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Original Article

Influence of the intraperitoneal administration of antitumor *Abarema auriculata* extract on mice behavior

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ABSTRACT

The organic extract EB689, obtained from the stem of *Abarema auriculata* (Benth.) Barneby & J.W.Grimes, Fabaceae, commonly known as "saboeiro-ferro", was chemically studied, as well as its influence over behavioral effects such as locomotion, emotionality and anxiety, after intra-peritonial administration were assessed. The open-field and elevated-plus maze were used in experiments divided into two stages. The first stage aimed for the identification of the main effects over behavior using a reduced number of animals against half-fold diluted doses of EB689. The same variables were also tested in a second stage of the experiment using the non-lethal intra-peritoneal dose of 4.8 mg/kg in a larger number of animals. It was observed that EB689 clearly decreased locomotion, which was probably caused by internal hemorrhage causing hypovolemic shock. Although it is the first time lupeol and eucryphin are described in *A. auriculata*, it is still not clear if they are involved in the toxicology of *A. auriculata*. The undesirable effects of EB689 are better understood, the basis for further pharmacological assays aiming antitumor activity are supported.

Introduction

Abarema auriculata (Benth.) Barneby & J.W.Grimes (crude extract also known as EB689) is a plant found in the Amazon rainforest that belongs to the Fabaceae family and is locally known as "saboeiro-ferro" (Salomão et al., 2007). The organic extract obtained from the stem of the plant, named here as EB689, showed a notable cytoxicity against prostate cancer cell lines (growth inhibition -32.66%; in Suffredini et al., 2006b), human central nervous system cancer cell lines (growth inhibition -24.69%; in Suffredini et al., 2007) and against head-and-neck tumor cell lines. They displayed significant growth inhibition, when compared to control groups (Ozi et al., 2011) *in vitro*. EB689 was selected amongst more than 1,300 plant extracts screened (Suffredini et al., 2006a, Younes et al., 2007). As there are no reports on the traditional use or toxic effects of the

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plant, it is imperative that information regarding its possible cytotoxic effects be evaluated. In the present study, the influence over locomotion, anxiety and emotionality in male mice was assessed after intraperitoneal (*i.p.*) administration of the crude extract.

Previously, our group determined the lethal dose 50% and evaluated the influence of the extract over general activity (Gusmão et al., 2013) in an optimized protocol using few animals, with the purpose of avoiding unnecessary animal suffering. The present study, which is an extension of the previous one, aims to evaluate the influence of different doses of EB689 on multiple parameters related to anxiety, locomotion, and emotionality after intraperitoneal (*i.p.*) administration, over Balb-c male mice (Botham, 2004).

Materials and methods

Plants were collected in the Brazilian Amazon rainforest, under Brazilian Government (Environment Ministry) licenses (CGen/MMa#12A/2008) for collecting genetic resources in protected areas of the Brazilian forests. Plants were collected in Manaus, AM, in the *Igapó* forest, (seasonally flooded forests from the Rio Negro Basin), in January 22 1999. The voucher specimen was deposited at UNIP Herbarium (*A.A.Oliveira, 3353* (UNIP Herbarium)). Specimen was authenticated by Curator Prof. Dr. Mateus L. B. Paciencia.

Plant material

The stems of *Abarema auriculata* (Benth.) Barneby & J.W.Grimes, Fabaceae, were collected, dried in a ventilated drying oven (Fanem) at 40°C, and ground in a hammermill crusher (Holmes). About 545 g of the ground material was placed in a glass percolator (Kontes) where it was macerated for 24 h with 1.80 l of dichloromethane and methanol (1:1) (Suffredini et al., 2006b) (Merck), resulting in 26.56 g (4.87% yield) of crude extract. Solvents were evaporated under vacuum (Büchi) and were kept in a freezer (Revco) until use. The extract was suspended in almond oil for animal administration.

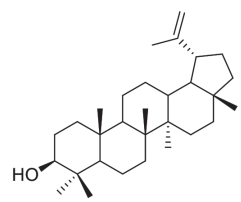
Assays

Extract fractionation and isolation of compound

Organic extract EB689 (14.09 g) was solubilized in methanol (15 ml) and chloroform (10 ml), followed by the addition of water (20 ml). The solubilized extract was transferred to a glass column (2.5 cm diameter, 90 cm length). Chloroform (100 ml) was added to the column, and as the chloroform is heavier than the aqueous phase, it passes through the polar phase in an intimate contact eluting low polarity substances. The procedure was repeated once more. The chloroform extract was air evaporated, resulting in a dark-brown chloroform residue ($RCHCl_3$) (2.63 g; 18.45% yield). The remaining solvent in the aqueous phase was evaporated. The aqueous phase was then subjected to a butanol partition, resulting in the production of a brown butanolic residue (RBuOH) (7.02 g; 50.07% yield). The aqueous phase was lyophilized (RH_2O) (4.44 g; 31.51% yield). $RCHCl_3$was subjected to a Sephadex LH20 (22 g) column chromatography (CC) (2.5 cm diameter, 90 cm length) with 300 ml of hexane, 250 ml dichloromethane and 200 ml methanol used to elute the column, resulting in FrHEX (653.0 mg; 4.63% yield), FrDCM (464.9 mg; 17.68% yield) and FrMeOH (1.51 g; 10.72% yield). FrHEX (219.5 mg; 1.56% yield) and FrDCM (147.6 mg; 1.05% yield) were further fractionated by CC using silica gel (60-200 µm particle size) eluted with solvent mixtures of increasing polarity such as *n*-hexane, ethyl acetate and methanol; this resulted in 28 and 27 fractions, respectively. Fractions were combined according to analytical thin layer chromatographic (TLC) similarity after visualization with 25% sulfuric acid followed by heating. As a result spinasterol was isolated (Gusmão et al., 2013). From 12-14FrHex the compound named lupeol was isolated after purification in preparative layer chromatography and Hex:AcOEt (9.5:0.5) as eluent.

