Antinociceptive and anti-inflammatory activities of the hexanic extract of *Echinodorus macrophyllus* (Kunth) Micheli in mice

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**Abstract**

Introduction: *Echinodorus macrophyllus* (Kunth) Micheli, Alismataceae, commonly known as “chapéu de couro”, is used in the treatment of various inflammatory conditions. The aim of this study was to evaluate the antinociceptive and anti-inflammatory neurogenic potential and perform the phytochemical analysis of its hexanic extract (HEEm). Material and methods: The HEEm was obtained by maceration of dried leaves with hexane (100 g d.w./2 L). Its composition was determined by GC-MS (DB1 column) by comparison of retention indices in the database and literature. The antinociceptive potential was evaluated in SW or DBA/1 male mice using chemical (acetic acid and formalin), thermal (tail immersion and hot plate tests) and topical (xylene) nociception models, all approved by the Ethics Committee (CEA-IBRAG). Results: HEEm presented antinociceptive activity in the model of: acetic acid-induced writhing (52%; 25mg/kg); tail immersion (60 and 90 minutes; 50 mg/kg); hot-plate in 60 minutes (25 and 100mg/kg) and 120 minutes (25mg/kg); formalin tests, at the neurogenic (63.4%, 100mg/kg) and inflammatory (50%; 50 and 100mg/kg) phases; and in neurogenic inflammation induced by xylene (88.3%; 100mg/kg). These activities seem to be related to the terpene and fatty acid derivatives evidenced by GC-MS. Discussion: HEEm presented antinociceptive activity, as well as anti-inflammatory activity by central and peripheral mechanisms, it consists of terpene and fatty acid derivatives, described in the literature as antioxidants, anti-inflammatory, and antinociceptives. Conclusions: HEEm showed antinociceptive activity in all models, which can be related to the presence of terpene and fatty acid derivatives.

**Keywords**: *Echinodorus macrophyllus*; Nociception; Neurogenic inflammation; Phytochemistry.

**Resumo**

Atividade antinociceptiva e anti-inflamatória do extrato hexânico de *Echinodorus macrophyllus* (Kunth) Micheli em camundongos

Introdução: *Echinodorus macrophyllus* (Kunth) Micheli, Alismataceae, conhecida como chapéu de couro, é utilizada no tratamento de diversas condições inflamatórias. O objetivo do estudo foi avaliar o potencial antinociceptivo e anti-inflamatório neurogênico e realizar a análise fitoquímica de seu extrato hexânico (EHEm). Material e métodos: O EHEm foi obtido por maceração das folhas em hexano (100 g p.s./2 L). Sua composição foi determinada por GC-MS (coluna DB1) por comparação dos índices de retenção do banco de dados e da literatura. O potencial antinociceptivo foi avaliado em camundongos SW ou DBA/1 machos, utilizando modelos de nocicepção química (ácido acético; formalina), térmica (teste de imersão da cauda; placa quente) e tópica (xileno), aprovados pelo comitê de ética (CEA-IBRAG). Resultados: O EHEm apresentou atividade antinociceptiva nos modelos de contorções induzidas pelo ácido acético (52%; 25mg/kg); teste de imersão da cauda (60 e 90 min; 50 mg/kg); placa quente em 60 min (25 e 100 mg/kg) e 120 min (25 mg/kg); formalina, na fase neurogênica (63,4%; 100 mg/kg) e na inflamatória (50%; 50 e 100 mg/kg) e na inflamação neurogênica induzida pelo xileno (88,3%; 100 mg/kg). Estas atividades parecem estar relacionadas aos derivados de terpeno e ácidos graxos evidenciados por GC-MS. Discussão: O EHEm mostrou atividade antinociceptiva por mecanismos centrais e periféricos, além de anti-inflamatória. É composto por derivados terpênicos e de ácidos graxos, descritos na literatura como antioxidantes, anti-inflamatórios e antinociceptivos. Conclusões: O EHEm mostrou atividade antinociceptiva em todos os modelos, a qual pode estar relacionada à presença de derivados terpênicos e de ácidos graxos.

