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ORIGINAL ARTICLE

Mice Behavioral Phenotype Changes after Administration of *Anani (Symphonia globulifera*, Clusiaceae), an Alternative Latin American and African Medicine

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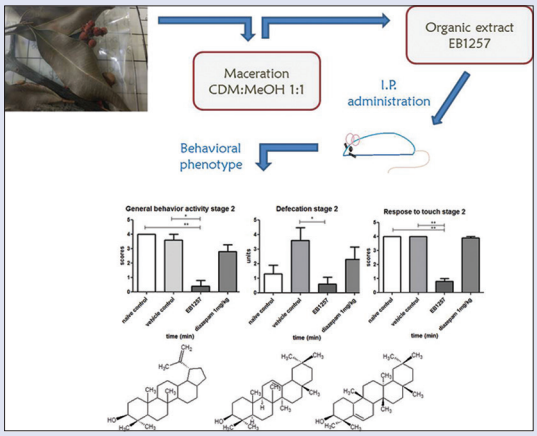
ABSTRACT

Background: *Anani*, (*Symphonia globulifera,* Clusiaceae), known as chewstick, is a traditional plant occurring in Africa and in Central and South Americas that is used against parasites and microorganisms. Although its use is popular in some of these countries, there is a lack of information related to its influence over behavioral phenotype (BP). **Objective:** The objective of this study is to evaluate the influence of the administration of the extract obtained from the aerial organs of *Anani* (EB1257) to male Balb-c mice over BP. **Materials and Methods:** Open cage observation, open field, and elevated-plus maze apparatuses were used. Evaluations were done 15, 30, 60, 120, and 180 min after intraperitoneal administration of *Anani* extract. **Results:** Impairment of general behavior activity, response to touch, tail squeeze, defecation, locomotion and rearing frequency were observed although no signs of hemorrhage or macroscopical alterations of internal organs. *Anani* is harmful, but not toxic if used in the appropriate doses, yet to be determined to male mice. Impairment of locomotion and defecation was observed, indicating some degree of influence over locomotion, but no alterations in anxiety levels were assessed. Three compounds were previously found in the plant-lupeol (1), β-amyrin (2) and 3-β-hydroxyglutin-5-ene (3), and one is being described for the first time to occur in the species: oleanolic acid (4). **Conclusions:** The present work contributes in the support of the rational use of *Anani*, an important Latin American and African alternative medicine, presenting findings that are being reported for the first time.

Key words: Behavioral phenotype, general activity, locomotion, *Symphonia globulifera*

SUMMARY

- *Symphonia globulifera* impairs locomotion and defecatin in behavior analyses
- No alterations in anxiety was observed
- Oleanolic acid occurs in the species.



Abbreviations used: BP: Behavioral phenotype; OF: Open field, EPM: Elevated-plus maze, MMA/ICMBio/SISBIO: Ministério do Meio Ambiente/Instituto Chico Mendes de Conservação da Biodiversidade/Sistema de Autorização e Informação em Biodiversidade, IBAMA/MMA/CGen: Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis/Ministério do Meio Ambiente/Conselho de Gestão do Patrimônio Genético, AM: Amazonas State, UNIP: Universidade Paulista, mg: milligram, kg: kilogram, I.P: Intraperitoneal, CEUA/ICS/UNIP: Comissão de Ética no Uso de Animais/Instituto de Ciências da Saúde/Universidade Paulista, LD: Lethal dose, NLD: Nonlethal dose, GBA: General behavior activity, $FCHCL_3$: Fraction chloroform, FBuOH: Fraction buthanol, FH_2O: Fraction water, FrHEX: Fraction hexane, FrDCM: Fraction dichloromethane, FrMeOH: Fraction methanol, ^{13}C NMR: Carbon nuclear magnetic resonance, EPA: United States Environmental Protection Agency.

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INTRODUCTION

Symphonia globulifera L.f. (Clusiaceae), also named *Moronobea globulifera* (L.f.) Schltdl. and *Symphonia utilissima* R. E. Schultes is a plant native to the Latin America and tropical Africa. It is a timber tree, also used as medicinal and ornamental, due to the beauty of its flowers, known as chewstick. In Brazil, its common names are *Anani, anani-da-mata, anani-da-terra-firme, bacuri, bulandi, oanani, canadi, guanadi, pitia-da-lagoa, pitomba-de-guariba, uanani, vanani* (www.

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ibama.gov.br/lpf/madeira/caracteristicas.php?ID=243andcaracteristica=177, assessed in August 11, 2011). It is also known as *agheribedi, boncillo, karamanni, leche amarilla, mataaki, manni, numgundo, paletuvier jaune, tshilunga noir*, *usonghia, waika, yellow mangue*, among others. In traditional medicine, it is used against parasitic disorders in Africa[1] and in South America[2] and to relief pain.[3] The plant is traditionally used as laxative for pregnant women, in Cameroon.[4] The leaves are toasted and used as cataplasm in wounds derived from snake bite, while stem bark is used in infected wounds, the sap is used in vision-related diseases and decoction made with stem bark is used against cutaneous leishmaniasis.[2] The species is highly consumed and are considered endangered species or even extinct in some African countries. The previous studies on biogeographic history related to the origin and dispersion of *S. globulifera* using fossil records were done, and as a conclusion, authors relate that this is the only *Symphonia* species occurring outside Madagascar.[5]

S. globulifera is a tropical tree that first appeared approximately 45 million years ago, in Africa, and can be interestingly considered a live fossil. Its pollen is used in oil prospection by the oil industry in stratigraphic studies, and for that reason, it is profoundly known. The pollen was first found in Nigeria, in sediment layers from Eocene. After that, registers of pollens aging 30 million-year-old were found in Venezuelan coast, in sediment layers dating from Miocene, and after that, registers in Mexico and Costa Rica dating from Pliocene.[5]

Ethyl acetate extract obtained from the root bark of *S. globulifera* showed antiplasmoidal activity related to polycyclic prenylated acylphloroglucinol compounds.[6] Methanolic leaf extract of *S. globulifera* showed a significant leishmanicidal and antimalaric activities, a low trypanomicidal activity and cytotoxicity.[7] Although the biology, pharmacology, and chemistry of *S. globulifera* have been widely assessed in the last decades and its popular use disseminated across Africa and Latin America, there is an urgent need of prospecting on the influence of *S. globulifera* extracts over behavioral phenotypes (BPs), as to provide some degree of understanding the effects at the central and peripheral nervous system related to the level of exposure people are submitted to when using this medicinal plant. For that reason, the present work aims to report a series of 27 parameters[8] that were herein used in the analysis of general behavioral activity, plus five parameters related to emotionality and locomotion, accessed in open field (OF), and five parameters related to anxiety, accessed by elevated-plus maze (EPM) apparatus, in male Balb-c mice, after treatment with the organic extract made with the aerial organs of *S. globulifera*, the so-called EB1257.

