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Research paper

Therapeutic effects of *Carissa edulis* aqueous extract against L-glutamic acid-induced neurotoxicity in brain mice

Sabine Adeline Fanta Yadang [a,*], Yvette Nguezeye [a], Germain Sotoing Taiwe [b], Gabriel Agbor Agbor [a], Elisabeth Ngo Bum [c]

[a] *Laboratory of Pharmacology and Drug Discovery, Centre for Research on Medicinal Plants and Traditional Medicine, Institute of Medical Research and Medicinal Plants Studies, Yaounde, Cameroon*
[b] *Department of Animal Biology and Conservation, Faculty of Science, University of Buea, Buea, Cameroon*
[c] *Department of Biological Sciences, Faculty of Science, University of Maroua, Maroua, Cameroon*

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ABSTRACT

The over-stimulation of N-methyl-d-aspartate glutamate receptors causes an excitotoxic neuronal death which plays an important role in many neurodegenerative diseases. *Carissa edulis*, a medicinal plant used in African pharmacopeia has been shown to have many therapeutic effects. In this study, the therapeutic effects of *Carissa edulis* aqueous extract on L-glutamic acid-induced neurotoxicity in mice were investigated. Two-month-old mice received an intraperitoneal injection of L-glutamic acid (2 g/kg) for seven consecutive days and were treated with an aqueous extract of *Carissa edulis*. Mice were monitored for behavioural studies including locomotion, muscle strength, and memory. Oxidative stress was determined by measuring lipid peroxidation and the level of antioxidant enzymes. Elisa kits were used to evaluate the levels of the proinflammatory cytokines. Hippocampal histopathology was examined using cresyl violet staining. *Carissa edulis* administration exhibited a protective effect on L-glutamic acid-induced abnormal locomotor activity and increased the mice's muscle strength. Also, it increased the memory of treated mice. *Carissa edulis* aqueous extract administration decreased the malondialdehyde level and increased the catalase activity and glutathione level. Furthermore, it significantly decreased the levels of IL-1β and TNF-α compared to the L-glutamic acid group. There were no significant pathological changes in the hippocampus of the *Carissa edulis*-treated group compared to the L-glutamic acid group. Our results indicated that *Carissa edulis* aqueous extract showed therapeutic effects by alleviating memory impairment, decreasing oxidative stress, and proinflammatory cytokines in the brains of mice treated with L-glutamic acid. Therefore, *Carissa edulis* treatment may help reduce glutamatergic neurotoxicity in neurodegenerative diseases.

1Introduction

Glutamate is the principal and most abundant excitatory neurotransmitter present in the central nervous system (CNS). It is involved in several central functions and metabolic pathways (Wang et al., 2018). It also plays a fundamental role during development with the formation of neural networks and brain functions, including synaptic plasticity, learning and memory processes (Jenner and Caccia, 2019). The action of glutamate at the central level is paradoxical. Even though this neurotransmitter is necessary for the functioning of the CNS, the over-activation of its receptor can have harmful effects. Excessive activation of NMDA glutamate receptors impairs Ca^{2+} homeostasis, leading to a considerable influx of Ca^{2+} and Na^{+} ions into the cell, thus generating several cascades, such as the formation of free radicals, dysfunction of mitochondrial and calcium homeostasis, and the increase of nitric oxide (NO) production, which can directly damage the neuron and cause its death (Anaïs, 2014; Maya et al., 2018). This phenomenon is called "excitotoxicity".

Glutamate is particularly concentrated in brain regions essential for cognitive processes, especially in the cerebral cortex, hippocampus, striatum, and dentate gyrus (Abass and El-Haleem, 2011). Glutamate receptors, such as N-methyl-d-aspartate (NMDA) receptors, are widely distributed in the central nervous system, including the amygdala, hippocampus, and hypothalamus, and participate in regulating energy metabolism and autonomic function. When the ionotropic and metabotropic receptors of this neurotransmitter are over-stimulated in these

* Correspondence to: Institute of Medical Research and Medicinal Plants Studies, PO Box 13033, Yaounde, Cameroon.
E-mail address: fantayadang@gmail.com (S.A.F. Yadang).

