

## Comparative Ultrastructural and Biochemical Studies of Four Demosponges from Gulf of Mannar, India

Ramjee Pallela<sup>1,2</sup>✉, Venkateswara Rao Janapala<sup>1</sup>✉

1. Toxicology Unit, Biology Division, Indian Institute of Chemical Technology, Hyderabad-500007, India

2. Synthetic Biology and Biofuels Group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi-110067, India

✉ Corresponding author email: [rpallela@gmail.com](mailto:rpallela@gmail.com); [jvraoiict@gmail.com](mailto:jvraoiict@gmail.com)

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**Abstract** Marine sponge explorations at Gulf of Mannar (GoM), India in the past few years (2003-2009) revealed around forty sponge species that lead to various comprehensive studies on chemocobiological molecules including collagens and their applications. Basing on the preliminary evaluations on collagen content, four marine sponges *Hyattella cribriformis*, *Fasciospongia cavernosa*, *Callyspongia fibrosa* and *Dysidea fragilis* belong to the families Spongiidae, Thorectidae, Callyspongiidae and Dysideidae of the class Demospongiae, were chosen for macromolecular and micro-anatomical comparison. Microscopic examinations were performed to understand the form and arrangement of skeletal components in each sponge, where unique skeletal composition and spongin distribution is typical for all the sponges except *D. fragilis*, which is highly embedded with diversified oxeae. Biochemical analyses were performed to resolve the macromolecular content for each sponge species. Sponge *D. fragilis* was found to possess less protein content ( $57.26 \pm 3.36$  mg/g freeze-dried sponge wt) when compared to other sponges and significantly, *C. fibrosa* possess high protein ( $209.55 \pm 9.53$  mg/g) and other macromolecular contents, except pentose ( $24.12 \pm 1.21$  mg/g). Different forms of collagens viz., Acid soluble (AS), salt soluble (SS) and insoluble (Ins) collagens are estimated to understand the variation in collagen make up in their respective tissues. The higher collagen content in *C. fibrosa* could propose to use this species as a potential model for targeting collagenous molecules towards human health benefits.

**Keywords** Marine sponges; Demospongiae; Biochemical; Collagen; Electron microscopy; Gulf of Mannar

### Introduction

Sponges (Phylum: Porifera) are one of the better-known, diverse multi-cellular invertebrates diverged from the common ancestor of monophyletic origin, Eumetazoa (Müller et al., 2006; Ereskovsky et al., 2010). Although phylogenetically crucial among all the sponges of Porifera, class Demospongiae has been overlooked for various anatomical and developmental studies (Brusca and Brusca, 2003). Connective tissue in Demosponges is found to be well developed and typically forms a complex and often elegant skeleton, whose rigidity varies widely among species and growth forms. The skeleton of the Demosponges was formed by gelatinous mesohyl supported with fine collagen fibers and/or a collagenous matrix (spongin) embedded with mineral spicules or oxeae. Included in the class are the bath sponges, which obviously lack spicules, but have a well-developed spongin skeleton. With respect to their body organization, cell

differentiation and embryogenesis, the representatives of Porifera are characterized as model systems due to their pronounced plasticity in the determination of cell lineages (Koziol et al., 1998). These simple organisms possess numerous structural elements like collagen that are involved in cell adhesion and recognition, similar to the characteristics of more complex animals (Boute et al., 1996; Fernández-Busquets, 2008; Rao et al., 2011; Pallela et al., 2012).

The composition and analyses of various macromolecular and biochemical content of each sponge species is very valid in choosing potential candidate of interest, because the spicule formation is dependent on the apposition and deposition of macromolecules like protein (spongin/collagen), lipids, carbohydrates etc. (Aizenberg et al., 1996). In addition, detailed morphological studies that demosponges infer a considerable polymorphism within the species by

means of their collagenous skeleton (Garrone, 1999; Garrone, 1985). Successful applications of collagens from various terrestrial sources is as vast as in the treatment of hypertension, urinary incontinence, pain associated with osteoarthritis, wound healing, tissue engineering for human organ implants etc. However, the outbreaks of contagious diseases through the highly antigenic collagens could lead to health complications like BSE and TSE (Swatschek et al., 2002). Hence, current researchers are targeting safer collagen from nature, more importantly from marine sources, as life originated from ocean. In this context, marine sponges are the ideal role models for several collagen explorations in order to meet the recent medical demands of human beings.

Despite of having an attractively simple basic organization that remains fairly constant throughout all species, they do manage to show a great variety of forms and shapes to give around 11,000 known species of sponges worldwide among which, only 8500 are considered valid (Van Soest et al., 2012). More than 300 species of sponges have been recorded from Gulf of Mannar (GoM) and Palk Bay region located on the southeast coast of India, which is considered to be one of the hotspots for marine sponges in the world (Venkataraman and Wafar, 2005). GoM possess a coastline of 315 km starting from the pilgrim town of Rameshwaram and ending at industrial town of Tuticorin. Taking their remarkable biomedical and pharmacological applications into account (Chairman et al., 2012), preliminary studies on the Demosponges from GoM were carried out in our laboratory. In the perspective of the microscopic and biochemical analyses, four marine sponges *Fasciospongia cavernosa*, *Hyattella cribriformis*, *Dysidea fragilis* and *Callyspongia fibrosa* were taken into consideration as all of them are prominent at the shallow and deep collection sites of GoM. The present paper depicts an over view of the biochemical content including the collagen of collected demosponges. This is the latest report of listing recently available sponge species in GoM area, where specific focus has been given to the macromolecular and ultrastructural differences between the experimental sponges for emphasizing the importance of these collagenated organisms, although intensive studies on collagen from *Ircinia fusca* were studied previously by our

group. Present study may prove to be the ideal vault for further chemicobiological explorations on remaining available marine sponges in the same area; and also for the isolation, purification and analysis of therapeutically important, safer protein molecules like collagens from different sponge species of GoM.

