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ORIGINAL ARTICLE

Picrolemma sprucei Quassinosides Inhibits Breast and Prostate Cell Growth and Impairs Behavioral Phenotype in Mice

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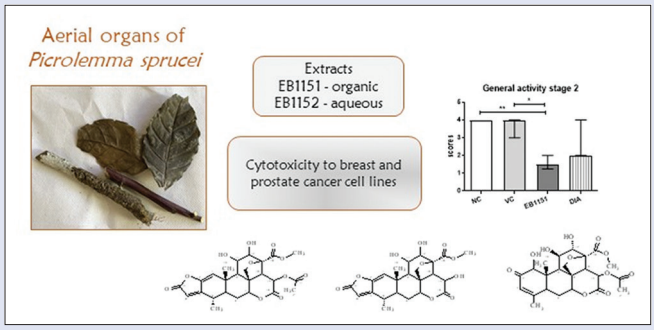
ABSTRACT

Background: It is estimated that in Brazil 59,700 new cases of breast and 68,220 new cases of prostate cancer will occur, in 2018, in Brazil. For that reason, there is a need for introducing new antitumor drugs in therapy. **Objective:** The objective of the study is to bioguide fractionate the organic/aqueous extracts from the aerial organs of *Picrolemma sprucei* (EB1151 and EB1152) aiming the identification of antitumor compounds and to evaluate the influence of EB1151 on mice behavioral phenotype. **Materials and Methods:** The breast (MCF-7) and prostate (PC-3) cancer cell lines cytotoxicity assay were assessed using the sulforhodamine B method. Fractionation was carried out by thin layer and column chromatographic techniques. The influence of the intraperitoneal administration of nonlethal dose (NLD, 39.1 mg/kg) of EB1151 to Balb-c male mice was assessed in open cage and open-field (OF) apparatuses. **Results:** $FCHCl_3$ partition phases from EB1151 and EB1152 showed cytotoxic activity against both cancer cells, as did fractions UNIP343, which contains sergeolide and isobruceine B, and UNIP344, which contains sergeolide and 15-deacetylsergeolide. Higher doses of EB1151 showed significant alterations in response to touch, hindquarter fall, irritability, auricular and corneal reflexes, ataxia, micturition, ptosis, piloerection, hypothermia, and cyanosis, and NLD showed alterations in general activity and response to touch, but recovery was obtained up to the end of the experiment. **Conclusions:** Quassinosides from the aerial organs of *P. sprucei* showed significant cytotoxicity against MCF-7 and PC-3 cell lines. Extract EB1151 has impaired general activity and activity in the OF apparatus, but recovery was observed.

Key words: Antitumor, isobruceine B, quassinosides, sergeolide, *Simaroubaceae*

SUMMARY

- Cytotoxic activity was observed for the partition phases and purified fractions of *Picrolemma sprucei*
- Sergeolide, 15- deacetylsergeolide, and isobruceine B were identified in the active fractions of *P. sprucei*
- Organic extract of *P. sprucei* impaired phetotype behavior of Balb-c male mice, but recovery was observed by the end of the experiment.



Abbreviations used: IBAMA/CGen/MMA: Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis/Conselho de Gestão do Patrimônio Genético/Ministério do Meio Ambiente; UNIP: Universidade Paulista; g: Gram; h: Hour; mL: Milliliters; $FCHCl_3$: Fraction chloroform; FBuOH: Fraction butanol; FH_2O: Fraction water; FHEX: Fraction hexane; FDCM: Fraction dichloromethane; FMeOH: Fraction methanol; FACN15: Fraction acetonitrile 15%; FACN50: Fraction acetonitrile 50%; FAcEt: Fraction ethyl acetate; $CDCl_3$: Deuterated chloroform; NH_4OH: Amonium hydroxide; DOXO: Doxorubicin; M: Molar; TCA: Trichloroacetic acid; µg: Microgram; IC_{50}: 50% inhibitory concentration; CEUA/ICS/UNIP: Comissão de Ética no Uso de Animais/Instituto de Ciências da Saúde/Universidade Paulista; kg: cm^3: Cubic centimeters; Kilogram; BP: Behavioral phenotype; OF: Open-field apparatus; NLD: Nonlethal dose; mL: Microliters; ANOVA: Analysis of variance; FAkl: Fraction alkaloids; mM: Millimolar; DNA: Desoxyribonucleic acid.

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INTRODUCTION

In Brazil, breast and prostate cancers are the most prevalent types of cancer in women and men, respectively, if not considering nonmelanoma skin cancer. It is expected that 59,700 new cases of breast and 68,220 new cases of prostate cancers will occur, in 2018.[1] Despite recent progress in the treatment of metastatic disease, the availability of new options is mandatory, mainly for the frequent cases of resistance to currently available therapies.

Brazilian flora, the most diverse in the world, has become an interesting spot to prospect for new chemical leads or hits due to its species diversity and associated chemical richness. Screening programs have been established in Brazil[2] as a strategy to identify potentially active substances.[3-5] From random screening, the extract (here designated as EB1151) obtained from the stem of *Picrolemma sprucei* Hook.f. (synonym Picrolemma *pseudocoffea* Ducke), a plant belonging to

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Simaroubaceae family and found in the Amazon rainforest, has previously shown significant antitumor activity in *in vitro* assays against head-and-neck cancer cell lines[6] and against leukemia cell line RPMI-8226 showing growth inhibition of −37.56%.[7]

Although previous reports show stimulating pharmacological and biological activities, studies regarding its cytotoxicity against MCF-7 and PC-3 cell lines as well as its influence over behavioral phenotype (BP) are needed as was not achieved before. *P. sprucei* is popularly known as "caferana," in Brazil, and it is conventionally used against gastritis, febrifuge, anthelmintic, and as antimalarial.[8] Furthermore,"sacha café," as it is known in Peru, is also used to similar conditions.[9]

In the present work, the cytotoxicity of extracts EB1151 and EB1152 and purified fractions of EB1151 was assessed as well as was the influence of EB1151 over BP in Balb-c male mice.

MATERIALS AND METHODS

Plant collection and extracts preparation

Aerial organs of *P. sprucei* were collected in the Brazilian Amazon rainforest, under IBAMA/CGen/MMA licenses (12-A/2008), in the surrounds of Manaus city, state of Amazonas, in a seasonally flooded forest from Rio Negro Basin (the so-called *igapó* forest). The voucher is deposited at UNIP Herbarium (*A.A.Oliveira, 3582* [UNIP]). The plant material was used to obtain the crude extract used in the present assays, and the preparation is described elsewhere.[10,11] Briefly, 202.0 g of the aerial organs of the plant were ground in a hammermill (Holmes), and the powder was macerated with dichloromethane and methanol (1:1) for 24 h. Macerate was drained and solvents removed by rotavaporation (Buchi). A second 24-h maceration was carried out with Milli-Q water, and then, the water was lyophilized. Organic and aqueous extracts were named EB1151 and EB1152, respectively. Both extracts were submitted to fractionation and cytotoxic assays although identification of compounds was performed only for EB1151. The influence over behavior was assessed for EB1151.

