

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

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### Abstract:

Three distinct fractions containing polar glycolipids (**PF**<sub>1,3</sub>) were isolated from the chloroform soluble fraction of crude methanolic extract of red alga *Chondria armata* (Kütz.) Okamura on gel chromatography over Sephadex LH<sub>20</sub>. Their structure was elucidated by multidimensional nuclear magnetic resonance (NMR) techniques like <sup>1</sup>H, <sup>1</sup>H correlation spectroscopy (COSY), <sup>1</sup>H, <sup>1</sup>H total correlation spectroscopy (TOCSY), <sup>1</sup>H, <sup>13</sup>C heteronuclear multiple quantum coherence (HMQC) and <sup>1</sup>H, <sup>13</sup>C heteronuclear multiple bond correlation (HMBC) complemented by electrospray ionization mass spectrometry (ESI-MS) in the positive ion mode. An exclusive configuration of the sugar molecules in the glycerolipids was indicated by the coupling constant of the anomeric proton in <sup>1</sup>H NMR spectrum. Major glycolipids were identified as (2R)-2-*O*-(5,8,11,14-eicosatetraenoyl)-3-*O*- $\alpha$ -D-galactopyranosyl-sn-glycerol (**GL**<sub>2</sub>), its pentacetate (**GL**<sub>1</sub>) and (2R)-1-*O*-(palmitoyl)-2-*O*-(5,8,11,14,17-eicosapentanoyl)-3-*O*- $\beta$ -D-galactopyranosyl-sn-glycerol (**GL**<sub>3</sub>). Each was methanolysed to give the same galactosylglycerol which on ESI-MS provided a pseudomolecular ion at m/z 309 representing deacylated glycolipid with the sodiated sugar moiety. Additionally, six minor glycolipids were also identified on the basis of ESI-MS. These include, a 1,2-di-*O*-acyl-3-*O*-(acyl-6'-galactosyl)-glycerol (**GL**<sub>1a</sub>), sulfonoglycolipids 2-*O*-palmitoyl-3-*O*-(6'-sulfoquinovopyranosyl)-glycerol (**GL**<sub>2a</sub>) and its ethyl ether derivative (**GL**<sub>2b</sub>), 1-oleoyl-2-palmitoyl-3-*O*-galactosyl glycerol (**GL**<sub>3a</sub>) and 1,2-diacyl phosphatidyl glycerol (**GL**<sub>3b</sub>). **GL**<sub>1</sub>, **GL**<sub>1a</sub> and **GL**<sub>2b</sub> are new to the literature. The novelty of the remaining identified compounds lies in the diversity of their fatty acid composition.

Antimicrobial properties of these glycolipids against pathogens were evaluated. The yeast, *Candida albicans* and the bacteria, *Klebsiella sp.* were as sensitive as the standard Nystatin and antibiotic Streptomycin against **PF**<sub>3</sub>. Considerable activity was expressed by the same metabolite against the fungus, *Cryptococcus neoformans* as compared to the control. Weak activity against the bacteria *Shigella flexineri* and *Vibrio cholerae* and the fungus *Aspergillus fumigatus* was also observed. Fraction **PF**<sub>2</sub> was weakly active against some strains whereas all of them were resistant to its acetyl derivative **PF**<sub>1</sub>. Antimicrobial activity of glycolipids is being reported here for the first time.

**Running title:** Glycolipids from the alga *Chondria armata* (Kütz.) Okamura

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## Introduction

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

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

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

## Results

### *Extraction and fractionation of glycolipids*

Fresh alga was exhaustively treated with methanol and the chloroform soluble fraction on column chromatography gave, in order of polarity, fractions **PF<sub>1-3</sub>**, apparently homogenous on TLC, yielding purplish pink spots on spraying with methanolic sulphuric acid. Their <sup>1</sup>H and <sup>13</sup>C NMR spectra (Table-I) closely resembled those reported for galactosyl glycerolipids. ESI-MS was useful to characterize molecular ions and the sequence of groups in the studied molecules and to distinguish lipid structures that gave similar NMR spectra.

### Structural characterisation of **PF**<sub>1</sub>

The purified **PF**<sub>1</sub> gave protonated molecular ion [M+H]<sup>+</sup> at m/z 751 in its ESI-MS spectrum (Figure 1C). The presence of spin systems corresponding to one hexose, glycerol and fatty acid were readily identified from the 1D and 2D homonuclear <sup>1</sup>H correlation spectroscopy (COSY) NMR spectra. Thus, the <sup>1</sup>H NMR spectrum (300MHz, CDCl<sub>3</sub>) 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<sub>2</sub>)<sub>n</sub>] 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 methylene 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  $\delta$  4.23 and 4.35 correspond to the substitution at C-1 ( $\delta$  62.3) by an *O*-acyl group and the doublet at  $\delta$  3.56 and 3.93 was assigned to C-3 ( $\delta$  68.2) of glycerol substituted by the  $\alpha$ -galactose residue. The glycerolipid structure was confirmed by the presence of a characteristic signal at 70.3/5.23 (C-2) having a distinct  $\alpha$ -shift to lower field for <sup>13</sup>C and <sup>1</sup>H nuclei when substituted by an *O*-acyl group, this being a fingerprint for glycolipids containing glycerol as alcohol rather than sphingosine (Sasaki *et al.*, 1999).

<sup>1</sup>H-<sup>1</sup>H COSY, TOCSY (total correlation spectroscopy) and HMQC correlations allowed assignment of sugar carbons and protons (Table-I). <sup>1</sup>H-<sup>1</sup>H COSY and TOCSY correlation of the anomeric proton at  $\delta$  4.178 with the sn-3 protons at  $\delta$  3.83 established connectivity of the sugar moiety with the glycerol. The anomeric proton at  $\delta$  4.178 with a coupling constant of 2.1 Hz indicated  $\alpha$ -glycosidic configuration of the sugar linkage with the glycerol (Dabrowski *et al.*, 1980).

