

## Chemotaxonomic Study of the Demosponge *Cinachyrella cavernosa* (Lamarck)

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**Abstract-**Sterols as acetates and phthalate esters were identified by GC-MS in demosponge *Cinachyrella cavernosa*. The sponge contained mainly C<sub>27</sub>, C<sub>28</sub> and C<sub>29</sub> Δ<sup>5</sup> conventional sterols and minor amounts of C<sub>27</sub> and C<sub>28</sub> Δ<sup>0</sup> analogues. This study also reports the presence of an ester, clionasterol oleate, 16-dehydropregnenolone (DPA), relatively rare cyclopropane containing sterols such as gorgosterol, 4α-methyl gorgostanol, phthalate esters, and nucleoside, 9β-D- ribofuranosyl adenine in *C. cavernosa*. 4α-Methyl gorgostanol, DPA, phthalate esters: 1,2 benzene dicarboxylic acid bis-(2-methyl propyl) ester and nucleoside, adenosine, are described for the first time from this genus. Dibutyl phthalate and 1,2 benzene dicarboxylic acid bis 2-ethyl hexyl ester have previously been identified in *Cinachyrella australiensis*. To our knowledge, this is the first report of 4α-methyl gorgostanol from a sponge and DPA from a marine source. The probable origin and chemotaxonomic importance of some of the metabolites is discussed.

**Keywords:** Adenosine; *Cinachyrella cavernosa*; 16-dehydropregnenolone; GC/MS; phthalates; sterols.

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## 1. Introduction

Sponges represent a rich and the most diverse source of unconventional sterols, with unusual structural features (Kerr and Baker, 1991). The presence of sterols in sponges is not only interesting from a biochemical and functional point of view, but also useful for the classification of these marine invertebrates (Bergquist et al., 1991). In fact, sterols were considered as the first class of components which could be used for chemotaxonomic studies of sponges (Bergmann, 1949). As consequence, particular combinations of sterols appear to characterize some orders, families and genera of the phylum (Bergquist et al., 1978, 1980, 1991).

Morphological characteristics of these invertebrates are extremely variable and sometimes difficult to use for taxonomic purposes (Santalova et al., 2007). Sponges of the genus *Cinachyrella* within the family Tetillidae have been particularly studied since precise specific identification on the basis of morphological characteristics is difficult. For example, the genus *Cinachyrella* found from the Mediterranean has been initially identified as *Cinachyrella tarentina* (Pulitzer-Finali, 1983) and also as *Chrotella cavernosa* (Lamarck 1815) by (Tsumamal, 1969). The latter species from Alexandria was recorded earlier by Burton (1936) as *Cinachyra australiensis* (Carter 1886) but without providing a description. However, both Burton and Tsumamal found it to be a migrant species with excessive synonymy for *Chrotella cavernosa* and thus concluded that *Cinachyra australiensis* and *Chrotella cavernosa* are one and the same species identical to *C. tarentina*. All the three species are now accepted as *C. tarentina* (Pulitzer-Finali 1983). Similarly, *Cinachyrella cavernosa* of present investigation was earlier identified as *Chrotella australiensis* (Mishra et al., 1997).

Surprisingly, literature on the chemical investigation of *C. cavernosa* (= *C. tarentina*/ *C. australiensis*) is scarce. *C. australiensis* from the South China Sea is known to produce secondary metabolites including nucleosides, phthalates, fatty acids (Li et al., 2004) and (3E)-cholest-4-en-3,6-dione-3-oxime (Xiao et al., 2005). Three common  $3\beta$ -hydroxysterols, mono and diketo steroids are known from Mediterranean *Cinachyra tarentina* (Aiello et al., 1991). Recently, Lakshmi et al. (2008) identified two new ceramides and tetillapyrone from an Indian specimen of *C. cavernosa*. In view of our research interest in marine natural products from marine invertebrates particularly sponges, we herein investigated *C. cavernosa* (belonging to the class Demospongiae, family Tetillidae). The main objective was to study sterol chemistry of *C. cavernosa* and compare it with the reported sterol profiles of other members of genus *Cinachyrella*, which shall provide new insights into the systematic of these marine invertebrates. Additionally, other non-steroidal

constituent of this sponge has also been reported wherein, the nucleoside, adenosine (10) was identified for the first time from this particular natural source.