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regions, they can cause neuronal damage and thus impair learning and memory functions (Park et al., 2016). Moreover, excessive stimulation of these receptors induces lipid peroxidation altering the antioxidant status in different brain regions (Thonda et al., 2014). The induced oxidative stress then induces calcium overload that impairs mitochondrial function, thereby inhibiting cysteine uptake, causing intracellular glutathione (GSH) deprivation and accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), ultimately leading to necrosis or apoptosis (Prentice et al., 2015). These phenomena are involved in neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases (Ezza and Khadrawyb, 2014; Qiang et al., 2020).

Glutamate-induced excitotoxicity can directly affect inflammation through its ability to stimulate immune cells (Holton, 2021). Recently, the combination of excitotoxicity and inflammation, called immuno-excitotoxicity, has received increased attention. Microglia, the primary immune cell in the CNS, when activated, produces a large amount of pro-inflammatory mediators such as IL-6, IL-1β, and TNF-α and also up-regulate the expression of inflammatory mediators such as inducible nitric oxide synthase (Wang et al., 2020; Alfarhan et al., 2022). The microglia activation induces neuroinflammation and evenly boots the excessive release of glutamate, which in turn lead to glutamate toxicity and exacerbate further neuroinflammation (Wang et al., 2020). Glutamatergic receptors are also found on microglia cells. When glutamate binds with these receptors in the synaptic clef, the microglia can stimulate or inhibit the production and release of cytokines (Haroon et al., 2017). Activation of NMDA and Kainate receptors enhances the microglial release of TNF-α, IL-1β, and NO (Haroon et al., 2017; Iovino et al., 2020). The accumulation of extracellular glutamate can also strengthen inflammation (Blaylock, 2017). Indeed, regulation of proinflammatory cytokines could help in inhibiting glutamate toxicity by reducing glutamate release, mitochondrial dysfunction, and apoptosis.

Plants around us contain many natural compounds which have been reported to be capable of protecting the body against the development of excitotoxicity induced by glutamate and other neurotoxins (Neveen and Iman, 2010). Also, natural plants have been found to possess various bioactive properties such as antioxidant and anti-inflammatory activities (Qiang et al., 2020). *Carissa edulis* is a plant used in traditional African medicine against several diseases including malaria and fever. Previous studies have shown that *C. edulis* possess polyphenols, flavonoids and tannins contents that exhibit strong antioxidant and antiinflammatory activities (Woode et al., 2008; Fowsiya and Madhumitha, 2017; Yadang et al., 2019; Yadang et al., 2020). It also shows anticonvulsant activity through different mechanisms such as GABAergic pathways (Ya'u et al., 2008), anti-plasmodial activity against the *Plasmodium falciparum* parasite (Kebenei et al., 2011), and hepatoprotective effects (Yahya and Omar, 2019). In this study, we investigated the protective role of *Carissa edulis* aqueous extract on L-glutamic acid-induced behavioural impairment, oxidative stress and neuroinflammation in mice brains. We hypothesized that *Carissa edulis* with its antioxidant and antiinflammatory properties could be significantly effective against L-glutamic acid-induced neurotoxicity by inhibiting the free radical formation, decreasing IL-1β and TNF-α production, and reducing hippocampal neurodegeneration.

2Materials

2.1. Plant material and extraction

Carissa edulis (C. edulis) fresh leaves were collected in Bogo in the far northern region of Cameroon (August 2016) and identified at the Yaounde National Herbarium (Cameroon) under reference specimen number 2965/SRFK. Washed leaves were shade-dried, crushed, and sifted to obtain a fine powder. The preparation of the extract followed the procedures used by the traditional healers. Ten grams (10 g) of this powder were mixed with 60 ml of distilled water and boiled on a heating plate set at 100 °C for 20 mins to obtain the aqueous extract (Yadang et al., 2020). After cooling down, the resulting mixture was filtered using N° 1 Wattman Paper. The amount of dry matter in the extract was determined by evaporating water in a drying oven (80 °C) and a 3.9 g of dry extract was obtained. The stock solution (Dose = 628 mg/kg) was diluted to 1/2, 1/4, and 1/10 to obtain different doses of 628 mg/kg, 314 mg/kg, 157 mg/kg, and 62.8 mg/kg respectively. The various doses were orally administered to animals in a volume of 10 ml/kg of body weight. The choice of the doses of the extract in the study has been done according to the procedure of the traditional healer and the use of the doses has been validated and used in our previous study (Yadang et al., 2020).

