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Artigo

Antinociceptive activities of crude methanolic extract and phases, *n*-butanolic, chloroformic and ethyl acetate from *Caulerpa racemosa* (Caulerpaceae)

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RESUMO: "Atividade antinociceptiva do extrato metanólico bruto e das fases *n*-butanólica, clorofórmica e acetato de etila de *Caulerpa recemosa* (Caulerpaceae)". Neste estudo, tentamos identificar a atividade antinociceptiva do extrato metanólico bruto e das fases *n*-butanólica, clorofórmica e acetato de etila provenientes da alga *Caulerpa racemosa*. Esta alga é cosmopolita no mundo, principalmente em regiões tropicais. O extrato metanólico bruto e as fases n-butanólica, clorofórmica e acetato de etila foram administrados por via oral, na concentração de 100 mg/kg. Estes foram capazes de reduzir a nocicepção produzida pelo ácido acético, sendo 47,39%, 70,51%, 76,11% e 72,24%, respectivamente. No ensaio da placa quente as fases clorofórmica e acetato de etila foram ativas neste modelo. Na fase neurogênica do teste de formalina, foi observado que o extrato metanólico bruto (51,77%), fase *n*-butanólica (35,12%), fase clorofórmica (32,70%) e indometacina (32,06%) foram eficazes em inibir a resposta nociceptiva. Na fase inflamatória, apenas a fase acetato de etila (75,43%) e indometacina (47,83%) foram capazes de inibir significativamente a resposta nociceptiva. Com base nestes dados, podemos sugerir que o a fase acetato de etila apresenta um significativo efeito anti-inflamatório, cuja potência ainda não foi determinada. No entanto, estudos farmacológicos e químicos serão necessários, a fim de caracterizar o mecanismo responsável pela ação antinociceptiva e também para identificar outros princípios ativos presentes na alga *Caulerpa racemosa*.

Unitermos: *Caulerpa racemosa*, Caulerpaceae, antinociceptivo, anti-inflamatório, alga.

ABSTRACT: In this study, we attempted to identify the possible antinociceptive actions of *n*-butanolic phase, chloroformic phase, ethyl acetate phase and crude methanolic extract obtained from *Caulerpa racemosa*. This seaweed is cosmopolitan in world, mainly in tropical regions. The *n*-butanolic, chloroformic, ethyl acetate phases and crude methanolic extract, all administered orally in the concentration of 100 mg/kg, reduced the nociception produced by acetic acid by 47.39%, 70.51%, 76.11% and 72.24%, respectively. In the hotplate test the chloroformic and ethyl acetate phase were activite in this models. In the neurogenic phase on formalin test, were observed that crude methanolic extract (51.77%), *n*-butanolic phase (35.12%), chloroformic phase (32.70%) and indomethacin (32.06%) were effective in inhibit the nociceptive response. In the inflammatory phase, only the ethyl acetate phase (75.43%) and indomethacin (47.83%) inhibited significantly the nociceptive response. Based on these data, we can infer that the ethyl acetate phase shows a significant anti-inflammatory profile, whose power has not yet been determined. However, pharmacological and chemical studies are continuing in order to characterize the mechanism(s) responsible for the antinociceptive action and also to identify other active principles present in *Caulerpa racemosa*.

Keywords: *Caulerpa racemosa*, Caulerpaceae, antinociceptive, anti-inflammatory, seaweed.

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INTRODUCTION

Marine organisms represent a valuable source of new compounds. The biodiversity of the marine environment and the associated chemical diversity constitute a practically unlimited resource of new active substances in the field of the development of bioactive products. The marine pharmacy currently holds more than 35,000 marine-derived biological samples, with approximately 150 compounds to be cytotoxic against the tumor cells (Arif et al., 2004). It was also observed a large increase of studies about the anti-inflammatory pharmacology of the marine compounds, such as astaxanthin, bolinaquinone, cacospongionolide B, clathriol B, conicamin, cycloamphilectene 2, elisabethadione, plakohypaphorine, pourewic acid A, methylpourewate B, cadlinolide C, petrocortyne A, petrosaspongiolides M-R, pseudopterosin N, pseudopterosin R, seco-pseudopterosin E (Mayer et al., 2007).

