

Effects of Sterculia Apetala Seed Oil on Anxiety-like Behavior and Neuronal Cells in the Hippocampus in Rats

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Abstract The present study evaluated anxiety-like behavior and neuronal changes in the CA1, CA3, and dentate gyrus subregions of the hippocampus that were induced by chronic sterculic oil administration in Wistar rats. The study included two experiments. The first experiment evaluated doses-response curves of sterculic oil (low dose, 0.06 g; medium dose, 0.12 g; high dose, 0.24 g). The second experiment evaluated the effects of sterculic oil on the number of adult hippocampal neurons. For all experiments, we employed a behavioral test battery that included the elevated plus maze, locomotor activity test, and forced swim test. All sterculic oil doses produced anxiogenic-like effects, without significant changes in locomotor activity or behavior in the forced swim test. We observed a reduction of neurons in the hippocampus in rat groups that were exposed to behavioral tests compared with a test-free vehicle group. Sterculia apetala seed oil administration produced anxiogenic-like effects, with a smaller effect of the medium dose on neurons in the CA1 and dentate gyrus regions of the hippocampus.

Keywords: anxiety, behavior, hippocampus, neurons, Sterculia apetala

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1. Introduction

Seeds are the main basis for human alimentary livelihood [1]. In diverse countries, seed-based foods are a part of cultural heritage, and these population frequently consume them as part of their diet [2]. In Mexico, seed consumption is common and part of the identity and cultural tradition of settlers. *Oecopetalum mexicanum* (locally known as "cachichin"; [3], *Leucaena spp.* (locally known as "guaje"; [4], and *Sterculia apetala* (locally known as "bellota," "castana," or "tepetaca"; [5] are some examples of seeds that are locally consumed.

Sterculia apetala seeds are consumed in boiled or roasted form or used as a chocolate flavoring in the region of Tuxtla, Veracruz, Mexico. Beneficial effects and side effects have been identified in rats that receive supplementation with sterculic oil extract from *Sterculia apetala* seeds [6]. These effects are associated with the

presence of sterculic acid (8-[2-octyl-1-cyclopropenyl] octanoic acid) and malvalic acid (7-[2-octyl-1-cyclopropenyl] heptanoic acid), a $\Delta 9$ desaturase (stearoyl-CoA desaturase) inhibitor [7]. Beneficial effects include decreases in blood pressure, triglycerides, insulin resistance, body mass, and liver weight, among others [8,9]. Additionally, the consumption of sterculic oil in obese Zucker rats decreased exploration latency to enter the light compartment in the light/dark test, without producing nonspecific motor effects, suggesting an anxiolytic-like effect [10].

Seed consumption-related neurotoxic effects have been reported previously. The chronic administration of cycad seeds (*Dioon spinulosum*) produced motor impairment and neurodegenerative effects in rats [11]. In humans, the chronic consumption of *Lathyrus sativus* seeds produced neurolathyrism, and the consumption of *Datura stramonium* seeds caused delirium and hallucination symptoms [12].

Neurotoxic effects that are produced by natural chemical compounds that are found in seeds have been

detected in several brain structures. For example, a microinjection of methylazoxymethanol, a toxic substance present in cicada, into the CA1 and CA3 areas of the hippocampus produced motor impairment [13]. Similarly, a microinjection of acetone cyanohydrin in the CA1 area of the hippocampus produced motor impairment in rats [14]. These results suggest that the hippocampus could be a target site of chemical compounds in seeds that produce neurotoxic effects.

The anxiolytic-like effects of stercuric oil in obese Zucker rats were explained by their beneficial effects on parameters of obesity and not an anxiolytic-like effect per se. The effects of this oil in healthy subjects remain unexplored. Therefore, the present study evaluated the effects of chronic treatment with stercuric oil from *Sterculia apetala* seeds on anxiety-like behavior, spontaneous locomotor activity, and neurons in distinct subregions of the hippocampus in rats.

2. Materials and Methods

2.1. Stercuric Seed Oil Extraction

Mature *Sterculia apetala* seeds were collected in Los Tuxtlas, Veracruz, Mexico (18°27'54" N, longitude 94°59'11" W). The seeds were peeled, crushed, and mixed with hexane to extract stercuric oil. Fatty acid methyl esters were analyzed as reported previously [6].

2.2. Ethics

The experimental procedures were performed according to international ethical guidelines, based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals [15] and official Mexican Guidelines for the Use and Care of Laboratory Animals [16]. All efforts were made to minimize animal discomfort during the study, based on the Reduce, Refine, Replace (3R) principles [17].

2.3. Subjects

Forty-four adults male Wistar rats, weighing 200-250 g, were maintained under a 12 h/12 h light/dark cycle (lights on at 8:00 AM) at 25°C ± 1°C. The rats were housed in Plexiglas cages (two rats per cage) and provided with purified water and a standard rodent diet ad libitum.

2.4. Experiment 1: Dose-response Curve

To generate the dose-response curve, 26 rats were divided into four groups: one vehicle group received water (1.5 ml, $n = 6$) and three groups received different doses of stercuric oil (low dose [SOL], 0.06 g, $n = 6$; medium dose [SOM], 0.12 g, $n = 7$; high dose [SOH], 0.24 g, $n = 7$; Figure 1a). The stercuric oil doses were calculated according previous studies by our group [8]. All treatments were administered orogastrically for 6 weeks. To prepare stercuric oil, an emulsion with 3% Tween-20 (Sigma-Aldrich, Mexico) was prepared. At the end of the experimental diet period, the rats were sequentially evaluated in the elevated plus maze (EPM; 5 min),

locomotor activity test (LAT; 5 min), and forced swim test (FST; 6 min) [18].

