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Crotalic and emarginellic acids: Two triterpenes from *Crotalaria emarginella* and anti-inflammatory and anti-hepatotoxic activity of crotalic acid

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Abstract

The aerial parts of *Crotalaria emarginella* Vatke (Leguminosae) has afforded two triterpenes, characterized as 3α-hydroxy-arbor-12-ene-28-carboxylic acid, designated as crotalic acid (**1**), and 2β,3β,21-trihydroxy-arbor-12-ene-29-carboxylic acid, designated as emarginellic acid (**2**). The structures of the isolated products were elucidated on the basis of spectral and chemical studies. On screening the biological activity, the crotalic acid (**1**) exhibited a significant anti-inflammatory activity (dose: 10 mg/kg), which showed 53% inhibitory effect. Whereas, the standard oxyphenyl butazone (100 mg/kg) exhibited 69% inhibition with respect to carrageenan (0.05 ml, 1%) used to cause inflammation in rat paw method. In addition, it also showed anti-hepatotoxic activity by 13–30% with respect to standard silybon-70 (35–57%) against CCl_4 induced toxicity in Wistar rats.

Keywords: *Crotalaria emarginella*; Crotalic acid; Arborene triterpene; Anti-inflammatory activity; Anti-hepatotoxic activity

1. Introduction

The plant *Crotakria emarginella* Vatke (Leguminosae) is distributed in the Tropical East Africa, Yemen and Saudi Arabia including Najad, Asir and eastern region (Chaudhary, 2001). The plant is woody-based bushy, leafy, much branched, perennial herb, and is used in traditional medicine in scabies, impetigo, fever, and as a colic remedy. In India the root is used as a haemoptysis remedy and skin diseases (Mossa et al., 1987). The plants of the genus *Crotalaria* are also used for cooling and purifying blood in impetigo and psoriasis (Dymock et al., 1890). A literature survey indicated that no phytochemical work has been done so far on the plant. However, pyrrolizidine alkaloids (Mahran et al., 1979; Arseculeratne et al., 1981; Bhakuni and Chaturvedi, 1984), flavonoids (Yoo et al., 2004), chalcones (Narender et al., 2005) lectins (Pando et al., 2004), and polysaccharides (Gupta and BeMiller, 1990) have been reported from other species of *Crotalaria* genus. We have isolated two new arborane type triterpenes from the acetone insoluble part, of the alcoholic extract of the aerial parts of *Crotalaria emarginella*. Other fractions such as petroleum ether, and acetone soluble parts were not investigated. The isolation of arborane type triterpenes from this plant is new class found in the genus. However, the arborane type triterpenes have also been found in the family Gramineae (Ohmoto et al., 1970; Nishimoto et al., 1968) and Rubiaceae (Hui and Lam, 1965). The isolated triterpenes were characterized as 3α-hydroxy-arbor-12-ene-28-carboxylic acid, designated as crotalic acid (**1**), and 2β,3β,21-trihydroxy-arbor-12-ene-29-carboxylic acid, named as emarginellic acid (**2**). The crotalic acid was found a major component (13.0 g) of the plant, which exhibited a significant anti-inflammatory activity, showing 53%

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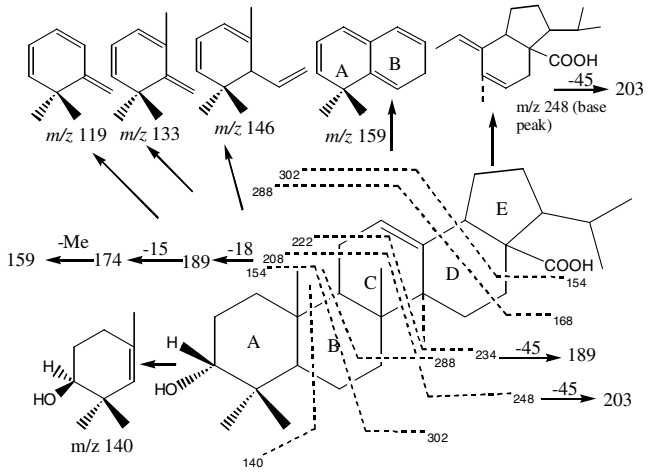
inhibitory effect in comparison to standard oxyphenyl butazone (69%), with respect to carrageenan in rat paw method. Moreover, it also showed anti-hepatotoxic activity against CCl_4 induced toxicity in Wistar rats, by about 13–30% with respect to standard drug silybon-70 (35–57%), a commercial product of Micro Labs Limited, prepared from the crude extract of *Silybum marianum*. However, the anti-diabetic activity was not observed.

