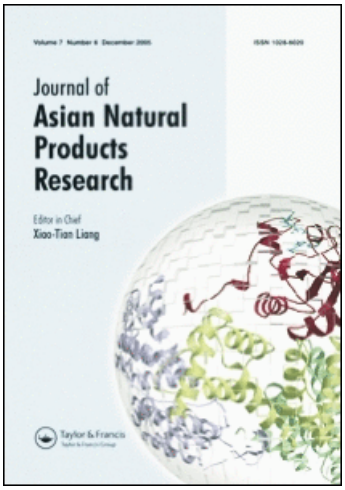

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

Irritant potential of some constituents from the seeds of *Caesalpinia bonducella* (L.) Fleming

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Online publication date: 09 September 2010

To cite this Article Saeed, M. Asif and Sabir, A. W.(2003) 'Irritant potential of some constituents from the seeds of *Caesalpinia bonducella* (L.) Fleming', Journal of Asian Natural Products Research, 5: 1, 35 — 41
To link to this Article: DOI: 10.1080/1028602031000080441
URL: http://dx.doi.org/10.1080/1028602031000080441

Journal of Asian Natural Products Research, 2003 VOL. 5 (1), pp. 35–41

IRRITANT POTENTIAL OF SOME CONSTITUENTS FROM THE SEEDS OF *CAESALPINIA BONDUCELLA* (L.) FLEMING

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(Received 27 May 2002; Revised 20 June 2002; In final form 22 July 2002)

The irritant potential of four triterpenoids, isolated for the first time from the seeds of *Caesalpinia bonducella*, identified as α-amyrin [12-ursen-3β-ol], β-amyrin [12-oleanex-3β-ol], lupeol [lup-20(29)-en-3β-ol] and lupeol acetate [lup-20(29)-en-3β-yl acetate] was investigated by open mouse ear assay, evaluating their ID_{50} (irritant dose in 50% animals) after acute effects and by irritant units (IU) after chronic effects. α-Amyrin, lupeol acetate and β-amyrin were the most potent and persistent irritant compounds with red weals of 1.5–2.1 cm diameter areas of the animal skin and with lowest $ID_{50} = 0.078$, 0.186 and 0.190 mg/10 μl after 1.5, 2.10 and 3.5 h, respectively. Their reactions lasted for 24 h with IU = 2.5; 0.312 and 1.25 mg/10 μl, respectively. Lupeol was the least irritant and least persistent compound with $ID_{50} = 0.603$ mg/10 μl after 4.5 h. Its reaction subsided before 24 h.

Keywords: Irritant potentials; Triterpenoids; *Caesalpinia bonducella*

INTRODUCTION

Caesalpinia bonducella (L.) Fleming, belonging to the family Caesalpiniaceae, is a small tree and commonly known as fever nut (Katkaranja) among the local population. This plant is widely distributed in subtropical and temperate regions of Pakistan and abundantly found in the Punjab Province during the rainy season [1]. The husk of the tree yields large amounts of tanning material which is used in vegetable tanning and retaining of leather [2]. The leaves and seeds of this plant were used as a folk medicine to treat asthma and chronic fever [3]. They were found to be very potent as an antiperiodic, antipyretic and utilized as febrifuge, antispasmodic, an antirheumatic and mild purgative [3,4].

A number of diterpenoid compounds, such as α-caesalpin, β-caesalpin, γ-caesalpin [5–10], ε-caesalpin, δ-caesalpin [11,12], cassane [13], caesalpinitol, D-insoil, D-(+)-pinitol [14], caesalpinin [15], bonducellpins A–D [16] and bondenolide [17], from the seeds and other parts of this plant have previously been isolated and characterized. Many fatty acid

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ISSN 1028-6020 print/ISSN 1477-2213 online
DOI: 10.1080/1028602031000080441

triglycerides, including palmitic, stearic, octadec-4-enoic and octadeca-2,4-dienoic acids from the seed kernels of this species have also been isolated and identified [18].

