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Original Article

Antinociceptive activity of *Sargassum polyceratium* and the isolation of its chemical components

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ABSTRACT

Marine algae have been the focus of important studies over the past fifty years, with a considerable number of components important to chemists and taxonomists having been isolated and characterized. The scientific data available on *Sargassum polyceratium* are extremely limited. The objective of the present study was to evaluate the antinociceptive activity of an ethanol extract of *S. polyceratium* and to isolate its components. Intraperitoneal treatment with ethanol extract of *S. polyceratium* reduced the number of acetic acid-induced writhes and the amount of time spent in paw-licking in the second phase of the formalin test. Ethanol extract of *S. polyceratium* also reduced the amount of time spent in paw-licking in the glutamate test; however, there was no difference in the reaction time in the hot plate test at any of the doses tested. The chemical components isolated from ethanol extract of *S. polyceratium* were identified using one- and two-dimensional spectroscopic methods such as infrared spectroscopy, mass spectrometry and ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. The analytical results were also compared with data obtained in the literature. The following porphyrin derivatives were isolated from *S. polyceratium*: 13^2-hydroxy-(13^2-*R*)-pheophytin-*a*, 13^2-hydroxy-(13^2-*S*)-pheophytin-*a*, pheophytin-*a*, and the steroid fucosterol. The present results indicate that the ethanol extract of *S. polyceratium* has antinociceptive activity. In addition, four new substances were isolated from the species evaluated.

Introduction

Algae represent one of the most biologically active resources in nature, since they are rich in primary bioactive compounds and secondary metabolites (O'Sullivan et al., 2010). Marine algae have captured the attention of the scientific community due to their great potential as producers of chemical substances that interest the industrial, economic and medical/pharmaceutical sectors. These substances exert a variety of pharmacological activities that have already been described in the literature. These include antibacterial (Lima-Filho et al., 2002; Freile-Pelegrín and Morales, 2004), antitumoral (Yamamoto et al., 1974; Mayer and Hamann, 2005), antiangiogenic (Dias et al., 2005), hemagglutinating (Nishino and Nagumo, 1991; Freitas et al., 1997) and antiviral activities (Damonte et al., 1996; Carlucci et al., 1999; Romanos et al., 2002).

The importance of the genus *Sargassum* C. Agardh, family Sargassaceae, as a component of the marine flora in tropical and subtropical regions of the world is indisputable. It is one of the most representative of the 41 genera of the order Fucales, with an estimated 485 species (Coimbra, 2006). The species of the *Sargassum* genus are distributed predominantly in coastal areas of consolidated substrate, both in tropical and subtropical regions, frequently forming what is known as *Sargassum* banks (Széchy and Paula, 2000). Some recent studies suggest that the polysaccharides, alginates and fucoidans isolated from marine algae of the genus *Sargassum* exert important biological activities with therapeutic relevance due to their antioxidant activity in endothelial cells and immunoregulatory activity in natural killer (NK) cells, macrophages and T-cells (Chen et al., 2007). This genus appears promising from a biological point of view; however, studies are sparse and initial results require greater scientific support.

Sargassum polyceratium is a benthic microalgae species found in regions that stretch from the coastal waters of the Caribbean in southwest Florida to northeastern Brazil (Engelen et al., 2001). Its ecological importance relies on the fact that it acts as a biofilter, reducing pollution in the marine environment, accumulating toxic metals and thus reducing their harmful effects on the local ecosystem of coastal regions (Murugadas et al., 1995). *S. polyceratium* is

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also used for human consumption in some countries such as Cuba where it is eaten fresh, cooked or preserved (Engelen et al., 2001).

Although there are various drugs that act by modulating nociceptive response, the scientific community has been seeking more powerful drugs with fewer toxic effects (Le Bars et al., 2001). Therefore, the present study was developed to evaluate the antinociceptive activity of *S. polyceratium* and to identify its chemical components, as well as to contribute to the chemotaxonomy of the species in question.

Material and methods

Plant materials

Sargassum polyceratium was collected on Bessa beach in the municipality of João Pessoa in the state of Paraíba, Brazil in March 2009. The marine material was identified by Professor George Emmanuel Cavalcanti de Miranda of the Systematics and Ecology Department of the Federal University of Paraíba and three exsiccates were deposited in the *Professor Lauro Pires Xavier* herbarium of the Federal University of Paraíba under the following codes: JPB 13994, JPB 13995 and JPB 13996.

