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ORIGINAL ARTICLE

Irritant and co-carcinogenic diterpene esters from the latex of *Euphorbia cauducifolia* L.

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Ten (**1**–**10**) irritant and mild co-carcinogenic diterpene esters were isolated from the latex of *Euphorbia cauducifolia* L. using bioassay-guided countercurrent distribution and other chromatographic techniques. The isolated compounds were characterized on the basis of spectroscopic results and mass measurements. As an outcome, the ingenane-type esters were established with the following structures: 3-*O*-angeloyl-17-*O*-palmatoylingenol (**1**), 3-*O*-palmatoyl-5-*O*-angeloylingenol (**2**), 5-*O*-angeloyl-17-*O*-palmatoylingenol (**3**), 3-*O*-angeloyl-5-*O*-palmatoylingenol (**4**), 17-*O*-(2*Z*,4*E*,6*Z*)-2,4,6-tetradecatrienoyl-20-*O*-palmatoylingenol (**5**), 5-*O*-angeloyl-17-*O*-benzoylingenol (**6**), 5-*O*-angeloyl-17,20-diacetoxyingenol (**7**), 3-*O*-angeloyl-17-*O*-benzoyl-20-acetoxyingenol (**8**), 3-acetoxy-5-*O*-angeloyl-17-*O*-benzoylingenol (**9**), and 5-*O*-angeloyl-3,17,20-triacetoxyingenol (**10**). Their biological screening revealed that they are moderate irritants, and low to moderate tumor promoters compared to TPA, but hardly showed any solitary carcinogenic activity. The isolated esters represent new compounds and were not reported before from any source.

Keywords: *Euphorbia cauducifolia*; irritant; tumor promoters; diterpenes; 17-hydroxyingenol esters

1. Introduction

Due to complexity in diseases, limited resources, high cost of synthetic drugs, and their side-effects, 80% of the world population relies on plant extracts, natural products, and their derivatives which are biodegradable, safe to use, and innocuous to the environment [1–7].

Pakistan is very rich in diversity of plants and Euphorbiaceae is one of the largest families of the angiosperm grown in Pakistan, which has 300 genera and 7500 species. The genus *Euphorbia* has 1600 species including herbs, shrubs, and trees, [8–10], which are well known for containing potential toxic, carcinogenic, co-carcinogenic, co-carcinostatic, anti-inflammatory, anticancer, dermatitis producing, antitumor compounds [10–13]. The plants are equally popular among the local people as folk medicine against various ailments such as an antidote for scorpion sting, anticancer, expectorant, amoebicidal, against cough, asthma, healing wounds, sore eyes, and throat [14,15].

Euphorbia cauducifolia L. *syn. Euphorbia nerifolia* L. is a shrub with a thick-armed cylindrical stem and large oval-shaped leaves having leafless green branches and very common in coastal, arid, and desert areas of Sind [14]. Its latex is highly irritant and toxic, on contact; it induces inflammation, produces conjunctivitis, scouring and blistering in eyes. The

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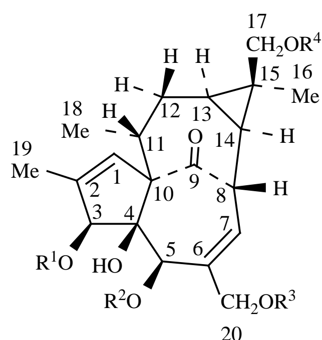

	R_1	R_2	R_3	R_4
1.	Ang	H	H	Palm
2.	Palm	Ang	H	H
3.	H	Ang	H	Palm
4.	Ang	Palm	H	H
5.	H	H	Palm	Tetradec
6.	H	Ang	H	Bz
7.	H	Ang	Ac	Ac
8.	Ang	H	Ac	Bz
9.	Ac	Ang	H	Bz
10.	Ac	Ang	Ac	Ac

Abbreviations: Ac = Acetyl; Ang = Angeloyl; Bz = Benzoyl; Palm = Palmitoyl and Tetradec = –(*2Z,4E,6Z*)– (*2,4,6*)- Tetradecatrienoyl.

Figure 1. Structures of compounds **1–10**.

literature reveals that no proper attention has been paid to this plant and very few inactive diterpenes [14,15] and triterpenes are isolated [13–16]. Therefore, these aspects prompted us to investigate its biologically active constituents. In previous studies, we reported biologically active compounds from the latex [8,9]. In the present investigation, 10 compounds (**1–10**) were isolated and their structures were elucidated (Figure 1), and their biological activity was assessed.

2. Results and discussion

During the preliminary studies, fresh latex extracts showed irritancy (ear redness) and disturbance in animals. Therefore, a systematic study was carried out as given in the experimental section. The latex was collected and extracted with different organic solvents, and their irritant activity was assessed. Diethyl ether and ethyl acetate extracts showed IU_{50}^{25}: 43.4 and 31.7 μg/ml, respectively.

The fractions were further separated using silica gel column chromatography. The active portion of the material was identified by testing against mouse ear. The selected crude was subjected to HPLC to get pure compounds (**1–10**).

The 2D NMR spectral data (COSY-45°, HOHAHA, and TOCSY) of compounds **1–10** revealed the spin sequence (CH^7—CH^8—CH^{13}—CH^{14}—CH_2^{12}—CH^{11}—CH_3^{18}) typical for 17-hydroxyingenol esters [11,16–19] and was further confirmed through double resonance decoupling experiments.

