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Anticonvulsant Effects of *Chrysanthellum americanum* L. (Vatke) Aqueous Extract in Mice Pilocarpine Model of Epilepsy and Associated Memory Impairment: Role of Antioxidant Defense System and Cholinergic Transmission

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Abstract

Chrysanthellum americanum (L.) Vatke is a medicinal plant used by the traditional healers to treat epilepsy and associated memory impairment. This work aims at evaluating the anticonvulsant effects of *Chrysanthellum americanum* aqueous extract in mice pilocarpine model of epilepsy and associated memory loss. Mice were administered orally *Chrysanthellum americanum* aqueous extract (27.69, 69.22, 138.45, 276.9 mg/kg, prepared from the whole part) for test groups, intraperitoneally 300 mg/kg sodium valproate for the positive control group or orally 10 mL/kg distilled water for the negative control group, respectively, during a period of seven consecutive days. On the first day, temporal lobe epilepsy was induced by intraperitoneal injection of 360 mg/kg pilocarpine one hour after the administration of different treatment to mice, and the occurrence of status epilepticus was evaluated. On the second day, the anticonvulsant property was measured after the intraperitoneal injection of a sub-convulsive dose of picrotoxin (1 mg/kg). On the seventh day, the anti-amnesic properties of the extract were evaluated in the epileptic mice using the T-maze and open field paradigms. The results show that *Chrysanthellum americanum* extract significantly ($p < 0.01$) increased the latency to clonic and generalized clonic-tonic convulsions, and decreased the number and duration of seizures. The plant extract induced a significant ($p <$

0.001) increase in the number of entries and time spent into preferred arm of the T-maze test. The number of crossing and the time spent in the centre of the open field were increased by the extract. *Chrysanthellum americanum* (276.9 mg/kg) likewise sodium valproate (300 mg/kg) significantly ($p < 0.01$) alleviated pilocarpine-induced increase in the hippocampal malondialdehyde and nitric oxide levels; increased the reduced glutathione and acetylcholine rates, and the activities of catalase and super oxide dismutase. In summary, these results indicate that *Chrysanthellum americanum* aqueous extract has anticonvulsant effects against pilocarpine induced-epileptic seizures and memory impairment. These properties could be mediated by the amelioration of antioxidant defense system and cholinergic neurotransmission in epileptic mice, which could partly justify the use of *Chrysanthellum americanum* in the traditional medicine for the treatment of epilepsy.

Keywords

Chrysanthellum americanum, Epilepsy, Memory Impairment, Oxidative Stress, Cholinergic Transmission

1. Introduction

Epilepsy is a chronic disease considerably affecting the central nervous system which induces abnormal brain functions directly depending on the different implicated brain region; consequently, causing epileptic seizures and associated behavioural disorders. These behavioural disorders can be categorised as memory impairment, depressions or anxiety [1] [2]. In approximately 70 million people that are affected by epilepsy worldwide, about 85% live in developing countries. The incidence of epilepsy in Sub-saharan Africa is 64 to 215/1000 [3]. Inappropriately, despite the availability of a diverse array of antiepileptic drugs, approximately half of the patients treated with modern medications continue to experience seizures [4]. A significant number of epilepsy patients in low- and middle-income countries do not receive therapy. In the adult mammalian brain, acetylcholine is one of the main excitatory neurotransmitters. It modifies neuronal excitability, affects synaptic transmission, promotes synaptic plasticity, and synchronises the firing of neural networks [5]. Its perturbations, particularly in the synapse culminated hyperexcitability in the postsynaptic neurons and sustained seizure activity with consequential excitotoxicity and neuronal cell death.

However, the several heavy comorbidities accompany this disease such as memory impairment. These abnormalities are related to multiple factors, including seizure type, age of onset, location of focus, and seizure frequency [6]. Another major factors that can affect cognition are antiepileptic drugs as chronic use of most antiepileptic drugs (AEDs) has been shown to predispose to cognitive impairment [7]. Moreover, for the use of existing treatments, convulsions still occur in 30% of people [8] [9]. As a result, research is being carried out to find a medical alternative from natural plant extracts. Because plants today constitute a

natural bank of bioactive constituents thanks to the secondary metabolites that it abounds.

Chrysanthellum americanum (L.) Vatke is a plant that belongs to the Asteraceae family. It is an herbaceous that grows in the tropical zone of Africa from Senegal to Nigeria and tropical America from southern Mexico to northern Brazil preferring the wastelands, dry and rocky [10]. This plant extract is used in west-African traditional medicine, known for its flavonoid and saponin richness and for its strong antioxidant potential [11]. Most of the therapeutical properties of medicinal plant are attributed to the presence of active compounds, such as: chrysanthellin A and B, from the family of saponins; and luteolin 7-O-glucoside, eriodictyol 7-O-glucoside, isookanin 7-O-glucoside or flavonomarein, okanin 4-O-glucoside or marein, and maritimetin 6-O-glucoside or maritimein from the family of flavonoids, respectively [12]. In Cameroonian traditional medicine the whole plant of *Chrysanthellum americanum* is used as a decoction for the treatment of epilepsy, schizophrenia, memory impairment, depression and infantile convulsions [10]. Till date, study reporting on the experimental or clinical efficacy of *Chrysanthellum americanum* aqueous extract in the treatment of temporal lobe epilepsy and associated comorbidities is not clarified. However, a six-day oral administration of *Chrysanthellum americanum* polyphenolic extract is reportedly effective after mood and cognitive disturbances related to stress-induced in an irritable bowel syndrome rat model [13].

The aim of this study was to evaluate the effects of an aqueous extract of *Chrysanthellum americanum* on pilocarpine-induced epilepsy, cognitive impairment and oxidative stress in mice. Its effects on brain butyrilcholinesterase and acetylcholinesterase activity and the regulation of acetylcholine rate were also evaluated.

2. Materials and Methods

2.1. Plant Material

The whole plant of *Chrysanthellum americanum* was collected from Garoua, the headquarter of the North Region of Cameroon, in April 2019. The species was identified at the National Herbarium of Cameroon where the voucher specimen was deposited under the reference number 7728/SRF/Cam.

2.2. Preparation of the Aqueous Extract

The plant extract used in the different experiments was obtained as follow: 10 g of dried whole plant of *Chrysanthellum americanum* were boiled in 100 mL distilled water for 20 min. The supernatant was collected, filtered using a Whatman paper N˚1 to obtain 66.52 mL. This 66.52 mL is the stock solution used at different concentrations in the present study. The stock solution corresponds to a concentration of 27.69 mg/mL, that is 1.842 g of extract in 66.52 mL distilled water and represent a 5.43% yield. The extract was administered orally (*per os* (*p.o.*)) to mice at the volume of 10 mL/kg, 1 h before the experiment [14]. The

following doses of the extract were used: 27.69, 69.22, 138.45 and 276.9 mg/kg.

2.3. Preliminary Phytochemical Analysis

Preliminary and qualitative phytochemical characterisation of the decoction of *Chrysanthellum americanum* was done using methods already described for the determination of alkaloids, anthraquinones, flavonoids, glycosides, phenols, glycosides, saponins, sterol, coumarins and tannins [15] [16] [17].

