An Ethanolic Extract of *Boehmeria caudata* Aerial Parts Displays Anti-inflammatory and Anti-tumor Activities

**Authors**
Paula Pereira de Paiva¹, ², ³, Fabiana Regina Nonato², Ana Lúcia Tasca Gois Ruiz², Ilza Maria de Oliveira Sousa², ³, Rafael Rosolen Teixeira Zafred¹, ², Diogo Noin de Oliveira³, Rodrigo Ramos Catharino³, Mary Ann Foglio³, João Ernesto de Carvalho³

**Affiliations**
1 Institute of Biology, University of Campinas, Campinas, Brazil
2 Chemical, Biological and Agricultural Research Center, (CPQBA), University of Campinas, Paulínia, Brazil
3 Faculty of Pharmaceutical Sciences, University of Campinas, Campinas, Brazil

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Boehmeria caudate, Urticaceae, inflammation, antineoplastic agents, Ehrlich tumor carcinoma, drug screening assays

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**ABSTRACT**
The tumor microenvironment presents several therapeutic targets, with inflammation being one of them. In search of new drugs, plants have shown to be an effective source of potent anti-inflammatory and anticancer agents. This study aimed to evaluate the antitumoral and inflammatory activities of *Boehmeria caudata* aerial parts extract. Bioguided in vitro antiproliferative screening showed that phenanthroquinolizidine obtained from the aerial *B. caudata* ethanolic extract had a straight relationship with activity. Moreover, the orally administered ethanolic extract reduced Ehrlich solid tumor growth and displayed an anti-inflammatory effect in both evaluated experimental models (carrageenan-induced paw edema and croton oil-induced ear edema). These results suggest that the antitumor activity of the ethanolic extract could be explained by antiproliferative effects associated with anti-inflammatory action.

Supporting Information for this article is available online at http://www.thieme-connect.de/products.
Introduction

Considered one of the hallmarks of cancer, inflammation substantially contributes to the development and progression of malignancies [1]. Many plant-derived natural products have been described as potent anti-inflammatory and anticancer agents, in which they provide alternative strategies for the development of new drugs [2–4].

*Boehmeria caudata* Sw (Urticaceae) aerial parts extract had showed a promising antiproliferative effect (total growth inhibition lower than 0.25 μg mL⁻¹) in a preliminary screening done by our research team [5]. This species is widely spread across the neotropical region, that is, throughout Central and South America [6]. Found in the Southeast and Southern regions, and in the State of Mato Grosso do Sul in Brazil, *B. caudata* is well known as “lixa-da-folha-larga” and “assa-peixe” [7].

Despite the traditional usage, there is only one study aiming at the investigation of the pharmacological potential of *B. caudata* that describes the cytotoxic activity of *B. caudata* stem wood ethanolic extract upon human epidermoid carcinoma of the nasopharynx [8]. Considering the *Boehmeria* genus, there is evidence of anti-inflammatory and antitumor effects in addition to the presence of phenanthroquinolizidine alkaloids, such as cryptopleurine, in some extracts [9–14]. Therefore, the present study prompted the in vitro and in vivo evaluation of anti-inflammatory and antitumor effects of the ethanolic extract of *B. caudata* aerial parts.

Results and Discussion

DE, EE, and AE of *B. caudata* aerial parts and AEF were evaluated on four human cell lines (one non-tumor and three tumor cell lines) using doxorubicin as a positive control. Expressed as the sample concentration required for TGI (μg mL⁻¹), the extracts and fraction were classified as active when TGI ≤ 50 μg mL⁻¹ against at least two tumor cell lines in a panel of three tumor cell lines [15]. DE weakly inhibited breast (MCF-7, TGI: 35.26 μg mL⁻¹) and lung (NCI-H460, TGI: 38.19 μg mL⁻¹) tumor cell lines while AE showed weak and moderate activity on glioma (U251, TGI: 27.73 μg mL⁻¹) and lung (NCI-H460, TGI: 6.67 μg mL⁻¹) tumor cell lines in the order given. The most active samples were EE and AEF, which potently inhibited (TGI < 0.25 μg mL⁻¹) the three tumor cell lines. Further, all extracts inhibited the non-tumor cell line HaCat (▶ Table 1).

