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Preliminary evaluation of the acute toxicity related to *Abarema auriculata* to mice and investigation of cytotoxicity of isolated flavonones

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Summary

Abarema auriculata crude extract EB689 showed cytotoxic activity against prostate, central nervous system and head-and-neck cancers, and may be useful in veterinary and human care in the future. For the first time, the presence of steroids and phenolic compounds were related to *A. auriculata*. Spinasterol, a bioactive steroid, was isolated from the non polar fractions of EB689, and the phenolic compounds neoastilbin, astilbin, isoastilbin, neoisoastilbin and engeletin were isolated from polar fractions of EB689. Preliminary toxicological results showed that EB689 exhibited a non-lethal dose of 4.90 mg/kg and a LD50 of 15.0 mg/kg. Higher doses of EB689 IP administered influenced general activity of male mice, provoking significant alterations in sensory system functions, including corneal reflex and tail squeeze and touch responses. Effects were also observed in psychomotor system function, particularly in hindquarter fall, surface-righting reflex, grasp reflex and body tone and in the autonomic nervous system, where piloerection, defecation, hypothermia, cyanosis and breathing were influenced. Significant alterations were observed in general activity, cyanosis and breath after the administration of the non-lethal dose (4.90 mg/kg), corroborating the initial observations. Diminishment of general activity and breathing and appearance of cyanosis are possibly related to intestine hemorrhage observed in necropsy, which led to animal death. The occurrence of the isolated compounds in EB 689 is unlikely related to animal death and it is the first time they are isolated from *Abarema auriculata*.

Key words: Toxicity, Abarema auriculata (Fabaceae), steroid, flavanones, Amazon plant extract

Introduction

Plants have played a significant role in the treatment of cancer and infectious diseases for the last four decades (1,2). Natural products have been rediscovered as important tools for drug development, as they play an important role in combinatorial chemistry approaches based on natural products scaffolds that may resemble drug-like compounds.

The Brazilian flora, the most diverse in the world, has become an interesting source for the prospecting of new chemical leads due to its species diversity and associated chemical richness. Screening programs have been established in Brazil (3,4) as a strategy to identify potentially active substances (5). The extract obtained from the stem of *Abarema auriculata* (Benth.) Barneby & J.W.Grimes (designated as EB689), a plant found in the Amazon rain forest, has shown significant antitumor activity in *in vitro* assays against the PC3 prostate cancer cell line showing growth inhibition of 32.66 % of an untreated control, and against SF268 central nervous system cancer cell line, showing growth inhibition of 24.69 % (6). EB 689 has also been cytotoxic to squamous cell carcinoma, *in vitro* (7)[Ozi et al., 2011]. Although *in vitro* results showed significant activity against tumor cell lines, further studies are needed to assess pharmacological, toxicological and chemical properties of *A. auriculata*.

Before pharmacological assays can be developed, a wide array of the general signs of toxicity was assessed in the present study. The establishment of LD50 values is not well accepted today due to the misuse of a large number of animals frequently submitted to severe assays, and for that reason, analysis of toxicology is being conducted according to recent guidelines (8).

These guidelines describe methods that have been developed to minimize animal use and animal suffering and were introduced as a tool to assess the toxicological index of an unknown compound (9). In order to evaluate toxicity after the administration of plant extracts, a previously described method (10,11) was performed using a small number of animals (8). In addition, testosterone concentration in serum was evaluated in order to overview the possible alterations caused by the administration of EB689 to male mice.

The present study aims to evaluate the general activity and acute toxicity after the administration of different doses of the extract EB689 obtained from *A. auriculata* species, assessing the general signs of toxicity, including the influence on sex hormone levels in an animal model, and to isolate and identify compounds from the active extract.

Methods

Plant collection and extract preparation

Plant material was collected in the Brazilian Amazon rain forest, under Brazilian Government licenses (license CGen/MMA#12A/2008) for collecting and bioprospecting genetic resources in protected areas of Brazilian forests. The collection was made in the surrounds of Manaus city, state of Amazonas, in a seasonally flooded forest from Rio Negro Basin (Igapó forest). The voucher is deposited at UNIP Herbarium [A.A.Oliveira, 3353 (UNIP)] and was identified by Mateus L. B. Paciencia, curator from Herbarium UNIP. The stem of *A. auriculata* was dried in an air circulating oven (Fanem) at 40°C and was ground in a hammer-mill (Holmes). Next, 545 g of the dried plant ground material was placed in a glass percolator (Kontes) and macerated for 24h with 1.80 L of dichloromethane and methanol (1:1) (5)[Younes et al., 2007] (Merck), Solvents were evaporated under vacuum (Büchii) resulting in 26.56 g of crude extract, which was maintained in a freezer (Revco) until use.

Preparation of extract for administration to mice

Extract EB689 was suspended in almond oil and the following doses were administered IP: 5,000, 2,500, 1,250, 625, 312.5, 156.3, 78.1, 39.1, 19.5, 9.7, 4.9 mg/kg. Almond oil was used in the extract formulation because of its non polar origin and non-toxic profile, and for that reason, it is compatible as a

vehicle for the administration of drugs to mammals. To avoid alterations of extract composition, the extract suspensions were not sterilized or filtered. The intraperitoneal route was chosen due to the absence of bioavailability loss.

Animals

Male Balb-C mice (*Mus musculus*) weighing 25-30 g, 6 to 9 weeks old, were obtained from São Paulo University Animals Facilities After arrival in the laboratory, animals were randomly selected, individually marked, and housed in groups of five in isolated units (polypropylene cages 38 x 32 x 16 cm) with controlled temperature (22 ± 2°C) and humidity (65–70%). Artificial lighting was provided (12 h light/12 h dark cycle, lights on at 8:00 a.m.), as well as 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, allowing for adaptation to the new laboratory environment and conditions. Animals were fasted for a period of one hour before receiving treatments. Animals were observed for toxic responses, and if lethality occurred during a period of observation, a necropsy was performed. On the other hand, if animals survived until the end of the observation period, they were humanely sacrificed in a gas chamber with CO_2 gas, according to the Ethics Committee directions. Moreover, the sacrificed animals were necropsied and individual records containing the alterations observed were kept. All experiments with mice were subjected to Ethics Committee protocol (CEP/ICS/UNIP 025/08 and CaPPesq 1109/090).

