European Journal of Medicinal Chemistry 44 (2009) 3188–3194

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Bio-active compounds from *Euphorbia cornigera* Boiss

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ARTICLE INFO

Article history:
Received 25 May 2008
Received in revised form
24 January 2009
Accepted 16 March 2009
Available online 26 March 2009

Keywords:
Euphorbia cornigera Boiss
Euphorbiaceae
Ingenol esters
2D ^{1}H ^{13}C NMR spectra of ingenol esters
Molluscicidal activity

ABSTRACT

Euphorbia cornigera Boiss. (*Euphorbiaceae*) roots extracted in various organic solvents were tested against *Biomphalaria glabrata* snails as molluscicide using Bayluscide as a control. Among these, acetone extract was found to be the most active ($LC_{50} = 17.5\ \mu g\ L^{-1}$) as compared to Bayluscide. The application of HPLC fractionation yielded ten (**1**–**10**) *N*-(2-aminobenzoyl)anthraniloy esters. Structure and the relative configuration of all the compounds were established through spectroscopic (UV, IR ^{1}H, ^{13}C NMR, 2-D NMR, HSQC, HMQC, HMBC, COSY-45°, TOCSY, HOHAHA, HOESY, ROESY, NOESY, SECSY, NOE and mass measurements) techniques. On these basis the esters are named as: 3-*O*-[*N*-(2-aminobenzoyl)]-5-*O*-acetyl-20-*O*-angelylingenol (**1**), 3-*O*-[*N*-(2-aminobenzoyl)]anthraniloyl-5-*O*-angelyl-20-*O*-acetylingenol (**2**), 3-*O*-acetyl-5-*O*-[*N*-(2-aminobenzoyl)]anthraniloyl-20-*O*-angelylingenol (**3**), 3-*O*-acetyl-5-*O*-angelyl-20-*O*-[*N*-(2-aminobenzoyl)]anthraniloylingenol (**4**), 3-*O*-angelyl-5-*O*-acetyl-20-*O*-[*N*-(2-aminobenzoyl)]-anthraniloylingenol (**5**), 3-*O*-angelyl-5-*O*-[*N*-(2-aminobenzoyl)]anthraniloyl-20-*O*-acetylingenol (**6**), 3,20-*O*-diacetyl-5-*O*-[*N*-(2-aminobenzoyl)]anthraniloylingenol (**7**), 5,20-*O*-diacetyl-3-*O*-[*N*-(2-aminobenzoyl)]anthraniloylingenol (**8**), 3-*O*-[*N*-(2-aminobenzoyl)]anthraniloyl-20-*O*-acetylingenol (**9**) and 20-*O*-[*N*-(2-aminobenzoyl)]anthraniloyl-3-*O*-acetylingenol (**10**). The literature reveals that compounds **1**–**8** are new from plant kingdom, whereas **9** and **10** are known but not reported from this source earlier. Their molluscicidal activity (in terms of LC_{50}) showed that all the compounds were 1.3–2.2 times more toxic than Bayluscide except **5** and **6**.

1. Introduction

The Schistosomiasis is spreading very fast in tropic, sub-tropic, African and South American countries by *Schistosoma mansoni* [1–4] and considered to be one of the most dangerous diseases in terms of morbidity burden [5]. Niclosamide an ethanolamine salt of 2,5-dichloro-4-nitrosalicylanide trade name bayluscide is being widely used to control Schistosomiasis in these days. However, this drug is not only expensive and beyond the approach of the poor endemic countries but also has a negative impact over the environment [6–10]. The problem is becoming even more severe due to resistance developed by the organisms against the drug. This is the reason that 80% of the world population relies on traditional medicine to meet their daily health care requirements which is increasing with the passage of time [11–14]. Hence, it is the need of the day to find biologically active natural products which might be friendly to environment and ecological systems being bio-degradable, and specific to application. Unfortunately in this field very limited work has been carried out and need a lot to be done [12,15–18].

Pakistan is considered to be among those countries which are very rich in medicinal plants. The *Euphorbiaceae* family also grows in Pakistan in abundance whose constituents are biologically very active but not yet well explored [12,15–24]. The literature also reveals that plants of the *Euphorbiaceae* family are natural molluscicides [15–18] and big source of bio-active compounds [19–23]. The prevailing state of affairs prompted us to investigate one of the most common and regionally available species of *Euphorbia cornigera* Boiss. The aim of the present study is to search for bio-active diterpene esters, and to evaluate their toxicity. In this study ten (**1**–**10**) bioactive compounds are being reported from *E. cornigera* Boiss. and their structure has been confirmed.

2. Results

2.1. Chemistry

The spectral data ^{1}H, ^{13}C NMR (Tables 1–3), UV, IR and mass measurements of compounds (**1**–**10**) as displayed in Fig. 1 are very close to each other. 2D NMR data obtained from COSY-45°, HOHAHA, HMQC and HMBC, NOE and NOESY of **1**–**10** displayed in Fig. 2a are consistent with an ingenol skeleton with change of acid

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0223-5234/$ – see front matter © 2009 Published by Elsevier Masson SAS.
doi:10.1016/j.ejmech.2009.03.019

Table 1
^{1}H NMR data for diterpenoids **1**–**4**.[a]

