Chapter 3

TRITERPENOIDS FROM *Psidium guajava* AND THEIR BIOCIDAL ACTIVITY

Introduction

The Himalayan region of Darjeeling and Terai are well natured with diversified plants having pronounced medicinal activities as evidenced by recent literature reports[146-148] as well as by the tribal medicinal practice in this region. Plants of the family *Myrtaceae* are extensively used in indigenous medicine from prehistoric ages. *Psidium guajava* is an important representative of this family. Present day reports about *P. guajava* are attracting because of their highly encouraging biological activities[148-156]. Different parts of these plants are used in the traditional system of medicine for the treatment of various human ailments such as ulcers, bronchitis, eye sores, bowels, diarrhoes and cholera[148-150]. It is reported in the literature that the leaf extract of *P. guajava* has anti tussive, antibacterial, hemostatic, antioxidant and narcotic properties[152, 155]. Recently Abreu *et. al.* has reported that guava extract can alter the labelling of blood with technetium-99m* [156].

In view of the attributed medicinal properties and in an ongoing search for bioactive triterpenoids from plants of *Myrtaceae* available in Darjeeling foothills, the toluene extract of leaves of *P. guajava* was selected for further investigation. In continuation of our studies on the phytochemical investigation of medicinal plants available in the foothills of Darjeeling and Terai, The author report herein the isolation of two triterpenoids betulinic acid and lupeol from the leaf extract of *Psidium guajava* and their potential antimicrobial and phytotoxic activities. All the structures of the isolated compounds were confirmed by spectral (IR, NMR) analysis and by comparison with the literature reports. The leaf extract of *P. guajava* was found to contain two new triterpenoids betulinic acid (A) and lupeol (B) along with earlier reported guajanoic acid (C)[151], β-sitosterol (D), ursolic acid (E) and oleanolic acid (F). Compounds A and B have been characterized as betulinic acid and lupeol,respectively. This is the first report of the isolation of these two triterpenoids from the leaf extract of *P. guajava* available plenty in

the foothills of Darjeeling. In addition to that preliminary studies towards the antimicrobial and phytotoxic activities of these compounds, which have not yet reported so far from this source, have also been carried out against some fungal and bacterial pathogens (Table 1, 2, 3 and 4).

Results and Discussion

Isolation and purification of the Compounds present in the leaf of the plant *Psidium guajava*

The dried and powdered plant materials were extracted with toluene using soxhlet apparatus for 72 hours. The solvents were then removed under reduced pressure and a sticky brown residue was obtained. This residue of the compounds present in the leaf of the plant was than purified by column chromatography using silica gel (60-120) mesh and suitable proportions of petroleum ether and ethyl acetate were used as the eluent.

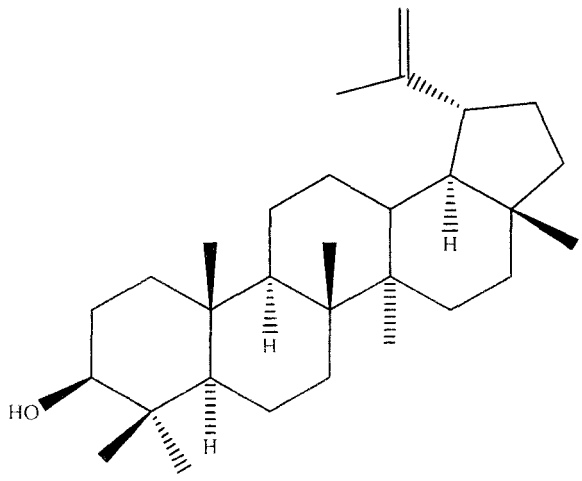
Characterization of compound A (betulinic acid)

Compound A was isolated as white gummy solid ($CHCl_3$ + MeOH) of m.p 299-301°. IR spectrum has exhibited hydroxyl at ν_{max} 3610, 1020 cm^{-1} and exomethylene at ν_{max} 3060, 1630, 880 cm^{-1}. The details of its characterization have been discussed in part I of chapter 2.

