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TOXICITY OF *Ipomoea alba* L. (CONVOLVULACEAE)

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Summary

Objective: To evaluate the influence of plant extract (EB1493) obtained from *Ipomoea alba*, Convolvulaceae, over general activity and over toxicity of central nervous system, autonomous nervous system, sensorial and psychomotor systems in mice and in rats.

Methods: Acute toxicity assay was done with animals that received different doses of EB1493, intraperitonealy. For each dose, more than 25 parameters related to central and autonomous nervous systems, sensorial and psychomotor systems were evaluated, as well as LD50 was determined A multi-dose assay (MDTA) was also performed, in which the animals topically received EB1493 three times/day in the mouth mucosa, for 11 days, and were evaluated for the same parameters considered in the first assay at day 1, day 2, day 5 and day 11.

Results: LD50 is 2.19 g/kg, considered harmful. General activity, touch response, tail squeeze, hindquarter fall, body tone and grip reflex were significantly altered after administration of both higher doses in the first stage of the experiment, as well as ptosis, auricular and corneal reflexes and defecation were altered in the second stage of the experiment. Alterations in general activity, touch response, piloerection, micturition and defecation were observed in MDTA, in some sessions. Differences in body weigh were observed as well.

Conclusions: The first findings related to the toxicity profile of EB1493 were optimistic for have shown low toxicity. Further pharmacological, chemical and toxicological analyses are going to be made, in order to gather additional information on EB1493 antimicrobial potential.

Keywords: *Ipomoea alba*, drug toxicity, general activity, central nervous system, autonomous system

Introduction

Ipomoea alba L. (Convolvulaceae) is a plant used in ancient Mesoamerica to vulcanize rubber, sometimes also considered as weed in agricultural areas. Our team has implemented a bioprospection program (license CGen/MMA n° 012A/2008) aiming the identification of plant extracts active against human tumor cell lines and against pathogenic microorganisms, such as *Streptococcus mutans* and *S. sanguinis*.

Streptococci microorganisms are involved in the earlier stages of several oral diseases and have also been found in infective endocarditis (1) and in bacteremia (2) and *I. alba* was identified as an extremely successful antibacterial agent that defeated both *Strepotococci* (3) and *Enterococcus* faecalis (4) *in vitro* at concentrations as low as 40 µg/mL. Further studies related the extract activity on induced periodontitis, *in vivo* (5) were also assessed.

In Brazil, *I. alba* is known as "dama-da-noite" ('lady-of-the-night', in a free translation) or "boa-noite" ('good-night', in a free translation). In other countries, the plant is known as tropical white morning-glory, moonflower vine, moon vine and evening glory.

The plant is native to the tropical Americas, from Argentina to Florida (USA). Due to its beauty, it is used in gardening. The reports that relate the use of this plant in manufacturing rubber together with latex from other species (6) show that the presence of the sulfur-containing material may be involved in the rubber vulcanization.

Although the plant has a traditional use as rubber vulcanizer and has shown a significant antibacterial activity against *Streptococcus* sp., no toxicological reports are found. The acute and multi-dose toxicity and the evaluation of the general activity of plant extract obtained from *I. alba* (the so-called EB1493) were assessed (7) for the first time in the present work, both in mice and rats.

Methods

Plant collection and extract preparation

Plant was collected in the Brazilian Amazon rain forest, under Brazilian Government licenses for collecting and bioprospecting genetic resources in protected areas of Brazilian forests. The studied species was collected in the surrounds of Manaus city, state of Amazonas, in an area of confluence of Rio Negro and Rio Solimões, known as Janauari Lake. The voucher is deposited at UNIP Herbarium [*A.A.Oliveira, 4031* (UNIP)].

Aerial parts of *I. alba* was collected, dried in air-circulating stove (Fanem) at 40 °C and ground in a hammer-mill (Holmes). The ground material was placed in a glass percolator (Kontes) and a 24h-maceration proceeded with dichloromethane and methanol 1:1 (Merck). Solvents were evaporated under vacuum (Büchi) and were kept in freezer (Revco) until use. Extract was suspended in almond oil to be administered to animals during experiments (8).

The extract was suspended in almond oil and the following doses were I.P. administered: 5.0, 2.5 and 1.25 g/kg to be used in the acute toxicity test. Also, EB1493 was diluted in 10% tween 80/water to a concentration of 400 mg/mL, to be used in the multi-dose toxicity assay, being topically administered.

Animals

All the experiments done with mice and rats were subjected to UNIP Ethic Committee (CEP/ICS/UNIP 025/08 and 036/10). Male Balb-C mice (*Mus domesticus*) weighing 25-30 g, obtained from São Paulo University, were used. Male adult Wistar rats (*Rattus novergicus*), obtained from the same institution, weighing 210-320 g, were used. After arrival in the laboratory, animals were housed in groups of six in polypropylene cages (38 x 32 x 16 cm) with controlled room temperature (22 ± 2°C), humidity (65–70%), 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 animals arrived in for habituation to the new laboratorial conditions. Animals were divided into control group, test group and naïve group.

General activity – Acute toxicity

Acute toxicity was assessed (9), with modifications. Parameters related to the general activity, to the sensorial system (such as vocal tremor, irritability, auricular reflex, corneal reflex, tail squeeze, response to touch), to psychomotor system (contortion, hindquarter fall, surface-righting reflex, body tone and grip reflex), to central nervous system (as convulsions, ataxia, anesthesia, hypnosis, straube tail, tremor, stimulation and sedation) and to autonomous nervous system (lacrimation, breath, ptosis, piloerection, micturition, defecation, hypothermia and cyanosis) were assessed and a score from 0 to 4 was given for each parameter, except to micturition and defecation, which numbers of urination and boli were counted.