2.4. Drugs and Chemicals

Sodium valproate was obtained from SANOFI, AVENTIS, France. Ethyl acetate, ethylenediamineteraacetic acid, glutamic acid, n-butanol, nicotinamide adenine dinucleotide, nitro blue tetrazolium, N-naphthyl ethylene diamine, pilocarpine, picrotoxin, pyridine, pyridoxal phosphate, sodium dodecyl sulphate, sodium chloride, sulfanilamide, thiobarbituric acid, tris-HCl, α-oxoglutarate and 5'5-dithiobis (2-nitrobenzoic acid), and all the other reagents used for biochemical analyses were purchased from Sigma Chemical-Corporation, USA.

2.5. Animal

Adult male mice (Mus musculus Swiss; 20 - 25 g; 6 per group) were used for this experiment. The animals were housed in standard cages at 25˚C on a 12 h light-dark cycle. They were supplied with food and water ad libitum. These animals were purchased from the National Veterinary Laboratory (LANAVET) in Garoua (North - Cameroon) and then acclimatised in the animal house of the University of Ngaoundere, Cameroon. The study was carried out in accordance with the Cameroon National Ethical Committee (Ref No. FW-IRB00001954, 22 October 1987). The authorization number (UB-IACUC N˚06/2022) was given and the study was done also in conformation with the international regulation, minimizing the number of mice used and their suffering. The mice were organized into eight groups of six animals each.

2.6. Pharmacological Analysis

2.6.1. Pilocarpine Induced Status Epilepticus in Mice and Anticonvulsant Test

Animal were randomly grouped into eight groups of six animals each. The first day of experiment, animals were treated with distilled water (10 mL/kg; p.o.) for the normal group and the negative control group, the aqueous extract of *Chrysanthellum americanum* (27.69, 69.22, 138.45, 276.9 mg/kg; *p.o.*) for the test groups, sodium valproate (300 mg/kg; intraperitoneally, i.p.) and piracetam (200 mg/kg; *p.o.*) for the positives controls groups, respectively. To reduce the peripheral effects of pilocarpine, forty min after the administration of the various treatments to mice, they were injected intraperitoneally by a single dose of scopolamine (1 mg/kg) [18]. Thereafter, twenty minutes later, *status epilepticus* characterised by tonic and clonic convulsions, were induced to mice by intraperitoneal injection of pilocarpine (360 mg/kg) except the normal and positive control group which

received the piracetam. Seizure severity of *status epilepticus* was measured in mice immediately after the injection of pilocarpine according to the Racine scale ranging from zero to five [19], the strength of the convulsions was recorded as no response (Phase 0); vibrissae twitching, restlessness, and hyperactivity (Phase 1); myoclonic jerks, clonus, and head nodding (Phase 2); bilateral or unilateral limb clonus (Phase 3); clonic seizures of forelimbs (Phase 4); and generalized tonic-clonic seizures and falling (Phase 5), this during a duration of six hours [20]. The latency time of *status epilepticus* was evaluated 24 h after administration of pilocarpine and one hour after administration of the various treatments, the following parameters were evaluated; the number and durations of tonic and clonic seizures in each mouse for a period of 30 min (**Figure 1**). Latency times are expressed as scores, calculated as follows: Score = 1-Latency of tonic-clonic seizures in the negative group [21].

To evaluate the anticonvulsant test, twenty-three hours after the induction of *status epilepticus* with intraperitoneal injection of pilocarpine, animals received once again the different treatments (with distilled water for the normal group (lot 1) and the negative control group (lot 2), the plant extract (lots 3 to 6) for the test groups, sodium valproate (lot 7) and piracetam (lot 8), respectively). After 1 h, a sub-convulsive dose of picrotoxin (1 mg/kg) were injected intraperitoneally to mice and the behavioural parameters were evaluated for each mouse for a period of 30 min (**Figure 1**). The parameters observed were the latency time to the first tonic-clonic seizures, the duration of tonic-clonic seizures, and the number of tonic-clonic seizures. From the third to the seventh day, animals received the respective treatments as on the second day [22].

2.6.2. Behavioural Tests in Pilocarpine-Treated Mice Seven Day after the Induction of Status Epilepticus

1) Memory test in the T-maze

T-maze is device that is consists of a departure compartment and two arrival corridors measuring 30 cm long, 10 cm wide and 25 cm high. Animals were placed one after the other for a period of five min in the starting arm of the T-maze one hour after the administration of the different substances. This test takes place in three phases: habituation, acquisition, and retention. In the first phase or habituation phase, the mice are familiarized with the device [23]. The parameters recorded are the latency time to choose one of the arms (the arm that constitutes the animal's preferred arm throughout the test), the time spent and the number of entries in the preferred arm and the discriminated arm, and finally the number of returns in the starting arms. The second phase, or acquisition phase, begins 24 hours after the habituation phase. The following parameters are noted: the latency time, the time spent and the number of entries into the preferred arms the number of returns to the starting arm. Finally comes the retention phase 24 hours after the acquisition phase. The parameters recorded are the latency time to find the preferred arm, the time spent and the number of entries in the preferred arm and the discriminated arm, the number of returns to the starting arm [23].

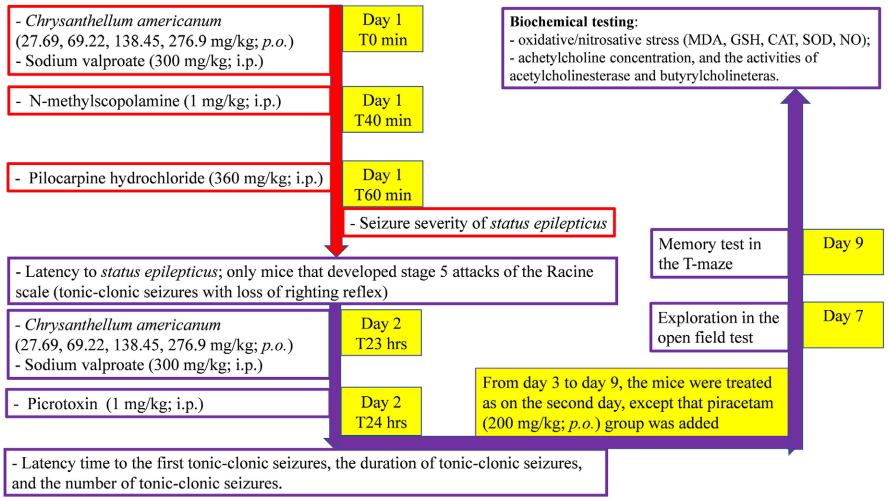

Figure 1. Schematic representation of the pharmacological analysis of *Chrysanthellum americanum*. Malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), Nitrite Oxide (NO).