2.2. Animals

Adult *Mus musculus* Swiss mice (n = 35) weighing between 25 and 30 g and approximately 2 months old were used for this study. These mice were obtained from the National Institute of Health in Islamabad, Pakistan. They were acclimatized for one week in the animal facility of the Department of Pharmacy, COMSATS Institute of Information Technology, Abbottabad, Pakistan where the temperature was maintained at 25 °C ± 2 under a 12 h light/dark cycle. These animals were fed and drank water ad libitum. The experiments have been approved by COMSATS Institute of Information Technology Ethics Committee, Abbottabad, Pakistan (Reg. No. 73 Phm. Eth/FA17-CS-M10/17–010–71) according to the Guidelines for Care and Use of Laboratory Animals from the National Institutes of Health (NIH Publications No. 8023, revised 1978).

2.3Chemicals

The substances used in our experiments were L-glutamic acid, vitamin C, Ellman's reagent, acetylthiocholine iodide, and thiobarbituric acid obtained from Sigma Aldrich St Louis, USA. Phosphate buffer, Tris-HCl buffer, trichloroacetic acid, hydrogen peroxide and paraformaldehyde were obtained locally. Rats IL-1β and TNF-α Elisa kits were obtained from Elabscience Biotechnology Co., Ltd (Texas, USA).

3Methods

3.1Experimental design

The mice were divided into seven groups of 5 mice each: control group, L-glutamic acid group (L-glu), positive control group, and four test groups. Mice were gradually deprived of food to maintain them at 80–85 % of their normal weight. L-glutamic acid (2 g/kg) was prepared in a physiological solution (0.9 % NaCl) and administered intraperitoneally for seven days to induce memory impairment and neurotoxicity.

Various treatments were carried out as follows: The control group received orally distilled water; the L-glutamic acid group (L-glu) received L-glutamic acid (2 g/kg, i.p.) followed by the oral administration of distilled water; Four test groups received L-glutamic acid (2 g/kg, i.p.) followed by the oral administration of *Carissa edulis* aqueous extract at the doses of 62.8; 157; 314 and 628 mg/kg respectively and the positive control group received L-glutamic acid (2 g/kg, i.p.), followed by the administration of vitamin C (250 mg/kg orally administered).

The dose of L-glutamic acid was selected according to previous studies (Ramanathan et al., 2007; Ganesan et al., 2013). The treatment with L-glutamic acid and the plant's aqueous extract was done every day for seven days. The time between the administration of L-glutamic acid and the different treatments was 1 hour. The animals were observed for possible behavioural changes throughout the various treatments, and from day 8, they were assessed for locomotor activities and memory functions.

3.2Neurological score for locomotor activity assessment

A neurological score test was used to measure the impairment of locomotor functions induced by the over-activation of Ca^{2+}-NMDA receptors by glutamate. Glutamate acting as a neurotoxin is associated with several locomotor disorders that prevent normal animal movements (Thonda et al., 2014). The severity of motor impairment in the different groups was assessed using a quantitative neurological scale adapted from Guo et al. (2012). This score was determined for each animal on the last day, 4 hours after administration of the different treatments. Briefly, mice were given a score of 0 (normal) to 5 (severely impaired) for motor activity. Score = 1: general slowness of movement resulting from slight impairment of the hind limbs, score = 2: incoordination and marked gait abnormalities, score = 3: paralysis of the hind limbs, score = 4: inability to move, impaired fore and hind limbs, score = 5: lying down (Ahuja et al., 2008; Guo et al., 2012).

3.3Hanging wire test

The hanging wire test was used to assess muscle strength in rodents (Abou-Donia et al., 2001). The hanging wire apparatus is a device made of steel wire 2 mm in diameter suspended on two vertical wooden bars placed 50 cm high from the ground. For this test, each mouse was held by the tail and allowed to hand the steel wire with its forelimbs. The time the mouse was able to hold the wire was recorded. The latency time to fall off the wire was taken as an indirect measure of gripping force.

