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Phytochemical and pharmacological evaluation from leaves of *Costus spicatus* Swartz. (Costaceae)

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ABSTRACT

The species Costus spicatus Swartz, known popularly as "cana-do-brejo," is widely used in folk medicine in treatment of various health problems, such as pain and inflammation. The present study aimed to identify the main chemical classes present in the crude methanol extract and in the hexane, chloroform, ethyl acetate and n-butanol phases from leaves of C. spicatus, as well as to assess the antinociceptive profile of the organic phases in two tests of nociception: acetic acid-induced writhing and formalin-induced nociception in mice. Phytochemical screening revealed the presence of tannins, flavonoids, steroids and triterpenoids in the crude methanol extract and in all organic phases, and the absence of alkaloids only in the n-butanol phase. The tests were negative for saponins and quinones. In the pharmacological evaluation, at the doses of 50, 100 and 200 mg/kg (by oral route), all organic phases showed antinociceptive activity in the acetic acid-induced writhing test ($p < 0.001$). In the formalin test, during the first phase, only treatment with the chloroform fraction demonstrated the antinociceptive response ($p < 0.05$). In the second phase, the hexane, chloroform and ethyl acetate fractions showed an antinociceptive activity ($p < 0.05$). Furthermore, the organic phases did not impair motor coordination of the animals. Therefore, the results showed antinociceptive activity in all organic phases, except in the n-butanol phase, indicating that these effects were probably due to the presence of secondary metabolites.

Key words: *Costus spicatus;* Costaceae; antinociceptive activity

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INTRODUCTION

Pain is a complex, multivariate perceptual experience, defined according to the IASP as "an unpleasant sensory and emotional experience associated with actual or probable tissue damage, or described in terms that suggest such damage" [1]. Considering this aspect, pain is considered a common symptom of many clinical conditions and is probably the main reason for procuring medical consultation.

Many treatments can be considered for the treatment of painful conditions. However, when considering pharmacological treatment, some problems limit the chronic use of many medications. The side effects described for many non-steroidal, anti-inflammatory drugs (NSAIDs) [2] and the phenomena of dependence and tolerance caused by continuous use of opioids are among the main obstacles currently encountered in their clinical use [3].

The main alternatives to the use of currently available medications are natural products, the main source for the discovery of new chemical entities with biological action. Pharmaceutical companies have used crude plant extracts to produce therapeutic formulations for this purpose [4]. According to Nodari and Guerra [5], plants are a rich source of biologically active substances that are widely used as models for synthesis of a large number of drugs.

Therefore, the pharmaceutical industry enthusiastically explores the contribution of medicinal plants in the development of new pharmaceutical products.

Therefore, the use of plants with antinociceptive, anti-inflammatory effects could be pharmacologically beneficial without the undesirable effects of synthetic drugs. Thus, discovering new compounds with analgesic activity, in which there are less pronounced side effects, has been systematically the objective of several studies over the years [6].

In the search for pharmacological characterization of the therapeutic action of several species, there is the study of *Costus spicatus* Swartz, belonging to the family Costaceae, popularly known as "cana-do-brejo." In folk medicine, this species is used to treat inflammation of the bladder (cystitis), urination pain and difficulty, kidney stones and inflammation of the urethra [7].

Studies on some species of the genus *Costus*, using different parts of the plant, have reported antinociceptive, anti-inflammatory activity in rodents. Rodriguez and collaborators [8] demonstrated the antinociceptive activity of *C. pictus* in a study of the extract from the leaves and stem. Bhattacharya and Nagaich [9] suggest the antinociceptive activity of rhizomes from the species *C. speciosus*. Other study demonstrated the anti-inflammatory and antinociceptive activity of the crude methanol extract obtained from *C. spicatus* (MECs) in different models of nociception in rodents [10]. Thus, because of the wide use of this plant species in northeastern Brazil traditional medicine for the treatment of painful conditions and pharmacological studies on genus *Costus*, the objective of this work was the phytochemical investigation of the MECs and the hexane, chloroform, ethyl acetate and *n*-butanol phases from the leaves of *C. spicatus* (HEXPh, CLPh, ETACPh and *n*-BUPh, respectively), aiming to identify their chemical classes and to assess the antinociceptive profile of these phases in rodents.

