

Bioactivity and Structure of Metabolites From Marine Organisms

*Thesis submitted for the degree of
Doctor of Philosophy in Chemistry
to
Goa University*

By

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Declaration

As required under the University ordinance 0.19.8 (vi), I state that the present thesis entitled "Bioactivity and structure of metabolites from marine organisms" is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge the present study is the first comprehensive work of its kind from the area mentioned. The literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

Swt. dulla

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This is to certify that all suggestions and corrections made by the viva-voce examination board have been incorporated in the thesis.

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Certificate

This is to certify that the thesis entitled "Bioactivity and structure of metabolites from marine organisms", submitted by Mr. Ammar A. Al-Fadhli for the award of the degree of Doctor of Philosophy in Chemistry is based on his original studies carried out by him under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any University or Institution.



**Dr. Solimabi Wahidulla
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The happiest moments of my life are the ones, which I have passed at home in the bosom of my family. Good, honest, hardheaded character is a function of home. If the proper seed is sown there and properly nourished for few years, it will not be easy for that plant to be uprooted. All that I am today is what my parents have given me. I have no adequate words to express my love and gratitude to my parents therefore I dedicate this thesis to my dear parents as an expression of my love and care for them.

Finally to all those that have consciously and sub-consciously helped friends, acquaintances, colleagues and me, I wish to thank all of them for the support, confidence and love given to me over the years to fulfill my dreams.

Ammar A. Al-Fadhli

GENERAL REMARKS

Compounds used are commercially available. All the solvents used were dried and freshly distilled. All melting points were measured on a digital melting point apparatus (Electothermal 9100) and were uncorrected.

Silica gel 60 F₂₅₄ (Merck, 0.2mm) TLC plates (aluminium sheets) were used. Silica gel (Merck, 60-120 mesh, 200-400 mesh) was used for column chromatography.

UV-Vis spectrophotometer (Shimadzu-2401) was used to record λ_{\max} . Infra red spectra were taken on Shimadzu FTIR-8201 spectrophotometer while ¹H NMR and ¹³C NMR spectra including 2D experiment (COSY, TOCSY, HMQC and HMBC) were recorded on Bruker 300 MHz spectrometer using TMS as internal standard unless otherwise stated. ESI-MS/MS spectra were recorded on QSTARXL (Applied Biosystems, Canada) mass spectrometer. Optical polarimeter (ADP220 polarimeter, Bellingham and Stanley Ltd.) was used to record optical rotation.

All figures, tables, structures numbers and references in a section refer to that particular section only.

Abbreviations

α	Alpha
Amu	Atomic mass unit
br s	broad singlet
β	Beta
c	Concentration (g/100 ml)
$^{\circ}\text{C}$	Degrees celsius
CAD	Collisionally activated dissociation
CDCl_3	Deuterated chloroform
CD_3OD	Deuterated methanol
CHCl_3	Chloroform
CH_2Cl_2	Dichloromethane
CID	Collision induced dissociation
cm	10^{-2} metre
COSY	Correlated spectroscopy
d	doublet
dd	Doublet of doublet
δ	NMR chemical shift (ppm)
D	Dextra rotatory isomers
1D	One dimensional
2D	Two dimensional
Da	Dalton
DCM	Dichloromethane
DEPT	Distortionless enhancement by polarisation transfer
ED_{50}	Effective dose 50%
e.g.	example given
ESI-MS	Electrospray ionisation-Mass spectrometry
EtOAc	Ethyl acetate
FAB	Fast atom bombardment
g	gram
GC	Gas chromatography
GI_{50}	Growth inhibitory power
HIV-1	Human immunodeficiency virus 1
HMBC	Hetero nuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
HSQC	Heteronuclear single bond correlation
HPLC	High performance liquid chromatography
HR	High resolution
Hz	Hertz
I_2	Iodine
IC_{50}	Inhibitory concentration 50%
IR	Infrared spectroscopy
J	Spin-spin coupling constant [Hz]
$^2J_{\text{CH}}/^3J_{\text{CH}}$	1,2 and 1,3 carbon-proton correlations
KB cells	Human carcinoma of the nasopharynx
KBr	Potassium bromide
Kg	Kilogram
LCB	Long chain base
L	Laevo rotatory isomer

L-1210	Lymphocytic leukemia
LSI-MS	Liquid secondary ionization-Mass spectrometry
m	multiplet
MeOH	Methanol
mg	Milligram
MHz	Megahertz
min	minute
MIC	Minimal inhibitory concentration
ml	10 ⁻³ litre
mM	10 ⁻³ Mol
m.p.	melting point
m/z	mass to charge ratio (amu)
μg	10 ⁻⁶ gram
μL	10 ⁻⁶ litre
μm	10 ⁻⁶ metre
μM	10 ⁻⁶ Molar
ε	Molar absorptivity
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
nm	10 ⁻⁹ metre
NMR	Nuclear magnetic resonance
nOe	Nuclear Overhauser enhancement
NOESY	Nuclear Overhauser enhancement spectroscopy
ODS	Octadecylsilane
[α] _D	Optical rotation
%	Percentage
P-388	Mouse leukemia
ppm	Parts per million
q	quartet
RP	Reversed phase
RT	Room temperature
R _t	Retention time
RF	Retardation factor
s	singlet
sp.	species
t	triplet
TAG	Triacylglycerol
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
TOCSY	Total correlated spectroscopy
TOF	Time of flight
UV	Ultra Violet
V	Volt
λ	Wavelength (nm)
v	Wave number (cm ⁻¹)

Introduction

The oceans have enormous potential to provide new therapeutic agents mainly because they cover approximately 71% of earth's surface with more than 300,000 known species of fauna and flora comprising 34 out of 36 living phyla so far identified¹. Research over the past 30 years has shown that marine organisms are indeed a good source of natural products with exceptionally diverse chemical structures. This diversity of chemical compounds is believed to be the consequence of the competition between organisms for space and resources in most marine habitats.

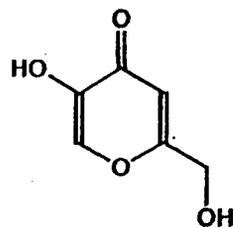
Marine natural products being reported encompass a wide variety of chemical structures including acetogenins, terpenes, steroids, alkaloids, peptides and many other compounds of mixed biogenesis. The structures of compounds derived from marine environment vary from simple to highly complex. Therapeutic potential of these compounds has been explored with few of them reaching drug stage.

The discovery of marine natural products with potential as therapeutic agents require a multidisciplinary team approach involving biologists, pharmacologists, virologists, microbiologists and natural product chemists.

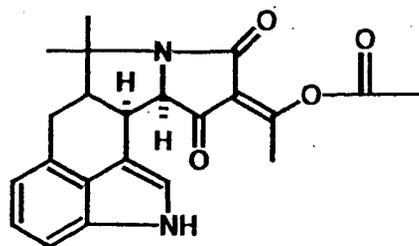
Research program at NIO under the title "Bioactive Substances from the sea" is relevant to defining the roles and biomedical applications of the unique molecules produced by marine life. Program is integrated mainly to isolate, identify and assess the potential of these unique molecules in the treatment of human diseases, the main focus being on anti-infectives and anticancer agents.

A part of this program explores associated marine microorganisms, bacteria and fungi, as an unexplored chemical diversity. This study has illuminated an entirely new source of naturally derived anti-infective compounds. Over the past few years' studies led to the discovery of new sources of industrially important chemicals besides identification of a number of new molecules. Thus, kojic acid (1), a product being widely used in cosmetics as whitening agent and as flavouring agent in foods was obtained from *Aspergillus-sulphureus*². This fungus also yielded a new antifungal metabolite, acetyl derivative of cyclopiazonic acid (2). *Fusarium nivale* (Fres.) Ces was a new source of immunosuppressive cyclosporin A (3)³. (+)-(R, R)- Terrein(4) and (-)-(S, S)-Terrein(5) was yet another metabolic

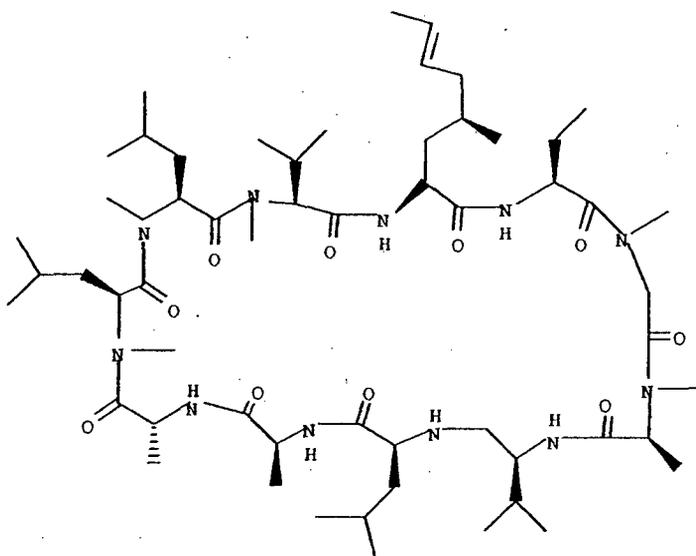
product of *Aspergillus terreus*⁴; with the nephrotoxic antibiotic citrinin(6)⁵ and neoechinulin A(7)⁶ being obtained from *Penicillium chrysogenum*.



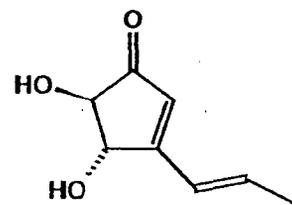
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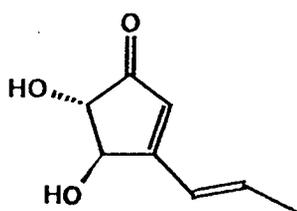
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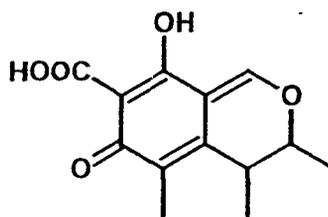
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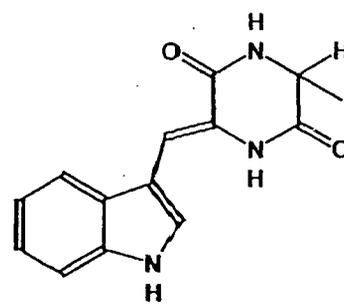
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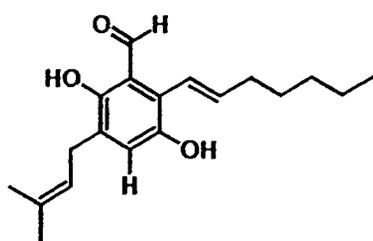


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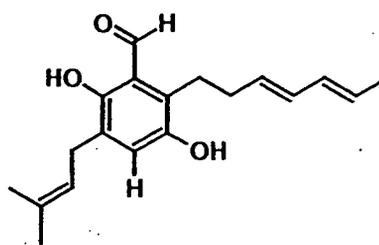


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Fungal strain of *Eurotium sp.* yielded tetrahydroauroglaucin [2-(1E-heptenyl)-3,6-dihydroxy-5-(3-methyl-2-butenyl)-benzaldehyde](8) and isodihydroauroglaucin [2-(3E,5E-heptadienyl)-3,6-dihydroxy-5-(3-methyl-2-butenyl)-benzaldehyde](9)⁷.



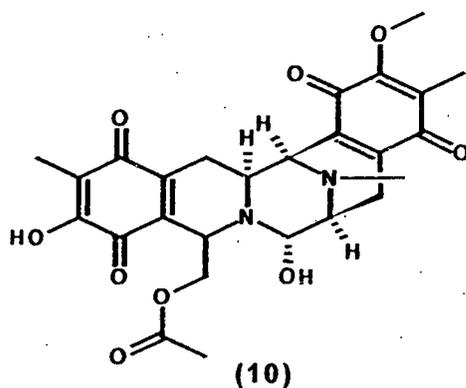
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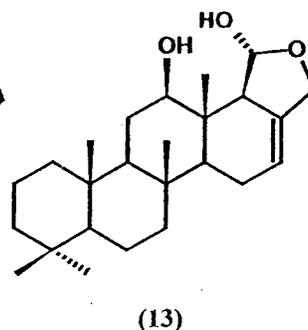
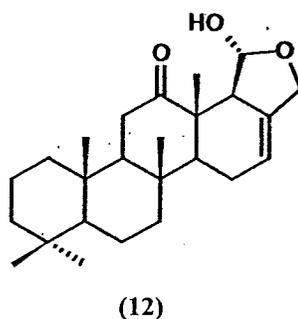
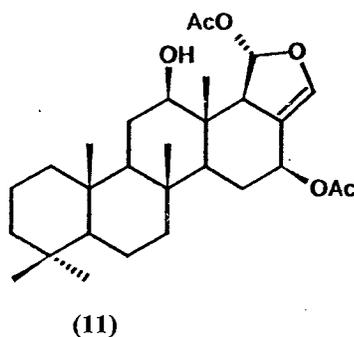
Compounds 8 & 9 were known to possess anti-oxidant properties and exhibit synergism with tocopherol.

A collaborative program with the CNR Institute of Biomolecular Chemistry, Naples uses different strategy. It studies the bioactivity of the molecules based on the chemical defense of the organisms specially the nudibranchs against the predators. These investigations led to the development of a molecule, an antitumor isoquinoline alkaloid, jorumycin (10) identified from the Pacific nudibranch *Jorunna funebris* as anticancer agent⁸. It is antitumor against HT29-human colon carcinoma, A549-human lung carcinoma, Me129-human melanoma, P 388-mouse lymphoma with IC₅₀ of 1.5 ng/ml. It has been licensed to pharmaceutical industry and is in clinical trial stage 1 under the trade name of Zalypsis⁹. Zalypsis is a novel chemical entity related to the marine natural compound jorumycin and the family of renieramycins, obtained from molluscs and blue sponges, such as *Reniera*, *Haliclona cibricutis* and *Xestospongia* sp. Zalypsis binds to DNA and is cytotoxic; however, it does not activate the "DNA damage checkpoint" response. Thus, Zalypsis has cytotoxic effects dependent on DNA binding that are not associated with DNA damage. In pre-clinical trials, Zalypsis demonstrated strong *in vitro* and *in vivo* antitumour activity in a wide variety of solid and haematological tumour cell lines and human transplantable breast, gastric, prostate and renal xenografted tumours. Zalypsis also demonstrated a manageable and reversible preclinical toxicology profile.

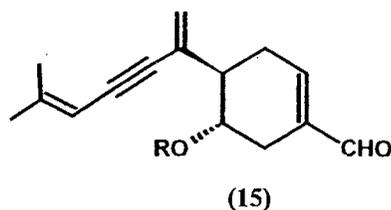
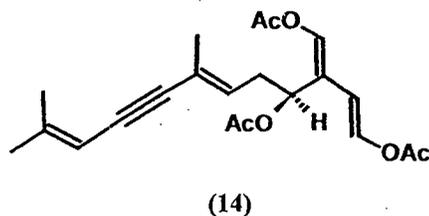


Jorumycin is also antimicrobial against *Bacillus subtilis* and *Staphylococcus aureus* at a concentration of 0.050 $\mu\text{g/ml}$, with an inhibition zone of 16mm but inactive against *Escherichia coli* at the same concentration.

This collaboration has also resulted in identification of a series of novel molecules. Thus, nudibranch *Glossodoris atomarginatum*¹⁰ yielded the known sesterterpene heteronemin (11) and two new scalarans (12-13).

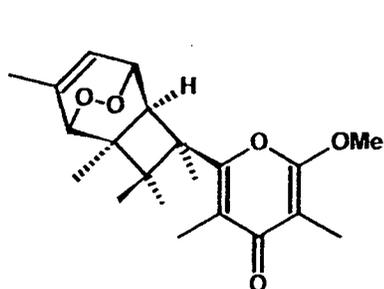


The sacoglossan *Volvatella* (Pease 1860), found grazing on siphonacean algae, genus *Caulerpa* contained the known farnesane sesquiterpenoid, caulerpenyne (14), a caulerpalean metabolite, in addition to a more polar and highly unstable caulerpenyne derivative characterized as volvatellin (15)¹¹.

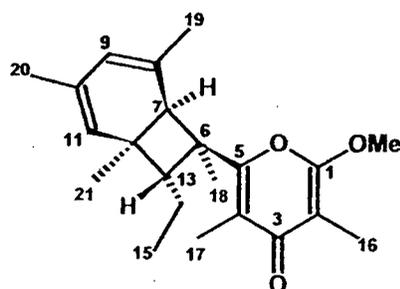


Sacoglossans of genus *Elysia* that feeds upon the green algae of genus *Bryopsis* like *Elysia rufescens*, *Elysia ornata* and *E. grandifolia* are able to accumulate and modify toxic depsipeptides kahalalides from the algal diet. Kahalalide F (16) is a major depsipeptide of *Bryopsis* sp, which the mollusk acquires via its diet. It is the

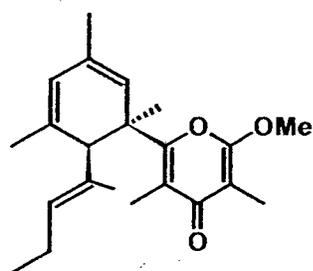
ring¹⁵ together with the known propionates 9,10-deoxy-tridachione (22)¹⁶, photodeoxytridachione(23)¹⁷, tridachiahypopyrone B(24) and C (25)¹⁸ and iso-9,10-deoxy-tridachione (26)¹⁹ reported earlier from the same mollusk of Pacific region. Photodeoxy-tridachione (23) has shown activity in an ichthyotoxicity assay at 5 ppm. These compounds are antioxidants.



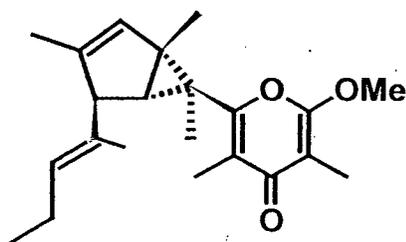
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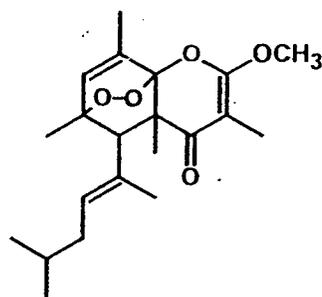
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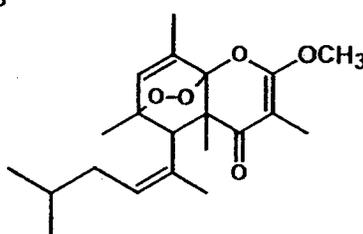
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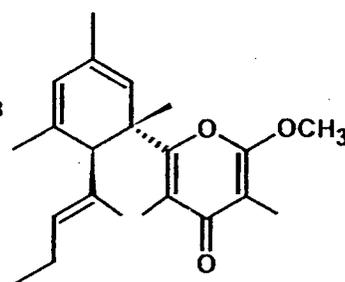
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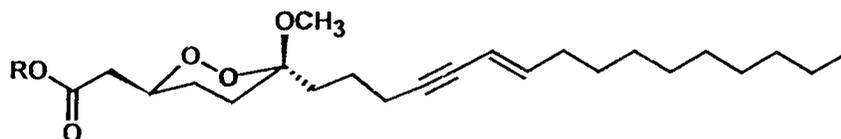


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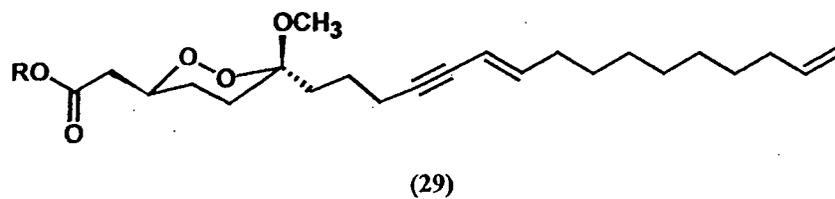
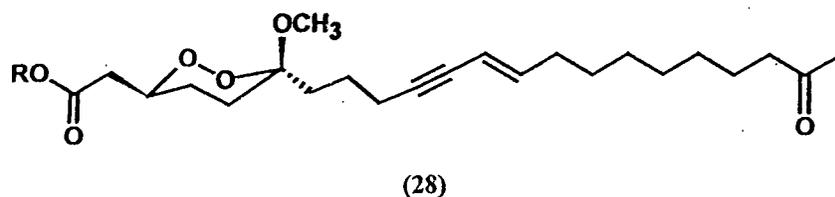


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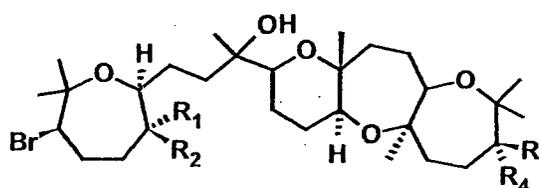
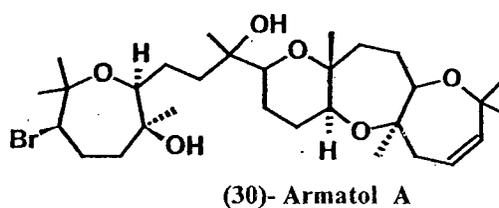
The sponge *Acarinus bicladotylota* yielded two new acetogenin peroxides named peroxyacarnic acid C (27) and D (28) besides the known peroxyacarnic acid A(29)²⁰.



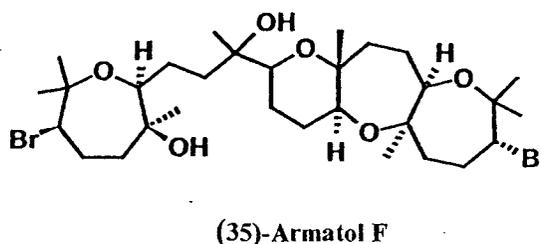
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Cytotoxic and antiviral alga, *Chondria armata* led to novel cytotoxic triterpenoid polyethers designated as armatols A-F²¹ besides known pigment caulerpin and lipids.



- (31)-R1=Me, R2=OH, R3=H, R4=Br (armatol B)
 (32)-R1=OH, R2=Me, R3=H, R4=Br (armatol C)
 (33)-R1=Me, R2=OH, R3=Br, R4=H (armatol D)
 (34)-R1=OH, R2=Me, R3=Br, R4=H (armatol E)



A part of this program funded by Department of Ocean Development searches the bioactive molecules from marine flora and fauna in collaboration with Central Drug Research Institute, Lucknow; Post Graduate Institute of Biological and Medical Sciences, Madras; Indian Institute of Chemical Technology, Hyderabad and six other laboratories. The main objectives are: to isolate and characterize

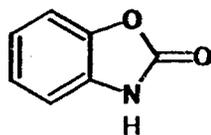
chemical components and to assess the pharmaceutical potential of these compounds through microbiological and pharmacological tests and to use such biologically active compounds as models for laboratory synthesis.

Under this program NIO has a sample acquisition team that is responsible for the collection of marine organisms. The macro organisms like seaweeds, sponges, soft corals and sea anemones in our collection have been obtained from depths ranging from intertidal to 30 meters depth by hand picking and scuba diving respectively. So far we have collected more than 800 organisms.

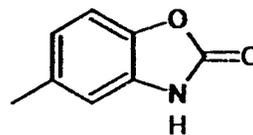
A team of pharmacologists, virologists and biochemists at CDRI, Lucknow evaluates the potential drug targets and design appropriate *in vitro* and *in vivo* models for evaluating the pharmaceutical potential of crude alcoholic extracts of the organisms collected. The team at Madras and Hyderabad are engaged in testing for cytotoxic and antimicrobial and insecticidal activities respectively.

A team of natural product chemists is engaged in purifying and determining the structure of the natural organic compounds including the active principles present in the organisms.

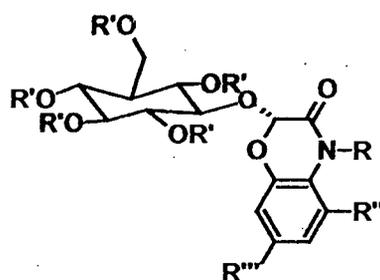
This study has provided a base line data on the potency of different marine organisms. Investigations under this program led to the identification of analgesic and anti-inflammatory benzoxazolin-2-one(36)²² and the benzoxazinoids(37-38)^{23,24} from the mangrove *Acanthus illicifolius*. Benzoxazolin-2-one(36) analogous were synthesized in order to increase its potency as the lead molecule against the protozoa *Leishmania donovani*²⁵ being as effective as pentamidine, the only drug though toxic being used clinically against *Kala-azar*.



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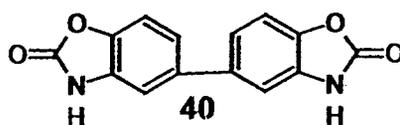
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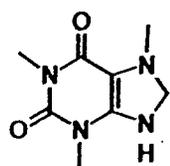
(37) R=OH ; R'=R'' = R'''=H.

(38) R=H ; R'=R'' = R'''=H

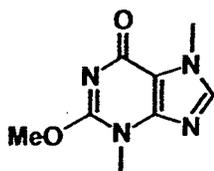
The synthetic analog 5-methyl benzoxazolinone-2-one(39) was antispasmodic against spasmogens acetyl choline, histamine, 5-hydroxytryptamine and barium chloride²⁶. A bis-benzoxazolinone(40) has also been identified as a metabolites of *Acanthus illicifolius*²⁷.



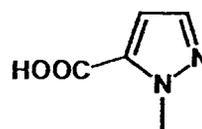
Antifouling xanthine derivative caffeine (1,3,7-trimethylxanthine)(41) and its 2-O-methyl analog (42) was identified from the gorgonian *Echinomuraceae splendens* which also was found to contain N-methyl-pyrazole-5-carboxylic acid (43)²⁸.



(41)

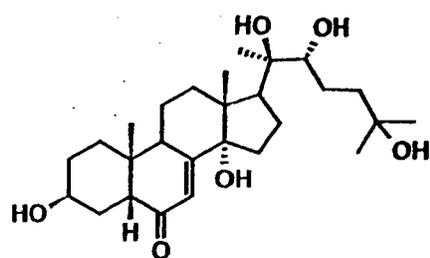


(42)

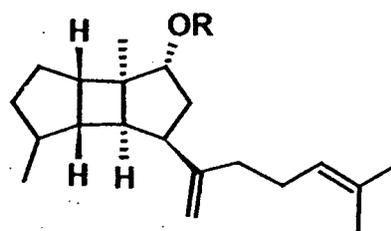


(43)

A steroidal hormone, 2-deoxy-hydroxyecdysone (44), with promising oxytocic activity, and potency higher than the clinical standard, oxytocin and PGF_{2α}, was identified from a marine *Zoanthus sp*²⁹. Novel antibacterial diterpenoid stoechospermol (45) and its acetate (46) have been reported from the alga *Stoechospermum marginatum*^{30,31}.

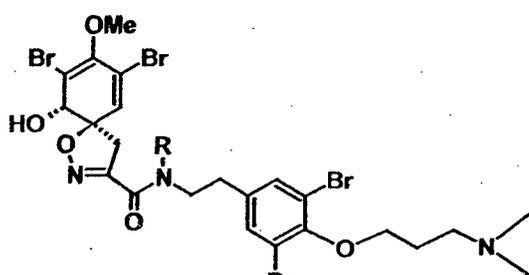


(44)

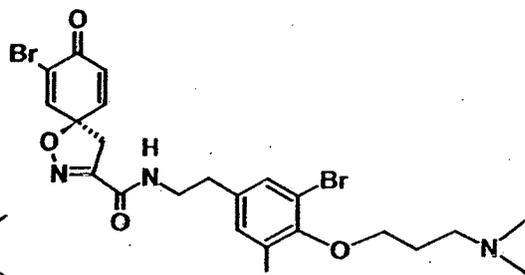


(45) R = H, (46) R = Ac.

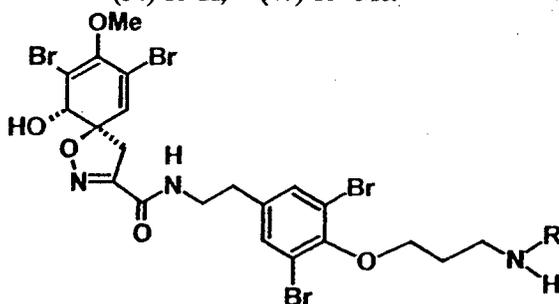
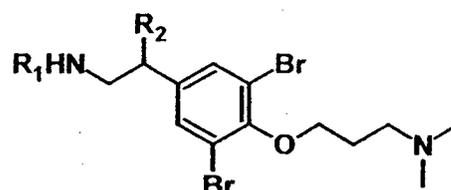
Seven new bromotyrosine alkaloids purpurealidin A, B, C, D, F, G and H (47-53) along with the known compounds purealidin Q (54), purpurealidin E (55), 16-debromoaplysamine-4 (56) and purpuramine I (57) have been identified from marine sponge *Psammaplysilla purpurea*. Purpurealidin B, 16-debromoaplysamine-4 and purpuramine I were antibacterial against *E. Coli*, *S. aureus*, *V. cholerae*. Purpurealidin B, 16-debromoaplysamine-4 were also active against *Shigella flexineri* and *Salmonella typhi* while purealidin Q was bactericidal only against *Salmonella typhi*³².

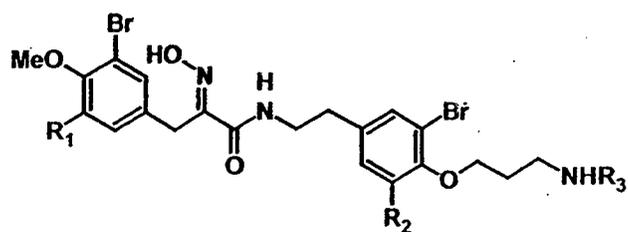


(54)-R=H, (47)-R= Me.



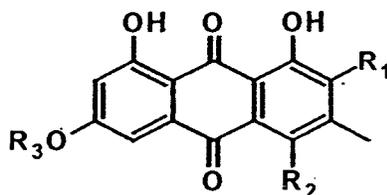
(48)

(49) - R=CO(CH₂)₁₁CH(CH₃)₂
(50) - R=CO(CH₂)₁₂CH₂CH₂CH₃(51) - R₁=H, R₂=OH
(52) - R₁=COCH₂CH₃, R₂=OH
(55) - R₁=R₂=H.

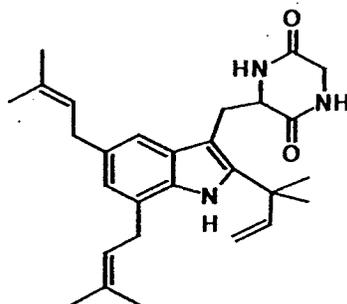


(53) - $R_1 = \text{Br}$, $R_2 = \text{H}$, $R_3 = \text{Me}$. (56) - $R_1 = \text{Br}$, $R_2 = R_3 = \text{H}$.
 (57) - $R_1 = \text{H}$, $R_2 = \text{Br}$, $R_3 = \text{Me}$.

Fungus, *Eurotium* sp, isolated from the mangrove plant *Porteresia coarcatata* (Roxb) when grown on potato dextrose agar (PDA) produced known biologically active anthraquinones: physcion(58), fluoroglucin(59), catenarin(60) and alaternin(61) beside a cyclic dipeptide echinulin(62) with a triprenylated indole moiety. These compounds are fungal/angiosperm metabolites known to be anti-bacterial, anti oxidant and cytotoxic³³.

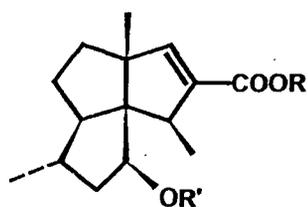


(58) - $R_1 = R_2 = \text{H}$, $R_3 = \text{CH}_3$; (59) - $R_1 = \text{H}$, $R_2 = \text{OH}$, $R_3 = \text{CH}_3$.
 (60) - $R_1 = R_3 = \text{H}$, $R_2 = \text{OH}$; (61) - $R_1 = \text{OH}$, $R_2 = R_3 = \text{H}$.



(62).

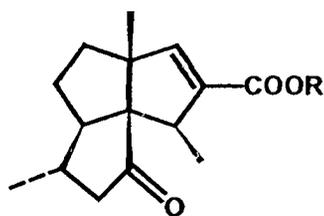
The cardiotoxin, subergoric acid and its analogs(63-67) were identified from the soft coral *Subergorgia suberosa* from Mandapam coast³⁴. Same group identified two heteroaromatic acids, pyrazole-3(5)-carboxylic acid and 4-methylpyrazole-3(5)-carboxylic acid and β carboline from *Tedania anhelans*³⁵.



(64); R = Me, R' = H

(66); R = Me, R' = COMe

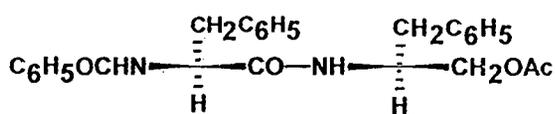
(67); R = R' = H



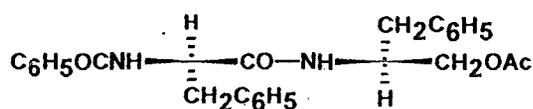
(63); R = H.

(65); R = Me.

The red alga *Acantophora spicifera* with anti-fertility activity contained rare stereoisomeric dipeptides aurantiamide acetate (68) and dia-aurantiamide acetate(69).

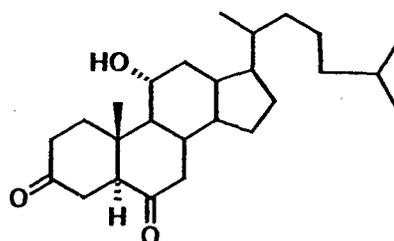


(68)



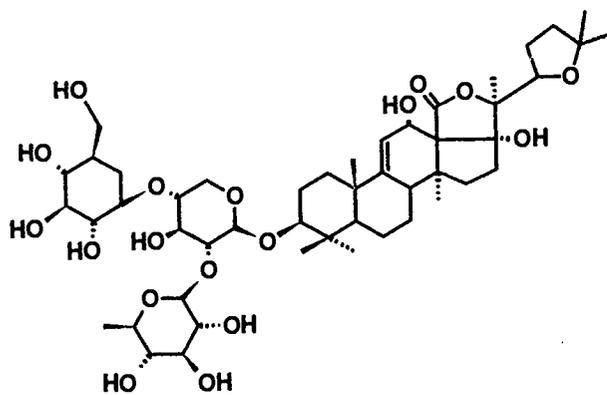
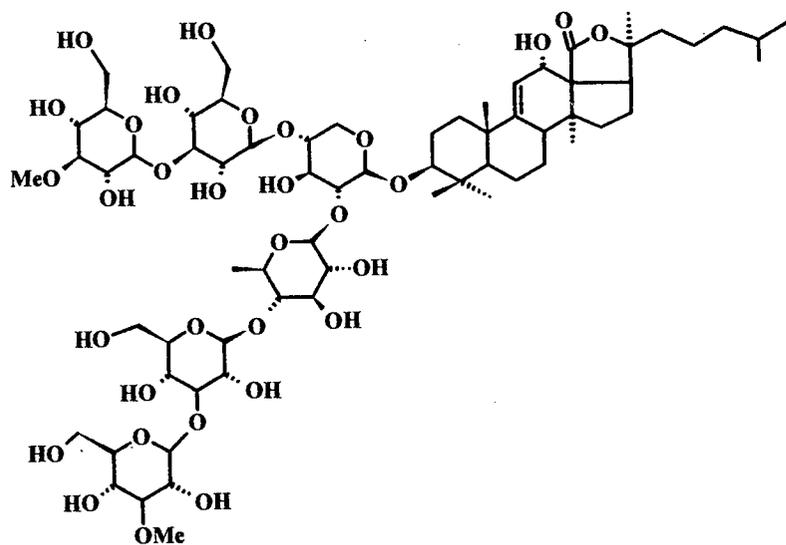
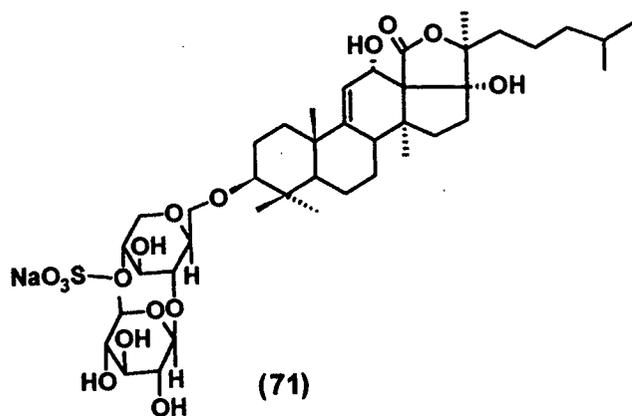
(69)

This was the first report of the natural occurrence of dia-aurantiamide acetate (69) and also the presence of two isomers of the same compound from the same source³⁶. The alga was also found to be a source of 5 α - cholstane-11 α - hydroxy-3,6-dione(70)³⁷ beside 5 α - cholestane-3,6-dione³⁸.



(70)

Toxic principle of methanolic extract of the sea cucumber from Lakshadweep, *Actinopyga mauritiana* was identified as a glycoside, echinoside-B(71)³⁹. The same group identified two novel triterpene glycosides (72-73) as selective inhibitors of chemokine receptor-5 from the sea cucumber *Telenata ananas*⁴⁰.



As a student of Goa University author had an opportunity to work with the Marine Natural Product Chemists at NIO. During the course of the research author was involved in the isolation, structural elucidation and screening for anti-infective

properties of metabolites identified from marine organisms. The investigations carried out on some selected marine organisms are the subject of present thesis. The work presented in the thesis has been divided into three chapters.

Chapter I deals with the chemical investigations of the red alga *Chondria armata* a seaweed belonging to family Rhodomelaceae. It has been subdivided into two sections:

Section 1 : Lipids – Glycerolipids and steroids are discussed here.

Section 2 : Squalene derived triterpene- polyethers have been described in this section.

Chapter II- This chapter deals with the identification of secondary metabolites from the mangrove plants. It has been divided into three sections.

Section 1: Antimicrobial constituents of *Lumnitzera racemosa*. This section has been divided into two parts:

Part-1: Antimicrobial activity of the tonga mangrove, *Lumnitzera racemosa*.

Part-2: Chemical investigation of the active n-butanol fraction by Tandem mass spectrometry.

Section 2: Metabolites of mangrove plant *Aegiceras corniculatum*.

Section 3: Chemical constituents of *Sesuvium portulacastrum*.

Chapter III - Chemical investigations of the sponge *Cinachyra cavernosa* has been incorporated in this chapter.

It has been divided into two sections.

Section 1: Steroids identified from the chloroform fraction, as steryl acetates, by GC-MS have been included in this section.

Section 2: Peptides from the butanol fraction tentatively identified solely on the basis of analysis of peptide rich fraction by ESI-MS/MS have been dealt within this section.

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Chapter I

Chemical investigations of the red alga Chondria armata

Chondria armata: A review.

Marine environment has proven to be a rich source of natural products with novel structures; it is also a potentially rich source of therapeutically useful agents. Among the marine organisms marine algae are one of the largest producers of biomass in the marine environment that produce a wide variety of chemically active metabolites in their surroundings, potentially as an aid to protect themselves against other settling organisms. These active metabolites, also known as biogenic compounds, produced by several species of marine macro- and micro-algae, have antibacterial, antialgal, antimacrofouling and antifungal properties, which are effective in the prevention of biofouling and have other likely uses, e.g. in therapeutics. The isolated substances with potent antifouling activity belong to groups of fatty acids, lipopeptides, amides, alkaloids, terpenoids, lactones, pyrroles and steroids. These biogenic compounds have the potential to be produced commercially using metabolic engineering techniques. Therefore, isolation of biogenic compounds and determination of their structure could provide leads for future development of not only, environmentally friendly antifouling agents, but also serve as new and more effective therapeutic agents.¹

Algae are important as primary producers of organic matter at the base of the food chain. They also provide oxygen for other aquatic life. Algae may contribute to mass mortality of other organisms, in cases of algal blooms, but they also contribute to economic well-being in the form of food, medicine and other products. In tropical regions, coralline algae can be as important as corals in the formation of reefs. Approximately there are about 30,000 known species of algae, but the actual number of species probably exceeds this. Today the algae are classified into seven Phyla, based on their colour, type of chlorophyll, form of food storage substance and cell wall composition² (Table 1):

Table 1: Classification of Algae

SEVEN PHYLA OF ALGAE				
PHYLUM	THALUS FORMAT	PHOTOSYNTHETIC PIGMENTS	FORM OF FOOD STORAGE	CELL WALL COMPOSITION
<i>Chlorophyta</i> (Green Algae)	Unicellular, Colonial, Filamentous, and Multicellular	Chlorophylls <i>a</i> and <i>b</i> , Carotenoids	Starch	Polysaccharides, Primarily Cellulose
<i>Phaeophyta</i> (Brown Algae)	Multicellular	Chlorophylls <i>a</i> , and <i>c</i> , Carotenoids, Fucoxanthin	Laminarin (an o carbohydrate)	Cellulose with Alginic Acid
<i>Rhodophyta</i> (Red Algae)	Multicellular	Chlorophylls <i>a</i> , Phycobilins, Carotenoids	Starch	Cellulose or Pectin, many with Calcium Carbonate
<i>Bacillariophyta</i> (Diatoms)	Mostly Unicellular, Some Colonial	Chlorophylls <i>a</i> and <i>c</i> , Carotenoids, Xanthophyll	Leucosin (an oi carbohydrate)	Pectin, many with Silicon Dioxide
<i>Dinoflagellata</i> (Dinoflagellates)	Unicellular	Chlorophylls <i>a</i> and <i>c</i> , Carotenoids	Starch	Cellulose
<i>Chrysophyta</i> (Golden Algae)	Mostly Unicellular, Some Colonial	Chlorophylls <i>a</i> and <i>c</i> , Xanthophyll, Carotenoids	Laminarin (an o carbohydrate)	Cellulose
<i>Euglenophyta</i> (Euglenoids)	Unicellular	Chlorophylls <i>a</i> and <i>b</i> , Carotenoids, Xanthophyll	Paramylon (a Starch)	No Cell Wall, Protein-rich Pellicle

Traditionally, the red algae (*Rhodophyta*) were divided into two classes the *Bangiophyceae* and *Florideophyceae*. Alternatively, a single class, the *Rhodophyceae* and two subclasses, *Bangiophycidae* and *Florideophycidae* are used. Based on ultrastructure and molecular evidence the *Bangiophyceae* is now accepted as a paraphyletic group, while the *Florideophyceae* is considered to be monophyletic based on two synapomorphic characters presence of a filamentous gonimoblast and tetrasporangia³. Since this chapter deals with the identification of chemical constituents of the alga *Chondria armata*, collected off Goa coast (India) during the low tides a review of literature on the metabolites from this alga is presented here. The alga belongs to phylum *Rhodophyta*, class *Florideophyceae* and family *Rhodomelaceae*.

Domoic acid (DA) (1) an insecticidal agent, was the first compound to be isolated from *Chondria armata* in Japan⁴, and is named after the Japanese word for this seaweed, "domoi". It was later identified in the rhodophytes, *Alsidium corallinum*, from the east coast of Sicily⁵, and *Chondria baileyana*, from southern Nova Scotia and PEI, Canada⁶. It is also known to be a constituent of *Amansia glomerata*, *Digenea simplex* and *Vidalia obtusiloba*, all belonging to the family *Rhodomelaceae*⁷. DA belongs to a group of amino acids called the kainoids, which are classed as neuroexcitants or excitotoxins that interfere with neurotransmission mechanisms in the brain. The first structure of domoic acid was proposed in 1958, which was later revised by NMR study in 1966⁸. However, it was not until 1982 that the correct structure with absolute configuration for (-)-domoic acid (1) was finally determined by stereospecific total synthesis⁹ and confirmed by X-ray analysis¹⁰. DA, compound responsible for the insecticidal activity of *C. armata*, was 14 times more potent than DDT when administered subcutaneously into the abdomen of American cockroach¹¹. Additional related compounds, isodomoic acids (2-5)¹² and domoic lactone (6-7) were also discovered from the insecticidal fraction of the alga^{13,14}. The insecticidal activities of isodomoic acids were much weaker than that of domoic acid but comparable with that of DDT¹³ while both lactones were found to be inactive¹⁴. Zaman and co-workers¹⁵ reported two new isomers of isodomoic acid (8) and (9), along with the

known isodomoic acids (2,3,10) and (11) from Kyushu Island. Their structure was deduced on the basis of ESI-MS and ^1H NMR spectral analysis including ^1H - ^1H correlation spectroscopy and NOE correlation spectroscopy. Domoic acid is also known to be vermifuge in a single dose as low as 20mg and inhibits ovulation. It also exterminates *Oxyris* and *Ascaris*^{4,16}. These useful properties of domoic acid are associated with certain disadvantages. Domoic acid is also present in edible mussels *Mytilus edulis* and whenever there has been episodes of shellfish poisoning, domoic acid has been identified as the causative substance¹⁷. It acts by causing neuronal depolarization; the resultant short-term memory loss is symptomatic of domoic acid poisoning. Other symptoms include dizziness, nausea and vomiting, ultimately leading to coma and brain damage or death in the most severe cases.

Chondria armata from the Japanese waters is also reported to contain hypoxanthine (12), L-glutamic acid (13) and D-aspartic acid (14)¹⁸. Hypoxanthine is a naturally occurring purine derivative and one of the products of the action of xanthine oxidase on xanthine. It is occasionally found as a constituent of nucleic acid where it is present in the anticodon of tRNA in the form of its nucleoside inosine. L-Glutamic acid is found in virtually all living organisms. It is one of the major amino acids in plant and animal proteins, and is also involved in many physiological functions. It acts as neurotransmitters in the brain. Humans readily metabolize ingested L-glutamic acid so that concentration in the body remain constant.

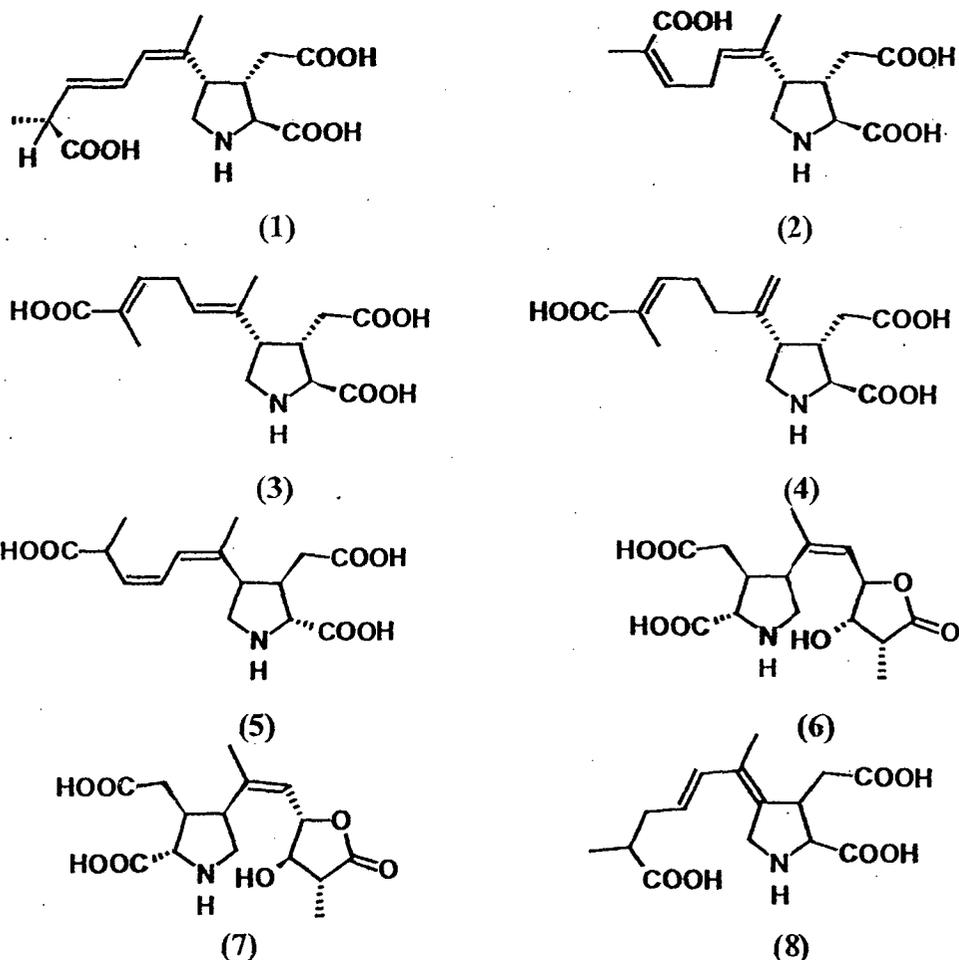
An α -amino acid, citrulline (15), [2-amino-5-(carbamoyl amino]pentanoic acid and isoglutamic acid (16) (3-amino glutaric acid) are also known to be a constituent of this alga¹⁹. The name is derived from *citrullus*, the Latin word for watermelon, from which it was first isolated²⁰. It is made from ornithine and carbamoyl phosphate in one of the central reactions in the urea cycle. Glutamic acid is present in a wide variety of foods and is responsible for one of the five basic tastes of the human sense of taste (umami), especially in its physiological form, the sodium salt of glutamate at neutral pH. Ninety-five percent of the dietary glutamate is metabolized by intestinal cells in a first pass²¹. Overall,

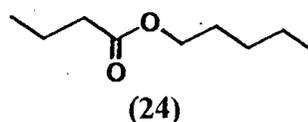
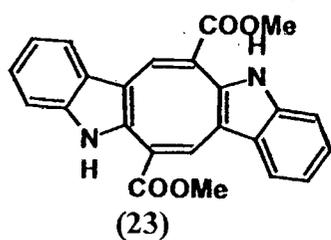
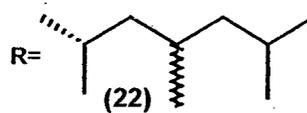
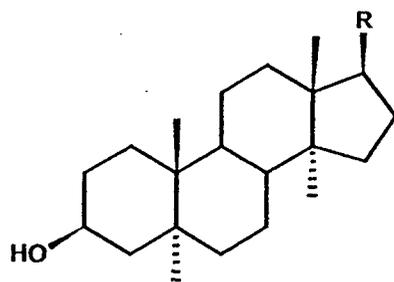
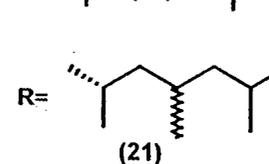
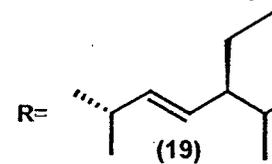
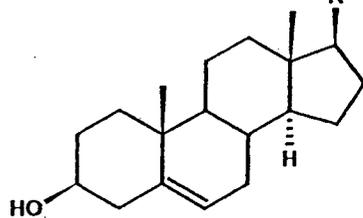
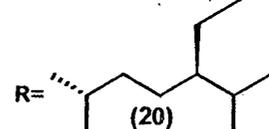
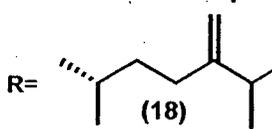
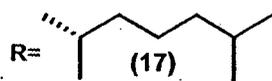
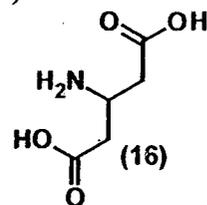
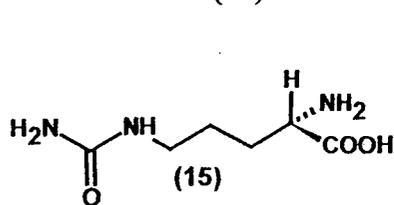
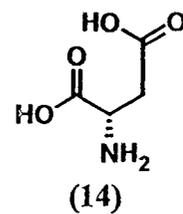
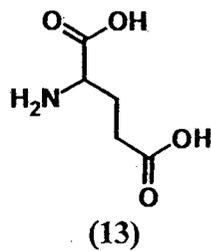
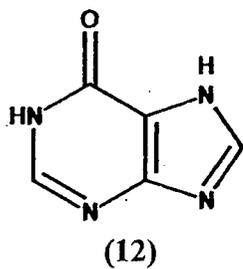
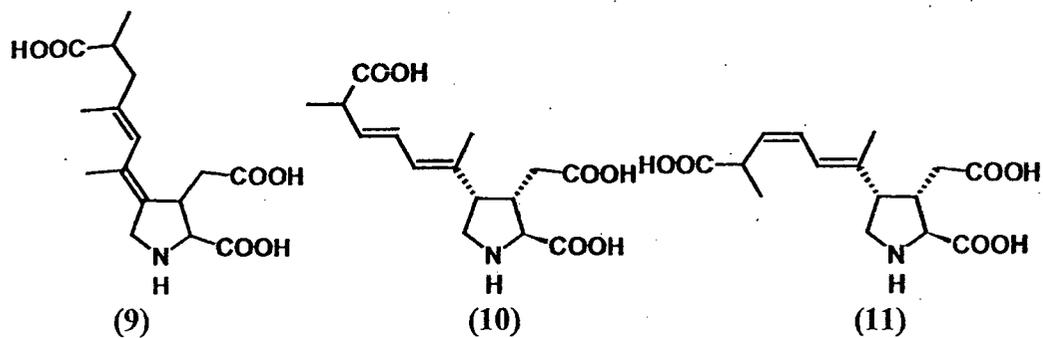
glutamic acid is the single largest contributor to intestinal energy. As a source for umami, the sodium salt of glutamic acid, monosodium glutamate (MSG) is used as a food additive to enhance the flavor of foods, although an identical effect can be achieved by mixing and cooking together different ingredients rich in this amino acid and other umami substances as well.

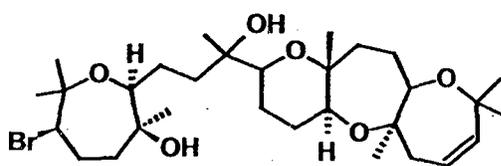
Preliminary screening of the chloroform extract of *C. armata* collected from Goa (west coast of India) showed antiviral, antibacterial, and antifungal activities^{22,23}. Continued research aimed at the chemistry and bioactivity of this alga by Govenkar et al resulted in the isolation of fatty acids, a novel ester, steroids and an alkaloid. The fatty acids were identified as myristic acid (C₁₄H₂₈O₂), pentadecylic acid (C₁₅H₃₀O₂), palmitic acid (C₁₆H₃₂O₂), stearic acid (C₁₈H₃₆O₂), 5-palmitoleic acid (C₁₆H₃₀O₂), 4-palmitoleic acid (C₁₆H₃₀O₂) and oleic acid (C₁₈H₃₄O₂) using gas chromatograph-mass spectrometer (GC-MS) equipped with a cross linked methyl silicone capillary Hewlett-Packard column (L=25 m & i.d 0.2 mm)²⁴. The free sterols, were possessing Δ^5 , 3 β -hydroxy nucleus and were identified as cholest-5-en-3 β -ol(17), 24-methylene-cholest-5-en-3 β -ol(18), 24 β -ethyl cholest-5,22-diene-3 β -ol(19), 24 β -ethyl cholest-5-en-3 β -ol(20), 23 ξ -methyl cholest-5-en-3 β -ol (22) and 23 ξ -methyl 5 α -cholestan-3 β -ol(22). Acetylation of the sterol mixture was also carried out and the corresponding steryl acetates obtained were analyzed by GC-MS²⁵.

Caulerpin (23), a dimer of indole-3-acetic acid is also present in this alga along with a fatty ester, pentyl hentriacontanoate(24)²⁶. The pigment caulerpin is a well known constituent of the green algae of genus *Caulerpa*²⁷⁻²⁹. It displays a moderate in vitro antitumor activity, acts as a plant growth regulator like its monomeric counterpart and indole-3-acetic acid (auxin)³⁰ and inhibits the multidrug resistance (MDR) pump in algae³¹. In the root elongation test with germinated lettuce seedlings, the activity of caulerpin was slightly weaker than that of auxin but stronger than those of indole-3-pyruvic acid and indole-3-acrylic acid. The corresponding dicarboxylic acid form of (23) also showed similar potency.³⁰

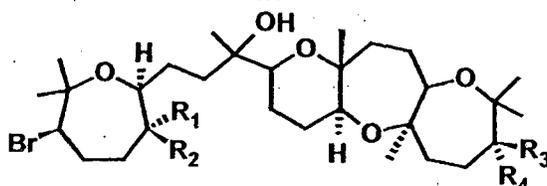
Subsequently, Cimino group³² reported a new class of bromotriterpenes, Armatols A-F. Their structures were characterized by spectroscopic techniques, in particular 1D- and 2D-NMR including HMQC and HMBC experiments. They also concluded that the triterpenoids polyethers identified from *Laurencia* and the armatols could arise from (6S,7S,10R,11R,14R,15R,18S,19S)-squalene tetraepoxide, a common precursor. However, from a biogenetic point of view, the discoveries of several molecules with different stereocenters suggest the hypothesis that the biosynthesis of these molecules may occur in a not concerted way. Interestingly, Fernandez et al, also reported the strong cytotoxic properties of these squalene-derived compounds, suggesting that further biological assay should be directed to an evaluation of this activity³³.







Armatol A

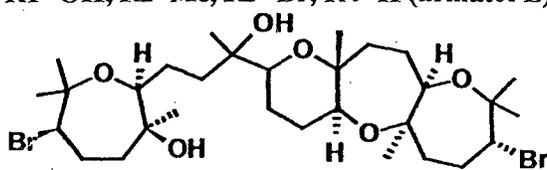


R1=Me, R2=OH, R3=H, R4=Br (armatol B)

R1=OH, R2=Me, R3=H, R4=Br (armatol C)

R1=Me, R2=OH, R3=Br, R4=H (armatol D)

R1=OH, R2=Me, R3=Br, R4=H (armatol E)



Armatol F

Section 1

*Lipids – Glycerolipids and steroids of the
red alga Chondria armata.*

1.1: Lipid constituents of the red alga *Chondria armata*

Marine organisms produce a variety of lipids because of their characteristic living environments. Lipids are major source of metabolic energy and essential materials for the formation of cell and tissue membranes. They are very important in physiology, reproductive processes of marine animals and reflect the special biochemical and ecological condition of the marine environment. The interest of chemist, biochemist and biotechnologists in lipids from marine organisms has been stimulated, in particular, by the recognition that polyunsaturated fatty acids (FA) are important for human health and nutrition. They are required for reproduction and growth. The relative proportion and composition of FA in marine organisms are characteristic for every species and genus and also depends on the environmental conditions.

The principal role of neutral lipids, which in marine organisms consist predominantly of triacylglycerols and wax esters, is as an energetic reserve of FA that are destined either for oxidation to provide energy (ATP) or for incorporation into phospholipids. Phospholipids are the building blocks for the membrane lipid bilayer. FA provide the hydrophobic interior of all cell membranes, forming an impermeable barrier to water and polar molecules and separating the cell contents from the extracellular medium. The physical properties of the membranes are determined by the individual lipids within the FA components of the lipids and their interaction with proteins and sterols. Membrane lipids other than phospholipids are the glycolipids.

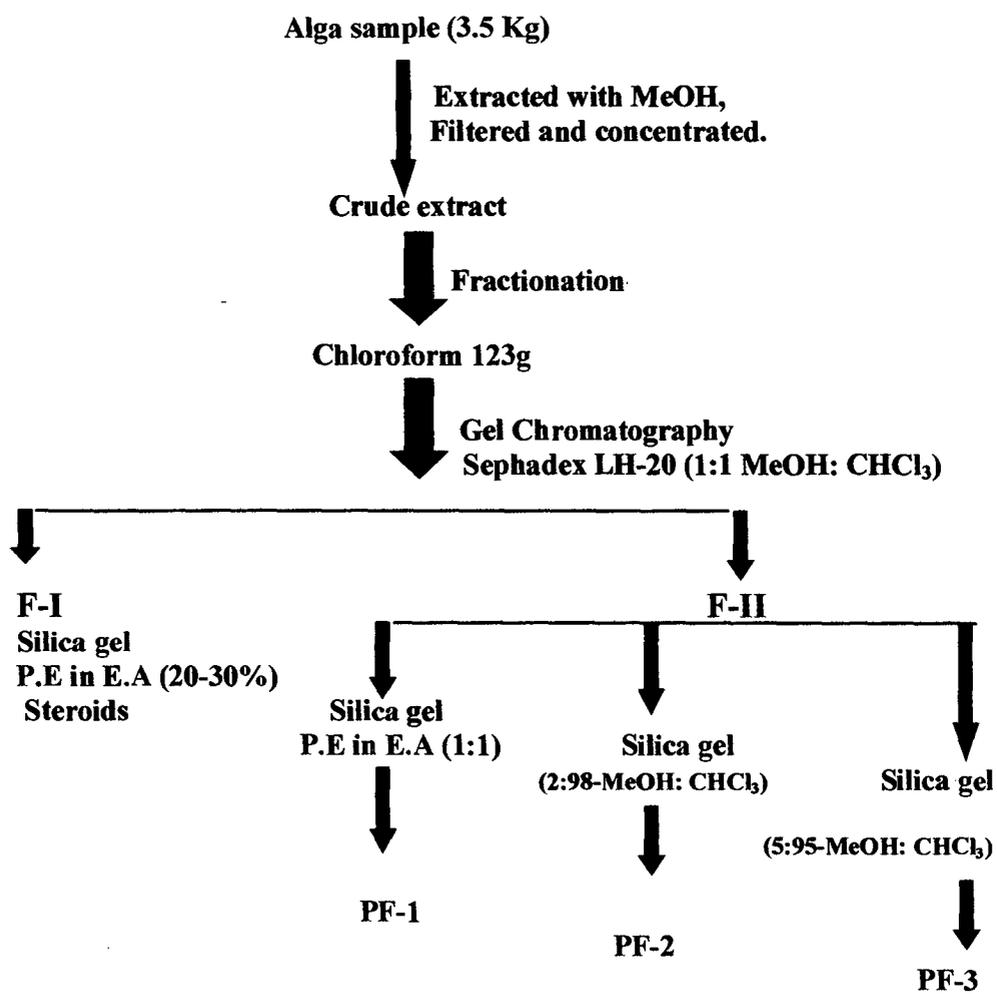
Glycolipids as mentioned are ubiquitous compounds in the cell membrane of most cell types. There are two major classes of glycolipids: glycosphingolipids and glyco glycerolipids. Glycosphingolipids, in which the carbohydrate moiety is linked to a ceramide lipid moiety, have been more widely studied. Glycosylceramides play an important role in many fields of cell biochemistry such as molecular recognition. In addition, ceramides from marine organisms have excited great attention as signal transducers, and some of them have been recognized as possessing antimicrobial and cytotoxic activities.

Glycosphingolipids, are tumor markers for various neoplasms and are markers of maturation or differentiation of cells in adults and embryonic tissues. Changes in composition, metabolism and organization of glycosphingolipids in the cell membrane are some of the most common biological changes associated with neoplastic transformation³⁴⁻³⁵

In contrast, the class of glycolipids (i.e. glycosyl glycerides) has received less attention in the recent literature. In these glycolipids the carbohydrate is O-glycosidically linked to carbon-3 of diacyl or monoalkyl-monoacyl glycerolipid.³⁶ Glycolipids are common components of various plant tissues and bacterial cell walls. In bacteria, mono and diglycosyldiacyl glycerols containing glucose galactose and mannose are most commonly seen³⁶. In plant cells, galactosyl and digalactosyldiacyl glycerols are the most common glycolipids. Although acylated and sulfonated variants, as well as trigalactosyldiacyl glycerols have also been found³⁶.

Glycolipids constitute an important class of membrane lipid that are synthesized by both prokaryotic and eukaryotic organisms³⁷. They are reported to exhibit diverse biological functions. There is currently considerable interest in both, intracellular and extracellular glycolipids specially galactosyl glycolipids as antitumor promoters in cancer chemoprevention.

This section presents a full account of the structural elucidation of major galactosylglycerols isolated from the chloroform soluble fraction of crude methanolic extract of red alga *Chondria armata* (Kütz.) Okamura. The chloroform fraction, which was subjected to gel chromatography over Sephadex LH20 using methanol as mobile phase gave, in order of polarity fractions PF₁₋₃, apparently homogenous on TLC, yielding purplish pink spots on spraying with methanolic sulphuric acid. This resulted in the isolation of three major glycolipids. The flow chart and TLC of the purified fractions PF₁₋₃ is given below (Scheme I, II). Their structure was elucidated by multidimensional nuclear magnetic resonance (NMR) techniques like ¹H, ¹H correlation spectroscopy (COSY), ¹H, ¹H total correlation spectroscopy (TOCSY), ¹H, ¹³C heteronuclear multiple quantum coherence



Scheme I: Sequential organic extraction, isolation and purification of the polar glycolipids.

Scheme II: TLC of the polar glycolipids (PF₁₋₃).

(HMQC) and ^1H , ^{13}C heteronuclear multiple bond correlation (HMBC) complemented by electrospray ionization mass spectrometry (ESI-MS) in the positive ion mode.

Major glycolipids were identified as (2R)-2-O- (5,8,11,14-eicosatetraoyl)-3-O- α -D-galactopyranosyl-sn-glycerol (**GL₂**), its pentacetate (**GL₁**) and (2S)-1-O-(palmitoyl)-2-O-(5,8,11,14,17-eicosapentaoyl)-3-O- β -D-galactopyranosyl-sn-glycerol (**GL₃**). Additionally, six minor glycolipids were also identified on the basis of ESI-MS. These include, a 1,2-di-Oacyl-3-O-(acyl-6'-galactosyl)-glycerol (**GL_{1a}**), sulfonoglycolipids 2-O-palmitoyl-3-O- (6'-sulfoquinovopyranosyl)-glycerol (**GL_{2a}**) and its ethyl ether derivative (**GL_{2b}**), 1- oleoyl-2-palmitoyl-3-O-galactosyl glycerol (**GL_{3a}**), 1,2-diacyl phosphatidyl glycerol (**GL_{3b}**) and 3-digalactosyl-2-palmitoyl glycerol (**GL_{3c}**).

Structural characterization of PF₁:

The IR spectrum (Fig 1.1) of the purified PF₁ showed absorption bands at 2925, 2856 cm^{-1} for aliphatic chain and 1747, 1224 cm^{-1} for the presence of ester group. It also gave protonated molecular ion peak $[\text{M}+\text{H}]^+$ at m/z 751 in its ESI-MS spectrum (Fig 1.7). The presence of spin systems corresponding to one hexose, glycerol and fatty acid were readily identified from the 1D and 2D homonuclear ^1H correlation (COSY) NMR spectra. Thus, the ^1H NMR spectrum (Fig1.2) (300MHz, CDCl_3) and ^{13}C NMR data including DEPT experiments (Fig1.3, Table 1) was in agreement with diacylated monogalactosyl glycerol (MGDG) with the fatty acyl chain being evident by the presence of a triplet due to a terminal methyl at δ 0.827, a broad methylene signal at δ 1.202 $[(\text{CH}_2)_n]$ of aliphatic chain, multiplets at δ 2.268, 1.967 and 1.562 assigned to three methylenes linked α , β and γ to the ester carbonyl functionality. A broad multiplet at δ 2.7 arises from allylic methylene protons and the olefinic methine protons were evident at δ 5.293. A sharp singlet at δ 2.12 was attributed to acetyl methyls.

The presence of glycerol moiety was also confirmed by heteronuclear multiple HMQC (Heteronuclear multiple quantum coherence) experiment, which showed

two doublets arising from C-3 and C-1. The signals at δ 4.22 and 4.35 correspond to the substitution at C-1 (δ 62.2) by an *O*-acyl group and the doublet at δ 3.56 and 3.96 was assigned to C-3 (δ 68.2) of glycerol substituted by the α -galactose residue. The glycerolipid structure was confirmed by the presence of a characteristic signal at δ 70.0/5.23 (C-2) having a distinct α -shift to lower field for ^{13}C and ^1H nuclei when substituted by an *O*-acyl group, this being a fingerprint for glycolipids containing glycerol as alcohol rather than sphingosine³⁸.

^1H - ^1H COSY, TOCSY (Fig1.4) (total correlation spectroscopy) and HMQC (Fig1.5) correlations allowed assignment of sugar carbons and protons (Table-1). ^1H - ^1H COSY and TOCSY correlation of the anomeric proton at δ 4.178 with the sn-3 protons at δ 3.56 and 3.96 established connectivity of the sugar moiety with the glycerol. The anomeric proton at δ 4.178 with a coupling constant of 2.1 Hz indicated α glycosidic configuration of the sugar linkage with the glycerol³⁹. TOCSY correlations are illustrated in Fig 1a.

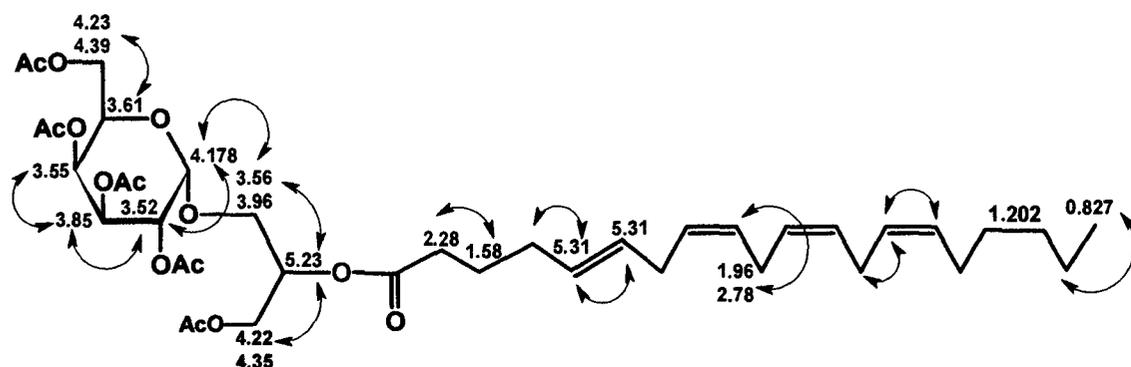


Fig 1a: TOCSY correlations of GL₁

Long-range heteronuclear multiple bond correlation (HMBC)(Fig 1.6) diagnostic correlations were observed between the ester carbonyls at δ 173.8 and δ 173.5 and C-1 and C-2 of glycerol indicating the linkage. The complete assignments of all the HMBC correlations are shown in Fig 1b.

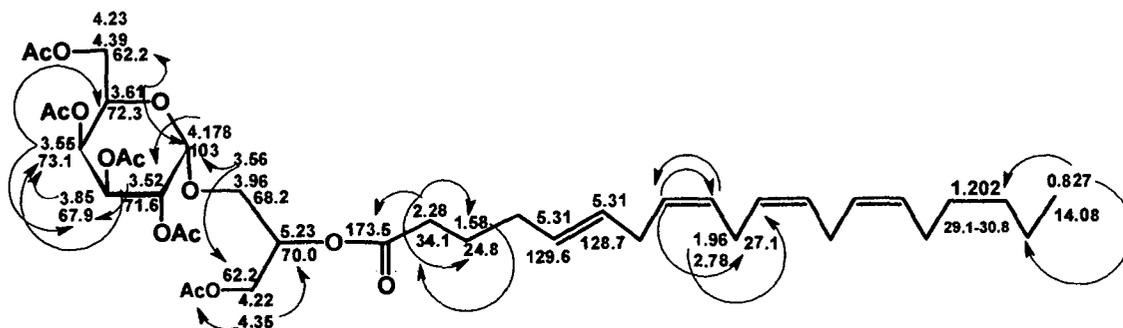


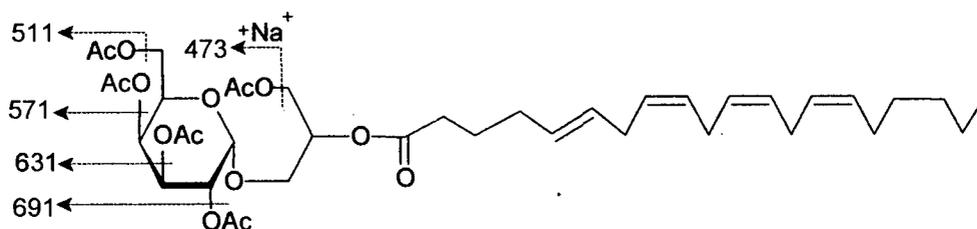
Fig 1b: HMBC correlations of GL_1

Table 1: 1H , $^{13}CNMR$, TOCSY and HMBC of GL_1

Carbon No.	1H NMR δ_H , ppm	^{13}C NMR δ_C , ppm	TOCSY Correlations	HMBC Correlations
1	4.22,4.35	62.2	H2	C1''', C2
2	5.23(m)	70.0	H1, H3a, H3b	-
3	3.56(d, 3.6Hz) 3.96(d, 6.0Hz)	68.2	H2	C1, C1'
1'	4.17(d, 2.1Hz)	103.0	H2', H2	C2'
2'	3.52	71.6	H1', H3'	C3', C4'
3'	3.85(b, s)	67.9	H2', H4'	C4'
4'	3.55(d, 3.6Hz)	73.1	H3'	C3', C5'
5'	3.61(m)	72.3	H6'	C1', C6'
6'	4.23, 4.39	62.2	H5'	C5'
1''	-	173.5	-	-
2''	2.28	34.1	H3''	C3'', C1'', C4''
3''	1.58	24.8	H2''	C2''
7''	1.96, 2.78	27.1	H8''	C8'', C9''
8''	5.31(d, 5.4Hz)	129.6	H7'', H9''	C7''
9''	5.31	128.7	H8'', H10''	C10''
18''	0.82(t, 6.9Hz)	14.0	H17''	C16'', C17''
(CH ₂) _n	1.20(bs)	29.1-30.8		
1'''	-	173.8		C2'''
2'''	2.12(s)	22.6		C1'''
O=C-CH ₃	2.12	22.6		Acetyls -Sugar.

The stereochemistry at C-2 was assigned to be *R* by comparison of the coupling constant values between H-2/H-3a ($J=3.6\text{Hz}$) and H-2/H-3b ($J=6.0\text{Hz}$) respectively with those of published data^{40,41,42}. On the basis of the above data the major component of PF_1 was identified as pentacetate of (2*R*)- 2-*O*-(5,8,11,14-icosatetraenoyl)-3-*O*- α -D-galactopyranosyl-sn-glycerol (GL_1). The fragmentation

observed in MS/MS spectrum of GL_1 (Fig 1.7), is well in agreement with the structure assigned. The pseudomolecular ion at m/z 751 generated a series of daughter ions at m/z 691, 631, 571 and 511 reflecting successive loss of four acetic acid molecules. The presence of fifth acetyl group was evident from the elimination of yet another acetic acid molecule yielding sodiated fragment at m/z 473. Alternately, the ion at m/z 473 might have originated, as diprotonated sodiated fragment ion, after the elimination of arachidonate ion. This ion on elimination of fifth acetic acid molecule would lead to ion at m/z 413. This confirmed the presence of acetylated hexose linked to the glycerol moiety, with the latter being diesterified by acetic acid and eicosatetraenoic acid. The proposed structure of GL_1 along with identified fragments is represented in (Scheme1).



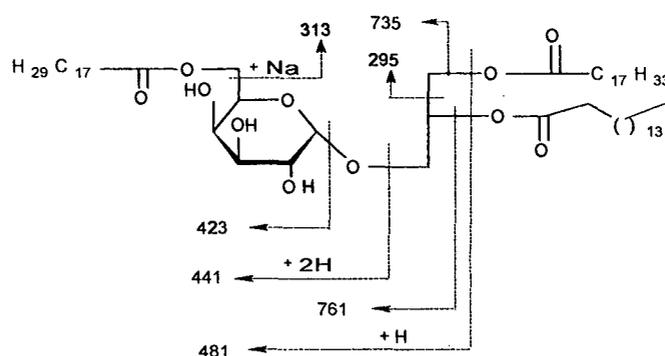
Scheme1: Mass fragmentation of GL_1

There is a solitary reference in the literature on the identification of 2-*O*- α -D-galactopyranosyl glycerol hexacetate from *Ruellia britoniana* E. Leonard (*Acanthaceae*)⁴³. The acetylated galactoglycerolipid is being reported here for the first time from a marine source.

ESI-MS of PF_1 , though apparently homogenous on TLC, showed some heterogeneity by the presence of an additional related molecular species with m/z 1017. Based on the fragmentation pattern observed in MS/MS (Fig 1.8) it was characterized as 1,2-di-*O*-acyl-3-*O*- (6-acylgalactosyl)-glycerol GL_{1a} .

MS/MS studies of the $[M+H]^+$ ion at m/z 1017 (Fig1.8) resulted in three major diagnostically important daughter ions at m/z 481, 735 and 761. The ions at m/z 735 and 761 reflect the neutral losses of the sn-1 and sn-2 substituent as free $C_{18:1}$ and $C_{16:0}$ carboxylic acid respectively is supporting the presence of palmitic and oleic acyl moieties in the molecule. The intensity differences of these various ions indicated the position of the different fatty acid moieties, as the substituent position at sn-2 fragments comparatively easily⁴⁴. This leaves a mass for the core

of the molecule of 481 amu. Such a mass can be explained by a substituted hexose connected to a glycerol backbone after elimination of fatty acyl groups from the protonated molecular ion $[M + H]^+$. This is further supported by the presence of an additional fragment ion at m/z 441, which reflects the loss of acyl groups ($C_{18:1}$ and $C_{16:0}$) from the molecular ion along with the glycerol backbone together corresponding to a total mass of 577 amu. Fragment ion at m/z 423 results from the cleavage between C-1 of hexose and C-3 of glycerol. Cleavage of the molecule between C5-C6 of the sugar leads to sodiated fragment at m/z 313 which corresponds to the third acyl substituent ($C_{18:3}$) along with C-6 of sugar which possibly seems to be galactose. The ion at m/z 295 results from the cleavage between C1-C2 of glycerol. Furthermore, there were a number of fragments in the upper mass region at intervals of about 14 amu. These correspond to fragmentation along the fatty acid acyl chains. On the basis of this fragmentation pattern of the molecular species with the pseudomolecular ion at m/z 1017 we propose the structure of the molecule as being 1-oleoyl-2-palmitoyl-3-*O*-(linolenyl-6'-galactosyl)-glycerol (GL_{1a}) that along with identified fragments is illustrated in Scheme 2.



Scheme2: Mass fragmentation of GL_{1a} .

Structural characterization of PF_2 :

A similar approach was adopted for PF_2 that showed physicochemical characteristics of glycolipids. The IR spectrum (Fig 2.1) of the purified PF_2 showed absorption bands at 3409.9 cm^{-1} for the presence of a hydroxyl groups (-OH), 2922.0 , 2852.5 cm^{-1} for the aliphatic chain and 1737.7 , 1172.6 cm^{-1} for the presence of ester group. Its $^1\text{H NMR}$ (Fig2.2) and $^{13}\text{C NMR}$ (Fig2.3) data including

DEPT experiments differed from that of PF₁ only by the absence of signals for the acetyl groups (Table-2) indicating GL₂ to be deacetylated derivative of GL₁.

Table 2: ¹H, ¹³CNMR, COSY and HMBC of GL₂

Carbon No.	¹ HNMR δ _H , ppm	¹³ CNMR δ _C , ppm	COSY Correlations	HMBC Correlations
1	4.00, 4.39	62.2	H2	1'', 2
2	5.30	70.0	H1, H3a, H3b	-
3	3.51 (b) 3.91 (b)	67.9	H2	1, 1'
1'	4.25(d, 4.5 Hz)	103.6	H2'	2'
2'	3.63	72.4	H1', H3'	3', 4'
3'	3.90	67.9	H2', H4'	4'
4'	3.69	73.1	H3'	3', 5'
5'	3.50 (b)	71.6	H6'	1', 6'
6'	4.30, 4.39	62.2	H5'	5'
1''	-	173.9	-	-
2''	2.35	34.1	H3''	3'', 1'', 4''
3''	1.63	27.2	H2''	2'', 4''
7''	2.04 (b)	29.3	H8''	8'', 9''
8''	5.39 (b)	128.8	H7'', H9''	7''
9''	5.39 (b)	130.0	H8'', H10''	10''
18''	0.82	14.1	H17''	16''
(CH ₂) _n	1.28 (b)	29.3-31.9		

The structure is also confirmed by COSY (Fig 2.4), HMQC (Fig 2.5) and HMBC (Fig 2.6) spectral data (Table 2). COSY and HMBC correlations are illustrated in Fig 2a and Fig 2b respectively.

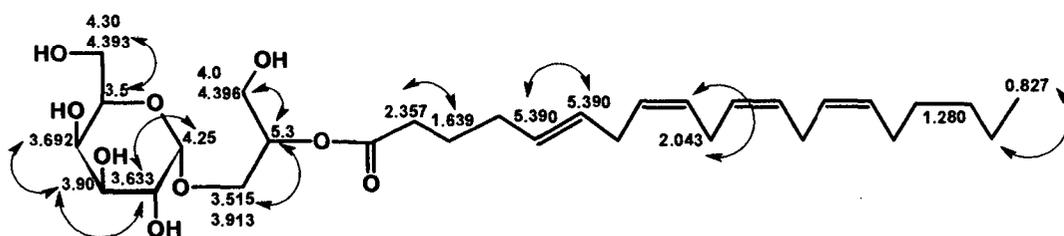


Fig 2a: COSY correlations of GL₂

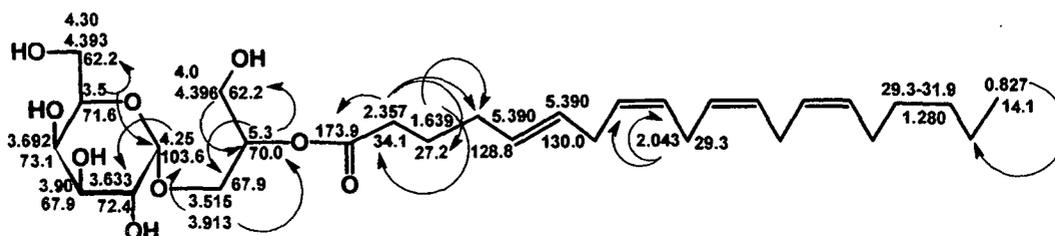
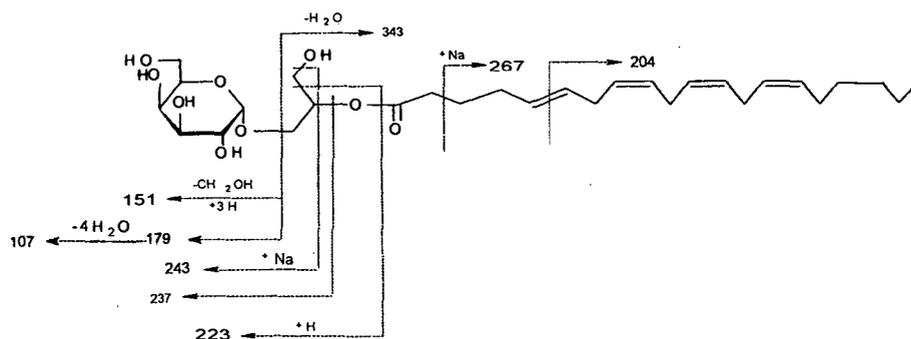


Fig 2b: HMBC correlations of GL_2

This was further supported by its ESI-MS (in MeOH) which exhibited pseudomolecular ion $[M + H]^+$ at m/z 541 consistent with the molecular formula of $C_{29}H_{49}O_9$ (PF_2). The MS/MS at m/z 541 (Fig2.7) showed peak at m/z 179 for loss of a sugar unit. Subsequent loss of the four water molecules from the hexose led to the base peak at m/z 107. The cleavage of the molecule between C-3 of glycerol and oxygen linking it to the hexose gives the fragment ion at m/z 343 with simultaneous elimination of water molecule. The sodiated ion at m/z 204 results from the attachment of two hydrogens to the hexose moiety.

Elimination of the fatty acyl chain and hydroxyl group at C-1 of glycerol leads to the sodiated ion at m/z 243, which is characteristic of monogalactosyl glycerols.

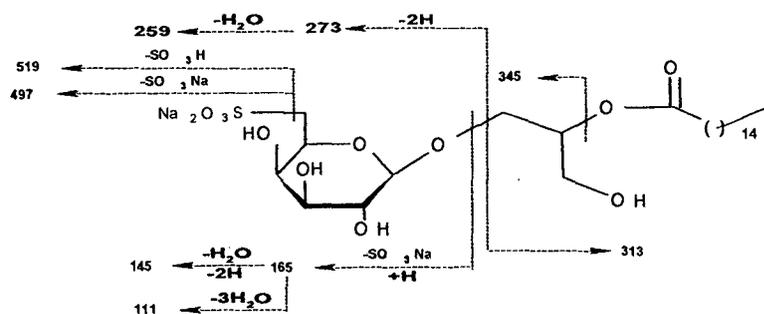
Fragmentation of the ester bond leads to the ion at m/z 239. Similarly the fragment at m/z 223 could be explained as being formed by cleavage of C2-C3 bond of glycerol backbone and cleavage between the oxygen and carbonyl of carboxylate group. The ion at m/z 267 results from the addition of sodium to the fragment derived from the McLafferty rearrangement in the acyl moiety. Thus the structure of major component from PF_2 was established as 2-*O*-(5,8,11,14-eicosatetraenyl)-3-*O*- α -D-galactopyranosyl-*sn*-glycerol GL_2 . The fragment ions peaks observed for GL_2 are illustrated in Scheme 3.



Scheme 3: Mass fragmentation of GL₂

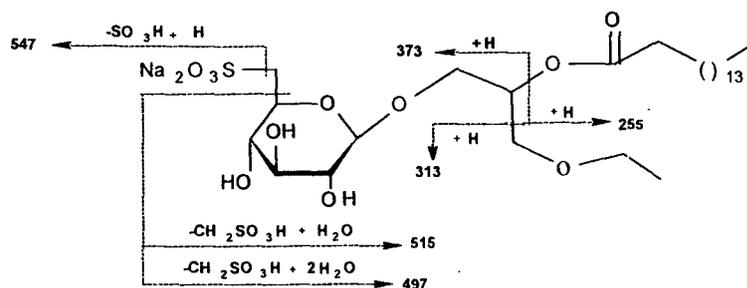
The ESI-MS examination of PF₂ when taken in a dilution solvent (as given under experimental section) showed additional peaks at *m/z* 601 and 629 corresponding to pseudomolecular ions of the sodium salt (Na⁺ form) of sulfonoglycolipids [M – H + 2Na]⁺. An effort was made to elucidate their structure by tandem mass spectrometry of these molecular species.

Thus, MS/MS of the pseudomolecular ion at *m/z* 601 (**fig 2.8**) exhibited the most abundant product ions at *m/z* 519 and 497 that has a mass difference corresponding to likely loss of sulfonyl group (82 amu) as sulfonic acid group (SO₃H) and as sodium salt (SO₃Na) respectively. The product ion observed at *m/z* 345 appears to have originated by the loss of fatty acyl side chain as corresponding acid (palmitic acid, C16:0). Cleavage between C-3 of glycerol and the oxygen at the anomeric carbon of hexose results in the simultaneous formation of the fragments at *m/z* 313 and 273. The later ion is formed with the loss of two hydrogens. The ion at *m/z* 273 losses one water molecule to yield the fragment 259amu. The ion at *m/z* 165 results from the elimination of sodium sulphonate group from the sulfonoquinovopyranosyl moiety and cleavage between C-3 of glycerol and the oxygen at the anomeric carbon with the attachment of three hydrogens. Subsequent elimination of three water molecules leads to the ion at *m/z* 111. Based on fragmentation pattern the glycolipid with pseudomolecular ion [M – H + 2Na]⁺ at *m/z* 601 was characterized as 2-*O*-palmitoyl-3-*O*-(6'-sulfoquinovopyranosyl)-glycerol GL_{2a}. The proposed structure along with its identified fragments is shown in **Scheme3**.



Scheme3: Mass fragmentation of GL_{2a} .

A similar fragmentation pattern was observed for the sulfonoquinovosyl molecular species with pseudomolecular ion at m/z 629 led to the structure GL_{2b} , as represented in (Fig 2.9). From the fragmentation observed it is interesting to note that the difference of 28 amu observed between the two sulfonolipids is not because of the difference in the fatty acid chain length as expected but seems to be due to the ethoxy group at C-1 of glycerol. The presence of sulfono group is further reinforced by the presence of ^{13}C NMR signal for CH_2 attached to sulphur at 53.6 ppm, as an impurity in PF_2 . The glycerolipids MGDG (monogalactosyldiacylglyceride) and DGDG (digalactosyl diacylglyceride) are uncharged species while SQDG (Sulfoquinovosyldiacylglyceride) is negatively charged at neutral pH. This explains their presence in admixture as sodiated adducts. The fragment ions peaks observed for GL_{2b} are illustrated in Scheme 4.



Scheme4: Mass fragmentation of GL_{2b} .

Structural characterization of PF_3 :

ESI-MS of the major component of this fraction was consistent with the sodiated molecular ion $[M+Na]^+$ at m/z 799 [calc. 799.5336, obsr. 799.5532] corresponding to the molecular formula of $C_{45}H_{76}O_{10}Na$. Hydroxyl and ester carbonyl

functionalities were indicated by IR absorption at 3413.8, 1732.0 and 1166.8 cm^{-1} (fig.3.1). Its ^1H NMR (Fig.3.2), ^{13}C NMR and DEPT(Fig 3.3) (Table-3) closely resembled those of PF₂ except that the ^{13}C signals due to the unsaturation in the fatty acid moiety were more distinct.

The tandem MS/MS spectrum of ion at m/z 799 is illustrated in Fig.3.7 and it represents (2S)-1-O-palmitoyl-2-O-eicosapentanoyl-3-O- β -D-galactosyl-sn-glycerol GL₃. HMQC, TOCSY and HMBC spectra of GL₃ are represented in (Figs. 3.4, 3.5, 3.6) respectively. As evident, the TOCSY spectrum is characteristic of glyceroglycolipid with the spin systems of glycerol, sugar and the constituent fatty acids. TOCSY and HMBC correlations are illustrated in Fig 3a and Fig 3b respectively.

