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ORIGINAL ARTICLE

Phenotype Behavioral Impairment after the Administration of *Xylopia Aromatica* to Male Balb-c Mice and Cytotoxicity to Breast and Cancer Cell Lines

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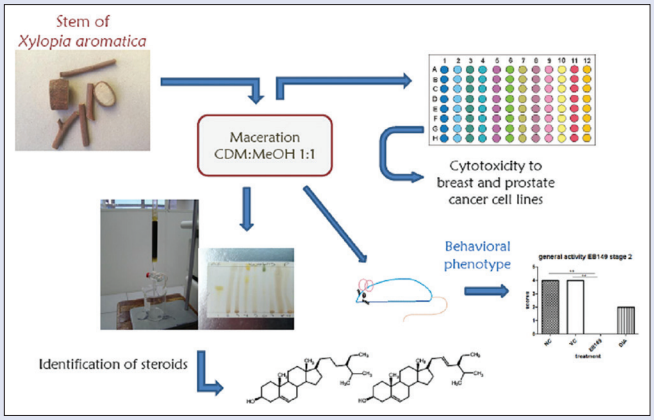
ABSTRACT

Background: Crude extract obtained from the stem of *Xylopia aromatica* (Annonaceae, EB149) known as *pimenta-de-macaco*, a traditional Brazilian medicinal and edible plant, showed cytotoxicity against leukemia cell lines. Despite the initial findings, no information regarding its influence on behavioral phenotype (BP) has been previously reported. **Objective:** The objective was to assess the effect on EB149 on BP in male mice and to perform a bioguide-fractionation aimed at the verification of the cytotoxic potential against breast and prostate cancer cell lines. **Materials and Methods:** BP was assessed by observation in an open cage and subsequent analysis in an open field (OF) at 15, 30, 60, 120, and 180 min, after intraperitoneal administration of *X. aromatica*, in a two-stage experimental delineation. **Results:** EB149 impaired general activity, piloerection, defecation, breathing, auricular and corneal reflexes, tail squeeze, response to touch, hindquarter fall, surface-righting reflex, body tone, and grip reflex in the first stage. In the second stage, general activity, tail squeeze, touch response, and breathing were impaired, and a decrease in locomotion frequency in OF was also observed. All behavioral changes were recovered during the period of experiment in both stages. The LD50 of EB149 was 1.944 g/kg. Two fractions obtained from EB149 named FSIST, which contains sitosterol and stigmasterol as major compounds, and total alkaloid fraction, which contains total alkaloids, showed cytotoxicity against breast and prostate cancer cell lines. **Conclusions:** The traditional plant *X. aromatica* EB149 organic extract showed cytotoxicity against breast and prostate cancer cell lines and the recovery of behavioral impairment related to general activity, tail squeeze, touch response, breath, and locomotion frequency, causing no harm to male lab mice.

Keywords: Annonaceae, antiproliferative, behavioral phenotype, cytotoxicity, *Xylopia aromatica*

SUMMARY

- The objective was to assess the effect on EB149 on BP in male mice and to perform a bioguide-fractionation aimed at the verification of the cytotoxic potential against breast and prostate cancer cell lines. The traditional plant X. aromatica EB149 organic extract showed cytotoxicity against breast and prostate cancer cell lines and the recovery of behavioral impairment related to general activity, tail squeeze, touch response, breath, and locomotion frequency, causing no harm to male lab mice.



Abbreviations used: MMA/ICMBio/SISBIO: Ministério do Meio Ambiente/Instituto Chico Mendes de Conservação da Biodiversidade/Sistema de Autorização e Informação em Biodiversidade; CGen/IBAMA/MMA: Conselho de Gestão do Patrimônio Genético/Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis/Ministério do Meio Ambiente; AM: Amazonas State; UNIP: Universidade Paulista; g: Gram; mg: Milligram; µg: Microgram; kg: Kilogram; I. P: Intraperitoneal; CEUA/ICS/UNIP: Comissão de Ética no Uso de Animais/Instituto de Ciências da Saúde/Universidade Paulista; BP: Behavioral phenotype; OF: Open-field apparatus; NLD: Nonlethal dose; LD: Lethal dose; ANOVA: analysis of variance; $FCHCl_3$: Fraction chloroform; FBuOH: Fraction buthanol; FH_2O: Fraction water; FHEX: Fraction hexane; FDCM: Fraction dichloromethane; FMeOH: Fraction methanol; TLC: Thin-layer chromatography; U. V.: Ultraviolet; TAF: Total alkaloid fraction; nm: Nanometer; CC: Column chromatography; ATLC: Analytical thin-layer chromatography; NMR: Nuclear magnetic resonance apparatus; FSIST: Fraction containing sitosterol and stigmasterol; NC: Naive control; VC: Vehicle control; DIA: Diazepam

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INTRODUCTION

Cancer is a global issue. It is estimated that 22.2 million new cases of cancer will be identified worldwide, in 2030, primarily due to population growth and aging.[1] According to the *Instituto Nacional de Câncer José Alencar Gomes da Silva–INCA*, it is estimated that, in Brazil, more than 600,000 people will be affected by cancer in 2016–2017. Although prostate cancer is supposed to be the most frequent in men, as breast cancer is in women, reports show that cancer of lung, colon, stomach, and oral cavity will be the most frequent in men, while colon, cervical, lung, and stomach cancers will predominate in women, this is in spite of nonmelanoma cancers, the incidence of which is elevated for both men and women, corresponding to 30% of all cancers.[2]

Brazil has one of the richest biodiversity hotspots in the world, such richness may be used in the development of future medicines.[3] A wide number of drugs have been obtained from plants, such as paclitaxel, vinca alkaloids, camptothecin, podophylotoxin, and others.[4] These findings have enabled the establishment of new research programs aimed at the search for new natural product antitumor drugs. From a large screening program,[5] plant extracts were identified as active against the leukemia cell line RPMI-8226,[6] one of the cytotoxic extracts was obtained from *Xylopia aromatica* (Lam.) Mart (Annonaceae), popularly known as *pimenta-de-macaco*, which is found in Cerrado and the Amazon rainforest, in Brazil. Although studies concerning some of its chemical and biological properties are available, no information on its effect on behavioral phenotype (BP) or on cytotoxicity against breast and prostate cancer cell lines has been accessed so far.

