Involvement of Opioid System, TRPM8, and ASIC Receptors in Antinociceptive Effect of *Arrabidaea brachypoda* (DC) Bureau

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Abstract: *Arrabidaea brachypoda* (DC) Bureau is a medicinal plant found in Brazil. Known as “cipó-una” it is popularly used as a natural therapeutic agent against pain and inflammation. This study evaluated the chemical composition and antinociceptive activity of the dichloromethane fraction from the roots of *A. brachypoda* (DEAB), its mechanism of action and potential acute toxicity. The chemical composition was characterized by high-resolution mass spectrometry. The antinociceptive effect was evaluated in the formalin and hot plate tests after oral administration (10-100 mg/kg) in mice. We also investigated the involvement of TRPV1 (transient receptor potential vanilloid 1), TRPA1 (transient receptor potential ankyrin 1), TRPM8 (transient receptor potential melastatin 8), and ASIC (acid-sensing ion channel), as well as the opioidergic, glutamatergic, and supraspinal pathways. The fraction is composed only by dimeric flavonoids, exhibited no sign of acute toxicity in vivo or locomotor performance interference. Moreover, it could reduce the nociceptive response (30 mg/kg) in the early and late phase of the formalin test. DEAB activity appears to involve the opioid system, TRPM8 and ASIC receptors clearly showing that the DEAB alleviates acute pain in mice and suggest involvement of the TRPM8 and ASIC receptors, and the opioid system in acute pain relief.

Keywords: *arrabidaea brachypoda* (DC) bureau; bignoniaceae; antinociceptive effect; pain

1. Introduction

Historically the study of plants used for medicinal purposes accompanied and promoted the discoveries and development of pure drug substances and, consequently, new therapeutics. Although medicinal plants have been explored for an extensive period, they still present themselves as a remarkable source of new bioactive substances with therapeutic potential [1,2].
Brazilian flora is distributed in extremely diverse ecosystems. Contributing to this diversity, the Cerrado (neotropical savanna) extends mainly over the northeast, middle west, and southeast of Brazil and contains more than 12,000 plant species [3].

Arrabidaea brachypoda (DC.) Bureau is a medicinal plant of the Cerrado; its roots are used in traditional medicine for the treatment of joint pain [4]. There are few pharmacological and phytochemical studies in the literature using this species. A previous study revealed that a dichloromethane extract of *A. brachypoda* roots had anti-*Trypanosoma cruzi* activity in assays *in vitro* and *in vivo* [5]. Furthermore, a hydroethanolic extract of the root of *A. brachypoda* showed gastroprotective and gastrointestinal healing properties, as well as anti-inflammatory and antinociceptive activities in animal models [6,7]. Although these previous studies have shown that polar extracts of *A. brachypoda* display antinociceptive effects, the underlying mechanisms whereby this medicinal plant reduces pain are not well understood. The hydroethanolic extract contains a very large variety of phytochemical compounds (e.g., flavonoids, saponins, coumarins, tannins, cardiac glycosides, steroids, and phenolic compounds), and the pharmacological action of the extract is widely distributed among them. However, in this study, we propose to investigate the antinociceptive effect of the apolar (dichloromethane) fraction *A. brachypoda*, which has a smaller composition of phytochemical compounds (mainly unusual dimeric flavonoids). Furthermore, this study will determine the antinociceptive mechanism of action of this dichloromethane root fraction.

2. Results and Discussion

Previous studies have already demonstrated antinociceptive activity with hydroethanolic root extracts of *A. brachypoda* in rodents, but the underlying mechanisms of pain reduction are not well understood [6]. In the present study, we extend previous data using a DCM (dichloromethane) fraction extracted from the hydroethanolic root extract. In contrast to the hydroethanolic extract that contains saponins, cardiac glycosides, phenolic compounds, tannins, steroids, and flavonoids, HPLC-PDA-SEI-MS (high-performance liquid chromatography-photodiode array detection-electrospray ionization mass spectrometry) analysis revealed only three major compounds in the UV (ultraviolet) trace of the DEAB. The resulting PDA-UV spectra revealed the presence of three phenolic compounds with similar UV patterns and a common chromophore (Figure 1).

![Figure 1](A) HPLC-PDA chromatogram of dichloromethane fraction of samples obtained from the roots of *Arrabidaea brachypoda*. (B) UV spectrum of compounds 1-3.

