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Saline Extract from *Moringa oleifera* Leaves Has Antidepressant and Anxiolytic Effects in Mouse Models

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Abstract: Plant extracts can be therapeutic alternatives for depression and anxiety. However, some plant-derived preparations can also be toxic. *Moringa oleifera* leaves are used in human nutrition due to their high nutritional value and antioxidant activity. This study investigated a saline extract from *M. oleifera* leaves (MoLE) for secondary metabolites, proteins, cytotoxicity, hemolytic activity, in vivo acute oral toxicity, and neurobehavioral effects. MoLE contains flavonoids (rutin and vitexin), lectin, and a trypsin inhibitor. It is neither cytotoxic nor hemolytic for human cells and did not present acute oral toxicity (2000 mg/kg) to mice. The elevated plus maze test showed that MoLE (500, 1000, and 2000 mg/kg, p.o.) significantly increased the number of entries as well as the time spent by mice in open arms, while it decreased the number of entries and the time spent in closed arms when compared to the control. MoLE (500, 1000, and 2000 mg/kg, p.o.) reduced immobility time of mice in the tail suspension and forced swimming tests, compared to the control. The anxiolytic-like effect of MoLE is possibly mediated by a GABA mimetic action once it is prevented by pre-treatment with flumazenil. The present study demonstrated that MoLE has antidepressant and anxiolytic effects in mice and is a promising herbal medicine.

Keywords: plant protein; flavonoids; acute toxicity test; nervous system

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1. Introduction

Anxiety and depression are neurological disorders that constitute a group of pathologies with a high and growing prevalence in the general population [1]. According to the World Health Organization (WHO), in 2019, about 970 million people (1 in every 8 people) around the world were living with a mental disorder, of which 301 million people were living with anxiety and approximately 280 million had depression. Moreover, more than 700,000 people die due to suicide associated with depression every year [2].

The neurobehavioral changes found in these disorders share similar pathophysiological patterns, such as oxidative stress [3], neurotransmitter dysfunction [4], and neuroinflammation [5], among others. Monoaminergic signaling, which involves dopaminergic, noradrenergic, serotonergic, and histaminergic circuitries, is one of the impaired pathways of anxiety and depression [4]. Current treatments are effective in treating some of the symptoms, but there is still no effective therapy capable of completely mitigating the negative factors caused by these illnesses [6]. Medications currently used for the long-term

treatment of these diseases cause several adverse effects and chemical dependency, making it important to search for safer and more effective treatment alternatives [7].

Phytotherapy has emerged as an alternative since compounds present in plant preparations or isolated active ingredients have the potential to be used as medicines to treat anxiety and depression [8]. For example, *Passiflora incarnata* (passion fruit) has pharmacological properties in the central nervous system (CNS) and is being used for anxiety disorders in pharmacotherapy [9].

Moringa oleifera (Lam.) tissues contain more than 90 nutritional compounds, such as proteins, lipids, vitamins, minerals, carbohydrates, and dietary fiber, and are widely used as dietary supplements [10]. The leaf is a reliable source of essential amino acids, minerals, and phenolic compounds [11,12]. All parts of *M. oleifera* are traditionally used for medicinal purposes; however, studies report that the leaves can act against convulsions [13], as well as pain-relieving, anti-inflammatory, immunomodulatory, antidepressive, and neuroprotective activities [14,15]. It has been demonstrated that phytochemicals present in *M. oleifera* leaves, such as isothiocyanate and flavonoids, are capable of modulating critical signal transduction pathways involved in cancer, inhibiting metastasis and inducing apoptosis [16]. The leaves are widely used for their nutraceutical benefits in improving fitness and overall health [10,16]. Metabolites derived from *M. oleifera* leaves, such as isorhamnetin and quercetin, have been reported to provide antioxidant activity and promote anti-inflammatory and anti-Alzheimer's activity [12,17].

M. oleifera acts against many neurological diseases including epilepsy, fatigue, memory impairment, and seizures. Methanolic and hexanic extracts (500 mg/kg p.o.) from *M. oleifera* leaves had an anxiolytic-like effect in mice [18]. Mahmoud et al. [19] demonstrated that ethanolic extract from *M. oleifera* leaves (400 mg/kg/day p.o.), applied preventively for 14 days, played a neuroprotective role against neurotoxicity induced by carbon tetrachloride and significantly improved anxiety and depression conditions due to antioxidant and anti-inflammatory effects. Additionally, a diet supplemented with pellets of moringa leaves (1.0, 5.0 and 10.0%) significantly improved oxidative stress and restored cholinergic neuronal transmission in the brain of mice in an Alzheimer's model [20]. A bioactive protein with lectin activity isolated from *M. oleifera* seeds (WSMoL) showed an anxiolytic effect through the serotonergic, noradrenergic, and dopaminergic pathways, demonstrating that these proteins can also have an effect on the CNS [21].

Protein solubility is affected by the ionic strength of the medium. At low salt concentrations (<0.5 M), protein solubility increases in a phenomenon known as salting-in [22]. Thus, the preparation of a saline extract using salts such as 0.15 M NaCl can promote the greater extraction of bioactive proteins, such as lectins [23,24] and trypsin inhibitors [25], from *M. oleifera* leaves. This study evaluated, for the first time, the neurobehavioral effects of saline extract from *M. oleifera* leaves (MoLE), a preparation containing both secondary metabolites and proteins (trypsin inhibitor and lectins). Additionally, the hemolytic and cytotoxic activities in human cells and the acute oral toxicity in mice were assessed.

2. Materials and Methods

2.1. Preparation of M. oleifera Leaf Extract (MoLE)

The leaves of *M. oleifera* were collected in Recife, Brazil (8°02′56.1″ S; 34°56′48.8″ W). After drying (27 ± 2 °C), they were crushed. The leaf flour (7.5 g) was homogenized (4 h at 28 °C) on a magnetic stirrer (Fisatom, São Paulo, Brazil) with citrate–phosphate buffer pH 3.0 containing 0.15 M NaCl (100 mL). Then, the mixture was filtered (through filter paper) and centrifuged (9000× *g*; 15 min; 4 °C). The supernatant corresponded to the *M. oleifera* leaf extract (MoLE), which had a yield of 29.3% (*w*/*w*).

For protein quantification, the sample (0.2 mL) was incubated (10 min, 28 °C) with 1 mL of an alkaline copper reagent and then 0.1 mL of the Folin–Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA), diluted 1:1 with water. After 30 min, absorbance at 720 nm was measured. A standard curve (31.25 to 500 µg/mL) of bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) was prepared [26].