EXPERIMENTAL SECTION

Collection of the plant material

Leaves of *C. spicatus* were collected at the University Campus "Prof. Aloísio de Campos," in the municipality of São Cristóvão, Sergipe, in November 2009. A voucher specimen of the species was deposited in the Herbarium of the Department of Biology, Federal University of Sergipe (UFS), Sergipe, Brazil, under number ASE 11453.

Preparation of the crude methanol extract of C. spicatus

The plant material was dried in a greenhouse with circulating air at 40 °C and triturated in a knife mill, obtaining 2.100 kg of powder. The powder was subjected to thorough maceration in methanol for 72 hours at ambient temperature. Then, the extract was filtered and concentrated in a rotary evaporator, yielding 90 g of the MECs (4.28% yield).

Fractionation of the methanol extract

The MECs (24.0 g) was subjected to liquid-liquid partition using organic solvents in order of increasing polarity: hexane, chloroform, ethyl acetate and *n*-butanol. After evaporation of the solvents in a rotary evaporator, the following phases were obtained, respectively: HEXPh, 6.680 g; CLPh, 2.600 g; ETACPh, 2.500 g; and *n*-BUPh, 1.700 g.

Phytochemical screening

The MECs and the HEXPh, CLPh, ETACPh and *n*-BUPh were submitted to a preliminary screening, through chemical reactions, to detect the presence of the following classes of secondary metabolites [11]: alkaloids (Dragendorff, Bouchardat and Mayer), quinones (Borntrager direct), tannins (lead acetate and potassium dichromate), saponins (foam index), flavonoids (alkali metal hydroxides and iron salts), steroids and triterpenoids (Liebermann-Buchard).

Drugs

Glacial acetic acid PA, Tween 80, sodium chloride, morphine hydrochloride and Diazepam (DZP) were purchased from Sigma Chemical Company (USA). Formaldehyde 37% was purchased from Synth (Brazil).

Animals

Male Swiss mice weighing 25-30 g were used in all experiments. The animals were housed in polypropylene cages under controlled temperature (23 ± 2 °C) and light (light-dark cycle of 12 hours), and with food and water *ad libitum*. The mice were acclimated in the laboratory at least eight hours before the experiments, and on the day of the experiment were fasted for six hours. All experimental protocols were approved by the Ethics Committee for Animal Research at the Federal University of Sergipe (CEPA/UFS 20/2010).

Acetic acid-induced writhing test

This test was performed according to the methodology described by Koster and collaborators [12] and modified by Broadbear and collaborators [13]. Initially, the mice were divided into 14 groups (n = 6) and pretreated with vehicle (saline/Tween 80 0.2%, p.o.), HEXPh, CLPh, ETACPh, *n*-BUPh (50, 100 and 200 mg/kg, p.o.) and MOR (3 mg/kg, i.p.). After 60 minutes, an acetic acid solution (0.85%) was administered at a proportion of 0.1 ml per 10 g of animal body weight (i.p.). Five minutes after acetic acid injection, the animals were observed individually, and the number of writhings was recorded for a period of 15 minutes.

Formalin test

This study used the formalin test described by Hunskaar and Hole [14]. The animals were divided into 14 groups (n = 6) and pretreated with vehicle (saline/Tween 80 0.2%, p.o.), HEXPh, CLPh, ETACPh, *n*-BUPh (50, 100 and 200 mg/kg, p.o.) and MOR (5 mg/kg, i.p.). After 60 minutes, 20 ml of a solution of 1% formalin was injected into the dorsal surface of the right hind paw of the animal. The nociceptive response was evaluated by measuring the time that the animal spent licking the paw that had received the formalin injection, in two periods: the first period, 0-5 minutes (first phase); and the second period, 15-30 minutes (second phase).

