Evaluation of bioactivity in Marine Sponge *Sigmadocia pumila* collected from the South Eastern Region of India

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**ABSTRACT**

The present study explores the potential of marine invertebrates as a source for new antimicrobials. The preliminary investigation for the methanolic crude extracts of *Sigmadocia pumila* showed various in vitro analysis of the sponge *Sigmadocia pumila*. It includes larvicidal activity against *Aedes egyptii*, hemolytic activity, brine shrimp cytotoxicity, and immunomodulatory study using NBT assay. These findings indicate that *Sigmadocia pumila* have therapeutic applications.

**Key words:** *Sigmadocia pumila*, larvicidal, hemolytic activity, NBT

**INTRODUCTION**

Marine sponges have numerous bioactive compounds with promising pharmaceutical properties. From the discovery of sponge derived drugs in the Caribbean sponge *Tectitethya crypta* (Cryptotethya), many compounds have entered the preclinical and clinical trials [1]. The incidence and the structural diversity of secondary metabolites in marine sponges are larger than in any other marine phylum. Meanwhile, many of the extracted compounds have shown anticancer, antiinflammatory, antibacterial and antiviral properties [2]. Marine sponges are known as chemical factories because they produce hundreds of unique chemical compounds that have been isolated and their structures are determined. *Ircinia felix*, *Pandaros acanthifolium*, *Topsentia ophiraphidites*, *Verongula rigida* are the abundant source of chemical compounds. The compounds from *Topsentia* sp. show strong hemolytic activity on fresh bovine erythrocytes [3].

Two unique glycosphingolipids belonging to a new class of prenylated glycolipids, Plakoside A and B, have been isolated from the marine sponge *Plakortis simplex*. These plakosides are strongly immunosuppressive on activated T cells and proved to be useful natural model for an improved comprehension of the structural requirements for immunomodulating activity of glycosphingolipids [4]. One agelasphrin from Okinawan sponge *A. mauritianus* analogue KRN 7000 showed interesting anticancer activity and it is now under phase I clinical trials as anticancer agent. The compound was reported to reduce pancreatic inflammation and diabetes [5]. New ceramide homologues, possessing iso- and anteiso-long chain, were isolated from the marine sponge *Clathria fasciculate* from the South China Sea near Hainan Island, China. The fatty acid part was mainly composed of (4E)-2-hydroxy-4-docosenic, tricosenic, tetracosenic, and pentacosenic acids [6]. The *in vitro* antiviral activity of mycalamide A and mycalamide B was studied from a New Zealand sponge of the genus *Mycale*. The crude extract containing 2% mycalamide A
was found to be active against A59 corona virus. Micalamide A also inhibited the Herpes simplex type I and Polio type I viruses. The property of protein synthesis inhibition may be attributed to their biological activity as antiviral agents [7]. Halichondrin B, a compound from the sponge *Lissodendoryx* spp. acted as the potential anticancer agent entered in to the clinical phase level [8].

Marine sponges belonging to the genus *Ircinia* are known to be a very rich source of terpenoids Eg: Variabilins, which were polypropyl – hydroquinones, had analgesic and anti-inflammatory properties. Among the halogenated alkaloids, bromoalkaloids form the most widely distributed group of natural compounds, which are predominantly found in marine eukaryotes like sponges, are significantly rarer in prokaryotic micro plants and animals [9]. The high biological activity of *Aplysina cavernicola*, a much studied sponge which produces aeroplysinin and aethionin and other dibromo and dichlorotyrosine derivatives, with some antibiotic activity against *Bacillus subtilis* and *Proteus vulgaris* [10]. The growing interest in bioactive compounds for new drugs resulted in a notable research boom on secondary metabolites from marine invertebrates, especially from sponges. Some sponge species display promising antibiotic and antitumour activities.

