Revista Brasileira de Farmacognosia 26 (2016) 216–224

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

Casinga-cheirosa organic extract impairment over Balb-c male mice behavioral phenotype

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ARTICLE INFO

Article history:
Received 14 April 2015
Accepted 13 October 2015
Available online 8 December 2015

Keywords:
Laetia suaveolens
Salicaceae
Open field
Elevated-plus maze
Locomotion
Emotionality

ABSTRACT

Laetia suaveolens (Poepp.) Benth., Salicaceae, popularly known as "casinga-cheirosa", "caferana", or "laranjinha", is native to Brazil but not endemic to this country. A crude organic extract was obtained from the leaves and stem and intraperitoneally administered in male Balb-c mice. Its behavioral effects were evaluated in the open field and elevated plus maze in a two-stage experiment that assessed ten different parameters related to behavior as locomotion, emotionality, and anxiety. In the first stage of the experiment, intraperitoneal the crude organic extract administration dose-dependently impaired locomotion and emotionality 30–120 min after administration. A significant decrease in defecation was observed, which was related to emotionality. No alterations in the elevated plus maze were found; thus, this apparatus was not used in the next stage of the experiment. In the second stage, the previously determined non-lethal dose of 0.1563 g/kg was intraperitoneally administered, which impaired locomotion and rearing frequency and increased immobility time. Necropsy revealed smooth intestine hemorrhage. Rutin, leucoside, nicotiflorin, guaijaverin, and astragalin were isolated from the crude organic extract. This is the first time that these compounds have been identified in *L. suaveolens*. In conclusion, the crude organic extract impaired locomotion and emotionality and caused hemorrhage in male Balb-c mice, indicating that its consumption can be harmful to humans and animals. The present results provide a basis for further studies on the pharmacology, toxicology, and natural product chemistry of the crude organic extract.

Introduction

Laetia suaveolens (Poepp.) Benth. belongs to the Salicaceae family. It is popularly known as "casinga-cheirosa", "caferana", or "laranjinha" in Brazil (Gama et al., 2007). Although native to Brazil, it is not endemic to this country (Boletim Museu Paraense Emilio Goeldi, 2013). It is widely distributed in South America, especially Brazil, Bolivia, Colombia, Venezuela, Guyana, and Peru. According to the Missouri Botanical Garden, its basyonim is *Samydia suaveolens* Poepp., but it may also be known as *Casinga suaveolens* Griseb. ex Benth., *Guidonia calophylla* Kuntze, *G. suaveolens* Kuntze, *L. calophylla* Eichler, and *S. petiolaris* Spruce ex Eichler. In the present study, we adopted the nomenclature proposed by the Missouri Botanical Garden (2014). *L. suaveolens* is also popularly known as *yutubanco-del-bajo* in Spanish Amazonia (Revilla, 2002) and is commonly used in construction. The first reports on this species indicated that an organic extract that was obtained from the leaves and stem of *L. suaveolens* (designated EB719) showed cytotoxic activity (30.05% lethality in relation to cell growth control) against prostate cancer cell lines (Suffredini et al., 2006) and squamous cell carcinoma (Ozi et al., 2011). The effects of EB719 on general activity, including an analysis of 27 parameters related to the central nervous system, autonomic nervous system, and sensorial and psychomotor systems, were previously reported, and seven molecules contained in this species were described (Estork et al., 2014).

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http://dx.doi.org/10.1016/j.bjp.2015.10.004
0102-695X/

In the present study, we further evaluated the behavioral effects of EB719 in male Balb-c mice, as it has never been done before and no further information could be retained from popular uses. We also identified five molecules in *L. suaveolens* that were not reported previously.

Materials and methods

Plant material

The leaves and stem of *Laetia suaveolens* (Poepp.) Benth., Salicaceae, were collected in the Brazilian Amazon rain forest under Brazilian government licenses (no. CGen/MMA#12A/2008 and no. MMA/ICMBio/SISBIO#14895). The collection was made in the surrounding area of Manaus city (Lat 2°58′, Long 60°26′), Amazonas, in a seasonally flooded forest from Rio Negro Basin (Igapó forest). A voucher specimen was deposited at the UNIP Herbarium [A.A. Oliveira, 3383 (UNIP)], collected in April/1999, including voucher specimens for the first and second collections [M.B.P., 3093 (UNIP)], collected in March/2009, and [M.B.P., 3203 (UNIP)], collected in March/2010, respectively. EB719 was obtained by maceration as previously described (Suffredini et al., 2007).

Preparation of extract and diazepam for administration

The technique for preparing EB719 for administration in animals was described elsewhere (Gusmão et al., 2013b; Estork et al., 2014). Briefly, plant material was subjected to 24 h maceration with dichloromethane:methanol (1:1). The solvents were removed by rotary evaporation, and the extract was kept in a freezer until use. The extracts were dissolved in almond oil, which was also used as the vehicle control. Diazepam (Hipolabor; lot no. AO011/11; validity: 10/13; concentration, 5 mg/ml; injectable medication) was administered at a dose of 1 mg/kg.

Animals

Adult male Balb-c mice (*Mus musculus*), weighing 19–23 g, were obtained from the Animal Facilities of São Paulo University. After arrival in the laboratory, the animals were randomly selected, individually marked, and housed in groups of five in isolated polypropylene cages (38 cm × 32 cm × 16 cm) under controlled temperature (21 ± 2 °C) and humidity (55–60%). Artificial lighting was provided (12 h/12 h light/dark cycle, lights on at 8 am), with free access to Nuvilab rodent chow (Nuvital Company, São Paulo, Brazil) and an unlimited supply of filtered water. The experiments began one week after the mice arrived in the laboratory, allowing for adaptation to the new environmental conditions. The animals were fasted for 1 h before receiving the treatments. The animals were observed for toxic responses. If lethality occurred during the period of observation, then necropsy was performed. If the animals survived until the end of the 14 day observation period, then they were humanely euthanized in a CO_2 gas chamber according to Ethics Committee directives. The euthanized animals also underwent necropsy, and individual records of the necropsy were kept. All of the experiments received Ethics Committee approval (CEP/ICS/UNIP 025/08, February 12, 2009).