Preparation of the crude ethanol extract

The marine material collected (30 kg) was kept in an ice bath and later frozen at a temperature below 0 °C to conserve its chemical components. Prior to extracting its compounds, the algae were washed with distilled water and dried in the shade. After drying, 3 kg of marine alga were obtained, corresponding to 10% of the material collected. Next, the material was macerated and the chemical components of the alga were extracted with 95% ethanol (EtOH) over seven days. This process was repeated three times to obtain the extracted solution, which was then concentrated in a rotating evaporator under reduced pressure at 40 °C. This resulted in 210 g of crude ethanol extract (SpEE), a yield of 7% in relation to the dry weight of the algae.

Animals

Male Swiss mice weighing 25–35 g, obtained from the Thomas George animal laboratory of the Federal University of Paraíba, were maintained under controlled temperature conditions (21 ± 2 °C) with free access to water and feed pellets (Purina). Prior to initiating the study, all the experimental procedures were reviewed and approved by the Federal University of Paraíba's Ethics Committee on Animal Research under approval certificate number 1006/13.

Drugs

Glutamate and MK-801 were acquired from Sigma–Aldrich (St. Louis, MO, USA). A solution of 37% formaldehyde and morphine hydrochloride was purchased from Vetec, Brazil. All the drugs were diluted in distilled water except for the crude ethanol extract of *S. polyceratium* (SpEE), for which Tween 80 was required in addition to distilled water. All the other reagents used in this study were of the highest analytical grade available or of high-performance liquid chromatography (HPLC) grade.

Acetic acid writhing tests

The animals were divided into five groups ($n = 8$) and allocated to receive the vehicle, SpEE (50, 100 and 200 mg/kg, *i.p.*), or morphine (6 mg/kg, *i.p.*) as a positive control. Thirty minutes after treatment, the animals received an intraperitoneal injection of 1% acetic acid (10 μl/g of weight) (Koster et al., 1959). The number of writhes was counted over a 10 min period for each animal. A writhe is characterized by a contraction of the abdominal muscle and stretching of the hind paws.

Hot plate test

Thirty, 60 and 120 min after receiving their respective treatment with the vehicle, SpEE (50, 100 and 200 mg/kg, *i.p.*) or morphine (10 mg/kg, *i.p.*), the mice were placed individually on a hot plate at a temperature of 55 ± 1 °C. The parameter recorded was the latency period until the animal began licking its hind paw or jumping from the plate. The cut-off time was defined at 15 s to avoid tissue injury.

Formalin test

The procedure was performed as described by Hunskaar and Hole (1987), with some modifications. Thirty minutes after treatment with the vehicle, SpEE (50, 100 and 200 mg/kg, *i.p.*) or morphine (10 mg/kg, *i.p.*), formalin (20 μl, 2.5%) was administered into the plantar region of the animal's right hind paw. The total amount of time spent in paw-licking was recorded in two phases: the early phase (0–5 min after the formalin injection) and the late phase (15–30 min after the formalin injection).

Glutamate test

The role of the glutamatergic system was investigated in accordance with the descriptions of Beirith et al. (2002). The mice were divided into five groups ($n = 8$) and treated intraperitoneally with the vehicle, SpEE (50, 100 and 200 mg/kg) or MK-801 (0.03 mg/kg) 30 min prior to the injection of 20 μl of glutamate solution (30 μmol) into the animal's right hind paw. Immediately after the injection of the phlogistic agent, the mice were individually observed in a transparent box for 15 min and the amount of time that the animal spent licking the glutamate-injected paw was considered the nociceptive reaction.

Rotarod test

With the objective of evaluating the sedative or muscle relaxant effects of SpEE, the mice were submitted to the rotarod test (Duham and Miya, 1957; Santos et al., 2011). The device consists of a rod that rotates at 7 r.p.m. The animals were pretreated with the vehicle, with SpEE (50, 100 and 200 mg/kg, *i.p.*) or with diazepam (4 mg/kg, *i.p.*). The total time they remained on the rotating bar was evaluated at 30, 60 and 120 min following treatment, up to a maximum of 3 min.

