BioChem

MDPI

Article

Neurotransmitter Availability and Anti-Inflammatory and Antioxidant Effects of Subacute Administration of *Aloysia gratissima* (Gillies & Hook) Tronc. and Rutin in Female Wistar Rats

Gênifer Erminda Schreiner [1,*], Elizandra Gomes Schmitt [1], Gabriela Escalante Brittes [2], Laura Smolski dos Santos [1], Luana Tamires Maders [2], Itamar Luís Gonçalves [3], Sílvia Muller de Moura Sarmento [4], Nessana Dartora [5] and Vanusa Manfredini [1,2,4,*]

1 Pós-Graduate Program in Biochemistry, Federal University of Pampa, BR 472, Km 585, Uruguaiana 97508-000, Brazil; elizandraschmitt.aluno@unipampa.edu.br (E.G.S.); laura_smolski@hotmail.com (L.S.d.S.)
2 Pharmacy Course, Federal University of Pampa, BR 472, Km 585, Uruguaiana 97501-970, Brazil; gabibrites20@gmail.com (G.E.B.); luanamaders.aluno@unipampa.edu.br (L.T.M.)
3 Health Sciences Department, Regional Integrated University of Upper Uruguai and Missions, 7 of September Avenue, 1621, Erechim 99709-910, Brazil; itamar3141@yahoo.com.br
4 Multicentric Pós-Graduate Program in Physiological Sciences, Federal University of Pampa, BR 472, Km 585, Uruguaiana 97508-000, Brazil; silviasarmento.aluno@unipampa.edu.br
5 Biological Sciences Course, Federal University of South Frontier, Av. Jacob Reinaldo Haupenthal, 1580, Bairro São Pedro, Cerro Largo 97900-000, Brazil; nessa.dartora@gmail.com
* Correspondence: geniferschreiner.aluno@unipampa.edu.br (G.E.S.); vanusa@unipampa.edu.br (V.M.)

Abstract: Background: Levels of chronic inflammation, oxidative stress, and neurotransmitter availability are altered in depressed patients and can be used as biological markers. This study aimed to analyze these markers in female Wistar rats under chronic inflammation induced by *E. coli* lipopolysaccharide (LPS), treated with aqueous extract of *A. gratissima* and rutin, the major flavonoid of its extract. Methods: Thirty female Wistar rats under a chronic inflammatory regimen induced by 1 mg/kg i.p. of LPS were divided into six experimental groups: control (1), treated with fluoxetine 5 mg/kg (2), rutin at 50 mg/kg (3) or 100 mg/kg (4), aqueous extract of *A. gratissima* 100 mg/kg (5), and co-treatment with 50 mg/kg of extract and 10 mg/kg of rutin (6). Treatments were administered by gavage for 15 days. Results: Oxidative damage to proteins and lipids was lower in group 6 compared to group 2. Pro- and anti-inflammatory cytokines increased in group 1 but not in group 2, indicating a relationship with depression. Similar effects were observed in the treated groups, showing no significant differences from group 2. Neurotransmitter levels of dopamine and serotonin were low in group 1, and all treatments effectively increased them. Additionally, *A. gratissima* extract at 100 ppm increased locomotor activity in planarians. Conclusions: This study demonstrates the effectiveness of the LPS induction model in subacute experimental designs and the potential antidepressant effect of the treatments due to their antioxidant and anti-inflammatory properties, and ability to increase neurotransmitter levels.

Keywords: depression; flavonoid; medicinal plant

check for updates

Citation: Schreiner, G.E.; Schmitt, E.G.; Brittes, G.E.; dos Santos, L.S.; Maders, L.T.; Gonçalves, I.L.; de Moura Sarmento, S.M.; Dartora, N.; Manfredini, V. Neurotransmitter Availability and Anti-Inflammatory and Antioxidant Effects of Subacute Administration of *Aloysia gratissima* (Gillies & Hook) Tronc. and Rutin in Female Wistar Rats. *BioChem* **2024**, *4*, 252–267. https://doi.org/10.3390/biochem4030013

Academic Editor: Mihail Lucian Birsa

Received: 3 August 2024
Revised: 31 August 2024
Accepted: 9 September 2024
Published: 20 September 2024

Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

In recent years, as a result of the COVID-19 pandemic, cases of major depression, which were already high, increased 27.6%, rising from around 193 million cases in 2020 to 246 million in the following year. A bigger increase was noted in women, with a growth of 29.8% in the new cases number, while in men this increase was 24.0% [1], which follows the trend already exposed by the WHO (2017) in which females have higher rates of the disease [2].

Despite having already been the focus of much research, the pathophysiology of depression is still not fully elucidated. In the 1990s, Smith (1991) proposed a theory regarding the interaction of inflammatory processes with the disease, criticizing the model in which mental and physical diseases were separated and presenting evidence of the relationship between both aspects. Since then, there has been further progress in studies relating to both [3].

For example, a significant number of patients with major depression, bipolar depression, and schizophrenia exhibit low degrees of chronic inflammation. In fact, many of the inflammatory cytokines produced during chronic inflammation processes have cytotoxic and antineurogenic properties: they are able to affect brain development and functioning [4–10].

This interaction is explored in the laboratory to induce a depressive-like state in animals. A common way to do this is using an exogenous toxin, *E. coli* lipopolysaccharide (LPS), for example. It causes neuroinflammation by activation of microglial cells, which leads to chronically elevated production of pro-inflammatory mediators such as IL-1β, IL-6, and TNF-α, inducing depressive-like behavioral symptoms [8].

Another factor that is unregulated in cases of depression is the production of reactive oxygen species (ROS), the decrease of the body's antioxidant capacity and, consequently, the increase in oxidative stress levels. These can even be a cause of the inflammatory process [7]. The brain is a richly vascularized region, so it has a high oxygen consumption, is rich in easily oxidizable polyunsaturated lipids, and with a lag in antioxidant indices, it naturally ends up being a target for oxidative stress, being an important aggravating factor for depressive patients [7,11,12].

Dopamine and serotonin are neurotransmitters related to the mechanism of pleasure, reward and motivation [13,14], and the regulation of physiological processes such as sleep, appetite, sexual pleasure, behavior and mood [14], respectively. The deregulation of many of these aspects is a symptom in depressed patients, and can be seen as a reflection of their low availability in these individuals. Most treatments for depression have an action precisely on the reuptake of these neurotransmitters by the presynaptic neurons, increasing their levels [5,8,15,16].

Due to the difficulty of treatment, research on the subject remains relevant. Because of their abundant pharmacological properties, and being theoretically compounds with fewer toxic effects and easy to obtain, many research efforts focus on medicinal plants. The native plant *A. gratissima* is known for its anti-inflammatory [17,18], antioxidant [19,20] and neuroprotective [21] potential, which explains its antidepressant capacity in the acute form [21,22], and subchronic form [23] as well as its main flavonoid, rutin [24,25].

Therefore, the objective of the present paper was to evaluate the ability of subacute treatment using *A. gratissima* extract and the flavonoid rutin to improve oxidative and inflammatory parameters and the availability of neurotransmitters, parameters related to depression, in female Wistar rats submitted to an inflammation model, induced by LPS administration. Another goal of our study was to evaluate the effects of *A. gratissima* extract on planarians, an alternative animal model widely used in neuropharmacological research.

2. Methodology

2.1. Reagents

All chemicals used to perform the tests were of high analytical grade, 95% pure, and purchased from Sigma Chemical Corp® (St. Louis, MO, USA).

2.2. Plant Material

The collection of plant material was carried out in February 2021, in the city of Uruguaiana-RS, at coordinates 29°49′17.6″ S 57°05′59.8″ W. One exsiccates was prepared and can be found in the Bruno Edgar Irgang herbarium under registration number HBEI 1639 for future consultations. The leaves were separated and dried at room temperature in the shade; then, they were macerated and mixed with distilled water, in a ratio of 50 g of

leaves to 1 L of distilled water and stored in the refrigerator, in amber bottles, for seven days, mixing daily. Afterwards, the extract was filtered with cotton and lyophilized to obtain the pure extract, which was stored in a freezer at −20 °C until used, a technique adapted from Feksa et al. (2018) [26].

2.3. Phytochemical Analysis

The technique of high-performance liquid chromatography (HPLC) was used coupled with a diode array detector (DAD) and a C18 column, using an LC-2020 system (Shimadzu®), composed of an automatic sampler, a column oven and a binary pump, and the solvents used were water (solvent A) and acetonitrile (solvent B), both containing 0.1% and formic acid. The mobile phase was formed by different concentrations of both solvents. First, the concentration of solvent B was increased from 0 to 35% over 10 min, then increased to 100% from 10 to 15 min, then maintained for another 3 min and returned to the initial condition (100% A) in 18 min. The flow used was 600 µL/min and the column was re-equilibrated for 3 min before each injection [27]. Samples were prepared using MeOH-H_2O (1 mg/mL) and subsequently injected into the column.

