Chapter 2

TRITERPENOIDS FROM *Schleichera oleosa* OF DARJEELING FOOTHILLS AND THEIR ANTIMICROBIAL ACTIVITY

Introduction

Darjeeling hills and Tarai region of West Bengal are full of flora and more than 6000 different plant species having medicinal value. The tribal medicinal practice in the above region provides the evidence of the utilization of medicinal plants by the local people as a folk lore. In this region peoples believing these medicinal plants more rather than as usual medicines. A good number of them, such as *Schleichera oleosa, Psidium guajava, Biscofia javanica, Xanthoxylum budrunga Dioscoria prazziri, Dysoxylum proceru* etc. reported to synthesis terpenoids / triterpenoids in them.

However despite their widespread existence in the vicinity of our University and their direct and indirect defensive activities, systematic study regarding their biocidal activity has not been attempted so far. As a consequence of that the author has undertaken the present investigation on some of the medicinal plants to make a systematic study of biocidal activity of the isolated triterpenoids as well as on some of their prepared derivatives (that will be described in the next part of the thesis).

Schleichera oleosa, commonly known as Ceylon Oak belongs to the family of sapindaceae[1] occurs naturally in the foot hills of Darjeeling and used in traditional medicine for a long time. It is generally used as analgesic, antibiotic and against dysentery [125]. Oken is a well known medicinal plant [2] in the Teak forest of east Java and is also available this northern part of West Bengal India and is used as a commercial lac host for obtaining sticklac for production of seedlac/shellac [126]. In parts of southern India, *Schleichera oleosa* is a prominent bee plant for nectar and is used for production of kosum cake, and animal feed stock. The oil obtained from its seed , called Kosum oil or Macassar oil is traditionally used for the cure of itch, acne, burns, other skin troubles, rheumatism (external massage), hair dressing and promoting hair growth [127]. Very recently Mohapatra and Sahoo have reported the use of its bark along with water to treat

menorrhoea [128]. Previous studies have revealed the presence of several bioactive triterpenoids in the extract of this plant as a whole. In an ongoing search for bioactive triterpenoids from *Schleichera oleosa* collected from the foothills of Darjeeling the benzene extract was selected for further investigation. Two novel triterpenoids, taraxerone and tricadenic acid were isolated from the benzene extract of the outer bark of *Schleichera oleosa* available in Darjeeling foothills. A preliminary study on their biological activities was also done against some fungal and bacterial specimen. The structure of these compounds was determined by chemical and spectral data and by comparison with the spectral data of the already reported compounds. This is the first report of the existence of taraxerone (**A**) and trichadenic acid A (**B**) in the above plant.

Results and Discussion

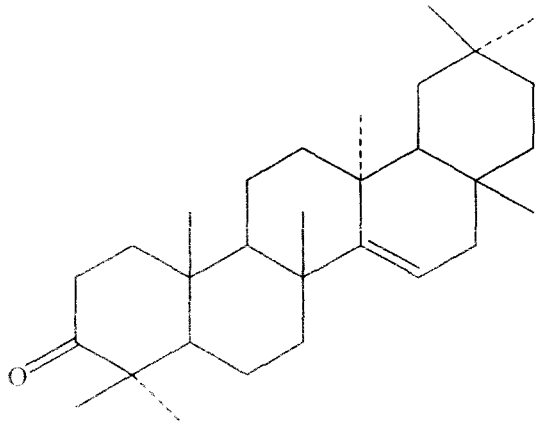
Isolation of the compounds

The powdered plant material was extracted with benzene in a soxhlet apparatus for 72 hours. The solvent was recovered that yielded a deep brown gummy residue. This crude benzene extract of the plant was then purified over a column of silica gel of 60-120 mesh using petroleum ether (PE) and PE-ethyl acetate (EA) with increasing concentration of the eluent. Four components were separated (A – D) of which identified as taraxerone (**A**) and trichadenic acid A (**B**) along with betulinic acid and betulin (for details please see experimental). The structures of the compounds were determined based on their chemical and spectral data (IR, UV and NMR). All these compounds evaluated for their antimicrobial activities.

Characterization of compound A (taraxerone)

Compound A was crystallized from chloroform methanol mixture to afford white needle shaped crystals of m. p. 237-239°C. It showed I.R absorption at ν_{max} 3047.3, 2924.8, 2851.6, 1708.7 (carbonyl gr.), 1375.2 and 955.2cm^{-1}. ^{1}H NMR spectra (δ, ppm) showed peaks between 0.82 to 1.21 ppm for eight tertiary methyls and at 5.51 (dd, 1H, J = 8, 3.2 Hz) for the olefinic proton at C-15. All other peaks are for the presence of saturated methylene and methine protons. ^{13}C NMR (δ, ppm) spectra showed the presence of all the 30 carbons in which eight methyl peaks appeared at 28.1(C-23), 15.60(C-24),

14.26(C-25), 29.58(C26), 30.0(C-27), 29.88(c-28), 33.23(C-29) 21.4 (C-30) ; ten primary carbons at 40.65(C-1), 33.1(C-2), 33.59(C-6), 34.18(C-7), 35.12(C-11),36.69(C-12), 41.4(C-19), 36.26(C-21),27.29(C-22) ; three secondary carbons at 55.8(C-5), 48.8(C-9), 48.88(C-18) ; six tertiary carbons at 47.72(C-4), 37.83(C-8), 38.49(C-10), 37.6(C-13), 33.89(C-17), 29.5(C-20) and two olefinic carbons at 157.7(C-14),117.0(C-15) ppm. The carbonyl carbon at C-3 appeared at 210.1 ppm. These data were found identical with already reported data for taraxerone [23, 50].