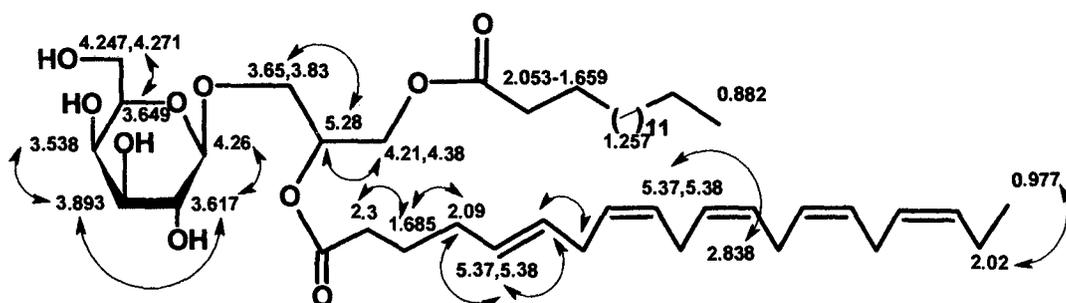


Fig 3a: TOCSY correlations of GL₃

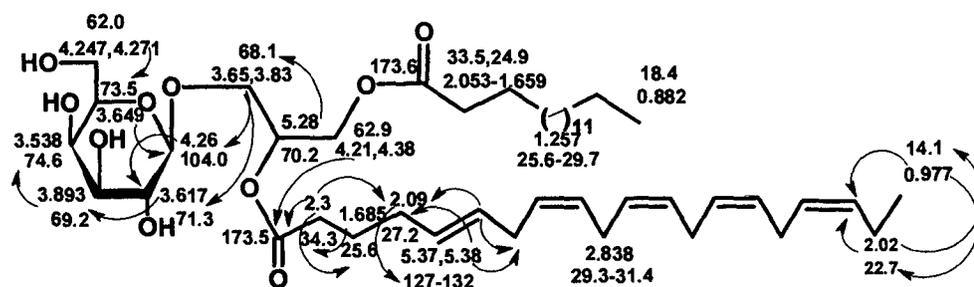
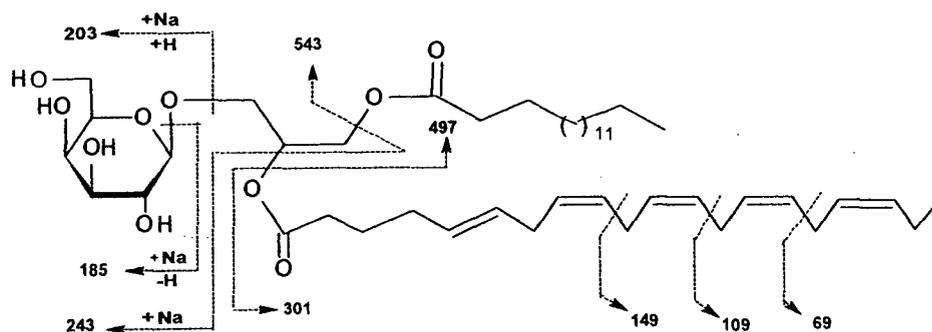


Fig 3b: HMBC correlations of GL₃

Table 3: ^1H , ^{13}C NMR, COSY and HMBC of GL_3

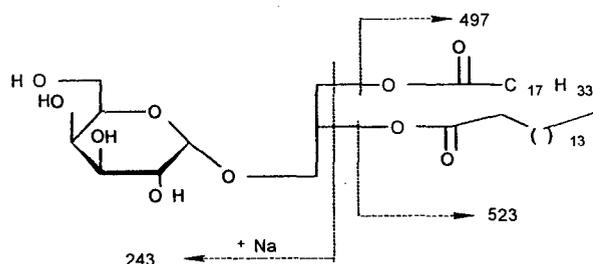
Carbon No.	^1H NMR δ_{H} , ppm	^{13}C NMR δ_{C} , ppm	COSY Correlations	HMBC Correlations
1	4.21, 4.38	62.9	H2	1'''
2	5.28	70.2	H1, H3a, H3b	3
3	3.65 3.83 (d, 6Hz)	68.1	H2	1', 2'
1'	4.26(d, 7.2 Hz)	104.0	H2'	2'
2'	3.61	71.3	H1', H3'	3'
3'	3.89	69.2	H2', H4'	
4'	3.53	74.6	H3'	
5'	3.64	73.5	H6'	1'
6'	4.24, 4.27	62.0	H5'	5'
1''	-	173.5	-	-
2''	2.30(d, 7.8Hz)	34.3	H3''	3'', 1'', 4''
3''	1.68m	25.6	H2''	2'', 4''
4''	2.09(q, 6Hz)	27.2		3'', 5''
5'', 6'', 8'', 9'', 11''---	5.37-5.38 (cluster)	127.0-132.0 (10 d)		4'', 7''
7'', 10''---	2.83(br dd)	29.3-31.4 (4t)	H8''	5'', 6''
19''	2.02(m)	22.7		18'', 20''
20''	0.97(t, 7.5)	14.1	H19''	18'', 19''
1'''	-	173.6		
2''', 3'''	2.05-1.65(m)	33.5, 24.9		
4''', --- 13'''	1.25	25.6-29.7		
16'''	0.88(t, 6.9Hz)	18.4		

The main fragmentation pathway corresponding to concomitant elimination of two fatty acyl moieties yielded ion at m/z 243 characteristic of MGDG^{45} . Ions reflecting neutral loss of eicosapentanoate and palmitate as free fatty acids are evident from the fragment ions at m/z 497 and m/z 543 respectively. Elimination of the palmitoyl acyl group as an acid from the pseudomolecular ion $[\text{M}+\text{H}]^+$ led to the ion at m/z 521. Additional ions are observed due to loss of three molecules of water from the sugar moiety yielding protonated ion at m/z 109. Loss of two water molecules along with hydroxymethyl group from the sugar moiety produced ion at m/z 97 and loss of arachidonate ion results in ion at m/z 301. The fragment ions peaks observed for GL_3 are illustrated in **Scheme 5**.



Scheme 5: Mass fragmentation of GL_3 .

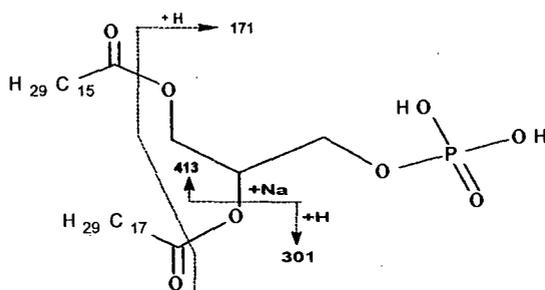
ESI-MS of PF_3 , though apparently homogenous on TLC, showed some heterogeneity in its mass as evidenced by the presence in the ESI-MS of several other ions of related molecular species besides the main component. The MS/MS of sodiated molecular ion $[M+Na]^+$ at m/z 779 (**Fig. 3.8**) yielded fragments at m/z 497 and m/z 523 indicative of loss of C18:1 and C16:0 fatty acyl groups from the molecule as free fatty acids respectively. The presence of galactosyl sugar moiety was evident from the ion at m/z 243. The intensity of the signals led to the placement of palmitic acid at sn2 position^{46,47,48}. Taken together, the structural analysis for the molecular species with ion at m/z 779 is consistent with 1-oleoyl-2-palmitoyl-3-*O*-galactosyl glycerol GL_{3a} . The proposed structure along with its identified fragments is shown in **Scheme 6**.



Scheme 6: Mass fragmentation of GL_{3a}

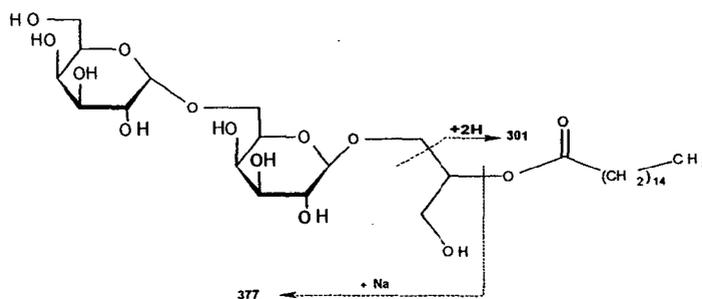
The CID daughter ion spectrum of the molecular species at m/z 691 is illustrated in (**Fig 3.9**) and it represents 1,2-diacyl phosphatidyl glycerol. The main fragmentation pathway observed here is the formation of ion at m/z 413 originating from the loss of 278 amu corresponding to the loss of C18:3 as free fatty acid. The ions at m/z 171 and m/z 189 are consistent with the cleavage at C12 of γ -linolenic acid as free acid and as ketene respectively⁴⁹. The fragment at

m/z 171 could also arise from phosphoglycerol moiety. The most intense ion at m/z 301 was attributed to the concomitant elimination of palmitoleoyl and phosphatidyl groups along with the glycerol backbone as depicted in (Fig 3.9) or elimination of linoleic acid as sodium salt. The abundance of the ion at m/z 301 as compared to the ion at m/z 413 is consistent with the notion that neutral loss of the fatty acid at sn-2 is sterically more favorable than the analogous loss at sn-1 position^{46,47,48}. Thus structure **GL_{3b}** was proposed for the molecular species with $[M+Na]^+$ ion at m/z 691. The schematic fragmentation pattern is shown in scheme 7.



Scheme 7: Mass fragmentation of **GL_{3b}**

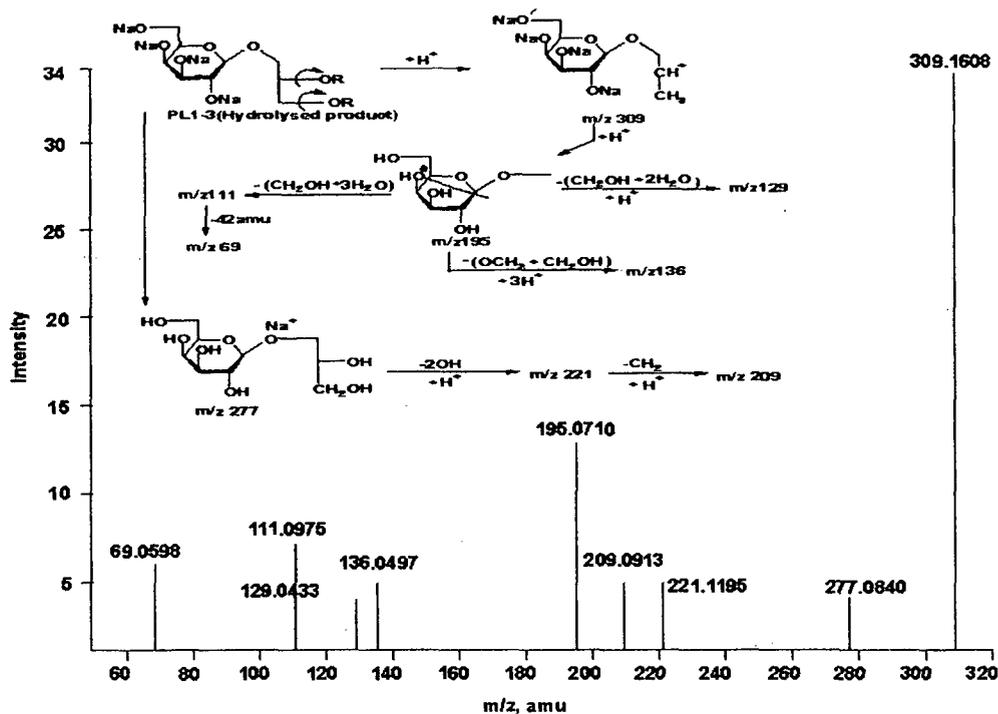
Tandem MS scanning experiment of protonated molecular species at m/z 655 yielded the most prominent ion at m/z 301 reflecting loss of 354 amu which is probably due to digalactosyl unit present and a much less intense fragment at m/z 377 corresponding to the loss of palmitoyl group as sodium palmitate from the molecule. The relative abundance of the ions placed the palmitoyl group at sn-2 position. The spectrum is consistent with 3-digalactosyl-2-palmitoylglycerol **GL_{3c}** represented in (Fig.3.10). The proposed structure along with its identified fragments is shown in Scheme 8.



Scheme 8: Mass fragmentation of **GL_{3c}**

Methanolysis of PF₁₋₃:

In order to identify the acid substituents at C-1 and C-2 of component glycolipids of PF₁₋₃, methanolysis was performed in anhydrous methanol with excess of Na₂CO₃. All the three fractions yielded the same glycoside 3-*O*-D-galactopyranosyl-*sn*-glycerol and methyl esters of corresponding fatty acids. The mixture of the reaction product was analyzed by ESI-MS in the positive ion mode. Thus, for example, the ESI-MS of PF₃ gave pseudomolecular ions at *m/z* 183, 277, 309, 301, 334 and 389. Analysis of each of these ions by tandem mass spectrometry established their identity. Thus the ion at *m/z* 183 corresponded to the attachment of two hydrogens to the sugar moiety [M+2H]⁺. 3-*O*-D-galactopyranosyl glycerol as sodium adduct was observed at *m/z* 277. Deacylated glycolipid with the sodiated sugar moiety was evident as protonated molecular ion at *m/z* 309. The fragment at *m/z* 301 represented the presence of eicosapentanoate. Thus, the fragmentation observed in MS/MS of ion at *m/z* 309, a fragment common as product of hydrolysis of PF₁₋₃, is shown in (Scheme 9). In order to establish the nature of the sugar moiety as D-galactose, the glycolipids were subjected to acid hydrolysis and the compound identified by TLC with standard sugars as described in experimental section. The optical rotation of the sugar obtained by hydrolysis was well in agreement with the values reported for D-galactose.



Scheme 9. ESI-MS/MS of hydrolysed product with m/z 309 from the fraction PF₁₋₃ along with the fragmentation pattern

Antimicrobial activity of PF₁₋₃:

Bergsson *et al.* (2001)⁵⁰ have studied the susceptibility of *Candida albicans* to several fatty acids and their 1-glycerides. They observed that capric acid, 10 carbon saturated fatty acid, causes the fastest and most effective killing of all the three strains of *C. albicans* tested. Lauric acid, a 12 carbon saturated fatty acid, was the most active acid at lower concentrations. Subsequently, Frentzen *et al.* (2003)⁵¹ reported on the medium chain fatty acids of 8-12 carbon atoms exhibiting antibacterial and antifungal properties, which are enhanced when these acids are esterified with glycerol. The same authors also state sucrose esters as being less effective in inhibiting the fungal growth. Based on these reports it is expected that pathogens would be sensitive to glycolipids. This led us to evaluate the pure fractions PF₁₋₃ of the present investigation, isolated and identified from the red alga, *Chondria armata*, against different strains of pathogenic microorganisms, for antibacterial and antifungal activities and compares them with the commercially available antibiotics (Table-4).

Table-4: Antimicrobial activity of glycolipids from *Chondria armata*

Fractions	Antibacterial							Antifungal						
	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>S. typhi</i>	<i>S. flexineri</i>	<i>Klebsiella sp.</i>	<i>V. cholerae</i>	<i>A. fumigatus</i>	<i>Fusarium sp.</i>	<i>C. neoformans</i>	<i>A. niger</i>	<i>Rhodotorula sp.</i>	<i>Nocardia sp.</i>	<i>C. albicans</i>
PF ₁ .	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PF ₂ .	+(st)	-	+(st)	+	+(st)	-	1	1	-	1	1	+	-	-
PF ₃ .	-	-	-	-	+	4	1	1	-	2	-	-	-	4
Standard	10	-	11	+	6	4	10	-	-	-	-	-	-	4

Numbers indicate the zone of inhibition in mm from center of imbued disk

(-) No activity, (+) Weak activity.

+(st) It shows activity but zone of inhibition is not very clear

As evident, from the above Table-4 all the bacteria and fungi tested were resistant to PF₁ at the dose tested (65 µg/ml). PF₂ showed mild inhibitory activity against the bacteria tested except *P. aeruginosa* and *K. pneumoniae*, at 250 µg/disc being also weakly active against the fungi, *A. fumigatus*, *C. neoformans*, *A. niger* and *Rhodotorula sp.* PF₃, at 130 µg/disc, was as effective as standard Nystatin and antibiotic Streptomycin, against the yeast *Candida albicans* and bacteria *Klebsiella sp.* respectively. Considerable activity was also expressed by PF₃ against the fungus, *Cryptococcus neoformans*, strain resistant to Nystatin. PF₃ showed mild activity against the bacteria *Shigella flexineri* and *V. cholerae* and the fungus *Aspergillus fumigatus*. All the three compounds were ineffective against the multidrug resistant strains tested. Results indicate that acetylation inactivates the molecule and the activity is greatly influenced by the anomeric configuration of glycosidic linkage. Compounds with β configuration being more effective than the glycosides with α configuration. Antimicrobial activity of glycolipids is being reported here for the first time.

Discussion:

Three major galactoglycerolipids have been isolated and identified, in the native form, from the red alga *C. armata* using NMR complemented with mass spectrometry. Six minor glycolipids have also been identified on the basis of electrospray ionization tandem MS/MS spectrometry alone. Methanolysis of the glycolipids yielded galactosylglycerol, which on ESI-MS provided a pseudomolecular ion at m/z 309 representing deacylated glycolipid with the sodiated sugar moiety. Recently, Shao *et al.* (2002)⁹⁷ reported the presence of a new sulfonoglycolipid, crassicaulisine, with palmitoyl and myrsitoyl as acyl groups, from the red alga belonging to the same genus *Chondria crassicaulis*. Acyl groups in PF1–3 were characterized as the corresponding acids or carboxylate ions (ESI-MS), and the principal components were arachidonic acid in PF1–2, palmitic acid, and eicosapentaenoic acid in PF3. There were minor components, which include C16:1, C18:1, and C18:3 acids.

It is of interest to note that polyunsaturated fatty acids eicosapentaenoic acid (EPA) and arachidonic acid (AA) are present in the alga in bound form as acyl substituents in galactosyl acyl glycerols. In agreement with previous reports, palmitic acid seems to be the major fatty acid in sulfonoglycolipids of marine algae. Contrary to the reports of Choi *et al.* (1999)⁹⁸ in glycolipids of marine algae, the glycosidic linkage could be α/β and the sugar moiety is attached, mainly, to C-3 of sn-glycerol.

GL1a is the first example of the natural occurrence of acyl glycerol acylated at the sn-1, sn-2 and 6' positions. The presence of acyl glycerol acylated at the sn-1 and 6' positions of mannobiosyl is known from the bacteria *Arthrobacter atrocyaneus* and *Microcoleus luteus*.^{99,100}

In recent years, glycolipid analogues have gained importance in cancer chemo prevention because of the promising inhibitory effect exhibited by them on tumor promoting activity. The fatty acyl chain length, its position and the nature of sugar moiety influence the activity. Galactosyl glycerols are reported to be

more potent than the corresponding glucosylglycerols with the same structural features^{101,102}. The anomeric configuration does not seem to affect the activity¹⁰³.

MGDGs, containing (7Z, 10Z)-hexadecadienoic acyl group, from the green alga *Chlorella vulgaris* are reported to exhibit anti-tumor promoting effect¹⁰⁴. SQDG from algae inhibits DNA-polymerase and HIV-reverse transcriptase^{105,106,107}. It is well known that biological activity of marine macrophytes is related to the essential polyunsaturated fatty acids (PUFAs), which are the abundant components of macrophytic glycolipids^{108,109,110}.

The red algae are reported to have high levels of polyunsaturated fatty acids, mainly EPA and AA¹¹¹, but the contents vary within the same genus. *Chondria dasyphylla* (Wood) Ag. is reported to have equal contents of EPA and AA whereas in *Chondria decipiens* EPA predominates¹¹². Further, in red algae PUFAs belonging to C20 series are reported to be mainly concentrated in MGDG¹¹³. This has in fact been observed in the present investigation, with EPA and AA being the constituent fatty acids of major glycolipids identified in PF1–3, and is well in agreement with our earlier communication on the fatty acids from the alga *C. armata*, where C20 acids were not detected as free fatty acids¹¹⁴.

Glycoglycerolipids occur widely and copiously in vascular plants¹¹⁵, certain green seaweeds^{116,117,118} cyanobacteria¹¹⁹, marine dinoflagellates¹²⁰, and the freshwater alga *C. vulgaris*¹⁰⁴. As to the glycoglycerolipids of red algae, hydroxyeicosapentaenoyl galactosyl glycerols are known from the temperate red alga *Gracilariopsis lemaneiformis*¹²¹, and MGDG, DGDG, and SQDG are reported from *Gracilaria verrucosa*¹²², which is also known to contain sulfoquinovosylmonogalactosyl glycerol (SQMG) (GL2a). This SQMG is also reported to be a constituent of cyanobacterium *Synechocystis* PCC 6803¹²³ and lichenized basidiomycetes, *Dictyonema glabratum*¹²⁴. 2-O- α -D-galactopyranosylglycerol is a metabolite of *Laurencia pinnatifida*¹²⁵ and 2,3-dipalmitoyl sulfonoglycolipid has been identified in *Laurencia pedicularioides* and is reported to be the major glycolipid in red algae¹²⁶. Recently, Shao *et al.* (2002)⁹⁷ reported the presence of a new sulfonoglycolipid, crassicaulisine, in the red alga *C. crassicaulis*. Taxonomically, genus *Laurencia* and *C. armata* belong

to the same family, Rhodomelaceae, but in the present investigation *C. armata* did not contain either of the glycolipids.

Interestingly, palmitic acid has been found to be the most abundant fatty acid present in the sulfonoglycolipids of marine origin^{127,128,105,122,129}. The two sulfonoglycolipids of the present investigation provide yet another example of a glycolipid which contains palmitic acid as the only fatty acid component. Palmitic acid was described as having hemolytic activity in sea urchin eggs¹³⁰ and was presumed to be playing a unique role in algal physiology¹²⁷.

Sulfonoquinovosyl acyl glycerols, in particular compounds with C18 fatty acid on the glycerol moiety, may be clinically promising antitumor or immunosuppressive agents¹³¹.

EXPERIMENTAL SECTION:

General experimental procedures:

Sephadex LH20 (Pharmacia) and silica gel (60-120 mesh) [Qualigens] were used for gel filtration and column chromatography respectively. Precoated Kieselgel 60 F₂₅₄ TLC plates (Merck) were used for analytical TLC. Compounds were visualized as purplish spots on spraying with 5% methanolic sulphuric acid followed by heating at 100°C. Solvent system for TLC I and II was light petrol/ethyl acetate (6:4) and (1:1) respectively and TLC III was methanol: chloroform (5:95).

Mass spectrometry:

Mass spectra were recorded, in the positive mode, on a QSTARXL MS/MS Applied Biosystems, Switzerland equipped with Analyst Software. The declustering potential and the collision energy were optimised for MS/MS experiments so as to cause fragmentation of the selected molecular ion species as

evident by the appearance of fragment ions and decrease in the intensity of the molecular ion. ESI-MS was carried out by dissolving the compounds in methanol as solvent. ESI-MS of PF₂ was taken in methanol as well as dilution solvent.

Dilution Solvent:

It was prepared as follows: 15.4 milligrams of ammonium acetate was dissolved in 49.9 ml of water. To this solution was added a mixture of 49.9ml of methanol, 0.1 ml of formic acid and 0.1 ml of acetonitrile.

NMR:

¹H, ¹³C, COSY, HMQC and HMBC experiments were recorded, in CDCl₃, on a Bruker (Avance 300) spectrometer with TMS (tetramethylsilane) as internal standard.

Biological material:

The alga was collected during the low tides from coastal waters of Goa, west coast of India [15° 51' N to 15°54' N and 73° 51' E to 73° 52' E] during the pre-monsoon periods. The alga, sample no. 1316, identified by Geeta Deshmukh, CIFE, Mumbai has been deposited at NIO Repository and Taxonomic Center.

Extraction and isolation of glycolipids:

The red alga, *Chondria armata* (3.5kg, dry wt.) was cleaned and extracted thrice with methanol using a sonicator (15mins) at room temperature. The combined methanolic extracts were evaporated under reduced pressure at 37°C temperature to a certain minimum volume (~200ml), and then partitioned into chloroform, n-butanol and water-soluble fractions.

The chloroform fraction (123g) was fractionated, initially on a column of Sephadex LH20 with methanol (500ml) as eluant collected in fractions of 20ml each. The fractions obtained were examined by TLC (solvent:light petrol:ethylacetate, 1:1,v/v, spray: 5% methanolic sulfuric acid) and combined according to their profile. Fractions yielding purplish spots were then purified by

repeated silica gel chromatography using petroleum ether (60-80°C): ethyl acetate (1:1) to give PF₁ (4mg, R_f = 0.52 in solvent I), and methanol: chloroform (2:98) yielded PF₂, [α]_D = -16° (c = 0.02, CHCl₃, R_f = 0.45 in solvent II; yield 13mg). Further elution of the same column with methanol: chloroform (5:95) yielded PF₃ [α]_D = -20° (c = 0.02, CHCl₃, R_f = 0.175 in solvent III, yield 23mg). Final purification was done on RP-18 column with methanol as eluant. As the neutral glycolipids yielded purplish pink spots with methanolic sulphuric acid, all the constituents, from chloroform soluble fraction, showing purplish pink spots on TLC were purified.

Methanolysis of glycolipids (PF₁₋₃):

PF₁₋₃, 2 mg each were dissolved in anhydrous methanol (1ml) and an excess of sodium carbonate was added. The solution was stirred at room temperature overnight, filtered and the solvent evaporated. The residue was analysed by ESI-MS in methanol. Tandem mass was taken at collision energy between 30-35 eV.

Acid hydrolysis of glycolipids (PF₁₋₃):

Each fraction (4-8mg) in 5 ml of 2% H₂SO₄ in methanol was refluxed for 3 hours. This was followed by the addition of 4 ml of water to the reaction mixture. Methanol was removed in vacuo and the aqueous solution extracted with chloroform and then neutralized with barium hydroxide. Precipitated barium sulphate was filtered through celite, water removed in vacuo and the residue dissolved in 1 ml of water. TLC (butanol:acetic acid: water; 5:1:4) showed a single major spot identical with D-galactose. The NMR data do not distinguish between L and D forms of the glycosyl moieties. The D form of the monosaccharide dominates in living organisms; the only occurrence of L galactose is in agar-agar⁵². For confirmation of configuration of sugar residue PF₁₋₃ were hydrolysed with 2M TFA (trifluoroacetic acid) at 110°C for 3 hours, following concentration to dryness under stream of nitrogen. The product was then filtered through Sephadex G-10 (Pharmacia) using MeOH:H₂O (1:1) as the mobile phase. Fractions (5 ml) were collected and monitored on TLC plates using

butanol: acetic acid: water (5:1:4) as the solvent system for development. Rf value of the sugar thus obtained was equivalent to the standard D galactose . Fractions containing sugar (galactose) were combined, concentrated on a rotavapor and their optical rotation measured. It was found to be (+)150° [Literature(+150.7°)] in case of hexose from PF₁₋₂ and +52°(literature: (+)52.8°) for sugar from PF₃. These results indicated that all the three samples yielded D-galactose having α configuration in PF₁₋₂ and β configuration for sugar in PF₃.

Antibacterial assays:

Antibacterial activity was determined against six Gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella flexneri*, *Klebsiella pneumoniae* and *Vibrio cholerae*) and one Gram positive bacteria (*Staphylococcus aureus*) using the paper disk assay method⁵³. The sterile paper disk of 6mm diameter impregnated with 65µg/disk of PF₁ and 130µg/disk of PF₂ were placed on agar plates containing the test microorganisms. In all cases, the concentration was approximately 1.2x10⁸ CFU/µl. The impregnated disks were placed on the medium suitably spaced apart and the plates were incubated at 37°C for 24 hrs. Disk of Streptomycin (10µg/ml) was used as a positive control. The diameter (mm) of the growth inhibition halos caused by the sample was examined.

Antifungal assay:

Antifungal activity was determined against *Aspergillus fumigatus*, *Fusarium sp.*, *Cryptococcus neoformans*, *Aspergillus niger*, *Rhodotorula sp.*, *Nocardia sp.* and *Candida albicans* using the paper disk assay method as previously described in the antibacterial assay. The sterile disk was impregnated with the compound (65µg/disk of PF₁ and 130 µg/disk of PF₂). The inoculum concentration was 0.5x10³-2.8x10³ CFU/ml. Nystatin (100 µg/disk) was used as positive control. The plates were incubated at 24°C for 18 h. The diameter (mm) of growth inhibition halos caused by the compound was examined.

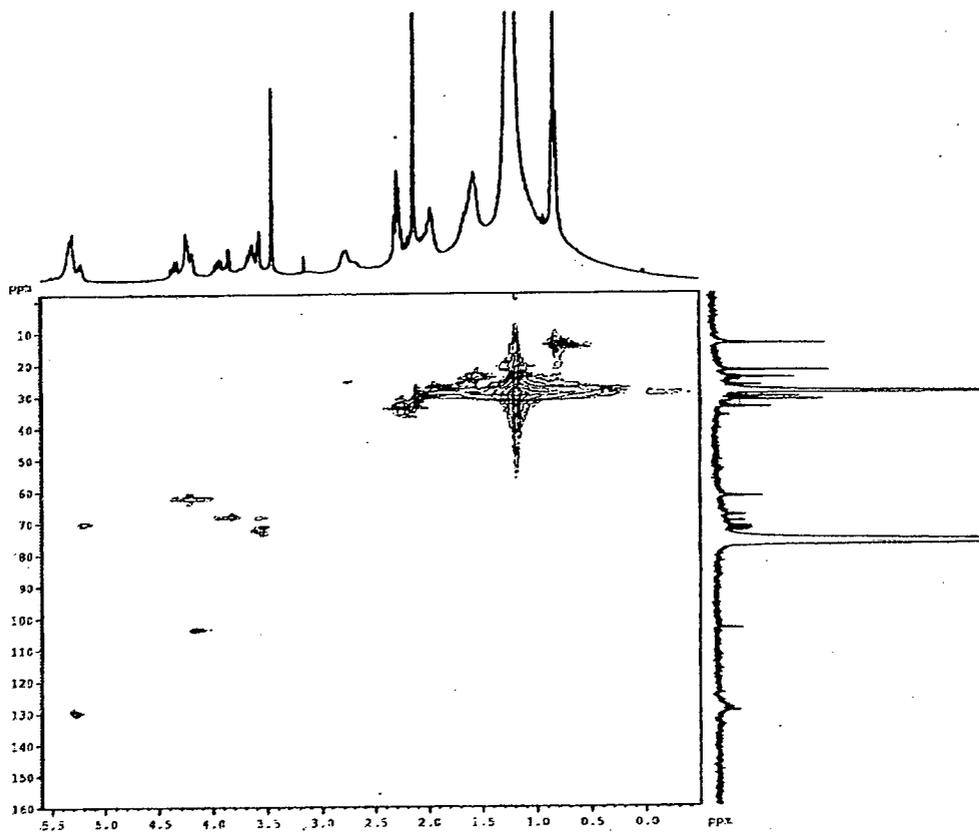


Fig1.5: HMQC spectrum of GL₁

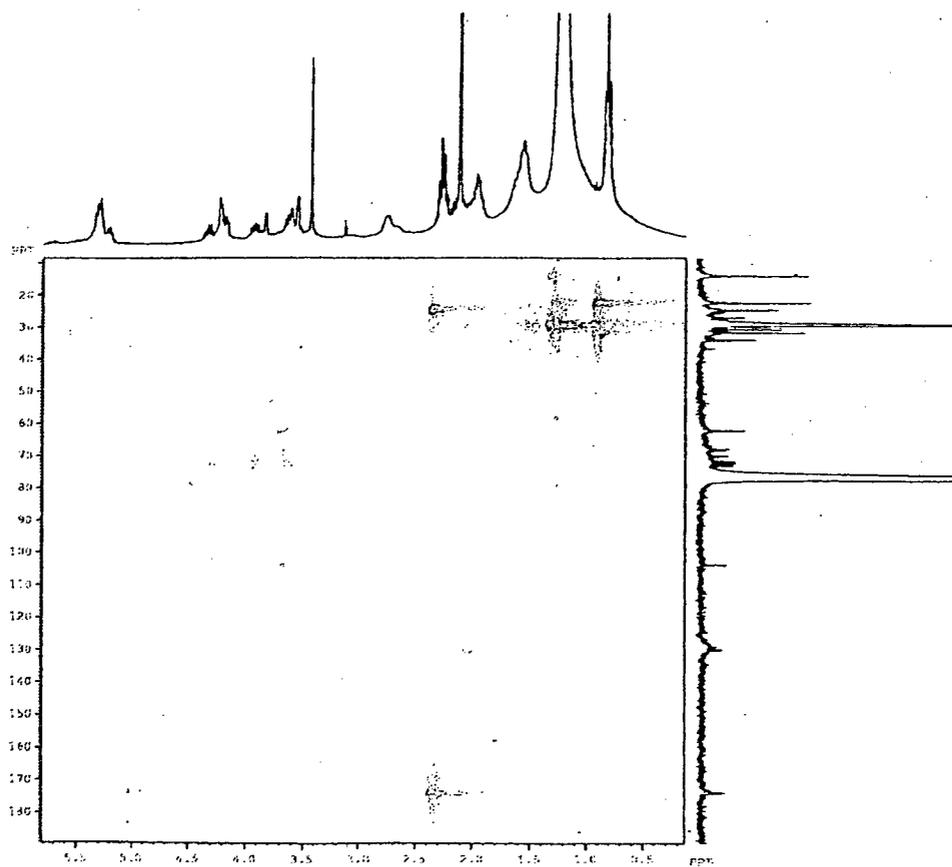


Fig1.6: HMBC spectrum of GL₁

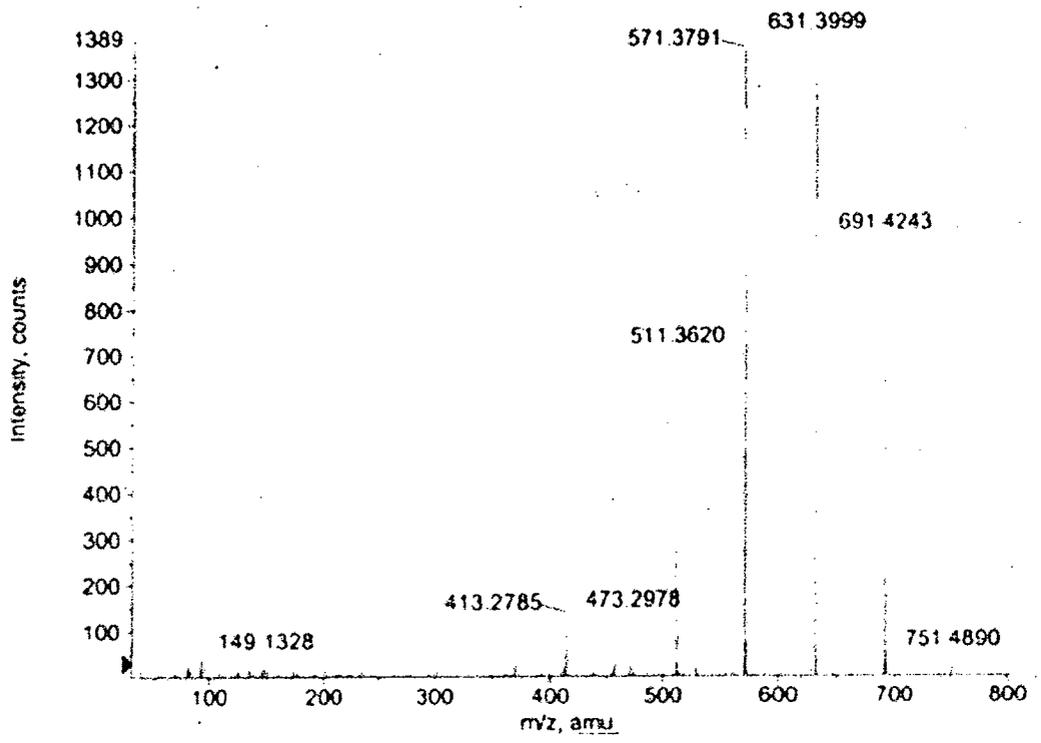


Fig 1.7:ESI-MS spectrum of GL₁

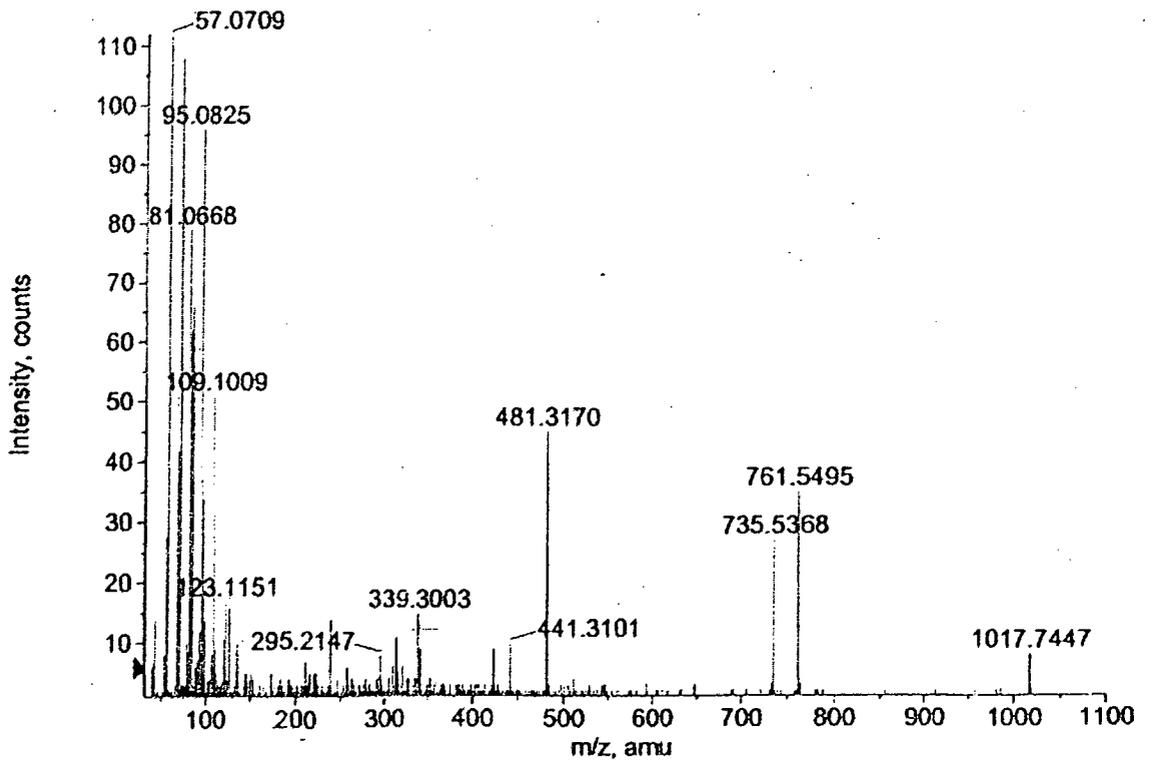


Fig1.8: MS/MS spectrum of GL_{1a}

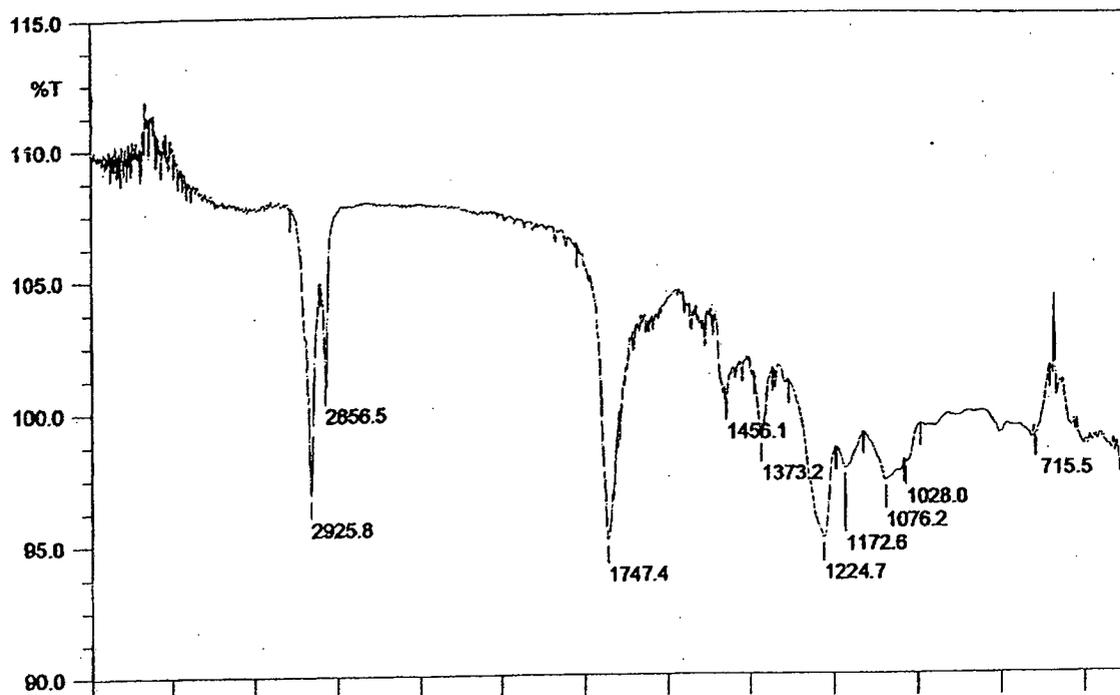


Fig 1.1: IR spectrum of GL₁

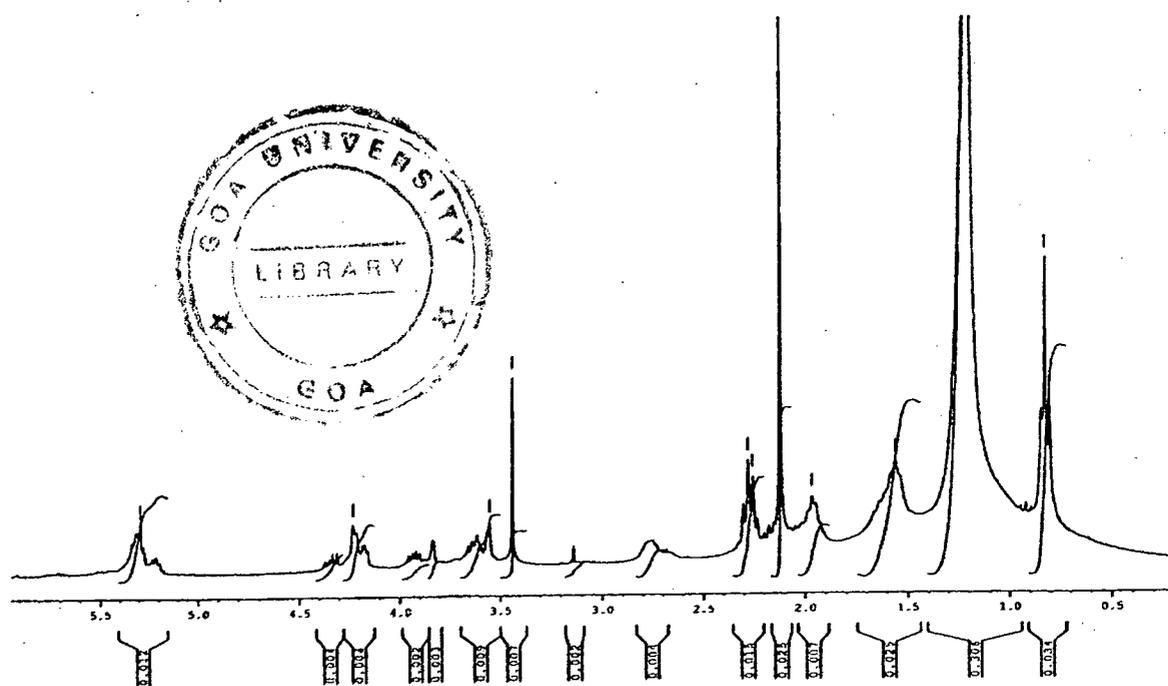


Fig 1.2: ¹H NMR spectrum of GL₁

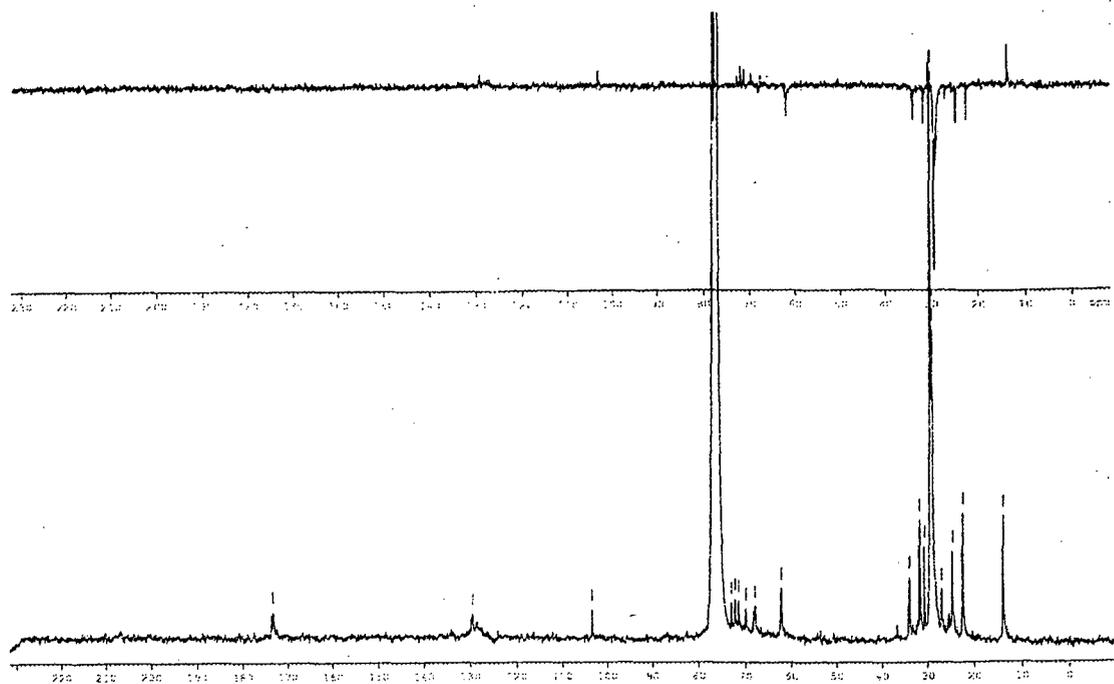


Fig 1.3: ^{13}C NMR and DEPT spectra of GL_1

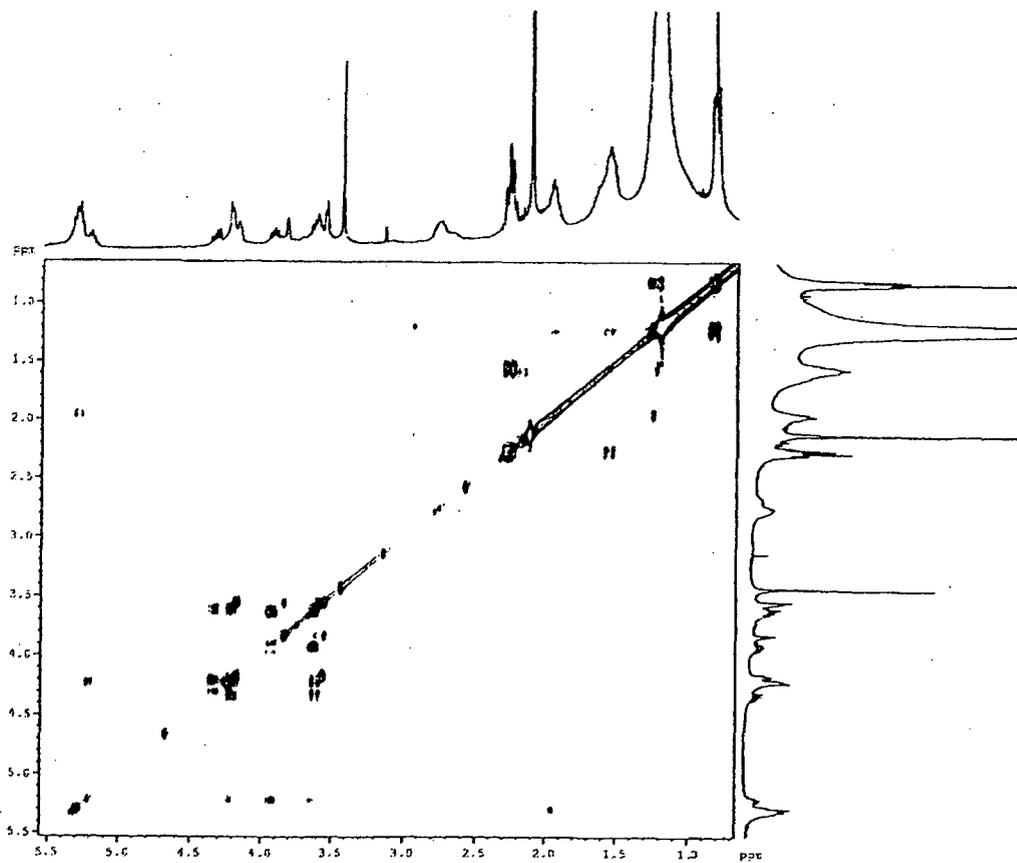


Fig 1.4: TOCSY spectrum of GL_1

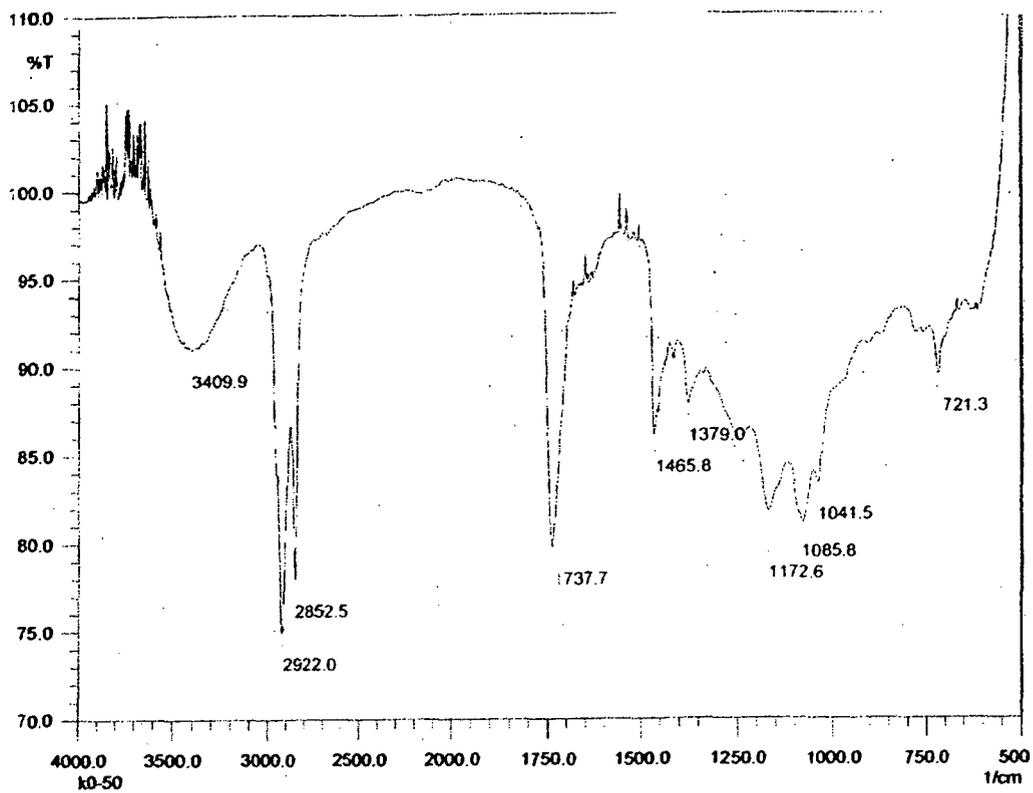


Fig 2.1: IR spectrum of GL₂

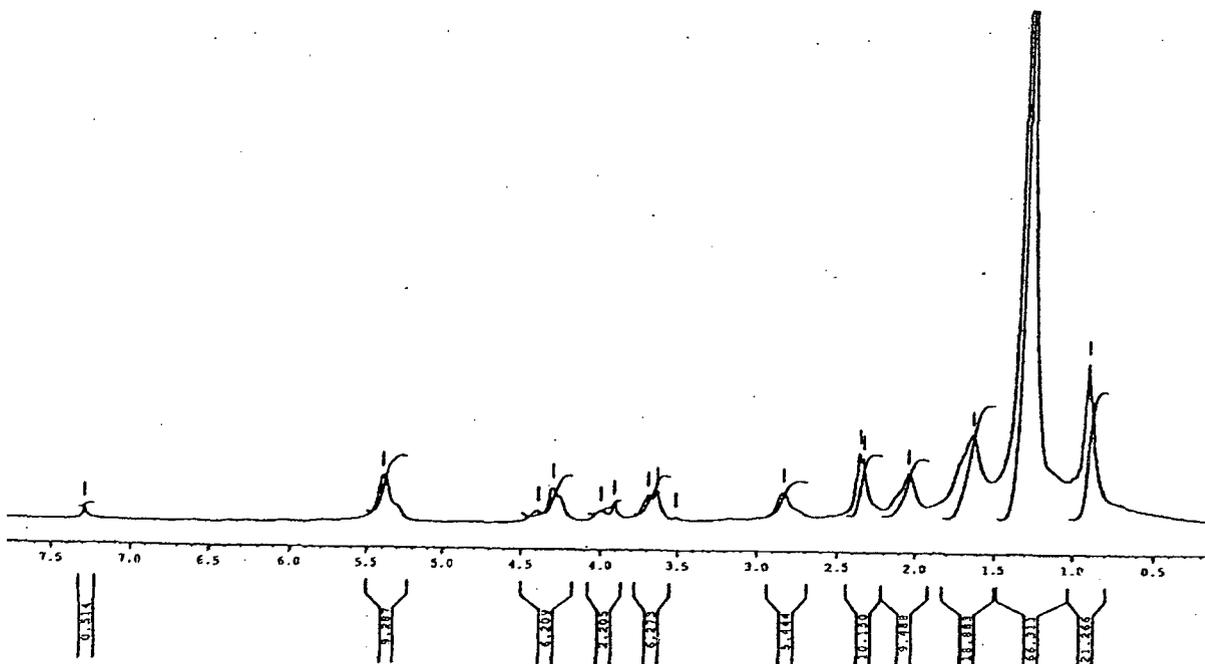


Fig 2.2: ¹H NMR spectrum of GL₂

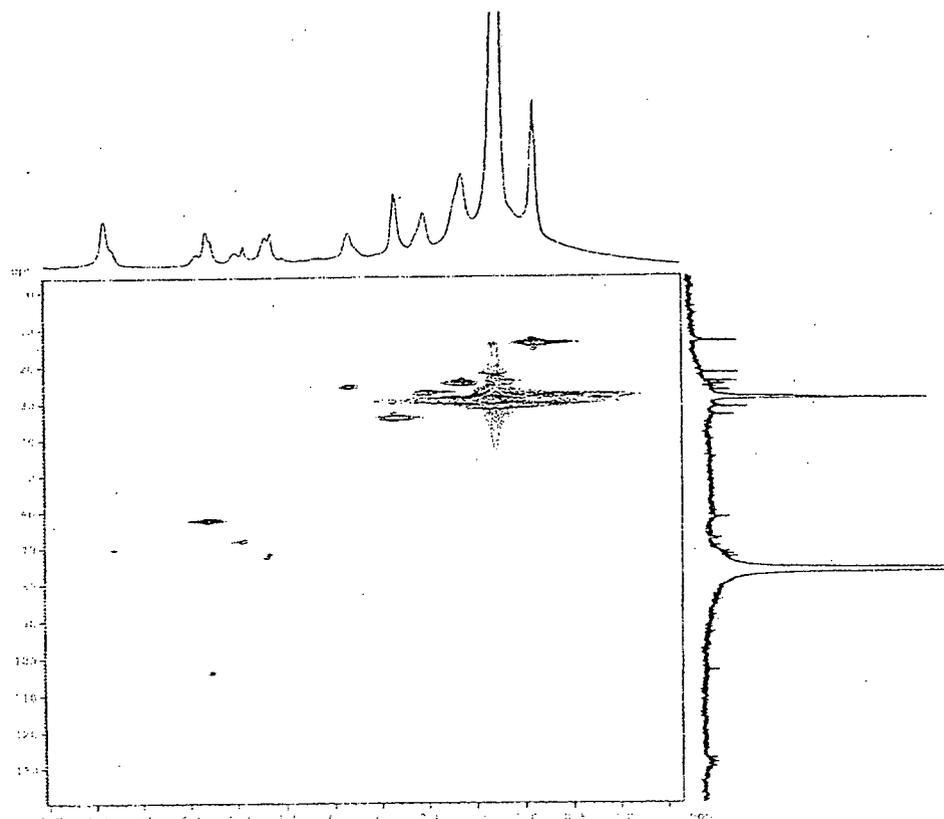


Fig2.5: HMQC spectrum of GL₂

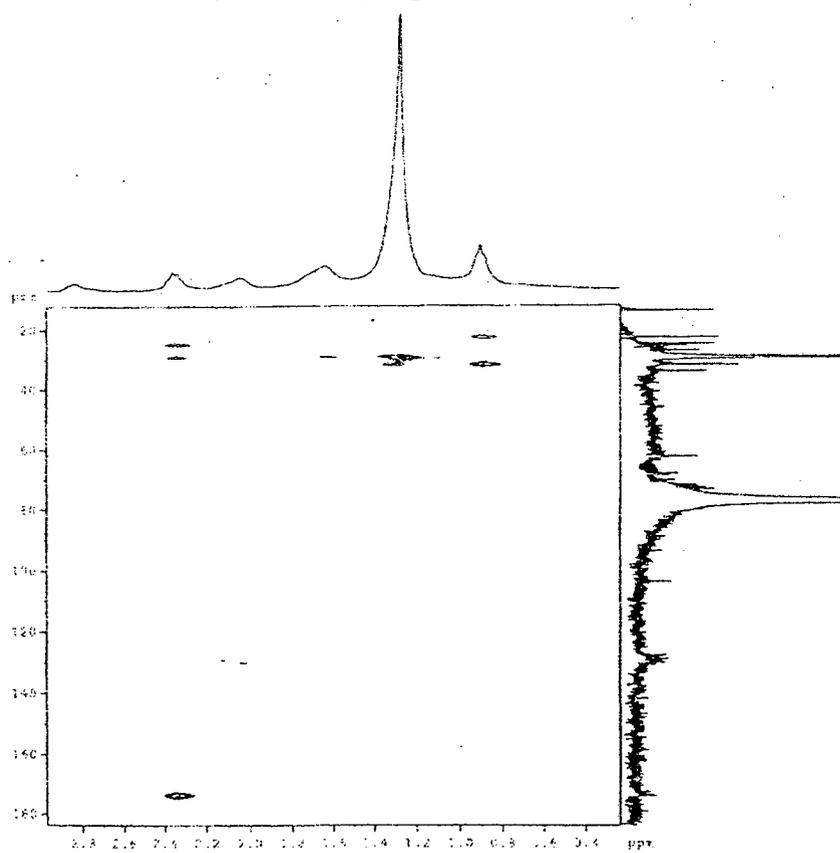


Fig2.6: HMBC spectrum of GL₂

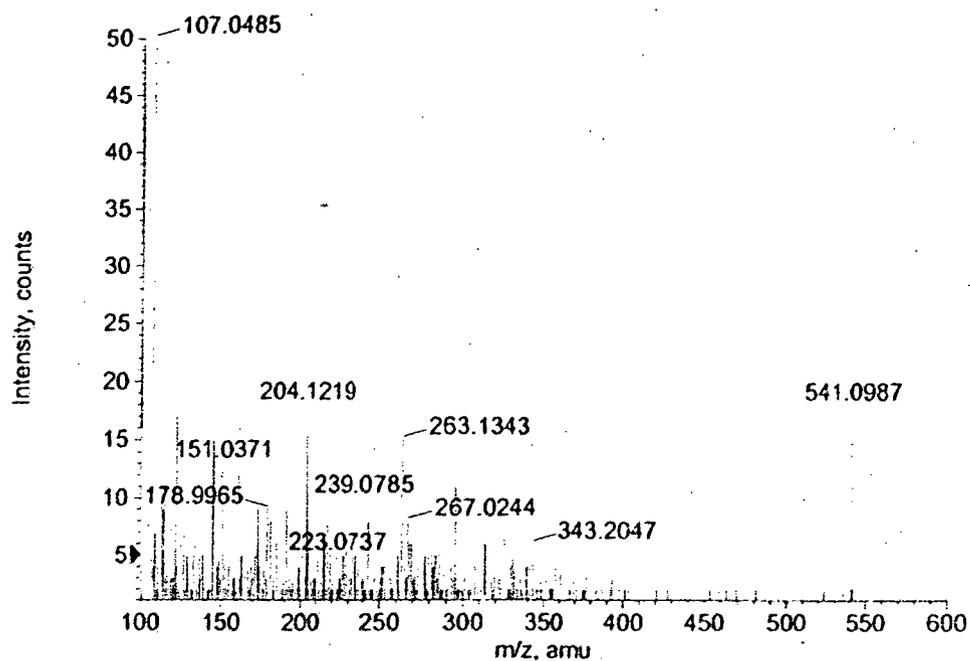


Fig 2.7: ESI-MS spectrum of GL₂

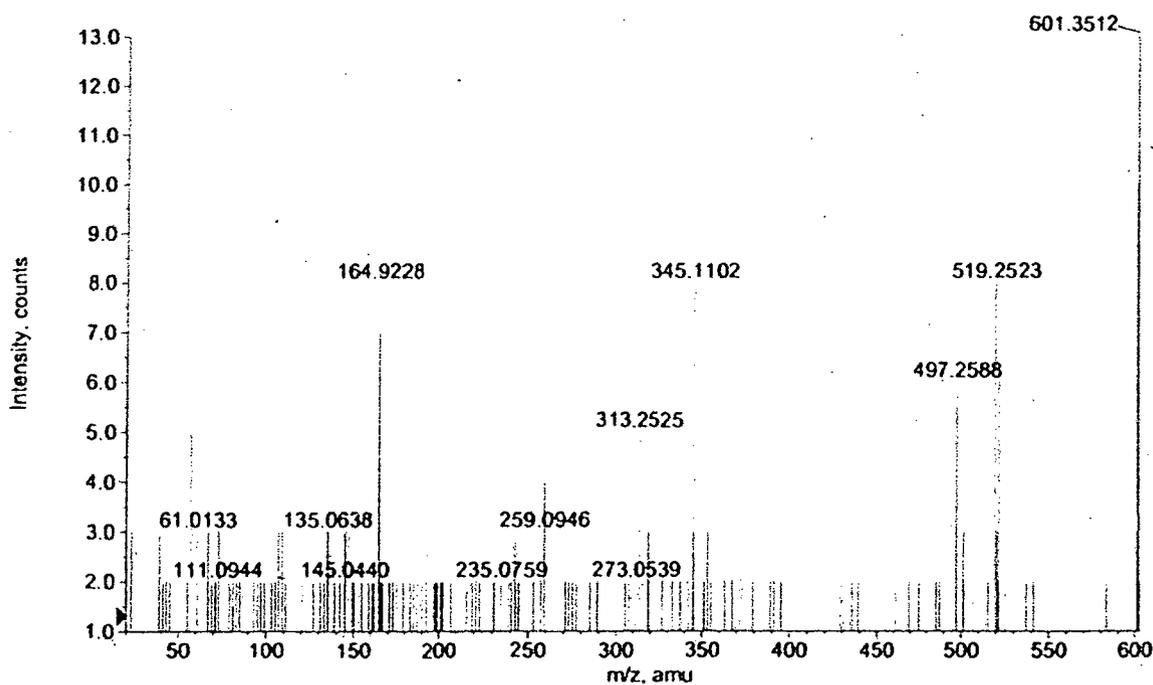


Fig2.8: MS/MS spectrum of GL_{2a}

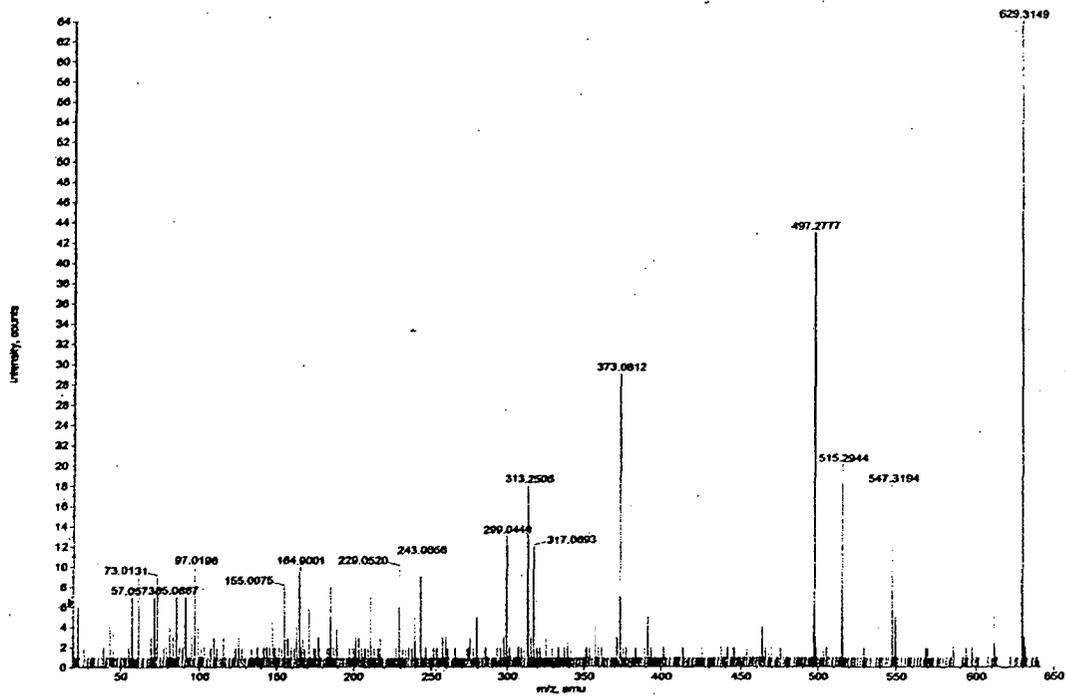


Fig 2.9: MS/MS spectrum of GL_{2b}

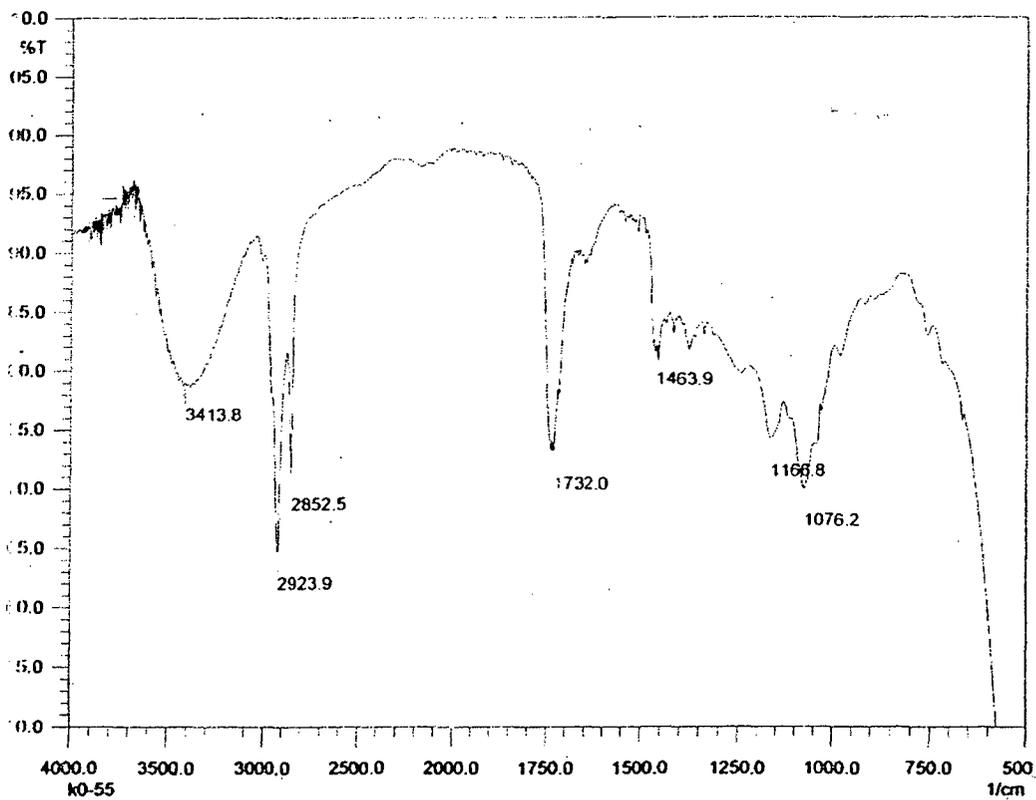


Fig 3.1: IR spectrum of GL₃

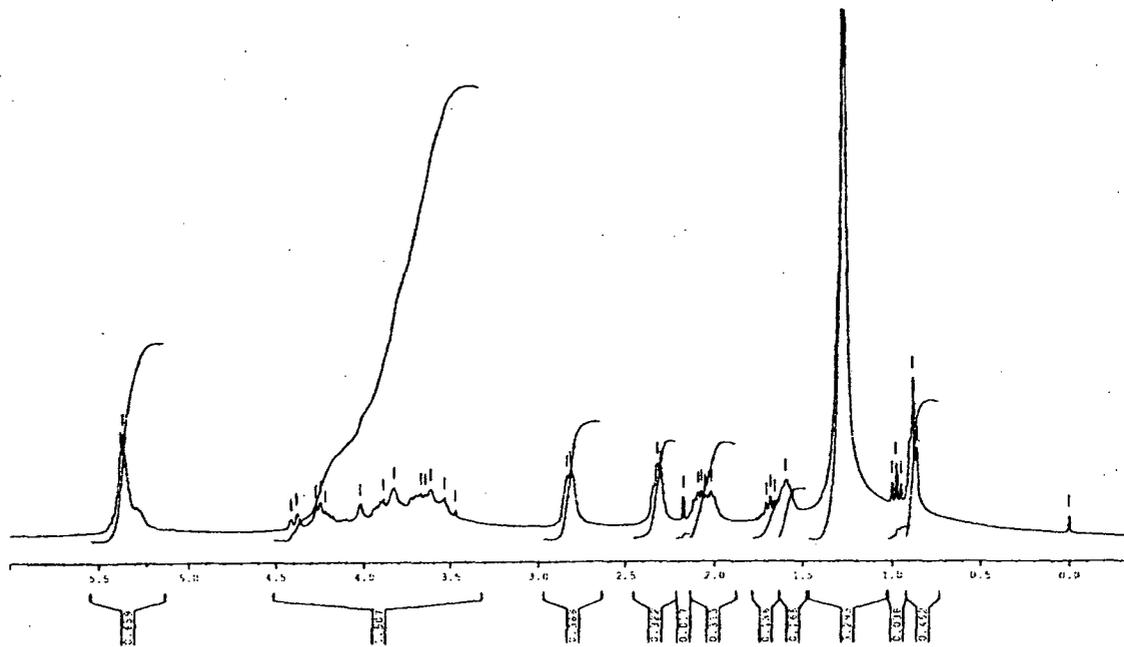


Fig 3.2: ^1H NMR spectrum of GL_3

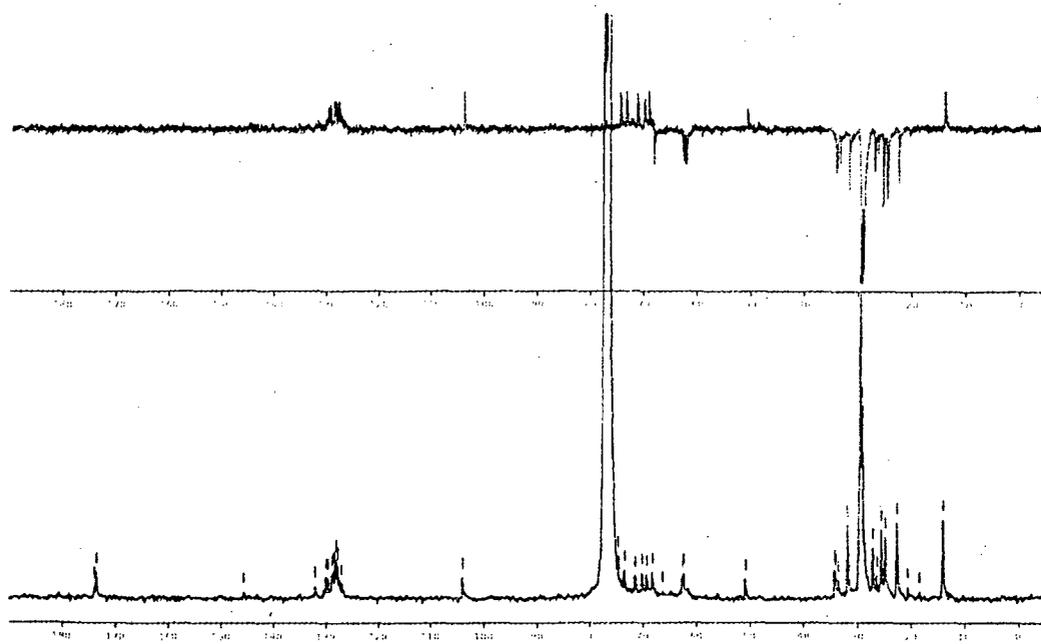


Fig 3.3: ^{13}C NMR spectrum of GL_3

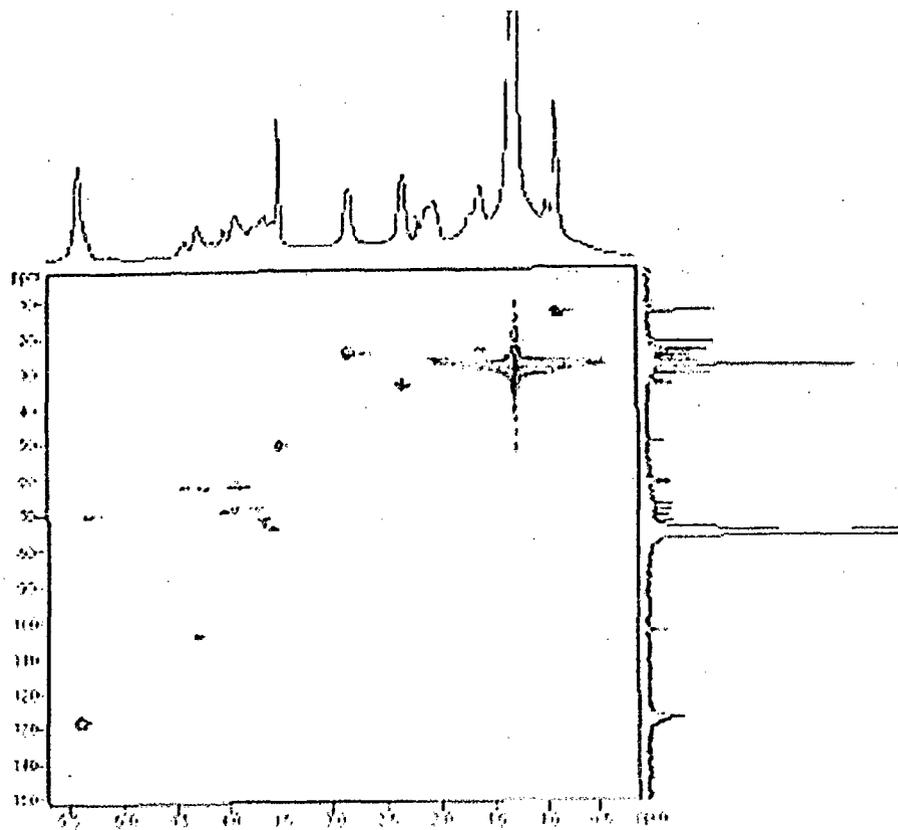


Fig3.4: HMQC spectrum of GL₃

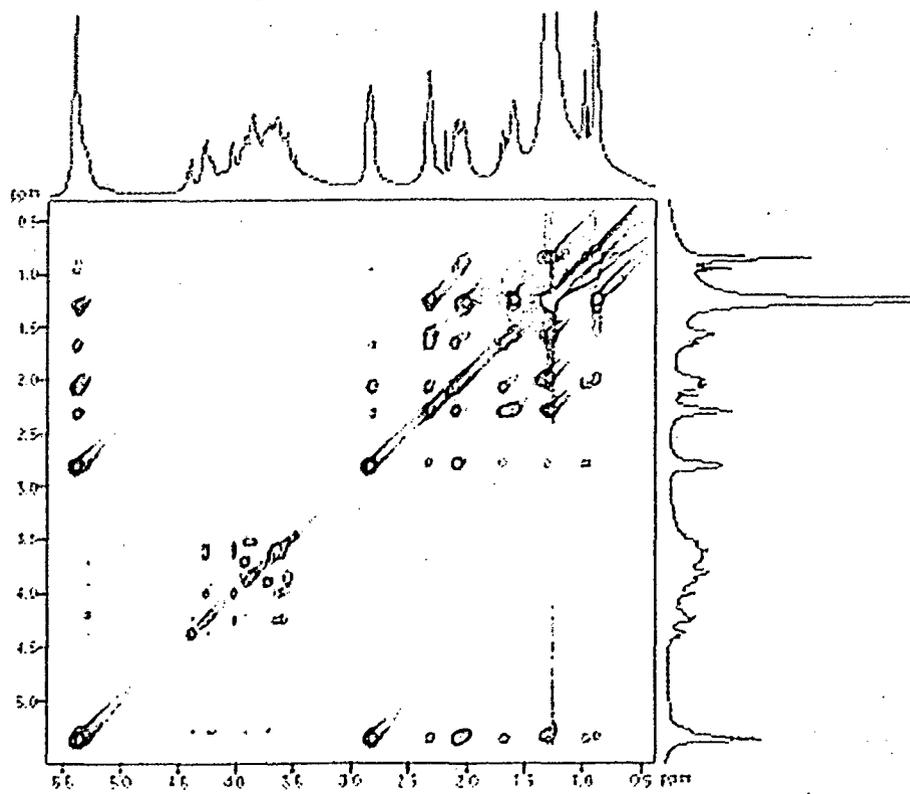


Fig 3.5: TOCSY spectrum of GL₃

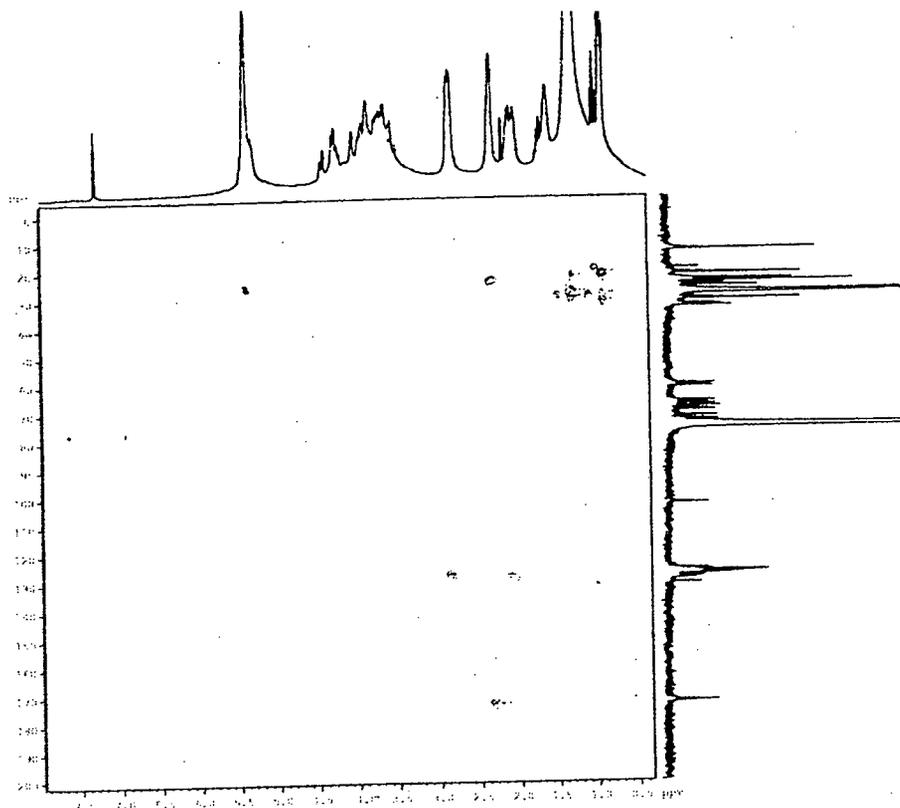


Fig3.6: HMBC spectrum of GL₃

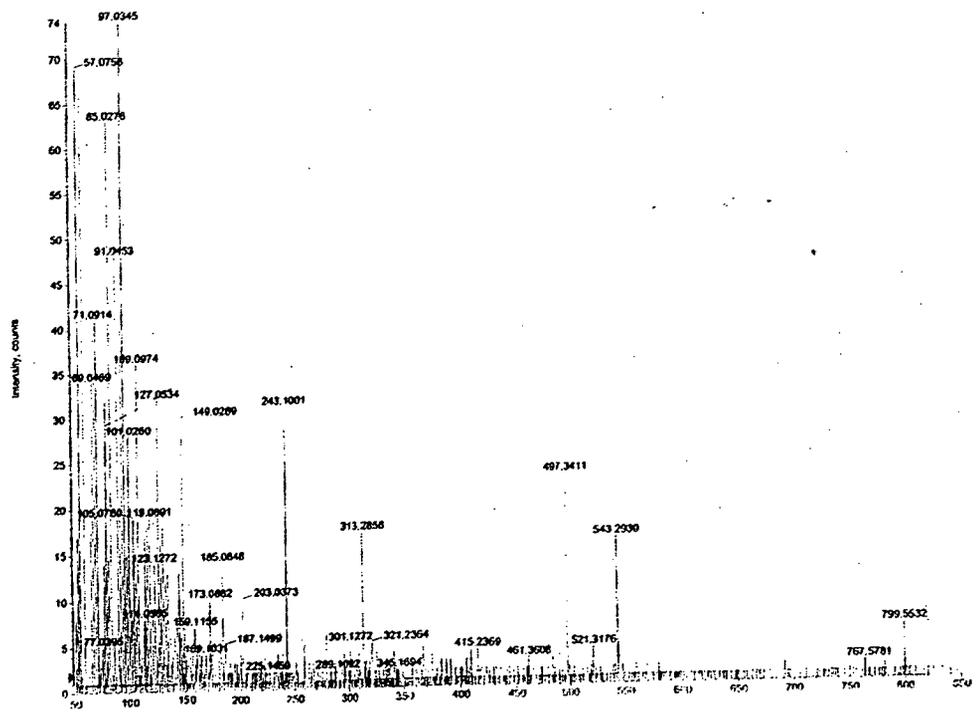


Fig 3.7:ESI-MS spectrum of GL₃

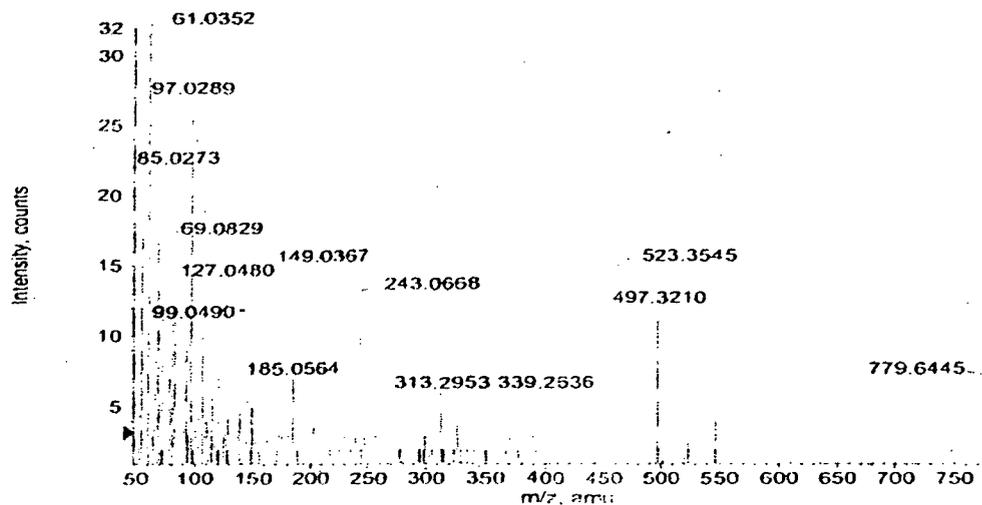


Fig3.8: MS/MS spectrum of GL_{3a}

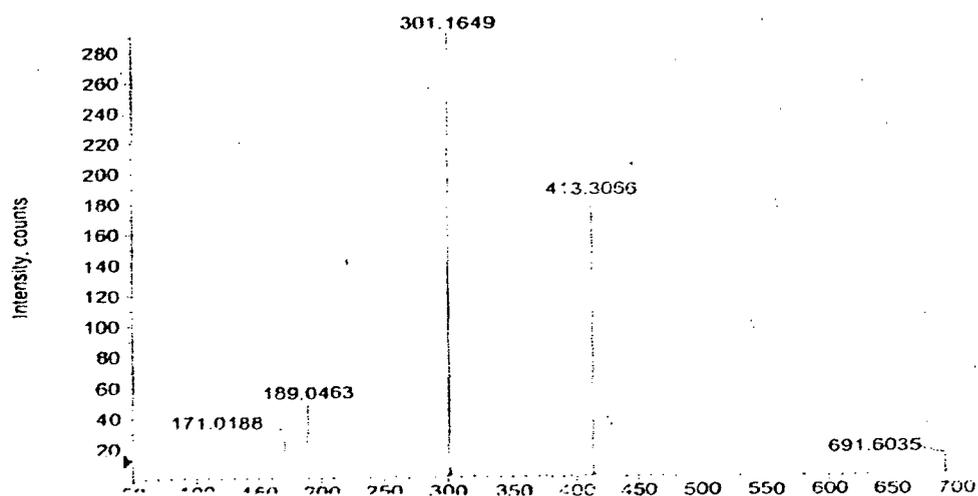


Fig3.9: MS/MS spectrum of GL_{3b}

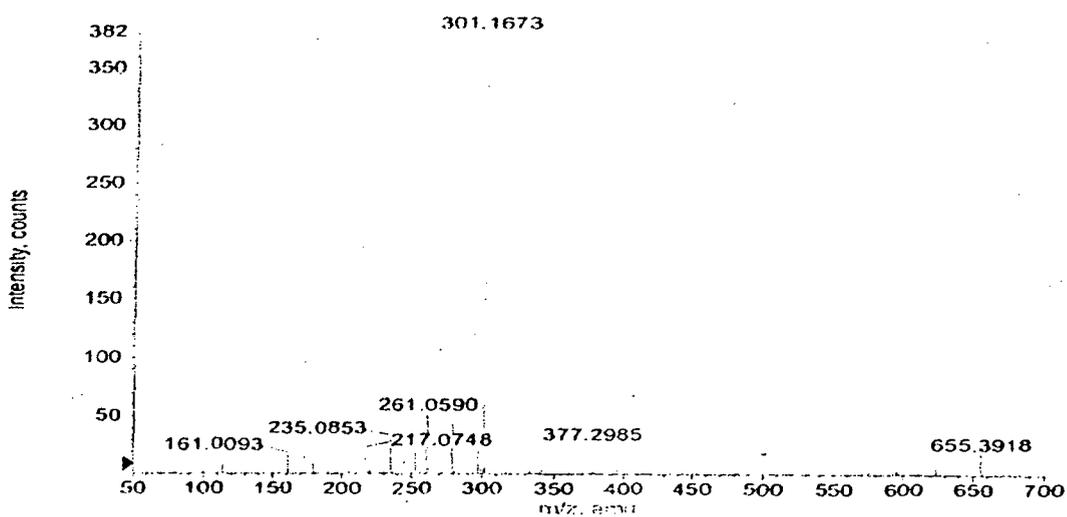


Fig3.10: MS/MS spectrum of GL_{3c}

1.2: Sterols constituents of the red alga *Chondria armata*:

Plants produce variety of different sterols, which represent a group of compounds that are alcoholic derivatives of cyclopentanoperhydrophenanthrene. These are essential constituents of cell membranes in animals and plants. Cholesterol is the sterol of mammalian cells, whereas multiple sterols, or phytosterols, are produced by plants, with sitosterol, campesterol, and stigmasterol being most common. Plant sterols, although structurally similar to cholesterol, are not synthesized by the human body. They are very poorly absorbed by the human intestine. The specific plant sterols that are currently incorporated into foods intended to lower blood cholesterol levels are extracted from soybean oil or tall (pine tree) oil. Additional sources of plant sterols may be available in the near future. The plant sterols currently incorporated into foods are esterified to unsaturated fatty acids (creating sterol esters) to increase lipid solubility, thus allowing maximal incorporation into a limited amount of fat. Some plant sterols currently available are saturated, to form the stanol derivatives, sitostanol and campestanol, which after esterification form stanol esters.⁵⁴

Oxysterols are oxygenated derivatives of cholesterol with a very short half-life relative to cholesterol. As a consequence they are present in very low concentrations in all mammalian systems, almost invariably accompanied by 10^3 - to 10^6 -fold excess of cholesterol. Oxysterols are important intermediates in a number of hepatic and extrahepatic catabolic pathways, most of which generate water-soluble bile acids as final products. Based on largely indirect evidence, and in spite of their low levels in vivo, oxysterols are generally believed to be important physiological mediators of cholesterol-induced effects. Perhaps the best support for this model is the existence of nuclear receptors that bind these compounds with high affinity and the fact that oxysterols potently regulate the expression of sterol-sensitive genes in vitro.⁵⁵

The occurrence of sterols in marine organisms can be discussed in various ways. Schmitz in his review⁵⁶ simply used a chemical approach by discussing the sterol structures in terms of carbon content. While convenient from a chemical standpoint, such a presentation has no bearing on biosynthesis or biological function and none was intended in that review. Goad⁵⁷ used a taxonomic approach starting at the bottom of the evolutionary tree with algae and fungi, and then proceeding via sponges, coelenterate animals and other intermediate phyla to the chordates. In addition to emphasizing the chemotaxonomic potential of marine sterol analyses - demonstrated in a striking fashion in sponges by Bergquist⁵⁸, this approach offers important clues to the possible origin of certain sterols in the food chain. The taxonomic approach offers some assistance in designing appropriate experiments for biosynthetic studies, which are much more complicated than in terrestrial organisms. To paraphrase an infamous limerick, when wondering about the origin of marine sterols we usually do not know "who is doing what, with which, and to whom." As Goad correctly pointed out⁵⁷, the existence of a given sterol in a specific marine organism may be due to one or more of the following four processes:

- (1) *De novo* sterol biosynthesis via acetate, mevalonate and squalene.
- (2) Dietary origin without further chemical modification.
- (3) Dietary origin of sterols followed by chemical modification.
- (4) Result of symbiotic relationship between host and symbiont (e.g. algae, fungi, bacteria).

Over the last decades, there have been many investigations on the chemical composition of algal lipids, including the composition of sterols. However, taxonomic classifications based on these data were not always fully substantiated, especially earlier ones, obtained by unsophisticated analytical methods. Still, some taxonomic conclusions based on sterol composition are available⁵⁹. Different classes of algae have a distinct sterol composition. For the evolutionary lower red algae (*Rhodophyceae*), cholesterol and in some cases, its biogenetic precursor cholesta-5,24(25)-dien-3 β -ol are the major sterols, while those of the same class

considered more advanced contain 24-methylenecholesterol, the C-24 alkylated sterols and sterols with Δ^{22} -double bond being present in low concentrations.

In brown (*Phaeophyceae*) and green (*Chlorophyceae*) algae, which are regarded as evolutionary more advanced, methylation products of 24-methylenecholesterol are the major sterols – fucosterol ((*E*)-stigmasta-5,24(28)-dien-3 β -ol) in the former and isofucosterol ((*Z*)-stigmasta-5,24(28)-dien-3 β -ol) in the latter, while in advanced green algae, the $\Delta^{24(28)}$ double bond is reduced, leading to an accumulation of sitosterol ((24*R*)-stigmast-5-en-3 β -ol)⁵⁹.

The prognosis for the future of marine sterol chemistry is excellent. It is quite likely that additional novel structures will be encountered, which will serve to delineate even further the range (in terms of carbon content) and variety of substitution patterns possible in the side chain. Unique nuclear variations seem less abundant, but when they are found, they are likely to be of considerable taxonomic significance.

Present section gives a full account of the structural elucidation of major oxygenated polar keto steroids identified as cholest-4-en-3,6-dione (**1**), 6 β -hydroxy cholest-4-en-3-one (**2**) and cholest-4,24-dimethyl-6 β -hydroxy-4-en-3-one (**3**) (**Fig 2a**) and reports antimicrobial activity exhibited by **1** & **2**.

Despite the importance of steroids, the literature on ESI-MS of neutral sterols is limited mainly because it does not have the required sensitivity for trace analysis, and the ionization efficiencies of most sterols are relatively low. Reports available are mainly on the derivatised sterols as glucuronated and sulfated metabolites^{60,61}. Here, effort to elucidate structure of the additional related molecular species, with the same R_f value on TLC and were inseparable from the purified major steroids, was made based on tandem mass spectrometry. The structures proposed for these minor constituents have also been incorporated. These were identified as cholest-4-en-3-one (**4**), 25-hydroperoxy-24-methyl-cholest-4-en-3,6-dione (**5**), 5 α -cholesta-3,6-dione (**6**), 24-ethyl-4-methyl-6-hydroxy-cholest-4-en-3-one (**7**) and cholesta-4,24-dimethyl-6 β -hydroxy-3-one (**8**) (**Fig 2a**).

