December 30, 2017

PharmacologyOnLine

Archives • 2017 • vol.3 • 91-104

PRACAXI IMPAIRS GENERAL ACTIVITY AND LOCOMOTION IN MALE MICE

Ivana B. Suffredini,[1,2,*] Sergio A. Frana[2], Áurea M. M. Santos[3], Ingrit E. C. Díaz[4], Maria M. Bernardi[1]

[1]*Graduate Program in Environmental and Experimental Pathology,Graduate Program in Dentistry, Graduate and Research Vice-Dean Office, Paulista University, R. Dr. Bacelar, 1212, Vila Clementino, São Paulo, São Paulo, 04026-002, Brazil.*

[2]*Center for Research in Biodiversity, Extraction laboratory, Botany Laboratory and Herbarium UNIP, Paulista University, Av. Paulista, 900, Bela Vista, São Paulo, São Paulo, 01310-100, São Paulo, São Paulo, 01310-100, Brazil.*

[3]*Faculty of Pharmacy, Paulista University, R. Amazonas da Silva, 737, Vila Guilherme, São Paulo, São Paulo, 02051-001, Brazil.*

[4]*Facultad de Ingeniería Química y Textil de la Universidad Nacional de Ingeniería, Lima, Peru*

*ibsuffredini@yahoo.com.br

Abstract

Pracaxi, or *Pehtaclethra macroloba* (Willd.) Kuntze, Fabaceae-Mimosoideae, is a medicinal plant used in Brazil against snakebite, as healing and as insecticide. Its stem organic extract showed relevant cytotoxicity. Reports concerning alterations in behavioral phenotype are not known, nor were compounds isolated from *pracaxi* stem. Organic extract (EB827) were administered to Balb-c male mice and general behavioral changes related to motor, sensorial and central/autonomous nervous systems were evaluated. Both 50% lethal (LD50) and non-lethal doses (NLD) tendencies were determined. EB827 was chemically studied using partition, column chromatography and thin layer chromatography to isolate four compounds that were H^1 and C^{13} nuclear magnetic resonance analyzed. LD50 tendency was 228.25 mg/mL for EB827 and NLD was 78.1 mg/mL. Impairment in general activity was observed, as it did in tail squeeze, response to touch, defecation, cyanosis and breath. Then, behavioral changes in general activity, response to touch, tail squeeze, defecation and breath were observed after NLD administration. Locomotion was diminished by the administration of EB827, as immobility time has increased, in the open field apparatus. Necropsy showed that mice ended up with pale liver and opaque internal organs, despite the occurrence of haemorrhage, which could have been the cause of amimals death. Friedelin, epifriedelanol, sitosterol and stigmasterol were isolated from *pracaxi* stem. Behavioral phenotype after administration of the organic extract of *pracaxi* was determined by the subsequent analyses proposed in the present work, and displays the basis of further pharmacological studies. Also, four molecules were described to occur in the stem of the plant for the first time.

Keywords: *Laetia suaveolens, Salicaceae, behavior, general activity, open field, locomotion.*

Introduction

Pehtaclethra macroloba (Willd.) Kuntze, Fabaceae-Mimosoideae, is popularly known as *pracaxi*, *paracaxi* or *pashaco* (1), in Brazil. According to this author, the plant is widely distributed in South and Central America, especially in Trinidad and Tobago, Costa Rica, Nicaragua, Panama, Guyana, Suriname, Venezuela and Brazil (http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?27302). The species is popularly used against snakebite, as healing and insecticide. Oil is extracted from seeds to be used in both culinary and cosmetics and the wood is used in furniture (1). Seed extracts are being studied as insecticide, as well as compounds that were isolated from this extract, particularly proteins, were patented (2). Those proteins are also tripsin inhibitors (3). Triterpenoid saponins that occurred in the species inhibited metalloproteinase of snake venom (4). Also, antihemorrhagic, antinucleolytic and antiophidian properties of aqueous extract from *pracaxi* were reported (5). The presence of monodesmosidic saponins in the species has been reported (6). Larvicidal activity and the presence of triterpen saponins have also been assessed for the plant (7). Chemical studies performed with other species from the same genus show the presence of alkaloids, saponins, flavonoids and phenolics, and a series of vitamins, as riboflavin, niacin, and others (8).

Our group has studied the cytotoxic and antibacterial activity of the organic and aqueous extracts (9) obtained from the stems of *P. macroloba*, and results show that the organic extract was cytotoxic to prostate cancer cell lines (percentage of growth inhibition= -22.67%) (10). The species has never been studied before, in terms of its influence over behavioral phenotype, and for that reason, new findings may support further pharmacological assays, as it was done before for other compounds (11). The present study aims the description of the first signs of behavior alterations expressed by mice under influence of EB827, which is based on the evaluation of general activity plus 26 other parameters associated with motor, sensorial and central/autonomous nervous systems. Plus, parameters related to the analysis of mice behavior and locomotion in the open field apparatus, using the least number of animals, are also reported. In addition, the identification of some of the non-polar compounds is also reported.

Materials and Methods

Plant collection and extract preparation

P. macroloba (scientific synonym P. brevipila or P. filamentosa) was collected in the Brazilian Amazon rain forest, under Brazilian Government licenses for collecting and bioprospecting genetic resources in protected areas of Brazilian forests (IBAMA-CGen/MMA#012A-2008). The studied species was collected in the surrounds of Manaus city, state of Amazonas, in a seasonally flooded forest from Rio Negro Basin (the so called igapó forest). The voucher is deposited at UNIP Herbarium [A.A.Oliveira, 3481 (UNIP)].

Stem of P. macroloba was dried in air-circulating stove (Fanem) at 40°C - temperature which is usually employed to dry plant crude material that does not interfere in the active compounds. Stem was ground in a hammer-mill (Holmes), and it was subsequently placed in a glass percolator (Kontes), where a 24h-maceration proceeded with dichloromethane and methanol 1:1 (12) (Merk) was made. Solvents were evaporated under vacuum (Buchii), in order to eliminate organic solvents from the extract, and were kept in freezer (Revco) until use. Extract was suspended in almond oil to be administered to animals during experiments.

Preparation of extract and reference substance to be administered

The extract EB827 was suspended in almond oil, and the following doses were administered: 2,500, 1,250, 625.0, 312.5, 156.3 and 78.1 mg/kg by intraperitoneal (I.P.) route, according to the propositions of OECD 423 (Guideline for testing of chemicals. https://ntp.niehs.nih.gov/iccvam/suppdocs/feddocs/oecd/oecd_gl423.pdf, accessed in 05/27/2016.) with adaptations. Almond oil was used in the extract dilution because of the absence of toxicity (13, 14), which makes it compatible to mammalian organisms. Diazepam (Hipolabor; lot no. AO011/11; validity: 10/13; concentration, 5 mg/ml; injectable medication) was administered at a dose of 1 mg/kg. The I.P. route was chosen due to need of absence of first-pass effect for EB827, so as supposedly a less reduction in bioavailability will occur as a result of drug metabolites, which could interfere with behavior changes. Also, according to Ministério da Ciência e Tecnologia guidelines, I.P. route is extremely commonly applied in rodents experimentation and can be used as it is justified (http://www.mct.gov.br/upd_blob/0238/238343.pdf, assessed in 05/30/2016).