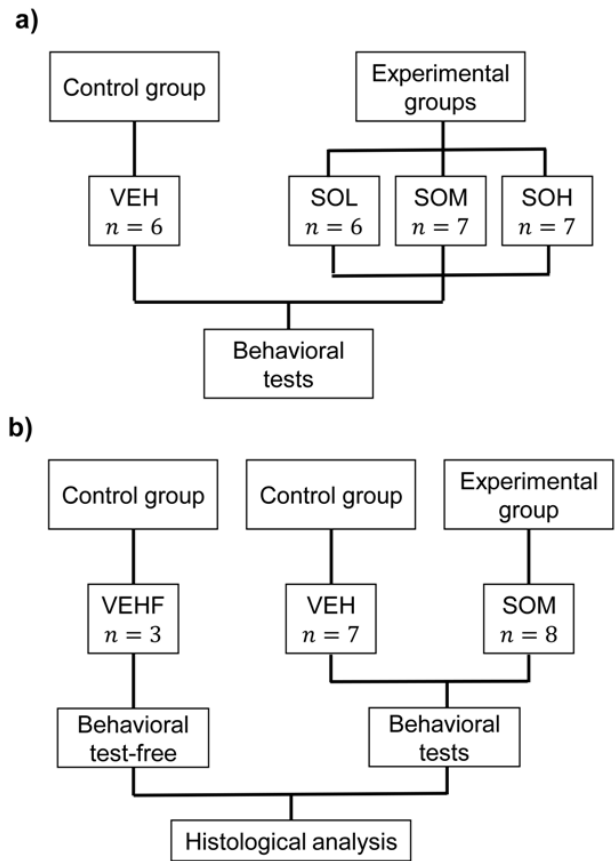


Figure 1. Experimental design. a) Experiment 1. Doses-response curve; b) Experiment 2. Effect of stercuric oil administration doses (VEH: vehicle; SOL: stercuric oil low dose; SOM: stercuric oil medium dose; SOH: stercuric oil high dose; VEHF: vehicle group behavioral tests-free)

2.4.1. Behavioral test

Elevated plus-maze

To identify whether stercuric oil consumption produces anxiolytic- or anxiogenic-like effects, we used the EPM [19], which consists of two opposite open and closed arms that are set in a plus configuration. The test was conducted in an isolated room that was illuminated at 40 lux. The open arm dimensions were 50 cm length × 10 cm width. The closed arm dimensions were 50 cm length × 10 cm width with 40 cm high walls. The entire maze was elevated 50 cm above the floor. The following variables were evaluated: (a) time spent on open arms (s), (b) number of entries into open arms, (c) total number of entries into both arms (open arms plus closed arms), (d) percentage of entries into open arms, (e) Anxiety Index ($AI = 1 - [(time\ spent\ on\ open\ arms / test\ duration) + (entries\ into\ open\ arms / total\ number\ of\ entries) / 2]$; [20], (f) frequency and time spent head-dipping (s), and (g) frequency and time spent in attempts (s). A digital video camera (Sony Handycam model DCR-DVD108) was installed above the EPM to record the rats' activity for 5 min.

Locomotor activity test

This test evaluated the effects of the treatments on spontaneous locomotor activity and identify or discard hypo- or hyperactivity that was caused by the treatments [21,22], which could interfere with the rats' behavior in

the EPM and FST. The rats were individually and gently placed in an opaque Plexiglas cage (44 cm × 33 cm × 40 cm). The floor was divided into 12 squares (11 cm × 11 cm each) that were drawn on the floor. The following variables were evaluated: (a) number of squares crossed (crossings), (b) frequency of rearing, (c) time of rearing (s), (d) frequency of grooming, and (e) time of grooming (s). This behavioral test was conducted for 5 min after the EPM test. A Sony DCR-SR85 video camera was used (2000× optical zoom). At the end of each test, the box was cleaned with a 15% ethanol solution. Approximately 2 min after the LAT, the rats were evaluated in the FST.

Forced swim test

This test detects the antidepressant- and antistress-like activity of natural compounds and drugs [22,23]. The rats were individually forced to swim in a rectangular glass tank (40 cm × 30 cm × 50 cm) that contained 25°C ± 1°C water to a depth of 25 cm. The present study used the method of Borsini [24]. Each swim session lasted 6 min, but data from only the last 4 min of the session were analyzed. The following variable was evaluated: a) immobility time (the sum of all periods in which the rat performed minimal movements to allow it to float without displacement) (s). The experimental sessions were recorded with a Sony model DCR-SR85 video camera (2000× optical zoom). For this test, the variable was evaluated by two independent observers using a behavior analysis program until they reached 95% agreement.

2.4.2. Statistical Analysis (Experiment 1)

The data from Experiment 1 were analyzed using one-way analysis of variance (ANOVA). Normality and homogeneity assumptions were verified using the Shapiro-Wilks and Bartlett tests. The Student-Newman-Keuls post hoc test was used to analyze differences between groups. If any variable violated any of the assumptions, then the Kruskal-Wallis nonparametric test was used. The results are expressed as mean ± standard error of the mean. For nonparametric statistics, the median was used as a measure of central tendency. All analyses were performed using SigmaPlot 12 and GraphPad Prism 8.0.1 software to compare treatment groups. Values of $p < 0.05$ were considered statistically significant.

2.5. Experiment 2: Effects of Sterculic Oil on Behavior and Neuronal Cells in the Hippocampus

Once the minimal effective dose was determined in Experiment 1, we assigned 18 male Wistar rats to three independent groups: vehicle group that did not undergo the behavioral tests (VEHF group; 1.5 ml water, $n = 3$), vehicle group that underwent the behavioral tests (VEH group; 1.5 ml water, $n = 7$), and a group that received the minimal effective dose of sterculic oil from Experiment 1 (SOM group; 0.12 g, $n = 8$). All treatments were administered orogastrically for 6 weeks. Sterculic oil emulsion was prepared as described above. At the end of the diet period, only the VEH group and SOM group were evaluated in the same battery of behavioral tests under the same conditions as described in Experiment 1 (Figure 1b). At the end of the behavioral tests, the rats were perfused,

and brains were extracted to perform histological analyses of the hippocampus.

2.5.1. Histological Analysis

Perfusion

The rats received an overdose of sodium pentobarbital (80 mg/kg, i.p., PiSA Agropecuaria, veterinary use) and were transcardially perfused with 200 ml of saline solution followed by 200 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (27°C, pH 8.3). Immediately afterward, brains were removed and preserved in 20% formalin for the histological analysis [25]. For cryoprotection, the brains were immersed in 10%, 20%, and 30% sucrose solutions. Each change of sucrose was performed until the brains touched the bottom of the container (~24 h; [26]).

