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INVESTIGATION / RESEARCH

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Sedative and anxiolytic effect of the methanolic extract of *Erythrina edulis* Triana ex Micheli in mice

Efecto sedante y ansiolítico del extracto metanólico de *Erythrina edulis* Triana ex Micheli en ratones

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ABSTRACT

The traditional use of medicinal plants and its link with pharmacological studies is essential in order to corroborate the effect scientifically. The present work was an experimental study designed to investigate the sedative and anxiolytic effect of the methanol extract of the leaves of *Erythrina edulis*. The extract was prepared by percolation. Different concentrations: 500, 250 and 125 mg/kg of the extract and diazepam as a controlled drug were administered intraperitoneally to Swiss CD1 mice for both, sedation and anxiety tests. The sleep enhancement test with pentobarbital and the traction test were used for the sedative activity. Results showed that the extract did not possess sedative activity in any of the administered doses. However, the possibility of an opposite effect was observed as an activation of the central nervous system probably. The anxiolytic activity was evaluated by the open field test and the elevated plus maze test, where a behavior with a low level of anxiety was observed. Altogether, the results suggest that the extract has an anxiolytic activity without sedation, which supports traditional use. But, its responses are not dose-dependent, and its mechanism of action is unknown.

Besides, toxicity assay was performed in *Artemia salina* nauplii, resulting in a lethal

concentration 50 of 16.570 ug/mL corresponding to zero toxicity. Finally, the phytochemical analysis of the extract revealed the presence of flavonoids (flavonols, flavones and phenolic carboxylic acids), anthrones, anthranols, terpenes (mono and sesquiterpenes), saponins and hydrolysable tannins.

Key words: anxiety, citotoxicity, sedation, *Erythrina edulis*.

RESUMEN

El uso tradicional de plantas medicinales y su relación con estudios farmacológicos que demuestren su actividad es muy importante. El presente trabajo fue un estudio experimental diseñado para investigar los efectos sedante y ansiolítico del extracto metanólico de las hojas de *Erythrina edulis*. El extracto fue preparado por percolación. Diferentes concentraciones: 500, 250 y 125 mg/kg y diazepam como droga control fueron administradas por vía intraperitoneal a los ratones Swiss CD1 tanto para los ensayos de sedación como ansiedad. Para la actividad sedante se usaron las pruebas de inducción del sueño con pentobarbital y la de tracción. Los resultados obtenidos demostraron que el extracto no tuvo actividad sedante a las dosis administradas, observándose más bien el efecto contrario, probablemente por activación del sistema nervioso. La actividad ansiolítica fue analizada con las pruebas de campo abierto y laberinto elevado en cruz, observándose un efecto ansiolítico. En conclusión, el extracto tiene potencial ansiolítico sin producir sedación, lo cual soporta el uso tradicional de la planta, sin embargo, este efecto no se presentó dosis dependiente en las dosis utilizadas. Además, el ensayo de toxicidad fue realizado con nauplios de *Artemia Salina*, obteniéndose una dosis letal 50 de 16.570 ug/mL, lo cual corresponde a toxicidad nula. Finalmente, el screening fitoquímico general demostró la presencia de flavonoides (flavonoles, flavonas and ácidos fenólicos carboxílicos,), antranas, antranoles, terpenos (mono y sesquiterpenos), saponinas y taninos hidrolizables.

INTRODUCTION

Ecuador is a small country with *excellent* plant biodiversity being in the list of megadiverse countries around the world. *Erythrina edulis* is a native plant from it that belongs to the *Fabaceae* family. It is locally known as “cañaro”, and widely used as a source of nutrients and also as a medicine to treat different health problems ¹; for example, in the country, the maceration of the leaves has been used for managing nervousness ².