Isolation and purification of the chemical components

Part of the SpEE (180 g) was dissolved in a solution of methanol (MeOH):H_2O (7:3, v/v) and homogenized under mechanical agitation for 60 min, obtaining a hydroalcoholic solution that was partitioned in a separation funnel with 3000 ml of hexane, 1500 ml of dichloromethane, 1500 ml of ethyl acetate (AcOEt) and 1000 ml of *n*-butanol (*n*-BuOH). Four phases were obtained, with the following yields: 83.5 g from the hexane phase, 15.5 g from the dichloromethane phase, 3.7 g from the ethyl acetate phase and 12.4 g from the *n*-butanol phase. The isolation, purification and analysis of the chemical components of *S. polyceratium* were performed using chromatographic methods: column chromatography (CC), preparative thin layer chromatography (PTLC) and analytical thin layer chromatography (TLC), respectively. A 20 g aliquot from the hexane phase was submitted to CC using silica gel 60 as the stationary phase, eluted initially with hexane and finalizing with a mixture of AcOEt and MeOH in an increasing gradient of

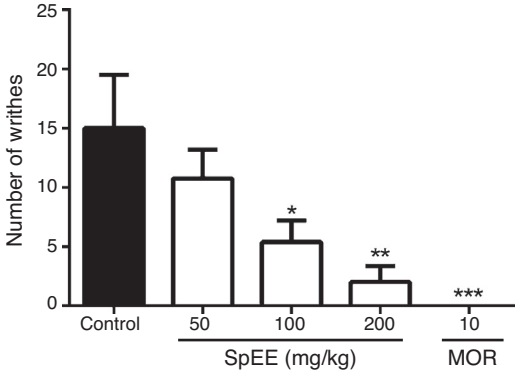


Fig. 1. Effect induced by the ethanol extract of *Sargassum polyceratium* (50, 100 and 200 mg/kg, *i.p.*) and morphine (MOR: 10 mg/kg, *i.p.*) in the acetic acid writhing test. Each bar represents the mean ± SEM of eight animals. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ when compared to the control group (ANOVA followed by Dunnett's test).

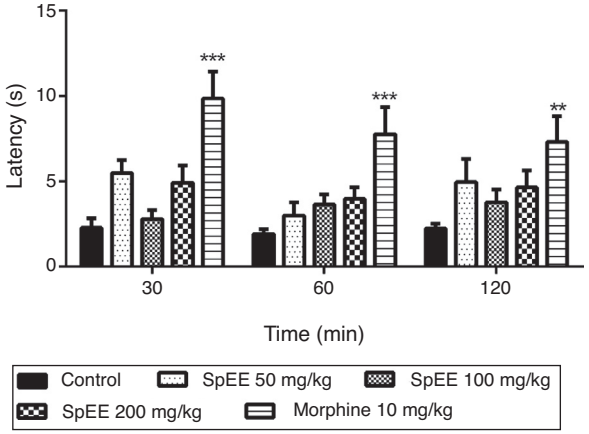


Fig. 2. Effect of the ethanol extract of *Sargassum polyceratium* (50, 100 and 200 mg/kg, *i.p.*) and morphine (10 mg/kg, *i.p.*) in the hot plate test. Each bar represents the mean ± SEM of eight animals. $^{**}p < 0.01$; $^{***}p < 0.001$ when compared to the control group (ANOVA followed by Dunnett's test).

polarity. From this CC, 480 fractions of 150 ml each were collected and concentrated in a rotary evaporator. The fractions were analyzed by TLC using different elution systems and grouped together, when similar, into 16 groups in accordance with their rate of flow (R_f), following analysis by ultraviolet light and impregnation with iodine vapor. The chemical structure of the substances isolated was characterized using infrared spectroscopy, ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry, and additionally, by determining their melting points.

Statistical analysis

The results are presented as the means ± standard error of the mean (SEM) obtained in each individual experiment in relation to the control values. Statistical analysis of the data was performed using one-way analysis of variance (ANOVA) followed by Dunnett's test. *p*-Values < 0.05 were considered statistically significant.

Results

The effect of SpEE in the acetic acid writhing test

At the doses of 100 and 200 mg/kg, the extract significantly reduced the number of writhes (5.3 ± 1.8 and 2.0 ± 1.3, respectively) compared to the control group (15.0 ± 4.5). In the morphine group (10 mg/kg, *i.p.*), there were no writhes at all (0.0 ± 0.0) (Fig. 1).

