Ethanolic Extract of the Red Algae Meristiella echinocarpa (Areschoug) Confers Neuroprotection in Mice

Extrato Etanólico da Alga Vermelha Meristiella echinocarpa (Areschoug) Confere Neuroproteção em Camundongos

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Abstract

Objectives: This study aimed to investigate the neuroprotective effects of the Ethanolic extract obtained from red algae marine Meristiella echinocarpa (Aracheoungiaceae) – EEMe. Methods: EEMe was used in doses ranging from 10 to 40 mg/kg, administered intraperitoneally in mice. Behavioral tests were performed to assess locomotor activity (open field), anxiety (elevated plus maze), depression (tail suspension), and motor coordination (rota-rod). The anticonvulsant effect of the algae extract was evaluated in two models of seizures induced by strychnine and pentylenetetrazol. The level of oxidative stress was also evaluated in the following brain areas: the prefrontal cortex, hippocampus, and striatum. Statistical analysis was performed applying ANOVA followed by the Bonferroni test. Results: EEMe reduced significantly the number of crossing (36%) and rearing (54%) in the open field test and increased 1.3x the immobility time in the tail suspension test. In brain areas EEMe also reduced significantly malondialdehyde levels (striatum: 45%, hippocampus: 38%, prefrontal cortex: 37%) and nitrite levels (striatum: 72%, hippocampus: 79%, prefrontal cortex: 63%), and increased the reduced-glutathione levels (striatum: 72%, hippocampus: 73%, prefrontal cortex: 42%). In addition, the extract significantly prolonged the latency of seizures induced by strychnine (38%) or pentylenetetrazol (57%), and the latency of death induced by pentylenetetrazol (6.1x). Conclusion: EEMe exhibits antioxidant and anticonvulsant effects, probably involving GABAergic and glycinegic pathways.

Keywords: Marine alga; Meristiella echinocarpa; Brain; Anticonvulsant; Oxidative Stress.

INTRODUCTION

Free radical production in excess leading to damage in cellular structures of the central nervous system has been closely associated with pathological conditions, such as Parkinson's disease, stroke, dementia, and epilepsy. The antioxidant system plays defensive mechanisms against free radicals, as reactive oxygen (ROS) and nitrogen (RNS) species. In homeostatic conditions the production of these species is well regulated, but when this balance is shifted towards pro-oxidants, a state of oxidative stress occurs.

The number of people in the world affected by neurodegenerative diseases has increased, mainly because of an aging population, which has encouraged the search for new therapies from natural sources. The literature highlights marine algae as an important source of compounds structurally diverse, that possess pharmaceutical and biomedical potential, including neuroprotective activities, as described for ethanol extracts obtained from red alga, such as Gelidium amansii, Gracilariosps chorda, and Porphyra yezoensis.

Regarding the red algae Meristiella echinocarpa (Areschoug), widely found in the coast of Southeast and Northeast Brazil, and...
the in vitro antioxidant activity of its ethanol extract had been demonstrated\textsuperscript{11}. However, so far, no report had referred to the in vivo effects of \textit{M. echinocarpa} neither to those of other algae belonging to the same genera or family in the central nervous system.

Thus, this study aimed to investigate the central effects of the ethanol extract of \textit{M. echinocarpa} (EEMe) in behavioral tests and alterations of oxidative stress markers in brain areas.

**MATERIALS AND METHODS**

**Algae Collection**

The algae collection was performed during low tide in Paracuru (São Gonçalo do Amarante, Ceará, Brazil) under authorization of the Brazilian organ SISBIO/IBAMA (n° 33913-1). The botanical identification was carried out by Dr. Alexandre H. Sampaio and a voucher specimen was deposited (n° 53175) at the Herbarium Prisco Bezerra of the Biology Department, Federal University of Ceará (UFC).

**Preparation of Ethanol extract**

Algae was washed with distilled water, grounded, lyophilized, and submitted to double extraction with 70\% ethanol at 1:20 (m/v). The extracted material (EEMe) was concentrated by reduced-pressure distillation and dissolved in sterile saline before assay\textsuperscript{11,12}.