Phytochemical analysis performed on the genus *Erythrina* have determined the presence of alkaloids, flavonoids, and proteoids¹; and, specifically in *E. edulis* has been reported the existence of quaternary alkaloids, steroids, and terpenes³. These different compounds have shown a potential use for anxiety treatment and sedation because of their possible effects on the gamma-aminobutyric acid (GABA) system which is directly associated to these conditions^{4,5}. So, the phytochemical characteristics of the plant and traditional uses reported in Ecuador by Torres and Ríos suggest that *E. edulis* could be appropriate for the treatment of nervousness, anxiety, and also has a potential sedative effect. Nevertheless, there are no scientific studies that validate or support the usage of the species and define its specific pharmacological response. Thus, this research is intended to determine the sedative and anxiolytic effects of the methanolic extract of the leaves of *E. edulis* in Swiss CD1 mice.

MATERIALS AND METHODS

Plant material: *E. edulis* leaves were collected in Gualaceo – Chordeleg (Ecuador) at 2332 meters above sea level, (2054'4.''S, 78046'28.3''W). The plant was taxonomically identified at Azuay University Herbarium, and the voucher was deposited under the number 11980.

Preparation of plant extracts for biological activities: the leaves of *E. edulis* were dried in an oven at 40°C for 24 hours. The material was pulverized and soaked in methanol for 12 hours in order to obtain the methanolic extract by percolation. The obtained extract was concentrated with nitrogen and then lyophilized. The extract was stored under -20 °C in a bio freezer until use. The reconstitution of the material was done in distilled Water-Tween 20 (1%) solution (vehicle) for all in vivo tests.

Animals: male Swiss CD1 mice obtained from the bioterium of the National Institute of Investigation in Public Health in Ecuador were used. Mice were maintained under international protocols for the housing and care of laboratory animals. They were under controlled environmental conditions of light (12 hours' light/dark cycle) and temperature (25°C ± 2°C). Animals had food and water *ad libitum*. Before performing each test, the animals were fasted for 15 hours and acclimated to the test environment for 30 minutes⁶. Mice were randomly assigned

in groups of 6 for each test using five groups distributed as follows: **negative control group** treated with vehicle; **positive control group** treated with Diazepam (DZ) in a dose of 1mg/kg, and three **test groups** treated with the methanolic extract of *E. edulis* leaves at a dose of 500, 250 and 125 mg/kg of weight, respectively. All mice were injected intraperitoneal (i.p.) with the different solutions. All tests were carried out between 8am to 1pm. Every subject was used only once.

Sedative activity

Pentobarbital-induced sleeping time: each group of animals was given (i.p.) a single dose of vehicle, diazepam or the extracts as described before. Thirty minutes later pentobarbital was administered to all animals to induce sleep in a dose of 40 mg/kg. Mice were put in individual cages for observation by recording them. Latency time and sleeping time were recorded; the first being the time needed for the mice to lose his ability to stand on his four paws and the second being the time needed for the mice to get back on his paws.

Traction test - horizontal bar: the apparatus consists of a horizontally arranged metal bar 38 cm long and 2 mm diameter supported by two 49 cm long bars⁷, and they were swung in front of the horizontal bar letting them grab the bar by its center point with their front legs releasing their tail by the operator immediately after it. The time needed for the mice to place the rear leg on the bar was recorded. The cut-off time was set to 30 seconds. All mice were previously tested to ensure an excellent locomotor activity; mice that were not able to grab the metallic rod with their hind limbs in 5 seconds or less were discarded. The test was executed at 30, 60 and 90 minutes' post i.p. Injection to assess the effect of the drug on neuromuscular function over time.

Anxiolytic activity

Open Field: the apparatus consists of a 50 cm square glass box with 30 cm tall walls and a white grid base. The arena was divided in two segments: the outside one consisted of 16 squares along the walls, and the inner segment had nine squares including the central square marked in red. After 30 minutes of the i.p. treatment mice were individually placed in the center of the apparatus and left in the arena for 5 minutes. Total number of crossed lines, the total number of rearing, total time spent in the center and the total number of center entries were recorded. This test was filmed with a video camera due to the possible interaction of the mice with the

experimenter.