Using TLC with Dragendorff reagent (▶ Fig. 1a–m), the expectable presence of alkaloids was confirmed in the *B. caudata* aerial parts extract by the regard of fluorescent spots under UV, which were brownish due to the Dragendorff reagent. Acid-based extraction of EE afforded the AEF (▶ Fig. 1j–m). Thus, the presence of alkaloids in both EE and AEF could explain the likeness in the antiproliferative profile of these samples.

High-resolution electrospray ionization mass spectrometry (HRESI-MS) analysis of EE allowed for the putative identification and relative quantification of five phenanthroquinolizidine alkaloids (cryptopleurine, boehmeriasin A, boehmeriasin B, julandine, hydroxycryptopleurine), one quinolizidine alkaloid [3-(4-hydroxyphenyl)-4-(3-methoxy-4-hydroxyphenyl)-3,4-dehydroquinolizidine], and two acetonaphone alkaloids (3,4-dimethoxy-ω-(2'-piperidyl) acetonaphone and 2',4'dimethoxyacetofenone) (▶ Table 2). Except for cryptopleurine, seven alkaloids were identified for the first time in *B. caudata* aerial parts extract.

Due to the antiproliferative effect of EE, the anti-inflammatory [18] and antitumor [8, 11, 18] activities of the identifiable alkaloids (▶ Table 2), and the availability of test material, further assessments were conducted on in vivo antitumor and anti-inflammatory models to establish the in vivo correlation between the two activities.

Following the acute toxicity protocol [19], the higher administrated dose of EE that promotes no clinical adverse effects was determined using two different routes of administration. Nevertheless, after 48 h exposition, TGI values were calculated by nonlinear regression analysis using ORIGIN 8.0 (OriginLab Corporation). Human tumor cell lines: U251 (glioma), MCF-7 (breast), NCI-H460 (lung, non-small cells); human non-tumor line: HaCat (immortal keratinocyte), DE: dichloromethane extract, EE: ethanolic extract, AE: aqueous extract; AEF: alkaloid enriched fraction; DOXO: doxorubicin (positive control). CSIR's criteria: inactive (I, TGI > 50 μg mL⁻¹), weak activity (W, 15 μg mL⁻¹ < TGI < 50 μg mL⁻¹), moderate activity (M, 6.25 μg mL⁻¹ < TGI < 15 μg mL⁻¹), and potent activity (P, TGI < 6.25 μg mL⁻¹) [15].
less, when administrated via the i.p. route, EE (500 mg kg\(^{-1}\)) promoted depression, piloerection, tachypnea, and palpebral ptosis up to 4 h after administration, which suggested central nervous system effects. After 24 h, the EE-treated mice showed normal behavior, which persisted throughout the experiment. In addition, at a higher dose (1000 mg kg\(^{-1}\), i.p.), EE promoted signs and symptoms of central nervous system depression, which resulted in the death of mice within the first 24 h. Based on these results, we chose 150 mg kg\(^{-1}\) (30 % of the lower toxic dose on acute evaluation) of EE as the highest dose on the pharmacological experiments using the i.p. route.

In the same manner, after oral treatment, EE at 1000 mg kg\(^{-1}\) produced piloerection, tachypnea, and palpebral ptosis in mice during the first 4 h after treatment. All these symptoms disappeared after 24 h, with mice exhibiting normal behavior during the following 14 days. Thus, to avoid possible cumulative adverse effects, the highest dose on pharmacological assays of EE using the o.r. route was established as 300 mg kg\(^{-1}\) (30 % of 1000 mg kg\(^{-1}\)). The result also suggested that the toxic substances present in EE had low bioavailability by the o.r. route, not reaching a sufficient systemic concentration to trigger the physiological changes that led to the death of the mice when compared to EE administration via the i.p. route.