Experimental design for toxicity assay

Considering that *1)* extracts obtained from plants native to the Amazon rain forest are hard to obtain, due to the limited access to plant collection related to bioprospection purposes, and *2)* the modern guidelines for evaluation of the signs of toxicity tends to use a limited number of animals (7), we conducted a two-stage experiment in order to prospect the lethality, the general activity and the toxicity of EB1493 *in house*. In the first stage, the lethal dose 50% was obtained with a reduced number of animals and observations prospecting the toxicity and general activity were assessed. The second stage of the experiment was conducted using the non-lethal dose against groups of 18 animals each, when the accuracy of general activity and toxicity could be observed. Animals were sacrificed in a CO_2 gas chamber at the end of the experiment. If death occurred, a gross necropsy was performed on the animal.

Different doses of extract were administered, starting from the limit dose of 5.0 g/kg. Each dose was administered and if lethality appears in at least one among three mice, a lower dose, prepared to the half of the preceding one, was administered to a new group of three animals, and observations were again done. This procedure was repeated until no death was observed. Mice were individually observed in a glass cage for toxic reactions and/or lethality at 7-10, 25-30, 55-60, 115-120 and 175-180 min after administration or until dead; mice who survive were observed every 24 hours in the subsequent 14 days. Two parameters were obtained from the experiments conducted in the first stage: the lethal dose 50% and the non-lethal dose (NLD). NLD was used to test a large group of animals (n=10) in the second stage of the tests, when a group of naïve mice was introduced with the purpose of controlling the possible influence of intraperitoneal injection.

Multi-dose toxicity assay

The multi-dose toxicity assay (MDTA) was performed using rats that were induced periodontal disease (10,11) and received topical application three times a day during 11 days in order to evaluate this substances ability to reduce the development of periodontitis in rats. Groups of 18 animals, determined by previous studies, were divided into (G1; n=16) control group which received tween 80%dissolved to 10% in water; (G2; n=16) extract group which received EB1493 diluted to 400 mg/mL and (G3; n=16) Periogard® group, clorhexidine gluconate 0.12%. Substances were locally administered in the mandibular first molars of each animal in a volume of 0.3 mL. Animals were observed in a glass cage for toxic reactions and/or lethality at one day before animals were submitted to periodontitis induction and topical administration (session 1), one day after the induction and topical administration (session 2), after 5 days (session 3) and at the end of the experiment at day 11 before sacrificing (session 4). The same parameters related to the general activity, to the sensorial system, to psychomotor system, to central nervous system and to autonomous nervous system (such as lacrimation, breath, ptosis, piloerection, micturition, defecation, hypothermia and cyanosis) were accessed and a score from 0 to 4 was given for each parameter, except to micturition

and defecation, which was counted.

Statistical analysis

Most of the parameters observed in the general activity, acute toxicity and multi-dose toxicity were based on scores ranked from 0 to 4, except for micturition and defecations, which number of micturition and boli were counted. In order to organize statistical analysis of non parametric data, the scores of each group were summed in the first stage of the experiment and formed a new group to be ranked. So, analysis of variance by ranks Kruskal-Wallis followed by Dunn post test (12) was then applied. In the second stage of the experiment, the median was used in the Kruskal-Wallis ranking. Micturition and defecation were analyzed by ANOVA, followed by Bonferroni's post test. Body weight was analyzed by two-way ANOVA followed by Bonferroni post-test or by one-way ANOVA followed by Tukey's post test. All analysis were run under 0.05 significance level (á=0.05). Statistical procedures were conducted by the software Prism 5.0® (GraphPad Software 2010) and the LD50 curve was obtained using GraphPad Instat3.0® (GraphPad Software 2009).

Results

The lethal dose 50 to EB1493 I.P. administered is 2.19 g/kg. The toxicity provoked by the administration of different doses of EB1493 to groups of three mice each, in the stage one of experiments, was analyzed and the results are statistically described, as follows.

Figure 1 shows results related to the influence of the administration of EB1493 over general activity in the first stage of experiments. The administration of higher doses significantly influences the general activity ($H\sim\chi^2_{0.05,(3)}$ = 13.62; p<0.01 for dose 5.0 g/kg). General activity is recovered in relation to control group (p>0.05) when lower doses are administered. Fig. 1 also shows the influences of the administration of EB1493 over touch response ($H\sim\chi^2_{0.05,(3)}$ = 14.27; p<0.001 for dose 5.0 g/kg), tail squeeze ($H\sim\chi^2_{0.05,(3)}$ = 15.78; p<0.01 for dose 5.0 g/kg), hind-quarter fall ($H\sim\chi^2_{0.05,(3)}$ = 19.00; p<0.01 for doses 5.0 and 2.5 g/kg), body tone ($H\sim\chi^2_{0.05,(3)}$ = 18.63; p<0.01 for dose 5.0 g/kg), and grip reflex ($H\sim\chi^2_{0.05,(3)}$ = 12.87; p<0.01 for dose 5.0g/kg)

Figure 2 shows the influence of the administration of EB1493 over autonomous nervous system in the first stage of the experiment. Piloerection was observed only at dose of 1.25 g/kg ($H\sim\chi^2_{0.05,(3)}$ = 7.242; p<0.05), ptosis showed alterations in relation to control group at dose 5.0 g/kg ($H\sim\chi^2_{0.05,(3)}$ = 10.00; p<0.05), animals of the 5.0 g/kg group showed alterations in auricular reflex ($H\sim\chi^2_{0.05,(3)}$ = 11.41; p<0.01). Alterations in corneal reflex parameter were observed at dose of 5.0 and 2.5 g/kg ($H\sim\chi^2_{0.05,(3)}$ = 10.42; p<0.01 for the group that received 5.0 g/kg and p<0.05 for the group that received dose of 2.5 g/kg). At last, alterations in defecation were observed at doses 5.0, 2.5 and 1.25 g/kg ($F_{(3,16)}$=8.147; p<0.01; X^2=0.6044).