The ^1H and ^{13}C NMR spectral data of **1–10** (Tables 2–4) showed a peak at δ 4.22 instead of δ 1.13, indicating the oxidation of Me-17 to CH_2-17-O-R. This was confirmed through DEPT by the presence of a triplet at δ 66.3 instead of the usual quartet at ca. δ 25. In addition, the protons of CH_2-17 displayed cross-peak correlations with C-15 and Me-16 in the HMBC spectrum. The presence of another oxymethylene group, as an AB doublet at δ 4.42, 4.72, was due to CH_2-20-O-R, which showed cross-peak correlations with C-5, C-6, and C-7 in the HMBC spectrum (Figure 2). The position of the various ester groups in **1–10** was deduced from the increase in chemical shift of *gem*-protons and that of respective carbons [H-C-3-O, H-C-5-O, CH_2-17-O, CH_2-20-O], as well as by cross-peak correlations between the carboxyl carbon of the esters and the respective protons of the diterpene core (HMBC evidence). The multiplicities of the carbon atoms in **1–10** compounds were revealed by performing DEPT experiments with the last polarization pulse angle set at 45°, 90°, and 135°.

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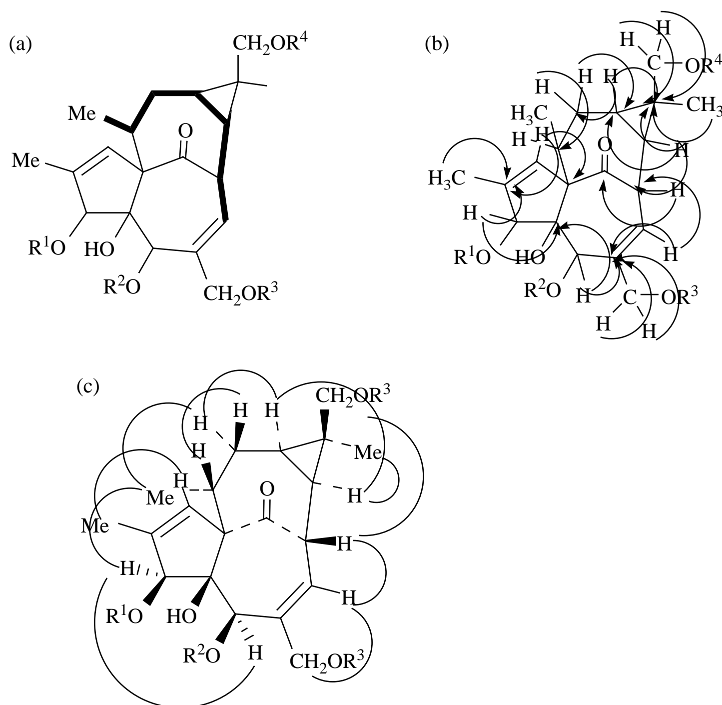

Figure 2. (a) Key COSY (bold bonds), (b) HMBC, and (c) NOESY correlations of compounds **1–10**.

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The nature of the various acid residues in compounds **1–10** was identified after hydrolysis and comparing ^{1}H and ^{13}C NMR spectral data (Table 1) with the authentic samples using the GC-MS technique.

The UV, IR, and mass fragmentation pattern of compounds **1–4** were very similar to each other and showed a close resemblance to the already reported compounds from the same source [11]. The molecular formula was established on the basis of peak in the HR-EI-MS molecular ion peak $[M]^+$ observed at *m/z* 684.4602. Judging from the ^{1}H and ^{13}C NMR spectral data (Table 2), they were constitutional isomers. In their EI-MS mass spectra **1–4** showed peaks at *m/z* 428 $[M - 256]^+$ and 584 $[M - 100]^+$, characteristic for palmatic and angelic acid residues.

The DEPT experiments revealed 41 carbon atoms as 6 CH_3, 17 CH_2, 8 CH, and 10 C, which consist of one ketone group, three alcohol groups (tertiary, secondary, and primary), and two ester groups.

In the ^{1}H NMR spectrum of **1** (Table 2), the H-3-*O* resonated at δ 5.43 as singlet, and CH_2-17-O as AB doublets at δ 4.53 and 4.36 with a *gem*-coupling constant of 12.5 Hz. In the HMBC spectrum, the H-3-*O* of ingenol displayed an interaction with the C=O at δ 166.3 of the angelic acid and CH_2-17-*O* at δ 4.53, 4.36 with C=O of palmatoyl at δ 174.8.

The NOE spectrum showed interactions between H-3 of ingenol and H-3 of the angelic acid, indicating the presence of angelate moiety at C-3. The spectra also showed the interaction between H_2-17 of ingenol and H_2-2 of palmitic acid, indicating the attachment of palmitate moiety at C-17. In the light of the above evidence, we concluded that the structure of **1** is 3-*O*-angeloyl-17-*O*-palmatoyl-ingenol. The above spectral data estab-

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Table 1. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectral data of acids present in **1**–**10** (δ values, in $CDCl_3$).

Position	Palmatic acid		Tetradecanoic acid		Benzoic acid		Angelic acid		Acetic acid	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		174.3		175.3		167.2		166.3		174.2
2	2.30 t (8.0)	34.5	2.31 t (8)	34.5		130.3		128.0	2.21 s	22.5
3	1.28 br s	24.9	1.27 br s	24.3	8.10 d (7)	129.5	6.13 qq (7.0, 1.2)	137.3		
4	1.27 br s	29.7	1.27 br s	29.7	7.48 t (8.0)	128.1	2.01 d (7.0)	15.9		
5	1.27 br s	29.7	1.27 br s	29.7	7.59 t (7.0)	133.1	1.93 d (1.2)	20.6		
6	1.26 br s	29.7	1.26 br s	29.6	7.48 t (8.0)	128.3				
7	1.26 br s	29.7	1.26 br s	29.5	8.10 d (7.0)	129.5				
8	1.25 br s	29.6	1.25 br s	29.5						
9	1.25 br s	29.4	1.25 br s	29.5						
10	1.25 br s	29.3	1.25 br s	27.6						
11	1.24 br s	29.3	1.24 br s	29.7						
12	1.24 br s	29.2	1.24 br s	31.2						
13	1.24 br s	29.5	1.24 br s	22.7						
14	1.23 br s	31.9	0.88 t (8.0)	14.2						
15	1.23 br s	22.7								
16	0.88 t (8)	14.1								

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Table 2. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectral data of **1–3** (δ values, in $CDCl_3$).

