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# Analyses of Methanol Extracts of Two Marine Sponges, *Spongia officinalis var. ceylonensis* and *Sigmadocia carnosa* from Southwest Coast of India for their Bioactivities

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**A B S T R A C T**

Marine sponges proved to be the richest source of bioactive compounds . In this study, methanol extracts of two marine sponges, *Sigmadocia carnosa* and *Spongia officinalis var. ceylonensis* were examined for their various bioactivities. The chemical constituents of these extracts were analyzed by routine phytochemical methods showed the presence of alkaloids, phenols, steroids, triterpenoids, reducing sugars and aromatic acids in both the sponge extracts. Antioxidant activity assayed using DPPH radical scavenging activity and total antioxidant activity indicated that both sponge extracts possessed antioxidant activity. Immunomodulatory activity analyzed by calculating phagocytic index and Nitro Blue tetrazolium assay indicated that the extract of Sigmadocia was more immunomodulatory. Acetylcholinesterase inhibition assay revealed that Sigmadocia extract was more active. Anticancer effect of the methanolic extracts on colon cancer cell line HT-29 was confirmed by MTT assay. Thus the biomedical potential of the two species has been confirmed and further purification, bioactivity evaluation and chemical investigation of these extracts will yield potential bioactive molecules.

## Introduction

Marine environment is a great reservoir of novel compounds with pharmacological relevance. Among 36 known living phyla, 34 of them are found in marine environment (Dhinakaran *et al*., 2014). Of these diverse marine organisms, marine sponges are the prolific source of bioactive molecules with novel chemical structure. Sponges- the most primitive sessile filter feeders - produce these compounds as secondary metabolites as part of chemical defense against predators, space competitors and fouling

since they lack physical defense mechanisms. They produce a wide array of secondary metabolites ranging from derivatives of aminoacids and nucleosides to macrolides, porphyrins, terpenoids, aliphatic cyclic peroxides and sterols (Joseph & Sujatha, 2011). According to the composition of the skeleton of the sponges, they are mainly divided into three main classes namely, Calcarea, glass sponges (Hexactinellidae) and Demosponges. Demosponges have been reported to possess large number of bioactive compounds with pharmaceutical relevance.

Bioactive compounds from sponges have been reported to possess an array of activity including anti-inflammatory, anticancer, immunosuppressive, antiviral, antibacterial and antifouling activities. In the last decade, most number of new compounds with bioactive potential has been isolated from marine sponges (Mehbub *et al*., 2014). At present there are a number of compounds from marine sponges which are under investigation and/ or are being developed as new pharmaceuticals.

The peninsular coast of India is a hotspot of diverse marine floral and faunal assemblages particularly sponges, sea anemones, sea cucumber, sea urchin, soft corals and diverse number of sea weeds (Sathiyanarayanan *et al.,* 2014). In this study bioactive property of the extracts from two marine sponges of the class Demospongiae- *Spongia officinalis var. ceylonensis* and *Sigmadocia carnosa* collected from Southwest coast of India has been investigated.

*Spongia officinalis var. ceylonensis* confined only to the Indian seas is massive, subglobular, subpyriform in appearance with surface projections. The sponge belongs to the order Keratosida which is unique in having only spongin fibres in its skeleton. The second species *Sigmadocia carnosa* belong to the order Haplosclerida having a skeleton of hydrated silica and spongin. This species bears small tubular processes bearing terminal oscules. There were reports on the antibacterial, antifungal, insecticidal and larvicidal activities of the extracts of both the species indicating that the organic extracts of these species exhibited good activity (Sujatha & Joseph, 2011; Lakshmi & Shukla, 2014).

## Materials and Methods

### Sample Collection

The marine sponge samples were collected from Southwest coast of India with the help of SCUBA divers at depth of 8-10 feet and were identified as *Spongia officinalis var. ceylonensis* and *Sigmadocia carnosa* with the help of Dr. P. A Thomas, Retd. Principal Scientist, CMFRI, Vizhinjam. The samples were kept in sterile plastic bags and transferred to lab and stored at -20°C.