Elevated cross maze: the apparatus consists of two open arms (25 x 5 x 15 cm) and two closed arms (25 x 5 x 1 cm) elevated to a height of 50 cm above the ground. After thirty minutes of the i.p. treatment mice were individually placed in the center of the apparatus and left for 5 minutes. The total number of entries and time spent in open and closed arms were recorded. This test was filmed with a video camera due to the possible interaction of the mice with the experimenter.

All experiments described were conducted in accordance with accepted principles of the guide for the care and use of laboratory animals ⁸.

Toxicity assay: lethal concentration 50 (LC50) was determined following Meyer method (1982) modified by Molina and collaborators (2006). The following concentrations of the *E. edulis* extract were used: 25000, 22500, 20000, 17500, 15000 and 12500 µg/mL, and the control group was treated with vehicle. *Artemia salina* nauplii were used in this assay. The plate was incubated under dim light at 25°C for 24 hours; nauplii were observed under a stereomicroscope and they were considered dead if they stayed immobile for a lapse of 10 seconds. LC50 was estimated using Probit analysis, and it was compared to Clarkson's toxicity index ⁹.

Preliminary phytochemical screening: thin layer chromatography (TLC) was performed for the detection of alkaloids, flavonoids, anthraquinones, terpenes, saponins, and tannins following the methodology described by H. Wagner and S. Bladt ¹⁰.

Statistical analysis: data was analyzed using One-way ANOVA followed by Tukey's posthoc test using R 3.4.3 64 bits' version software. Toxicity assay was analyzed using linear regression with Finney Probit analysis.

RESULTS

Erythrina edulis was collected in the province of Azuay in order to process the material to obtain the methanolic extract. After the procedures, the yield of the extraction was 9:1 (dry drug/ lyophilized).

Sedative activity

Pentobarbital-induced sleeping test was used at the beginning to evaluate sedation, obtaining the following results: the positive drug, diazepam, produced a decrease ($p < 0.001$) in the latency time and a definite increase ($p < 0.001$) in the sleeping time compared to the control group which validates the test. The administered doses of the extract presented no significant variation in the latency time. Doses of 250 and 500 mg/kg produced a decrease ($p < 0.01$) in the sleeping time, which is the opposite response to the one expected (Figure 1).

