Chapter I

Chemical investigations of the red alga
Chondria armata
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):

---

¹ Chondria armata: A review.

²
### Table 1: Classification of Algae

<table>
<thead>
<tr>
<th>PHYLUM</th>
<th>THALUS FORMAT</th>
<th>PHOTOSYNTHETIC PIGMENTS</th>
<th>FORM OF FOOD STORAGE</th>
<th>CELL WALL COMPOSITION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chlorophyta</strong></td>
<td>Unicellular, Colonial, Filamentous, and Multicellular</td>
<td>Chlorophylls a and b, Carotenoids</td>
<td>Starch</td>
<td>Polysaccharides, Primarily Cellulose</td>
</tr>
<tr>
<td>(Green Algae)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Phaeophyta</strong></td>
<td>Multicellular</td>
<td>Chlorophylls a, and c, Carotenoids, Fucoxanthin</td>
<td>Laminarin (an o carbohydrate)</td>
<td>Cellulose with Alginic Acid</td>
</tr>
<tr>
<td>(Brown Algae)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Rhodophyta</strong></td>
<td>Multicellular</td>
<td>Chlorophylls a, Phycobilins, Carotenoids</td>
<td>Starch</td>
<td>Cellulose or Pectin, many with Calcium Carbonate</td>
</tr>
<tr>
<td>(Red Algae)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bacillariophyta</strong></td>
<td>Mostly Unicellular, Some Colonial</td>
<td>Chlorophylls a and c, Carotenoids, Xanthophyll</td>
<td>Leucosin (an o carbohydrate)</td>
<td>Pectin, many with Silicon Dioxide</td>
</tr>
<tr>
<td>(Diatoms)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dinoflagellata</strong></td>
<td>Unicellular</td>
<td>Chlorophylls a and c, Carotenoids</td>
<td>Starch</td>
<td>Cellulose</td>
</tr>
<tr>
<td>(Dinoflagellates)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Chrysophyta</strong></td>
<td>Mostly Unicellular, Some Colonial</td>
<td>Chlorophylls a and c, Xanthophyll, Carotenoids</td>
<td>Laminarin (an o carbohydrate)</td>
<td>Cellulose</td>
</tr>
<tr>
<td>(Golden Algae)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Euglenophyta</strong></td>
<td>Unicellular</td>
<td>Chlorophylls a and b, Carotenoids, Xanthophyll</td>
<td>Paramylon (a Starch)</td>
<td>No Cell Wall, Protein-rich Pellicle</td>
</tr>
<tr>
<td>(Euglenoids)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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 tetrasporangia. 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 Japan, 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 Sicily, and *Chondria baileyana*, from southern Nova Scotia and PEI, Canada. It is also known to be a constituent of *Amansia glomerata*, *Digenea simplex* and *Vidalia obtusiloba*, all belonging to the family *Rhodomelaceae*. 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 1966. However, it was not until 1982 that the correct structure with absolute configuration for (-)-domoic acid (1) was finally determined by stereospecific total synthesis and confirmed by X-ray analysis. 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 cockroach. Additional related compounds, isodomoic-acids (2-5) and domoilactone (6-7) were also discovered from the insecticidal fraction of the alga. The insecticidal activities of isodomoic acids were much weaker than that of domoic acid but comparable with that of DDT while both lactones were found to be inactive. Zaman and co-workers 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 $^1$H NMR spectral analysis including $^1$H-$^1$H 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\textsuperscript{4,16}. These useful properties of domoic acid are associated with certain disadvantages. Domoic acid is also present in edible mussels \textit{Mytiulis edulis} and whenever there has been episodes of shellfish poisoning, domoic acid has been identified as the causative substance\textsuperscript{17}. 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.

\textit{Chondria armata} from the Japanese waters is also reported to contain hypoxanthine (12), L-glutamic acid (13) and D-aspartic acid (14)\textsuperscript{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 $\alpha$-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 alga\textsuperscript{19}. The name is derived from \textit{citrullus}, the Latin word for watermelon, from which it was first isolated\textsuperscript{20}. 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 pass\textsuperscript{21}. 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 \textit{C. armata} collected from Goa (west coast of India) showed antiviral, antibacterial, and antifungal activities\textsuperscript{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 (C\textsubscript{14}H\textsubscript{28}O\textsubscript{2}), pentadecylic acid (C\textsubscript{15}H\textsubscript{30}O\textsubscript{2}), palmitic acid (C\textsubscript{16}H\textsubscript{32}O\textsubscript{2}), stearic acid (C\textsubscript{18}H\textsubscript{36}O\textsubscript{2}), 5-palmitoleic acid (C\textsubscript{16}H\textsubscript{30}O\textsubscript{2}), 4-palmitoleic acid (C\textsubscript{16}H\textsubscript{30}O\textsubscript{2}) and oleic acid (C\textsubscript{18}H\textsubscript{34}O\textsubscript{2}) 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)\textsuperscript{24}. The free sterols, were possessing Δ\textsuperscript{5}, 3β-hydroxy nucleus and were identified as cholest-5-en-3β-ol (17), 24-methylene-cholest-5-en-3β-ol (18), 24β-ethyl cholest-5,22-diene-3β-ol (19), 24β-ethyl cholest-5-en-3β-ol (20), 23ξ-methyl cholest-5-en-3β-ol (22) and 23ξ-methyl 5α-cholestan-3β-ol (22). Acetylation of the sterol mixture was also carried out and the corresponding steryl acetates obtained were analyzed by GC-MS\textsuperscript{25}.