Long range HMBC diagnostic correlations were observed between the ester carbonyls at  $\delta$  173.8 and  $\delta$  173.5 and C-1 and C-2 of glycerol indicating the linkage. The stereochemistry at C-2 was assigned to be *R* by comparison of the coupling constant values between H-2/H-3a (*J*=3.6Hz) and H-2/H-3b (*J*=6.0Hz) respectively with those of published data (Murikami *et al.*, 1995; Oshima *et al.*, 1995; Reshef *et al.*, 1997). On the basis of the above data the major component of **PF**<sub>1</sub> was identified as pentacetate of (2*R*)-2-*O*-(5,8,11,14-eicosatetraenoyl)-3-*O*- $\alpha$ -D-galactopyranosyl-sn-

glycerol (**GL<sub>1</sub>**). The fragmentation observed in MS/MS spectrum of **GL<sub>1</sub>** (Figure 1C), is well in agreement with the structure assigned. The pseudomolecular ion at m/z 751 generated a series of daughter ions at m/z 691, 631, 571 and 511 reflecting successive loss of four acetic acid molecules. The presence of fifth acetyl group was evident from the elimination of yet another acetic acid molecule yielding sodiated fragment at m/z 473. Alternately, the ion at m/z 473 might have originated, as diprotonated sodiated fragment ion, after the elimination of arachidonate ion. This ion on elimination of fifth acetic acid molecule would lead to ion at m/z 413. This confirmed the presence of acetylated hexose linked to the glycerol moiety, with the latter being diesterified by acetic acid and eicosatetraenoic acid. The proposed structure of **GL<sub>1</sub>** along with identified fragments is represented in Figure 1C.

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

ESI-MS (Figure 1A) of **PF<sub>1</sub>**, 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 (Figure 1B) it was characterized as 1,2-di-*O*-acyl-3-*O*-(6-acylgalactosyl)-glycerol **GL<sub>1a</sub>**.

MS/MS studies of the [M+H]<sup>+</sup> ion at m/z 1017 (Figure 1B) resulted in three major diagnostically important daughter ions at m/z 481, 735 and 761. The ions at m/z 735 and 761 reflect the neutral losses of the sn-1 and sn-2 substituent as free C<sub>18:1</sub> and C<sub>16:0</sub> carboxylic acid respectively supporting the presence of palmitic and oleic acyl moieties in the molecule. The intensity differences of these various ions indicated the position of the different fatty acid moieties, as the substituent position at sn-2 fragments comparatively easily (Murphy and Harrison, 1994). This leaves a mass for the core of the molecule of 481 amu. Such a mass can be explained by a substituted hexose connected to a glycerol backbone after elimination of fatty acyl group from the protonated molecular ion (M + H)<sup>+</sup>. This is further supported by the presence of an additional protonated fragment ion at m/z 441, which reflects the loss of acyl groups (C<sub>18:1</sub> and C<sub>16:0</sub>) 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<sub>1</sub> 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<sub>18:3</sub>) along with C-6 of sugar which possibly seems to be galactose. The ion at m/z 295 results from the cleavage between C1-C2 of

glycerol. Furthermore, there were a number of fragments in the upper mass region at intervals of about 14 amu. These correspond to fragmentation along the fatty acid acyl chains. On the basis of this fragmentation pattern of the molecular species with the pseudomolecular ion at  $m/z$  1017 we propose the structure of the molecule as being 1-oleoyl-2-palmitoyl-3-*O*- (linolenyl-6'-galactosyl)-glycerol (**GL<sub>1a</sub>**) that along with identified fragments is illustrated in Figure 1B.

*Structural characterization of PF<sub>2</sub>:*

A similar approach was adopted for **PF<sub>2</sub>** that showed physicochemical characteristics of glycolipids. Its NMR data differed from that of **PF<sub>1</sub>** only by the absence of signals for the acetyl groups (Table-I) indicating it to be deacetylated derivative of **GL<sub>1</sub>**. 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<sub>29</sub>H<sub>49</sub>O<sub>9</sub> (**PF<sub>2</sub>**). The MS/MS at  $m/z$  541 (Figure 2D) showed peak at  $m/z$  179 for loss of a sugar unit. 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 ion at  $m/z$  204 results from the cleavage of the fatty acyl chain between C5-C6. 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. Thus the structure of major component from **PF<sub>2</sub>** was established as 2-*O*-(5,8,11,14-eicoasatetranoyl)-3-*O*- $\alpha$ -D-galactopyranosyl-sn-glycerol **GL<sub>2</sub>** .

The ESI-MS examination of **PF<sub>2</sub>** (Figure 2A) when taken in a dilution solvent (as given under material and methods) showed additional peaks at  $m/z$  601 and 629 corresponding to pseudomolecular ions of the Na salt (Na<sup>+</sup> 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 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 (SO<sub>3</sub>H) and as sodium salt (SO<sub>3</sub>Na) respectively. The product ion observed at  $m/z$  345 and 313 appears to have originated by the loss of fatty acyl side chain as corresponding acid (palmitic acid, C<sub>16:0</sub>) and by simultaneous cleavage of fatty acyl group along with hydroxymethine of glycerol respectively. 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<sub>2a</sub>**. The proposed structure along with its identified fragments is shown in Figure 2C.

A similar fragmentation pattern was observed for the sulfonoquinovosyl molecular species with pseudomolecular ion at  $m/z$  629 led to the structure **GL<sub>2b</sub>** as represented in Figure 2B. 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  $^{13}\text{C}$  NMR signal for  $\text{CH}_2$  attached to sulphur at 53.6 ppm, as an impurity in **PL<sub>2</sub>**. The galactosylglycerolipids MGDG and DGDG are uncharged species while SQDG is negatively charged at neutral pH. This explains their presence in admixture as sodiated adducts.

#### *Structural characterization of PF<sub>3</sub>:*

The ESI-MS profile of **PF<sub>3</sub>** is illustrated in Figure 3A. ESI-MS of the major component of this fraction was consistent with the sodiated molecular ion  $[\text{M}+\text{Na}]^+$  at  $m/z$  799 corresponding to the molecular formula of  $\text{C}_{45}\text{H}_{76}\text{O}_{10}\text{Na}$ . Its  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR (Table-I) closely resembled those of **PF<sub>2</sub>** except that the  $^{13}\text{C}$  signals due to the unsaturation in the fatty acid moiety were more distinct. The tandem MS/MS spectrum of ion of at  $m/z$  799 is illustrated in Figure 3B and it represents 1-*O*-palmitoyl-2-*O*-eicosapentanoyl-3-*O*- $\beta$ -D-galactosyl-*sn*-glycerol **GL<sub>3</sub>**. The main fragmentation pathway corresponding to concomitant elimination of two fatty acyl moieties yielded ion at  $m/z$  243 characteristic of MGDG (Wettiet *et al.*, 2003). Ions reflecting neutral loss of eicosapentanoate and palmitate as free fatty acids are evident from the fragment ions at  $m/z$  497 and  $m/z$  543 respectively. Elimination of one of the acyl groups with simultaneous cleavage of the other acyl at position  $\alpha$  to the carbonyl group leads to the loss of fragment with 278 amu giving ion at  $m/z$  521. Additional ions are observed due to loss of 3 molecules of water from the sugar moiety yielding protonated ion at  $m/z$  109. Loss of two water molecules along with hydroxymethyl group from the sugar moiety produced ion at  $m/z$  97. HMQC and TOCSY spectra of **GL<sub>3</sub>** are represented in Figure 4(A and B). As evident the TOCSY spectrum is characteristic of glyceroglycolipid with the spin systems of glycerol, sugar and the constituent fatty acids.