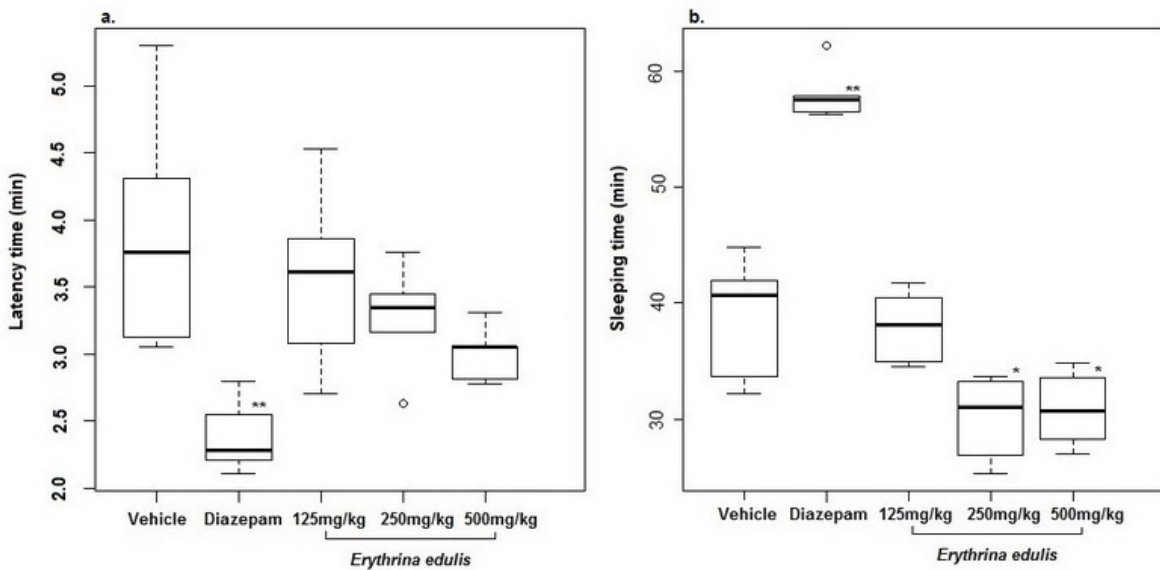


Figure 1. Latency and sleeping time of the methanolic extract of *E. Edulis* and diazepam compared with the control group. All data are expressed by mean \pm SEM (n=6); * $p < 0.05$, ** $p < 0.01$. One-way ANOVA and Tukey's post hoc test were applied.

Traction test was also performed to evaluate the potential sedative activity of *Erythrina edulis*, which was measured as the recovery time after 30, 60 and 90 minutes of the administration of the extract. This test has to do with muscular relaxation, which probably is not the mechanism of action of the extract because all doses at different times did not show an increase in the reincorporation time. *E. edulis* at doses of 125 and 250 mg/kg decreased ($p < 0.001$ and 0.01) the recovery time at 30 minutes in comparison to the control group. Similar results were shown by *E. edulis* at doses 250 and 500 mg/kg at 60 minutes ($p < 0.01$) (Figure. 2). No

changes were observed at the third exposure at 90 minutes (data not shown).

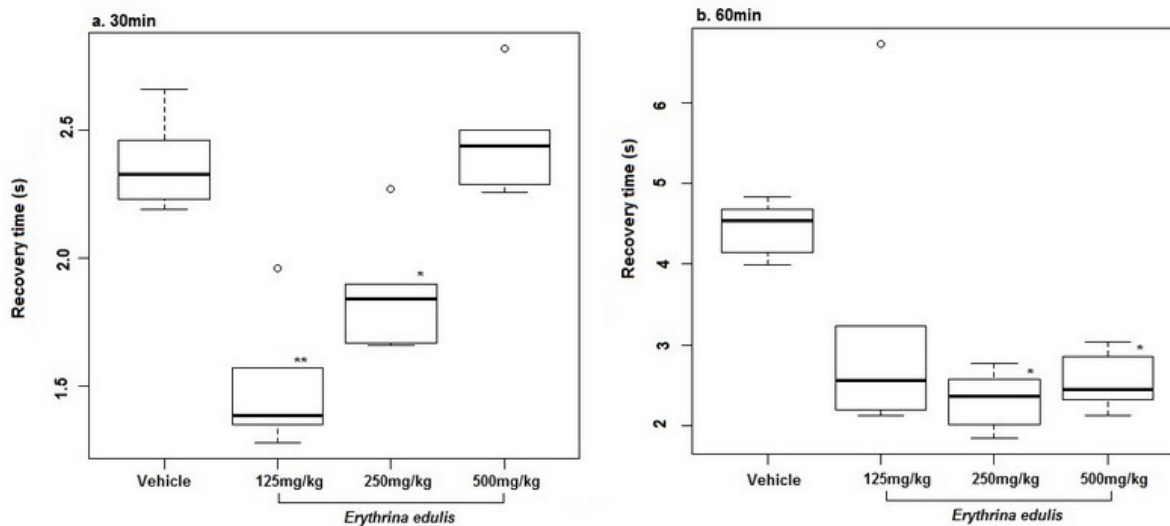


Figure 2. The recovery time of *E. Edulis* compared with the control group at 30 and 60 min after the extract administration. All data are expressed as mean \pm SEM (n=6); *p<0.05, **p<0.01. One-way ANOVA and Tukey's post hoc test were applied.