Caulerpin (23), a dimer of indole-3-acetic acid is also present in this alga along with a fatty ester, pentyl hentriacontanoate (24)\textsuperscript{26}. The pigment caulerpin is a well known constituent of the green algae of genus \textit{Caulerpa}\textsuperscript{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)\textsuperscript{30} and inhibits the multidrug resistance (MXR) pump in algae\textsuperscript{31}. 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.\textsuperscript{30}
Subsequently, Cimino group\textsuperscript{32} 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 \textit{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 activity\textsuperscript{33}. 

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{chemical_structures.png}
\caption{Chemical structures of Armatols A-F.}
\end{figure}
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)
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 glycoglycerolipids. 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 transformation. In contrast, the class of glycoglycerolipids (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. Glycoglycerolipids 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 seen. In plant cells, galactosyl and digalactosyldiacyl glycerols are the most common glycoplycerolipids. Although acylated and sulfonated variants, as well as trigalactosyldiacyl glycerols have also been found.

Glycolipids constitute an important class of membrane lipid that are synthesized by both prokaryotic and eukaryotic organisms. 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 LH2O 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. Their structure was elucidated by multidimensional nuclear magnetic resonance (NMR) techniques like $^1$H, $^1$H correlation spectroscopy (COSY), $^1$H, $^1$H total correlation spectroscopy (TOCSY), $^1$H, $^{13}$C heteronuclear multiple quantum coherence
Alga sample (3.5 Kg)

Extracted with MeOH, Filtered and concentrated.

Crude extract

Fractionation

Chloroform 123g

Gel Chromatography
Sephadex LH-20 (1:1 MeOH: CHCl₃)

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

Scheme II: TLC of the polar glycolipids (PF₁,₃).
(HMOC) and $^1$H, $^{13}$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-eicosatetrayl)-3-O-$\alpha$-D-galactopyranosyl-sn-glycerol (GL2), its pentacetate (GL1) and (2S)-1-O-(palmitoyl)-2-O-(5,8,11,14,17-eicosapentanoyl)-3-O-$\beta$-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 (GL1a), sulfonoglycolipids 2-O-palmitoyl-3-O-(6'-sulfoquinovopyranosyl)-glycerol (GL2a) and its ethyl ether derivative (GL2b), 1-oleoyl-2-palmitoyl-3-O-galactosyl glycerol (GL3a), 1,2-diacyl phosphatidyl glycerol (GL3b) and 3-digalactosy1-2-palmitoyl glycerol (GL3c).

**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 -M+1- 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 $^1$H correlation (COSY) NMR spectra. Thus, the $^1$HNMR spectrum (Fig1.2) (300MHz, CDCl3) and $^{13}$CNMR 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 $\delta$ 0.827, a broad methylene signal at $\delta$ 1.202 [[CH$_2$]$_n$] of aliphatic chain, multiplets at $\delta$ 2.268, 1.967 and 1.562 assigned to three methylenes linked $\alpha$, $\beta$ and $\gamma$ to the ester carbonyl functionality. A broad multiplet at $\delta$ 2.7 arises from allylic methylene protons and the olefinic methine protons were evident at $\delta$ 5.293. A sharp singlet at $\delta$ 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 $^1$H nuclei when substituted by an O-acyl group, this being a fingerprint for glycolipids containing glycerol as alcohol rather than sphingosine.

$^1$H-$^1$H COSY, TOCSY (Fig 1.4) (total correlation spectroscopy) and HMQC (Fig 1.5) correlations allowed assignment of sugar carbons and protons (Table-1). $^1$H-$^1$H 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 a glycosidic configuration of the sugar linkage with the glycerol.