Acute toxicity signs and delayed death observations

Acute toxicity profiles were assessed (10), with modifications to the number of animals used in the first stage of experiments. Parameters related to general activity, sensory system (such as vocal tremor, irritability, auricular reflex, corneal reflex, tail squeeze, response to touch), psychomotor system (contortion, hindquarter fall, surface - righting reflex, body tone and grasp reflex), central nervous system (convulsions, ataxia, anesthesia, hypnosis, straub tail, tremor, stimulation and sedation) and to autonomic nervous system (lacrimation, breathing, ptosis, piloerection, micturition, defecation, hypothermia and cyanosis) were assessed and a score from 0 to 4 was given for each parameter, considering that 0 is the absence of the effect and 4 is the complete manifestation of the parameter, except to micturition and defecation, which numbers of urination and boli were recorded, respectively. Body weight for each animal was measured immediately before administration of extract and at least twice during the observation period.

Toxicology Experimental design

A two-stage experiment was designed in order to probe the lethality, general activity, and the toxicity of EB689. In the first stage, the LD50 was determined utilizing three mice per dose and observations of general activity and toxicity were assessed. The second stage of the experiment was conducted using the non-lethal dose against groups of ten animals each, in which the accuracy of general activity and toxicity could be observed.

In the first stage of the experiments, control group animals received IP administration of almond oil, treatment groups received the corresponding dose of EB689 IP, and the naïve group received no treatment.

Two parameters were obtained from the experiments conducted in the first stage: the LD50 and the non-lethal dose (NLD). Ten animals were used per extract dose in the second stage of experiments. The assays were initiated at 1:00 p.m. and terminated before 5:00 p.m., in order to circumvent circadian influences.

EB689 dosing for stage 1 toxicity assays

Varying doses of extract were administered, starting at 5,000 mg/kg. If death occurred in at least one of the three treated mice, a lower dose, corresponding to the half of the preceding one, was administered to a new group of three animals, and observations were repeated. This procedure was repeated until no death was observed. Mice were individually observed in a glass cage for toxic reactions and/or lethality at 10min, 30min, 1h, 2h and 3h after administration or until death occurred; mice that survived were observed every 24 hours for a 14 additional days, in the event of delayed death.

Determination of serum testosterone

Male Balb-C mice weighing 25-30 g, 6 to 9 weeks-old were used. Individual weights were taken immediately before extract administration, and at least twice during the 45 days of observation. Mice were divided into groups of 12 animals (n=12; N_{total}=48), subdivided in two groups of six each, and housed in two different cages. All animals received 490 µg/mL of EB689 diluted in almond oil, orally, for 22 consecutive days. A subgroup of six animals was submitted to euthanasia after the 22nd day. Blood was removed by i.v. puncture and immediately submitted to centrifugation in order to obtain serum. Serum was frozen until use. The other mice remained in the cage without receiving treatment up to the 45th day from experiment start, with free access to water and food. By the end of the 45th day, animals were submitted for euthanasia, their blood was removed and the serum was obtained and storage in freezer until use. Almond oil was used as vehicle in the control group (n=12; N_{total} =48); 1 mg/kg of testosterone was administered orally or intraperitoneally as positive controls (n=12 each; N_{total} =48).

Serum testosterone quantification

The concentration of testosterone in mouse serum was determined by Elisa Kit (Life Science, Inc., Missouri City, United States), code E90458Mu. The procedure was conducted in 96-well microtiter plates, following the manufacturer's instructions.

Statistical analysis

In order to organize statistical analysis of non parametric data, the scores (0-4) of each group were summed and formed a new group to be ranked. The Kruskal-Wallis analysis of variance by ranks followed by Dunn post test (13) was then applied. Micturition and defecation were analyzed by one-way ANOVA, followed by Tukey's multiple comparison test. Concentration of serum testosterone was analyzed using two-way ANOVA followed by the Bonferroni post-test. All analyses were run under significance level of $p<0.05$. Statistical procedures were conducted with GraphPad Prism 5.0®, as well as IC50 calculation; LD50 curve was obtained using GraphPad Instat3.0®.

Extract fractionation and isolation of compounds

Liquid-liquid partition

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 intern diameter, 90 cm length). One hundred mL of chloroform were added to the column, and as the chloroform is heavier than the water 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). Organic solvent that remained 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). RCHCl3 was subjected Sephadex LH20 (22 g) column chro-

matography (CC) (2.5 cm intern diameter, 90 cm length) with 300 mL of hexane, 250 mL dichloromethane and 200 mL methanol used to elute the column. This produced FrHEX (653.0 mg; 4.63% yield), FrDCM (464.9 mg; 17.68% yield) and FrMeOH (1.51 g; 10.72% yield). The same fractionation procedure was performed for FrHEX (219.5 mg; 1.56% yield) and FrDCM (147.6 mg; 1.05% yield). These were submitted to further fractionation by CC using silica gel (60-200 um particle size) eluted with mixtures composed of hexane, ethyl acetate and methanol in order of increasing polarity. From these procedures, 28 and 27 fractions were obtained from FrHEX and FrDCM, respectively. Fractions were combined according to analytical thin layer chromatographic (TLC) similarity after visualization with 25% sulfuric acid followed by heating. Spinasterol was isolated after purification on silica gel preparative TLC of fractions 1/16-18FrHex + 1/9-13FrDCM, using a mixture of hexane and ethyl acetate (9:1).

Fraction RBuOH was subjected to flash column chromatography (3.5 cm intern 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 water acidified 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 RH_2O, named Fr15%ACNRH_2O (2.62 g; 18.60% yield), Fr50%ACNRH_2O (1.81 g; 12.85% yield) and FrMeOHRH_2O (21.0 mg; 0.15% yield).