H	1	2	3	4
H-1	6.11 (d, J = 1.3)	6.14 (d, J = 1.4)	6.23 (d, J = 1.3)	6.15 (d, J = 1.5)
H-3	5.82 (s)	5.87 (s)	5.84 (s)	5.86 (s)
H-5	4.92 (brd, J = 7.7)	4.82 (brd, J = 7.7)	4.87 (brd, J = 7.7)	4.95 (brd, J = 7.7)
H-7	6.24 (brd, J = 4.5)	6.29 (brd, J = 4.5)	6.18 (brd, J = 4.5)	6.17 (brd, J = 4.5)
H-8	4.13 (dd, J = 11.4, 4.5)	4.14 (dd, J = 11.4,		
4.5)	4.13 (dd, J = 11.4, 4.5)	4.13 (dd, J = 11.4, 4.5)		
H-11)	2.76 (ddd, J = 7.3, 6.4, 3.2)	2.75 (ddd, J =7.2, 6.3, 3.1)	2.59 (ddd, J = 7.3, 6.4, 3.2)	2.46 (ddd, J = 7.3, 6.4, 3.2)
CH_2-12α	1.76 (ddd, J = 12.4, 6.2, 6.4)	1.74 (ddd, J = 12.2, 6.2, 6.3)	1.73 (ddd, J = 12.2, 6.3, 6.3)	1.74 (ddd, J = 12.4, 6.2, 6.4)
CH_2-12β	2.18 (ddd, J = 12.2, 8.5, 3.1)	2.38 (ddd, J = 12.2, 8.5, 3.1) (ddd, 12, 8.5, 3)	2.48 (ddd, J = 12.2, 8.5, 3.3)	2.48 (ddd, J = 12.2, 8.5, 3.2)
H-13	0.73 (ddd, J = 8.5, 8.5, 6.2)	0.71 (ddd, J = 8.5, 8.5, 6.3)	0.72 (ddd, J = 8.5, 8.5, 6)	0.73 (ddd, J = 8.5, 8.5, 6.3)
H-14	0.95 (dd, J = 11.6, 8.5)	0.97 (dd, J = 11.6, 8.5)	0.94 (dd, J = 11.6, 8.5)	0.93 (dd, J = 11.6, 8.5)
Me-16	1.06 (s)	1.03 (s)	1.09 (s)	1.08 (s)
Me-17	1.04 (s)	1.04 (s)	1.09 (s)	1.07 (s)
Me-18	1.05 (d, J = 7.5)	1.03 (d, J = 7.5)	1.04 (d, J = 7.3)	1.07 (d, J = 7.5)
Me-19	1.83 (d, J = 1.3)	1.84 (d, J = 1.3)	1.83 (d, J = 1.3)	1.84 (d, J = 1.3)
CH_2-20α	4.43 (d, J = 12.5)	4.4 7 (d, J = 12.5)	4.49 (d, J = 12.5)	4.46 (d, J = 12.5)
CH_2-20β	4.75 (d, J = 12.5)	4.73 (d, J = 12.5)	4.71 (d, J = 12.5)	4.74 (d, J = 12.5)

[a] For numbering see Fig. 1.

Table 2
^{1}H NMR δ: for diterpenoids **5**–**8**.[a]

	5	6	7	8
H-1	6.11 (d, J = 1.3)	6.14 (d, J = 1.3)	6.23 (d, J = 1.3)	6.15 (d, J = 1.3)
H-3	5.82 (s)	5.87 (s)	5.84 (s)	5.85 (s)
H-5	4.92 (brd, J = 7.7)	4.82 (brd, J = 7.7)	4.87 (brd, J = 7.7)	4.95 (brd, J = 7.7)
H-7	6.12 (brd, J = 4.5)	6.13(brd, J = 4.5)	6.11 (brd, J = 4.5)	6.13 (brd, J = 4.5)
H-8	4.15 (dd, J = 11.4, 4.5)	4.19 (dd, J = 11.4, 4.5)	4.43 (dd, J = 11.4, 4.5)	4.53 (dd, J = 11.4, 4.5)
H-11	2.76 (ddd, J = 7.3, 6.4, 3.2)	2.75 (ddd, J = 7.3, 6.4, 3.2)	2.59 (ddd, J = 7.3, 6.4, 3.2)	2.46 (ddd, J = 7.3, 6.4, 3.2)
CH_2-12α	1.73 (ddd, J = 12.3, 6.2, 6.3)	1.71 (ddd, J = 12.3, 6.2, 6.3)	1.73 (ddd, J = 12.2, 6, 6.3.2)	1.71 (ddd, J = 12, 6.3, 6.2)
CH_2-12β	2.18 (ddd, J = 12, 8.5, 3.4)	2.38 (ddd, J = 12, 8.5, 3.3) (ddd, 12, 8.5, 3)	2.48 (ddd, J = 12, 8.5, 3.2)	2.48 (ddd, J = 12, 8.5, 3.3)
H-13	0.75 (ddd, J = 8.5, 8.5, 6.2)	0.71 (ddd, J = 8.5, 8.5, 6.3)	0.77 (ddd, J = 8.5, 8.5, 6.3)	0.73 (ddd, J = 8.5, 8.5, 6.3)
H-14	0.95 (dd, J = 11.5, 8.5)	0.97 (dd, J = 11.6, 8.3)	0.94 (dd, J = 11.4, 8.4)	0.93 (dd, J = 11.6, 8.5)
Me-16	1.14 (s)	1.10 (s)	1.09 (s)	1.08 (s)
Me-17	1.14 (s)	1.24 (s)	1.09 (s)	1.07 (s)
Me-18	1.25 (d, J = 7.4)	1.03 (d, J = 7.3)	1.15 (d, J = 7.3)	1.09 (d, J = 7.5)
Me-19	1.87 (d, J = 1.3)	1.85 (d, J = 1.3)	1.8 7 (d, J = 1.3)	1.89 (d, J = 1.3)
CH_2-20α	4.43 (d, J = 12.5)	4.4 7 (d, J = 12.5)	4.49 (d, J = 12.5)	4.46 (d, J = 12.5)
CH_2-20β	4.75 (d, J = 12.5)	4.73 (d, J = 12.5)	4.71 (d, J = 12.5)	4.74 (d, J = 12.5)

[a] For numbering see Fig. 1.