Animals

Male Balb-C mice weighing 25-30 g, obtained from São Paulo University, were used. After arrival in the laboratory, animals were housed in groups of five in microisolators (38 x 32 x 16 cm) with controlled room temperature (22 ± 2°C), humidity (55–65%), and artificial lighting (12 h light/12 h dark cycle, lights on at 8:00 a.m.), and free access to Nuvilab® rodent chow (Nuvital Company, São Paulo, Brazil) and filtered water. The experiments began one week after the mice arrived. All the experiments done with mice were subjected to Ethic Committee (CEP/ICS/UNIP 025/08). The experimental delineation proposed in the present study is in

accordance with the 3R's principle proposed as long as 50 years ago (15; www.nc3rs.org.uk, accessed in 05/27/2016), particularly the "reduction" proposition which in the

present study is represented by the use of the reduced number of animals to prospect LD50 tendency, NLD and behavioral phenotype changes analyses described as the subsequent observation of locomotion and anxiety.

Parameters related to the refinement of evaluating general activity

General activity and 26 other parameters were assessed (16), with modifications (17-20). The evaluation was made by giving scores for each of the parameters.

Locomotion and behavior alterations signs

Open field

The open field (OF) was used to assess the influence of the extract over locomotion and emotionality. The apparatus was built in accordance to descriptions done elsewhere (21) and was adapted to fit mice experimentation, as described elsewhere (18). Locomotion frequency, rearing frequency, immobility time, grooming and the number of fecal boli were assessed. Experiments were conducted according to directions described elsewhere (17-20).

Experimental design

A two-stage experiment was conducted in order to access tendency of lethal dose 50 (LD50) and non-lethal dose (NLD; figure 1). Based on the OECD guideline 423 (with adaptations as described elsewhere (17-20), LD50 tendency and NLD were accessed by using a reduced number of animals (n=3 per dose), which was in accordance to the principles of the 3Rs (replacement, reduction and refinement), here proposing a reduction in the number of animals to the initial prospection of its influence over behavior parameters, as well as refinement of the assay, diminishing animal suffering. Animals were observed for lethality and for alterations in behavior in five sessions distributed by the first three hours of experiment After that period animals were observed each other day for the following 14 days.

Statistical analysis

Parameters observed in the general behavioral changes prospection were based on scores ranked from zero to four, so analysis of variance by ranks Kruskal-Wallis followed by Dunn post test (22) was applied. In order to organize statistical analysis of non parametric data in relation to the five sessions (time), the scores of each group were summed and formed a new group to be ranked, as a previous mathematical treatment. Micturition and defecation were analyzed by one-way ANOVA followed by Tukey's post-test. Analyses related to open field apparatus parameters were done using two-way repeated measure ANOVA followed by Bonferroni's post-test. All analysis were run under 0.05 significance level (α=0.05). Statistical procedures were conducted by the software Prism 5.0® (GraphPad Software 2010) and the LD50 tendency curve was obtained using Finney's probit analysis.

Extract fractionation and isolation of compounds

Fractionation and isolation were made based on previous reports (23). Organic extract EB827 (5g) was solubilized in a mixture of methanol (10 mL) chloroform (3 mL) and water (8 mL). The solubilized extract was transferred to a glass column (2.5 cm intern diameter, 90 cm length). Then, 50 mL of chloroform were added to the column, and as the chloroform shows a higher density when compared to water, it passes through the polar phase in an intimate contact eluting low polarity substances as descending through the sample. The procedure was again repeated. The chloroform extract was air-evaporated, resulting in a dark brown chloroform residue ($RCHCl_3$) (1.53 g; 30.6% yield). Organic solvent that remained in the aqueous phase was evaporated. The aqueous phase was subjected to a butanol partition, resulting in the production of a brown butanolic residue (RBuOH) (2.56 g; 51.2% yield). The aqueous phase was lyophilized (RH_2O) (0.91 g; 18.2% yield). $RCHCl_3$ was subjected to Sephadex LH20 column chromatography (CC) (1 cm intern diameter, 70 cm length) with 100 mL of hexane, 70 mL dichloromethane and 50 mL methanol as eluents, resulting in FrHEX (462.3 mg; 30.2% yield), FrDCM (105.5 mg; 6.9% yield) and FrMeOH (972 mg; 63.5% yield) fractions, respectively. After that, FrHEX was then submitted to fractionation by CC using silica gel (60-200 um particle size) and was eluted with mixtures composed of hexane, ethyl acetate and methanol, according to increasing polarity. From these procedures, 19 fractions were obtained from FrHEX. All fractions were combined according to analytical thin layer chromatographic (TLC) similarities after visualization with 25% sulfuric acid followed by heating. preparative TLC was used in the purification of some fractions from FrHEX, resulting in 5.3 mg of friedelin (**1**), obtained from fractions 5-6, identified by H^1 and C^{13} nuclear magnetic resonance spectra, or NMR, spectra (24, 25), 11.4 mg of epifriedelanol (**2**), obtained from fractions 3-11, identified H^1 and C^{13} NMR spectra (24), 18 mg of sitosterol (**3**) and stigmasterol (**4**), obtained as a mixture from fractions 3-11 (figure 2), identified H^1 and C^{13} NMR spectra (26). A mixture of hexane and ethyl acetate (9:1) was used in the TLC purification of compounds.

Results

General behavioral changes evaluation

LD50 tendency calculated for EB827 was 228.25 (522.07–102.73) mg/mL, and NLD was 78.1 mg/mL. In the first stage of the experiment, three mice were used to prospect the alterations in behavioral phenotype caused by EB827 using general activity plus 26 parameters

related to general behavior changes. EB827 administration has significantly diminished general activity (Figure 3A) in mice ($H\sim\chi^2_{0.05,(7)}$ = 18.22; p<0.01) after administration of doses 1,250 mg/kg, 625.0 mg/kg and 312.5 mg/kg. Sensorial system parameters as tail

squeeze (Figure 3B) ($H\sim\chi^2_{0.05,(7)}$ = 22.92; p<0.001) showed diminish in response after administration of doses 312.5 mg/kg, 625.0 mg/kg and 2,500 mg/kg, while response to touch (Figure 3C) ($H\sim\chi^2_{0.05,(7)}$ = 21.92; p<0.01) were diminished after administration of doses 2,500 mg/kg down to 625.0 mg/kg. Recovery could be observed after administration of lower doses, in both cases, when compared to vehicle control. Corneal reflex (Figure 3E) ($H\sim\chi^2_{0.05,(7)}$ = 24.03; p<0.001) diminish was observed in mice that received doses of 2, 500 mg/kg down to 625.0 mg/kg, followed by recovery at lower doses.

