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Original article

Anti-tumor 12-deoxyphorbol esters from *Euphorbia cornigera*

Imam Bakhsh Baloch [a], Musa Kaleem Baloch [a,*], Qazi Najam us Saqib [b]

[a] *Department of Chemistry, Gomal University, Dera Ismail Khan, Pakistan*
[b] *Department of Pharmacognosy, Gomal University, Dera Ismail Khan, Pakistan*

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Abstract

Nine (**1**–**8** and **10**) new and two (**9** and **11**) known compounds have been isolated from roots of *Euphorbia cornigera* Boiss. Their structure and relative stereochemistry were acquired through NMR (^{1}H, ^{13}C, COSY-45, HOHAHA, HMQC, HMBC, NOE and HMBC) spectroscopic measurements. Compounds **1**–**10** were identified as diesters of 13,20-*O*-diacyl and **11** as 13-*O*-acetyl of 12-deoxyphorbol. Cytotoxic activity of the compounds was investigated on human KB cells by reduction of MTT. Compounds **8**–**10** displayed IC_{50} of 0.8, 0.5, and 1.0 μg mL^{-1}, respectively, whereas the activity of rest of the compounds (**1**–**7**) was either very low or (**11**) zero even up to 1000 μg mL^{-1}. The inhibition of DNA synthesis through Trypan blue exclusion and Brd-U assay was investigation to figure out the role of compounds **8**–**10** and concluded that these were responsible for the death of KB cells. Significant correlation has been found between the cytotoxicity and DNA cross-link and DNA strand-break formation.

Keywords: *Euphorbia cornigera* Boiss; 13,20-*O*-Diacyl-12-deoxyphorbol; Cytotoxic activity; KB human cell lines; Inhibition of DNA synthesis

1. Introduction

Cancer is one of the most dangerous, fast propagating with quite high mortality rate diseases of present century even in the developed part of the world. The situation is even worse in the developing countries due to lack of knowledge, poverty and non-availability of quality drugs. Therefore, most of the peoples rely over folk/indigenous medicines derived from plants, etc. Plants of Euphorbiaceae have been used for remedy against cancer since ancient times. A variety of anti-bacterial, anti-viral, molluscicidal and tumor-promoting to tumor-inhibiting compounds have been isolated from these plants [1–5]. On the one hand, *Euphorbia cornigera* Boiss. is considered to be one of the most toxic species of the Euphorbiaceae family but on the other hand its leaves, roots, shoots and fruit are used for the treatment of various ailments [1–4] and are very common in hilly areas of Pakistan [1]. Esters of ingenol, phorbol and ingol have already been reported from this species [2–4] and now esters of 13,20-*O*-diacyl (**1**–**10**) and as 13-*O*-acetyl (**11**) of 12-deoxyphorbol are presented. The structural elucidation of these (**1**–**11**) compounds has been established through spectroscopic (^{1}H, ^{13}C NMR, 2D NMR, HMQC, COSY-45°, HMBC, HOHAHA, SECSY, NOESY and NOE, UV, IR and mass) measurements. It has also been noted that **1**–**8** and **10** are new while **9** and **11** are known compounds. Biological screenings of the title compounds are investigated with the help of ear-mice activities (IC_{50}, ID_{50}) [15] and cytotoxic assays (MTT, Trypan blue dye exclusion, Brd-U test and DNA inhibition synthesis) on human KB cell lines [16–20].

2. Chemistry

Based on spectroscopic results, compounds **10** and **11** were identified as 13,20-didecanoylphorbol [10,11] and 13-*O*-acetyl-12-deoxyphorbol [3]. The UV, IR, NMR (Tables 1 and 2)

* Corresponding author. Tel.: +92 966 750424; fax: +92 966 750255.
E-mail address: musakaleem2001@yahoo.com (M.K. Baloch).

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Table 1
^{1}H NMR data for diterpenoids **1**–**10**