## 1 Materials and Methods

### 1.1 Sampling

Marine sponges were collected from the shallow and sub-tidal regions between 15 to 20 feet by skin-diving during our collection trips from the year 2006 to 2009 at Mandapam coast, Gulf of Mannar (Lat. 9°5' N, Long. 79°5' E), India. Immediately after collection, the samples were frozen and kept at -20°C until the analytical work was started. Few sponge specimens were placed into sampling bags under water to avoid contact with air and transported to the laboratory. The voucher specimens kept in small zip lock bags were submitted to National Institute of Oceanography (NIO), Goa for depository purpose and were identified by Dr. P.A. Thomas, Vizhinjam Research Centre of Central Marine Fisheries Research Institute (ICAR-CMFRI), Vizhinjam, Thiruvananthapuram, India. The identification of these Demosponges was based on the classical and recent bibliography. Four marine sponges belonging to four families viz., Thorectidae [*Fasciospongia cavernosa* (Schmidt, 1862)], Spongiidae [*Hyattella cribriformis* (Hyatt, 1877)], Callyspongiidae [*Callyspongia (Cladochalina) fibrosa* (Ridley & Dendy, 1886)] and Dysideidae [*Dysidea fragilis* (Montagu, 1818)] of the class Demospongiae were chosen for the present study, based on the available information on their possible ecological significance and were analyzed microscopically and biochemically. All the reagents used in the present study are of analytical grade and used without further purification.

### 1.2 Gross study of the sponges

Sponge specimens collected from the sea were photographed digitally (SONY) and with a CCD camera (SONY CCD IRIS, model no. SSC-M370CE, Japan) attached to a computer.

### 1.3 Microscopic Study

#### Video microscopy

Video microscopy was employed to examine the form and arrangement of skeletal components in intact

specimens of each sponge species. Thin, transverse sections of sponge tissue were obtained using sharp scalpel blade and observed under Hirox (model: MX-2010Z) video microscope attached to computer.

#### ***Light microscopy***

Prior to the washing of each sponge species in distilled water, squashed suspension of sponge tissue was subjected to microscopic slide and observed under POLYVAR (REICHERT-JUNG) compound microscope to understand the structural array of spongin and spicules.

#### ***Electron microscopy***

Scanning Electronic Microscopy was done according to the method of Bozzola and Russell (1999). Briefly, distilled water cleaned sponge tissues were dissected with thin scalpel blade and the sections were mounted over the stubs with double-sided carbon tape and were fixed with a 4% aqueous Osmium tetroxide vapours for 2 hr. A thin layer of Platinum (palladium) coat was then applied over the samples using an automated sputter coater (JEOL JFC-1600) for about 4 min. Then the samples were scanned under Scanning Electron Microscope (Model: JOEL-JSM 5600) at various magnifications.

#### **1.4 Macromolecular analysis**

Each specimen of collected sponge species was washed under running tap water for two to three times and then washed finally with 0.1M phosphate buffer (pH 7.5). For protein estimation, each experimental sponge tissue was chopped and minced using sharp dissection scissors and homogenized (10% w/v) in ice-cold 0.1M phosphate buffer (pH 7.5) using Heidolph DIAX 900 homogenizer. The homogenates were centrifuged at 500 g for 10 min and the supernatant was further recentrifuged at 5000 g for 10 min using refrigerated centrifuge (Kubota; Model: 6930). The resultant supernatant of sponge homogenates were used to estimate the total protein content by spectrophotometer (SpectraMAX Plus, Molecular Devices; Model: S/NP02512) assisted with software, SoftMax Pro ver. 5.0. following the method of Bradford (1976), using bovine serum albumin (BSA) as standard.

Total carbohydrate content was measured using the amended phenol-sulfuric acid method of Taylor (1995), whereas the total lipid content was measured

by using a mixture of chloroform:methanol (1:2) according to the previous method of Brooks et al (1998).

For carbohydrate estimation, 1 g of each test sponge tissue was homogenized with distilled water and the homogenate was taken into a test tube, in which, 100  $\mu$ L of 8% phenol was added. 1 mL of concentrated sulfuric acid was then added rapidly, the steam of acid being directed against the liquid surface rather than against the side of the test tube in order to obtain good mixing. The tubes were thoroughly mixed and later they were placed in a water bath for 10 to 20 minutes at room temperature to obtain yellow orange colored complex. The color was found to be stable for several hours and readings may also be taken later, accordingly. The absorbance was measured at 490 nm for hexoses and 480 nm for pentoses by using UV-Visible Spectrophotometer (Molecular Device, USA; Softmax pro 5.0). Reference standard curve constructed for the particular sugar were used to determine the amounts of carbohydrate present in each sponge species. All solutions were prepared in triplicate to minimize errors resulting from any accidental contamination.

For lipid analysis, 1 g of each experimental sponge tissue was homogenized with 10 mL distilled water and the resultant pulp was transferred to a conical flask containing 30 mL of chloroform-methanol mixture and mixed well. The extraction was left overnight at room temperature in a dark place. After this, equal volumes (20 mL) of chloroform and distilled water were added and the suspension was gently mixed by vortexing and subjected to centrifugation, to obtain three layers. The methanol layer was discarded and the lower layer of chloroform containing all the lipids was carefully collected free of interphase, by sucking out with a fine capillary tube. The organic layer was carefully evaporated by leaving the extraction in warm water (around 50°C) with a flow steam of nitrogen gas on the surface. As some lipids get polymerized or decomposed on exposure to light, heat and oxygen; the sample was covered with a dark paper to protect from light. When the solution was free of organic solvents, the total lipid content was determined and the results are expressed in terms of weight in micrograms of total lipid per gram dry tissue.

