Full Length Research Paper

Phytochemical investigation, antinociceptive activity and cytotoxicity of crude extracts of *Calea uniflora* Less.

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Calea uniflora Less. is a medicinal plant used in the treatment of inflammation and haematomas in southern Brazil. The aim of this study was to investigate the antinociceptive effects and cytotoxicity of *C. uniflora*. Regarding phytochemical evaluation, the crude extracts of plant were analysed by high-performance liquid chromatography (HPLC). Antinociceptive activities utilised models on chemical and thermal stimuli *in vivo*. To evaluate cytotoxic activity, the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide method (MTT test) was utilised *in vitro*. Phytochemical analyses verified the presence of flavonoids and sesquiterpenes. Regarding antinociceptive activity, the models produced significant results that correspond to chemical at doses of 100 and 300 mg/kg of the crude extract as compared to the control groups, respectively. The rota rod model showed satisfactory results, since the extract did not cause motor incoordination and sedation in this experiment. In the *in vitro* cytotoxic tests, the crude extracts and ethyl acetate and butanolic fractions produced IC_{50} values greater than 58 µg/mL with the HaCaT lineage and 48 µg/mL with the B16-F1 lineage; thus, these values did not produce cytotoxic effects. According to these results, flavonoids and alkaloids were found in *C. uniflora* extracts. Pharmacological activities were also detected as reported by the local population that uses this plant in traditional medicine, especially antinociceptive and cytotoxic activities.

Key words: Antinociception, cytotoxicity, phytochemistry, *Calea uniflora*, medicinal plant.

INTRODUCTION

Medicinal plants are a means of obtaining molecules to be exploited therapeutically, and many compounds isolated from plants remain promising as new drugs (Atanasov et al., 2015). Many species are used

empirically, without scientific support for efficacy and safety, which shows that in a country like Brazil, with enormous biodiversity, there is a huge gap between the supply of plants and little research (Oliveira et al., 2014; Santos et al., 2014). Among the great diversity of plants, there is the Astereaceae family, which includes different *Calea* genera (Youssef et al., 2013). The genus *Calea* consists of 110 species that are distributed from northern to southern Brazil (Moura and Roque, 2014).

Moreover, some *Calea* species have been studied for their biological activity, which suggests that great scientific interest in this genus exists (Mondin et al., 2015; Nascimento and Oliveira, 2004; Nascimento et al., 2002). Some species of this genus were investigated for their medicinal properties including the following: *Calea pinnatifida* (Lima et al., 2015) with leishmanicidal activity; *Calea urticifolia* (Yamada et al., 2004) used to treat oliguria and gastroenteritis; *Calea zacatechichi* (Leonti et al., 2003) used for dermatological/respiratory ailments and gastrointestinal purposes; *Calea serrata* Less. (Ribeiro et al., 2008) used to treat ulcers and liver problems and *Calea glomerata* (Guerrero et al., 2002) used as an antihypertensive.

Among these species, *Calea uniflora* has gained popularity due to its use for the treatment of diseases (Ferraz et al., 2009). *C. uniflora* Less. is native to the southern region but is also found in the southeast and midwest regions of Brazil (Mato Grosso do Sul, Minas Gerais and São Paulo), Argentina, Uruguay and Paraguay (Nakajima and Semir, 2001). This plant, popularly known as 'arnica da praia' or arnica, is used as a medicine in the southern region of Santa Catarina (Mondin et al., 2015). The local population of the region uses the plant for anti-inflammatory activity, pain, bruising and rheumatism (Rossato et al., 2012).

A study has shown that the most commonly used pharmaceutical form in this region is the flowers in an ethanol tincture (Rossato et al., 2012). *C. uniflora* has been little studied. Previous studies showed that this plant is rich in constituents such as lactones, sesquiterpenes, flavonoids, saponins and derivatives of p-hydroxyacetophenone (Lima et al., 2015). Other phytochemical studies by Nascimento and Oliveira (2004) showed that *C. uniflora* contains the compounds, glucoside-5-deoxyflavone, 3',4',7-trihydroxyflavone-7-O-β-glucopyranoside, 2',4-dihydroxy-3-methoxychalcone-40-O-β-glucopyranoside and quercetin-3-O-β-galactopyranoside. Regarding biological activities of *C. uniflora*, leishmanicidal activities (Nascimento et al., 2007), antifungal properties (Nascimento and Oliveira, 2004), trypanocidal action (Nascimento et al., 2002) and genotoxicity (Ferraz et al., 2009) have been studied.