No attempt has been made to isolate and evaluate the harmful effects of its constituents. Our phytochemical and biological investigations of local natural products have led to the isolation of triterpenoid compounds for the first time from the seeds of *C. bonducella*. In the present communication, we describe the irritant effects of the seeds of this species on albino mice, followed by fractionation to isolate and characterize its active compounds, whose irritant potencies were evaluated by calculating their ID_{50}.

RESULTS AND DISCUSSION

Four triterpenoids (**1**–**4**) α-amyrin [12-ursen-3β-ol] (**1**), β-amyrin [12-oleanex-3β-ol] (**2**), lupeol [lup-20(29)-en-3β-ol] (**3**) and lupeol acetate [lup-20(29)-en-3β-yl acetate] (**4**) were isolated from the seeds of *C. bonducella*. These compounds were isolated for the first time from this source but they have been already reported in the literature from other sources. They were identified with the help of spectral data after comparison with the published data of similar compounds from other sources [19–27].

In many experiments, rabbits and guinea pigs have been preferred for testing skin irritancy [28–30]. However, both these species lack the varied human responses to skin irritants, partly due to a reduced microvasculature [31]. Both these species react more strongly than humans to mild or moderate irritants that originate from plants and often produce a diffused picture of irritant reactions that is difficult to evaluate [30,31]. On the other hand, the traditional mouse ear test for irritancy [34] is known to be useful for screening the extracts of higher plants for inflammatory reaction [34]. For these reasons, mouse ear assay [35] was used for evaluating the irritant responses of the compounds isolated from the seeds of *C. bonducella*. To compare the irritant potencies of these compounds with the euphorbium, the mouse ear assay involved measurement of the ID_{50} at the time when the maximum acute irritant effect was observed. An estimate of the irritancy after 24 and 48 h of application is also included in the data, being measured as irritancy units (IU) as defined by Hecker [36].

Among the four isolated compounds from the seeds of *C. bonducella*, α-amyrin (**1**), lupeol acetate (**4**) and β-amyrin (**2**) were more potent and persistent irritant compounds than the third compound lupeol with lower ID_{50} (i.e. 0.078, 0.186 and 0.190 mg/10 μl) after 1.5, 2.10 and 3.5 h, respectively, when compared with the effect of euphorbium on the mice ears. The effects of the four isolated compounds from the seeds of *C. bonducella* lasted for 24 h, indicating IU = 2.5, 0.312 and 1.25 mg/10 μl after 24 h, respectively (Table I). Lupeol (**3**) on the other hand, was the least irritant and least persistent compound, with $ID_{50} = 0.603\,mg/10\,\mu l$ after 4.50 h. Its effect did not last more than 12 h under the concentrations used (Table I). The inflammatory effects for α-amyrin, lupeol acetate and β-amyrin with ++ intensities appeared as red weals that spread in 1.5–2.1 cm diameter areas of the skin of the animal ears.

Since two of the isolated triterpenoids (α-amyrin and β-amyrin) had similar types of pentacyclic nuclei in their structures, with minor variations in side chains (Fig. 1), it was assumed that the potent and persistent inflammation was probably due to penetration of such triterpenoids in the skin, which causes some tissue damage in the animal skin. The other two isolated triterpenoids (lupeol and lupeol acetate) also had a pentacyclic nucleus with five carbons instead of six in one of the ring with variations in their side chains (Fig. 1). The least persistent inflammation due to lupeol was possibly due to the direct action at some skin receptor sites, whose reaction appeared as temporary and subsided after about 12 h.

TABLE I Irritant responses of the compounds isolated from the seeds of *C. bonducella* on albino mice

Dose levels (mg/10 μl)		Compounds				
		α-Amyrin (**1**)	β-Amyrin (**2**)	Lupeol (**3**)	Lupeol acetate (**4**)	Euphorbium
1.0		11*/12†	8/12	7/12	10/12	12/12
0.5		10/12	8/12	7/2	8/12	12/12
0.25		8/12	7/12	5/12	6/12	12/12
0.125		8/12	6/12	5/12	4/12	11/12
0.0625		6/12	4/12	3/12	4/12	11/12
0.03125		4/12	4/12	1/12	3/12	10/12
0.015625		3/12	1/12	–	2/12	9/12
0.0078125		0/12	0/12	–	1/12	9/12
ID_{50}	mg/10 μl	0.078	0.190	0.603	0.186	0.0020
	U.C.L.	0.1352	0.457	0.941	0.447	0.0081
	L.C.L.	0.0446	0.101	0.380	0.098	0.0000
IU (μg/10 μl) after	24 h	0.25	0.125	0.5	0.0312	0.0156
	48 h	>1.0	>0.5	>0.5	>1.0	0.0625

