) Andrews (the orange cultivar), *G. nivea* DC. and *D. ecklonis* DC. were supplied from the Experimental and Research Station of Faculty of Pharmacy, Cairo University, Giza, Egypt. Identification of the plant materials was carried out by Prof. Dr. Monier M. Abdel Ghani, Prof. of Plant Taxonomy, Botany Department, Faculty of Science, Cairo University, Egypt. Voucher specimens were deposited at the Museum of the

Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

Extraction and Isolation

The air-dried remaining aerial parts (1.5 kg) and flowers (1 kg.) of each of *H. bracteatum*, *G. nivea* and *D. ecklonis* were separately extracted by cold maceration with ethanol (95 %) (7 X 10 L) till exhaustion, followed by partitioning with petroleum ether (10 x 200 ml) to yield 44, 63, 74, 84, 50 and 26 g of light petroleum ether extracts of the remaining aerial parts and flowers of *H. bracteatum*, *G. nivea* and *D. ecklonis* respectively. The petroleum ether extracts of the remaining aerial parts and flowers of each plant were separately chromatographed over a vacuum liquid chromatography column (VLC) (Si gel H, 70 g, 5 x 20 cm). Gradient elution was carried out with petroleum ether-chloroform mixtures and chloroform-ethyl acetate mixtures and fractions 200 ml each were collected.

The petroleum ether extract of the remaining aerial parts of *H. bracteatum* (30 g) yielded four main fractions (A_{HA}-D_{HA}). Fraction $\mathbf{A}_{HA}$ (30-35 % chloroform/petroleum ether, 1.5 g) was evaporated, washed with acetone and crystallized from *n*-hexane to give white needle crystals of compound **1** (100 mg). Fraction $\mathbf{B}_{HA}$ (40 % chloroform/petroleum ether, 2.96 g) was rechromatographed over a Si gel 60 column (40 x 3.5 cm, 100 g), using *n*-hexane-ethyl acetate (9.5: 0.5 v/v) as an eluent to give white needle crystals of compound **2** (271 mg). Rechromatography of fraction $\mathbf{C}_{HA}$ (45 % chloroform/petroleum ether, 3 g) on series of Si gel columns, using 1% methanol/chloroform yielded compound **3** (121 mg) as a viscous oily substance. Fraction $\mathbf{D}_{HA}$ (75-100% ethyl acetate / chloroform, 3 g) was purified over a Si gel 60 column (40 x 3.5 cm, 100 g) using 4 % methanol/chloroform as an eluent to give a white powder of compound **4** (114.6 mg).

The petroleum ether extract from the flowers of *H. bracteatum* (25 g) yielded four main fractions (A_{HF}-D_{HF}). Fraction $\mathbf{B}_{HF}$ contained major compound **3** which was previously isolated from the aerial parts. The fractions $A_{HF,}$ C_{HF} and D_{HF} were rechromatographed over a Si gel 60 columns eluted with *n*-hexane/ethyl acetate or methanol/Chloroform mixtures to afford compounds **5** (42 mg, white needle crystals), **6** (60 mg, white powder) and **7** (73 mg, white powder).

The petroleum ether extract from the remaining aerial parts of *G. nivea* (20 g) yielded four main fractions (A_{GA}-D_{GA}). Each fraction was rechromatographed over a Si gel 60 column eluted with *n*-hexane-ethyl acetate or chloroform-methanol mixtures. Fraction $\mathbf{A}_{GA}$ (0 – 15% chloroform/petroleum ether, 3 g) yielded two main subfractions, which were further purified by preparative TLC on Si gel G_{60} plates developed with (*n*-hexane-ethyl acetate 9.5:0.5, and *n*-hexane-ethyl acetate, 9:1), respectively, to give white powder of compound **8** (20 mg) and white needle crystals from *n*-hexane of compound **9** (18 mg). Fraction $\mathbf{B}_{GA}$ yielded compounds **1** (88.3 mg) and **10** (339.3 mg), respectively. Fraction $\mathbf{C}_{GA}$ (55–70% chloroform/petroleum ether, 2.2 g) was further purified by preparative TLC on Si gel G_{60} developed with chloroform-methanol (9.8: 0.2) as solvent system to yield white powder of compound **11** (30 mg). Fraction $\mathbf{D}_{GA}$ (80–100% chloroform/petroleum ether, 2 g) yielded white powder of compound **12** (35 mg).