2.4. Antioxidant Capacity of the Extract

The in vitro antioxidant capacity of the extract was quantified by evaluating the discoloration produced by the inhibition of the ABTS*+ cation [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)]. For this, the ABTS*+ radical was previously produced for the reaction between ABTS 7 mM and potassium persulfate 140 mM, which produces a dark blue chromophore. The antioxidant capacity of our compound was directly proportional to its ability to reduce ABTS*+ to its stable ABTS form, which is colorless. For the test, 1 mL of a diluted ABTS 42.77 µM solution was added to 10 µL of the diluted sample, incubated for 6 min in the dark, with the difference in shade being measured using a spectrophotometer, model SP-22, single beam (Biospectro), at a wavelength of 734 nm. The result was expressed by the 50% radical inhibition capacity (IC_{50}), which was calculated from the equation of the straight line, produced with six different concentrations of the compound, being 5, 2.5, 2, 1, 0.5 and 0.25 mg/mL [28].

Another method used to evaluate the antioxidant potential of the in vitro extract was the scavenging of the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical. For the test, an aliquot of 1 mL of DPPH 50.5 µM, solubilized in methanol, was added to 10 µL of the sample at the desired dilution, incubated in the dark for 30 min and read in a spectrophotometer (SP-22 single beam from Biospectro), at 517 nm. The result was also expressed in IC_{50}, based on use of five different concentrations of the compound (5, 2.5, 2, 1 and 0.5 mg/mL) [29]. All reagents used in the tests were prepared on the day the test was carried out, except for the stock solution of ABTS 0.3763 mM, which was prepared 16 h before in order to allow the formation of the ABTS*+ radical. The procedures were carried out in the dark and the flasks used were covered with aluminum foil to reduce contact with light [28].

2.5. In Vivo Activity in Rats

This study was approved by the Unipampa Animal Ethics Committee (CEUA-UNIPAMPA), under protocol number 021/2021. For the experiment, 36 adult female Wistar rats, aged 8 weeks, were purchased from the Biopampa bioterium, from Unipampa, where they were also kept, receiving commercial food and water on demand, with a controlled temperature of 21 ± 1 °C, and a 12 h light/dark cycle for 15 days for acclimatization.

The inflammatory state was induced by an intraperitoneal (i.p.) injection of LPS Sigma®, at a dose of 1 mg/kg. After 24 h of observation, they were randomly separated into 6 groups with 6 animals in each, differing in terms of the treatment, which was administered by gavage daily, for 15 consecutive days, configured as a subacute administration (CONCEA, 2016). The experimental groups formed were: (1) control group, treated with 0.9% saline solution; (2) positive control group, treated with fluoxetine at a dose of 5 mg/kg; (3) group treated with rutin at a concentration of 50 mg/kg; (4) group treated with rutin at

a dose of 100 mg/kg; (5) group treated with aqueous extract of *A. gratissima* leaves at a dose of 100 mg/kg; and (6) group treated with a co-treatment of both compounds, with a dose of 50 mg/kg of *A. gratissima* extract and 10 mg/kg of rutin. The choice of administered doses of *A. gratissima* extract [23] and rutin [30,31] were based on previous studies. All treated compounds were diluted in distilled water before administration, so the total ingestion volume did not exceed the recommended 1 mL. The schematic design of the development of the experiment is shown in Figure 1.

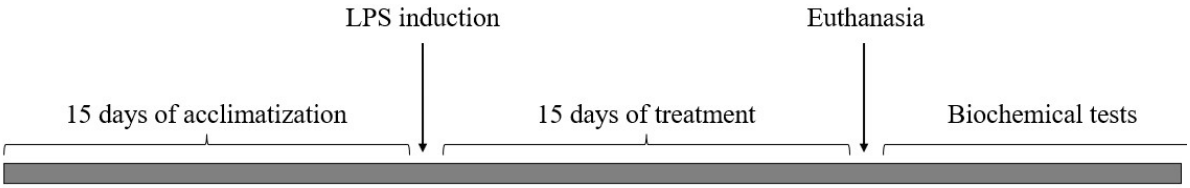


Figure 1. Experimental design, including 15 days for acclimatization, the induction of chronic inflammation by the administration of LPS, followed by 15 days of treatment, euthanasia and, finally, the performance of laboratory tests.

At the end of the 15 days of treatment, the animals were anesthetized with Xylazine® (10 µ/kg) and Ketamine® (30 µ/kg) i.p., followed by cardiac puncture. The collected blood sample was placed in one EDTA tube and one without anticoagulant for analyses of oxidative stress and inflammatory parameters, respectively. The brain mass was quickly and carefully removed and the hippocampal region separated, being stored at −80 °C until it was transported in liquid nitrogen to the Hospital de Clínicas de Porto Alegre, where analyses of inflammatory parameters and the availability of neurotransmitters was carried out.

2.5.1. Determination of Carbonyl Groups

To quantify the protein carbonylation, the carbonyl technique described by Levine (1990) was performed. For this test, 100 µL of trichloroacetic acid (28%) was added to the same amount of a sample of plasma, causing the proteins present in the sample to precipitate. Their breaking up and marking was performed using 1 mL of DNPH or 3% HCl, for the blank sample, which was subtracted from the sample value at the end of the reading. Afterwards, the mixture was incubated for 90 min at 37 °C. Then, the interferents were washed using 1 mL of ethanol-ethyl acetate (1:1 v/v) and the proteins were resuspended using 200 µL of guanidine hydrochloride (6 M), prepared using 20 mM PBS buffer. The reading was performed with a SpectraMax M5 microplate reader (Molecular Devices®, Sunnyvale, CA, USA) at 370 nm [32].

2.5.2. Determination of Thiobarbituric Acid Reactive Species (TBARS)

Lipid peroxidation levels were measured using the TBARS method [33]. For this, 200 µL of sample was conditioned with 1 mL of orthophosphoric acid (0.2 M), 500 µL of distilled water and 250 µL of trichloroacetic acid reagent (0.1 mol/L) for 120 min at a temperature of 95 °C. After cooling the sample, the reading was taken in a spectrophotometer, model SP-22, single beam (Biospectro, Shimadzu®, Kyoto, Japan) at 532 nm.

2.5.3. Determination of Ascorbic Acid

Vitamin C was determined by HPLC following the methodology of Karatepe (2004) [34]. One hundred microliters of 0.1 M perchloric acid and 1 mL of distilled water were added to a 100 mL aliquot portion of plasma and acid was then added. The samples were centrifugated at 4.500 rpm for 5 min and used for HPLC analysis. The mobile phase was monobasic potassium phosphate (pH 3.6, 30 mM): methanol 82.5:17.5 (v/v) while the flow rate was 1.2 mL/min. A C18 reverse phase column was used and the chromatograms were monitored at 250 nm. The concentrations were calculated according to external standardization.

2.5.4. Determination of Total Antioxidant Status (TAS)

TAS was quantified in the serum samples using a commercial kit produced by Randox, according to the manufacturer's instructions. The absorbance was read using a spectrophotometer at 600 nm.

2.5.5. Determination of Phenolic Compounds

The serum sample was diluted with Milli-Q water in a proportion of 1:100. An aliquot of 125 µL was mixed with the same volume of Folin–Ciocalteu reagent, 1.5 mL of Milli-Q water and 1.25 mL of 7% sodium carbonate. The mixture was incubated for 90 min at room temperature, and then the absorbance was read at 760 nm using a spectrometer (model SP-22, Biospectro) [35].

2.5.6. Inflammatory Assays

Inflammatory profiles from samples of hippocampus tissue were quantified by the determination of IL-1β, IL-6, IL-8, IL-10, TNF-α and CRP-us using kits from Thermo Fisher Scientific®. The analyses were performed by the ELISA method using 50 or 100 µL aliquots of the samples.

2.5.7. Dopamine and Serotonin Levels Measurement

The hippocampal dopamine (DA) levels were determined by high-performance liquid chromatography (HPLC, Shimadzu®, Kyoto, Japan) [36]. Homogenates prepared from the hippocampi were measured using a reverse-phase HPLC system. The rats' hippocampus was quickly removed in an iced surface and homogenized in 50 mM TrisHCl, pH 7.4 (1/10, *w*/*v*). Posteriorly, samples were centrifuged at 2000× *g* for 15 min, and supernatants were stored at −20 °C until use. To separate DA, we used the programming isocratic with a flow rate of 0.8 mL/min. The samples were filtered through 0.22 µm syringe filters; then, we injected 70 µL samples into the HPLC system using an auto sampler device (YL9150). The detection was at 198 nm by DAD. Chromatograms were recorded and integrated by PC integration software (YL Clarity 8.6.1.). The analytical parameters were as follows: linear range, 0.1–10.0 µg/mL; determination coefficient, 0.999; and calibration equation, y = 625.11x − 34.342. Dopamine for HPLC was supplied by Sigma-Aldrich Brazil. All analyses were run in triplicate. Serotonin neurotransmitter levels from samples of hippocampus tissue were measured using Leac® (Santana, SP, Brazil) kit. The assays were performed according to the manufacturer's recommendations and quantifications were performed by the ELISA method (Fully Automated Elisa Analyzer- MCL-5033A, Guangzhou, China) using 50 µL aliquots of the samples. All samples were run in triplicate.