Lupeol (**1**): beige powder (5.2 mg). ^{1}H NMR ($CDCl_3$): δ 0.77 (Me-24, *s*), 0.79 (Me-28, *s*), 0.84 (Me-25, *s*), 0.96 (Me-27, *s*), 0.98 (Me-23, *s*), 1.04 (Me-26, *s*), 1.69 (Me-30, *s*), 2.39 (H-19, *ddd J* = 11.16, 11.16, 5.71), 3.20 (H-3α, *dd J* = 11.42, 4.67), 4.58 (H-29a, *m*), 4.70 (H-29b, *m*). ^{13}C NMR ($CDCl_3$): δ 14.55 (C-27), 15.36 (C-24), 15.98 (C-26), 16.11 (C-25), 18.01 (C-28), 18.32 (C-6), 19.31 (C-30), 20.94 (C-11), 25.16 (C-12), 27.43 (C-2, 15), 27.99 (C-23), 29.86 (C-21), 34.29 (C-7), 35.59 (C-16), 37.18 (C-10), 38.07 (C-13), 38.72 (C-1), 38.86 (C-4), 40.01 (C-22), 40.85 (C-8), 42.84 (C-14), 43.00 (C-17), 47.99 (C-19), 48.32 (C-18), 50.46 (C-9), 55.31 (C-5), 79.02 (C-3), 109.31 (C-29), 150.98 (C-20). Comparison of the obtained results can be revised elsewhere (Mahato and Kundu, 1994).

Eucryphin (**2**) (10 mg). ^{1}H NMR (CD3OD): δ 1.28 (Me-Rham, *d J* = 6.16), 5.33 (H-1Rham, *d J* = 0.99), 6.22 (H-6, *d J* = 1.99), 6.34 (H-8, *d J* = 1.79), 8.11 (H-2, *s*). ^{13}C NMR ($CDCl_3$): δ 18.13 (C-6´), 71.32 (C-4´), 71.76 (C-2´), 72.05 (C-5´), 73.68 (C-3´), 95.11 (C-1´), 100.18 (C-8), 102.24 (C-6), 106.63 (C-10), 140.53 (C-3), 148.11 (C-2), 159.46 (C-5), 163.59 (C-9), 166.3 (C-7), 178.86 (C-4). Comparison of the obtained results can be revised elsewhere (Tschesche et al., 1979). Li et al. (1996) proposed the structure of a compound isolated from *Smilax glabra*, which was named smiglanin. Smiglanin has an identical molecular structure of that proposed to eucryphin by Tschesche years before. In the present work we use eucryphin as the name of the identified molecule.



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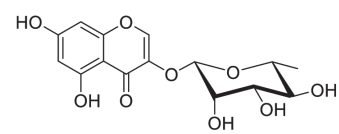

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Fraction RBuOH was subjected to flash column chromatography (3.5 cm diameter and 25 cm length), using 60 g of C-18 silica as stationary phase and eluted with 400 ml of 15% acetonitrile (ACN) in acidified water with 0.1% trifluoroacetic acid (TFA), 500 ml of 50% ACN in 0.1% TFA acidified water and 200 ml of MeOH (0.1% TFA). Three fractions were obtained from RBuOH, named:

Fr15%ACNRBuOH (2.18 g; 15.47% yield); Fr50%ACNRBuOH (4.64 g; 32.93% yield); and FrMeOHRBuOH (194.8 mg; 1.38% yield). Three fractions were obtained from the C-18 chromatography of RH2O named: Fr15%ACNRH2O (2.62 g; 18.60% yield); Fr50%ACNRH2O (1.81 g; 12.85% yield); and FrMeOHRH2O (21.0 mg; 0.15% yield). Five flavonones named neoastilbin, astilbin, isoastilbin, neoisoastilbin and engeletin were previously isolated from Fr15%ACNRBuOH of EB689 (Gusmão et al., 2013), their spectral information can be found elsewhere. The HPLC chromatogram at 254 nm fraction contains isolated eucryphin (**2**); it can be seen in Fig. 1. Eucryphin was isolated at 18% ACN, the LC/MS/MS (18-30% de ACN $t_{0\rightarrow30min}$, 30-50% ACN $t_{30\rightarrow40min}$, 50%-100% ACN $t_{40\rightarrow50min}$.) chromatogram displayed the eucryphin containing fraction at negative mode, as well as its ESI- mass spectrum, the negative mode MS2 spectra of $(2M-H)^-$ and the negative mode MS^2 spectra of $(M-H)^-$ can be seen in the supplementary material. Eucryphin (1 mg/ml) was dissolved in methanol, membrane-filtered (0.45 µm), and analyzed by $LC/ESI-MS^n$. A Shimadzu HPLC System consisting of a CBM 20 A, LC 20AD binary-pump, SPD 20A detector, a SIL 20AC auto sampler, and a C-18 Luna HPLC column, (5 µm, 250 mm x 4.6 mm; Phenomenex) were used. The column was run at 40 °C. The mobile phase consisted of A: H_2O w/0.1% CH_3COOH; B: MeCN; isocratic 18% ACN for 30 min; flow rate: 1 ml/min. A Bruker Daltonics Esquire 3000 ion trap mass spectrometer, equipped with an electrospray ionization (ESI) source was used. Instrument control and data acquisition were performed using Esquire 5.2 software. The ion source temperature was 320 °C and capillary voltage was set at 4000V and end plate offset at 500V. Nitrogen served as nebulizer gas regulated at 27 psi and at a flow rate of 7 l/min. The mass spectrometer was operated in full- scan, negative-ions mode.