The aims of the present work were to evaluate the influence of *X. aromatica* extract EB149 on BP in male mice, to verify its cytotoxic potential against breast and prostate cancer cell lines and to isolate major compounds.

MATERIALS AND METHODS

Plant collection and extract preparation

X. aromatica was collected from the Amazon rainforest, under Brazilian Government licenses for collecting and bioprospecting genetic resources in protected areas (no. CGen/IBAMA/MMA#12A/2008 and no. MMA/ICMBio/SISBIO #14895). Collection took place in the surroundings of Manaus, AM, Brazil, in a seasonally flooded forest from Rio Negro Basin (the so-called *igapó* forest). The voucher was deposited at UNIP Herbarium (I. B. S., 008 [UNIP]).

Stem (706.50 g) of *X. aromatica* was collected, dried in an air-circulating stove (Fanem, Diadema, São Paulo, Brazil) at 40°C, and ground in a hammer mill (Holmes, Danville, Illinois, USA). The ground material was placed in a glass percolator (Kontes, New Jersey, USA) where a 24 h-maceration was performed with a 1:1 mixture of dichloromethane and methanol (Merk, Darmstadt, Germany). After that, solvents were evaporated under vacuum (Buchi, Flawil, Switzerland) and the dried organic extract, here named EB149, was kept in a freezer at −20°C (Revco, Thermofisher, Marietta, Ohio, USA) until use.

Preparation of extract to be administered

EB149 (43.60 g) was suspended in almond oil and doses of 2500, 1250, and 625.0 mg/kg were intraperitoneally (I. P) administered. Almond oil was used in the extract dilution because of its nonpolar origin and nontoxic profile; for that reason, it is suitable for use in mammalian organisms. The I. P. route was chosen due to the absence of bioavailability loss.

Cytotoxic activity

Cell culture technique

Tumor cell line MCF-7 (estrogen receptor-positive breast adenocarcinoma) was cultivated in tissue-culture flasks (Costar, Corning New York, USA) supplemented with RPMI-1640 plus 5% fetal bovine serum (both Cambras, Campinas, São Paulo, Brazil) and 1% glutamine (Sigma, Saint Louis, Missouri, USA), and was kept in an incubator (Forma, Thermofisher, Marietta, Ohio, USA) at 37°C with 5% CO_2 and 100% relative humidity. Cells were passaged weekly (Trypsin-EDTA, Cambras, Campinas, São Paulo, Brazil). Cell densities were obtained with a hemocytometer chamber, using the Trypan blue exclusion method. Tests were carried out in 96-well microplates, and the density of 10,000 cells per well was considered in the screening experiment. Cells were incubated for 24 h before the drug/extract was added, and the drug/extract remained in contact with the cells for 48 h in the microculture assay. After that, end points were obtained by the sulforhodamine B (SRB) assay.[7]

Doxorubicin (DOXO; Sigma) was used as the standard drug in the assays at a concentration of 2.5×10^{-5} M. Extracts were tested in one dose of 100 μg/mL, and a percentage of cell lethality <15 was considered selective in the assays, when compared to cells without treatment.

The SRB cytotoxicity test[7] was performed with breast (MCF-7) and prostate (PC-3) human cancer cell lines at a density of 10,000 cells/well or 7500 cells/well, respectively, in the 96-well microplates. Samples of EB149, fractions, and the total alkaloid fraction (TAF) were tested at a final concentration of 100 μg/mL. The alkaloid sample and fraction were added to a final concentration of 100 μg/mL. All plates were read at 515 nm in a spectrophotometric reader (Biotek, Winooski, Vermont, USA) after 48 h of sample contact with the cell in a CO_2 incubator at 37°C and 100% relative humidity. The inhibitory concentration (%IC) was obtained by the following formulae: $\%IC = ([T - T_0]/[C - T_0]) \times 100$, where T is the optical density value related to samples, T_0 is the optical density related to time zero, and C refers to the optical density of the cell growth control.

Animals

Male Balb-c mice weighing 25–30 g, aged 6–9 weeks, obtained from São Paulo University, were used. Animals were housed for 1 week under laboratory conditions for habituation before experimental procedures.[8] Experiments conducted with animals were approved by the Ethics Committee (CEUA/ICS/UNIP 025/08).

Assessment of behavioral phenotypes

BP parameters were assessed based on a previous work,[9] with modifications.[10-12] Thus, 27 parameters related to the general activity, the sensorial system, the psychomotor system, the central nervous, and the autonomous nervous systems were assessed by scoring from 0 to 4, except for micturition and defecation, for which occurrences of urination and fecal *boli* were counted. Experiments followed a two-stage protocol.