These compounds (i.e., brachydin A, B, and C) possess unusual structural features compared to the substances previously reported in the genus *Arrabidaea* [8]. To identify these constituents and assess their biological activities, the extract was fractionated using reversed-phase MPLC (medium pressure liquid chromatography), thus purifying hundreds of milligrams of each compound (1-3) in
one step. The structures were elucidated using UV spectroscopy, NMR (nuclear magnetic resonance), ECD (electron capture detection), and HRMS (high-resolution mass spectrometry) [5,7]. There is only one report about the pharmacological action of these compounds (i.e., anti-T. cruzi activity) using *in vitro* and *in vivo* assays. Further studies to elucidate the mode of action and toxicity of these compounds are required [5].

Therefore, the first step in this study was to ensure the safety of this nonpolar fraction obtained from the hydroethanolic extract of *A. brachypoda*. Although medicinal plants and natural products are generally considered safe, they may present risks of intoxication when taken in large doses or over a long period of time [9,10]. Hippocratic screening in a toxicity study is a valuable tool to detect toxic and pharmacologic effects via behavioral indicators [11]. Complementary monitoring of the changes in body weight of the animals is also an important method to assess toxic metabolites [12]. The oral administration of DEAB (2000 mg/kg) did not produce behavioral signs of toxicity, apart from a mild analgesia both in male and female mice (data not shown), when compared to the effect of vehicle. Furthermore, there was no mortality associated with DEAB administration, and it did not alter body or organ weights (Figure 2; Table 1; Table 2). Therefore, similar to that of the crude hydroethanolic extract [6], the non-polar fraction obtained from the roots of *A. brachypoda* showed no acute toxicity *in vivo*.

![Figure 2. Bodyweight changes in (A) female and (B) male mice after treatment with the dichloromethane fraction of samples obtained from the roots of *Arrabidaea brachypoda* (DEAB). The results are expressed as the mean of the values obtained in 10 animals. Student's *t*-test; *P* > 0.05.](image)

<table>
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<th>Treatment</th>
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<th>Kidneys</th>
<th>Lungs</th>
<th>Liver</th>
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<th>Testicles</th>
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<td>Control</td>
<td>3.79 ± 0.09</td>
<td>6.05 ± 0.11</td>
<td>4.15 ± 0.05</td>
<td>12.47 ± 0.22</td>
<td>3.65 ± 0.16</td>
<td>3.96 ± 0.09</td>
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<tr>
<td>DEAB</td>
<td>3.87 ± 0.08</td>
<td>6.03 ± 0.13</td>
<td>4.13 ± 0.04</td>
<td>12.55 ± 0.18</td>
<td>3.37 ± 0.08</td>
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The results are expressed as the mean ± S.E.M (standard error of the mean) of the relative organ/total body weight of the animals. The ratio was converted into arcsine for statistical adjustment. The statistical significance was determined by Student’s *t*-test (*P* > 0.05).

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<th>Treatment</th>
<th>Heart</th>
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*Table 2. Toxicological parameters after the acute oral administration (2000 mg/kg) of DEAB in female Swiss mice*
The results are expressed as the mean ± S.E.M. of the relative organ/total body weight of the animals. The ratio was converted into arcsine for statistical adjustment. The statistical significance was determined by Student’s t-test ($P > 0.05$).

There is evidence that myorelaxant drugs or sedatives can promote changes in locomotor performance of mice, resulting in false positive results for drugs having antinociceptive effects [13]. Thus, to exclude this possibility we evaluated the integrity of locomotor performance of the mice treated with DEAB on a rotarod apparatus. Pretreatment with DEAB did not significantly affect the locomotor performance of the animals after oral administration of 300 mg/kg. Only the positive control (diazepam, 2 mg/kg) group ($P < 0.01$) significantly decreased the endurance time on the rotating rod when compared with the time of the control group (Figure 3). Therefore, after ensuring that the DEAB was not toxic and would not cause muscle relaxation or sedation when acutely administered, its possible antinociceptive effect could then be tested.

Figure 3. Effect of the dichloromethane fraction of samples obtained from the roots of *Arrabidaea brachypoda* (DEAB) on the locomotive performance of mice in a rotarod test. The results are the number of falls in each group of animals expressed as the mean of the values obtained in 8 animals ± S.E.M. One-way ANOVA (analysis of variance) followed by Dunnett’s test (all vs. vehicle) and Student’s t-test (Diazepam vs. DEAB); *$P < 0.05$, **$P < 0.01$.