Motor system parameters were also altered. Body tone (Figure 3F) ($H\sim\chi^2_{0.05,(7)}$ = 15.42; p<0.05) has diminished after administration of dose 2,500 mg/kg, but no differences in relation to vehicle control were observed for the other doses. Grip reflex (Figure 3G) ($H\sim\chi^2_{0.05,(7)}$ = 19.14; p<0.01) was also reduced after administration of doses 2,500 mg/kg and 1,250 mg/kg, but recover occurred after administration of the other doses. Hindquarter fall (Figure 3H) ($H\sim\chi^2_{0.05,(7)}$ = 29.90; p<0.001) was reduced in mice that received dose of 2,500 mg/kg, but not observed in the other doses.

Alterations were also shown in parameters related to autonomous nervous system (ANS), as defecation (Figure 3D) ($F_{(6,34)}$ = 6.070; χ^2= 0.5654;p<0.01), that has diminished in mice receiving any dose of EB827. Lastly, breath (Figure 3I) ($H\sim\chi^2_{0.05,(7)}$ = 34.00; p<0.001) decreased in mice that received any dose of the extract. The other parameters did not show significant alterations in relation to vehicle control.

In the second stage of experiment,four groups were tested:naïve and control groups, NLD of EB827 and diazepam, which was used as standard drug (figure 4). The general activity (figure 4A) ($H\sim\chi^2_{0.05,(4)}$ = 14.90; p<0.01) and the response to touch (figure 4B) ($H\sim\chi^2_{0.05,(4)}$ = 11.62; p<0.01) were decreased in mice that received EB827 compared to naïve and vehicle controls. Tail squeeze (figure 4C) ($H\sim\chi^2_{0.05,(4)}$ = 13.17; p<0.01) was diminished in mice that received diazepam. Defecation (figure 4D) ($F_{(3,19)}$ = 3.689; χ^2= 0.4089;p<0.05) was diminished in the group that received EB827. Finally, breath (figure 4E) ($H\sim\chi^2_{0.05,(4)}$ = 19.00; p<0.001) was also reduced in relation to both naïve and vehicle groups and to diazepam group. *Open field evaluation*

In the first stage of locomotion analysis (table 1), mice (n=3; n_{total}=21) were evaluated in OF apparatus. In the analysis of locomotion frequency, treatment accounted for 48.08% of the total variance ($F_{6,14}$=5.85; p<0.01). Time accounted for 5.71% of the total variance ($F_{4,56}$=5.76; p<0.0001), while the interaction between treatment and time accounted for 13.13% of the total variance (($F_{24,56}$=2.21; p<0.01). Locomotion frequency significantly has diminished after administration of doses 2,500 mg/kg down to dose 625.0 mg/kg of EB827, and partial recovery was also observed after administration of lower doses (312.5 mg/kg down to 78.1 mg/kg). Observations related to immobility time are in accordance to the locomotion results. Treatment accounted for 29.30% of the total variance ($F_{6,14}$=4.47; p<0.01), time accounted for 7.89% of total variance ($F_{4,56}$=4.20; p<0.01), interaction between treatment and time accounted for 21.22% of total variance ($F_{24,56}$=1.88; p<0.05). It is possible to observe a tendency of diminishing immobility time in a dose-dependent way, but the higher dose clearly highlight immobilization of the animal. Rearing frequency is influenced only by the interaction between treatment and time ($F_{24,56}$=1.85; p<0.05) and this condition accounted for 25.77% of the total variance. Both treatment and time alone did not account for variance (p>0.05). A slight increase in grooming could be observed after 120-125 min of administration of dose 0.3125 g/kg, where treatment accounted for 19.37% of the variance ($F_{4,14}$=3.33; p<0.05) and time accounted for 9.91% of the total variance ($F_{4,56}$=4.01; p<0.01). No significant differences were observed in defecation (p>0.05). In the second stage (table 2), it was observed that treatment has significantly diminished locomotion frequency ($F_{(3,28)}$=6.63; p<0.01; 15.62% of the total variance, after adjusting for matching), as well as time, that was extremely significant in diminishing locomotion frequency ($F_{(4,112)}$=17.34; p<0.001; 11.51% of the total variance, after adjusting for matching). It was observed that the interaction between time and treatment accounted for 30.25% of the total variance ($F_{(12,112)}$=15.18; p<0.001). In the first section of 10-15 min, animals that received EB827 have significantly reduced locomotion (p<0.001), in relation to control groups. A similar diminish in locomotion was still observed in the second session (30-35 min; p<0.05). Animals seem to recover locomotion one hour after having received EB827. Immobility time supports the findings related to locomotion. Both treatment ($F_{(3,28)}$=7.33; p<0.001; 17.39% of the total variance, after adjusting for matching) and time ($F_{(4,112)}$=18.53; p<0.01; 14.19% of the total variance, after adjusting for matching) have significantly increased immobility time, the interaction between treatment and time accounted for 12.80% of the total variance ($F_{(12,112)}$=9.49; p<0.001). EB827 administration induced immobilization in the first session (10-15 min; p<0.001), second session (30-35 min; p<0.05) and third session (p<0.01), when compared to naïve, vehicle controls and diazepam, respectively. Recovery of locomotion was observed after two hours from EB827 administration. Meanwhile, time was not considered significant in rearing

frequency, as was treatment ($F_{(3,28)}$=5.25; p<0.01; 21.48% of the total variance, after adjusting for matching). Interaction between time and treatment ($F_{(12,112)}$=3.94; p<0.001; 11.31% of the total variance). EB827 has diminished rearing frequency when compared to diazepam (p<0.001) and to naïve control (p<0.05), in the first and second sessions, respectively. Treatment was not considered significant for causing alterations in grooming (p>0.05), nonetheless, increase could be seen after the fifth session (180-185 min; p<0.05), if compared to diazepam. Finally, treatment was extremely significant in diminishing defecation ($F_{(3,28)}$=13.83; p<0.001; 21.26% of the total variance, after adjusting for matching) when compared to naïve control (p<0.001; first session) and diazepam (p<0.05; fourth and fifth sessions).

Compounds isolated from EB827

Triterpens friedelin and epifriedelanol and steroids sitosterol and stigmasterol were isolated from the non-polar fractions of EB827, the organic extract obtained from the stem of *Pentaclethra macroloba*. This is the first report on the occurrence of these compounds in *pracaxi*.

Additional remarks

Necropsy showed that all dead animals presented pale liver and opaque internal organs, as kidneys, intestines and abdominal cavity. A lack of hemorrhage was observed in the animals submitted to necropsy. Latency to death for animals receiving 2,500 mg/kg was after 360 min and before the sunrise of day 2. Dead animals that received lower doses starting from 1,250 mg/kg had their latency to death overnight from day 1 to day 2.