Tissue processing

The brains were dehydrated with an ascending series of ethanol solutions (70%, 80%, 96%, and 100%), cleared in xylene, and embedded in paraffin. Serial coronal sections (10 µm thick) at the level of the hippocampus were obtained [27]. In all experimental groups, the following dorsal hippocampal regions were analyzed: CA1 (-3.30 mm to -4.30 mm anterior, 1.2 mm lateral, and -3.00 mm depth from bregma), CA3 (-3.30 mm to -4.30 mm anterior, 2.6 mm lateral, and 3.80 mm depth from bregma), and unilateral dental gyrus (-3.30 mm to -4.30 mm anterior, 2.6 mm lateral, and 3.80 mm depth from bregma).

Stains and preparation of brain slices

The hippocampus brain slices were stained with a modified Cresyl violet staining technique and dried at room temperature [28]. The slides were examined under a Leica LED DM1000 light microscope. Images were acquired using a Leica DFC450C camera (Nussloch, Germany) at 20× magnification.

Cell counting

Cell counts were performed by two observers who were blind to the experimental groups using 10 × 10 grids (500 µm × 500 µm) in ImageJ software. All values are expressed as an average for each group. Only cells with a cytosol, nucleus, and well-defined cellular membrane were considered [29]. Gray scale and sepia images were obtained, the latter with the differentiation of cell nuclei in white. Each micrograph that was obtained from Cresyl violet staining corresponded to a surface area of 0.36 µm².

2.5.2. Statistical Analysis (Experiment 1)

The behavioral and histological data were statistically analyzed as the following:

- Behavioral analysis: Student's t-test was used to compare the VEH group and the minimal effective dose of sterculic oil in the SOM group. In addition to the traditional statistical analysis, the aforementioned groups were compared using machine learning techniques to assess the time spent in each condition and the frequency of interactions between behavioral states.

- Histological analysis: One-way ANOVA was used to compare all experimental groups that were included in this experiment. All statistical tests were subjected to a criterion of significance of $\alpha = 0.05$.

To further evaluate possible relationships among variables, we used two machine learning techniques: decision trees (DTs) and transitional behavior graphs (Markov chains) for behavioral data.

Decision trees. This technique creates decision rules about the analyzed variables, forming a visually explanatory graph [30]. It consists of nodes (attributes) and arcs (discriminative values). Open-source WEKA 2.2 machine learning software was used to construct DTs using the J48 algorithm [31].

Transitional behavior graphs (Markov chains). These graphs are models that are used to observe transitions from one state to another. This representation shows sequences of changes in a given timeline [32]. The construction of these graphs is based on Markov chain processes, computing the conditional probability of moving from one behavior (state) to another [33].

$$P_{ij} = P(E_i | E_j) = \frac{f_{ij}}{F_j} \quad (1)$$

Where:

P_{ij} = conditional probability

E_i = current event

E_j = future event (to which it changes)

f_{ij} = event frequency ij

F_i = total events j

Likewise, the percentages of the relative frequency of each behavioral change during the period of the time evaluated were computed using the following equation:

$$F_r = \frac{f_{ij}}{N} \quad (2)$$

Where:

F_r = relative frequency

f_{ij} = event frequency ij

N = total events.

3. Results

3.1. Sterculic Seed Oil Extraction

As shown in Table 1, sterulic oil contained 26.3% sterulic acid (8-2-octyl-1-cyclopropenyl) and 2.04% malvalic acid (7-2-octyl-1-ciclopropenil). Palmitic acid, oleic acid, and linoleic acid were also present in the *Sterculia apetala* seed oil.

Table 1. Fatty acid composition of *Sterculia apetala* seeds oil

Fatty acids	% of total fatty acids
SFA	
Palmitic acid, C16:0	27.70
Stearic acid, C18:0	2.64
Total	30.34
MUFA	
Miristic acid, C16:1	2.25
Oleic acid, C18:1 <i>cis</i>	26.54
Elaidic acid, C18:1 <i>trans</i>	3.58
Total	32.37
PUFA	
Linoleic acid, C18:2	6.51
Total	6.51
Sterculic acid	26.30
Dihydrosterculic	2.44
Malvalic acid	2.04
Total	30.78

Values are expressed as a percentage of total fatty acids. Presence of cyclopropene ring. C18:1, malvalic acid [7-(2-octyl-1-clopropenyl) heptanoic acid]; C19:1, sterulic acid [8-(2-octyl-1-cyclopropenyl) octanoic acid].

3.2. Experiment 1. Dose-response Curve

3.2.1. Elevated Plus-maze

Significant differences ($F_{3,22} = 5.07$, $p < 0.001$) were found in the time spent on the open arms among groups. The post hoc test showed that this variable significantly decreased in all sterulic oil-treated groups compared with the vehicle group (Figure 2a). Similarly, the analysis of head-dipping revealed significant differences ($F_{3,22} = 4.11$, $p < 0.01$) between groups. The post hoc test showed that this variable significantly decreased in the sterulic oil-treated groups compared with the vehicle group (Figure 2b). This effect was more evident in rats that were treated with the medium dose of sterulic oil (Figure 2b). The time spent head-dipping was also significantly different ($F_{3,22} = 5.20$, $p < 0.001$) between groups. The post hoc test revealed that this variable significantly decreased in all sterulic oil-treated groups compared with the vehicle group (Figure 2c).