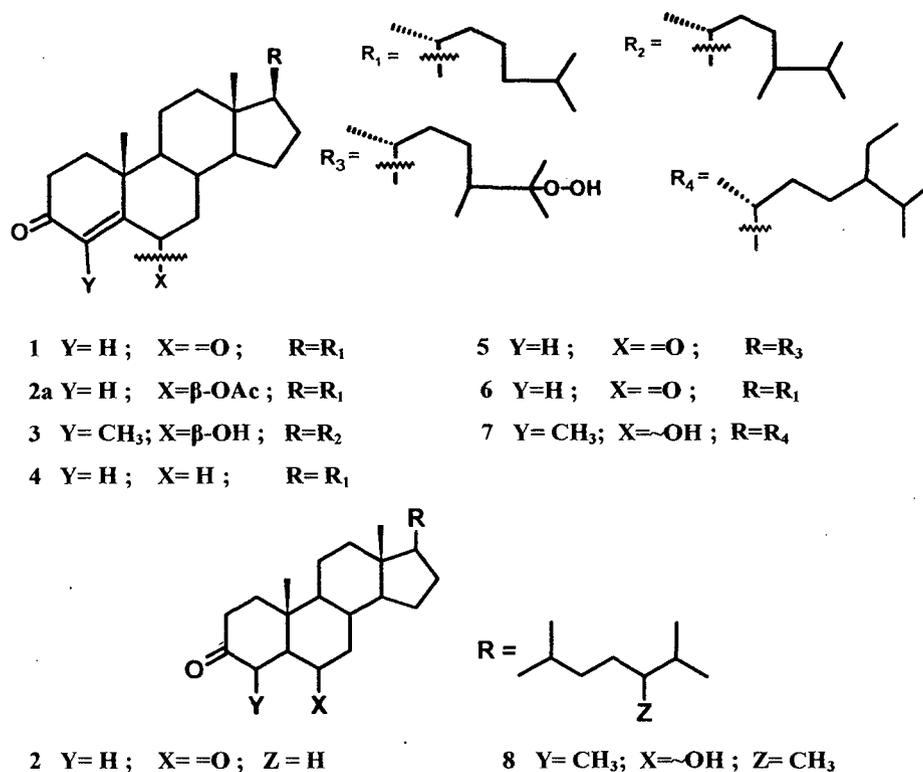


Fig. 2a: Structures of the compounds 1 to 8 from *Chondria armata*

Results and discussion:

The chloroform soluble fraction on filtration over Sephadex LH20 followed by chromatography over a silica gel column gave, in order of their polarity, compounds 1-2 apparently homogeneous on TLC. Compound 1, a crystalline solid, $R_f = 0.76$ [solvent system, 30:70 (ethyl acetate:petroleum ether)] m.p. 124°C, [124-125°C]⁶² coupled with a $[M+H]^+$ peak at m/z 399 in the ESI/MS spectrum IR(Fig.2.1) and NMR(Fig.2.2) data suggested a molecular formula of $C_{27}H_{42}O_2$, indicating seven degrees of unsaturation. The ¹³CNMR spectrum of 1(Fig.2.3, Table-1) together with the information from a DEPT spectrum (Fig.2.3), showed the presence of 27 well-resolved signals of which 5 were methyls, 10 methylenes, 7 methines (one olefinic) and 5 non-protonated carbons (one olefinic and two ketonic). These data were consistent for a monounsaturated diketosteroid. A comparison of these data with those of cholest-4-en-3,6-dione reported for the diketosteroid synthesized from cholesterol tetrahydropyranyl

under non aqueous conditions⁶³ established the structure of compound **1** as cholest-4-en-3,6-dione. The structure is well in agreement with the fragmentation observed in its tandem mass spectrum of ion at m/z 399.3.

ESI-MS/MS of compound **1** with the pseudomolecular ion $[M+H]^+$ at m/z 399 (Fig. 2.4,C) exhibited a base peak at m/z 109 (corresponding sodium and potassium adducts were observed at m/z 131 and 147 respectively) resulting from the simultaneous cleavage of C₅-C₆ and C₉-C₁₀ bonds in ring B. The base peak on elimination of C-19 methyl led to the sodiated adduct ion at m/z 117. Cleavage of C₆-C₇ bond along with C₉-C₁₀ bond yielded protonated fragment at m/z 137 (corresponding sodium adduct appeared at 159) which is characteristic of steroidal 4-en-3,6 diketones⁶⁴. Ring C cleavage (fission of C₉-C₁₁ and C₈-C₁₄ bonds) produced protonated fragments at m/z 177 (sodiated ion at 199) and m/z 223. Fission of C₁₁-C₁₂ and C₈-C₁₄ bonds of ring C generated the protonated ion at m/z 191. Ring D cleavage (fission of C₁₄-C₁₅ and C₁₃-C₁₇ bonds) resulted in protonated ion at m/z 155. Ring A cleavage (fission of C₁-C₁₀ and C₃-C₄ bonds) yields the protonated fragment ion at m/z 341. Thus, the structure of compound (**1**) was confirmed as cholest-4-en-3,6-dione. Positive ESI-MS spectrum of compound (**1**) showed additional pseudomolecular ions at m/z 385, 401 and 445 besides its protonated pseudomolecular at m/z 399 (Fig. 2.4,A). Based on the fragmentation observed, these molecular species have been identified and the structures are proposed.

The pseudomolecular ion peak at m/z 385 identified as cholest-4-en-3-one (**4**) when subjected to CID at collision energy of 40 KeV (Fig.2.5,A) showed the most abundant ion at m/z 123, characteristic of Δ^4 -3-keto-steroids, and ion at m/z 261 which loses the side chain to produce fragment at m/z 149. The base peak on elimination of C-19 methyl group yields protonated ion at m/z 109. The ions at m/z 367 and 357 represent ions generated by the loss of water and alkene respectively from the protonated molecule. Ring A cleavage at C₁-C₁₀ and C₃-C₄ resulted in the formation of ion at m/z 329. Fragment ion at m/z 177 is formed by splitting of C₁₁-C₁₂ and C₈-C₁₄ bonds. Similar cleavage but now involving C₁₂-C₁₃ and C₈-C₁₄ bonds gave ion at m/z 189. Ion at m/z 259 represents loss of side chain along with C₁₈ methyl. Ring D cleavage yields ion at m/z 247.

CID spectrum of the pseudomolecular ion at m/z 445 is represented in (Fig.2.5,B). Initial loss of oxygen from the peroxy group in the side chain (ion at m/z 413) is associated with the loss of either one methyl (ion at m/z 399) or two methyls (ion at m/z 385). The latter fragment in turn loses either one water molecule or two water molecules resulting in the formation of ions at m/z 367 and 349 respectively. Concomitant elimination of side chain along with one water molecule gives ions at m/z 269; this fragment could also result from ring C cleavage along C₈-C₁₄ and C₁₂-C₁₃ bonds. Fission of C₈-C₁₄ and C₁₁-C₁₂ gives rise to ion at m/z 255 and when cleavage of ring B at C₉-C₁₀ is associated with the fission of C₅-C₆, it yields ion at m/z 339, whereas cleavage associated with C₆-C₇ bond or C₈-C₉ bond leads to fragments m/z 137 or m/z 151 respectively. Ring D cleavage along the C₁₃-C₁₇ and C₁₄-C₁₅ produces ion at m/z 201 which in turn loses two methyls with the formation of ion at m/z 171; same cleavage associated with the loss of two water molecules and a methyl generated ion at m/z 259. Based on this fragmentation structure 25-hydroperoxy-24-methyl-cholest-4-en-3,6-dione was proposed for the compound (5).

The CID spectrum of the [M+H]⁺ precursor ion at 401 (Fig.2.4,B) produced key ions at m/z 383 and 365 by successive elimination of one and two water molecules respectively. Loss of side chain followed by elimination of water generates ions at m/z 289 and m/z 271 respectively. Ring D cleavage at C₁₃-C₁₇ and C₁₄-C₁₅ accompanied by elimination of one water molecule gives ion at m/z 229, which subsequently loses two methyl groups resulting in the formation of fragment at m/z 199. Ring C cleavage at C₁₁-C₁₂ and C₈-C₁₄ produced sodiated ion at m/z 215 whereas cleavage of the same ring at C₁₂-C₁₃ and C₈-C₁₄ resulted in the formation of the ion at m/z 207 which generated ion at m/z 189 with the loss of one water molecule. Ring B cleavage between C₉-C₁₀ and C₇-C₈ results in the sodiated ion at m/z 175 and the fission of C₉-C₁₀ and C₅-C₆ bonds leads to the protonated ion at m/z 111 which in turn loses a methyl group to produce fragment at m/z 95. The structure 5 α -cholesta-3,6-dione (6) is well in agreement with the fragmentation observed for this molecule.

Compound (2) was obtained also as crystalline solid, next to compound (1) in elution, melting point 194°C [lit 192-195°C]⁶⁵ analysed for C₂₇H₄₄O₂ which was

supported by pseudomolecular ion $[M+H]^+$ at m/z 401 in its ESI-MS spectrum (Fig.2.13, B). It showed hydroxylic (3479.3 cm^{-1}), and α - β unsaturated ketonic absorption (1681.8 cm^{-1}) in its IR spectrum (Fig. 2.6). The ^1H (Fig.2.7) and ^{13}C NMR (Fig.2.8) spectra were typical of a sterol. The ^{13}C NMR indicated a secondary alcohol function with a doublet at δ 73.245 in addition with a carbonyl resonating at 200.443 ppm and olefinic singlet and doublet at 168.528 and 126.282 ppm respectively.

The ^1H NMR (300MHz, CDCl_3 , Table-1) of (2) showed in addition to hydroxymethine proton at δ 4.35, the presence of an olefinic proton singlet at δ 5.81(H-4), two tertiary methyls resonating as singlets at δ 0.744 (H_3 -18) and δ 1.379 (H_3 -19). A doublet at δ 0.929 ($J=6.3\text{Hz}$) was assigned to C_{21} methyl group and a pair of doublets at δ 0.876 and at δ 0.873 due to isopropyl methyls. These data suggested that compound (2) is a hydroxyketosteroid.

The location of the functional groups was deduced by a combination of COSY (Fig.2.9), HMQC(Fig.2.10) and HMBC(Fig.2.11), 2D NMR experiments. From the ^1H - ^1H COSY H-4-H-6-H₍₂₎-2—H₍₂₎-1 and H-6-H₍₂₎-7 spin system were inferred and by TOCSY additional H-6-H₍₂₎-7-H-8-H9 system was deduced. HMBC correlation of H_3 -19 at δ 1.379 allowed identifying C-1, C-5, C-9 and C-10. The olefinic proton at δ 5.81 showed HMBC connectivity with C-10 (37.084) and C-6 (73.245) while the hydroxymethine proton at δ 4.35 correlated with C-5(168.528), C-7 (38.557) and C-8 (29.709). HMBC correlations are illustrated in Fig 2b.

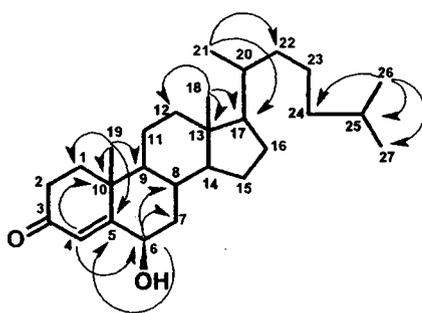


Fig 2b : HMBC correlations of compound 2

^1H - ^1H COSY had already established H₍₂₎-2—H₍₂₎-1 connectivity, thus, completing the assignments of rings A and B with the placement of the carbonyl

and hydroxyl functionalities at C-3 and C-6 respectively. HMBC correlation was also observed for H₍₃₎-18 at δ 0.744 with C-12 (39.479), C-13(42.501) and C-17 (56.151).

The side chain linked to C-17 was assigned by the long range correlation observed for H₃-21 at δ 0.929 with C-22 (36.114) and C-17(56.151) carbons and H₃-26 with C-27(22.541), C-25(27.996) and C-24 (39.479). Thus, based on the above data the structure of (2) was established as cholest-6-hydroxy-3-one.

The stereochemistry was assigned on the basis of NOESY data. An NOE was observed between H-6 at δ 4.3 and H-7 proton at δ 1.379 which had NOE correlation with H-9 at δ 0.858 which served to assign a β orientation to the –OH group. NOE was also observed between H-4 at δ 5.81 and H-6 further confirming the β orientation of the –OH group. Additional evidence comes from the melting point 194°C and the optical rotation $[\alpha]_D = +42.5$ ($c = 09, \text{CHCl}_3$) observed for compound (2) well in agreement with the reported values⁶⁶ for cholest-6 β -hydroxy-4-en-3-one. Fragmentation observed in ESI/MS/MS of compound (2) further confirmed the structure assigned.

Tandem mass spectrometry of protonated compound (2), $[\text{M}+\text{H}]^+$ 401.3 (Fig.2.13, B), gave a base peak at m/z 383 resulting from dehydration of the molecule. Subsequent elimination of side chain produced fragment at m/z 271. Elimination of a second water molecule originated by protonation of the ketonic group and abstraction of the hydrogen from the adjacent carbon, led to the fragment m/z 365. which yielded ion at m/z 253 on the loss of side chain. Loss of two water molecules associated with the cleavage of ring C (C₈₋₁₄ and C₉₋₁₁) generated the sodiated ion at m/z 243 and loss associated with ring A cleavage (C₃H₄) produced ion at m/z 325. Loss of ketene from ring A along with two methyl groups from the pseudomolecular ion gave ion at m/z 329. Ion at m/z 109 resulted from the cleavage of the bond at C₅-C₆ and C₉-C₁₀ producing ion at m/z 81 on the loss of ethylene from ring A and ion at m/z 325 on simultaneous loss of water and methyl group. Loss of methyl group alone with the attachment of two hydrogens yielded ion at m/z 95.

Table-1 ¹³C NMR (300MHz) data of compounds 1 and 2 in CDCl₃

Carbon No.	Compound 1		Compound 2	
	¹³ CNMR	¹³ CNMR	¹ HNMR	HMBC
1	35.5	37.9	2.02	
2	33.9	34.2	2.41	
3	199.4	200.4	-	
4	125.4	126.2	5.81	C10, C6
5	161.0	168.5	-	
6	202.2	73.2	4.35	C5, C7, C8
7	46.7	38.5	1.37	
8	34.2	29.7	1.25	
9	50.9	53.6	0.85	
10	39.7	37.0	-	
11	20.8	20.9	1.37	
12	39.1	39.4	1.12	
13	42.5	42.5	-	
14	56.5	55.8	0.88	
15	23.9	24.1		
16	27.9	27.9		
17	55.9	56.1	0.88	
18	11.8	12.0	0.74	C12, C13, C17
19	17.4	19.4	1.37	C1, C5, C9, C10
20	35.5	35.7	1.50	
21	18.6	18.6	0.92, J = 6.3Hz	C22, C17
22	36.0	36.1		
23	23.7	23.8		
24	39.4	39.4	1.12	
25	27.9	27.9		
26	22.5	22.7	0.87	C27, C25, C24
27	22.7	22.5	0.87	

The ¹HNMR data of compound (2) from repeat collection showed the presence of an additional sharp singlet at δ 1.601. This was attributed to a methyl group on a double bond and hence it was placed at C₄. Since there was no separate doublet for C₂₈ methyl it was presumed that it coincides with C₂₁ doublet centred at δ

0.914. This assumption is reinforced by the presence of additional signal for C₂₄ carbon at δ 41.3 in the ¹³CNMR of compound (2). The positioning of the second methyl group at C₂₄ stems from the fact that biogenetically 24-methylene cholesterol is precursor of 24 alkylated steroids and it is one of the major steroids in *Chondria armata*⁶⁷. On the basis of NMR signals observed it was concluded that the compound (2) contained cholest-4,24-dimethyl-6-hydroxy-4-en-3-one (3) in minor quantities. This is further supported by the presence of additional molecular species with [M+H]⁺ ions at *m/z* 429 corresponding to the molecular mass of (3) and ions at *m/z* 431 and 465 besides the pseudomolecular ion [M+H]⁺ at *m/z* 401 for compound (2) in its ESI-MS (Fig.2.12,A). The fragmentation observed for molecular species with protonated molecular ion *m/z* 429 is well in agreement with cholest-4,24-dimethyl-6-hydroxy-4-en-3-one.

The genesis of some of the major peaks observed in the mass spectrum of steroid (3) has been briefly discussed. The MS/MS spectrum of molecular species with [M+H]⁺ ion at *m/z* 429 at collision energy of 40 V is shown in (Fig.2.13,A). The ions at *m/z* 411 and 393 represent [(M+H)⁺-H₂O] and [(M+H)⁺-2H₂O]. The fragment ion at *m/z* 359 and 73 seems to have originated from the cleavage of C₂₃-C₂₉ bond of the side chain with the rearrangement of a hydrogen atom. Loss of one and two water molecules from the former ion results in the formation of ions at *m/z* 341 and 323 respectively. Elimination of the side chain produces ion at *m/z* 301. Ring D cleavage (C₁₃-C₁₇ and C₁₄-C₁₅) along with the side chain produces appreciable amount of ion at *m/z* 247. Ring B cleavage at C₉-C₁₀ and C₇-C₈ with the initial loss of one water molecule gives rise to ion at *m/z* 149 but with simultaneous loss of methyl group leads to ion at *m/z* 135. Cleavage of C₉-C₁₀ and C₅-C₆ bonds with simultaneous elimination of alkene from ring A produces ion at *m/z* 95. Thus, based on the fragmentation pattern observed the molecule with pseudomolecular ion at *m/z* 429 were confirmed as 4,24 dimethyl-6-hydroxy cholest-4-en-3-one. Further confirmation of the presence of this molecular species arises from the presence of the sodiated ion at *m/z* 493 in the mass spectrum of acetylated compound (2).

Product ion spectrum of sodiated molecular ion at *m/z* 493 is illustrated in (Fig.2.13, C). Initial loss of acetic acid from the sodiated molecular ion (ion at *m/z*

433) is followed by the loss of ketene (ion at m/z 391), which in turn loses C_5H_{11} from the side chain leading to the formation of the fragment at m/z 320. The ion at m/z 184 was interpreted as being formed by the loss of acetic acid with the concomitant cleavage of side chain and ring B at C_5-C_6 and C_9-C_{10} . Two additional fragment ions at m/z 449 and 434 were formed presumably by the loss of an acyl group followed by loss of methyl from the molecule. Fission of ring B at C_5-C_6 and C_9-C_{10} produces the sodiated ion at m/z 145. Same cleavage gave ions at m/z 107 and m/z 105 (base peak) with the loss of methyl and water respectively. The protonated side chain ion is evident at m/z 128. The ion at m/z 241 is derived from the loss of side chain, ketene from ring A and acetic acid from the molecule. Ring D cleavage at $C_{13}-C_{17}$ and $C_{14}-C_{15}$ bonds result in the sodiated ion at m/z 325. Cleavage of ring C at $C_{12}-C_{13}$ and C_8-C_{14} as well as fission of ring B at C_7-C_8 and C_9-C_{10} leads to the ion at m/z 209. Cleavage of ring C at C_8-C_{14} and C_9-C_{11} with initial loss of acetic acid produced ion at m/z 175. Ring B fission at C_9-C_{10} and C_6-C_7 bonds yielded ion at m/z 195 which in turn loses either acyl or methyl groups to generate ions at m/z 135 and m/z 181 respectively.

The MS-MS analysis of the $[M+H]^+$ ion at m/z 431 is shown in (Fig.2.12,C). The protonated molecular species at m/z 431, itself representing the base peak, on successive loss of water molecule and methyl group generated ions at m/z 413 and 399 respectively. The loss of an isopropyl group from the side chain with simultaneous elimination of water molecule produces ion at m/z 369. The ion at m/z 395, representing removal of two water molecules from the pseudomolecular ion, yields ion at m/z 353 when associated with the cleavage at $C_{24}-C_{25}$.

$C_{23}-C_{24}$ cleavage followed by elimination of water produced ions at m/z 361 and 343 respectively. Splitting of side chain with initial loss of two water molecules gave rise to ion at m/z 269, whereas splitting with elimination of a methyl group and a water molecule led to the formation of ion at m/z 271. Another ion at m/z 243 resulted from the ring D cleavage between $C_{13}-C_{17}$ and $C_{14}-C_{15}$ with the initial loss of water. Ring C cleavage at $C_{12}-C_{13}$ and C_8-C_{14} produced ion at m/z 209. The cleavage at C_9-C_{11} and C_8-C_{14} with initial loss of one and two water molecules yielded sodiated ion at m/z 199 and protonated ion at 159. Ring B cleavage at C_5-C_6 and C_9-C_{10} generates the protonated ion at m/z 125 which losses

ethylene from ring A yielding fragment at m/z 97. From the foregoing discussion the molecular species with the $[M+H]$ ion at m/z 431 was identified as 4,24-dimethyl-cholestan-3-one-6-ol (8).

Molecular species with sodiated molecular ion at m/z 465 was identified as 24-ethyl-4-methyl-6-hydroxy-cholest-4-en-3-one (7) on the basis of fragmentation observed. The most intense signal i.e. sodiated ion at m/z 334 results from the cleavage of the side chain at $C_{20}-C_{22}$ with the initial loss of one molecule of water which decomposes further with the loss of a methyl group to give product ion at m/z 319. Dehydration of the sodiated molecule with the loss of a water molecule results in the formation of ions at m/z 447 and loss of two water molecules from the parent ion produced ion at m/z 406. Loss of ketene and a methyl group along with two hydrogens gives ion at m/z 421, which further losses either one methyl or an isopropyl to give ions at m/z 406 and m/z 378 respectively. The latter decomposes further with elimination of either one water molecule to yield m/z 361 or ethyl group from the side chain to produce ion at m/z 351. A characteristic feature that has been observed in the fragmentation of steroids of present investigation is that oxygen when present in the molecule either as a ketonic group or a hydroxyl group it is eliminated as water molecule.

In our previous paper⁶⁷ we have reported the presence of cholesterol (12.02%) and its alkylated derivatives 24-methylene cholesterol (11.5%), 23-methyl cholesterol (9%), 23-methyl cholestanol (6.7%), 24- β -ethylcholest-5,22-diene-3- β -ol (4%) and 24- β -ethylcholesterol (18.02%) in the alga *Chondria armata*. In the present investigation, four additional new oxysterols cholest-4,24-dimethyl-6 β -hydroxy-4-en-3-one (3), 25-hydroperoxy-24-methylcholest-4-en-3,6-dione (5), 24-ethyl-4-methyl-6-hydroxy-cholest-4-en-3-one (7) and 4,24-dimethyl-cholestane-3-one-6-ol (8) together with four known ketosteroids cholest-4-en-3,6-dione (1), 6- β -hydroxy cholest-4-en-3-one (2) and cholest-4-en-3-one (4), 5 α -cholest-3,6-dione (6), are being reported from the same source. It is clear from the observations that this red alga is rich in 24 alkylated steroids with the dominance of 24 β -ethyl cholesterol. Usually cholesterol, and in some cases, its biogenetic precursor cholesta-5,24(25)-diene-3 β (desmosterol) dominate in the

lower red algae^{68,57,69}. Some of the evolutionary higher red algae contain 24-methylene cholesterol, a precursor of the sterols alkylated at C₂₄, and the sterol with a C₂₂ double bond, in low concentrations⁷⁰. But, in the present investigation 24-methylene cholesterol is one of the major sterols in this alga and this explains the presence of sterols with alkylated side chain in *Chondria*.

4-Methyl sterols occur widely in sediments being main contribution from dinoflagellates. Virtually all marine and fresh water dinoflagellate species biosynthesise 4-methyl sterols with different unsaturated patterns for the nucleus [(Δ^5 , Δ^7 , $\Delta^{8(14)}$, Δ^{14} , $\Delta^{17(20)}$], for the side chain [(Δ^{22} , $\Delta^{24(28)}$] and different alkyl substitution patterns for the side chain [(no substituent, 23-methyl, 24-methyl, 23,24-dimethyl, 24-ethyl, $\Delta^{22(23)}$ -23,24-dimethyl]. There is a solitary reference in the literature on the presence of 4-methyl sterol in the freshwater plant *Utricularia neglecta* L⁷¹ (*Lactibulariaceae*) that is reported to contain high proportions of 4-methyl sterols with the predominance of citrostanol (4- α -methyl-24-ethyl-5 α -cholestan-3 β -ol). 4-methyl sterols are being reported here for the first time from a marine alga.

Steroid al ketones with 4-en-3-one or 4-en-3,6-dione were isolated mainly from marine sponges⁷²⁻⁷⁹, the hard coral *Dendrophyllia cornigera*⁸⁰, the callus tissue of plants^{81,82}, *Phoenix dactylifera*⁸³, *Typha latifolia*⁸⁴, and queen bee ovary⁸⁵. Oxygenated fucosterols are also known from the brown alga, *Turbinaria conoides*⁸⁶ and the sea grass *Cymodocea nodosa*⁸⁰. 5 α -Cholestane-3,6-dione-11-hydroxy-5 α -cholestane-3,6-dione are also known as constituents of the red alga *Acantophora spicifera*^{87,88}. However, they are being reported for the first time from algae of genus *Chondria*.

There are recent reports of the identification of 6 α isomer of compound (2) from the red alga *Hypnea musciformis*⁸⁹ and of the presence of 6 β isomer in a marine sponge *Iotrochoto birotulata*⁷⁹. Ours is the first report of natural occurrence of the β isomer of compound (2) in an alga. These metabolites are known as microbial transformation products of cholesterol by the microorganisms *Coriolus hirstus*⁶³, *Pseudomonas* strain ST-200⁹⁰ and enzymatic oxidation product of Δ^4 -cholest-3 β -ol by soyabean lipxygenase⁹¹.

The origin of oxidized sterols has been questioned for a long time ⁹². As the work was carried out with fresh organisms rather than air dried material in order to avoid possible autoxidation and autoxidation of cholesterol is known to give 7 α and 7 β hydroxy cholesterol, it is possible that this oxysterol could be biosynthesized from cholesterol via photosensitized oxygenation or a related mechanism ⁹⁰. Alternately, bromoperoxidases which are widespread in the marine algae and are found particularly in red algae ⁹³ could be responsible for the oxidation of sterols of the present investigation. Sheu and coworkers (1999) have studied the cytotoxic activity of the oxygenated fucosterols from the brown alga *Turbinaria conoides*, the oxygenated desmosterols from the red alga *Glaxaura marginata* ^{94,95} and the oxygenated clerosterols from green alga *Codium arabicum* ⁹⁶ using P388, KB, A-549 and HT-29 cell lines. Based on ED₅₀ values against these four cell lines they concluded that the compounds with 4-en-3,6-dione moiety seem to be more potent growth inhibitors than the compounds with 4-en-3-one moiety.

As mentioned, the crude methanolic extract with a LD₅₀ value of 17.8 mg/kg exhibited promising antiviral activity. It is now well known that sterols with ketonic function in 3,6 position are cytotoxic. In analogy with the literature reports cytotoxic activity is expected for these compounds and it would also partly explain the antiviral activity observed in the crude extract of the alga.

The absence of the facilities for the cytotoxic activity, precluded any possibility of obtaining bioactivity data to allow a full assessment of its toxicological significance to be made but, the antimicrobial activity of the major steroids 1-2 has been evaluated (Table-2). Compound (1) showed mild antibacterial activity as compared to the standard *Streptomycin* against all the bacteria tested including multidrug resistant strains. Considerable activity was expressed by the metabolite against the yeast, *Candida albicans* and fungi *Aspergillus fumigatus* and *Rhodotorula* as compared to the standard, Nystatin, which was ineffective against both. It was a better candidate against the fungus, *Cryptococcus neoformans* and *Aspergillus niger* as compared to the control. Weak activity against the fungi *Fusarium* and *Nocardia* was also observed. Compound (2) was practically

inactive against all the microbes tested. Antimicrobial activity of these steroids is being reported here for the first time and since it is not promising it was not pursued further.

Table-2: Antimicrobial activity exhibited by compounds 1 and 2.

Bacteria	Antibacterial activity		Fungi	Antifungal activity		MDR Bacteria	MDR Activity	
	1	2		1	2		1	2
<i>E. coli</i>	+	+	<i>A. fumigatus</i>	4	-	<i>S.pyogenes</i>	-	+
<i>P. aeruginosa</i>	+	-	<i>Fusarium</i>	1	-	Acinetobacter	1(st)	-
<i>S. aureus</i>	1-2(st)	-	<i>C. neoformans</i>	4(st)	-	<i>S. typhi</i>	1(st)	-
<i>S. typhi</i>	1	-	<i>A. niger</i>	3-4	-			
<i>S. flexineri</i>	1	-	<i>Rhodotorula</i>	1-2	1-2(st)			
<i>Klebsiella sp.</i>	2-3(st)	-	<i>Nocardia sp.</i>	1	+			
<i>V. cholerae</i>	1	-	<i>C. albicans</i>	1.5	-			

Numbers indicate the zone of inhibition in mm from center of imbued disk

(-) indicates No activity,(+) indicates Weak activity.

+(st) indicates that it shows activity but zone of inhibition is not very clear.

Experimental:

General:ESI/MS and NMR spectrometry:

NMR was recorded on Bruker Avance 300MHz spectrometer in CDCl₃ with TMS as internal standard. Electrospray ionization experiments were performed on a QSTARXL MS/MS Applied Biosystems/MDS Sciex Instruments (Canada) equipped with Analyst Software. Recording of ESI/MS and MS/MS spectra was done as given in section 1 under glycolipids of this chapter.

Isolation of sterols from *Chondria armata* (Kütz):

After extraction and fractionation of the crude methanolic extract as given in section 1 under glycolipids of this chapter, the chloroform fraction (123g) was filtered, initially on a column of Sephadex LH20 with methanol (500ml) as eluant collected in fractions of 20ml each. The fractions obtained were examined by TLC (solvent - light petrol:ethyl acetate, 7:3, v/v, spray: 5% methanolic sulfuric acid) and combined according to their profile. Fractions yielding bluish spots were then purified by repeated silica gel chromatography using petroleum ether (60-80°C):ethyl acetate (7:3) to give the above mentioned sterols. The only difference being that the steroids were eluted from silica gel column using ethyl acetate:petroleum ether (30:70) and same solvent system was used for TLC.

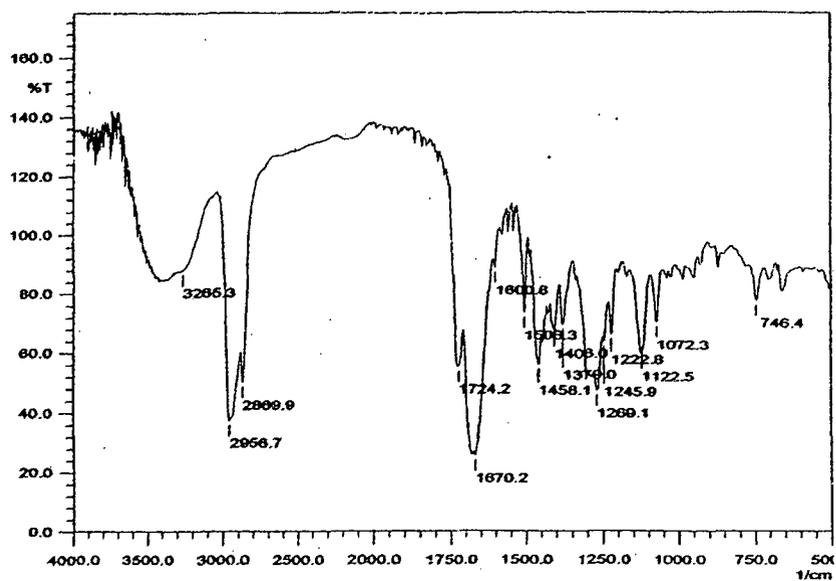


Fig 2.1: IR spectrum of compound 1

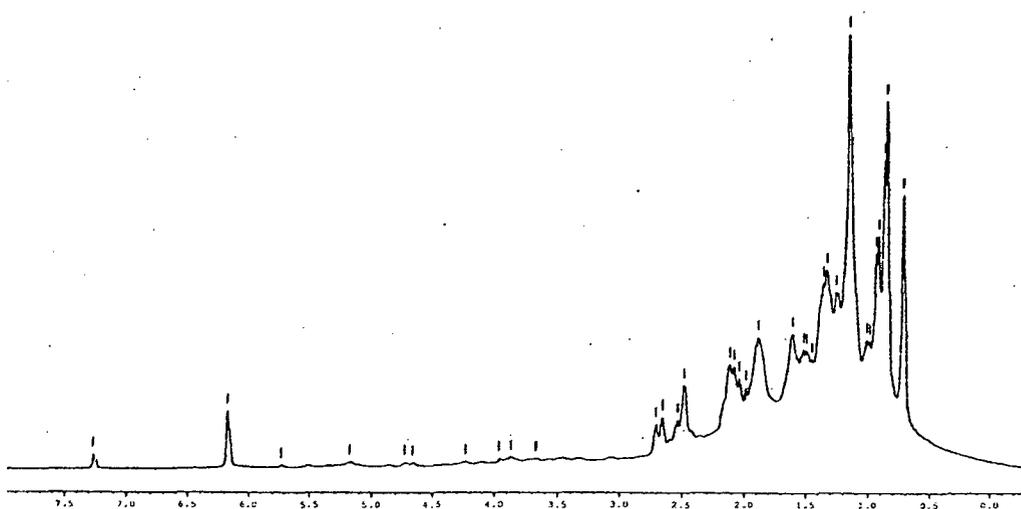


Fig 2.2: ¹H NMR spectrum of compound 1

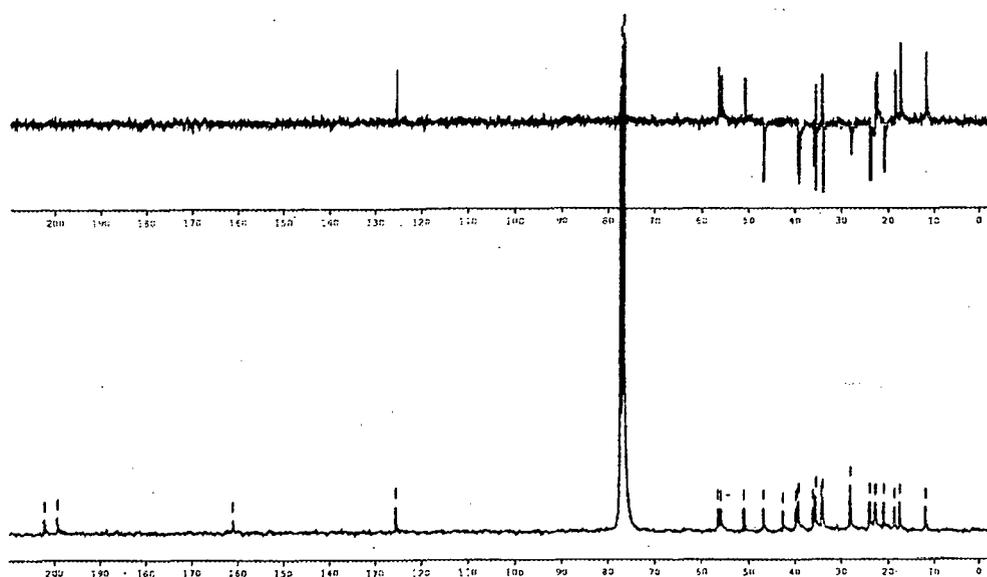


Fig 2.3: ¹³C NMR and DEPT spectra of compound 1

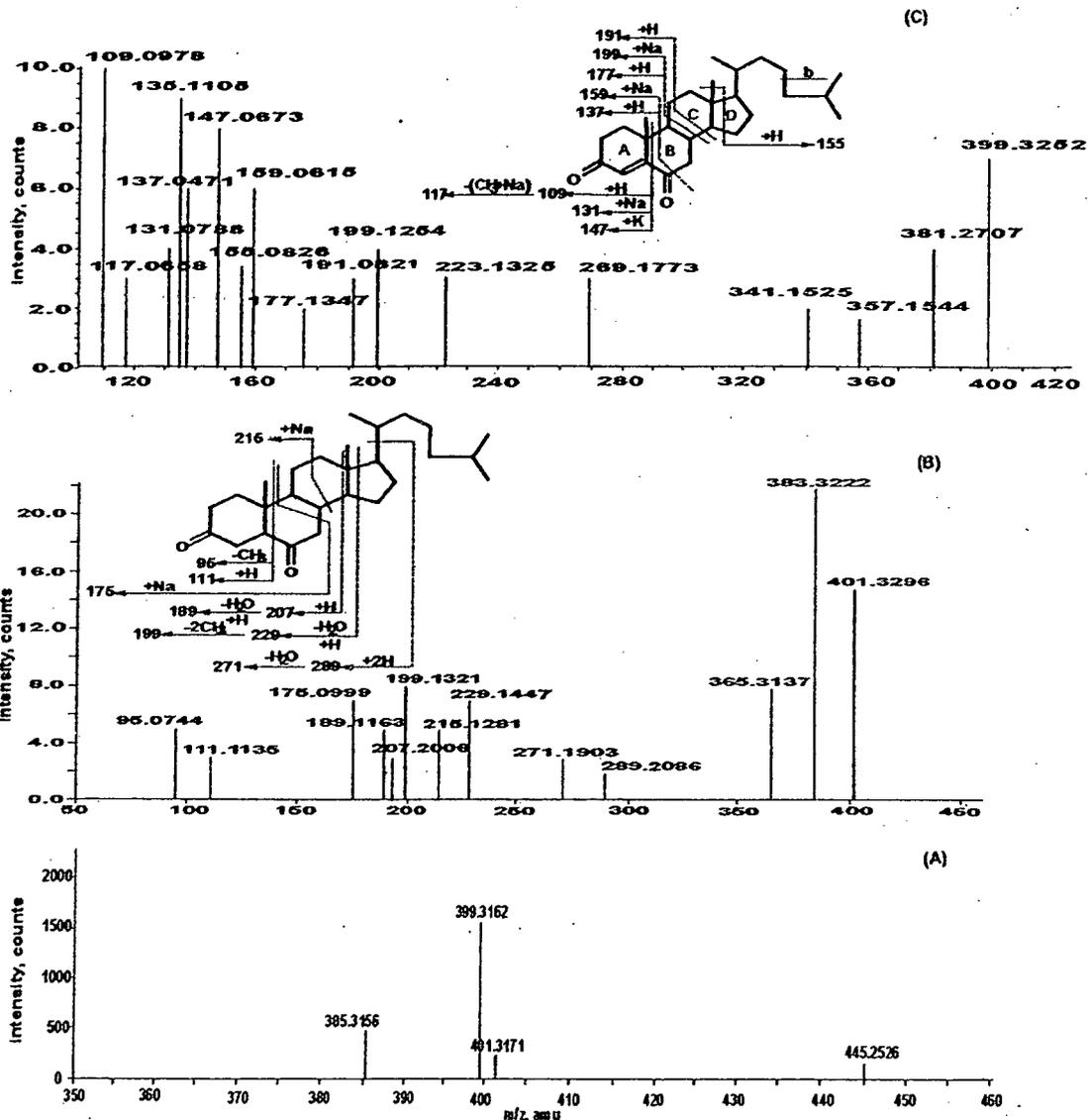


Fig. 2.4: (A) - Positive ESI-MS of the compounds from *Chondria armata*; (B & C) MS/MS of the ions at m/z 401.3[M+H]⁺ and 399.3[M+H]⁺ of the sterols along with their proposed structures

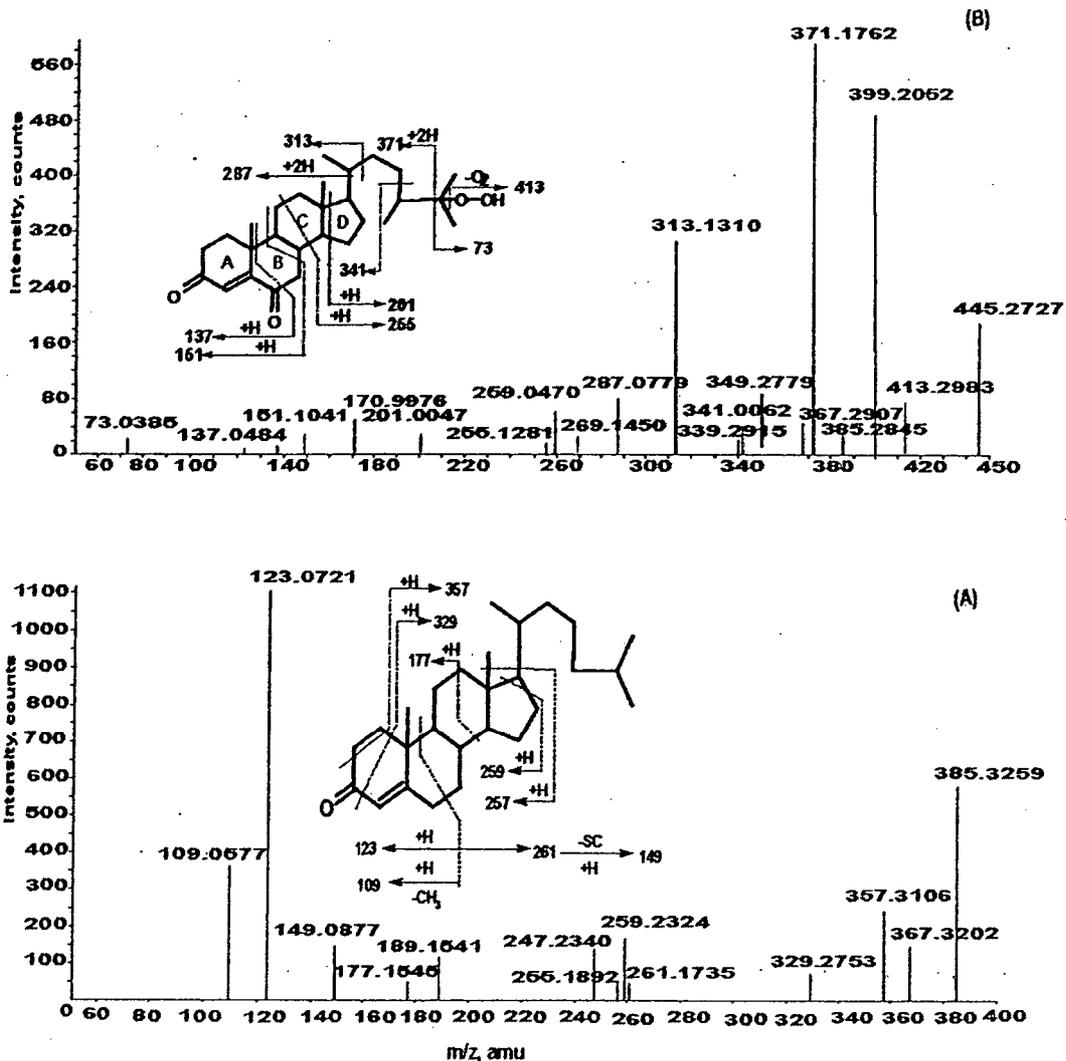


Fig. 2.5: (A & B) MS/MS of the ions at m/z 385 and m/z 445 of the sterols along with their proposed structures.

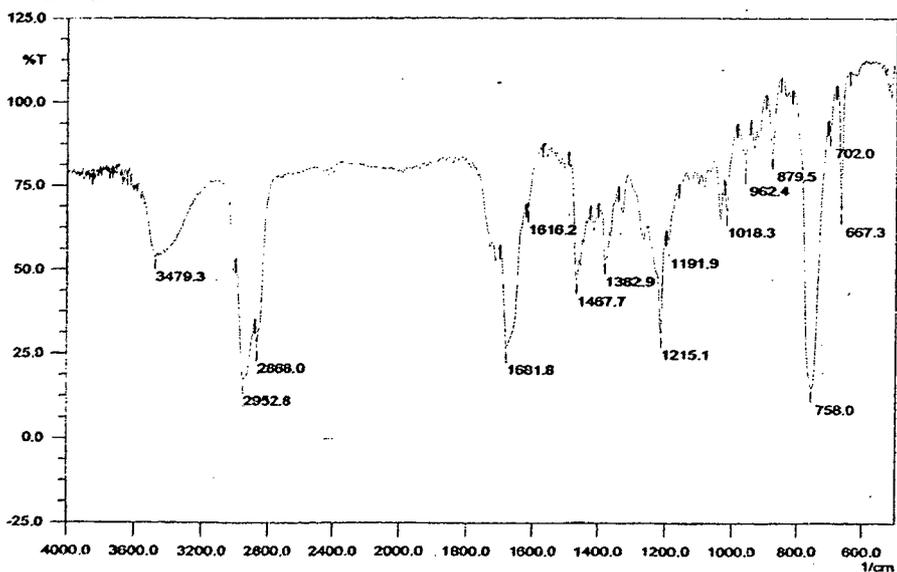


Fig 2.6: IR spectrum of compound 2 .

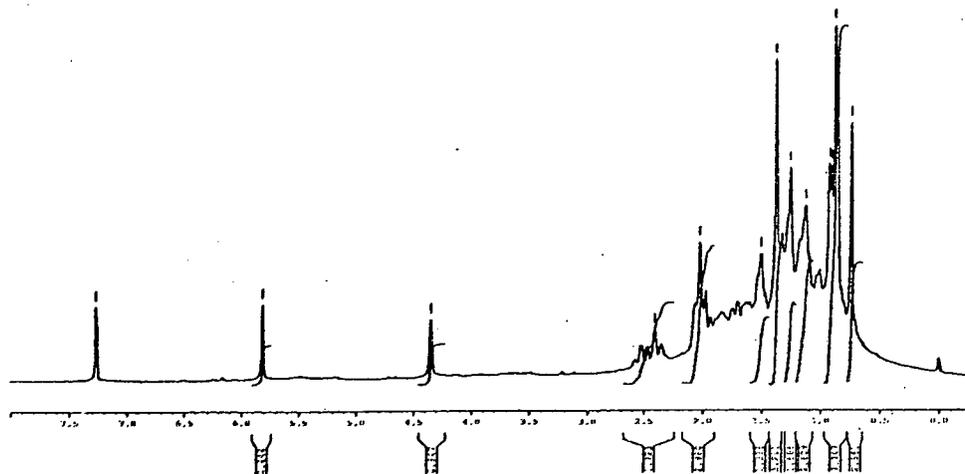


Fig 2.7: ^1H NMR spectrum of compound 2

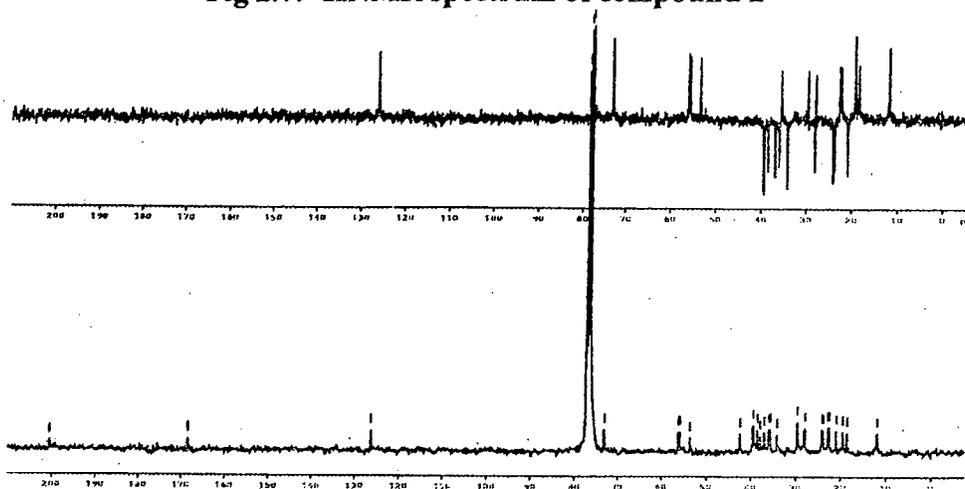


Fig 2.8: ^{13}C NMR and DEPT spectra of compound 2

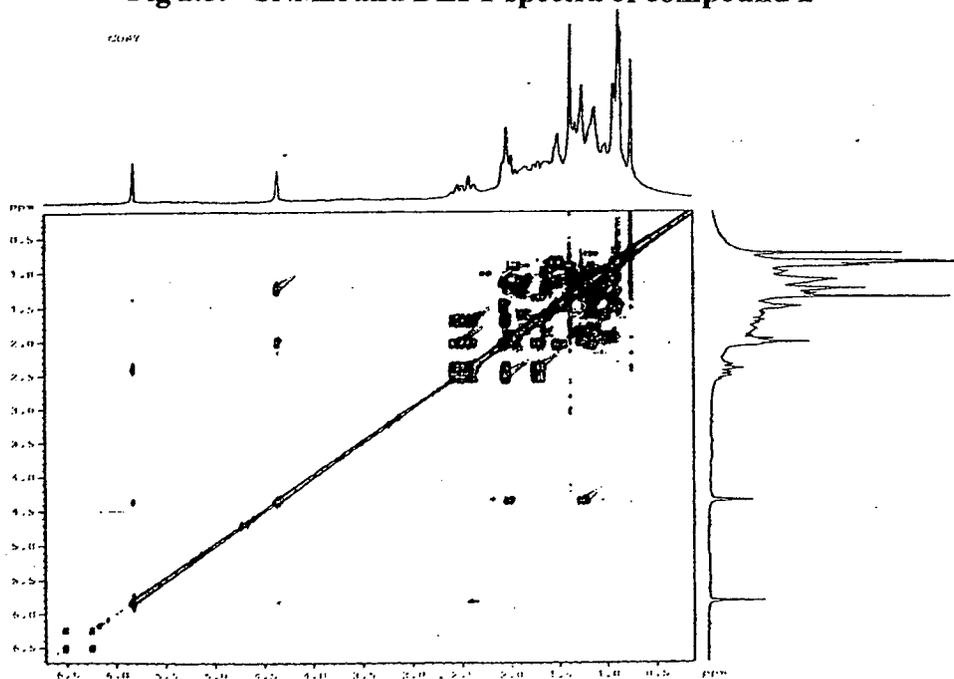


Fig 2.9: COSY spectrum of compound 2.

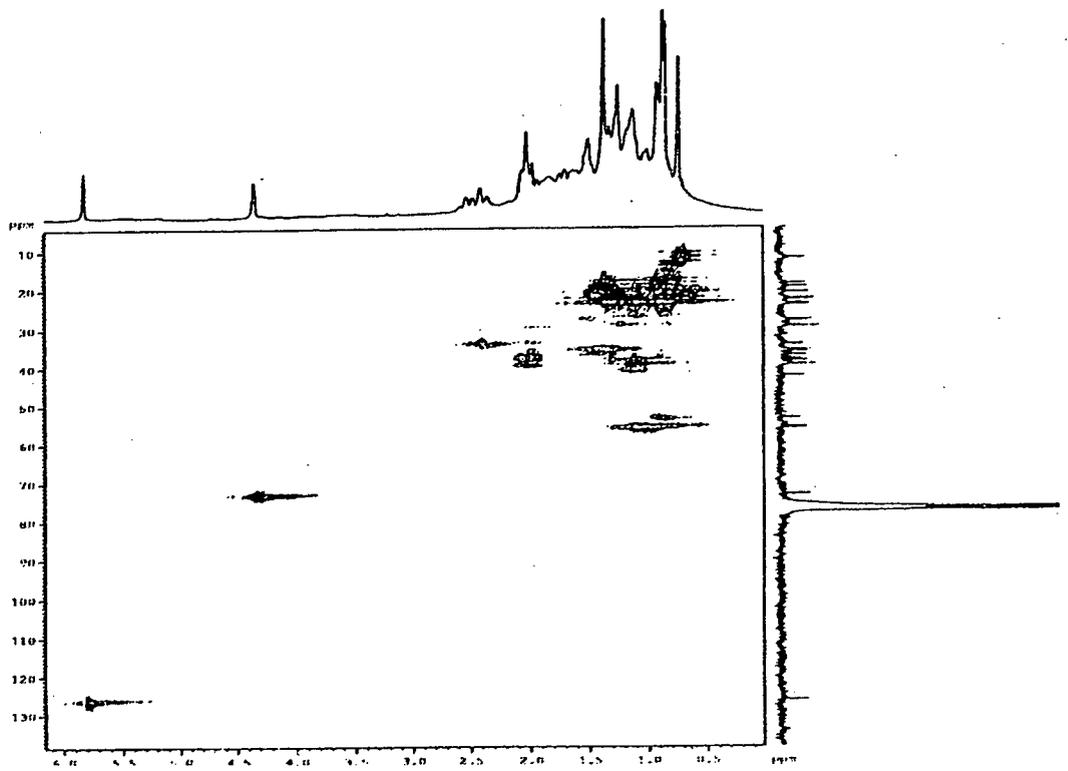


Fig 2.10: HMQC spectrum of compound 2.

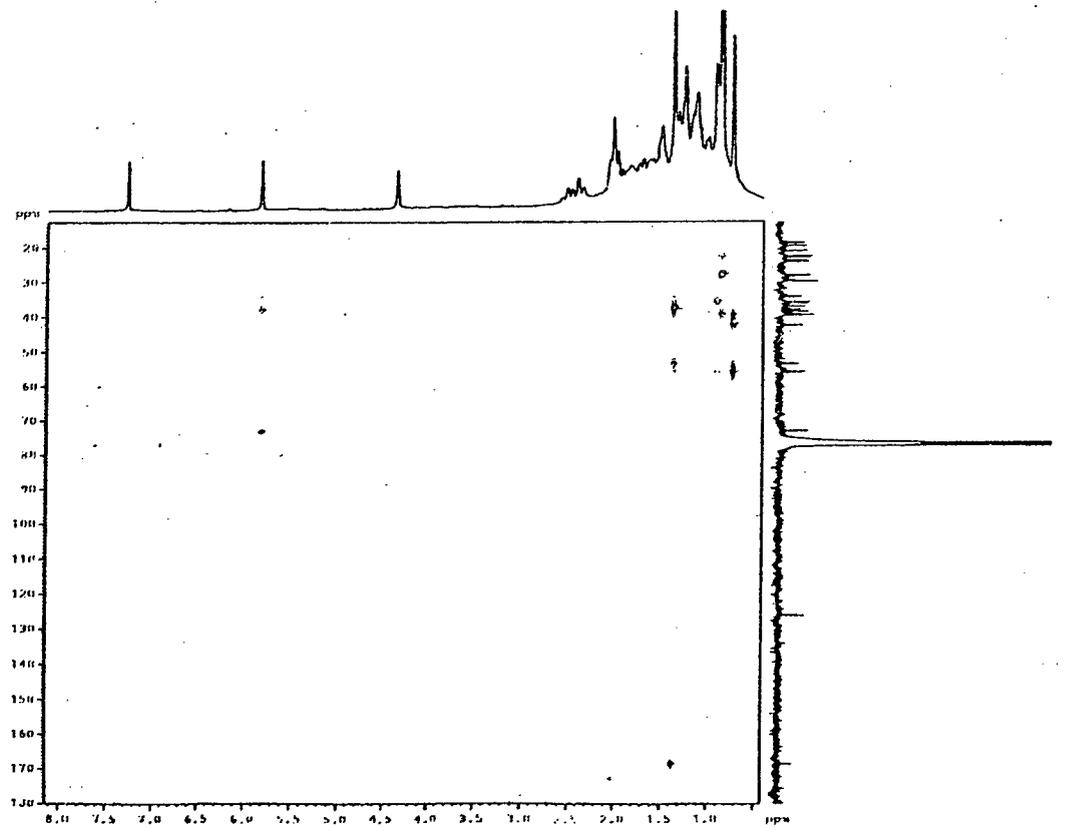


Fig 2.11: HMBC spectrum of compound 2.

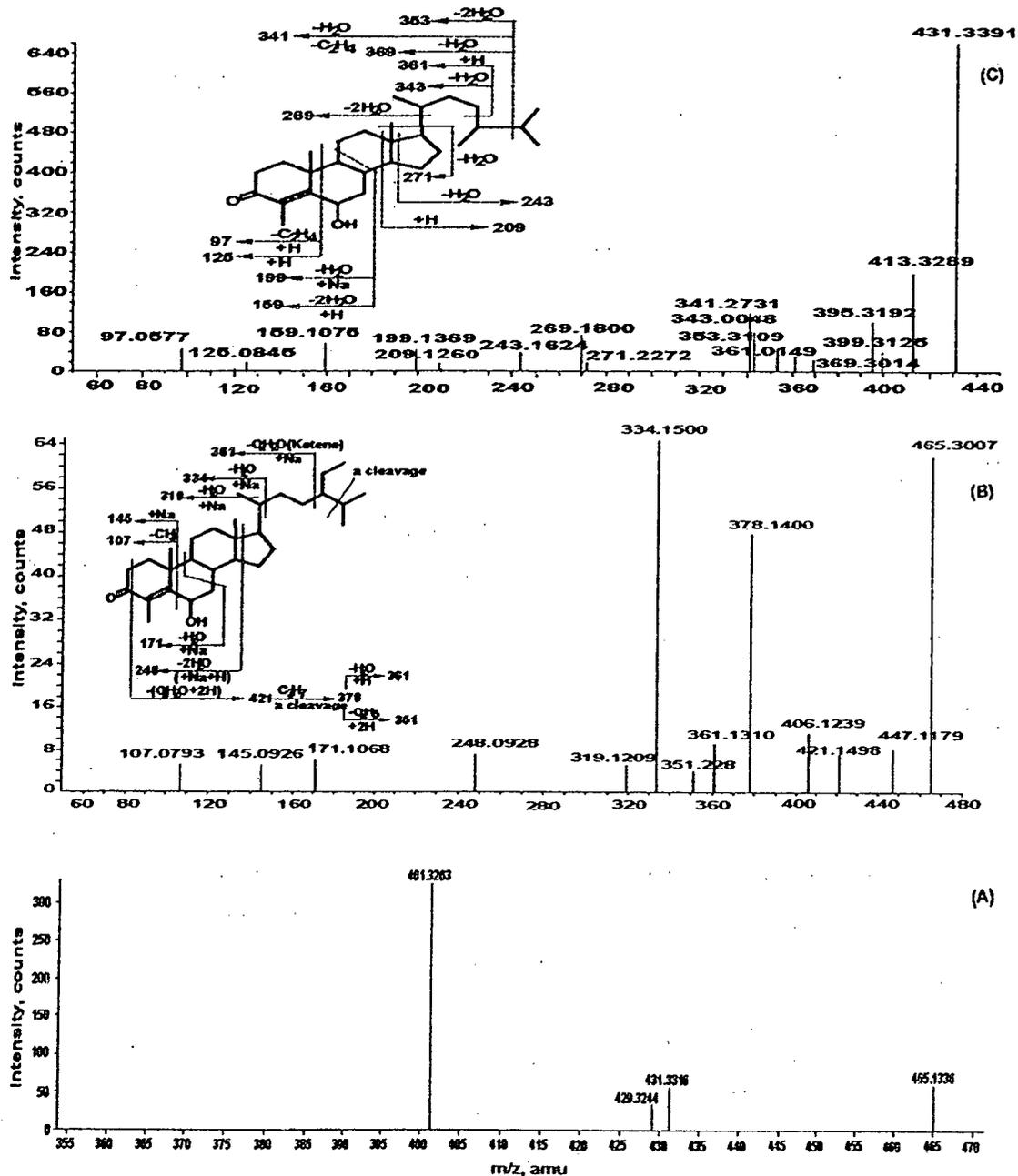


Fig.2.12: (A) - Positive ESI-MS of the compounds from *Chondria armata*; (B & C) MS/MS of the ions at m/z 465 [M+Na]⁺ and 431 [M+H]⁺ of the sterols along with their proposed structures.

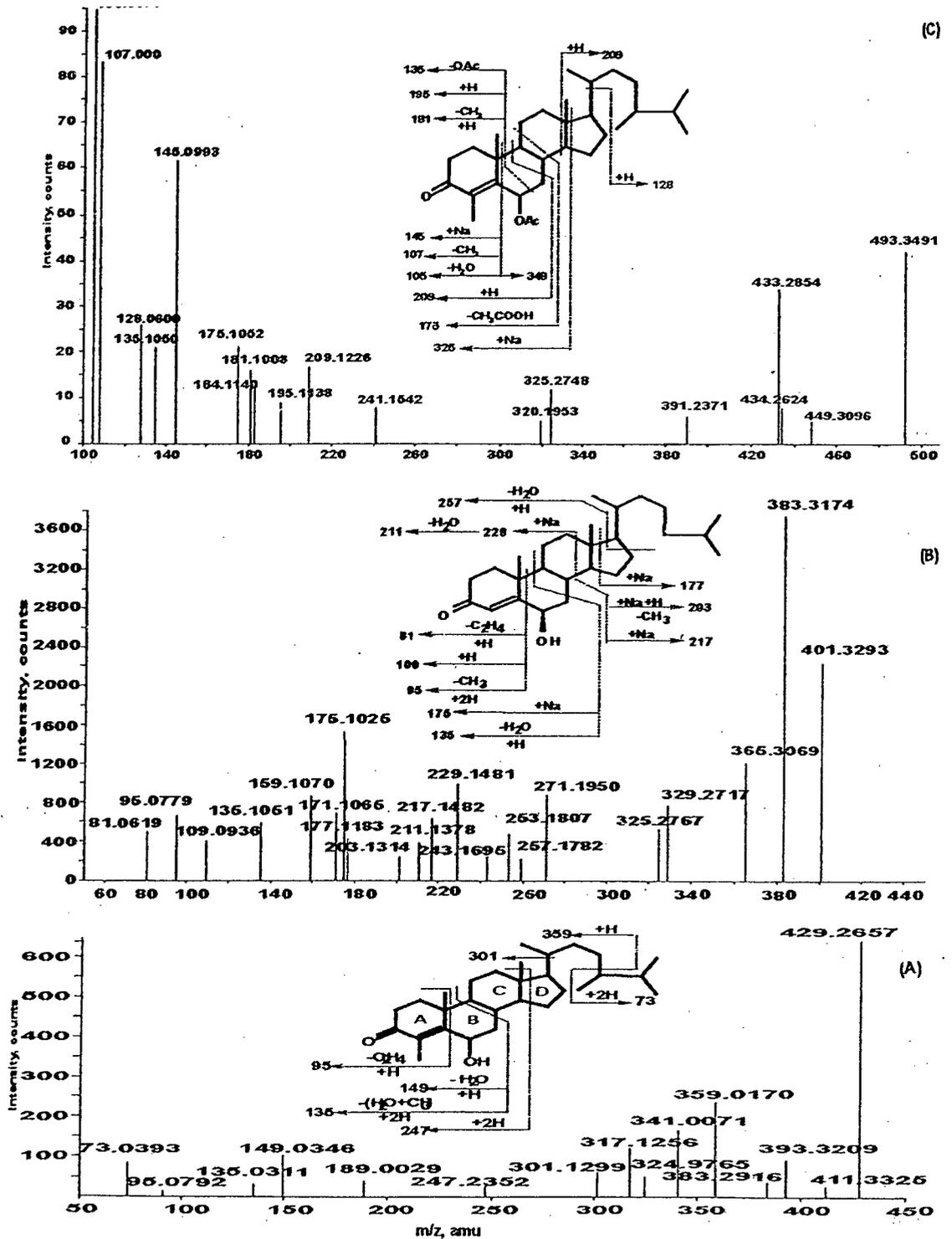


Fig.2.13: (A, B & C) MS/MS of the ions at m/z 429 $[M+H]^+$, 401 and 493 $[M+Na]^+$ of the sterols along with their proposed structures.

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Section II

*Triterpenoids- Polyethers of the red alga
Chondria armata.*

2.1. Polyether squalene derivatives-a Review:

The marine ecosystem has revealed a multitude of bioactive natural products. These marine-derived compounds are found primarily among soft corals, sponges, algae, and bacteria. Their therapeutic properties include significant activities in antitumor, anti-inflammatory, analgesia, allergy, and anti-viral assays. Although there is no single marine-derived natural product that has become a pharmaceutical drug as of 2004, a large number of compounds of marine origin are currently undergoing clinical trials^{1,2}.

Hence, marine natural products represent a valuable source for the discovery of novel biologically active compounds. The potential therapeutic applications provided by these molecules along with their unique structural features have encouraged substantial scientific interest and investigations.

The living environment of marine organisms differs much from that of terrestrial organisms. Owing to this difference as well as many other factors, the chemical constituents in the marine organisms differ considerably from those of the terrestrial organisms. Thus, the marine organisms (among others algae) contain abundantly halogenated organic compounds in particular brominated compounds even though the concentration of bromine in seawater is substantially less than that of the chloride, it is nevertheless about 1mM and its incorporation into organic compounds is catalyzed selectively by halogenating enzyme bromoperoxidase. This enzyme catalysis the oxidation of bromide by H₂O₂ accompanied by either further oxidation to produce singlet oxygen or bromination of an available organic substrate³. Bromoperoxidases are widespread in the marine algae and are found particularly in red algae and to a lesser extent in brown algae⁴.

Many marine organisms contain polyoxy or polyether constituents their characteristic feature being their chemical reactivity and bioactivity in particular, toxicity. In this section of Chapter I, squalene derived triterpene polyethers, isolated and identified from the red alga, *Chondria armata*, have been discussed but, before going into the details of this investigation a review of literature,

particularly on the triterpene polyethers with a squalene carbon skeleton, and derived mainly from the red alga belonging only to genus *Laurencia* is presented. The algae of genus *Laurencia*, taxonomically belongs to the same class (*Rhodophyta*), order (*Ceramiales*) and family (*Rhodomelaceae*) as the alga, *Chondria armata*, of the present investigation.

Marine polyethers are characterized, from the structural point of view, by the presence of rings of diverse size which form spiro or trans-fused systems. This type of active metabolites includes the marine polyether triterpenes isolated from red algae of the genus *Laurencia*, the green microalga, *Botryococcus braunii*⁵, in sponge of *Axinellidae* family and mollusk *Dolabella auricularia*⁶. The studies of these metabolites began with the isolation of thyriferol (**1**) from *Laurencia thyrifera* twenty five years ago⁷, and since that initial discovery, further examples have been isolated from the red algae of the genus *Laurencia*. The unique structural characteristic of thyriferol is that it consists of a central *trans*-fused pyranopyran unit, an appended cyclic bromo ether and an aliphatic side chain connecting the central unit to a *trans*- tetrahydrofuran ring.

The genus *Laurencia* has proved to be a rich source of natural products yielding interesting bioactive metabolites such as sesquiterpenes, diterpenes and polyether squalene derivatives². The third group, although they are not the major constituents of this genus, had generated a high degree of interest with reports of thyriferol (**1**) and its congeners showing potent cytotoxic, antiviral and inhibitory effects on protein phosphatase type 2A.^{6,8-10}

Isolation and spectroscopic characterization of thyriferol (**1**) was first carried out by Munro *et al* in 1978⁷. An x-ray crystallographic analysis of its C₁₈ -acetate derivative established its chemical structure and assigned each of the relative stereocentres. The absolute stereochemistry was not determined at the time of the isolation, but it was elucidated a few years later when venustatriol (**2**) was characterized¹¹. The X-ray crystallographic analysis of thyriferol 18-acetate (**5**) revealed a strained tetrahydropyran ring C in a twist-boat conformation so as to

avoid 1,3-diaxial interactions between the methyl groups at C₁₀ and C₁₅. Initial biological studies of the natural product by Munro *et al* did not reveal any significant pharmacological activity⁷.

Dehydrothysiferol (3), an analogue of thysiferol (1), was the next marine metabolite discovered from *Laurencia pinnatifida* from the Canary Island of Spain. Its chemical structure was verified via chemical transformation into thysiferol (1). A year later, the crude extracts of *Laurencia obtusa*, obtained off the coast of Japan, were shown to exhibit a strong, *in vitro*, cytotoxic activity against P-388 cells (ED₅₀=0.3µg/ml) which was associated with the presence of thysiferol 23-acetate (4)¹². Treatment of (4) with K₂CO₃ in MeOH yielded a product whose spectroscopic data were identical to thysiferol (1).

Venustatriol (2), a diastereomeric isomer of (1) was found as a metabolite of the red algae *Laurencia venusta* by Higa's group in 1986¹¹. This compound was shown to possess many of the structural features of thysiferol (1), with the exception of the stereocenters at C₁₈ and C₁₉. This tetracyclic polyether, venustatriol (2) was reported to display significant anti-viral activity against vesicular stomatitis virus (VSV) and herpes simplex virus type 1 (HSV-1). The absolute configuration of venustatriol (2) was verified by x-ray crystallography, an experiment that facilitated the assignment of the absolute stereochemistry of thysiferol (1) and its congeners.

Similar bromine-containing polyethers continued to be isolated in the ensuing years. From *L. obtusa* for instance, compounds 15(28)-anhydrothysiferyl diacetate (7), 15(16)-anhydrothysiferyl diacetate (8), and magireols A (9), B (10), and C (11) were discovered.¹³

In 1996, Norte and co-workers reported two new antitumor polyoxygenated squalene derivatives related to (1) and (2) from the acetone extracts of *Laurencia viridis*¹⁴. Isodehydrothysiferol (12) possesses an appended tetrahydropyran unit

instead of the common tetrahydrofuran moiety observed in previous chemical structures.

10-*epi*-Dehydrothysiferol (13) differed from other congeners in that the two central pyran rings must be *cis*-rather than *trans*-fused¹⁴ as revealed by ROESY correlation among the hydrogens at C₇ and at C₁₁, and between C₁₄ and C₂₇.

During the course of research into the secondary metabolites of *Laurencia* sp. from the same *L. viridis* algae, Norte's research group reported isolation of five new polyether triterpenes structurally related to both thysiferol (1) and venustatriol (2)¹⁵. These new compounds include: dehydrovenustatriol (14), 15,16-dehydrovenustatriol (15), predehydrovenustatriol acetate (16), 16-hydroxydehydrothysiferol (17) and 10-*epi*-15,16-dehydrothysiferol(18). Predehydrovenustatriol acetate (16) presents an attractive structural feature in regards to its biogenesis. The absence of the A-ring and the presence of the trisubstituted double bond between C₂ and C₃ points to a step-wise biosynthetic pathway; *Laurencia viridis* has also been the source of thyrseol A (19) and thyrseol B (20). Both of these compounds display an unusual enol ether moiety in the C-ring¹⁶.

Callicladol (21) was identified by Suzuki et al. from a Vietnamese species, *Laurencia calliclada*,¹⁷. This compound constitutes the first example of a halogenated squalene derived polyether from the genus *Laurencia* that contains a hydroxyl group at C₅.

Laurencia viridis from Canary Islands in Macaronesia is the most prolific source of this type of metabolites. Continued research by Norte's group resulted in isolation and characterization of yet another series of cytotoxic squalene triterpenoids which include clavidol (22), martiriol (23), dioxepandehydrothysiferol (24), lactodehydrothysiferol (25), 16-*epi* hydroxydehydrothysiferol (26), dehydrothysiferol (27), pseudodehydrothysiferol (28)^{18,19}. The compounds though arising from a common precursor they show some divergence from the common backbone structures previously observed. For example, clavidol (22) lacks the C-ring, dioxepandehydrothysiferol (24) contains

a framework with two *trans*-fused 7-membered ether rings, and lactodehydrothysiferol (25) contains a lactone in place of the A-ring bromoether.

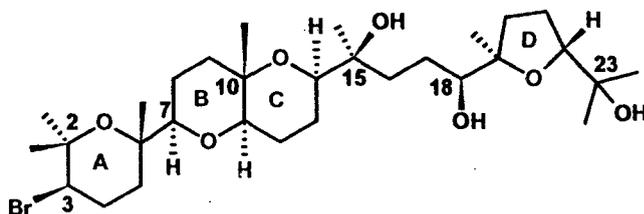
Enshuol(29) is the first example of a pentacyclic triterpene polyether from *L. omaezakiana* from Japan²⁰. Its structure is formed by a 2,8-dioxobicyclo [5.4.0] undecane ring (A-B ring) and three isolated oxolane rings (C-D-E rings). Aurilol (30), a novel cytotoxic bromotriterpene, was isolated from the Japanese sea hare *Dolabella auricularia*. The structure of 30, including the absolute stereochemistry of the five stereocenters, was determined by spectroscopic and chemical analyses. Aurilol (30) exhibited cytotoxicity against HeLa S₃ cells with an IC₅₀ of 4.3 μg /mL.²¹ The origin of these metabolites have been attributed to algal diet of mollusc. It is proposed that accumulation of these metabolites in the animal gut may have a defensive role against predation.

The highly symmetric squalene-derived triterpene polyether, Teurilene (31) was isolated from the red alga *Laurencia obtusa* along with thysiferol 23-acetate (4) by Kurosawa et al¹². Its stereostructure was elucidated by X-ray crystallography analysis. Teurilene (31) exhibit prominent cytotoxic activities on KB cells(IC₅₀ = 7.0 μg/ml).

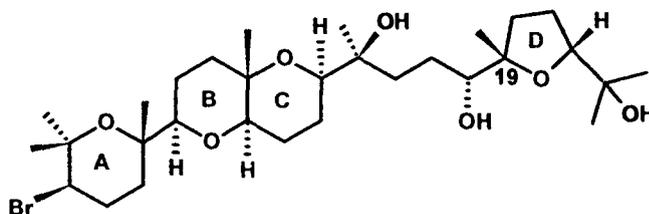
Intricatetraol (32) was isolated from the red alga *Laurencia intricata* from Japan in 1993, and a crude fraction including intricatetraol (32) as the major component exhibited cytotoxic activity against P388 with an IC₅₀ of 12.5 μg mL⁻¹. The structural analysis was mainly carried out by NMR methods. Although it has been found that the molecule has C₂ symmetry, *cis* configuration within the THF ring, and *R* configuration at the C₁₁ (C₁₄) position, the stereochemistries between C₆ and C₇ (C₁₈ and C₁₉) and C₁₀ and C₁₁ (C₁₄ and C₁₅) and at the bromine-attached C₃ (C₂₂) position remain to be determined²².

There have also been many other types of oxasqualenoids; however, it is often difficult to determine their stereostructures even by the current, highly advanced

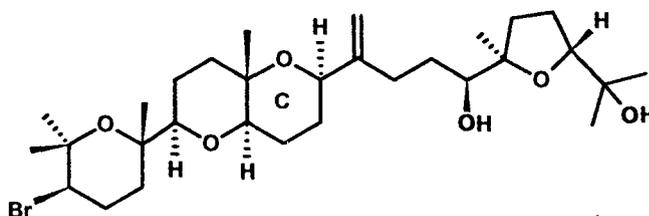
spectroscopic methods, especially in acyclic systems including stereogenic quaternary carbon centers such as C₆-C₇ (C₁₈-C₁₉) and C₁₀-C₁₁ (C₁₄-C₁₅) in (32). This section deals with the structural elucidation of the major halogenated as well as non halogenated squalene derived polyether, isolated from the red alga *Chondria armata*.



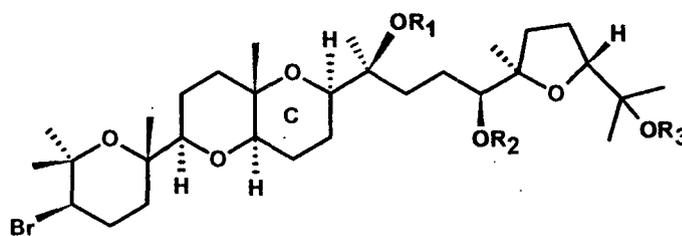
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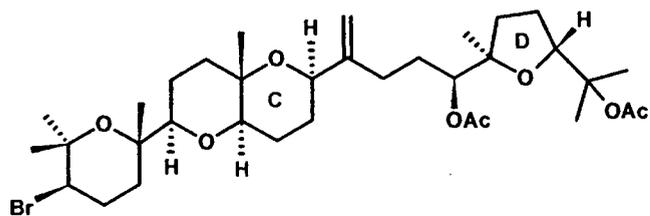
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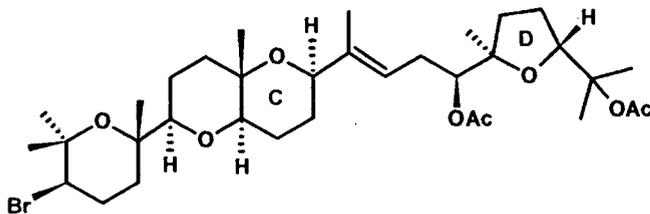
4 R₁ = R₂ = H, R₃ = Ac.

5 R₁ = R₃ = H, R₂ = Ac.

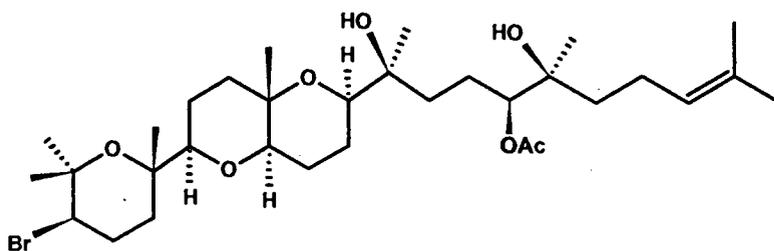
6 R₁ = H, R₂ = R₃ = Ac.



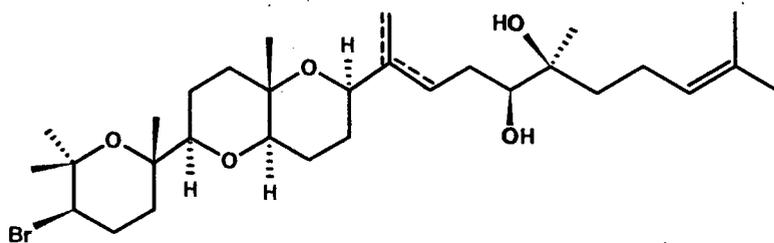
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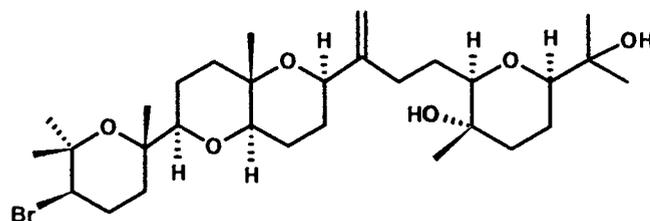
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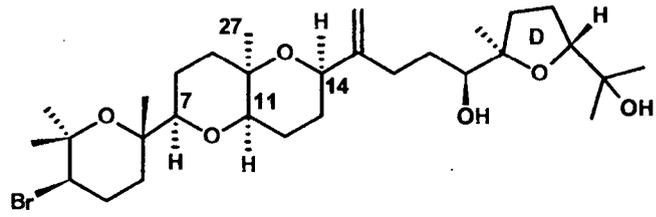
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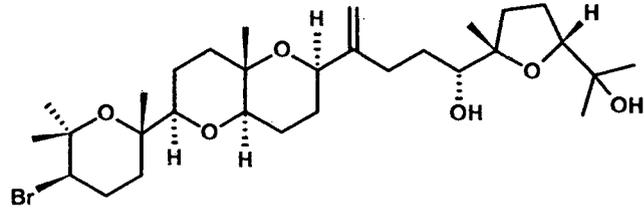
10, $\Delta^{15,28}$ magireol B. - 11, $\Delta^{15,16}$ magireol C.



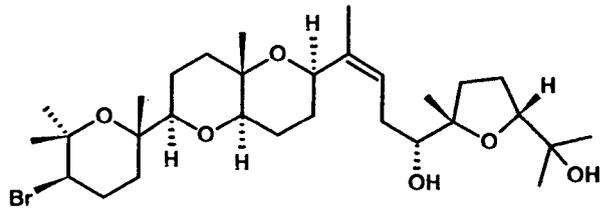
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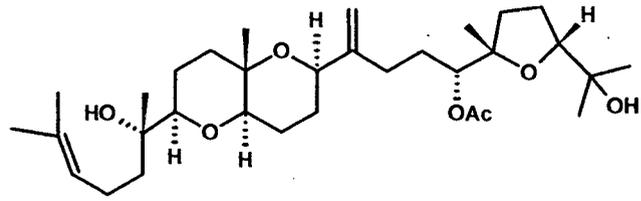
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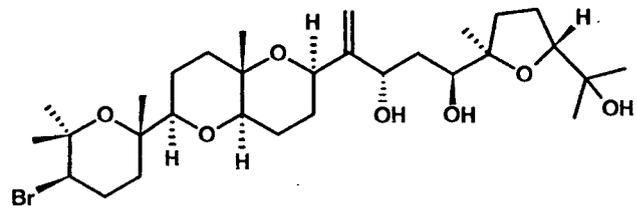
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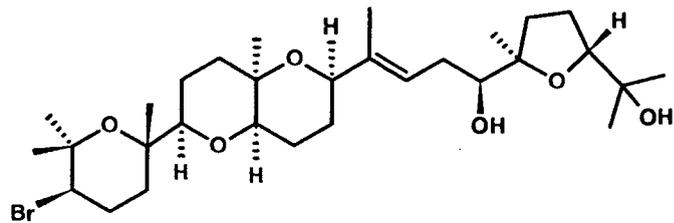
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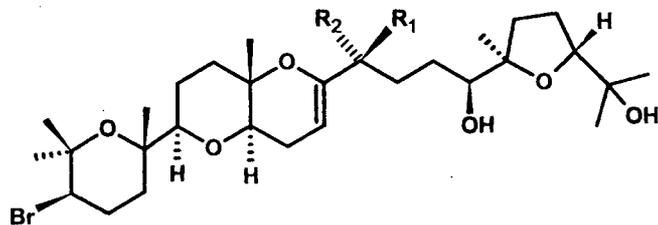
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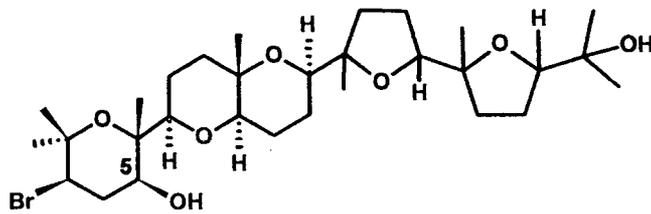


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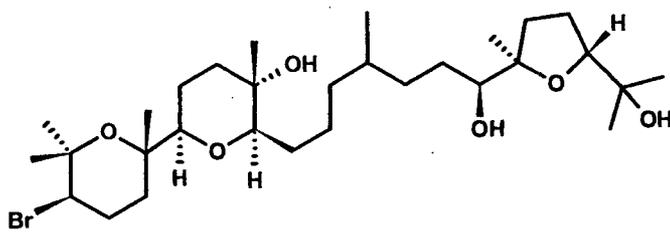


19 R₁ = OH, R₂ = CH₂OH

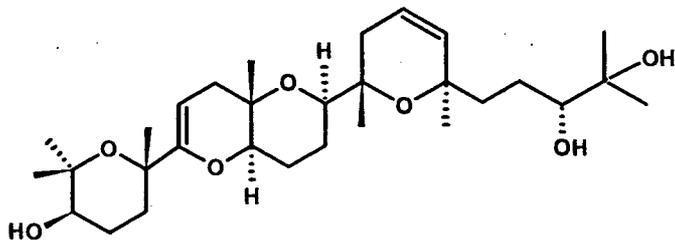
20 R₁ = CH₂OH, R₂ = OH



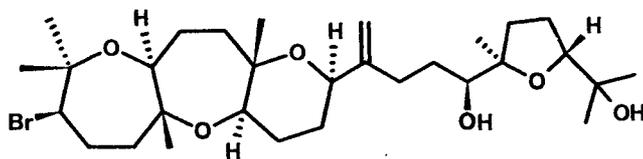
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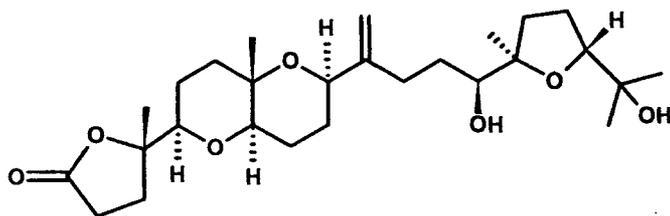
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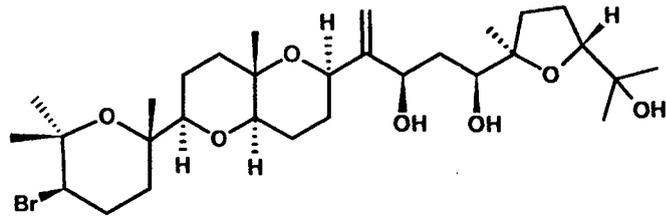
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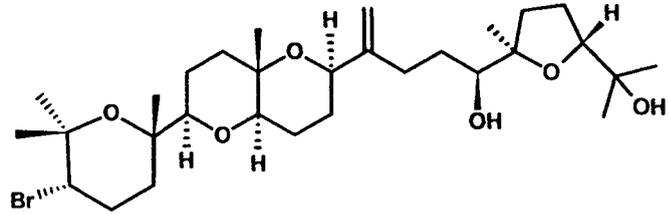
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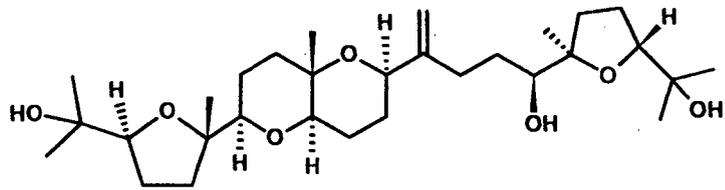
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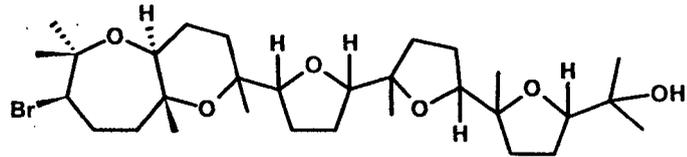
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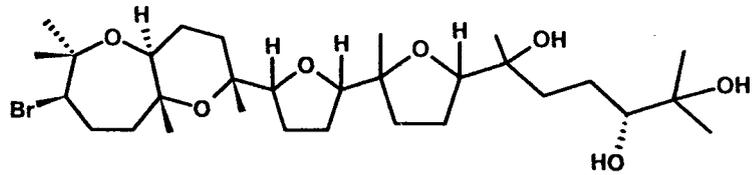
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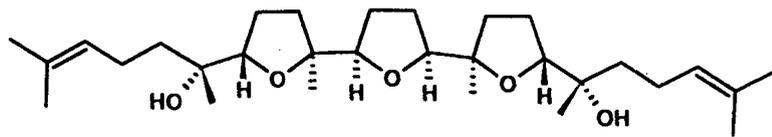
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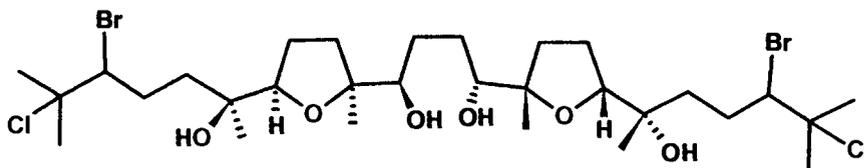
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2.2. Squalene derived triterpene polyethers from *Chondria armata*

Chondria armata (Kuetz) Okamura, as mentioned earlier, is a red seaweed taxonomically placed within the order *Ceramiales* and family *Rhodomelaceae*. It is inhabitant of shallow waters and intertidal community, particularly in the tropical and subtropical ecosystems. It has been known as antihelmintic since ancient times being effective against threadworms, and *Oxyris* as well as *Ascaris*. It is found in abundance, intermingled with other algae along the Anjuna Coast (Goa, West coast of India) during the pre monsoon periods.

In our earlier sections of this Chapter we have discussed about the glycolipids and steroids from the chloroform soluble fraction of this alga. We have already communicated several squalene derived triterpenoids polyethers designated as armatols A-F from this source²³. Herein, we wish to discuss structure elucidation of additional novel squalene derived triterpenoids, isolated from the chloroform soluble fraction, based on spectral data including 2D NMR techniques.

The chloroform soluble fraction, on chromatography over Sephadex LH20 with $\text{CHCl}_3:\text{MeOH}$ (1:1) yielded compounds 1 and 2. Each of these compounds were subjected to final purification on ODS column using 20:80($\text{H}_2\text{O}:\text{CH}_3\text{CN}$) solvent system for elution.

Structure elucidation of compound 1:

Compound 1, a viscous oil, $[\alpha]_D = +27.8$ ($c = 0.27, \text{CHCl}_3$), showed a sodiated molecular ion peak at m/z 689 ($\text{M}^+ + \text{Na}$) [calc. 689.2028 obsr. 689.2019]. Its molecular formula was deduced as $\text{C}_{30}\text{H}_{52}\text{Br}_2\text{O}_6$ from its ESI-MS (Fig 1.2.1a, Fig 1.2.1b) and ^{13}C NMR spectra (Fig 1.2.2). The presence of two bromine atoms was deduced from the characteristic 1:2:1 pattern of the molecular ion in the mass

spectrum (689: 691: 693) and by the presence of the fragment ion at m/z 609/611 with the intensities in the ratio 1:1 and m/z 529 corresponding to the loss of 2HBr consecutively from the molecule. This was further reinforced by the presence in its ^{13}C NMR spectrum of the doublets at δ 58.08 and δ 63.83 due to the two-bromomethine carbons (CH-Br). Elimination of two water molecules from the sodiated ion at m/z 529 led to the formation of protonated fragment at m/z 471, suggestive of the presence of two -OH groups in the molecule. This was further corroborated by the presence of absorbance at 3438 cm^{-1} in its IR spectrum (Fig 1.2.3). The spectrum also displayed strong bands at 1062 and 1137 cm^{-1} , with no carbonyl absorption, indicating the remaining four oxygen to be ether groups.

The ^1H NMR spectrum (Fig.1.2.4) featured signals for eight methyls at δ 1.09, 1.11, 1.22, 1.23, 1.27, 1.36, 1.4(6H), six methine protons of which two methines one at δ 4.32 (broad triplet, $J = 2.9\text{ Hz}$) and the other at δ 3.9 (d, $J = 10.9\text{ Hz}$) were attributed to two CH-Br and the remaining four were oxygenated methine protons at δ 3.27 (dd, $J = 10.9\text{ Hz}$, 2.97 Hz), δ 3.3 (dd, $J = 11.7\text{ Hz}$, 3.0 Hz), δ 3.75 (dd, $J = 11.0\text{ Hz}$, 3.3 Hz) and δ 3.44 (dd, $J = 10.08\text{ Hz}$, 5.08 Hz). The ^{13}C NMR spectrum revealed signals for 30 C-atoms that on the basis of DEPT experiments confirmed the presence of eight methyls besides showing additional signals for ten methylenes, six methines (2 bearing bromine atoms and four bearing oxygen) and six oxygenated quaternary carbons. Proton carbon chemical shift correlation was established by HSQC experiments (Table 1.2.1). The cross peaks from the HSQC spectrum (Fig. 1.2.5) identified the sets of directly bonded carbons and hydrogens.

Most of the connectivity's were established by careful interpretation of combined ^1H - ^1H COSY (Fig.1.2.6), TOCSY (Fig.1.2.7) and HSQC (Fig. 1.2.8) experiments. In ^1H - ^1H COSY spectrum the signal at δ 4.32 (H-22) showed coupling with the methylene protons at δ 2.03-1.48 (H₂-21) which in turn was linked to another methylene at δ 2.069-1.63 (H₂-20). Similarly, the methine signal at δ 3.90 (H-3) showed connectivity with the methylene at δ 2.31-2.0 (H₂-4) which in turn correlated with another methylene at δ 1.71-1.48 (H₂-5); the methine signal at δ 3.75 (H-18) showed coupling to the methylene protons at δ 1.187-1.5 (H₂-17)

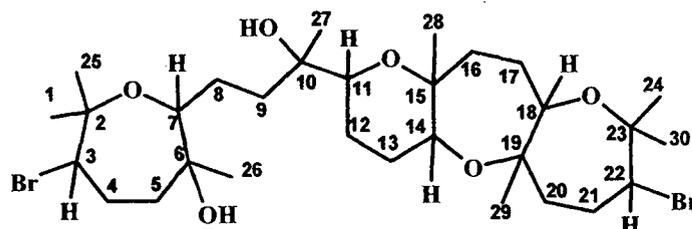
which in turn was linked to the methylene at δ 1.77 (H₂-16); the signal at δ 3.3 (H-7) was linked to the methylene signal at δ 1.63-1.48 (H₂-8) which in turn correlated with the methylene at δ 1.5 (H₂-9) and finally the methine signal at δ 3.44 (H-14) was coupled to the methylene protons at δ 1.63 (H₂-13) which in turn correlated to the methylene at δ 1.6 (H₂-12) which in turn showed connectivity to the other methine signal at δ 3.27 (H-11).