Anxiolytic activity

The open field test is one of the most critical experiments used to value the emotionality in rodents. It exposes to rodents to an unknown and broad place. This test assesses some parameters. The most representative ones are shown. Treatments with *E. edulis* at doses of 125 and 500 mg/kg increased significantly ($p < 0.01$, 0.001) the number of crossed lines compared with the control group, which is an indicator of the stimulation of neuromuscular system. The number of entries to the central square increased ($p < 0.001$) by *E. edulis* at doses of 125, 250 and 500 mg/kg; results that suggests an exploratory behavior which is an indicative of low levels of anxiety (Figure 3). The number of assisted rearings was decreased by *E. edulis* treatment ($p < 0.01$) (Figure 4). The total time spent in the center shows that neither *E. edulis* nor diazepam administrations induced changes.

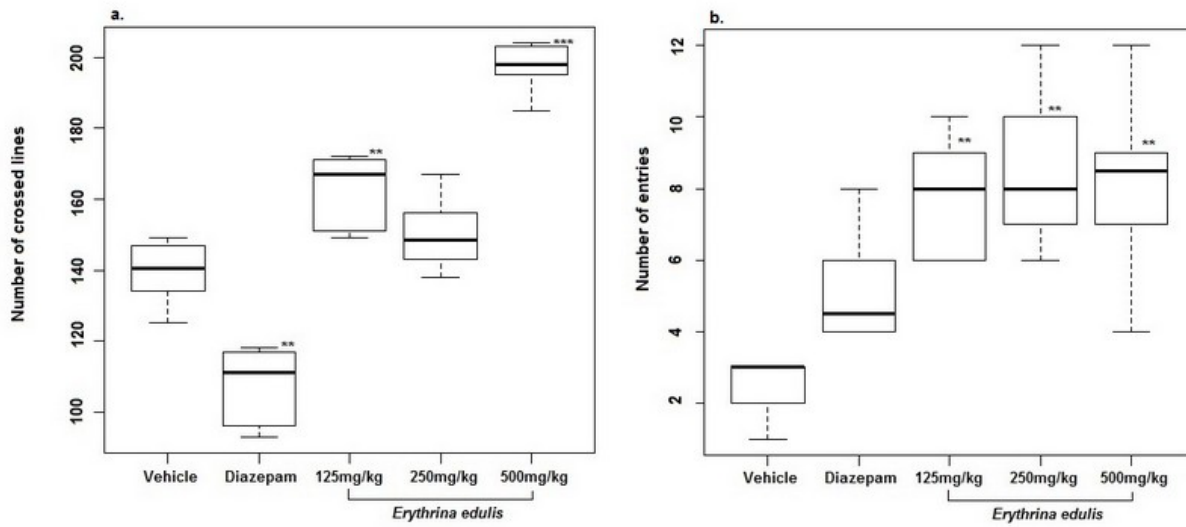


Figure 3. The number of crossed lines and entries in the central square. All data are expressed by mean \pm SEM (n=6); *p<0.05, **p<0.01, ***p<0.001. One-way ANOVA and Tukey's post hoc test were applied.