### 1.5 Hydroxyproline assay and collagen quantification

Different forms of collagens viz., salt soluble (SS), acid soluble (AS) and insoluble (Ins) collagens were extracted by the modified method of Prockop (1964). The content of hydroxyproline (Hyp) and total collagen was quantified according to the slightly modified methods as described earlier (Pallela et al., 2011; Jamall et al., 1981; Siddiqi and Alhomida, 2003). Briefly, the sponge tissues were cut into small pieces and homogenized in sufficient distilled water to yield 10% homogenate (w/v). Equal volume of 12 N HCl was added to each aliquot (2 mL) of homogenate and hydrolyzed at 105°C for 18 h. Aliquots of 25 µL sponge tissue hydrolyzate, in triplicate, were added to separate vials and subjected to evaporation leading to dryness under vacuum. Sets of samples were spiked with 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.6 µg of standard hydroxyproline (Hyp). To each of the hydrolyzate and Hyp standard vials, 1.2 mL of 50% isopropanol was added, followed by 0.2 mL of 0.56% buffered chloramine-T solution. One ml of Ehrlich's Reagent (ER) solution was then added after an interval of 10 mins. The solutions obtained were mixed and incubated at 50°C for 90 mins. Samples were cooled and the absorbance was measured spectrometrically at 558 nm, using water as reference and corrected for reagent blank. The absolute value of the negative intercept on the X-axis found by linear regression analysis of Hyp standards was taken to represent the Hyp content of the tissue. Quantification of total and fractionated forms (AS, SS and INS) of collagen was performed according to the standardized calculation by Neuman and Logan (1950).

The following formula was used to calculate the collagen content.

Collagen content =  $7.46 \times$  [mg of Hydroxyproline per g freeze-dried sponge weight].

Where, the factor, 7.46 = ratio of the weight of collagen and weight of hydroxyproline

Note: Collagen content is expressed as mg collagen / g freeze-dried sponge weight.

### 1.6 Statistical analyses

All the data in triplicates (n=3) was analyzed using the student's *t*-test to quantify macromolecules and multiple replicates of sponge tissue samples were used for microscopic observations. Statistical significance for all the biochemical analyses was defined as  $P < 0.05$ .

## 2 Results

According to our earlier explorations from the year 2006, there are a total of 40 species were collected in the Mandapam, Palk Bay and Rameswaram regions of Gulf of Mannar (GoM) (Table 1). Depending on the abundance and past pharmacological significance, out of all the collected marine sponges, four experimental sponges *Hyattella cribriformis*, *Fasciospongia cavernosa*, *Callyspongia fibrosa* and *Dysidea fragilis* were compared to bring out their ultrastructural and biochemical analysis in the current studies.

While collecting, the sponges *Hyattella cribriformis*, *Fasciospongia cavernosa* and *Dysidea fragilis* were found to be comparatively more fragile and easy to be ripped off. A possible reason for the fragile nature of these sponges could be due to the lesser content of collagen or spongin in the matrix owing to less amount of strength in the skeletal network, differentiating them from *Callyspongia fibrosa*, where the situation is in contrary and they possess greater amount of collagen matrix. The morphotypic features and key characters along with the whole mount images of these sponges at the time of collection are presented in the Table 2. The specimen of *Hyattella cribriformis* is brittle and lightweight, yellowish-white or pale in color. This sponge is available at around 10 to 15 f from the seashore. Another sponge, *Dysidea fragilis*, is available at the depth of 15 to 20 f. Morphologically, this species appears as a mass of greenish black to yellow color. Yet another member of Demospongiae, but belonging to a different order family Callyspongiidae, *Callyspongia fibrosa*, is composed of finger shaped or flattened branches, surface is with strong conules and has prominent conules at growing tips. The osculae irregularly distributed, terminal, marginal, rounded or elliptical, shallow and compound. They appear to be pale yellow to brown when alive. Dermal skeleton is reticulate, meshes triangular or subdivided by tertiary fibres, which are either uni- or multispicular.

### 2.1 Microscopic Study

#### Video microscopy

The four sponges showed markedly different skeletal alignment and distinctive arrangement of bundle of spicules or oxeae along with the network of spongin, when observed under video microscope (Figure 1). In these sponges, the major canals are strengthened with

Table 1 List of Demosponges collected at Gulf of Mannar (GoM), India from the year 2006-2009




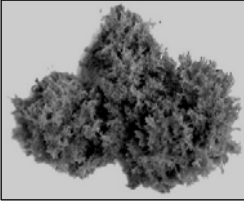
S. No.	Order	Family	Name of the Demosponge*
1	Agelasida	Agelasidae	<i>Agelas mauritiana</i> (Carter, 1883)
2	Astrophorida	Ancorinidae	<i>Rhabdastrella globostellata</i> (Carter, 1883)
3	Dendroceratida	Darwinellidae	<i>Spongionella nigra</i> (Dendy, 1889)
4	Dictyoceratida	Dysideidae	<i>Dysidea fragilis</i> (Montagu, 1818)
5			<i>Lamellodysidea herbacea</i> (Keller, 1889)
6		Irciniidae	<i>Ircinia fusca</i> (Carter, 1880)
7		Spongiidae	<i>Spongia officinalis</i> (Linnaeus, 1759)
8			<i>Hyattella cribriformis</i> (Hyatt, 1877)
9		Thorectidae	<i>Fasciospongia cavernosa</i> (Schmidt, 1862)
10			<i>Lendenfeldia chondrodes</i> (De Laubenfels, 1954)
11			<i>Hyrtios erectus</i> (Keller, 1889)
12	Hadromerida	Clionaidae	<i>Sphaciospongia vagabunda</i> var. <i>trincomaliensis</i> (Ridley, 1884)
13			<i>Sphaciospongia inconstans</i> (Dendy, 1887)
14	Haplosclerida	Callyspongiidae	<i>Haliclona (Gellius) cellaria</i> (Rao, 1941)
15			<i>Callyspongia (Cladochalina) diffusa</i> (Ridley, 1884)
16			<i>Callyspongia (Cladochalina) fibrosa</i> (Ridley & Dendy, 1886)
17		Chalinidae	<i>Haliclona (Reniera) cribricutis</i> (Dendy, 1922)
18			<i>Haliclona (Gellius) fibulata</i> (Schmidt, 1862)
19			<i>Haliclona pigmentifera</i> (Dendy, 1905)
20			<i>Haliclona tenuiramosa</i> (Burton, 1930)
21			<i>Gelliodes carnosa</i> (Dendy, 1889)
22			<i>Gelliodes pumila</i> (Lendenfeld, 1887)
23		Petrosiidae	<i>Neopetrosia similis</i> (Ridley & Dendy, 1886)
24			<i>Petrosia (Petrosia) nigricans</i> (Lindgren, 1897)
25			<i>Xestospongia testudinaria</i> (Lamarck, 1815)
26	Halichondrida	Axinellidae	<i>Axinella donnani</i> (Bowerbank, 1873)
27			<i>Dragmacidon agariciforme</i> (Dendy, 1905)
28			<i>Phycopsis</i> sp. (Carter, 1883)
29		Dictyonellidae	<i>Stylissa carteri</i> (Dendy, 1889)
30		Heteroxyidae	<i>Myrmekioderma granulatatum</i> (Esper, 1794)
31	Poecilosclerida	Chondropsidae	<i>Chondropsis lamella</i> (Vacelet, Vasseur & Lévi, 1976)
32		Iotrochotidae	<i>Iotrochota baculifera</i> (Ridley, 1884)
33		Microcionidae	<i>Clathria (Thalysias) reinwardti</i> (Vosmaer, 1880)
34			<i>Clathria (Thalysias)</i> sp. (Duchassaing & Michelotti, 1864)
35		Mycalidae	<i>Mycale (Mycale) grandis</i> (Gray, 1867)
36		Myxillidae	<i>Myxilla (Ectyomyxilla) arenaria</i> (Dendy, 1905)
37		Raspailiidae	<i>Echinodictyum clathratum</i> (Dendy, 1905)
38		Tedaniidae	<i>Tedania (Tedania) anhelans</i> (Vio in Olivi, 1792)
39	Spirophorida	Tetillidae	<i>Cinachyrella tarentina</i> (Pulitzer-Finali, 1983)
40	Verongida	Pseudoceratinidae	<i>Pseudoceratina purpurea</i> (Carter, 1880)