2) Exploration, locomotion and motor coordination of mice in the open field

The device used was similar to that described by Taiwe *et al.* [24]. It was a square enclosure (40 × 40 cm), and 45 cm high. The exploration surface was divided into 17 squares of equal dimensions (10 × 10 cm): 16 squares which divided the interior surface, and one central square. After passing through the T-maze, the mice were immediately introduced into the open field. The test consisted of placing the mice one after the other in the centre of the device, so as to allow them free exploration. The number of "crossings", the number of "grooming", the number of "rearing", the time spent in the centre and the mass of the stools produced in the device were noted for a period of 5 min for each animal. After each observation, the mouse was returned to its cage, then the device was cleaned with ethyl alcohol (70˚C).

3) Biochemical tests

At the end of the open field test, all the animals were sacrificed by cervical decapitation, and their hippocampus were removed, dissected and introduced into tubes containing Tris buffer (1 mL, 50 mM HCl, 150 mM KCl, pH 7.4). Thereafter, the mixtures were centrifugated at 10,000 rpm for 15 minutes duration using an Eppendorf centrifuge 5810/5810 R series. The supernatant of each tube was pipetted and introduced into Eppendorf tube which was then stored at −20˚C for biochemical assays. Biochemical determinations were performed by using a microplate spectrophotometer/spectrofluorometer (ThermoFisher Scientific, Multiskan™ FC,) and/or a standard spectrophotometer (ThermoFisher Scientific, NanoDrop™ Lite).

a) Nitrosative and oxidative stresses assay in the hippocampus

Biomarkers of oxidative stress were assay in hippocampi homogenates using established and previously described protocols: malondialdehyde (MDA) with

thiobarbituric acid [25] [26], reduced glutathione (GSH) with 2,2'-dithio-5,5'-dinitrobenzoic acid [27], superoxide dismutase (SOD) with adrenaline [28], catalase (CAT) with Hydrogen peroxide [29] and nitrite with griess reagent [30] [31], respectively. Concentrations or activities were measured in triplicate.

b) Achetylcholine concentration and the activities of acetylcholinesterase and butyrylcholineterase in the hippocampus

Achetylcholine concentration was determined using Hestrin's method with trichloroacetic acid [32]. The activities of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were estimated according to the method of Ellman respectively with acetylthiocholine iodide and butyrylthiocholine iodide [26] [33]. Concentrations or activities were measured in triplicate.

2.7. Statistical Analysis

Data were expressed as mean ± standard error of the means (S.E.M.) per group. Statistical differences between control and treated groups were tested by the analysis of variance (ANOVA), followed by Tukey's post hoc multiple comparison test. The differences were considered significant at $P < 0.05$. The statistical package used for the analysis was Graphpad Prism 5.01 for Window (Graphpad Prism Software, San Diego, CA, USA.

3. Results

3.1. Phytochemical Constituents of *Chrysanthellum americanum* Aqueous Extract

The preliminary phytochemical assays revealed that *Chrysanthellum americanum* aqueous extract contains alkaloids, anthraquinones, flavonoids, glycosides, phenols, glycosides, saponins, coumarins, sterol and tannins are also present.

3.2. Effects of *Chrysanthellum americanum* Aqueous Extract on the Latency Time of Status Epilepticus, The Number and Duration of Clonic Seizures, The Number and Duration of Clonic-Tonic Seizures and Score of Seizures in Pilocarpine-Treated Mice

Intraperitoneal injection of pilocarpine induced s*tatus epilepticus* in mice. In contrast, all the animals from the normal group did no present *status epilepticus*. The latency time of *status epilepticus* is 21.09 ± 2.82 sec in the negative control group. This latency time significantly increased to 31.45 ± 3.80 sec ($p < 0.05$), 44.03 ± 2.90 sec ($p < 0.05$) and 45.27 ± 3.50 sec ($p < 0.05$) in the group of mice treated with the plant extract at the doses of 69.22, 138.45 and 276.9 mg/kg, respectively. Sodium valproate, an antiepileptic drug induced a significant ($p < 0.01$) increase of the latency of status epilepticus to 52.04 ± 4.35 sec (**Figure 2**).

From **Table 1**, it can be depicted that the latency to the first tonic clonic seizure significantly increased from 45.66 ± 5.57 sec to 653.33 ± 32.51 sec ($p < 0.01$), 1020.83 ± 155.60 sec ($p < 0.01$) and 1462.50 ± 112.11 sec ($p < 0.001$) in the

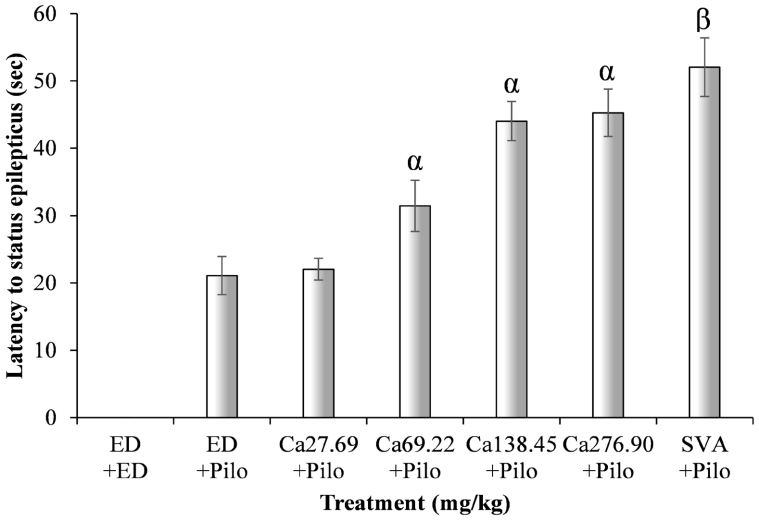

Figure 2. Effects of *Chrysanthellum americanum* aqueous extract the latency time of *status epilepticus*. Values are expressed as mean ± SEM (n = 6). $^{\alpha}p < 0.05$, $^{\beta}p < 0.01$ *versus* negative control group (ED + Pilo), ANOVA followed by Tukey's post hoc multiple comparison test. Ca27.69: 27.69 mg/kg *Chrysanthellum americanum* aqueous extract; ED: 10 mL/kg distilled water; Pilo: 360 mg/kg pilocarpine; SVA: 300 mg/kg sodium valproate.

Table 1. Effects of *Chrysanthellum americanum* aqueous extract on the latency time of status epilepticus, the number and the duration of clonic seizures, the number and duration of clonic-tonic seizures and score of seizures in pilocarpine-treated mice.