Discussion

The value obtained in the calculation of the LD50 tendency for EB827 indicates that it can be considered as toxic (27). The first stage of the experiment gave us a general view of the behavioral phenotype influence over mice that received EB827. General activity diminish was accompanied of a diminish in response to touch, tail squeeze, defecation, cyanosis and breath parameters in a dose-dependent way. It was observed that the administration of NLD EB827 reduced the general activity, as well as response to touch, defecation and breath when compared to both naïve and vehicle controls. These observations are in accordance with results obtained in the first stage of experiment. Although it is premature to determine if EB827 caused depression in central nervous system, or even if the extract has provoked any alterations in autonomous nervous system, as in adrenergic or gabaergic systems, the introduction of diazepam as a drug reference, in the second stage of experiment, may support further pharmacological prospections on the EB827 activity. EB827 impaired general activity and defecation, and although diazepam did not show statistical differences related to general activity and to defecation, it is possible to observe a downward bias in the group receiving diazepam, when compared to naïve and control groups. At this point, it is important to establish if both general activity and defecation parameters are being equally influenced by EB827 and diazepam, and further behavioral analysis shall be done to support this theory. According to the results obtained in OP during the first stage of experiment, it was observed that EB827 has impaired mice locomotion, in a dose-dependent way, and that recovery was not observed before three hours from extract administration, but only after this period. Immobility time was recovered after the third hour, when animals started developing some kind of movement on OP. The first observations related to behavioral phenotype in OF indicate that EB827 caused some kind of depressive-like behavior. Observations made in the second stage of experiments showed that non-lethal dose caused impairment in locomotion, in OF, and recovery happened after 1 h from EB827 administration. Immobility time has been also observed after I.P. administration of EB827, supporting first locomotion findings. Locomotor alterations that occurred in both first and second stages of OF experiment suggest that the dose-response related loss of locomotion, as well as the recovery of locomotion after a significant immobility period. As immobility could have been reverted to locomotion depending on dose and time, a suggestion of a reversible bond to dopaminergic sites may be considered (28), yet to be confirmed. It is clear that more detailed neuropharmacological assays shall be done, in order to specify how EB827 influences behavioral phenotype. Although some propositions are speculative so far, the method proposed in the present work supports further pharmacological strategies to propose new experiments related to the pharmacological activity of EB827 over TMA's. Information based on the necropsied dead mice supports better the behavioral phenotype and the cause of mice death. During necropsy, pale livers and opaque internal organs were observed. Maybe some component of the extract could have led to a chemical liver failure – which would explain animal death - occurring concomitantly to a systemic inflammation (29), which could explain the alterations observed in general activity, tail squeeze and response to touch. Liver failure can be described as the sudden loss of hepatic function in a person (animal) without preexisting liver diseases, and concerning chemical induced liver failure, acetaminophen is the responsible for 46% of the cases, in the United States (30). In Brazil, the inadvertent use of some medicinal plants (exotic or native) may lead to liver failure or even other kinds of liver diseases as hepatitis. Some of the plants are *cavalinha* (*Teucrium chamaedrys*),

chaparral (*Larrea tridentate*), *efedra* (*Ma huang*), *erva-de-são-cristóvão* (*Cimicifuga racemosa*), *kava-kava* (*Piper methysticum*), *poejo* (*Hedeoma pulegoides* and *Mentha pulegium*), *cardo-do-visco* (*Atractylis gummifera*), *cassia-amarela* (*Cassia siamea*), *sacaca* (*Croton cajucara*), *unha-de-gato* (*Uncaria tomentosa*), *mãe-boa* (*Cissampelos fasciculata*), *espinheira-santa* (*Maytenus ilicifolia*), *confrei* (*Symphytum officinale*) and *maria-mole* (*Senecio* sp.). Toxicity caused by the incorrect use of plants led to 1.728 cases of intoxication caused by plants, in 2002 (31).

Friedelin, epifriedelanol, sitosterol and stigmasterol were isolated from EB827, although the alterations in behavior here reported were not proved to be related to any of those. Friedelin is reported to be insecticidal against *Musca domestica* and *Aedes albopictus* (32) and cytotoxic to breast, cervical, ovarian and blood human cancer cell lines (33). Epifriedelanol was reported to inhibit on adriamycin-induced cellular senescence in human fibroblasts, which could be used in supplements or cosmetics that modulate tissue aging or aging-associated diseases (35). Previously, triterpen saponins macrolobin-A and –B were isolated from *P. macroloba* (4), as well as two monodesmoside saponins (6) and triterpen saponins (7). Friedelin, epifriedelanol, sitosterol and stigmasterol are being reported in *P. macroloba* for the first time.

Conclusions

The administration of EB827 to mice has altered behavioral phenotype, specifically diminishing general activity, defecation, response to touch, tail squeeze, auricular reflex, piloerection and breath. Also, the plant extract may have caused death due to liver failure. Friedelin, epifriedelanol, sitosterol and stigmasterol is being reported to occur in *P. macroloba* for the first time.

Acknowledgements

Authors want to thank Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP for Grant #2008/58706-8.

Authors' contributions

SAF and AMMS contributed to plant collection, herbarium confection, and the laboratory work. IECD contributed to the chromatographic analysis and molecule identification. MMB and IBS designed the study, supervised the laboratory work, wrote the manuscript, performed the laboratory work, and critically read the manuscript. All of the authors read the final manuscript and approved the submission.

References

1- Revilla J. Plantas úteis da Bacia Amazônica. Manaus, INPA e SEBRAE, Manaus. 2002; v.1-2, 858p.

2- Mccutchen BF, Abad AR. (Pioneer Hi-Bred International, Inc., USA, E.I. Dupont De Nemours and Company). Combined uses of Bt insecticidal protein and insecticidal lipase for enhancing insect resistance in plants U.S. Pat. Appl. Publ. (2005), 59pp., which. CODEN: USXX COUS 2005 188439A 120050825 Patent written in English. Application: US2005-6189420050218. Priority: US 2004-546533 20040220, US2004-546845 20040223. CAN 143:243460AN2005:904416 CAPLUS (Copyright (C) 2009 AC Son SciFinder (R)).

3- Chen R, Schubert K R, Czapla TH. (Board of Regents for the University of Oklahoma, USA). Trypsin inhibitors with insecticidal properties obtained from *Pentaclethra macroloba*. U.S. (1999), 20 pp., Cont.-in-part of U.S.5,672,680. CODEN: USXXAMUS5981722A19991109 Patent written in English. Application: US97-93897519970926. Priority: US95-560727 1995 1120. CAN 131:319318AN 1999: 719023 CAPLUS (Copyright (C) 2009 AC Son SciFinder(R)).