The formalin test is a widely used model of persistent nociception and is a mainstay for the development of novel agents for the treatment of acute and chronic pain [14,15]. The test shows a biphasic response. The first phase begins immediately after the formalin injection and is caused by direct action of the solution on the local sensory C-fibers, resulting in the release of calcitonin gene-related peptide (CGRP) and substance P; the first phase is inhibited by narcotic drugs such as morphine [16,17]. The second phase (15-30 minutes after injection) is associated with inflammatory pain due to the release of inflammatory mediators, such as prostaglandins and nitric oxide, and is responsive to non-steroidal anti-inflammatory drugs (NSAIDs) [15,16,18,19].

The results depicted in Figure 4 show that the intraplantar injection of formalin resulted in a typical biphasic nocifensive behavior. Mice spent approximately 90 s and 250 s displaying nociceptive behaviors during the first 5 min (neurogenic pain, phase 1) and the subsequent 15–30 min (inflammatory pain, phase 2) of the assay, respectively (Figure 4 A and B). In the neurogenic phase, morphine (2.5 mg/kg) and DEAB (30 mg/kg) reduced nociceptive behavior by 72% ($P < 0.001$) and 50.1% ($P < 0.001$), respectively, when compared to that of the vehicle-treated group (Figure 4 A). During the inflammatory phase, the positive control (piroxicam, 30 mg/kg) and DEAB at 30 mg/kg reduced hind-paw licking by 47.2% ($P < 0.05$) and 42.7% ($P < 0.05$), respectively, compared to that of the negative control (Figure 4 B). These results show that, in contrast to the crude polar extract of *A. 
*brachypoda* that shows antinociceptive activity only during the second phase (inflammatory pain), the DCM fraction can induce antinociceptive effects during both phases. Hence, the subsequent experiments with DEAB were carried out at the effective dose of 30 mg/kg.

**Figure 4.** Effect of DEAB (10–100 mg/kg, i.g.), morphine (2.5 mg/kg, s.c.) or piroxicam (30 mg/kg, i.g.) on nocifensive behavior induced by intraplantar injection of formalin in mice. The total time spent licking the hind paw was measured during the (A) neurogenic phase (0–5 min) and the (B) inflammatory phase (15–30 min). The results are expressed as mean of the values obtained in 8-10 animals ± S.E.M. One-way ANOVA followed by Dunnett’s test (all vs. vehicle); *P < 0.05, ***P < 0.001.

After verifying the antinociceptive activity of the DEAB, its mechanisms of action were investigated. The hot plate test is an easy and reliable method that is capable of evoking supraspinal responses [20]. However, DEAB showed no antinociceptive activity against supraspinal responses. The DEAB-treated group showed the same latency response as the vehicle-treated group did (P > 0.05) throughout the observation period (Figure 5).
Figure 5. Effects of the dichloromethane fraction of samples obtained from *Arrabidaea brachypoda* root (DEAB) or morphine (5 mg/kg) on the supraspinal reflexes of mice in the hot plate test. The results are the time to exhibit a nociceptive response to a hot plate expressed as the mean of the values obtained in 8 animals ± S.E.M. Two-way ANOVA with a Bonferroni correction for multiple comparisons (all vs. vehicle); *P* < 0.05, **P** < 0.01.

Noxious thermal, mechanical, or chemical stimuli evoke pain through excitation of the peripheral terminals called nociceptors. Many kinds of ionotropic and metabotropic receptors are involved in this process, such as transient receptor potential (TRP) and ASIC channels [21]. The TRP channels are divided into seven subfamilies and four of them are involved with nociception [22]. TRPV1 is sensitive to capsaicin and noxious heat, TRPA1 is activated by a wide variety of chemical agents such as isothiocyanates and cinnamaldehyde, and TRPM8 responds to noxious cold as well as menthol [23,24]. ASICs are cationic channels from the degenerin/epithelial sodium channel (DEG/ENaC) family, which are involved in the nociception to elevated extracellular H⁺ concentrations in both the central and peripheral nervous systems [25].