Characterization of compound B (lupeol)

Compound (B) was isolated as white crystals from $CHCl_3$ + MeOH mixture and gave m.p. 210-212° $[\alpha]_D$ = +30.4 (conc. 0.58 in $CHCl_3$). Its IR spectrum exhibited hydroxyl at ν_{max} 3610, 1020 cm^{-1} and exomethylene at ν_{max} 3070, 1640, 887 cm^{-1} absorption. The ^1H NMR exhibited six tertiary methyl signals at δ_H 0.75, 0.77, 0.80, 0.92, 0.94 and 1.02, a vinyl methyl group at δ_H 1.66 (broad d J = 0.5 Hz)], a secondary carbinol group at δ_H 3.20 (dd, J = 9.6 and 6.2 Hz) and an exomethylene group at δ_H 4.58 (1H, triterpenoid[15-16] of lupeol (B). The structural assignment of (B) was further substantiated by its ^{13}C NMR spectrum which showed seven methyl groups at δ_C 28.0 (C-23), 19.3 (C-30), 18.0 (C- 28), 16.1 (C-25), 15.9 (C-26), 15.4 (C-24), 14.5 (C-27), an exomethylene group at δ_C 150.8 (C-20), 109.3 (C-29) and a secondary hydroxyl bearing carbon at δ_C 78.9 (C-3) in addition to ten primary carbons, five secondary and five tertiary carbons. The shielding of C- 23 methyl of

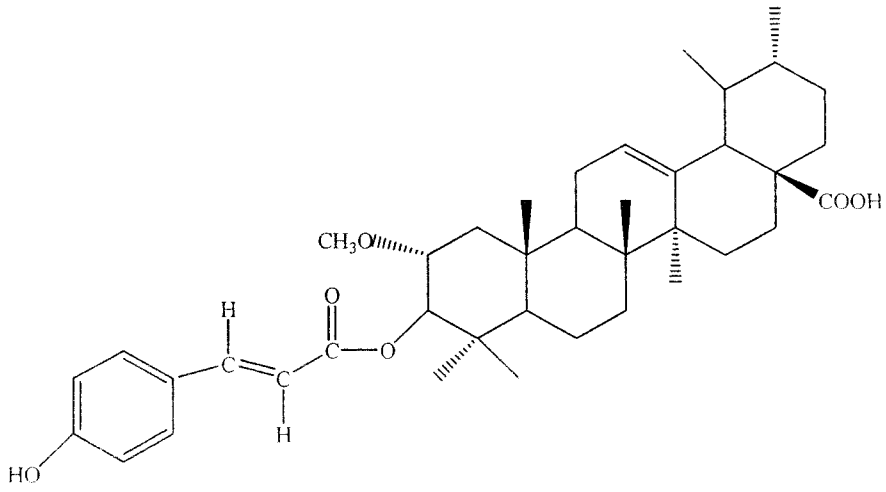
(B) could be due to the influence of the adjacent C-3 hydroxyl group. These data were in close agreement with those reported for lupeol (B) [159-161].



B

Characterization of compound C (guajanoic Acid)

Compound (C) was isolated as MeOH mixture and gave fine colorless needles from MeOH, m.p. 195-196°C; $[\alpha]_D$ = +5.1° (conc. 0.041 in MeOH). The elemental analysis revealed that the compound contains 75.76% of C, 8.81% of H and 15.43% of O. Based upon the functional group analysis it was found that the nature of oxygen was hydroxyl, carboxyl and ester also supported by IR spectroscopy. The absorptions bands appeared at 3420-2610 (COOH, OH), 2910, 2840 (CH), 1720 (ester C=O); 1700 (acid C=O), 1610-1380(C=C and aromatic ring), 1130 (C—O); UV λ_{max}(MeOH) nm: 204, 305, and 316; Mass spectra of this compound suggested that its molecular mass is 632.87 having characteristic fragments observed at m/z: EIMS *m/z* (rel. int.%): 468[M^+—*p*-coumaric acid] (8), 423 (15), 248 (100), 219 (18), 203 (64), 187 (14), 164 (50), 147 (15), 133 (32); HREIMS *m/z*: 468.3601 [$C_{31}H_{48}O_3$; requires for 468.3603; M^+—*p*-coumaric]$^+$, 423.3624 $[C_{30}H_{47}O]^+$ 248.1774 $[C_{15}H_{23}O]^+$, 203.1796 $[C_{15}H_{23}]^+$, 187.1484$[C_{14}H_{19}]^+$, 164.0472$[C_9H_8O_3]^+$, 147.0441$[C_9H_7O_2]^+$, 133.1012$[C_{10}H_{13}]^+$. These data were identical to those reported for guajanoic acid [172].