Treatments	Latency to the first tonic-clonic seizure (sec)	Number of clonic seizures	Duration of clonic seizures (sec)	Number of clonic-tonic seizures	Duration of clonic-tonic seizures (sec)	Score of seizures
ED + ED	---	---	---	---	---	1
ED + Pilo	45.66 ± 5.57	31.33 ± 4.36	56.33 ± 5.43	10.33 ± 0.82	13.16 ± 0.98	00 ± 00
Ca27.69 + Pilo	217.33 ± 51.91*	27.83 ± 1.16	29.83 ± 3.97*	8.16 ± 0.75	12.33 ± 1.21	0.79 ± 0.02*
Ca69.22 + Pilo	653.33 ± 32.51**	14.16 ± 1.47*	17.83 ± 1.16*	7.16 ± 0.75*	10.66 ± 0.81	0.93 ± 0.01*
Ca138.45 + Pilo	1020.83 ± 155.60**	6.50 ± 1.37**	13.83 ± 3.31**	5.16 ± 0.40 **	7.83 ± 0.75*	0.96 ± 0.06*
Ca276.90 + Pilo	1462.50 ± 112.11***	3.16 ± 0.75 ***	11.16 ± 1.60**	2.33 ± 0.51*	4.66 ± 0.51**	0.97 ± 0.04*
SVA + Pilo	1537.33 ± 90.60***	2.17 ± 0.75***	11.17 ± 1.8**	2.60 ± 0.51*	3.33 ± 0.51*	0.97 ± 0.02*

Values are expressed as mean ± SEM (n = 6). *p < 0.05, **p < 0.01, ***p < 0.001 versus negative control group (ED + Pilo), ANOVA followed by Tukey's post hoc multiple comparison test. Ca27.69: 27.69 mg/kg *Chrysanthellum americanum* aqueous extract; ED: 10 mL/kg distilled water; Pilo: 360 mg/kg pilocarpine; SVA: 300 mg/kg sodium valproate.

groups of mice administered respectively the doses of 69.22, 138.45 and 276.9 mg/kg *Chrysanthellum americanum* aqueous extract.

The number of clonic seizures significantly reduced from 31.33 ± 4.36 in pilocarpine-treated animals to 14.16 ± 1.47 ($p < 0.05$), 6.50 ± 1.37 ($p < 0.01$) and 3.16 ± 0.75 ($p < 0.001$) in the groups of mice treated by the respective doses of 69.22, 138.45 and 276.9 mg/kg *Chrysanthellum americanum* aqueous extract. The number of clonic seizures significantly reduced in pilocarpine-treated sodium valproate group to 2.17 ± 0.75 ($p < 0.001$) (Table 1).

The duration of clonic seizures significantly decreased from 56.33 ± 5.43 sec in pilocarpine-treated distilled water group to 17.83 ± 1.16 sec ($p < 0.05$), 13.83 ± 1.16 sec ($p < 0.01$) and 11.16 ± 1.60 sec ($p < 0.01$) in the test groups treated with the plant extract at the doses of 69.22, 138.45 and 276.9 mg/kg, respectively. Similarly, sodium valproate administered at a dose of 300 mg/kg induced a significant ($p < 0.01$) reduction in the duration of clonic seizures (Table 1).

The aqueous extract of *Chrysanthellum americanum* induced a significant reduction in the number of clonic-tonic seizures from 10.33 ± 0.82 in the negative control group of mice to 7.16 ± 0.75 ($p < 0.05$), 5.16 ± 0.40 ($p < 0.01$) and 2.33 ± 0.51 ($p < 0.05$) in the test groups treated with the respective doses of 69.22, 138.45 and 276.9 mg/kg (Table 1).

The duration of clonic-tonic seizures is 13.16 ± 0.98 sec in the negative control group of mice injected by pilocarpine and treated with distilled water. The plant extract administered at the doses of 138.45 and 276.9 mg/kg induced a significant reduction in the duration of clonic-tonic seizures from 13.16 ± 0.98 sec in the negative control group to 7.83 ± 0.75 ($p < 0.05$) and 4.66 ± 0.51 ($p < 0.01$), respectively. The duration of clonic-tonic seizures is significantly reduced to 3.33 ± 0.51 ($p < 0.05$) for the positive control group of mice administered the reference drug sodium valproate (300 mg/kg) (Table 1).

The score of seizures significantly increased from 00 ± 00 in the negative control group of mice to 0.93 ± 0.01 ($p < 0.05$), 0.96 ± 0.06 ($p < 0.05$) and 0.97 ± 0.04 ($p < 0.05$) in pilocarpine-treated *Chrysanthellum americanum* aqueous extract mice administered with the respective doses of 69.22, 138.45 and 276.9 mg/kg. The score of seizures is also ameliorated to 0.97 ± 0.02 ($p < 0.05$) in the positive control group of mice treated with 300 mg/kg of sodium valproate (Table 1).

3.3. Effects of *Chrysanthellum americanum* Aqueous Extract on Memory in Pilocarpine Treated-Mice Placed on the T-Maze

During the habituation phase, animals were allowed to explore the T-maze for 5 minutes duration. The preferred arm for each animal was identified based on the higher number of entries into the two arrival corridors of T-maze (Figure 3).

The latency time to reach preferred arm significantly increased from 73.33 ± 2.33 sec in the distilled water-treated pilocarpine mice to 27.17 ± 2.17 sec ($p < 0.05$) and 23.83 ± 2.17 sec ($p < 0.001$) in the test groups treated with the respective doses of 138.45 and 276.90 mg/kg *Chrysanthellum americanum* aqueous extract, during the acquisition phase. The number of entries into the preferred arm significantly ($p < 0.05$) decreased from 8.16 ± 0.67 in the normal group of mice treated only with distilled water to 1.67 ± 0.67 in the negative control group injected with pilocarpine (Figure 3(a)). *Chrysanthellum americanum* aqueous extract induced a significant increase of this number to 6.83 ± 0.55 and 5.83 ± 1.67 for the test groups treated with the doses of 138.45 and 276.90 mg/kg, respectively. The spent time into the preferred arm significantly increased from 6.50 ± 0.83 sec to 24.16 ± 3.56 sec ($p < 0.01$) and 41.83 ± 5.17 sec ($p < 0.01$) respectively for the test groups treated with the doses of 138.45 and 276.90 mg/kg plant

