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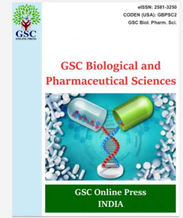

(Research Article)

Antiepileptic and antioxidant effects of aqueous extract of *Lippia multiflora* Moldenke (Verbenaceae) on pilocarpine-induced seizures in *Mus musculus* Swiss mice

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Abstract

Epilepsy is a chronic neurological disorder that affects the central nervous system. Approximately 3% of the population at some point in their lives would be affected by epileptic disorders in the world, that is to say nearly 70 million people and therefore about 85% live in developing countries. The objective of this study is to determine the anticonvulsant effects of *Lippia multiflora* on the animal model of epileptogenesis induced by pilocarpine injection in mice during the acute phase. Mice were treated with different doses of the aqueous extract of *L. multiflora* or sodium valproate. The anticonvulsant effects were evaluated 24 after the injection of pilocarpine by referring to the Racine scale. Then a daily treatment was done for one week corresponding to the acute stage of the disease. Oxidative stress parameters were measured from brain samples taken on the last day of treatment. The injection of pilocarpine induced the *Status epilepticus*, which is translated by the appearance of convulsive seizures while the administration of different doses of the aqueous extract of *L. multiflora* led to a significant decrease of the seizures induced by pilocarpine in mice. In addition, *L. multiflora* extract restored endogenous levels of oxidative stress markers (MDA and NO) and increased the activity of oxidative stress enzymes (SOD, CAT and GSH). *L. multiflora* possesses anticonvulsant properties mediated by the involvement of $GABA_A$ receptors and antioxidants.

Keywords: *Lippia multiflora*; Epilepsy; Oxidative stress; Traditional medicine

1. Introduction

Epilepsy is a chronic neurological disorder that affects the central nervous system [1]. The disease is characterized by the periodic and unpredictable occurrence of seizures. These seizures are spontaneous and unprovoked, caused by an imbalance in the cortex [2, 3]. The causes are diverse, and epilepsy can occur as a result of vascular, tumor, or toxic trauma, or can result from a genetic mutation in an ion channel or neurotransmitter gene that alters brain activity [4] or oxidative stress. Reactive oxygen species (ROS) are also highly involved in the development of pilocarpine-induced seizures [5]. Neuronal hyperexcitability is associated with a calcium-dependent activation of intracellular oxidant systems, including NOX2, which is the major NMDAR-regulated source of superoxide [6]. The initial disturbances, generally of abrupt onset, will immediately extend to both cerebral hemispheres defining generalized seizures or be

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localized in a single cerebral hemisphere defining partial or focal seizures. This could result in altered consciousness, motor, sensory, cognitive or psychic disorders [7]. This pathology affects approximately 3% of the population at some point in their lives, nearly 70 million people would be affected by epileptic disorders in the world, and thus approximately 85% live in developing countries [8]. A recent study showed that, approximately 60% of epilepsy patients present with partial epilepsy, the origin of which is most often localized in the temporal lobe. Temporal lobe epilepsy is therefore the most common form of epilepsy of focal origin [9] and is characterized by lesions in the limbic system (hippocampus, amygdala, entorhinal cortex), which is considered the epileptic focus [10]. In the temporal lobe, a focal decrease in $GABA_A$ receptors is one of the diagnostic signs of temporal lobe epilepsy with hippocampal sclerosis [11]. In about 40% of adult patients with temporal lobe epilepsy, seizures will persist despite the various antiepileptic treatments available and the usual antiepileptic drugs present numerous side effects such as sedation, hepatotoxicity, anxiety, and depression. However, the search for a natural pharmacological treatment with anticonvulsant properties from medicinal plants would help prevent the onset of epilepsy. Such a medicine could be the aqueous extract of the leaves and stems of *Lippia multiflora*, a medicinal plant that is widely used in traditional medicine in many regions. Previous scientific studies have shown that the aqueous extract of *L. multiflora* leaves has analgesic, antipyretic and anti-inflammatory properties, antipyretic and anti-inflammatory properties [12]. This plant is also described for its pesticidal properties [13]. Several pharmacological pharmacological studies have been carried out, demonstrating the antifungal and antiviral properties of volatile compounds, as well as antimalarial and diarrheal diarrheal activity of *L. multiflora* decoction or infusion [14]. Another study was carried out on essential oils, in which twenty-nine compounds were characterized in the essential oil of *L. multiflora* representing a total of 97.3%, with p-cymene (21.3%), thymol (14%), β-caryophyllene (12.9%), carvacrol (9.3%) and carvone (8.6%) the main compounds, and 1,8-cineole (5%), α-humulene (3.5%), α-amorphene (3.3%), α-phellandrene (3.1%), β-myrcene (2.4%), α-thujene (2.3%), α-terpinene (1.9%), thymol acetate (1.6%), bicyclogermacrene (1.3%) and δ-cadinene (1.2%), the minor compounds [15]. On the other hand, 1,8-cineole (48.9%), geranial (15.1%), α-terpineole (8.5%) and sabinene (5.1%) were identified as major compounds [16]. Linalool (46.6%) and (E)-nerolidol (16.5%) have also been identified as the majority compounds in the essential oil of this plant [17]. The results of this study could help us to better understand the mechanisms by which these natural medicines could help prevent and treat epilepsy, with a view to developing safer and more effective drugs for the treatment of epilepsy. Hence, the interest in determining the anticonvulsant effects of *Lippia multiflora* on the animal model of epileptogenesis of the mesial face of the temporal lobe induced by pilocarpine administration during the acute phase.

2. Material and methods

2.1. Biological plant material

The leaves and stems of *Lippia multiflora* used in our study were collected in the morning precisely at Ngaoundéré-Dang (07°25'17.23 "N, 13°32'25.06 "E) in the Adamaoua region of Cameroon. This plant has been identified in the national herbarium of Cameroon under number 7753/SRF/Cam. These leaves and stems were washed, shade and air dried, then crushed and the powder was stored at room temperature.

2.2. Animal biological material

We used during our manipulation the white mice *Mus musculus* Swiss of both sexes, aged about two (02) months having a mass of about 20 to 25 g as biological model for the various pharmacological tests. These mice were raised in the animal house in cages with a litter of shavings and were fed with pellets composed of corn bran, soybean meal, corn meal, cotton cake, palm oil, fish, salt and drinking water.

2.3. Preparation of the decoction of the dry powder of leaves and stems of *Lippia multiflora*

We used 10 g of dry powder of the leaves and stems of *L. multiflora*, which we introduced into a beaker containing 100 mL of distilled water. The mixture was boiled in a Beaker for 20 min. After decoction and cooling, the mixture was filtered and we obtained a filtrate of 39 mL volume.

2.4. Determination of the doses of the aqueous extract of leaves and stems of *Lippia multiflora*

Knowing the mass of the dry extract (0.9 g) and the volume of the decoction (39 mL) obtained after the preparation of 10 g of dry powder of the leaves and stems of *L. multiflora*, we can determine our different doses of the aqueous extract while first calculating the concentration of our extract using the following formula: Concentration = (Mass of dry extract)/ (Volume of decoctate)

We thus obtained a concentration of 23.078 mg/mL and as the volume of administration is 10 mL/kg, we obtained a dose of 230.78 mg/kg using the following formula: Dose = Concentration × Administration Volume. This dose was diluted to 1/2, 1/4, and then 1/10th to obtain the respective doses of 115.39 mg/kg, 57.69 mg/kg, and 23.07 mg/kg.