MATERIALS AND METHODS

Plant material

Collection of the plant was made in the Amazon rain forest (licenses no. Ministério do Meio Ambiente [MMA]/Instituto Chico Mendes de Conservação da Biodiversidade/Sistema de Autorização e Informação em Biodiversidade#14895 and Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis/MMA/Conselho de Gestão do Patrimônio Genético#012A-2008). Plant was collected in Manaus, AM, in *igapó* forest, (*A. A. Oliveira*, *3713* [Universidade Paulista [UNIP]), in October 8, 2001 and was identified by Dr. Mateus L. B. Paciencia. A voucher was deposited at UNIP herbarium under register no. 4942.

Aerial organs of *S. globulifera* was collected, dried at 40°C (Fanem) and ground in a hammer-mill (Holmes).[9] The ground material was placed in a glass percolator (Kontes) and was 24 h-macerated with dichloromethane and methanol (1:1) (Synth). Solvents were evaporated under vacuum (Buchi) and were kept in freezer (Revco) until use.[10,11] Extract was suspended in almond oil to be intraperitoneally (I.P) administered to animals at doses of 1,250, 625, and 312.5 mg/kg. Almond oil as used as vehicle control.[12,13] Almond oil and the I.P route were chosen due to the absence of bioavailability loss.[14] Commercial intramuscular diazepam (1 mg/mL; Hipolabor) was used as drug control.

Animals

Male Balb-c mice (*Mus domesticus*) weighing 25–30 g were used. Animals were housed in groups of five, in polypropylene cages (38 cm × 32 cm × 16 cm), under controlled room temperature (22°C ± 2°C), humidity (55%–65%), artificial lighting (12 h light/12 h dark cycle, lights on at 8:00 a.m.), with free access to rodent chow (Nuvilab®, Nuvital Company, São Paulo, Brazil) and filtered water.[15] The experiments began 1 week after the mice arrived in the laboratory for habituation to the new conditions. The experiments currently executed were subjected and approved by the Ethic Committee (Comissão de Ética no Uso de Animais/Instituto de Ciências da Saúde/Universidade Paulista 025/08).

Behavior evaluation

BP can be considered as the emotional, locomotor, fearfulness, and anxiety alterations suffered by laboratory animals exposed to some drug, changing behavior parameters. General behavior activity, sensorial system parameters (auricular reflex, corneal reflex, irritability, response to touch, tail squeeze, and vocal tremor), psychomotor system parameters (body tone, contortion, grip reflex, hindquarter fall, and surface-righting reflex), central nervous system (ataxia, anesthesia, convulsions, hypnosis, tremor, sedation, and straube tail) and autonomous nervous system parameters (lacrimation, breath, ptosis, piloerection, micturition, defecation, hypothermia, and cyanosis) were accessed,[8] and a score from 0 to 4[12-15] was given for each parameter, except to micturition and defecation, that was counted.

Open field

OF was used to assess the influence of the extract overemotionality, motility, and anxiety.[16,17] The experiment was performed as previously described.[12-15]

Elevated-plus maze

EPM is an apparatus first conceived by the British psychologist Sheila Handley as a model to evaluate anxiety, and it is one of the most used for that purpose.[18] The experiment was performed as previously described.[12-15]

Experiment design

The present study aims the prospection of the general behavior activity after the administration of different doses of EB1257, using a limited number of animals.[13,19] The experiment was performed in two phases [Figure 1]. The first one was conducted with groups of $n = 3$ animals each (OECD, 1996). Animals of control group received only almond oil; treatment groups received the corresponded dose of EB1257. The first phase of the experiment was also used in the determination of the lethal dose 50 (LD_{50}) and the nonlethal dose (NLD), which was the lower dose animals did not perish. Different doses of extract were administered, starting from 1250 mg/kg (limit dose). Each dose was administered and if lethality appears in at least one among three mice, a lower dose, prepared to the half of the preceding dose, was administered to a new group of three animals, and observations were again done. This procedure was repeated until no dead was observed. Mice were individually observed in a glass cage for behavior reactions and/or lethality as described before or until dead; mice which survived were observed every 24 h in the subsequent 14 days.

In the second phase of the experiment, the same tests were again performed, but using NLD, and ten mice were used to reach accuracy of data.[14,15]

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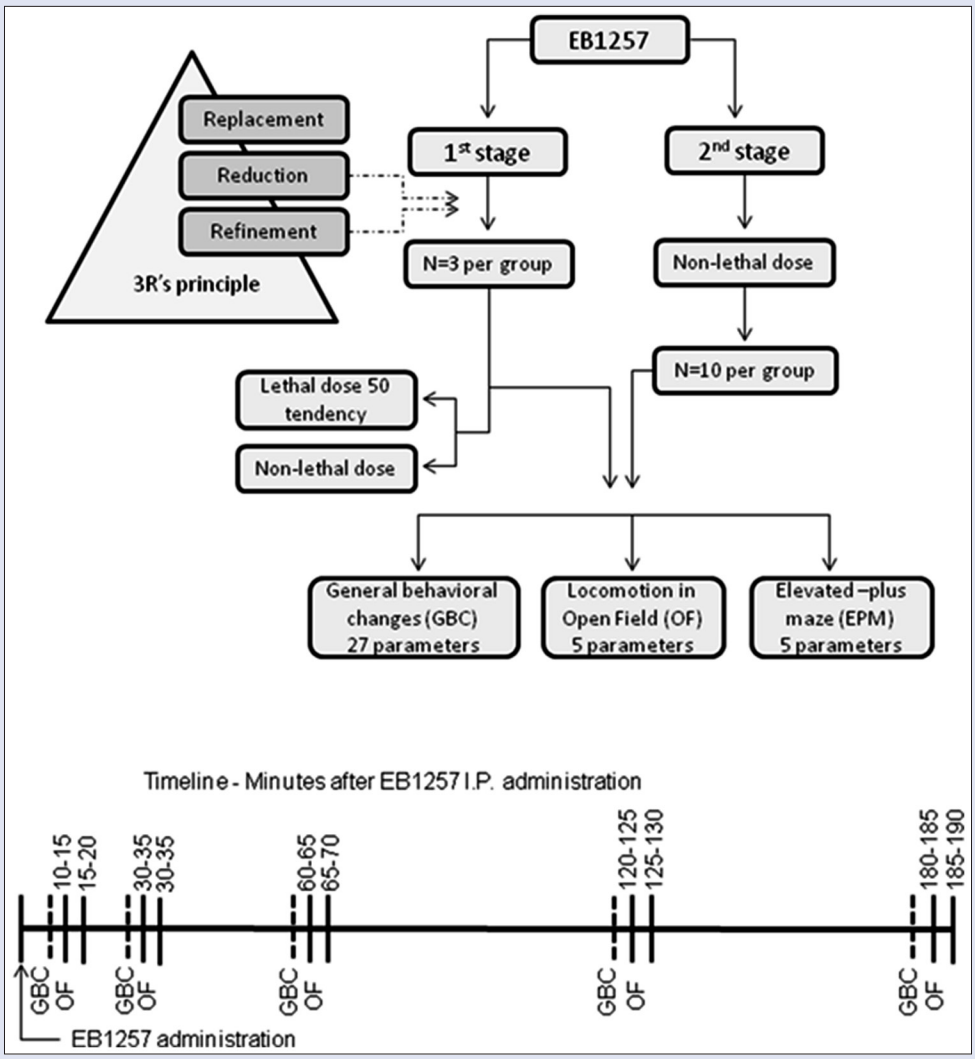

