PHYTOCHEMICAL INVESTIGATION OF SOME MEDICINAL PLANTS, TRANSFORMATIVE REACTION ON THE ISOLATED ORGANIC COMPOUNDS AND STUDIES ON THEIR BIOLOGICAL ACTIVITIES.

THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY (SCIENCE) OF
THE UNIVERSITY OF NORTH BENGAL

BY
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WEST BENGAL, DARJEELING, INDIA
2010

DECLARATION

The research work embodied in this thesis entitled **"Phytochemical investigation of some Medicinal Plants, Transformative reaction on the isolated Organic compounds and studies on their Biological Activities"** has been carried out in the Department of Chemistry, University of North Bengal, Darjeeling and partly in the Raiganj College (University College) under the supervision of Dr. Pranab Ghosh, Department of Chemistry and Dr Aniruddha Saha, Department of Botany, University of North Bengal, Darjeeling. To my belief this thesis or any part of it has not been submitted before at any University or Institution for Ph.D. or any other degree or diploma.

Date: 23.11.10

Mohammed Golam Rasul

Place: N B U

(Mohammed Golam Rasul)

Acknowledgement

The present thesis embodies the results of research work carried out and completed with the excellent facilities made accessible to me by Department of Chemistry and Department of Botany, University of North Bengal and some part was carried out in the Department of Chemistry, Raiganj (University) College, Raiganj, North Dinajpur.

I would like to express my deep sense of gratitude and thankfulness to my supervisor Dr. Pranab Ghosh, Associate Professor in Chemistry and Co-supervisor Dr. Aniruddha Saha, Associate Professor in Botany, University of North Bengal, for their valuable suggestions, guidance and continued interest during the progress of the work.

The work embodies in this thesis would have never been accomplished without invaluable continuous encouragement, keen interest and precious attention of my research Supervisor Dr. Pranab Ghosh without him I would not been able to complete the work.

This is a hard job to include the names of all the persons who have involved directly or indirectly in the accomplishment of this work. However, I am very grateful of all my teachers since my School days to University. I think without being trained enough, I would have not been able to enter into the area of investigation of Natural Products.

I would like to express my thanks to the authorities of the North Bengal University for providing Laboratory and Library facilities. I am indebted to Dr. A. K. Nanda, Associate Professor in Chemistry for most of the ^{1}H NMR and ^{13}C NMR spectra recorded in this thesis; to Dr. A. Patra, Department of Chemistry, University college of Science, Kolkata for some of the spectral data. I am also grateful to the authorities of the Sukhna forest, Darjeeling, for supplying the plant materials.

I would also like to express my sincere thanks to Prof. B. P. Pradhan and Prof. S. N. Bose for their valuable suggestions.

I would like to thanks to my fellow research workers, my colleagues and friends for their kind help, suggestions and word of encouragement for the compilation of the thesis.

Finally, I would like to express my deepest appreciation to my parents and family members for their love, support, constant care and encouragement during the work.

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Summary

Investigation embodied in this present thesis entitled "**Phytochemical investigation of some medicinal plants, transformative reaction on the isolated organic compounds and studies on their biological activities**" were initiated under the supervision of Dr. Pranab Ghosh and Dr. Anirudha Saha at the Department of Chemistry and Department of Botany, respectively, University of North Bengal, Darjeeling-734013.

The work carried out in this thesis is divided into two parts, Part I and Part II. The principal theme of this thesis is the phytochemical investigation of the medicinal plants which are available in Darjeeling district and used as folk lore, transformative reactions of the isolated compounds and studies on their biocidal activities.

Part 1

Phytochemical investigation of some medicinal plants and studies on the biological activities of the isolated compounds.

Part I has been divided into four chapters.

Chapter I

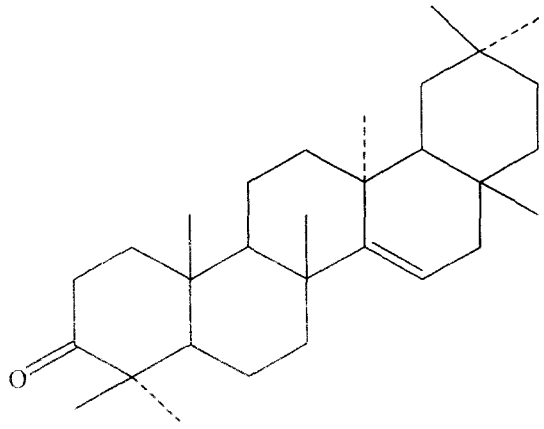
This chapter comprises a short review on the phytochemical investigation of medicinal plants and their biological activity of isolated materials from medicinal plants. This chapter is divided into two sections, Section A and Section B.

Section A comprises a short review on the phytochemical investigation of medicinal plants and **Section B** comprises a Short review on the biological activity of isolated materials from medicinal plants

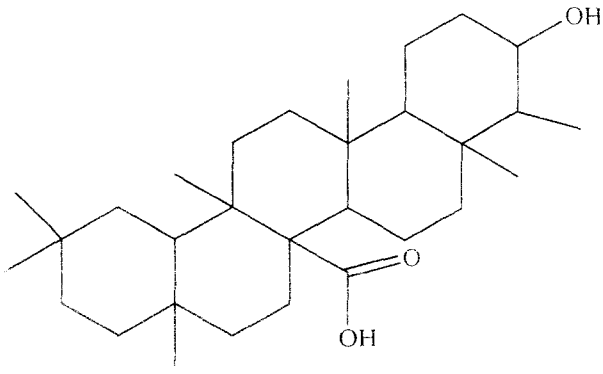
Chapter II

This chapter contains isolation and characterization of triterpenoids isolated from *Schleichera oleosa* commonly known as Ceylon Oak of Darjeeling foothills and studies their antimicrobial activity. A total of four triterpenoids taraxerone (A), trichadenic acid A (B), betulinic acid (C) and betulin (D) have been isolated from this plant. Out of these four terpenoids, taraxerone (A) and trichadenic acid A (B) were detected for the first time from

Schleichera oleosa. The preliminary study on their biological activities were studied against some fungal and bacterial specimen such as *Colletrichum Gleosproides*, *Fussarium equisitae*, *Curvularia eragrostidies*, *Alterneria alternata* , *Calletotricheme camellie*, *B. subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Enterobactor*. The structure of these compounds was determined by chemical and spectral data and by comparison with the spectral data of the already reported compounds.



A



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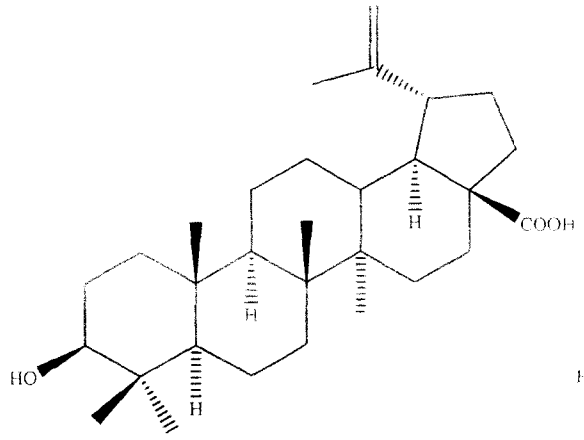

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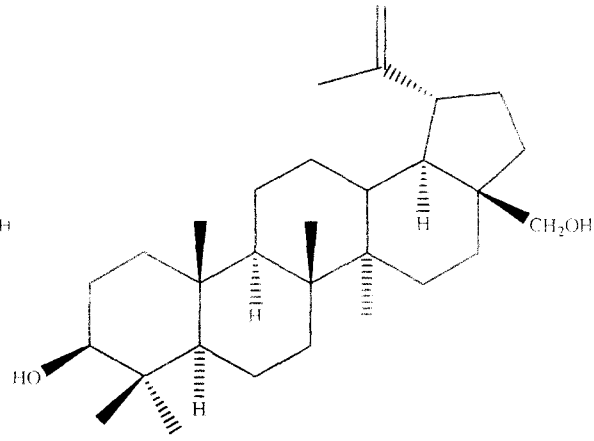

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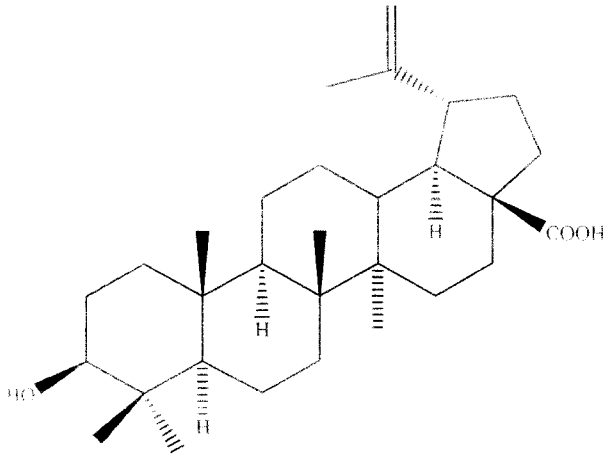
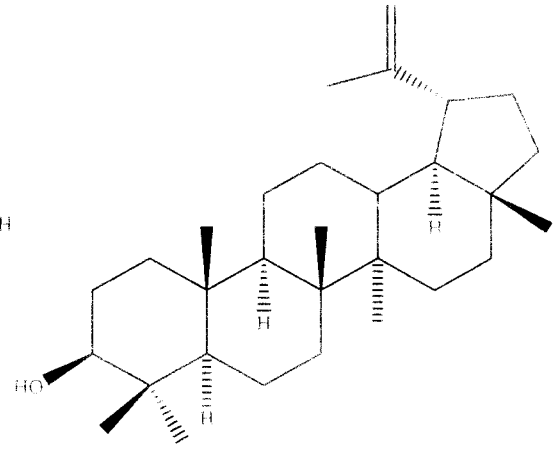
Chapter III

This chapter contains isolation and characterization of triterpenoids isolated from *Psidium guajava* of Darjeeling foothills and studies their antimicrobial and phytochemical activity. A total of six triterpenoids such as betulinic acid (**A**), lupeol (**B**), guajanoic acid (**C**), β-sitosterol (**D**), ursolic acid (**E**) and oleanolic acid (**F**) have been isolated from this plant. Out of these six compounds, betulinic acid (**A**) and lupeol (**B**) is the first report of isolation from the leaf extract of *P. guajava* available plenty in the foothills. The preliminary studies towards the antimicrobial and phytotoxic activities of these isolated triterpenoids, which have also been carried out against some fungal and bacterial pathogens such as *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Enterobactor*, *Colletrichum gleosporioides*, *Fussarium equisitae*, *Curvularia eragrostidies*, *Alterneria alternate*, *Colletotrichum camellie*. The phytotoxicity were carried out against seeds of rice (*Oriza sativa*), wheat (*Triticum aestirium*), and pea (*Pisum sativum*) and reported their results. All the structures of the isolated compounds were confirmed by spectral (IR, NMR) analysis and by comparison with the literature reports.



A



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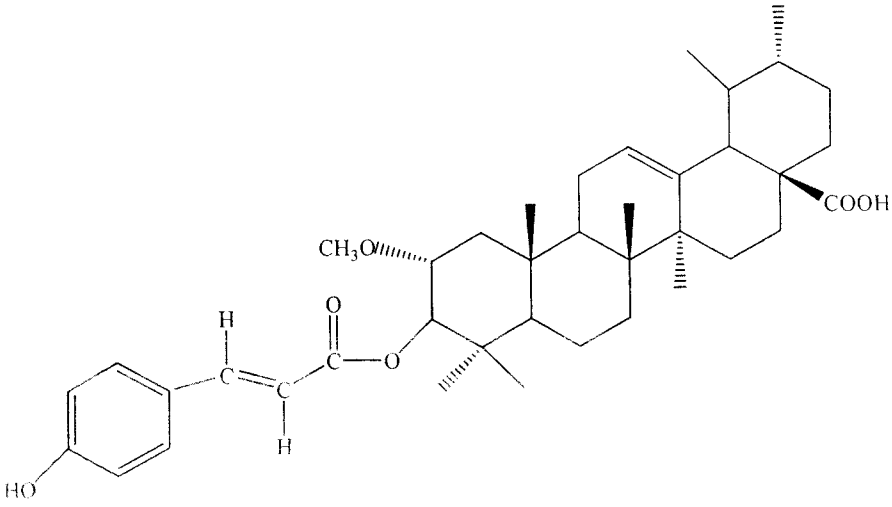

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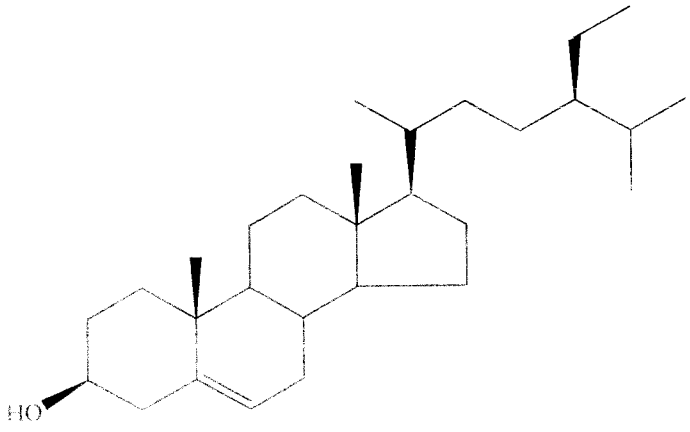

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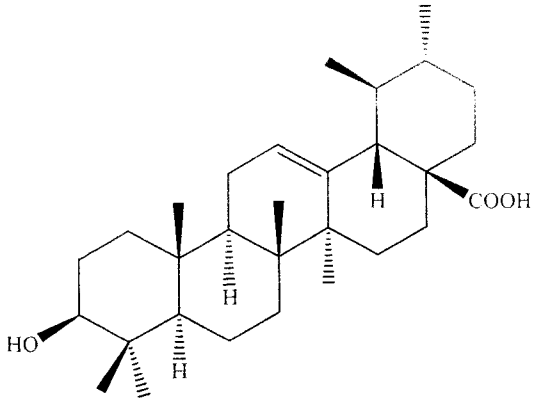

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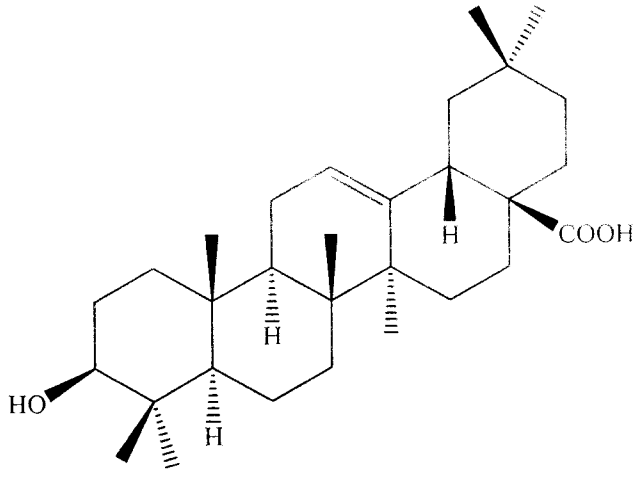

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Chapter IV

This chapter is divided into two sections, Section A and Section B that comprises extraction, isolation, synthesis, formulation, bioassay of isolated natural products and references.

Section A deals with the experimental of Chapter II and III which constitutes the extraction of compounds from the different parts of plants, elucidation of the structures, chromatography of the neutral part, examination of different fractions, isolation of the natural products and methods for the investigation of biocidal activity of the isolated compounds. **Section B** constitutes collection of plants for extraction of botanicals, details of the source of the microbial cultures, maintenance of the stock cultures, the common laboratory reagents used during the work, the media/solutions used during the work along with their standard compositions , assay of antifungal activity, Spore germination bioassay, the antibacterial sensitivity test by the disc diffusion method, phytotoxicity, antibacterial assay.

Part 2

TRANFORMATIVE REACTIONS OF TRITERPENOIDS AND THE BIOCIDAL ACTIVITY OF THE DERIVED COMPOUNDS

Part 2 of this thesis is concerned with transformative reactions of the pentacyclic triterpenoids and biological activity of the derived compounds. Part 2 has been divided into four chapters.

Chapter I

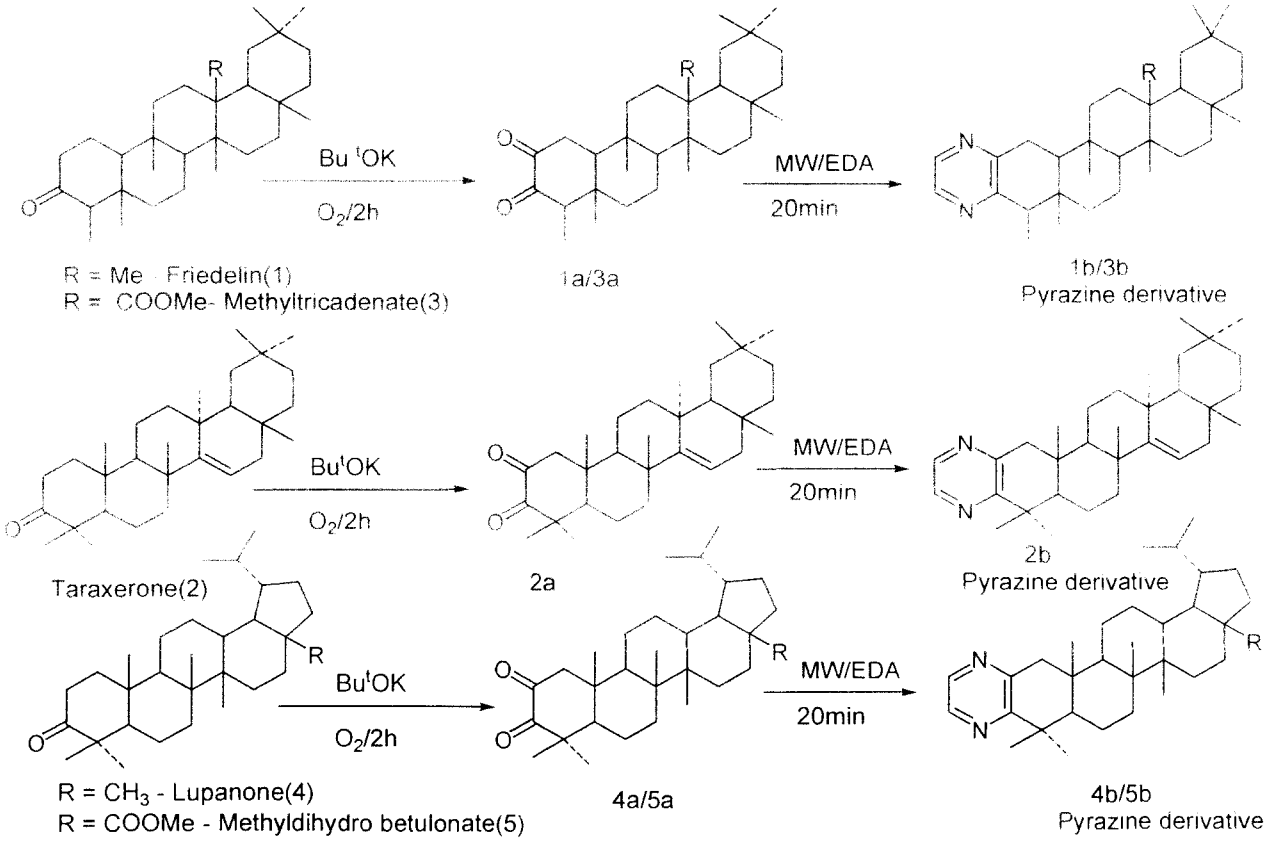
This chapter comprises a short review on transformative reaction of triterpenoids and biocidal activity of the prepared derivatives of pentacyclic triterpenoids.

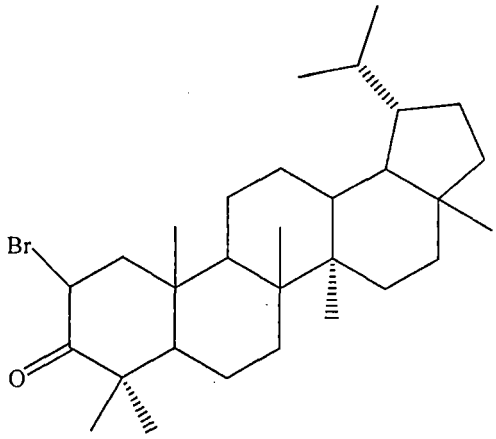
Chapter II

This chapter contains microwave assisted one pot synthesis of pyrazine derivatives of pentacyclic triterpenoids (Scheme I) and their biological activity. The 1,4-pyrazine derivatives of the triterpenoids were prepared in a mono-mode microwave oven at

100W(100°C) in only 20 minutes reaction time by adding dry ethylene diamine and Li and their anti microbial activity were studied against some fungal and bacterial specimen such as *Aspergillus niger* (AN), *Candida albicans* (CA), *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *dysenteries* (SD). A total of five 1,4-pyrazine derivatives have been synthesized and chracterised. These are 1,4-pyrazine derivative of friedelin (**1b**), 1,4-pyrazine derivative of taraxerone (**2b**), 1, 4-pyrazine derivative of methyltrichadenate (**3b**), 1, 4-pyrazine derivative of lupanone (**4b**), pyrazine derivative of methyldihydrobetulinate (**5b**). All the structures of the pyrazine derivatives were confirmed by spectral (IR, NMR) analysis and by comparison with the literature reports.

Scheme I





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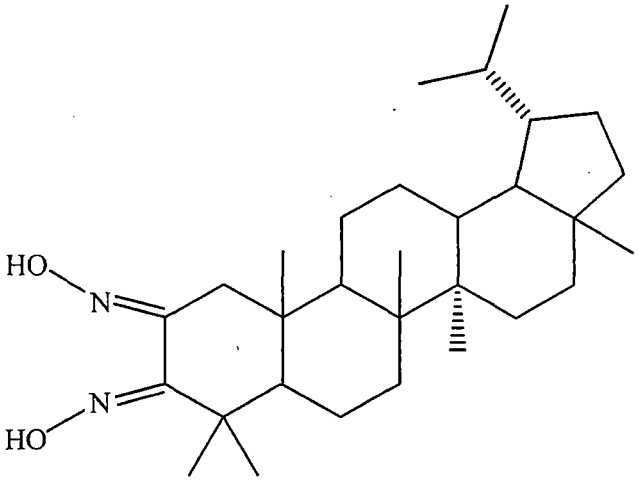

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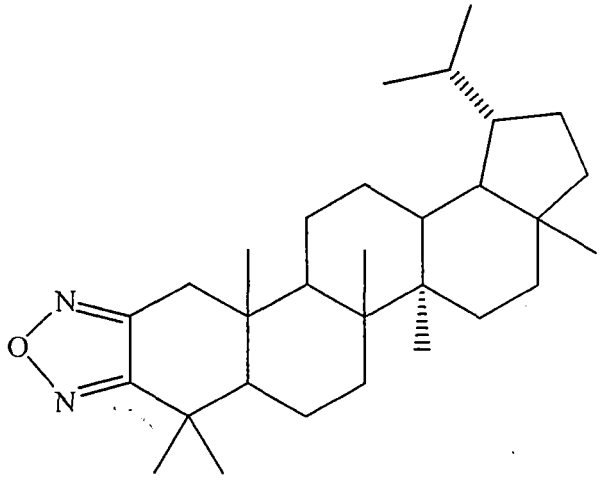

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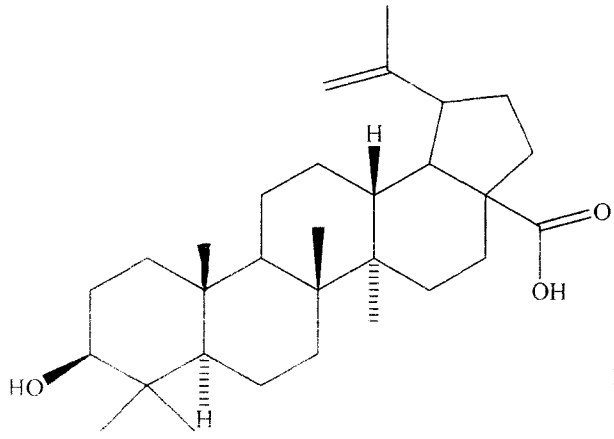
Section B: This section constitutes the preparation of 28-carbomethoxy-2,3-dioximinolupane and the subsequent cyclization to 28-carbomethoxy lupan[2,3-C]-1′, 2′, 5′-oxadiazole a) Esterification of betulinic acid (**6**) yielded methylbetulinate (**7**) which on hydrogenation yield methyldihydrobetulinate (**8**). Jone's oxidation of methyldihydrobetulinate (**8**) furnishes methyldihydro betulonate (**9**).

b) Treatment of methyl dihydrobetulonate (**9**) with N-bromosuccinimide furnished two compounds which were chracterised as 2, 2-dibromomethyldihydrobetulonate (**10**) and 2α-bromomethyl dihydrobetulonate (**11**).

c) Treatment of 2,2-dibromomethyl dihydrobetulonate (**10**) with hydroxylamine hydrochloride yielded 28-carbomethoxy-2,3-dioximinolupane **12** which on subsequent cyclization under MW irradiation afforded 28-carbomethoxy lupan[2,3-C]-1′, 2′, 5′-oxadiazole (**13**). The structures of 6, 7, 8, 9, 10, 11, 12, 13 have been established on the basis of spectral analysis (IR, UV, ^{1}H NMR, ^{13}C NMR) and by direct comparison with authentic sample.



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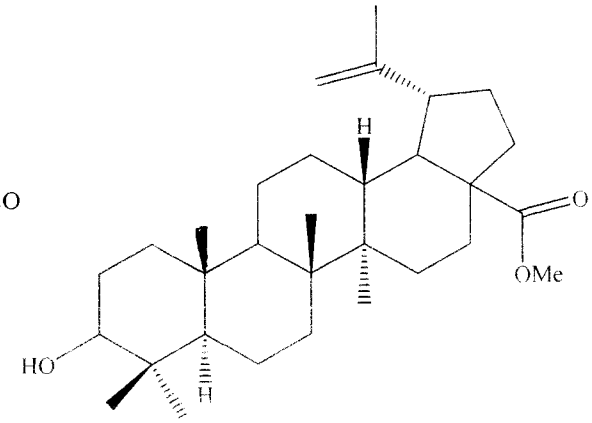

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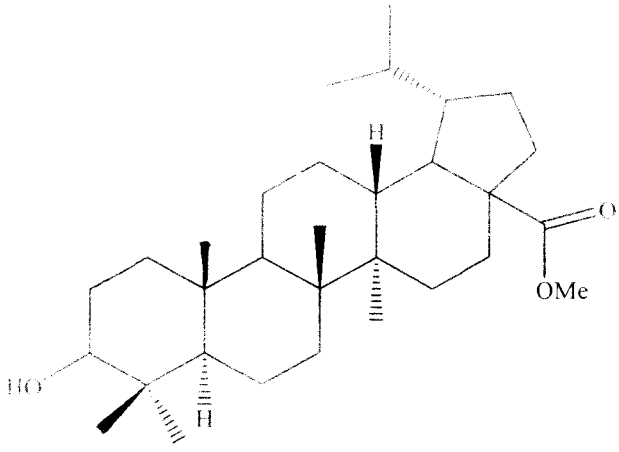

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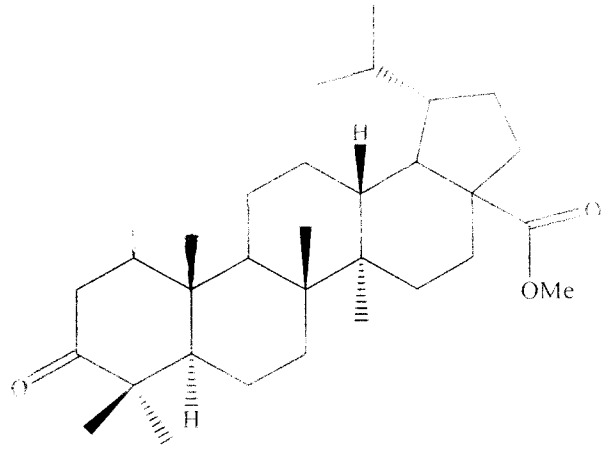

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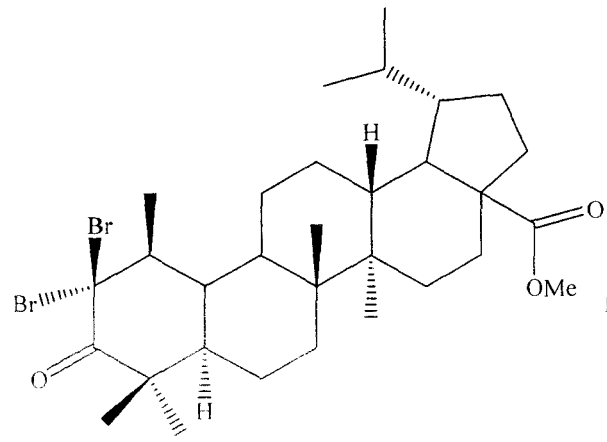

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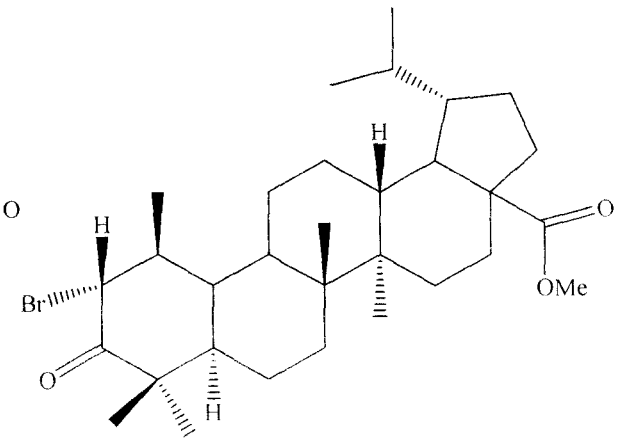

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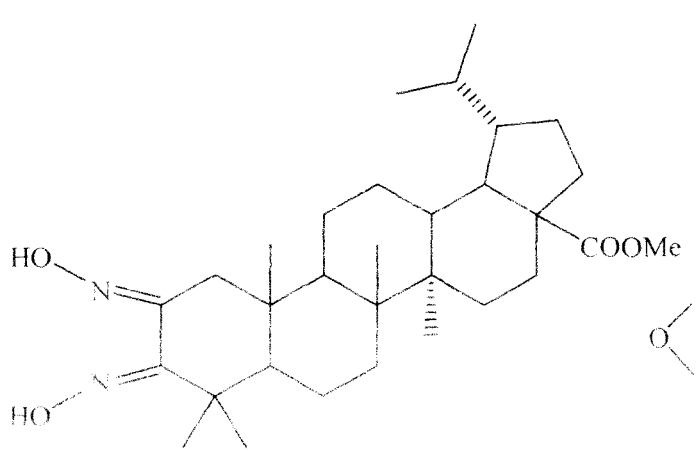

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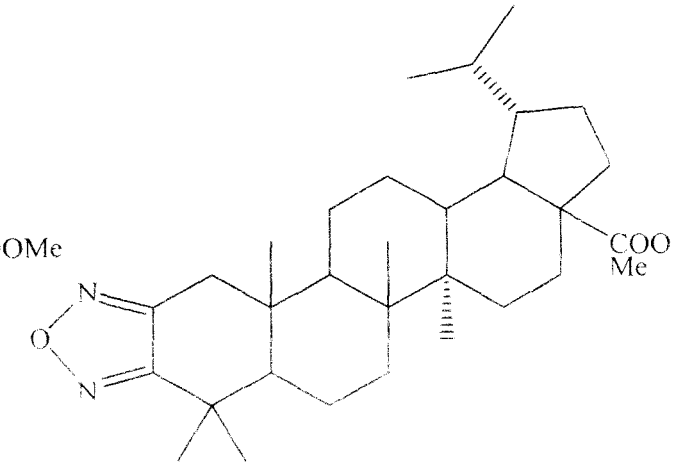

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The preliminary studies towards the antimicrobial and phytotoxic activities of oxadiazole derivative and lupeol have been carried out against five bacterial and fungal pathogens such as *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Lactobacillus*, *Pseudomonas* and *F. solani*. The phytotoxicity were carried out against seeds of rice (*Oriza sativa*), wheat (*Triticum aestirium*), and pea (*Pisum sativum*) and reported their results.

Chapter IV

This chapter contains experimental and references of Part 2 and deals the experimental of chapter II which constitutes the microwave applications in Organic synthesis, theory behind microwave heating, equipment needed in microwave synthesis, microwave reactors, elucidation of the structures, general procedure for the synthesis of 2, 3- diketo compounds and 1, 4-pyrazine derivatives, general procedure for the synthesis of 1, 4-pyrazine derivatives, examination of different fraction and Isolation of 1, 4-pyrazine derivative of friedelin, 1, 4-pyrazine derivative of taraxerone, 1, 4-pyrazine derivative of methyl trichadenate, 1, 4-pyrazine derivative of lupanone, 1, 4-pyrazine derivative of methyl dihydrobetulinate, extraction of *Xanthoxylum budrunga* and *Biscofia javanica* and isolation and synthesis of different compounds and all the references of Chapter I, Chapter II and Chapter III of part 2.

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PART II

TRANFORMATIVE REACTIONS OF TRITERPENOIDS AND THE BIOCIDAL ACTIVITY OF THE DERIVED COMPOUNDS

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Part II

List of Publications:

1. Triterpenoids from Schleichera oleosa of Darjeeling foothills and their antimicrobial activity. Pranab Ghosh, Prasanta Chakraborty, Amitava Mandal, **Md. Golam Rasul** and Aniruddha Saha. *Indian J. Pharm.* Sci.MS ID No.10-117, 2010, aceepted for publication.

2. Microwave assisted one pot synthesis of pyarazine derivatives of pentacyclic triterpenoids and their biological activities. Pranab Ghosh, **Md. Golam Rasul**, Amitava Mandal, Madhumita Chakraborty and Aniruddha Saha, Communicated to *Indian Journal of Chemistry*, Section-B: MS ID No. ¾ (SCCB-1332) / 2009.

3. Triterpenoids from Psidium Guajava Collected From Darjeeling Foothills And Their Biocidal Activity. Pranab Ghosh, Amitava Mandal, **Md. Golam Rasul**, Madhumita Chakraborty and Aniruddha Saha, Communicated to *Indian J. Pharm. Sci.* MS ID No 8: 1261, 2009.

4. Transformative Reaction and Biological Activity of pentacyclic Triterpenoids: Part 1: Microwave assisted one pot synthesis of pyrazine derivative and their biocidal activity Pranab Ghosh, **Md. Golam Rasul**, Amitava Mandal, Madhumita Chakraborty and Aniruddha Saha, Proceedings of the 3rd Mid - year symposium of the Chemical Research Society of India, NIPER, Mohali, Punjab, India, Page 48, 2008.

5. Synthesis and biological activity of (2, 3-C)1′,2′,5′ oxadiazole derivative of pentacyclic triterpenoids, **Md. Golam Rasul** and Pranab Ghosh. Abstract of the annual IIT Madras Chemistry Symposium, page 88, 2006.

6. Biological properties of some prepared derivatives of pentacyclic triterpenoids. Pranab Ghosh, **Md. Golam Rasul,** Madhumita Chakraborty, Sandip Choudhury, Proceedings of the 7th CRSI national Symposium in Chemistry, IACS, Kolkata, page 364, 2005.

7. Biological Properties of Some Derivatives of Pentacyclic Triterpenoid, Madhumita Chakraborty **Md. Golam Rasul**, Sandip Choudhury, Prabir Kumar Roy, B. P. Pradhan and Pranab Ghosh, Proceedings of the seminar on Health and Environment Hazards and Remedies, Raiganj college (University College), West Bengal, India, page . 6, 2004.

8. Novel triterpenoids from two different plants available in Darjeeling foothills and their bioactivity against bacterial and fungal pathogen, Amitava Mandal, **Md. Golam Rasul**, Madhumita Chakraborty Aniruddha Saha and Pranab Ghosh, Chemistry in North Bengal University, 3(1), page 31, 2009

Part 1

PHYTOCHEMICAL INVESTIGATION OF SOME MEDICINAL PLANTS AND STUDIES ON THE BIOLOGICAL ACTIVITIES OF THE ISOLATED COMPOUNDS

Chapter 1

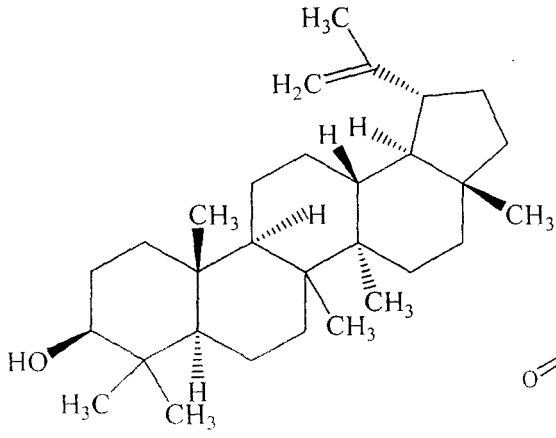
A SHORT REVIEW ON THE PHYTOCHEMICAL INVESTIGATION OF MEDICINAL PLANTS AND BIOLOGICAL ACTIVITY OF THE ISOLATED PHYTOCONSTITUENTS.

This chapter is divided into two sections, Section A and Section B

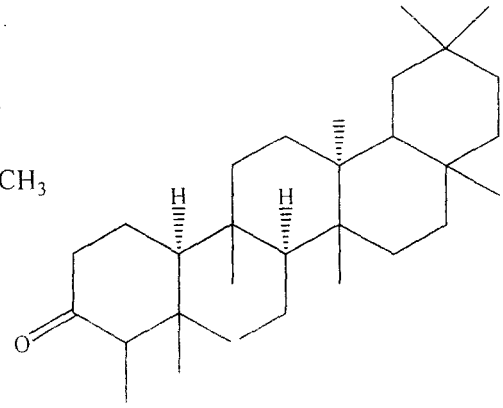
Section A: A short review on the Phytochemical Investigation of medicinal plants

The northern part of West Bengal commonly called as North Bengal is endowed with diverse natural resources. 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. Our laboratory is actively involved in chemical investigation on Medicinal plants of Darjeeling hill and Tarai region. As a result a number of new di and triterpenoids have been isolated and chracterised so far. Potent pharmacological activity of such type of compounds has been documented recently by some group of workers. The observation of the previous workers in concord with the present line of investigation is being presented, in a selective manner, in the following paragraphs.

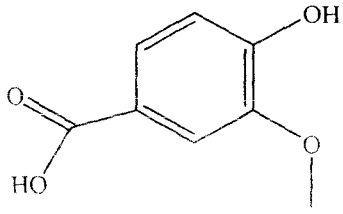
Rahaman *et al.* [1] reported that two new triterpenoids, 18 alpha, 19 beta-20(30)taraxasten-3 beta, 21 alpha-diol (cichoridol) (1) and 17-epi-methyl-6-hydroxyangolensate (intybusoloid) (2) obtained from the methanolic extract of seeds Cichorium intybus (Asteracae) along with eleven known compounds, lupeol (3), friedelin (4), betunaldehyde (9), syrginic acid (10), vanillic acid (11), 6,7-dihydroxycoumarin (12) and methyl-alpha-D-galactopyranoside (13). Compound 1 and 11 reported to exhibit a good alpha-glucosidase inhibitory activity.



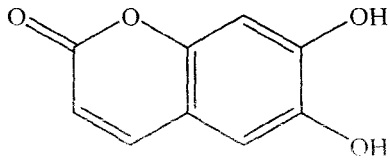
1(lupeol)



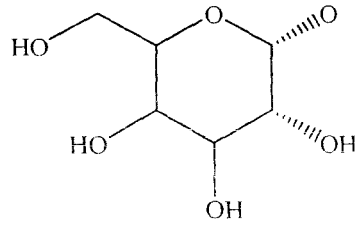
friedelin



Vanillic acid

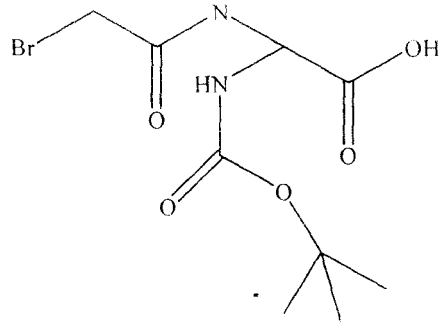


6, 7-dihydroxycoumarin

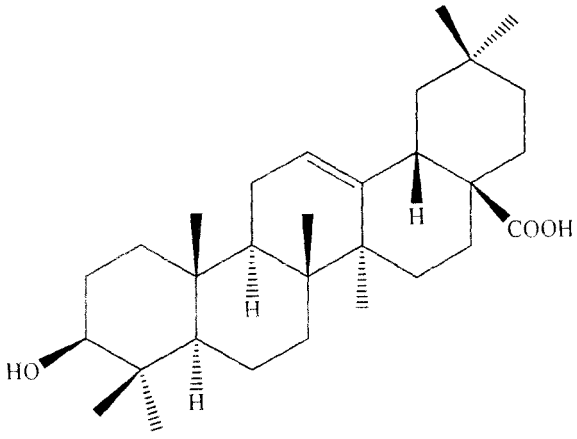


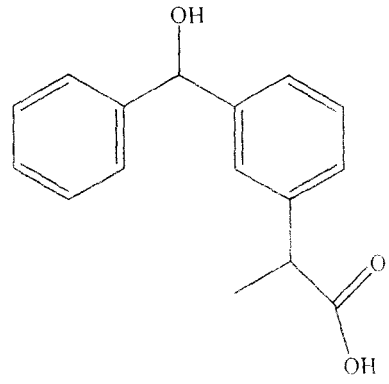
Methyl-α--D-galactose

Choudhury *et al.* [2] reported that biotransformation of a pentacyclic triterpene, oleanolic acid (1), with *Fusarium lini* afforded two oxidative metabolities, 2α, 3β–dihydroxy-olean-12-en-28-oicacid (2), and 2α, 3β, 11β–trihydroxyolean-12-en-28-oicacid(3). They also found that metabolities 3 is a new compound. The structures were characterized on the basis of spectroscopic studies. These metabolities exhibited a potent inhibition of α–glucosidase enzyme and thus are effective in diabetes by delaying the glucose absorption.



2α, 3β, 11β–trihydroxyolean-12-en-28-oic acid

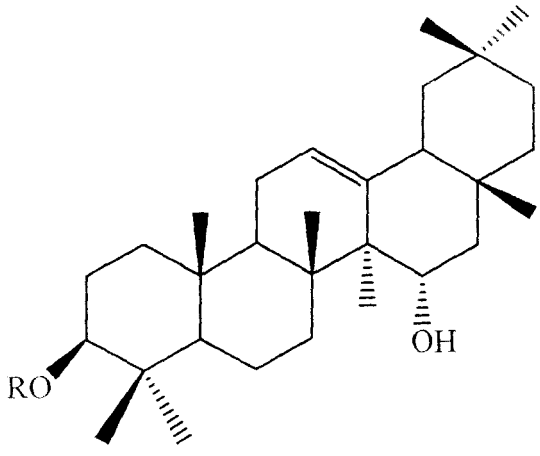


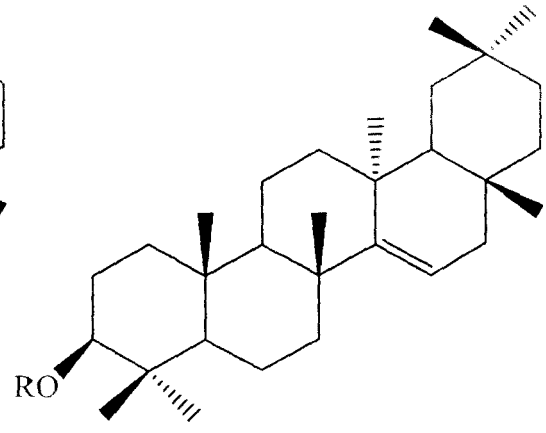


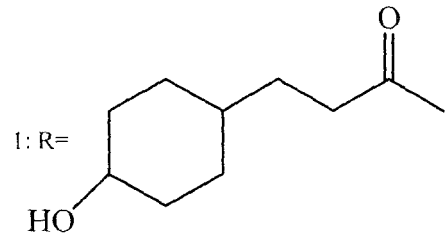
2 (Oleanolic acid)

2α, 3β–dihydroxy-olean-12-en-28-oicacid

Li *et al.* [3] isolated seven pentacyclic triterpenoids including 3β–o–coumaryl (1) 15α–hydroxy, β–amyrin (2), 3β–taraxerol (3), 3β–taraxerol formate (4), 3β–taraxerol acetate (5), 3β–o–(E)–coumaryl-taraxerol (6) and 3-β-o-(Z)-coumaroyl-taraxerol (7) from the stems and twigs of the mangrove plant *Rhizophora stylosa* (Rhizophorace). The structures of the isolated compounds were determined by extensive analysis of their spectroscopic data. They found that among these metabolites, compound 1 is a new olenane type terpenoid coumaroyl ester, while compound 4 is a new natural product obtained for the first time.







1. R = 3β–o–coumaryl

2. R=H=15α–hydroxy, β–amyrin

3. R=H=3β–taraxerol

4. R=Formyl=3β–taraxerol formate

5. R=Acetyl=3β–taraxerol acetate

6. R=E-coumaryl=3β–o–(E)–coumaryl-taraxerol

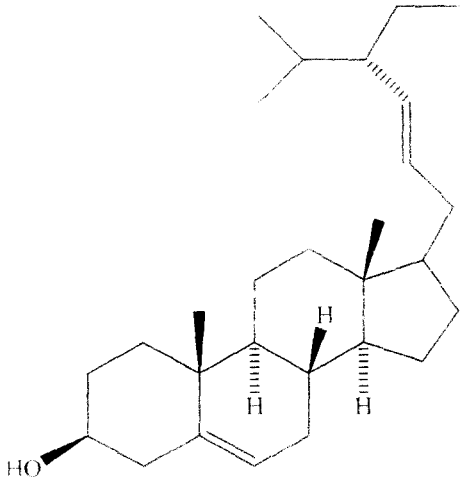
7. R=Z-coumaryl=3–β-o- (Z)-coumaroyl-taraxerol

He *et al.* [4] isolated the chemical constituents of the roots of *Aconitum taipaicum* (Ranunlaceae) and purified using silica gel column chromatography. They found new norditerpenoid alkaloids, isodelelatine along with five known alkaloids. The structure of the new compound was elucidated on the basis of spectral data.

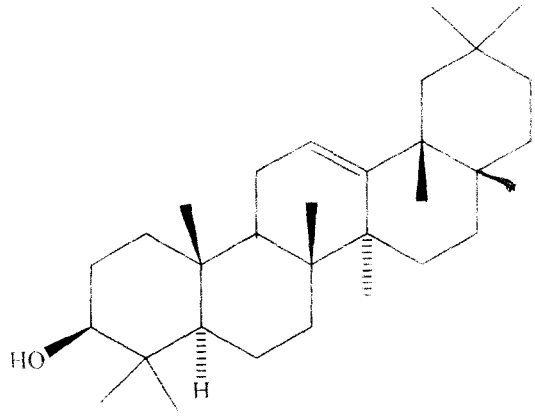
Srikrishna *et al.* [5] carried out antibacterial activity using cup plat method by pet. ether, chloroform, methanol and water extract of the bark of *Aporosa lindleyana* (Euphorbiaceous). They observed that the compounds showed moderate to very good activity against *Bacillus subtilis*, *Escherichia coli* and compared with the standard drug tetracycline. They studied the antifungal activity against *Penicillium chrysogenous*, *Candida albicans*, *Aspergilus niger* and *Trichoderma vridar* and compared with the standard drug fluconazole. The pet.ether extract showed considerable activity towards all the four fungal organism. Analgesic activity has been carried out on Swiss albino male mice by abdominal constriction method. All the extracts showed moderate analgesic activity while methanol extract showed very good activity.

Ohtsu *et al.* [6] isolated four known and four abietane diterpenes from the $CHCl_3$ extract of the *Larix kaempferi*. A known compound 13, 14-seco-13, 14-et-13-en-18-oic acid was isolated from natural sources for the first time. Their structure was determined by chemical and spectropic methods and crystallographic analysis. They studied the inhibitory effects of these compounds on EBV-EA activation induced by tumor promoter and results are reported.

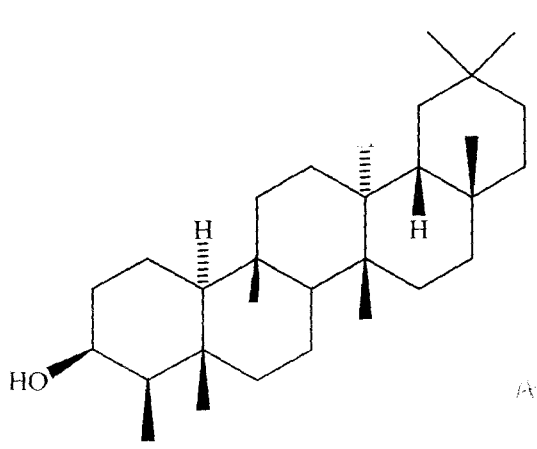
Singh *et al.* [7] extracted a mixture of triterpenoids: β–sitosterol, stigmasterol, β–amyrin, friedelan-3β–ol (epifridelenol), cycloartenone, β–amyrin acetate, friedelin and epi-friedenyl acetate *Heliotropium marifolium* using hexane as a solvent. They tested the isolated triterpenoids against selected pathogenic bacteria and fungi, e.g. *Escherichia coli, Staphylococcus aureus, Aspergillus niger and Penicillium chrysogenum.* They also discussed the quantification and assessment of their growth inhibitory potency and found that cycloartenone was the major triterpenoids in both in *vivo* and *vitro* cell culture.



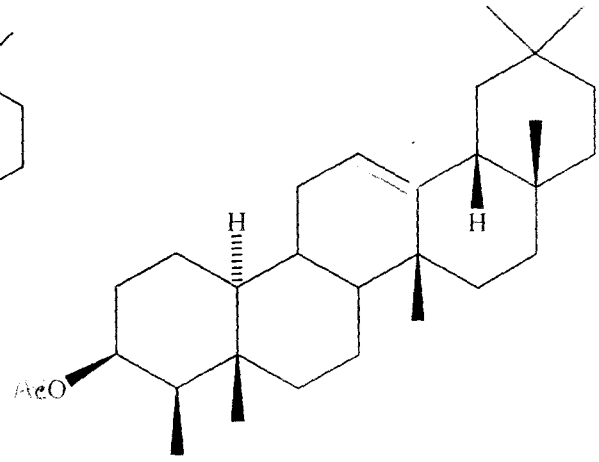
Stigmasterol



Betaamyrin



Friedelan-3β-ol



β–amyrin acetate

Larshini *et al.* [8] extracted 12 plants selected on the basis of the folk-medicine reports and examined their anti bacterial effects against eight pathogenic bacteria. They found that the n-butanol extract of *Calotropis procera* flowers and the aqueous extract of *Eugenia caryophyllata* were the most effective against the bacteria tested.

Shrimali *et al.* [9] extracted compounds from the dried stem bark of *Ailanthus excelsa* using different solvent and studied their antibacterial activity against different bacterial strains. The ethyl acetate (EA) fraction inhibited the growth of all test bacteria. The MIC of the EA fraction was found to be 6 mg/disc.

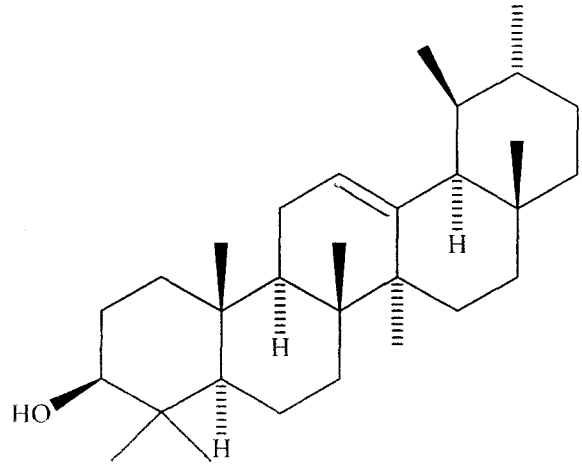
Pichai *et al.* [10] extracted the powdered material of "Vidattali" equated to *Dichrostachys cinerea* and separated n-octacosanol, β–sitosterol, friedelin, epifridelinol, α-amyrin and β–sitosterol-3-β-D-glucopyranoside in the aerial part. They were studied antibacterial and antifungal activities of n-hexane and chloroform extracts on four bacteria *Escherichia coli*, *Pseudomonas aeruginasa*, *Staphylococcus aureus* and two fungi *Aspergillus flavus* and *Mucor* sp. at 1.25, 2.5, 5.0 and 10 mg/ml concentration levels in nutrient agar and SDA mediums respectively by steak method. They observed that the chloroform extract showed moderate inhibition of *E.coli* and *Staphylococcus* at higher concentrations of 5-10 mg/ml. Antifungal activities of these extracts against *Aspergillus* and *Mucor* were observed at higher concentration.

Xu *et al.* [11] isolated Geumonoid, a new triterpene from *Geum japonicum* and its structure was elucidated on the basis of 1D, 2D NMR and MS spectroscopic analysis. They observed that Geumonoid showed inhibitory activity against HIV-1 protease.

Sukul and Chaudhuri [12] extracted the leaves of *Lantana canara* using different solvents. They observed that four fractions of petroleum ether extract showing significant antibacterial activity against some human pathogens under in *vitro* conditions. The MIC of the methanol fraction, containing triterpenoids, active against these pathogens was found to be comparable with those of some therapeutically used antibiotics.

Panizzi *et al.* [13] isolated some constituents from the flowering aerial parts of *Geum rivale* and studied their antimicrobial activity on bacteria and fungi. The activity was concentrated in the triterpenes fraction and, for Gram-positive and Gram-negative bacteria, and also in the flavonoids fraction.

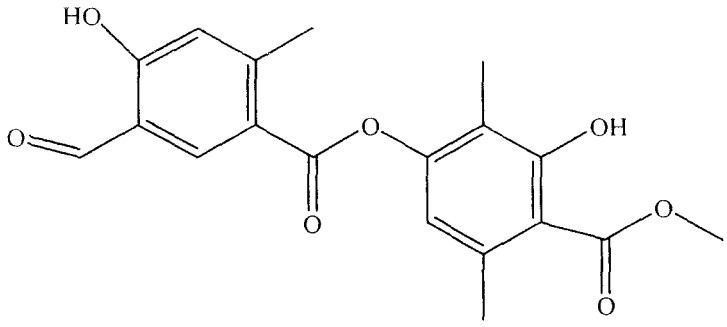
Pitchai and Saraswath [14] extracted n-octacosanol, β–sitosterol, friedelin, epifridelinol, α-amyrin and β–sitosterol-3-β-D-glucopyranoside from the aerial part of *Dichrostachys cinerea* using different solvents. They studied the antibacterial and antifungal activities of n-hexane and chloroform extracts in four bacteria at 1.25, 2.5, 5.0 and 10 mg/ml concentration in nutrient agar medium by streak method. They observed that n-hexane showed 100 percent inhibition to the growth of *Escherichia coli* and *Pseudomonas aeruginosa* in all concentrations whereas *Staphylococcus aureus* and *S.albus* were moderately affected at 5.0 and 10 mg levels. The $CHCl_3$ extract showed moderate inhibition at higher concentration to *S.albus* and *E.coli.* Antifungal activities of these extracts against *Aspergillus flavus* and *Mucor* sp. were found at higher concentration.



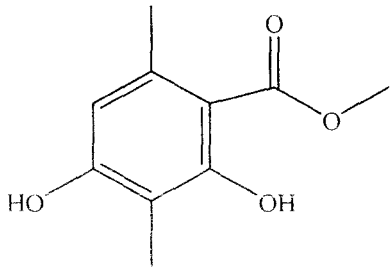
α-amyrin

Chizozem *et al.* [15] isolated two new friedelane-type triterpenes named 12alpha-hydroxyfridelane-3,15-dione and 3beta-hydroxyfriedelan-25-al, together with six known compounds from the stems of *Drypetes paxii* Hutch.(Euphorbiaceae) and established their structures. They also tested the antimicrobial activity of the five friedelane-type triterpenes, one olean-12-ene triterpene saponin against some Gram-positive and Gram-negative bacteria and they appeared to be modestly active.

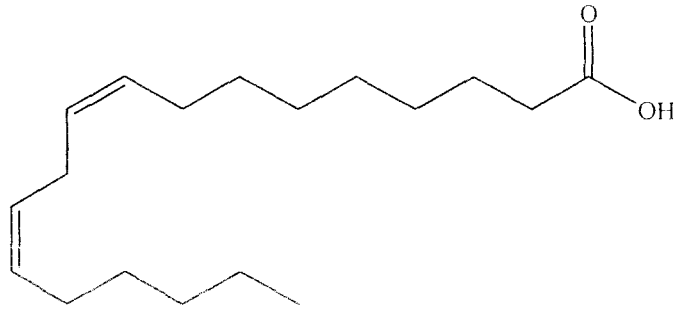
Mutai *et al.* [16] isolated three new pentacyclic triterpenoids : (20R)-3-oxolupan-30-al (1), (20*S*)-3-oxolupan-30-al (2) and (20*R*)-28-hydroxylupen-30-al-3-one (3), along with (20*S*)-3β-hydroxylupan-30-al (4) and the known metabolites 30-hydroxylup-20-(29)-en-3-one (5), 30-hydroxylup-20-(29)-en-3β-ol (6), atranorin, methyl 2,4-dihydroxy-3,6-dimethylbenzoate, sitosterol-3β-O-glucoside and linoleic acid from *Acacia mellifera* . The structures of the new metabolites were elucidated by extensive spectroscopic analyses and their relative stereochemistry was determined by NOESY experiments. They observed that the new metabolite 3 exhibited significant cytotoxic activity against the NSCLC-N6 cell line, derived from a human non-small-cell bronchopulmonary carcinoma.



Atranorin

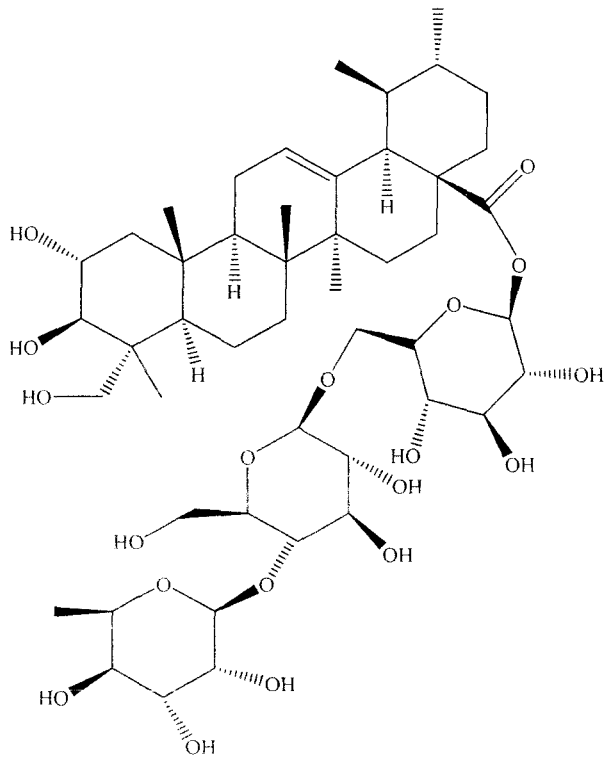


Methyl2,4-dihydroxy-3,6-dimethylbenzoate

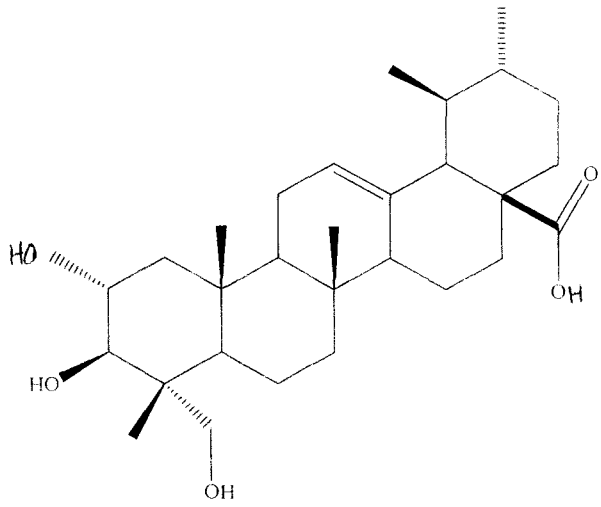


Linoleic acid

James and Dubery [17] accumulated large quantities of pentacyclic triterpenoid saponins collectively known as centelloids from Centella asiatica. These terpenoids include asiaticoside, centelloside, madecassoside, brahmoside, brahminoside, thankuniside, sceffoleoside, centellose, asiatic-, brahmic-, centellic- and madecassic acids. They studied biological activity of these compounds, the Centella triterpenoids can be regarded as phytoanticipins due to their antimicrobial activities and protective role against attempted pathogen infections. They reported that these plant-derived pharmacologically active compounds have complex structures, the production of secondary metabolities by cultured cell provides a particularly important benefit of manipulate and improve the production of the desired compounds.



Asiaticoside



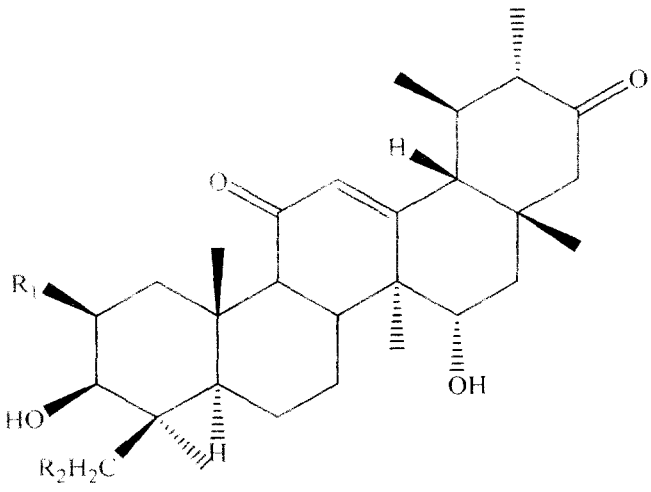
Asiatic acid

Antonia *et al.* [18] extracted lupane triterpenoid lupeol, the ursane triterpenoid α–amyrin and esters of these compounds from the bark of roots of *Alstonia boonei* and observed that these compounds have anti-inflammatory properties. They found that □amyrin is a competitive inhibitor of bovine trypsin and chymotrypsin, lupeol linoleate, lupeol palmitate and α–amyrin linoleateare non-competitive inhibitor of chymotrypsin. They also found that lupeol, α–amyrin, palmitic and linoleic acid esters of these compounds are very weak inhibitors of porcine pancreatic elastase and of *Lucilia cuprina* and *Helicoverpa punctigera* leucine aminopeptidases.