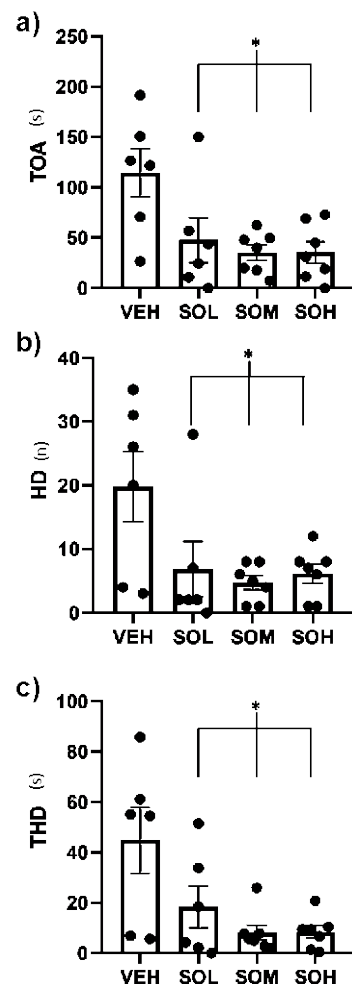


Figure 2. D Effects of sterulic oil administration on anxiety-like behavior in the elevated plus maze. TOA: time spent on the open arms, (a) HD: head-dipping (b), THD: time spent in head-dipping (c). * $p < 0.05$ vs vehicle group (one-way ANOVA followed by Student-Newman-Keuls post hoc test; VEH: vehicle; SOL: sterulic oil low dose; SOM: sterulic oil medium dose; SOH: sterulic oil high dose)

The number of entries into the open arms and total arm entries (closed arms plus open arms) were not significantly different among groups, but the percentage of entries into the open arms was significantly different. The

statistical analysis did not reveal significant differences in the number of attempts or the time spent in attempts between groups. Significant differences in the AI were found between the vehicle and sterculic oil groups ($H = 7.87$, $p < 0.05$), in which sterculic oil-treated groups had a higher AI (Table 2).

3.2.2. Locomotor Activity Test

The number of crossings was not significantly different among groups. The time spent grooming was significantly different between groups. No significant differences in the frequency of grooming or the frequency and duration of rearing, were found (Table 2).

3.2.3. Forced Swim Test

The analysis of the total time of immobility revealed no significant differences between groups (Table 2).

3.3. Experiment 2. Effects of Sterculic Oil on Behavior and Neuronal Cells in the Hippocampus

3.3.1. Elevated Plus-maze

The statistical analysis showed no significant differences in the time spent on the open arms or the number of entries into the open arms. Although the total number of entries into the open arms and the percentage of entries into the open arms decreased, these changes were not significantly different from the vehicle group. The sterculic oil group exhibited a significant increase in the number of attempts and time spent in attempts compared with the vehicle group. No significant differences in the number of head-dipping, the time spent head-dipping, or the AI were found between sterculic oil- and vehicle-treated groups (Table 3).

Table 2. Effect of sterculic oil administration on behavioral tests in experiment 1, dose-response curve

BT	Var	Groups				Statistics	
		VEH	SOL	SOM	SOH	F/H	p-value
EPM	EOA †	13.33 ± 2.99	7.17 ± 2.13	8.43 ± 1.71	9.43 ± 1.81	1.41	0.26
	TE †	26.33 ± 5.94	15.00 ± 4.25	17.43 ± 3.49	19.57 ± 3.53	1.19	0.33
	PEOA □	0.50	0.47	0.48	0.47	9.07	0.02*
	AT †	8 ± 1.36	7.5 ± 1.18	8.43 ± 0.92	7.29 ± 1.19	0.20	0.88
	TAT□	4.64	4.5	4.94	4.21	0.37	0.94
	AI □	0.54	0.70	0.68	0.69	7.87	0.04*
LAT	C †	56 ± 10.35	63.33 ± 6.71	54 ± 9.14	59 ± 3.38	0.26	0.84
	GF †	2.5 ± 0.56	2.83 ± 1.01	1.28 ± 0.52	1.42 ± 0.52	1.32	0.29
	GD □	12.71	2.56	0.33	1.4	7.89	0.04*
	RF □	47	47	36	39	2.61	0.45
	RD †	87.79 ± 22.68	85.07 ± 10.79	95.15 ± 17.08	93.04 ± 8.78	0.05	0.98
FST	IT □	12.91	24.09	31.78	33.41	3.71	0.28

† Variables analyzed by one-way ANOVA test, reporting mean ± standard error; □ variables analyzed by Kruskal-Wallis test, reporting medians. * $p < 0.05$ vs VEH; BT: behavioral test; EPM: elevated plus-maze; LAT: locomotor activity test; FST: forced swim test; Var: variables; VEH: vehicle; SOL: sterculic oil low; SOM: sterculic oil medium; SOH: sterculic oil high; EOA: number of entries in open arms; TE: total entries; PEOA: percentage of entries into the open arms; AT: attempts; TAT: time spent in attempts; AI: Anxiety index; C: crossing; GF: grooming frequency; GD: grooming duration; RF: rearing frequency; RD: rearing duration; IT: immobility time.

Table 3. Effect of sterculic oil administration on behavioral tests in experiment 2

BT	Var	Groups		Statistics	
		VEH	SOM	t/U	p-value
EPM	TOA†	42.47 ± 10.59	21.97 ± 9.99	1.39	0.18
	EOA †	6 ± 1.25	3.14 ± 0.85	1.82	0.09
	TE †	12.75 ± 2.39	7.28 ± 1.71	1.80	0.09
	PEOA □	46.66	42.85	16	0.18
	AT □	6	11	8.50	0.02*
	TAT□	4.51	8.25	1	0.00*
	HD†	3.75 ± 1.04	2.57 ± 0.92	0.83	0.42
	THD□	4.54	4.12	27	0.95
	AI †	0.51 ± 0.04	0.64 ± 0.06	1.67	0.11
LAT	C †	37.12 ± 3.82	32.71 ± 3.51	0.83	0.41
	GF †	5.62 ± 0.94	5 ± 1	0.45	0.65
	GD □	32.3	16.6	15	0.15
	RF †	36 ± 2.13	33.42 ± 4.36	0.55	0.59
	RD †	82.65 ± 6.49	71.19 ± 10.47	0.95	0.36
FST	IT †	82.75 ± 13.85	119.81 ± 19.24	1.59	0.13

† Variables analyzed by t of Student test, reporting mean ± standard error; □ variables analyzed by U of Mann-Witney test, reporting medians. * $p < 0.05$ vs VEH; BT: behavioral test; EPM: elevated plus-maze; LAT: locomotor activity test; FST: forced swim test; Var: variables; VEH: vehicle; SOM: sterculic oil medium; TOA: time spent in open arms; EOA: number of entries in open arms; TE: total entries; PEOA: percentage of entries into the open arms; AT: attempts; TAT: time spent in attempts; HD: head-dipping; THD: time spent in head-dipping; AI: Anxiety index; C: crossing; GF: grooming frequency; GD: grooming duration; RF: rearing frequency; RD: rearing duration; IT: immobility time.