Analytical LC-DAD and semi-preparative LC-UV analysis

Polar fractions were submitted for analytical HPLC using a Shimadzu LC-20 system consisting of an auto sampler, a LC-20AD high-pressure binary pump, a Shimadzu Shimpack ODS HPLC column (5 µm, 250 x 4.6mm), a series 8042717 oven, and a SPD-M20A photodiode array detector. The HPLC mobile phase conditions were: A=H_2O (0.1%TFA), B=ACN; 18% A 0-30 min, gradient to 100% B over 45 min, then 100% B for 5 min; flow rate 1 mL/min; injection volume 20 µL; sample concentration 2 mg/mL in MeOH. DAD was set to 210 nm, 254 nm or for full spectra acquisition from 190-800 nm (2 nm resolution). Samples submitted for semi-preparative HPLC were purified using a Shimadzu LC20 system consisting of a LC-6AD series high-pressure pump, a manual injector, a Shimpack-prep ODS HPLC column (5ìm 25 x 2 cm) and a SPD-20A UV detector operated at 254 nm.

The column was eluted isocratically with 18% ACN in H_2O (0.1%TFA), for 60 min, flow rate 10 mL/min; injection volume =1 mL; sample concentration = 50 mg/mL dissolved in MeOH; monitored at 254 nm. From the semi-preparative analysis performed with Fr15%ACNRBuOH, a total of 14 fractions were collected, the organic solvent was then evaporated, before being frozen at 70°C for lyophilization. An amorphous powder was obtained and five additional structures (**2-6**), were identified by comparison to existing data from the literature.

Spinasterol. (Fig. 5.1) 8.5 mg (0.060% yield) of a clear crystal were isolated from $RCHCl_3$ and submitted for ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) analysis (14,15,16).

Neoastilbin (Fig. 5.2) 45.8 mg (0.33% yield) was isolated from RBuOH as a cream-colored powder and submitted for ^{1}H NMR (500 MHz) analysis, and ^{13}C NMR (125 MHz) (17). ESI MS (pos. ion mode) *m/z* 450.9 [M+H]$^+$; ESI MS (neg. ion mode) *m/z* 449 [M-H]$^-$.

Astilbin (Fig 5.3) 80.0 mg (0.57% yield) was isolated from RBuOH as a cream-colored powder, and was submitted for ^{1}H NMR (500 MHz) analysis, (17), and to ^{13}C NMR (125 MHz) analysis, (18)[de Britto et al., 1995]. ESI MS (pos. ion mode) *m/z* 450.9 [M+H]$^+$; ESI MS (negative ion mode) *m/z* 448.9 [M-H]$^-$.

Isoastibin (Fig 5.4) and Neoisoastilbin (Fig.5.5) 30 mg (0.21% yield, in relation to RBuOH + RH_2O) of the mixture were isolated from RBuOH and analyzed by ^{1}H NMR (500 MHz). (17). Samples were also analy-

zed by ^{13}C NMR (125 MHz) and resulting data were compared to those reported in the literature to isoastilbin and to neoisoastilbin (18). ESI MS (pos. ion mode) *m/z* 451 [M+H]$^+$; ESI MS (neg. ion mode) *m/z* 449 [M-H]$^-$ to isoastilbin and ESI MS (pos. ion mode) *m/z* 451 [M+H]$^+$; ESI MS (negative ion mode) *m/z* 449 [M-H]$^-$ to neoastilbin.

Engeletin (Fig. 5.6) 6.3 mg (0.05% yield) of a cream-colored powder was isolated from RBuOH and submitted for ^1H NMR (500 MHz) and to ^{13}C NMR (125 MHz) (19). ESI MS (pos. ion mode) *m/z* 435.1 [M+H]$^+$; ESI MS (neg. ion mode) *m/z* 433.1 [M-H]$^-$.

Liquid Chromatography - Mass Spectrometry (HPLC/ESI-MS^n) analysis

Fraction 15%ACNRBuOH (1.3 mg/mL) was dissolved in methanol, membrane-filtered (0.45ìm), and analyzed by LC/ESI-MS^n. A Shimadzu HPLC System consisting of a SCL 10A VP binary-pump, a DAD, a SPD MI10A VP, an SIL 10AF auto sampler, and a C-18 Luna HPLC column, (5 µm, 250 mm x4.6; Phenomenex). The column was maintained at room temperature. The mobile phase consisted of A = H_2O w/ 0.1% HCOOH, B = MeCN; gradient 18-30% B over 0–30 min, 30-50% B over 30-40 min, 50-100% B over 40-50 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 (neg. ion mode) (20), and 4000V (ionpos. mode) (21) and plat offset 500V. Nitrogen served as nebulizer gas regulated at 27 psi and a flow rate of 7 L/min. The mass spectrometer was operated in full-scan mode monitoring positive or negative ions. Fragmentation of [M+H]$^+$ and [M-H] molecular ions into specific product ions was performed in enhanced product ion (EPI) mode by collision induced dissociation.

Results

The LD50 calculated for EB689 is 15.0 mg/kg (22,23). Results related to the influence of the administration of EB689 over general activity are given (Fig. 1). General activity is described as the usual behavior of the animal in the observation cage, represented by the exploration, locomotion and stimuli caused by a treatment. Specific alterations, if occurred, will appear in parameters subsequently observed (10). The administration of higher doses significantly reduced the general activity ($H\sim\chi^2_{0.05,(11)}$ = 41.63; $p<0.05$ for doses 2,500, 625.0, 156.3 and 78.1 mg/kg and $p<0.01$ for dose 1,250 mg/kg). General activity is recovered in relation to the control group ($p>0.05$) when lower doses are administered. The significance upon autonomic nervous system parameters is also illustrated (Fig. 1). Piloerection showed significant differences ($H\sim\chi^2_{0.05,(11)}$ = 48.39; $p<0.05$) at doses of 5,000, 2,500, 156.3 and 78.1 mg/kg. Defecation ($H\sim\chi^2_{0.05,(11)}$ = 43.56) was significantly altered at doses of 5,000, 2,500, 156.3 and 78.1 mg/kg ($p<0.01$) and at 625.0 mg/kg ($p<0.05$). The incidence of hypothermia significantly appeared ($H\sim\chi^2_{0.05,(11)}$ = 24.47; $p<0.01$) after administration of 1,250 mg/kg as well as cyanosis ($H\sim\chi^2_{0.05,(11)}$ = 35.50) at doses of 1,250 ($p<0.01$), 625.0 mg/kg ($p<0.01$), 312.5 and 9.8 mg/kg ($p<0.05$). Breathing was altered ($H\sim\chi^2_{0.05,(11)}$ = 38.84; $p<0.05$) at doses 5,000, 2,500, 156.3 and 78.1 mg/kg. No significant changes were observed in lacrimation, ptosis and micturition for any of the doses administered. Figure 1 also shows alterations observed in central nervous system functions, specifically in ataxia ($H\sim\chi^2_{0.05,\,(11)}$ = 19.18; $p<0.05$ for dose 1,250 mg/kg) and tremors ($H\sim\chi^2_{0.05,(11)}$ = 27.62; $p<0.01$ for doses 156.3 and 78.1 mg/kg). No significant effect upon convulsions, ataxia, anesthesia, hypnosis, straube tail or sedation was observed.