So, in this study, the preliminarily phytochemical profile of crude extracts and fractions, antinociceptive and muscle relaxant activity and cytotoxic potential of crude extracts and fractions obtained from flowers of *C. uniflora* were analysed.

MATERIALS AND METHODS

Plant material

The aerial parts (inflorescence) of *C. uniflora* were identified by Dr. Vanilde Citadini Zanette and Mara Rejane Ritter and were collected in January 2013 in Balneário Rincão (GPS position 28°48'20.0" S 49°14'45.3" W). The voucher specimen was deposited in the CRI Herbarium of Dr. Raulino Reitz of the Universidade do Extremo Sul Catarinense (UNESC-SC), Brazil, CRI 10304.

Preparation of extracts

The plant material was dried at approximately 50 to 60°C and cut into small pieces. The flowers and leaves were crushed on a type mill, and the resulting matter was extracted with ethanol (70%) for 15 days with occasional stirring, followed by filtration and concentration of the filtrate by rotary evaporation. The flower extract was subsequently subjected to a process of liquid-liquid partition with solvents of increasing polarity: ethyl acetate, dichloromethane and *n*-butanol. So, three fractions were obtained for use in the experiments earlier. The partition methods were based on that adapted from Cechinel-Filho and Yunes (2009).

Phytochemistry

Crude extracts of leaves and flowers of *C. uniflora* fractions were subjected to HPLC to investigate the compounds present. For the high-performance liquid chromatography (HPLC) analysis, measurements were performed on a Waters Spherisorb ODS-2 C18 column (150 × 4.6 mm, 5 µm particle size) (Waters Technology Ireland, Ltd., Wexford, Ireland) protected by an in-line filter and set at room temperature. Peak detection was performed online using a diode array detector (HPLC 540 DAD, Kontron instruments, Montigny-le-Bretonneux, France) at 280 and 310 nm, and absorption spectra (210 to 400 nm) were recorded every second directly on the HPLC-separated peaks. The solvents used for separation were HPLC-grade acetonitrile (solvent A) and 1% phosphoric acid (concentrated)/10% acetic acid (glacial)/5% acetonitrile (v/v/v) in water (solvent B). The linear gradient elution program was ran as follows: 0 min, 100% (B); 30 min, 70% (B); 40 min, 100% (A) (Giusti et al., 1999).

An amount of 1 mg of crude extract of *C. uniflora* was mixed with 1 mL of ethanol at room temperature, producing a freshly prepared solution of 1 g/L. The extract was stirred in a vortex for 10 min until diluted, filtered with a single-use filter unit, and directly injected (20 µL in volume) into the HPLC system.

Pharmacological assays

Animals

Swiss mice weighing between 20 and 30 g were purchased from the vivarium at UNESC. The animals were housed under controlled light (12:12 h light-dark cycle) and temperature conditions (23 ± 1°C) with access to water and food *ad libitum*. The groups received treatments of appropriate doses of *C. uniflora* crude flower extracts or vehicle, administered orally (p.o.). The experimental protocol was approved by the local ethics committee (Ethics Committee on Animal Use, CEUA of UNESC 019/2013).

Antinociceptive activity

Acetic acid-induced writhing in mice

Acetic acid (0.6%) was injected into the peritoneal cavities of mice,

which were placed in a large glass cylinder. The intensity of nociceptive behaviour was quantified by counting the total number of writhes occurring between 0 and 20 min after stimulus injection. *C. uniflora* was administrated at doses of 30, 100 and 300 mg/kg (p.o.) given 60 min prior to acetic acid injection. The antinociceptive activity was expressed as writhing scores during a 20 min period (Choi et al., 2003; Koster et al., 1959).

Formalin test

Mice were pre-treated with a vehicle (saline) or *C. uniflora* (30, 100 or 300 mg/kg p.o.) 60 min before the start of the experiment. The positive control, morphine (10 mg/kg s.c.), was administrated 30 min before the start of the experiment. For nociception induction, an intraplantar injection of a 2.5% formalin solution (20 µL) was injected into the hind paw plantar surface, and the animals were individually placed in transparent observation chambers. The time spent licking the injected paw was recorded and expressed as the total licking time in the early phase (phase 1; 0 to 5 min) and late phase (phase 2; 20 to 30 min) after formalin injection (Hunskaar and Hole, 1987).