Open field

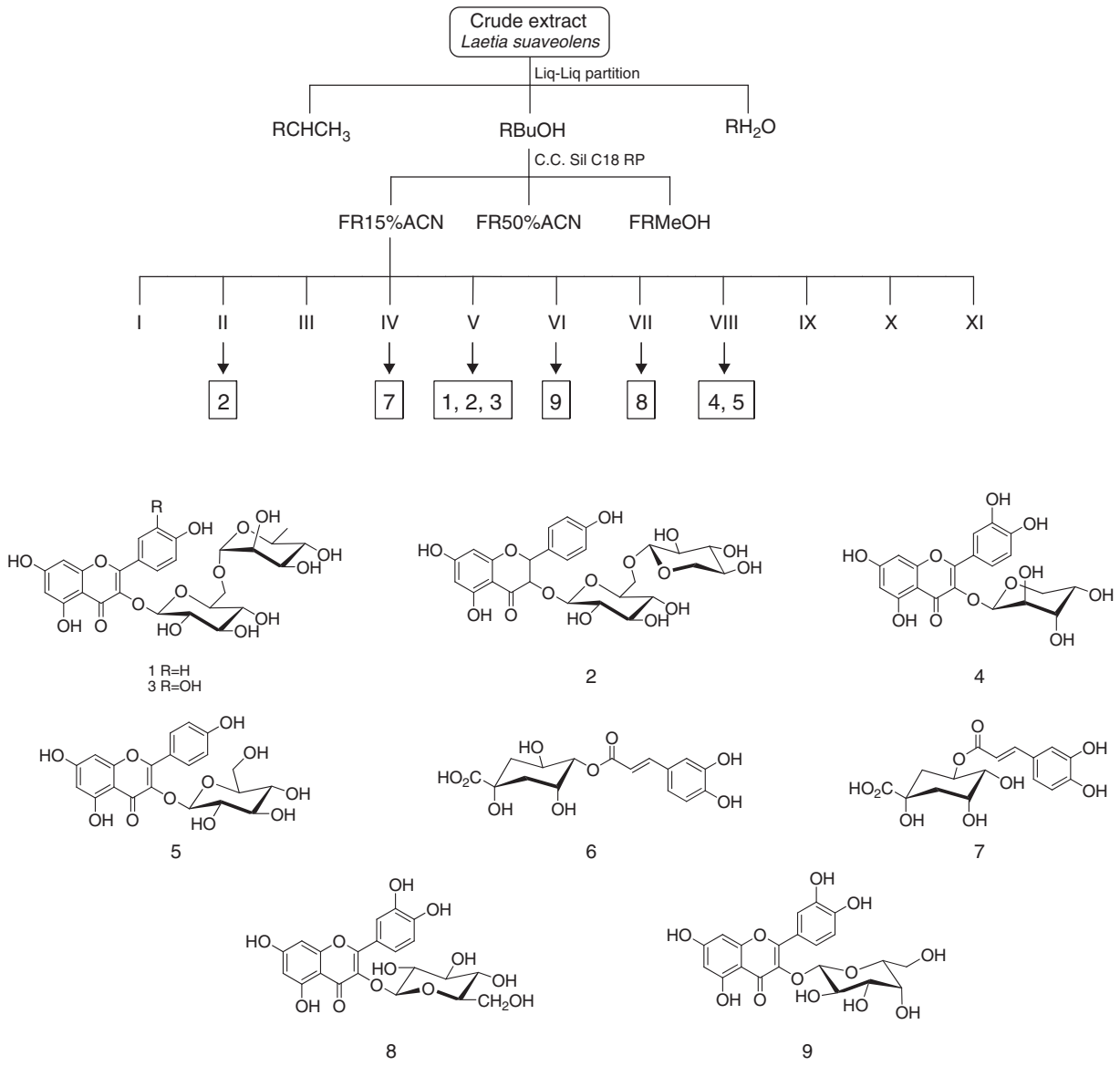
An open field (OF) was used to assess the effects of the extract on emotionality and motility and constructed according to Broadhurst (1960). The apparatus was adapted to the size of mice. Emotionality can be defined as the observable behavioral and physiological components of mice emotion, yet to be used in behavioral studies for both humans and animals. When studies are made in animals, OF is one of the recommended apparatus to evaluate emotionality. The OF apparatus was a circular wooden box (40 cm diameter, 50 cm height) with an open top and floor divided into 19 squares. Illumination (400 lx) was provided by a 100 W lamp at the floor of the apparatus. Each animal was individually placed in the center of the OF arena, and the parameters were measured in five 5-min periods: 10–15 min, 30–35 min, 60–65 min, 120–125 min, and 180–185 min after extract administration. Hand-operated counters were used to count locomotion frequency (*i.e.*, the number of squares crossed with all four paws), rearing frequency (*i.e.*, one unit of rearing corresponded to a standing position on the hindlimbs, with the trunk perpendicular to the floor, head tilted up, and forelimbs in the air, either touching or not touching the walls of the arena), and the number of fecal boli at the end of the session. A chronometer was used to measure the duration of immobility (*i.e.*, the total time in seconds without spontaneous movements, when the head, trunk, and limbs were still) and duration of grooming (*i.e.*, the total time of washing movements over the head, licking the paws and fur, and tail/genital cleaning). To minimize the possible influence of circadian changes on behavior in the 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 possible bias caused by odors left by previous animals. The experimental sessions began at 1:00 pm and ended before 5:00 pm to prevent circadian influences.

Elevated plus maze

The elevated plus maze (EPM) was first conceived by British psychologist Sheila Handley's group as a model to evaluate anxiety, and it is one of the most used apparatus for that purpose (Lapiz-Bluhm et al., 2008). The EPM was made of wood and had two open arms (23.5 cm × 8 cm) and two closed arms of the same size with 20 cm high walls. The apparatus was elevated 80 cm above the floor. It was located in a sound-proof room with a 100 W lamp at the floor of apparatus that provided 400 lx illumination. Two behaviors are readily noticeable in the EPM: avoidance of the open arms while staying in the closed arm and escape from an open arm directly to a closed arm (Pinheiro et al., 2007). In the present study, the apparatus was used to assess anxiety-like behavior in the first stage of the experiment. The animals were tested in the EPM after being tested in the OF. Exploratory behavior was defined as the number of entries into the closed arms and number of crosses in the central area of the apparatus. The animals were first placed in the center of the maze, which was previously cleaned with 5% alcohol. The number of open-arm and closed-arm entries, time spent in each arm, and number central area crossings were measured. Observations were made in five 5-min sessions: 15–20 min, 35–40 min, 65–70 min, 125–130 min, and 185–190 min after extract administration. Because reexposure to the EPM may represent spatial memory of an anxiolytic/anxiogenic condition, the mice were observed in the remained sessions (Onodera et al., 1998; Miyazaki et al., 1995; Sharma and Kulkarni, 1992).

Experimental design

To investigate the effects of EB719 on the behavioral phenotype of mice, a two-stage experiment was performed. In the first stage, the mice were divided into four groups: vehicle group, 0.625 g/kg EB719 treatment group, 0.3125 g/kg EB719 treatment group, and 0.1563 g/kg EB719 treatment group. The ability of EB719 to cause alterations in behavioral phenotype after *i.p.* administration was assessed in stage 1 with a reduced number of animals ($n = 3$ per



Scheme 1. Fractionation of crude organic extract EB719, obtained from the leaves and stem of *Laetia suaveolens*.

group). Assessments in the OF and EPM were performed immediately after assessing general behavioral changes (Gusmão et al., 2013a). Stage 1 of the experiment began with administration of 0.625 g/kg EB719 in a group of three animals. The mice were observed for general behavioral activity in the OF and EPM and death. Because death occurred in the group of mice that received the higher dose of EB719, a half-fold dilution was subsequently administered in a second group of animals, and the observations were made again. This procedure was repeated until a non-lethal dose (NLD) was determined, defined as the dose that caused no death in mice after *i.p.* administration. In the second stage of the experiment, the NLD was administered *i.p.* in ten male mice that were divided into four groups: naive, vehicle (almond oil) control, diazepam, and NLD. The animals were again subjected to behavioral observations. To avoid circadian influences on the experimental results, the assays began at 1 pm and ended before 5 pm. All of the mice underwent necropsy after they died.

Statistical analysis

The results were analyzed using two-way repeated-measures analysis of variance (ANOVA; Zar, 1999) followed by the Bonferroni *post hoc* test. The statistical analysis was performed using GraphPad Prism 5.0 software. Values of $p < 0.05$ were considered statistically significant.