4- Silva JO, Fernandes RS, Ticli FK, Oliveira CZ, Mazzi MV, Franco JJ, Giuliatti S, Pereira PS, Soares AM, Sampaio SV. Triterpenoid saponins, new metalloprotease snake venom inhibitors isolated from *Pentaclethra macroloba*. Toxicon 2007;50:283-291.

5- Silva JO, Coppede JS, Fernandes VC, Sant'ana CD, Ticli FK, Mazzi MV, Giglio JR, Pereira PS, Soares AM, Sampaio SV. Antihemorrhagic, antinucleolytic and other antiophidian properties of the aqueous extract from *Pentaclethra macroloba*. J Ethnopharmacol 2005;100:145-152.

6- Viana FA, Pouliquen YB, Andrade-Neto M, Santiago GM, Pessoa OD, Rodrigues-Filho E, Braz-Filho R. Complete 1H and 13C NMR assignments for two new monodesmoside saponins from *Pentaclethra macroloba* (Willd.) Kuntze. Magn Reson Chem 2004;42:695-699.

7- Santiago GMP, Viana FA, Pessoa ODL, Santos RP, Pouliquen YBM, Arriaga AMC, Andrade-Neto M, Braz-Filho R. Avaliação da Atividade Larvicida de Saponinas Triterpênicas Isoladas de *Pentaclethra macroloba* (Willd.) Kuntze (Fabaceae) e *Cordia piauhiensis* Fresen (Boraginaceae) sobre *Aedes aegypti*. Rev Bras Farmacogn 2005;15:187-190.

8- Okwu DE, Aluwuo CJ. Studies on the phytochemical composition and fermentation of the seeds of African oil bean tree (*Pentaclethra macrophylla* Benth.) Intern J Chem Sci 2008;6:773-788.

9- Suffredini IB, Varella AD, Younes RN. Cytotoxic molecules from natural sources. Tapping the Brazilian biodiversity. Anti-Cancer Ag Med Chem 2006a;6:367-375.

10- Suffredini IB, Paciencia MLB, Varella AD, Younes RN. *In vitro* prostate cancer cell growth inhibition by Brazilian plant extracts. Pharmazie 2006b;61:722-724.

11- Teodorov E, Camarini R, Bernardi MM, Felicio LF. Treatment with steroid hormones and morphine alters general activity, sexual behavior, and opioid gene expression in female rats. Life Sci 2014;104:47-54.

12- Younes RN, Varella AD, Suffredini IB. Discovery of new antitumoral and antibacterial drugs from Brazilian plant extracts using high throughput screening. Clinics 2007;62:763-768.

13- Peleva E, Mourad S, Citra D, Daniel SJ. Assessment of the ototoxicity of almond oil in a chinchilla animal model. Laryngoscopy 2011;121:2661-2664.

14- Saidi H, Shojaie S. Effect of sweet almond oil on survival rate and plasma cholinesterase activity of aluminum phosphide-intoxicated rats. Hum Exper Toxicol 2012;31:518-522.

15- Russell WMS, Burch RL. The principles of humane Experimental Technique. London, Methuen 1959.16- Brito AS. Manual de ensaios de toxicologia. Campinas: Editora da Unicamp. 1994:122p.

17- Estork DM, Gusmão DF, Paciencia MLB, Díaz IEC, Varella AD, Younes RN, Reis LFL, Montero EFS, Bernardi MM, Suffredini IB. First chemical and toxicological evaluation of *Casinga-cheirosa* in Balb-c male mice. Molecules 2014;19:3973-3987.

18- Estork DM, Gusmão DF, Paciencia MLB, Frana SA, Díaz IEC, Varella AD, Younes RN, Reis LFL, Montero EFS, Bernardi MM, Suffredini IB. *Casinga-cheirosa* organic extract impairment over Balb-c male mice behavioral phenotype. Rev Bras Farmacogn 2016;26:216-224.

19- Gusmão DF, Estork DM, Paciencia MLB, Diaz IEC, Frana SA, Rodrigues PA, Suffredini IB, Varella AD, Younes RN, Reis LFL, Montero EFS, Bernardi MM. Preliminary evaluation of the acute toxicity related to *Abarema auriculata* to mice and investigation of cytotoxicity of isolated flavonones. Pharmacologyonline (Salerno) 2013a ;1:113-127.

20- Gusmão DF, Estork DM, Paciencia MLB, Díaz IEC, Suffredini IB, Varella AD, Younes RN, Reis LFL, Montero EFS, Bernardi MM. Influence of the intraperitoneal administration of antitumor *Abarema auriculata* extract on mice behavior. Rev Bras Farmacogn 2013b;23:903-912.

21- Broadhurst PL. Experiments in psychogenetics: application of biometrical genetics to the inheritance of behavior. In H.J. Eysenck, ed. *Experiments in personality* Vol 1, London, Routledge & Kegan Paul. 1960;1-256.

22- Zar JH. Biostatistical Analysis. 4th Ed. New Jersey, Prantice-Hall Inc 1999.

23- Castilho AL, Silva JPC, Saraceni CHC, Díaz IEC, Paciencia MLB, Varella AD, Suffredini IB. *In vitro* activity of Amazon Plant Extracts against *Enterococcus faecalis* Braz J Microbiol 2014;45:769-779.

24- Li YZ, Li ZL, Yin SL, Shi G, Liu MS, Jung YK, Hua HM. Triterpenoids from *Calophyllum inophyllum* and their Growth Inhibitory Effects on Human Leukemia HL-60 Cells, Fitoterapia 2010;81:586-589.

25- Omeje EO, Khan MP, Osadebe PO, Tewari D, Khan MF, Maurya R, Chattopadhyay N. Analysis of contituents of the eastern Nigeria mistletoe, *Loranthus micranthus* Linn revealed presence of new classes of osteogenic compounds. J Ethnopharmacol 2014;151:643-651.

26- Holland HL, Diakow PRP, Taylor GJ. ^{13}C Nuclear Magnetic Resonance Spectra of Some C-19-Hydroxy, C-5,6-epoxy, C-24-ethyl and C-19-norsteroids. Can J Chem 1978;56:3121–127.

27- Barros SBM, Davino SC. Avaliação da toxicidade. In: Oga S, Camargo MMA, Batistuzzo JAO. Ed. Fundamentos de toxicologia. 3rd Ed. São Paulo: Atheneu. 2008;59-70.

28- Stahl SM. Role of α1 adrenergic antagonism in the mechanism of action of iloperidone: reducing extrapyramidal symptoms. CNS Spectr 2013;18:285-8.

29- Marques PE, Amaral SS, Pires DA, Nogueira LL, Soriani FM, Lima BHF, Lopes GAO, Russo RC, Ávila TV, Melgaço JG, Oliveira AG, Pinto MA, Lima CX, Paula AM, Cara DC, Leite MF, Teixeira MM, Menezes GB. Chemokines and mitochondrial products activate neutrophils to amplify organ injury during mouse acute liver failure. Hepatol 2012;56:1971-1982.