Open-field apparatus

Observations of five parameters in open-field (OF) apparatus were immediately performed after observations in a glass cage, in order to assess alterations in locomotion and anxiety, in a two-stage protocol, as described previously.[8,10-13]

Experimental design for *in vivo* studies

Experimental design of the study has been described elsewhere.[9-12] Briefly, different doses of EB149 were administered to groups of three animals, starting from 2500 mg/kg. A subsequent half-dose was administered

if death occurred to one or more of the animals. The procedure was repeated until no deaths were observed. The dose which did not cause death was named the nonlethal dose (NLD) and was subsequently used in the second stage. Thus, a total of 12 animals (n = 3, n_{total} = 12) were used in the first stage of the experiments. After receiving a dose of EB149, mice were individually observed in a glass cage for behavioral reactions and/or lethality at 7–10, 25–30, 55–60, 115–120, and 175–180 min or until death; mice that survived were observed every 24 h for the following 14 days. The tendency of lethal dose 50% and NLD was also obtained in the first stage. In the second stage of the experiment, four groups were tested: naïve control group (NC), vehicle control group (VC), NLD group (EB149), and diazepam group (DIA) (n = 10, n_{total} = 40). The NC group was introduced with the purpose of controlling the possible influence of I. P. injection. DIA (Hipolabor; lot no. AO011/11; validity: 10/13; concentration: 5.0 mg/ml; injectable medication) at a dose of 1 mg/kg was introduced as a reference drug. Based on physiological issues, the assays started at 1:00 p.m. and ended before 5:00 p.m., in order to prevent circadian influences.

Statistical analysis

Kruskal–Wallis test followed by the Dunnett's posttest analyses of variance by ranks was used to verify differences between medians; if $\alpha < 0.05$, the result was considered significant,[14] and the LD50 tendency curve was also obtained (GraphPad, Software Instat3.0®, 2009, San Diego, CA, USA). Parameters obtained from the OF apparatus were analyzed by two-way repeated measures ANOVA followed by Bonferroni's posttest to compare means; significance was set at $P < 0.05$ (GraphPad Prism 5.0® Software 2010, San Diego, CA, USA).

Liquid–liquid partitioning of EB149

EB149 was suspended in 90% methanol in water. The suspended extract was transferred to a glass column. Then, chloroform was added to the column and, as the chloroform went down the column through the polar phase, the nonpolar components were solubilized and separated from methanol phase. The chloroform phase was separated and evaporated, in order to obtain the fraction chloroform ($FCHCl_3$). Then, butanol was added to the column and the process was repeated, resulting in the fraction butanol (FBuOH). Finally, the methanolic/aqueous phase was removed from the column and the solvent was evaporated and lyophilized, resulting in the fraction water (FH_2O). The fraction $FCHCl_3$ was submitted to column chromatography (CC) (Sephadex LH-20, Sigma, Saint Louis, Missouri, USA as stationary phase, and hexane, dichloromethane, and methanol as eluents). Then, three new fractions were obtained from $FCHCl_3$, named FHEX, FDCM, and FMeOH. The fractions FBuOH and FH_2O were submitted to CC using silica gel C-18 as the stationary phase, and 15% acetonitrile, 50% acetonitrile, and 100% methanol as eluents; this generated three fractions from FBuOH and three fractions from FH_2O, named 10% ACN, 50% ACN, and 100% MeOH, as eluents. Fractions FHEX and FDCM were submitted to CC and then to thin-layer chromatography (TLC).

Analytical and preparative thin-layer chromatography

FHEX and FDCM were submitted to an open column, generating new fractions that were then submitted to analytical TLC using silica gel PF_{254} as stationary phase. Visualization was performed under ultraviolet (UV) light at 254 and 366 nm and by using 25% H_2SO_4 plus heating as reagent. Fractions were grouped according to their similarity and were then submitted to preparative TLC. Preparative plates were done with silica gel 60 PF_{254} (5–40 μm; Merck®). Two compounds were isolated, sitosterol and stigmasterol. Structures were confirmed based on ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra.

Alkaloid extraction

EB149 (27.49 g) was solubilized with 0.1 mol/L phosphoric acid. The solution was transferred to a separating funnel. Then, hexane was used to extract the organic phase containing grease material and to separate it from the alkaloidal organic phase. The alkaloidal organic phase was alkalinized with NH_4OH 1N to obtain free alkaloids, which were then extracted with $CHCl_3$, to obtain the TAF.

RESULTS

LD50 tendency obtained for EB149 was 2.344 g/kg. According to the European Community, harmful chemicals show an LD50 ranging from 200 to 2000 mg/kg. Therefore, EB149 is classified as a harmful product. The NLD was established as the dose where no death was observed and was determined to be 650 mg/kg.

Evaluation of behavioral phenotype alterations in the first stage

Results related to the administration of different doses of EB149 are shown in Figures 1 and 2, obtained in the first stage of the experiment. General activity was impaired by the administration of EB149, observed after the administration of doses of 625 mg/kg ($P < 0.01$) and 2500 mg/kg ($P < 0.05$) [Figure 1a; $H \sim \chi^2_{0.05,(3)} = 12.51$; $P < 0.001$]. Response to touch also decreased after the administration of doses of 1250 mg/kg ($P < 0.01$) [Figure 1b; $H \sim \chi^2_{0.05,(3)} = 11.09$; $P < 0.05$]. Tail squeeze decreased after the administration of doses of 1250 mg/kg ($P < 0.01$) and 2500 mg/kg ($P < 0.01$) [Figure 1c; $H \sim \chi^2_{0.05,(3)} = 13.76$; $P < 0.01$]. Hindquarter fall alterations were observed [Figure 1d; $H \sim \chi^2_{0.05,(3)} = 19.00$; $P < 0.001$] and were impaired after the administration of doses 2500 mg/kg ($P < 0.01$). Decrease in surface-righting reflex was observed after the administration of doses 2500 and 1250 mg/kg ($P < 0.05$) [Figure 1e; $H \sim \chi^2_{0.05,(3)} = 14.16$; $P < 0.01$], as was body tone after the administration of a dose of 2500 mg/kg ($P < 0.05$) [Figure 1f; $H \sim \chi^2_{0.05,(3)} = 12.75$; $P < 0.01$]. An impairment of grip reflex was observed after the administration of the dose of 1250 mg/kg of EB149 ($P < 0.05$) [Figure 1g; $H \sim \chi^2_{0.05,(3)} = 12.08$; $P < 0.01$].