2.2. Characterization of MoLE by Thin Layer Chromatography (TLC)

MoLE (1 mg) and standards (1 mg), solubilized in 1 mL of methanol (Tedia, Fairfield, OH, USA), were manually applied to silica gel 60-F254 chromatographic plates (Macherey-Nagel, Dueren, Germany). The plates were developed in vats after saturation (15 min at 28 °C) with the mobile phase. After elution, the plates were dried at 28 °C and then observed under ultraviolet light at 254 nm and under visible light at 365 nm. They were then revealed with specific reagents for each metabolite. The following standards (Sigma-Aldrich) were used: gallic acid for hydrolysable tannins; catechin for condensed tannins; rutin and quercetin for flavonoids; chlorogenic acid and caffeic acid for cinnamic derivatives; β-sitosterol for terpenes and steroids; coumarin; escin for saponins; sennoside A for quinones; atropine for alkaloids; and D-maltose for reducing sugars.

2.3. Characterization of MoLE Using High-Performance Liquid Chromatography (HPLC)

MoLE (50 mg) was solubilized in 5 mL of HPLC-grade methanol (Tedia, Fairfield, OH, USA) and then diluted in ultrapure water (Purelab Classic UV, ELGA LabWater, High Wycombe, UK) to obtain a 5 mg/mL solution. The analysis was managed on an UltiMate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an autosampler with a 20 µL loop (ACC-3000), a binary pump (HPG-3x00RS), a diode array detector, and a degasser. The absorbance at 350 nm was monitored in order to detect flavonoids. Chromatography was performed at a flow rate of 0.8 mL/min and 25 ± 2 °C in a C18 column (250 mm × 4.6 mm i.d., particle size 5 m; Dionex Corp., Sunnyvale, CA, USA) assembled to a guard column (C18, 4 mm × 3.9 µm; Phenomenex, Torrance, CA, USA). The mobile phases were purified water (A) and methanol (B), both acidified with 0.05% (*w*/*v*) trifluoroacetic acid. The following gradient scheme was utilized: 0–10 min, 5–20% B; 10–13.5 min, 20–25% B; 13.5–18 min, 25–40% B; 18–25 min, 40–80% B; 25–30 min, 80% B; 30–34 min, 80–5% B. Chromeleon™ software version 6 (Dionex/Thermo Fisher Scientific) was used. Standards (vitexin, rutin, quercetin, and kaempferol; Sigma-Aldrich) were dissolved in methanol and diluted in purified water to obtain 10 µg/mL solutions. These were submitted for analysis under the same conditions described for the sample.

2.4. Investigation of MoLE for Hemagglutinating Activity (Lectin)

Aliquots (50 µL) of MoLE was serially diluted in 0.15 M NaCl before adding 50 µL of suspension (2.5% *v*/*v*) of glutaraldehyde-fixed rabbit erythrocytes (collection method approved by the Ethics Committee for Animal Experimentation of UFPE; process 23076.033782/2015-70). The assay was carried out in 96-well microtiter plates according to Procópio et al. [27]. One unit of hemagglutinating activity (titer^{-1}) was defined as the inverse of the highest dilution of the sample that caused a hemagglutination visible to the naked eye. Specific hemagglutinating activity (SHA) was defined by the ratio between the titer and the concentration of proteins (mg/mL).

2.5. Determination of Trypsin Inhibitor Activity

The assay was performed in a 96-well flat-bottom plate according to Pontual et al. [28]. Aliquots (10 or 20 µL) of MoLE were added to the wells containing 0.1 M Tris-HCl buffer pH 8.0 in 0.15 M NaCl (180 or 170 µL). Then, 5 µL of 0.1 mg/mL porcine trypsin (Sigma-Aldrich) and 8 mM N-Benzoyl-DL-arginine-4-nitroanilide hydrochloride (BApNA; Sigma-Aldrich) substrate (5 µL) were added. The absorbance at 405 nm was determined before (time zero) and after incubation at 37 °C for 2 h. In the control, the extract was commutated by 0.1 M Tris-HCl buffer pH 8.0 in 0.15 M NaCl. The test was performed in triplicate. One unit of trypsin inhibitor activity (U/mg) was established as the amount of inhibitor that reduced absorbance by 0.01 in comparison with control.

2.6. Isolation of Peripheral Blood Mononuclear Cells (PBMCs) and Evaluation of the Effect of MoLE on Lymphocyte Viability

Forty-five milliliters of human blood was taken through venipuncture from healthy and non-smoking donors (n = 15), and deposited in heparin tubes (Vacuette, Greiner AG, Kremsmünster, Austria). All donors signed an informed consent form, and the experimental protocols were approved by the Ethics Committee of UFPE (CAAE 33719520.9.0000.5208). PBMCs were separated using Ficoll-Paque Plus (GE Healthcare Life Sciences, Uppsala, Sweden), and cell viability was checked using trypan blue (Sigma-Aldrich). Cells were used when viability was greater than 98%. PBMCs were cultured in RPMI 1640 medium (Sigma-Aldrich) with 10% (w/v) fetal bovine serum (Sigma-Aldrich) in 24-well plates to achieve a density of 10^6 cells/well.

PBMCs were cultured in the absence (control) or presence of MoLE (protein concentration in the wells ranging from 6.25 to 50.0 μg/mL) for 24 h. After the centrifugation (450× g, 4 °C, 10 min) and discarding of the supernatant, 1.0 mL of PBS was added to the precipitate. The cells were resuspended and centrifuged one more time under the same conditions. The pellet was resuspended in 300 μL of 10.0 mM HEPES (pH 7.4), 150.0 mM NaCl, 5.0 mM KCl, 1.0 mM $MgCl_2$, and 1.8 mM $CaCl_2$. Annexin V (AnnV) conjugated to fluorescein isothiocyanate (FITC) (1:500) and propidium iodide (PI, 20 μg/mL) were added. Data were acquired on a FACSCalibur platform (BD Biosciences, San Jose, CA, USA). A gate was created to exclude cell debris and define the cell population of interest. A minimum of 20,000 events in the gate were collected per sample. FL1 filter (excitation 488 nm, emission 530/30 nm) was used (Ann V-FITC detection), and FL3 filter (excitation 488 nm, emission 585/40 nm) was used (PI detection). The results were analyzed using CellQuest Pro software version 5.1 (BD Biosciences). FL1 and FL3 quadrants were established considering the intrinsic fluorescence of the cells from negative controls unstained with Annexin V-FITC and propidium iodide. Negative control cells stained only with Ann V-FITC or only with PI were used to setup compensation parameters. AnnV−/PI+ cells were considered necrotic and AnnV+/PI− cells were assumed to be in the first stages of apoptosis. AnnV−/PI− cells were designed to be viable.