2. Results and discussion

2.1. Phytochemical part

The compound **1**, named as crotalic acid obtained as colorless needles, had a molecular formula $C_{30}H_{48}O_3$ as established on the basis of HR-MS (456.3603), elemental analysis, ^{13}C NMR and DEPT spectra. It gave a positive Lieberamnn–Buchard test indicating it to be a triterpene. Its IR spectrum indicated the presence of hydroxyl group (3500, 1050 cm^{-1}), carboxylic group (3310–3090, 1758, 1437, 1320 cm^{-1}) and double bond (1603, 780 cm^{-1}). The ^{13}C NMR and DEPT spectra (Pegg et al., 1982) showed 30 carbon atoms for the molecule consisting of seven methyls, nine methylenes, seven methines, five quaternary, one olefinic and one carboxyl carbon atom (in total $C_{30}H_{46}$). The sequential assignments of proton and carbon atoms were made with the help of ^1H–^1H-COSY and HMQC experiments (Nakanishi, 1990) starting with the easily distinguishable olefinic and carbinolic protons. The carbinolic proton appeared at δ_H 3.55 (*dd*, $J = 5.0$, 5.5 Hz, δc 702) attributable to position-3 biogenetically and on the basis of mass fragmentation pattern (Scheme 1). The small coupling constants of the carbinolic proton indicated the β-orientation of proton and α-orientation of hydroxyl group (Silverstein et al., 1981). The compound **1** gave a monoacetate on acetylation, which showed absorption bands in IR at V_{max} 1750 (C=O) and 1245 (C–O, ester) due to acetyl moiety, as well as exhibited a three proton singlet in ^1H NMR at δ_H 2.06 (δ_C 20.9) due to methyl group, and a sharp peak at 171.1 due to carbonyl carbon atom in ^{13}C NMR spectrum indicating clearly only one hydroxyl group in the molecule. It showed correlations in ^1H–^1H-COSY spectrum with methylene protons at δ_H 1.64 and 1.91 (δ_C 23.2) assignable at position-2, which in turn, correlated with another methylene protons at δ_C 1.99 and 2.11 (δ_C 24.1) attributable to position-1. These positions were further substantiated with long-range coupling in HMBC spectrum, wherein the H-3 correlated with C-2, C-1 and C-4; H_2-2 with C-1 and C-3. The methyl groups appeared in ^1H–^1H NMR spectrum at δH 0.88 (δ 28.1) and 0.96 (15.5) exhibited long-range coupling with C-4 (δ_C 36.8), C-3 and C-5 (δ_H 0.84, δ_C 55.5) substantiating the placement of these methyl groups at position 23 and 24, respectively. The H-5 displayed correlation in ^1H–^1H-COSY spectrum with H_2-6, and in turn H_2-6 with H_2-7 indicating their consecutive assignments. The long-range coupling in HMBC spectrum exhibited correlation of H-5 with C-6 (δ_C 30.9), C-3 and C-9 (52.5); H-6a with C-7 (δ_C 32.9) and C-10 (475); H-6b with C-5 and C-6 (39.5); whereas the methyl group at δ_H 0.89 (δ_C 15.8) placeable at position-26 showed long-range correlation with C-8, C-7, C-9, and C-14 (42.1).

In addition, the proton appeared at δ_H 2.19 (*dd*, $J = 7.5$, 5.5 Hz; δ_C 52.5) attributable to position-9 showed long-range coupling with C-8, C-10, Me-26, C-11 (δ_C 18.1), C-12 (δ_C 122.6), C-13 (139.1), Me-25 (δ_C 212) and C-14 substantiating strongly the proposed structure of rings-A, B and C. Moreover, the H-9 exhibited correlations in ^1H–^1H-COSY spectrum with methylene protons assignable at position-11, whereas H_2-11 in turn, coupled with an olefinic proton H-12 (δ_H 5.26). The H-12, consequently displayed long-range correlations in HMBC spectrum with C-11, C-14, C-18 (δ_C 47.1) and C-19 (23.8) confirming further the structure of rings C and D. The methyl group appeared at δ_H 1.06 (δ_C 23.9) showed long-range correlation with C-13, and hence could be attached with C-14. The methylene group appeared at δ_H 1.09, 1.68 (δ_C 36.9) showed correlations in COSY spectrum with another methylene group at δ_H 1.02, 1.68 (δ_C 37.8) indicating their consecutive position, which could be assigned at position 15 and 16, respectively. Moreover, H_2-15 displayed long-range coupling with C-14; H_2-16 with C-15, C-14 and C-17 (δ_C 38.2); whereas H-18 (δ_H 15.3, *dd*, $J = 8.5$, 5.5 Hz; δ_C 47.1) correlated with C-14, C-16, C-17, and C-21 (δ_C 39.1) substantiating the above positions of rings D and E. In the same way the consecutive positions of CH-18, CH_2-19, CH_2-20, and CH-21 of ring E could be assigned with the help of ^1H–^1H-COSY spectrum (Table 1), and further supported with the help of long-range couplings in HMBC spectrum (Table 1, Fig. 1), wherein, H-21 showed correlations with C-17, C-18, C-20 and C-28 (δ_C 183.1, COOH) indicating the placement of carboxylic group at position-28. In addition, the two methyl groups appeared at δ_H 0.86 (*d*, $J = 6.0$ Hz; δ_C 16.9) and 0.87 (*d*, $J = 6.0$ Hz; δ_C 17.1) attributable to positions 29 and 30, respectively, showed correlations in ^1H–^1H COSY spectrum with a



Scheme 1. Crotalic acid (**1**): $C_{30}H_{48}O_3$ (M^+ 456).

Table 1
1D- and 2D NMR data of crotalic acid (**1**)