C

Characterization of compound D: (β-sitosterol)

Compound D on repeated crystallization from $CHCl_3$-MeOH mixture gave white crystal, m.p. 136-137°C; $[\alpha]_D$ -34°. The elemental analysis revealed that the compound contains 83.86% of C, 12.25% of H and 3.89% of O. The compound gave positive Libermann-Burchard color test for sterol. So, Based upon the functional group analysis it was found that the nature of oxygen was hydroxyl, also supported by IR spectroscopy. IR absorptions bands appeared at 3549.99 cm^{-1} (OH), 2935.73 cm^{-1} (CH_2), 2867.38 cm^{-1} (CH), 1637.63 cm^{-1} (C=C), 1063.34cm^{-1} (C-O) (Fig. 7). Mass spectra of this compound (Fig. 10) suggested that its molecular mass is 414 (M.F.$C_{29}H_{50}O$) having characteristic fragments observed at m/z: 414, 396, 381, 329, 303, 289, 273, 255, 231, 213, 199, 173, 159, 145, 119, 95, 81, 69, 55. The NMR spectrum of this compound showed that this compound having six methyl, eleven primary carbons and three secondary carbons with a hydroxyl group. The carbons of alkenes conjugated are at 140.78 ppm (C5) and 121.72 ppm (C6) which was confirmed from the ^{13}CNMR (Fig. 9). These data were found identical with already reported data for β-sitosterol [7, 148-150]. So, Based on the melting point and other related data (IR, NMR and Mass) the structure of the compound was identified as β-sitosterol.

