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Protective effects of ethanolic extract from the red algae *Amansia multifida* on experimental inflammation, nociception and seizure experimental models

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ABSTRACT. This study aimed to investigate the EEAm effect in mice models of nociception, inflammation and in behavioral tests evaluating the central nervous system. EEAm had inhibitory effects in the following tests: acetic acid-induced writhing (78%); formalin (62% - inflammatory phase); open field (46%). EEAm increased the nociceptive latency (56%) in tail flick test and increased the death-latency by 36% in the pentylenetetrazole-induced seizure model. Moreover, EEAm inhibited paw edema (82%) and peritonitis (45%) induced by carrageenan. In conclusion, EEAm presents antinociceptive, anti-inflammatory and anticonvulsant effects involving peripheral and central-acting mechanisms in mice.

Keywords: acute inflammatory process, antinociception, epilepsy, red seaweed.

Efeito protetor do extrato etanólico da alga marinha vermelha *Amansia multifida* em modelos experimentais de inflamação, nocicepção e convulsão

RESUMO. Neste estudo objetivou-se investigar o efeito do EEAm em modelos de nocicepção e inflamação, e em testes comportamentais que avaliam o sistema nervoso central em camundongos. EEAm exibiu efeitos inibitórios nos testes comportamentais de contorções abdominais induzidas por ácido acético (78%); formalina (62% - fase inflamatória) e campo aberto (46%). EEAm aumentou a latência de nocicepção no teste de retirada da cauda (56%) e a latência de morte 36% no modelo de convulsões induzidas por pentilenetetrazol. Além disso, EEAm inibiu o edema de pata (82%) e a peritonite (45%) induzidos por carragenana. Como conclusão, EEAm apresenta efeitos antinociceptivo, anti-inflamatório e anticonvulsivante em camundongos por mecanismos periféricos e centrais.

Palavras-chave: Processo inflamatório agudo, antinocicepção, epilepsia, alga vermelha.

Introduction

Although the oceans represent rich sources of bioactive compounds, they only began to attract the interest of pharmaceutical companies and research institutions 50 years ago (Proksch, Edrada, & Ebel, 2002). Seaweeds are classified into three categories: Chlorophyta (green algae), Phaeophyta (brown algae) and Rhodophyta (red algae) (O'Sullivan et al., 2010). Red algae are considered the most important source of structurally diverse compounds with valuable pharmaceutical and biomedical potential (El Gamal, 2010).

Extracts of red algae present several biological activities, such as antifungal (*Symphyocladia latiuscula* and *Laurencia papillosa*) (Xiuli, Liyuan, Junhai, Lijie, & Fuhang, 2014, Alarif, Al-Lihaibi, Abdel-Lateff, & Ayyad, 2011); antioxidant (*Neorhodomela aculeate* and *Rhodomela confervoides*) (Lim et al., 2006, Li, Li, Gloer, & Wang, 2011); antitumor-antimicrobial (*Laurencia obtusa*) (Alarif, Al-Lihaibi, Ayyad, Abdel-Rhman, & Badria., 2012); leishmanicidal (*Bostrychia tenella*) (Felício, Albuquerque, Young, Yokoya, & Debonsi, 2010) and anti-inflammatory/antinociceptive (*Bryothamnion triquetrum*) (Cavalcante-Silva et al., 2012).

In recent years, the mechanisms and mediators involved in nociceptive and inflammatory processes have been the target of several studies (Hua & Cabot, 2010, Gris et al., 2010). However, the various natural or synthetic compounds used for treatment in these processes have not been identified as ideal anti-inflammatory or analgesic agents, especially due to the magnitude of adverse effects. Many anti-

inflammatory/analgesic drugs currently available cause acute gastrointestinal damage, kidney and platelet disorders, hepatotoxicity, pancreatitis (Kummer & Coelho, 2002), sedation, tolerance development (Chang, Chen, & Mao, 2007, Gris et al., 2010), among others. Thus, the search for substances with anti-inflammatory and antinociceptive activities with few adverse effects is still quite encouraged by the scientific community.