Figure 2 shows that auricular reflex was impaired after the administration of dose 2500 mg/kg ($P < 0.01$) [Figure 2a; $H \sim \chi^2_{0.05,(3)} = 12.20$; $P < 0.01$], as was corneal reflex at dose 2500 mg/kg ($P < 0.05$) [Figure 2b; $H \sim \chi^2_{0.05,(3)} = 11.18$; $P < 0.05$]. Stimulation increased after administration of dose 2500 mg/kg ($P < 0.01$) [Figure 2c; $H \sim \chi^2_{0.05,(3)} = 19.00$; $P < 0.0001$], as well as defecation at doses 1250 mg/kg ($P < 0.01$) and 2500 mg/kg ($P < 0.05$) [Figure 2e; $H \sim \chi^2_{0.05,(3)} = 11.78$; $P < 0.01$]. Piloerection has improved after administration of dose 2500 mg/kg ($P < 0.01$) [Figure 2d; $H \sim \chi^2_{0.05,(3)} = 12.88$; $P < 0.01$]. Cyanosis appeared after administration of dose 1250 mg/kg ($P < 0.001$) [Figure 2f; $H \sim \chi^2_{0.05,(3)} = 15.82$; $P < 0.01$]. Finally, breathing was decreased after administration of all doses ($P < 0.001$) [Figure 2g; $H \sim \chi^2_{0.05,(3)} = 19.00$; $P < 0.0001$].

Evaluation of behavioral phenotype alterations in the second stage

Figure 3 shows the general activity and evaluation of parameters after the administration of NLD in the second stage of the experiments. General activity was impaired after the administration of EB149 in relation to the NC group ($P < 0.01$) and VC group ($P < 0.01$) [Figure 3a; $H \sim \chi^2_{0.05,(3)} = 16.49$; $P < 0.001$]. Tail squeeze decreased after the administration of DIA ($P < 0.01$) when compared to both NC and VC groups [Figure 3b; $H \sim \chi^2_{0.05,(3)} = 13.44$; $P < 0.01$]. No statistical differences were noticed in tail squeeze after the administration of EB149, in tail squeeze ($P > 0.05$). A significant decrease in touch response was observed after administration of EB149 when compared to NC and VC groups ($P < 0.05$) [Figure 3c; $H \sim \chi^2_{0.05,(3)} = 11.56$; $P < 0.01$]. Finally, breathing decreased after the administration of EB149,

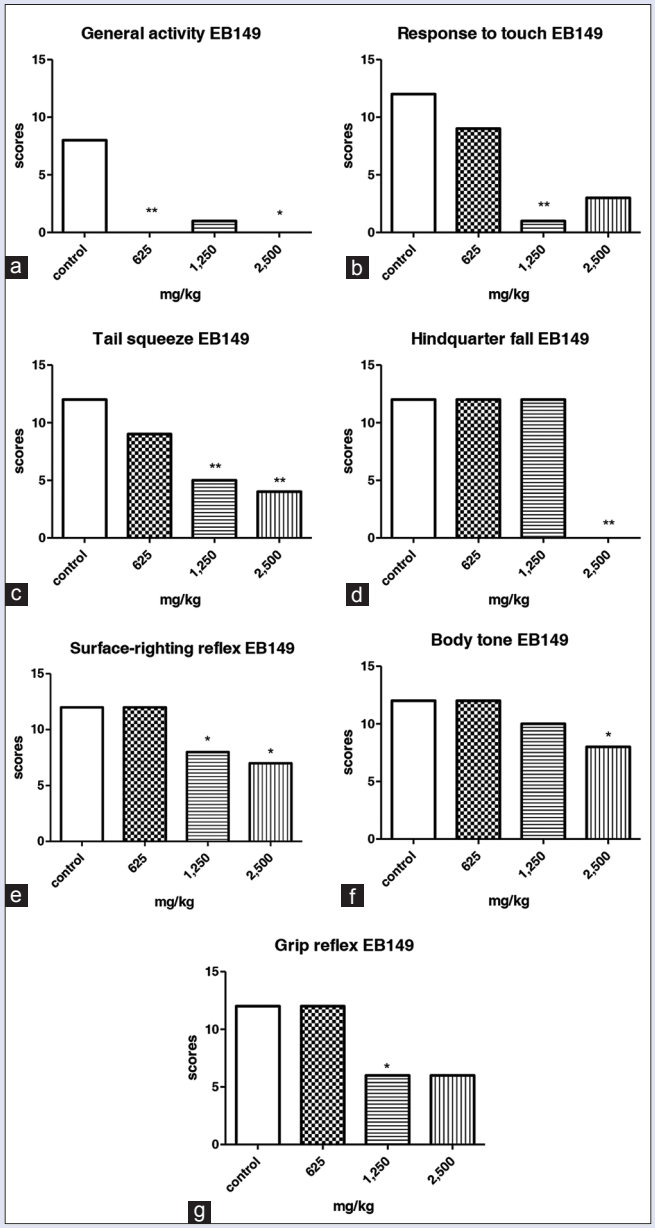

Figure 1: Effect of the administration of the organic extract made with the stem of *Xylopia aromatica* (EB149) to male mice. Impairment results for (a) general activity, (b) response to touch, (c) tail squeeze, (d) hindquarter fall, (e) surface-righting reflex, (f) body tone, and (g) grip reflex in the first stage of experiment are shown. Kruskal–Wallis statistics (n = 3; n_{total} = 12) were used for all the parameters, except for defecation, which was analyzed by one-way ANOVA followed by Bonferroni´s posttest. Differences among means after Dunnett's multiple comparison tests are given and were significant if $P < 0.05$

when compared to both NC and VC groups and to DIA ($P < 0.01$) [Figure 3d; $H \sim \chi^2_{0.05,(3)} = 18.75$; $P < 0.001$].