The antitumor activity of EE was evaluated by the Ehrlich solid tumor model, which is used for mouse experimentation worldwide [20]. Derived from murine breast adenocarcinoma with aggressive and fast-growing characteristics, the Ehrlich tumor cells can grow in ascitic and solid forms depending on the inoculation in cavities or tissue, respectively [21]. Therefore, EE treatment significantly decreased tumor growth by 48.51 % [75 mg kg\(^{-1}\), i.p., RTW = 0.00155 ± 0.0006, p < 0.01] and 35.5 % (150 mg kg\(^{-1}\), i.p., RTW = 0.00194 ± 0.0004, p < 0.05) in comparison to vehicle-treated animals (RTW = 0.00301 ± 0.0007), while doxorubicin promoted a reduction of 33.88 % (3 mg kg\(^{-1}\), RTW = 0.00199 ± 0.0003, p < 0.05. There was no statistically significant difference among the treated groups (▶ Fig. 2). Furthermore, EE doses did not produce any clinical signs of toxicity during treatment.

It is well known that Ehrlich cell growth generates a local inflammatory response characterized by increased vascular permeability, edema formation, cell migration, and recruitment of the immune response [21]. As we take the tumor microenvironment into consideration as a therapeutic target, EE was evaluated by two different inflammation models in mice. Since i.p. administration can induce the local inflammatory process, the o.r. route was chosen for these assays.

CG-induced paw edema is a well-established in vivo inflammation model, commonly used to evaluate the anti-edematous effect of natural products [22]. When injected, CG-induced inflammatory edema result in a sequential and integrated actions of several inflammatory mediators. Two hours after CG injection, there is the release of histamine, 5-hydroxytryptamine, bradykinin, and serotonin, among other substances. Afterwards, increased COX-2 expression and prostaglandin production, monocyte mobilization, macrophage migration, and nitric oxide production maintain the edema up to 4 to 6 h after CG injection [23, 24]. In this model, oral administration of EE inhibited CG-induced edema with an inverse dose-effect relationship (▶ Table 3). Thus, the major inhibitory effect of EE was observed at 75 mg kg\(^{-1}\), from the first hour of CG edema induction (p < 0.001), while at 150 mg kg\(^{-1}\), the inhibitory effect of EE was significant only after 4 h of edema induction. Moreover, at 300 mg kg\(^{-1}\), EE had a slight increase in CG-induced edema (p < 0.05) at 6 h in comparison to the vehicle group. As expected, dexamethasone (positive control) inhibited edema development in a significant manner in relation to the vehicle group 2 h after CG injection.
The proinflammatory effect observed for EE at 300 mg kg\(^{-1}\) could be attributed, in part, to the vesicant properties of cryptopleurine, which resemble those of nitrogen mustard [25], an alkylating agent used as a chemotherapeutic agent [26]. As the other identified phenanthroquinolizidine alkaloids shared chemically similarities with cryptopleurine, all these alkaloids together in high dose, could present this vesicant properties and induced the edema. Other alkaloids used in cancer chemotherapy also have vesicant properties, such as vinca and taxol, which can cause severe irritation with vesicle formation, edema, and tissue destruction when in contact with intact skin [27].

Based on the report that the NSAIDs were unable to inhibit the first 2 h after CG edema induction [28], our results suggested that EE anti-edematogenic activity at 75 mg kg\(^{-1}\) can be partially explained as similar to that observed for steroidal anti-inflammatory drugs. To support this hypothesis, we decided to select the croton oil-induced ear edema model in mice. Used as a screening assay for substances with potential anti-inflammatory action [29], the croton oil-induced ear edema model allowed for the evaluation of both topical and systemic routes of steroidal and nonsteroidal anti-inflammatory drugs [30]. Croton oil, obtained from croton seeds (Croton tiglium L.), is a phorbol ester enriched phlogistic agent, with TPA as the major compound. Since phorbol esters activate the arachidonic acid inflammatory cascade through COX and LOX activities, substances with LOX and/or COX inhibitory effects can be evaluated in this model [31].