Table 1.2.1: NMR data in CDCl₃ of compound 1, δ in ppm, J in Hz

Position	δ ¹ H m Hz	δ ¹³ C m	HMBC
1	1.36(s)	25.2q	-
2	-	77.7s	-
3	3.90 (d, 10.9)	58.0d	C1, C4, C5, C25
4	2.31, 2.00	30.3t	-
5	1.71, 1.48	44.2t	C26
6	-	72.4s	-
7	3.30 (dd, 11.7, 3.0)	75.3d	C26
8	1.48, 1.63	25.1t	-
9	1.50	33.4t	C27
10	-	73.0s	-
11	3.27(dd, 10.9, 3.0)	74.1d	C27
12	1.60	27.0t	-
13	1.63	27.1t	-
14	3.44 dd	70.4d	C28
15	-	76.6s	-
16	1.77	39.5t	-
17	1.87, 1.50	27.8t	-
18	3.75(dd, 11, 3.3)	74.9d	C29
19	-	75.8s	-
20	2.06, 1.63	40.3t	-
21	2.03, 1.48	28.2t	-
22	4.32 t 2.9	63.8d	C20, C21, C24, C30
23	-	76.9s	-
24	1.27	30.0q	-
25	1.40	25.6q	-
26	1.11	24.9q	-
27	1.09	23.3q	-
28	1.22	18.8q	-
29	1.23	17.2q	-
30	1.40	25.6q	-

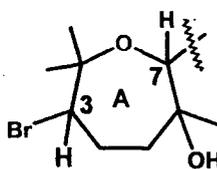
Thus the COSY spectrum revealed the presence of the following partial structures confirmed by TOCSY: H-3-H-5, H-7-H-9, H-11-H-14, H-16-H-18, H-20-H-21.

These subunits were linked together with the help of a long range correlation HMBC experiment (Fig. 1.2.9) establishing the following gross structure for compound 1.



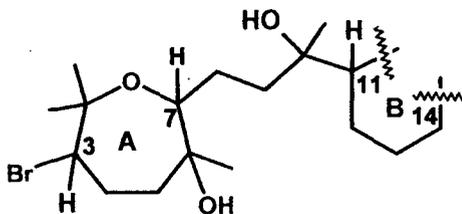
Compound 1

The ^1H - ^{13}C HMBC cross peaks were observed from C-3 to H₃-1, H₃-25, H₂-4, H₂-5 and C-7 and C-5, both have correlation with H₃-26. A three bond HMBC correlation from C-3 to H₃-1 necessitates attachment of C-1 to C-2. Carbons C-2 and C-7 must be attached to oxygen due to their chemical shift forming the oxepane ring A. The data is fully consistent with part structure I as shown.



I

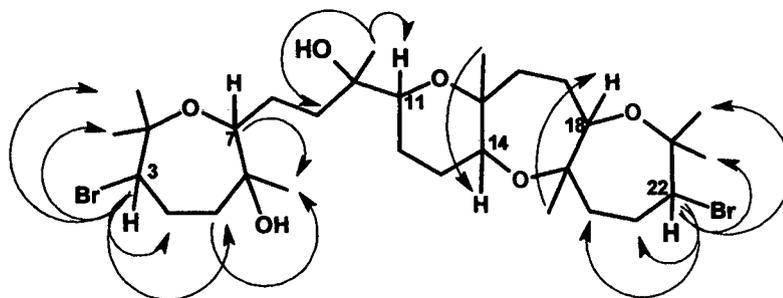
Linking the two subunits H-7-H-9 and H-11-H-14 through a three bond HMBC correlation from C27 to H-11 and H-9 led to the extension of part structure I to II.



II

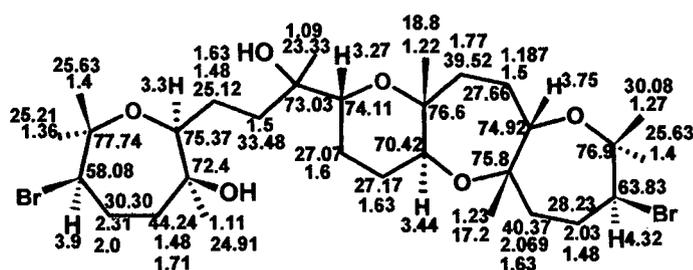
A three bond HMBC correlation from H₃-28 to C-14 necessitates the attachment of C-14 to C-15, which is a singlet. Carbon C-11 and C-15 must be attached to oxygen due to their chemical shift forming the ring B.

Similarly HMBC cross peaks from C-22 to H₂-21, H₂-20, H₃-24, H₃-30 and long-range correlation between each methyl and the other atoms completed the overall connectivity assignment, which is shown in Scheme 1.2.1 with all HMBC correlation observed.



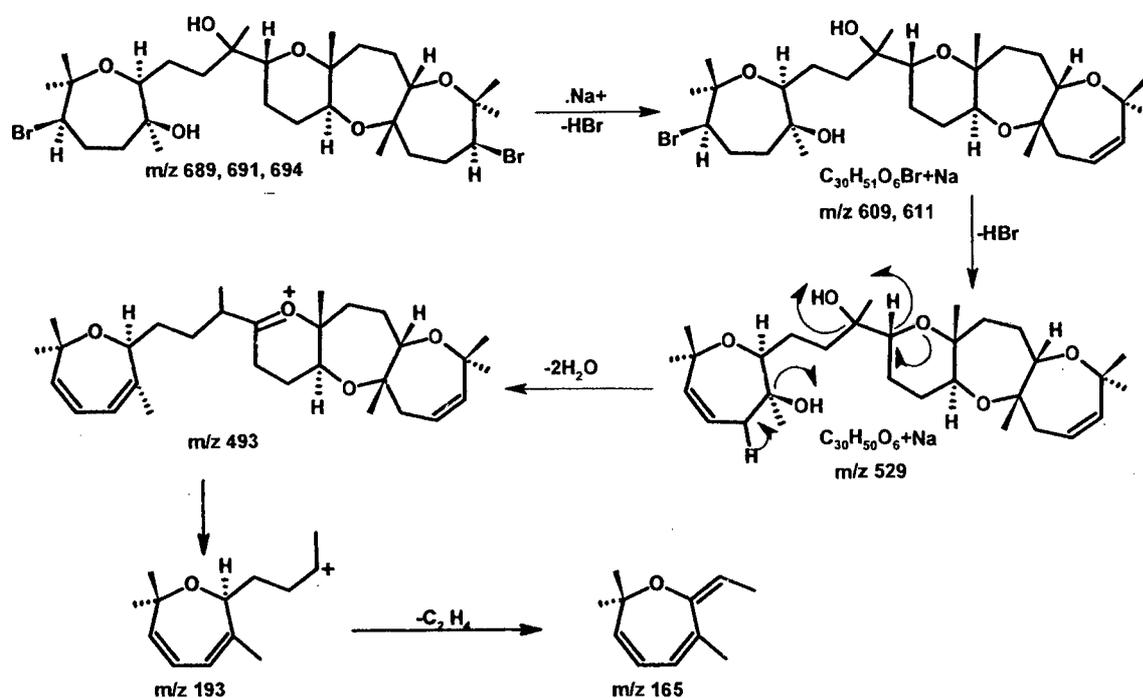
Scheme 1.2.1: HMBC correlation for compound 1

Careful inspection of the foregoing NMR data of compound (1) with armatol B-E reported by Cimino¹ and his group revealed that (1) possesses the same array of oxepane ring. It's spectral data being very close to armatol D suggesting a similar planar structure. The relative stereochemistry was established as the same in the A-B-C ring system as that of armatol D. However, the chemical shifts of H-17, C-18, C-19 together with the coupling constant values of H-22 methine established the cis fusion for the ring C-D. Thus compound (1) was finally assigned structure with the relative stereochemistry as shown and was designated as Armatol J.



Compound (1)

The genesis of various major fragments observed in the mass spectrum of armatol J could be easily explained on the basis of structure assigned as shown in Scheme 1.2.2.



Scheme 1.2.2 Mass spectral fragmentation of Compound 1

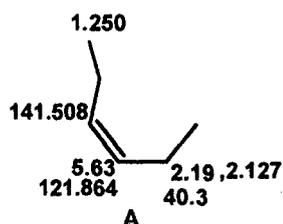
Compound (2) was isolated as an amorphous solid $[\alpha]_D = + 21.6$ ($c = 0.12, \text{CHCl}_3$). Mass and ^{13}C NMR spectral analysis of this metabolite were consistent with molecular formula C₃₀H₅₀O₇. The ^{13}C NMR (Fig. 1.2.10) shows the presence of eight methyls, nine methylenes and six methines together with seven quaternary carbon centres, which are α to oxygens, one of them being ketonic. The presence of ketonic group is further reinforced by the presence in its IR spectrum (Fig. 1.2.11) of carbonyl absorption at 1712cm^{-1} and a broad absorption at 3402cm^{-1} was indicative of -OH groups. ^1H NMR (Fig. 1.2.12) spectrum also displayed in the low field region of ^1H NMR four methines signals at δ 3.583, 3.180, 3.567, 3.628 which were assigned to protons H-7, H-11, H-14, H-18.

A broad signal at δ 5.637, integrated for two protons, due to olefinic protons and an allylic methylene group at δ 2.19 and 2.12 were also observed.

HMQC(Fig. 1.2.13) experiments established proton-carbon connectivity (Table-1.2.2) Assignment of the structure for this metabolite was also aided by the considerable spectroscopic analogy to armatol A²³.

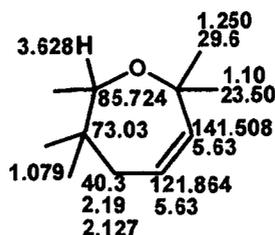
Table 1.2.2: NMR data in CDCl₃ of compound 2, δ in ppm, J in Hz

Position	δ ¹ H Hz	δ ¹³ C	HMBC
1	1.27	28.0	-
2	-	84.2	-
3	-	212.6	C1, C4, C5, C25
4	2.17	41.0	-
5	2.21	36.1	C26
6	-	72.4	-
7	3.58	75.2	C26
8	1.62	21.5	-
9	1.30	34.0	C27
10	-	71.0	-
11	3.18	84.2	C27
12	1.76, 1.51	25.8	-
13	2.04	28.0	-
14	3.56	77.1	C28
15	-	71.0	-
16	1.66	38.5	-
17	1.47	35.6	-
18	3.62	85.7	C29
19	-	73.0	-
20	2.19, 2.12	40.3	-
21	5.63	121.8	-
22	5.63	141.5	C20, C21, C24, C30
23	-	70.4	-
24	1.25	29.6	-
25	1.05	24.1	-
26	1.23	29.6	-
27	1.05	16.5	-
28	1.14	21.5	-
29	1.07	21.1	-
30	1.10	23.5	-



A long range HMBC (Fig. 1.2.14) correlation of methyl at δ 1.250 with the olefinic carbon at δ 141.508, quaternary carbon at δ 70.48 and C-24 methyl carbon at δ 29.68 further established the part structure A.

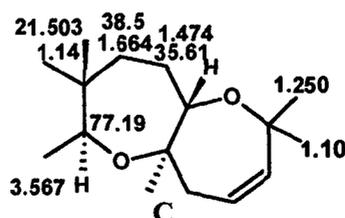
The allylic protons showed HMBC correlations with the olefinic carbon and the quaternary carbon at δ 73.03



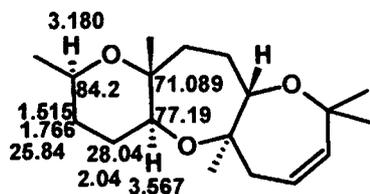
B

A TOCSY (Fig. 1.2.15) correlation was also observed for the olefinic protons with the methyl at δ 1.10 (C-30) leading to the extension of part structure A to B. The methyl protons at δ 1.079 showed long-range correlations with the allylic carbon at δ 40.38, quaternary carbon at δ 73.03 and the carbon signal at δ 85.72(C-18). The methine (H-18) showed 2D-NMR(TOCSY) connectivity with the methylenes at (δ 1.664-m) H2-16 and H2-17 (1.474).

A long-range correlation of the methyl at δ 1.14 with C-16 at δ 38.52, quaternary carbon at δ 71.08 and the secondary carbon at δ 77.19 led to the extension of B to part structure C.

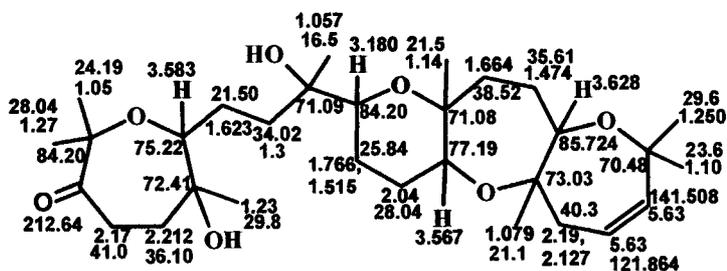
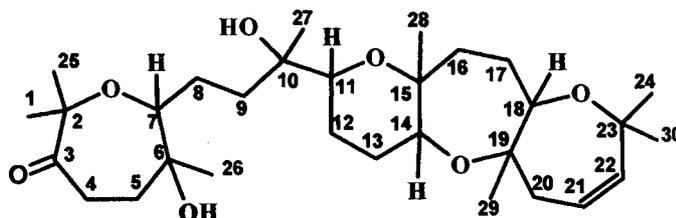


A TOCSY correlation of H-14 at δ 3.567 with H-13 at δ_H 2.04(C δ_C 28.0) and H-12 at δ_H 1.766(C δ_C 25.8) which in turn couples with the C-11 methine at δ_H 3.180(C δ_C 84.2) leading to the further extension of the part structure C to D.



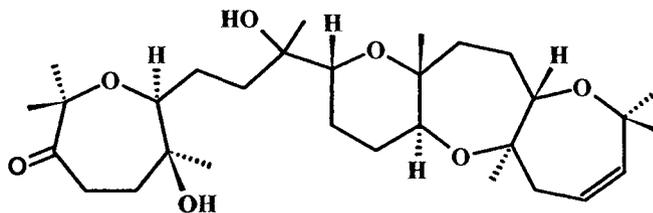
D

A long-range correlation was observed of a carbonyl at δ 212.64, a quaternary carbon at δ 84.20, a doublet at δ 75.22 and the methylenes at δ 36.10 and 41.0. These correlations together with the COSY (Fig. 1.2.16) correlation of the methine proton at δ 3.180 with proton at δ 1.515 and 1.766 and the NOESY (Fig. 1.2.17) correlation established the gross structure of the squalene polyether as compound-2:

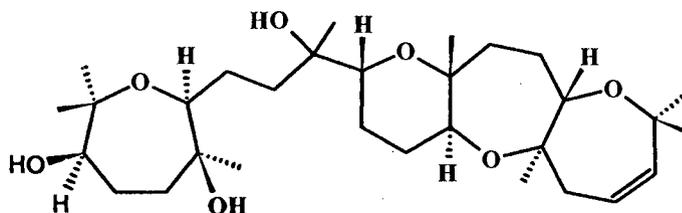


Compound 2

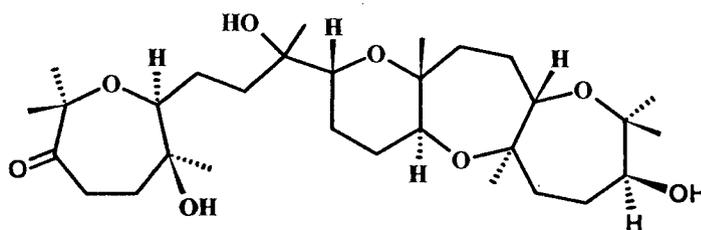
The relative stereochemistry of the molecule was established as shown on the basis of NOESY and comparison with the reported armatols from this source²³. The NMR values assigned for this armatol needs confirmation by High Resolution NMR as there is overlapping of signals in the high field region.



The ESI-MS(Fig. 1.2.18) of this metabolite also contained additional signals at m/z 547 and 563 indicative of the presence of the following metabolites.



m/z 547



m/z 563

Experimental:

The chloroform fraction (123g) was filtered, initially on a column of Sephadex LH20 with chloroform:methanol (1:1) as eluant. The fractions obtained were combined according to their TLC profile. Further purification was done by repeated silica gel chromatography and elution with increasing amounts of ethyl acetate in petroleum ether. Increase in the concentration of ethyl acetate to 25% resulted in the elution of the first polyether as a colourless oil(**Compound-1**) (13mg) with a R_f value of 0.54 in 30% ethyl acetate in petroleum ether. Elution of the column and TLC with 30% of the above solvent system yielded (**Compound-2**) with a R_f value of 0.32.

+TOF Product (691.2): 77 MCA scans from MT20080522113111.wiff
a=3.56242412115625640e-004, t0=-4.15046737971060790e+001

Max. 1835.0 counts

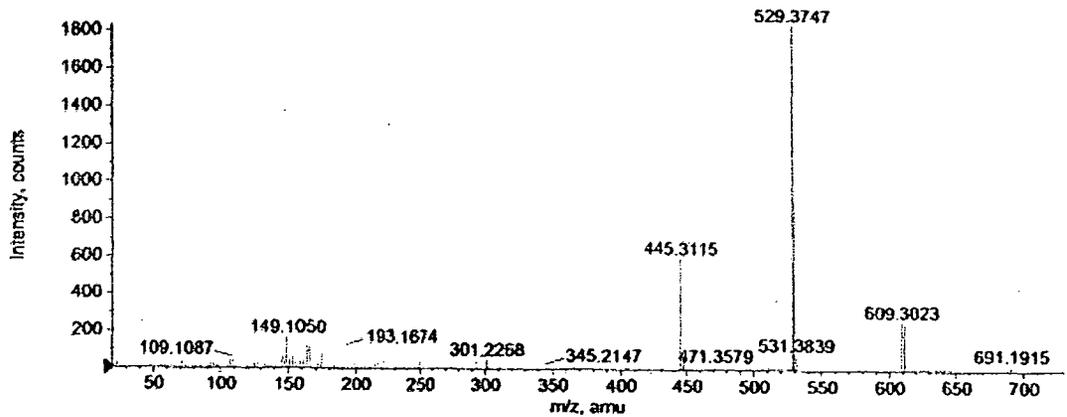


Fig 1.2.1a: ESI-MS of Compound (1)

+TOF Product (691.5): 68 MCA scans from MT200502231529...
a=3.56404782097534190e-004, t0=-4.09604417039008690e+...

Max. 499.0 counts.

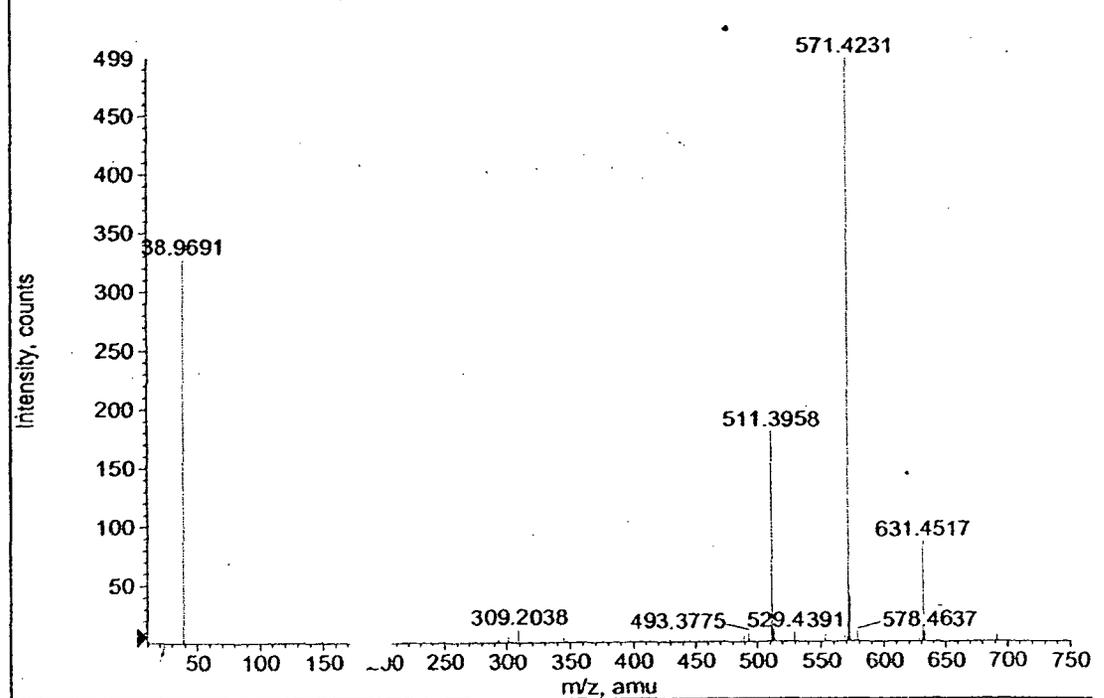


Fig 1.2.1b: ESI-MS of Compound (1)

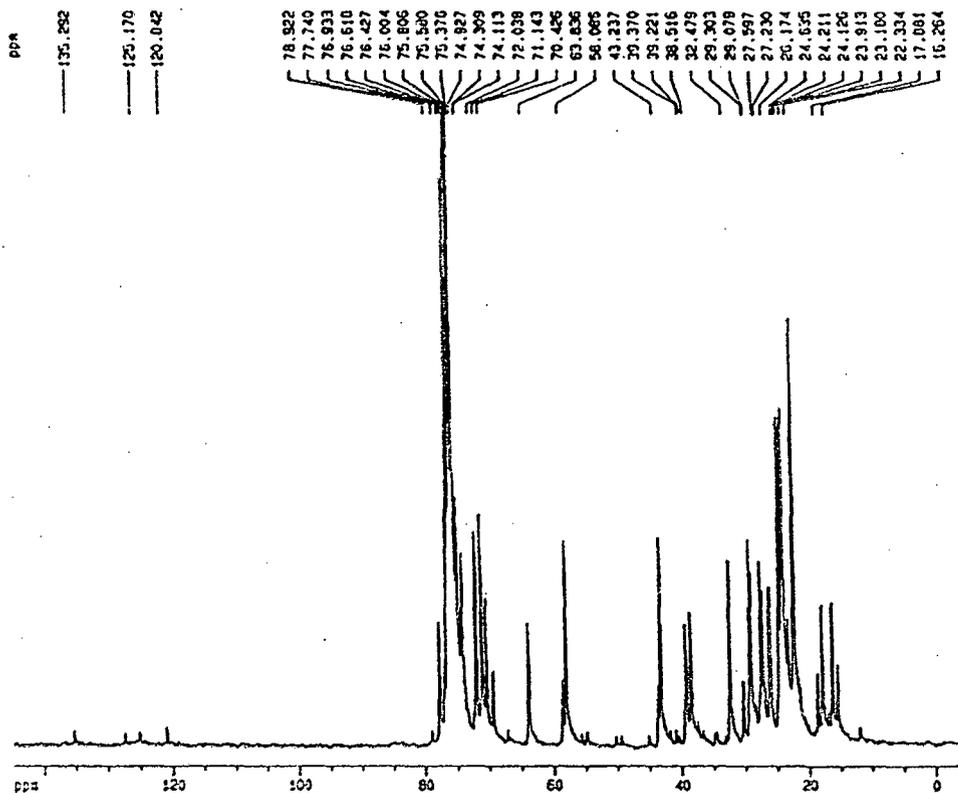


Fig 1.2.2: ¹³CNMR of Compound (1).

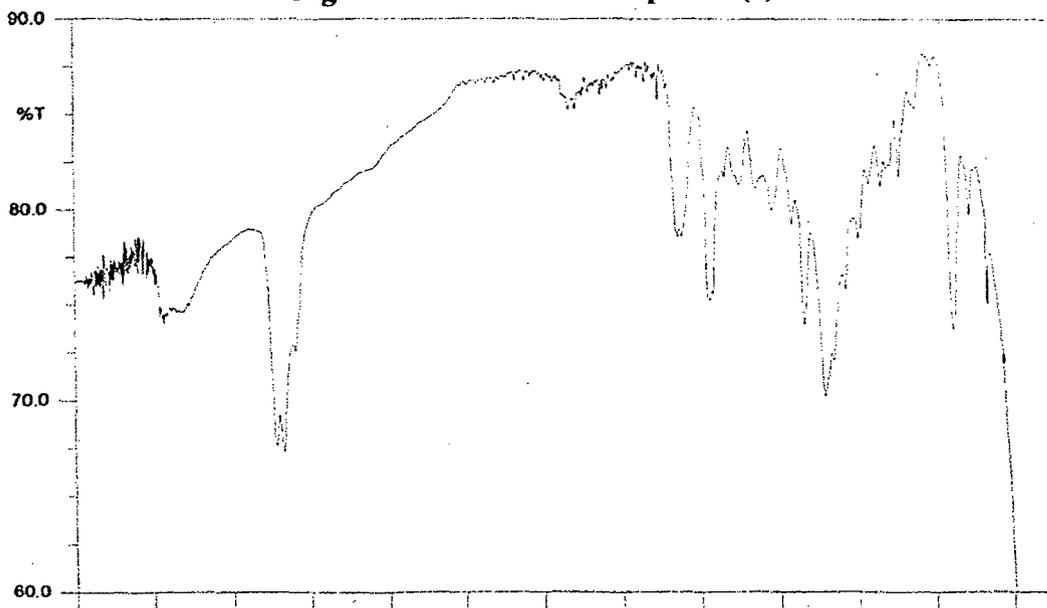


Fig 1.2.3: IR of Compound (1).

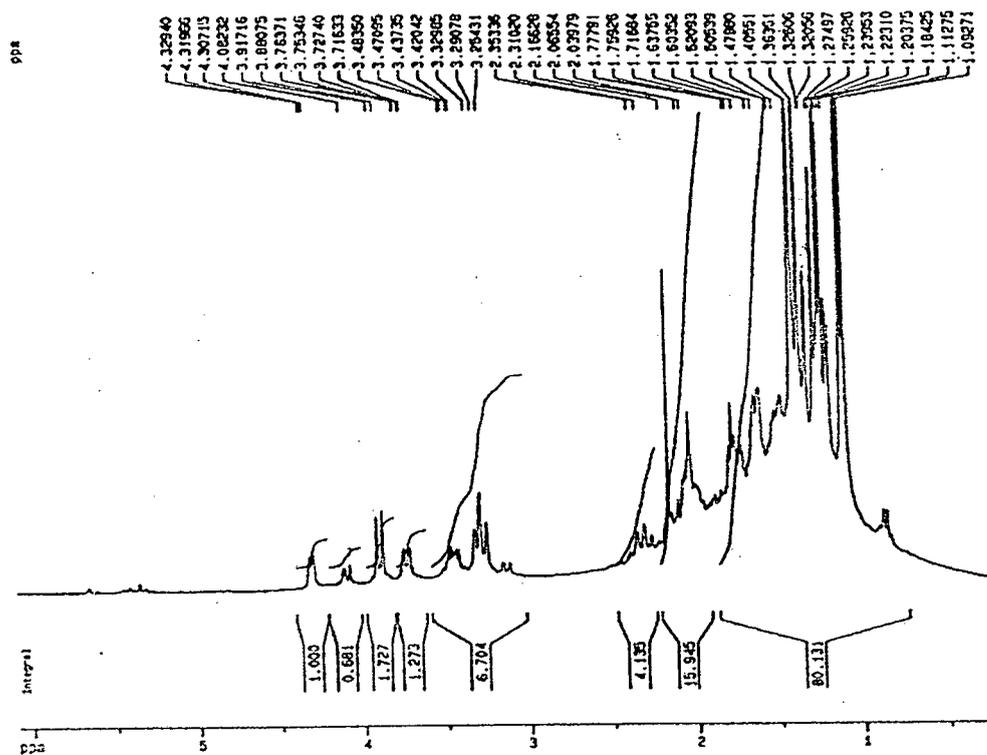


Fig 1.2.4: ¹H NMR of Compound (1).

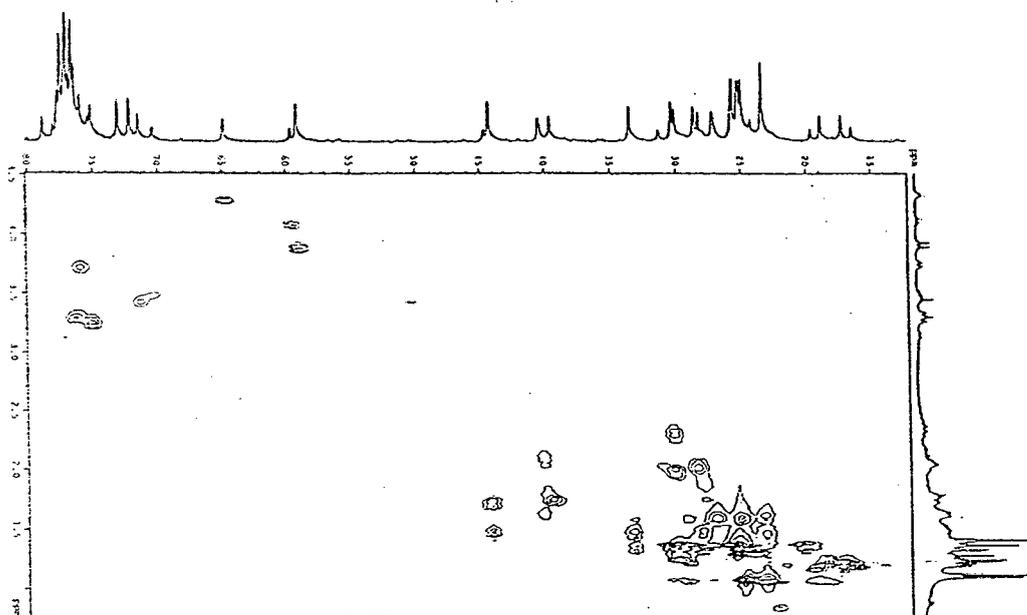


Fig 1.2.5: HSQC of Compound (1).

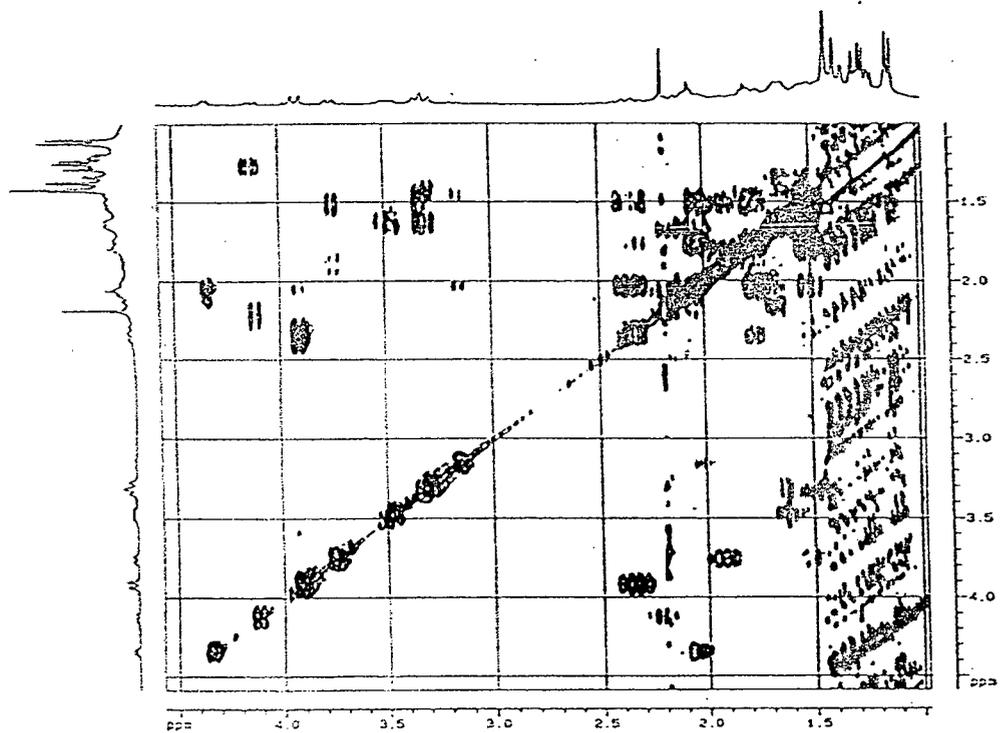


Fig 1.2.6: ^1H - ^1H COSY of Compound (1).

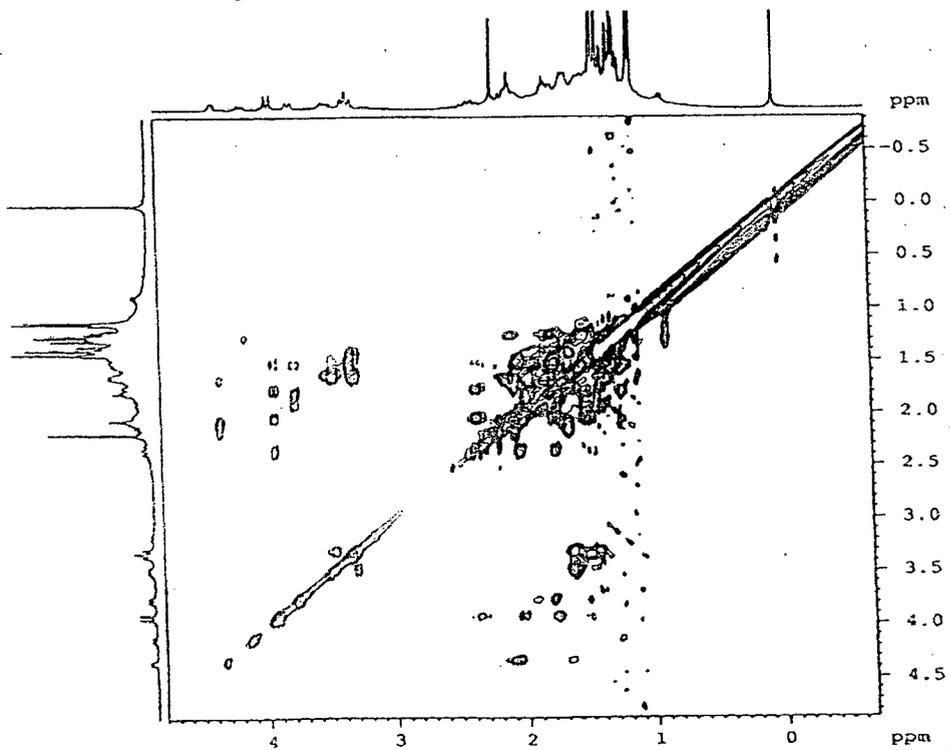


Fig 1.2.7: TOCSY of Compound (1).

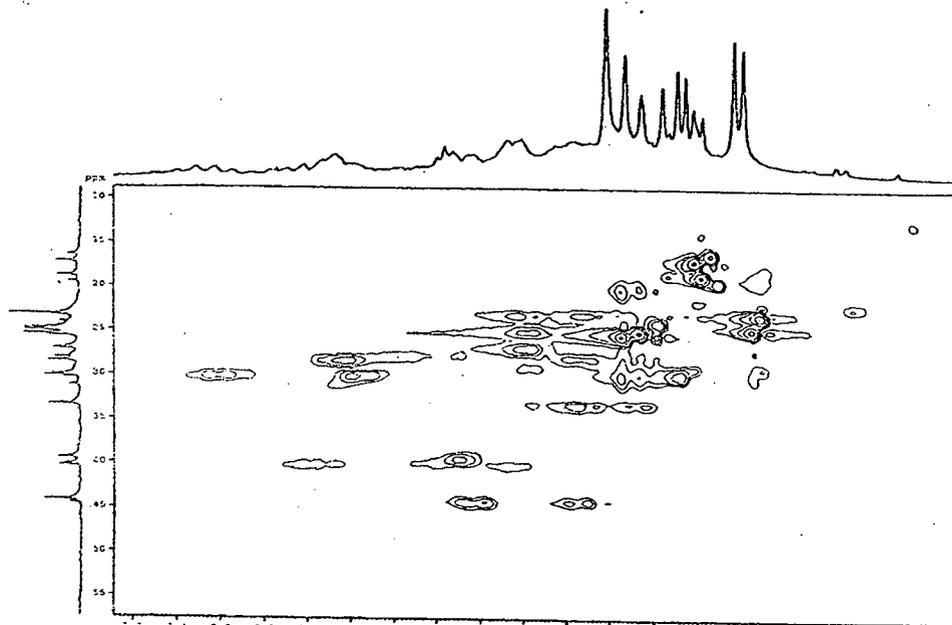


Fig 1.2.8: HSQC of Compound (1).

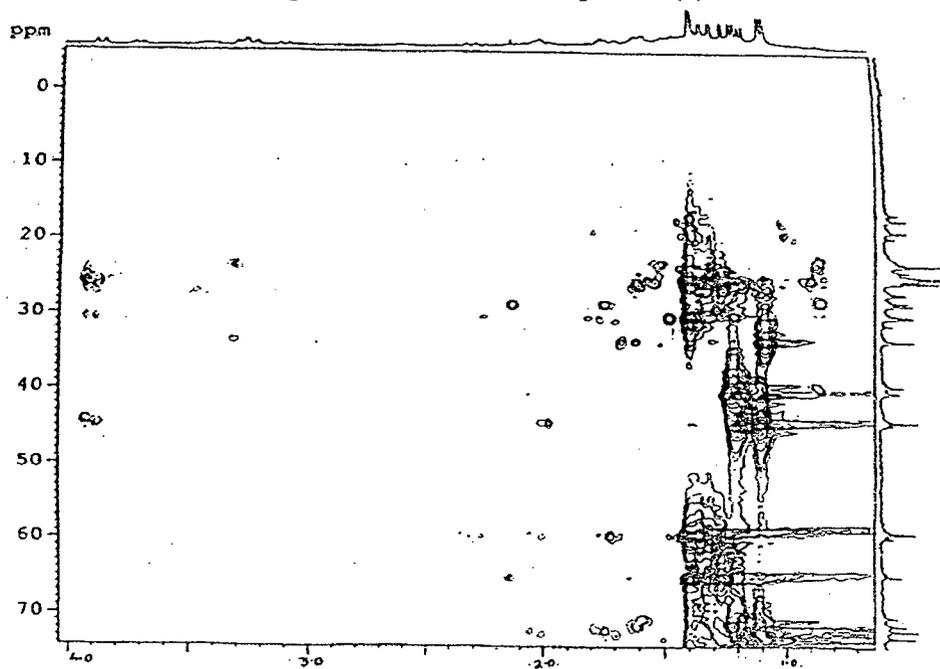


Fig 1.2.9: HMBC of Compound (1).

Chapter II

*Studies on the bioactivity and chemistry of
some selected mangrove plants*

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BIOACTIVE SUBSTANCES FROM THE MANGROVES- A REVIEW.

The mangrove ecosystem is one of the important ecosystems in the tropics and has great economic and ecological value. The mangrove forests provide habitats for species, which are adapted to a saline tidal environment and a large number of diverse migratory waterfowl and terrestrial animals and are very important as habitat of aquatic organisms. The term mangrove is also used to designate halophytic and salt resistant marine tidal forests comprising of trees, shrubs, palms, epiphytes, ground ferns and grasses, which are associated in stands or groves¹. Mangroves are usually found only in tropical climates, as they need consistently warm conditions for development and survival.

Mangrove flora can be classified into true mangroves and mangrove associates. True mangrove species consist of plants which are absolutely confined to salt or brackish water, while mangrove associates are plants which belong to the littoral zone and/inland vegetation but can frequently be found with back mangrove. The mangrove associates are salinity tolerant plant species, which are not found exclusively in the proximity of mangroves and may occur only in transitional vegetation, landwards and seawards. However, they do interact with true mangroves^{2,3}. Mangroves (mangroves, mangrove minors and mangrove associates) are highly productive ecosystem with various important economic and environmental functions. The uses of mangroves fall in two major categories: First is its indirect use in control of coastal erosion and protection of coastal land, stabilization of sediment, natural purification of coastal water from pollution. Second use of economic importance is associated with prawn fisheries and many other species including crabs, shrimp, oysters, lobsters and fish. Traditionally, besides their use in folklore medicine, the mangroves have been exploited for firewood and charcoal and their uses also include construction of dwellings, furniture, boats and fishing gear and production of tannins for dyeing and leather production. The mangrove leaves are useful contributors to the nutrient system of the mangrove environment. It is known that mangrove leaves contain sufficient amounts of minerals, vitamins and amino acids, which are essential for the

growth, and nourishment of marine organisms and livestock. Mangrove foliage plays an important role in the formation of detritus, which is utilized by several estuarine and marine detritivorous organisms, and mangrove leaves make a superior fodder due to their high salt and iodine content.^{2,4,5}

Two basic factors justify the study of the chemical constituents of mangrove plants. First is their ability to thrive in a very peculiar environment under stressful conditions such as violent environments, high concentration of moisture, high and low tides of water, and abundant living microorganisms and insects and serve as a bridging ecosystem between freshwater and marine systems. These have imposed several modifications in these plants and due to these reasons one expects them to contain chemical compounds, which protect them from these destructive elements. The second and the most important reason is that numerous mangrove plants are being used in folklore medicine, and recently, extracts from mangroves and mangrove-dependent species have proven activity against human ailments and animal and plant pathogens. These plants can be used as alternative medicine, but scientific proof of their effectiveness and research into possible side effects are still needed before they can be widely recommended. Only limited investigations have been carried out to identify the metabolites responsible for their bioactivities.

Metabolites, some with novel chemical structures, and belonging to a diversity of 'chemical classes', have been characterized from mangroves and mangal associates. But, here we will restrict only to molecules with unusual structures and products of secondary metabolism having toxicological, pharmacological and ecological importance identified from selected mangroves and mangal associates.

Medicinal uses, bioactivity of extracts, and metabolites characterized from selected mangroves and mangal associates:

Acanthus illicifolius, is an evergreen spiny herb used traditionally in the treatment of paralysis, asthma, rheumatic pains and possessing analgesic, anti-inflammatory and, leishmanicidal activities. Chinese use this plant to cure skin itches and abscesses. It is a rich source of long chain alcohols, triterpenes, steroids and triterpenoidal saponins. Stigmasterol (1), a common plant steroid, abundantly present in *A. illicifolius*, has been shown to have hypercholesterolemic effects. 2-

Benzoxazoline (2), used extensively as a central nervous system depressant, also exhibiting antipyretic, hypnotic, and muscle relaxant activity was first identified as anti *Fusarium* factor from this plant. Its precursors, benzoxazinoids, have also been identified as anti-inflammatory agents⁶. Furthermore, the ribose derivatives of this compound are active as anticancer and anti-viral agents⁷⁻⁹. Jongsuvat (1981)¹⁰ found that the extracts of the plant were non-toxic to experimental mice but displayed significant anti-leukemic activity. Minocha and Tiwari report besides triterpenoidal saponins a novel alkaloid, acanthicifolin, from the root of this spiny herb¹¹. Benzoquinones have been identified from *Aegicers corniculatum* and *Kandelia candel*. Decoctions made from the rhizome of the reed grass *Arundo donax* has been used as emollients and diuretics and are said to stimulate menstrual discharge and diminish secretion of milk. Triterpenes, sterols, alkaloids, and the novel compound, N-(4'-bromophenyl)-2,2- diphenylacetanilide (3), hitherto known only as a synthetic compound, has been isolated from different parts of this plant. Significant anti-feedant activity was exhibited by the isolates tricentanol (4), flavonoid triclin (5), and tetramethyl-N, N-bis (2,6-dimethylphenyl) cyclobutane-1,3-diimine (6)¹². Known triterpenes, steroids, and a novel triterpenoid esters have been isolated from *Acrostichum aureum* and *Rhizophora apiculata*, a mangrove fern and tree respectively. The extracts of these plants are being used in folklore medicine^{11,13,14}. Rocaglamide (7), a substituted benzofuran, along with its congeners, has been identified as the active insecticidal constituent of the Chinese rice flower bush *Aglaia odorata*. Clopentabenzofuran, and aglaiastatin, two protein synthesis inhibitors have also been identified¹⁵⁻¹⁷. Anti-plasmodial and cytotoxic activities of *Alstonia macrophylla* are due to an array of alkaloids present in the extracts. *Avicennia alba* is a rich source of naphthoquinones. An associate of *A.marina* fungus belonging to the genus *Phytophthora*, produced three chemically related phytoalexins (8). Naphthoquinone derivatives occurring in the *Diospyros* species have potent anti-tumor promoting activity. Tannin from species of *Diospyros* has anti-hemorrhages (snakebite) effects. Plants of the family *Rhizophoraceae* may be generally divided into mangrove species and inland species. Either group of the plants contains organic sulfur containing compounds. The alkaloids brugine (9) and gerradine

(10) are 1,2-dithiolane (sulfur containing) compounds isolated from *Bruguiera sexangula* (mangrove species) and *Cassipourea gerrardii* (mangal associate) respectively. Extracts of *B. sexangula* bark were active against, Sarcoma 180 and Lewis Lung Carcinoma cell lines. The activity was partly associated with tannins and partly with tannin-free aqueous residue containing the alkaloid brugine (9) as well as tropine and its acetic acid ester. The alkaloids were found to be toxic¹⁸⁻¹⁹. Kato and coworkers²⁰⁻²¹ identified 1,2-dithiolane compounds, brugierol (11), isobrugierol, and 4-hydroxy-1,2-dithiolane-1-oxide, from the mangrove species *B. conjugata* with antibacterial and insecticidal activities. The bark of *Gymnotroches axillaris* has yielded hygrolin (12).

Metabolites belonging to different 'chemical classes' have been identified as antifungal agents and in chemical narcosis of fish. Antifungal metabolites include alkaloids, flavonoids and related compounds, modified fatty acids, oxygen heterocyclics, proanthocyanidins, quinones, stilbenes, terpenoids, triterpenoids and saponins. The bark of the molluscicidal and piscicidal plant *Balanites aegyptiaca*, besides being used for the treatment of abdominal pains, as a purgative, and as an anthelmintic, is also employed as a detergent, fish poison, and also as a remedy for malaria and syphilis²². The leaf is edible and has been once regarded as an effective medicine for sleeping sickness. The piscicidal effect of *B. aegyptiaca* to the Nile *Tilapia* and the molluscicidal activity is due to the metabolites balanitin, 1,2, and 3²³⁻²⁴. The saponins are the main constituents responsible for the piscicidal activity of *Aegiceras majus*, *Derris trifoliata*, *D. elliptica* and *D. urucu*. Rotenone (13), a well-known fish poison and a natural insecticide²⁵, is found among tropical plant species such as, *Derris*, *Lonchocarpus*, and *Tephrosia*. The sesquiterpenes heritianin, heritol, heritonin, vallapin (14) and vallapianin are the ichthyotoxins isolated from the mangrove plant *Heritiera littoralis*. Vallapin and vallapianin also showed activity against Boll Weevils²⁶. A triterpene ester isolated from *H. littoralis* showed significant anti-fungal and Boll Weevil anti-feedant activities¹¹. The piscicidal activity of the extracts of *Aegiceras corniculatum* is due to the benzoquinones embelin (15) and 5-O-methyl embelin (16). 5-O-Methyl embelin also inhibited the growth of the fungi *Pythium ultimum*. Fagaronine (17), an antileukemic alkaloid, has been isolated from *Fagara*

zanthoxyloides. The alkaloid was found to be bactericidal but not mutagenic. *Caesalpinia bonducella*, extensively used in Jamaican folk medicine is a rich source of furanoditerpenes collectively referred to as caesalpins (18)²⁷. The oleoresin from the bark of *Callophyllum inophyllum* (Guttiferae) is used as a cicatrisant, whereas an infusion or decoction of the leaves has been traditionally used for the treatment of eye diseases and as an ingredient in aromatic powders and liniments. Anti-bacterial, anti-inflammatory, and phagocytosis stimulant activities have been reported for this plant. *Guttiferaceous* species are a rich source of xanthenes, biflavonoids, benzophenones, neo-flavonoids, and coumarin derivatives²⁸. Recently, various bioactivities such as cytotoxic, and antitumour, anti-inflammatory, antifungal, enhancement of choline acetyltransferase activity, and inhibition of lipid peroxidase due to xanthenes have been revealed. Two new xanthenes, calaxanthenes A (19) and B have been isolated from the root bark of *C. inophyllum*. The giant African snail, *Achatina fulica*, feeds on the leaves of *C. inophyllum* and ingest inophyllums and calophyllolides (20) from the plant. A xanthone derivative, subelliptenone, and related compounds showed strong inhibitory effect against topoisomerases I and II, in *in vitro* experiments. These xanthenes are claimed to be prospective lead compounds for anticancer drugs and inophyllums as active against HIV-1 in cell culture²⁸⁻³⁰. Earlier phytochemical studies had revealed that *C. inophyllum* to be a rich source of benzopyrans, coumarins, steroids, triterpenes, and xanthenes. Plants of the genus *Clerodendron* are well known for their pesticidal properties³¹. They are used as armyworm antifeedants and to arrest bleeding from cuts, wounds, as well as post-partum hemorrhage. *Clerodendron inerme*, a mangal associate, is recognized medicinal plant exhibiting antipyretic, larvicidal, antiviral and uterine stimulant activities. Extracts of *C. inerme* were effective as surface protectants for cowpea seeds against pulse beetle infestation. The anti-viral resistance-inducing protein isolated from the plant is a polynucleotide³²⁻³³. A number of flavonoids, a neolignan (21), and novel complex iridoids, phenyl propanoids, sterols and known terpenes, a new diterpene acid cleroinermin (22), have been characterized from the plant³⁴⁻³⁶. *Cyprus rotundus* was used in traditional control of insect pests, the effectiveness being due to the presence of novel sesquiterpene alkaloids³⁷⁻⁴⁰. Triterpenes,

steroids, long chain aliphatic carboxylic acids are responsible for the antifeedant activity of *Eleocharis dulcis*⁴¹. The sap of *Hippomane mancinella* is known to cause a reaction characteristic of a burn and contact with eye, produce severe conjunctivitis, which, if complicated by secondary infection, could result in loss of sight⁴². Surprisingly, the poisonous latex, a source of various metabolites has been used as an ingredient in many native preparations. 2-Hydroxy-2, 6-dimethoxyacetophenone, mono, di and trimethyl ethers of ellagic acid (23), and a novel alkaloid have been isolated from various parts of the plant. The toxic principle of the extracts of leaves and twigs was identified as tannins, hippomanin A and B. The irritant factor was assigned to esters of deoxyphorbol (24), resiniferonol, and 13-hexadeca-2, 4, 6-trienoic acid⁴³. Apart from its folk medicinal utilization, *Excoecaria agallocha* contains toxic principles injurious particularly to the skin. The latex though mildly active against certain fungi and inactive against bacteria and yeast it is well known for its biocidal effects on marine organisms and phytoplankton. It causes metabolic depression of the rice field crab, *Oziotelphusa senex* and is used as an uterotonic, fish poison, dart poison, and contains novel chalcones and piperidine alkaloids. Soil bacteria and yeast actively degrade the latex, which probably helps in the detoxification of the latex in nature. The infusion of leaves posses antioxidant and anti-tumor promoting properties. Bioassay guided isolation led to the characterization of excoecarin, an irritant and a tumor promoter and excoecariatoxin (25), piscicidal agent with activity comparable to natural rotenone. A novel phorbol ester was isolated as the anti-HIV principle of the leaves and stems⁴⁴⁻⁴⁹. Sesquiterpenoid quinones, the hibiscones (26), hibiscoquinones and benzoquinones (27) are the major constituents of *Hibiscus tileaceous*. *Ipomoea pes-caprae* is a traditional medicinal plant used in the treatment of headache and various types of inflammation including jellyfish sting dermatitis. The extracts from the leaves exhibits anti-inflammatory activity, reduce prostaglandin synthesis *in vitro*, and inhibit smooth muscle contraction. 2-Hydroxy-4, 4, 7-trimethyl-1 (4H)-naphthalenone, mellein, eugenol, and 4-vinylguaicol, were the inhibitors of prostaglandin synthesis. The antispasmodic and anti-nociceptive activities⁵⁰⁻⁵² was exhibited by the presence of the isoprenoids β -damascenone (28) and E-phytol

(29). *Melaleuca leucadendron* exists in three chemotypes, the volatile leaf oil of two of which is characterized by very high content of phenylpropanoids. In addition to small amounts of known mono-, di-, sesqui-, and tri- terpenes, stilbene glycosides, novel triterpenoid esters, and hydrolysable tannins, have been isolated from the plant, whose extracts possess antifungal properties. Terpenoids along with stilbenes (30), inhibited histamine release from rat mast cells and were active against *Bacillus* and *Staphylococcus*⁵³. Known glycosides, fatty acid esters, and a novel trisaccharide have been characterized from ripe fruits of *Morinda citrifolia*, which is toxic to nematodes and *Drosophila*. Octanoic acid, toxic to many insect species, along with hexanoic and other carboxylic acids, are the main toxic compounds isolated from the extracts⁵⁴. Hirazumi and Furusawa⁵⁵ reported the presence of a 'polysaccharide-rich substance' with antitumor activity in the fruit juice of *M. citrifolia*, which also showed anticancer and analgesic activity. A polysaccharide extracted from the leaves of *B. cylindrica*, *E. agallocha*, *R. apiculata*, *R. mucronata*, *Salicornia brachiata*, *Sesuvium portulacastrum*, *Sueda maritima* and *S. monica* showed positive activity against human immunodeficiency viruses⁵⁶⁻⁵⁷.

Antioxidant activity of extractives of *Pandanus odoratissimus* has been demonstrated as due to phenolics, lignans, and a benzofuran derivative⁵⁸. A number of *Pluchea* species are noted for their ethnomedical properties, of which the reputed viper venom neutralization activities of *P. odorata* and *P. indica* are probably the best known. Neuropharmacological actions (including viper venom neutralization) of the shrub *P. indica* have been investigated⁵⁹⁻⁶¹. The leaves and roots of the shrub have been reported to possess anti-inflammatory and anti-ulcer, especially gangrenous ulcers, astringent and antipyretic properties and are used as a diaphoretic in fevers. The crushed leaves and young shoots mixed with alcohol, are used to relieve lumbago and rheumatic pains and in baths to treat scabies. A number of known compounds and a new eudesmane derivative (31) have been identified from the leaves⁶².

(Z)-5-Tetradecenyl acetate and tetradecyl acetate were identified as sex pheromone components of an unnamed *Planotortrix* leaf roller moth species found in *Avicennia resinifera*. All parts of the plant *Pongamia pinnata*, used as a

crude drug for the treatment of tumors, piles, skin diseases, wounds, ulcers, is a rich source of flavonoid and related compounds⁶³⁻⁶⁴. Extracts of the plant showed positive activity against human and simian immunodeficiency viruses⁶⁵⁻⁶⁷.

The indole derivative, rhizophorine (32) is a major constituent of the leaves of *Rhizophora mucronata* and a novel type of water soluble polymer has been isolated from the leaves of *R. stylosa*¹¹. Triterpenoids from *R. mangle* possess insecticidal properties and has clinical use in the control of diabetes. Warm aqueous extract of the bark of *R. apiculata* is used as an astringent for diarrhoea, nausea, and vomiting, and as an antiseptic. The extract is also used to stop bleeding in fresh wounds and for the treatment of chronic typhoid fever. A nitrogen containing phorbol ester, sapintoxin A (33), a piscicidal agent, has been isolated from the poisonous plant *Sapium indicum*. Sapinine, a diterpene ester (a phorbol derivative), and a non-biologically active metabolite was isolated using traditional purification techniques⁶⁸. Skin irritant and tumor promoting diterpene ester, 12-deoxyphorbol, has been identified from *Sapium sebiferum*⁶⁹. *Sesuvium portulacastrum*, a salt marsh halophyte, is a rich source of an array of amino acids⁷⁰. An unusual secondary metabolite, 2-nitro-4-(2'-nitroethenyl) phenol (34) has been isolated from the fruits of *Sonneratia acida*. The fruits are used as poultice in swelling and sprains. Fermented juice of the fruit is useful for arresting haemorrhage. The wood has yielded three anthraquinones and the leaves contain plant growth regulators, the diterpenoid gibberellins (35)⁷¹. *Bruguiera gymnorhiza*, *Rhizophora mucronata* and *Sonneratia apetala* were also found to contain gibberellins⁷²⁻⁷³

Terminalia catappa is used in folk medicine for preventing hepatoma and treating hepatitis and is a rich source of tannins. Antioxidant and hepatoprotective activity, anti-sickling potential, and the effects of the major tannin components, punicalagin and punicalin of *T. catappa* on carrageenan-induced inflammation in rats, bleomycin-induced genotoxicity in rabbits, have been evaluated^{47,74,75}. The triterpene lupeol is the antibacterial principle of the leaves of *Thespesia populnea*, and gossypol was the active ingredient in the flowers, which accounted for its antifertility activity⁷⁶. Naturally occurring quinones, the mansonones, extracted from the heartwood of *T. populnea* showed cytotoxicity, antibacterial and anti-

steroidogenic activities⁷⁷⁻⁷⁸. The limonoid ester, xylocensins (36), the esters of alcohols, isobutyrate and alpha-methylbutyrate, methyl angolensate, gedunin, phragmalin were the novel constituents of *X. moluccensis*⁷⁹⁻⁸⁰. Alvi et al.⁸¹ characterized two new limonoids, xylocensin 1 and 2, devoid of any biological activity, from the Fijian species of *X. granatum* and *X. moluccensis*. The fruit of *X. molluscensis* is used in folk medicine in East Africa as aphrodisiacs. The bitter principle of young fruits, an unusual monoterpenoid having a nonglycosidic hemiacetal function⁸² xylomollin (37), tested positive as an antifeedant and strongly inhibited the respiratory reactions of mitochondria from rat liver. Insect antifeedant bioassays employing African armyworms and Mexican bean beetle has led to the isolation and characterization of N-methylflindersine (38) and several benz [C] phenanthridone alkaloids from the extracts of *X. granatum*. The former metabolite has been identified as the principle responsible for insect antifeedant activity⁸³.

Drugs of 'natural' origin, either the 'original' natural product, products derived 'Semi-synthetically' from natural products or synthetic products based on natural products models, play an invaluable role in the drug discovery process. Marine organisms and plants produce novel metabolites unique to the environment. Mangroves and mangal associates living in yet another different environment to that of marine and terrestrial plants, produce metabolites, which are unique to these plants and are of interest to the 'curious' chemist. Although the chemistry of the natural products of mangrove plants is little known, there have been some examples in recent years to support the need to study the chemistry of the mangroves. This belief is well supported by the illustrated examples. The chemistry of mangrove plants tends to establish that they are not only source of novel compounds but also provide a new source for many already known biologically active compounds including toxic compounds. Rotenoids, alkaloids, terpenoids are among the classes of natural products, which provide numerous toxins. Toxin in plants often has the role of feeding repellents. A remarkable number of insecticidal plants seem to have been recognized first as fish poisons. Knowledge of the toxins in higher plants has led to a variety of useful drugs. Metabolites though toxic, are still used clinically for the treatment of diseases.

Typical examples are those of the toxic drugs, sodium stibogluconate and pentamidine, used in the treatment of *Leishmania donovani* infection⁷. Though numerous mangroves and mangal associates are recommended in traditional medicine as active against various diseases, very little attempts have been made to investigate the veracity of these assertions in controlled experiments. Few workers have investigated the reputation of such plants by performing *in vitro* and *in vivo* experiments in order to demonstrate whether there are any protective effects, using drugs or mixtures of drugs prepared using traditional formulae.

In our ongoing program on 'Bioactive substances from Indian Ocean' we have directed our efforts towards detecting pharmacological activities of the extracts of marine organisms including mangrove plants and towards isolating and characterizing those active substances which show promise as potential therapeutic agents. During these investigations we had an opportunity to study the bioactivity and chemistry of mangrove plants, *Lumnitzera racemosa*, *Aegiceras corniculatum* and *Sesuvium portulacastrum*. Accordingly this chapter has been divided into three sections:

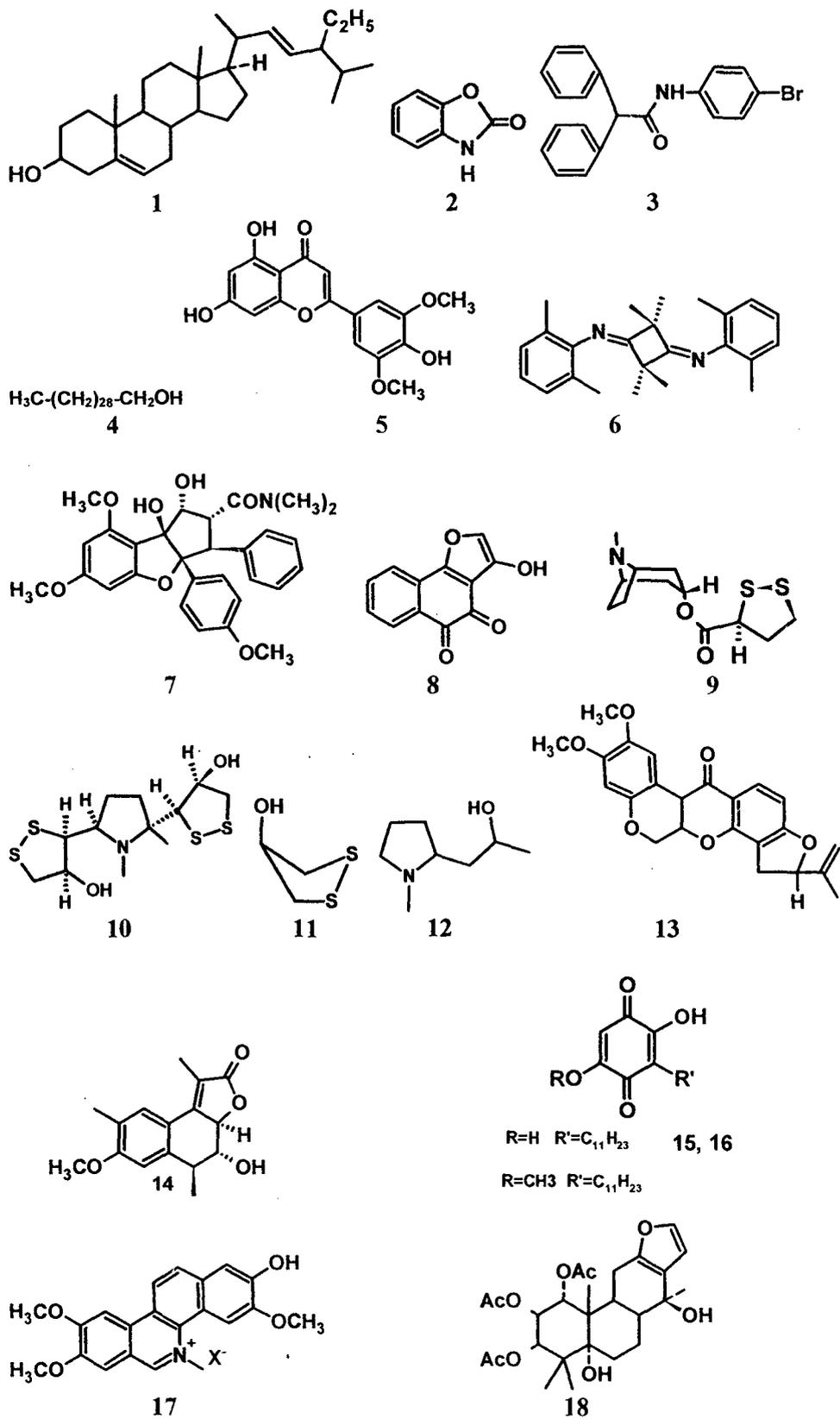
SECTION-I- Antibacterial flavonoids identified from *Lumnitzera racemosa* are described. This section has been divided into two parts:

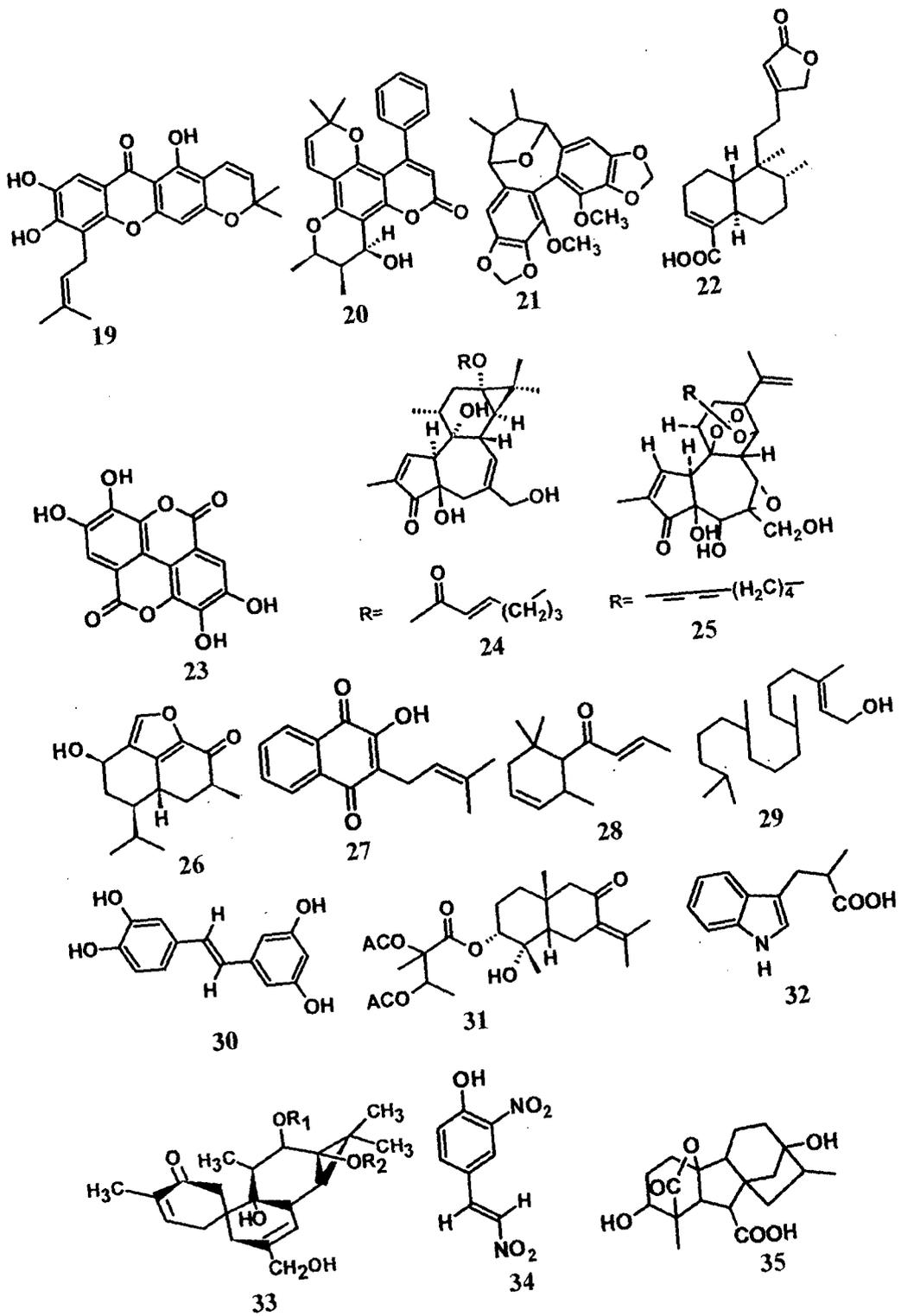
Part-1: Antimicrobial activity of the tonga mangrove, *Lumnitzera racemosa*.

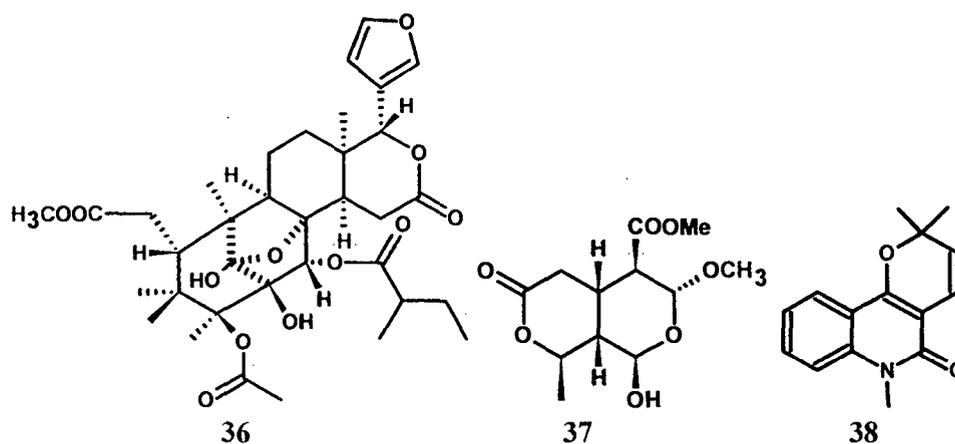
Part-2: Chemical investigation of the active n-butanol fraction by Tandem mass spectrometry.

SECTION-II- Deals with the antimicrobial and CNS depressant properties of *Aegiceras corniculatum*.

SECTION-III-Chemical investigation on *Sesuvium portulacastrum* exhibiting oxytocic activity has been discussed.







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Introduction:

Infectious diseases are still a major scourge of human life, and recent emergence of multidrug resistance to antibiotics by bacteria due to genetic mutation or gene acquisition has left physicians with little recourse for the treatment of what were once routine infections. Current threats posed by *Staphylococcus aureus* are disturbing, with deaths associated with infection by methicillin-resistant *S. aureus*¹ and the increased prevalence of drug-resistant *S. pneumoniae* in community-acquired pneumonia². While attention commonly focuses on resistance to antibacterial agents, resistance to antifungal³, antiparasitic⁴ and antiviral drugs⁵ is also on the rise. This has created an urgent need for the rapid and continued development of new antimicrobial agents to replace the current regimens.

During the general screening of the extracts for biological activity the crude methanolic extract of *Lumnitzera racemosa* Willd exhibited considerable activity against antibacterial infection. Fractionation of the crude extract, by partitioning into solvents of increasing polarity i.e. successively with petroleum-ether, chloroform, n-butanol and the residue is considered as water-soluble. Testing of these fractions located the activity in n-butanol soluble fraction.

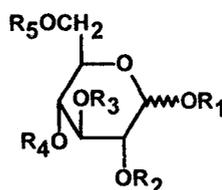
This section has been further divided into two subsections:

The first one relates to the antibacterial activity observed in methanolic extracts of this mangrove, and the bioassay guided identification of the antibacterial principle from the n-butanol fraction of the methanolic extract of the plant *Lumnitzera racemosa* Willd.

The second subsection deals with the flavonoids identified from the active n-butanol fraction by ESI-MS/MS.

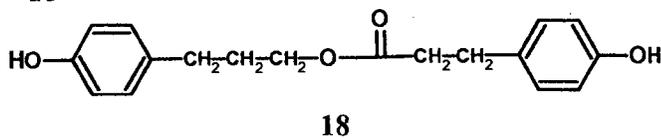
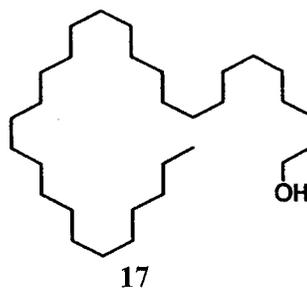
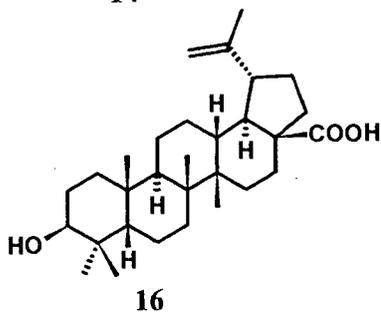
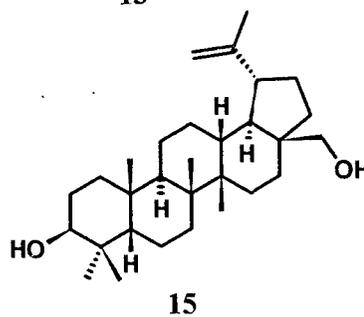
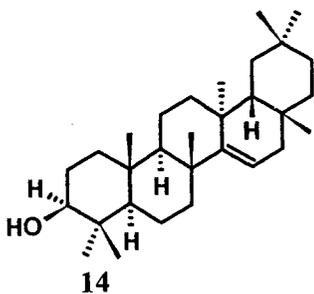
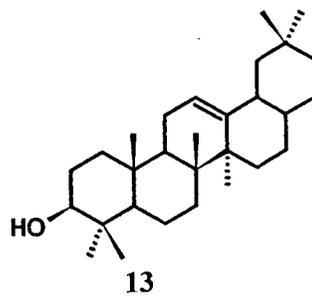
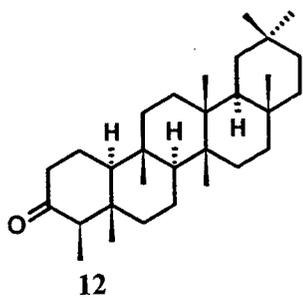
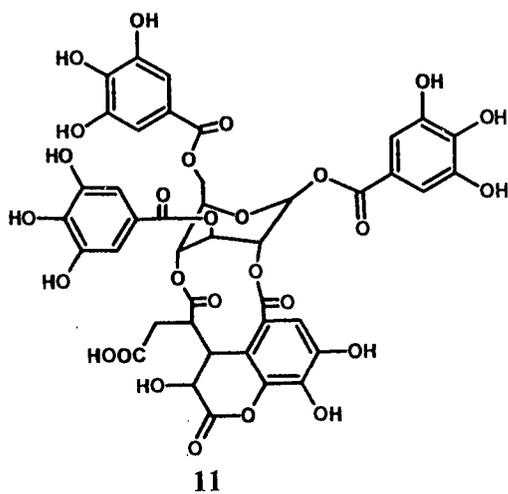
2.1.1 Antimicrobial activity of the tonga mangrove, *Lumnitzera racemosa* (Willd):

The plants of *Lumnitzera racemosa* Willd , a mangrove belonging to family *Combretaceae* are large glabrous shrubs, growing on the salt marshes along with mangroves on the coast of India and on the Andaman and Nicobar Islands. The wood of *L.racemosa* is used as a fuel for its calorific value and the leaves of the plant are eaten in South Pacific Island during periods of scarcity. The reddish brown bark contains 15-19% tannin while the leaves and wood contain smaller quantities. A fluid obtained from incisions made in the stem was reported to be employed as an external application for the treatment of herpes and itches⁶. According to the folk medicine, the fruits of this plant are curative in skin disorders. Antihypertensive activity has been reported for the aqueous acetone extract of the plant⁷. The blood pressure lowering activity was due to the presence of hydrolysable tannins.(1-11)²². Chemical examination of this plant occurring in various parts of the world was reported to give a large number of compounds, long chain rubber like polyisoprenoid alcohols in leaves⁸, flavonoids and long chain fatty acids⁹ and low molecular weight carbohydrates¹⁰ . Chemical examination of Indian species was reported to give friedlin (12), β-amyrin (13), taraxerol (14), betulin (5), betulinic acid (16) and tricontanol (17)¹¹ besides a new aromatic ester (18)¹² and an enzyme cinnamyl alcohol-NADPH- dehydrogenase¹³. The presence of trace elements is also reported¹⁴.



G =galloyl. HHDP = hexahydroxydiphenoyl. CHEB = chebuloyl.
 GALA = gallayl. NECH = neochebuloyl

	R1	R2	R4	R3	R5		R1	R2	R3	R4	R5
1	G	H	H	HHDP		9	H	HHDP	H	H	
2	G	CHEB		HHDP		10	H	HHDP		GALA	
3	G	CHEB		G	G						
4	G	NECH		G	G						
5	H	G	H	G	H						
6	G	G	H	G	G						
7	H	G	G	G	G						
8	G	G	G	G	G						



Results:

Crude methanolic extract of the tonga mangrove (*Lumnitzera racemosa*) was screened *in vitro* against fungi (*Candida albicans*, *Aspergillus fumigatus*, *Mucor sp.*), virus (*Hepatitis B*) and pathogenic gram-positive (*Staphylococcus aureus*) as well as gram-negative bacteria (*Salmonella typhi*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Proteus mirabilis*, *Vibrio cholerae*). The extract and the fractions were ineffective against fungi and the *Hepatitis B* virus tested even at 500 µg/ml concentration, but the crude methanolic extract and the n-butanol fraction were bactericidal with an inhibition zone greater than 10mm at 50µg/ml against all the pathogenic bacteria tested (**Table-1**). The active n-butanol fraction on bioassay-guided fractionation and chromatography over sephadex LH20 with methanol yielded two yellow compounds identified as flavonoids, quercetin (**1**), M^+ 302 and myricetin (**2**), M^+ 318, on the basis of spectral data (**Table-2**) and these data compared well with the literature values¹⁵.

Compound **1** was obtained as yellow amorphous powder with an elemental composition of $C_{15}H_{10}O_7$ as evidenced by its electron impact mass spectra (EI-MS, **Fig-2.1.1**), which showed molecular weight of 302. The infrared spectrum (**Fig-2.1.2**) indicated the presence of hydroxyl group (3294.2 cm^{-1}) and α,β unsaturated carbonyl groups (1668.3 cm^{-1}). Aromaticity was evident from the absorption bands at $1614.3, 1517.9\text{ cm}^{-1}$. Its nuclear magnetic resonance (NMR) data was characteristic of flavonoids with ^1H NMR (**Fig-2.1.3**)(**Table-2**) showing two meta coupled protons, as doublets at δ 6.183 ($J=2.1\text{ Hz}$) and 6.38 ($J=2.1\text{ Hz}$) ascribed to H-6 and H-8 and are indicative of 5,7 disubstitution in ring A. The aromatic protons on the ring B of **1** showed an ortho coupling doublet δ 6.88 ($J=8.7\text{ Hz}$), a meta coupling doublet at δ 7.73 ($J=2.1\text{ Hz}$) and a double doublet at δ 7.64dd ($J= 2.1, 8.4\text{ Hz}$) assigned to H-5', H-2' and H-6' respectively.

able-1. Antibacterial activity of the methanolic extract, fractions, and flavonoids.

	Concentration($\mu\text{g/ml}$)	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. mirabilis</i>	<i>P.aeruginosa</i>	<i>S. typhi</i>	<i>S. fle.</i>	<i>S. aureus</i>	<i>V. cholerae</i>
Extract	500	+++	+++	+++	+++	+++	+	+++	+++
Petroleum ether	50	-	-	-	-	-	-	-	-
Chloroform	50	-	-	-	-	-	-	-	-
n-butanol	50	+++	+++	++	+++	+++	+	++	+++
Quercetin	30	-	-	-	++++	-	++	+++	-
Myricetin	30	+++	++++	++++	++++	-	++	+++	-
(Quercetin+ Myricetin) 1:1	30	-	-	+++	-	-	+	+++	-

-) No zone of inhibition (inactive); (++) 2-3mm zone of inhibition (moderately active); (+++) 3-5mm zone of i
++++) 5-7mm zone of inhibition (strongly active).

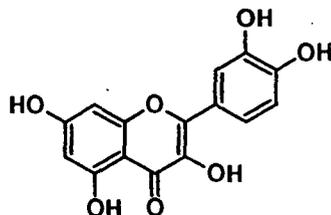
bioassays that were carried out on the extract against the fungi and virus below showed no inhibitory activity.

[*Aspergillus fumigatus*. J *Mucor* sp. K *Candida albicans*.

[*Hepatitis B virus*

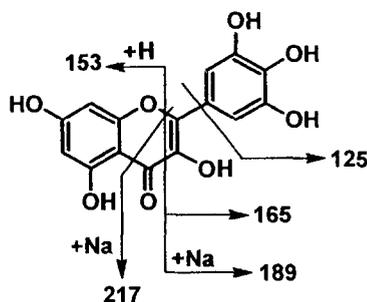
tion (significantly active);

The compound also exhibited singlets at δ 13.4, 13.6, 14.6 and 14.9 due to D₂O exchangeable phenolic hydroxyl protons. This ¹H NMR data coupled with its ¹³C NMR values (Fig-2.1.4)(Table-2) identified compound 1 as quercetin, the data being well in agreement with the literature reports¹⁵.



Compound 1

Compound 2 with molecular formula of C₁₅H₁₀ O₈, as evidenced by ESI-MS (M⁺ 318) (Fig-2.1.5a), MS/MS (Fig-2.1.5b) had spectral characteristics similar to that of compound 1. The only difference observed was that the protons in tetrasubstituted ring B appeared as a two-proton singlet at δ 7.3 indicating their equivalence. This value was assigned to H-2' and H-6' of ring B. On the basis of spectral data observed and its comparison with the literature values the compound was identified as myricetin¹⁵. The structure of compound 2 was confirmed by its IR (Fig-2.1.6), ¹HNMR(Fig-2.1.7) and ¹³C (Fig-2.1.8) (Table-2). The fragmentation pattern observed in its MS/MS spectrum is well in agreement with the structure of myricetin (2).



Compound 2

It is evident from bioassay-guided fractionation of the active n-butanol fraction and identification of the active constituents that the observed activity was due to the presence of flavonoids identified as quercetin (1) and myricetin (2). Quercetin was effective only against three bacterial strains (*P. aeruginosa*, *S. flexineri* and

Section I

*Antibacterial flavonoids from Lumnitzera
racemosa*

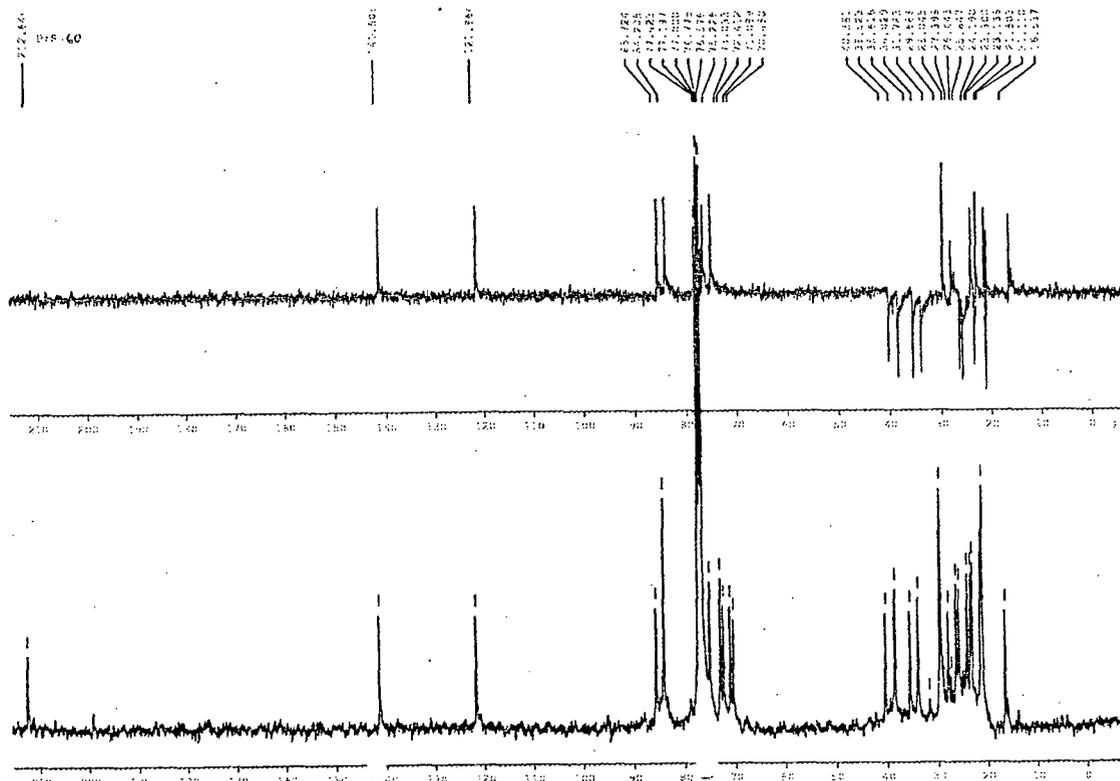


Fig 1.2.10: ^{13}C NMR of Compound (2).

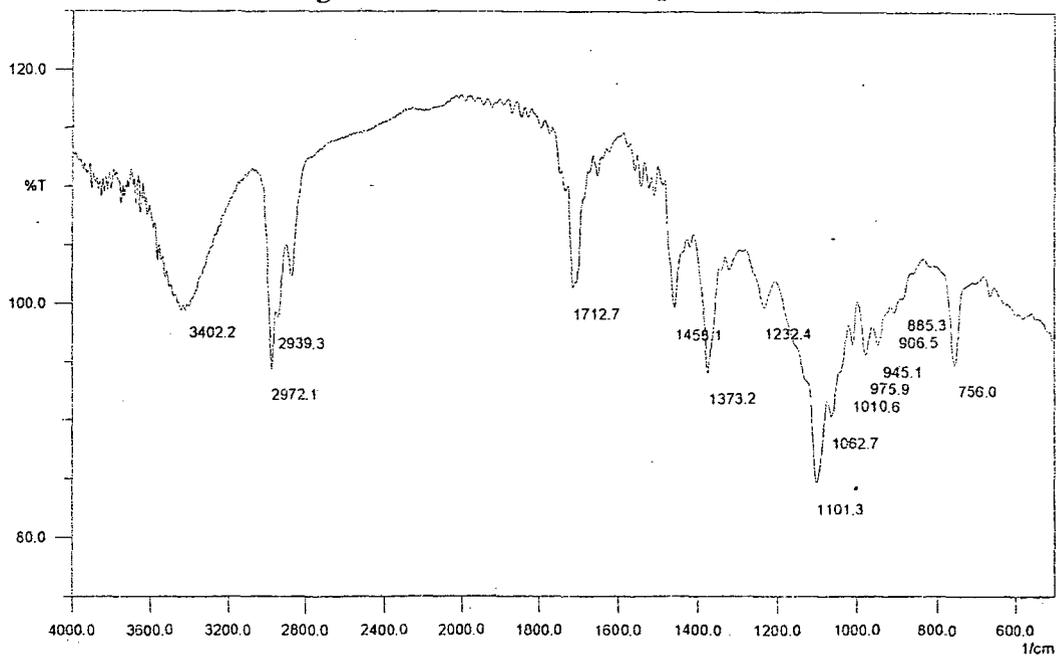


Fig 1.2.11: IR of Compound (2).

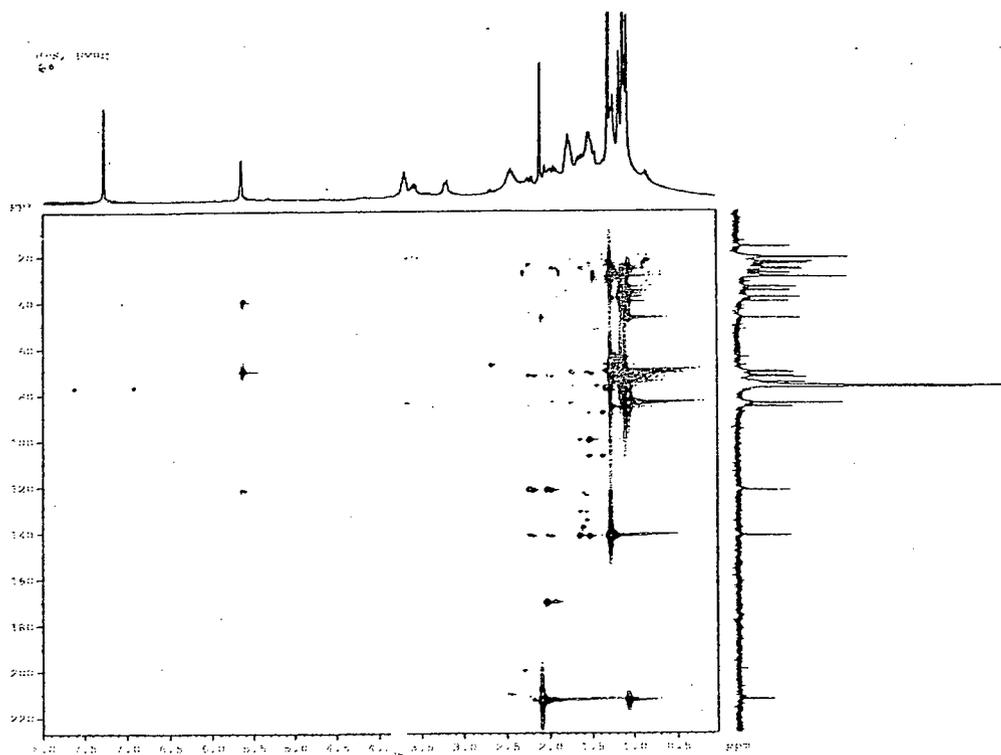


Fig 1.2.14: HMBC of Compound (2).

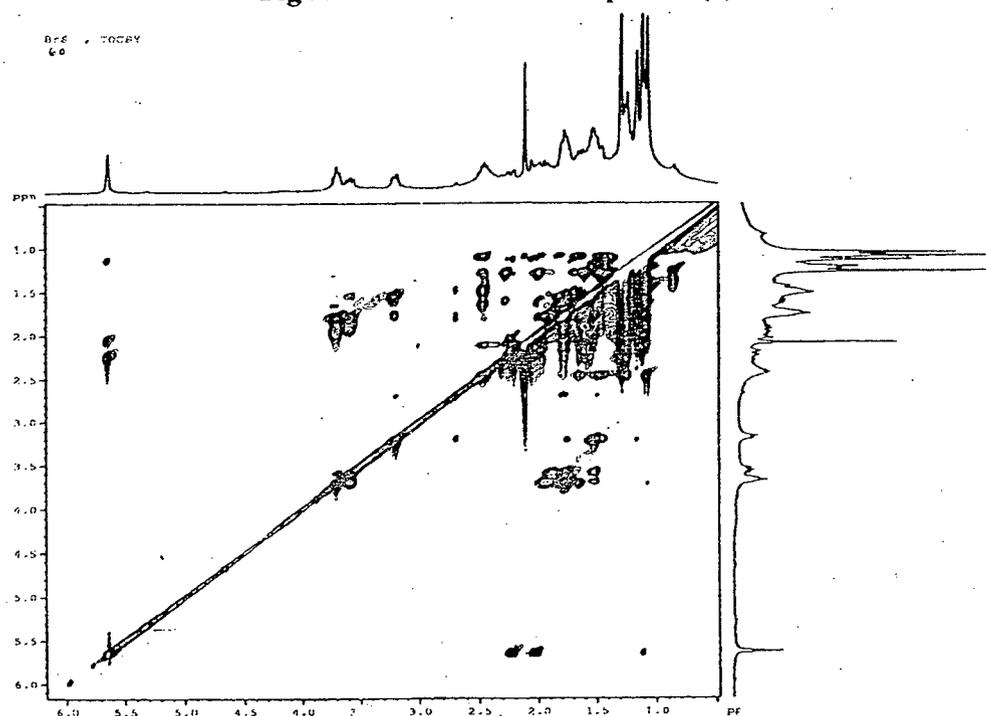


Fig 1.2.15: TOCSY of Compound (2).