### MATERIALS AND METHODS

**Collection and preparation of extracts of sponges**

Specimens of the marine sponge *Sigmadocia pumila* were collected from the coast of Kanyakumari, Tamilnadu, India by “by-Catch method” during active fishing season. Sponges were cut into small pieces and extracted thrice with distilled methanol and the pooled organic solution was filtered through Whatmann No.1 filter paper fitted in a Buchner funnel using suction. Solvents were removed by rotary vacuum evaporator (Buchi-type) under reduced pressure so as to get the crude methanol extract. The concentrated crude extract was collected in airtight plastic containers and kept in the refrigerator for further use.

**Larvicidal activity**

The larvicidal activity of methanolic extracts of marine sponge was evaluated against the third instar stage of *Aedes egyptii*. The egg cards of *Aedes egyptii* were obtained from the Centre for Research in Medical Entomology, Indian Council of Medical Research, Madurai. The crude extract was tested to determine the larval bio-efficacy by diluting the original extract to 1.0 to 5.5% levels. The bioassays were performed at a room temperature of 27 ± 1°C by exposing 20 larvae in a final volume of 250 ml water in 500 ml glass beaker with minimum of four replicates for each concentration. Simultaneously, control groups were also maintained in beakers without addition of extract but with the solvent alone. Observations were made after 12, 24, 36, 48, and 60 hrs of treatment for larvicidal activity. Based on the percentage mortality the LC$_{50}$ values of the extracts were determined using Probit Scale Analysis.

**Brine shrimp cytotoxicity**

The brine shrimp (*Artemiasalina*) lethality assay was performed. For the bioassay, the dried cysts of 1g were allowed to hatch in a beaker filled with filtered seawater (32 ppt) under constant aeration. After 48h, the phototropic brown coloured nauplii were siphoned out using a glass pipette. The stock extracts were prepared at the concentration of 5mg/ml. The nauplii were counted against an illuminated background and ten nauplii were transferred to each cavity cup containing 2 ml of filtered seawater dissolved with varying dilutions of sponge extract ranging from 0.1% to 1% from the stock. To ensure that the mortality observed in the bioassay could be attributed to bioactive compounds and not due to starvation, control was maintained without adding the extract. The cavity cups were maintained under constant illumination. Experiments were carried out in triplicates to get statistically significant results and the mean value was recorded as mortality after 24 h. Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation. Based on the percent mortality, LC$_{50}$ value of the extract was determined using the probit scale analysis.

**Hemolytic activity**

The hemolytic activity of the crude methanolic extracts of marine sponge on human red blood cells (RBC) was tested by a micro-hemolytic method. Human “ B” positive blood was obtained, from the Vivek Institute of Laboratory Medicine, Nagercoil, (Kanyakumari District, Tamilnadu) in EDTA solution as an anticoagulant at 5% of the blood volume and brought to the laboratory. Then 1% erythrocyte suspension was prepared by adding PBS, pH 7.4, to 1 ml of packed RBC. The micro-hemolytic test was performed on 96-well U-bottom microtiter plates. Serial two fold dilutions of the extracts were carried out in 100 µl of PBS, pH 7.4. For the control set 100µl of

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distilled water was added, to 3% RBC suspension. The plate was gently shaken and allowed to stand for two hours at room temperature. Uniform red color suspension in the wells was considered positive hemolysis while sedimentation on the bottom was considered lack of hemolysis. The percentage of the hemolytic cells was calculated using the formula

\[
\% \text{ Hemolysis} = \frac{\text{Absorbance of sample} - \text{Absorbance of blank}}{\text{Highest Absorbance for positive control}} \times 100
\]