3.4Elevated plus maze (EPM)

Memory impairment was assessed using the elevated plus maze. It is a device consisting of a crossed shape with two opened and two closed arms of equal dimensions (25 cm x 5 cm x 12 cm) connected to a central square (5 cm x 5 cm) elevated 50 cm above the ground. This device is used to assess anxiety and often memory in animals (Morales-Delgado et al., 2018). Memory acquisition task was evaluated on day 7 after the different treatments. Mice were placed individually at the start of one open arm then the initial latency time (ITL), which is the time taken by the animal to enter one of the closed arms was recorded. When in 90 seconds the animal had not entered any of the closed arms, it was gently introduced there and the ITL was assigned to 90 seconds. Mice were allowed to explore the maze for 2 min after ITL recording and then returned to their cages. Twenty-four hours later, which means day 8, the retention of this learned task was examined and a retention latency time (RTL) was recorded. The effect of treatment on latency time was expressed by the retention index (RI) ratio. The RI was calculated using the following formula (Kulkarni et al., 2011; Otari et al., 2012):

$$RI = \frac{ITL - RTL}{RTL} x \quad 100$$

3.5Measurement of antioxidant enzyme activities and malondialdehyde content

After the behavioural studies, animals were anesthetized and sacrificed by cervical decapitation, and the brain tissues were collected, weighed, and rinsed with cold saline. A 10 % (w/v) homogenate of the whole brain was prepared in 0.1 M phosphate-buffered saline (pH 7.4) and then centrifuged at 10,000 rpm per min for 15 min at 4 °C. The supernatant collected was used for further biochemical analysis.

Malondialdehyde (MDA) level, a marker of lipid peroxidation, in the brain homogenate was determined by the biochemical method according to Wilbur (Wilbur et al., 1949. During the process, MDA reacted with Thiobarbituric acid to form a pink color complex which was measured using a spectrophotometer set at 532 nm. The MDA level was expressed in nmol/g of tissue using the Beer-Lambert formula with a molar extinction coefficient of 1.56 $\times 10^5$ mmol/cm.

Catalase is an antioxidant enzyme that catalyzes the rapid decomposition of H_2O_2 into water and oxygen. Catalase activity was assessed by the method described by Aebi (Aebi, 1974) and the rate of decomposition of H_2O_2 was measured using a spectrophotometer at 240 nm. Catalase activity was expressed in μM H_2O_2/min/mg of tissue.

Reduced glutathione (GSH) was measured according to Ellman's method (Ellman, 1959). In brain supernatant, GSH reacted with 5, 5′-dithio-bis-2-nitrobenzoic acid (Ellman's reagent) to generate a yellow chromophore which was then measured at 412 nm using a spectrophotometer UV. The glutathione level was calculated using the value of the molar extinction coefficient of 13,600 mol-1.cm-1 and was expressed in μmol/g of tissue.

3.6Estimation of proinflammatory cytokines

The determination of cytokine levels IL-1β (Cat No E-EL-R0012) and TNF-α (Cat No E-EL-R0019) in mice brain homogenates was measured according to the protocols of mouse ELISA kits (Elabscience Biotechnology Co., Ltd, Texas, USA) following the manufacturer's recommendations and expressed in pg/ml.

3.7Histological observation

For assessing the histological damage, collected brain samples were fixed for one week in 10 % (v/v) paraformaldehyde dissolved in phosphate-buffered saline (0.1 M, pH = 7.4). Then, the brain block was embedded in paraffin and cut into 5 μm coronal sections. Tissue sections were stained with cresyl violet for Nissl staining. Finally, the tissues of each group were examined under an optical microscope and photographed at magnifications of 100 and 40x. The number of normal neurons was quantified by Image-Pro plus 6 Software.

3.8Statistical analysis

All data were expressed as mean ± standard deviation (S.D.). The statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison tests with GraphPad Prism version 8.0 Software to analyze the differences between groups. A *p-value* < 0.05 was considered statistically significant. All figures were generated by using GraphPad Prism 8 software.

