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Antinociceptive and Anti-inflammatory Activities of Marine Sponges *Aplysina Caissara, Haliclona* sp. and *Dragmacidon Reticulatum*

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ABSTRACT

Marine sponges are a rich source of bioactive natural products with multiple pharmacological properties. In this study, the anti-inflammatory and antinociceptive effects of extracts obtained from Aplysina caissara, Haliclona sp. and Dragmacidon reticulatum were evaluated by using the writhing test and formalin-induced mouse paw edema model in mice. All extracts were administered via oral pathway in the doses of 60 and 90 mg/kg. In the writhing test the pre-treatment with all sponges resulted in significant inhibition of the acetic acid-induced response, suggesting an antinociceptive effect. The formalin test showed that the extracts from A. caissara, Haliclona sp. and D. reticulatum, in the tested doses, did not affect the first formalin phase, however, they were effective in the late phase. To assess the potential anti-inflammatory activity of the extracts, the test of formalin-induced paw edema was used. The oral administration of A. caissara, Haliclona sp. and D. reticulatum extracts significantly reduced the formalin-induced paw edema in mice. In conclusion, our data show that marine sponges can be an important source of anti-inflammatory and antinocicpetive products that can be promising therapeutical leads. Furthermore, pharmacological and chemical studies have been developed not only to characterize the mechanism(s) that is/are responsible for the antinociceptive and anti-inflammatory action but also to identify the active principles of sponges.

Keywords: inflammation; nociception; marine sponges.

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INTRODUCTION

Marine organisms (bacteria, fungi, micro-algae, sponges, mollusks and other invertebrates) are sources of numerous new compounds with multiple pharmacological properties [1–5]. Regarding the diversity of marine organisms, the phylum of sponges is the most productive. The phylum Porifera, which is found in both sea surface and deep waters, besides having a few freshwater species, is traditionally known to be a promising source of bioactive metabolites [6].

Sponge-derived bioactive substances have possessed antibacterial, antiviral, antifungal, antimalarial, antihelminthic, immunosuppressive, muscle relaxants, anti-inflammatory and analgesic activities [7]. The search for new anti-inflammatory and analgesic agents from marine sources has yielded several promising therapeutic leads. Several sponges' species and isolated sponges derived compounds have been described by their effects. Some examples of sponge species with anti-inflammatory activity includes *Fasciospongia cavernosa, Petrosia contignata, Cacospongia linteiformis* [5], *Luffariella variabilis* [8], *Xestospongia testudinaria* [9] and *Aplysina fistularis* [10]. Some of the reported mechanisms involved in the anti-inflammatory effects of marine sponges metabolites were the inhibition of phospholipase A2, inhibition of interleukin-1 mediated prostaglandin synthesis, inhibition of cicloxigenase and reduction of superoxide production by neutrophils [11,12]. The analgesic potential of sponges is less explored and was already evidenced for *Aplysina caissara.* [13]

Our study was conducted with three species of marine sponges, *Haliclona sp.*, *Dragmacidon reticulatum* and *Aplysina caissara.* Sponges of the genus Haliclona are well-known for producing a variety of secondary metabolites, many of them are cytotoxic [14,15], antifungal [16], antimicrobial [17] and anti-inflammatory [18,19]. The specie *D. reticulatum* produce some secondary metabolites that are cytotoxic, antimicrobial, antiprotozoal and antiviral [20].

The specie *A. caissara*, an endemic Brazilian specie of marine sponges, is rich in active peptides and alkaloids [21,22]. Our laboratory studies have indicated that aqueous extracts of *A. caissara*, administered intraperitoneally show antinociceptive and anti-inflammatory profiles [13]. Besides, the anti-tuberculosis and cytotoxic activity was also shown *in vitro* [22]. More recently, Medeiros et al. [10] showed that an isolated compound from *Aplysina fistularis* present anti-inflammatory effects in macrophages cell line.

The purpose of this study was to investigate the anti-inflammatory and antinociceptive activities of extracts from *A. caissara, Haliclona* sp. and *D. reticulatum*, sponges that are abundant in Brazil, by using the writhing test and formalin-induced mouse paw edema model. We have also compared the effects of the extracts between them and with a non-steroidal anti-inflammatory drug (diclophenac) and morphine.