2.5. Chemical substances

Diazepam: Roche, France; Sodium valproate: Sigma Aldrich Inc, St Louis, USA; N-methyl-scopolamine: Sigma Aldrich Inc, St Louis, USA; Pilocarpine hydrochloride: Sigma Aldrich Inc, St Louis, USA.

2.6. Induction of status epilepticus by pilocarpine

The injection protocol was similar to those of Turski et *al*. [18] and Curia et *al*. [19]. Mice received the different treatments: distilled water for the normal and negative controls, the different doses of the aqueous extract of *L. multiflora* (23.078, 57.69, 115.39 and 230.78 mg/kg, *per os*) for the test groups and sodium valproate (300 mg/kg; intraperitoneal (i.p)) for the positive control. Forty minutes after these different treatments, a low dose of methyl-scopolamine nitrate (1 mg/kg, i.p.), a cholinergic antagonist, was administered to all treated groups except the normal control in order to reduce the cholinergic effects in the periphery. Then, 20 methyl-scopolamine nitrate, *status epilepticus* was induced by the injection of a single dose of pilocarpine (400 mg/kg, i.p.) dissolved in 0.9% sterile saline. After pilocarpine injection, mice were observed for 6 hours to determine the severity and duration of acute seizures with reference to the Racine scale [20] ranging from 0 to 5 (level 0: no response; level 1: Hyperactivity and vibrissae clonus; level 2: head nodding, head clonus, and myoclonic jerks; level 3: unilateral forelimb clonus; level 4: pitching and bilateral forelimb clonus; level 5: tonic-clonic seizures with loss of motor control reflex). Twenty-four hours after induction of *status epilepticus*, the different substances were administered to the animals according to groups. One hour after these administrations, the following parameters were assessed individually in each mouse for a duration of 30 minutes: the latency time of onset of the first tonic or clonic seizure, the duration and number of tonic or clonic seizures. Latency times are expressed as scores, calculated as follows: Score = 1- (tonic-clonic seizure latency time in the negative control batch/tonic-clonic seizure latency time in the test batch or positive control batch). The score is 0 in the negative control and 1 in those who did not develop seizures.

After administration of pilocarpine and observation, the animals were treated daily for 7 days (corresponding here to the acute stage of the disease). On the last day of treatment, the animals were sacrificed by decapitation with a dissection kit, the brains were removed and the hippocampus was harvested for the determination of different biochemical parameters. For each animal, the hippocampus thus removed was introduced into an EDTA tube containing 1 mL of Tris buffer (HCl 50 mM, KCl 150 mM, pH 7.4) and the contents of each tube were centrifuged at 10 000 rpm for 15 minutes. The supernatant was pipetted and introduced into a labeled Eppendorf tube which was then stored at -20 °C in the freezer.

2.7. Evaluation of the activity of some biochemical parameters

2.7.1. Evaluation of the activity of gamma amino butyric acid (GABA)

The concentration of GABA in the hippocampus was determined by the colorimetric assay technique of mouse brain homogenates described by [21]. The working reagent consisted of a mixture of 0.2 mL of 0.14 M ninhydrin solution prepared in carbonate-bicarbonate buffer solution (0.5 M; pH 9.9), and 0.1 mL of glacial trichloroacetic acid (10%). A 100-µL sample of homogenate was taken and introduced into the working reagent; the mixture was incubated at 60 °C in a water bath for 30 minutes. After cooling, the mixture was introduced into 5 mL of copper tartrate solution prepared from 0.16% disodium carbonate, 0.03% copper sulfate and 0.0329% tartaric acid. This was kept at a temperature of 25 °C for 10 minutes. The fluorescence resulting from the reaction between ninhydrin and GABA in the basic medium was measured using a spectrofluorometer (377/451 nm). The measured absorbance was proportional to the concentration of GABA in the homogenates. A standard GABA solution was prepared in parallel from different masses of GABA (100, 150, 200, 250, 300, 350, and 400 µg) each mixed with 1.5 mg of glutamate dissolved in 0.1 mL of 10% TCA ice solution. The concentration of GABA in hippocampal homogenate samples was determined with reference to the following GABA calibration curve:

2.7.2. Assessment of GABA-transaminase activity

GABA-transaminase (GABA-T) activity was assessed by the colorimetric assay method of Nayak and Chatterjee [22]. Graduated 10-mL tubes were used for this assay. Into tubes were introduced 15 µmol of α-oxoglutarate, 15 µmol of GABA, 10 µg of pyridoxal phosphate, 0.1 mL of homogenate supernatant (test tubes), and 0.1 mL of 5% methanol (blank tube). The final volume of the mixture was made up to 3 mL with Tris-HCl buffer. The tubes were incubated at 37 °C for 30 min in a 96-well microplate. The reaction was finalized by adding 0.5 mL of glacial 20% TCA. The succinic semi-

aldehyde acid produced during the incubation of the mixture was estimated spectrophotometrically and the absorbance was read at 610 nm after 30 and 90 s against the blank. The staining of the complex formed by succinic semi-aldehyde acid and 3-methyl-2-benzothia-zolone-2-hydrazone in the presence of 12% $FeCl_3$ was measured and was proportional to the concentration of GABA-T in the homogenates. GABA-T activity was estimated in pg/min/mg of tissue according to the Beer-Lambert law:

$$\text{GABA} - \text{T activity (pg/min/mg tissue)} = \frac{\text{Am}.10^3 \text{ x Vt}}{\varepsilon \text{ x l x Ve x m}}$$

Am: mean absorbance at 610 nm; ε: molar extinction coefficient = 40 $M^{-1}.cm^{-1}$; Vt: total volume of reaction medium; Ve: volume of homogenate; t: reaction time (min); l: optical path of the cell = 1 cm; m: mass of tissue used. $Am.10^3$ x Vt İ x l x Ve x m.

2.7.3. Assessment of oxidative stress marker and enzyme activity.

For each animal, the mass of the hippocampus thus removed was evaluated, and then into a sample of this hippocampus (0.1 g) were added 1 mL of Tris buffer (HCl 50 mM, KCl 150 mM, pH 7.4). After grinding in a poter, the mixture was introduced into a labeled tube and centrifuged at 10,000 rpm for 15 minutes. The supernatant was pipetted and introduced into a labeled Eppendorf tube which was then stored at -20 °C in the freezer.

Determination of superoxide dismutase (SOD) activity

SOD activity was measured according to the method of Misra & Fridovish [23]. The presence of SOD in the sample inhibits the oxidation of adrenaline to adrenochrome. The increase in absorbance is proportional to the activity of SOD. This absorbance is measured at 20 s and 80 s at 480 nm. In a spectrophotometer cell, 134 µL of organ homogenate for the assay was introduced. Then, 1666 µL of carbonate buffer (0.05 M, pH 10.2) was introduced into the white tube. After calibrating the spectrophotometer, the reaction was initiated by adding 200 µL of epinephrine (0.3 mM) to each tube. The mixture was homogenized by rapid inversion of the tube. The optical density at 480 nm was noted after 20 s and 80 s. Specific SOD activity was expressed as units (U) of SOD/g of brain tissue. One unit (1 U) of SOD activity is defined as the amount of SOD required causing 50% inhibition of the oxidation of adrenaline to adrenochrome for 1 minute in the brain tissue sample. SOD activity was calculated according to the following relationship: SOD activity = n SOD units/m. Where: n SOD units is the number of SOD units = [100 - (ΔDOessay x 100) / ΔDOblank] = % inhibition (ΔDO is the change in OD per min = OD20s - OD80s); m is the brain tissue mass (g).