As reported by our research group [32], independent of the administration route (o.r., i.p., or t.t. treatment), EE reduced croton oil-induced edema in mice ears by 41.39 % (6.3 mg ± 1.7, t.t.), 57.48 % (4.2 mg ± 1.34, i.p.), and 62.02 % (4.31 mg ± 0.71, o.r.) when compared to the control group (n = 6 male Balb/c mice per group).

### Table 2: Chemical composition of the alkaloid enriched fraction obtained by B. caudata aerial parts.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular formula</th>
<th>[M + H]+ (^a)</th>
<th>(m/z) measured</th>
<th>(m/z) experimental</th>
<th>Error (ppm)</th>
<th>DBE (^b)</th>
<th>Reference</th>
<th>Relative intensity (%)</th>
<th>Mass (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-Cryptopleurine, boehmeriasin A</td>
<td>C(<em>{24})H(</em>{28})O(_3)N</td>
<td>378.20637</td>
<td>378.20599</td>
<td>-1.01</td>
<td>11.5</td>
<td>0.442</td>
<td>[11, 16–17]</td>
<td>0.7</td>
<td>0.7</td>
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<td></td>
<td>1.719</td>
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<td>2.7</td>
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<td>0.513</td>
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<td>0.82</td>
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<td>0.369</td>
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<td></td>
<td>1.123</td>
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<td>1.80</td>
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<tr>
<td>Julandine</td>
<td>C(<em>{24})H(</em>{28})O(_3)N</td>
<td>380.22068</td>
<td>380.22159</td>
<td>-1.13</td>
<td>10.5</td>
<td>2.30</td>
<td>[16]</td>
<td>3.68</td>
<td>3.68</td>
</tr>
<tr>
<td>(-)-C(15R)-Hydroxycryptopleurine</td>
<td>C(<em>{24})H(</em>{28})O(_3)N</td>
<td>394.20128</td>
<td>394.20058</td>
<td>-1.87</td>
<td>11.5</td>
<td>0.47</td>
<td>[17]</td>
<td>0.75</td>
<td>0.75</td>
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<td></td>
<td></td>
<td>0.42</td>
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<td>0.75</td>
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<td>0.51</td>
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<td>0.36</td>
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<td></td>
<td>1.12</td>
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<td>1.80</td>
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<tr>
<td>Boehmeriasin B</td>
<td>C(<em>{24})H(</em>{28})O(_3)N</td>
<td>364.19072</td>
<td>364.19001</td>
<td>-1.95</td>
<td>11.5</td>
<td>1.80</td>
<td>[11]</td>
<td>2.88</td>
<td>2.88</td>
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<td></td>
<td>1.62</td>
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<td>2.0</td>
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<tr>
<td>3-(4-Hydroxyphenyl)-4-(3-methoxy-4-hydroxyphenyl)-3,4-dehydroquinolizidine</td>
<td>C(<em>{24})H(</em>{28})O(_3)N</td>
<td>352.19072</td>
<td>352.19067</td>
<td>-0.20</td>
<td>10.5</td>
<td>0.4</td>
<td>[11]</td>
<td>0.64</td>
<td>0.64</td>
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<td></td>
<td>0.36</td>
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<td>0.44</td>
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<tr>
<td>3,4-Dimethoxy-o-(2-piperidyl) acetophenone</td>
<td>C(<em>{24})H(</em>{28})O(_3)N</td>
<td>264.15942</td>
<td>264.15899</td>
<td>-1.67</td>
<td>5.5</td>
<td>72.21</td>
<td>[18]</td>
<td>115.54</td>
<td>115.54</td>
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<td></td>
<td>65.0</td>
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<td>79.5</td>
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<tr>
<td>2',4'Dimethoxyacetophenone</td>
<td>C(<em>{24})H(</em>{30})O(_3)</td>
<td>n.i.</td>
<td>n.i.</td>
<td>n.i.</td>
<td>n.i.</td>
<td>7.12</td>
<td>-</td>
<td>11.39</td>
<td>11.39</td>
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<td></td>
<td>6.4</td>
<td></td>
<td>Higher: 7.83</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Results obtained by HRESI-MS analysis; \(^b\)DBE = double bonds equivalent; \(^c\)results obtained by GC/MS analysis; yield calculated on relative ratio; n.i. = not identified by HRESI-MS.
Table 3  Evaluation of the edema variation and inhibition rate (%) promoted by EE in the carrageenan-induced paw edema model.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg kg⁻¹)</th>
<th>1 h</th>
<th>2 h</th>
<th>4 h</th>
<th>6 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MV ± SD (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>10</td>
<td>7.35 ± 3.29</td>
<td>-</td>
<td>11.69 ± 7.72</td>
<td>-</td>
<td>20.36 ± 6.05</td>
</tr>
<tr>
<td>Dexamethasone (Dexa)</td>
<td>25</td>
<td>1.96 ± 3.04</td>
<td>73.33</td>
<td>1.96 ± 3.04</td>
<td>83.23</td>
<td>3.60 ± 3.29</td>
</tr>
<tr>
<td>Crude ethanolic extract (EE)</td>
<td>75</td>
<td>0.00 ± 0.00</td>
<td>100</td>
<td>100</td>
<td>2.46 ± 4.05</td>
<td>78.95</td>
</tr>
<tr>
<td>150</td>
<td>0.93 ± 2.27</td>
<td>87.34</td>
<td>7.69 ± 4.90</td>
<td>74.21</td>
<td>7.83 ± 5.09</td>
<td>61.54</td>
</tr>
<tr>
<td>300</td>
<td>2.22 ± 4.97</td>
<td>69.79</td>
<td>12.60 ± 4.93</td>
<td>-7.78</td>
<td>23.30 ± 9.87</td>
<td>-14.44</td>
</tr>
</tbody>
</table>

