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Phytochemical Investigation of *Sapium baccatum*: Identification of 3α-hydroxy-1α, 2α-epoxy lupan

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Abstract Phytochemical investigation was carried with the crude toluene extract of the stem bark of *Sapium baccatum*. The isolated compounds were purified by column chromatography followed by crystallization. The structure of all compounds was established by chemical characterization and by the analysis of spectroscopic (IR, NMR and Mass) data and in some cases by comparison with authentic samples. Five compounds, namely 3, 3′ -di-o-methyl ellagic acid, taraxerone, 1-hexacosanol, taraxerol, baccatin along with a new compound 3 α-hydroxy-1 α, 2 α-epoxy lupan were identified.

Keywords *Sapium baccatum*, phytochemical investigation, natural products, biocidal activity

Introduction

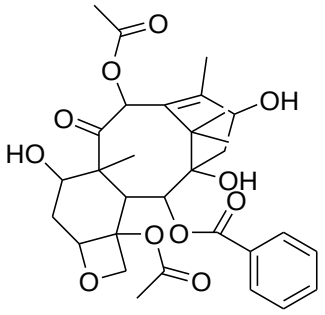
Natural products (NPs) are an incredibly diverse group of small (usually molecular weight less than 1500 Da) organic compounds isolated from a variety of natural sources, principally from plants. The reason that NPs capture the imagination of organic chemists and pharmaceutical scientists is because of their well documented and wide ranging biological activities and their skeletal diversity and intriguing functional group characteristics, which render them as indispensable leads for probing biological systems status and for drug discovery with new bioassay systems. The Himalayan region of Darjeeling and the Terai region of the northern part of West Bengal are well natured with floras and faunas having medicinal value [1-3]. The tribal medicinal practice in this region provides the evidence of utilization of medicinal plants by the local people as folklore. Plenty of medicinal plants have so far been screened for pharmacological activities. The interesting biological activity and pharmacological prospects of naturally occurring compounds [4-12] is one of the emerging areas of the present day natural product research. Since the medicinal plants of Darjeeling hill is also reported as a rich source of bioactive molecules, so in addition to the phytochemical investigation of the medicinal plants in this area, there also exists ample scope to make a systematic study on the biocidal activity of phytoconstituents as well as on their prepared derivatives. Keeping these views in mind for last couple of years, we are actively engaged in isolation of these phytoconstituents particularly triterpenoids and steroids, characterization of them and further functionalization of bioactive frameworks. Our studies have resulted in the syntheses of several new organic molecules - some of them have been assayed for antimicrobial activities and found to be promising [13-18].

Keeping this view in mind, phytochemical investigation was carried with the crude toluene extract of the stem bark of *Sapium baccatum* ROXB from Darjeeling foothills. The isolated compounds were purified by column chromatography followed by crystallization. The structure of all the compounds was established by chemical characterization and by the analysis of spectroscopic (IR, NMR and Mass) data and in some cases by comparison with authentic samples.

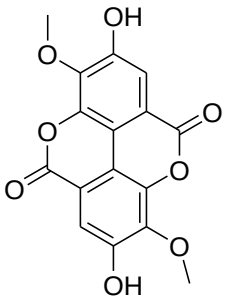
Isolation Process:

Dried and powdered stem bark of *Sapium baccatum* ROXB (2kgs) was extracted with toluene in a soxhlet apparatus for 20 hrs. On cooling the toluene extract, a yellow insoluble compound separated out, this was collected by filtration. This was purified and identified as 3, 3-di-o-methyl ellagic acid [19]. From the clear filtrate, toluene was distilled off and the residual gummy solid (30gms) was taken up in ether (2 ltrs). The clear ether solution was washed with cold water till washings were neutral and dried over anhydrous Na_2SO_4.