Hot plate test

Mice were pre-treated with *C. uniflora* (30, 100 or 300 mg/kg, p.o.) 60 min earlier, or morphine (10 mg/kg, s.c.), the positive control, 30 min before being placed on a metal plate warmed to 52 ± 0.5°C. The time that elapsed between the start of the experiment and the appearance of reactions (latency, in seconds) to the thermal stimulus, such as lifting or licking the paws, was recorded as an index of nociception. To avoid damage to the animals, the maximal time standing on the plate was limited to 30 s (Le Bars et al., 2001).

Rotarod

The animals were treated with *C. uniflora* (30, 100, or 300 mg/kg, p.o.) 60 min after being subjected to the equipment, according to the methodology of Oliveira et al. (2008). The cut-off time used was 60 s. The animals had been selected 24 h previously by eliminating those mice that did not remain on the bar for 60 s.

Cytotoxicity assay

Cytotoxicity bioassay and cell culture

Murine cancer (B16-F1) and keratinocyte-type cell lines (HaCaT) were used. The cells were maintained as previously described except for the use of RMPI as medium culture with 5% FBS instead of 10% FBS. Diluents (300 mM) of test compounds were prepared in dimethyl sulfoxide (DMSO) and added to each well 1 day after seeding. The amount of DMSO was adjusted to give a final concentration lower than 0.1%. Cells were cultured according to the methodology described previously (Millot et al., 2007).

Cytotoxic assay

Cytotoxic activity was determined in B16 cells seeded at 20 000 cells/mL at day 0. Compounds were serially diluted in RPMI 1640 at day 1 in a 96-well plate; with concentrations ranging from 2.5 to 300 µM. Incubation was performed at 37°C in an atmosphere of 10% CO_2. After 48 h of incubation, corresponding to day 3, compounds were added a second time. After a new 48 h incubation period, cell growth and viability were measured at day 5, using 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) (Millot et al., 2007). Each experiment was repeated at least three times, and three different wells were used for each concentration.

Statistical analysis

The results are expressed as means ± SEM. Statistically significant differences between groups were measured using one-way analysis of variance (ANOVA) followed by Dunnett's test. *$p < 0.05$ or **$p < 0.01$ was considered statistically significant. The geometric mean IC_{50} values were determined by nonlinear regression from individual experiments using GraphPad Prism software.

RESULTS

Phytochemistry

It was found that the crude extracts of flowers had more peaks (Figure 1A and B), which indicated the presence of more compounds in this extract.

In addition, the crude extract of *C. uniflora* flowers was subjected to preparative thin-layer chromatography (TLC). The results showed the presence of flavonoids and sesquiterpenes, compared with the references used in HPLC analysis.

Antinociceptive activity

Acetic acid-induced writhing in mice

The treatment of mice with *C. uniflora* crude extract given by the oral route, at doses of 100 and 300 mg/kg, significantly reduced the writhing response induced by injection of 0.6% acetic acid, as shown in Figure 2.

Formalin test

In the formalin test (Figure 3), the pre-treatment of animals with *C. uniflora* promoted a significant reduction in the nociceptive response in the second phase only at higher doses (B), while in the first phase, it was ineffective (A).

Hot plate

The treatment with *C. uniflora* at doses of 300 mg/kg by the oral route showed significant results of tolerance to pain on the hot plate (52°C) compared to the group treated with morphine (10 mg/kg, s.c.; positive control), since morphine and *C. uniflora* extract (300 mg/kg) increase the latency time, as shown in Figure 4.