Extract fractionation and compound identification

The following techniques were used for chemical isolation: liquid–liquid partition (LLP), analytical liquid chromatography–diode array dispositive (LC–DAD), and semi-preparative liquid chromatography coupled with ultraviolet spectrophotometer (LC–UV) as described elsewhere (Estork et al., 2014). In the present work, the fractionation of fraction Fr15%ACNRBuOH, obtained from the FBuOH LLP-fraction of EB719 (Scheme 1) is described. Using the semi-preparative LC–UV technique, Fr15%ACNRBuOH was fractionated to yield eleven fractions, which were analyzed by LC–DAD. From fraction II, 3-*O*-caffeoylquinic (6) acid was isolated. From fraction IV, 5-*O*-feruloylquinic acid (7) was isolated. From fraction VI, hyperoside (9) was isolated. From fraction VII, isoquercitrin (8) was isolated. These four molecules (Scheme 1) were identified by hydrogen and carbon nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR,

respectively) analyses and high-performance liquid chromatography/electron spray ionization-mass spectrometry (HPLC/ESI-MS^n) as described elsewhere (Estork et al., 2014). Fractions V (18.6 mg) and VIII (27.7 mg) were also analyzed by ^{1}H NMR, ^{13}C NMR, and HPLC/ESI-MS^n, and the results are presented herein.

The ^{1}H NMR and ^{13}C NMR spectra of fraction V showed chemical shifts that corresponded to a mixture of the following flavonoids: quercetin-3-*O*-rhamnopyranosyl-(1 → 6)-glucopyranoside (1) (Yang et al., 2011), also called rutin or quercetin 3-*O*-rutinoside, kaempferol-3-*O*-xylopyranosyl-(1 → 6)-glucopyranoside (2) (Hubner et al., 1999; Chung and Lee, 2012), also called leucoside or kaempferol 3-*O*-sambubioside, and kaempferol-3-*O*-rhamnopyranosyl-(1 → 6)-glucopyranoside (3) (Olennikov et al., 2012; Lee et al., 2010), also called nicotiflorin or kaempferol 3-*O*-rutinoside. The ESI-mass spectra of fraction V in negative ion mode showed three peaks at 7.6 min that corresponded to molecular ions $[M-H]^-$ at *m*/*z* 609.2, 579.2, and 369 and three peaks at 7.7 min that corresponded to molecular ions $[M-H]^-$ at *m*/*z* 593.2, 567.3, and 413.2. Further investigation of the ion at *m*/*z* 609.2 (1) in the LC–MS^2 experiment yielded several fragment ions at *m*/*z* 591.1, 463, 342.9, 300.9, 270.9, 254.9, and 179.1. The daughter ion at *m*/*z* 591.1 directly originated from the parent ion at *m*/*z* 609.2 by the loss of H_2O $[M-H-H_2O]^-$. The ion at *m*/*z* 463 was produced from the neutral loss of a rhamnose $[M-H\text{-}Rham]^-$, which was in agreement with previous data (Cuyckens and Claeys, 2004). The ion at *m*/*z* 342.9 was produced by cross-ring cleavages of the glucose residue [M−H-Rham-120], which was also in agreement with the literature (Cuyckens and Claeys, 2004; Shi et al., 2007). The additional loss of 162 (glucose) or direct loss of the rutinose residue resulted in the aglycone quercetin ion at *m*/*z* 300.9 $[M-H\text{-}Rham\text{-}Glc]^-$. These data were also in agreement with the literature (Cuyckens and Claeys, 2004). The ion at *m*/*z* 270.9 was produced by the loss of CH_2O from aglycone $[M-H\text{-}Rham\text{-}Glc\text{-}CH_2O]^-$, and the ion at *m*/*z* 254.9 was produced by the loss of the H_2O and CO from aglycone $[M-H\text{-}Rham\text{-}Glc\text{-}H_2O\text{-}CO]^-$. These findings were in full agreement with the results of a previous study (Cuyckens and Claeys, 2004). The ion at *m*/*z* 179 differed by a mass of 122 Da (neutral molecule) from its corresponding ion of aglycone (*m*/*z* 300.9), which is also consistent with the literature (Chen et al., 2002). The LC–MS^2 investigations of the ion at *m*/*z* 579.2 (2) yielded several fragment ions at *m*/*z* 447, 356.9, 326.9, 284.9, 255.0, 200.1, and 169.5. The daughter ion at *m*/*z* 447 was produced directly from its parent ion at *m*/*z* 579.2 by the neutral loss of xylose (132 Da) $[M-H\text{-}Xyl]^-$, which was also consistent with previous data (Abu-Reidah et al., 2012). The ions at *m*/*z* 356.9 and 326.9 were produced by cross-ring cleavages of the glucose residue by the loss of $[M-H\text{-}Xyl\text{-}90]^-$ and $[M-H\text{-}Xyl\text{-}120]^-$, respectively, which was in agreement with the literature (Chen et al., 2002). The additional loss of 162 (glucose) or direct loss of the sambubioside residue resulted in the aglycone kaempferol ion at *m*/*z* 284.9 $[M-H\text{-}Xyl\text{-}Glc]^-$, which was consistent with the literature (Abu-Reidah et al., 2012). The ion at *m*/*z* 255 differed by a mass of 30 Da (neutral molecule) from its related ion of aglycone (*m*/*z* 284.9), which was consistent with the literature (Ye et al., 2005). Further LC–MS^2 investigation of the ion at *m*/*z* 593.2 (3) showed several ion fragments at *m*/*z* 447, 428.9, 326.9, 284.9, and 254.9. The ion at *m*/*z* 447 was produced directly from the parent ion at *m*/*z* 593.2 by the neutral loss of rhamnose $[M-H\text{-}Rham]^-$. The ion at *m*/*z* 428.9 was generated by the loss of neutral molecules of rhamnose and water $[M-H\text{-}Rham\text{-}H_2O]^-$, and the ion at *m*/*z* 326.9 was produced by cross-ring cleavages of the glucose residue, which was in agreement with the literature (Cuyckens and Claeys, 2004; Shi et al., 2007). The additional loss of 162 (glucose) or direct loss of the rutinose residue resulted in the aglycone kaempferol ion at *m*/*z* 284.9 $[M-H\text{-}Rham\text{-}Glc]^-$, which was consistent with the literature (Cuyckens et al., 2001). The ion at *m*/*z* 254.9 differed by a mass of 30 Da (neutral molecule) from the ion of aglycone (*m*/*z* 284.9), which was consistent with the literature (Ye et al., 2005).

The ^{1}H NMR and ^{13}C NMR spectra of fraction VIII showed chemical shifts of the flavonoid mixture: quercetin 3-*O*-arabinose or guaijaverin (4) (Yoshida et al., 1992; Prabu et al., 2006) and kaempferol 3-*O*-glucoside (5) or astragalin (Demirezer et al., 2006; Yang et al., 2005). The ESI-mass spectra of fraction VIII in negative ion mode showed three peaks at 8.8 min of the molecular ions $[M-H]^-$ at *m*/*z* 867.2, 447.1, and 433. Further LC–MS^2 investigation of the ion at *m*/*z* 867.2 yielded two fragment ions at *m*/*z* 432.9 and 300.9, indicating that it corresponded to the molecular ion of the dimer $[2M-H]^-$ from the compound with a molecular ion at *m*/*z* 433 $[M-H]^-$. The LC–MS^2 analysis of the ion at *m*/*z* 433 yielded several fragment ions at *m*/*z* 342.9, 300.9, and 179. The ion at *m*/*z* 342.9 was produced by cross-ring cleavages of the glucose residue [M−H-Rham-90], which was in agreement with the literature (Cuyckens and Claeys, 2004). An additional neutral loss of 132 (arabinose, neutral molecule) resulted in the aglycone quercetin ion at *m*/*z* 300.9 $[M-H\text{-}Ara]^-$, which was also consistent with the literature (Prabu et al., 2006). The LC–MS^2 analysis of the ion at *m*/*z* 447.1 revealed fragment ions at *m*/*z* 431.9, 401.9, 357, 326.9, 283.9, and 255. The ions at *m*/*z* 431.9 and 401.9 were produced by the loss of 15 and 45 Da, respectively. The ions at *m*/*z* 357 and 326.9 were produced by cross-ring cleavages of the glucose residue (loss of 90 and 120 Da, respectively), which was consistent with the literature (Cuyckens and Claeys, 2004). The mass spectra showed abundantions at *m*/*z* 284.9 and 283.9 as the base peak through fission of the glycosidic bond to form an aglycone ion (*m*/*z* 284.9) $[M-H\text{-}Glc]^-$ and radical aglycone ion (*m*/*z* 283.9) $[M-H\text{-}Glc\text{-}H]^-$ by hemolytic cleavage, respectively, which were also in agreement with the literature (Kite and Veitch, 2009). The ion at *m*/*z* 283.9 was the precursor of the ion at *m*/*z* 255 $[M-H\text{-}Glc\text{-}H\text{-}CO\text{-}H]^-$ (Ablajan et al., 2006).