Determination of malondialdehyde (MDA) activity

The presence of malondialdehyde (MDA) in a sample results in the formation of aldehydes in an acidic and warm environment (100 °C), including malonic aldehyde, which reacts with thiobarbituric acid (TBA) to form a pink complex that absorbs at 530 nm. This complex accounts for MDA in the sample. The MDA assay was performed according to the protocol described by Wilbur et *al*. [24]. For this assay, 250 µL of homogenate was introduced into the test tubes, and 250 µL of Tris buffer (HCl 50 mM; KCl 150 mM; pH 7.4) into the blank tube. To each tube was then added 125 µL of 20% trichloroacetic acid (TCA), followed by 250 µL of 0.67% thiobarbituric acid (TBA). The tubes were capped with glass beads and incubated for 10 minutes at 90 °C in a water bath. They were then left in the open air to cool before being centrifuged at 3,000 rpm for 15 minutes at room temperature. The supernatant was pipetted and the absorbance was read with a spectrophotometer (Fisher) at 530 nm against the blank. The concentration of MDA was determined using the following formula:

$$[\text{MDA}] \text{ (mmol/g tissue)} = ((\text{ODx Vt}) / (\varepsilon \text{ x L x Vi x m})) \text{ x } 2$$

OD: optical density; Vt: total volume of supernatant (mL); Vi: volume of supernatant used for the assay (mL); m: mass of tissue used (g); L: length of the cell = 1 cm; ε: molar extinction coefficient = 1.56×105 mmol-1.cm-1.

Determination of catalase activity (CAT).

The determination of CAT was performed following the protocol described by Sinha et *al*. [25]. Hydrogen peroxide is disrupted in the presence of CAT. The residue (peroxide) binds to potassium dichromate to form a blue-green precipitate of unstable perchromic acid. This colored substance will be decomposed by heat and form a green complex (chromic acid). The amount of peroxide degraded will be determined by measuring the absorbance at 570 nm with a spectrophotometer. In the test tubes, 12.5 µL of the homogenate and 187.5 µL of phosphate buffer (0.1 M, pH 7.5) were introduced. The timer was started after addition of 50 µL of hydrogen peroxide (50 mM). One minute later, the reaction

was stopped by adding 500 µL of dichromate/acetic acid solution. The solutions were then heated (100 °C for 10 min) and cooled. The optical density was read with a spectrophotometer at 570 nm against the blank. The amount of hydrogen peroxide remaining in the solution after addition of perchromic acid was evaluated using a calibration curve. The specific activity of catalase was determined and then expressed as mmol H_2O_2 consumed/min/mg protein in brain tissue according to the following formula: CAT activity = [(OD Ech - OD blank) × f) / (a × t × m)]. Where: OD Blank is the optical density of the sample; f is the dilution factor; a is the coefficient of the equation line of the calibration curve (a = 0.003582); t is the reaction time (t = 1 min); m is the mass of brain tissue corresponding to the assay volume (g).

Determination of reduced glutathione (GSH) activity

2,2'-dithio-5,5'-dinitrobenzoic acid (DTNB) reacts with the -SH (thiol) groups of glutathione present in the homogenate to form a yellow colored complex that shows an absorption maximum at 412 nm. The determination of reduced glutathione was performed according to the protocol described by Ellman [26]. To perform this assay, 1.5 mL of Ellman's reagent was introduced into test tubes previously containing 100 µL of homogenate (test tubes), and 100 µL of Tris buffer (HCl 50 mM, KCl 150 mM, pH 7.4) (control tube). The tubes were shaken and allowed to stand for incubation for one hour at room temperature, and then the absorbance was read with a spectrophotometer (Fisher) at 412 nm against the blank. The amount of reduced glutathione was determined using the following formula: [Reduced Glutathione] (mol/g tissue) = ((ODx Vt)/(εx Lx Vi x m)) x 2

OD: optical density; Vt: total volume of supernatant (mL); Vi: volume of supernatant used for the assay (mL); m: mass of tissue used (g); L: length of the cell = 1 cm; ε: molar extinction coefficient = 13600 $mol^{-1}.cm^{-1}$.

Determination of the activity of Nitric oxide (NO)

The determination of NO by the method of Griess (1959) is based on two diazotization reactions. Acidified nitric oxide produces a nitrosating agent which reacts with sulphanilic acid to produce the diazonium ion. The latter will couple to naphthylenediamide to form a chromophoric nitrogen derivative that absorbs at 540 nm. The assay was performed according to the method described by Grand et *al*. [27]. A serial dilution of the NaNO2 solution was made in 13 test tubes. The highest concentration being 1 mM. The dilutions were made by the half. In the first tube, 200 µL of $NaNO_2$ was introduced and in each of the remaining 12 tubes, 200 µL of distilled water. Subsequently, 200 mL of NaNO2 was added to tube 2, and the whole was homogenized by vortexing. Two hundred microliters were taken and added to tube N° 3. This experiment was carried out until tube N° 13. For tube 13, the 200 µL of the mixture was removed and discarded. To the contents of tubes 1 to 13, 200 µL of Griess reagent was added. The absorbance of each tube was read after 10 min at 570 nm by a spectrophotometer. This first set of tubes was used to establish a calibration curve. To assess the amount of NO in different samples, 200 µL of hippocampal homogenate and 200 µL of Griess reagent were introduced into each test tube in order. After homogenization of the mixture, the optical density of each tube was read by spectrophotometer after 10 min at 570 nm. The amount of nitrite was determined from the calibration curve.

2.8. Statistical analysis

To record the effect of *L. multiflora*, the means of the different parameters obtained in the mice treated with the extract were compared with those obtained in the control mice. The parameters measured in the pharmacological tests were expressed as mean ± standard error on the mean (S.E.M.) or as a percentage. Values were compared using the analysis of variance (ANOVA) test and when differences existed, Tukey's multiple comparison tests were used to separate them and Fisher's exact test was used for comparison of seizure scores. From $p<0.05$, the values were considered significant. The different statistical analyses of the results were performed using Graphpad prism version 5.03 and Microsoft Office Excel 2016.