Alterations in psychomotor system are displayed in figure 2. All four parameters (hindquarter fall, surface-righting reflex, body tone and grasp reflex) were significantly altered at higher doses. Hindquarter fall ($H\sim\chi^2_{0.05,(11)}$ = 532.37; presented $p<0.05$ for doses 5,000 and 2,500 mg/kg and $p<0.01$

for doses 156.3 and 78.1 mg/kg, in surface-righting reflex ($H \sim \chi^2_{0.05,(11)}$ = 43.55), significance ($p<0.05$) appeared only in dose 5,000 mg/kg and $p<0.01$ in doses 2,500, 156.3 and 78.1 mg/kg, body tone ($H \sim \chi^2_{0.05,(11)}$ = 40.71) revealed significant differences ($p<0.05$) at doses 5,000, 156.3 and 78.1 mg/kg and grasp reflex ($H \sim \chi^2_{0.05,(11)}$ = 43.10) showed significance ($p<0.01$) at doses of 5,000 and 2,500 mg/kg and $p<0.05$ at 156.3 and 78.1 mg/kg. Contortion was not affected by extract administration in relation to the control group ($p>0.05$; results not shown). Results related to the influence of the administration of EB689 over the sensory system are also given (Fig. 2). A similar response to that observed in general activity was evident in corneal reflex ($H \sim \chi^2_{0.05,(11)}$ = 42.62; $p<0.05$ for doses 5,000, 2,500, 156.3 and 78.1 mg/kg and $p<0.01$ for dose 625.0 mg/kg), response to touch ($H \sim \chi^2_{0.05,(11)}$ = 45.60; $p<0.01$ for doses 5,000, 2,500, 156.3 and 78.1 mg/kg) and tail squeeze ($H \sim \chi^2_{0.05,(11)}$ = 44.87; $p<0.05$ for dose 5,000 mg/kg; $p<0.01$ for dose 2,500 mg/kg and $p<0.001$ for doses 156.3 and 78.1 mg/kg) and auricular reflex ($H \sim \chi^2_{0.05,(11)}$ = 45.71; $p<0.05$ for doses 156.3 and 78.1 mg/kg). Vocal tremor and irritability did not show significant alterations in relation to the control group ($p>0.05$; results not shown).

Statistical differences observed in general activity (Fig. 3) ($H \sim \chi^2_{0.05,(11)}$ = 7.973; $p<0.05$) after administration of the non-lethal dose of EB689 (4.9 mg/kg) confirmed observations from stage one of the experiments. Severe cyanosis was evident after administration of EB689 ($H \sim \chi^2_{0.05,(11)}$ = 10.18; $p<0.05$) as was breathing EB689 ($H \sim \chi^2_{0.05,(11)}$ = 14.00; $p<0.05$) in relation to naïve control.

Necropsy was performed on all experimental mice, either after receiving a lethal dose or at the end of an observation period. All the animals of the treatment groups showed extensive reddish portions in the small intestine endothelium as well as significant hemorrhage, possibly due to dilated endothelial veins and capillaries and edema. These conditions were present after the administration of all doses, excluding the non-lethal doses. Further hystopathological analysis is ongoing.

Results obtained from the analysis of the testosterone concentration in serum are presented (Fig 4). Two-way ANOVA indicated that the relationship between treatment and time is significant ($F_{(3,40)}$=3.35; $p<0.05$) and that time was also significant ($F_{(1,40)}$=4.25; $p<0.05$). The difference between the vehicle control group (45^{th} day) and the testosterone IP group (45^{th} day) was significant ($p<0.01$) as was the difference between vehicle control group (45^{th} day) and EB689 (45^{th} day) ($p<0.01$). No differences were observed in the vehicle control group and the orally-administered testosterone group.

Discussion

The study of the chemical composition of EB689 led to the isolation of spinasterol. Spinasterol was isolated in 1964 from *Medicago sativa* (Fabaceae) (24). Biological and pharmacological activities of spinasterol include antigenotoxic activity (25), cytotoxic activity against MCF-7, ZR-75-1, MDA-MB-231, SK-BR-3, and Hs578T human cancer cell lines (26) and antitpoliferative activity against human cervix adenocarcinoma (HeLa), skin carcinoma (A431), and breast adenocarcinoma (MCF-7) cells (27). Moreover, spinasterol showed anti-*Helycobacter pylori* (28), anti- *Enterococcus faecalis* and anti-*Candida tropicalis* activity (29). Modulation of oxidative and anti-inflammatory activity *in vitro* via up regulation of heme oxygenase HO^{-1} has been reported (30), as well as antioxidant activity (31).

This work is the first report of the finding and isolation of spinasterol from the genus *Abarema*. Pharmacological and chemical reports in the literature support the cytotoxic activity of spinasterol which was observed in EB689 , though it is still unclear if the cytotoxic activity from EB689 is due exclusively to spinasterol or if other cytotoxic compounds play a role as well. It is not yet possible to confirm whether spinasterol is responsible for the observed toxicity or the hemorrhaging, and no information related to endothelial toxicity was found in the literature.