The petroleum ether extract (20 g) from the flowers of *G. nivea* yielded four main fractions A_{GF}-D_{GF}. Fractions $\mathbf{A}_{GF}$ and $\mathbf{C}_{GF}$ contained major compounds **1** and **11,** respectively, which were isolated previously from the aerial parts of the same plant. Fraction $\mathbf{B}_{GF}$ (40 - 55% chloroform- petroleum ether, 1.45 g) was evaporated and washed with acetone and crystallized from *n*-hexane to yield compound **5** (55 mg) as white needle crystals. Fraction $\mathbf{D}_{GF}$ (75 % - 100% ethyl acetate-chloroform, 3.6 g) was rechromatographed over a Si gel 60 column (40 x 3.5 cm, 100 g) eluted with methanol-chloroform (3.5 %) to yield white powder of compound **7** (22 mg).

The petroleum ether extract (20 g) from the remaining aerial parts of *D. ecklonis* yielded three main fractions A_{DA}-C_{DA}. Each fraction was rechromatographed over a Si gel 60 column eluted with *n*-hexane-ethyl acetate or ethyl acetate-chloroform mixtures. Fraction $\mathbf{A}_{DA}$ (25-35 % chloroform-petroleum ether, 2.96 g) was further purified using preparative TLC technique using Si gel G_{60} plates and solvent system (*n*-hexane-ethyl acetate 9 : 1 v/v) to yield white needle crystals (from *n*-hexane) of compound **1** (38 mg). Fraction $\mathbf{B}_{DA}$ (40% - 50 % chloroform-petroleum ether, 2.2 g) gave white needle crystals of compound **5** from *n*-hexane (40 mg). Fraction $\mathbf{C}_{DA}$ (70 % - 100% ethyl acetate-chloroform, 3 g) yielded white powder of compound **7** (163 mg).

The petroleum ether extract (11 g) from the flowers of *D. ecklonis* DC. yielded four main fractions. Each fraction was rechromatographed over a Si gel 60 column eluted with *n*-hexane-ethyl acetate or chloroform-methanol mixtures to give white needle crystals of compounds **1** (103 mg), **9** (10 mg), **10** (37mg), **12** (50 mg) and **13** (12 mg).