The effect of SpEE in the hot plate test

Treatment with SpEE failed to alter the latency time until nociceptive response in the hot plate test at any of the doses tested. However, morphine (10 mg/kg) increased the reaction time at 30 (9.9 ± 1.6 s), 60 (7.8 ± 1.6 s) and 120 min (7.3 ± 1.5 s) compared to the control group (2.3 ± 0.6 s, 1.9 ± 0.3 s and 2.2 ± 0.3 s) (Fig. 2).

The effect of SpEE in the formalin test

Pretreatment with SpEE was found to significantly reduce the amount of time the animals spent in paw-licking; however, only in the second phase of the test (Fig. 3B). This inhibitory effect was found at the doses of 100 (0.0 ± 0.0 s) and 200 (0.0 ± 0.0 s) mg/kg compared to the negative control group (125.2 ± 18.2 s). As expected, in the group treated with morphine (10 mg/kg, *i.p.*), pain was significantly inhibited in both phases in relation to the negative control group (Fig. 3A and B).

The effect of SpEE in the glutamate test

Evaluating the possible participation of the peripheral glutamatergic system, results showed that at the doses of 100 and 200 mg/kg, SpEE significantly reduced glutamate-induced nociception (76.8 ± 9.8 s and 79.6 ± 11.1 s, respectively) compared to the negative control group (145.9 ± 14.5 s) (Fig. 4). Similarly, in the positive control group treated with MK-801 there was also a significant

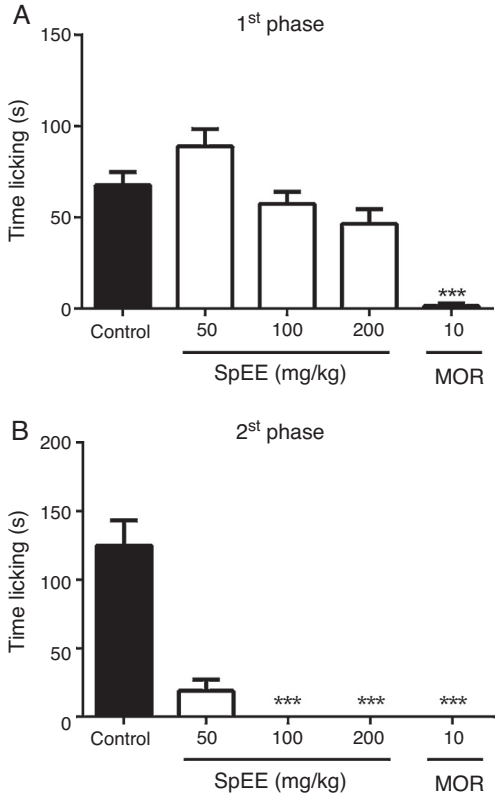


Fig. 3. The effect of the ethanol extract of *Sargassum polyceratium* (50, 100 and 200 mg/kg, *i.p.*) and morphine (MOR: 10 mg/kg, *i.p.*) in the first (panel A) and second (panel B) phases of the formalin test. Each bar represents the mean ± SEM of eight animals. $^{***}p < 0.001$ when compared to the control group (ANOVA followed by Dunnett's test).

reduction in the amount of time the animals spent in paw-licking (9.9 ± 3.0 s).

The effect of SpEE in the rotarod test

Treatment of the animals with SpEE at the doses of 50, 100 and 200 mg/kg did not alter motor coordination in the rotarod test at any of the moments evaluated. Diazepam (4 mg/kg) reduced behavioral response at all the moments evaluated (Fig. 5).