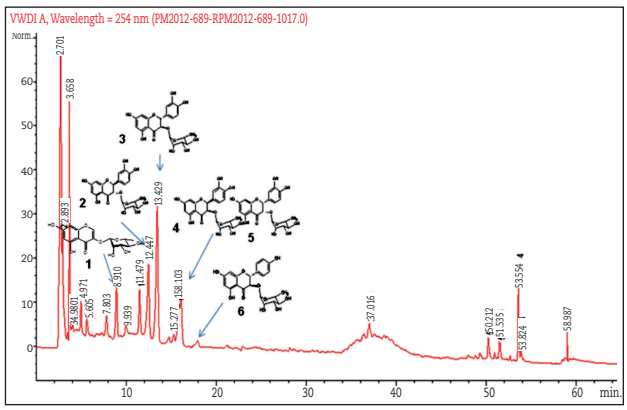

Figure 1 - HPLC chromatogram of fraction 15% ACN BuOH from EB689 (obtained from *Abarema auriculata*), at 254 nm. (18-30% de ACN $t_{0\rightarrow30min}$, 30-50% ACN $t_{30\rightarrow40min}$, 50%-100% ACN $t_{40\rightarrow50min}$.) **1**. eucryphin; **2**. neoastilbin; **3**. astilbin; **4**. isoastilbin; **5**. neoisoastilbin; **6**. engeletin. Identification and elucidation of flavonones **2** to 6 can be found elsewhere (Gusmão et al., 2013).

Preparation of extract to be administered

EB689 was suspended in almond oil and the following doses were administered: 5 000, 2 500, 1 250, 625, 312.5, 156.3, 78.1, 39.1, 19.5, 9.7, 4.8 mg/kg *i.p.* The extract was diluted in almond oil, due to its non-polar origin, and because it is compatible with human organism as well as for its non-toxicity. The *i.p.* route was chosen due to the absence of bioavailability loss. Vehicle control group was assessed (0.1 ml/10 g of almond oil) as well as a naïve group, which did not receive any treatment.

Animals

Male Balb-C mice (*Mus domesticus*) weighing 25-30 g were used; they were obtained from São Paulo University Medical School. After arrival in the laboratory, animals were housed in groups of five in microisolation 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 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 to the lab for habituation to the new conditions. All the experiments done with mice were subjected and approved by the Ethic Committees (CEP/ICS/UNIP 025/08 and CaPPesq 1109/090).

Open field

The open field (OF) test used to assess the influence of the extract over emotionality and motility was built as early proposed (Broadhurst, 1960), adapted to the size of mice. The open-field used in the present study was a circular wooden box (40 cm in diameter and 50 cm high) with an open top and a floor divided into 19 squares. Illumination was provided by a room lamp of 100W (at the floor of apparatus 400 lx). Each animal was individually placed in the center of the arena of the OF, and parameters were measured over a period of 5 min in five sessions starting assessment 10-15, 30-35, 60-65, 120-125 and 180-185 min after the administration of the extract. Hand-operated counters were employed to count locomotion frequency (*i.e.*, number of squares crossed with the four paws), rearing frequency (one unit of rearing corresponds to a standing position on the hindlimbs, with the trunk perpendicular to the floor, head tilted up and the forelimbs touching, or not, the walls of the arena with its forelegs in the air) and the number of *fecal boli* at the end of experiments. A chronometer was used to measure the immobility duration (*i.e.*, total time in seconds without spontaneous movements, *i.e.*, when head, trunk and limbs were still) and grooming duration (total time of washing movements over the head, licking the paws, fur licking, and tail/genital cleaning). To minimize the possible influences of circadian changes on OF, control and experimental animals were alternated. The device was cleaned with a 5% alcohol/water solution before placing the animals in it to eliminate the possible bias caused by odors left by the previous subject. The assays started at 1 p.m. and ended before 5 p.m., in order to prevent the circadian influences.

Elevated-plus maze

The elevated-plus maze (EPM) is an apparatus first conceived by the British psychologist Sheila Handley's group as a model to evaluate anxiety and it is one of the most used (Lapiz-Bluhm et al., 2008). The elevated plus-maze used was made of wood and had two open arms (23.5 cm × 8 cm) and two enclosed arms of the same size with 20 cm high walls; the apparatus was elevated 80 cm above the ground, it was placed in a sound-proof room with room lamp of 100W (at the floor of apparatus 400 lx). Basically, two strategies can be easily noticed: avoidance of the open arms while staying in the closed arm, and escaping behavior from the open arm directly to the closed arm (Pinheiro et al., 2007). In the present study, the maze was used to assess anxiety, and the animals were evaluated after being tested in the OF. Exploratory behavior was determined by the number of entries into the closed arms and the number of crosses in the center of the EPM. The animals were allocated in the center of the maze, which was previously cleaned with 5% alcohol. The number of entrances in the open arm and in the closed arm, as well as the time remaining in each arm and the number of center crossing were measured. Observations were done at 15-20, 35-40, 65-70, 125-130 and 185-190 min after the administration of the extract and immediately after observation in the OF.

Experiment design

In order to investigate the effects of EB689 administration over mice we performed a two-stage experiment. At the first stage, the tendency to provoke alterations in anxiety and psychomotricity levels on mice after the administration of known doses of EB689 was assessed; this stage was performed with a reduced number of animals (n = 3 per group). Evaluation in open field and elevated-plus maze were done right after assessing general activity (results in Gusmão et al., 2013) The initial dose employed was 5,000 mg/kg and doses were half-fold diminished according to lethality observed in at least one animal/dose previously reported by Gusmão et al. (2013). The second stage of the experiment was conducted using the non-lethal dose (NLD) of 4.8 mg/kg, using ten mice/group. Mice were individually observed in the OF followed by the EPM test in stage 1, as shown in Fig. 2, but extracts were not tested in EPM in the second stage. Based on physiological issues, the assays started at 1 p.m. and ended before 5 p.m., in order to prevent the circadian influences.

Statistical analysis

Parametric measures were analyzed by two-way ANOVA (Zar, 1999) followed by Bonferroni's posttests (GrahpPad Prism 5.0). A probability of p <0.05 was considered significant to show differences among means.