^{1}H	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
H-1	7.56 (br s)	7.53 (br s)	7.54 (br s)	7.53 (br s)	7.55 (br s)	7.56 (br s)	7.56 (br s)	7.53 (br s)	7.54 (br s)	7.53 (br s)
CH_2-5	2.01 (dd, $J = 18.2$, 10.5 Hz)	1.98 (dd, $J = 18.2$, 10.5 Hz)	2.10 (dd, $J = 18.2$, 10.5 Hz)	2.02 (dd, $J = 18.2$, 10.5 Hz)	2.03 (dd, $J = 18.2$, 10.5 Hz)	2.05 (dd, $J = 18.2$, 10.5 Hz)	2.01 (dd, $J = 18.2$, 10.5 Hz)	1.98 (dd, $J = 18.2$, 10.5 Hz)	2.10 (dd, $J = 18.2$, 10.5 Hz)	2.02 (dd, $J = 18.2$, 10.5 Hz)
CH_2-5	2.8 (br dd, $J = 18.1$, 9.0 Hz)	2.8 (br dd, $J = 18.2$, 9.0 Hz)	2.82 (br dd, $J = 18.2$, 9.2 Hz)	2.86 (br dd, $J = 18.2$, 9.1 Hz)	2.81 (br dd, $J = 18.2$, 9.0 Hz)	2.83 (br dd, $J = 18.2$, 9.0 Hz)	2.8 (br dd, $J = 18.1$, 9.0 Hz)	2.8 (br dd, $J = 18.2$, 9.0 Hz)	2.82 (br dd, $J = 18.2$, 9.2 Hz)	2.86 (br dd, $J = 18.2$, 9.1 Hz)
H-7	5.23 (br d, $J = 5.5$, 2.2 Hz)	5.17 (br dq, $J = 5.5$, 2.1 Hz)	5.18 (br d, $J = 5.5$, 2.3 Hz)	5.20 (br dq, $J = 5.5$, 2.2 Hz)	5.21 (br dq, $J = 5.5$, 2.3 Hz)	5.19 (br dq, $J = 5.5$, 2.3 Hz)	5.23 (br d, $J = 5.5$, 2.2 Hz)	5.17 (br dq, $J = 5.5$, 2.1 Hz)	5.18 (br d, $J = 5.5$, 2.3 Hz)	5.20 (br dq, $J = 5.5$, 2.2 Hz)
H-8	2.37 (br t, $J = 5.5$ Hz)	2.34 (br t, $J = 5.6$ Hz)	2.33 (br t, $J = 5.4$ Hz)	2.34 (br t, $J = 5.3$ Hz)	2.35 (br t, $J = 5.5$ Hz)	2.37 (br t, $J = 5.5$ Hz)	2.37 (br t, $J = 5.5$ Hz)	2.34 (br t, $J = 5.6$ Hz)	2.33 (br t, $J = 5.4$ Hz)	2.34 (br t, $J = 5.3$ Hz)
H-10	3.27 (dddq, $J = 4.5$, 2.5, 2.5, 1.5 Hz)	3.28 (dddq, $J = 4.5$, 2.5, 2.5, 1.5 Hz)	3.28 (dddq, $J = 4.5$, 2.5, 2.5, 1.5 Hz)	3.29 (dddq, $J = 4.5$, 2.5, 2.5, 1.5 Hz)	3.27 (dddq, $J = 4.5$, 2.5, 2.5, 1.5 Hz)	3.26 (dddq, $J = 4.5$, 2.5, 2.5, 1.5 Hz)	3.27 (dddq, $J = 4.5$, 2.5, 2.5, 1.5 Hz)	3.28 (dddq, $J = 4.5$, 2.5, 2.5, 1.5 Hz)	3.28 (dddq, $J = 4.5$, 2.5, 2.5, 1.5 Hz)	3.29 (dddq, $J = 4.5$, 2.5, 2.5, 1.5 Hz)
H-11	1.58 (dq, $J = 9.5$, 6.5 Hz)	1.56 (dq, $J = 9.5$, 6.5 Hz)	1.53 (dq, $J = 9.5$, 6.5 Hz)	1.52 (dq, $J = 9.5$, 6.5 Hz)	1.55 (dq, $J = 9.5$, 6.5 Hz)	1.56 (dq, $J = 9.5$, 6.5 Hz)	1.58 (dq, $J = 9.5$, 6.5 Hz)	1.56 (dq, $J = 9.5$, 6.5 Hz)	1.53 (dq, $J = 9.5$, 6.5 Hz)	1.52 (dq, $J = 9.5$, 6.5 Hz)
CH_2-12	1.94 (br d, $J = 9.2$ Hz)	1.94 (br d, 9.3 Hz)	1.94 (br d, $J = 9.2$ Hz)	1.94 (br d, $J = 9.1$ Hz)	1.94 (br d, $J = 9.1$ Hz)	1.94 (br d, $J = 9.1$ Hz)	1.94 (br d, $J = 9.2$ Hz)	1.94 (br d, 9.3 Hz)	1.94 (br d, $J = 9.2$ Hz)	1.94 (br d, $J = 9.1$ Hz)
H-14	0.95 (d, $J = 5.5$ Hz)	0.97 (d, $J = 5.5$ Hz)	0.96 (d, $J = 5.5$ Hz)	0.98 (d, $J = 5.5$ Hz)	0.97 (d, $J = 5.5$ Hz)	0.96 (d, $J = 5.5$ Hz)	0.95 (d, $J = 5.5$ Hz)	0.97 (d, $J = 5.5$ Hz)	0.96 (d, $J = 5.5$ Hz)	0.98 (d, $J = 5.5$ Hz)
Me-16	1.21 (s)	1.23 (s)	1.20 (s)	1.22 (s)	1.22 (s)	1.20 (s)	1.21 (s)	1.23 (s)	1.20 (s)	1.22 (s)
Me-17	1.21 (s)	1.24 (s)	1.22 (s)	1.24 (s)	1.23 (s)	1.21 (s)	1.21 (s)	1.24 (s)	1.22 (s)	1.24 (s)
Me-18	0.89 (d, $J = 6.5$ Hz)	0.88 (d, $J = 6.5$ Hz)	0.87 (d, $J = 6.5$ Hz)	0.9 (d, $J = 6.5$ Hz)	0.91 (d, $J = 6.5$ Hz)	0.88 (d, $J = 6.5$ Hz)	0.89 (d, $J = 6.5$ Hz)	0.88 (d, $J = 6.5$ Hz)	0.87 (d, $J = 6.5$ Hz)	0.9 (d, $J = 6.5$ Hz)
Me-19	1.72 (br s)	1.71 (br s)	1.72 (br s)	1.74 (br s)	1.72 (br s)	1.71 (br s)	1.72 (br s)	1.71 (br s)	1.72 (br s)	1.74 (br s)
CH_2-20α	4.41 (d, $J = 12.8$ Hz)	4.42 (d, $J = 12.8$ Hz)	4.42 (d, $J = 12.8$ Hz)	4.43 (d, $J = 12.8$ Hz)	4.42 (d, $J = 12.8$ Hz)	4.43 (d, $J = 12.8$ Hz)	4.41 (d, $J = 12.8$ Hz)	4.42 (d, $J = 12.8$ Hz)	4.43 (d, $J = 12.8$ Hz)	4.43 (d, $J = 12.8$ Hz)
CH_2-20β	4.73 (d, $J = 12.8$ Hz)	4.72 (d, $J = 12.8$ Hz)	4.73 (d, $J = 12.8$ Hz)	4.741 (d, $J = 12.8$ Hz)	4.73 (d, $J = 12.8$ Hz)	4.72 (d, $J = 12.8$ Hz)	4.74 (d, $J = 12.8$ Hz)	4.73 (d, $J = 12.8$ Hz)	4.73 (d, $J = 12.8$ Hz)	4.73 (d, $J = 12.8$ Hz)