*aTime after edema induction, b dose expressed in mL kg⁻¹, c dose expressed in mg kg⁻¹. Edema variation (%): results expressed as the mean ± standard deviation (MV ± SD). Edema inhibition rate (%): difference of the edema variation of the treated groups in relation to the edema variation of the vehicle group divided by the edema variation of the vehicle group multiplied by one hundred. Groups: negative control (vehicle: PBS pH 7 + Ttween 80 5 %, orally), positive control (dexamethasone: 25 mg kg⁻¹, orally), and experimental groups (EE 75, 150, and 300 mg kg⁻¹, orally). EE: ethanolic extract.  * P < 0.05,  ** p < 0.01, and  *** p < 0.001, significant difference in relation to the vehicle group by statistical means (two-way ANOVA followed by Bonferroni’s test).

Fig. 3  Effect of Boehmeria caudata ethanolic extract and positive control on ear edema induced by croton oil (Croton tiglium L.). The results expressed as the mean ± standard error media; weight difference (mg) = weights differences of equal portions obtained from the treated and untreated ears of the animals from each experimental group. Groups: negative control: vehicle (acetone 70 % to group with application topical and 10 mL kg⁻¹ of the PBS pH 7 + Ttween 80 5 % to group with intraperitoneal route); positive control: dexamethasone (dexa) and experimental group: crude ethanolic extract (EE). a Treatment by application topical: t.t., b treatment by intraperitoneal route: i.p, and c treatment by oral route: o.r.
pared to the vehicle group (10.75 mg ± 3.31, t.t; 9.88 mg ± 1.78, i.p.; and 11.35 mg ± 2.8, o.r.). In all experiments, dexamethasone (5 mg mL⁻¹, t.t.; 5 mg kg⁻¹, i.p.; or 50 mg kg⁻¹, o.r.) was able to inhibit from 88.05% (1.18 mg ± 1.13, i.p.) to 88.55% (1.23 mg ± 1.07, t.t.) and 88.89% (1.26 mg ± 1.04, o.r.) of the croton oil-induced edema in mice ears (▶ Fig. 3).