A

Characterization of compound B (trichadenic acid A)

Compound B was isolated as a white solid. Crystallization from $CHCl_3$ - MeOH mixture afforded white needle shaped crystals of m. p 294°C, $[\alpha]_D$= +25.0°. IR spectra exhibited peaks at ν_{max} 3300 cm^{-1} for OH group, and at 1685 cm^{-1} for the carboxylic acid group (Fig. 1), corresponding methyl ester has the m. p. 200-201°C. ^1H spectra showed the presence of six tertiary methyl groups that appear as singlets at δ 0.81, 0.85 (d, 3H J = 6Hz), 0.93, 0.99, 1.11, 1.20 and a secondary methyl that appear as a doublet at 0.74 (d, 3H, J = 7Hz); 3.76 (s, 3H) for -$COOCH_3$ group. Mass spectra of this compound suggested that its molecular mass is 458.58 (M.F.$C_{30}H_{50}O_3$) having characteristic fragments observed at m/z: 458.38 (100.0%), 459.48% (34.2%), 460.38%. The elemental analysis revealed that the compound contains 78.48% of C, 10.80% of H and calculated for $C_{30}H_{50}O_3$ 78.69%, 10.92%. The

physical and spectral data of B found in complete agreement to those data published for trichadenic acid A130.

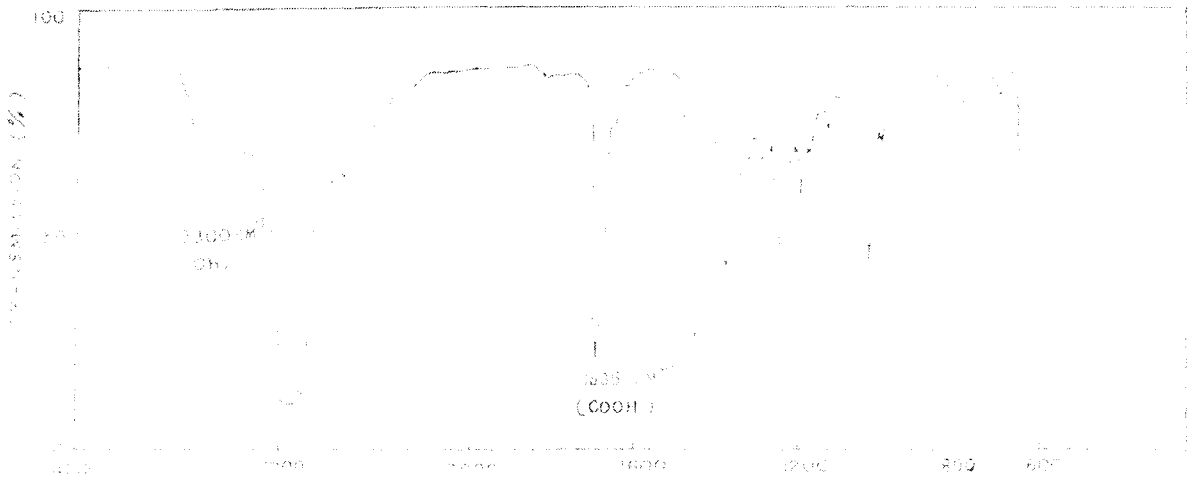
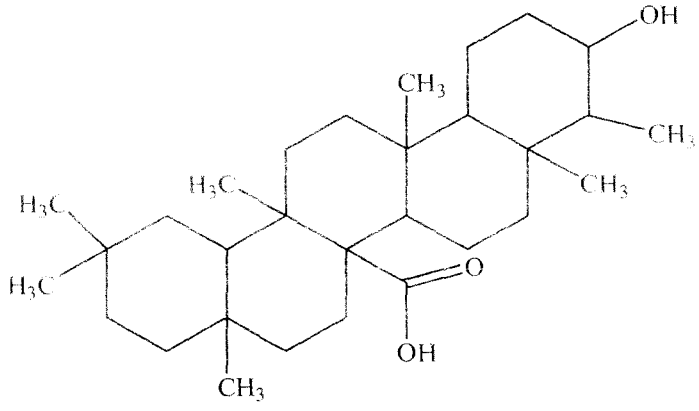


Fig.1 IR spectrum of trichadenic acid



B

Characterization of compound C (betulinic acid)

Compound C 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 ^1H NMR spectrum (Fig. 2) revealed signals for five tertiary methyls δ_H 0.65, 0.75, 0.90, 0.96 and 0.98, a vinyl methyl δ_H = 1.97 (broad d, J = 0.5 Hz), a

secondary carbinol $\delta_H = 3.16$ (dd, J = 9.5 and 6.0 Hz) and $\delta_H = 2.95$ (dd, J = 9.0, 6.0and 0.5 Hz) an exomethylene group $\delta_H = 4.55$ (1H, d, J = 0.4 Hz) and $\delta_H = 4.65$(1H, d, J = 0.4 Hz). These data indicated a pentacyclic triterpenoid of betulinic acid, confirmed by comparison with already published data [126-127]. The ^{13}C NMR spectrum (Fig. 3) of (C) showed six methyl group at δ_C 27.9 (C-23), 15.4 (C-24), 16.2 (C-25), 16.3 (C-26), 14.6 (C-27), 19.6 (C-30) and exomethylene group at δ_C 150.0 (C-20), 108.8 (C-29) and a secondary carbon bearing hydroxyl at δc 79.0 (C-3) and a carboxyl group at $\delta_C = 180.6$ (C-28) in addition to ten primary carbon atom, five secondary carbon atom and five tertiary carbon atom. These data were identical to those reported for betulinic acid [158-161].