Table 2
^{13}C NMR data for diterpenoids **1**–**10**

C	1	2	3	4	5	6	7	8	9	10
1	160.26 (d)	160.26 (d)	160.26 (d)	160.23 (d)	160.25 (d)	160.28 (d)	160.28 (d)	160.26 (d)	160.25 (d)	160.28 (d)
2	136.27 (s)	136.26 (t)	136.24 (t)	136.23 (t)	136.24 (t)	136.22 (t)	136.27 (t)	136.26 (t)	136.23 (t)	136.25 (t)
3	210.22 (s)	210.26 (d)	210.28 (d)	210.29 (d)	210.26 (d)	210.27 (d)	210.26 (d)	210.25 (d)	210.27 (d)	210.25 d)
4	44.5 (s)	44.54 (s)	44.49 (s)	44.45 (s)	44.46 (s)	44.49 (s)	44.55 (s)	44.53 (s)	44.56 (s)	44.56 (s)
5	34.0 (t)	34.02 (t)	34.05 (t)	34.08 (t)	34.09 (t)	33.98 (t)	34.02 (t)	34.04 (t)	34.05 (t)	34.05 (t)
6	139.56 (s)	139.61 (s)	139.63 (s)	139.65 (s)	139.67 (s)	139.65 (s)	139.62 (s)	139.61 (s)	139.64 (s)	139.65 (s)
7	125 (d)	125.66 (d)	125.65 (d)	125.62 (d)	125.67 (d)	125.68 (d)	125.66 (d)	125.64 (d)	125.63 (d)	125.56 (d)
8	42.2 (d)	42.21 (d)	42.23 (d)	42.24 (d)	42.2 (d)	42.23 (d)	42.19 (d)	42.18 (d)	42.19 (d)	42.22 (d)
9	77.9 (d)	77.9 (d)	77.9 (d)	77.9 (d)	77.9 (d)	77.9 (d)	77.9 (d)	77.9 (d)	77.9 (d)	77.9 (d)
10	54.3 (s)	54.33 (d)	54.34 (d)	54.32 (d)	54.34 (d)	54.36 (d)	54.31 (d)	54.32 (d)	54.31 (d)	54.33 (d)
11	42.3 (d)	42.33 (d)	42.35 (d)	42.36 (d)	42.31 (d)	42.33 (d)	42.31 (d)	42.33 (d)	42.35 (d)	42.36 (d)
12	56.7 (t)	56.74 (t)	53.76 (t)	56.77 (t)	54.76 (t)	56.75 (t)	56.73 (t)	56.73 (t)	56.74 (t)	52.71 (t)
13	65.0 (s)	65.01 (s)	65.04 (s)	65.06 (s)	65.0 (s)	65.07 (s)	64.98 (s)	65.01 (s)	65.02 (s)	65.04 (s)
14	35.9 (d)	35.79 (d)	35.99 (d)	35.946 (d)	35.9 (d)	35.97 (d)	35.92 (d)	35.94 (d)	35.97 (d)	35.96 (d)
15	25.8 (s)	25.8 (s)	25.8 (s)	25.8 (s)	25.8 (s)	25.8 (s)	25.8 (s)	25.8 (s)	25.8 (s)	25.8 (s)
16	23.9 (q)	23.92 (q)	23.94 (q)	23.97 (q)	23.94 (q)	23.98 (q)	23.92 (q)	23.95 (q)	23.9 (q)	23.93 (q)
17	16.97 (q)	16.98 (q)	16.95 (q)	16.94 (q)	16.92 (q)	16.93 (q)	16.95 (q)	16.92 (q)	16.92 (q)	16.79 (q)
18	15.12 (q)	15.13 (q)	15.17 (q)	15.15 (q)	15.14 (q)	15.18 (q)	15.12 (q)	15.17 (q)	15.18 (q)	15.01 (q)
19	10.22 (q)	10.24 (q)	10.26 (q)	10.28 (q)	10.26 (q)	10.29 (q)	10.23 (q)	10.26 (q)	10.27 (q)	10.28 (q)
20	62.23 (t)	66.4 (t)	67.2 (t)	65.9 (t)	67.4 (t)	68.2 (t)	66.9 (t)	66.7 (t)	66.5 (t)	65.9 (t)

data of compounds **1**–**10** were in complete agreement with 13,20-diester of 12-deoxyphorbol [6–14]. The HREIMS of compound **1** displayed $[M]^+$ at *m/z* 494.4374, corresponding to molecular composition $[C_{29}H_{34}O_7]^+$ and EIMS showed fragments at *m/z* 372 [M−122, $C_6H_5CO_2H]^+$, 434 [M−60, $CH_3COOH]^+$, indicating the presence of benzoic and acetic acids in the molecule. Three-spin system of phorbol skeleton was established with the help of COSY-45°, HOHAHA, HMQC and HMBC spectra (Fig. 1). The ^{1}H, ^{13}C NMR spectra (Tables 1 and 2) gave peaks at δ values 4.42, 4.72 d, $J = 12.8$ Hz and at δ 65.23 triplet in DEPT, suggested *gem*-ester moiety at CH_2–O-20 and peak at δ 65.2 (s in DEPT) was assigned to C-13. High δ value revealed ester functionality at this carbon, concluding the presence of acetyl and benzoyl groups in the molecule. Position of benzoyl and acetyl moieties was deduced through HMBC and NOE interactions at CH_2–O-20, C–O-13, respectively [6]. ^{13}C NMR (APT and DEPT) spectra showed 29 carbon atoms with multiplicity of five CH_3, three CH_2, 12 CH and nine C, out of these five were oxygenated (one ketone, two tertiary alcohols and two ester) groups. Based on spectral data analysis, **1** was proposed as 13-*O*-acetyl-20-*O*-benzoyl-12-deoxyphorbol and concluded to be a novel natural product.

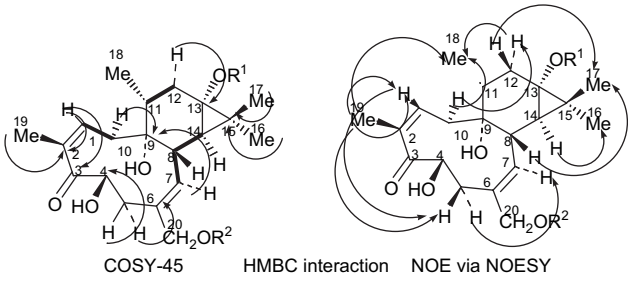
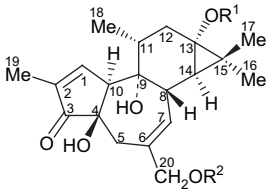


Fig. 1. Results of some 2D NMR experiments.