3. Results

3.1. Effects of *Lippia multiflora* on pilocarpine-induced seizures during the acute phase

Pilocarpine injection induced a 50.00% ($p<0.01$) increase in the percentage of mortality in the negative control compared to the normal control. The plant extract at doses of 115.39 and 230.78 mg/kg and sodium valproate induced a significant decrease ($p<0.001$) in this mortality compared to the negative control by protecting 100% of the animals from SE-induced death (figure 1)

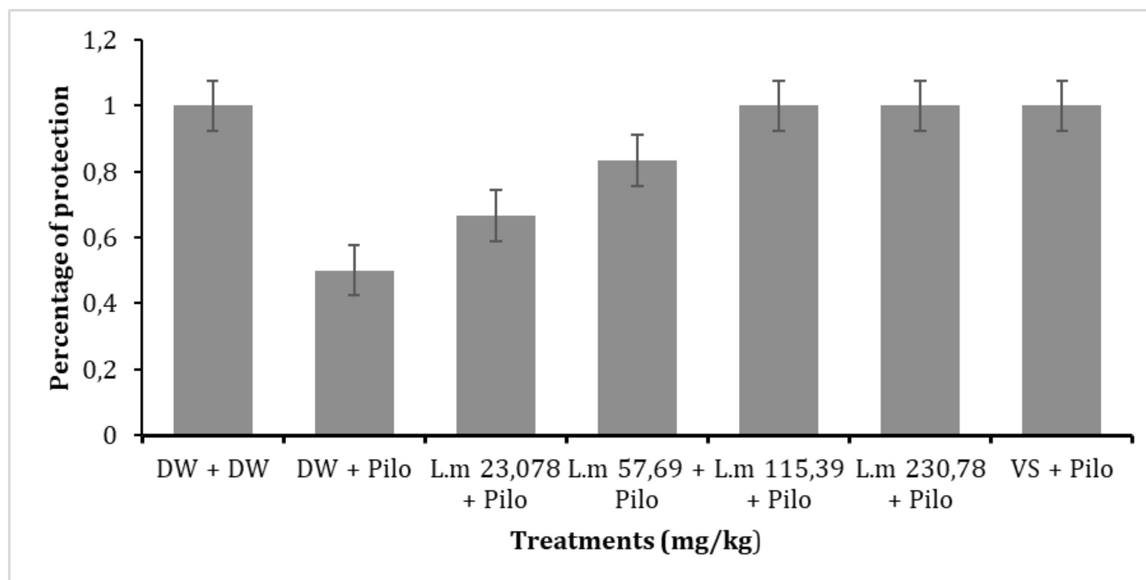

Figure 1 effects of *Lippia multiflora on* percentage of protection

DW+DW: normal control; DW+Pilo: negative control; DW : Distilled water (10 mL/kg); PILO: Pilocarpine (400 mg/kg); Lm: *Lippia multiflora*; VS: Sodium Valproate (300 mg/kg) positive control;

3.2. Effects of *Lippia multiflora* on pilocarpine-induced *status epilepticus*

The effects of *L. multiflora* on the latency time of status epilepticus in mice given pilocarpine are shown in Figure 2 below. It can be seen that the latency time is significantly increased in the negative control which is 21.45 ± 2.99 min compared to the normal control in which this time is zero. Aqueous extract of *L. multiflora* delayed the time of onset of convulsions by increasing this time in a dose-dependent and significant manner [F (6, 35) = 148.4, P<0,001] by 29.686, 41.305, 42.84 min respectively in the 57.69, 115.39 and 230.78 mg/kg dose groups compared to the negative control. Similarly, sodium valproate significantly increased this time to 46.32 min compared to the negative control.

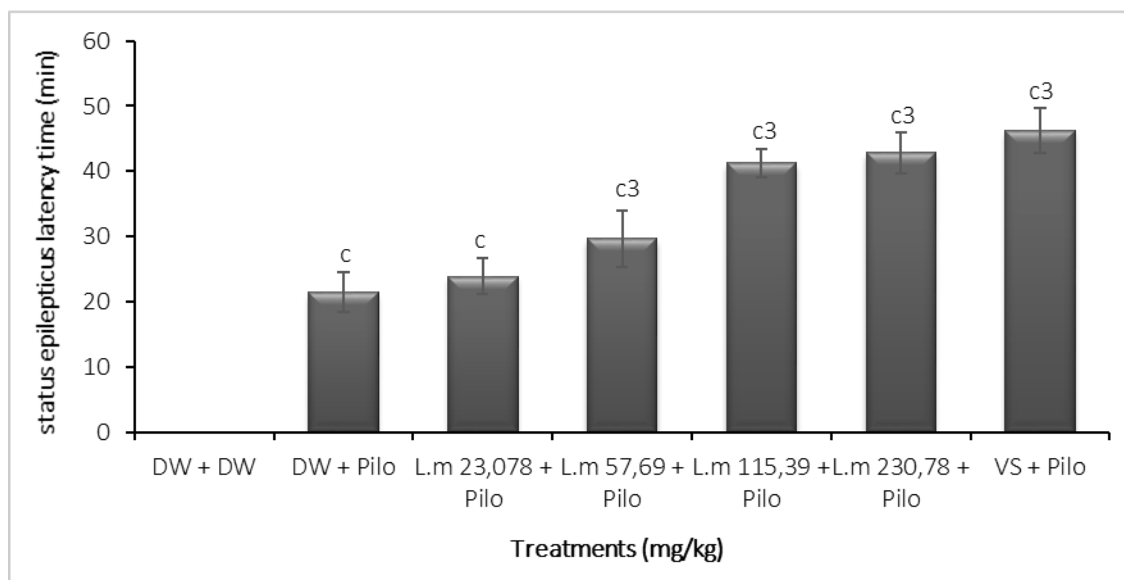

Figure 2 Effects of *Lippia multiflora* on pilocarpine-induced *status epilepticus* latency

Each bar represents the mean ± MSE of the group, n = 6. Data were analyzed with one-way ANOVA followed by Tukey's multiple post-test. $^{c}p<0.001$; significant difference compared to normal control (DW+DW) and $^{3}p<0.001$ compared to negative control (DW+pilo); DW: Distilled Water (10 mL/kg); PILO: Pilocarpine (400 mg/kg); Lm: *Lippia multiflora*; VS: Sodium Valproate (300 mg/kg) positive control;

3.3. Effects of *Lippia multiflora* on post-*status epilepticus* seizures induced by pilocarpine during the acute phase

3.3.1. Effects of Lippia multiflora on latency time of the first tonic-clonic seizure

Pilocarpine induced seizures manifested by the onset of tonic-clonic seizures in animals. The time of onset of the first tonic-clonic seizures increased significantly [F (6, 35) = 232.9, p<0.001] and in a dose-dependent manner (from 569.5 sec at a dose of 57.69 mg/kg to 1508.67 sec at a dose of 230.78 mg/kg) in mice treated with *L. multiflora* extract compared to the negative control in which this time was 41.17 sec. The same was true for sodium valproate, which delayed this onset time to 1520.67 seconds (Figure 3).

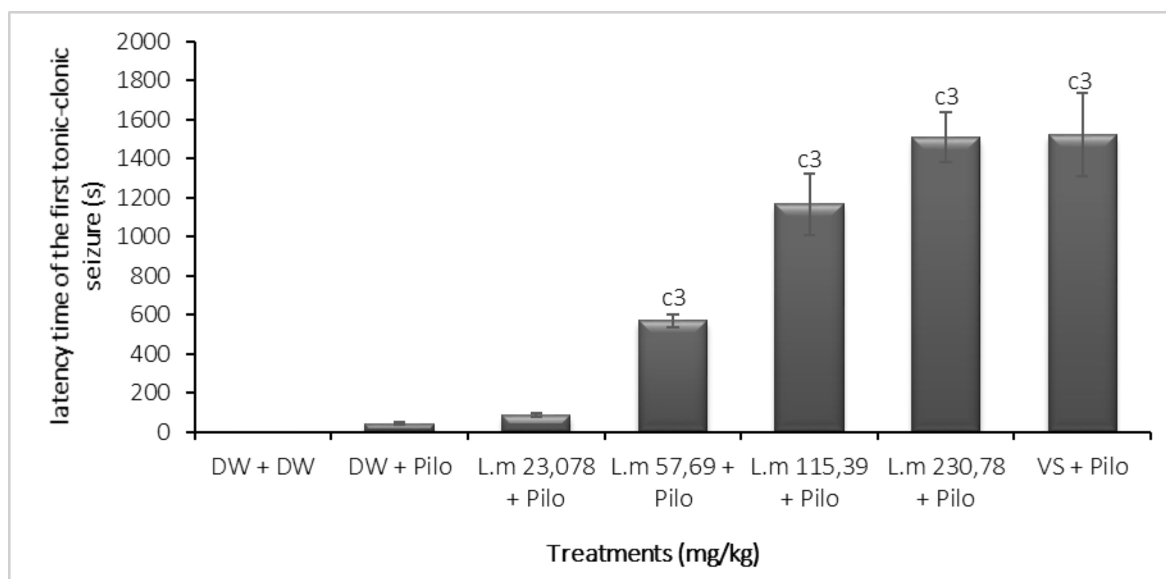

Figure 3 Effects of *Lippia multiflora* on latency time of the first tonic-clonic seizure