	1		**2**		**3**	
Position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	6.18 q (1.2)	132.7	6.27 q (1.2)	134.7	6.21 q (1.2)	132.5
2	–	135.3	–	137.7	–	135.5
3	5.45 s	80.8	5.47 s	82.7	3.85 s	76.8
4	–	74.8	–	76.8	–	75.8
5	3.85 br s	76.0	4.98 s	81.3	5.45 s	82.0
6	–	137.0	–	137.0	–	138.2
7	6.21 d (4.0)	128.5	5.63 d (4.0)	128.7	7.03 d (4.0)	129.1
8	4.12 dd (12.5, 4.5)	42.8	4.14 dd (12.5, 4.5)	42.9	4.15 dd (12.5, 4.5)	43.3
9	–	205.3		205.6		205.7
10	–	72.1	–	72.2		72.3
11	2.43 ddq (7.5, 5.2, 4.3)	37.5	2.42 ddq (7.5, 5.2, 4.3)	37.4	2.45 ddq (7.5, 5.2, 4.3)	37.7
12α	2.33 ddd (15.5, 6.2, 4.2)	30.4	2.35 ddd (15.5, 6.2, 4.2)	30.3	2.37 ddd (15.5, 6.2, 4.2)	30.5
12β	1.85 dd (15.5, 6.2)		1.87 (15.5, 6.2)		1.83 dd (15.5, 6.2)	
13	1.03 br s	23.7	1.05 br	23.8	1.04 br s	23.9
14	1.30 dd (12.4, 8.5)	23.4	1.31 dd (12.4, 8.5)	23.5	1.33 dd (12.4, 8.5)	23.3
15	–	27.7	–	27.3	–	27.5
16	1.09 s	24.5	1.12 s	24.6	1.13 s	24.7
17α	4.22 (13.3)	66.6	4.29 brs	62.3	4.22 (13.0)	66.7
17β	4.26 (13.3)				4.28 (13.0	
18	0.94 d (7.5)	18.3	0.97 d (7.5)	18.3	0.98 d (7.5)	18.2
19	1.77 d (2)	15.5	1.86 d (2)	15.6	1.83 d (2.0)	15.7
20α	3.98 br s	62.2	3.94 brd (5.0)	62.4	3.97 br s	62.3
20β						

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Table 3. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectral data of **4–6** (δ values, in $CDCl_3$).

	4		**5**		**6**	
No.	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	6.13 d (1.2)	132.3	6.01 q (1.2)	132.9	6.07 q (1.2)	135.7
2	–	136.3	–	136.3	–	136.3
3	5.45 s	81.8	3.85 s	74.7	3.98 s	76.8
4	–	76.8	–	74.8	–	75.8
5	4.98 br s	82.0	3.83 s	73.0	5.83 s	82.0
6	–	137.0	–	135.0	–	138.3
7	6.21 d (4.0)	128.4	5.63 d (4.0)	127.4	5.63 d (4.0)	131.4
8	4.12 dd (12.5, 4.5)	42.8	4.14 dd (12.5, 4.5)	42.3	4.14 dd (12.5, 4.5)	44.8
9	–	205.7		205.3		206.2
10	–	72.0		72.03		74.2
11	2.44 ddq (7.5, 5.2, 4.3)	37.7	2.44 ddq (7.5, 5.2, 4.3)	37.4	2.44 ddq (7.5, 5.2, 4.3)	38.4
12α	2.37 ddd (15.5, 6.2, 4.2)	30.5	2.36 ddd (15.5, 6.2, 4.2)		2.35 ddd (15.5, 6.2, 4.2)	31.7
12β	1.87 dd (15.5, 6.2)		1.85 dd (15.5, 6.2)	30.3	1.86 dd (15.5, 6.2)	
13	1.13 br s	23.8	1.03 br	23.7	1.07 br s	25.2
14	1.31 dd (12.4, 8.5)	23.4	1.30 dd (12.4, 8.5)	23.5	1.32 dd (12.4, 8.5)	24.7
15	–	27.5	–	27.6	–	29.3
16	1.17 s	24.5	1.22 s	24.6	1.22 s	31.5
17	4.29 br s	62.2	4.22 (13.0) 4.28 (13.0)	66.4	4.22 (13.0) 4.28 (13.0)	66.3
18	0.94 d (7.5)	18.2	0.97 d (7.5)	18.3	0.96 d (7.5)	19.7
19	1.77 d (1.2)	15.4	1.76 d (1.2)	15.5	1.86 d (1.2)	17.3
20α	3.93 br s	62.2	4.45 d (13.0)	66.4	3.98 br s	62.3
20β			4.73 d (13.0)			

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Table 4. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectral data of **7–10** (δ values, in $CDCl_3$).