Phytochemical studies

Ten grams of EB1151 and 7 g of EB1152 were weighed. A liquid–liquid partition in a glass column was performed for each of the extracts, as was previously described.[12] EB1151 was partitioned with chloroform and butanol, resulting in fractions chloroform (F1CHCl$_3$), butanol (F1BuOH), and aqueous (F1H$_2$O), which were obtained from the remaining water, after solvent evaporation. EB1152 was partitioned with the same solvents, resulting in the same fractions, named F2CHCl$_3$, F2BuOH, and F2H$_2$0. Both F1CHCl$_3$ and F2CHCl$_3$ were fractionated in column chromatography using Sephadex LH-20, by an initial solubilization of the fractions in hexane, which was the first solvent used in the elution (200 mL), resulting in fraction F1Hex and F2Hex, respectively. Then, 200 mL of dichloromethane was used as the second solvent in the elution, resulting in fractions F1DCM and F2DCM, respectively. Methanol (150 mL) was the third solvent to be used in the elution, which resulted in fractions F1MeOH and F2MeOH, respectively. F1BuOH and F2BuOH were fractionated in column chromatography using silica gel C18; fractions were initially solubilized in methanol; and 300 mL of 15% acetonitrile in water was used as the first eluent, resulting in fractions F1ACN15 and F2ACN15. Then, 350 mL of 50% acetonitrile in water was used as the second eluent, resulting in fractions F1ACN50 and F2ACN50. A third eluent was used, so 300 mL of 100% methanol resulted in fractions F1MeOH and F2MeOH, respectively. Ethyl acetate (300 mL) was used as the last eluent, resulting in fractions F1AcEt and F2AcEt.

The fraction F1Hex was column chromatographed in silica gel, resulting in 41 fractions. The fraction F1DCM was column chromatographed in silica gel, resulting in 45 fractions. All the resulting fractions were thin layer chromatographed to compare similarities among them. Similar fractions from F1Hex were reunited, resulting in fractions named UNIP324, UNIP328, UNIP335, and UNIP336, and similar fractions obtained from F1DCM were reunited and resulted in fractions UNIP343 and UNIP344. UNIP fractions were sent to be analyzed by H^1 and C^{13} nuclear magnetic resonance (500 MHz and 125 MHz NMR, Bruker, $CDCl_3$).

Total alkaloid fractionation

One gram of each extract, EB1151 and EB1152, were used in the alkaloidal separation. Degreasing of the extract was done by the addition of 45 mL of Hex. EB1151 was solubilized in $CHCL_3$ and BuOH and kept in a funnel. Then, two aliquots of 20 mL and 40 mL of 1% phosphoric acid were added to the solution. Acidic phase was filtered and alkalinized with NH_4OH up to pH 10. Then, 400 mL of $CHCl_3$ was added to the funnel and shaken. Chloroformic phase was separated, and the solvent was evaporated. The alkaline phase was lyophilized. Both chloroform and alkaline phases were analyzed for the presence of alkaloids by the use of Dragendorff reagent. The same procedure was adopted in the alkaloid separation of EB1152.

Cell culture technique

Tumor cell lines (MCF-7 breast adenocarcinoma and PC-3 prostate carcinoma) were cultivated in 150 cm^3 tissue-culture flasks (Costar), in RPMI-1640 medium supplemented with 5% fetal bovine serum (both Cambras) and 1% glutamine (Sigma), kept in an incubator (Forma) at 37°C with 5% CO_2 and 100% relative humidity. Cell lines were weekly passaged (Trypsin-EDTA, Cambras) and a split ratio of 1:10 to breast cells and 1:4 to prostate cancer cells.[13]

Cytotoxicity assay

For the toxicity assay, the same growth conditions were maintained during the assay. Cell densities per well varied according to the cell line, as follows: MCF-7 (10,000) and PC-3 (7500). Cells were transferred according to previous densities to 96-well microplates (Costar). Plates 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, the endpoints were obtained by the sulforhodamine B (SRB) assay, as follows. Viable cells were fixed to the 96 microplates with cold trichloroacetic acid (TCA) solutions (50 μL/well of 50% TCA). Microplates were washed with water five times until complete the removal of dead cells, which were not fixed. A hundred μL of SRB 0.4% in acetic acid were added to each well and were kept in contact with the cells for 10 min. After that, unbound SRB (SRB binds to proteins of viable cells) was removed from plate by washing four times the wells with 0.1% acetic acid solution. The remaining stain was then resuspended with the addition of 100 μL of Trizma buffer. The variation in the amount of remaining proteins bound to the stain was measured by obtaining the optical densities of the wells in a spectrophotometer reader at 515 nm.[14] The inhibitory concentration 50% (IC_{50}) was obtained for the crude extracts and fractions. Doxorubicin (DOXO) was used as standard drug in the assay. The highest DOXO concentration in the test was 2.5×10^{-5} M. Five 10-fold dilutions were made, and the concentration X response curves obtained show the cytotoxic trend observed for each extract/fraction.

Behavioral phenotype studies

Animals

Male Balb-c mice weighing 25–30 g were used. After arrival in the laboratory, animals were housed in groups of five in polypropylene

cages (38 cm × 32 cm × 16 cm) with controlled room temperature (22°C ± 2°C), humidity (55%–65%), and artificial lighting (12 h light/12 h dark cycle, lights on at 8:00 a.m.), and free access to Nuvilab® rodent chow (Nuvital Company, São Paulo, Brazil) and filtered water. The experiments began 1 week after the mice arrived in for habituation to the new laboratorial conditions. All the experiments done with mice were subjected to the ethics committee (CEUA/ICS/UNIP 025/08 and CaPPesq 1109/090).

Plant extract preparation and control drug

EB1151 was suspended in almond oil, and the described doses of 5000, 2500, 1250, 625.0, 312.5, 156.3, 78.1, and 39.1 mg/kg were administered by intraperitoneal route. Almond oil was used in the extract dilution because of its low polar origin and because of the absence of toxicity, which makes it fully compatible with mammalian organisms. The intraperitoneal route was chosen due to the absence of bioavailability loss.

Behavior phenotype alterations

BP was assessed,[15] with modifications.[12,16-18] Parameters related to the general activity, to the sensorial system (such as vocal tremor, irritability, auricular reflex, corneal reflex, tail squeeze, and response to touch), to psychomotor system (contortion, hindquarter fall, surface-righting reflex, body tone, and grip reflex), to central nervous system (convulsions, ataxia, anesthesia, hypnosis, straube tail, tremor, stimulation, and sedation), and to autonomous nervous system (lacrimation, breath, ptosis, piloerection, micturition, defecation, hypothermia, and cyanosis) were assessed, and a score from 0 to 4 was given for each parameter, except to micturition and defecation, which numbers of urination and fecal boli were counted.

Evaluation in open-field apparatus

Open-field (OF) apparatus was used as a tool to evaluate the influence of EB1151 over locomotion and anxiety and was immediately done after observations in open cage, as previously described.[12,16-18]

Experimental design

The evaluation of BP alterations and variations in locomotion and anxiety was made using a limited number of animals in a two-stage experiment, as described before.[12,16-18] Different doses of extract were administered, starting from the limit dose of 2500 mg/kg, and subsequently administrations of EB1151 in ½-fold dilution paths, if lethality occurred in at least one among three mice. Observations in open cage followed by observations in OF were done at 7–10, 25–30, 55–60, 115–120, and 175–180 min after administration or until dead; mice who survive were observed every 24 h in the subsequent 14 days. The tendency of lethal dose 50% and the tendency of nonlethal dose (NLD) were obtained in the first stage of the experiment. NLD was used to test a large group of animals (n = 10; n_{total} = 40) in the second stage of the tests, when a group of naïve mice was introduced with the purpose of controlling the possible influence of intraperitoneal injection and a group which received 1 mg/kg diazepam (Hipolabor; lot no. AO011/11; validity: 10/13; administered concentration: 5.0 mg/mL; and injectable medication), as control drug. Based on physiological issues, the assays started at 8:00 p.m. and ended before noon, to prevent the circadian influences.