According to Carlson et al. [31], COX inhibitor drugs such as indomethacin, aspirin, and piroxicam show no anti-inflammatory effect when administered orally by TPA-induced and arachidonic acid-induced ear edema assays, while mixed COX/LOX inhibitors as well as steroidal anti-inflammatory drugs are more effective by the oral route.

Analyzing our results, we observed that EE was more effective when administered by a systemic route than t.t. (oral route. In comparison to selective inhibition [33, 34].

In conclusion, the antitumor activity of EE from B. caudata aerial parts could be explained by the antiproliferative effects associated with the anti-inflammatory effects.

Material and Methods

Chemical and equipment

Dichloromethane and ethanol were purchased from Labsynth. Trichloroacetic acid, Tween 80, dexamethasone (purity > 90 %), DMSO, CG, and croton oil were obtained from Sigma-Aldrich. All solvents were of analytical grade. Culture medium RPMI 1640 and fetal bovine serum were from Gibco. Penicillin/streptomycin (1000 U/mL:1000 mg/mL) was acquired from Vitrocell. Doxorubicin (doxorubicin chloride 50 mg; purity > 90 %) was purchased from Eurofarma. TLC plates with silica gel 60 were from Agilent, and the Plethysmometer apparatus (7140) was from Ugo Basile.

Plant material

The aerial parts of the B. caudata Sw. were collected at the CPQBA – UNICAMP experimental field, in which the botanical identification was performed by MSc Jorge Yoshio Tamashiro, researcher of the Department of Plant Biology, Institute of Biology/UNICAMP. A voucher specimen was deposited at the UNICAMP Herbarium under number UEC 107966. B. caudata is a Brazilian native genetic material. Hence, the present study had been approved by the Genetic Patrimony Management Board (CGEN/MMA) through the Access and Shipment Component of Genetic Heritage for scientific research purposes (number: 010672/2012–5).

Extract preparation

Milled dried aerial parts of B. caudata (5 g) were extracted with dichloromethane, 95 % ethanol, and distilled water by a Soxhlet extraction system (1:5 plant:solvent ratio, w/v) in succession. After solvent evaporation under vacuum at 40 °C and freeze-drying, the DE (5 % yield), EE (4.6 % yield), and AE (6.7 % yield) were stored at −20 °C until further analysis.

Acid-base extraction

An aliquot of EE diluted in distillate water (1:4 plant:solvent ratio, w/v) was acidified with 10 % hydrochloric acid until pH ≈ 1. After 24 h at 4 °C, the mixture was vacuum filtered to separate a dark green precipitate from the red supernatant in which was extracted by liquid-liquid partition with ethyl acetate (3:1, v/v, 3 times) affording the aqueous acidic solution (AAS), and the ethyl acetate solution. Furthermore, the AAS was partitioned with ethyl ether (3:1, v/v, 3 times) providing ethyl ether solution, and the final aqueous acidic solution (AAS₃). After pH adjustment (pH = 11) with ammonium hydroxide, AAS₃ was partitioned with dichloromethane (3:1, v/v, 3 times) giving forth the aqueous basic and dichloromethane (DCM) solutions. After being washed with distilled water (3:1, v/v, 3 times) and filtered through anhydrous sodium sulfate, the DCM solution was evaporated to dryness under vacuum at 40 °C. After HRESI-MS analysis, the DCM fraction was renamed as AEF (0.9 % yield).

