CHAPTER II

EXPERIMENTAL AND REFERENCES

1. Experimental

1.1 General experimental procedure

Melting points were determined by open capillary method and were uncorrected. IR spectra were measured on Shimadzu 8300 FT-IR spectrophotometer and Perkin Erlmer FT-IR spectrophotometer. NMR spectra were recorded on Bruker-Avance 300 and 400 MHz FT-NMR spectrometers respectively. ESIMS was obtained on Applied Biosystem API 2000 and FAB MS were obtained on Jeol SX 102 mass spectrometer from CDRI Lucknow. TOF MS was recorded in 4800 (ABSciex) MALDI-TOF/TOF Tandem Mass Spectrometer.

1.2 Collection of Plant Material

Plants of *C. bonplandianum* used in this experiment were collected from North Bengal, India in May, 2008. After collection all the plants were washed thoroughly by plenty of water and the roots were separated by simple cutting through a knife in wet condition and separated those from the rest parts.

1.3 Drying and grinding

The plant's materials were shade dried and were cut into small pieces. It was then grinded in small lots to powdered form in a mechanical grinder and used for the extraction process.

1.4 Extraction and Isolation *C. bonplandianum* Bail

The air dried powdered roots of *C. bonplandianum* (2 Kg) was extracted with MeOH (2 L) in a soxhlet extractor for 7 days and MeOH was recovered in *vacuo*. The deep brown extracted mass (350 g) was suspended in distilled water (1L) and was partitioned successively between hexane (300 x 3), EtOAc (300 x 3) and n-BuOH (300 x 3) to obtain four fractions (hexane phase, ethyl acetate phase, n-butanol phase and water phase). The ethyl acetate phase (1.5 g) was purified by repeated column chromatography (silica gel) using petroleum ether (PE) and PE:ethyl acetate (EA) of varying concentrations as eluent.

Table 5 Purification of the crude extract of *C. bonplandianum* Bail

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-3	Nil	--
2	PE-EA (9.9:0.1)	4-10	Oil	--
3	PE-EA (9.8:0.2)	10-12	Nil	--
4	PE-EA (9.6:0.4)	13-18	White solid (≈ 0.2 g)	244-246 °C
5	PE-EA (9.5:0.5)	19-23	White solid (≈ 0.05 g)	306-308 °C
6	PE-EA (9.4:0.6)	24-31	White solid (≈ 0.05 g)	280-282 °C
7	PE-EA (9.3:0.7)	32-41	Nil	--
8	PE-EA (9.2:0.8)	42-66	White solid (≈ 0.3 g)	136-137 °C[a]
9	PE-EA (9.1:0.9)	66-82	Nil	--

Further elution with more polar solvent mixture did not afford any solid material

[a]Describes melting point of the simple isolated compound as the it was hard to crystallize.

1.4.1 Characterization of compound 1

Compound **1** was obtained as a white powder of melting point (mp) 244-246 °C, $[\alpha]_D$ + 4.0, UV inactive, showed strong absorption band at 3422 (-OH), 2850, 1461, 1376 (gem dimethyl), 1053, 970, 959, 838 cm^{-1}, ESIMS data [m/z = 425 (M+1)]. ^1H NMR spectra of compound **1** (Table 1) showed characteristic proton signals due to six tertiary methyl groups at δ_H (proton chemical shift) 0.68 (3H, s), 0.69 (3H, s), 0.76 (3H, s), 0.84 (3H, s), 1.01 (3H, s), 1.25 (3H, s), and two secondary methyl signals as doublet centered at 0.79 (3H, d, J = 6.3 Hz) and 0.92 (3H, d, J = 6.3 Hz).Elemental analysis: C, 84.82% (cald.

84.84), O, 3.68% (cald. 3.77) and H 11.50 (cald. 11.39). Thus compound **1** is 3α-hydroxy-urs-12,15-dien.

1.4.2 Characterization of compound 2

Compound **2** after complete purification was obtained as a white powder of mp 306-308 °C and its molecular formula was suggested to be $C_{30}H_{50}O_3$ on the basis of FAB MS data [m/z = 458.2 $(M)^+$]. The ^1H NMR spectra) showed characteristic proton signals due to seven tertiary methyl groups at δ_H 1.24 (3H, s, C-23), 1.00 (3H, s, C-24), 0.98 (3H, s, C-25), 1.04 (3H, s, C-26), 1.30 (3H, s, C-27), 0.97 (3H, s, C-29), and 1.02 (3H, s, C-30). An oxygenated methine signal appeared at δ_H 3.44 (1H, m) and a trisubstituted olefinic signal at δ_H 5.49 (1H, s). Therefore compound **2** is oleanolic acid. Finally it was confirmed by comparison with an authentic sample of oleanolic acid (mixed melting point, co-IR, co-tlc).