Each bar represents the mean ± MSE of the group, n = 6. Data were analyzed with one-way ANOVA followed by Tukey's multiple post-test. ^{c}p<0.001; significant difference compared to normal control (DW + DW) and ^{3}p<0.001 compared to negative control (DW + pilo); DW: Distilled water (10 mL/kg); PILO: Pilocarpine (400 mg/kg); Lm: *Lippia multiflora*; VS: Sodium Valproate (300 mg/kg) positive control;

3.3.2. Effects of Lippia multiflora on number and duration of clonic seizures induced by pilocarpine

The numbers of clonic convulsions increased (p<0.001) in the negative control compared to the normal control in which this number is zero. The aqueous extract of *L. multiflora* and sodium valproate therefore caused a reduction [F (6, 35) = 95.02 p<0.001] respectively in this number compared to the negative control (Figure 4).

A significant increase (p<0.001) in the duration of clonic convulsions was observed in the negative control group (56.33 sec) compared to the normal control. Aqueous extract of *L. multiflora* caused a significant decrease [F (6, 35) = 251.8, p<0.001] in the duration of clonic seizures from 29.83 sec (23.078 mg/kg) to 7.17 sec (230.78 mg/kg) compared to the negative control. Sodium valproate also decreased this time to 6.17 sec (Figure 4).

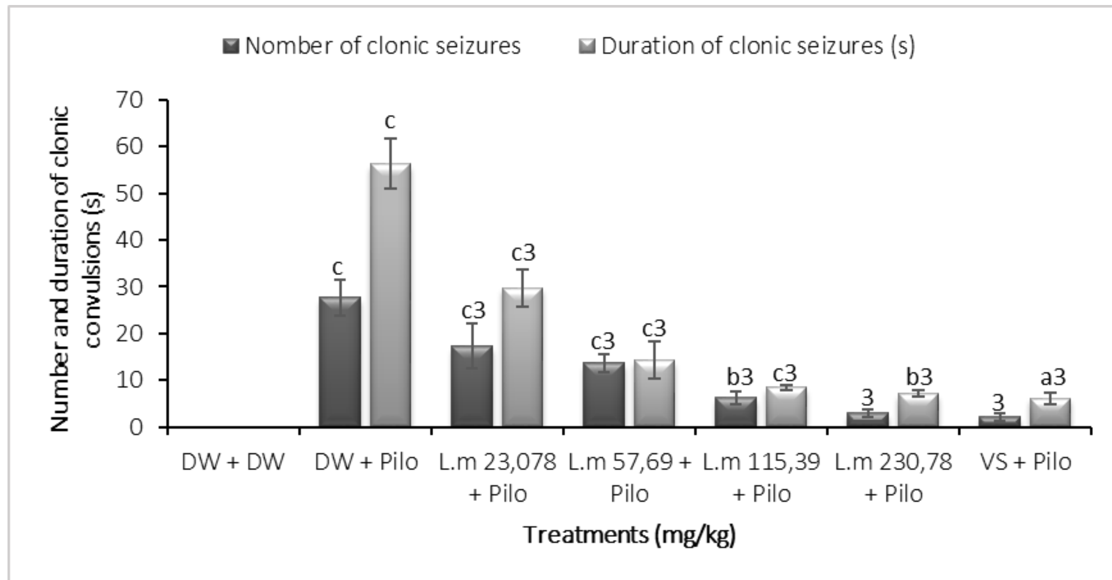

Figure 4 Effects of *Lippia multiflora* on number and duration of clonic convulsions induced by pilocarpine

Each bar represents the mean ± MSE of the group, n = 6. Data were analyzed with one-way ANOVA followed by Tukey's multiple post-test. [a]p<0.05, [b]p<0.01, [c]p<0.001; significant difference compared to normal control (DW + DW) and [3]p<0.001 compared to negative control (DW + pilo); DW: Distilled water (10 mL/kg); PILO: Pilocarpine (400 mg/kg); Lm: *Lippia multiflora*; VS: Sodium Valproate (300 mg/kg) positive control;

3.3.3. Effects of Lippia multiflora on number and duration of tonic seizures induced by pilocarpine

The numbers of tonic convulsions increased ($p<0.001$) in the negative control compared to the normal control in which this number is zero. The aqueous extract of *L. multiflora* and sodium valproate therefore caused a reduction F (6, 35) = 120.8, $p<0.001$] in this number compared to the negative control.

Aqueous extract of *L. multiflora* significantly [$F (6, 35) = 150.2$, $p<0.001$] reduced the duration of tonic convulsions to 8.17 ± 0.75 sec (57.69 mg/kg) up to 3.83 ± 0.75 sec (230.78 mg/kg) compared to the negative control (12.83 sec). The same was true for sodium valproate, which reduced this time to 3.5 ± 0.84 sec (Figure 5).

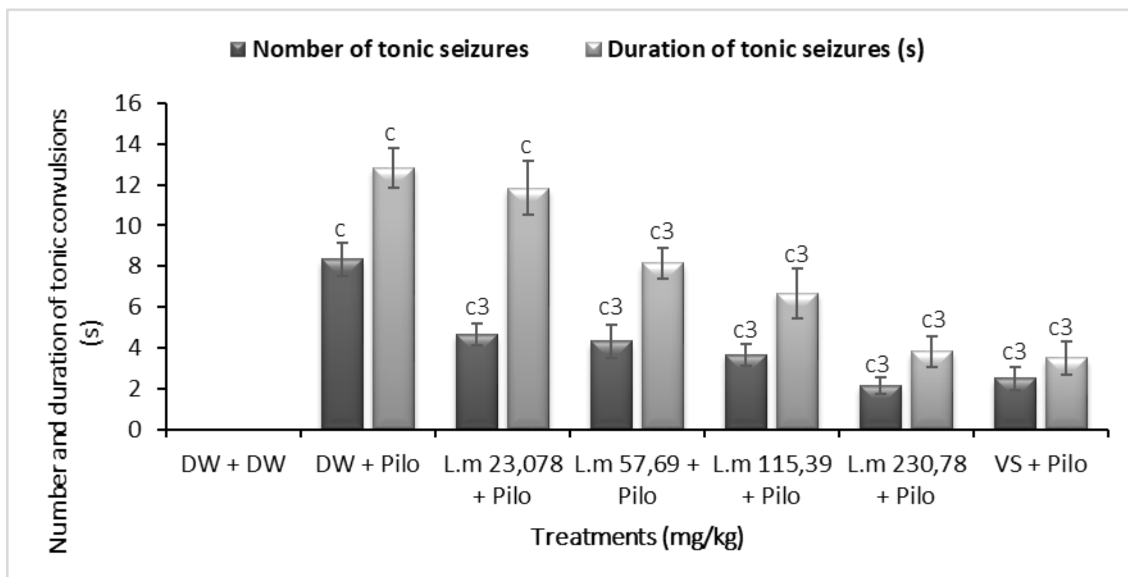

Figure 5 Effects of *Lippia multiflora* on number and duration of tonic convulsions induced by pilocarpine