Figure 3 shows the alterations observed after administration of 10% of the non-lethal dose of EB1493 in the second stage of experiments. Only four out of all the parameters observed were altered. General activity was altered in the experimental group, in relation to naïve control group and to control group ($H\sim\chi^2_{0.01,(10)}$ = 13.64; p<0.01), as well as breath ($H\sim\chi^2_{0.05,(3)}$ = 14.00; p<0.01); defecation was altered in relation to naïve control group ($F_{(2,12)}$=4.192; p<0.05; X^2=0.4113, as well as piloerection ($H\sim\chi^2_{0.05,(3)}$ = 12.64; p<0.01).

Figures 4 and 5 represent the results related to the MDTA statistics studies that were performed in a two-step pattern: first step was the comparison of results obtained in the four sessions for the same group (control, Periogard® or EB1493) and to evaluate the occurrence of differences among all groups; the second step was to compare control, Periogard® and EB1493 groups in each one of the four sessions, separately. Only four out of 27 parameters were altered and are here represented. So, figure 4 shows alterations related to general activity in the MDTA. Firstly, alterations were significantly observed among sessions, for all three groups. Control group showed significant differences

between sessions 1 and 2, sessions 1 and 3 and sessions 1 and 4, respectively ($H\sim\chi^2_{0.001,(16)}$ = 19.11; p<0.01; p<0.05 and p<0.001). Similar alterations occurred in test group EB1493 between sessions 1 and 2, sessions 1 and 3 and sessions 1 and 4, respectively ($H\sim\chi^2_{0.001,(16)}$ = 21.53; p<0.01; p<0.05 and p<0.001). Differences occurred in Periogard® group between sessions 1 and 2, sessions 1 and 3 and sessions 1 and 4, respectively ($H\sim\chi^2_{0.001,(16)}$ = 15.62; p<0.01; p<0.01 and p<0.05). Considering the differences of the three groups in each of the sessions, no significant differences were observed. Differences observed in touch response in MDTA is also related in figure 4. Firstly, control group did not show significant differences among sessions (p>0.05). Test group showed differences between sessions 1 and 3 and between sessions 3 and 4 ($H\sim\chi^2_{0.01,(16)}$ = 14.21; p<0.05 and p<0.01). Periogard® group showed differences between sessions 1 and 2 and between sessions 1 and 3, respectively ($H\sim\chi^2_{0.01,(16)}$ = 15.68; p<0.01 and p<0.05). No differences were observed among the three groups in each session separately. Differences in piloerection in the MDTA is also related in figure 4. Control group showed significant differences between session 2 and 4 ($H\sim\chi^2_{0.05,(16)}$ = 8.842; p<0.05). Test group EB1493 and Periogard® group did not show significant differences among groups (p>0.05). Also, there were no differences observed in the comparison of the three groups in each sessions (p>0.05).

Statistical differences were observed in micturition, in figure 5, in the control group between sessions 3 and 4 ($H\sim\chi^2_{0.05,(16)}$ = 11.32; p<0.01). No differences were observed in the comparison of the three groups in each session separately (p>0.05). Also, Periogard® group showed significant difference between sessions 1 and 3 ($H\sim\chi^2_{0.05,(16)}$ = 10.08; p<0.05). No differences were observed in the comparison of the three groups in each session separately (p>0.05), in the MDTA. Also, figure 5 relates differences in defecation between sessions 1 and 3 ($H\sim\chi^2_{0.05,(16)}$ = 3.13; p<0.05). No differences were observed in the comparison of the three groups in each session separately (p>0.05).

Figure 6 represents the variation of body weight of the animals under treatment in multi-drug toxicity assay. Statistical differences were observed in Periogard® group in the first session of the experiment (p<0.001).

Discussion

Reports describe *I. alba* being used to vulcanize rubber. Although the use of the plant is millennial, no reports relating its toxicity was found, despite the known toxicity of other *Ipomoea* species. This plant was randomly collected in the Brazilian Amazon Rain Forest and its aqueous extract was submitted to an antibacterial and antitumor screening. Our first findings relate the significant anti-*Streptococcus* activity. But is the plant toxic to animals or to the man, despite its *in vitro* antibacterial activity?

The methodologies applied in the present work were guided by previous works (9) and by Ethical principles that mastered to minimize animal suffering. According to the LD50, EB1493 is not considered harmful (13). EB1493 altered general activity and some of the psychomotor and autonomous nervous system parameters when the higher dose or the subsequent lower dose was administered in the stage one of the acute toxicity experiment. Stage two of the acute toxicity experiment, which was performed with the non-lethal dose, showed that general activity, defecation, piloerection and breath suffered alterations in relation to both vehicle and naïve controls. So, the first findings related to the toxicity profile of EB1493 were optimistic and further analyses were done. Subsequently, the MDTA was performed following a treatment protocol for periodontitis in which EB1493 was locally administered three times a day after induction of the disease.