Fig. no. 7 IR spectrum of β-sitosterol

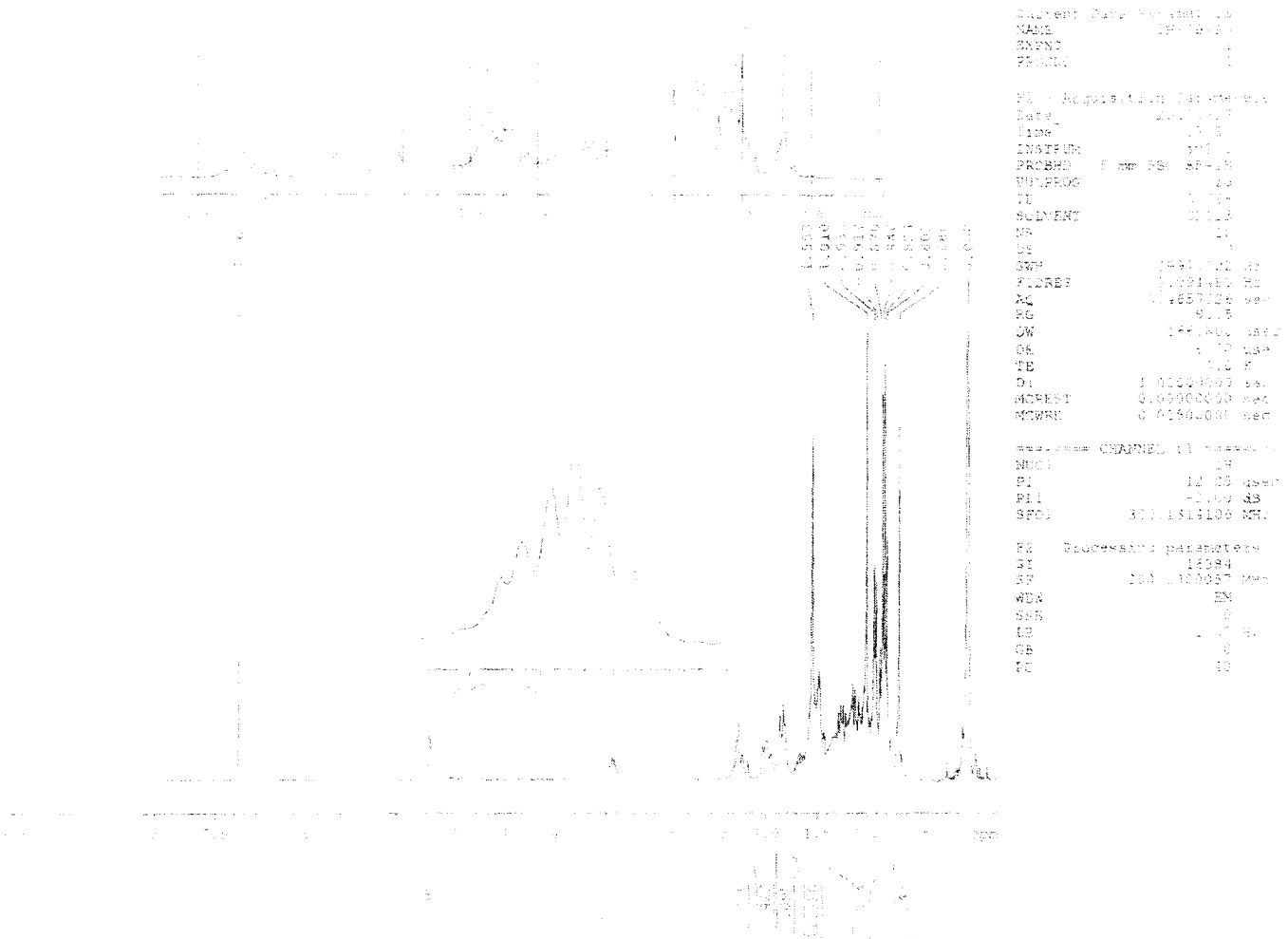


Fig.. 8 ^{1}H NMR spectrum of β-sitosterol

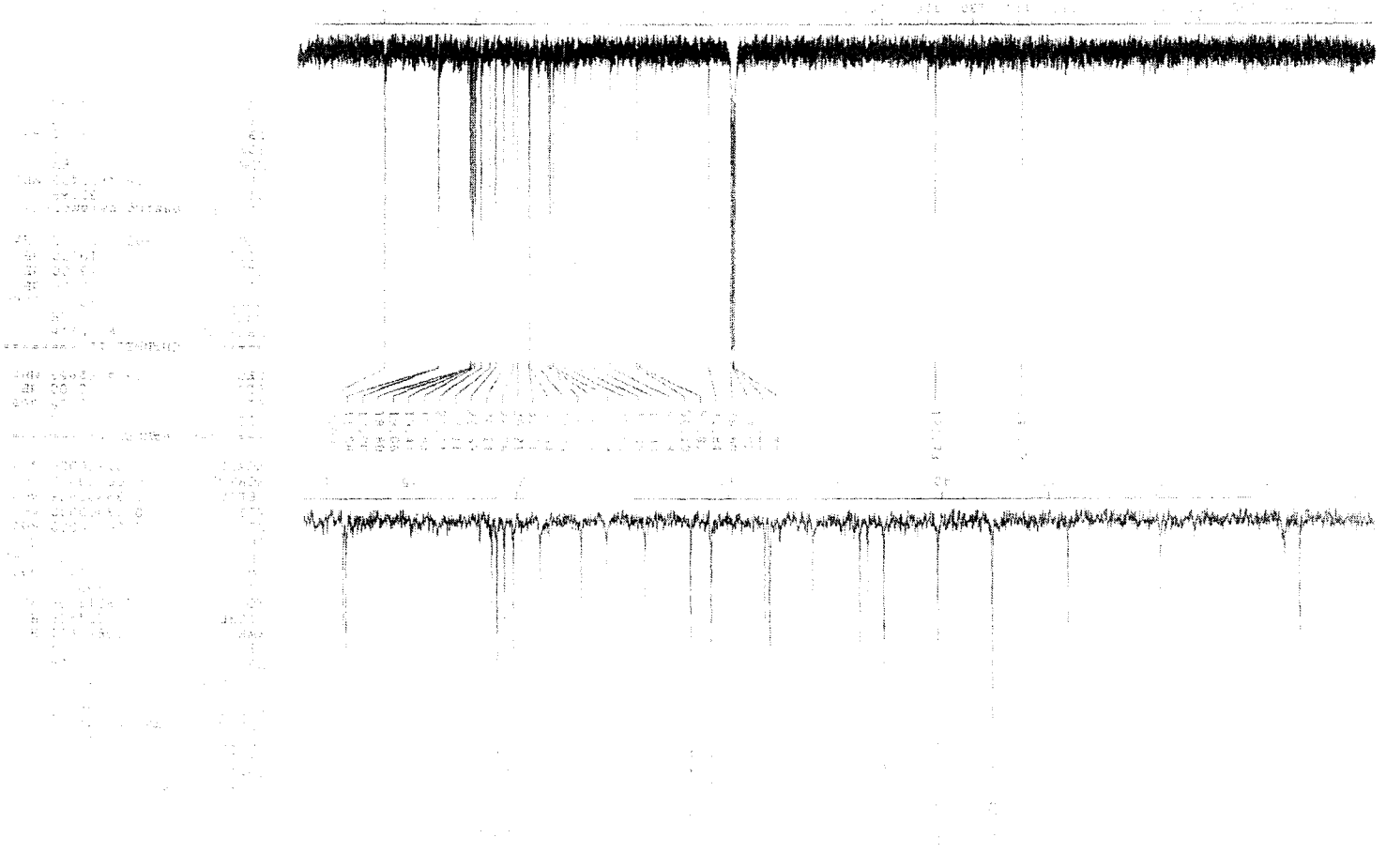


