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Phytochemical investigation of the toluene extract of the root of *Croton bonplandianum* Bail

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ABSTRACT

Triterpenoid, lup-1, 2-ene-3-one (1), lupeol (2), oleanolic acid (3), ursolic acid (4) and steroid, β-sitosterol (5) was isolated from the toluene extract of the root of *Croton bonplandianum* Bail. Their chemical structures were established on the basis of spectroscopic analysis (UV, IR, NMR, Mass *etc*.) and by chemical means. A probable biosynthetic pathway of compound 1 was also proposed. Compound 1 and 2 is reporting first time to obtain from this plant. The triterpenpoids (1, 2, 3 and 4) have also been screened for their antimicrobial activity. Preliminary investigation showed potent activity of 1against a series of fungal and bacterial pathogens.

KEYWORDS

Triterpenoid;
Bioactive natural products;
Antimicrobial activity;
Biosynthetic pathway.

INTRODUCTION

Croton bonplandianum Bail is the plant of Euphorbiaceae family and is native to southwestern Brazil, northern Argentina, southern Bolivia and Paraguay. In India it was first reported by Kaul[1] during late 1890. It occurs widely along roadsides, railway abandoned field in wide open ravines, paddy or sugarcane fields and on sandy or sandy clay soils. Because of resemblance of the leaves and flower cymes to that of Tulsi, this plant is often called Ban Tulsi locally. *C. bonplandianum* was reported to have many medicinal uses including the repellent property against the insects,[2-5] mosquito, *A. aegypti*[6]. People in the remote area of West Bengal as well as in other parts of India are using its root against snake bite and the leaf extract against high fever. In addition the plant is used both as a fuel and as detergent. The methanol extract of *C. bonplandianum* has been found to exhibit antitumor properties using *Agrobacterium tumefaciens* and has larvicidal activities.

Phytochemically Croton is rich in secondary metabolites including alkaloids and terpenoids[7,8]. Diterpene resins found in many species of croton have been used experimentally in the studies of tumor initiation and conceivably prove to be useful in cancer therapy[9]. Apart from the above limited reports, no systematic study has yet been initiated for the total phytochemical investigation of *C. bonplandianum* Bail.

The present author has submitted two specimens of *Croton bonplandianum* with the tag numbers of R-1706, R-1707 to Taxonomy and Environmental Biol-

