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Pharmacological and Genotoxic Evaluation of *Calea clematidea* and *Calea uniflora*

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SUMMARY. *Calea clematidea* and *Calea uniflora* are native shrubs found in the southern Brazil. In previous study, *C. zacatechichi* extracts showed psychopharmacologic properties. The aim of this paper is to investigate the effect of these two plants on CNS and genotoxic effects in rats. Methanolic extracts of *C. clematidea* and *C. uniflora* showed apparent efficacy in decreasing the number of entries in closed arms, but no species tested affected the number of entries or time spent in the open arms in the elevated plus-maze test. *C. clematideae* and *C. uniflora* did not change the number of crossings and rearings performed in the open field task. Both extracts did not induce DNA damage in brain tissue from treated animals, assessed by comet assay. The results suggest *C. clematidea* and *C. uniflora* do not induce anxiolytic and genotoxic effects, nor do they alter locomotor activity in rats.

INTRODUCTION

Anxiety disorders are the most common type of mental illness in humans, and have become a very important area of research interest in psychopharmacology [1]. Pharmacological treatment with benzodiazepines has been the most widely used, and these are relatively safe drugs for short-term treatment of anxiety, despite their drug dependence potential and side effects [2]. Plant products used in this way are not as potent as synthetic drugs, but they do not have as many disadvantages as their synthetic counterparts, which are often recommended for short-term use [3]. For this reason, there is considerable interest in the development of new anxiolytic drugs derived from traditional herbs, and the use of phytopharmaceuticals is very popular among the general public [2].

Calea L. is a large genus belonging to the Heliantheae tribe, and to the Asteraceae family. It comprises nearly 110 species and is found in Mexico, and in Central and South America [4,5]. *Calea clematidea* Baker and *Calea uniflora* L., Asteraceae, are native shrubs found in the southern region of Brazil and in Uruguay. In Rio Grande do Sul (Brazil) *C. clematidea* is traditionally used by local people as an anti-influenza agent and for the treatment of catarrh [5]. However, *C. uniflora is* not used in popular medicine [4]. In general, species of this genus are used to treat stomach disease [6-8], and in other studies, crude extracts displayed several biological reactions, such as antifungal [5], anti-inflammatory [9], antimicrobial [10], leishmanial [11], acaricidal [12] and trypanocidal [7]. *C. zacatechichi* has extensive popular use in Mexico, where it is employed as an appetizer cholagogue, a cathartic antidysentry remedy, an insecticide febrifuge, and is used by Chontal Indians to receive divine messages during dreaming [13]. Previous studies showing psychopharmacologic properties with *C. zacatechichi* extracts, particularly, caught our attention to this genus. Mayagoitia *et al.* [13] observed that this plant induces sleep in cats; in-

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creases the superficial stages of sleep and the number of spontaneous awaking in humans. The effects demonstrated on the central nervous system (CNS) by *C. zacatechichi,* and the complete lack of studies of the effects of other species of *Calea* on the CNS, were the prime motivation for our studies. In this work we have studied the phytochemical, neuropharmacological (anxiety and locomotor activity) and neurotoxicological (genotoxicity) effects of *C. clematidea* and *C. uniflora* in rats.

MATERIAL AND METHODS

Plant collection

The upper parts of both *Calea clematidea* Baker and *C. uniflora* Less were collected in January 2006, in Bom Jesus, Rio Grande do Sul State, Southern Brazil, and identified by Prof. Dr. Sérgio Bordignon. The specimens were deposited in the Herbarium of the Lutheran University of Brazil (HERULBRA).

Preparation of plant extracts

A 10 g sample of dried and powdered plant material was macerated in 100 ml methanol for 24 h. The sample was then filtered through Whatman Nº 1 filter paper and the marc was washed with another 100 ml of methanol. This procedure was repeated for 10 days, after which the methanolic solutions were combined and evaporated to dryness under reduced pressure.

Phytochemical screening

The phytochemical analysis (flavonoids, tannins, anthraquinones, alkaloids, saponins, volatile coumarins and cardiac glycosides) of the upper parts of *Calea clematidea* Baker and *Calea uniflora* Less was carried out using methods described by Harborne [14]. The thin layer chromatography analyses were performed following systems and developers indicated by Wagner & Bladt [15].