Ghosh *et al.* [19] extracted seeds of *Swietenia mahagoni* using methanol as a solvent and reported pharmacological activity including anti-inflammatory activity of the extract. They evaluated the anti-inflammatory activity using acute, sub-chronic, and chronic models of inflammation in rodents. The anti pyretic and analgesic activities were evaluated in mice models. They studied the acute toxicity of the extract using different doses and the effect was compared with the standard drug, ibuprofen. The results revealed

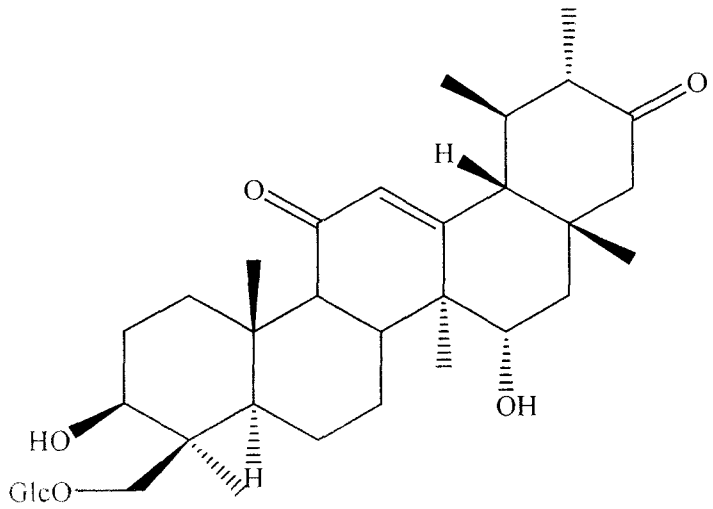
that the extract produces anti-inflammatory activity through dual inhibition of the cyclo-oxygenase and lipo-oxygenase pathways of archidonic acid metabolism.

Zhou *et al.* [20] isolated three new triterpenoids, 11,21-dioxo-2beta,3beta,15alpha-trihydroxyurs-12-ene-2-O-beta-D-glucopyranoside (1), 11,21-dioxo-3beta,15alpha,24-trihydroxyurs-12-ene-24-O-beta-D-glucopyranoside (2), and 11,21-dioxo 3beta, 15alpha,24-trihydroxyolean-12-ene-24-O-beta-D-glucopyranoside (3), and two new flavonoids, apigenin-7-O-[2''-O-(5'''-O-feruloyl)-beta-D-apiofuranosyl]-beta-D-glucopyranoside (4) and chrysoeriol-7-O-[2''-O-(5'''-O-feruloyl)-beta-D-apiofuranosyl]-beta-d-glucopyranoside (5) from the whole plant of fresh *Apium graveolens* together with 10 known flavonoids. The structures of the new compounds were elucidated by analysis of spectroscopic data. The evaluated the inhibitory effects of the compounds isolated on nitric oxide production in lipopolysaccaride-activated macrophages.

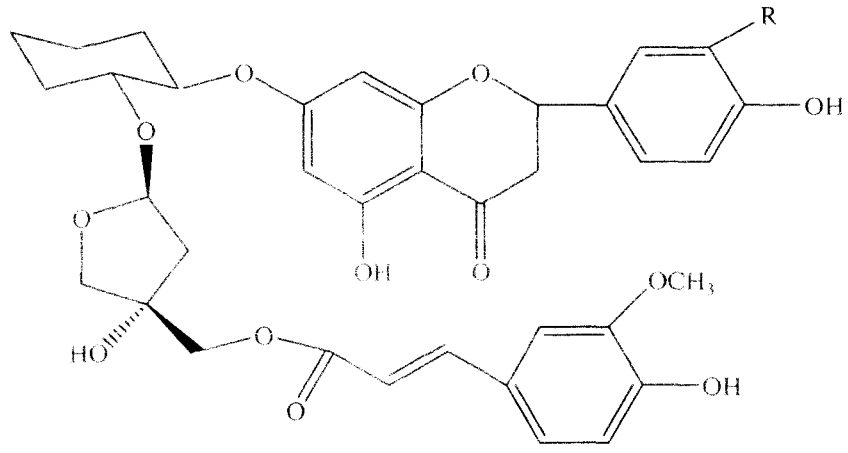


1 R_1=OGlc, R_2=H ; 11,21-dioxo-2beta,3beta,15alpha-trihydroxyurs-12-ene-2-O-beta-D-glucopyranoside

2R_1=H R_2=OGlc ; 11,21-dioxo-3beta,15alpha,24-trihydroxyurs-12-ene-24-O-beta-D-glucopyranoside



3 = 11,21-dioxo-3beta,15alpha,24-trihydroxyolean-12-ene-24-O-beta-D-glucopyranoside



4 R=H; apigenin-7-O-[2"-O-(5"'-O-feruloyl)-beta-D-apiofuranosyl]-beta-D-glucopyranoside

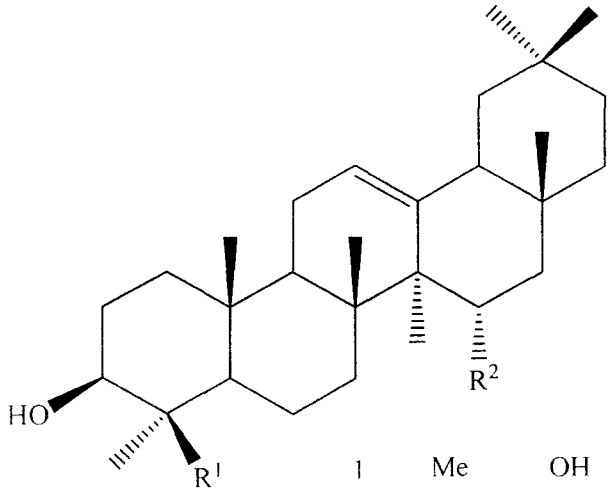
5 R=OCH_3; chrysoeriol-7-O-[2"-O-(5"'-O-feruloyl)-beta-D-apiofuranosyl]-beta-d-glucopyranoside

Angeh *et al.* [21] isolated four known triterpenoids, 1alpha,3beta-dihydroxy-12-oleanen-29-oic (1), 1-hydroxy-12-olean-30-oic acid (2), 3,30-dihydroxyl-12-oleanen-22-one (3), and 1,3,24-trihydroxyl-12-olean-29-oic acid (4) along with a new pentacyclic triterpenoid (1alpha,23-dihydroxy-12-oleanen-29-oic acid-3beta-O-2,4-di-acetyl-L-rhamnopyranoside) 5 through a bioassay-guided procedure from the leaves of *Combretum imberbe*. The structures of the compounds were elucidated on the basis of 1D and 2D

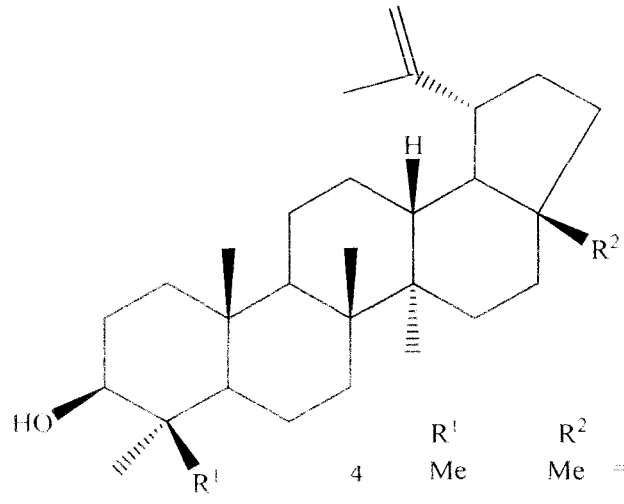
NMR experiments, as well as mass spectrometric data. They observed that all the isolated compounds have moderate (62 microg/ml) to strong (16 microg/ml) antibacterial activity (MIC values) against *Staphylococcus aureus* and *Escherichia coli*, with 1 and 5 being most active. The results of this study give credence to the ethnomedicinal use of Combretum imberbe and expand our knowledge on the biological activity of its metabolites.

Mathabe *et al.*[22] extracted four known compounds, two triterpenoids, compound 1 [d-friedoolean-14-en-oic acid (3-acetyl aleuritolic acid)] and compound 2 (lupeol), and two diterpenes, compound 3 [ent-2,6alpha-dihydroxy-norbeyer-1,4,15-trien-3-one (diosphenol 2)] and compound 4 (ent-3beta-hydroxy-beyer-15-ene-2-one) from the bark of *Spirostachys africana* using ethanol as a solvent . They were tested the antibacterial activity of the isolated compounds using micro-dilution method and observed that Compound 1, exhibited minimum inhibitory concentration (MIC) of 50 microg/ml against *Staphylococcus aureus, Salmonella typhi, Vibrio cholera, Escherichia coli* and *Shigella dysentery*.

Wada *et al.* [23] isolated lupane- and oleanane- type triterpenoids from the bark of *Phyllanthus flexusus* and screened inhibitory activity on human Topos (topoisomerases) I and II. They found that olean-12-en-3β, 15α-diol (1), olean-12-en-3β, 15α, 24-triol (3), lupeol (4), and betulin (6) were selective catalytic inhibitors of human Topo II activity with IC_{50} values in the range of 10-39 μM.



		1	Me	OH	= olean-12-en-3 15 -diol
R^1	R^2	3	CH_2OH	OH	= olean-12-en-3 , 15 , 24-triol

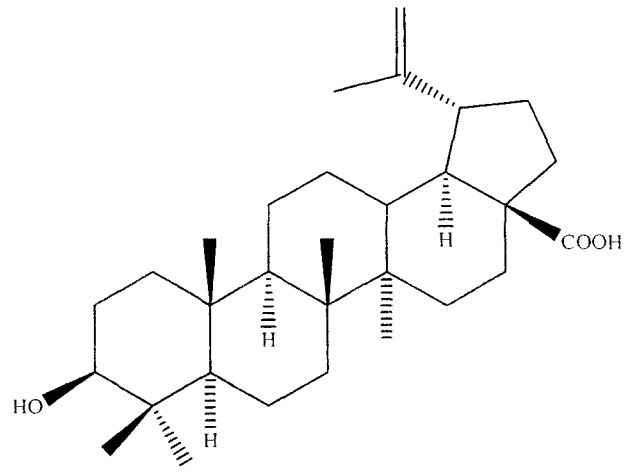


	R^1	R^2	
4	Me	Me	= Lupeol
6	Me	CH_2OH	= Betulin

Li *et al.* [24] extracted a new lupane type triterpenoid, 3β, 11α–dihydroxy-30-norlupan-20-one and six known lupane triterpenoids from the whole plant of Salvia roborowskii Maxim using petroleum ether as a solvent. They elucidated their structures by means of spectral methods including NMR and MS techniques.

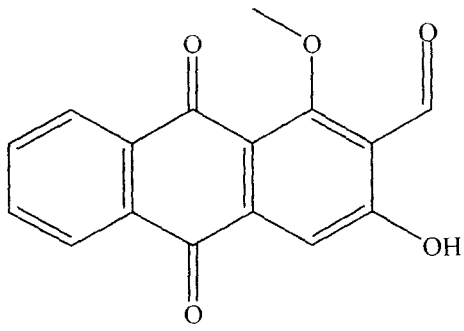
Tostikoya *et al.* [25] studied the biological activity of natural and semi synthetic lupane triterpenoid and discussed in two-part review. The first part was devoted to the pharmacological properties of natural lupane triterpenoids. They reported that betulinic

acid proven to be the most effective antitumor agent among more than fifty natural lupanes.

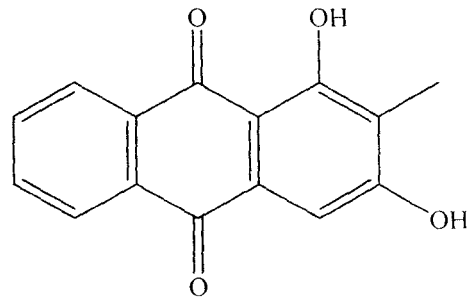


betulinic acid

Kanokmedhakul *et al.* [26] isolated a new 1,3-dihydroxy-2-methyl-5,6-dimethoxyanthraquinone **(1)**; six known anthraquinones, nordamnacanthal **(2)**, damnacanthal **(3)**, rubiadin **(4)**, rubiadin-1-methyl ether **(5)**, lucidin-ω-methyl ether **(6)**, and 1-hydroxy-2-hydroxymethyl-3-methoxyanthraquinone **(7)**; a β-sitosterol **(8)**; together with two known triterpenoids, β-acetylolean-12-en-28-olic acid **(9)**, and 3β-O-acetyl-11α,12α-epoxyolean-28,13-olide **(10)** from the roots and stems of *Prismatomeris fragrans*. Their structures were established on the basis of spectral data. This was the first isolation of compounds **2, 6, 7, 9** and **10** from *Prismatomeris* genus. They studied the antiplasmodial, antituberculosis, antifungal and anticancer cell lines tests of the isolated compounds and the bioactivity assays showed that only **9** exhibited moderate antimalarial activity, **2** and **3** exhibited antifungal activity while **2, 3, 4, 7** and **9** showed antituberculosis activity. In addition, compounds **2, 3** and **7** exhibited cytotoxicity to BC cell line while **1, 1a** (the methyl ether derivative of **1**), **2, 3, 4, 5,** and **9** exhibited cytotoxicity to NCI-H187 cell line.

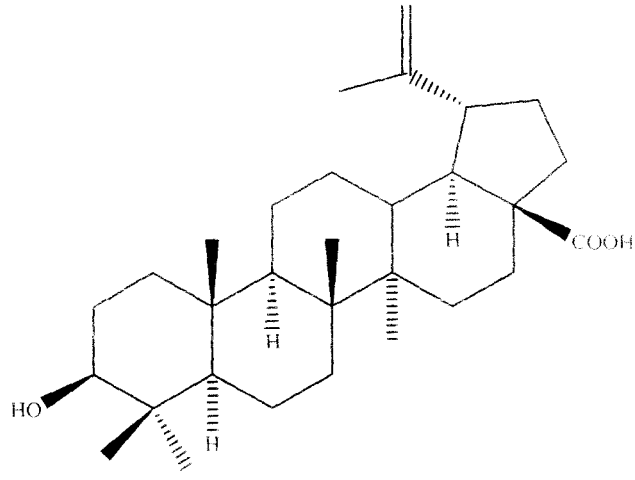


Damnacanthal



Rubiadin

Setzer *et al.* [27] extracted the crude from the bark of Syncarpia glomulifera using chloroform as a solvent and reported antibacterial and cytotoxic activity. They isolated oleanolic acid-3-acetate, ursolic acid-3-acetate and betulinic acid from the bark. They observed that the relatively large abundance (10 % of the crude extract) and high degree of activity of betulinic acid were responsible for the bioactivity of the crude bark extract.

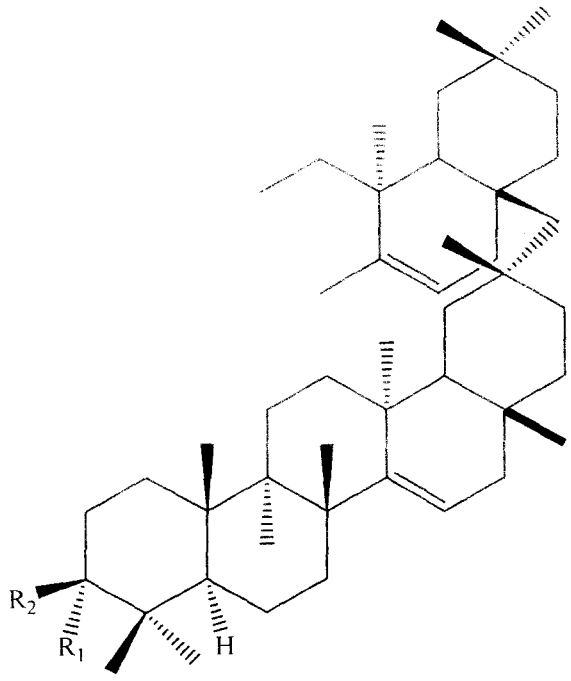


betulinic acid

Lutskii *et al.* [28] isolated triterpenoids from the plants of the *Thalictrum genus* and the structural, chemical and spectral properties were systematized for the first time. They discussed the features of the ^{13}C NMR spectra of cycloartane triterpenoids and also gave the data for the biological activities of certain cycloartane and oleanane triterpenoids.

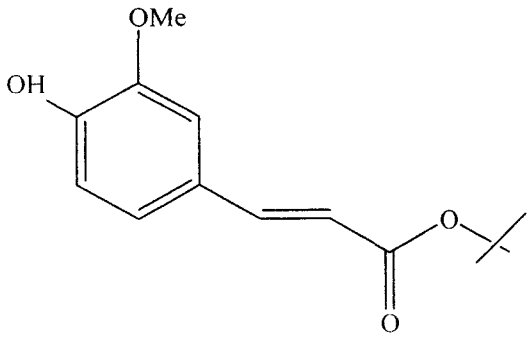
Li *et al.* [29] isolated seven pentacyclic triterpenoids including 3β-*O*-(*E*)-coumaroyl-15α-hydroxy-β-amyrin (**1**), 15α-hydroxy-β-amyrin (**2**), 3β-taraxerol (**3**), 3β-taraxerol formate (**4**), 3β-taraxerol acetate (**5**), 3β-*O*-(*E*)-coumaroyl-taraxerol (**6**), and 3β-*O*-(*Z*)-coumaroyl-taraxerol (**7**) from the stems and twigs of the mangrove plant *Rhizophora stylosa*. The structures of the isolated compounds were determined by extensive analysis of their spectroscopic data. They reported that among these metabolites, compound **1** is a new oleanane-type triterpenoid coumaroyl ester, while compound **4** is a new natural product obtained here as an isolated substance for the first time.

Laphookhieo *et al.* [30] isolated six new pentacyclic triterpenoids esters (1-6) together with 3α- and 3β-tarexerol from the fruits of *Bruguiera cylindrica*. The structures of the new compounds were characterized as 3α-E-feruloyltaraxerol (1), 3α-Z-feruloyltaraxerol (2), 3 β-E-feruloyltaraxerol (3), 3 β–Z-feruloyltaraxerol (4), 3α-E-coumaroyltararaxerol (5), and 3α-Z-coumaroyltaraxerol (6). They reported that compounds 2 and 6 exhibited weak cytotoxicity against the NCl-H187 cell line.

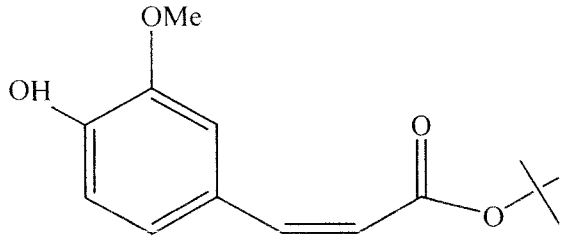


[redacted] 235171

R_1 R_2

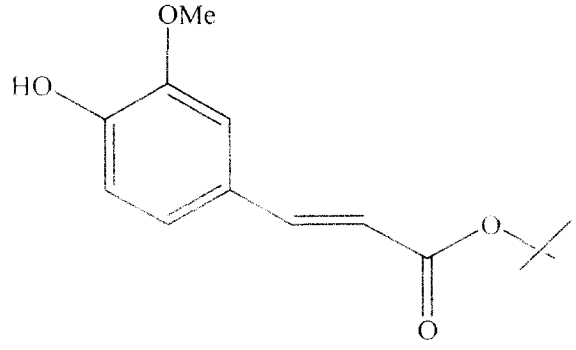


H = 3α–E-feruloyltaraxerol



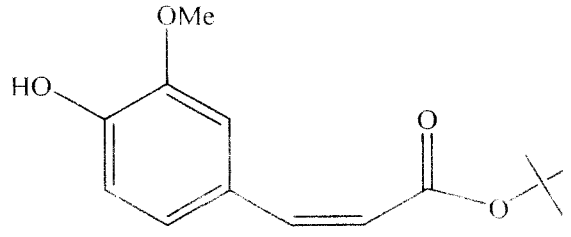
H =3α–Z-feruloyltaraxerol

H

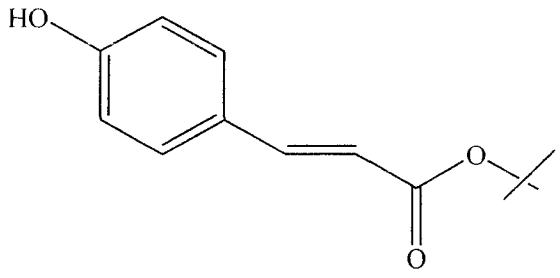


= 3β–E-feruloyltaraxerol

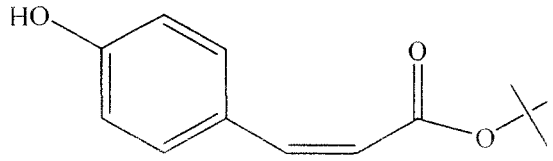
H



=3β–Z-feruloyltaraxerol

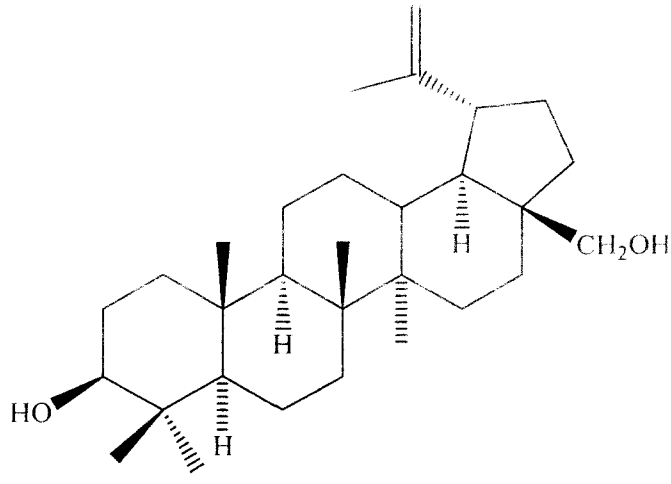


H = 3α–E–coumaroyltaraxerol



H = 3β–Z–coumaroyltaraxerol

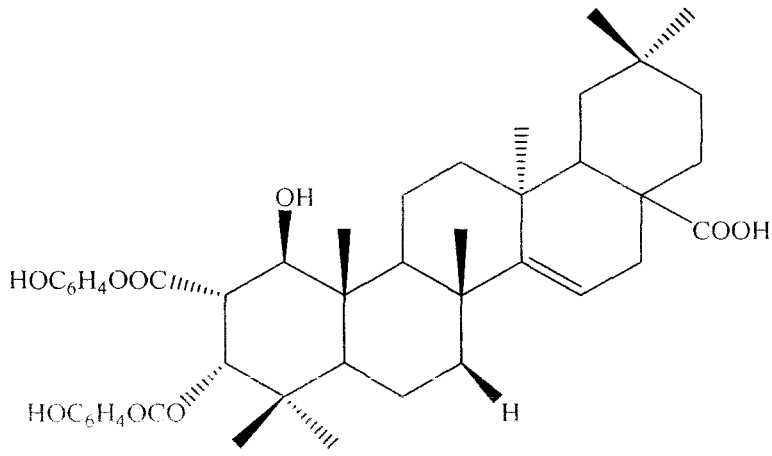
Araujo and Chaves [31] isolated eleven oleanane, ursane and lupane-type triterpenes daturadiol (3β,6β–dihydroxy-olean-12-ene), 3β-hydroxy-30-norlupan-20-one, lupenone, β-amyrenone, α–amyrenone, lupeol, β-amyrin, α–amyrin, betulin, erythrodiol and uvaol, in addition to squalene, sitosterol and α–tocopherol from the leaves of *Terminalia brasiliensis* Camb. They identified the structures of these compounds by ^{1}H and ^{13}C NMR spectral analysis.



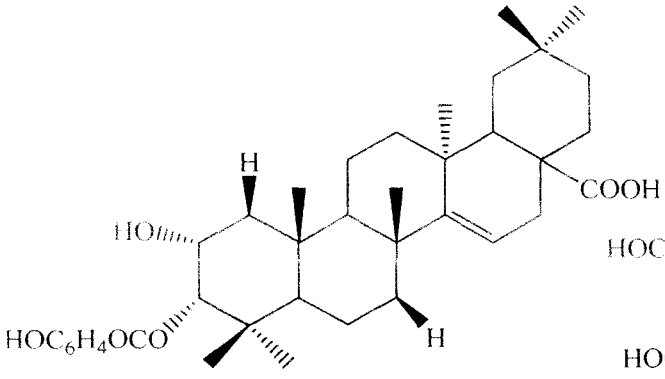
4 (Betulin)

Chaudhuri *et al.* [32] isolated pentacyclic triterpenoids based on the taraxer-14-ene skeleton with a C-28 attached carboxylic acid group from the roots of *Maprounea*

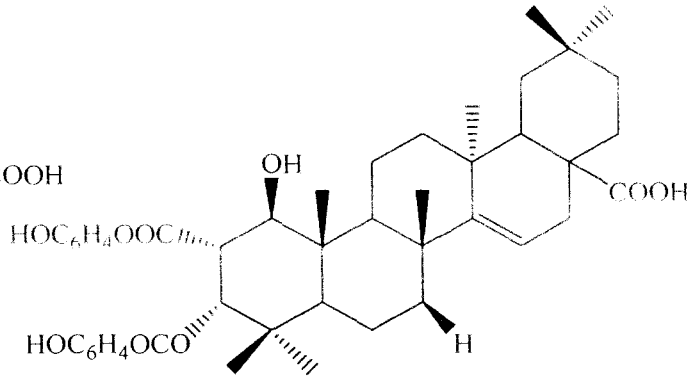
africana. They identified these compounds as 1β, 2α-dihydroxyaleuritolic acid 2,3-bis-hydroxybenzoate[1], 2□-hydroxyaleuritolic acid 3-p-hydroxybenzoate [2], 2α-hydroxyaleuritolic acid 2,3-bis-p-hydroxybenzoate[4], aleuritolic acid 3-p-hydroxybenzoate[5], aleuritolic acid [6], and aleuritolic acid 3-acetate [7]. They reported that compounds 1 and 2 are new triterpene esters.



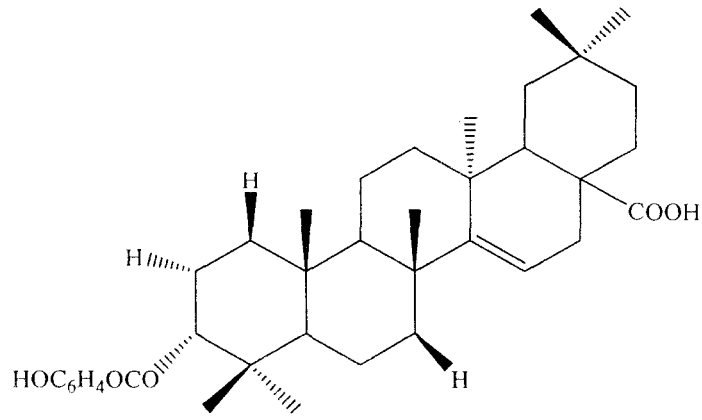
1β, 2α-dihydroxyaleuritolic acid 2,3-bis-hydroxybenzoate



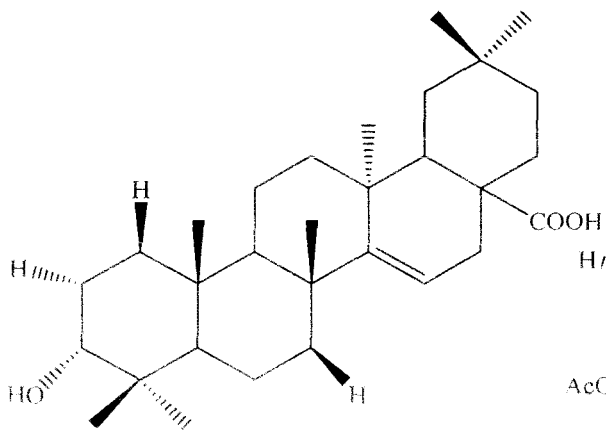
2α-hydroxyaleuritolic acid 3-p-hydroxybenzoate



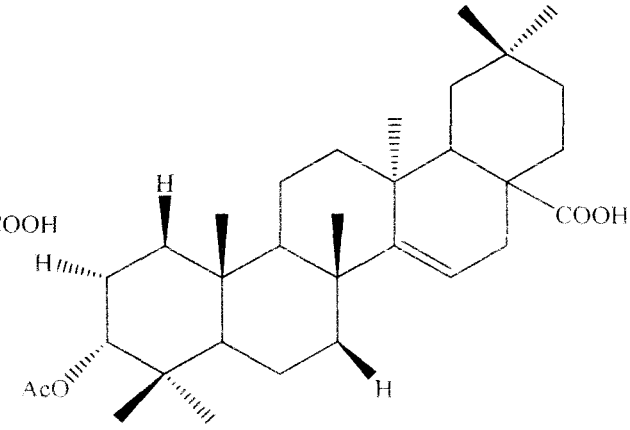
2α-hydroxyaleuritolic acid 2,3-bis-p-hydroxy benzoate



Aleuritolic acid 3-p-hydroxybenzoate

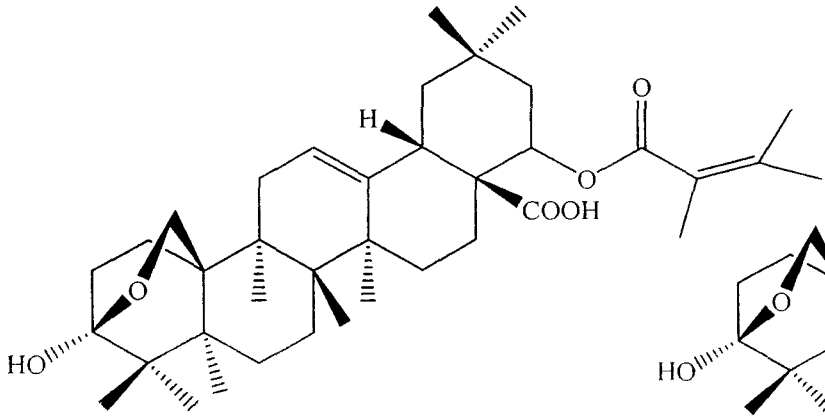


Aleuritolic acid

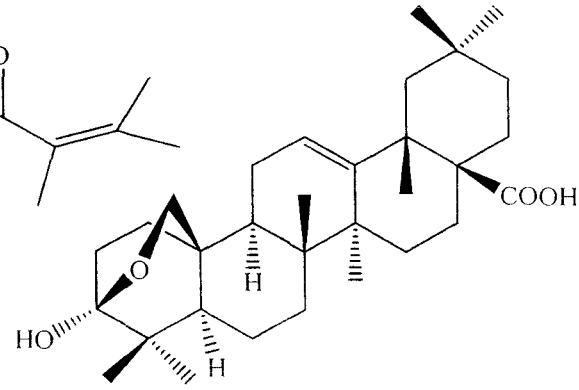


Aleuritolic acid 3-acetate

Begum *et al.* [33] isolated three new pentacyclic triterpenoids, camaryoloic acid (1), methylcamaralate (2) and camangeloyl acid (3) with six known compounds, □-sitosterol 3-O-□-D-glucopyranoside (4), octadecanoic acid (5), docosanic acid (6), palmitic acid (7), camaric acid (8) and lantanolic acid (9) from the aerial parts of *Lantana camara*. They elucidated the structures of the new compounds by spectroscopic and chemical methods.



Camaric acid



lantanolic acid

Machocho *et al.* [34] isolated five oleanane-type pentacyclic triterpenoids by chromate graphic separation from the stem bark of Embelia schimperi using chloroform solvent. They reported that three compounds have a methyleneoxy bridge and two compounds ,embelinone and schimperinone were first time extracted from natural source. Their structures were determined by spectroscopic techniques, among which 2-D NMR were useful for complete characterization. They observed that three of the triterpenoids exhibited mild antibacterial properties against the Gram-positive bacterial strain Rhodococcus sp.

Siddiqui *et al* [35] isolated nine pentacyclic triterpenoids along with a coumarin from a fresh, undried and uncrushed spring leaves of *Plumeria obtuse*. They characterized the new triterpenes obtusin and obtusilic acid as the 24-E and 27-Z p-coumaric esters of the novel 3β, 24-dihydroxyrus-12-en-28-oic acid and 3β,27-dihydroxyrus-12-en-30-oic acid respectively through chemical and spectral studies while the other eight compounds identified were known kaneroside, oleandrin, α-amyrin, neriucoumaric acid, isoneriucoumaric acid, alphitolic acid, oleanonic acid, methyl p-E-coumarate and scopoletin.

Karalai and Laphookhieo [36] isolated three new pentacyclic triterpenoid esters 1-3 together with six known lupane-type triterpenoids from *Bruguiera cylindrica*. They elucidated the structures of the new compounds by spectroscopic methods and were

characterized as 3α-E-coumaroyllupeol 1, 3α-Z-coumaroyllupeol 2 and 3α-E-caffeoyltaraxerol 3.

Begum *et al.* [37] isolated three pentacyclic triterpenoids including one new guajavanoic acid (2) and two known obtusinin (1) and goreishic acid I (3) from the leaves of *Psidium guajava* . They characterized the new constituent 2 as 2 α-hydroxy-3 β-p-E -coumaroyloxyurs-12, 18-dien-28-oic acid through 1 H-NMR and 13 C-NMR. They isolated compound 1 and 3 first time from the genus *Psidium.*

Laphookhieo *et al.* [38] isolated a new sesquiterpene (1) and two new pentacyclic triterpenoid esters (2,3) together with three known compounds (4-6) from the fruits of *Rhizophora mucronta.* They elucidated the structures of the isolated compounds and characterized as 3-hydroxy-3, 7, 11-trimethyl-9-oxododeca-1,10-diene(1),3β-E-caffeoyltaraxerol (2) and 3β-Z-caffeoyltaraxerol (3).

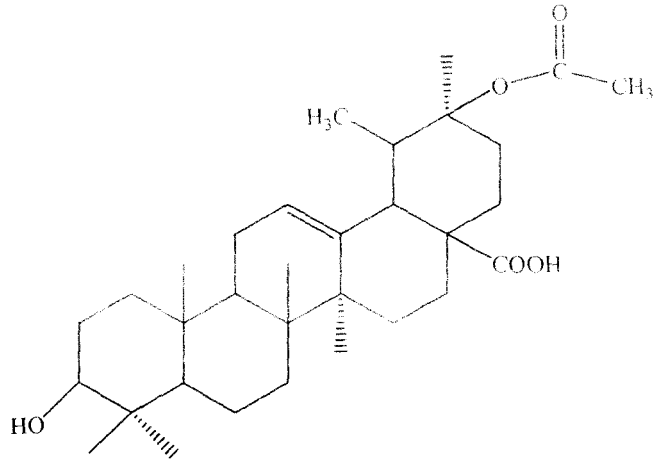
Ibrahim and Ali [39] isolated a long chain ketone, a pentacyclic triterpenoid coupled with fatty acid moiety, and an acyclic diterpenoid from the ethanol soluble part of *Nepeta crassifolia* collected from Kangavar, Iran. They elucidated the structures of all the metabolities with the aid of spectroscopic techniques, including 2D NMR experiments.

Tabopda *et al.* [40] isolated four new triterpene glucosides (1-4) using methanol as a solvent from the stem bark of *Terminalia superba.* The structures of the new compounds were established by spectroscopic method and characterized as alpha,3 beta-dihydroxyolean-12-en-28-oic acid 28-O-beta-D-glucopyranoside (1), 2 alpha,3 beta, 21 beta-trihydroxyolean-12-en-28-oic acid 28-O-beta-D-glucopyranoside (2), 2 alpha,3 beta, 29-trihydroxyolean-12-en-28-oic acid 28-O-beta-D-glucopyranoside (3) and 2 alpha,3 beta,23,27-tetrahydroxyolean-12-en-28-oic acid 28-O-beta-D-glucopyranoside (4) together with the known triterpene 2 alpha,3 beta,23-trihydroxyolean-12-en-28-oic acid (5). They investigated the antibacterial activity of 1-5 against two gram-positive bacteria (*Bacillus subtilis, Staphylococcus aureus*), and four Gram-negative (*Escherichia coli, Shigella flexenari, Pseudomonas aeruginosa, Salmonella typhi*) bacterial strains.

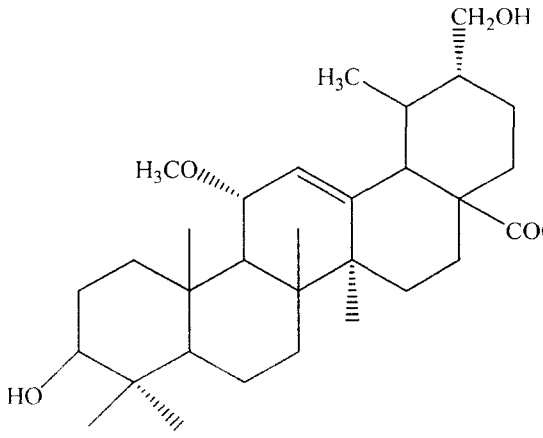
Siddiqui *et al.* [41] investigated the constituents of the fresh, uncrushed leaves of *Eucalyptus camaldulensis* var. obtusa and isolated a triterpenoid amirinic acid with four known triterpenoids ursolic acid lactone, betulinic acid, oleanolic acid and ursolic acid. They transformed amirinic acid into amirolide in deuterated chloroform at room temperature. The new products were characterized by exhaustive spectroscopic studies.

Siddiqui *et al.* [42] studied the fresh leaves of *Carissa carandas* collected from the Karachi Region in Pakistan and isolated four pentacyclic triterpenoids (1-4) including one new constituent carissin (1) and two hitherto unreported compounds 2 and 3. They elucidated the structure of the new compounds as 3beta-hydroxy-27-E-feruloyloxyurs-12-en-28-oic acid.

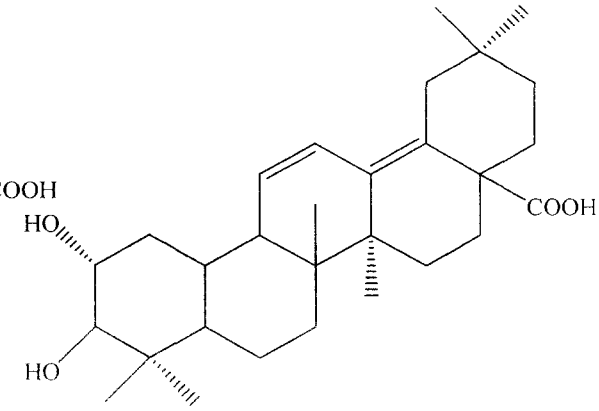
Begum and Farhat [43] investigated the constituents of fresh, uncrushed leaves of *E. camaldulensis* var. obtuse and isolated a known and 3 new triterpenoids. They characterized the new compounds by chemical and spectroscopic studies as camaldulic acid (20 beta-acetoxy-3 beta-hydroxyrus-12-en-28-oic acid), camaldulensic acid (3 beta, 30-dihydroxy-11 alpha-methoxyrus-12 en-28-oic acid) and camaldulenic acid (2 alpha, 3 beta-dihydroxyolean-11,13(18)-dien-28-oic acid)



Camaldulic acid



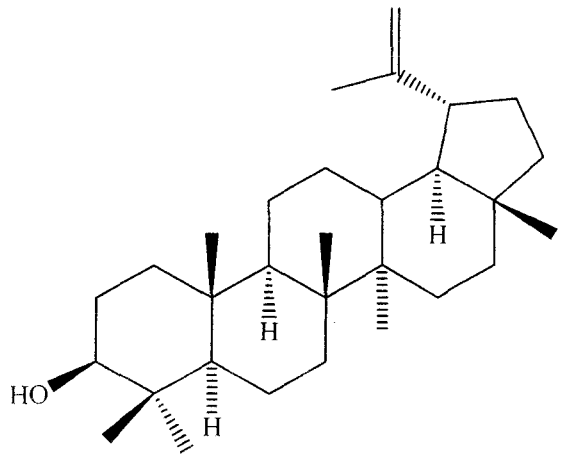
Camaldulensic acid



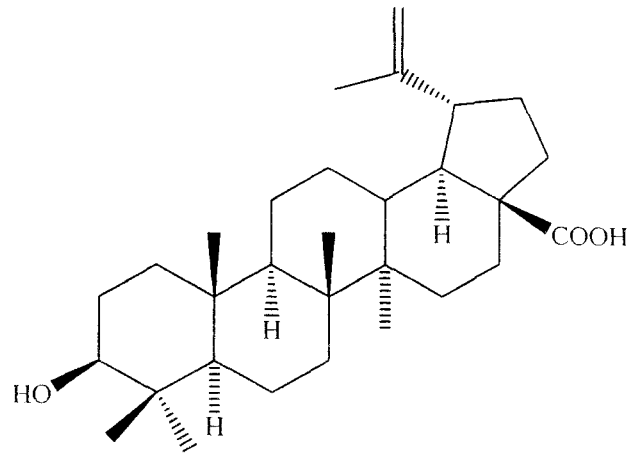
Camaldulenic acid

Begum *et al.* [44] isolated two triterpenoids, 20 beta-acetoxy-2 alpha,3 beta-dihydroxyrus-12-en-28-oic acid (guavanoic acid, 3) and 2 alpha,3 beta-dihydroxy-24-p-z-coumaroyloxyrus-12-en-28-oic acid (guavacoumaric acid,7) along with six known compounds 2 alpha-hydroxyursolic acid (1), jacoumaric acid (2), isoneriucoumaric acid (4), asiatic acid (5), ilelatifol D and (6) beta-sitosterol-3-O-beta-D-glucopyranoside (8) from the leaves of *Psidium guajava*. They determined the structures of the isolated compounds through spectroscopic methods.

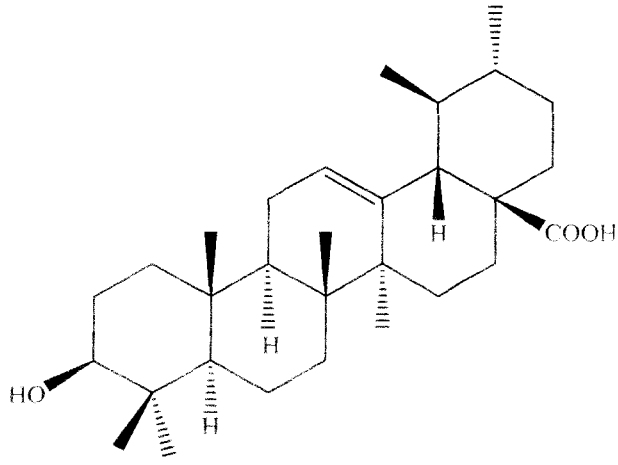
Shai *et al.* [45] isolated four compounds lupeol (1), betulinic acid (2), ursolic acid (3) and 2 alpha-hydroxyursolic acid (4) from the leaves of *Curtisia dentate*. They studied the antibacterial and antifungal activity using broth microdilution assay and bioautography method and found that betulinic acid, ursolic acid and 2 alpha-hydroxyursolic acid appreciably inhibited fungal growth with MIC values ranging from 8 to 63μg/ml.



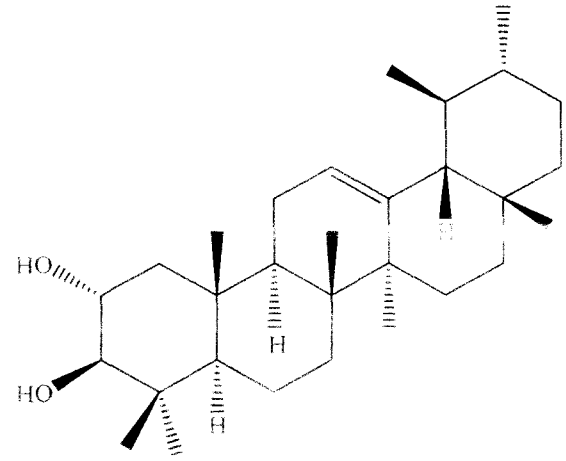
Lupeol



Betulinic acid

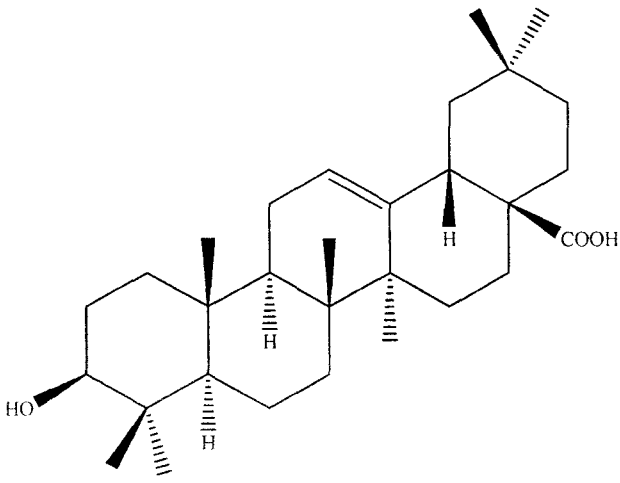


Ursolic acid

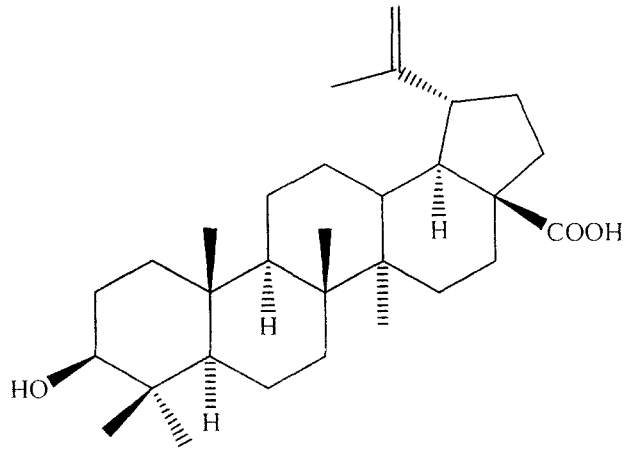


2-Alpha-hydroxyursolic acid

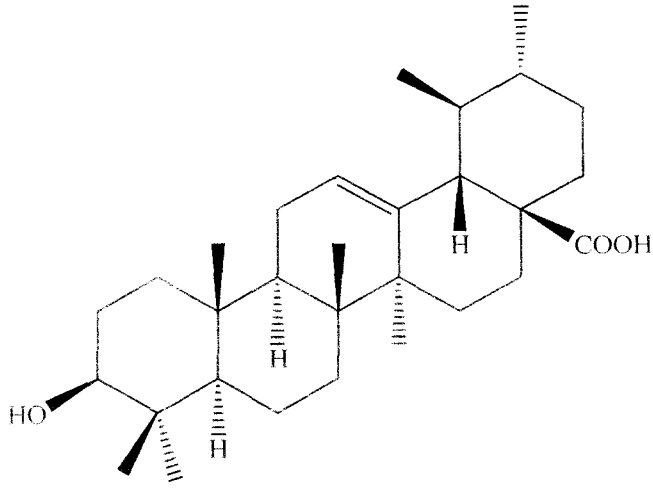
Gu *et al.* [46] derived three pentacyclic triterpenoids from plant as oleanolic acid (**1**), betulinic acid (**2**) and ursolic acid (**3**) and found that this triterpenoids exhibit moderate anti-tubercular activity in a microplate alamar blue assay.



Oleanolic acid

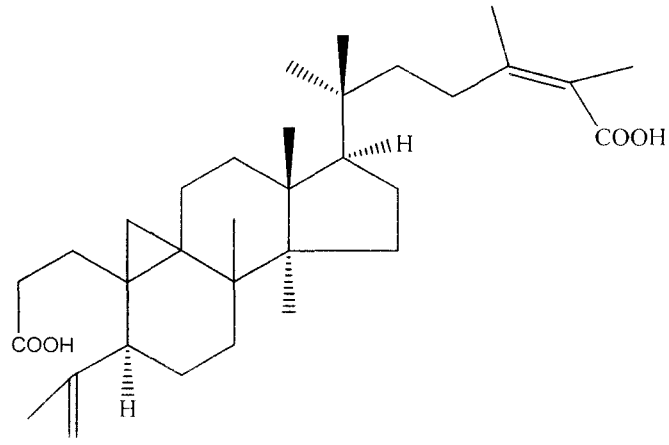


Betulinic acid



Ursolic acid

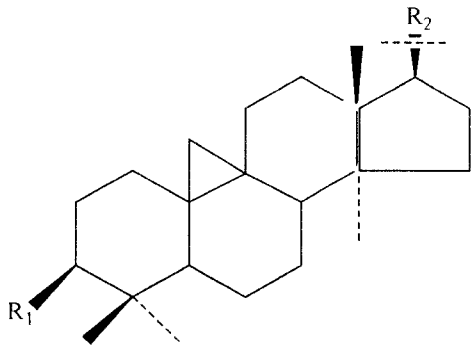
Sun *et al.* [47] isolated a ring-secocycloartene triterpenoid, nigranoic acid (3,4-secocycloarta-4(28),24-(Z)-diene-3,26-dioic acid from the stem of Schisandra sphaerandra, a Chinese traditional medicinal plant and its structure elucidation and unambiguous NMR spectral assignment were achieved by the combination of 1D- and 2D-NMR techniques with the aid of computer modeling. They found that nigranoic acid showed activity in several anti-HIV reverse transcriptase and polymerase assays.



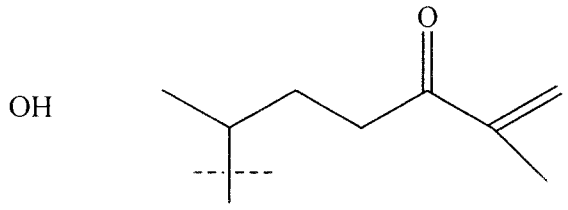
Nigranoic acid (3,4-secocycloarta-4(28),24-(Z)-diene-3,26-dioic acid

Takeoka *et al.* [48] isolated three triterpenoids betulinic acid, oleanolic acid and ursolic acid as their methyl esters from diethyl ether extracts of almond hulls using flash chromatography and preparative high performance liquid chromatography. They were characterized triterpenoids using chromatographic and spectroscopic methods and these studies demonstrated that almonds hills are a rich source of triterpenoids. They reported anti-inflammatory, anti-HIV and anti-cancer activities of these triterpenoids.

Madureira *et al.* [49] carried out phytochemical reinvestigation of the whole plant of *Euphorbia segetalis* and isolated five tetracyclic triterpenes: 3β-hydroxy-cycloart-25-en-24-one (**1**), cycloart-25-ene-3β,24-diol (**2**), cycloart-23-ene-3β,25-diol (**3**), lanosta-7,9(11),24-trien-3β-ol (**4**) and lanosta-7,9(11),24(31)-trien-3β-ol (**5**). 3β-acetoxy-cycloart-25-en-24-one (**1a**) and glutinol (**6**), lupenone (**7**), friedelin (8) dammaranodienol (**9**), cycloartenol acetate (**10**), 24-methylenecycloartanol acetate (**11**) and β-sitosterol (**12**). They were studied for their antiviral activities against Herpes simplex virus (HSV) and African swine fever virus (ASFV) and observed that lupenone exhibited strong viral plaque inhibitory effect against HSV-1 and HSV-2. The *in vitro* antifungal and antibacterial activities of **1a**, cycloart-23-ene-3β,25-diol, 3-acetate (**3a**) and **6-12** were also investigated.

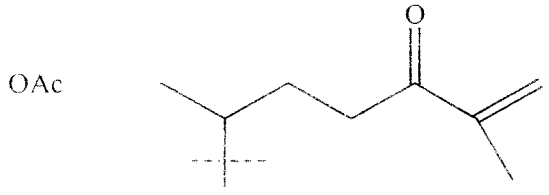


R1 R2

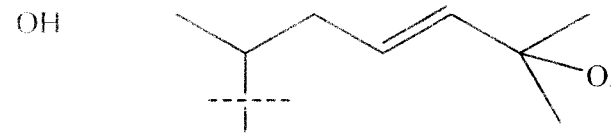


= 3 β-hydroxy-cycloart-25-en-24-one

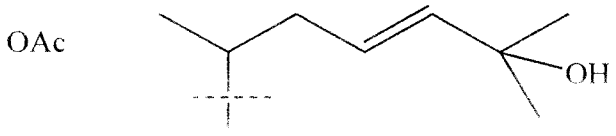
R1 R2



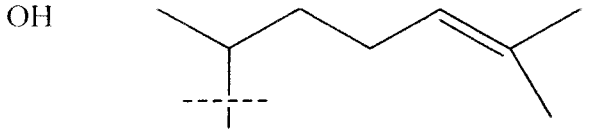
= 3β-acetoxy-cycloart-25-en-24-one



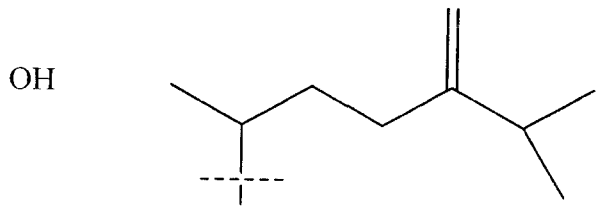
= cycloart-25-ene-3β,24-diol



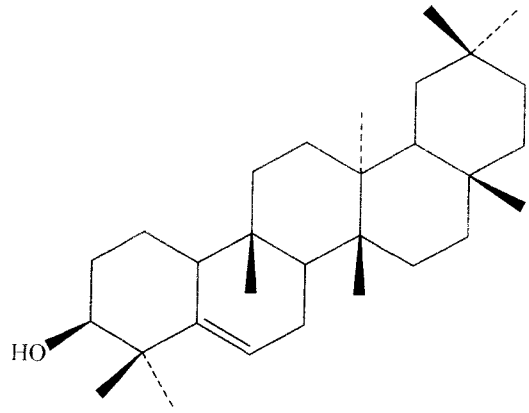
= cycloart-23-ene-3β,25-diol



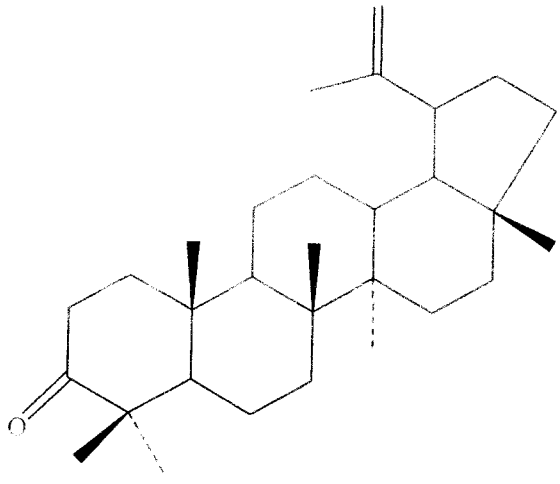
= cycloartenol



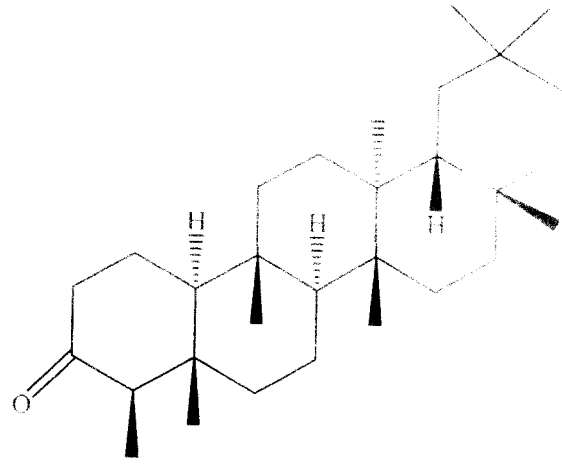
= 24-methylenecycloartanol



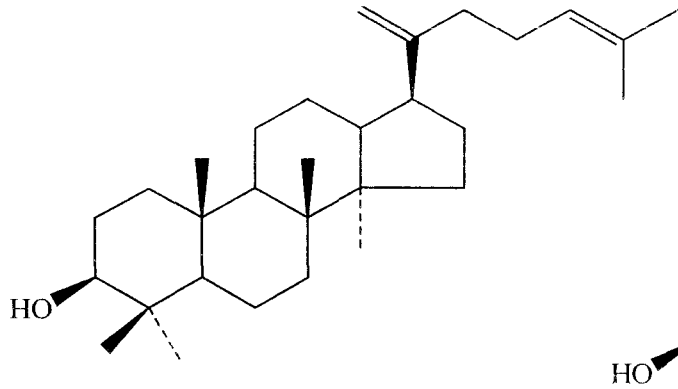
Glutinol



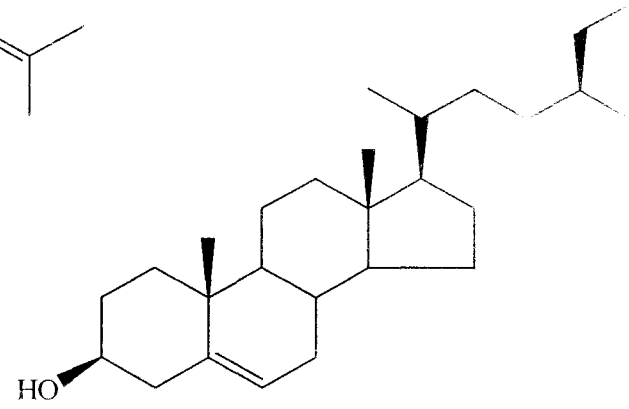
lupenone



friedelin

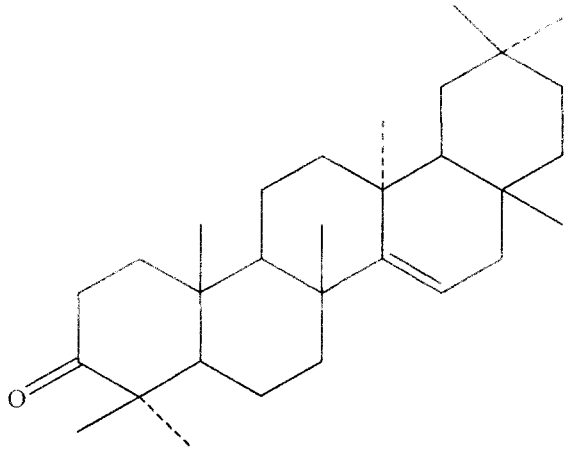


Dammaranodienol

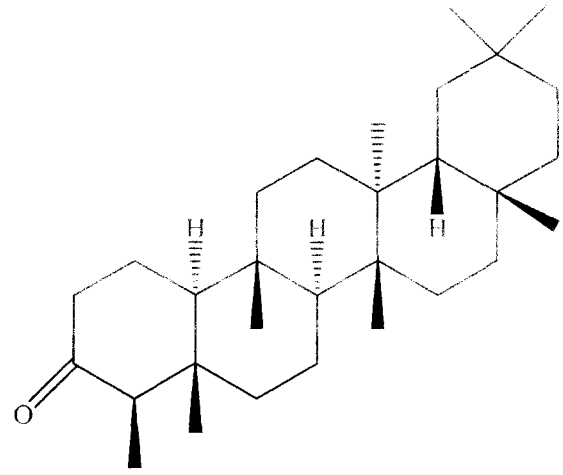


β-sitosterol

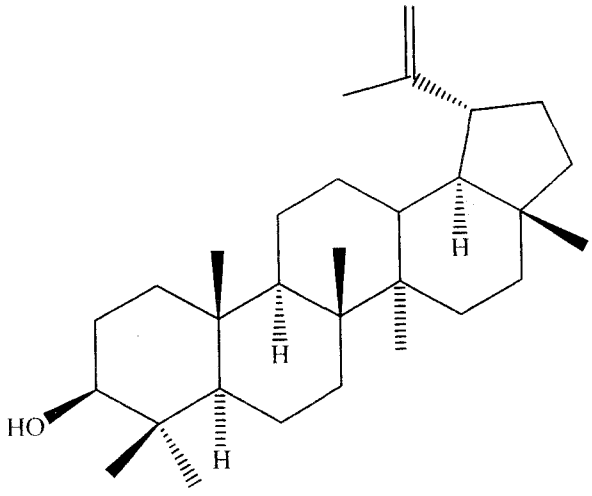
Gunaskera et al. [50] isolated new lupine derivative 3β–hydroxy-28-p-coumaroyloxy-lup20(29)-27-oic acid from *Camipa densifolia* and whose structure was deduced by chemical correlation with betulin (6) Simiarenol (l) taraxerone (2) friedelin (3) lupeol (4) betulinic acid (5) betulin (6) and β-sitosterol-g-D-glucoside.



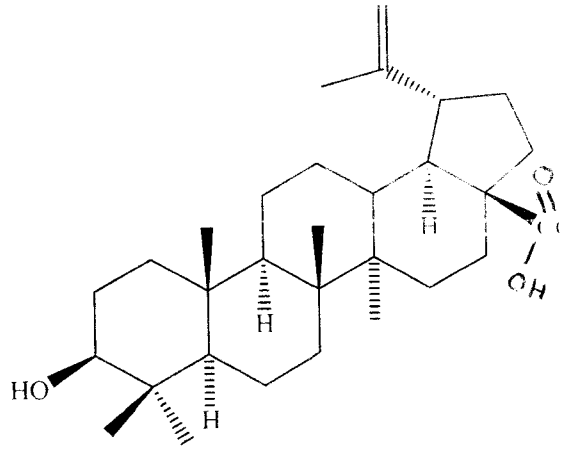
Taraxerone



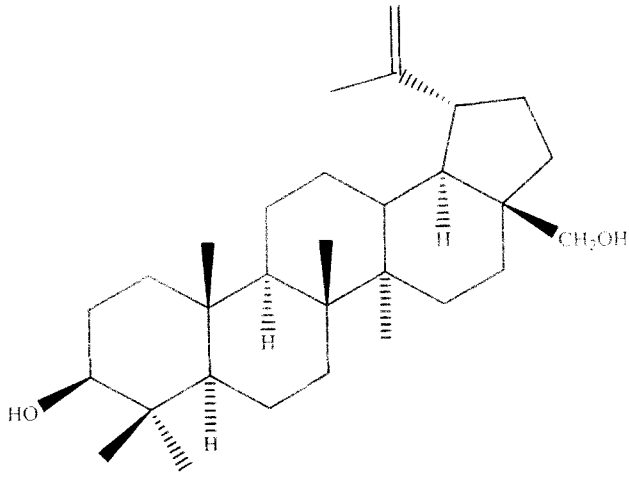
Friedelin



Lupeol



Betulinic acid



Betulin

Section B: A Short Review on the Biological activity of isolated materials from medicinal plants

Throughout history, mankind has always been interested in naturally occurring compounds from prebiotic, microbial, plants and animals sources. Various extracts of different parts of plants have been widely used in folk medicines and perfumes as well as in food flavor and preservatives and are more commonly utilized in chronic diseases like cancer, diabetes and asthma [104,105].

The ancient Egyptians have described several useful preparations such as opium and castor oil. They also used "rotten bread" for treating infections which resembles our use of antibiotics from moulds and fungi.

The Chinese are considered as leaders in using natural products for healing. The oldest compilation of Chinese herbs is Shen Nung Pen Ts'ao. Which lists 385 materials. 5267 medicinal herbs were used in China in 1967. One of the most famous herbs among them is the ginseng root, *Panax ginseng* is used for health maintenance and for the treatment of various diseases. Another popular folk drug is the extract of the Ginkgo tree, *Ginkgo biloba* which can improve memory and mental alertness.

During the 17th century, the Jesuite brought with them from South America the bark of the China tree for the treatment of malaria. In 1820, Pelletia and Caventou isolated from the China tree the active compound, quinine. American Indians used the powerful hallucinogen, mescaline for a long period. The Indian hemp plant, *Cannabis sativa*, has been used since 3000 BC, and it is used as marijuana or hashish.