30- Lee WM, Squires Jr RH, Nyberg SL, Doo E, Hoofnagle JH. Acute liver failure, summary of a workshop. Hepatol 2008;47:1401-1415.

31- Silveira PF, Bandeira MAM, Arrais PSD. Farmacovigilância e reações adversas às plantas medicinais e fitoterápicos: uma realidade. Rev Bras Farmacogn 2008;18:618-626.

32- Huang JG, Zhou LJ, Xu HH, Li WO. Insecticidal and cytotoxic activities of extracts of *Cacalia tangutica* and its two active ingredients against *Musca domestica* and *Aedes albopictus*. J Econ Entom 2009;102:1444-1447.

33- Ee GC, Lim CK, Rahmat A, Lee HL. Cytotoxic activities of chemical constituents from *Mesua daphnifolia*. Trop Biomed 2005;22: 99-102.

34- Yang HH, Son JK, Jung B, Zheng M, Kim JR. Epifriedelanol from the root bark of *Ulmus davidiana* inhibits cellular senescence in human primary cells. Planta Med 2011;77:441-449.

Table 1. Locomotion influence observed in male mice after receiving the administration of EB827, obtained from the stem of *Pentaclethra macroloba*, and exposed to open field apparatus, in the first stage of experimentation. Two-way repeated measures ANOVA was used, as Bonferroni's post test, considering significance if $p<0.05$.

Open field evaluation stage 1							
Locomotion frequency							
	control	2,500	1,250	625	312.5	156.3	78.1
15-20	116.30(42.71)	13.67(10.79)	10.67(5.03)	18.00(12.77)	16.00(13.45)	19.00(11.14)	11.00(3.61)
30-35	98.67(87.96)	18.67(21.22)	6.00(2.0)	24.00(33.78)	16.33(20.03)	71.33(63.01)	67.33(9.29)
60-65	91.67(69.62)	19.67(12.66)	26.33(30.62)	46.00(22.07)	28.00(21.38)	128.30(87.49)	72.67(60.10)
120-125	167.70(79.26)	6.67(4.73)***	11.67(1.53)***	35.00(18.52)**	66.00(33.60)***	126.00(74.18)	74.00(38.94)
180-185	178.70(61.34)	2.67(2.31)****	6.33(4.93)****	8.00(10.58)****	41.33(25.89)	54.00(23.81)*	87.00(59.57)
Rearing frequency							
	control	2,500	1,250	625	312.5	156.3	78.1
15-20	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.67(0.58)	0.00(0.00)
30-35	3.33(5.77)	0.00(0.00)	0.00(0.00)	0.33(0.58)	0.33(0.58)	2.00(2.00)	0.00(0.00)
60-65	2.33(4.04)	0.00(0.00)	0.67(1.16)	1.00(1.73)	0.00(0.00)	6.33(5.69)	10.33(17.90)
120-125	7.33(7.09)	0.00(0.00)	0.00(0.00)	0.00(0.00)	1.00(1.73)	13.67(16.50)	4.00(6.93)
180-185	20.00(20.95)	0.00(0.00)**	0.00(0.00)**	0.00(0.00)**	0.00(0.00)**	1.33(1.53)**	0.33(0.58)***
Defecation							
	control	2,500	1,250	625	312.5	156.3	78.1
15-20	0.00(0.00)	0.67(1.16)	0.00(0.00)	0.00(0.00)	0.00(0.00)	1.00(1.00)	0.00(0.00)
30-35	0.67(0.58)	0.33(0.58)	0.00(0.00)	0.00(0.00)	0.33(0.58)	0.00(0.00)	0.33(0.58)
60-65	1.00(1.00)	0.00(0.00)	0.00(0.00)	1.00(1.73)	0.00(0.00)	0.00(0.00)	0.67(1.16)
120-125	1.00(1.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.33(0.58)
180-185	0.67(1.16)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	1.3(1.53)
Grooming							
	control	2,500	1,250	625	312.5	156.3	78.1
15-20	0.33(0.58)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.33(0.58)	0.00(0.00)	0.00(0.00)
30-35	2.67(3.79)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.33(0.58)	0.33(0.58)	2.00(2.00)
60-65	1.00(1.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	1.67(1.52)	5.00(4.36)	0.33(0.58)

120-125	1.33(0.58)	0.00(0.00)	0.33(0.58)	0.67(0.58)	8.33(3.79)*	4.00(6.08)	1.00(1.00)
180-185	3.00(2.65)	0.00(0.00)	0.00(0.00)	0.00(0.00)	6.33(8.39)	4.00(2.00)	4.00(5.29)

immobility time

	control	2,500	1,250	625	312.5	156.3	78.1
15-20	82.33(76.51)	178.00(64.09)	237.30(28.94)	233.30(44.06)	248.00(6.08)*	258.70(22.19)*	263.70(13.43)*
30-35	182.70(77.14)	216.30(45.24)	285.70(9.29)	253.00(51.12)	216.70(111.64)	169.30(113.18)	180.70(14.19)
60-65	176.00(38.31)	239.30(17.01)	231.30(29.94)	199.00(41.39)	250.70(27.97)	122.70(93.39)	203.70(77.15)
120-125	42.67(54.46)***	206.70(45.94)	266.70(22.03)	161.70(114.69)	155.70(57.85)	115.00(118.77)	141.70(72.43)
180-185	91.33(67.35)*	291.30(15.01)	260.70(32.58)	221.70(82.40)	216.00(40.34)	212.00(39.00)	125.30(76.81)

Table 2. Locomotion influence after the administration of the organic extract (EB827) obtained from the stem of *Pentaclethra macroloba* to male mice, in the second stage of the experiment. Two-way repeated measures ANOVA and Bonferroni's post test were used, considering significant if p<0.05.

Open field evaluation stage 2

Locomotion frequency

	NC	VC	EB827	DIA
15-20	129.50(29.20)	174.30(81.63)	21.00(13.00)****	236.20(92.13)
30-35	129.50(15.26)	58.50(45.51)	9.40(12.09)***	82.80(66.66)
60-65	124.20(17.66)	83.50(50.77)	50.20(40.33)	74.80(41.61)
120-125	124.30(19.11)	82.00(67.79)	111.00(41.30)	90.70(48.64)
180-185	127.20(22.00)	83.17(39.80)	81.60(38.63)	53.40(34.99)

Rearing frequency

	NC	VC	EB827	DIA
15-20	22.33(17.10)	5.33(6.38)	0.00(0.00)****	31.10(32.03)*
30-35	25.17(18.02)	2.50(4.18)	0.00(0.00)*	12.80(21.25)
60-65	20.33(11.34)	10.17(12.35)	0.30(0.68)	8.50(12.49)
120-125	18.33(8.76)	19.33(24.26)	3.90(3.45)	14.00(11.47)
180-185	23.33(8.31)	21.83(15.36)	4.20(3.23)	9.10(8.44)