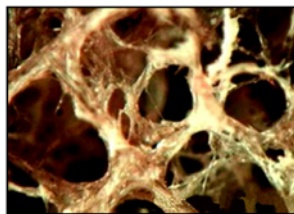
Note: \*According to the latest nomenclature of World Register of Marine Species (WoRMS) and World Porifera Database (WPD)

spicules or detritus material embedded in collagen (spongin) fibers to perform the same functional role. Collagen is an important extracellular matrix (ECM) component in binding spicules and other amorphous material to increase the structural rigidity of the mesohyl. The canals in *Callyspongia fibrosa* were surrounded by

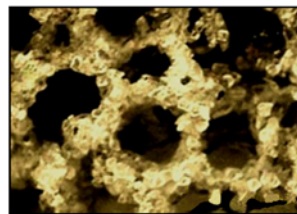
very thick and broad walls consisting of a collagen matrix embedded with solid particles, being either spicules or incorporated foreign matter. The ECM of *Hyattella cribriformis*, *Fasciospongia cavernosa* and *Dysidea fragilis* possesses bigger cavities with minute detritus material embedded in the spongin network.

Table 2 Identification and key characteristic features of the four experimental marine Demosponges

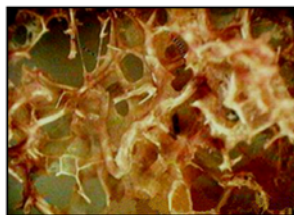
Gross Image	Species Name and Classification	Key Characteristic Features
	<b><i>Fasciospongia cavernosa</i></b> Phylum: Porifera Class: Demospongiae Order: Dictyoceratida Family: Thorectidae	Massive, keratose sponge with peduncles. Conulose surface is in dark green to purple brown and internally grayish yellow. Some forms resemble <i>Ircinia species</i> even in the symbiotic hostage to many organisms. Body is of fleshy consistency.
	<b><i>Callyspongia (Cladochalina) fibrosa</i></b> Phylum: Porifera Class: Demospongiae Order: Haplosclerida Family: Callyspongiidae	Irregular, ramose sponge with cobweb-like uneven surface appears in brownish purple to pale yellow. Sub-cylindrical branches and prominent osculate appear most of the forms. Body consistency and texture is hard and brittle.
	<b><i>Hyattella cribriformis</i></b> Phylum: Porifera Class: Demospongiae Order: Dictyoceratida Family: Spongiidae	Growth form tubular, erect and repent. Flat encrustations of the body, without any proper shape due to repeated folds. Externally pale yellow to orange brown or brownish yellow to green. Body texture is hard and little compressible.
	<b><i>Dysidea fragilis</i></b> Phylum: Porifera Class: Demospongiae Order: Dictyoceratida Family: Dysideidae	Cobweb-like surface formed by huge number of spicules and sand particles, irregularly encrusting or massive and lobe-shaped. Consistency is very brittle to hold and externally appears pale pinkish to pale yellow or brownish to grayish-white.



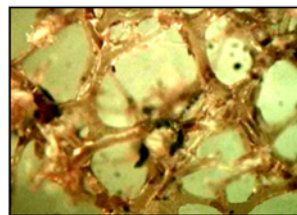
*Callyspongia fibrosa*



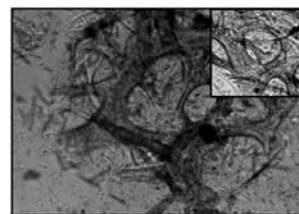
*Dysidea fragilis*



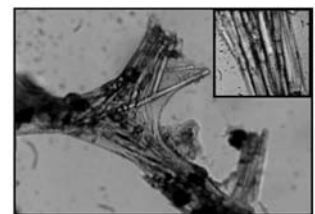
*Fasciospongia cavernosa*



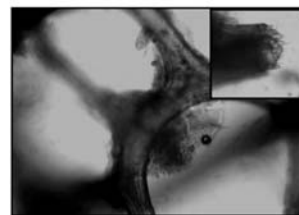
*Hyattella cribriformis*



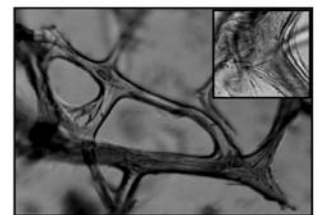
*Callyspongia fibrosa*



*Dysidea fragilis*



*Fasciospongia cavernosa*



*Hyattella cribriformis*

Figure 1 Video micrographs of tangential sections of four marine sponges

Note: Image magnification at 220× for *F. cavernosa* and at 300× for *C. fibrosa*, *H. cribriformis* and *D. fragilis*

### Light Microscopy

The sponge skeletal matrix was observed under light microscope to evaluate the specular arrangement and the branching pattern of each sponge species (Figure 2).