## 2. Material and methods

### 2.1. Extraction, purification and acetylation of sterols

*C. cavernosa* collected off Goa (India), was identified by Dr. P A Thomas (Central Marine Fisheries Research Institute, Trivandrum, India). The voucher specimen (NIO-41) is deposited at NIO Repository and Taxonomic Center. Sponge was extracted thrice with methanol at room temperature, and the alcoholic extract was concentrated *in vacuo*. The residue was suspended in H<sub>2</sub>O and partitioned successively with chloroform and n-butanol. The chloroform fraction was subjected to column chromatography on silica using petroleum ether : EtOAc gradient, as eluent. Fractions, distinguished as bluish spots on spraying with 5% H<sub>2</sub>SO<sub>4</sub> in MeOH, were monitored by TLC [*R*<sub>f</sub>=0.52, petroleum ether: ethyl acetate (80:20, v/v)] to afford sterol rich fraction (221 mgs). A part of the sterol fraction thus obtained was subjected to spectral data (NMR & ESI-MS; Supplementary Fig.1a, b, c) and the remaining (25mgs) was acetylated at room temperature for 20 h using anhydrous Ac<sub>2</sub>O/pyridine mixture (1:1, v/v). Acetates were analyzed (21 mgs) by GC-MS.

Silica gel chromatography of n-butanol fraction and gradual elution with MeOH: CHCl<sub>3</sub> resulted in the isolation of an UV active fraction which was further purified by filtration through sephadex LH-20 to yield 10 mgs of the nucleoside (10).

### 2.2. Analytical Procedures

Analyses of sterol acetates were carried out on a Shimadzu GC-MS QP2010 System using capillary column, XTI-5 ( 30m X 0.25mm, film thickness 0.25µm, crossbound-5% diphenyl-95% dimethyl polysiloxane) at oven temperature programmed from 60 to 280°C at 5°C/min; helium at 1.0 ml /min as the carrier gas; injector temperature 300°C, the ionizing voltage was of 70eV and the mass range was m/z 40-700 Da. Structural assignments were based on interpretation of mass spectrometric fragmentations and reference library (Itoh et al., 1982; Hochmut et al., 2004). NMR spectra were recorded on Bruker Avance 300 MHZ spectrometer in CDCl<sub>3</sub> with TMS as internal reference. TLC was performed on silica gel 60 F<sub>254</sub> plates (Merck). Sephadex LH-20 (Pharmacia) and silica gel (120-250 microns) (Qualigens) were used for gel filtration and column chromatography respectively.

### 3. Results and discussion

The chromatographic profile of the volatile chemical constituents of purified sterol fraction of *C. cavernosa* as obtained by GC-MS showed the presence of 22 compounds including 18 steroids, 3 phthalate esters and a fatty acid (Table-1, Fig.1). As evident, among  $\Delta^5$  sterols identified in *C. cavernosa*, stands out as major constituents cholesterol (35.3%) and clionasterol (29.7%) followed by brassicasterol (6.1%) , 22-dihydrobrassicasterol (5.9%) . A remarkable amount of cholesta- 5, 22(E)-diene -3 $\beta$ -ol (3.8%) with minor amount of the corresponding (22Z) isomer (0.6%) was also present in the mixture. This is coherent with those reported previously for other sponges of genus *Cinachyrella* (Bergquist et al.,1980, Barnathan et al., 1992, 2003), except for variation in their proportion (Table-2). However,  $\Delta^5$  sterols do not provide sufficient taxonomic information since they are common in marine invertebrates (Barnathan et al.,1992).