Defecation				
	NC	VC	EB827	DIA
15-20	1.67(1.03)	1.00(0.89)	0.10(0.32)***	0.90(0.99)
30-35	0.83(0.41)	1.00(0.89)	0.00(0.00)	0.20(0.42)
60-65	0.33(0.52)	1.00(1.10)	0.10(0.32)	0.40(0.70)
120-125	1.00(0.63)	0.83(0.75)	0.10(0.32)*	1.10(0.88)
180-185	0.50(0.55)	0.83(0.98)	0.00(0.00)*	1.10(0.88)

Grooming				
	NC	VC	EB827	DIA
15-20	21.83(13.75)	39.33(50.14)	0.40(0.84)	5.70(5.91)
30-35	19.50(7.26)	13.67(15.57)	1.90(5.04)	10.30(14.99)
60-65	19.17(12.66)	10.67(8.09)	10.20(16.66)	20.70(50.83)
120-125	19.50(9.40)	43.00(56.46)	35.20(29.20)	22.60(23.81)
180-185	24.83(10.94)	34.83(30.60)	51.40(56.42)	8.90(10.93)*

Immobility time				
	NC	VC	EB827	DIA
15-20	38.83(26.86)	64.67(44.53)	204.70(30.77)****	35.70(69.06)
30-35	48.17(25.21)	191.50(65.30)	255.00(39.71)****	166.50(94.85)*
60-65	38.33(16.60)	135.50(88.68)	144.30(67.48)*	165.60(77.14)*
120-125	40.67(21.42)	89.83996.39)	75.50(47.48)	105.90(69.34)
180-185	51.33(23.62)	51.33981.36)	59.90(40.30)	144.00(74.910

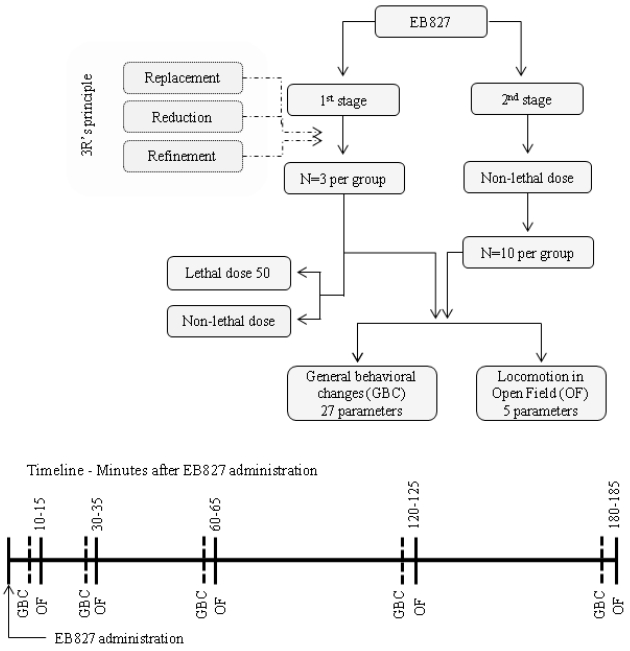

Figure 1. Experiment design used in the evaluation of behavioral changes in mice after I.P. administration of the extract made with stem of *Pentaclethra macroloba* EB827. GBC-general behavioral changes; OF-open field. Sessions given in minutes.

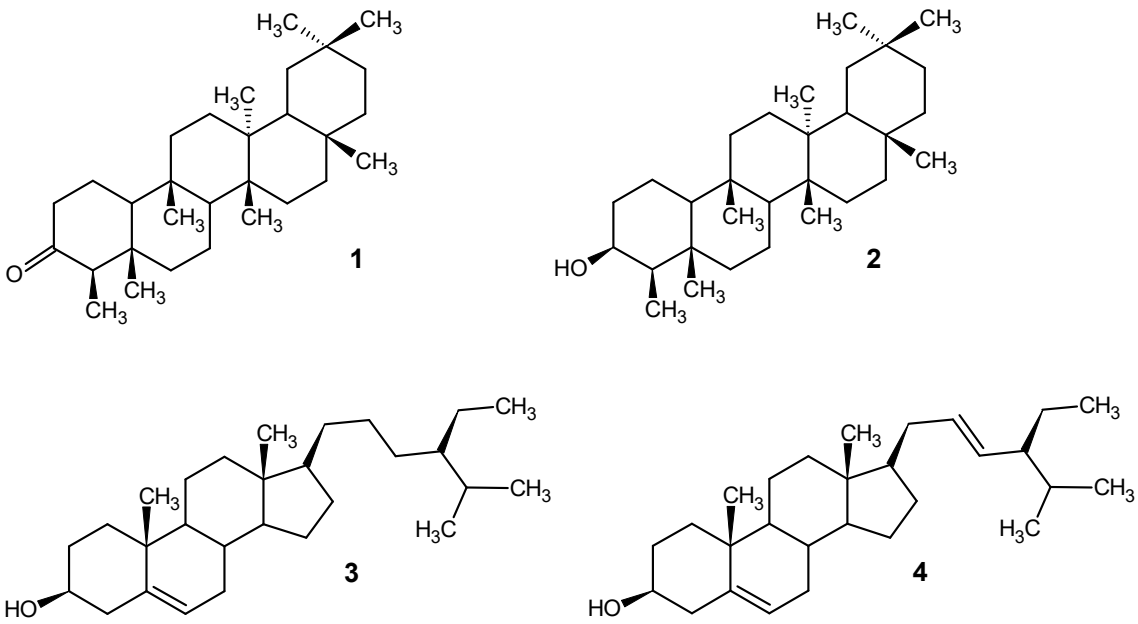

Figure 2. Triterpenes (friedelin **1** and epifriedelanol **2**) and steroids (sitosterol **3** and stigmasterol **4**) that were isolated and identified from the stem of *Pentaclethra macroloba*.