The involvement of these channels in the antinociceptive mechanism of DEAB was evaluated. Our results showed that DEAB, at the dose that was effective in reducing inflammatory and neurogenic pain in the formalin test, did not reverse the nociception caused by capsaicin (an activator of the TRPV1 channel) or cinnamaldehyde (an activator of the TRPA1 channel) when compared to that of the control group (*P* > 0.05) (Figure 6 A and B). DEAB also was ineffective against the nociceptive behavior caused by glutamate (*P* > 0.05) (Figure 7). Interestingly, DEAB significantly reduced the nociceptive behavior induced by menthol (an activator of the TRPM8 channel, 38.7%, *P* < 0.05) and acidified saline (an activator of the ASIC channel, 57.9%, *P* < 0.01) when compared to that of the vehicle-treated group (Figure 6 C and D).
Figure 6. Effects of the dichloromethane fraction of samples obtained from *Arrabidaea brachypoda* root (DEAB) on the nocifensive behavior of mice induced by an intraplantar injection of (A) capsaicin (2 µmol/paw), (B) cinnamaldehyde (40 nmol/paw), (C) menthol (2 µmol/paw), and (D) acidified saline (pH 2.0/paw). The results are the time the animals spent licking their right hindpaw expressed as the mean of the values obtained in 8 animals ± S.E.M. Student’s *t*-test; *P* < 0.05, **P** < 0.01.

Figure 7. Effects of the dichloromethane fraction of samples obtained from *Arrabidaea brachypoda* root (DEAB) on the nocifensive behavior of mice induced by an intraplantar injection of glutamate (30 µmol/paw). The results are the time the animals spent licking their right hindpaw expressed as the mean ± S.E.M. Student’s *t*-test; *P* > 0.05.

These results confirm the data in which DEAB decreased the nociception induced by formalin in both phases, since ASIC receptors are activated by extracellular acidosis, which is caused by tissue damage and inflammation [26]. In 2007 Andersson et al. [27] described that the inhibition of phospholipase A2 (PLA2) can prevent TRPM8 activation by cold, icilin, and menthol. The inhibition of this pro-inflammatory enzyme would also explain a reduced activation of ASIC receptors because the administration of anti-inflammatory drugs is capable of reducing the acidosis-induced nociception mediated by this ion channel [28]. This would explain the antinociceptive effect of DEAB observed in the second phase of the formalin test. In addition, TRPM8 channels have been associated with modulation of inflammatory pain hypersensitivity because they are activated by a wide range of temperatures, from harmless to noxious cold [29].

The opioid system plays an important role in the transmission and modulation of noxious stimuli. This occurs via peptides and receptors expressed throughout the nervous system [30].
The opioid system acts by modulating the ion channels of the neurons in the descending pathways of nociception. When activated, the opioid receptors inhibit the influx of Ca^{2+} in the presynaptic fibers, reducing the release of neurotransmitters, while hyperpolarizing the postsynaptic fibers due to K^{+} efflux [30].

We have also studied whether the opioid system contributes to the important antinociceptive effect of DEAB. Based on the results shown in Figure 8, we have confirmed the above findings that DEAB inhibits the nociceptive response induced by intraplantar injection of formalin, and have also shown that naloxone (a non-selective antagonist of opioid receptors) completely reversed the antinociception caused by morphine ($P < 0.01$) and DEAB ($P < 0.05$). This finding confirms that the opioid system also contributes to the analgesic effect of the DEAB.

![Figure 8](image.png)

**Figure 8.** Involvement of opioid system in antinociceptive effect of the dichloromethane fraction of samples obtained from *Arrabidaea brachypoda* root (DEAB) in mice previously treated with saline (10 mL/kg, i.p.) or naloxone (1 mg/kg, i.p., a nonselective opioid receptor antagonist) against nocifensive behavior induced by intraplantar injection of formalin in mice. The total time spent licking the hind paw was measured during the neurogenic phase (Early phase, 0–5 min). The results are expressed as mean of the values obtained in 8 - 10 animals ± S.E.M. One-way ANOVA followed by Dunnett’s test (all vs. vehicle within pre-treatment groups) and Student’s t-test (similar treatments of different pre-treatment groups); *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

### 3. Materials and Methods

#### 3.1. Chemicals and reagents

The chemicals that were used are as follows: acetic acid (Labimpex, Diadema, Brazil), formaldehyde (Chemco, Campinas, Brazil), capsaicin, cinnamaldehyde, menthol, L-glutamic acid hydrochloride (Sigma-Aldrich, St. Louis, MO, USA), naloxone (Tocris Cookson Ltd., Bristol, UK), morphine (Cristália, Itapira, Brazil), diazepam (Hipolabor, Belo Horizonte, Brazil), piroxicam (Pfizer, São Paulo, Brazil), methanol (MeOH, Sigma-Aldrich, HPLC grade), dichloromethane (DCM, Tedia, Rio de Janeiro Brazil), and formic acid (Tedia, Rio de Janeiro, Brazil). A saline solution (0.9% NaCl) was used as vehicle for the drugs, and the solutions were adjusted to pH 7.0 with 3 M NaOH, if necessary.