At the onset of the present study it was considered to review the reports presented by the earlier workers regarding the biocidal activity of various plant extracts tested on different organisms, especially on them selected for the present investigation. The observation of the previous workers in concord with the present line of investigation is being presented, in a selective manner, in the following paragraphs.

Mansouri [51] found new antibacterial agents from ethanolic extracts of ten plants. The agents were effective against *Staphylococcus aureus*. Several samples (489 samples) of *S.aureus* were isolated from healthy carriers (nose and throat) or clinical samples. Out of 489 isolates 98.6% were sensitive to trimethoprim-sulfamethoxazole. The extracted compounds from the plants were screened for antibacterial activity. *Myrtus communis* L. (leaves) showed the greatest activity, inhibiting the growth of 99% of the isolates. *Glycyrrhiza glabra* L., *Eucalyptus globolus Labill* and *Menta vividis L.* were also active against the isolates and inhibited the growth of 90, 59.5 and 48.7% of the isolates respectively.

Reddy *et al.* [52] studied the antibacterial activity of the pure isolates from *Piper longum* (L) (black pepper) and *Taxus bacata* (L) (yew). Three isolates of black pepper were active against Gram positive bacteria and moderately active against Gram negative bacteria. They reported that each isolates was highly active against at least one particular species of bacteria: Piperlongumine against *Bacillus subtilis*, Piperine against *Staphylococcus aureus*, and Pellitorine against *Bacillus sphaericus*. 3-(3'-4'-5'-Trimethoxyphenyl). Propionic acid did not show any bacterial activity. From the results they showed that most of the isolates of *piper longum* had antibacterial activity.

Samy and Ignacimuthu [53] reported the antifungal activity of crude drug from the tree bark of *T. arjuna* which was tested against bacteria using the hole-plate diffusion method with concentrations of 5-25 mg/mL. The effective results of bacteria were confirmed by the dilution method (1.25-2.0 mg/ml) in MIC. The results were supported by pathochemical analysis. The specific activity against pathogenic bacterium, *Bacillus subtilis* and *Staphylococcus aureus* showed the traditional usage of bark of *T. arjuna*.

Khan *et al.* [54] fractionated extracts of leaves, stem bark and root bark of *Eupomatia laurina* and performed test against 13 Gram-positive and 12 Gram-negative bacteria, a protozoan and four fungi. They found that all the extracts were active against most of the bacteria and fungi and the dichloromethane and ethyl acetate extracts of the stem bark and the dichloromethane extract of the root bark exhibited superior levels of antibacterial activity.

Ramesh *et al.* [55] isolated Friedelin, epi-Friedelin, n-Octacosanol, α–Amyrin, Sitosterol, Sitosterol-3- -D-glucopyranoside and luteoforol from Bridelia crenulaa Roxb. The aqueous and methanolic extracts and their fractions were tested against ten human pathogenic bacteria and four fungal strains. They observed that inhibitory activities were maximum in the chloroform-methanol (1:1) fraction of the methanolic extract against *E.coli, K.pneumoniae* and *P.aeruginasa*, which were responsible for the pathogenesis of urinary tract infection. The above study provided scientific evidence for the efficacy of the use.

Murillo-Alvarez *et al.* [56] extracted compounds from plants used in the traditional medicine of Baja California 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.

Smith *et al.* [57] performed influence of medium type inoculum density and a cold incubation on antimicrobial assay sensitivity test. The largest and most distinct zones were produced using nutrient agar and the $1/10^4$ inoculum density for *Pseudomonas aeruginasa* and *Escherichia coli*. The greatest number of zones was detected without cold incubation. Using this method eight plants from Belize were screened for antibacterial activity. They reported that six plants showed activity against the four organisms tested. Both inoculum density and medium type played important roles in assay sensitivity.

Srikrishna *et al.* [58] carried out antibacterial activity using cup plate method. They observed that pet. ether, chloroform, methanol and water extract of the bark of *Aporosa lindleyana* (Euphorbiaceae) showed moderate to very good activity against bacteria such as *Bacillus subtilis, Escherichia coli.* They studied antifungal activity such as *Penicillium chrysozenous, Candida albicans, Aspergilus niger* and *Trichoderma vridar* and compared with the standard drug fluconazole. The pet. ether extract showed considerable activity towards all the four fungal organisms.

Akinpelu [59] observed that *Anacardium occidentale* bark 60 percent methanolic extract exhibited antimicrobial activity against 13 out of 15 bacterial isolates at a concentration of 20 mg/ml.

Audu *et al.* [60] extracted components from *Annona senegalensis* (root), *Nauclea latifolia* (stem bark) and *Ziziphus abyssinica* (root bark) using methanol, diethyl ether and cold water as solvent. They studied their activity on *Candida albicans*, *Escherichia coli*, *Salmonella* spp. and *Staphylococcus aureus* at different concentrations and found that all these components inhibited the growth of microbes.

Kamalakanman *et al.* [61] extracted 20 plant leaf and screened their inhibitory effect against the rice blast pathogen. They reported that *Prosopis juliflora* followed by *Zizyphus jujube* and *Abutilon indicum* significantly inhibited the mycelial growth and biomass as well as toxin production and spore germination under laboratory conditions.

Mehmood *et al.* [62] studied the antimicrobial potential of some Indian medicinal plants and their formulations. They tested twenty five different formulations based on five alcoholic extracts against several pathogenic micro-organisms. They observed that ten formulations showed higher potency compared to their constituents and good synergistic activity leading to significant reduction in the MIC values.

Ragasa *et al.* [63] extracted the air dried leaves of *Vitex negundo* which afforded vitexilactone and casticin by silica gel chromatography. Their structures were elucidated by extensive 1D and 2D NMR spectroscopy. They studied their activity and found to inhibit the growth of the fungi: *Candida albicans* and *Aspergillus niger* and the bacteria: Staphyllococcus aureus and Pseudomonas aeruginasa, but inactive against *Escherichia coli* and *Bacillus subtilis*.

Habtemariam and Macpherson [64] investigated the cytotoxic and antibacterial activity of an ethanol extract of leaves of a herbal drug *Eupatorium perfoliatum*. They observed that the extract showed a potentcytotoxicity and weak antibacterial activity against gram positive test organisms *Staphylococcus aureus* and *Bacillus megaterium*.

Mackeen *et al.* [65] reported that the crude ethanol extracts exhibited predominantly antibacterial activity with the root extract showing the strongest inhibition against the test bacteria at a minimum inhibitory dose (MID) of 15.6 microg/disc. They observed that most of the extracts failed to inhibit the growth of fungi but the root, leaf, trunk and stem bark extracts showed strong antioxidant activity. Antitumour-promoting activity was shown by the fruit, leaf, stem, and trunk bark extracts.

Lall and Meyer [66] observed that the water and acetone extracts of roots of *Euclea natalensis* inhibited the growth of *Bacillus cerus, Bacillus pumilus, Bacillus subtilis, Micrococcus kristinae* and *Staphylococcus aureus* at concentration ranging between 0.1 and 6.0 mg/ml. They found that the water extract did not exert any inhibitory action on Gram-negative bacteria while the acetone extract showed inhibitory activity at a concentration of 5.0 mg/ml against all the Gram-negative bacteria investigated. The antibacterial activity of acetone extract was also investigated by a direct bioassay on TLC plates against *S. aureus*.

Pichai *et al.* [67] extracted the leaves of *Tabebuia rosea* using n-hexane, chloroform and aqua as a solvents and screened the antibacterial activities against the pathogens Escherichia coli, Salmonella typhi and Staphylococcus aureus by agar dilution method. They observed that the aqueous extract exhibited potential antibacterial activity against *E. coli, S. Typhi* and *S. aureus* the hexane extracts had no effect on the three bacteria.

Ahmad and Beg [68] extracted 45 Indian plants traditionally used in medicine using ethanol as a solvent and studied their antimicrobial activity against certain drug-resistant bacteria and a yeast *Candida albicans*. They observed that of these 40 plant extracts showed varied levels of antimicrobial activity against one or more test bacteria. Anticandidal activity was detected in 24 plant extracts.

Savikin *et al.* [69] investigated the antimicrobial activity of the methanolic extracts of flowers and leaves of *Gentiana lutea* L together with the isolated compounds mangiferin, isogentisin and gentiopicrin. They studied the activity against a Gram-positive and a Gram-negative bacteria as well as the yeast *Candida albicans* and observed that both

extracts and isolated compounds showed antimicrobial activity with MIC values ranging from 0.12-0.31 mg/ml.

Al-Hussaini and Mahasneh [70] studied the antimicrobial and antiqurorum sensing activities of fourteen ethanolic extracts of different parts of eight plants against four Gram-positive, five Gram-negative bacteria and four fungi. They were recorded variable activities depending on the plant part extract and microorganism at 3 μg/disc. They found that among the Gram-positive bacteria tested, the activities of *Laurus nobilis* bark extract ranged between a 9.5 mm inhibition zone against Bacillus subtilis up to a 25 mm one against methicillin resistant *Staphylococcus aureus*. They also found that *Staphylococcus aureus* and *Aspergillus fumigatus* were the most susceptible among bacteria and fungi tested towards plants parts. However, minimum inhibitory concentrations (MIC's) for both bacteria and fungi were relatively high (0.5-3.0mg).

Ettebong and Nwafor [71] 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*. They also reported that none of the extracts showed any inhibitory effect against *Pseudomonas aeruginosa* and the fungal strains of *Candida albicans* and *Tinea capitis* and the most potent of these extracts was chloroform extract with Minimum Inhibitory Concentration (MIC) of 25 mg/ml for bacteria. The Phytochemical screening of the root of *C. lutea* revealed the presence of saponins, anthraquinones, flavonoids, cardiac glycosides, simple sugar and terpenes.

Alves *et al.* [72] evaluated the antimicrobial, antifungal and antiadherent activity of aroeira-do-sertao, mallow and guava tree on oral biofilm microorganisms and oral *candidiasis* in vitro. They found that the extracts were shown to be effective the inhibition the growth of bacteria of the oral biofilm and fungi of oral *candidiasis*.

Qadrie *et al.* [73] studied the antibacterial activity of the ethanolic extract of *Indoneesiella echioides* (L) nees by filter paper disc method, this method was based on the diffusion of an antibiotic from a filter paper disc through the solidified culture media of a Petri dish. They observed that the growth was inhibited entirely in a circular area "zone around the filter" paper disc containing a solution of antibiotic and the plant extract. The used microorganisms were: *Staphylococcus aureus* and *Escherichia coli* and the organisms were maintained on nutrient agar slants. They tested the organisms using nutrient broth, one loop full of the respective cultures was taken in slants which were inoculated below 40 degree C and incubated at 37 degrees C for 24 hrs and observed the growth with naked eye for their turbid nature and compared with that of sterile broth.

Duraipandiyan *et al.* [74] studied the antimicrobial activity of 18 ethnomedicinal plant collected from Palni hills of Southern Western Ghats against nine bacterial strains (*Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Ervinia sp, Proteus vulgaris*) and one fungal strain(*Candida albicans*) using paper disc diffusion method. They reported that out of 18 plants, 10 plants exhibited antimicrobial activity against one or more of the tested microorganisms at three different concentrations of 1.25, 2.5and 5 mg/disc. The study evaluated the antimicrobial activity of the some ethnomedicinal plants used in folkloric medicine.

Gangoue-Pieboii *et al.* [75] investigated the antimicrobial activities in Vitro of 10 plant species (*Voacanga africana, Crepis cameroonica, Plagiostyles africana, Crotalaria retusa, Mammea africana, Lophira lanceolata, Ochna afzelii, Ouratea elongate, Ou. flava* and *Ou. sulcata*) each of which used in the traditional medicine in Cameroon. They 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 and no activity was found against the Gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae*

Pseudomonas aeruginosa) and Plagiostyles africana showed greatest antimicrobial activity.

Yasunaka *et al.* [76] studied the antibacterial activity of the thirty two extracts from 22 Mexican medicinal plants of 15 different families against *Escherichia coli and Staphylococcus aureus.* They reported that seventeen plants showed antibacterial, while five plants showed no activity against both bacteria and all of the extracts showed higher activity against *Staphylococcus aureus* than *Escherichia coli* except one.

Kumar *et al.* [77] carried out antimicrobial properties of a series of 61 medicinal plants belonging to 33 different families used in various infectious disorders at 1000 and 500 microg/ml concentration by agar dilution method against *Bacillus cereus, Bacillus pumilus, Bacillus subtilis, Bordetella bronchiseptica, Micrococcus luteus, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa , Streptococcus faecali, Candida albicans, Aspergillus niger and Saccharomyces cerevisiae.* They found that 28 plant extracts showed activity against at least one of the test organisms used. The crude extracts of *Dorema ammoniacum, Sphaeranthus indicus, Dracaena cinnabari, Mallotus philippinessis, Jatropha gossypifolia, Aristolochia indica, Lantana camara, Nardostachys jatamansi, Randia dumetorum* and *Cassia fistula* exhibited significant antimicrobial activity and property that support the folkloric use in the treatment of as broad-spectrum antimicrobial agents.

Adamu *et al.* [78] carried out a survey of medicinal plants used locally in the treatment of various diseases in Bauchi State-Nigeria and total 84 medicinal plants were listed. They investigated the antimicrobial activity of the aqueous extracts of the plants and found that out of 84 plants, 75 exhibited antimicrobial activity against one or more of the test organisms at a concentration of 200 mg/ml. They found that the extracts showed potentially interesting activity against *Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli.*

Bonjar [79] studied the antibacterial activities of the 45 species of 29 plant families used in the traditional medicine by Iranian people against *Bacillus cereus, Bacillus*

pumilus, Bordetella bronchiseptica, Escherichia coli, Klebsiella pneumoniae Micrococcus luteus, Pseudomonas aeruginosa, Pseudomonas fluorescens, Serratia marcescens, Staphylococcus aureus, Staphylococcus epidermidis. He found that no plant showed activity against *Serratia marcescens and Bordetella bronchiseptica* was the most susceptible species. All the extracts showed the same activity 18 months later.

Saleh *et al.* [80] isolated the known triterpenoids lantic acid, camarinic acid and lantanilic acid from *Lantana camara* (L) cultivated in Egypt and carried out the antibacterial activity of lantic acid using bioautography assays for Gram-positive and Gram-negative bacteria. They found that lantic acid possess strong antibacterial activity against *Escherichia coli* and *Bacillus cereus* in which 0.08 and 0.1 μg were the minimum inhibition doses compared to 0.05 and 0.005 μg for chloramphenicol. The results showed that lantic acid has broad spectrum antibacterial activity and may hold potential as a non-selective antimicrobial agent.

Mathabe *et al.* [81] isolated four known compounds from the stem bark of *Spirostachys africana* using ethanol as a solvent which is used traditionally for the treatment of diarrhoea and dysentery in Limpopo province of South Africa. The isolated compounds were, two triterpenoids, compound 1 [d-Friedoolean-14-en-oic acid (3-acetyl aleuritolic acid)] and compound 2 (lupeol), and two diterpenes, compound 3[ent-2, 6alpha-hydroxy-norbeyer-1,4,15-trien-3-one (diosphenol2)] and compound 4 (ent-3beta-hydroxy-beyer-15-ene-2-one). They tested the antibacterial activity using micro dilution method and found that compound 1, exhibited MIC of 50 microg/ml against *Staphylococcus aureus, Salmonella typhi, Vibrio cholera, Escherichia coli* and *Shigella dysentery*, compound 2 was not active against all tested microorganisms at 200 microg/ml and at 200 microg/ml all four compounds were not active against *Shigella sonnei.*

Angeh *et al.* [82] isolated four known triterpenoids, 1alpha,3beta-dihydroxy-12-oleanen-29-oic acid (1), 1-hydroxy-2-olean-30-oic acid (2), 3,30-dihydroxyl-12-oleanen-22-one (3), and 1,3,24-trihydroxyl-12-olean-29-oic acid (4) along with a new pentacyclic

triterpenoids (1alpha,23-dihydroxy-12-oleanen-29-oicacid-3beta-O-2,4-di-acetyl-L-rhamnopyranoside) 5 through a bioassay-guided procedure from the leaves of *Combretum imberbe*. They found that all the isolated compounds had moderate (62 μγ/ml) to strong (16 μg/ml) antimicrobial activity (MIC values) against *Staphylococcus aureus*, *Escherichia coli*, and compound 1 and 5 was most active. The results of this study gave credence to the ethnomedicinal use of *Combretum imberbe* and biological activity of its metabolities.

Mothana *et al.* [83] studied the antiproliferative activity against three human cancer cells, antimicrobial activity against antibiotic susceptible three Gram-positive, three Gram-negative bacterial and one fungal stains and three multiresistant Staphylococcus strains by the agar diffusion method and the determination of MIC against three Gram-positive bacteria with the broth micro-dilution assay, as well as for their antioxidant activity using the DPPH radical scavenging method of sixty four methanolic and aqueous extracts of thirty Yemeni plants used in traditional medicine. They found that 12 plants showed growth inhibitory effect against all cancer cells with IC50 values< 50 μg/ml, 9 plants showed pronounced antimicrobial activity against Gram-positive bacteria among them multiresistant bacteria with inhibition zones>15 mm and MIC values < 500 μg.

Shai *et al.* [84] isolated four compounds lupeol (1), betulinic acid (2), ursolic acid (3) and 2-alpha-hydroxyursolic acid (4) from the leaves of *Curtisia dentate*. They studied the antibacterial and antifungal activity using broth microdilution assay and bioautography method and found that betulinic acid, ursolic acid and 2-alpha-hydroxyursolic acid appreciably inhibited fungal growth with MIC values ranging from 8 to 63μg/ml.

Khan *et al.* [85] extracted the leaves, seeds, stem and root barks, stem and root heart-woods of *Michelia champaca* using methanol, petrol, dichloromethane, ethyl acetate, butanol as a solvent and observed that different fractions exhibited antibacterial activity. They also observed that fractionation drastically enhanced the level of activity particularly in all fractions of the stem bark and dichloromethane fraction of the root bark and some fractions of the leaves, stem and root barks demonstrated activity against some of the tested moulds. They found that among all the fractions liriodenine was the active

constituent of the root bark, with a broader and in some cases, better level of activity as compared to the standard.

Khan and Omoloso [86] extracted different fractions from the leaves, stem and root barks of *Dracantomelon dao* using methanol, petrol, dichloromethane, ethyl acetate, butanol as a solvent and found that they demonstrated a very good level of broad spectrum antibacterial activity. They reported that the dichloromethane and butanol fractions of the leaf were the most active and they had antifungal activity.

Khan and Omoloso [87] reported that the methanolic extracts and the fractions (petrol, dichloromethane, ethyl acetate, butanol) obtained from the leaves, seeds, stem and root barks of *Sarcocephalus coadunatus* exhibited a high level of broad spectrum antibacterial activity. They found that the activity was more pronounced in the dichloromethane, ethyl acetate fractions of the leaves, ethyl acetate and butanol fractions of the seeds, dichloromethane fractions of the stem bark and the ethyl acetate fractions of the root bark and none of the fractions showed any antifungal activity.

Dulger *et al* [88] extracted compounds from three *Verbascum* L. species (*Verbascum olympicum* Boiss., *Verbascum prusianum* Boiss., and *Verbascum bombyciferum* Boiss.) and *Klebsiella pneumonia Klebsiella pneumonia Klebsiella pneumonia* investigated their antimicrobial activity using the agar disc diffusion assay against *Escherichia coli* ATCC 11230, *Micrococcus luteus* La 2971, *Staphylococcus aureus* ATCC 6538P, *Salmonella thyphi* ATCC 19430, *Klebsiella pneumonia* UC57, *Pseudomonas aeroginosa* ATCC 27893, *Corynebacterium xerosis* CCM 2824, *Bacillus cereus* ATCC 7064, *Bacillus megaterium* DSM 32, *Mycobacterium smegmatis* CCM 2067, *Proteus vulgaris* ATCC 8427,*Candida albicans* ATCC 10231, *Rhodotorula rubra*, and *Saccharomyces cerevisiae* ATCC 9763. They found that *Verbascum* L. species showed antimicrobial activity against the Gr (+) bacteria and yeasts, but no activity was seen against the Gr (-) bacteria used in this study.

Kirmizigul *et al.* [89] reported antimicrobial and antifungal activities of the MeOH extract from the flowers of Cephalaria transsylvanica and three triterpenic acid glycosides, transsylvanoside A-C by MeOH using an agar-disc diffusion method. They observed that

both the MeOH extract and the glycosides possess antimicrobial and antifungal activities against *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Corynebacterium xerosis*, *Klebsiella pneumonia*, *Candida utilis*, *Kluyveromyces fragilis*, *Aspergillus oryzae* and *Aspergillus flavus* respectively.

Sudharmini and Ashalatha [90] isolated triterpenoids from *Myxopyrum smilacifolium* leaf and found the presence of ursolic acid (0.175mg/g). They reported that the tritrpenoids showed antimicrobial activity in gram positive bacteria and *Candida sps.*

Horiuchi *et al.* [91] isolated the effective compound and identified it as oleanolic acid, a triterpenoid from *Salvia officinalis* (Sage) leaves and tested antimicrobial activity against vancomycin-resistant enterococci (VRE). They also tested the antimicrobial activity of similar triterpenoids, ursolic acid, uvaol, betulinic acid and betulin and found that ursolic acid also showed antimicrobial activity against VRE. The minimum inhibitory concentrations (MICs) of oleanolic acid and ursolic acid were 8 and 4 μg/ml, respectively and these two compounds also showed antimicrobial activity against *Streptococcus pneumoniae* and methicillin-resistant *Staphylococcus aureus* (MRSA). They found that these compounds also showed bactericidal activity against VRE at least for 48 h when added at concentrations that were two-times higher than their MICs.

Khan *et al.* [92] isolated amblyone, a triterpenoid from *Amorphophallus campanulatus* and studied in vitro antibacterial, antifungal and cytotoxic activities using disc diffusion technique and minimum inhibitory concentration was determined using serial dilution technique. They observed large zones of inhibition in disc diffusion antibacterial screening against four Gram-positive bacteria (*Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus* and *Streptococcus pyogenes*) and six Gram-negative bacteria (*Escherichia coli*, *Shigella dysenteriae*, *Shigella sonnei*, *Shigella flexneri*, *Pseudomonas aeruginosa* and *Salmonella typhi*) and the MIC values against these bacteria ranged from 8 to 64 μg/ml. In antifungal screening, the compound showed small zones of inhibition against *Aspergillus flavus*, *Aspergillus niger*, *Rhizopus aryzae*, and *Candida albicans* was resistant against the compound.

Leite *et al.* [93] obtained various organic and aqueous extracts from the leaves of *Indigofera suffruticosa* Mill (Fabaceae) by infusion and maceration and screened their

antibacterial and antifungal activities. They were tested the extracts against 5 different species of humanpathogenic bacteria and 17 fungal strains by the agar-solid diffusion method. They observed that most of the extracts were devoid of antifungal and antibacterial activities, except the aqueous extract of leaves of *I. suffruticosa* obtained by infusion, which showed strong inhibitory activity against the Gram-positive bacteria *Staphylococcus aureus* with a minimal inhibitory concentration (MIC) of 5000 μg ml^{-1}. The MIC values to dermatophyte strains were 2500 μg ml^{-1} against *Trichophyton rubrum* (LM-09, LM-13) and *Microsporum canis*.

Mbwambo *et al.* [94] extracted compounds from stem bark, wood and whole roots of *Ternimalia brownii* using solvents of increasing polarity, namely, pet ether, dichloromethane, dichloromethane: methanol (1:1), methanol and aqua, respectively and the extracts were tested for antifungal and antibacterial activity. They observed that the extracts of the stem bark, wood and whole roots of *T. brownii* exhibited antibacterial activity against standard strains of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Salmonella typhi* and *Bacillus anthracis* and the fungi, *Candida albicans* and *Cryptococcus neoformans*. They found that aqueous extracts exhibited the strongest activity against both bacteria and fungi.

Escalante *et al.* [95] isolated three monodesmosidic triterpenoid saponins from the butanolic extract of *Phytolacca tetramera* and established their structures. They reported that the three saponins belong to the olean-type triterpenoid saponins, with 28,30 dicarboxylic groups and an olefinic double bond on C-12. They observed that phytolaccosides B and E showed antifungal activities against a panel of human pathogenic opportunistic fungi but phytolaccoside F did not show any activity. The most sensitive fungus was *Trichophyton mentagrophytes*.

Ofodile *et al.* [96] extracted compounds from the four species of *Ganoderma* available in Nigeria using n-hexane: diethyl ether, chloroform:acetone and methanol as a solvent and tested their antimicrobial activity. They found that all the three solvent extracts of all the species of *Ganoderma* were active against Pseudomonas syringae and *Bacillus subtilis*, whereas none of the extracts were active against Cladosporium herbarum.

Bouzada *et al.* [97] isolated 44 methanol extracts from 37 Brazilian traditional medicinal plants and evaluated for their antibacterial activity and toxicity to brine shrimp using agar-well diffusion method against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhimurium, Shigella sonnei* and *Bacillus cereus.* They were subjected to serial dilution assay by the extracts for determination of the minimal inhibitory concentration (MIC) and reported that extracts of Baccharis dracunculifolia, Cajanus cajan, Eugenia uniflora, Solanum palinacanthum and Solanum concinnum presented strong antibacterial activity with MIC values below 10 μg/ml for some bacterial strains. The phytochemical analysis showed extracts of the major groups were phytoconstituents.

Khan *et al.* [98] extracted crude from the leaves, stem bark, stem heart wood, root bark and root heart wood of *Euroschinus papuanus* and isolated fractions on partitioning with petrol, dichloromethane (D), ethyl acetate (E) and butanol (B) and studied antibacterial and antifungal activity. They observed that E fractions of the stem heart wood, D of root bark and E of root heart wood demonstrated excellent antibacterial activity and B fractions of leaves; stem heartwood and root bark demonstrated antifungal activity.

Ramesh *et al.* [99] tested the antimicrobial efficiency of aqueous, methanol, chloroform and hexane extracts of *Swertia corymbosa* and noticed maximum inhibitory activity against *Staphylococcus aureus* and *Salmonella typhi.*

Khan and Omoloso [100] extracted the *Breynia cernua* leaves, stem and root barks and heart-woods with petrol, dichloromethane, ethyl acetate, butanol and methanol which gave various fractions. They studied antimicrobial activity of these fractions and found that the best activity was exhibited by the methanol extract of the root bark followed by its butanol fraction and the dichloromethane fraction of the stem bark also demonstrated good activity.

Lauk *et al.* [101] investigated antifungal activity of methanolic extract and alkaloidal fraction of Berberis aetnensis against Candida species. They observed that the crude extracts were active against Candida species and this activity was higher than that of the alkaloidal fraction and berberine.

Aqueveque *et al.* [102] isolated a new biologically active triterpenoid favolon B from fermentation broths of *Mycena* sp. Strain 96180. They found that favolon B showed antifungal activities against *Botrytis cinerea, Mucor miehei, Paecilomyces variotii* and *Penicillium notatum*. No activities were observed against bacteria and yeast.

Ragasa *et al.* [103] extracted the essential oil of *Cymbopogon citrates* Stapf. by the supercritical fluid extraction process and fractionation of the oil afforded cymbopogonol(1) and citral(2). Antimicrobial tests on 1 and 2 indicated that they had moderate activity against *C. albicans* and low activity against *P. aeruginosa, E. coli, S. aureus* and *T. mentagrophytes* and both compounds were inactive against *B. subtilis* and *A. niger*.

From the above literature work the author has found some discrete work on phytochemical investigation of the plants particularly which are available in this region and no systematic study regarding their biocidal activity has so far been carried out. Thus it was felt necessary to undertake thorough study towards the phytochemical investigation of medicinal plants available in this region of West Bengal and also to make a systematic study of the biocidal activity of the isolated plant materials.

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

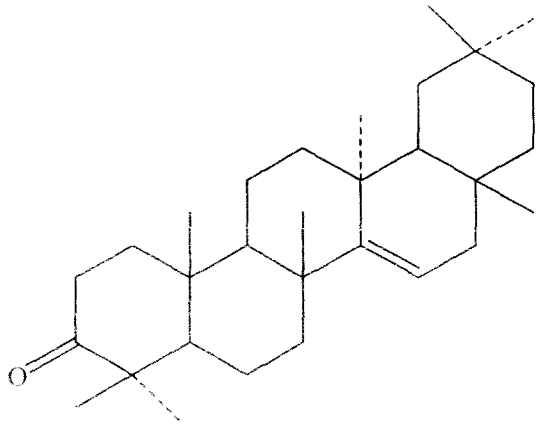
Isolation of the compounds

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.

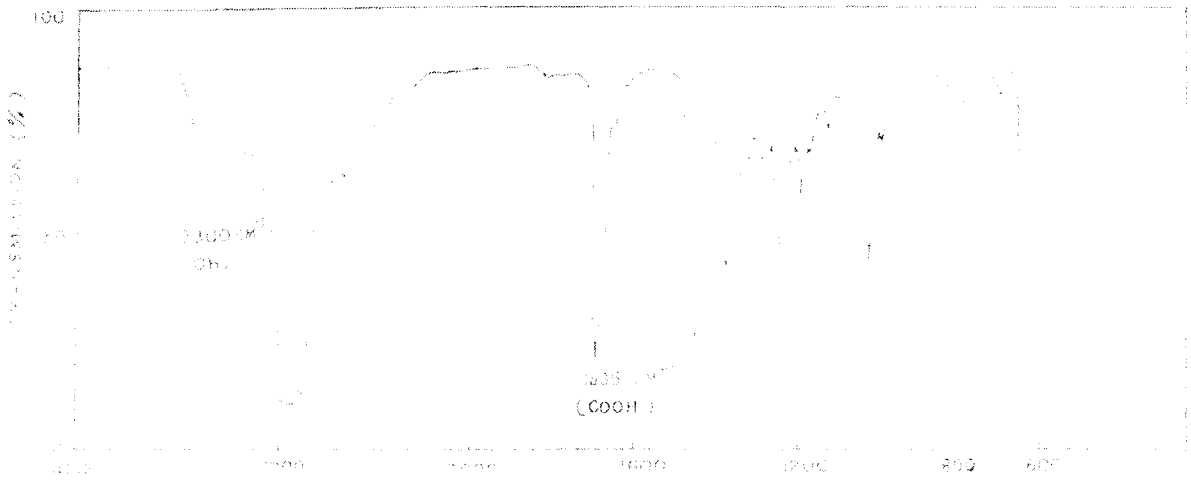
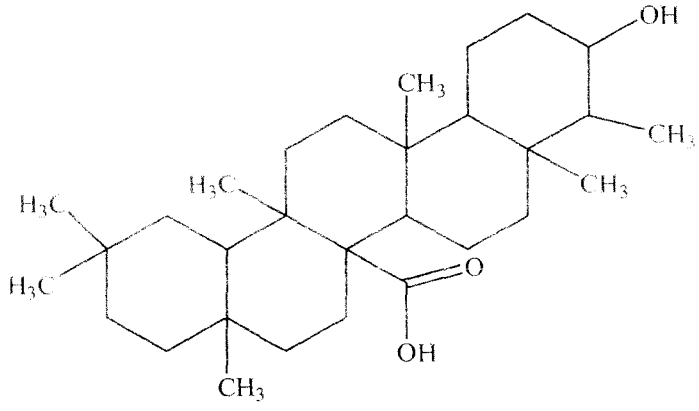


Fig.1 IR spectrum of trichadenic acid



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].

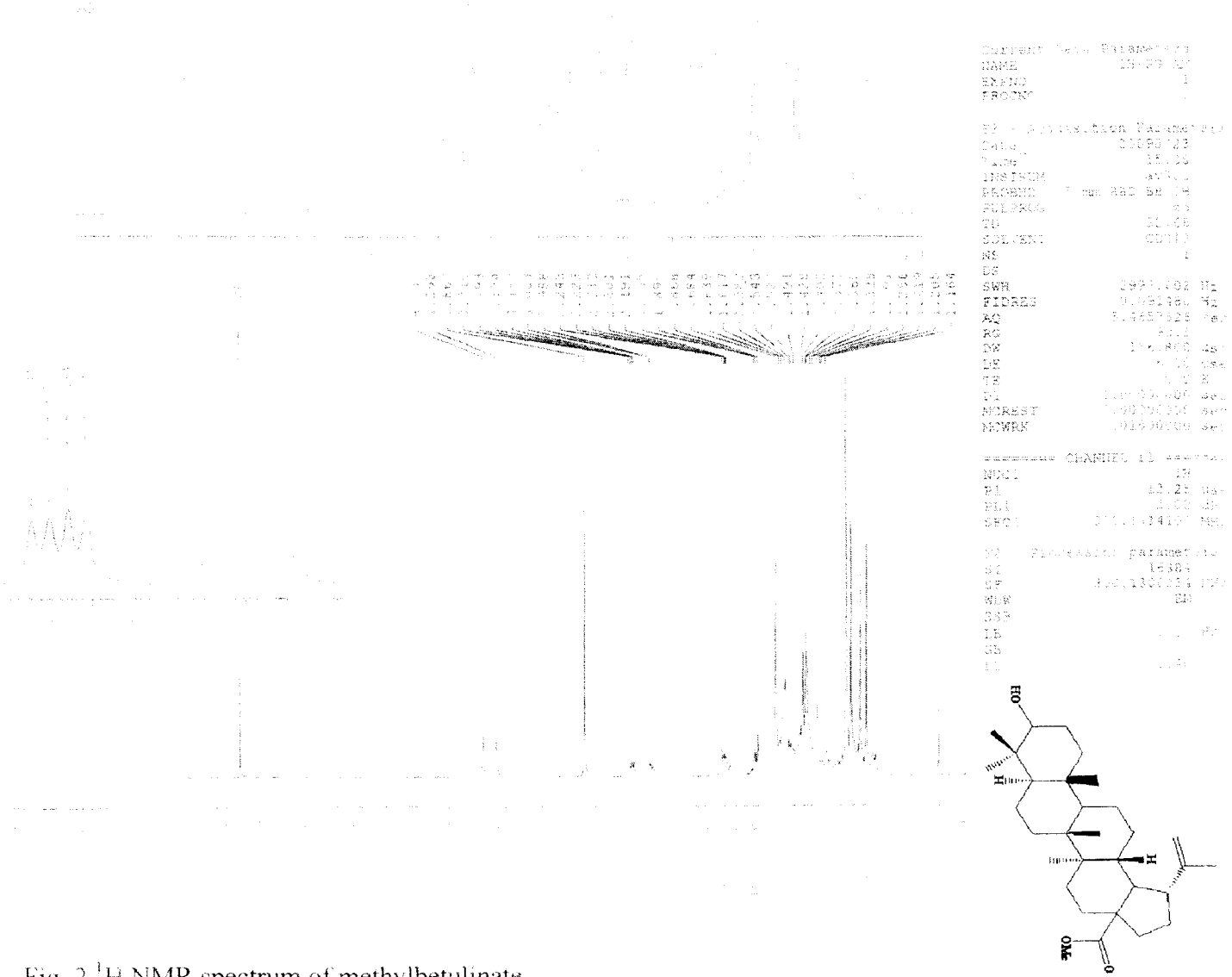


Fig. 2 ^{1}H NMR spectrum of methylbetulinate

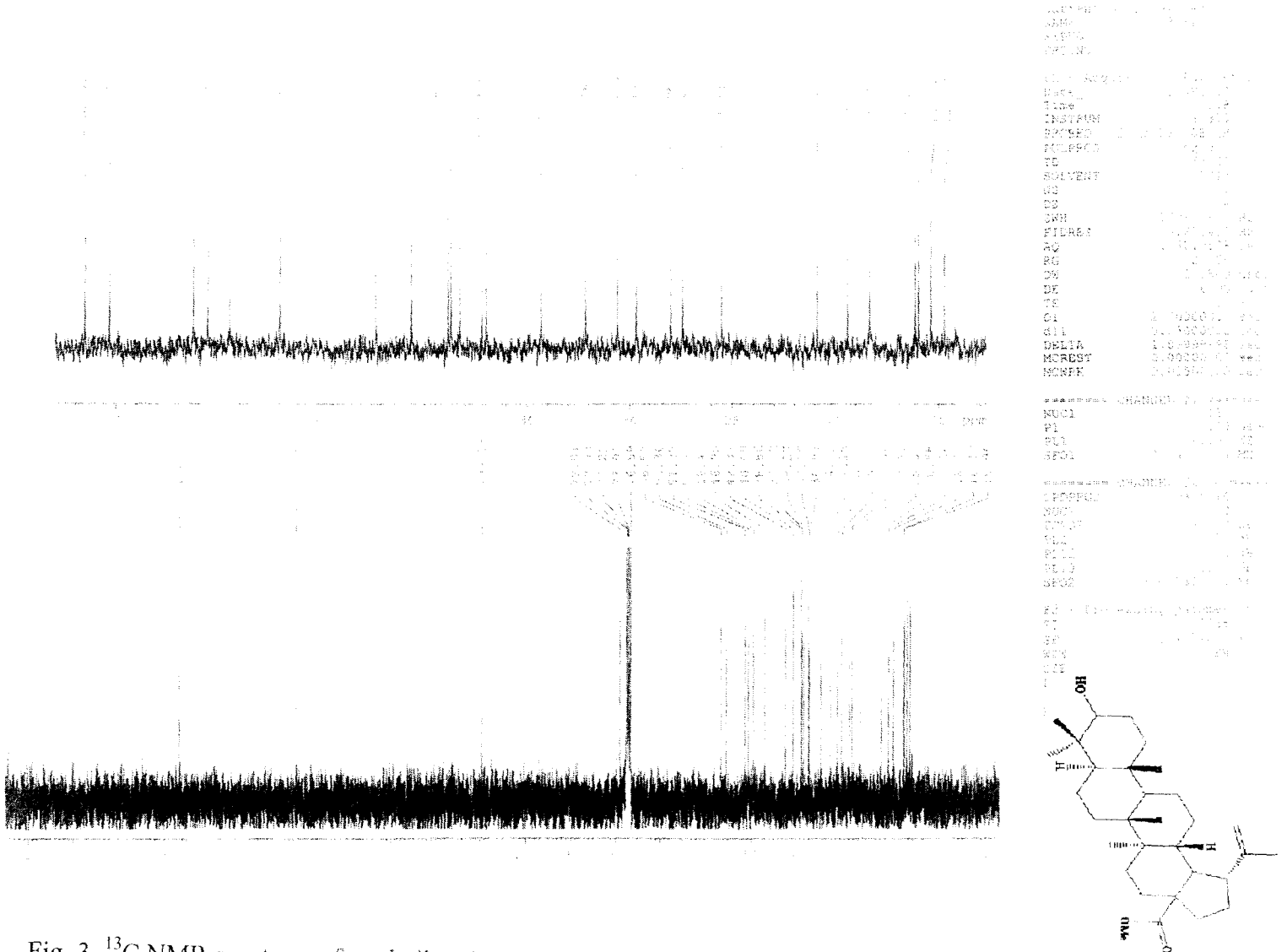
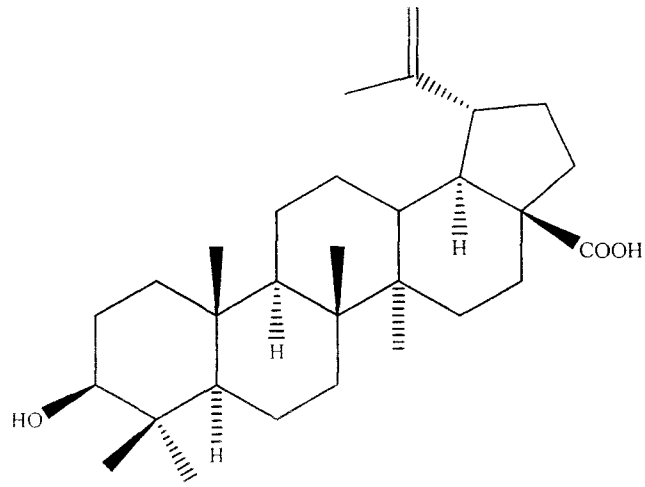


Fig. 3. ^{13}C NMR spectrum of methylbetulinate



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].

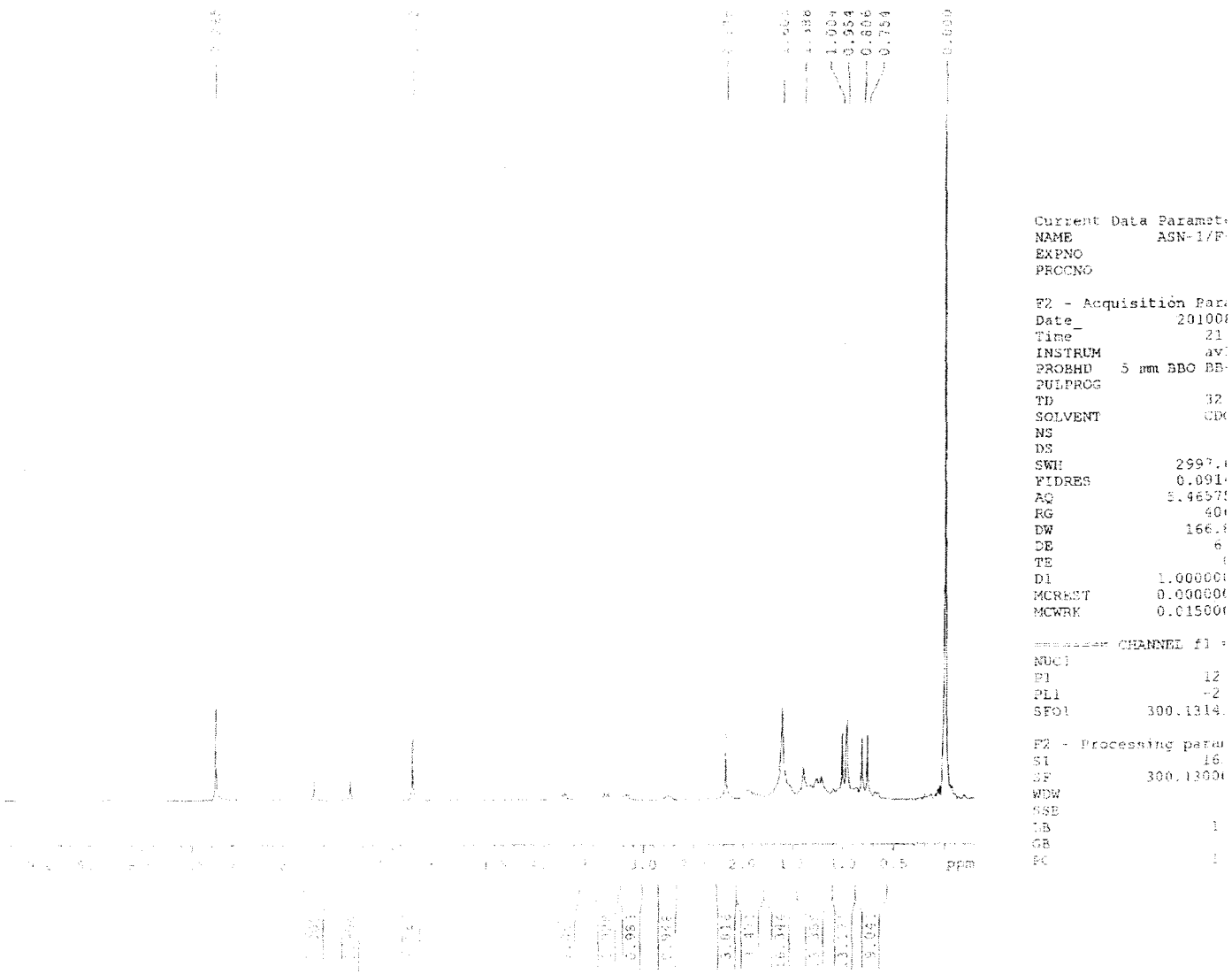


Fig. 4. ^{1}H NMR spectrum of betulin

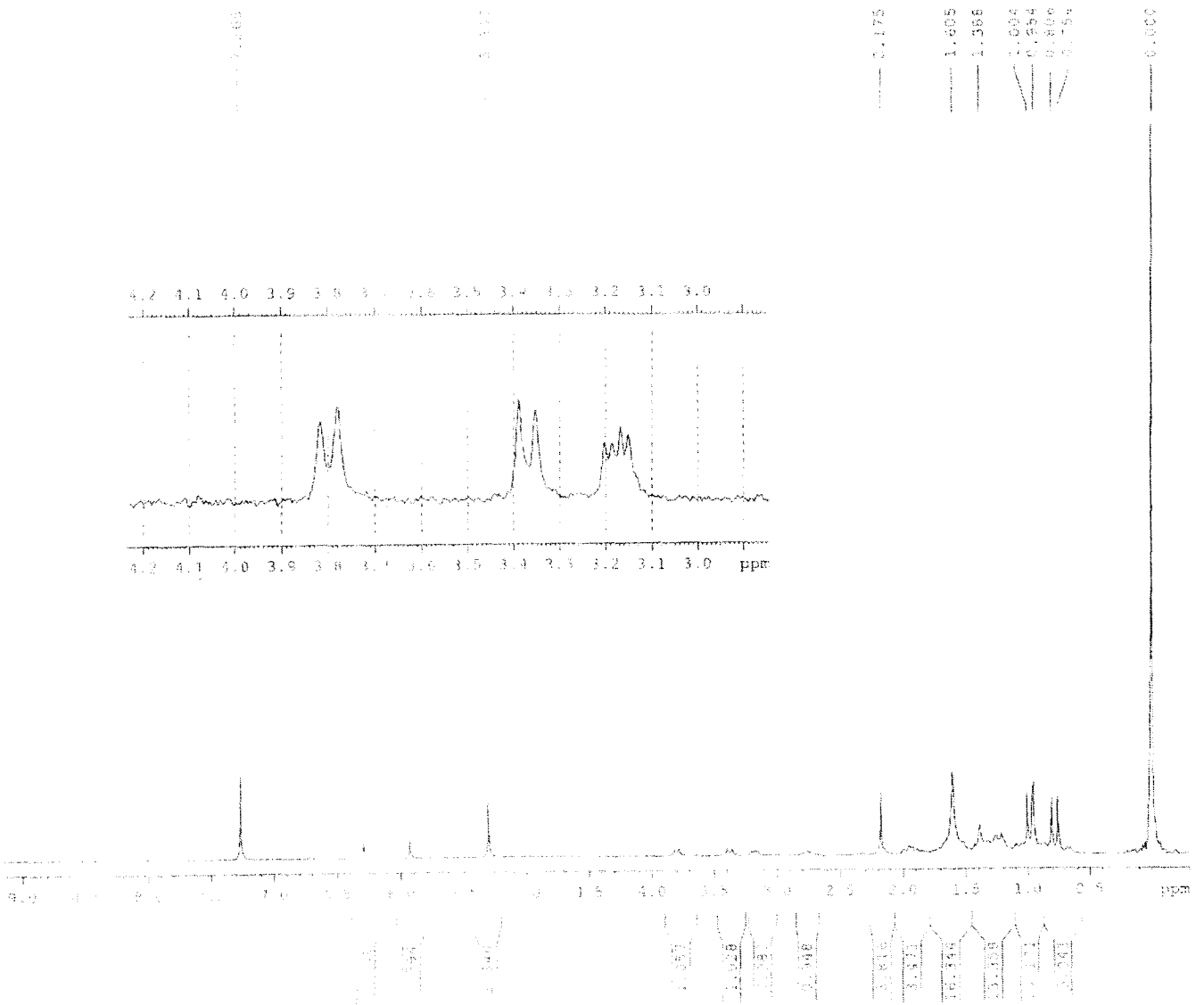


Fig. 5. ^{1}H NMR spectrum of betulin

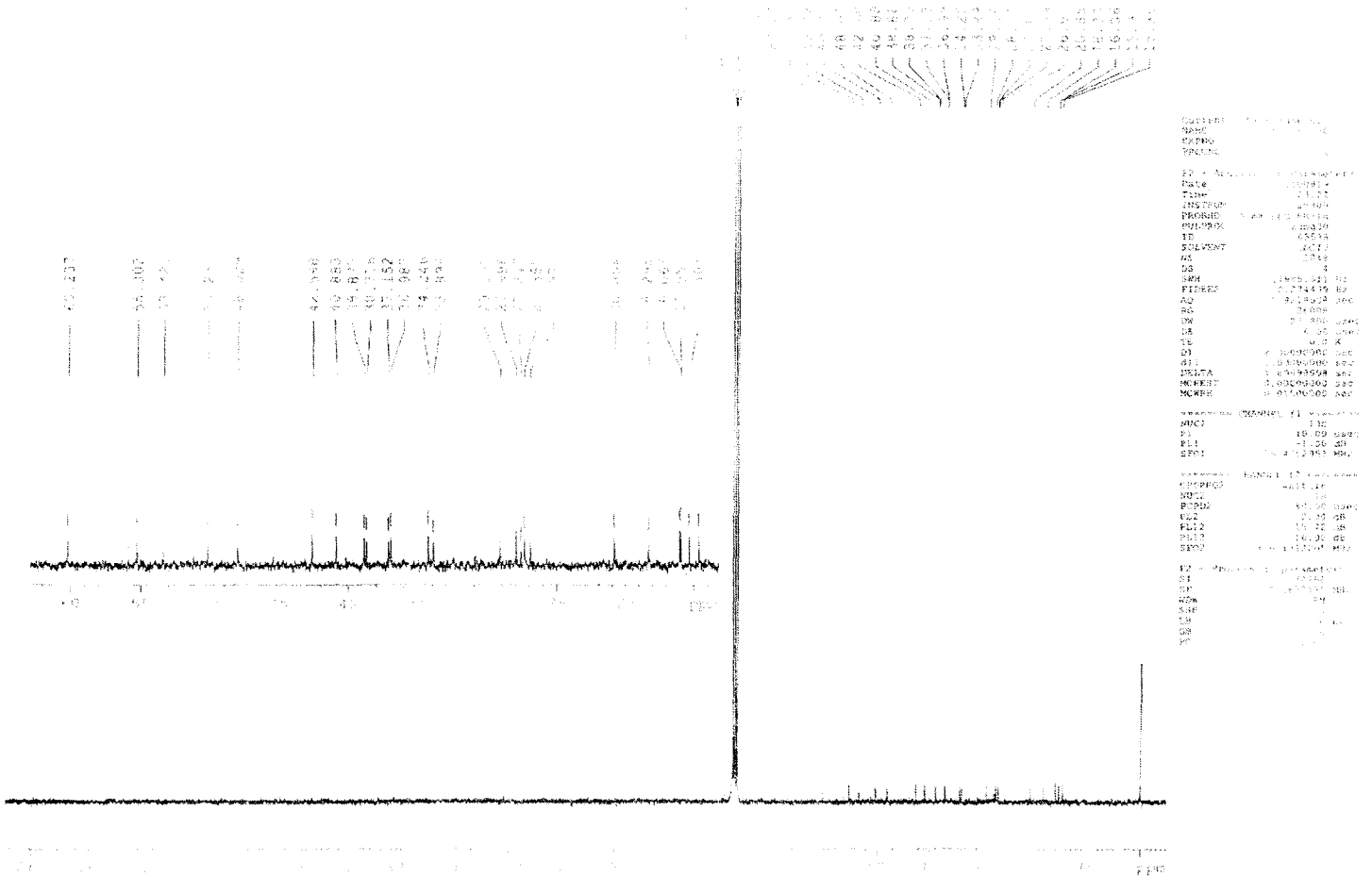
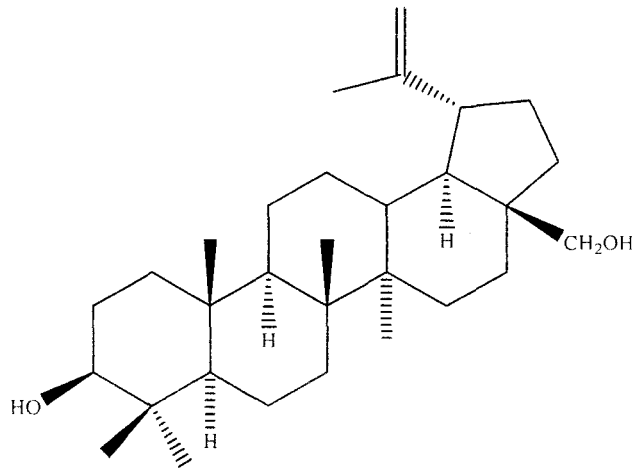


Fig. 6. ^{13}C NMR spectrum of betulin



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.

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.

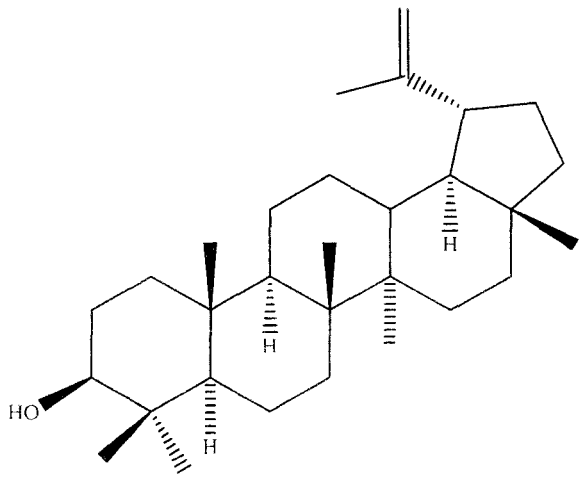
Characterization of compound A (betulinic acid)

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

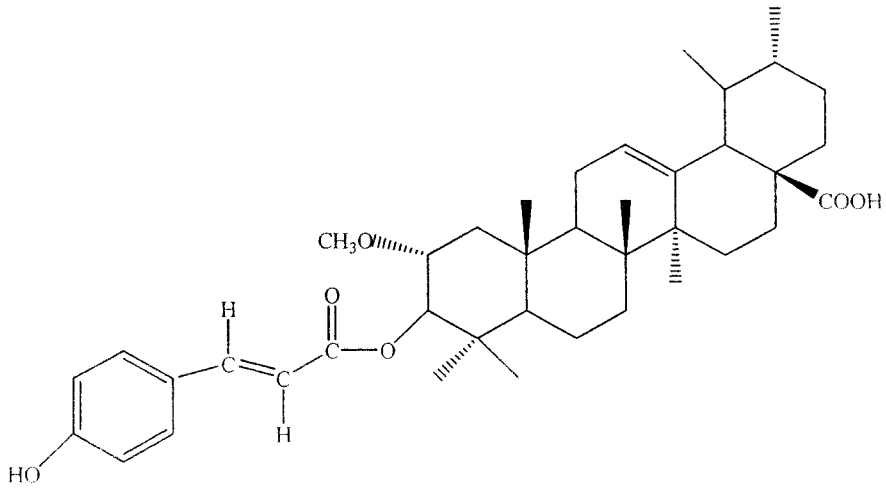
(B) could be due to the influence of the adjacent C-3 hydroxyl group. These data were in close agreement with those reported for lupeol (B) [159-161].



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

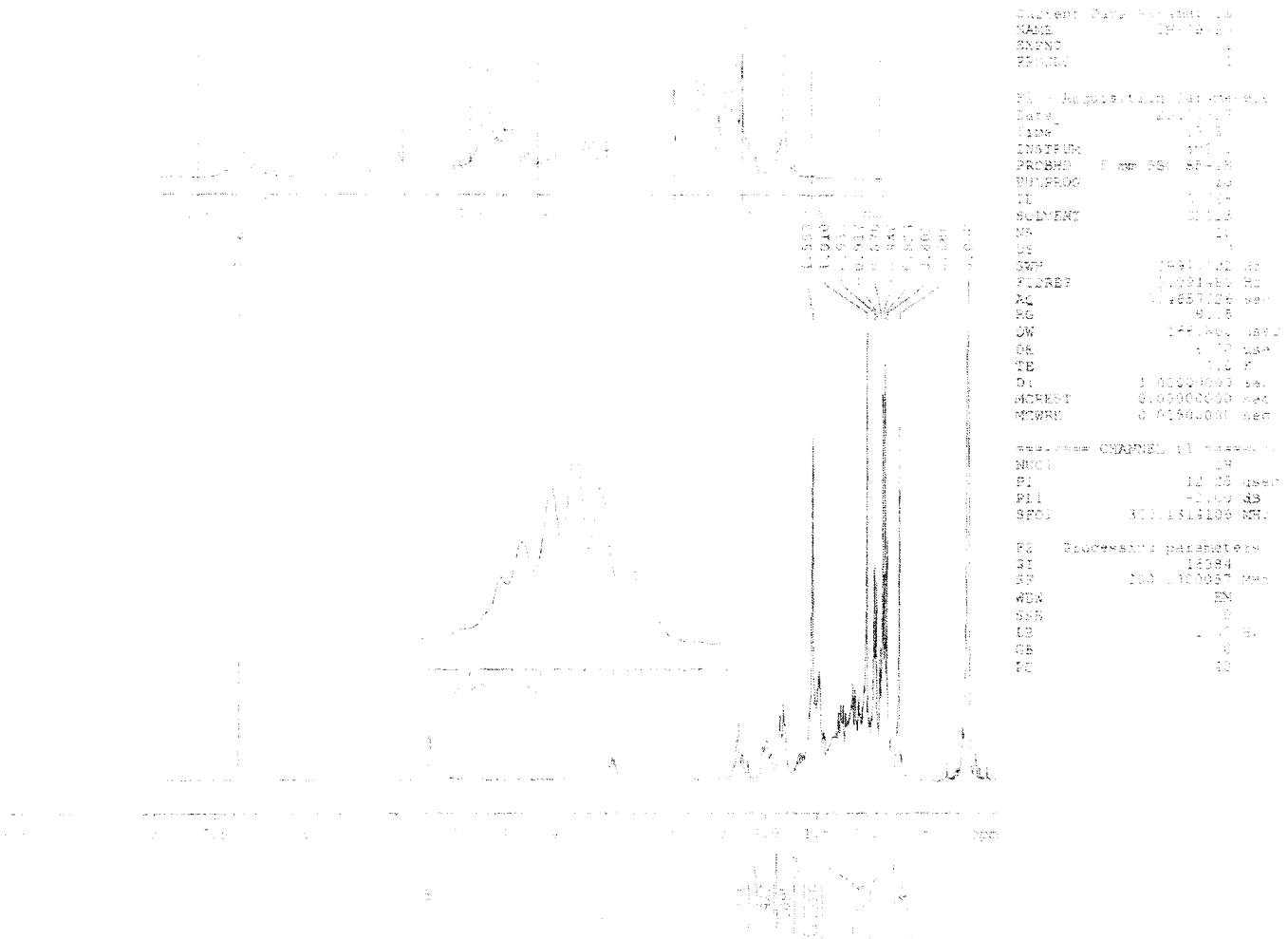


Fig.. 8 ^{1}H NMR spectrum of β-sitosterol

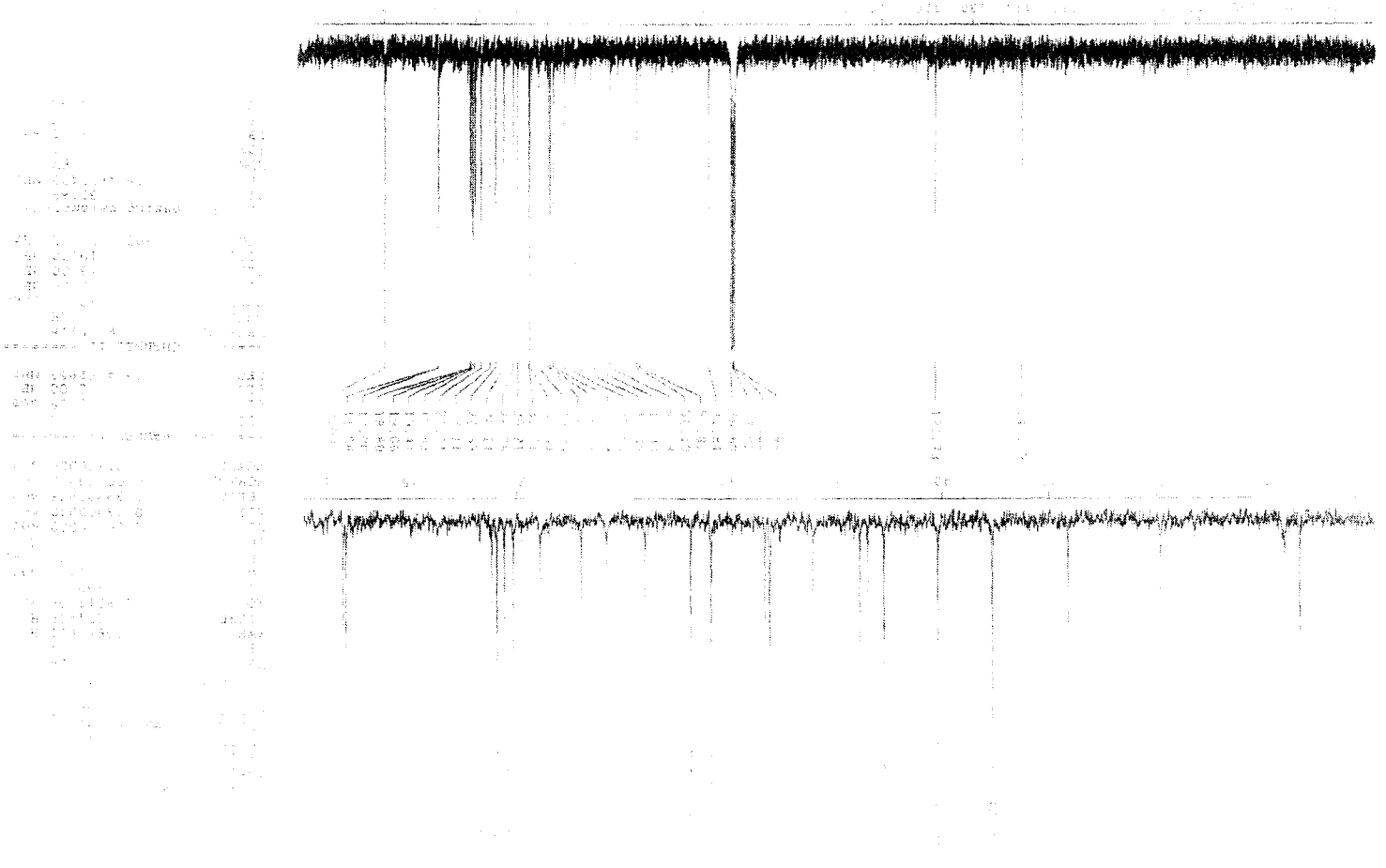


Fig.. 9 ^{13}C NMR spectrum of ß-sitosterol

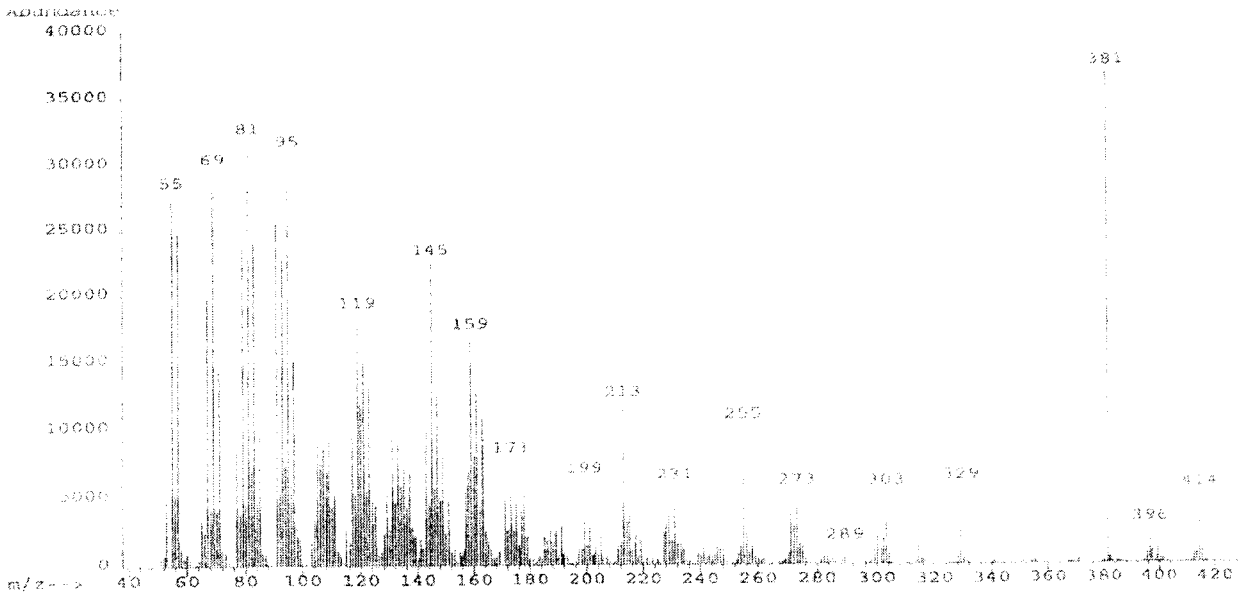
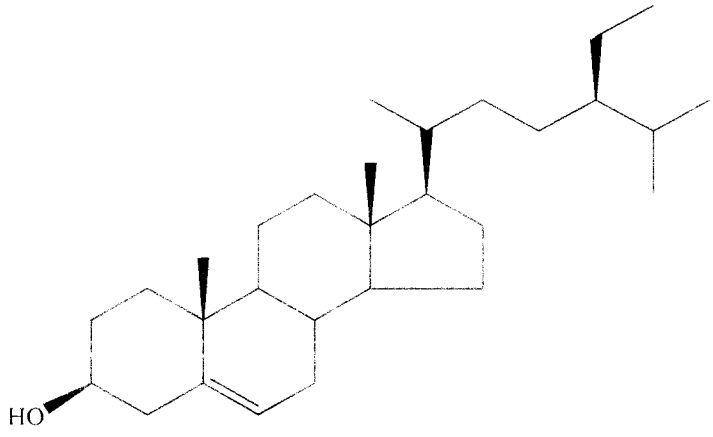


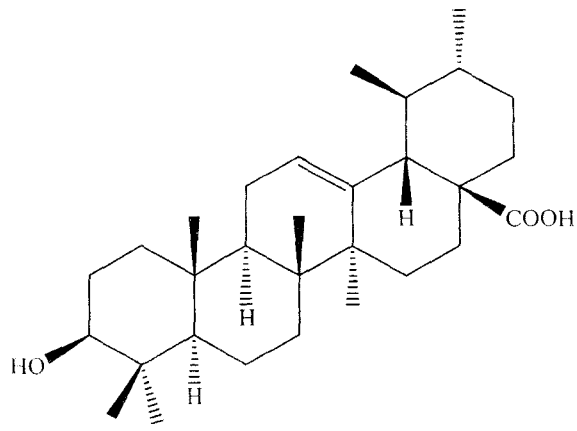
Fig. 10 Mass spectrum of β-sitosterol



D

Characterization of compound E: (ursolic acid)

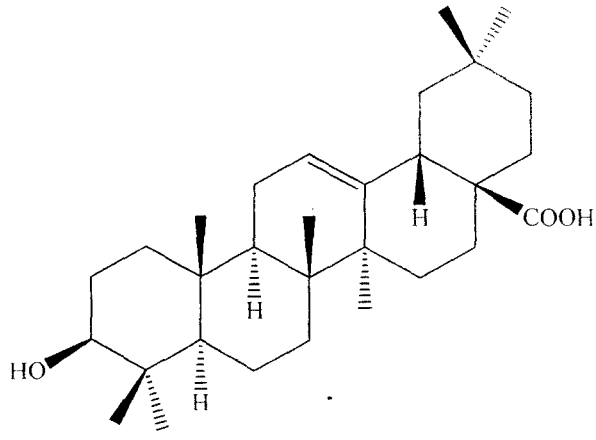
Compound (E) was obtained as a white powder with a melting point of 267-269°C. The elemental analysis revealed that the compound contains 78.90% of C, 10.59% of H and 10.51% of O. Mass spectra of this compound suggested that its molecular mass is 456.36 (M.F.$C_{30}H_{48}O_3$) having characteristic fragments 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.