Figure 2 Light micrographs of the spongin network of four experimental sponges (magnification 100×)

Note: Insets representing the high magnification (330×) images

Sponge skeletal network is formed by the intervening of spongin fibers, spicules and some amorphous material to give a unique distribution of the spongin frames. These spongin frames are of different shapes

due to the multiple interventions of the spongin fibers in regular as well as irregular fashion. Among the four sponges, the skeletal arrangement was almost similar for *F. cavernosa* and *H. cribriformis*, whose skeletal network was formed to be branched in multiple directions. The terminal portion of the spongin fiber in *F. cavernosa* (Inset) is highlighted to expose the bunch of oxeae and collagen packed by an outer cuticular layer. The junctional region of the spongin network in *H. cribriformis* (Inset) is covered with a membrane like material. Ramifying spongin fibers were seen in *C. fibrosa*, where native spongin network was observed to be thicker and internal striations of the spongin were clearly visible at high magnification (Inset). The spongin network observed in *D. fragilis* was unique in possessing multiple types of fibres loaded with sand grains, broken spicules and other foreign material covered by a very thin transparent layer of amorphous material, softly holding the bunch of parallelly arranged detritus material (Inset) with the support of tiny collagenous material.

### Scanning electron microscopy

Ultrastructural orientation of the differential skeletal make up and varied distribution of spongin in extra cellular matrix of four experimental marine sponges was analyzed by Scanning Electron Microscopy (SEM) (Figure 3-6). SEM analyses depict the branching and pattern of spongin network and the lattice distribution of silicon layers cemented around the interlacing skeletal fibers. At higher magnifications, *C. fibrosa* exhibited a distinct skeleton formed of thick walls of silicon based sheets layered along with the spongin and uniform sized spicules (Figure 3). The peeled off, fragmented material at the broken portions of the tissue seem to appear as collagenous layers packed at different densities. On the other hand, SEM analysis of *D. fragilis* depicted a number of spacious cavities called atria, formed of bundles of different variety of material composed of detritus and fragmented spicules intercalated with amorphous siliceous material rather than much fibrous protein (Figure 4).

It is clearly observed from the SEM results that carpets of amorphous material are seen much in the case of *F. cavernosa* (Figure 5). The skeleton found to be majorly formed of large spongin fibers supported

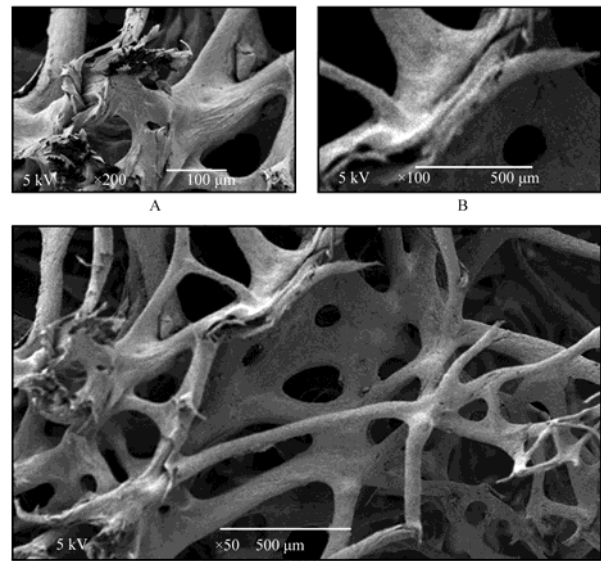


Figure 3 Scanning electron micrographs of *Callyspongia fibrosa* showing the much interlacing skeletal network. A) Disrupted spongin network exposing the collagenous form of terminal portions and B) Enlarged view of spongin network showing peeled off amorphous layer

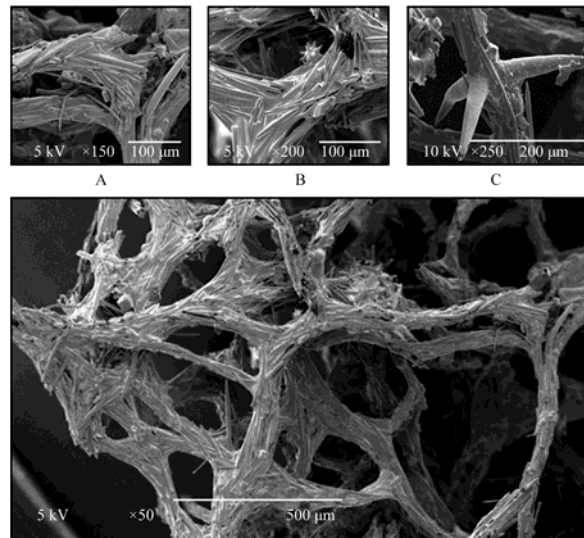


Figure 4 Scanning electron micrographs of *Dysidea fragilis*. A) Enlarged view of skeletal branches formed by high density of detritus material; B) Enlarged view of a branch of skeleton formed of diversified spiraster like material and C) Image showing a variety of large spiraster

by the proteinaceous sheets of extracellular material, mainly impregnated with spicule like fragments intercalated with collagen fibrils passing through the sponge atria. Although the cellular (pinacocytes and choanocytes) appearance in the experimental sponge sections was not remarkable because of the processing