### Organic Extraction

Extraction was carried out using methanol as solvent by some modification of method of Sepcic *et al.,* 2010. The sponge samples were lyophilized and then 1g of sponge tissue was soaked in 30ml of methanol in sealed container and was shaken overnight at 37°C. The extracts were filtered and remaining material was repeatedly extracted for three days at 37°C with constant shaking. The solvent was evaporated and the extracts were dissolved in DMSO.

### Screening for Chemical Constituents

Preliminary screening for chemical constituents in the extracts was performed with slight modifications of the method of Harborne (1998).

## Antioxidant Activity

### Total Antioxidant Activity Assay

The total antioxidant activities of the extracts were evaluated by the phosphomolybdenum method (Prieto *et al*., 1999). This assay is based on the reduction of Mo (VI) to Mo(V) by the antioxidant compound and the subsequent formation of a green phosphate / Mo(V) complex at acid pH. A 0.3 mL extract solution was dispensed into screw capped test tubes. A 3mL reagent solution (6M $H_2SO_4$, 28mM sodium phosphate, 4mM ammonium molybdate) was added and the tubes were capped and incubated at 95°C for 90 minutes. After cooling to room temperature, the absorbance was measured at 695nm using a spectrophotometer. A blank test was done using the solvent used. The antioxidant activity was expressed as ascorbic acid equivalents using ascorbic acid as reference standard.

### DPPH Radical Scavenging Activity Test

The stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined using the method of Mensor *et al*., 2001. The samples and the reference were mixed with DPPH solution. Remaining DPPH amount was measured at 517nm using spectrophotometer. Ascorbic acid was employed as the reference. Inhibition of DPPH in percentage was calculated as given below:

I%= [(A blank- A sample)/ A blank] ×100, where A blank is the absorbance of the control reaction and A sample is the absorbance of the extracts.

## Immunomodulatory Activity

### Phagocytic Activity Assay

Immunomodulatory activity was analysed by a slide method (Wadekkar *et al*., 2008) through *in vitro* phagocytosis of *Saccharomyces cerevisiae*. This assay was performed using blood from healthy donors and placing 100µl of it into each glass slide. The slide was incubated for the cells to get attached and then the clot was removed and the slide was flood with test sample and incubated for half an hour. The slide was then drained and flooded with a suspension of heat killed opsonised yeast cells and incubated for 1 hour. After draining the yeast suspension, slides were fixed in absolute ethanol. Then it was stained with Giemsa stain.

The phagocytic index was calculated as the number of yeast cells phagocytosed by 100 granulocytes and the percentage of phagocytosis was calculated using the equation [PI (test)-PI (control)/PI (control) ×100].

### NBT Assay

The assay was performed with a modification of the method of Weiss *et al*., 1998. For the assay, $1x10^4$ leucocytes/ ml were incubated with 0.1% Nitro Blue Tetrazolium dye in phosphate buffered saline (pH-7.2) and the test samples for 30 minutes at 37°C with 5% $CO_2$. A set of cells treated with crude filtrate of *E.coli* broth culture was used as a positive control. After incubation, a drop from each sample was carefully transferred onto a grease free glass slide and a coverslip smear was made. The smear was allowed to dry and fixed with ethanol for three minutes. Then the slides were stained with saffranin for three minutes, air dried and observed under 40X objective for calculating percentage of neutrophils with formazan granules.

### Acetylcholinesterase Inhibition Assay

The acetylcholinesterase assay was

performed according to Sepcic *et al*., 2010. Briefly AChE from electric eel (Sigma, USA), was dissolved in 100mM phosphate buffer (pH7.3) to achieve 500EU/mL. Prior to the test, the enzyme was 100 fold diluted in the same buffer. To each microtiter plate well 140µL of the Ellman reagent (5,5-dithiobis-2-nitrobenzoic acid) in 25mM phosphate buffer (pH 7.0), 10µL acetylcholine (ACh) in1mM final concentration, 20µL of sponge sample (aqueous or organic), and finally 50µL of AChE was added to start the reaction. Deionized (20µl) water or ethanol (20µl) was used as controls. The time course of the enzymatic reaction was monitored for 12 minutes at 412 nm at 25°C.