Fig.. 9 ^{13}C NMR spectrum of ß-sitosterol

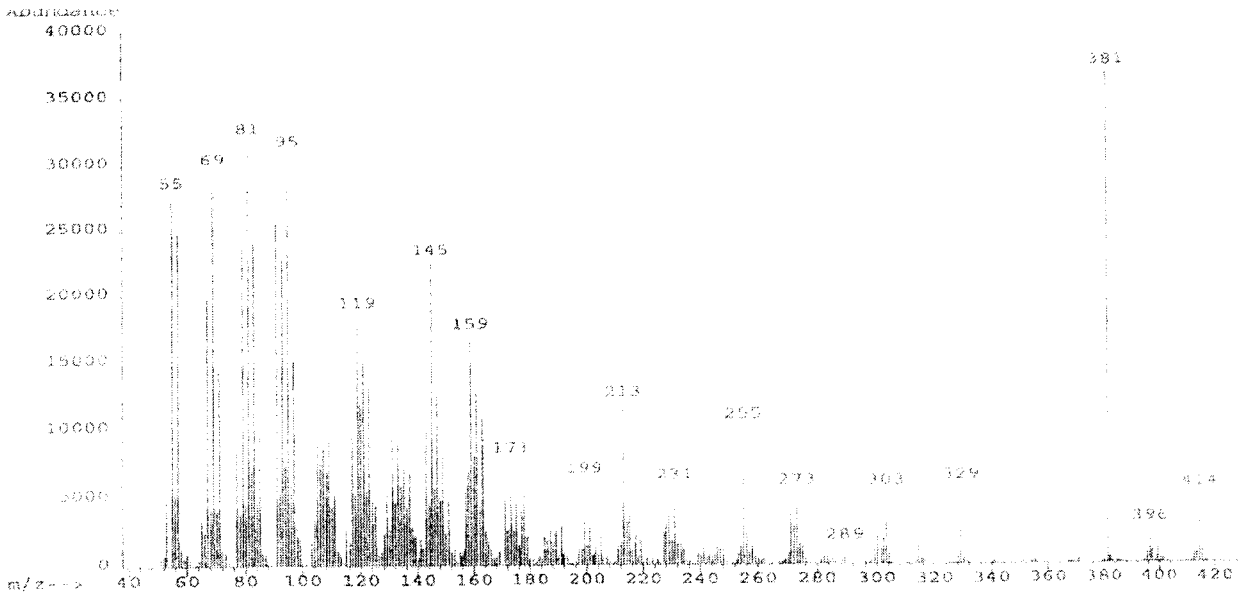
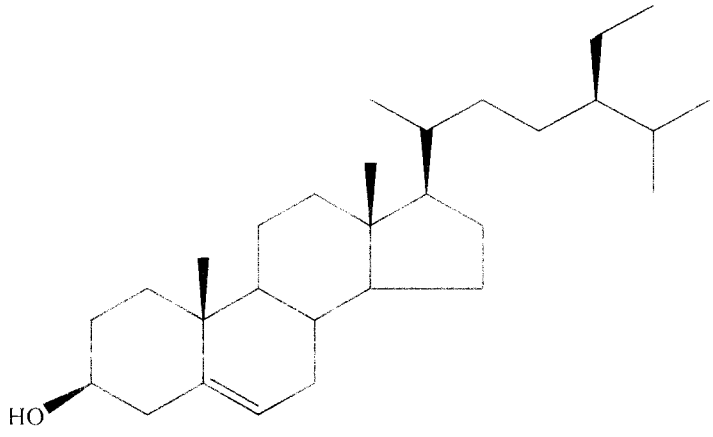