Motor coordination test

The animals were selected 24 hours before the experiment, excluding those that did not remain 180 s (at 9 rpm) on the Rota-rod apparatus (AVS®, Brazil). The selected animals were divided into 6 groups (n = 6) and pretreated with vehicle (saline/Tween 80 0.2%, p.o.), HEXPh, CLPh, ETACPh, *n*-BUPh (200 mg/kg, p.o.) and DZP (3 mg/kg, i.p.). After 30, 60 and 120 min, the animals were again subjected to the Rota-rod test, and the results were expressed as the time (in seconds) that the animals remained on the rotating bar up to a maximum of 180 seconds.

Statistical analysis

All results were presented as mean ± standard deviation of the mean, and the differences between control and the groups treated with the organic phases were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's or Dunnett's test. Differences were considered significant for $p < 0.05$.

The percentage inhibition was calculated by the following formula [15]:

% inhibition = 100 x (control - experiment)/control.

RESULTS AND DISCUSSION

This study presented a knowledge of the main secondary metabolite classes found in the MECs and in the organic phases of the species *C. spicatus* and conducted an evaluation of the antinociceptive profile of HEXPh, CLPh, ETACPh and *n*-BUPh in two tests of nociception in rodents: acetic acid-induced writhing and the formalin test.

Of the seven classes of chemical substances evaluated in the preliminary screening, our study detected the presence of tannins, flavonoids, steroids and triterpenes and the absence of saponins and quinones in the MECs and in all organic phases. As for the presence of alkaloids, *n*-BUPh was the only phase that was found negative for this constituent. The absence of saponins in the MECs and in the organic phases is not consistent with the literature data for the species *C. spicatus* [16] which can be explained by several factors that affect the secondary metabolism of plants, such as seasonality, soil type, temperature, water availability, and climatic factors, among others [17].

Figures 1, 2, 3 and **4** show the results from HEXPh, CLPh, ETACPh and *n*-BUPh, respectively, in the acetic acid-induced writhing test. Treatment of the animals with the organic phases significantly reduced the number of writhings induced by the administration of acetic acid solution (i.p.) compared to animals in the control group.

HEXPh (**Figure 1**) showed an antinociceptive effect at all doses tested (50 mg/kg, $p < 0.01$; 100 and 200 mg/kg, $p < 0.001$). The same occurred with the ETACPh fraction (**Figure 3**) (50 mg/kg, $p < 0.05$; 100 and 200 mg/kg, $p < 0.001$). On the other hand, CLPh (**Figure 2**) and *n*-BUPh (**Figure 4**) required higher doses (100 and 200 mg/kg) to significantly reduce ($p < 0.001$) the number of writhings.

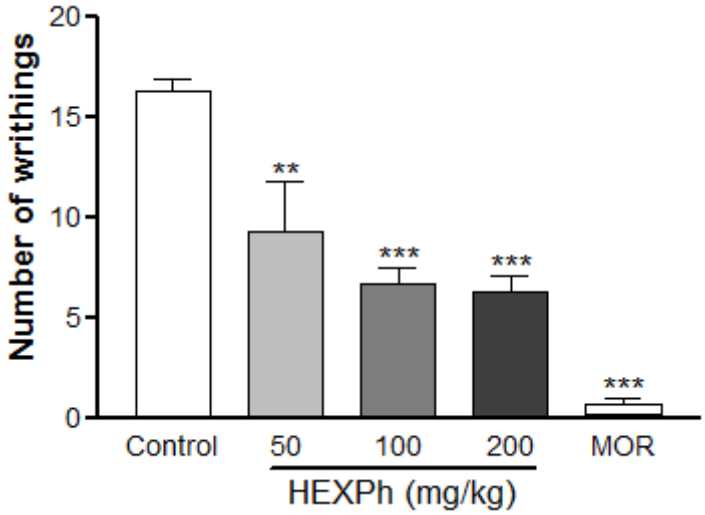

Figure 1. Effect of administration of vehicle, HEXPh (50, 100 and 200 mg/kg, p.o.) and MOR (3 mg/kg, i.p.) on the nociceptive behavior in the test for writhing induced by 0.85% acetic acid in mice (n = 6)