No.	**7**		**8**		**9**		**10**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	6.08 d (1.2)	131.7	6.12 q (1.2)	131.7	6.12 q (1.2)	133.7	6.17 q (1.2)	134.2
2	–	136.3	–	136.3	–	136.7	–	137.3
3	3.85 s	75.2	5.45 s	80.8	5.47 s	80.8	5.49 s	81.2
4	–	75.8	–	74.8	–	75.3	–	76.1
5	5.45 s	80.0	3.83 br s	75.0	4.93 s	81.0	4.98 s	81.3
6	–	137.0	–	137.0	–	138.3	–	137.5
7	6.21 d (4.0)	128.4	5.63 d (4.0)	128.4	5.73 d (4.0)	129.2	5.83 d (4.0)	129.7
8	4.12 dd, (12.5, 4.5)	43.4	4.14 dd (12.5, 4.5)	42.8	4.17 dd (12.5, 4.5)	43.3	4.19 dd (12.5, 4.5)	44.1
9	–	205.7		206.3		206.7		207.3
10	–	72.0		72.3		73.3		73.9
11	2.44 ddq (7.5, 5.2, 4.3)	37.7	2.42 ddq (7.5, 5.2, 4.3)	37.7	2.44 ddq (7.5, 5.2, 4.3)	38.7	2.47 ddq (7.5, 5.2, 4.3)	39.2
12α	2.38 ddd (15.5, 6.2, 4.2)	30.7	2.43 ddd (15.5, 6.2, 4.2)	30.5	2.44 ddd (15.5, 6.2, 4.2)	31.3	2.48 ddd (15.5, 6.2, 4.2)	31.7
12β	1.85 dd (15.5, 6.2)		1.85 dd (15.5, 6.2)		1.85 dd (15.5, 6.2)		1.85 dd (15.5, 6.2)	
13	1.02 br s	23.7	1.05 br s	23.8	1.02 br s	24.3	1.02 br s	25.1
14	1.31 dd (12.4, 8.5)	23.4	1.33 dd (12.4, 8.5)	23.7	1.34 dd (12.4, 8.5)	24.7	1.35 dd (12.4, 8.5)	25.4
15	–	27.5	–	27.7	–	27.9	–	28.1
16	1.07 s	24.5	1.12 s	24.7	1.17 s	25.5	1.22 s	26.1
17	4.22 (13.0)4.27 (13.0)	66.7	4.22 (13.0)4.27 (13.0)	66.7	4.22 (13.0)4.27 (13.0)	66.3	4.22 (13.0)4.27 (13.0)	66.6
18	0.94 d (7.5)	18.3	0.95 d (7.5)	18.2	0.97 d (7.5)	18.7	0.98 d (9.0)	18.8
19	1.77 d (1.2)	15.5	1.86 d (1.2)	15.5	1.86 d (1.2)	16.5	1.86 d (1.2)	17.3
20α	4.43 d (13.0)	66.4	4.42 d (13.0)	66.4	3.91 br s	62.4	4.48 d (13.0)	66.4
20β	4.73 d (13.0)		4.72 d (13.0)				4.78 d (13.0)	

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Table 5. ID_{50} values (μg/mL) of compounds **1**–**10** isolated from the latex of *E. cauducifolia* L.

Compounds	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	TPA
ID_{50} (μg/ml)	3.10	2.8	3.1	2.5	3.5	4	4.1	4.4	3.5	7.9	2.5
Standard deviation	1.27	1.22	1.27	1.3	1.24	1.28	1.21	1.23	1.27	1.31	1.31

lished that **1** is a novel compound, which has not been reported earlier from the plant kingdom.

To differentiate the isolates from each other, HMBC and NOE experiments were performed. The structures of other isolates were suggested as 3-*O*-palmatoyl-5-*O*-angeloylingenol for **2**, 5-*O*-angeloyl-17-*O*-palmatoylingenol for **3**, and 3-*O*-angeloyl-5-*O*-palmatoylingenol for **4**. The literature reveals that **2**–**4** are new metabolites from the plant kingdom.

The EI-MS spectrum of **5** displayed peaks at *m/z* 552 $[M - 256]^+$ and 586 $[M^+ - 222]^+$ compatible with the respective loss of palmatic and tertadeca-2,4,6-trienoic acid moieties, respectively, from the parent molecule. NMR spectral data (^{1}H and ^{13}C; Table 3) were almost superimposable to compound **2** except the NOE experiment in which H_2-17 displayed the interaction with H-2 (acyl) at δ 5.63 and in the HMBC spectrum, the carbonyl carbon at δ 166.3 of acyl (2,4,6-tetradecatrienoyl) with H_2-17, concluding that angelic acid in **2** has been replaced by tetradeca-2,4,6-trienoic acid in **5**. The ^{13}C NMR (APT) and DEPT spectra of **5** showed 50 carbon atoms, including 5 CH_3, 19 CH_2, 18 CH, and 8 C, of which eight are oxygenated (one keto, one tertiary alcohol, two secondary, two primary alcohols, and two ester carboxyls). On the basis of these evidences, the structure of **5** was proposed as 17-*O*-(2*Z*,4*E*,6*Z*)-2,4,6-tetradecatrienoyl-20-*O*-palmatoylingenol. Compound **6** was found to be 5-*O*-angeloyl-17-*O*-benzoylingenol and both **5** and **6** represent new compounds from the plant kingdom.

On the basis of EI-MS fragmentation patterns and ^{1}H and ^{13}C NMR spectral data (Table 4), compound **7** was suggested as tri-acyloxyingenols. The presence of one angelate and two acetates in **7** was deduced with the help of EI-MS fragmentation. The ^{1}H and ^{13}C NMR spectral data (Table 4) of **7** revealed that the positions C-O-5, C-O-17, and C-O-20 were esterified. With the help of HMBC and NOE experiments, the

Table 6. Tumor-promoting and co-carcinogenic activities of compounds **1–10** and the number of mice used for the experiment was 28.

Compounds	Single dose (nmol)	Tumor rate/number of survivors		Tumor yield/number of survivors		Survival rate (%) after 48 weeks
		24 weeks	48 weeks	24 weeks	48 weeks	
1	40	0/27	0/27	0/27	0/27	96
2	65	1/25	2/25	4/23	11/23	82
3	40	1/27	2/27	4/27	3/27	96
4	80	5/27	9/27	15/27	17/27	96
	5[a]	0/27	0/27	0/27	0/27	96
5	40	0/26	0/26	0/23	0/23	82
6	40	1/27	0/27	0/27	0/27	96
7	40	0/27	0/27	0/27	0/27	96
8	40	2/27	7/27	3/26	7/26	92
9	40	4/27	3/27	5/27	8/27	96
10	40	2/27	3/27	3/28	4/28	100
TPA	5	3/27	20/28	4/27	112/27	96

Note: [a]Number of initiation with DMBA (7,12-dimethylbenz(a)anthracene); $i = 0.1$ml of acetone. For initiation of tumor promotion, 100 nmol of DMBA was used twice a week.