Results

Results obtained from the first stage of the experiments in the OF can be seen in Table 1. Animals that received doses of 5 000 down to 625.0 mg/kg were deadly affected by *i.p.* administration of EB689, for the entire duration of the experiment. For that reason, the main effects of EB689 on multiple parameters related to anxiety, locomotion and emotionality could in fact be observed after *i.p.* administration of doses 312.5 down to 4.8 mg/kg. Table 1 shows the locomotion and rearing frequencies and the immobility time, as well as defecation and grooming parameters. Locomotion frequency suffers significantly by the treatment ($F_{7;80} = 12.98$; $p < 0.001$), as well as the interaction between treatment and time ($F_{28;80} = 1.77$; $p < 0.05$). It was observed that locomotion significantly decreased after *i.p.* administration of doses 312.5 down to 19.8 mg/kg in all 4th and 5th sessions ($p < 0.01$), excluding those sessions observed after administration of doses 9.8 and 4.8 mg/kg. Treatment ($F_{7;79} = 3.34$; p <0.01) showed to be the parameters that diminished rearing frequency after *i.p.* administration of doses 312.5 down to 19.8 mg/kg in all 5th sessions ($p < 0.05$), excluding those sessions observed after administration of doses 9.8 and 4.8 mg/kg. However, immobility time, grooming and defecation in treated animals did not exhibit significant alterations in relation to control group ($p > 0.05$).

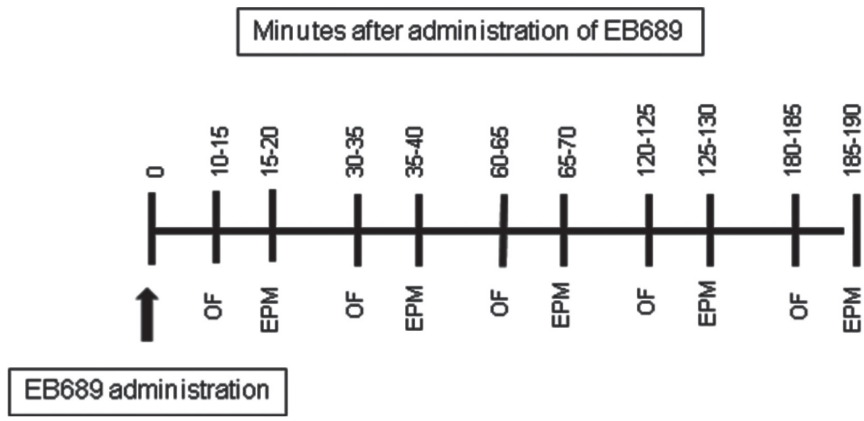

Figure 2 - Experiment design diagram of the first and second stage. Behavioral effects of EB689 after intraperitoneal injection, in mice: time line related to the experimental design of first stage of experiments. OF, open field; EPM, elevated-plus maze.

Table 1
Locomotion and rearing frequencies (units), immobility time (seconds), grooming behavior (units) and defecation (units), open field parameters in the first stage of experiments (n = 3), after intraperitoneal administration of different doses of EB 689, obtained from *Abarema auriculata*. Five sessions of five minutes each were assessed at 15, 30, 60, 120 and 180 minutes after administration of the extract. Data are presented as mean ± SEM, calculated by two way ANOVA followed by Bonferroni test; *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$), in relation to control group.