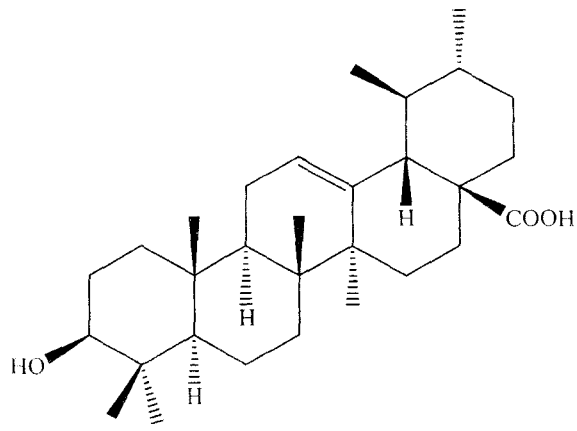
Fig. 10 Mass spectrum of β-sitosterol



D

Characterization of compound E: (ursolic acid)

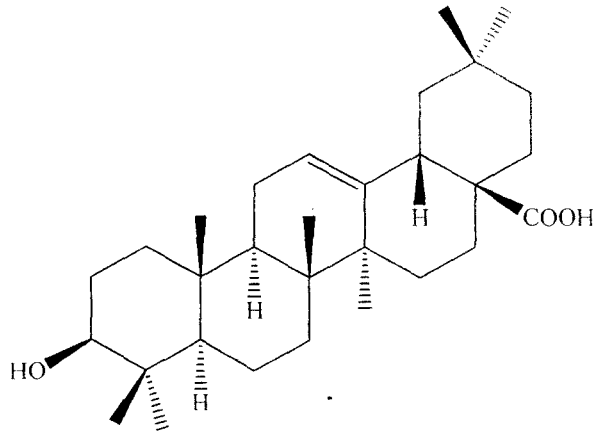
Compound (E) was obtained as a white powder with a melting point of 267-269°C. The elemental analysis revealed that the compound contains 78.90% of C, 10.59% of H and 10.51% of O. Mass spectra of this compound suggested that its molecular mass is 456.36 (M.F.$C_{30}H_{48}O_3$) having characteristic fragments observed at *m/z*: 456.36 (100.0%), 457.36 (33.5%), 458.37% (5.7%). The ^{1}H NMR and ^{13}C NMR data of this compound were consistent with the reported data of ursolic acid [12-14]. It showed no depression in melting point when mixed with authentic sample of ursolic acid.