Acetate of pregna-5, 16-diene-3-ol-20-one also known as 16-dehydropregnenolone acetate (DPA), is present in considerable quantities (5.0%). Although the identification of DPA is described for the first time in this genus as well marine source, its first natural occurrence was reported from a terrestrial plant, *Solanum lyratum* (Sun et al., 2006). Other interesting sterols found in *C. cavernosa* are gorgosterol (9) and 4 $\alpha$ -methyl gorgostanol (7). Gorgosterol was recently reported from *Cinachyrella* sponges of Saudi Arabian Red Sea (Barnathan et al., 2003) and 4 $\alpha$ -methyl gorgostanol, a sterol characteristic of corals and other Coelenterates (Goad, 1978; Djerassi, 1981), is also found in dinoflagellates particularly zooxanthellae and Pyrrophyta. Compound (7) has not been reported to date in a sponge where it could originate through its food or *de novo* biosynthesis by symbiotic pyrrophytic algae. Though there are indications that the association between marine sponges and zooxanthellae appears less frequent (Bergquist, 1978), *C. cavernosa* is described as a free living sponge with endosymbiotic zooxanthallae, referred to as

*Symbiodinium microadriaticum* (Scalera-Liaci et al., 1999) which is known to contain complex mixture of 4-methyl sterols and 4-desmethyl sterols (Mansour et al, 2002). Sterols (7) and (9) are reported as two of the five major steroids in dinoflagellate *Gonyaulax monilata* (Wengrovitz et al., 1981) and *Peridinium foliaceum* (Withers et al., 1979).

The sterol, 24-oxocholesterol, also present in *C. cavernosa*, was originally described from a marine brown alga *Pelvetia canaliculata* (Motzfeldt, 1970) and marine microalga *Isochrysis galbana* (Won et al., 2002). Brassicasterol is a plant sterol synthesized by phytoplankton and used as a biomarker for the presence of marine algal matter in the environment (Volkman, 1986). Diatoms and dinoflagellates are known to be important components of the phytoplankton and thus play an

important role in marine food webs. They also occur as symbionts in marine invertebrates. Thus, it seems likely that occurrence of brassicasterol and 24-oxocholesterol in marine animals such as sponges, corals, gorgonians and some mollusks could be due to their association with macro and microalgae.

The acetylated mixture, also contained clionasterol as oleate, in addition to 1,2-benzenedicarboxylic acid bis (2-methyl propyl) ester, palmitic acid, dibutyl phthalate and 1,2 benzene dicarboxylic acid bis 2-ethyl hexyl ester (Table-1). The last two phthalate esters are known as constituents of sponges, *C. australiensis* (Li et al., 2004), and *Biemna fortis* (Topsent) (Xiao-Chun et al., 2008). These are likely of bacterial origin as they are reported as fermentation products of soil isolates belonging to genus *Streptomyces* (Roy et al., 2005; Al-Bari et al., 2005). The presence of bacteria in *Cinachyrella* sponges is known (Barnathan et al., 2003).

Sponges of genus *Cinachyrella* harbour mono as well as diketo steroids either as free or derivatized at 3/6 position as hydroxyimino compounds (Table-2). Keto steroids were not detected in *C. cavernosa* mainly because the free sterol fraction was esterified and taken for GC-MS analysis. However, we have reported the presence of  $\Delta^4$ -3-keto-steroids in our earlier investigation on *C. cavernosa* (= *Chrotella australiensis*) (Mishra et al., 1997). The hydroxyimino derivatives seem to be potential marker of the genus *Cinachyrella* (Erpenbeck and van Soest, 2006), and particularly for *C. tarentina* (= *C. australiensis*) (Xiao et al., 2005). However, spectral data of the sterol fraction (Supplementary Fig.1a, b, c) of *C. cavernosa* was indicative of absence of hydroxyimino derivatives when compared with the data reported by Rodríguez et al. (1997) for 6-hydroximino-4-en-3-one steroids from a mixture of two morphospecies of the *Cinachyrella* (*C. alloclada* versus *C. apion*). Thus, the signals in  $^1\text{H}$ NMR (Fig.1a) for trisubstituted double bond [ $\delta$  6.43(1H, s), oxime -OH [ $\delta$  9.88 (1H, bs)] and 2 diastereotopic protons [ $\delta$  3.42 (1H,dd, J=16.0 and 4.7Hz) and 1.55(1H,m)] were absent.  $^{13}\text{C}$ NMR (Fig.1b) showed the presence of signals at 157.63 and 110 ppm due to C24 methylene of 24 methylene-cholesterol which is a known constituent of *Chrotella australiensis* (= *C. cavernosa*) and not due to C-6 quaternary carbon carrying hydroxyimino group which is reported at 156.0 ppm. Further confirmation arises by the lack of signals in ESI-MS profile (Fig.1c) of the sterol fraction at m/z 442 as well as at m/z 414, as expected for 6-hydroximino-4-en-3-one steroids. The absence of hydroxyimino steroid in the sponge of present investigation may be either due to biotic as well as abiotic factors including season and temperature or else sterol complement is not a useful feature at taxonomical level suggesting that *C. cavernosa* and *C. tarentina* (= *C. australiensis*) are two distinct biological species. Further, it is also to be noted that Rodríguez et al. (1997) report 6-

