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RESEARCH ARTICLE

EVALUATION OF *IN VITRO* ANTI-INFLAMMATORY ACTIVITY OF FIVE SELECTED MARINE SPONGES AGAINST DENATURATION OF PROTEIN-A PILOT STUDY

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ABSTRACT

Background: Marine sponges are well renowned for producing bioactive secondary metabolites with drug leads. Screening of anti-inflammatory compounds from marine sponges is highly appreciated in the field of marine pharmacognosy due to their effectiveness and specificity over the most of synthetic non-steroidal anti-inflammatory drugs. *In vitro* models to test anti-inflammatory activity are considered obligatory prior to pre-clinical studies.
Objective: To evaluate *in vitro* anti-inflammatory effect of crude extracts of five marine sponge samples (N=5), collected from Dehiwala, Colombo, Sri Lanka.
Methodology: Identification of sponge species were based on morphology, spicule and skeleton analysis, using light microscopy. Each sponge crude extract (SCE) was tested for selected zoo-chemicals and against the denaturation of albumin to assess the anti-inflammatory activity. Diclofenac sodium was used as the reference drug.
Results: Sponge samples were identified as 1) *Stylissa* sp, 2) *Stylissa carteri*, 3) *Axinella* sp., 4) *Phakellia* sp. and 5) Family Axinellidae. Zoo-chemical analysis indicated the presence of alkaloids, saponins, terpenoids, and sterols in sponge extracts in varying degree. Heat induced egg albumin denaturation was inhibited by 4 SCEs specifying marked anti-inflammatory activity. Accordingly, the 3 sponge crude extracts were more potent (IC50 = 22.74 for Sp. 02, 3.98 for Sp. 03 and 63.665µgmL -1for Sp. 05) than the of standard reference drug, Diclofinac sodium (IC50=147.02 µg/mL).
Conclusions: Thus, the present study for the first time investigated *in vitro* anti-inflammatory activity of crude extract of 5 selected marine sponge species from Sri Lanka, out of which 3 were more potent than the reference diclofenac sodium. Therefore, isolation and characterization of bioactive compounds which are responsible for anti- inflammatory activity will lead to discover novel marine derived anti-inflammatory drugs in the future.

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INTRODUCTION

Inflammation, the response of cells and tissues to injury, infection or irritation is a complex process associated with pain, vascular permeability, membrane alteration and increase of protein denaturation (Alhakmani *et al.,* 2013; Calixto *et al.,* 2004). It is a defensive response characterized by redness, pain, heat, swelling and loss of function and are orchestrated by a highly modulated interaction between inflammatory cells and mediators of inflammation (Chandra *et al.,* 2012; Farnsworth, 1996). Mast cells, macrophages, granulocytes, platelets, lymphocytes and complement activation factors play an important role in the process of inflammatory response by generating an array of signaling molecules.

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The activation of these immune cells can be achieved by the release of chemical mediators from damaged tissue and migrating cells. The process generally leads to resurgence from infection and to healing. However, if targeted destruction and assisted repair are not properly phased, inflammation can lead to persistent tissue damage by lymphocytes, leukocytes or collagen (Hooper *et al.,* 2002). Since inflammatory diseases have become one of the leading causes of health issue throughout the world it has a considerable influence on health-care costs. A wide class of drugs such as non-steroidal anti-inflammatory drugs (NSAID) and glucocorticoids which are currently used to the control of inflammatory conditions are available in the market, but all suffer from adverse side effects such as intestinal tract ulcers, erosions of the stomach lining and intestinal tract, chronic renal and hepatotoxicity (Hutagalung *et al.,* 2014). Thus, the importance of screening natural anti-inflammatory compounds of plants and animal origin is highlighted. Despite the fact that the biodiversity in