2.7. Investigation of MoLE for Hemolytic Activity

Human blood (9 mL) was collected in heparinized tubes through venipuncture from healthy volunteers (n = 3), after they had signed an informed consent form (approved by the Ethics Committee in Human Research of UFPE; protocol 33719520.9.0000.5208). Erythrocytes were isolated by centrifugation (452× g, 10 min at 4 °C) and washed five times with 0.15 M NaCl. Each tube received 1.1 mL of erythrocyte suspension (2%, v/v) and 0.4 mL of MoLE (0.125, 0.25, 0.5, 1.0 or 2.0 mg/mL) resuspended in saline solution. For controls, saline solution (negative) and 0.0025% (w/v) saponin (positive) were used. After 1 h of incubation, centrifugation ensued and the absorbance at 540 nm of the supernatant was recorded. Hemolytic activity was expressed as a percentage of hemolysis using the following formula:

$$\text{Hemolytic Activity (\%)} = (X - Y) \times 100/(Z - Y)$$

where X = sample absorbance; Y = absorbance of the negative control; and Z = absorbance of the positive control.

2.8. In Vivo Assays

2.8.1. Animals

Female (n = 12) and male (n = 60) Swiss mice (90 days, ca. 30 g) were obtained from the vivarium of the *Instituto Keizo Asami* (iLIKA) of UFPE. All animals were maintained under a controlled temperature (22 ± 2 °C), with standard food and water ad libitum, in polypropylene boxes with a maximum of 10 animals, with a 12 h scotophase. For oral administration, 1 mL syringes and an oral cannula (0.1 cm × 4 cm) were used. At the end of

the experiments, all animals were euthanized through an overdose of ketamine (300 mg/kg i.p.; Sigma-Aldrich) and xylazine (30 mg/kg i.p.; Sigma-Aldrich). The procedures were carried out only after the approval of the Ethics Committee on the Use of Animals from UFPE (process no. 0018/2021).

2.8.2. Acute Oral Toxicity

Groups of female mice (n = 3 per group) were randomly separated and received the following treatments *per os*: MoLE (2000 mg/kg diluted in 0.15 M NaCl) or 0.15 M NaCl (control), following the recommendations of the Organization for Economic Cooperation and Development [29]. Two independent assays were performed, totaling n = 6 per treatment. Immediately and for up to 4 h, mice were individually observed for the presence of stimulating and depressing signals in the central nervous system. Subsequently, the animals were allocated to their respective groups and their food consumption, water, and body weight were monitored daily for 14 days [29]. At the end of the test (15th day), the animals (fasted for 6 h) were anesthetized with a solution of ketamine (100 mg/kg i.p.) and xylazine (10 mg/kg i.p.) to collect blood from the inferior vena cava for the analysis of hematological and biochemical parameters. Part of the blood was homogenized in a tube containing anticoagulant (ethylenediamine tetra-acetic acid, EDTA) and subjected to an automatic analyzer for the evaluation of the following hematological parameters: total erythrocytes, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, total and segmented leukocytes, lymphocytes, and monocytes. Another part of the blood was transferred to a tube containing a separator gel and subjected to centrifugation (452× g for 10 min) to obtain the serum, which was evaluated for the following: albumin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma-glutamyl transferase, total protein, creatinine, bilirubin, total cholesterol, and triglycerides. All tests were conducted using kits (Labtest Diagnóstica S.A., Lagoa Santa, Brazil) according to the manufacturer's instructions.

After blood collection, exploratory laparotomy was performed, followed by the collection of the kidneys, liver, and spleen. The organs were washed with distilled water, fixed in 10% buffered formalin for 48 h, and then dehydrated in ethanol (70%, 80%, 90% and 100%). After this step, they were enclosed in paraffin, cut (5 µm) using a RM 2035 microtome (Leica, Wetzlar, Germany) and mounted on slides. The sections were stained with hematoxylin and eosin, covered with a coverslip, and fixed in balm. The slides were checked, along their entire length, with a BX-49 optical microscope (Olympus, Tokyo, Japan).

2.8.3. Measurement of Antioxidant Enzyme Levels

For the measurement of antioxidant enzyme levels, liver homogenates (10% w/v) were prepared by grinding the organ fragment in 50 mM Tris-HCl pH 7.4 containing 1 mM EDTA, 1 mM sodium orthovanadate, and 2 mM phenylmethanesulfonyl fluoride. The material was centrifuged at 2500× g for 10 min at 4 °C. The supernatant was collected and used to determine the levels of thiobarbituric acid reactive substances (TBARS) and to estimate the activities of superoxide dismutase (SOD) and catalase (CAT). Total protein content was estimated, as described by Bradford [30].

To determine the levels of TBARS, the reaction was developed by adding 100 µL of homogenate to 10 µL of 30% (w/v) trichloroacetic acid and 390 µL of 10 mM Tris-HCl pH 7.4. The mixture was centrifuged at 2500× g for 10 min. The supernatant was reassigned to another tube, and 0.8% (v/v) thiobarbituric acid (10 µL) was added in the sequence. The reaction was boiled in a water bath for 30 min, and after cooling, the absorbance of the organic phase was read at 535 nm. The results are expressed as nmol of malondialdehyde (MDA) per mg of protein [31].

The determination of total tissue SOD activity was performed by incubating (37 °C water bath) the homogenate (80 µL) with 880 µL of 0.05% (w/v) sodium carbonate (pH 10.2) containing 0.1 mM EDTA and 20 µL of 30 mM epinephrine in 0.05% (v/v) acetic acid. The kinetics of inhibition of epinephrine autoxidation were monitored by reading

the absorbance at 480 nm. One unit of SOD activity was defined as the amount of protein required to inhibit the autoxidation of 1 µmol of epinephrine per minute. The SOD activity was expressed as U/mg of protein.

To measure CAT activity, the homogenate (80 µL) was added to potassium phosphate-buffered solution (pH 7.4, 1 mL) and centrifuged at 3000 rpm for 15 min. In a quartz cuvette, 0.1 mL of the supernatant was added to 1 mL of 0.059 M hydrogen peroxide solution and 1.9 mL of distilled water. Enzyme activity was measured at 240 nm to monitor the change in absorbance between the first and sixth minutes [32]. One unit of CAT activity was defined as the amount of protein required to convert 1 µmol of H_2O_2 per minute into H_2O. Tissue CAT enzymatic activity was expressed as mU/mg of protein.

2.8.4. Evaluation of MoLE on Symptoms of Anxiety and Depression in Mice

Male mice were divided into five groups (n = 6 per group) for each neurobehavioral evaluation (anxiety or depression). MoLE was administered orally at doses of 500, 1000, and 2000 mg/kg, according to Barros et al. [33]. Diazepam (2 mg/kg, *per os*) and fluoxetine (10 mg/kg *per os*) were used as positive standards for anxiety and depression tests, respectively. The negative control received the vehicle (distilled water *per os*, 10 mL/kg). All drugs were diluted in distilled water. The elevated plus maze (EPM), forced swimming test (FST) and the tail suspension test (TST) were performed 1 h after treatment.