E

Characterization of compound F: (oleanolic acid)

Compound (F) was isolated as white colorless needles from $CHCl_3$ + MeOH mixture and gave m.p. 305-306°C and $[\alpha]_D$ +78.9°($CHCl_3$). It gave positive colour reaction of triterpenes. The IR spectrum showed absorption bands for hydroxyl group (2640-3400 cm^{-1}), carbonyl of the carboxyl group (1700 cm^{-1}) and trisubstituted double bond (1660 and 820 cm^{-1}). The ^{1}HNMR spectrum of F showed signals for seven methyls as singlets at δ 0.89, 0.90, 0.91, 0.97, 0.98, 1.03 and 1.12. The signal at δ 5.24(1H, J=3.45 Hz) was due to the olefinic proton while the proton germinal to the hydroxyl group was observed at δ 3.60 (dd, J=4.1 and 9.9 Hz). The ^{13}CNMR assignments of various C atoms were substantiated by DEPT experiment which revealed the presence of six methyls, ten primary carbons, five secondary carbons, six tertiary carbons and two olefinic carbons. The physical and spectral data of compound D found in complete agreement to those data published for oleanolic acid [2].



F

Biocidal activity of the isolated compounds

In this present work the *in vitro* antifungal, antibacterial activities and the phytotoxicity of the two isolated triterpenoids have been studied. Five different fungal pathogens namely, *Colletotrichum camelliae*, *Fusarium equiseti*, *Alterneria alternata*, *Curvularia eragrostidis*, *Colletotrichum gloeosporioides* were used for the antifungal study. For antibacterial study *Escherichia Coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Enterobactor* were used as bacterial pathogen. Suitable strains of these organisms were procured from the microbiology laboratory of our institute (for details see experimental). MICs (minimum inhibitory concentration) of the triterpenoids against bacterial and fungal pathogens are reported in Table 1 and 2 respectively.

Table 1. MICs of A, B, C, D, E & F against different bacteria

Compounds	MIC in μg/mL against different strains of bacteria			
	EC	BS	SA	EB
A	150	<100	100	100
B	200	100	200	100
C	150	90	180	80
D	180	80	150	90
E	180	90	170	90
F	170	80	160	90
Amicillin	128	64	64	128

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

Table 2. MICs OF A, B, C, D, E & F against different fungi

Compounds	MIC in μg/mL against different fungi				
	CG	FE	CE	AA	CC
A	4	2.5	10	5	4
B	10	5	10	5	5
C	8	4	8	5	4
D	6	3	4	4	3
E	5	4	4	5	3
F	5	3	3	4	3
Streptomycin	1.25	2.5	2	2.5	2.5

CG- *Colletrichum gloeosporioides*, FE- *Fusarium equisitae*, CE- *Curvularia eragrostidies*, AA- *Alterneria alternata*, CC- *Colletotrichum camelliae*.

Table 3. Antifungal properties A, B, C, D, E & F based on spore germination bioassay

Fungal	Compounds					
	A	B	C	D		F
	PG[a] PI AL[b] (μm)	PG[a] PI AL[b] (μm)	PG[a]PIAL[b] (μm)	PG[a] PI AL[b] (μm)	PG[a]PI AL[b] (μm)	PG[a] PI AL[b] (μm)
CC	00 100 00	0.5 95 4.5	0.4 100 05	00 100 00	04 90 06	05 90 05
FE	00 100 00	00 100 00	00 95 05	08 90 02	05 100 00	00 90 08
AA	00 100 00	00 100 00	00 90 10	0.4 96 3.5	02 90 08	04 92 07
CG	00 100 00	00 100 00	05 90 05	07 85 08	00 95 05	00 100 00
CE	00 95 05	10 95 9.0	8 95 00	10 90 00	05 90 05	10 90 00

CG- *Colletotrichum Gleosproides*, FE- *Fusarium equisitae*, CE- *Curvularia eragrostidis*, AA- *Alterneria 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. All data were taken after 48 h of incubation.

Table 4. Phytotoxicity of the compounds based on the length (cm) of roots after 7 days.