**Nitro Blue Tetrazolium assay**

The *in vitro* immunomodulation study was done on both the crude methanolic extracts of *Sigmadocia pumila*. In five test tubes the reaction mixture consisted of leucocytes suspension (0.4ml), endotoxin activated plasma as standard PBS solution at 0.1ml was used as control. To each test tube 0.1 ml different concentrations of test samples (25, 50, 100, 200 µg/ml) were added and 5% NBT solution (0.8ml) was added and incubated at 37°C in water bath with shaker for 30 min and reaction was stopped with cold PBS. Then it was centrifuged at 1000 rpm for five minutes. After discarding the supernatant, a drop of PBS was added and gently the cells were resuspended. A drop of this reaction mixture was spread on a clean glass slide, dried and fixed in methanol for two minutes stained with 0.8% aqueous safranin for 2 min. The smear was washed, dried and mounted. NBT Positive cells represented by dark blue colour were counted under 100X in a Nikon (E200) binocular microscope.

**RESULTS**

Larvicidal potentials using the extracts of Marine sponge *Sigmadocia pumila* against the third instar stage of *Aedes aegypti* was detected. The results of LC\(_{50}\) values evaluated based on the potency of the extracts at various concentrations form 1.0 to 5.5% and at different intervals of time from 12,24,36,48, and 60 h using the mortality rate against the third instar stage of *Aedes aegypti* are presented in (Table 1). The crude extracts of the sponge *Sigmadocia pumila* exhibited high toxicity against *Artemia salina* nauplii. Results of artemia cytotoxicity bioassay of marine sponge *Sigmadocia pumila* are depicted in (Table 2). The sponge extracts were added at various concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0%. Results indicate 50% mortality at 0.4% concentration of the extracts and 100% mortality happened at the higher dose of 10.0% of the extracts during 24 h interval. (Table 3) showed the hemolytic activity using the methanolic crude extracts of *Sigmadocia pumila*. The optical density value for the extract at (5mg/ml) concentration was 0.492. The calculated hemolytic activity was 96.47%. In the *in vitro* model of Qualitative NBT test significant immunostimulant activity of the sponge *Sigmadocia pumila* was found at a dose of 50% respectively (Table 4).

![Table 1. LC\(_{50}\) values for 12,24,36,48 and 60h with their 95% fiducial (lower and upper) limits, regression equation, Chi-square and P-levels of marine sponge *Sigmadocia pumila* against the 3rd instar larvae of *Aedes aegypti*](image)

The methanolic fractions of the sponge had significantly increased the phagocytic function of human neutrophils, when compared with control and increase the movement of neutrophils towards the foreign body which is the most

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important step in the phagocytosis. Fig 1. describes the presence of NBT positive cells at 100x magnification in blue colour due to the effect of the methanolic crude extracts of marine sponge *Sigmadocia pumila*. It is assumed that the sponge extracts have significant immunomodulatory activity.

Table 2. Mortality of Brine shrimp exposed for 24 hours to different concentrations of methanol extract from sponge *Sigmadocia pumila* ($\pm$ represents standard deviation)

<table>
<thead>
<tr>
<th>Conc of extracts (%)</th>
<th>Mortality (24 hrs) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Nil</td>
</tr>
<tr>
<td>0.1</td>
<td>20 ±0.10</td>
</tr>
<tr>
<td>0.2</td>
<td>30 ±0.24</td>
</tr>
<tr>
<td>0.4</td>
<td>50 ±1.52</td>
</tr>
<tr>
<td>0.6</td>
<td>70 ±2.20</td>
</tr>
<tr>
<td>0.8</td>
<td>90 ±3.16</td>
</tr>
<tr>
<td>1.0</td>
<td>100 ±0.0</td>
</tr>
</tbody>
</table>

Table 3 Hemolytic activity of *Sigmadocia pumila*

<table>
<thead>
<tr>
<th>Sigmadocia pumila</th>
<th>Absorbance at 541nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0.164</td>
</tr>
<tr>
<td>Sample (1mg/ml)</td>
<td>0.492</td>
</tr>
<tr>
<td>Positive control</td>
<td>0.340</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.265</td>
</tr>
</tbody>
</table>