4Results

4.1Neurological score evaluation

The neurological scores evaluated 4 h after the treatment in control, L-glu and *C. edulis*-treated groups (n = 5) were presented in Fig. 1. The results showed that the administration of L-glutamic acid causes disturbances in locomotion and behaviour. The neurological score increased significantly in the L-glu group compared to the control group which had a score of 0, exhibiting normal behaviour ($p < 0.001$). The L-glu group presented a score of 3.6 showing disturbances from slight impairment of the hind limbs, incoordination and marked gait abnormalities, paralysis of the hind limbs and inability to move with impaired fore and hind limbs. The scores of 2.4 and 0.6 presented by *C. edulis* aqueous extract-treated groups with the respective doses of 314 and 628 mg/kg were significantly lower than the L-glu group ($p < 0.001$). These groups exhibited less abnormal behaviour. Vit C, the standard drug, prevents significantly the locomotion disturbances induced by the L-glu administration ($p < 0.001$).

4.2Hanging wire test

The muscle strength of mice was measured using the hanging wire test. The results are shown in Fig. 2. It is observed that the administration of L-glutamic acid to mice for 7 days decreases their muscle strength

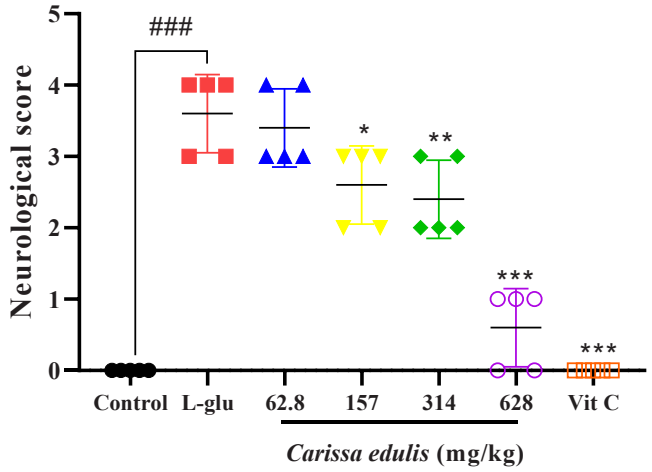

Fig. 1. Effects of *C. edulis* aqueous extract on neurological score. Data are expressed as mean ± SD, n = 5. $^{###}p < 0.001$, L-glu group vs. control group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. L-glu group. One-way ANOVA followed by Tukey multiple comparison tests. L-glu: L-glutamic acid, Vit C: Vitamin C.

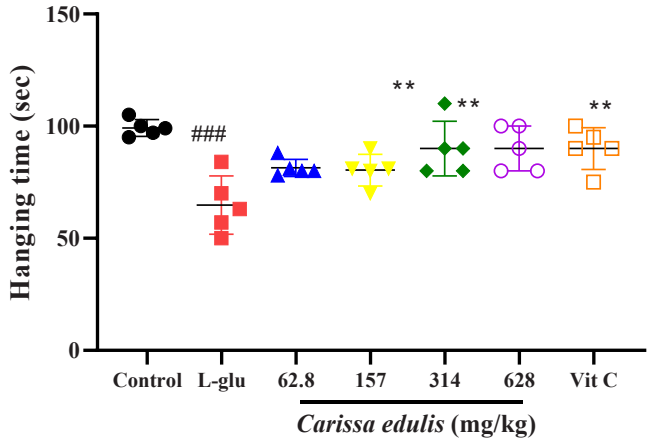

Fig. 2. Effects of *C. edulis* aqueous extract on hanging wire test for muscle strength. Data are expressed as mean ± SD, n = 5. $^{###}p < 0.001$ L-glu group vs. control group. $^{**}p < 0.01$ vs. L-glu group. One-way ANOVA followed by Tukey's multiple comparison tests. L-glu: L-glutamic acid, Vit C: Vitamin C.

compared to the control group. The hanging time of the L-glutamic group was significantly reduced compared to the control group ($p < 0.001$). While the treatment with *C. edulis* aqueous extract significantly increased this hanging time at the dose of 314 and 628 mg/kg ($p < 0.01$) showing an improvement in muscle strength.

4.3Latency time

The elevated plus maze test was used to measure the learning and memory of mice. The results of the latency time to enter into the closed arm are presented in Fig. 3. It can be seen that during the acquisition trial, latency time was not significantly different compared to the control. However, during the retention trial, 24 hours after acquisition, it was observed that the latency time significantly increased for the L-glu group compared to the control group ($p < 0.001$). Treatment with *C. edulis* aqueous extract significantly reduced this latency transfer time compared to the L-glu group ($p < 0.001$) at the different doses of 157, 314, and 628 mg/kg showing significant improvement in the learning and memory performance. The reference substance vitamin C also reduced significantly ($p < 0.001$) this latency time when compared to the L-glu group, thus showing a similar result to the control group.