Elevated plus Maze (EPM) Test

A plus maze apparatus (Insight, Ribeirão Preto, Brazil), divided into two open arms and two closed arms, both elevated at a height of 40 cm above the floor, in a perpendicular position [34], was used for this test. Once placed on the central platform of the device, the animals were observed, and the following parameters were recorded for 5 min: number of entries into the open and closed arms; time spent in the open and closed arms [35].

A second set of experiments was carried out to evaluate the involvement of GABA/benzodiazepine receptors in the anxiolytic effect of MoLE. For this purpose, the animals were pre-treated with flumazenil (FMZ; 2.5 mg/kg i.p.; Sigma-Aldrich) 15 min before the administration of diazepam (2 mg/kg *per os*) or MoLE (2000 mg/kg *per os*). After 1 h, the animals were analyzed for 5 min for the same parameters described above.

Forced Swimming Test

Each mouse was individually placed in a plastic cylinder (10 cm in diameter and 24 cm high) filled with water up to a 15 cm height, at a temperature of 25 ± 1 °C and the duration of immobility events were measured for 6 min [36]. When the mouse floated or only made movements necessary to keep its head above the water it was considered immobile.

Tail Suspension Test

The mice, acoustically and visually isolated, were suspended at a height of 50 cm above the floor into an apparatus using adhesive tape placed at the end of their tails. The duration of immobility events was recorded for 6 min [37].

2.9. Statistical Analysis

The obtained data were analyzed using GraphPad Prism® version 8.0 (GraphPad Software Inc., La Jolla, CA, USA) and expressed as mean values ± mean standard error (SEM). Statistically significant differences were calculated using t test or one-way analysis of variance (ANOVA) followed by the Tukey test. Values were considered significantly different at $p < 0.05$.

3. Results

MoLE showed a protein concentration of 8.12 mg/mL and had lectin and trypsin inhibitor activities (Table 1).

Table 1. Hemagglutinating and trypsin inhibitor activities of *M. oleifera* leaf extract (MoLE).

	Protein (mg/mL)	HA (Titer^{-1})	SHA	TIA (U/mg)	STIA
MoLE	8.12	16	1.97	9.81	54.5

HA: Hemagglutinating activity. SHA: Specific hemagglutinating activity. TIA: Trypsin inhibitory activity. STIA: Specific trypsin inhibitory activity.

TLC analysis showed the presence of flavonoids in MoLE. The HPLC profile contained eight peaks (Figure 1a), confirming the presence of vitexin and rutin at retention times of 24.70 (peak 2) and 25.75 (peak 5) min, respectively (Figure 1b). The contents of rutin and vitexin were 0.27 and 0.20 g%, respectively. Figure 2 presents scan spectra of the other peaks, which also correspond to flavonoids.

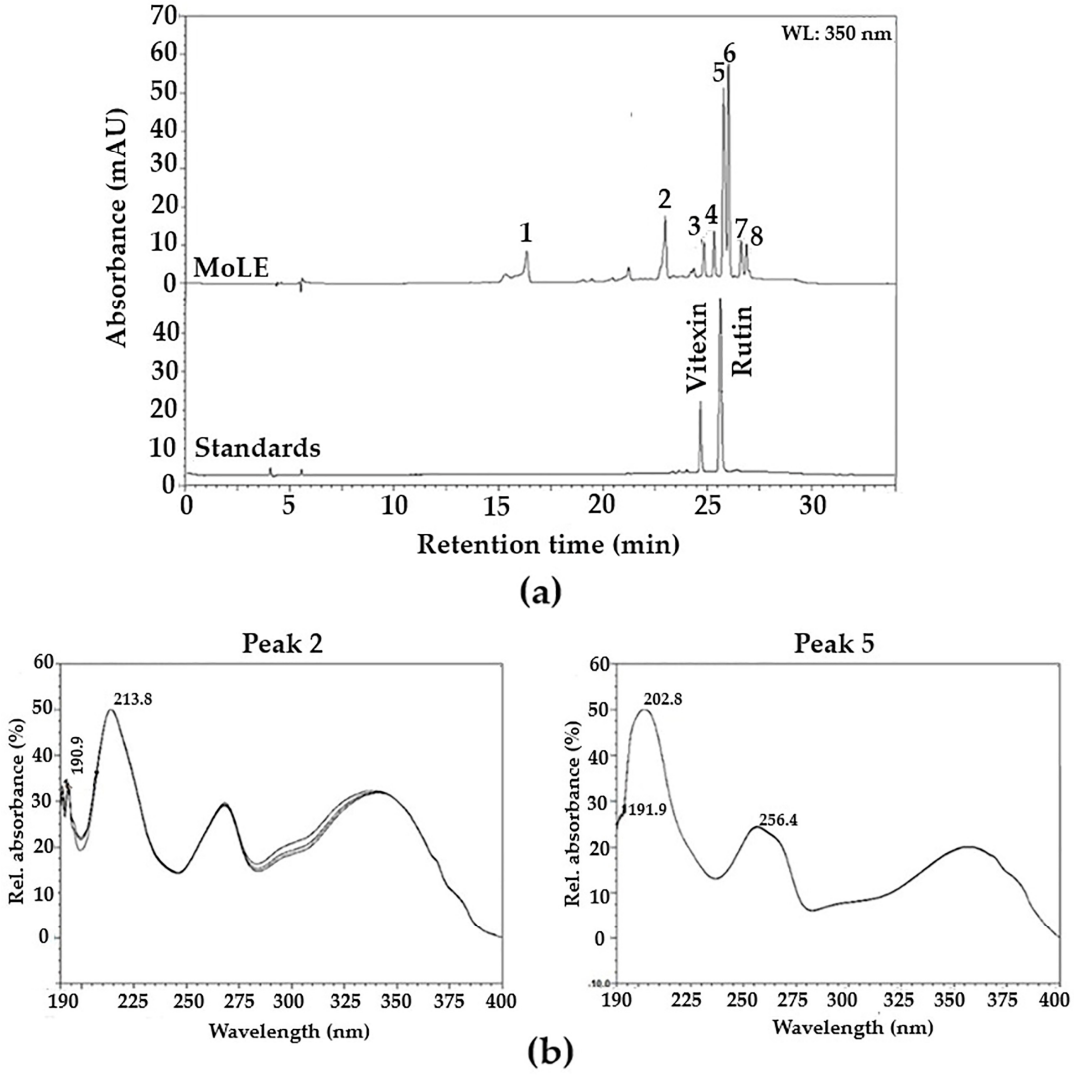


Figure 1. Chemical characterization of the *M. oleifera* leaf extract (MoLE). (**a**) High-performance liquid chromatography (HPLC) was performed for the standards vitexin and rutin and of MoLE at a wavelength of 350 nm. Eight peaks were indicated in the chromatogram. (**b**) Overlayed scan spectra of vitexin, rutin, and their corresponding peaks (2 and 5) in MoLE.