#### 3.2. Collection and identification of plant samples

Samples of *A. brachypoda* roots were collected in April 2013 at the Sant’Ana da Serra farm in João Pinheiro, Minas Gerais, Brazil (Location: 17°44’45’’S, 46°10’44’’W). The plant was identified at the Instituto de Ciências Exatas e Biológicas (ICEB) by Prof. Maria Cristina Teixeira Braga Messias from...
the José Badine Herbarium of the Federal University of Ouro Preto. A voucher specimen (no. 17935) was deposited at the Herbarium of the Federal University of Ouro Preto, Minas Gerais, Brazil.

3.3. Preparation of the fraction and isolation

The dried roots (300 g) were successively extracted by percolation, at room temperature, with 70% ethanol. The crude hydroethanolic extracts were filtered and evaporated to dryness under vacuum at approximately 40°C, yielding 11.8 g of dried hydroethanolic extract. The hydroalcoholic extract was partitioned with DCM and a methanol-water (7:3) mixture. The crude DCM fraction was obtained after decantation and evaporation to dryness under vacuum at approximately 40°C, yielding 37.7% (4.44 g) based on the dry mass. The DCM fraction (DEAB, 2.5 g) was initially fractionated using MPLC with a Zeoprep® C18 column as the stationary phase (15-25 µm, 460 x 49 mm i.d.) (Zeochem, Uetikon am See, Switzerland). The mobile phase was methanol and 0.002% formic acid in water, which was applied as a linear gradient from 5% to 100% methanol over 50 h. The flow rate was 3.5 mL/min, and the compounds were detected by their UV absorbance at 217 nm. The MPLC generated 235 fractions. Each fraction was analyzed further by HPLC-PDA.

3.4. Animals

Adult male and female Swiss mice (20-35 g) were obtained from the Anilab Laboratory Animal Creation and Trade Ltd. (Paulínia, São Paulo, Brazil). All animals were housed collectively in cages and were kept in a controlled environment (22 ± 2 °C, with a 12 h light/dark cycle, lights on at 06:00) with access to water and food (Presence®, Brazil) ad libitum. Animals were allowed to acclimatize to housing conditions for at least seven days before experiments, and all experiments were performed during the light phase of the light/dark cycle. All experiments conducted were in accordance with the Brazilian legislation regulated by the National Council for the Control of Animal Experimentation (CONCEA) and ethical principles in animal research formulated by the Brazilian Society of Science in Laboratory Animals. The animal protocol was approved by the Biosciences Institute/ UNESP Ethics Committee on Use of Animal (approval no. 728-CEUA).

3.5. Acute toxicity analysis and Hippocratic screening

Male and female Swiss mice were divided into four groups (n = 10 per group), according to sex and whether they received saline solution as the control (10 mL/kg) or DEAB (2000 mg/kg) orally. After administration, the acute toxicity and behavioral parameters (observational, Hippocratic screening) were assessed 30, 60, 120, 240, and 360 min after oral administration; the recording and grading of symptoms were in accordance with the method of Malone and Robichaud (1962). After behavioral assessment, body weights were monitored daily for 14 days. On the fifteenth day, the animals were euthanized, and the hearts, lungs, livers, spleens, kidneys, testicles, ovaries, and uteruses were collected, weighed, and subjected to macroscopic analyses as described by Souza Brito in 1994 [12].

3.6. Locomotor performance

To evaluate the possible non-specific muscle relaxant effect of DEAB, mice were tested with a rotarod apparatus (Insight Ltd., Ribeirão Preto, Brazil), based on the method of Dunham and Miya (1957). Twenty-four hours before the test, male Swiss mice that were capable of remaining on the rotarod (4 cm in diameter, 6 rpm) for three periods of 60 s without falling were preselected. For the test, the animals (n = 8) received vehicle (saline, 10 mL/kg, oral by gavage), DEAB (300 mg/kg, oral), or diazepam (2 mg/kg, intraperitoneal) as a positive control. One hour after oral treatment with saline or DEAB and 30 min after diazepam injection the mice were placed on the apparatus. The number of falls from the apparatus was recorded with a stopwatch for 180 s.
3.7. Formalin-induced nociception

The model that was used has been described by Hunskaar and Hole in 1987 [16], with a few modifications. The mice (n = 8-10) were treated with DEAB (10, 30, or 100 mg/kg, oral), vehicle (10 mL/kg, oral), morphine (2.5 mg/kg, subcutaneous) as a positive control for the neurogenic phase, or piroxicam (30 mg/kg, oral) as positive control for the inflammatory phase. One hour after oral treatments and 30 min after morphine administration, the mice received an intraplantar injection in their right hind paw of a formalin/saline solution (20 µL, 1% formaldehyde). After formalin injection, the animals were immediately placed into 20-cm glass cylinders, and the time (seconds) spent licking the injected paw was recorded with a chronometer as an indicator of nociception. The mice were observed during the first 5 min (neurogenic phase) and between the fifteenth and thirtieth minute (inflammatory phase).