## MTT Assay

Ht-29 colon cancer cell lines were purchased from NCCS Pune were maintained in Dulbecco's modified eagles media supplemented with 10% FBS and grown to confluency at 37°C in 5 % $CO_2$ in a humidified atmosphere in a $CO_2$ incubator. The cells were trypsinized (500µl of 0.025% Trypsin in PBS/ 0.5mM EDTA solution for 2 minutes) and passaged to T flasks in complete aseptic conditions. Extracts were added to grown cells at a final concentration of 6.25µg/ml, 12.5µg/ml, 25 µg/ml, 50 µg/ml and 100µg/ml from a stock of 1mg/ml and incubated for 24 hours. The % difference in viability was determined by standard MTT assay after 24 hours of incubation. The cells was washed with 1x PBS and then added 30 µl of MTT solution to the culture (MTT -5mg/ml dissolved in PBS). It was then incubated at $37^0$C for 3 hours. MTT was removed by washing with 1x PBS and 200µlof DMSO was added to the culture. Incubation was done at room temperature for 30 minutes until the cell got lysed and colour was obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2 minutes to precipitate cell debris. Optical density was read at 540 nm using DMSO as blank in a microplate reader. % inhibition = [(OD of Control - OD of Test)/OD of Control] X 100

## Statistical Analysis

The experiments were done in triplicates and error bars in graphs represents standard error of means per triplicate samples. The results were analyzed by Student's t test and were considered statistically significant when $p<0.05$. IC 50 values were calculated in MS Excel.

## Results and Discussion

Marine sponges are a rich source of structurally unique natural compounds, several of which have shown a wide variety of biological activities (De Rosa *et al*., 2003). The concentration of many highly active compounds in marine invertebrates are often minute, accounting for less than a millionth of wet weight (Proksch *et al*., 2002). Excellent drug candidates from sponges are often not well developed because the sponges are difficult to collect and their extraction and purification of active compound is very challenging. The sponges are naturally in close association with a variety of microorganisms and this symbiotic relation provides a rich source of biologically active secondary metabolites.

## Identification of Samples

The collected samples were identified as *Spongia officinalis* var. *ceylonensis* (Fig 1A) and *Sigmadocia carnosa* (Fig 1B) with the help of Dr. P.A. Thomas, Retd. Principal Scientist, CMFRI, Vizhinjam, Kerala. The species *Spongia* used in this study is the widely used commercial bath sponge. In addition to this, *Spongia officinalis* has

medicinal uses. It has been used as medicine in Israel, Syria and Lebanon and also for treatment of dry and asthmatic cough in Western world (Lev, 2003; Sipkema *et al*., 2005).

## Screening of Chemical Constituents (Table 1)

Triterpenoids , aromatic acids, phenolics, steroids and reducing sugars were present in the extracts of the two sponge species. All these chemical constituents singly or in combination contribute to the bioactivities exhibited by the extracts. There were reports of steroids and alkaloids from methanolic extract of *Spongia officinalis* (Thale *et al.*, 2002; Lakshmi & Ghoshal, 2014). Kohamaic acids A and B are known to be constituents of Ircinia species from Okinawa (Kokubo *et al.*, 2001). Triterpenoids such as stelletins, sodwanones, raspascionins, and sipholanols were isolated from various sponge species such as *Stellata tenuis, Axinella wltneri, Raspasciona aculata and Siphonochalina siphonella* respectively. All these triterpenoids exhibited cytotoxic and anticancer activity (Cimino *et al*., 1994). Cytotoxic activity of triterpenoids from various marine sponges has been reported (Li *et al*., 2013).

## Antioxidant Activity

Antioxidant assays revealed that organic extracts of both the species possessed significant activity.

## Total Antioxidant Activity Assay

This assay is employed for quantitative determination of antioxidant capacity through the formation of phosphomolybdenum complex. The results are expressed as mg/g equivalence of ascorbic acid. The results (Table 2) show that methanolic extract of Spongia is more potent than that of Sigmadocia.