Evaluation of locomotion alterations in the open-field parameters in the first and second stages

Table 1 shows the results obtained from the observation in the OF apparatus, in both stages 1 and 2. The results described for stage 1 are given. In locomotion frequency, both treatments ($F_{(3,8)} = 13.40$; $P < 0.01$) and time ($F_{(4,32)} = 2.90$; $P < 0.05$) were significant and accounted for 62.43% and 4.98% of the total variance, respectively. In rearing frequency, time was significant ($F_{(4,32)} = 4.03$; $P < 0.01$) and the interaction between time and treatment was also significant ($F_{(12,32)} = 2.28$; $P < 0.05$). These variables accounted for 12.42% and 21.08% of the total variance, respectively. Treatment was significant in defecation ($F_{(3,8)} = 11.79$; $P < 0.01$) and accounted for 31.48% of the total variance, but did not influence the variances in the experiment. No significant alterations were observed for grooming ($P > 0.05$). Finally, immobility time showed that treatment ($F_{(3,8)} = 18.57$; $P < 0.001$) and time ($F_{(4,32)} = 5.99$; $P < 0.01$) were significant and accounted for 43.75% and 17.64% of the total variance, respectively. The results of Bonferroni posttest analyses for all the five OF parameters, considering treatments compared to the control group, are shown in Table 1.

Table 1 also reports the results obtained for stage 2 of the OF analysis. In locomotion frequency, both treatments ($F_{(3,28)} = 16.79$; $P < 0.001$) and time ($F_{(4,112)} = 14.70$; $P < 0.001$) were extremely significant, as was the interaction between these two variables ($F_{(12,112)} = 6.50$; $P < 0.001$), accounting for 33.89%, 10.92%, and 14.47% of the total variance, respectively. Furthermore, treatment was extremely significant ($F_{(3,28)} = 9.30$; $P < 0.001$), time was significant ($F_{(4,112)} = 2.47$; $P < 0.05$), and interaction between both time and treatment was also extremely significant ($F_{(12,112)} = 4.21$; $P < 0.001$). These variables accounted for 34.51%, 1.77%, and 9.05% of the total variance, respectively. Treatment was extremely significant for defecation ($F_{(3,28)} = 10.29$; $P < 0.001$) and accounted for 16.76% of the total variance, while time was not significant ($F_{(4,112)} = 0.34$; $P > 0.05$), accounting for only 0.67% of the total variance, and interaction between treatment and time was considered significant ($F_{(12,112)} = 1.99$; $P < 0.05$), accounting for 11.84% of the total variance. Only treatment was considered significant in grooming ($F_{(3,28)} = 6.22$; $P < 0.01$) and accounted for 20.80%, but time ($F_{(4,112)} = 1.37$; $P > 0.05$) and the interaction between both variables ($F_{(12,112)} = 1.37$; $P > 0.05$) were not significant and accounted for 1.97% and 5.90% of the total variance, respectively. Finally, treatment ($F_{(3,28)} = 15.78$; $P < 0.001$), time ($F_{(4,112)} = 4.99$; $P < 0.001$), and the interaction between them ($F_{(12,112)} = 3.23$; $P < 0.001$) were extremely significant and accounted for 39.42%, 4.14%, and 8.03% of the total variance, respectively. The Bonferroni posttest analyses performed for all the five OF parameters in stage 2, considering treatments compared to control group, are shown in Table 1.

Fractionation of EB149

Three fractions resulted from the fractionation of 14.656 g of EB149 and were named fractions $FCHCl_3$ (6.079 g), FBuOH (3.165 g), and FH_2O (5.411 g). $FCHCl_3$ was again fractionated with hexane, dichloromethane, and methanol, yielding three new fractions, named FHex (0.333 g), FDCM (1.642 g), and FMeOH (4.105 g). After separation of fractions FHex and FDCM using CC and following analysis in analytical TLC (ATLC), the major compounds stigmasterol and sitosterol [Figure 4] were isolated and identified by the analysis of ^1H and ^{13}C NMR spectra as well as in comparison to the literature, resulting in the fraction named FSIST.

^1H NMR spectrum of both substances showed characteristic signals of steroids, such as a double-double-double-doublet at δ 3.52 (J = 9.5, 4.8, 11.2, and 4.6 Hz), attributed to H-3, and a large triplet at δ 5.35, which indicates the presence of an olephinic hydrogen (H-6). Signals at δ 5.0 and 5.14 indicate the presence of hydrogen atoms bonded to carbons 22 and 23. In the ^{13}C NMR spectrum, it was possible to observe several signs related to aliphatic carbons (region between δ 11 and 57) and a sign of carbinolic carbon (C-OH) at δ 71.79. The signs at δ 140.74,