MATERIALS AND METHODS

Chemicals

Diclofenac (Voltaflan®) and morphine (Dinomorf®) were kindly provided by the University Hospital Dr. Miguel Riet Corrêa Jr from Universidade Federal do Rio Grande. Formaldehyde and acetic acid were purchased from Delaware® (Porto Alegre, RS, Brazil). The extracts and drugs were dispersed or dissolved in saline solution (0.9% NaCl) for administration.

Sponge Material

Samples of *A. caissara*, *Haliclona* sp. e *D. reticulatum* were collected on *Arvoredo* Island (Florianópolis, SC, Brazil) in April 2006 at a depth of 7 m. They were washed in sea water and all visible surface debris was removed. Afterwards, they were rapidly washed in freshwater and immediately frozen. The frozen samples were also immersed in ethanol and maintained at -20ºC. The specimens were identified by Clea Lerner, PhD, and kept in the *Museu de Ciências Naturais, Fundação Zoobotânica of Rio Grande do Sul*, Brazil – Porífera collection (MCNPOR).

Extract Preparation

The extract was prepared according to the following procedure: the sponges were extracted in ethanol and the remaining material was sequentially extracted four times with methanol (0.3 g/mL) by maceration for 4 days. After the fourth day, the ethanol and methanol solutions were blended and filtered. After filtration, the extracted material was concentrated in a rotary evaporator (Fisaton, Brazil) and the final extract was partitioned against hexane (1 : 1 v/v). The final extract was dried in SpeedVac (SPD1010; ThermoSavant, NY, USA) [13].

Animals

Male Swiss albino mice (25–35 g) were provided by the Animal House of the *Universidade Federal do Rio Grande* (FURG), were housed in rooms at controlled temperature (20–22^{0}C), in 12-h : 12-h light/dark cycles. Standard rodent diet and tap water were provided *ad libitum*. The experiments were performed after approval of the protocol by the Institutional Ethics Committee in agreement with the guidelines of the Brazilian National Council for Control of Animal Experimentation.

Antinociceptive Activity

Writhing Test

The abdominal writhing response to the acetic acid administration (0.6%, 10 mL/kg, i.p.) consists of contractions of the hind limbs [23]. For the writhing test, mice got the acetic acid injection 30 min after getting their respective treatments (n = 8 animals/group). Animals were treated with the *A. caissara* extract (60 and 90 mg/kg), *Haliclona* sp. extract (60 and 90 mg/kg), *D. reticulatum* extract (60 and 90 mg/kg) or saline (control group) (0.9%; 0.1 mL/10 g) by oral pathway (gavage). One group was treated with the reference opioid analgesic, morphine (2.0 mg/kg) 60 min prior to the acetic acid injection by intraperitoneal pathway (i.p.) and another group received diclofenac (5 mg/kg; i.p.), also 60 min prior to the acetic acid injection, an anti-inflammatory non-steroidal drug. The number of abdominal writhing was counted cumulatively for 25 min, starting 5 min after the administration. Antinociception was calculated as a percentage of inhibition of writhing constrictions by using the formula [(control group mean – test group mean)/(control group)] 100%.

Formalin Test

In this test, 20 μL of 2.5% formalin was injected into the left hind paws of mice 30 or 60 min after they had been submitted to their respective treatments (n=8 animals/group). Animals were treated with the *A.* caissara extract (60 and 90 mg/kg), *Haliclona* sp. extract (60 and 90 mg/kg), *D. reticulatum* extract (60 and 90 mg/kg) or saline (control group) (0.9%; 0.1 mL/10 g) by oral pathway (gavage) 30 min prior to formalin injection. One group was treated (60 min prior to the formalin injection) with the opioid analgesic morphine (2.0 mg/kg; i.p.) and another group received diclofenac (5 mg/kg; i.p.), also 60 min prior to the formalin injection, an anti-inflammatory non-steroidal drug. The formalin-induced paw licking was considered an indicator of nociceptive behavior. The amount of time that each animal spent licking the paw was recorded during two 5-min intervals: the first one began immediately after the injection (first phase) and the second one began 20 min after the injection (second phase) [24]. The total time spent in licking the injected paw was recorded and used for quantifying the nociceptive behavior.