moieties at different positions [25–28]. Since all the compounds have common skeleton therefore compound **1** is discussed as representative for all the compounds, hence the chemistry of compound **1** is discussed in detail. It is the angeloyl derivative of known compound 3-*O*-[*N*-(2-aminobenzoyl) anthraniloyloxy]-20-*O*-acetylingenol (**9**) [17,25,26]. On the basis of molecular ion peak [M^+] in HR-EI-MS at *m*/*z* 712.6321 the molecular formula of **1** is proposed as $C_{41}H_{48}N_2O_9$. The EI-MS fragmentation pattern displaying peaks at *m*/*z* 652 [M^+-60] for the fragment $[C_{39}H_{46}N_2O_8]^+$, *m*/*z* 612 [M^+-100 for $C_{36}H_{40}N_2O_8]^+$ and *m*/*z* 457 [M^+-255 for $C_{37}H_{40}O_3]^+$ suggested that **1** is a tri-ester of acetic, angelic and *N*-(2-aminobenzoyl) anthranilic acid respectively. It was supported by the peaks at *m*/*z* 255, 83 and 43 due to formation of $[C_{14}H_{10}N_2O_3]^+$, $[C_4H_7CO]^+$ and $[CH_3CO]^+$ moieties. Further base peak at *m*/*z* 105 attributed to $[C_6H_5CO]^+$ indicated the presence of *N*-(2-aminobenzoyl) anthranilic acid in the molecule. These acidic moieties in **1** were further verified by ^{1}H, and ^{13}C NMR spectral data (Tables 3 and 4) for aromatic *N*-(2-aminobenzoyl) anthranilic acid, angelic acid and acetyl for acetic acid.

Table 3
^{1}H NMR δ: for *N*-(2-aminobenzoyl)anthraniloyl moiety in diterpenoids **1**–**8**.[a]

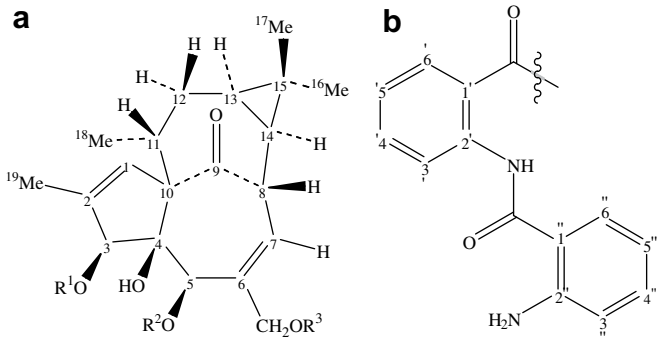
H-3′	8.76 (dd, 8.6, 1.1)	H-3″	6.68 (dd, 8.2, 1.2)
H-4′	7.56 (ddd, 8.6, 7.5 1.1)	H-4″	7.23 (ddd, 8.2, 7.2, 1.1)
H-5′	7.10 (ddd, 8.6, 7.4, 1.1)	H-5″	6.71 (ddd, 8, 7.2, 1.2)
H-6′	8.0 (dd, 8.6, 1.5)	H-6″	7.64 (dd, 8, 1.3)
H-N	11.63 (s, slow exchangeable)	H_2-N	5.74 (brs, slow exchangeable)

[a] For numbering see Fig. 1.

Position of the acyloxy groups was deduced by carrying out HMBC experiment which showed correlations between carbonyl carbon (δ 166.50) of anthraniloyl moiety and CH-O-3 (δ 5.82), and acetyl carbonyl carbon (δ 170.23) and CH-O-5 (δ 4.92). The 3rd acyloxy moiety was placed at C-5. Similarly the NOE spectrum suggested the interaction between CH-3′ (δ 8.76) and CH-O-3 (δ 5.83), and CH-O-5 with methyl proton of acetyl (δ 2.12). Keeping in view these experimental results it was concluded that anthraniloyl moiety is present at C-O-3, acetyl group at CH-O-5 and the third ester of angelic acid at CH_2-O-20. Hence the structure for the compound **1** is proposed as 3-*O*-N-2-aminobenzoylanthraniloyl-5-*O*-acetyl-20-*O*-angeloylingenol.

Similarly compound **2** is the angeloyl derivative of known compound **10** reported from *Euphorbia milli* [17,25,26]. Thus the structure for **2** is 3-*O*-[*N*-(2-aminobenzoyl) anthraniloyloxy-5-*O*-angeloyl-20-*O*-acetylingenol.

HR-EI-MS mass measurement gave molecular ion peak $[M]^+$ at *m*/*z* 712.6321 and displayed super imposable spectral (UV, IR, Mass,



Compounds	R^1	R^2	R^3
1	*N*-(2-aminobenzoyl) anthraniloyl	Acetyl	Angeloyl
2	*N*-(2-aminobenzoyl) anthraniloyl	Angeloyl	Acetyl
3	Acetyl	*N*-(2-aminobenzoyl) anthranilol	Angeloyl
4	Acetyl	Angeloyl	*N*-(2-aminobenzoyl) anthraniloyl
5	Angeloyl	Acetyl	*N*-(2-aminobenzoyl) anthraniloyl
6	Angeloyl	*N*-(2-amino benzoyl) anthraniloyl	Acetyl
7	Acetyl	*N*-(2-aminobenzoyl) anthraniloyl	Acetyl
8	*N*-(2-aminobenzoyl) anthraniloyl	Acetyl	Acetyl
9	*N*-(2-aminobenzoyl) anthraniloyl	H	Acetyl
10	Acetyl	H	*N*-(2-aminobenzoyl) anthraniloyl

Fig. 1. Schematic presentation of (a) compounds **1**–**10** and (b) Anthraniloyl.

^{1}H NMR, ^{13}C NMR) data to compound **1**, hence compounds **3**–**6** are constitutional isomers of **1** and possess $C_{41}H_{48}N_2O_9$ as the molecular composition. However the ester position of each differs as per 2D NMR data.