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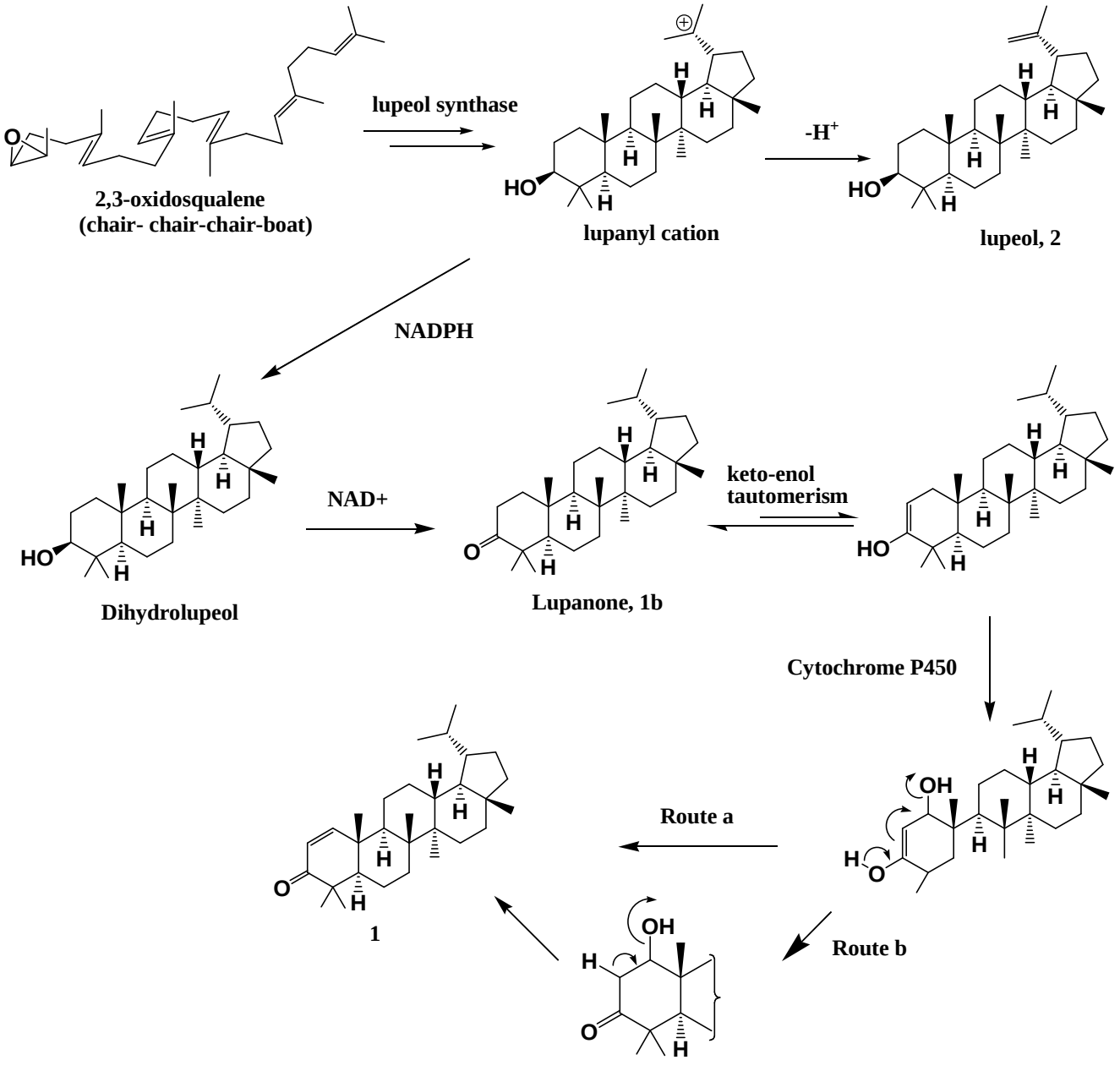

Scheme I

ogy Laboratory, Department of Botany, University of North Bengal, Darjeeling, India. Accession numbers of the submitted specimens were 9629 and 9630 and were stored in NBU Herbarium, Department of Botany, University of North Bengal, Darjeeling, India.

RESULTS AND DISCUSSION

During our search towards the isolation of bioactive natural products in tropical plants[10], we have investigated the chemical constituents of the toluene extract of roots of *C. bonplandianum*. The investigation yielded the isolation of lup-1, 2-ene-3-one (1), along with three other triterpenoids, lupeol (2), oleanolic acid (3), ursolic acid (4) and steroidal skeleton β-sitosterol (5). The triterpenpoid compounds (1, 2, 3 and 4) have been screened for their antimicrobial activity. Compound 1 and 2 is reporting first time to obtain from this plant.

The toluene extract of the roots of *C. bonplandianum* (1.5 kg) was distilled under reduced pressure to get a reddish gummy mass (1.2 gm). It was purified (through repeated column chromatography over

TABLE 1 : MICs of 1 to 4 against different bacteria

Compounds	MIC in µg/ml against different bacterial strains			
	EC	BS	SA	EB
1	100	<150	150	100
2	100	100	200	100
3	100	130	150	150
4	130	170	130	100
Ampicillin	128	64	64	128

BS- *Bacillus. subtilis*, EC- *Escherichia coli*, SA- *Staphylococcus. aureus*, EB-*Enterobactor*, MIC- Minimum inhibitory concentration.