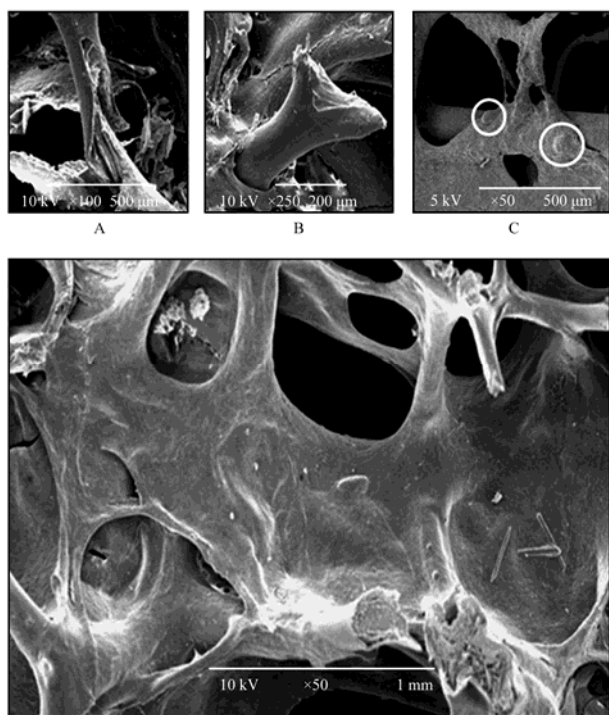


Figure 5 Scanning electron micrographs of *Fasciospongia cavernosa*. A) Broken junctions of spongin network exposing the layers of spongin; B) Enlarged view of broken spongin network engulfed by laminated silicon cement and C) Amorphous laminated silicon material spread as a mesh or thin membrane (circles indicate the appearance of sponge cells)

of the sponge tissue towards the spongin analysis, certain sponge cells appear in the section of *F. cavernosa* (Figure 5 C). In *H. cribriformis*, the skeletal network was characterized by typical spongin network rather than free spongin fibers or collagen like filaments, and the multi-junctional regions of the network were overlapped with layers of silicon and amorphous cuticular material (Figure 6). *H. cribriformis* contains fibrillar extensions at the terminal fragmented portions in the skeletal network, indicating that, silicon cementing around the spongin fibers is very important in order to form the fibrillar extensions throughout the skeleton of sponge body (Figure 6 C).

## 2.2 Biochemical analysis

All the results for macromolecular analysis are reported

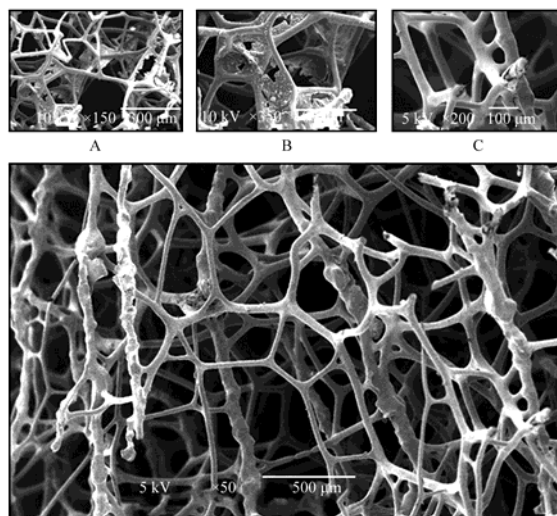


Figure 6 Scanning electron micrographs of *Hyattella cribriformis*. A) Skeletal junctions filled with amorphous material; B) Sheets of the collagenous and amorphous cuticular material disrupted at the junctional regions of the skeletal network and C) Enlarged view of broken skeletal network exposing the spongin fibrils and the peeled off layer at the junctions

on freeze-dried weight basis (Table 3). *C. fibrosa* have high protein content ( $209.6 \pm 9.53$  mg/g freeze dried sponge wt) followed by *H. cribriformis* ( $162.72 \pm 8.90$  mg/g) than other experimental sponge species. It could be a possible indication that the high protein content dominated the other macromolecular content like carbohydrates (pentoses and hexoses) and lipids in these two sponges. It is very clear that sponge *D. fragilis* has less protein content ( $57.26 \pm 3.36$  mg/g) when compared to other sponges. Regarding the carbohydrate content, it is observed that *C. fibrosa* and *H. cribriformis* possess relatively high content of pentose and hexose than the other two sponges. Interestingly, although the pentose content is slightly varied in both the species of *D. fragilis* and *F. cavernosa*, the hexose content ( $9.6 \pm 0.5$  mg/g) is similar in each species. Similarly, total lipid content of *C. fibrosa* and *H. cribriformis* is proportionately higher among the four experimental sponges ( $21.2 \pm 1.1$  and  $19.5 \pm 0.9$  mg/g, respectively).

Table 3 Macromolecular comparison of four marine demosponges

Sponge Species	Carbohydrate		Protein	Lipid	Differential Collagen			Total Collagen
	Hexose	Pentose			SS	AS	Ins	
<i>C. fibrosa</i>	14.9±0.7	24.1±1.2	209.6 ±9.5	21.2 ±1.1	29.7±1.4	27.8±1.3	37.9±2.8	160.2±8.0
<i>D. fragilis</i>	9.6 ±0.5	15.8±0.8	57.3 ±3.4	10.2 ±0.7	16.5±0.8	17.8±0.9	34.8±1.7	44.8±2.2
<i>F. cavernosa</i>	9.6 ±0.5	16.1± 0.8	101.6±5.1	16.4±0.8	18.2±0.9	14.8±0.7	42.8±2.1	80.2±4.0
<i>H. cribriformis</i>	13.5±0.7	24.9±1.3	156.4 ±7.8	19.5 ±0.9	19.0±1.1	17.8±0.9	35.0±1.8	127.7±6.4

Note: All the data expressed as Mean ± SE of each biochemical component in mg / g freeze-dried sponge wt



### 2.3 Hydroxyproline and collagen content

Estimation of the amino acid, hydroxyproline (Hyp) is diagnostic of collagens in any organism. According to our preliminary explorations of Hyp content in all the collected sponges, six sponges along with *I. fusca* and *S. officinalis* possess high amount of Hyp than the remaining sponges and the differences between these selected sponges in their Hyp content was analyzed by spectrophotometric method. However, specific important has been given to *I. fusca* and *S. officinalis* to study separately to emphasize on the collagenous network; and four sponges considered for ultrastructural and biochemical comparison in the present study. Collagen content (based on Hyp concentration) significantly differed in all the four experimental sponges. The sponge, *C. fibrosa* seems to possess highest collagen content ( $169.19 \pm 9.12$  mg/g freeze-dried sponge wt) among all the four sponges (Table 3).