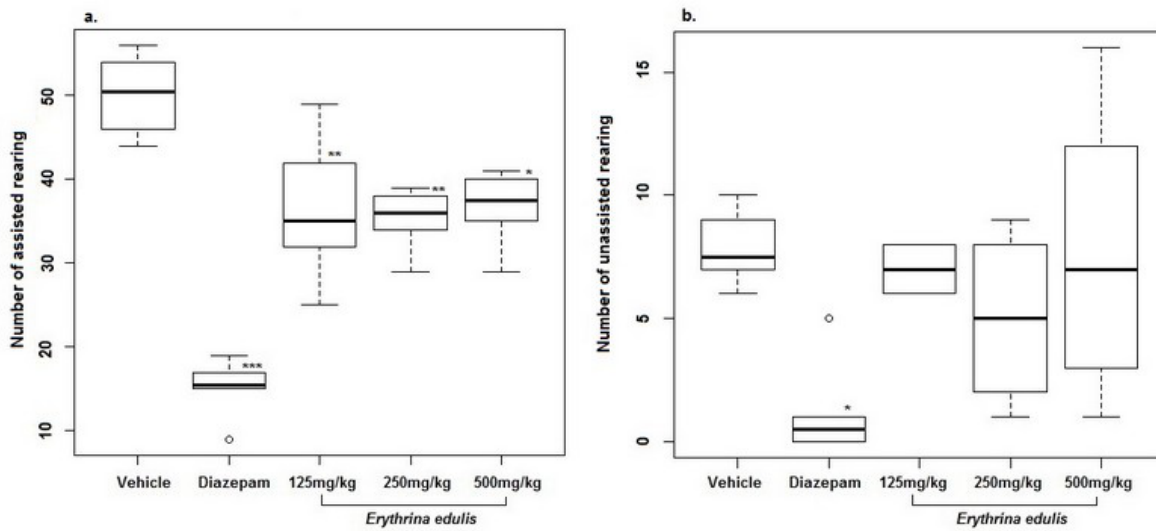


Figure 4. The number of rearings. All data are expressed as mean \pm SEM (n=6); *p<0.05, **p<0.01, ***p<0.001. One-way ANOVA and Tukey's post hoc test were applied.

The elevated cross maze was also performed. It evidenced that the number of entries to the open arms increased with the doses of 125 mg/kg ($p < 0.01$), and 250 mg/kg ($p < 0.05$). Also, the time permanence of mice in the open arms increased with 125 and 250 mg/kg ($p < 0.01$) in the time of permanence in the open arms. It is important to mention that the protocol developed is excellent because diazepam produced an increase ($p < 0.01$) in the activity within the open arms and produces a decrease ($p < 0.001$) in the time of permanence in the closed arms (Figure 5).

