Neuropharmacological and genotoxic evaluation of ethanol extract from *Erythrina falcata* leaves, a plant used in Brazilian folk medicine

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Abstract: The aim of the present study was to determine neurobehavioral and genotoxic activities of ethanol extract of *Erythrina falcata* Benth., Fabaceae, leaves on rats. Animals were treated with ethanol extract of *E. falcata* (100, 300 or 500 mg/kg; i.p.) and the open field and elevated plus-maze tasks were used as behavioral models to investigate a possible effect on the locomotor and exploratory activity and anxiety, respectively. Genotoxic effect was investigated using the Comet assay. Ethanol extract of *E. falcata* leaves decreased the number of crossings and rearings in the open field task and increased the latency to start locomotion, though it was not able to affect habituation to apparatus measured 24 h after the first session. Behavioral parameters in the plus-maze test were not affected by *E. falcata*. Ethanol extract did not increase damage index and damage frequency in blood or brain, indicating no genotoxic effect. The results suggest that ethanol extract of *E. falcata* leaves was able to affect locomotion, exploration, and motivation of animals without anxiolytic/anxiogenic effect, indicating a possible depressant action on the central nervous system. Furthermore, the lack of DNA damage in brain is an indicative that ethanol extract of *E. falcata* leaves may not induce neurotoxic effects.

Keywords: behavior, central nervous system, Comet assay, elevated plus-maze, *Erythrina falcata*, open field

Introduction

The historical relevance of medicinal properties of the *Erythrina* genus in Brazil can be proved by the mention of *Erythrina mulungu* in the first Brazilian Pharmacopoeia (Silva, 1929). The genus *Erythrina* is used in Brazilian folk medicine for the treatment of central nervous system (CNS) disorders, especially the species *Erythrina velutina* in the northern and *Erythrina mulungu* in the Southern regions of the country. These species are frequently used in some Brazilian communities to treat insomnia and other CNS disorders (Dantas et al., 2004; Raupp et al., 2008; Vasconcelos et al., 2007). Similarly, in Argentina, the aerial parts of *Erythrina crista-galli* are used as analgesic (Etcheverry et al., 2003). Based on the popular use, many studies have demonstrated that species of the *Erythryna* genus exert CNS effects such as analgesic (Etcheverry et al., 2003; Vasconcelos et al., 2003), anxiolytic (Flausino et al., 2007a; Flausino et al., 2007b; Onusic et al., 2003; Raupp et al., 2008) and anticonvulsant (Jesupillai et al., 2008; Vasconcelos et al., 2007).

The medicinal properties of *Erythrina falcata* Benth., Fabaceae, have been reported in ethnobotanical surveys (Botrel et al., 2006) and like the other species of this genus, is also used as sedative and anxiolytic (Almeida, 2010). Considering the popular use of *E. falcata* and the lack of scientific studies providing its efficacy and safety, the aim of the present study was to evaluate some pharmacological properties, using the open field and elevated plus-maze tests. The genotoxic effect of ethanol extract from *E. falcata* leaves was also investigated using the comet assay in brain and blood cells, after acute treatment in rats.

Material and Methods

**Animals**

Male Wistar rats (2-3 months of age; 200-250 g) were used in this study. All animals were maintained in a controlled temperature environment. Five animals were kept in cages under 12-h light/dark cycles. The animals were allowed free access to food and water.
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A minimum of nine rats were used for each treatment group. All procedures involving animals were conducted in accordance with the Ethics Committee of Lutheran University of Brazil (CEP/ULBRA 2006-002A) and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH).

**Plant material**

The leaves of *Erythrina falcata* Benth., Fabaceae, were collected in April 2008, in Osório. This city is located in Rio Grande do Sul state, Southern Brazil, and the plant was identified by Prof. Dr. Sérgio Bordignon. The specimens were deposited in the Herbarium of the Lutheran University of Brazil, recorded under number 4662. The leaves were dried under the shade for several days and then powdered.

**Preparation of ethanol extract**

Fifty grams of dried and a powdered leaves of *E. falcata* were treated with 500 mL of ethanol for 24 h. The samples were then filtered through Whatman number 1 filter paper and the biomass was extracted with another 300 mL of ethanol. This procedure was repeated for five days, after that the ethanol solutions were combined and evaporated in a rotary evaporator at 45 °C until dry.