The formalin-induced nociception is widely used as a model of persistent pain (Hunskaar, Fasmer, & Hole, 1985), in which the injection of formalin produces a biphasic behavioral response (Phase 1 and Phase 2). Phase 1 is characterized by neurogenic pain caused by direct chemical stimulation of nociceptors and involves mainly the release of substance P and bradykinin. Phase 2 is characterized by inflammatory pain, triggered by a combination of stimuli, involving inflammation of peripheral tissues and central sensitization by histamine, serotonin, prostaglandins, nitric oxide and bradykinin, released from damaged cells (Lopes et al., 2010, Tjølsen, Berge, Hunskaar, Rosland, & Hole, 1992).

The red algae *Amansia multifida* J. V. Lamourouxis is widely found in the southeast and northeast coast of Brazil. Its ethanolic extracts, containing phenolic compounds as main constituents, present *in vitro* antioxidant activity and no toxicity (Alencar et al., 2014), which encouraged the investigation of effects of *A. multifida* ethanolic extracts on experimental models of nociception, inflammation and in behavioral tests of central nervous system.

Material and methods

Preparation of EEAm

The red seaweed *Amansia multifida* J. V. Lamouroux was collected during low tide in Paracuru (São Gonçalo do Amarante, Ceará, Brazil) under authorization of the Brazilian Institutions SISBIO/IBAMA (33913-1). The identification was carried out by Dr. Alexandre H. Sampaio and a voucher specimen was deposited (n° 53175) at the Herbarium Prisco Bezerra, Department of Biology, *Universidade Federal do Ceará*. Fresh macroalgae was washed with distilled water, to remove epiphytes and other organisms, ground and lyophilized. The lyophilized sample was subjected to double extraction with 70% ethanol at 1:20 (m v^{-1}). Ethanolic extracts of *A. multifida* (EEAm) were concentrated by reduced-pressure distillation before use in biological assays (Alencar et al., 2014).

Animals

Male Swiss mice (25-30 g; N=231), maintained under 12/12h - light/dark cycle at 25°C, were given food and water *ad libitum*. Experimental protocols were conducted according to the guide for the care and use of Laboratory Animals for National Research Council of the National Academy (revised in 2011) and approved by the Institutional Animal Care and Use Committee of the *Universidade Estadual do Ceará* - Brazil (CEUA 331951512914).

Drugs and reagents

λ-carragenan, formalin, morphine, indomethacin, acetic acid and pentylenetetrazole (PTZ) were purchased from Sigma (St. Louis, MO, USA) and diazepam by Novaquímica (São Paulo, SP, Brazil). Drugs were solubilized directly in sterile saline (NaCl 0.15 M), except for indomethacin, that was first dissolved in dimethyl sulfoxide up to 10% of the total volume, then in saline.

Behavioral Tests

EEAm at 2.5, 5 and 10 mg kg^{-1} (v v^{-1}; 0.1 mL 10 g^{-1} body mass) was dissolved in sterile saline and injected by intraperitoneal (i.p.) route in mice before being assayed in behavioral tests of nociception (writhing, formalin and tail flick test) or to evaluate alterations of the central nervous system (open field and seizure test induced by PTZ). Control animals received saline (i.p.) instead of EEAm.

Formalin: Formalin (20 µL 2.5% v v^{-1}.) was injected by subcutaneous (s.c.) route in the right hind paw of mice and the time (s) that the animal spent licking paws was recorded at initial or neurogenic (Phase 1: 0-5 min) and late or inflammatory (Phase 2: 15-30 min) phases (Hunskaar, et al. 1985). Animals were treated with saline, EEAm or morphine (5 mg kg^{-1}; s.c.) 30 min before formalin.

Writhing: Acetic acid (0.8%) was injected i.p. in mice (v v^{-1}; 0.1 mL 10 g^{-1} body mass) 10 min before recording the number of writhes (typical contractions of the abdominal musculature followed by hind limb stretches) and evaluated for 20 min (Koster, Anderson, & Debeer, 1959). Animals were treated with saline, EEAm or indomethacin (10 mg kg^{-1}, i.p.) 30 min before acetic acid.