In Brazil, research in natural products of marine origin has been initiated for more than 30 years, according Kelecom (1997), however it is still incipient. Although the Brazilian coast is the second largest in the world, after Australia, with approximately 8,000 km in length, the development of chemistry of natural products from marine organisms in Brazil has been neglected for many years, because the main focus of the chemistry of natural products Brazil was directed to the study of plants (Berlinck et al., 2004). Thus, the Brazilian marine fauna and flora remain unexplored regarding bioprospecting for active natural products.

The macroscopic marine algae have attracted the attention of the scientific community for the great potential as producers of chemicals, medical and pharmacological interest (Mayer and Hamann, 2004, 2005; Blunt et al., 2005; Dresch et al., 2005; Lhullier et al., 2006; Mayer et al., 2007; Rocha et al., 2007; Seleghim et al., 2007; Rozas & Freitas, 2008a,b). To date, more than 2,400 natural products have been isolated from algae, especially the divisions rhodophytes (red algae), pheophytes (brown algae or brown) and chlorophytes (green algae), and most of them, from tropical and subtropical populations. In general, algae synthesize lipophilic non-polar secondary metabolites such as terpenoids and acetogeninas as compounds of mixed biosynthesis occur in low concentrations. Polar polyphenols may occur in high concentrations (Pereira et al., 2003).

Caulerpa racemosa (Forsskal) J. Agardh (Caulerpaceae), a pan-tropical to temperate-warm water species widely distributed throughout the world, is a green alga (Bryopsidales) that was collected for the first time in 1926 in the Mediterranean Sea by Hamel in the Sousse harbour, Tunisia (Verlaque et al., 2000; Piazzi et al., 2001). This seaweed has a number of pharmacological activities described in the literature, among which we mention: antitumor (Cavas et al., 2006), anti-viral (Ghosh et al., 2004; Chattopadhyay et al., 2007) and antioxidant (Cavas and Yurdakoc, 2005). However, up to date there are a few investigations supporting the pharmacological properties of this seaweed.

As the seaweed up an important biological source as biologically active natural products and structurally unusual and few studies conducted with this purpose, mainly in Brazil, then this study was intended to evaluate the antinociceptive and anti-inflammatory activities of *n*-butanolic phase, chloroformic phase, ethyl acetate phase and crude methanolic extract from *C. racemosa* in nociceptive animal models.

MATERIAL AND METHODS

Marine material

Seaweed *C. racemosa* (Forsskal) J. Agardh was collected of the coastal region of Bessa (7º03´52´´S / 34º49´51´´), João Pessoa, State of Paraiba, Brazil in April 2008. The specimen was identified by Dr George Emmanuel Cavalcanti de Miranda. A voucher specimen (JPB 13999) has been deposited at the Herbarium Lauro Pires Xavier of the Universidade Federal da Paraíba, Brazil.

Extraction and fractionation

The fresh sample of *C. racemosa* (5.4 kg) was exhaustively extracted with MeOH at room temperature. The solvent was removed under reduced pressure at <40 °C, a dark green residue was obtained. Part of the crude methanol extract was submitted to solid-liquid partition successively with hexane, chloroform, ethyl acetate and *n*-butanol. The solutions produced were evaporated under reduced pressure producing the hexane (0.13 g), chloroformic (3.58 g), ethyl acetate (1.53 g) and *n*-butanolic (3.53 g) phases. Chloroformic, ethyl acetate and *n*-butanolic phase and crude methanolic extract were separated to be evaluated for their analgesic and anti-inflammatory activities. Hexane phase did not present sufficient quantity to perform the pharmacological tests.

Biological activity tests

Drugs and reagents

Acetic acid and indomethacin (Merck), arabic gum and dipyrone (Sigma Chemical), and morphine sulphate (Dimorf-Cristalia-BR) were purchased from commercial sources. A solution of formalin 2.5 % was prepared with formaldehyde (from Merck) in saline (NaCl 0.9%). The marine material was used as suspension in arabic gum in all the experiments.