Spectral data (UV, IR and mass fragmentation) of compound **2** were almost identical to compound **1**, with the difference of *p*-methoxybenzoyl moiety. ^{1}H and ^{13}C NMR displayed peaks in aromatic region (δ 6.9–7.2 4H, 128–131 6C) and DEPT confirmed 2C and 4 CH. Sharp singlets at δ 3.5 and 61.34 (three protons and carbon) were assigned to methoxy group. Position of the –OMe was confirmed at *para* through HMBC and NOE experiments (Fig. 2).

Compounds **3** and **4** were found to be diesters of decanoic acid, angelic acid and tiglic acid. HREIMS of these displayed common $[M]^+$ peak at *m/z* 584.6178 indicating the composition as $C_{35}H_{52}O_7$. EIMS spectrum provided peaks at *m/z* 484 [M−100, $C_4H_7CO_2H]^+$, 412 [M−172, $C_9H_{19}CO_2H]^+$, indicating the presence of angelic/tiglic acid and decanoic acid. Tiglic acid and angelic acid were differentiated through



Formula Collection

Compound	R^1	R^2
1	Acetyl	Benzoyl
2	Acetyl	p-Methoxybenzoyl
3	Decanoyl	Angeloyl
4	Decanoyl	Tigloyl
5	Acetyl	Decanoyl
6	Butanoyl	Decanoyl
7	Hexanoyl	Decanoyl
8	Octanoyl	Decanoyl
9	Decanoy	Decanoyl
10	Dodecanoyl	Decanoyl
11	Acetyl	H

Fig. 2.

NMR δ 6.13 and 6.17, respectively. The decanoyloxy moiety was confirmed in both **3** and **4** at C-13. The angelyloxy in **3** and tiglyloxy in **4** were identified at C–O-20.

HREIMS spectra of **5–10** exhibited [M^+] peaks at *m/z* 712.8273, 740.7263, 768.7253, 796.6253, 824.6253, 852.4253, 880.7243 and 908.8043 indicating a difference of 28 amu from the preceding one. EIMS demonstrated peak at *m/z* 172 for each (**5–10**) compound indicating an ester of decanoic acid, while the others were higher homologues of acetic acid having two methylene groups extra in each case. On the basis of HMBC results the decanoyloxy moiety was deduced at C-20 and rest of the acyloxy species at C-13. The literature reveals that compounds **1–8** and **10** are novel metabolites while compounds **9** and **11** were identified as 13,20-didecanoylphorbol and 13-*O*-acetyl-12-deoxyphorbol, respectively, which have already been reported [3,10,11].

3. Results

3.1. Skin irritancy tests

The skin irritant activity of the pure compounds was determined on the ears of SIM and NMRI mice by following the standard protocol [15]. On the basis of irritant activity results, all the isolates (**1–11**) were divided into (i) highly irritant (**1**), (ii) moderately irritant (**1–7**) and (iii) non-irritant (**8–10**) compounds.

3.2. Cytotoxicity assay

Cytotoxicity of the samples was evaluated on human KB cells by enzymatic reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide). Though all the isolates except **11** showed activity but **8–10** were more toxic and displayed $IC_{50} = 0.8,\ 0.5,\ 1.0\ \mu g\ mL^{-1}$, respectively (Table 3). Keeping the toxicity of **8–10** in view, the mechanism of their action in the inhibition of DNA synthesis was investigated. The morphological changes were examined microscopically after the treatment of KB cells with compounds **8–10** and a number of changes related to apoptosis like reduction in cell volume, chromatin condensation and fragmentation of the cells' nucleus were noted. Viable KB human cells were determined using Trypan blue exclusion test in which 3×10^5 cells mL^{-1} concentration was used and incubated with the compounds for 24 h [17]. The viability of untreated and DMSO treated cells was assessed and reported in Table 4.

The KB cells (3×10^5 cells mL^{-1}) were stained with acridine orange/ethidium bromide (AO/EB) and the apoptotic pattern induced by compounds **8–10**, after 24 h incubation was observed. KB cells were treated with compounds **8–10** and incubated for 24 h, the proportion of viable, apoptotic and necrotic was determined and reported in Table 4. The study concluded that the number of apoptotic cells was decreased and necrotic cells increased with the increase in the concentration of these compounds and that these were responsible for the death of KB cells by causing apoptosis [18]. Brd-U incorporated cells were counted microscopically by treating the KB cells with compounds **8–10** for 24 h [19]. The results obtained concluded that the percentage inhibition of DNA synthesis was increased with the concentration of the tested compounds and it was maximum for compound **9** (Table 5). In addition, a correlation was developed between the cytotoxicity, DNA cross-link and DNA strand-break formation, and found to be significant [20].

Table 4
Determinations of the proportion of necrotic and apoptotic KB leukemic cells treated for 24 h with increasing concentrations of **8–10**

Compound	Concentration ($\mu g\ mL^{-1}$)	Viable cells (%)	Apoptotic cells (%)	Necrotic cells (%)
Control	–	97.7 ± 0.64	2.23 ± 0.67	0.00 ± 0.00
Doxorubicin	0.3	37.67 ± 2.67	61.33 ± 3.17	1.00 ± 0.75
8	1.0	74.69 ± 1.73	25.23 ± 1.73	0.00 ± 0.00
	2.5	65.00 ± 1.71	34.00 ± 1.71	1.00 ± 0.00
	5.0	26.11 ± 1.06	70.89 ± 1.32	3.00 ± 0.00
9	1.0	58.76 ± 1.76	39.24 ± 1.75	2.00 ± 0.00
	2.5	36.71 ± 1.72	60.29 ± 1.71	3.00 ± 0.00
	5.0	15.87 ± 1.23	78.13 ± 1.13	6.00 ± 0.53
10	1.0	78.76 ± 1.76	11.24 ± 1.75	0.00 ± 0.00
	2.5	46.67 ± 1.72	52.23 ± 1.71	1.00 ± 0.00
	5.0	27.87 ± 1.23	70.13 ± 1.13	2.00 ± 0.53

Key: Doxorubicin as a positive control. Data represent means ± SEM obtained from three different fields using fluorescence microscopy (4×). $p < 0.05$, ANOVA followed by Student–Newman–Keuls.