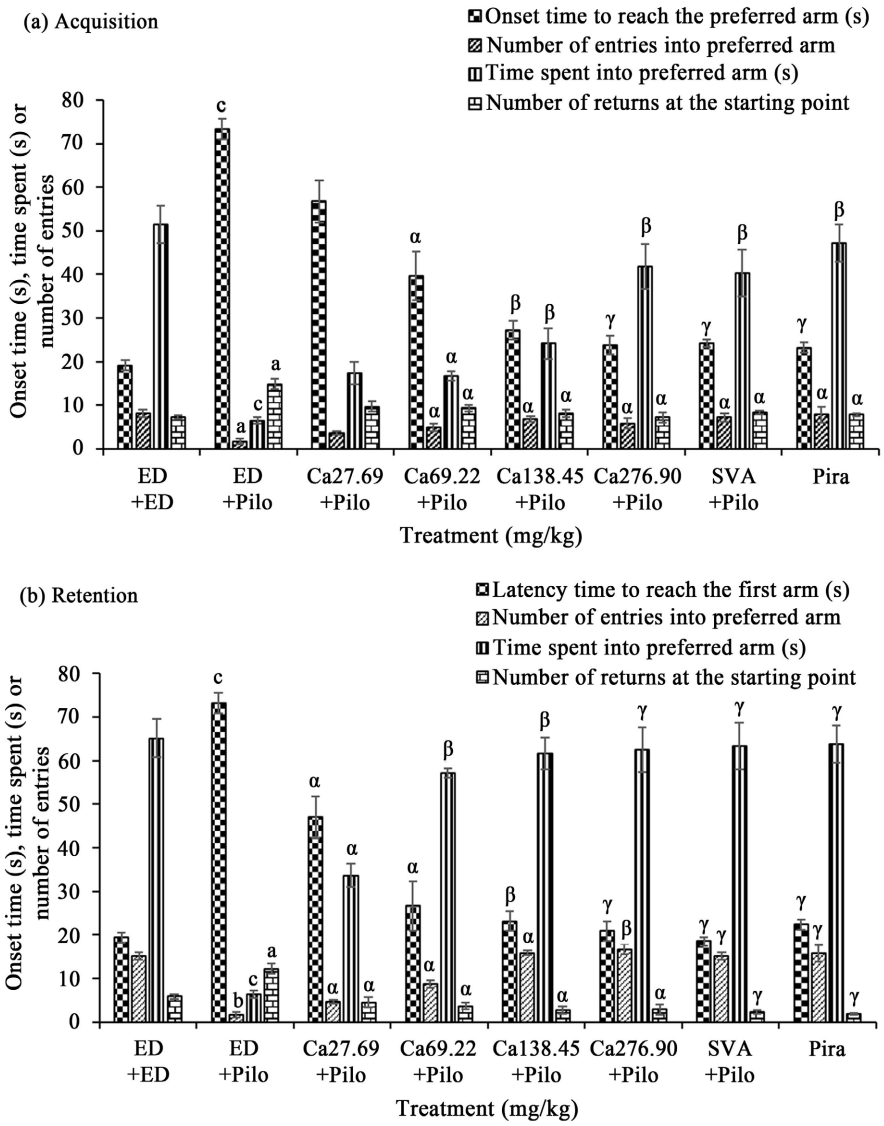

Figure 3. Effects of *Chrysanthellum americanum* aqueous extract on the latency time to reach the first arm, number of entries into preferred arm, time spent into preferred arm, number of entries into discriminated arm, time spent into discriminated arm and number of returns at the starting point during the acquisition and retention phases in the T-maze task. Values are expressed as mean ± SEM (n = 6). $^{a}p < 0.05$, $^{b}p < 0.01$, cp < 0.001 normal group (ED + ED) versus negative control group (ED + Pilo), and $^{\alpha}p < 0.05$, $^{\beta}p < 0.01$, $^{\gamma}p < 0.001$ plant extract versus negative control group (ED + Pilo), ANOVA followed by Tukey's post hoc multiple comparison test. Ca27.69: 27.69 mg/kg *Chrysanthellum americanum* aqueous extract; ED: 10 mL/kg distilled water; Pilo: 360 mg/kg pilocarpine; SVA: 300 mg/kg sodium valproate; Pira: 200 mg/kg piracetam.

extract. Pilocarpine treatment induced a significant increase in the number of returns at the starting point from 7.33 ± 0.44 in the distilled water treated group to 14.83 ± 1.16. *Chrysanthellum americanum* aqueous extract ameliorated the number of returns at the starting point by reducing this number to 8.16 ± 0.83 ($p < 0.05$) and 7.16 ± 1.16 ($p < 0.05$) for the respective doses of 138.45 and 276.90 mg/kg (**Figure 3(a)**).

During the retention time of the T-maze task the latency time to reach the first arm significantly decreased from 73.16 ± 3.44 sec in the distilled water-treated pilocarpine to 23.16 ± 1.44 sec ($p < 0.01$) and 20.83 ± 1.55 sec ($p < 0.001$), respectively for the test group administered 138.45 and 276.90 mg/kg *Chrysanthellum americanum* aqueous extract. This effect was comparable (18.50 ± 2.33 sec; $p < 0.001$) to that of sodium valproate administered at a dose of 300 mg/kg. The number of entries into preferred arm is 15.16 ± 0.55 in the distilled water-treated group. Pilocarpine injection significantly ($p < 0.001$) induced a significant reduction in the number of entries into preferred arm to 1.66 ± 0.6 in the negative control group, and was out of the normal range (**Figure 3(b)**). Interestingly, the plant extract also induced a significant increase of this number to 15.83 ± 0.88 ($p < 0.05$) and 16.66 ± 2.11 ($p < 0.05$) at the doses of 138.45 and 276.90 mg/kg *Chrysanthellum americanum* aqueous extract. The time spent into preferred arm significantly increased from 6.33 ± 0.66 sec in the distilled water-treated pilocarpine group to 61.66 ± 6.38 sec ($p < 0.01$) and 62.50 ± 3.71 sec ($p < 0.01$) for the test groups of mice administered of 138.45 and 276.90 mg/kg *Chrysanthellum americanum* aqueous extract. In addition, the number of returns at the starting point significantly decreased from 12.16 ± 0.55 in the distilled water-treated pilocarpine group to 2.66 ± 0.44 ($p < 0.05$) and 2.83 ± 0.27 ($p < 0.05$) in the test groups administered of 138.45 and 276.90 mg/kg *Chrysanthellum americanum* aqueous extract. Sodium valproate administered as a reference antiepileptic drug significantly lower this number to 2.33 ± 0.66 (**Figure 3(b)**).

3.4. Effects of *Chrysanthellum americanum* Aqueous Extract on Exploration, Locomotion and Motor Coordination of Pilocarpine-Treated Mice and Placed in the Open Field

The open field test revealed that *Chrysanthellum americanum* aqueous extract significantly increased in a dose-dependent manner, the number of crossing and grooming from 3.83 ± 0.55 and 0.83 ± 27 in the distilled water-treated pilocarpine groups to 28.33 ± 0.66 ($p < 0.001$) and 3.66 ± 0.44 ($p < 0.001$), respectively, at the dose of 276.90 mg/kg (**Table 2**). The increase was also observed in the time spent by mice in the centre from 3.50 ± 0.55 s in the distilled water-treated pilocarpine group to 25.33 ± 0.66 s ($p < 0.01$) at the dose of 276.90 mg/kg. At the same dose of 276.90 mg/kg *Chrysanthellum americanum* aqueous extract decreased the number of rearing (1.66 ± 0.44; $p < 0.001$) and the mass of fecal boli (0.01 ± 0.03; $p < 0.001$), respectively when compared with the negative control group (11.50 ± 0.66 and 0.60 ± 0.19). The effect was the same with soduim valproate (300 mg/kg) (**Table 2**).

3.5. Effects of *Chrysanthellum americanum* Aqueous Extract on Oxidative/Nitrosative Stress in the Hippocampus of Pilocarpine-Treated Mice

The activities of *Chrysanthellum americanum* aqueous extract on the occurrence

Table 2. Effects of *Chrysanthellum americanum* aqueous extract on rearing, crossing, grooming, center time and quantity of fecal boli in the open field test.