Chromatography of the 15%ACNRBuOH fraction yielded astilbin, neoastilbin, isoastilbin, neoisoastilbin and engeletin. The flavonones also occurred in Fr50%ACNRBuOH, according to HPLC-UV chromatogram. The LC-MS/MS chromatogram at positive mode from Fr5%ACNBuOH and LC-MS/MS chromatogram at negative mode of Fr5%ACNBuOH confirmed the molecular weight of the isolated flavonones, which showed no significant cytotoxicity against prostate cancer cell line, when compared to IC50 obtained for EB689 (IC50=1.24 µg/ml). According to the literature, a wide range of pharmacological properties is related to these flavonoids. Astilbin decreased serum uric acid level by increasing the urinary uric acid level and fractional excretion of urate, recovering renal parameters as serum creatinine and blood urea nitrogen; also, astilbin prevented kidney damage by interfering with cytokines related to inflammation (32)[Chen et al., 2011]. Astilbin has been shown to be an anti-infective agent against *Malassezia pachydermatis*, *Staphylococcus intermedius* and *Pseudomonas* sp. (33), and against skin microflora as an agent to be added to skin care products (34). Other bioactivities reported for astilbin include roles as an antioxidant (35), an inhibitor of angiotensin converting enzyme (36), an antidepressant (37) and an ameliorant to contact hypersensitivity (38). Engeletin, and astilbin were found to aid in the prevention of immunological hepatocyte damage (39), as well as possessing anti-inflammatory properties (19). Finally, astilbin, isoastilbin, engeletin and other analogs have been submitted for investigations related to their estrogen and anti-estrogen activity (40).

The experimental model applied in the present work (10) was adapted to fit the modern perspectives related to toxicological analysis. Discussion regarding the value of LD50 determinations took place decades ago, and addressed assay variability, reproducibility and – recently – the involvement of a large number of animal species subjected to tortuous experiments (8). Using acute toxicity assays, determination of the LD50 was introduced in 1927 (41) and rapidly became the standard test to establish the toxicity of chemical substances. In all protocols developed since, lethality was the main parameter, and approximately 100 animals were killed to construct lethality curves (42). Although such issues must be taken into account, it is reasonable to propose alternative methods to assess the toxicity of new drugs, especially those derived from natural sources for use in humans or in veterinarian medicine. In the 1980's and 1990's, new criteria have been proposed to reach the estimation of LD50.

In the present work, the use of three animals per extract dose was adopted (totaling 36 animals), starting from the high dose of 5,000 mg/kg, that was subsequently reduced by half-fold dilution, according to the mortality observed in at least one out of three animals (43). When no deaths occurred within a dosage group, the non-lethal dose was obtained. EB689 showed a non-lethal dose of 4.9 mg/kg and an LD50 of 15.0 mg/kg.

New recommendations for minimizing animal suffering and death in toxicity assays is what guides toxicological analysis today. In order to maximize the amount of toxicological information obtained in these experiments, the effect of IP administration of extract EB689 on the murine central nervous system, autonomic nervous system, psychomotor and sensory systems were assessed (10).

A decrease in general activity was observed in the first stage of our experiments at many of the doses. The administration of doses 156.3 mg/kg, 78.1 mg/kg and 19.8 mg/kg did not elicit a perfect dose-response relationship, probably due to individual traits leading the most susceptible animals to a moribund situation or to death. The fact that only three animals were used in each dose level (or group) may also have contributed to a wide variation in the results.

The psychomotor and sensory systems were affected by treatment with EB689. In relation to the sensory system, response to touch, tail squeeze, corneal reflex and, to a lesser degree, auricular reflex decreased. Dose-dependent alterations in psychomotor parameters such as hindquarter fall, surface-righting reflex, body tone and grasp reflex

were also observed and may be related to the decay of general activity. No major differences were observed in central nervous system functions, but ataxia (dose of 1,250 g/kg) and tremors (doses of 156.3 and 78.1 g/kg) were observed and maybe considered a secondary sign of toxicity. Finally, signs of autonomic system stimulation were detected. Piloerection increased, and cyanosis and hyperthermia also increased after administration of higher doses. Defecation and breathing diminished in relation to the control group when higher doses were administered.

A *post-mortem* gross necropsy was performed on all animals that died or that were sacrificed during the experiment. Hemorrhaging was observed at all lethal doses but was not evident in animals receiving the non-lethal dose. Thus, a relationship between toxic signs observed and the hemorrhagic shock could be made. Thus, the decreased general activity, in several reflexes and psychomotor parameters could be attributed to hemorrhaging. More closely linked with this process are increased cyanosis, piloerection, low breathing and hypothermia. Breathing also diminished when higher doses were administered, suggesting that the influence of the extract may be due to hemorrhaging process, as well as hypothermia and cyanosis associated to higher doses. The analysis of the second stage experiments, in which a group of ten animals received non-lethal doses, confirmed the observations and reflected the strong influence of EB689 on general activity. The occurrence of cyanosis suggested the possible toxic mechanism of action that induces intestine hemorrhage. Besides the increased cyanosis and the decreased general activity, reduced breathing and ptosis were detected. The presence of ptosis might also reflect an autonomic interference on an oculomotor nerve level induced bycholinergic hyperstimulation (44). In this respect, cholinergic activation produces bronchoconstriction which could also contribute to increased cyanosis (45). Further studies related to the confirmation of the mechanism of toxicity and to the chemical composition of extract EB689 are needed to assess the toxicological process as a whole.

Serum testosterone levels were evaluated according to the described protocol. It was observed that the vehicle control group showed increased levels of testosterone after the recuperation period (day 23-45). This increase may have been caused by physiological maturation of the animals over the course of the experiment, considering that they were 6 to 9 weeks in age, and at the end of the 45^{th} day, had aged to 12 to 15 weeks. Also, oral administration of testosterone followed the same pattern of response, with no significant differences in relation to the control group. However, IP administered testosterone reduced serum testosterone levels relative to the vehicle control group. It is evident that animals did not recuperate hormone levels during the recovery period. The IP route may have caused a direct effect over hypophysis, producing a negative feed-back response. It was also observed that EB689 influenced the levels of serum hormone during the recovery period. This may have been caused by the direct toxicity of the extract to animal gonads or to hypophysis. Further work is needed t in order to confirm either hypothesis.