Phytochemical study of the crude ethanol extract of Sargassum polyceratium

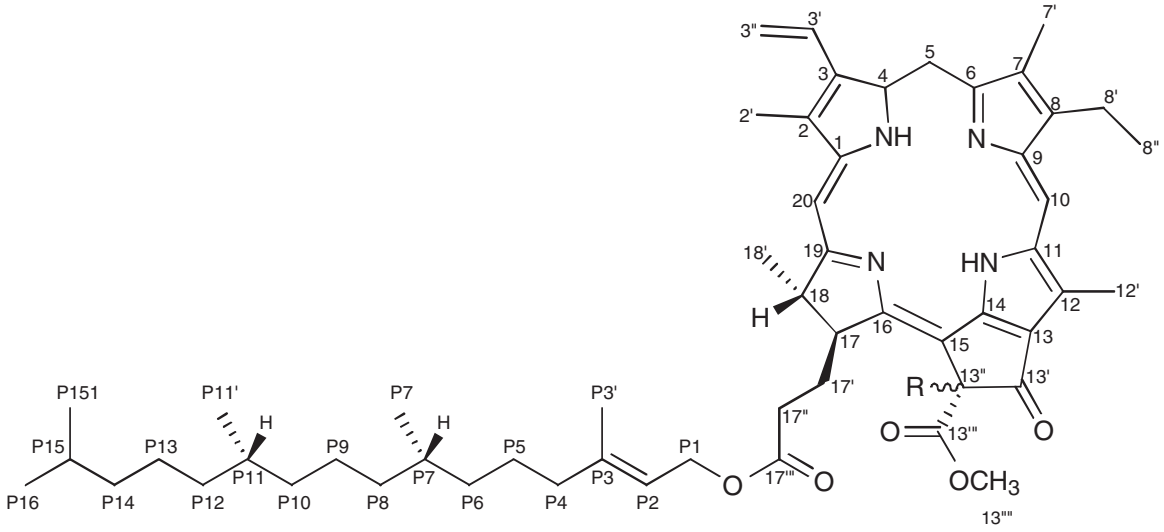
From the hexane phase, 13^2-hydroxy-(13^2-*S*)-pheophytin-*a* (**1**); a pheophytin-*a* (**2**); 13^2-hydroxy-(13^2-*R*)-pheophytin-*a* (**3**); and fucosterol (**4**) were obtained.

Compound **1** composed 0.012% of the dry weight of the alga, with a melting point of 171–172 °C. Analysis of the spectrums of IV, ^{1}H and ^{13}C NMR (200 MHz and 50 MHz, respectively, chloroform) permitted us to infer that **1** is a mixture of the optic isomers *R* and *S*.

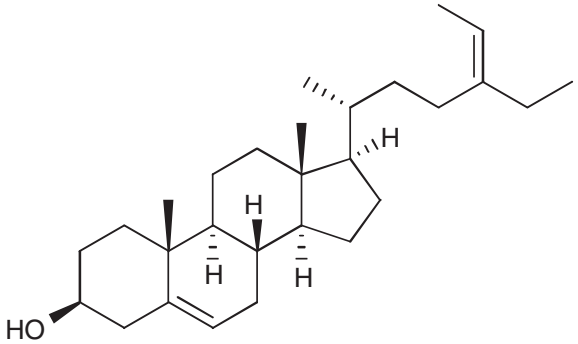
Compound **2**, forming 0.006% of the dry weight of the alga, was identified as being a pheophytin-*a*, following analysis of the one-dimensional spectrum of IV and ^{1}H and ^{13}C NMR and compared to data in the literature.

Compound **3** was found to make up 0.001% of the dry weight of the alga, with a melting point of 183–184 °C. The spectrums of IV and ^{1}H and ^{13}C NMR and their expansions permitted us to speculate that **3** is a substance belonging to the pheophytin group, since signs were seen of the porphyrin skeleton, of the methyl group inserted in carbon C-7 and of the phytyl chain in C-173.

Compound **4** corresponded to 0.004% of the dry weight of the alga and had a melting point of 123–124 °C. The spectral data, the features of the spectrums and the comparisons with data from the literature, all suggest that it has a steroid nucleus of the stigmastane type, and it was identified as fucosterol.



1 R=OH (*S*)
2 R=H
3 R=OH (*R*)



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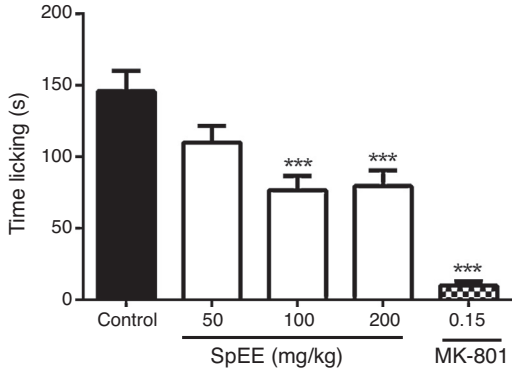

Fig. 4. Effect of the ethanol extract of *Sargassum polyceratium* (50, 100 and 200 mg/kg, *i.p.*) and MK-801 (0.15 mg/kg, *i.p.*) in the glutamate test. Each bar represents the mean ± SEM of eight animals. ***$p < 0.001$ when compared to the control group (ANOVA followed by Dunnett's test).