ID_{50}, Irritant dose in 50% animals, calculated by probit analysis.
U.C.L., Upper confidence limit; L.C.L., Lower confidence limit.
* Number of animal ears reacted to irritant compound.
† Total number of animals used.

The $-CH_3COO$ group in the side chain of lupeol acetate also appeared to be active in expressing inflammatory reaction on the animal's skin. Probably an acetate group enhances the penetration of this compound into the skin or reacts strongly with the skin protein, causing much more potent and persistent inflammation than lupeol.

We concluded from this investigation that the seeds of *C. bonducella* from our local source contained closely related triterpenoids, which have potent irritant potential on mouse skin. Four of these irritant triterpenoids (α-amyrin, β-amyrin, lupeol and lupeol acetate) were isolated and identified with the help of spectral data. Further work is imperative to amplify

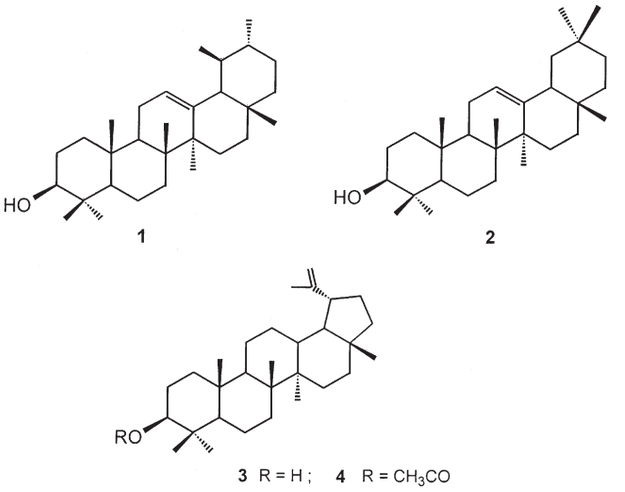

FIGURE 1 Triterpenoids isolated from the seeds of *C. bonducella.*

this property through the preparation of their derivatives, which would possibly lead to the structure–activity relationship of such irritant compounds from our natural sources.

EXPERIMENTAL SECTION

General Experimental Procedures

Unless otherwise stated, all the chemicals used were of analytical grade. Concentrations were performed under reduced pressure. Melting points were uncorrected. UV spectra were measured on a Hitachi 270-30 spectrophotometer in MeOH and IR spectra of the compounds were obtained as KBr disc or as thin films on NaCl discs on a Pye-Unicam SP-8-400 spectrophotometer. ^{1}H NMR spectra were acquired in DMSO-d_6 solvent at 300 MHz and ^{13}C NMR spectra at 75 MHz on a Bruker AM-300 NMR spectrophotometer, using tetramethylsilane as an internal reference. EI mass spectra were recorded on a Varian MAT-312 double focusing mass spectrometer using the direct inlet method. Column chromatography was carried out on silica gel 60 (70–230 mesh) and TLC was performed on silica gel F_{254} with 0.25 mm thicknesses. The spots were visualized either by exposure to UV light (365/254 nm) or with I_2 vapors or with anisaldehyde/H_2SO_4 spraying reagent [32] or with Liebermann–Burchard spraying reagent [33].

Plant Material

Seeds of *C. bonducella* were collected from the waste and uncultivated areas of Punjab (central plain areas of Pakistan), in July–August 2001. These were authenticated by Professor Dr A. A. Bhutta, Department of Botany, University of Punjab, Lahore. A voucher specimen of the sample (P-cog. 041) was kept in the Herbarium of the Pharmacognosy Section, Department of Pharmacy, University of Punjab, Lahore. Air-dried seeds were pulverized.