3-Ethyl-6-(7'-hydroxy-7'-methyl-nonyl)-2-methoxy-5-methyl-pyran-4-one (3) was obtained as a viscous yellow oily substance (121 mg) UV (MeOH): l max (log e) 253 (1.068) nm; IR (KBr) nmax 3420 (OH), 2962, 2931, 2887 (CH) and 1665 (C=O) cm^{-1}; ^{1}H-NMR: δ (300 MHz, $CDCl_3$) 3.99, 3H, s, **(-OC$\underline{H}_3$),** 1.00, 3H, t, *J*=7.5 Hz **(C$\underline{H}_3$-13`),** 2.35, 2 H, q, *J*=7.5 Hz **(C$\underline{H}_2$-12`),** 1.93, s, **(C$\underline{H}_3$-11`), C$_6$-(7-`hydroxy-7`-methyl-nonyl**); [2.55, 2H, t, *J*=7.5 Hz**, C$\underline{H}_2$-1'**, 1.61, 2H, m, **C$\underline{H}_2$-2'**, 1.26-1.43, 8H, m, **C$\underline{H}_2$-3'- C$\underline{H}_2$-6'**, 1.47, 2H, q, *J*=7.5 Hz**, C$\underline{H}_2$-8'**, 0.86, 3H, t, *J*=7.5 Hz**, C$\underline{H}_3$-9'**, 1.14, 3H, s, **(C$\underline{H}_3$-10`)**]. ^{13}C-nmr: δ (75 MHz, DMSO): γ-pyrone nucleus; 162.03 **(C$_2$),** 105.17 **(C$_3$),** 182.28 **(C$_4$),** 118.38 **(C$_5$),** 158.22 **(C$_6$), C$_6$-(7'-hydroxy-7'-methyl-nonyl**); 30.21 **(C-1')**, 26.79 **(C-2')**, 23.5, 28.8, 29.6, 41.03 **(C-3'-6')**, 72.48 **(C-7'),** 34.06 **(C-8')** and 8.05 **(C-9'),** 26.1 **(C-10')**, 9.71 **(C-11'), C$_3$-ethyl**; [12.69 **(C-13')**, 15.04 **(C-12')**], 55.12 **(-OC$\underline{H}_3$).** EI-MS: (70 ev, rel. int.), m/z 324 $[M]^+$ (57.4), 309 (34.6), 295 (100), 279 (22.7), 252 (11), 237 (7.5), 223 (17.5), 209 (20), 195 (44.4), 182 (34.8), 167 (37.9), 151 (19.5), 149 (74.7), 123 (19.9), 113 (31), 95 (18.8), 81 (18.8), 73 (70.9) and 55 (63.9).

Cytotoxicity in-vitro assay using Sulphorhodamine B (SRB) method

The cytotoxicity assay was performed following the method of Skehan *et al* (1990) [18] at the National Cancer Institute of Egypt on compound **3** against four cell lines; MCF7 (Breast cancer cell line), Hela (Cervix cancer cell line), HCT 116 (Colon carcinoma cell line) and HEPG2 (Liver cancer cell line). The cells were plated in 96-multiwell plate (104 cells/ well) for 24 hrs. before treatment with the compound to allow attachment of cells to the wall of the plate. Different concentrations of compound **3** (0, 1, 2.5, 5 and 10 µg/ml in DMSO) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with compound **3** for 48 hrs. at 37°C, in an atmosphere of 5% CO_2. After 48 hrs. cells were fixed, washed and stained with sulphorhodamine B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer. Colour intensity was measured in an ELISA reader. The relation between surviving fraction and the compound concentration is plotted to get the survival curve of each tumor cell line after treatment with the compound. Cisplatin was used as a reference drug. Data fitting and graphics were performed by means of the Prism 3.1 computer program (Graph Pad software, USA). Data are given in the mean ± S.E.M.

HIV protease assay [19]

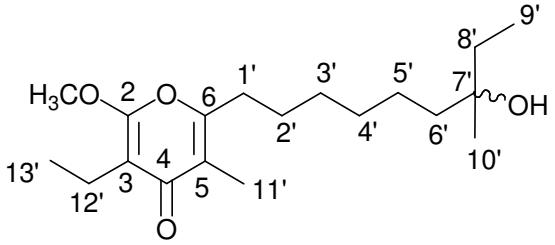
Twenty three µl of HIV-protease assay buffer (50 mM NaOAc, PH 4.9) containing 10µl of the substrate [His-Lys-Ala-Arg-Val-Leu-(*p*NO_2-Phe)-Glu-Ala-Nle-Ser-NH_2] were mixed with 2µl of a compound solution (using DMSO as a solvent), then 8 µl of rec HIV-protease (0.02 mg/ml) were added.