Rotarod

In addition, the extract of *C. uniflora* had no significant

A

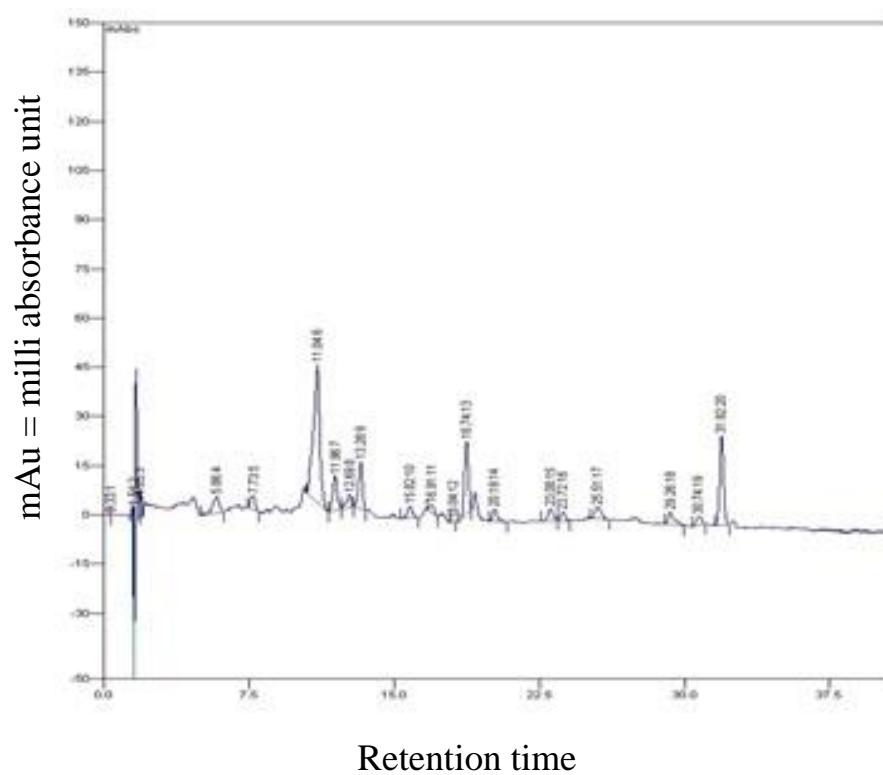Retention time

B

Retention time
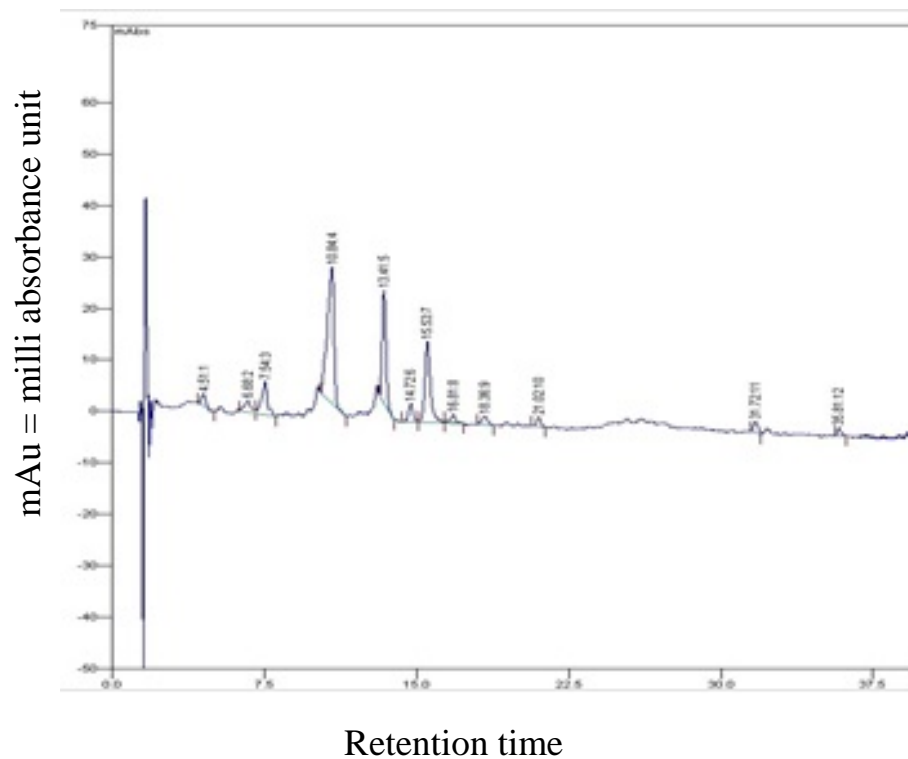
Figure 1. Chromatogram of the crude extracts of leaves (A) and flowers (B) of *C. uniflora* detected with a DAD 540 HPLC at 280 nm with a runtime of 60 min. Mobile phase: solvent A, 100% acetonitrile; solvent B, phosphoric acid (1%), acetic acid (10%), acetonitrile (5%) and water (84%).
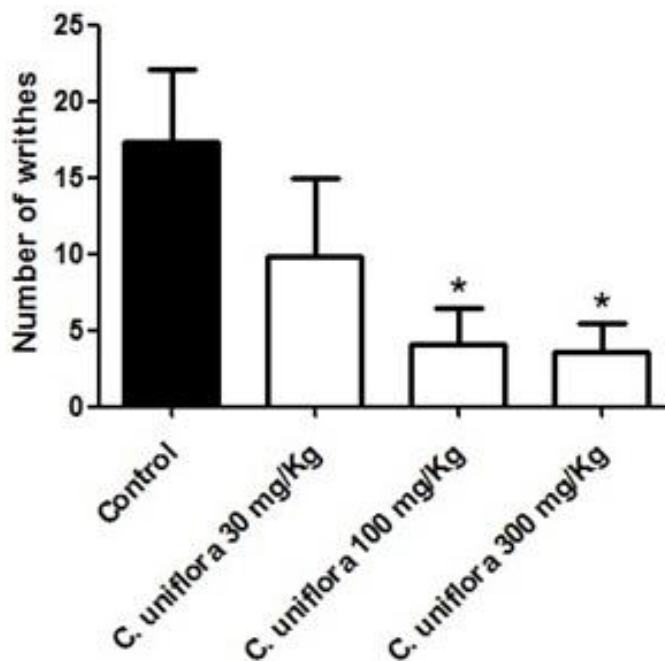
Figure 2. Effects of *C. uniflora* (30, 100 and 300 mg/kg) given orally 60 min before the administration of acetic acid to induce abdominal constriction. Each column represents the mean ± SEM of 8 to 10 animals. Control values indicate animals injected with the vehicle. Asterisks indicate statistically significant differences: *$p < 0.05$ and **$p < 0.01$ as compared to respective control values (via analysis of variance [ANOVA] and Dunnett's test).