Open field evaluation stage 1								
Ñocomotion frequency (Mean ± SEM)								
	control 0.3 ml/30 g	**312.5 mg/kg**	**156.3 mg/kg**	**78.1 mg/kg**	**39.1 mg/kg**	**19.8 mg/kg**	**9.8 mg/kg**	**4.8 mg/kg**
session 1	116.30 ± 24.66	50.67 ± 25.18	20.67 ± 16.68	30.67 ± 12.44	10.33 ± 4.10	41.67 ± 17.36	62.33 ± 9.49	76.33 ± 14.84
session 2	98.67 ± 50.78	18.33 ± 5.78	13.67 ± 10.67	67.67 ± 12.25	74.00 ± 6.81	164.00 ± 21.78	64.67 ± 34.11	62.00 ± 10.07
session 3	91.67 ± 40.19	14.33 ± 2.33	26.67 ± 14.84	27.67 ± 11.26	18.00 ± 7.81	64.33 ± 27.69	68.33 ± 37.84	132.70 ± 25.39
session 4	167.70 ± 45.76	18.00 ± 6.51**	4.33 ± 1.20***	12.00 ± 2.08**	4.67 ± 0.67***	2.67 ± 1.33***	69.33 ± 27.19	108.30 ± 28.90
session 5	178.70 ± 35.41	10.33 ± 7.97***	8.33 ± 6.44***	4.33 ± 1.20***	3.67 ± 2.73***	5.33 ± 4.37***	110.30 ± 96.02	96.33 ± 25.99
Rearing frequency (Mean ± SEM)								
	control 0.3 ml/30 g	**312.5 mg/kg**	**156.3 mg/kg**	**78.1 mg/kg**	**39.1 mg/kg**	**19.8 mg/kg**	**9.8 mg/kg**	**4.8 mg/kg**
session 1	0.00 ± 0.00	0.33 ± 0.33	0.50 ± 0.50	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.67 ± 0.67	0.33 ± 0.33
session 2	3.33 ± 3.33	1.00 ± 1.00	0.00 ± 0.00	0.33 ± 0.33	7.33 ± 6.36	0.00 ± 0.00	5.67 ± 1.20	0.33 ± 0.33
session 3	2.33 ± 2.33	0.00 ± 0.00	0.33 ± 0.33	0.00 ± 0.00	0.33 ± 0.33	0.670.33	13.33 ± 10.48	7.67 ± 5.78
session 4	7.33 ± 4.10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	8.00 ± 5.69	8.67 ± 7.22
session 5	20.00 ± 12.10	0.33 ± *0.33	0.00 ± *0.00	0.00 ± *0.00	0.00 ± *0.00	0.00* ± 0.00	9.00 ± 9.00	5.67 ± 5.17
Defecation								
	control 0.3 ml/30 g	**312.5 mg/kg**	**156.3 mg/kg**	**78.1 mg/kg**	**39.1 mg/kg**	**19.8 mg/kg**	**9.8 mg/kg**	**4.8 mg/kg**
session 1	0.00 ± 0.00	0.00 ± 0.00	0.50 ± 0.50	0.67 ± 0.67	0.33 ± 0.33	0.33 ± 0.33	0.00 ± 0.00	0.33 ± 0.33
session 2	0.67 ± 0.33	0.33 ± 0.33	0.00 ± 0.00	0.00 ± 0.00	0.33 ± 0.33	0.67 ± 0.33	0.67 ± 0.33	0.67 ± 0.33
session 3	1.00 ± 0.58	0.00 ± 0.00	0.67 ± 0.33	0.33 ± 0.33	0.33 ± 0.33	0.33 ± 0.33	0.33 ± 0.33	1.00 ± 0.58
session 4	1.00 ± 0.58	0.00 ± 0.00	0.00 ± 0.00	0.33 ± 0.33	0.67 ± 0.67	0.00 ± 0.00	1.00 ± 0.58	0.33 ± 0.33
session 5	0.67 ± 0.67	0.00 ± 0.00	0.67 ± 0.33	0.00 ± 0.00	0.33 ± 0.33	0.00 ± 0.00	1.00 ± 0.58	0.33 ± 0.33
Grooming								
	control 0.3 ml/30 g	**312.5 mg/kg**	**156.3 mg/kg**	**78.1 mg/kg**	**39.1 mg/kg**	**19.8 mg/kg**	**9.8 mg/kg**	**4.8 mg/kg**
session 1	0.33 ± 0.33	0.33 ± 0.33	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.67 ± 0.67	0.00 ± 0.00
session 2	2.67 ± 2.19	0.67 ± 0.67	0.00 ± 0.00	0.33 ± 0.33	4.00 ± 1.53	4.00 ± 3.00	2.67 ± 2.19	1.33 ± 0.88
session 3	1.00 ± 0.58	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3.33 ± 2.85	2.33 ± 2.33	2.33 ± 1.45
session 4	1.33 ± 0.33	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.67 ± 0.67	3.33 ± 2.85
session 5	3.00 ± 1.53	2.33 ± 2.33	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.67 ± 0.67	9.67 ± 5.55
Immobility time								
	control 0.3 ml/30 g	**312.5 mg/kg**	**156.3 mg/kg**	**78.1 mg/kg**	**39.1 mg/kg**	**19.8 mg/kg**	**9.8 mg/kg**	**4.8 mg/kg**
session 1	82.33 ± 44.18	159.30 ± 66.23	236.00 ± 29.0	249.00 ± 10.82	241.30 ± 10.84	238.70 ± 17.34	137.70 ± 50.716	76.33 ± 41.37
session 2	182.7 ± 44.54	189.00 ± 47.96	259.30 ± 14.85	202.30 ± 26.30	93.67 ± 10.20	116.00 ± 39.00	169.00 ± 72.062	125.00 ± 13.58
session 3	176.00 ± 22.12	154.70 ± 77.20	215.00 ± 45.35	266.70 ± 13.64	243.30 ± 16.90	250.30 ± 39.94	163.70 ± 66.356	61.33 ± 20.84
session 4	42.67 ± 31.44	253.30 ± *24.01	284.70 ± **4.63	278.70 ± **10.11	254.30 ± *12.44	296.70 ± ***2.85	210.70 ± 25.828	55.33 ± 16.91
session 5	91.33 ± 38.89	273.70 ± 19.33	264.00 ± 27.50	290.70 ± *1.76	272.00 ± 15.89	194.70 ± 97.44	184.30 ± 84.175	114.00 ± 38.55

Fig. 3 shows the results observed in the second stage of the experiments, and it's relation to the results of OF (n = 10), after administration of the non-lethal dose of 4.8 mg/kg, in comparison to administration of diazepam 1 mg/kg. Considering locomotion frequency, treatment ($F_{3;140}$ = 14.69; p < 0.001; 12.55% influence on the total variance) and time ($F_{4;140}$ = 23.65; p < 0.0001; 26.93% influence on the total variance) have significantly affected the results, as well as did the interaction ($F_{12;140}$ = 3.49; p < 0.0001; 11.94% of total variance). Statistical differences in locomotion could be observed in the group treated with EB689, particularly at the 4th and 5th sessions. Treatment accounts for 15.19% of the total variance in rearing frequency and is considered extremely significant ($F_{3;140}$ = 10.24; p < 0.001) and interaction between treatment and time accounts for 11.20% of total variance ($F_{12;140}$ = 1.89; p < 0.05). Alterations in rearing were observed after administration of 1 mg/kg diazepam, compared to vehicle control group. Differently from stage 1 results, immobility time increased in stage 2: Treatment ($F_{3;140}$ = 18.36; p < 0.0001; 19.50% of the total variance), time ($F_{4;140}$ = 8.54; p < 0.001; 12.10% of the total variance) and interaction between factors ($F_{12;140}$ = 3.48; p < 0.001; 14.81% of the total variance). Differences in defecation could be observed between naïve control and EB689 groups (p < 0.05), and treatment was considered extremely significant ($F_{3;140}$ = 5.45; p < 0.001; 8.83% of the total variance), as well as did time ($F_{4;140}$ = 2.53; p < 0.001; 5.46% of the total variance). No significant differences were observed in grooming (p > 0.05).