1.4.3 Characterization of compound 3

Compound **3** was also obtained as a white powder of mp 280-282 °C and its molecular formula was suggested to be $C_{30}H_{50}O_3$ on the basis of FAB MS data [m/z = 458.5 $(M)^+$]. The ^1H NMR spectra of **3** showed characteristic proton signals due to five tertiary methyl signals at δ_H 0.92 (3H, s), 1.02 (3H, s), 1.06 (3H, s), 1.24 (3H, s), 1.24 (3H, s), and two secondary methyl signals as doublet centered at 0.97 (3H, d, J = 6.5 Hz) and 1.02 (3H, d, J = 6.5 Hz) of the triterpenoid moiety. An oxygen methine signal at at δ_H 3.44 (1H, m, H-3) and a trisubstiyuted olefinic signal at δ_H 5.49 (1H, t-like, H-12), suggesting that **3** is a 3β-hydroxy-urs-12-en type triterpenoid. Finally it was confirmed by comparison with an authentic sample of ursolic acid (mixed melting point, co-IR, co-tlc).

1.4.4 Characterization of compound 4

Purification of the most polar fraction afforded white crystals of m.p. 136-137°C, M^+ 414, and was identified as β-sitosterol. Finally it was confirmed by comparison with an authentic sample of β-sitosterol (mixed melting point, co-IR, co-tlc).

1.5 Preparation of acetyl derivative of compound 1

Compound **1** (100 mg, 0.23 mmol) in a 50 mL round bottom flask was warmed over a water bath with 10mL of dry pyridine and 2 mL of acetic anhydride for 6 hours. The reaction mixture was then poured into 50 mL of ice cold water; a milky white precipitate appeared that was worked up with ether, dried over anhydrous sodium sulfate. In the tlc chromatogram, developed by vaporized iodine showed a single compound. Solvent ether was recovered to make a minimum volume and to that silica gel (60-120 mesh) was added. The dried silica adsorbed compound was added over a column of silica gel (60-120 mesh) and the column was run using petroleum ether (PE) and PE:ethyl acetate (EAA) of varying concentrations as eluent.

Table 6 Purification of the acetylated derivative of compound **1**

Entry	Eluent	Fractions 20 mL each	Residue On evaporation	mp after crystallization
1	PE (60-80 °C)	1-6	Nil	--
2	PE-EA (9.95:0.05)	7-11	Oli	--
3	PE-EA (9.9:0.1)	12-18	White solid ($\approx$ 0.085 g)	223-224 °C
4	PE-EA (9.8:0.2)	19-22	Nil	--

Further elution with more polar solvent mixture did not afford any solid material

1.5.1 Characterization of acetyl derivative of compound 1

IR spectrum showed peaks at 2850, 1461, 1376, 1248, 1053, 959, 970, 838 cm^{-1}. In the mass spectrum it showed a molecular ion peak at 467. The ^1H NMR spectrum of the acylated derivative showed characteristic signals due to five tertiary methyl groups at at δ_H 0.68 (3H, s, C-23), 0.69 (3H, s, C-24), 0.76 (3H, s, C-25), 0.84 (3H, s, C-26), 1.01 (3H, s, C-27), 1.25 (3H, s, C-28), and two secondary methyl signals as doublet centered at 0.79 (3H, d, J = 6.3 Hz) and 0.92 (3H, d, J = 6.3 Hz). The acetate methyl appeared as a

singlet centered at 2.33 (3H, s). Elemental analysis: C, 82.28% (cald. 82.35), O, 6.78% (cald. 6.86) and H 10.94 (cald. 10.80). Thus compound **1** is 3α-hydroxy-urs-12,15-dien.

1.6 Bioassay

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 petridishes and were incubated at 37 °C for 48 h. The bacterial growth was confirmed by a change of yellow to purple colour. Bacterial nutrient media was prepared by using agar, beef extract and bacto peptone in distilled water and the pH of the solution (6.8-7.0) was adjusted. Culture media for fungal strains were prepared by mixing in suitable proportions of potato extract, dextrose and agar powder. All glass apparatus, culture media were autoclaved before use. The whole process was carried out in inoculation chamber. Additionally slide germination method was also used for determination of antifungal activity (Table 3). The antifungal activities between these compounds and streptomycin and antibacterial activity with ampicillin, a β–lactam antibiotic were compared.