***p < 0.01, ***p < 0.001 significantly different from control (one-way ANOVA followed by Tukey's test)*

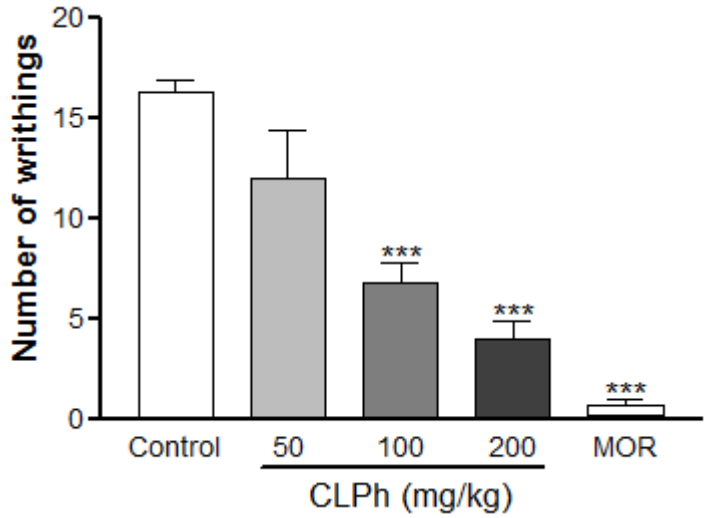

Figure 2. Effect of administration of vehicle, CLPh (50, 100 and 200 mg/kg, p.o.) and MOR (3 mg/kg, i.p.) on the nociceptive behavior in the test for writhing induced by 0.85% acetic acid in mice (n = 6)

***p < 0.01, ***p < 0.001 significantly different from control (one-way ANOVA followed by Tukey's test)*

Secondary metabolites are widely described in the literature to exhibit various pharmacological properties. The correlation between the antinociceptive activities displayed by all the organic phases in the acetic acid-induced writhing test is probably associated with the presence of these chemical constituents (alkaloids, flavonoids, tannins, steroids and triterpenes), with the exception of the alkaloids for *n*-BUPh.

The test for writhing is a widely used model for screening compounds with potential analgesic, anti-inflammatory activity. The intraperitoneal injection of acetic acid causes a characteristic behavioral response, considered a reflection of visceral pain [18,19].

Inflammation causes the release of prostaglandins, enough to cause spasms translated into writhing [20-22]. It is believed that acetic acid acts indirectly causing the release of endogenous mediators involved in modulation of nociception, including bradykinin, serotonin, histamine and prostaglandins [23,24]. Furthermore, nociception induced by acetic acid depends on the release of cytokines such as IL-1β, TNF-α and IL-8 from resident macrophages and basophils in the abdominal cavity and, in conjunction with other mediators, can induce the characteristic nociception observed in this test [25,26]. Another important contributing factor is the direct activation of nociceptors by protons through the opening of a wide variety of channels of non-selective cations, such as the TRPV1 receptors present in the cutaneous and visceral peripheral terminations of the primary afferents [26,27].