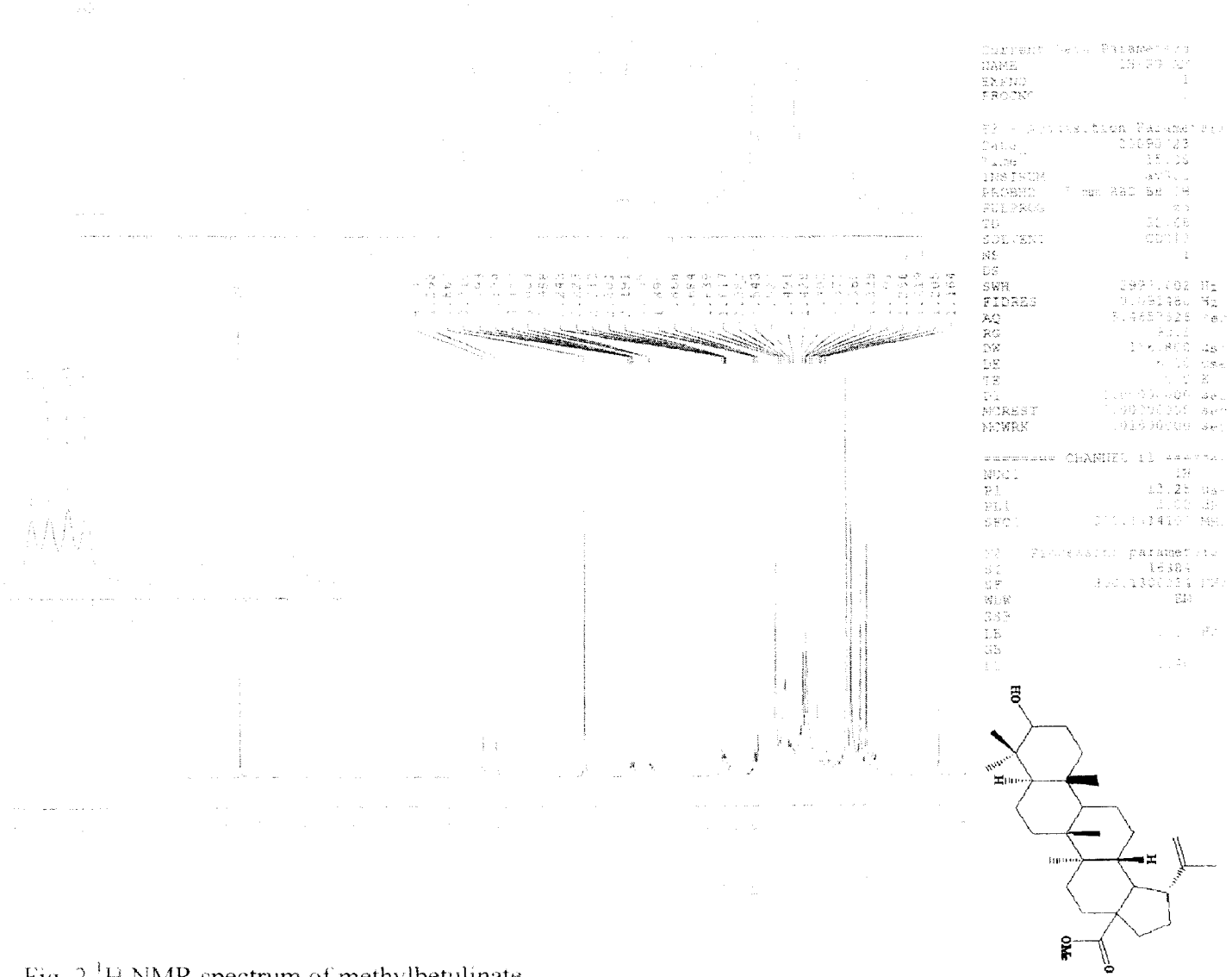


Fig. 2 ^{1}H NMR spectrum of methylbetulinate

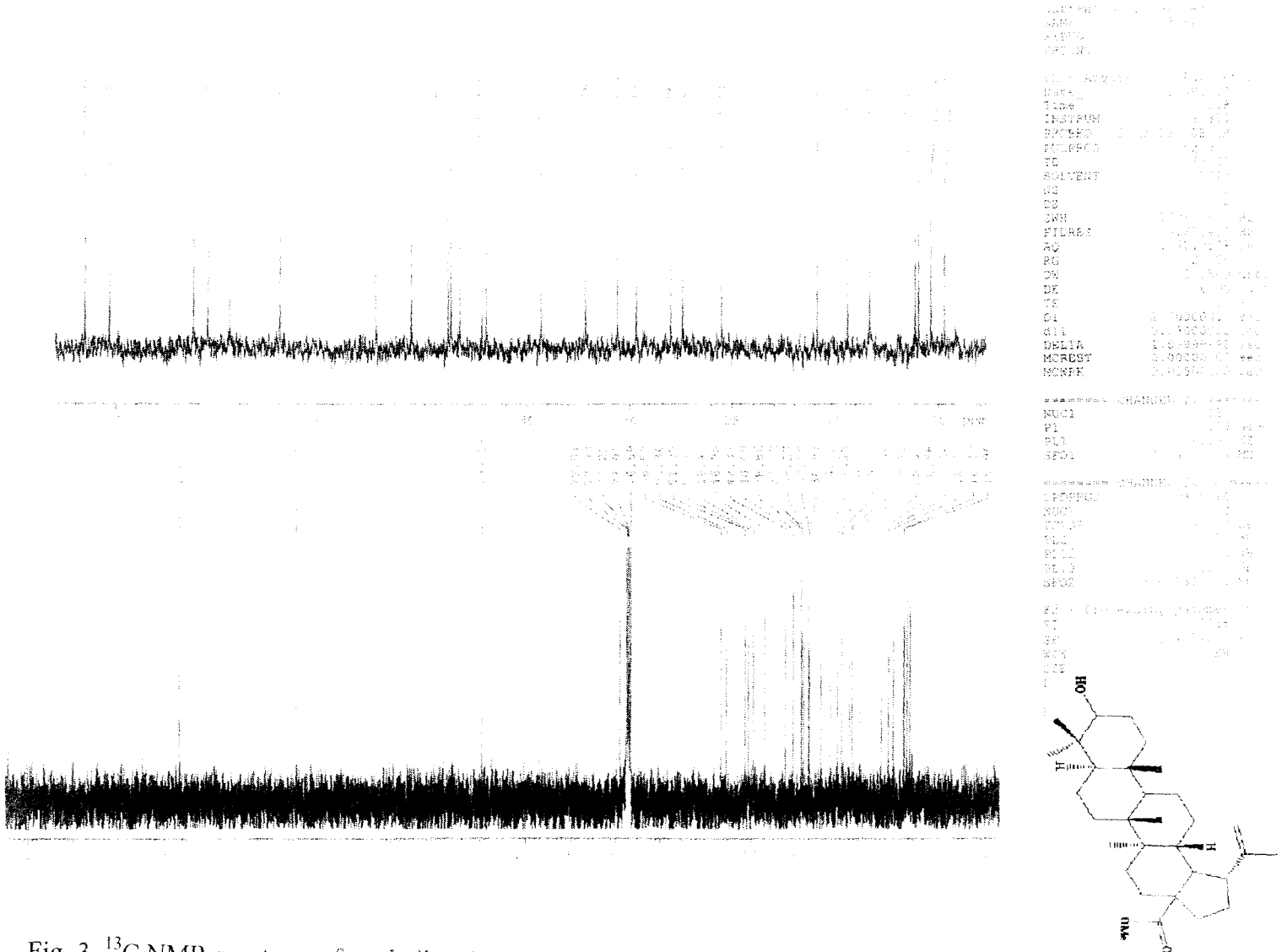
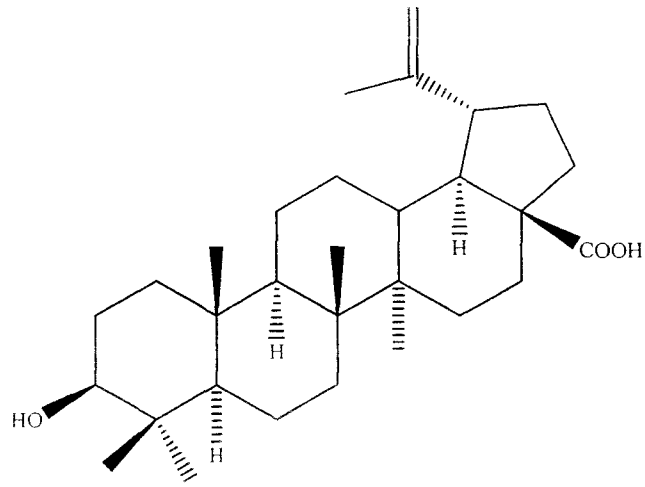


Fig. 3. ^{13}C NMR spectrum of methylbetulinate



C

Characterization of compound D (betulin)

The pet. ether: benzene fractions of compound D was further purified by crystallization method using chloroform and methanol as solvent to obtain colourless needles of D, m.p. 251-252°C and $[\alpha]_D$ +20°($CHCl_3$). The HRMS exhibited the moleculer ion peak at *m/z* 442.3562. The IR spectrum showed absorption bands at 3430(OH), 3070, 1635 and 880 (terminal methylene group). The ^{1}HNMR spectrum (Fig. 4 & 5) displayed signals due to six tertiary methyl groups at δ 0.68, 0.72, 0.80, 0.91, 0.97 and 0.99 (3H, each s). It also showed a signal due to carbinolic proton at δ 3.25 (dd, J=9.6, 1.2 Hz). Its chemical shift and coupling constant led us to assign β-- and equatorial configuration of hydroxyl gr. at C-3. A further signal at δ 3.91 (2H, s) could be assigned to the methylene carbon attached to a hydroxyl gr. The ^{13}CNMR assignments of various C atoms were substantiated by DEPT experiment. The ^{13}C NMR (δ, ppm) spectra (Fig. 6) showed the presence of all the 30 carbons in which six methyl peaks appeared at δ_C 28.0 (C-23), 15.9 (C-24), 16.0 (C-25), 18.3 (C-26), 15.3 (C-27), 20.8 (C-30) and exomethylene group at δ_C 150.0 (C-20), 108.8 (C-29) and a secondary carbon bearing hydroxyl at δc 78.9 (C-3) and a in addition to ten primary carbon atom, five secondary carbon atom and five tertiary carbons. These data were identical to those reported for betulin [23, 50].