For studying the inhibitory effect of the two triterpenoids against test fungal pathogens following slide germination method, the spores of the pathogens were allowed to germinate in presence of the prepared and the 50% ethanol extracts. Compound solution was placed on the centre of the grease free microscope slide. In control the corresponding solvent, either sterile distilled water or 50% ethanol was placed. Thirty micro litre spore suspension prepared from ten days culture of the fungal pathogens were added to the spots in both experimental and control slides. In case of 50% ethanol extract, spore suspension was added after ethanol was evaporated. Three experimental slides were taken for each compound. The slides were then incubated at 28 °C in a humid chamber. Two small glass rods (60 mm in length) were placed in a 90 mm petridish and a slide was placed on the rods in a uniformly balanced position. Then the petridish was filled with sterile distilled water so that the bottom of the slide remained just above the water surface. The petridish was then covered and incubated at 28 °C. Following 48 h of incubation, the slides were stained with lacto phenol-cotton blue mixture and observed in each slide for germination. Numbers of aspersoria formed were also observed and lengths of 50 germ tubes were measured. The entire experiment was repeated thrice.

1.6.1 Source of microbial cultures

Fungal and bacterial cultures used during the work were either isolated from the field or procured from Microbial type culture collection (MTCC), Chandigarh, India. The details of the source of the fungal cultures are given in Table 7.

Table7 List of cultures used during the present study

Name of the microorganisms	Source	Number
Staphylococcus aureus	MTCC,Chandigarh, India	MTCC–26
Escherichia coli	MTCC,Chandigarh, India	MTCC–739
Pseudomonas aeruginosa	MTCC,Chandigarh, India	MTCC–2453
Escherichia coli	MTCC,Chandigarh, India	MTCC–2939
Candida albicans	MTCC,Chandigarh, India	MTCC–227
Penicillium chrysogenum	MTCC,Chandigarh, India	MTCC–160
Colletrichum gloeosporioides	Plant pathology lab. NBU	Identification No. 5446.02
Fusarium equiseti	Plant pathology lab. NBU	Identification No. 6566.07
Curvularia eragrostidis	Plant pathology lab. NBU	Identified by Dr. A. Saha[22]
Alterneria alternata	Plant pathology lab. NBU	Identification No. 7065.08
Colletotrichum camelliae	Plant pathology lab. NBU	Identified by Dr. A. Saha[22]
Bacillus subtilis	Plant pathology lab. NBU	Identified by NBU[22]
Enterobactor sp.	Plant pathology lab. NBU	Identified by NBU[22]
S. dysenteriae	Plant pathology lab. NBU	Identified by NBU[22]
Aspergillus niger	Plant pathology lab. NBU	Identified by NBU[22]

1.6.2 Maintenance of stock cultures

Freshly prepared sterile slants of PDA medium were used for maintenance and preservation of fungal cultures. After four to eight weeks of sub culturing, the fungal pathogens grown on sterile PDA slants were stored in two different conditions, viz. at room temperature (25 °C) and in refrigerator (4-8 °C). Apart from weekly transfer for experimental purpose, the cultures were also examined at regular intervals under microscope.

1.6.3 Major Chemicals used

Table 8 In addition to the common laboratory reagents, following chemicals were used during the work

Chemicals	Company
$C_6H_{12}O_6$	Glaxo Laboratories (India) Ltd
$NaNO_3$	S d fine-CHEM limited
KCl	S d fine-CHEM limited
$MgSO_4$, $7H_2O$	Merck, India
$FeSO_4$, $7H_2O$	Merck, India
K_2HPO_4	Merck, India
$C_{12}H_{22}O_{11}$	Glaxo Laboratories (India) Ltd

1.6.4 Composition of media and solutions used

Some of the media/solutions used during the work are listed below along with their standard compositions.

Table 9 Nutrient Agar (NA)

Components	Amount
Beef extract	1.0 g
Yeast	2.0 g
Peptone	5.0 g
NaCl	5.0 g
Agar	15 g
Distilled water	1 L

Table 10 Potato Dextrose Agar (PDA)

Component	Amount
Potato	40 g
Dextrose	2 g
Agar agar	2 g
Distilled water	100 mL

Table 11 Malt yeast Agar

Components	Amount
Malt extract	3.0 g
Yeast extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g
Agar	20.0 g
Distilled water	1.0 g
pH	7.0

Table 12 Czapek concentrate

Components	Amount
$NaNO_3$	30.0 g
KCl	5.0 g
$MgSO_4$, 7 H_2O	5.0 g
$FeSO_4$, 7 H_2O	0.1 g
Distilled water	1.0 L
K_2HPO_4	1.0 g
Yeast extracts	5.0 g
Sucrose	30.0 g
Agar	15.0 g

1.6.4.1 ***Staphylococcus aureus*** were grown in nutrient agar media at 37±1 °C in an incubator for 48 h. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 48 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

1.6.4.2 ***Escherichia coli*** were grown in nutrient agar media at 37±1 °C in an incubator for 12 h. The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 12 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

1.6.4.3 ***Pseudomonas aeruginosa*** were grown in nutrient agar media at 37±1 °C in an incubator for 24 h. The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 24 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals

1.6.4.4 ***Candida albicans*** were grown in nutrient agar media at 25±1 °C in an incubator for 48 h. The growth condition was malt yeast agar. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 48 h old freshly grown cultures were used. Routine subcultures were made at 60 days intervals.