**Drugs and Reagents**

Pentyleneetrazol and Strychnine were purchased from Sigma (St. Louis, MO, USA). Diazepam from Nova Quimica (São Paulo, SP, Brazil). Phenobarbital, Imipramine and Flumazenil were purchased from União Química (Brasília, DF, Brazil). Drugs were solubilized directly in sterile saline (NaCl 0.9\%). All other reagents were of analytical grade.

**Animals and Ethics Committee**

Male Swiss mice (25–30 g) were provided by the Central Animal House from the Federal University of Ceará – Brazil maintained with free access to food and water at 22 ± 2 °C and 12 h light-dark cycle and allowed to adapt to the laboratory for at least 1 h before experiments. The experimental protocols were performed during the light phase of the cycle according to the guidelines of the Brazilian College of Animal Experimentation (COBEA) and the Ethics Committee for Animal Use (CEUA/UECE N°. 331951512914).

**Experimental Design**

EEMe (10, 20, 40 mg/kg v/v; 0.1 mL/10 g body mass) or saline was injected by intraperitoneal (i.p.) route in mice, 30 min before behavioral assays. Diazepam, imipramine, phenobarbital, or flumazenil were used as reference drugs. Animals were sacrificed and brain areas removed to quantify malondialdehyde (MDA), nitrite, and reduced-glutathione (GSH).

**Locomotor Activity (Open Field Test)**

Mice were individually placed in the open-field apparatus, consisting of an acrylic box (30 x 30 x 15 cm) with the floor divided into 9 squares. The number of rectangles crossed by all animal paws (crossing), elevations (rearing), and grooming were counted during 6 min\textsuperscript{13}. Animals received diazepam (2 mg/kg; i.p.) 30 min before evaluation.

**Anxiety (Elevated Plus Maze Test)**

Mice were placed in the center of the elevated plus-maze, with its head turned to one of the closed arms. The equipment consists of two opposing open arms (30 x 5 x 25 cm) and two closed arms (30 x 25 x 5 cm). The frequency of entries and the time spent in the open arms were observed during 5 min\textsuperscript{14}. Control animals received diazepam (1 mg/kg; i.p.) 30 min before evaluation.

**Depression (Tail Suspension Test)**

Mice were suspended 50 cm from the ground, fixed by a tape placed at 1 cm from the tip of the tail, and the immobility time was counted during 5 min\textsuperscript{15}. Control animals received imipramine (10 mg/kg; i.p.) 30 min before evaluation.

**Motor Coordination (Rota-Rod Test)**

Mice were placed in the rotator bar (2.5 cm in diameter), distant 25 cm from the system floor at a constant speed of 18 rpm. The time in which animals remained on the rota-rod and the number of falls were registered during 60 sec\textsuperscript{16}. Control animals received diazepam (2 mg/kg i.p.) 30 min before evaluation.

**Seizure Test**

Mice received i.p. the convulsant stimuli (pentyleneetrazol at 100 mg/kg or strychnine at 20 mg/kg) and the latencies to the first seizure and for death were evaluated during 20 min\textsuperscript{17,18}. Control animals received diazepam (2 mg/kg; i.p.) or phenobarbital (2 mg/kg; i.p.) 30 min before convulsant drugs. Flumazenil (2.5 mg/kg, i.p.) was injected 15 min before EEMe.

**Tissue Preparation**

Brain tissue samples (striatum, prefrontal cortex, and hippocampus) were homogenized in 0.1 M phosphate buffer (pH 7.4), centrifuged (13.000 rpm, 10 min) and the supernatant was used for evaluation of oxidative stress.

**Nitrite (Griess Reaction)**

The homogenate supernatant (100 μL) was incubated with 100 μL of Griess reagent [1% sulfanilamide, 1% H₃PO₄, 0.1% N-(1-
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naphthyl)-ethylenediamine dihydrochloride, distilled water (1:1:1:1) at room temperature for 10 min. Nitrite content was determined based on the standard NaNO<sub>2</sub> (0.75 - 100 mM) curve at A540 nm.

**Malondialdehyde (T-BARS)**

The homogenate supernatant (63 µL) was mixed with 100 µL of 35% perchloric acid, centrifuged (5,000 rpm/10 min) and the final supernatant (150 µL) removed, mixed with 50 µL of 1.2% thiobarbituric acid and heated in boiling water bath for 30 min. Malondialdehyde (MDA) was quantified at A535 nm.