Statistical analysis

Most of the parameters observed in the general activity prospection were based on scores ranked from 0 to 4. To organize statistical analysis of nonparametric data, the scores of each group were summed and formed a new group to be ranked. Hence, analysis of variance (ANOVA) by ranks Kruskal–Wallis followed by Dunn posttest[19] was then applied. Two-way ANOVA and two-way repeated measures ANOVA followed by Bonferroni posttest were applied in the evaluation of OF parameters. All analysis ran under 0.05 significance level (α =0.05). Statistical procedures were conducted by the software Prism 5.0® (GraphPad Software, 2010; San Diego, CA, USA).

RESULTS

Compounds that were identified in EB1151 are shown in Figure 1. Sitosterol[1] and stigmasterol[2] were identified in the fraction UNIP324. H^1 NMR (in $CDCl_3$) shows signs that are characteristic related to steroid skeleton as the double-double-double-doublet at J = 9.5, 4.8, 11.2, and 4.6 Hz, attributed to H-3 and a large singlet at δ 5.36 which indicates the presence of an olefinic hydrogen (H-6), also shows the double-doublets at δ 5.16 and 5.02, characteristic of stigmasterol (H-23 and H-22). Chemical shifts can be seen in Table 1, where the comparison to the literature was done.[20] Compounds (*14S, 17S, 20S*)-*14, 17, 20*-trihydroxy-24-malabaricen-3-one[3] and (*17R, 20R, 24R*)-*17, 20, 24, 25*-tetrahydroxy-14[18]-malabaricen-3-one[4] were identified from fraction UNIP328. Chemical shifts were obtained: H^1 NMR (in $CDCl_3$) shows singlets at δ 0.96–1.25 and at δ 1.6–1.74, corresponding to the methyl. H17, H18, and H21 from the structure are at δ 3.30–3.50. Chemical shifts for C^{13} are described in Table 1 and were compared to the literature.[21] Cathin-6-one was identified in fraction UNIP335. Table 1 shows C^{13} NMR and $CDCl_3$ chemical shifts for cathin-6-one,[5] compared to the literature.[22]

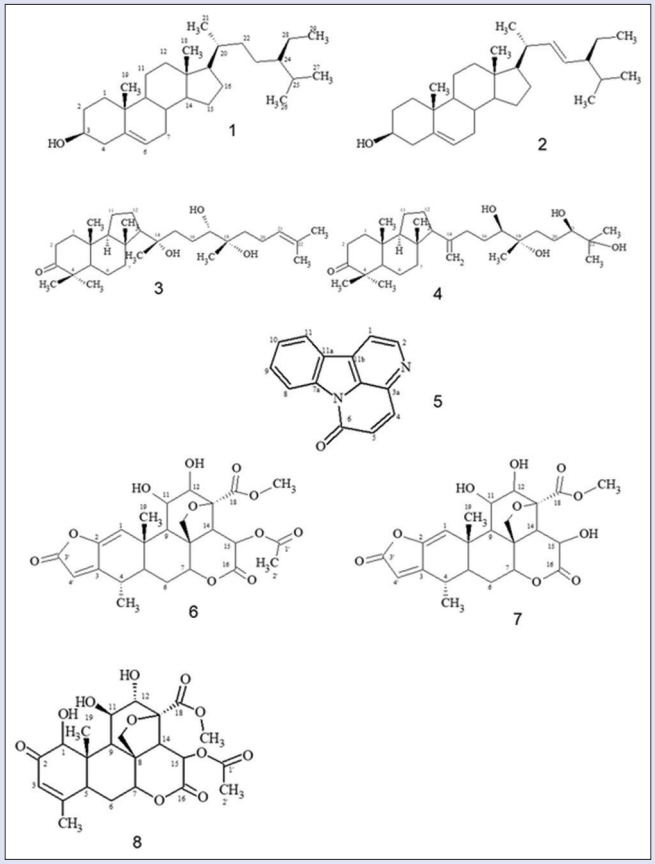

Figure 1: Molecules identified from fractions of the extract EB1151, which was obtained from the aerial organs of *Picrolemma sprucei* (Simaroubaceae). (1) Sitosterol, (2) stigmasterol, (3) (14S, 17S, 20S)-14,17,20-trihydroxy-24-malabaricen-3-one, (4) (17R, 20R, 24R)-17,20,24,25-tetrahydroxy-14 (18)-malabaricen-3-one, (5) cathin-6-one, (6) sergeolide, (7) 15-deacetylsergeolide and (8) isobruceine B

Table 1: Chemical shifts for compounds that were identified, after fractionation of EB1151, extract obtained from aerial organs of *Picrolemma sprucei*

n	^{13}C-NMR ($CDCl_3$)		^{13}C-NMR ($CDCl_3$)		*n°*	^{13}C-NMR (CD_3OD)		^{13}C-NMR ($CDCl_3$)		*n*	^{1}H-NMR $CDCl_3$	^{1}H-NMR $CDCl_3$	*n°*	^{1}H-NMR $CDCl_3$	^{1}H-NMR $CDCl_3$	^{13}C-NMR	^{13}C-NMR
	Sitosterol	Stigmasterol	UNIP324			(*14S,17S,20S*)-14,17,20-trihydroxy-24-malabaricen-3-one	(*17R,20R,24R*)-17,20,24,25-tetrahydroxy-14 (18)-malabaricen-3-one	UNIP328			Cathin-6-one	UNIP335		Cathin-6-one	UNIP-336	Cathin-6-one	UNIP-336
1	37.3	37.3	37.23	37.23	1	39.34	38.95	38.54	38.54	1	7.59 d J=5.0	7.99 d J=5.19	1	7.59 d J=5.0	7.93 d J=5.19	115.37	116.42
2	31.6	31.6	31.63	31.63	2	33.71	33.72	33.83	33.21	2	8.58 d J=5.0	8.85 d J=5.19	2	8.58 d J=5.0	8.76 d J=5.19	144.84	145.62
3	71.8	71.8	71.78	71.78	3	218.97	218.00	217.87	217.79	4	7.77 d J=9.7	8.06 d J=9.76	3a	-	-	135.23	136.81
4	42.2	42.3	42.19	42.26	4	47.14	47.13	46.71	47.83	5	6.75 d J=9.7	7.01 d J=9.66	4	7.77 d J=9.7	8.01 d J=9.76	138.57	139.38
5	140.8	140.8	140.73	140.73	5	55.02	55.35	55.46	55.61	8	8.28 d J=7.7	8.71 d J=8.24	5	6.75 d J=9.7	6.95 d J=9.76	127.98	128.86
6	121.7	121.7	121.69	121.69	6	20.57	20.22	19.51	19.69	9	7.45 t J=7.7	7.73 ddd J=7.32, 7.32, 1.22	6	-	-	158.21	159.49
7	31.9	31.9	31.88	31.88	7	36.81	35.55	36.70	36.32	10	7.28 t J=7.7	7.55 ddd J=7.32, 7.32, 1.22	7a	-	-	138.24	139.38
8	31.9	31.9	31.88	31.88	8	44.18	44.81	44.61	44.72	11	7.73 d J=7.7	8.14 d J=8.24	8	8.28 d J=7.7	8.62 d J=8.29	116.29	117.20
9	51.2	51.2	51.22	51.22	9	59.79	54.31	60.83	54.39				9	7.45 t J=7.7	7.68 ddd J=7.32, 7.32, 1.22	129.84	129.49
10	36.5	36.5	36.49	36.49	10	36.46	36.02	36.16	36.74				10	7.28 t J=7.7	7.49 ddd J=7.62, 7.62, 0.95	124.69	124.93
11	21.1	21.1	21.04	21.04	11	21.09	20.13	20.95	20.95				11	7.73 d J=7.7	8.07 d J=7.63	121.61	122.68
12	39.8	39.7	39.75	39.66	12	23.68	26.93	22.61	26.65				11a	-	-	123.33	124.58
13	42.3	42.3	42.26	42.26	13	59.79	56.71	60.83	55.46				11b	-	-	128.99	130.40
14	56.8	56.9	56.74	56.84	14	75.40	155.81	74.08	155.00				11c	-	-	130.91	131.28
15	24.3	24.4	24.28	24.35	15	39.01	36.42	39.27	36.99								
16	28.3	28.4	28.23	28.89	16	25.17	30.03	25.56	29.68								
17	56	56.1	55.93	55.93	17	78.25	77.52	78.84	78.84								
18	11.9	11	11.96	11.83	18	74.17	72.55	73.87	73.95								
19	19.4	21.2	19.61	21.20	19	37.97	35.39	37.12	36.16								
20	36.2	40.5	36.16	40.47	20	24.38	20.58	24.76	20.98								
21	18.8	21.2	18.87	21.20	21	124.70	79.03	124.59	79.41								
22	33.9	138.3	33.93	138.29	22	130.56	74.16	127.96	73.87								
23	26.1	129.3	26.04	129.25	23	21.65	24.52	20.98	24.76								
24	45.9	51.2	45.80	51.22	24	14.88	13.08	14.19	13.81								
25	29.2	31.9	29.12	31.88	25	20.85	25.63	20.95	25.56								
26	19.8	21.2	19.79	21.2	26	24.47	24.56	24.76	25.82								
27	19.3	19	19.38	19.02	27	16.33	24.08	16.62	25.32								
28	23.1	25.4	23.04	25.39	28	20.04	20.08	19.39	20.95								
29	12.2	12.1	12.23	12.02	29	25.70	107.90	25.82	108.66								
					30	25.07	23.35	25.32	22.67								