Animals

Male Wistar rats (a total of 83 animals; aged 2-3 months; weight 200-250 g) from our breeding colony were used (Lutheran University of Brazil). They were housed in plastic cages, with "ad libitum" access to water and food, under a 12 h light/dark cycle at a constant temperature of 23.0 °C. All experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and the Brazilian Society for Neuroscience and Behavior (SBNeC) Recommendations for Animal Care.

Drugs and pharmacological procedures

The plant extracts were dissolved in tween 10 %. Diazepan (Compaz®, Cristália) was diluted in saline solution (NaCl 0.9%). Thirty min prior to the behavioural procedures, the animals were given an intraperitoneal (i.p.) injection of saline 0.9%, tween 10%, diazepan 1 mg/kg, *Calea clematidea* 100 or 150 mg/kg, or *Calea uniflora* 100 or 150 mg/kg, in a volume of 2 ml/kg body weight.

Elevated plus-maze test

The elevated plus-maze test is described in detail elsewhere [16]. Briefly, the apparatus consist of two open arms (50 x 10 cm) and two enclosed arms (50 x 10 x 40 cm), arranged in such a way that the two arms of each type were opposite to each other, and to a platform (10 x 10 cm). The height of the maze was 50 cm, and the tests were conducted under red light. The animals received the injections thirty mins before the test. After this, they were placed individually on the central platform of the plus-maze. During a 5-min test period, the following statistics were recorded: the number of entries, and the time spent in the open and enclosed arms. The anxiolytic compounds reduce the animals' natural aversion to the open arms, and promote exploration of these. Diazepam (1 mg/kg, i.p.) was used as a positive control for the anxiolytic effect [17].

Open field behaviour

The animals were exposed to a 40 x 50 x 60 cm open field divided into 12 equal white squares divided by black lines. They were placed in the rear left square and allowed to explore freely for 5 mins. Crossings of the black lines and rearings performed were counted and used as measures of locomotion and exploration [18].

Comet assay

Alkaline comet assay was carried out as described by Tice *et al.* [19], with minor modifications [20]. Rats were sacrificed by decapitation 3 hours after injections. Each brain specimen was placed in 0.5 ml cold phosphate-buffered saline solution (PBS), and minced into fine pieces in order to obtain cell suspensions. Brain cell suspensions (5 µl) were embedded in 95 µl of low-melting-point agarose at 0.75% (Gibco BRL), and spread on agarose-precoated microscope slides. After solidification, the slides were placed in lysis buffer solution (2.5 M NaCl, 100 mM EDTA, and 10 mM Tris, pH 10.0), with freshly

added 1% Triton X-100 (Sigma) and 10% DMSO for 24 h at 4 °C. The slides were subsequently incubated in freshly prepared alkaline buffer (300 mM NaOH and 1 mM EDTA, pH >13) for 20 mins, at 4 °C. An electric current of 300 mA and 25 V (0.90 V/cm) was applied for 15 mins to induce DNA electrophoresis. The slides were then neutralized (0.4 M Tris, pH 7.5), stained with silver as described by Nadin *et al.* [21] , and analyzed under a microscope.

Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed from each animal. Cells were also visually scored according to tail size into five classes, ranging from undamaged (0) to maximally damaged (4), resulting in a single DNA damage score for each animal, and consequently, for each studied group. Therefore, the damage index (DI) ranged from 0 (completely undamaged, 100 cells x 0) to 400 (with maximum damage, 100 x 4). Damage frequency (%) was calculated based on the number of cells with tail versus those with no tail [22].

Statistical analysis

Data from the elevated plus-maze and open field tests are expressed as mean ± S.E.M. These data were examined by one-way ANOVA followed by Duncan's test. The statistical evaluation of data from the comet assay was carried out using the Tukey`s test. In all comparisons, $p < 0.05$ indicated statistical significance.