The solubility of collagens is a factor of extracting different forms of collagen viz., salt soluble (SS), acid soluble (AS) and Insoluble (Ins). It is clear that the AS and SS collagen content is not much varied but the content of Ins collagen differed much among the four sponges (Table 3). In all the sponges, the insoluble (Ins) form of the collagen was more than the amount of SS and AS forms of the collagen. Although the total collagen content of *F. cavernosa* is less than *C. fibrosa* and *H. cribriformis*, the collagen (Ins) is higher ( $42.8 \pm 2.1$  mg/g) when compared to other sponges. However, the AS and SS collagen of *C. fibrosa* is higher among all the experimental sponges.

### 3 Discussion

This is the first extensive work on the skeletal architecture, isolation and characterization of collagens from Demosponges available at Gulf of Mannar (GoM) India. The GoM falls in the Indo-Pacific region, which is considered one of the world's richest in marine biological resources. It has more than 3600 species of plants and animals that make it the biologically richest coastal region in India (Kumaraguru et al., 2006).

Although the collected sponge species are localized to specific habitat locations of GoM, there is much variation in the morphology of each species due to the seasonal changes and habitat disturbances by the local trawlers. Since our studies mainly focus on the highly important protein molecules like collagen, *Ircinia*

*fusca* was previously been well explored based on the remarkable collagen like fiber content (Pallela et al., 2011). While the general structural features of sponges are visible through video microscopy, a more elaborated picture on the presence of collagen was obtained through electron microscopy. Gross and Diehl-Seifert were the first to prove the existence of collagen in marine as well as in freshwater sponges, electron microscopically (Gross et al., 1956). Furthermore, the orientation of spongin along with the fine fibrillar material embedded in extra cellular matrix of sponges is a major feature which forms the basis of differentiation for the marine sponges. Cowden (1970) has histologically proven the collagen occurrence in marine sponge tissue. Besides this, spongin skeleton formed of fine fibrils of collagen interwoven along with other connective tissue components like spicules and particulate matter could be observed through SEM.

The macromolecules are the major constituents of different forms of life and form the basis to determine the structure of these life forms, to a certain extent. The macromolecular content of different experimental sponges was estimated to reach to the sponge species with the highest amount of collagen as it is the main structural component. It is well known that sponge tissue protein content dominates other macromolecular content ranging around 40% of the total dry tissue weight (Hadas et al., 2005). Except for hexoses, *C. fibrosa* has shown higher collagen to relative protein, pentose and lipid content than other sponge species. Analysis of sponge biochemical components reveals that not all the organic components were resolved in the methods applied in the present research.

Collagen is the only intercellular organic framework and amounts to approximately 10% of the total organic matter in Demospongiae (Wiens et al., 1999). Genomic and complementary DNA studies showed that proteinaceous fibrous materials (collagen and spongin) contain the classic collagenous Gly-Xaa-Yaa motif (Exposito and Garrone, 1990; Boute et al., 1996), where Hydroxyproline (Hyp) occupies any one of the positions in the triplet motif other than Gly (Glycine) position. Since the ultimate goal of the project is to target the collagens, standard methods of estimating Hyp to quantify collagen from marine sponges was employed. Though it is a differential

extraction for the isolation and estimation of these forms rather the total collagen, the quantification procedures are same to deduce the collagen quantity in all the sponges. As observed in the present study, collagen content is higher in *Callyspongia*, explanation to this may lie in the presence of high collagenous material in the heavily packaged proteinaceous sheets in the sponge tissue. Based on the present data, the potential candidate for the characterization of this collagen and collagenous nature will be verified further by various biophysical and molecular techniques.

The precipitated collagen is completely soluble in the cold acid and salt extraction buffers. However, more percentage of the precipitated collagenous material remains insoluble, which gives the Ins collagen content of the sponge tissue of all the four sponges. It is understood from the present results that the high percentage of the Ins form of the collagen in the sponges are much responsible for the tissue integrity and unique skeletal framework of the individual sponges, this fact may be responsible for the presence of high Ins collagen than AS and SS collagen forms.

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#### References

Aizenberg J., Ilan M., Weiner S., and Addadi L., 1996, Intracrystalline macromolecules are involved in the morphogenesis of calcitic sponge spicules, *Connective Tissue Research*, 34: 255-261  
<http://dx.doi.org/10.3109/03008209609005269>

Boute N., Exposito J.Y., Boury-Esnault N., Vacelet J., Noro N., Miyazaki K., Yoshizato K., and Garrone R., 1996, Type IV collagen in sponges, the missing link in basement membrane ubiquity-, *Biology of the Cell*, 88: 37-44  
[http://dx.doi.org/10.1016/S0248-4900\(97\)86829-3](http://dx.doi.org/10.1016/S0248-4900(97)86829-3)

Bozzola J.J., and Russell L.D., 1999, *Electron microscopy: principles and techniques for biologists* 2<sup>nd</sup> Edn., Jones and Bartlett Publishers, Sudbury, MA, p. 670

Bradford M.M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Analytical Biochemistry*, 72: 248-254  
[http://dx.doi.org/10.1016/0003-2697\(76\)90527-3](http://dx.doi.org/10.1016/0003-2697(76)90527-3)

Brooks S.P.J., Ratnayake W.M.N., Lampi B.J., and Hollywood R., 1998, Measuring total lipid content in -rat carcasses: A comparison of commonly employed extraction methods, *Journal of Agricultural and Food Chemistry*, 46: 4214-4217  
<http://dx.doi.org/10.1021/jf980052w>

Brusca R.C., Brusca G.J., 2003, *Invertebrates*, 2<sup>nd</sup> Edn. Sinauer Associates, Inc., Sunderland, MA, p. 936

Chairman K., Ranjit Singh A.J.A., and Ramesh M., 2012, Screening twelve species of sponges for biomedical activity in Gulf of Mannar Tuticorin coast, *International Journal of Marine Science*, 2: 43-50

<http://dx.doi.org/10.5376/ijms.2012.02.0006>

Cowden R.R., 1970, Connective tissue in six marine sponges: a histological and histochemical study, *Zeitschrift für Mikroskopisch-anatomische Forschung*, 82: 557-569