3.8. Hot plate test

Thermal hypersensitivity after DEAB administration was evaluated with a hot plate test [33]. The animals (n = 8) were treated with DEAB (30 mg/kg, oral), vehicle (10 mL/kg, oral), or morphine as a positive control (5 mg/kg, subcutaneous). One hour after the oral and 30 min after the subcutaneous treatments the mice were placed on a heated metal plate (Ugo Basile, Italy) with the temperature set at 56 ± 1 °C. The time (in seconds) until the mouse manifested a nociceptive behavior (lifting or licking its hind-paw) was considered the latency response to the thermal stimuli. A cut-off time of 30 s was chosen to avoid tissue injury. This latency response was recorded 60, 90, and 120 min following oral treatment.

3.9. Involvement of transient receptor potential cation channel subfamily V member 1, A member 1, and M member 8 (TRPV1, TRPA1, and TRPM8, respectively) and acid-sensing ion channel (ASIC)

To evaluate the involvement of TRPV1, TRPA1, TRPM8, and ASIC channels in the antinociceptive activity of DEAB, we used specific activators of each channel. Male Swiss mice (n = 8) were pretreated with DEAB (30 mg/kg) or saline (10 mL/kg) orally one hour before algogenic injections. Then, the mice received 20 µL (intraplantar injection) of capsaicin (2 µmol/paw), cinnamaldehyde (40 nmol/paw), menthol (2 µmol/paw), or acidified saline (3% acetic acid, pH = 2) into the ventral surface of the right hind paw. Animals were placed individually in a glass cylinder and were observed for 6 min (TRPV1 and TRPA1) and for 20 min (TRPM8 and ASIC), according to the procedures outlined in previous publications [34,35], with modifications. The amount of time (seconds) spent licking the injected paw was recorded and considered indicative of nociception.

3.10. Involvement of glutamatergic system

To evaluate the involvement of the glutamatergic system, the animals (n = 8-10) received a 20 µL glutamic acid injection (30 µmol/paw, pH=7, intraplantar injection) in their right hind paw, one hour after DEAB (30 mg/kg) or saline (10 mL/kg) oral treatments. After the injection, the animals were observed for 15 min. The time, in seconds, that each mouse spent licking its right hind paw was used as the nociception indicator [36].

3.11. Involvement of the opioid system

To assess whether the opioid system mediated the antinociceptive effect of DEAB, mice (n = 8-10) received naloxone, a non-selective opioid receptor antagonist (1 mg/kg, intraperitoneal), and after 30 min were treated with DEAB (30 mg/kg, oral), vehicle (10 mL/kg, oral), or morphine (2.5 mg/kg, subcutaneous) as a positive control. One hour after oral treatments and 30 min after morphine administration the mice received 20 µL of a formalin/saline solution (1% formaldehyde, intraplantar injection) in their right hind paw, and they were observed during the first 5 min (neurogenic phase).
The time, in seconds, that each mouse spent licking its right hind paw was used as the nociception indicator [37,38].

3.12. Statistical analyses

The results are expressed as means ± standard error of the mean of the parameters obtained. The parameters were analyzed using one-way ANOVA followed by Dunnett’s test to compare three or more groups, using Student’s t-test to compare two groups, or using two-way ANOVA followed by Bonferroni’s test to compare three groups with repeated measures. The minimal significance level considered was P < 0.05.

5. Conclusions

Arrabidaea brachypoda (DC) Bureau is a medicinal plant that is popularly used in Brazil as a natural therapeutic agent to treat pain and inflammation. This study evaluates the antinociceptive activity of the DEAB obtained from the roots of this plant. We found that this nonpolar fraction (consisting only by brachydin A, B, and C) alleviates acute pain in mice, and our data suggest the involvement of the TRPM8 channels and ASIC receptors, as well as the opioid system in the acute pain relief. We believe that our study makes a significant contribution to the literature because it provides a mechanistic insight into the pain-alleviating activities of this important medicinal plant and may pave the way for the discovery of new antinociceptive drugs.

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