TABLE 2 : MICs of 1 to 4 against different fungi

Compounds	MIC in µg/ml against different fungal strains				
	CG	FE	CE	AA	CC
1	3.83	10.0	30.0	10.0	<5.0
2	3.77	10.5	25.0	15.5	30.0
3	4.50	20.0	35.0	20.0	39.0
4	4.00	19.0	35.0	20.0	40.0
Bavistan	3.50	3.50	3.70	4.00	4.20

CG- *Colletotrichum gloeosporioides*, FE- *Fusarium equisiti* CE- *Curvularia eragrostidis*, AA- *Alternaria alternata*, CC- *Colletotrichum camelliae*.

silica gel) to isolate the constituents *viz*. lup-1, 2-ene-3-one (1, 0.1 gm), along with three known triterpenoids, lupeol (2, 0.2 gm) oleanolic acid (3, 0.25gm) and ursolic acid (4, 0.12 gm) and β-sitosterol (5, 0.08 gm). The structures of all the compounds were elucidated on the basis of chemical and spectroscopic analysis and by comparison (for compounds 2, 3, 4 and 5) with the authentic sample (compound 2) / spectral data of already reported compounds.

Crystallization of compound 1, furnished fine needle shape crystal, analysed for $C_{30}H_{48}O$, m.p.178-79^{0}C $[\alpha]_D$ +20.6^0. UV absorption of the compound showed strong absorption band at 228 nm (ϵ =19,000) that indicated the presence of a α, β-unsaturated carbonyl group which is further supported by its IR spectrum that showed a sharp peak at 1680 cm^{-1} (α, β-unsaturated ketone) along with other peaks. ^{1}H NMR spectrum of the compound showed the presence of six tertiary methyls appeared as singlets at 0.79, 0.94, 1.07, 1.08, 1.11 and 1.14 ppm, two secondary methyls appeared as doublet centered at 0.77 and 0.87 (d, J=8Hz) ppm respectively, two doublets each at 5.80 (1H, J=10Hz) ppm and at 7.11(1H, J=10Hz) ppm indicating the presence of grouping –CO–CH=CH–C<. [13]C NMR spectrum of the compound showed the presence of 30 carbons and DEPT experiment showed the presence of eight methyl carbon as quartets; eight methylene carbon as triplets, eight methine carbon as doublets and six tertiary carbon as singlets; two olefinic carbons at C-1 and C-2 appeared as doublets at 159.8 and 125.2 ppm respectively and the carbonyl carbon at C-3 appeared as a singlet at 205.6 ppm. The downfield shift (about 19 ppm compare to that of parent ketone, 1b) was due to shielding effect of the olefinic double bond at the α, β-position. Carbon-13 NMR data of 1 are presented along with those of already reported dihydroglochidone[11] and glochidone[12] (1a) in TABLE 3. Mass spectral data established the molecular formula of the compound as $C_{30}H_{48}O$ [M^+ 424 m/z]. Besides the molecular ion at m/z 424, the mass spectrum also showed prominent fragmentation peaks at m/z 381[M^+ - $CH(CH_3)_2$], 288, 287, 231, 150 (base peak),137, 95, 69, 55. On the basis of the above spectral analysis the compound has been assigned the structure as 1(2) - dehydrolupanone (1) and was found to be identical with dihydro glochidone[11].