3.3.2. Locomotor Activity Test

The analysis of crossings, frequency, and grooming revealed no significant differences between the two experimental groups (Table 3).

3.3.3. Forced Swim Test

The statistical analysis did not reveal significant differences in total immobility time between groups (Table 3).

3.3.4. Decision Tree

To analyze differences between the sterculic oil-treated group and vehicle-treated group, a DT method was used. The first DT was constructed using the time spent in attempts and time spent head-dipping. The structure of the DT showed that the time spent in attempts was the most relevant attribute for classifying the groups, with a classification accuracy of 80%. When time was longer than 4.89 s, the subjects belonged to the sterculic oil-treated group (Figure 3a).

Likewise, we were able to discriminate between groups with regard to LAT variables, including grooming duration and rearing duration, with a classification accuracy of 60%. In this case, the time spent grooming produced the main division between groups. When grooming time was ≤ 16.76 s, the subjects belonged to the sterculic oil-treated group. For grooming times that were > 16.76 s, the DT required another division for classification, represented by the time spent rearing. When the time spent rearing was > 56.33 s, the subjects belonged to the vehicle group; otherwise, the subjects belonged to the sterculic oil-treated group (Figure 3b).

3.3.5. Transitional Behavior Graphs (Markov Chains)

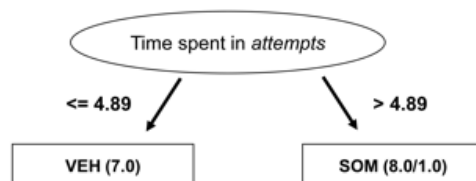
In a previous analysis, traditional tests like ANOVAs were used to compare the time spent in different behaviors

by the subjects. Using this approach, however, interactions among different states were not explored because it evaluates only the total amount of time for each behavior. To study transitions among behaviors that were expressed by the rats in the behavioral tests, a transitional behavior graph was constructed. The probabilities of behavioral state transitions (see equation 1) were compared between the vehicle group and sterculic oil group. In the EPM test, it only showed significant differences ($p = 0.028$), assessed using Student's t-test when the subjects were kept in the behavior of attempts, showing that the sterculic oil-treated group (probabilities: 0.02 for VEH group vs. 0.18 for SOM group) was more likely to remain in such a behavioral state (Table 4). In the LAT, the transition state that showed differences between groups was the change from grooming to resting behavior ($p = 0.01$), showing that the sterculic oil-treated group was more likely to perform this transition (probabilities: 0.12 for VEH group vs. 0.26 for SOM group).

We used relative frequencies (see equation 2) to represent percentages of state changes. In the EPM, we observed significant differences in behavioral transitions from closed arms to attempts ($p = 0.01$), which was higher in the sterculic oil-treated group (1.73% for VEH group vs. 3.67% for SOM group). We observed a significant difference in remaining in the behavioral state of attempts ($p = 0.02$), which was higher in the sterculic oil-treated group (0.04% for VEH group vs. 0.72% for SOM group; Figure 4).

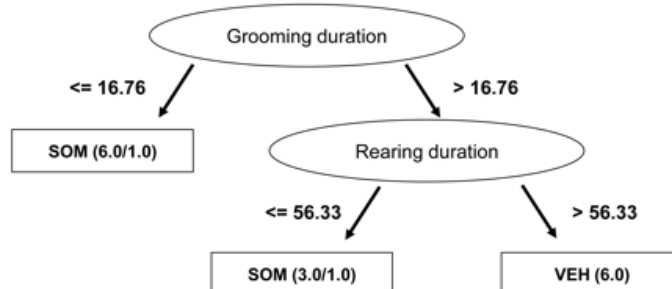
Similarly, differences were found when the subjects were in the open arm state and remaining in that state ($p = 0.02$), which was lower in the sterculic oil-treated group (10.46% for VEH group vs. 3.67% for SOM group). A significant decrease ($p = 0.04$) in transitions from open arm behavior to attempts was observed in the sterculic oil-treated group (1.01% for VEH group vs. 0.13% for SOM group; Figure 4).

a)



Confusion matrix		
	a	b
a=VEH	3	0
b=SOM	1	1

b)



Confusion matrix		
	a	b
a=VEH	2	1
b=SOM	1	1

Figure 3. Decision trees for the classification of the control and sterculic oil groups evaluated in the behavior tests (a: times in elevated plus maze, b: times in locomotor activity test; VEH: vehicle; SOM: sterculic oil medium dose)

Table 4. Effect of sterculic oil administration on behavior transitions in experiment 2

VEH			SOM			Student test
State "i"	State "j"	Prob	State "i"	State "j"	Prob	p-value
CA	CA	0.95	CA	CA	0.95	† 0.98
	OA	0.02		OA	0.01	† 0.09
	AT	0.02		AT	0.03	▣ 0.06
	HD	0.00		HD	0.00	▣ 0.87
OA	CA	0.14	OA	CA	0.20	▣ 0.57
	OA	0.76		OA	0.69	▣ 0.16
	AT	0.03		AT	0.02	▣ 0.27
	HD	0.07		HD	0.09	† 0.97
AT	CA	0.76	AT	CA	0.59	▣ 0.32
	OA	0.22		OA	0.23	▣ 0.13
	AT	0.02		AT	0.18	▣ 0.02*
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HD	CA	0.12	HD	CA	0.14	▣ 0.79
	OA	0.36		OA	0.24	▣ 0.79
	AT	0.08		AT	0.03	▣ 0.95
	HD	0.44		HD	0.59	▣ 0.50

† Variables analyzed by t of Student test, reporting mean ± standard error; ▣ variables analyzed by U of Mann-Witney test, reporting medians. *< p 0.05 vs VEH; VEH: vehicle; SOM: sterculic oil medium; CA: closed arms; OA: open arms; AT: attempts; HD: head-dipping.