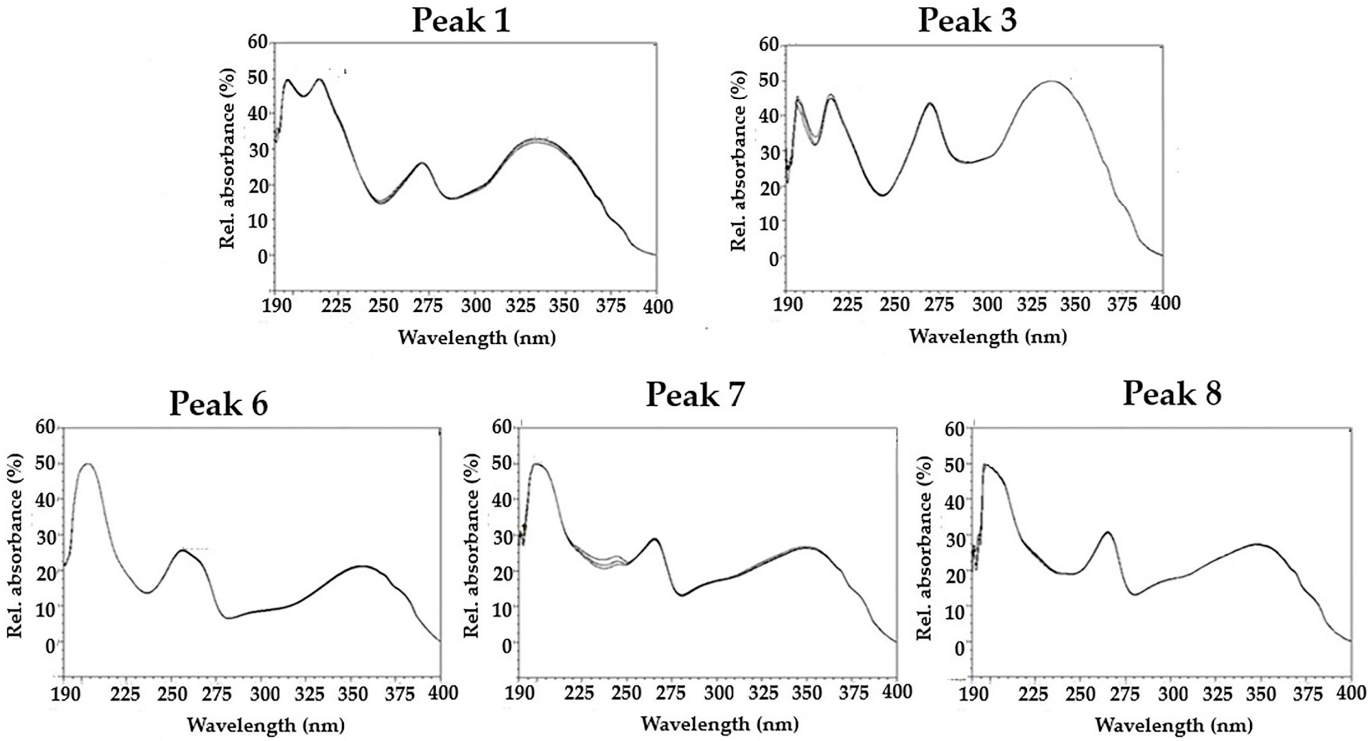


Figure 2. Scan spectra of the peaks 1, 3, 6, 7, and 8 (see Figure 1a) found in *M. oleifera* leaf extract (MoLE), which corresponded to flavonoids.

Flow cytometry analysis showed that MoLE was not cytotoxic to human PBMCs at all tested concentrations, with a cell viability greater than 98%. Additionally, MoLE did not cause hemolysis in human erythrocytes.

The administration of a single dose (2000 mg/kg *per os*) of MoLE did not result in mortality or behavioral changes in the initial four hours and during 14 days after administration. The animals also did not have significant changes in weight variation as well as water and food consumption (Table 2) when compared to the control group.

Table 2. Assessment of weight as well as food and water consumption of mice treated with pr without *M. oleifera* leaf extract (MoLE) at 2000 mg/kg p.o.

Treatment	Parameters		
	Weight (g)	**Food Consumption (g)**	**Water Consumption (mL)**
Control	36.45 ± 2.58	13.04 ± 1.23	25.11 ± 2.24
MoLE	37.33 ± 3.02	14.10 ± 1.10	24.16 ± 2.08

Values represent the mean ± SEM (n = 6 animals/group). No significant differences ($p > 0.05$) were found by t test.

Table 3 shows the hematological evaluation of animals treated with or without MoLE. The data revealed that erythrocyte and leukocyte parameters were similar in both groups. No significant differences were found in the serum levels of total cholesterol and triglycerides and in markers of liver and kidney function when MoLE-treated and control groups were compared (Table 4).

Figure 3 shows the histological analysis of the kidneys, spleen and liver of animals treated with MoLE and from controls, evidencing that morphological changes were not observed in the organs. Finally, Table 5 shows that there were no changes in TBARS levels and the activity of antioxidant enzymes in animals treated with MoLE when compared to control animals. Therefore, the administered dose of the saline extract was classified as safe. Then, for neurobehavioral tests, doses of 500, 1000 and 2000 mg/kg *per os* were used.

Table 3. Hematological parameters of mice treated or not with *M. oleifera* leaf extract (MoLE).

Parameter	Control	MoLE (2000 mg/kg)
Red cells (10^6/mm^3)	5.21 ± 0.58	5.74 ± 0.39
Hematocrit (%)	34.23 ± 2.09	35.69 ± 3.34
Hemoglobin (g/dL)	14.11 ± 0.26	14.10 ± 0.24
Mean corpuscular volume (%)	46.45 ± 3.98	43.70 ± 4.55
Mean corpuscular hemoglobin (%)	16.52 ± 1.31	16.34 ± 1.15
Mean corpuscular hemoglobin concentration (%)	36.10 ± 3.16	37.28 ± 3.22
Leukocytes (10^3/mm^3)	7.65 ± 0.56	7.34 ± 0.45
Segmented (%)	68.89 ± 4.66	72.25 ± 5.74
Lymphocytes (%)	27.94 ± 1.35	26.03 ± 1.74
Monocytes (%)	3.43 ± 0.30	3.33 ± 0.35
Basophils (%)	0.25 ± 0.05	0.20 ± 0.05
Eosinophils (%)	1.34 ± 0.19	1.38 ± 0.18

Values represent the mean ± SEM (n = 6/group). No differences ($p > 0.05$) found by t test.

Table 4. Biochemical parameters of mice treated or not with *M. oleifera* leaf extract (MoLE).