1.6.4.5 ***Penicillium chrysogenum*** were grown in Czapek concentrate media at 25±1 °C in an incubator for 7 days .The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 7 days old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

1.6.4.6 ***Colletotrichum gloeosporioides*** were grown in nutrient agar media at 37±1 °C in an incubator for 12 h. The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 12 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

1.6.4.7 ***Fusarium equiseti*** were grown in nutrient agar media at 37±1 °C in an incubator for 48 h. The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 12 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

1.6.4.8 ***Curvularia eragrostidis*** were grown in nutrient agar media at 37±1 °C in an incubator for 12 h. The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 24 h old freshly grown cultures were used. Routine subcultures were made at 45 days intervals

1.6.4.9 ***Alterneria alternata*** were grown in nutrient agar media at 37±1 °C in an incubator for 24 h. The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 24 h old freshly grown cultures were used. Routine subcultures were made at 60 days intervals

1.6.4.10 ***Colletotrichum camelliae*** was grown in nutrient agar media at 37±1 °C in an incubator for 48 h. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 48 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

1.6.4.11 ***Bacillus subtilis*** were grown in Czapek concentrate media at 25±1 °C in an incubator for 7 days .The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 7

days old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

1.6.4.12 ***Enterobactor sp.*** was grown in nutrient agar media at 37±1 °C in an incubator for 48 h. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 48 h old freshly grown cultures were used. Routine subcultures were made at 30 days intervals.

1.6.4.13 ***S. dysenteriae*** was grown in nutrient agar media at 25±1 °C in an incubator for 48 h. The growth condition was malt yeast agar. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 48 h old freshly grown cultures were used. Routine subcultures were made at 60 days intervals.

1.6.4.14 ***Aspergillus niger*** were grown in nutrient agar media at 37±1 °C in an incubator for 12 h. The growth condition was aerobic. Finally the cultures were kept in a refrigerator for storage and short term maintenance. Throughout the present study, 24 h old freshly grown cultures were used. Routine subcultures were made at 45 days intervals

1.6.5 Assay of Antifungal activity

Fungi were grown on potato dextrose agar (PDA) medium at 28 ± 1 °C for mycelial growth. The fungicidal activities were determined using agar cup bioassay and spore germination bioassay.

1.6.6 Spore germination bioassay[23]

The purified eluents (10 µL) were placed on two spots 3 cm apart on a clean grease free slide and the solvent was allowed to keep for some time to evaporate. One drop (0.02 mL) of spore suspension (10spores/mL) prepared from 15 days old culture of the test fungi was added on the same place where the purified eluents were placed and subsequently evaporated. Various compounds of five different concentrations were prepared (500 ppm, 400 ppm, 300 ppm, 200 ppm, 100 ppm). The studies were performed at 28±1 °C for 24 h under humid conditions in Petri plates. Finally after proper incubation period, one drop of a cotton blue-lacto phenol mixture was added to each spot to fix the germinated spores. The number of spores germinated was compared with that of germinated spores of control (where no chemicals were used). Moist chamber was used for germination of spores. The number of germinated spores was calculated on the basis

of an average of 300 spores per treatment. The minimum inhibitory concentration required for complete inhibition was recorded in units of microgram per milliliter (μg/mL).

1.6.7 Antibacterial sensitivity test by the disc diffusion method[23]

One ml of 48 h old culture of the test bacterium was taken in a petridish of 90 mm diameter. Then 20 mL of sterile NA medium was poured in the petridish and shaken carefully to mix the bacterial suspension with the medium. The petridishes were allowed to cool. Filter paper discs of 5 mm diameter containing desired concentration of the test samples were placed on the surface of the solidified media and incubated at 37 °C in an incubator for 48 h. Diameter of the inhibition zones was noted. In control sets no chemicals were used in the filter paper discs but sterile distilled water was used to soak the filter papers.

1.6.8 Antibacterial Assay

One ml of bacterial suspension was taken in a sterilized petridish of 90 cm diameter. Then Nutrient agar was poured in it. Sterilized filter paper discs (5 mm in diameter) were soaked in the desired test sample and finally placed on the surface of nutrient agar medium. The petridishes were incubated at 30 ± 2 °C in an incubator. Results were observed after 48 h of incubation

2. Notes and References

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