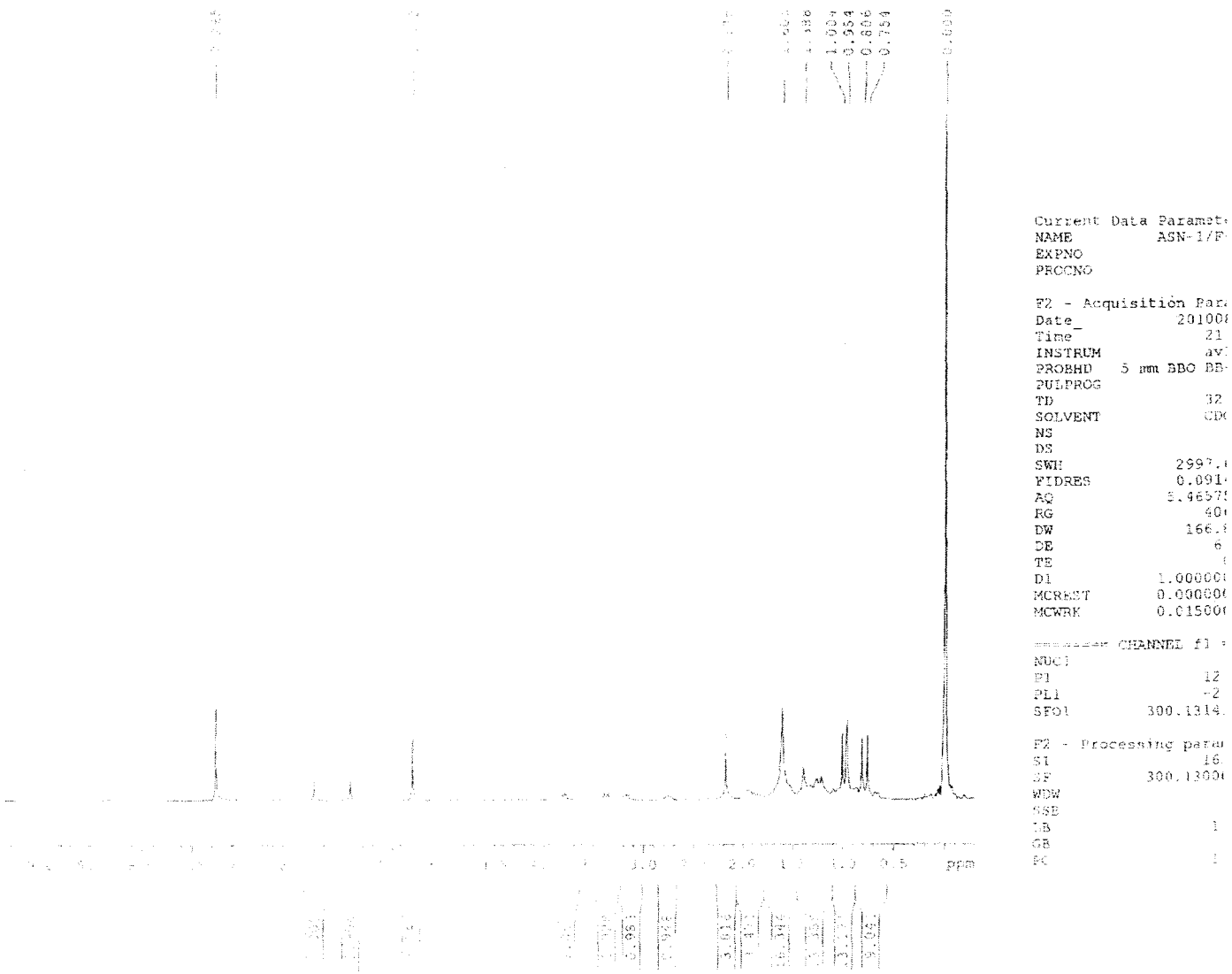


Fig. 4. ^{1}H NMR spectrum of betulin

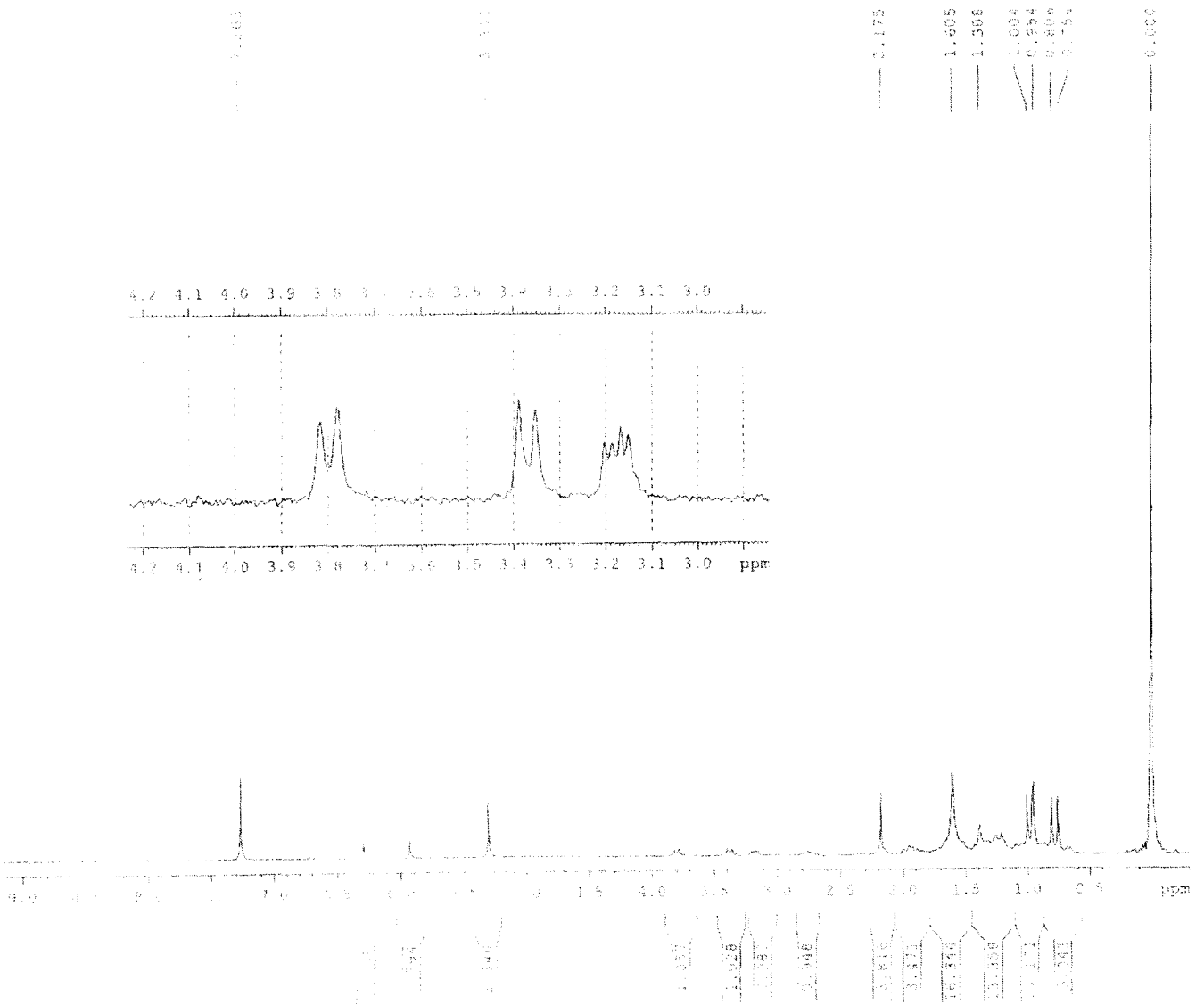


Fig. 5. ^{1}H NMR spectrum of betulin

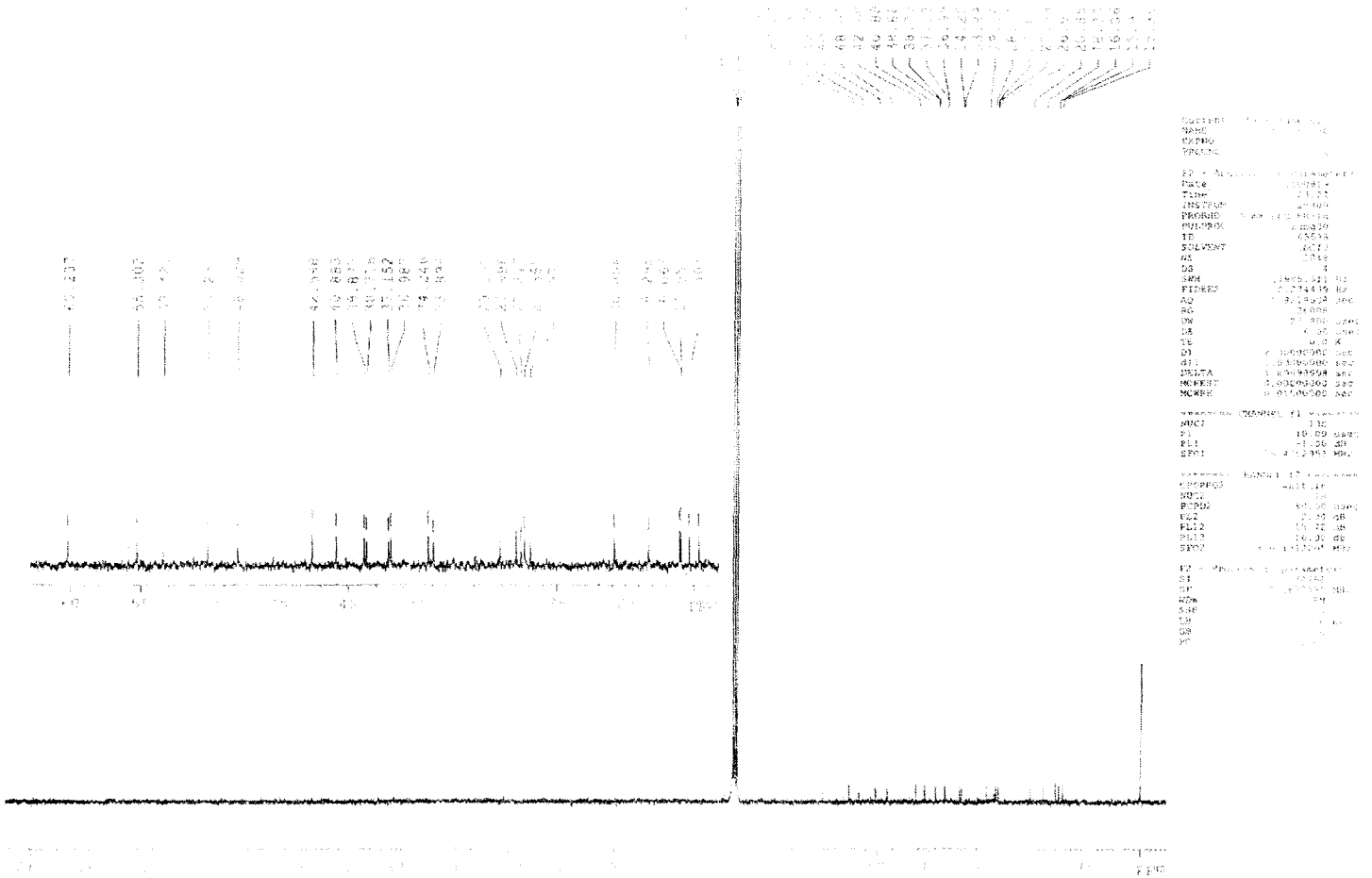
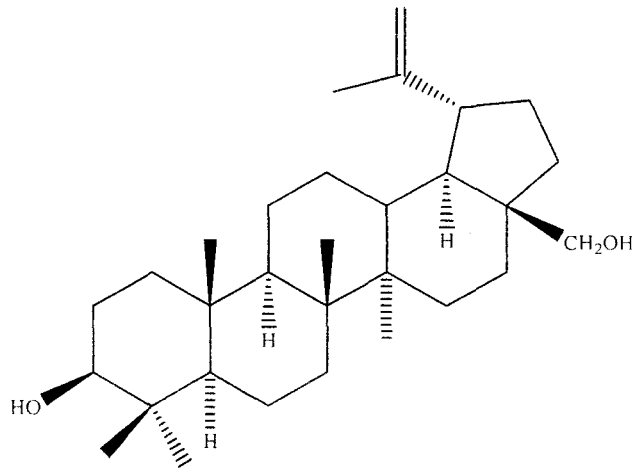


Fig. 6. ^{13}C NMR spectrum of betulin



D

Biocidal activity

We studied both the *in vitro* antifungal and antibacterial activity of all isolated triterpenoids. Five different fungal pathogens namely, *Colletotrichum camelliae*, *Fusarium equiseti*, *Alternaria alternata*, *Curvularia eragrostidis*, *Colletrichum gloeosporioides* were used for the antifungal study and for antibacterial study *E. Coli*, *B. Subtilis*, *S. aureus*, *Enterobactor* were used as bacterial pathogen. 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 pathogen are reported in table 1& 2 respectively. 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 48h. 