**Descritores**: *Echinodorus macrophyllus*, Nocicepção; Inflamação neurogênica; Fitoquímica.
The use of products and supplements from medicinal plants has increased in recent decades. The World Health Organization has estimated that approximately 65% of the world’s populations rely mainly on plant-derived traditional medicines for their primary health care. However, the effectiveness and toxicity of many of these products are not ensured, requiring more studies.

The species of this study, *Echinodorus macrophyllus* (Kunth) Micheli, popularly known as “chapéu de couro”, belongs to the family Alismataceae, which is composed by 14 genus and 60 species, occurring mainly in tropical areas, of which the genus *Echinodorus* is the most abundant in Brazil. The infusion of its leaves is used in folk medicine as a diuretic and to treat inflammatory conditions.

Previous studies with the aqueous extract of *E. macrophyllus* (AEEm) showed no mutagenicity or cytotoxicity on renal epithelial cell lines and hepatoma. Treatment of mice with AEEm for six weeks at the recommended dose for humans (23 mg d.w./kg) showed no changes. However high doses (297 mg d.w./kg or 2.22 g/kg crude extract) promoted a reduction of body weight, plasma changes suggestive of subclinical liver toxicity and genotoxic activity in the kidneys, which constitutes a warning signal for treatments with high doses. AEEm was effective in the suppression of T-cell immune response in mice, in the reduction of nitric oxide in J774 cells stimulated in vitro with LPS, and in anti-inflammatory activity in vitro on RAW 264.7 cells and in vivo in air pouch model. Acute and subchronic anti-inflammatory effects were observed for the ethanolic extract of *E. macrophyllus*. On the other hand, only a few reports exist of *E. macrophyllus* antinociceptive action for the essential oil and hydroethanolic extract in the acetic acid-induced writhes model.

Although *E. macrophyllus* is listed in the Brazilian Pharmacopoeia (both in 1926 and in 1959), it is essential to promote research for its further use as a phytotherapeutic drug, also due to its commercial importance.

In this work, the antinociceptive properties of the hexanic extract of *E. macrophyllus* were demonstrated, using different models of nociception and neurogenic inflammation.
95% (100g/2 L) and evaporation at 35°C on a rotary evaporator (802D Fisatom).

The HEEm was solubilized in dichloromethane (1µg/µL) and analyzed by gas chromatography coupled with mass spectrometry (GC-MS) using a DB-1 capillary column (30m x 0.25mm x 0.25µm) and nitrogen (flow 1.0mL/min) as a carrier gas. The temperature of the injector and the interface were 260°C and 200°C, respectively, with an operating temperature range of 100°C to 300°C (7°C/min) and a flow rate of 1 mL/min. The identification of HEEm proceeded by comparing retention indices and mass spectra (MS) with data from the published literature and with the WILEY and NIST 275 3.0 library, provided by equipment (Shimadzu 17A-Shimadzu QP 2010Plus). The results were also confirmed by comparing the elution order of compounds with their relative retention indices (Rlilis) reported in the literature. Retention indices (RI) were calculated for all volatile components using the data retention of n-alkanes with C9-C30 linear. HEEm was diluted with a vehicle (15% ethanol, 1.25% Tween 20, 1.5mL/kg) for the antinociceptive tests.

In vivo assays

Male Swiss Webster (SW) mice (3-4 months, 25-35g) or DBA/1J mice (3-4 months, 25-30g) were obtained from Department of Biochemistry of the State University of Rio de Janeiro or the Vital Brazil Institute. The mice were housed in a climate-controlled room at constant temperature (23±2°C), under a 12h light/dark period, and free access to food and water before use. At the end of each experimental protocol, the animals were euthanized in the CO2 chamber. All experiments were in agreement with guidelines for ethical standards of the members (CEA-IBRAG committee/protocol 07/2013, 07/2017, CEA-IBRAG committee/protocol 07/2013, 07/2017, 013/2018) approved this study, which was performed under the norms of the National Council for Animal Experimentation Control (CONCEA).