4. Discussion

The cytotoxic effects of isolated compounds over human KB cells are displayed in Table 3. Depending upon their activity, the isolated compounds have been divided into three classes: (i) (**1–7**) irritant, non-cytotoxic; (ii) (**8–10**) non-irritant, cytotoxic; (iii) (**11**) irritant, non-cytotoxic compounds. The results further revealed that the esters formed from fatty acids having alkyl group in the range of C_7–C_{11} and phorbol through its 13-C–OH were more active and responsible for cytotoxicity as concluded by others [10]. These results are also in accord with the existing hypothesis about cytotoxicity/irritancy which states that the irritant and cytotoxic activities of phorbol diesters depend upon their liposolubility and

Table 3
Growth inhibitory concentrations of diterpenoids **1–11** from *E. cornigera*

Isolates	Control	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
IC_{50} [$\mu g\ mL^{-1}$]	10	8.2	8.5	6.4	7.6	8.5	7.8	7.2	0.8	0.5	1.0	1000

Table 5
Inhibition of 5-bromo-2′-deoxyuridine (Brd-U) incorporation with **8–10** on KB human leukemic cell lines

Compound	Concentration ($\mu g\,mL^{-1}$)	Brd-U incorporation (%)	T/C
Control	–	67.98	–
Doxorubicin	0.3	37.67 ± 2.67	0.57
8	1.0	74.69 ± 1.73	0.87
	2.5	85.00 ± 1.71	0.71
	5.0	23.00 ± 1.06	n.d
9	1.0	78.76 ± 1.76	0.57
	2.5	86.00 ± 1.72	0.71
	5.0	27.87 ± 1.23	n.d
10	1.0	58.76 ± 1.76	0.47
	2.5	76.00 ± 1.72	0.51
	5.0	25.87 ± 1.23	n.d

Key: Doxorubicin as a positive control. Data are presented as percent of Brd-U incorporation per 200 cells. T/C ratio was calculated using the % of labeled cells; treated/control. $p < 0.05$ compared by test. ANOVA followed by Student, n.d. = not determined because most of the cells were non-viable.

the activity increases with the increase in liposolubility of the compounds that allows them to enter into the cells and reach their target [10].

5. Conclusion

Nine new bioactive diterpene esters were isolated from *E. cornigera* Boiss. Their structure and relative stereochemistry were successfully deduced from 2D NMR techniques. The toxicity was noted to be structure and lipophilicity dependent.

6. Experimental

6.1. Chemistry

Melting point was determined using Gallenkamp (UK) melting point apparatus and reported without correction. All the reagents were AR grade and used as such. All the solvents were double distilled prior to use. Solvents for extraction and chromatography (MeOH, EtOH, Et_2O, Me_2CO, $CHCl_3$, C_6H_{14}, CCl_4, CH_2Cl_2, EtOAc, etc.) were commercial and double distilled. While solvents for HPLC experiments (MeCN, MeOH, EtOAc) were purchased from Fluka/Riedel-deHaen, Germany.

The ^1H (300 MHz) and ^{13}C NMR (75.0 MHz) spectra were recorded on Bruker Biospin AMX 300 MHz FT NMR spectrometer in $CDCl_3$ using TMS for ^1H and chloroform-*d* for ^{13}C as internal references. EIMS were obtained on double-focusing Finnegan MAT 112 spectrometer. HREIMS were recorded on JEOL HX 110 spectrometer. IR spectra (ν_{max}) of the pure samples were obtained on Tensor-27 FTIR spectrophotometer Bruker, Switzerland (4000–500 cm^{-1} range). UV spectra were obtained in absolute MeOH using UV/vis-spectrophotometer Model U 2020 Germany and presented in λ_{max} (nm) (log ε). Optical rotation was measured using digital polarimeter supplied by OSK OGAWA Seiki Co. Ltd, Japan.

Column chromatography was performed on silica gel (230–400 mesh, E. Merck, Germany). All the HPLC experiments were performed on HPLC Perkin Elmer, USA, Model 250 with Binary LC Pump 250, equipped with UV/vis LC 290 detector. Separation was performed on pre-coated thin layer chromatography (TLC) using polyethylene sheets with silica gel (F_{254} 0.20 mm, E. Merck, Germany). Preparative thin layer chromatography (PTLC) was carried out using pre-coated polyethylene plates with silica gel (F_{254} 5.0 mm thickness, E. Merck, Germany). Chromatographic fractions and pure compounds were monitored by TLC, detected by UV light source of 250 nm (UV GL-25 Mineralight lamp) wave length or color reactions by spraying a solution of vanillin/perchloric acid/EtOH followed by 5 min heating at 110 °C.

Human KB cells, bovine serum, glutamine, gentamycin, adriamycin hydrochloride, MTT solution, fetal bovine serum, Trypan blue dye, doxorubicin, eocin–hematoxylin, acridine orange/ethidium bromide (AO/EB), 5-bromo-2′-deoxyuridine (Brd-U), DNA cells, chromogen DAB were from Sigma and Eagle's essential medium from Gibco Co. Ltd.

6.2. Plant material

Roots of *E. cornigera* Boiss. were collected from Murree Hills, Pakistan. They were botanically authenticated with the retained specimens (EC no.1274) at the Herbarium of Department of Botany, University of Peshawar, Pakistan, and identified by Prof. Dr. Qazi Najam us Saqib, Dean Faculty of Pharmacy, Gomal University, Dera Ismail Khan, Pakistan.