Ereskovsky A.V., Konyukov P.Y., and Tokina D.B., 2010, Morphogenesis accompanying larval metamorphosis in *Plakina trilopha* (Porifera, Homoscleromorpha), *Zoomorphology*, 129: 21-31  
<http://dx.doi.org/10.1007/s00435-009-0097-5>

Exposito J.Y., and Garrone R., 1990, Characterization of a fibrillar collagen gene in sponges reveals the early evolutionary appearance of two collagen gene families, *Proceedings of the National Academy of Sciences of the United States of America*, 87: 6669-6673  
<http://dx.doi.org/10.1073/pnas.87.17.6669>

Fernández-Busquets X., 2008, The sponge as a model of cellular recognition. In: P. Michael Conn (Ed.), *Sourcebook of Models for Biomedical Research*, Humana Press Inc., Totowa, NJ, pp. 75-83

Garrone R., 1985, The collagen of the Porifera. In: Bairati A., and Garrone R., (Eds.), *Biology of Invertebrate and Lower Vertebrate Collagens*. Plenum Press, London, pp. 157-175.  
[http://dx.doi.org/10.1007/978-1-4684-7636-1\\_12](http://dx.doi.org/10.1007/978-1-4684-7636-1_12)

Garrone R., 1999, Collagen, a common thread in extracellular matrix evolution, *Journal of Chemical Sciences*, 111: 51-56

Gross J., Sokal Z., and Rougvie M., 1956, Structural and chemical studies on the connective tissue of marine sponges, *Journal of Histochemistry and Cytochemistry*, 4: 227-246  
<http://dx.doi.org/10.1177/4.3.227>

Hadas E., Shpigel M., and Ilan M., 2005, Sea ranching of the marine sponge *Negombata magnifica* (Demospongiae, Latrunculiidae) as a first step for latrunculin B mass production, *Aquaculture*, 244: 159-169  
<http://dx.doi.org/10.1016/j.aquaculture.2004.11.052>

Jamall I.S., Finelli V.N., and Que Hee S.S., 1981, A simple method to determine nanogram levels of 4-hydroxyproline in biological tissues, *Analytical Biochemistry*, 112: 70-75  
[http://dx.doi.org/10.1016/0003-2697\(81\)90261-X](http://dx.doi.org/10.1016/0003-2697(81)90261-X)

Kozioł C., Borojevic R., Steffen R., and Müller W.E.G., 1998, Sponges (Porifera) model systems to study the shift from immortal to senescent somatic cells: the telomerase activity in somatic cells, *Mechanisms of Ageing and Development*, 100: 107-120  
[http://dx.doi.org/10.1016/S0047-6374\(97\)00120-6](http://dx.doi.org/10.1016/S0047-6374(97)00120-6)

Kumaraguru A.K., Joseph V.E., Marimuthu N., and Wilson J.J., 2006, Scientific information on Gulf of Mannar - A bibliography, Centre for Marine and Coastal Studies, Madurai Kamaraj University, Madurai, Tamilnadu, India, pp. 655

Müller W.E.G., Müller I.M., and Schröder H.C., 2006, Evolutionary relationship of Porifera within the eukaryotes, *Hydrobiologia*, 568: 167-176  
<http://dx.doi.org/10.1007/s10750-006-0318-6>

Neuman R.E., and Logan M.A., 1950, The determination of collagen and elastin in tissues, *Journal of Biological Chemistry*, 186: 549-556

Pallela R., Bojja S., and Janapala V.R., 2011, Biochemical and biophysical characterization of collagens of marine sponge, *Ircinia fusca* (Porifera: Demospongiae: Irciniidae), *International Journal of Biological Macromolecules*, 49: 85-92  
<http://dx.doi.org/10.1016/j.ijbiomac.2011.03.019>

Pallela R., Venkatesan J., Janapala V.R., and Kim S.K., 2012, Biophisicochemical evaluation of chitosan-hydroxyapatite-marine sponge collagen composite for bone tissue engineering, *Journal of Biomedical Materials Research Part A*, 100A: 486-495  
<http://dx.doi.org/10.1002/jbm.a.33292>

Prockop D.J., 1964, Isotopic studies on collagen degradation and the urine excretion of hydroxyproline, *Journal of Clinical Investigation*, 43: 453-460

Rao J.V., Pallela R., and Prakash G.V.S.B., 2011, Prospects of marine sponge collagen and its applications in cosmetology. In: Kim S.K (Ed.), *Marine Cosmeceuticals: Trends and Prospects*: 77-103

Siddiqi N.J., and Alhomida A.S., 2003, Hydroxyproline concentrations in ocular tissues of Arabian camel (*Camelus dromedarius* Linn.), *Indian Journal of Biochemistry and Biophysics*, 40: 451-454

- Swatschek D., Schatton W., Kellermann J., Müller W.E.G., and Kreuter J., 2002, Marine sponge collagen: isolation, characterization and effects on the skin parameters surface-pH, moisture and sebum, European Journal of Pharmaceutics and Biopharmaceutics, 53: 107-113  
[http://dx.doi.org/10.1016/S0939-6411\(01\)00192-8](http://dx.doi.org/10.1016/S0939-6411(01)00192-8)
- Taylor K.A.C.C., 1995, A modification of the phenol/sulfuric acid assay for total carbohydrates giving more comparable absorbances, Applied Biochemistry and Biotechnology, 53: 207-214  
<http://dx.doi.org/10.1007/BF02783496>
- Van Soest R.W.M., Boury-Esnault N., Vacelet J., Dohrmann M., Erpenbeck D., De Voogd N.J., Santodomingo N., Vanhoorne B., Kelly M., and Hooper J.N.A., 2012, Global diversity of sponges (Porifera), PLoS ONE, 7: e35105  
<http://dx.doi.org/10.1371/journal.pone.0035105>
- Venkataraman K., and Wafar M., 2005, Coastal and marine biodiversity of India, Indian journal of marine sciences, 34: 57-75
- Wiens M., Koziol C., Batel R., and Müller W.E.G., 1999, Prolidase in the marine sponge *Suberites domuncula*: Enzyme activity, molecular cloning, and -phylogenetic relationship, Marine Biotechnology, 1: 191-199  
<http://dx.doi.org/10.1007/PL00011767>