Thin-layer chromatography analyses

DE, EE, AE, and AEF were analyzed by TLC plates with silica gel 60 as the stationary phase and two mobile phases [dichloromethane:methanol 97:3 (v/v) and BAW (butanol:acetic acid:distilled water 4:1:5, v/v)]. After complete elution, the TLC plates were visualized under UV light (254 and 366 nm) followed by detection with spray reagents anisaldehyde/sulfuric acid (lipophilic substances, sugars, and glycosides) and Dragendorff (nitrogen-containing compounds) [35].

Characterization of the ethanolic extract and alkaloid enriched fraction by high-resolution electrospray ionization mass spectrometry

One aliquot (10 μL) of EE and AEF solution (20 mg mL⁻¹, in methanol) was diluted in separate methanol/water (1:1, v/v) with 0.1 % formic acid (99%, 990 μL). To utilize one syringe pump, the sample solution was injected by direct infusion into the ESI-Obitrap mass spectrometer for positive ion mode ionization in which the total acquisition time for each spectrum was fixed at 1 min. ESI-MS (full scan) spectra were acquired in the range of m/z 150 to 800 for the EE, and the alkaloids were identified in the range of 200–400 m/z of AEF. The ESI-MS/MS spectra were acquired with a collision energy of 10–40 eV. The general operating conditions of the equipment were: 3500 V of spray voltage, 320°C to capillary temperature, 10 psi of sheath gas pressure, and 50 V to 5-lens. Data treatment was carried out using Xcalibur software. (Thermo Scientific).

Alkaloid enriched fraction analysis by gas chromatography coupled to mass detector

A sample (1 μL) of AEF solution (20 mg mL⁻¹) was subject to scientific analysis by capillary gas chromatography coupled to a mass
selective detector, equipped with a silica column (30 m × 0.25 mm, HP-5). The overall operating conditions of the equipment were: temperature of the injector: 250 °C; detector temperature: 300 °C; temperature program: 110 (2 min) − 240 °C, 5 °C/min, 240−300 °C, 10 °C/min; with or without split ratio 1:100.

**Cell lines**

Human tumor cell lines [U251 (glioma), MCF-7 (breast), NCI-H460 (lung, non-small cells)] were provided by the National Cancer Institute. The non-tumor cell line HaCat (human keratinocytes) was donated by Professor Dr. Ricardo Della Coletta, FOP/ UNICAMP.

**Cell culture**

All cell lines were maintained in complete medium [RPMI 1640 medium supplemented with 5 % fetal bovine serum and 1 % of a penicillin:streptomycin mixture (v/v) (1000 U mL\(^{-1}\);1000 g mL\(^{-1}\))]. For incubation conditions, the temperature was set at 37 °C in a humid atmosphere with 5 % of CO\(_2\), and the in vitro experiments were conducted under the same conditions.

**In vitro antiproliferative activity assay**

Cells in 96-well plates (100 µL cells/well) were exposed to sample concentrations (0.25, 2.5, 25, and 250 µg mL\(^{-1}\)) in DMSO/complete medium, in triplicate) for 48 h. The final DMSO concentration (≤ 0.25 %) was unaffected by cell viability. Doxorubicin was the positive control (0.025; 0.25, 2.5 and 25 µg mL\(^{-1}\)). Before (T0 plate) and after the sample addition (T1 plates), cells were fixed with 50 % trichloracetic acid, and cell proliferation was determined by spectrophotometric quantification (540 nm) of the cellular protein content employing sulforhodamine B. Using the concentration-response curve for each cell line, TGI was determined through non-linear employing sulforhodamine B. Using the concentration-re-

**Acute toxicity evaluation**

Swiss mice were distributed (n = 5 animals/group) into negative control (vehicle, 10 mL kg\(^{-1}\)) and experimental (EE, 1000 mg kg\(^{-1}\), o.r.; 500 and 1000 mg kg\(^{-1}\), i.p) groups. After administration, animals were observed during the first 4 h, and daily for 14 days. Afterwards, all animals were euthanized by cervical dislocation [19].