effect in the rotarod test, dismissing possible unspecific effects such as motor incoordination in mice (Figure 5).

Cytotoxicity assay

The crude extracts of leaves and flowers did not show a high degree of cytotoxicity as compared to the controls. The ethyl acetate and butanol fractions of the flowers did not show a high degree of cytotoxicity, but the dichloromethane fraction showed a significant inhibition in both B16-F1 and HaCaT cells as compared to the vincristine and doxorubicin controls (Figure 6). The controls used have different mechanisms of action: vincristine acts in the inhibition of the mitotic spindle by binding to the microtubule proteins and consequently disrupting cell division in metastasis, while the doxorubicin mechanism of action specifies on cell cycle arrest by intercalating DNA and inhibiting topoisomerase II.

DISCUSSION

C. uniflora is widely known and used in Brazilian

population as a medicinal plant in wound healing, muscle pain, bumps and hematomas, flu and colds, insect bites and toothache. Despite its vast use in southern Brazil, this plant has been the subject of only a few studies and so we decided to analyse the crude extract in order to ensure safe use of the species and validate its popular use.

In the phytochemical analysis, it was observed that the chromatograms obtained by HPLC showed differences in the amounts of compounds of the crude extracts of flowers as compared to the crude extracts of the leaves (the extracts of flowers showed more peaks than the leaf ones). These differences may be related to the plant organs in which the compounds are stored (Gobbo-Neto and Lopes, 2007). Chromatographic analyses by TLC of the ethyl acetate fraction of *C. uniflora* after purification in preparative TLC indicated the presence of some chemical constituents. The ethyl acetate fraction included predominantly flavonoids and sesquiterpenes.