**Reduced-Glutathione (DTNB Reaction)**

The homogenate supernatant (66 µL) was mixed with 50% trichloroacetic acid and centrifuged (5,000 rpm, 15 min). The final supernatant was collected, mixed with 0.4 M Tris-HCl buffer (131 µL, pH 8.9) and 0.01 M DTNB (3.3 µL), incubated for 1 min, and the reduced-glutathione (GSH) was quantified at A412 nm.

**RESULTS**

**Behavioral Effects of EEMe**

EEMe reduced the n° of crossing by 23% at 10 (40.28 ± 4.04), 36% at 20 (33.00 ± 1.30) and 35% at 40 mg/kg (33.57 ± 3.06) versus saline (52.86 ± 3.80) (Figure 1A). EEMe also reduced the n° of rearing by 52% at 20 (10.57 ± 1.15) and 49% at 40 mg/kg (11.29 ± 1.42) versus saline (22.00 ± 2.53) (Figure 1B). However, the n° of grooming was similarly increased by 75% at 20 (2.00 ± 0.21) and 40 mg/kg (2.00 ± 0.21) versus saline (1.14 ± 0.14) (Figure 1C). The reference drug diazepam (2 mg/kg) inhibited all behaviors (crossing by 54%: 24.20 ± 1.71; rearing by 81%: 4.10 ± 0.76; grooming by 75%: 0.28 ± 0.18).

**Statistical Analysis**

Results were expressed as Mean ± Standard Error of the Mean (SEM) and analyzed by ANOVA (One-Way) followed by Bonferroni’s multiple comparisons test. The level of significance was set for p<0.05.

The anxiolytic drug diazepam (1 mg/kg) increased the time of permanence (116.70 ± 11.75 s vs. saline: 55.00 ± 3.76 s) and the n° of the animal entries (9.00 ± 0.32 vs. saline: 4.57 ± 0.64) in the open arms, while EEMe reduced the animal permanence time by 55% (24.63 ± 4.67) and 67% (17.86 ± 5.73 s) at 10 and 20 mg/kg, respectively. EEMe reduced the n° of entries by 57% at 20 (2.00 ± 0.61) and 53% at 40 mg/kg (2.14 ± 0.26) (Figure 1D and 1E).

In the tail suspension test, EEMe increased the immobility time by 1.9 fold at 20 (129.1 ± 8.16 s) and 1.3 fold at 40 mg/kg (103.6 ± 5.00 s) versus saline (44.86 ± 8.19 s). However, the antidepressant drug imipramine (10 mg/kg) reduced by 60% the immobility time (17.71 ± 4.24 s) (Figure 1F). In addition, EEMe did not alter the animal permanence time or the number of falls in the rota-rod test, while the depressor drug diazepam (2 mg/kg) reduced by 38% the permanence time (35.43 ± 3.16
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vs. saline: 57.57 ± 2.42 s) and increased in 4.7 fold the number of falls (4.85 ± 0.34 vs. saline: 0.85 ± 0.14) (data not shown).

EEMe Reduces Pro-oxidant and Increases Antioxidant Markers in the Brain

EEMe (40 mg/kg) reduced by 37% malondialdehyde levels in prefrontal cortex (168.0 ± 21.83 vs. saline: 269.0 ± 11.74 µmol MDA/g), 38% in hippocampus (205.10 ± 36.78 vs. saline: 335.1 ± 25.08 µmol MDA/g) and 45% in striatum (180.3 ± 13.85 vs. saline: 328.6 ± 30.41 µmol MDA/g) (Figure 2A).

EEMe also reduced nitrite in hippocampus, striatum and prefrontal cortex: a) hippocampus by 45% at 10 (7.90 ± 1.64), 55% at 20 (6.53 ± 1.53) and 80% at 40 mg/kg (2.92 ± 0.60) versus saline (14.56 ± 2.09 mM/g tissue); b) striatum by 44% at 20 (5.27 ± 1.55) and 72% at 40 mg/kg (2.63 ± 0.18) versus saline (9.55 ± 0.73 mM/g tissue); c) prefrontal cortex by 63% at 40 mg/kg (2.59 ± 0.33 vs. saline: 7.08 ± 1.51 mM/g tissue) (Figure 2A). On the other hand, EEMe increased GSH in all brain areas: a) hippocampus by 68% at 10 (941.8 ± 83.12) and 73% at 40 mg/kg (966.3 ± 93.21) versus saline (558.30 ± 45.81 ng GSH/g tissue); b) prefrontal cortex by 42% at 40 mg/kg (871.5 ± 65.23) versus saline (710.5 ± 39.98 ng GSH/g tissue); c) striatum by 72% at 40 mg/kg (1338 ± 146.20) versus saline (774.0 ± 72.01 ng GSH/g tissue) (Figure 2C).