Each bar represents the mean ± MSE of the group, n = 6. Data were analyzed with one-way ANOVA followed by Tukey's multiple post-test. [c]p<0.001; significant difference compared to normal control (DW + DW) and [3]p<0.001 compared to negative control (DW + pilo); DW: Distilled water (10 mL/kg); PILO: Pilocarpine (400 mg/kg); Lm: *Lippia multiflora*; VS: Sodium Valproate (300 mg/kg) positive control;

3.4. Effects of *Lippia multiflora* on pilocarpine-induced seizure score in the acute phase

Pilocarpine significantly (p<0.001) reduced the seizure score to 0 in the negative control compared to the normal control which was 1 (Figure 2). Aqueous extract of *L. multiflora* thus increased (p<0.001) this score from 0.52 (at 23.078 mg/kg) to 0.97 (230.78 mg/kg dose) compared to the negative control. Sodium valproate also increased (p<0.001) this score to 0.97 (Figure 6).

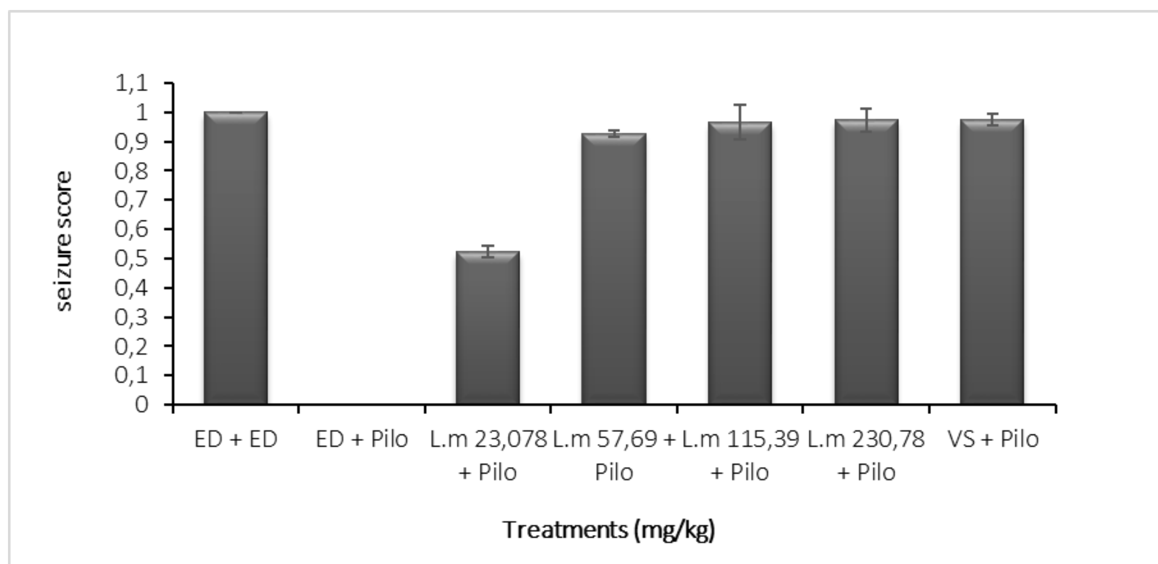

Figure 6 Effects of *Lippia multiflora* on pilocarpine-induced seizure scores

Each bar represents the mean ± MSE of the group, n = 6. Data were analyzed with one-way ANOVA followed by Tukey's multiple post-test. $^{c}p<0.001$; significant difference compared to normal control (DW + DW) and $^{3}p<0.001$ compared to negative control (DW + pilo); DW: Distilled water (10 mL/kg); PILO: Pilocarpine (400 mg/kg); Lm: *Lippia multiflora*; VS: Sodium Valproate (300 mg/kg) positive control;

3.5. Effects of *Lippia multiflora* on GABA and GABA-transaminase activity

Figure 7 shows the effects of *L. multiflora* on GABA and GABA-transaminase levels in the hippocampus of mice during pilocarpine-induced epileptogenesis. It results from this figure that the amount of GABA in the hippocampus of control batch mice was reduced to 267.667 µg/g tissue (32.52% reduction and at p<0.001) significantly compared to the normal control whose amount is 396.667 µg/g tissue. Oral administration of *L. multiflora* extract induced a significant increase of 9.21% (p<0.05) in mice at 23.078 mg/kg dose and also 20.61, 33.87 and 43.52% [F (7, 40) = 91.44, p<0.001] of this amount respectively in the 57.69, 115.39 and 230.78 mg/kg dose groups compared to the negative control. Sodium valproate also induced this increase by 46.38 compared to the negative control.

The amount of GABA-transaminase was significantly increased by (p<0.001) in the negative control compared to the normal control and ranged from 44.833 ± 0.94 pg/min/mg tissue in the latter to 108.833 ± 5.16 pg/min/mg tissue in the negative control. *L. multiflora* induced a dose-dependent decrease in this activity of 26.18, 35.37, 42.42, and 59.26% [F (7, 40) = 150.8, p<0.001] in mice in the 23.078, 57.69, 115.39, and 230.78 mg/kg dose groups respectively, compared with the negative control. Sodium valproate also induced a significant decrease in this activity of 60.34% (p<0.001) compared to the negative control.