oxime derivatives from morphospecies of the *Cinachyrella* (*C.alloclada* versus *C. apion* ) whereas Xiao et al. (2005) report 3-oxime derivative from *C. australiensis*.

Chemical investigation of n-butanol fraction yielded a nucleoside, readily identified as 9 $\beta$ -D-ribofuranosyl adenine (10) by comparison of its spectral data with those reported in the literature (Ciuffreda et al., 2007). This is the first report of a nucleoside adenosine from the sponge *C. cavernosa*. Corresponding 2'-deoxy adenosine has been reported by Li et al. (2004) from *C. australiensis*. However, various bioactive nucleosides of marine origin has been previously reported which has been reviewed by Bhakuni and Rawat (2005).

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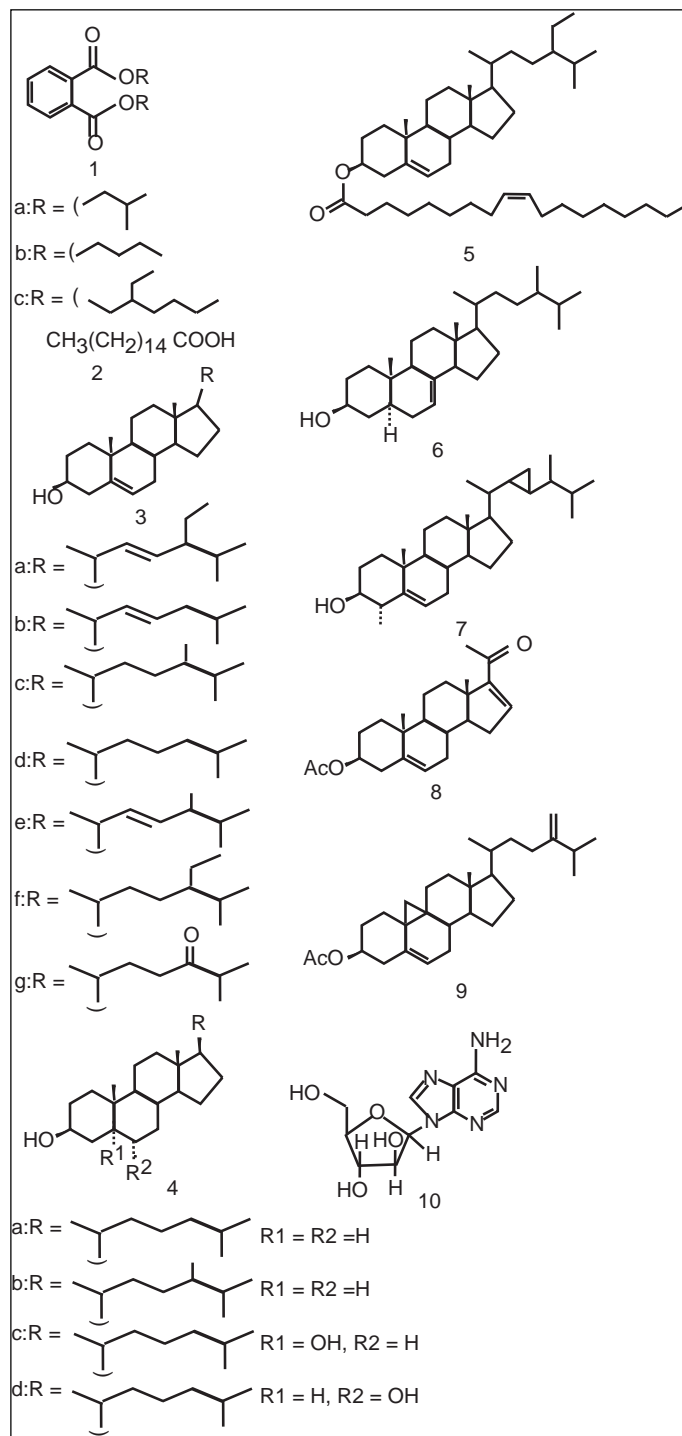
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**Figure 1**