As evident from Figure 3A, **PF<sub>3</sub>** 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 (Figure 3C) yielded fragments at  $m/z$  497 and  $m/z$  523 indicative of loss of C18:1 and C16:0 fatty acyl groups from the molecule as free fatty acids respectively. The presence of galactosyl sugar moiety was evident from the ion at  $m/z$  243. The intensity of the signals led to the placement of palmitic acid at sn2 position (Hankin *et al.*, 1997; Blair, 1990; Waugh and Murphy, 1996). Taken together, the structural analysis for the molecular species with ion at  $m/z$  779 is consistent with 1-oleoyl-2-palmitoyl-3-*O*-galactosyl glycerol **GL<sub>3a</sub>**.

The CID daughter ion spectrum of the molecular species at  $m/z$  691 is illustrated in Figure 3D 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 C<sub>12</sub> of  $\gamma$ -linolenic acid as free acid and as ketene respectively (Kim *et al.*, 1999). The fragment at  $m/z$  171 could also arise from phosphoglycerol moiety. The most intense ion at  $m/z$  301 was attributed to the concomitant elimination of palmitoleoyl and phosphatidyl groups as depicted in the Figure 3D. 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 (Hankin *et al.*, 1997; Blair, 1990; Waugh and Murphy, 1996). Thus structure **GL<sub>3b</sub>** was proposed for the molecular species with  $[M+Na]^+$  ion at  $m/z$  691.

Tandem MS scanning experiment of protonated molecular species at  $m/z$  655 yielded the most prominent ion at  $m/z$  301 reflecting 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 from the molecule. The relative abundance of the ions placed the palmitoyl group at sn-2 position. The spectrum is consistent with 3-digalactosyl-2-palmitoyl glycerol represented in Figure 3E.

#### *Methanolysis of PF<sub>1-3</sub>:*

In order to identify the acid substituents at C-1 and C-2 of components glycolipids of **PF<sub>1-3</sub>** methanolysis was performed in anhydrous methanol with excess of Na<sub>2</sub>CO<sub>3</sub>. 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 **PF<sub>3</sub>** 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 diprotonated sugar moiety  $[M+2H]^+$ . 3-*O*-D-galactopyranosyl glycerol as sodium adduct was observed at  $m/z$  277. Deacylated glycolipid with the sodiated sugar moiety was evident as protonated molecular ion at  $m/z$  309. The fragment at  $m/z$  301 represented the presence of eicosapentanoate. Thus, the fragmentation observed in MS/MS of ion at  $m/z$  309, (Figure 5) a fragment common as product of hydrolysis of **PF**<sub>1-3</sub>, is shown in Figure 5.

In order to establish the nature of the sugar moiety as galactose, the glycolipids were subjected to acid hydrolysis and the compound identified by TLC with standard sugar as described in material and methods.

#### *Antimicrobial activity of PF*<sub>1-3</sub>:

Bergsson *et al.* (2001) have studied the susceptibility of *Candida albicans* to several fatty acids and their 1-glycerides. They observed that capric acid, 10 carbon saturated fatty acid, causes the fastest and most effective killing of all the three strains of *C. albicans* tested. Lauric acid, a 12 carbon saturated fatty acid, was the most active acid at lower concentrations. Subsequently, Margarit *et al.* (2003) reported on the medium chain fatty acids of 8-12 carbon atoms exhibiting antibacterial and antifungal properties, which are enhanced when these acids are esterified with glycerol. The same authors also state sucrose esters as being less effective in inhibiting the fungal growth. Based on these reports it is expected that pathogens would be sensitive to glycolipids. This led us to evaluate the pure fractions **PF**<sub>1-3</sub> 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-II).

As evident, from the above Table-II all the bacteria and fungi tested were resistant to **PF**<sub>1</sub> at the dose tested (65 $\mu$ g/ml). **PF**<sub>2</sub> showed mild inhibitory activity against the bacteria tested except *P. aeruginosa* and *K. pneumoniae*, at 250  $\mu$ g/disc being also weakly active against the fungi, *A. fumigatus*, *C. neoformans*, *A. niger* and *Rhodotorula sp.* **PF**<sub>3</sub>, at 130  $\mu$ g/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 **PF**<sub>3</sub> against the fungus, *Cryptococcus neoformans*, strain resistant to Nystatin. **PF**<sub>3</sub> 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  $\beta$  configuration being more effective than the glycosides with  $\alpha$  configuration. Antimicrobial activity of glycolipids is being reported here for the first time.

### **Discussion:**

Three major galactoglycerolipids have been isolated and identified, in the native form, from the red alga *Chondria 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 gave rise to the same 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) 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 PF<sub>1-3</sub> were characterized as the corresponding acids or carboxylate ions (ESI-MS), and the principal components were arachidonic acid in PF<sub>1-2</sub>, palmitic acid and eicosapentaenoic acid in PF<sub>3</sub>. 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 and arachidonic acids 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) in glycolipids of marine algae the glycosidic linkage could be  $\alpha/\beta$  and the sugar moiety is attached, mainly, to C-3 of glycerol.

1-oleoyl-2-palmitoyl-3-O- (linolenyl-6'-galactosyl)-glycerol (**GL<sub>1a</sub>**) 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' position of mannobiosyl is known from the bacteria *Arthrobacter atrocyaneus* and *Microcoleus luteus* (Niepel *et al.* 1997; Ponce- Bultel *et al.*, 1997).

In recent years, glycolipid analogues have gained importance in cancer chemoprevention due to 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 (Colombo *et al.*, 1996, 1999). The anomeric configuration does not seem to affect the activity (Colombo *et al.*, 2000).

Monogalactosyldiacylglycerols [MGDG], containing (7Z, 10Z)-hexadecadienoic acyl group, from the green alga *Chlorella vulgaris* are reported to exhibit anti-tumor promoting effect (Morimoto *et al.*, 1995). Sulfoquinovosyl diacylglycerol [SQDG], from algae inhibits DNA-polymerase and HIV-reverse transcriptase (Gustafson *et al.*, 1998; Loya *et al.*, 1998; Ohta *et al.*, 1998). It is well known that biological activity of marine macrophytes is related to the essential polyunsaturated fatty acids [PUFAs], which are the abundant components of macrophytic glycolipids (Khotimchenko, 1993; Goncharova *et al.* 2000; Sanina *et al.*, 2000).