**Phytochemical analysis**

The phytochemical analysis of ethanol extract from leaves of *E. falcata* was carried out according to methods described by Harborne (1998). The thin layer chromatography analyses were performed following systems and developers indicated by Wagner & Bladt (1996).

**Drugs and pharmacological procedures**

*E. falcata* ethanolic extract was dissolved in 5% polysorbate 80 and saline. Thirty minutes prior to the behavior experiment, animals were given an intraperitoneal injection of saline, tween (5% polysorbate 80 solution), *E. falcata* 100, 300 or 500 mg/kg (volume of injection of the 0.1 mL/100 g body weight). Doses were chosen based on LD50 results. The same visual observer participated in all behavioral tests to try to decrease possible variables.

**Acute toxicity determination (LD50)**

The determination of acute toxicity (LD50) was carried out as previously described (Tice et al., 2000) with minor modifications (Rodrigues et al., 2009). The animals were euthanized after behavioral tasks (24 h after the injection). Blood samples (50 μL) were placed in 15 μL anticoagulant (heparin sodium 25.000 IU- Liquemine®). Each total brain was placed in 0.5 mL cold phosphate-buffered saline solution (PBS) and minced into small pieces in order to obtain cell suspension. Cell suspensions from brain and blood (5μL) were embedded in 95 μL of 0.75% low melting point agarose (Gibco BRL) and spread on agarose-precoated microscope slides. After solidification, slides were placed in lysis buffer (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 kg as intraperitoneal injections of the plant extracts. The mortality of animals was noted for a 14-day period.

**Open field behavior and habituation**

Animals were exposed to a 40 x 50 x 60 cm open field divided into twelve identical white squares described by black lines. Animals were placed in the rear left square and allowed freedom to explore the environment for 5 min. Latency to start the locomotion, crossings of black lines and rearings performed were counted and used as measures of motivation, locomotion and exploration (Viana et al., 2007).

The habituation test was conducted after 24 h, when the same animals were tested again for open field behavior, for 5 min. Long-term retention of habituation to a novel environmental can be considered a type of learning. The decrease in the number of rearings performed between the first and the second exploration sessions was considered as a measure of habituation (Viana et al., 2007).

**Elevated plus-maze test**

The apparatus consists of a platform (10 x 10 cm), two open arms (50 x 10 cm) and two closed arms (50 x 10 x 40 cm), arranged in such a way that the two arms of each type are opposite to each other. The maze wall was 50 cm high, and the tests were conducted under dim red light. The animals received the injections 30 min before the test. They were then placed individually on the central platform of the plus-maze. During a 5-min test period, the numbers of entries and the time spent in open and closed arms were recorded. Benzodiazepine diazepam (Valium®, Roche; 1 mg/kg *i.p.*) was utilized as positive control. It is a standard anxiolytic and is also employed in behavior pharmacology as a reference drug (Rex et al., 2002).

**Comet assay**

The alkaline comet assay was carried out as previously described (Tice et al., 2000) with minor modifications (Rodrigues et al., 2009). The animals were euthanized after behavioral tasks (24 h after the injection). Blood samples (50 μL) were placed in 15 μL anticoagulant (heparin sodium 25.000 IU- Liquemine®). Each total brain was placed in 0.5 mL cold phosphate-buffered saline solution (PBS) and minced into small pieces in order to obtain cell suspension. Cell suspensions from brain and blood (5μL) were embedded in 95 μL of 0.75% low melting point agarose (Gibco BRL) and spread on agarose-precoated microscope slides. After solidification, slides were placed in lysis buffer (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 kg as intraperitoneal injections of the plant extracts.
for 48 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 min, at 4 °C. An electric current of 300 mA and 25 V (0.90 V/cm) was applied for 15 min to perform DNA electrophoresis. The slides were then neutralized (0.4 M Tris, pH 7.5), stained with silver and analyzed using 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 to each animal, and consequently to each studied group. Therefore, the damage index (DI) can range from 0 (completely undamaged, 100 cells x 0) to 400 (with maximum damage, 100 cells x 4) (Pereira et al., 2009).