4.4Retention index

The retention index presented in Fig. 4 clearly shows that the animals which received only L-glutamic acid have a significantly low retention index compared to the control group ($p < 0.001$). Thus, the L-glu group performed poor retention of memory, indicating a cognitive dysfunction. The treatment with the different doses of *C. edulis* increased significantly and, in a dose-dependent manner, this retention index ($p < 0.001$) showed significant memory-enhancing activity.

4.5Effect of Carissa edulis on antioxidant enzymes activities and malondialdehyde content

MDA content: The results of the antioxidative effects of *C. edulis* aqueous extract are presented in Table 1. After seven days of L-glutamic acid administration followed by the treatment with *C. edulis*, and vitamin C, it emerges from this table that the administration of L-glutamic acid leads to a significant increase in malondialdehyde level of 389.82 ± 10.73 nmol/g of tissue compared to the control ($p < 0.001$). However, treatment with the different doses of *C. edulis* attenuated significantly in a dose-dependent manner L-glutamic acid-induced increase in lipid peroxidation reaching the value of 221.36 ± 15.22 nmol/g of tissue for the dose of 628 mg/kg ($p < 0.001$) compared to the L-glutamic group. Vitamin C also significantly ($p < 0.001$) decreased this concentration of MDA (Table 1).

Catalase activity: The effects of *C. edulis* aqueous extract administration on catalase activity in the brain homogenate of L-glutamic acid-treated mice are presented in Table 1. It appears that the L-glu group significantly reduced catalase activity (42.16 ± 5.88 μmol H_2O_2/min/mg of tissue ($p < 0.01$)) compared to the control group. Oral administration of *C. edulis* aqueous extract significantly increased the catalase activity to 62.05 ± 3.58 μmol H_2O_2/min/mg of tissue ($p < 0.05$) and 66.05 ± 4.98 μmol H_2O_2/min/mg of tissue ($p < 0.01$) for the groups of mice receiving the different doses of 314 mg/kg and 628 mg/kg respectively compared to the L-glu group. Catalase activity also was increased by the administration of vitamin C ($p < 0.001$).

Reduced glutathione: The results of the brain glutathione levels are presented in Table 1. The results show that there is a depletion of brain GSH in the L-glu-treated group compared to the control group. This depletion is inhibited by the treatment with *C. edulis* aqueous extract. The GSH levels decrease in the group receiving only L-glutamic acid from 1.57 ± 0.16 μmol/g of tissue ($p < 0.001$) in the control group to 0.85 ± 0.14 μmol/g of tissue. The administration of the different doses of 157, 314, and 628 mg/kg of *C. edulis* aqueous extract significantly increased ($p < 0.001$) this glutathione level to 1.28 ± 0.12 ($p < 0.05$), 1.40 ± 0.09 ($p < 0.01$) and 1.52 ± 0.11 μmol/g of tissue respectively when compared to the L-glu group.

4.6Effect of C. edulis aqueous extract on brain cytokines levels

The effect of *C. edulis* on the level of proinflammatory cytokines in the brain tissue of mice injected with L-glutamic acid was evaluated by the expression of proinflammatory cytokines levels such as TNF-α and IL-1β, in the brain homogenate. The results presented in Fig. 5A and B show that the TNF-α and IL-1β levels in the brain homogenates of the L-glu-treated mice were significantly higher than the control group, demonstrating that L-glutamic acid can lead to the occurrence of inflammation and the release of proinflammatory cytokines. The level of brain IL-1β increased significantly ($p < 0.001$) in the L-glutamic acid-treated group compared to the control group. Administration of *C. edulis* aqueous extract at the doses of 314 and 628 mg/kg significantly decreased this IL-1β concentration ($p < 0.001$), respectively when compared to the L-glutamic group. It emerges from Fig. 5B that treatment with L-glutamic acid significantly increases ($p < 0.001$) the TNF-α concentration (558.80 ± 7.21 pg/ml) compared to the control (416.80 ± 12.16 pg/ml). The concentration of TNF-α has significantly reduced