Contd...

Table 1: Contd...

n	^{13}C-NMR ($CDCl_3$ - 5%pyridine-d_5)	^{13}C-NMR ($CDCl_3$)	^{1}H-NMR ($CDCl_3$)	^{1}H-NMR ($CDCl_3$)	*n*	^{13}C-NMR ($CDCl_3$ - 5%pyridine-d_5)		^{13}C-NMR ($CDCl_3$)	
	Sergeolide	UNIP-343	15-deacetylsergeolide	UNIP-343		-	Sergeolide	UNIP344	
1	113.2	113.21	6.23 s	6.24 s	1	81.3	113.2	80.54	113.21
2	160.8	160.51	-	-	2	197.6	160.8	197.01	160.51
3	148.4	148.44	-	-	3	124.5	148.4	124.23	148.44
4	30.7	30.65	2.38 m	2.37 m	4	162.6	30.7	163.05	30.65
5	38.8	38.86	1.80 ddd J=13;2;9	1.85 m	5	43.4	38.8	43.41	38.86
6	28.5	28.53	1.69 ddd J=15; 2.5;2.5	1.68 m	6	28.2	28.5	28.42	28.53
			2.24 ddd J=15;2;2	2.23 m	7	81.7	82.8	81.04	82.97
7	82.8	82.97	4.63 s	4.65 s	8	45.8	46.4	45.55	46.35
8	46.4	46.35	-	-	9	42.4	44.7	42.58	44.66
9	44.7	44.66	2.28 d J=5	2.28 m	10	47.7	41.6	47.48	41
10	41.6	41	-	-	11	74.3	72.9	73.76	72.87
11	72.9	72.87	4.54 d J=5	4.50 m	12	75.1	76	75.63	76.07
12	76	76.07	4.17 s	4.23 s	13	81.7	81.1	81.04	81.04
13	81.1	81.04	-	-	14	52.3	53.1	52.93	53.26
14	53.1	53.26	2.81 dd J=13;2	2.89 dd J=12.5;1.5	15	67.8	66.9	66.67	66.67
15	66.9	66.67	5.16 d J=13	4.9 d J=12.85	16	167.6	167.1	166.95	166.80
16	167.1	166.80	-	-	18	169.5	170.9	169.25	171.24
18	170.9	171.24	-	-	30	73.0	73.8	73.31	73.76
30	73.8	73.76	4.75 d J=8	4.77 d J=8.25	OMe	49.8	51.2	53.02	52.93
OMe	51.2	52.93	3.83	3.84	Me-4	22.4	18.0	22.51	17.98
Me-4	18.0	17.98	1.18 d J=7	1.19 d J=6.65	Me-10	11.3	19.4	11.48	19.43
Me-10	19.4	19.43	1.57 s	1.57 s	1’	170.7	169.4	170.32	169.25
1’	169.4	169.25	-		2’	20.5	20.4	20.32	20.84
2’	20.4	20.84	-		3’		171.4		171.55
3’	171.4	171.55	-		4’		161.1		160.51
4’	161.1	160.51	5.72 s	5.69 s					

The same molecule[5] was identified in fraction UNIP336. Sergeolide[6] and 15-deacetylsergeolide[7] were identified in fraction UNIP343, and Table 1 describes chemical shifts obtained for UNIP343 and its comparison to the literature.[23,24] Isobruceine B[8] and sergeolide[6] were identified in fraction UNIP344, and Table 1 describes the chemical shifts for C^{13} NMR, $CDCl_3$, and its comparison to the literature.[23]

Table 2 and Figure 2 show results obtained from cytotoxic assay which show that partition phases $FCHCl_3$ obtained from both EB1151 and EB1152 showed to be more cytotoxic against breast (0.128 μg/mL and 0.011 μg/mL) and prostate (0.388 μg/mL and 0.029 μg/mL) cancer cell lines. Then, fractions FBuOH were subsequently active against breast (3,573 μg/mL and 0.370 μg/mL) and prostate, respectively (9,528 μg/mL and 2.519 μg/mL). Both partition phases FH_2O were less active although still significant against breast (42,740 μg/mL and 28.740 μg/mL) and prostate (937.800 μg/mL and 40,580 μg/mL) cancer cell lines. Fraction FAlk from EB1152 was more effective against breast cancer cell line (3,716 μg/mL), in relation to Falk from EB1151 (26.130 μg/mL). Both alkaloidal fractions, from EB1151 and EB1152, showed a similar cytotoxic activity against prostate cancer cell line (25,3800 μg/mL and 28.080 μg/mL). Fractions UNIP343 (breast = 0.093 μg/mL; prostate 0.229 μg/mL) and UNIP344 (breast = 0.031 μg/mL; prostate 0,158 μg/mL), obtained from partition phase $FCHCL_3$, showed to be the most effective against both cancer cell lines, while fraction UNIP324 was not active against the cell lines. Fractions UNIP328, UNIP335, and UNIP336 were also significantly active against the cancer cells, as seen for breast (17.510 μg/mL, 33.820 μg/mL, and 19.260 μg/mL) and prostate (23.570 μg/mL, 34.640 μg/mL, and 21.970 μg/mL) cell lines.