Tail Flick: Mice tails were immersed in water bath (50°C) and the reaction latency of thermal stimuli (time spent before appearance of tail flick reaction) was recorded. Two basal latencies were measured up to 10 s before test (D'amour, & Smith 1941). The nociceptive latency (spinal reflex) was

recorded at zero, 30, 60, 90, 120, 150 and 180 min. Animals were treated with saline or EEAm 30 min before tail immersion.

Open Field: Mice were individually placed in the open-field apparatus, consisting of an acrylic box (30 × 30 × 15 cm) with floor divided into 9 squares. The number of crossed with all paws was counted during 6 min (Montgomery, 1955). Animals were treated with saline, EEAm or diazepam (2 mg kg^{-1}; i.p.) 30 min before evaluation.

Seizures: Mice received pentylenetetrazole (PTZ; 85 mg kg^{-1}; i.p.) and the latencies to the first seizure and to death (anticonvulsant response) were evaluated during 20 min (Erdogan, Golgeli, Arman, & Ersoy, 2004). Animals were treated with saline, EEAm or diazepam (2 mg kg^{-1}; i.p.) 30 min before pentylenetetrazole.

Inflammation Models

EEAm (2.5, 5 or 10 mg kg^{-1}) was dissolved in sterile saline and administered i.p. in mice, 30 min before the inflammatory agent carrageenan (1%; s.c.), for evaluation of its anti-inflammatory activity in the models of paw edema and peritonitis. Control animals received saline (v v^{-1}; 0.1 mL 10 g^{-1} body mass) in substitution of EEAm.

Paw Edema: Paw edema was induced in mice by 1% carrageenan (50 μL paw^{-1}; s.c.) and evaluated by plethysmometry (PanlabLE-7500) at the following intervals: zero, 30 min and from 60 to 240 min thereafter. Edema was expressed by the difference in paw volume displacement (μL) between the different time measurements and zero time or by the area under the curve (AUC) (arbitrary units) (Landucci et al., 1995). Indomethacin (10 mg kg^{-1}) was injected s.c. in the contralateral paw as reference drug.

Peritonitis: Leukocyte migration was induced by 1% carrageenan (50 μL; i.p.) and evaluated 4 h later. After sacrifice, peritoneal cells were harvested by injection of 3 mL PBS, containing 0.1% heparin, for total and differential cell counts (Souza & Ferreira, 1985).

Statistical Analysis

Results were tested by ANOVA and Bonferroni's test, except for total cell counts, in which Newman-Keuls was used as post-hoc test. Values of $p < 0.05$ were considered significant.

Results and discussion

EEAm inhibited the licking time (s) in both phases. In Phase 1 (neurogenic), EEAm was inhibitory at all doses: 2.5 mg kg^{-1} (30.00 ±5.20) by 59%, 5 mg kg^{-1} (39.33 ± 7.34) by 46% and 10 mg kg^{-1} (40.33 ± 5.96) by 45% compared to saline (72.64 ± 5.89). In Phase 2 (inflammatory), EEAm was inhibitory at 2.5 mg kg^{-1} (68.29 ± 14.85) by 62% and at 5 mg kg^{-1} (94.33 ± 26.00) by 50%, compared to control (187.3 ± 14.14). The analgesic control morphine (5 mg kg^{-1}) inhibited both phases: Phase 1 (10.50 ± 4.27) by 85% and Phase 2 (1.62 ± 1.25) by 99% (Figure 1A). These effects suggest a dual action, either central or peripheral, like morphine (Czuczwar & Frey, 1986).