The red algae are reported to have high levels of polyunsaturated fatty acids mainly eicosapentaenoic (EPA) and arachidonic acids (AA) (Khotimchenko and Svetashev, 1987) but the contents vary within the same genus. *Chondria dasyphylla* (Wood) Ag. is reported to have equal contents of eicosapentaenoic (EPA) and arachidonic acids (AA) whereas in *Chondria decipiens* EPA predominates (Khotimchenko and Vaskovsky, 1990). Further, in red algae PUFAs belonging to C20 series are reported to be mainly concentrated in MGDG (Sanina *et al.*, 2004). This has in fact been observed in the present investigation with EPA and AA being the constituent fatty acids of major glycolipids identified in **PF**<sub>1-3</sub>, and is well in agreement with our earlier communication on the fatty acids from the alga *Chondria armata* where C20 acids were not detected as free fatty acids (Govenkar and Wahidulla, 1999).

Glycoglycerolipids occur widely and copiously in vascular plants (Van Hummel, 1975), certain green seaweeds (Falsone *et al.*, 1994; Arao and Yamada, 1989; Mancini *et al.*, 1998), cyanobacteria (Reshef *et al.*, 1997), marine dinoflagellates (Oshima, 1994) and the freshwater alga *Chlorella vulgaris* (Morimoto *et al.* 1995). As to the glycoglycerolipids of red algae hydroxyeicosapentaenoyl galactosyl glycerols are known from the temperate red alga, *Gracilariopsis lemaneiformis* (Jiang and Gerwick, 1990) and MGDG, DGDG and SQDG are reported from *Gracilaria verrucosa* (Son, 1990) who also reports the presence of SQMG (**GL**<sub>2a</sub>) from the same source. This SQMG is also known to be a constituent of cyanobacterium *Synechocystis* PCC 6803 (Kim *et al.*, 1999), and lichenized basidiomycetes, *Dictyonema glabratum* (Sasaki *et al.*, 2001). 2-O- $\alpha$ -D-galactopyranosylglycerol is a metabolite of *Laurencia pinnatifida* (Aplin *et al.*, 1967) and 2,3-dipalmitoyl sulfonoglycolipid has been identified in *Laurencia pedicularioides* and is reported to be the most abundant component in red algae

(Siddhanta *et al.*, 1995). Recently, Shao *et al.* (2002) reported the presence of a new sulfonoglycolipid, crassicaulisine, in the red alga *Chondria crassicaulis*. Taxonomically, genus *Laurencia* and *Chondria armata* belong to the same family, Rhodomelaceae but in the present investigation *Chondria 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 (Araki *et al.* 1989, Fusetani and Hashimoto, 1975; Gustafson *et al.* 1989; Siddhanta *et al.* 1991, Son 1990). The two sulfonoglycolipids of the present investigation provide yet another example of a glycolipid which contains palmitic acid as the only fatty acid component. Palmitic acid was described as having hemolytic activity in sea urchin eggs (Fusetani and Hashimoto, 1976) and presumed to be playing unique role in algal physiology (Fusetani and Hashimoto, 1975).

Sulfoquinovosyl acyl glycerols, in particular compounds with C<sub>18</sub> fatty acid on the glycerol moiety, may be clinically promising antitumor or immunosuppressive agents (Aoki *et al.*, 2005).

The precise physiological functions of these glycolipids are not clear and since they are present in the alga in trace amounts, they are unlikely to play any major role in stabilizing the algal membranes in contrast to the vascular plants.

## **Material and methods**

### *Biological material*

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

### *General procedures*

Sephadex LH-20 (Pharmacia) and silica gel (60-120 mesh) [Qualigens] were used for gel filtration and column chromatography respectively. Precoated Kieselgel 60 F<sub>254</sub> TLC plates (Merck) were used for analytical TLC. Compounds were visualized as purplish spots on spraying with 5% methanolic sulphuric acid followed by heating at 100°C. Solvent system for TLC II and I was light petrol/ethyl acetate (1:1) and (1:4) respectively.

### *Mass spectrometry*

Mass spectra were recorded, in the positive mode, on a QSTARXL MS/MS Applied Biosystems 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 **PF**<sub>2</sub> was taken in methanol as well as dilution solvent.

### *Dilution Solvent*

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

### *NMR*

<sup>1</sup>H, <sup>13</sup>C, COSY, HMQC and HMBC experiments were recorded, in CDCl<sub>3</sub>, on a Bruker (Avance 300) spectrometer with TMS (tetramethylsilane) as internal standard.

### *Extraction and isolation of glycolipids*

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

The chloroform fraction (123g) was fractionated, initially on a column of Sephadex LH<sub>20</sub> with methanol (500ml) as eluant collected in fractions of 20ml each. The fractions obtained were examined by TLC (solvent:light petrol:ethylacetate, 1:1,v/v, spray: 5% methanolic sulfuric acid) and combined according to their profile. Fractions yielding purplish spots were then purified by repeated silica gel chromatography using petroleum ether (60-80°C):ethyl acetate (1:1) to give **PF**<sub>1</sub> (4mg), and methanol: chloroform (2:98) yielded **PF**<sub>2</sub>, [ $\alpha$ ]<sub>D</sub>-16 (c 0.02, CHCl<sub>3</sub>, R<sub>f</sub>-0.52 in solvent I; yield 13mg). Further elution of the same column with methanol: chloroform (5:95) yielded **PF**<sub>3</sub> [ $\alpha$ ]<sub>D</sub>-20 (c 0.02, CHCl<sub>3</sub>, R<sub>f</sub>-0.12 in solvent II, 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 (PF<sub>1-3</sub>)*

**PF<sub>1-3</sub>**, 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 (PF<sub>1-3</sub>)*

Each fraction (4-8mg) in 5 ml of 2% H<sub>2</sub>SO<sub>4</sub> 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 sulphate. 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 (Jonet *et al.*, 1995), the only occurrence of L galactose is in agar-agar (Mathur and van Holde, 1990).

### *Antibacterial assays*

Antibacterial activity was determined against six Gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella flexneri*, *Klebsiella pneumoniae* and *Vibrio cholerae*) and one Gram positive bacteria (*Staphylococcus aureus*) using the paper disk assay method (El-Masry *et al.* 2000). The sterile paper disk of 6mm diameter impregnated with 65µg/disk of **PF<sub>1</sub>** and 130µg/disk of **PF<sub>2</sub>** were placed on agar plates containing the tested microorganisms. In all cases, the concentration was approximately 1.2x10<sup>8</sup> CFU/µl. The impregnated disks were placed on the medium suitably spaced apart and the plates were incubated at 37°C for 24 hrs. Disk of Streptomycin (10µg/ml) was used as a positive control. The diameter (mm) of the growth inhibition halos caused by the sample was examined.