HMBC and NOE showed cross-peak correlation of carbonyl carbon of *N*-(2-aminobenzoyl)anthraniloyloxy moiety (δ 166.50) with CH-O-5 (δ 4.92), and angeloyl carbonyl (δ 166.70) with CH_2-O-20 (δ 4.42) concluded the structural formula of compound **3** as 3-*O*-acetyl-5-*O*-[*N*-(2-aminobenzoyl)anthraniloyloxy]-20-*O*-angeloylingenol. Similarly the formula for the compounds **4**–**6** are designated as 3-*O*-acetyl-5-*O*-angeloyl-20-*O*-[*N*-(2-aminobenzoyl)anthraniloyloxy] (**4**), 3-*O*-angeloyl-5-*O*-acetyl-20-*O*-[*N*-(2-aminobenzoyl)anthraniloyloxy]ingenol (**5**) and 3-*O*-angeloyl-5-*O*-[*N*-(2-aminobenzoyl)anthraniloyloxy]-20-*O*-acetylingenol (**6**) respectively.

The compounds **7** and **8** are observed as acetyl derivatives of known compounds 3-*O*-[*N*-(2-aminobenzoyl)anthraniloyloxy]-20-*O*-acetylingenol (**9**) and 20-*O*-[*N*-(2-aminobenzoyl) anthraniloyloxy]-3-*O*-acetylingenol (**10**) respectively [17,25,26]. Additional acetate group was identified by HMBC and NOE and placed at CH-O-5 in **7** and at CH_2-O-20 in **8**; thus the suggested structure for **7** and **8** are 5,20-*O*-diacetyl-3-*O*-[*N*-(2-aminobenzoyl)anthraniloyloxy]ingenol and 3,20-*O*-diacetyl-5-*O*-[*N*-(2-aminobenzoyl) anthraniloyloxy]ingenol respectively. The relative stereochemistry at various stereogenic centers was established by NOESY and NOE measurements as displayed in Fig. 2b.

2.2. Molluscicidal activity

Preliminary study was conducted to examine molluscicidal activity of roots of *E. cornigera* on the fresh water snail *Biomphalaria glabrata* (Pulmonata: Planorbidae) intermediate host of *Schistosoma mamsoni* of 8–10 mm in diameter for the purpose to screen out the active fraction in a specific solvent. The extracts were obtained in water and various organic solvents (CCl_4, Et_2O, $CHCl_3$, Me_2CO, EtOAc, EtOH and MeOH), and molluscicidal activity of each extract was tested over the snails. The activity was carried out using a bioassay that employed 10 adult snails submerged for 24 h at room temperature in beaker containing 500 mL of 10.0 $\mu g\,L^{-1}$ solution of each extract. Control experiments were performed containing equivalent concentration of each solvent (CCl_4, Et_2O, $CHCl_3$, Me_2CO, EtOAc, EtOH and MeOH) and run side by side with the test experiments. Mortality was recorded every 24 h, and the snails which did not show movement and foot irritation with a needle up to 72 h were considered as dead, and so removed immediately. All the compounds showing at least 50% mortality during this period were considered potent molluscicidal. Among all the extracts the acetone soluble material showed a significant activity ($LC_{50} = 17.5\ \mu g\,L^{-1}$) (see Table 6). This motivated us in conducting further investigation on purification and isolation leading to **1–10** pure compounds. For comparison purpose Bayluscide was used as a reference drug. LC_{50} of isolated compounds and the reference drug are provided in Table 7. Though all the isolated compounds were more active than the Bayluscide but the

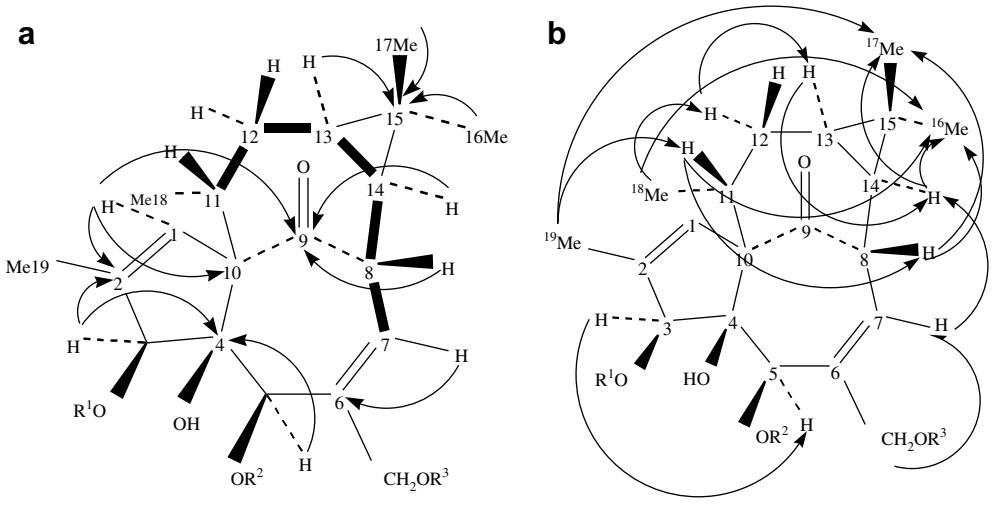


Fig. 2. (a) COSY 45° and HOHAHA bold bonds HMBC interactions by arrows. (b) NOE interactions by arrows via NOESY spectrum.

Table 4
^{13}C NMR δ: for *N*-(2-aminobenzoyl)anthraniloyl moiety in diterpenoids **1**–**8**.[a]

C	1′	2′	3′	4′	5′	6′	7′	1″	2″	3″	4″	5″	6″	7″
Δ	117.6	142.6	142.6	137.6	127.6	132.6	167.6	121.6	127.6	157.6	121.6	121.6	120.6	159.6

[a] For numbering; see Fig. 1.