Treatments	Number of rearing	Number of crossings	Number of grooming	Centre time, (s)	Fecal boli, (g)
ED + ED	7.83 ± 0.56	7.66 ± 0.66	3.33 ± 0.44	11.50 ± 0.66	0.49+0.34
ED + Pilo	11.50 ± 0.66a	3.83 ± 0.55a	0.83 ± 27a	3.50 ± 0.55a	0.60 ± 0.19a
Ca27.69 + Pilo	3.50 ± 0.83**	12.50 ± 0.66*	2.16 ± 0.55*	11.66 ± 0.56*	0.10 ± 0.03***
Ca69.22 + Pilo	3.66 ± 0.55**	18.16 ± 0.83*	2.83 ± 0.55*	13.83 ± 0.61*	0.03 ± 0.04***
Ca138.45 + Pilo	2.66 ± 0.44**	23.16 ± 0.88**	3.33 ± 0.44*	21.16 ± 1.55**	0.03 ± 0.05***
Ca276.90 + Pilo	1.66 ± 0.44***	28.33 ± 0.66***	3.66 ± 0.44*	25.33 ± 0.66**	0.01 ± 0.03***
SVA + Pilo	1.67 ± 0.44***	31.83 ± 0.83***	3.50 ± 0.66*	26.67 ± 0.66***	0.01 ± 0.02***
Pira	1.50 ± 0.50***	36.33 ± 0.77***	3.33 ± 0.44*	22.16 ± 0.88**	0.02 ± 0.03***

Values are expressed as mean ± SEM (n = 6). [a]$p < 0.05$ normal group (ED + ED) versus negative control group (ED + Pilo), and *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ plant extract versus negative control group (ED + Pilo), ANOVA followed by Tukey's post hoc multiple comparison test. Ca27.69: 27.69 mg/kg *Chrysanthellum americanum* aqueous extract; ED: 10 mL/kg distilled water; Pilo: 360 mg/kg pilocarpine; SVA: 300 mg/kg sodium valproate; Pira: 200 mg/kg piracetam.

of oxidative/nitrosative stress (MDA, GSH and NO levels, and SOD and CAT activities) in pilocarpine-injected animals (Table 3).

It can be depicted from Table 3 that the concentration of MDA significantly increased in the distilled water-treated pilocarpine mice (negative control; 0.48 ± 0.02 µmol/g; $p < 0.001$) when compared with the normal control of mice administered only distilled water (0.18 ± 0.03 µmol/g). *Chrysanthellum americanum* aqueous extract induces a significant dose-dependent decrease in this concentration to 0.24 ± 0.01 µmol/g ($p < 0.001$) and 0.18 ± 0.03 µmol/g ($p < 0.001$) in the treated animals with the respective doses of 138.45 and 276.90 mg/kg (Table 3).

The activity of CAT was significantly ($p < 0.05$) lessened from 0.31 ± 0.07 U/min/mg in the distilled water-treated group to 0.19 ± 0.03 U/min/mg in the distilled water-treated pilocarpine group. This decrease in CAT activity was significantly raised by *Chrysanthellum americanum* extract. The plant extract significantly ($p < 0.05$) increased the activity of CAT from 0.19 ± 0.03 U/min/mg in the distilled water-treated pilocarpine group to 0.33 ± 0.04 U/min/mg, and 0.34 ± 0.04 U/min/mg at the doses of 138.45 and 276.90 mg/kg, respectively. Sodium valproate (300 mg/kg) also increased the CAT activity to 0.33 ± 0.03 U/min/mg ($p < 0.05$) (Table 3).

Mice treated with pilocarpine in the distilled water-treated pilocarpine group experienced a significant reduction in the hippocampal concentration of GSH. *Chrysanthellum americanum* aqueous extract significantly reversed this pilocarpine's effect in all doses of the treatment groups. The increases in the level of GSH were significant ($p < 0.05$) from 0.27 ± 0.06 µmol/mg in the distilled water-treated pilocarpine group to 0.70 ± 0.15, 0.79 ± 0.07 and 0.75 ± 0.12 µmol/mg in the plant at the doses of 138.45 and 276.90 mg/kg and sodium valproate (300 mg/kg), respectively (Table 3).

Table 3. Effects of *Chrysanthellum americanum* aqueous extract on oxidative/nitrosative stress in hippocampus of pilocarpine-treated mice.

Treatments	MDA (µmol/g)	CAT (U/min/mg)	GSH (µmol/mg)	SOD (U/min/mg)	NO (µM/mg)
ED + ED	0.18 ± 0.03	0.31 ± 0.07	0.78 ± 0.05	17.29 ± 1.3	2.04 ± 0.26
ED + Pilo	0.48 ± 0.02c	0.19 ± 0.03a	0.27 ± 0.06b	13.20 ± 0.97a	4.11 ± 0.35a
Ca27.69 + Pilo	0.34 ± 0.02	0.21 ± 0.03	0.38 ± 0.06	13.61 ± 1.64	3.36 ± 0.5
Ca69.22 + Pilo	0.27 ± 0.02*	0.26 ± 0.04	0.44 ± 0.36*	15.04 ± 0.77	2.94 ± 0.46*
Ca138.45 + Pilo	0.24 ± 0.01**	0.33 ± 0.04*	0.70 ± 0.15*	16.57 ± 0.86*	2.12 ± 0.15*
Ca276.90 + Pilo	0.18 ± 0.03**	0.34 ± 0.04*	0.79 ± 0.07*	16.24 ± 0.98*	2.08 ± 0.51*
SVA + Pilo	0.18 ± 0.00**	0.33 ± 0.03*	0.75 ± 0.12*	16.26 ± 0.86*	2.01 ± 0.02*
Pira	0.18 ± 0.02**	0.34 ± 0.02*	0.80 ± 0.06*	17.87 ± 1.94*	2.04 ± 0.05*

Values are expressed as mean ± SEM (n = 6). [a]$p < 0.05$ normal group (ED + ED) versus negative control group (ED + Pilo), and *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ plant extract versus negative control group (ED + Pilo), ANOVA followed by Tukey's post hoc multiple comparison test. Ca27.69: 27.69 mg/kg *Chrysanthellum americanum* aqueous extract; ED: 10 mL/kg distilled water; Pilo: 360 mg/kg pilocarpine; SVA: 300 mg/kg sodium valproate; Pira: 200 mg/kg piracetam.

Chrysanthellum americanum aqueous extract significantly increased the activities of SOD in the hippocampus of mice that received acute intraperitoneal pilocarpine with distilled water and this same elevation was noted in the group that received sodium valproate as an antiepileptic treatment. The activity of SOD was significantly ($p < 0.001$) increased from 13.20 ± 0.97 U/min/mg in the distilled water-treated pilocarpine group to 16.57 ± 0.86, 16.24 ± 0.98 and 16.26 ± 0.86 U/min/mg in the plant at the doses of 138.45 and 276.90 mg/kg, and sodium valproate (300 mg/kg) groups, respectively (Table 3).