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angelate residue was deduced at C-5, and hence acetate groups were placed at CH_2-17 and CH_2-20. Therefore, the structure of **7** was established as 5-*O*-angeloyl-17,20-*O*-diacetylingenol. Compounds **8** and **9** were concluded to be triester of angelic, acetic, and benzoic acid. HMBC and NOE experiments suggested that **8** and **9** were 3-*O*-angeloyl-17-*O*-benzoyl-20-*O*-acetylingenol and 3-*O*-acetyl-5-*O*-angeloyl-17-*O*-benzoylingenol, respectively.

According to the spectral evidence obtained through mass measurement and 1D and 2D NMR (^{1}H and ^{13}C; Table 4) spectral data, compound **10** is 5-*O*-angeloyl-3,17,20-triacetoxylingenol, which is reported for the first time from the plant kingdom.

All compounds showed $ID_{50} = 2.5$–4.4 µg/ml, indicating moderate irritant activity when compared to TPA (Table 5). Compounds **1–4** are positional isomers of each other but different in activities. Due to the presence of free OH groups at C-3 in compounds **1** and **3**, they are less irritant than **2** and **4**, which is the same as for **5** and **6**, whereas **2** and **4** are equal to TPA in activity. Compound **2** is the most irritant in the series as it possesses palmitate at C-3, while the other compounds, triesters **7–9** and tetraester **10**, show less irritant activity which may be due to esterification of C-5 or C-20.

All the compounds exhibited no tumor promoting activity up to a dose of 40 nmol/application after 48 weeks when compared with the standard TPA. Compounds **2** and **4** showed weak tumor promoting activity with a dose of 65 nmol/application after 48 weeks (Table 5). A moderate activity was observed for **4** at a dose of 80 nmol/application giving 15/27 tumor rate after 24 weeks and 19/27 after 48 weeks (Table 6). However, no solitary carcinogenic activity was detected with compounds **2** and **4**.

3. Experimental

3.1 General experimental procedures

Optical rotation was measured on a digital polarimeter supplied by OSK Ogawa Seiki Co. Ltd (Tokyo, Japan). UV spectra were recorded in absolute MeOH using λ_{max} in nm ($\log \varepsilon$) on an IRMECO UV/Vis spectrophotometer Model U-2020

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(Geesthacht, Germany). IR spectra were recorded in nujol mull ν_{max} in cm^{-1} on TENSOR 27 FT-IR spectrophotometer supplied by Bruker (Switzerland). ^{1}H and ^{13}C NMR (300 and 75 MHz) spectra were obtained in $CDCl_3$ at room temperature, with TMS as an internal standard using a Bruker Biospin-AMX 300-MHz FT NMR spectrometer; δ in ppm, coupling constant J in Hz. The ^{1}H and ^{13}C NMR correlation experiments, i.e. COSY-45°, HOESY, ROESY, HSQC, HMQC, HMBC, TOCSY, HOHAHA, COLOC, NOE, and NOESY spectra were recorded on the same instrument. The isolated carbon–hydrogen bond(s) was identified by hetero-COSY (HMQC). The linkage of the structural fragment with quaternary carbons was established through $^{\delta 2}J$CH, $^{\delta 3}J$CH-HMBC experiments. One bond coupling was avoided by employing BIRD, a modified long-range COLOC experiment. The carbon to carbon connectivity was established by recording INADEQUATE spectra. The relative stereochemistry at the various stereogenic centers was established with the help of NOE difference measurements and recording 2D NOE (NOESY) spectra. Mass measurements were made on double-focusing Finnegan MAT 112 spectrometer (Bremen, Germany); and recorded in *m/z* (relative intensity, %). HR-EI-MS measurements were obtained on JEOL HX 110 spectrometer (JEOL, Tokyo, Japan).

Biological assays were performed on adult (male and female) Swiss Webster NMRI mice strain, which are very sensitive and have been used in a variety of biological studies. The mice were taken from the local market, kept in an animal house under standard conditions, and fed a control diet obtained from Lab-Blox (Allied Mills, Inc., Chicago, IL, USA). The standard drugs 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and 7,12-dimethylbenz[*a*]anthracene (DMBA) were obtained from Sigma Chemical Co. (St Louis, MO, USA), and used as a control and initiator, respectively.

3.2 Plant material

The plant of *E. cauducifolia* L. was collected from the campus of Karachi University (Karachi, Pakistan), and was identified by taxonomist Prof. Dr S.I. Ali, Head Department of Botany, Karachi University. A voucher specimen (No. 1271) has been deposited in the herbarium of the department for verification of the specimen and latex was collected directly in a flask containing 100% methanol by cutting plant leaves and stem and transported to Dera Ismail Khan.

3.3 Extraction and isolation

The latex (5000 g) trickled in 95% methanol (10,000 ml) was allowed to soak (with occasional shaking) for 7 days at room temperature and then filtered. The filtrate was evaporated to dryness under vacuum, re-extracted with acetone (1000 ml, thrice), and the solvent was evaporated at a reduced pressure to obtain a semi-solid black mass of approximately 230 g. The material was taken up in aqueous methanol (10%) and successively extracted with diethyl ether (37 g) and ethyl acetate (36 g).

The dried substances from both the extracts were separately partitioned with 500 ml petroleum ether/MeOH/H_2O (15/10/0.5) and the irritant hydrophilic fractions were named as F_1 and F_2 (17.5 and 13.5 g), respectively. F_1 and F_2 were subjected to various Craig's distributions and monitoring the biological activity at each stage to obtain the most active fractions $F_{1.1}$ and $F_{2.1}$ (2.2 and 1.5 g), respectively.