### *Antifungal assay*

Antifungal activity was determined against *Aspergillus fumigatus*, *Fusarium sp.*, *Cryptococcus neoformans*, *Aspergillus niger*, *Rhodotorula sp.*, *Nocardia sp.* and *Candida albicans* using the paper disk assay method as previously described in the antibacterial assay. The sterile disk was impregnated with the compound (65µg/disk of **PF<sub>1</sub>** and 130µg/disk of **PF<sub>2</sub>**). The inoculum

concentration was  $0.5 \times 10^3$ - $2.8 \times 10^3$  CFU/ml. Nystatin (100 $\mu$ g/disk) was used as positive control. The plates were incubated at 24°C for 18 h. The diameter (mm) of growth inhibition halos caused by the compound was examined.

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### **Abbreviations**

COSY,  $^1\text{H}$ ,  $^1\text{H}$  correlation spectroscopy; TOCSY,  $^1\text{H}$ ,  $^1\text{H}$  total correlation spectroscopy; HMQC,  $^1\text{H}$ ,  $^{13}\text{C}$  heteronuclear multiple quantum coherence; HMBC,  $^1\text{H}$ ,  $^{13}\text{C}$  heteronuclear multiple bond correlation; TLC, thin layer chromatography; NMR, nuclear magnetic resonance; PF<sub>1-3</sub>, polar fractions 1 to 3; GL<sub>1-3</sub>, glycolipids 1 to 3; ESI-MS, electrospray ionization mass spectrometry; MS/MS, tandem mass spectrometry; CID, collision induced dissociation; SFV, semiliki forest virus; CDCl<sub>3</sub>, Deuterated chloroform; MGDG, monogalactosyldiacylglycerol; SQMG, sulfoquinovosylmonogalactosyl glycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol

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## Legends to figures

Fig. 1: (A) Positive ESI-MS of the fraction PF<sub>1</sub> from *Chondria armata*; (B & C) MS/MS of the ions at m/z 1017[M+H]<sup>+</sup> and 751[M+H]<sup>+</sup> of the glycolipids along with their proposed structures

Fig. 2: (A) ESI-MS spectra, positive ion mode of glycolipids PF<sub>2</sub>, (B) MS/MS of the positive ion GL<sub>2b</sub> at m/z 629, (C) GL<sub>2a</sub> at m/z 601 and (D) GL<sub>2</sub> with [M + Na]<sup>+</sup> at m/z 541

Fig 3: (A) Positive ESI-MS of glycolipid fraction PF<sub>3</sub> ranging from m/z 0-900, (B) MS/MS of glycolipid GL<sub>3a</sub> at m/z 799; (C) m/z 779 [M + Na]<sup>+</sup>; (D) of phospholipid with [M + Na]<sup>+</sup> 691; (E) of glycolipid with m/z 655

Fig. 4: HMQC (A) and TOCSY (B) spectra of the glycolipid, GL<sub>3</sub> at 300MHZ in CDCl<sub>3</sub>

Fig. 5: ESI-MS/MS of glycolipid with m/z 309 from the fraction PF<sub>1-3</sub> along with the fragmentation pattern.

**Table-I: NMR (300MHz) data of PF<sub>1-3</sub> in CDCl<sub>3</sub>**

PF <sub>1</sub>				PF <sub>2</sub>			PF <sub>3</sub>			
Carbon No.	<sup>1</sup> H 1	<sup>13</sup> C 1	HMBC 1	<sup>1</sup> H 2	<sup>13</sup> C 2	HMBC 2	Carbon No.	<sup>1</sup> H 3	<sup>13</sup> C 3	HMBC 3
1	4.22, 4.35	62.2	1''', 2	4.0,4.396	62.2		1	4.21, 4.38	62.9	1''
2	5.23 (m)	70.0	-	5.3	70.0	-	2	5.28	70.2	3
3	3.56(d, 3.6Hz) 3.96(d, 6.0Hz)	67.9	1, 1'	3.515 (b) 3.913 (b)	67.9		3	3.65 3.83(d, 6Hz)	68.1	1', 2'
1'	4.178(d, 2.1Hz)	103	2'	4.25(d,4.5Hz )	103.6		1'	4.26(d,7.2Hz)	104.0	2'
2'	3.52	71.6	3', 4'	3.633	72.4		2'	3.617	71.3	3'
3'	3.85(b, s)	68.2	4'	3.90	67.9		3'	3.893	69.2	
4'	3.55(d, 3.6Hz)	73.1	3', 5'	3.692	73.1		4'	3.538	74.6	
5'	3.61(m)	72.3	1', 6'	3.5(b)	71.6		5'	3.649	73.5	1'
6'	4.23 4.39	62.2	5'	4.30 4.393	62.2		6'	4.247 4.271	62.0	5'
1''	-	173.5		-	173.9		1''	-	173.5	
2''	2.28	34.1	3'',1'',4''	2.357	34.1	1'', 3'', 4''	2''	2.3(d, 7.8 Hz)	34.3	1'', 3'',4''
3''	1.58	24.8	2''	1.639	27.2	2'', 4''	3''	1.685m	25.6	2'', 4''
8''	1.96 2.78	27.1 -	9'', 10'' -	2.043(b)	29.3	9'', 10''	4''	2.09 (q, 6Hz)	27.2	5'',3''
9''	5.31 (d, 5.4Hz)	129.6	8''	5.390(b)	128.8	8''	5'',6'',8'' ,9'',11''---	5.37-5.38 (cluster)	127-132 (10 d)	4'', 7''---
10''	5.31	128.7	11''	5.390(b)	130.0	11''	7'',10''--	2.838 (br dd)	29.3-31.4 (4 t)	5'',6''---
18''	0.827(t, 6.9Hz)	14.08	16'', 17''	0.827	14.1	16''	19''	2.02 (m)	22.7	18'',20''
(CH <sub>2</sub> )n	1.202 (bs)	29.1- 30.8		1.280(b)	29.3- 31.9		20''	0.977(t, 7.5)	14.1	18'', 19''
1'''	-	173.8	2'''				1'''	-	173.6	
2'''	2.12 (s)	22.6	1'''				2''',3'''	2.053-1.659 (m)	33.5,24.9	
O=C- CH <sub>3</sub>	2.12	22.6	Acetyls -sugar.				4'''--- 13'''	1.257	25.6-29.7	
							16'''	0.882 (t, 6.9Hz)	18.4	

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

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

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

(-) indicates No activity

(+) indicates Weak activity.