Figure 1: Experimental design diagrams. Behavioral effects of EB1257 intraperitonealy injected in mice: flowchart and timeline related to the experimental design of the experiments. GBA: General behavior activity; OF: Open field; EPM: Elevated-plus maze

Subsequent observations in a glass cage in OF apparatus and in EPM were done as seen in Figure 1. Based on physiological issues, the assays started at 1:00 p.m. and ended before 5:00 p.m., to prevent the circadian influences.

Statistical analysis

Nonparametric results related to general behavioral activity were analyzed by Kruskal–Wallis test followed by Dunn's posttest, except for defecation and micturition that were tested by one-way ANOVA followed by Bonferroni posttest as variances were parametric. Mann–Whitney test was also used in the analysis of general activity and behavioral parameters in the second stage of the experiment, except for defecation, which was analyzed by Student's *t*-test. For OF and EPM assays, two-way repeated measures ANOVA followed by Bonferroni posttest was used to fit the experimental design as proposed. $P < 0.05$ was considered able to show differences among means by the use of GraphPad Prism 5.0(R) (GraphPad Software, La Jolla, CA, USA).[20] The tendency of LD_{50} was obtained using GraphPad Instat 3.0® GraphPad INstat 3.0(R) (GraphPad Software, La Jolla, CA, USA).

EB1257 fractionation

Organic extract EB1257 (16.76 g) originated fractions methanol, chloroform and water, named fraction chloroform ($FCHCL_3$), fraction butanol, and FH_2O (7.2 g/43%, 3.8 g/22.8% and 5.7 g/34.2%, respectively) by the use of liquid-liquid partition. $FCHCL_3$ was column chromatographed in Sephadex LH20 (1 cm intern diameter, 70 cm length) with 100 mL of hexane, 70 mL dichloromethane, and 50 mL methanol as eluents, resulting in fraction hexane (FrHEX) (3.0 g/42.0% yield), fraction dichloromethane (1.2 g/17.0% yield), and FrMeOH (3.0 g/40.9% yield) fractions, respectively. After that, FrHEX was chromatographed in column using silica gel (60–200 um particle size) and was eluted with solvent mixtures composed of hexane, ethyl acetate, and methanol, according to increasing polarity. From these procedures, 51 fractions were obtained from FrHEX. All fractions were combined according to analytical thin layer chromatographic (TLC) similarities after visualization with 25% sulfuric acid followed by heating. Preparative TLC was used in the purification of some fractions from FrHEX, and one of the fractions was sent to carbon nuclear magnetic resonance analyses. Three compounds were previously isolated from the organic extract of *S. globulifera* and were identified[21] as lupeol (1), β-amyrin (2) and 3-*β*-hydroxyglutin-5-ene (3) [Figure 2]. In the present work, oleanolic acid (4) was identified and is being described to occur in *S. globulifera* for the first time. Oleanolic acid (4) was elucidated by hydrogen and carbon magnetic nuclear resonance, according to comparison of chemical shifts with the literature [Table 1].

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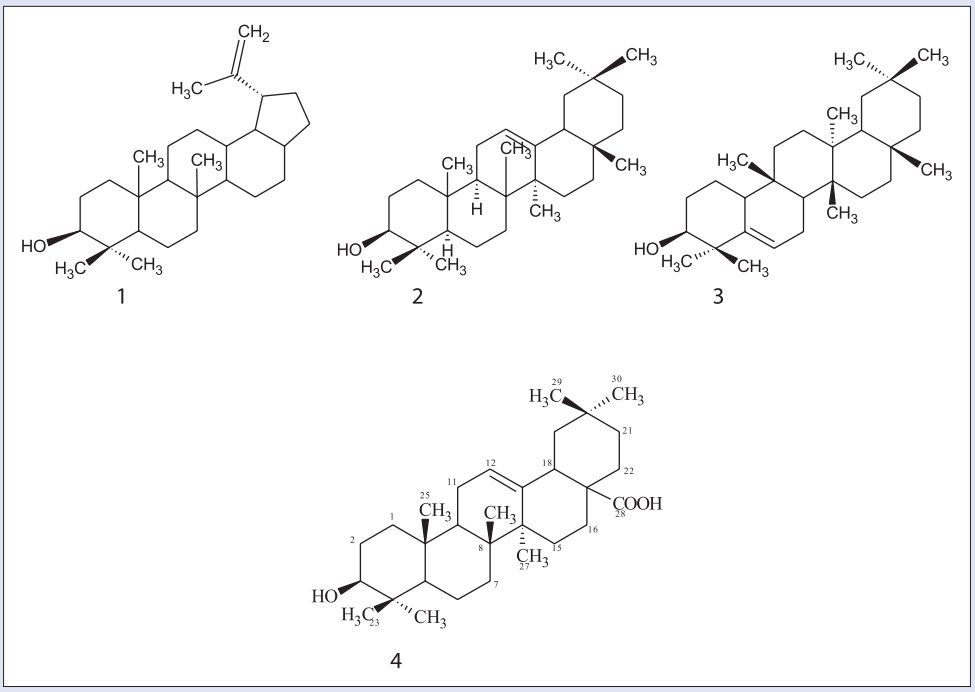

Figure 2: Compounds isolated from *Symphonia globulifera* organic extract. Lupeol (1), β-amyrin (2), 3-*β*-hydroxyglutin-5-ene (3), and oleanolic acid (4)