Extraction and Isolation

The ground seed material (8.0 kg) was soaked in MeOH for three weeks. It was filtered and the filtrate was concentrated under reduced pressure. The concentrated extract (128 g) was partitioned between EtOAc and H_2O. The aqueous layer was again shaken with *n*-BuOH. The EtOAc extract was condensed by removing the solvent under the reduced pressure and the material thus obtained was subjected to column chromatography using cyclohexane, cyclohexane–$CHCl_3$, $CHCl_3$ and $CHCl_3$–MeOH as eluting solvents.

Compound 1 (α-Amyrin)

Compound **1** (α-amyrin) was obtained as light yellow needles, eluted from cyclohexane/$CHCl_3$ (9:1), recrystallization from hot EtOH, 61 mg, mp 183–184°C. EIMS, *m/z* (rel. intens. %): 426 [$C_{30}H_{50}O$, M^+] (24), 411 [$M - Me$]$^+$ (18), 408 [$M - H_2O$]$^+$ (24), 218 [$M - C_{14}H_{24}O$]$^+$ (100), 207 [$M - C_{16}H_{27}O$]$^+$ (15), 203 [$M - C_{15}H_{27}O$]$^+$ (56) and 189 [$M - C_{16}H_{29}O$]$^+$ (68); IR (thin film): 3512 (OH), 3058, 1638 and 822 (trisubstituted double bond); ^{1}H NMR, δ 5.24 (1 H, m, H-12), 3.22 (1 H, dd, $J_{ax,ax} = 10.2$ Hz, $J_{ax,eq} = 4.3$ Hz, H-3), 1.16 (3 H, s, Me-27), 1.02 (3 H, s, Me-26), 0.99 (3 H, s, Me-23), 0.96 (3 H, s, Me-25), 0.88 (6 H, s, Me-29 and Me-30), 0.84 (3 H, s, Me-28) and 0.81 (3 H, s, Me-24); ^{13}C NMR, δ 38.50 (C-1), 27.00 (C-2), 78.00 (C-3), 38.00 (C-4), 55.12 (C-5), 18.34 (C-6), 32.66 (C-7), 40.02 (C-8), 47.54 (C-9), 37.00 (C-10), 23.01 (C-11), 122.54 (C-12), 143.52 (C-13), 41.54 (C-14),

28.34 (C-15), 26.25 (C-16), 32.54 (C-17), 47.22 (C-18), 46.80 (C-19), 31.14 (C-20), 34.82 (C-21), 37.22 (C-22), 26.02 (C-23), 15.57 (C-24), 15.52 (C-25), 16.82 (C-26), 26.00 (C-27), 27.34 (C-28), 33.22 (C-29) and 23.70 (C-30) (Fig. 1).

Compound 2 (β-Amyrin)

Compound **2** (β-amyrin) was obtained as light yellow needles, eluted from cyclohexane/$CHCl_3$ (8:2) and further from cyclohexane/EtOAc (7:3), re-crystallized from hot EtOH, 47 mg, mp 197–198°C. EIMS, *m/z* (rel. intens. %): 426 [$C_{30}H_{50}O$, M^+] (16), 411 [$M - Me]^+$ (17), 408 [$M - H_2O]^+$ (18), 393 [$M - Me - H_2O]^+$ (34), 257 [$M - C_{11}H_{21}O]^+$,(20), 218 [$M - C_{14}H_{24}O]^+$ (100), 207 [$M - C_{16}H_{27}O]^+$ (11), 203 [$M - C_{15}H_{27}O]^+$ (46) and 189 [$M - C_{16}H_{29}O]^+$ (58); IR (thin film): 3510 (OH), 3055, 1636 and 820 (trisubstituted double bond); ^{1}H NMR, δ 5.10 (1 H, m, H-12), 3.18 (1 H, dd, $J_{ax,ax} = 10.0$ Hz, $J_{ax,eq} = 4.6$ Hz, H-3), 1.09 (3 H, s, Me-27), 1.02 (3 H, s, Me-26), 1.01 (3 H, s, Me-23), 0.96 (3 H, s, Me-25), 0.91 (3 H, d, $J = 6.6$ Hz, Me-30), 0.81 (6 H, s, Me-24) and 0.80 (3 H, d, $J = 6.8$ Hz Me-29); ^{13}C NMR, δ 39.00 (C-1), 27.30 (C-2), 78.99 (C-3), 39.00 (C-4), 55.23 (C-5), 18.31 (C-6), 33.00 (C-7), 41.01 (C-8), 47.82 (C-9), 37.00 (C-10), 17.45 (C-11), 124.34 (C-12), 139.32 (C-13), 42.04 (C-14), 28.70 (C-15), 26.65 (C-16), 33.74 (C-17), 59.02 (C-18), 96.65 (C-19), 39.54 (C-20), 31.22 (C-21), 41.52 (C-22), 28.11 (C-23), 15.61 (C-24), 15.95 (C-25), 16.80 (C-26), 23.30 (C-27), 28.02 (C-28), 24.32 (C-29) and 20.80 (C-30) (Fig. 1).