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

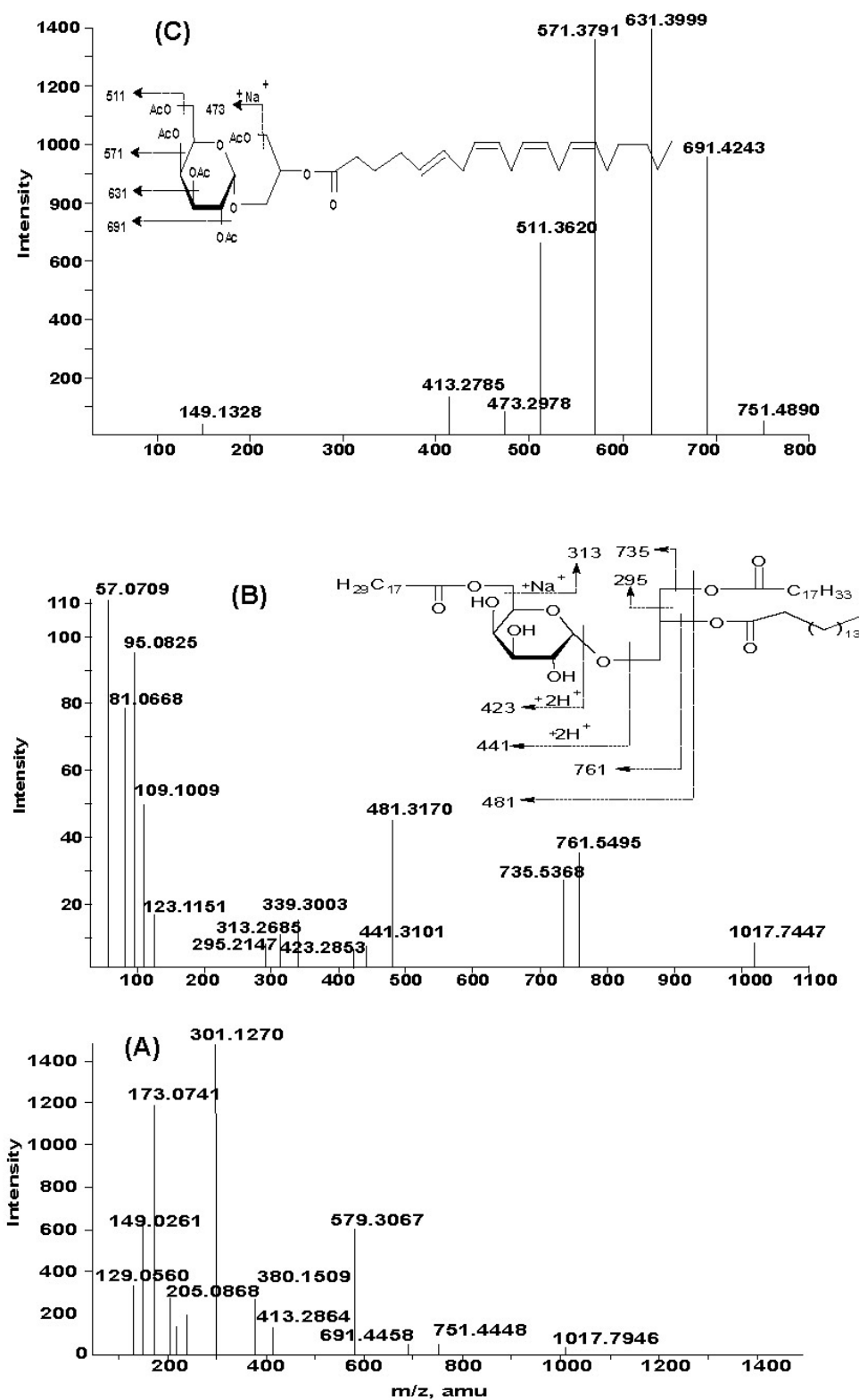


Fig. 1: (A) - Positive ESI-MS of the fraction PF<sub>1</sub> from *Chondria armata*; (B & C) MS/MS of the ions at m/z 1017[M+H]<sup>+</sup> and 751[M+H]<sup>+</sup> of the glycolipids along with their proposed structures