CHAPTER 4

MATERIALS AND METHODS RELATED TO EXTRACTION, ISOLATION, SYNTHESIS, FORMULATION AND BIOASSAY OF ISOLATED NATURAL PRODUCTS

SECTION A:

EXPERIMENTAL FOR CHEMICAL WORK

Extraction

Fresh outer bark of *Schleichera oleosa* was collected from Sukna belt of foothills of Darjeeling in an early summer. The plants collected were shade dried at room temperature and mechanically reduced to coarse powder. Then from these powder compounds extracted using benzene as a solvents in a soxhlet apparatus for 72 hours. Benzene was distilled off and brown gummy residue was obtained. The residue was extracted with ether, washed with aqueous NaOH solution and then with ether till neutral. The ether solution was dried over sodium sulphate and evaporated when a gummy residue was obtained. This residue dissolved in benzene and placed over a column of silica gel of 60-120 mesh using petroleum ether and ethyl acetate with increasing concentration as eluent.

Elucidation of the structures

All the melting points were determined by open capillary method and are uncorrected. The NMR spectra were recorded in $CDCl_3$ solutions at ambient temperature on a Bruker Avance 300 MHz-FT NMR spectrometer using 5mm BBO probe. The chemical shift δ are given in ppm related to tetra methyl silane (TMS) as internal standard. The coupling constant (J) are reported in Hz. The IR spectra were recorded in Shimadzu FT-IR spectrophotometer in KBr discs.

Chromatography of the neutral part

The above gummy neutral part was chromatographed over silica gel and four components were separated. Out of these four components we found two new compounds (Table 1) along with two others reported earlier. Both structures of the compounds were determined based on their chemical and spectral data (IR, UV, and NMR).These two compounds were evaluated for their antifungal and antibacterial activities.

Table 1. Chromatography of the *Schleichera oleosa* residue

Eluent	Fractions 50 ml each	Residue	M.P.	M.P. after crystallization
Petroleum ether	1-8	Solid	_	235-237°C
Pet. ether + benzene (80:20)	9-15	Solid	_	294°C
Pet. ether + benzene (70:30)	16-22	Solid	299-301°C	-
Pet. ether + benzene (60:40)	23-30	Solid	_	251-252°C

Examination of fraction no. 1-8 and isolation of compound A

Fractions 1–8 on rechromatography over silica gel and several crystallization from chloroform-methanol mixture furnished white crystals having constant m.p. 235-237°C, $[\alpha]_D = +9^\circ$. Elemental analysis coupled with mass spectrometric determination of molecular weight established the molecular formula of the compound to be $C_{30}H_{48}O$ (M^+ 424). It developed a yellow colour with tetranitromethane indicating unsaturation in the compound. It gave a violet coloration Libermann-Burchard reaction and gave a positive test in

Zimmermann colour reaction showing the compound is a triterpene ketone, the keto group being at C-3 position. It showed no depression in melting point when mixed with authentic sample of taraxerone and was found identical with the sample of taraxerone (mmp, CO IR, CO TLC and spectral data 23,50).

	% C	% H
Analysis report found	84.95	11.58
Calculated for $C_{30}H_{48}O$	84.84	11.39

IR: ν_{max}^{Nujol} 3047.3, 2924.8, 2851.6, 1708.7 (carbonyl gr.), 1375.2 and 955.2cm^{-1}.

^{1}H NMR spectra showed eight tertiary methyls between 0.82 to 1.21 ppm 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 group.

^{13}C NMR($CDCl_3$):

40.65(C-1), 33.1(C-2), 210.1(C-3), 47.72(C-4), 55.8(C-5), 33.59(C-6), 34.18(C-7), 37.83(C-8), 48.8(C-9), 38.49(C-10),35.12(C-11),36.69(C-12),37.6(C-13),157.7(C-14),117.0(C-15),33.49(C-16),33.89(C-17), 48.88(C-18),41.4(C-19),29.5(C-20),36.26(C-21),27.29(C-22),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) and 21.4(C-30).

Examination of fraction no. 9-15 and isolation of compound B

Fractions 9–15 (Table 1) on rechromatography over alumina and several crystallization from chloroform-methanol mixture furnished white needle shaped crystals having constant m.p. 294°C, $[\alpha]_D$= +25.0° [Lit. m.p. 294°C $[\alpha]_D$= +25.0°]. IR spectra exhibited peaks at 3300 cm^{-1} for OH group, and at 1685 cm^{-1} for the carboxylic acid group, 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. It showed no depression in melting point when mixed with authentic sample of trichadenic acid A and was found identical with the sample of trichadenic acid A (mmp, CO IR, CO TLC and spectral data 130).

	% C	**% H**
Analysis report found	78.48	10.80
Calculated for $C_{30}H_{50}O_3$	78.69	10.92

Nujol

IR: ν_{max} 3300 cm^{-1} (OH group)

1685 cm^{-1} (-COOH group)

^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: 458.58 (M.F.$C_{30}H_{50}O_3$) m/z: 458.38 (100.0%), 459.48%(34.2%), 460.38%.

Examination of fraction no. 16-22 and isolation of compound C

The gummy solid compound obtained from the fractions 16 — 22 (Table 1) were mixed which gave a white gummy solid of betulinic acid, m.p. 299-301°C

Esterification of betulinic acid: Preparation of methylbetulinate

The crude gummy acid (8 g) dissolved in ether (300 ml) and then added to a solution of diazomethane in ether prepared from nitromethylurea (3 gm) and was kept overnight. Next day excess of diazomethane was destroyed by acetic acid (CH_3COOH, 2 ml). The ether solution was washed with water, 10% sodium bicarbonate solution and

again with water until neutral and dried by using sodium sulphate. Evaporation of the ether yielded a gummy residue (2 gm). This crude ester dissolved in benzene (20 ml) was placed over a column of silica gel (100 g) developed with petroleum ether and was eluted with the following solvents.

Table 2. Chromatography of the esterified betulinic acid residue

Eluent	Fractions 50 ml each	Residue on evaporation	M. P
Petroleum ether	1—6	Oil	—
Petroleum ether + benzene (90:10)	7—10	Nil	—
Petroleum ether + benzene (85:15)	11—14	Nil	—
Petroleum ether + benzene (80:20)	15—20	Solid	221°—223°C

Further elution with more polar solvent did not yield any solid materials.

Examination of fractions 15 —20 (Table 2): Isolation of methylbetulinate

The solid compound obtained from the fractions 15—20 (Table 2) were mixed and crystallised from a mixture of chloroform and methanol to afford a colourless needle shaped methylbetulinate, m.p. 221—223°C, $[\alpha]_D$ +5.0°, identical with the original sample (m.m.p, CO IR and CO TLC48) (Lit m.p 224—225°C, $[\alpha]_D$ +5.0°)

	% C	% H
Analysis report found	78.71	10.59
Calculated for $C_{31}H_{50}O_3$	79.10	10.71

Nujol

IR: ν_{max} 3540 cm^{-1} (—OH)

1730 cm^{-1} ($-COOCH_3$)

1660,

890 cm^{-1} ($=CH_2$)

^{1}H NMR: 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), δ_H = 3.16 (dd, J = 9.5 and 6.0 Hz) and δ_H = 2.95 (ddd, J = 9.0, 6.0and 0.5 Hz) δ_H = 4.55 (1H, d, J = 0.4 Hz) and δ_H = 4.65(1H, d, J = 0.4 Hz).

^{13}C NMR: δ_C 27.9 (C-23), 15.4 (C-24), 16.2 (C-25), 16.3 (C-26), 14.6 (C-27), δ_C 150.0 (C-30), 108.8 (C-29) δc 79.0 (C-3) δ_C = 180.6 (C-28)

Examination of fraction no. 23 - 30 and isolation of compound D

The solid compound obtained from the fractions 23 - 30 (Table 1) were mixed and crystallised from a mixture of chloroform and methanol to afford a white solid, m.p. 251-252°C, $[\alpha]_D$ +20°. This compound was found to be identical with an authentic specimen of betulin. (m.m.p, CO IR, and CO TLC 23, 50)

IR ν_{max} (KBr): 3430, 3070, 1635 and 880 cm^{-1}.

^{1}HNMR: δ 0.68, 0.72, 0.80, 0.91, 0.97 and 0.99 (3H, δ 3.25 (dd, J=9.6, 1.2 Hz).

^{13}C NMR: 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) δ_C 150.0 (C-20), 108.8 (C-29) δc 78.9 (C-3).

Extraction of *Psidium guajava*

First collected the fresh leaves of *Psidium guajava* in bulk from young mature plants at the Sukna belt of Darjeeling foothills during early summer, washed, shade dried and milled into coarse powder by a mechanical grinder. The powdered plant material was then 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 was then purified by column chromatography using silica gel (60-120) mesh and suitable proportions of petroleum ether and ethyl acetate were used as the eluent.

Elucidation of the structures

All the melting points were determined by open capillary method and are uncorrected. The NMR spectra were recorded in $CDCl_3$ solutions at ambient temperature on a Bruker Avance 300 MHz-FT NMR spectrometer using 5 mm BBO probe. The chemical shift δ are given in ppm related to tetra methyl silane (TMS) as internal standard. The coupling constant (J) are reported in Hz. The IR spectra were recorded in Shimadzu FT-IR spectrophotometer in KBr discs.

Chromatography of the neutral part

The above gummy neutral part was chromatographed over silica gel and eight components were separated. Out of these eight components we found two new compounds (Table 3) along with six others reported earlier. The structures of the compounds were determined based on their chemical and spectral data (IR, UV, and NMR).These two compounds were evaluated for their antifungal and antibacterial activities.

Table 3.Chromatography of the *Psidium guajava* residue

Eluent	Fractions 50 ml each	Residue	M.P.	M.P. after crystallization
Petroleum ether	1-4	oil	—	—
Pet. ether + benzene (90:10)	5-12	gummy solid	299– 301°C	
Pet. ether + benzene (80:20)	13-18	solid	—	212-213°C
Pet. ether + benzene (70:30)	19-24	solid	—	195-196°C
Pet. ether + benzene (60:40)	25-31	solid	—	267-269°C
Pet.ether + benzene (60:40)	32-40	solid	—	305-306°C

Examination of fractions 5-12: Isolation of betulinic acid

The gummy solid compound obtained from the fractions 5—12 (Table 3) were mixed and crystallised from a mixture of chloroform and methanol to afford a white gummy solid of betulinic acid, m.p. 299-301°C.

Esterification of betulinic acid: Preparation of methylbetulinate:

Already discussed above (Table 2).

Examination of fractions 13-18: Isolation of lupeol

Fractions 13— 18 (Table 3) were mixed and crystallised by chloroform and methanol mixture. The compound isolated had m.p. 212– 213°C and was found to be identical (m.m.p, CO IR, CO TLC and spectral data 7, 159-161) with authentic specimen of lupeol.

	% C	% H
Analysis report found	84.18	12.02
Calculated for $C_{31}H_{53}O$	84.29	12.09

Nujol

IR: ν_{max} 3610, 1020 cm^{-1}

3070, 1640, 887 cm^{-1}

Examination of fractions 13-18: Isolation of guajanoic acid

Fractions 13-18 on crystallization from chloroform and methanol mixture had m.p. 195-196°C, $[\alpha]_D$ +5.1°. Elemental analysis showed the moleculer formula as $C_{40}H_{56}O_6$. On hydrolysis and subsequent methylation it afforded to methyl nepotate. It showed no depression in melting point when mixed with an authentic sample of guajanoic acid and was found identical with original sample of guajanoic acid (mmp, CO IR, CO TLC and spectral data, [172]).

	% C	% H
Analysis report found	75.76	8.81
Calculated for $C_{31}H_{53}O$	75.91	8.92

IR ν_{max} (KBr) cm^{-1}: 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)

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[$C_{10}H_{13}$]$^+$.

Examination of fractions 19-24: Isolation of β–sitosterol.

Fractions 19-24 on crystallization from chloroform and methanol mixture to afford white crystals had m.p. 136-137°C, $[\alpha]_D$–32°. Elemental analysis showed the moleculer formula as $C_{29}H_{50}O$. It showed no depression in melting point when mixed with an authentic sample of β–sitosterol. On treatment with acetic anhydride and pyridine it afforded an acetate, $C_{31}H_{52}O_2$, m.p. 127-128°C, $[\alpha]_D$–40°. The acetate was identified as β–sitosterol acetate by direct comparison with authentic specimen of β–sitosterol acetate. Hence the parent alcohol was found identical with an authentic sample of β–sitosterol (mmp, CO IR, CO TLC and spectral data 7, 148-150).

IR ν_{max} (KBr): 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).

Mass: 414 (M.F. $C_{29}H_{50}O$) m/z: 414, 396, 381, 329, 303, 289, 273, 255, 231, 213, 199, 173, 159, 145, 119, 95, 81, 69, 55

Examination of fractions 25-31: Isolation of ursolic acid

Fractions 25-31 on crystallization from chloroform and methanol mixture to afford white crystals had m.p. 267-269°C. Elemental analysis showed the moleculer formula as $C_{29}H_{50}O$. It showed no depression in melting point when mixed with an authentic sample

of ursolic acid and was found identical with original sample of ursolic acid (mmp, CO IR, CO TLC and spectral data [31]).

Examination of fractions 32-40: Isolation of oleanolic acid

Fractions 32-40 on crystallization from chloroform and methanol mixture to afford white colorless needles had m.p. 305-306°C and $[\alpha]_D$ +78.9°($CHCl_3$). Elemental analysis showed the moleculer formula as $C_{29}H_{50}O$. It showed no depression in melting point when mixed with authentic sample of oleanolic acid and was found identical with the sample of oleanolic acid (mmp, CO IR, CO TLC and spectral data [2]).

SECTION B:

Experimental related to Biocidal activity of the isolated compounds

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 [157] 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°Cin 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°. 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.

Seeds of rice (*Oryza 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 filter paper as support. Test solutions (5 ml) was added to the filter paper in the petridish and dried completely *in vacuo* at 40°C. Five seeds from each category were placed on the filter paper and incubated for 7 days at 25° in the dark. The effects of the pure compounds were determined by measuring the elongation of roots and averaged for each concentration.

Collection of plants for extraction of botanicals

Plant parts (bark, root, stem, leaf etc.) of four different plants (*Schleichera oleosa, Psidium guajava, Biscofia javanica, Xanthoxylum budrunga*) were collected from foothills of Darjeeling and Terai region (West Bengal, India). The plants were selected on the basis of their folk medicinal value in these areas. The plants were collected, identified and voucher specimens have been deposited in the departmental herbarium of the Department of Botany, University of North Bengal.

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 the Table 11.

Table 11. 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
Alterneria alternata	Plant pathology lab. NBU	Identification No. 7065.08
Colletotrichum camelliae	Plant pathology lab. NBU	Identified by Dr. A. Saha
Bacillus subtilis	Plant pathology lab. NBU	Identified by NBU
Enterobactor sp.	Plant pathology lab. NBU	Identified by NBU
S. dysenteriae	Plant pathology lab. NBU	Identified by NBU
Aspergillus niger	Plant pathology lab. NBU	Identified by NBU

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.

Major Chemicals used

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
$FeSO_4, 7H_2O$	Merck
K_2HPO_4	Merck
$C_{12}H_{22}O_{11}$	Glaxo Laboratories (India) Ltd

Composition of media and solutions used

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

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 lit

Potato Dextrose Agar (PDA)

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

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

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 lit
K_2HPO_4	1.0 g
Yeast extracts	5.0 g
Sucrose	30.0 g
Agar	15.0 g

Staphylococcus aureus 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.

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.

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

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.

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.

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.

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.

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

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

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.

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.

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.

S. dysenteriae 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.

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

The biological activities of the compounds which extracted from *Schleichera oleosa, Psidium guajava* and Friedelin plants against fungal pathogen (*F.solani*), Bacteria (*E. coli, B. subtilis, S. aureus, Lactobacillus, Pseudomonas*) and Crops (rice, wheat, pea) were investigated. The experiments used in the study were done in the plant pathology laboratory Department of Botany, North Bengal University.

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.

Spore germination bioassay

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, 300ppm, 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).

Antibacterial sensitivity test by the disc diffusion method

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 5mm 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 48h. 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.

Phytotoxicity

In order to show phytotoxic activities of the compounds solution of different concentrations of different compounds were prepared and applied to check germination of both root and shoot of the germinating seeds. Phytotoxicities of the isolated compounds were determined on the healthy seeds of wheat (Sonalika variety), rice (IR-20, Jaya variety) and pea purchased from Anup seed company, Bidhan Market, Siliguri, West Bengal. These healthy seeds were dipped in acetone-water suspensions of the compounds of different concentration(500 ppm, 250 ppm, 100 ppm) and incubated for 1,4 and 8 hours. The treated seeds of wheat, rice and pea were allowed to germinate on a mat of moist filter paper. The roots and shoots of germinated seeds were kept in a covered Petri plates. Each experiment were based on 80 seeds of each varieties or plants. After five days of incubation the germinated seeds (treated with compounds) were counted. Treated experimental sets were compared with that of control sets where no compounds were used to treat the seeds. Each experiment was repeated in triplicate. All apparatus and materials used were sterilized where necessary.

Antibacterial Activity

Standard cultures of *Escherichia coli* (MTCC No.), *Bacillus subtilis* (MTCC No.), *Staphylococcus aureus*(MTCC No.) *Lactobacillus* (MTCC No.) and *Pseudomonas* (MTCC No.) were collected from Microbial type culture collections, Chandigarh, India. The cultures were maintained in Nutrient Agar (NA) media in the laboratory. The cultures were sub cultured routinely and used for experimental purposes.

Bacterial suspension was made from 48 hr old cultures of the bacteria. Initially 1 ml of sterile distilled water was poured in a NA slant of a bacterium and rinsed mildly. The

suspension was taken in a sterile tube for use in the experiments. The bacterial suspension was always used fresh.

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.

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PART 2

TRANFORMATIVE REACTIONS OF TRITERPENOIDS AND THE BIOCIDAL ACTIVITY OF THE DERIVED COMPOUNDS

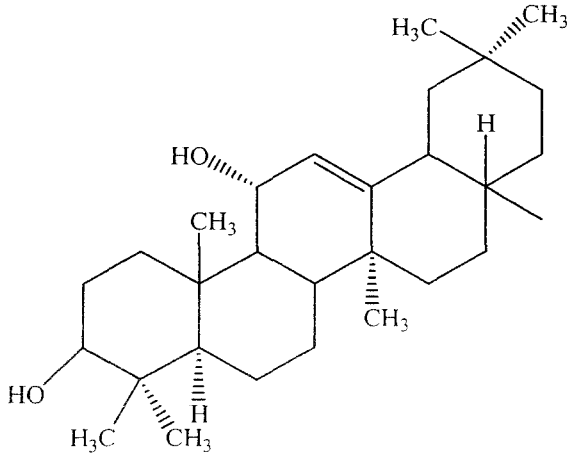
Chapter 1

SHORT REVIEW ON TRANFORMATIVE REACTION OF TRITERPENOIDS AND BIOCIDAL ACTIVITY OF THEIR PREPARED DERIVATIVES

Triterpenoids represent a varied class of natural products. Thousands of structures have been reported with hundreds of new derivatives discovering each year. Among these are included the pentacyclic lupane type triterpenes which are represented by a diverse assemblage of bioactive natural products. The author extracted several triterpenes from plants and studied their biological activities against different microorganisms and seeds and observed some interesting results (Part 1).

So the author interested to introduce some groups or atoms to triterpenes through transformative reactions including microwave assisted methodology. For this the author has undertaken these surveys to summarize the available literature. A number of computer based databases, journals, abstracts were utilized in literature search. Based on this search it was observed that more than 1000 publications were identified in which triterpenes were mentioned but very fewer publications mentioned the transformative reactions of triterpenoids and biocidal activities of the derived compounds. A short review of it is presented in this chapter.

Corey *et al.* [1] established that the 3α, 11β-dihydroxy pentacyclic triterpenoid (**4**) on treatment in methylene chloride with a solution of 30% hydrogen peroxide and p-toluene sulphonic acid in tertiary butanol forms an epoxide, 11α,12α-epoxide and undergoes a skeletal rearrangement by C_{14}-C_{13} methyl migration and shift of the double bond (**5**).



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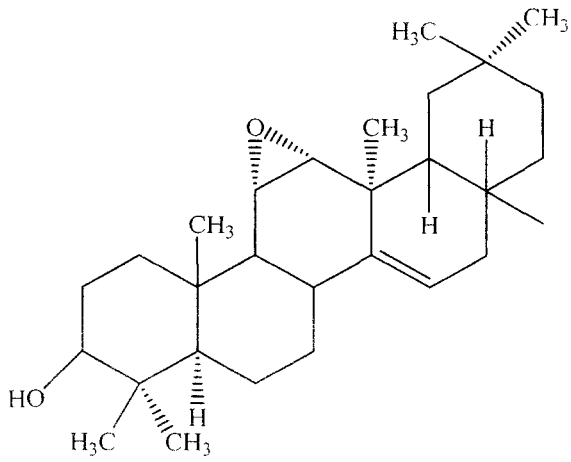
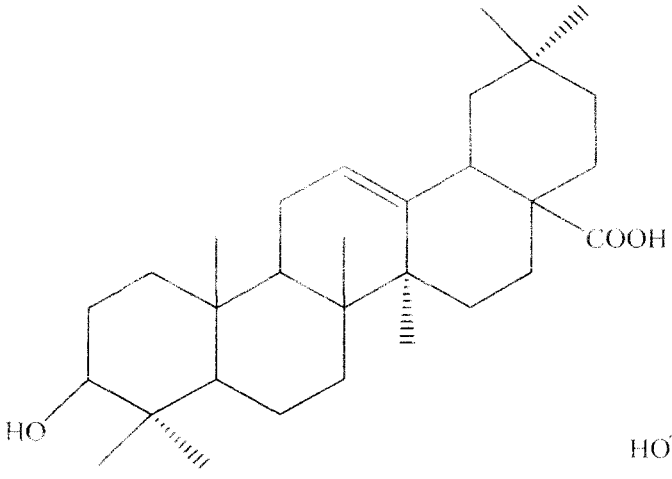


5

Kitagawa *et al.* [2] studied photooxidation of oleanolic acid and reported that irradiation of oleanolic acid **(6)** and reported that irradiation of the acidified ethanolic solution of 7 for 80 hours afforded products **8, 9** together with starting material.



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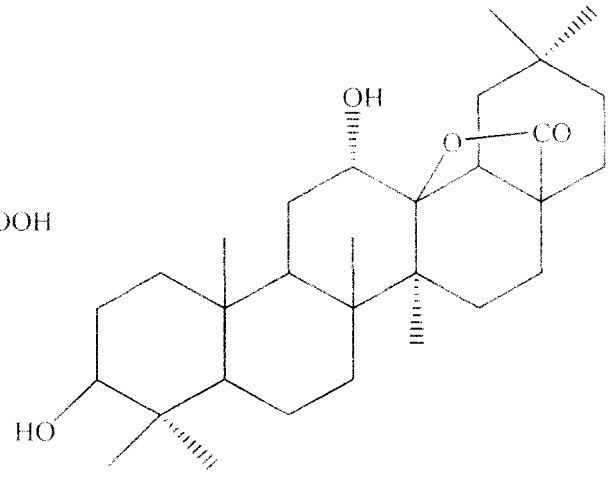

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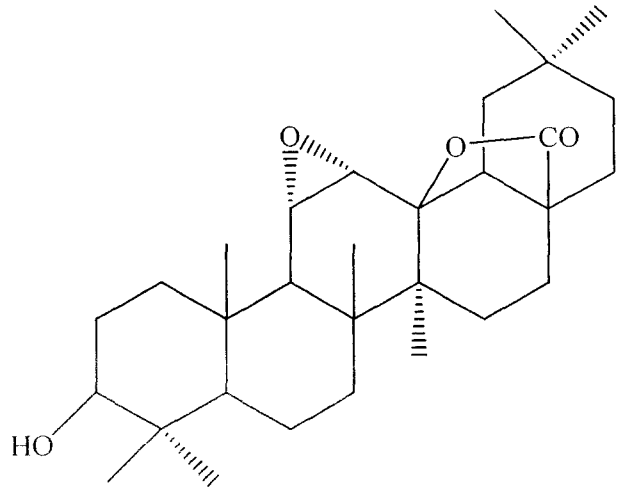
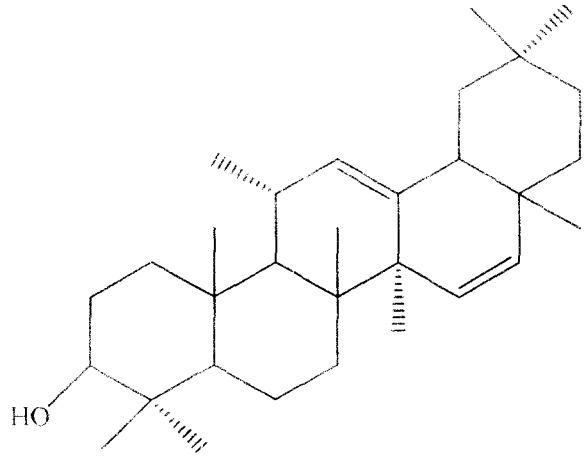
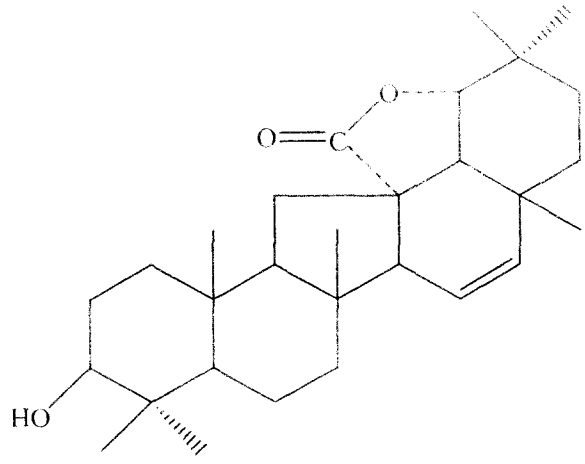


9

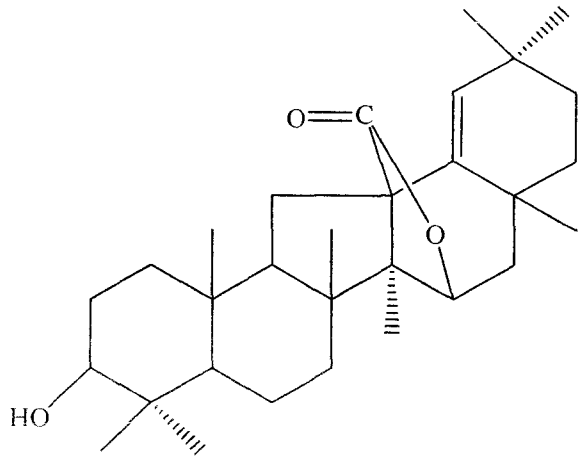
Pradhan *et al.* [3] carried out the reaction of olean-12, 15-dien-3, 11-diol **10** with hydrogen peroxide containing p-toluene sulphonic acid under identical conditions of corey *et al.*[1] with a view to producing the multiflorenol derivative **12**. But they isolated two isomeric γ-lactones identified as 3β–acetates of C-12-nor-olean-15-en-13α-carb— 19α-olide **12** and C-12-nor-olean-18(19)-en-13 -carb— 15 –olide **13**.



olean-12, 15-dien-3, 11-diol (11)



12



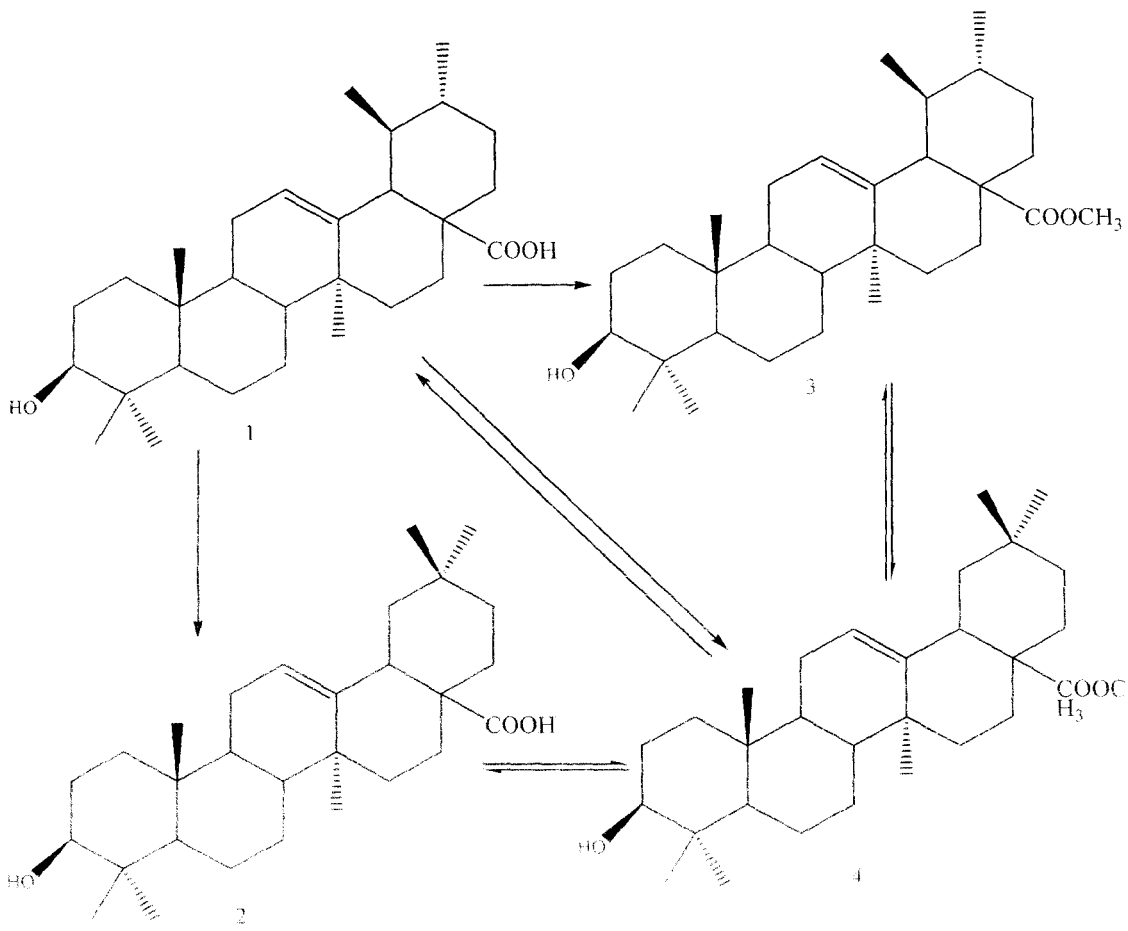
13

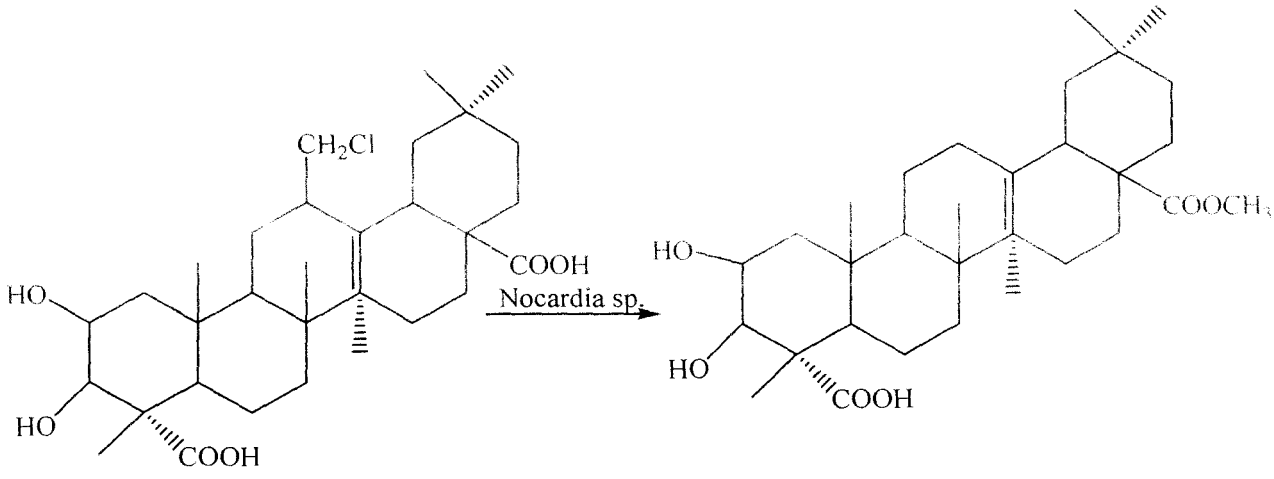
Yadava and Chakravarti [4] isolated a new bioactive triterpenoid saponin 3β-O-[β-D-xylopyranosyl(1 → 3)-O-β-D-galactopyranosyl]-lup-12-ene-28 oic acid-28-O-α-L-rhamnopyranosyl ester compound (**A**) from the methanolic fraction of the roots of *carthamus tinctorius* linn by various colour reactions, chemical degradations and spectral analysis. They reported that compound (**A**) showed anti-inflammatory activity.

Haytsu *et al.* [5] elucidated the possible natural evolutionary pathways for the transformation of pentacyclic triterpenoids. They heated three terpenoids samples, Δ^2-allobetulene, tetranormethylallobetulheptaene and fernenes independently at 150°C for 7 weeks with montmorillonite clay. As a result of this transformation they obtained di, tri-, tetra-, and pentacyclic hydroaromatic and aromatic hydrocarbons, which are commonly found in higher rank coals.

Zhang *et al.* [6] converted six pentacyclic triterpene acids, ursolic acid, oleanolic acid, betulinic acid, 23-hydroxybetulinic acid, glycyrrhetinic acid, and senegenin to their corresponding 28-methyl esters through novel biotransformation by the microbe *Nocardia* sp. NRRL 5646. They reported that notably, ursolic acid (**1**) was converted to oleanolic acid methyl ester (**4**) via two intermediates, oleanolic acid (**2**), and ursolic acid methyl ester (**3**), which are formed by participation of 'retro-biosynthetic' methyl migration from C-19 to C-20. They also reported that senegenin (**11**) was selectively converted to a

nortriterpene methyl ester, senegenic acid methyl ester (**12**), via an unprecedented C–C bond cleavage.





senegenin (**11**)

senegenic acid methyl ester (**12**)

Chatterjee *et al.* [7] studied the microbial transformation of the antimelanoma agent betulinic acid. The main objective of this study was to utilize microorganisms as in vitro models to predict and prepare potential mammalian metabolites of the compound. The resultant product obtained of this biotransformation with resting-cell suspensions of *Bacillus megaterium* ATCC 13368 identified as 3-oxo-lup-20(29)-en-28-oic acid, 3-oxo-11α-hydroxy-lup-20(29)-en-28-oic acid, 1β-hydroxy-3-oxo-lup-20(29)-en-28-oic acid, and 3β,7β,15α-trihydroxy-lup-20(29)-en-28-oic acid based on nuclear magnetic resonance and high-resolution mass spectral analyses.

Guirado *et al.* [8] established an efficient synthetic method for dichloromethylated pyrazolines. They efficiently prepared aryl-4,4-dichlorobut-3-en-1-ones **4** by treatment of acetophenones with anhydrous chloral, followed by dehydration and reductive dechlorination. They reported that compounds 4 reacted with hydrazine hydrate and methylhydrazine to give the respective 5-dichloromethyl-2-pyrazolines in high to quantitative yields and determined the molecular structure of 5-dichoromethyl-1-methyl-3-(2-naphthyl)-2-pyrazoline by X-ray crystallography.

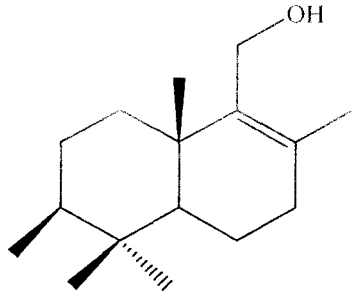
Wael *et al.* [9] has been designed a dual action prodrug based on the imidazo-[1, 2-a]-pyrazin-3-(7H)-one scaffold for combining antioxidant and anti-inflammatory activities, possibly unmasked upon oxidation. The construction of the target-molecule requires two building blocks, namely a 2-amino-1, 4-pyrazine and a 2-ketoaldehyde. They reported that attempts to synthesize the 2-ketoaldehyde (**5a**) derived from ibuprofen failed, but led to the corresponding 2-ketoaldoxime (**7a**) which could not be condensed with the pyrazine synthons. They observed that a model compound, i.e. phenylglyoxal aldoxime, reacted well under microwave activation to furnish novel imidazo [1, 2-a]-pyrazine-3-(7H)-imine derivatives (**18a,b**) and this heterobicycles behave as antioxidants by inhibiting the lipid peroxidation, and one compound (**18b**) is endowed with a significant anti-inflammatory effect in a cellular test.

Neto *et al.* [10] performed Retro-Michael reactions by super-heated steam distillation of 1, 5-diketones with a basic catalyst. They observed that dammarane, hopane and lupane derivatives gave yields in the range of 85 to 100% using sodium hydroxide deposited on glass wool as a catalyst.

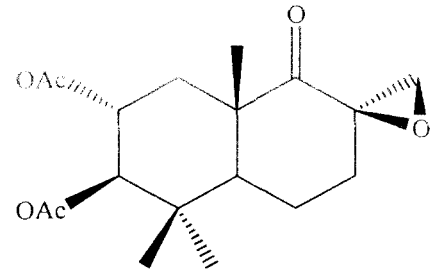
Parra et al. [11] obtained new derivatives of triterpenoids that are potentially useful for pharmacological studies by microbial transformation. They reported that In these biotransformation processes, several reactions that are difficult to achieve by chemical means have been accomplished, such as: introduction of hydroxyl groups into remote positions of the molecules; selective cleavage of the side chains of tetra-cyclic terpenoids to produce C19 steroids; regioselective glycosidic transfer reactions; selective ring cleavage through a Baeyer-Villiger-type oxidation to render *seco*-triterpenoids; and carbon skeleton rearrangements involving a methyl group migration. They observed that these biotransformations have been used as *in vitro* models to mimic and predict the mammalian metabolism of biologically active triterpenoids.

Yogeeswari *et al.*[12] reported that a naturally occurring pentacyclic triterpenoid betulinic acid exhibit a variety of biological activities including inhibition of human immunodeficiency virus (HIV), antibacterial, antimalarial, antiinflammatory, anthelmintic and antioxidant properties. They carried out various structural modifications and their biological and pharmacokinetic profiles are also incorporated.

Garcı a-Granados *et al.* [13] obtained three *seco*-C-ring triterpenic compounds from oleanolic or maslinic acids from olive-mill solid wastes by photochemical and chemical reactions. They also obtained different remarkable sesquiterpene and *nor*-sesquiterpene fragments such as 3β- hydroxydrimenol (**13**) and epoxydecalone (**16**) from these oleantriene compounds through oxidative cleavages of the double bonds in the opened C-ring.



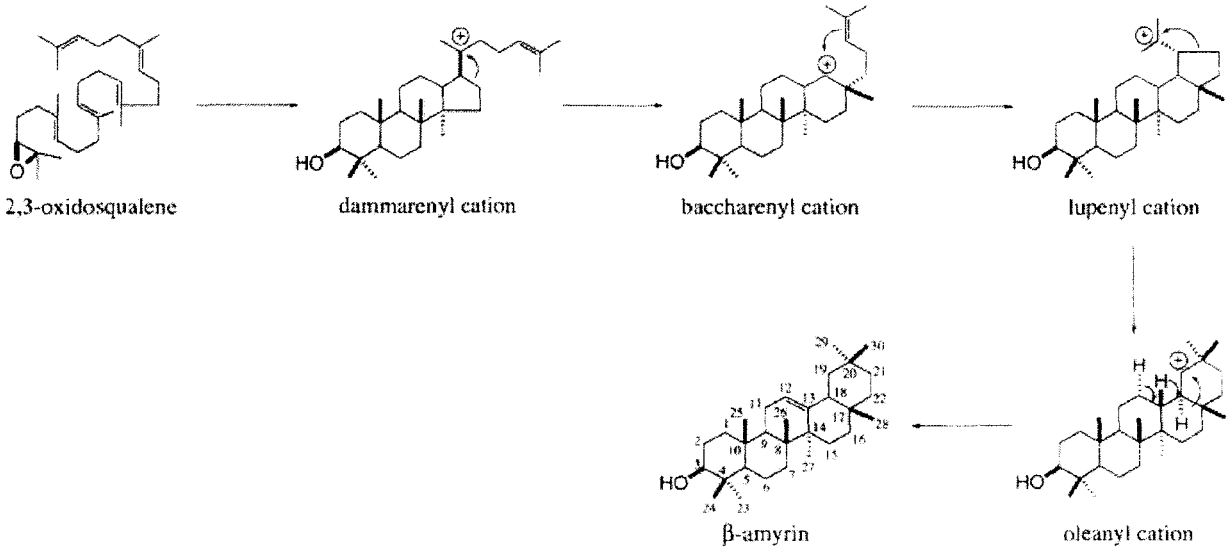
3β-hydroxydrimenol (13)



epoxydecalone (16)

Kushiro *et al.* [14] biosynthesised β-amyrin, a typical pentacyclic triterpene having an oleanane skeleton from (3*S*)- 2, 3-oxidosqualene. The enzyme, β-amyrinsynthase,

catalyzing the cyclization of oxidosqualene into β-amyrin, generates five rings and eight asymmetric centers in a single transformation.



Ahmad *et al.* [15] isolated a new *C*-glycoside, symcososide (**1**) along with one known compound β-sito-glycoside (**2**) from the re-investigation of the chemical constituents of *Symplocos racemosa* Roxb. They observed that the glycoside **1** displayed *in vitro* inhibitory activity against butyrylcholinesterase (BChE) enzyme with *IC*50 value of 21.2 ± 0.01 M.

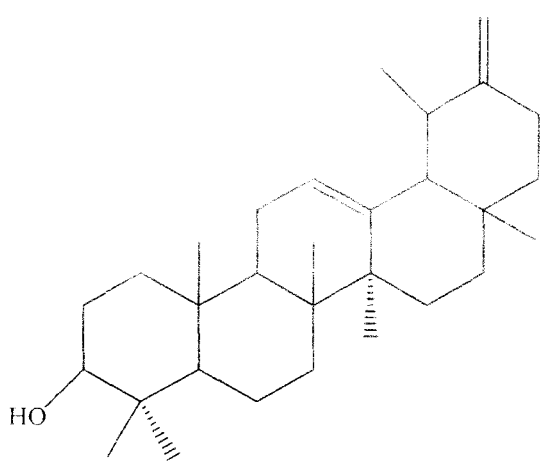
Pomarnacka *et al.* [16] were synthesized of 1-(6-chloro-1,1-dioxo-1,4,2-benzodithiazin-3-yl)-4-hydroxysemicarbazides **11–19**, hydroxybenzaldehyde *N*-(6-chloro-7-methyl-1,1-dioxo-1,4,2-benzodithiazin-3-yl) hydrazones **21–24** and 8-chloro-2-(1-naphthylamino)-5,5-dioxo[1,2,4] triazolo[2,3-*b*][1,4,2]benzodithiazine-7-carbonitrile (**26**). All compounds were tested for their *in vitro* cytotoxic potency against 12 human cancer cell lines at the Institute of Pharmacy, University of Greifswald. They observed that compounds **11–19** were inactive, whereas **22** and **24** exhibited weak tumor growth inhibitory properties. The compound **26** was screened at the National Cancer Institute and showed reasonable anticancer activity.

Sahaa *et al.* [17] extracted compounds from the leaves of *Mimusops elengi* Linn. and evaluated reducing power and total antioxidant capacity by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay. They reported that the extract showed significant

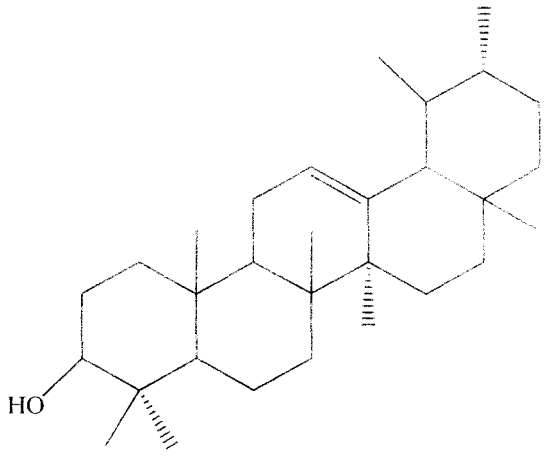
activities in all antioxidant assays compared to the reference antioxidant ascorbic acid in a dose dependent manner. They also reported that DPPH scavenging assay the IC50 value of the extract was found to be 43.26μg/ml while the IC50 value of the reference standard ascorbic acid was 58.92 μg/ml. Total antioxidant activity was also found to increase in a dose dependent manner. Moreover, *M. elengi* extract showed strong reducing power. These results suggest that *Mimusops elengi* may act as a chemopreventative agent, providing antioxidant properties and offering effective protection from free radicals.

Nishikawa *et al.* [18] efficiently synthesized of L-α-phosphatidyl-D-*myo*-inositol 3,5-bisphosphate from 1,2,5,6-diisopropylidene-D-glucose by utilizing ring-closing metathesis and catalytic OsO_4 dihydroxylation.

Corey *et al.* [19] used Li in presence of ethylene diamine for selective reduction of Olefinic double bond on triterpenoid. They obtained α-amyrin (1) from ursa-12:20(30)-dien-3β-ol (1a).



α-amyrin



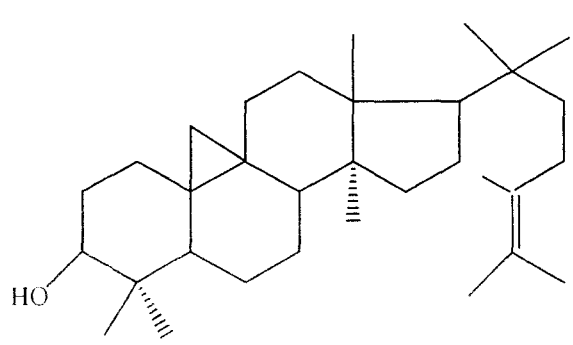
ursa-12:20 (30)-dien-3β-ol

Reggel *et al.* [20] also studied the isomerisation of olefins and dehydrogenation of cyclic dienes with Li in presence of ethylenediamine.

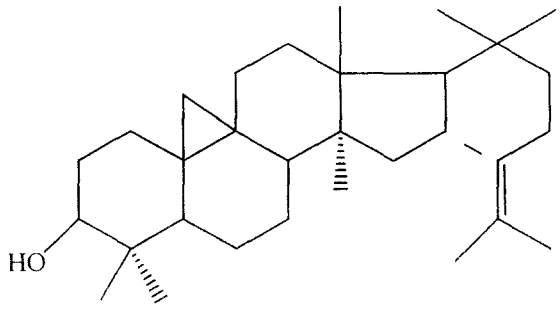
Tyagi *et al.* [21] studied the behavior of cyclopropane and cyclobutane rings on many triterpenoids towards the Li-ethylenediamine.

Smith *et al.* [22] reported that primary and secondary alcohols were dehydrogenated to carbonyl compounds in presence of Li metal in ethylene diamine.

Sukhdev *et al.* [23] reported that cyclolaudenol 2 on exposure to N-lithioethylenediamine at 120-125oC gave the isopropylidene isomer 2a in 92% yield.

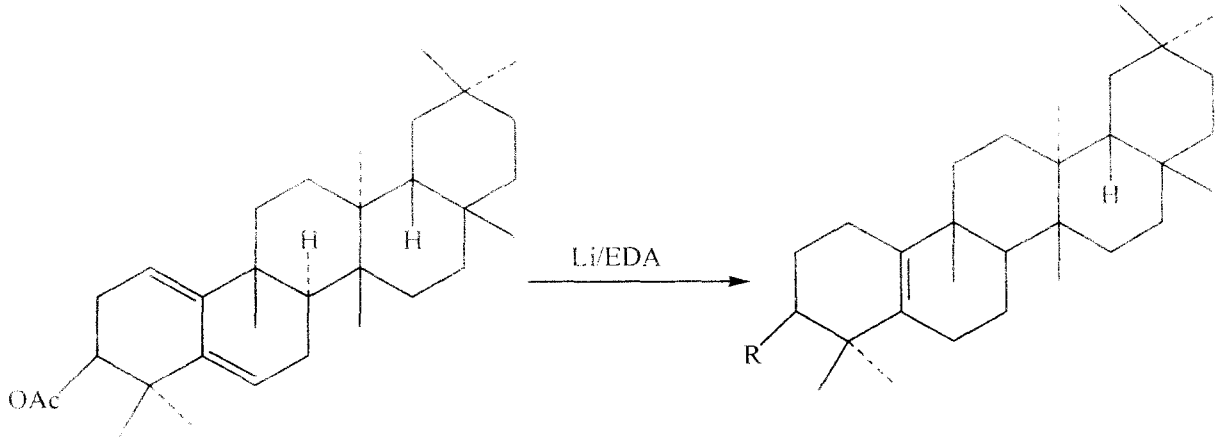


Cyclolaudenol



isopropylidene

Sengupta *et al.* [24] studied the reaction of Li in ethylene diamine on triterpenoid heteroannular 1,3-dienes and observed that gluto-1(**10**), 5-dienyl-3β–acetate **4** gave a mixture of deoxygenated product, glut-5(10)-ene **5a** and hydrolysed product, glut-5(10)-en-3β-ol **5b.**

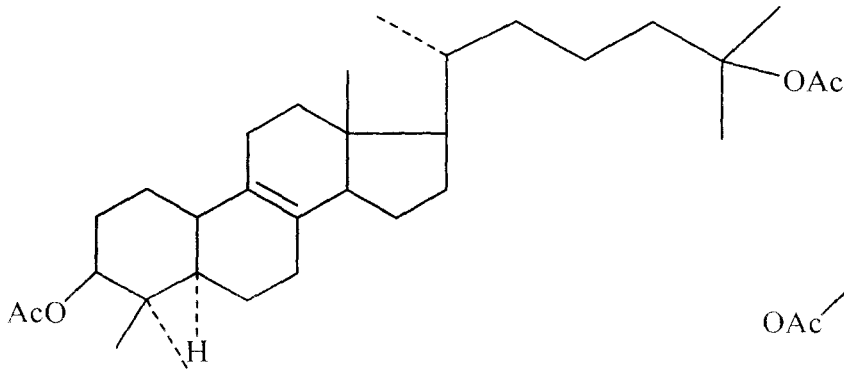


4

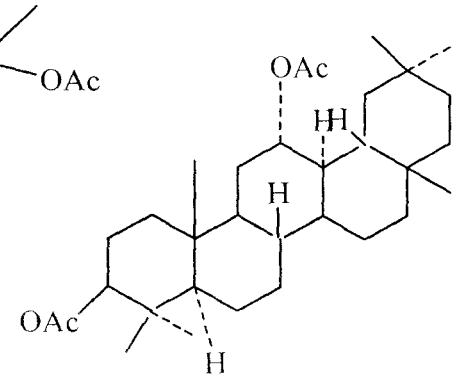
5a. R=H
5b. R=OH

Barton *et al.* [25] studied the reduction on sterol system contained two ester groups in sterically different environments. The compounds **6** and **7** when treated with Li in

ethylene diamine the products **8, 9, 10** were obtained respectively



6



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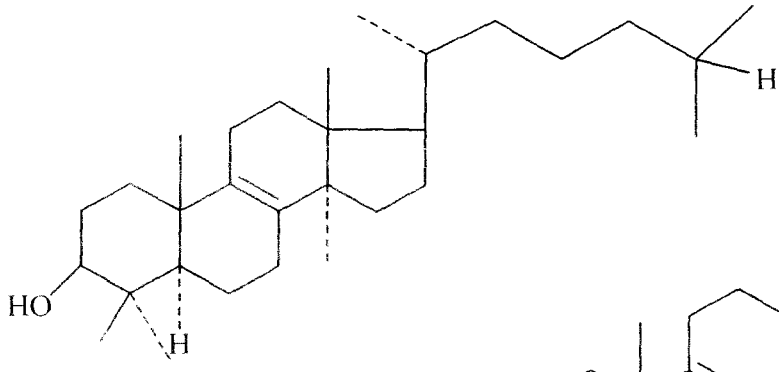

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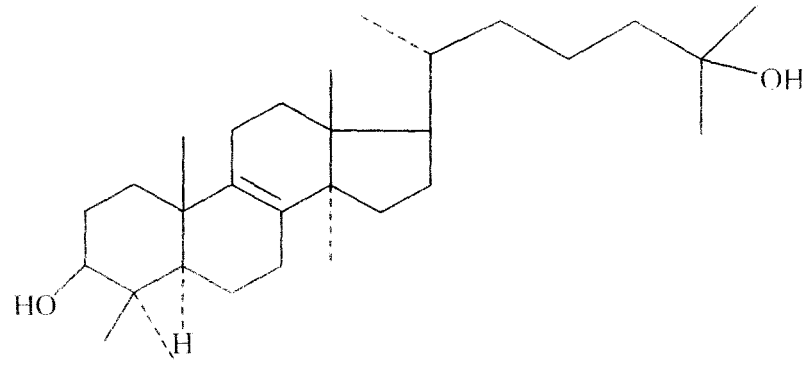

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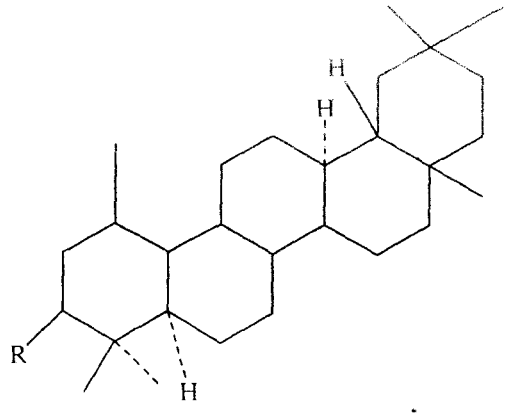


10

From the above literature work it appears that limited chemical work has so far been attempted both on the transformative reaction of triterpenoids as well as on the systematic studies of the biological activities of the derived compounds. Hence, there exists an ample opportunity to study the transformative reactions on the isolated triterpenoids using various reagents and also the biocidal activities of each of them in comparison to the parent compound. These investigations will not only enrich the little known triterpenoid chemistry but at the same time may yield a number of very useful pharmacologically important derivatives.

Chemical transformations using MW irradiation has been used extensively by various groups of researchers and a short review of which is described below.

Ma *et al.* [26] has been developed an efficient one-pot method to generate structurally diverse and medicinally interesting pyrazolone derivatives in good to excellent yields of 51–98% under microwave irradiation and solvent-free conditions.

Gaina *et al.* [27] applied a dynamic microwave power system in the chemical synthesis ofsome phenothiazine and quinoline derivatives is described. They compared the microwave-assisted synthesis with the conventional synthetic methods and found advantages related to shorter reaction time and in some cases better reaction yields in the case of microwave-assisted system.

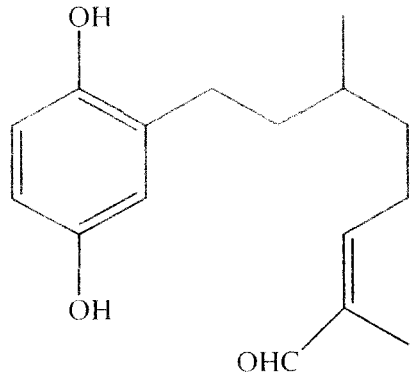
Chen and Hu [28] were synthesized a series of 1,2,3,4-tetrahydrocarbazoles by the reaction of substituted 2-bromocyclohexanones with appropriate anilines under microwave irradiation without any other catalysts.

Wu *et al.* [29] was developed a microwave-assisted reaction to facilitate the construction of 4,5-disubstituted pyrazolopyrimidines and reported that using microwave irradiation leads to high product conversion, low side product formation, and shorter reaction.

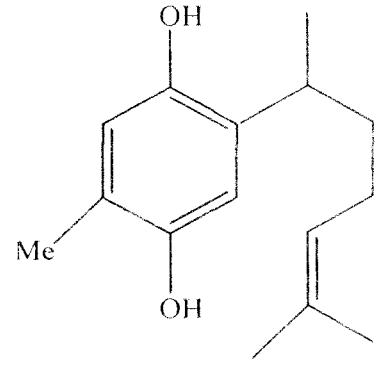
Raghunandan *et al.* [30] synthesized bio functionalized silver nanoparticles from guava (*Psidium guajava*) leaf by microwave-assisted process. The reaction was completed within 90 s. They selected the microwave-assisted route for synthesis to carry out the reaction fast, suppress the enzymatic action and to keep the process environmentally clean and green.

Bai *et al.* [31] has been developed a simple and rapid microwave-assisted extraction (MAE) procedure optimized for extracting triterpenoids (TTP) from the *Actinidia deliciosa* root. They reported that several variables that could potentially affect the extraction efficiency, namely extraction time (min), ethanol fraction (%), liquid: solid ratio (volume per mass) and microwave power. They also reported that under the optimum operating conditions (ethanol fraction 72.67 %, microwave power 362.12 W, liquid: solid ratio 15:1 and extraction time 30 min) the percentage of extracted TTP was 84.96 %, and MAE showed significantly higher recoveries than those obtained by the conventional extraction methods

Kad *et al.*[32] readily synthesized terpenoids **1** and **2** from readily available starting materials using Li_2CuCl_4-catalyzed coupling of Grignard reagents with alkyl/aryl bromides and microwave assisted oxidation of allylic methyl groups using SeO_2/ BuOOH adsorbed over SiO_2 as key steps.



1



2

More *et al.* [33] have been synthesized various thymyl ethers and esters by reactions of thymol with alkyl halides and acid chlorides, respectively, in aqueous medium using microwave irradiation and reported that the products are important as potent pest managing agents.

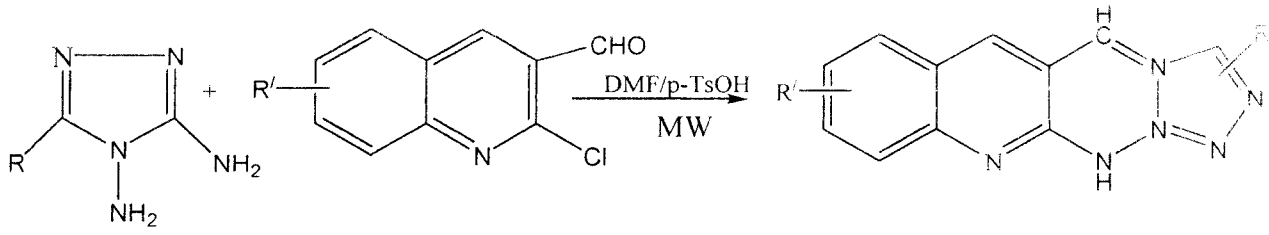
Gopalakrishnan *et al.* [34] oxidized Tetranortriterpenoids from *Azadirachta indica* A. Juss and *Soymida febrifuga* (Meliaceae) to single major products which exhibited bioactivity higher than the parent compound azadirachtin A. They reported that the reaction completed in less than 15 min and 1 min on being assisted by ultrasound and

microwave irradiation, respectively. They also reported a rapid and selective oxidation of the furan moiety of some limonoids employing microwave and ultrasound irradiations.