The reaction mixture was incubated for 30 min at 37°C and then terminated by addition of 3 µl of 10% trifluoroacetic (TFA). The hydrolysate (*p*NO_2-Phe-Glu-Ala-Nle-Ser-NH_2) and the remaining substrate were quantitatively analyzed by reversed phase HPLC. HPLC conditions: column, TSK gel ODS-80Ts column (21.5x300 mm, Tosoh Co.); solvent: gradient of acetonitrile (20-40%) in 0.1% TFA in water; flow rate, 1ml/min. UV: 280 nm. The substrate was eluted at 9.5 min while the hydrolysates were eluted at 6.3 and 7 min. The HIV-protease inhibitory activity of a compound was calculated as follows:

% inhibition = ($A_{control}$-A_{sample}) x 100/$A_{control}$

(Where A is relative peak area of the hydrolysate)

RESULTS AND DISCUSSION



Compound 3

Seven compounds were isolated from the petroleum ether extract of *H. bracteaum*. Compounds **1-4** were isolated from the remaining aerial parts (after separation of flowers), while compounds **5-7** were isolated from the flowers. Eight compounds were isolated from the petroleum ether extract of *G. nivea*. Compounds **1** and **8-12** were isolated from the remaining aerial parts meanwhile compounds **5** and **7** were isolated from the flowers. On the other hand, eight compounds were isolated from the petroleum ether extract of *D. ecklonis*. Compounds **1, 5** and **7** were isolated from the remaining aerial parts and compounds **1, 9, 10, 12** and **13** were isolated from the flowers.

The isolated compounds except compound **3** gave positive Liebermann and Salkoviski tests indicating their possible steroidal or triterpenoidal nature. In addition, compounds **4, 7** and **12** gave positive reaction with Molisch's test indicating their glycosidal nature.

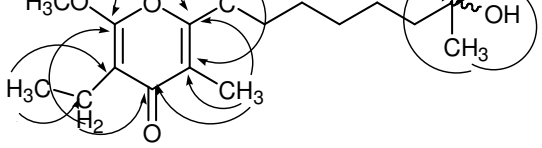
Compounds **1, 6, 8, 9** and **13** developed a reddish purple colour, while compounds **2, 3** and **4** gave a bluish colour and compounds **5, 7, 10, 11** and **12** showed a violet colour when sprayed with *p*-anisaldehyde-sulphuric acid reagent. On the other hand, compound **3** gave an orange colour with Dragendorff's reagent and a negative test for the presence of nitrogen [6].

Six triterpenes *viz*. α-amyrin (**1**) [7-9], oleanolic acid (**6**) [10,11], lupeol acetate (**8**) [7], ß-amyrin (**9**) [7,8], ursolic acid (**11**) [10, 12, 13] and lupeol (**13**) [7, 8] as well as three steroidal compounds namely; spinasterol (**2**) [7], stigmasterol (**5**) [7] and ß-sitosterol (**10**) [7] were identified by their m. p., TLC and GLC comparison with authentic reference samples, mass spectrometry, ^{1}H-NMR and ^{13}C-NMR. Compounds **4, 7** and **12** were identified as spinasterol-3-O-ß-D-glucoside [7], stigmasterol-3-O-ß-D-glucoside [7] and ß-sitosterol-3-O-ß-D-glucoside [7, 14].

This is the first report for the isolation of compounds **1, 5, 6** and **7** from *H. bracteatum*, compounds **2** and **4** from genus *Helichrysum*, compounds **1, 8** and **10** from *G. nivea.*, compounds **5, 7, 9, 11** and **12** from genus *Gazania* and compounds **1, 5, 7, 9, 10, 12** and **13** from genus *Dimorphotheca*.