In this study, the isolation and structural analysis of the extract constituents revealed the presence of spinasterol and five known flavanones from EB 689, from *A. auriculata*, all of which are reported to possess biological or pharmacological activities, corroborating the first findings of cytotoxicity. Also, the toxic effects of IP administration of the extract have been observed in mice. The lethality of EB689 was assessed and the toxicity of EB689 regarding general activity and the influence over sensorial, psychomotor and nervous system was observed and quantified. Much has still to be done in order to determine the toxic molecule, and to verify if the toxic molecule is the same that causes cytotoxicity to human cancer cells, as well as to verify the causes of the influence in the concentration of serum testosterone. We determined that the isolated compounds, although present in active fractions of the extract, are not responsible for toxic effects, *in vitro*. Future *in vivo* studies are warranted in order to confirm and provide further

information on the antitumor or toxic activity of the compounds here isolated.

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Conflict of Interest

The authors state that there is no conflict of interest in this manuscript.

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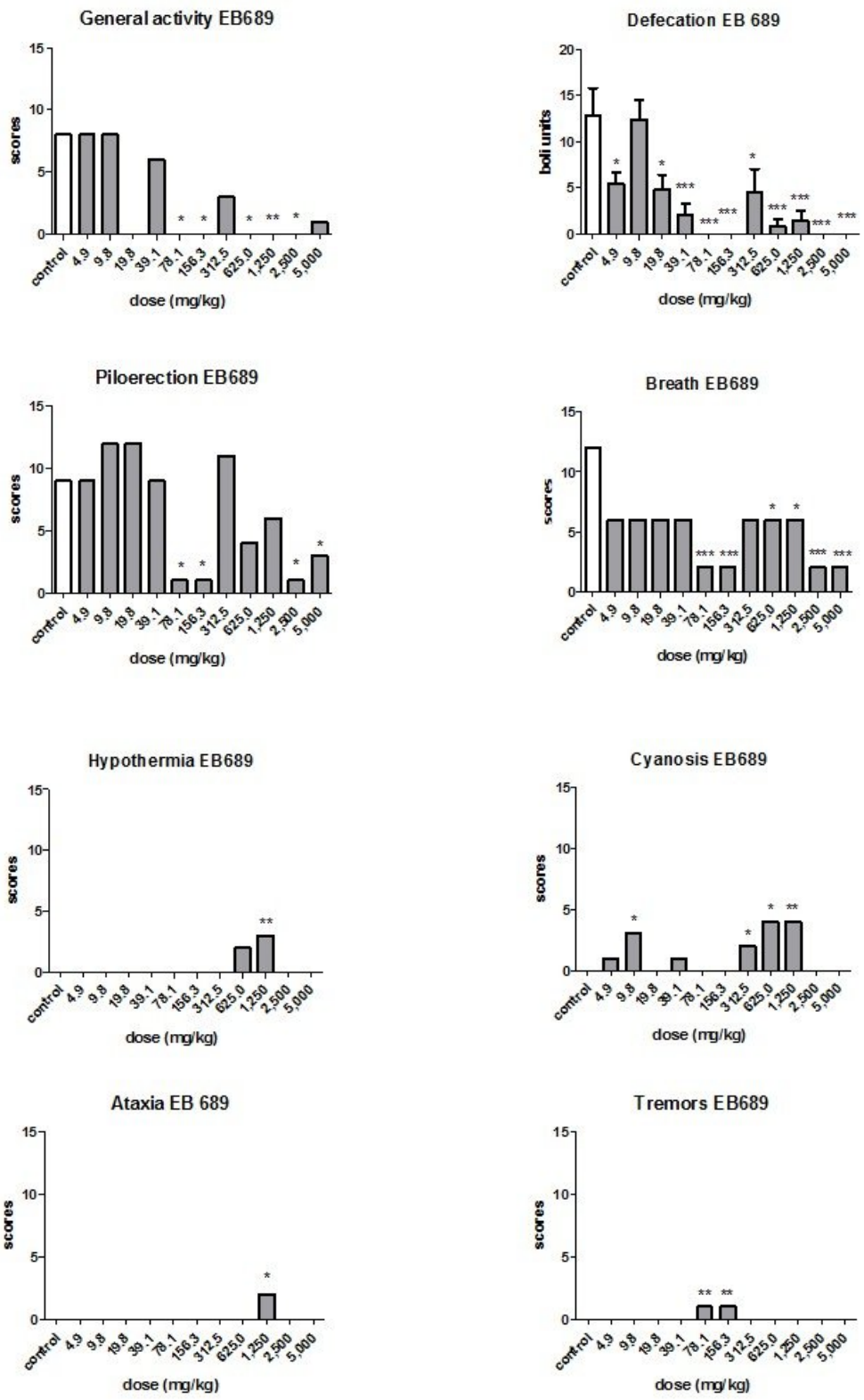

Figure 1. Effect upon murine general activity, autonomic and central nervous systems after administration of EB689, obtained from *Abarema auriculata*, in stage one of the experiment. Kruskall-Wallis statistics (n=3; N_{total}=36) were used for all the parameters, except for defecation. Differences among medians after Dunn''92s multiple comparison tests are given and were significant if * ($p<0.05$) or ** ($p<0.01$). For defecation parameter, one-way ANOVA statistics (n=3; N_{total}=36) was used, and differences among means were obtained by Tukey''92s multiple comparison tests, significant if * ($p<0.05$) or *** ($p<0.001$).