The alterations in BP originated from the administration of different doses of extract to three mice each group in the stage one of experiments were analyzed, and the results are statistically described, as shown in Table 3. Results related to the influence of the administration of EB1151 over BP in the first stage of the experiment are given [Figure 3a]. The administration of different doses has not significantly influenced the general activity ($H \sim \chi^2_{0.05}$, (7) = 7.989; $P > 0.05$). Diminish in the response to touch

Table 2: Results obtained from cytotoxic assay made with breast (MCF-7) and prostate (PC-3) human cancer cell lines after treatments with partition phases $FCHCl_3$, FBuOH, FH_2O, FAlk, and for purified fractions from $FCHCl_3$, named UNIP324, UNIP328, UNIP335, UNIP336, UNIP 343, and UNIP344, expressed as IC_{50}, in μg/mL and reference drugs curcumin, a natural product, and doxorubicin, expressed as IC_{50}, in μg/mL

Samples	IC_{50} (μg/mL)	
	Breast (MCF-7)	Prostate (PC-3)
F1$CHCl_3$	0.13	0.39
F1BuOH	3.57	9.53
F1H_2O	42.74	937.80
EB1151 FAlk	26.13	25.38
F2$CHCl_3$	0.01	0.03
F2BuOH	0.37	2.52
F2H_2O	28.74	40.58
EB1152 FAlk	3.72	28.08
UNIP324	256.70	177.10
UNIP328	17.51	23.57
UNIP335	33.82	34.64
UNIP336	19.26	21.97
UNIP343	0.09	0.30
UNIP344	0.03	0.16
Curcumin	132.62	158.40
Doxorubicin	77.51	1488.1

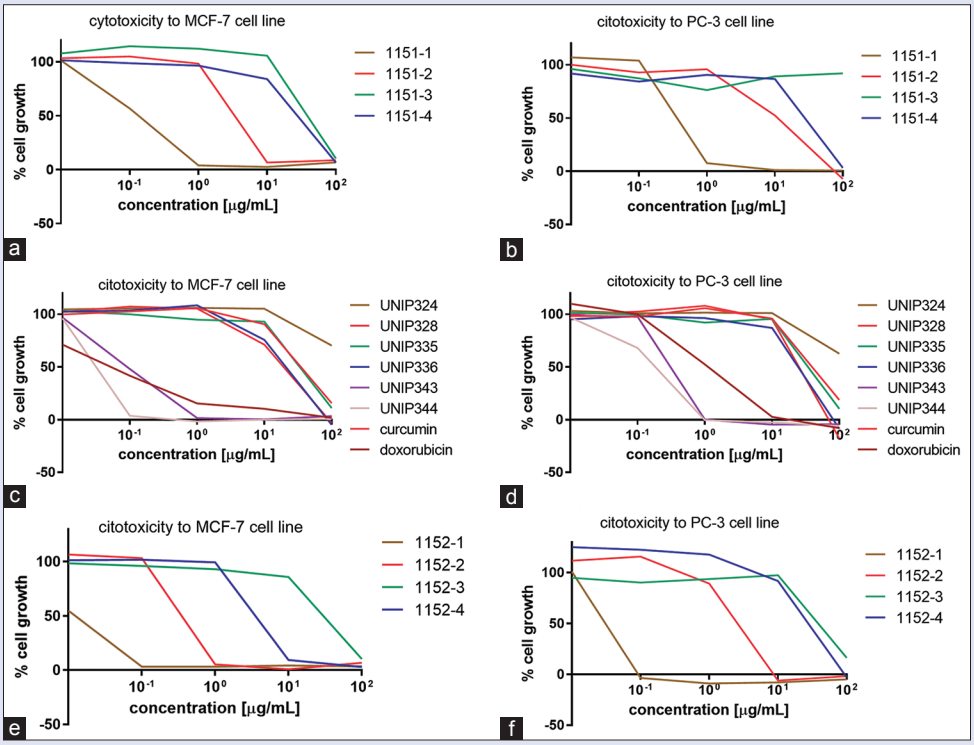

Figure 2: Results obtained from the cytotoxic assay done with fractions and isolates from the aerial organs of *Picrolemma sprucei*. (a) Cytotoxicity to MCF-7 cell lines treated with EB1151; (b) Cytotoxicity to PC-3 cell lines treated with EB1151; (c) Cytotoxicity to MCF-7 cell lines from EB1151 purified fractions; (d) Cytotoxicity to PC-3 cell lines from EB1151 purified fractions; (e) Cytotoxicity to MCF-7 cell lines treated with EB1152; (f) Cytotoxicity to PC-3 cell lines treated with EB1152. Legend: 1151-1 and 1152-1 = $FCHCl_3$ partition phases; 1151-2 and 1152-2 = FBuOH partition phases; 1151-3 and 1152-3 = FH_2O partition phases; 1151-4 and 1152-4 = total alkaloid fractions

Table 3: Behavioral phenotype on open-field parameters of male mice treated with EB1151, in Stages 1 and 2 of the experiment. Doses of EB1151 are given in mg/kg

Open-field evaluation Stage 1									Open-field evaluation Stage 2				
Locomotion frequency									Locomotion frequency				
Minutes	Control	2500	1250	625	312.5	156.3	78.1	39.1	Minutes	NC	VC	EB1151	DIA
15-20	165.00	61.33	148.00	122.00	61.00	102.00	176.70	81.00	15-20	129.50	174.30	132.10	236.20
30-35	171.70	62.67	106.30	44.33*	103.70	70.00	49.00*	66.33	30-35	129.50	58.50	83.00	82.80
60-65	166.00	70.00	140.70	65.00	139.70	61.67	137.70	104.30	60-65	124.20	83.50	88.00	74.80
120-125	183.00	26.67**	122.00	82.67	163.30	118.00	162.30	134.30	120-125	124.30	82.00	97.00	90.70
180-185	91.00	22.33	35.33	65.33	72.33	67.00	113.30	100.70	180-185	127.20	83.17	78.70	53.40
Rearing frequency									Rearing frequency				
Minutes	Control	2500	1250	625	312.5	156.3	78.1	39.1	Minutes	NC	VC	EB1151	DIA
15-20	4.33	0.67	4.33	4.667	3.333	2.667	10	1.667	15-20	22.33	5.33	5.80	31.10*
30-35	5.67	2.00	4.00	2.333	3	1.667	1.667	9.667	30-35	25.17	2.50	6.40	12.80
60-65	13.33	10.00	14.33	3	14.33	11.33	17.67	6.667	60-65	20.33	10.17	5.50	8.50
120-125	18.33	1.33	12.33	4.667	28.67	14	21	22.67	120-125	18.33	19.33	9.70	14.00
180-185	20.67	2.00	6.67	14	5	14.33	17.33	7.667	180-185	23.33	21.83	13.50	9.10
Defecation									Defecation				
Minutes	Control	2500	1250	625	312.5	156.3	78.1	39.1	Minutes	NC	VC	EB1151	DIA
15-20	0.67	0.00	0.00	0.67	0.00	0.67	1.67	0.00	15-20	1.67	1.00	0.30	0.90
30-35	1.33	0.00	0.00	0.33	0.67	0.67	0.33	1.00	30-35	0.83	1.00	0.40	0.20
60-65	2.33	0.33*	0.33*	1.00	0.67	1.00	0.67	2.00	60-65	0.33	1.00	1.00	0.40
120-125	0.67	0.00	0.33	1.00	0.00	0.67	0.67	0.67	120-125	1.00	0.83	0.20	1.10
180-185	0.67	0.33	0.00	0.00	0.00	0.00	0.33	0.67	180-185	0.50	0.83	0.40	1.10
Grooming									Grooming				
Minutes	Control	2500	1250	625	312.5	156.3	78.1	39.1	Minutes	NC	VC	EB1151	DIA
15-20	4.00	0.00	3.67	0.33	2.33	0.00	1.00	0.67	15-20	21.83	39.33	18.60	5.70
30-35	5.00	1.00	2.67	1.00	2.67	0.33	0.00	1.33	30-35	19.50	13.67	18.80	10.30
60-65	16.33	2.00**	4.33	3.67*	15.00	5.33	12.67	1.67**	60-65	19.17	10.67	40.70	20.70
120-125	14.33	2.00*	10.67	0.33*	9.33	6.00	15.67	4.33	120-125	19.50	43.00	37.60	22.60
180-185	14.67	0.00**	3.33	2.00*	3.33	2.00*	15.67	3.67	180-185	24.83	34.83	21.70	8.90
Immobility time									Immobility time				
Minutes	Control	2500	1250	625	312.5	156.3	78.1	39.1	Minutes	NC	VC	EB1151	DIA
15-20	79.67	209.70	108.30	120.70	126.30	151.30	76.00	113.00	15-20	38.83	64.67	30.60	35.70
30-35	148.70	203.00	164.00	209.00	128.30	187.70	161.70	125.70	30-35	48.17*	191.50	95.70	166.50
60-65	78.67	142.70	42.00	182.70	53.33	125.70	68.67	73.67	60-65	38.33	135.50	79.00	165.60
120-125	95.00	116.30	120.70	136.30	58.33	116.30	55.00	49.67	120-125	40.67	89.83	99.80	105.90
180-185	101.00	137.30	193.00	131.70	115.00	220.00	116.70	129.30	180-185	51.33	51.33	117.90	144.00