Fig.1: Structures of compounds identified in *Cinachyrella cavernosa*

Table-1: Sterols and Phthalate esters identified in *Cinachyra cavernosa* by GC-MS

Peak No.	Compound	RT <sup>a</sup>	M <sup>+</sup>	%Are <sup>b</sup>
1	bis-(2-methyl propyl) phthalate ( <b>1a</b> )	19.60	278	0.4
2	n-hexadecanoic acid ( <b>2</b> )	20.90	256	0.9
3	Dibutyl phthalate ( <b>1b</b> )	21.03	278	0.4
4	bis-(2-ethyl hexyl)phthalate ( <b>1c</b> )	28.59	390	0.7
5	Unidentified	34.16	243	0.2
6	Stigmasta-5,22 (Z)-diene-3 β-ol ( <b>3a</b> )	34.29	412	0.5
7	Stigmasta-β-5en-3β-ol /clionasterol oleate ( <b>5</b> )	34.49	678	0.6
8	Cholestane-3β,5α-diol( <b>4c</b> )	34.70	404	0.4
9	Cholestane-3β,6α-diol ( <b>4d</b> )	34.80	404	0.4
10	Cholest-5,22 (Z)-diene-3β-ol ( <b>3b</b> )	35.46	384	0.6
11	Cholest-5,22 (E)-diene-3β-ol ( <b>3b</b> )	35.61	384	3.8
12	24-Methyl-cholest-7-ene-3β-ol//campest-7en3-ol( <b>6</b> )	35.72	400	0.5
13	Cholesterol ( <b>3d</b> )	36.02	386	35.3
14	Cholestanol ( <b>4a</b> )	36.09	388	3.2
15	Brassicasterol/crinosterol ( <b>3e</b> )	36.40	398	6.1
16	4α-Methylgorgostanol ( <b>7</b> )	36.50	442	1.1
17	22 dihydrobrassicasterol/campestrol ( <b>3c</b> )	36.98	400	5.9
18	Ergostan-3β-ol ( <b>4b</b> )	37.08	402	0.3
19	Unidentified	37.20	410	1.7
20	Stigmasta-5,22(E)-diene-3 β-ol ( <b>3a</b> )	37.27	412	0.8
21	Stigmasta-5ene-3β-ol (clionasterol) ( <b>3f</b> )	37.91	414	29.7
22	16-Dehydropregnenolone acetate ( <b>8</b> )	38.06	356	5.0
23	24-Oxo cholesterol ( <b>3g</b> )	38.29	400	0.5
24	Gorgosterol ( <b>9</b> )	38.46	426	1.1

a-Gas chromatographic retention time of acetylated sterols and phthalate esters

b-Quantification of the sterols acetates and phthalate esters based on the area of the GC peak