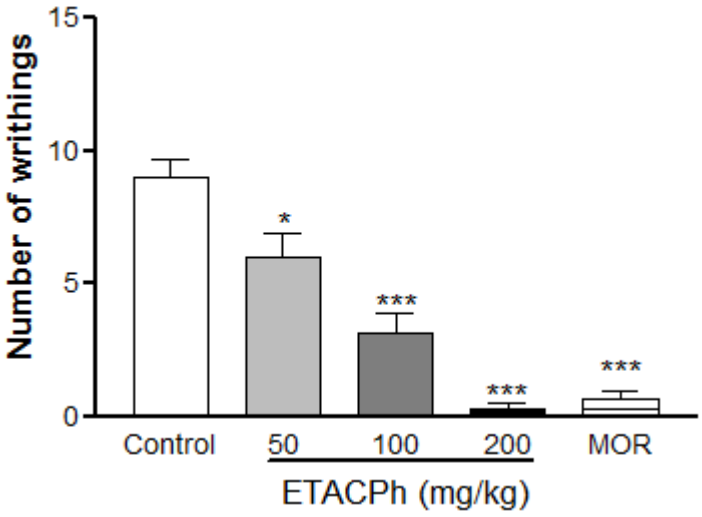

Figure 3. Effect of administration of vehicle, ETACPh (50, 100 and 200 mg/kg, p.o.) and MOR (3 mg/kg, i.p.) on the nociceptive behavior in the test for writhing induced by 0.85% acetic acid in mice (n = 6)
**p < 0.05, ***p < 0.001 significantly different from control (one-way ANOVA followed by Tukey's test)*

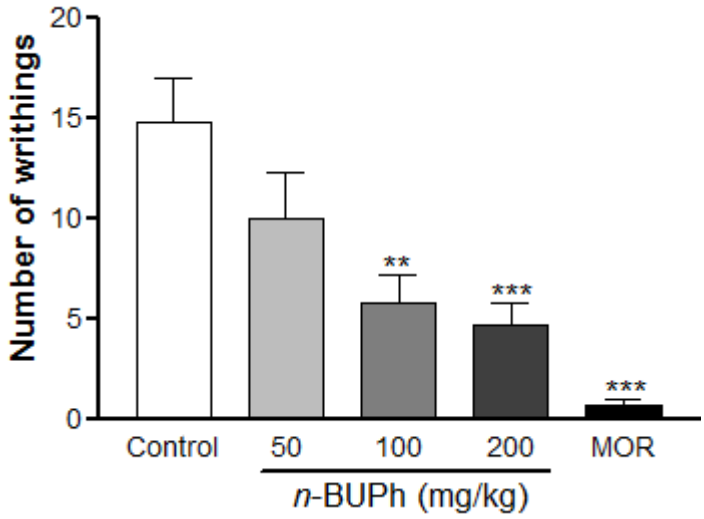

Figure 4. Effect of administration of vehicle, *n*-BUPh (50, 100 and 200 mg/kg, p.o.) and MOR (3 mg/kg, i.p.) on the nociceptive behavior in the test for writhing induced by 0.85% acetic acid in mice (n = 6)
***p < 0.01, ***p < 0.001 significantly different from control (one-way ANOVA followed by Tukey's test)*

The results support the hypothesis that the organic phases participate in the inhibition of prostaglandin synthesis, since the nociceptive mechanism of writhing induced by acetic acid involves the process or the release of arachidonic acid metabolites via cyclooxygenase (COX) and the biosynthesis of prostaglandins [28].

This method, despite being considered sensitive enough to allow the identification of substances with analgesic power, is not very specific. In part, this is because the test is sensitive to substances with antinociceptive action that act in both the CNS and peripheral nervous system, making any distinction difficult. It is also commonly used as a model of visceral inflammatory pain [29].

To elucidate the effect of the organic phases, our study used a more specific test, the formalin test, which permits assessing two phases of pain sensitivity. The first phase occurs during the first 5 minutes after injection of formalin (nociception of neurogenic origin) and is produced by a direct activation of primary afferent sensory neurons. Formalin excites these neurons by chemical stimulation of the peripherally located TRPA-1 channels [30]. The second phase occurs 15-30 minutes after injection of formalin, which causes release of inflammatory mediators formed in the peripheral tissues - such as prostaglandins, serotonin, histamine and bradykinin - and induces

functional changes in the dorsal horn neurons that, over time, promote transmission at the spinal level and result in increased primary afferent conduction, followed by sensitization of the spinal nociceptive neurons [31].

