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Original Article

Cycloartane Glycoside: A New Lactate Dehydrogenase Inhibitor, from the Aerial part of Agriculture Waste of Watermelon

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ABSTRACT

4*β*,14*α*-dimethyl-5*α*-ergosta-9*β*,19-cyclo-24(31)-en-3*β*-*O*-glucopyranosyl-4*α*-carboxylic acid; a new cycloartane glucoside (**10**) and thirteen other known compounds; chrysophanol (**1**), physicon (**2**), lupeol (**3**), *β*-sitosterol (**4**), *β*-sitosterol-3-*O*-*β*-D-glucoside (**5**), emodin (**6**), aloe-emodin (**7**), 22,23-dihydrospinasterol-3-*O*-*β*-D-glucoside (8), succinic acid (**9**), gallic acid (**11**), 2-*O*-cinnamoyl-1-*O*-galloyl- *β* -D-glucopyranoside (**12**), chrysophanol-8-*O*-*β*-D-glucoside (**13**) and emodin-8-*O*-*β*-D-glucoside (**14**) were isolated from watermelon aerial part. The isolated compounds were identified using different spectroscopic techniques (1D and 2D-NMR, MS, UV and IR). Compounds were tested against human mammary cancer cell line (MCF-7) using *in vitro* Lactate Dehydrogenase (LDH) assay. Several compounds exhibit similar and/or better inhibitory activity than cisplatin. This is the first report for isolation of cycloartane glycoside and anthraquinones from the aerial part of watermelon.

Keywords: *Citrullus lanatus*, agricultural waste, cycloartane glucoside, LDH

INTRODUCTION

The vast development in industry, agriculture and the human civilization resulted in intensive production of a huge amount of agro-industrial wastes. The amount of agricultural wastes in Egypt range from 30-35 million tons a year [1]. 18% of the agricultural wastes is used directly as fertilizer and another 30% is used as animal food. The remainder is burnt directly [2]. The burning not only is considered an economic loss but also has harmful effects on the environment [1]. Watermelon embodies one of the most widely cultivated crops in the world. The production capacity is about 29.7 million tons from 1.8 million ha. Egypt is ranked the fifth country in watermelon production with about 1.6 million tons [3]. Due to this, a huge amount of wastes resulting from the cultivation of this plant. Watermelon fruit was found to contain an array of diverse phytochemical components such as saponin, flavanoides, anthraquinone, terpenoides, phlobatannin, alkaloids and cardiac glycosides [4], citrulline, phenolic acids, iridoids, coumarins, lignan, and other phenolic derivatives [5]. Medicinally, watermelons are mildly diuretic, being effective in the treating dropsy and renal stones, reducing hypertension, acting as an antioxidant, and treating enlarged liver, jaundice and giardiasis [5]. The leaf of Watermelon reported to contain bioactive compounds such as flavonoid, phenolic compound, tannin, triterpenes, sterols, alkaloids and vitamins. Anti-diabetic, anti-inflammatory, analgesic, gonorrhoea, mosquitocidal and anti-microbial property of the leaves [6] were previously reported.

Watermelon wastes like rind [7] and seed [8] can be used more efficiently for production of bioactive compounds. The rind is utilized as a natural source of dietary fiber and antioxidants in cake [7]. The seeds [9] and the wasted watermelons (specifically the estimated 20% of the annual watermelon crop which isn't suitable for sale because of blemishes or unusual shapes) are utilized for production of bio-ethanol [10]. In the last decade, our research group was successful in developing new potential therapeutic agents targeted a wide array of diseases [11, 12]. This study aims to utilize the aerial parts of watermelon as an agriculture waste to produce valuable chemicals and/or pharmaceuticals.

MATERIALS AND METHODS

General: Melting points were determined on Stuart® melting point apparatus model SMP10 and

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are uncorrected. Ultraviolet spectral data was performed on a UV/Visible spectrophotometer (Shimadzu 1601 PC, model TCC-240 A; Shimadzu, Kyoto, Japan).^{1}H and ^{13}C-NMR spectra were obtained in $CDCl_3$, DMSO-d_6 solutions with TMS as internal standard at 400,500 MHz for ^{1}H-NMR and 100,125 MHz for ^{13}C-NMR on JEOL Eclipse NMR or BRUKER Ascend^{TM} 200 spectrometer. Chemical shifts (δ) are reported in ppm relative to the solvent signal and coupling constants are given in Hz. Column chromatography was carried out on silica gel G 60-230 mesh (Merck, Germany). Analytical thin layer chromatography (TLC) is performed on precoated silica gel 60 GF_{254} (20 x 20 cm, 0.2 mm thick) on aluminium sheets (Merck, Germany). Whereas UV, vanillin/sulfuric acid and alcoholic potassium hydroxide spray reagent were used as revealing agents. Organic solvents were distilled prior use.

Collection of Agro-Waste Material: The aerial part of watermelon was collected in July, 2012 from Zayan in Egypt and identified as *citrullus lanatus* (Cucurbitaceae) by Prof. Dr. Taha El-gazar, Depratment of Fruits and Vegetables, Faculty of Agriculture, Mansoura University, Egypt. A voucher specimen (No. 1001) was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University, Mansoura 35516 Egypt.

Extraction and Isolation of compounds: The air dried powdered *C. lanatus* aerial part (3kg) were extracted by cold maceration in a glass jar with distilled methanol (5 X 6L) at room temperature. The solvent of combined methanol extract was evaporated under reduced pressure at 45°C then allowed to dry in a desiccator over anhydrous $CaCl_2$ to a constant weight (350 g). The dried methanolic extract was suspended in 1/2 L distilled water and partitioned successively with petroleum ether (10 × 1/2 L), methylene chloride (6 × 1/2 L) and ethyl acetate (8 × 1/2 L). The solvent, in each case, was distilled under reduced pressure at 45 °C to afford the petroleum ether fraction (110 g, 31.4%), methylene chloride fraction (25 g, 7.1%) and ethyl acetate fraction (60 g, 17.1%).

The petroleum ether fraction (110 g) was chromatographed on a normal phase silica gel column (5 x 98 cm, 650g) and eluted using mixture increased polarity from (0%-70%) ethyl acetate-petroleum ether. Fractions of 250 mL were collected and monitored by TLC using ethyl acetate/petroleum ether (5%, 10%, 20%, 30% and 100%) as developing systems and vanillin/H_2SO_4 acid as a spray reagent followed by heating at 110 °C for 1 min. Similar fractions were pooled to afford five major fractions (A1 - A5). Fraction A1 (5.3 g), A2 (4.8 g) and A3 (2.6 g) were eluted with 10% ethyl acetate/petroleum ether and purified by repeated crystallization from the same solvent system to afford compounds **1** (219 mg), **2** (85 mg) and **3** (40 mg), respectively. Fraction A4 (3.3 g) was eluted by 15% ethyl acetate/petroleum ether and purified by repeated crystallization from the same solvent system to afford compound **4** (560 mg). Fraction A5 (2.2 g) was eluted with 70% ethyl acetate/petroleum ether and purified by repeated crystallization from the same solvent system to afford compound **5** (197 mg).

The methylene chloride fraction (25 g) was chromatographed on a normal phase silica gel (52 × 4.5 cm, 350 g) and eluted using successive gradient elution with petroleum ether - ethyl acetate followed by elution with methanol/ethyl acetate (0%-10%). Fractions of 250 mL were collected and monitored by TLC using ethyl acetate/petroleum ether (20%, 30% and 60%) and methylene chloride/methanol (10%) as developing systems and vanillin/H_2SO_4 acid as a spray reagent followed by heating at 110 °C for 1 min. Similar fractions were pooled to afford five fractions (B1-B5). Fraction B1 (130 mg) and B2 (95 mg) were eluted with 20% ethyl acetate/petroleum ether and purified by repeated crystallization from the same solvent system to afford compounds **6** (22 mg) and **7** (43 mg), respectively. Fraction B3 (32 mg) was eluted with 25% ethyl acetate/petroleum ether and purified by repeated crystallization from the same solvent system to afford compound **8** (20 mg). Fraction B4 (250 mg) was eluted with 60% ethyl acetate/petroleum ether and purified by repeated crystallization from the same solvent system to afford compound **9** (132 mg). Fraction B5 (123 mg) was eluted with 10% methanol/ ethyl acetate and purified by repeated crystallization from the same solvent system to afford compound **10** (86 mg).

The ethyl acetate fraction (30 g) was chromatographed on a normal phase silica gel (63 × 4.5 cm, 400g) and eluted using successive gradient elution with petroleum ether- ethyl acetate and then the elution was continued using methanol/ethyl acetate (0%-50%). Fractions of 250 mL were collected and monitored by TLC using ethyl acetate/petroleum ether (20%–90%) as developing systems and vanillin/H_2SO_4 acid as a spray reagent followed by heating at 110 °C for 1 min. Similar fractions were pooled to afford three fractions (C1-C3), eluted with 60%, 90% ethyl acetate/petroleum ether and 10% methanol/ethyl acetate, respectively. Fraction C1 (1.2g) was rechromatographed over a silica gel column (70 × 2.0 cm, 150g) and eluted with ethyl acetate in petroleum ether (50%–55% v/v) to afford

compound **11** (36 mg). Fraction C2 (3g) was rechromatographed over a silica gel column (20 × 1.0 cm, 22g) and eluted with ethyl acetate in petroleum ether (80%–85% v/v) to afford compound **12** (45 mg). Fraction C3 (13 g)was rechromatographed over a silica gel column (73 × 3.0 cm) and eluted with methanol in methylene chloride (7%–10% v/v) to afford compound **13** (197 mg) and compound **14** (49 mg).

The thirteen known compounds were identified were identified using different spectroscopic techniques (1D and 2D-NMR, MS and IR). As well as by comparison of their spectral data with those in the literature. These compounds were identified as:

Chrysophanol (**1**), physicon (**2**), lupeol (**3**), *β*-sitosterol (**4**), *β*-sitosterol-3-*O*-*β*-D- glucoside (**5**), emodin (**6**), aloe-emodin (**7**), 22,23-dihydrospinasterol-3-*O*-*β*-D-glucoside (**8**), succinic acid (**9**), gallic acid (**11**), 2-*O*-Cinnamoyl-1-*O*-galloyl- *β* -D-glucopyranoside (**12**), chrysophanol-8-*O*- *β*-D- glucoside (**13**) and emodin-8-*O*- *β*-D-glucoside (**14**).

Compound (**10**): white needles (86 mg). m.p = 256 °C. R_f= 0.21[silica gel plates GF_{254}, methylene chloride/methanol (9:1, pink colour after heating with vanillin-sulfuric acid spray reagent]. IR (KBr disc, υmax cm^{-1}): λ_{max} =3444, 2935, 1639, 1462, 1378, 1285, 1032.^{1}H and APT NMR: see table I

Cell line and culture conditions: The non-aggressive human mammary epithelial breast cancer cell line MCF-7 was purchased from VACSERA (Cairo, Egypt) and cultured in RPMI (Roswell Park Memorial Institute; Buffalo, New York, USA) medium supplemented with 100 µg/mL penicillin-streptomycin, 2.5 µg/mL fungizone, 10% heat-activated foetal calf serum, and 2 mM glutamine. Cells were allowed to grow at 37 ºC in a humidified atmosphere of 5% CO_2 and 95% air to form a monolayer. At 60 - 70% confluence, cells were subcultured; first they were washed with phosphate-buffered saline (PBS), then trypsinized with 3 mL of 0.25% trypsin in 0.03% EDTA, then washed with fresh medium and seeded at 1 x 10^4 cells/well in a 96-well microplate. The reagent kit for the assay of LDH **(**lactate dehydrogenase) was purchased from Biorex Diagnostics (Antrim, UK). Cisplatin was kindly supplied as a gift from the Oncology Center, Mansoura University, Mansoura 35516 Egypt.

Cell treatment: MCF-7 cells were treated with the isolated compounds at concentrations from 0 - 250 µM. All chemicals were dissolved in RPMI medium and filtered through a membrane filter (0.2 µm) before cell treatment. LDH activities were measured at a period intervals of 1, 24, 48, and 72 h.

Cytotoxicity assay: Cytotoxicity was determined through monitoring the release of LDH into the medium. Fifty µl of the supernatant were drawn off from each cell culture well and assayed for LDH activity by measuring the absorbance at 340 nm [13, 14].

RESULTS AND DISCUSSION

In this work a new cycloartane glycoside, 4*β*, 14*α*-dimethyl-5α-ergosta-9*β*, 19-cyclo-24(31)-en-3*β* -*O*-glucopyranosyl-4*α*-carboxylic acid (**10**), was isolated from *C. lanatus* along with thirteen known compounds (Figure 1).

Compound (**10**) was isolated as a white needles, with m.p.= 256 °C. The molecular formula was established as $C_{37}H_{60}O_8$ by FABMS. The 1H-NMR spectral data revealed the presence of a characteristic pair of doublets (each assigned for one proton) at δ 0.36 (*J*= 4.0 Hz) and δ 0.55 (*J*= 4.0 Hz), corresponding to the methylene protons at position 19 of the cyclopropane ring of a cycloartane triterpene [15]. It also featured three secondary methyl proton peaks at δ 0.87 (d, *J*= 6.5 Hz, H-21), 0.99 (d, *J*=7 Hz, H-27), 1.04 (d, *J*=7 Hz, H-26); three tertiary methyl proton peaks at δ 0.93 (H-30), 0.98 (H-18), 1.25 (H-29); one anomeric proton peak at δ 4.16 (d, *J* =7.4 Hz, H-1`); two *exo*-olefinic proton peaks at δ 4.65 (1H, br.s, H-24), 4.70 (1H, br.s, H-31); one oxygen substituted methine proton peak at δ 3.98 (br.s, H-3) [16, 17]. APT spectral data revealed the presence of 37 carbon signals categorized to 16 (CH_3 and CH) and 21 (CH_2 and qC). the downfield carbon signal δ 178.1 (C-28) indicated the presence of free carboxylic group [17], the two carbon signals at δ 106.9 (C-13), 156.2 (C-24) confirmed the presence of methylene *exo*-cyclic group, the six oxygenated glucose carbon peaks at δ 103.9 (C-1`), 74.1 (C-2`), 77.1 (C-3`), 70.5 (C-4`), 77.3 (C-5`) and 61.6 (C-6`) are similar to those of *β*-D-glucose [18]. The high coupling constant value 7.4 Hz of the anomeric proton H-1`confirmed the *β* configuration of the glucose moiety. The glycosidation at position 3 of the aglycon was deduced from biosynthetic aspect and was confirmed from H^1-NMR signal at δ 3.98 (br.s, H-3), APT downfield shifted signals atδ 83.3 (C-3) [19] and C-O stretching absorption band at 1032 cm^{-1} in its IR spectrum. The structure elucidation of **10** based on NMR data was further confirmed by mass spectral analysis. From the previous discussion, compound **10** was assigned the structure (4*β*,14α-dimethyl-5*α*-ergost-9*β*,19-cyclo-24(31)-en-3*β*-*O*-glucopyranosyl carboxylic acid.

The known compounds were identified by comparing their spectral data with those in the literature as follows: chrysophanol (**1**) [20], physicon (**2**) [20], lupeol (**3**) [21], *β*-sitosterol (**4**) [22], *β*-sitosterol-3-*O*-*β*-D- glucoside (**5**) [23], emodin (**6**) [20], aloe-emodin (**7**) [24], 22,23-dihydrospinasterol-3-*O*-*β*-D- glucoside (8) [25], succinic acid (**9**) [26], gallic acid (**11**) [27], 2-*O*-cinnamoyl-1-*O*-galloyl- *β*-D-glucopyranoside (**12**) [28], chrysophanol-8-*O*- *β*-D- glucoside (**13**) [29] and emodin-8-*O*-*β*-D-glucoside (**14**) [30].

Extracellular LDH is a reliable marker for cytotoxicity because damaged cells are fragmented completely during the course of prolonged incubation and thereby release LDH. In addition, the LDH assay is used to assess plasma membrane integrity. Generally, in cancer cells the release of LDH is absent due to the propagation of the cells [31]. Cells of the MCF-7 line were incubated in medium containing the isolated compounds at different concentrations, and their growth was compared with that of untreated cells and of cells treated with a well known cytotoxic compound (cisplatin) commonly used in the chemotherapy of breast cancer (Table II). Compounds **3** and **14** exhibited higher cytotoxic activity than Cisplatin (IC_{50} 56.4 and 55.7 µM respectively) against the breast cancer cell line MCF-7. Compounds **2**, **6**, **10** and **13** were moderate in cytotoxicity as determined by LDH activity (69.4, 64.1, 61.3 and 65.7 µM respectively). Finally, other compounds showed the lowest cytotoxicity at all periods.

Table I: ^{1}H-NMR (400 MHz, DMSO-d_6) and APT (100 MHz, DMSO-d_6) spectral data of Compound **10**.

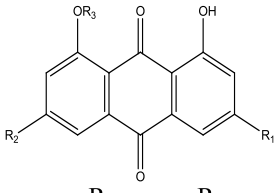
	Atom No.	APT Chemical shift (δ)	^{1}H-NMR Chemical shift (δ)
	1	26.3	
	2	31.2	
	3	83.3	4.86 (1H, br.s)
	4	53.3	
	5	44.5	
	6	22.9	
	7	28.1	
	8	47.4	
	9	19.8	
	10	28.8	
	11	25.5	
	12	32.9	
	13	45.3	
	14	48.8	
	15	35.4	
	16	25.2	
	17	52.1	
	18	18.1	0.98 (3H,s)
	19	29.3	0.36 (1H, d, *J*= 4.0 Hz), 0.55 (1H, d, *J*= 4.0 Hz)
	20	35.9	
	21	18.6	0.87(3H, d, J= 6.5 Hz)
	22	34.9	
	23	31.4	
	24	156.2	
	25	33.5	
	26	22.2	1.04 (3H, d, J= 7 Hz)
	27	22.1	0.99 (3H, d, J= 7 Hz)
	28	178.1	
	29	10	1.25 (3H, s)
	30	19.4	0.93 (3H, s)
	31	106.9	4.65 (1H, br.s), 4.70 (1H, br.s)
Glucose moiety	1'	103.9	4.16 (1H, d, J =7.4 Hz)
	2'	74.2	
	3'	77.1	
	4'	70.5	
	5'	77.3	
	6'	61.6	

Table II: IC_{50} (µM) of Compounds 1- 14 on MCF-7 by LDH Assay, after 72 hrs.

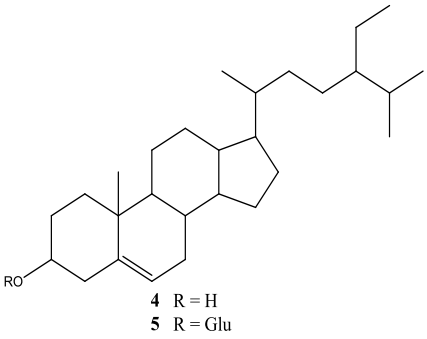
Compound	IC_{50} (µM)[a]	Positive control	IC_{50} (µM)[a]
[b]**1**	93.3±0.0 µg /mL	Cisplatin	60 ± 0.03
2	69.4±0.14		
3	56.4± 0.05		
4	120±0.08		
5	89±0.017		
6	64.1 ± 0.07		
7	ND		
8	ND		
[b]**9**	95.8 ± 0.12		
10	µg/mL		
	61.3± 0.11		
11	95.7 ± 0.11		
[b]**12**	93.3 ± 0.01		
	µg /mL		
13	65.7 ± 0.11		
14	55.7 ± 0.02		

[a]Data shown are the mean ± SD of three experiments. The means were significantly different across the samples

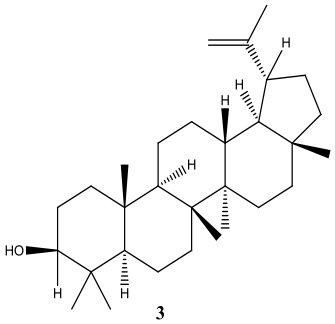
[b]Compounds were expressed as µ/mL as the M.Wts were not determined



	R_1	R_2	R_3
1	CH_3	H	H
2	CH_3	OCH_3	H
6	CH_3	OH	H
7	CH_2OH	H	H
13	CH_3	H	Glu
14	CH_3	OH	Glu



4 R = H
5 R = Glu



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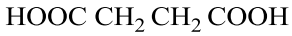

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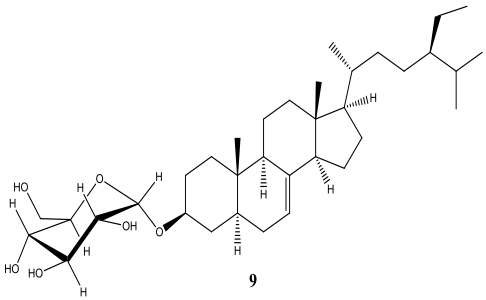

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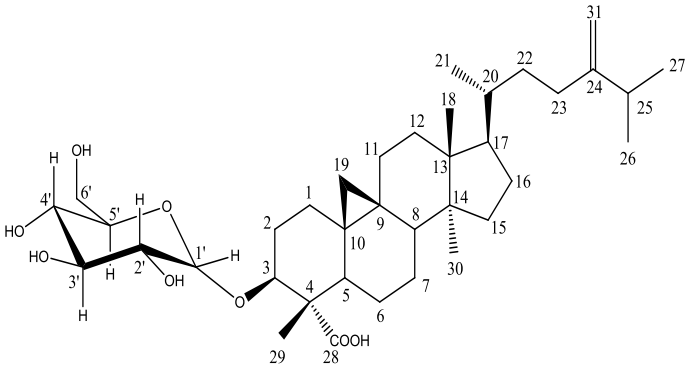

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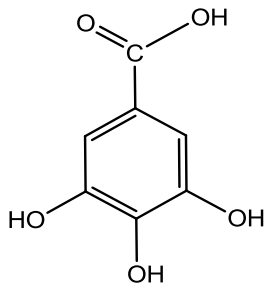

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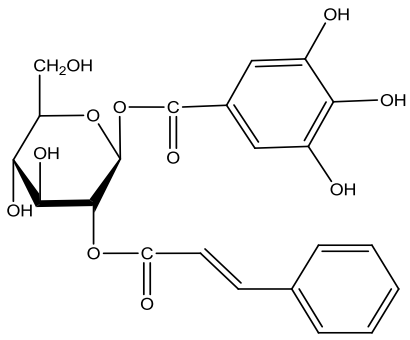

12

Fig. 1. Chemical structures of the isolated compounds **1**– **14**.

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