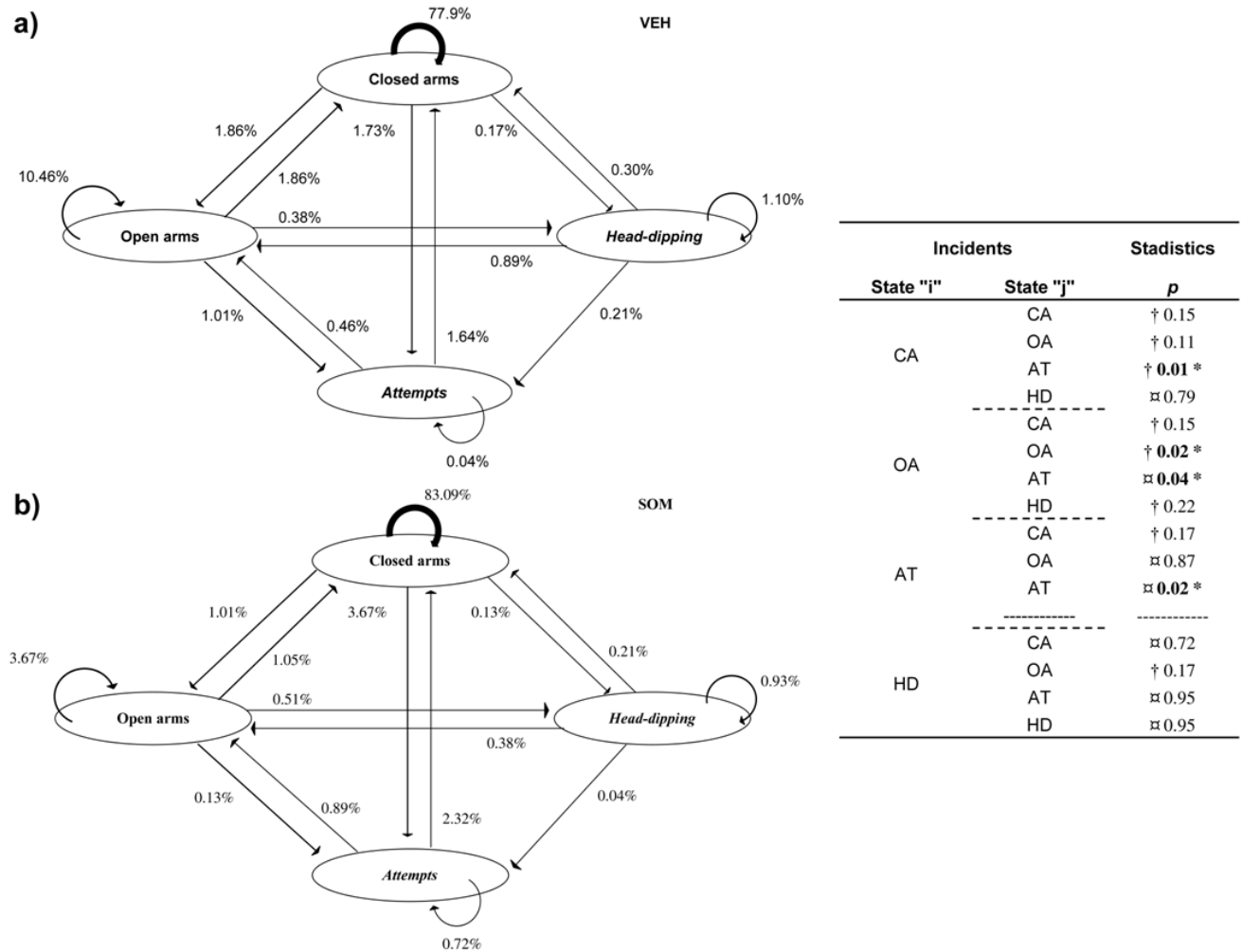


Figure 4. Transitional behavior graphs of the effect of sterculic oil administration in the elevated plus maze, represented by relative frequency († Variables analyzed by t of Student test, reporting mean ± standard error; ▣ variables analyzed by U of Mann-Witney test, reporting medians. *< p 0.05; Veh: vehicle; SOM: sterculic oil medium; CA: closed arms; OA: open arms; AT: attempts; HD: head-dipping)