Compound	Concentration (μgm/ml)	Rice	Wheat	Pea
	Control	0.5	1.0	1.64
A	100	0.5	1.12	1.64
	250	0.5	1.12	1.67
	500	0.5	1.12	1.64
	100	0.5	1.21	1.56
	250	0.5	1.22	1.55
B	500	0.5	1.25	1.56
	100	0.4	1.10	1.50
	250	0.5	1.10	1.55
C	500	0.4	1.05	1.48
	100	0.3	1.12	1.55
	250	0.5	1.15	1.57
D	500	0,4	1.15	1.55
	100	0.4	1.10	1.50
	250	0.5	1.12	1.55
E	500	0.4	1.12	1.50
	100	0.4	1.10	1.50
	250	0.4	1.12	1.53
F	500	0.5	1.15	1.55

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

Discussion

The antibacterial activities of six different compounds (A, B, C, D, E, F against bacteria) were tested against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Enterobactor*. Antibacterial activity of amicillin was also tested. The results of the six different compounds were compared with amicillin and the results have been presented in Table-1.

From the result it is evident that all the compounds were effective against bacterial specimen but 'compound A' exhibited better activity in comparison to other five compounds.

Ettebong and Nwafor [174] studied the antimicrobial activities of n-hexane, chloroform, ethyl acetate and methanol extract of *Carpolobia lutea* root which were used as a folk medicine in southern Nigeria against four typed cultures of bacteria namely, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis* and two clinical strains of fungi, namely *Candida albicans* and *Tinea capitis* using agar well diffusion method. They reported that the ethyl acetate extract gave the widest zone of inhibition (21.0 mm) followed by chloroform when tested on *E.coli*.

Murillo-Alvarez *et al.* [175] extracted compounds from plants used in the traditional medicine of *Baja californi* sur (Mexico) using ethanol as a solvent. They tested antimicrobial activities of the isolated compounds. The antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Candida albicans* and *Escherichia coli* was determined and *Aristolochia monticola*, *A. brevipes*, *Hymenoclea sp.* were found to be the most active.

Gangoue-Pieboii *et al.* [176] investigated studied the activities of methanol extract of each plant in disc diffusion assays against 37 species or laboratory strains of seven species of microorganism (*Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus hirae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Candida albicans*). They observed that each of the 10 methanol extracts displayed some degree of antimicrobial activity against at least one species of microorganisms.

The antifungal activities of six different compounds (A, B, C, D, E, F) and there spore germination bioassay were also tested against *Colletotrichum camelliae*, *Fusarium equiseti*, *Alternaria alternata*, *Curvularia eragrostidis*, *Colletotrichum gloeosporioides.* Antifungal activity of Streptomycin was also tested. The results of the six different compounds have been presented in Table-2 and 3.

From the result it is evident that all the compounds are active against all the tested fungi but compound A demonstrated better result with respect to other compounds. The observations are in accordance with the structure activity relationship as reported elsewhere[17-20]

Hassanein *et al.* [177] studied leaf extracts of neem (*Azadirachta indica*) and chinaberry (*Melia azedirach*) against two tomato pathogenic fungi *Alternaria solani* and *Fusarium oxysporum*, the causal agents of early blight and wilt diseases of tomato plant respectively. Usha *et al.* [178] reported that floral malformation caused by *Fusarium mangiferae* is a serious threat to mango cultivation in various countries.

In the present study, six different compounds have been tested to determine their efficacy against the five pathogens, *Colletotrichum camellie*, *Fusarium equisitae*, *Alternaria alternata*, *Curvularia eragrostidis*, *Colletotrichum gloeosporioides*. The objective of use of fungicides in the present study is to compare the efficacy of fungicides with that of botanicals.

In order to show phytotoxic activities of the compounds solution of different concentrations of different compounds were prepared and applied to check germination of root. The phytotoxic effects of compound A, B, C, D, E, F, on the germination of *Triticum aestirium* (wheat), *Oryza sativa* (rice) and *Pisum sativum* (pea) seeds have been summarized in Table 4.

In case of rice all concentration showed very less effects on the root germination compared to control set. For wheat all the compounds at different concentration showed root germination but compound D showed better result in comparison to other compounds.

In case of pea all the compounds showed activity on the root germination in comparison to control set but compound A showed better result with respect to other compounds.

Therefore, the outcome of the investigation not only would enrich the understanding of structure and their biological activities among the lupane type of triterpenoid groups of natural products, but at the same time would provide a scientific base to the folk medicine culture in the tribal area.