Flavonoids exhibit a great variety of therapeutic properties including antioxidant, anti-inflammatory, antifungal, antimicrobial and anticancer (Favarin et al., 2013; Kim et al., 2004; López-Posadas et al., 2008; Nijveldt et al., 2001). According to the Pastoral da Saúde Regional Sul IV, *C. uniflora* is popularly used for
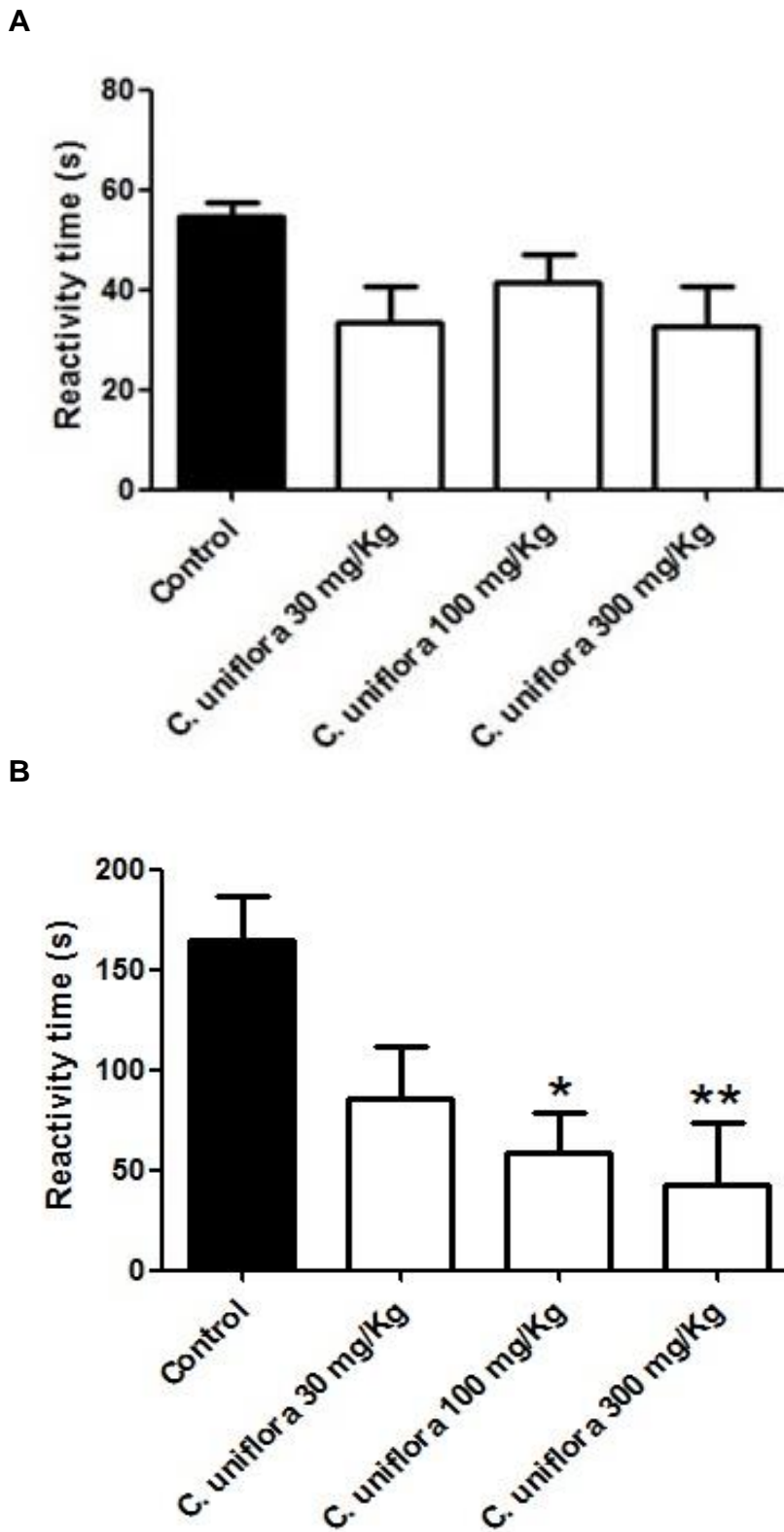
A

B

Figure 3. Effects of *C. uniflora* (30, 100, and 300 mg/kg) on the formalin test in the first (A) and second phases (B) administered orally 60 min before formalin-induced nociception. Each column represents the mean ± SEM of eight animals. Control values indicate animals injected with the vehicle. Asterisks indicate statistically significant differences: *$p < 0.05$ and **$p < 0.01$ as compared to respective control values (using ANOVA and Dunnett's test).
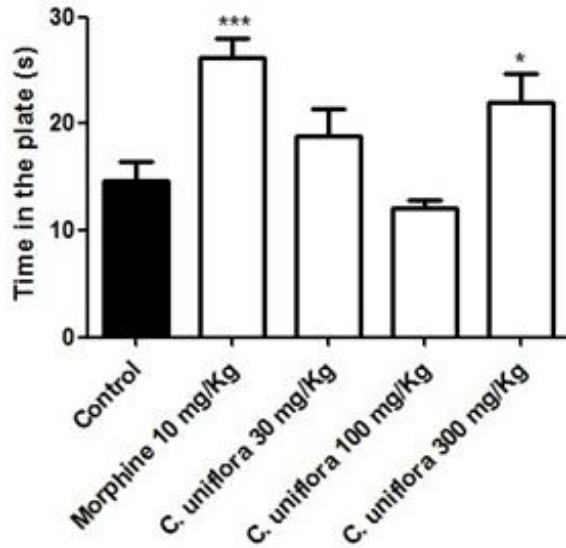
Figure 4. Effects of *C. uniflora* administered orally in the hot plate test. Animals were pre-treated orally with a vehicle, morphine (10 mg/kg, s.c.), or crude extracts of *C. uniflora* (30, 100, or 300 mg/kg) on a hot plate at 52°C. Each column represents the mean ± SEM of latency time of eight animals per group. Control values indicate animals injected with the vehicle. Asterisks indicate statistically significant differences: *$p < 0.05$ and **$p < 0.01$ as compared to respective control values (using ANOVA and Dunnett's test).
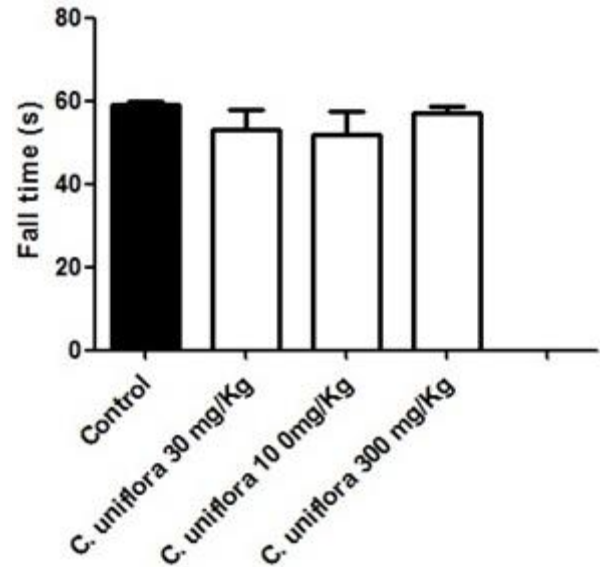