*P<0.05; **P<0.01. Two-way ANOVA and Bonferroni posttest was adopted, α<0.05. ANOVA: Analysis of variance

[Figure 3b] ($H\sim\chi^2_{0.05}$, (7) = 14.64; $P < 0.05$; after administration of dose 2,500 mg/kg). Hindquarter fall was observed after administration of dose 2,500 mg/kg [Figure 3c] ($H\sim\chi^2_{0.05}$, (7) = 34.33; $P < 0.01$). Irritability appeared [Figure 3d] ($H\sim\chi^2_{0.05}$, (7) = 22.11; $P < 0.01$; after administration of dose 1,250 mg/kg). Auricular reflex [Figure 3e] ($H\sim\chi^2_{0.05}$, (7) = 27.00; $P < 0.01$; diminished after administration of dose 2,500 mg/kg. Corneal reflex [Figure 3f] ($H\sim\chi^2_{0.05}$, (7) = 15.35; $P < 0.05$; diminished after administration of dose 153.8 mg/kg).

Figure 4 shows that ataxia appeared [Figure 4a] ($H\sim\chi^2_{0.05}$, (7) = 38.89; $P < 0.001$) in animals that received dose of 1,250 mg/kg. Micturition was more frequent in animals that received dose of 2.500 mg/kg [Figure 4b] ($H\sim\chi^2_{0.05}$,(7) = 25.30; $P < 0.01$). Ptosis emerged after administration of dose 625.0 mg/kg [Figure 4c] ($H\sim\chi^2_{0.05}$, (7) = 30.33; $P < 0.01$). Piloerection was observed after administration of all tested doses, except 2,500 and 625.0 mg/kg [Figure 4d] ($H\sim\chi^2_{0.05}$, (7) = 27.89; $P < 0.01$). Finally, hypothermia emerged [Figure 4e] ($H\sim\chi^2_{0.05}$,(7) = 22.11; $P < 0.01$) after the administration of dose 153.8 mg/kg, and cyanosis [Figure 4f] ($H\sim\chi^2_{0.05}$,(7) = 23.58; $P < 0.01$) increased after administration of doses 153.8 and 78.1 mg/kg.

Figure 5 shows which parameters were altered in Stage 2 of the experiment, done with the NLD. General activity of animals treated with EB1151 was significantly diminished in relation to control and to the naïve groups [Figure 5a] ($H\sim\chi^2_{0.05}$, (8) = 12.97; $P < 0.01$) and response to touch [Figure 5b] ($H\sim\chi^2_{0.05}$, (8) = 11.94; $P < 0.01$).

Table 3 reports behavioral changes related to locomotion and anxiety, evaluated in OF. In the first stage of analysis, locomotion frequency was reduced, and interaction ($F_{(28,64)} = 1.87$; $P < 0.05$), treatment ($F_{(7,16)} = 4.11$; $P < 0.01$), and time ($F_{(4,64)} = 8.10$; $P < 0.001$) accounted for 19.54%, 28.50%, and 12.13%, respectively, of the total variance. No differences between groups were observed in immobility time although time ($F_{(4,64)} = 6.65$; $P < 0.001$) accounted for 15.02% of the total variance. The same was observed for rearing frequency ($F_{(4,64)} = 8.32$; $P < 0.001$; accounted for 17.40% of the total variance). Diminish in defecation was observed, and variances were accounted by time ($F_{(4,64)} = 4.61$; $P < 0.01$; 10.87%) and treatment ($F_{(7,16)} = 2.88$; $P < 0.05$; 17.98%). Finally, grooming has diminished, and variances were accounted by time ($F_{(4,64)} = 13.26$; $P < 0.001$; 18.68%), treatment ($F_{(7,16)} = 5.62$; $P < 0.01$; 28.70%), and interaction ($F_{(28,64)} = 1.87$; $P < 0.05$; 18.42%).

In the second stage, the following statements could be done. Although interaction ($F_{(12,140)} = 2.98$; $P < 0.001$) and time ($F_{(4,140)} = 13.00$; $P < 0.001$), no differences among means were observed in locomotion frequency after administration. Concerning immobility time, interaction ($F_{(12,140)} = 2.11$;