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 spore germination by wet chamber method was also used for determination of antifungal activity [108] (Table 3). We compared the antifungal activities of the compounds with streptomycin and antibacterial activity with ampicillin, a β-lactam antibiotic.

Table 1. MICs of compound A, B, C and D against different bacteria (Agar cup method).

Compounds	MIC in μg/mL against different strains of bacteria			
	EC	BS	SA	EB
A	100	<100	100	100
B	150	100	200	100
C	140	100	150	130
D	130	100	140	120
Ampicillin	128	64	64	128

BS- *B. subtilis*, EC- *E. coli*, SA- *S. aureus*, EB-*Enterobactor*, MIC- Minimum inhibitory concentration.

Table 2. MICs of compound A, B, C and D against different fungi. (Agar cup method)

Compounds	MIC in μg/mL against different strains of fungi				
	CG	FE	CE	AA	CC
A	<5	20	40	10	<5
B	4.87	19.5	40	19.5	39
C	4.50	19.0	38	18	25
D	4.40	18.0	38	17	26
Streptomycin	1.25	2.5	<2.5	2.5	2.5

CG- *Colletrichum Gleosproides*, FE- *Fussarium equisitae*, CE- *Curvularia eragrostidies*, AA- *Alterneria alternata* , CC- *Calletotricheme camellie*.

Table 3. Evaluation of antifungal properties of reported triterpenoids against five virulent pathogens by spore germination bioassay (wet chamber method) after 48 h of incubation [108].

Fungal pathogen	Compounds							
	A			B			C			D		
	PG[a]	PI	AL[b] (μm)	PG[a]	PI	AL[b] (μm)	PG[a]	PI	AL[b] (μm)	PG[a]	PI	AL[b] (μm)
CC	00	100	00	05	95	4.5	00	95	04	00	96	05
FE	00	100	00	00	100	00	00	100	03	00	100	04
AA	00	100	00	00	100	00	00	100	00	00	100	05
CG	00	100	00	00	100	00	00	100	00	00	96	00
CE	05	96	06	00	100	00	00	95	00	05	100	05

CG- *Colletrichum gleosporioides*, FE- *Fussarium equisitae*, CE- *Curvularia eragrostidis*, AA- *Alternaria alternate*, CC- *Calletotricheme camellie*. PG-Percent germination, PI-Percent Inhibition, AL-Average germ tube length, [a]Based on 200 spores, [b]Based on 25 germ tubes.