2.6. In Vivo Activity in Planarians

Planarians of the *Girardia tigrina* species were used for the behavioral evaluation. The planarians were kept in water for planarian cultivation, composed of deionized water with ion replenishment provided by commercially available inorganic salts, and maintained at 23 °C. The planarians were exposed to 100 ppm of *A. gratissima* extract and were monitored during the first 5 min of exposure via video recording. The videos in AVI format were uploaded to the ToxTrack 2.98 software [37], and calibration was performed using rulers positioned orthogonally. After calibration and arena definition, the animals were tracked, and the parameters of velocity, distance, exploration rate, mobility rate, and acceleration were measured.

2.7. Statistical Analysis

The results were expressed as mean ± standard deviation and the normality was investigated using the Shapiro–Wilk test. The means were compared among the experimental groups using variance analysis followed by the Tukey test or *t*-test. To allow better understanding of the dataset structure, principal component analysis was performed. All the analyses were realized using the GraphPad Prism 9.2 software.

3. Results

3.1. Phytochemical Analysis

The phytochemical composition of *A. gratissima* was determined using liquid chromatography coupled to a mass spectrometer. From the chromatogram obtained (Figure 2), and comparing the results with standards and data from the literature, it was possible to identify 25 compounds in the aqueous extract of the leaves of the *A. gratissima*, namely gallic acid, protocatechuic acid, theobromine, chlorogenic acid, benzoic acid, arenarioside, verbascoside, hofmaniacetone, rutin, p-coumaric acid, ferulic acid, kaempferol/luteolin diglycoside, hydroxymethoxyluteolin, hydroxy-dimethoxypigenin, luteolin, genkwanin, guaiol, bisabolol, spathulenol, zeaxanthin, α-amyrin, lupeol acetate, betulin, ursolic and oleanoic acid (Table 1).

Table 1. Putative identification of the compounds observed in *Aloysia gratissima* leaves obtained from LC-MS analysis.

Peak	Rt (min)	$[M-H]^-$ (*m*/*z*)	Compounds	Identification
1	4.400	-	n.i.	-
2	4.501	-	n.i.	-
3	4.692	-	n.i.	-
4	5.009	-	n.i.	-
5	5.147	-	n.i.	-
6	5.318	169	Gallic acid	analytical standard
7	5.991	153	Protocatechuic acid	[18,20]
8	6.452	179	Teobromine	analytical standard
9	6.683	-	n.i.	-
10	6.855	-	n.i.	-
11	6.973	-	n.i.	-
12	7.060	179	n.i.	-
13	7.362	-	n.i.	-
14	7.758	-	n.i.	-
15	8.127	353	Chlorogenic acid	analytical standard
16	8.503	-	n.i.	-
17	8.688	121	Benzoic acid	analytical standard
18	8.891	755	Arenarioside	[18,20]
19	9.096	623	Verbascoside	[18,20]
20	9.542	319	Hoffmaniacetone	[18,20]
21	10.269	609	Rutin	analytical standard
22	10.520	163	*p*-Coumaric acid	analytical standard
23	11.008	193	Ferulic acid	analytical standard
24	11.328	609	Kaempferol/luteolin diglicoside	MS^2
25	11.640	311	Hydroxy-metoxyluteolin	[18,20]
26	11.798	301	Hidroxy-dimethoxyapigenin	[18,20]
27	13.224	-	n.i.	-
28	13.583	285	Luteolin	[18,20]
29	14.468	283	Genkwanin	[18,20]
30	14.913	221	Guayol	[18,20]
31	15.037	283	Bisabolol	[18,20]
32	15.210	219	Sphathulenol	[18,20]
33	15.499	567	Zeaxanthin	[18,20]
34	15.691	425	α-Amyrin	[18,20]
35	15.890	467	Lupeol acetate	analytical standard
36	16.173	441	Betuline	analytical standard
37	17.962	455	Ursolic acid	analytical standard
		455	Oleanolic acid	analytical standard

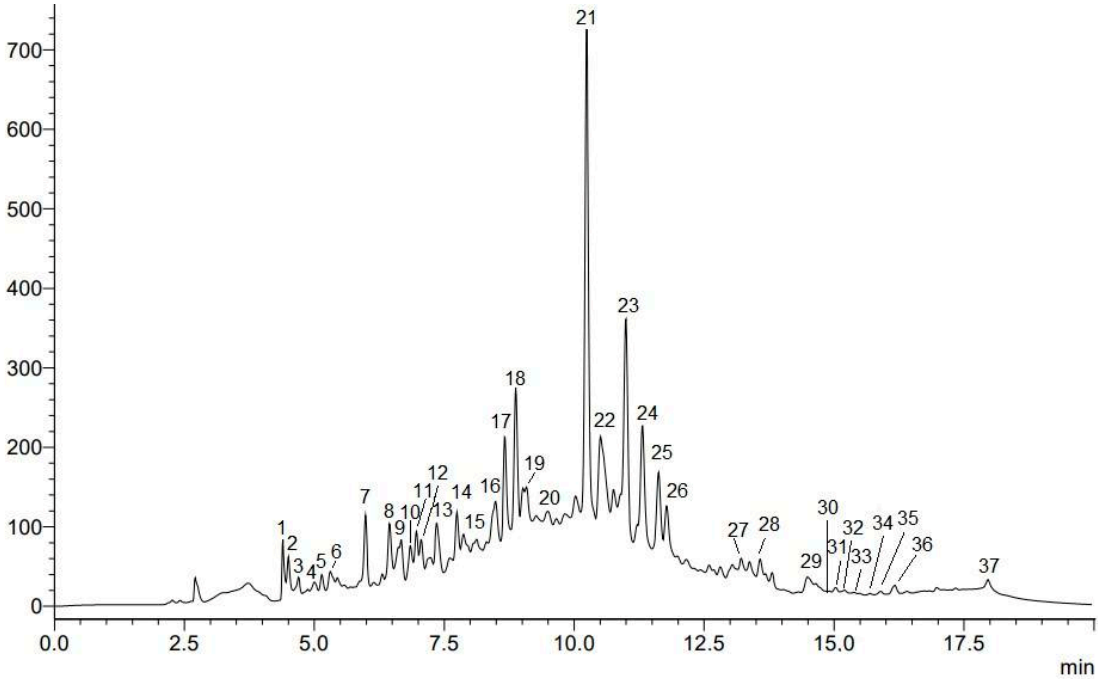


Figure 2. Chromatogram obtained from the analysis of the aqueous extract of the leaves of *Aloysia gratissima* in HPLC.

3.2. Antioxidant Capacity of the Extract

As can be seen in Figure 3a, in the ABTS technique, from reading the different concentrations of the extract of A. gratissima, a straight line was constructed with $r^2 = 0.9900$ and equation $y = 16.752x + 3.11$, making it possible to calculate the inhibition of 50% of the radical (IC_{50}), which was 0.279 mg/mL, and the percentage of inhibition ranged between 85.76% and 5.42%. Similarly, for DPPH, as shown in Figure 3b, the calculated IC_{50} value was 0.781 mg/mL, obtained from the equation $y = 6.3468x + 0.421$, with $r^2 = 0.9955$, and the percentage of inhibition ranged between 31.92% and 3.58%.

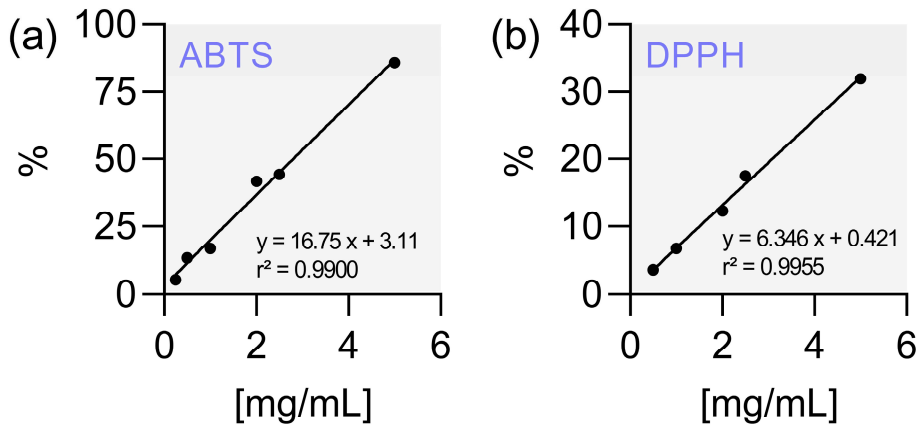


Figure 3. In vitro antioxidant assessment (**a**) showing the ABTS scavenging activity against the extract concentration, and (**b**) the DPPH scavenging activity against the extract concentration.