In the acetic acid-induced writhing model,11 SW mice were treated orally (p.o.) with HEEm, vehicle or 50mg/kg dipyrone, one hour before intraperitoneal (i.p.) injection of 0.6% acetic acid (10µL/g b.w.). The contortions (abdomen, trunk and/or pelvis, extension of the members) were observed after 5 minutes of irritant injection for 10 minutes.

In the hyperalgesia induced by formalin12 SW mice were treated (v.o.) 60 minutes before with the vehicle or HEEm, and 30 minutes earlier with dipyrone 50mg/kg (v.o.) or morphine 10mg/kg (subcutaneous, s.c.). After this period, all animals received 20µL 2.5% formalin in phosphate-buffer saline pH 7.4 (PBS), in the right hind paw (sub-plantar, s.p.). Lifting/licking of the injected paw, recorded as nociceptive responses, was measured between 0-5 minutes (first phase, neurogenic) and 15-25 minutes (second phase, inflammatory) after formalin injection.

The hot-plate analgesia test13 was performed with SW mice previously selected with a cut-off of 5-8 seconds of nociceptive response. After 1h of fasting, the animals (n = 5/group) were submitted to treatments (p.o.) with samples (AEEm), vehicle, or with the control drug morphine (10mg/kg, s.c.). After that, mice were placed individually on the plate heated to 55±1°C at 30, 60, and 120 minutes. The latency time, considered as a nociceptive response (reflex of lifting or licking the hind paw), was determined until 30 seconds of cut-off in each instance, to prevent damage.

In the immersion test,14 the SW mice were gently immobilized, and 1/3 of their tails was immersed in a bath with water at 55±1°C. The time (s) between the immersion of the tail and its withdrawal of the water (latency) was determined, with a cut-off of 10 seconds, to avoid tissue damage. Each animal used was its own control, the latencies, before treatment with the samples, being determined three times with intervals of 15 minutes, being selected groups (n = 5) with latency between 1.5 and 3.5 seconds. After 1h of the treatment (v.o.) with the HEEm or the vehicle, or 45 minutes in the morphine control group (10mg/kg, i.p.), the response in the immersion test was evaluated. The responses obtained in this model were converted to a maximum percentage of effect, according to the following formula: %MPE = [(post-treatment latency - baseline latency)/(cut-off time - baseline latency)] x 100.

The xylene-induced mouse ear edema, an experimental model of neurogenic inflammation,15 was carried out in DBA/IJ mice (n = 5/group) sedated (s.c.) with phenobarbital 10mg/kg. After 30 minutes, treatments with HEEm (i.p.), vehicle (i.p.), and indomethacin (10mg/kg, i.p.) were performed. Acute inflammation was induced 1h later by the topical application of 20µL/ear of xylene on the anterior and posterior surfaces of the right ear. After 30 minutes, the animals were euthanized, and the ear punches (6mm diameter) were taken and immediately weighed. The edema was evaluated by comparing the increment of right ear punch weight with the left ear punch used as a control for each animal.
Antinociceptive activity

The treatment with HEEm produced a significant reduction in the number of acetic acid-induced writhing in all doses tested (Figure 2), with the percentage of inhibition of 28% (5mg/kg), 52% (25mg/kg), 32% (50mg/kg), and 35% (100mg/kg), compared to the control group, while dipyrone (50mg/kg) inhibited 51% of contortions.

Antinociceptive activity of HEEm was evaluated in two models of thermal analgesia. In the tail immersion test (Figure 3a), treatment with HEEm showed antinociceptive activity in 60 and 90 minutes with 50mg/kg, increasing the latency time in 2.9x and 3.6x, respectively. On the hot plate (Figure 3b), treatment with HEEm induced significant response in doses of 25mg/kg (2.9x) and 100mg/kg (7.1x) in 60 minutes and 25mg/kg (7.2x) in 120 minutes.