Position	^{1}H NMR[a]	^{13}C NMR/HMQC	DEPT[b]	COSY	HMBC[c]	
					$^2J_{CH}$	$^3J_{CH}$
1a	1.99 *ddd* (14.0, 9.5, 4.5)	24.1 *t*	CH_2	H-1b, H_2-2	C-2, C-10	C-25
1b	2.11 *ddd* (14.0, 5.5, 4,5)	–	–	H-1a, H_2-2	C-2,	C-25
2a	1.64 *ddd* (14.5, 8.5, 5.5)	23.2 *t*	CH_2	H-2b,H_2-1,H-3	C-3	C-10
2b	1.91 *ddd* (14.5, 9.0, 5.0)	–	–	H-2b ,H-3	C-3, C-1	C-4
3β	3.55 *dd* (5.0, 5.5)	70.2 *d*	CH	H_2-2, H-1a	C-2, C4	
(Acetate)[d]	4.51 *dd* (5.0, 5.5)	80.1 *d*	CH	–	–	C-1′
4	–	36.8 *s*	C	–	–	–
5	0.84 *dd* (8.5, 4.5)	55.5 *d*	CH	H-6a	C-6	C-9, C-3
6a	1.35 *ddd* (15.0, 8.5, 5.5)	30.9 *t*	CH_2	H-6b, H_2-7, H-5	C-7	C-10
6b	1.51 *ddd* (15.0, 10.5, 4.5)	–	–	H-6a, H-5	C-5	C-8
7a	1.64 *m*	32.9 *t*	CH_2	H-7a, H2-6	C-6, C-8	–
7b	1.17 *m*	–	–	H-7b, H-6b	C-8	–
8	–	39.5 *s*	C	–	–	–
9	2.19 *dd* (7.5, 5.5)	52.5 *d*	CH	H_2-11	C-8, C-10, C-11	C-12, C-25, C-26
10	–	47.5 *s*	C	–	–	–
11a	1.34 *ddd* (13.5, 7.5, 6.0)	18.1 *t*	CH_2	H-11b,H-9,H-12	C-9, C-12	C-13
11b	1.56 *ddd* (13.5, 5.5, 4.5)	–	–	H-11a, H-9, H-12	C-12	C-8
12	5.26 *t* (7.0)	122.6 *d*	CH	H_2-11	C-11, C-13	C-18, C-19, C-9, C-14
13	–	139.1 *s*	C	–	–	–
14	–	42.1 *s*	C	–	–	–
15a	1.09 *m*	36.9 *t*	CH_2	H-15a, H_2-16	C-14, C-16	–
15b	1.68 *m*	–	–	H-15b, H_2-16	C-14, C-16	–
16a	1.02 *m*	37.8 *t*	CH_2	H-16b, H_2-15	C15, C-17	C-18
16b	168 *m*	–	–	H-16a, H_2-15	C-17	–
17	–	38.2 *s*	C	–	–	–
18	153 *dd* (8.5, 5.5)	47.1 *d*	CH	H_2-19	C-17	C-14
19a	1.67 *m*	23.8 *t*	CH_2	H-19b, H18, H_2-20	C-18, C-20	–
19b	1.92 *m*	–	–	H-19a, H_2-20	C-20	–
20a	1.74 *m*	28.0 *t*	CH_2	H-20b, H-21, H_2-19	C-21	–
20b	1.89 *m*	–	–	H-20a, H-21	C-19	C-17
21	0.95 *m*	39.1 *d*	CH	H-20a, H-22	C-20, C-22	C-18
22	1.33 septet ($W\frac{1}{2}$ 25.0)	28.5 *d*	CH	H-21, Me-29, Me-30	C-21, Me-9, Me-30	–
23	0.88 *s*	28.1 *q*	CH_3	–	C-4	C-3
24	0.96 *s*	15.5 *q*	CH_3	–	C-4	C-3, C-5
25	0.96 *s*	21.2 *q*	CH_3	–	C-10	–
26	0.89 *s*	15.8 *q*	CH_3	–	C-8	C-7, C-10
27	1.06 *s*	23.9 *q*	CH_3	–	C-14	C-13
28	–	183.1 *s*	C	–	–	–
29	0.87 *d* (6.0)	16.9 *q*	CH_3	H-22	C-22	C-21, Me-30
30	0.87 *d* (6.0)	17.1 *q*	CH_3	H-22	C-22	C-21, Me-29
1′(Acetate)[d]	–	171.1 *s*	C	–	–	–
2′(Acetate)[d]	2.06 *s*	20.9 *q*	CH_3	–	–	C-1′

[a] Assignments were based on ^{1}H–^{1}H COSY, and HMQC experiments; coupling constants in Hertz are given in parentheses; *s*: singlet, *d*: doublet, *m*: multiplet.
[b] DEPT chemical shifts are presented at $\theta = 3\pi/4$ when methylene groups reaches negative maximum. C-multiplicities were established by DEPT experiment; $s = C$, $d = CH$, $t = CH_2$, $q = CH_3$.
[c] The correlations in HMBC have been shown from protons to carbons.
[d] Only pertinent data in case of acetate are given, there is no need to give all data.

methine proton at δ_H 1.33 (septet, W1/2 25.0 Hz; δ_C 28.5) assignable at position-22, which indicated the presence of an isopropyl side chain. The H-22 and H-21 correlated with each other in ^{1}H–^{1}H-COSY spectrum indicating the linkage of isopropyl side chain at position-21, which was further substantiated with the help of long-range coupling of Me-29 and Me-30 with C-22 and C-21, respectively.

The structure of the compound **1** was also confirmed by fragmentation pattern in its mass spectrum, which displayed prominent peaks at *m/z* 456 (M^+), 248 (base peak), 222, 203, 208, 174, 189 and 159 due to molecular ion and rupture of ring C with other minor fragmentations; 302, 288, 168 and 154 due to rupture of ring D, and 302, 146, 140, 133, and 119 due to rupture of ring B supporting the proposed structure of the compound. Other peaks in the spectrum were also in good agreement with the structure (Scheme 1).

Thus, based on above chemical and spectral evidences, the structure of the compound was elucidated as 3α-hydroxy-arbor-12-ene-28-carboxylic acid, and has been designated as crotalic acid (**1**).

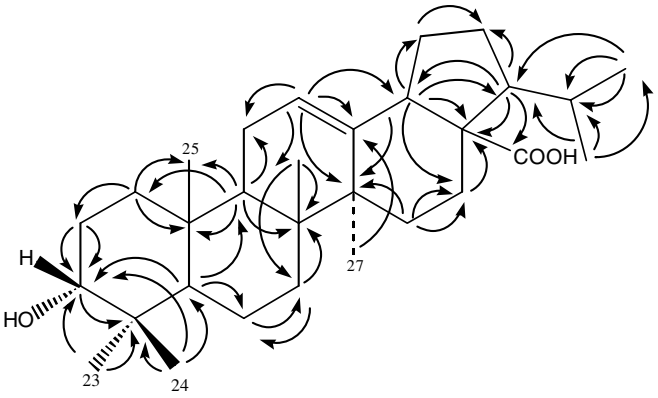


Fig. 1. Significant heteronuclear multiple bond correlations (HMBC) for crotalic acid (**1**). Arrows point from proton to carbon.