Parameter	Control	MoLE (2000 mg/kg)
Albumin (g/dL)	39.19 ± 3.76	38.75 ± 3.65
Alanine aminotransferase (U/L)	67.32 ± 4.51	68.57 ± 4.75
Aspartate aminotransferase (U/L)	89.10 ± 4.31	90.04 ± 5.79
Alkaline phosphatase (U/L)	13.24 ± 0.45	13.52 ± 0.45
Gamma-glutamyl transferase (U/L)	12.44 ± 0.41	12.17 ± 0.42
Total protein (g/dL)	70.26 ± 5.14	71.33 ± 5.48
Urea (mg/dL)	0.35 ± 0.04	0.34 ± 0.05
Creatinine (mg/dL)	4.57 ± 0.50	4.42 ± 0.39
Bilirubin (mg/dL)	0.42 ± 0.09	0.43 ± 0.09
Total cholesterol (mg/dL)	70.65 ± 6.34	74.84 ± 6.11
Triglycerides (mg/dL)	90.12 ± 7.53	92.35 ± 8.20

Values represent the mean ± SEM (n = 6/group). No differences ($p > 0.05$) found by t test.

Table 5. Assessment of oxidative stress in liver of mice treated or not with *M. oleifera* leaf extract (MoLE) at 2000 mg/kg p.o.

Treatment	Parameter		
	Malondialdehyde (nM/mg of Protein)	Superoxide Dismutase (U/mg of Protein)	Catalase (nM/mg of Protein)
Control	6.19 ± 0.52	10.12 ± 1.25	3.10 ± 0.15
MoLE	6.10 ± 0.43	10.27 ± 1.12	3.17 ± 0.26

Values represent the mean ± SEM (n = 6/group). No significant differences were found ($p > 0.05$) in the t test.

Figure 4 shows the data obtained from the elevated plus maze (EPM) test. The extract, at all doses, significantly increased the number of entries into the open arm compared to control groups (Figure 4a; $F_{4,25}$: 3.294, p: 0.0315), as well as the time spent in the open arms (Figure 4b; $F_{4,25}$: 10.13, p: 0.0001). It was also possible to observe a decrease in the number of entries into the closed arms in treatments with all doses of MoLE compared to the control group (Figure 4c; $F_{4,25}$: 7.300, p: 0.0009) and a decrease in the time spent in the closed arms (Figure 4d; $F_{4,25}$: 6.657, p: 0.0014). Diazepam also showed an anxiolytic effect (Figure 4).

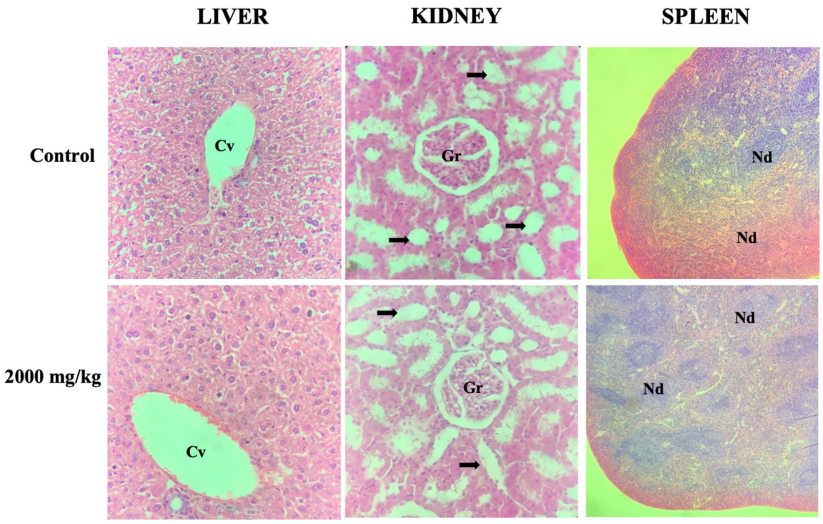


Figure 3. Representative photomicrographs of the livers, kidneys, and spleens of animals treated and not treated with *M. oleifera* leaf extract (MoLE). Livers: the centrilobular vein (Cv) is seen in all images with the presence of well-organized hepatocyte cords. Kidneys: Renal glomeruli (Gr) and the contorted tubes (arrows) are preserved in order. Bowman's intracapsular space is well defined and has a normal diameter. Spleen: Lymph nodes (Nd) are well defined in the control and treated groups. We can visualize the pulps of the organ with no hyperactivation and with well-defined contours. Hematoxylin and eosin staining was used. Magnification: liver and kidney (400×) and spleen (100×).

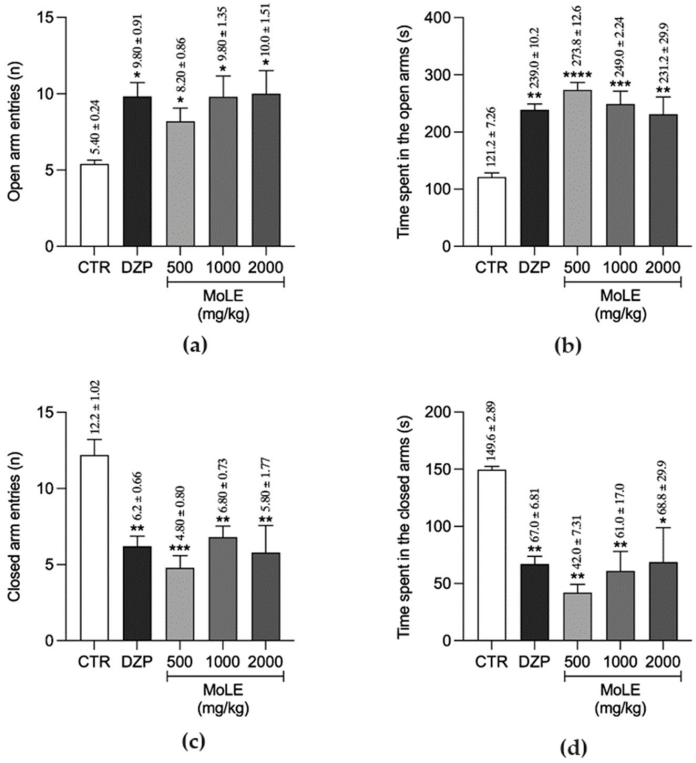


Figure 4. Acute effect of *M. oleifera* leaf extract (MoLE) in mice evaluated using the elevated plus maze (EPM) test. Mice were treated orally with a vehicle (control, CTR), diazepam (DZP, 3 mg/kg) and MoLE (500, 1000 and 2000 mg/kg). The values represent the mean ± SEM (n = 6) of the number of open arm entries (**a**), time spent in the open arms (**b**), number of closed arm entries, (**c**) and time spent in the closed arms (**d**) evaluated for 5 min. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ indicate significant differences between treatments according to ANOVA followed by Tukey's multiple comparisons test.