**Figure 2.** EEMe reduces MDA and nitrite, and increases GSH in the brain. Mice received saline or EEMe (10, 20, 40 mg/kg) 30 min before evaluation. Mean ± SEM (n=8). ANOVA and Bonferroni test. *p<0.05, **p<0.01 and ***p<0.001 vs. saline. MDA: malondialdehyde, GSH: reduced-glutathione.

EEMe Inhibits Seizures Induced by Strychnine and Pentylenetetrazol

EEMe increased the seizure latency induced by strychnine by 27% at 10 (33.80 ± 1.57 s), 28% at 20 (33.90 ± 1.82 s) and 38% at 40 mg/kg (36.60 ± 1.99 s) versus saline (26.50 ± 1.85 s). However, there was no significant difference between groups in respect to the latency of death. The reference drug phenobarbital (2.0 mg/kg) increased the seizure latency by 60% (42.38 ± 2.13 s vs. saline: 26.50 ± 1.85 s) and the death latency in 1.3 fold (32.73 ± 4.76 s vs. saline: 14.00 ± 0.84 s) (Table 1).

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Table 1. EEMe protects against strychnine-induced seizures in mice

| Group (mg/kg) | Latency of seizure (s) | % Seizure animals | Latency death (s) | % Surviving animals |
|--------------|------------------------|-------------------|-------------------|-------------------|
| Saline       | 26.50 ± 1.85           | 100               | 14.00 ± 0.84      | 0                 |
| EEMe (10)    | 33.80 ± 1.57*          | 100               | 13.70 ± 1.23      | 0                 |
| EEMe (20)    | 33.90 ± 1.82*          | 100               | 12.60 ± 0.74      | 0                 |
| EEMe (40)    | 36.60 ± 1.99*          | 100               | 17.10 ± 2.48      | 0                 |
| PHB (2.5)    | 42.38 ± 2.13*          | 100               | 32.73 ± 4.76*     | 0                 |

EEMe = Ethanolic Extract of *Meristiella echinocarpa*. PHB = phenobarbital. ANOVA and Bonferroni Test *p<0.05 compared to saline.

Table 2. EEMe protects against pentylenetetrazol-induced seizures in mice

| Group (mg/kg) | Latency of seizure (s) | % Seizure animals | Latency death (s) | % Surviving animals |
|--------------|------------------------|-------------------|-------------------|-------------------|
| Saline       | 40.80 ± 3.17           | 100               | 40.70 ± 3.90      | 0                 |
| EEMe (10)    | 57.60 ± 2.50           | 100               | 159.30 ± 17.36*   | 0                 |
| EEMe (20)    | 65.10 ± 6.21*          | 100               | 283.90 ± 19.66*   | 0                 |
| EEMe (40)    | 63.30 ± 2.93*          | 100               | 250.70 ± 23.01*   | 0                 |
| FLU + EEMe (20) | 49.86 ± 5.69#        | 100               | 85.00 ± 22.10#    | 0                 |
| DZP (1)      | 122.5 ± 9.44*          | 100               | 1007 ± 98.98*     | 70                |

EEMe = Ethanolic Extract of *Meristiella echinocarpa*. FLU = Flumazenil. DZP = Diazepam. ANOVA and Bonferroni Test. *p<0.05 vs. Saline. #p<0.05 vs. EEMe 20 (mg/kg).

DISCUSSION

In this study, the inhibitory effect of EEMe in mice exploratory activity was demonstrated, although devoid of anxiolytic or antidepressant activities. Besides, EEMe was shown to reduce per se oxidative stress and provide protection against seizures induced by pentylenetetrazol or strychnine.