6.3. Extractions and isolation

Air-dried powdered roots (5 kg) were extracted with acetone (10 L) at room temperature. The plant material was filtered off and further dried to semi-solid by heating under vacuum. The extract (50 g) was then suspended in H_2O (1 L) and re-extracted with ethyl acetate (2 L). The ethyl acetate soluble crude (36.2 g) was partitioned between petroleum ether/MeOH/H_2O (15:10:0.5). After drying the MeOH extract (17.5 g) was subjected to various Craig's distributions to get bioactive fractions. The most active fraction (10.2 g) was further subjected to silica gel column (12 × 50 cm) and eluted with C_6H_{14}/EtOAc gradient (12 mL min^{-1}). As an outcome of it, following twelve (1–12) 1 [2000 mL, C_6H_{14}/EtOAc (19:1)], 2 [4000 mL, C_6H_{14}/EtOAc (9:1)], 3 [5000 mL, C_6H_{14}/EtOAc (8:2)], 4 [4000 mL, C_6H_{14}/EtOAc (7:3)], 5 [5000 mL, C_6H_{14}/EtOAc (1:1)], 6 [5000 mL, C_6H_{14}/EtOAc (1:3)], 7 [5000 mL, EtOAc], 8 [5000 mL, EtOAc/MeOH (19:1)], 9 [5000 mL, EtOAc/MeOH (9:1)], 10 [3000 mL, EtOAc/MeOH (7:3)], 11 [3000 mL, EtOAc/MeOH (1:1)] and 12 [500 mL, 100% MeOH] sub-fractions were collected. The sub-fraction 4.8 (6.6 g) was further fractionated on a silica gel column (5 × 50 cm) using C_6H_{14}/EtOAc as an eluant to get four sub-fractions 4.8.1–4.8.4 (eluant volume: 800 mL/fraction). The most active fraction 4.8.4 (4 g), showing a single spot by an analytical TLC [silica gel GF_{254}, *n*-C_6H_{14}/Et_2O/

EtOAc (4:3:3)], was loaded on HPLC RP-18 column (250 × 2.5 cm) using (H_2O/MeCN) gradient system (2 mL min^{-1}) and 88% H_2O/MeCN as an eluant for 30 min. The polarity of the solvent was increased up to 96% in 1 h keeping the flow rate as 1 mL min^{-1} (eluant volume: 800 mL $fraction^{-1}$). Such arrangement resulted in pure **1–8** (15.5, 12.7, 10.2, 13.71, 21.3, 17.9, 14.6, and 10.7 mg) compounds with 5, 6.5, 7.3, 8.2, 10.3, 11.4, 12.2, and 13.5 min retention time. Fractions containing mixture of compounds were further loaded on the same column using same eluant yielded **9–11** (7.4, 8.3, 10.9 mg) compounds with 8.2, 10.3, 11.4 min retention time, respectively.

6.4. Characteristics of new compounds *1–8* and *10*

6.4.1. 13-O-Acetyl-20-O-benzoyl-12-deoxyphorbol *1*

Pale yellow oil (0.000067%); $[\alpha]_D^{25}$ +44 ($CHCl_3$, *c* 0.57); UV: 264 (2.9) and 254 (2.4) (aromatic), 243 (4.3) (α,β-unsaturated ketone); IR: 3456, 1696, 1723, 3306; ^1H and ^{13}C NMR: (Tables 1 and 2); EIMS (*m/z*) 494 (M^+, 2%), 476 (7), 458 (12), 434 (15), 389 (9), 372 (12), 330 (5), 294 (70), 190 (35), 105 (100), 71 (85), 65 (55), 43 (65); HREIMS: 494.413, obsd 494.576. Anal. Calcd for $C_{29}H_{34}O_7$: C, 68.94; H, 8.10; O, 22.96; found: C, 68.92; H, 8.07; O, 22.93.

6.4.2. 13-O-Acetyl-20-O-p-methoxybenzoyl-12-deoxyphorbol *2*

Resinous (0.00023%); $[\alpha]_D^{25}$ +46 ($CHCl_3$, *c* 0.54); UV: 243 (4.3); IR: 3456, 1734, 1696, 3306, 690; ^1H and ^{13}C NMR: (Tables 1 and 2); EIMS (*m/z*) 524 (6), 506 (15), 488 (M^+, 28%), 464 (12), 388 (12), 372 (23), 294 (70), 190 (35), 135 (100), 95 (33); HREIMS: 524.4582, obsd 524.602. Anal. Calcd for $C_{30}H_{36}O_8$: C, 70.43; H, 6.93; O, 22.64; found: C, 70.41; H, 6.91; O, 22.62.

6.4.3. 13-O-Decanoyl-20-O-angelyl-12-deoxyphorbol *3*

Pale yellow oil (0.00023%); $[\alpha]_D^{25}$ −46 ($CHCl_3$, *c* 0.74); UV: 244 (4.1); IR: 3458, 1697, 1737, 3308, 993; ^1H and ^{13}C NMR: (Tables 1 and 2); EIMS (*m/z*) 584 (M^+, 3%), 566 (12), 548 (23), 501 (24), 412 (12), 312 (5), 354 (22), 336 (21), 294 (70), 190 (35), 83 (100), 71 (80); HREIMS: 584.6834, obsd 584.783. Anal. Calcd for $C_{35}H_{52}O_7$: C, 71.89; H, 8.92; O, 19.15; found: C, 71.86; H, 8.90; O, 19.12.

6.4.4. 13-O-Decanoyl-20-O-tiglyl-12-deoxyphorbol *4*

Yellow oil (0.00023%); $[\alpha]_D^{25}$ +36 ($CHCl_3$, *c* 0.74); UV: 245 (4.2); IR: 3459, 1737, 1694, 3303, 993; ^1H and ^{13}C NMR: (Tables 1 and 2); EIMS (*m/z*) 584 (M^+, 8%), 566 (14), 548 (25), 484 (26), 412 (22), 312 (100), 294 (74), 190 (45), 71 (90); HREIMS: 584.6173, obsd 584.783. Anal. Calcd for $C_{35}H_{52}O_7$: C, 71.89; H, 8.92; O, 19.15; found: C, 71.87; H, 8.90; O, 19.13.