Animals

Adult male and female Swiss albino mice (20-35 g) were used in the experiments. They were housed in single-sex cages under a 12-h light:12 h dark cycle (lights on at 6 h) in a controlled temperature room (22 ± 2 ºC). They had free access to food and water. Groups of 6-8 animals were used in each test group and control animals received vehicle only. The experiments were performed after the approval of the protocol by the local Institutional Ethics Committee. All experiments were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983).

Writhing test

The methods described by Koster et al. (1959) were used with few modifications. In brief, the selected groups of animals, consisting of six mice per dose of phases, extracts or drug, were used in the test. Animals were pretreated (60 min before acid acetic injection) with *n*-butanolic phase, chloroformic phase, ethyl acetate phase and crude methanolic extract (all at the dose 100 mg/kg, body wt., p.o). Positive control mice groups received standard analgesics for comparison, dipyrone 60 min prior to the i.p. injection of acetic acid 0.6% (0.25 mL). The negative control animals not received anything prior to the i.p. injection of acetic acid. Five minutes after the i.p. injection of acetic acid, the number of writhing exhibited by each mouse was counted for 20 min. The antinociceptive activity was expressed as the reduction on the number of abdominal writhing.

Hot-plate test

The hot plate test was used to measure response latency according to the method described by Eddy and Leimbach (1953), with minor modifications. In these experiments, the hot plate apparatus (Ugo Basile, Model-DS 37) was maintained at 55.5 ± 1 ºC. Animals were placed on the heated surface and the time between placement and licking of the paws or jumping was recorded as latency. Latency was recorded for vehicle control groups (10 mL/kg) or pre-treated groups with *n*-butanolic phase, chloroformic phase and ethyl acetate phase (all at the dose 100 mg/kg, p.o.) or morphine (15 µmol/kg, body wt., i.p). The test compounds were administered after animal selection on time of 30 min. The selection was made on the basis of the reactivity on the test. Pre-treatment times 0 and 30 min were used for assay adaptation and selection of the animals, respectively. Only mice showing a reaction time within the range of 4-10 sec. were used in this test. The latency of the reaction to nociception was measured at time 0 and then at 30 min intervals up to the 180 th min. The phases and the extrats were administered at time 30 min and treatment latencies were recorded at times 60, 90, 120 and 150 min.

Formalin-induced nociception in mice

The formalin test was performed according to the method of Hunskaar and Hole (1987). Briefly, 20 µl of a 2,5% (v/v) solution of formalin in saline was injected into the sub plantar region of the right hind paw and the quantification of the time that the animal spent licking the right hind paw during the first 5 min (first phase) and from 15 to 30 min (second phase) of post-injection time was performed. The test was performed at ambient temperature of 22-26 ºC and care was taken to exclude environmental disturbances (high temperature, noise and excessive movement) that might interfere with the animal's response (Tjolsen and Hole, 1997).

Statistical analysis

Data obtained from animal experiments were expressed as the mean standard error (Mean ± S.E.M.). Statistical differences between the treated and the control groups were evaluated by ANOVA and Dunnett hoc tests. $P < 0.05$ was considered to be significant (*$P < 0.05$; **$P < 0.01$).

RESULTS

Writhing test

The results depicted in Figure 1 show that the crude methanolic extract and *n*-butanolic, chloroformic and ethyl acetate phase from *C. racemosa*, given 40 min beforehand, produced an inhibition of acetic acid-induced abdominal constrictions in mice. The treatment of mice with crude methanolic extract, *n*-butanolic phase, chloroformic phase and ethyl acetate phase (all at the dose 100 mg/kg, p.o) produced marked inhibition of acetic acid induced writhing response. The inhibitions were 72.24%, 47.39%, 70.51% and 76.11% respectively.

Hot-plate test

The results in Figure 2 show that the treatment of animals with morphine (15 µmol/kg, i.p.) induced a marked increase in the latency of the animals at all the reading (60, 90, 120 and 150 min). Moreover, the chlorofomic phase (at the 150 min) and ethyl acetate phase (at 90 and 120 min) increased the latency time of response in animal model of plate, suggesting a supra-spinal activity to these phases.