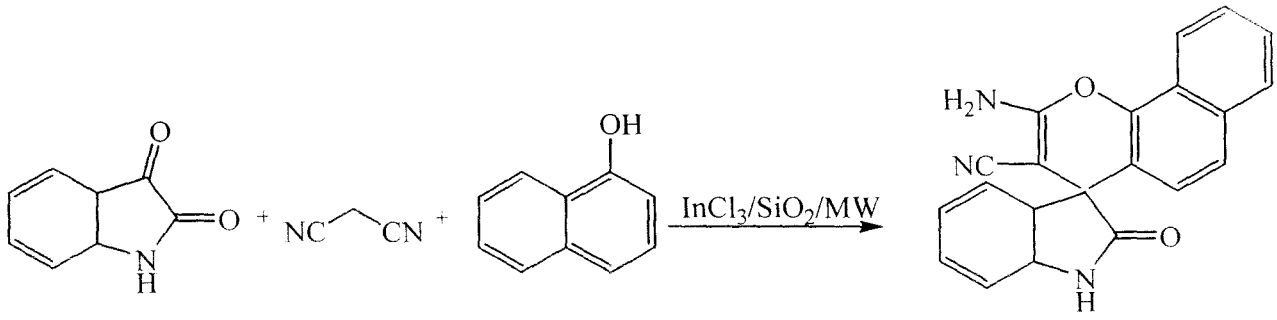
Yan *et al.* [35] were obtained volatile oils from *Marchantia convolute* by microwave extraction (ME) and phytosol extraction (PE) methods. The attained extracts were analyzed by gas chromatography with mass spectrometric detector. They identified a total of fourteen compounds in ME extract accounting for 80.72% of total peak area. Isolongifolene (24.588%), 1, 2-benzenedicarboxylic acid, butyl cyclohexyl ester (10.768%), pyrene (9.328%) and hexadecanoic acid, ethyl ester (8.570%) were the major compounds identified from ME extract.

Azizian *et al.* [36] introduced a microwave assisted one-pot three-component procedure for preparation of some dicyanomethylene derivatives of indenoquinoxaline and tryptanthrin under solvent free conditions.

Gupta *et al.* [37] synthesized 9-substituted -3-aryl-5H, 13 aH-quino-linol[3,2-f]triazolo[4,3-b][1,2,4]triazepines **8** from 5-aryl-3,4-diamino-1,2,4-triazoles **5** and 2-chloro-3-formyl quinolines **7** using catalytic amount of p-TsOH and N,N-dimethyl formamide as an energy transfer medium using microwave heating as well as solvent using oil bath heating at 80°C. The product were obtained in the good to moderate yield and in the state of high purity.



Shanthi *et al.* [38] described a simple and efficient method for the one-pot three-component synthesis of new spirooxindoles under conventional and solvent free microwave irradiation is described.



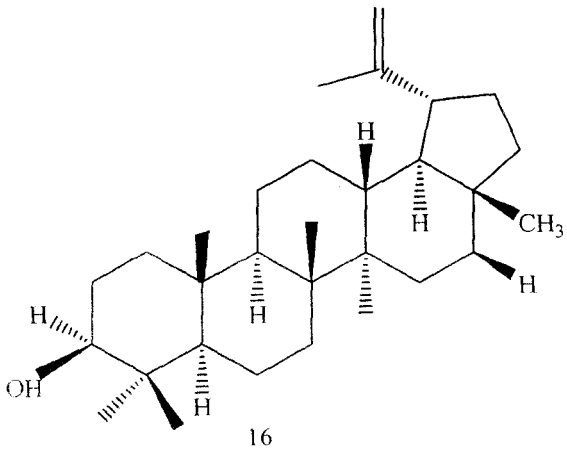
More *et al.* [39] have been synthesized various thymyl ethers and esters by reactions of thymol with alkyl halides and acid chlorides, respectively, in aqueous medium using microwave irradiation and reported that the products are important as potent pest managing agents.

It appears from the above that transformation under microwave irradiation has not yet been attempted on terpenoids and more precisely on pentacyclic triterpenoids. This encouraged the author to carry out the microwave assisted transformation of some of the isolated triterpenoids.

REVIEW ON BIOCIDAL ACTIVITY OF THE DERIVATIVES OF PENTACYCLIC TRITERPENOIDS

Akihisa *et al.* [40] isolated twenty-eight 3-hydroxy triterpenoids from the non-saponifiable lipid fraction of the flower extract of *chrysanthemum* (*Chrysanthemum morifolium*) and one lupane-type 3 alpha-hydroxy triterpenoid (**17**) derived from **16** were tested for their antitubercular activity against Mycobacterium tuberculosis strain H37Rv using the Microplate Alamar Blue Assay (MABA). They observed that Cytotoxicity of compound **17** against Vero cells gave an IC50 value of over 62.5 microg/ml, suggesting some degree of selectivity for M.

tuberculosis.



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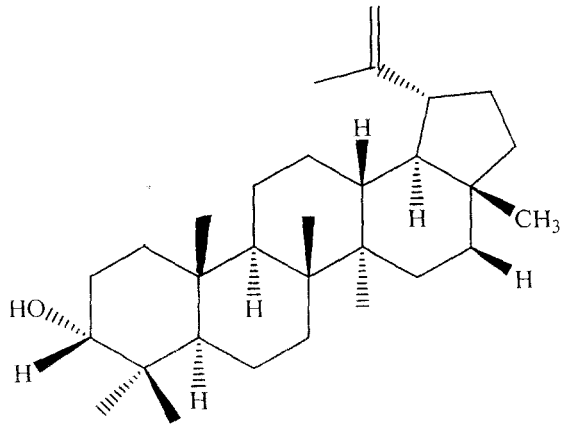


17

Ryu *et al.* [41] studied antiviral activity of triterpenoid derivatives and observed that 3-*oxo* or/and 11-oxo derivatives of natural 3-*hydroxy* triterpenes *i.e.*, 3-oxoursolic acid I a, 11-oxoursolic acid I b, 3,11-dioxoursolic acid I c, 3-oxobetulinic acid II a and 3-oxopomolic acid VI a were exhibited to show an increased anti-HSV-1 activity *in vitro*, four to ten times with respect to corresponding parent 3-*hydroxy* compounds.

Liby *et al.* [42] observed that synthetic oleanane triterpenoids have profound effects on inflammation and the redox state of cells and tissues, as well as being potent anti-proliferative and pro-apoptotic agents. Rexinoids are ligands for the nuclear receptor transcription factors known as retinoid X receptors. They found that both classes of agents can prevent and treat cancer in experimental animals and these drugs have unique molecular and cellular mechanisms of action and might prove to be synergistic with standard anti-cancer treatments.

Tamura *et al.* [43] reported that the leaf beetle *Ophraella communa* infests almost exclusively *Ambrosia artemisiifolia* in the fields of Japan and a filter paper bioassay showed that the feeding of *O. communa* is strongly stimulated by methanolic extracts of *A. artemisiifolia.* They also reported that triterpenoid derivatives (α-amyrin acetate or β-amyrin acetate) and caffeic acid derivatives (3, 5-dicaffoylquinic acid or 5-caffeoylquinic acid) showed feeding stimulant activity when mixed together.

Reddy *et al.* [44] isolated lupeol from the leaves extract of *Aegle marmelos* and synthesized few novel derivatives (**2–13**) from the naturally occurring lupeol (**1**) and

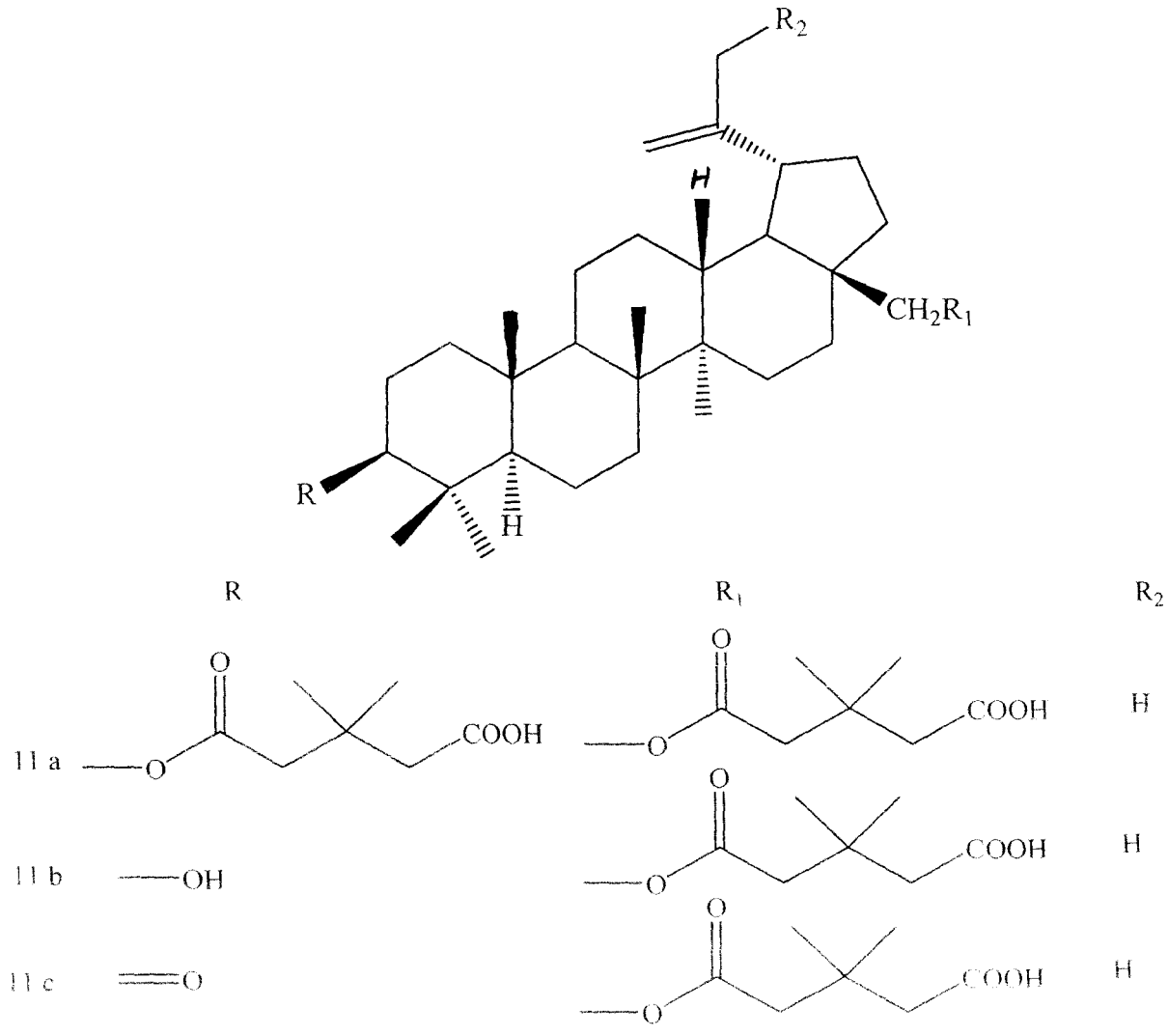
screened for their antihyperglycemic activity (**2–11**) and antidyslipidemic activity (**2–4** and **12–13**). They found that derivative **4** lowered the blood glucose levels by 18.2% and 25.0% at 5 h and 24 h, respectively, in sucrose challenged streptozotocin induced diabetic rats (STZ-S) model at the dose of 100 mg/kg body weight and the compound **4** also significantly lowered 40% (P <0.001) in triglycerides, 30% (P <0.05) in glycerol, 24% (P <0.05) in cholesterol quantity and also improved the HDL–cholesterol by 5% in dyslipidemic hamster.

Meng *et al.* [45] were synthesized and designed a series of boswellic acid derivatives in order to search for new potent anti cancer agents and six of them were identified by IR, NMR and MS.

Woldmichael *et al.* [46] detected at least 16 saponins in the seeds of *Chenopodium quinoa*. They studied antifungal activity and hemolytic activity on erythrocytes of these compounds and derived monodesmosides against *Candida albicans*. They found that both bidesmosides and derived monodesmosides showed little antifungal activity whereas a comparatively higher degree of hemolytic activity could be determined for monodesmosides.

Tolstikov *et al.* [47] systematized the data on natural source of betulin and methods of its extraction, transformation and its available derivatives. They presented the data on the biological activity of betulin, its natural and synthetic analogs. The reported the promising character of the compounds based on betulin for creation of antiviral and antitumour agents.

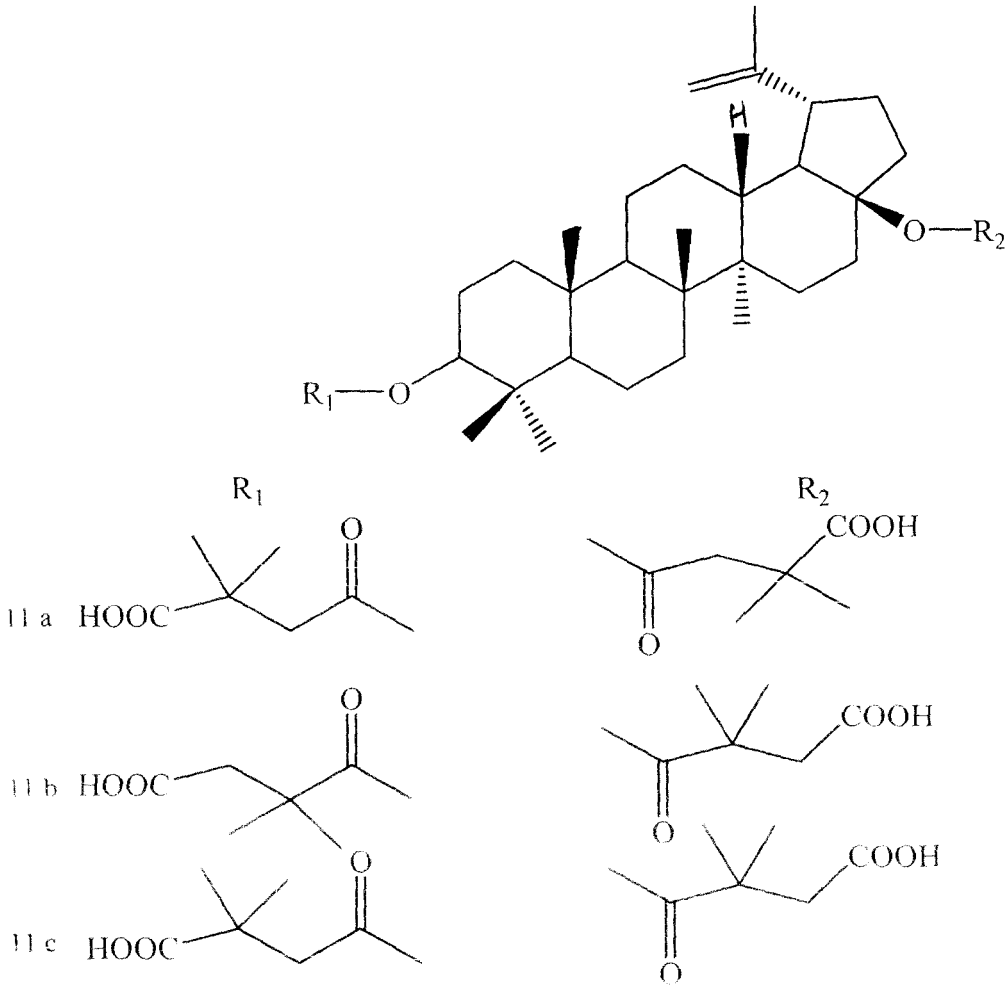
Su *et al.* [48] synthesized various o-acyl betulinic and dihydro betulin derivatives. Among them the most potent compound **11a** with two 3′, 3′-dimethyl glutaryl groups displayed anti-HIV activity with an EC_{50} value of 0.66 nM and SI of 21,515. The dihydro betulin derivative of **11a** showed a SI of 2253. Monoacyl betulin (**11b**), containing a substitutd glutaryl group only at C_{28} position, had an EC_{50} value of 36 μM and SI of 7.8. Conversion of the 3β-hydroxy gr. of **11b** to the mono keto derivative led to the **11c** and 0.46 μM respectively.



Schuhly *et al.* [49] isolated betulinic acid from the stem bark of Brazilian medicinal plant Zizypus jaazerio and its three new derivatives namely 7β-(4-hydroxybenzoyloxy) betulinic acid and 27-(4-hydroxy-3-methoxybenzoyloxy) betulinic acid and 27-(4-hydroxy-3-methoxybenzoyloxy) showed considerable activity against Gram-positive bacteria.

Kashiwada *et al.* [50] prepared four isomeric 3, 28-di-O-(dimethylsuccinyl) betulin derivatives and evaluated their anti-HIV potency. Among these derivatives, **11c** demonstrated the highest activity in acutely infected H9 cells with an EC_{50} value of 0.87 nM and inhibited uninfected H_9 cell growth with an IC_{50} value of 36.9 μM. Its calculated SI value (42,400) was comparable to that of zidovudine (41,622). Compound **11a** was also

extremely potent with an EC_{50} value of 0.02 μM and SI of 1680. Compound 11**b** displayed fair activity (EC_{50}=0.4 μM; SI=96.5) while 12**b** was toxic.

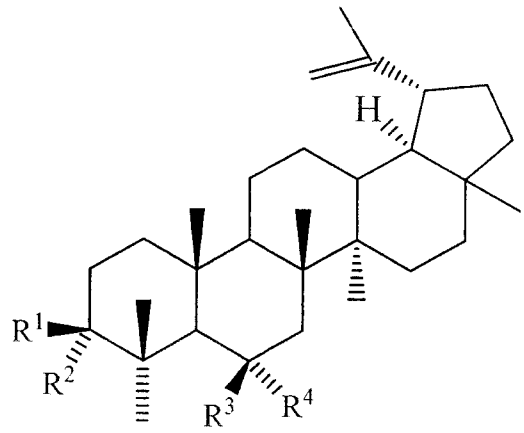


Su *et al.* [51] studied a series of triterpene derivatives for quantitative structure-activity relationship with multiple linear regression (MLR) and artificial neural networks (ANN). They observed that the linear model with MLR performed poorly while the nonlinear model with ANN preformed well. For the ANN model with architecture of 5-6-1, the root mean square error for the training set, validation set and the prediction set were 0.2019, 0.2214 and 0.2883, respectively. In this study they used different methods to select the most relevant descriptors for MLR and ANN and the result indicates these descriptors are playing an important role on the anti-HIV activity of triterpene derivatives.

Suh *et al.* [52] reported that the new synthetic oleanane triterpenoid 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) is a potent, multifunctional molecule. It induces monocytic differentiation of human myeloid leukemia cells and adipogenic differentiation of mouse 3T3-L1 fibroblasts and enhances the neuronal differentiation of rat PC12 pheochromocytoma cells caused by nerve growth factor. They found that CDDO inhibited proliferation of many human tumor cell lines, including those derived from estrogen receptor-positive and -negative breast carcinomas, myeloid leukemias, and several carcinomas bearing a *Smad4* mutation and suppresses the abilities of various inflammatory cytokines.

Baltina *et al.* [53] modified betulin and betulinic acid at the C-3 and C-28 positions and evaluated in vitro for antiviral activity. It was found that simple modifications of the parent structure of lupane triterpenes produced highly effective agents against influenza A and herpes simple type 1 viruses.

Mustafa *et al.* [54] reported that lupeol derivatives **(III-V)** containing functional groups in the ring B displayed a high inhibiting activity toward α-glucosidase and a moderate antibacterial activity. Lupeol ester **(VI)** was found to display cytostatic activity against JB6 cells Gao *et al.* [55].



	R^1	R^2	R^3	R^4
III	OH	H	OH	H
IV	–	O –	–	O –
V	$COC_{17}H_{35}$	H	H	OH
VI	$OCOCHCHC_6H_{13}(OH)_2$	H	H	H

Chapter 2

MICROWAVE ASSISTED ONE POT SYNTHESIS OF PYRAZINE DERIVATIVES OF PENTACYCLIC TRITERPENOIDS AND THEIR BIOLOGICAL ACTIVITY

Introduction

Triterpenoids are distributed widely in nature and recent literature has reported their wide spectrum of biological activities [56]. It is well known and well documented that there exists a connection between the wide spectrum of biological activities and the molecules having pyrazine nucleus [57]. They possess varieties of activities like antimicrobial [57], anti filarial [57], anti leukemia [57] in mice against i.p. P388 and several pyrazines were more active than the corresponding oxazines or thiazines [57]. Also a series of pyrazine-carboxymides has been described as eukalemic agents possessing diuretic and natriuretic properties. Hence pyrazine is a lead compound for designing potential bioactive agents. Thus it is anticipated that incorporation of a pyrazine ring into a molecule like triterpenoids may induce biological activity or may enhance the same if already present in the latter. So a study can be taken up to incorporate a pyrazine ring into the pentacyclic triterpenoids and to study the biological activity of the derivatives. But the conventional method of pyrazine synthesis [58, 59, and 60] involves hazardous, expensive, polluting organic solvents; a prolonged reaction time and tedious working procedures also produce significant amount of side products. Again reports regarding the one pot synthesis of pyrazine derivatives of pentacyclic triterpenoids are very limited. Thus, a one pot method involving milder, more selective, inexpensive and eco-friendly reaction condition is still in demand.

The potential application of microwave (MW) technology in organic synthesis is increasing [61] rapidly because of the reaction simplicity, less polluting and minimum reaction time providing rapid access to large libraries of diverse molecules. This technology has been implemented since the middle of 1980s in the field of organic chemistry. The increasing number of related publications in recent years indicates that this technique is a widely accepted unconventional energy source for performing organic

synthesis due to substantial reduction in reaction time [62, 63], better yields [64] and easier work up procedures [65]. In addition, the high selectivities of the reactions contribute to the prevention of waste formation [66]. Although a very few reports of microwave assisted transformative reactions of terpenoids, flavonoids [67] and steroids [68] are known, current literature is lack about the microwave assisted transformative reaction of pentacyclic triterpenoids.

In continuation of the studies on the transformative reactions of pentacyclic triterpenoids, the author has developed a one pot synthesis of 1, 4-pyrazine derivative of pentacyclic triterpenoids under microwave irradiation. The structures of the compounds have been confirmed by means of spectral data (IR, NMR). Compounds 1b, 2b and 3b have been reported for the first time. The anti microbial potential associated with them has also been investigated.

Results and Discussion

Synthesis of 2, 3- diketo triterpenoids

2, 3-diketo triterpenoids were prepared by auto oxidation of the respective terpenoids (for details please see experimental).

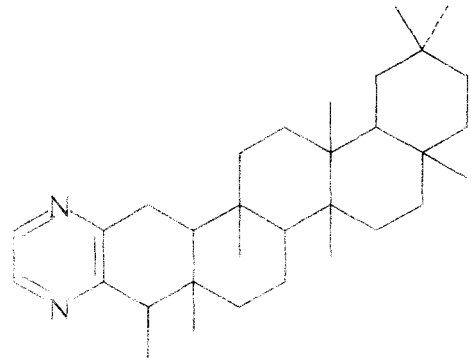
Synthesis of 1, 4-pyrazine derivatives

1,4-pyrazine derivatives of the triterpenoids were prepared (Scheme 1) in a mono-mode microwave oven at 100W(100°C) in only 20 minutes reaction time by adding dry ethylene diamine and Li(for details please see experimental).

Characterization of compound 1b: (1,4-pyrazine derivative of friedelin)

2, 3-diketo friedelin (**1a**) prepared by auto oxidation of friedelin (**1**) was subjected to microwave irradiation (100W, 100°C) for 20 minutes with small pieces of metallic Li and dry ethylene diamine (EDA). The product obtained after usual work up showed a single spot in TLC and was purified over a column of silica gel (60-120 mesh). Crystallization of the compound from $CHCl_3$ – MeOH mixture furnished a compound A, analyzed for $C_{32}H_{50}N_2$, m.p. 228 °C. IR spectrum of the compound showed peaks at 1650 – 70, 1430, 1120 cm^{-1} for pyrazine ring [69]. UV spectrum showed peaks at 272 (ε = 5800)

and 278 (ε = 5450) nm. Mass spectrum of A showed molecular ion peak at m/z 462 as base peak, which is the characteristic feature of pyrazine compounds [69]. The other peaks appeared at m/z 447,420, 271, 241, 227, 163, 149, 125, and 69. The ^{1}H NMR spectrum of A was indicative of the presence of seven tertiary methyls which appeared as sharp singlets (3H each) between 0.82 – 1.22 ppm (7s, 21H, 7t CH_3), the doublet centered at 0.99 ppm was due to the presence of secondary methyl protons (d, J = 6.5Hz), two aromatic protons at 8.40 and 8.27 ppm appeared as a doublet with J = 3Hz. ^{13}C NMR spectrum of the compound A, showed the presence of 32 carbons, two singlets at 150.8 and 150.9 ppm and two doublets at 141.4 and 142.3 ppm were due to heterocyclic ring carbons typical to 2, 3 – disubstituted pyrazine skeleton [70]. All the above facts lead us to assign structure **1b** to compound A. The formulation of structure 1b for compound A is further supported by mass fragmentation pattern that gives similar fragmentation pattern as that of friedelin skeleton as observed earlier [71].

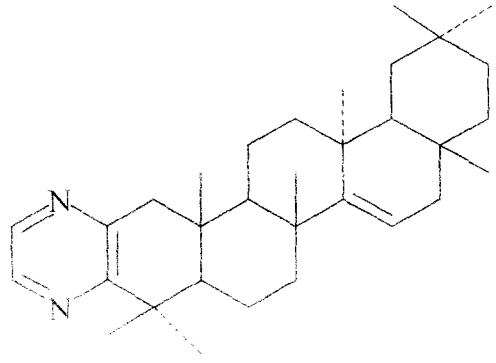


1b

Characterization of compound 2b: (1,4-pyrazine derivative of taraxerone)

2, 3-diketo taraxerone (**2a**) was also treated with Li – dry EDA in the similar way. The single compound obtained was purified over a column of silica gel (60-120 mesh) followed by crystallization from $CHCl_3$ – MeOH mixture to afford a compound, B. It was analyzed for $C_{32}H_{48}N_2$, m.p. 262°C. IR spectrum of B showed peaks at 1600 and 810 cm^{-1} due to the presence of trisubstituted (R_2C = CHR) double bond, the peaks at 1650, 1430 and 1120 cm^{-1}were characteristic for a pyrazine derivative [69]. The UV spectrum of compound B showed absorption at 272 nm (ε = 6150) and at 278 nm (ε = 5200),

characteristic for a pyrazine skeleton. Mass spectrum showed molecular ion peak at m/z 460 as base peak. Its ^{1}H NMR spectrum showed the presence of eight tertiary methyls which appeared as sharp singlets (3H each) in the region 0.81 to 1.25 ppm, the doublet of a doublet centered at 5.54 ppm was due to one olefinic proton at C-15, C-1 protons appeared as a multiplet at 2.30 ppm and two aromatic protons appeared at 8.40 and 8.27 ppm as a doublet with J = 3Hz. The ^{13}C NMR spectrum of B accounted for all 32 carbons and APT experiment indicated the existence of 8 $-CH_3$ as quartets, 9 $-CH_2$ as singlets. The singlet at 156.9 ppm and doublet at 117.4 ppm were due to olefinic carbons at C-14 and C-15 typical of the taraxerone skeleton [72] and the aromatic ring carbons appeared as doublets at 141.4 and 142.3 ppm and singlets at 150.8 and 150.9 ppm. All the above facts lead to the assignment of structure **2b** for compound B.

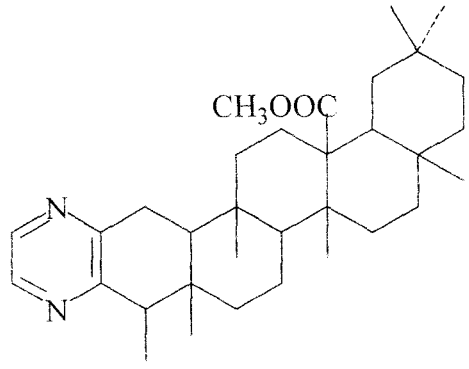


2b

Characterization of compound 3b: (1, 4-pyrazine derivative of methyltrichadenate)

1, 4-pyrazine derivative of methyltrichadenate (**3b**) was also prepared starting from methyltricadenate (**3**). The single compound, C, obtained by the usual procedure was analyzed for $C_{33}H_{50}N_2O_2$, m.p. 198°C. IR spectrum showed peaks at 1730cm^{-1} for carbomethoxy group and at 1650–70, 1430, 1120 cm^{-1} for pyrazine ring [69]. It also showed characteristic UV absorption at 272 nm (ε = 5785) and 278 nm (ε = 5600) for a pyrazine ring. Mass spectrum showed molecular ion peak at m/z 506 as base peak. ^{1}H NMR spectrum of compound C showed the presence of six tertiary methyls that appeared as singlets at 0.81, 0.85, 0.93, 0.99, 1.11 and 1.20 ppm and a secondary methyl as a doublet centered at 0.74 (d, 3H, $CHCH_3$, J = 7Hz) ppm. A singlet at 3.67 ppm indicated the

presence of carboxy methyl group, C-1 protons appeared as a multiplet at 2.30 ppm and two aromatic protons appeared at 8.40 and 8.27 ppm as a doublet with J = 3Hz. Thus from spectral analysis the structure for C has been assigned as **3b**. Encouraged by these findings, and the recent report about the anti HIV activity of triterpenoid lupane skeleton [73] has prompted us to study the same reaction on such skeleton e.g. lupanone and dihydromethyl betulonate and to make a preliminary study on their biocidal activities.



3b

Characterization of compound 4b: (1, 4-pyrazine derivative of lupanone)

Auto oxidation of lupanone yielded 2, 3-diketo lupane which upon treatment with Li – dry EDA in the similar manner followed by purification yielded a single compound. Crystallization of the compound from $CHCl_3$ – MeOH mixture furnished D, analyzed for $C_{32}H_{50}N_2$. $[\alpha]_D$ +19.6°. IR spectrum (Fig. 2) of the compound showed peaks at 1650, 1430 and 1120 cm^{-1}, probably due to the presence of a heterocyclic ring system in compound. UV absorption maxima (Fig. 1) at 272 nm (ε= 5831) and 278 nm (ε= 5792) also suggested presence of aromatic moiety in 4b. The mass spectrum (Fig. 4) of the compound showed molecular ion peak at m/z 462. PMR spectrum (Fig. 3) of the compound D showed the presence of eight tertiary methyl groups resonated at 0.78, 0.83,0.98, 1.11, 1.29, 1.31 ppm (6s, 18H, 6t-CH3), 0.77 and 0,86 ppm (2d, 6H, $CH(CH_3)2$, J=7 Hz): two doublets at 2.47 and 3.04 ppm with germinal coupling of 16 Hz could be assigned to the methylene proton at C-1 that have no protons in the vicinal alpha carbons; two olefinic protons that appeared at 8.405 and 8.27 ppm as doublets with J= 3Hz, the former being further splitted by long rang 1,4 coupling with C-1 proton appearing

at 2.47 ppm; the large downfield shift of these protons indicates that these are present in a pyrazine ring. Hence doublet centered at 8.27 ppm (J=3 Hz) and a doublet of a doublet centred at 8.41 ppm (J= 3 and 1 Hz) were probably due to the aromatic protons (C=C), the nature of (dd) of the peak centred at 8.14 ppm could also be explained by considering the long range coupling of one of the aromatic proton with the methyl proton of the isopropyl group at C-19 position. Thus from the spectral analysis the structure for the compound was identified as pyrazine derivative **4b** of lupanone [74].

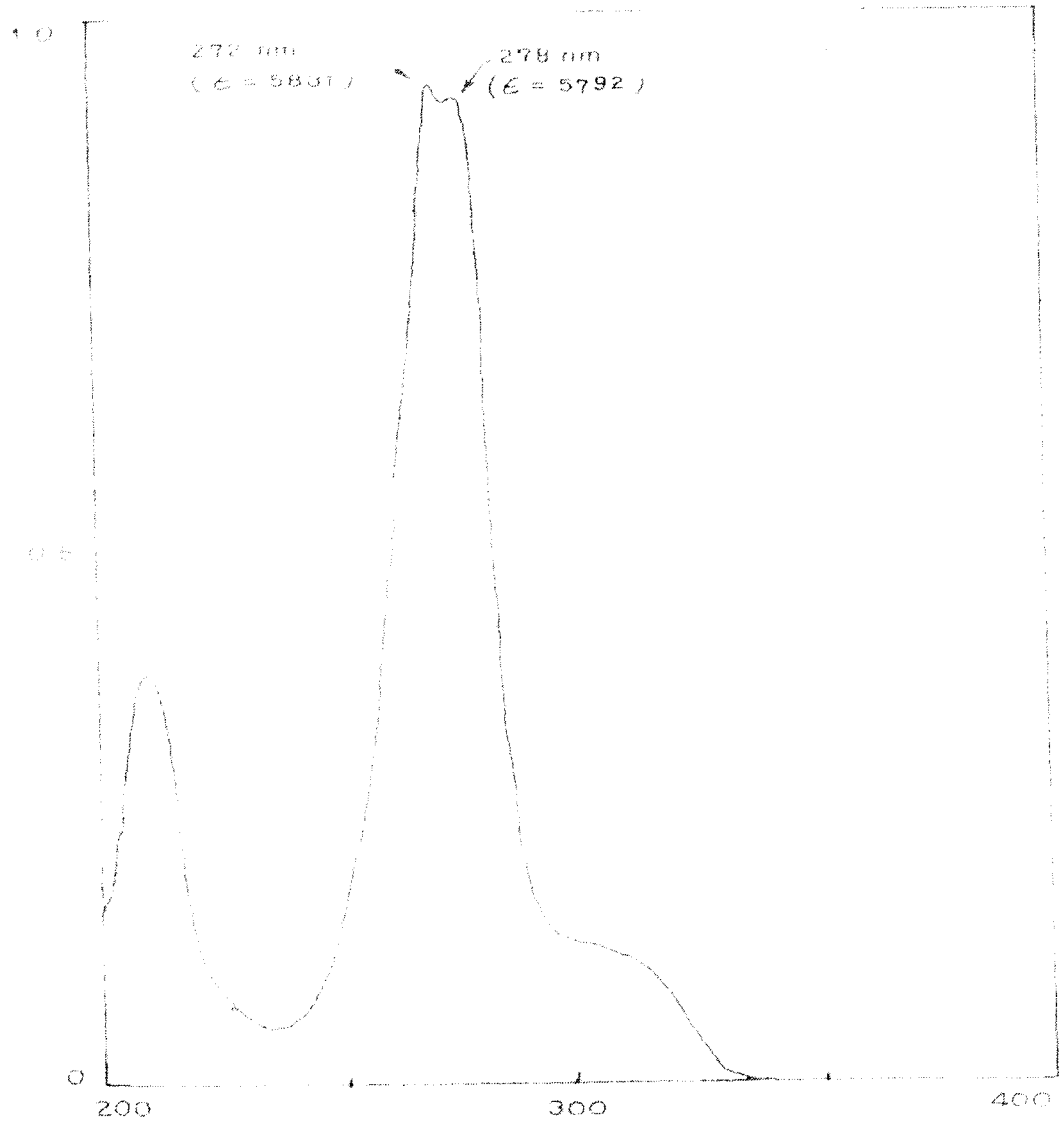


Fig. 1. UV. spectrum of the pyrazine derivative of lupanone

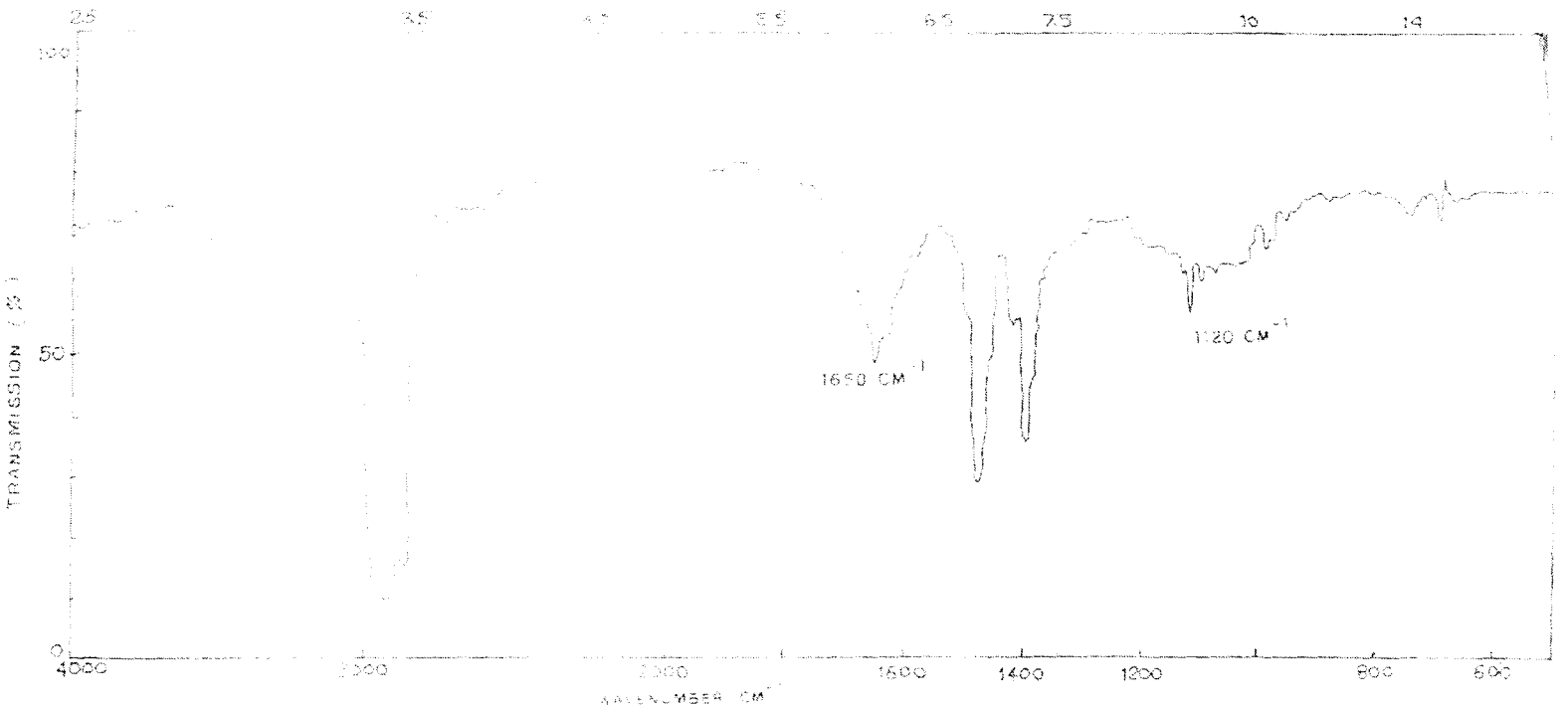


Fig. 2. IR spectrum of the pyrazine derivative of lupanone

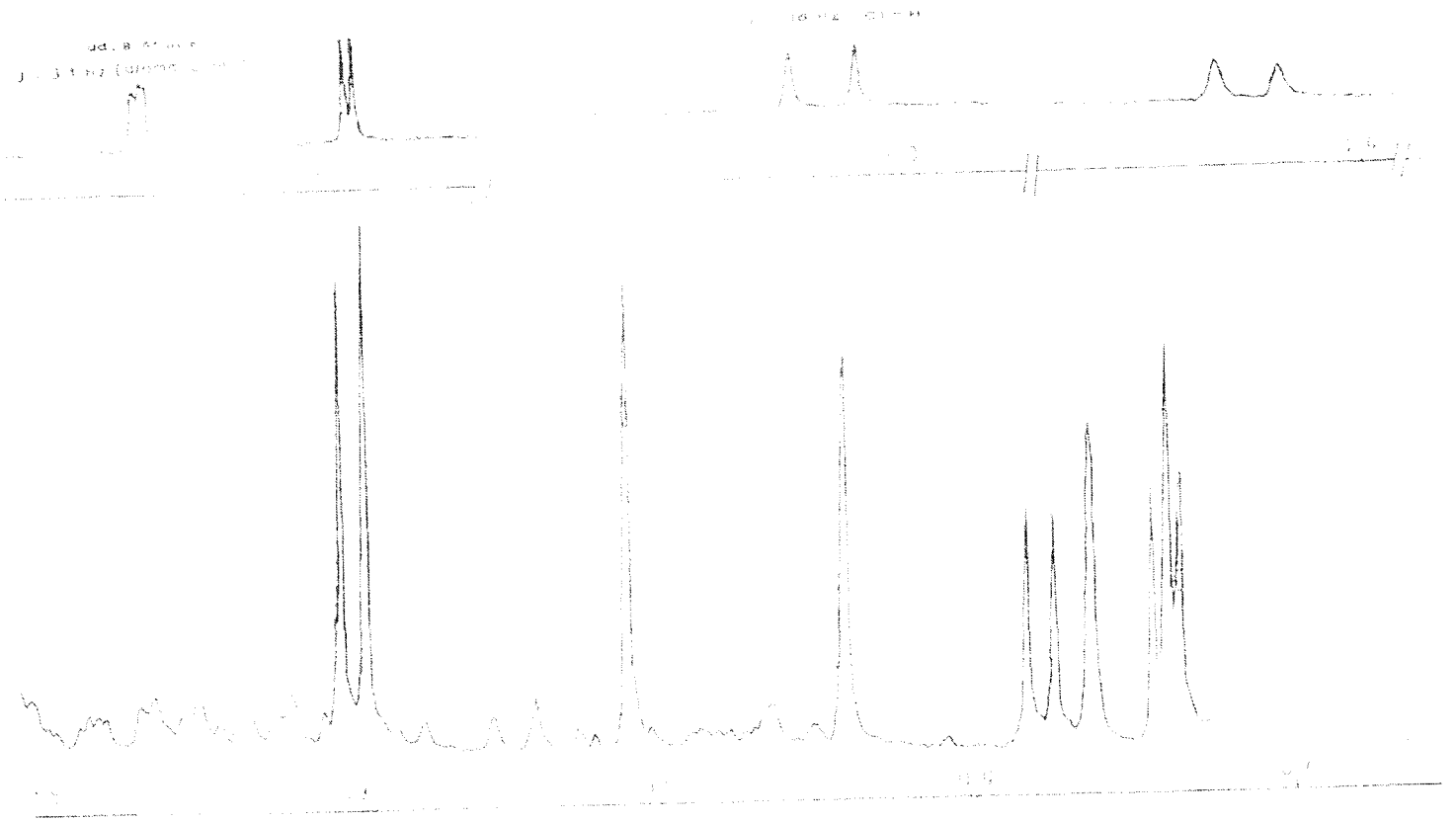


Fig. 3. ^{1}H NMR spectrum of the pyrazine derivative of lupanone

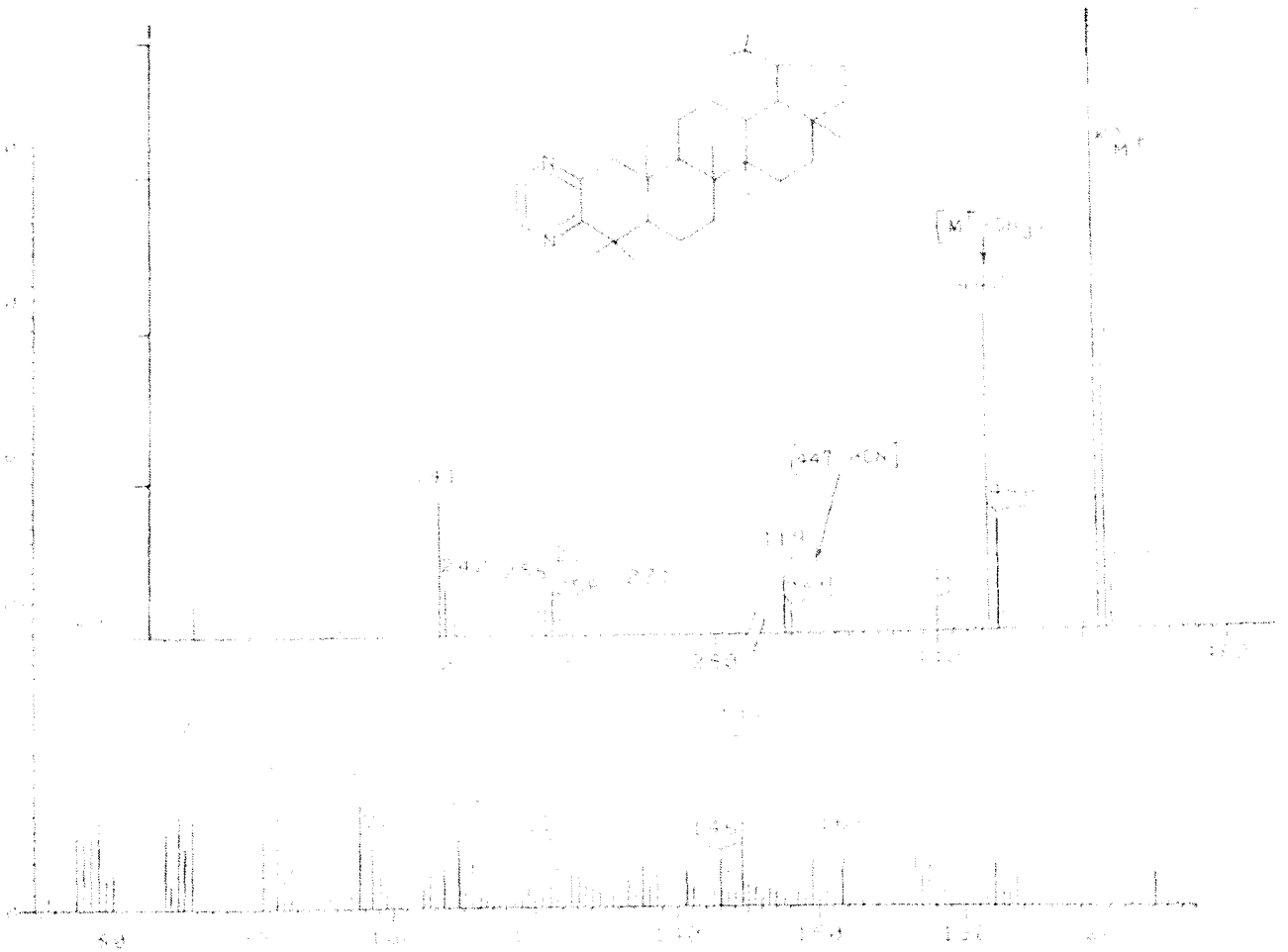
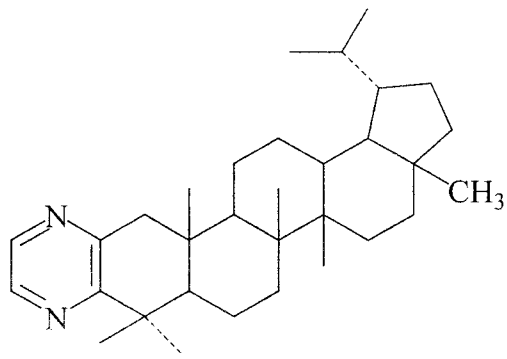


Fig. 4. Mass spectrum of the pyrazine derivative of lupanone



4b

Characterization of compound 5b: (Pyrazine derivative of methyldihydrobetulonate)

Pyrazine derivative **5b** of 28-carbomethoxy lupane was also prepared from 2, 3-diketo 28-carbomethoxy lupane. Crystallization of the compound from chloroform-methanol mixture furnished a compound E, analyzed for $C_{33}H_{50}O_2N_2$ m.p. 220^0. IR spectrum (fig. 6) of the compound showed peaks at 1710-20 (CO_2Me) and 1650-70, 1430, 1120 cm-1 for pyrazine ring. UV spectrum (fig. 5) of compound E showed peaks at 272nm (ε=5712) and 278 nm. It did not respond to the TNM test for active unsaturation. Mass spectrum (fig. 8) of compound E showed molecular ion peak at m/z 506 as base peak which is the characteristic feature of pyrazine compound the others peaks appeared at 491[M – CH_3]$^+$, 463 [M – $CH(CH_3)_2$]$^+$, 447[M – $COOCH_3$]$^+$, 432, 431, 258, 256, 241, 191, 187, 175, 159, 147, 133, 95, 55.

PMR spectrum (Fig. 7) of the compound E showed the presence of eight tertiary methyls resonated at 0.82, 0.985, 0.99, 1.28, 1.305, 0.76 and 88 (2d, 6H, $CH(CH_3)_2$, J = 7 Hz) ppm; two doublet at 2.48 and 3.04 ppm with germinal coupling of 16 Hz which has been assigned to C–1 methylene protons, besides the two doublet at 8.27 and 8.41 ppm with J = 3 Hz for the two aromatic protons and the ester methyl as a sharp singlet at 3.66 ppm. Thus from spectral analysis, the structure for the compound E has been assigned as Pyrazine derivative of methyldihydro betulonate and was found identical with the already reported compound [74].

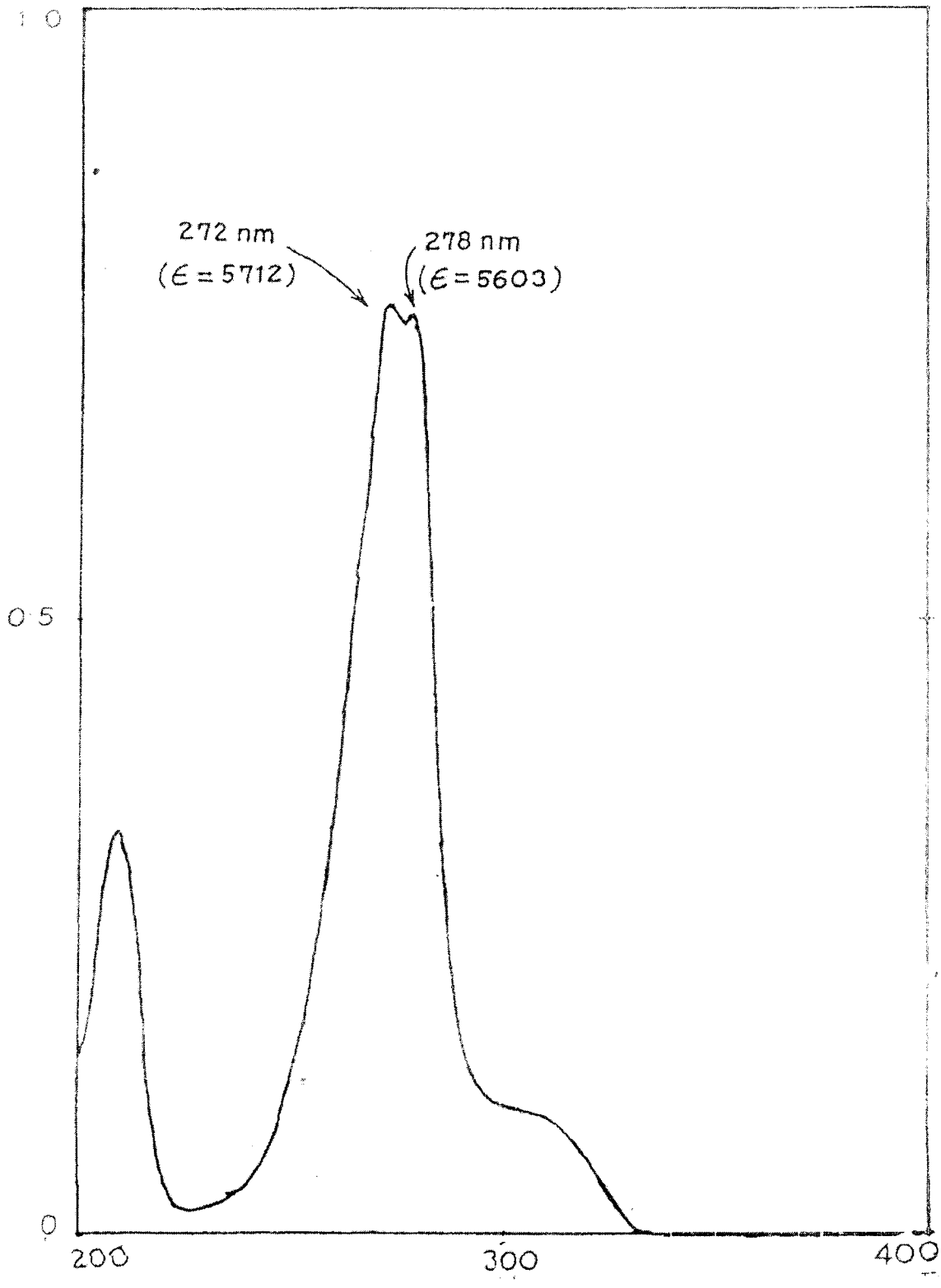


Fig. 5. UV spectrum of the pyrazine derivative of methyldihydrobetulonate

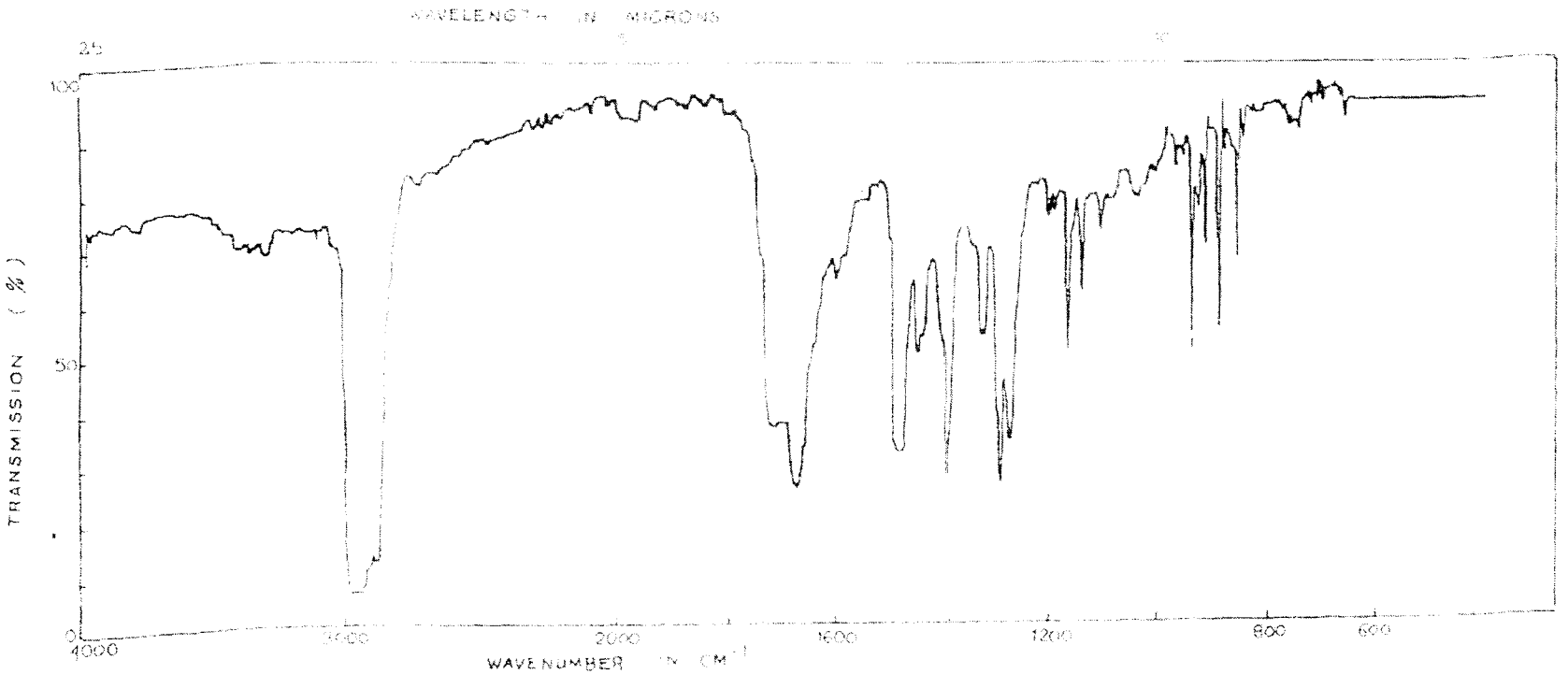


Fig. 6. IR spectrum of the pyrazine derivative of methyldihydrobetulonate

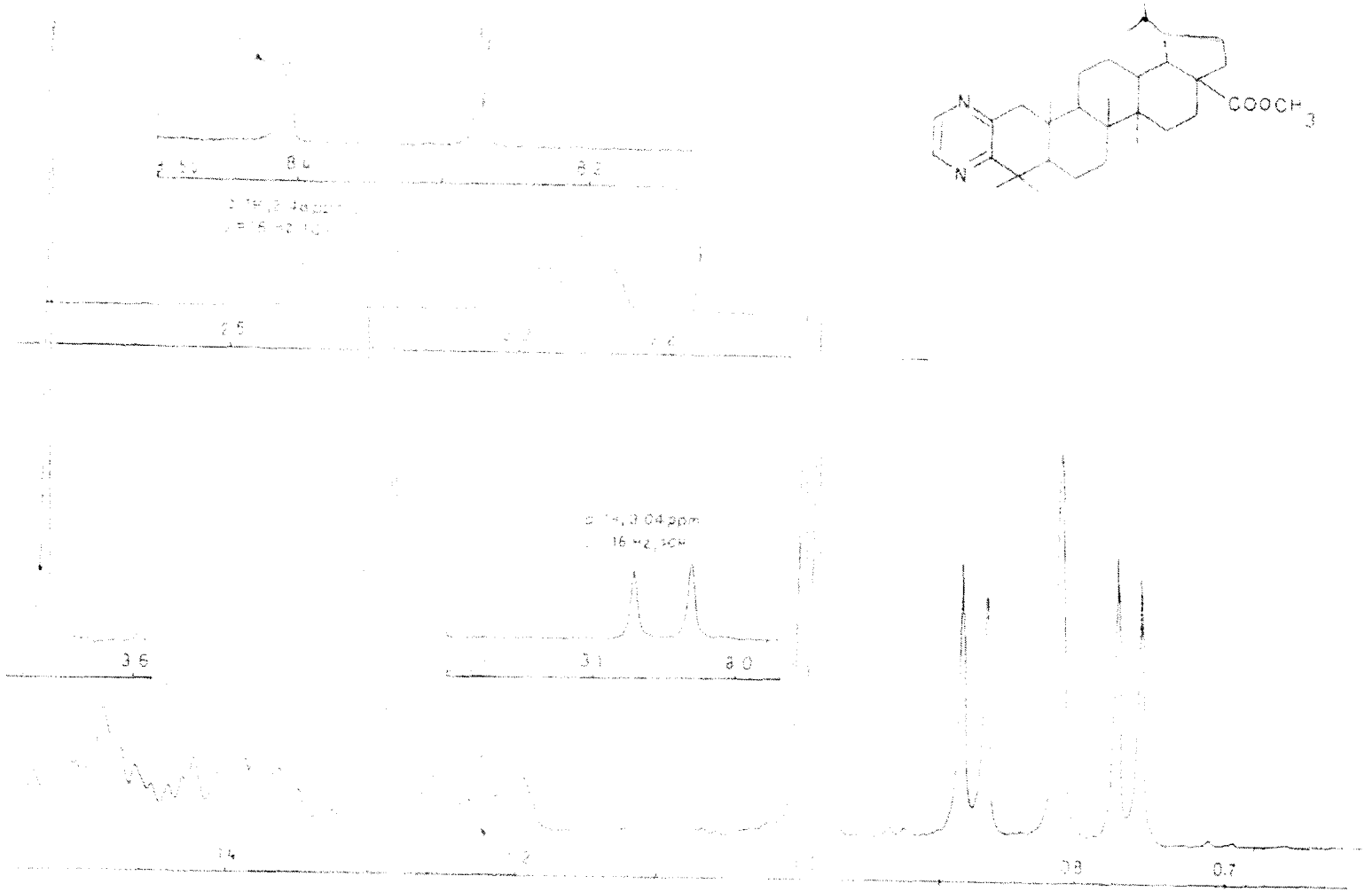


Fig.7. ^{1}H NMR spectrum of the pyrazine derivative of methyldihydrobetulonate

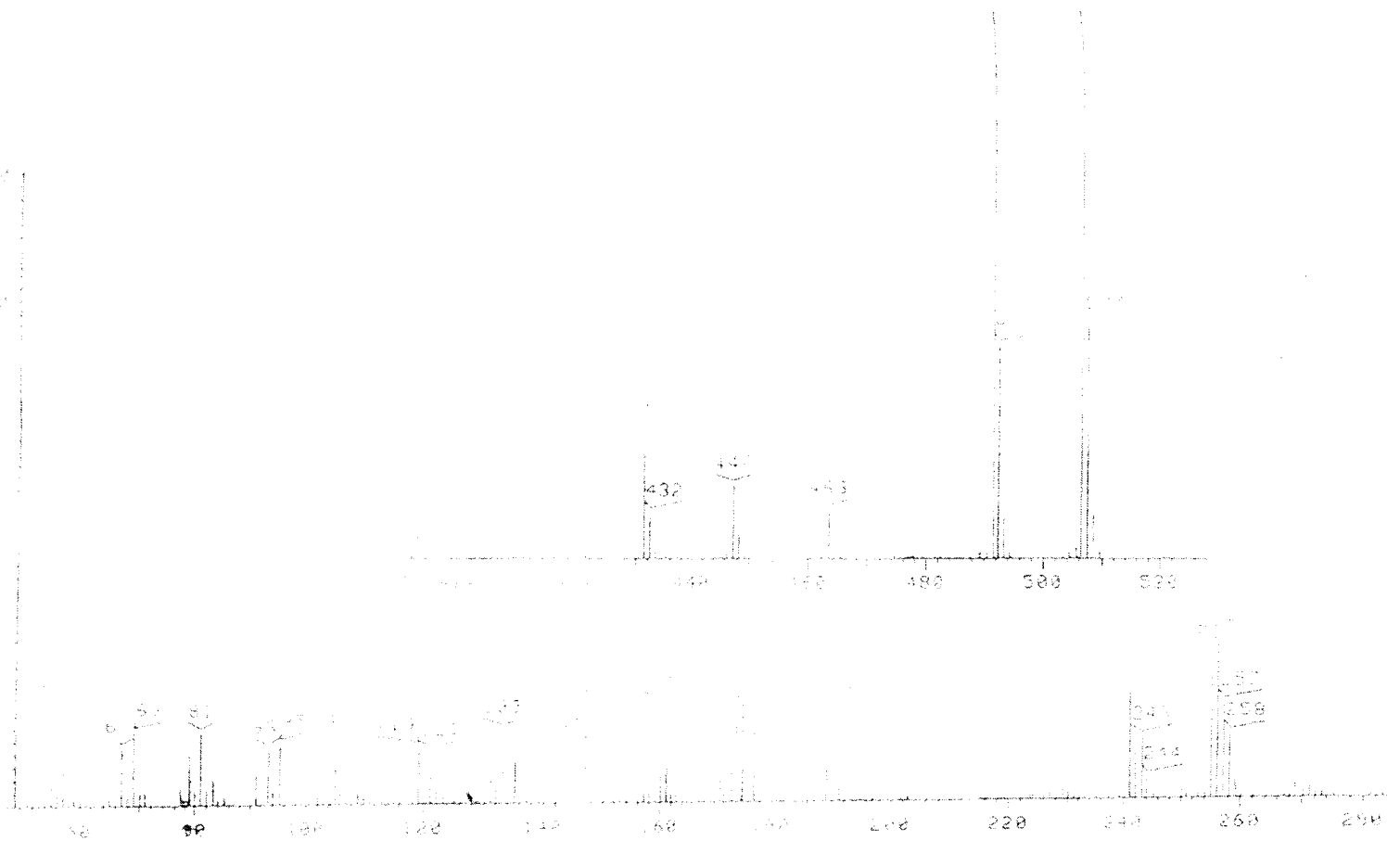
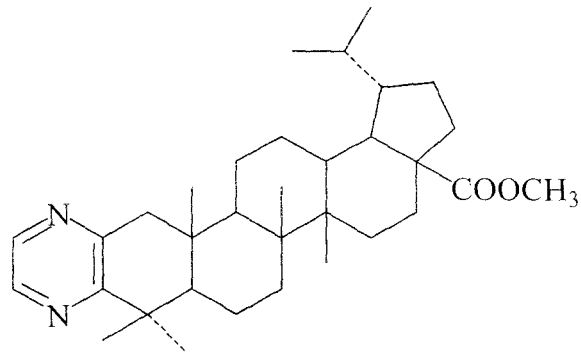
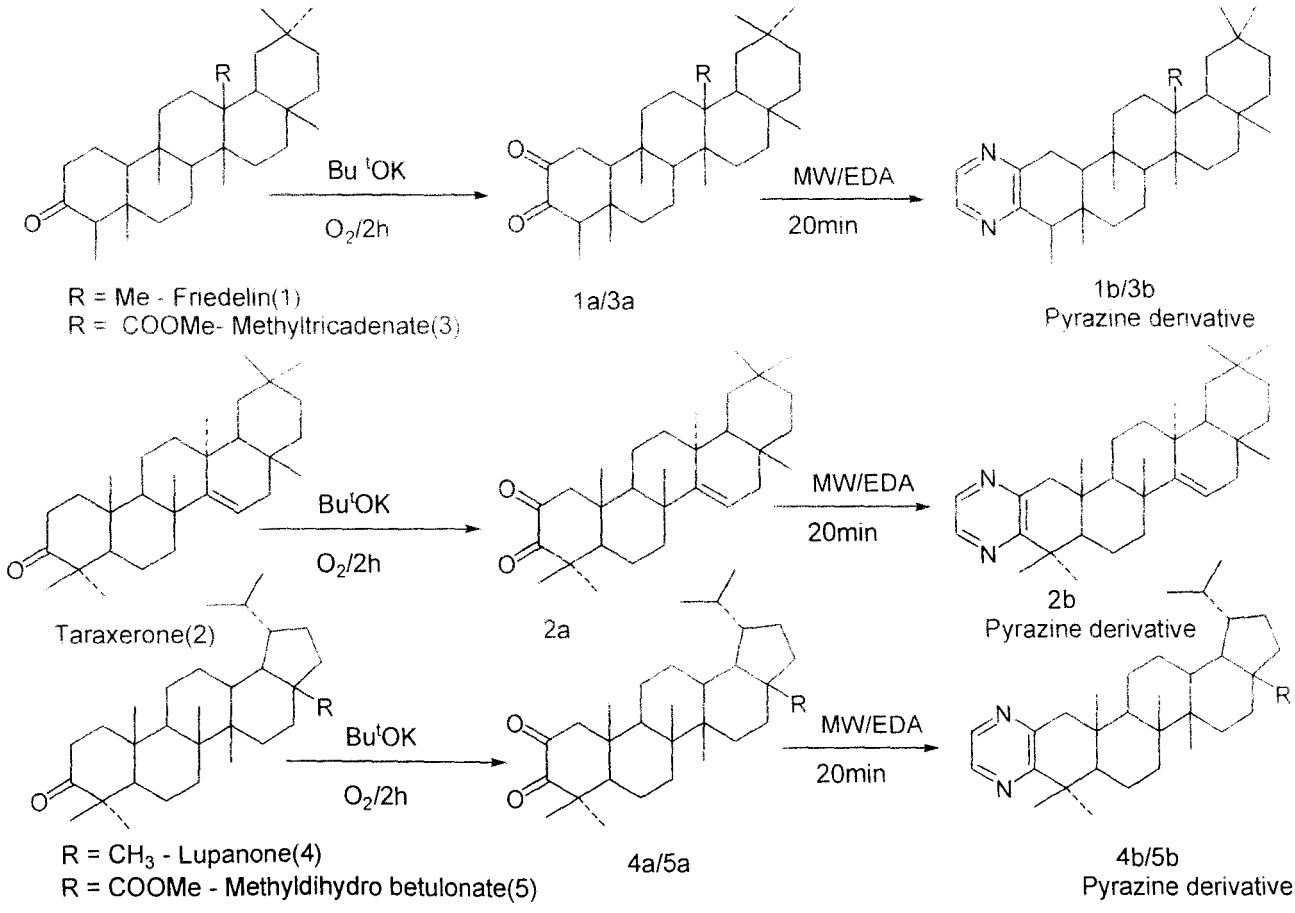


Fig.8. Mass spectrum of the pyrazine derivative of methyldihydrobetulonate


COOCH3
N
N

5b

Scheme 1


R
Bu tOK
O2/2h
MW/EDA
20min
R = Me - Friedelin(1)
R = COOMe- Methyltricadenate(3)
1a/3a
1b/3b
Pyrazine derivative
Taraxerone(2)
BuᵗOK
O2/2h
2a
MW/EDA
20min
2b
Pyrazine derivative
R = CH3 - Lupanone(4)
R = COOMe - Methyldihydro betulonate(5)
4a/5a
4b/5b
Pyrazine derivative

Biological activity

The parent compounds isolated from plants and their pyrazine derivatives were tested against the bacterial species *E. coli* (EC), *S. dysenteries* (SD) (gram negative) and *S. aureus* (SA), *B. subtilis* (BS) (gram positive). Similarly the antifungal activity against *Aspergilus niger* (AN), *Candida albicans* (CA) were also determined. Suitable strains of these organisms were procured from the microbiology laboratory of our institute. MICs (Minimum inhibitory concentration) of the compounds against bacterial and fungal pathogen are reported in table 1. 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 slide germination method was also used for determination of antifungal activity [77].