Table 1: List of chemical shifts obtained from carbon-13 nuclear magnetic resonance (125 MHz, $CDCl_3$) data of compound 1, isolated from FHex, obtained from EB1257, the organic extract from *Symphonia globulifera*

n	Oleanolic acid[22]	UNIP-153	
	NMR-^{13}C	NMR-^{1}H (500 MHz)	NMR-^{13}C (125 MHz)
1	38.5		38.40
2	27.4		27.17
3	78.7	3.23 *dd*, *J*=11.4, 4.5	79.03
4	38.7	-	38.75
5	55.2		55.21
6	18.3		18.30
7	32.6		32.63
8	39.3	-	39.27
9	47.9		47.62
10	37.0	-	37.06
11	23.1		22.95
12	122.1	5.29 *t*	122.64
13	143.4	-	143.57
14	41.6	-	41.62
15	27.7		27.67
16	23.4		23.39
17	46.6	-	46.49
18	41.3	2.83 *dd*, *J*=13.9, 4.7	41.04
19	45.8		45.87
20	30.6	-	30.67
21	33.8		33.79
22	32.3		32.42
23	28.1		28.09
24	15.6	0.8 *s* (CH_3)	15.54
25	15.3	0.93 *s* (CH_3)	15.31
26	16.8	0.79 *s* (CH_3)	17.06
27	26.0	1.01 *s* (CH_3)	25.91
28	181.0	-	No
29	33.1	0.94 *s* (CH_3)	33.05
30	23.6	0.96 *s* (CH_3)	23.53

UNIP: Universidade Paulista; ^{13}C NMR: Carbon-13 nuclear magnetic resonance

RESULTS

LD_{50} for EB1257 is 547.0 mg/kg, and the extract is considered harmful by the European Community (oecd.org/ehs/test/testlist.htm) and by the United States Environmental Protection Agency (http://www.epa.gov).

Figure 3 shows how I.P. administration of EB1257 has influenced general behavior activity and psychomotor activity, in stage one of the experiments. Figure 3 shows how EB1257 I.P. administration has diminished mice general behavior activity [Figure 3a; $H\sim\chi^2 0.05,(3) = 12.17$; $P = 0.0068$], but in a significantly way at doses 625.0 mg/kg and 2,500 mg/kg. Significant reduction on response to touch [Figure 3b; $H\sim\chi^2 0.05,(3) = 9.832$; $P = 0.0201$] were observed after the administration of doses 1250 mg/kg and 312.5 mg/kg. Tail squeeze [Figure 3c; $H\sim\chi^2 0.05,(3) = 11.85$; $P = 0.0079$] was diminished after administration of all doses ($P < 0.05$). Diminish in corneal reflex [Figure 3d; $H\sim\chi^2 0.05,(3) = 14.27$; $P = 0.0026$] was observed in treatment made with EB1257 at 1,250 mg/kg and At 312.5 mg/kg of EB1257 ($P < 0.05$). Ataxia was significant increased [Figure 3e; $H\sim\chi^2 0.05,(3) = 13.72$; $P = 0.0033$] in mice that received 1250 mg/kg ($P < 0.01$). Extremely significant differences were observed in defecation [Figure 3f; $F_{(5,24)} = 13.71$; $R^2 = 0.7199$; $P = 0.0001$; Bartlett's test for equal variance $P < 0.0001$] observed for all treatments, from 1250–312.5 mg/kg ($P < 0.0001$).

Figure 4 shows statistical analyses by Mann–Whitney test that were obtained after treatment with the NLD of 156.3 mg/mL, in stage 2 of the experiment. EB1257 group was compared to vehicle control. General behavior activity [Figure 4a] was significantly diminished in the treated group ($P < 0.05$). Significant differences were observed in response to touch [Figure 4b], once EB1257 diminished response if compared to vehicle control ($P < 0.01$). In tail squeeze parameter [Figure 4c], diminish appeared after the administration of EB1257 in relation to vehicle control ($P < 0.01$). Ptosis [Figure 4d] has increased after the administration of EB1257, when compared to control ($P < 0.05$). Defecation [Figure 4e] was diminished in the treated group ($P < 0.05$).

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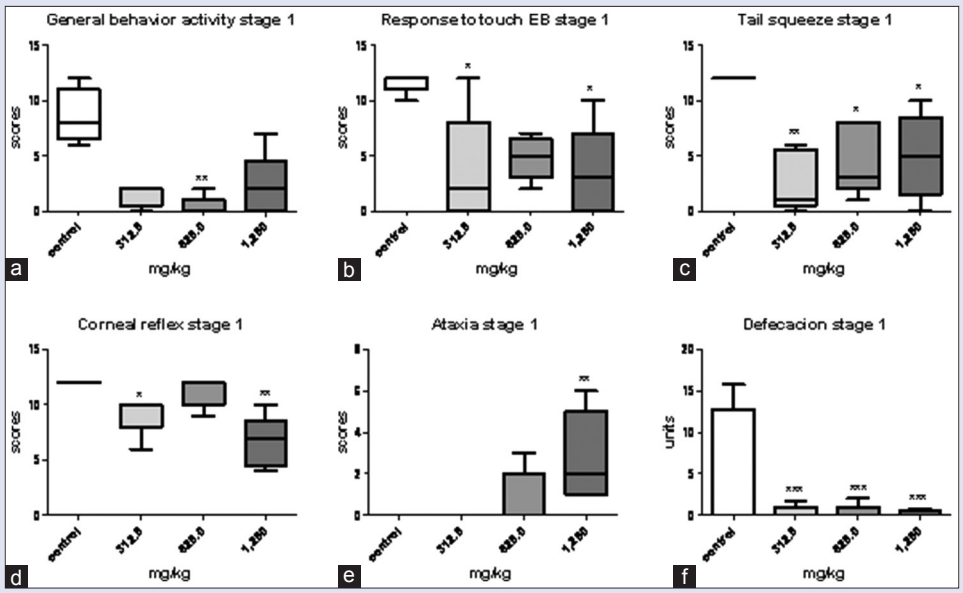

Figure 3: Behavior alterations observed in male Balb-c mice after administration of organic extract EB1257, obtained from *Symphonia globulifera* (Clusiaceae), in stage 1 of the experiment. the parameters that were observed was (a) General behavior; (b) Response to touch; (c) Tail squeeze; (d) Corneal reflex; (e) ataxia and (f) defecation. Kruskal-Wallis nonparametric variance test was performed ($n = 3$; $n_{total} = 12$), followed by the Dunnet posttest, median ± range is represented; significance among medians was considered if $P < 0.05$. For defecation analysis, a parametric one-way ANOVA was performed ($n = 3$; $n_{total} = 12$), followed by Bonferroni posttest, mean ± standard error is represented and significance among means was achieved if $P < 0.05$. *=P<0.05, **=P<0.01, ***P<0.001