3.3. Oxidative Stress Studies in Rat Plasma

Figure 4 shows the levels of lipid peroxidation (a), protein carbonylation (b), and markers of antioxidant status (c, d, e) in the plasma of female Wistar rats with chronic inflammation induced by LPS. A significant decrease in the lipid peroxidation index was observed in group 3, treated with rutin 50 mg/kg ($p = 0.0113$), and in group 5, treated with *A. gratissima* extract ($p = 0.0079$), compared to group 1. Compared to fluoxetine (group 2), all

groups showed some level of decrease in lipid peroxidation. In the carbonyl test (Figure 4b), no statistically significant changes were observed in the treatments compared to the control (group 1), although fluoxetine (group 2) caused a slight increase in protein carbonylation. Treatments with rutin 100 mg/kg in group 4 ($p = 0.0296$) and the extract in group 6 ($p < 0.001$) mitigated oxidative damage to proteins compared to group 2. Regarding the levels of TAS (Figure 4c), ascorbic acid (Figure 4d) and phenols (Figure 4e), all treatments with rutin or *A. gratissima* extract, alone or combined, resulted in an increase in these parameters. Consistent with our results, fluoxetine treatment did not produce changes in the levels of TAS, ascorbic acid and phenols compared to group 1.

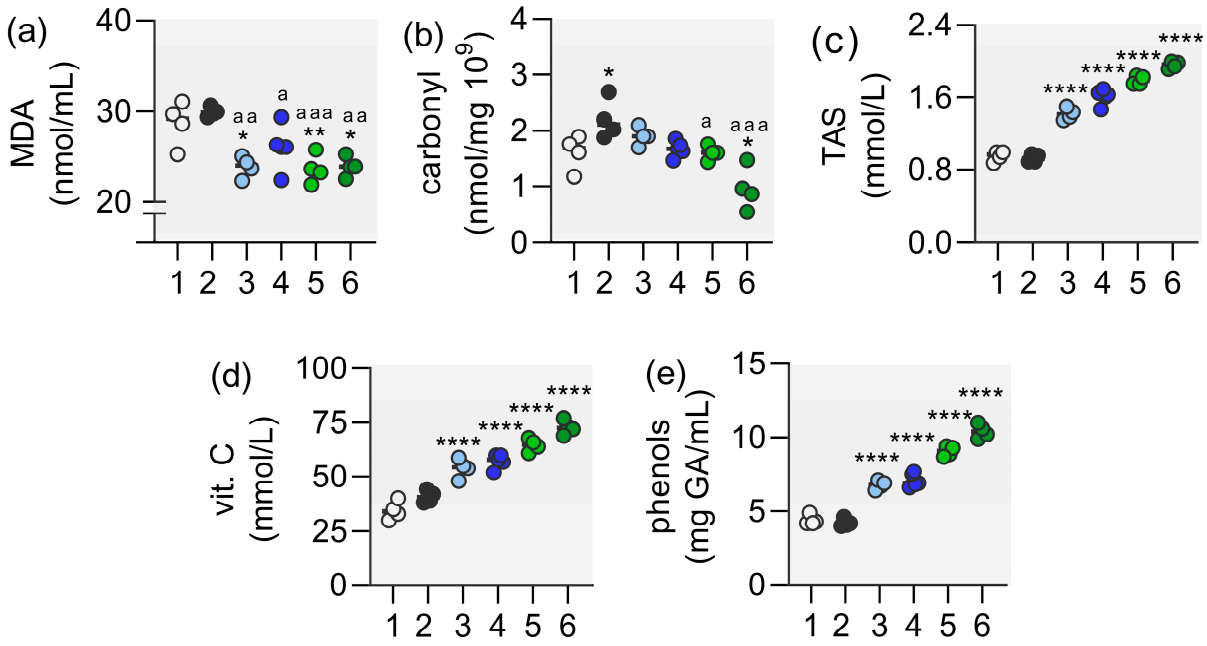


Figure 4. Plasma oxidative stress markers in rats with LPS-induced inflammation across different experimental groups. (**a**) TBARS, (**b**) carbonyl, (**c**) TAS, (**d**) ascorbic acid and (**e**) total phenol levels in the plasma of female rats exposed to LPS and treated. (1) Control group; (2) fluoxetine group; (3) rutin 50 mg/kg group; (4) rutin 100 mg/kg group; (5) *A. gratissima* 100 mg/kg group and (6) *A. gratissima* 50 mg/kg + rutin 10 mg/kg group. The values are expressed as mean ± standard deviation, and ANOVA followed by Tukey's test was performed. **** $p < 0.0001$ and * $p < 0.05$ compared to group (1); aaa $p < 0.001$, aa $p < 0.01$, and a $p < 0.05$ compared to group (2). TAS: total antioxidant status; GA: gallic acid.

3.4. Inflammatory Cytokines in the Rat Hippocampus

Figure 5 shows the levels of inflammatory cytokines quantified in the hippocampus of animals; high levels of all can be observed in animals that received LPS endotoxin and were treated only with saline solution (group 1), showing that a chronic infection was established, remaining relevant even after two weeks of contact with the LPS. The treatment with the commercial antidepressant, fluoxetine (group 2), was effective in significantly decreasing the levels of all pro-inflammatory cytokines (Figure 5a–c,e,f), and increase the anti-inflammatory IL-10 (Figure 5d), proving to be a positive control ($p < 0.0001$). The other treatments tested obtained the same result, not differing statistically from the positive control in most cases, except for IL-1β, a parameter in which some treatments had less anti-inflammatory activity; namely, group 3 (rutin 50 mg/kg), with $p = 0.0207$, group 4 (rutin 100 mg/kg), with $p = 0.0150$, and group 5 (*A. gratissima* 100 mg/kg), with $p = 0.0207$. It should be highlighted here that only group 5 showed no significant difference compared to fluoxetine (group 2).

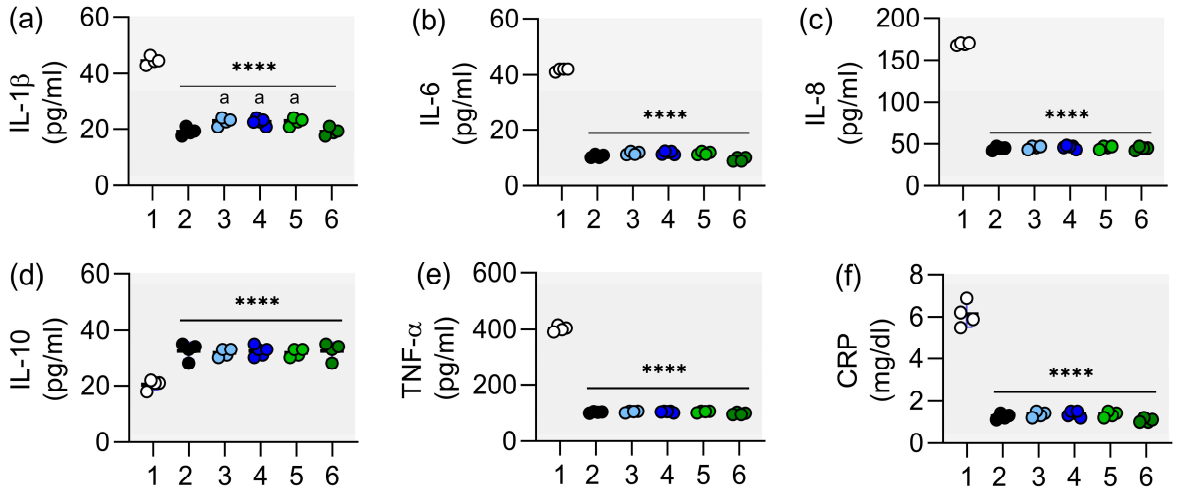


Figure 5. Levels of inflammatory markers in the rat hippocampus. (**a**) IL-1β, (**b**) IL-6, (**c**) IL-8, (**d**) IL-10, (**e**) TNF-α and (**f**) C-reactive protein. The numbers used are as follows: (1) control group, (2) fluoxetine group, (3) rutin 50 mg/kg group, (4) rutin 100 mg/kg group, (5) *A. gratissima* extract 100 mg/kg group and (6) combination of extract 50 mg/kg with rutin 10 mg/kg group. **** denotes $p < 0.00001$ in comparison to the control group. a $p < 0.05$ if compared to group 2.

3.5. Quantification of Neurotransmitters in the Rat Hippocampus

Figure 6 shows the levels of dopamine and serotonin present in the hippocampus of animals. It can be noted that group 1 (control), which received only saline solution as treatment, presented neurotransmitters at significantly lower levels than those found in the fluoxetine group (2), with $p < 0.0001$, as well as in the tested treatments. Even if able to increase the levels of neurotransmitters, the treatments continued to be significantly lower than for the fluoxetine group (2), both for serotonin and dopamine levels ($p < 0.0001$), except for group 6, whose significance difference was lower, both for dopamine ($p = 0.0029$) and for serotonin ($p = 0.0008$).