The compound **2**, named as emarginellic acid obtained as colorless needles, had a molecular formula $C_{30}H_{18}O_5$ as established on the basis of HR-MS (488.3502), elemental analysis, ^{13}C NMR and DEPT spectra. It gave a positive Lieberamnn–Buchard test indicating it to be a triterpene. Its IR spectrum indicated the presence of hydroxyl group (3530, 1060 cm^{-1}), carboxylic group (3315–3090, 1761, 1439, 1318 cm^{-1}) and double bond (1605, 785 cm^{-1}). The ^{13}C NMR and DEPT spectra (Pegg et al., 1982) showed 30 carbon atoms for the molecule consisting of seven methyls, eight methylenes, seven methines, six quaternary, one olefinic and one carboxyl carbon atom (in total $C_{30}H_{48}$). The sequential assignments of proton and carbon atoms were made with the help of ^1H–^1H-COSY and HMQC experiments (Nakanishi, 1990) starting with the easily distinguishable olefinic and two carbinolic protons. One carbinolic proton appeared at δ_H 3.53 (*d*, $J = 10.5$ Hz, δ_C 71.2) was attributed to position-3 biogenetically, and on the basis of mass spectrum fragmentation pattern (Table 2). Another carbinolic proton appeared at δ_H 3.65 (*ddd*, $J = 10.5$, 5.0, 5.5 Hz, δ_C 40.1), which exhibited correlation in ^1H–^1H COSY spectrum with the first carbinolic proton at position-3 indicating its location at position-2. It was further substantiated with the help of long-range correlations in HMBC spectrum, wherein the H-3 displayed correlations with C-2, C-4 and C-5; whereas, H-2 coupled with C-3, C-l and C-10. The high values of coupling constants of H-2 ($J = 10.5$ Hz, due to coupling with H-3) and H-3 ($J = 10.5$ Hz, due to coupling with H-2) indicated their α-orientations, and hence β-orientations of hydroxyl groups (Silverstein et al., 1981). The compound **2** gave a diacetate on acetylation, which showed absorption bands in IR at ν_{max} 1748, 1743 (C=O) and 1248 (C–O, ester) due to acetyl groups, as well as exhibited two singlets of three protons each in ^1H –^1H NMR spectrum at δ_H 1.85 (δ_C 20.9), and 1.93 (21.2) due to two methyl groups, and sharp peaks at δ_C 170.6 and 170.8 due to two carbonyl carbon atoms in ^{13}C NMR spectrum, indicating unambiguously the presence of two secondary hydroxyl groups in the molecule. The ^{13}C NMR spectrum displayed a quaternary carbon atom at δ_C 73.0 indicating a carbon containing an angular/tertiary hydroxyl group that could not be acetylated on acetylation with Ac_2O–pyridine. It was assigned at position-21 based on long-range correlations in HMBC spectrum, wherein H-22, Me-28, Me-30 and H-18 showed correlation with C-21 substantiating the location of this hydroxyl group at position-21. The ^1H NMR spectrum displayed a three proton doublet ($J = 6.5$ Hz) at δ_H 0.82 (δ_C 16.3) due to a methyl group attributable to position-30, which showed correlation with H-22 (*d*, $J = 65$ Hz δ_C 41.0) in COSY spectrum substantiating its location at position-30. It also showed long-range correlations in HMBC spectrum with C-22, C-21, and a carbonyl group, which allowed placing the carboxylic acid at position-29. A methyl group appeared at δH 1.06 (δ_C 27.4) in ^1H NMR spectrum showed long-range couplings with C-17, C-18 and C-21, which thus could be placed at position-28. Other data of the compound **2** were found similar to that of compound **1** indicating the similarities with rings B, C and D (see Fig. 2).

The structure of the compound **2** was also confirmed by fragmentation pattern in its mass spectrum, which displayed prominent peaks at *m/z* 488 (M^+), 224, 266, 248 (base peak), 238, 219, 203, 189, 159 and 187 due to molecular ion and rupture of ring C, with other minor fragments; 304, 318, 184 and 170 due to rupture of ring D; and 170, 304, 119, 146, 133, 159, 156 and 119 due to rupture of ring B supporting the proposed structure of the compound. Other peaks in the spectrum were also in good agreement with the structure (Scheme 2).

Thus, based on above chemical and spectral evidences, the structure of the compound was elucidated as 2β,3β,21-trihydroxy-arbor-12-ene-28-carboxylic acid, and has been designated as emarginellic acid (**2**).

2.2. Pharmacological section

2.2.1. Anti-inflammatory activity

The results have been presented in Table 3. The crotalic acid **1**, has shown a significant anti-inflammatory activity, as it has decreased the edema by 53% at a dose level of 10 mg/kg in comparison to standard oxyphenyl butazone (69%) at the dose level of 100 mg/kg, and by 53% with respect to carrageenan (0.05 ml, 1%) used to cause inflammation in the paw of the rat. The test compound produced a significant ($P < 0.05$ and $P < 0.01$) inhibition of inflammation after 3 h, as compared in the case of standard drug oxyphenyl butazone ($P < 0.01$). In addition, it was also observed that the test compound **1** has exhibited a significant activity on lower doses (1/10th, 10 mg/kg) in comparison to reference drug (100 mg/kg). However, the acetate derivative showed a decrease in activity, by 33% indicating the negative effect of acetylation on anti-inflammatory activity. Further, the activity did not enhance on increasing the dose level (20 mg/kg) of the test compound, which indicated the dose independent effect of crotalic acid.