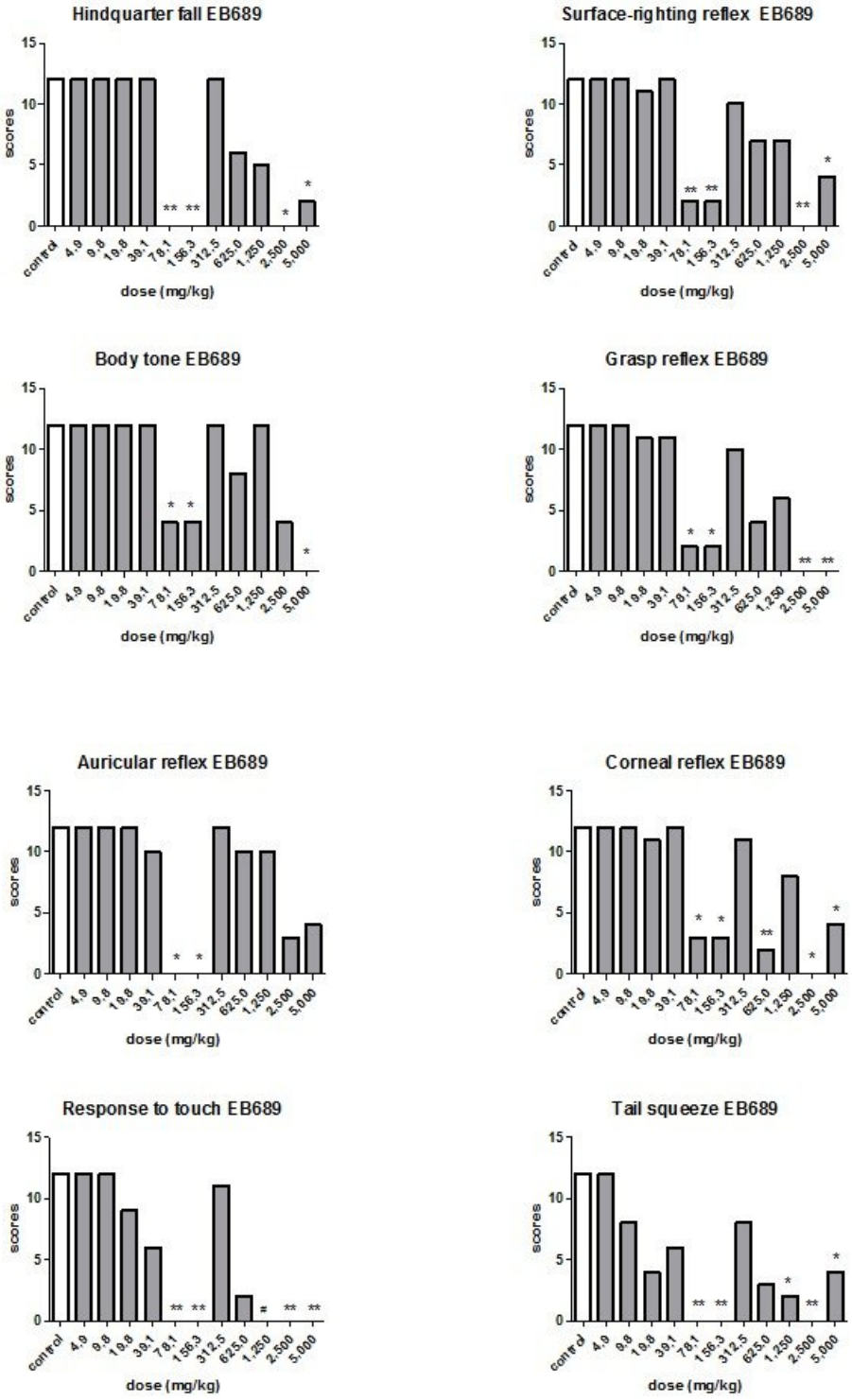

Figure 2. Observation over murine psychomotor and sensory systems after administration of EB689, obtained from *Abarema auriculata* in stage one of the experiment. Kruskall-Wallis statistics (n=3; N_{total}=36) were used for all the parameters (n=3; N_{total}=36). Differences among medians after Dunn's multiple comparison tests are given and significant if * ($p<0.05$) or ** ($p<0.01$).