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 GL₁

Table 1: \(^1\)H, \(^{13}\)CNMR, TOCSY and HMBC of GL₁

<table>
<thead>
<tr>
<th>Carbon No.</th>
<th>(^1)HNMR (\delta), ppm</th>
<th>(^{13})CNMR (\delta), ppm</th>
<th>TOCSY Correlations</th>
<th>HMBC Correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.22, 4.35</td>
<td>62.2</td>
<td>H₂</td>
<td>C₁, C₂</td>
</tr>
<tr>
<td>2</td>
<td>5.23 (m)</td>
<td>70.0</td>
<td>H₁, H₃a, H₃b</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>3.56 (d, 3.6 Hz)</td>
<td>68.2</td>
<td>H₂</td>
<td>C₁, C₁'</td>
</tr>
<tr>
<td>1'</td>
<td>4.17 (d, 2.1 Hz)</td>
<td>103.0</td>
<td>H₂', H₂</td>
<td>C₂'</td>
</tr>
<tr>
<td>2'</td>
<td>3.52</td>
<td>71.6</td>
<td>H₁', H₃'</td>
<td>C₃', C₄'</td>
</tr>
<tr>
<td>3'</td>
<td>3.85 (b, s)</td>
<td>67.9</td>
<td>H₂', H₄'</td>
<td>C₄'</td>
</tr>
<tr>
<td>4'</td>
<td>3.55 (d, 3.6 Hz)</td>
<td>73.1</td>
<td>H₃'</td>
<td>C₃', C₅'</td>
</tr>
<tr>
<td>5'</td>
<td>3.61 (m)</td>
<td>72.3</td>
<td>H₆'</td>
<td>C₁', C₆'</td>
</tr>
<tr>
<td>6'</td>
<td>4.23, 4.39</td>
<td>62.2</td>
<td>H₅'</td>
<td>C₅'</td>
</tr>
<tr>
<td>1''</td>
<td>-</td>
<td>173.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2''</td>
<td>2.28</td>
<td>34.1</td>
<td>H₃''</td>
<td>C₃'', C₁'', C₄''</td>
</tr>
<tr>
<td>3''</td>
<td>1.58</td>
<td>24.8</td>
<td>H₂''</td>
<td>C₂''</td>
</tr>
<tr>
<td>7''</td>
<td>1.96, 2.78</td>
<td>27.1</td>
<td>H₈''</td>
<td>C₈'', C₉''</td>
</tr>
<tr>
<td>8''</td>
<td>5.31 (d, 5.4 Hz)</td>
<td>129.6</td>
<td>H₇'', H₉''</td>
<td>C₇''</td>
</tr>
<tr>
<td>9''</td>
<td>5.31</td>
<td>128.7</td>
<td>H₈'', H₁₀''</td>
<td>C₁₀''</td>
</tr>
<tr>
<td>18''</td>
<td>0.82 (t, 6.9 Hz)</td>
<td>14.0</td>
<td>H₁₇''</td>
<td>C₁₆'', C₁₇''</td>
</tr>
<tr>
<td>(CH₃)₅n</td>
<td>1.20 (bs)</td>
<td>29.1-30.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 PF₁ was identified as pentacetate of \((2R\)-2-\(O\)-(5,8,11,14-eicosatetranoyl)-3-\(O\)-\(\alpha\)-D-galactopyranosyl-sn-glycerol (GL₁). The fragmentation
observed in MS/MS spectrum of GL₁ (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 GL₁ along with identified fragments is represented in (Scheme 1).

Scheme 1: Mass fragmentation of GL₁

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 PF₁, 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 GL₁ₐ. MS/MS studies of the [M+H]⁺ ion at m/z 1017 (Fig 1.8) 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 C₁₈:₁ and C₁₆:₀ 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 (GL1a) that along with identified fragments is illustrated in Scheme 2.

Scheme 2: Mass fragmentation of GL1a.

**Structural characterization of PF2:**

A similar approach was adopted for PF2 that showed physicochemical characteristics of glycolipids. The IR spectrum (Fig 2.1) of the purified PF2 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 \(^1\)HNMR (Fig 2.2) and \(^{13}\)CNMR (Fig 2.3) 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: $^1$H, $^{13}$CNMR, COSY and HMBC of GL2