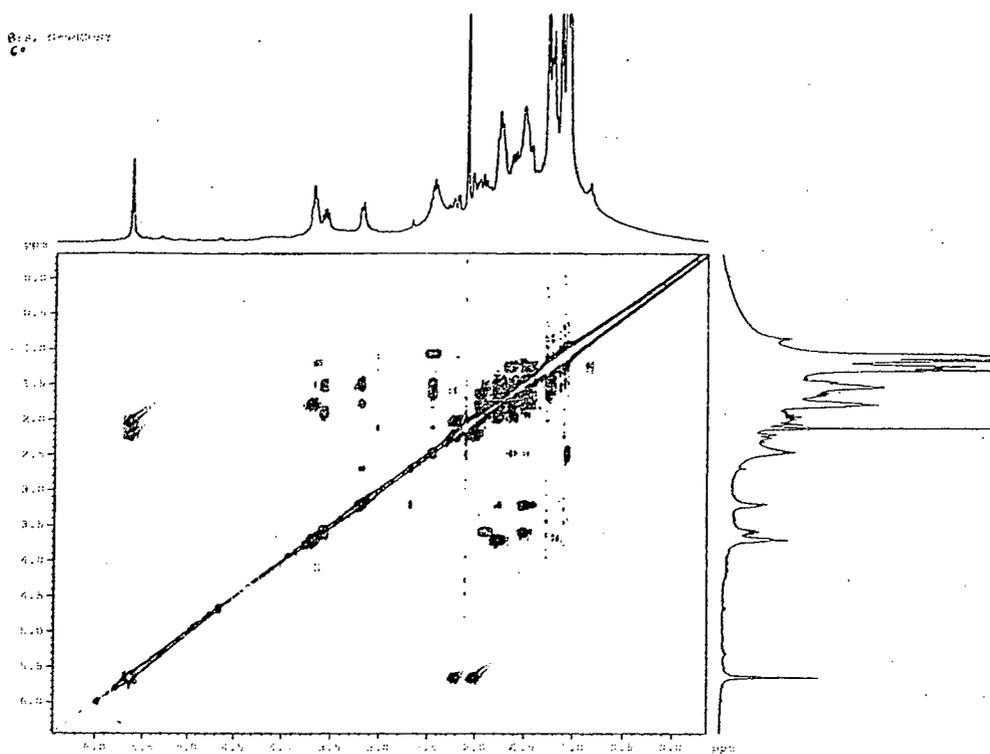


Fig 1.2.16: ^1H - ^1H COSY of Compound (2).

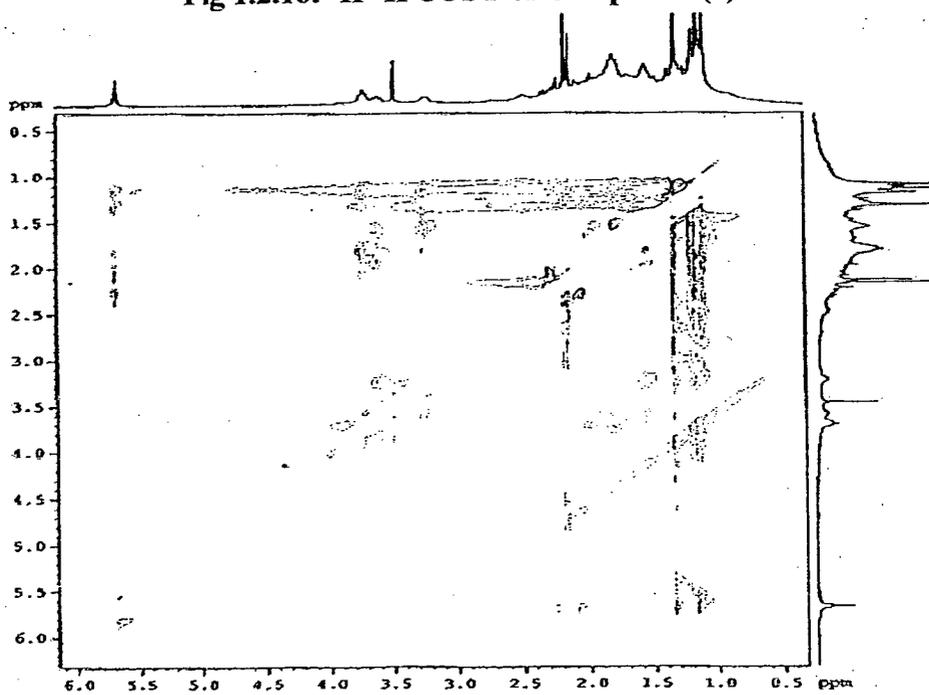


Fig 1.2.17: NOESY of Compound (2).

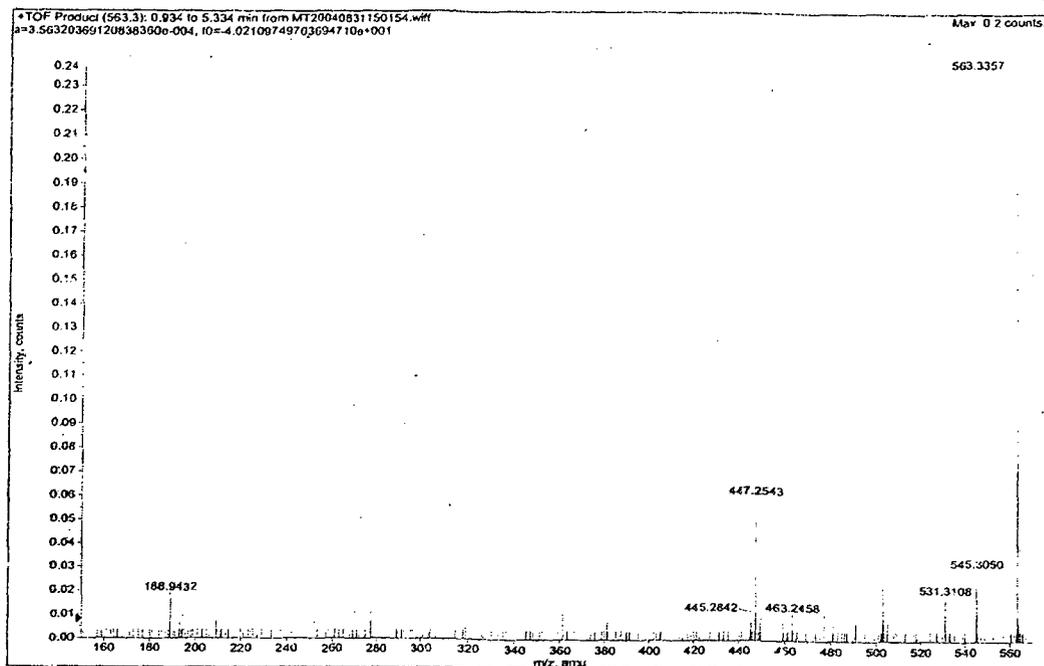


Fig 1.2.18: ESI-MS of Compound (2).

S.aureus) among eight tested at 30 µg/ml (MICs, 6µg/ml; Table 3). At the same concentration, myricetin was effective in inhibiting all of the gram-positive as well as gram-negative bacteria (MIC - 6µg/ml), with the strongest activity being observed against *P. aeruginosa* (MIC of 1.5µg/ml). *Salmonella typhi* and *V. cholerae* were not susceptible to myricetin.

Table- 2: NMR spectral data (300 MHz; DMSO) of Flavonoids.

Carbon No.	1		2	
	¹³ C	¹ H	¹³ C	¹ H
C-2	148.0	-	156.5	-
C-3	137.2	-	135.9	-
C-4	177.3	-	175.6	-
C-5	162.5	-	160.9	-
C-6	99.2	6.18(d, J=2.1Hz)	98.5	6.17(d, J = 2.1Hz)
C-7	165.5	-	164.0	-
C-8	94.4	6.38(d, J=2.1Hz)	93.6	6.37(d, J = 2.1Hz)
C-9	158.2	-	156.5	-
C-10	104.5	-	103.2	-
C-1'	121.6	-	121.7	-
C-2'	116.0	7.73(d, J=2.1Hz)	107.6	7.34(s)
C-3'	146.2	-	145.4	-
C-4'	148.7	-	135.3	-
C-5'	116.2	6.88(d, J=8.7Hz)	145.4	-
C-6'	124.1	7.52 (dd, J=8.4, 2.1Hz)	107.6	7.31(s)

Table 3. MIC (µg/ml) of flavonoids against one strain each of eight medically important bacteria.

Compound	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. mirabilis</i>	<i>P. aeruginosa</i>	<i>S. typhi</i>	<i>S. flexneri</i>	<i>S. aureus</i>	<i>V. cholerae</i>
Quercetin	-	-	-	6	-	6	6	-
Myricetin	6	6	6	1.5	-	6	6	-
Q + M.	-	-	6	-	-	6	6	-

Q (Quercetin) + M (Myricetin)

A strong inhibitory activity of myricetin against the most resistant organism, *P. aeruginosa* and methicillin-resistant organism, *S. aureus* have significant clinical implications. Based on the current findings, it can be concluded that this plant has antibacterial activity, which is being reported here for the first time.

Discussion

In this study, strong antibacterial activity by quercetin from *Lumnitzera racemosa* was observed only against the bacteria *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Shigella flexneri*, with MICs at 6 µg/ml. Quercetin is reported by Aziz et al.¹⁶ to inhibit microorganisms such as *Escherichia coli*, *Klebsiella pneumoniae*, *Bacillus cereus*, *Aspergillus parasiticus*, *Aspergillus flavus* at a concentration of 100-200 µg/ml. Sensitivity of *Staphylococcus aureus*, *Staphylococcus epidermis*, *Bacillus subtilis*, *Mucor luteus* and *Escherichia coli* to quercetin is also reported¹⁷ at higher concentrations (500 µg/ml). Surprisingly, no significant activity against *Pseudomonas aeruginosa* ATCC 9027 and *Escherichia coli* ATCC 8739 was noted by Rauha et al.¹⁷ even at 500 µg/ml, perhaps because the strain used (*P. aeruginosa* ATCC9027) was a genetically mutated, resistant strain in comparison to the strain used in the present investigation. Gatto et al.¹⁸ reported that quercetin and its 3-O-acyl derivatives did not exhibit significant activity against gram-positive strains of bacteria (*S. aureus*, *B. subtilis*, *Listeria ivanovi*, *Listeria monocytogenes*, *Listeria serligeri*) as well as gram-negative strains (*E. coli*, *S. flexneri*, *Shigella sonnei*, *Salmonella enteritidis*, *Salmonella tiphymurium*) and yeasts (*Candida albicans* and *Candida glabrata*) tested, and attributed the inactivity to the lower concentrations used for screening (100 µg/ml). In the present study, myricetin was found to be more inhibitory than quercetin, and was effective against six of eight bacterial species (one strain each) tested. *Salmonella typhi* and *Vibrio cholerae* were the two strains that showed resistance to myricetin. These data are supported by Puupponen et al.¹⁹, who found that myricetin inhibited growth of all lactic acid bacteria derived from gastrointestinal tract flora, but did not affect a *Salmonella*

enterica sv. *typhimurium* E-981151 strain. The flavonoid, myricetin is also known to inhibit other medically important, multidrug resistant bacteria in addition to methicillin-resistant *Staphylococcus aureus*, by inhibiting their ability to synthesize essential proteins²⁰.

To check whether mixing the two flavonoids could enhance the antibacterial activity, the mixture in 1:1 proportion was tried. It was observed that, quercetin and myricetin, in 1:1 combination had no effect on the MIC value (6 µg/ml) when *Shigella flexneri* and *Staphylococcus aureus* were used as the test bacteria. Quercetin alone did not show any activity against *Proteus mirabilis*, and its addition had no effect on MIC for myricetin. Although both flavonoids were strongly inhibitory to *Pseudomonas aeruginosa* (MIC for myricetin, 1.5 µg/ml), mixing rendered them inactive. In contrast, Arima et al²¹ reported enhancement of antibacterial activities of flavonoids by combining or mixing them, especially when quercitrin was mixed either with quercetin, morin or rutin. Better potency of myricetin as compared to quercetin seems to be associated with the additional phenolic hydroxyl in ring B of myricetin.

Conclusion

In the present investigation, extract from the tonga mangrove, *Lumnitzera racemosa*, as well as a n-butanol fraction inhibited eight species (one strain each) of medically important gram-positive and gram-negative bacteria tested. Two flavonoids were partly responsible for the observed activity. *L. racemosa*, a plant belonging to *Combretaceae*, has been reported to contain large amount of tannins castalgin, punicalin and punicalagin²². These tannins, and/or synergistic action of the constituents of flavonoids from the active fraction and other tannins present in the plant could be responsible for the activity against *Salmonella typhi* and *Vibrio cholerae*, which were resistant to the two flavonoids isolated and tested in this study. Tannins have antimicrobial activity although, paradoxically, some organisms (e.g. the fungus, *Candida* sp.) are capable of using tannins as a carbohydrate source^{23,24}.

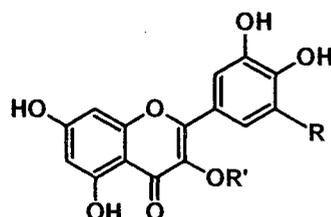
Tea extract in combination with β -lactam antibiotic is reported to have a synergistic effect with methicillin against methicillin-resistant *Staphylococcus aureus* (MRSA), due to the presence of catechin compounds and polyphenols including quercetin and myricetin²⁵. A pharmaceutical composition containing 0.412% quercetin derived from St John's wort was shown to be suitable for clinical or veterinary use²⁶. Commercially the above-mentioned composition is known as Novoimamine, and is effective against *S. aureus* infection²⁶. Quercetin is a better bactericidal agent against *S. aureus* as compared to conventionally used sulfanilamide. In this study, the extract and n-butanol fraction from *Lumnitzera racemosa* showed broad-spectrum antibacterial activity against all the pathogenic bacterial strains tested, suggesting that this mangrove species has similar pharmacological value.

2.1.2: Chemical investigation of the active n-butanol fraction by Tandem mass spectrometry:

1. Characterization of flavonol glycosides from *L. racemosa*:

The active n-butanol fraction from the methanol extract of leaves of *L. racemosa* was analyzed by ESI-MS and Tandem mass spectrometry. It showed mass spectral characteristics of flavonoids, flavonoid glycosides, and biflavonoids. (Table 4) lists their molecular masses and fragmentation observed.

First order ESI-MS(Fig.2.1.9a,b) spectrum of the flavonoid rich fraction indicated that quercitrin (quercetin-3-O- α -L-rhamnopyranoside) (3) is a major constituent of the fraction followed by quercetin 3-O-hexoside (4).



Compound 3 : R = H; R' = Rha.

(Fig 2.1.10) represent the MS/MS spectrum of a protonated flavonol glycoside $[M+H]^+$ at m/z 471. It was identified as quercitrin (3). As evident from the spectrum compound (3) loses its terminal rhamnose unit to yield sodiated fragment at m/z 325 and also to produce a product ion at m/z 301 followed by Retro Diels-Alder (RDA) fragmentation $^{1,3}A$ to give ion at m/z 151.

RDA reaction of flavonoids is an important fragmentation reaction, which may occur in six membered cyclic structures containing a double bond and involves the relocation of three pairs of electrons in the cyclic ring. The net result of these rearrangements is the cleavage of two-bonds and the formation of two-bonds, for example, cyclohexene will fragment into butadiene and ethylene. Two (complementary) fragments, $^{1,3}A^+$ and $^{1,3}B^+$, are formed and charge retention can occur on either side of the cleavages, as depicted in Fig. A

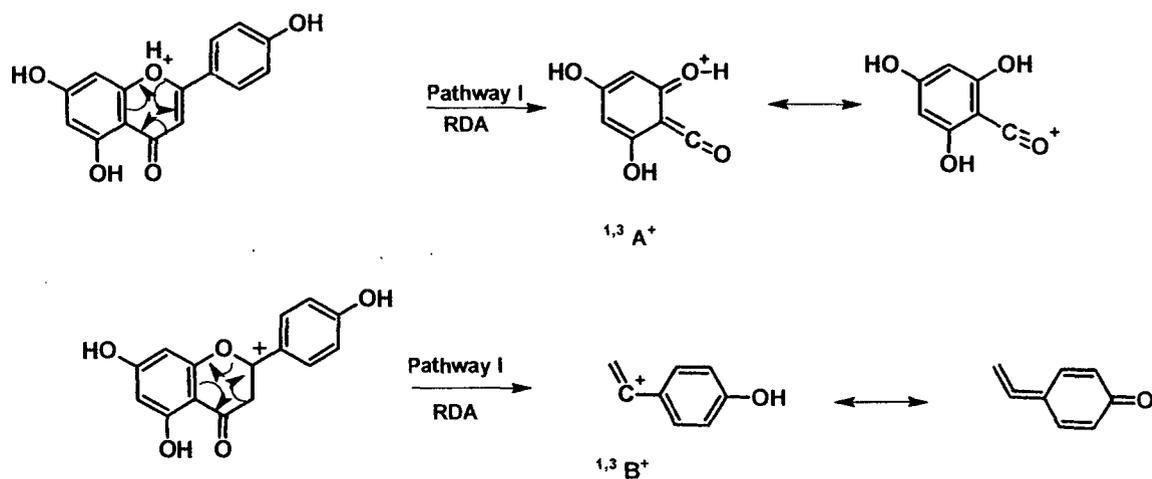
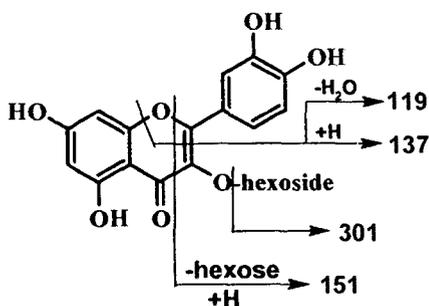


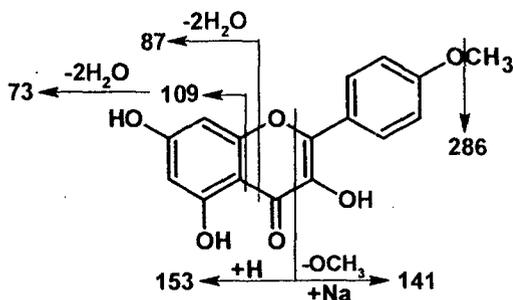
Fig. A

The MS/MS spectrum (Fig 2.1.11) $[M + Na]^+$ peak at m/z 487 / $[M+H]^+$ at m/z 465 was attributed to the flavonoid, Quercetin-3O-hexoside (glucoside/galactoside) (4). The fragmentation observed could be explained as shown below. The loss of hexose moiety from the molecular ion yields a fragment at m/z 301, which corresponds to quercetin aglycone suggesting that this compound is either quercetin glucoside or galactoside.

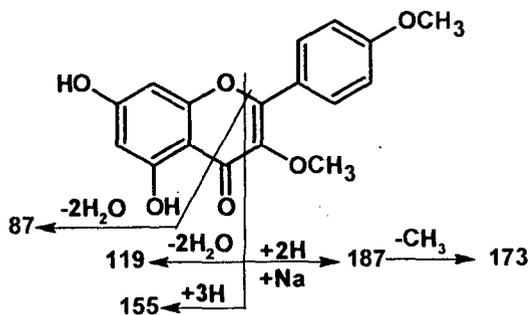


Compound 4

The aglycones kaempferol 4'-methylether (5) M^+ 301.1345 (Fig.2.1.12), kaempferol 3,4'-dimethylether (6) M^+ 315.148 (Fig.2.1.13), have also been identified in the fraction solely on the basis of fragmentation pattern observed in their tandem mass spectra (Table-4) and comparison with the literature values^{34, 35}



Compound 5



Compound 6

Table-4: Tandem mass of Flavonoids

Compound	[M+Na] ⁺ / [M+H] ⁺	ESI-MS/MS (% base peak)- Major ions
Quercitrin(Quercetin-3-O-rhamnoside)[3].	471	325(100), 301(9.6), 119(40), 197(24), 165(17.6), 105(12), 151(8), 129(6.4)
Quercetin-3-O-hexoside. (glucoside/galactoside) [4]	487 465	119(100), 87(28), 105(20), 151(28), 137(5), 245(7), 301(7), 343(6). 119(100), 87(28), 151(28), 137(5), 455(15), 473(2), 301(7).
Kaempferol-3,4'-dimethylether [6]	315	87(100), 55(38), 73(25), 155(32), 119(5), 187(12.5), 173(6).
Kaempferol-4'methylether[5]	301	55(100), 286(15), 153(11), 141(25), 109(0.5), 73(15), 87(50).
Bi-isorhamnetin[7]	615	87(100), 301(50), 209(20), 187(10), 105(20).
Myricetin-7-O-methyl ether(3→8'')quercetin-3-O-rhamnoside[8]	763	119(100), 87(50), 105(44), 151(16), 195(22), 467(20), 617(12).

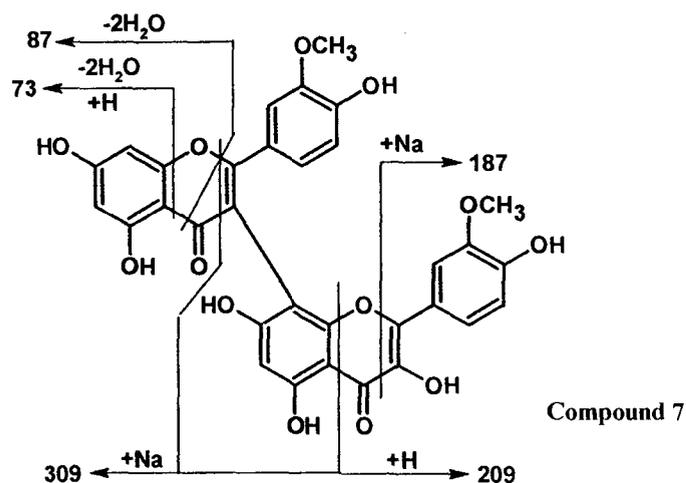
The product ion mass spectrum (Fig 2.1.12) of isosakurametin (5) (kaempferol 4' methyl ether) contained ions at m/z 286 corresponding to the fragments [M + H – CH₃]. The RDA fragmentation products wherein bonds 1 and 3 undergo scission leading to the formation of the ^{1,3}A ion at m/z 153 and the sodiated ion ^{1,3}B at m/z 141 with simultaneous elimination of –OCH₃ group were also evident.

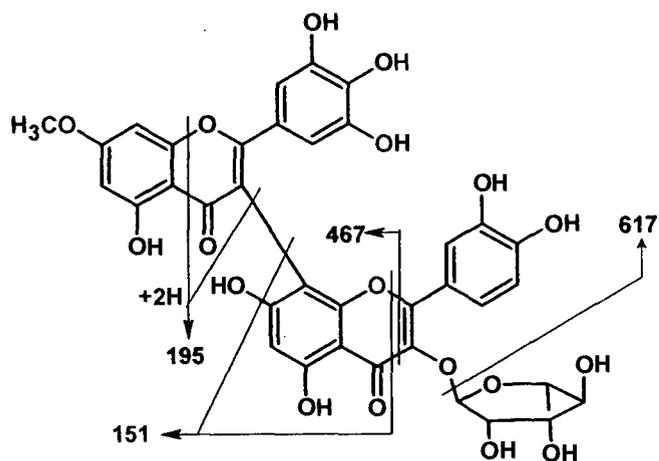
Kaempferol 3,4' dimethyl ether (6) exhibited fragmentation (Fig 2.1.13) with the RDA fragmentation producing ions at ^{1,3}A m/z 155 and ^{1,3}B sodiated ion at m/z 187 which produces ion at m/z 173 with the loss of –CH₂ group. Cleavage ^{1,2}B led to the formation of ion at m/z 119. These product ion mass spectra were similar to those observed in other mass spectrometry studies of flavonoids and provided unequivocal identification of the relevant flavonols.

Quercetin besides being antibacterial is an effective antioxidant. Its glycosidic form should also be equally effective since in biological system glycosides undergo enzymatic hydrolysis to the corresponding aglycone³⁸. Quercetin is known to get metabolized to 3-O-methyl quercetin in intact rat lens. Interestingly, both the aglycone and its methyl ether are known to inhibit hydrogen peroxide induced sodium and calcium influx and lens opacification and thus play a role in the prevention of cataract formation³⁹.

2.Characterization of biflavonoids:

The majority of naturally occurring biflavonoids contain C-C linked monomers with ring A usually being involved in the inter flavonoid linkage^{37,38}. The combination so far found in nature are C-6→C8; C-3'→C6''; C8→C8''; C3'→C8'' and C-3→C8''. In the present investigation the tandem mass spectra of the flavonoid constituents of the active n-butanol fraction was also indicative of the presence of biflavonoids, bi-isoramnethin (7) (MS/MS spectrum (Fig 2.1.14)) 5,7,4'-trihydroxy-3'methoxy (3→8'')-3'',5'',7'',4'''-tetrahydroxy-3'''-methoxy flavone and myricetin-7-O-methylether (3→8'') quercetin-3-O-rhamnoside (8) (MS/MS spectrum (Fig 2.1.15)) 5,3',4',5'-tetrahydroxy-7-methoxy flavone (3→8'')-5'',3''',4'''-trihydroxy-3'''-O-rhamnoside in the active fraction, the fragmentation pattern observed being well in agreement with the structure assigned to these biflavonoids.





Compound 8

Biflavonoids are a series of naturally occurring compounds that include flavone-flavone, flavanone-flavone and flavanone-flavanone subunit linkages. More than 100 biflavonoids have been identified from plants since the isolation of ginetin in 1929⁴⁰. A variety of biological activities for biflavonoids have been published including anti inflammatory, antimicrobial, anti-oxidants and others^{41,42}. Biflavonoids *Rheedia gardneriana* volkensiflavone, fukugetin, fukugiside are antibacterial against *E.coli*, *P.aeruginosa*, *S aureus* and *B. cerrus* with MIC ranging from 0.15-1.0mgml⁻¹ concentrations⁴³. Antimicrobial biflavonoids from the aerial parts of *Ouratea sulcata* are reported active against *S aureus*, *B subtilis*, *V anguillarum* and *E coli* at MIC ranging from 0.85-12.5 µg/ml it being almost as effective as the standard streptomycin used. They were inactive against *E.coli*. *L racemosa*, a plant belonging to *Combretaceae*, has been reported to contain large amount of tannins castalgin, punicalin and punicalagin besides corilagin²². In the present investigation, we have identified flavonoids and biflavonoids from the active n-butanol fraction and the antimicrobial activity observed in this fraction could partly be due to the presence of the tannins. Corilagin is known to cause marked potentiation of β-lactams against methicillin-resistant *Staphylococcus aureus*⁴⁴.

EXPERIMENTAL:

Plant material:

The tonga mangrove, *L. racemosa* as authenticated by Dr T. G. Jagtap, National Institute of Oceanography, Goa was collected from Ratnagiri, Maharashtra, along the West coast of India. A voucher specimen is deposited at National Institute of Oceanography Herbarium, Dona Paula, Goa, India, bearing the number NIO/DOD/DIO-1466. For this study, leaves and stems of 2 small plants (2.5Kg wet weight) were cut into small pieces. Extracts were prepared by percolation with 90% aqueous methanol²⁷ that yielded 11.2gms of the crude extract on removal of aqueous methanol. Fractionation of the crude methanolic extract was done by partitioning successively with crude extract:petroleum ether (1:2, v/v, thrice), chloroform (1:2, v/v, thrice), n-butanol (1:1, v/v, thrice), and the insoluble aqueous residue and thus the petroleum ether, chloroform, n-butanol and aqueous fractions were obtained.

Microorganisms:

The microorganisms used in this study were one strain each of two moulds (*Aspergillus fumigatus* and *Mucor* sp.), a yeast (*Candida albicans*), a virus (*Hepatitis B*), and eight bacterial species: one gram-positive bacterial strain (*Staphylococcus aureus*), and seven gram-negative strains (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Staphylococcus typhi*, *Shigella flexneri* and *Vibrio cholerae*). All microorganisms were clinical isolates obtained from the stock cultures maintained in the microbiology laboratory of Goa Medical College, Goa, India.

Antibacterial and antifungal assays:

The active crude extract and fractions were subjected to bioassay-guided fractionation and the flavonoids to quantitative bioassays for the determination of the minimum inhibition concentration (MIC), defined as the lowest concentration

of the compounds showing no visible microbial growth after incubation time²⁸. Initially, antimicrobial spectrum of the extract was determined by the Agar well diffusion method²⁸. One loop full of a 24-hr-old culture containing 10^4 - 10^6 cells²⁹ was spread on the surface of the Mueller-Hinton agar plates. Wells 5-7mm in diameter were dug in the medium using a sterile borer, and were filled with a 500 μ g/ml concentration of the *L. racemosa* crude extract. Zones of inhibition were measured after overnight incubation, and expressed as the diameter of zone in millimeters. Crude extracts with an inhibition zone greater than 10mm were selected for further investigations. Bioassays of the fractions and active constituents with concentrations are given in **Tables 1** and **2**. Ampicillin and nystatin were used as antibacterial and antifungal controls.

The MIC was determined by tube dilution method³⁰, using Mueller-Hinton broth. Tubes that had been inoculated with bacterial cultures (10^4 - 10^6 cells/tube) which were found to be sensitive to the testing material were incubated overnight at a temperature of 37°C. Graded dilution of the testing material, which can inhibit growth of these organisms, was assessed.

Antiviral assay:

Since the *Hepatitis B* virus cannot be grown on tissue cultures³¹ human plasma positive for hepatitis B surface antigen (HbsAg) was used as the virus source and the extract at 500 μ g /ml concentration was mixed with *hepatitis B* surface antigen (HbsAg) positive plasma. This mixture was retested for *hepatitis B* surface antigen (HbsAg) by enzyme-linked immunosorbent assays (ELISAs)³² after regular intervals of incubation (2hrs) with suitable controls (antigen-enzyme II from Abbott Laboratories). Negative antigen activity indicates promising anti HBV property.

Isolation and purification of active constituents:

The active n-butanol fraction (3.5g/300ml of butanol) was resuspended in methanol (100%) and loaded on to a column of Sephadex LH20, obtained from

Amersham Pharmacia Biotech Asia Pacific Ltd., Hong Kong, which was equilibrated with methanol³³. Bioassay-guided fractionation led to the isolation of two yellow solids, identified as quercetin (38mg/2.5Kg wet wt. of plant material) and myricetin (23mg/2.5Kg wet wt. of plant material) on the basis of spectral data (Table 3). The retardation factor [(Rf) - defined as the distance travelled by the compound divided by the distance travelled by the solvent] values on thin layer chromatography (TLC) silica gel F₂₅₄ plates obtained from E. Merck (Germany) developed in methanol:chloroform solvent (1:4 ratio) were 0.76 and 0.58, respectively, and in agreement with the standards obtained from Sigma-Aldrich Company.

ESI-QSTARXL MS/MS spectrometry:

Full scan positive ion ESI mass spectra were obtained for each of the flavonols by direct infusion of diluted methanol mixture. In addition to full scan mass spectra, collision induced dissociation was undertaken in the MS/MS mode to yield diagnostic product ion mass spectra, which were characteristic of the structural moieties present in the analyte. While infusing the mixture, the collision energy was varied from 20V-50V so as to obtain optimum product ion mass spectra

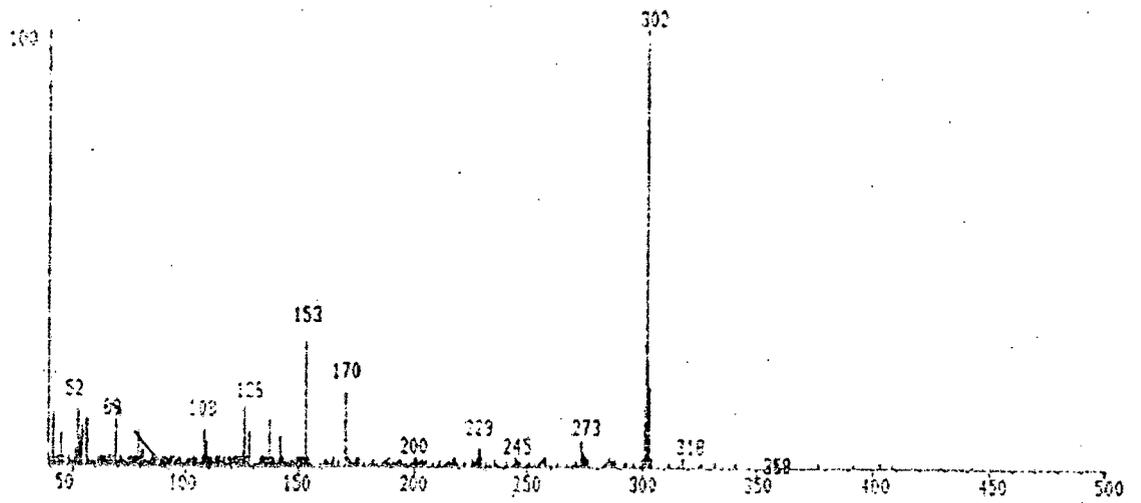


Fig 2.1.1: ESI-MS spectrum of compound 1

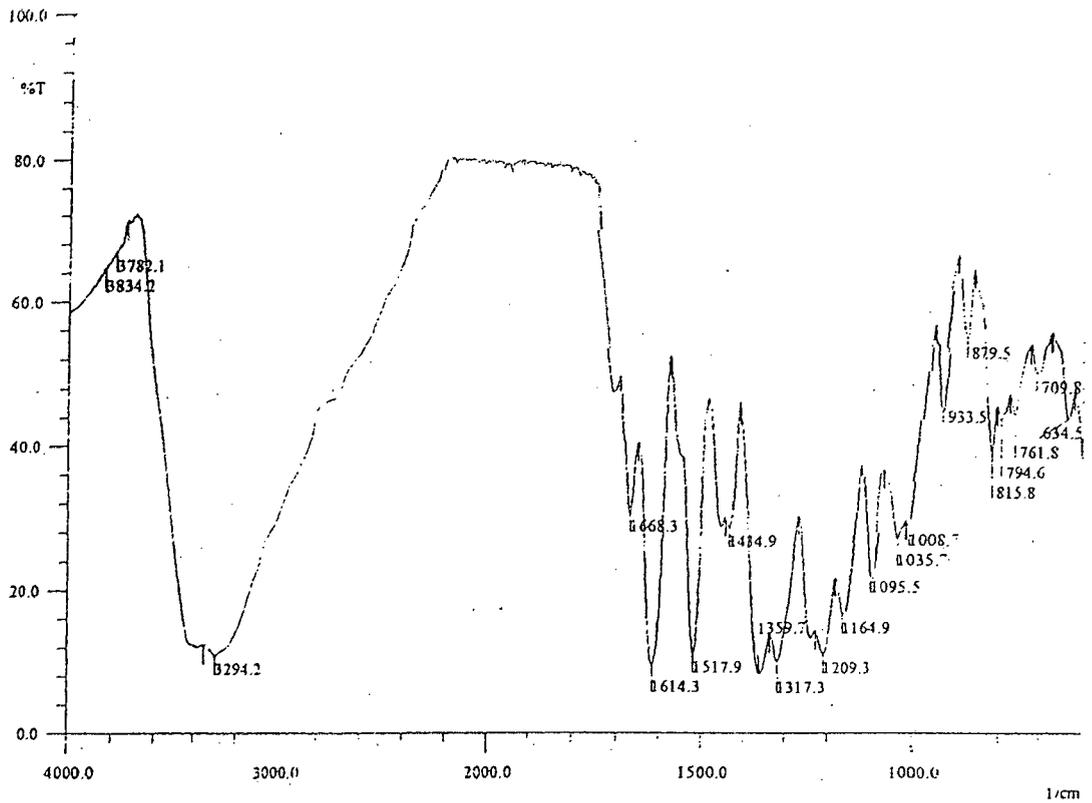


Fig 2.1.2: IR spectrum of compound 1

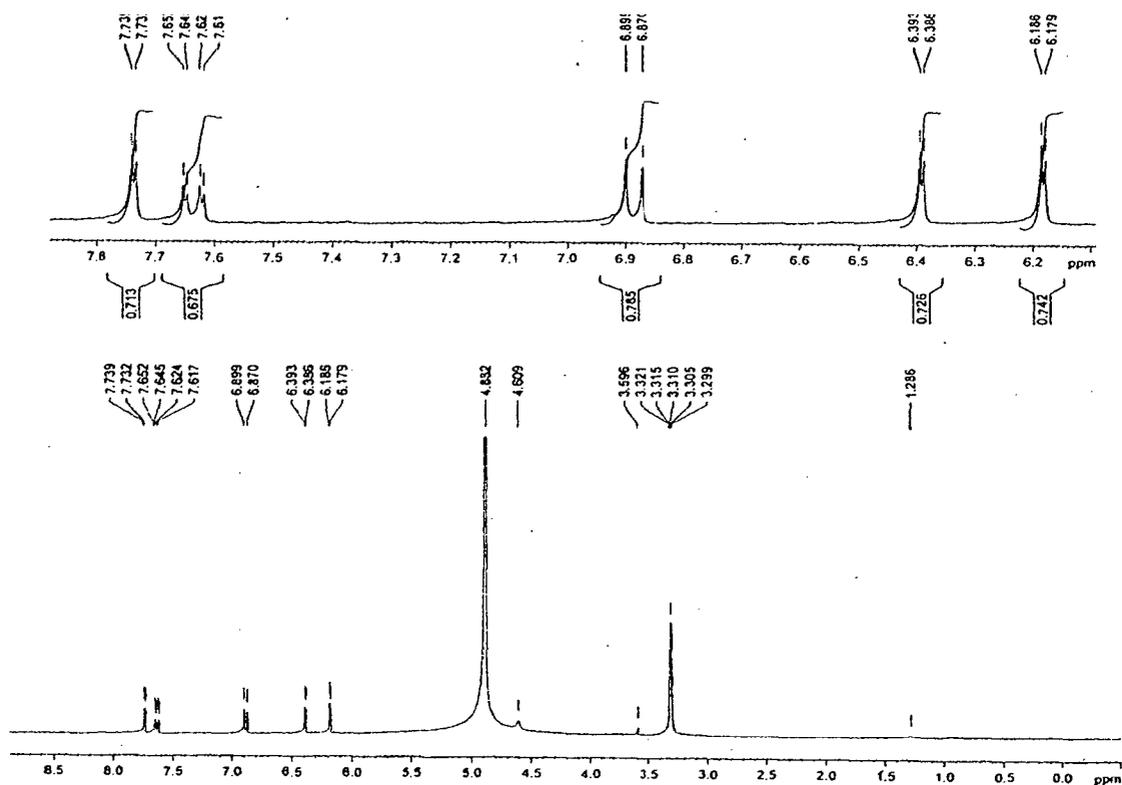


Fig 2.1.3: ¹H NMR of compound 1

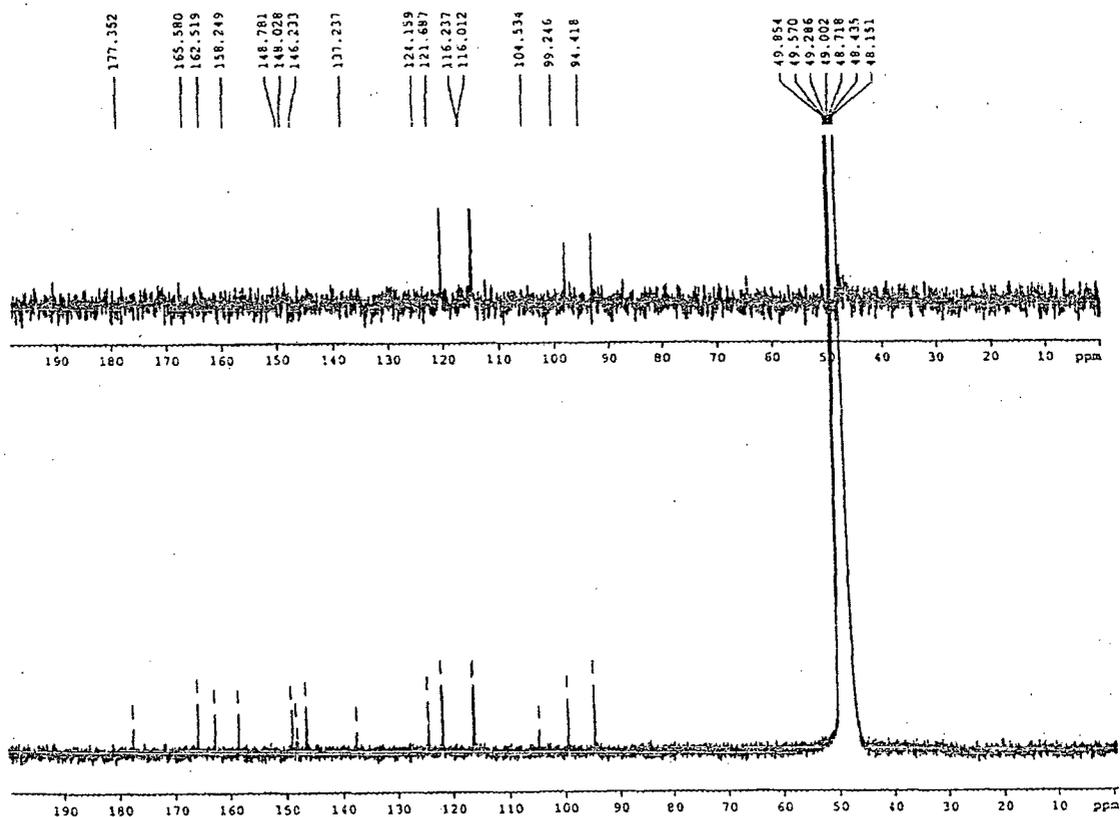


Fig 2.1.4: ¹³C NMR of compound 1

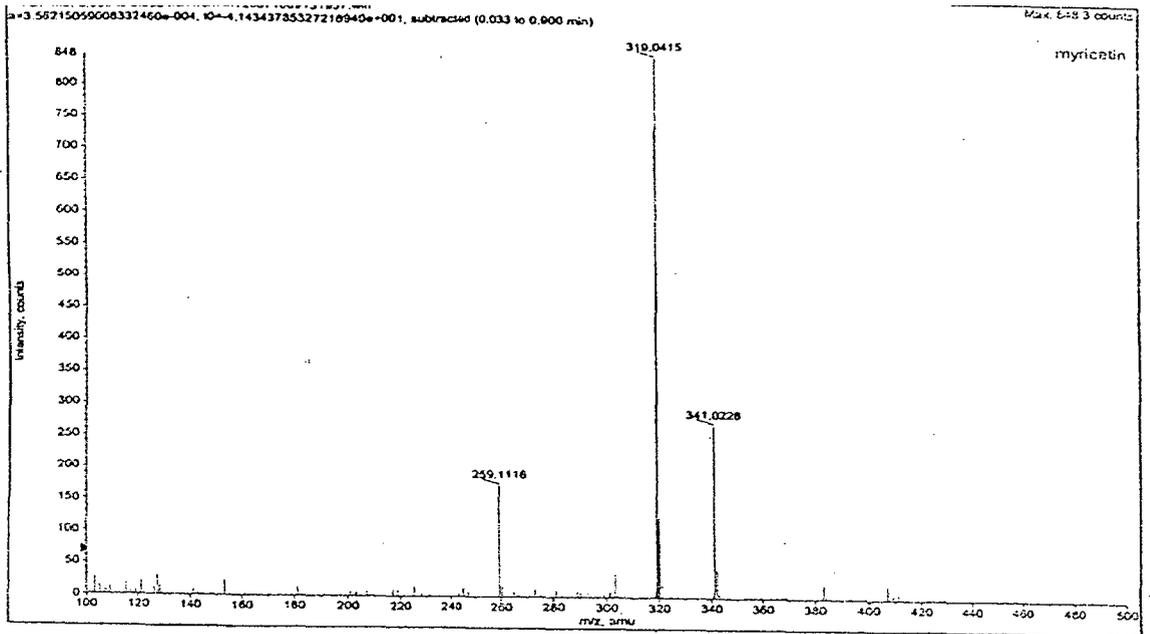


Fig 2.1.5a: ESI-MS spectrum of compound 2

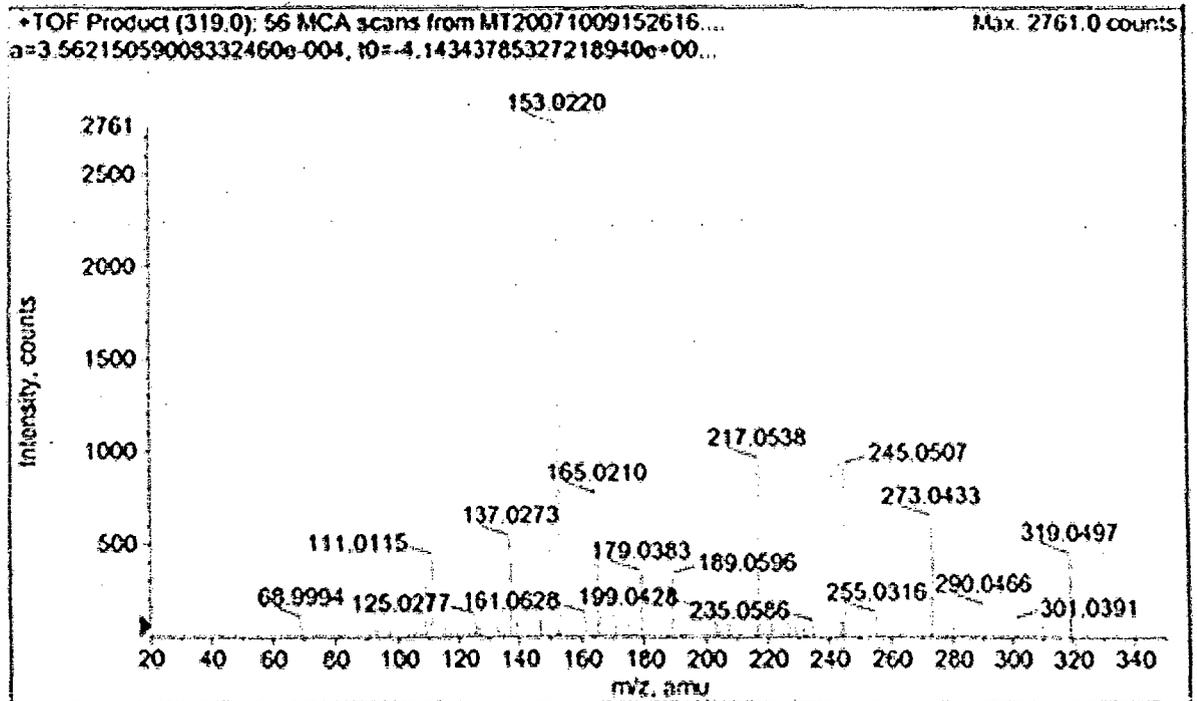


Fig 2.1.5b: MS/MS spectrum of compound 2

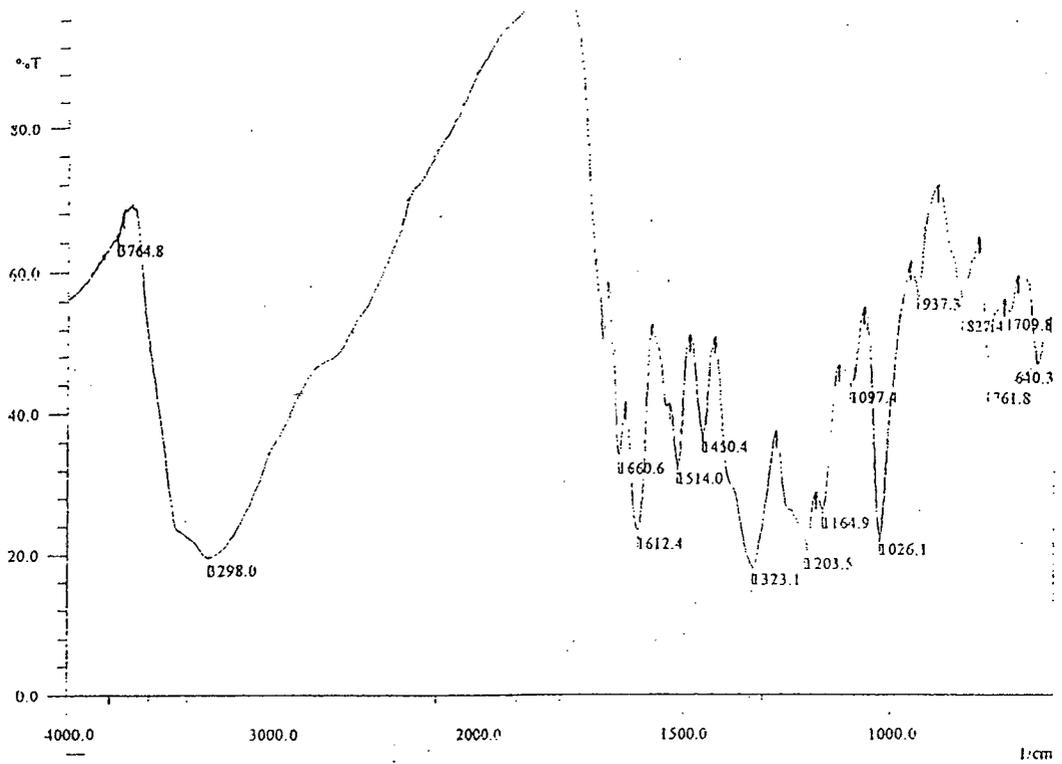


Fig 2.1.6: IR spectrum of compound 2

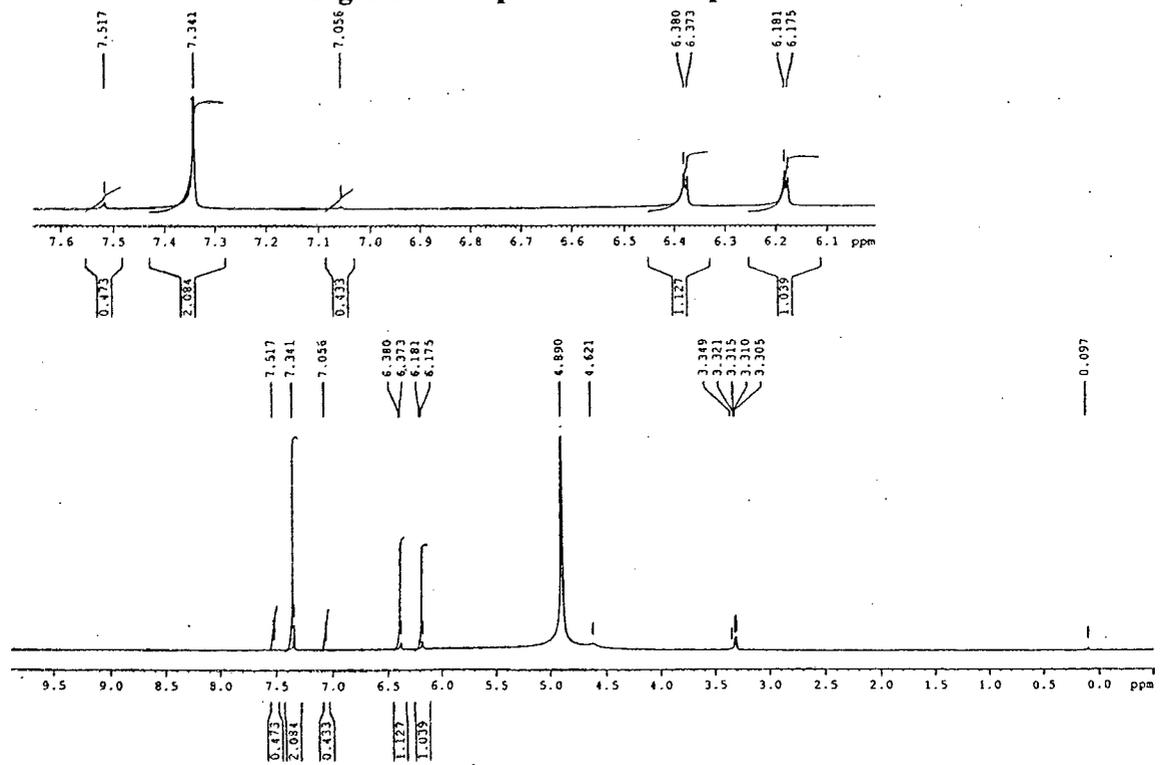


Fig 2.1.7: ¹H NMR of compound 2

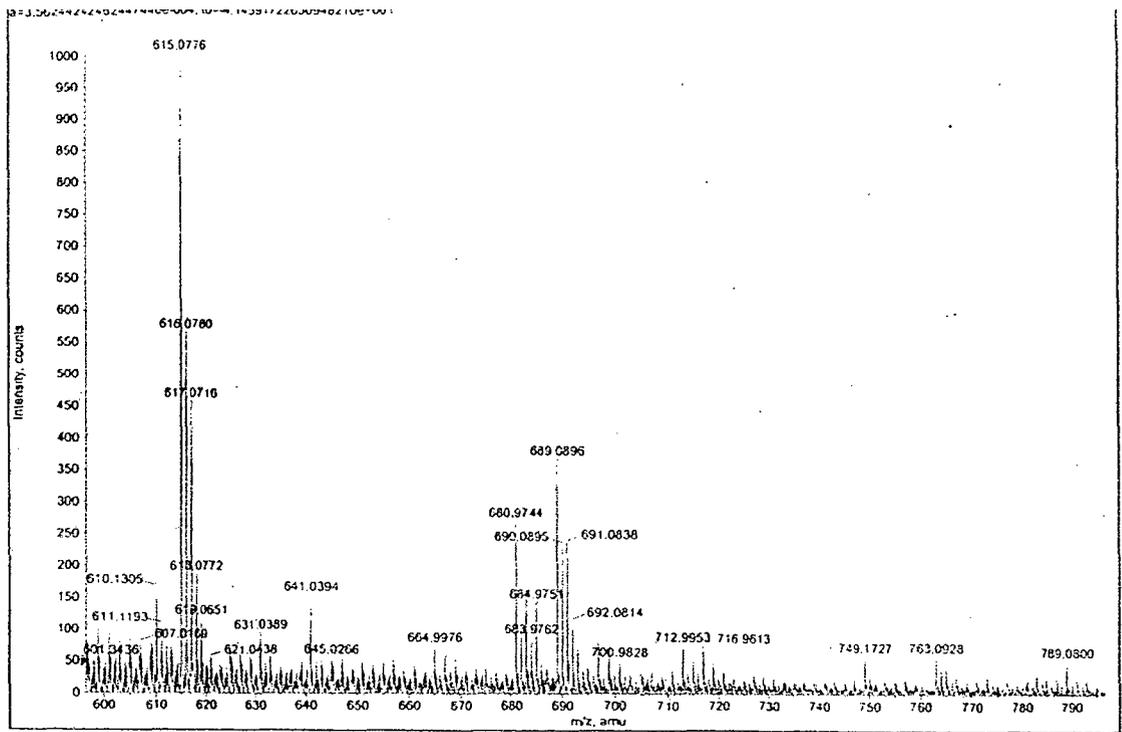


Fig 2.1.9b: ESI-MS spectrum of the flavonoid rich fraction

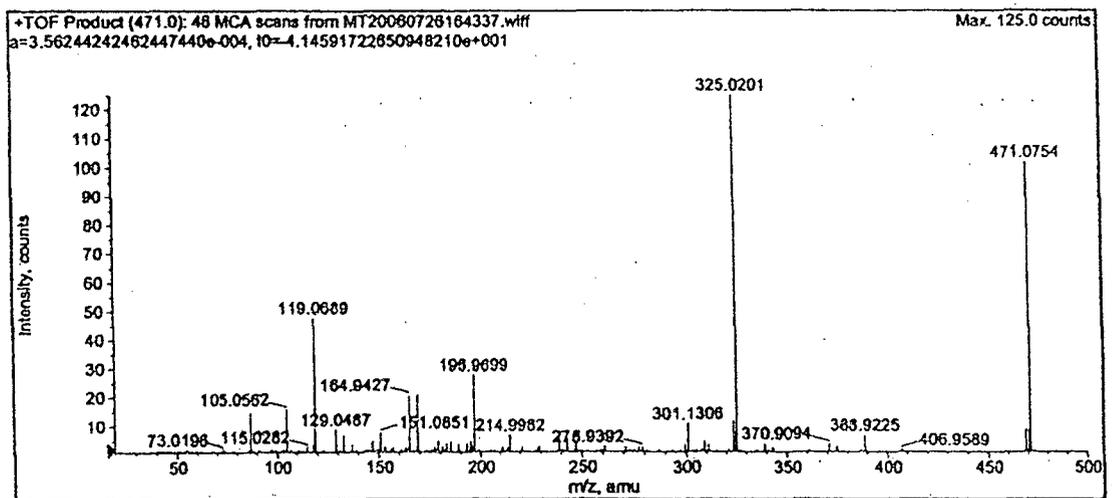


Fig 2.1.10: MS/MS spectrum of compound 3

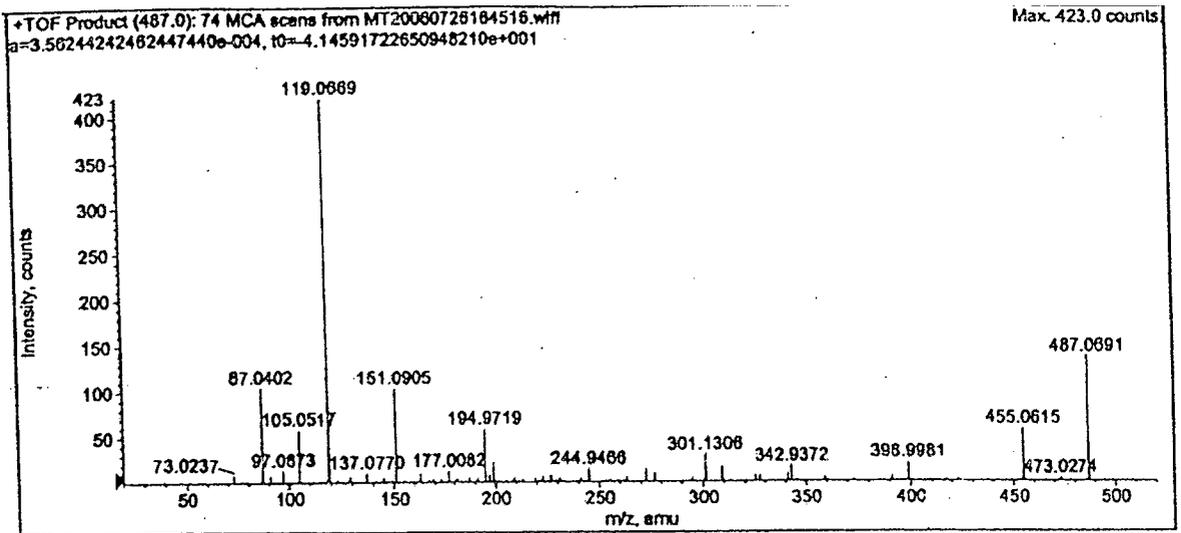


Fig 2.1.11: MS/MS spectrum of compound 4

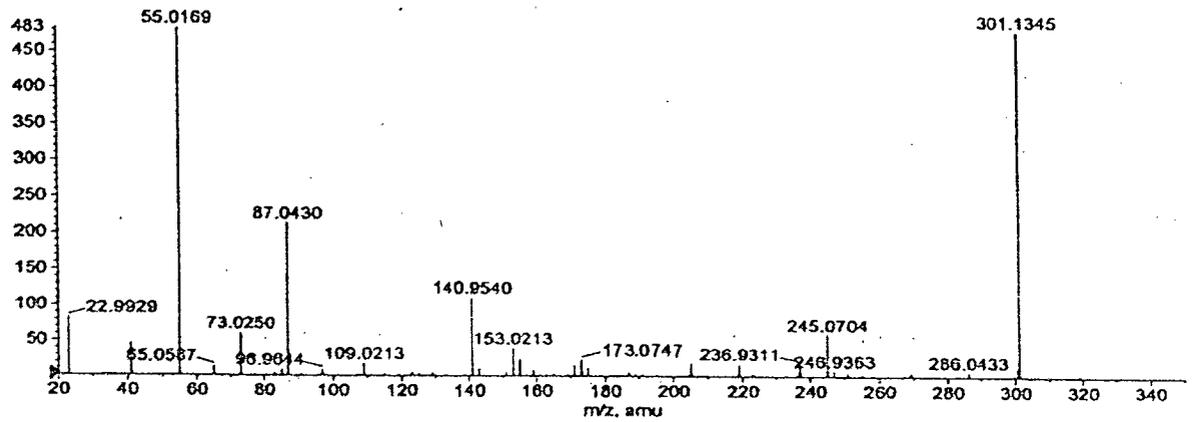


Fig 2.1.12: MS/MS spectrum of compound 5

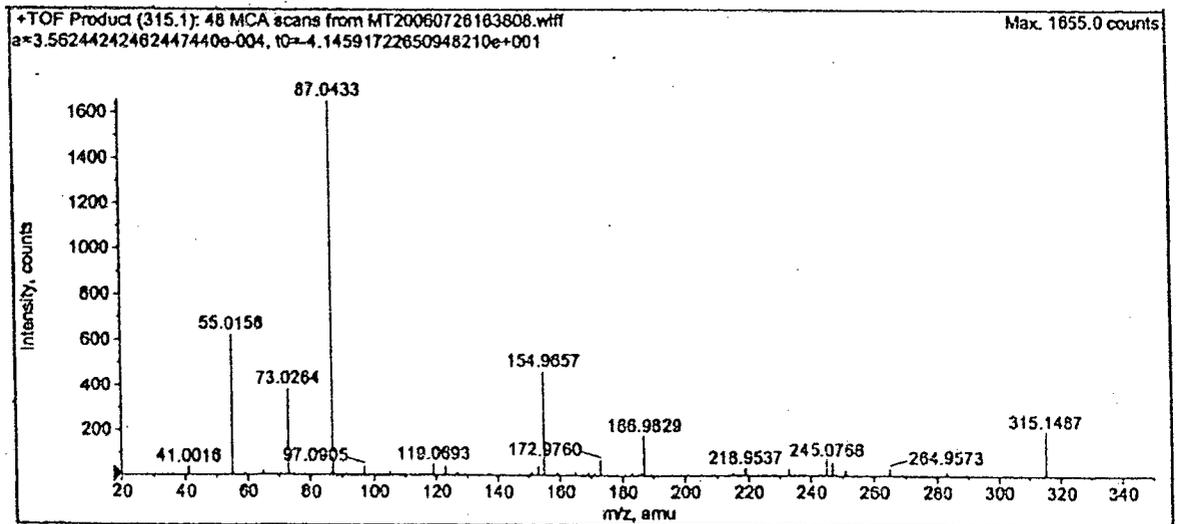


Fig 2.1.13: MS/MS spectrum of compound 6

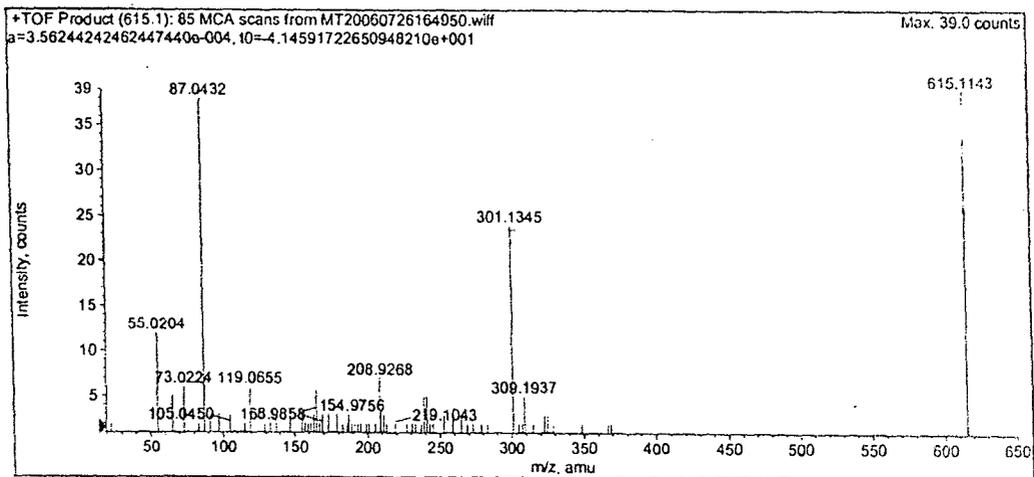


Fig 2.1.14: MS/MS spectrum of compound 7

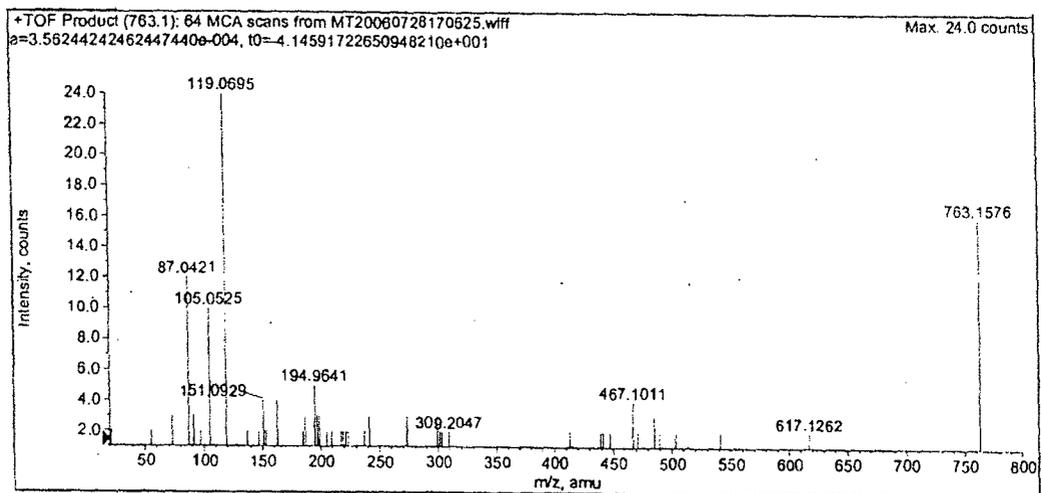


Fig 2.1.15: MS/MS spectrum of compound 8

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Section II

*The antimicrobial and CNS depressant
properties of *Aegiceras corniculatum* .*

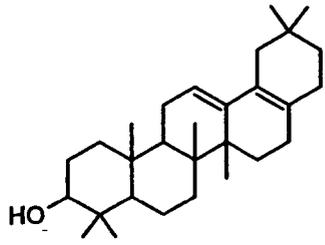
Introduction:

Aegiceras majus Gaerten (Syn. *A. corniculatum* ,Blanco) a shrub, commonly found along the eastern and Western coasts of India, is a mangrove plant belonging to family Myrsinaceae.

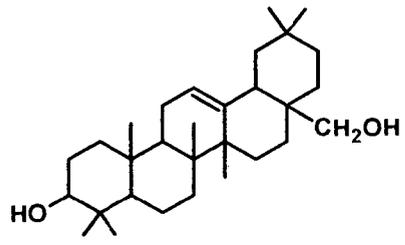
Extensive literature survey did not reveal any information regarding medicinal uses of this plant except for the studies on the toxicity of its aqueous extract to fish using juvenile *Nile tilapia*, *Saroth erodon niloticus* as test organisms¹. It was selected for chemical investigation on the basis of observation made during preliminary screening of this mangrove plant for a wide range of biological activities.

There are some reports on the phytochemical investigations of this mangrove. Rao and coworkers describes the isolation from the bark of *A. majus* (Gaerten) (Syn. *A. corniculatum*), of triterpene sapogenins, aegiceradienol (1)², genin A (2)³, aegiceradiol (3)⁴ and norechinocystadienol (4)⁵ as products of usual acid hydrolysis of saponin mixture. Later on, Henses and Lewis⁶ demonstrated that aegiceradiol (3) or aegiceradienol (1) are artifacts formed during the fairly vigorous hydrolysis conditions used for the crude glycosidic mixture. Isorhamnetin (5), echinocystic acid (6) and rapanone(7) are also known to be constituents of *A. corniculatum*⁷. Nonglycosidic extract of the same plant was found to contain triterpenoid, aegicerin (8)⁸ besides the usual triterpenoids lupeol (9), amyirin (10), oleanolic acid (11) and ursolic acid (12) and the steroids campesterol (13), stigmasterol (14) and sitosterol (15)⁹. Benzoquinones, embelin and the fish toxin 5-O-methyl embelin are also known to be present in the plant¹⁰. These benzoquinones are also responsible for the piscicidal activity of the extracts of *Aegiceras corniculatum*. 5-O-Methyl embelin also inhibited the growth of the fungi *Pythium ultimum*. This mangrove is known to photosynthesize aspartate and alanine as major products of short time photosynthesis¹¹.

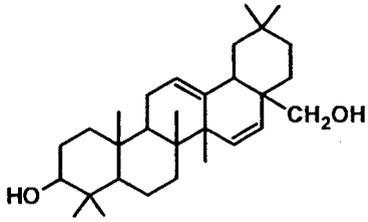
A recent report has indicated aegicerin, isolated from the Peruvian plant *Clavija procera*, a member of the rare *Theophrastaceae* family, to be a powerful antimycobacterial agent against sensitive as well as resistant *Mycobacterium tuberculosis* strain with MIC values ranging between 1.6 and 3.12 µg/ml¹².



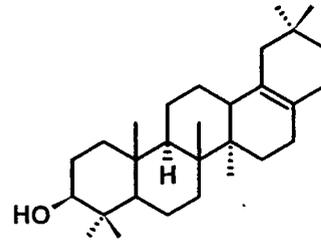
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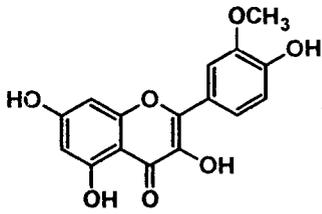
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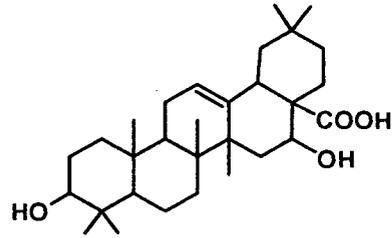
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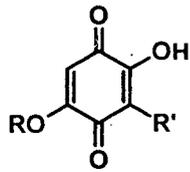
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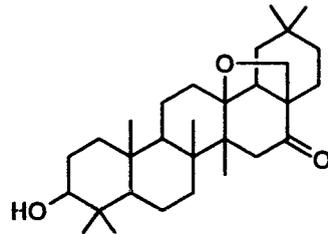


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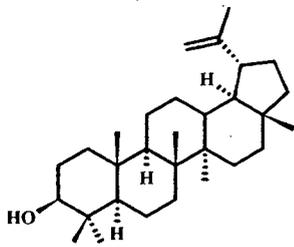


$R = H, R' = C_{13}H_{27}$

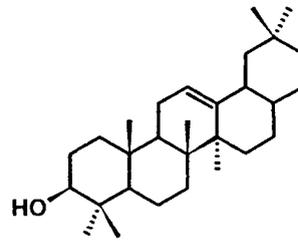
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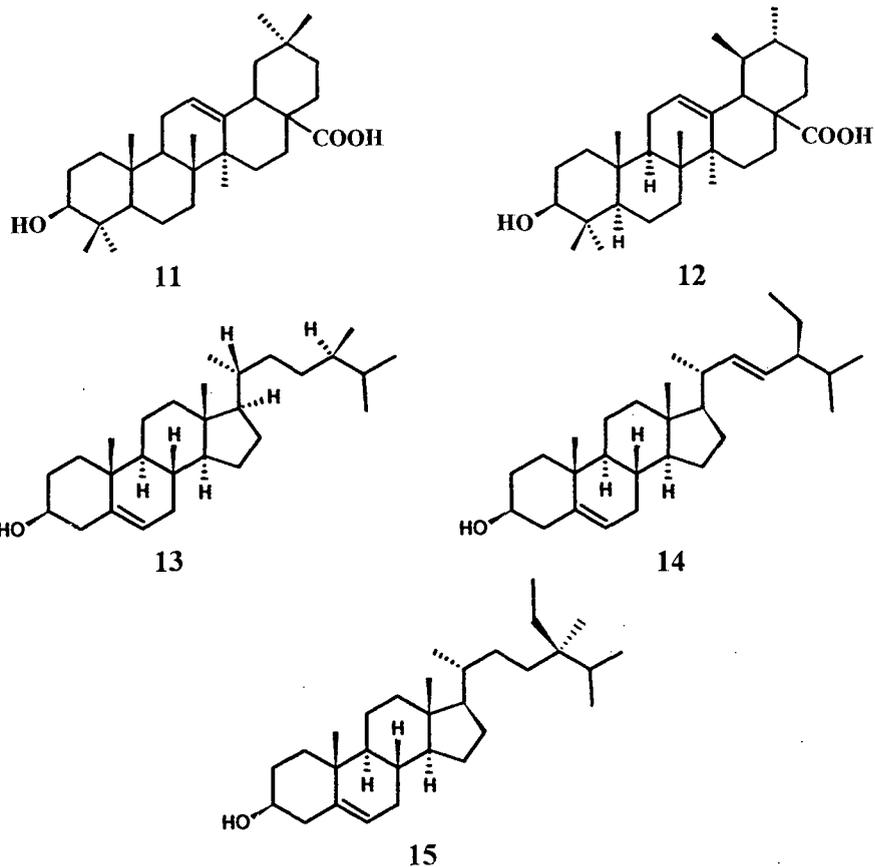
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In our continuous search for biologically active metabolites from marine organisms this plant was investigated to ascertain biological activities of this plant in search for new molecules having medicinal properties. The crude extract was tested on animals (guinea pigs) for a wide range of biological activities. It was observed that the crude extract of this mangrove exhibited promising central nervous system depressant activity. Its pharmacological action includes hypnotic sedative action, anticonvulsant properties and reduction in amphetamine-induced hyperactivity in mice. This property of this extract led us to investigate further leading to the isolation of the active principle. It was found that methanolic extract of twigs of a mangrove plant *A. corniculatum* (Blanco) also inhibited the growth of the bacterium *E. coli* and fungi *A. fumigatus* and *Fusarium sp.* The isolation and identification of antimicrobial constituent from this mangrove has also been discussed in this section.

Results:

The crude extract (**Table-1**) as mentioned above caused considerable depression of central nervous system. To simplify the isolation of the active principle the active crude extract was subjected to bioassay guided fractionation, which includes partitioning of the extract successively, with chloroform and n-butanol. The residue, which remained insoluble, was considered as water-soluble. During the partitioning it was observed that lot of emulsion was formed making the separation of the two layers difficult. The viscosity of the extract depended on the concentration of water in the crude extract. Higher the concentration of water in the extract greater is the extent of emulsion formed. To reduce the emulsion the extract was neither filtered through celite nor treated with sodium chloride as it might interfere with the pharmacological testing. The addition of sodium chloride would increase its concentration in aqueous fraction and while testing on animals it might lead to an increase in the blood pressure, thus, misleading the results. Similarly filtering through celite might cause lost of some component or decrease its concentration. To avoid all this, the amount of solvent was increased and after shaking in a separatory funnel it was left overnight for the separation of two layers. On testing of the fractions (**Table-2**) it was observed that the activity gets distributed in all the fractions in decreasing order, it being highest in the chloroform fraction followed by butanol, the aqueous fraction being the least active.

The details of the pharmacological testing are given under experimental procedures. All the experiments were conducted at room temperature, $24 \pm 1^\circ\text{C}$.

Table 1: Effect of crude extract on CNS (Central Nervous System).

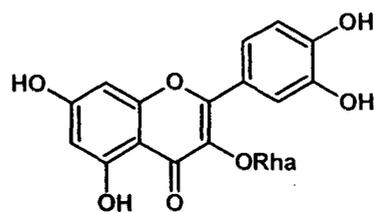
	Activity %			
	Antimetrazol Seizure	Barbiturate Hypnosis	Analgesia	Antielectroshock Seizure
Crude Extract	80	18	+ve	+ve
Dose	12.5mg/kg	12.5mg/kg	100 mg/kg	100 mg/kg

Table 2: Effect of fractions on CNS.

Fractions	Dose	Activity %			
		Antimetrazol test	Barbiturate Hypnosis	Rotarod test	CAR blocking
Chloroform	12.5mg/kg	80	60	I	80
n-Butanol	12.5mg/kg	60	33	-	-
Aqueous	12.5mg/kg	60	30	-	-

The active n-butanol fraction (80g) was repeatedly chromatographed over silica gel (60-120 mesh; methanol:chloroform gradient system) and Sephadex LH20 (methanol:chloroform, 1:1, eluent) columns. This led to the isolation of the active compound identified as, flavonoid glycoside quercitrin (**1**), an hexose sugar identified as Iditol (**2**) and an inorganic mixture.

The molecular formula $C_{21}H_{20}O_{11}$ of compound **1** was determined by ESI-MS. Negative ESI-MS (**Fig.2.2.1**) exhibited peaks at m/z 447 $[M-H]^-$ and m/z 299.9 $[Aglycone-H]^-$. Its IR spectrum (**Fig.2.2.2**) showed a broad $-OH$ absorption at 3278.8 cm^{-1} , a peak at 1656.7 cm^{-1} was attributed to the α - β unsaturated carbonyl group and the absorption at 1604.7 and 1502.4 cm^{-1} to the presence of aromaticity. Its 1H NMR was typical of monoglycosyl flavonol. The 1H NMR spectrum (**Fig.2.2.3**) showed five aromatic signals at δ 6.02 (1H), 6.21 (1H), 7.20 (1H), 6.75 (1H), and 7.15 (1H) which were assigned respectively to H-6,, H-8, H-5', H-6' and H-2' in a tetrasubstituted flavonol unit (**Table-3**). Along with the aromatic signals, sugar signal (0.80-5.20 ppm) could be assigned to deoxyhexose moiety. The methyl doublet δ 0.80 which is unresolved and the anomeric proton at δ 5.20 suggested an α -rhamnopyranosyl unit. The deoxyhexose moiety was confirmed by both ^{13}C (**Fig.2.2.4**) and DEPT (**Fig.2.2.5**) spectral analysis and by the presence of an anomeric carbon at 103.50, four-sugar carbons at δ 71.88-73.20 and a methyl at δ 17.64. Besides the sugar signals, 15 carbons from 94.7 to 179.5 were assigned to tetrahydroxylated flavonol unit (**Table-3**). The chemical shifts of compound **1** were in good agreement with that published for quercitrin¹³.

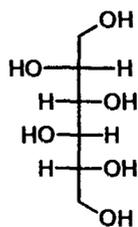


Compound 1

Table-3: NMR data of compound 1

Carbon No.	¹³ CNMR δ _H , ppm	¹ HNMR δ _c , ppm
Aglycone		
2	159.2	-
3	136.2	-
4	179.5	-
5	163.1	-
6	99.7	6.02(s)
7	165.8	-
8	94.7	6.21(s)
9	158.4	-
10	105.8	-
1'	122.8	-
2'	116.9	7.20(d)
3'	146.3	-
4'	149.7	-
5'	116.3	6.75(d)
6'	122.8	7.15(dd)
Rhamnose		
1''	103.5	5.20(s)
2''	73.2	4.08 (s)
3''	71.8	3.6(dd)
4''	72.0	3.21-3.30(m)
5''	72.0	3.10-3.21(m)
6''	17.6	0.80(bs)

Compound 2, was obtained as a white crystalline solid and was identified as Iditol based on the spectral data of its acetate. Its polyhydroxy nature was evident from its IR (Fig 2.2.6). The ¹HNMR and ¹³CNMR data is presented in Table 4 and (Fig 2.2.7), (Fig 2.2.8).



Compound 2

Table 4: NMR data of hexacetate of compound 2

Carbon No.	¹³ C(ppm)	¹ H (δ)
1,6	61.9	4.06 (dd, 5.1, 12Hz, 2H) 4.10 (dd, 2.7, 2.7Hz, 2H)
2,5	67.9	5.07 (m, 2H)
3,4	67.5	5.44 (d, 8.7Hz)
Acetyl-		
C=O	170.5, 169.9, 169.7	
-CH ₃	20.8, 20.6, 20.6	2.07 – 2.15

These NMR values are well in agreement with those reported for Iditol hexaacetate^{14,15}. The effect of both the compounds 1 and 2 on CNS are shown in Table-5.

Table 5: Effect of compound 1 and 2 on CNS

Compounds	Dose	Activity %			
		Antimetrazol test	Barbiturate Hypnosis	Rotarod test	SCR blocking
1	25mg/kg	80	60	I	100
2	25mg/kg	60	33	-	100
Inorganic mixture	12.5mg/kg	60	30	-	-

Antimicrobial activity of *Aegiceras corniculatum*

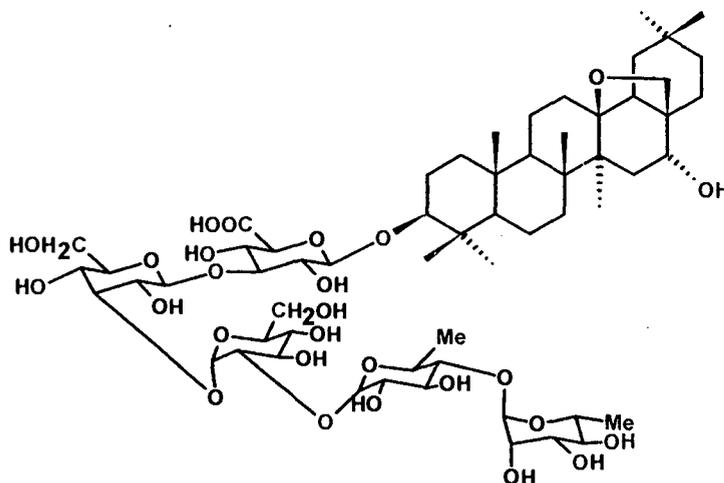
The methanolic extract, as mentioned, also displayed antimicrobial activity against *Cryptococcus neoformans*, *Aspergillus niger* and *Fusarium sp.* Fractionation, located the activity in n-butanol fraction. Activity guided fractionation of the fractions afforded 3 in 0.058% yield, antibacterial against *E. coli* and fungistatic against *A. fumigatus*.

Compound 3 obtained as colourless amorphous powder had a molecular formula of C₆₀H₉₈O₂₇ deduced from the detailed analysis of NMR and HRESI-MS data, m/z 1273.717 (calculated for C₆₀H₉₈O₂₇Na) in +ve mode and m/z1249.165 in -ve mode as M⁺-1.

Electrospray ionization in the positive as well as the negative ion mode, especially in combination with MS/MS techniques, has been used for sugar sequence analysis. Positive ion mode ESI mass spectrum (Fig.2.2.9) of 3 afforded a less intense molecular ion at m/z 1273 (M + Na)⁺ and a base peak of disodium

molecular ion at m/z 1295 $[M + 2Na - H]^+$. This parent ion was selected using the quadrupole mass analyzer and collision induced decomposition gave daughter ions those were separated in the time of flight mass analyzer. The resulting MS/MS spectrum (Fig.2.2.9) showed fragment ions at m/z 1149 $[(M + 2Na - H) - dhex]$, m/z 1003 $[M + 2Na - H - 2 \times dhex]^+$, m/z 841 $[(M + 2Na - H) - 2 \times dhex - hex]^+$, m/z 679 $[(M + 2Na - H) - (2 \times dhex + 2 \times hex)]^+$, m/z 503 $[(M + 2Na - H) - (2dhex + 2hex + GlcA)]^+$. These fragments were generated through loss of the respective saccharides. Thus MS/MS analysis indicated that compound is a pentasaccharide with the sugar sequence (dhex – dhex – hex – hex – GlcA) linked to the oxygen at C-3 of the aglycone.

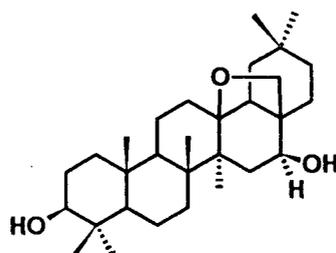
Fragment ions were also observed at m/z 477 (dhex + dhex + hex + Na), hexose + GlcA + 2Na – H = 383, m/z 177 [GlcA +H]⁺, m/z 131 being GlcA-COOH. The MS/MS experiment with a collision energy of 96, selecting ion of m/z 1273 revealed signals at m/z 815, which indicated that the aglycone of the saponin had a molecular mass of 458amu $[M + Na - 815] \cong [1273 - 815]$. Thus the combined ESI-MS data indicated that the sugar sequence in **3** is dhex – dhex – hex – hex – GlcA- and the carbohydrate moiety is linked to the aglycone having a molecular mass of 458 amu.



Compound 3

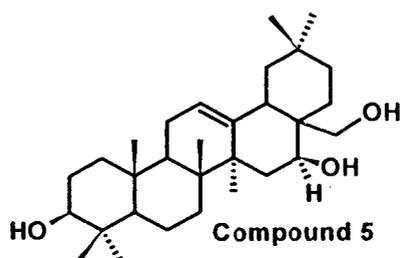
¹H NMR (300MHz, C₅D₅N)(Fig.2.2.10) spectrum of **3** revealed the presence of seven tertiary methyl groups at δ 0.738, 0.932, 0.986, 1.026, 1.089, 1.279, 1.51 and a doublet at 1.58 (J = 5.4Hz) assigned to the secondary methyl of deoxy sugar.

In addition, there are doublets at δ 3.229 ($J=6.9\text{Hz}$) and δ 3.275 ($J=6.9\text{Hz}$) typically observed for 13β , 28 epoxy oleanane compounds (Hegde et al., 1995)¹⁶. This is supported by the presence in its ^{13}C NMR (Fig.2.2.11) spectrum of oxygenated quarternary carbon signal at δ 86.5 and a triplet at δ 77.8. This was suggestive of aglycone being protoprimulagenin A (4). The attachment of the carbohydrate moiety at C-3 position of the aglycone was evident from the ^{13}C NMR glycosylation shift at C-3 of 1 and its β configuration was established from the coupling constant of $J=7.8\text{Hz}$ observed for H-3. The signal due to $16\beta\text{-H}$ was observed as a broad singlet at δ 4.97. These observations led to the formulation of **3** as shown. Glucuronide saponins are common in *Primulaceae* and *Myrsinaceae*.



Compound 4

Acid hydrolysis of **3** with HCl yielded artifactual aglycone **5** besides sugars, glucose, rhamnose and glucuronic acid in the proportion of 2:2:1. These sugars were identified by comparison on TLC with standard sugars. Aglycone **5** was identified as primulagenin A by ^1H (Fig.2.2.12) and ^{13}C NMR (Fig.2.2.13) comparison of the ^{13}C NMR spectra of **3** and **5** further supported that **5** is not a genuine aglycone of **3**. The spectrum of **3** lacked signals due to unsaturated carbon at δ 123.3 and 145.3 but an additional oxygenated quaternary carbon signal was observed at δ 86.5; this confirmed that the genin of **3** has the structure of protoprimulagenin A with the ^{13}C NMR data of aglycone moiety of **3** being well in agreement with that of protoprimulagenin A (Table 6).



As mentioned the saponin is a pentasaccharide, and the presence of five sugars was further evidenced from the five anomeric protons at δ 4.4(d, $J=7.8\text{Hz}$, 2H), 5.2(d, $J=6.6\text{Hz}$, 1H) and 5.4(brs, 2H) and carbons (104.8, 103.75, 102.54, 100.69 and 100.69). The magnitude of coupling constant indicated that three of the sugars have β -configuration and in the remaining two the anomeric protons were equatorial. The sequential loss of sugar moieties observed in ESI-MS spectrum were due to sequential cleavage of terminal rhamnose, rhamnose, glucose, glucose and glucuronic acid. This data established the sugar series in the molecule. Further evidence of the nature of the linkages within the pentasaccharide unit was forthcoming from 2D NMR experiments, especially HMQC, ^1H - ^1H COSY (Fig.2.2.14), TOCSY (Fig.2.2.15) and HMBC(Fig.2.2.16). HMQC was used to establish one-bond carbon-proton connectivities. Proton-proton connectivities were established using ^1H - ^1H COSY and TOCSY experiments. HMBC was used to establish long- range (2 or 3 bond) carbon proton connectivities. Some of the important connectivities observed were as follows: the proton at C-3 (δ 3.15) of the triterpene showed a long range correlation with the carbon resonating at 104.58 (δ 4.97) which must be the C-1 of glucuronic acid. The glucuronic acid H-1 was coupled to H-2 at δ 4.02 (TOCSY) enabling glucuronic C₂ to be identified at 75.9ppm. TOCSY experiment showed connectivity of H-2 to δ 3.72. A HMQC correlation of this proton identified C-3 carbon of the sugar at δ 81.2. The downfield C-3 value shift as compared to pyranose sugar established the first intersugar linkage of glucose anomeric proton to C-3 of glucuronic acid. Similarly the anomeric proton of the terminal rhamnose at δ 5.4 (100.69) was coupled to the proton at δ 4.6 (72.5), which showed connectivity to δ 4.5 (71.6) in ^1H - ^1H COSY. The terminal rhamnose anomeric proton showed HMBC correlation with the C-5 carbon of inner rhamnose at δ 67.96 indicating the

linkage between the two rhamnose units. Thus the compound (3) is 3-O-[[α -L-rhamnopyranosyl-(1 \rightarrow 4)-O- β -D-rhamnopyranosyl(1 \rightarrow 2)-O- α -D-glucopyranosyl-(1 \rightarrow 3)-O- β -D-glucopyranosyl-(1 \rightarrow 3)]- β -glucuronopyranosyl]]protoprimulagenin A.

Table 6: ^{13}C NMR of Compounds 3, 4 and 5

	Primulagenin A(5)	Protoprimulagenin* A (4)	Compound 3	3-O-sugar moiety
C-1	39.8	38.7	39.2	GlcA 1 -104.8
C-2	27.5	27.3	26.6	2 - 75.9
C-3	79.6	79.0	90.0	3 - 81.2
C-4	39.8	38.9	39.8	4 - 72.1
C-5	56.8	55.1	55.8	5 - 76.7
C-6	19.4	17.7	18.29	6 - 176.2
C-7	33.5	31.0	31.9	Glc 1 - 103.7
C-8	40.0	42.0	42.5	2 - 76.0
C-9	48.1	50.1	50.5	3 - 79.7
C-10	38.0	36.9	36.8	4 - 72.1
C-11	24.5	18.6	19.2	5 - 76.9
C-12	123.3	34.0	34.5	6 - 63.2
C-13	145.3	86.4	86.5	Glc 1 - 100.6
C-14	40.9	43.9	44.6	2 - 78.0
C-15	34.0	36.8	36.9	3 - 71.4
C-16	74.8	77.4	77.2	4 - 76.0
C-17	48.7	44.1	44.6	5 - 77.0
C-18	48.4	50.6	51.5	6 - 61.6
C-19	49.5	38.8	39.1	Rha 1 - 102.5
C-20	33.5	31.5	31.9	2 - 71.4
C-21	38.0	36.6	36.8	3 - 73.7
C-22	28.7	32.3	32.9	4 - 77.0
C-23	27.9	27.9	28.0	5 - 69.7
C-24	16.2	16.1	16.6	6 - 18.2
C-25	17.5	15.3	16.4	Rha 1 - 100.6
C-26	19.4	18.1	18.4	2 - 72.5
C-27	17.5	19.4	19.7	3 - 71.6
C-28	71.1	78.1	77.8	4 - 74.2
C-29	34.0	33.3	33.8	5 - 69.8
C-30	27.9	24.3	24.8	6 - 18.4

* (Alex et al)¹⁷

Antimicrobial activity

Compound 3 was evaluated for in vitro antimicrobial activity (Table 7) against six Gram -ve bacteria [*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella flexneri*, *Klebsiella pneumoniae*, *Vibrio cholerae* and one gram +ve bacteria *Staphylococcus aureus*]. In vitro fungistatic activity was determined against the fungal strains of *Aspergillus fumigatus*, *Fusarium sp.*, *Candida neoformans*, *Aspergillus niger*, *Rhodotorula*, *Nocardia sp.* and *Candida albicans*. The cells of these strains, which were all clinical isolates were used in the assay.

Compound 3 was found to be as effective as the standard antibiotic streptomycin used against *E. coli* and exhibited mild activity against *Aspergillus fumigatus* at 15µg/disk. The standard nystatin was ineffective against *A. fumigatus*.

Saponins with 16α-hydroxy-13β,28 epoxyoleananes or protoprimulagenin A saponins are reported to be biologically active. Phospholipase D inhibitors are known from *Myrsine australis*. Molluscidal against *Oncomelania nosophora* at 800ppm and lethal to the snail at 2ppm are sakurasaponins from *Lysimachia sikokiana*¹⁸. Amoros and Girre¹⁹ found them antiviral against *herpes simplex* virus type I and polio virus. Molluscidal activity (50ppm) against the schistosomiasis transmitting snail *Biomphalaria glabrata* and antifungal activity against the plant pathogenic fungus *Cladosporium*²⁰. Oleanane oligoglycoside of present investigation is antimicrobial against pathogenic bacteria, *E. coli* and fungus, *A. fumigatus*. Saponin from this source is being reported for the first time. The hydrolysed product, Protoprimulagenin A / Aegicerin has been reported recently to exhibit a powerful antimycobacterial activity against sensitive as well as resistant *Mycobacterium tuberculosis* strain with MIC values ranging between 1.6 and 3.12 µg/ml¹².