Formalin-induced nociception in mice

In assessing the antinociceptive and antiinflammatory activity in this teste of nociception induced by formalin depicted in Figures 3A and 3B, the antinociceptive activity was observed in the neurogenic phase to the crude methanolic extract (51.77%, *n*-butanolic phase (35.12%), Chloroformic phase (32.70%) and indomethacin (32.06%), exception to ethyl acetate phase. In the inflammatory phase only the ethyl acetate phase (75.43%) and indomethacin (47.83%) induced significant inhibition of response in this model. From these data can be suggested that the crude methanol extract, *n*-butanolic phase, and phase Chloroformic presents a profile antinociceptive while the ethyl acetate phase presents an anti-inflammatory profile whose power has not been determined yet.

DISCUSSION

The antinociceptive activities of *n*-butanolic phase, chloroformic phase, ethyl acetate phase and crude methanolic extract from *C. racemosa*, all givem orally at the concentration 100 mg/kg, were evaluated using chemical (acetic acid and formalin) and thermal (hot plate test) stimuli. Methods for investigating antinociception were selected so that both the central and peripheral activity were investigated. The acetic acid-induced abdominal constriction and hot-plate methods elucidated peripheral and central activity, respectively, while the formalin test investigated both.

The acetic acid-induced writhing model is normally used to study the peripheral antinociceptive effects of drugs. This method is simple and reliable, and affords rapid evaluation of peripheral analgesic action (Amendoeira et al., 2005). The extract and phases of *C. racemosa* tested in this study showed significant effect on this viscero-somatic model (tonic pain) reflected in a significant reduction of the acetic acid-induced abdominal writhing. The acetic acid- induced abdominal writhing involves the production and release of arachidonic acid metabolites via cycloxygenase (COX) and prostaglandin biosynthesis (Dongmo et al., 2005). However, this test is a nonspecific model (e.g. anticholinergic, tricyclic antidepressants and antihistaminic and other agents show activity in this test) it is widely used for analgesic screening and involves local peritoneal receptors (cholinergic and histaminic receptor) and the mediators acetylcholine and histamine (Alexandre-Moreira et al., 1999; Miranda et al., 2001). Thus, the *n*-butanol phase, chloroformic phase, ethyl acetate phase crude methanol extract from *C. racemosa* may be modulated the nociception by any of these mechanisms above.

The hot plate test is a central pain model that has a selectivity for painkillers such as morphine and other centrally active drugs. And the method has several advantages, particularly the strong sensitivity to pain and limited tissue damage (Deraedt et al., 1980). The heat induces an effect of termonociceptive skin and the integration of stimulus is due to stimulation of myelinated C fibers not driving slow (Hendry et al., 1999). The point is that the chloroformic and ethyl acetate phases increase the latency time of response in animal model of hot plate, and shows that these phases showed activity of supra-spinal analgesia.

To better assess the antinociceptive profile, extracts and phases were subjected to the test of nociception induced by formalin. This test is characterized by two phases: the first phase which is characterized by intense neurogenic pain immediately after injection and seems to be caused by stimulation of C-fibers after peripheral stimulation (direct stimulation of nociceptors). For a period there is a reduction of nociceptive activity (Hunskaar and Hole, 1987). The second phase appears to be caused by tissue and functional changes in dorsal horn and the caudal spine and is accompanied by release of inflammatory mediators and cannot be interpreted as a consequence of the first phase. It also originates from peripheral mechanisms and appears to be mediated from central sensory neurons due to peripheral inflammation as well as the activation of primary afferent neurons. For this reason the formalin test is used to evaluate the analgesic activity of substances, and also elucidate the mechanisms of analgesia in the first (neurogenic) and the second (inflammatory) phase (Hunskaar and Hole, 1987).

It has been well documented that several inflammatory mediators such as substance P and bradykinin participate in the manifestation of the response in the first phase (Shibata et al., 1989), where prostaglandins (Hunskaar and Hole, 1987; Shibata et al., 1989), serotonin, histamine and cinines are involved in the response of the second phase of formalin test (Tjolsen and Hole, 1997). In assessing the antinociceptive and antiinflammatory activity in the test of hyperalgesia induced by formalin (Figure 3), activity was observed in the neurogenic phase of crude methanolic extract and its phases, with the exception of ethyl acetate phase. In the inflammatory phase only the ethyl acetate phase and indomethacin induced significant inhibition of response in this model. From these data it can be suggested that the ethyl acetate phase has shown a significant anti-inflammatory profile.