<table>
<thead>
<tr>
<th>Carbon No.</th>
<th>1HNMR $\delta_{HH}$, ppm</th>
<th>$^{13}$CNMR $\delta_{C}$, ppm</th>
<th>COSY Correlations</th>
<th>HMBC Correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.00, 4.39</td>
<td>62.2</td>
<td>H2&quot;</td>
<td>1'&quot;&quot;, 2&quot;</td>
</tr>
<tr>
<td>2</td>
<td>5.30</td>
<td>70.0</td>
<td>H1, H3a, H3b</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>3.51 (b)</td>
<td>67.9</td>
<td>H2&quot;</td>
<td>1', 1&quot;</td>
</tr>
<tr>
<td>1'</td>
<td>4.25 (d, 4.5 Hz)</td>
<td>103.6</td>
<td>H2&quot;</td>
<td>2&quot;</td>
</tr>
<tr>
<td>2'</td>
<td>3.63</td>
<td>72.4</td>
<td>H1', H3'</td>
<td>3', 4'</td>
</tr>
<tr>
<td>3'</td>
<td>3.90</td>
<td>67.9</td>
<td>H2', H4'</td>
<td>4'</td>
</tr>
<tr>
<td>4'</td>
<td>3.69</td>
<td>73.1</td>
<td>H3'</td>
<td>3', 5'</td>
</tr>
<tr>
<td>5'</td>
<td>3.50 (b)</td>
<td>71.6</td>
<td>H6'</td>
<td>1', 6'</td>
</tr>
<tr>
<td>6'</td>
<td>4.30, 4.39</td>
<td>62.2</td>
<td>H5'</td>
<td>5'</td>
</tr>
<tr>
<td>1'&quot;</td>
<td>-</td>
<td>173.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2'&quot;</td>
<td>2.35</td>
<td>34.1</td>
<td>H3&quot;&quot;</td>
<td>3'&quot;&quot;, 1&quot;&quot;, 4&quot;&quot;</td>
</tr>
<tr>
<td>3'&quot;</td>
<td>1.63</td>
<td>27.2</td>
<td>H2&quot;&quot;</td>
<td>2&quot;&quot;, 4&quot;&quot;</td>
</tr>
<tr>
<td>7&quot;&quot;</td>
<td>2.04 (b)</td>
<td>29.3</td>
<td>H8&quot;&quot;</td>
<td>8&quot;&quot;, 9&quot;&quot;</td>
</tr>
<tr>
<td>8&quot;&quot;</td>
<td>5.39 (b)</td>
<td>128.8</td>
<td>H7&quot;&quot;, H9&quot;&quot;</td>
<td>7&quot;&quot;</td>
</tr>
<tr>
<td>9&quot;&quot;</td>
<td>5.39 (b)</td>
<td>130.0</td>
<td>H8&quot;&quot;, H10&quot;&quot;</td>
<td>10&quot;&quot;</td>
</tr>
<tr>
<td>18&quot;&quot;</td>
<td>0.82</td>
<td>14.1</td>
<td>H17&quot;&quot;</td>
<td>16&quot;&quot;</td>
</tr>
<tr>
<td>(CH$_2$)$_n$</td>
<td>1.28 (b)</td>
<td>29.3-31.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The structure is also confirmed by COSY (Fig 2.4), HMQC (Fig 2.5) and HMBC (Fig2.6) spectral data (Table 2). COSY and HMBC correlations are illustrated in Fig 2a and Fig 2b respectively.

Fig 2a: COSY correlations of GL2
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$_{40}$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-eicosatetranoyl)-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 GL$_2$

The ESI-MS examination of PF$_2$ 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 sulfono group (82 amu) as sulfonic acid group (SO$_3$H) and as sodium salt (SO$_3$Na) 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.
Scheme 3: Mass fragmentation of GL₂a.

A similar fragmentation pattern was observed for the sulfonoquinovosyl molecular species with pseudomolecular ion at m/z 629 led to the structure GL₂b 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 CH₂ attached to sulphur at 53.6 ppm, as an impurity in PF₂. 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 GL₂b are illustrated in Scheme 4.

Scheme 4: Mass fragmentation of GL₂b.

Structural characterization of PF₃:
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. 799.5332] corresponding to the molecular formula of C₄5H₇6O₁₀Na. Hydroxyl and ester carbonyl
functionalities were indicated by IR absorption at 3413.8, 1732.0 and 1166.8 cm$^{-1}$ (fig.3.1). Its $^1$HNMR (Fig.3.2), $^{13}$CNMR 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 GL$_3$. HMQC, TOCSY and HMBC spectra of GL$_3$ 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 GL$_3$](image)

![Fig 3b: HMBC correlations of GL$_3$](image)
Table 3: $^1$H, $^{13}$CNMR, COSY and HMBC of GL₃