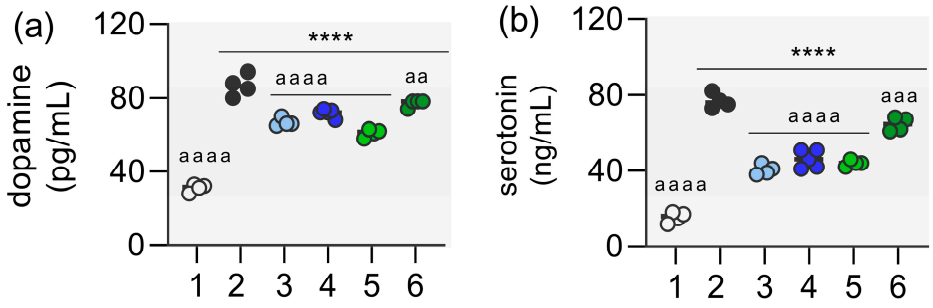


Figure 6. Neurotransmitter dopamine (**a**) and serotonin (**b**) levels in the hippocampus of female rats exposed to LPS, untreated and treated. (1) Control group; (2) fluoxetine group; (3) rutin 50 mg/kg group; (4) rutin 100 mg/kg group; (5) *A. gratissima* 100 mg/kg group and (6) *A. gratissima* 50 mg/kg + rutin 10 mg/kg group. The values are expressed as mean ± standard deviation and was performed ANOVA followed by Tukey test. **** $p < 0.0001$ if compared to group (1); aaaa $p < 0.0001$ if compared to group (2); aaa $p < 0.001$ if compared to group 2; aa $p < 0.01$ if compared to group 2.

3.6. Principal Component Analysis

Aiming to summarize our results, we performed a principal component analysis with the data obtained from serum and hippocampus rats. The plot depicted in Figure 7a shows four well-defined groups produced by the PC1 and the PC2 values. Low PC1 values were obtained for the control group, due to their high inflammatory markers (C-RP, IL-1β, IL-6, IL-8 and TNF-α), yielding separation along the PC1 axis. All groups except the control group were positioned on the right side of the plot. These groups were characterized

by an improved antioxidant balance (indicated by vectors TAC, PF and vitamin C), an inflammatory profile (IL-10), and neurotransmitter levels (dopamine and serotonin).

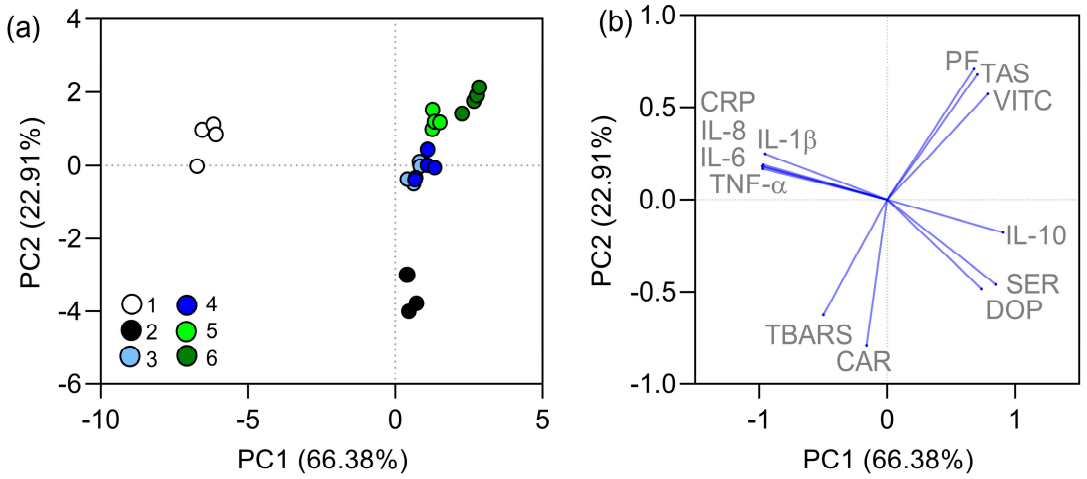


Figure 7. PCA analysis of serum and hippocampus parameters in rats. (**a**) PC1 vs. PC2 scatter plot showing the distribution of the rats based on their PC1 and PC2 values. Each point represents an individual rat, and the positioning reflects its similarities and differences. (**b**) Vector plots showing the vectors of the variables that influence the clustering of the rats. Each vector represents a different parameter measured in the serum and hippocampus. The direction and length of the vectors indicate the contribution and impact of each parameter on the principal components. Parameters with longer vectors have a greater influence on the differentiation of the rats via the principal components, while the direction of each vector shows how the parameter correlates with the principal components. (1) Control group, (2) fluoxetine group, (3) rutin 50 mg/kg group, (4) rutin 100 mg/kg group, (5) *A. gratissima* extract 50 mg/kg group and (6) combination of the extract with rutin 10 mg/kg group. Abbreviations: TAS: total antioxidant status; PF: total polyphenol; VITC: vitamin C; CAR: carbonyl protein; TBARS: thiobarbituric acid reactive substances; SER: serotonin; DOP: dopamine; CRP: C-reactive protein.

Separation along the y-axis was primarily driven by measurements related to oxidative stress. Group 2 (treated with fluoxetine) exhibited lower PC2 values, which were mainly attributed to the higher levels of hippocampal oxidative parameters. Supporting our analysis, the vector plot in Figure 7b demonstrates an inverse correlation between IL-10 and inflammatory markers, as the vectors are positioned in completely opposite regions. A similar pattern can be observed for antioxidant parameters (TAS, vitamin C, and phenols) compared to oxidative stress parameters (TBARS and carbonyl).

3.7. Activity of A. gratissima Extract on Planarians

Aiming to investigate the neuropharmacological *in vivo* effects of the *A. gratissima* extract, planarians were used as an alternative invertebrate animal model. The aqueous extract of *A. gratissima* at 100 ppm was able to produce changes in the behavior of the planarians during the first 5 min of exposure (Figure 8). It is worth highlighting that no toxicity was observed during the experiment, as the animals monitored after the experiment were still alive. The extract produced an increase in the velocity of the planarians from 0.7275 ± 0.1415 cm/s to 1.768 ± 0.7260 cm/s ($p = 0.0007$), as observed in Figure 8a. Significant changes in the distance traveled were identified (from 121.4 ± 27.23 cm to 420.4 ± 203.4 cm, $p = 0.0013$), as shown in Figure 8b. Significant changes were also observed in the parameters of average acceleration (Figure 8d, $p = 0.004$) and mobility rate (Figure 8e, $p = 0.0022$). Mobility rate is defined as the percentage of the experimental time during which the organisms stay above the mean velocity. The exploration rate (percentage of the Petri plate explored by the planarian) changed to a lesser extent. Another finding to highlight is the changes produced in the trajectories' shape, as observed in Figure 8f–j,l–p.