The presence of 4-*O*-caffeoylquinic acid (6), 5-*O*-feruoylquinic acid (7), and isoquercitrin (8) was also found, although these results were reported previously (Estork et al., 2014).

Results and discussion

In the first stage of the behavioral phenotyping, we evaluated various parameters in the OF and EPM. The OF results are shown in Fig. 1 ($n = 3$, $n_{total} = 12$). Locomotion frequency (Fig. 1A) was influenced by the treatment, which accounted for 31.12% of the total variance ($F_{3,8} = 15.06$, $p < 0.01$), and time accounted for 32.37% of the total variance ($F_{4,32} = 15.52$, $p < 0.001$). The interaction between time and treatment accounted for 14.30% of the total variance ($F_{12,32} = 2.29$, $p < 0.05$). Thirty minutes after EB719 administration, a significant dose- and time-dependent decrease in locomotion was observed at all three concentrations tested. A significant reduction of locomotion was observed 30–120 min after *i.p.* administration of 0.625 g/kg EB719 ($p < 0.05$ at 30 min; $p < 0.01$ at 60 min; $p < 0.001$ at 120 min) compared with the control group. The immobility time results (Fig. 1B) showed a significant effect of time, which accounted for 29.13% of the total variance ($F_{4,32} = 9.66$, $p < 0.001$), but no effect of treatment was found, with no interaction between time and treatment. Although no significant differences were obtained, a tendency toward immobilization was observed in mice 30–120 min after *i.p.* administration of EB719. The differences in rearing frequency were not significant ($p > 0.05$; Fig. 1C), although a tendency was observed in the animals' staying more frequently in a standing position after administration of the smaller dose of EB719; the opposite was observed after administration of the higher dose. The different doses of EB719 accounted for 41.35% of the total variance in grooming ($F_{3,8} = 10.21$, $p < 0.01$; Fig. 2A), and the interaction between treatment and time accounted for 21.66% of the total variance ($F_{12,32} = 2.87$, $p < 0.01$).

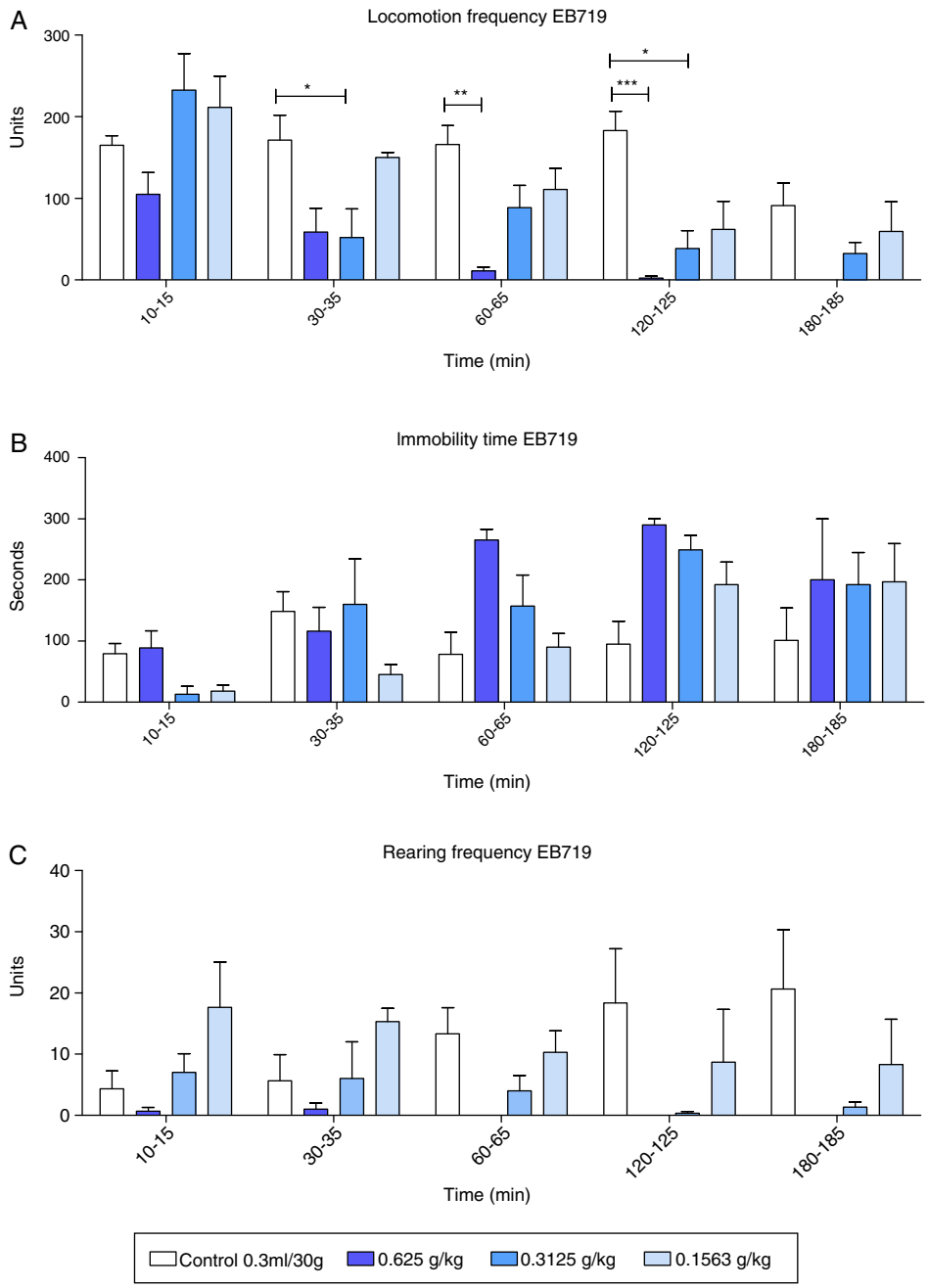


Fig. 1. Effects of intraperitoneal administration of different doses of EB719, the organic extract obtained from *Laetia suaveolens*, in male mice in the open field in stage 1 of the experiment. (A) Locomotion frequency (mean ± standard error). (B) Immobility time (mean ± standard error). (C) Rearing frequency (mean ± standard error). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, vehicle control group compared with the three treatment groups (two-way repeated-measures ANOVA followed by Bonferroni *post hoc* test).