Anti-Inflammatory Activity

Formalin-Induced Paw Edema

The anti-inflammatory activity of *Haliclona* sp. and *D. reticulates* extracts were assessed by paw edema test in mice [25]. Before formalin injection (20 μL; 2.5%,), the volume of each mouse paw was measured separately by means of a plethysmometer (Letica, Barcelona, Spain). Thirty minutes after the administration (by gavage) of the *A. caissara* extract (60 and 90 mg/kg), *Haliclona* sp. extract (60 and 90 mg/kg), *D. reticulatum* (60 and 90 mg/kg) or saline (control group) (0.9%; 0.1 mL/10 g) to the mice, acute inflammatory edema was induced by sub plantar injection of formalin into the right hind paws of mice (n = 8 animals/group). The edema caused by formalin was measured at 30 and 180 min after formalin injection. Diclofenac (5 mg/kg; i.p.) was used as positive control. The volume of the edema was expressed for each animal as the difference between before and after formalin injected paws.

Statistical Analysis

Results were expressed as mean ± SEM (standard error of mean). Data were analyzed by one way analysis of variance followed by Tukey's *post hoc* test. Values of $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

This study reports the antinociceptive and anti-inflammatory effects induced by extracts obtained from three sponges, *A. caissara*, *Haliclona* sp. and *D. reticulatum*, in mice. The antinociceptive and anti-inflammatory activities were evaluated by the acetic acid-induced writhing response and the formalin test.

To evaluate the possible analgesic effect of the sponges, we used the writhing test, that is commonly used for screening peripherally active analgesic. In this test, acetic acid acts indirectly inducing the release of endogenous mediators that stimulate the nociceptive neurons which are sensitive to non-steroidal anti-inflammatory drugs and opioids. In the

writhing test, the acetic acid-induced writhing response, the nociceptive response appears to result from the release of TNF-α, interleukin-1β and interleukin-8 by resident peritoneal macrophage and mast cells [26], the release of biogenic amines (e.g., histamine and serotonin), cyclooxygenases and their metabolites (e.g., PGE_2 and PGF2α) [27] and opioid mechanisms [28]. The participation of eicosanoids and sympathomimetic amines in the nociceptive responses induced by acetic acid has also been demonstrated [29,30].

Our results showed that pre-treatment with all sponges resulted in significant inhibition of the acetic acid-induced writhing response. All extracts were evaluated at doses of 60 and 90 mg/kg by oral pathway. Results in Table 1 show that the oral administration of both doses of aqueous extract of *A. caissara* inhibited the acetic acid-induced abdominal constriction (62.8 and 60.1% - $p<0.001$, respectively). Similar results were obtained by both oral doses of aqueous extract of *Haliclona* sp. in the acetic acid-induced abdominal constriction (50.2 and 71.2% - $p<0.001$, respectively). The two doses of the extract of *D. reticulatus* also significantly inhibited the acetic acid-induced abdominal constriction (27.5 and 29.3% - $p<0.05$), however it was less effective than *A. caissara* and *Haliclona* sp. Furthermore, the antinociceptive effects in the test were also observed for diclofenac (62.4% - $p<0.001$), used as the reference peripheral analgesic drug and for morphine (97.1%- $p<0.001$), used as the reference central analgesic drug (Table 1). These data suggest the possible antinociceptive action of the extracts from *A. caissara*, *Haliclona* sp. and *D. reticulatum*. In addition, they also corroborate a previous study of the antinociceptive activity of *A. caissara* [13]. Here, both doses of *A. caissara* and *Haliclona* sp. were as potent as diclofenac in the inhibition of constrictions in mice.

Table 1. Effect of *A. caissara*, *Haliclona* sp. and *D. reticulatum* extracts on acetic acid-induced writhing behavior in mice.

Group	***Number of abdominal constrictions (during 25 min)***	***% of writhes inhibition***
Control	101.3 ± 5.5	-
Morphine (2 mg/kg)	2.9 ± 1.6***	97.1
Diclofenac (5 mg/kg)	38.1 ± 2.1***	62.4
A. caissara (60 mg/kg)	37.7 ± 6.3***	62.8
A. caissara (90 mg/kg)	40.5 ± 5.5***	60.1
Haliclona sp. (60 mg/kg)	50.4 ± 5.8***	50.2
Haliclona sp. (90 mg/kg)	29.2 ± 6.1***	71.2
D. reticulatus (60 mg/kg)	73.4 ± 7.3*	27.5
D. reticulatus (90 mg/kg)	71.6 ± 8.9*	29.3

The number of abdominal constrictions are expressed as mean ± SEM. All extracts and saline (control group) were administered by oral pathway (n = 8 animals/group). Morphine and diclofenac were administrated by intraperitoneal pathway. ***p <0.001; *p<0.05 indicates statistical difference when compared to control (one-way ANOVA followed by Tukey *post hoc* test).

Neurogenic and inflammatory pain was evaluated by using the formalin test. In this test, the initial pain (early phase) is explained as a direct stimulation of nociceptors and reflects centrally mediated pain, whereas the late phases are thought to be secondary to the inflammatory reactions [24]. The first phase corresponds to acute neurogenic pain while the second phase corresponds to inflammatory pain. Experimental results have demonstrated that several mediators, such as substance P and bradykinin, participate in the early phase, while histamine, serotonin, prostaglandins, nitric oxide and bradykinin, released from damaged cells, take part in the inflammatory response. Besides, they are involved in the late phase of the formalin test [31]. Drugs that act primarily on the central nervous system inhibit both phases equally, while peripherally acting drugs inhibit the late phase [32]. Therefore, second phase behaviors are selectively attenuated by cyclooxygenase inhibitors whereas first and second phase behaviors are attenuated by opioids [33].

The extracts from *A. caissara, Haliclona* sp. and *D. reticulatum*, in the tested doses, did not affect the first formalin phase, however, they were effective in the late phase ($p < 0.001$), as demonstrated in the Figure 1 and Table 2. It suggests that peripheral mechanism is involved in its effects and may also exhibit an associated anti-inflammatory effect since the anti-inflammatory drugs exhibited some effect in this phase. This is consistent with our results that show the effectiveness of diclofenac (as positive control in this study) in the late phase (74.2 % of inhibition), but not in the early one. Previous results showed that the i.p. administration of the extract of *A. caissara* in the formalin test inhibited the licking behavior significantly [13], during both early and late phases. In contrast, in this study, the extract administrated by oral pathway was not capable to reduce the first phase of formalin test. It seems that the systemic administration can be more effective in reduce the acute neurogenic pain (first phase) in mice. There are no previous data in the literature showing the antinociceptive effects of *Haliclona* sp. and *D. reticulatum*. The results from the writhing and formalin tests indicate the potential antinociceptive effect of the three sponges extracts.

Since the reduction of the second phase in the formalin test implied that there might be an anti-inflammatory mechanism, we decided to evaluate the effects of extracts from sponges on peripheral inflammation. By using formalin as a stimulus, we could produce an acute inflammatory response after 3 h in the paws of mice. The role played by inflammatory mediators, such as bradykinin, interleukin (IL)-1β, IL-6, IL-8 and tumor-necrosis factor-α (TNF-α)[34], eicosanoids and nitric oxide [24,35] in the response induced by formalin has been demonstrated. Anti-inflammatory compounds are likely to have suppressed the edema formation by the inhibition of the inflammatory mediator substances.

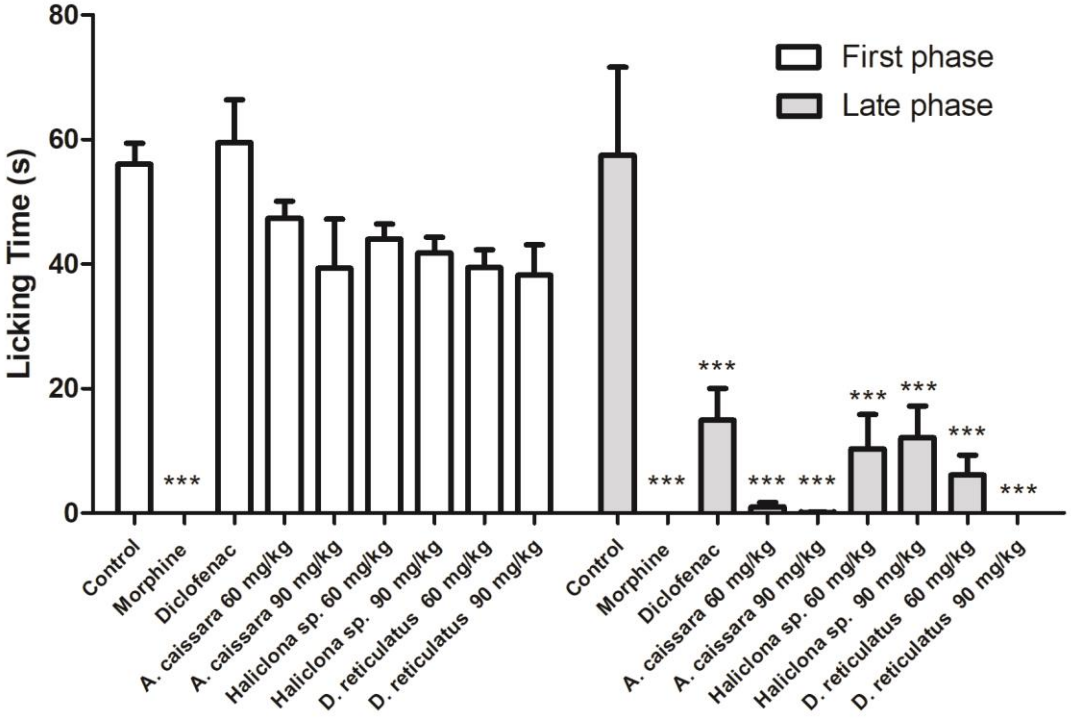

Figure 1: Antinociceptive effect of *A. caissara*, *Haliclona sp*. and *D. reticulatum* extracts on formalin-induced pain in mice. The amount of time spent in licking the injected paw was recorded in two phases: first phase: 0–5 min post-formalin injection; second phase: 20–25 min post-injection. Values are expressed as mean ± SEM. All extracts and saline (control group) were administered by oral pathway (n = 8 animals/group). Morphine and diclofenac were administrated by intraperitoneal pathway. ***p <0.001 indicates statistical difference when compared to control (one-way ANOVA followed by Tukey post hoc test).

Table 2: Percentage of inhibition of *A. caissara, Haliclona sp*. and *D. reticulatum* extracts on formalin-induced pain in mice.

Group	**First phase** ***Inhibition (%)***	**Late phase** ***Inhibition (%)***
Control	-	-
Morphine (2 mg/kg)	100	100
Diclofenac (5 mg/kg)	-	74.2
A. caissara (60 mg/kg)	15.5	98.3
A. caissara (90 mg/kg)	29.8	99.8
Haliclona sp. (60 mg/kg)	21.5	82.1
Haliclona sp. (90 mg/kg)	25.3	79.0
D. reticulatus (60 mg/kg)	29.5	89.3
D. reticulatus (90 mg/kg)	31.8	100

The amount of time spent in licking the injected paw was recorded in two phases: first phase: 0–5 min post-formalin injection; second phase: 20–25 min post-injection. Resuls are expressed as percentage of inhibition of the licking time. All extracts and saline (control group) were administered by oral pathway (n = 8 animals/group). Morphine and diclofenac were administrated by intraperitoneal pathway.

In this study, oral administration of *A. caissara*, *Haliclona* sp. and *D. reticulatum* extracts reduced the formalin-induced paw edema in mice. Table 3 shows that *A. caissara* (60 and 90 mg/kg) and *Haliclona* sp. (60 and 90 mg/kg) significantly reduced the formalin-induced hind paw edema in rats in 28.8 ($p<0.001$) and 24.4% ($p<0.01$); and 20.0 ($p <0.05$) and 28.9% ($p< 0.001$), respectively. Under the same experimental conditions *D. reticulatum* significantly only reduced the formalin-induced edema in the higher dose (90 mg/kg) in 26.7 % ($p<0.001$). The effects were similar to the one obtained by diclofenac (positive control).

Table 3. Effect of *A caissara*, *Haliclona* sp. and *D. reticulatum* extract on formalin-induced hind paw edema in mice.

Group	***Paw edema (mL) 3h after formalin***	***% of inhibition***
Control	0.45 ± 0.01	
Diclofenac (5 mg/kg)	0.35 ± 0.03**	22.2
A. caissara (60 mg/kg)	0.32 ± 0.02***	28.8
A. caissara (90 mg/kg)	0.34 ± 0.02**	24.4
Haliclona sp. (60 mg/kg)	0.36 ± 0.02*	20.0
Haliclona sp. (90 mg/kg)	0.32 ± 0.02***	28.9
D. reticulatus (60 mg/kg)	0.37 ± 0.01	17.8
D. reticulatus (90 mg/kg)	0.33 ± 0.01***	26.7

The paw edema (mL) values are expressed as mean ± SEM. All extracts and saline (control group) were administered by oral pathway (n = 8 animals/group). Diclofenac was administrated by intraperitoneal pathway. ***p <0.001; **p<0.01; indicates statistical difference when compared to control (one-way ANOVA followed by Tukey *post hoc* test).

Marine sponges to the order Verongida, that includes *Aplysina* species are a rich source of naturally occurring bromide-containing alkaloids, derived from the tyrosine (bromotyrosine-derived alkaloids) [36–38]. These metabolites have been described as microbicides and antitumorals [36], however, the anti-inflammatory effects of these compounds are poorly studied. Some years ago, Medeiros et al. [10] reported the anti-inflammatory activity of 11-oxoaerothionin, a bromotyrosine-derivated alkaloid isolated from the marine sponge *Aplysina fistularis* in culture of macrophages. Moreover, the authors showed that the anti-inflammatory mechanism of this compound seems to be related to the inhibition of NO production, inflammatory cytokines and PGE_2. Chemical studies from the crude extract of *A. caissara* showed the presence of bromotyrosine-derivated alkaloids: caissarine A, B and C; agelocaissarines A1, A2, B1, B2; fistularin-3 and 11-hydroxyaerothionin [37,39]. Based on this studies we can suggest that this class of compounds may be responsible for the anti-inflammatory and antinociceptive effects of *A. caissara* observed in this study and in our previous work from Azevedo et al [13].

In addition to *A. caissara*, the extract of *Haliclona* sp also presented anti-inflammatory activity in the experimental model. Corroborating our results, Koh and Shin [40] recently demonstrated that an extract from *Haliclona* sp. decreases the NO production and IL-1β in macrophages stimulated with lipopolysaccharide (LPS) *in vitro*. Previous chemical studies of marine sponges belonging the genus *Haliclona* lead to the isolation of several secondary metabolites including alkaloids, macrolides, steroids, peptides, polyacetylenes, polyketides and halogenated derivates [41]. Randazzo et al. [18] attributed the anti-inflammatory activity of *Haliclona* sp. to two peptides (halipeptins A and B) that showed a very strong anti-inflammatory activity, causing 60% reduction of edema in mice at the dose of 300 mg/kg. Another study investigated the effects of β-carboline alkaloid manzamines isolated from *Haliclona* sp. in LPS-activated rat microglia. They showed that

this compound modulates the thromboxane A_2 and superoxide anion production and can be a potentially useful anti-inflammatory agent to treat neurodegenerative disease [42].
The specie *D. reticulatum* is less studied among the species analyzed in this study. Literature data show that sponges of the genus *Dragmacidon* present piperazine alkaloid derivates and β-carboline alkaloids, some of them are characterized by possessing potent anti-inflammatory and antitumour activities [43]. Recently, Abou-Hussein et al.[44] isolated one new nucleoside (dragmacidoside) and other compounds (adenosine, iosine, deoxycytidine, methyl-a-D-glucopyranoside, clionasterol, stigmasterol, campesterol and brassicasterol) from the Red Sponge *Dragmacidon coccinea*. The authors also showed that a chloroform fraction demonstrated significant anti-inflammatory activity in in the carrageenan-induced hind paw oedema in rats. To our knowledge, this is the first experimental study that demonstrates that *D. reticulatum* present anti-inflammatory and antinociceptive effects.

CONCLUSIONS

In summary, our results demonstrate that the extracts obtained from marine sponges *A. caissara*, *Haliclona* sp. and *D. reticulatum* exhibit analgesic and anti-inflammatory effects against some classical models of nociception and inflammation in mice. Even though a more exhaustive pharmacological investigation on the extracts of *A. caissara, Haliclona* sp. and *D. reticulatus* is needed to characterize the exact target(s) of the compounds, it is clear that the extracts display anti-inflammatory effect *in vivo*. These findings encourage further pharmacological studies not only to evidence the action mechanism of the extract but also to isolate active compounds found in sponge extracts.

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