Table 7: Antimicrobial activity of n-butanol fraction, compound 3 & control

Compound	Microorganisms : antibacterial							Microorganisms : antifungal						
	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>S. typhii</i>	<i>S. flexneri</i>	<i>Klebsiella sp.</i>	<i>V. cholerae</i>	<i>A. fumigatus</i>	<i>Fusarium sp.</i>	<i>C. neoformans</i>	<i>A. niger</i>	<i>Rhodotorula</i>	<i>Nocardia</i>	<i>C. albicans</i>
Butanol fr.	-	-	-	-	-	-	-	-	+	-	+	-	-	-
1	4	-	-	-	-	-	-	1	-	-	-	-	+	-
Control	4	15	5	-	10	3	6	11	-	+	-	-	11	4

(-) No activity; (+) mild activity

Numbers indicate the halo of zone of inhibition in mm.

EXPERIMENTAL

Preparation of crude extract

Aegiceras corniculatum (Blanco) collected from West Coast of India and authenticated by Dr Sayeeda Wafar, biologist from the Institute, was used in the present investigation. A voucher specimen is deposited at Taxonomy Reference Centre of the institute soon after collection. About 10kg of the fresh specimen was soaked in methanol for a week and filtered. From the filtrate solvent was vacuum evaporated at 45°C to obtain crude extract (about 256gms).

Fractionation of crude extract

The crude extract was fractionated into different fractions by partitioning into solvents of increasing polarity. It was thus fractionated into chloroform, n-butanol and the aqueous residue. All the three fractions were again subjected to the activity test. All the fractions were found to be active but the activity decreased from non-polar to polar fraction. n-butanol fraction was chosen for follow up studies. The methodologies adopted for activity testing are given below. This example illustrates the activity tests with the crude extract and fractions.

TESTING FOR CNS ACTIVITY*:

Drug induced hyperactivity in animals:

These studies were conducted in mice with amphetamine sulphate (dexedrine). Hyperactivity was created in a group of five mice by injecting 5mg/kg amphetamine persulphate subcutaneously. After about 20 minutes this mice became aggressive, hostile and hyper motile characterized by fast and continuously fast and multidirectional movement. This factor lasted for more than five hours. Male mice weighing between 100 to 150gms were divided into five groups of five animals each. This experiment was conducted at room temperature ($24\pm 1^{\circ}\text{C}$).

Sleeping time potentiation:

Albino rats of same sex weighing 100-150gms were used. The animals were divided into two groups: control group received sodium pentobarbitone (35mg/kg i.p.) alone and treated group received the drug (25mg/kg p.o) 30 minutes prior to pentobarbitone. Time interval between loss and regain of righting reflex was noted. The experiment was carried out at room temperature ($24\pm 1^{\circ}\text{C}$).

Rotarod test:

Mice were placed one hour after i.p. (intraperitoneal) administration of saline on a horizontally rotating rod (diameter 32mm; rotating at 5rpm). Animals remaining on the rod for 3 minutes or more in two successive trials were selected and placed in-group of 10 animals each. The animals in each group then received test material or control vehicle and 30 minutes later were placed on the rod at intervals of 30 minutes up to 2.5 hours. If an animal failed more than once to remain on the rod for 3 minutes, the test was considered to be positive i.e. motor in coordination present. The experiment was conducted at room temperature ($24\pm 1^{\circ}\text{C}$).

*We thank Director CDRI (Central Drug Research Institute) for providing testing results.

Spontaneous motor activity:

Mice (20-25g) were divided into two groups (6 mice in each group). One group served as control and other group was fed with drug (25mg/kg). Both groups were kept in actophotometer initially for two minutes, after that spontaneous activity was recorded at 0, 30, 60, 90, 120, 180, 240, and 300 minutes. The experiment was carried out at room temperature ($24\pm 1^{\circ}\text{C}$).

ESI/MS and NMR spectrometry:

NMR was recorded on Bruker Avance 300MHz spectrometer in CD_3OD and $\text{C}_5\text{D}_5\text{N}$ with TMS as internal standard. Electrospray ionization experiments were performed on a QSTARXL MS/MS system, Applied Biosystems/MDS Sciex Instruments (Canada). The instrument was operated in the positive as well as negative mode. All the analysis were performed using ion spray source with the following settings: ion spray voltage was set to 5.5kV in positive mode and – 4.5kV in negative mode, nebuliser gas (N_2) 20 arbitrary units and curtain gas (N_2) 20 arbitrary units. The declustering potential (DP) and the focusing potential were optimized around 60 and 300 respectively during infusion experiment and collision energy was optimized from 15-130 for MS/MS experiments. The sample was directly injected at a constant flow rate of $10\mu\text{l}/\text{min}$ into the electrospray source using infusion syringe pump. Data acquisition and processing was carried out using a Dell Optiplex computer and PE Sciex Analyst QS 1.0 software version SP7 supplied with the instrument. The MS range was m/z 0 - 2000 with scan duration of 1.0s. Each mass spectrum was recorded over a period of 1.0s and mass spectra were accumulated over a period of at least 5 minutes for both single analysis profiles and CID experiments.

Purification of compounds 1-3

The n-butanol fraction was column chromatographed over silica gel (60-120mesh size) and Sephadex LH20 using increasing amounts of methanol in chloroform and methanol respectively as the eluents. Homogeneity of fractions was tested on

E. Merck precoated silica gel F₂₅₄ TLC plates using B:A:W (5:1:4) or CHCl₃:MeOH:H₂O (65:35:10) as the developing solvent. The spots were visualized, by spraying with 5% methanolic sulphuric acid followed by heating at 100°C for 3mins. Compound 1 was the least polar in B:A:W as solvent system and gave yellowish orange spots with methanolic sulphuric acid as the visualizing agent. Compound 2 was eluted from silica gel column as white crystals, m.p.169°C and was the most polar spot when developed in B:A:W and yielded pink spot with methanolic sulphuric acid as the detecting agent and compound 3 was obtained as purplish pink spots when sprayed with methanolic sulphuric acid and had a RF value of 0.56, when developed in the second solvent system i.e.CHCl₃:MeOH:H₂O (65:35:10) as the developing solvent

Acid hydrolysis of saponin 3:

Compound 3 (30 mg) was dissolved in 10 mL 2N HCl solution (H₂O-MeOH 1:1) and the mixture was refluxed while stirring for 3h. After evaporation of the methanol in vacuo, the solution was extracted with EtOAc (3x4mL). The combined organic layers were washed with H₂O and then evaporated to dryness to give an amorphous powder. The H₂O layer was concentrated and compared with standard monosaccharides by silica gel TLC, using (butanol:acetic acid: water; 5:1:4) and visualization by spraying with methanolic H₂SO₄(5%).

Antibacterial activity

Antibacterial activity was determined against bacterial strains tested, using the paper disk assay method. The paper disk (2mm) impregnated with the sample was placed on agar plate containing bacterium and the plates were incubated for 24hrs at 37°C, and observed for zone of inhibition halos. Streptomycin was used as broad-spectrum control.

Antifungal activity

Antifungal activity was determined against strains mentioned in text. The paper disk (6mm) impregnated with the sample was placed on agar plate containing fungus and plates were incubated for 18hrs at 24°C. Nystatin was used as control. A control experiment without the test substance was carried out for verification of the microbial growth.

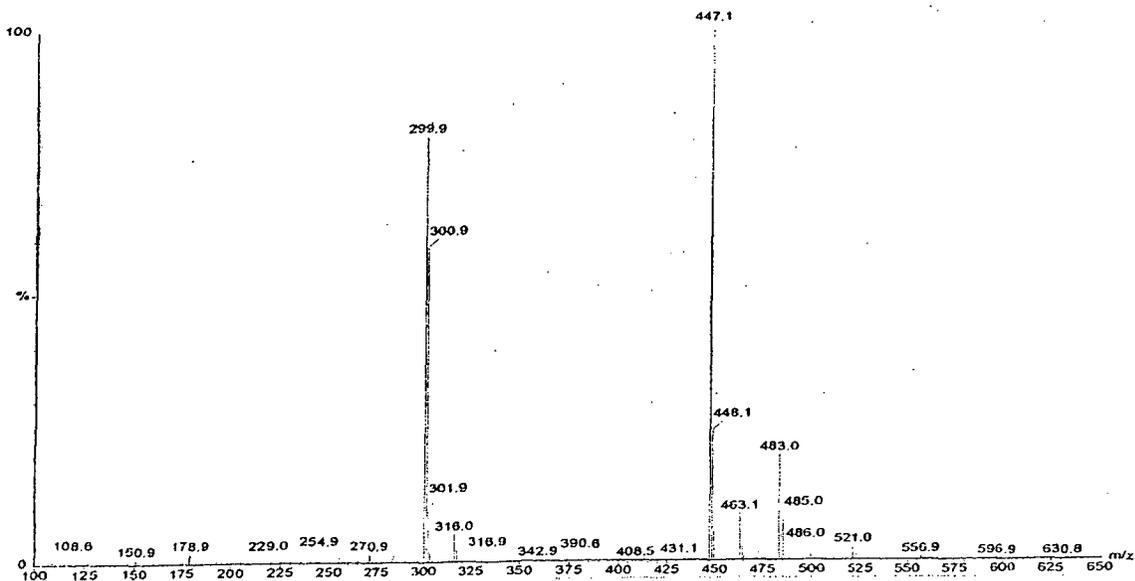


Fig.2.2.1 ESI-MS of compound 1

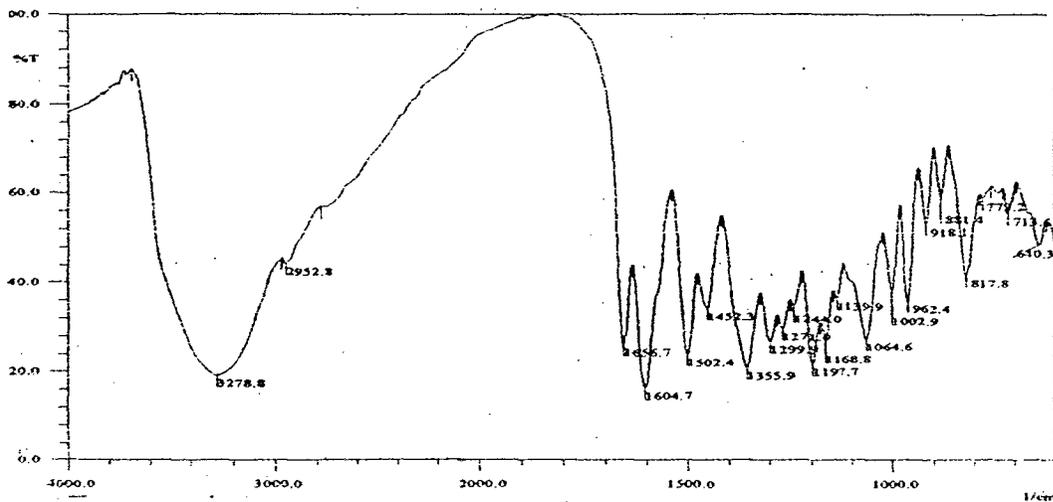


Fig.2.2.2 IR of compound 1

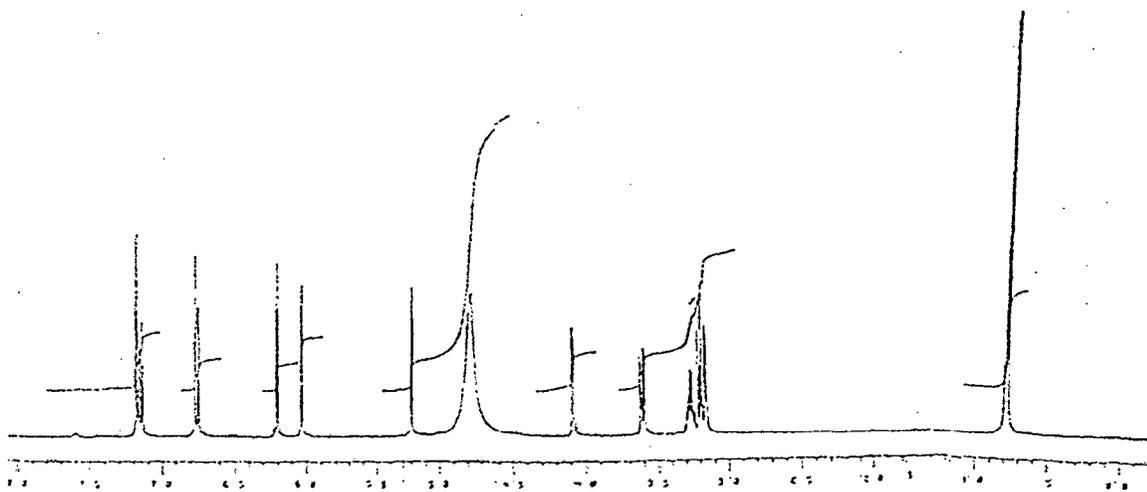


Fig.2.2.3 ^1H NMR of compound 1



Fig.2.2.4 ^{13}C NMR of compound 1

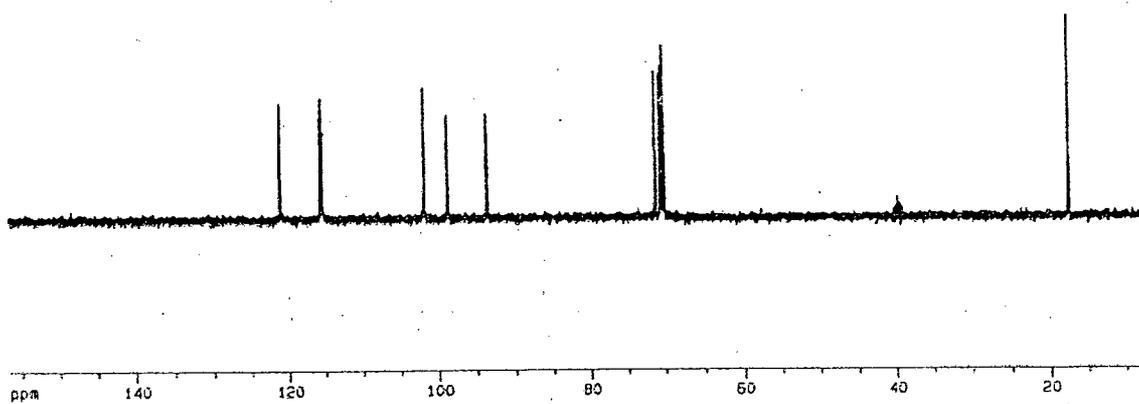


Fig.2.2.5 DEPT spectra of compound 1

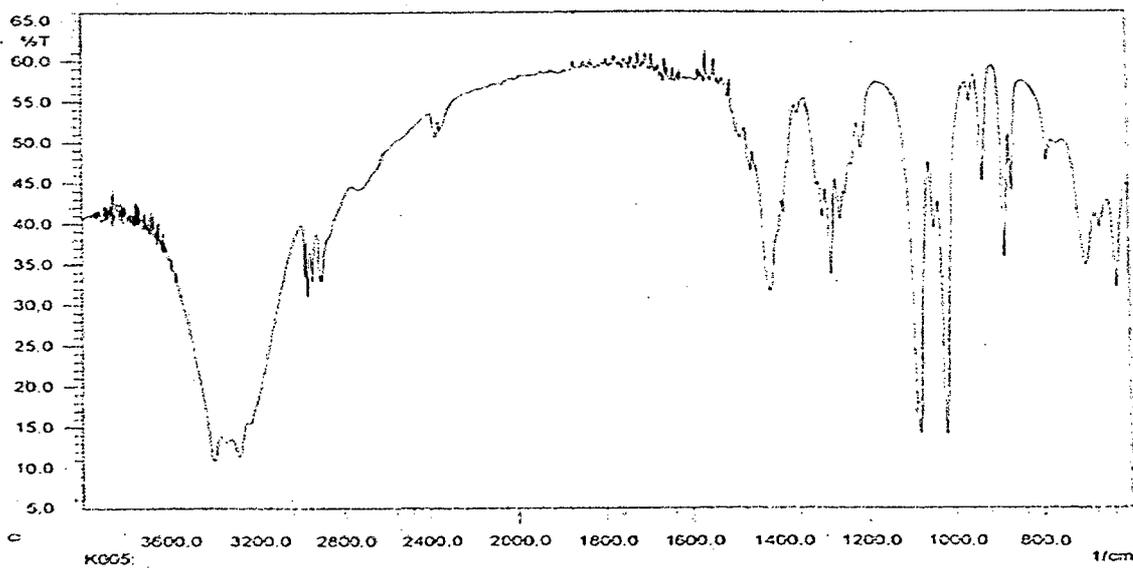


Fig.2.2.6 IR of compound 2

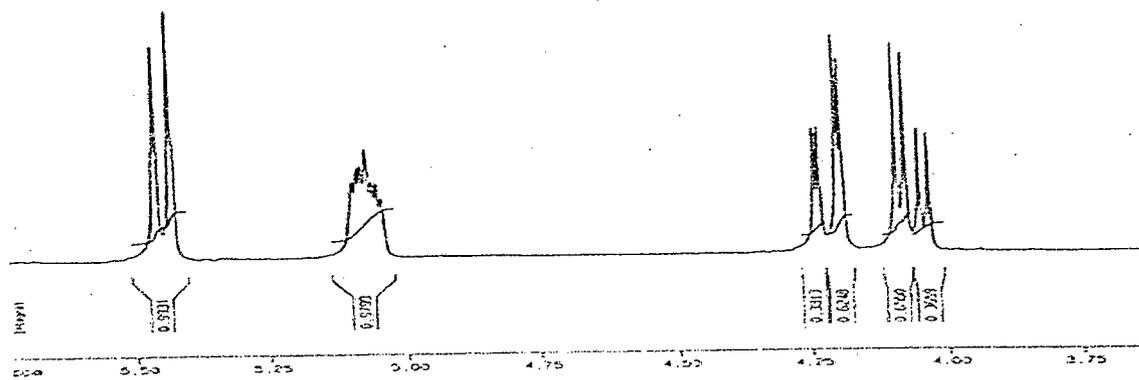


Fig.2.2.7 ^1H NMR of compound 2

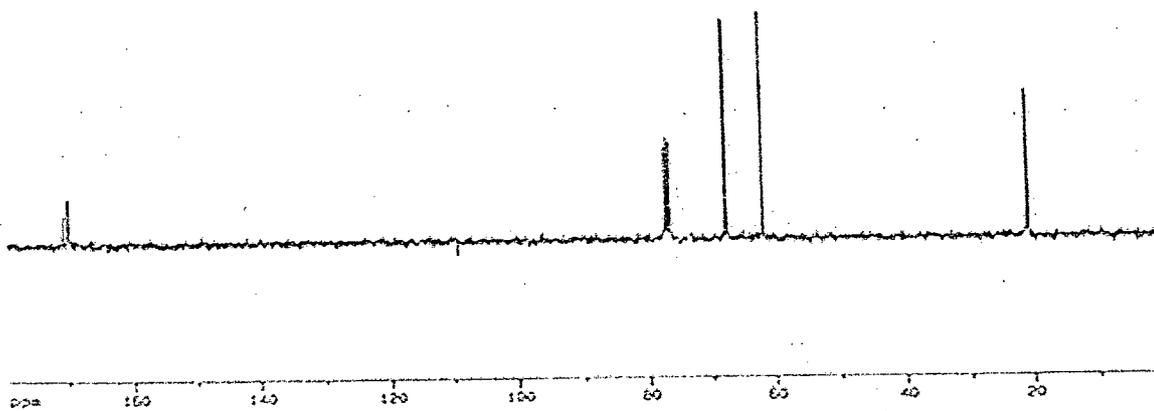


Fig.2.2.8 ^{13}C NMR of compound 2

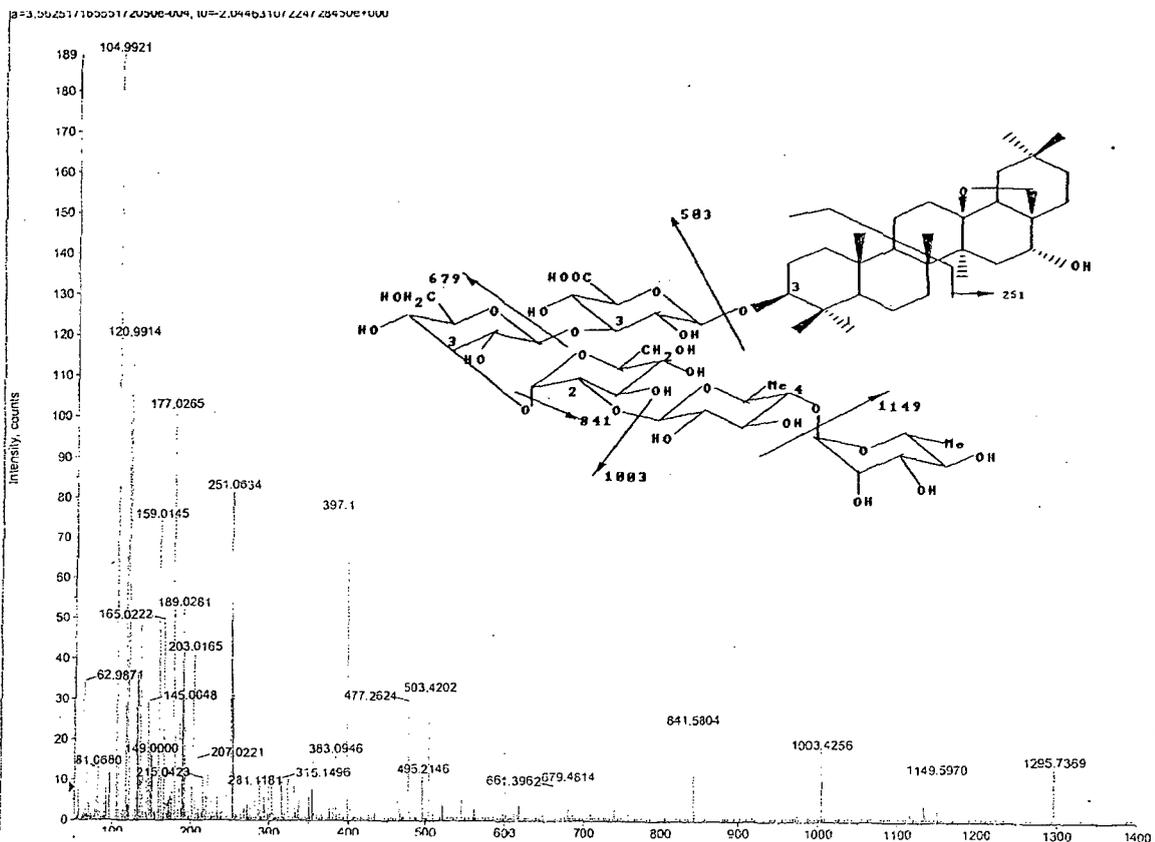


Fig.2.2.9 ESI-MS/MS of the ion 1295.7 of Compound 3

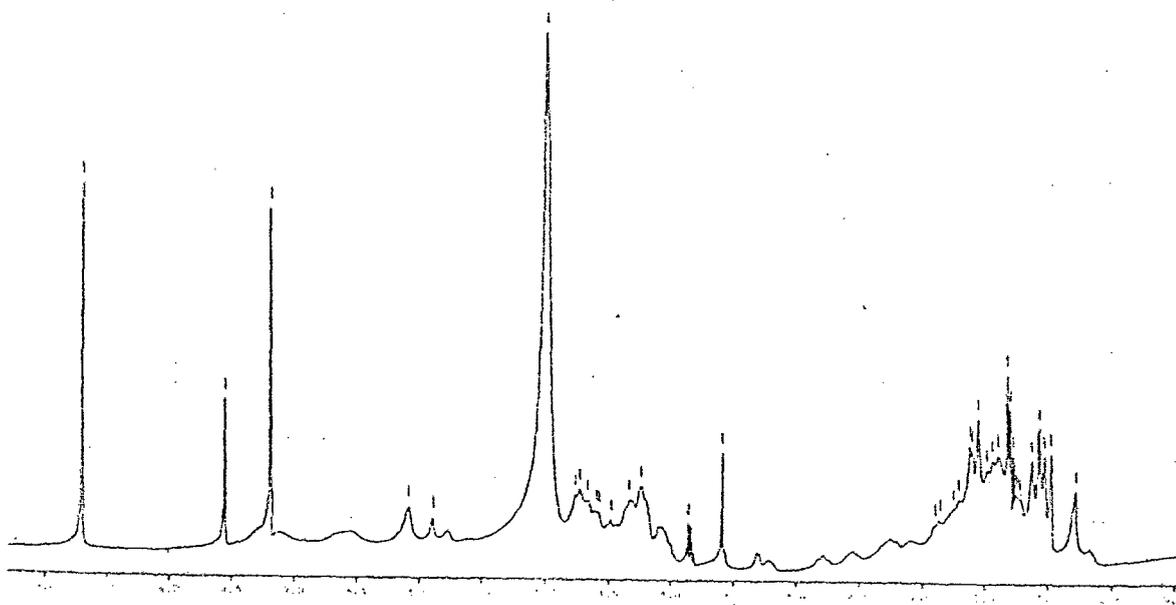


Fig.2.2.10 ^1H NMR of compound 3

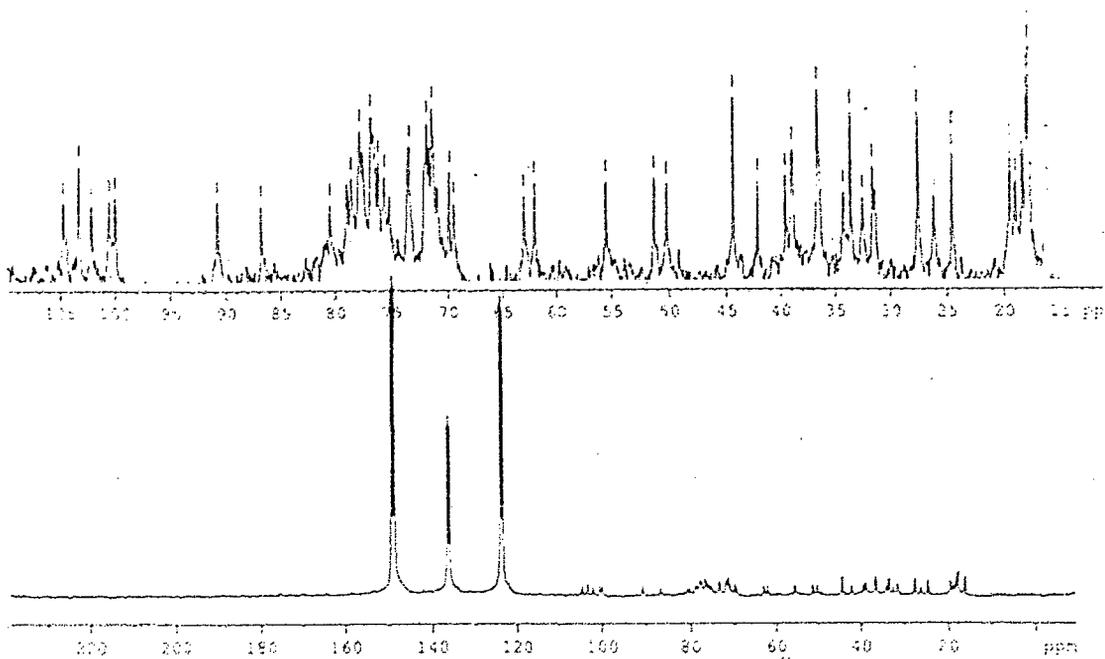


Fig.2.2.11 ^{13}C NMR of compound 3

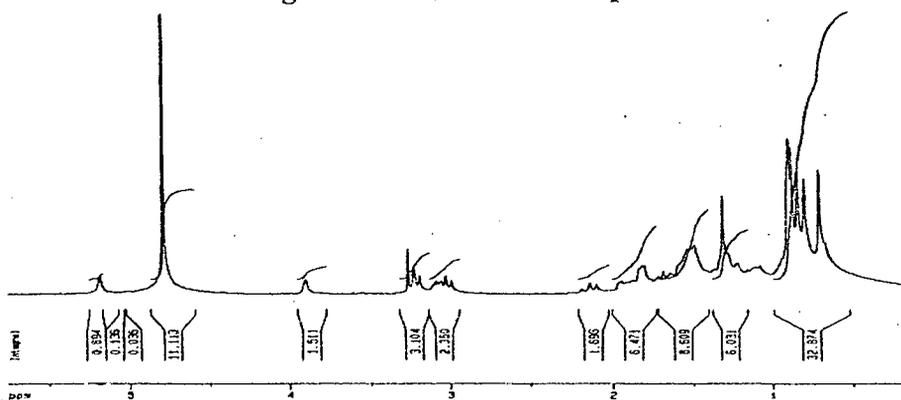


Fig.2.2.12 ^1H NMR of compound 5

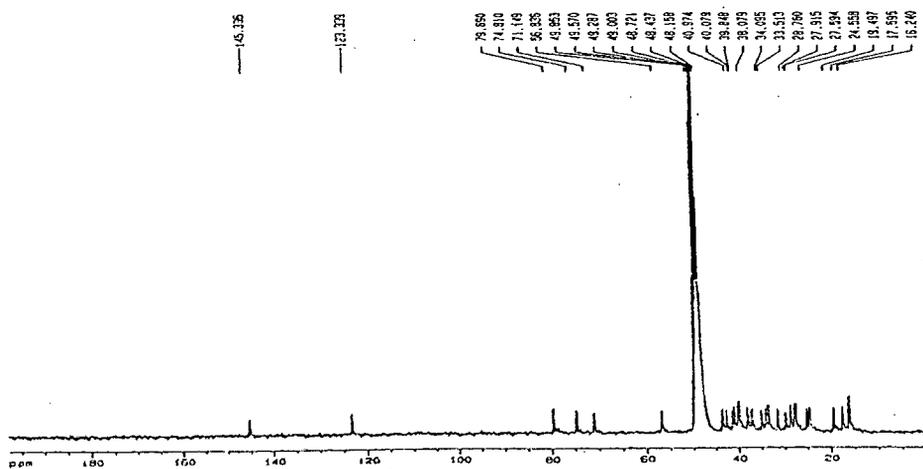


Fig.2.2.13 ^{13}C NMR of compound 5

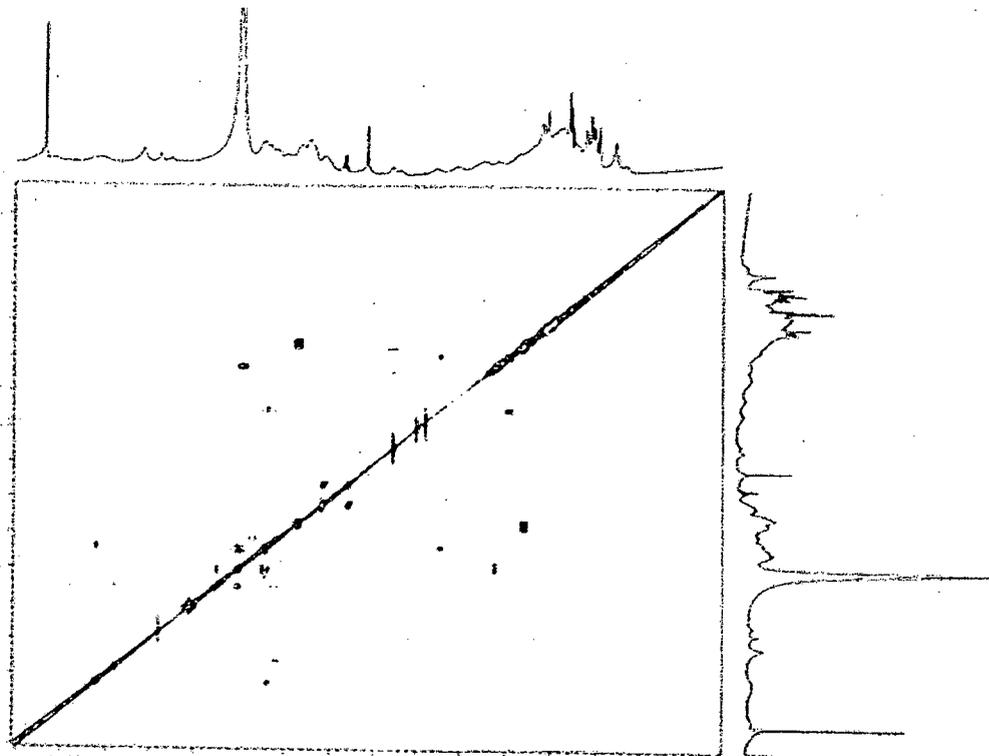


Fig.2.2.14 COSY spectrum of compound 3

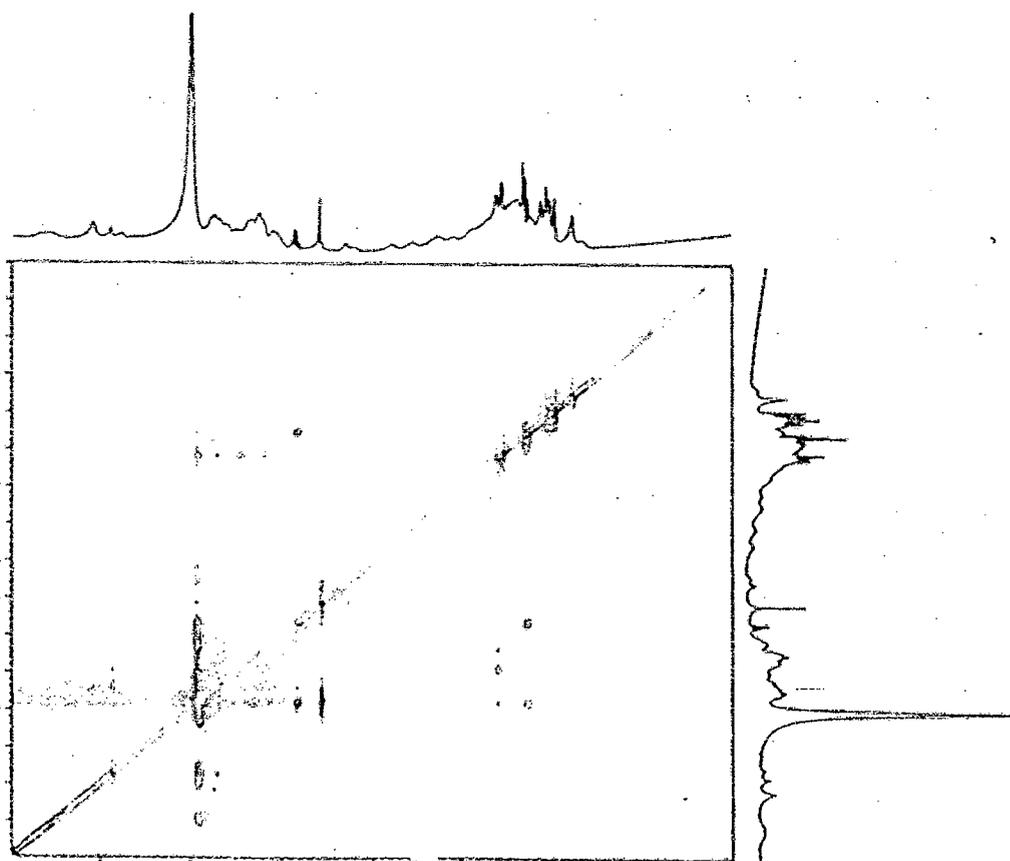


Fig.2.2.15 TOCSY spectrum of compound 3

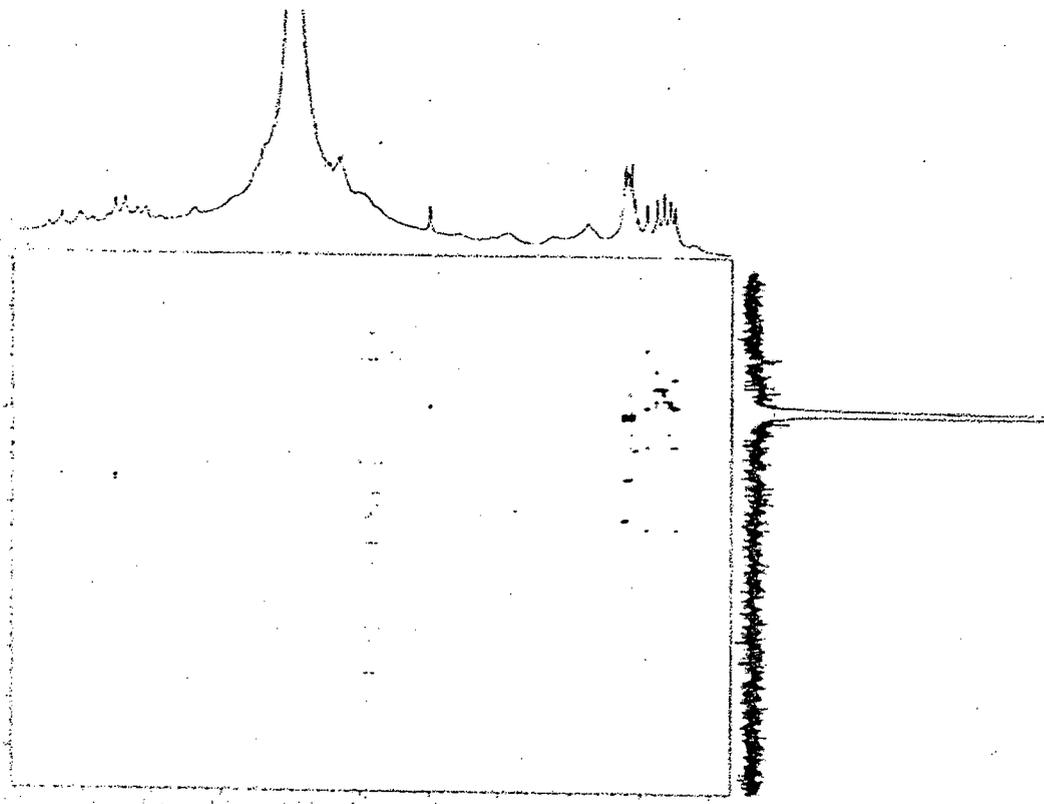


Fig.2.2.16 HMBC spectrum of compound 3

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Section III

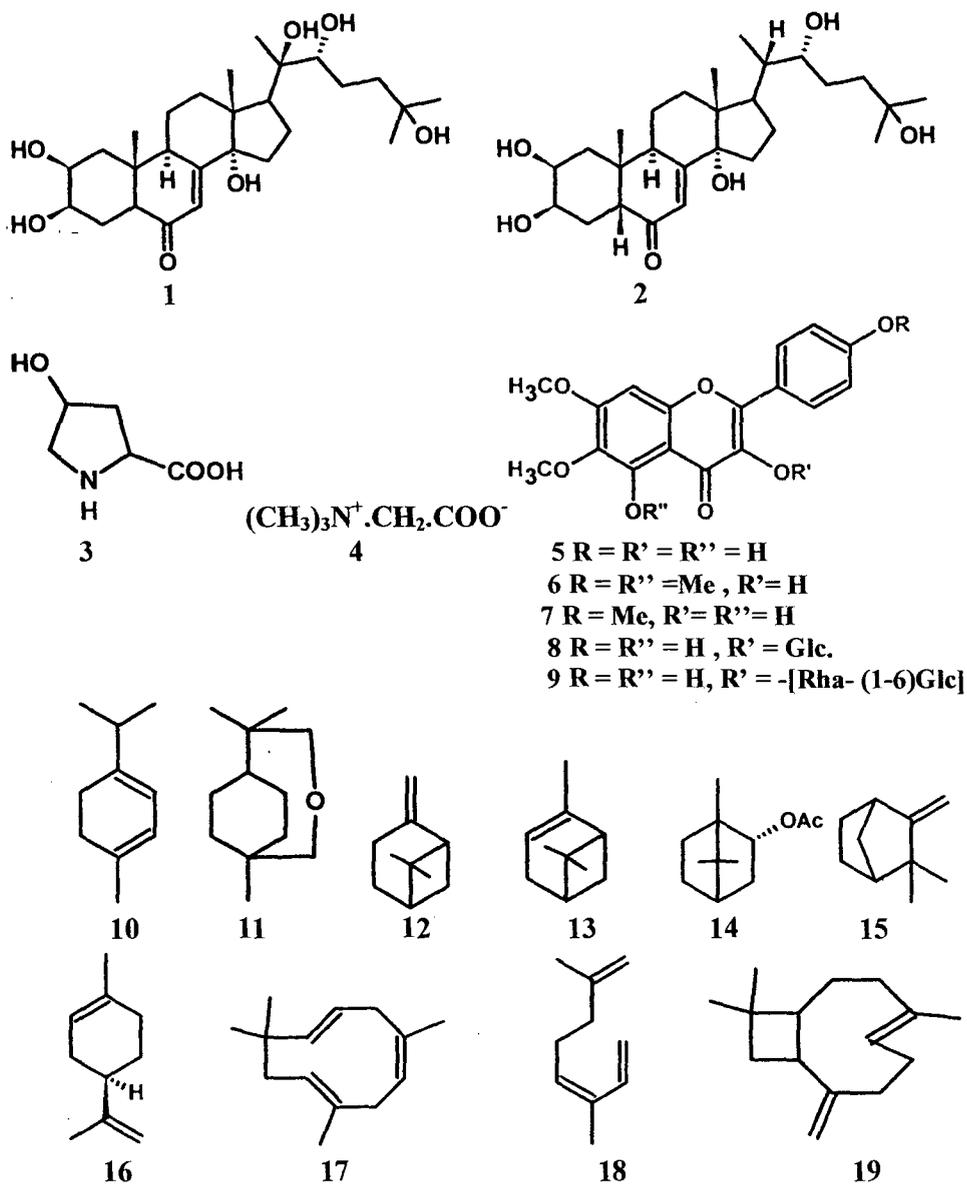
*Chemical constituents of Sesuvium
portulacastrum..*

Introduction:

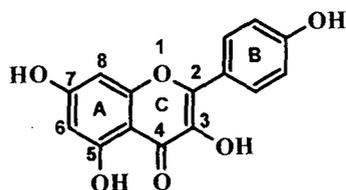
Sesuvium portulacastrum commonly known as Sea Purslane and belonging to family Aizoaceae is a herbaceous perennial plant usually growing in sand. It grows on the ocean side of the dunes down to the high tide mark. The thick, fleshy leaves are borne on succulent; reddish-green stems that branch regularly forming dense stands close to the ground. Small, showy pink flowers are borne more or less continually throughout the year. Each flower opens for only a few hours each day. These plants help build the dunes by catching sand in between stems and leaves. The plant is closely related to the more familiar purslane commonly found in garden centers. It is high salt tolerant herb, with the increase in the salt concentration being associated with the reduction in the growth rate¹ and increased sulfolipid content². Starch, proline as well as glycinebetaine concentrations are reported to increase considerably under salt stress conditions³. The mangrove is also known to be hyper accumulator of cadmium⁴.

Sesuvium portulacastrum has a history of use in the both traditional and folklore medicine as a remedy for fever, kidney disorders scurvy and in the treatment of various infections^{5,6}.

The chemistry of the constituents of *S. portulacastrum* has been of interest for at least last three decades. Ecdysterones^{7,8}(1-2), amino acids (3-4) and mineral constituents⁹, flavonols and flavonol glycosides^{10,11}(5-9), and very recently chemical composition and biological activities of the essential oil (10-19) from the leaves of this mangrove have been reported⁶. The oil exhibited antifungal activity against *Candida albicans*, *Aspergillus niger*, *Aspergillus flavus* and *Penicillium notatum* and antibacterial activity against *Acetobacter calcoacetica*, *Bacillus subtilis*, *Clostridium sporogenes*, *Clostridium perfringens*, *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus* and *Yersinia enterocolitica*. The essential oil also showed antioxidant activity threshold of 15.9 mm mean zone of colour retention.



During the course of our search for bioactive molecules from marine organisms, we have examined the 90% aqueous methanolic extract of the whole plant, collected from Goa coast, for a wide range of pharmacological activities. It showed marked inhibition of oxytocin-induced contractions. This led us to the chemical investigation of this mangrove, which resulted in the identification of novel flavonol glycosides 1-4, and a glycolipid 5. This section gives an account of identification of these chemical constituents from the methanolic extract of this halophyte. The numbering system in flavonols is as shown below:



Numbering system in flavonols.

Results and discussion:

Methanolic extract of the leaves of *Sesuvium portulacastrum* was chromatographed on sephadex LH-20 using methanol as eluent to afford new flavonol glycosides 1-4 and a glycolipid 5.

Compound 1 was obtained as yellow amorphous powder, $[\alpha]_D = -17.6^\circ$ ($c = 0.17, \text{MeOH}$), with an elemental composition of $\text{C}_{29}\text{O}_{16}\text{H}_{34}$ as judged from ESI-MS with a sodiated adduct $[\text{M}+\text{Na}]^+$ ion at m/z 661 (Fig.2.3.1) and NMR data. Its flavonoid nature was indicated by intensification of the yellow colour on spraying the silica gel TLC spot with H_2SO_4 and ammonia solution. This is further confirmed by its UV absorption (Fig.2.3.2), which showed λ_{max} (MeOH) at 212, 270 and 341 nm. The absorption at 270nm (band II) determined by A ring and 341 nm (band I) that reflects the substitution patterns in B and C rings. A bathochromic shift of about 49nm observed for band I after addition of sodium methoxide was indicative of presence of free $-\text{OH}$ group at the 3-position¹³. This is further confirmed by the bathochromic shift of 31nm observed for band I on the addition of AlCl_3 ¹⁴. The up field shift on the addition of AlCl_3 , is also an indication that the $-\text{OH}$ group at position 5 is unsubstituted¹⁶. The bathochromic shift of band I observed, as compared to the spectrum in MeOH, on addition of AlCl_3+HCl is indicative of $-\text{OMe}$ group at the C-6 position¹⁸. The lack of shift of band II with CH_3COONa suggested that C-7 $-\text{OH}$ was substituted¹⁵. Infra-red spectrum (Fig.2.3.3) with ν_{max} in KBr at 3404, 1651, 1598 and 1461 cm^{-1} indicated that hydroxyl group, α - β unsaturated carbonyl group and aromaticity could be present in the molecule. Strong absorption at 1056 and 1132 cm^{-1} reflected the glycosidic nature of the compound¹². The ^1H NMR (300MHz, CD_3OD , Fig.2.3.4) spectrum showed doublets ($J=8.4\text{Hz}$) at δ 6.835 and 8.087 integrated for two

protons each representing A_2B_2 system in ring B. This is also confirmed in the 1H - 1H -COSY spectrum of the compound. A broad singlet at δ 6.664 was assigned to C-8 proton. The presence of two methoxyl group was inferred by the presence of singlets at δ 3.791 and 3.902. In the sugar region a well resolved doublet ($J=7.5$) appeared at δ 5.053 ($H-1''$) which was attributed to the anomeric proton of inner glucose and suggestive of β -D-glucosyl residue on the basis of the coupling constant. The terminal sugar unit of this molecule was identified as rhamnose and its anomeric proton was evident at δ 4.472 as broad singlet, in agreement with the α configuration of α -L-rhamnose. The other sugar protons gave unresolved signals between δ 3.239-3.921. The ^{13}C NMR (Table-1, Fig.2.3.5) of compound 1 clearly confirmed the nature of the sugars, the linkages and the identity of aglycone. It contained a carbonyl signal at δ 179.7 and five signals between δ 161.6 and 153.1 representing aromatic carbon atoms directly connected to oxygen atoms. Careful interpretation of the cross signals in the 1H - 1H COSY (Fig.2.3.6), HMQC (Fig.2.3.7) and HMBC (Fig.2.3.8) spectra combined with the coupling constants of the signals in the 1H NMR spectrum resulted in the structure 1 for the compound.

The position of attachment of rhamnose was deduced to be at C-6 of glucose as the chemical shift of this carbon was downfield shifted to 67.5 caused by the glucosylation while the adjacent anomeric carbon of rhamnose underwent an upfield shift of 2.7 ppm at 101.9 ppm. The HMBC spectrum was utilized to identify the position of methoxy groups and disaccharide moieties. A long range correlation between H-8/C-6, H-2'/C-4', H-3'/C-4' established the position of the two methoxyl groups. Other long range correlation observed are as shown in the Fig-1a and Table-1.

Observation of 1H NOESY cross peak (Fig.2.3.9) between the proton at δ 8.07 and the proton at δ 6.8 and the methoxy protons at δ 3.9 further confirmed the position of $-OCH_3$ group at the position 4' of ring B. The decrease in the band I intensity on the addition of NaOMe is an additional evidence that position 4' is methoxylated¹⁶.

HMBC correlation observed between the anomeric carbon at δ 105.2 and the H-8 proton at δ 6.664 led to the placement of disaccharide moiety at C-7 position and this is further supported by the ^1H - ^1H COSY correlation observed between the $-\text{OCH}_3$ at δ 3.791 and the anomeric proton at δ 5.053.

Table-1: NMR data of compound 1

Carbon No.	^{13}C NMR δ_c , ppm	^1H NMR δ_H , ppm	COSY Correlations	HMBC Correlations
Aglycone				
2	160.6	-	-	-
3	135.6	-	-	-
4	179.7	-	-	-
5	153.9	-	-	-
6	130.8	-	-	-
7	159.7	-	-	-
8	92.1	6.66 (b s)	-	C-6,C-7,C-9,C-10
9	153.1	-	-	-
10	106.9	-	-	-
1'	122.5	-	-	-
2'	132.5	8.08(d, 8.4Hz)	H-3', H-5'	C-2, C-4'
3'	116.1	6.86(d, 8.4Hz)	H-2', H-6'	C-1', C-4'
4'	161.6	-	-	-
5'	116.1	6.86(d, 8.4Hz)	H-2', H-6'	C-1', C-4'
6'	132.5	8.08(d, 8.4Hz)	H-3', H-5'	C-2, C-4'
OCH_3	61.1	3.79		C-6
OCH_3	57.0	3.90		C-4'
Glucose				
1''	105.2	5.05(d, 7.5Hz)	$-\text{OCH}_3$	C-8
2''	70.1	3.73		
3''	75.4	3.53		
4''	74.9	3.47		
5''	72.0	3.27		
6''	67.5	3.30	H-5''	
Rhamnose				
1'''	101.9	4.47(bs)		C-6''
2'''	72.9	3.79		
3'''	69.7	3.47		
4'''	73.8	3.23		
5'''	72.2	3.47(m)		
6'''	17.9	1.12(d,6Hz)		

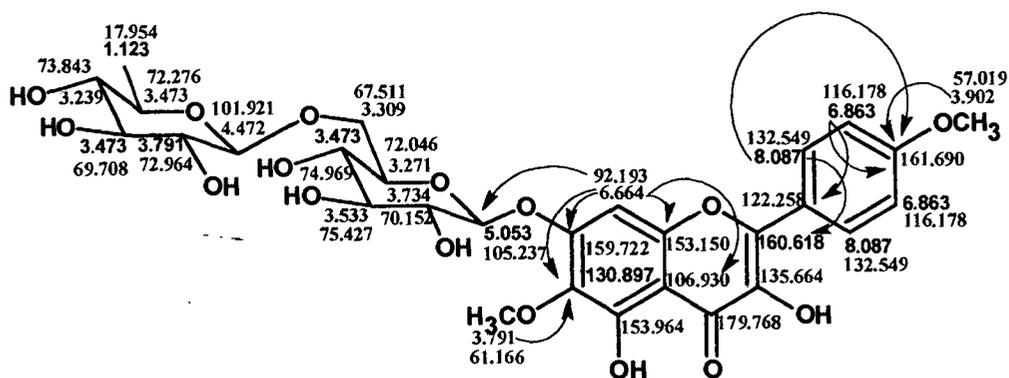


Fig.1a: HMBC correlation in compound 1

As mentioned the ESI-MS of compound 1 showed $[M+Na]^+$ ion at m/z 661 (Fig.2.3.10). The CID mass spectrum of the sodiated flavonoid glycoside molecule is shown in (Fig.2.3.10) and the fragmentation pathway observed in the CID spectrum of the molecule is as shown in Fig-1b. A prominent peak at m/z 353 in the spectrum corresponds to the loss of 308 amu from the sodiated ion, which is equivalent to the mass of rutin (rhamno-glucopyranosyl) moiety from the parent ion.

The fragment ion m/z 515 correspond to the loss of a terminal rhamnose (146 amu) from the sodiated molecule, the protonated aglycone molecule as well as sodiated rhamnoglucosyl moiety corresponded to the fragment m/z 331. Fission with the removal of O-rutinose (O-glucosyl-rhamnosyl) from the sodiated molecule results in the formation of the base peak at m/z 336.9. Additional peaks are seen at m/z 169 and m/z 185 due to the sodiated rhamnose and glucose sugar moieties respectively. Thus, compound 1 was established to be 3,5 dihydroxy 6, 4' dimethoxy-flavone-7-O-[α -L-rhamnopyranosyl-(1 \rightarrow 6)- β -D-glucopyranoside].

The fragmentation pattern observed in ESI-MS/MS spectrum of compound 1 is well in agreement with the structure assigned (Fig.-1b)

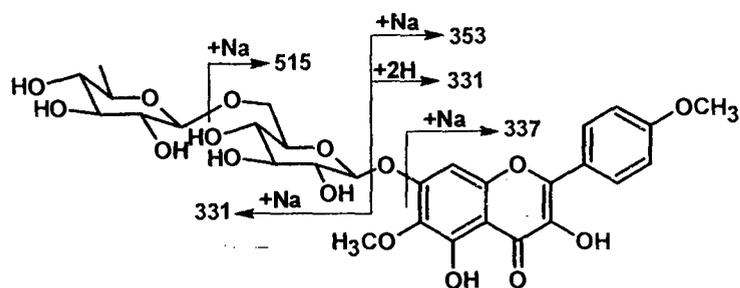


Fig-1b: Fragmentation observed in the ESI-MS/MS of compound 1

Since more of compound 1 was needed for substitution pattern studies, more material was collected. ESI-MS/MS (Fig.2.3.10) of compound 1 of repeat collection showed the presence of an additional signal as sodiated ion $[M+Na]^+$ at m/z 691 besides $[M+Na]^+$ 661 for compound 1.

The structure of compound with $[M+Na]^+$ at m/z 691 and designated as compound 2 was established as 3,5-dihydroxy-6,3',4'-trimethoxy-flavone-7-O- $[\alpha$ -L-rhamnopyranosyl-1(1 \rightarrow 6)- β -D-glucopyranoside. Identification of compound 2 was possible from the chemical shifts obtained by spectra subtraction using spectral data for mixture of 1+2 and that of pure 1.

The ^1H NMR and ^{13}C NMR spectrum (Fig.2.3.11, Fig.2.3.12 Table-2) of compound 1 of repeat collection showed the presence of an additional flavonoid with trisubstituted ring B. Aromatic B ring protons were evident as doublets at δ 7.58 (H-6') and 6.88 (H-5') and a broad singlet at δ 8.02 (H-2'). A long range HMBC correlation was observed for the proton at δ 8.02 with the carbon at 160.65 and the methoxy at δ 4.09. The latter showed HMBC connectivity with the carbon at δ 148.421. All these spectral data was indicative of an additional $-\text{OCH}_3$ group at H-3' position of ring B in compound 1. In the substitution pattern studies of compound 1 it was observed that on addition of NaOMe, the UV spectrum, showed an additional band at 247nm. Two absorption maxima of band II is indicative of a 3',4' oxygenated ring B system¹⁷ further supporting the presence of two $-\text{OCH}_3$ in ring B. The ^1H NMR of the admixture also showed the presence of an additional anomeric proton as a doublet at δ 5.34.

Table-2: NMR data of compound 2

Carbon No.	¹³ CNMR δc, ppm	¹ HNMR δH, ppm	COSY Correlations	HMBC Correlations
Aglycone				
2	160.6	-	-	-
3	135.4	-	-	-
4	179.6	-	-	-
5	153.9	-	-	-
6	133.4	-	-	-
7	159.7	-	-	-
8	92.2	6.88(bs)	-	C-6,C-7,C-9,C-10
9	153.2	-	-	-
10	107.0	-	-	-
1'	122.5	-	-	-
2'	114.6	8.02(d, 2.1Hz)	-	C-2
3'	148.4	-	-	-
4'	159.2	-	-	-
5'	116.1	6.92(d, 8.4Hz)	H-6'	C-1', C3'
6'	123.9	7.58(dd, 2.1, 8.4Hz)	H-5'	C-2, C-6'
OCH ₃	61.1	3.73	-	C-6
OCH ₃	57.0	3.91	-	C-4'
OCH ₃	61.1	4.09	-	C-3'
Glucose				
1''	104.6	5.34(d, 7.5Hz)	-	-
2''	70.1	3.60	-	-
3''	75.5	3.57	-	-
4''	74.9	3.17	-	-
5''	72.0	3.15	-	-
6''	67.5	3.34	H-5''	-
Rhamnose				
1'''	101.9	4.60(bs)	-	C-6''
2'''	73.0	3.86	-	-
3'''	70.0	3.82	-	-
4'''	73.8	3.17	-	-
5'''	72.2	3.91(m)	-	-
6'''	17.9	1.05(d, 6Hz)	-	-

The fragmentation pathways observed in the ESI-MS/MS (Fig.2.3.13) compound 2 is shown in Fig.-1c. The fragmentation is similar to that observed for compound 1 with the elimination of rhamnosyl moiety leading to the fragment at m/z 545 which further losses hexose to generate sodiated aglycone at m/z 383. Cleavage of the C₇-O bond produced sodiated adduct ion at m/z 367 which further dissociated with the loss of CO to form ion at m/z 339.

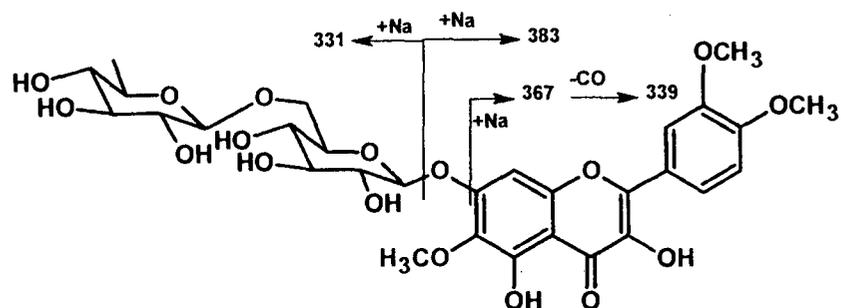


Fig.-1c: ESI-MS/MS fragmentation of compound 2

On the basis of foregoing evidence, compound **2** was identified as 3,5-dihydroxy-6,3',4'-trimethoxy-flavone-7-O- $[\alpha$ -L-rhamnopyranosyl-1(1 \rightarrow 6)- β -D-glucopyranoside.

Compound **3**, next to compound **1** in polarity, was isolated as amorphous yellow powder, $[\alpha]_D = -30.1^\circ$ ($c = 0.14$, MeOH). The molecular formula $C_{28}H_{32}O_{17}$ for **3** was deduced from its pseudomolecular ion $[M+Na]^+$ at 663 in its ESI-MS (Fig.2.3.14) and NMR data. UV spectral maxima (Fig.2.3.15, Table-3) in MeOH at 204, 260 and 349 nm indicated that **3** was a C3-O-substituted flavonol¹⁵. The bathochromic shift of band I with $AlCl_3+HCl$ (20nm) is a characteristic feature of a 5-hydroxy-3O-substituted flavonol, it is also indicative of the presence of -OMe group at the C-6 position. This is further evidenced by the presence of a pronounced shoulder at around 410nm in the long wavelength side of the band I in the $AlCl_3+HCl$ spectrum¹⁸.

A bathochromic shift of 53nm without decrease in intensity observed for band I after addition of NaOMe was indicative of free -OH group in the 4' position¹⁹. On the basis of mass and UV spectral data, **3** was assigned to be a sodium salt of a flavonol glycoside.

Table-3: UV data of flavonoid glycosides*

Solvent	Compound (1)	Compound (3)
MeOH	341, 270, 212	349, 260, 204
MeONa	390, 276, 247, 216	402, 340sh 273, 204
AcONa	342, 270, 212	355, 270, 205
AlCl ₃	372, 278, 213	400, 273, 206
AlCl ₃ + HCl	361, 274, 213	410sh, 369, 276, 206

*Expressed as λ_{\max} (nm).

The infrared spectrum (Fig.2.3.16) showed the presence of hydroxyl (3408cm^{-1}), α,β - unsaturated ketone (1647cm^{-1}) and aromatic rings ($1556, 1506\text{cm}^{-1}$). The ^1H NMR spectrum (300MHz, CD_3OD , Fig.2.3.17) was characteristic of a flavonol with four broad signals in the region of δ 6-8. The signal at δ 6.55 was assigned to H-8 proton of ring A and the remaining three aromatic signals were diagnostic of ABX substitution pattern in ring B with the absorption at δ 6.86 (d, $J=8.4\text{Hz}$) being assigned to H-5', the signal at δ 7.86 (d, $J=2.0\text{Hz}$) was attributed to H-2' and the doublets of doublet at δ 7.51 to H-6' (dd, $J=8.4, 2.0\text{Hz}$).

These data were confirmed by ^1H - ^1H COSY (Fig.2.3.18) correlation of dd at δ 7.51 with the doublet δ 6.8. Along with aromatic signals, unresolved sugar protons appeared as overlapping broad multiplets in the region (δ 3-4.1) with the methyl group of rhamnosyl moiety being evident as a doublet at δ 1.2 ($J=6.2\text{Hz}$).

The two anomeric signals at δ 5.1 (d, $J=7.5\text{Hz}$) and broad signal at δ 4.526 respectively, indicated the presence of two linked sugar units with β and α configuration respectively. Sugar protons assignment was not possible due to overlapping of signals with the $-\text{OCH}_3$ methoxy signals. The ^{13}C resonances of the sugars were assigned by the one bond heteronuclear HMQC experiment (Table-4, Fig.2.3.19).

Table-4: NMR data of compound 3

Carbon No.	¹³ CNMR δ _c , ppm	¹ HNMR δ _H , ppm	COSY Correlations	HMBC Correlations
Aglycone				
2	159.1	-	-	-
3	135.5	-	-	-
4	179.7	-	-	-
5	153.7	-	-	-
6	132.4	-	-	-
7	158.8	-	-	-
8	95.0	6.55(bs)	-	C-6,C-7,C-9,C-10/C-1''
9	153.5	-	-	-
10	105.8	-	-	-
1'	123.0	-	-	-
2'	117.9	7.86(d,2.0Hz)	H-6'	C-2, C-1', C-3'
3'	149.9	-	-	-
4'	145.7	-	-	-
5'	116.1	6.86 (d,8.4Hz)	H-6'	C-1', C-3',C-4'
6'	122.8	7.51(dd, 8.4,2.0 Hz)	H-2', H-5'	C-2'
OCH ₃	60.7	3.88	-	C-6
Glucose				
1''	105.8	5.10(d, 7.5Hz)	-	C-3
2''	70.1	} 3 – 4.1	-	-
3''	75.3		-	-
4''	74.3		-	-
5''	72.0		-	-
6''	67.3		H-5''	-
Rhamnose				
1'''	101.9	4.52(bs)	-	C-6''
2'''	73.1	} 3 – 4.1	-	-
3'''	71.2		-	-
4'''	73.8		-	-
5'''	72.2		-	-
6'''	17.9		1.20 (d,6Hz)	-

Thus based on ¹³C and ¹HNMR shift values and coupling constants, the sugar units were identified as β-glucose and α -rhamnosyl respectively. The crosspeak at 5.1/135.52 ppm in 2D long range HMBC experiment (Fig.2.3.20) between the anomeric glucosyl proton and C-3 showed that the sugar moiety was linked to the 3 position of the aglycone. The linkage point between the two sugars units was indicated to be at the glucosyl 6''-hydroxyl by the downfield shift of the C-6'' (67.36ppm) in the DEPT spectrum (Fig.2.3.21). The cross peaks at 4.52/67.3ppm in the long range HMBC spectrum further confirming this linkage point. A sodiated molecular ion at [M+Na]⁺ at m/z 662.9988 confirmed the identity of

compound 3 to be 5,7,3',4'-tetrahydroxy-6-methoxy-flavone-3-O-[α -L-rhamnopyranosyl-1(1 \rightarrow 6)- β -D-glucopyranoside]-5,7,3',4'-tetrahydroxy-6-methoxy 3-O-rutinoside.

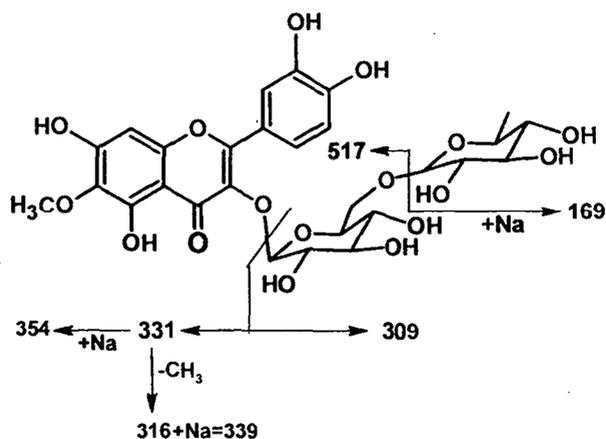


Fig.-1d: ESI-MS/MS fragmentation of compound 3

The structure assigned was well in agreement with the fragmentation pathway observed in its MS/MS (Fig.2.3.22) spectrum as shown in Fig.-1d.

The ESI-MS of compound 3 (Fig.2.3.14) displayed another intense signal at m/z 647, which is 16 amu less than the molecular mass of compound 3 indicating it to have one $-OH$ hydroxyl group less than compound 3. It produced $[M+Na-308]$ ion at m/z 337.9789 in its MS/MS (Fig.2.3.23). The fragmentation observed in its tandem mass spectrum was well in agreement with the identification of compound 4 as 5,3',4'-trihydroxy-6-methoxy-flavone-3-O-[α -L-rhamnopyranosyl-1(1 \rightarrow 6)- β -D-glucopyranoside]/5,3',4',trihydroxy-6-methoxy-flavone-3-O-rutinoside (Fig.-1e).

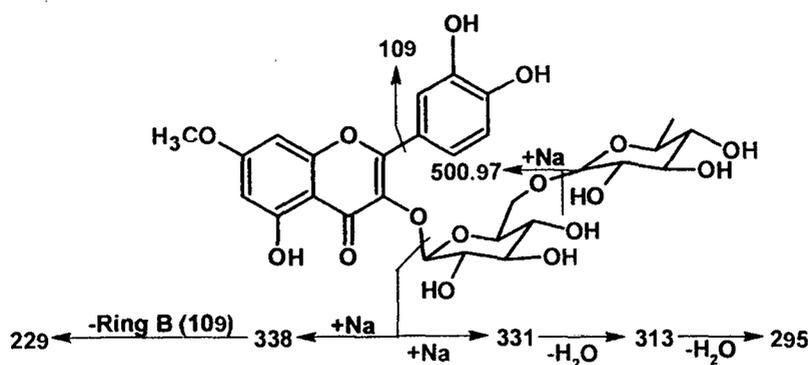


Fig.-1e: ESI-MS/MS fragmentation of compound 4

C-Flavonoids and O-flavonoids have been used chemotaxonomically as a distinguishing characteristic for plants belonging to family *Molluginaceae* and *Aizoaceae*²⁴. *Sesuvium portulacastrum* belongs to *Aizoaceae* and as such it is expected to contain O-flavonoids and not C-flavonoids. This has in fact been observed in the present case well in agreement with the reports of Richardson(1981)²⁴.

The most polar component, compound 5, was identified as a glycolipid on the basis of following spectral data. Its infra-red spectrum (Fig.2.3.24) was typical of a polyhydroxy compound with a broad band at 3382 cm⁻¹ due to the hydroxyl functionality. A pronounced signal at 1732 cm⁻¹ was assigned to the carbonyl group of the ester. The peaks at 2927 and 2856 cm⁻¹ for aliphatic CH stretching were also present in the spectrum. ESI-MS/MS spectrum (Fig.2.3.25) of compound 5 exhibited a pseudomolecular ion [M+Na]⁺ at m/z 775.5978 indicating a molecular formula of C₄₃H₇₆O₁₀Na. The ¹HNMR spectrum (Fig.2.3.26; Table-5) was typical of a glycolipid, monogalactosyldiacyl glycerol with signals for C-1 protons of glycerol, substituted by an O-acyl group, being evident at δ 4.36 and δ 4.21 as doublets, a multiplet at δ 5.28 was assigned to C-2 and C-3 protons of glycerol substituted by the β-galactose residue appeared as doublets at δ 3.71 and δ 3.89. This conclusion was supported by the ¹³C NMR spectrum (Fig.2.3.27), wherein the resonances of the glycerol C-1, C-2 and C-3 were observed at δ 62.8, δ 70.1 and 68.03 respectively which is in agreement with the relevant literature data.

Its ¹HNMR spectrum also exhibited two terminal methyl signals, one as a deformed triplet at δ 0.874 (3H, t, J= 6.9Hz) due to the presence of saturated fatty acyl chain and a terminal methyl residue at δ 0.959 (3H, t, J= 7.5 Hz) of unsaturated fatty acyl chain; a broad signal, split into two, the less intense one at δ 1.242 and the more intense at δ 1.289 were assigned to the methylene protons of the aliphatic chains of the two acyl groups; multiplets centered at δ 2.3, 2.0 and 1.58 were attributed to the methylene groups linked α,β, and γ respectively to the

ester functional group; a triplet at δ 2.78 (4H, m) is usually observed for the allylic methylene group of the type present in unsaturated fatty acyl group such as linolenic acid. The olefinic methine protons were evident as cluster at δ 5.31- δ 5.33. The sugar protons were apparent in the region between δ 3.71-4.36 there being some overlapping of the signals with the glycerol protons.

The ^{13}C NMR spectrum showed two quaternary carbons at δ 173.8 and 173.4 for ester carbonyl functions; terminal methyls were evident at δ 14.06, methylene carbons were evident in the region between δ 20.5 and 34.22. Anomeric carbon appeared as a doublet at δ 103.9 with the corresponding anomeric proton being evident at δ 4.23 as a doublet with a coupling constant of 7.2 Hz indicative of β -linkage of sugar with glycerol. On the basis of foregoing evidence compound 5 was identified as a monogalactosyldiacyl glycerol. The glycerolipid structure was confirmed by the presence of a characteristic signal at δ 70.1/5.28 (C-2) having a distinct α -shift to lower field for ^{13}C and ^1H nuclei when substituted by an O-acyl group, this being a fingerprint for glycolipids containing glycerol as alcohol rather than sphingosine²⁵. In addition, the C-2 configuration in the glycerol moiety of 5 is presumed to be *R* on the basis of a comparison of the specific rotation with the reported values, $[\alpha]_{\text{D}} = -6.95^{\circ}$ ($c = 0.58$, MeOH):²⁰.

Total COSY (Fig.2.3.28), and HSQC (Fig.2.3.29) correlations allowed assignment of sugar carbons and protons (Table-5). TOCSY correlation of the anomeric proton at δ 4.23 with the sn-3 protons at δ 3.71 and δ 3.89 established connectivity of the sugar moiety with the glycerol. The anomeric proton at δ 4.23 with a coupling constant of 7.2 Hz was suggestive of β - configuration of the sugar linkage with the glycerol²¹. Long range HMBC correlation allowed the identification of fatty acyl group at C-1 of glycerol as α -linolenic acid i.e. 9,12,15 octadecatrienoic acid. (Table-5).

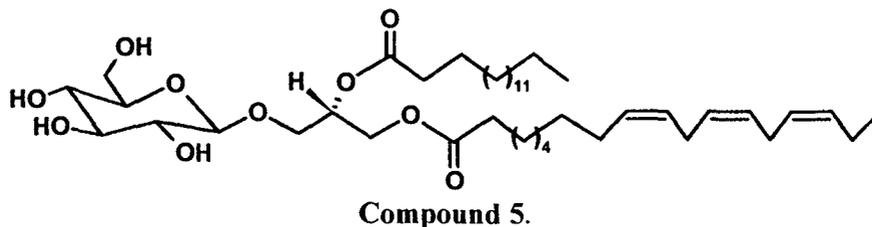


Table-5: NMR data of compound 5

Carbon No.	¹ HNMR δ_H , ppm	¹³ CNMR δ_C , ppm	HMBC Correlations
1a	4.36(d, 9.9Hz)	62.8	-
1b	4.21(d, 6.6Hz)	62.8	-
2	5.28 m	70.1	-
3a	3.71(d, 6.6Hz) 3.89 (d, 4.5Hz)	68.0	-
1'	4.23(d, 7.2 Hz)	103.9	-
2'	3.60	71.1	-
3'	3.88	68.9	-
4'	3.71	74.5	-
5'	3.66	73.3	-
6'	3.89, 3.91	61.5	-
1''	-	173.8	-
2''	2.30(d, 4.2Hz)	34.2	3'', 1'', 4''
3''	1.58	25.4	2'', 4''
4''- 7''	1.28	29.1- 29.6	3'', 5''
8''	2.05	27.1	9'', 10''
11'', 14''	2.78(t, 5.7Hz)	25.5	12'', 13''
9'', 10'', 12'', 13'', 15'', 16''	5.31-5.33 (Cluster)	127.0-131.9 (6d)	-
17''	1.23(s)	31.8	18''
18''	0.95(t, 7.5Hz)	14.2	17''
1'''	-	173.4	-
2'''- 3'''	2.03-1.58(m)	24.8-27.1	-
4'''- 15'''	1.28	22.5-25.5	-
16'''	0.87(t, 6.9Hz)	14.0	-

The ESI-MS/MS of the sodiated ion at m/z 775.5 (Fig.2.3.25) showed the presence of a fragment at m/z 243.1, characteristic of MGDG²³ resulting from the concomitant elimination of two fatty acyl moieties. Ions reflecting neutral loss of palmitate and linolenate (C_{18:3}) as free fatty acids are evident from the fragment ions at m/z 519.3 and m/z 497.3 respectively. The abundance of ion at m/z 519.3 as compared to the ion at 497.3 is consistent with the notion that neutral loss of the fatty acid at sn-2 position is sterically more favorable than the analogous loss at the sn-1 position²². Thus, compound 5 was identified as (2R)-1-O-linolenyl-2-O- (palmitoyl) 3-O- β -D-galactopyranosyl-sn-glycerol.

Sesuvium portulacastrum is a potential halophytic plant known to accumulate more Na⁺ in comparison with K⁺ and under such salt stress conditions the plant is reported to increase its sulfolipid content which helps in osmoregulation². In the present investigation the major glycolipid was found to be (2R)-1-O-linolenyl-2-

O- (palmitoyl) 3-O-β-D-galactopyranosyl-sn-glycerol, a monogalactosyl diacyl glycerol and not sulfoquinovosyl diacyl glycerol as expected probably because of the solubility of the latter in water as it mostly occurs as mono/disodium salt. The aqueous fraction is under investigation for the presence of sulfonoglycosides.

EXPERIMENTAL

General: column chromatography was carried out on sephadex LH20 (25-100 μm) (Pharmacia). Final purification was carried out on HPLC using RP-18 column. NMR was recorded on a Bruker Avance 300MHz instrument in deuterated methanol and deuterated chloroform with TMS as the internal standard. ESI-MS spectra were recorded, in the positive mode, on a QSTARXL MS/MS Applied Biosystems instrument, Switzerland equipped with Analyst Software.

Collection of biological material:

Sesuvium portulacastrum was collected at Sinqerim beach and identified by Dr Sayeeda Wafar of Biological Oceanography Division of NIO. The specimen has been deposited at Taxonomic Reference Centre, NIO.

Isolation of constituents:

The sea pursulane (3 Kgs) was extracted thrice with methanol and the crude extract partitioned successively with chloroform and n-butanol. Flavonoids were isolated from the n-butanol fraction by gel filtration over sephadex LH20 using methanol as eluent. The eluted fractions were combined on the basis of their TLC profile using butanol: acetic acid: water (5:1:4) as the solvent system. This was followed by column chromatography over silica gel and gradient elution with increasing concentration of methanol in chloroform. Compound 1 eluted first in 10% Methanol, followed by compound 3 in polarity, which eluted in 15%

methanol. Final purification was achieved by chromatography of the partially purified material over ODS column with (MeOH: CHCl₃)(15%: 85%) as eluent.

UV spectra in methanolic reagents:

The reagents and the procedure used for obtaining UV spectra of flavonoids were those described by Mabry et al. 1970. In **Table-3** UV spectra in methanol and four methanolic shift reagents are given. Flavonoids are identified by the presence or the absence of spectra shifts that occur in the presence of these shift reagents.

UV/Vis spectra were measured in methanol, sodium methoxide, methanolic aluminium chloride, methanolic aluminium chloride –hydrochloric acid, and methanolic sodium acetate. All spectra were recorded using Shimadzu spectrophotometer. As an example in one such experiment the sample of purified flavonoid was dissolved in methanol. Using 1 ml absorption cell, the spectrum of methanolic solution of purified flavonoid was recorded first. This was followed by the addition of shift reagent; say one drop of sodium methoxide, to 0.5 ml of flavonoid solution and the spectrum recorded again. The process was repeated with other shift reagents.

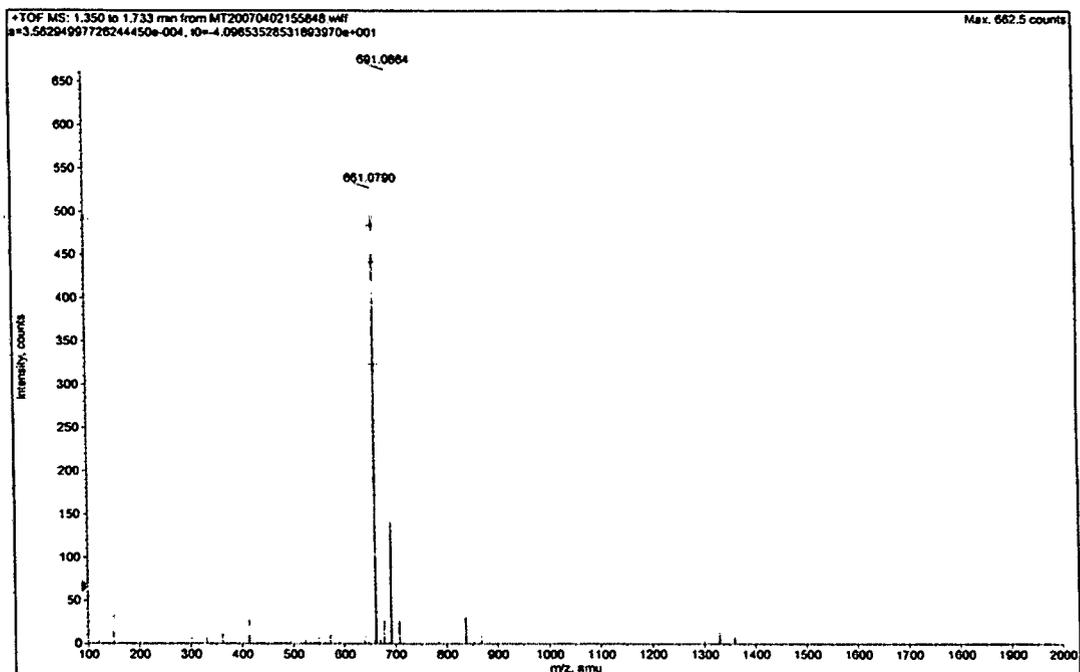


Fig.2.3.1: ESI-MS of repeat collection.

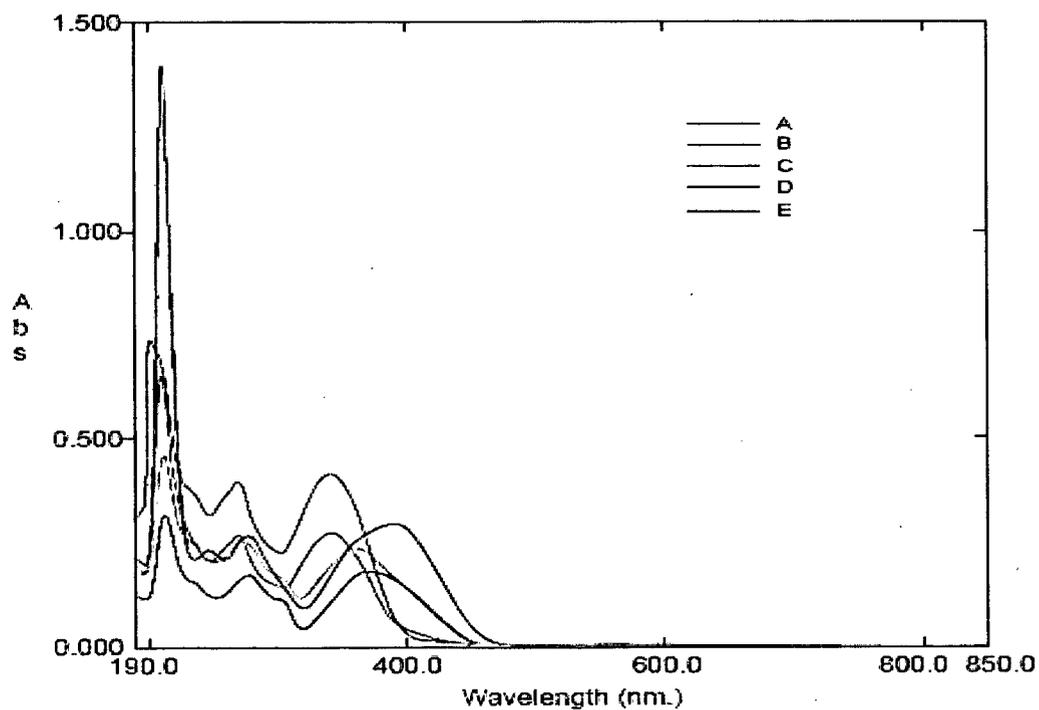


Fig.2.3.2: UV spectrum of compound 1.

A- MeONa: 390, 276, 247, 216.

B- MeOH: 341, 270, 212.

C- AcONa: 342, 270, 212.

D- AlCl₃: 372, 278, 213.

E- AlCl₃+ HCl: 361, 274, 213.

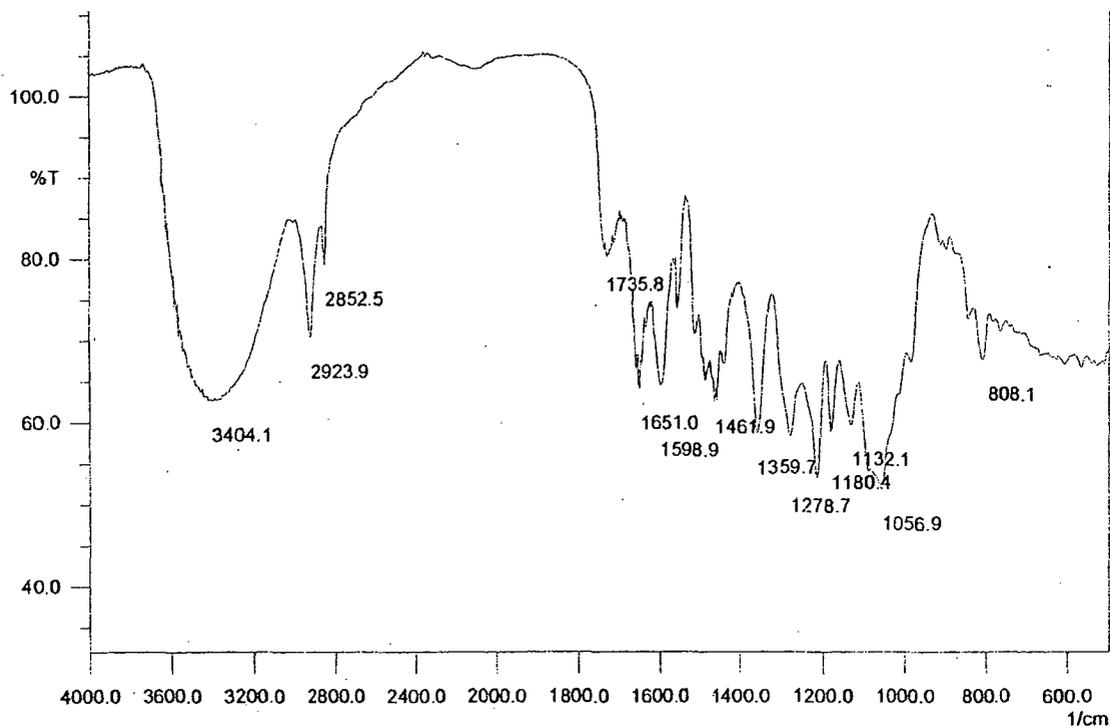


Fig.2.3.3: IR spectrum of compound 1.

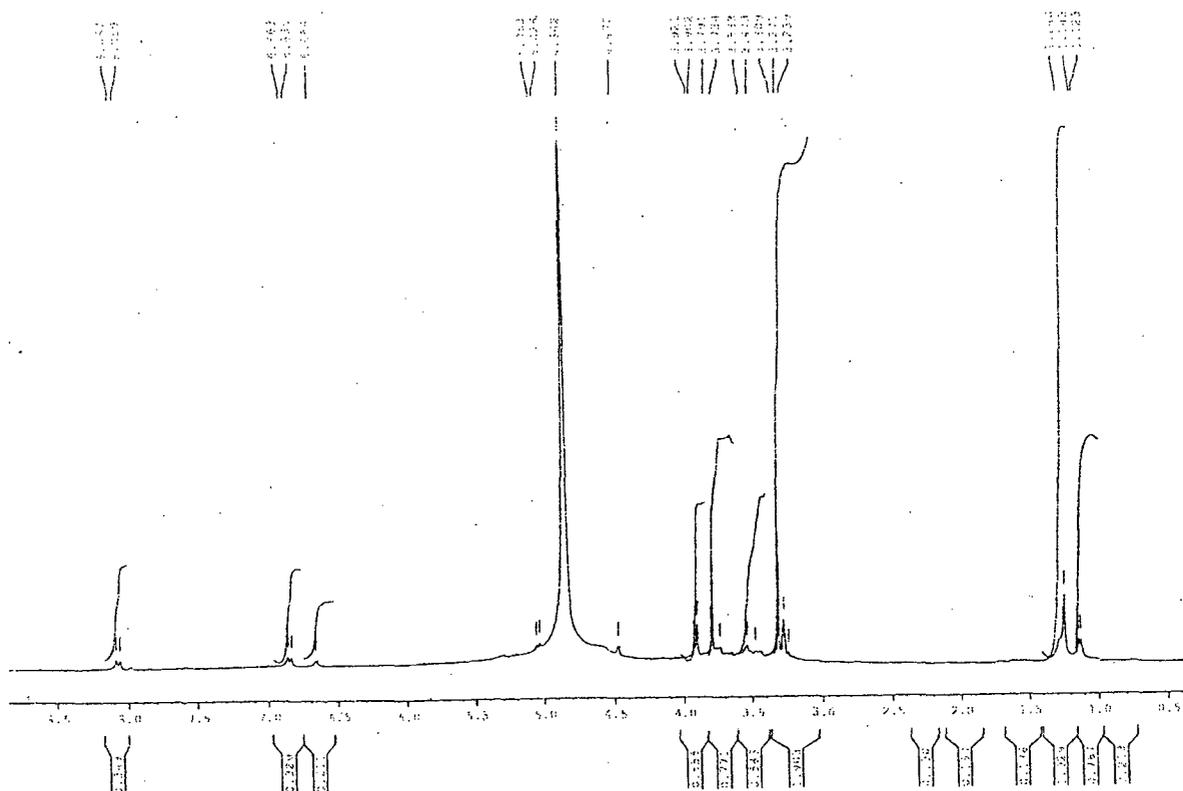


Fig.2.3.4: ¹H NMR of compound 1.

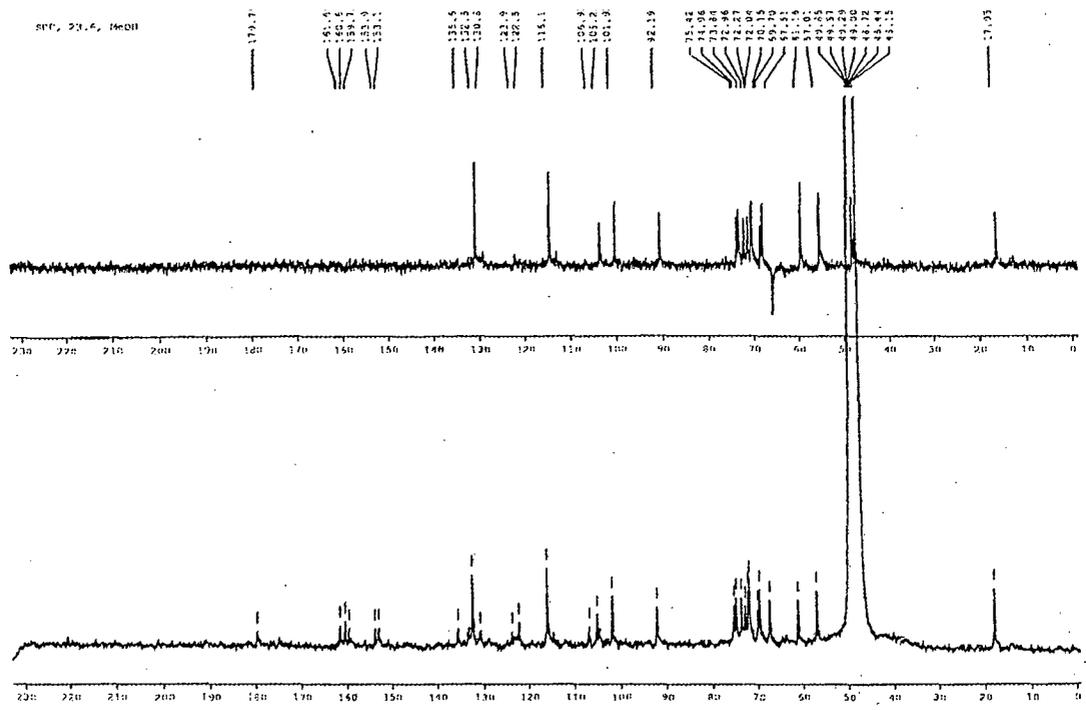


Fig.2.3.5: ¹³CNMR and DEPT of compound 1.

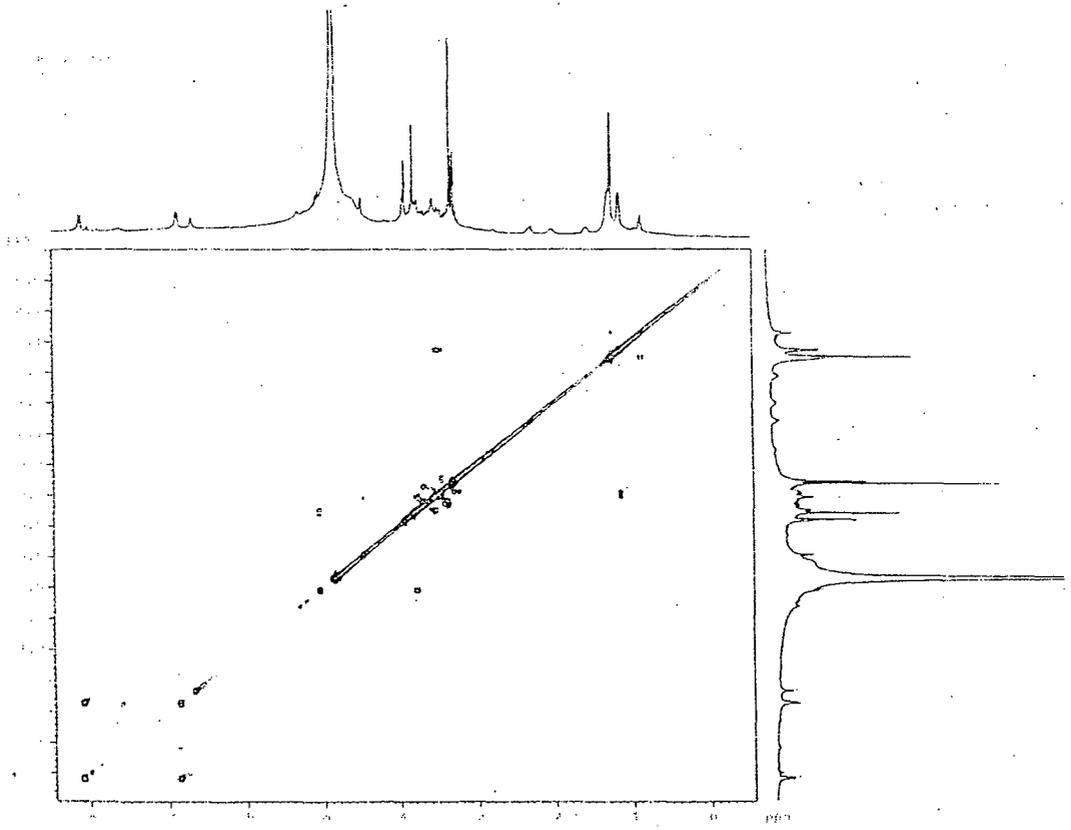


Fig.2.3.6: ¹H-¹H COSY of compound 1.