the oceanic environment far exceeds that of the terrestrial environment, research into the employment of marine natural products as pharmaceutical agents is still in its infancy (Jha *et al.,* 2004). Despite the obstacles in exploring the marine environment, a vast majority of marine organisms are reported as potent producers of bioactive compounds (Keyzers *et al.,* 2015). Invertebrates such as sponges, mollusks, bryozoans, tunicates are among those bio synthesizers. Of all these taxa, Phylum Porifera (sponges) remains the most prolific phylum, concerning novel pharmacologically active compounds (Leelaprakash and Dass, 2011). Over 60% of potentially useful bioactive compounds discovered so far from living organisms have been obtained from marine fauna, 70% of which comes from sponges (Mayer and Hamann, 2005). Therefore, sponges are ranked at the top of the hierarchy of all marine organisms with bioactive secondary metabolites. Marine sponges are responsible for producing most of the marine derived anti-inflammatory compounds (Mayer *et al.,* 2011). As the secondary metabolite composition of sponges is dominated by terpenoid compounds, it is not surprising that anti-inflammatory sponge natural products are also dominated by isoprenoid derived metabolites, especially sesterterpenes (Nathan, 2012). However, there is a great variation in the structures of these compounds produced by marine sponges. Being an island nation, Sri Lanka harbors a massive coastal area with enormous diversity of marine sponges which are rarely screened for their bioactive properties. This present study reports the morphological identification, zoo chemical analysis and *in vitro* anti-inflammatory activity by means of egg albumin denaturation of crude extracts of five marine sponge samples.

MATERIALS AND METHODS

Sponge material: Five sponge samples belong to Class Demospongiae with approximate weights less than 500g were harvested by a commercial scuba diver at depth of 9-20 m on 12.04.2017 from Dehiwala, Sri Lanka. Samples were packed in separate concealed plastic bags and transported to the Department of Zoology, University of Sri Jayewardenepura, Nugegoda, Sri Lanka and stored at -20°C till extraction.

Drugs and chemicals: Dichlofenac sodium (Voltaren® 50, Switzerland) was used as the standard anti-inflammatory drug and purchased from a pharmaceutical shop (Union Chemists Private Limited, Colombo, Sri Lanka). All the other chemicals were of analytical grade obtained commercially.

Preparation of sponge crude extracts (SCE): Diced sponge material was extracted in methanol/dichloromethane (1:1 v/v) by incubating for 24 -72 hours (Prakash, 2017). The extracts were collectively filtered through Whatmann No 1 filter paper, and subjected to rota evaporation (BUCHI Rota vapor R-124, Germany) at 40^0C.

Preliminary qualitative zoo-chemical analysis: In the absence of established protocol, for screening of zoo chemical constituents in animal tissue, modified methods from Farnsworth, 1966 were carried out to analyze chemical compounds present in SCEs (Purushottama *et al.,* 2009). Thus, each SCE was tested for alkaloids, flavonoids, saponins, terpenoids, quinones, anthraquinones, tannins, sterols, unsaturated sterols and proanthocyanidin.

Preparation of SCEs: Accurately, 100µgmL^{-1} concentrated sample of each SCE was prepared by dissolving in 5% ethanol. The samples were vortexed and a concentration series of 3.125, 6.25, 12.5,25 and 50 µg/mL were prepared followed by proper dilution. Similarly, a concentration series of 1250, 625, 312.5, 156.25 and 78.125µg/mL was prepared for standard drug, diclofenac sodium.

Evaluation of *in vitro* anti-inflammatory activity: Modified methods of Alhakmani *et al*., 2013 was carried out to evaluate anti-inflammatory activity (Sadique *et al.,* 1987). The reaction mixture consisted of 2mL of different concentrations of SCEs or standard drug, 2.8mL of phosphate buffered saline (PBS) (pH 6.4) and 2mL of egg albumin obtained from fresh hen's egg. Followed by an incubation at 27+1 ^{0}C for 15 minutes the reaction mixture was further incubated at 70 ^{0}C for 10 minutes to induce the albumin denaturation. The samples were cooled to room temperature and the absorbance was measured by UV-visible spectrophotometer at 660nm (UVD-2960) using PBS as a blank. The absorbance of the negative control was measured which consisted of 5% ethanol only. Each absorbance was taken as triplicates and the average and percentage inhibition of albumin denaturation was calculated as below.

The percentage inhibition (PI) was calculated using the following formula.

$$\text{Percentage Inhibition} = \frac{Ab_t - Ab_c}{Ab_c} \times 100$$

Where,

Ab_t =Absorbance of test sample
Ab_c =Absorbance of control

The inhibition concentration (IC_{50}) was calculated for each SCE and compared with the IC_{50} of diclofenac sodium.

RESULTS

Identification of the sponge species

The collected sponge materials were identified according to the guidelines in Hooper and Soest (2002) and classified under the updated nomenclature in the World Porifera Database (http://marinespecies.org/porifera/) (Sipkema *et al.,* 2015; Thakur *et al.,* 2004). Accordingly, the sponge species 01 was identified as *Stylissa* sp, while species 02 as *Stylissa carteri*. Species 03 and 04 were identified as Axinella sp and *Phakellia* sp respectively. The sponge species 05 was belong to the family Axinellidae (Fig 1).

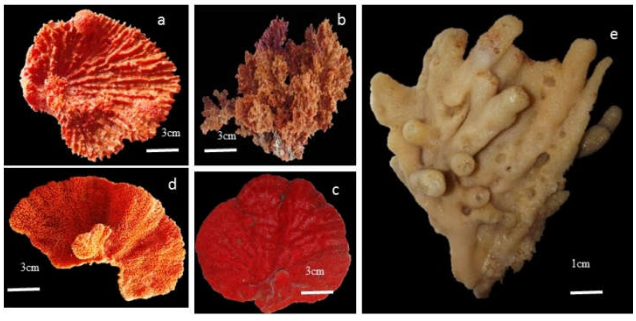

Fig. 1. Sponge species a). Stylissa sp, b). Stylissa carteri, c). Axinella sp, d). Phakellia sp, e). Family Axinellidae

Table 1. Qualitative zoo-chemical analysis

Zoochemical	Sp. 01	Sp.02	Sp. 03	Sp.04	Sp. 05
Alkaloids	-	-	+	+	+
Quinones	-	-	-	-	-
Anthraquinones	-	-	-	-	-
Saponins	+	-	-	+	-
Flavonoids	-	-	-	-	-
Terpenoids	+	-	+	+	+
Tannins	-	-	-	-	-
Proanthocyanidin	-	-	-	-	-
Triterpenes	-	-	-	-	-
Unsaturated sterol	-	-	+	+	-

Sp- species,
+ Present, appreciable amount (Positive within 5 minutes)
- Absent

Table 2. Effect of SCEs on protein denaturation

Concentration (μgmL^{-1})	Percentage inhibition %				
	SP. 01	SP. 02	SP. 03	SP. 04	SP. 05
200					458.8
100	-17.64	210.49		223.24	273.82
50	25.49	97.35	150.98	108.32	77.45
25	29.41	55.58	121.56	80.098	83.33
12.5	33.33	31.37	90.19	93.431	190.19
6.25	43.13	17.94	48.03	119.6	361.76
3.125			29.088		
IC_{50} (µg/mL)	-	22.74	3.98	63.665± 37.043	- 5.807*

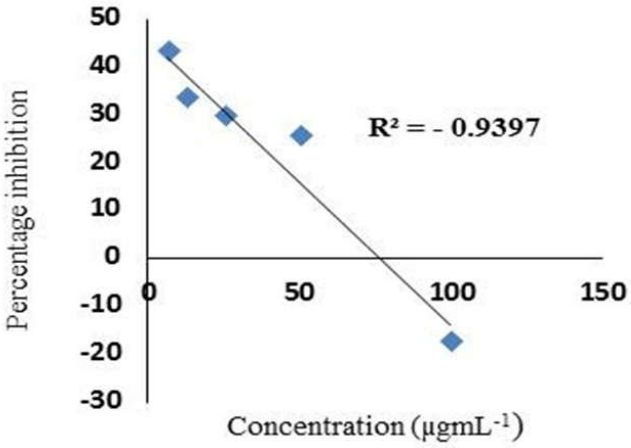

Fig. 2. Effect of SCE of species 1 on inhibition of albumin denaturation data presented as mean± SEM (n=3)

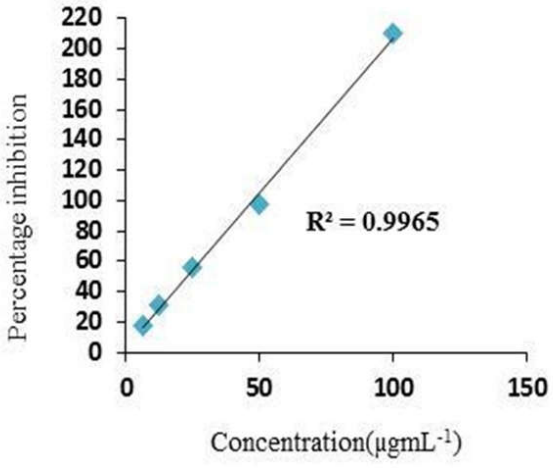

Fig. 3. Effect of SCE of species 2 on inhibition of albumin denaturation data presented as mean± SEM (n=3)

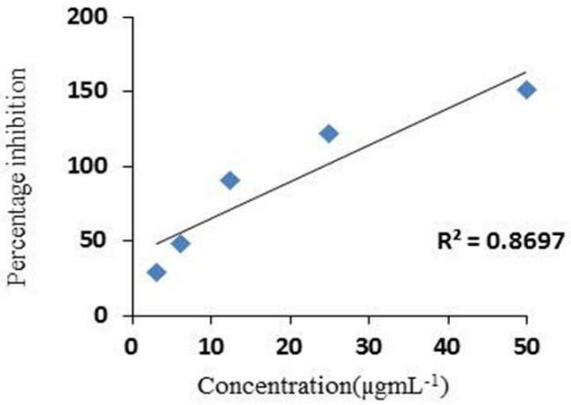

Fig. 4. Effect of SCE of species 3 on inhibition of albumin denaturation data presented as mean± SEM (n=3)

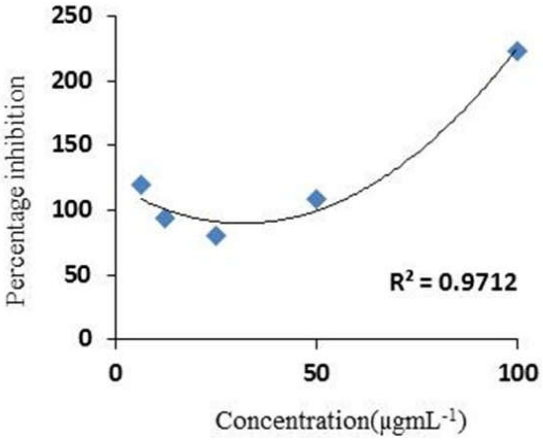

Fig. 5. Effect of SCE of species 4 on inhibition of albumin denaturation data presented as mean± SEM (n=3)

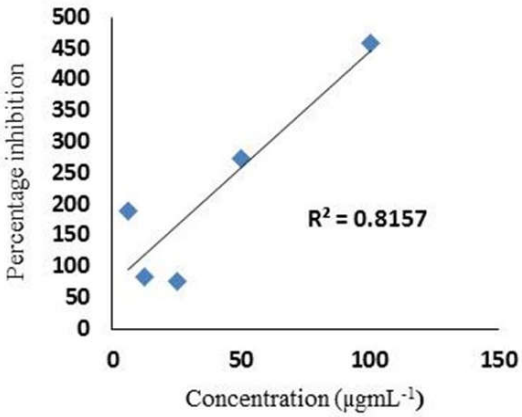

Fig. 6. Effect of SCE of species 5 on inhibition of albumin denaturation data presented as mean± SEM (n=3)

Qualitative zoo - chemical analysis

The zoo chemical analysis resulted the presence of some major classes of zoo – chemicals; alkaloids, saponins, terpenoids and unsaturated sterols. Alkaloids were present in three SCEs, species 03, 04 and 05 while species 01 and 04 revealed the presence of saponins. Terpenoids were present in all SCEs except species 02. Unsaturated sterols were present in only two SCEs (species 03 and 04). Anthraquinones, quinones, tannins, triterpenes, Proanthocyanidins and flavonoids were not detected in any SCEs (Table 01).

***In vitro* egg albumin denaturation assay**

The denaturation of egg albumin was inhibited by all SCEs except specie 01 (Fig 2). A dose dependent inhibition was detected by SCEs of species 02 (Fig 3), 03 (Fig 4) and 05(Fig 6). However, the inhibition by SCE of species 4 was not dose dependent (Fig 5). The inhibitory effect was further confirmed by comparing its IC_{50} values (Table 2). Accordingly, the IC_{50} of species 02, 03 and 04 were more potent than the IC_{50} of standard reference drug, Diclofinac sodium (IC_{50}-147.02 µg/mL)

DISCUSSION

Concerning ethical issues associated with animal use in experimental pharmacological research, it is highly encouraged to conduct in *vitro* assays to assess bioactivities of new chemical compounds. Hence, in the present study the protein denaturation bioassay was selected for *in vitro* assessment of anti-inflammatory property of five sponge crude extracts. Denaturation of tissue proteins is one of the well-documented causes of inflammatory and arthritic diseases (Van Soest *et al.,* 2017). Agents that can prevent protein denaturation therefore, would be worthwhile for anti-inflammatory drug development. Thus, in the present pilot study, investigations were made to evaluate the anti-inflammatory activity of five marine sponge samples using denaturation of egg albumin protein. Secondary metabolites produced by marine sponges play a major role in modulating bioactivity. Terpenoids, alkaloids, saponins and peptides are some of those metabolites which have been studied comprehensively using various *in vitro* and *in vivo* models (Nathan, 2002; Zivanovic *et al.,* 2011). The present study too, supported those previous findings by possessing abundant alkaloids, terpenoids, steroids and saponins in the samples which showed anti-inflammatory properties. Among the species studied *Phakellia* sp. which possess saponins, alkaloids, terpinoids and sterols was the richest source of secondary metabolites followed by *Axinella* sp. which having alkaloids, terpenoids and sterols. Species 01 possessed saponins and terpenoids whereas species 05 possessed alkaloids and terpenoids. SCE of Species 02 was lack of tested zoo chemocals. Therefore, the reported anti-inflammatory activity of this sponge species may be due to some other group of zoo chemicals which have not been detected by the present analysis.

Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound, such as strong acid, strong base, a concentrated inorganic salt, an organic solvent or heat. Most biological proteins lose their biological function when they are denatured [3]. Denaturation of tissue proteins is a well-documented cause of inflammation and arthritic diseases, through auto antigen production. Therefore, as a part of the study of anti-inflammatory activity of sponges, ability of SCEs to inhibit heat induced protein denaturation was studied. This experiment was carried out at pH 6.4, a pH which could be attained in strongly inflamed tissue. Heat was provided to induce the denaturation of proteins. The increments in absorbance of test samples with respect to control indicated the stabilization of protein by sponges and the reference drug diclofenac sodium. From IC_{50} values, it becomes evident that some of the SCEs (SCEs of species 02, 03 and 05 were more potent than the standard drug diclofenac sodium, being effective in lower concentrations. Though SCE of species 05 showed inhibition of albumin denaturation it was not dose dependent, suggesting further experimentation. Of all SCEs tested, species 03 (*Axinella* sp) showed the maximum inhibition (IC_{50}=3.98µgmL^{-1}) of heat induced protein denaturation revealing it is the most potent SCE with respect to inhibition of protein denaturation. However, SCE of species 01 was not effective as an anti-inflammatory bioactive donor, as it showed pro inflammatory activity. Being sedentary at maturity, sponges lack defense against predators except the metabolites they secrete into the surrounding environment. However, it is unclear whether the sponges themselves produce these bioactive molecules or the molecules are synthesized by the organisms living inside of them in symbiosis. Further, all the sponge species except species 2 proved that they were rich in secondary metabolites. Finally, the anti-inflammatory potential of these sponges can be attributed to the secondary metabolites they produced. The anti-inflammatory activity is undoubtedly, cannot be due to single zoo-chemical constituent, but may be due to the synergistic effect of different chemicals in SCEs. Therefore, a comprehensive chemical characterization of these SCEs is strongly warranted, followed by a pre-clinical study using *in vivo* models. However, the resent study holds a landmark in studying anti-inflammatory activity of Sri Lankan marine sponge crude extracts, which can be used as lead compounds for designing potent anti-inflammatory drugs.

Conflicts of interest: The authors declare there is no conflicts of interest regarding the content in the manuscript.

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