3.4 Identification of 17-hydroxyingenol

After hydrolyzing F_1 and F_2 fractions with 0.5 M methanolic KOH for 20 min at 25°C, the products were subjected to separation on HPLC (Perkin-Elmer) with an RP-18 column, an LC pump and LC 290 UV/Vis detectors, using acetonitrile/water gradient

(flow rate: 1 ml/min; solvent A: 100% H_2O; solvent B: 88% aqueous acetonitrile; 5 min A, linear gradient to B in 25 min, 5 min B, linear gradient back to A in 2 min) and the basic skeleton of the diterpenoid pentanol was identified as 17-hydroxyingenol already isolated from the same source [11] by comparing ^1H and ^{13}C NMR and mass spectrometric data.

3.5 Isolation of tumor promoting compounds (1–10)

Fraction F_1 was separated on an open silica gel column (12 × 150 cm) after gradient elution with *n*-C_6H_{14}/EtOAc (12 ml/min). After collecting 500 ml of each fraction with an increasing level of solvent polarity (19:1, 9:1, 8:2, 7:3, 1:1, 1:3, 0:1), no activity appeared in the eluates and these were hence discarded. The extraction was continued with successive increase in the polarity within the ethyl acetate–methanol system (19:1, 9:1, 8:2, 7:3, 1:1, 1:3, 0:1) until pure methanol, and each fraction was examined for biological activity. The fractions having the same level of activity were combined to yield fraction $F_{1.1}$ (1.2 g), which was further fractionated on a silica gel column (5 × 80 cm) using *n*-C_6H_{14}/EtOAc as the eluent to obtain four fractions (eluent volume: 800 ml/fraction). The most active fraction $F_{1.1.4}$ (1.0 g) showed a single spot on analytical TLC [silica gel PF_{254}, C_6H_{14}/-Et_2O/EtOAc (4/3/3)]. The material being unseparable was loaded on HPLC with an RP-18 column (2.5 × 250 mm) using a (H_2O/MeCN) gradient system (2 ml/min). Out of the eight fractions including $F_{1.1.4.1\text{-}8}$ [5000 ml (1/19); 7000 ml (1/9); 5500 ml (2/8); 7000 ml (3/7); 8500 ml (1/1); 9500 ml (3/1); 5000 ml (19/1); and 5000 ml (100% water)], the biologically active fractions ($F_{1.1.4.4}$–$F_{1.1.4.6}$) were combined together (700 mg) and reloaded on an HPLC (1.5 × 150 mm) column with a gradient system (flow rate: 1 ml/min; 100% H_2O, 7 min; 88% MeCN with H_2O, 25 min) to yield seven pure compounds **1** (80.3 mg), **2** (27.3 mg), **3** (121.3 mg), **4** (19.4 mg), **5** (53 mg), **6** (29 mg), and **7** (45 mg) with respective retention times of 12, 13, 13.5, 14, 15.2, 15.7, and 16.1 min.

Fraction F_2 was likewise subjected to a silica gel column (12 × 150 cm), eluted gradiently with *n*-C_6H_{14}/EtOAc (12 ml/min), and 12 fractions ($F_{2.1}$–$F_{2..12}$) were collected. The most active fraction $F_{2.7}$ (500 mg) was further loaded on HPLC (1.5 × 150 mm) using gradient 62% aqueous acetonitrile for 60 min followed by 88% aqueous acetonitrile to obtain pure compounds **8** (71 mg), **9** (32 mg), and **10** (41 mg) after respective retention times of 13, 13.5, and 14 min.

*3.5.1 3-O-Angeloyl-17-O-palmatoylingenol (**1**)*

Colorless oil, $[\alpha]_D^{25} + 39.5$ ($c = 0.19$, MeOH). UV (MeOH) λ_{max} (nm, log ε): 208 (4.19), 301.5 (3.45). IR (dry) ν_{max} (cm^{-1}): 3678, 3567, 3517, 1716, 1627, 1608. ^1H (300 MHz, $CDCl_3$) and ^{13}C (75 MHz, $CDCl_3$) NMR spectral data are listed in Table 2. EI-MS *m/z* (rel. int.): 684 (M^+, 3), 666 (6), 669 (9), 648 (8), 601 (47), 584 (19), 445 (23), 428 (15), 416, 376, 310 (100), 294 (30), 239 (45) 237 (23), 197 (47), 83 (30). HR-EI-MS: *m/z* 684.4602 $[M]^+$ (calcd for $C_{41}H_{64}O_8$, 684.4601).

*3.5.2 3-O-Palmatoyl-5-O-angeloylingenol (**2**)*

Colorless oil, $[\alpha]_D^{25} + 39.5$ ($c = 0.19$, $CHCl_3$); UV (MeOH) λ_{max} (nm, log ε): 207 (4.19), 302.5 (3.45); IR (dry) ν_{max} (cm^{-1}): 3668, 3415, 3423, 1717, 1637, 1610; ^1H (300 MHz, $CDCl_3$), ^{13}C (75 MHz, $CDCl_3$) spectral data are listed in Table 2. EI-MS *m/z* (rel. int.): 684 (M^+, 3), 666 (6.7), 669 (11), 648 (8), 648 (20), 601 (47), 584 (21), 445 (29), 428 (17), 416, 376, 310 (100), 294 (30), 239 (45) 237 (23), 197 (47), 83 (37). HR-EI-MS: *m/z*

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684.4598 [M]$^+$ (calcd for $C_{41}H_{64}O_8$, 684.4601).