Time did not have a significant effect ($p > 0.05$). Grooming decreased in mice that received 0.625 g/kg EB719 60 min after administration ($p < 0.01$). In mice that received 0.625 and 0.3125 g/kg EB719, grooming decreased 120 min after administration ($p < 0.05$). In mice that received 0.625 g/kg EB719, grooming decreased 180 min after administration ($p < 0.05$). Treatment accounted for 31.98% of the total variance in defecation ($F_{3,8} = 14.04$, $p < 0.01$), and time accounted for 14.98% of the total variance ($F_{4,32} = 4.55$, $p < 0.01$). The interaction between treatment and time accounted for 20.65% of the total variance ($F_{12,32} = 2.09$, $p < 0.05$; Fig. 2B). A significant decrease in defecation was observed in mice that received 0.625 g/kg ($p < 0.001$) and 0.3125 g/kg ($p < 0.001$) EB719 60 min after *i.p.* administration.

No significant differences were observed in the EPM in the first session that evaluated anxiolytic/anxiogenic-like effects or remaining sessions that evaluated learning/memory in an anxiolytic/anxiogenic context (Supplemental Fig. S1) in the first stage of the experiment.

In the second stage of the experiment, the NLD of 0.1563 g/kg was administered *i.p.* in male mice, and the animals were evaluated in the OF. Treatment accounted for 19.24% of the total variance in the analysis of rearing frequency ($F_{3,28} = 4.29$, $p < 0.05$; Fig. 3A). Time did not significantly influence the total variance (0.55%). The interaction between treatment and time accounted for 11.99% of the total variance ($F_{12,112} = 4.20$, $p < 0.001$). The first observation that occurred 10 min after treatment showed that the group that received diazepam exhibited a behavioral phenotype that was statistically identical to the naive control group ($p > 0.05$), whereas the vehicle control group exhibited a decrease in rearing compared with the naive control group ($p < 0.05$). EB719

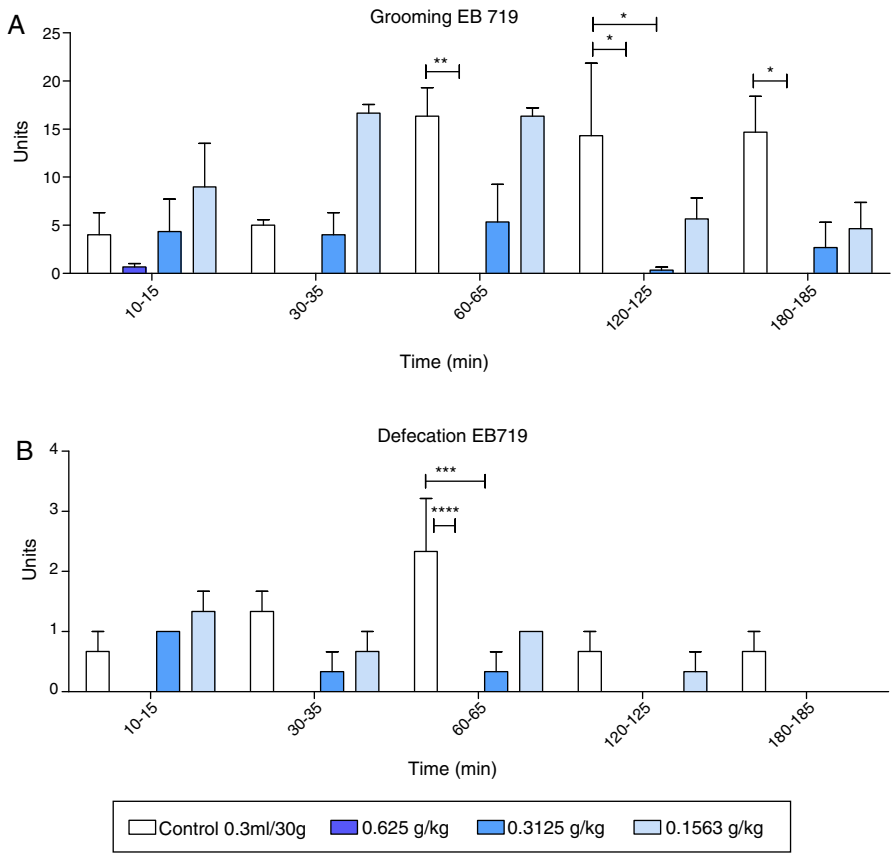


Fig. 2. Effects of intraperitoneal administration of different doses of EB719, the organic extract obtained from *Laetia suaveolens*, in male mice in the open field in stage 1 of the experiment. (A) Grooming (mean ± standard error). (B) Defecation (mean ± standard error). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, vehicle control group compared with the three treatment groups (two-way repeated-measures ANOVA followed by Bonferroni *post hoc* test).

administration decreased rearing frequency compared with the naive group 10 min ($p < 0.05$) and 180 min ($p < 0.01$) after administration compared with the diazepam group ($p < 0.05$). The immobility time data showed that treatment accounted for 18.52% of the total variance ($F_{3,28} = 5.89$, $p < 0.01$), and time accounted for 4.55% of the total variance ($F_{4,112} = 4.54$, $p < 0.01$). The interaction between treatment and time accounted for 17.24% of the total variance ($F_{12,112} = 5.74$, $p < 0.001$). EB719 clearly increased immobility time (Fig. 3B) compared with the naive control group 120 min ($p < 0.05$) and 180 min ($p < 0.001$) after *i.p.* administration and compared with the vehicle control group at the same time points ($p < 0.01$ and $p < 0.001$, respectively). Treatment accounted for 17.87% of the total variance ($F_{3,28} = 6.62$, $p < 0.01$) in locomotion frequency (Fig. 3C), and time accounted for 18.26% of the total variance ($F_{4,112} = 20.96$, $p < 0.001$). The interaction between treatment and time accounted for 10.72% of the total variance ($F_{12,112} = 4.10$, $p < 0.001$). EB719 decreased locomotion compared with the vehicle control group 10 min ($p < 0.01$), 120 min ($p < 0.05$), and 180 min ($p < 0.01$) after administration and compared with the naive control group 180 min after administration ($p < 0.05$). For grooming, treatment accounted for 12.07% of the total variance ($F_{3,28} = 5.10$, $p < 0.01$), and the interaction between treatment and time accounted for 13.49% of the total variance ($F_{12,112} = 2.65$, $p < 0.01$; Fig. 3D). The animals that received EB719 exhibited an increase in grooming compared with the animals that received diazepam 30 min after *i.p.* administration. For defecation, treatment accounted for 14.67% of the total variance ($F_{3,28} = 8.83$, $p < 0.001$), and the interaction between treatment and time accounted for 12.65% of the total variance ($F_{12,112} = 2.11$, $p < 0.05$; Fig. 3E). Naive control animals accounted for most of the differences in this parameter, particularly the decrease in fecal boli 30 min ($p < 0.001$) and 120 min ($p < 0.05$) after *i.p.* EB719 administration and 30 min after diazepam administration ($p < 0.001$).

The EPM test was not performed in the second stage of the experiment because no significant differences were observed in the first stage.