<table>
<thead>
<tr>
<th>Carbon No.</th>
<th>$^1$H NMR $\delta_{H}$, ppm</th>
<th>$^{13}$CNMR $\delta_{C}$, ppm</th>
<th>COSY Correlations</th>
<th>HMBC Correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.21, 4.38</td>
<td>62.9</td>
<td>H2</td>
<td>1‴‴‴</td>
</tr>
<tr>
<td>2</td>
<td>3.86</td>
<td>70.2</td>
<td>H1, H3a, H3b</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>3.65</td>
<td>68.1</td>
<td>H2</td>
<td>1′, 2′</td>
</tr>
<tr>
<td></td>
<td>3.83 (d, 6Hz)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1′</td>
<td>4.26 (d, 7.2 Hz)</td>
<td>104.0</td>
<td>H2′</td>
<td>2′</td>
</tr>
<tr>
<td>2′</td>
<td>3.61</td>
<td>71.3</td>
<td>H1′, H3′</td>
<td>3′</td>
</tr>
<tr>
<td>3′</td>
<td>3.89</td>
<td>69.2</td>
<td>H2′, H4′</td>
<td></td>
</tr>
<tr>
<td>4′</td>
<td>3.53</td>
<td>74.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5′</td>
<td>3.64</td>
<td>73.5</td>
<td>H6′</td>
<td>1′</td>
</tr>
<tr>
<td>6′</td>
<td>4.24, 4.27</td>
<td>62.0</td>
<td>H5′</td>
<td>5′</td>
</tr>
<tr>
<td>1″</td>
<td>-</td>
<td>173.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2″</td>
<td>2.30 (d, 7.8Hz)</td>
<td>34.3</td>
<td>H3″″</td>
<td>3″″, 1″″, 4″″″</td>
</tr>
<tr>
<td>3″</td>
<td>1.68m</td>
<td>25.6</td>
<td>H2″″</td>
<td>2″″, 4″″</td>
</tr>
<tr>
<td>4″</td>
<td>2.09 (q, 6Hz)</td>
<td>27.2</td>
<td></td>
<td>3″″, 5″″</td>
</tr>
<tr>
<td>5″″, 6″″, 8″″</td>
<td>5.37-5.38 (cluster)</td>
<td>127.0-132.0 (10 d)</td>
<td></td>
<td>4″″, 7″″</td>
</tr>
<tr>
<td>7″″, 10″″</td>
<td>2.83 (br dd)</td>
<td>29.3-31.4 (4t)</td>
<td>H8″</td>
<td>5″″, 6″″</td>
</tr>
<tr>
<td>19″</td>
<td>2.02 (m)</td>
<td>22.7</td>
<td></td>
<td>18″, 20″″</td>
</tr>
<tr>
<td>20″</td>
<td>0.97 (t, 7.5)</td>
<td>14.1</td>
<td>H19″</td>
<td>18″, 19″</td>
</tr>
</tbody>
</table>
| 1‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴‴Nonnull
ESI-MS of PF₃, 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 position⁴⁶,⁴⁷,⁴⁸. 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 GL₃a. The proposed structure along with its identified fragments is shown in Scheme 6.

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. 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.
Antimicrobial activity of PF1_3:

Bergsson et al. (2001)\textsuperscript{50} 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)\textsuperscript{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*

<table>
<thead>
<tr>
<th>Fractions</th>
<th><strong>Antibacterial</strong></th>
<th><strong>Antifungal</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. coli</em></td>
<td><em>P. aeruginosa</em></td>
</tr>
<tr>
<td><strong>PF1</strong></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>PF2</strong></td>
<td>+ (st)</td>
<td>-</td>
</tr>
<tr>
<td><strong>PF3</strong></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Standard</strong></td>
<td>10</td>
<td>-</td>
</tr>
</tbody>
</table>

Numbers indicate the zone of inhibition in mm from center of imbedded 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 glycoglycerolipids 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)\(^9^7\) 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)\(^9^8\) 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.

GLla 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*.\(^9^9,10^0\)

In recent years, glycoglycerolipid analogues have gained importance in cancer chemoprevention 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\textsuperscript{101,102}. The anomeric configuration does not seem to affect the activity\textsuperscript{103}.

MGDGs, containing (7Z, 10Z)-hexadecadienoic acyl group, from the green alga \textit{Chlorella vulgaris} are reported to exhibit anti-tumor promoting effect\textsuperscript{104}. SQDG from algae inhibits DNA-polymerase and HIV-reverse transcriptase\textsuperscript{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\textsuperscript{108,109,110}.

The red algae are reported to have high levels of polyunsaturated fatty acids, mainly EPA and AA\textsuperscript{111}, but the contents vary within the same genus. \textit{Chondria dasyphylla} (Wood) Ag. is reported to have equal contents of EPA and AA whereas in \textit{Chondria decipiens} EPA predominates\textsuperscript{112}. Further, in red algae PUFAs belonging to C20 series are reported to be mainly concentrated in MGDG\textsuperscript{113}. 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 \textit{C. armata}, where C20 acids were not detected as free fatty acids\textsuperscript{114}.

Glycoglycerolipids occur widely and copiously in vascular plants\textsuperscript{115}, certain green seaweeds\textsuperscript{116,117,118} cyanobacteria\textsuperscript{119}, marine dinoflagellates\textsuperscript{120}, and the freshwater alga \textit{C. vulgaris}\textsuperscript{104}. As to the glycoglycerolipids of red algae, hydroxyeicosapentaenoyl galactosyl glycerols are known from the temperate red alga \textit{Gracilariopsis lemaneiformis}\textsuperscript{121}, and MGDG, DGDG, and SQDG are reported from \textit{Gracilaria verrucosa}\textsuperscript{122}, which is also known to contain sulfoquinovosylmonogalactosyl glycerol (SQMG) (GL2a). This SQMG is also reported to be a constituent of cyanobacterium \textit{Synechocystis} PCC 6803\textsuperscript{123} and lichenized basidiomycetes, \textit{Dictyonema glabratum}\textsuperscript{124}. 2-O-\alpha-D-galactopyranosylglycerol is a metabolite of \textit{Laurencia pinnatifida}\textsuperscript{125} and 2,3-dipalmitoyl sulfonoglycolipid has been identified in \textit{Laurencia pedicularioides} and is reported to be the major glycolipid in red algae\textsuperscript{126}. Recently, Shao \textit{et al.} (2002)\textsuperscript{97} reported the presence of a new sulfonoglycolipid, crassicaulisine, in the red alga \textit{C. crassicaulis}. Taxonomically, genus \textit{Laurencia} and \textit{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\textsuperscript{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 eggs\textsuperscript{130} and was presumed to be playing a unique role in algal physiology\textsuperscript{127}.