CONCLUSION

In conclusion, this study has shown that the crude methanolic extract and phases from *C. racemosa* possess significant antinociceptive effect in animals models, administrated by oral route. Moreover, pharmacological studies are continuing in order to characterize the mechanism(s) responsible for the antinociceptive action. Further studies are being conducted to isolate, characterize and evaluate the antinociceptive activity of

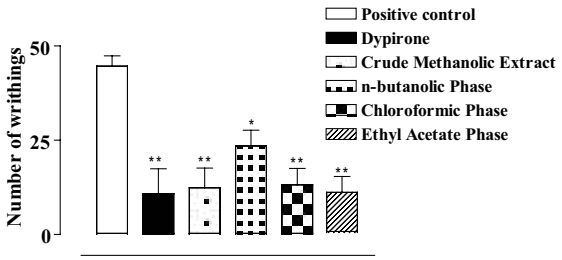

Figure 1. Antinociceptive effect of *n*-butanolic phase, chloroformic phase, ethyl acetate phase, crude methanolic extract (all at the dose 100 mg/kg) and dypirone (100 µmol/kg) on the acetic acid-induced writhings in mice. Each column represents the mean ± sem of 6-8 animals. The asterisks denote the significance levels in comparison with control groups, **P* < 0.05, ***P* < 0.01.

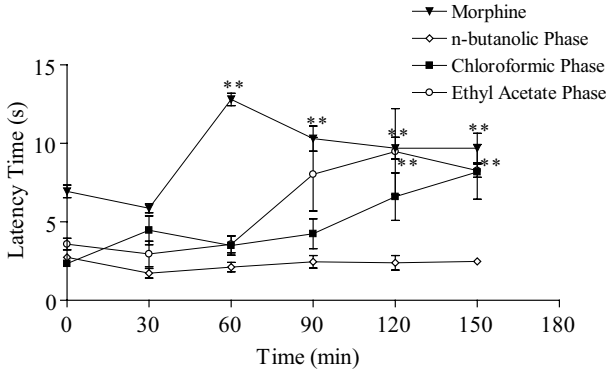

Figure 2. Time-course of the effects of *n*-butanolic phase, chloroformic phase, ethyl acetate phase (all at the dose 100 mg/kg, p.o.) and morphine (15 µmol/kg, s.c.) on thermal nociception. Each point represents the mean ± sem of 6-8 animals. The asterisks denote the significance levels in comparison with control groups, ***P*<0.01.

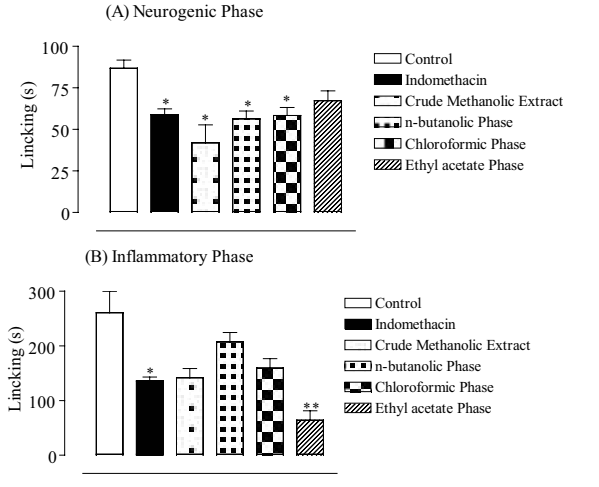

Figure 3. Antinociceptive of *n*-butanolic phase, chloroformic phase, ethyl acetate phase, crude methanolic extract (all at the dose 100 mg/kg) and indomethacin (100 µmol/kg), against the early-phase (0-5 min, panel A) or late-phase (15-30 min, panel B) of formalin-induced nociception in mice. Each column represents the mean ± sem of 6-8 animals. The asterisks denote the significance levels in comparison with control groups, **P*<0.05, ***P*<0.01.

constituents from *n*-butanolic, chloroform and acetate phase, which may be responsible for antincceptive activity described in this work. Furthermore, this work shows for the first time the antinociceptive activity of the seaweed *C. racemosa*.

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