Alterations in mice exploratory behavior evaluated in the open field, such as reduction in the n° of crossing and rearing or increase in the n° of grooming, indicate central inhibitory effect. In another study, the ethanolic extract of the red algae *Amansia multifida* also decrease the exploratory activity in mice in the open field test, presenting central inhibitory effects. Drugs that inhibit the CNS, such as the anxiolytic benzodiazepine, are used to treat anxiety, convulsions, and seizures, in cases of emergency, or chronic epilepsy in long-term treatment.

The tail suspension test is widely used to determine the therapeutic effects of antidepressant drugs. In this test, the observed increase in immobility time elicited by EEMe discard the antidepressive effect. In addition, the lack of evidence of motor disorders or muscle relaxant effects observed in the Rota Rod test suggests that the EEMe inhibitory effects occur via central nervous system modulation.

The antioxidant system of living organisms is the main defensive mechanism against free radicals (ROS, RNS) naturally formed during metabolic processes in the brain. If well regulated contributes to the homeostasis in healthy cells. However, the increased levels of free radicals, creating a state of oxidative stress, leads to the development of depression, anxiety, schizophrenia, and epilepsy. In this study, EEMe inhibited pro-oxidants (MDA, nitrite) and stimulated the antioxidant marker GSH in brain areas (striatum, hippocampus, prefrontal cortex), suggesting a potent antioxidant effect. The in vivo antioxidant activity of EEMe is in line with previous in vitro demonstration for the red algae *M. echinocarpa*, evidenced by its ability to scavenge electrons using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and to treat hypersomnia and narcolepsy. Anxiogenic drugs such as caffeine and modafinil in high doses can also reduce locomotor activity, an effect that was also observed for EEMe, as seen in the open field test.

The antioxidant system of living organisms is the main defensive mechanism against free radicals (ROS, RNS) naturally formed during metabolic processes in the brain. If well regulated contributes to the homeostasis in healthy cells. However, the increased levels of free radicals, creating a state of oxidative stress, leads to the development of depression, anxiety, schizophrenia, and epilepsy. In this study, EEMe inhibited pro-oxidants (MDA, nitrite) and stimulated the antioxidant marker GSH in brain areas (striatum, hippocampus, prefrontal cortex), suggesting a potent antioxidant effect. The in vivo antioxidant activity of EEMe is in line with previous in vitro demonstration for the red algae *M. echinocarpa*, evidenced by its ability to scavenge electrons using 2,2-diphenyl-1-picrylhydrazyl (DPPH)
radicals\(^1\), as well by other studies performed with red algae extracts or with its bioactive compounds, showing antioxidant effect\(^2\,\,^2\). Besides, neuroprotective effects of homotaurine, a small aminosulfonate molecule, which is present in different species of red algae have been demonstrated in vitro and in vivo models of neurodegenerative diseases\(^2\)\(^9\).

It is well known that strychnine increases the activity of excitatory synapses via inhibition of glycine receptors present in motoneurons and the spinal cord\(^1\), and pentyleneetetrazol inhibits the chloride channel conductance via GABA-A receptor\(^2\) and increases the density of glutamate receptors\(^2\)\(^6\). Our results demonstrated that EEMe increased the seizure latency induced by strychnine, but did not alter the latency of death.

In respect to pentyleneetetrazol-induced seizures, EEMe increased both seizures and death latency, and when associated with the antagonist of benzodiazepine receptors flumazenil, showed protective effects, reducing the latency of convulsion and the death latency. These data altogether suggest that EEMe triggers the GABA-A pathway in order to exert its protective effect in convulsion. The anticonvulsant effect of EEMe is in accordance with the protective effects of the ethanolic extract obtained from the red algae Amansia multifida on seizure experimental models\(^1\)\(^2\).

In conclusion, we demonstrate that EEMe exhibits a protective effect against pentyleneetetrazol-induced seizures by increasing the seizure latency. The anticonvulsant activity of EEMe is associated with antioxidant activity and a modulatory effect on the central nervous system via GABAergic and glycinergic pathways. The results presented in this investigation suggest that EEMe possesses a pharmacological potential to treat seizures due to its antioxidant activity and modulator effect on the central nervous system via GABAergic and glycinergic pathways. The antioxidant activity could be associated with the elevated concentration of phenolic compounds found in EEMe ethanolic extract\(^1\) as also described for another red macroalgae\(^7\).

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