Figure 5. Effects of *C. uniflora* given 60 min prior to rotarod motor incoordination after administration of crude extract (30 to 300 mg/kg, p.o.) or vehicle. Each column represents the mean ± SEM of eight animals. Control values indicate animals injected with the vehicle. Asterisks indicate statistically significant differences: *$p < 0.05$ and **$p < 0.01$ as compared to respective control values (using ANOVA and Dunnett's test).

inflammatory processes and has a healing action.

The phytochemical composition and popular use, it was proposed to study the antinociceptive effects of *C. uniflora* extracts. The pharmacological studies were performed with the crude extracts of the flowers of *C. uniflora* to verify antinociceptive activity. Some experimental models based on chemical stimuli (writhing and formalin) and thermal stimuli (hot plate) can detect this effect. The writhing test is based on the number of twists that occur in response to peritoneal irritation produced by acetic acid, a similar inflammatory pain to peritonitis (Le Bars et al., 2001). The data obtained in this model were satisfactory for antinociceptive potential because crude extracts of *C. uniflora*, administered orally at doses of 100 and 300 mg/kg, reduced the number of writhes as compared to the controls. According to Rodrigues et al. (2011), plants indicated by 26 indigenous groups in Brazil are used for analgesic properties and contain predominantly alkaloids but also triterpenoids, phenolic compounds and coumarins in their chemical composition. Alvarenga et al. (2013) reported that metabolites, such as saponins and flavonoids, can be directly related to peripheral analgesia. According to the phytochemical experiments in this study, the chemical composition of *C. uniflora* contains alkaloids and flavonoids, and these compounds may be related to the antinociceptive action observed in the writhing model.

The formalin model allows for evaluation of two distinct phases of pain. In the first phase (up to 5 min after injection), called the neurogenic phase, activation of C and Aδ fibres occurs. After the first phase, the silent phase is initiated (5 to 15 min after injection), where the animal has no nociceptive behaviour. In the second phase, an inflammatory reaction in the peripheral tissue, referred to as the neuropathic phase, occurs (15 to 30 min after injection) (Hunskaar and Hole, 1987). In this experiment, the plant extracts showed significant results only in the neuropathic phase at doses of 100 and 300 mg/kg. The second phase is characterised by inflammation, which is caused by local inflammation and release of hyperalgesic and inflammatory mediators that can cause inflammatory responses. Thus, it can be concluded that *C. uniflora* can exhibit anti-inflammatory activity which is related to the presence of secondary metabolites such as flavonoids. As reported by Mutoh et al. (2000), flavonoids, such as quercetin and apigenin, showed anti-inflammatory action, as these compounds cause inhibition of cyclooxygenase (COX-2) and nitric oxide synthase.