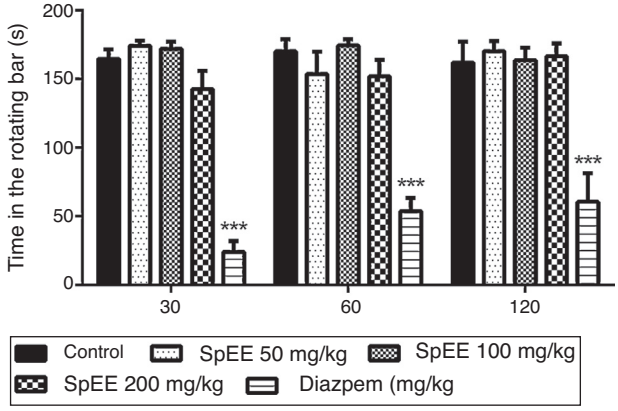

Fig. 5. Effect of the ethanol extract of *Sargassum polyceratium* (50, 100 and 200 mg/kg, *i.p.*) and diazepam (4 mg/kg, *i.p.*) in the rotarod test. Each bar represents the mean ± SEM of eight animals. ***$p < 0.001$ when compared to the control group (ANOVA followed by Dunnett's test).

Discussion

A multitude of studies have focused on the treatment of pain. Plants and their derivatives have been used extensively for therapeutic purposes. Their use is increasing worldwide, with the pharmaceutical market reporting rising sales of phytotherapeutics over the past ten years. Attention is now being paid to natural marine products, as they represent new perspectives for novel drugs and because of the complexity of their chemical structures, which are unlike any found in land-based plants (Souza et al., 2012).

The phytochemical evaluation of the crude ethanolic extract of *S. polyceratium* resulted in the isolation of four compounds from the hexane fraction: three porphyrin derivatives and fucosterol. Porphyrins are natural compounds with a range of biological activities including anti-inflammatory and antinociceptive activity (Alonso-Castro et al., 2015). Fucosterol, a phytosterol identified in some species of the genus *Sargassum*, exerts antidiabetic, antioxidant and anti-inflammatory effects (Lee et al., 2003, 2004). From these results, the pharmacological study of SpEE appears highly promising.

The antinociceptive activity of SpEE was evaluated using chemical models of nociception (the acetic acid writhing test, the formalin test and the glutamate test) as well as a thermal model (the hot plate test). The acetic acid writhing test has been described as a classic model of visceral inflammatory nociception that provokes an irritation in the peritoneum, characterized by an abdominal constriction and by stretching of the hind paws (Collier et al., 1968). The results suggest that at the doses of 100 mg/kg and 200 mg/kg, SpEE inhibited nociception in the mice by significantly reducing the number of abdominal constrictions induced by the acetic acid when compared to the control group. Morphine completely inhibited the abdominal constrictions (100%).

To differentiate between central and peripheral antinociception, the effect of SpEE was evaluated in the hot plate test. This test is used to study a noxious thermal stimulus mediated by central sensitization (Gunn et al., 2011). SpEE failed to alter the latency time in response to the nociceptive stimulus in the hot plate test, eliminating a possible central involvement in the analgesic activity of SpEE. Nevertheless, the extract of *Sargassum fulvellum* and *Sargassum thunbergii* was found to exert supraspinal antinociceptive activity, as evaluated using the tail-flick test (Kang et al., 2008).