Compound 3 (Lupeol)

Compound **3** (lupeol) was obtained as white needles from cyclohexane/$CHCl_3$ (7:3) and cyclohexane/EtOAc (6:4), re-crystallized from hot Me_2CO–MeOH (1:1), 142 mg, mp 214–215°C; EIMS, *m/z* (rel. intens. %): 426 [$C_{30}H_{50}O$, M^+] (21), 411 [$M - Me]^+$ (26), 408 [$M - H_2O]^+$ (32), 393 [$M - Me - H_2O]^+$ (36), 385 [M-41]$^+$ (14), 220 [$M - C_{15}H_{26}]^+$ (82), 218 [$M - C_{14}H_{24}O]^+$ (56), 207 [$M - C_{16}H_{27}]^+$ (24), 189 [$M - C_{16}H_{29}O]^+$ (100) and 139 [$M - C_{21}H_{35}]^+$ (71); IR (Thin film): 3454 (OH), 3078, 1646 and 880 (exomethylene group); ^{1}H NMR, δ 4.75 and 4.63 (2 H, br. s, 1H each, H-29), 3.22 (1 H, dd, $J_{ax,ax} = 9.9$ Hz, $J_{ax,eq} = 4.4$ Hz, H-3), 1.66 (3 H, br. s, Me-30), 1.05 (3 H, s, Me-26), 0.98 (3 H, s, Me-23), 0.94 (3 H, s, Me-27), 0.84 (3 H, s, Me-25), 0.79 (3 H, s, Me-28) and 0.78 (3 H, s, Me-24); ^{13}C NMR, δ 38.61 (C-1) 27.52 (C-2), 78.80 (C-3), 38.74 (C-4), 55.32 (C-5), 18.30 (C-6), 34.26 (C-7), 40.82 (C-8), 50.42 (C-9), 37.10 (C-10), 20.96 (C-11), 25.25 (C-12), 38.15 (C-13), 42.84 (C-14), 27.39 (C-15), 35.50 (C-16), 92.90 (C-17), 48.20 (C-18), 47.78 (C-19), 150.64 (C-20), 92.89 (C-21), 39.87 (C-22), 28.02 (C-23), 15.47 (C-24), 16.12 (C-25), 15.88 (C-26), 14.50 (C-27), 18.13 (C-28), 109.26 (C-29) and 19.24 (C-30) (Fig. 1).

Compound 4 (Lupeol Acetate)