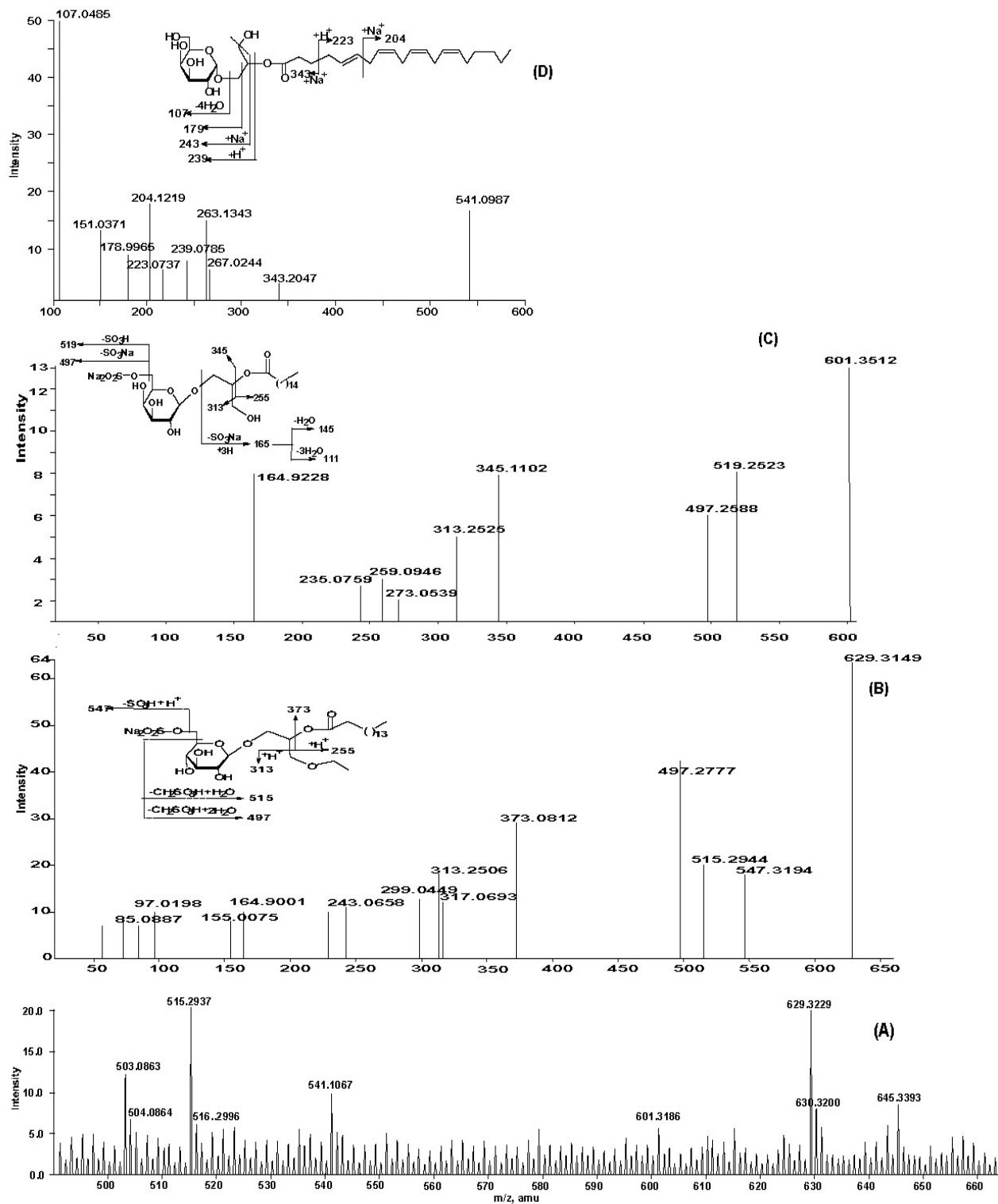


Fig. 2. (A) ESI-MS spectra, positive ion mode of glycolipids PF<sub>2</sub>, (B) MS/MS of the positive ion GL<sub>2b</sub> at m/z 629, (C) GL<sub>2a</sub> at m/z 601 and (D) GL<sub>2</sub> with [M + Na]<sup>+</sup> at m/z 541

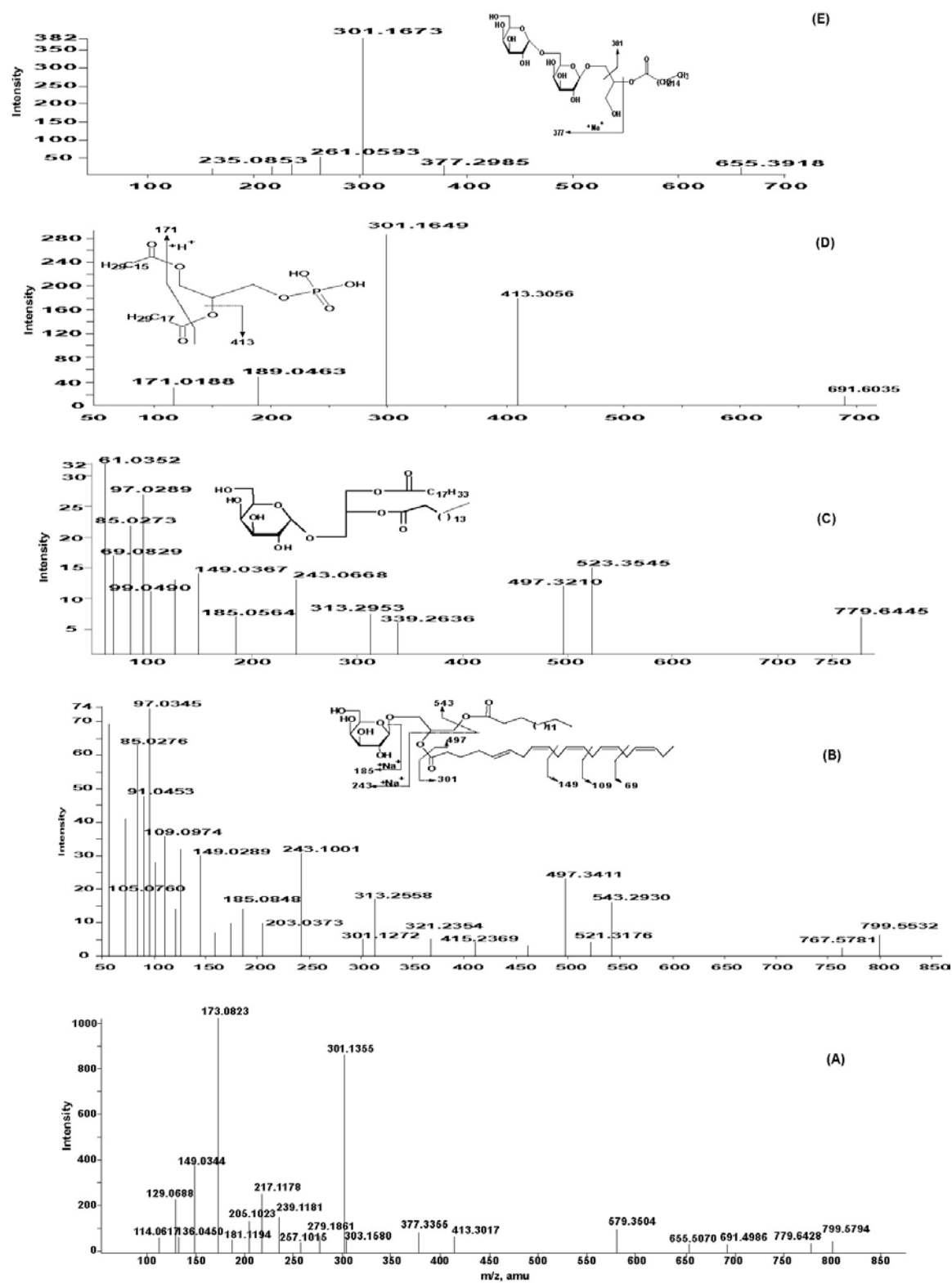


Fig 3: (A) Positive ESI-MS of glycolipid fraction PF<sub>3</sub> ranging from m/z 0-900, (B) MS/MS of glycolipid GL<sub>3a</sub> at m/z 799; (C) m/z 779 [M + Na]<sup>+</sup>; (D) of phospholipid with [M + Na]<sup>+</sup> 691; (E) of glycolipid with m/z 655