Table 1. ***In vitro*** **Antimicrobial screening results of the compounds.**

Microorganis-	MIC in g/mL of the compounds									
ms	1	1b	2	2b	3	3b	4	4b	5	5b
SA	25	<25	100	25	100	25	200	50	200	25
BS	50	25	100	25	50	50	100	25	200	25
EC	200	25	100	<25	200	25	100	<25	100	25
SD	200	50	200	<25	200	50	200	<25	100	50
AN	100	50	100	25	100	>100	100	<25	100	<25
CA	50	<50	200	25	50	50	50	25	100	<25

SA- *S. aureus*, BS- *B. subtilis*, EC- *E. coli*, SD- *S. dysenteriae*, AN- *Aspergillus niger*, and CA- *Candida albicans*, MIC- Minimum inhibitory concentration.

Discussion

All the compounds showed prominent antimicrobial activities against the tested fungal specimens (*Aspergillus niger*, *Candida albicans*) and bacterial pathogens (*E. coli*, *S. dysenteries* *S. aureus*, *B. subtilis*) as evident from table 1.

MIC record of the compounds (Table 1) clearly indicates that in comparison to the parent compounds, derivatives 2b and 5b were highly active against *S. dysenteries*, *B. subtilis* and *E. coli*. Compound 3b showed high activity against *S. aureus* and *E. coli* and moderate activity against *B. subtilis* except *S. dysenteries* 5b showed high activity against all the bacterial organisms studied in comparison to the respective parent compounds 1, 2, 3 and 4. In addition, all the derivatives showed high to moderate activity against the fungi *Aspergilus niger* and *Candida albicans*.

Srikrishna *et al.* [78] carried out antibacterial activity using cup plate method. They observed that pet. ether, chloroform, methanol and water extract of the bark of *Aporosa lindleyana* (Euphorbiaceae) showed moderate to very good activity against bacteria such as *Bacillus subtilis*, *Escherichia coli*. They studied antifungal activity such as *Candida albicans*, *Aspergilus niger* and compared with the standard drug fluconazole. The pet. ether extract showed considerable activity towards all the four fungal organisms.

Audu *et al.* [79] extracted components from *Annona senegalensis* (root), *Nauclea latifolia* (stem bark) and *Ziziphus abyssinica* (root bark) using methanol, diethyl ether and cold water as solvent. They studied their activity on *Candida albicans*, *Escherichia coli*, *Salmonella* spp. and *Staphylococcus aureus* at different concentrations and found that all these components inhibited the growth of microbes.

Ragasa *et al.* [80] extracted the air dried leaves of *Vitex negundo* which afforded vitexilactone and casticin by silica gel chromatography. They studied their activity and found that the compounds inhibited the growth of the fungi: *Candida albicans* and *Aspergillus niger* and the bacteria: *Staphyllococcus aureus* and *Pseudomonas aeruginasa*.

Kumar *et al.* [81] carried out antimicrobial properties of a series of 61 medicinal plants belonging to 33 different families used in various infectious disorders at 1000 and 500 microg/ml concentration by agar dilution method against *Bacillus cereus, Bacillus pumilus, Bacillus subtilis, Bordetella bronchiseptica, Micrococcus luteus, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa , Streptococcus faecali, Candida albicans, Aspergillus niger and Saccharomyces cerevisiae.* They found that 28 plant extracts showed activity against at least one of the test organisms used.

Mbwambo *et al.* [82] were extracted compounds from stem bark, wood and whole roots of *Ternimalia brownii* using solvents of increasing polarity, namely, pet ether, dichloromethane, dichloromethane: methanol (1:1), methanol and aqua, respectively and the extracts were tested for antifungal and antibacterial activity. They observed that the extracts of the stem bark, wood and whole roots of *T. brownii* exhibited antibacterial activity against standard strains of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella typhi* and *Bacillus anthracis* and the fungi *Candida albicans* and *Cryptococcus neoformans*. They found that aqueous extracts exhibited the strongest activity against both bacteria and fungi.

Chapter 3

SYNTHESIS AND BIOLOGICAL ACTIVITY OF (2, 3-C)1′,2′,5′ OXADIAZOLE DERIVATIVE OF PENTACYCLIC TRITERPENOIDS

Introduction

Fusion of heterocyclic ring system at position 2 and 3 of the steroidal nucleus has been found to afford chemically useful compounds [83-85]. Encouraged by these findings and with a view that the fusion of heterocyclic system to ring A of triterpenes may give interesting biologically active compounds, the author selected lupanone (1b) as the starting material to introduce oxadiazole moiety to ring-A of the triterpenoid. The structure of the compound was established on the basis of spectroscopic (UV, IR, NMR) analysis. The same protocol was repeated on 28-carbomethoxy lupan-3-one to obtain the similar oxadiazole derivatives which was identified as 28-carbomethoxy lupan (2,3-C)1′,2′,5′-oxadiazole by spectroscopic method (UV, IR, NMR).

The derivatives obtained have been selected for their biological, toxicological, fungicidal and phytotoxic properties.

Results and discussion

Section A

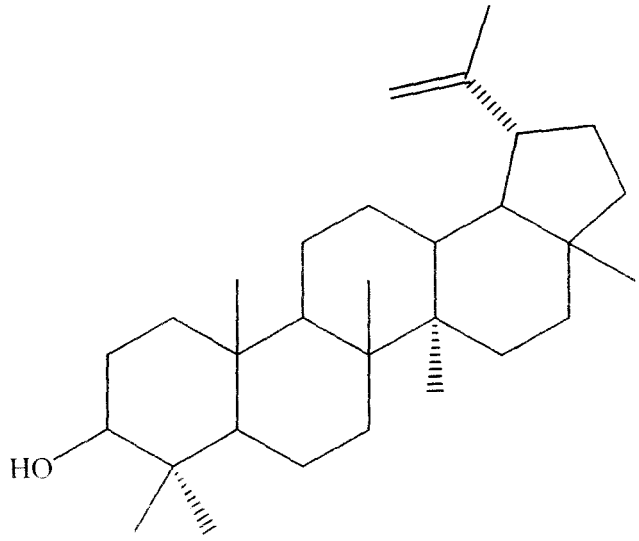
Isolation of the Compounds from the barks of *Xanthoxylum budrunga*

The powdered plant materials (barks of *Xanthoxylum budrunga*) were extracted with benzene using soxhlet apparatus for 72 hours (for details see experimental). The solvents were then removed under reduced pressure and a sticky brown residue was obtained. This residue was then purified by column chromatography [2] using silica gel (60-120) mesh and the chromatogram on elution with pet. ether: benzene (4:1) furnished a single compound.

Characterization of the isolated compound as lupeol (1)

Different fractions of compound 1 were mixed and crystallised by chloroform and methanol mixture which gave white crystals, m.p. 210– 212°C; $[\alpha]_D$ = +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 and was identified as lupeol [88] from other spectral data (NMR, Mass) and by comparison with authentic sample of lupeol (1).

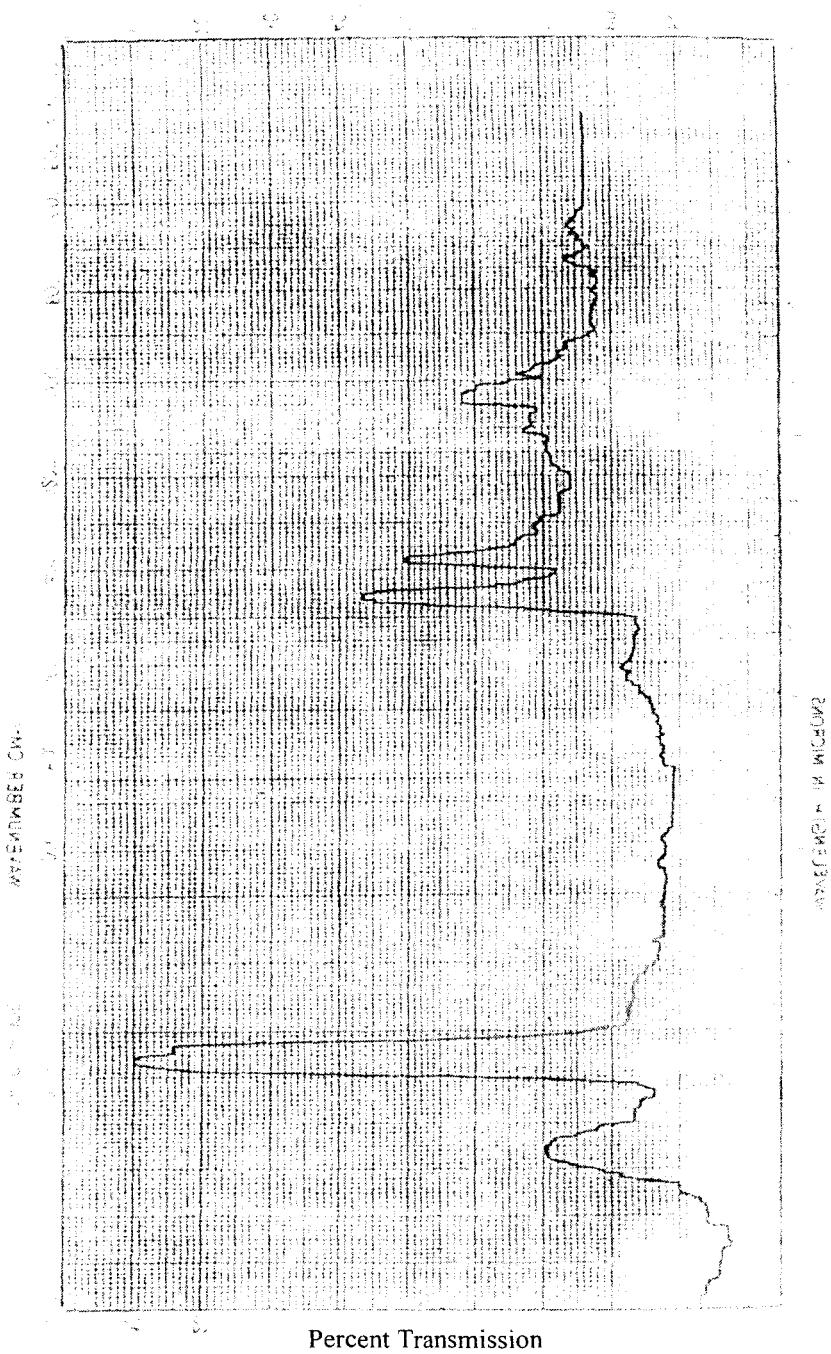


1

Hydrogenation of lupeol: Preparation of lupanol (1a)

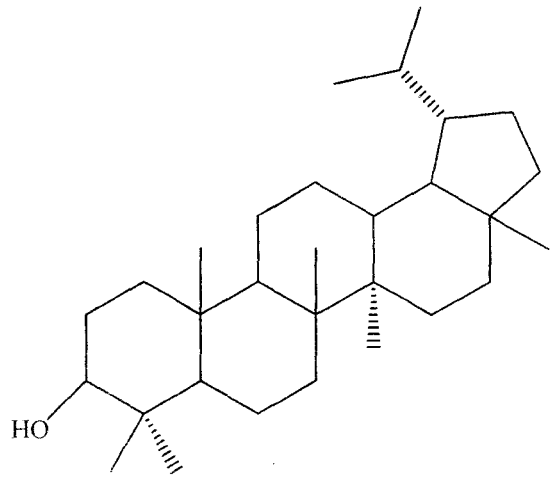
Lupeol dissolved in a mixture of ethyl acetate and acetic acid (100 ml each) was shaken in an atmospheric hydrogen in presence of PtO_2 catalyst (for details see experimental). The solid obtained (1a) after crystallization by using a mixture of chloroform and methanol had m.p. 204°C. [α] −15°. IR spectrum (Fig. 9) of 1a showed peak at 3330 $cm^{-1}$for hydroxy functional group. This compound 1a was found to be identical with an authentic sample of lupanol (m.m.p, CO TLC, CO IR) [Lit91 m.p. 206°C, [α] −17.8°]

Percent Transmission



Percent Transmission

Fig. 9. spectrum of lupanol



1a

Jone's oxidation of lupanol: Preparation of lupanone (1b)

To a solution of lupanol in pure acetone Jone's reagent was added until complete the reaction. The resulted mixture dissolved in benzene and chromatographed over a column of silica gel developed with petroleum ether. The chromatogram on elution with pet. ether: benzene (70:30) furnished different fractions of a single compound. Different fractions were mixed and crystallised by using chloroform and methanol mixture. The crystallization furnished colourless solid 1b, m.p. 208-9°C, $[\alpha]_D$ +15°, IR 1710 cm^{-1} for carbonyl group (Fig. 10). It did not respond to the Beilstein test for halogen and to the TNM test for unsaturation. The compound was found to be identical (m.m.p., CO IR, CO-TLC) with an authentic sample of lupanone [Lit89 m.p. 210-11°C, $[\alpha]_D$ +16.2°].

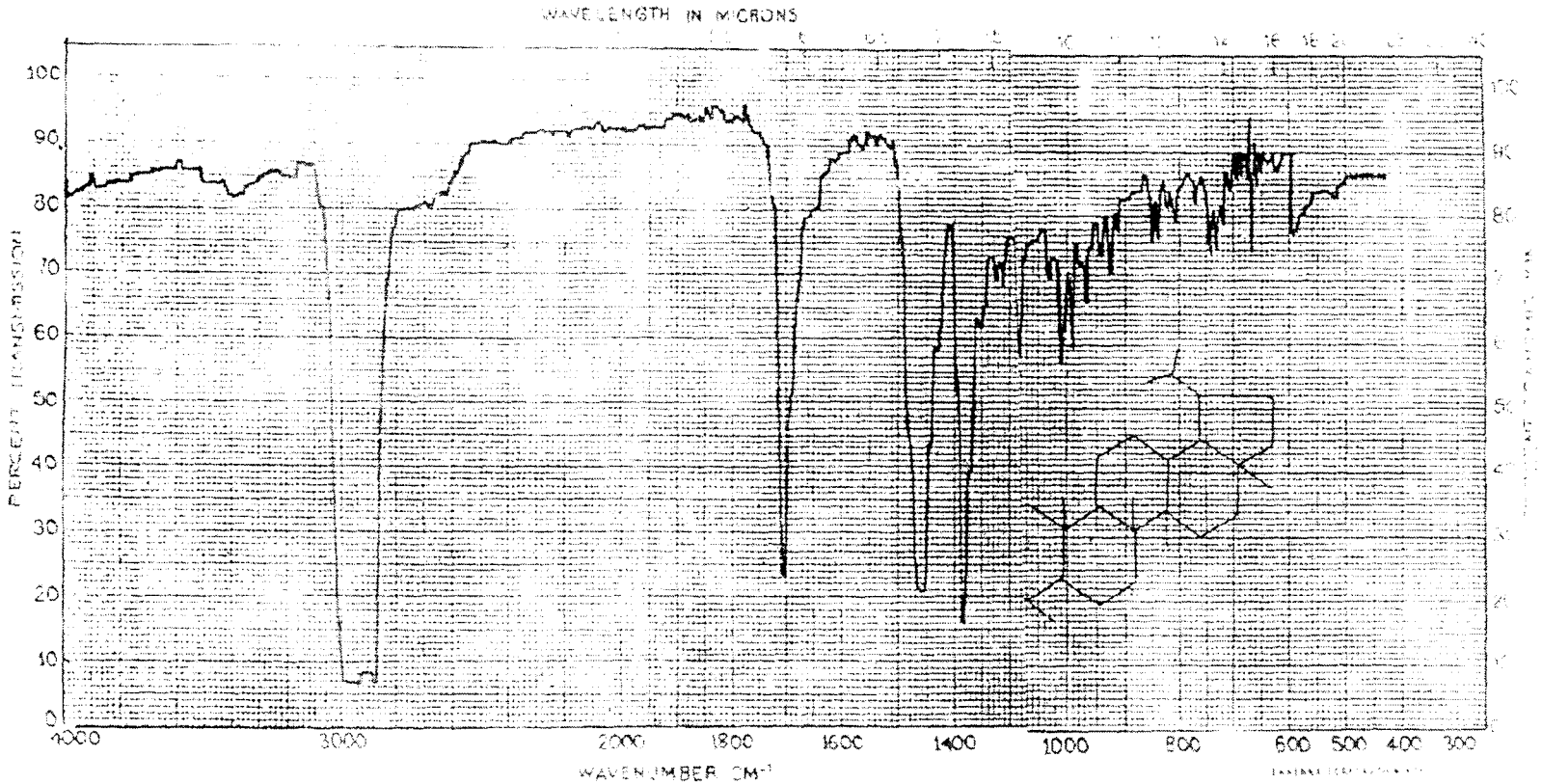
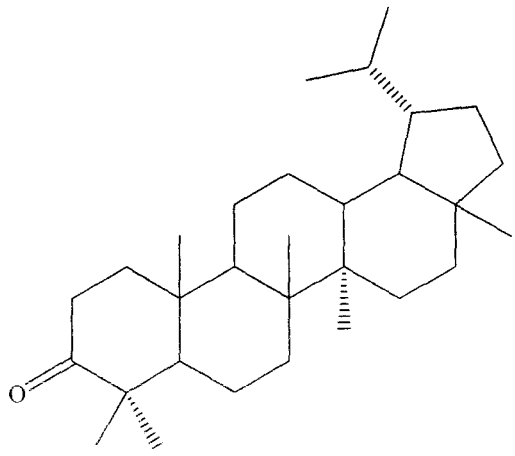


Fig. 10. IR spectrum of lupanone



1b

Treatment of lupanone with N-bromosuccinimide: Formation of 2, 2-dibromolupanone (2) and 2α–bromolupanone (3).

A solution of lupanone was mixed with dimethylsulphoxide. N-bromo succinimide was added (for details please see experimental). The resulted mixture was chromatographed over a column of silica gel. The chromatogram on elution with petroleum ether only furnished a single compound 2 and further elution with pet. ether: benzene (80: 20) furnished another single compound 3.

Examination of petroleum ether fractions: Isolation and characterization of 2, 2-dibromolupanone (2).

The different fractions of compound 2 showed homogeneity on TLC plate, hence these were mixed and crystallised from a mixture of chloroform and methanol to afford a needle shaped crystals, m.p. 209—210°C gave an intense green flame in Beilstein test showing the presence of bromine in compound 2. It gave no coloration with TNM. Elemental analysis indicated the moleculer formula to be $C_{30}H_{48}OBr_2$ which was supported by the existence of three ion peaks (Fig. 15) at 586, 584 and 582 in the ratio of 1:2:1, proving the presence of two Br atoms in compound 2. The other ion peaks appeared at m/z 567, 569, 571 $[M\text{-}CH_3]^+$, 539,541, 543$[M\text{-}CH(CH_3)_2]^+$, (1:2:1), 504, 506 $[M\text{-}HBr]^+$, 489, 491$[M\text{-}HBr\text{-}CH_3]^+$ (1:1), 461, 463$[M\text{-}HBr\text{-}CO]^+$(1:1), 424, 425, 426 $[M\text{-}2Br]^+$(1:1:1), 409,

283, 285, 274, 231, 206, 205, 191, 171, 163, 123. Its IR spectrum showed (Fig. 12) showed a peak at 1722 cm^{-1} for the carbonyl stretching vibration which is 17 cm^{-1} higher frequency than the parent ketone due to the electronic repulsion of equatorial bromine atom. Its UV spectrum showed a hump at 312 nm and the CD curve (Fig. 11) showed a very prominent negative cotton effect curve with its trough at 320 nm (= −8977.85), the other peak 239 nm gave a positive sign (€= +4590.18).This negative CD value is evidently due to the axial bromine atom on C-2, the chromophor carbonyl group being at C-3, thus from IR and CD spectrum the existence of the two bromine atoms at the same carbon at C-2 is obvious. This is further substantiated by ^{1}H NMR spectrum (Fig. 13) of compound 2, that showed two doublets at 3.64 and 3.13 ppm with germinal coupling of 16 Hz which could be assigned to the methylene protons at C-1 that have no protons in the neighboring α-carbons, the other signals due to methyl groups appeared at 0.77 (2s, 6H, 2Me), 0.78+ 0.860 (2d, 6H, J=7 Hz, -$CHMe_2$), 0.942, 0.9675, 1.098, 1.240 (4s, 15H, 5 Me) ppm. ^{13}C NMR of 2 (Fig.14) showed 30 peaks of which eight were quartets, nine-triplets, six doublets and seven singlets, the peak at 204.38 ppm is due to the carbonyl group that is deshielded by the two alpha bromine atoms causing an upfield shift of 13.5 ppm. Thus from spectral analysis the structure of the compound 2 has been established as 2,2-dibromolupanone

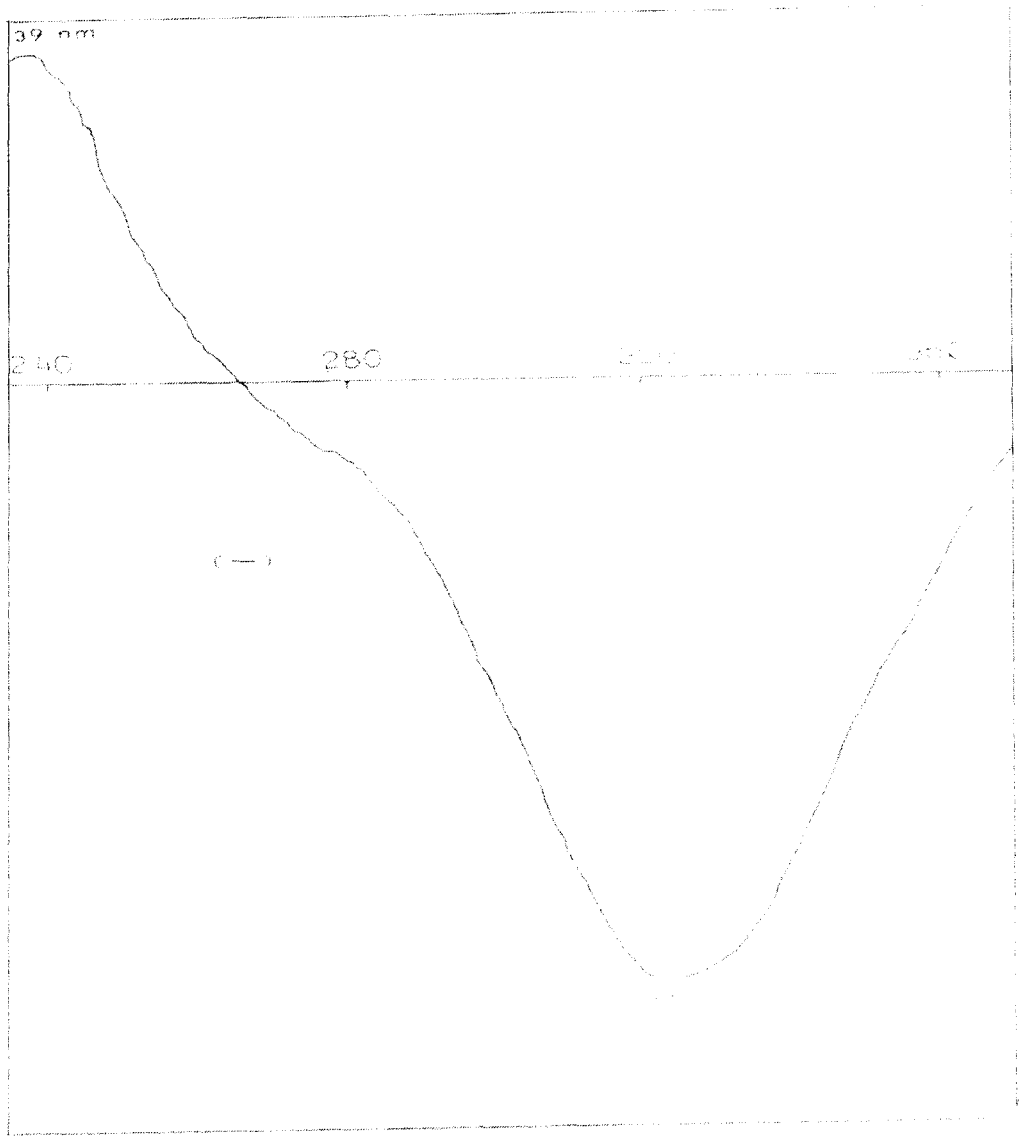


Fig. 11. CD spectrum of 2,2-dibromolupanone

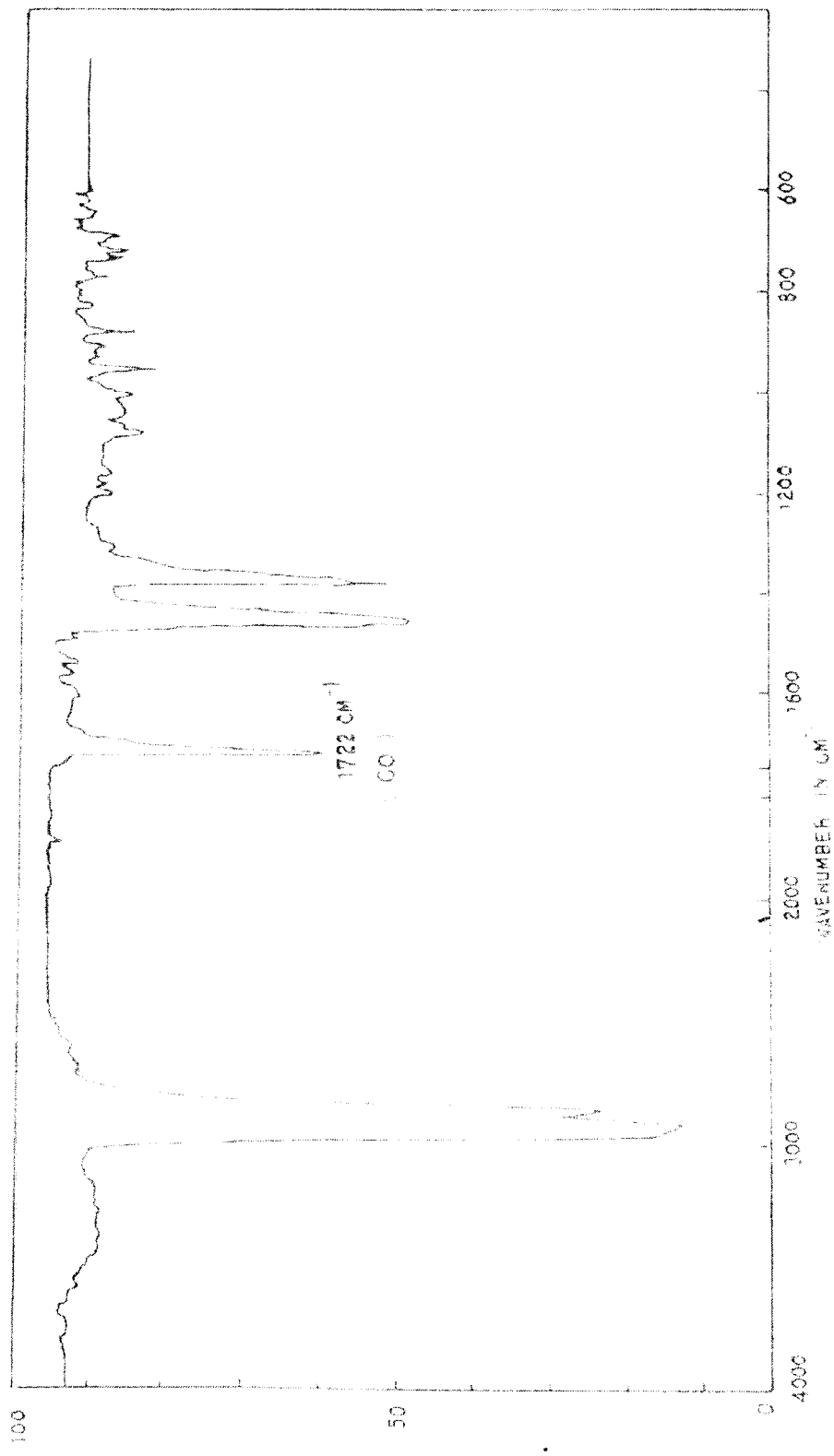


Fig. 12. IR spectrum of 2,2-dibromolupanone

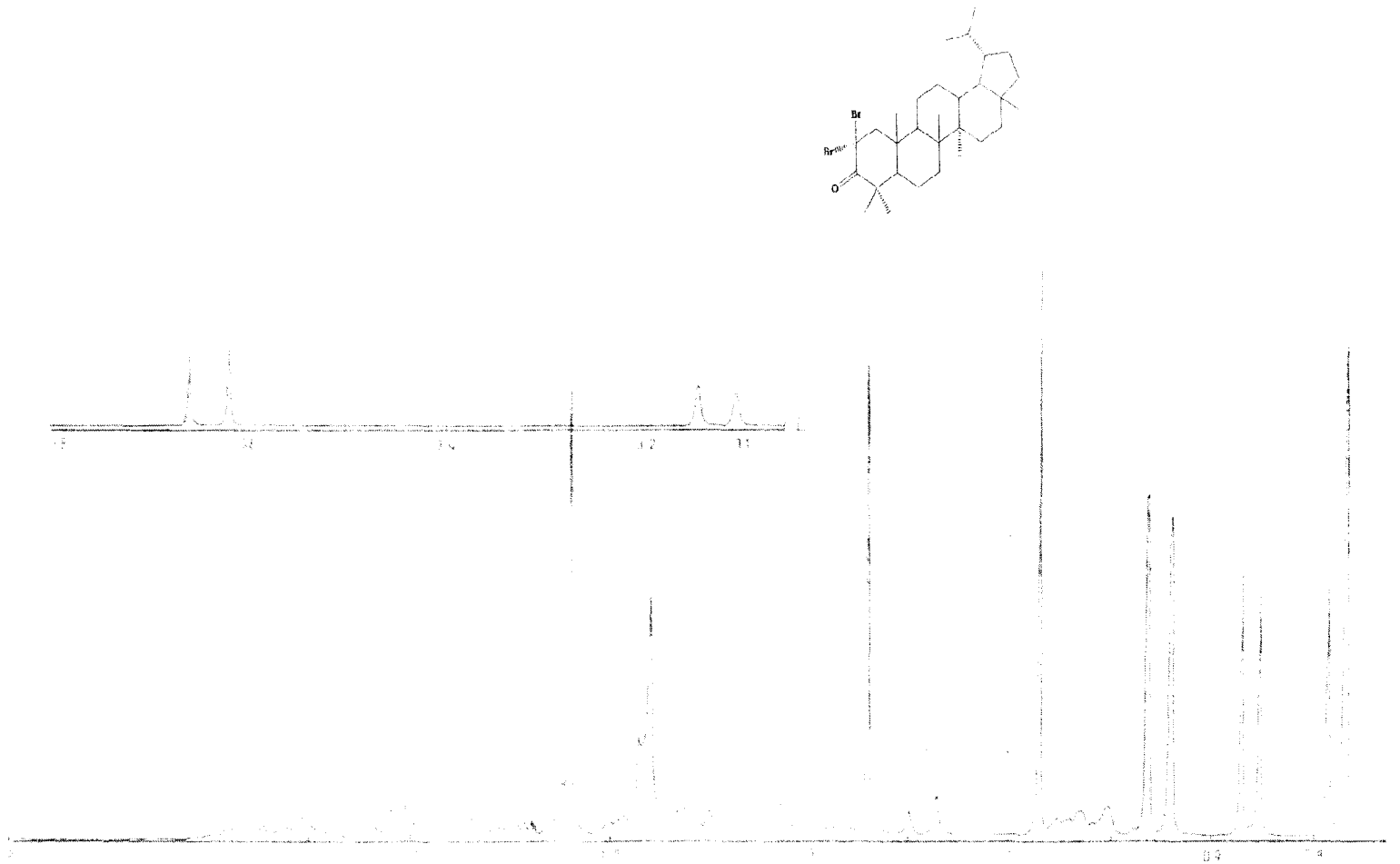


Fig. 13. ^{1}H NMR spectrum of 2,2-dibromolupanone

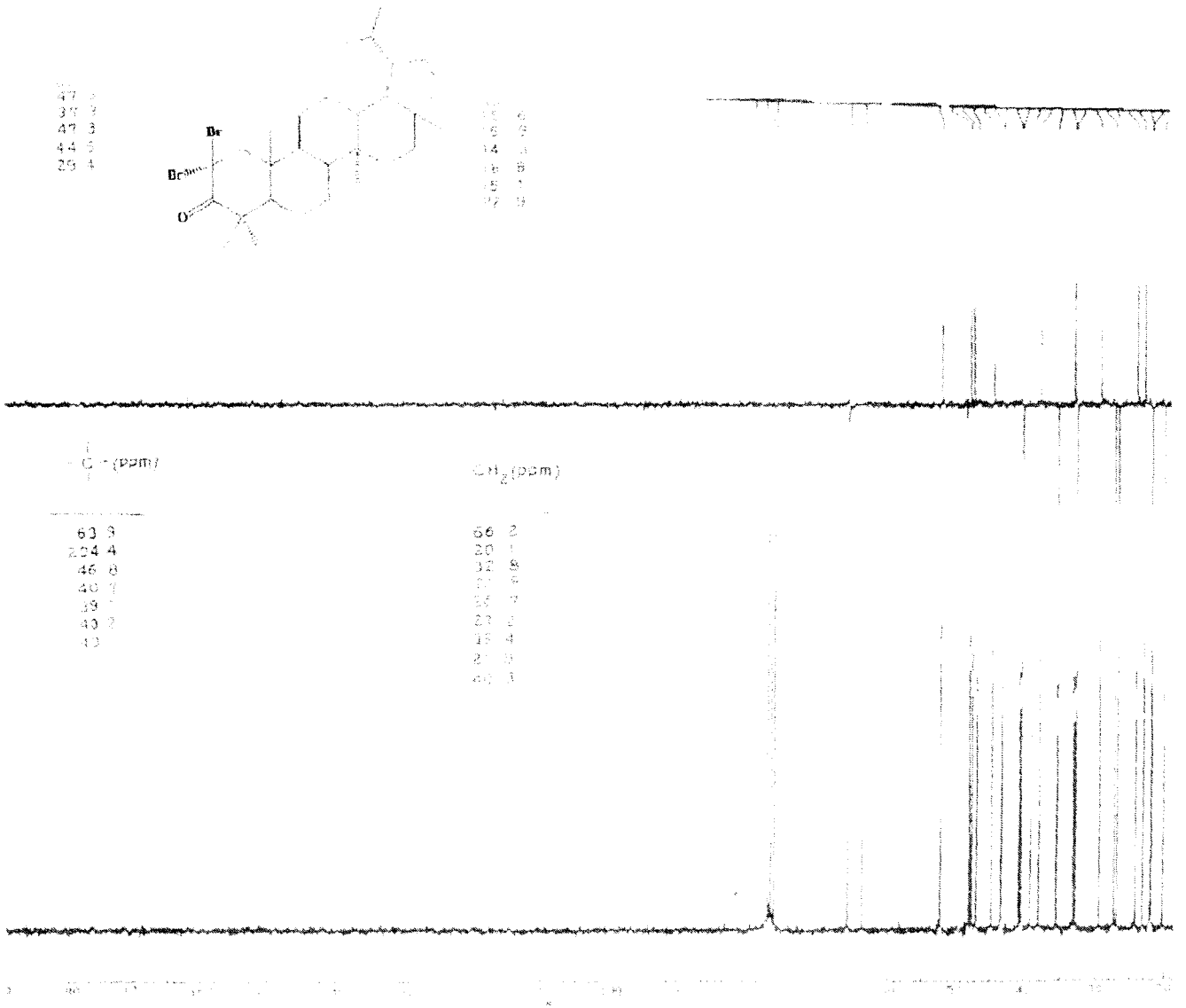


Fig. 14. ^{13}C NMR spectrum of 2,2-dibromolupanone

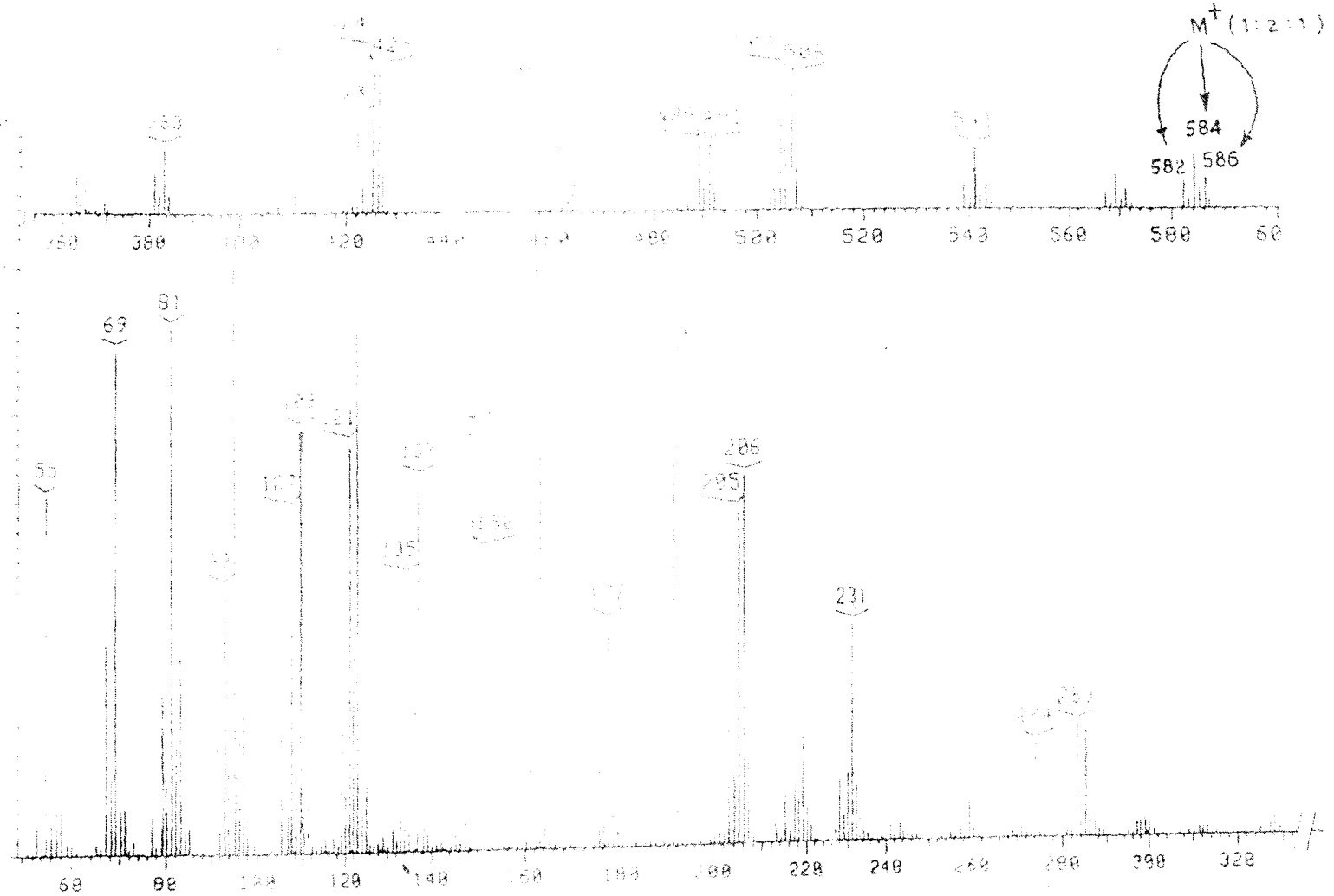
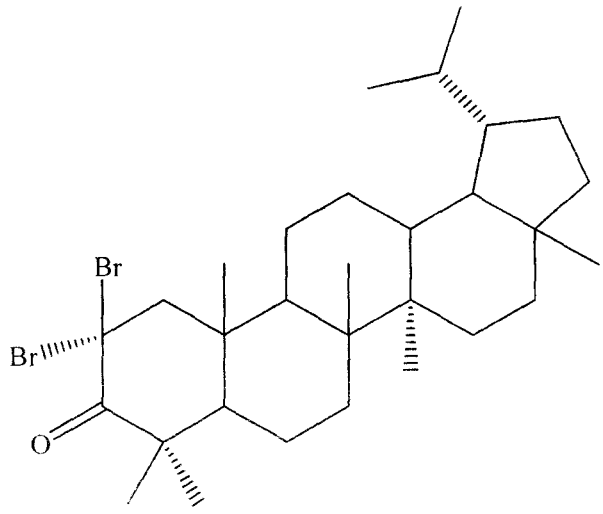


Fig. 15. Mass spectrum of 2,2-dibromolupanone



2

Examination of pet. ether and benzene fractions: Isolation and characterization of 2α–bromolupanone (3)

The different fractions of compound 3 showed homogeneity on TLC plate, hence these were mixed and crystallised from chloroform and methanol mixture to afford amorphous white solid with constant m. p. at 224-25°C, Beilstein test showed a persistant green flame indicating the introduction of bromine in lupanone. Elemental analysis showed the molecular formula to be $C_{30}H_{49}OBr$ which was corroborated by mass spectral analysis that showed the molecular ions (Fig. 18) at m/z 506 and 504 of almost equal heights due to the isotopic mass difference of bromine atom of 79 and 81.The other peaks of prominence appeared at m/z 491, 489, $[M\text{-}CH_3]^+$ (1:1), 463, 461 $[M\text{-}CH(CH_3)2]^+$ (1:1), 426,425 [M-HBr] $^+$, 285, 283, 274, 206, 191, 163, 149, 123 (100%). The IR spectrum (Fig. 16) of compound of 3 showed a peak at 1723 cm^{-1} which is 15cm^{-1} higher frequency than the parent ketone showing that the bromine introduced at C-2 position is lying in the same plane as carbonyl group thereby pointing the stereochemistry of bromine as 2α-equatorial. Finally, the ^1H NMR spectrum (Fig. 17) settled the stereochemistry of 3 which showed a doublet at 5.06 with axial–axial and axial-equatorial coupling of 12 and 6 Hz respectively. The C-1 equatorial proton appeared at 2.6475 ppm as doublet of a doublet with germinal coupling of 12 Hz and vicinal coupling of 6 Hz , the C-1 axial proton appeared at 2.4675 ppm has a triplet with J = 12 Hz , the other methyl peaks resonated at 0.760 and 0.850 (2d, 6H, $(CH_3)_2$ –CH-, J= 7 Hz) 0.770 (s, 6H , 2 Me) , 0.920 , 1.100, 1.135, 1.20 (4s, 12H,

4Me) ppm. Its ^{13}C NMR spectrum showed the presence of eight quartets, nine triplets. seven doublets and six singlets ,one of which that appeared at 207.01 ppm, about 10.8 ppm upfield than the parent ketones 4a , is due to the electronic repulsion of equatorial bromine atom. Thus the compound 3 has been fully charcterised by spectral analysis as 2α-bromolupanone.

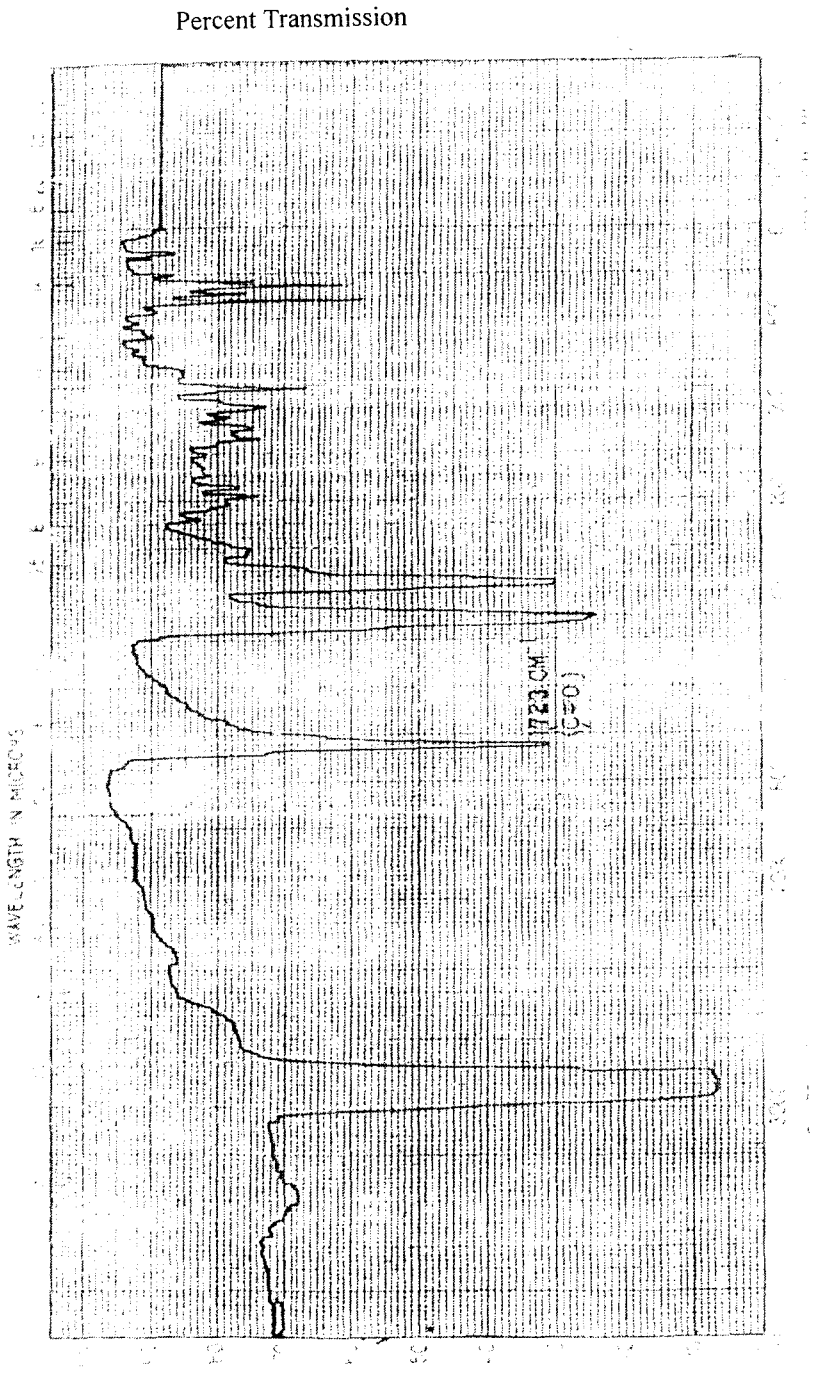


Fig. 16. IR spectrum of 2α-bromolupanone

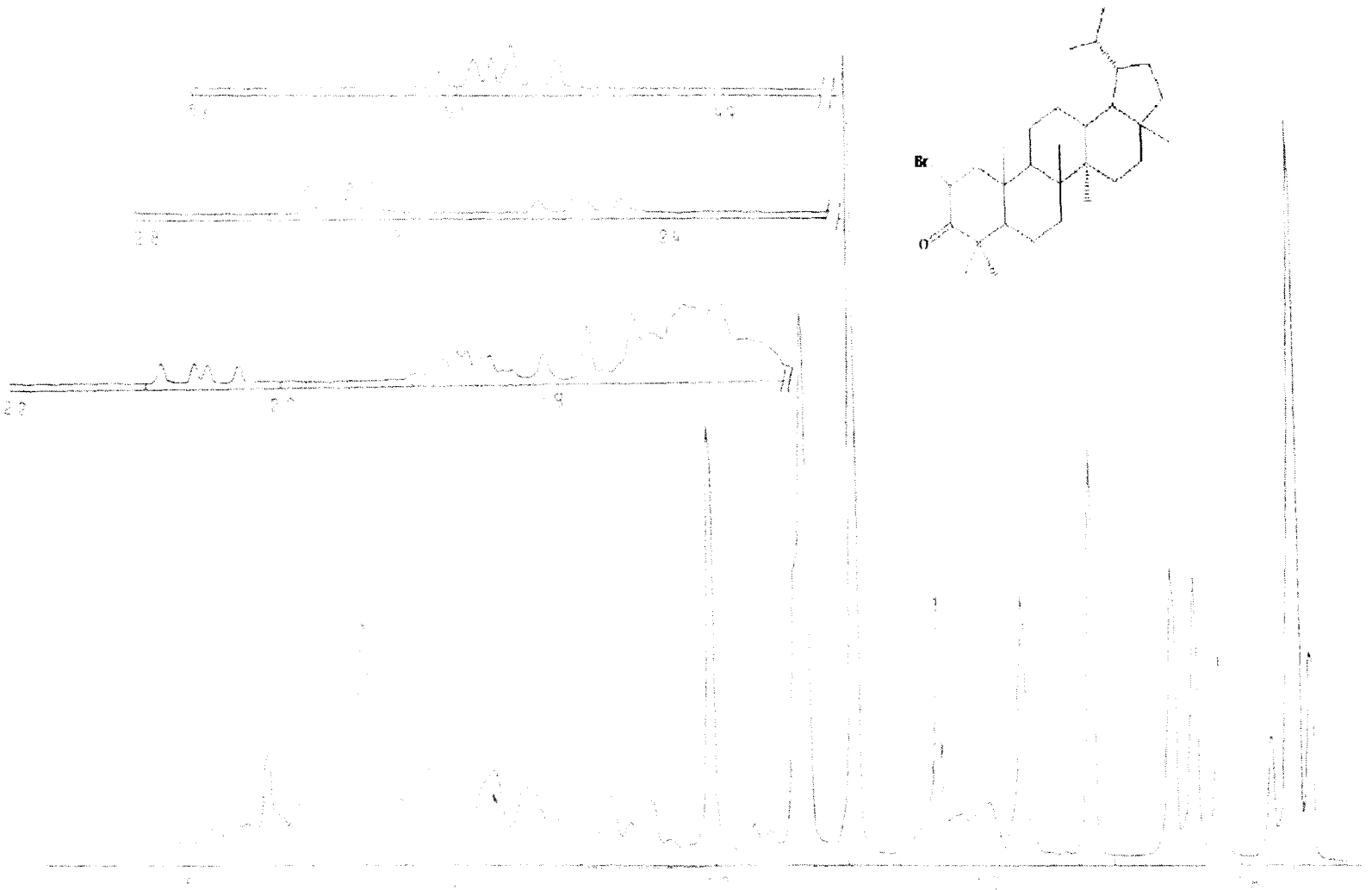


Fig. 17.^{1}H NMR spectrum of 2α-bromolupanone

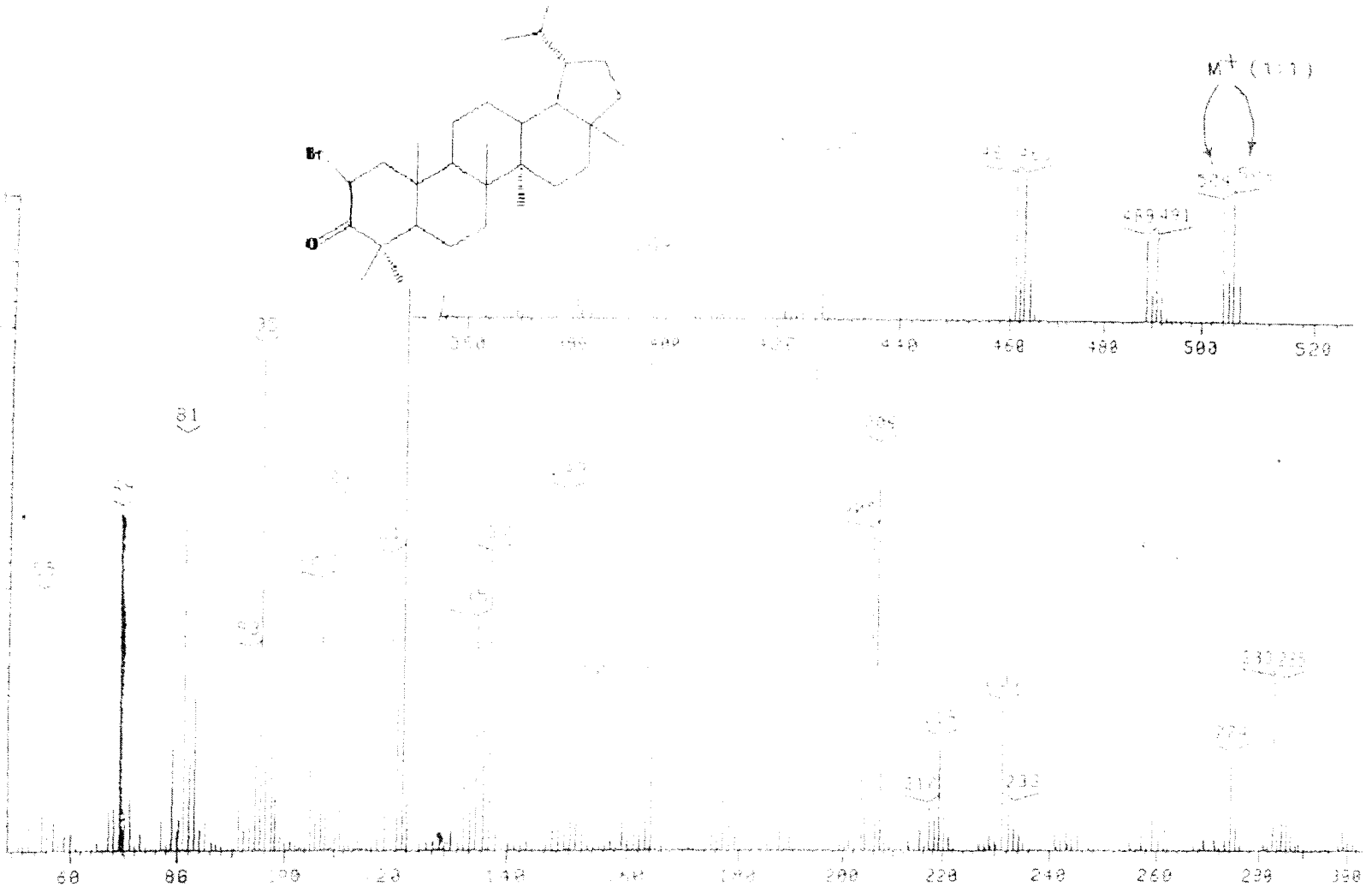
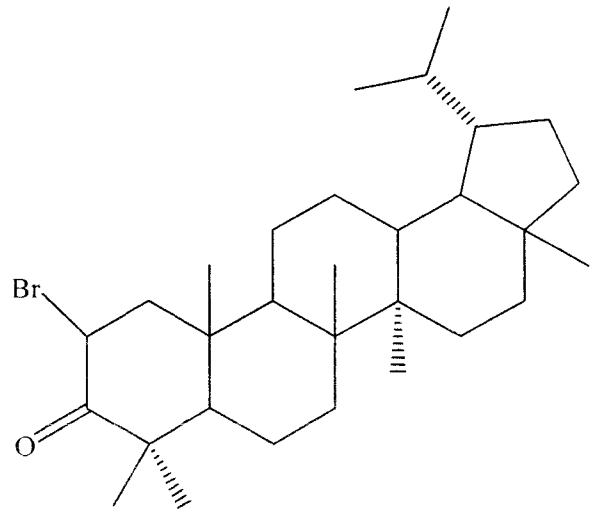


Fig.18. Mass spectrum of 2α-bromolupanone



3

Treatment of 2, 2-dibromolupanone (2) with hydroxylamine hydrochloride: Preparation of 2, 3-dioximino lupane (4) and the subsequent cyclization of the dioximino derivative to lupan[2,3-C]-1′,2′,5′-oxadiazole (5) under MW irradiation

2, 2-dibromo lupanone (**2**) dissolved in pyridine was refluxed with hydroxyl amine hydrochloride in ethanol (see experimental). The compound obtained from the reaction was purified by repeated crystallization from chloroform-methanol mixture to obtain a white amorphous powder of compound A, analyzed for $C_{30}H_{50}O_2N_2$, m.p. 193°C, $[\alpha]_D$ +21.6°. IR spectrum showed (Fig. 19) peaks at 3200-3400 cm^{-1} (C=N). It exhibited UV absorption maximum at 220 nm (€=5100) (Fig.21). Mass spectrum of the compound showed moleculer ion peak (Fig. 20) at m/z 469$[M]^+$ the other peaks at prominence appearance at m/z 441, 439, 425, 424(base peak), 422, 380, 341, 340, 299, 231, 191, 163, 149, 136, 122, 121, 95, 81, 69. PMR spectrum of A could not be taken owing to solubility problem. Thus on the basis of the above spectral data compound A was identified as 2, 3-dioximino lupane 4.