Sulfonoquinovosyl acyl glycerols, in particular compounds with C18 fatty acid on the glycerol moiety, may be clinically promising antitumor or immunosuppressive agents\textsuperscript{131}.

**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 F\textsubscript{254} 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\degree 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 49.9 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.

**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* (3.5 kg, dry wt.) was cleaned and extracted thrice with methanol using a sonicator (15 mins) at room temperature. The combined methanolic extracts were evaporated under reduced pressure at 37°C temperature to a certain minimum volume (~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 (4mg, Rf = 0.52 in solvent I), and methanol: chloroform (2:98) yielded PF₂, [α]D = -16° (c = 0.02, CHCl₃, Rf = 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, Rf = 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-agar. 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 typhii*, *Shigella flexineri*, *Klebsiella pneumoniae* and *Vibrio cholerae*) and one Gram positive bacteria (*Staphylococcus aureus*) using the paper disk assay method. 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/ml. 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.
Fig 1.5: HMQC spectrum of GL₁

Fig 1.6: HMBC spectrum of GL₁
Fig 1.7: ESI-MS spectrum of GL₁

Fig 1.8: MS/MS spectrum of GL₁
Fig 1.1: IR spectrum of GL₁

Fig 1.2: $^1$HNMR spectrum of GL₁
Fig 1.3: $^{13}$CNMR and DEPT spectra of GL₁

Fig 1.4: TOCSY spectrum of GL₁
Fig 2.1: IR spectrum of GL$_2$

Fig 2.2: $^1$HNMR spectrum of GL$_2$
Fig 2.5: HMQC spectrum of GL2

Fig 2.6: HMBC spectrum of GL2
Fig 2.7: ESI-MS spectrum of GL$_2$

Fig 2.8: MS/MS spectrum of GL$_{2a}$
Fig 2.9: MS/MS spectrum of GL2b

Fig 3.1: IR spectrum of GL3
Fig 3.2: $^1$HNMR spectrum of GL$_3$

Fig 3.3: $^{13}$CNMR spectrum of GL$_3$
Fig 3.4: HMQC spectrum of GL\textsubscript{3}

Fig 3.5: TOCSY spectrum of GL\textsubscript{3}
Fig 3.6: HMBC spectrum of GL₃

Fig 3.7: ESI-MS spectrum of GL₃
Fig 3.8: MS/MS spectrum of GL₃a

Fig 3.9: MS/MS spectrum of GL₃b

Fig 3.10: MS/MS spectrum of GL₃c
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.\(^5^4\).

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.\(^5^5\)
The occurrence of sterols in marine organisms can be discussed in various ways. Schmitz in his review\textsuperscript{56} 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. Goad\textsuperscript{57} 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 Bergquist\textsuperscript{58}, 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 out\textsuperscript{57}, the existence of a given sterol in a specific marine organism may be due to one or more of the following four processes:

1. \textit{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 available\textsuperscript{59}. Different classes of algae have a distinct sterol composition. For the evolutionary lower red algae (\textit{Rhodophyceae}), cholesterol and in some cases, its biogenetic precursor cholesta-5,24(25)-dien-3β-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 \( \Delta^{22} \)-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\(\beta\)-ol) in the former and isofucosterol ((Z)-stigmasta-5,24(28)-dien-3\(\beta\)-ol) in the latter, while in advanced green algae, the \( \Delta^{24(28)} \) double bond is reduced, leading to an accumulation of sitosterol ((24R)-stigmast-5-en-3\(\beta\)-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\(\beta\)-hydroxy cholest-4-en-3-one (2) and cholest-4,24-dimethyl-6\(\beta\)-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 Rf 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\(\alpha\)-cholesta-3,6-dione (6), 24-ethyl-4-methyl-6-hydroxy-cholest-4-en-3-one (7) and cholesta-4,24-dimethyl-6\(\beta\)-hydroxy-3-one (8) (Fig 2a).
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, RF = 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 C27H42O2, 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 conditions 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 C5-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 diketones. 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 cholesta-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 Δ4-3-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 losses 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 C\(_8\)-C\(_{14}\) and C\(_{12}\)-C\(_{13}\) bonds. Fission of C\(_8\)-C\(_{14}\) and C\(_{11}\)-C\(_{12}\) gives rise to ion at \( m/z \ 255 \) and when cleavage of ring B at C\(_9\)-C\(_{10}\) is associated with the fission of C\(_5\)-C\(_6\), it yields ion at \( m/z \ 339 \), whereas cleavage associated with C\(_6\)-C\(_7\) bond or C\(_8\)-C\(_9\) bond leads to fragments \( m/z \ 137 \) or \( m/z \ 151 \) respectively. Ring D cleavage along the C\(_{13}\)-C\(_{17}\) and C\(_{14}\)-C\(_{15}\) produces ion at \( m/z \ 201 \) which in turn losses 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 C\(_{13}\)-C\(_{17}\) and C\(_{14}\)-C\(_{15}\) accompanied by elimination of one water molecule gives ion at \( m/z \ 229 \), which subsequently losses two methyl groups resulting in the formation of fragment at \( m/z \ 199 \). Ring C cleavage at C\(_{11}\)-C\(_{12}\) and C\(_8\)-C\(_{14}\) produced sodiated ion at \( m/z \ 215 \) whereas cleavage of the same ring at C\(_{12}\)-C\(_{13}\) and C\(_8\)-C\(_{14}\) 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 C\(_9\)-C\(_{10}\) and C\(_7\)-C\(_8\) results in the sodiated ion at \( m/z \ 175 \) and the fission of C\(_9\)-C\(_{10}\) and C\(_5\)-C\(_6\) 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\(\alpha\)-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 C\(_{27}\)H\(_{44}\)O\(_2\) 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 \(\alpha-\beta\) unsaturated ketonic absorption (1681.8 cm\(^{-1}\)) in its IR spectrum (Fig. 2.6). The \(^1\)H(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 \(\delta\) 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 \(^1\)HNMR (300MHz, CDCl\(_3\), Table-1) of (2) showed in addition to hydroxymethine proton at \(\delta\) 4.35, the presence of an olefinic proton singlet at \(\delta\) 5.81(H-4), two tertiary methyls resonating as singlets at \(\delta\) 0.744 (H\(_{2}\)-18) and \(\delta\) 1.379 (H\(_{3}\)-19). A doublet at \(\delta\) 0.929 (J=6.3Hz) was assigned to C\(_{21}\) methyl group and a pair of doublets at \(\delta\) 0.876 and at \(\delta\) 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 \(^1\)H-\(^1\)H 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 \(\delta\)1.379 allowed identifying C-1, C-5, C-9 and C-10. The olefinic proton at \(\delta\) 5.81 showed HMBC connectivity with C-10 (37.084) and C-6 (73.245) while the hydroxymethine proton at \(\delta\) 4.35 correlated with C-5(168.528), C-7 (38.557) and C-8 (29.709). HMBC correlations are illustrated in Fig 2b.

\[\text{Fig 2b : HMBC correlations of compound 2}\]

\(^1\)H-\(^1\)H 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(18) at $\delta$ 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 H-21 at $\delta$ 0.929 with C-22 (36.114) and C-17 (56.151) carbons and H-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 $\delta$ 4.3 and H-7 proton at $\delta$ 1.379 which had NOE correlation with H-9 at $\delta$ 0.858 which served to assign a $\beta$ orientation to the –OH group. NOE was also observed between H-4 at $\delta$ 5.81 and H-6 further confirming the $\beta$ orientation of the –OH group. Additional evidence comes from the melting point 194°C and the optical rotation $[\alpha]_D = +42.5$ (c = 0.9, CHCl$_3$) observed for compound (2) well in agreement with the reported values for cholest-6-$\beta$-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), [M+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 (C$_8$-14 and C$_9$-11) generated the sodiated ion at $m/z$ 243 and loss associated with ring A cleavage (C$_3$H$_4$) 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 C$_5$-C$_6$ and C$_9$-C$_{10}$ 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 $^{13}$C NMR (300MHz) data of compounds 1 and 2 in CDCl$_3$

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

The $^1$HNMR 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 C$_4$. Since there was no separate doublet for C$_{28}$ methyl it was presumed that it coincides with C$_{21}$ doublet centred at δ
0.914. This assumption is reinforced by the presence of additional signal for C_{24} carbon at δ 41.3 in the $^{13}$C NMR of compound (2). The positioning of the second methyl group at C_{24} 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)^+ -H_2O] and [(M+H)^+ -2H_2O]. The fragment ion at m/z 359 and 73 seems to have originated from the cleavage of C_{23}-C_{29} 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 (C_{13}-C_{17} and C_{14}-C_{15}) along with the side chain produces appreciable amount of ion at m/z 247. Ring B cleavage at C_{9}-C_{10} and C_{7}-C_{8} 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 C_{9}-C_{10} and C_{5}-C_{6} 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 losses C₅H₁₁ 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 C₅-C₆ and C₉-C₁₀. 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 C₅-C₆ and C₉-C₁₀ 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 C₁₃-C₁₇ and C₁₄-C₁₅ bonds result in the sodiated ion at m/z 325. Cleavage of ring C at C₁₂-C₁₃ and C₈-C₁₄ as well as fission of ring B at C₇-C₈ and C₉-C₁₀ leads to the ion at m/z 209. Cleavage of ring C at C₈-C₁₄ and C₉-C₁₁ with initial loss of acetic acid produced ion at m/z 175. Ring B fission at C₉-C₁₀ and C₆-C₇ bonds yielded ion at m/z 195 which in turn losses 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 C₂₄-C₂₅.