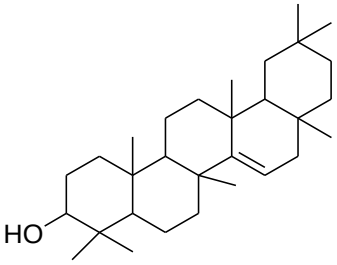
The solvent was evaporated when the neutral material (11gms) was obtained as a yellow gummy solid, which after chromatography and crystallisation from chloroform-methanol mixture gave shining crystals (1.3 gm) having m.p 238-240 ºC and was found identical in all respect with authentic sample of taraxerone [20] (mixed m.p. Co-1R ; Co-TLC). Other compounds isolated were 1-hexacosanol [21], taraxerol [20] and baccatin [22] (fig 1) along with the new compound F. 1-hexacosanol, taraxerol and baccatin were identified by comparison (mixed m.p. Co-IR ; Co-TLC) with respective authentic samples.



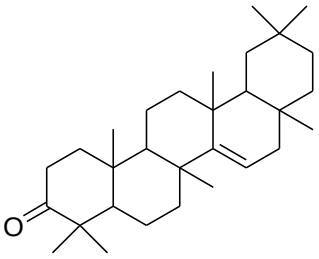
Baccatin



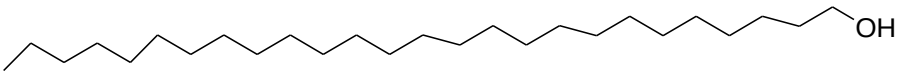
3, 3/-dimethyl ellagic acid



Taraxerol



Taraxerone



1-hexacosanol

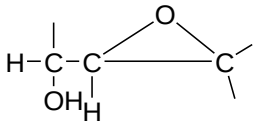
Figure 1: Structure of the isolated compounds

Characterization of compound F: Identification of 3 α-hydroxy-1α, 2α-epoxy lupan.

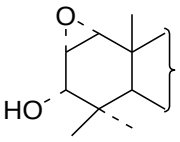
Compound F was purified by crystallisation from chloroform – methanol mixture to afford white crystal of m.p. 203-204 ºC. IR absorption peaks at 3530 cm^{-1} and 880 cm^{-1} indicated the presence of hydroxyl group and epoxide ring respectively in F. It did not respond to TNM test for the presence of unsaturation and the absence of any halogen was also indicated by Beilstein test. UV spectrum showed no absorption in the region 220 -300 nm. The mass spectral analysis indicated the molecular ion peak at 442 $[M]^+$ with other peaks at m/z 427 $[M\text{-}Me]^+$, 424 $[M\text{-}H_2O]^+$, 409 $[427\text{-}H_2O]^+$, 406 $[424\text{-}H_2O]^+$, 399 $[M\text{-}CHMe_2]^+$, 381 $[399 - H_2O]^+$, 331, 272, 231, 217, 191, 169 ,163, 149, 55 (base peak). From elemental analysis and mass spectral data the compound F was shown to have the molecular formula $C_{30}H_{50}O_2$. ^1H NMR spectrum of compound F showed the presence of eight methyl groups in the region 0.75 to 1.17 ppm, a doublet of triplet centered at 1.88 ppm and another at 2.04 ppm each integrate for one proton; it further showed two doublets centered at 2.31 (J= 3.5 Hz) and 3.05 (J=3.5 Hz) ppm, a sextet (ddd) centered

at 3.53 ppm (*J*=3.5 and 4.47), another doublet of doublet at 3.64 ppm with *J*=6.04 and 8.7 Hz all integrated for a single proton each. The nature and relationship of these protons were established by carrying out the following experiments.