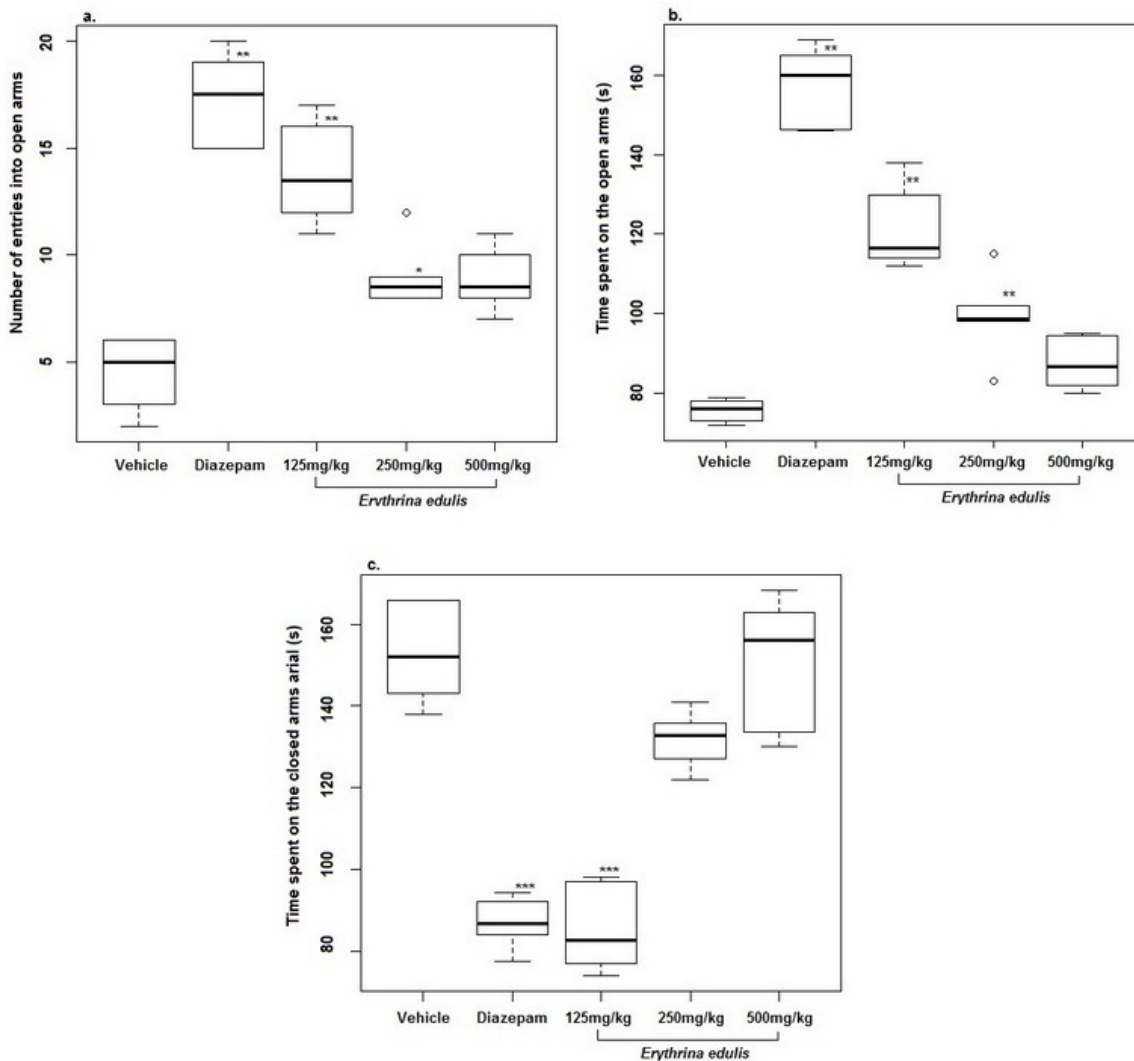


Figure 5. Number of entries into open arms and time spent in open and closed arms. All data are expressed as mean \pm SEM (n=6); * $p < 0.05$, ** $p < 0.01$, * $p < 0.001$. One-way ANOVA and Tukey's post hoc test were applied.**

Toxicity assay: All data obtained from this test is shown in Table 1.

Dose (ug/mL)	Log Dose	Dead nauplii	Total naupli	% Mortality	Probit
25000	4,097	65	65	00	-
22500	4,176	63	68	93	6,48
20000	4,243	54	69	78	5,77
17500	4,301	46	67	69	5,5
15000	4,352	35	70	50	5
12500	4,398	2	64	3	3,12

Table 1. Toxicity assay in *Artemia Salina* nauplii.

The LC50 obtained from the extract was 16571.78µg/mL calculated with a linear regression equation: $y = 12,0x + (-45,70)$. This value corresponds to zero toxicity in *Artemia salina* according to the Clarkson toxicity index ⁹.

Phytochemical analysis of the extract revealed the presence of flavonoids (Flavonols, flavones and phenolic carboxylic acids), anthrones, anthranols, terpenes (mono and sesquiterpenes), saponins and hydrolysable tannins.

DISCUSSION

In the present study, the sedative and anxiolytic effect of the acute administration via intraperitoneal of the methanolic extract of *Erythrina edulis* in mice were studied.

It is well known that the central nervous system (CNS) depressant drug in the pentobarbital-induced sleeping test causes a decrease in the latency time and an increase in the sleeping time. We observed that the extract of *E. edulis* produced the contrary effect. It is also important to mention that doses of 250 and 500 mg/kg decreased the sleeping time even lower than the control group. These results suggest that there could be a nervous system stimulation due to the extract which should be confirmed with another type of studies. Also, these can be attributed to other factors such as an interaction of antagonism between the extract and pentobarbital or induction of the cytochrome P450 produced by the extract stimulating the pentobarbital metabolism ¹¹.