In order to verify the analgesic action at the central level, the hot plate test was performed. This model is characterised by producing a rapid response to noxious stimuli. The heat stimulates thermoreceptors, and these activate an unalterable activation sequence. In practice,
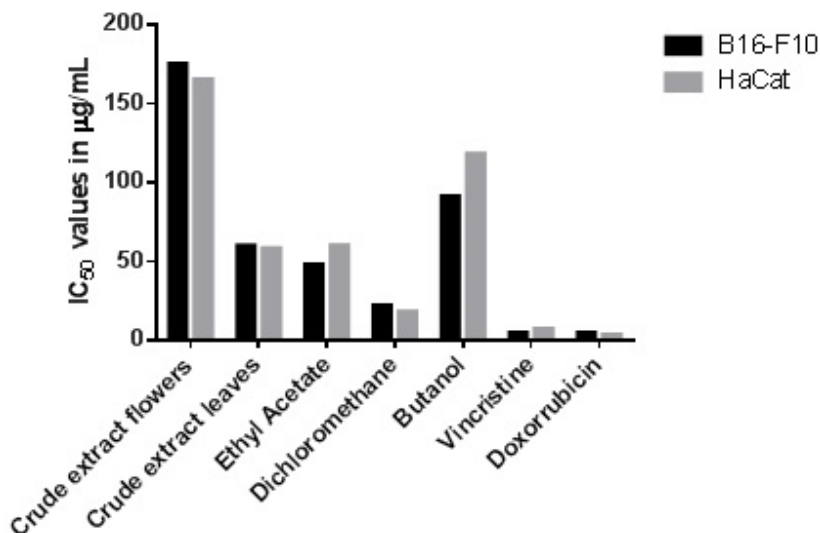
Figure 6. IC_{50} values of leaf and flower crude extracts of *C. uniflora* and of the ethyl acetate, dichloromethane and butanol fractions of flowers compared to the standards doxorubicin and vincristine of two cell lines, B16-F1 and HaCaT, made in triplicate. The IC_{50} was calculated using linear regression.

an animal quickly withdrawing its paw from the stimulus is a result of action at the central level. This model evaluates the antinociceptive activity of opioid drugs (Ankier, 1974; Hiruma-Lima et al., 2000; Le Bars et al., 2001). The results of this test indicated that *C. uniflora* has a central analgesic effect at doses of 300 mg/kg as compared to morphine. In studies of analgesic substances, possible alterations of motor performance, which can be produced by some potentially analgesic drugs, must be disclosed. The largest source of error in studies of drugs that interfere with the transmission of nociceptive response is the change in motor performance of the animal (Millan, 2002). Therefore, motor incoordination was evaluated through the rotarod test. The results showed that the crude extracts of *C. uniflora* did not alter the motor performance of animals at the doses tested (30, 100 and 300 mg/kg). Thus, it was shown that the reduction of nociceptive behaviour in animals was due to the analgesic effect and not changing motor performance.

In addition to the phytochemical and antinociceptive analysis, the cytotoxic potential of extracts and fractions of *C. uniflora* was evaluated, since this plant is widely used in the local region. The *in vitro* culture of cells is an important tool for studying the cytotoxic activity of substances with potential therapeutic activity (Freshney, 2001).

The evaluation of the cytotoxicity of *C. uniflora* showed that crude extracts of flowers showed a lower cytotoxic potential than the crude extract of leaves. The ethyl acetate and butanol fractions also showed no significant degree of cytotoxicity according to the National Cancer Institute of the United States criteria, which confirms the

plant is safe for popular use, especially important since it is widely used in the region (Geran et al., 1972).

The screening program of plants requires that plant extracts with IC_{50} less than 20 mg/mL for crude extracts and 4 µg/mL for pure compounds exhibit cytotoxic potential. However, the dichloromethane fraction has more affinity with these types of compound, extracting more lipophilic substances. This may be responsible for exhibiting a greater cytotoxic potential when compared with other extracts and fractions (Geran et al., 1972). Therefore, the dichloromethane fraction becomes more interesting as it may present a promising effect.

Conclusion

Results from this study indicate that the crude extracts of *C. uniflora* flowers have more chemical compounds than the crude extracts of leaves. Flavonoids and alkaloids were identified in the ethyl acetate fractions; these plant compounds have been demonstrated to have pharmacological action.

The results also showed that the extracts and fractions of ethyl acetate and butanol of *C. uniflora* had IC_{50} values greater than 58 mg/mL for the HaCaT lineage and 48 mg/mL for strain B16-F1. Thus, these values exhibited no cytotoxicity, allowing people to use the plant safely. The dichloromethane fraction exhibited an IC_{50} of 18 mg/mL, showing significant inhibition as compared to the controls, vincristine and doxorubicin. Because dichloromethane showed a potential cytotoxicity higher than the other fractions, it requires further study.

The antinociceptive activities for both thermal and

chemical stimuli showed significant results for crude extracts of *C. uniflora* flowers at doses of 100 and 300 mg/kg. These effects can be related to the chemical compounds present, with sesquiterpenes at the central level and flavonoids at the peripheral level. The extracts of *C. uniflora* do not induce motor incoordination or muscle relaxant activities.

Conflicts of Interests

The authors have not declared any conflict of interests.

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