The hippocampal nitric oxide level raised from 2.04 ± 0.26 µM/mg of tissue in the distilled water-treated group (normal group) to 4.11 ± 0.35 µM/mg in the distilled water-treated pilocarpine group ($p < 0.05$). *Chrysanthellum americanum* aqueous extract administered at a dose of 276.90 mg/kg as well as sodium valproate (300 mg/kg) significantly ($p < 0.05$) reduced this amount to 2.08 ± 0.51 and 2.01 ± 0.02 µM/mg of tissue, respectively (Table 3).

3.6. Effects of *Chrysanthellum americanum* Aqueous Extract on the Level of Acetylcholine, And the Activities of Acetylcholinesterase and Butyrylcholinesterase in the Hippocampus of Pilocarpine-Treated Mice

Figure 4 shows the results of the effects of *Chrysanthellum americanum* aqueous extract on cholinergic status in the hippocampus of mice. Mice were injected intraperitoneally with pilocarpine resulted to a decrease in the level of acetylcholine in the distilled water-treated pilocarpine group (496.93 ± 6.19 µmol/g of tissue) when compared with the normal group of mice treated only with distilled water (812.14 ± 25.99 µmol/g of tissue). The concentration of acetylcholine in hippocampus lowered by acute pilocarpine injection in the distilled water-treated pilocarpine group (496.93 ± 6.19 µmol/g of tissue) was significantly

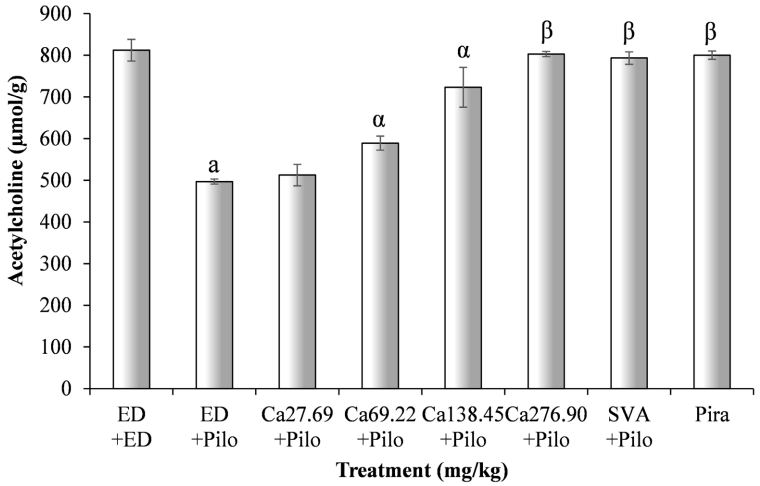

Figure 4. Effects of *Chrysanthellum americanum* aqueous extract on the level of acetylcholine in the hippocampus of pilocarpine-treated mice. Values are expressed as mean ± SEM (n = 6). $^{a}p < 0.05$ normal group (ED + ED) versus negative control group (ED + Pilo), and $^{a}p < 0.05$, $^{\beta}p < 0.01$ plant extract or Pira versus negative control group (ED + Pilo), ANOVA followed by Tukey's post hoc multiple comparison test. Ca 27.69: 27.69 mg/kg *Chrysanthellum americanum* aqueous extract; ED: 10 mL/kg distilled water; Pilo: 360 mg/kg pilocarpine; SVA: 300 mg/kg sodium valproate; Pira: 200 mg/kg piracetam.

raised by the oral administration of *Chrysanthellum americanum* aqueous extract at a dose of 276.90 mg/kg to a value of 802.79 ± 6.18 µmol/g of tissue ($p < 0.01$) (**Figure 4**).

The plant extract at the dose of 276.90 mg/kg significantly ($p < 0.05$) decreased the hippocampal activity of acetylcholinesterase to 12.56 ± 0.59 µmol/min/mg of tissue which was increased in the distilled water-treated pilocarpine group to 17.11 ± 0.66 µmol/min/mg of tissue. Sodium valproate also significantly ($p < 0.05$) lowered acetylcholinesterase activity to a value of 12.73 ± 0.81 µmol/min/mg of tissue (**Figure 5**). The group that received an acute intraperitoneal injection of pilocarpine (negative control group) was found to significantly ($p < 0.05$) increase butyrylcholinesterase activity (18.07 ± 0.82 µmol/min/mg) in the hippocampus of mice when compared with the normal control group of mice treated only with distilled water in which the hippocampal butyrylcholinesterase activity was 12.47 ± 0.87 µmol/min/mg of tissue. The activity of butyrylcholinesterase was significantly ($p < 0.001$) lowered from 18.07 ± 0.82 µmol/min/mg of tissue in the distilled water-treated pilocarpine group to 12.53 ± 0.78 and 12.54 ± 1.26 µmol/min/mg at the dose of 276.90 mg/kg of *Chrysanthellum americanum* aqueous extract and sodium valproate, respectively (**Figure 5**).

4. Discussion

Temporal lobe epilepsy (TLE) is the most common form of pharmaco-resistant epilepsy, and several rodent models are used to study pathogenesis, physiology and pharmacology of the disease [34]. The pilocarpine model is commonly used

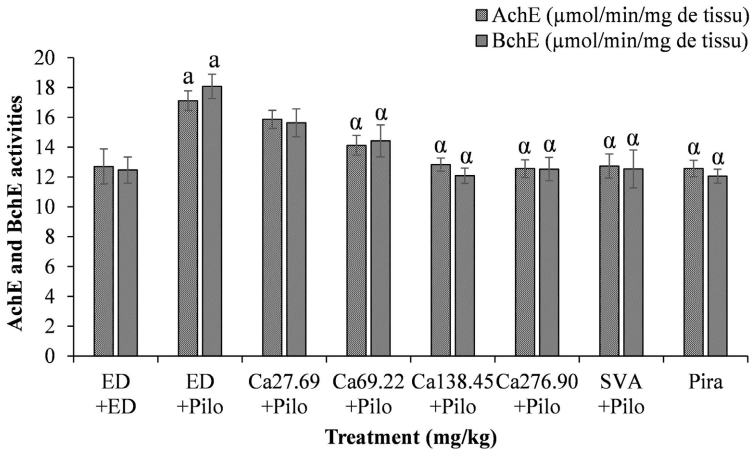

Figure 5. Effects of *Chrysanthellum americanum* aqueous extract on the activities of acetylcholinesterase and butyrylcholinesterase in the hippocampus of pilocarpine-treated mice. Values are expressed as mean ± SEM (n = 6). $^{a}p < 0.05$ normal group (ED + ED) versus negative control group (ED + Pilo), and $^{\alpha}p < 0.05$ plant extract or Pira versus negative control group (ED + Pilo), ANOVA followed by Tukey's post hoc multiple comparison test. Ca27.69: 27.69 mg/kg *Chrysanthellum americanum* aqueous extract; ED: 10 mL/kg distilled water; Pilo: 360 mg/kg pilocarpine; SVA: 300 mg/kg sodium valproate; Pira: 200 mg/kg piracetam; AchE, acetylcholinesterase; BchE, butyrylcholinesterase.