Table 5
^{13}C NMR Chemical Shifts for diterpenoids **1**–**8**.[a]

C	1	2	3	4	5	6	7	8
1	133.16 (d)	133. 06 (d)	133.1 (d)	133.12(d)	133.1 (d)	133.12 (d)	133.1 (d)	133.16 (d)
2	135.42 (s)	135.45 (s)	135.4 (s)	135.38 (s)	135.5 (s)	135.35 (s)	135.35 (s)	135.45 (s)
3	83.75 (d)	84.03 (d)	84.03 (d)	84.07 (d)	84.06 (d)	82.98 (d)	83.05 (d)	83.23 (d)
4	85.2 (s)	85.22 (s)	85.23 (s)	85.25 (s)	85.22 (s)	85.27 (s)	85.2 (s)	85.22 (s)
5	74.53 (d)	74.52 (d)	74.53 (d)	74.55 (d)	74.51 (d)	74.52 (d)	74.53 (d)	74.54 (d)
6	135.57 (s)	135.6 (s)	135.65 (s)	135.6 (s)	135.65 (s)	135.56 (s)	135.63 (s)	135.46 (s)
7	130.1 (d)	130.1 (d)	130.0 (d)	130.21 (d)	130.11 (d)	130.12 (d)	130.13 (d)	130.1 (d)
8	43.69 (d)	43.68 (d)	43.71 (d)	43.69 (d)	43.67 (d)	43.68 (d)	43.69 (d)	43.68 (d)
9	205.2 (d)	205.49 (d)	205.19 (d)	205.12 (d)	205.1 (d)	205.1 (d)	205.2 (d)	205.4 (d)
10	71.95 (s)	71.92 (s)	71.97 (s)	71.88 (s)	71.87 (s)	71.89 (s)	71.93 (s)	71.87 (s)
11	39.12 (t)	39.1 (t)	39.09 (t)	39.07 (t)	39.06 (t)	39.05 (t)	39.05 (t)	39.1 (t)
12	31.11 (t)	31.12 (t)	31.11 (t)	31.12 (t)	31.13 (t)	31.1 (t)	31.12 (t)	31.11 (t)
13	23.08 (d)	23.09 (d)	23.1 (d)	23.11 (d)	23.12 (d)	23.13 (d)	23.10 (d)	23.09 (d)
14	22.9 (d)	22.93 (d)	22.93 (d)	22.92 (d)	22.91 (d)	22.92 (d)	22.89 (d)	22.92 (d)
15	24.22 (s)	24.23 (s)	24.22 (s)	24.21 (s)	24.23 (s)	24.2 (s)	24.21 (s)	24.22 (s)
16	28.43 (q)	28.39 (q)	28.41 (q)	28.38 (q)	28.37 (q)	28.39 (q)	28.38 (q)	28.37 (q)
17	15.54 (q)	15.51 (q)	15.52 (q)	15.48 (q)	15.49 (q)	15.52 (q)	15.54 (q)	15.53 (q)
18	17.22 (q)	17.21 (q)	17.22 (q)	17.23 (q)	17.23 (q)	17.19 (q)	17.21 (q)	17.23 (q)
19	15.69 (q)	15.73 (q)	15.69 (q)	15.68 (q)	15.74 (q)	15.72 (q)	15.69 (q)	15.73 (q)
20	66.93 (t)	66.89 (t)	66.93 (t)	66.94 (t)	66.95 (t)	66.96 (t)	66.88 (t)	66.94 (t)

[a] For numbering see Fig. 1.

Table 6
Molluscicidal activity of the various extracts of *E. cornigera* roots on *B. glabrata*.

Root extract	CCl_4	Et_2O	$CHCl_3$	Me_2CO	EtOAc	EtOH	MeOH
LC_{50} ($\mu g\,L^{-1}$)	73.4	55.3	52.7	17.5	35.7	27.4	21.3

Table 7
LC_{50} of all the isolated compounds (**1**–**10**) extracted from acetone fraction no. 10 of *E. cornigera*.

Compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	Baylucide
Dose ($\mu g\,L^{-1}$)	12.5	13.5	14.5	15.5	61.5	75.4	18.3	11.4	14.3	19.2	25.0
Dose (nM)	17.54	18.95	20.35	21.75	86.30	112.27	27.21	16.95	22.77	30.57	84.18

one esterified at C-O-3 with *N*-(2-aminobenzoyl) anthranilic acid or acetic acid (**1–4** and **7–10**) showed relatively high activity than the rest of the compounds (**5**–**6**).

3. Discussion

Ten compounds (**1**–**10**) were isolated from the roots of *E. cornigera* Boiss., out of these eight (**1**–**8**) were new diterpene esters and two (**9** and **10**) were known but isolated first time from this plant source. All the reported compounds showed molluscicidal activity of significant level. The highly toxic compounds obtained from the hydrophilic fraction of the roots of *E. cornigera* Boiss. were identified as the esters of ingenol C-O-3.

The esters of ingenol are likely to activate protein kinase C similar to the action of phorbol esters which play an important role in maintaining the integrity of the Schistosome surface [5]. Activation of protein kinase C by ingenol esters may lead to phosphorylation of different proteins and reorganization of the cell cytoskeleton [5] as a consequent. Further the enzyme being competent regulating activity of ion channels which may lead to vesicle formation on the parasite surface, as observed for Schistosomes treated with praziquantel or with pore-forming toxins [5]. Thus it is hypothesized that the ingenol esters in the extract of *E. cornigera* roots probably induced osmosnailic instability, surface vesiculation and subsequent death of snails.

Acetone extract from roots of *E. cornigera* was much toxic against intermediate snails possibly due to hydrophilic components in *E. cornigera* such as diterpene ingenol esters. Diterpene peptidal esters are known to possess molluscicidal properties [17,25–28]. Comparison of molluscicidal activity of the already isolated compounds from the *Euphorbiaceae* family concludes that the compounds show a wide range of activity some of these are highly toxic ($LD_{100} = 3.0\ \mu g\,mL^{-1}$) [18] whereas others are very mild ($LC_{100} = 100\ \mu g\,mL^{-1}$) [17]. Therefore the compounds isolated and reported in this article are considered to be significantly toxic.

4. Conclusion

Probing into the roots of *E. cornigera* Bioss. brought forth eight (**1**–**8**) new bio-active diterpene esters. The structural formulae and relative stereochemistry have been established with the help of modern

spectroscopic techniques. Their molluscicidal activity was also examined and concluded that peptidal esters were responsible for this activity.