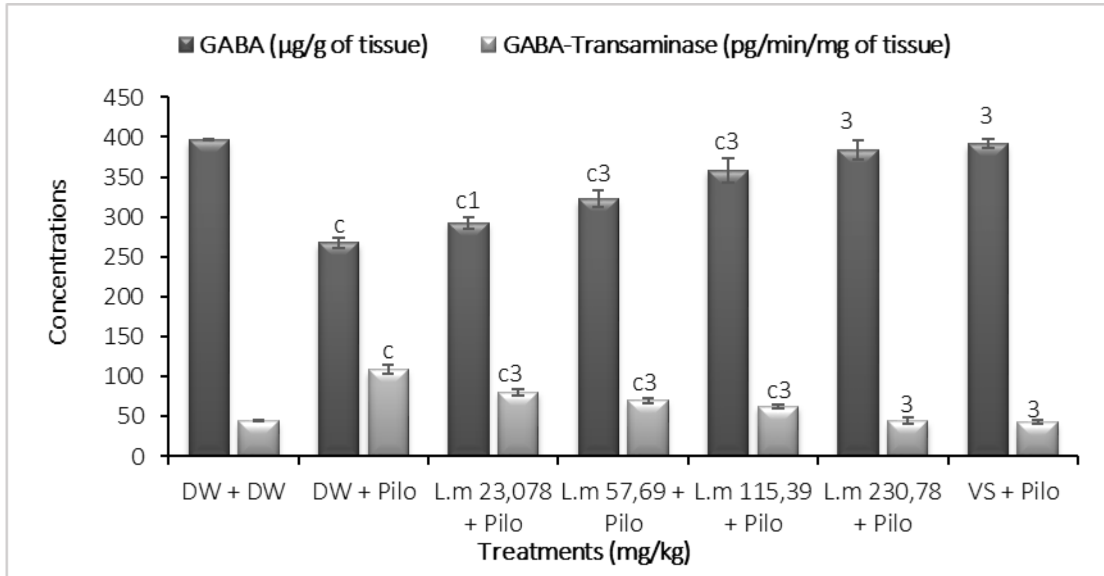

Figure 7 Effects of *Lippia multiflora* on GABA and GABA-transaminase activity

Each value represents the mean±SME of the group, n=6. Data were analyzed with one-way ANOVA followed by Tukey's multiple post-test. [c]p<0.001; significant difference compared to normal control (DW + DW) and [1]p<0.05, [3]p<0.001 compared to negative control (DW + pilo). DW: Distilled water (10 mL/kg); PILO: Pilocarpine (400 mg/kg); Lm: *Lippia multiflora*; VS: Sodium Valproate (300 mg/kg) positive control;

3.6. Effects of *Lippia multiflora* on the activity of oxidative stress markers and enzymes

3.6.1. Effects of Lippia multiflora on the activity of superoxide dismutase (SOD) and malondialdehyde (MDA)

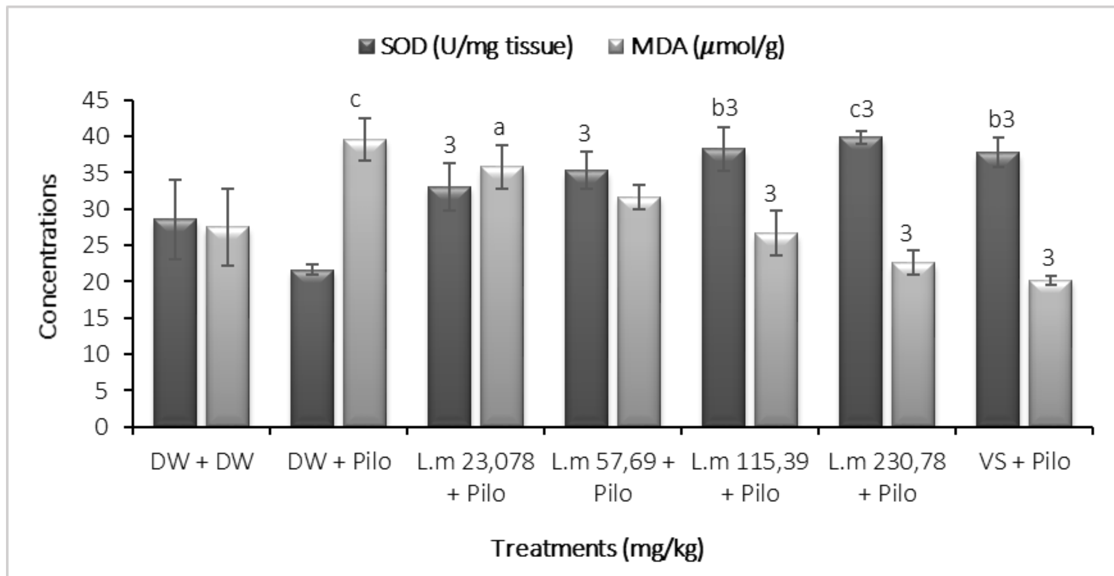

Figure 8 Effects of *Lippia multiflora* on the activity of superoxide dismutase and malondialdehyde

Each value represents the mean±SME of the group, n=6. Data were analyzed with one-way ANOVA followed by Tukey's multiple post-test. [a]p<0.05, [b]p<0.01, [c]p<0.001; significant difference compared to normal control (DW + DW) and [3]p<0.001 compared to negative control (DW + pilo). DW: Distilled water (10 mL/kg); PILO: Pilocarpine (400 mg/kg); Lm: *Lippia multiflora*; VS: Sodium Valproate (300 mg/kg) positive control; SOD: Superoxide Dismutase; MDA: Malondialdehyde.

The concentration of superoxide dismutase (SOD) was decreased in the negative control compared to the normal control. The plant extract induced a significant [F (7, 40) = 11.07, $p<0.001$] and dose-dependent increase in this concentration compared to the negative control. Sodium valproate also induced an increase ($p<0.001$) in this concentration to 37.79 ± 2.08 U/mg tissue compared to the negative control. The effect of *L. multiflora* at 115.39 and 230.78 mg/kg exceeded that of valproate in which this concentration is 38.34 ± 3.00 U/mg tissue (Figure 8).

Pilocarpine during epileptogenesis induces a significant ($p<0.001$) increase in malondialdehyde (MDA) levels. *L. multiflora* extract induced a significant [$F (7, 40) = 12.81$, $p<0.001$] decrease of 26.68 ± 3.04 and 22.56 ± 1.69 U/mg tissue respectively at 115.39 and 230.78 mg/kg compared to the negative control. The same is true for sodium valproate which induced a decrease in this rate in a very significant way ($p<0.001$) compared to the negative control (Figure 8).

3.6.2. Effects of Lippia multiflora on the activity of catalase (CAT), reduced glutathione (GSH) and nitric oxide (NO)

The concentration of catalase decreased significantly to 0.20 ± 0.05 U/mg tissue in the negative control compared to the normal control, which had a level of 0.31 ± 0.10 U/mg tissue. This concentration increased with the plant extract and was significant [$F (7, 40) = 4.186$, $p<0.01$] at 115.39 and 230.78 mg/kg compared to the negative control. Sodium valproate also increased this concentration significantly ($p<0.01$) compared to the negative control (Figure 9).

GSH concentration decreased during epileptogenesis in the negative control compared with the normal control. In contrast to the negative control, this concentration increased [$F (7, 40) = 3.762$, $p<0.001$] in a dose-dependent manner with administration of the aqueous extract of *L. multiflora* as well as with sodium valproate (Figure 9).

The effect of pilocarpine in mice given only distilled water was reflected in an increase ($p<0.001$) in nitric oxide concentration compared with the normal control. This concentration increased from 2.06 ± 0.35 U/mg tissue in the normal control to 4.02 ± 0.19 U/mg tissue in the negative control. All doses of the plant extract significantly decreased this concentration but 115.39 mg/kg and 230.78 mg/kg decreased [$F (7, 40) = 11.98$, $p<0.001$] to 2.36 U/mg tissue and 2.01 U/mg tissue respectively compared to the negative control. Sodium valproate decreased ($p<0.001$) this concentration to 2.17 U/mg tissue compared to the negative control (Figure 9).