Purification of compound 2 yielded a white powder of m.p 215 ^{0}C, $[\alpha]_D$ +33.0^0, showed strong absorption band at 3385 (-OH), 2851, 1460, 1354 (gem dimethyl), 1034, 826,765 cm^{-1} in the IR spectrum indicative of the presence of hydroxyl, olefinic and gem dimethyl groups. On the basis of elemental analysis and MS data [m/z = 426 $(M)^+$] its molecular formula was assigned as $C_{30}H_{50}O$ and finally the structure was elucidated as lupeol (2) by comparison with an authentic sample (co-tlc, ir, m.m.p)

Purification of compound 3 yielded a white powder of m.p 306-308 ^{0}C, molecular formula was found to be $C_{30}H_{50}O_3$ on the basis of FAB MS data [m/z = 458.2 $(M)^+$]. Finally its structure was elucidated as oleanolic acid by ^{1}H and ^{13}C NMR data and by comparison with the data already reported in literature[14]. Crystallization ($CHCl_3$-MeOH) of compound 4 also yielded a white powder of m.p 280-282 ^{o}C. Molecular formula, $C_{30}H_{50}O_3$ (on the basis of FAB MS data [m/z = 458.5, M^+) and was finally identified as ursolic acid by ^{1}H and ^{13}C NMR data and that already reported in literature[14].

Purification of the most polar fraction (10% ethyl acetate in pet. ether) afforded white crystals of m.p 136-137 ^{o}C, Mass spectral data showed m/z = 414 (M^+)

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and was identified as β -sitosterol 5 by spectral analysis and by comparison with an authentic sample of β -sitosterol (mixed m.p, co-IR, and co-tlc etc.).

A probable biosynthetic pathway for the formation of the new triterpenoid 1 has depicted in scheme 1 on the basis of the formation of an ene diol[15] in presence of cytocrome P450 and its subsequent rearrangement to 1.

Although the natural products (1 to 4) do not show any significant phytotoxicity when tested on a number of specimens (TABLE 3) but all the isolated compounds showed moderately good antimicrobial activities against the tested fungal and bacterial pathogens as evidenced from the TABLES 1 and 2. Compound 1 to 4 showed better activity against all the microorganisms and their activity is comparable to that of Ampicillin against *E. coli* and *Enterobactor*. The activity of compound 1 was nearly comparable to that of Bavistan, when the compound was tested against *Colletotrichum gloeosporioides* and *Colletotrichum camelliae*. Finally it can be concluded that the present study will be extremely helpful to enrich the present knowledge about different types of triterpenoids and also help the researchers to design newer generation of drugs based on such information about triterpenoids.

EXPERIMENTAL

General experimental procedure

Melting points were determined by open capillary method and were uncorrected. IR spectra were measured on Shimadzu 8300 FT-IR spectrophotometer. NMR spectra were recorded on Bruker-Avance 300 and 400 MHz FT-NMR spectrometer. MS were recorded on varian mat 711(70eV) by E1/C1 method, ESIMS was obtained on Applied Biosystem API 2000 and TOFMS were obtained on 4800 (ABSciex) MALDI-TOF/TOF Tandem Mass Spectrometer.

Plant materials

Plants of *C. bonplandianum* used in this experiment were collected from North Bengal, India in May, 2008.

Extraction and isolation

The air dried roots of *C. bonplandianum* (1.5 Kg) was chopped into small pieces and extracted with toluene (2 l) in a soxhlet extractor for 7 days and toluene was recovered in *vacuo*. The extracted mass (350 g) was purified by repeated column chromatography (silica gel) using petroleum ether (PE) and PE:ethyl acetate of varying concentrations as eluent.

Bioassay

Five different fungal pathogens (C*olletotrichum camelliae*, *Fusarium equisiti*, *Alternaria alternata*, *Curvularia eragrostidis*, *Colletotrichum gloeosporioides)* were used for *in vitro* antifungal assay[16-17]. Antibacterial assay were performed against four bacterial pathogens (*Escherichia Coli*, *Bacillus Subtilis*, *Staphylococcus aureus*, *Enterobactor).* Suitable strains of these organisms were procured from the microbiology laboratory of our institute. MICs (Minimum inhibitory concentration) of the triterpenoids against bacterial and fungal pathogens have been presented in TABLE 1 and 2 respectively. The antifungal and antibacterial media used are as follows. For nutrient agar 28 gm of media (HiMedia) was suspended in 1000 ml of distilled water according to the manufacturer's protocol. It was boiled to dissolve the medium completely at sterilized by autoclaving at 15 lbs pressure (121^0 for 15 min.). The nutrient agar contained peptic digest of