Table 2 presents the results obtained in the elevated-plus maze after treatments. Treatment is considered extremely significant over the number of entrances in the open arm ($F_{7;80}$ = 10.50; p < 0.001; accounting for 36.24% of the total variance) as well as the effect of treatment vs. time ($F_{28;80}$ = 1.64; p < 0.05; accounting for 22.64% of the total variance). On the other hand, time did not significantly account for the total variance (p > 0.05). Treatment accounts for most of the variance and the effect is considered extremely significant ($F_{7;80}$ = 16.29; p < 0.001; 45.36% of the total variance) over the number of entrances in closed arms, time accounts for some of the variance and is considered significant ($F_{4;80}$ = 3.60; p < 0.01; 5.73% of the total variance) and 4th and 5th sessions of groups treated with doses 312.5 down to 19.8 mg/ml showed an elevated tendency of preferring the closed arms (p < 0.001). All five sessions of groups treated with the last lower doses seemed to behave as control group (p > 0.05). In relation to the time spent in closed arms, treatment ($F_{7;80}$ = 10.07; p < 0.001; 28.91% of the total variance) and interaction between treatment and time ($F_{28;80}$ = 3.18; p < 0.001; 36.53% of the total variance) influenced the alterations. Specifically the 4th sessions of groups treated with doses 312.5 down to 19.8 mg/ml (p < 0.05) and session 1 of the group treated with doses 39.1 mg/ml. No significant differences were observed in the groups treated with doses 9.8 and 4.8 mg/ml nor significant differences were observed in the time spent in open arms (p > 0.05).

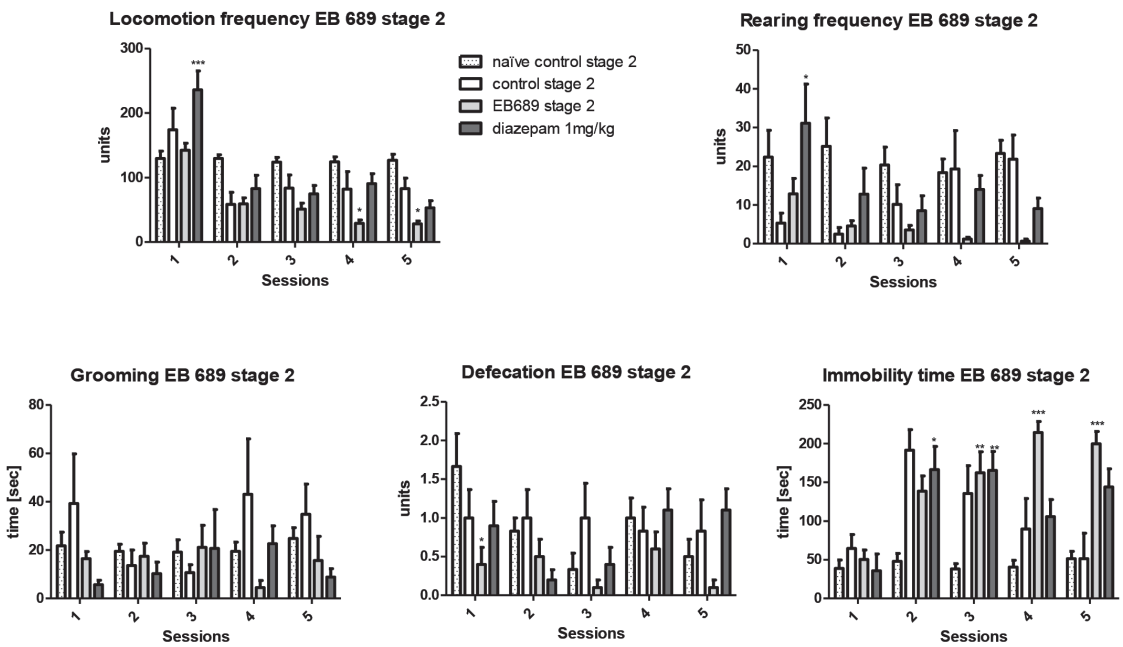

Figure 3 - Open field behavior of mice (n = 10/group) in the second stage of experiments after administration of 4.8 mg/kg of EB 689 obtained from *Abarema auriculata*, and diazepam 1 mg/kg, via intraperitoneal injection. Session 1 = 10-15 min, session 2 = 30-35 min, session 3 = 60-65 min, session 4 = 120-125 min and session 5 = 180-185 min. Two way ANOVA followed by Bonferroni test. $^{*}p$ < 0.05 in relation to control group; $^{\#}p$ < 0.05 in relation to control naïve group.

Table 2
Elevated plus-maze anxiety indexes obtained from intraperitoneal administration of EB689 to male Balb-c mice at different half-doses of 312.5 down to 4.8 mg/kg, and observed for 5 min in sessions of 15, 30, 60, 120 and 180 min after administration. Results are given in number of entries in closed or open arms and amount of time spent in open or closed arms. Data are presented as mean ± SEM, calculated by two way ANOVA followed by Bonferroni test; *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$).