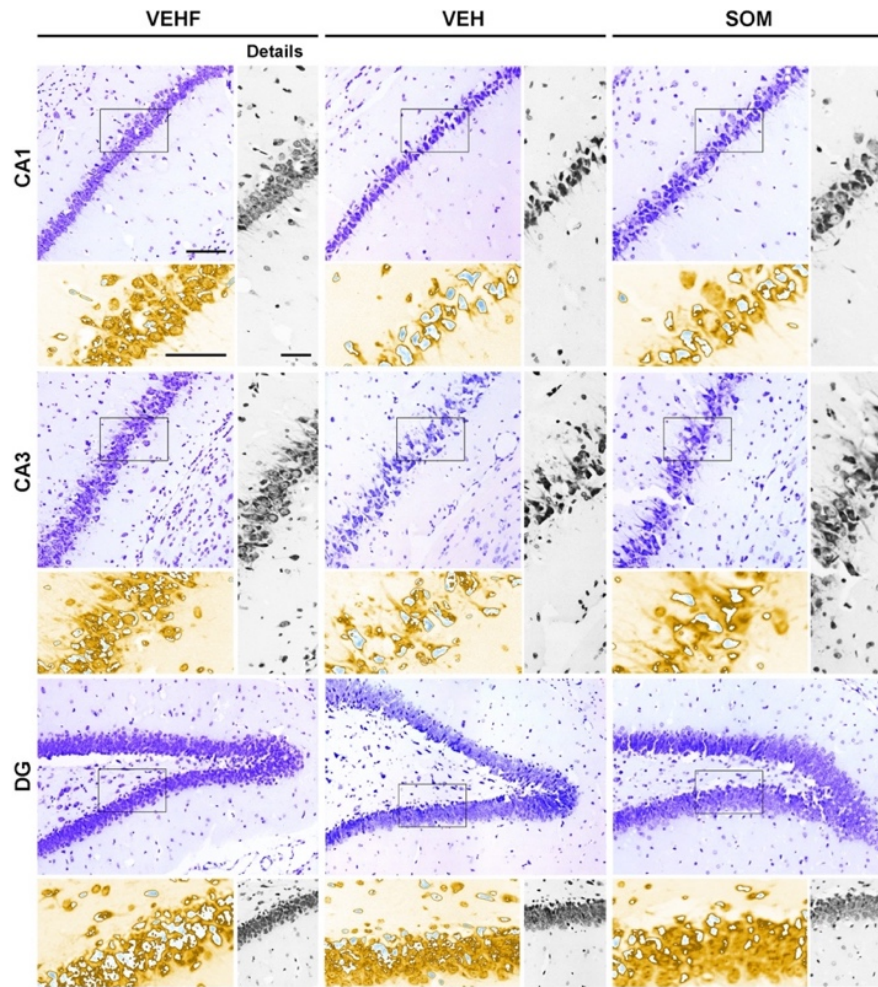


Figure 5. Cellular effects of sterculic oil in rat hippocampus. Representative micrographs of CA1, CA3 and Dentate gyrus (DG) stained by cresyl violet solution followed by a gradient-map-based image analysis (gray and light brown scales). VEHF: vehicle group behavioral tests-free; SOM: sterculic oil medium dose. The details correspond to the area highlighted with the rectangle. Bars = 500 μ m (cresyl violet images) and 100 μ m (details section)

3.3.6. Neuronal Quantification in the CA1, CA3, and Dentate Gyrus Regions of the Hippocampus

In the analysis of cell counts in the CA1 area of the hippocampus, significant differences were found between groups ($F_{2,43} = 136.17$, $p < 0.001$). The post hoc test showed that all groups were different from each other. The VEH group (70.18 ± 5.22) exhibited fewer neurons than the VEHF group (184.41 ± 4.99). The sterculic oil-treated group (151.04 ± 3.61) exhibited an intermediate number of neurons between the mentioned groups (Figure 5). In the CA3 area, differences were found between groups ($H = 28.75$, $p < 0.001$). The post hoc test showed a significant reduction of the number of neurons in the groups that underwent the behavioral tests (medians: 121 for VEHF group, 54 for VEH group, 76 for SOM group).

The dentate gyrus analysis showed significant differences among groups ($F_{2,37} = 30.83$, $p < 0.001$). The post hoc test indicated that all groups were different from each other. Both groups that underwent the behavioral tests exhibited a reduction of the number of neurons compared with the VEHF group (536.80 ± 15.87). The SOM group (471.70 ± 10.24) had a higher number of neurons than the VEH group (334 ± 20.49 ; Figure 5).

Interestingly, the grayscale view revealed hyperchromatic cell nuclei and the absence of macrophage, polymorphonuclear, or lymphocyte-like cellular infiltrates, indicating the absence of neuroinflammation in the three hippocampal

areas. Finally, the analysis of the gradient map of the CA1 and CA3 regions indicated differential contrast between the nucleus and neuronal soma, suggesting transient alterations of cell morphology, and highlighted the presence of some pyramidal neurons in the VEH group and globular in the SOM group. In the dentate gyrus, differential contrast between the nucleus and neuronal soma did not show alterations of cell morphology.

4. Discussion

The principal results of the present study showed that sterculic oil administration produced anxiogenic-like effects in the EPM. We observed fewer neurons in the CA1 area and dentate gyrus, which could be associated with specific fatty acids that were present in sterculic oil that participate in the structure of cell membranes. These results suggest that the chronic consumption of sterculic oil in healthy subjects may produce negative effects on emotional state, specifically producing anxiety.

4.1. Behavioral Effects of Sterculic Oil

In Experiment 1, sterculic oil administration significantly decreased the time spent on the open arms and number of entries into the open arms, suggesting an

anxiogenic-like effect [34]. The number of head-dippings and time spent head-dipping significantly decreased, which are related to stressful and anxiogenic situations [35], in rats that were treated with SOM, for example, with the administration of *Passiflora incarnata* it has been observed that it promotes anxiogenic-type effects, reflected in shorter exposure times in open arms in EPM (Elsas et al., 2010).

The AI identifies a general tendency of the anxiety grade (from 0 to 1). Anxiety Index values near 1 reflect higher levels of anxiety [20], for example, animals that were exposed to natural predator scent, showed which higher AI values were found [36]. In the present study, SOM administration increased the AI, indicating an anxiogenic-like state. This finding is similar to studies that evaluated that received natural extracts, for example, the *Myristica fragrans* seeds that evaluated in male Albino mice where found a anxiogenic effect showed in a reduction in time spent in open arms on elevated plus-maze [37].

Grooming behavior is related to an animal's motivation [38]. Grooming behavior increases in mildly stressful situations but is eliminated under severe stress conditions [39]. In the present study, grooming behavior significantly decreased in the sterculic oil-treated groups. We also previously observed this behavior in Zucker rats that received sterculic oil supplementation [10], therefore, it suggests the possibility that consumption of SOM induces anxiogenic-type conditions in Wistar and Zucker rats.

In the FST, antidepressant drugs are well known to exhibit a reduction of the total time of immobility [40]. In the present study, no significant effects of sterculic oil were observed in the FST, particularly immobility time. However, the administration of natural foods (e.g., cicada, *Dioon spinulosum*) in rats can generate motor alterations, reflected by turning behavior [9,25]. In the present study, impairments in motor coordination were not detected in sterculic oil-treated rats, suggesting that this oil has no negative effects on motor coordination.

In Experiment 2, significant differences in the frequency and duration of attempts were observed in the SOM group, which exhibited increases in this behavior. These variables are used to evaluate the risk of situations and is exhibited more by "anxious" animals [41]. Rodents have an innate propensity to avoid open arms in the EPM. Increases in entries into the closed arms and time spent on the close arms may be a more useful baseline to measure the effects of anxiogenic compounds, where attempts are an important variable to observe anxiety-like behavior.