The results of the antinociceptive effect of the organic phases of *C. spicatus* in the formalin test are summarized in **Table 1**. Only the CLPh fraction, at its highest dose (200 mg/kg), showed an antinociceptive profile in the first phase of the test because it significantly reduced ($p < 0.05$) the pain response when compared to the control group. In the second phase of the test, the HEXPh fraction significantly reduced the pain response at all three doses tested (50 and 100 mg/kg, $p < 0.01$; 200 mg/kg, $p < 0.001$), while the ETACPh fraction was able to significantly reduce the nociceptive response only at doses of 100 ($p < 0.05$) and 200 mg/kg ($p < 0.01$), and CLPh at a dose of 200 mg/kg ($p < 0.05$). However, *n*-BUPh had no antinociceptive effect in this test.

The formalin test produces a nociceptive stimulus of tonic and moderate character that persists for a few minutes, and from which the animal cannot escape. Therefore, this test, which closely resembles the clinical condition of pain, is a useful model for investigating potential analgesic drugs [19]. Drugs that inhibit the first phase of this test are centrally acting, such as the opioid analgesics, since this phase is caused by direct stimulation of nociceptors [14,32]. The second phase of nociception can be inhibited either by NSAIDs or by substances that act on the CNS [33], as it is characterized by production of inflammatory mediators.

In this context, the results showed that pretreatment with the organic phases reduced the pain response in the second phase of the formalin test, indicating an antinociceptive action through inhibition of inflammatory mediators such as prostaglandins, histamine and cytokines.

Since only CLPh, at the highest dose, was able to significantly reduce the pain response in the first phase of the test, it is possible to suggest an effect mediated by central mechanisms. However, further experiments are needed to confirm this hypothesis.

Conversely, *n*-BUPh did not reduce the pain response at any of the doses tested (50, 100 and 200 mg/kg) in any of the phases. To make a correlation of this result with the phytochemical analysis suggests that this is due to the absence of alkaloids in this organic phase, since the tests performed for this chemical class were positive for the other organic phases, which were able to reduce the formalin-induced nociceptive response. This result is consistent with several studies in the literature that show that the alkaloids have important pharmacological activities, highlighting their antinociceptive, anti-inflammatory activity [34,35].

Table 1 Effect of the organic phases or morphine on the nociception induced by formalin

Treatment	Dose (mg/kg)	Licking time (s)			
		0-5 min		15-30 min	
		Pain response[a]	% inhibition	Pain response[a]	% inhibition
Vehicle	-	88.0 ± 23.7	-	93.83 ± 20.7	-
HEXPh	50	65.7 ± 5.4	13.4	38.33 ± 12.4[c]	65.0
HEXPh	100	67.8 ± 7.6	10.5	38.80 ± 18.2[c]	64.6
HEXPh	200	54.6 ± 7.2	27.9	11.83 ± 5.8[d]	89.9
CLPh	50	56.3 ± 10.3	35.9	34.16 ± 10.1	63.6
CLPh	100	38.8 ± 8.3	55.8	4 8.66 ± 22.7	48.1
CLPh	200	29.5 ±8.4[b]	66.4	22.50 ± 7.3[b]	76.1
ETACPh	50	60.0 ± 67.5	31.8	56.50 ± 13.6	39.7
ETACPh	100	64.5 ± 11.1	26.7	32.16 ± 13.3[b]	65.7
ETACPh	200	67.6 ± 15.8	23.1	25.33 ± 8.3[c]	73.0
n-BUPh	50	48.6 ± 7.6	44.7	67.33 ± 12.7	28.2
n-BUPh	100	60.1 ± 6.9	31.6	52.50 ± 13.5	44.1
n-BUPh	200	63.5 ± 11.7	27.8	55.50 ± 10.1	40.8
MOR	5	1.1± 0.8[d]	98.6	2.5± 2.5[d]	98.3

n = 6

a Values represent the mean ± standard deviation of the mean.
b $p < 0.05$ (one-way ANOVA followed by Tukey's test), significantly different from control.
c $p < 0.01$ (one-way ANOVA followed by Tukey's test), significantly different from control.
d $p < 0.001$ (one-way ANOVA followed by Tukey's test), significantly different from control.