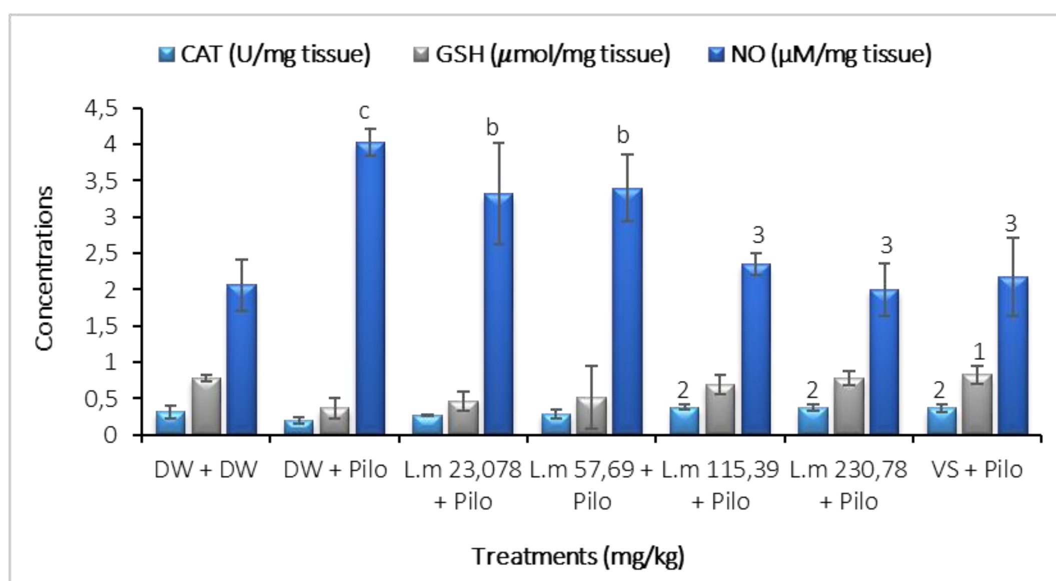

Figure 9 Effects of *Lippia multiflora* on the activity of catalase, reduced glutathione and nitric oxide

Each value represents the mean±SME of the group, n=6. Data were analyzed with one-way ANOVA followed by Tukey's multiple post-test. [b]$p<0.01$, [c]$p<0.001$; significant difference compared to normal control (DW + DW) and [1]$p<0.05$; [2]$p<0.01$, [3]$p<0.001$ compared to negative control (DW + pilo). DW: Distilled water (10 mL/kg); PILO: Pilocarpine (300 mg/kg); Lm: *Lippia multiflora*; VS: Sodium Valproate (300 mg/kg) positive control; CAT: Catalase; GSH: Reduced Glutathione; NO: Nitric oxide.

4. Discussion

The results obtained show that the aqueous extract of *L. multiflora* increased the latency time of *status epilepticus* (SE) and the latency and score of seizures. These results suggest that the plant extract would effectively inhibit pilocarpine-induced SE and thereby have a preventive effect on nerve cell damage in the hippocampus. Induction of SE by pilocarpine also leads to severe and widespread cell loss in several areas of the hippocampus [28]. Indeed, during status epilepticus, pilocarpine acts on the muscarinic M1 receptor by activating phospholipase C which produces diacylglycerol and inositol triphosphate which will allow Ca^{2+} ions to enter the cell and increase brain excitability [29]. The high concentration of Ca^{2+} favors the release of glutamate inducing the *status epilepticus* with the consequent activation of lipases, proteases and nucleases that will cause the death of nerve cells by necrosis or apoptosis [30]. The

effects of *L. multiflora* at high dose (230.78 mg/kg) are similar to those of sodium valproate, a reference antiepileptic drug that acts on cation channels by blocking T-type (transient) calcium channels and voltage-dependent sodium channels in thalamic neurons, which also promotes GABA action. The reduction of seizures could be due to the presence of certain compounds such as triterpenoids, saponins, and phenolic compounds in the extract of *L. multiflora*, which would antagonize the effect of glutamate by suppressing the activity of the neurons that release glutamate. Triterpenoids and phenolic compounds protect animals from tonic and clonic seizures [31] and possess significant GABA-T inhibitory activity [32]. Saponins have been reported to possess inhibitory action on different types of Ca^{2+} channels [33]. Thus, the protection against pilocarpine-induced tonic and clonic seizures in mice suggests that the aqueous extract of *L. multiflora* would possess antiepileptic properties against partial epilepsy and particularly temporal lobe epilepsy [34]. Anticonvulsant, anxiolytic and sedative (e.g. sodium valproate) are known to exert their pharmacological action by causing an increase in GABA acid content in mice cerebral hemisphere [35]. This plant would also have effects on the inhibition of voltage-gated sodium channels and the increase of GABA concentration in synapses facilitating GABAergic neurotransmission [36]. It was found that the aqueous extract of *Lippia multiflora* and sodium valproate significantly enhanced the brain GABA concentration, which again is suggestive of an anticonvulsant action of the extract and the reference drug. Such substances have been shown to possess anticonvulsant and antiepileptic properties in several animal models of epilepsy [37]. Histochemical studies have shown that in the absence of sufficiently efficient antioxidant systems, an overproduction of free radicals is capable of causing lesions due to the cytotoxic and mutagenic character of the metabolites resulting from oxidative stress, in particular from lipid oxidation [38]. Thus, the rather high oxidative stress in people with epilepsy has serious consequences on their lifestyle [39]. This stress is reflected in the increase of MDA and NO in negative control mice. The increase in MDA is a marker of lipid peroxidation [40]. Furthermore, there was a significant decrease ($p<0.001$) in the activity of these markers in mice treated with aqueous extract of *L. multiflora* at the doses of 115.39 and 230.78 mg/kg. These results are related to those of Taiwé et *al*. [34] who hold the fact that substances that antagonize the activity of lipid peroxidation markers in the cell have anti-oxidative properties. An increase in superoxide dismutase, catalase, and reduced glutathione activity in mice treated with aqueous extract of *L. multiflora* was observed. Animals with SE or spontaneous seizures have reduced SOD activity and increased levels of hydroperoxides (products of lipid peroxidation) in the hippocampus of animals with the pilocarpine model of epilepsy [41]. As the brain is vulnerable compared to other tissues, the reduced SOD activity could be related to the cell death and brain damage that was found in the hippocampus of these animals [30]. Catalase and reduced glutathione are antioxidants that prevent the production of reactive oxygen species by limiting the initiation phase of oxidation reactions. The increase in the level of reduced glutathione would be due to the effects of the extract of *L. multiflora* which would have transformed the reactive free radicals into stable compounds in the hippocampus thanks to the presence of compounds such as alkaloids, polyphenols, flavonoids, tannins which play the role of scavengers of the free radicals [42]. These substances have antioxidant properties [43] playing a neuroprotective role by preventing lipid peroxidation of neuronal cell membranes.

5. Conclusion

The objective of this study was to evaluate the effects of aqueous extract of *L. multiflora* on complex partial recurrent seizures of temporal lobe epilepsy induced by acute administration of pilocarpine. It appears that the extract of *L. multiflora* prevents the appearance of convulsive seizures by increasing the latency time of *status epilepticus* and reduces tonic and clonic seizures induced by pilocarpine. The study also revealed that the extract could act on the GABAergic system by the potentiation of GABAA receptors and the increase of the activity of GABA receptors, the inhibition of glutamatergic transmission at the level of the neurons and thus decreasing seizure activity. Moreover, this plant prevents the peroxidation of membrane lipids by decreasing the level of stress markers (MDA and NO) and increases the level of oxidative stress enzymes (SOD, CAT and GSH). All these results confirm its anticonvulsant, antiepileptogenic and antioxidant effects.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

Statement of ethical approval

The protocols were performed in concordance with the International Guide for the Care and Use of Laboratory Animal (National Institute of Health; publication No. 85-23, revised 1996) and the Cameroon National Ethical Committee, Yaounde (No. FW-IRB00001954).

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