On the other hand, the traction test was completed to assess muscle strength and neuromuscular function in rodents using the restoration reflex as a guide to determine the level of sedation. Mice that are exposed to a drug with a sedative effect do not have such a reflex or it takes them longer to reestablish throughout 3 expositions¹². The mice exposed to *E.edulis* methanolic extract showed a decrease in recovery time. During the first exposure mice treated with 125 and 250 mg/kg doses have a shorter recovery time. This behavior is repeated in the second exposure (60min) with the 250 and 500 mg/kg doses discarding the muscle relaxant effect of the extract.

The reason for performing those two tests together was because the sedative effect of a substance is measured by its capacity to produce hypnosis, muscle relaxation or both. According to what was obtained in both pentobarbital-induced sleeping and horizontal bars, the extract produces an action opposite to hypnosis and muscle relaxation, which suggests a possible neuromuscular stimulation produced by the extract.

The technique of open field (OF) is one of the most used ones to evaluate emotionality in mice. The exposure of mice to bright unknown spaces produces fear and anxiety responses in the animals changing their behavior and locomotive activity^{13,14}. The number of crossed lines measures locomotor activity, and it is used to measure the sedative activity in mice^{15,16} roughly. Foyet and colleagues define it as horizontal movement. Foyet suggests that an increase in this parameter discards a sedative effect because an increase in the horizontal movement is an indicator of stimulation in the central nervous system. Another behavior analyzed in the OF is exploration. Thus, the number of entries to the central square is an indicator of exploratory behavior; therefore, of low levels of anxiety^{13,16 17}. Anxiety can also be measured with this parameter if we consider thigmotaxis behavior. Thigmotaxis refers to the influence that a solid object has on the behavior of the rodent. In this case, the walls of the apparatus provide a safe area inciting mice to stay close to them indicating high levels of anxiety.

Consequently, mice that have entered the central quadrant with more frequency show signs of low anxiety¹⁸. Finally, an anxiolytic effect is also reflected in a decrease in the assisted rearing and an increase in the unassisted rearing. These parameters are closely connected to the fight-flight reflex and exploration. Assisted rearing occurs when the mice stand on their hind legs leaning against the walls of the apparatus, and it is an indicator that mice are looking for an escape route. On

the other hand, unassisted rearing is a sign of exploratory behavior; mice stand on their hind legs far from the walls in order to know and get acquainted with the arena 13,17

E. edulis presented essential results in the tests related to anxiety. OF showed an increment of the number of crossed lines by mice at doses of 125 and 500 mg/kg. The extract also increased the number of entries to the central square at doses of 125, 250 and 500 mg/kg. The number of assisted rearings is also affected by the extract producing a decrease with all doses. Ergo findings obtained in the OF test suggest a possible non-dose-dependant anxiolytic effect produced by the extract. Elevated cross maze proposes that the number of open arms entries and the time spent in them is higher in mice with less or no anxiety^{19,21}. As expected, diazepam produces an increase in open arms entries ($p < 0.001$) when compared to the control group. All doses of *E. edulis* extract produced an increase in the number of entries into the open arms. The extract also increased permanence time in the open arms at doses of 125 and 250 mg/kg. These results corroborate what was reported by Ribeiro et al. who determined that two species of *Erythrina* exerted anxiolytic-like effects associated with generalized anxiety disorders²⁰. Probably the effect could be mediated by the alkaloids present in the extract what corresponds with the reports of erythrinian alkaloids from *E. mulungo*²¹. In future studies, it would be interesting to have a complete phytochemical profile of *E. edulis*.

All data exposed before leads to a state that the methanolic extract of *Erythrina edulis* has a potential anxiolytic effect being no sedative. These results give us the possibility to develop in the future an alternative treatment for anxiety without sedation as a side effect, contrary to the drugs used for this disease today and which could cause some problems in patients.

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Conflict of interest

The authors declare that there is no conflict of interest.

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