Table 2
1D- and 2D NMR data of emarginellic acid-b (**2**)

Position	^{1}H NMR[a]	^{13}C NMR/HMQC	DEPT[b]	COSY	HMBC[c]	
					$^2J_{CH}$	$3J_{CH}$
1a	0.96 *ddd* (15.0, 9.0, 4.5)	43.8 *t*	CH_2	H-1b, H-2	C-2, C-10	C-25
1b	1.89 *ddd* (15.0, 5.5, 4.5)	–	–	H-1a, H-2	C-2	C-25
2α	3.65 *ddd* (10.5, 5.0, 5.5)	68.1 *d*	CH	H_2-1, H-3	C-3, C-1	C-10
(Acetate)[d]	4.97 *ddd* (10.5, 5.0, 5.5)	70.1 *d*	CH	H_2-1, H-3	C-3	C-10, C-3′
3α	3.53 *dd*(10.5)	712 *d*	CH	H-2	C-2, C-4	C-5
(Acetate)[d]	4.62 *dd* (10.5)	80.6 *d*	CH	–	C-2, C-4	C-1′, C-5
4	–	37.4 *s*	C	–	–	–
5	0.85 *dd*(8.0, 4.5)	52.8 *d*	CH	H-6a	C-6, C-4	C-9, C-3
6a	0.97 *m*	28.4 *t*	CH_2	H-6b, H_2-7, H-5	C-7	C-10, C-8
6b	1.71 *ddd* (14.5, 10.5, 4.5)	—	—	H-6a, H-5	C-5	C-8
7a	1.20 *ddd* (14.0, 10.5, 4.5)	32.5 *t*	CH_2	H-7a, H_2-6	C-6, C-8	C-5
7b	1.44 *ddd* (14.0, 9.5, 5.5)	–	–	H-7b, H-6b	C-8	C-9
8	–	39.3 *s*	C	–	–	–
9	1.57 *dd* (8.0, 5.0)	47.1 *d*	CH	H_2-11	C-8, C-10, C-11	C-12, C-25, C-26
10	–	47.7 *s*	C	–	–	–
11a	1.59 *ddd* (14.5, 7.0, 5.5)	253 *t*	CH_2	H-11b, H-9, H-12	C-9, C-12	C-13
11b	2.4 *ddd* (13.5,5.5,5.0)	–	–	H-11a, H-9, H-12	C-12	C-8
12	5.20 *t* (7.0)	128.8 *d*	CH	H_2-11	C-11, C-13	C-18, C-9, C-14
13	–	138.0 *s*	C	–	–	–
14	–	42.1 *s*	C	–	–	–
15a	1.44 *ddd* (14.5, 9.5, 5.0)	37.4 *t*	CH_2	H-15a, H_2-16	C-14, C-16	–
15b	1.68 *ddd* (14.5, 5.5, 4.5)	–	–	H-15b, H_2-16	C-14, C-16	–
16a	1.47 *ddd* (15.0, 9.5, 4.5)	25.9*t*	CH_2	H-16b, H_2-15	C-15, C-17	–
16b	1.66 *m*	–	–	H-16a, H_2-15	C-17	–
17	–	39.9 *s*	C	–	–	–
18	1.53 *dd* (8.0, 5.0)	54.8*d*	CH	H_2-19	C-17	C-14
19a	1.75 *m*	17.6*t*	CH_2	H-19b, H-18, H_2-20	C-18, C-20	C-17
19b	1.38 *m*	–	–	H-19a, H_2-20	C-20	–
20a	1.41 *m*	23.7 *t*	CH_2	H-20b, H-21, H_2-19	C-21	–
20b	1.67 *m*	–	–	H-20a, H-21	C-19	C-17
21	–	73.0 *s*	C	–	–	–
22	1.25 *d* (6.5)	41.0 *d*	CH	Me-30	C-21, C-29, Me-30	–
23	0.77 *s*	16.9 *q*	CH_3	–	C-4	C-3
24	0.77 *s*	28.4 *q*	CH_3	–	C-4	C-3, C-5
25	0.93 *s*	16.1 *q*	CH_3	–	C-10	–
26	0.59 *s*	17.6 *q*	CH_3	–	C-8	C-7, C-9
27	1.12 *s*	24.9 *q*	CH_3	–	C-14	C-13
28	1.06 *s*	27.4 *q*	C	–	C-17	C-21, C-18
29	–	183.3 *s*	–	–	–	–
30	0.82 *d* (6.5)	16.3 *q*	CH_3	H-22	C-22	C-29, C-21
1′(Acetate)[d]	–	170.6 *s*	C	–	–	–
2′(Acetate)[d]	1.85 *s*	20.9 *q*	CH_3	–	–	C-1′
3′(Acetate)[d]	–	170.8 *s*	C	–	–	–
4′(Acetate)[d]	1.93 *s*	21.2*q*	CH_3	–	–	C-1′

[a] Assignments are based on COSY, and HMQC experiments; coupling constants in Hertz are given in parentheses.
[b] DEPT chemical shifts are presented at $\theta = 3\pi/4$ when methylene groups reaches negative maximum. C-multiplicities were established by DEPT experiment; $s = C$, $d = CH$, $t = CH_2$, $q = CH_3$.
[c] The correlations in HMBC have been shown from protons to carbons.
[d] On1y pertinent data in case of acetate are given, there is no need to give all data.

2.2.2. Anti-hepatotoxic activity

As shown in Table 4, activities of liver enzymes SGPT, SGOT alkaline phosphatase and bilirubin were markedly elevated in CCl_4 treated animals in comparison to normal values. Administration of silymarin (standard drug), and crotalic acid (**1**) at the dose level 10, 10 and 20 mg/kg body weight, respectively, had prevented CCl_4 induced elevation of serums GPT, GOT, alkaline phosphatase and bilirubin. The silymarin (10 mg/kg) had significantly decreased the levels of SGOT, SGPT, alkaline phosphatase and bilirubin by 107.56, 71.6, 199.66, and 1.35 units/ml, respectively. Whereas, crotalic acid (**1**) had a considerable decrease by 177.80, 160.50 units/ml in SGOT; 126.33, 144.16 units/ml in SGPT; 298.66, 212.0 units/ml in alkaline phosphatase; and 1.45, 1.58 units/ml in bilirubin levels in the dose of 10 and 20 mg/kg, respectively.