Statistical analysis

Data from LD50 were examined using the Probit’s analysis. Data from elevated plus-maze and open field test are expressed as mean±SEM. These data were examined using the one-way ANOVA followed by the Duncan’s test. Habituation results were analyzed using the Paired t-test. The statistical evaluation of data from comet assay was carried out using the Tukey’s test. In all comparisons, p≤0.05 was considered as indicating statistical significance.

Results

Phytochemical analysis

The phytochemical analysis of ethanol extract from E. falcata leaves revealed the presence of alkaloids, flavonoids and saponins, while other phytochemicals such anthraquinone, cardiac glycosides, cumarins and tannins were not detected.

Acute toxicity studies (LD50)

The LD50 of ethanol extract from E. falcata in rats was estimated at 3,309 mg/kg during an observation period of fourteen days.

Open field behavior and habituation

The behavioral patterns of the groups given saline or ethanol extract from E. falcata (100, 300 and 500 mg/kg) extracts, 30 min prior to the test during a 5-min exploration of an open field are shown in Figure 1. The number of crossings performed by the groups that received 300 or 500 mg/kg significantly decreased (p<0.05); however, this was not observed for the group treated with 100 mg/kg (mean±SEM = 60±8.5 p>0.05; Figure 1A). The ethanol extract in all the administered doses was able to decrease the number of rearings in the open field test, when compared with the control group (p<0.05; Figure 1B). The administration i.p. of 300 and 500 mg/kg of E. falcata ethanol extract was shown to change motivation, increasing latency to start locomotion (p<0.05; Figure 1C).
When the animals were exposed again to the open field apparatus (24 h after the training) the groups that received ethanol extract from *E. falcata* (all doses) did not show any difference when compared to the first exposure (*p* >0.05; Figure 2). Saline and tween groups showed a decrease in number of rearings after a 24-h period, suggesting that these animals were habituated to the environment (*p*<0.05).

*Figure 2. Effect of pretraining administration* *Erythrina falcata* ethanolic extract (100, 300 and 500 mg/kg) administration on habituation to an open field (24 h after training). Animals received an *i.p.* injection of saline solution, tween or *Erythrina falcata* extract 30 min prior the training. White columns: training; gray columns: test. * *p*<0.05; Paired T-test. Data are expressed as mean±SEM (n=10 animals per group).

**Elevated plus-maze test**

In the plus-maze test ethanol extract from *E. falcata* leaves was not able to produce effect both in the number of entries and time spent in the open and closed arms, compared to control group (*p* >0.05; Figure 3). Only diazepam, used as positive control, exerted anxiolytic effect on rats performing this task.

The total number of entries in the arms (open and closed) after treatment with *E. falcata* extract was lower than that in the saline group (Figure 3).

**Comet assay**

The extract did not increase the damage index (DI) and the damage frequency (DF) in blood and brain tissues collected 24 h after the administration (Table 1).

**Discussion**

This study was designed to evaluate behavioral and toxicological effects of *Erythrina falcata* Benth., Fabaceae, ethanol extract on rats. LD50 was determined as 3,309 mg/kg. According to Veerappan et al. (2007), an LD50 of 1,000 mg/kg, calculated based on intraperitoneal administration, may indicate a relatively safe use of a given compound or extract. The values of LD50 found in this study are above this value, and thus may indicate that ethanol extract of *E. falcata* enjoys a wide safety margin.

The acute administration of 300 and 500 mg/kg of the extract of *E. falcata* decreased the number of crossings performed in the open field task. A decrease in the number of rearings in all groups that received the extracts was observed as compared to the control.
group. These results suggest that *E. falcata* extract was able to decrease locomotor and exploratory activities in rats. The time to start locomotion was increased only in the groups that received *E. falcata* 300 or 500 mg/kg, indicating a decrease in the motivation of the animals exposed to higher doses. The effects of other species of *Erythrina* on the CNS have been evaluated. In the sodium pentobarbital sleeping time test, *E. velutina* and *E. mulungu* extracts (200 and 400 mg/kg) promoted an increase in sleeping time, suggesting a depressant effect on the CNS (Vasconcelos et al., 2007).

We evaluated if ethanol extract from *E. falcata* was able to affect habituation in the open field 24 h after training. When the animals were exposed again to the apparatus, an increase in the number of rearings was not observed in all groups treated with ethanol extract. The group that received saline or tween showed a significant decrease in the number of rearings after 24 h, demonstrating the habituation to the apparatus only in these control groups.