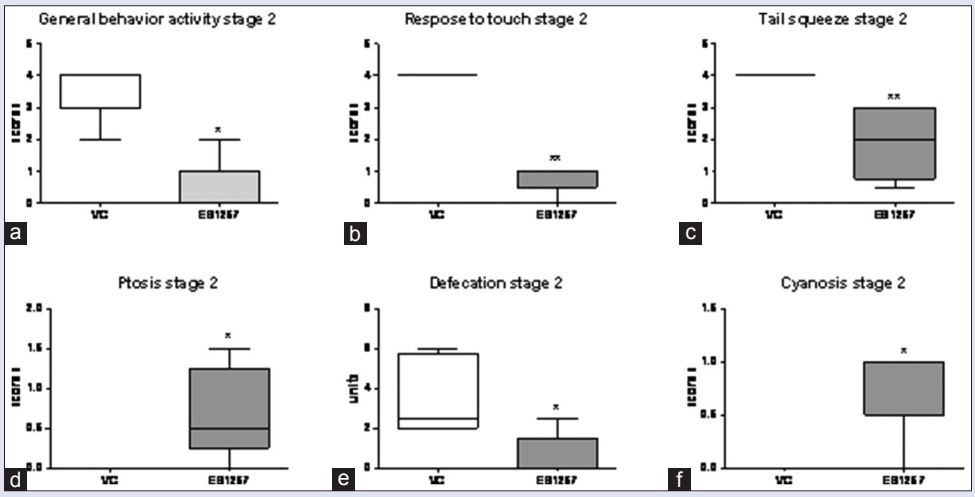

Figure 4: Behavior effects over parameters that were influenced by the intraperitoneal administration of nonlethal dose of organic extract EB1257, obtained from *Symphonia globulifera* (Clusiaceae), in male Balb-c mice. The following parameters were observed in stage 2; (a) General behavior; (b) Response to touch; (c) Tail squeeze; (d) ptosis; (e) Defecation and (f) Cyanosis. Mann-Whitney test was performed ($P < 0.05$). VC = Vehicle control. *=P<0.05, **=P<0.01

Cyanosis [Figure 4f] was significantly higher in the treated group than in the vehicle control group ($P < 0.05$).

Table 2 shows the influence of treatments over locomotion observed in OF apparatus, experiment stage 1. Locomotion frequency has been significantly diminished by the administration of any dose of EB1257, after 120 min (time $F_{4,32} = 0.89$, total variance of 1.40%; $P > 0.05$; treatment $F_{3,8} = 11.04$, total variance of 60.35%; $P < 0.01$; interaction $F_{12,32} = 2.35$; $P < 0.05$, total variance of 11.09%). Confirmation of the lack of locomotion is based on the immobility time parameter, which was significantly altered after 120 min, as well (significances ranged from $P < 0.05$ to $P < 0.001$, depending on time and dose, starting from dose 1,250 mg/mL; (time $F_{4,32} = 4.12$, total variance of 7.57%; $P < 0.01$; treatment $F_{3,32} = 20.61$, total variance of 55.47%; $P > 0.0001$; interaction $F_{12,32} = 2.74$; $P < 0.05$, total variance of 15.09%). Rearing frequency was also significantly diminished (time $F_{4,32} = 2.90$, total variance of 8.06%,; $P < 0.05$; treatment $F_{3,32} = 2.31$, total variance of 20.95%; $P > 0.05$; interaction $F_{12,32} = 2.94$; $P < 0.01$, total variance of 24.56%) 3 h after the administration of EB1257. Both grooming and defecation have not been affected by EB1257 administration ($P > 0.05$).

Table 3 shows results obtained in EPM. EB1257 treatment significantly influenced the number of entries in open arm 2 h after extract administration (time $F_{4,32} = 1.19$, total variance of 2.56%, $P > 0.05$; treatment $F_{3,8} = 5.38$, total variance of 47.66%; $P < 0.05$; interaction $F_{12,32} = 1.39$; $P > 0.05$, total variance of 8.95%). Mice entered the closed

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Table 2: Parameters related to open field analysis in the first stage of the experiment, after intraperitoneal administration of the organic extract, obtained from *Symphonia globulifera* (clusiaceae), to male Balb-c mice

Dose (mg/kg)	Minutes of observations				
	10-15	30-35	60-65	120-125	180-185
Locomotion frequency					
Control	116.33 (42.71)	98.67 (87.96)	91.67 (69.62)	167.67 (79.25)	178.67 (61.34)
1.250	22.33 (20.50)	5.33 (6.66)	7.00 (6.24)	3.33 (0.58)***	5.00 (1.73)****
0.625	27.33 (10.97)	3.33 (1.53)	49.67 (55.77)	10.33 (10.02)***	2.33 (3.21)****
0.3125	15.67 (12.50)	31.33 (21.01)	61.00 (48.12)	43.00 (22.91)**	15.00 (13.53)***
Rearing frequency					
Control	0.00 (0.00)	3.33 (5.77)	2.33 (4.04)	7.33 (7.09)	20.00 (20.95)
1.250	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)***
0.625	0.00 (0.00)	0.00 (0.00)	0.33 (0.58)	0.00 (0.00)	0.00 (0.00)***
0.3125	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)***
Grooming					
Control	0.33 (0.58)	2.67 (3.79)	1.00 (1.00)	1.33 (0.58)	3.00 (2.65)
1.250	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
0.625	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
0.3125	0.00 (0.00)	0.00 (0.00)	0.67 (1.15)	1.33 (2.31)	1.67 (2.08)
Immobility time					
Control	82.33 (76.51)	182.67 (77.14)	176.00 (38.31)	42.67 (54.45)	91.33 (67.35)
1.250	212.67 (35.95)*	270.00 (18.03)	271.00 (11.14)	263.67 (20.55)****	252.33 (20.31)***
0.625	211.00 (20.66)*	284.67 (13.01)	216.00 (28.62)	242.00 (80.57)****	291.67 (8.02)****
0.3125	237.00 (35.59)**	222.00 (25.98)	208.00 (60.51)	217.00 (33.72)***	281.00 (17.78)****
Defecation					
Control	0.00 (0.00)	0.67 (0.58)	1.00 (1.00)	1.00 (1.00)	0.67 (0.15)
1.250	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)*	0.00 (0.00)	0.00 (0.00)
0.625	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)*	0.00 (0.00)	0.00 (0.00)
0.3125	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)*	0.67 (0.58)	0.00 (0.00)