Discussions

All the compounds showed prominent antimicrobial activities against the tested fungal and bacterial pathogens as were observed from the experimental results (Table 1, 2 & 3).

In case of MICs against different bacteria, compound-A showed better activity against all the tested microorganisms (*B. subtilis*, *E. coli*, *S. aureus*, *Enterobactor*) than that of compound B, C, D and their activity was compared with ampicillin.

Murillo-Alvarez *et al.* [132] tested some isolated compounds which showed antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus, Streptococcus faecalis, Candida albicans* and *Escherichia coli.*

In case of MICs against five different fungi (*Colletrichum gloeosproides, Fusarium equiseti, Curvularia eragrostidis, Alternaria alternata, Calletotrichum camelliae*) it was found that the activity of compound A is nearly comparable to Streptomycin and compound A and B showed better activity against all the microorganisms in comparison to C and D (Table 2).

Kanauchi *et al.* [133] showed that the extract of cocklebur (*X. strumarium)* contains xanthatin, an antibacterial substance. Minimum inhibitory concentrations (MICs) of xanthatin were 12.5-100µg/ml against *Bacillus* sp. MICs of xanthatin were 25-100 µg/ml against *Candida* sp., *Pichia* sp., *Saccharomycopsis* sp. and *Torulaspora* sp. They also suggested that xanthatin from cocklebur leaf extract against *Bacillus* sp. and some film-forming yeast may be used to prevent contamination in koji and during production of alcoholic beverages. Compound from *Terminalia arjuna* were found to be effective against *Fusarium equiseti* and other two pathogens by Digrak *et al.* [134]. The leaf extract of *Clerodendrum viscosum* completely checked the radial growth of the test fungus *Curvularia lunata*. The leaf extract 1:10 dilution was the most successful for the inhibition of the test fungus in term of its growth (Parimelazhagan and Franchis ,[135]).

In the present study, four different compounds (A, B,C and D) have been tested to determine their efficacy against the five pathogens, *Colletrichum gleosproides*, FE-*Fussarium equisitae*, *Curvularia eragrostidies*, *Alterneria alternata*, *Colletotrichum camellie*. Minimum inhibitory concentrations (MIC) were determined against the fungal pathogens. The objective of use of fungicides in the present study is to compare the efficacy of fungicides with that of botanicals. It was found that MICs of compound A against different fungi was comparable to Streptomycin. Among the four compounds, compound A and B showed better activity against all the other microorganisms (Table 2). Raghavendra [136] compared the antifungal activity of alkaloid extract of *Prosopis juliflora* (Sw.) DC. (Mimosaceae) against *A. alternata* with synthetic fungicides viz., blitox, captan, dithane M-45 and thiram. They found that alkaloid extract was effective even at the dosage lesser than the synthetic fungicides.

In case of antifungal activities against five virulent pathogens by spore germination bioassay it was found that compound B and D only showed germination against *C. camellie* (5%) and *C. eragrostidies* (5%) at 100 ppm and 500 ppm concentration respectively. From the result it was found that all the compounds inhibited the spore germination against all the tested microorganisms and some compounds also have effect on the germ tube length elongation.

Initially 80 plant extracts (50% ethanolic) were screened *in vitro* for their antifungal properties against the four pathogens (*F. equiseti, C. gloeosporioides, A. alternata* and *P. theae*) following spore germination technique. Spore germination is a determining factor for the pathogen during the early phase of host colonization (Egley, 137). Use of alcoholic extracts for screening of antifungal properties has been reported by several authors (Shalini and Srivastava, [138]; Veljic *et al.* [139]; Vukovic *et al.* [140]; Singh and Karnwal, [141]). In the present study, benzene extract of compound A (from *Schleichera oleosa* plant) was effective in controlling spore germination (100% control) of CC, FE, AA, CG; similarly, compound B was effective against FE, AA, CG, CE; compound C was effective against CC, FE, AA and compound D was effective against FE, AA, CA. About seventeen other plants have also shown antifungal potentiality (>80% inhibition) against these two pathogens. Leaf extract of *Datura metel*, *X. strumarium* and *D. stramonium* exhibited 100% of inhibition of spore germination of *A. alternata*. Spore germination of *P. theae* was completely inhibited by leaf extract of *Polyalthia longifolia*, *X. strumarium* and *D. stramonium*. Many authors have reported the antifungal potentiality of several plant extracts through spore germination bioassay (Prusky *et al.* [142]; Rahmani *et al.* [143]; Abou-Jowdah *et al.* [144]; Kim *et al.* [145]) showed that crude extracts of *Xanthium strumarium* inhibited zoospore germination of *Phytophthora drechsleri*, the causal agent of Atractylis rot, *in vitro*.

Finally, it can be concluded that the present study will be extremely helpful to enrich the present knowledge about the structure activity relationship for these type of triterpenoid skeleton and also help the researchers to design newer generation of drugs based on such triterpene skeleton as well.