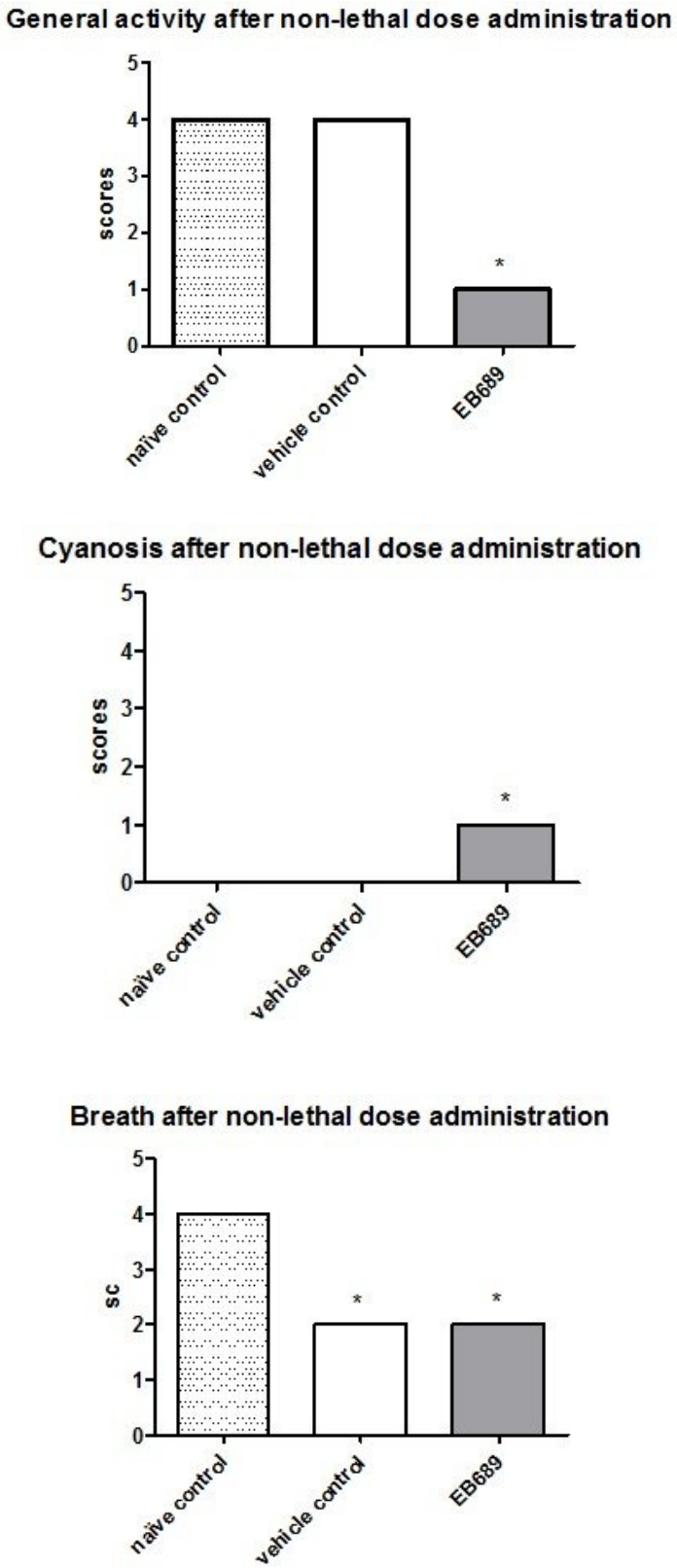

Figure 3. Observations after intraperitoneal administration of the non-lethal dose of EB689 (4.8 mg/kg), obtained from *Abarema auriculata* after IP administration of the non-lethal dose of 4.90 mg/kg. Kruskall-Wallis statistics (n=3; N_{total}=36) were used for all the parameters (n=10; N_{total}=30). Differences among medians after Dunn''92s multiple comparison tests are given and significant if * ($p<0.05$).

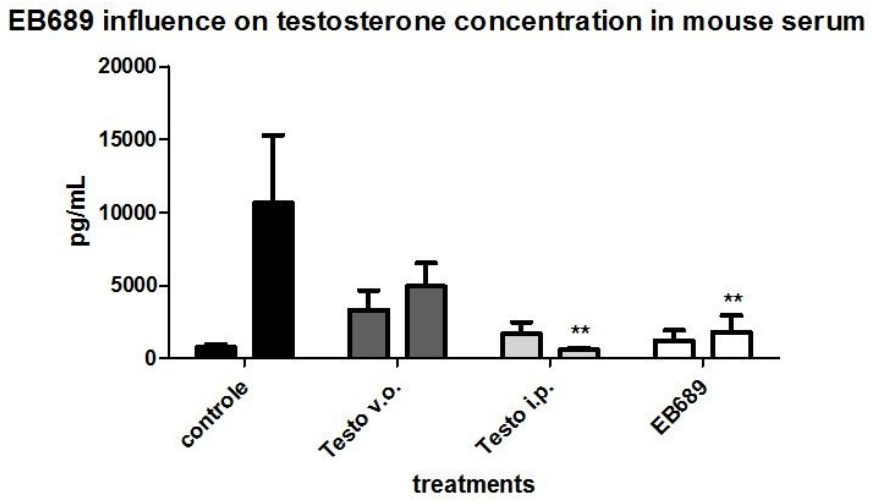

Figure 4. Results obtained from the analysis of serum testosterone after multi-dose administration of EB689. Testosterone was administered either orally (v. o.) or IP as controls.

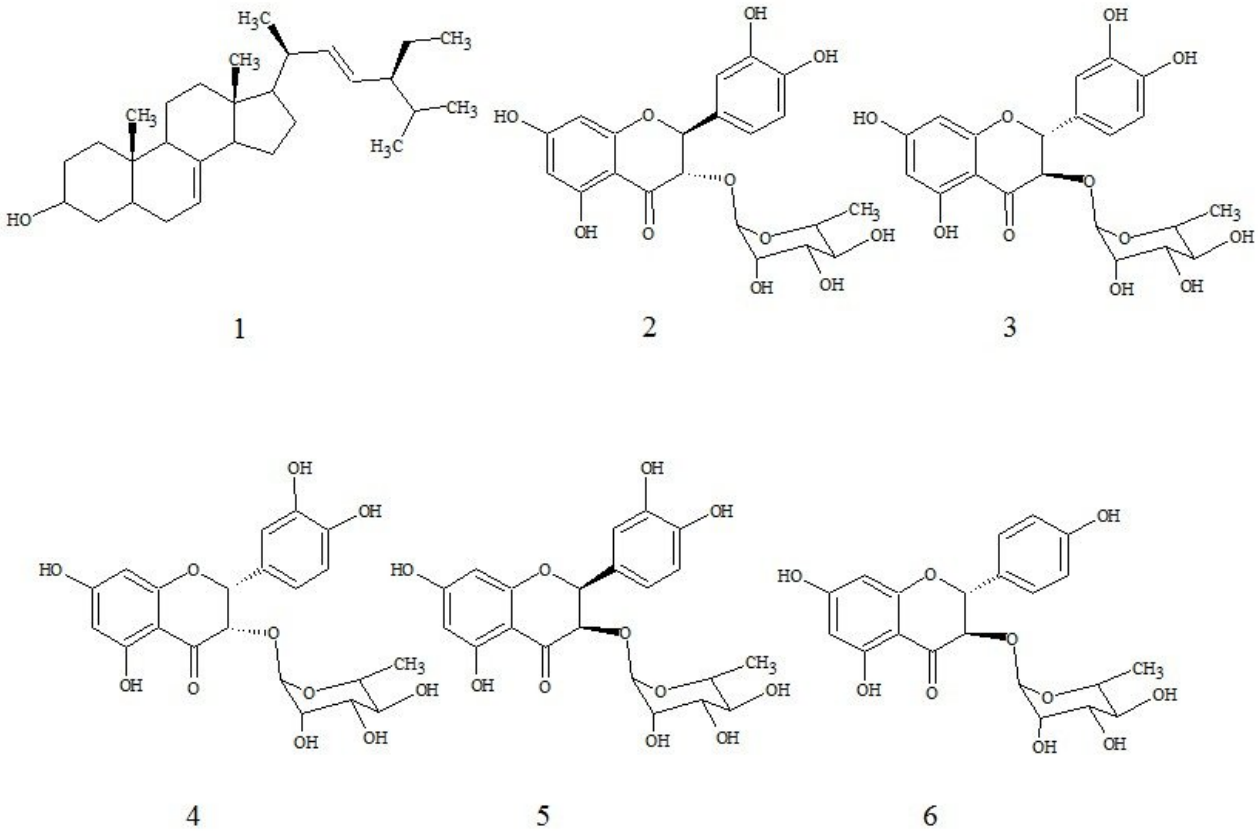

Figure 5. Chemical structures of (1) Spinasterol; (2).Neoastilbin; (3) Astilbin; (4) Isoastilbin; (5) Neoisoastilbin; (6) Engeletin, isolated from the crude extract of *Abarema auriculata*.