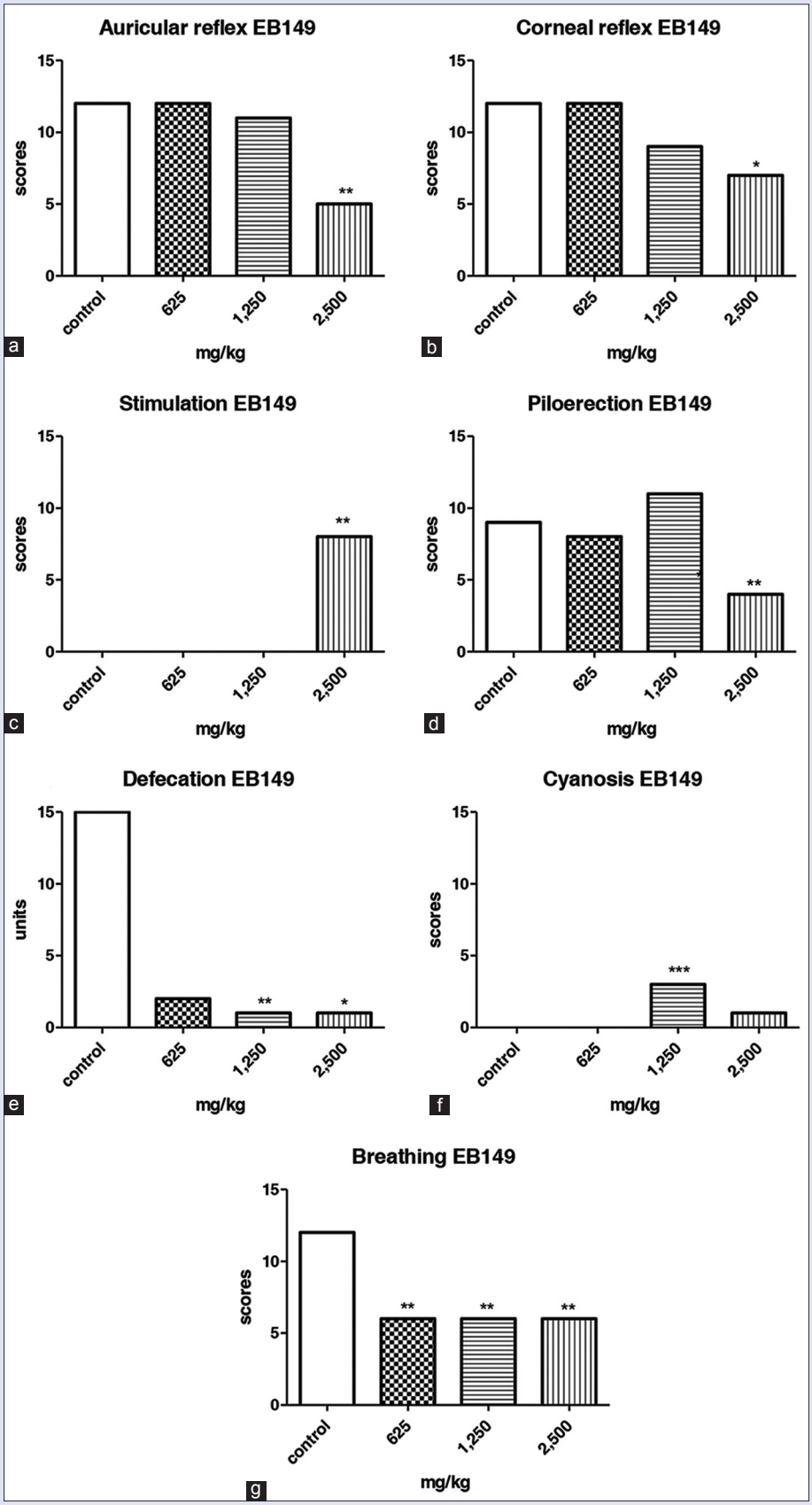

Figure 2: Effect of the administration of EB149 to male mice. Impairment results for (a) auricular reflex, (b) corneal reflex, (c) stimulation, (d) piloerection, (e) defecation, (f) cyanosis, and (g) breathing in the first stage of experiment are shown. Kruskal–Wallis statistics ($n = 3$; $n_{total} = 12$) followed by Dunn's post-test were used for all the parameters. Defecation was analyzed by ANOVA followed by Bonferroni's post-test. Differences among means were Significant if $P < 0.05$.

Table 1: Open-field evaluation of male mice treated with the organic extracts obtained from the stem of *Xylopia aromatica* (EB149), in stages 1 and 2 of the experiment. Two-way analysis of variance and Bonferroni posttest were adopted, considering $P<0.05$