RESULTS

The phytochemical analyses of the upper parts of *C. clematidea* and *C. uniflora* indicated the presence of saponins and flavonoids and no evidence of alkaloids, coumarins, tannins, anthraquinones, or cardiac glycosides. The flavonoids analyses indicated for *C. clematidea* the same chromatographic behaviour as for kaempferol, as the main compound, and for *C. uniflora* mainly phenolic acids and flavonoids in low concentration were detected.

We verified the effect of pre-test administration of *C. clematidea* and *C. uniflora* on plus-maze in rats (Fig. 1). *C. clematidea* 100 mg/kg (5.22 ± 1; $p < 0.05$) and 150 mg/kg (4.11 ± 0.67; $p < 0.05$), and *C. uniflora* 100 mg/kg (5,66 ± 1,1; $p < 0.05$) showed apparent efficacy in decreasing the number of entries in the closed arms when compared with the saline group, but did not affect the number of entries in the open arms. The group that received diazepam 1 mg/kg showed a decrease in the number of entries in the closed arms (5.44 ± 1.2; $p < 0.05$) and increased the number of entries and time spent in the open arms (6.55 ± 0.9; $p < 0.05$). The tween control group showed no effect in this test when compared to the saline control group.

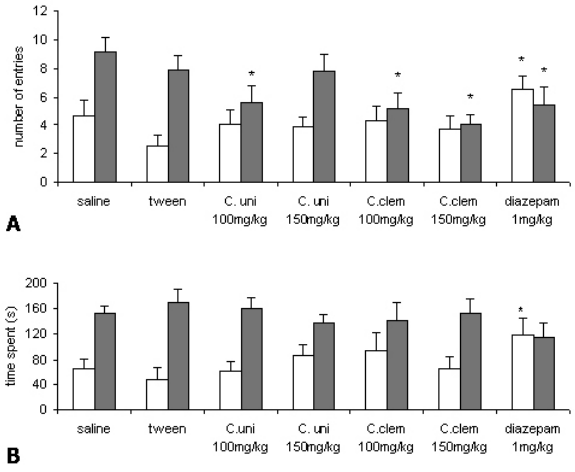

Figure 1. Effect of pretest administration of *C. clematideae, C. uniflora* and diazepam on the (**A**) number of entries and (**B**) time spent (s) in the open and closed arms. Animals received an i.p. injection of saline, tween, *C. clematideae, C. uniflora* or diazepam 30 min prior being exposed to the plus maze. White columns: open arms, gray columns: closed arms. Data are expressed as means ± S.E.M. *N* = 9 animals per group; * $p < 0.05$ compared to the saline group; ANOVA/Duncan's test.

The behavioral patterns of the groups given saline, *C. clematidea* or *C. uniflora* extracts, or tween 30 min prior to the test during a 5-min exploration of an open field are shown in Figure 2. There were no significant differences between the groups regarding the number of rearings ($p = 0.249$) and crossings ($p = 0.416$) performed, suggesting that *C. clematidea* or *C. uniflora* extracts did not affect the exploration or locomotion of the animals in this task.

Table 1 shows the effects of *C. clematidea* and *C. uniflora* on damage index (DI) and damage frequency (DF), as measured by DNA damage in rat brain tissue, using the comet assay. The brains of the rats treated with 150 mg/kg of the extracts and sacrificed 3 hours later showed no significant difference in either of the parameters used to assess DNA damage in comparison with the tween- control group.

DISCUSSION

Phytochemical analyses of the upper parts of *C. clematidea* and *C. uniflora* indicate the pres-