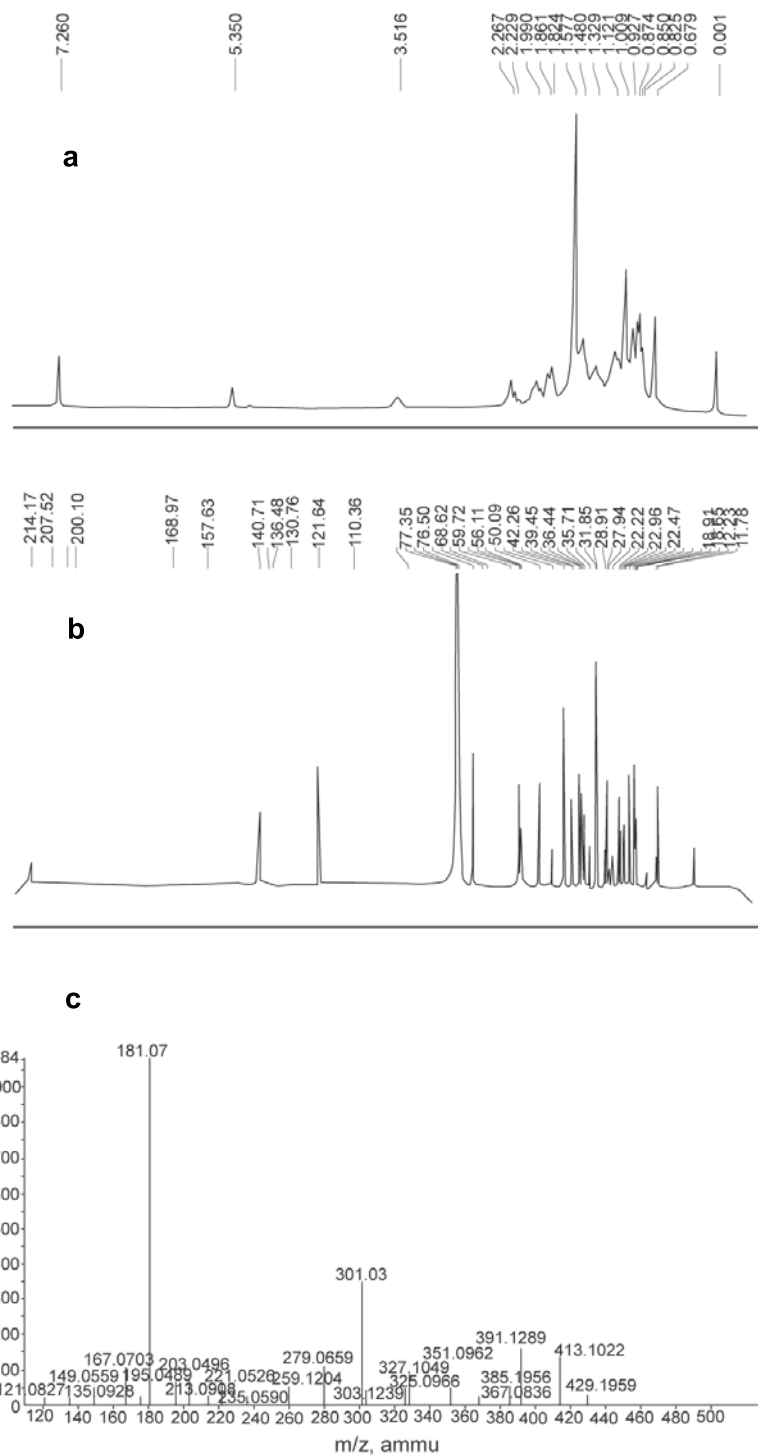
Table-2: Sterol compositions of *Cinachyrella* sponges from different locations