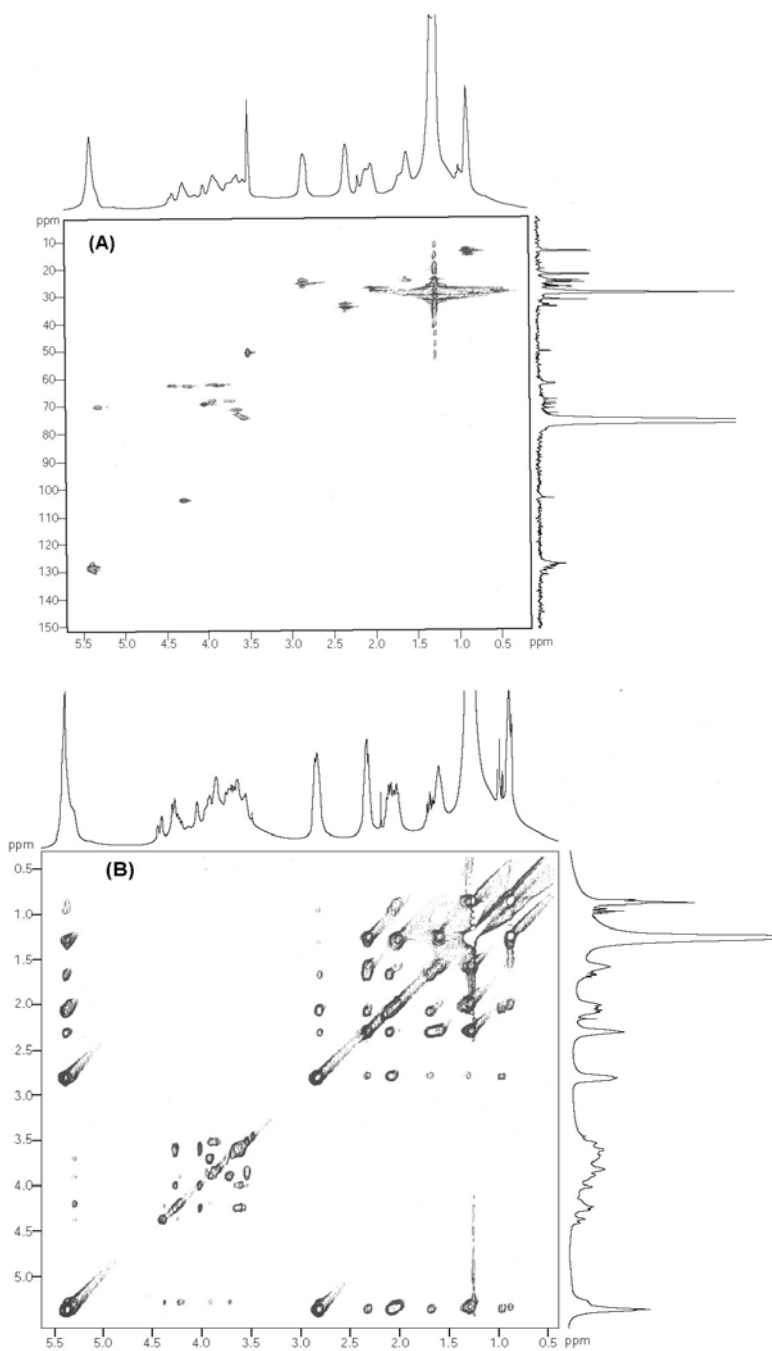


Fig. 4. HMQC (A) and TOCSY (B) spectra of the glycolipid, GL<sub>3</sub> at 300MHz in CDCl<sub>3</sub>

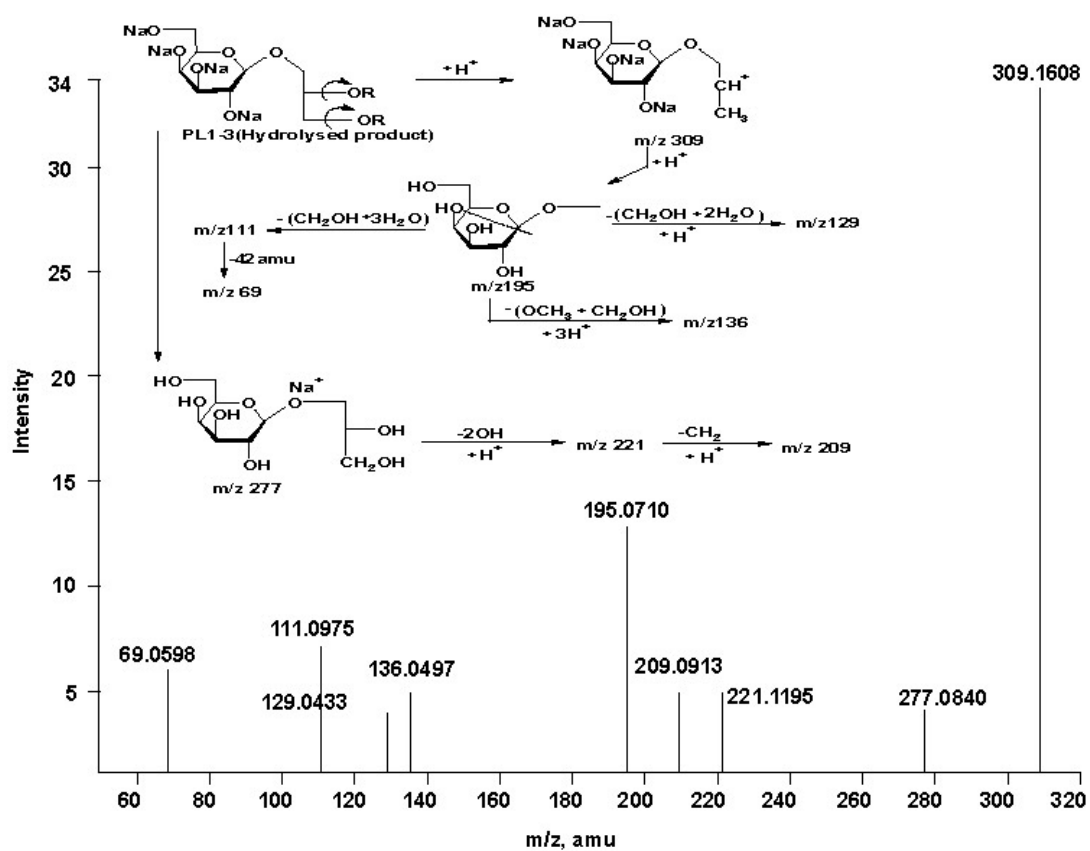


Fig. 5. ESI-MS/MS of hydrolysed product with  $m/z$  309 from the fraction PF<sub>1-3</sub> along with the fragmentation pattern