Anxiety Indexes								
Entries in open arms								
	control 0.3 ml/30 g	**312.5 mg/kg**	**156.3 mg/kg**	**78.1 mg/kg**	**39.1 mg/kg**	**19.8 mg/kg**	**9.8 mg/kg**	**4.8 mg/kg**
session 1	**1.33 ± 0.88**	**0.00 ± 0.00**	**0.00 ± 0.00**	**0.33 ± 0.00**	**2.00 ± 2.00**	**0.00 ± 0.00**	**5.33 ± 2.85**	**0.33 ± 0.33**
session 2	4.33 ± 3.84	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	2.67 ± 0.88	0.33 ± 0.33	4.33 ± 1.76	1.67 ± 1.67
session 3	5.00 ± 2.65	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00 ± 1.00	0.00 ± 0.00	1.33 ± 0.88	11.0 ± 5.13
session 4	6.33 ± 3.18	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	7.67 ± 4.10	4.00 ± 2.52
session 5	7.00 ± 1.16	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3.33 ± 2.40	9.33 ± 0.33
Entries in closed arms								
	control 0.3 ml/30 g	**312.5 mg/kg**	**156.3 mg/kg**	**78.1 mg/kg**	**39.1 mg/kg**	**19.8 mg/kg**	**9.8 mg/kg**	**4.8 mg/kg**
session 1	8.33 ± 1.86	3.67 ± 2.67	1.00 ± 0.00	2.67 ± 0.88	5.33 ± 2.33	1.67 ± 0.33	11.00 ± 1.53	10.67 ± 3.53
session 2	6.67 ± 2.85	1.67 ± 0.33	1.00 ± 0.00	1.00 ± 0.00	6.00 ± 0.58	8.33 ± 0.33	9.33 ± 1.20	5.00 ± 2.65
session 3	6.00 ± 3.22	1.67 ± 0.67	1.00 ± 0.00	1.00 ± 0.00	2.67 ± 1.67	3.00 ± 1.16	4.33 ± 1.20	6.67 ± 1.45
session 4	11.33 ± 0.88	0.67 ± ***0.33	1.00 ± ***0.00	1.00 ± ***0.00	1.00 ± ***0.00	1.00 ± ***0.00	8.33 ± 3.18	5.00 ± 1.00
session 5	10.33 ± 3.33	1.00 ± **0.00	1.00 ± **0.00	1.00 ± **0.00	1.00 ± **0.00	0.67 ± **0.33	4.33 ± 3.84	4.33 ± 1.33
Time spent in open arms								
	control 0.3 ml/30 g	**312.5 mg/kg**	**156.3 mg/kg**	**78.1 mg/kg**	**39.1 mg/kg**	**19.8 mg/kg**	**9.8 mg/kg**	**4.8 mg/kg**
session 1	16.33 ± 12.99	0.00 ± 0.00	0.00 ± 0.00	4.00 ± 4.00	12.33 ± 12.33	0.00 ± 0.00	67.00 ± 42.19	1.67 ± 1.67
session 2	76.3 ± 373.35	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	34.00 ± 15.62	3.00 ± 3.00	48.33 ± 22.51	75.33 ± 75.33
session 3	72.33 ± 45.74	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	19.67 ± 19.67	0.00 ± 0.00	12.00 ± 6.43	167.30 ± 78.84
session 4	103.30 ± 53.49	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	110.70 ± 57.98	99.67 ± 71.75
session 5	139.30 ± 32.46	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	200.00 ± 100.00	0.00 ± 0.00	153.70 ± 86.68	230.70 ± 46.37
Time spent in closed arms								
	control 0.3 ml/30 g	**312.5 mg/kg**	**156.3 mg/kg**	**78.1 mg/kg**	**39.1 mg/kg**	**19.8 mg/kg**	**9.8 mg/kg**	**4.8 mg/kg**
session 1	199.00 ± 75.08	300.00 ± 0.00	294.00 ± 6.00	296.30 ± 3.67	12.33* ± 12.33	300.00 ± 0.00	228.00 ± 47.57	299.00 ± 1.00
session 2	208.30 ± 80.92	300.00 ± 0.00	300.00 ± 0.00	300.00 ± 0.00	34.00 ± 15.62	295.00 ± 5.00	260.30 ± 30.33	228.30 ± 71.67
session 3	217.30 ± 56.61	300.00 ± 0.00	300.00 ± 0.00	300.00 ± 0.00	280.30 ± 19.67	300.00 ± 0.00	283.00 ± 8.89	132.00 ± 79.78
session 4	111.70 ± 34.65	300.00* ± 0.00	300.00* ± 0.00	300.00* ± 0.00	300.00* ± 0.00	300.00* ± 0.00	189.00 ± 58.59	201.70 ± 71.93
session 5	160.00 ± 30.12	300.00 ± 0.00	300.00 ± 0.00	300.00 ± 0.00	300.00 ± 0.00	200.00 ± 100.00	169.30 ± 88.73	70.67 ± 44.28

Discussion

The open field test was developed to track alterations in emotionality in lab rodents. The measurement was stated as the amount of defecation of an emotional rodent compared to a non-emotional rodent, where the emotional rodent tends to defecate more in the apparatus (Hall, 1934 cited by Prutt and Belzung, 2003). Historically, OF defecations and activity has been used to assess emotionality and fearfulness (Hall, 1934) and also to analyze for hyperactivity and exploratory behaviors. Usual parameters for rodent behavior would be that the number of fecal boli deposited, as well as their activity pattern, vary in relation to the levels of fear and emotional reactivity; where a large number of boli and little activity indicate a fearful individual. Also, rodents tend to avoid brightly illuminated novel open spaces and the Open Field would work as an anxiogenic stimulus, allowing for the development of anxiety related locomotor activity - and exploratory behaviors, resulting in the approach-avoidance conflict. The intensity of locomotion can be related to an anxiety-like or anxiolytic-like

effect promoted by some drug, and in the present report, it helped in the establishment of a behavioral pattern related to the administration of EB689. The first exposure of the animals to the device has a more significant emotional and locomotor component than the subsequent exposures (Batatinha et al., 1995; Lazarini et al., 2000; Massoco et al., 1995; Moniz et al., 1994), although controversial (Carobrez and Bertoglio, 2005), which may lead to habituation. In the present work, no significant differences in defecation were observed during the first stage of experiments. This may indicate that mice receiving treatment were not influenced by EB689 over fearfulness or emotionality parameters. The analysis of locomotive behaviors showed that control groups followed an usual response to emotionality in response to a novel environment, as animals got used to the apparatus after 1st session. Locomotion in open field significantly decreased after treatment, however, after lower doses of EB689, animals moved more across the squares after the administration in a dose-dependent relationship. After the 4th session, the treatment group showed improvement in locomotive behaviors when compared to the control group, which happened 120 min after *i.p.* administration of 9.8 mg/kg of EB689. A similar pattern of behavior was observed for both rearing and immobility time. In the second stage of experiment, diazepam was introduced as reference drug. Then, mice were observed after the administration of a non-lethal dose (4.8 mg/kg) of EB689 and the administration of 1 mg/kg diazepam. Locomotion was clearly impaired by the extract administered, misleading the usual square crosses pattern seen to the other three groups. EB689 administration significantly showed reduced number of square crosses (in an inversely proportional relationship) and increased immobility time (in a direct relationship), maybe due to a sedative effect. It was observed the anxiolytic activity related to diazepam in the first session, but no alterations related to EB689. It is possible that more than 120 min are needed for the extract to carry out its effect. Prutt and Belzung (2003) proposed that rearing frequency diminishes after the administration of lower doses of diazepam to rats, but to Balb-c mice we observed that rearing frequency was significantly increased. Apparently, rearing frequency decreases after EB689 administration, in relation to naïve mice, but no significance was observed. The significant alteration in defecation caused by EB689 in the second session may indicate a decrease in emotionality, but this result is not in agreement to the first stage of experiments and it remains inconclusive so far. As the first stage of experiments is more indicative than conclusive, the second stage of the experiment tends to support a more conclusive approach to determine the real influence of EB689 over mice.