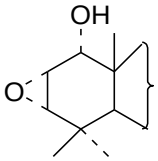
D_2O exchange experiments caused collapse of the doublet at 2.31 ppm and the doublet of doublet at 3.64 ppm appeared as a doublet with *J* value of 6 Hz. This clearly indicated that the doublet at 2.31 ppm is due to hydroxyl proton of the grouping -CH-OH and the methine proton germinal to -OH group has a neighbouring proton with small coupling constant of 6 Hz. This proton (at 3.64 ppm) on irradiation caused the appearance of a singlet at 2.31 ppm and collapse of the doublet of a doublet at 3.53 ppm to a broadened singlet, thus this proton (i.e. at 3.64 ppm) is α to the -CH-OH proton (F-1); irradiation at 3.53 ppm caused collapse of the quartet at 3.64 ppm to a flattened doublet, and the doublet at 3.05 ppm to a sharp singlet. Hence, the proton centred at 3.05 ppm is related to that centred at 3.53 ppm. Thus the above experiments indicated the presence of partial skeleton F-1 in F. Two such structures of ring – A can be drawn as F-2 and F-3.



F-1

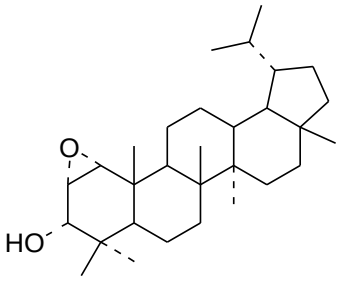


F-2

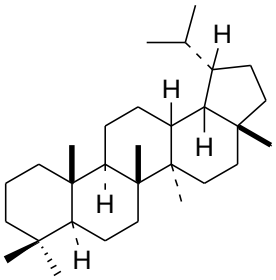


F-3

Construction of drieding models showed that the epoxide ring at 1α, 2α- position with the –OH group at C-3 position as quasi-equatorial is the most probable conformation with least interaction. Thus the structure of compound F has been assigned as lup-1α, 2α-epoxy-3α-ol (7).



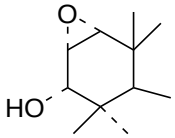
F



Lupane

HETCOR NMR spectrum showed that the carbon at 67.56 ppm is attached to proton at 3.64 ppm, carbon at 56.06 ppm is related to the proton at 3.53 ppm and that at 65.06 ppm is attached to proton appearing at 3.05 ppm. ^{13}C NMR spectrum displayed the presence of 30 carbons in the region 14.6 to 67.6 ppm and the DEPT experiment showed the presence of eight-CH_3 as quartets, eight-CH_2 as triplets nine-doublets (for –CH carbon) and five singlets (for quaternary carbons). The ^{13}C NMR signals are assigned on the basis of assignments to lupane skeleton and the present XH correlation spectrum (See Table 1).

Construction of drieding model showed that the epoxide ring with the least interaction is only possible if it is attached to the ring - A in it boat form at 1α, 2α position. In this conformation the C-4β methyl is quasi-equatorial and the C-10β methyl is quasi axial with flag pole interaction with C-3β hydrogen. The 1, 3 – methyl-methyl interactions between C-24 and C-25 methyl groups become minimize in this arrangement. It is further apparent that in order to have minimum flag pole interaction the substituent at C-3 should be quasi equatorial. Thus it is assumed that the compound F will have the epoxide ring in ring – A of lupane skeleton and should have the structure as given below:



F

The structure F is proved by LAH reduction of lup -1α, 2 α-epoxy-3-one [prepared by treatment of lup-1 (2)-en -3 one with alkaline H_2O_2] which furnished a compound identical with F (^{1}H NMR, CO-TLC, CO-IR, m.m.p).