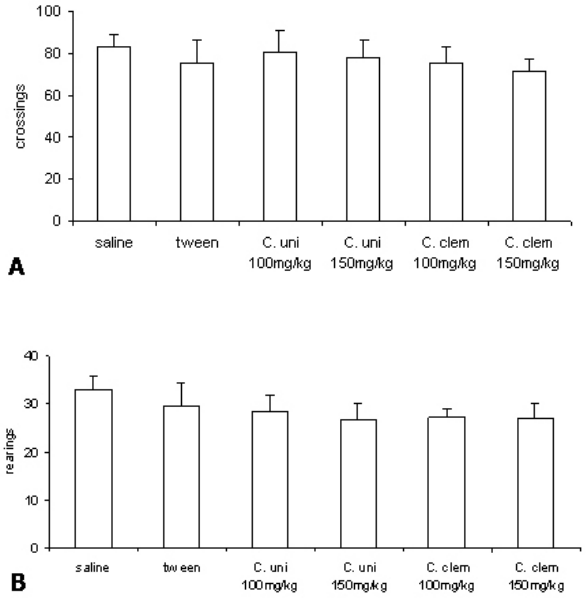

Figure 2. Effect of pretest administration of *C. clematideae, C. uniflora* on (**A**) number of crossings performed and (**B**) number of rearings performed during a 5-min exploration of an open field. Animals received an i.p. injection of saline, tween, *C. clematideae, C. uniflora* 30 min prior being exposed to the locomotor behavior task in the open field. Data are expressed as means ± S.E.M. *N* = 9 animals per group. There were no significant differences among groups.

ence of saponins and flavonoids. These groups of compounds are frequently related with the Asteraceae species, and substances not related with this family such as anthraquinones and cardiac glycosides, were not detected.

This study employed animal models of anxiety and locomotor activity to assess the effects of *C. clematidea* and *C. uniflora* on the behaviour of rats using the elevated plus-maze and open-field tasks. These tests are classic models for screening central nervous system actions providing information about psychomotor performance and anxiety [23]. We observed that *C. uniflora* (100 mg/kg) and *C. clematidea* (100 and 150 mg/kg) decreased the number of entries in the closed arms, but did not affect the number of entries in the open arms. These data suggest that, the doses used of *C. clematidea* and *C. uniflora,* did not show anxiolytic-like effects in this behavioral model in rats. The decrease in the number of entries in the closed arms showed that these extracts were able to change the animal behaviour, however this did not reflect in any increase in the number of entries in open arms in comparison to the control group. This effect could be related to the flavonoids, detected by phytochemical screening, due to their ability to cross the blood-brain barrier [24-26]. Conversely, using diazepam 1 mg/kg, both parameters changed, indicating anxiolytic activity, which is in accordance with previous studies [23,27].

Group	Parameters of DNA damage	
	DI [a]	**DF** [b]
Saline	101.0 ± 69.96	32.87 ± 19.67
Tween	87.62 ± 64.92	29.5 ± 19.31
C. clematidea	120.62 ± 90.60	37.37 ± 23.64
C. uniflora	63.12 ± 14.87	23.37 ± 4.33

Table 1. Comet assay in brain tissue from rats treated with 150 mg/kg of *C. clematidea* or *C. uniflora*. *N* = 5 animals by group. [a] Damage index- can range from 0 (completely undamaged, 100 cells x 0) to 400 (with maximum damaged 100 x 4). [b] Damage frequency- was calculated based on number of cells with tail versus those with no tail.

In order to evaluate possible DNA damage induced by *C. clematidea* and *C. uniflora* in the brain, we used the alkaline comet assay, which detects DNA strand breaks, alkali-labile sites and incomplete excision repair events in individual cells [28]. The comet assay is particularly useful as a tool for the evaluation of local genotoxicity, especially for organs/tissue types which can not be easily evaluated by other standard tests [29]. As shown in Table 1, both extracts did not induce brain DNA damage in the higher dose. In line with the absence of DNA damage in the treated animals, the extracts did not affect locomotion and motivation as shown on the open field (Fig. 2), suggesting *C. clematidea* and *C. uniflora* do not induce psychomotor impairments or genotoxic effects in the tested doses.

The crude extracts from *C. clematidea* or *C. uniflora* were prepared using methanol. This solvent extracts flavonoids, detected by the phytochemical screening, and other natural products related to *Calea* genus, like benzofurans [30,31], chromenes [6,29], chromanones [11] and germacrolides [8], which could cross the blood-brain barrier, acting on CNS due to their low polarity.

Based on the decrease in the number of entries in the closed arms in the elevated plus-maze test, the lack of genotoxicity in brain tissue, in addition to the diversity of natural products present in the crude extracts, guided bioassays should be carried out to identify the active

percentage, and isolate the main compound. Clearly, these results warrant further studies to characterize possible neuropharmacological effects from both *Calea* species.

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