After the habituation task, the animals were euthanized and blood and brain samples were collected to evaluate possible genotoxic effects using the comet assay, which detects DNA strand breaks, alkali-labile sites and incomplete excision repair events in individual cells (Hartmann et al., 2003). The ethanol extract was not able to induce DNA damage in either tissue, suggesting no genotoxic activity (Table 1).

Recent studies have shown that some drugs able to impair neurobehavioral performance can induce DNA damage in brain tissue (Kaefér et al., 2010). Apart from the transitory impairment of locomotion, in the present study ethanol extract from *E. falcata* doses tested did not impair the non-associative memory assessed by habituation. Similarly, no increase in brain DNA damage was observed in treated rats (Table 1).

Previous studies reported the presence of flavonoids (Jesupillai et al., 2008; Tanaka et al., 2001), alkaloids (Etcheverry et al., 2003; Tanaka et al., 2001) and saponins (Carvalho et al., 2009) in the *Erythrina* genus. In this work, the phytochemical screening showed the presence of flavonoids, and alkaloids in *E. falcata*, corroborating the findings of Almeida (2010). The presence of alkaloids has been associated to neuromuscular blocking action elicited by some species of *Erythrina* (Megirian et al., 1995). In this study, ethanol extract from *E. falcata* leaves disturbed locomotion and exploration in the open field. It is possible that this effect might have been caused by the presence of alkaloids characteristic of this genus.

The possible anxiolytic effects of the species of *Erythrina* are all imputable to the presence of erythrinian alkaloids. Flausino et al. (2007a) evaluated three alkaloids isolated from *E. mulungu* in animal models of anxiety in mice after acute oral administration. The results obtained suggested that the alkaloids erythravine and (+)-11α-hydroxy-erythravine are responsible for the anxiolytic effects of *E. mulungu* extract. In the elevated plus-maze test, chronic, but not acute, *E. velutina* water-alcohol extract (100 mg/kg) administration increased the percentage of open arm entries, suggesting an anxiolytic-like effect on mice (Raupp et al., 2008). In this study we investigated the acute effect of ethanol extract from *E. falcata* leaves using the same task. The groups that received *E. falcata* extract did not show significant difference of the number of entries or time spent in the open arms, when compared to the group control, suggesting no anxiolytic effect. Similarly, acute treatment with *E. mulungu* (200-800 mg/kg) was not able to produce effect in the plus-maze task (Vasconcelos et al., 2004). However, using this behavioral model we could observe in our study a decrease in the total number of entries in the arms (open and closed) after treatment with all doses tested, suggesting a possible depressant effect of ethanol extract from *E. falcata* leaves on the CNS.

Studies about the mechanism of action of *Erythrina* genus are scarce. Carvalho et al. (2009) showed that *E. velutina* extract was able to produce a contractile response in the guinea pig ileum. This result was related to GABA<sub>A</sub> receptors activation, acetylcholine release, muscarinic receptor activation and calcium action in the cells. Our study investigated if *E. falcata* was able to affect pharmacological and toxicological parameters. The results obtained suggest the importance to know the compounds of ethanol extract, as well as its action mechanism in the effects observed here.

In conclusion, this study showed that ethanol extract from *E. falcata* leaves affects the locomotion, exploration and motivation of animals in the open field test, suggesting an activity in the CNS of rats. In this sense, all doses of ethanol extract tested impaired the habituation of animals, but with no significant difference. Thus, this extract should be tested in other memory models, like inhibitory avoidance or recognition of objects task, to confirm the effect observed in the present study on the habituation and memory processes. Acute treatment with *E. falcata* did not exert an anxiolytic effect, but the experimental protocol used here suggest a depressant action of this species on the CNS, manifested as the reduction in the total number of entries in the arms (open and closed). *E. falcata* did not exert genotoxic effect on blood and brain samples as measured using the comet assay. Further studies about this species are necessary to evaluate different doses and behavioral parameters to confirm the depressant effect on CNS observed here.
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**Authors' contributions**

SAD performed behavioral and genotoxic tests. AEMON performed behavioral tests. ABFF wrote and revised the paper. JNP performed genotoxic tests, analyzed the data on genotoxic parameters and revised the paper. PP designed the research, analyzed the data on behavior and wrote and revised the paper.

**References**


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