TABLE 3 : Phytotoxicity of the compounds based on the length (in cm) of roots after 7 days

Compounds	Concentration (μg/ml)	Rice	Wheat	Pea
	Control	0.6	1.0	1.50
1	100	0.6	1.12	1.50
	250	0.6	1.23	1.50
	500	0.6	1.12	1.50
	100	0.6	1.21	1.51
2	250	0.6	1.12	1.53
	500	0.6	1.15	1.55
	100	0.5	1.09	1.60
3	250	0.5	1.10	1.65
	500	0.6	1.12	1.60
	100	0.6	1.22	1.55
4	250	0.6	1.13	1.60
	500	0.6	1.15	1.62

Seeds of rice (*Oriza sativa*), wheat (*Triticum aestirium*) and pea (*Pisum sativum*) were collected from local market and used after washing.

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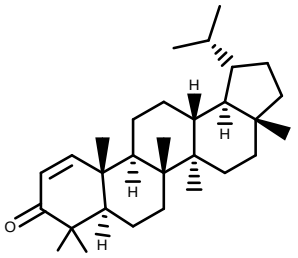

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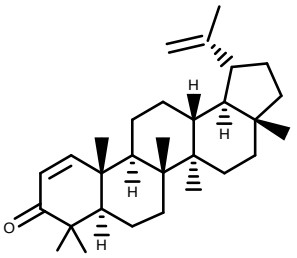
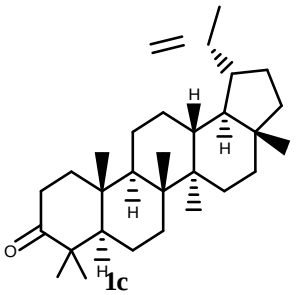
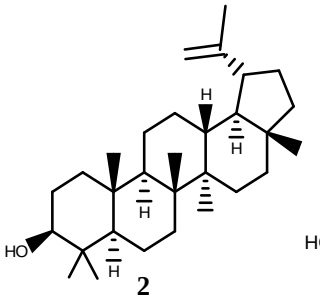


1a



1c



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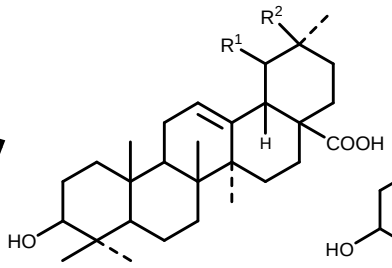
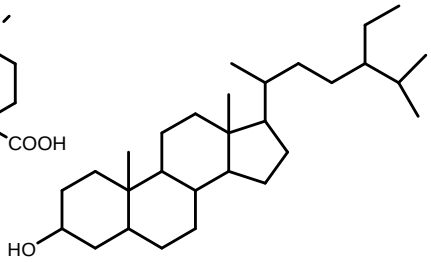


3: R^1 = H, R^2 = Me

4: R^1 = Me, R^2 = H



5

animal tissue (5 gm), sodium chloride (5 gm), beef extract (1.5 gm), yeast extract (1.5 gm), agar (15 gm) and dissolved water (1000 ml). P^H was adjusted to 7.2. For preparation of PDA (potato-dextrose-agar) peeled potato was cut into small pieces and boiled in required volume of dissolved water. The mixture was filtered through muslin cloth and the extract was mixed with dextrose and agar. The resultant mixture was heated in order to dissolve. Finally the media was sterilized at 15 lbs (121^0 for 15 min.). Composition of the media was peeled potato (400 gm), dextrose (20 gm), agar (20 gm) and dissolved water (1000 ml). P^H was adjusted to 6.0. DMSO (Dimethyl sulfoxide) was used as solvent to prepare different concentrations of the triterpenoids. Solvent control (DMSO) was also maintained throughout the experiment. All experiments were performed in Petri dishes and were incubated at 37°C for 48 h. The required media (either PDA or NA) was poured in a Petri dish and allowed for solidification. After solidification wells or cups were made by inserting a cork borer in the media. The numbers of wells were made according to the requirement of the experiment. Fungal spores were suspended on the PDA media before well or cup formation. Test solution (100 µl/well) was poured in the well or cup. We compared the antifungal activities of these compounds with that of Bavistan and antibacterial activity with that of Ampicillin, a β -lactam antibiotic.