**The Ehrlich solid tumor model**

Ehrlich tumor cells were maintained in the ascitic form by weekly i.p. transplantation in Swiss mice. For the experiments, the ascitic suspension was collected and the tumor cell suspension was prepared at a density of 5 × 10\(^{6}\) cells/60 µL/animal after cell viability evaluation with trypan blue.

Ehrlich cells suspension was inoculated subcutaneously in the flank of Balb/c mice (n = 6 animals/group, 60 µL/animal). On the 3rd day, animals with palpable tumors were randomly divided into negative control (vehicle, 10 mL kg\(^{-1}\), i.p.), positive control (doxorubicin, 3 mg kg\(^{-1}\), i.p.), and experimental groups (EE, 75 and 150 mg kg\(^{-1}\), i.p.). The negative control and EE groups were treated every day, while the positive control group was treated every 2 days. On the 10th day, animals were euthanized, and tumors were removed and weighted. The procedures used for this study were similar to those previously described [21].

**Carrageenan-induced paw edema**

Balb/c mice (n = 6/group) were weighed, randomly distributed, and orally treated with vehicle (10 mL kg\(^{-1}\), negative control), dexamethasone (25 mg kg\(^{-1}\), positive control) or EE (75, 150, and 300 mg kg\(^{-1}\)). After 1 h, inflammation was induced by carrageenan inoculation (3 % in PBS, 30 µL/paw) into the subplantar region of the right hind footpad. The hind paw volume was evaluated using a plethysmometer at 0 (basal volume), 1, 2, 4, 6, and 24 h after CG inoculation, and the edema volume was obtained by the difference between basal and experimental hind paw volumes. The procedures used for this study were similar to those described previously, with minor modifications [10, 36].

| Table 4 | Group distribution in the croton oil-induced ear edema assay in mice. |
|---|---|---|---|
| **Route of administration** | **Vehicle** | **Dexamethasone** | **EE** |
| Topical | 20 µL | 5 mg mL\(^{-1}\) | 75 mg mL\(^{-1}\) |
| Oral | 10 mL kg\(^{-1}\) | 50 mg kg\(^{-1}\) | 75 mg kg\(^{-1}\) |
| Intraperitoneal | 10 mL kg\(^{-1}\) | 5 mg kg\(^{-1}\) | 75 mg kg\(^{-1}\) |

\(n = 6\) Balb/c mice/group; \(^{c}\)negative control (70 % acetone on topical administration and PBS with 5 % Tween 80 by oral administration; \(^{d}\)positive control; \(^{e}\)ethanolic extract. |
Croton oil-induced ear edema

Balb/c mice (n = 6 animals/group) were weighed and distributed into the experimental groups (► Table 4). One hour after each treatment, mice received croton oil topical application (20 µL/ear, 5 % in acetone 70 %) in the right ear, while the left ear was treated with 70 % acetone (20 µL/ear). Four hours after croton oil application, the animals were euthanized, and the ear edema was determined by weight difference between the left and right ears [36].

Statistical analysis

The experimental results are expressed as the mean ± standard error media (SEM) or mean ± standard deviation (SD). Statistical significance was evaluated by analysis of variance (ANOVA), one-way or two-way, followed by Tukey’s or Bonferroni’s test in the order given using GraphPad Prism version 5.0 software. Statistical significance is represented by ’p < 0.05, ’’p < 0.01, and ’’’p < 0.001.

Supporting information

The ethanolic extract and alkaloid enriched fraction spectrum by HRESI-MS is available as Supporting Information (Fig. 1S and Fig. 2S).

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Conflict of Interest

The authors declare that they have no conflict of interest.

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