It was observed that crotalic acid (**1**) decreased the levels of alkaline phosphatase and bilirubin ($P < 0.05$) comparable with that of standard drug silymarin, exhibiting 32% and 30% decrease in comparison to silymarin (36%) against

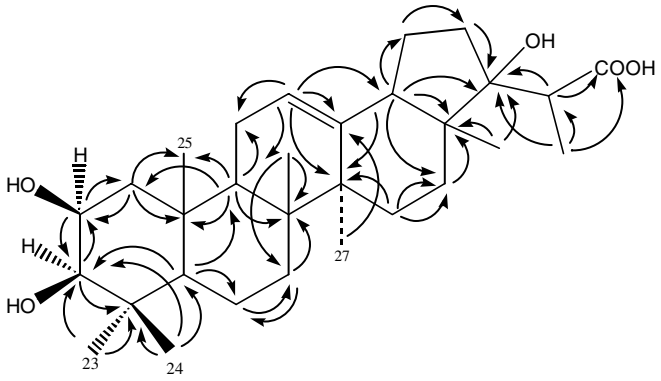


Fig. 2. Significant heteronuclear multiple bond correlations (HMBC) for emarginellic acid (**2**). Arrows point from proton to carbon.

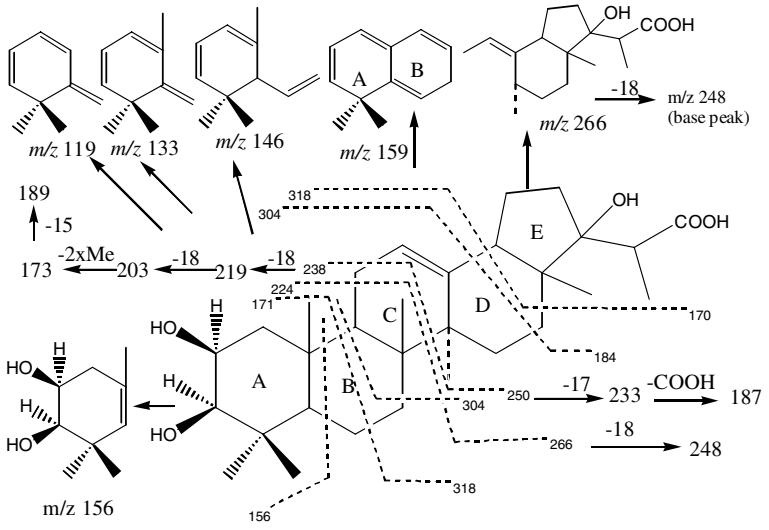
intoxicated control. Whereas, the levels of SGOT and SGPT were also decreased considerably in comparison to standard and intoxicated control (Table 4). However, no significant effect on increasing the dose was noticed indicating the dose independent effect of the test drug.

3. Experimental

3.1. Part A: Chemistry

3.1.1. General

Melting points were determined on Metier 9100 Electro thermal apparatus by open capillary method and are



Scheme 2. Emarginellic acid (**2**): $C_{30}H_{48}O_5$ (M^+ 488).

Table 3
Effect of crotalic acid on right rat paw swelling induced by carrageenan

Group ($n = 4$)	Dose mg/kg orally	Volume of paw (ml) after carragenan-induced edema				Total increase in paw vol. after 3 h	(%) Inhibition
		0 h	1 h	2 h	3 h		
Control (only carrageenan)	0.05 ml (1%)	1.01 ± 0.05	1.32 ± 0.03	1.58 ± 0.03	$1.95 \pm 0.06^{**}$	$0.94 \pm 0.05^{**}$	–
Crotalic acid + carrageenan	10 mg	0.99 ± 0.05	1.28 ± 0.03	1.42 ± 0.06	$1.43 \pm 0.03^{**}$	$0.44 \pm 0.03^{**}$	53%
Crotalic acid + carrageenan	20 mg	0.96 ± 0.07	1.35 ± 0.03	1.43 ± 0.02	$1.42 \pm 0.05^{**}$	$0.45 \pm 0.04^{**}$	52%
Acetyl crotalic acid + carrageenan	100 mg	1.02 ± 0.04	1.36 ± 0.02	1.56 ± 0.06	$1.65 \pm 0.03^{**}$	$0.65 \pm 0.03^{**}$	33%
Oxy phenyl butazone + carrageenan	100 mg	0.99 ± 0.05	$1.18 \pm 0.01^{*}$	$1.25 \pm 0.03^{**}$	$1.28 \pm 0.04^{**}$	$0.29 \pm 0.03^{**}$	69%

Values are expressed as mean ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$ (Dunnett's test).

Table 4
Effect of crotalic acid (**1**) on liver enzymes in CCl_4 induced liver damage in rats

Groups ($N = 4$)	Treatment	Dose (p.o.)	SGOT[a] (Units/ml)	SGPT[a] (Units/ml)	ALKP[a] (Units/ml)	Bilirubin[a] (Units/ml)
1	Normal/control	Nil	52.7 ± 8.44	31.03 ± 8.69	174.33 ± 14.01	1.04 ± 0.12
2	CCl_4 intoxicated control	0.5 ml/kg	$205.66 \pm 27.84^{**}$, $t = 5.32$	$167.8 \pm 30.08^{**}$, $t = 4.37$	$312.33 \pm 32.80^{*}$, $t = 3.87$	2.09 ± 0.11
3	Silybon-70 + CCl_4	10 mg/kg	$107.56 \pm 9.41^{**}$, $t = 5.32$ (47%)	$71.6 \pm 9.55^{*}$, $t = 3.05$ (57%)	$199.66 \pm 16.19^{*}$, $t = 3.08$ (36%)	$1.35 \pm 0.15^{**}$, $t = 6.45$ (35%)
4	Crotalic acid + CCl_4	10 mg/kg	177.8 ± 22.03 (13%)	126.33 ± 31.62 (24%)	298.66 ± 22.59 (5%)	$1.45 \pm 0.19^{*}$, $t = 2.91$ (30%)
5	Crotalic acid + CCl_4	20 mg/kg	160.5 ± 33.04 (21%)	$144.16 \pm 35.43^{*}$ (13%)	$212.0 \pm 18.54^{*}$, $t = 2.96$ (32%)	$1.58 \pm 0.13^{*}$, $t = 2.99$ (24%)