According to MDTA findings, alterations were evident when groups were individually evaluated among sessions. All three groups showed the same pattern of alterations that can be related not directly to EB1493, Periogard® or vehicle control administration, but can be related to the periodonti-

tis induction procedure, which consists in mimicking established biofilm with dental floss for 11 days, when inflammation appears together with local bone loss. This is clear when the alterations appear in sessions 2 (1 day after periodontitis induction), 3 (6 days after periodontitis induction) and 4 (11th and last day of periodontitis induction and before sacrifice) and do not appear before periodontitis induction (session 1). On the other hand, when groups were compared to each other in one session, no differences could be observed, which confirms that the periodontitis induction procedure did influence the alterations over treatments. According to the two-way ANOVA applied to analyze changes in rats' weight, no alterations could be seen, but it was observed in the Periogard® group in the first session, before the induction of periodontitis induction, which means that the procedure followed by treatments did not influenced food intake by any of the groups, once groups were randomly composed.

Previous studies reported the toxicity of some *Ipomoea* species. *I. carnea* is considered a toxic plant growing in tropical areas. For that reason, more studies relating its toxicity than studies relating its pharmacological properties were found. The toxic effects over murine offsprings is documented (14,15), and such intoxication may be related to swainsonine, an indolizidine alkaloid. Some authors relate the toxicity of *I carnea* to other molecules than swainsonine, as calystegines B1, B2 and B3 (16) and 2-epi-lentiginoside (17). *I. batatas* or purple sweet potato is widely studied, both pharmacologicaly and toxicologicaly. The antimutagenicity of mono-, di- and tricafeoylquinic acid derivatives was studied (18). Some authors claimed that treatment with purple sweet potato may improve spatial learning and memory impairment (19). Antioxidant activity attenuating induced hepatotoxicity was also assessed (20), as well as its anti-inflammatory activity (21), the ability of inhibiting apoptosis (22) and of anti-fibrotic effects (23). Pharmacological activities are associated to anthocyanins (24), polyphenols (25) and caffeoyiquinic acid derivatives (18). Toxicity of *I. batatas* is also related and is related to sesquiterpenoids (26). Other *Ipomoea* species are also reported. No reports concerning toxicity of *I. alba* was found, and the present study was mandatory to support further pharmacological studies. Nonetheless, the literature does not support the use of the plant in vulcanizing rubber due to its sulfur content, as there are no reports concerning the presence of any sulfated compounds in *Ipomoea* species.

Initial signs of toxicity were not beheld in the present study, and for that reason, EB1493 can be still considered a promising extract to be used against initial manifestations of oral diseases.

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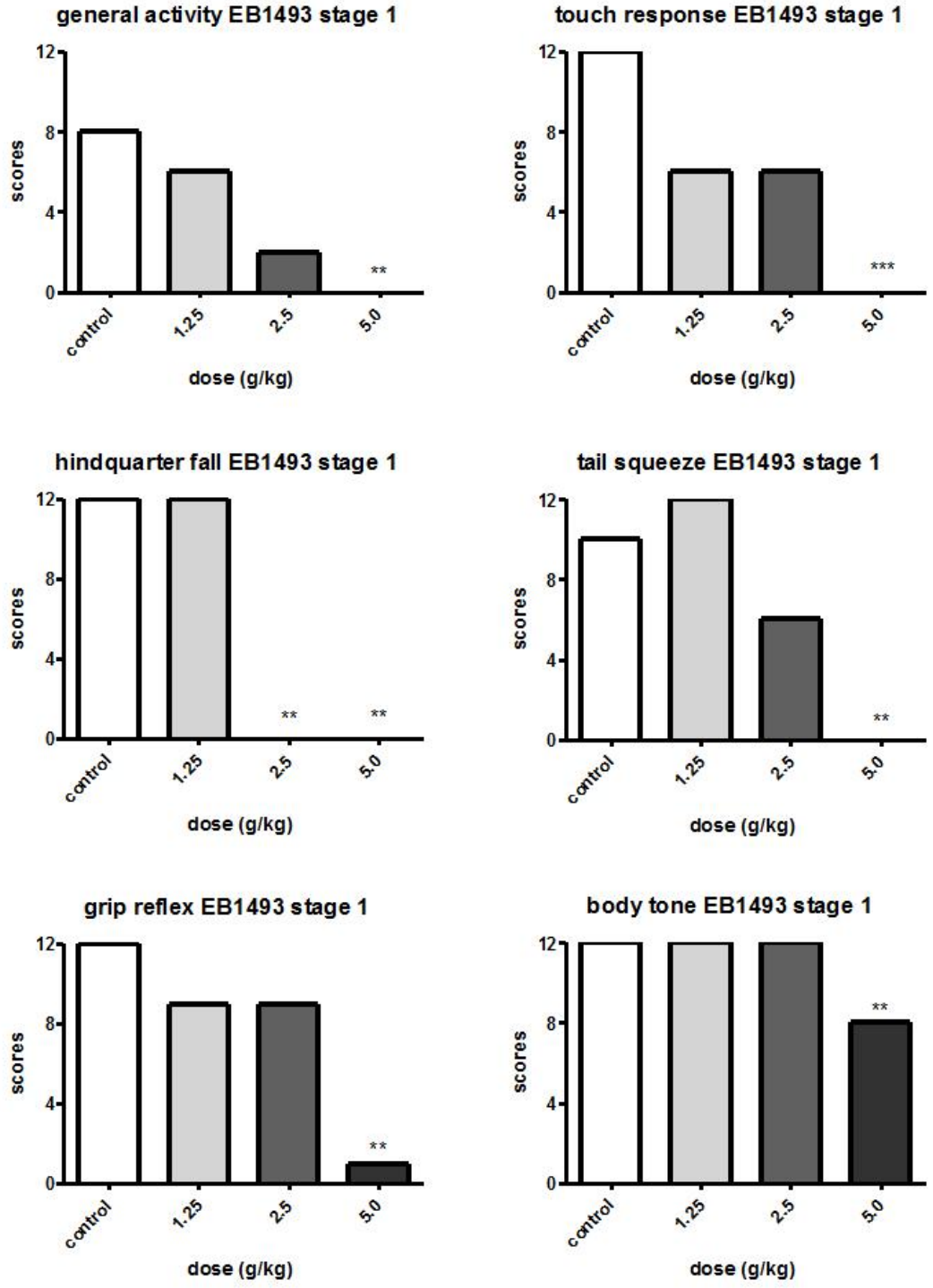