to study the mechanisms of epileptogenesis as well as the anticonvulsant and antiepileptic properties of new pharmacological agents [35]. Some findings suggest that the mechanism by which pilocarpine induces convulsions is that pilocarpine is a muscarinic-type cholinergic receptor agonist. At the cellular level, this chemical is able to induce neuronal excitability by blockage of potassium conductance and eventually increase neuronal depolarisation. However, the systemic effects of pilocarpine can be effectively inhibited by pretreatment with the muscarinic antagonist, methylscopolamine [36]. As indicated by the results obtained, *Chrysanthellum americanum* aqueous extract induced a significant reduction in the duration and number of clonic and clonic-tonic seizures and an increase in the latency time for the onset of seizures induced by pilocarpine when compared with the disease group. *Status epilepticus* causes severe neuronal alterations leading to hippocampal cell death [22] [37]. The protection of mice by *Chrysanthellum americanum* aqueous extract against *Status epilepticus* could be explained by an antagonization of muscarinic receptors because the ability of pilocarpine to induce *Status epilepticus* is linked to the activation of these receptors [38]. Likewise, these pharmacological activities attributed to *Chrysanthellum americanum* may be due to the presence of polyphenols, flavonoids and saponins in the aqueous extract, classified as large families of natural products, which have also demonstrated significant properties on the central nervous systems such as an affinity for $GABA_A$ complex receptors, inducing antiepileptogenesis and anticonvulsant effects [13] [39].

Hence, according to the qualitative phytochemical analysis of *Chrysanthellum americanum* aqueous extract, it can be depicted the occurrence of alkaloids and phenols. Interestingly, these compounds possess anticonvulsant properties through

the activation of a $GABA_A$ complex receptor [40] [41]. The protection conferred by *Chrysanthellum americanum* aqueous extract was near to that provided by sodium valproate use as an antiepileptic reference, recognised as a broad-spectrum antiepileptic [42]. Memory impairment is generally associated with temporal lobe epilepsy due to the implication of the temporal lobe in establishment of memory, and eventually damage to the hippocampus [7] [43] [44]. Certainly, the analysis of the results obtained during the T-maze paradigm based on the tendency to explore and memorize a new environment makes it possible to determine several behavioural parameters in rodents. From these results during the retention phase, *Chrysanthellum americanum* aqueous extract induced a significant decrease in the number of returns to the starting arm, a significant decrease in the latency time to enter to the preferred arm, and a significant increase in time spent in the preferred arm. The decrease in the latency time to enter in the preferred arm suggests improvement of memory in mice [45]. In addition, the increase in the number of entries, the time spent in the preferred arm and the decrease in the number of returns to the starting arm indicates an increase in the exploratory behaviour, consequently amelioration of memory aptitudes [25]. The increase in time spent in preferred arms reveals excellent memory functions [46] [47].

The central cholinergic system plays an important role in the processes of memory [47]. A dysfunction of neurons containing acetylcholine in the elderly presents cognitive deficiencies [48]. From the results obtained in this experiment, the level of acetylcholine significantly lowered in the epileptic mice injected with pilocarpine. This reduction is accompanied by the alteration of memory in mice. Interestingly, *Chrysanthellum americanum* aqueous extract reverted this alteration in the concentration of acetylcholine indicating the ameliorative property of the plant extract against memory impairment in pilocarpine-treated mice [5] [47] [48].

In addition, the biochemical analysis for acetylcholinesterase and butyrylcholinesterase activities indicated that, *Chrysanthellum americanum* aqueous extract induced an inhibition of the activities of these enzymes; and consequently, an increase in the level of acetylcholine in the hippocampus of treated mice with the different doses of extract; and an improvement in memory observed during the T-maze task. These results suggest that *Chrysanthellum americanum* aqueous extract has ameliorative properties on memory process [49] which could be explained by the presence of certain active compounds in the aqueous extract, with capability to cross the hematoencephalitis barrier [13].

Finally, the locomotion, exploration and motor coordination of mice evaluated in the open field paradigm demonstrated an increase in the number of "crossings" and the time spent in the centre of the open filed in *Chrysanthellum americanum* aqueous extract-treated mice. The increase in the number of "crossings" and the time spent in the centre of the open field indicates the increase in the exploratory behaviour and locomotion activity in rodents, and represents an intrinsic manifestation of the reduction in anxiety [14] [50] [51] [52] [53].

Epilepsy has been described as a condition of excessive neuronal discharge associated with or resulting from oxidative stress [54]. Neurochemical and enzymatic studies have proposed that the glutamatergic excitotoxic activity induced by pilocarpine contributes to the production of reactive oxygen species or reactive nitrogen species, overriding the neutralizing capacity of neuronal glutathione [55], with resulting in neuronal cells loss related to the resulting oxidative and nitrosative stresses. Low glutathione levels are associated with many dysfunctions and even cell death [56]. Once released into the tissues, free radicals induce membranes damage and cells death through three main mechanisms: by protein oxidation [57], lipid peroxidation [58] or oxidation and degradation of DNA [59]. Previous findings show that, the excessive formation of free oxygen radicals has been linked to neuronal injury in mice injected intraperitoneally by pilocarpine, induced epileptic convulsions causing lipid peroxidation and elevated malondialdehyde and nitric oxide concentrations in the hippocampus of mice while decreasing in the level of reduced glutathione, and the activities of superoxide dismutase and catalase [60]. According to the results obtained in this study *Chrysanthellum americanum* extract could protect animals against oxidative and nitrosative stresses eventually by preventing lipid peroxidation and regulating the balance between free radicals and antioxidant defense system. This could be explained by the presence of active compounds such as flavonoids, tannins and phenols in *Chrysanthellum americanum* aqueous extract which are major contributors to the antioxidant activities of medicinal plants [61].

Phytopharmacological studies are continuing in order to characterize the mechanism(s) responsible for these anticonvulsant and anti-amnesic actions and also to identify the active compounds present in *Chrysanthellum americanum* extract. As limitation for this work, the effects of *Chrysanthellum americanum* extract on mice model of temporal lobe epilepsy without generalisation of seizures in the different regions of brain and with non-convulsive focal seizures was not evaluated, as our primary aim of this study is to assess the effects of *Chrysanthellum americanum* on mice pilocarpine model of epilepsy and associated memory loss.

5. Conclusion

These results ascertained that *Chrysanthellum americanum* aqueous extract has antiepileptic and anti-amnesic properties by reducing seizures and improving memory in epileptic mice. This protection would be on the one hand the consequence of the attenuation of certain dysfunctions due to crises such as oxidative stress and on the other hand the consequence of the regulation of the activity of the enzymes which hydrolyse acetylcholine and eventually the raised level of acetylcholine.

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Authors' Contributions

YN, GST and ENB conceived and designed the study. YN and FSAY conducted behavioural experiments. SP, VTJ, HMAYM, RBB, TAO and GST conducted the biochemical analyses. YN, FSAY and GST were responsible for data management, and interpreted the results and wrote the first draft of the manuscript. GST, GAA and ENB supervised and critically reviewed the manuscript for important and intellectual content.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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