3.5.3 5-O-Angeloyl-17-O-palmatoylingenol (3)

Colorless oil, $[\alpha]_D^{25} + 39.5$ ($c = 0.19$, MeOH); UV (MeOH) λ_{max} (nm, $\log \varepsilon$): 208 (4.19), 301.5 (3.45); IR (dry) ν_{max} (cm^{-1}): 3517, 3535, 3427, 1716, 1629, 1628. ^{1}H (300 MHz, CDCl$_3$), ^{13}C (75 MHz, CDCl$_3$) spectral data are listed in Table 2. EI-MS *m/z* (rel. int.): 684 (M$^+$, 4), 666 (6), 669 (11), 648 (7), 648 (23), 601 (43), 584 (39), 445 (27), 428 (15), 416, 376, 310 (100), 294 (30), 239 (45) 237 (23), 197 (47), 83 (30). HR-EI-MS: *m/z* 684.4611 [M]$^+$ (calcd for $C_{41}H_{64}O_8$, 684.4601).

3.5.4 3-O-Angeloyl-5-O-palmatoylingenol (4)

Colorless oil, $[\alpha]_D^{25} + 49.7$ ($c = 0.19$, MeOH). UV (MeOH) λ_{max} (nm, $\log \varepsilon$): 210 (4.19), 303.5 (3.45). IR (dry) ν_{max} (cm^{-1}): 3658, 3567, 3517, 1716, 1627, 1608. ^{1}H (300 MHz, CDCl$_3$), ^{13}C (75 MHz, CDCl$_3$) spectral data are listed in Table 3. EI-MS *m/z* (rel. int.): 684 (M$^+$, 3), 666 (6), 669 (9), 648 (9), 648 (22), 601 (43), 584 (19), 445 (23), 428 (17), 416, 376, 310 (100), 294 (37), 239(45) 237 (23), 197 (47), 83 (30). HR-EI-MS: *m/z* 684.4612 [M]$^+$ (calcd for $C_{41}H_{64}O_8$, 684.4601).

3.5.5 17-O-(2Z,4E,6Z)-Tetradeca-(2,4,6)-trienoyl-20-O-palmatoylingenol (5)

Colorless oil, $[\alpha]_D^{25} + 79.3$ ($c = 0.19$, MeOH). UV (MeOH) λ_{max} (nm, $\log \varepsilon$): 318 (4.19), 322.5 (3.45). IR (dry) ν_{max} (cm^{-1}): 3515, 3423, 1716, 1629, 1638. ^{1}H (300 MHz, CDCl$_3$), ^{13}C (75 MHz, CDCl$_3$) spectral data are listed in Table 3. EI-MS *m/z* (rel. int.): 804 (M$^+$, 3), 786 (12), 795 (7), 599 (20), 582 (23), 565 (47), 564 (23), 548 (19), 530 (23), 428 (15), 416, (29), 376 (54), 325 (22), 310 (100), 308 (23), 294 (30), 239 (45) 237 (23), 197 (47), 83 (30). HR-EI-MS: *m/z* 804.5443 [M]$^+$ (calcd for $C_{50}H_{76}O_8$, 804.5440).

3.5.6 5-O-Angeloyl-17-O-benzoylingenol (6)

Colorless oil, $[\alpha]_D^{25} + 79.8$ ($c = 0.19$, MeOH). UV (MeOH) λ_{max} (nm, $\log \varepsilon$): 367 (3.4), 348 (3.2), 315 (2.5), 208 (4.19), 301.5 (3.45). IR (dry) ν_{max} (cm^{-1}): 3678, 3515, 1717, 1637, 1648. ^{1}H (300 MHz, CDCl$_3$), ^{13}C (75 MHz, CDCl$_3$) NMR spectral data are listed in Table 3. EI-MS *m/z* (rel. int.): 550 (M$^+$, 3), 532 (6), 514 (9), 467 (8), 450 (20), 445 (56), 428 (47), 416, 376, 310 (100), 294 (30), 237 (23), 197 (47), 105 (67), 83 (30). HR-EI-MS: *m/z* 550.2564 [M]$^+$ (calcd for $C_{32}H_{38}O_8$, 550.2567).

3.5.7 5-O-Angeloyl-17,20-diacetoxyingenol (7)

Colorless oil, $[\alpha]_D^{25} + 59.4$ ($c = 0.19$, MeOH). UV (MeOH) λ_{max} (nm, $\log \varepsilon$): 208 (4.19), 301.5 (3.45). IR (dry) ν_{max} (cm^{-1}): 3517, 3515, 3423, 1719, 1637, 1648. ^{1}H (300 MHz, CDCl$_3$), ^{13}C (75 MHz, CDCl$_3$) NMR spectral data are listed in Table 4. EI-MS *m/z* (rel. int.): 530 (M$^+$, 3), 512 (6), 494 (9), 470 (7), 447 (14), 410 (23), 430 (8), 376 (23), 370 (34), 310 (100), 294 (30), 197 (47), 83 (30), 43 (34). HR-EI-MS: *m/z* 530.2512 [M]$^+$ (calcd for $C_{29}H_{38}O_9$, 530.2516).

3.5.8 3-O-Angeloyl-17-O-benzoyl-20-acetoxyingenol (8)

Colorless oil, $[\alpha]_D^{25} + 69.3$ ($c = 0.19$, MeOH). UV (MeOH) λ_{max} (nm, $\log \varepsilon$): 208 (4.19), 301.5 (3.45); IR (dry) ν_{max} (cm^{-1}): 3517, 3515, 1719, 1647, 1668. ^{1}H (300 MHz, CDCl$_3$), ^{13}C (75 MHz, CDCl$_3$) NMR spectral data are listed in Table 4. EI-MS *m/z* (rel. int.): 534 (M$^+$, 3), 498 (6),

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491 (12), 480 (9), 474 (11), 451 (8), 434 (20), 374 (17), 584 (19), 445 (23), 428 (15), 416 (23), 412 (34), 376, 310 (100), 294 (30), 237 (23), 197 (47), 83 (30). HR-EI-MS: *m/z* 592.2669 $[M]^+$ (calcd for $C_{34}H_{40}O_9$, 592.2672).