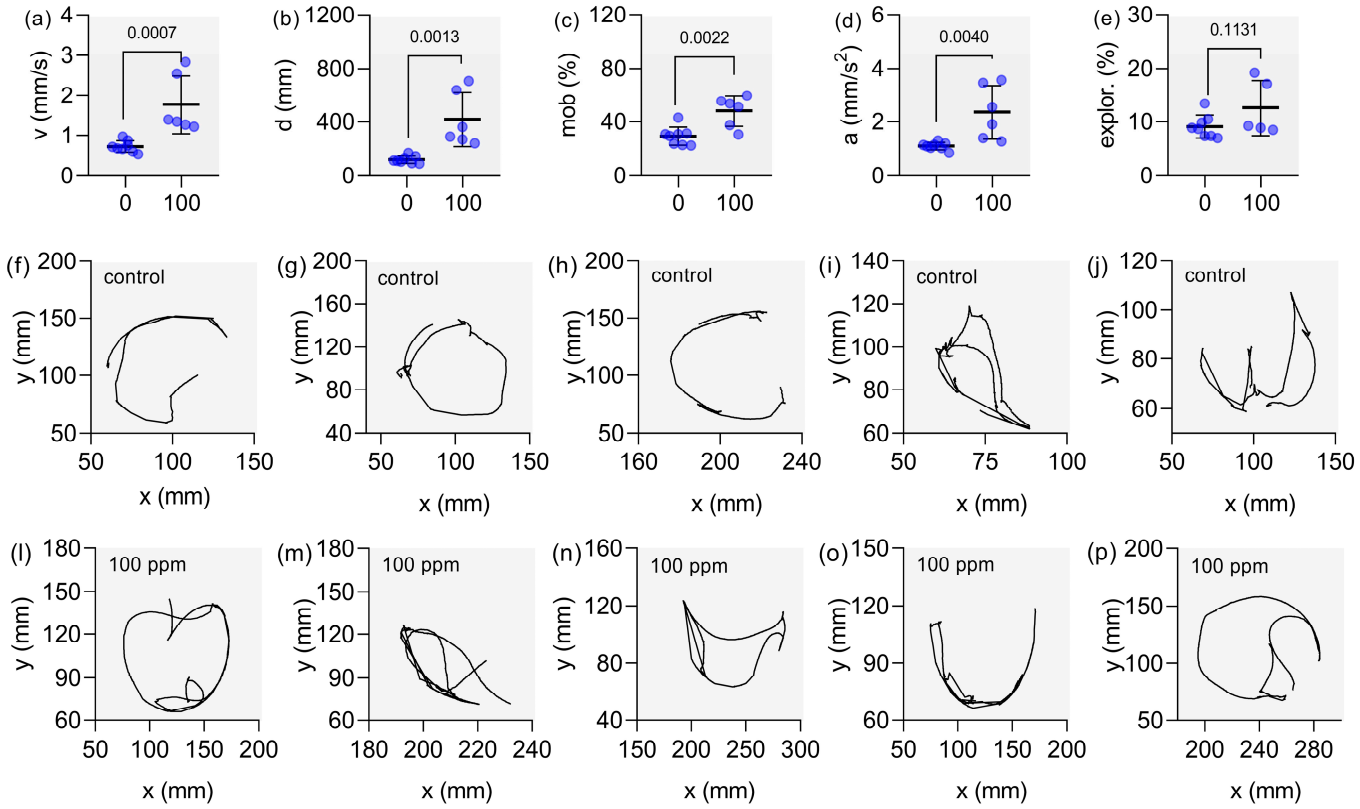


Figure 8. Effect of the *A. gratissima* extract on planarian behavior. In (**a**), the effect of the extract on average velocity is represented; in (**b**), on distance; in (**c**), on mobility rate; in (**d**), on average acceleration; and in (**e**), on exploration rate. Subfigures (**f**–**j**) show the representative trajectories of the planarians from the control group, while subfigures (**l**–**p**) show the representative trajectories of the planarians exposed to the extract at 100 ppm.

4. Discussion

In the present research, the aim was to work primarily with females, since there is still a gap in their use in experimental models. Mainly due to the hormonal changes that females present during their estrous cycle, these may respond differently to some treatments [38], and their use is important, not only to cover a more significant sample of the population, but to represent and understand pathologies that affect more women, including depression [2].

Seeking to analyze the possible antidepressant potential of the tested compounds, it was proposed to analyze biological markers related to the disease, such as oxidative stress and inflammatory levels, which are observed to increase in depressive patients, as well as dysregulation of neurotransmitter levels [7,9,12].

The relationship between oxidative stress and depression can be visualized by the neurodegeneration present in them, caused mainly due to the interruption of important signaling processes of proteolysis, gene expression and generation of mutations due to damage caused by oxidative stress to chemical structures, which in turn are recognized by the immune system, triggering an inflammatory process [7,8,12].

Such deregulation supports the results of known antioxidant species, such as *A. gratissima* and rutin, with an antidepressant role [21–23,25]. With that in mind, the identification of the secondary metabolites present in the aqueous extract of *A. gratissima* began, and a total of 25 compounds were identified, more than previous works, in which 22 compounds were identified, obtained through a different extraction technique [27]. The difference was due to the identification of gallic acid, protocatechuic acid, hydroxy-methoxyluteolin and α-amyrin and the absence of theophylline in our extracts.

Analyzing the identified compounds, it was possible to observe that many already had described antioxidant capacities, such as ferulic acid and arenarioside [39], which explains the antioxidant potential described for the extract [20,21] and observed through the DPPH and ABTS tests.

The protective action of lipid oxidation was evaluated using TBARS, and in the combination of the extract with rutin group (6), the rutin 50 mg/kg group (3) and *A. gratissima* extract group (5) it was also shown to be significantly effective when compared with the results of the fluoxetine group (2) and control group (1). This finding agrees with those of other authors [20], who discuss that the antioxidant action of *A. gratissima* and rutin is mainly related to the protection of lipids, which explains the better response obtained by the extract in the TBARS test than in the carbonyl assay.

As previously mentioned, high levels of oxidative stress may be related to the creation of new epitopes recognizable by the immune system, which can lead to the establishment of a chronic inflammatory process [7,8,12], explaining, at least in part, why this is another important marker observed in patients with depression [4–6,9].

Many cytokines have an effect on the modulation of hippocampal neurogenesis, being considered inhibitory factors, with IL-6 and TNF-α having a great part in this. This interference in neurogenesis can culminate in a decrease in hippocampal volume, observed in patients with major depression [6]. Highlighting their importance in the pathophysiology of the disease, both cytokine levels [5,6] and neurogenesis itself [40] can be normalized with the use of antidepressants.

This relationship explains why LPS is a good model for inducing depressive-like behavior. Since it acts on the body's immune system, it causes an increase in inflammatory markers in the CNS [41]. This specificity is such that, in the present study, it was not possible to quantify the cytokines in the blood plasma of the animals due to their low levels. This agrees with data previously published [41] that demonstrated an initial systemic increase of TNF-α after an administration of LPS; however, after a week, its action was only localized in the brain. This increase of pro-inflammatory cytokine levels in the brain region was also observed in the untreated group (group 2) of the present study.

All treatments tested here were effective in reducing the levels of pro-inflammatory cytokines caused by the administration of LPS, which agrees with previous studies. Arruda-Silva et al. (2014), observed the ability of rutin to decrease the synthesis and concentrations of the cytokines TNF-α and IL-1β [42]. This also agrees with in vitro tests, which demonstrated a decrease in IL-6, TNF-α and NF-κB levels after contact with the flavonoid [24]. For the aqueous extract of *A. gratissima*, no previous data were found about its action on inflammatory cytokines, as observed in the present work, but its anti-inflammatory action on edema has already been recognized [17,18].

High levels of inflammatory cytokines in patients with major depressive disorder are described by several meta-analyses [4–6,9], highlighting IL-1, IL-6 and TNF-α, as well as its soluble receptors and acute phase reagents, such as CRP, which is similar to the results found in the present study. The pro-inflammatory cytokine IL-10 was also affected by the treatments, being significantly ($p < 0.0001$) higher than in the control. Its relationship with depression has also been previously studied, and mice that do not express it (IL-10KO) present depressive-like symptoms, which can be corrected by administering it. It is hypothesized that this fact is due to its ability to increase neuronal survival, since the decrease in the hippocampus is a known sign in depressed patients. IL-10KO animals showed high levels of the hormone corticosterone, demonstrating the action of interleukin on the regulation of the HPA axis, a known stress pathway and modulator of depressive symptoms [43].

Other biochemical signals observed in depressed patients are low levels of neurotransmitters, mainly dopamine and serotonin, which are the targets for most available pharmacological treatments [16], as can be seen in Figure 6, in the which drug administered to the fluoxetine group (2) significantly raised its levels. This decrease may be linked to chronic inflammation itself and, jointly, to oxidative stress, already discussed, since

cytokines are related to impairments in the functioning of dopamine, including a decrease in synthesis of its receptors, impairing its packaging and release, in addition to increasing its reuptake [4,16].

Serotonin levels can also be related to the presence of pro-inflammatory cytokines, especially IL-6 and TNF-α, as these induce the expression of the indoleamine-2,3-dioxygenase enzyme, a limiting factor for the synthesis of quinurenine from dietary tryptophan. The increase in this pathway reduces the bioavailability of tryptophan, which is also the precursor of serotonin, and directly affects the production of this neurotransmitter, impairing neurogenesis and the behavioral processes in which serotonin is involved. Furthermore, the quinurenine produced is capable of inflicting damage on hippocampal neurons, leading them to apoptosis. Some of the compounds generated during the pathway may also act as antagonists or agonists of the glutamate NMDA (N-methyl-D-aspartate) receptor, which is also of interest in the context of depression physiopathology [5,6,44].

That said, in the present work, following the positive trend demonstrated by the previously presented results of related processes, all the treatments used were efficient in increasing the levels of neurotransmitters when compared to the control. Previous studies that investigated its potential as an acute antidepressant showed an interaction of *A. gratissima* with serotonergic and dopaminergic pathways [22]. The action of rutin on the tail suspension test has also been attributed to the serotonergic, dopaminergic and/or noradrenergic system, since the administration of pathway inhibitors inhibited this effect [25], which explains the action of rutin on neurotransmitter levels.

Due to the intrinsic relationship that oxidative stress, chronic inflammation and neurotransmitter production have, both among themselves and with depression itself, it is understood that they can be used, in a way, as quantitative measures of easy visualization for evaluation of antidepressant effects of new compounds. Taking into account this premise, and the fact that the treatments tested have been efficient in decreasing the levels of oxidative stress in some cases, normalizing the levels of inflammatory cytokines, often without significant difference when compared to the positive control, and increasing the levels of neurotransmitters, we can think of their potentials in combating depressive-like symptoms.