The participation of GABAergic receptors in the anxiety-like effect of MoLE was evaluated using the EPM test. The anxiolytic-like effect of MoLE was prevented by pre-treatment with flumazenil, as indicated by the data for time spent in the open arms (Figure 5a; $F_{4,25}$: 35.99, $p < 0.0001$) and in the closed arms (Figure 5b; $F_{4,25}$: 18.99, $p < 0.0001$).

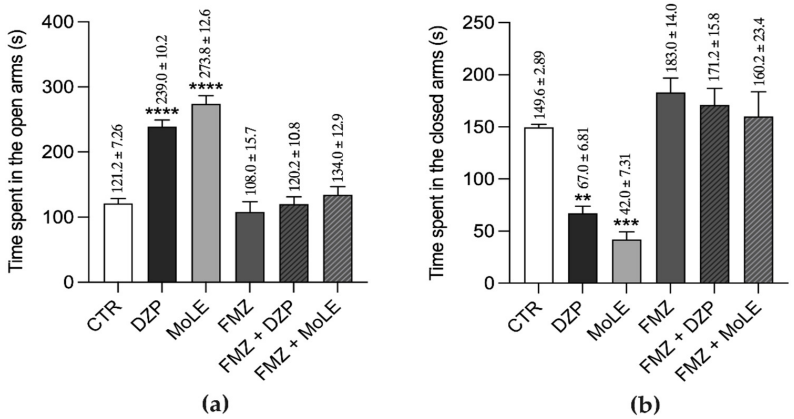


Figure 5. The participation of GABAergic receptors in the anxiety-like effect of *M. oleifera* leaf extract (MoLE) on mice submitted to the elevated plus maze (EPM) test. Mice were treated orally with vehicle (control, CTR), diazepam (DZP, 3 mg/kg), MoLE (2000 mg/kg), flumazenil (FMZ, 2.5 mg/kg) and diazepam (3 mg/kg) or MoLE (2000 mg/kg) after pretreatment with flumazenil (FMZ; 2.5 mg/kg) 15 min before the administration. The values represent the mean ± SEM (n = 6) of the time spent in the open arms (**a**) and time spent in the closed arms (**b**) in seconds for 5 min. ** $p < 0.01$, *** $p < 0.001$ or **** $p < 0.0001$ indicated significant differences between treatments according to ANOVA followed by Tukey's multiple comparisons test.

The data obtained in the tail suspension test (TST) and forced swimming test (FST) are presented in Figure 6. In the TST, animals treated with MoLE at all doses showed a reduction in immobility time in comparison with the control group (Figure 6a; $F_{4,25}$: 183.9, p <0.0001). Figure 6b shows the immobility time of the animals in the FST, where the groups treated with MoLE had a reduction in this parameter compared to the control ($F_{4,25}$: 8.282, p: 0.0004). Animals treated with fluoxetine showed a reduction in immobility time in both tests (Figure 6).

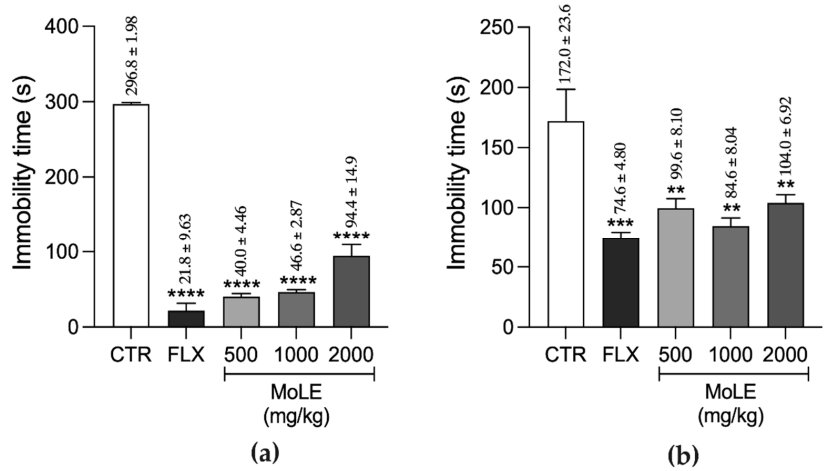


Figure 6. Acute effect of *M. oleifera* leaf extract (MoLE) in mice evaluated using the tail suspension test (**a**) and forced swimming test (**b**). Mice were treated orally with vehicle (control, CTR), fluoxetine (FLX, 10 mg/kg), and MoLE (500, 1000, and 2000 mg/kg). The values represent the mean ± SEM (n = 6) of the immobility time in seconds for 5 min. ** $p < 0.01$, *** $p < 0.001$, or **** $p < 0.0001$ indicated significant differences between treatments according to ANOVA followed by Tukey's multiple comparisons test.

4. Discussion

A phytochemical analysis of MoLE showed the presence of flavonoids, including rutin and vitexin. Flavonoids are secondary metabolites that have several biological activities, including effects on the central nervous system, such as anxiolytic, sedative, and ataxic activities [35,38–40]. In addition to flavonoids, the applied methodology was effective in solubilizing bioactive proteins such as lectin and trypsin inhibitor. The presence of bioactive proteins was not reported in a previous study on an aqueous extract of *M. oleifera* leaves that showed anxiolytic and antiepileptic effects [41].

Studies with PBMCs provide answers about the toxicity of potential new drug compounds in humans, especially their immune system. PBMCs are also critical tools for predictive studies that determine the dosage threshold of new drug compounds [42]. MoLE was not cytotoxic PBMCs or hemolytic to erythrocytes. Araújo et al. [43] showed that a water-soluble lectin from *M. oleifera* seeds (WSMoL) was not cytotoxic for PBMCs (IC_{50} > 100 μg/mL). The aqueous extract of *M. oleifera* leaves (IC_{50} > 500 μg/mL) did not show cytotoxicity to PBMCs [44]. The *M. oleifera* flower trypsin inhibitor, MoFTI, was not able to induce damage or interfere with cell proliferation in mouse splenocytes. Additionally, MoFTI did not cause hemolysis in murine erythrocytes [25].

After oral administration, xenobiotics can interact with different organs such as the esophagus, stomach, small intestine, and large intestine [45]. Each compartment presents distinct physiological parameters, such as pH, enzymes, pressure, and surface structure, which can increase toxicity during the digestive process [46]. Female mice orally administered with MoLE (2000 mg/kg) did not show weight loss or a decrease in water and food consumption and behavioral changes. MoLE did not cause any deaths until the end of the experiment. Madukwe et al. [47] evaluated the nutritional composition of the aqueous extract of the dry leaf of *M. oleifera* and showed elevated levels of crude fiber, proteins, carbohydrates, iron, and calcium, which usually have a positive impact on both human and animal health.