In order to distinguish between peripheral and central antinociceptive action, EEAm was assayed in the writhing test induced by acetic acid and in the tail flick test. The writhing test is widely used to search for new agents possessing peripheral analgesic and anti-inflammatory properties (Le Bars, Gozariu, & Cadden, 2001). The nociceptive activity of acetic acid is associated with the release of inflammatory mediators, such as prostaglandins, sympathomimetic amines and several cytokines (IL-1β, TNF-α, IL-8) released from resident peritoneal macrophages and mast cells (Ribeiro et al., 2000). EEAm inhibited the number of writhes induced by acetic acid at all doses: 2.5 mg kg^{-1} (18.63 ± 4.61) by 50%; 5 mg kg^{-1} (8.12 ± 2.40) by 78% and 10 mg kg^{-1} (14.86 ± 2.85) by 60% compared to saline (37.29 ± 3.88). The anti-inflammatory control indomethacin reduced by 45% the number of writhes (20.40 ± 2.94) (Figure 1B), reinforcing its effect on the inflammatory phase of the formalin test (Phase 2). Accordingly, the methanolic extract of the red algae *Bryothamnion triquetrum* showed inhibitory effect in the writhing test induced by acetic acid (Cavalcante-Silva et al., 2012). Also, there is a study demonstrating the inhibitory effect of lectin isolated from *Amansia multifida* in both phases with predominance in phase 2, causing 61 and 48% inhibition of licking time at the doses of 5 and 10 mg kg^{-1}, p.o., and 60, 88, and 69% inhibition after i.p. administration of 1, 2, and 5 mg kg^{-1}, respectively of the formalin test (Neves et al., 2007).

The tail flick test evaluates nociceptive activity mediated by central mechanisms, in which opioid agents exert analgesic effects via supra spinal and spinal receptors (Bannon & Malmberg, 2007). EEAm (10 mg kg^{-1}) increased the nociceptive latency (s) at 90 min (4.20 ± 0.55 *vs.* saline: 1.50 ± 0.26) by 64% and at 150 min (4.80 ± 0.57 *vs.* saline: 2.10 ± 0.60) by 56% (Figure 1C), supporting the results obtained in the neurogenic phase of the formalin test (Phase 1).

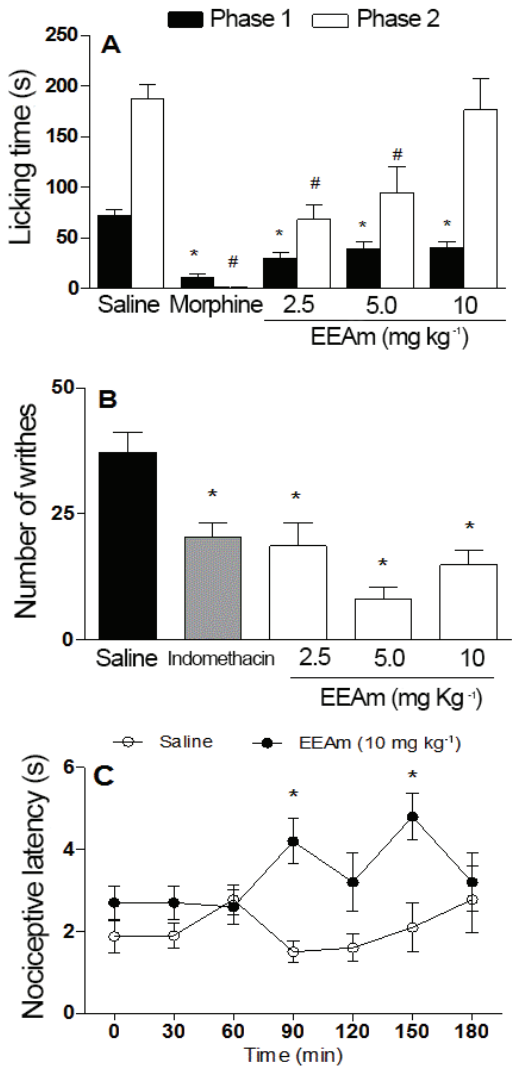

Figure 1. Effect of EEAm on nociception. Animals received saline or EEAm i.p. 30 min before nociceptive tests. (A) Formalin (2.5% v v^{-1}; i.p.) test; (B) Writhing test: acetic acid (0.8% v v^{-1}; i.p.); (C) Tail flick test (50°C). Indomethacin (10 mg kg^{-1}, i.p.) or morphine (5 mg kg^{-1}; s.c.) was administered 30 min before stimuli. Mean ± SEM (n=8). ANOVA and Bonferroni's test. ⋆p < 0.05 *vs.* saline; #p < 0.05 *vs.* saline (Phase 2).