Figure 1. Observations over general activity and psychomotor system after administration of different doses of EB1493, obtained from *Ipomoea alba* in the stage one of the experiment. Ordinates present the sum of scores per three mice's group (n=3; N_{total}=36). Differences among medians are marked by * ($p<0.05$) or ** ($p<0.01$)

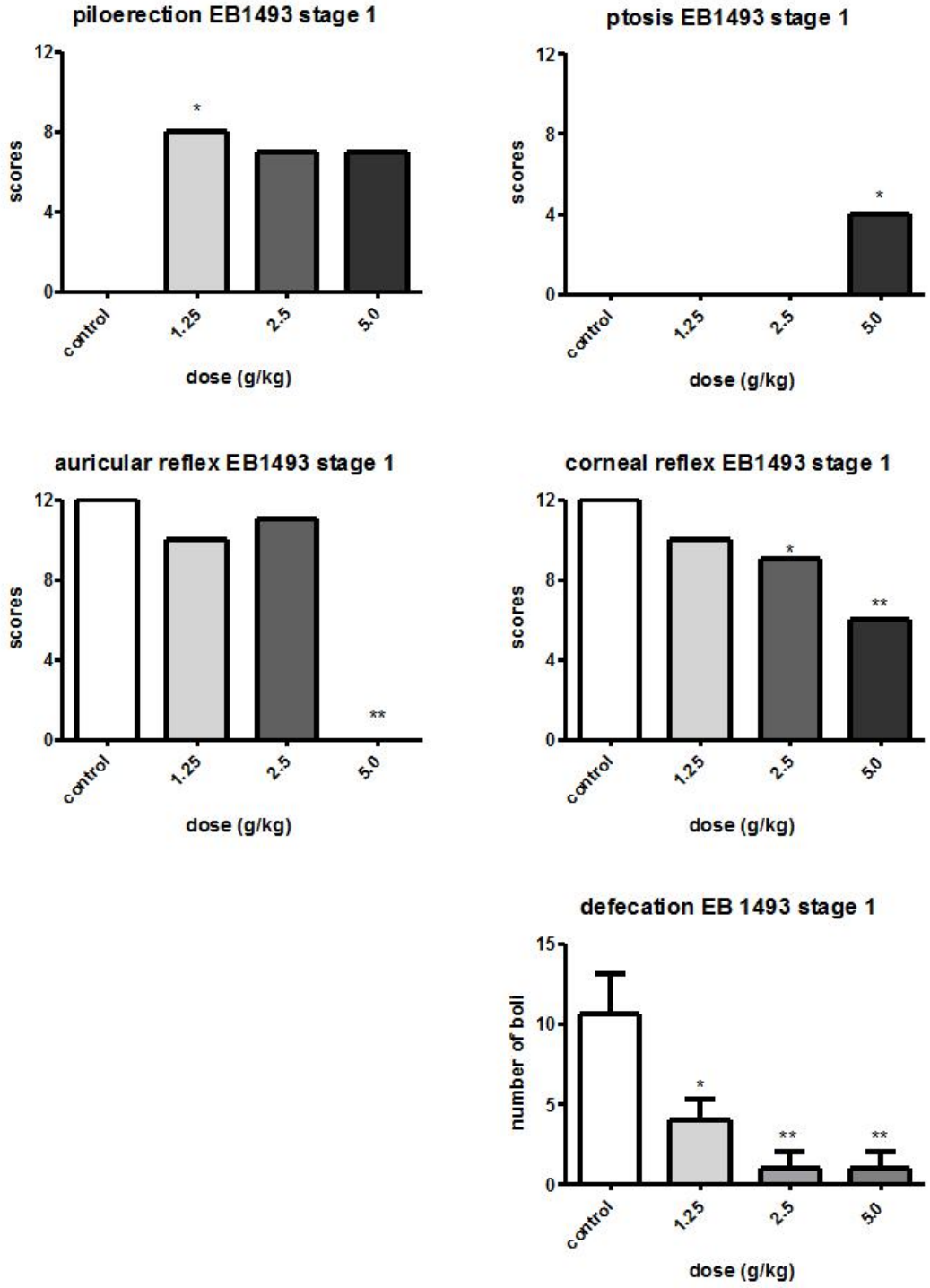

Figure 2. Observation over autonomous nervous system after administration of different doses of EB1493, obtained from *Ipomoea alba* in the stage one of the experiment. Ordinates present the sum of scores per three mice's group (n=3; N_{total}=36). Differences among medians are indicated * ($p<0.05$) or ** ($p<0.01$)