Open-field evaluation (Stage 1)					Open-field evaluation (Stage 2)				
minutes	Control	2500 mg/mL	1250 mg/mL	625 mg/mL	minutes	NC	VC	EB149	DIA
Locomotion frequency (uniys)					**Locomotion frequency (units)**				
15-20	116.30 (42.71)	28.33 (21.22)	12.67 (13.43)*	14.00 (10.39)*	15-20	184.00 (42.37)	261.30 (94.82)	36.50 (55.08)****	236.20 (92.13)
30-35	98.67 (87.96)	4.67 (0.58)	4.67 (1.53)	36.00 (28.35)	30-35	172.80 (39.39)	173.00 (60.42)	29.80 (47.93)****	82.80 (66.65)*
60-65	91.67 (69.62)	6.00 (1.73)	7.67 (8.14)	0.33 (0.58)	60-65	140.50 (69.93)	134.30 (43.25)	17.10 (15.00)**	74.80 (41.61)
120-125	167.70 (79.25)	10.00 (8.00)***	36.00 (31.95)**	50.33 (49.66)*	120-125	136.80 (66.33)	159.30 (40.61)	86.10 (78.61)	90.70 (48.63)
180-185	178.70 (61.34)	4.67 (1.53)****	11.33 (12.66)****	26.67 (32.33)***	180-185	152.80 (72.38)	176.30 (35.48)	75.00 (54.00)*	53.40 (34.99)**
Rearing frequency (units)					**Rearing frequency (units)**				
15-20	0.00 (0.00)	0.00 (0.00)	0.33 (0.58)	0.00 (0.00)	15-20	36.33 (13.78)	3.00 (2.68)**	1.20 (3.80)	31.10 (32.03)**
30-35	3.33 (5.77)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	30-35	35.83 (17.30)	8.67 (9.95)*	1.20 (2.90)	12.80 (21.25)
60-65	2.33 (4.04)	0.00 (0.00)	0.00 (0.00)	0.33 (0.58)	60-65	28.83 (14.63)	12.33 (9.18)	0.00 (0.00)	8.50 (12.49)
120-125	7.33 (7.09)	0.00 (0.00)	2.00 (2.00)	1.00 (1.73)	120-125	33.17 (30.90)	26.17 (15.54)	5.70 (1.75)*	14.00 (11.47)
180-185	20.00 (20.95)	0.00 (0.00)***	5.00 (7.00)*	0.00 (0.00)***	180-185	39.83 (33.13)	27.50 (14.53)	2.10 (2.64)*	9.10 (8.44)
Defecation (units)					**Defecation (units)**				
15-20	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	15-20	1.3 (1.21)	0.83 (0.75)	0.30 (0.68)	0.90 (0.99)
30-35	0.67 (0.58)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	30-35	2.50 (1.38)	0.50 (0.84)**	0.10 (0.32)	0.20 (0.42)
60-65	1.00 (1.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	60-65	1.17 (1.60)	1.33 (1.51)	0.00 (0.00)	0.40 (0.70)
120-125	1.00 (1.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	120-125	1.67 (2.74)	0.33 (0.52)	0.20 (0.42)	1.10 (0.88)
180-185	0.67 (1.15)	0.00 (0.00)	0.00 (0.00)	0.33 (0.58)	180-185	0.67 (1.21)	0.33 (0.52)	0.30 (0.48)	1.10 (0.88)
Grooming (seconds)					**Grooming (seconds)**				
15-20	0.33 (0.58)	0.00 (0.00)	0.67 (1.15)	0.00 (0.00)	15-20	30.83 (25.25)	20.17 (13.63)	5.70 (14.85)	5.70 (5.91)
30-35	2.67 (3.79)	0.00 (0.00)	0.00 (0.00)	0.67 (1.15)	30-35	40.00 (29.50)	38.50 (24.48)	3.80 (12.02)	10.30 (14.99)
60-65	1.00 (1.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	60-65	15.00 (15.99)	45.83 (18.39)	10.40 (31.51)	20.70 (50.83)
120-125	1.33 (0.58)	0.00 (0.00)	0.67 (1.15)	0.00 (0.00)	120-125	23.67 (38.01)	58.00 (41.65)	7.50 (9.55)**	22.60 (23.81)
180-185	3.00 (2.65)	0.00 (0.00)	0.67 (1.15)	0.00 (0.00)	180-185	26.00 (35.43)	44.17 (20.59)	11.20 (11.49)	8.90 (10.93)
Immobility time (seconds)					**Immobility time (seconds)**				
15-20	82.33 (76.51)	218.70 (35.80)	215.30 (57.57)	250.30 (17.16)	15-20	35.50 (25.36)	74.17 (56.28)	219.60 (72.12)**	35.70 (69.06)
30-35	182.70 (77.14)	260.30 (11.06)	273.70 (17.62)	253.30 (17.90)	30-35	30.83 (30.02)	86.00 (81.79)	243.50 (63.68)***	166.50 (94.85)
60-65	176.00 (38.31)	271.70 (16.92)	276.30 (26.95)	234.70 (40.05)	60-65	65.33 (89.63)	99.67 (56.41)	256.80 (51.21)***	165.60 (77.14)
120-125	42.67 (54.45)	201.00 (74.11)	182.00 (24.06)	196.00 (49.57)	120-125	81.50 (97.05)	70.00 (72.95)	159.40 (82.52)	105.90 (69.34)
180-185	91.33 (67.35)	278.30 (6.03)	163.00 (128.29)	228.30 (36.09)	180-185	49.33954.74)	52.83 (51.51)	173.70 (70.35)*	144.00 (74.910)

*$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$. NC: Naïve control group; VC: Vehicle control group; DIA: Diazepam group

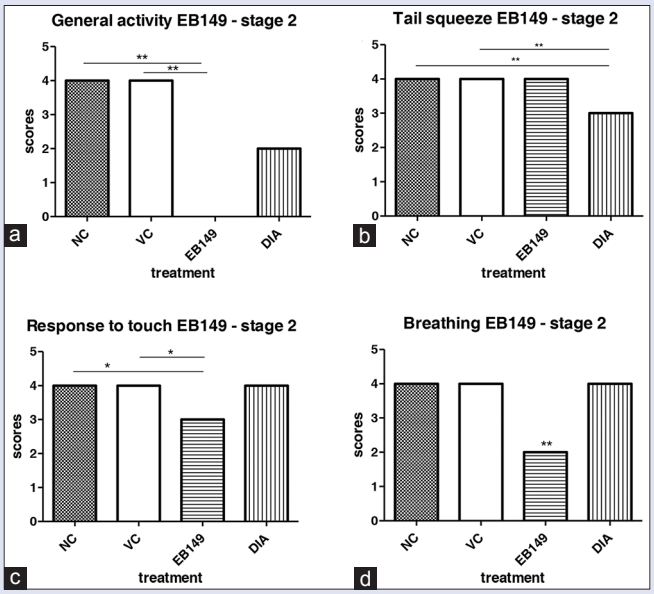

Figure 3: Effect over murine (a) general activity, (b) tail squeeze, (c) response to touch, and (d) breathing after administration of the nonlethal dose of EB149, obtained from the stem of *Xylopia aromatica*, in stage 2 of the experiment. Kruskal–Wallis statistical analysis ($n = 10$; $n_{total} = 40$) followed by Dunnett's multiple comparison tests was performed among means. Significances were given if $P < 0.05$