Elevated-plus maze was used to assess anxiety effects of treatments in the first stage of the experiments. Briefly, an increase in open arm activity duration reflects anti-anxiety - or anxiolytic-like - behavior over the approach-avoidance conflict well established for EPM (Walf and Frye, 2007; Carobrez and Bertoglio, 2005). In the present work, EPM analysis showed that animals remained longer in closed arms in a clear choice for the closed over the open arms, both for treated and control animals. It seems that EB689 does not influence the conflict between approach and avoidance, once animals did chose the closed arms as a normal expression of anxiety-like avoidance behavior. It was observed that although treated animals chose the closed arms, the number of center crosses was low, indicating that animals did not explore whatsoever. The results are in accordance with OF. On the other hand, this phenomenon was observed in the group that received 4.8 mg/kg, although the tendency was not statistically significant. These results bolster our findings. After the administration of doses 312.5 mg/kg down to 4.8 mg/kg there was a dose-dependent recovery of all parameters, when compared to the control. EPM was not assessed in the second stage of experiments, but only OF. Although OF and EPM are adequate and traditionally effectiv methods for the evaluation of possible influences of a treatment over locomotion, anxiety and emotionality; as it was made in the present work, further specific *in vivo* tests should be performed in order to refine the pharmacology and toxicology of EB689. The pronounced effects caused by the *i.p.* administration of the extract have been understood so far.

At the necropsy, it was observed the presence of hemorrhages at all administered doses, except for the non-lethal dose, which led us to disregard sedation as a possible effect of EB689. Thus, a relationship between the OF and EPMc signs first observed with hemorrhagic shock could be made. Hypovolemic shock refers to a medical or surgical condition in which rapid fluid loss results in multiple organ failure due to inadequate circulating volume and subsequent inadequate perfusion. Most often, hypovolemic shock is secondary to rapid blood loss. In this case, a general weakness, cyanosis, rapid breathing followed by a reduced breathing and the activation of the sympathetic autonomous nervous system occurs previous to death. The toxicity of EB689 over the intestine endothelium may be causing internal hemorrhage and pain, leading to hypovolemic shock. Maybe this could be the main cause of the lack of immobility in OF and EPM, misleading any interpretation of parameters related to anxiety and/or emotionality. Ubiquitously, there are plants interfering in bleeding, such as Gingko (*Gingko biloba*), excess garlic (*Allium sativum*), ginger (*Zingiber officinale*), ginseng (*Panax* spp.), horse chestnut (*Aesculus hyppocastanum*), turmeric (Curcuma longa) and white willow (*Salix alba*), affecting clotting in one way or another (http://umm.edu/health/medical/altmed/condition/hemophilia, accessed in 11/10/2013). As *A. auriculata* is now being introduced to the light of pharmacology and chemistry, its chemical composition has not been dilucidated so far; for that reason, it is still hard to propose a relationship between bleeding and the compounds that have been isolated.

Although lupeol and eucryphin were isolated from EB689, they were not tested in the toxicological assay. Further recollection of the plant species shall be made in order to perform *in vivo* evaluations of isolated compounds. Nonetheless, lupeol is widely studied in terms of biological or pharmacological activities, particularly as anticancer agents and as cytotoxic for tumor cell lines, as can be seen elsewhere. On the other hand, eucryphin has been poorly studied. Han et al. (1998) described that eucryphin enhanced lypolysis in rat fat cells. Also, Kimura and colleagues (2007) reported that eucryphin, isolated from *Astilbe* species, is traditionally used

to heal cuts and insect bites. Sumiyoshi and Kimura (2010) have described the influence of eucryphin (**2**) over burn wound healing and have reported that eucryphin would interfere with IL-1beta, MCP-1, VEGF, and TGF-beta1 levels in exudates from wounds; and increased the expression of VEGF, TGF-beta1, and HIF-1alpha of keratinocytes. Although such biological activities were reportedly associated to eucryphin there is no support that correlates such findings with the toxicity profile here reported.

EB689 was selected from a biological screening aiming to the identification of cytotoxic Brazilian Amazon plant extracts against cancer cell lines. In the present work, it was observed that EB689 significantly decreased locomotion in mice and that may be caused by an evident internal intestinal bleeding result of *i.p.* administration of the extract. It is unlikely that lupeol (**1**) and eucryphin (**2**) – here described for the first time as constituents of *A. auriculata* - could be involved in the toxicology of *A. auriculata*. The effects of EB689 have been further elucidated, and they make up the basis for further pharmacological assays aiming towards the analysis of antitumor activity.

Authors' contributions

DFG and DME (MsC students) carried out the laboratory work and biological studies. MLBP and ADV contributed by collecting plant samples, identification, and herbarium confection. IECD contributed in phytochemical isolation of compounds and chromatographic/spectrometric analysis. RNY, LFLR and EFSM contributed to critical reading of the manuscript and animal laboratory work. IBS and MMB designed the study, supervised the laboratory work, laboratory work itself, analysis of data, drafted the paper and performed critical reading/review of the manuscript. All the authors have read the final manuscript and approved the submission.

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