[a] SGPT, serum glutamic pyruvate transaminase; SGOT, serum glutamic oxaloacetic transaminase; ALKP, alkaline phosphatase; p.o, per oral; $^{*}P < 0.05$; $^{**}P < 0.01$, vs. intoxicated control using Student's t-test. Values are mean ± SE of four rats.

uncorrected. The IR spectra were recorded as KBr pellets on PYE UNICAM Spectrophotometer; mass spectra on a Finnegan MAT 300 mass spectrometer; ID and 2D NMR on Bruker DRX 500 spectrometer in $CDCl_3$ and MeOH-d_4 using TMS as internal standard reference, chemical shift in δ (ppm) and J values in Hz. The Centrifugal Preparative TLC was performed on a Chromatotron of Harrison Research Inc., USA using 4 mm rotor and silica gel PF-254 with $CaSO_4 1/2H_2O$ as an adsorbent.

3.1.2. Plant material

The aerial parts of *Crotalaria emarginella* Vatke were collected on April 22, 2004 from Agabat Tanha, Abha, Saudi Arabia, and identified by a taxonomist Dr. M.A. Rahman of the center, where a Voucher Specimen No. 14708 has been deposited in the herbarium for future reference.

3.1.3. Extraction and isolation

Air-dried aerial parts (2.6 kg) were crushed to coarse powder and extracted exhaustively in a Soxhlet apparatus with 95% ethanol. The extracts were concentrated under reduced pressure to yield viscous mass (96.0 g). The extract was extracted with petroleum ether, and then the residue was dissolved in acetone. The acetone insoluble part (15.0 g) was collected by filtration, which was chromatographed on a column using chloroform–methanol mixture as the eluent with increasing polarity. The eluent chloroform–methanol (99:1) afforded the compound **1** (13.0 g); whereas, the eluent chloroform–methanol (955) yielded the compound **2** (800 mg).

3.1.4. Crotalic acid (*1*)

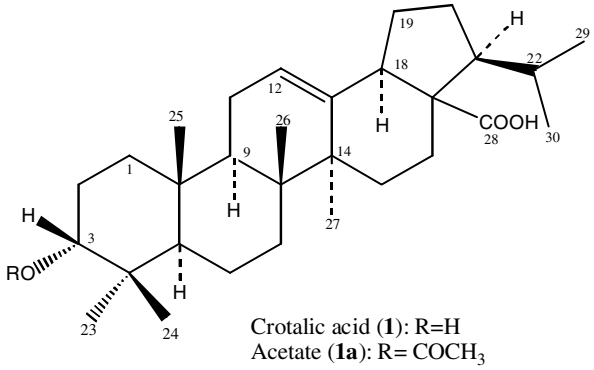
Colorless crystalline solid from methanol (13.0 g); m.p. 242–3 °C, eluent: 1% MeOH in $CHCl_3$; R_f 0.66 (50% $CHCl_3$–MeOH); $[\alpha]_D^{25} = +29.6$ ($c = 0.06$, MeOH); IR (KBr): ν_{max} 3500 (OH), 3310–3090 (COOH), 2960 (CH_3), 2865 (CH_2), 1758 (C=O), 1603 (C=C), 1542, 1437 (O–H, carboxylic acid), 1411, 1320 (C–O, carboxylic acid), 1161, 1050 (C–O–H, alcoholic), 910, 872, 780 (C=C) cm^{-1}; ID- and 2D NMR data: see Table 1; EIMS (probe) 70 eV, m/z (rel. int): 456 $[M]^+$ (20), 438 $[M^+-H_2O]$ (10), 441 $[M-CH_3]$ (15), 411 $[M^+-COOH]$ (13), 302 $[M^+-$ring E] (5), 288 $[M^+-$ring D and E] (6), 248 $[M^+-$ring A, B and C, rupture via bond 9(11)–8(14)] (100), 208 $[M^+-$ring C, D and E, rupture via bond 9(11)–8(14)] (30), 222 $[M^+-$ring C, D and E, rupture via bond 11(12)–8(14)] (15), 234 $[M^+-$ring A, B and C, rupture via bond 11(12)–8(14)] (10), 204 [222–18] (50), 189 [248-COOH] (15), 175 (10), 161 (10), 144 (7), 133 (50), 119 (20), 95 (25), 69 (30), 55 (45), 43 (80); HRMS: m/z 456.36913 $[M]^+$ (Calc. for $C_{30}H_{48}O_3$, 456.3603); Elemental analysis: Found: C, 7859; H, 10.60; O, 10.50%; required for $C_{30}H_{48}O_3$: C, 78.90; H, 10.59; O, 1051%.

3.1.5. Acetylation of crotalic acid (*1*)

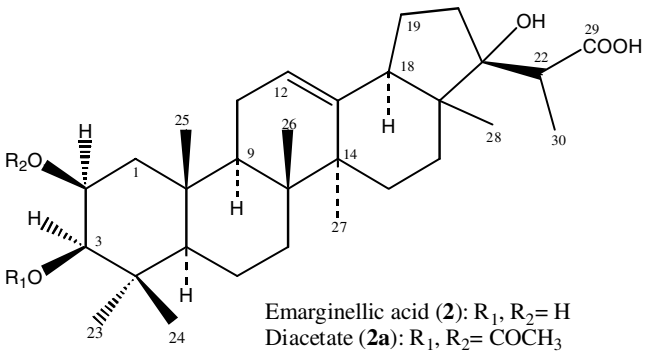
The compound **1** (200 mg) was acetylated with Ac_2O–pyridine (1:1), which on usual work up afforded acetate **2a** (180 mg), m.p. 275–76 °C, IR (KBr): ν_{max} 1760 (C=O), 1753 (C=O, acetate), 1245 (C–O, ester group); NMR data: see Table 1; EIMS (probe) 70 eV, m/z (rel. int): 498 $[M]^+$ (20), 456 (20), 439 (30).