We used DTs to complement the data analysis and observe integrations of variables. The classification model was adjusted for Experiment 2 data using the LAT variables. When comparing the sterculic oil group and vehicle group, we obtained classifications based on shorter grooming times and vertical behavior and an increase in the time spent in attempts. This analysis reinforces the anxiogenic-like effects of sterculic oil. Decreases in these behaviors were previously shown to be associated with higher levels of anxiety [38]. Importantly, only when we did Markov chains were significant interactions identified in the behavior, indicating an anxiogenic-like effect of sterculic oil.

To identify behavioral changes and complement the behavioral analysis, transitional behavior graphs were constructed. In the present study, a higher percentage of the incidence of behavioral changes from closed arms to attempts and from attempts to attempts was identified in the sterculic oil group. Similarly, a decrease in change incidence of open arms to open arms and from open arms to attempts was observed. These behaviors are identified as an anxiogenic-like effect because they reflect an increase in the anxiety state of the animals [19].

4.2. Impact on Neuronal Cells in the Hippocampus

In the present study, changes in the number of neuronal cells in CA1, CA3, and dentate gyrus areas were observed. Our data revealed a decrease in the number of neurons in both groups exposed to behavioral tests compared with the free-tests group, however, the SOM group showed an attenuated reduction of neurons in the CA1 and dentate gyrus regions. The forced swim test is a strong stressor for rodents, which quickly activates the hypothalamus-pituitary-adrenal (HPA) axis and raises the level of corticosterone in the blood plasma [42]. The hippocampus is a limbic structure involved in anxiety-like behavior. Recently, Bertagna et. al., [35] reported that the ventral hippocampus, but not the dorsal, plays a causal role for anxiety in the elevated-plus maze. According to Sapolsky et al., [43] activation of HPA axis involves the glucocorticoid cascade that leads to hippocampal neuronal loss. Stress is a mechanism of neuronal plasticity [44], specifically, the plasticity of hippocampal circuitry is principally vulnerable to stressors [45].

We found a decrease in number of neurons in the CA1 region in the groups that underwent the behavioral tests. However, in the group that received sterculic oil, we observed an attenuation decrease in the number of neurons, generate by stress that was induced in the behavioral tests, possibly because of the fatty acid composition of sterculic oil, such as stearic acid, oleic acid, and linoleic acid [6]. A neuroprotective effect of stearic acid administration was reported in rodents [46]. Beneficial cognitive and neurogenic effects and synapse arrangement were shown to be promoted by the consumption of oleic acid and linoleic acid [47].

In the CA3 region, we observed a reduction of the number of neurons in the groups that were treated with VEH and SOM and underwent the behavioral tests. In the CA3 region, chronic stress produced dendritic retractions [48]. Additionally, corticotropin-releasing factor (CRF), together with excitatory amino acids, promoted the atrophy of dendrites in the CA3 area of the hippocampus in male rats [49]. In this specific region in the present study, the sterculic oil group did not exhibit a protective effect of sterculic oil on neuronal cells. Our data are consistent with Drenska et al. [50], who reported that stress that was associated with light (via diurnal rhythm disruption) decreased neuronal density in the CA3 region in male Wistar rats.

The present study revealed a decrease in neuronal cells in the dentate gyrus in the groups that underwent the behavioral tests compared with the VEHF group.

However, the group that received sterculic oil exhibited an attenuation of this decrease. Neurogenesis occurs in the dentate gyrus of the hippocampus, and acute stressful experiences can suppress ongoing neurogenesis [51]. As mentioned above, this condition could be due to fatty acids composition present in the oil.

Interestingly, Arauchi et al. [52] reported an increase in the activation of microglia and astrocytes in the CA1, CA3, and dentate gyrus regions of the hippocampus in Gunn rats after they were subjected to the stress of the FST. The activation of microglia and astrocytes may be involved in the death of neuronal cells in the hippocampus. Moreover, the activation of these two types of glial cells has been shown to cause neurotoxicity in in vitro models [53]. Notably, the presence of antioxidant compounds from *Sterculia apetala* seeds, specifically gallic acid, has been reported [54]. Supplementation with these compounds in rats has been related to neuroprotective and anxiolytic-like effects [55]. Therefore, in the present study, we can hypothesize that both antioxidant compounds and fatty acids in sterculic oil could act additively or synergistically to generate neuroprotection against the impact of stress that is associated with the battery of behavioral tests, thereby preventing neuronal cell loss. Therefore, in the present investigation, we can hypothesize that both antioxidant compounds and fatty acids content present in sterculic oil, could be acting synergistically, generating neuroprotection against the impact of the stressor produced by forced swim test, avoiding a decrease in the neuronal cell. Although in our study some behavioral tests variables show anxiogenic-like effects, in the hippocampus this effect was not reflected, because we did not find evidence of a decrease in neurons, on the contrary, in two of the three regions evaluated, protection against stress exerted by behavioral battery tests was observed.

One limitation of the present study was that we did not explore behavioral effects of sterculic acid or malvalic acid isolates. We also did not include a separate group that received sterculic oil and did not undergo the behavioral tests in the histological analysis to exclude a possible decrease in the number of neurons. Further toxicological studies are necessary to investigate the safe consumption of *Sterculia apetala* seed oil. Some reports showed a neurotoxic effect of seed consumption [11,12]. In the present study, we administered natural seed oil, but populations mostly consume roasted seeds, which lowers the sterculic acid content [6]. Lastly, the mechanism of action of the anxiogenic effects of sterculic oil should be explored to understand the involvement of neuropharmacological processes.

5. Conclusions

In conclusion, *Sterculia apetala* seed oil administration in Wistar rats produced an anxiogenic-like effect at all tested doses. However, attenuated effect in CA1 region and hippocampus dentate gyrus was observed when we administer the dose medium, likely because of the fatty acid content in sterculic oil. These seeds are commonly consumed as a part of cultural tradition in populations that are not knowledgeable about its effects. The present findings will contribute to future investigations of the

effects of *Sterculia apetala* seed consumption on human health.

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Statement of Competing Interests

The authors declare no conflict of interest.

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