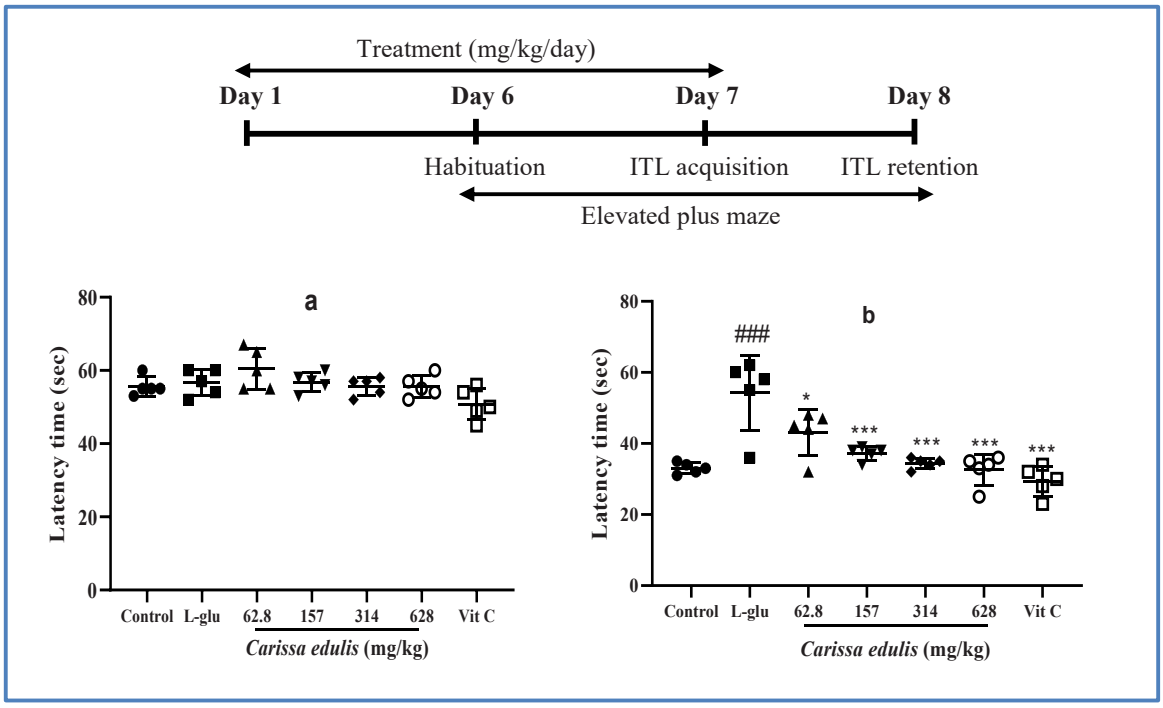

Fig. 3. Effects of *C. edulis* aqueous extract on latency time to enter in the closed arms in the elevated plus maze. a) Acquisition trial, b) Retention trial. Data are expressed as mean ± SD, n = 5. $^{###}p < 0.001$ L-glu group vs. control group. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. L-glu group. One-way ANOVA followed by Tukey multiple comparison tests. L-glu: L-glutamic acid, Vit C: Vitamin C.

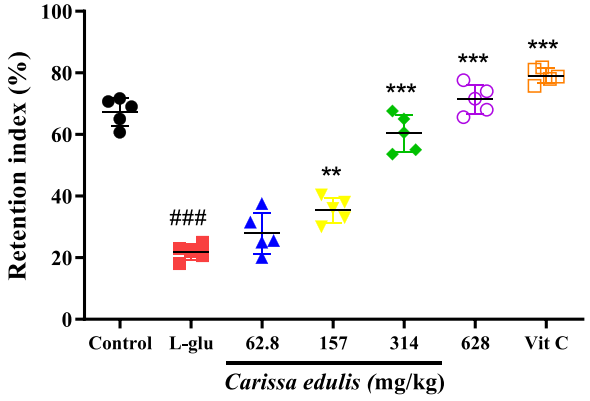

Fig. 4. Effects of *C. edulis* aqueous extract on retention index in the elevated plus maze. