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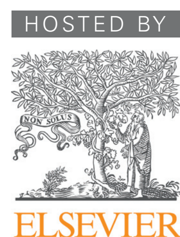

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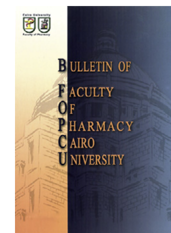

ORIGINAL ARTICLE

Chemical constituents and biological investigations of the aerial parts of Egyptian *Clerodendrum inerme*

Sabrin R.M. Ibrahim [a,*], **Khalid Z. Alshali** [b], **Mostafa A. Fouad** [c], **Ehab S. Elkhayat** [d], **Rwaida A. Al Haidari** [e], **Gamal A. Mohamed** [d]

[a] *Department of Pharmacognosy, Faculty of Pharmacy, Assiut University, Assiut 71526, Egypt*
[b] *Department of Medicine, Faculty of Medicine, King Abdulaziz University, Jeddah 21589, Saudi Arabia*
[c] *Department of Pharmacognosy, Faculty of Pharmacy, Minia University, Minia 61519, Egypt*
[d] *Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, Assiut Branch, Assiut 71524, Egypt*
[e] *Department of Pharmacognosy and Pharmaceutical Chemistry, Faculty of Pharmacy, Taibah University, Al Madinah Al Munawwarah 30001, Saudi Arabia*

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KEYWORDS

Clerodendrum inerme;
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Abstract B-friedoolean-5-ene-3-β-ol (**1**), β-sitosterol (**2**), stigmasta-5,22,25-trien-3-β-ol (**3**), betulinic acid (**4**), and 5-hydroxy-6,7,4′-trimethoxyflavone (**5**) were isolated from the aerial parts of *Clerodendrum inerme* L. (Verbenaceae). Their structures were established based on analyses of physical and spectroscopic data. Compounds **1**, **4**, and **5** were isolated for the first time from the plant. *C. inerme* L. was known as a rich source of terpenes, sterols, and phenolic compounds, so the antioxidant and anti-inflammatory activities were evaluated. The total methanolic extract (TME) and compound **5** showed scavenging activity with maximum inhibition of 61.84% for TME (100 μg/mL) and 37.19% for **5** (20 μM), respectively, using DPPH assay. In addition, the TME exhibited anti-inflammatory activity more than indomethacin at dose 200 mg/kg using the formalin induced hind paw edema method.
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1. Introduction

The genus *Clerodendrum* (Family: Verbenaceae) is very widely distributed in tropical and subtropical regions of the world. More than five hundred species of the genus were identified.[1] They include small trees, shrubs, and herbs.[1] *C. inerme* is a shrub distributed in South and South-east Asia, Australia, and Pacific islands. The plant is used in the treatment of scrofulous and venereal infections, and also as an antidote for poisoning from fish, crabs, and toadstools.[2] The fresh leaf juice is

* Corresponding author. Tel.: +20 88 2141330; fax: +20 882332776.
E-mail address: sabrinshaur@gmail.com (S.R.M. Ibrahim).
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used externally for treating skin diseases. Moreover, the roots are boiled in oil and used in rheumatic affections.[3,4] The plant had been shown to possess antimicrobial, anti-coagulants, uterine stimulant, hypertensive, and laxative effects.[3,4] Previous phytochemical studies of this plant lead to the isolation of sterols,[5,6] flavones,[7] clerodane diterpenes,[8,9] neolignans,[10] iridoid glycosides,[11,12] and triterpenes.[13] As part of our ongoing study on Egyptian plants, we investigated the constituents of *C. inerme* L. (Verbenaceae). The present study reports the isolation and structural elucidation of five compounds: B-friedoolean-5-ene-3-β-ol (**1**),[14] β-sitosterol (**2**),[15] stigmasta-5,22,25-trien-3-β-ol (**3**),[16,17] betulinic acid (**4**),[18,19] and 5-hydroxy-6,7,4′-trimethoxyflavone (**5**).[20] The anti-inflammatory activity of the TME at different doses using the formalin induced hind paw edema method and indomethacin as standard was evaluated. Also, the TME and compound **5** were tested for their antioxidant activity using DPPH assay compared with propyl gallate (standard synthetic antioxidant).

2. Experimental

2.1. General procedures

Melting points were carried out using an Electrothermal 9100 Digital Melting Point apparatus (Electrothermal Engineering Ltd, Essex, England). The UV spectrum was determined using a Perkin Elmer double beam spectrophotometer Model 550S, attached to a Hitachi recorder Model 561, using a 1 cm quartz cell. The IR spectra were measured on a Shimadzu Infrared-400 spectrophotometer (Kyoto, Japan). EIMS were recorded on a Finnigan MAT TSQ 7000 mass spectrometer. 1D and 2D NMR spectra (chemical shifts in ppm, coupling constants in Hz) were recorded on a Bruker BioSpin GmbH 400 MHz Ultrashield spectrometer using $CDCl_3$ as solvent, with TMS as the internal reference. Solvents were distilled prior to spectroscopic measurements. Column chromatographic separations were performed on SiO_2 60 (0.04–0.063 mm, Merck). TLC was performed on pre-coated TLC plates with SiO_2 60 F_{254} (0.2 mm, Merck). The solvent systems used for TLC analyses were *n*-hexane:EtOAc (90:10, S_1), *n*-hexane:EtOAc (85:15, S_2), and $CHCl_3$:MeOH (95:5, S_3). The compounds were detected by UV absorption at λ_{max} 255 and 366 nm followed by spraying with anisaldehyde:H_2SO_4 reagent and heating at 110 °C for 1–2 min.

2.2. Plant material

Clerodendron inerme L. aerial parts were collected in March 2009 from the botanical garden, Faculty of Agriculture, Assiut University and were authenticated by Dr. Abdulaziz. A. Fayed, Prof. of Plant Taxonomy, Faculty of Science, Assiut University, Assiut, Egypt. A voucher specimen was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Minia University, Minia, Egypt (Registration code CIS-2009).

2.3. Extraction and isolation

The air-dried powdered aerial parts of *C. inerme* (400 g) were extracted with 70% MeOH (4 × 1.5 L) at room temperature. The combined methanol extract was concentrated under reduced pressure to afford a dark green residue (27.5 g). The TME (2.5 g) was kept for biological study. The TME (25.0 g) was subjected to vacuum liquid chromatography (VLC) using *n*-hexane:EtOAc and EtOAc:MeOH gradients to afford six fractions: CI-1 (5.4 g; *n*-hexane, 100%), CI-2 (3.4 g; *n*-hexane:$CHCl_3$, 50:50), CI-3 (3.1 g; $CHCl_3$, 100%), CI-4 (2.9 g; $CHCl_3$:EtOAc, 50:50), CI-5 (2.1 g; EtOAc, 100%), and CI-6 (6.5 g; MeOH, 100%). CI-2 (3.4 g) was subjected to SiO_2 column chromatography (160 g × 50 cm × 2 cm) using *n*-hexane:EtOAc gradient to afford compounds **1** (8.7 mg, white crystals) and **2** (11.2 mg, colorless needles). Fraction CI-3 (3.1 g) was chromatographed over SiO_2 column (140 g × 50 cm × 2 cm) using *n*-hexane:EtOAc gradient to obtain compounds **3** (5.4 mg, colorless needles) and **4** (5.6 mg, colorless needles). SiO_2 column chromatography (120 g × 50 cm × 2 cm) of fraction CI-4 (2.9 g) using *n*-hexane:EtOAc as an eluent gave **5** (14 mg, yellow crystals). Other fractions were kept for further investigations.

D:B-friedoolean-5-ene-3-β-ol (**1**): It was obtained as white crystals (8.7 mg, MeOH); R_f 0.79 (S_1). m.p. 211–212 °C. EIMS: m/z 426 $[M]^+$. IR (KBr): γ_{max} 3440, 2890, 1040 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ_H 1.48 (2H, m, H-1), 1.85 (1H, m, H-2A), 1.72 (1H, m, H-2B), 3.48 (1H, t, J = 2.9 Hz, H-3), 5.64 (1H, d, J = 6.0 Hz, H-6), 2.02 (1H, m, H-7A), 1.68 (1H, m, H-7B), 1.55 (1H, m, H-8), 0.90 (1H, m, H-10), 1.38 (2H, m, H-11), 1.35 (1H, m, H-12A), 1.27 (1H, m, H-12B), 1.52 (1H, m, H-15A), 0.93 (1H, m, H-15B), 1.40 (1H, m, H-16A), 1.23 (1H, m, H-16B), 1.59 (1H, m, H-18), 1.53 (2H, m, H-19), 1.60 (2H, m, H-21), 1.39 (2H, m, H-22), 1.04 (3H, s, H-23), 1.14 (3H, s, H-24), 0.85 (3H, s, H-25), 1.09 (3H, s, H-26), 1.00 (3H, s, H-27), 1.16 (3H, s, H-28), 0.95 (3H, s, H-29), 0.99 (3H, s, H-30). ^{13}C NMR ($CDCl_3$, 100 MHz): δ_C 18.2 (C-1), 27.8 (C-2), 76.5 (C-3), 40.5 (C-4), 141.8 (C-5), 122.5 (C-6), 23.5 (C-7), 47.5 (C-8), 34.6 (C-9), 50.1 (C-10), 34.4 (C-11), 30.5 (C-12), 39.3 (C-13), 37.8 (C-14), 32.0 (C-15), 36.0 (C-16), 30.1 (C-17), 43.2 (C-18), 35.0 (C-19), 28.5 (C-20), 33.2 (C-21), 38.9 (C-22), 28.5 (C-23), 25.6 (C-24), 16.2 (C-25), 19.5 (C-26), 18.4 (C-27), 32.2 (C-28), 34.6 (C-29), 32.2 (C-30).[14]

β-Sitosterol (**2**): It was isolated as colorless needles (11.2 mg, acetone); R_f = 0.73 (S_1). m.p. 139–140 °C. IR (KBr): γ_{max} 3455, 2940, 1617, 1454, 1385, 1062 cm^{-1}. EIMS: m/z 414 $[M]^+$. NMR data (400 and 100 MHz, $CDCl_3$) are in good agreement with the published data.[15]

Stigmasta-5,22,25-trien-3-β-ol (**3**): It was obtained as colorless needles (5.4 mg, acetone); R_f 0.61 (S_1). m.p. 158–160 °C. IR (KBr): γ_{max} 3340, 2940, 1640, 1060 cm^{-1}. EIMS: m/z 410 $[M]^+$. ^1H NMR ($CDCl_3$, 400 MHz): δ_H 1.80 (1H, m, H-1A), 1.10 (1H, m, H-1B), 1.79 (1H, m, H-2A), 1.36 (1H, m, H-2B), 3.55 (1H, m, H-3), 1.70 (1H, m, H-4A), 1.26 (1H, m, H-4B), 5.35 (1H, d, J = 5.0 Hz, H-6), 1.62 (1H, m, H-7A), 1.28 (1H, m, H-7B), 1.55 (1H, m, H-8), 1.65 (1H, m, H-9), 1.72 (1H, m, H-11A), 1.49 (1H, m, H-11B), 2.04 (2H, m, H-12), 1.80 (1H, m, H-14), 1.52 (1H, m, H-15A), 1.40 (1H, m, H-15B), 1.66 (1H, m, H-16A), 1.25 (1H, m, H-16B), 1.24 (1H, m, H-17), 0.55 (3H, s, H-18), 0.80 (3H, s, H-19), 2.01 (1H, m, H-20), 1.02 (3H, d, J = 6.5 Hz, H-21), 5.25 (1H, dd, J = 15.5, 7.8 Hz, H-22), 5.21 (1H, dd, J = 15.5, 7.8 Hz, H-23), 2.42 (1H, m, H-24), 1.65 (3H, s, H-26), 4.69 (1H, s, H-27A), 4.67 (1H, s, H-27B), 0.84 (3H, t, J = 7.5 Hz, H-29). ^{13}C NMR ($CDCl_3$, 100 MHz): δ_C 37.2 (C-1), 38.1 (C-2), 71.1 (C-3), 39.5 (C-4), 140.7 (C-5), 121.6 (C-6), 31.6 (C-7), 40.5

(C-8), 49.5 (C-9), 34.2 (C-10), 21.5 (C-11), 29.6 (C-12), 43.5 (C-13), 55.1 (C-14), 23.0 (C-15), 28.5 (C-16), 55.9 (C-17), 12.0 (C-18), 13.1 (C-19), 40.3 (C-20), 20.9 (C-21), 138.3 (C-22), 129.2 (C-23), 52.1 (C-24), 148.5 (C-25), 20.2 (C-26), 109.5 (C-27), 25.6 (C-28), 12.2 (C-29).[16,17]

Betulinic acid (**4**): It was obtained as colorless needles (5.6 mg, acetone); R_f 0.41 (S_2), m.p. 292–293 °C. EIMS: m/z 456 $[M]^+$. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 1.19 (2H, m, H-1), 1.56 (2H, m, H-2), 3.19 (1H, m, H-3), 0.65 (1H, m, H-5), 1.42 (2H, m, H-6), 1.47 (2H, m, H-7), 1.25 (1H, m, H-9), 1.23 (2H, m, H-11), 1.68 (1H, m, H-12A), 1.30 (1H, m, H-12B), 2.20 (1H, dd, J = 9.5, 3.6, H-13), 2.27 (1H, m, H-15A), 1.54 (1H, m, H-15B), 2.37 (1H, m, H-16A), 1.50 (1H, m, H-16B), 1.61 (1H, m, H-18), 3.01 (1H, dt, J = 8.5, 4.0 Hz, H-19), 1.96 (1H, m, H-21A), 1.42 (1H, m, H-21B), 1.97 (2H, m, H-22), 0.96 (3H, s, H-23), 0.75 (3H, s, H-24), 0.82 (3H, s, H-25), 0.93 (3H, s, H-26), 0.97 (3H, s, H-27), 4.74 (1H, s, H-29A), 4.51 (1H, s, H-29B), 1.69 (3H, s, H-30). ^{13}C NMR ($CDCl_3$, 100 MHz): δ_C 38.7 (C-1), 27.4 (C-2), 78.9 (C-3), 38.8 (C-4), 55.3 (C-5), 18.2 (C-6), 34.3 (C-7), 40.7 (C-8), 50.5 (C-9), 37.2 (C-10), 20.8 (C-11), 25.5 (C-12), 38.3 (C-13), 42.4 (C-14), 30.5 (C-15), 32.1 (C-16), 56.3 (C-17), 46.9 (C-18), 49.2 (C-19), 150.4 (C-20), 29.7 (C-21), 37.0 (C-22), 28.0 (C-23), 15.3 (C-24), 16.0 (C-25), 16.1 (C-26), 14.7 (C-27), 180.1 (C-28), 110.0 (C-29), 19.3 (C-30).[18,19]

5-Hydroxy-6,7,4′-trimethoxyflavone (**5**): It was obtained as yellow crystals (14 mg, MeOH); R_f 0.69 (S_3). m.p. 189–190 °C. UV (MeOH): λ_{max} 272, 335. EIMS: m/z 328 $[M]^+$. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 6.58 (1H, s, H-3), 6.54 (1H, s, H-8), 7.83 (1H, d, J = 7.2 Hz, H-2′), 7.01 (1H, d, J = 7.2 Hz, H-3′), 7.01 (1H, d, J = 7.2 Hz, H-5′), 7.83 (1H, d, J = 7.2 Hz, H-6′), 12.77 (1H, s, 5-OH), 3.93 (3H, s, 6-OCH_3), 3.96 (3H, s, 7-OCH_3), 3.89 (3H, s, 4′-OCH_3). ^{13}C NMR ($CDCl_3$, 100 MHz): δ_C 164.0 (C-2), 104.1 (C-3), 182.7 (C-4), 153.0 (C-5), 132.6 (C-6), 158.7 (C-7), 90.5 (C-8), 153.2 (C-9), 106.1 (C-10), 123.5 (C-1′), 128.0 (C-2′), 114.5 (C-3′), 162.6 (C-4′), 114.5 (C-5′), 128.0 (C-6′), 60.8 (6-OCH_3), 56.3 (7-OCH_3), 55.5 (4′-OCH_3).[20]

2.4. Antioxidant activity

The antioxidant activity was evaluated using 2,2′-diphenylpicrylhydrazyl (DPPH) assay as previously outlined.[21] 1 mL of the TME (25, 50, and 100 µg/mL) and compound **5** (20 µM) were mixed with 1 mL of DPPH (4 mg was dissolved in 50 mL HPLC MeOH to obtain a concentration of 80 µg/mL) and allowed to stand for half an hour for any reaction to occur. The UV absorbance was recorded at 517 nm compared to DPPH in MeOH (blank). The experiment was

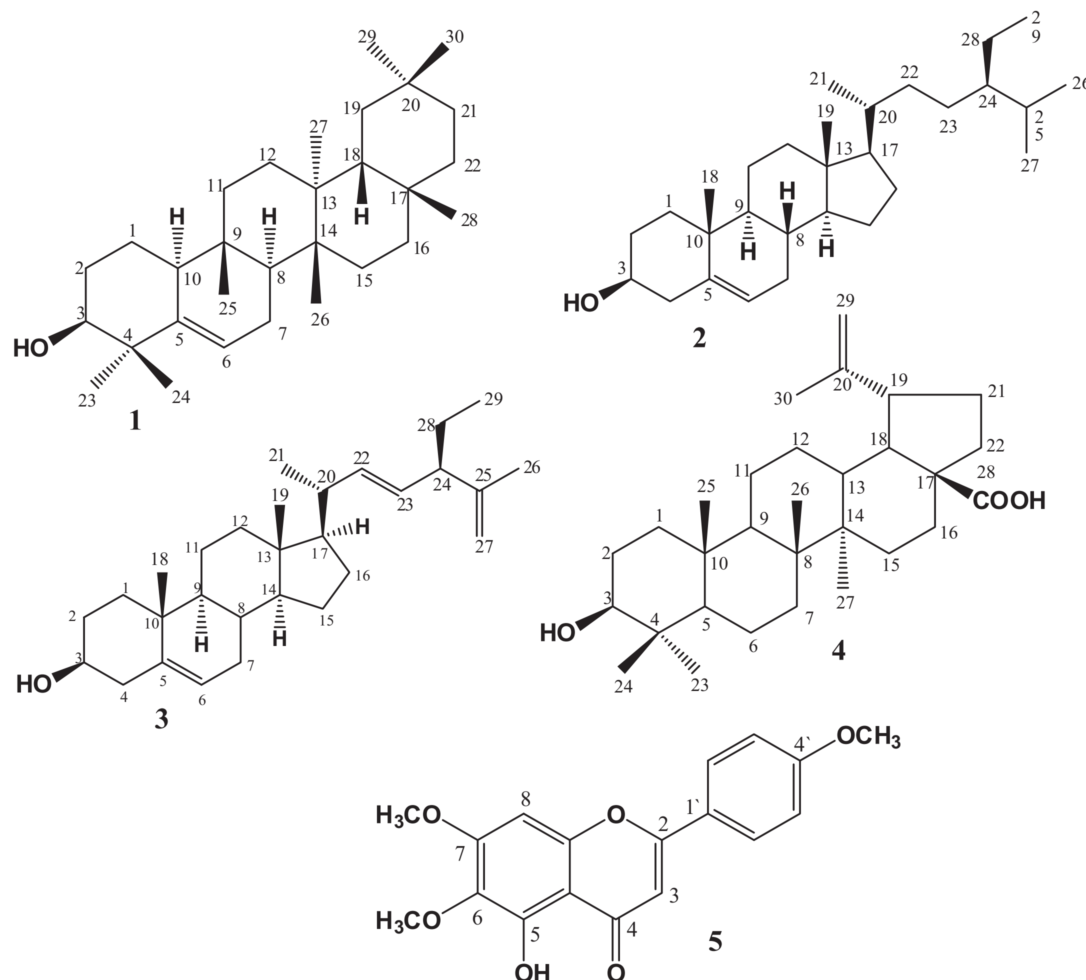

Figure 1 Structures of the isolated compounds **1**–**5**.

Table 1 The DPPH radical scavenging activity results.

Sample	Conc.	DPPH (% inhibition)
TME[*]	25	29.71
	50	47.24
	100	61.84
5[**]	20	37.19

Values are mean of 3 experimental.
[*] Conc. (μg/mL).
[**] Conc. (μM).

performed in triplicate and the average absorption was recorded for each concentration. The same procedure was followed for the standard propyl gallate (a known synthetic antioxidant) set as 100% antioxidant activity. The % free radical scavenging activity was calculated using the following formula:

$$\text{Antioxidant activity} = 100 \times \left(1 - \frac{\text{absorbance with the sample}}{\text{absorbance of the blank}}\right)$$

2.5. Pharmacological study

2.5.1. Animals

Adult male albino rats (100–120 g) were used. The animals were housed under standardized environmental conditions in the pre-clinical Animal House, Pharmacology Department, Faculty of Medicine, Assuit University, Assiut, Egypt. The animals were fed with standard diet and free access to water. They were kept at 24–28 °C temperature, 60–70% relative humidity, and light/dark cycle (12 h:12 h) for one week to acclimatize to environmental conditions. The work was conducted in accordance with the internationally accepted principles for laboratory animals' use and care as found in the European Community Guidelines, and approval of the Institutional Ethics Committee was obtained.[22]

2.5.2. Anti-inflammatory activity

Hind paw edema was induced by injection of 4% formalin (20 μL) solution into the subplanter region of the left hind paw of adult male albino rats (100–120 g).[23] The inflamed animals were divided randomly into five groups (6 for each): inflamed control group, inflamed treated with indomethacin (at a dose of 8 mg/kg subcutaneously), three groups of inflamed animals were treated with the TME individually at doses of 50, 100, and 200 mg/kg subcutaneously (the plant extract was dissolved in sterile distilled water). The change in paw thickness in all tested animals was measured with Plethysmometer 7150 (UGO, Basil, Italy) at 0, 1, 2, 4, and 6 h after formalin solution injection. The anti-inflammatory effect of the tested extract was calculated in comparison to inflamed control group as shown in Table 2. The percentage of edema (inflammation) was calculated according to the following equation:[24]

$$\text{Inhibition}(\%) = \frac{V_c - V_t}{V_c} \times 100$$

V_c = Volume of paw edema in control animals.
V_t = Volume of paw edema in treated animals.

Table 2 The anti-inflammatory activity results.

Groups $n = 6$	Dose mg/kg	0 h		1 h		2 h		4 h		6 h	
		PET[a]	% Inhibition	PET[a]	% Inhibition	PET[a]	% Inhibition	PET[a]	% Inhibition	PET[a]	% Inhibition
Formalin	–	2.96 ± 0.13	0.00	4.79 ± 0.12	0.00	5.11 ± 0.10	0.00	5.70 ± 0.19	0.00	4.85 ± 0.13	0.00
Formalin + Indometh[b]	8	2.93 ± 0.15	1.01	4.12 ± 0.16[*]	14.00	3.33 ± 0.11[*]	34.83	3.01 ± 0.05[*]	47.19	3.57 ± 0.10[*]	26.93
Formalin + TME	50	2.95 ± 0.11	0.33	4.71 ± 0.19[*]	1.67	4.42 ± 0.17	13.50	3.39 ± 0.11[*]	40.52	3.72 ± 0.21[*]	23.29
	100	2.93 ± 0.12	1.01	4.58 ± 0.21	4.38	4.13 ± 0.14[*]	19.17	3.10 ± 0.11[*]	45.61	3.58 ± 0.14[*]	26.19
	200	2.91 ± 0.13	1.68	4.18 ± 0.12[*]	12.73	3.36 ± 0.14[*]	34.25	2.83 ± 0.04[*]	50.35	2.99 ± 0.08[*]	38.35

Each value represents the mean ± S.E.M., $n = 6$.
[*] Significantly different from formalin only group at $P < 0.01$.
[a] PET: Paw edema volume (mm).
[b] Indometh: Indomethacin.

2.6. Statistical analysis

All data were expressed as mean ± standard error using Student's *t* test and the statistical significance was evaluated by a one-way analysis of variance (ANOVA). The values were considered to be significantly different when *P* values were less than 0.01.

3. Discussion and conclusion

Two sterols (**2** and **3**), two triterpene derivatives (**1** and **4**), and a methoxylated flavonoid (**5**) were isolated from the MeOH extract of *C. inerme* L. aerial parts cultivated in Egypt (Fig. 1). Compounds **1**, **4**, and **5** have been isolated for the first time from the plant under investigation. Their structures were established by physical and spectral data (UV, 1D, 2D NMR, and MS), as well as comparison with authentic samples and the literature.

The TME and **5** showed a concentration dependent scavenging activity by quenching DPPH radical (Table 1), maximum inhibition (61.84% for TME at 100 μg/mL and 37.19 for **5** at 20 μM) of DPPH. This antioxidant capacity of the TME may be due to its phenolic contents. Furthermore, the TME of the plant was tested for its anti-inflammatory effect using formalin induced hind paw edema test. It is well known that inhibition of formalin induced paw edema in rats is one of the most suitable test procedures to screen anti-arthritic and anti-inflammatory agents as it closely resembles human arthritis. Injection of formalin subcutaneously into hind paw of rats produced localized inflammation and pain. The nociceptive effect of formalin is biphasic. The earlier phase of formalin induced pain reflects the direct effect of formalin on nociceptors, whereas the late phase reflects inflammatory pain which appears to be attributable to prostaglandin synthesis.[25,26] Inflammatory disorders are also accompanied by the production of significant amounts of free radicals and nitrogen reactive species.[27] The TME extract of *C. inerme* showed anti-inflammatory activity more than indomethacin at dose 200 mg/kg after 4 h (Table 2). That may be due to the presence of flavonoids, terpenes, and sterols and also due to inhibition of main inflammatory mediators like histamine, serotonin, prostaglandins, bradykinin, angiotensin, tachykinin, platelet activating factor, and substance-P.[28] Our results show that *C. inerme* exerts significant inhibitory effects on nociceptive response of the late phase of the chemical and inflammatory pain model in formalin test. The presence of flavonoids in *C. inerme* may modify the action or production of free radicals and nitrogen reactive species.[27] Also, the flavonoids are known to inhibit the prostaglandin synthase enzyme, specially the endoperoxide and reported to produce anti-inflammatory activity (Table 2).[29,30]

4. Conflict of interest

None.

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