Antinociceptive activity of HEEm was also evaluated in the formalin test (Table 1) that displays two phases: the first phase, which occurs between 0-5 minutes corresponds to the acute pain due to the

Statistical analysis

Results are presented as mean values ± SD. Data were subjected to analysis of variance (One-way ANOVA) followed by the Tukey’s post-hoc test, using the program GraphPad Prism®. Differences between groups were considered significant at a level of p ≤ 0.05 for all comparisons.

Results

Phytochemical analysis

The maceration of dried leaves from *E. macrophyllus* with hexane produced an extract with low complexity by GC-MS and yield of 1.11%. The HEEm GC-MS analysis (Figure 1) showed six peaks of higher intensity, with retention times between 45 and 82 minutes, showing compounds with medium and low volatility. The comparison of RI with the NIST database and literature revealed the palmitic acid, squalene, (Z, Z)-9-12-ethyl octadecadienoate (ethyl linoleate) and the (E)-phytol, as the major compounds.
The painful process by the injection of irritant (neurogenic); the second phase, which comprises the 15-25 minutes period after formalin injection, corresponds to the inflammatory pain, with release of nociceptive mediators. Animals treated with HEEm exhibited predominant antinociceptive effect during the first phase, showing a reduction of 63.4% (100mg/kg) at this time, and a decrease of 50.0% (50 and 100mg/kg) during the second phase. Morphine inhibited both phases and dipyrone reduced mainly the second phase of this model.

The xylene-induced mouse ear edema was used for the study of neurogenic anti-inflammatory activity (Figure 4). HEEm presented a “U” response, with more significant reduction of edema with 100mg/kg (88.3%) and a 34.9% reduction at a higher dose (150mg/kg). Indomethacin reduced on average 54.8% of the neurogenic inflammation induced by xylene.

Discussion

The *Echinodorus macrophyllus* species exhibits anti-inflammatory activity, as suggested by its popular medicinal use. Castro reported the antiedematogenic and antinociceptive potential of its aqueous extract and Fernandes observed the neurogenic anti-inflammatory potential, in addition to detecting the presence of polyphenols, flavonoids and their antioxidant activity, which were taken as bases for this study.
The HEEm was evaluated for antinociceptive potential in experimental models of nociception, such as the abdominal constriction induced by acetic acid, formalin test, tail-immersion test, hot-plate test, and in the xylene-induced neurogenic inflammation.

The acetic acid-induced hyperalgesia is due to the release of endogenous mediators, such as histamine, serotonin, bradykinin, substance P, prostaglandins and some cytokines, which stimulate the nociceptive neurons, sensitive to non-steroidal anti-inflammatory drugs (NSAIDs) and opioids. This test is used to assess both peripherally and centrally acting analgesic activities of natural products. Thus, the antinociceptive effect of HEEm in this model may be related to the inhibition of the release of some of these mediators in response to acetic acid.

The formalin model is commonly used to evaluate acute inflammatory pain and produces two distinct phases. The first phase (neurogenic), characterized by intense pain, starts immediately after the formalin injection and seems to be caused predominantly by activation of C-fibers after peripheral stimulation (direct stimulation of nociceptors). The late phase of moderate pain (inflammatory pain) appears to be caused by tissue and functional changes in the dorsal horn of the spinal cord and is accompanied by the release of inflammatory mediators, via activation of N-methyl-D-aspartate (NMDA) receptors. This phase originates from peripheral mechanisms and seems to be mediated by the activation of central sensitized neurons due to peripheral inflammation, as well as the ongoing activity of primary afferents. Both phases are attenuated by central analgesic drugs, such as opioids, while the response in the second phase is decreased mainly by peripherally acting drugs, selective cyclooxygenase inhibitors, such as steroids (hydrocortisone, dexamethasone) and NSAIDs (aspirin). HEEm was active in all doses at both neurogenic and inflammatory phases, similarly with other compounds that act on the central nervous system (Table 1).