The crude organic extract of *L. suaveolens* (EB719) was previously reported to be cytotoxic against prostate cancer cells (Suffredini et al., 2006) and squamous cell carcinoma (Ozi et al., 2011), and its influence on general behavior was also evaluated (Estork et al., 2014). To complement the initial reports on the pharmacological and chemical profiles of EB719, the present study further investigated the effects of EB719 on the behavioral phenotype of male Balb-c mice. We also identified five flavonoids in *L. suaveolens* that were not reported previously. Intraperitoneal EB719 administration significantly impaired locomotion, with effects that were similar to diazepam but significantly different from the vehicle control, with the exception of the first observation 10 min after extract administration. Animals that are evaluated in the OF tend to exhibit an increase in locomotion frequency when they are first exposed to the apparatus, and such behavior was observed in the vehicle control group in the second stage of the experiment. A significant increase in immobility time was observed in the group that received EB719 35 min after administration. A decrease in rearing frequency and increase in grooming were observed 30 min after EB719 administration. Defecation was also influenced by EB719, reflecting emotionality in the mice. Necropsy was performed in animals that died after EB719 administration. Extensive reddish areas with hemorrhage and consequent bleeding were observed in the small intestine endothelium, which may be the likely reason for death. The ingestion of some plants can cause bleeding. Reports on leaf extracts of *Gingko biloba* showed

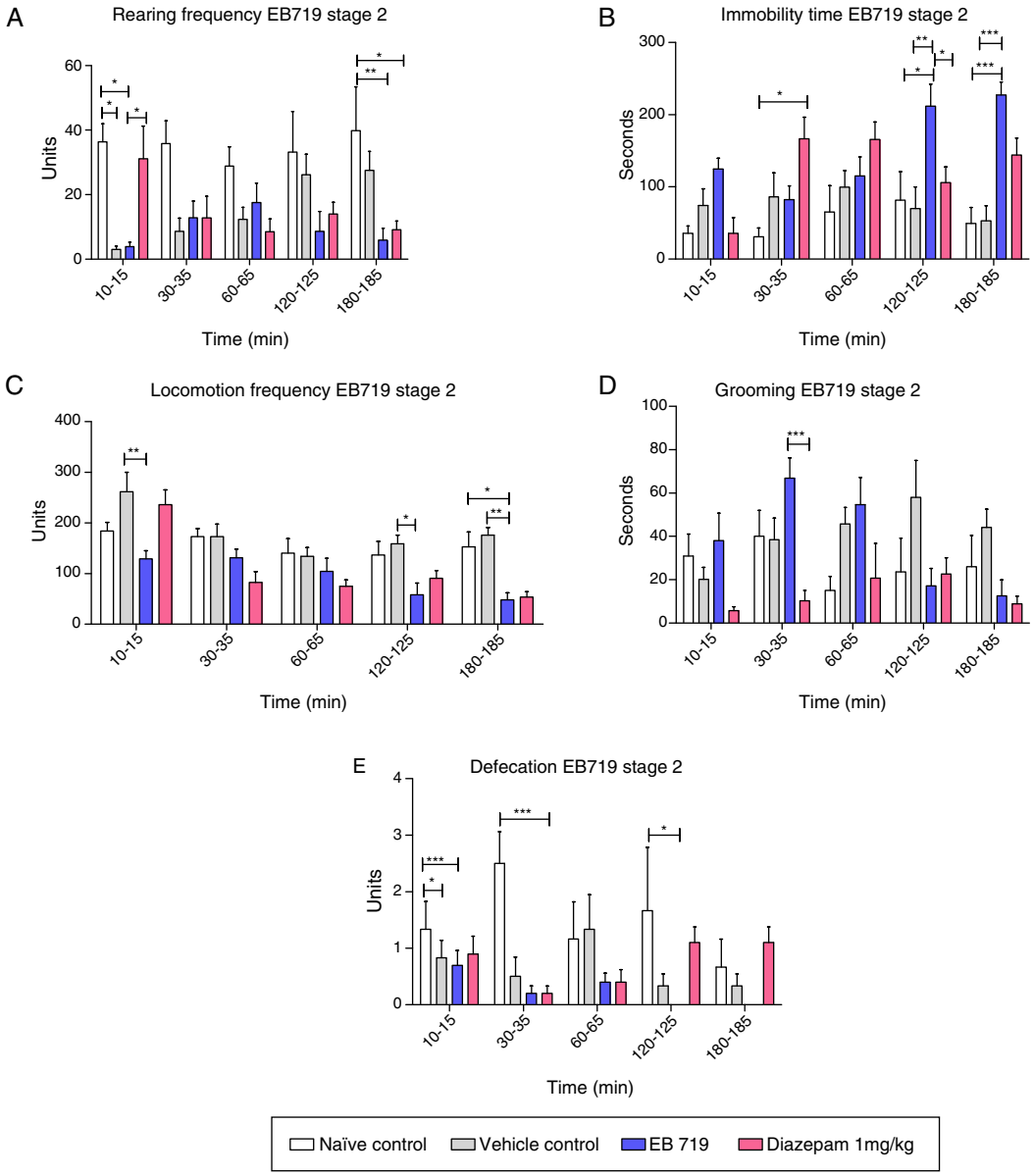


Fig. 3. Effects of intraperitoneal administration of the non-lethal dose of EB719, the organic extract obtained from *Laetia suaveolens*, in male mice in the open field in stage 2 of the experiment. (A) Rearing frequency (mean ± standard error). (B) Immobility time (mean ± standard error). (C) Locomotion frequency (mean ± standard error). (D) Grooming (mean ± standard error). (E) Defecation (mean ± standard error). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, vehicle control group, diazepam control group, and naive control group compared with non-lethal dose group (two-way repeated-measures ANOVA followed by Bonferroni *post hoc* test).

that they caused hemorrhage (Koch, 2005), in addition to other plant species (Moro et al., 2001). Considering the impairments in locomotion and emotionality and the fact that intraperitoneal administration of an organic extract of *casinga-cheirosa* can cause hemorrhage, its use as a popular medicine may be harmful and should be avoided.

Five flavonoids were isolated from EB719: rutin, leucoside, nicotiflorin, guaijaverin, and astragalin. No studies have reported the influence of these flavonoids on hemorrhage. Nonetheless, some of these compounds, such as rutin, are well known as vascular protective agents that act by reducing capillary permeability, reverting its fragility, maybe by an anti-thrombotic activity (Gryglewski et al., 1987). Such activity was not observed in animals that died, which may be attributable to other as-yet-unidentified compounds in EB719 that may have interfered with hemorrhage. Rutin was shown to interfere with the activity of racemic warfarin when they were concomitantly administered, reducing its coagulant effect by decreasing the elimination half-life of one specific enantiomer (Chan et al., 2009). Studies of plants and phenolic compounds with antithrombolytic and antiplatelet activity have recently been performed, particularly to evaluate their use as nutritional supplements in menopausal women and in some cardiovascular conditions by targeting plasma coagulation factors (Bijak et al., 2014a,b) or fibrinogen (Chen et al., 2013; Rios et al., 2008). Although indicative, no one of the five flavonoids that was isolated from *L. suaveolens* were indicated as antithrombolytic or antiplatelet active, in any of the articles.

EB719 interfered with locomotion and emotionality in male mice after *i.p.* administration, but its effects on anxiety-like behavior and spatial learning in the EPM were inconclusive. The mode of action of EB719 on behavior cannot be directly compared with diazepam because smooth intestine hemorrhage was observed and may have been the main reason for the decrease in locomotion and emotionality.

Conclusions

Intraperitoneal EB719 administration influenced the behavioral phenotype of male Balb-c mice by impairing locomotion and emotionality. EB719 also exerted a strong effect on the smooth intestine, causing hemorrhage. Rutin, leucoside, nicotiflorin, guaijaverin, and astragalin were isolated from EB719, which were not previously identified in *L. suaveolens*.Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

DME (M.Sc. student) and DFG (M.Sc. student) ran the laboratory work. MLBP contributed to plant identification and herbarium confection. SAF contributed to plant collection, herbarium confection, and the laboratory work. IECD contributed to the chromatographic analysis and molecule identification. LFLR critically read the manuscript and contributed to the HPLC analysis. EFSM contributed to maintaining the animal facilities. 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.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

The authors thank Fundação de Amparo à Pesquisa do Estado de São Paulo for financial support (grant no. 2008/58706-8) and Michele Sanchez for technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2015.10.004.

References

Ablajan, K., Abliz, Z., Shang, X.-Y., He, J.-M., Zhang, R.-P., Shi, J.-G., 2006. Structural characterization of flavonol 3,7-di-*O*-glycosides and determination of the glycosylation position by using negative ion electrospray ionization tandem mass spectrometry. J. Mass Spectrom. 41, 352–360.

Abu-Reidah, I.M., Arráez-Román, D., Quirantes-Piné, R., Fernández-Arroyo, S., Segura-Carretero, A., Fernández-Gutiérrez, A., 2012. HPLC–ESI-Q-TOF-MS for a comprehensive characterization of bioactive phenolic compounds in cucumber whole fruit extract. Food Res. Int. 46, 108–117.

Bijak, M., Ponczek, M.B., Nowak, P., 2014a. Polyphenol compounds belonging to flavonoids inhibit activity of coagulation factor X. Int. J. Biol. Macromol. 65, 129–135.

Bijak, M., Ziewiecki, R., Saluk, J., Ponczek, M., Pawlaczyk, I., Krotkiewski, H., Wachowicz, B., Nowak, P., 2014b. Thrombin inhibitory activity of some polyphenolic compounds. Med. Chem. Res. 23, 2324–2337.

Boletim Museu Paraense Emilio Goeldi, 2013. http://scielo.iec.pa.gov.br/img/revistas/bmpegscn/v1n1/pdf/1a07a1.pdf (accessed 05.02.15).

Broadhurst, P.L., 1960. Experiments in psychogenetics, application of biometrical genetics to the inheritance of behavior. In: Hysenck, H.J. (Ed.), Experiments in Personality, vol. 1. Routledge & Kegan Paul, London, pp. 1–256.

Chan, E., Hedge, A., Chen, X., 2009. Effect of rutin on warfarin anticoagulation and pharmacokinetics of warfarin enantiomers in rats. J. Pharm. Pharmacol. 61, 451–458.

Chen, M., Song, F., Guo, M., Lui, Z., Liu, S., 2002. Analysis of flavonoid constituents from leaves of *Acanthopanax senticosus* Harms by electrospray tandem mass spectrometry. Rapid Commun. Mass Spectrom. 16, 264–271.

Chen, X.Q., Wang, X.B., Guan, R.F., Tu, J., Gong, Z.H., Zheng, N., Yang, J.H., Zhang, Y.Y., Ying, M.M., 2013. Blood anticoagulation and antiplatelet activity of green tea (−)-epigallocatechin (EGC) in mice. Food Funct. 4, 1521–1525.

Chung, D.-W., Lee, S.B., 2012. Novel synthesis of leucoside by enzymatic hydrolysis of tea seed extract. J. Sci. Food Agric. 93, 362–367.

Cuyckens, F., Claeys, M., 2004. Mass spectrometry in the structural analysis of flavonoids. J. Mass Spectrom. 39, 1–15.

Cuyckens, F., Rozenberg, R., de Hoffmann, E., Claeys, M., 2001. Structure characterization of flavonoid O-diglycosides by positive and negative nano-electrospray ionization ion trap mass spectrometry. J. Mass Spectrom. 36, 1203–1210.

Demirezer, L.O., Gürbüz, F., Güvenalp, Z., Ströch, K., Zeeck, A., 2006. Iridoids, flavonoids and monoterpene glycosides from *Galium verum* subsp. verum. Turkish J. Chem. 30, 525–534.

Estork, D.M., Gusmão, D.F., Paciencia, M.L.B., Díaz, I.E.C., Varella, A.D., Younes, R.N., Reis, L.F.L., Montero, E.F.S., Bernardi, M.M., Suffredini, I.B., 2014. First chemical and toxicological evaluation of *Casinga-cheirosa* in Balb-c male mice. Molecules 19, 3973–3987.

Gama, J.R.V., de Souza, A.L., Calegário, N., Lana, G.C.R., 2007. Fitossociologia de duas fitocenoses de floresta ombrófila aberta no município de Codó, estado do Maranhão. Revista Árvore 31, 465–477.

Gryglewski, R.J., Korbut, R., Robak, J., Swies, J., 1987. On the mechanism of antithrombotic action of flavonoids. Biochem. Pharmacol. 36, 317–322.

Gusmão, D.F., Estork, D.M., Paciencia, M.L.B., Diaz, I.E.C., Frana, S.A., Rodrigues, P.A., Suffredini, I.B., Varella, A.D., Younes, R.N., Reis, L.F.L., Montero, E.F.S., Bernardi, M.M., 2013a. Preliminary evaluation of the acute toxicity related to *Abarema auriculata* to mice and investigation of cytotoxicity of isolated flavonones. Pharmacologyonline (Salerno) 1, 113–127.

Gusmão, D.F., Estork, D.M., Paciencia, M.L.B., Díaz, I.E.C., Suffredini, I.B., Varella, A.D., Younes, R.N., Reis, L.F.L., Montero, E.F.S., Bernardi, M.M., 2013b. Influence of the intraperitoneal administration of antitumor *Abarema auriculata* extract on mice behavior. Rev. Bras. Farmacogn. 23, 903–912.

Hubner, G., Wray, V., Nahrstedt, A., 1999. Flavonol oligosaccharides from the seeds of *Aesculus hippocastanum*. Planta Med. 65, 635–642.

Kite, G.C., Veitch, N.C., 2009. Assigning glucose or galactose as the primary glycosidic sugar in 3-*O*-mono-di- and triglycoside of kaempferol using negative ion electrospray and serial mass spectrometry. Rapid Commun. Mass Spectrom. 23, 3125–3132.

Koch, E., 2005. Inhibition of platelet activating factor (PAF)-induced aggregation of human thrombocytes by gingkolides, considerations on possible bleeding complications after oral intake of Gingko biloba extracts. Phytomedicine 12, 10–16.

Lapiz-Bluhm, M.D., Bondi, C.O., Doyen, J., Rodriguez, G.A., Bédard-Arana, T., Morilak, D.S., 2008. Behavioral assays to model cognitive and affective dimensions of depression and anxiety in rats. J. Neuroendocrinol. 20, 1115–1137.

Lee, H.-B., Kim, E.-K., Park, S.-J., Bang, S.-G., Kim, T.G., Chung, D.-W., 2010. Isolation and characterization of nicotiflorin obtained by enzymatic hydrolysis of two precursors in tea seed extract. J. Agric. Food Chem. 58, 4808–4813.

Missouri Botanical Garden (www.tropicos.org, accessed 10.12.14).

Miyazaki, S., Imaizumi, M., Machida, H., 1995. The effects of anxiolytics and anxiogenics on evaluation of learning and memory in an elevated plus-maze test in mice. Methods Find. Exp. Clin. Pharmacol. 17, 121–127.

Moro, P.A., Flacco, V., Cassetti, F., Clementi, V., Colombo, M.L., Chiesa, G.M., Menniti-Ippolito, F., Raschetti, R., Santuccio, C., 2001. Hypovolemic shock due to severe gastrointestinal bleeding in a child taking na herbal syrup. Ann. Inst. Super Sanita 47, 278–283.

Olennikov, D.N., Tankhaeva, L.M., Partilkhaev, V.V., Rokhin, A.V., 2012. Chemical constituents of *Caragana bungei* shoots. Braz. J. Pharmacogn. 22, 490–496.

Onodera, K., Miyazaki, S., Imaizumi, M., Stark, H., Schunack, W., 1998. Improvement by FUB 181, a novel histamine H3-receptor antagonist, of learning and memory in the elevated plus-maze test in mice. Naunyn Schmiedebergs Arch. Pharmacol. 357, 508–513.