Based on the demonstration of the neurogenic effect of EEAm, other probable central actions were investigated. In the open field test, EEAm reduced the number of crossing at all doses: 2.5 mg kg^{-1} (31.71 ± 1.47) by 31%, 5 mg kg^{-1} (28.14 ± 3.09) by 38% and 10 mg kg^{-1} (21.33 ± 4.40) by 46%compared to saline (46.09 ± 3.81). The central nervous system depressant diazepam was also inhibitory (24.20 ± 1.71) (Figure 2). In the seizure test induced by PTZ, EEAm did not alter the latency of first seizures. However, it increased by 36% the latency of death at 5 mg kg^{-1} (927.0 ± 240.3) compared to saline (332.5±75.98) and 25% of the animals survived (Table 1). It is known that PTZ induces seizures by inhibition of chloride channel conductance via GABA-A receptor binding sites (Macdonald & Barker, 1978, Huang, et al., 2001) and increases the density of glutamate receptors (Vasil'ev et al., 2015). Furthermore, one could speculate that EEAm triggers the GABA-A receptor pathway for the occurrence of its protective effect in convulsion.

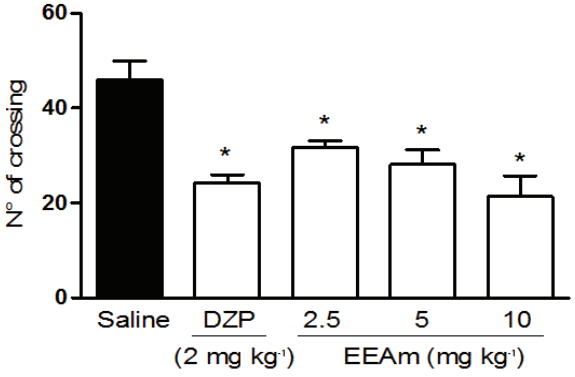

Figure 2. Effect of EEAm on open field. Animals received i.p. saline, EEAm or DZP: diazepam (2 mg kg^{-1}) 30 min before. Mean ± SEM (n=8). ANOVA and Newman-Keuls test. ⋆p < 0.05 *vs.* saline.

Moreover, in this present study, the peripheral action of EEAm was clearly demonstrated in two models of inflammatory nociception (formalin Phase 2 and acetic acid-induced writhes). It is known that paw edema induced by carrageenan in mice is characterized by two distinct phases: an initial inflammatory response that lasts 4-6 hours and a late response, which is maximal at 72 hours and lasts up to 96 hours (Posadas et al., 2004). EEAm inhibited the edema time-course elicited by carrageenan (Cg) at 5 mg kg^{-1} in the 3rd h by 71% (16.66 ± 4.94 μL) and in the 4thh by 91% (5.00± 5.00 μL) compared to Cg (3 h: 58.33 ± 7.03; 4 h: 58.33 ± 9.45 μL).

Table 1. Effect of EEAm on PTZ-induced seizures.

[a]Treatment (i.p.)	Latency of seizure (s)	Seizure (%)	Latency of death (s)	Survival (%)
Saline	61.8 ± 4.82	100	332.4 ± 64.22	25.0
Diazepam (2 mg kg^{-1})	122.5 ± 9.44⋆	100	1007.0 ± 98.98⋆	70.0
EEAm (2.5 mg kg^{-1})	79.47 ± 10.15	100	459.6 ± 112.06	12.5
EEAm (5 mg kg^{-1})	104.3 ± 14.78	100	941.3 ± 239.90⋆	25.0
EEAm (10 mg kg^{-1})	74.8 ± 15.27	100	336.1 ± 30.37	0.0

[a]30 min prior to test. Mean ± SEM (n=8). ANOVA and Newman-Keuls test. ⋆p<0.05 *vs*. Saline.