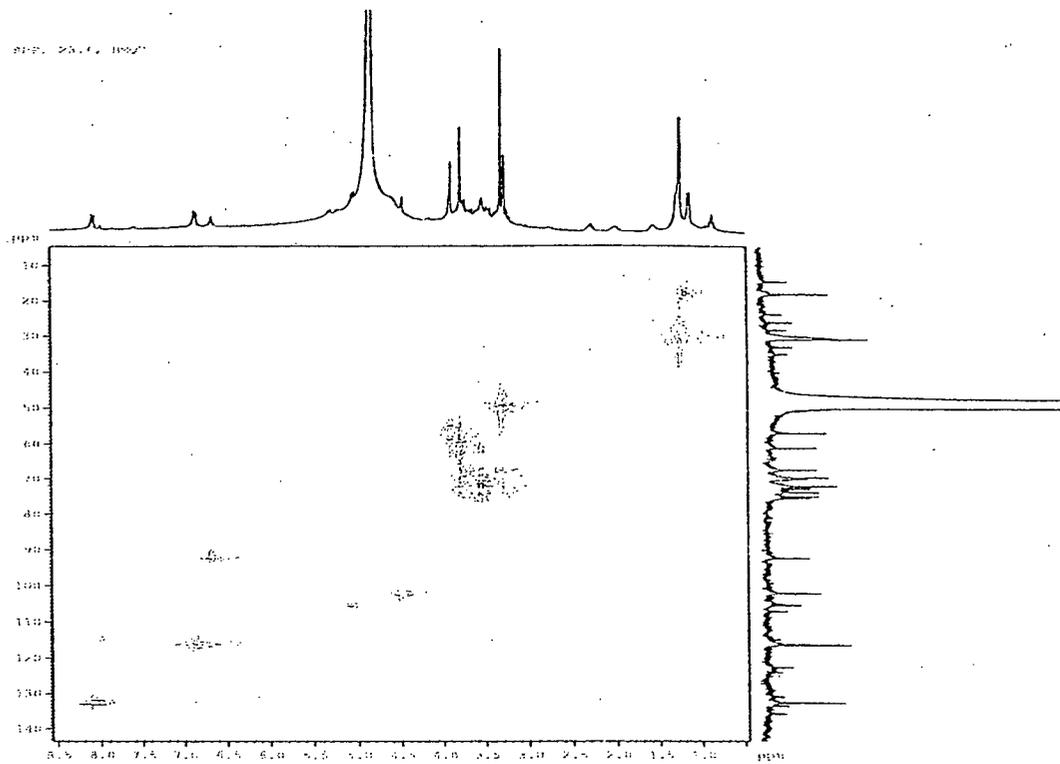


Fig.2.3.7: HMQC of compound 1.

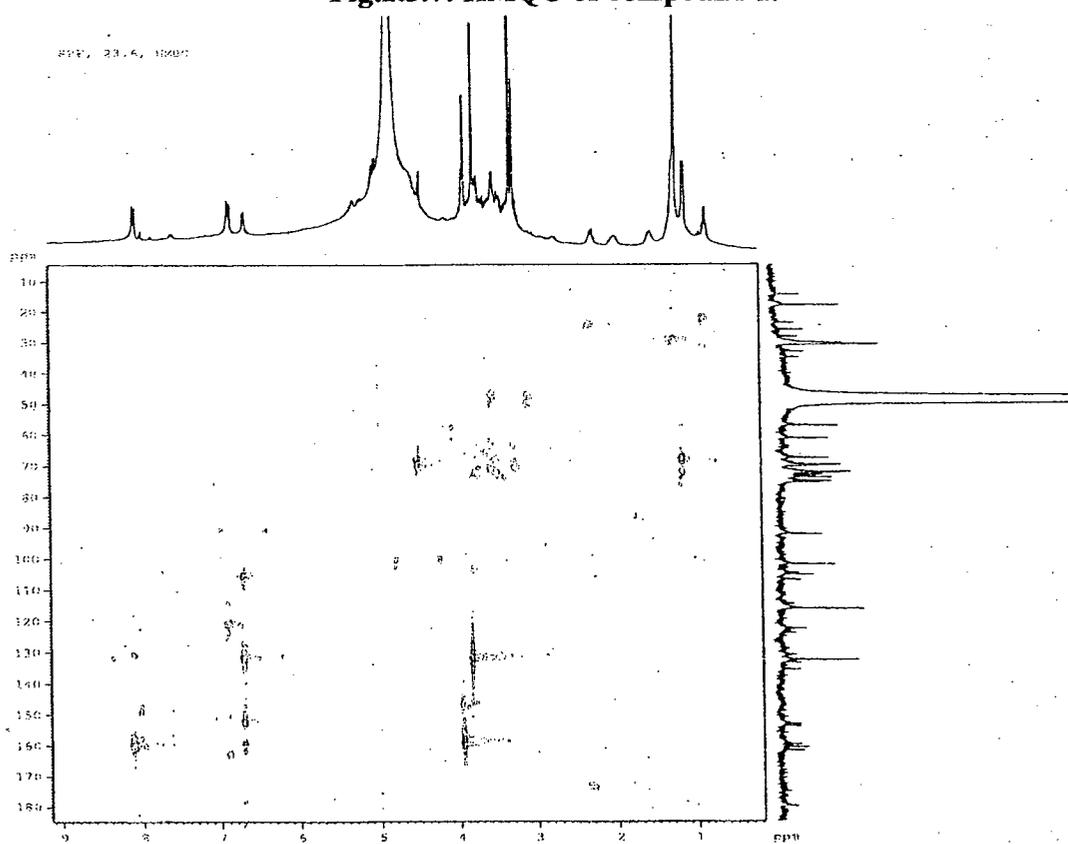


Fig.2.3.8: HMBC of compound 1.

SPF, NOESY

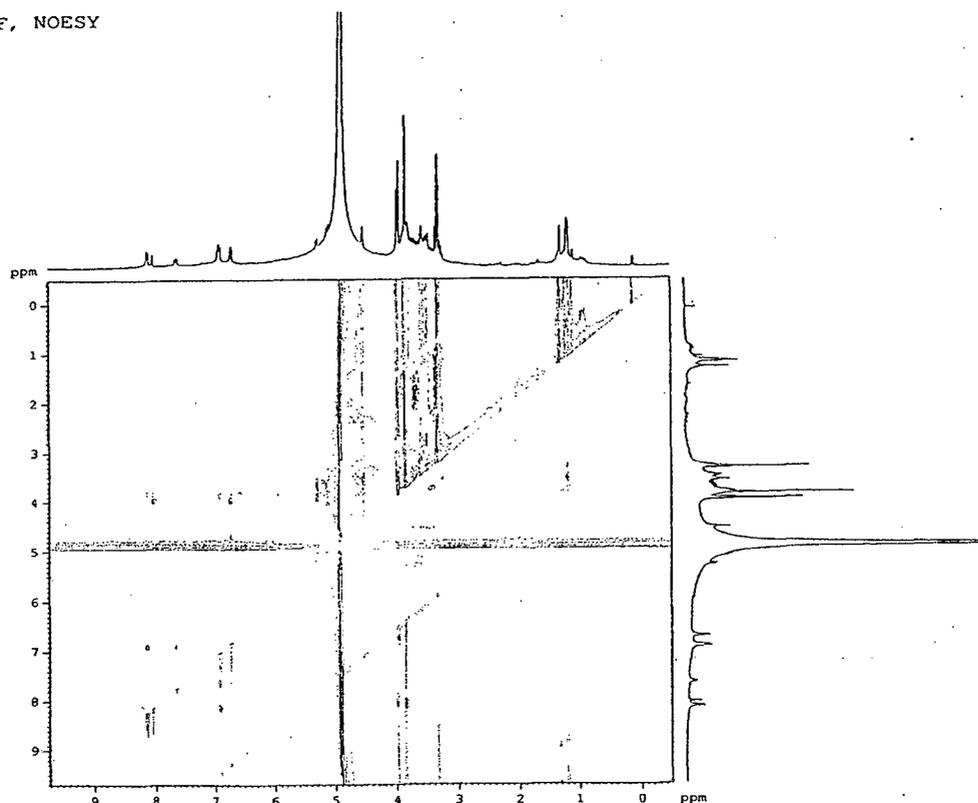


Fig.2.3.9: ^1H NOESY of compound 1.

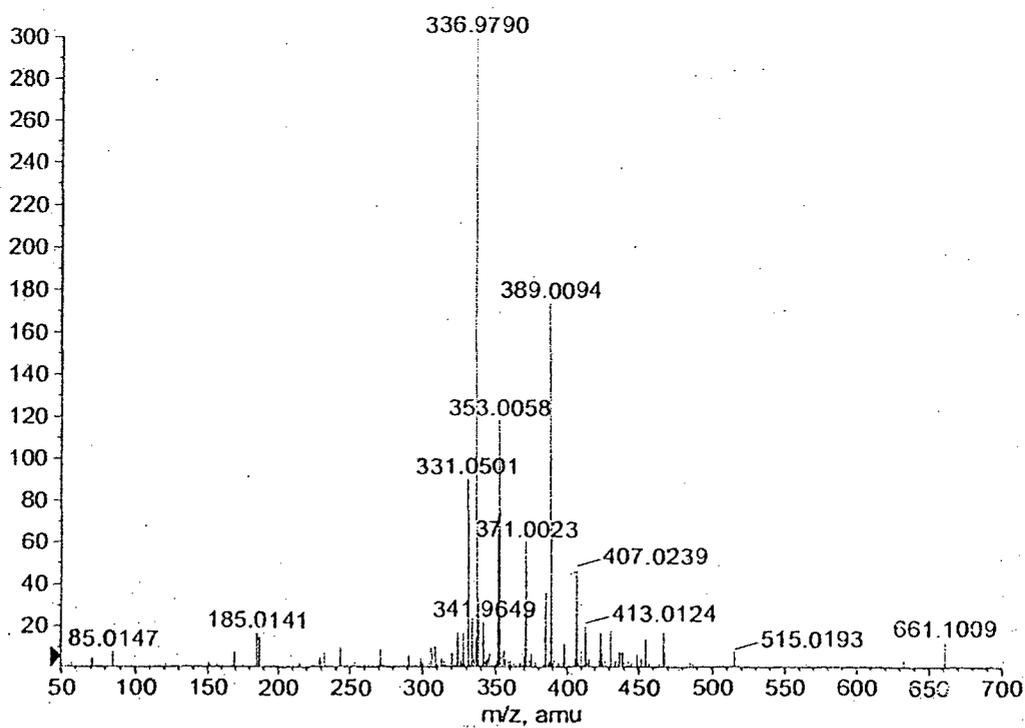


Fig.2.3.10: MS/MS spectrum of compound 1.

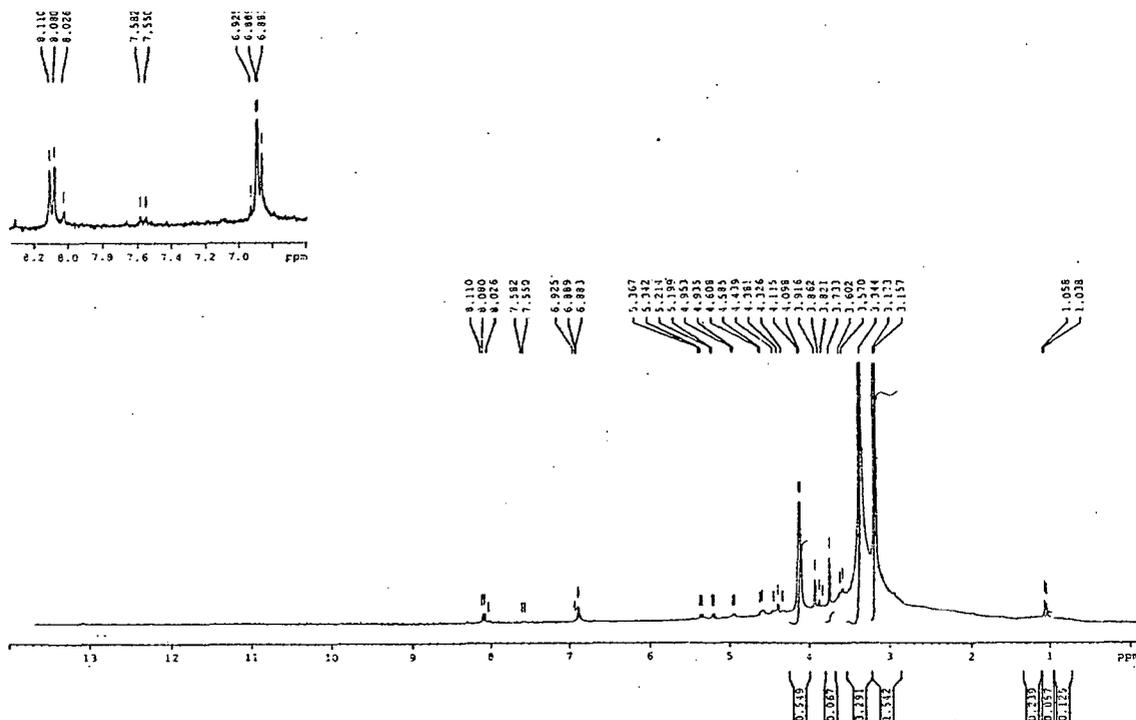


Fig.2.3.11: ^1H NMR spectrum of compound 1 of repeat collection.

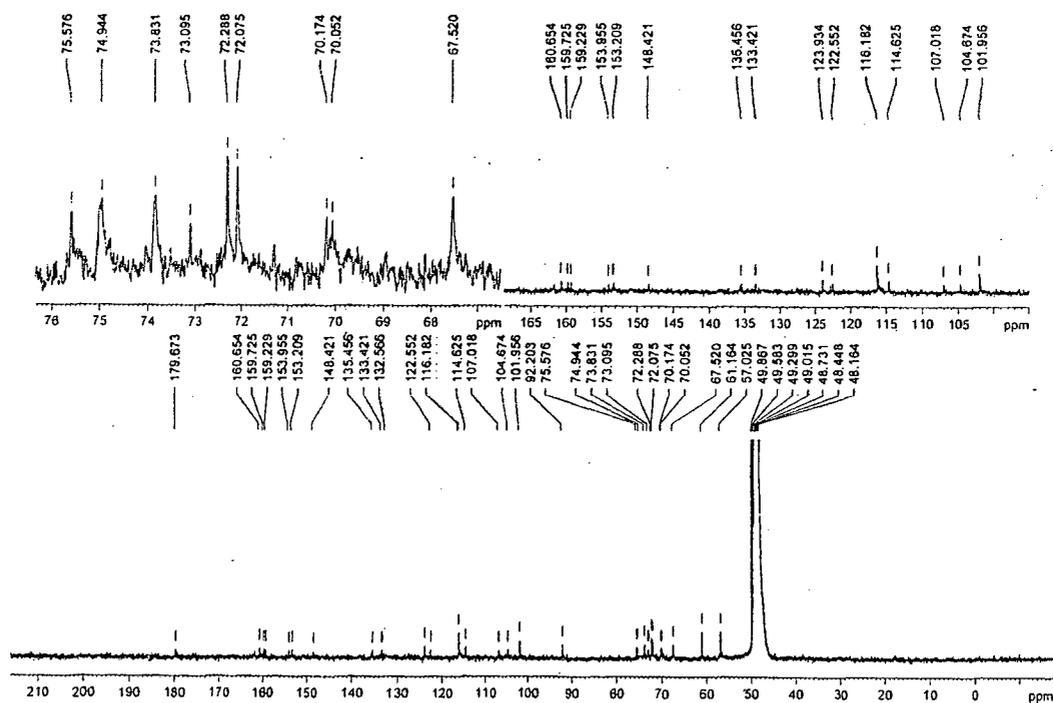


Fig.2.3.12: ^{13}C NMR spectrum of compound 1 of repeat collection.

z=3.56294997726244450e-004, t0=-4.09853528531893970e+001

Max. 662.0 counts

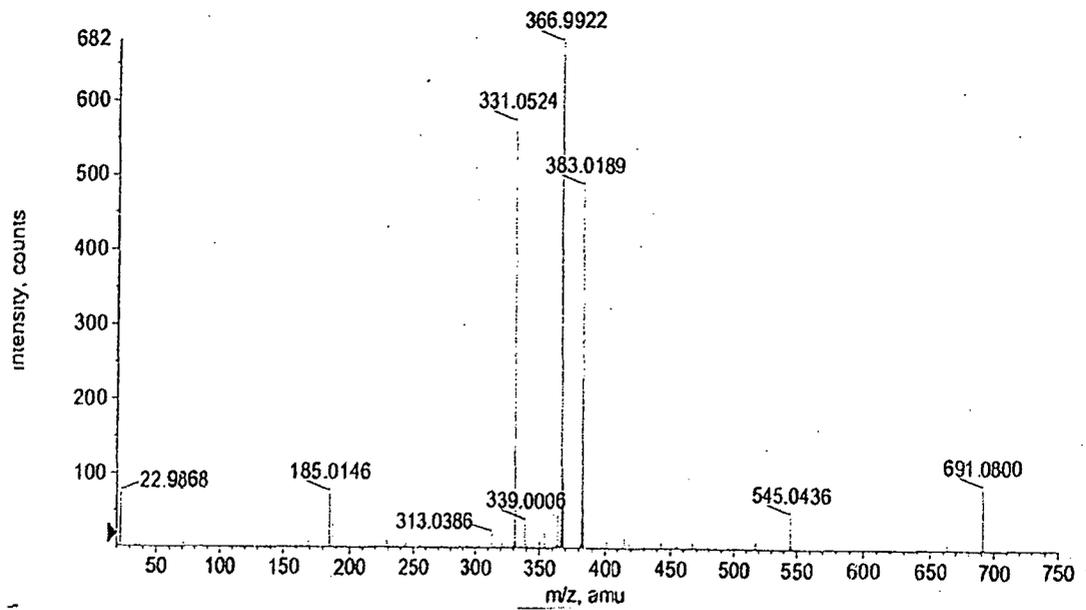


Fig.2.3.13: ESI-MS/MS of compound 2.

+TOF MS: 3.283 min from MT20070403103731.wiff
z=3.58294997726244450e-004, t0=-4.09853528531893970e+001

Max. 260.0 counts

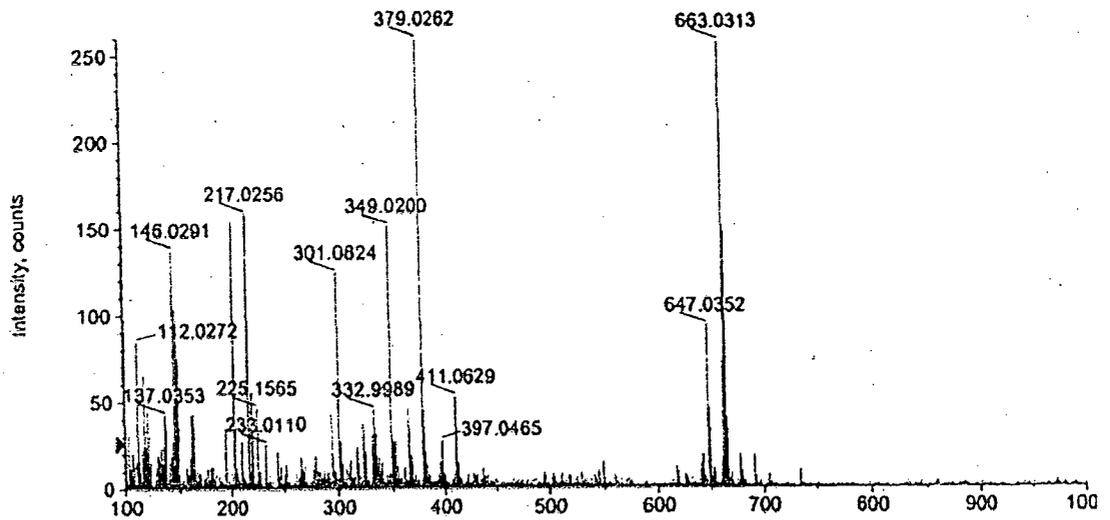


Fig.2.3.14: ESI-MS/MS of compound 3.

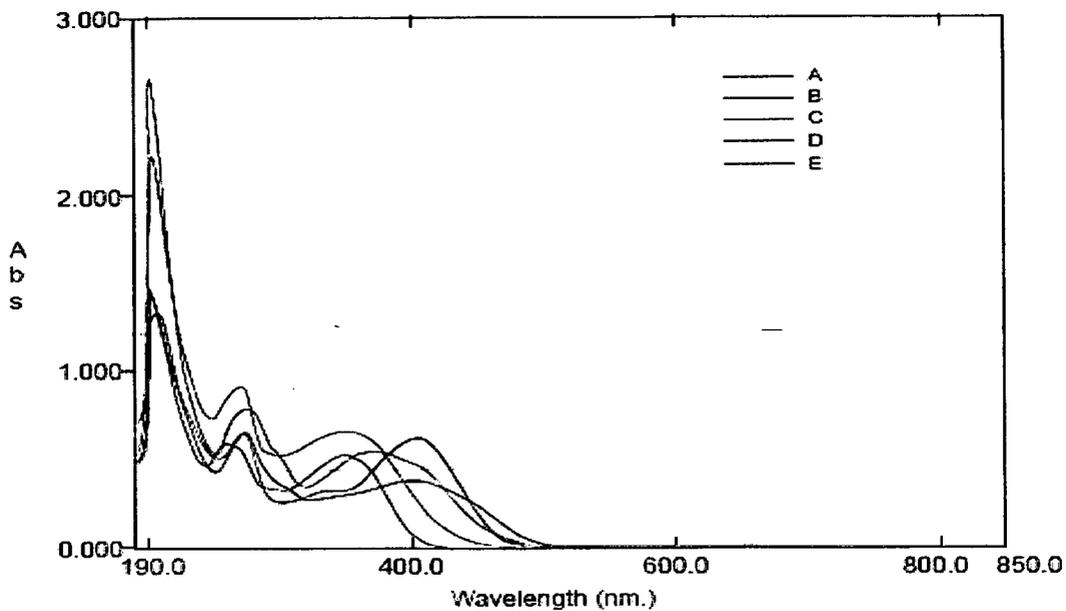


Fig.2.3.15: UV spectrum of compound 3.

A- AlCl_3 : 400, 273, 206.

B- MeOH: 349, 260, 204.

C- AcONa: 355, 270, 205.

D- MeONa: 402, 340sh, 273, 204.

E- $\text{AlCl}_3 + \text{HCl}$: 410sh, 369, 276, 206.

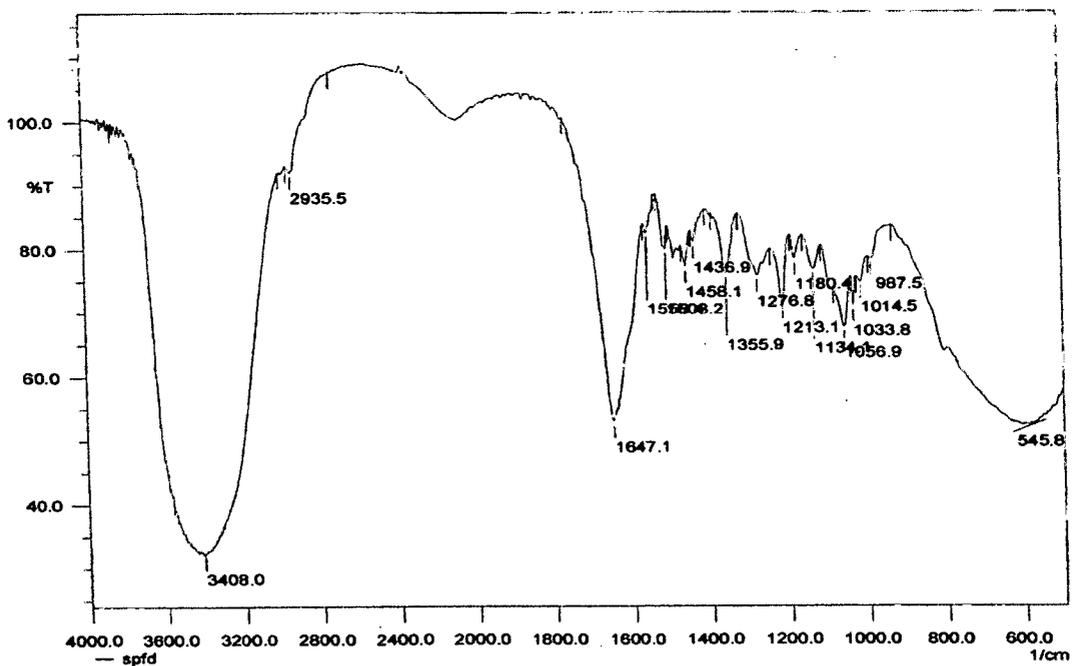


Fig.2.3.16: IR spectrum of compound 3.

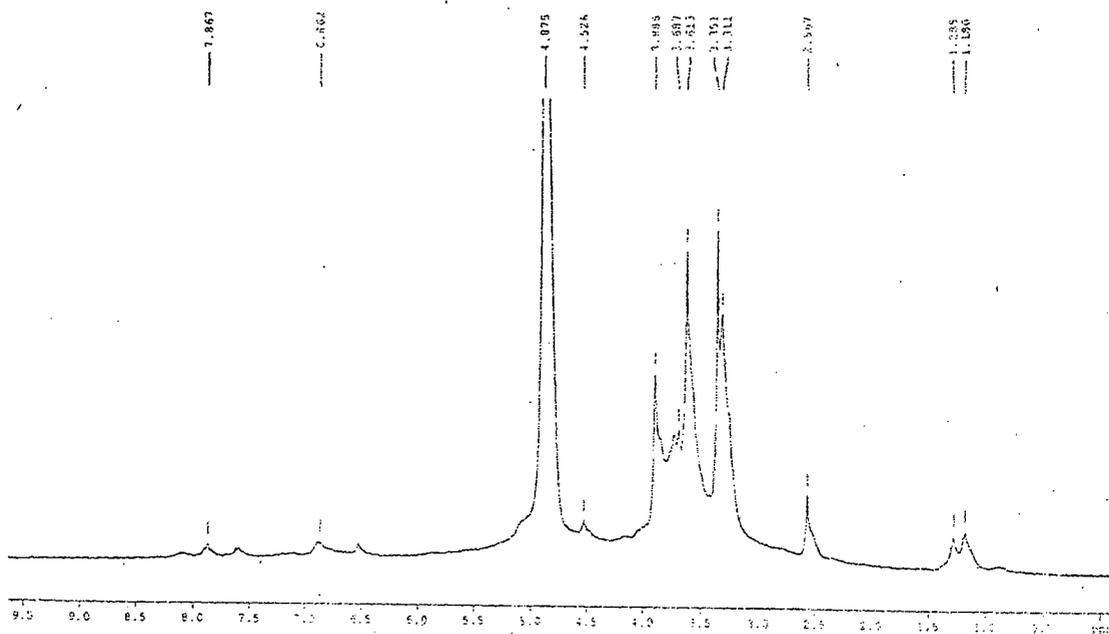


Fig.2.3.17: ¹H NMR spectrum of compound 3.

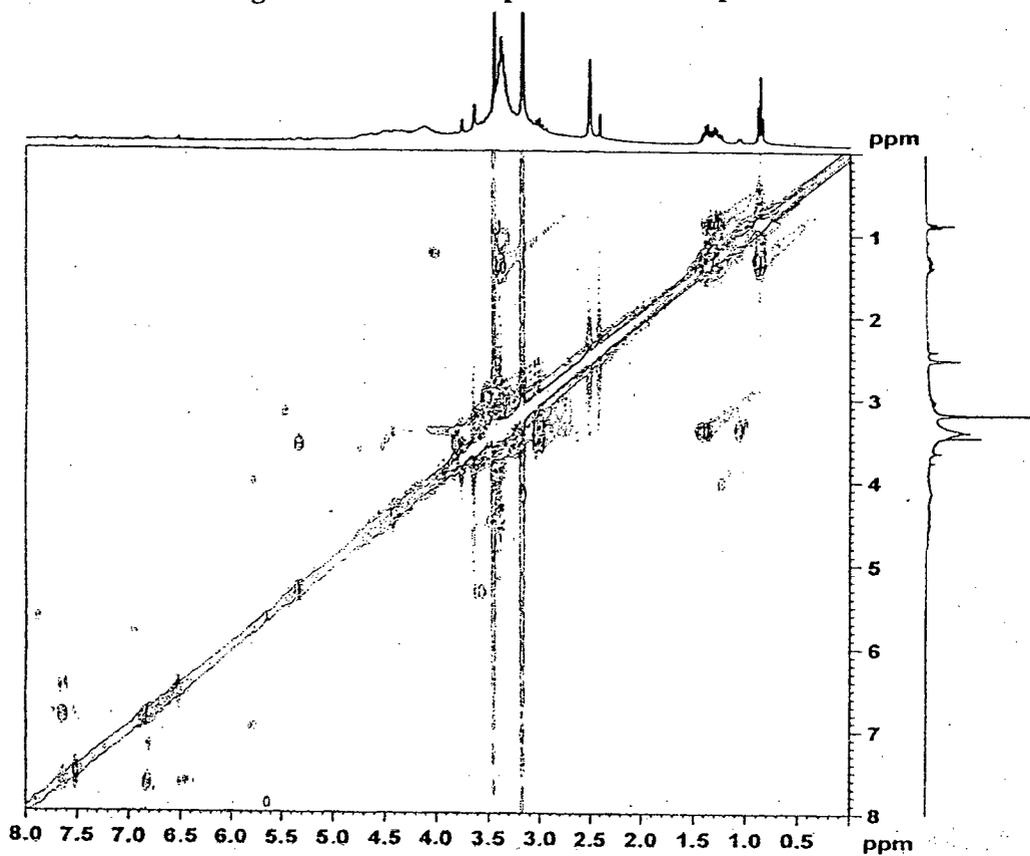


Fig.2.3.18: ¹H COSY of compound 3.

SPF-D, HMQC

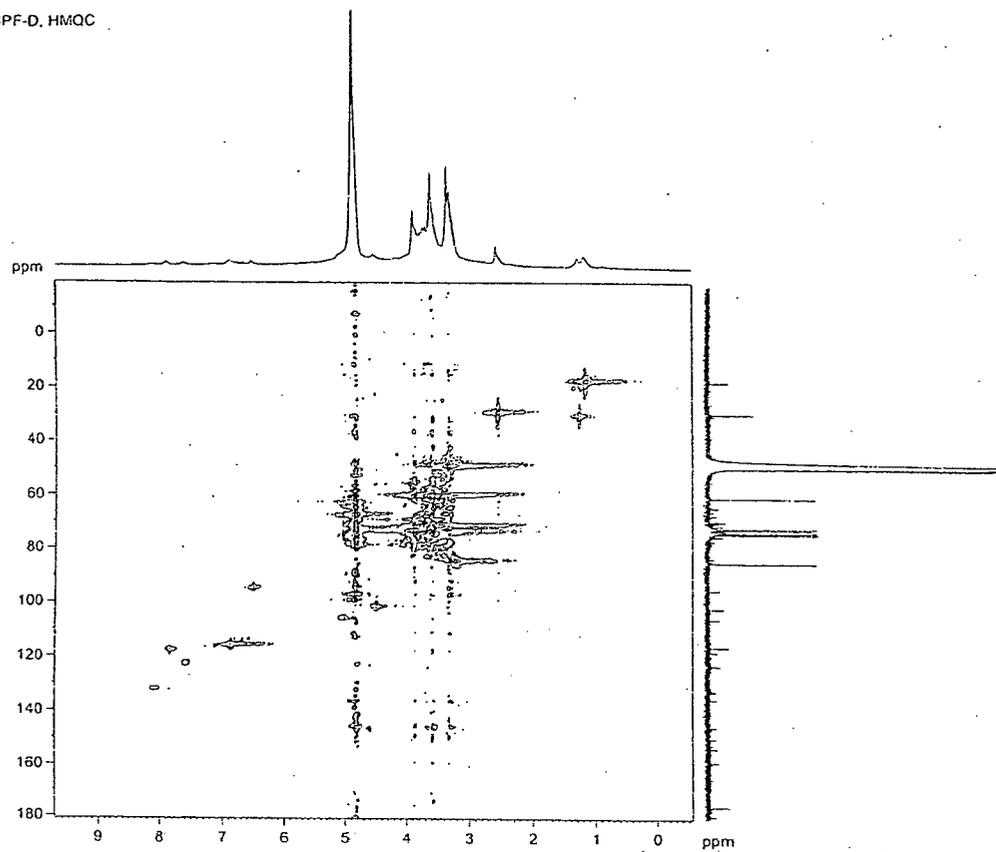


Fig.2.3.19: HMQC of compound 3.

SPF-D, HMBC

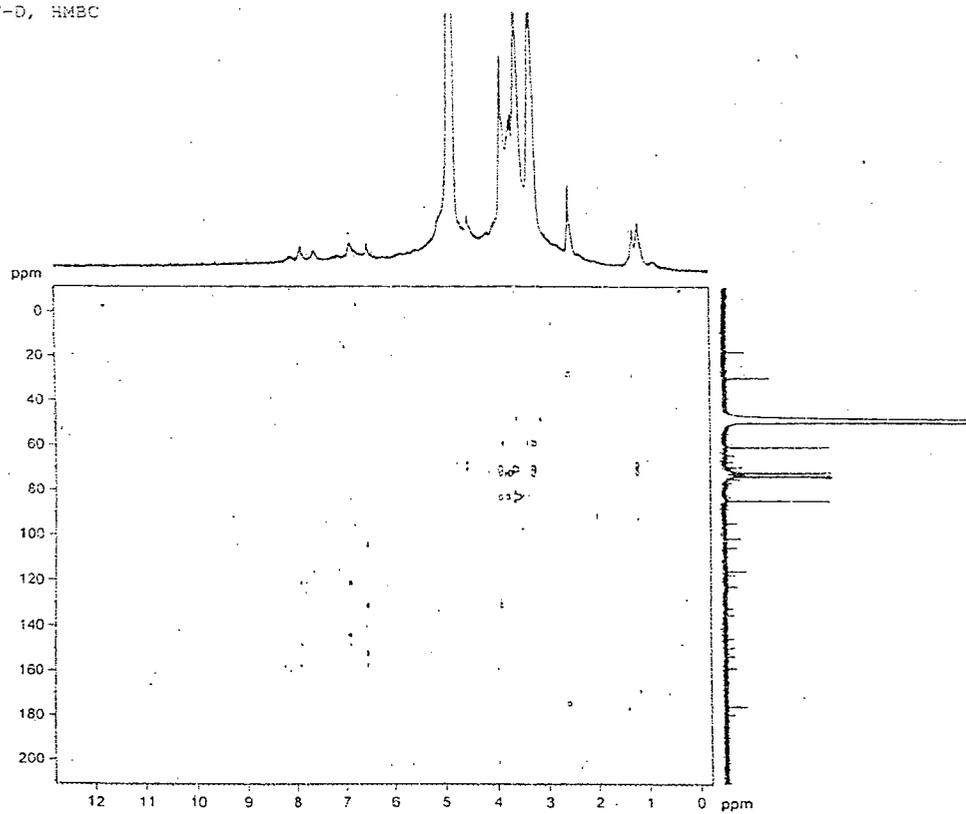


Fig.2.3.20: HMBC of compound 3.

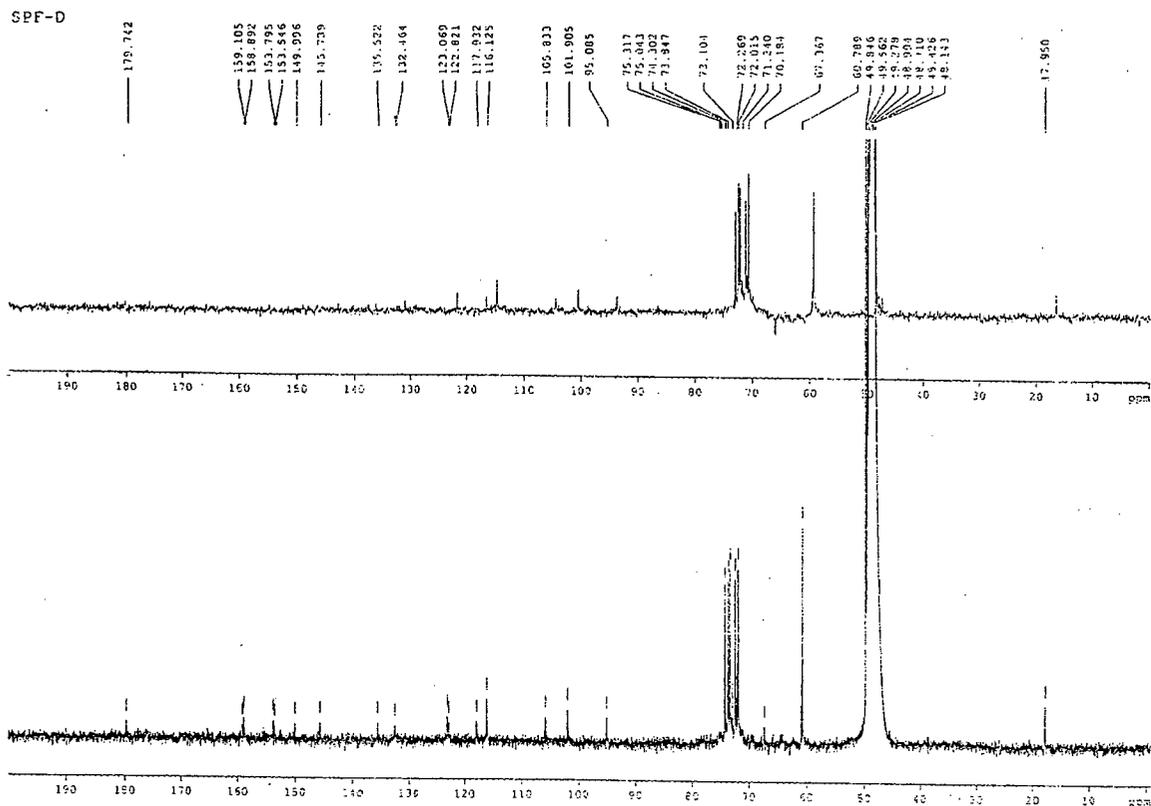


Fig.2.3.21: ^{13}C NMR and DEPT of compound 3.

+TOF Product (863.0): 39 MCA scans from MT20070403104325.wiff Max. 221.0 counts
 $z=3.56294997726244450e-004$, $t_0=-4.09853528531893970e+001$

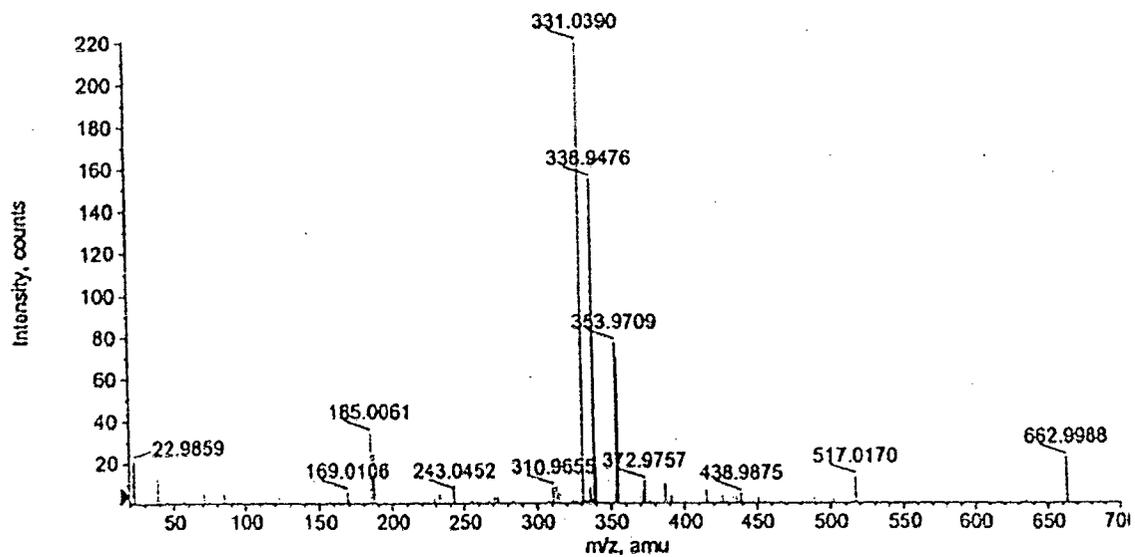


Fig.2.3.22: MS/MS of compound 3.

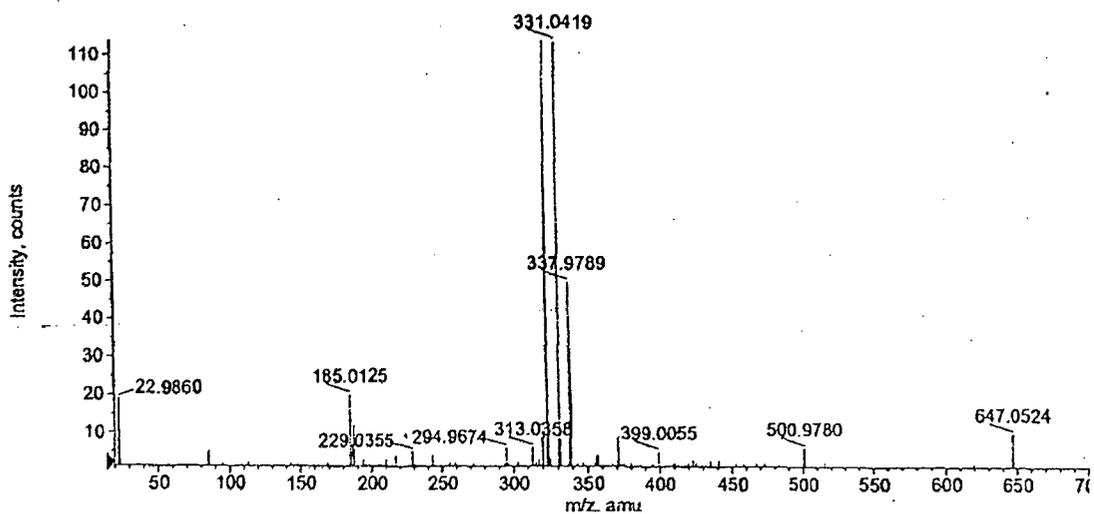


Fig.2.3.23: MS/MS of compound 4.

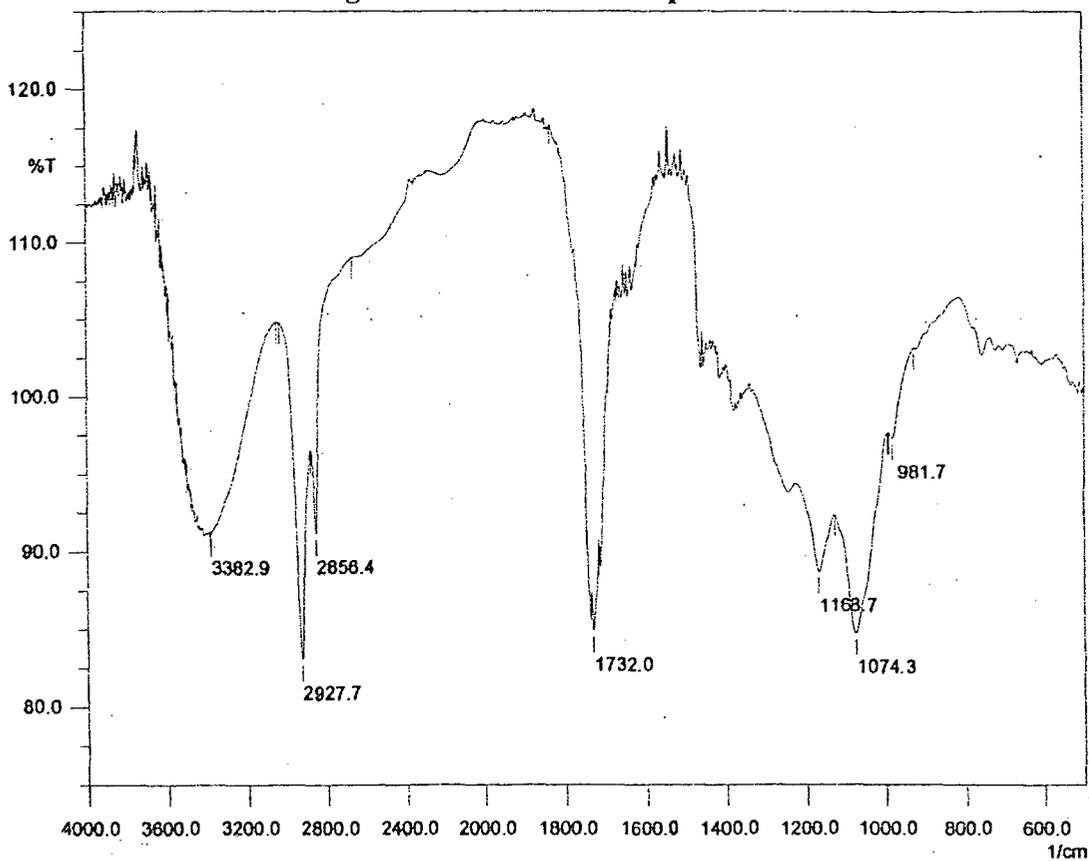


Fig.2.3.24: IR spectrum of compound 5.

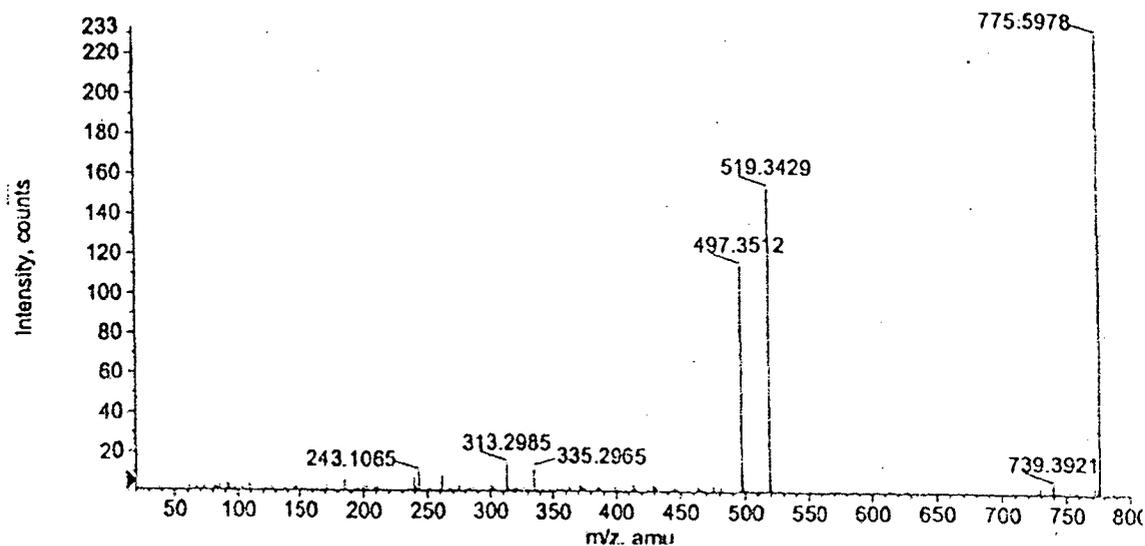


Fig.2.3.25: MS/MS of compound 5.

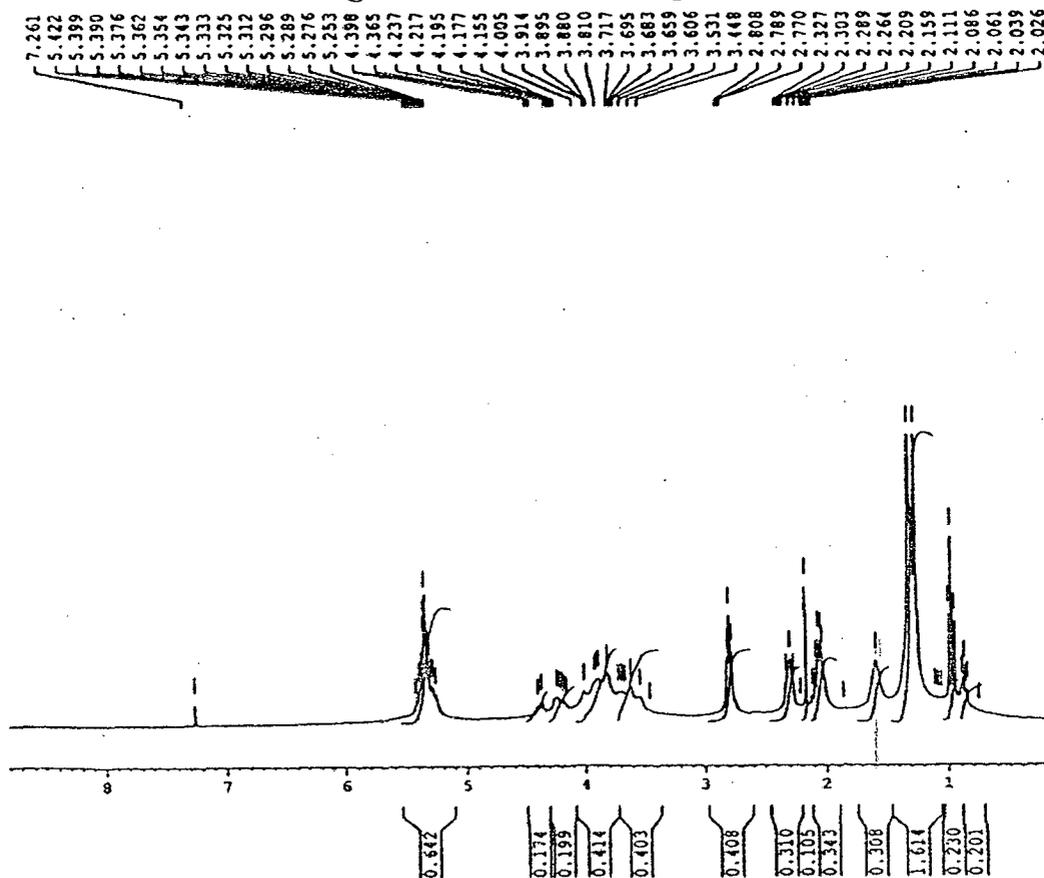


Fig.2.3.26: ¹H NMR spectrum of compound 5.

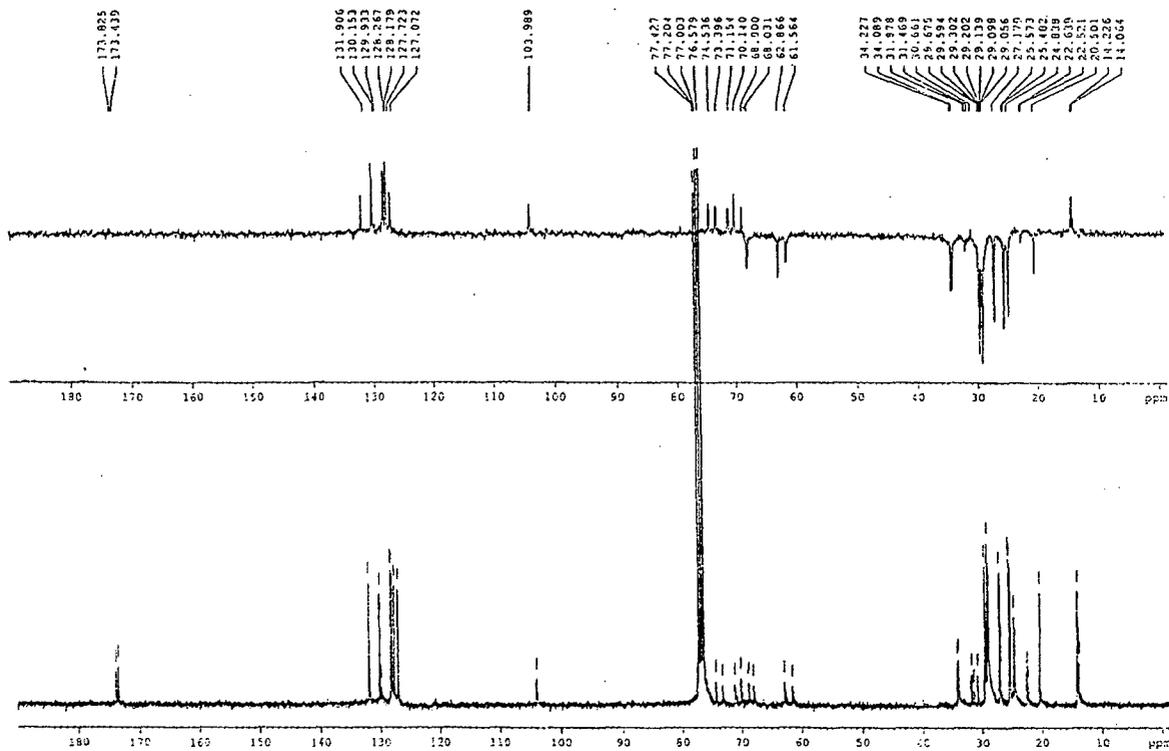


Fig.2.3.27: ^{13}C NMR spectrum of compound 5.

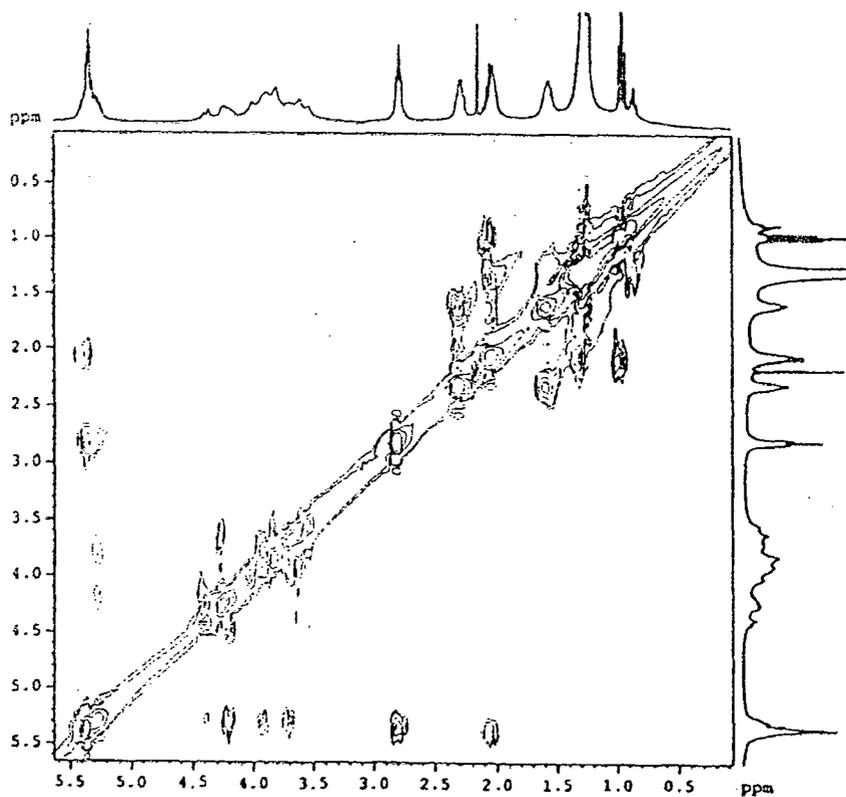


Fig.2.3.28: ^1H - ^1H COSY of compound 5.

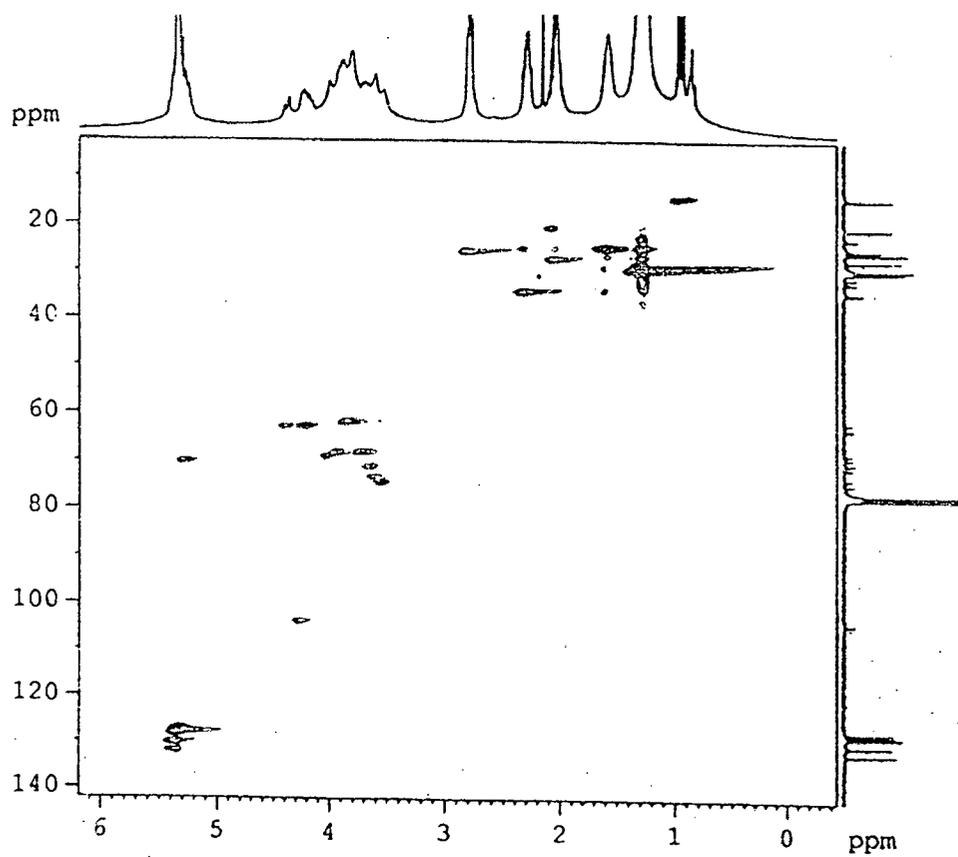


Fig.2.3.29: HSQC of compound 5.

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Chapter III

*Chemical investigation of the sponge
Cinachyra cavernosa*

Sponges are the oldest metazoan group, belonging to Phylum Porifera, with about 5000 species known across the world and having an outstanding importance as a living fossil¹. Though fresh water living species are known, sponges are primarily marine, inhabiting every type of marine environment, from polar seas to temperate and tropical waters and also thrive and prosper at all depths. They show an amazing variety of shapes, sizes and colors. Giant barrel sponges can reach up to 70 inches in height, while another tiny encrusting sponge may only be half of an inch long. Sponges are sessile organisms. However, due to their cellular plasticity, many sponges reorganize their bodies continuously and move during this process very slowly.² Except for the free-swimming larval stage (by means of cilia), sponges pass their whole subsequent existence fixed to a suitable substratum.

Sponges contain certain unifying attributes that are characteristic for the phylum Porifera. Sponges have cellular level organization, meaning that their cells are specialized so that different cells perform different functions, but similar cells are not organized into tissues and bodies are a sort of loose aggregation of different kind of cells. This is the simplest kind of cellular organization found among parazoans.

Sponges are found in virtually all-aquatic habitats, although they are most common and diverse in the marine environment. Many species contain toxic substances, probably to discourage predators. Certain other marine animals take advantage of this characteristic of sponges by placing adult sponge on their bodies, where the sponges attach and grow. The chemicals also probably play a role in competition among sponges and other organisms, as they are released by sponges to insure themselves space in the marine ecosystem. Some of these chemicals have been found to have beneficial pharmaceutical effects for humans, including compounds with respiratory, cardiovascular, gastrointestinal, anti-inflammatory, antitumor, and antibiotic activities. Sponges also provide a home for a number of small marine plants, which live in and around their pore systems. Symbiotic relationships with bacteria and algae

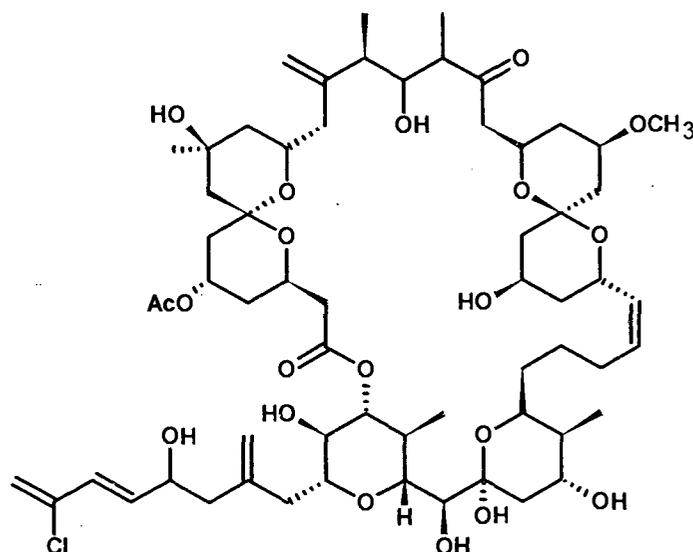
have also been reported, in which the sponges provide its symbionts support and protection and the symbionts provides the sponge with food. Some sponges (boring sponges) excavate the surface of corals and mollusks, sometimes causing significant degradation of reefs and death of mollusk. The corals and mollusks are not eaten; rather the sponge is probably seeking protection for itself by sinking into the hard structures it erodes. Even this process has some beneficial effects, in that it is an important part of the process by which calcium is recycled.

Chemicals i.e. secondary metabolites produced by the sponges not only play various roles in the metabolism of the producer but also in their strategies in the given environment. The diversity of secondary metabolites produced in sponges has been highlighted in several reviews^{1,2}. They range from derivatives of amino acids and nucleosides to macrolides, porphyrins, terpenoids to aliphatic cyclic peroxides and sterols.

In this chapter the sterols, identified from the chloroform fraction and peptides from the n-butanol fraction of the crude methanolic extract of sponge *Cinachyra cavernosa*, collected off the Goa coast (west coast of India) are described.

Sponge belonging to genus *Cinachyra* was found to be, by Fusetani and co-workers³ highly cytotoxic due to the presence of spiroketal macrolide cinachyrolide A. It is cytotoxic against L1210 murine leukemia cells with an IC₅₀ of <0.6 mg/mL.

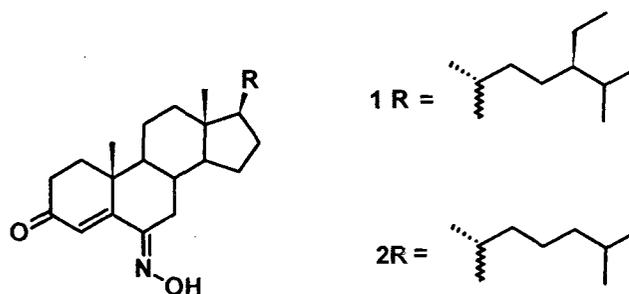
The aqueous extract of *Cinachyra alloclada* (formerly *Cinachyra cavernosa*) is known to be *in vivo* active not only in the Walker M and Walker 256 assays, but also against some solid tumor cell lines and in the differential DNA repair assay, exhibiting greatest inhibition against strain AB 1886[uvr A6]⁴. Bioassay guided purification have shown the activity to spread all over the chromatographed fraction and to be due to inseparable polyamines. Cardellina et al reported 17-Z- tetracosenyl 1-glycerol ether from the sponge *Cinachyra alloclada*⁵.



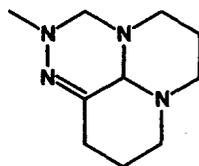
Cinachyrolide A

Cinachyrolide A discovered in *Cinachyra sp* is structurally similar to altohyrtin A. First reported in 1993 by three groups (Kobayashi and Kitagawa, Pettit, and Fusetani)⁶⁻⁹, the altohyrtins/ spongistatins/ cinachyrolides are a unique form of antimitotic macrolides¹⁰, which are obtained from various sponges in trace amount by bioassay guided isolation and display exceptional potency against wide variety of human cancer cell lines. They feature a highly substituted 42-membered macrolide ring which comprises two spiroacetals (AB and CD rings) and a bis(tetrahydropyran) unit (E and F rings) including a triene side chain along with 24 stereogenic centers. Cinachyrolide A, isolated by the Fusetani group from a genus of *Cinachyra* is assumed to have the same structure as 15-desacetylaltohyrtin A and spongistatin 4. Methanol-toluene extract of *Cinachyra antarctica* showed almost non-existent or very weak response against yeasts and fungi with the exception of the yeast *Candida tropicalis*, which was strongly inhibited by extracts of the sponges *Cinachyra antarctica*¹¹. Immunological assay have shown that *Cinachyra antarctica* is capable of rejecting tissues from congeners indicating that this sponge is capable of producing defensive chemicals that were toxic to genetically dissimilar tissues.

*Cinachyra tenuifolia*¹² is found to contain agglutinating proteins of a size similar to that of niphatevirin, a human-immunodeficiency-virus inhibitory glycoprotein, identified from *Niphates erecta*¹³. This agglutinin is inhibited by D-lactose but not by D-melibiose or other oligosaccharides indicating that they may react with terminal D-galactose beta-1,4- residues. Rodrigues et al¹⁴ reported the first natural occurrence of 6-hydroxyimino-4-en-3-one steroids, (24R,6E)-24-ethylcholest-6-hydroxyimino-4-en-3-one and cholest-6-hydroxyimino-4-en-3-one, along with the known cholest-4-en-3-one. This group of steroids was reported to show a high affinity for human placental aromatase, and function as competitive inhibitor of this enzyme¹⁵. *C. tenuifolia* has the ability to oxidize not only C-3 and C-6 position but also to introduce the hydroxyimino group exclusively at C-6 position. Wu et al.(2005)¹⁶ also reports the presence of cholest-4-en-3,6-dione-3-oxime in marine sponge *Cinachyrella australiensis* from the South China Sea



Cinachyramine, a novel alkaloid with an unprecedented cage system possessing a hydrazone and two aminals is reported from Okinawan *Cinachyrella sp.*



Section I

Steroids from Cinachyra cavernosa

All marine organisms have been proven to be a veritable cornucopia of unusual steroid metabolites, but some believe that marine sponges may provide the most diverse and biogenetically unprecedented array of unconventional steroids in the entire animal kingdom¹⁹. Marine sterols are associated with the polar lipid fatty acids in membranes. They have been studied in chemo taxonomic and ecological perspectives and in order to identify novel structures, especially those postulated as biosynthetic intermediates.

- Sterols from sponges belonging to Tetillidae family have been little investigated to date. Sterols of a *Cinachyra* sp. have been described in a comparative study with several other sponges from New Zealand²⁰. Steroid cholest-4-en-3,6-dione and (24R)-24-ethylcholest-4-en-3,6-dione in addition to three common 3 β -hydroxysterols are reported as constituents of Mediterranean marine sponge *Cinachyra tarentina*¹⁷. *Cinachyra cavernosa* (Lamarck, 1815 sensu Tsumamal, 1969) is now accepted as *Cinachyrella tarentina*¹⁸.

During the course of investigation on the bioactive metabolites from marine invertebrates particularly sponge we had an opportunity to investigate *Cinachyra cavernosa*, a sponge belonging to the class *Demospongiae*, family *Tetillidae*, order *Choristida*, genus *Cinachyra*, species *cavernosa*.

This sponge was found to be antiviral. In the first section of this chapter the identification of sterols from the chloroform fraction as steryl acetates by GC-MS are reported. Other non-steroidal constituents of this fraction having the same RF values as the sterols have also been identified.

Results and discussion:

The sterol composition of the sponge *Cinachyra cavernosa* is presented in (Table-1, Fig.3.1.1). Sixteen components were shown to be steroids with the highest

concentration of cholesterol (35.35%) followed by clionasterol (29.66%), 22-dihydrobrassicasterol (5.89%), brassicasterol (6.12%) and 16-dehydropregnenolone acetate (5.02%). Considerable amount of cholesta-5,22(E)-diene-3 β -ol (3.78%) with much less amount of the corresponding (22Z) isomer (0.55%) were also found in the mixture. The sterol composition reported for three *Cinachyrella* sp. from Senegal and for the *Cinachyra* sp. of New Zealand²⁰ is also similar, with clionasterol being major component followed by cholesterol, brassicasterol and dihydrobrassicasterol²¹. These differences found in the sterol composition could be due to differences in the life cycle and to the differences in the ecological conditions.

The presence of pregna-5,16-diene-3-ol-20-one as acetate also known as 16-dehydropregnenolone acetate (DPA) was also evident in considerable quantities (5.02%). It is a key intermediate in the industrial synthesis of hormones progesterone and cortisone derivatives. DPA was earlier synthesized from diosgenin as the starting material, which has now been replaced by solanidine, a potato alkaloid²². Sun et al. (2006)²³ reported the first natural occurrence of DPA and the corresponding glycoside 16-dehydropregnenolone 3-O- α -L-rhamnopyranosyl (1 \rightarrow 2) β -D glucopyranoside uronic acid from a terrestrial plant, *Solanum lyratum*. To the best of our knowledge it is the second report of its natural occurrence and first report from marine source.

The sterol, 24-oxocholesterol, has been described from a marine brown alga *Pelvetia canaliculata*²⁴. It was found in marine microalga *Isochrysis galbana* which is a food for Pacific oyster, *Crassostrea gigas*²⁵. Brassicasterol is also a plant sterol synthesized by phytoplankton and is used as a biomarker for the presence of marine algal matter in the environment²⁶. Diatoms and dinoflagellates are known to be important components of the phytoplankton and thus to form the foundation of marine food chains. They also occur as symbionts in marine invertebrates. Thus, it seem likely that brassicasterol and 24-oxocholesterol are widespread for attached marine animals such as sponges, corals, gorgonians and some mollusks. It has been indicated that the association between marine sponges and zooxathellae appears less frequent²⁷ but,

Cinachyra cavernosa/*Cinachyra tarentina* is reported to be a free living sponge with symbiotic dinoflagellate²⁸.

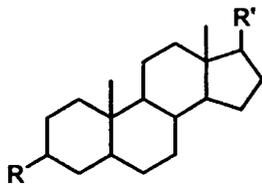
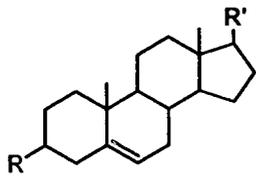
Cholesta-5,22-diene -3 β -ol is also known to occur in diatoms and has been detected in marine organisms including marine sponges^{29,30,31}. It probably originates from dietary planktonic sources.

It is evident from the results, Δ^5 sterols are the major sterols present in *Cinachyra cavernosa*. This is well in agreement with the sterol composition of three *Cinachyrella sp.* from Senegal reported by Barnathan et al.²¹

The acetylated mixture also contained clionasterol as oleate. Besides sterols present in the mixture were 1,2 benzenedicarboxylic acid bis (2-methyl propyl) ester, palmitic acid, dibutyl phthalate and 1,2 benzene dicarboxylic acid bis (2-ethyl hexyl) ester **Table-2**. The last two phthalic acid esters are known as constituents of Chinese sponge *Cinachyrella australiensis*.³².

Table-1: Sterol composition of *Cinachya cavernosa*:

Peak No.	Sterols	RT	M ⁺	%Area
6	Stigmasta-5,22-diene-3 β -ol, acetate(1)	34.29	454	0.46
7	Stigmasta- β -5en-3 β -ol oleate(2)	34.49	678	0.55
8	Cholest-5en-3 β -ol(3)	34.70	386	0.41
10	Cholesta-5,22 Z-dien-3 β -ol(4)	35.46	384	0.55
11	Cholesta-5,22 E-dien-3 β -ol(5)	35.61	384	3.78
13	Cholesterol acetate(6)	36.02	428	35.33
14	Cholestanol acetate(7)	36.09	430	3.18
15	Ergosta-5,22-dien-3 β -ol (brassicasterol)(8)	36.40	398	6.12
16	4 α -methylgorgostanol(9)	36.50	442	1.11
17	Ergost-5-ene-3 β -ol(22-dihydrobrassicasterol)(10)	36.98	400	5.89
18	Ergostan-3 β -ol acetate(11)	37.08	444	0.30
20	Stigmasta-5,22-diene-3 β -ol (22Z) acetate(12)	37.27	454	0.80
21	Stigmast-5ene-3 β -ol (clionasterol)(13)	37.91	414	29.66
22	16-dehydropregnenolone acetate (14)	38.06	356	5.02
23	24-oxo cholesterol(15)	38.29	442	0.46
24	9,19-Cycloergost-24(28)-en-3 β -ol,4,14 dimethyl acetate (4 α ,5 α)(16)	38.46	468	1.11



Compound	R	R'
1	OAc	
6	OAc	
12	OAc	
15	OAc	
3	OH	
4,5	OH	
10	OH	
13	OH	
8	OH	

Compound	R	R'
7	OAc	
11	OAc	

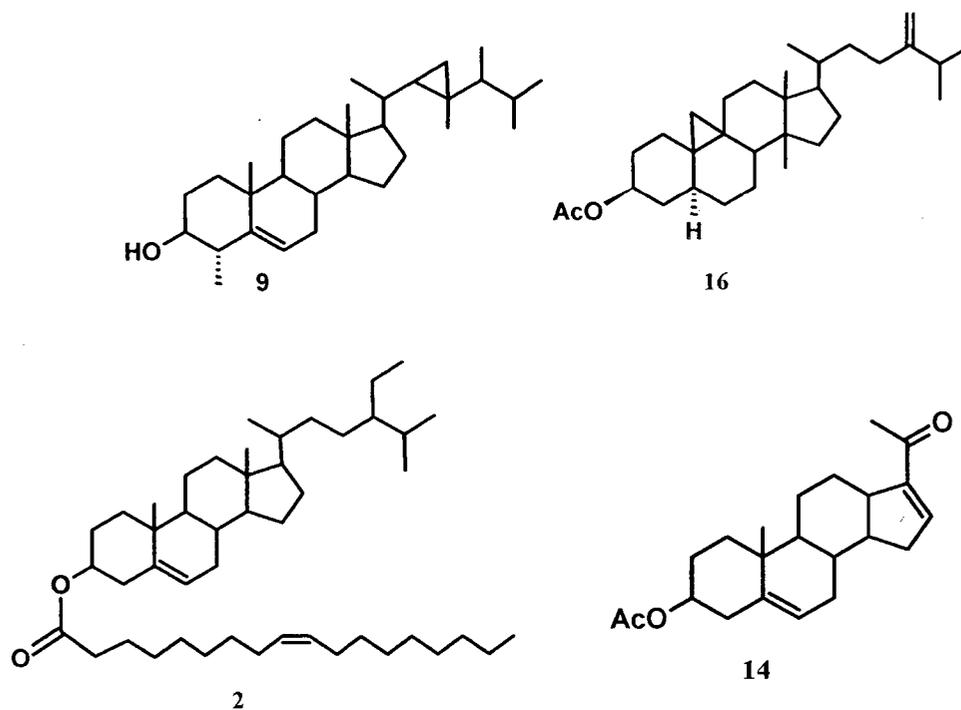
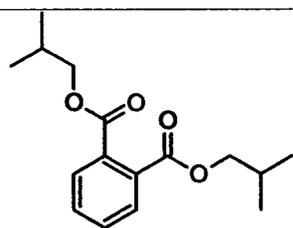


Table-2: Additional compounds identified from the acetylated mixture

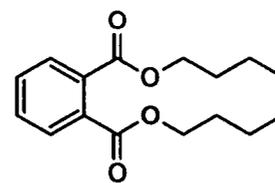
Peak No.	Compound	RT	M ⁺	Area
1	1,2 Benzene dicarboxylic acid bis (2-methyl propyl) ester. (17)	19.60	278	0.38
2	N-hexadecanoic acid. (18)	20.90	256	0.90
3	Dibutyl phthalate. (19)	21.03	278	0.37
4	1,2-Benzenedicarboxylic acid bis(2-ethyl hexyl)ester. (20)	28.59	390	0.75



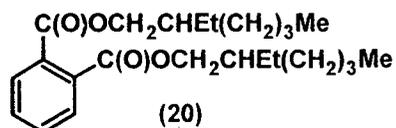
(17)

HO₂C(CH₂)₁₄Me

(18)



(19)



EXPERIMENTAL:

Instrumentation:

Shimadzu 2010 gas chromatograph fitted with XTI-5 (bonded, 5% phenyl) capillary column (30m X 0.32mm, film thickness 0.25 μ M) and coupled to a Shimadzu 2010 mass selective detector was used for the analysis. Oven temperature was programmed from 60° at 5°/min; helium was used as carrier gas at a flow rate of 1.00 ml/min.; ion source temp. and interface temp. were maintained at 230°C and 320°C respectively; solvent cut time 2.00 min, and detector voltage IKV was taken. Scanning range was from m/z 40-700 for 42.0 min. ACQ mode was used for analysis with isobutane as the reagent gas.

Biological material

The sponge identified by DR P A Thomas of Central Marine Fisheries Research Institute, Trivandrum was hand picked, in January 2006 from Goa coast (Anjuna), during low tide. The specimen has been deposited at NIO Repository and Taxonomic Center.

Extraction and isolation of sterols mixture:

The sponge specimen were washed in sea water, carefully cleaned, cut in small pieces, and extracted in acetone over night, at room temperature. The process was repeated thrice. The acetone extract was partitioned into chloform soluble fraction. The neutral lipids from this fraction were separated from other lipids by column chromatography over silica gel (60-120 mesh) and gradient elution with ethyl acetate in hexane as eluent. The free sterols (205 mg), generally obtained among the middle fraction and were combined, based on the TLC profile of the spots with Rf value of 0.52, concentrated under reduced pressure and the residue subjected to derivatization.

TLC was carried out on F₂₅₄ silica gel plates which were developed using petrol: ethyl acetate 80:20 v/v and visualized by spraying with 5% methanolic H₂SO₄ followed by heating in an oven at 100°C.

Gas Chromatographic analysis of the sterol mixture:

The free sterols (25 mg) were acetylated with acetic anhydride: pyridine (1:1_{v/v}) for 24 hours at room temperature. The reaction mixture was taken in chloroform, washed successively with water, dil hydrochloric acid and again with water. The chloroform layer was then dried over anhydrous sodium sulphate. Solvent removed and the residue(21mg) subjected to GC/MS analysis.

Identification of component was based on retention time and computer matching with the WILEY 7.LIB and NIST 147.LIB, as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature.

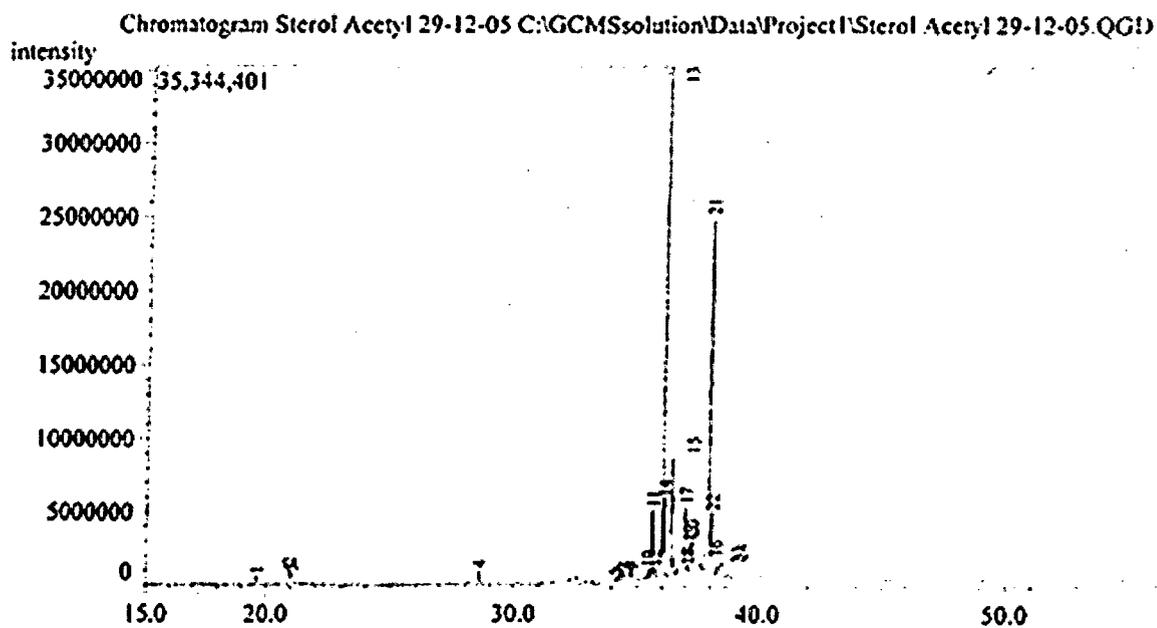


Fig.3.1.1: GC/MS of Sterols from *Cinachyra cavernosa*

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Section II

*Cyclic peptides from the sponge Cinachyra
cavernosa by ESI-MS/MS*

Sponges are a large and diverse group of colonial organisms that constitute the phylum Porifera with thousands of different species extensively distributed from superficial waters near the seashores up to deep waters of the ocean. Sponges have been traditionally known as a source of novel bioactive metabolites like terpenoids, alkaloids, macrolides, polyethers, nucleoside derivatives and many other organic compounds. Synthetic analogues of the C-nucleosides spongouridine and spongothymidine isolated from a Caribbean sponge led later to the development of cytosine arabinoside, an anticancer compound¹. More recently, the attention has been directed also to the search of bioactive peptides from sponges, being actually a well established sector in the research of marine natural products. The discovery of the bioregulatory role of different endogenous peptides in the organism as well as the understanding of the molecular mechanisms of action of some new bioactive peptides obtained from natural sources on specific cellular targets, contributed to consider peptides also as promising lead drug candidates. Recently marine peptides have opened a new perspective for pharmaceutical developments².

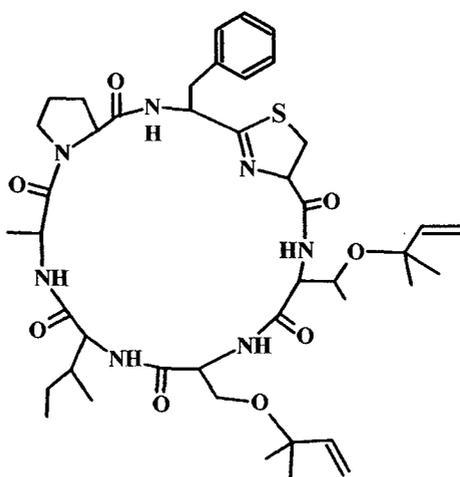
Active peptides from sponges most of them with unique unprecedented structures in comparison with these kind of compounds from other sources are often cyclic or linear peptides containing unusual amino acids which are either rare in terrestrial and microbial systems or even totally novel, and also frequently containing uncommon condensation between amino acids.

These peptides exhibit a variety of activities, including insecticidal, antimicrobial, antiviral, antitumor, tumor promotive, antiinflammatory, and immunosuppressive actions³⁻⁴. Some of these compounds are in advanced clinical trials, and others have proven useful in studies directed toward the elucidation of biochemical pathways⁵. Their significant pharmacological variety is a function of peptide or depsipeptide structure and conformational diversity, specifically, rare residues such as D-amino acids, N- or C-alkylated amino acids, α,β - dehydro amino acids, hydroxyl acids and structurally elaborate amino acids such as the reverse prenyl (rPr) of two residues of Ser and Thr in trunkamide A (1).

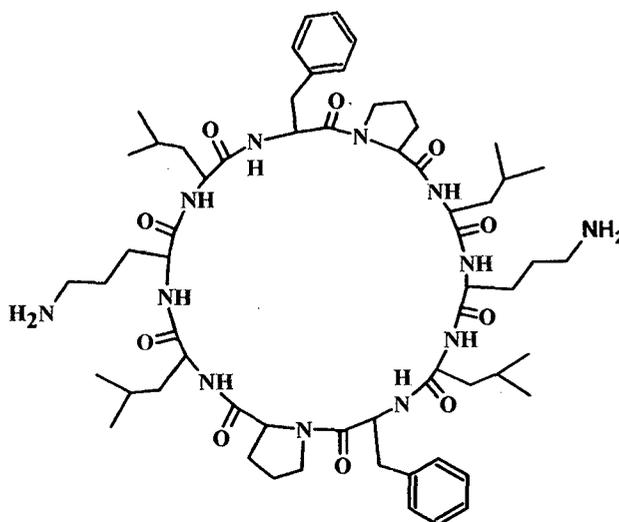
Cyclic and linear peptides discovered from marine animals have increased our knowledge about new potent cytotoxic, antimicrobial, ion channels specific blockers, and many other properties with novel chemical structures associated to original mechanisms of pharmacological activity. These facts introduce marine peptides as a new choice for the obtainment of lead compounds for biomedical research.

Apart from the occurrence in a variety of naturally occurring bioactive metabolites that possess strongly expressed and useful biological activity, cyclic peptides are often more stable *in vivo* than their linear counterparts and therefore often represent promising drug candidates. Another feature that contributes to the appeal of cyclic peptides is their reduced conformational mobility, which allows them to be used in the study and mimicry of protein folding and to present diverse functionalities in a well-defined and predictable manner.

Interest in cyclic peptides dates back half a century to the discovery that the antibiotic gramicidin S (2) is a cyclic decapeptide⁶. Since then numerous natural cyclic peptides acting as antibiotics and toxins have been found. A variety of biological studies have suggested that cyclic structures may exhibit improved metabolic stabilities, increased potencies, better receptor selectivity and more controlled bio-availabilities than linear peptides. Further, the constrained geometries of cyclic peptides favour conformational investigations and can help in locking one key structural element.⁶



(1)



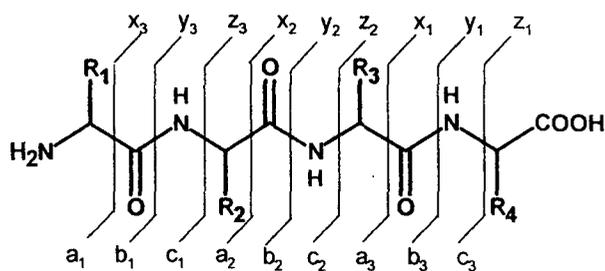
(2)

In a continuing search for structurally and pharmacologically interesting secondary metabolites, with special reference to peptides from the marine organisms, detailed chemical investigation of marine sponge *Cinachyra cavernosa* was undertaken. Here in, the analysis carried out using ESI-QTOF MS/MS of the peptides rich fraction from the butanol extract of the marine sponge *Cinachyra cavernosa* resulted in the identification of some cyclic peptides. Sequences of these cyclic peptides were elucidated on the basis of collision-induced dissociation (CID) experiments.

Tandem mass spectrometry for peptide sequencing:

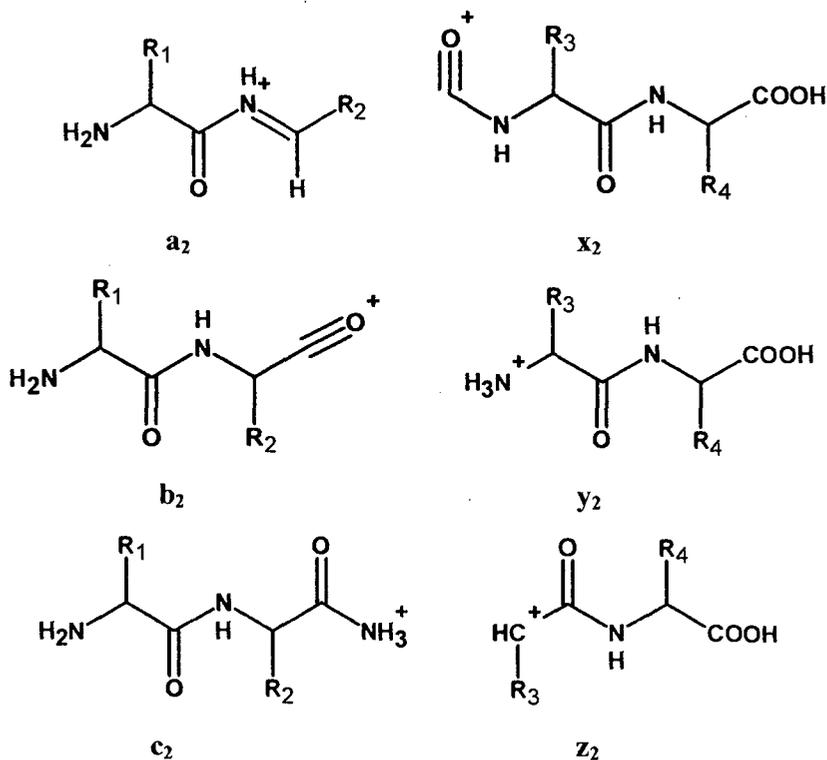
Mass spectrometry is becoming increasingly important in the life sciences due to its high sensitivity, high information content, and ability to study complex mixtures without purification. Peptide tandem mass spectrometry^{7,8} (MS/MS) with the aim of determining the amino acid sequence of peptides is in fact the key technology for protein identification in the rapidly expanding field of proteomics⁹. The most common instruments utilize collision-induced dissociation (CID) of protonated peptides, where collisions with a neutral background gas induce bond cleavages in the molecule. Under low collision energy conditions, protonated

peptides predominantly fragment to produce so-called N-terminal *b* and C-terminal *y* fragment ions^{10,11} Scheme 1.



Scheme 1: Peptide Fragmentation

Fragments will only be detected if they carry at least one charge. If this charge is retained on the N terminal fragment, the ion is classed as either *a*, *b* or *c*. If the charge is retained on the C terminal, the ion type is either *x*, *y* or *z*. A subscript indicates the number of residues in the fragment. In addition to the proton(s) carrying the charge, *c* ions and *y* ions abstract an additional proton from the precursor peptide. Thus, the structures of the six singly charged sequence ion are:



The appearance of peptides CID spectra, especially those of $(M+H)^+$ ions, are highly dependent on the collision energy¹². The CID spectra of peptides recorded at low collision energy (<100eV) tend to show abundant fragment ions formed by cleavage of the peptide bonds. In low energy CID peptide carrying a positive charge fragments, mainly along its backbone, generating predominantly a, b and y ions. In addition, peaks due to loss of ammonia (-17 Da) denoted a*, b* and y* and water (-18 Da) denoted a⁰, b⁰ and y⁰ are also observed. While high energy CID (KeV) spectra often contain fragment ions formed by other backbone x,c,z and also side chain cleavage are observed¹³. In case of cyclic depsipeptides, the fragmentation pattern is different. Das *et al* (1979)¹⁴ have suggested that the protonation of ester oxygen opens the ring to form a linear peptide with the C terminal carboxylic acid that fragments sequentially.

In an ideal fragmentation process the sequence of a peptide could be simply determined by converting the mass difference of consecutive ions in a spectrum to the corresponding amino acids. The ideal situation would occur if the fragmentation process could be controlled so that each peptide was cleaved between every two consecutive amino acids¹⁵.

Tandem Mass spectrometric analysis for peptides from the sponge *Cinachyra cavernosa*:

On the basis of TLC, the n-butanol fraction of the marine sponge *Cinachyra cavernosa* was found to be rich in ninhydrin positive spots indicating the possibility of containing peptides. When the fraction was chromatographed on a column of sephadex LH20 it was observed that none of the spots was present in amounts sufficient for NMR data. Hence, the peptide rich fraction as evident from TLC, from the n-butanol fraction was analyzed using tandem mass spectrometry (ESI-MS/MS), which resulted in the tentative identification of five cyclic peptides. The characterization of these peptides based on the fragmentation pattern observed in the ESI-MS/MS spectrum has been discussed.