Such a premise has been previously tested after inducing depressive-like behavior in rats by olfactory bulbectomy, producing observed increased levels of TNF-α and IL-1β in the hippocampus of these animals [45]. Moreover, after subjecting them to a treatment with a bioactive compound with anti-inflammatory potential, there was noted not only the control of this increase, but also the improvement of the results in behavioral tests, being statistically equal to the positive control used, exemplifying the relationship between depressive-like behavior and inflammatory markers [45].

Encouraged by the promising results on neurotransmitter levels achieved by the *A. gratissima* extract in the hippocampus of rats, we extended our research to assess the extract's effects on the behavioral activity of planarians. Planarians serve as a simple alternative animal model with a localized nervous system containing neurotransmitters also found in vertebrates [46]. Previous studies have reported or suggested the presence of dopamine [47], serotonin [48], aspartate and glutamate [49] in planarians, and complex behaviors induced by various central nervous system drugs have been observed in this model [50]. An opposite effect to that observed in this research was produced by sulpiride, a drug that acts as an antagonist of dopaminergic receptors [47]. Our findings revealed that *A. gratissima* extract increased locomotor activity in planarians, suggesting its neuropharmacological activity and corroborating the results obtained in rats.

5. Conclusions

It was possible to observe the antioxidant potential of the extract of *A. gratissima* supplemented with rutin, which was higher than that of the compounds administered alone, even if at lower doses. The anti-inflammatory action of all treatments employed produced positive interference in the levels of the neurotransmitters serotonin and dopamine. *A. gratissima* extract increased the locomotor activity of planarians, in agreement with its

neuropharmacological activity identified in rats. Due to these results, it is believed that the aqueous extract of *A. gratissima* and its main flavonoid, rutin, as well as its concomitant use, represent a possible alternative in the treatment of depression since they presented promising results in all biochemical markers tested, previously related to the physiopathology of depression.

Author Contributions: Conceptualization, V.M. and G.E.S.; methodology, E.G.S.; software, I.L.G.; validation, L.T.M., L.S.d.S. and G.E.B.; formal analysis, G.E.S. and E.G.S.; investigation, L.S.d.S.; resources, V.M.; data curation, N.D.; writing—original draft preparation, G.E.S. and V.M.; writing—review and editing, N.D. and V.M.; visualization, S.M.d.M.S.; supervision, V.M.; project administration, G.E.S.; funding acquisition, V.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The animal study protocol was approved by Unipampa Animal Ethics Committee (protocol 021/2021).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data available on corresponding author request.

Acknowledgments: The authors thank UNIPAMPA for the structure and support, CAPES, CNPq and FAPERGS for their support, and the research partners at UFFS.

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

References

1. Santomauro, D.F.; Herrera, A.M.M.; Shadid, J.; Zheng, P.; Ashbaugh, C.; Pigott, D.M.; Abbafati, C.; Adolph, C.; Amlag, J.O.; Aravkin, A.Y.; et al. Global prevalence and burden of depressive and anxiety disorders in 204 countries and territories in 2020 due to the COVID-19 pandemic. *Lancet* **2021**, *398*, 1700–1712. [CrossRef] [PubMed]
2. World Health Organization. *Depression and Other Common Mental Disorders: Global Health Estimates*; World Health Organization: Geneva, Switzerland, 2017.
3. Smith, R.S. The macrophage theory of depression. *Med. Hypotheses* **1991**, *35*, 298–306. [CrossRef] [PubMed]
4. Felger, J.C.; Treadway, M. Inflammation effects on motivation and motor activity: Role of dopamine. *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol.* **2017**, *42*, 216–241. [CrossRef] [PubMed]
5. Himmerich, H.; Patsalos, O.; Lichtblau, N.; Ibrahim, M.A.A.; Dalton, B. Cytokine research in depression: Principles, challenges, and open questions. *Front. Psychiatry* **2019**, *10*, 30. [CrossRef]
6. Dowlati, Y.; Herrmann, N.; Swardfager, W.; Liu, H.; Sham, L.; Reim, E.K.; Lanctôt, K.L. A meta-analysis of cytokines in major depression. *Biol. Psychiatry* **2010**, *67*, 446–457. [CrossRef]
7. Maes, M.; Galecki, P.; Chang, Y.S.; Berk, M. A review on the oxidative and nitrosative stress (o&ns) pathways in major depression and their possible contribution to the (neuro)degenerative processes in that illness. *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* **2011**, *35*, 676–692.
8. Maes, M.; Yirmyia, R.; Noraberg, J.; Brene, S.; Hibbeln, J.; Perini, G.; Kubera, M.; Bob, P.; Lerer, B.; Maj, M. The inflammatory & neurodegenerative (i&nd) hypothesis of depression: Leads for future research and new drug developments in depression. *Metab. Brain Dis.* **2009**, *24*, 27–53.
9. Osimo, E.F.; Pillinger, T.; Rodriguez, I.M.; Khandaker, G.M.; Pariante, C.M.; Howes, O.D. Inflammatory markers in depression: A meta-analysis of mean differences and variability in 5166 patients and 5083 controls. *Brain Behav. Immun.* **2020**, *87*, 901–909. [CrossRef]
10. Rengasamy, M.; Marsland, A.; McClain, L.; Kovats, T.; Walko, T.; Pan, L.; Price, R.B. Longitudinal relationships of cytokines, depression and anhedonia in depressed adolescents. *Brain Behav. Immun.* **2021**, *91*, 74–80. [CrossRef]
11. Behl, T.; Rana, T.; Alotaibi, G.H.; Shamsuzzaman, M.; Naqvi, M.; Sehgal, A.; Singh, S.; Sharma, N.; Almoshari, Y.; Abdellatif, A.A.H.; et al. Polyphenols inhibiting mapk signalling pathway mediated oxidative stress and inflammation in depression. *Biomed. Pharmacother.* **2022**, *146*, 112545. [CrossRef]
12. Black, C.N.; Bot, M.; Scheffer, P.G.; Cuijpers, P.; Penninx, B.W.J.H. Is depression associated with increased oxidative stress? A systematic review and meta-analysis. *Psychoneuroendocrinology* **2015**, *51*, 164–175. [CrossRef] [PubMed]
13. Burke, D.A.; Alvarez, V.A. Serotonin receptors contribute to dopamine depression of lateral inhibition in the nucleus accumbens. *Cell Rep.* **2022**, *39*, 110795. [CrossRef] [PubMed]
14. Frederick, A.L.; Stanwood, G.D. Drugs, biogenic amine targets and the developing brain. *Dev. Neurosci.* **2009**, *31*, 7–22. [CrossRef] [PubMed]