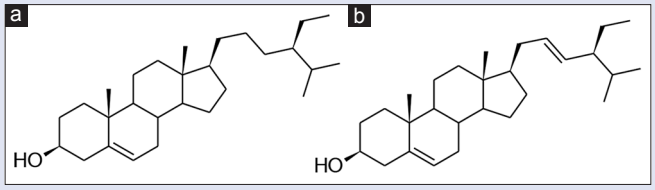

Figure 4: (a) Sitosterol; (b) Stigmasterol isolated from the stem of *Xylopia aromatica*

138.29, 129.26, and 121.69 permitted the structural propositions. The comparison of these data with those in the literature[15] enabled the deduction that the fraction is a mixture of both steroid constituents.

Cytotoxic response of EB149

Three samples were then analyzed for their cytotoxicity against breast and prostate cancer cell lines: FSIST (100 µg/mL), TAF (100 µg/mL), and doxorubicin (25 mM) as the standard drug. Results for breast cancer cell lines were as follows: FSIST showed a percentage of growth inhibition of 39.22% and an IC_{50} of 191.7 µg/mL, TAF showed a percentage of growth inhibition of 56.59% and an IC_{50} of 104.7 µg/mL, while doxorubicin showed a percentage of growth inhibition of 89.82% and IC_{50} of 0.287 mM. Results obtained for prostate cancer cell lines were as follows: FSIST showed a percentage of growth inhibition of 43.16% and IC_{50} of 123.7 µg/mL, TAF showed a percentage of growth inhibition of 55.03% and an IC_{50} of 106.4 µg/mL, while doxorubicin showed a percentage of growth inhibition of 98.49% and an IC_{50} of 1.148 mM.

DISCUSSION

Reports on the presence of volatile compounds in the fruits, flowers, and leaves of *X. aromatica* were previously published.[16-21] Although alkaloids have been found in *Xylopia* species as isoquinoline alkaloids in *X. parviflora*,[22-25] aporphine alkaloids in *X. benthamii* (Pimenta, 2012),[26] and oxoaporphine alkaloids in *X. aethiopica*,[27] no reports on the presence of alkaloids in *X. aromatica* were found. Kaurane diterpenes,[28] acetogenins,[29,30] volatile compounds[31] as sesquiterpenes,[32,33] and labdane dimmers[34] were found to occur in *X. aromatica*. Furthermore, *X. aromatica* extracts showed activity against *Leishmania* sp., *Trypanosoma cruzi*,[35] and *Plasmodium*[36] and against microbes[37] and *Aedes aegypti*.[38] It is popularly used as a diuretic, as it is indicated to treat skin edema.[37] EB149 showed cytotoxic activity against leukemia and RPMI-8226 cell lines (lethality of −94.40% over T_0 control).[6,39]

Despite a significant number of studies having been developed for *X. aromatica*, no groups have reported its influence over BP. The present findings report that LD_{50} obtained for EB149 is 2.344 g/kg, and the extract is relatively safe to use, although alterations in BP have been found.

It was observed that an impairment in general activity, piloerection, defecation, breathing, auricular and corneal reflexes, tail squeeze, response to touch, hindquarter fall, surface-righting reflex, body tone, and grip reflex was decreased after the administration of higher doses of EB149, but a significant recovery was observed after the administration of a dose of 625.0 mg/kg, in the first stage of the experiment. In the second stage, when NLD was administered, a significant decrease in general activity, tail squeeze, touch response, and breathing was still observed, but animals recovered well during the period of observation (14 days). Locomotion was decreased by EB149 I. P. administration, as a tendency toward immobility was observed; a situation that was observed in both the first and second stages of the experiment. Locomotion was recovered during the experimental period (14 days).

Cytotoxicity of the fractions FSIST and TAF against breast and prostate cancer cells was presently found, although EB149 has not shown cytotoxicity against the same cancer cells (results not shown). Nonetheless, a crude extract obtained from the African *X. aethiopica* was toxic to MCF-7 breast cancer cell lines.[40]

Sitosterol and stigmasterol are being reported to occur in *X. aromatica* for the first time and they were identified to occur as a mixture in the FSIST fraction, showing activity against breast ($IC_{50} = 191.7$ µg/mL) and prostate ($IC_{50} = 123.7$ µg/mL) cancer cell lines. Sitosterol and stigmasterol are known as phytosteroids. The structure of sitosterol is similar to cholesterol, although it is produced exclusively by plants; it is considered essential to the constitution of plant cell membranes.[41] Sitosterol is commonly found in plants. Its cytotoxic activity has been described against MCF-7 cell lines,[42,43] as well as its antiproliferative activity and apoptotic induction associated with downregulating Bcl-2. Also, the degradation of polymerase and phospholipase C and the activation of caspase-3 was observed in leukemia cell lines.[44] Stigmasterol was found in plant extracts showing cytotoxic activities against cancer cell lines.[43,45]

CONCLUSIONS

EB149 showed an impairment of general activity, tail squeeze, touch response, and breathing, and a decrease in locomotion frequency after being administered to male mice, but behavior recovered during the experimental period. Its influence over BP was considered not harmful, as mice have recovered during the experimental period. Two fractions obtained from the crude organic extract named FSIST, which contains sitosterol and stigmasterol as major compounds, and TAF, which is the total alkaloidal fraction, showed cytotoxic activity against breast and prostate cancer cell lines.

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Conflicts of interest

There are no conflicts of interest.

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