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ORIGINAL ARTICLE

Investigation of the anti-inflammatory and analgesic effects from an extract of *Aplysina caissara*, a marine sponge

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ABSTRACT

A variety of biologically active compounds with pharmacological applications has been reported to occur in marine sponges. The present study was undertaken to provide a set of data about an extract from *Aplysina caissara*, a Brazilian marine sponge. The antinociceptive and anti-inflammatory effects were investigated against different experimental models in mice. When evaluated against writhing test intraperitoneally (60 and 90 mg/kg), the extract significantly inhibited abdominal constriction by 33.7% and 41.4% respectively. In the formalin test (60 and 90 mg/kg), the extract of sponge inhibited 43.6% and 51.6% in the first phase and 98.2% and 97.2% in the second phase respectively. When evaluated against the hot plate test, both doses demonstrated activity. An increase in the hot plate latency was observed after 60 min. The anti-inflammatory effect was evaluated by formalin-induced mice paw edema. Extract from *A. caissara* (60 and 90 mg/kg) significantly reduced hind paw swelling. Mortality increased with increasing doses, with LD_{50} of 212.2 mg/kg for intraperitoneal administration. These results demonstrated that the extract of the marine sponge *A. caissara* possesses antinociceptive and anti-edematogenic effects.

INTRODUCTION

Marine organisms (bacteria and fungi, micro-algae, sponges, molluscs and other invertebrates) possess greater molecular diversity than terrestrial life, and the potential of these organisms as a source of novel molecules is immense [1]. Indeed, some of the most interesting natural products often characterized by novel molecular architecture have been isolated from Porifera [2] and are known to modulate various biological activities, and have anti-fungal [3], anti-cancer [4], antihelminthic, antiviral and antimicrobial properties [5].

Marine sponges are known for their anti-inflammatory activities: examples are the compound cavernolide isolated from *Fasciospongia cavernosa*, contignasterol isolated from *Petrosia contignata* and the compound cyclolinteinone from the sponge *Cacospongia linteiformis*. The actions of these compounds can be explained by the inhibition of enzymatic activities, like inhibition of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) gene expression, plasma exudation in vivo in response to ovalbumin and prostaglandin E_2 [6]. From the marine sponge *Haliclona* sp., compound halipeptin A was isolated, which has shown anti-inflammatory activity on mouse paw edema assay. This metabolite is more potent than standard anti-inflammatory drugs like indomethacin and naproxen for anti-inflammatory effects [2]. Petrosaspongiolide M, isolated from the

Caledonian marine sponge *Petrosaspongia nigra*, is a potent inhibitor in vivo and in vitro of phospholipase A_2 (PLA_2), demonstratin anti-inflammatory activity in models of acute and chronic inflammation [7].

Sponges of the Order Verongida have been the source of a variety of biologically active peptides and alkaloids [8,9]. From the *Aplysina caissara*, an endemic Brazilian species of marine sponge [10], new members of the dibromotyrosine derivatives, agelocaissarine A_1, A_2, B_1, B_2 and caissarine C, fistularine 3 and 11-hidroxyaerothionins were isolated [8,11]. Many of bromotyrosine-derived alkaloids display potent biological cytotoxic and antibacterial activities [8]. However, there are no previous studies of the anti-inflammatory and antinociceptive effects from the extract of the marine sponge *A. caissara*.

The purpose of the present study was to investigate the anti-inflammatory activity of an extract of *A. caissara* using the formalin-induced mice paw edema models. We have also undertaken an evaluation of antinociceptive properties of the extract and compared with the commonly used non-steroidal anti-inflammatory drug (NSAID), using the inhibition of the number of writhes induced by acetic acid, hot plate and formalin tests, and calculating its acute toxicity (LD_{50}).