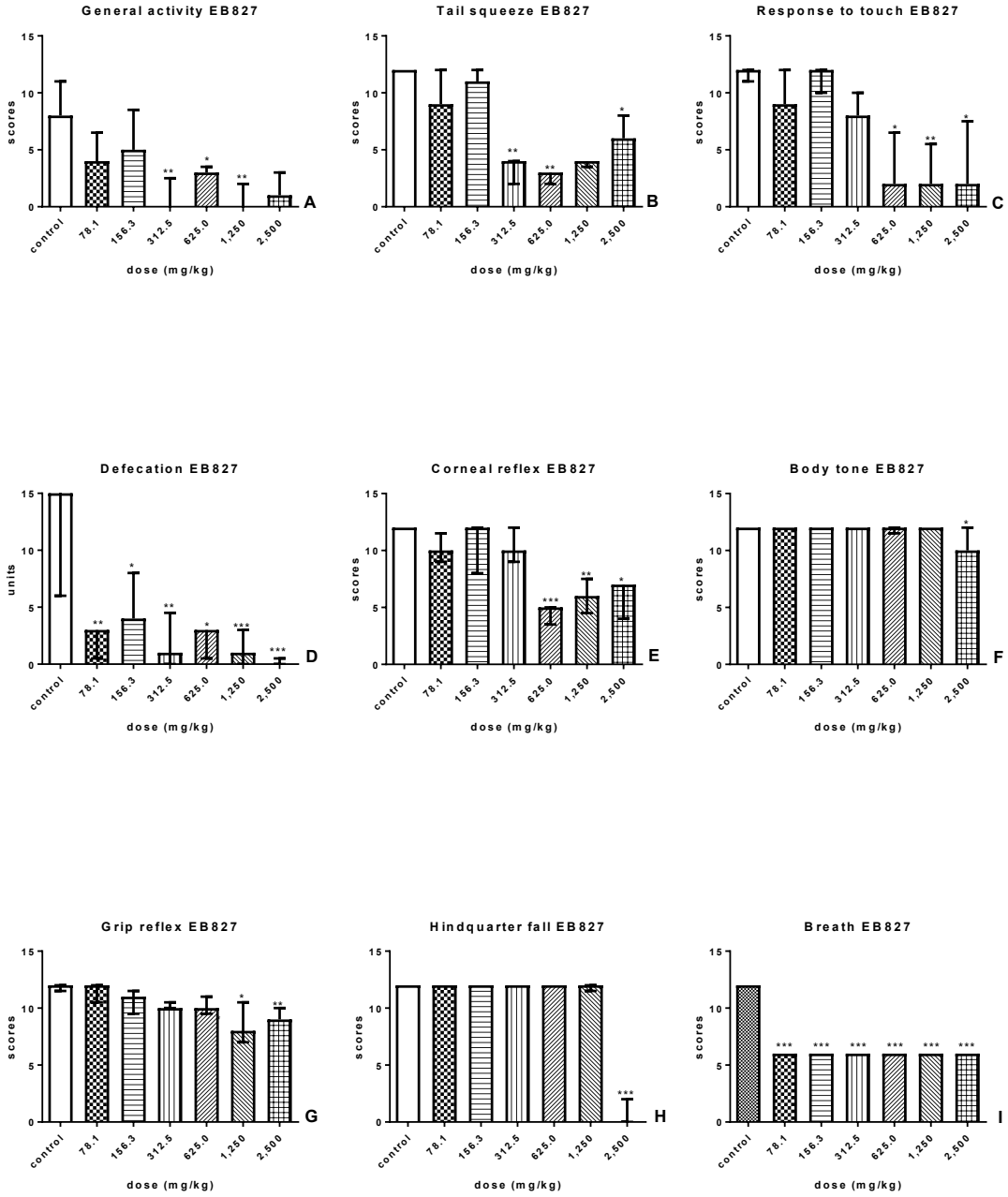

Figure 3. Effect over murine **(A).** General activity, **(B).** Tail squeeze, **(C).** Response to touch, **(D).** Defecation, **(E).** Corneal reflex, **(F).** Body tone, **(G).** Grip reflex, **(H).** Hindquarter fall and **(I).** breath after administration of organic extract EB827, obtained from *Pentaclethra macroloba*, in stage one of the experiment. Kruskall-Wallis statistics (n = 3; N_{total} = 21) followed by Dunn's post test were used for all the parameters but defecation, which was analyzed by one-way ANOVA followed by Tukey post test. Differences among medians/means were significant if $p < 0.05$.

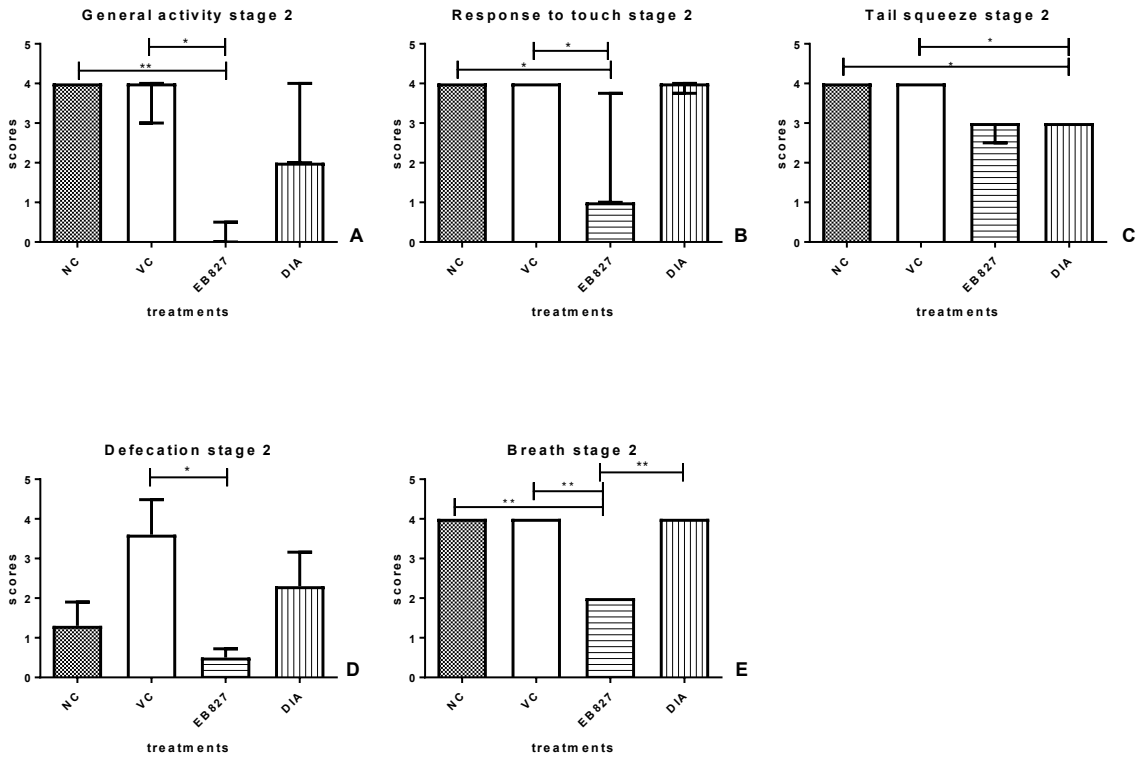

Figure 4. Effect on male mice Balb-c **(A).** General activity, **(B).** Response to touch, **(C).** Tail squeeze, **(D).** Defecation and **(E).** Breath after administration of the non-lethal dose of EB827, obtained from *Pentaclethra macroloba*, in stage two of the experiment. Kruskall-Wallis statistical analysis (n = 10; N_{total} = 40) followed by Dunn's multiple comparison tests were performed among medians, except for defection, which was analyzed by one-way ANOVA and Tukey's post-test, which were preformed among means. Significances were given if $p < 0.05$.