Seeds of rice (*Oriza sativa*), wheat (*Triticum aestirium*) and pea (*Pisum sativum*) were collected from local market. The assay seeds were shorted for uniformity of size and all damaged seeds were discarded. Before the bioassay seeds were washed with tap water and the surface were sterilized using NaCl (10% v/v) for 10 min followed by several washes in sterile distilled water. For testing phytotoxicity dehydrated ethanol was used as control. Bioassays were carried out using petridishes (90 mm diameter) containing a sheet of Whatman 1 filter paper as support. Test solutions (5 ml) was added to the filter paper in the petridish and dried completely *in vacue* at 40^0. Five seeds from each category were placed on the filter paper and incubated for 7 days at 25^0 in the dark. The effects of the pure compounds were determined by measuring the elongation of roots and average for each concentration.

CONCLUSION

Triterpenoids, lup-1, 2-ene-3-one (1) and lupeol (2), of lupane skeleton has been isolated for the first time from the root of *C. bonplandianum* along with triterpenoids, oleanolic acid (3) and ursolic acid (4) of friedelin skeleton and β-sitosterol (5) of steroidal skeleton. Antimicrobial potentiality of the triterpenoids was also detected against five different pathogens. A plausible biosynthetic pathway for the formation of 1was also suggested.

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TABLE 4 : Comparison of Carbon-13 chemical shifts (ppm) of 1 along with Dihydro glochidone and Glochidone

Carbon	Dihydro glochidone[11]	1	Glochidone(1a)[12]
1	159.8(d)	159.8(d)	159.8(d)
2	125.2(d)	125.2(d)	125.1(d)
3	205.6(d)	205.6(d)	205.5(s)
4	44.6(d)	44.6(d)	44.6(s)
5	53.4(d)	53.4(d)	53.4(d)
6	19.1(t)	19.1(t)	19.0(t)
7	33.8(t)	33.8(t)	33.8(t)
8	39.5(s)	39.5(s)	39.5(s)
9	44.1(d)	44.1(d)	44.4(d)
10	41.8(s)	41.8(s)	41.7(s)
11	21.2(t)	21.2(t)	21.4(t)
12	26.7(t)	26.7(t)	25.1(t)
13	38.0(d)	38.0(d)	38.2(d)
14	43.3(s)	43.3(s)	43.1(s)
15	27.3(t)	27.3(t)	27.3t)
16	35.4(d)	35.4(d)	35.5(t)
17	43.2(s)	43.2(s)	43.0(s)
18	47.5(d)	47.5(d)	48.1(d)
19	44.6(d)	44.6(d)	47.9(d)
20	29.4(d)	29.4(d)	150.7(s)
21	21.9(t)	21.9(t)	29.8(t)
22	40.4(t)	40.4(t)	40.0(t)
23	27.8(q)	27.8(q)	27.8(q)
24	21.4(q)	21.4(q)	21.3(q)
25	16.5(q)	16.5(q)	-
26	19.0(q)	19.0(q)	16.5(q)
27	14.3(q)	14.3(q)	14.4(q)
28	18.1(q)	18.1(q)	18.1(q)
29	15.1(q)	15.1(q)	109.5(t)
30	23.0(q)	23.0(q)	19.2(q)

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