MATERIALS AND METHODS

Animals

Male Swiss albino mice (25–35 g) were used in this study to minimize the effects of hormonal changes. The animals were provided by the Animal House of Universidade Federal do Rio Grande (FURG), and were housed in rooms with controlled temperature (20–22 °C), under 12-h : 12-h light/dark cycles. Standard rodent diet and tap water were provided ad libitum. The experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, by the Colégio Brasileiro de Experimentação Animal [12], and to the ethical guidelines for investigation of experimental pain in conscious animals [13]. The animals were kept in transparent glass observation chambers during the tests.

Chemicals and standard drugs

Diclofenac (Voltaflan®) and morphine (Dinomorf®) were obtained at the university hospital pharmacy. Indomethacin was obtained from Sigma (St Louis, MO, USA). 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%) for administration.

Sponge material

Samples of *A. caissara* were collected at Arvoredo Island (Florianópolis, SC, Brazil) in April 2006 at a depth of 7 m. They were washed with sea water, cleaned all visible surface debris, washed rapidly with freshwater, and immediately frozen. The frozen samples were also immersed in ethanol and maintained at −20 °C. The specimens were identified by Dr Clea Lerner and deposited in the Museu de Ciências Naturais, Fundação Zoobotânica of Rio Grande do Sul, Brazil – Porifera collection (MCNPOR).

Extract preparation

Aqueous extracts were prepared by the following procedure: the sponge *A. caissara* was extracted in ethanol and the remaining material was sequentially extracted four times with methanol (0.3 g/ml) by maceration over 4 days. The methanolic solution was reserved in each of these days. After the fourth day, the ethanol and methanol solutions were blended and filtered. After filtration, the extracted material was concentrated in a rotatory evaporator (Fisaton, Brazil) and the final aqueous extract was partitioned against hexane (1 : 1 v/v). The final extract was dried in SpeedVac (SPD1010; ThermoSavant, NY, USA).

Acute toxicity tests

The extract was suspended in water and administered intraperitoneally to four treated groups of mice. The control group received only the vehicle (saline solution, 0.9%) and the remaining groups ($n = 8$ per group) received increasing doses of extract of *A. caissara* administered intraperitoneally (58.75, 117.5, 235.0 and 470.0 mg/kg). Food and water were available freely during the experiment.

The general behavior of mice was observed continuously for 1 h after the treatment, and then after the first 6 h. After that, observations were made daily for the successive 72 h for short-term outcomes. Mice were further observed for up to 14 days following treatment for signs of toxicity and for the long-term possible outcome, which in this case was death. Behavioral manifestations of acute toxicity were also noted. All observations were systematically recorded and maintained for each mouse individually. The LD_{50} value was determined according to the method of Miller and Tainer [14]. In accordance with this acute toxicity test, the *A. caissara* extract was tested at concentrations of 60 and 90 mg/kg b.w. for the subsequent tests.

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. The number of abdominal writhing was counted cumulatively for a period of 25 min, 5 min after the administration [15]. For the writhing test, mice received the acetic acid injection 30 min after receiving their respective treatments ($n = 10$ per group); one group was pretreated with saline solution (0.9%; 0.1 mL/10 g body weight; i.p.). Two groups were pretreated with the *A. caissara* extract (60 and 90 mg/kg body weight; i.p.) Finally, one group was pretreated with the reference opioid analgesic, morphine (2.0 mg/kg body weight; i.p.), 60 min prior to the acetic acid injection. Antinociception was calculated as a percentage of inhibition of writhing constrictions using the formula [(control group mean – test group mean)/(control group)] × 100% [15].

Formalin test

In this test, 20 μL of 2.5% formalin solution was injected into the left hind paw of mice, in the five treatment groups ($n = 10$ per group), 30 or 60 min after they had been submitted to their respective treatments. The amount of time that each animal spent licking the paw was recorded during two 5-min intervals: the first beginning immediately after the injection (first phase) and the second 20 min after the injection [16] (second phase). One group, which served as the negative control group, was pretreated with saline solution (0.9%) in the appropriate volume (0.1 mL/10 g body weight; i.p.). Two groups were pretreated (30 min prior) with the *A. caissara* extract dose (60 and 90 mg/kg body weight; i.p.). Finally, two groups were pretreated (60 min prior to the formalin injection) with the reference drugs, with the first receiving morphine (2.0 mg/kg body weight; i.p.), the reference opioid analgesic, and the second receiving diclofenac (5 mg/kg body weight; i.p.), the reference anti-inflammatory non-steroidal drug. The formalin-induced licking of the paw was considered as an indicative of the nociceptive behavior. The total time spent in licking and biting the injected paw was recorded (chronometer), and used to quantify the nociceptive behavior.

Hot plate test

The hot plate test was used to measure the response latency according to the method described previously by Eddy and Leimbach [17], with minor modifications. In the current experiments, mice were placed individually on a hot plate (Insight®, Barcelona, Spain) maintained at 54 ± 1 °C before receiving their pretreatments. We recorded the time that elapsed until the animal jumped or licked one of its hind paws (latency time in seconds); this time was considered to be the reaction time. Mice showing a pretreatment reaction time greater than 12 s were not used in the subsequent test. A time limit of 30 s was imposed to avoid tissue damage. Reaction times were again measured in mice of the five groups ($n = 8$ per group) at 30, 60, 90, 120 and 150 min after pretreatment.

The groups were pretreated (30 min prior to the hot plate test) with the *A. caissara* extract (60 and 90 mg/kg body weight; i.p.) or saline (0.9%; 0.1 mL/10 g body weight; i.p.) and morphine group (2.0 mg/kg body weight; i.p.) was treated 60 min prior to the hot plate test.

Anti-inflammatory activity

Formalin-induced mice paw edema models

The anti-inflammatory activity of *A. caissara* extract (60 and 90 mg/kg body weight; i.p.) was assessed by the formalin [18] (2.5%, 20 μL) paw edema test in mice. Before formalin injection, the paw volume for each mouse was measured separately by means of a plethysmometer (Letica, Barcelona, Spain). Thirty minutes after the administration of the extract (60 and 90 mg/kg body weight; i.p.) or saline solution (0.9%; 0.1 mL/10 g body weight; i.p.) and 1 h after the administration of indomethacin (10 mg/kg body weight; i.p.) to mice, acute inflammatory edema was induced by subplantar injection into the right hind paw of mice ($n = 5$ per group) of freshly prepared suspensions of formalin.

The edema caused by formalin was measured at 30, 60, 90, 120, 150 and 180 min. The anti-inflammatory potential of the *A. caissara* extract was determined by comparing with the results of a group administered (i.p.) with indomethacin, and a control group, which was administered (i.p.) saline solution. The volume of the edema was expressed for each animal as the difference between before and after formalin- or carrageenin-injected paws.

Statistical analysis

The results were expressed as mean ± SEM. The data were analyzed by analysis of variance and complemented by Tukey's post hoc test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Acute toxicity

The mortality rate and the acute toxicity of the intraperitoneally administered *A. caissara* extract were progressive with the increasing dose (*Table I*): the mortality rate of 0% at and up to a dose of 58.75 mg/kg gradually rose to 100% at 470.0 mg/kg, the highest dose studied. The LD_{50} of *A. caissara* extract was 212.2 mg/kg for mice.

All mice died within 24 h after i.p. dose administration of 470 mg/kg. After autopsy, the appearance of organs and tissue, compared with the test control mice, were not significantly different.

Antinociceptive activity

Writhing test

The results presented in *Table II* show that the i.p. administered aqueous extract of *A. caissara* inhibited significantly ($P < 0.05$) the acetic acid-induced abdominal constriction. Both doses (60 and 90 mg/kg b.w.) were equally potent in inhibiting the constrictions in mice (33.7% and 41.4% respectively). However, significant difference was also found between morphine-administered mice and the extract groups. Morphine was more potent than the highest antinociceptive dose of the extract (90 mg/kg body weight; i.p.).

Formalin test

The i.p. administration of the aqueous extract of *A. caissara* in the formalin test significantly inhibited the licking behavior (*Table III*) during both early and late phases. Both 60 and 90 mg/kg doses were effective, inhibiting 43.6% and 51.6%, respectively, of the behavior in the first phase and 98.2% and 97.2%, respectively, in the second phase.

Hot plate test

Table IV shows the effects of the aqueous extract (60 and 90 mg/kg body weight; i.p.) of *A. caissara* in the hot plate test. A significant ($P < 0.05$) increase in reaction time was observed in the hot plate test in mice after 60 min of treatment with both doses of the extract of *A. caissara* when compared with the control. However, no significant effect was found at other times. Morphine, at a dose of 20 mg/kg, also showed a significant increase in reaction time at 30, 60 and 90 min following injection.

Table I Acute toxicity of a lyophilized extract of *Aplysina caissara* administered by intraperitoneal injection to mice.

Dose of extract (mg/kg)[a]	D/T	Mortality latency (h)	Toxic symptoms
0	0/8	–	None
58.7	0/8	–	Diarrhea
117.5	1/8	>48<72	Diarrhea, piloerection
235.0	4/8	>24<72	Piloerection, palpebral ptosis, hypoactivity
470.0	8/8	>24<24	Respiratory difficulties, piloerection, palpebral ptosis, hypoactivity

D/T, dead/treated mice; none, no toxic symptoms during the period; mortality latency, time to death (in days) after injection.

[a]The lyophilized extract of *A. caissara* was dissolved in saline solution (0.9%) and administered as a single intraperitoneal dose to groups of mice. Control group received saline solution (0.9%) only. Mice of each group ($n = 8$) were carefully examined for any signs of toxicity (behavioral changes and mortality) for 14 days.

Table II Effect of *Aplysina caissara* aqueous extract on acetic acid-induced writhing behavior in mice.

Treatment (mg/kg, i.p.)	Number of abdominal constrictions (during 25 min)	% of writhes inhibition
Control	58.7 ± 4.1	_
Morphine	0.8 ± 0.3*	98.6
Extract (60)	38.9 ± 4.4*	33.7
Extract (90)	34.4 ± 1.9*	41.4

Values are expressed as mean ± SEM. To the control group only saline solution (0.9%) was administered. i.p., intraperitoneal administration. $n = 10$ in each group. Differences between groups were statistically analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's test. *$P < 0.05$ compared with the control group.

Table III Antinociceptive effect of aqueous extract of *Aplysina caissara* on formalin-induced pain in mice.

Treatment group (mg/kg, i.p.)	First phase (s)	Inhibition (%)	Second phase (s)	Inhibition (%)
Control	53.7 ± 6.6	–	49.6 ± 10.6	–
Diclofenac (5)	61.3 ± 7.0	–	0.0 ± 0.0*	100
Morphine (20)	4.6 ± 1.7*	91.4	0.0 ± 0.0*	100
Extract (60)	30.3 ± 4.8*	43.6	0.9 ± 0.6*	98.2
Extract (90)	26.0 ± 4.9*	51.6	1.4 ± 0.9*	97.2

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. To the control group, only saline solution (0.9%) was administered. i.p., intraperitoneal administration. $n = 10$ in each group. Differences between groups were statistically analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's test. *$P < 0.05$ vs. control group.

Table IV Effect of aqueous extract of *Aplysina caissara* on heat-induced pain in mice (hot plate test).

	Latency (% increase)				
Treatment (mg/kg, i.p.)	0 min	30 min	60 min	90 min	120 min
Control	3.8 ± 0.5	4.4 ± 0.9 (15.8)	4.5 ± 0.7 (18.4)	6.6 ± 0.8 (73.7)	6.9 ± 1.3 (81.6)
Morphine (20)	4.8 ± 0.5	14.8 ± 2.6 (208.3)*	17.1 ± 3.2 (256.2)*	20.6 ± 3.1 (329.2)*	10.5 ± 3.7 (118.7)
Extract (60)	5.1 ± 0.6	11.8 ± 3.0 (131.4)	18.8 ± 2.7 (268.6)*	14.4 ± 2.3 (182.4)	13.9 ± 2.4 (172.5)
Extract (90)	5.0 ± 0.7	9.4 ± 0.8 (88)	15.9 ± 2.9 (218)*	14.7 ± 2.0 (194)	10.5 ± 2.1 (110)

Mice were placed on the hot plate at 0, 30, 60, 90 and 120 min after treatment. Latency values are expressed as mean ± SEM. The percentage of increase in hot plate latency from 0 min is given in parentheses. To the control group, only saline solution (0.9%) was administered. i.p., intraperitoneal administration. $n = 8$ in each group. Differences between groups were statistically analyzed by a one-way analysis of variance (ANOVA).

Table V Effect of *Aplysina caissara* extract on formalin-induced paw edema in mice.

Treatments	Edema rate (mL)						
(mg/kg; i.p.)	0 min	30 min	60 min	90 min	120 min	150 min	180 min
Control	0.270 ± 0.029	0.363 ± 0.015	0.380 ± 0.018	0.418 ± 0.013	0.430 ± 0.015	0.433 ± 0.018	0.453 ± 0.033
Indomethacin	0.245 ± 0.010	0.303 ± 0.018*	0.300 ± 0.016*	0.308 ± 0.013*	0.343 ± 0.023*	0.348 ± 0.024*	0.363 ± 0.028*
Extract (60)	0.225 ± 0.005	0.258 ± 0.013*	0.255 ± 0.019*	0.295 ± 0.022*	0.300 ± 0.023*	0.323 ± 0.018*	0.333 ± 0.031*
Extract (90)	0.255 ± 0.005	0.303 ± 0.009*	0.313 ± 0.008*	0.315 ± 0.005*	0.330 ± 0.006*	0.335 ± 0.006*	0.330 ± 0.006*

Values are expressed as mean ± SEM. To the control group, only saline solution (0.9%) was administered. i.p., intraperitoneal administration. $n = 5$ in each group. Differences between groups were statistically analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's test. $^*P < 0.05$ compared with the control group.

Anti-inflammatory activity – edema induced by formalin

Intraplantar injection of formalin to mice caused an inflammatory reaction. The intra-peritoneal pretreatment with *A. caissara* extract (60 and 90 mg/kg body weight; i.p.) reduced hind paw swelling (*Table V*). This inhibition effect was observed during the whole experiment and was as effective as indomethacin in this model.

DISCUSSION AND CONCLUSIONS

This study is the first report on antinociceptive and anti-inflammatory effects induced by aqueous extracts of *A. caissara* in mice. The antinociceptive effect of the extract was elicited regardless of the noxious stimulus used: heat (hot plate) or chemical agents (acetic acid and formalin).

The antinociceptive activity was evaluated by acetic acid-induced writhing response. The writhing response to an intraperitoneal injection of acetic acid (somewhat unspecific) is used to screen for both peripheral and central antinociceptive activities. Acetic acid acts indirectly by inducing the release of endogenous mediators that stimulate the nociceptive neurons that are sensitive to non-steroidal anti-inflammatory drugs and opioid [19].

Choi et al. [20] showed that indomethacin significantly inhibited the writhing response to acetic acid. In this model, the nociceptive response induced by endotoxin appears to result from the release of TNF-α, interleukin-1β and interleukin-8 by resident peritoneal macrophage and mast cells [21]. The participation of eicosanoids and sympathomimetic amines in the nociceptive responses induced by acetic acid has also been demonstrated [22]. We have shown that diclofenac decreased and morphine antagonized the amount of writhing induced in mice [23].

Aqueous extract of *A. caissara* reduced the amount of writhing and stretching induced by the 0.6% acetic acid solution. In this test, our findings showed a significant decrease in the number of acetic acid-induced abdominal constrictions. The mechanism of the analgesic effect of *A. caissara* extract may be due to the blockade or the release of endogenous substances that excite pain nerve endings similar to indomethacin, which is mediated via peripheral mechanism.

In the formalin 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 [16]. Experimental results have demonstrated

that several mediators, for example, 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, and are involved in the late phase of formalin test [24].

Drugs that act primarily on the central nervous system inhibit both phases equally, while peripherally acting drugs inhibit the late phase [25]. In previous studies, we have reported an inhibitory effect of morphine and diclofenac on formalin test in mice [23].

Aplysina caissara extract significantly inhibited both phases of the formalin test, but their effect was more pronounced in the late phase of the formalin test, and suggest that peripheral mechanism are involved and also may exhibit an associated anti-inflammatory effect, since the anti-inflammatory drugs exhibited 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 but not in the early one.

Our results showed the inhibitory effect of the extract mainly on the nociceptive response in the late phase of the formalin test, and suggest that the antinociceptive effect of the extract could be due to its peripheral (or anti-inflammatory) action. The peripheral analgesic effect of the extract may be mediated via inhibition of cyclooxygenases and/or lipoxygenases (and other inflammatory mediators).

It is well established that the use of methods such as the hot plate test to gauge reflex latency reactions to thermal stimulation is an appropriate means of measuring the antinociceptive effect of opioid analgesics. These methods, however, are insensitive to non-steroidal analgesics like cyclooxygenase inhibitors [26]. Although the extract of *A. caissara* presented antinociceptive activity in the first phase of the formalin test, this central protective effect was not corroborated in the hot plate test and other experiments are necessary to confirm these results. In the present study, morphine, a centrally active analgesic drug, produced antinociceptive effect more than the *A. caissara* extract in the hot plate test. This is consistent with our results that show the effectiveness of morphine in this test [23].

The formalin-induced inflammation usually involves two different phases: the initial phase is due to the direct stimulation of nociceptors, while the second may be associated with inflammation mediators [27–29]. Histamine, nitric oxide, serotonin, bradykinin, prostaglandin and substance P are involved in the increase in vascular permeability and paw edema [30]. Some studies have shown that substance P receptor antagonists inhibit the later phase of formalin-induced edema, and substance P has a role in this response [31].

In the present study, the effect of an extract from *A. caissara* on acute inflammation was investigated on formalin-induced paw edema. It was seen that both doses of *A. caissara* (60 and 90 mg/kg) reduced inflammation induced by formalin and the effect was higher than that by indomethacin.

The chemical studies with another species of marine sponges revealed the presence of many chemicals such as manoalide, an inhibitor of arachidonic acid from membrane phospholipids [32,33], cavernolide, a potent inhibitor of tumor necrosis factor-α (TNF-α), nitric oxide and prostaglandin E_2, [34], cyclolinteinone, an inhibitor of inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 [35] and halipeptin A, which have shown anti-inflammatory activity on mouse paw edema assay [2].

The different species of *Aplysina* (Porífera, Desmospongiae and Verongida) can be distinguished by the presence of carotenoids [36–38]. Astaxanthin is a carotenoid that is found in marine animals and vegetables, and previous studies have demonstrated that astaxanthin has a dose-dependent ocular anti-inflammatory effect by the suppression of NO (nitric oxide), PGE_2 and TNF-α production through directly blocking NOS enzyme activity [39].

The component(s) of the whole sponge extract responsible for the toxic manifestations after the intraperitoneal dose are not known. However, toxicity and lethality of the *A. caissara* extract may be due to one or more compounds present in the crude extract, some of which have been isolated and their cytotoxicity assessed [11]. Recently, we have demonstrated that the crude extracts of *A. caissara* are toxic against a hepatoma cell line and *Mycobacterium tuberculosis* and capable of inhibiting cellular growth [40].

While a more exhaustive pharmacological investigation on the extract of *A. caissara* is needed to characterize the exact target(s) of the compounds, it is clear that the extract display anti-inflammatory effect in vivo.

In summary, the results of the present study demonstrate that the extract obtained from marine sponge *A. caissara* exhibits analgesic and anti-inflammatory effects against some classical models of nociception and inflammation in mice.

These findings encourage further pharmacological studies, to evidence the mechanism of action of the extract, as well to isolated active compounds present in *A. caissara* extract.

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