## DPPH Radical Scavenging Activity

DPPH is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecule does not dimerize, as would be the case with most other free radicals. The delocalization of electrons also gives rise to deep violet colour and absorption is measured at 517nm. This assay was performed for different concentrations (62.5µg/ml, 125µg/ml, 250µg/ml, 500 µg/ml and 1000µg/ml) of the extracts. Both the extracts exhibited significant percentage inhibition (Fig 2). IC50 values (Table 2) were calculated and it showed that *Sigmadocia* has very low IC50 which indicates its higher antioxidant capability.

Total antioxidant activity was more for methanol extract of Spongia than for Sigmadocia. But DPPH scavenging activity was more pronounced in methanol extract of Sigmadocia. Recent studies reported that the methanolic extract of *Sigmadocia carnosa* exhibited good DPPH radical scavenging activity (Joseph & Kavimani, 2014). Extracts of Mediterranean sea sponges *Dysidea avara*, *Axinella cannabina, Axinella damicornis, Agelas oroides* and *Ircinia fasciculata* exhibited good DPPH activity (Aktas *et al*.2014). Phenolic metabolites from marine sponges have been reported to possess antioxidant activity (Utkina *et al*. 2004).

## Immunomodulatory Activity

Immunomodulatory activities of the extracts were analysed by phagocytosis assay and Nitroblue tetrazolium reduction assay. Assays indicate that both the extracts possess immunostimulatory activity.

## Phagocytic Activity

The phagocytic index (Fig.3 and Table 3) was calculated as the number of yeast cells phagocytosed by 100 granulocytes and the percentage of phagocytosis was calculated using the equation [PI (test)-PI (control)/PI (control) ×100]. The percentage of phagocytosis was greater for Sigmadocia than that of Spongia.

## NBT Assay

Immunomodulatory effects of the extracts were further confirmed by NBT assay. Nitro Blue Tetrazolium is an electron acceptor used to detect indirectly the production of superoxide by stimulated neutrophils. Superoxide reduces the yellow soluble NBT to the blue black formazan, an insoluble material that precipitate which can be seen microscopically within the cell. Both the extracts exhibited significant reduction (Fig 4, 5A and 5B). The results showed that the extract of Spongia is more active.

Both the extracts possessed considerable immunomodulatory activity. Significant immunostimulant activity of methanolic extract of sponge *Sigmadocia pumila* was reported (Dhinakaran *et al*. 2012).

**Table.1** Screening of Chemical Constituents

| Sample | Phytochemicals | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Alkaloid | Triterpenoid | Xanthoprotein | Phenolics | Reducing sugar | Aromatic acids | Steroid | Flavanoids |
| ***Spongia officinalis var. ceylonensis*** | - | + | - | + | + | + | + | - |
| ***Sigmadocia carnosa*** | - | + | - | + | + | + | + | - |

**Table.2** Antioxidant Activity of the Extracts-Total Antioxidant Activity Expressed in Milligram Per Gram Equivalents of Ascorbic Acid. IC 50 of DPPH Radical Scavenging Activity Expressed in Microgram

| Sample | Total antioxidant activity (mg/g equivalents of ascorbic acid) | DPPH radical scavenging activity assay (IC50 value in µg) |
|---|---|---|
| *Spongia officinalis* var. *ceylonensis* Methanol extract | 40.433±0.72188 | 832.56 |
| *Sigmadocia carnosa* Methanol extract | 66.33±3.38296 | 493.49 |

**Table.3** Phagocytic Activity Phagocytic Index and Percentage of Phagocytosis were Calculated and Represented as Mean ± sem ($p<0.05$)

| **ample** | **Phagocytic index** | **%of phagocytosis** |
|---|---|---|
| ***Spongia officinalis var. Ceylonensis*** | 1014.33 | 6.16623±0.15688 |
| ***Sigmadocia carnosa*** | 1078.66 | 10.28±0.88854 |

**Table.4** Acetylcholinesterase Inhibitory Activity. Percentage Inhibition per Triplicate Samples were Calculated and Expressed as Mean ± SEM . The values were Significant by Student's t-test ($p<0.05$). IC 50 values were Expressed in µg

| **Sample** | **% inhibition** | **IC 50** |
|---|---|---|
| *Spongia officinalis* var. *ceylonensis* | 22.63±1.16<br>26.39±2.15686<br>32.83±1.09303<br>40.67±1.20185<br>50.33±0.88192 | 780.25 |
| *Sigmadocia carnosa* | 27.70±0.8952<br>35.16±1.947<br>37.85±1.38378<br>47.56±1.90739<br>58.47±1.95633 | 667.57 |