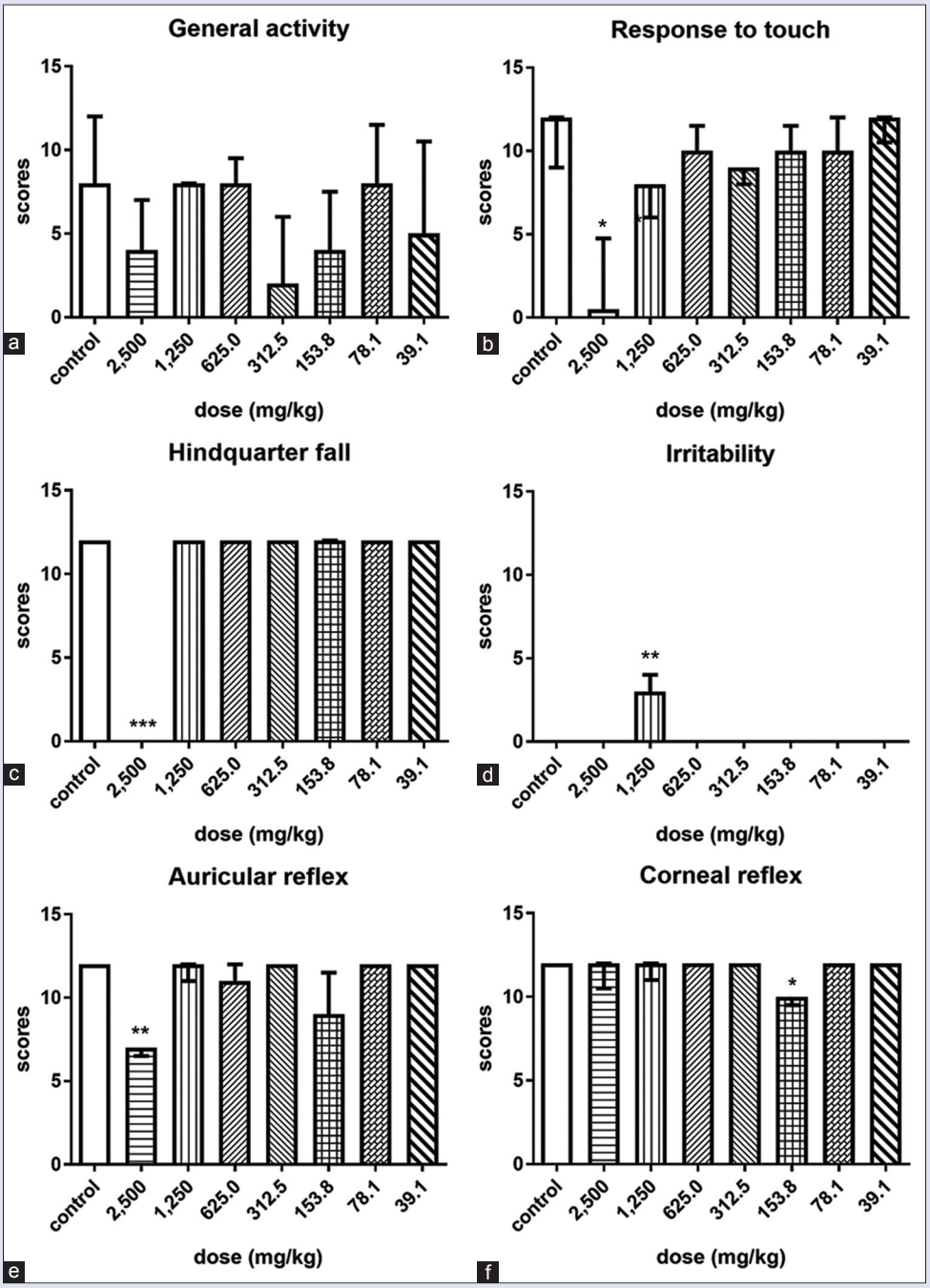

Figure 3: Impairment over behavioral phenotype parameters after the I.P. administration of EB1151 to male mice. (a) General activity, (b) Response to touch, (c) Hindquarter fall, (d) Irritability, (e) Auricular reflex, (f) Corneal reflex, in the first stage of experiment are shown. Kruskall–Wallis statistics ($n = 3$; $n_{total} = 24$) were used for all the parameters but defecation, which was analyzed. Differences among means after Dunn's multiple comparison tests are given, $\alpha<0.05$. **P*<0.05; ***P*<0.01

$P < 0.05$), time ($F_{(4,140)} = 6.66$; $P < 0.001$), and treatment ($F_{(3,140)} = 10.16$; $P < 0.001$) were considered statistically significant and accounted for 11.09%, 11.68%, and 13.36% of the total variance, respectively. Interaction ($F_{(12,140)} = 1.90$; $P < 0.05$) and treatment ($F_{(3,140)} = 5.99$; $P < 0.001$) affected the results and accounted for 12.28% and 9.67% of the total variance. Although interaction ($F_{(12,140)} = 2.16$; $P < 0.05$) and treatment ($F_{(3,140)} = 3.01$; $P < 0.05$) were considered statistically significant and accounted for 14.56% and 5.06% of the total variance, no differences among means were observed in the groups. Finally, no significant differences were observed in grooming ($P > 0.05$).

DISCUSSION

Simaroubaceae species are known for their biological activity and for the presence of alkaloids and quassinoids and other compounds as anthracenone C-glycosides, which occur in *Alvaradoa haitiensis* and have significant cytotoxic activity in the hollow fiber *in vivo* murine model using KB cell line and against murine tumors.[25] Previous works[26] showed multiple compounds isolated from *Simarouba glauca* by biomonitored fractionation using KB cancer cell line. They isolated alkaloids, triterpenes, coumarins, and triglycerides. *Simaba orinocensis*