5. Experimental

5.1. Chemistry

The final separation of the crude diterpene fractions was performed through HPLC (Perkin Elmer, USA) equipped with RP-18 column and UV detector by employing solvent gradient technique. M.P. of the compounds was determined using Gallenkamp (U.K.) melting point apparatus, optical rotation by digital polarimeter supplied by OSK OGAWA Seiki Co. Ltd., Tokyo, Japan, UV spectra taken in absolute MeOH recorded using IRMECO UV/VIS Model U-2020 spectrophotometer, Geesthacht/Germany, IR spectra recorded over TENSOR 27 FT-IR spectrophotometer supplied by Bruker, Switzerland, and ^{1}H and ^{13}C NMR, 1D, 2D, homo/heteronuclear (300 and 75 MHz with Bruker Biospin-AMX 300-MHz FT NMR) spectra were taken in $CDCl_3$ at room temperature using TMS as an internal standard. Mass measurements were made on a double-focusing Finnigan MAT 112, spectrometer and HR-EI-MS measurements made on JEOL HX 110 spectrometer.

5.1.1. Snails, chemicals and biochemicals

The fresh water snails of *B. glabrata* (Pulmonata: Planorbidae) of size 8–10 mm were collected from the Indus River, near Dera Ismail Khan, Pakistan, and were kept in two-liter aquarium manufactured locally. Molluscicidal drug Bayluscide (niclosamide) was got from Bayer India. The solvents (CCl_4, Et_2O, $CHCl_3$, Me_2CO, EtOAc, EtOH, CH_2Cl_2, MeO-CMe_3, PhMe, MeOH, etc.) used for chromatographic separations were acquired from E. Merck, Germany, and those required for plant extraction were purchased from the local market, and were of commercial grade however were double distilled before use.

5.2. Plant material

The plant *E. cornigera* Boiss. was collected from Murree Hills, Pakistan, and got authenticated botanically from the Department of Botany (the specimen no. EC 1274 retained in the Herbarium), University of Peshawar, (N.W.F.P.) Pakistan; and identified by Prof. Dr. Qazi Najam us Saqib, Dean Faculty of Pharmacy, Gomal University, Dera Ismail Khan, (N.W.F.P.) Pakistan. Roots of the plant were detached for further experimentation.

5.3. Extraction and isolation

Air dried roots (5 kg) and ground (20 mesh size) of *E. cornigera* Boiss. were extracted with methanol (7000 mL) after soaking with occasional shaking for 5 days at room temperature and the solvent removed under reduced pressure at 30 °C to get a crude extract (123.3 g). The extract suspended in methanol/water (1/9, 1000 mL) was extracted thrice with EtOAc (3 × 300 mL) and evaporated under vacuum at 35 °C to yield 65.3 g. After defating the substance with *n*-C_6H_{14} it was dissolved in Me_2CO (150 mL) to yield crude diterpene mixture (57.7 g).

The material (55.5 g) was loaded on silica gel column (192 × 5.4 cm), and $CHCl_3$ was allowed to run with flow rate of 7 mL min^{-1} (1500 mL). The polarity was increased gradually with EtOAc in $CHCl_3$ and then by MeOH in EtOAc (500 mL per composition), and 20 fractions were collected. The collection of the portions was made on the basis of bioassay (molluscicidal activity) and TLC (dichloromethane/methyl-*tert*-butylether/toluene: 7/6/6) guided fractionation. The most active fraction (8.12 g, 10–15% MeOH in EtOAc) was reloaded on silica gel column (72 × 4 cm), gradiently eluted with the same eluant and the fraction (25% MeOH in EtOAc: 3500 mL, 6.6 g) was re-chromatographed on a silica gel column (5 × 50 cm) using same eluant to obtain four fractions (eluant volume: 800 mL per fraction). The most active fraction (4.0 g) showed number of spots by analytical TLC [silica gel PF_{254}, *n*-C_6H_{14}/Et_2O/EtOAc: 4/3/3]. The substance (3.9 g) was loaded on HPLC RP-18 column (250 × 2.5 cm) using aqueous MeCN gradient system (2 mL min^{-1}), and the fraction (30–45% MeCN) containing semi pure compounds (2.7 g). The material was subjected to HPLC (150 × 1.5 cm) RP-18 column using 88% H_2O in MeCN, for 30 min; polarity of the eluant was increased gradually to 96% within 1 h at the flow rate of 1.0 mL min^{-1} (eluant volume: 800 mL/fraction). Eventually ten fractions (110.4, 70.5, 83.6, 92.5, 65.2, 70.5, 83.6, 92.5, 65.3, 130.7 mg) were obtained. Each fraction was further purified on preparative HPLC using MeOH-H_2O gradient system which resulted into pure compounds **1** (4.3 mg), **2** (3.4 mg), **3** (4.4 mg), **4** (4.6 mg), **5** (5.2 mg), **6** (4.4 mg), **7** (5.4 mg), **8** (10 mg), **9** (13.5 mg) and **10** (24.4 mg) with R_t 5.6, 6.3, 7.3, 7.7, 8.2, 8.7, 9.1, 9.7, 15.6 and 17.3 min respectively.

5.4. Characterization of new compounds ***1–8***

5.4.1. *3*-O-*[N-(2-Aminobenzoyl)]anthraniloyl-5*-O-*acetyl-20*-O-*angelylingenol [(=(1aR,2S,5R,5aS,6S,8aS,9R,10aR)-6-[N-(2-aminobenzoyl)anthraniloyloxy)]-1a,2,5,5a,6,9,10,10a-octahydro-5a-monohydroxy-4-(angelyloxymethyl)-1,1,7,9-tetramethyl-11-oxo-1H-2,8a, methanocyclopenta[a]cyclopropa[e]cyclodecen-5-yl] ethanoate **1***

Amorphous powder; M.P. 108–110 °C; $[\alpha]_D^{25}$: +3 (*c*1.4, $CHCl_3$); UV (MeOH) (log ε): 224 (4.5), 264 (3.7), 347 (4.7); IR (dry): 3100–3600, 2940, 2870, 1720, 1660, 1610, 1580, 1530, 1450, 1250, 760; ^{1}H and ^{13}C NMR (Tables 1 and 5); EIMS (*m*/*z*): 312 (5), 294 (3), 238 (100), 120 (80), 43 (45); D/CIMS: *m*/*z* = 671 (9), 313 (18), 257 (100), 239 (40), 120 (61); HREIMS: 712.6321 obsd. 712.6325 Anal. Calcd. for $C_{41}H_{48}N_2O_9$.