**Fig.1A** *Spongia Officinalis* Var.*Ceylonensis*

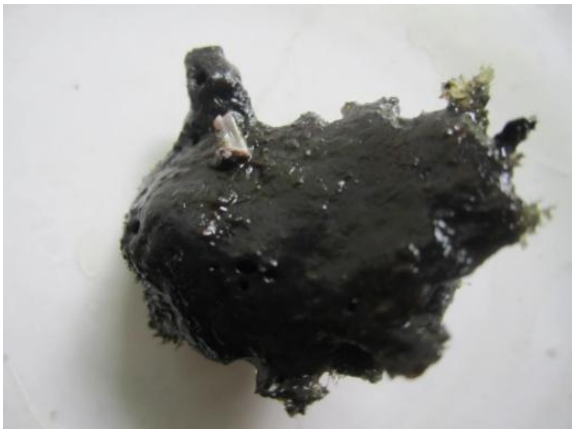


**Fig.1B** *Sigmadocia Carnosa*

**Fig.2** Dpph Radical Scavenging Activity of the Extracts. some- *Spongia Officinalis* var.*Ceylonensis* Methanol Extract; sc me- Sigmadocia Carnosa Methanol Extract.Mean ± Standard Error from the Tested Triplicate Samples were Represented as Mean Error Bars. p value by Students Test:p<0.05

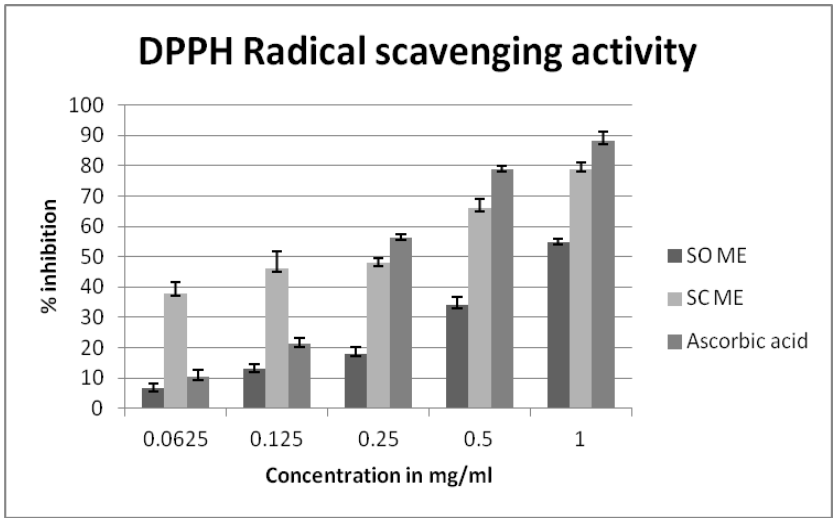



**Fig.3** Phagocytic Activity by Slide Method

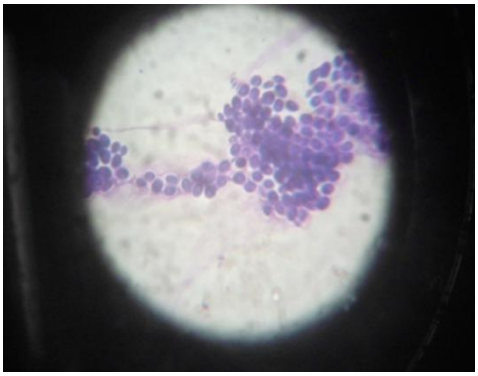


**Fig.4** Nbt Reduction. Percentage of Neutrophils by Nitroblue Tetrazolium Reduction Assay Mean ± Standard Error from the Tested Triplicate Samples were Represented as Mean Error Bars. P value by Students Test:p<0.05

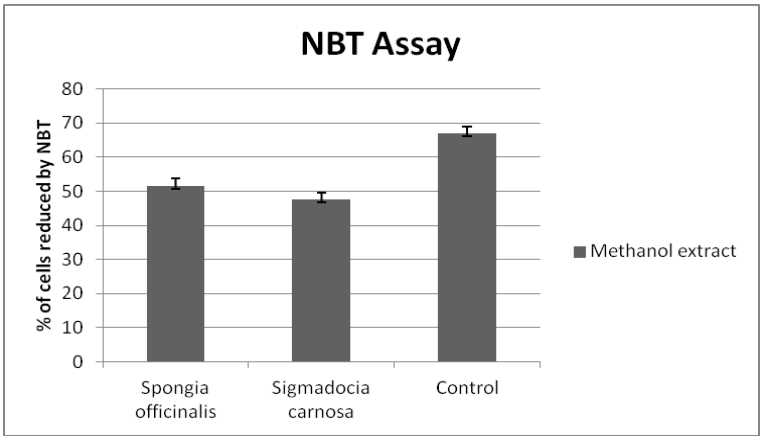



**Fig.5A** NBT Test

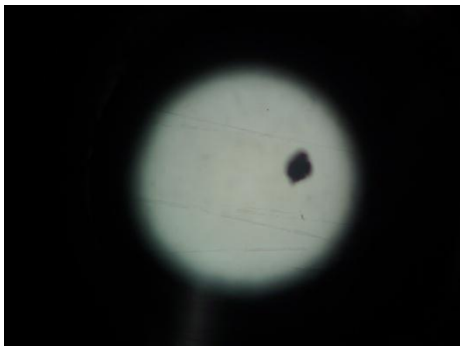


**Fig.5B** NBT Control

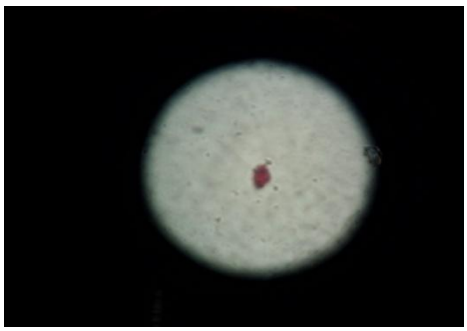


**Fig.6** Acetylcholinesterase Inhibitory Activity

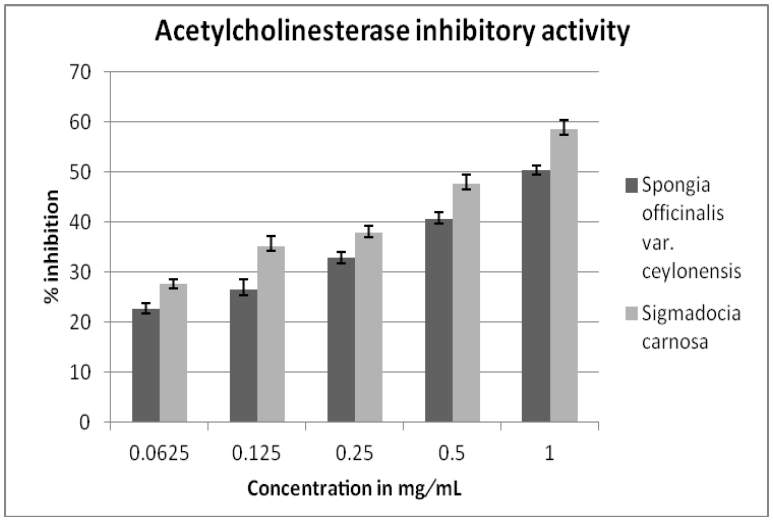
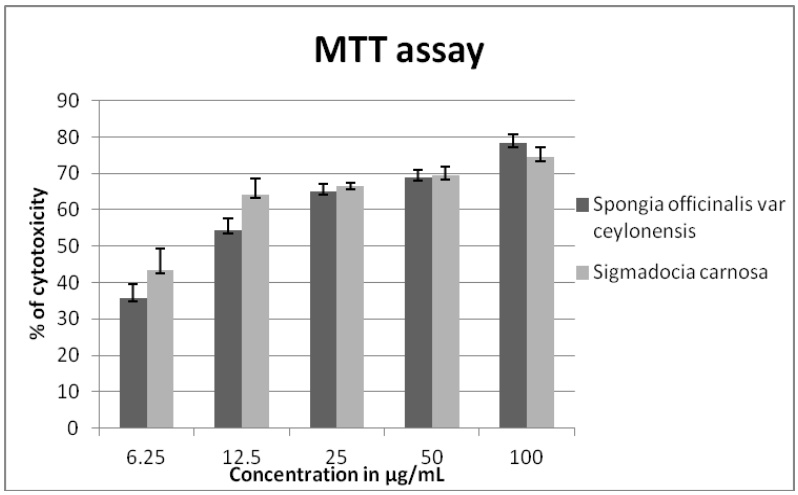



**Fig.7** Mtt Assay on ht-29 Cells. Mean ± Standard Error from the Tested Triplicate Samples were Represented as Mean Error Bars. P Value by Students Test:p<0.05




## Acetylcholinesterase Activity

AChE inhibiting compounds may act as anticancer compounds affecting cholinergic system expressed in a variety of cancer cell lines. AChE inhibitors have a significant role in the treatment of Alzheimer's disease. They help in retaining the neurotransmitters especially at the synaptic terminals through the inhibition of the hydrolytic enzymes ie. cholinesterases thereby compensating the deficiency of the cholinergic neurotransmitters in AD patients (Langjae et al, 2007).

The extracts of both the sponges exhibited significant percentage inhibition (Table 4 and Fig 6). IC 50 values and percentage inhibition indicated that Sigmadocia was more active. Methanolic extract of Sigmadocia showed a low IC 50 value of 684.36µg. AChE inhibitory activity

exhibited by both the extracts indicate that these extract may serve as good candidates for development of drugs against cancer and Alzheimer's disease since AChE inhibitors act on cholinergic system. Acetone, butanol and methanolic extracts of marine sponge *Agelas clathrodes* exhibited substantial AChE inhibitory activity (Sepcic, 2010). Methanolic extracts of *Ircinia* and *Dysidea* species displayed promising results in AChE inhibition test over 50% (Aktas *et al*., 2014). Steroidal alkaloid from purified methanol fractions of Corticium species exhibited potent AChE activity (Langjae *et al*., 2007).

## MTT Assay

The potent methanolic extracts subjected to MTT assay against HT-29 cells significantly reduced cell viability (Fig 6). The extracts exhibited 35-78% inhibition and the activity is concentration dependent. Methanolic extract of *Spongia* has an IC 50 value of 15.11µg and Sigmadocia with an IC 50 value of 14.08µg, indicated that both species would be good candidates for anticancer drug development. Anticancer activity by MTT assay of ethyl acetate extract of marine sponge, *Aurora globostellata* from Tuticorin coast have been reported (Chairman and Singh, 2013).

This study confirmed the medical potential of the two species. Though many reports were there on the antibacterial, antifungal, larvicidal and insecticidal activities of these two species, reports on antioxidant and anticholinesterase activities were scanty. This is the first report on the antioxidant, anticholinesterase and anticancer activities of the two species from the Southwest coast of India.

In conclusion, Sessile marine invertebrates such as sponges lack physical defense mechanism and hence they produce a variety of secondary metabolites as part of their chemical defense system – active immune mechanism in sponges. So it is not an astonishing fact that they produce potent antioxidant, cytotoxic and antibacterial compounds. The marine sponges have already proved to be the most important source of novel molecules with pharmacological properties. The bioactivity shown by sponges may be mainly due to the chemicals present in the sponge and its symbiotic microorganisms. But the secondary metabolites produced as part of defense may vary from species to species.

The results indicate that the extracts of both the sponge species possess significant bioactivity. This activity may be mainly due to the various chemical constituents present in the extracts which were revealed by chemical analysis. So it is expected that the purification and bioanalysis of these extracts will yield potent active compounds. Thus it was confirmed that further bioassay guided fractionation of these extracts and purification of them would provide compounds which can be used as novel drug candidates.

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