Table 1: Comparison of ^{13}C NMR of 7 with Lupane

Carbon no.	F	Lupane
1	56.1(d)	40.5(t)
2	65.1(d)	18.8(t)
3	67.6(d)	42.3(s)
4	40.4(s)	33.3(s)
5	43.2(d)	56.5(d)
6	18.8(t)	18.8(t)
7	33.1(s)	34.6(s)
8	40.6(s)	41.3(s)
9	40.4(d)	50.4(d)
10	32.8(s)	37.6(s)
11	21.4(t)	21.0(t)
12	24.7(t)	27.1(t)
13	38.1(d)	38.1(d)
14	43.5(s)	43.3(s)
15	27.5(t)	27.5(t)
16	35.5(t)	35.8(t)
17	43.2(s)	43.2(s)
18	47.6(d)	47.8(d)
19	44.8(d)	44.9(d)
20	27.9(d)	29.5(t)
21	22.2(t)	22.0(t)
22	40.1(q)	40.5(q)
23	21.9(q)	33.4(q)
24	29.3(q)	21.7(q)
25	16.3(q)	16.1(q)
26	15.9(q)	16.2(q)
27	14.6(q)	14.6(q)
28	18.1(q)	18.2(q)
29	15.1(q)	15.2(q)
30	23.0(q)	23.0(q)

Biocidal Activity of the Isolated Compounds:

In the present study, five different fungal pathogens were choosen (*Colletotrichum camelliae, Fusarium equisiti, Alternaria alternata, Curvularia eragrostidis, Colletotrichum gloeosporioides*) for *in vitro* antifungal assay. 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 3 α-hydroxy-1 α, 2 α-epoxy lupan (F) against bacterial and fungal pathogens have been presented in Table 1 and 2, respectively. The antifungal and antibacterial media used were as follows. For nutrient agar (NA) 28 g 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 (121º for 15 min). The nutrient agar contained peptic digest of animal tissue (5 g), sodium chloride (5 g), beef extract (1.5 g), yeast extract (1.5 g), agar (15 g) and distilled water (1000 ml). pH 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

distilled 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 ºC for15 min). Composition of the media was peeled potato (400 g), dextrose (20 g), agar (20 g) and distilled water (1000 ml). pH was adjusted to 6.0. DMSO (dimethyl sulfoxide) was used as solvent to prepare different concentrations of the 3 α-hydroxy-1 α, 2 α-epoxy lupan (F). Solvent control (DMSO) was also maintained throughout the experiment. All experiments were performed in Petri dishes and were incubated at 37º 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 ml/ well) was poured in the well or cup. Additionally slide germination method was also used for determination of antifungal activity (Table 3). We compared the antifungal activities of these compounds with that of Bavistan and antibacterial activity with that of ampicillin, a β-lactam antibiotic.

The result of antibacterial activity (table 2) indicated that compound F showed better bioactivity than the reference sample against two organisms, Escherichia coli and Enterobactor.

Table 2: MIC of isolated compounds against different Bacteria

Compounds	**MIC in mg/ml against different bacterial strains**			
	EC	**BS**	**SA**	**EB**
F	100	100	98	96
Ampicillin	128	64	64	128

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

The result of antifungal activity (table 3/4) with F was not encouraging as is evident from the data shown in table 3. However, in spore germination method some interesting result was obtained as is shown in table 4.

Table 3: MIC of isolated compounds against different fungi

Compounds	**MIC in mg/ml against different fungal strains**				
	CG	FE	**CE**	**AA**	**CC**
F	7	19	38	7	6
Ampicillin	3.5	3.5	3.7	4.0	4.2

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

Table 4: Antifungal properties of triterpenoids against five fungal pathogens by spore germination bioassay after 48 h of incubation

Fungal pathogen	**3 α-hydroxy-1α, 2α-epoxy lupan (F)**		
	PG[a]	**PI**	**AL[b](mm)**
CC	00	100	00
FE	00	100	00
AA	00	100	00
CG	00	100	00
CE	05	96	6.0

CG: *Colletotrichum gloeosporioides*, FE: *Fusarium equisiti*, CE: *Curvularia eragrostidis*, AA: *Alternaria alternata*, CC: *Colletotrichum camelliae*. PG: Percent germination, PI: Percent inhibition, AL: Average germ tube length, [a]Based on 200 spores, [b]Based on 25 germ tubes.

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