% Hemolysis = 96.47%

Table 4. Effect of the methanolic crude extracts of *Sigmadocia pumila* and *Holothuria atra jaeger* using qualitative nitroblue tetrazolium (NBT) test

<table>
<thead>
<tr>
<th>S.NO.</th>
<th>Groups</th>
<th>Conc. of extract 1mg/ml</th>
<th>% NBT positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control</td>
<td></td>
<td>23</td>
</tr>
<tr>
<td>2.</td>
<td>Endotoxin activated plasma (standard)</td>
<td>10%</td>
<td>85</td>
</tr>
<tr>
<td>3.</td>
<td><em>Sigmadocia pumila</em></td>
<td>10%</td>
<td>35%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25%</td>
<td>51%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50%</td>
<td>63%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50%</td>
<td>72%</td>
</tr>
</tbody>
</table>
DISCUSSION

Marine invertebrates offer a source of potential antimicrobial drugs. Studies of antimicrobial mechanisms and compounds of marine invertebrates may provide valuable information for new antibiotic discoveries and give new insights into bioactive compounds [11]. It is suggested that the Brine shrimp lethality assay is considered to be one of the most useful tools for the preliminary assessment of bioactivity and bioassay with cytotoxic activity against some human solid tumors. The antitumor activity of cell-free extracts from sponge-associated actinomycetes might be due to the presence of the active secondary metabolites alkaloids and terpenoids [12]. The brine shrimp larval mortality assay is widely accepted as a convenient probe for potential *in vitro* cytotoxicity and pharmacological activity in marine natural products [13]. The Indonesian sponge, *Callyspongia pseudoreticulata* yielded diyne which was found to be toxic in the brine shrimp assay [14]. Studies made by Zhang et al. [15] revealed that more than 10% of the investigated marine sponge species exhibited cytotoxic activity suggesting production of potential medicines for potent cytotoxic drugs. The present investigation indicates that the *Sigmadocia pumila* crude extracts showed significant LC$_{50}$ values of cytotoxic effect against the *Artemia salina*.

Mosquitoes such as *Aedes aegypti* act as vectors of dengue, yellow fever and chikungunya while *Anopheles stephensi* and *Culex sp* act as vectors of malaria and filariasis respectively. Most of the mosquito control programmes target the larval stage in their breeding sites with larvicides, as adulticides temporarily reduce the adult population only [16]. From the current study it is showed that the *Sigmadocia pumila* is having the highest larvicidal activity against the 3rd instar stage of *Aedes egyptii*. Detecting the mosquito larvicidal activity of the sponge extracts it is evident advantages were showed such as the possibilities of developing new antifilarial and antimalarial compounds. Manzamines are the most promising antimalarial compound that has been discovered in many marine sponges [17]. Cacospongionolide, B a new sesterpene, isolated from the sponge *Lascispongia covernosa* showed antimicrobial activity, brine shrimp cytotoxicity and ichthyotoxicity[18]. The sponge class Demospongiae is known for producing the largest number and diversity of secondary metabolites isolated from

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**Fig 1.** NBT Positive cells in the extract of *Sigmadocia pumila* (100x magnification)
marine invertebrates. There is a worldwide interest in marine natural products as one of the few de novo sources of drug discovery[19]. Sepcic et al. [20] described significant levels of hemolytic activities of the sponge Reniera sarai extracts compared to the moderate hemolytic activity of extracts from Saracotragus muscarum and Aplysina aerophoba. The sponge Sigmadocia pumila also belongs to the class Demospongiae.

CONCLUSION

Bioactive compounds from various marine sources have often been found to be promising pharmaceutical agents particularly sponges. In the present study it is revealed that the sponge crude extracts of Sigmadocia pumila showed various in vitro activities such as the larvicidal, hemolytic, cytotoxic and immunomodulatory effects. Hence it is assumed that the sponge Sigmadocia pumila act as the source of bioactive compounds and it can be used for drug development.

REFERENCES