C₂₃-C₂₄ 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 C₁₃-C₁₇ and C₁₄-C₁₅ with the initial loss of water. Ring C cleavage at C₁₂-C₁₃ and C₈-C₁₄ produced ion at m/z 209. The cleavage at C₉-C₁₁ and C₈-C₁₄ 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 C₅-C₆ and C₉-C₁₀ 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 C20-C22 with the initial loss of one molecule of water which decomposes further with the loss of a methyl group to gives 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 paper 67 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-6β-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-dimethylcholestane-3-one-6-ol (8) together with four known ketosteroids cholest-4-en-3,6-dione (1), 6β-hydroxy cholest-4-en-3-one (2) and cholest-4-en-3-one (4), 5α-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 24β-ethyl cholesterol. Usually cholesterol, and in some cases, its biogenetic precursor cholesta-5,24(25)-diene-3β (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 C\(_{24}\), and the sterol with a C\(_{22}\) double bond, in low concentrations \(^{70}\). 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 \textit{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 \([(\Delta^5, \Delta^7, \Delta^{8(14)}, \Delta^{14}, \Delta^{17(20)})]\), for the side chain \([(\Delta^{22}, \Delta^{24(28)})]\) and different alkyl substitution patterns for the side chain \([\text{no substituent, 23-methyl, 24-methyl, 23,24-dimethyl, 24-ethyl, \Delta^{22(23)-23,24-dimethyl}}]\). There is a solitary reference in the literature on the presence of 4-methyl sterol in the freshwater plant \textit{Utricularia neglecta} \(^{71}\) (Lactibulariaceae) that is reported to contain high proportions of 4-methyl sterols with the predominance of citrostanol (4-\(\alpha\)-methyl-24-ethyl-5\(\alpha\)-cholestan-3\(\beta\)-ol). 4-methyl sterols are being reported here for the first time from a marine alga.

Steroidal ketones with 4-en-3-one or 4-en-3,6-dione were isolated mainly from marine sponges \(^{72-79}\), the hard coral \textit{Dendrophyllia cornigera} \(^{80}\), the callus tissue of plants \(^{81,82}\), \textit{Phoenix dactylifera} \(^{83}\), \textit{Typha latifolia} \(^{84}\), and queen bee ovary \(^{85}\). Oxygenated fucosterols are also known from the brown alga, \textit{Turbinaria conoides} \(^{86}\) and the sea grass \textit{Cymodocea nodosa} \(^{80}\). 5\(\alpha\)-Cholestane-3,6-dione-11-hydroxy-5\(\alpha\)-cholestan-3,6-dione are also known as constituents of the red alga \textit{Acanthophora spicifera} \(^{87,88}\). However, they are being reported for the first time from algae of genus \textit{Chondria}.

There are recent reports of the identification of 6\(\alpha\) isomer of compound (2) from the red alga \textit{Hypnea musciformis} \(^{89}\) and of the presence of 6\(\beta\) isomer in a marine sponge \textit{Iotrochoto birotulata} \(^{79}\). Ours is the first report of natural occurrence of the \(\beta\) isomer of compound (2) in an alga. These metabolites are known as microbial transformation products of cholesterol by the microorganisms \textit{Coriolus hirstus} \(^{63}\), \textit{Pseudomonas} strain ST-200 \(^{90}\) and enzymatic oxidation product of \(\Delta^5\)-cholest-3\(\beta\)-ol by soyabean lipoxygenase \(^{91}\).
The origin of oxidized sterols has been questioned for a long time. 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 7α and 7β hydroxy cholesterol, it is possible that this oxysterol could be biosynthesized from cholesterol via photosensitized oxygenation or a related mechanism. Alternately, bromoperoxidases which are widespread in the marine algae and are found particularly in red algae could be responsible for the oxidation of steroids 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 *Glauxaura marginata* and the oxygenated clerosterols from green alga *Codium arabicum* using P388, KB, A-549 and HT-29 cell lines. Based on ED$_{50}$ 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 LD$_{50}$ 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 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.

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

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* (Kutz):

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: $^1$HNMR spectrum of compound 1

Fig 2.3: $^{13}$CNMR 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: $^1$HNMR spectrum of compound 2

Fig 2.8: $^{13}$CNMR 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.
References:


