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Antinociceptive activity and acute toxicological study of a novel sulfated polysaccharide from *Caulerpa cupressoides* var. *lycopodium* (Chlorophyta) in Swiss mice

José Ariévilo Gurgel Rodrigues[1], Edfranck de Sousa Oliveira Vanderlei[2], Ana Luíza Gomes Quinderé[2], Valdécio Silvano Monteiro[2], Silvânia Maria Mendes de Vasconcelos[3] and Norma Maria Barros Benevides[1*]

*[1]Laboratório de Carboidratos e Lectinas, Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Av. Mister Hull, s/n, 60455-970, Fortaleza, Ceará, Brazil. [2]Programa de Pós-graduação em Bioquímica, Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil. [3]Departamento de Fisiologia e Farmacologia, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil. *Author for correspondence. E-mail: nmbb@ufc.br*

ABSTRACT. *Caulerpa cupressoides* var. *lycopodium* (Chlorophyta) contains three sulfated polysaccharides (SPs) fractions (SP_1, SP_2 and SP_3); but, their pharmacological properties have been limited. We investigated the antinociceptive activity of non-anticoagulant fraction (SP_1) and then its acute toxicological study in male Swiss mice was performed. Animals (19-25 g) received i.v. SP_1 30 min. prior to injection 0.8% acetic acid (10 mL kg^{-1}, i.p.); 1% formalin (20 μL, i.pl.) or were subjected to thermal stimuli. Open-field test was also performed. Mice were treated i.p. with SP_1 or 0.9% saline (0.1 mL 10 g^{-1}) for 72h. On the 4th day, the animals were anesthetized and sacrificed in order to collect blood and organs. SP_1 (3, 9 or 27 mg kg^{-1}) reduced ($p < 0.05$) the number of writhes induced by acetic acid by 44.21, 47.72 and 90.87%, respectively. SP_1 inhibited ($p < 0.05$) the second phase of the formalin test, without antinociceptive effect in the hot-plate test, suggesting that its analgesic action occurs through of peripheral mechanisms. SP_1 did not modify the locomotor activity. SP_1 (27 mg kg^{-1}) did not cause hepatic or renal dysfunctions, but affected the spleen of animals ($p < 0.05$). Therefore, SP_1 has analgesic action with high tolerance by the animals, presenting its potential applicability in pain conditions.

Keywords: green alga, sulfated sugars, nociception, biochemical analysis.

Atividade antinociceptiva e estudo toxicológico agudo de um polissacarídeo sulfatado novo de *Caulerpa cupressoides* var. *lycopodium* (Chlorophyta) em camundongos Swiss

RESUMO. A Chlorophyta *Caulerpa cupressoides* var. *lycopodium* possui três frações de polissacarídeos sulfatados (PSs) (PS_1; PS_2 e PS_3). Entretanto, são limitadas suas propriedades farmacológicas. Investigou-se a atividade antinociceptiva da fração não-anticoagulante PS_1 e, em seguida, foi desenvolvido seu estudo toxicológico agudo em camundongos Swiss machos. Os animais (19-25 g) receberam i.v. PS_1 30 min. antes da injeção de ácido acético 0,8% (10 mL kg^{-1}; i.p.); formalina 1% (20 μL; i.pl.) ou foram submetidos a estímulo térmico. Também foi utilizado o teste de campo aberto. Os camundongos foram tratados i.p. com PS_1 ou salina 0,9% (0,1 mL 10 g^{-1}) durante 72h. No quarto dia, os animais foram anestesiados e sacrificados para coleta de sangue e órgãos. A PS_1 (3, 9 ou 27 mg kg^{-1}) reduziu ($p < 0,05$) o número de contorções induzidas por ácido acético em 44,21; 47,72 e 90,87%, respectivamente, além de inibir ($p < 0,05$) a segunda fase do teste da formalina. Entretanto, não se apresentou efeito antinociceptivo no teste da placa quente, sugerindo ação analgésica mediante mecanismo periférico. A PS_1 não modificou a atividade locomotora. A PS_1 (27 mg kg^{-1}) não causou disfunção hepática ou renal, mas afetou o baço dos animais ($p < 0,05$). Portanto, PS_1 possui ações analgésicas e mostrando-se tolerante aos animais, apresentando aplicabilidade potencial em condições dolorosas.

Palavras-chave: alga verde, açúcares sulfatados, nocicepção, análises bioquímicas.

Introduction

In recent years, the human consumption of seaweeds as food (thickening, gelling and stabilizing, nutrients, etc.) (CAMPO et al., 2009; MARINHO-SORIANO et al., 2006; SMIT, 2004), and the search for bioactive products (with anticoagulant, antinociceptive, anti-inflammatory effects etc.) (ARAÚJO et al., 2012; POMIN, 2012; RODRIGUES et al., 2011a; VANDERLEI et al., 2010) with minimum adverse effects (ALMEIDA-LIMA et al., 2011; ASSREUY et al., 2008; LI

et al., 2005; VANDERLEI et al., 2010) have increased worldwide.

In review of Smit (2004), natural products from seaweeds have attracted commercial significance and influenced the modern western societies. Marine algae have been mainly used in medical and biochemical research. Of all seaweed products, sulfated polysaccharides (SPs), including galactan (Rhodophyta) (CAMPO et al., 2009; POMIN, 2012), fucan or fucoidan (Phaeophyta) (ALMEIDA-LIMA et al., 2011; LI et al., 2005), and arabinogalactan (Chlorophyta) (GHOSH et al., 2004; HAYAKAWA et al., 2000), are the most frequently found. These anionic polymers comprise the extracellular matrix of these marine organisms playing an important protective role (ANDRADE et al., 2010).

Although widely studied as anticoagulant and antithrombotic agents (ALMEIDA-LIMA et al., 2011; LI et al., 2005; PEREIRA et al., 2005), SPs may modulate a large number of other biological activities, including antiviral (GHOSH et al., 2004), antinociceptive, pro-inflammatory (ARAÚJO et al., 2011; ASSREUY et al., 2008; VIANA et al., 2002), antitumor (LINS et al., 2009) and anti-inflammatory properties (ARAÚJO et al., 2012; COURA et al., 2012; SIQUEIRA et al., 2011). It has been accepted that enzymatic digestion of seaweed results in high bioactive yield and shows biological activity in comparison with water extract (RODRIGUES et al., 2011a). In addition, the use of enzymes may be useful to extract pharmaceutically important materials without toxicity (HEO et al., 2005).

The genus *Caulerpa* Lamouroux (1809) of green seaweed of the family Caulerpaceae encompasses about one hundred species, found in tropical and subtropical waters. Algae from this genus have highly invasive capacity in the marine environment, being important contributors to the algal biomass of coral reefs and lagoons (TRI, 2009). Some metabolites with chemotaxonomic and medicinal significance have been isolated (MAO et al., 2011; RODRIGUES et al., 2012). In Brazil, some species of this genus have already been registered (RODRIGUES et al., 2012; VANDERLEI et al., 2010). Anticoagulant, antithrombotic, antitumor, antiviral and pro-thrombotic activities of the *Caulerpa* SPs have been described in the literature (GHOSH et al., 2004; HAYAKAWA et al., 2000; JI et al., 2008; RODRIGUES et al., 2011b). However, there are few studies on the biological potential of *Caulerpa* SPs. In addition, report in animal models of nociception and locomotion of seaweeds SPs, to the best of our knowledge, has not been associated to data.

Pain promotes an uncomfortable sensation to patients, and may be caused by tissue lesions or by an independent manner, being thus a complex interaction between peripheral and central structure from skin to central cerebral cortex. Tissue damage (due to inflammation or injury) may result in chronic neuropathic pain caused by increased sensitivity to painful stimuli (MOALEM; TRACEY, 2006). Animal models of nociception have been widely used as important tools for discovering new natural analgesic compounds (ARAÚJO et al., 2011; ASSREUY et al., 2008; COURA et al., 2012; FARIAS et al., 2011; VANDERLEI et al., 2010; VIANA et al., 2002).

The non-steroidal anti-inflammatory drugs are commonly used in the suppression of the inflammatory reaction in medical clinic, having the property to inhibit initial or latter manifestations. However, the prolonged administration of these therapeutic agents is usually followed by complications (gastric perforations, stomach ulcers and bleeding) (IWALEWA et al., 2007).

Issues about the safety of SPs are still rarely reported (CAMPO et al., 2009; LI et al., 2005; RODRIGUES et al., 2011c). Almeida-Lima et al. (2011) examined the subchronic toxicity *in vivo* of a heterofucan (Fucan A) from *Spatoglossum schröderi* (Phaeophyta) and no toxicologically significant changes were observed in the biochemical or hematological parameters in treated Wistar rats. In the present study, it was investigated the effects of a novel antinociceptive non-anticoagulant SP from *C. cupressoides* var. *lycopodium* (Chlorophyta) using experimental animal models of nociception and locomotion. The safety of this compound was also evaluated using an acute toxicity model in Swiss mice.

Material and methods

Experimental design

The crude SP was extracted from *C. cupressoides* and the antinociceptive activity of a non-anticoagulant fraction (SP_1) was tested *in vivo* with experimental animal models of nociception and locomotion using male mice. The effects on some biochemical parameters were also evaluated in mice treated for 72h using an acute toxicity model.

Marine alga and isolation and chemical composition of SPs

Specimens of the green alga *C. cupressoides* var. *lycopodium* (Vahl) C. Agardh (Chlorophyta, Caulerpaceae) were collected (September, 2009) on the seashore of Flecheiras Beach on the Atlantic coast of Ceará State, Brazil. The algal samples were taken to the Carbohydrate and Lectins Laboratory

(CarboLec), Department of Biochemistry and Molecular Biology, Federal University of Ceará, Brazil, and cleaned out of epiphytes, washed with distilled water and stored at -20°C until use. A voucher specimen (4977) was classified and cataloged by Ana Cecília Fortes Xavier at the Prisco Bezerra Herbarium, Federal University of Ceará. The crude SP was extracted by proteolytic digestion (papain) and separated into three different SP fractions (SP_1, SP_2, and SP_3) by ion-exchange chromatography (DEAE-cellulose). The chemical composition (total sugars [TSs], sulfate [S] and contaminant protein [CPs] content) of the fractions was also measured as previously reported by Rodrigues et al. (2011b).

Animals

Male Swiss mice (19-25 g) were randomly selected from the Animal House of the Federal University of Ceará, maintained on a 12h light/dark cycle, at temperature-controlled rooms and received water and food *ad libitum*. For the experiments, a total of 246 animals were used. All procedures and animal treatments were conducted in accordance with the guidelines for Institutional Animal Care and Use of the Federal University of Ceará, Ceará State, Brazil, previously approved by 80/10 protocol. In addition, the tested doses and administration route of SP_1 used in the experimental models were determined as previously reported (COURA et al., 2012).

Acetic acid-induced writhing test

This test was performed according to Collier et al. (1968). Groups of six mice received intravenously (i.v.) 0.9% saline (0.2 mL) or SP_1 (3, 9 or 27 mg kg^{-1} body weight). After 30 min., animals received a 0.8% acetic acid injection (10 mL kg^{-1}, i.p.). The number of writhings, determined by abdominal muscle contractions and kind paw extension, was recorded for 30 min. Two groups (n = 6) of animals received 5 mg kg^{-1} (s.c) of either morphine (Dimorf®, Cristália; Itapira, São Paulo State, Brazil) or indomethacin (Indocid®, Merck Sharp and Dohme; Campinas, São Paulo State, Brazil) 30 min. before noxious stimulus, and were used as standard.

Formalin test

This test has been used as a model for tonic pain and localized inflammatory pain (HUNSKAAR et al., 1985). Groups of six mice received intravenously (i.v.) SP_1 (3, 9 or 27 mg kg^{-1} body weight) or 0.9% saline. After 30 min. of administration, 1% aqueous formalin (20 μL) (Sigma; St. Louis, Missouri State, U.S.A.) was injected into the right hind paw. The time that the animal spent licking the injected paw was measured during the first 5 min. (phase 1, neurogenic) and 20-25 min. after formalin injection (phase 2, inflammatory). Morphine (Dimorf®, Cristália; Itapira, São Paulo State, Brazil) or indomethacin (Indocid®, Merck Sharp and Dohme; Campinas, São Paulo State, Brazil), both 5 mg kg^{-1} (s.c.), were used as standards.

Hot-plate test

This test is also used to measure the antinociceptive activity (EDDY; LEIMBACH, 1953). Groups of six animals were used. Each mouse was dropped twice on the heated plate (51 ± 1°C), separated by a 30 min. interval. The first trial familiarized the animal with the test procedure and the second served as the control reaction time (licking the paw or jumping). Animals showing a reaction time longer than 10 s were discarded. Immediately after the second trial (control reaction time), groups of six mice each received intravenously (i.v.) 0.9% saline in a volume of 10 mL kg^{-1}, SP_1 (3, 9 or 27 mg kg^{-1} body weight), morphine (Dimorf®, Cristália; Itapira, São Paulo State, Brazil) or indomethacin (Indocid®, Merck Sharp and Dohme; Campinas, São Paulo State, Brazil), both 5 mg kg^{-1} (s.c.). Reaction times were measured at time zero (0 time) and 30, 60 and 90 min. after administration, with a cut-off time of 40 s to avoid paw lesions.

Open-field test

This test was performed according to Archer (1973). The open-field area was made of acrylic transparent walls and black floor (30 × 30 × 15 cm), divided into nine squares of equal area, to evaluate the locomotor activity of the animal for 5 min. The following parameters were observed: number of squares crossed and number of grooming and rearings. The animals were divided into five groups of 8 animals each. The groups were treated with: control saline (0.9%, i.v.), SP_1 (3, 9 or 27 mg kg^{-1}, i.v.) and Diazepam (2 mg kg^{-1}, i.p.) (União Química; Campinas, São Paulo State, Brazil).

Acute toxicity model

This assay was based on Monji et al. (2011). Body mass loss, organ weight change, and blood biochemical parameters (alanine aminotransferase (AST), aspartate aminotransferase (ALT) and urea) were evaluated after acute treatment with single doses of SP_1 (27 mg kg^{-1}, i.p.), with an important antinociceptive effect, or 0.9% saline (i.p.) for 72h. For the *in vivo* assay, the male mice (6 animals per group) were weighed before the SP_1 (dissolved in

0.9% saline) administrations. During the assay, all animals had free access to water and food. Also, the animals were daily observed considering the following aspects: body mass variation, survival rate, mucosa, eyes, hair erection, scratching or licking paws, freezing reactions, general behavior, among others. After treatment, mice were again weighed and peripheral blood was collected for biochemical dosage (determined by enzymatic and colorimetric tests-Labtest, São Paulo, São Paulo State, Brazil). After sacrificing the animal, liver, kidney, heart, spleen, thymus and lymph nodes were removed and weighed.

Statistical analyses

All results are presented as mean ± standard error of the mean (S.E.M.). Data from the animals submitted to nociception or open-field tests were analyzed by ANOVA followed by Bonferron's or Student-Newman-Keuls *post hoc* test. $p < 0.05$ or $p < 0.001$ was considered as significant. Student t-test was also used, considering $p < 0.05$ as significant, when the group of animals that received SP_1 for 72 h on acute assay was evaluated. For these analyses, the software GraphPad Prism (5.0 version) was used.

Results and discussion

The *C. cupressoides* SPs have been extracted and studied. This species contains three different SPs fractions (SP_1, SP_2, and SP_3), when eluted at different NaCl concentrations (0.5, 0.75 and 1 M, respectively) on DEAE-cellulose column (RODRIGUES et al., 2011b, 2012). The Table 1 shows the chemical composition of SPs fractions obtained by ion-exchange chromatography (DEAE-cellulose) of the *C. cupressoides* crude polysaccharide extract. According to the quantitative chemical analysis, the S and TSs contents were high in all SP fractions, being the lowest data found for SP_1, with about 16.93% S and 13.67% TSs, respectively. As also reported, CPs were not detected in the crude extract and SP fractions from this species (RODRIGUES et al., 2011b).

Table 1. Chemical composition of crude extract and SP fractions from *Caulerpa cupressoides* var. *lycopodium*.

Polysaccharide	NaCl[a] (M)	TSs[b] (%)	S[c] (%)	CPs[d] (%)
Crude extract	-	47.23	22.30	-
SP_1	0.50	13.67	16.93	-
SP_2	0.75	39.78	29.86	-
SP_3	1.00	18.61	24.79	-

a – SPs eluted at different NaCl concentrations (DEAE-cellulose); b, c and d – Dosages of total sugars [TSs], sulfate [S] and contaminant proteins [CPs], respectively. Data are the mean value of three determinations; - not detected.

In previous studies, it was demonstrated that only the SP_2 fraction from *C. cupressoides* showed an anticoagulant activity (*in vitro*) by the activated partial thromboplastin time test. Fractions SP_1 and SP_3 were not capable of prolonging the coagulation time *in vitro* (RODRIGUES et al., 2011b, 2012). Fraction SP_2 also presented antithrombotic and prothrombotic activities (*in vivo*) (RODRIGUES et al., 2011b). Li et al. (2005) reported that the use of anticoagulant SPs in animal studies significantly altered the clotting time, and it could be considered a adverse effect for treating patients subjected to chronic renal diseases, when administrated at high doses (900 and 2500 mg kg^{-1}, oral administration). Recently, a non-anticoagulant SP, but with *in vivo* anti-inflammatory effect from five sequential extractions, was obtained from the marine brown alga *Lobophora variegata* (SIQUEIRA et al., 2011). Based on these hypotheses, we chose the SP_1 (that had higher yield compared with SP_3) to be further investigated in nociception and locomotion animal models.

Effect of pretreatment with SP_1 on the acetic acid-induced writhing test

To verify the antinociceptive effect of *C. cupressoides*, we previously tested SP_1 fraction using the acetic acid-induced writhing test, as shown in Figure 1. The mice pretreated (i.v) with SP_1 (3, 9 or 27 mg kg^{-1}), injected 30 min prior to 0.8% acetic acid, reduced ($p < 0.001$) the number of writhes by 44.21% (26.5 ± 4.02), 47.72% (24.83 ± 3.28) and 90.87% (4.33 ± 1.05), respectively, compared with the saline group (47.5 ± 2.21). Also, the dose of 27 mg kg^{-1} produced a significantly ($p < 0.001$) analgesia among all doses tested. For this experiment, animals pretreated with morphine or indomethacin (both 5 mg kg^{-1}, s.c.) were used as positive controls, and also resulted in antinociceptive actions (98.59% (0.66 ± 0.49) and 63.15% (17.50 ± 2.66), respectively) ($p < 0.001$).

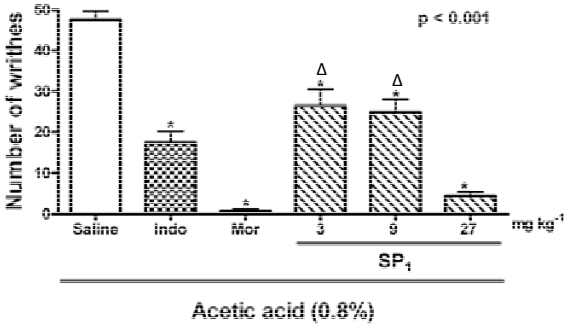

Figure 1. Effect of SP_1 on writhing response induced by acetic acid in mice. Data are expressed as mean ± S.E.M. (n = 6). *$p < 0.001$ compared with saline group; $^{\Delta}p < 0.001$ compared with the dose of 27 mg kg^{-1} (ANOVA, Bonferroni test).

This study corroborates the literature that shows seaweeds SPs as molecules capable of reducing the number of abdominal constrictions induced by the acetic acid (ARAÚJO et al., 2011; ASSREUY et al., 2008; VIANA et al., 2002). Interestingly, the antinociceptive profile of SP_1 (*C. cupressoides*), when evaluated by acetic acid-induced writhing test (Figure 1), was similar to that found for *Gracilaria cornea* (Rhodophyta) SPs by Coura et al. (2012). However, the antinociceptive effects are described uniquely for SPs from red and brown seaweeds (ASSREUY et al., 2008; FARIAS et al., 2011; VIANA et al., 2002), being rarely reported for SPs from Chlorophyta. SP_1 could inhibit the mediators released in response to chemical stimulus (acetic acid), such as bradykinin, substance P, prostaglandin, and some cytokines, such as IL-1β, TNF-α and IL-8 (COLLIER et al., 1968).

On the other hand, it has been reported the low specificity of writhing assays (COLLIER et al., 1968), requiring caution when interpreting the results until the performance of other tests. In order to support this hypothesis, two more complementary tests (formalin and hot-plate) were performed to prove the antinociceptive action of SP_1.

Effect of pretreatment with SP_1 on the formalin test

In the formalin test, characterized by two phases (Figure 2), the mice pretreated (i.v.) with SP_1 (27 mg kg^{-1}), injected 30 min prior to 1% formalin, have reduced ($p < 0.05$) by 51.61% (32.33 ± 3.19 s) the licking time in first phase (neurogenic) from control (67.5 ± 7.26 s) (Figure 2, panel A). Also, SP_1 (3, 9 or 27 mg kg^{-1}) produced a significant inhibition of the formalin response during the second phase (inflammatory) (Figure 2, panel B), by 56.41% (25.5 ± 2.26 s), 72.08% (16.33 ± 1.02 s) and 83.48% (12.67 ± 4.44 s), respectively ($p < 0.05$) compared with saline group (58.5 ± 7.37 s). Indomethacin (5 mg kg^{-1}, s.c.) led to a significant inhibition, especially on the second phase (31.85% (46 ± 7.04 s) -1st phase and 53.56% (27.17 ± 9.26 s) -2nd phase, respectively).

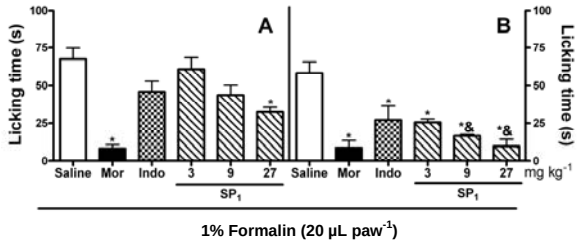

Figure 2. Effect of SP_1 on the licking time induced by formalin in mice. Data expressed as mean ± S.E.M. (n = 6). *$p < 0.05$ compared with saline group; &$p < 0.05$ compared with *C. cupressoides* SP_1 (3 mg kg^{-1}) (ANOVA, Bonferroni test).

As expected, morphine, used as another positive control, also caused a reduced licking time, during both phases (87.9% (8.16 ± 2.54 s) -1st phase and 85.16% (8.66 ± 4.8 s) -2nd phase, respectively ($p < 0.05$).

SP_1 presented a greater antinociceptive effect in the second phase, suggesting that its action is also related to inflammatory pain (Figure 2) (ARAÚJO et al., 2011; COURA et al., 2012; VANDERLEI et al., 2010). Moreover, the late phase seems to be an inflammatory response to inflammatory pain that can be modulated by anti-inflammatory drugs, which inhibit inflammatory mediators, such as serotonin, bradykinin and prostaglandin (HUNSKAAR et al., 1985). In recent studies, an anticoagulant SP from *Champia feldmannii* (Rhodophyta) produced a pro-inflammatory effect (ASSREUY et al., 2008), whereas a non-anticoagulant SP from the brown alga *L. variegata* was an anti-inflammatory compound (SIQUEIRA et al., 2011). Araújo et al. (2012) reported a crude extract containing ι-carrageenans from *Solieria filiformis* (Rhodophyta) without anti-clotting effect, but able to inhibit the cellular infiltrate in the peritoneal cavity of rats. There is a lack of structure-function relationship data concerning the anti-inflammatory action of these molecules (POMIN, 2012).

In this study, the SP_1 (27 mg kg^{-1}) also inhibited the first phase (Figure 2, panel A) compared with saline group, suggesting a possible interaction of this molecule with opioid systems. A similar result was suggested for a SP from *S. schroederi* (Phaeophyta) (FARIAS et al., 2011) and SPs extracted from *G. cornea* (Rhodophyta) (COURA et al., 2012).

Literature describes that the inflammatory hypernociception occurs at least in part as a phenomenon of sensitization of primary afferent nociceptors (MOALEM; TRACEY, 2006). In the inflammatory process, the neutrophils migration occurs in response to mechanical hypernociception, where the release of mediators is observed, such as prostaglandin (CUNHA et al., 2008). Formalin-induced nociception is attributed to an increase in peritoneal fluid levels of several mediators, such as histamine, serotonin, cytokine, and eicosanoid (HUNSKAAR et al., 1985). The greater antinociceptive effect of SP_1 in the second phase (Figure 2, panel B) could be the result of drugs related to inflammatory pain (ARAÚJO et al., 2011; VANDERLEI et al., 2010), inhibiting the pain in terms of synaptic transmission at the spinal level in response to formalin-action (HUNSKAAR et al., 1985).

Effect of pretreatment with SP_1 on the hot-plate test

To distinguish between peripheral and central action, SP_1 was also used in the hot-plate test, a specific test of central antinociception in which the opioid agents exert analgesic effects *via* supra spinal and spinal receptors (EDDY; LEIMBACH, 1953). *C. cupressoides* SP_1 did not increase ($p > 0.05$) the latency of animals (3 mg kg^{-1}– 30 (7.1 ± 1.1 s), 60 (8.2 ± 1.2 s) and 90 (8.7 ± 1.0 s) min.; 9 mg kg^{-1} – 30 (6.9 ± 1.2 s), 60 (7.2 ± 1.1 s) and 90 (8.5 ± 1.2 s) min.; and 27 mg kg^{-1} – 30 (7.3 ± 1.4 s), 60 (8.1 ± 1.2 s) and 90 (9.3 ± 1.3 s) min., respectively) when compared with the saline (at 30 (5.83 ± 1.01 s), 60 (4.5 ± 0.42 s) and 90 (5.16 ± 0.7 s) min., respectively), indomethacin (at 30 (8.4 ± 1.2 s), 60 (8.6 ± 1.3 s) and 90 (9.5 ± 1.1 s) min., respectively), and morphine (at 30 (33.4 ± 0.9 s), 60 (28.6 ± 1.2) and 90 (17 ± 1.1 s) min., respectively) groups, and corroborating thus with the data previously found in the formalin-test that showed antinociceptive effects *via* peripheral, rather than central-acting mechanisms (Figure 2, panel B).

Based on these findings, it was suggested that the SP_1 from green seaweed *C. cupressoides* exhibits antinociceptive effects predominantly through a peripheral mechanism similar to the lectin (protein) isolated from this same species by Vanderlei et al. (2010). These antinociceptive actions of SP_1 seemed not to be associated with central neurotransmission (FARIAS et al., 2011).

Effect of pretreatment with SP_1 on the open-field test

Once the SP_1 (*C. cupressoides*) showed antinociceptive effects, it was also explored this molecule on the open-field test, a classic animal model that evaluates autonomic effects and general activity (ARCHER, 1973). Our data showed that the SP_1 did not induce changes ($p > 0.05$) in the locomotor activity for number of squares, grooming, and rearing of mice. Diazepam (2 mg kg^{-1}) influenced ($p < 0.05$) the locomotor behavior of the animals (Table 2).

Table 2. Open-field test with mice treated with SP_1 from *Caulerpa cupressoides* var. *lycopodium* and diazepam.

Group	Parameter		
	NSC	G	R
Control	60.14 ± 4.95	1.75 ± 0.36	3.20 ± 0.73
Diazepam	22.40 ± 0.90⋆	0.89 ± 0.43⋆	0.73 ± 0.55⋆
3 mg kg^{-1}	53.17 ± 6.88	3.60 ± 0.92	2.77 ± 1.62
9 mg kg^{-1}	53.67 ± 6.34	3.20 ± 0.37	2.80 ± 1.67
27 mg kg^{-1}	54.78 ± 5.41	1.66 ± 0.33	2.89 ± 0.53

The parameters analyzed were: number of squares crossed (NSC), grooming (G) and rearing (R). The results are presented as means ± S.E.M. (n = 6). ⋆$p < 0.05$ compared to control group. (ANOVA and Student-Newman-Keuls *post hoc* test).

Toxicological evaluation

In order to investigate the safety for 'short term' use of *C. cupressoides* SP_1 (27 mg kg^{-1}, i.p., dose selected from antinociceptive assay data obtained in Figure 2), a toxicological assay was performed with male Swiss mice for 72h (Table 3). The administration with a single injection of SP_1 did not cause animal mortality as well as variation in body mass or wet weight of organs (heart, kidney, liver, thymus, and lymph nodes) compared with saline group.

During the experimental period, some physical and behavior parameters were also observed in animals, such as mucosa or eyes, hair erection, scratching or licking paws, freezing reactions and general behavior. All these aspects were considered normal (ALMEIDA-LIMA et al., 2011; ARAÚJO et al., 2011; ASSREUY et al., 2008; COURA et al., 2012; LI et al., 2005; LINS et al., 2009; MONJI et al., 2011; RODRIGUES et al., 2011c; SIQUEIRA et al., 2011). Except for the spleen that presented an increase more than 2.5-fold compared with control ($p < 0.05$) (Table 2), suggesting that SP_1 stimulated the immune function of mice (COURA et al., 2012). Probably, SP_1 induced a proliferation of T lymphocytes and production of specific antibodies (LINS et al., 2009; ZHANG et al., 2003). Furthermore, the histological structure of the spleen of animals treated with SP_1 was essentially preserved (data not shown), based on Coura et al. (2012).

Table 3. Body mass and weight of organs (w w^{-1}) of mice treated with SP_1 from *Caulerpa cupressoides* var. *lycopodium* after 72h.

Parameter	Treatment (i.p.)	
	Saline (0.9%)	SP_1 (27 mg kg^{-1})
Body mass before treatment	23.19 ± 0.39	22.75 ± 0.87
Body mass after treatment	27.34 ± 0.83	26.22 ± 0.55
Heart	0.142 ± 0.01	0.141 ± 0.02
Liver	1.307 ± 0.03	1.387 ± 0.07
Kidney	0.212 ± 0.04	0.209 ± 0.03
Thymus	0.11 ± 0.07	0.12 ± 0.05
Lymph nodes	0.06 ± 0.07	0.06 ± 0.09
Spleen	0.15 ± 0.02	0.39 ± 0.09⋆

Data expressed as mean ± S.E.M. (n = 6); ANOVA followed by Student's t-test. ⋆$p < 0.05$ significantly different.

Investigating the effects of a SP fraction isolated from *Porphyra haitanesis* (Rhodophyta), Zhang et al. (2003) observed an *in vivo* antioxidant activity (reducing the risks of lipid peroxidation) and increased size of spleen and thymus in mice. Spleen is an organ where T and B cells differentiate, and mature. Lins et al. (2009) examined a SP from the red alga *C. feldmannii* as antitumor agent. The authors noted that the compound increased the relative spleen weight, induced a hyperplasia of lymphoid follicles with nest of megakaryocytes in spleens, and reversed leucopenia, supporting the

hypothesis of an immunostimulant agent. In this way, our data suggest that the exact mechanism of SP_1 (*C. cupressoides*) related to immune function needs further investigation.

In respect to biochemical analyses, the SP_1 administrations did not cause any hepatic or renal dysfunctions (data not shown) based on Araújo et al. (2011) and Rodrigues et al. (2011c). Seaweeds have various phytochemicals (ANDRADE et al., 2010; MAO et al., 2011; MARINHO-SORIANO et al., 2006), including toxic secondary metabolites (MORALES et al., 2006). In this study, the lack of systemic toxicity *in vivo* of SP_1 over the considered biochemical parameters could be of pharmacological value (ALMEIDA-LIMA et al., 2011). Enzymatic extraction performed with papain digestion could also have eliminated any toxic compound in the SP_1, being consistent with the hypothesis of Heo et al. (2005).

Seaweeds SPs have also been extensively studied as anticoagulant and antithrombotic agents. Anticoagulant activity of *Caulerpa* SPs is positively correlated with the sulfate content and charge density (RODRIGUES et al., 2011b, 2012); but, the position of sulfate radicals and/or the occurrence of dissulfated units in the chemical structure of these molecules have also been suggested as requisite for anticoagulant action (PEREIRA et al., 2005). However, the toxicity *in vivo* of these compounds has been little investigated. Li et al. (2005) extracted fucoidan from *L. japonica* (Phaeophyta) and evaluated the toxicological effects in Wistar rats. The results indicated no toxicity when 300 mg kg^{-1} body weight per day fucoidan was orally given, but with 900 and 2500 mg kg^{-1} body weight per day, the clotting time was significantly altered. Fucoidan with strong anticoagulant activity in vivo may have clinical importance. However, it could be considered as limited therapeutic option for the treatment of chronic renal failure. An anticoagulant SP fraction from red alga *C. feldmannii* was evaluated in mice by i.v administration up to 30 mg kg^{-1}, and no adverse effect was observed on acute toxicity after 48h (ASSREUY et al., 2008).

Although SP_1 does not have *in vitro* anticoagulant effect (RODRIGUES et al., 2011b, 2012), algae SPs may also exhibit *in vivo* actions (LI et al., 2005). Almeida-Lima et al. (2011) investigated the acute and subchronic toxicity *in vivo* of a heterofucan (Fucan A), and observed that it practically devoid of anticoagulant activity (*in vitro)* and hemorrhagic effect (*in vivo*), but with antithrombotic effect (*in vivo*) when endovenously injected in the vena cava of Wistar rats. This compound had a pharmacological potential given the absence of toxicity *in vivo*.

In a previous study, it was demonstrated that an anticoagulant SP fraction (SP_2, Table 1) from *C. cupressoides* inhibited the thrombin activity by antithrombin (*in vitro*) (RODRIGUES et al., 2011b) and this interaction mechanism was different from other studied *Caulerpa* SPs (HAYAKAWA et al., 2000). In addition, this fraction also inhibited the venous thrombosis and produced prothrombotic activity and no hemorrhagic effect in Wistar rats (RODRIGUES et al., 2011b). The result of the present study is very important, considering that the heparin, a commercial anticoagulant SP widely used in medical clinic and recognized by its side effects, may induce discrete alterations in the enzymatic activity of aminotransferases in plasma (MAJERUS; TOLLEFSEN, 2005).

Overall, *C. cupressoides* features a non-anticoagulant SP fraction (SP_1) with antinociceptive effect which seems to be tolerable in mice systematically treated, and representing thus a novel potential source of analgesic compound to be explored in biomedical research. In addition, its analgesic action was observed to be completely dissociated from the absence of anti-clotting action (POMIN, 2012; RODRIGUES et al., 2012). New applications of *C. cupressoides* SPs in 'long term' doses could be of interest from oral administration of future dosage forms, registering the safety profile in repeated dose studies (MONJI et al., 2011). Additional studies are in progress by our group.

Conclusion

A non-anticoagulant sulfated polysaccharide from the green seaweed *Caulerpa cupressoides* var. *lycopodium* has interesting peripheral antinociception without important systemic alterations.

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References

ALMEIDA-LIMA, J.; DANTAS-SANTOS, N.; GOMES, D. L.; CORDEIRO, S. L.; SABRY, D. A.; COSTA, L. S.; FREITAS, M. L.; SILVA, N. B.; MOURA, C. E. B.; LEMOS, T. M. A. M.; LEITE, E. L.; ROCHA, H. A. O. Evaluation of acute and subchronic toxicity of a non-anticoagulant, but antithrombotic algal heterofucan from the *Spatoglossum schröderi* in Wistar rats. **Revista Brasileira de Farmacognosia**, v. 21, n. 4, p. 674-679, 2011.

ANDRADE, L. R.; LEAL, R. N.; NOSEDA, M.; DUARTE, M. E. R.; PEREIRA, M. S.; MOURÃO, P. A.

S.; FARINA, M.; FILHO, G. M. A. Brown algae overproduce cell wall polysaccharides as a protection mechanism against the heavy metal toxicity. **Marine Pollution Bulletin**, v. 60, n. 9, p. 1482-1488, 2010.

ARAÚJO, I. W. F.; RODRIGUES, J. A. G.; VANDERLEI, E. S. O.; PAULA, G. A.; LIMA, T. B.; BENEVIDES, N. M. B. *Iota*-carrageenans from *Solieria filiformis* (Rhodophyta) and their effects in the inflammation and coagulation. **Acta Scientiarum. Technology**, v. 34, n. 2, p. 127-135, 2012.

ARAÚJO, I. W. F.; VANDERLEI, E. S. O.; RODRIGUES, J. A. G.; COURA, C. O.; QUINDERÉ, A. L. G.; FONTES, B. P.; QUEIROZ, I. N. L.; JORGE, R. J. B.; BEZERRA, M. M.; SILVA, A. A. R.; CHAVES, H. V.; MONTEIRO, H. S. A.; PAULA, R. C. M.; BENEVIDES, N. M. B. Effects of a sulfated polysaccharide isolated from the red seaweed *Solieria filiformis* on models of nociception and inflammation. **Carbohydrate Polymers**, v. 86, n. 3, p. 1207-1215, 2011.

ARCHER, J. Tests for emotionality in rats and mice: A review. **Animal Behaviour**, v. 21, n. 2, p. 205-235, 1973.

ASSREUY, A. M. S.; GOMES, D. M.; SILVA, M. S. J.; TORRES, V. M.; SIQUEIRA, R. C. L.; PIRES, A. F.; CRIDDLE, D. N.; ALENCAR, N. M. N.; CAVADA, B. S.; SAMPAIO, A. H.; FARIAS, W. R. L. Biological effects of a sulfated polysaccharide isolated from the marine red algae *Champia feldmannii*. **Biological and Pharmaceutical Bulletin**, v. 31, n. 4, p. 691-695, 2008.

CAMPO, V. L.; KAWANO, D. F.; SILVA, D. B.; CARVALHO, I. Carrageenans: Biological properties, chemical modifications and structural analysis – A review. **Carbohydrate Polymers**, v. 77, n. 2, p. 167-180, 2009.

COLLIER, H. D. J.; DINNIN, L. C.; JOHNSON, C. A.; SCHNEIDER, C. The abdominal response and its suppression by analgesic drugs in the mouse. **Brazilian Journal of Pharmacology**, v. 32, n. 2, p. 295-310, 1968.

COURA, C. O.; ARAÚJO, I. W. F.; VANDERLEI, E. S. O.; RODRIGUES, J. A. G.; QUINDERÉ, A. L. G.; FONTES, B. P.; QUEIROZ, I. N. L.; MENEZES, D. B.; BEZERRA, M. M.; SILVA, A. A. R.; CHAVES, H. V.; JORGE, R. J. B.; EVANGELISTA, J. S. A. M.; BENEVIDES, N. M. B. Antinociceptive and anti-inflammatory activities of sulphated polysaccharides from the red seaweed *Gracilaria cornea*. **Basic and Clinical Pharmacology and Toxicology**, v. 110, n. 4, p. 335-341, 2012.

CUNHA, T. M.; VERRI JR., W. A.; SCHIVO, I. R.; NAPIMOGA, M. H.; PARADA, C. A.; POOLE, S.; TEIXEIRA, M. M.; FERREIRA, S. H.; CUNHA, F. Crucial of neutrophils in the development of mechanical inflammation hypernociception. **Journal of Leukocyte Biology**, v. 83, n. 4, p. 824-832, 2008.

EDDY, N. B.; LEIMBACH, D. Synthetic analgesics. II Dithienylbutenyl and dithienybutylamines. **Journal of Pharmacology Experimental**, v. 107, n. 3, p. 385-393, 1953.

FARIAS, W. R. L.; LIMA, P. C. W. C.; RODRIGUES, N. V. F. C.; SIQUEIRA, R. C. L.; AMORIM, R. M. F.; PEREIRA, M. G.; ASSREUY A. M. S. A novel antinociceptive sulphated polysaccharide of the brown marine alga *Spatoglossum schroederi*. **Natural Products Communications**, v. 6, n. 6, p. 863-866, 2011.

GHOSH, P.; ADHIKARI, U.; GHOSSAL, P. K.; PUJOL, C. A.; CARLUCCI, M. J.; DAMONTE, E. B.; RAY, B. *In vitro* anti-herpetic activity of sulfated polysaccharide fractions from *Caulerpa racemosa*. **Phytochemistry**, v. 65, n. 23, p. 3151-3157, 2004.

HAYAKAWA, Y.; HAYASHI, T.; LEE, J. B.; SRISOMPORN, P.; MAEDA, M.; OZAWA, T.; SAKURAGAWA, N. Inhibition of thrombin by sulfated polysaccharides isolated from green algae. **Biochimica et Biophysica Acta**, v. 1543, n. 1, p. 86-94, 2000.

HEO, S. J.; PARK, E. J.; LEE, K. W.; JEON, Y. J. Antioxidant activity of enzymatic extracts from brown seaweeds. **Bioresearch Technology**, v. 96, n. 14, p. 1613-1623, 2005.

HUNSKAAR, S.; FASMER, O. B.; HOLE, K. Formalin test in mice, a useful technique for evaluating mild analgesics. **Journal of Neurosciences Methods**, v. 14, n. 1, p. 69-76, 1985.

IWALEWA, E. O.; McGAW, L. J.; NAIDOO, V.; ELOOFF, J. N. Inflammation: the foundation of disease and disorders. A review of phytomedicines of South African origin used to treat pain and inflammatory conditions. **African Journal of Biotechnology**, v. 6, n. 25, p. 2868-2885, 2007.

JI, H.; SHAO, H.; ZHANG, C.; HONG, P.; XIONG, H. Separation of the polysaccharides in *Caulerpa racemosa* and their chemical composition and antitumor activity. **Journal of Applied Polymer Science**, v. 110, n. 3, p. 1435-1440, 2008.

LI, N.; ZHANG, Q.; SONG, J. Toxicological evaluation of fucoidan extracted from *Laminaria japonica* in Wistar rats. **Food and Chemical Toxicology**, v. 43, n. 3, p. 421-426, 2005.

LINS, K. O. A. L.; BEZERRA, D. P.; ALVES, A. P. N. N.; ALENCAR, N. M. N.; LIMA, M. W.; TORRES, W. M.; FARIAS, W. R. L.; PESSOA, C.; MORAES, M. O.; COSTA-LOTUFO, L. V. Antitumor properties of a sulfated polysaccharide from the red seaweed *Champia feldmannii* (Diaz-Pifferer). **Journal of Applied Toxicology**, v. 29, n. 1, p. 20-26, 2009.

MAJERUS, P. W.; TOLLEFESEN, D. M. Anticoagulantes, trombolíticos e fármacos antiplaquetários. In: GILMAN, A. G. (Ed.). **As bases farmacológicas da terapêutica**. Rio de Janeiro: McGraw-Hill, 2005. p. 1122-1155.

MAO, S. C.; LIU, D. Q.; YU, X. Q.; LAI, X. P. A new polyyacetylenic fatty acid and other secondary metabolites from the Chinese green alga *Caulerpa racemosa* (Caulerpaceae) and their chemotaxonomic significance. **Biochemical Systematic and Ecology**, v. 39, n. 4-6, p. 253-257, 2011.

MARINHO-SORIANO, E.; FONSECA, P. C.; CARNEIRO, M. A. A.; MOREIRA, W. S. C. Seasonal variation in the chemical composition of two tropical seaweeds. **Bioresource Technology**, v. 97, n. 18, p. 2402-2406, 2006.

MOALEM, G.; TRACEY, D. J. Immune and inflammatory mechanisms in neuropathic pain. **Brain Research Reviews**, v. 51, n. 2, p. 240-264, 2006.

MONJI, F.; TEHRANI, H. H.; HALVAEI, Z.; BIDGOLI, S. A. Acute and subchronic toxicity assessment of the hydroalcoholic extract of *Stachys lavandulifolia* in mice. **Acta Medica Iranica**, v. 49, n. 12, p. 769-775, 2011.

MORALES, J. L.; CANTILHO-CIAU, Z. O.; SÁNCHEZ-MOLINA, I.; MENA-REJÓN, G. J. Screening of antibacterial and antifungal activities of six marine macroalgae from coast of Yucatá Península. **Pharmaceutical Biology**, v. 44, n. 8, p. 632-635, 2006.

PEREIRA, M. G.; BENEVIDES, N. M. B.; MELO, M. R. S.; VALENTE, A. P.; MELO, F. R.; MOURÃO, P. A. S. Structure and anticoagulant activity of a sulfated galactan from the red alga, *Gelidium crinale*. Is there a specific structural requirement for the anticoagulant action? **Carbohydrate Research**, v. 340, n. 12, p. 2015-2023, 2005.

POMIN, V. H. Fucanomics and galactanomics: Current status in drugs discovery, mechanism of action and role of the well-defined structures. **Biochimica et Biophysica Acta**, v. 1820, n. 12, p. 1971-1979, 2012.

RODRIGUES, J. A. G.; QUINDERÉ, A. L. G.; QUEIROZ, I. N. L.; COURA, C. O.; BENEVIDES, N. M. B. Comparative study of sulfated polysaccharides from *Caulerpa* spp. (Chlorophyceae). Biotechnological tool for species identification? **Acta Scientiarum. Biological Sciences**, v. 34, n. 4, p. 381-389, 2012.

RODRIGUES, J. A. G.; ARAÚJO, I. W. F.; PAULA, G. A.; LIMA, T. B.; BESSA, E. F.; BENEVIDES, N. M. B. Carragenana da epífita *Hypnea musciformis* obtida do cultivo experimental de *Solieria filiformis* em Flecheiras, Estado do Ceará, Brasil. **Acta Scientiarum. Technology**, v. 33, n. 2, p. 137-144, 2011a.

RODRIGUES, J. A. G.; QUEIROZ, I. N. L.; QUINDERÉ, A. L. G.; VAIRO, B. C.; MOURÃO, P. A. S.; BENEVIDES, N. M. B. An antithrombin-dependent sulfated polysaccharide isolated from the green alga *Caulerpa cupressoides* has *in vivo* anti- and prothrombotic effects. **Ciência Rural**, v. 41, n. 4, p. 634-639, 2011b.

RODRIGUES, J. A. G.; ARAÚJO, I. W. F.; PAULA, G. A.; VANDERLEI, E. S. O.; QUEIROZ, I. N. L.; QUINDERÉ, A. L. G.; COURA, C. O.; BESSA, E. F.; LIMA, T. B.; BENEVIDES, N. M. B. Isolamento, fracionamento e avaliação toxicológica *in vivo* de polissacarídeos sulfatados de *Hypnea musciformis*. **Ciência Rural**, v. 41, n. 7, p. 1211-1217, 2011c.

SIQUEIRA, R. C. L.; SILVA, M. S. J.; ALENCAR, D. B.; PIRES, A. F.; ALENCAR, N. M. N.; PEREIRA, M. G.; CAVADA, B. S.; SAMPAIO, A. H.; FARIAS, W. R. L.; ASSREUY, A. M. S. *In vivo* anti-inflammatory effect of a sulfated polysaccharide isolated from the marine brown algae *Lobophora variegata*. **Pharmaceutical Biology**, v. 49, n. 2, p. 167-174, 2011.

SMIT, A. Medicinal and pharmaceutical uses of seaweed natural products: A review. **Journal of Applied Phycology**, v. 16, n. 4, p. 245-262, 2004.

TRI, P. H. Review of species of *Caulerpa* and *Caulerpella* (Chlorophyta, Bryopsidales) from Vietnam. **Marine Research in Indonesia**, v. 34, n. 1, p. 33-45, 2009.

VANDERLEI, E. S. O.; PATOILO, K. K. N. R.; LIMA, N. A.; LIMA, A. P. S.; RODRIGUES, J. A. G.; SILVA, L. M. C. M.; LIMA, M. E. P.; LIMA, V.; BENEVIDES, N. M. B. Antinociceptive and anti-inflammatory activities of lectin from the marine green alga *Caulerpa cupressoides*. **International Immunopharmacology**, v. 10, n. 9, p. 1113-1118, 2010.

VIANA, G. S. B.; FREITAS, A. L. P.; LIMA, M. M. L.; VIEIRA, L. A. P.; ANDRADE, M. C. H.; BENEVIDES, N. M. B. Antinociceptive activity of sulfated carbohydrates from the red algae *Bryothamnion seaforthii* (Turner) Kütz. and *B. triquetum* (S. G. Gmel) M. Howe. **Brazilian Journal of Medical and Biological Research**, v. 35, n. 6, p. 713-722, 2002.

ZHANG, Q. B.; LI, N.; ZHOU, G. F.; LU, X. L.; XU, Z. H.; LI, Z. *In vivo* antioxidant activity of polysaccharide fraction from *Porphyra haitanesis* (Rhodophyta) in aging mice. **Pharmacological Research**, v. 48, n. 2, p. 151-155, 2003.

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