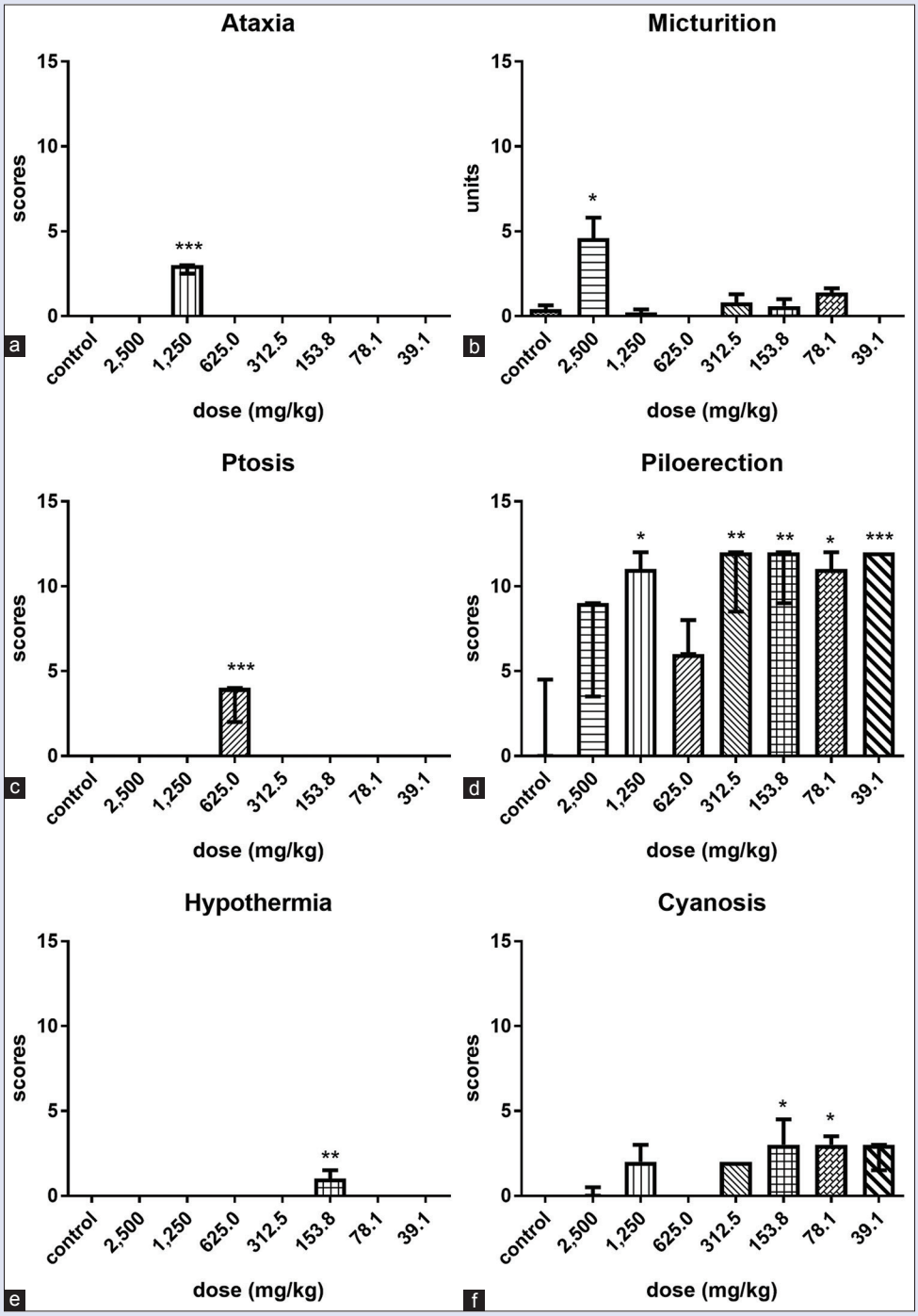

Figure 4: Impairment over behavioral phenotype parameters after the I.P. administration of EB1151 to male mice. (a) Ataxia, (b) Micturition, (c) Ptosis, (d) Piloerection, (e) Hypothermia, (f) Cianosis, in the first stage of experiment are shown. Kruskall–Wallis statistics (n = 3; n_{total} = 24) were used for all the parameters but defecation, which was analyzed. Differences among means after Dunn's multiple comparison tests are given, α <0.05. *P<0.05; **P<0.01

was studied for quassinoside antimalarial potential.[27] Finally, a flavonoid antihypertensive activity related to the leaves of *Ailanthus excelsa* was reported.[28]

P. sprucei belongs to *Simaroubaceae*, and in the present work, an aqueous (EB1151) and an organic (EB1152) extract were obtained from the aerial organs of the plant. The present findings describe the cytotoxicity that was observed for the extracts and their fractions against breast and prostate cancer cell lines. Previous report has described the cytotoxicity activity of *P. sprucei* extracts against KB-ADR-#12 squamous cell carcinoma of the mouth, *in vitro*,[6] and corroborates our findings.

In the present study, fraction UNIP344, which contains quassinoids sergeolide[6] and isobruceine B[8] was the most active against both breast and prostate cancer cell lines. Both compounds were first isolated from *Cedronia granatensis* (*Simaroubaceae*) and have shown activity against melanoma, colon, lung several other solid tumor lines, at concentrations as low as 10^{-2} mM to 10^{-5} mM.[29] Such previous results are in accordance with the results found in the present study. Furthermore,

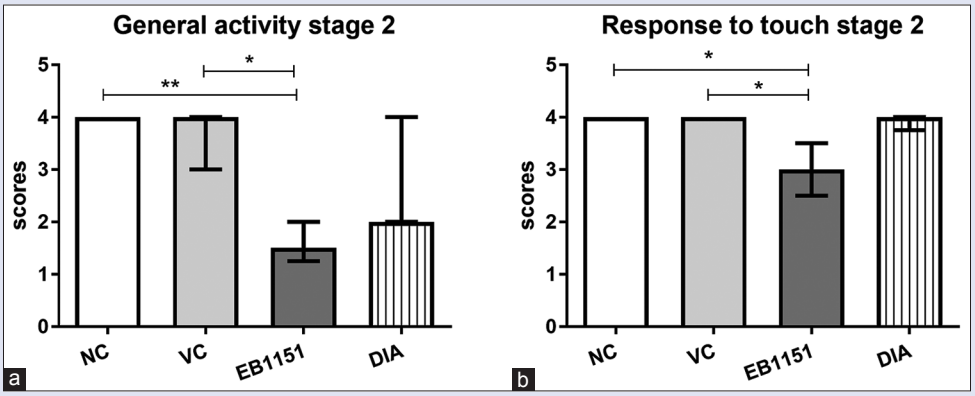

Figure 5: Impairment over behavioral phenotype parameters after the I.P. administration of EB1151 to male mice. (a) General activity, (b) Response to touch, in the second stage of experiment are shown. Kruskall–Wallis statistics (n = 10; n_{total} = 40) were used for all the parameters but defecation, which was analyzed. Differences among means after Dunn's multiple comparison tests are given, α<0.05. *P<0.05; **P<0.01

fraction UNIP343 has also shown na expressive effect against both breast and prostate cancer cell line. From this fraction, sergeolide[6] and 15-deacetylsergeolide[7] were identified. Previous reports showed that sergeolide,[6] 15-deacetylsergeolide,[7] and isobruceine B[8] showed activity against P-388 leukemia cell line.[24] On the other hand, fraction UNIP324, containing sitosterol[1] and stigmasterol,[2] did not show activity against both breast and prostate cancer cell lines.

Studies done with *P. sprucei*[30] led to the isolation of isobruceine B, neosergeolide, and 12-acetylneosergeolide. Later,[31] it was previously reported that the isolation of quassinosides and some semisynthetic derivatives that were tested against *Artemia franciscana* and against human tumor cell lines *in vitro* showed a strong activity against HL-60 leukemia cell line. Furthermore, the extract showed activity against *Aedes aegypti* larvae, hemolysis against murine erythrocytes, and against *Plasmodium falciparum*.[32] In addition, it was reported[33] that neosergeolide isolated from *P. sprucei* was tested against HL-60 leukemia cell line, showing that the compound mediated apoptosis by leading DNA damage for triggering its intrinsic pathways.

Studies[34] have reported the acute toxicity and brine shrimp toxicity assay of eurycomanone that was isolated from *Eurycoma longifolia* and was identified as the most toxic compound found in the species together with 13,21-dihydroeurycoma-none, eurycomanol, longilactone, 14.15 beta-dihydroxyklaineanone, and eurycomanol-2-O-beta-glucopyranoside against brine shrimps. Sergeolide[6] reported to be first previously isolated from *P. pseudocoffea* was first studied against malária, both in *in vitro* and *in vivo* assays;[35] also, 15-decacetylsergeolide was tested against leukemia cell lines[24] as was isobruceine B,[36] but no reports concerning their toxicity has been done so far. Those findings corroborate the cytotoxic results found in the present work.

EB1151 did not influence general activity in the first stage of the experiments, but alterations in ataxia, irritability, response to touch, and hindquarter fall may indicate that parts of the nervous system related to the control of movement and balance may be affected after administration of high doses of the extract. Animals were under stress after receiving intraperitoneal administration of treatment, so alterations in auricular and corneal reflexes, piloerection and ptosis may indicate that sympathetic nervous system might be involved. Micturition, hypothermia, and cyanosis were also altered and may indicate a decrease in the oxygenation. One important consideration to be made is that mice have recovered by the end of the experiments. In the second stage of the experiment, the NLD was administered, and alterations were seen in general activity and in response to touch. Animals recovered from symptoms in the end of the 14th day. Macroscopic observations of the organs did not reveal any changes in the lungs, liver, kidneys, or intestine. Signs of behavioral alterations prevail but faded up to the end of the experiment; administration of higher doses of *P. sprucei* extracts may cause injuries in murine behavior patterns.

CONCLUSIONS

Extracts from the aerial organs of *P. sprucei* and isolated compounds have shown significant cytotoxicity against breast and prostate human cancer cell lines. Extract EB1151 has impaired general activity of male Balb-c mice in the OF apparatus.

Acknowledgements

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Nil.

Conflicts of interest

There are no conflicts of interest.

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