*3.5.9 3-Acetoxy-5-*O*-angeloyl-17-*O*-benzoylingenol (**9**)*

Colorless oil, $[\alpha]_D^{25} + 49.5$ ($c = 0.19$, MeOH). UV (MeOH) λ_{max} (nm, $\log \varepsilon$): 208 (4.19), 301.5 (3.45). IR (dry) ν_{max} (cm^{-1}): 3678, 3517, 3515, 1716, 1627, 1608. ^1H (300 MHz, $CDCl_3$), ^{13}C (75 MHz, $CDCl_3$) NMR spectral data are listed in Table 4. EI-MS *m/z* (rel. int.): 592 (M^+, 3), 574 (4), 556 (6), 509 (8), 492 (20), 487 (47), 445 (23), 428 (15), 416, 376, 310 (100), 294 (30), 237 (23), 197 (47), 105 (34), 83 (30). HR-EI-MS: *m/z* 592.5217 $[M]^+$ (calcd for $C_{34}H_{40}O_9$, 592.5222).

*3.5.10 5-O-Angeloyl-3,17,20-triacetylingenol (**10**)*

Colorless oil, $[\alpha]_D^{25} + 19.7$ ($c = 0.19$, MeOH). UV (MeOH) λ_{max} (nm, $\log \varepsilon$): 208 (4.19), 301.5 (3.45). IR(dry) ν_{max} (cm^{-1}): 3678, 1716, 1627, 1608. ^1H (300 MHz, $CDCl_3$) and ^{13}C (75 MHz, $CDCl_3$) NMR spectral data are listed in Table 4. EI-MS *m/z* (rel. int.): 572 (M^+, 3), 554 (6), 536 (9), 512 (47), 489 (39), 445 (23), 428 (15), 416, 376, 310 (100), 294 (30), 237 (23), 197 (47), 83 (30). HR-EI-MS: *m/z* 572.25934 $[M]^+$ (calcd for $C_{31}H_{40}O_{10}$, 572.2622).

3.6 Biological activities

3.6.1 Determination of the ID_{50} value

The assay was performed by following the standard protocol developed by Hecker [11]. For this purpose, 30 mice of both sexes were taken and divided into five groups, each consisting of six animals. The experiment was performed by injecting 5 μl solution of extracts/pure compounds with the help of 'Drummond Microcap' in one ear of the mouse and the other ear as a control. The dose was expressed in terms of μg/ml of the original substances. The acetone was used as a positive control and TPA as a negative control.

3.6.2 Determination of tumor promoting and solitary activities

The assay was performed by following the standard protocol [11]. Twenty-eight mice (male and female) were taken with backs shaved with a razor. First of all, 100 nmol of DMBA was topically applied on the back of each mouse for initiation. Then, a single dose of the compounds was applied twice/week for a period of 24–48 weeks. The promoting activity was expressed as average tumor rate, which was calculated as the total number of tumor-bearing mice/number of survivors in percent and an average tumor yield obtained as the total number of tumors/numbers of survivors as a function of weeks of treatment. A reference TPA was used.

References

[1] S. Sultana, M. Ali, S.H. Ansari, and P. Bagri, *J. Asian Nat. Prod. Res.* **10**, 1123 (2008).
[2] Y.K. Qiu, T.G. Kang, D.Q. Dou, L. Liang, and F. Dong, *J. Asian Nat. Prod. Res.* **10**, 1109 (2008).
[3] S.X. Feng, L.D. Lin, H.H. Xu, and X.Y. Wei, *J. Asian Nat. Prod. Res.* **10**, 1155 (2008).
[4] J.Y. Ma, W.W. Jiang, Z.T. Zhou, J.M. Li, and H.Y. Wan, *J. Asian Nat. Prod. Res.* **10**, 1147 (2008).
[5] N. Nazir, S. Koul, M.A. Qurishi, S.C. Tanej, B. Purnima, and G.N. Qazi, *J. Asian Nat. Prod. Res.* **10**, 1137 (2008).
[6] J. Kang, R.Y. Chen, and D.Q. Yu, *J. Asian Nat. Prod. Res.* **7**, 729 (2005).
[7] W. Li, X. Li, J. Yang, D.L. Meng, and N. Li, *J. Asian Nat. Prod. Res.* **10**, 260 (2008).
[8] I.B. Baloch, M.K. Baloch, and Q.N. Saqib, *Planta Med.* **72**, 830 (2006).

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[9] I.B. Baloch, M.K. Baloch, and Q.N. Saqib, *Eur. J. Med. Chem.* **43**, 274 (2008).
[10] I.B. Baloch, M.K. Baloch, and A.K. Baloch, *Eur. J. Med. Chem.* **44**, 3188 (2009).
[11] I.B. Baloch, M.K. Baloch, and Q.N. Saqib, *Helv. Chim. Acta* **88**, 3541 (2005).
[12] I.B. Baloch, M.K. Baloch, Q.N. Saqib, and A. Mansoor, *Int. Chem. Pharm. Med. J.* **1**, 107 (2004).
[13] I.B. Baloch, M.K. Baloch, and A.K. Baloch, *Planta Med.* **75**, 1 (2009).
[14] S. Ahmad, O. Seligman, H. Wagner, and G. Hussain, *Phytochemistry* **16**, 1844 (1977).
[15] A.S. Ng, *Phytochemistry* **29**, 662 (1990).
[16] J. Marco, F. Juan, S. Cervera, J. Checa, E. Polmares, and B.M. Fraga, *Phytochemistry* **52**, 479 (1999).
[17] J. Jakupovic, T. Morgenster, J.A. Marco, and W. Berendsohn, *Phytochemistry* **47**, 1611 (1998).
[18] J.D. Connolly, C.O. Fakunle, and D.S. Pycroft, *Tetrahedron Lett.* **25**, 3773 (1984).
[19] Z.Q. Lu, M. Yang, J.Q. Zhang, G.T. Chen, H.L. Huang, S.H. Guan, C. Ma, X. Liu, and D.A. Guo, *Phytochemistry* **69**, 812 (2008).

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