3.1.6. Emarginellic acid (*2*)

Colorless crystalline solid from methanol (800 mg); eluent: 5% MeOH in $CHCl_3$; R_f 0.45 (40% $CHCl_3$ in MeOH); IR (KBr): ν_{max} 3530 (OH), 3315–3090 (COOH), 2965 (CH_3), 2860 (CH_2), 1761 (C=O), 1605 (C=C), 1540, 1439 (O–H, carboxylic acid), 1415, 1318 (C–O, carboxylic acid), 1160, 1060 (C–O–H, alcoholic), 915, 871, 785 (C=C) cm^{-1}; 1D- and 2D NMR data: see Table 2; EIMS (probe) 70 eV, m/z (rel. int): 488 $[M]^+$ (25), 470 $[M^+-H_2O]$ (12), 473 $[M-CH_2]$ (15), 443 $[M^+-COOH]$ (13), 318 $[M^+-$ring E] (15), 304 $[M^+-$ring D and E] (8), 266 $[M^+-$ring A, B and C, rupture via bond 9(11)–8(14)] (10), 248 [266-H_2O] (100), 224 $[M^+-$ring C, D and E, rupture via bond 11(12)–8(14)] (21), 238 $[M^+-$ring C, D and E, rupture via bond 9(11)–8(14)] (23), 304 $[M^+-$ring A, B and C rupture via bond 11(12)–8(14)] (10), 204 [222–18] (50), 189 [248-COOH] (15), 173 (10), 156 (10), 159 (25), 146 (7), 133 (45), 119 (22), 95 (15), 69 (20), 55 (40), 43 (80); HRMS: m/z 488.3515 $[M]^+$ (Calc. for $C_{30}H_{48}O_5$, 488.3502); Elemental analysis: Found: C, 73.75; H, 9.91; O, 16.38%; required for $C_{30}H_{48}O_5$: C, 73.73; H, 9.90; O, 16.37%.



Crotalic acid (**1**): R=H
Acetate (**1a**): R= $COCH_3$



Emarginellic acid (**2**): R_1, R_2= H
Diacetate (**2a**): R_1, R_2= $COCH_3$

3.1.7. Acetylation of emarginellic acid (*2*)

The compound **2** (200 mg) was acetylated with Ac_2O–pyridine (1:1), which on usual work up afforded acetate

2a (180 mg), m.p. 250–01 °C; $[\alpha]_D^{25} = -7.5$ ($c = 0.04$, MeOH); IR (KBr): ν_{max} 1762 (C=O), 1748 (C=O, acetate), 1743 (C=O, acetate), 1248 (C–O, ester group); NMR data: see Table 2; EIMS (probe) 70 eV, *m/z* (rel. int): 572 $[M]^+$(10), 526 (20), 488 [10], 454 (30).

3.2. Part B: Biological assay

3.2.1. Experimental animals

The animal experiments were performed in accordance with the ethical standards formulated in the Helsinki Declaration of 1964 (revised 2000), and guidelines issued by the Jamia Hamdard Animal Ethical Committee (JHAEC), registered (No. 173/CPCSEA) with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Chennai (Madras), India.

Wistar rats of either sex weighing 200–250 g were used for determining the different activity. The animals were maintained at 23 ± 2 °C with 12 h light and dark cycle, fed Purina-rat chow diet supplied by Grain Silos and Flour Mills Organization, Riyadh, Saudi Arabia; and had free access of diet and water.

3.2.2. Determinations of anti-inflammatory activity

Four rats each were allotted to different treatment groups. Edema was induced in the rats by injecting carrageenan (0.05 ml, 1% w/v in normal saline) into the subplantar tissue of the right hind paw using the reported method (Winter et al., 1962). Paw volume was measured with a plethysmometer (7140, Ugo Basile) before carrageenan injection and 0, 1, 2 and 3 h later. The edema was reported as the difference between the initial and final volume. The anti-inflammatory effect was expressed as the percentage inhibition as compared with carrageenan-treated animal. The standard oxyphenyl butazone (100 mg/kg) was used as a reference compound. The suspension of the test compounds with distilled water (0.1 ml/100 g rat) were administered orally 1 h before injection of the phlogistic agent.

3.2.3. Testing of anti-hepatotoxic activity

The animals were divided into five groups of four rats in each and were treated as follows: Group-1 (normal control without any treatment); Group-2 (intoxicated control) was given 0.5 ml CCl_4/kg body weight in liquid paraffin (1:1) by oral route for 7 days. Groups 3, 4 and 5 received 05 ml CCl_4/kg in liquid paraffin (1:1) followed by oral treatment with silymarin as standard dose (10 mg/kg), crotalic acid **1** (10 mg/kg), and (20 mg/kg), respectively, for 7 days.

After the treatment, the blood was obtained from all groups of rats by puncturing retro-orbital plexus. The blood samples were allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 2500 rpm at 30 °C for 15 min and analyzed for various biochemical parameters.

3.2.4. Assessment of the liver function

The biochemical parameters such as serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvate transaminase (SGPT) were estimated by reported method (Reitman and Frankel, 1957). The alkaline phosphatase and bilirubin were also measured according to the reported methods (Wooton, 1964; Kind and King, 1954).

3.2.5. Statistical analysis

The results of the inflammation and biochemical estimations are reported as mean ± SE. Total variation, present in a set of data was estimated by one way analysis of variance (ANOVA). Student's test and Dunnett's test were used for determining the significance (Woolson, 1987; Dunnett, 1964).

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