Compound **3** was obtained as a viscous oily substance. The EI-MS of compound **3** exhibited $[M]^{+}$ at m/z 324 calculated for $C_{19}H_{32}O_4$ (^{13}C-NMR and DEPT spectra). The UV spectrum of compound **3** showed one absorption maximum at λ 253 nm. Its IR spectrum showed absorption bands for a hydroxyl group (3420 cm^{-1}) and a carbonyl (1665 cm^{-1}) group of γ-pyrone ring (c.f. α-pyrone which absorbs at 1700-1740 cm^{-1}). ^{13}C-NMR and DEPT spectra showed the presence of 19 carbon signals; ascribed to six quaternary, eight methylene and 5 methyl groups. Singlet signal at δ_H 3.99 and δ_C 55.12 was due to a methoxy group while the singlet at δ_C 182.28 was due to a carbonyl signal of a γ-pyrone ring. The presence of the γ-pyrone ring was deduced from the presence of the strong band at 1665 cm^{-1} in IR spectrum [15], carbonyl signal at δ 182.28, and from the presence of four quaternary carbon signals at δ 162.03, 158.22, 118.38 and 105.17 ppm, in addition to the UV absorption maximum at λ_{max} 253 nm [15] (c.f. from α-pyrone nucleus at λ_{max} 300 nm). ^{1}H- and ^{13}C-NMR spectra showed the presence of two ethyl groups. This finding was established from signals at δ_H 1.00 (3H, *t*, *J*= 7.5 Hz, H-13') and 2.35 (2H, *q*, *J*= 7.5 Hz, H-12') and at δ_C 12.69 and 15.04, respectively, for the ethyl group attached to C-3 of the γ-pyrone ring. This was confirmed by the presence of the base peak in the mass spectrum at 295 (M- ethyl). A second ethyl group was deduced from the signals at δ_H 0.86 (3H, *t*, *J*= 7.5 Hz, H-9') and 1.47 (2H, *q*, *J*= 7.5 Hz, H-8') and at δ_C 8.05 and 34.06, respectively, for the ethyl group at C-7' of the aliphatic side chain. The two proton singlets at δ_H 1.93 and 1.14 which were directly correlated to carbon signals at δ_C 9.71 and 26.1, were assigned for Me-11` at C-5 of γ-pyrone ring and Me-10`, respectively. In addition, ^{1}H-NMR spectrum showed the presence of a methoxy group at δ_H 3.99, a downfield shifted methylene group appeared as a triplet at δ_H 2.55, a methylene group appeared as a multiplet at 1.61 and four methylene groups appeared as a multiplet at δ_H 1.26-1.43 ppm. A quaternary carbon bearing a hydroxyl group appeared at 72.48 ppm in ^{13}C-NMR spectrum.

The attachment of the methoxy group to C-2 of the γ-pyrone ring was confirmed from strong correlation between proton signals at δ_H 3.99 and δ_C 162.03 (C-2) in HMBC spectrum. The HMBC spectrum confirmed also the attachment of the methyl, ethyl groups and aliphatic chain to C-5, C-3 and C-6, respectively, from the strong correlation between proton signals at δ_H 1.93, 2.35 and 2.55 to carbon signals at δ_C 118.38, 105.17 and 158.22, respectively. The HMBC correlation between proton signal at δ_H 1.47 to carbon signal at δ_C 72.48 confirmed attachment of the second ethyl group to the quaternary oxy-carbon. From ^{1}H- ^{1}H-COSY, HMQC and HMBC the side chain at C-6 was found to be 7-hydroxy-7-methyl-nonyl. Therefore, the structure of compound **3** was deduced as 3-ethyl-6-(7'-hydroxy-7'-methyl-nonyl)-2-methoxy-5-methyl-pyran-4-one. This is the first report of this compound in nature.



The significant long range correlations detected in the HMBC spectrum of compound 3.

Gamma-pyrone compounds were considered as potential anti-cancer drugs. The γ-pyrone derivatives, cyclomorusin, dihydrocycloartomunin and artomunoxanthone exhibited potent inhibition of human PLC/PRF/5 and KB cells *in-vitro* [16]. Therefore, it was found of interest to test compound **3** for its antitumor activity on four human cancer cell lines *in-vitro*. The compound showed weak activity

against cervix and breast cancer cell lines (36 % and 35.6 % death of cervix and breast cancer cells respectively) at a concentration 10 µg/ml. Meanwhile, it showed no activity on colon carcinoma and liver cancer cell lines.

Four novel *bis*-(naphtho-gamma-pyrones) isolated from *Fusarium* species was found to be inhibitors of HIV-1 integrase [17]. Therefore, compound **3** was tested for the anti HIV activity and it showed about 61.7% inhibition at a concentration of 100 µg /ml which is considered a mild activity.

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