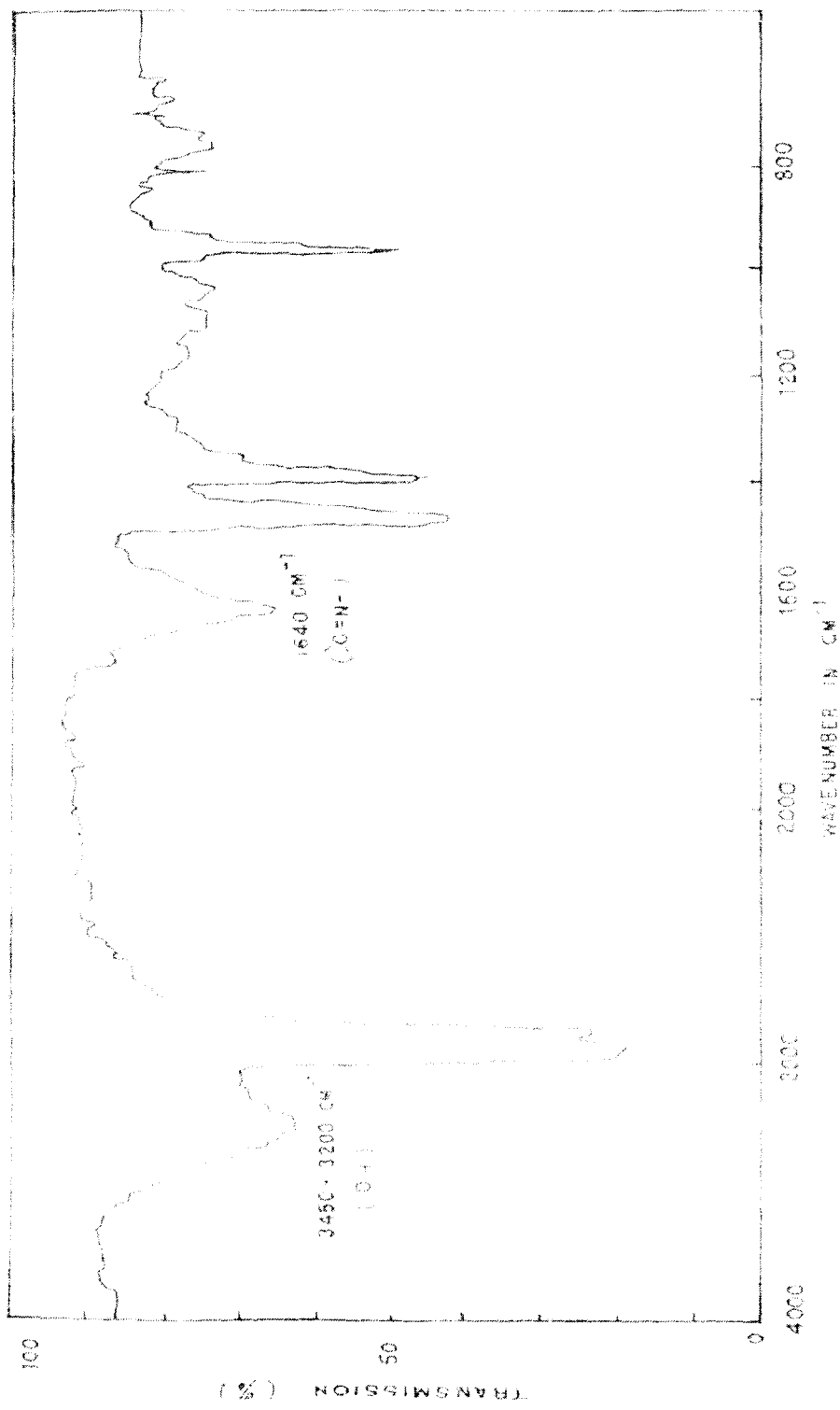


Fig. 19. IR spectrum of 2, 3-dioximinolupane

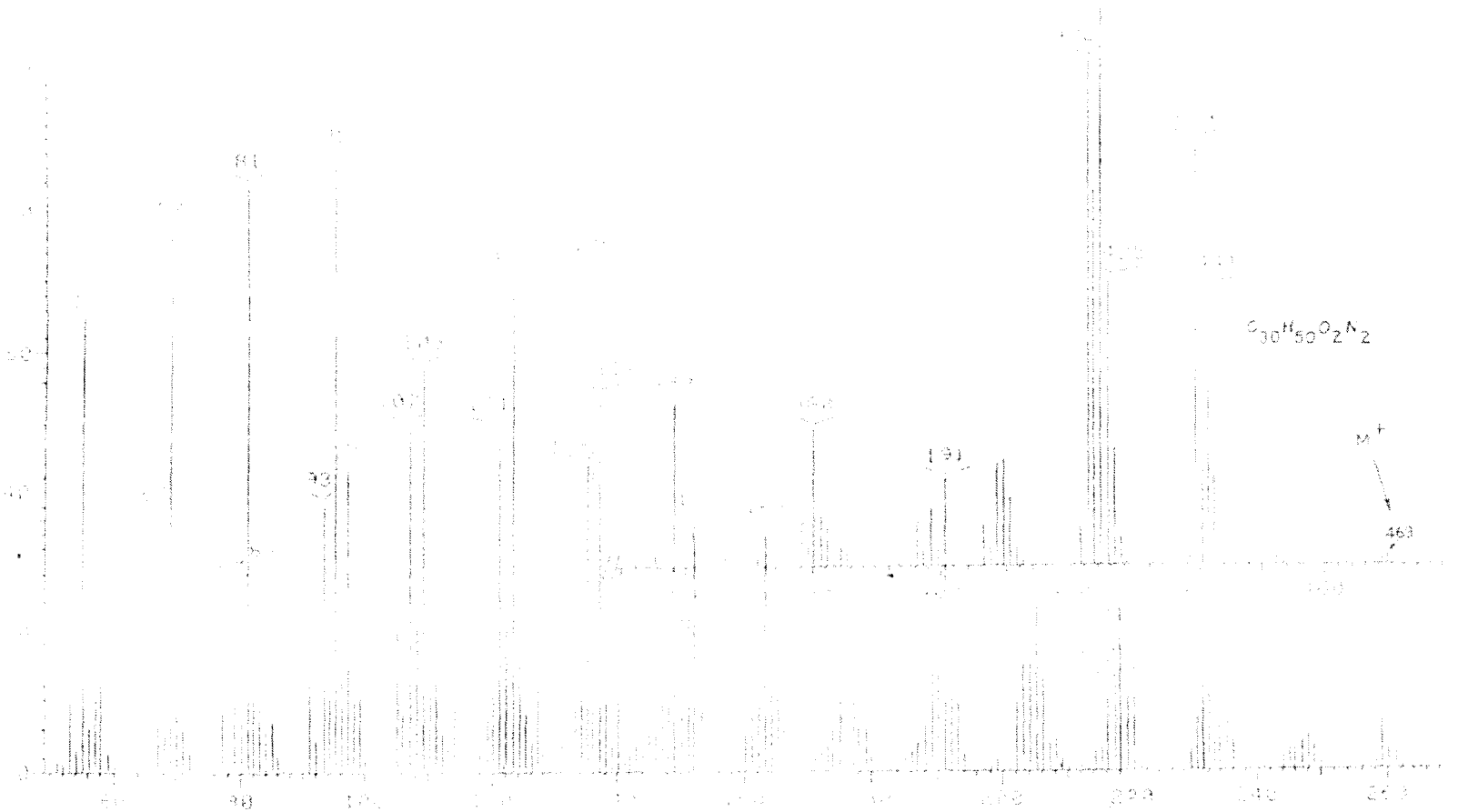
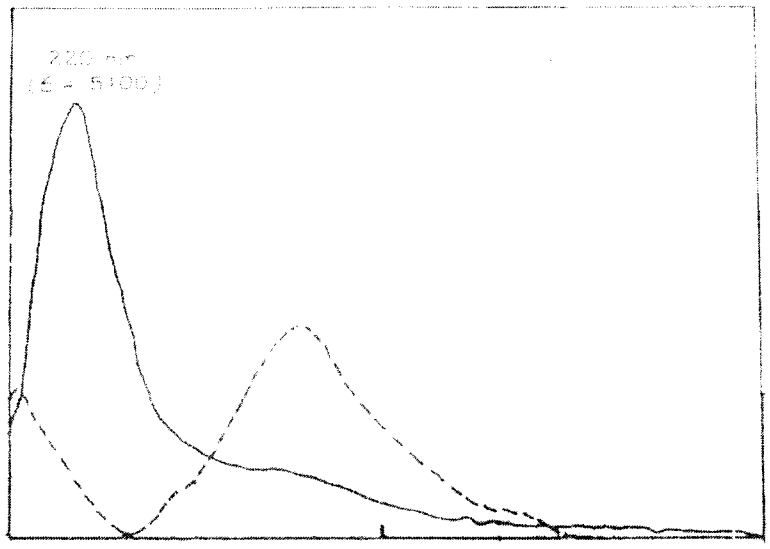


Fig. 20. Mass spectrum of 2, 3-dioximinolupane

Microwave assisted cyclisation of the dioxime (4) to lupan [2, 3-C]-1',2',5'-oxadiazole (5)

lupan[2,3-C]-1',2', 5'-oxadiazole (5) was prepared by cyclization of 2,3-dioximino lupane (4) in dry DMF under microwave irradiation (100W, 100°C) for 10 minutes (for details please see experimental). The compound obtained from the reaction was purified by repeated crystallization from chloroform-methanol mixture to obtain a compound 5, m.p.249-50°C, analyzed for $C_{30}H_{48}ON_2$, which was corroborated by mass spectral analysis that showed the moleculer ion at m/z 452. The other ions appeared at m/z 437 $[M\text{-}CH_3]^+$, 409 $[M\text{-}CH(CH_3)_2]^+$ (base peak), 367,271, 259, 245, 231, 206, 191, 163, 149, 123, 121, 109, 95, 81, 55 (Fig. 24). IR spectrum (Fig.22) of the compound showed peaks at 1620 cm^{-1} (—C=N-O) and 890 $cm^{-1}$for heterocyclic ring. It showed UV absorption maximum at 223 nm (€=5169) for disubstituted furazan derivative [96]. Interestingly in the NMR spectrum different methyl group signals got separated which may be due to the presence of heterocyclic ring attached with ring [97]. It showed the presence of (Fig. 23) six tertiary methyl of which four of them have been shifted downfield about 0.5 ppm in comparison to that of lupanone1b. A pair of one-proton doublets each at 2.1 (J=16 Hz) ppm and 3.15 (J= 16 Hz) ppm may be due to the germinal coupling of the C-1 proton which are adjacent to the furazan ring. Thus from spectral analysis the structure of the compound has been established as lupan [2,3-C]-1',2', 5'-oxadiazole (5)



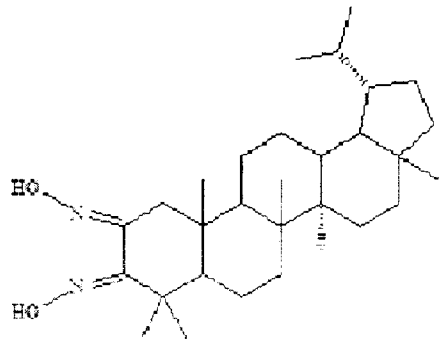
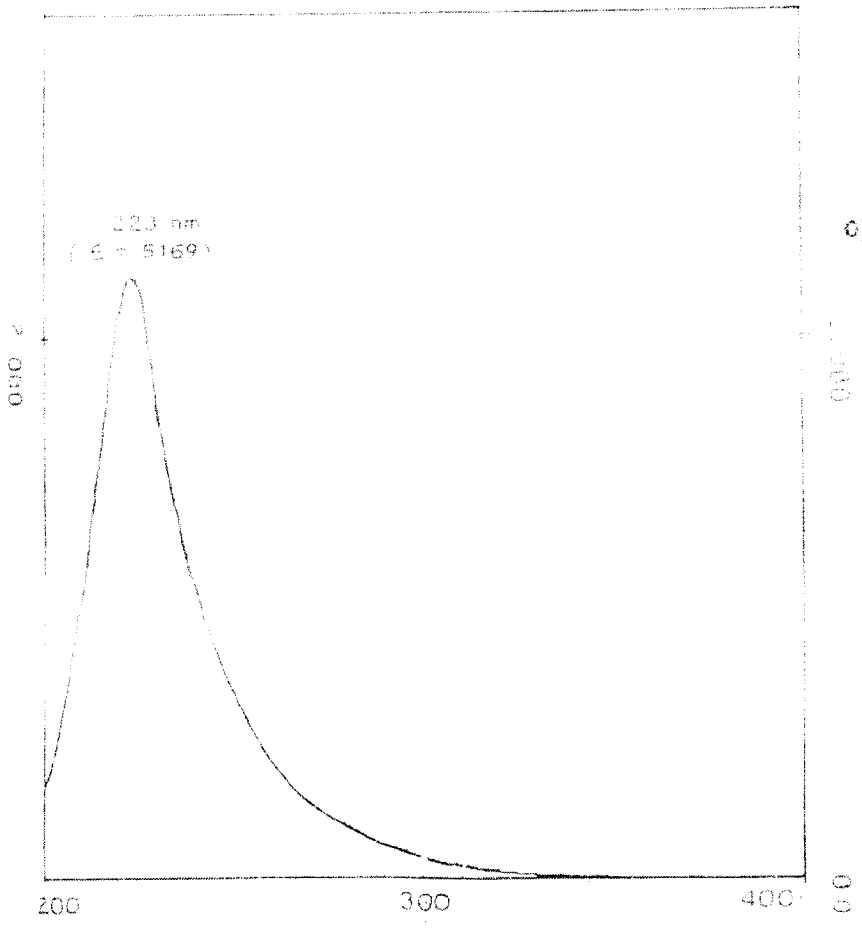


Fig. 21. UV spectrum of 2, 3-dioximino lupane



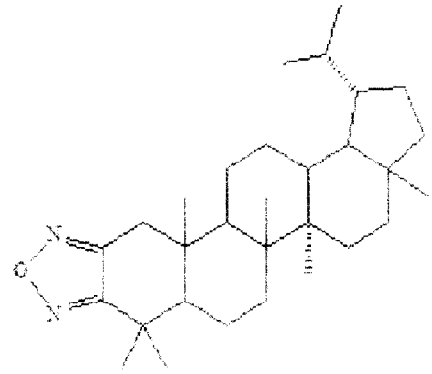


Fig. 22. UV spectrum of lupan[2,3-C]-1',2', 5'-oxadiazole

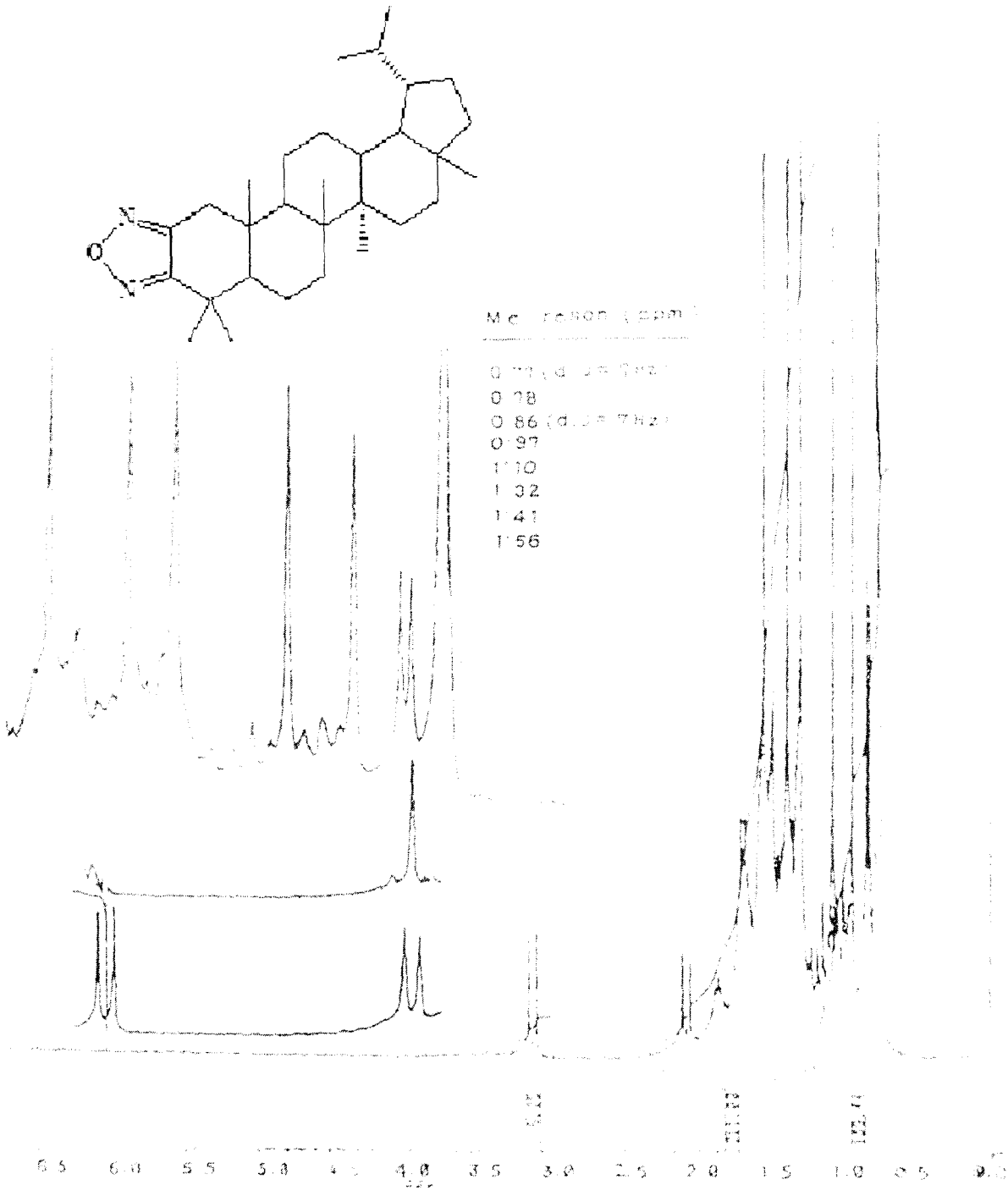


Fig. 23. ^{1}H NMR spectrum of lupan[2,3-C]-1',2', 5'-oxadiazole

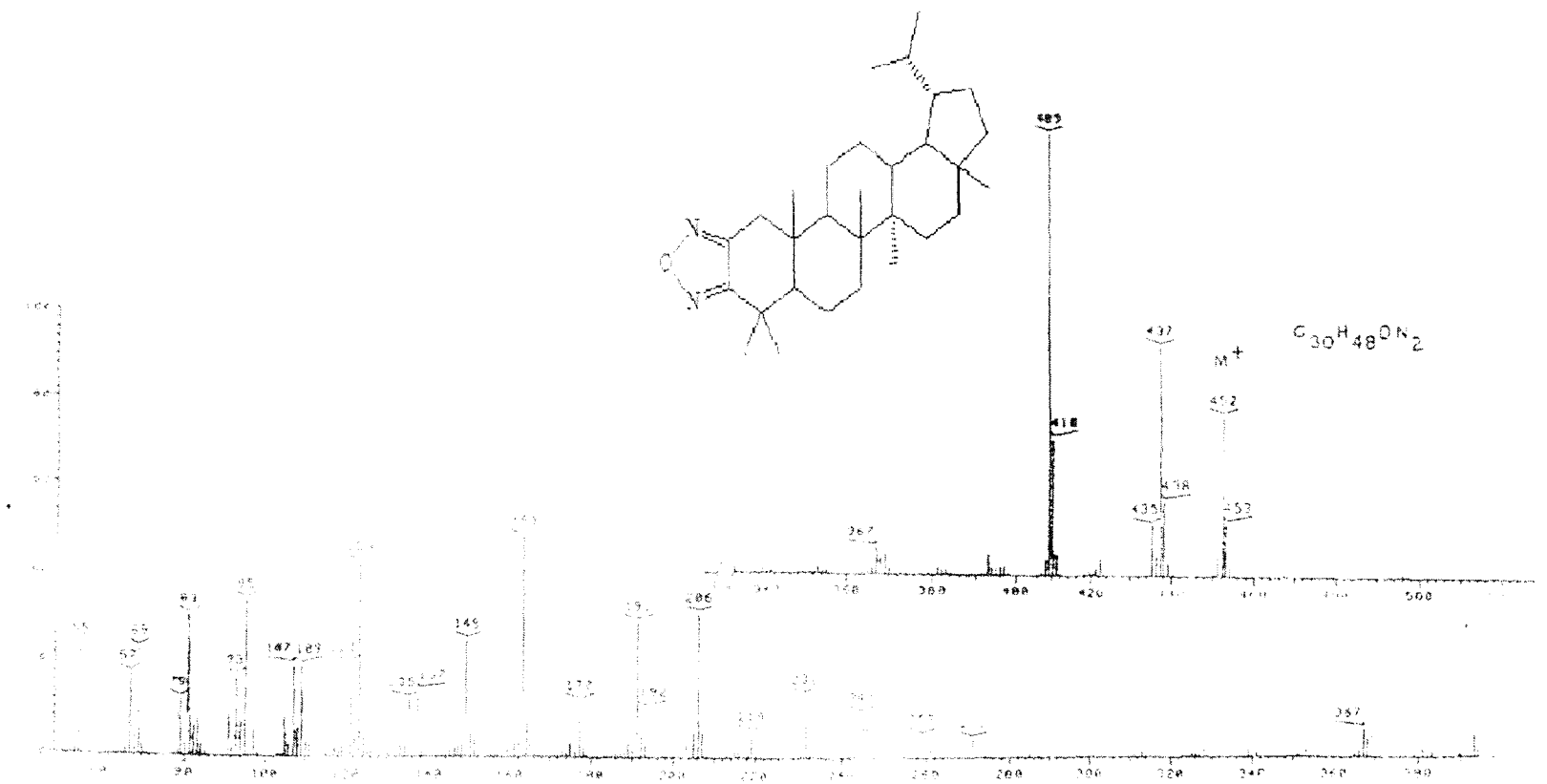
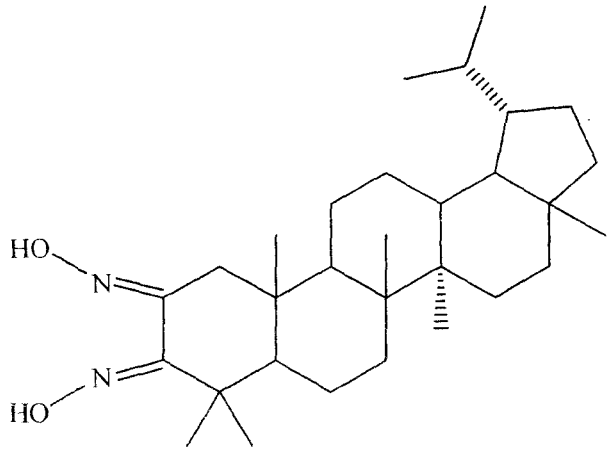


Fig. 24. Mass spectrum of lupan[2.3-C]-1`,2`, 5`-oxadiazole



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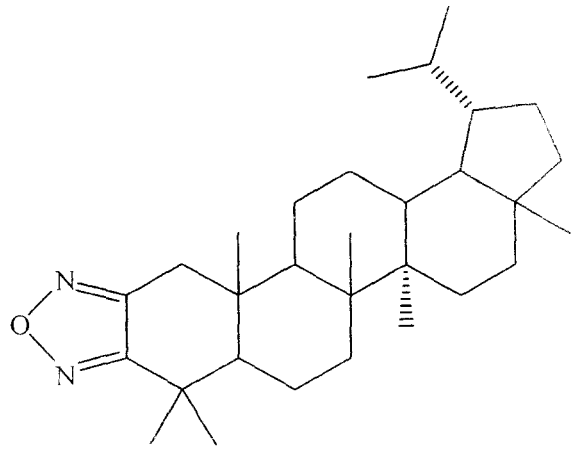


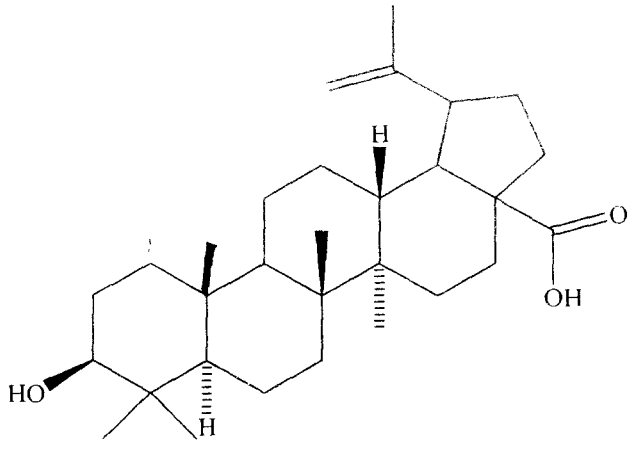
5

Structure 5 for compound D gets further support from its mass fragmentation pattern which is depicted in scheme XV1.

Section B

Extraction of *Biscofia javanica* blume: isolation of betulinic acid (6)

First collected the bark of Biscofia javanica blume from Darjeeling hilly region dried on sunlight and coarsely powdered. These powdered mass were extracted with benzene in a soxhlet apparatus for 36 hours (for details please see experimental). The solvents were then removed under reduced pressure and a sticky brown residue was obtained. This residue was then purified by column chromatography using silica gel (60-120) mesh and suitable proportions of benzene and ether (1:4) were used as the eluent which afforded betulinic acid, m.p. 301—303°C.



6

Esterification of betulinic acid: Preparation of methylbetulinate (7)

To the crude acid dissolved in ether was added to a solution of diazomethane in ether prepared from nitrosomethylurea and was kept overnight (for details see experimental). The crude ester obtained dissolved in benzene and placed over a column of silica gel (100 g). The column was developed with petroleum ether and the chromatogram on elution with pet. ether : benzene (80: 20) furnished a single compound.

Examination of fractions: Isolation and characterization of methylbetulinate (7)

The solid compound obtained from the different fractions were mixed (3.8 g) and crystallised from a mixture of chloroform and methanol to afford a colourless needle shaped compound, m.p. 221—223°C, $[\alpha]_D$ +5.0°. The details of its characterization have been discussed in part I of chapter 2.

Hydrogenation of methylbetulinate: Preparation of methyl-dihydrobetulinate (8)

Methylbetulinate dissolved in ethyl acetate was shaken in an atmosphere of hydrogen in presence of palladium in charcoal catalyst for three hours until absorption of hydrogen ceased (for details see experimental). Crystallization from a mixture of chloroform and methanol furnished colourless needle shaped of a compound m.p. 235—237°C, $[\alpha]_D$ −17.0°. IR spectrum (Fig. 25) showed peaks at 3540 cm^{-1} (OH) and 1705(CO) cm^{-1}. This compound was found to be identical with an authentic sample of methyldihydrobetulinate (m.m.p, CO TLC, CO IR).

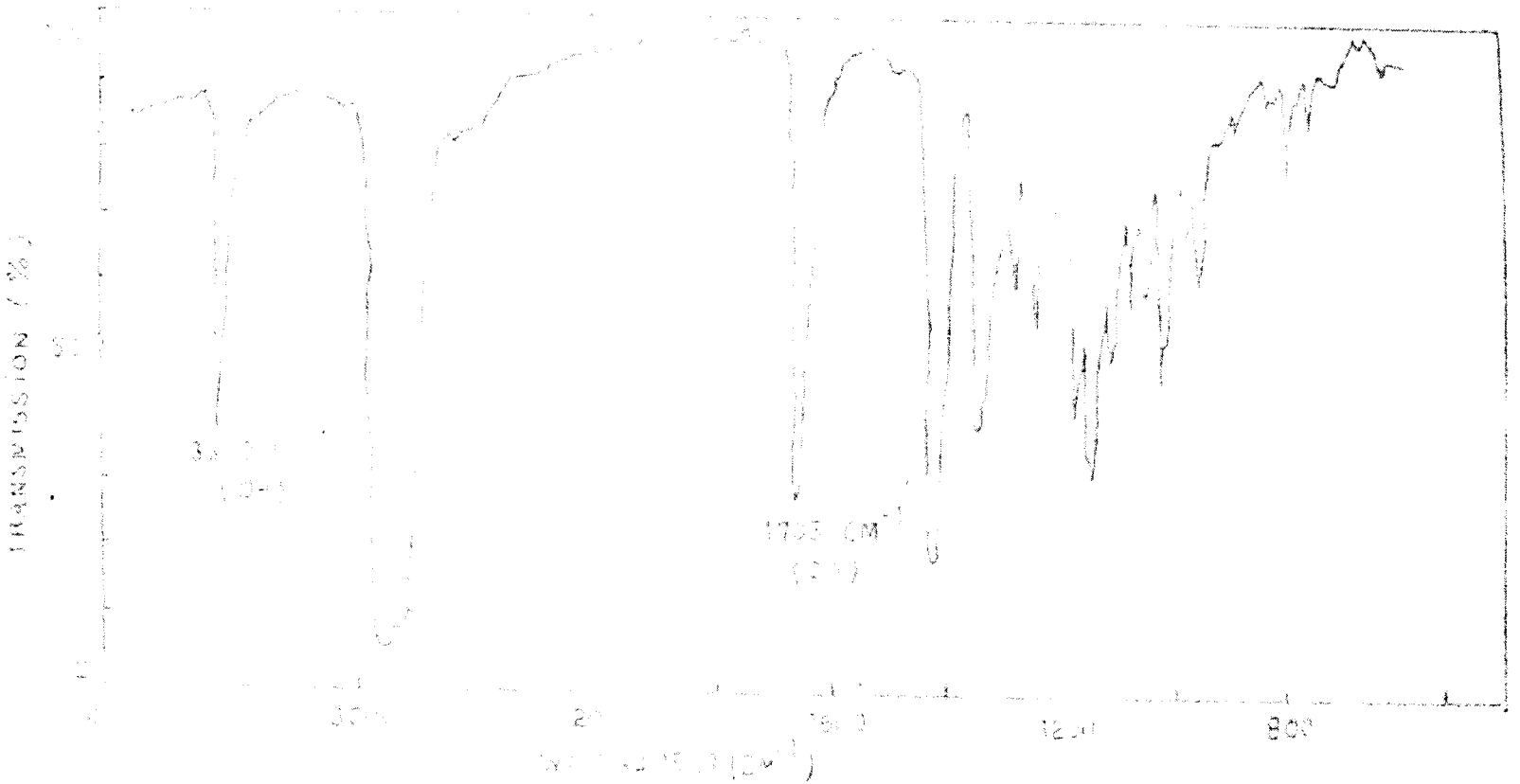
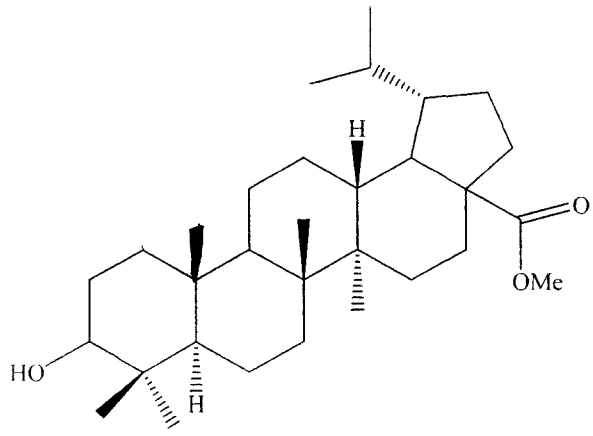


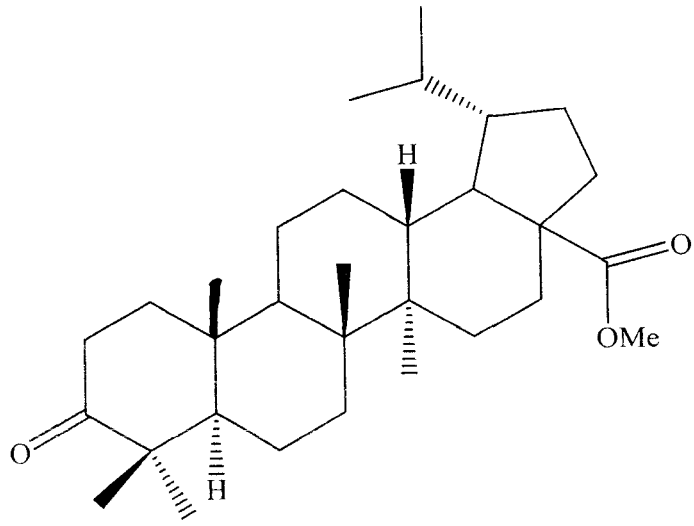
Fig. 25. IR spectrum of methyldihydrobetulinate



8

Jone's oxidation of methyldihydrobetulinate: Preparation of methyldihydrobetulonate (9)

To a solution of methyldihydrobetulinate (2.95 g) in pure acetone added Jone's reagent drop wise with constant shaking until a faint orange colour persisted (for details see experimental). The resulted product was chromatographed which developed with petroleum ether and elution with pet. ether: benzene (80: 20) furnished different fractions of a single compound. The different fractions were mixed and on crystallization from methanol furnished needle shaped crystals of methyldihydrobetulonate, m.p. 190—192°C, $[\alpha]_D$ + 8.2°. IR spectrum (Fig. 26) showed peaks at 1730 cm^{-1} (—COOMe) and 1708 cm^{-1} (CO) identical with an authentic sample of methyldihydrobetulonate (m.m.p, CO TLC, CO IR) [Lit m.p. 194°C, $[\alpha]_D$ + 8.4°]



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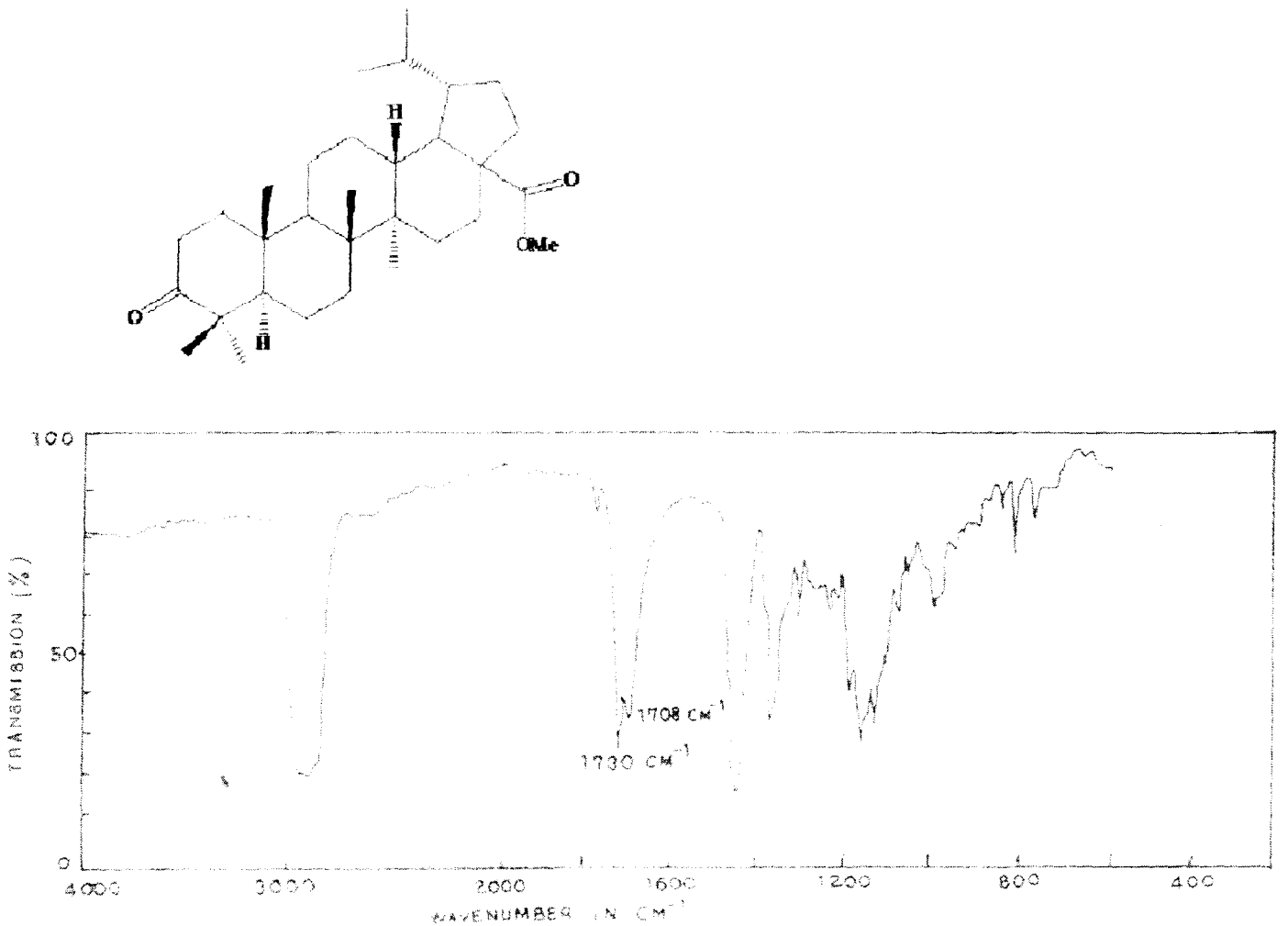


Fig. 26. IR spectrum of methyldihydrobetulonate

Treatment of methyl dihydrobetulonate (9) with N-bromosuccinimide: Formation of 2, 2-dibromomethyldihydrobetulonate (10) and 2α-bromomethyl dihydrobetulonate (11)

A solution of methyldihydrobetulonate in chloroform was mixed with dimethylsulphoxide. N-Bromosuccinimide was then added to the solution with constant shaking (for details see experimental). The residue obtained after usual workup two spots on chromatoplate. So the residue was chromatographed over silica gel column. The chromatogram was developed with petroleum ether and elution with pet. ether: benzene (90: 10) furnished a single compound **I** and further elution with pet. ether: benzene (75:25) gave **II**.

Examination of fractions I: Isolation and characterization of 2, 2-dibromomethyldihydro betulonate (10)

The different fractions showed homogeneity on TLC plate. They were mixed together and crystallised by using chloroform and methanol mixture of compound10, m.p. 160-162°C gave an intense green flame in Beilstein test showing the presence of bromine in the compound. It showed no yellow coloration with TNM. Elemental analysis showed the molecular formula to be $C_{31}H_{48}O_3Br_2$ which was supported by the existence of three ion peaks at 630, 628 and 626 in the ratio of 1:2:1, proving the presence of two bromine atoms, in the mass spectrum (Fig. 31) of 10 , the other ions appeared at m/z 571, 569, 567 $[M\text{-}COOCH_3]^+$ (1:2:1), 550, 548, 547 $[M\text{-}HBr]^+$, 533, 531$[M\text{-}HBr\text{-}CH_3]^+$, 525, 523 $[M\text{-}HBr\text{-}CH_3CO]^+$, 468, 470,471 $[M\text{-}2Br]^+$,453, 412, 411, 410, 409, 283,285, 274, 231, 205, 203, 191, 177(100%). IR spectrum (Fig. 28) showed peaks at 1725 cm^{-1} (—$COOCH_3$) and 1705(CO) cm^{-1}.

Its UV spectrum showed a hump at 310 nm (Fig. 27) and the CD curve showed a very prominent negative cotton effect similar to compound 3. Thus from IR and CD spectrum the existence of two bromine atoms at the same carbon at C-2 is obvious. This is further supported by ^1H NMR (fig. 29) of compound 10 that showed two doublets at 3.63 and 3.11 ppm with germinal coupling of 16 Hz due to methylene protons at C-1 that have no in the neighboring α-carbons, the other signals due to seven methyl groups appeared

between 0.76 to 1.22 ppm and an ester group at 3.65 (s, 3H, -$COOCH_3$) ppm. ^{13}C NMR of compound 10 (fig. 30) showed 31 peaks, of which eight were quartets, nine triplets, six doublets and seven singlets. The peak at 204.27 ppm is due to the carbonyl group at C-3 that is deshielded by the two alpha bromine atoms and the peak at 176.74 ppm is evidently due to carbomethoxy group. Thus the compound 10 has been fully characterized by spectral analysis as 2,2-dibromo methyl dihydrobetulonate.

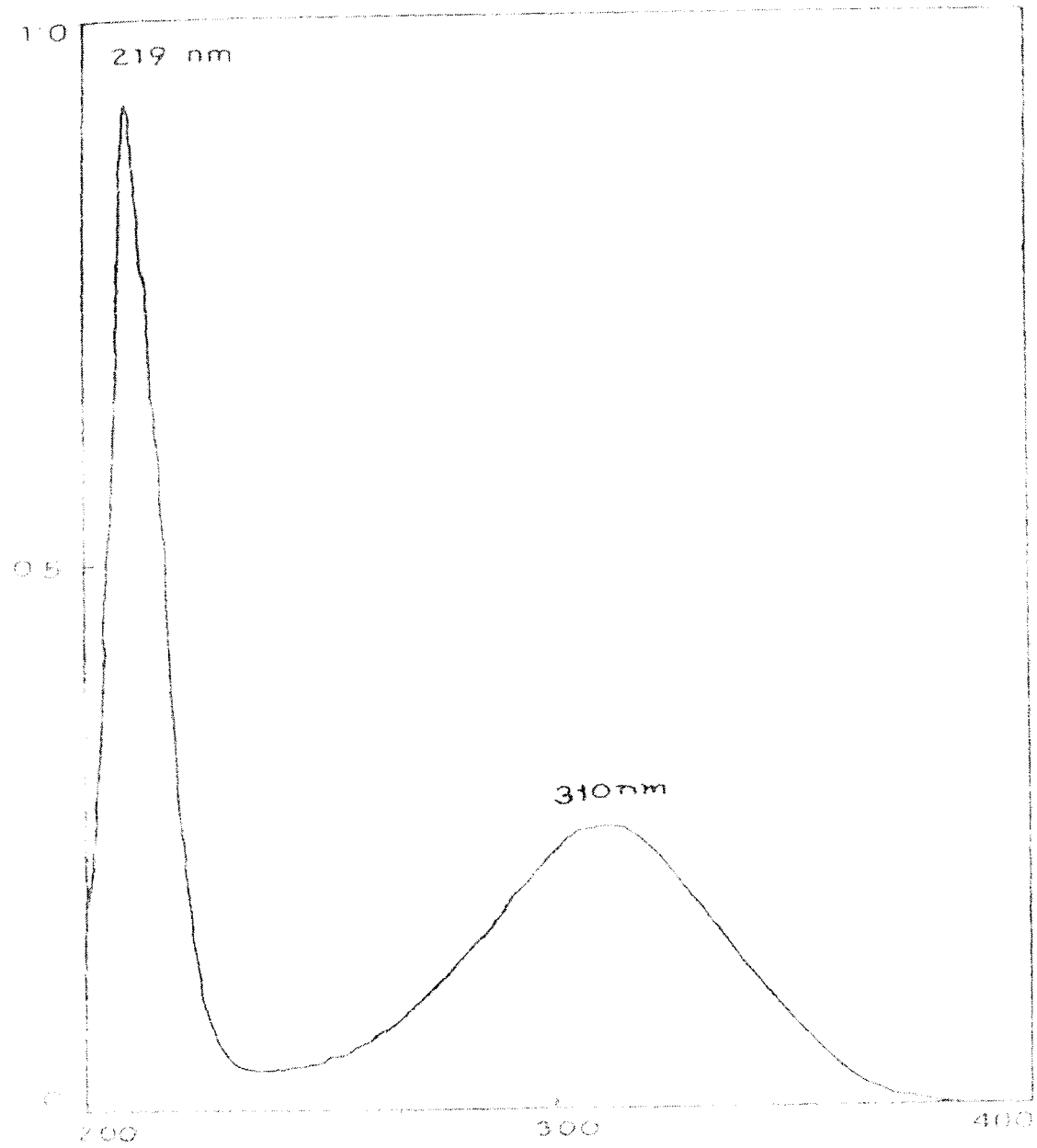


Fig. 27. UV spectrum of 2,2-dibromomethyldihydrobetulonate

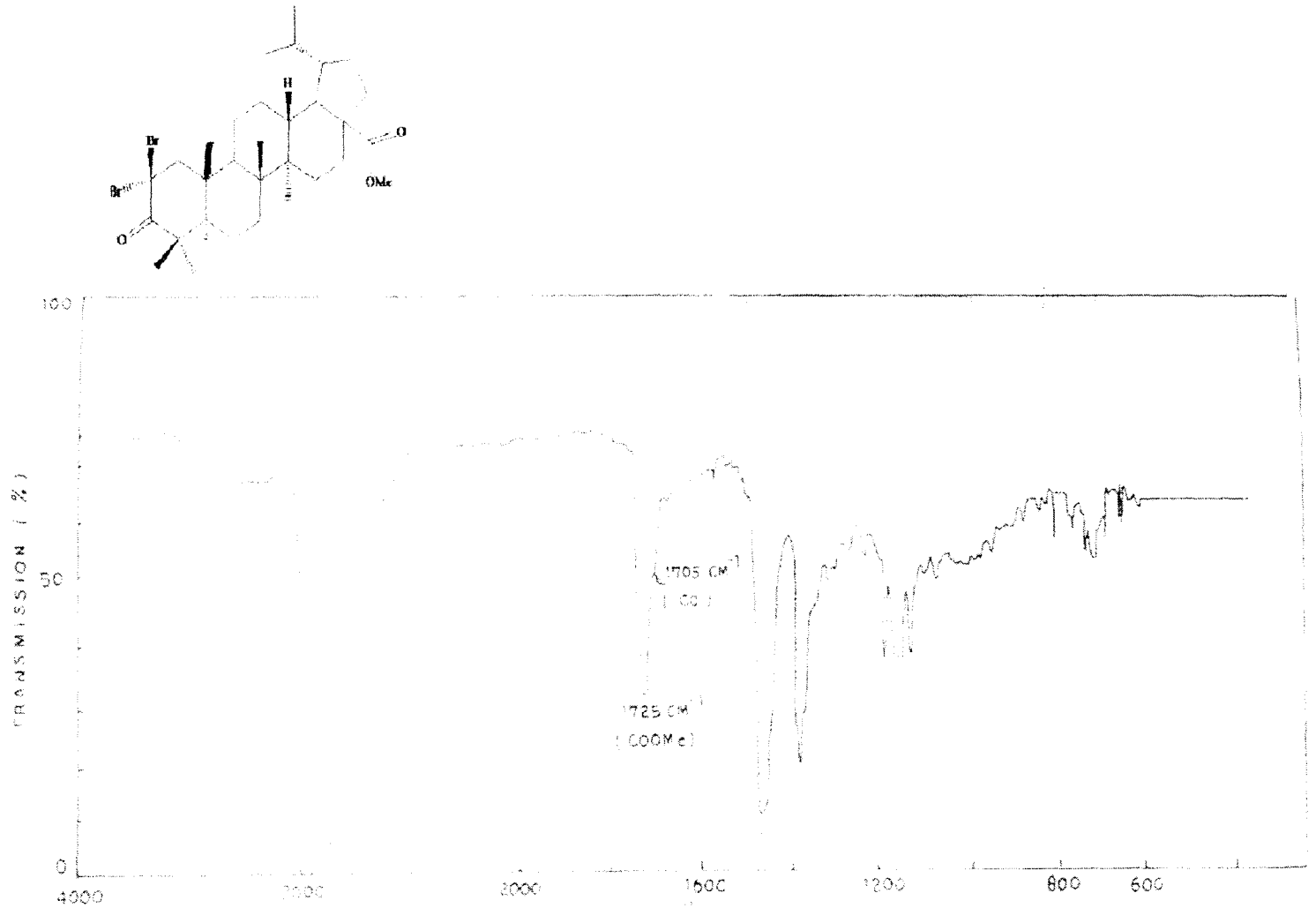


Fig. 28. IR spectrum of 2,2-dibromomethyldihydrobetulonate

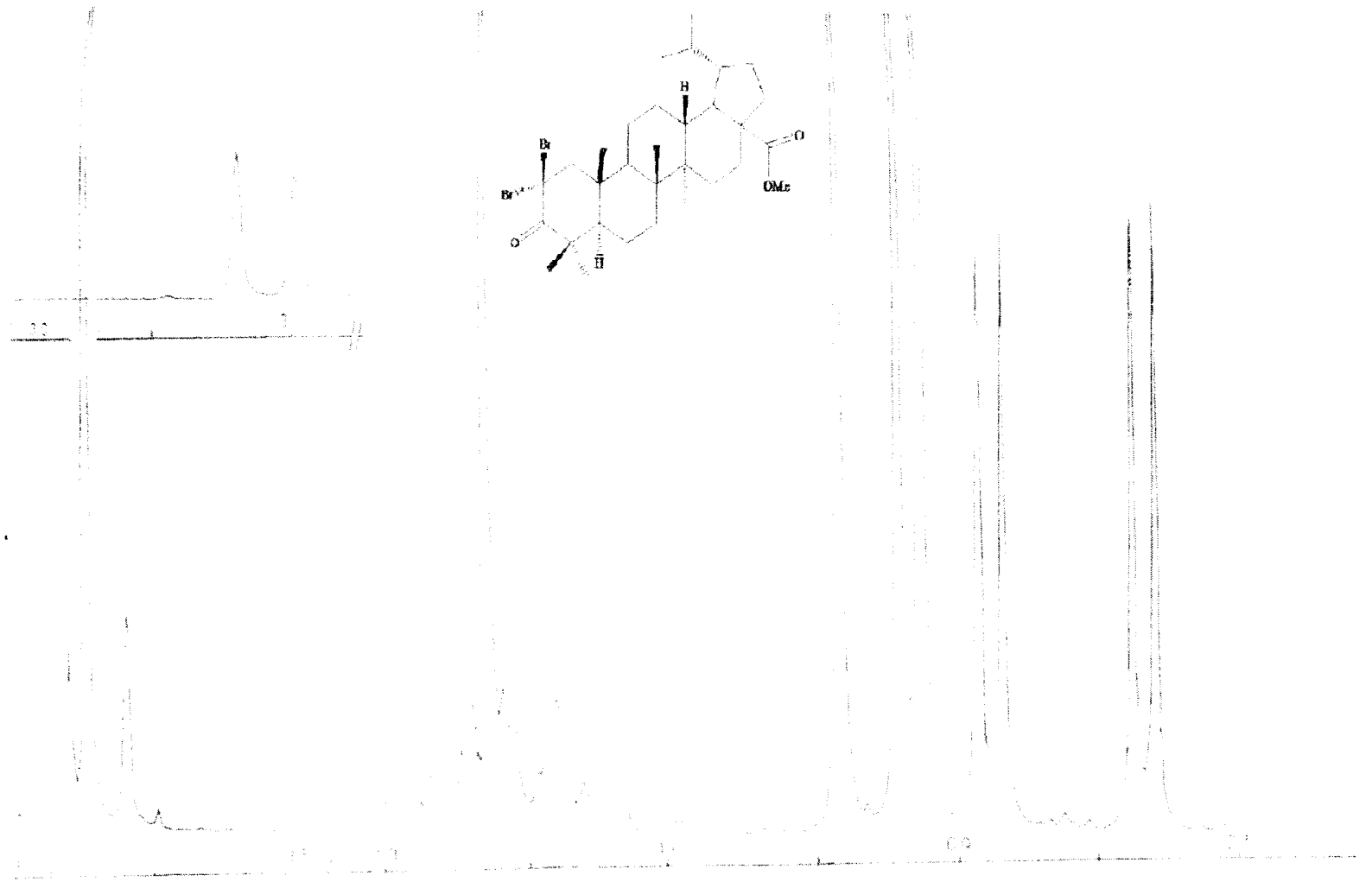


Fig. 29. ^{1}H NMR spectrum of 2, 2-dibrommethyldihydrobetulonate

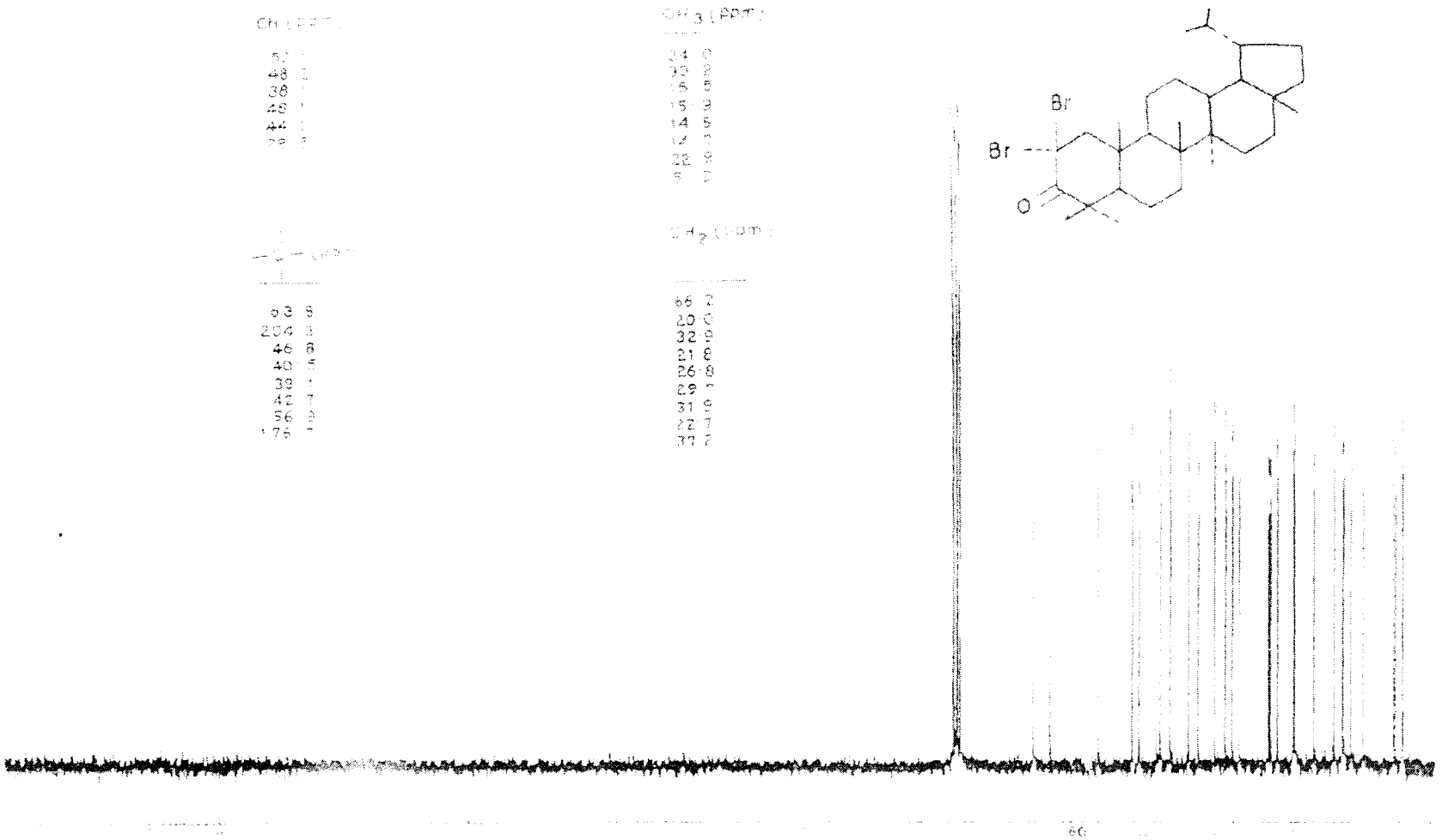


Fig. 30. ^{13}C NMR spectrum of 2, 2-dibrommethyldihydrobetulonate

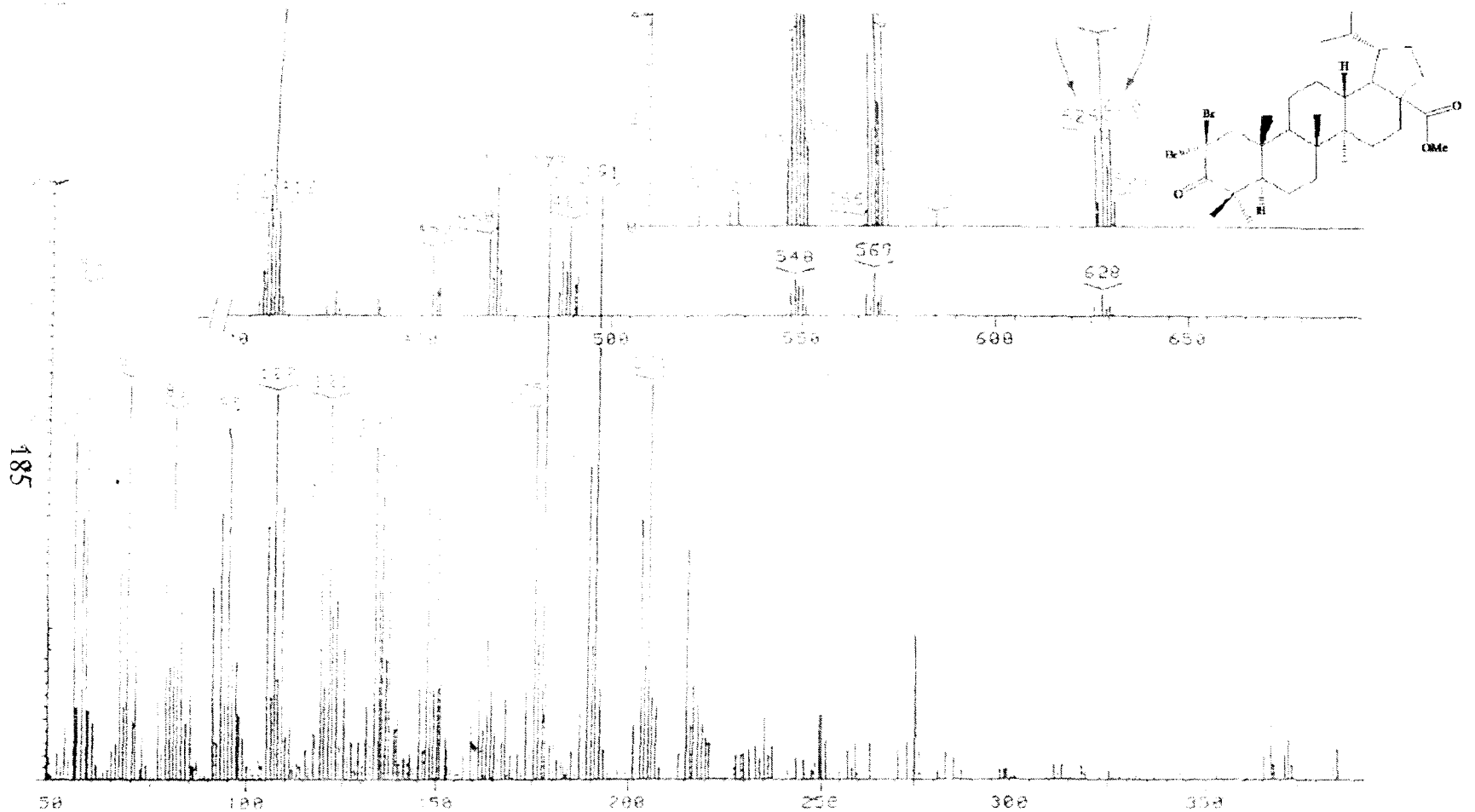
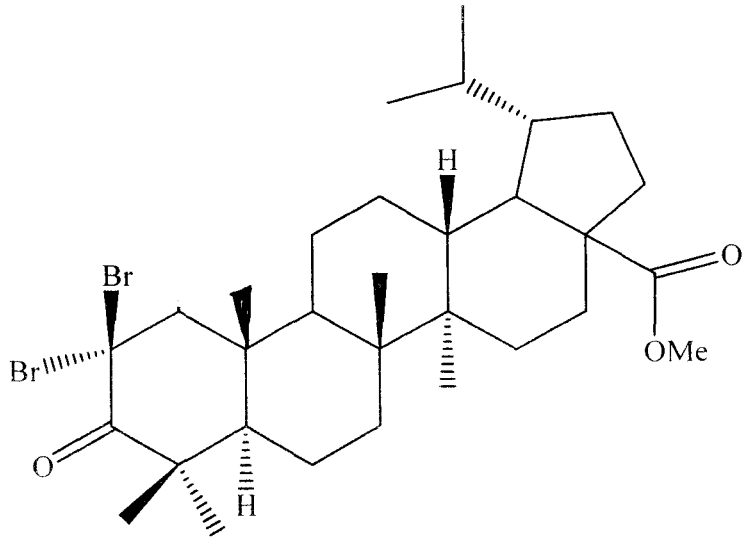


Fig. 31. Mass spectrum of 2, 2-dibrommethyldihydrobetulonate



10

Examination of fraction II: Isolation and characterization of 2α-bromomethyl dihydrobetulonate (11)

Fractions 2 were mixed (0.9 g) and on crystallization from chloroform methanol mixture afforded crystals of compound 11, m.p. 125-127°C which analyzed for $C_{31}H_{49}O_3Br$, showed positive Beilstein test for halogen. IR (Fig. 32) absorption spectrum of compound 11 showed peaks at 1725 cm^{-1} and 1705 cm^{-1} indicating the presence of -COOMe and C=O groups respectively. ^{1}HNMR spectrum (Fig. 33) of 11 showed a doublet of doublet at 5.06 ppm for C-2 proton with axial-axial and axial-equatorial coupling of 12 Hz and 6 Hz respectively . The C-1 equatorial proton appeared at 2.65 ppm as doublet of a doublet with germinal coupling of 12 Hz and vicinal coupling of 6Hz, the C-1 axial proton appeared at 2.47 ppm as a triplet with J=12 Hz, the other peaks appeared at 0.94, 0.97, 1.09, 1.23, 1.20 for five tertiary methyl groups and two doublets at 0.77 and 0.87 for two secondary methyl groups with J value equal to 7 Hz. Mass spectral analysis of compound 11 showed that molecular ions at m/z 550 and 548 (Fig. 34) of almost equal heights due to isotopic bromine atoms. The other peaks of prominence appeared at m/z 491, 489[M-$COOCH_3$]$^+$, 471, 469,470, 471 [M-Br]$^+$ (1:2:1); 412, 411(100%), 410, 395, 275, 260, 250, 205, 191, 177, 174, 119. Thus from above spectral analysis (PMR, IR and Mass) the compound 11 has been identified as 2α–bromomethyldihydrobetulonate.