15. Ang, B.; Horowitz, M.; Moncrieff, J. Is the chemical imbalance an 'urban legend'? An exploration of the status of the serotonin theory of depression in the scientific literature. *SSM Ment. Health* **2022**, *2*, 100098. [CrossRef]
16. Yeh, K.Y.; Shou, S.S.; Lin, Y.X.; Chen, C.C.; Chiang, C.Y.; Yeh, C.Y. Effect of ginkgo biloba extract on lipopolysaccharide-induced anhedonic depressive-like behavior in male rats. *Phytother. Res.* **2015**, *29*, 260–266. [CrossRef]
17. Souza, M.A.; Guzatti, J.G.G.; Martello, R.H.; Schindler, M.S.Z.; Calisto, J.F.F.; Morgan, L.V.; Aguiar, G.P.S.; Locateli, G.; Scapinello, J.; Müller, L.G.; et al. Supercritical CO_2 extraction of *Aloysia gratissima* leaves and evaluation of anti-inflammatory activity. *J. Supercrit. Fluids* **2020**, *159*, 104753. [CrossRef]
18. Vandresen, F.; Schmitt, E.; Kato, L.; Oliveira, C.M.A.d.; Amado, C.A.B.; Silva, C.C.d. Chemical composition and evaluation of antibacterial and antiedematogenic activities of *Aloysia gratissima* (Gillies & Hook.) Tronc. E *Aloysia virgata* (Ruiz & Pav.) pers., verbenaceae. *Rev. Bras. Farmacogn.* **2010**, *20*, 317–321.
19. Rosas-Romero, A.; Saavedra, G. Screening bolivian plants for antioxidant activity. *Pharm. Biol.* **2005**, *43*, 79–86. [CrossRef]
20. Zeni, A.L.B.; Albuquerque, C.A.C.d.; Gonçalves, F.; Latini, A.; Tasca, C.I.; Podestá, R.; Pagliosa, C.M.; Duarte, F.S.; Lima, T.; Maraschin, M. Phytochemical profile, toxicity and antioxidant activity of *Aloysia gratissima* (verbenaceae). *Quim. Nova* **2013**, *36*, 69–73. [CrossRef]
21. Zeni, A.L.B.; Zomkowski, A.D.E.; Dal-Cim, T.; Maraschin, M.; Rodrigues, A.L.S.; Tasca, C.I. Antidepressant-like and neuroprotective effects of *Aloysia gratissima*: Investigation of involvement of L-arginine-nitric oxide-cyclic guanosine monophosphate pathway. *J. Ethnopharmacol.* **2011**, *137*, 864–874. [CrossRef]
22. Zeni, A.L.B.; Zomkowski, A.D.E.; Maraschin, M.; Tasca, C.I.; Rodrigues, A.L.S. Evidence of the involvement of the monoaminergic systems in the antidepressant-like effect of *Aloysia gratissima*. *J. Ethnopharmacol.* **2013**, *148*, 914–920. [CrossRef]
23. Taboada, T.; Alvarenga, N.L.; Galeano, A.K.; Arrúa, W.J.; Campuzano-Bublitz, M.A.; Kennedy, M.L. In vivo antidepressant-like effect assessment of two aloysia species in mice and lcms chemical characterization of ethanol extract. *Molecules* **2022**, *27*, 7828. [CrossRef] [PubMed]
24. Liu, S.; Adewole, D.; Yu, L.; Sid, V.; Wang, B.; Karmin, O.; Yang, C. Rutin attenuates inflammatory responses induced by lipopolysaccharide in an in vitro mouse muscle cell (C2C12) model. *Poult. Sci.* **2019**, *98*, 2756–2764. [CrossRef] [PubMed]
25. Machado, D.G.; Bettio, L.E.B.; Cunha, M.P.; Santos, A.R.S.; Pizzolatti, M.G.; Brighente, I.M.C.; Rodrigues, A.L.S. Antidepressant-like effect of rutin isolated from the ethanolic extract from schinus molle l. In mice: Evidence for the involvement of the serotonergic and noradrenergic systems. *Eur. J. Pharmacol.* **2008**, *587*, 163–168. [CrossRef] [PubMed]
26. Feksa, D.L.; Coelho, R.P.; Aparecida da Costa Güllich, A.; Dal Ponte, E.S.; da Costa Escobar Piccoli, J.; Manfredini, V. Extract of citrus maxima (pummelo) leaves improve hepatoprotective activity in wistar rats submitted to the induction of non-alcoholic hepatic steatosis. *Biomed. Pharmacother.* **2018**, *98*, 338–346. [CrossRef]
27. Schreiner, G.E.; Eckert, G.L.; Schuster, M.F.; Baroni, S.; de Pelegrin, C.M.G.; Dartora, N. Cytotoxic and genotoxic effects of aqueous extracts of *Aloysia gratissima* (Gillies & Hook.) Tronc. Using *Allium cepa* L. Assay. *Pharmacol. Res. Nat. Prod.* **2024**, *2*, 100011.
28. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved abts radical cation decolorization assay. *Free Radic. Biol. Med.* **1999**, *26*, 1231–1237. [CrossRef]
29. Sharma, O.P.; Bhat, T.K. Dpph antioxidant assay revisited. *Food Chem.* **2009**, *113*, 1202–1205. [CrossRef]
30. Gul, A.; Kunwar, B.; Simjee, S.; Faizi, S.; Raza Shah, M.; Ahmed, D. Protective effect of rutin and its gold nanoparticles on oxidative stress markers and pro-inflammatory cytokine production in collagen induced arthritis (cia) model. *J. Pharmacol. Toxicol. Methods* **2017**, *88*, 205–206. [CrossRef]
31. Anesti, M.; Stavropoulou, N.; Atsopardi, K.; Lamari, F.N.; Panagopoulos, N.T.; Margarity, M. Effect of rutin on anxiety-like behavior and activity of acetylcholinesterase isoforms in specific brain regions of pentylenetetrazol-treated mice. *Epilepsy Behav.* **2020**, *102*, 106632. [CrossRef]
32. Levine, R.L. Carbonyl modified proteins in cellular regulation, aging, and disease. *Free Radic. Biol. Med.* **2002**, *32*, 790–796. [CrossRef] [PubMed]
33. Ohkawa, H.; Ohishi, N.; Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **1979**, *95*, 351–358. [CrossRef] [PubMed]
34. Karatepe, M. Simultaneous determination of ascorbic acid and free malondialdehyde in human serum by hplc-uv. *LC GC N. Am.* **2004**, *22*, 362–365.
35. Singleton, V.L.; Orthofer, R.; Lamuela-Raventós, R.M. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In *Methods in Enzymology*; Elsevier: Amsterdam, The Netherlands, 1999; Volume 299, pp. 152–178.
36. Vargas, L.S.; Ramires Lima, K.; Piaia Ramborger, B.; Roehrs, R.; Izquierdo, I.; Mello-Carpes, P.B. Catecholaminergic hippocampal activation is necessary for object recognition memory persistence induced by one-single physical exercise session. *Behav. Brain Res.* **2020**, *379*, 112356. [CrossRef] [PubMed]
37. Rodriguez, A.; Zhang, H.; Klaminder, J.; Brodin, T.; Andersson, P.L.; Andersson, M. Toxtrac: A fast and robust software for tracking organisms. *Methods Ecol. Evol.* **2018**, *9*, 460–464. [CrossRef]
38. Mora, S.; Dussaubat, N.; Díaz-Véliz, G. Effects of the estrous cycle and ovarian hormones on behavioral indices of anxiety in female rats. *Psychoneuroendocrinology* **1996**, *21*, 609–620. [CrossRef]

39. Skalski, B.; Pawelec, S.; Jedrejek, D.; Rolnik, A.; Pietukhov, R.; Piwowarczyk, R.; Stochmal, A.; Olas, B. Antioxidant and anticoagulant effects of phenylpropanoid glycosides isolated from broomrapes (orobanche caryophyllacea, phelipanche arenaria, and p. Ramosa). *Biomed. Pharmacother.* **2021**, *139*, 111618. [CrossRef]
40. Malberg, J.E.; Eisch, A.J.; Nestler, E.J.; Duman, R.S. Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J. Neurosci.* **2000**, *20*, 9104–9110. [CrossRef]
41. Qin, L.; Wu, X.; Block, M.L.; Liu, Y.; Breese, G.R.; Hong, J.S.; Knapp, D.J.; Crews, F.T. Systemic lps causes chronic neuroinflammation and progressive neurodegeneration. *Glia* **2007**, *55*, 453–462. [CrossRef]
42. Arruda-Silva, F.; Nascimento, M.V.P.S.; Luz, A.B.G.; Venzke, D.; Queiroz, G.S.; Fröde, T.S.; Pizzolatti, M.G.; Dalmarco, E.M. Polygala molluginifolia a. St.-hil. And moq. Prevent inflammation in the mouse pleurisy model by inhibiting nf-κb activation. *Int. Immunopharmacol.* **2014**, *19*, 334–341. [CrossRef]
43. Roque, S.; Correia-Neves, M.; Mesquita, A.R.; Palha, J.A.; Sousa, N. Interleukin-10: A key cytokine in depression? *Cardiovasc. Psychiatry Neurol.* **2009**, *2009*, 187894. [CrossRef] [PubMed]
44. Dantzer, R.; O'Connor, J.C.; Freund, G.G.; Johnson, R.W.; Kelley, K.W. From inflammation to sickness and depression: When the immune system subjugates the brain. *Nat. Rev. Neurosci.* **2008**, *9*, 46–56. [CrossRef] [PubMed]
45. Yang, S.-J.; Yu, H.-Y.; Kang, D.-Y.; Ma, Z.-Q.; Qu, R.; Fu, Q.; Ma, S.-P. Antidepressant-like effects of salidroside on olfactory bulbectomy-induced pro-inflammatory cytokine production and hyperactivity of hpa axis in rats. *Pharmacol. Biochem. Behav.* **2014**, *124*, 451–457. [CrossRef] [PubMed]
46. Göethel, G.; Augsten, L.V.; das Neves, G.M.; Gonçalves, I.L.; de Souza, J.P.S.; Garcia, S.C.; Eifler-Lima, V.L. The role of alternative toxicological trials in drug discovery programs. The case of caenorhabditis elegans and other methods. *Curr. Med. Chem.* **2022**, *29*, 5270–5288. [CrossRef]
47. Raffa, R.B.; Holland, L.J.; Schulingkamp, R.J. Quantitative assessment of dopamine d2 antagonist activity using invertebrate (planaria) locomotion as a functional endpoint. *J. Pharmacol. Toxicol. Methods* **2001**, *45*, 223–226. [CrossRef]
48. Kreshchenko, N.D. Immunocytochemical identification of serotoninergic neurons in planaria girardia tigrina. *Biochem. Suppl. Ser. A Membr. Cell Biol.* **2017**, *11*, 68–76. [CrossRef]
49. Vyas, C.A.; Rawls, S.M.; Raffa, R.B.; Shackman, J.G. Glutamate and aspartate measurements in individual planaria by rapid capillary electrophoresis. *J. Pharmacol. Toxicol. Methods* **2011**, *63*, 119–122. [CrossRef]
50. Raffa, R.B.; Finno, K.E.; Tallarida, C.S.; Rawls, S.M. Topiramate-antagonism of l-glutamate-induced paroxysms in planarians. *Eur. J. Pharmacol.* **2010**, *649*, 150–153. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.