Ozi, J.M., Suffredini, I.B., Paciencia, M.L.B., Frana, S.F., Dib, L.L., 2011. In vitro cytotoxic effects of Brazilian plant extracts on squamous cell carcinoma of the oral cavity. Braz. Oral Res. 25, 519–525.

Pinheiro, S.H., Zangrossi Jr., H., Del-Bem, C.M., Graeff, F.G., 2007. Elevated mazes as animal models of anxiety, effects of serotonergic agents. An. Acad. Bras. Cienc. 79, 71–85.

Prabu, G.R., Gnanamani, A., Sadulla, S., 2006. Guaijaverin – a plant flavonoid as potential antiplaque agent against *Streptococcus mutans*. J. Appl. Microbiol. 101, 487–495.

Revilla, R., 2002. Plantas úteis da Bacia Amazônica. Vol. 1. Manaus. Instituto Nacional de Pesquisas da Amazônia e Sebrae, pp. 371.

Rios, D.R., Rodrigues, E.T., Cardoso, A.P., Montes, M.B., Franceschini, S.A., Toloi, M.R., 2008. Effects of isoflavones on the coagulation and fibrinolytic system of postmenopausal women. Nutrition 24, 120–126.

Sharma, A.C., Kulkarni, S.K., 1992. Evaluation of learning and memory mechanisms employing elevated plus-maze in rats and mice. Prog. Neuropsychopharmacol. Biol. Psychiatry 16, 117–125.

Shi, P., He, Q., Song, Y., Qu, H., Cheng, Y., 2007. Characterization and identification of isomeric flavonoid *O*-diglycosides from genus *Citrus* in negative electrospray ionization by ion trap mass spectrometry and time-of-flight mass spectrometry. Anal. Chim. Acta 598, 110–118.

Suffredini, I.B., Paciencia, M.L.B., Frana, S.A., Varella, A.D., Younes, R.N., 2007. In vitro breast cancer cell lethality of Brazilian plant extracts. Pharmazie 62, 798–800.

Suffredini, I.B., Paciencia, M.L.B., Varella, A.D., Younes, R.N., 2006. *In vitro* prostate cell cancer cell growth inhibition by Brazilian plant extract. Pharmazie 61, 722–724.

Yang, H.-J., Song M.-Ch., Bang, M.-H., Lee, J.-H., Chung, I.-S., Lee, Y.-H., Jeong, T.-S., Kwon, B.-M., Kim, S.-H., Kim, D.-K., Park, M.-H., Baek, N.-I., 2005. Deveolopment of biologically active compounds from edible plant sources-XII. Flavonol glycosides from *Trigonotis penduncularis* Benth and its hACAT1 inhibitory activity. J. Korean Soc. Appl. Biol. Chem. 48, 98–102.

Yang, S., Park, S., Ahn, D., Yang, J.H., Kim, D.K., 2011. Antioxidative constituents of the aerial parts of *Galium spurium*. Biomol. Therap. 19, 336–341.

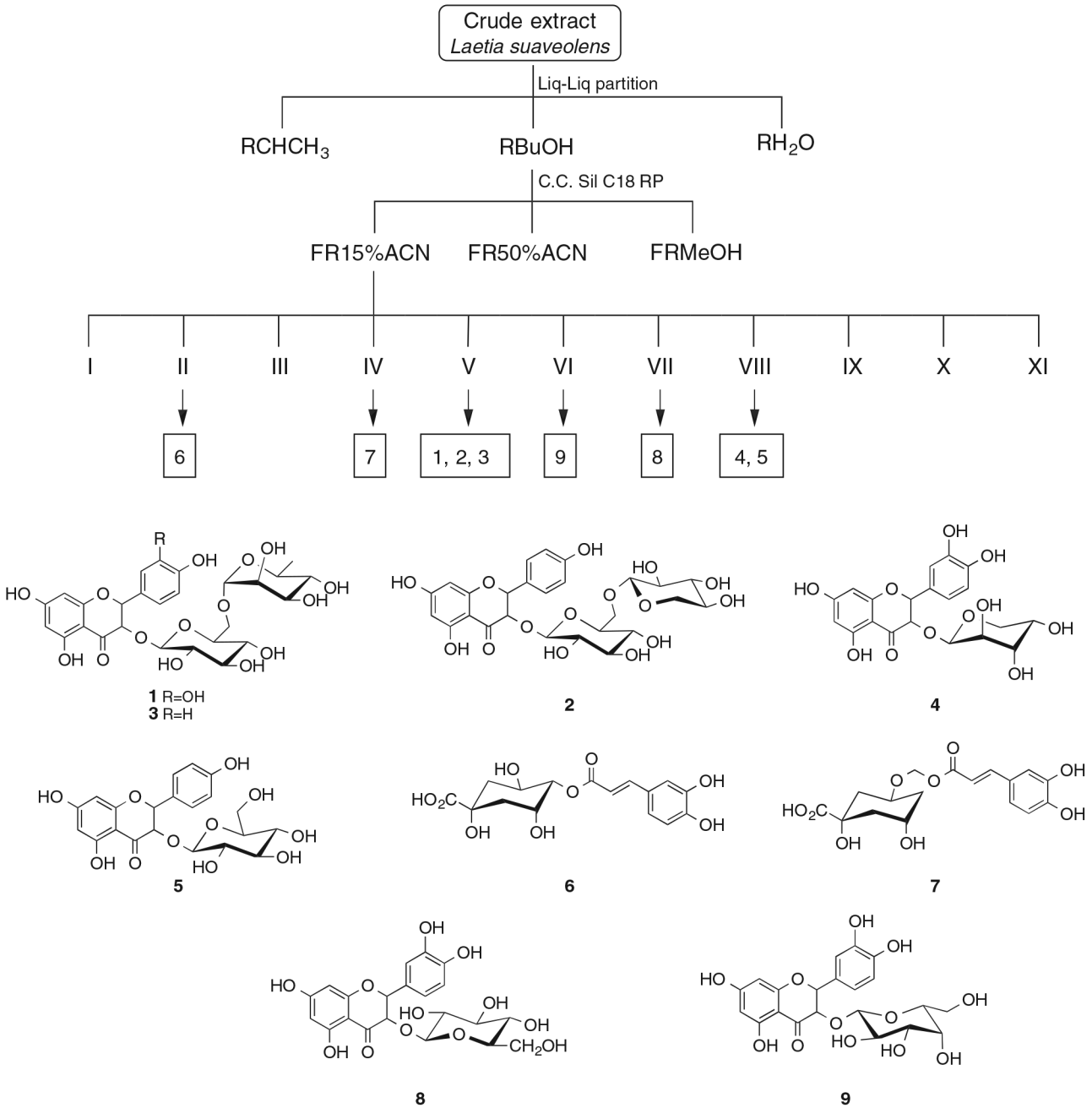
Ye, M., Yan, Y., Gou, D.-A., 2005. Characterization of phenolic compounds in the Chinese herbal drug Tu–Si–Zi by liquid chromatography coupled to electrospray ionization mass spectrometry. Rapid Commun. Mass Spectrom. 19, 1469–1484.

Yoshida, T., Maruyama, T., Nitta, A., Okuda, T., 1992. Eucalbanins A, B and C, monomeric and dimeric hydrolysable tannins from *Eucalyptus alba* Reinmw. Chem. Pharm. Bull. 40, 1750–1754.

Zar, J.H., 1999. Biostatistical Analysis, 4th ed. Prentice-Hall Inc., New Jersey, 663p. + 212app.

Erratum

The authors regret to inform that Scheme 1 appeared incorrectly in the original published article. The correct version of Scheme 1 is provided below.



Scheme 1. Fraction of crude organic extract EB719, obtained from the leaves and stem of *laetia suaveolens.*