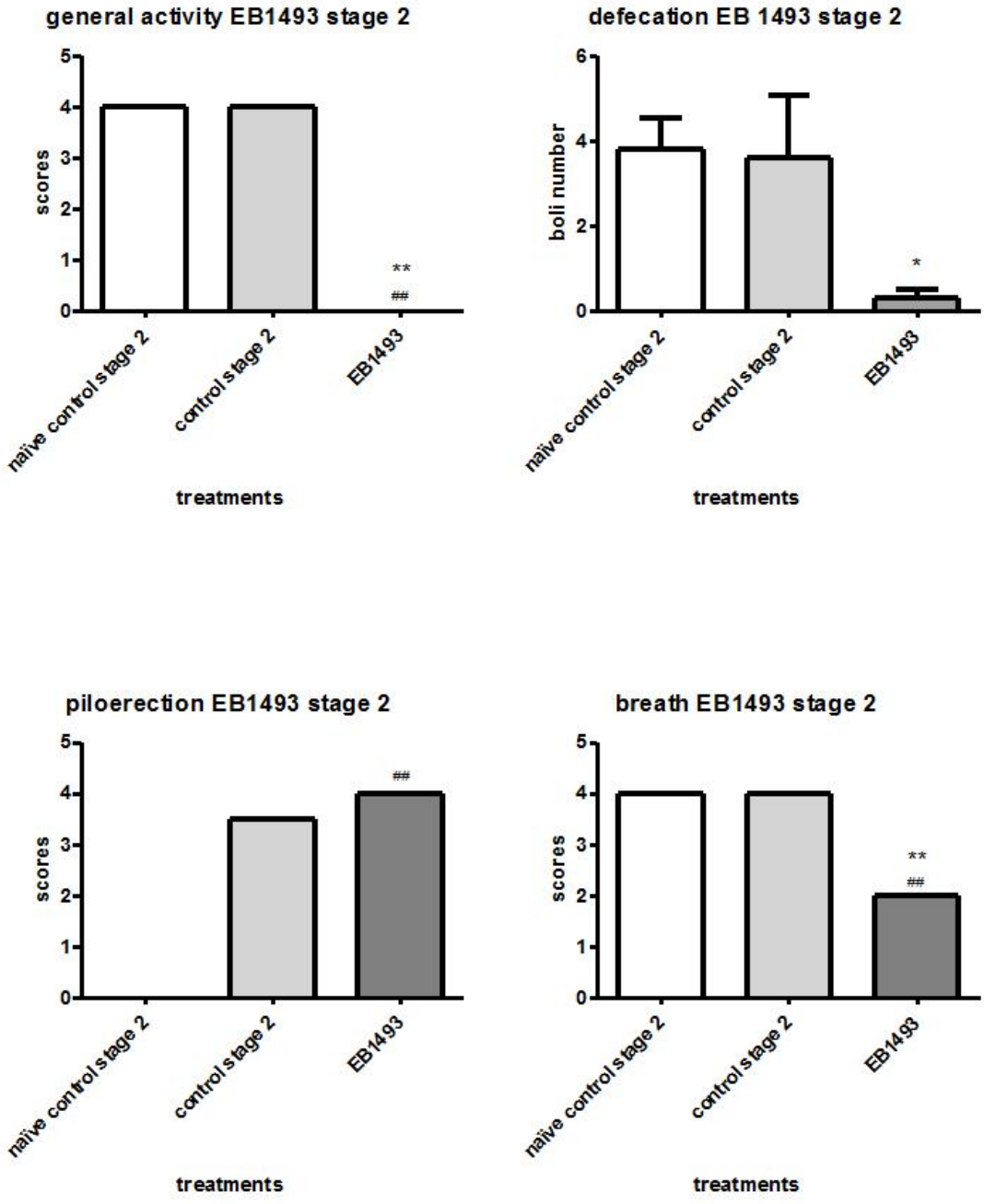

Figure 3. Observations after intraperitoneal administration of the non-lethal dose of EB1493 (1,250 mg/kg), obtained from *Ipomoea alba* in the stage two of the experiment (n=10; N_{total}=30)