6.4.5. 13-O-Acetyl-20-O-decanoyl-12-deoxyphorbol *5*

Pale yellow oil (0.00023%); $[\alpha]_D^{25}$ +46 ($CHCl_3$, *c* 0.54); UV: 242 (4.0); IR: 3453, 1692, 1738, 3309, 992; ^1H and ^{13}C NMR: (Tables 1 and 2); EIMS (*m/z*) 544 (M^+, 3%), 394 (12), 376 (5), 340 (13), 284 (15), 212 (91), 194 (70), 190 (35), 71 (100); HREIMS: 544.6827, obsd 544.719. Anal. Calcd for $C_{32}H_{48}O_7$: C, 70.56; H, 8.88; O, 20.56; found: C, 70.54; H, 8.83; O, 20.53.

6.4.6. 13-O-Butanoyl-20-O-decanoyl-12-deoxyphorbol *6*

Pale yellow oil (0.00023%); $[\alpha]_D^{25}$ +46 ($CHCl_3$, *c* 0.54); 244 (4.1); IR: 3458, 1692, 1733, 3305, 992; ^1H and ^{13}C NMR: (Tables 1 and 2); EIMS (*m/z*) 572 (M^+, 3%), 494 (12), 476 (5), 440 (13), 384 (15), 312 (91), 294 (70), 190 (35), 71 (100); HREIMS: 572.6827, obsd 572.772. Anal. Calcd for $C_{34}H_{52}O_7$: C, 71.30; H, 9.15; O, 19.55; found: C, 71.27; H, 9.13; O, 19.53.

6.4.7. 13-O-Hexanoyl-20-O-decanoyl-12-deoxyphorbol *7*

Pale yellow oil (0.00023%); $[\alpha]_D^{25}$ +46 ($CHCl_3$, *c* 0.54); UV: 242 (4.13); IR: 3452, 1693, 1731, 3307, 993; ^1H and ^{13}C NMR: (Tables 1 and 2); EIMS (*m/z*) 600 (M^+, 3%), 582 (12), 576 (5), 540 (13), 484 (15), 312 (91), 294 (70), 190 (35), 71 (100); HREIMS: 600.2733, obsd 600.826. Anal. Calcd for $C_{36}H_{56}O_7$: C, 71.97; H, 9.39; O, 18.64; found: C, 71.95; H, 9.37; O, 18.61.

6.4.8. 13-O-Octanoyl-20-O-decanoyl-12-deoxyphorbol *8*

Pale yellow oil (0.00023%) $[\alpha]_D^{25}$ +46 ($CHCl_3$, *c* 0.54); UV: 242 (4.1); IR: 3453, 1693, 1735, 3308, 995; ^1H and ^{13}C NMR: (Tables 1 and 2); EIMS (*m/z*) 628 (M^+, 3%), 610 (12), 576 (5), 540 (13), 484 (15), 312 (91), 294 (70), 190 (35), 71 (100); HREIMS: 628.7367, obsd 628.879. Anal. Calcd for $C_{38}H_{60}O_7$: C, 72.57; H, 9.62; O, 17.81; found: C, 72.55; H, 9.60; O, 17.79.

6.4.9. 13-O-Dodecanoyl-20-O-decanoyl-12-deoxyphorbol *10*

Pale yellow oil (0.00023%); $[\alpha]_D^{25}$ +46 ($CHCl_3$, *c* 0.54); UV: 245 (4.37); IR: 3454, 1693, 1735, 3307, 998; ^1H and ^{13}C NMR: (Tables 1 and 2); EIMS (*m/z*) 684 (M^+, 3%), 666 (12), 506 (5), 540 (13), 484 (15), 312 (91), 294 (70), 190 (35), 71(100); HREIMS: 684.8857, obsd 684.985. Anal. Calcd for $C_{42}H_{68}O_7$: C, 73.64; H, 10.01; O, 16.32; found: C, 73.61; H, 9.96; O, 16.31.

6.5. Preparation of solutions for estimation of irritation

The solutions for the determination of irritation on mouse ear were prepared by taking a small amount (ca. 1–2 μg) of a pure compound in 1 mL Me_2CO, a non-irritant solvent and diluted as per the requirement [15].

6.6. Estimation of irritation

A standard protocol described by Hecker et al. was followed to perform the irritation assays [10–15]. For this purpose, 30 female NMRI mice were taken and one ear of each mouse was treated with 6 μL of sample solution. Irritation was considered positive if it caused redness to the ear. The croton oil DAB7 was used as reference.

6.7. Cytotoxicity assays

6.7.1. MTT assay

Cytotoxicity of the samples was evaluated on human KB cells using reduced MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) with the help of mitochondrial dehydrogenase viable cells and measuring the concentration of the product (blue formazan) spectrophotometrically [16]. Briefly, the human KB cells ($\approx 10^{-4}$ cells mL^{-1}) from patients were seeded into each well in 100 μL of Eagle's essential medium containing 10% fetal bovine serum, 1% glutamine and gentamycin (100 μg mL^{-1}). The plates were cultured at 34 °C in a humidified atmosphere containing 5% CO_2 in 96-well plates. After 24 h, the cells were exposed to various test compounds. After four days, 20 μL (5 mg mL^{-1}) of MTT solution was added to each well and incubated for 6 h then 100 μL of DMSO was added to each well. The formazan crystals were dissolved in each well through vibration. The optical density was measured at 580 and 780 nm wavelengths by microplate autoreader (Perkin Elmer, USA). The IC_{50} values were calculated statistically using adriamycin hydrochloride salt as control.

6.7.2. Trypan blue exclusion

Cell viability was determined by the Trypan blue exclusion test over KB human leukemic cells, incubated for 24 h by following the standard protocol [17]. For this purpose, the cells (3×10^5 cells mL^{-1}) were incubated with **8–10** using 1, 2.5, 5.0 μg mL^{-1} concentrations. Viability of untreated and DMSO treated cells was assessed, which was greater than 95%. Doxorubicin (0.3 μg mL^{-1}) was used as a positive control.