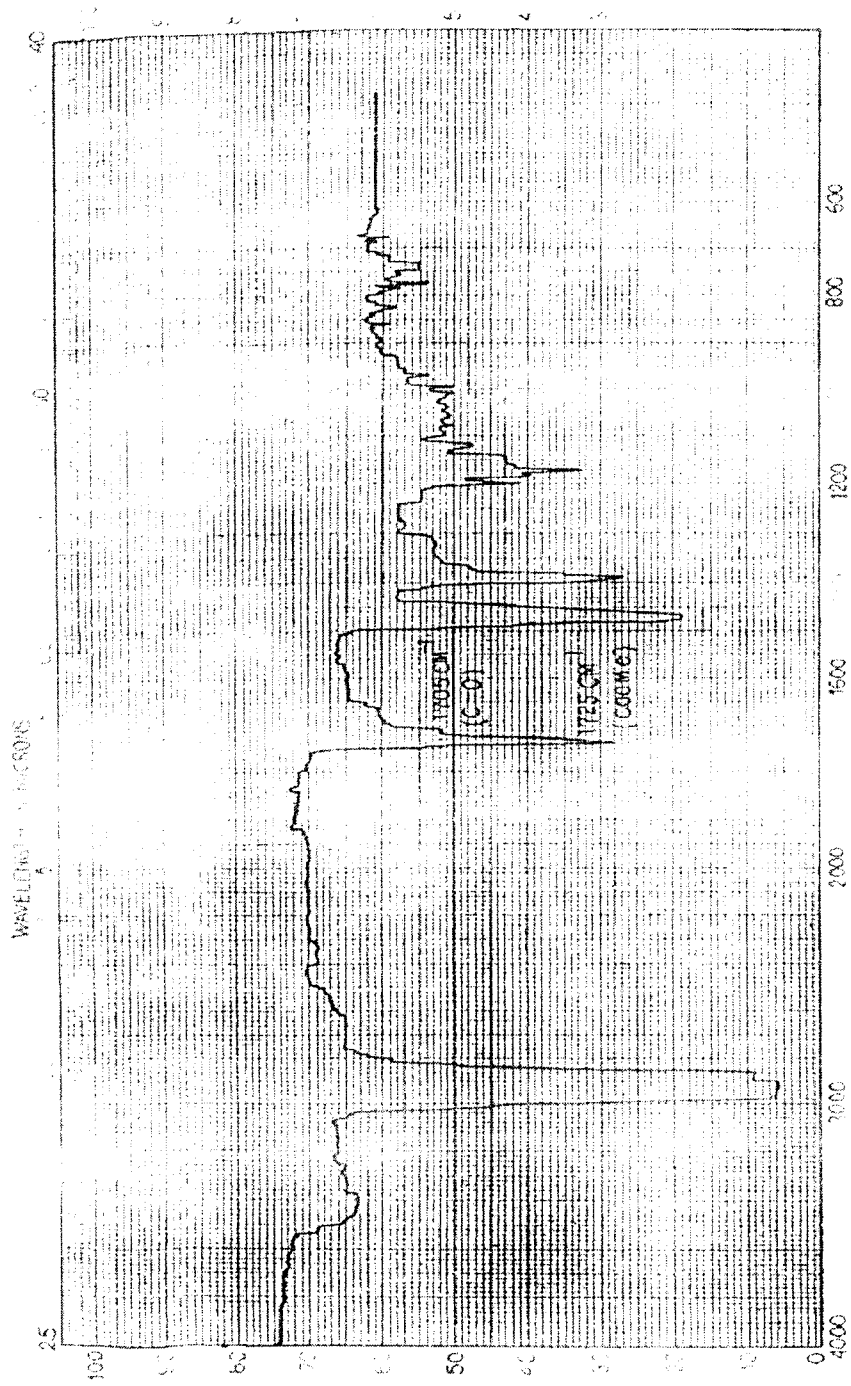


Fig. 32. IR spectrum of 2α-bromomethyldihydrobetulonate

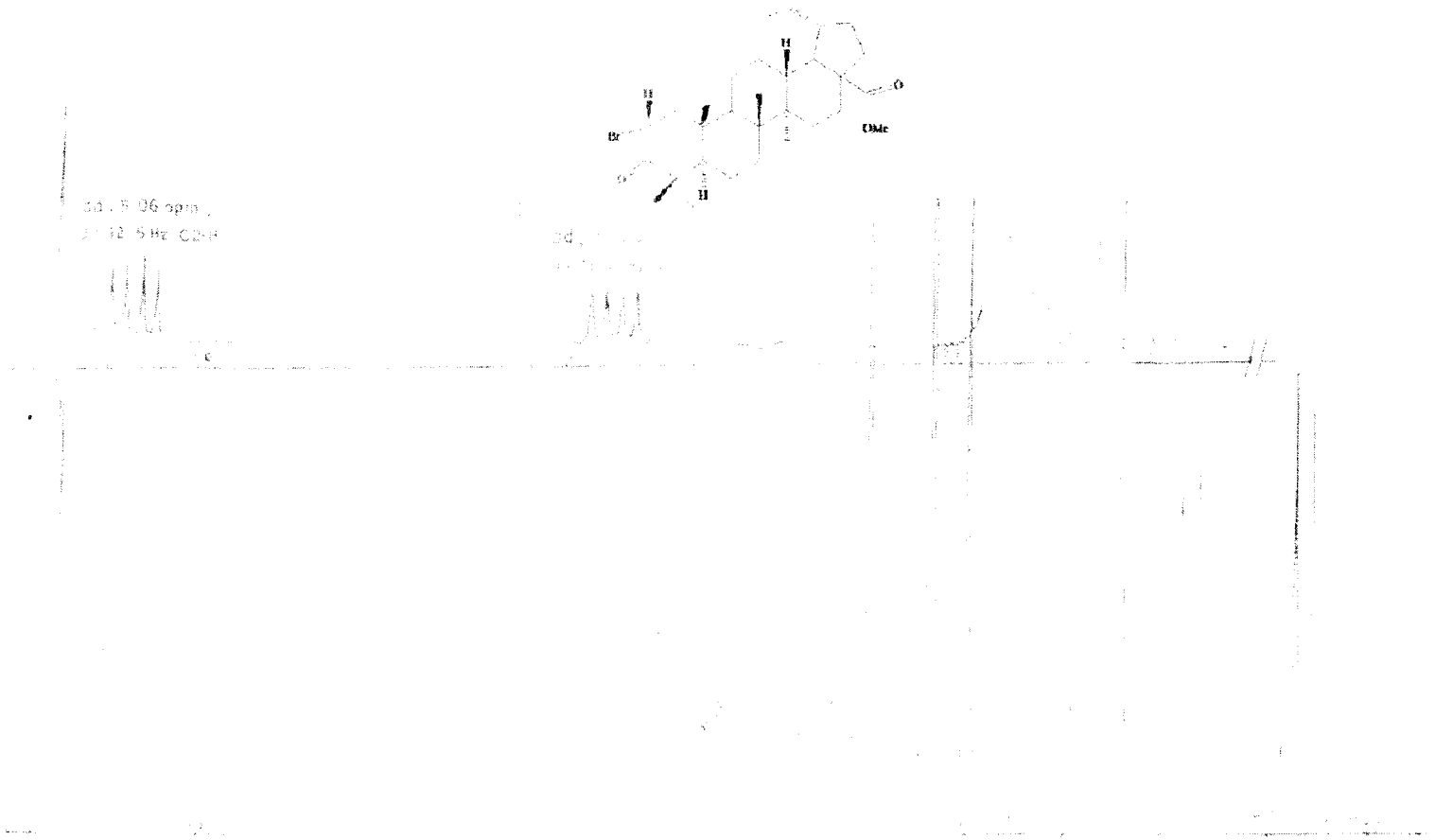


Fig. 33. ^{1}H NMR spectrum of 2α-bromomethyl dihydrobetulonate

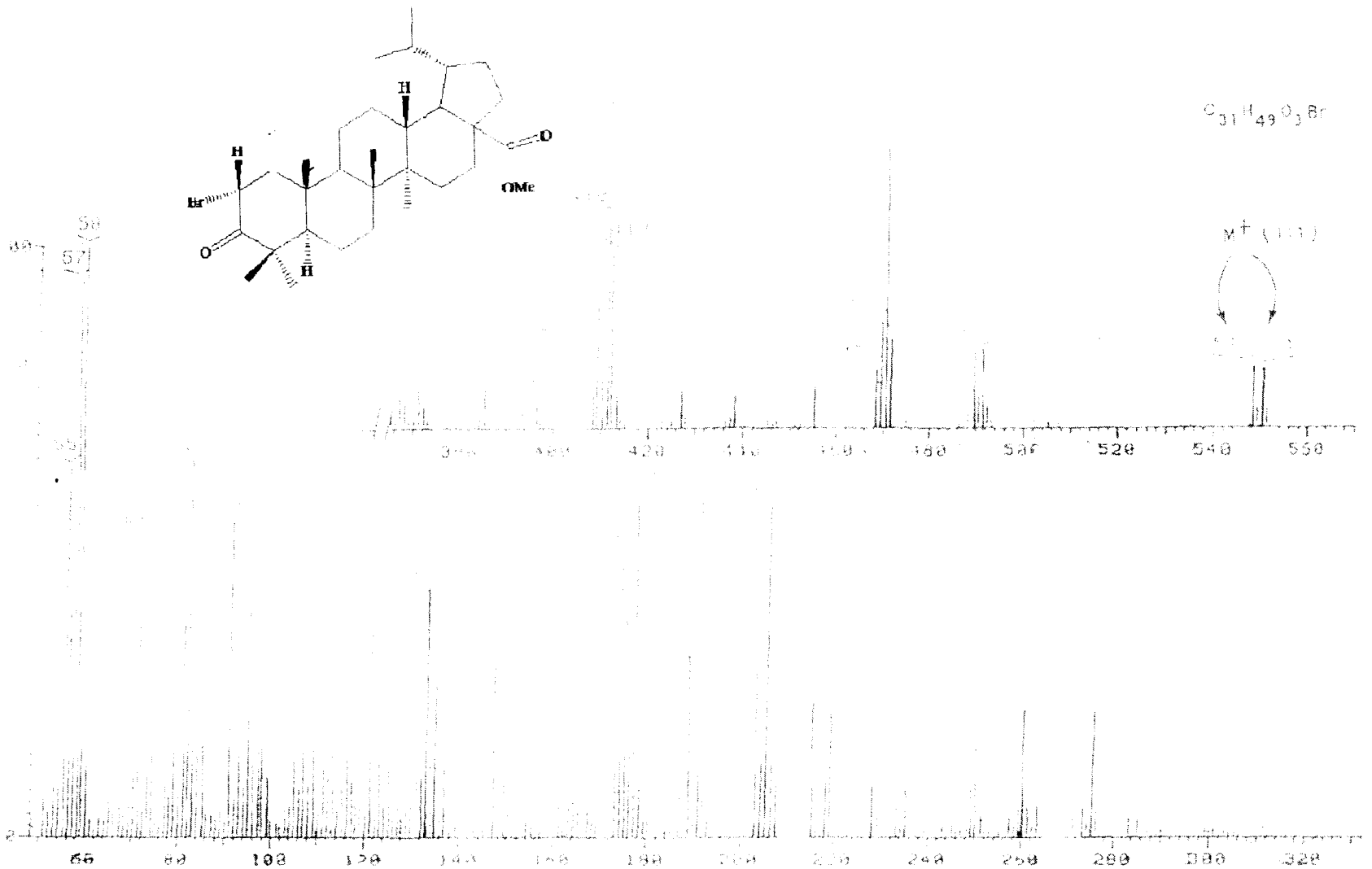
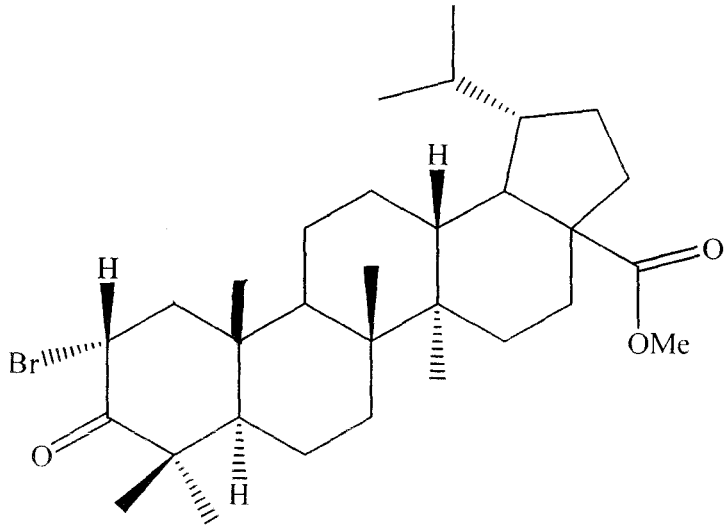


Fig. 34. Mass spectrum of 2α-bromomethyldihydrobetulonate



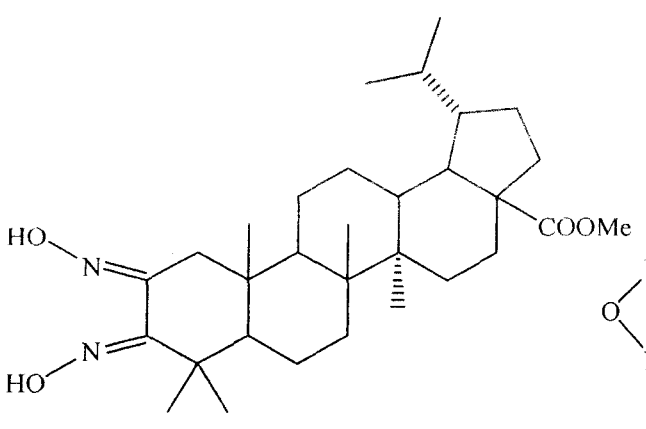
11

Treatment of 2,2-dibromomethyl dihydrobetulonate (10) with hydroxylamine hydrochloride: Preparation of 28-carbomethoxy-2,3-dioximinolupane (12) and the subsequent cyclization of the dioximino derivative to 28-carbomethoxy lupan[2,3-C]-1′, 2′, 5′-oxadiazole under MW irradiation (13)

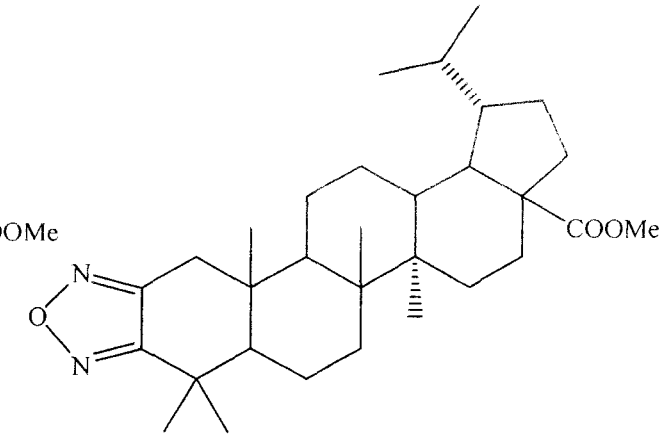
2,2-dibromomethyl dihydrobetulonate 10 dissolved in pyridine was refluxed with hydroxyl amine hydrochloride in ethanol (see experimental). The compound obtained from the reaction was purified by repeated crystallization from chloroform-methanol mixture to obtain a white amorphous powder of compound A, analyzed for $C_{32}H_{53}O_4N_2$.. IR spectrum showed peaks at 3200-3400 cm^{-1} (C=N) 1720 cm^{-1} (–COOMe). It exhibited UV absorption maximum at 220 nm (ϵ=5100). Mass spectrum of the compound showed moleculer ion peak at m/z 529 $[M]^+$ the other peaks at prominence appearance at m/z 530 531 (base peak), 424, 422, 380, 341, 340, 299, 231, 191, 163, 149, 136, 122, 121, 95, 81, 69.. PMR spectrum of A could not be taken owing to solubility problem. Thus on the basis of the above spectral data compound A was identified as 28-carbomethoxy-2,3-dioximinolupane (12).

Cyclisation of the dioxime 12 to 28-carbomethoxy lupan [2,3-C]-1′,2′,5′-oxadiazole (13)

28-carbomethoxy lupan[2,3-C]-1',2', 5'-oxadiazole was prepared by cyclization of 28-carbomethoxy-2,3-dioximinolupane in dry DMF under microwave irradiation (100W, 100°C) for 10 minutes (for details please see experimental Ch 4). The residue obtained after usual work up was purified by repeated crystallization from chloroform-methanol mixture and designated compound 13, analyzed for $C_{31}H_{48}O_4N_2$.Which was corroborated by mass spectral analysis that showed the moleculer ion at m/z 496. The other ions appeared at m/z 496.72 $[M\text{-}CH_3]^+$, 496 (base peak), 497, 367, 271, 259, 245, 231, 206, 191, 163, 149, 123, 121, 109, 95, 81, 55. IR spectrum of the compound showed peaks at 1620 cm^{-1} (—C=N-O) and 890 cm^{-1}, 1720 cm^{-1} (–COOMe) for heterocyclic ring. It showed UV absorption maximum at 223 nm (€=5169) for disubstituted furazan derivative (92). The ^{1}HNMR are 0.77 (d, 3H, J=7 Hz) 0.78, 0.86 (d, 3H, J=7 Hz), 0.97, 1.10. It showed the presence of six tertiary methyl. A pair of one-proton doublets each at 2.1 (J=16 Hz) ppm and 3.15 (J= 16 Hz) ppm may be due to the germinal coupling of the C-1 proton which are adjacent to the furazan ring. Thus from spectral analysis the structure of the compound 13 has been established as 28-carbomethoxy lupan [2,3-C]-1', 2', 5'-oxadiazole.



12



13

Biocidal activity of the derived compounds

a. Antibacterial activity

Two different compounds (oxadiazole derivative and lupeol) were tested for their antibacterial properties against five different bacteria (*E. coli, B. subtilis, S. aureus, Lactobacillus, Pseudomonas*) and the inhibition of growth have been summarized in Table 1.

Table 1. Results of antibacterial activity

Microorganism	Compounds under investigation	Inhibition zone (cm)		
		Concentration		
		100 ppm	250 ppm	500 ppm
E. coli	5 / 13	2.5 / 2.6	2.5/2.7	2.9/2.8
	1 / 7	2.4 / 2.3	2.5/2.4	2.7/2.5
B. subtilis	5 / 13	1.7/1.8	1.8/1.9	2.1/2.4
	1 / 7	1.4/1.6	1.7/1.8	2.1/2.3
S. aureus	5 / 13	1.6/1.7	1.7/1.9	1.8/1.9
	1 / 7	1.5/1.6	1.6/1.8	1.7/1.8
Lactobacillus	5 / 13	2.3/2.4	2.4/2.3	2.6/2.4
	1 / 7	1.7/1.9	1.9/2.0	2.2/2.3
Pseudomonas	5 / 13	NP/1.2	0.8/NP	1.0/1.2
	1 / 7	NP/1.1	NP/0.9	NP/1.0

NP= Not prominent; 1=lupeol; 7= methylbetulinate; 5= lupan [2,3-C]1',2', 5'-oxadiazole
13= 28-carbomethoxy lupan [2,3-C]-1', 2', 5'-oxadiazole

From the results presented in table-3 it is evident that significant inhibition of growth was observed in the case of *E. coli* and *Lactobacillus* by all the concentrations of the oxadiazole derivatives (5 and 13) and their corresponding parent compounds, Lupeol (1) and methyl betulonate (7, methyl ester of the parent compound) tested in disc diffusion method. For oxadiazole derivatives maximum inhibition was observed at 500 ppm when tested on *E. coli*; the inhibition zone was 2.9 cm and 2.8 cm respectively for the oxadiazole

derivative 5 and 7, but at 250 ppm and 100 ppm concentration the diameter of inhibition zones were 2.5 cm and 2.3 cm respectively. Thus there is a gradual increase in the zone of inhibition with the increase in concentration of the compounds. Similar results were also observed in case of the parent compounds, 1 and 7. Lupeol at 500 ppm, 250 ppm and 100 ppm concentrations the diameters of inhibition zones were 2.7 cm, 2.5 cm and 2.4 cm respectively.

Although the inhibition was found relatively less with *B. subtilis*, *S. aureus* in comparison to above two microorganisms but the trends of activity were found identical.

The growth of inhibition zone with *Pseudomonas* was not prominent for all the compounds tested.

b. Antifungal Activity

The antifungal activities of lupeol and oxadiazole derivative at different concentrations were tested against *F. solani*. The results of the antifungal activities have been presented in Table 2.

Table 2. Antifungal Activity on *F. solani*

Compounds	Concentration	% of germination	% of inhibition	Range of Germ tube length
	Control	96.77	0	3.0-5.0
1/7	100 ppm	83.88 /83.86	13.53 /13.51	2.4-5.4 / 2.6-5.9
	200 ppm	17.89 /17.75	81.56 /81.79	2.2-4.8 / 2.3-4.5
	300 ppm	16.19 /16.24	83.31 /83.51	2.2-4.4 / 2.4-4.5
	400 ppm	9.52 /9.54	91.19 /91.94	2.2-4.0 / 2.3-4.2
	500 ppm	4.43 /4.44	95.64 / 96.28	2.2-3.4 / 2.3-4.0
	Control	97.77	0	3.0-5.0
5/13	100 ppm	12.30 / 12.4	86.64 /86.65	2.4-4.8 / 2.4-4.9
	200 ppm	8.04 / 8.2	91.27 /91.34	2.0-4.4 / 2.2-4.6
	300 ppm	5.16 /5.10	94.40 / 94.51	2.0-4.2 / 2.3-4.5
	400 ppm	2.48 /2.56	97.31 /97.39	1.6-3.6 / 1.7-3.8
	500 ppm	1.02 / 1.00	98.90 / 98.72	1.6-3.0 / 1.5-3.4

The results revealed that both the parent compounds (1 and 7) and their respective oxadiazole derivatives (5 and 13) showed inhibition of spore germination and inhibitory effect on the growth of *F. solani*. Lupeol at 100 ppm concentration showed 83.88% germination where as 96.77% germination was experienced in control set. Similar results were also observed with methyl betulonate at the same concentrations. However, at 500 ppm concentration level both of them (1 and 7) significantly reduced germination of spores. They showed 95.64% and 96.28% inhibition of spore germination respectively

Oxadiazole derivatives (5 and 13) showed 12.30% and 12.4% germination respectively at their 100 ppm concentration. Whereas 97.77% germination was experienced in control set. The 500 ppm concentration of oxadiazole derivatives (5 & 13) significantly reduced germination of spores. They showed 98.90% and 98.72% inhibition of spore germination respectively.

c. Results of Phytotoxicity

The phytotoxic effects of lupeol and oxadiazole derivative on the germination of *Triticum aestirium* (wheat), *Oryza sativa* (rice) and *Pisum sativum* (pea) seeds have been summarized in Table 3.

Table 3. Results of phytotoxicity

Compound	Seeds of plants	Concentration	Germination of seeds	
			Length of Root (cm)	Length of Shoot(cm)
1/7	Rice	Control	GM, 0. 68	GM, 0.30
		100 ppm	GM, 0.59 / 0.61	GM, 0.24 / 0.26
		250 ppm	GM, 0.52 / 0.54	GM, 0.21 / 0.22
		500 ppm	GM, 0.43 /0.43	GM, 0.17 /0.18
	Wheat	Control	GM, 2.62	GM,1.1
		100 ppm	GM, 2.52 / 2.60	GM, 0.77 / 0.85
		250 ppm	GM, 2.4 / 2.38	GM, 0.65 / 0.64
		500 ppm	GM, 2.10 /2.15	GM,0.45 / 0.48
	Pea	Control	GM, 2.6	GM ,1.16
		100 ppm	GM, 1.65 /1.40	GM,0.74 / 0.69
		250 ppm	GM,1.36 / 1.14	GM, 0.56 / 0.45
		500 ppm	GM, 1.00 / 0. 94	GM 0.45 / 0.36
5/13	Rice	Control	GM, 0.68	GM, 0.30
		100 ppm	GM,0.60 / 0.65	GM 0.25 / 0.29
		250 ppm	GM,0.55 / 0.57	GM . 0.20 / 0.21
		500 ppm	GM,0.52 / 0.55	GM,0.10 / 0.12
	Wheat	Control	GM, 2.62	GM,1.1
		100 ppm	GM, 2.5 / 2.46	GM, 0.91 / 0.89
		250 ppm	GM, 2.12 / 2.10	GM, 0.75 / 0.77
		500 ppm	GM, 1.18 / 1.41	GM, 0.43 / 0.50
	Pea	Control	GM, 1.63	GM, 1.16
		100 ppm	GM, 0.65 / 0.69	GM, 0.52 / 0.49
		250 ppm	GM, 0.39 /0.43	GM, 0.33 / 0.38
		500 ppm	GM, 0.30 / 0.33	GM,0.22 /0.26

GM= Germinated; 1=lupeol; 7= methylbetulinate; 5= lupan [2,3-C]1',2', 5'-oxadiazole

13= 28-carbomethoxy lupan [2,3-C]-1', 2', 5'-oxadiazole

The above experimental results indicated that, in case of rice, the parent compounds (1 and 7) at 100 ppm concentration showed 0.59 cm and 0.61 cm root germination and 0.24 cm and 0.26 cm shoot germination respectively, whereas 0.68 cm root and 0.30 cm shoot germination was observed in the case of control set. Higher concentrations of the compounds gradually reduce the germination of root as well as shoot.

In case of wheat, the parent compounds (1 and 7) at 100 ppm concentration showed 2.52 and 2.60 cm root germination and 0.77 cm and 0.85 cm shoot germination respectively, in comparison to 2.62 cm root and 1.1 cm shoot germination experienced in control set where as 0.68 cm root and 0.30 cm shoot germination was observed in the case of control set. The rate of germinations (root and shoot) was found to be reduced at a higher concentrations of the compounds.

In case of pea, the parent compounds (1 and 7) at 100 ppm concentration showed 1.65 and 1.51 cm root germination and 0.74 cm and 0.69 cm shoot germination respectively, in comparison to 2.6 cm root and 1.16 cm shoot germination observed in control set. The rates of germinations (root and shoot) were found to be reduced at higher concentrations of the compounds.

Similar results were observed when the experiments were carried out with the respective oxadiazole derivatives (Table 3) In case of rice, the oxadiazole derivatives (5 and 13) at 100 ppm concentration showed 0.60 cm and 0.65 cm root germination and 0.25 cm and 0.29 cm shoot germination respectively, whereas 0.68 cm root and 0.30 cm shoot germination was experienced in control set. The 500 ppm concentration of Oxadiazole derivatives slightly reduced the germination of both root and shoot. It showed 0.52 cm and 0.55 cm root germination and 0.10 cm and 0.12 cm shoot germination respectively.

In case of wheat, the oxadiazole derivatives (5 and 13) at 100 ppm concentration showed 2.5 cm and 2.46 cm root germination and 0.91 cm and 0.89 cm shoot germination respectively, whereas 2.62 cm root and 1.1 cm shoot germination was observed in control set. The 500 ppm concentration of oxadiazole derivatives slightly reduced the germination of both root and shoot. They showed 1.18 cm and 1.11 cm root germination and 0.43 cm and 0.50 cm shoot germination respectively.

In case of pea, the oxadiazole derivatives (5 and 13) at 100 ppm concentration showed 0.65 cm and 0.69 cm root germination and 0.52 cm and 0.49 cm shoot germination

respectively, whereas 1.63 cm root and 1.16 cm shoot germination was observed in control set. The 500 ppm concentration of oxadiazole derivatives slightly reduced the germination of both root and shoot. They showed 0.30 cm and 0.33 cm root germination and 0.22 cm and 0.26 cm shoot germination respectively.

Thus it can be concluded that the phytotoxic behavior of the parent natural product and their respective oxadiazole derivatives are similar on the seeds of the plants studied.

Chapter 4

EXPERIMENTAL & REFERENCES

Experimental – Related to Chemical work

All the melting points were determined by open capillary method and are uncorrected. All the microwave reactions were carried out in a 10ml sealed glass tubes in a focused mono-mode microwave oven,"Discover" by CEM Corporation, Matthews, NC at 100W(100°C). The NMR spectra were recorded in $CDCl_3$ solutions at ambient temperature on a Bruker Avance 300 MHz NMR spectrometer using 5mm BBO probe. The chemical shift δ are given in ppm related to tetra methyl silane (TMS) as internal standard. The coupling constant (*J*) are reported in Hz. The IR spectra were recorded in Shimadzu FT-IR spectrophotometer in KBr discs.

General procedure for the synthesis of 2, 3- diketo compounds

3-keto triterpenoids (1mol) suspended in potassium tertiary butoxide (prepared from 6g of potassium and 60ml of tertiary butanol) was shaken in a stream of oxygen for two hours. The reaction mixture was then diluted with water and then 6N HCl was added till the solution was acidic. It was then extracted with $CHCl_3$ (100ml) and the combined extract was dried (Na_2SO_4) and the solvent was removed under reduced pressure to yield a colourless solid which after crystallization from $CHCl_3$-MeOH mixture afforded crystals (0.5g). Some of the prepared diketones were compared with the authentic samples prepared earlier [75, 76].

General procedure for the synthesis of 1, 4-pyrazine derivatives

A solution of the substrate (0.001mol) in dry ethylene diamine was taken in a 10 ml sealed tube. Small pieces of Li (0.001mol) metal were then added to the solution of the sample. The reaction mixture was then irradiated under microwave. The reaction mixture was cooled, excess lithium was destroyed by solid ammonium chloride, diluted with cold water, extracted with ether and purified by column chromatography (over silica gel) followed by crystallization.

Extraction of Friedelin

2 kgs of finely powdered cork was extracted with petroleum ether in a soxhlet apparatus for 24 hours. After removal of the solvent, a white solid separated out. The solid was dissolved in minimum volume of benzene and chromatographed over silica gel column developed with petroleum ether. Elution of the column with petroleum ether gave shining crystals of friedelin [93], m.p. 261-263°C, $[\alpha]_D$ –48.7°.

IR: 1720 cm^{-1}

Auto oxidation of Friedelin

The oxidation was carried out following the general method as described above. The compound obtained was purified (column chromatography) and crystallised from a mixture of $CHCl_3$ – MeOH, m.p. 263-264°C, $[\alpha]_D$ + 16° [Lit 94, m.p. 265-67°C, $[\alpha]_D$ + 16.5°] characterized as 2,3-diketofriedelin by comparison with an authentic sample (m.m.p, CO TLC, CO IR)

	%C	%H
Analysis report found	81.8	10.90
Calculated for $C_{30}H_{48}O2$	81.80	10.90

TNM test: Positive

UV: 269 nm (ε=4.07)

IR: 3600, 3200(OH), 1685 (C=O), 1665, 1610, 840 cm^{-1}

Preparation of 1, 4-pyrazine derivative of friedelin (1b)

The reaction was carried out following the general method as described above. The compound obtained was purified by column chromatography followed by crystallization from a mixture of $CHCl_3$ - MeOH and characterized by spectral analysis.

Table 1. Chromatography of 1, 4 -pyrazine derivative of Friedelin

Eluent	Fractions 50 ml each	Residue	M.P.
Petroleum ether	1- 4	—	—
Pet. ether + ethyl acetate (96:04)	5-10	Solid	228°C

Examination of fraction 5-10 and isolation of compound 1b (1, 4-pyrazine derivative of friedelin)

Crystallization of the compound from $CHCl_3$-MeOH mixture furnished A, analyzed for $C_{32}H_{50}N_2$, m.p. 228°C, IR at 1650-70, 1430, 1120 cm^{-1} for pyrazine ring [69]. UV absorption maxima at 272 nm (ε = 5800) and 278 nm (ε = 5450). Anal. calc.: 83.12% C, 10.82% H; found 83.10% C, 10.81% H. It showed no depression in melting point when mixed with authentic sample of 1, 4-pyrazine derivative of friedelin and was found identical with the original sample of 1, 4-pyrazine derivative of friedelin (mmp, CO IR, CO TLC and spectral data)

UV: λ_{max}^{MeOH} 272 nm (ε= 5800)
278 nm (ε=5450)

IR: ν_{max}^{Nujol} 1650-70, 1430, 1120 cm^{-1}

^{1}H NMR ($CDCl_3$): 0.82-1.22 ppm (7s, 21H, 7t CH_3)
0.99 ppm (d, J = 6.5 Hz)
8.40 and 8.27 ppm (d, 2H, J = 3Hz)

Mass: m/z at 462.40[M^+], 463.40, 464.40.

Preparation of 1, 4 pyrazine derivative of taraxerone (2b)

The reaction was carried out following the general method as described above. The compound obtained was purified by column chromatography followed by crystallization from a mixture of $CHCl_3$ - MeOH and characterized by spectral analysis.

Table 2. Chromatography of 1,4-pyrazine derivative of taraxerone (2b)

Eluent	Fractions 50 ml each	Residue	M.P.
Petroleum ether	1-4	Nil	—
Pet. ether + ethyl acetate (96:04)	5-10	Nil	-
Pet. ether + ethyl acetate (94:06)	11-15	Solid	262°C

Examination of fraction 11-15 and isolation of compound 2b (1, 4-pyrazine derivative of taraxerone)

Crystallization from $CHCl_3$-MeOH mixture afforded B, analyzed for $C_{32}H_{48}N_2$, m.p. 262°C. IR peaks at 1600, 810, 1650, 1430 and 1120 cm^{-1}. The UV absorption maxima at 272nm (ε = 6150) and at 278 nm (ε = 5200). Anal. Calc.: 83.48% C, 10.43% H; found 83.40% C, 10.31% H. Mass spectrum showed molecular ion peak at m/z 460 as base peak. It showed no depression in melting point when mixed with authentic sample of 1, 4-pyrazine derivative of taraxerone and was found identical with the original sample of 1, 4-pyrazine derivative of taraxerone (mmp, CO IR, CO TLC and spectral data).

MeOH

UV: λ_{max} 272nm (ε = 6150)

278 nm (ε = 5200)

Nujol

IR: ν_{max} 1600, 810, 1650, 1430 and 1120 cm^{-1}

^{1}H NMR($CDCl_3$) : 0.81 to 1.25 ppm (8s, 24H, 8t CH_3), at 5.54 ppm (dd, 1H), 2.30 ppm (m, 1H), 8.40, 8.27 ppm (d, 2H, J = 3Hz).

Mass: 460.38, 461.39, 462.39.

Preparation of 1, 4 pyrazine derivative of methyl trichadenate (3b)

The reaction was carried out following the general method as described above. The compound obtained was purified by column chromatography followed by crystallization from a mixture of $CHCl_3$ - MeOH and characterized by spectral analysis.

Table 3. Chromatography of 1, 4-pyrazine derivative of methyl trichadenate (3b)

Eluent	Fractions 50 ml each	Residue	M.P.
Petroleum ether	1-4	Nil	—
Pet. ether + ethyl acetate (96:04)	5-10	Nil	-
Pet. ether + ethyl acetate (94:06)	11-15	Nil	-
Pet. ether + ethyl acetate (90: 10)	16-20	Solid	198°C

Examination of fraction 16-20 and isolation of compound 3b (1, 4-pyrazine derivative of methyl trichadenate)

Crystallization from $CHCl_3$-MeOH mixture afforded C, analyzed for $C_{33}H_{50}N_2O_2$, m.p.198°C. IR peaks at 1730, 1650–70, 1430, 1120 cm^{-1}. UV absorption at 272 nm (ε = 5785) and 278 nm (ε = 5600). Anal. Calc.: 78.26% C, 9.88% H, 5.53% N; found 78.25% C, 9.73% H, 5.50% N. Mass spectrum showed molecular ion peak at m/z 506 as base peak. ^1H NMR spectrum at 0.81, 0.85, 0.93, 0.99, 1.11 and 1.20 ppm (6s, 18H, 6t CH_3), at 0.74ppm (d, 3H, $CHCH_3$, J = 7Hz), at 3.67 ppm(s, 3H), at 2.30 ppm (m, 1H) and at 8.40 and 8.27 ppm (d, 2H, J = 3Hz). Thus from spectral analysis the structure for C has been assigned as 3b. It showed no depression in melting point when mixed with authentic sample of 1, 4-pyrazine derivative of methyl trichadenate and was found identical with the original sample of 1, 4-pyrazine derivative of methyl trichadenate (mmp, CO IR, CO TLC and spectral data)

Extraction of *Xanthoxylum budrunga*: Isolation of lupeol (1).

First collected barks of *Xanthoxylum budrunga* (3 Kg) plant from Darjeeling hilly region and dried it on sunlight and coarsely powdered. Then from these powdered materials the compounds were extracted using benzene as a solvent in a soxhlet apparatus for 30 hours. Benzene was distilled off and the gummy residue (15 g) was taken with ether solution (2 lit). The ether solution was washed by 10% aqueous sodium hydroxide (1.5 lit) solution. The aqueous alkaline layer was thoroughly shaken with ether to remove any neutral components that might be present on it. The portion was washed with water till neutral and dried by using sodium sulphate (Na_2SO_4). Ether was removed when a gummy residue of Lupeol was obtained. This residue dissolved in benzene (45 ml) and placed over a column of silica gel developed with petroleum ether and was eluted with the following solvents (Table 4).

Table 4. Chromatography of the *Xanthoxylum budrunga* extract

Eluent	Fractions each 100 ml	Residue on evaporation	Melting point
Petroleum ether	1—5	Oil	—
Petroleum ether + benzene (80:20)	6—8	Nil	—
Petroleum ether + benzene (70:30)	9—11	Nil	—
Petroleum ether + benzene (60:40)	12—19	Solid	212—213°C

Further elution with more polar solvents did not afford any solid compounds.

Fractions 12—19 (Table 8) were mixed and crystallised by chloroform and methanol mixture. The white powdered compound (m.p. 212– 213°C) obtained was found to be identical[88] (m.m.p, CO IR, and CO TLC) with authentic specimen of lupeol (1).

IR: ν_{max}^{Nujol} 3610, 1020 cm^{-1}

3070, 1640, 887 cm^{-1}

^{1}H NMR($CDCl_3$,): 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) .

^{13}C NMR($CDCl_3$): 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 methylene, five methine and five quarternary carbons.

Hydrogenation of lupeol: Preparation of lupanol (1a)

Lupeol (7 g) dissolved in a mixture of ethyl acetate and acetic acid (100 ml each) was shaken in an atmosphere of hydrogen in presence of PtO_2 catalyst for three hours until absorption of hydrogen ceased. Ethyl acetate was removed by distillation and the solution was diluted with water. A white solid (6.5 g) separated out which was collected by filtration. The solid obtained crystallised by using a mixture of chloroform and methanol which furnished colourless components 1a, m.p. 204°C, $[\alpha]_D$ +15°. The compound did not respond to the TNM test for unsaturation and Beilstein test for halogen indicating the absence of them in 1a. IR spectrum of the compound 1a, showed peak at 3330 cm^{-1} for hydroxyl functional group. The compound 1a was identified as lupanol by comparison (m.m.p., CO IR and CO TLC) with an authentic specimen of lupanol and by preparation of its acetate $C_{32}H_{54}O_2$, m.p. 243-244°C, $[\alpha]_D$ −1.6° [Lit 91 m.p. 245-246°C].

Jone's oxidation of lupanol: Preparation of lupanone (1b)

To a solution of lupanol (6 g) in pure acetone (600 ml) Jone's reagent was added drop wise with constant shaking until a faint orange colour persisted. The mixture was kept at room temperature for 1 h, diluted with water and extracted with ether. The ether layer was washed thoroughly with water, dried (Na_2SO_4) and evaporated. The residue (5.6 g) dissolved in benzene was chromatographed over a column of silica gel (150 g) developed with petroleum ether and then eluted with the following solvents (Table 5)

Table 5. Chromatography of oxidized lupanol residue

Eluent	Fraction 50 ml each	Residue on evaporation	Melting point
Petroleum ether	1–6	Nil	—
Petroleum ether +			

benzene (90:10)	7—11	Nil	—
Petroleum ether + benzene (80:20)	12—16	Nil	—
Petroleum ether + benzene (70:30)	17— 24	solid	209°—210°C

Further elution with more polar solvents did not yield any solid materials.

Fractions 17—24 were mixed and crystallised by using chloroform and methanol mixture. The crystallization furnished colourless solid 1c, m.p. 208°C, $[\alpha]_D$ + 15° [Lit m.p. 210°C, $[\alpha]_D$ +16.2°]

	%C	%H
Analysis report found	84.11	11.82
Calculated for $C_{30}H_{50}O$	84.52	11.74

TNM test: No yellow coloration.

IR: ν_{max}^{Nujol} 1712 cm^{-1} (CO)

Auto oxidation of lupanone

The oxidation was carried out following the general method as described above. The compound obtained was purified (column chromatography followed by crystallization from $CHCl_3$ – MeOH mixture) had m.p. 210-213°C, $[\alpha]_D$ 70.9°C and characterized as 2, 3 diketo lupanone by spectral data and by comparison with the data reported in literature 92[Lit m.p. 210-13°C]

	%C	%H
Analysis report found	81.79	10.90
Calculated for $C_{30}H_{50}O_2$	81.82	10.91

MeOH

UV: λ_{max} 270 nm (ε= 7932)

310 nm (in KOH)

Nujol

IR: ν_{max} 3640 (OH),

1670, 1650, 860 cm^{-1}

Neutral $FeCl_3$ colour test: Positive

Mass: m/z at 440 $[M^+]$, 425 $[M\text{-}CH_3]^+$, 397 $[M\text{-}CH(CH_3)_2]^+$, 312,231, 191, 154, 137, 123, 71, 57 (base peak).

Preparation of 1, 4 pyrazine derivative of lupanone (4b)

The reaction was carried out following the general method as described above. The compound obtained was purified by column chromatography followed by crystallization from a mixture of $CHCl_3$ - MeOH and characterized by spectral analysis.

Table 6. Chromatography of 1, 4 pyrazine derivative of lupanone

Eluent	Fractions 50 ml each	Residue	M.P.
Petroleum ether	1-4	Nil	—
Pet. ether + ethyl acetate (96:04)	5-10	Nil	—
Pet. ether + ethyl acetate (94:06)	11-16	Nil	—
Pet. ether + ethyl acetate (90: 10)	17-24	solid	220°C

Further elution with more polar solvents did not yield any solid materials.

Examination of fraction 17-24 and Isolation of compound 4b (1, 4-pyrazine derivative of lupanone)

Crystallization afforded fine needle shaped crystals of compound 4b, m.p. 220°C. It showed no depression in melting point when mixed with authentic sample of 1, 4-pyrazine derivative of lupanone and was found identical with the original sample of 1, 4-pyrazine derivative of lupanone [89] (mmp, CO IR, CO TLC and spectral data).

	%C	%H
Analysis report found	83.03	10.80
Calculated for $C_{30}H_{50}N_2$	83.12	10.91

UV: λ_{max}^{MeOH} 272 nm (ε= 5831)
278 nm (ε=5063)

IR: ν_{max}^{Nujol} 1650, 1430, 1120 (cm^{-1})

^{1}H NMR($CDCl_3$) : 0.78, 0.83, 0.98, 1.11, 1.29, 1.31, (6S, 18H,6t-CH_3)
0.77 and 0.86(2d, 6H, $CH(CH_3)$, 7 Hz)
2.47 and 3.04(2d, 2H, 2-CH_2, J=16Hz)
8.27 (d, 1H, J= 3 Hz)
8.41(dd, 1H, J=1 and 3 Hz) ppm

Mass: m/z at 462[M^+], 447, 419, 271, 258, 257, 256, 242, 241, 149, 123, 57.

Extraction of *Biscofia javanica* blume: Isolation of betulinic acid (6)

First collected the bark of Biscofia javanica blume from Darjeeling hilly region dried on sunlight and coarsely powdered (2.5 kg). These powdered materials were extracted with benzene in a soxhlet apparatus for 36 hours. Benzene was distilled off and

the gummy residue (12 g) was taken up in ether (1 lit). The ether solution was washed with 10% aqueous sodium hydroxide solution. The aqueous alkaline layer was thoroughly shaken with ether to remove neutral materials present in it. The aqueous layer was acidified (1 lit) when some insoluble solids separated out. The acidified portion was extracted with ether, washed with water until neutral and dried using sodium sulphate. Ether was removed when a gummy residue of betulinic acid (8 g) obtained and chromatographed. Elution by a mixture of benzene and ether (1:4) and crystallised from aqueous methanol afforded betulinic acid, m.p. 301—303°C.

Esterification of betulinic acid: Preparation of methylbetulinate.

To the crude acid (8 g) dissolved in ether was added to a solution of diazomethane in ether prepared from nitrosomethylurea (4 g) and was kept overnight. Next day excess of diazomethane was destroyed by acetic acid (CH_3COOH, 2 ml). The ether solution was washed with water, 10% sodium bicarbonate solution and again with water until neutral and dried by using sodium sulphate. Evaporation of the ether yielded a gummy residue (4g). This crude ester dissolved in benzene (20 ml) was placed over a column of silica gel (100 g) developed with petroleum ether and was eluted with the following solvents (Table 7)

Table 7. Chromatography of the esterified betulinic acid residue

Eluent	Fractions 50 ml each	Residue on evaporation	M. P
Petroleum ether	1—6	Oil	—
Petroleum ether + benzene (90:10)	7—10	Nil	—
Petroleum ether + benzene (85: 15)	11—14	Nil	—
Petroleum ether + benzene (80:20)	15—20	Solid	221°—223°C

Further elution with more polar solvent did not yield any solid materials.

Examination of fractions 15—20: Isolation of methylbetulinate (7).

The solid compound obtained from the fractions 15—20 (Table 11) were mixed (3.8 g) and crystallised from a mixture of chloroform and methanol to afford a colourless needle shaped methylbetulinate, m.p. 221—223°C, $[\alpha]_D$ +5.0°, identical with the original sample (m.m.p, CO IR and CO TLC) (Lit m.p 224—225°C, $[\alpha]_D$ +5.0°)

	% C	% H
Analysis report found	78.71	10.59
Calculated for $C_{31}H_{50}O_3$	79.10	10.71

IR: ν_{max}^{Nujol} 3540 cm^{-1} (—OH)
1730 cm^{-1} ($-COOCH_3$)
1660,
890 cm^{-1} ($=CH_2$)

Hydrogenation of methylbetulinate: Preparation of methyldihydrobetulinate (8).

Methylbetulinate (3.8 g) dissolved in ethyl acetate (200 ml) was shaken in an atmosphere of hydrogen in presence of palladium in charcoal catalyst (200 mg)for three hours until absorption of hydrogen ceased. Ethyl acetate was removed by distillation after filtering off the catalyst. The solution was diluted with water whereas a white solid (3.1 g) Separated out which was collected by filtration. Crystallization from a mixture of chloroform and methanol furnished colourless needle shaped of a compound m.p. 235—237°C, $[\alpha]_D$ +17.0°. This compound was found to be identical with an authentic sample of methyldihydrobetulinate (m.m.p, CO TLC, CO IR).

IR: ν_{max}^{Nujol} 3540 (—OH), 1705 cm^{-1} ($COOCH_3$)

Jone's oxidation of Methyldihydrobetulinate: Preparation of methyldihydrobetulonate (9)

To a solution of Methyldihydrobetulinate (2.95 g) in pure acetone added Jone's reagent drop wise with constant shaking until a faint orange colour persisted. The mixture was kept at room temperature for 1 h, diluted with water and extracted with ether. The ether layer was washed thoroughly with water, dried (Na_2SO_4) and the ether evaporated. The residue (2.5 g) dissolved in minimum volume of benzene was chromatographed over a column of silica gel (50 g). The chromatogram was developed with petroleum ether and eluted with the following solvents (Table 8)

Table 8. Chromatography of the oxidized methyldihydrobetulinate residue

Eluent	Fractions 50 ml each	Residue on evaporation	M.P.
Petroleum ether	1—5	Nil	—
Petroleum ether + benzene (90:10)	6—9	Nil	—
Petroleum ether + benzene (85:15)	10—13	Nil	—
Petroleum ether+ benzene (80:20)	14—23	Solid	196°—198°C

Further elution with more polar solvent did not afford any solid materials.

Fractions 14—23 (Table 8) were mixed and on crystallization from methanol furnished needle shaped crystals of methyldihydrobetulonate, m.p. 190—192°C, $[\alpha]_D$ +8.2°, identical with an authentic sample of methyldihydrobetulonate (m.m.p, CO TLC, CO IR) [90] [Lit m.p. 194°C, $[\alpha]_D$ + 8.4°].

	% C	% H
Analysis report found	79.22	10.56
Calculated for $C_{31}H_{50}O_3$	79.10	10.71

Nujol

IR: ν_{max} 1730 (—COOMe)
1708 cm^{-1} (CO)

Auto oxidation of methyldihydrobetulonate (9)

The oxidation was carried out following the general method as described above. The compound obtained was purified (column chromatography) followed by crystallization from $CHCl_3$ – MeOH mixture afforded colourless crystals, m.p. 131-132oC, $[\alpha]_D$ - 1.94° [95] [Lit m.p. 131-133°, $[\alpha]_D$ - 1.96°]. It gave a positive ferric chloride coloration for diosphenol and was identified as 2, 3-diketomethyldihydrobetulonate (5b).

Nujol

IR: ν_{max} 3460, 1730, 1670, 860 cm^{-1}

MeOH

UV: λ_{max} 271 nm (ε=7829), 310 nm (alkali shift)

Preparation of 1, 4-pyrazine derivative (5b) of methyldihydrobetulonate (9).

The reaction was carried out following the general method as described above. The compound obtained was purified by column chromatography followed by crystallization from a mixture of CHCl3 - MeOH and characterized by spectral analysis.

Table 9. Chromatography of 1,4-pyrazine derivative of methyl dihydrobetulonate (5)

Eluent	Fractions 50 ml each	Residue	M.P.
Petroleum ether	1-4	—	—
Pet. ether + ethyl acetate (96:04)	5-10	nil	
Pet. ether + ethyl acetate (94:06)	11-15	nil	-
Pet. ether + ethyl acetate (90: 10)	16-20	-	-
Pet. ether + ethyl acetate (86: 14)	21-25	-	220°C

Examination of fraction 21-25 and Isolation of compound 5b (1, 4-pyrazine derivative of methyldihydrobetulonate)

Crystallization afforded compound E, $C_{33}H_{50}O_2N_2$. m.p. 220°C. IR spectrum showed peaks at 1710 cm^{-1}(CO_2Me); 1665, 1430 and 1120 cm^{-1}. UV spectrum showed absorption maximum at 272nm (ε = 5712) and 278 nm (ε = 5603). Anal. Calc.: 78.26% C, 9.88% H, 5.53% N; found 78.25% C, 9.73% H, 5.50% N. It showed no depression in melting point when mixed with authentic sample of 1, 4-pyrazine derivative of methyldihydrobetulonate and was found identical with the original sample of 1, 4-pyrazine derivative [98] of methyldihydrobetulonate (mmp, CO IR, CO TLC and spectral data)

MeOH

UV: λ_{max} 272 nm (ε= 5712)

278 nm (ε=5603)

Nujol

IR: ν_{max} 1710 cm^{-1}(CO_2Me); 1665, 1430 and 1120 cm^{-1}

^1H NMR($CDCl_3$): 0.82, 0.985, 0.99, 1.28, 1.305, 0.76 and 0.88 ppm (2d, 6H, $CH(CH)_3$, J = 7 Hz); 2.48, 3.04 ppm (2d, j = 16 Hz); 8.27, 8.41 ppm (2d, J = 3 Hz) and at 3.66 ppm (1s, ester methyl)

Mass: 491$[M\text{-}CH_3]^+$, 463$[M\text{-}CH(CH_3)_3]^+$, 447 $[M\text{-}COOCH_3]^+$, 432,431, 258, 256, 241, 191, 187, 175, 159, 147, 133, 95, 55.

Treatment of lupanone 1c with N-bromosuccinimide: Formation of 2, 2-dibromolupanone 2 and 2α–bromolupanone (3)

A solution of 1c (4 g) was mixed with dimethylsulphoxide (100 ml). N-bromosuccinimide was then added to it in small lots in order to keep the temperature of the reaction mixture below 25°C and the mixture was kept in dark place for 12 days. The mixture was extracted with chloroform and it washed several times with water, dried (Na_2SO_4) and the solvent removed under reduced pressure. The residue (3.7 g) was chromatographed over a column of silica gel (100 g). The chromatogram was developed with petroleum ether and eluted with the following solvents (Table 10).

Table 10 .Chromatography of the lupanone and N-bromosuccinimide mixture

Eluent	Fraction 50 ml each	Residue on evaporation	Melting point
Petroleum ether	1—4	Nil	—
Petroleum ether	5—10	Solid	209°–210°C
Petroleum ether + Benzene (90:10)	11—14	Nil	—
Petroleum ether + Benzene (85:15)	15—18	Nil	—

Petroleum ether + Benzene (80:20)	19—25	Solid	220°—222°C

Examination of fractions 5—10: Isolation of 2, 2-dibromolupanone (2)

The fractions 5—10 (Table 10) showed homogeneity on TLC plate, hence these were mixed (1.7 g) and crystallised from a mixture of chloroform and methanol to afford a needle shaped crystals, m.p. 209—210°C and was identified as 2,2-Dibromolupanone (**2**).

	% C	**% H**
Analysis report found	61.53	13.87
Calculated for $C_{30}H_{48}OBr_2$	61.41	13.32

UV: λ_{max}^{MeOH} 221 nm (ε= 7925) 313 nm (ε=25)

IR: ν_{max}^{Nujol} 1725 cm^{-1} (CO)

CD: λ_{max}^{CHCl3} 239 nm (ϕ= +4590.18), 320 nm (ϕ= −8977.85)

^{1}H NMR ($CDCl_3$): 0.77, 0.94, 1.09, 1.24 (5S, 15H, 5t—CH_3), 0.78 and0.86 (2d, 6H, 2S—CH_3, J= 7 Hz), 3.13 and 3.64 (2d, 2H, CH_2, J=16 Hz) ppm

Mass: m/z at 586, 584, 582 (M+), 567, 569, 571,539,541, 543, 504, 506, 489, 491, 461,463, 426, 425, 424, 409, 285, 283, 274, 231, 206, 205, 191, 171, 163, 123

Examination of fractions 19—25 (Table 10): Isolation of 2α–bromolupanone (3)

The fractions 19—25 (Table 10) were mixed (2.0 g) and crystallised from chloroform and methanol mixture to afford amorphous white solid, m.p. 222—223°C. It showed positive Beilstein test for bromine.

	% C	% H
Analysis report found	71.58	9.33
Calculated for $C_{30}H_{49}OBr$	71.15	9.68

UV: λ_{max}^{MeOH} 226 nm (ε=7015 nm)
309 nm (ε=40 nm)

IR: ν_{max}^{Nujol} 1723 cm−1

CD: λ_{max}^{MeOH} 297 nm (ϕ= 2625.80)

^{1}H NMR ($CDCl_3$): 0.77 (S, 6H, 2—CH_3), 0.92, 1.10, 1.13, 1.2 (4S, 12H, 4t-CH_3), 0.76 and 0.85 (2d, 6H-2S-CH_3, J=7 Hz), 2.65 (dd, 1H, 1-C-H, J= 12 and 6 Hz), 5.06 (dd, 1H, 2—CH, J=12 and 6 Hz) ppm.

Mass: m/z at 506, 504 (M), 491, 489, 463, 461, 426, 425, 285, 283, 274, 206, 191, 163, 149, 123, (base peak)

Treatment of 2,2-dibromo lupanone with hydroxylamine hydrochloride: Preparation of 2, 3-dioximino lupane (4) and the subsequent cyclization of the dioximino derivative to lupan[2,3-C]-1′,2′, 5′-oxadiazole (5)

2,2-dibromo lupanone **2** (1.4 g) dissolved in pyridine was refluxed with hydroxyl amine hydrochloride in ethanol. The compound obtained from the reaction was purified by

repeated crystallization from chloroform-methanol mixture to obtain a white amorphous powder of compound A, analyzed for $C_{30}H_{50}O_2N_2$, m.p. 193°C, $[\alpha]_D$ +21.6°.

IR: ν_{max}^{Nujol} 3200-3400 cm^{-1} (C=N).

UV: λ_{max}^{MeOH} 220 nm (€=5100).

Mass: m/z 469$[M]^+$ 441, 439, 425, 424(base peak), 422, 380, 341, 340, 299, 231, 191, 163, 149, 136, 122, 121, 95, 81, 69

Cyclisation of the dioximino derivative (4) to lupan[2,3-C]-1',2', 5'-oxadiazole (5)

The dioximino derivative, 4 (1 g) and dry DMF (3 ml) was taken in a 10 ml sealed glass tubes in a focused mono-mode microwave oven ("Discover" by CEM Corporation, Matthews, NC) at 100W(100°C) in only 10 minutes reaction time. Small pieces of Li (0.001mol) metal were then added to the solution of the sample. The reaction mixture was then irradiated under microwave. The reaction mixture was cooled, excess lithium was destroyed by solid ammonium chloride and the residue obtained after usual work up was crystallised by chloroform-methanol mixture which afforded compound **5**, analyzed for $C_{30}H_{48}ON_2$ m.p.249-50°C.

Mass: m/z 452, 437 $[M-CH_3]^+$, 409 $[M-CH(CH_3)_2]^+$,(base peak), 367,271, 259, 245, 231, 206, 191, 163, 149, 123, 121, 109, 95, 81, 55

Extraction of *Biscofia javanica* blume: isolation of betulinic acid (6)

First collected the bark of Biscofia javanica blume from Darjeeling hilly region dried on sunlight and coarsely powdered (2.5 kg). These powders were extracted with benzene in a soxhlet apparatus for 36 hours. Benzene was distilled off and the gummy residue (12 g) was taken up in ether (1 lit). The ether solution was washed with 10% aqueous sodium hydroxide solution. The aqueous alkaline layer was thoroughly shaken with ether to remove neutral materials present in it. The aqueous layer was acidified (1 lit) when some insoluble solids separated out. The acidified portion was extracted with ether, washed with water until neutral and dried using sodium sulphate. Ether was removed when a gummy residue of betulinic acid (8 g) obtained and chromatographed. Elution by a mixture of benzene and ether (1:4) and crystallised from aqueous methanol afforded betulinic acid, m.p. 301—303°C.

Treatment of methyldihydrobetulonate with N-bromosuccinimide: Formation of 2, 2-dibromomethyldihydrobetulonate (10) and 2α-bromomethyldihydrobetulonate (11)

A solution of methyldihydrobetulonate (2.2 g) in chloroform (100ml) was mixed with dimethylsulphoxide (50 ml). N-bromosuccinimide (2.5 g) was then added to the solution with constant shaking in order to keep the temperature of the reaction mixture below 25°C and the mixture was kept in dark place for 10 days. The residue (2 g) obtained after usual workup two spots on chromatoplate. So the residue was chromatographed over silica gel column. The chromatogram was developed with petroleum ether and eluted with the following solvents (Table 11).

Table 11. Chromatography of the methyldihydrobetulinate and N-bromosuccinimide residue.

Eluent	Fractions 50 ml each	Residue on evaporation	M.P.
Petroleum ether	1—5	Nil	—
Petroleum ether +	6—12	White solid	157—158°C

benzene (90:10)			
Petroleum ether + benzene (85:15)	13—18	Nil	—
Petroleum ether + benzene (80:20)	19—22	Nil	—
Petroleum ether + benzene (75:25)	23--28	White solid	121—123°C

Further elution with more polar solvent did not yield any solid material.

Examination of fractions 6-12 (Table 15): Isolation of 2,2-dibromomethyldihydrobetulonate (10)

Fractions 6--12 (Table 11) showed homogeneity on TLC plate. They were mixed together (0.7 g) and crystallised by using chloroform and methanol mixture which afforded 2,2-Dibromomethyldihydrobetulonate m.p.161—163°C. It gave positive Beilstein test for halogen.

	% C	% H
Analysis report found	58.86	12.75
Calculated for $C_{31}H_{48}O_3Br_2$	59.05	12.90

IR: ν_{max}^{Nujol} 1725 cm⁻¹ (COOMe)
1705 cm⁻¹ (CO)

UV: λ_{max}^{MeOH} 219 nm (ε=7879)

^{1}HNMR ($CDCl_3$): 0.76 to 1.22 for seven methyl
3.11 and 3.63 (2d, 1H, 1-CH2, J= 16 Hz)
3.65 (s, 3H, COOCH3) ppm.

Mass: m/z at 628,626, 624(M^+), 571569, 567 $[M=COOCH3]^+$ 550,548.547 533, 531, 525, 523, 468, 470,471,453, 412, 411, 410, 409, 283,285, 274, 231, 205, 203, 177(base peak) .

Examination of fractions 23—28 (Table 10): Isolation of 2α-bromomethyl dihydrobetulonate (11).

Fractions 23—28 (Table 11) were mixed (0.9 g) and on crystallization from chloroform methanol mixture afforded crystals of 2α—Bromomethyldihydrobetulonate, m.p. 126-128°C. responded Beilstein test for halogen.

	% C	% H
Analysis report found	67.46	13.40
Calculated for $C_{31}H_{49}O_3Br$	67.64	13.77

IR: ν_{max}^{Nujol} 1725 (COOMe), 1705 cm^{-1} (CO)

^{1}HNMR ($CDCl_3$): 0.94, 0.97, 1.09, 1.13, 1.20 (4s, 12H, 4t-CH_3)
0.77 and 0.87 (2d, 6H, 2S-CH_3, 1-CH_{-e}, J=7 Hz)

2.65 (dd, 1H, 1-CH_{-a}, J=12 Hz)
5.06 (dd, 1H, 2-CH, J=12 and 6 Hz) ppm

Mass: m/z at 550, 548, (M^+) (1:1), 491, 489 $[M=COOCH_3]^+$, 471, 470, 469 [M—Br]; 412, 411 (100%) 410, 395, 275, 260, 250, 205, 191, 177, 174, 119.

Treatment of 2, 2-dibromomethyl dihydrobetulonate with hydroxylamine hydrochloride: Preparation of 28-carbomethoxy-2,3-dioximinolupane (12) and the subsequent cyclization of the dioximino derivative to 28-carbomethoxy lupan[2,3-C]-1',2', 5'-oxadiazole (13)

2,2-dibromomethyldihydrobetulonate (0.8 g) dissolved in pyridine was refluxed with hydroxyl amine hydrochloride in ethanol. The compound obtained from the reaction was purified by repeated crystallization from chloroform-methanol mixture to obtain a white amorphous powder (0.7 g), analyzed for $C_{32}H_{53}O_4N_2$.

UV: λ_{max}^{MeOH} 220 nm (€=5100).

IR: ν_{max}^{Nujol} 3200-3400 cm^{-1} (C=N) 1720 cm^{-1} (–COOMe)

Mass: m/z 529[M]+ m/z 530, 531, (base peak), 424, 380, 341 340, 299, 231, 191, 163, 149, 136, 122, 121, 95, 81, 69.

Cyclisation of the dioxime to 28-carbomethoxy lupan [2,3-C]-1',2', 5'-oxadiazole

The dioximino derivative (0.7 g) and dry DMF was taken in a 10 ml sealed glass tubes in a focused mono-mode microwave oven ("Discover" by CEM Corporation, Matthews, NC) at 100W(100°C) in only 10 minutes reaction time. Small pieces of Li (0.001mol) metal were then added to the solution of the sample. The reaction mixture was then irradiated under microwave. The reaction mixture was cooled, excess lithium was destroyed by solid ammonium chloride and the residue obtained after usual work up was crystallised by chloroform-methanol mixture which afforded compound D analyzed for $C_{31}H_{48}O_3N_2$.

UV: λ_{max}^{MeOH} 223 nm (€=5169)

Nujol

IR: ν_{max} 1620 cm^{-1} (—C=N-O)

890 cm^{-1}, 1720 cm^{-1} (–COOMe)

^{1}HNMR ($CDCl_3$): 77 (d, 3H, J=7 Hz) 0.78, 0.86 (d, 3H, J=7 Hz), 0.97, 1.10

Mass: 498,(base peak) 496 m/z 449.7 $[M-CH_3]^+$, 367,271, 259, 245, 231, 206, 191, 163, 149, 123, 121, 109, 95, 81, 55.

Biocidal work

Details of the experimental procedure have been described in the Experimental section of Part I, Chapter 4.

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