5.4.2. *3*-O-*[N-(2-Aminobenzoyl)]anthraniloyl-5*-O-*acetyl-20*-O-*angelylingenol [(=(1aR,2S,5R,5aS,6S,8aS,9R,10aR)-6-[N-(2-aminobenzoyl)anthraniloyloxy)]-1a,2,5,5a,6,9,10,10a-octahydro-5a-monohydroxy-4-(angelyloxymethyl)-1,1,7,9-tetramethyl-5-(acetyloxy)-11-oxo-1H-2,8a,methanocyclopenta[a]cyclopropa[e]cyclodecen-5-yl]ethanoate **2***

Amorphous powder; M.P. 108–109 °C; $[\alpha]_D^{25}$: +5 (*c*1.4, $CHCl_3$); UV (MeOH) (log ε): 224 (4.7), 264 (4.4), 347 (4.7); IR (dry): 3100–3560, 2940, 2873, 1723, 1664, 1610, 1580, 1530, 1450, 1250, 760; ^{1}H and ^{13}C NMR (Tables 1 and 5); EIMS (*m*/*z*): 312 (7), 294 (8), 238 (100), 120 (83), 43 (47); D/CIMS (*m*/*z*): 671 (9), 313 (17), 257 (100), 239 (45), 120 (71); HREIMS: 712.6325 observed 712.6325 Anal. Calcd. for $C_{41}H_{48}N_2O_9$.

5.4.3. *3*-O-*Acetyl-5*-O-*[N-(2-aminobenzoyl)]anthraniloyl-20*-O-*angelyingenol [(=(1aR,2S,5R,5aS,6S,8aS,9R,10aR)-5-[N-(2-aminobenzoyl)anthraniloyloxy)]-1a,2,5,5a,6,9,10,10a-octahydro-5a-monohydroxy-4-(angelyloxymethyl)-1,1,7,9-tetramethyl-11-oxo-1H-2,8a,methanocyclopenta[a]cyclopropa[e]cyclodecen-6-yl]ethanoate **3***

Amorphous powder; M.P. 108–109 °C; $[\alpha]_D^{25}$: +7 (*c* 1.4, $CHCl_3$); UV (MeOH) (log ε): 224 (5.4), 264 (3.4), 347 (4.7); IR (dry): 3100–3460, 2940, 2876, 1727, 1663, 1613, 1583, 1533, 1454, 1257, 760; ^{1}H and ^{13}C NMR (Tables 1 and 5); EI-MS (*m*/*z*) 312 (6), 294 (7), 238 (100), 120 (77), 43 (47); D/CIMS (*m*/*z*) 671 (11), 313 (15), 257 (100), 239 (43), 120 (63); HREIMS (*m*/*z*): 712.6321 observed 712.6325 Anal. Calcd. for $C_{41}H_{48}N_2O_9$.

5.4.4. 3-O-Acetyl-5-O-angelyl-20-[N-(2-aminobenzoyl)]anthranilylingenol [(=(1aR,2S,5R,5aS,6S,8aS,9R,10aR)-5-angelyloxymethyl)-1a,2,5,5a,6,9,10,10a-octahydro-5a-monohydroxy-1,1,7,9-tetramethyl-4-[N-(2-aminobenzoyl)anthraniloyl-oxy]-11-oxo-1H-2,8a,methanocyclopenta[a]cyclopropa[e]cyclodecen-6-yl]ethanoate ***4***

Amorphous powder; M.P. 108–111 °C; $[\alpha]_D^{25}$: +9 (*c*1.4, $CHCl_3$); UV (MeOH) (log ε): 224 (3.7), 264 (4.7), 347 (4.3); IR (dry): 3100–3360, 2940, 2877, 1712, 1660, 1621, 1578, 1523, 1425, 1252, 763; ^1H and ^{13}C NMR (Tables 1 and 5); EIMS (*m*/*z*) 312 (7), 294 (13), 238 (100), 120 (78), 43 (43); D/CIMS (*m*/*z*) 671 (19), 313 (17), 257 (100), 239 (42), 120 (71); HREIMS: 712.6321 observed 712.6325 Anal. Calcd.for $C_{41}H_{48}N_2O_9$.

5.4.5. 3-O-Angelyl-5-O-acetyl-20-O-[N-(2-aminobenzoyl)]anthraniloylingenol [(=(1aR,2S,5R,5aS,6S,8aS,9R,10aR)-6-angelyloxymethyl)-1a,2,5,5a,6,9,10,10a-octahydro-5a-monohydroxy-1,1,7,9-tetramethyl-4-[N-(2-aminobenzoyl) anthraniloyloxy]-11-oxo-1H-2,8a,methanocyclopenta[a]cyclopropa[e]cyclodecen-5-yl]ethanoate ***5***

Amorphous powder; M.P. 107–109 °C; $[\alpha]_D^{25}$: +8 (*c*1.4, $CHCl_3$); UV (MeOH) (log ε): 224 (3.7), 264 (3.4), 347 (4.7); IR (dry): 3100–3560, 2940, 2770, 1722, 1666, 1621, 1568, 1523, 1435, 1245, 760; ^1H and ^{13}C NMR (Tables 2 and 5); EIMS (*m*/*z*) 312 (6), 294 (4), 238 (100), 120 (81), 43 (43); D/CIMS (*m*/*z*): 671 (11), 313 (16), 257 (100), 239 (42), 120 (51); HREIMS: 712.6321 observed 712.6325 Anal. Calcd. for $C_{41}H_{48}N_2O_9$.