Compound **4** (lupeol acetate) was obtained as white needles from cyclohexane/$CHC1_3$ (1:1) and cyclohexane/EtOAc (8:2), re-crystallized from hot Me_2CO–MeOH (1:1), 52 mg, mp 214–215.5°C; EIMS, *m/z* (rel. intens. %): 468 [$C_{23}H_{52}O_2$, M^+] (56),453 [$M - Me]^+$ (12), 427 [$M - C_3H_5$] (8), 408 [$M - AcOH]^+$ (21), 393 [$(M - Me) - AcOH]^+$ (4), 249 [$M - C_{16}H_{27}]^+$ (26), 218 [$M - C_{16}H_{26}O_2]^+$ (39), 189 [$(M - C_{16}H_{27}) - AcOH]^+$ (65), 181 [$M - C_{21}H_{35}O]^+$ (16) and 121 [$(M - C_{21}H_{35}O) - AcOH]^+$ (49); IR (Thin film): 1710 (ester carbonyl), 3075, 1646 and 880 (exomethylene group); ^{1}H NMR, δ 4.72 and 4.62 (2 H, br, s, 1 H each, H-29), 4.26 (1 H, dd, $J_{ax,ax} = 9.7$ Hz, $J_{ax,eq} = 4.3$ Hz, H-3), 2.10 (3 H, s, CH_3COO), 1.66 (3 H, dd, $J = 1.25$ Hz, Me-30), 1.05 (3 H, s, Me-26), 0.96 (3 H, s, Me-23),

0.94 (3 H, s, Me-27), 0.87 (3 H, s, Me-25), 0.79 (3 H, s, Me-28) and 0.76 (3 H, s, Me-24); ^{13}C NMR, δ 38.42 (C-1), 23.76 (C-2), 81.06 (C-3), 37.82 (C-4), 55.42 (C-5), 18.23 (C-6), 34.32 (C-7), 40.92 (C-8), 50.46 (C-9), 37.10 (C-10), 21.05 (C-11), 25.15 (C-12), 38.12 (C-13), 42.94 (C-14), 27.50 (C-15), 35.66 (C-16), 43.08 (C-17), 48.08 (C-18), 48.30 (C-19), 152.10 (C-20), 30.12 (C-21), 40.01 (C-22), 28.02 (C-23), 16.57 (C-24), 16.23 (C-25), 16.04 (C-26), 14.52 (C-27), 18.06 (C-28), 19.32 (C-29), 109.51 (C-30), 21.32 (CH_3COO) and 170.84 (CH_3COO) (Fig. 1).

Animals

Albino mice weighing 20–25 g were housed in cages on wood shavings in an animal house at a temperature of 30 ± 2.5°C and relative humidity 40 ± 4.1%. Palette food and de-ionized water were available *ad libitum.*

Irritant Activity

Ten milligrams of the test compound were dissolved in 10 ml of acetone to prepare 10 mg/10 ml (w/v) solution. It was further diluted according to the method of Evans and Schmidt [34] and Kinghorn and Evans [35]. Eight dilutions were prepared for the main assay. The procedure for assessing the irritancy on mouse ears was also adopted from Evans and Schmidt [34] and Kinghorn and Evans [35]. For the main assay, a group of 12 animals was used for each dilution. 10 μl of the solution under test were applied to the inner surface of one of the mouse ears using Drummond Microcaps (Drummond Scientific Co. USA). Similarly, another eight successive dilutions of 0.2 mg/ml of euphorbium (a resin from *Euphorbia helioscopia*) [34,35] in acetone were used for positive control groups. The untreated ear was used as negative control. The ears were examined for redness after 30 min and then at 15 min intervals until two observations displayed that further redness would not occur. The time of maximum erythema was noted. The number of ears eliciting the degree of redness corresponding to at least ++ intensity on Hecker's scale at peak irritancy [36], which was also mentioned by Evans and Schmidt [34], was noted and expressed in mg/10 μl per ear. The animals were also examined after 24 and 48 h to find out the chronic irritant effects of the test compound. The number of red ears with at least ++ intensity after 24 and 48 h were recorded and denoted by irritant units (IU on Hecker's scale) [36]. If no redness was observed after either the acute or chronic stage, the procedure was repeated with higher concentrations of the test solution on the ears of another group of animals. The total number of red ears per dilution was tabulated. ID_{50} (irritant doses in 50% individuals) along with the upper and lower confidence limits of the compound were calculated by probit analysis [37], using a computer program [38].

The numbers of inflamed red mouse ears induced by the four isolated compounds from the seeds of *C. bonducella* and euphorbium, and their ID_{50}, with the upper and lower confidence limits, have been outlined in Table I.

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