Two-way repeated measures ANOVA (n=3; n_{total}=12) followed by Bonferroni posttest were used in the analysis. Results are expressed as mean±SE; *=P<0.05, **=P<0.01, ***P<0.001, ****P<0.0001, relative to control group. SE: Standard error

arms and rarely left during the whole experiment (time $F_{4,32} = 0.57$, total variance of 1.06%; $P > 0.05$; treatment $F_{3,8} = 19.37$, total variance of 68.18%; $P < 0.001$; interaction $F_{12,32} = 1.16$; $P > 0.05$, total variance of 6.48%). Crossings in the center indicated that mice preferred to transit from one open arm to the other open arm, or from one closed arm to the other closed arm, other than to circulate between open and closed arms (time $F_{4,32} = 1.00$, total variance of 1.40%, $P > 0.05$; treatment $F_{3,8} = 13.86$, total variance of 68.00%, $P < 0.01$; interaction $F_{12,32} = 1.48$, $P > 0.05$, total variance of 6.25%). Time in both open and closed arms were not considered statistically significant ($P > 0.05$).

Figure 5 shows the results observed results obtained for OF apparatus in the second stage of the experiments (n = 10; n_{total} = 20), after administration of NLD. A significant diminish in locomotion was observed in the group treated with EB1257 (time $F_{4,56} = 7.88$, total variance of 11.42%, $P < 0.001$; treatment $F_{1,14} = 17.81$, total variance of 32.71%, $P < 0.001$; interaction $F_{4,56} = 9.96$, $P < 0.001$, total variance of 14.43%) at session 10–15 min. A significant improvement of immobility time was observed in the animals that received EB1257, in relation to control group in sessions 10–15 min, 120–125 min, and 180–185 min (time $F_{4,56} = 4.98$, total variance of 10.67%, $P < 0.01$; treatment $F_{1,14} = 19.09$, total variance of 29.69%, $P < 0.001$; interaction $F_{4,56} = 5.08$, $P < 0.01$, total variance of 10.88%). A diminish in rearing frequency (time $F_{4,56} = 6.25$, total variance of 11.43%, $P < 0.001$; treatment $F_{1,14} = 12.75$, total variance of 26.72%, $P < 0.01$; interaction $F_{4,56} = 6.25$, $P < 0.001$, total variance of 11.44%) was observed in mice that received EB1257 in sessions 120–125 min and 180–185 min. EB1257 diminished grooming (time $F_{4,56} = 1.99$, total variance of 5.65%, $P > 0.05$; treatment $F_{1,14} = 42.40$, total variance of 20.92%, $P < 0.001$; interaction $F_{4,56} = 1.68$, $P > 0.05$, total variance of 7.40%) at session 10–15 min and 120–125 min, in relation to vehicle control group. Last defecation (time $F_{4,56} = 0.05$, total variance of 0.22%, $P > 0.05$; treatment $F_{1,14} = 13.10$, total variance of 19.33%, $P < 0.01$; interaction $F_{4,56} = 0.30$, $P > 0.05$, total variance of 1.24%) was not influenced by the treatment, and differences among means were not considered statistically significant.

No internal hemorrhage was observed after the administration of the plant extract neither was macroscopical alterations in internal organs.

DISCUSSION

The introduction of the first reports on the influence of a potential alternative medicine is fundamental to establish good medicine practices. The previous reports relating the influence of alternative medicines over behavior have been done, such as to honey,[22] to the sino-japanese wakan-yaku,[23] to uncaria hook[24] and others.[25,26] Lupeol, β-amyrin and 3 β-hydroxyglutin-5-ene were previously isolated from *S. globulifera*,[21] and molecules were described to occur in the species for the first time, then. Before that, nine polycyclic polyprenylated acylphloroglucinol compounds and oxidized derivatives having a slight antiplasmoidal activity were isolated from the root barks,[6] xanthones (ugaxanthone, barraxanthone, and gentisein), polyoxygenated xanthone (globulixanthone F), and biflavonoids (GB2 and manniflavanone GB3) were isolated from the stem bark, also, globulixanthone F were tested against a series of microorganisms.[27] Leishmanicidal activity was also assessed by bioguided-fractionation and led to the isolation of one benzophenone named guttiferone A and one xanthone named xanthone V1 from the leaves.[28] Gaboxanthone was isolated from the seed shells of *S. globulifera* and was active against *Plasmodium falciparum*[29] and an antimicrobial active prenylated xanthone was also isolated from the seeds.[30] Cytotoxic globulixanthones A and B[31]

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Table 3: Parameters related to elevated-plus maze analysis in the first stage of the experiment, after intraperitoneal administration of the organic extract, obtained from *Symphonia globulifera* (clusiaceae), to male Balb-c mice

Dose (mg/kg)	Minutes of observations				
	15-20	35-40	65-70	125-130	185-190
Entries in open arms					
Control	1.33 (1.53)	4.33 (6.66)	5.00 (4.58)	6.33 (5.51)	7.00 (2.00)
1.250	0.33 (0.58)	0.00 (0.00)	0.33 (0.58)	0.00 (0.00)*	0.00 (0.00)*
0.625	0.33 (0.58)	0.33 (0.58)	0.33 (0.58)	0.33 (0.58)	0.33 (0.58)*
0.3125	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)*	0.00 (0.00)*
Entries in closed arms					
Control	8.33 (3.21)	6.67 (4.93)	6.00 (5.57)	11.33 (1.53)	10.33 (5.77)
1.250	1.33 (0.58)*	1.33 (0.58)	1.00 (1.00)	1.00 (0.00)***	1.00 (0.00)***
0.625	0.67 (0.58)**	0.67 (0.58)*	0.67 (0.58)	0.67 (0.58)****	0.67 (0.58)***
0.3125	1.00 (0.00)*	1.00 (0.00)	2.67 (2.89)	1.00 (0.00)***	1.00 (0.00)***
Time spent in open arms					
Control	16.33 (22.50)	76.33 (127.05)	72.33 (79.22)	103.33 (92.65)	139.33 (56.23)
1.250	98.33 (170.32)	0.00 (0.00)	97.33 (168.59)	0.00 (0.00)	0.00 (0.00)
0.625	100.00 (173.21)	100.00 (173.21)	100.00 (173.21)	100.00 (173.21)	100.00 (173.21)
0.3125	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Time spent in closed arms					
Control	199.00 (130.05)	208.33 (140.15)	217.33 (98.05)	111.67 (60.01)	160.00 (52.16)
1.250	194.00 (159.96)	300.00 (0.00)	195.33 (169.27)	299.67 (0.58)	299.67 (0.58)
0.625	200.00 (173.21)	200.00 (173.21)	200.00 (173.21)	200.00 (173.21)	200.00 (173.21)
0.3125	300.00 (0.00)	300.00 (0.00)	293.00 (12.12)	300.00 (0.00)	300.00 (0.00)
Crossing in the center					
Control	9.67 (4.62)	10.67 (11.02)	10.67 (9.45)	17.33 (4.04)	17.67 (7.23)
1.250	1.00 (1.00)	0.33 (0.58)*	0.33 (0.58)*	0.00 (0.00)****	0.00 (0.00)****
0.625	1.00 (0.00)	1.00 (0.00)*	1.00 (0.00)*	1.00 (0.00)****	1.00 (0.00)****
0.3125	1.00 (0.00)	1.00 (0.00)*	2.33 (2.31)	1.00 (0.00)***	1.00 (0.00)***