5.4.6. 3-O-Angelyl-5-O-[N-(2-aminobenzoyl)]anthraniloyl-20-O-acetylingenol [(=(1aR,2S,5R,5aS,6S,8aS,9R,10aR)-6-[N-(2-aminobenzoyl)anthraniloyloxy)]-1a,2,5,5a,6,9,10,10a-octahydro-5a-monohydroxy-1,1,7,9-tetramethyl-5-(anthraniloyloxy)-11-oxo-1H-2,8a,methanocyclopenta[a]cyclopropa[e]cyclodecen-4-yl]ethanoate ***6***

Amorphous powder; M.P. 106–108 °C; $[\alpha]_D^{25}$: +11 (*c*1.4, $CHCl_3$); UV (MeOH) (log ε): 224 (4.1), 264 (4.2), 347 (4.5); IR (dry): 3100–3360, 2940, 2867, 1722, 1656, 1613, 1578, 1523, 1445, 1253, 760; ^1H and ^{13}C NMR (Tables 2 and 5); EIMS (*m*/*z*) 312 (8), 294 (13), 238 (100), 120 (78), 43 (55); D/CIMS (*m*/*z*) 671 (19), 313 (17), 257 (100), 239 (42), 120 (81); HREIMS: 672.5643 observed 672.5720 Anal. Calcd. for $C_{38}H_{44}N_2O_9$.

5.4.7. 3,20-O-Diacetyl-5-O-[N-(2-aminobenzoyl)]anthraniloylingenol [(=(1aR,2S,5R,5aS,6S,8aS,9R,10aR)-5-[N-(2-aminobenzoyl) anthraniloyloxy)]-1a,2,5,5a,6,9, 10,10a-octahydro-5a-monohydroxy-1,1,7,9-tetramethyl-11-oxo-1H-2,8a,methanocyclopenta [a]cyclopropa[e]cyclodecen-4,6-yl] diethanoate ***7***

Amorphous powder; M.P. 106–108 °C; $[\alpha]_D^{25}$: +6 (*c* 1.4, $CHCl_3$); UV (MeOH) (log ε): 224 (5.1), 264 (4.3), 347 (4.1); IR (dry): 3100–3556, 2943, 2867, 1721, 1663, 1612, 1583, 1533, 1453, 1252, 764; ^1H and ^{13}C NMR (Tables 2 and 5); EIMS (*m*/*z*) 312 (7), 294 (13), 238 (100), 120 (68), 43 (55); D/CIMS (*m*/*z*) 671 (7), 313 (13), 257 (100), 239 (43), 120 (51); HREIMS: 672.5643 observed 672.5720 Anal. Calcd. for $C_{38}H_{44}N_2O_9$.

5.4.8. 5,20-O-Diacetyl-3-O-[N-(2-aminobenzoyl)]anthraniloylingenol [(=(1aR,2S,5R,5aS,6S,8aS,9R,10aR)-6-[N-(2-aminobenzoyl)anthraniloyloxy)]-1a,2,5,5a,6,9,10,10a-octahydro-5a-monohydroxy-1,1,7,9-tetramethyl-11-oxo-1H-2,8a,methano cyclopenta[a]cyclopropa[e]cyclodecen-4,5-yl]diethanoate ***8***

Amorphous powder; M.P. 107–109 °C; $[\alpha]_D^{25}$: +7 (*c* 1.4, $CHCl_3$); UV (MeOH) (log ε): 224 (3.5), 264 (4.1), 347 (4.3); IR (dry): 3130–3517, 2944, 2867, 1722, 1656, 1613, 1583, 1533, 1453, 1252, 767; ^1H and ^{13}C NMR (Tables 2 and 5); EIMS (*m*/*z*) 312 (3), 294 (5), 238 (100), 120 (78), 43 (55); D/CIMS (*m*/*z*) 671 (11), 313 (13), 257 (100), 239 (41), 120 (51); HREIMS: 672.5643 observed 672.5720 Anal. Calcd. for $C_{38}H_{44}N_2O_9$.

5.5. Preparation of samples for bioassay

Appropriate amount of extract from roots of *E. cornigera* Boiss. was used for bioassay evaluation after diluting it with water up to 2 L volume. In the case of pure compounds (**1**–**10**), 100 μg of each compounds was dissolved in 2 mL of DMSO, which was diluted to 2000 mL with distilled water. The various working solutions were prepared through further dilution. The solutions of the reference drugs (Bayluscide) were also obtained in the same way.

5.6. Determination of molluscicidal activity

Molluscicidal activity of the compounds under test was evaluated using snails (*B. glabrata*) of 8–10 mm size following the standard protocol [5]. The bioassay test compounds (**1**–**10**) were dissolved in minimum quantity of DMSO and then adding distilled water to get solution 0.1% in DMSO. Groups of 10 snails were kept at room temperature in aquarium having 500 mL of test/blank solutions for 24 h under normal diurnal light. Movement of the snails was controlled by suspension of a stainless steel mesh kept just above the water surface. Each test concentration was set in triplicate. The snails were also exposed to the potential molluscicide (niclosamide). The suspension was decanted; the snails were washed with fresh water and offered fresh lettuce leaves as food. The snails were then left in water for another 24 h and then examined to assess mortality. The snails were considered dead if they either remained motionless or did not respond to foot irritation and to the presence of food. To verify the snails' susceptibility two control sets were used: one with cupric carbonate at 50 ppm and other containing 0.1% DMSO distilled water. The collected data were analyzed, and the LC_{50} values determined (Table 6 and 7) and their 95% confidence intervals (CIs 95%) were obtained through non-linear regression using Finney software.

5.7. Statistical analysis

For the molluscicidal activity assay of *B. glabrata* snails of size 8–10 mm, the LC_{50} values and their 95% confidence intervals (CIs 95%) were obtained through non-linear regression using Finney software.

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