At 10 mg kg^{-1} EEAm inhibited the paw-edema in the 3rd h by 60% (23.33 ± 4.94 μL) and in the 4thh by 82% (10.00 ± 4.47 μL) compared to Cg (3 h: 58.33 ± 7.03; 4 h: 58.33 ± 9.45 μL) (Figure 3A). The anti-inflammatory control indomethacin was also inhibitory (1200 ±173.2 *vs* Cg: 3500 ± 475.4 AUC).

The suggestion of anti-inflammatory effect of EEAm was corroborated in the peritonitis model, in which EEAm inhibited the leukocyte migration at all doses: at 2.5 mg kg^{-1} by 45% (1288 ± 154 cells mL^{-1}), at 5 mg kg^{-1} by 41% (1371 ± 184.7 cells mL^{-1}) and at 10 mg kg^{-1} by 27% (1711 ± 278 cells mL^{-1}) compared to carrageenan (2347 ± 293 cells mL^{-1}) (Figure 3B).

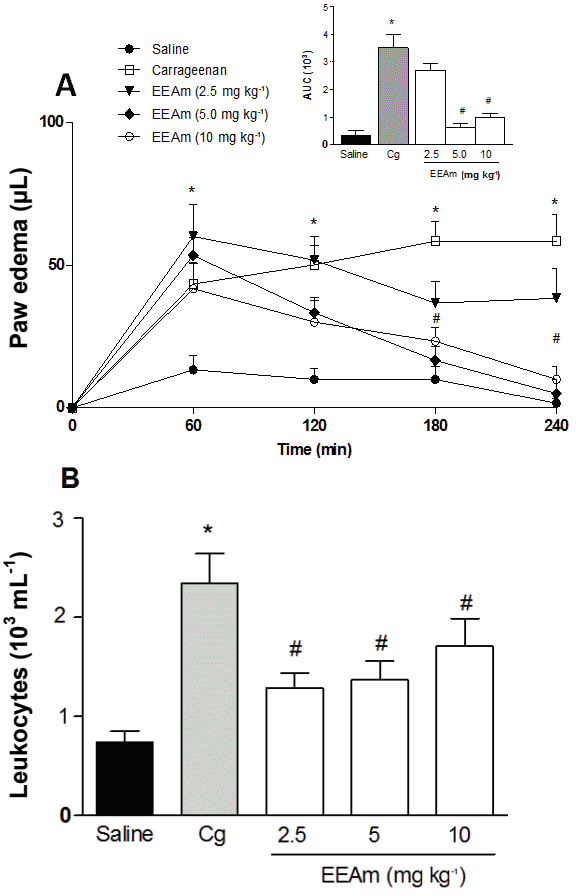

Figure 3. *Effect of EEAm in acute inflammatory parameters*. Animals received i.p. saline, EEAm or indomethacin (10 mg kg^{-1}; i.p.) 30 min before carrageenan (Cg; 1%) for (A) evaluation of paw edema and (B) leukocyte migration 4 h afterward. Mean ± SEM (n= 6-8). ANOVA and Bonferroni's test. $^{\star}p < 0.05$ *vs.* saline, $^{\#}p < 0.05$ *vs.* carrageenan.

Biological activities (anti-inflammatory, anti-hypernociceptive and antioxidant) of red marine algae extracts, such as *Lithothamnion muelleri* (Costa et al., 2015) *Neorhodomela aculeate* (Lim et al. 2006) and *Bryothamnion triquetrum* (Cavalcante-Silva et al., 2012) had already been demonstrated in vivo. Galactans from the red seaweed *Amansia multifida* present effects on inflammation, angiogenesis, coagulation and cell viability (Souza et al., 2012). *In vitro* antioxidant activity of *A. multifida* ethanolic extract (EEAm) was also described, together with its lack of toxicity (Alencar et al., 2014). The antioxidant effect is in accordance with our present demonstration of the EEAm anti-inflammatory effect.

These novel finding described in this study highlights the importance of red seaweed as alternative sources of anti-inflammatory, antinociceptive and anticonvulsant therapy possessing limited adverse effects.

Conclusion

In conclusion, EEAm presents antinociceptive, anti-inflammatory and anticonvulsant effects in mice involving peripheral and central-acting mechanisms.

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