Two-way repeated measures ANOVA (n=3; n_{total}=12) followed by Bonferroni posttest were used in the analysis. Results are expressed as mean±SE; *=P<0.05, **=P<0.01, ***P<0.001, ****P<0.0001, relative to control group. SE: Standard error

and antimicrobial globulixanthones C, D, and E[31,32] were isolated from the root barks of the plant, as well as was globulixanthone F.[27] Oleanolic acid, a pentacyclic terpenoid compound, is being reported in the species for the first time. Little information on the influence of oleanolic acid over behavioral changes has been reported, as its use as alternative antidepressant due to the anti-inflammatory activity through upregulating antioxidants and for inhibiting pro-inflammatory sinaling;[33] anti-inflammatory drugs are being strongly considered as a concomitant medicine to be used in depression based on the inflammation hypothesis, which says that pro-inflammatory cytokines are overexpressed in depressed people. Oleanolic acid is also known for their antidiabetis activity,[34] antitumor activity against several cancer types,[35] and multiple sclerosis.[36] Antimicrobial activity of *Anani* extracts was reported against *Staphylococcus aureus*[37,38] and against *Enterococcus faecalis*,[27,39] but not against *Pseudomonas aeruginosa*. Finally, reports on the ethnopharmacology of the plant both in Africa and in Central and South Americas introduced a list with >40 compounds that were already isolated from different organs of the plant and indicated those that were tested against parasites and microorganisms,[40] but no reports on the influence of *S. globulifera* on mice behavior phenotype have been reported so far.

EB1257 showed a NLD of 156.3 mg/kg and a LD50% of 547.0 mg/kg and is considered harmful. The classical methodologies applied in the present work, as OF and EPM,[41] was based on the previous works of the group,[12-15] and the parameters that were evaluated in general behavior activity were previously described.[8] After administration of the extract, it was observed a significant influence over general behavior activity and autonomous nervous systems parameters, as tail squeeze, response to touch, corneal reflex, and ataxia.

The prospection of EB1257 effects (stage 1) shows a significant decrease in general behavior activity, defecation, corneal reflex, and in the response to touch. An increase in tail squeeze and ataxia was also observed. The increased in tail squeeze suggest an increased sensitivity to pain.[42] Ataxia could reduce the motor performance leading to the decreased general activity. Thus, this scenario suggests a decreased exploratory behavior, diminishing of the autonomous and sensorial systems activities, except for pain sensitivity.

To confirm these data, we performed the second stage using the nonlethal EB1257 dose and a greater number of mice. We employed a blank group, i.e., nonmanipulated mice, as a negative control and a diazepam treated mice as a positive control group. The lack of differences between the blank group and the vehicle group indicates that the saline injection did not influence our results. The positive control group confirms the quality of our model because the data are in according to the previous results[43] observed in the literature.

The EB1257 NLD shows a similar profile of the phenotypic behaviors observed in the first stage of experiments. In fact, a decreased in general activity, autonomous, and sensorial systems were observed. In addition, an increased ptosis and reduced piloerection and increased cyanosis reinforced the hypothesis that the EB1257 extract acts at the autonomous system levels. Moreover, these signs could be related to toxicity of the extract because we examined the NLD but not the nontoxic dose. Interesting, in this new phase of experiments, the tail squeeze was reduced suggesting a reduced response to pain.

General behavior activity is an index for evaluating induced behavioral changes in animals not only by physiological and genetic manipulation but also by the influence of the administration of a drug. Among the techniques used to assess general behavior activity, OF stands out, making

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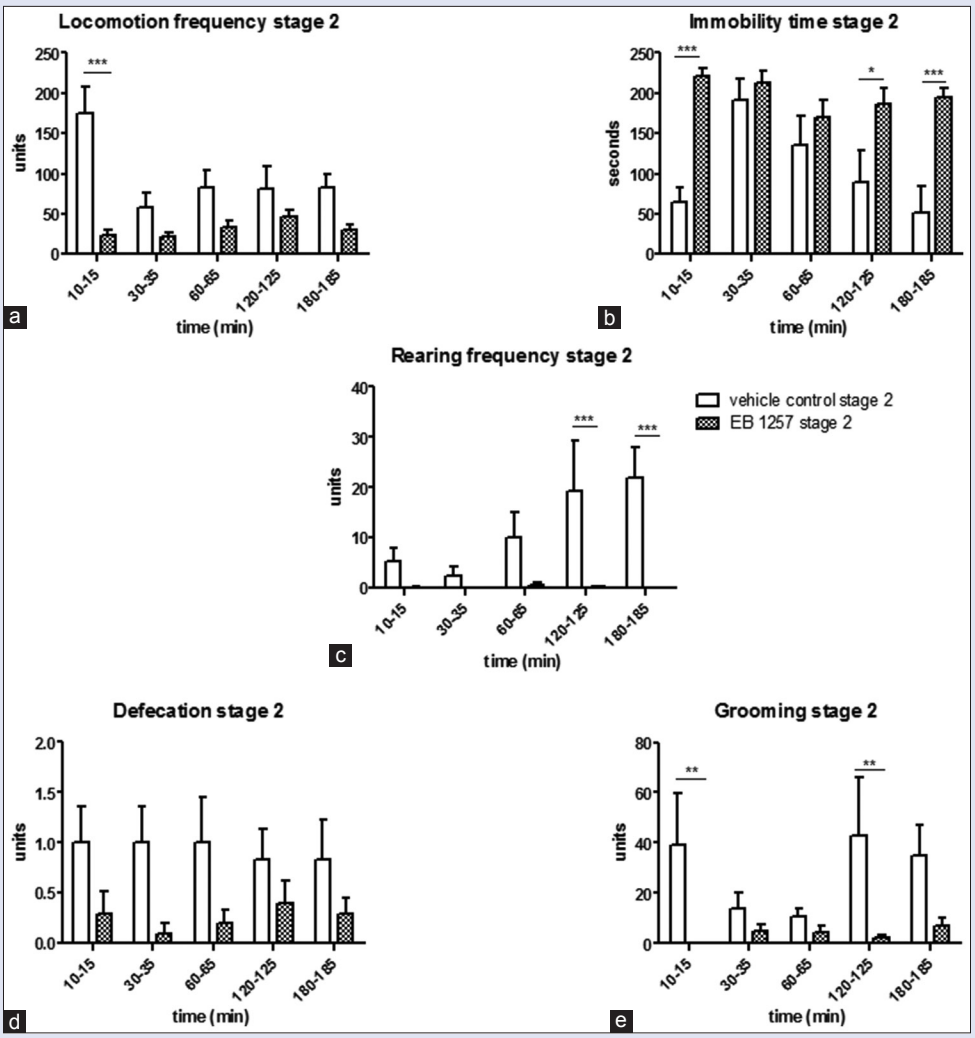