The latency of the heat-activated tail flick reflex is dependent upon activation of cutaneous nociceptors; afferent conduction to the dorsal horn; conduction within the central nervous system (central delay); and conduction from the ventral horn to and activation of tail muscles, and might be a complicated movement involving higher neural structures. The increased latency time at 60 and 90 minutes after administration of HEEm 50mg/kg in this experimental model (Figure 3), may be related to the inhibition of agents that activate the release of the endogenous peptide.

The analgesic effect of HEEm in the hot-plate test could result from modulation of the medullary or central level of pain, since this test has mediated for both, or from the direct inhibitory activity on nerve endings or transmission pathways. Thus, they may be acting either at the peripheral or the central level, or both.

The neurogenic inflammation induced by xylene is related to cellular mechanisms involved in the release of pro-inflammatory substances by sensory

**Table 1. Effects of reference drugs, HEEm, AEEm, Fr20 and Fr40 on formalin-induced nociception in SW male mice**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose mg/kg</th>
<th>1st Phase</th>
<th>2nd Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%Licking time (s)</td>
<td>%Inhibition</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>99.2 ± 5.2</td>
<td>-</td>
</tr>
<tr>
<td>Morphine</td>
<td>10 mg</td>
<td>20.4 ± 2.1***</td>
<td>79.4</td>
</tr>
<tr>
<td>Dipyrone</td>
<td>50 mg</td>
<td>61.0 ± 7.6***</td>
<td>38.5</td>
</tr>
<tr>
<td>HEEm</td>
<td>25 mg</td>
<td>47.4 ± 8.6***</td>
<td>52.2</td>
</tr>
<tr>
<td></td>
<td>50 mg</td>
<td>44.7 ± 7.2***</td>
<td>54.9</td>
</tr>
<tr>
<td></td>
<td>100 mg</td>
<td>36.3 ± 8.7***</td>
<td>63.4</td>
</tr>
</tbody>
</table>

*Mice (n=5/group) were treated with the vehicle (control), HEEm doses (p.o.), 60 minutes before formalin injection or with dipyrone (p.o.), or morphine (s.c.) 30 minutes before formalin injection. 1Mean of licking time ± S.D. between 0-5 minutes (1st phase) and 15-25 minutes (2nd phase) after formalin injection of two experiments. 2Inhibition was calculated in relation to control group. *p<0.05, **p<0.01 vs. control (ANOVA followed by Tukey’s test).
neurons and is useful for the evaluation of topical anti-inflammatory steroids and nonsteroidal antiphlogistic agents, especially those inhibiting phospholipase A2.

Application of xylene causes vasodilatation, increases vascular permeability and plasma extravasations leading to swelling of the ear. This inflammation process is initiated by the action of mediators, such as serotonin, acetylcholine, histamine, bradykinin, and prostaglandins, which release neuropeptides like substance P and activate its receptors, causing neurogenic inflammation. HEEm reduced the edema produced by topical application of this irritant by up to 88% (100 mg/kg), suggesting inhibition of neuropeptides and/or pro-inflammatory mediators action or release in the antinociceptive response.

Phytochemical studies indicate that crude extracts obtained from medicinal plants are rich in a series of secondary metabolites, whose complexity and function may be related to the pharmacological activity of these molecules. The composition of HEEm determined by GC-MS showed that it is composed mainly by terpene derivatives (48.87%) and fatty acid derivatives (63.33%).

Squalene, the major terpene derivative in the HEEm (23.28%), has considerable potential for several pharmaceutical applications. It is used as a protective agent, reducing the side effects induced by chemotherapy, improves the immune response, reduces the effect of reactive oxygen species.

The antinociceptive and anti-inflammatory activities of HEEm, as well as the presence of Squalene, indicate the presence of a range of secondary metabolites that may be responsible for the therapeutic potential.

Conclusion

The present study showed the antinociceptive effect of the hexanic extract of Echinodorus macrophyllus in different nociceptive responses generated by a chemical, harmful thermal or topical stimulus and suggested that terpene and fatty acid derivatives may be responsible for the therapeutic potential.

Acknowledgments

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Referências