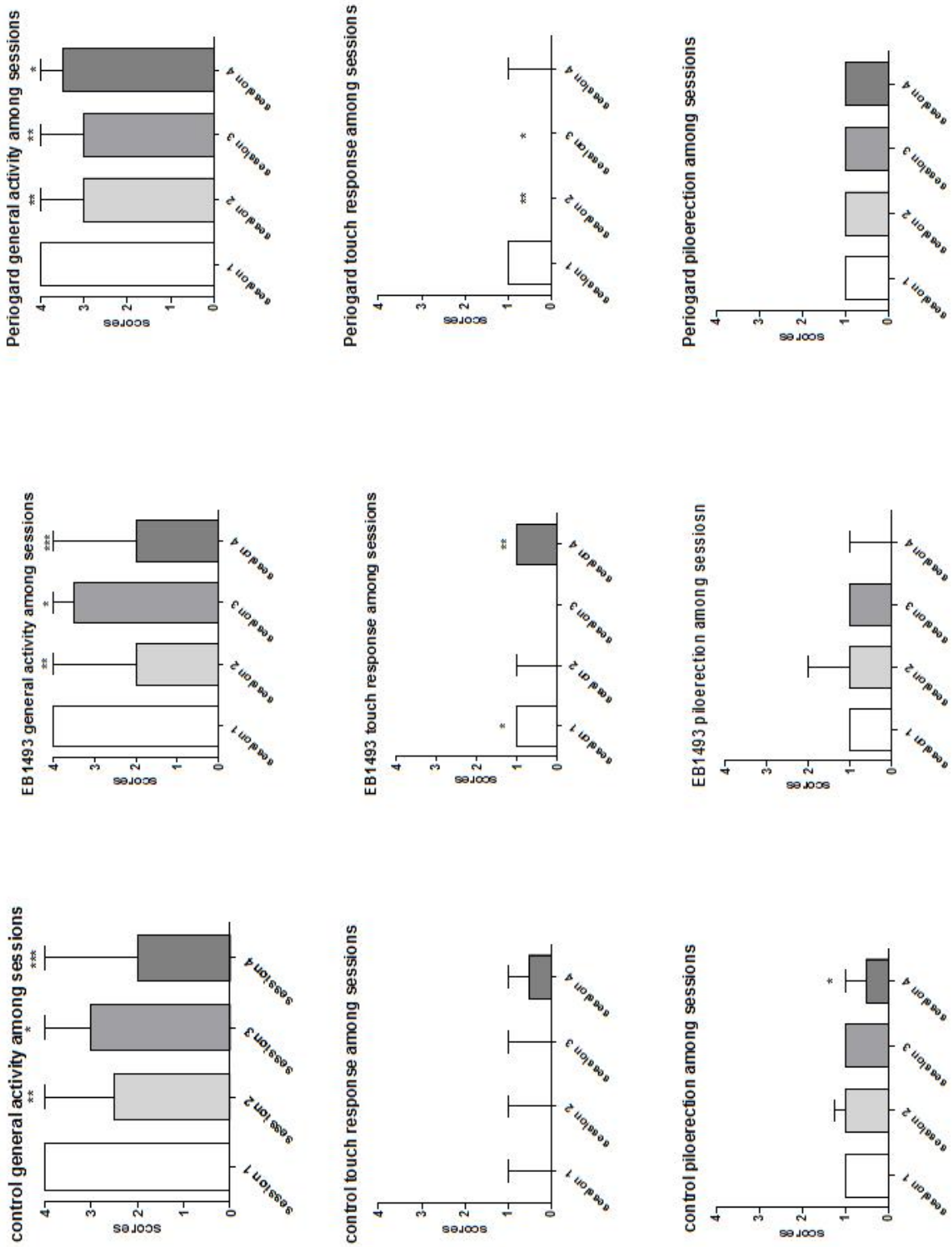

Figure 4. Observations over general activity, touch response and piloerection after administration of EB1493, obtained from *Ipomoea alba*, in the multi-dose toxicology assay. Ordinates present the scores per eighteen rats's group (n=18; N_{total}=54). Differences among medians are indicated * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$)