Figure 5: Results related to open field analysis in the second stage of the experiment, after intraperitoneal administration of the nonlethal dose of organic extract, obtained from *Symphonia globulifera* (Clusiaceae), to male Balb-c mice. The following parameters were observed in stage 2: a. locomotion frequency; b. immobility time; c. rearing frequency; d. Defecation; e. Grooming. Data are are presented as means ± standard error ($n = 10$; $n_{total} = 20$). Two-way repeated measures ANOVA followed by Bonferroni posttest were used in the analyses. Significance was observed if $P < 0.05$. *=P<0.05, **=P<0.01, ***P<0.001

it possible to measure various behavioral parameters, among which are those related to emotional, exploratory, and motor behavior. The first exposure of the animal to the device has a more marked emotional component than the remaining agents of exposure.[26,44-46] Historically, OF defecations and activity have been used to assess emotionality and fearfulness[16,17] and also to analyze for hyperactivity and exploratory behaviors. Usual parameters for rodent behavior would be that the number of fecal boli deposited as well as their activity pattern varied directly onto the levels of fear and emotional reactivity, where a large number of boli and little activity indicated a fearful individual. In addition, rodents tent to avoid brightly illuminated novel open spaces and the apparatus would work as an anxiogenic stimulus allowing for anxiety related to locomotor activity and exploratory behaviors. Despite discussions on the standardization of the OF test, it is still largely used today due to its simplicity in routine behavior testing. It is also reported that OF emotional and fearfulness evaluation can be related to genetic adaptations of rodent lineages, and variance in responses may occur.[47] Control group showed a usual behavior in OF, in the first session. In the first stage of the experiment, animals did not demonstrate increased emotionality or interferences with motility although they were placed in a strange brightly illuminated closed environment. This observation is confirmed by the analysis of the locomotion, which was high in all sessions, particularly increasing in the last two sessions, demonstrating a possible adaptation of the animals of the control group to the apparatus. In fact, in these sessions, the immobility duration was decreased in the first three sessions suggesting a reduced response to emotionality in response to novelty of the environment, and an adaptation in the last two sessions, indicating that the animal has adapted to the apparatus. Locomotion was significantly diminished by EB1257 in a dose-dependent relationship and the usual pattern of locomotion could not be recovered during the whole experiment. In addition, these findings are inversely related to the results achieved in the parameter immobility time, supporting first findings for locomotion. Immobility time is significantly pronounced during the whole experiment, maybe indicating a depressive or sick behavior, yet to be determined. The absence of significant differences in defecation related to EB1257 administration may indicate that mice receiving treatment was not influenced over fearfulness or emotionality parameters during exposition to OF. In addition, results observed in OF, stage 1, confirmed the first observations of diminishing in general activity. As OF is a prior apparatus to evaluate locomotion, which is the main parameter implicit in general activity evaluation, it is noted that the lack of locomotion frequency and the diminishing in rearing frequency in relation to the

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control group indicates that no horizontal or vertical exploration was made by the animals, in the apparatus, as first demonstrated.

EPM was used to assess anxiety effects of treatments in stage 1 of the experiments. Briefly, an increase in open arm activity duration reflects anti-anxiety behavior and the anxiety that takes the animals to choose the closed arm is confronted to the instinct to exploration caused by the open arm.[48] In the present work, animals remained longer in closed arms and chose the closed arm over the open arms. Results confirm a minor time spent in open arms. The treatment accounted for an expressive amount of the variance, which means the extract exerted a deleterious effect in the animals preventing them of exploring EPM apparatus and stimulating them in remaining in a protected and safe place. Data from the entrances in the closed and open arms as well as the crossings in the center of EPM are related to motor activity in the EPM. Our data are in accordance with a decreased exploratory activity induced by any dose of EB1257 and confirms observations made in the OF apparatus and in the general activity observations. OF and EPM data suggest an overall reduction in the activity of mice treated with the EB1257, in the first stage of the experiment. In this case, the decreased locomotion and rearing frequencies parallel to an increased immobility time indicates that the animals did not suffer from the environmental novelty, but the treatment itself represented a significant influence over immobilization. At the necropsy, it was not observed the presence of extensive hemorrhage, nor alterations in internal organs.

In the second stage of the experiment, after the administration of the NLD, only general behavior activity and OF assays were performed. In this stage, a larger number of animals (10 per group) were used to test the NLD of EB1257, and alterations in the most significant parameters could be observed. It is noticed that mice have their locomotion and rearing frequencies diminished in relation to controls and increased immobility duration. These parameters are in complete accordance with the previous observations and highlight the influence of EB1257 over locomotion parameters. Defecation and grooming showed no differences between control and experimental groups. On the other hand, no signs of hemorrhage were observed in the second stage of the experiment, when the NLD was administered. Results observed in the general behavior activity analysis indicated that although NLD was administered, cyanosis, and ptosis appeared after EB1257 administration as well as were response to touch, tail squeeze, and defecation impaired.

CONCLUSIONS

EB1257 is harmful but not toxic. The presence of lupeol, β-amyrin, 3-*β*-hydroxyglutin-5-ene, and oleanolic acid seems not to influence over phenotypic behavior. Although the plant is of common use in both Africa and Latin America, it use by population shall be made with some reservations, once it has been shown that effects over behavior could appear, as impairment of general behavior activity, response to touch, tail squeeze, defecation, locomotion, and rearing frequency was concisely observed. No signs of hemorrhage were observed after the administration of the NLD. The present work contributes in the support of the rational use of this important traditional alternative medicine, presenting findings that are being reported for the first time.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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