MoLE did not alter the hematological and biochemical profiles of the animals. Most in vivo toxicity research uses rodent models due to the similarity of their digestive and circulatory systems to those of humans, allowing researchers to accurately extrapolate the results of hematological and biochemical tests to humans and their correlations with xenobiotics [46]. The infusion and powder of *M. oleifera* leaves administered orally at concentrations of 2000 mg/kg and 5000 mg/kg once in 14 days were unable to alter hematology and biochemical markers [33]. Here, a single oral administration of 2000 mg/kg of MoLE was also considered safe.

Histological analysis revealed that animals treated with MoLE did not show changes in weight or macroscopic and microscopic morphology. According to Barros et al. [33], when applying the infusion or powder of the leaves at a concentration of 5000 mg/kg, it was neither able to cause changes in the histological pattern of the animals. Okumu et al. [48] showed that the aqueous–methanolic extract of *M. oleifera* leaf (1000 mg/kg) successfully protected toxin-induced liver injury.

MoLE did not promote oxidative stress in vivo. Some xenobiotics are capable of exerting adverse effects on the body and increasing the production of oxidizing compounds, such as reactive oxygen species (ROS), and reducing antioxidants, such as glutathione (GSH), leading to the oxidation of biomolecules causing tissue damage and, consequently, interfering with biological functions [49]. However, Gbadamosi et al. [50] analyzed the effect of an aqueous extract of *M. oleifera* leaves (200 mg/kg. p.o.; 28 days) on rats and showed the action of the plant material as a free radical scavenger in the central nervous system, reducing the effects of superoxide dismutase (SOD) and catalase (CAT) in the analyzed samples. Shousha et al. [51] stated that components of the *M. oleifera* plant are known for their ability to defend against free radicals. Fakurazi et al. [52] administered to animals extracts of leaves and flowers of *M. oleifera* at doses of 200 or 400 mg/kg and found significantly reduced levels of malondialdehyde. The set of results from the MoLE toxicity

assessment revealed that oral use at a dose of 2000 mg/kg was not toxic, making it possible to use it in behavioral assessment experiments.

The chemical constituents present in plants are targeted by the pharmaceutical industry due to their effectiveness in treating various diseases generally associated with few side effects [53]. MoLE contains primary and secondary constituents, which have promising properties for the treatment of illnesses linked to anxiety and depression, possibly due to their modulating role in the activity of neurotransmitters and their antioxidant activity [21,54]. Once the absence of toxicity of MoLE has been validated, we propose the investigation of its possible anxiolytic and antidepressant-like effects in mice.

The forced swim test (FST) and tail suspension test (TST) are the most applicable animal models for antidepressant screening. The present study showed that MoLE exhibited antidepressant capacity in both TST and FST and some of the compounds detected in the extract may be related to this activity. For example, rutin is a compound found in medicinal plants used to reduce anxiety, and a study showed that this flavonoid had anxiolytic and antidepressant effects in rats. The author also described that the effects were mediated by increased serotonin, norepinephrine, and dopamine levels in the cortical and hippocampal regions, and linked to antioxidant and vasodilator properties of rutin [40]. Vitexin is the main constituent responsible for the sedative activity of an aqueous extract from *Passiflora quadrangularis* [55] and has shown antidepressant effects in BALB/c mice mediated through serotonergic, noradrenergic, and dopaminergic receptors [56]. In addition, MoLE contains lectin, and this is interesting from the point of view of using the extract in studies that evaluate the effect on the nervous system. Sakaguchi et al. [57] related the use of lectins as specific ligands for receptors present in the central nervous system, showing promising results in experimental models and strategies aimed at repairing brain damage and neuroprotection against glutamatergic toxicity. Additionally, lectins also showed anxiolytic and antidepressant effects in mice [21,58].

Our data corroborate the study developed by Kaur et al. [59] who showed the antidepressant action of the ethanolic extract of *M. oleifera* (100 and 200 mg/kg) when administered both orally and combined with fluoxetine in mice tested using TST and FST, following acute or 14-day chronic treatments.

The anxiolytic-like activity of MoLE was assessed using the EPM test, known as a well-established experimental model for testing anxiolytic drugs [60]. This test verified an anxiolytic effect for MoLE at all doses and associated this with the effects of MoLE on γ-aminobutyric acid (GABA) receptors, possibly mediating the increase in the central inhibitory mechanism developed by this receptor in the central nervous system (CNS).

Low levels of GABA in the CNS are one of the neurological characteristics most commonly linked with anxiety disorders [61]. Several natural drugs present anxiolytic action by acting mimetically to GABA, improving GABAergic signaling [38,39]. Flumazenil is a $GABA_A$ receptor antagonist widely used in research to block these receptors and enable the verification of their involvement in the mechanism of action of new anxiolytic drugs. Once pre-treatment with flumazenil has been carried out, it is expected that if the drug under study presents an anxiolytic action via GABAergic receptors, its effect will be lost [62,63].

Bhat and Joy [64] also report that the ethanolic extract of the leaves of *M. oleifera* (200 mg/kg, i.p.) demonstrated anxiolytic activity in mice submitted to the EPM test. Ingale and Gandhi [41] evaluated the aqueous extracts of *M. oleifera* leaves and detected an anxiolytic effect at intraperitoneal doses of 250, 375, and 500 mg/kg on mice submitted to the EPM test, possibly mediated via of GABA mimetic action. Recently, the aqueous extract of *M. oleifera* leaves also caused an anxiolytic effect in mice orally treated (500 and 625 mg/kg) and tested in EPM test [65].

A large amount of flavonoids in *M. oleifera* leaves can interact with GABA receptors like a benzodiazepine molecule [54,65]. Additionally, evidence has suggested the possible binding of plant lectins to GABAergic receptors [66]. Together, both constituents of MoLE may be responsible for improving the inhibitory signaling of GABA receptors.

Although the GABA mimetic action of MoLE was found to be involved in anxiolytic actions; the mechanisms through which MoLE exhibited its antidepressant-like effect have not been explored in this study. However, the presence of some compounds of the extract was revealed, which is important for demonstrating aspects that can be addressed in future research. For example, the antidepressant-like effect of some secondary metabolites (e.g., flavonoids) and lectins occurs via noradrenergic, serotonergic, and dopaminergic pathways, being associated with an improvement in monoaminergic signaling [3,57]. Thus, the ability of MoLE to modulate monoaminergic signaling may be the object of investigations in the future. In addition, considering that *M. oleifera* leaves have been used for animal and human nutrition, the data described in the present paper could lead to translational studies regarding the use of MoLE in veterinary and human medicine.

5. Conclusions

The present study demonstrated that the saline extract of *M. oleifera* leaves (MoLE), containing secondary metabolites and proteins, did not present acute toxicity. MoLE has an anxiolytic-like effect in mice submitted to the elevated plus maze test, possibly mediated via GABA mimetic action. MoLE also presents an antidepressant-like action in mice evaluated in both forced swim test and tail suspension test. Thus, MoLE is a promising herbal medicine for treating anxiety and depression disorders.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

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