Parent Ion Scan (ESI-MS) for the detection of cyclic peptides:

The ESI-MS, in the positive ion mode, of ninhydrin positive spot is shown in **Fig-3.2.1**. It showed presence of several molecular species in the range of m/z 401 to 545. Five of these cyclic peptides with pseudomolecular ions $[M+H]^+$ 545,527,472,433,401 were selected for identification.

The fragment ion peaks are labeled according to the nomenclature proposed by Roepstorff and Fohlman (1984)¹⁰, subsequently modified by Johnson *et al* (1987)¹⁶. To retrieve a sequence from the mass spectrometric data of the new peptides the criteria that the mass difference between two adjacent peaks should precisely fit the mass of an amino acid residue was applied. Initially the MS/MS of the protonated molecular ion of all the peptides has been discussed. Depending upon the fragmentation pattern observed the sequence of amino acids for new peptides has been assigned.

Collision induced dissociation (CID) or MS/MS of each molecular ion peaks:

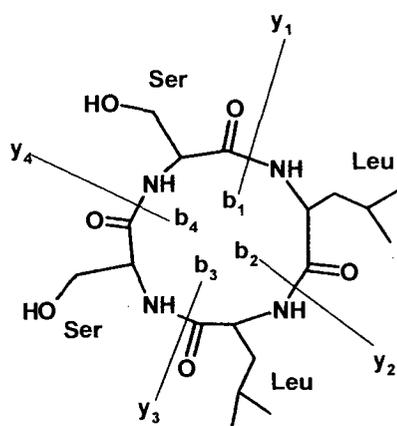
1) MS/MS at m/z 401.2679 of cyclic tetrapeptide (1):

For any cyclic peptides the first dissociation step involves the opening of the cyclopeptide ring by cleavage of the either lactone group or one of the amide bonds. In most cases the basic site gets protonated first resulting in the weakening of the C-N or C-O bond. Sequencing of cyclic peptides is complicated by the fact that a multiplicity of initial ring cleavages is possible, each of which can produce a series of sequence peaks¹⁷.

The **Fig 3.2.2** shows the MS/MS spectrum of a peptide with pseudomolecular ion at m/z 401.2697 which corresponds to the $[M+H]^+$ of the cyclic tetrapeptide (1) with the molecular formula $C_{18}H_{32}O_6 N_4$. The cyclic tetrapeptide (1) is one of the smallest cyclic peptide identified from *Cinachyra cavernosa* and is made up of four amino acids, Serine(Ser), Serine(Ser), Leucine(Leu), Leucine(Leu).

It is evident from the CID spectrum (**Fig 3.2.2**) that the cyclic peptide rings opens up and the amino acid residues are sequentially deleted. Thus, the loss of NH_3

from the side chain gives peak at m/z 384.2395 designated as a_1^0 . The combined loss of two water molecules from the two-side chains (there are two serine residues) gives peak at m/z 366.2263 designated as b_1^0 . The first major fragment peak y_2 at m/z 288.1850 involves the loss of 113 daltons, indicative that this ring cleavage produces an N-terminal leucine. The fragment ions b_3 and y_3 were significantly observed at m/z 227.1633 and 175.1055 for the cleavage between the Serine moiety and N-terminal of the leucine. The corresponding b_4 is observed at m/z 313.1836. The peaks at m/z 199.1662 are due to loss of CO from the fragment ion b_3 while m/z 183.1338 is for the combined loss of CO and NH_2 . The detailed fragment ions are given in the Table 1.



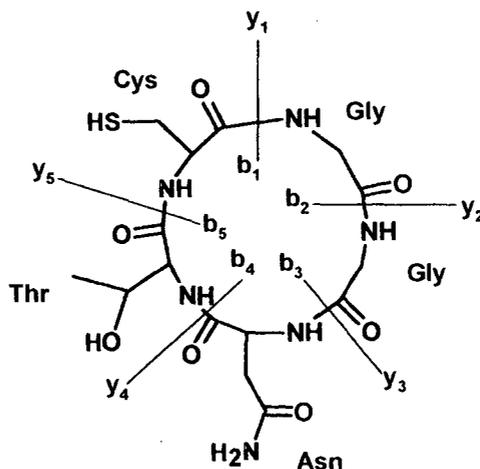
Scheme 1: Fragmentation pattern of cyclic tetrapeptide (1)

Table-1 : Assignment of fragment ions observed from the MS/MS spectra of cyclic tetrapeptide (1):

Fragment ions of m/z 401.2697	m/z	Fragment ions of m/z 401.2697	m/z
a_1^0	384.2395	$[y_2 - H_2O]^+$	270.1815
b_1^0	366.2263	$[y_2 - 2H_2O]^+$	253.1507
y_2	288.1850	$[y_2 - NH_3]^+$	271.1582
b_3	227.1633	$[y_2 - (H_2O + Ser)]^+$	183.1338
y_3	175.1055	$[y_2 - (H_2O + Leu)]^+$	157.0969
b_4	313.1836		
$[b_3 - CO]^+$	199.1662		
$[b_3 - (CO + NH_2)]^+$	183.1338		

2) MS/MS at m/z 433.2217 of cyclic pentapeptide (2) :

The Fig 3.2.3 shows the MS/MS spectrum of a protonated peptide at m/z 433.2217 which corresponds to the $[M+H]^+$ of the cyclic pentapeptide (2) with the molecular formula $C_{15}H_{24}O_7 N_6 S$. The five amino acids present are Glycine(Gly), Glycine(Gly), Asparagine(Asn), Threonine(Thr) and Cysteine(Cys). The loss of water molecule from the molecular ion gives peak at m/z 415.1106 designated as a_1^0 , subsequent loss of Gly (57) gives peak at m/z 359.0405 and loss of another Gly(57) gives peak at m/z 302.9788. The masses 377.0548, 321.1138, 208.9986 correspond to losses of Gly (57), Gly (57) and Asn (114), providing the sequence of three of the five amino acids. These fragment ions were assigned as y_2 , y_3 , and y_4 respectively. The corresponding b_4 is observed at m/z 225.0106. The combined loss of CO and NH_3 from the fragment ion y_3 gives peak at m/z 277.0957. Similarly m/z 163.0120 results from the combined loss of CO and NH_3 from the fragment ion y_4 . The loss of water molecule from the fragment ion y_4 gives peak at m/z 190.9861. The combined loss of Cys (103), Thr (101) from the fragment ion y_2 gives peak at 173.0111. Similarly m/z 265.0545 results from the loss of Gly (57) from the fragment ion y_3 . A summary of the CID fragmentation of this peptide is presented in Table 2 and Scheme 2:



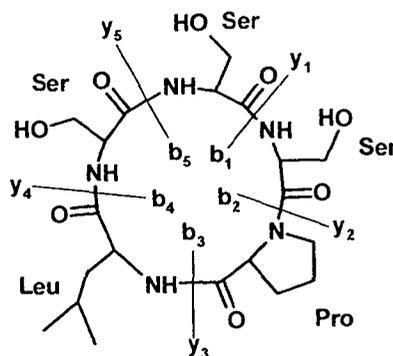
Scheme 2: Fragmentation pattern of cyclic pentapeptide (2).

Table-2 : Assignment of fragment ions observed from the MS/MS spectra of cyclic pentapeptide (2):

Fragment ions of m/z 433.2217	m/z	Fragment ions of m/z 433.2217	m/z
a_1^0	415.1106	$[a_1^0 - (H_2O + Gly)]^+$	359.0405
y_2	377.0548	$[a_1^0 - (H_2O + 2Gly)]^+$	302.9788
y_3	321.1138	$[y_3 - (CO + NH_2)]^+$	277.0957
y_4	208.9986	$[y_4 - (CO + NH_2)]^+$	163.0120
b_4	225.0106	$[y_4 - H_2O]^+$	190.9861
$[y_3 - Gly]^+$	265.0545	$[y_2 - (Thr + Cys)]^+$	173.0111
		$[Thr + Asn]^+$	216.9915

3) MS/MS at m/z 472.2947 of cyclic pentapeptide (3):

The CID spectrum of $[M+H]^+$ at m/z 472.2947 **Fig.3.2.4** of the cyclic pentapeptide (3) was studied and was shown to have the molecular formula $C_{20}H_{33}O_8N_5$. The amino acid sequence of 3 was determined to be Serine (Ser), Proline (pro), Leucine (Leu), and two moieties of Serine (Ser) residues based on the fragmentation showing sequential loss of amino acid residues. The spectrum showed peaks at m/z 384.2275 (y_2), m/z 288.1874 (y_3), m/z 175.1055 (y_4) and m/z 86.0873 (y_5). The corresponding b ions were observed at m/z 183.1331 (b_3), m/z 296.1298 (b_4). Fragment ion peaks b_2 and b_5 , could not be detected or they may be less intense (**Scheme 3**). The combined loss of CO and NH_3 gives peak at m/z 426.2799. The peaks at m/z 271.1582 and 158.0760 are due to loss of NH_3 from the fragment ions y_3 and y_4 respectively. The loss of water molecule from the fragment ion y_4 gives peak at m/z 157.1186 and the combined loss of CO and NH_3 resulted in the fragment at m/z 130.0876. The fragmentations observed are presented in **Table 3** and **Scheme 3**.



Scheme 3: Fragmentation pattern of cyclic pentapeptide (3).

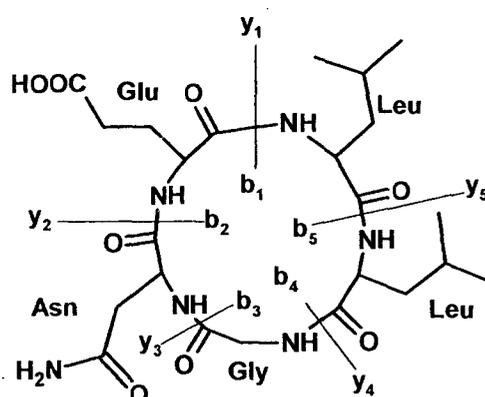
Table-3 : Assignment of fragment ions observed from the MS/MS spectra of cyclic pentapeptide (3):

Fragment ions of m/z 472.2947	m/z	Fragment ions of m/z 472.2947	m/z
y ₂	384.2275	[M+H- (CO+NH ₂)] ⁺	426.2799
y ₃	288.1874	[y ₃ - NH ₃] ⁺	271.1582
y ₄	175.1055	[y ₄ - H ₂ O] ⁺	157.1186
y ₅	86.0873	[y ₄ - NH ₃] ⁺	158.0760
b ₃	183.1331	[y ₂ - (2H ₂ O+NH ₂)] ⁺	331.1298
b ₄	296.1298	[y ₂ - Ser] ⁺	296.1298
[y ₄ - (CO+NH ₂)] ⁺	130.0876	[Ser+ Pro] ⁺	185.1132

4) MS/MS at m/z 527.2556 of cyclic pentapeptide (4):

The Fig 3.2.5 shows the MS/MS spectrum of a protonated peptide at m/z 527.2556, which corresponded to the molecular formula, C₂₃H₃₈O₈N₆. The cyclic pentapeptide 4 consists of five amino acids, Glutamic acid (Glu), Asparagine(Asn), Glycine(Gly) and two Leucine(Leu) moieties. The loss of water molecule from the molecular ion gives a peak at m/z 509.2072 designated as a₁⁰. The loss of Glycine(Gly) moiety from the fragment ion a₁⁰ gives a peak at m/z 451.2525. The combined loss of Asparagine(Asn) and Glycine(Gly) from a₁⁰ gives peak at m/z 339.1244 designated as x₁⁰ which yields fragment at m/z 321.1149 with elimination of water whereas elimination of CO from x₁⁰ produces

peak at m/z 311.0545. The corresponding y ions are observed at m/z 397.0502 (y_2), 284.9591 (y_3), 225.0071 (y_4) but the (y_5) fragment is observed at m/z 70.9488 due to loss of CO and NH_3 from the leucine residue. Similarly, the fragments at m/z 302.9765, 415.0906, assigned as b_4 , b_5 respectively, while the fragment ion b_3 is observed at m/z 227.0021 due to loss of NH_3 . The peak at m/z 433.1099 is due to loss of Glycine(Gly) moiety followed by loss of water from the fragment ions a_1^0 . The combined loss of $-\text{CONH}_2$ and H_2O and from the fragment ion b_5 gives peak at m/z 353.0993. The structure and the fragmentation pattern is summarized below in Table 4 and Scheme 4.



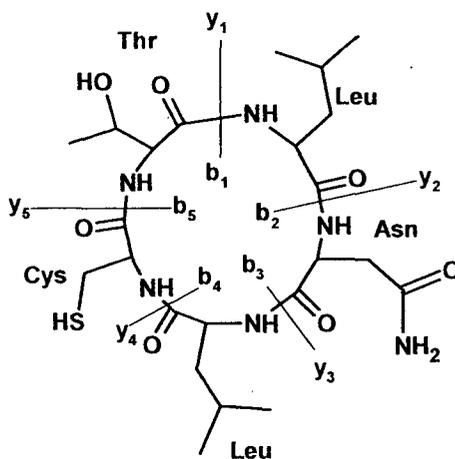
Scheme 4: Fragmentation pattern of cyclic pentapeptide (4).

Table-4 : Assignment of fragment ions observed from the MS/MS spectra of cyclic pentapeptide (4):

Fragment ions of m/z 527.2556	m/z	Fragment ions of m/z 527.2556	m/z
y_2	397.0502	a_1^0	509.2072
y_3	284.9591	$[a_1^0 - (\text{Gly})]^+$	451.2525
y_4	225.0071	$[a_1^0 - (\text{Gly} + \text{H}_2\text{O})]^+$	433.1099
y_5	70.9488	$[a_1^0 - (\text{Asn} + \text{Gly})]^+$ (x_1^0)	339.1244
b_4	302.9765	$[x_1^0 - \text{H}_2\text{O}]^+$	321.1149
b_5	415.0906	$[x_1^0 - \text{CO}]^+$	311.0545
$[b_3 - \text{NH}_3]^+$	227.0021	$[b_5 - (\text{H}_2\text{O} + \text{NH}_2\text{CO})]^+$	353.0993

5) MS/MS at m/z 545.0943 of cyclic pentapeptide (5) :

In a similar fashion, interpretation of the MS/MS of $[M+H]^+$ at m/z 545.0943 (**Fig 3.2.6** and **Table 5**) which corresponded to the molecular formula, $C_{23}H_{40}O_7N_6S$ showed the amino acid sequence in peptide 5 to be Leucine(Leu), Asparagine(Asn), Leucine (Leu), Cysteine (Cys) and Threonine(Thr). The loss of water from the pseudomolecular ion $[M+H]^+$ gives peak at m/z 527.0943 designated as a_1^0 whereas the combined loss of CO and NH_2 gives peak at m/z 501.2460. The corresponding y ions are observed at m/z 433.1116 (y_2), 320.9854 (y_3), 208.0015 (y_4). The corresponding b ions appeared at m/z 227.0002 (b_3) m/z while the fragment ion b_4 is observed at m/z 302.9756 due to loss of water. Fragment ion peaks b_2 , b_5 and y_5 could not be detected or they may be less intense (**Scheme 5**). The peaks at m/z 415.1074 and 190.9799 resulted from the loss of NH_3 from the fragment ions y_2 and y_4 respectively. The structure and the fragmentation pattern are summarized below in **Table 5** and **Scheme 5**.



Scheme 5: Fragmentation pattern of cyclic pentapeptide (5).

Table-5 : Assignment of fragment ions observed from the MS/MS spectra of cyclic pentapeptide (5):

Fragment ions of m/z 545.0943	m/z	Fragment ions of m/z 545.0943	m/z
y ₂	433.1116	a ₁ ⁰	527.2106
y ₃	320.9854	[M+H- (CO+NH ₂)] ⁺	501.2460
y ₄	208.9915	[y ₂ - H ₂ O] ⁺	415.1074
b ₃	227.0002	[y ₄ - NH ₃] ⁺	190.9799
[b ₄ - H ₂ O] ⁺	302.9765	[y ₄ - (H ₂ O+NH ₂ +CO)] ⁺	144.9947
		[Leu + Leu + Asn] ⁺	339.1279

As evident from the results, the sponge *Cinachyra cavernosa* seems to be rich in cyclic pentapeptides. The structures assigned exclusively on the basis of ESI-MS/MS are to be confirmed by the other spectral data specially NMR. This work is to be undertaken for which more material is to be collected and processed for the isolation of peptides in sufficient quantities.

Cyclic peptides have become increasingly important because of their wide range of pharmacological activities¹⁸ and interesting chemical structures^{19,20} they show therapeutic potential due to greater resistance to in vivo enzymatic degradation as well as greater bioavailability than non cyclic analogues¹⁹. Cyclisation helps reduce conformational flexibility of the peptide backbone, which enables systematic manipulation of the 3D structure to understand receptor bindings in biological targets or for improvement of biological activity.

The proline rich compounds usually containing seven or eight amino acid residues represent an interesting class of marine cyclic peptides. The role of proline in these molecules has been linked to the control of conformation of the molecule in solution because of the restricted ϕ of proline,^{21,22} a group of examples are the hymenamides²³, stylopeptide,²⁴ axinellins,^{25,26} axinastatins,²⁷ and phakellistatins.²⁸

Experimental Section:

Material: The sponge *Cinachyra cavernosa* was collected during the low tides from coastal waters of Goa, west coast of India [15° 51'N to 15°54'N and 73° 51' E to 73° 52'E] during the pre-monsoon periods

Extraction:

The collected sponge species were homogenized in an atmosphere of liquid nitrogen by crushing the sponge tissues in a mortar and pestle. The samples were extracted first with methanol and then partitioned with n-butanol. The organic solvents from the extracts were evaporated to dryness on a rotavapor under vacuum at 35⁰C and reconstituted with appropriate solvent before applying to the column. The water-soluble residues were lyophilized to remove all the water. The n-butanol fraction was filtered, initially on a column of Sephadex LH₂₀ with methanol as eluant and monitored by Thin-layer chromatography (TLC), alumina backed sheets (Si gel 60 F₂₅₄, 0.25mm thick) using Butanol: Acetic Acid: Water (5:1:4) mobile phase and sprayed with ninhydrin reagent. The ninhydrin positive fractions were then purified by repeated silica gel chromatography using mixtures of methanol and chloroform.

ESI-QTOF MS/MS spectrometry:

The mass spectrometer used was a QSTARXL MS/MS applied Biosystem instrument (Canada) equipped with Analyst software application. The instrument was operated in positive ionization mode. The sample dissolved in MeOH: H₂O containing trace of 0.1%TFA was directly infused at a constant flow rate of 10µl/min into the ion spray source using integrated syringe pump. The MS/MS products were produced by collision dissociation (CID) of selected precursor ions at collision energy between 25-40eV and mass analyzed using TOF analyzer of the instrument.

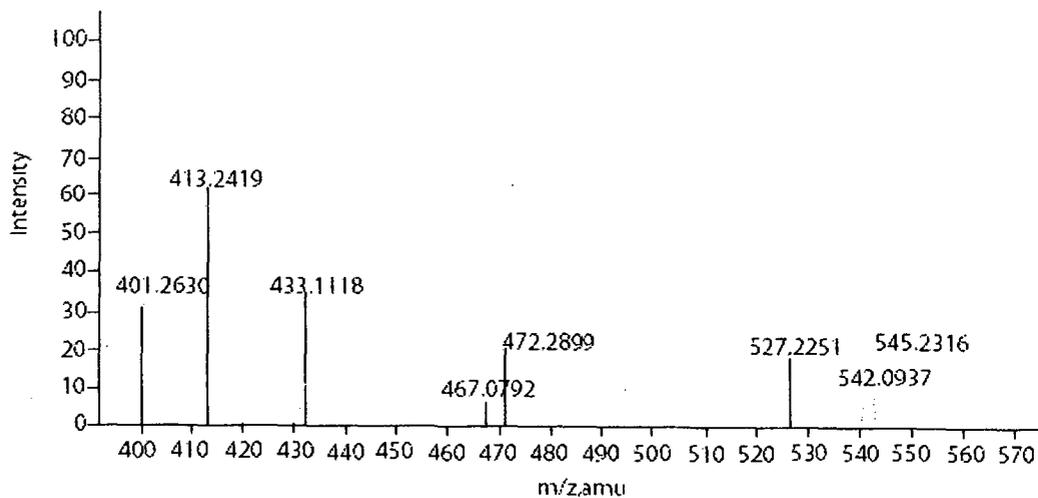


Fig-3.2.1.

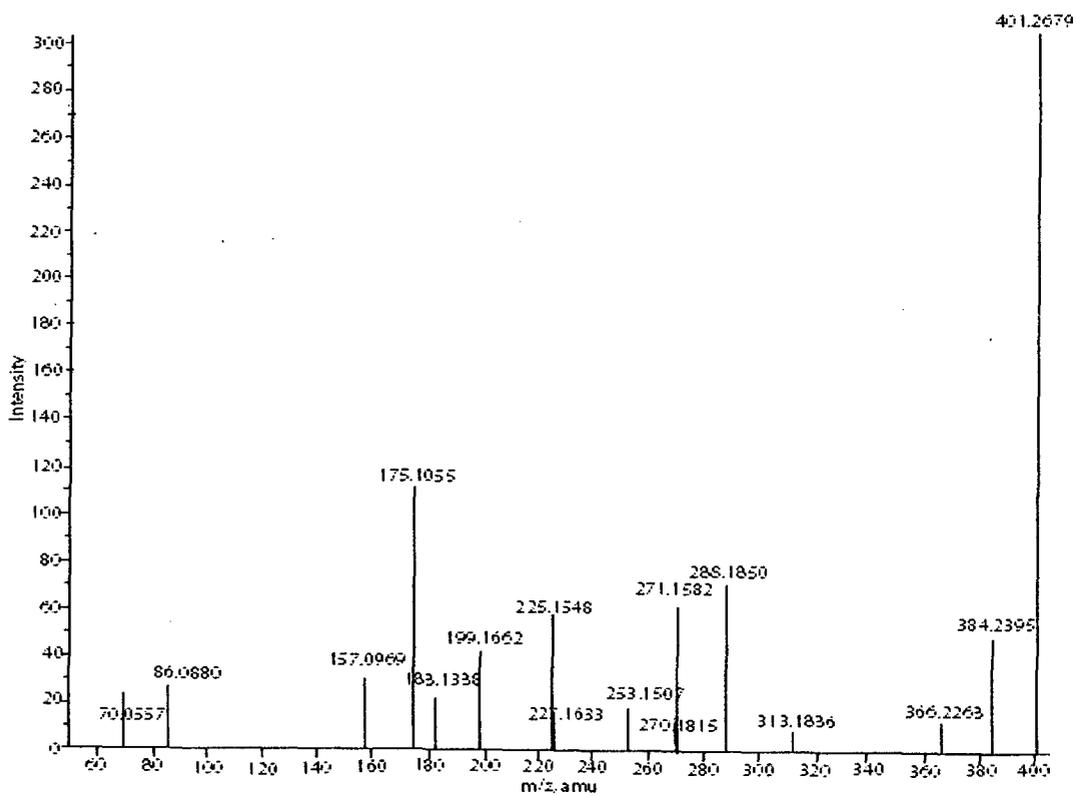


Fig-3.2.2.

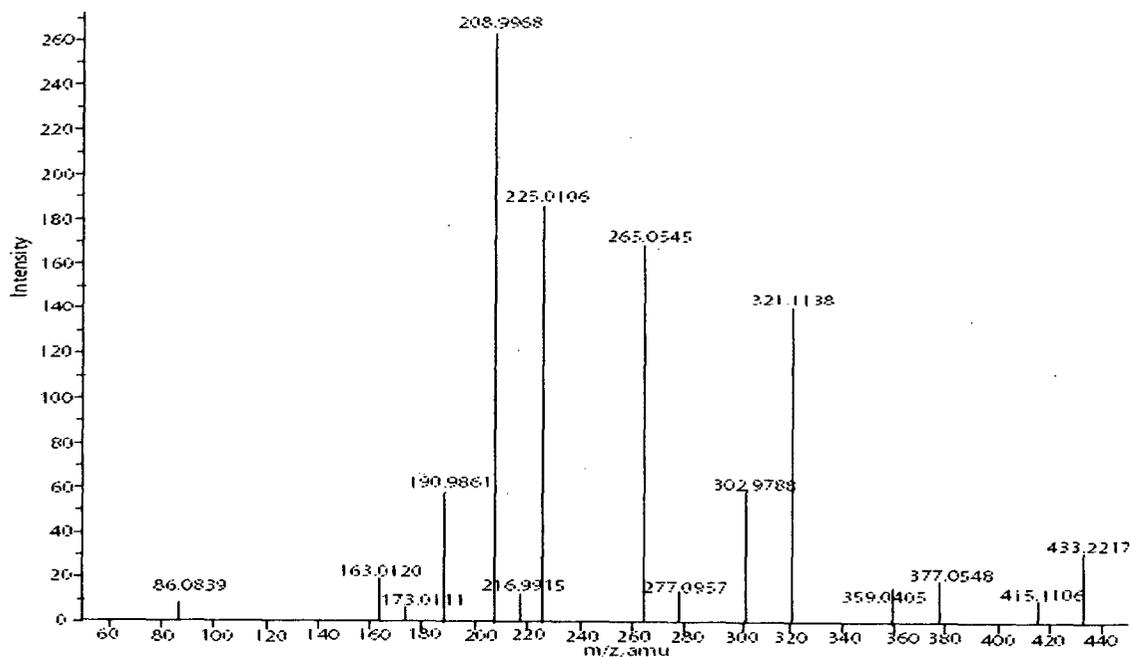


Fig-3.2.3.

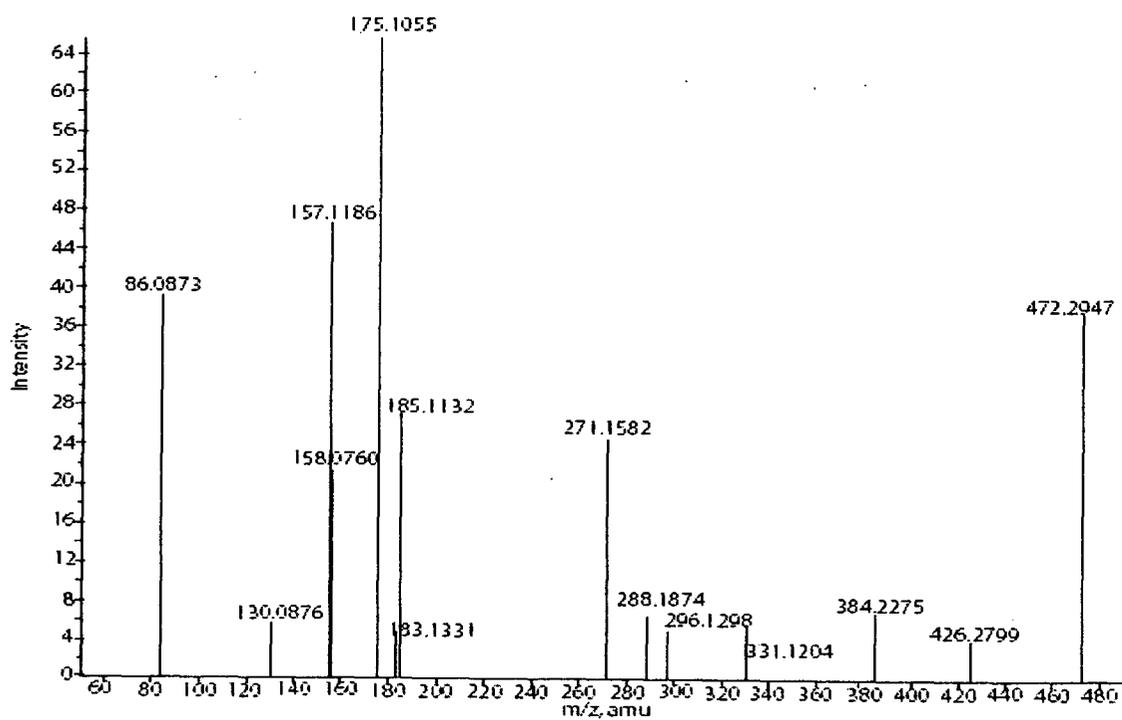


Fig-3.2.4.

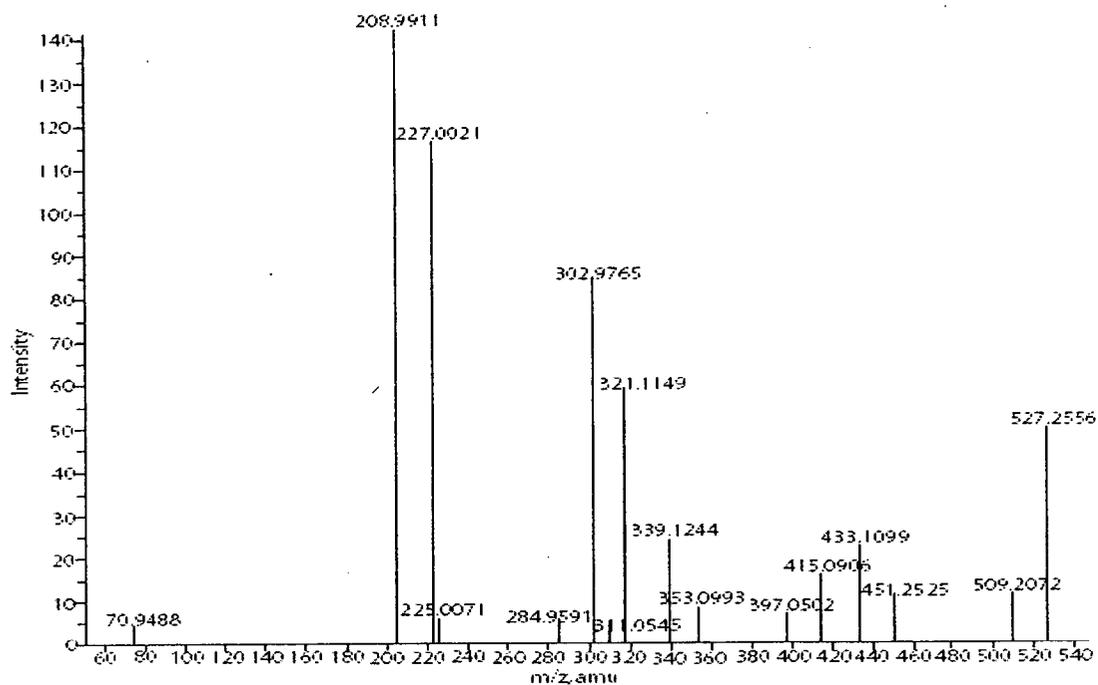


Fig-3.2.5.

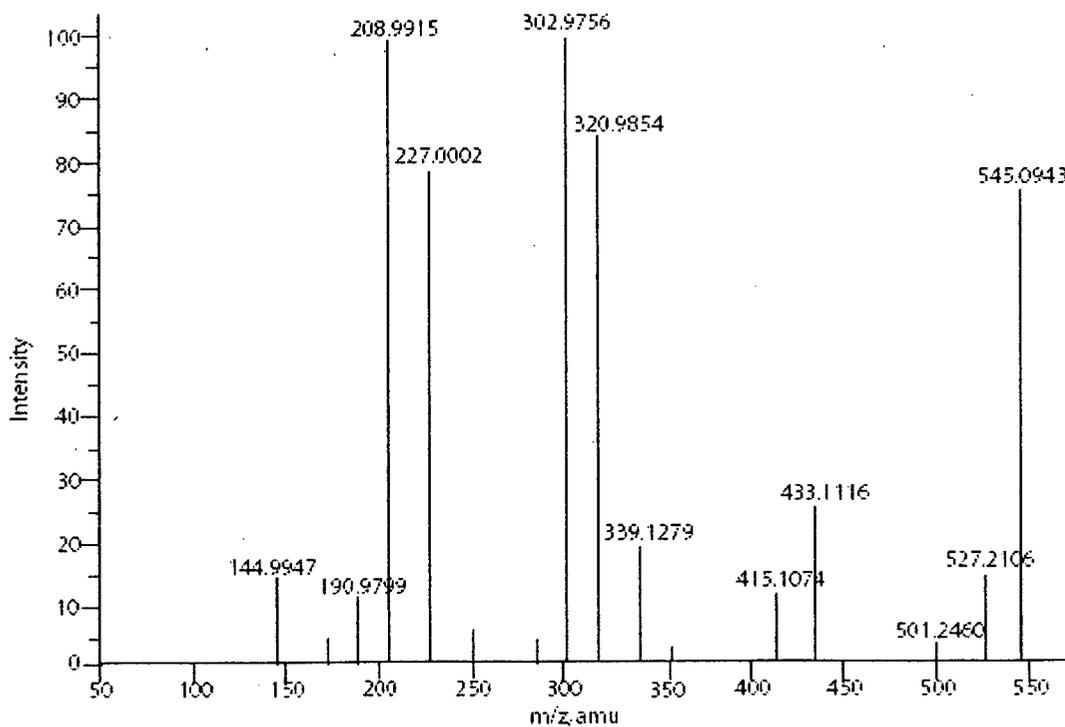


Fig-3.2.6.

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The oceans have been recently described to be the "Medicine Chest of the New Millennium". There is a gold rush amongst pharma and biotech companies to tap this new found resource. Traditionally many medicines have terrestrial origin however, with most of the plants and animals on land been discovered and many of the curative properties of the chemicals, they contain, been found, the man has diverted the attention towards the oceans as a source of therapeutic agents.

A strong reason to search drugs from marine source is that many organisms spend their life anchored to the seabed and have poisonous spines, claws and nails to ward off preys like fishes. These poisonous toxins are the most sought after, as cures against cancer and other diseases. It is believed that the same method, by which these toxins are able to harm the predators without harming the organisms itself, could be used to treat cancer. For example, killing cancerous cells selectively while not harming healthy cells, or killing disease causing bacteria without killing the host.

Secondly, many molecules found in marine organisms have novel structures differing totally from the molecules of medicinal value found in terrestrial plants. These marine compounds can lead to new curative methods in the human body, and thus opening up the possibility of treating drug resistance diseases like the tuberculosis.

With the aim of searching bioactive substances from the seas around India a program was initiated at the National Institute of Oceanography in eighties. The introductory section reviews the work done since then, under the programme, giving a list of compounds isolated and identified not only from macro-organisms but also from the associated micro-organisms.

In this thesis the results of chemical investigation of the red alga *Chondria armata*, the mangrove plants *Lumnitzera racemosa*, *Aegiceras corniculatum*, *Sesuvium portulacastrum* and the sponge *Cinachyra cavernosa* have been discussed. Chemical investigation of the above marine organisms has led to the isolation of some new as well as known compounds. The biological activities of

some of the compounds have been presented. The thesis has been divided into three chapters.

The first chapter narrates the chemical investigation of the active chloroform fraction of the red alga *Chondria armata*. NMR spectroscopy complemented with mass spectrometry has been used for structural elucidation of major lipids belonging to the class of glycolipids namely, galactosylglycerols and dioxygenated steroids including 4-methyl sterols with alkylated side chain, usually present in dinoflagellates. The minor ones, present in admixture with the major lipids and detected from their ESI-MS profiles have also been discussed. They were identified solely on the basis of electrospray ionization tandem mass spectrometry. Antimicrobial properties of these chemical constituents against pathogens have also been evaluated for the first time. This chapter also includes characterization of squalene-derived polyethers also present in the same fraction. Polyethers squalene derivatives are known to show strong cytotoxicity against p-388 murine leukemia *in vitro*, and to exhibit significant antiviral activity against Vesicular Stomatitis Virus and Herpes Simplex Virus type-I.

In our ongoing program on 'Bioactive substances from Indian Ocean' we had an opportunity to study the bioactivity and chemistry of mangrove plants, *Lumnitzera racemosa*, *Aegiceras corniculatum* and *Sesuvium portulacastrum*. Studies conducted on the bioactivity and chemistry of these selected mangrove plants have been incorporated in the second chapter.

The antibacterial principle of *Lumnitzera racemosa* which showed effectiveness against all the pathogenic bacterial strains tested was located in the butanol soluble fraction. Flavonoids were partly responsible for the observed activity. This mangrove and other plants belonging to *Combretaceae* are rich in tannins like corilagin, castalgin, punicalin, and punicalagin. Corilagin is reported to enhance the inhibition of methicillin resistant *Staphylococcus aureus*. Tannins have antimicrobial activity although, paradoxically, some organisms (e.g. the fungus, *Candida* sp.) are capable of using tannins as a carbohydrate source.

Tandem mass spectrometry (ESI-MS/MS) approach was also used for the identification of flavonoid glycosides, and biflavonoids from the active fraction.

Chemical investigation of the mangrove *Aegiceras corniculatum*; the crude extract of which exhibited promising central nervous system depressant activity resulted in the isolation and identification of flavonoid glycoside (quercitrin) and hexose sugar (iditol) as the active principle. Besides *A. corniculatum* (Blanco) also inhibited the growth of the bacterium *E. coli* and fungi *A. fumigatus* and *Fusarium sp.* which was found to be due to a new saponin, present in 0.058% yield, antibacterial against *E. coli* and fungistatic against *A. fumigatus*. The compounds were identified on the basis of 2D NMR and ESI-MS/MS.

The halophyte *S. portulacastrum*, exhibited marked inhibition of oxytocin-induced contractions. Chemical investigation of this mangrove resulted in the identification of four novel flavonol glycosides, and a glycolipid. Structure elucidation of these molecules using modern spectroscopic techniques has been discussed in this section.

Chemical studies of the hypotensive methanolic extract of the sponge *Cinachyra cavernosa* belonging to the class Demospongiae forms the content of third chapter. Sterols were identified on the basis of GC-MS of their acetates from the chloroform soluble fraction and amino acid sequences of cyclic peptides were elucidated on the basis of collision-induced dissociation (CID) experiments i.e. tandem mass spectrometry.

Publications

Glycolipids from the red alga *Chondria armata* (Kütz.) Okamura

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Three distinct fractions containing polar glycolipids (PF_{1–3}) were isolated from the chloroform soluble fraction of crude methanolic extract of red alga *Chondria armata* (Kütz.) Okamura on gel chromatography over Sephadex LH₂₀. Their structure was elucidated by multidimensional nuclear magnetic resonance (NMR) techniques like ¹H, ¹H correlation spectroscopy (COSY), ¹H, ¹H total COSY (TOCSY), ¹H, ¹³C heteronuclear multiple quantum coherence (HMQC), and ¹H, ¹³C heteronuclear multiple bond correlation (HMBC) complemented by electrospray ionization mass spectrometry (ESI-MS) in the positive ion mode. The coupling constant of the anomeric proton in ¹H NMR spectrum and sign of rotation indicated an exclusive configuration of the sugar molecules in the glycerolipids. Major glycolipids were identified as (2R)-2-O-(5,8,11,14-eicosatetranoyl)-3-O- α -D-galactopyranosyl-sn-glycerol (GL₂), its pentacetate (GL₁), and (2R)-1-O-(palmitoyl)-2-O-(5,8,11,14,17-eicosapentanoyl)-3-O- β -D-galactopyranosyl-sn-glycerol (GL₃). Each was methanolysed to give the same galactosylglycerol which on ESI-MS provided a pseudomolecular ion at *m/z* 309 representing deacylated glycolipid with the sodiated sugar moiety. Additionally, six minor glycolipids were also identified on the basis of ESI-MS. These include a 1,2-di-O-acyl-3-O-(acyl-6'-galactosyl)-glycerol (GL_{1a}), sulfonoglycolipids 2-O-palmitoyl-3-O-(6'-sulfoquinovopyranosyl)-glycerol (GL_{2a}) and its ethyl ether derivative (GL_{2b}), 1-oleoyl-2-palmitoyl-3-O-galactosyl glycerol (GL_{3a}), and 1,2-diacyl phosphatidyl glycerol (GL_{3b}). GL₁, GL_{1a}, and GL_{2b} are new to the literature. The novelty of the remaining identified compounds lies in the diversity of their fatty acid composition. Antimicrobial properties of these glycolipids against pathogens were evaluated. The yeast *Candida albicans* and the bacteria *Klebsiella sp.* were as sensitive as the standard Nystatin and antibiotic Streptomycin against PF₃. Considerable activity was expressed by the same metabolite against the fungus *Cryptococcus neoformans* as compared to the control. Weak activity against the bacteria *Shigella flexneri* and *Vibrio cholerae* and the fungus *Aspergillus fumigatus* was also observed. Fraction PF₂ was weakly active against some strains whereas all of them were resistant to its acetyl derivative PF₁. Antimicrobial activity of glycolipids is being reported here for the first time.

Key words: antimicrobial/*Chondriaarmata*/ESI-MS/ glycolipids/NMR

Introduction

Glycoglycerolipids are glycolipids in which one or more saccharide residues are linked by a glycosyl linkage to a lipid moiety containing a glycerol residue. They constitute an important class of membrane lipids that are synthesized by both prokaryotic and eukaryotic organisms (Kates, 1990). They are reported to exhibit diverse biological functions. There is currently considerable interest in both intracellular and extracellular glycolipids, especially galactosyl glycolipids as antitumor promoters in cancer chemoprevention (Colombo *et al.*, 2001).

As a part of our systematic search for potentially useful biomedical agents of marine origin, we have been investigating metabolites of the red alga *Chondria armata* (Kütz.) Okamura, belonging to the family Rhodomelaceae. The methanolic extract of this alga exhibited 75% antiviral activity against SFV (Semiliki Forest Virus) (Kamat *et al.*, 1992) and hypotensive activity (Naqvi *et al.*, 1981). Earlier we have reported pigment caulerpin, novel ester pentyl hentriacontanoate, fatty acids and sterols (Wahidulla, 1999, 2000a, 2000b), and novel polyethers, armatols (Ciavatta *et al.*, 2001), from this alga. This article presents a full account of the structural elucidation of major galactosylglycerols identified as (2R)-2-O-(5,8,11,14-eicosatetranoyl)-3-O- α -D-galactopyranosyl-sn-glycerol (GL₂), its pentacetate (GL₁), and 2R-1-O-(palmitoyl)-2-O-(5,8,11,14,17-eicosapentanoyl)-3-O- β -D-galactopyranosyl-sn-glycerol (GL₃) from the same source and reports antimicrobial activity exhibited by GL₂ and GL₃ (Figure 1A).

An effort to elucidate the structure of the additional related molecular species, with the same *R_f* values on thin layer chromatography (TLC) and were inseparable from the purified major glycolipids GL_{1–3}, was made based on tandem mass spectrometry. The structures proposed for these minor constituents have also been incorporated.

Results

Extraction and fractionation of glycolipids

Fresh alga was exhaustively treated with methanol and the chloroform soluble fraction on column chromatography gave, in order of polarity, fractions PF_{1–3}, apparently homogenous on TLC, yielding purplish pink spots on spraying with methanolic sulfuric acid. Their ¹H and ¹³C nuclear magnetic resonance (NMR) spectra (Table I) closely resembled those reported for galactosyl glycerolipids. Electrospray ionization mass spectrometry (ESI-MS) was useful to characterize molecular ions and the sequence

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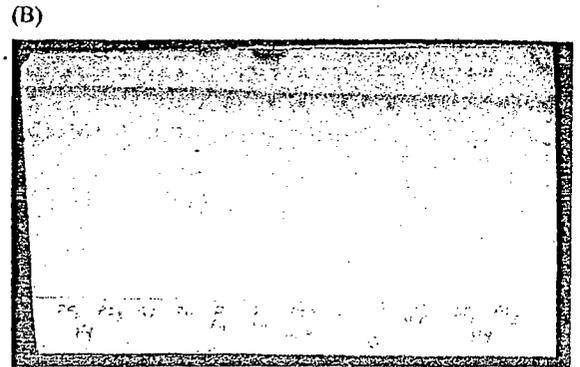
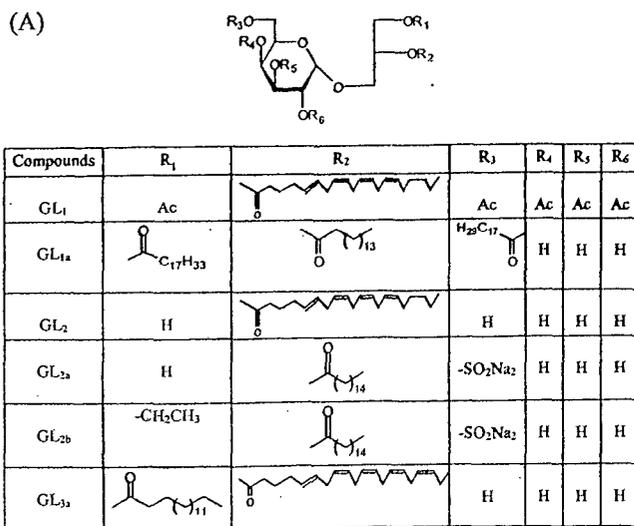


Fig. 1. (A) Table of structures of glycolipids, GL₁₋₃ isolated from *Chondria armata* (B) Thin Layer Chromatography of the hydrolysed fractions PF₁₋₃ the sugar standards [Galactose, Rhamnose, D & L Fucose, Deoxyglucose, Lactose and β-D-Glucose] in Butanol:Acetic acid:Water(5:1:4) solvent system.

of groups in the studied molecules and to distinguish lipid structures that gave similar NMR spectra.

Structural characterisation of PF₁

The purified PF₁ gave protonated molecular ion [M + H]⁺ at *m/z* 751 in its ESI-MS spectrum (Figure 2A). The presence of spin systems corresponding to one hexose, glycerol and fatty acid was readily identified from the 1D and 2D homonuclear ¹H correlation spectroscopy (COSY) NMR spectra. Thus, the ¹H NMR spectrum (300 MHz, deuterated chloroform [CDCl₃]) was in agreement with monogalactosyldiacylglycerol (MGDG), with the fatty acyl chain being evident by the presence of a triplet due to a terminal methyl at δ 0.827, a broad methylene signal at δ 1.202 [(CH₂)_n] of aliphatic chain, multiplets at δ 2.268, 1.967, and 1.562 assigned to three methylenes linked α, β, and γ to the ester carbonyl functionality. A broad multiplet at δ 2.7 arises from allylic methylene protons and the olefinic methine protons were evident at δ 5.293. A sharp singlet at δ 2.12 was attributed to acetyl methyls.

The presence of glycerol moiety was also confirmed by a heteronuclear multiple quantum coherence (HMQC) experiment, which showed two doublets arising from C-3 and C-1. The signals at δ 4.22 and 4.35 correspond to the substitution at C-1 (δ 62.2) by an *O*-acyl group and the doublet at δ 3.56 and 3.96 was assigned to C-3 (δ 68.2) of glycerol substituted by the α-galactose residue. The glycerolipid structure was confirmed by the presence of a characteristic signal at 70.0/5.23 (C-2) having a distinct α-shift to lower field for ¹³C and ¹H nuclei when substituted by an *O*-acyl group, this being a fingerprint for glycolipids containing glycerol as alcohol rather than sphingosine (Sasaki *et al.*, 1999).

¹H-¹H COSY, total COSY (TOCSY), and HMQC correlations allowed assignment of sugar carbons and protons (Table I). ¹H-¹H COSY and TOCSY correlation of the anomeric proton at δ 4.178 with the sn-3 protons at δ 3.56

and 3.96 established connectivity of the sugar moiety with the glycerol. The anomeric proton at δ 4.178 with a coupling constant of 2.1 Hz indicated α-glycosidic configuration of the sugar linkage with the glycerol (Dabrowski *et al.*, 1980).

Long-range heteronuclear multiple bond (HMQC) diagnostic correlations were observed between the ester carbonyls at δ 173.8 and δ 173.5 and C-1 and C-2 of glycerol, indicating the linkage. The stereochemistry at C-2 was assigned to be *R* by comparison of the coupling constant values between H-2/H-3a (*J* = 3.6 Hz) and H-2/H-3b (6.0 Hz), respectively, with those of published data (Oshikawa *et al.*, 1994; Murikami *et al.*, 1995; Reshef *et al.*, 1997). On the basis of the above data the major component of PF₁ was identified as pentacetate of GL₂ (GL₁). The fragmentation observed in tandem mass spectrometry (MS/MS) spectrum of GL₁ (Figure 2C) is well in agreement with the structure assigned. The pseudomolecular ion at *m/z* 751 generated a series of daughter ions at *m/z* 691, 631, 571, and 511, reflecting successive loss of four acetic acid molecules. The presence of a fifth acetyl group was evident from the elimination of yet another acetic acid molecule, yielding sodiated fragment at *m/z* 473. Alternately, the ion at *m/z* 473 might have originated, as diprotonated sodiated fragment ion, after the elimination of arachidonate ion. This ion on elimination of the fifth acetic acid molecule would lead to ion at *m/z* 413. This confirmed the presence of acetylated hexose linked to the glycerol moiety, with the latter being diesterified by acetic acid and eicosatetraenoic acid. The proposed structure of GL₁ along with identified fragments is represented in Figure 2C.

There is a solitary reference in the literature on the identification of 2-*O*-α-D-galactopyranosyl glycerol hexacetate from *Ruellia brittoniana* E. Leonard (Acanthaceae) (Ahmed *et al.*, 1990). The acetylated galactoglycerolipid is being reported here for the first time from a marine source.

ESI-MS (Figure 2A) of PF₁, though apparently homogeneous on TLC, showed some heterogeneity by the presence

Table I. NMR (300MHz) data of PF₁₋₃ in CDCl₃

PF ₁				PF ₂			PF ₃			
Carbon no.	¹ H	¹³ C	HMBC	¹ H	¹³ C	HMBC	Carbon no.	¹³ C	HMBC	
1	4.22, 4.35	62.2	1''', 2	4.0, 4.396	62.2		1	4.38	62.9	1''
2	5.23 (m)	70.0	-	5.3	70.0	-	2		70.2	3
3	3.56(d, 3.6Hz), 3.96(d, 6.0Hz)	68.2	1, 1'	3.515 (b), 3.913 (b)	67.9		3	3.83 (d, 6Hz)	68.1	1', 2'
1'	4.178(d, 2.1Hz)	103	2'	4.25 (d, 4.5Hz)	103.6		1'	(d, 7.2Hz)	104.0	2'
2'	3.52'	71.6	3', 4'	3.633	72.4		2'		71.3	3'
3'	3.85(b, s)	67.9	4'	3.90	67.9		3'		69.2	
4'	3.55(d, 3.6Hz)	73.1	3', 5'	3.692	73.1		4'		74.6	
5'	3.61(m)	72.3	1', 6'	3.5(b)	71.6		5'		73.5	1'
6'	4.23 (4.39)	62.2	5'	4.30, 4.393	62.2		6'	4.271	62.0	5'
1''	-	173.5	-	-	173.9		1''		173.5	
2''	2.268	34.1	3'', 1'', 4''	2.357	34.1	1'', 3'', 4''	2''	1, 7.8 Hz)	34.3	1'', 3'', 4''
3''	1.967	25.6	2''	2.043(b)	27.2	2'', 4''	3''	im	25.6	2'', 4''
4''	1.562	24.8		1.639	25.04		4''	(q, 6Hz)	27.2	5'', 3''
7''	2.78	27.1	9'', 10''	2.836(b)	29.3	9'', 10''				
8''	5.31 (d, 5.4Hz)	129.6	8''	5.390(b)	128.8	8''	5'', 6'', 8'', 9'', 11''	-5.38 (cluster)	127-132 (10 d)	4'', 7''
9''	5.31	128.7	11''	5.390(b)	130.0	11''	7'', 10''	3 (br dd)	29.3-31.4 (4 t)	5'', 6''
20''	0.827(t, 6.9Hz)	14.08	16'', 17''	0.827	14.1	16''	19''	(m)	22.7	18'', 20''
(CH ₂)n	1.202 (bs)	29.1-30.8		1.280(b)	29.3-31.9		20''	7(t, 7.5)	14.1	18'', 19''
1'''	-	173.8	2'''				1'''		173.6	
2'''	2.12 (s)	22.6	1'''				2''', 3'''	3-1.659 (m)	33.5, 24.9	
O=C-CH ₃	2.12	22.6	Acetyls-sugar				4''', 13'''	7	25.6-29.7	
							16'''	2 (t, 6.9Hz)	18.4	

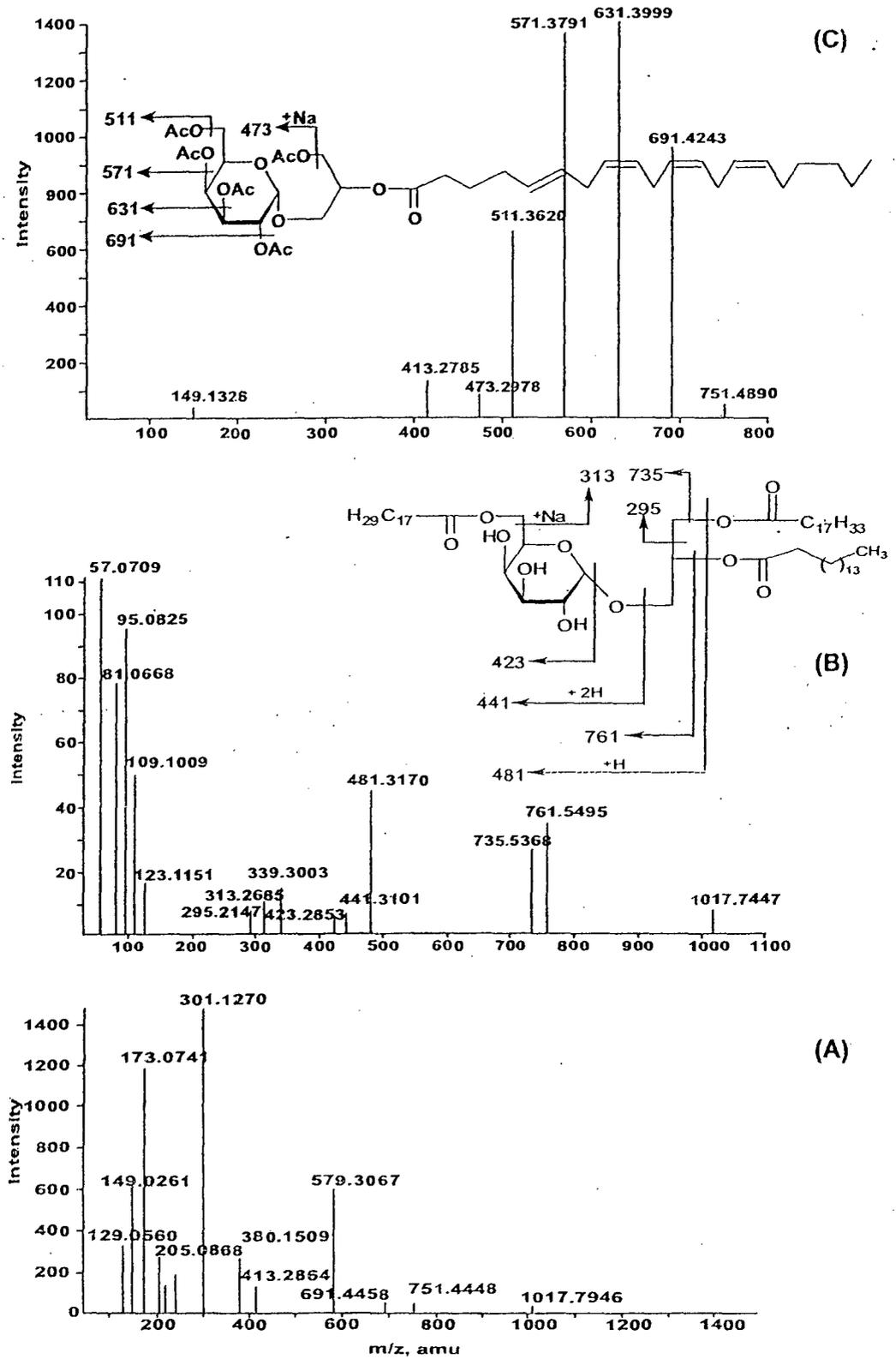


Fig. 2. (A) Positive ESI-MS of the fraction PF₁ from *Chondria armata*; (B and C) MS/MS of the ions at m/z 1017[M+H]⁺ and 751[M+H]⁺ of the glycolipids along with their proposed structures.

of an additional related molecular species with m/z 1017. Based on the fragmentation pattern observed in MS/MS (Figure 2B) it was characterized as 1,2-di-*O*-acyl-3-*O*-(6-acylgalactosyl)-glycerol GL_{1a}.

MS/MS studies of the $[M + H]^+$ ion at m/z 1017 (Figure 2B) resulted in three major diagnostically important daughter ions at m/z 481, 735, and 761. The ions at m/z 735 and 761 reflect the neutral losses of the sn-1 and sn-2 substituents as free C_{18:1} and C_{16:0} carboxylic acid, respectively, supporting the presence of palmitic and oleic acyl moieties in the molecule. The intensity differences of these various ions indicated the position of the different fatty acid moieties, as the substituent position at sn-2 fragments comparatively easily (Murphy and Harrison, 1994). This leaves a mass for the core of the molecule of 481 amu. Such a mass can be explained by a substituted hexose connected to a glycerol backbone after elimination of fatty acyl groups from the protonated molecular ion $[M + H]^+$. This is further supported by the presence of an additional fragment ion at m/z 441, which reflects the loss of acyl groups (C_{18:1} and C_{16:0}) from the molecular ion along with the glycerol backbone, together corresponding to a total mass of 577 amu. Fragment ion at m/z 423 results from the cleavage between C₁ of hexose and C-3 of glycerol. Cleavage of the molecule between C5 and C6 of the sugar leads to sodiated fragment at m/z 313 which corresponds to the third acyl substituent (C_{18:3}) along with C-6 of sugar, which possibly seems to be galactose. The ion at m/z 295 results from the cleavage between C1 and C2 of glycerol. Furthermore, there were a number of fragments in the upper mass region at intervals of about 14 amu. These correspond to fragmentation along the fatty acid acyl chains. On the basis of this fragmentation pattern of the molecular species with the pseudomolecular ion at m/z 1017, we propose the structure of the molecule as being 1-oleoyl-2-palmitoyl-3-*O*-(linolenyl-6'-galactosyl)-glycerol (GL_{1a}), which along with identified fragments is illustrated in Figure 2B.

Structural characterization of PF₂

A similar approach was adopted for PF₂, which showed physicochemical characteristics of glycolipids. Its NMR data differed from that of PF₁ only by the absence of signals for the acetyl groups (Table I), indicating it to be a deacetylated derivative of GL₁. This was further supported by its ESI-MS (in MeOH), which exhibited pseudomolecular ion $[M + H]^+$ at m/z 541 consistent with the molecular formula of C₂₉H₄₉O₉ (PF₂). The MS/MS at m/z 541 (Figure 3D) showed peak at m/z 179 for loss of a sugar unit. Subsequent loss of the four water molecules from the hexose led to the base peak at m/z 107. The cleavage of the molecule between C-3 of glycerol and oxygen linking it to the hexose gives the fragment ion at m/z 343 with simultaneous elimination of water molecule. The sodiated ion at m/z 204 results from the attachment of two hydrogens to the hexose moiety. Fragmentation of the ester bond leads to the ion at m/z 239. Similarly, the fragment at m/z 223 could be explained as being formed by cleavage of C2-C3 bond of glycerol backbone and cleavage between the oxygen and carbonyl of carboxylate group. The ion at m/z 267 results from the addition of sodium to the fragment derived from

the McLafferty rearrangement in the acyl moiety. The structure of the major component from PF₂ was established as GL₂.

The ESI-MS examination of PF₂ (Figure 3A) when in a dilution solvent (as given under *Materials and methods*) showed additional peaks at m/z 601 and 629 corresponding to pseudomolecular ions of the Na salt (Na⁺ form) of monoglycolipids $[M-H + 2Na]^+$. An effort was made to elucidate their structure by tandem mass spectrometry of molecular species.

Thus, MS/MS of the pseudomolecular ion at m/z 601 exhibited the most abundant product ions at m/z 514 and 497, which have a mass difference corresponding to loss of sulfono group (82 amu) as sulfonic acid (SO₃H) as sodium salt (SO₃Na) respectively. The product observed at m/z 345 appears to have originated by the loss of fatty acyl side chain as corresponding acid (palmitic C_{16:0}). Cleavage between C-3 of glycerol and the oxygen of the anomeric carbon of hexose results in the simultaneous formation of the fragments at m/z 313 and 273. The ion at m/z 273 loses one water molecule to yield the fragment at m/z 259. The ion at m/z 165 results from the elimination of sodium sulfonate group from the sulfoquinovopyranose moiety and cleavage between C-3 of glycerol and the oxygen at the anomeric carbon with the attachment of two hydrogens. Subsequent elimination of three water molecules leads to the ion at m/z 111. Based on fragment pattern, the glycolipid with pseudomolecular ion $[M + 2Na]^+$ at m/z 601 was characterized as 2-*O*-palmitoyl (6'-sulfoquinovopyranosyl)-glycerol (GL_{2a}). The proposed structure along with its identified fragments is shown in Figure 3C.

A similar fragmentation pattern (GL_{2a}) observed for sulfoquinovosyl molecular species with pseudomolecular ion at m/z 629 led to the structure GL_{2b} as represented in Figure 3B. From the fragmentation observed it is interesting to note that the difference of 28 amu observed between the two sulfolipids is not because of the difference in fatty acid chain length as expected but seems to be due to the ethoxy group at C-1 of glycerol. The presence of an ethoxy group is further reinforced by the presence of an NMR signal for CH₂ attached to sulfur at 53.6 ppm, indicating impurity in PL₂. The galactosylglycerolipids MGDG (digalactosyldiacylglycerol) and SQDG (sulfoquinovosyl diacylglycerol) are uncharged species while sulfoquinovosyl diacylglycerol (SQDG) is negatively charged at neutral pH. This explains their presence in the admixture as sodiated adducts.

Structural characterization of PF₃

The ESI-MS profile of PF₃ is illustrated in Figure 4A. MS of the major component of this fraction was consistent with the sodiated molecular ion $[M + Na]^+$ at m/z 799 corresponding to the molecular formula of C₄₅H₇₆O₁₀Na. Its ¹H NMR and ¹³C NMR (Table I) closely resembled those of PF₂ except that the ¹³C signals due to the unsaturation in the fatty acid moiety were more distinct. The tandem MS spectrum of ion at m/z 799 is illustrated in Figure 4B and it represents GL₃. The main fragmentation pattern corresponding to concomitant elimination of two fatty

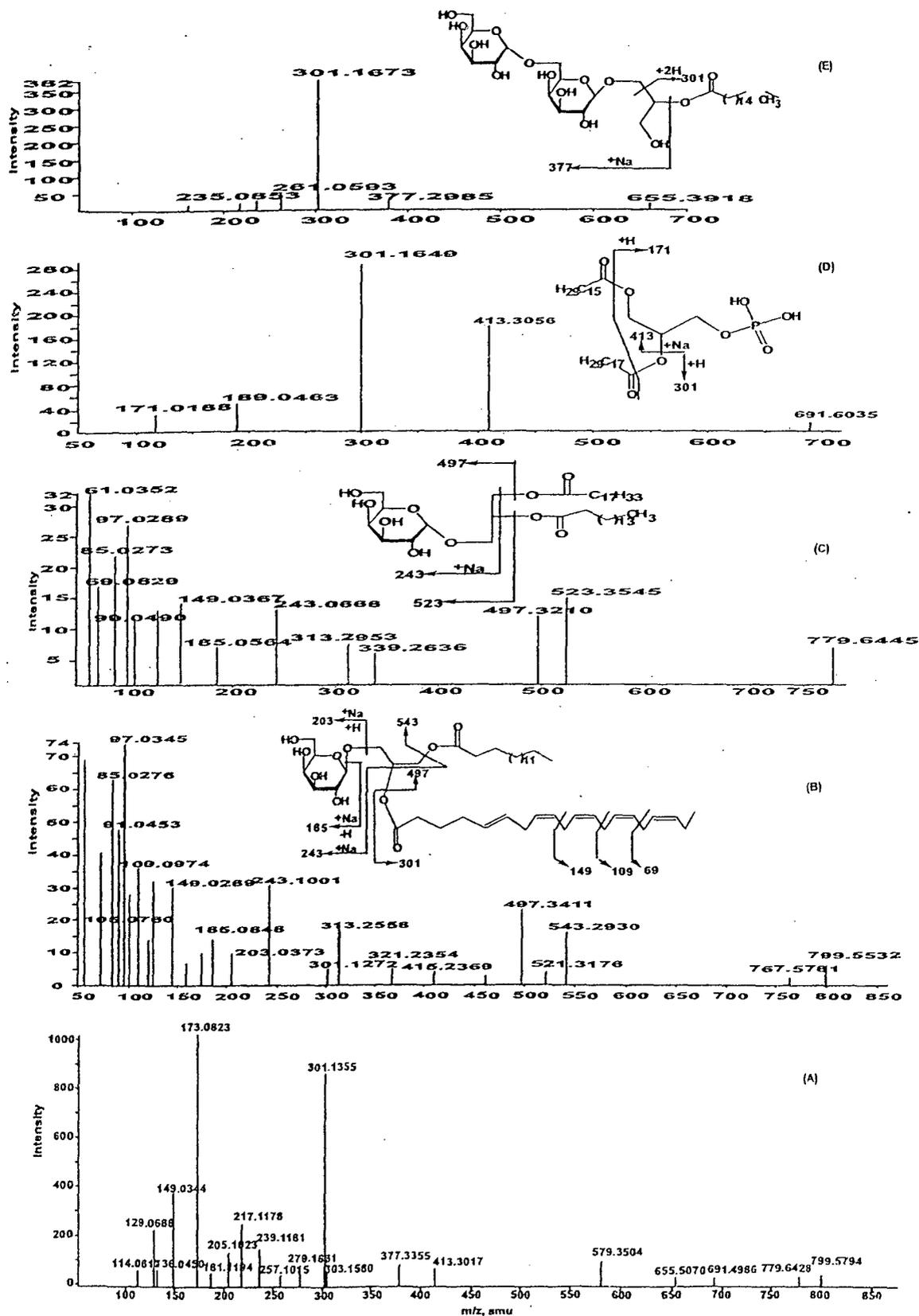


Fig. 4. (A) Positive ESI-MS of glycolipid fraction PF₃ ranging from *m/z* 0–900, (B) MS/MS of glycolipid GL_{3a} at *m/z* 799; (C) *m/z* 779 [M + Na]⁺; (D) of phospholipid with [M + Na]⁺ 691; (E) of glycolipid with *m/z* 655.

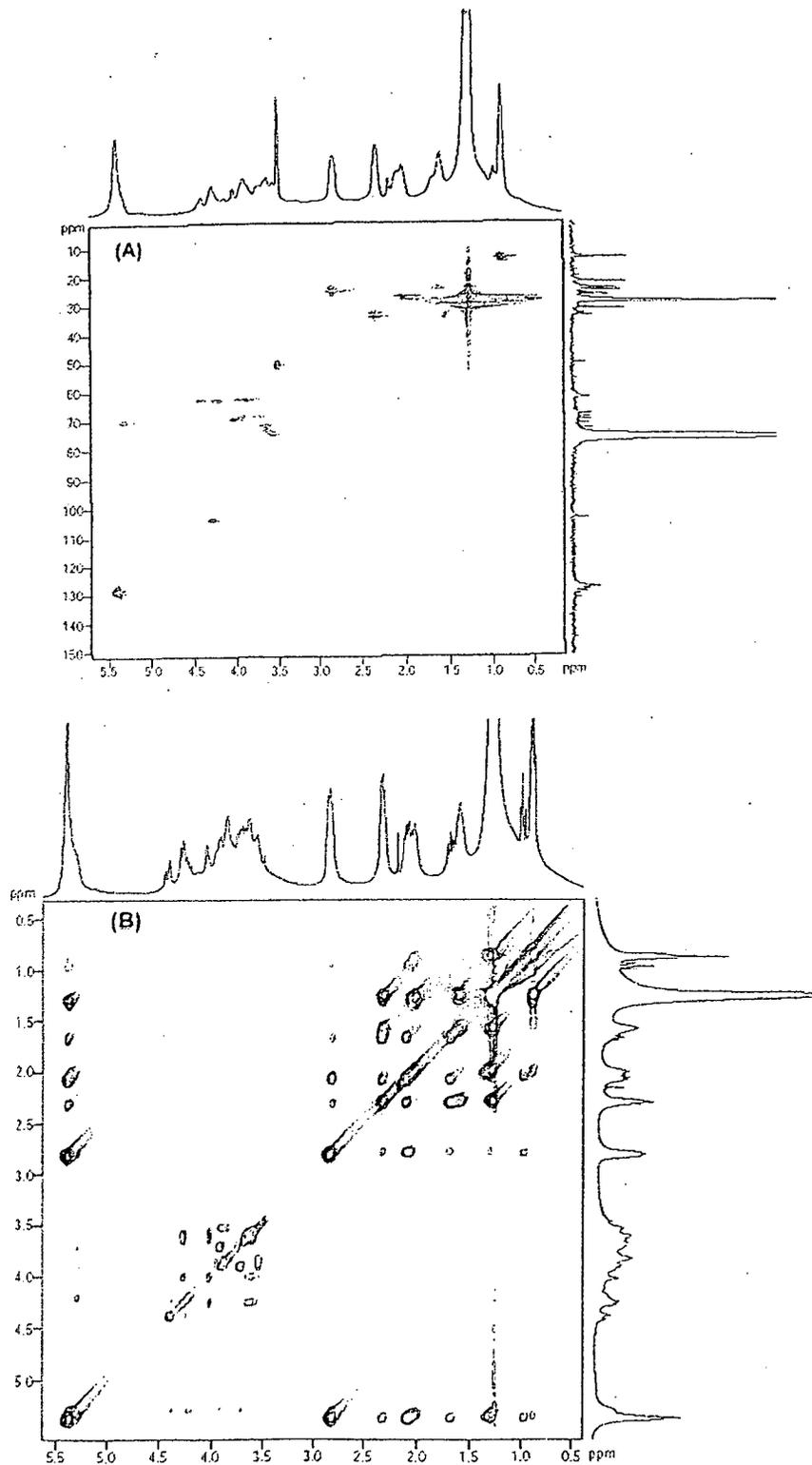


Fig. 5. HMQC (A) and TOCSY (B) spectra of the glycolipid, GL_3 at 300MHz in $CDCl_3$.

other ions of related molecular species besides the main component. The MS/MS of sodiated molecular ion $[M + Na]^+$ at m/z 779 (Figure 4C) yielded fragments at m/z 497 and m/z 523, indicative of loss of $C_{18:1}$ and $C_{16:0}$ fatty acyl

groups from the molecule as free fatty acids, respectively. The presence of galactosyl sugar moiety was evident from the ion at m/z 243. The intensity of the signals led to the placement of palmitic acid at sn-2 position (Blair, 1991

Waugh and Murphy, 1996; Hankin *et al.*, 1997). Taken together, the structural analysis for the molecular species with ion at m/z 779 is consistent with 1-oleoyl-2-palmitoyl-3-*O*-galactosyl glycerol (GL_{3a}).

The collision-induced dissociation (CID) daughter-ion spectrum of the molecular species at m/z 691 is illustrated in Figure 4D and it represents 1,2-diacyl phosphatidyl glycerol (GL_{3b}). The main fragmentation pathway observed here is the formation of ion at m/z 413 originating from the loss of 278 amu, corresponding to the loss of C_{18:3} as free fatty acid. The ions at m/z 171 and m/z 189 are consistent with the cleavage at C₁₂ of γ -linolenic acid as free acid and as ketene, respectively (Kim *et al.*, 1999). The fragment at m/z 171 could also arise from phosphoglycerol moiety. The most intense ion at m/z 301 was attributed to the concomitant elimination of palmitoleoyl and phosphatidyl groups along with the glycerol backbone as depicted in Figure 4D or elimination of linoleic acid as sodium salt. The abundance of the ion at m/z 301 as compared to the ion at m/z 413 is consistent with the notion that neutral loss of the fatty acid at sn-2 is sterically more favorable than the analogous loss at sn-1 position (Blair, 1990; Waugh and Murphy, 1996; Hankin *et al.*, 1997). Thus structure GL_{3b} was proposed for the molecular species with $[M + Na]^+$ ion at m/z 691.

Tandem MS scanning experiment of protonated molecular species at m/z 655 yielded the most prominent ion at m/z 301, reflecting the loss of 354 amu, which is probably due to the presence of a digalactosyl unit and a much less intense fragment at m/z 377 corresponding to the loss of palmitoyl group as sodium palmitate from the molecule. The relative abundance of the ions placed the palmitoyl group at sn-2 position. The spectrum is consistent with 3-digalactosyl-2-palmitoyl glycerol, represented in Figure 4E.

Methanolysis of PF₁₋₃

To identify the acid substituents at C-1 and C-2 of component glycolipids of PF₁₋₃, methanolysis was performed in anhydrous methanol with excess of Na₂CO₃. All the three fractions yielded the same glycoside, 3-*O*-D-galactopyranosyl-sn-glycerol, and methyl esters of corresponding fatty acids. The mixture of the reaction product was analyzed by ESI-MS in the positive ion mode. Thus, for example, the ESI-MS of PF₃ gave pseudomolecular ions at m/z 183, 277, 309, 301, 334, and 389. Analysis of each of these ions by tandem MS established their identity. Thus the ion at m/z 183 corresponded to the attachment of two hydrogens to the sugar moiety $[M + 2H]^+$. 3-*O*-D-galactopyranosyl glycerol as sodium adduct was observed at m/z 277. Deacylated glycolipid with the sodiated sugar moiety was evident as protonated molecular ion at m/z 309. The fragment at m/z 301 represented the presence of eicosapentanoate. Thus, the fragmentation observed in MS/MS of ion at m/z 309 (Figure 6), a fragment common as product of hydrolysis of PF₁₋₃, is shown in Figure 6.

To establish the nature of the sugar moiety as D-galactose, the glycolipids were subjected to acid hydrolysis and the compound identified by TLC with standard sugars as described in *Materials and methods* (Figure 1B). The optical rotation of the sugar obtained by hydrolysis was well in agreement with the values reported for D-galactose.

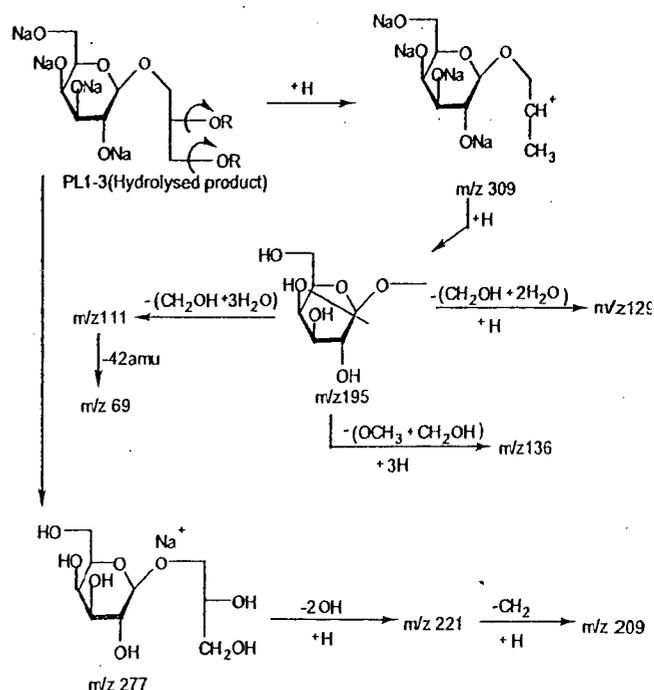


Fig. 6. ESI-MS/MS of glycolipid spectrum with m/z 309 from the fraction PF₁₋₃ along with the fragmentation pattern.

Antimicrobial activity of PF₁₋₃

Bergsson *et al.* (2001) have studied the susceptibility of *Candida albicans* to several fatty acids and their 1-glycerides. They observed that capric acid, a 10-carbon saturated fatty acid, causes the fastest and most effective killing of all the three strains of *C. albicans* tested. Lauric acid, a 12-carbon saturated fatty acid, was the most active acid at lower concentrations. Subsequently, Frentzen *et al.* (2003) reported on the medium-chain fatty acids of 8–12 carbon atoms exhibiting antibacterial and antifungal properties, which are enhanced when these acids are esterified with glycerol. The same authors also state sucrose esters as being less effective in inhibiting the fungal growth. Based on these reports, it is expected that pathogens would be sensitive to glycolipids. This led us to evaluate the pure fractions PF₁₋₃ of the present investigation, isolated and identified from the red alga *C. armata*, against different strains of pathogenic microorganisms, for antibacterial and antifungal activities and compare them with the commercially available antibiotics (Table II).

As evident from Table II, all the bacteria and fungi tested were resistant to PF₁ at the dose tested (65 $\mu\text{g}/\text{mL}$). PF₂ showed mild inhibitory activity against the bacteria tested except *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, and at 250 $\mu\text{g}/\text{disc}$ being also weakly active against the fungi *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Aspergillus niger*, and *Rhodotorula sp.* PF₃, at 130 $\mu\text{g}/\text{disc}$, was as effective as standard Nystatin and antibiotic Streptomycin, against the yeast *C. albicans* and bacteria *Klebsiella sp.*, respectively. Considerable activity was also expressed by PF₃ against the fungus *C. neoformans*, strain

Table II. Antimicrobial activity of glycolipids from *Chondria armata*

Fractions	Antifungal													
	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>S. typhii</i>	<i>S. flexneri</i>	<i>Klebsiella sp.</i>	<i>V. cholerae</i>	<i>A. fumigatus</i>	<i>Fusarium sp.</i>	<i>C. neoformans</i>	<i>A. niger</i>	<i>Rodhotorula sp.</i>	<i>Nocardia sp.</i>	<i>C. albicans</i>
PF ₁	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PF ₂	+(st)	-	+(st)	+	+(st)	-	1	1	1	1	+	-	-	-
PF ₃	-	-	-	-	+	4	1	1	-	2	-	-	-	4
Standard Streptomycin	10	-	11	+	6	4	10							
Standard nystatin														4

Numbers indicate the zone of inhibition in mm from center of imbedded disk.

-, indicates no activity.

+, indicates weak activity.

+(st), indicates that it shows activity but zone of inhibition is not very clear.

resistant to Nystatin. PF₃ showed mild activity against bacteria *Shigella flexneri* and *V. cholerae* and the fungus *fumigatus*. All the three compounds were ineffective against the multidrug-resistant strains tested.

Results indicate that acetylation inactivates the molecule and the activity is greatly influenced by the anomeric configuration of glycosidic linkage—compounds with β configuration being more effective than the glycosides with α configuration. Antimicrobial activity of glycolipid is being reported here for the first time.

Discussion

Three major galactoglycerolipids have been isolated and identified, in the native form, from the red alga *C. armata* using NMR complemented with mass spectrometry. Several minor glycolipids have also been identified on the basis of electrospray ionization tandem MS/MS spectrometry alone. Methanolysis of the glycolipids yielded galactosyl glycerol, which on ESI-MS provided a pseudomolecular ion at m/z 309 representing deacylated glycolipid with the associated sugar moiety. Recently, Shao *et al.* (2002) reported the presence of a new sulfonoglycolipid, crassicaulisine, with palmitoyl and myristoyl as acyl groups, from the red alga belonging to the same genus *Chondria crassicaulis*. Acyl groups in PF₁₋₃ were characterized as the corresponding acids or carboxylate ions (ESI-MS), and the principal components were arachidonic acid in PF₁₋₂, palmitic acid, and eicosapentaenoic acid in PF₃. There were minor components, which include C_{16:1}, C_{18:1}, and C_{18:3} acids.

It is of interest to note that polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and arachidonic acid (AA) are present in the alga in bound form as acyl substituents of galactosyl acyl glycerols. In agreement with previous reports, palmitic acid seems to be the major fatty acid of sulfonoglycolipids of marine algae. Contrary to the report of Choi *et al.* (1999) in glycolipids of marine algae, the glycosidic linkage could be α/β and the sugar moiety attached, mainly, to C-3 of sn-glycerol.

GL_{1a} is the first example of the natural occurrence of acyl glycerol acylated at the sn-1, sn-2 and 6' positions. The presence of acyl glycerol acylated at the sn-1 and 6' positions of mannobiosyl is known from the bacteria *Arthrobacter atrocyaneus* and *Microcoleus luteus* (Bultel-Por *et al.*, 1997; Niepel *et al.*, 1997).

In recent years, glycolipid analogues have gained importance in cancer chemoprevention because of their promising inhibitory effect exhibited by them on tumor promoting activity. The fatty acyl chain length, its position and the nature of sugar moiety influence the activity. Galactosyl glycerols are reported to be more potent than the corresponding glucosylglycerols with the same structural features (Colombo *et al.*, 1996, 1999). The anomeric configuration does not seem to affect the activity (Colombo *et al.*, 2000).

MGDGs, containing (7Z, 10Z)-hexadecadienoic acid group, from the green alga *Chlorella vulgaris* are reported to exhibit anti-tumor promoting effect (Morimoto *et al.*, 1995). SQDG from algae inhibits DNA-polymerase α and HIV-reverse transcriptase (Gustafson *et al.*, 1989; Loya *et al.*

1998; Ohta *et al.*, 1998). It is well known that biological activity of marine macrophytes is related to the essential polyunsaturated fatty acids (PUFAs), which are the abundant components of macrophytic glycolipids (Khotimchenko, 1993; Goncharova *et al.*, 2000; Sanina *et al.*, 2000).

The red algae are reported to have high levels of polyunsaturated fatty acids, mainly EPA and AA (Khotimchenko and Svetashev, 1987), but the contents vary within the same genus. *Chondria dasyphylla* (Wood) Ag. is reported to have equal contents of EPA and AA whereas in *Chondria decipiens* EPA predominates (Khotimchenko and Vaskovsky, 1990). Further, in red algae PUFAs belonging to C20 series are reported to be mainly concentrated in MGDG (Sanina *et al.*, 2004). This has in fact been observed in the present investigation, with EPA and AA being the constituent fatty acids of major glycolipids identified in PF₁₋₃, and is well in agreement with our earlier communication on the fatty acids from the alga *C. armata*, where C20 acids were not detected as free fatty acids (Govenkar and Wahidulla, 1999).

Glycoglycerolipids occur widely and copiously in vascular plants (Van Hummel, 1975), certain green seaweeds (Arao and Yamada, 1989; Falsone *et al.*, 1994; Mancini *et al.*, 1998), cyanobacteria (Reshef *et al.*, 1997), marine dinoflagellates (Oshima *et al.*, 1994), and the freshwater alga *C. vulgaris* (Morimoto *et al.*, 1995). As to the glycoglycerolipids of red algae, hydroxyecosapentaenoyl galactosyl glycerols are known from the temperate red alga *Gracilariopsis lemaneiformis* (Jiang and Gerwick, 1990), and MGDG, DGDG, and SQDG are reported from *Gracilaria verrucosa* (Son, 1990), which is also known to contain sulfoquinovosylmonogalactosyl glycerol (SQMG) (GL_{2a}). This SQMG is also reported to be a constituent of cyanobacterium *Synechocystis* PCC 6803 (Kim *et al.*, 1999) and lichenized basidiomycetes, *Dictyonema glabratum* (Sassaki *et al.*, 2001). 2-O- α -D-galactopyranosylglycerol is a metabolite of *Laurencia pinnatifida* (Aplin *et al.*, 1967) and 2,3-dipalmitoyl sulfonoglycolipid has been identified in *Laurencia pedicularioides* and is reported to be the major glycolipid in red algae (Siddhanta *et al.*, 1995). Recently, Shao *et al.* (2002) reported the presence of a new sulfonoglycolipid, crassicaulisine, in the red alga *C. crassicaulis*. Taxonomically, genus *Laurencia* and *C. armata* belong to the same family, Rhodomelaceae, but in the present investigation *C. armata* did not contain either of the glycolipids.

Interestingly, palmitic acid has been found to be the most abundant fatty acid present in the sulfonoglycolipids of marine origin (Fusetani and Hashimoto, 1975; Araki *et al.*, 1989; Gustafson *et al.*, 1989; Son, 1990; Siddantha *et al.*, 1991). The two sulfonoglycolipids of the present investigation provide yet another example of a glycolipid which contains palmitic acid as the only fatty acid component. Palmitic acid was described as having hemolytic activity in sea urchin eggs (Fusetani and Hashimoto, 1976) and was presumed to be playing a unique role in algal physiology (Fusetani and Hashimoto, 1975).

Sulfoquinovosyl acyl glycerols, in particular compounds with C₁₈ fatty acid on the glycerol moiety, may be clinically promising antitumor or immunosuppressive agents (Aoki *et al.*, 2005).

Materials and methods

Biological material

The alga was collected during the low tides from coastal waters of Goa, west coast of India [15°51'N to 15°54'N and 73°51'E to 73°52'E], during the pre-monsoon periods. The alga, sample no. 1316, identified by Geeta Deshmukh CIFE, Mumbai, has been deposited at NIO Repository and Taxonomic Center.

General procedures

Sephadex LH20 (Pharmacia) and silica gel (60–120 mesh [Qualigens]) were used for gel filtration and column chromatography, respectively. Precoated Kieselgel 60 F254 TLC plates (Merck) were used for analytical TLC. Compounds were visualized as purplish spots on spraying with 5% methanolic sulfuric acid followed by heating at 100°C. Solvent system for TLC I and II was light petrol/ethyl acetate (6:4) and (1:1), respectively, and TLC III was methanol/chloroform (5:95).

Mass spectrometry

Mass spectra were recorded, in the positive mode, on a QSTARXL MS/MS, Applied Biosystems, Switzerland equipped with Analyst Software. The declustering potential and the collision energy were optimized for MS/MS experiments so as to cause fragmentation of the selected molecular ion species as evident by the appearance of fragment ions and decrease in the intensity of the molecular ion. ESI-MS was carried out by dissolving the compounds in methanol as solvent. ESI-MS of PF₂ was taken in methanol, as well as dilution solvent.

Dilution solvent

It was prepared as follows: 15.4 mg of ammonium acetate was dissolved in 49.9 mL of water. To this solution was added a mixture of 49.9 mL of methanol, 0.1 mL of formic acid, and 0.1 mL of acetonitrile.

NMR

¹H, ¹³C, COSY, HMQC, and HMBC experiments were recorded, in CDCl₃, on a Bruker (Avance 300) spectrometer with TMS (tetramethylsilane) as internal standard.

Extraction and isolation of glycolipids

The red alga, *C. armata* (3.5 kg, dry weight) was cleaned and extracted thrice with methanol using a sonicator (15 min) at room temperature. The combined methanolic extracts were evaporated under reduced pressure at 37°C temperature to a certain minimum volume (\approx 200 mL), and then partitioned into chloroform, n-butanol and water-soluble fractions.

The chloroform fraction (123 g) was fractionated, initially on a column of Sephadex LH20 with methanol (500 mL) as eluant collected in fractions of 20 mL each. The fractions obtained were examined by TLC (solvent: light petrol: ethylacetate, 1:1, v/v, spray: 5% methanolic sulfuric acid) and combined according to their profile. Fractions yielding purplish spots were then purified by repeated silica gel

chromatography using petroleum ether (60–80°C) : ethyl acetate (1:1) to give PF₁ (4 mg, R_f 0.52 in solvent I), and methanol : chloroform (2:98) yielded PF₂, [α]_D-16° (c 0.02, CHCl₃, R_f 0.45 in solvent II; yield 13 mg). Further elution of the same column with methanol: chloroform (5:95) yielded PF₃ [α]_D-20° (c 0.02, CHCl₃, R_f 0.175 in solvent III, yield 23 mg). Final purification was done on RP-18 column with methanol as eluant. As the neutral glycolipids yielded purplish pink spots with methanolic sulfuric acid, all the constituents, from chloroform soluble fraction, showing purplish pink spots on TLC were purified.

Methanolysis of glycolipids (PF₁₋₃)

PF₁₋₃, 2 mg each, were dissolved in anhydrous methanol (1 mL), and an excess of sodium carbonate was added. The solution was stirred at room temperature overnight, filtered, and the solvent evaporated. The residue was analysed by ESI-MS in methanol. Tandem mass was taken at collision energy between 30 and 35 eV.

Acid hydrolysis of glycolipids (PF₁₋₃)

Each fraction (4–8 mg) in 5 mL of 2% H₂SO₄ in methanol was refluxed for 3 h. This was followed by the addition of 4 mL of water to the reaction mixture. Methanol was removed in vacuo and the aqueous solution extracted with chloroform and then neutralized with Barium hydroxide. Precipitated barium sulfate was filtered through celite, water removed in vacuo, and the residue dissolved in 1 mL of water. TLC (butanol:acetic acid: water; 5:1:4) showed a single major spot identical with D-galactose. The NMR data do not distinguish between L and D forms of the glycosyl moieties. The D form of the monosaccharide dominates in living organisms (Hauksson *et al.*, 1995), and the only occurrence of L galactose is in agar-agar (Mathews and van Hold, 1990).

For confirmation of configuration of sugar residue, PF₁₋₃ were hydrolyzed with 2M TFA (trifluoroacetic acid) at 110°C for 3 h, following concentration to dryness under stream of nitrogen. The product was then filtered through Sephadex G-10 (Pharmacia) using MeOH:H₂O (1:1) as the mobile phase. Fractions (5 mL) were collected and monitored on TLC plates using butanol: acetic acid: water (5:1:4) as the solvent system for development. R_f value of the sugar thus obtained was equivalent to the standard D-galactose (Figure 1B). Fractions containing sugar (galactose) were combined, concentrated on a rotavapor, and their optical rotation was measured. It was found to be (+)150° [Lit.(+)150.7°, (Takahashi and Ono, 1973)] in case of hexose from PF₁₋₂ and +52°(literature: (+)52.8°) for sugar from PF₃. These results indicated that all the three samples yielded D-galactose having alpha configuration in PF₁₋₂ and beta configuration for sugar in PF₃.

Antibacterial assays

Antibacterial activity was determined against six Gram-negative bacteria (*Escherichia coli*, *P. aeruginosa*, *Salmonella typhi*, *S. flexineri*, *K. pneumoniae* and *V. cholerae*) and one Gram-positive bacteria (*Staphylococcus aureus*) using the paper disk assay method (El-Masry *et al.*, 2000). The

sterile paper disk of 6 mm diameter impregnated with μg/disk of PF₁ and 130 μg/disk of PF₂ were placed on plates containing the tested microorganisms. In all cases the concentration was approximately 1.2×10^8 CFU. The impregnated disks were placed on the medium suitably spaced apart and the plates were incubated at 37°C for 18 h. Disk of Streptomycin (10 μg/mL) was used as a positive control. The diameter (mm) of the growth inhibition halos caused by the sample was examined.

Antifungal assay

Antifungal activity was determined against *A. fumigatus*, *Fusarium sp.*, *C. neoformans*, *A. niger*, *Rhodotorula*, *Nocardia sp.*, and *C. albicans* using the paper disk assay method as previously described in the antibacterial assay. The sterile disk was impregnated with the compound (10 μg/disk of PF₁ and 130 μg/disk of PF₂). The inoculum concentration was 0.5×10^3 – 2.8×10^3 CFU/mL. Nystatin (10 μg/disk) was used as positive control. The plates were incubated at 24°C for 18 h. The diameter (mm) of growth inhibition halos caused by the compound was examined.

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Conflict of interest statement

None declared.

Abbreviations

AA, arachidonic acid; CDCl₃, deuterated chloroform; COSY, ¹H, ¹H correlation spectroscopy; DGDG, digalactosyldiacylglycerol; EPA, eicosapentaenoic acid; ESI-MS, electrospray ionization mass spectrometry; GL₁₋₃, glycolipids 1–3; HMBC, ¹H, ¹³C heteronuclear multiple bond correlation; HMQC, ¹H, ¹³C heteronuclear multiple quantum coherence; MGDG, monogalactosyldiacylglycerol; MS, tandem mass spectrometry; NMR, nuclear magnetic resonance; PF₁₋₃, polar fractions 1–3; SQDG, sulfoquinovosyldiacylglycerol; SQMG, sulfoquinovosylmonogalactosyl glycerol; TLC, thin layer chromatography; TOCSY, ¹H total COSY.

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