6.7.3. Analysis of morphological changes

Untreated and phorbol-treated KB cells with three different (1.0, 2.5, 5.0 μg mL^{-1}) concentrations of compounds **8–10** were examined after 24 h for morphological changes, microscopically [18]. The nuclear morphology assay was performed, placing the harvested cells on a glass slide using cytospin, which was fixed with 96% ethanol for 1 h and stained with eocin–hematoxylin. Doxorubicin (0.3 μg mL^{-1}) was used as a positive control.

6.7.4. Assessment of apoptosis

This assay was performed over the KB cells (3×10^5 cells mL^{-1}) following the standard protocol [19]. The cells were stained with the mixture of acridine orange/ethidium bromide (AO/EB) and compounds **8–10**, using concentration of the compounds as 1, 2.5, 5.0 μg mL^{-1}. After 24 h of incubation, the cells were pelleted and re-suspended in 25 μL of PBS. Later, each sample was mixed with 1 μL AO/EB (1 part of 100 μg mL^{-1} of AO in PBS solution; 1 part of 100 μg mL^{-1} of EB in PBS) just before the microscopic study and quantification. The cell suspension (10 μL) was placed on a microscopic slide, covered with a glass cover slip, and minimum 300 cells were studied under the fluorescence microscope using a fluorescent filter and a 40× objective lens. The percentage of viable, apoptotic and necrotic cells was determined and presented in Table 4. Doxorubicin (0.3 μg mL^{-1}) was used as a positive control.

6.7.5. Inhibition of DNA synthesis

Inhibition of DNA synthesis of the human KB cell lines was studied with three different concentrations of **8–10** by following the standard procedure [20]. The cells (3×10^5 cells mL^{-1}) were treated with three different concentrations (1, 2.5, 5.0 μg mL^{-1}) for 24 h and placed in to a 24-well (2 mL $well^{-1}$) tissue culture. Then 10 μL of 5-bromo-2′-deoxyuridine (Brd-U, 10 mM) was added to each well and incubated for 3 h at 37 °C. To assay the amount of Brd-U incorporated in cell, DNA cells were harvested, placed on a glass slide using cytospin and left to dryness for about 2 h at room temperature. Cells that had been treated with Brd-U were labeled by direct peroxidase-cytochemistry, utilizing the chromogen DAB. Slide counterstained with hematoxylin was mounted and covered by cover slip. Evaluation of incorporated Brd-U was accomplished by microscopic study. Results are compiled in Table 5. Doxorubicin (0.3 μg mL^{-1}) was used as a positive control. Two hundred cells were counted per sample to obtain the percentage of positive cells.

6.8. Fatty acids

Acetic acid: 2.2 (3H, s, CH_3-2); ^{13}C NMR: 170.2 (C-1), 23.1 (C-2). *Butanoic acid*: 2.2 (2H, t, $J = 8.0$ Hz, CH_2-2), 1.63 (2H, m, CH_2-3), 0.96 (3H, t, $J = 7.4$ Hz, CH_3-4); ^{13}C NMR: 171.8 (C-1), 36.1 (C-2), 16.2 (C-3), 13.7 (C-4). *Hexanoic acid*: 2.2 (2H, t, $J = 8.0$ Hz, CH_2-2), 1.63 (2H, m, CH_2-3), 1.2–1.25 ($(CH_2)_3$), 0.96 (3H, t, $J = 7.4$ Hz, CH_3-6); ^{13}C NMR: 172.13 (C-1), 36.1 (C-2), 16.2 (C3-5), 13.7 (C-6). *Octanoic acid*: 2.2 (2H, t, $J = 7.3$ Hz, CH_2-2), 1.63 (2H, m, CH_2-3), 1.2–1.25 ($(CH_2)_5$), 0.96 (3H, t, $J = 7.4$ Hz, Me-8); ^{13}C NMR: 172.18 (C-1), 36.1 (C-2), 26.2 (C3-7), 13.7 (C-8). *Decanoic acid*: 2.3 (2H, t, $J = 8.0$ Hz, CH_2-2), 1.1–1.2 m, $(CH_2)_8$, 0.90, t, $J = 7$ Hz, Me-10. *Dodecanoic acid*: 2.25 (2H, t, $J = 8.0$ Hz, CH_2-2), 1.1–1.2 (m, $(CH_2)_{10}$), 0.90, (t, $J = 7$ Hz, Me-10), 172.75 (C-1), 34.32 (C-2), 24.97 (C-3), 29.70 (C-4), 29.67 (C-5, C-6), 29.32 (C-7), 29.29 (C-8), 29.15 (C-9), 29.47 (C-10), 22.70 (C-11), 14.10 (C-12). *p-Methoxybenzoic acid*: 6.8 (4H, s, aromatic), 3.86 OMe. *Benzoic acid*: 8.10 (2H, d, $J = 7.2$ Hz, aromatic protons, H-2 and H-6), 7.48 (2H, t, $J = 7.6$ Hz, aromatic protons, H-3 and H-5), 7.59, (1H, t, $J = 7.2$ Hz, aromatic proton, (H-4); ^{13}C NMR: 129.71 (C-1), 129.82 (C-2, 6), 128.12 (C-3, 5), 133.1 (C-4). *Tiglic acid*: ^{1}H NMR: 1.84 (3H, br, d, $J = 1.5$ Hz, CH_3), 1.80 (3H, dq, $J_1 = 7.0$ Hz, $J_2 = 1.5$ Hz, CH_3), 6.89 (1H, q, $J = 6.99$ Hz, H-3); ^{13}C NMR: 168.3 (s, C-1), 128.0 (s, C-2), 137.3 (d, C-3), 15.9 (q, C-4), 20.6 (q, C-5). *Angelic acid*: ^{1}H NMR: 6.13 (1H, qq, $J = 6.6$ Hz, H-3), 2.01 (3H, dq, CH_3), 1.93 (3H, dqs, CH_3); ^{13}C NMR: 168.3 (s, C-1), 128.0 (s, C-2), 137.3 (d, C-3), 15.9 (q, C-4), 20.6 (q, C-5).

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