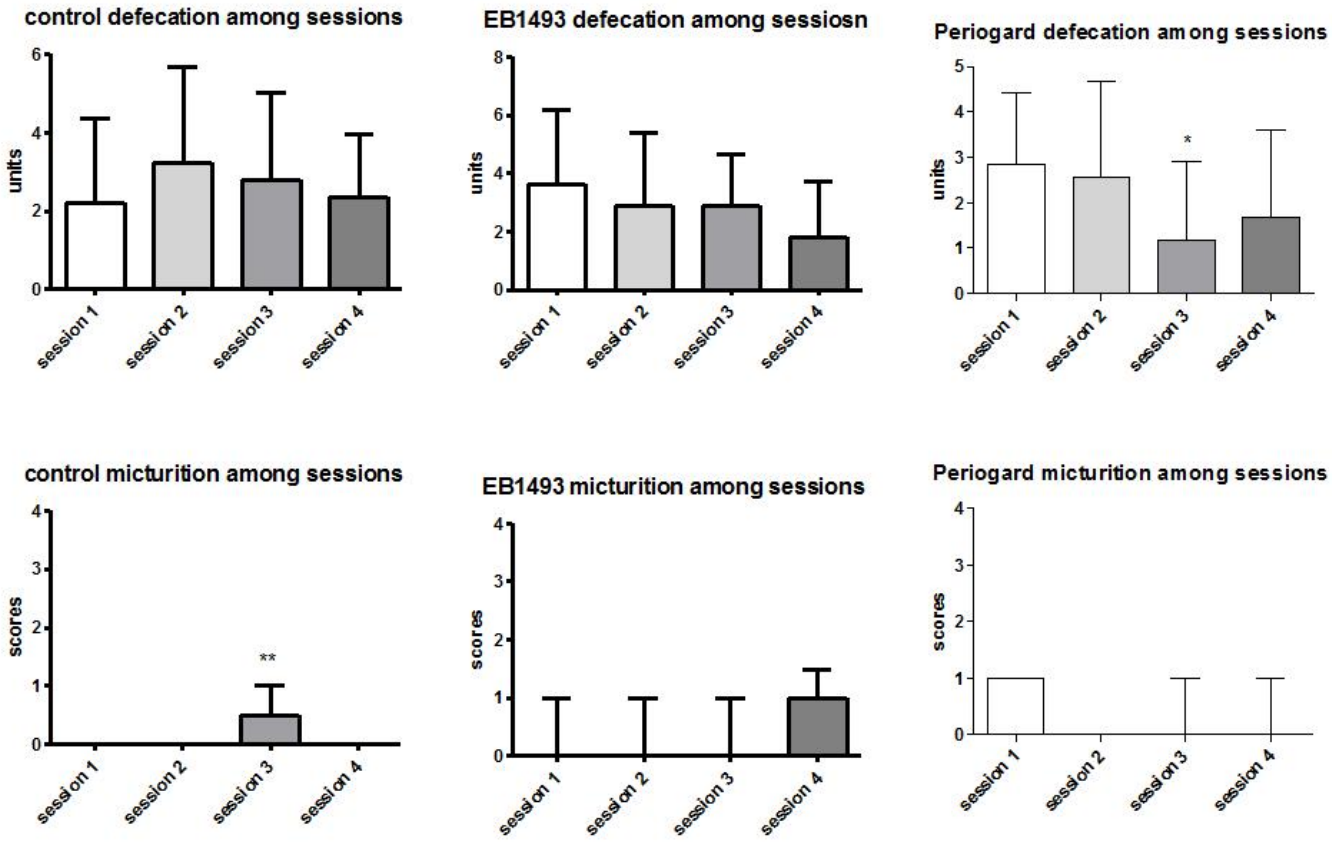

Figure 5. Observations over defecation and micturition after administration of EB1493, obtained from *Ipomoea alba*, in the multi-dose toxicology assay. Ordinates present the scores per eighteen rats' group (n=18; N_{total}=54). Differences among means obtained by two-way ANOVA and Bonferroni's post test are indicated by * ($p<0.05$), ** ($p<0.01$) or ***($p<0.001$)

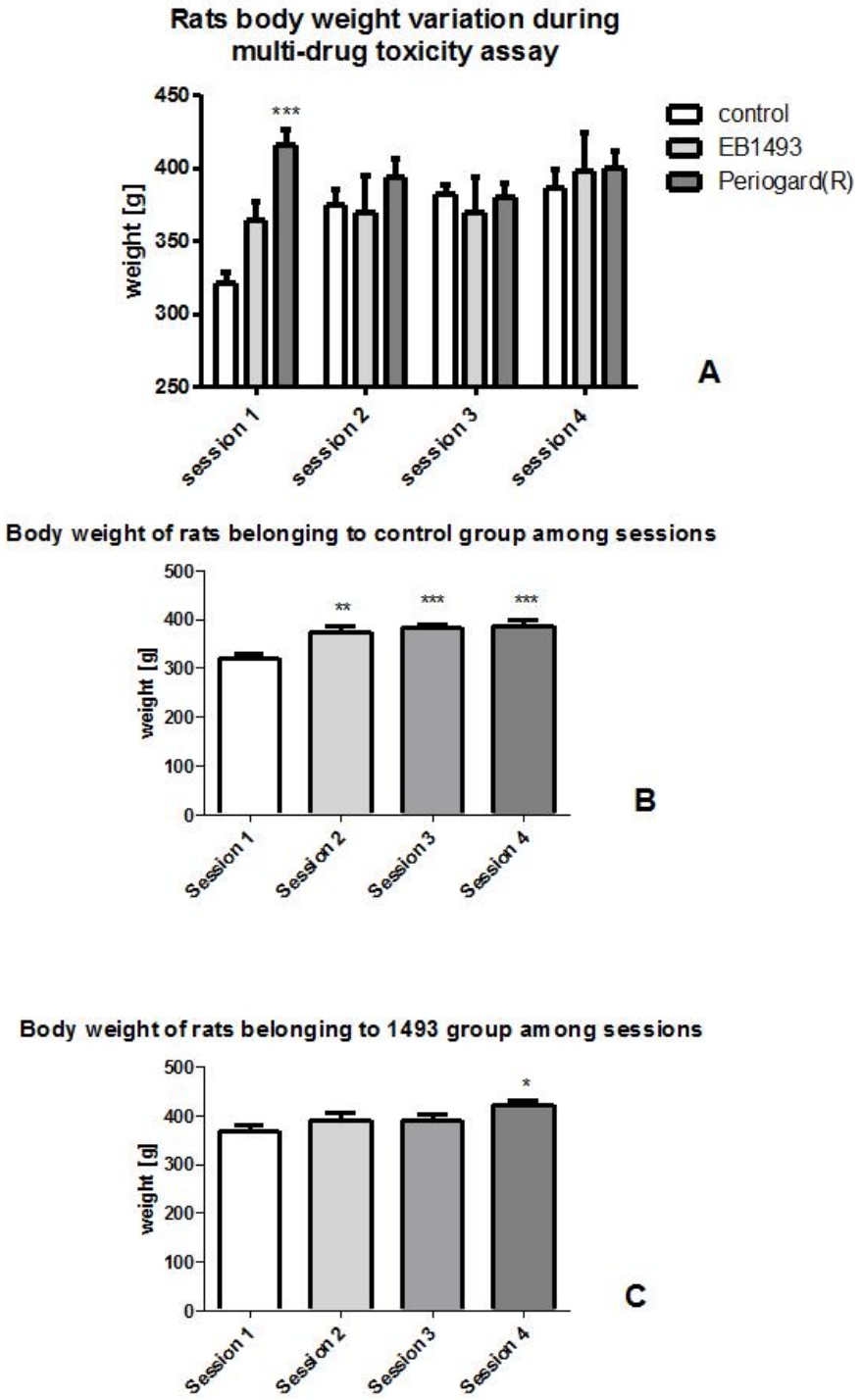

Figure 6. Variation of rats body weight during the multi-drug toxicity assay. A. Body weoght alterations observed for all groups during the period of experiment. Ordinates present the body weights per group (n=18; N_{total}=54). Differences among means obtained from two-way ANOVA and Bonferroni's post test. B. Differences of body weight of control group during all sessions (n=18; N_{total}=54). Differences among means obtained from one-way ANOVA and Tukey's post test. C. Differences of body weight of 1493 group during all sessions (n=18; N_{total}=54). Differences among means obtained from one-way ANOVA and Tukey's post test. Significance for all tests are indicated by * ($p<0.05$), ** ($p<0.01$) or ***($p<0.001$)