Sterol	<i>M<sub>w</sub></i>	Composition (%)						
		<i>C.alloclada</i>			<i>C. kükenti</i>	<i>C.australiensis/C. tarentina</i>		
		Red Sea Pacific	Senegal	Atlantic	Red Sea (Brazil)	Mediterranean	Arabian Sea	China
24-Norcholesta5,22-dienol	370	-	0.3/0.5	-	-	-	-	-
22(Z)-Dehydrocholesterol	384	2.3	0.4/0.8	-	1.4	-	0.6	-
22(E)-Dehydrocholesterol	384	5.3	8.4/7.0	-	4.8	-	3.8	-
22-Dehydrocholestanol	386	-	-/-	-	1.2	-	-	-
Cholesterol	386	14.5	22.4/22.0	-	11.3	(+)	35.3	-
Lathosterol	386	-	0.9/1.3	-	-	-	-	-
Cholestanol	388	3.1	2.1/3.2	-	5.4	-	3.2	-
Brassicasterol/crinosterol	398	24.3	15.8/13.8	-	18.8	(+)	6.1	-
24-Methylenecholesterol	398	0.3	Trace/0.3	-	0.4	(+)	(+)	-
24-oxocholesterol	400	-	-	-	-	-	0.5	-
Campesterol/dihydrobrassicasterol	400	11.0	8.1/9.3	-	14.2	-	5.9	-
Campesten-7-ol	400	-	-/-	-	2.1	-	0.5	-
22-Dehydrocampestanol	400	-	-/-	-	3.4	-	-	-
Campestanol	402	1.1	-/-	-	2.8	-	0.3	-
Poriferasterol/stigmasterol	412	0.8	4.5/4.4	-	1.0	-	0.5/0.8	-
23,24-Dimethylcholestadien-5,22-ol	412	-	2.2/-	-	-	-	-	-
Clerosterol	412	11.8	-/-	-	10.7	-	-	-
Fucosterol	412	-	1.8/2.6	-	-	-	-	-
Isofucosterol	412	-	-/-	-	-	-	-	-
Poriferasten-7-ol	412	-	-/-	-	0.7	-	-	-
Stigmasten-7-ol	412	-	-/-	-	1.0	-	-	-
Clionasterol	414	18.4	33.0/30.0	-	13.7	-	29.7	-
Sitosterol	414	0.7	-/-	-	0.9	-	-	-
22-dehydrostigmasterol	414	1.6	-/-	-	2.0	-	-	-

Stigmastanol	416	4.2	-/-	-	3.6	-	-	-
Unidentified C <sub>30</sub>	426	0.3	-/-	-	0.5	-	-	-
Gorgosterol	426	0.3	-/-	-	0.1	-	1.1	-
4 $\alpha$ -Methylgorgostanol	442	-	-/-	-	-	-	1.1	-
16-Dehydropregnenolone acetate	356	-	-/-	-	-	-	5.0	-
Cholest-4ene-3-one	384	-	-	(+)	-	(+)	(+)	(+)
24-Ethylcholest-4-ene-3-one	412	-	-	-	-	-	(+)	-
24-methylcholesta-4,24(28)diene-3-one	396	-	-	-	-	(+)	(+)	-
Cholest-4-ene-3,6-dione	398	-	-	-	-	(+)	-	-
Cholest-6-hydroxyimino-4-ene-3-one	413	-	-	(+)	-	-	-	-
24-Ethyl-cholest-4ene-3,6-dione	426	-	-	-	-	(+)	(+)	-
24R,6E-Ethylcholest-6-hydroxyimino-4-ene-3-one	441	-	-	(+)	-	-	-	-
Cholestane-3 $\beta$ ,5 $\alpha$ -diol	404	-	-	-	-	-	0.4	-
Cholestane-3 $\beta$ ,6 $\alpha$ -diol	404	-	-	-	-	-	0.4	-
Stigmasta- $\beta$ -5en-3 $\beta$ -ol /clionasterol oleate	678	-	-	-	-	-	0.6	-
(3E)Cholest-3-hydroxyimino-4-ene-6-one	413	-	-	-	-	-	-	(+)

(+) Indicates present; (-) Indicates absent

Table-2 (continued)



**Figure S1:**

**a:**  $^1\text{H}$ NMR spectrum of sterol fraction of *Cinachyrella cavernosa*

**b:**  $^{13}\text{C}$ NMR spectrum of sterol fraction of *Cinachyrella cavernosa*

**c:** ESI-MS spectrum of sterol fraction of *Cinachyrella cavernosa*