According to Rodrigues [36], alkaloids, terpenoids, phenolic compounds and coumarins predominate among the plants indicated as analgesic for 26 indigenous groups in Brazil, indicating that these secondary metabolites have an important action against pain processes. According to Barbosa-Filho and collaborators [37], of the 171 evaluated alkaloids, 137 have anti-inflammatory activity.

To assess an animal's behavioral response in accordance with the intensity of an applied stimulus, it is necessary to observe the integrity of locomotor function [38]. The Rota-rod test is used to verify if a drug interferes with animal

locomotor activity, either by a depressant effect on the CNS and/or by muscle relaxation [14]. If the test substance reduces the spontaneous motor coordination response of the animal by one of these mechanisms, the nociceptive behavioral tests are invalidated [39].

Figure 5 shows the Rota-rod test results. The mice treated with HEXPh, CLPh, ETACPh and *n*-BUPh at a dose of 200 mg/kg showed no significant changes in motor coordination compared to the control group. As expected, DZP reduced the residence time of the animals on the Rota-rod at a dose of 3 mg/kg (i.p.).

As mentioned above, systemic treatment with HEXPh, CLPh, ETACPh and *n*-BUPh at a dose of 200 mg/kg produced no significant change in the performance of the animals to remain on the Rota-rod, confirming that the antinociceptive action of the organic phases does not occur because of inhibitory effects on the CNS.

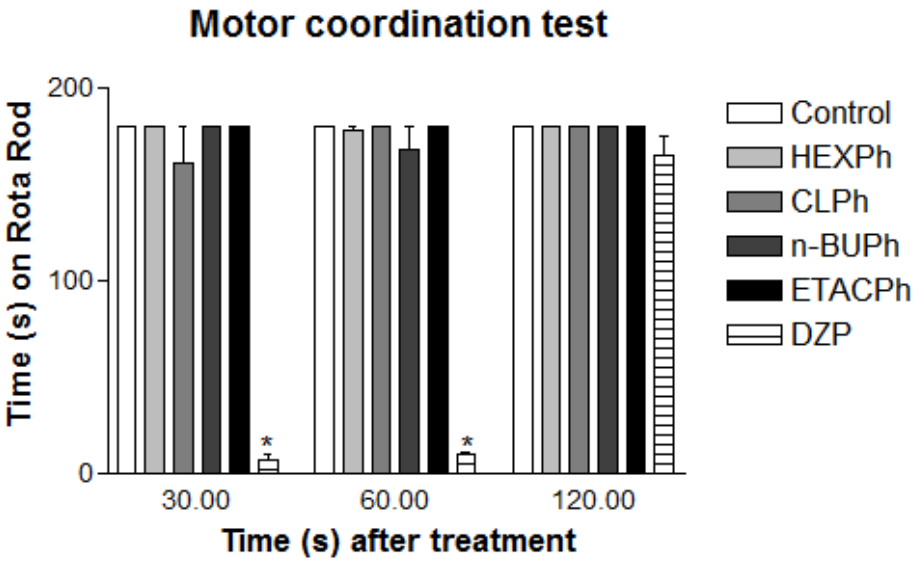

Figure 5. Effect of administration of vehicle, HEXPh, CLPh, ETACPh and *n*-BUPh (200 mg/kg, p.o.) and DZP (3 mg/kg, i.p.) in the Rota-rod test in mice (n = 6). Values represent mean ± standard deviation of the mean
**p < 0.001 significantly different from control (one-way ANOVA followed by Dunnett's test).*

CONCLUSION

The data from this work demonstrated that all organic phases presented antinociceptive activity in the acetic acid-induced writhing test, and only *n*-BUPh failed to show an antinociceptive effect in the formalin test. A correlation with the phytochemical analysis suggests that the chemical classes found in the phases are responsible for this pharmacological activity.

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