The results obtained in the acetic acid writhing test highlighted the antinociceptive potential of SpEE. The formalin test was then carried out to provide a better characterization of this profile. This methodology involves two different phases in which the animal licks the paw that received the nociceptive chemical stimulus intensely. The mechanism behind the nociceptive action in the first phase of the test, which begins immediately following the administration of formalin, is characterized by direct activation of sensory C-fibers through the transient receptor potential A1 (TRPA1) receptors (McNamara et al., 2007). It has also been reported that mediators such as substance P and bradykinin participate in this neurogenic mechanism (Parada et al., 2001). Drugs that act at central level (opioids) are the principal agents acting in this neurogenic phase of nociception (Ferreira et al., 2006). In the second phase of the test, which occurs after 15–30 min, inflammatory mediators resulting from the administration of formalin are released, including prostaglandins, serotonin, bradykinin, histamine, sympathomimetic amines, tumor necrosis factor-alpha (TNF-α) and interleukins (Hunskaar and Hole, 1987; Tornos et al., 1999; Milano et al., 2008). With the extract evaluated, an effect was only found in the second phase of the formalin test, referred to as the inflammatory phase. This finding suggests that SpEE may have a pronounced anti-inflammatory effect, since at the doses of 100 and 200 mg/kg it resulted in a reduction in the amount of time the animals spent in paw-licking in the peripheral pain inhibition phase. Sofidiya et al. (2014) also reported the anti-inflammatory effect of an ethanol extract of the leaves of *Alafia barteri* Oliv., a plant used in popular medicine, using the formalin test. Likewise, another study that corroborates the pharmacological potential of plants emphasized the antinociceptive and anti-inflammatory effects of the extract and fractions of *Eugenia jambolana* Lam. (Saha et al., 2013).

Glutamate is the predominant excitatory neurotransmitter in the central and peripheral nervous system, being released in response to nociception, inflammation or tissue damage (Yashpal et al., 2001). This excitatory amino acid may contribute to the onset of pain by acting on small-diameter afferent fibers in the peripheral terminations. The intra-plantar administration of glutamate promotes nociception by stimulating glutamate receptors in peripheral, medullary and supra-medullary sites and this effect may or may not be mediated by mechanisms involving *N*-methyl-D-aspartate receptors (Beirith et al., 2002; Miller et al., 2011; Sakurada et al., 2003). Furthermore, other excitatory amino acids, prostaglandin E2, nitric oxide, protons, substance P and also glutamate are released in the dorsal horn starting at the moment of the initial intra-plantar administration of glutamate. The results with SpEE in the glutamate test corroborate its efficacy as shown in the acetic acid writhing test and in the formalin test, since with this methodology the doses of 100 and 200 mg/kg resulted in the inhibition of nociception, as also shown in a study conducted by Moniruzzaman et al. (2015) with an ethanol extract of *Hedyotis corymbosa* L. It is reasonable, therefore, to suggest that SpEE may act via the glutamatergic system to promote antinociception.

In order to exclude a possible motor disruption caused by SpEE, the rotarod test was performed. Muscle relaxants, sedatives and central nervous system depressants are effective in tests such as the hot plate test and in glutamate-induced nociception (Cavalcante Melo et al., 2012). SpEE appeared to exert no activity at all in the rotarod test, indicating that it has no significant effect as a relaxant or as a sedative, and confirming its true antinociceptive effect.

The chemical study of the marine alga *S. polyceratium* Montagne led to the isolation and structural characterization of four substances: the mixture of 13^2-hydroxy-(13^2-*R*)-pheophytin-*a* and 13^2-hydroxy-(13^2-*S*)-pheophytin-*a* (**1**); the porphyrin derivative pheophytin-*a* (**2**); the porphyrin derivative 13^2-hydroxy-(13^2-*R*)-pheophytin-*a* (**3**); and the steroid fucosterol (**4**). All of these substances were as yet unknown in the species *S. polyceratium*. Although the majority of the substances isolated from *S. polyceratium* have already been reported in the literature, they are being reported for the first time in these species.

Therefore, the present findings show that SpEE is effective in different antinociceptive models without altering the animal's motor coordination; however, the molecular mechanisms behind the activity of SpEE remain unknown and further studies need to be conducted. The phytochemical analysis enabled four new substances to be isolated from SpEE, contributing to the chemical study of marine organisms.

Authors' contributions

AKFSS, DVF, VMM and PRRS contributed to biological studies. PAT contributed to analysis of the data. NSL, CSD and LCMP contributed in collecting plant samples and identification. JMBF and RNA designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved for submission.

Conflicts of interest

The authors declare no conflicts of interest.

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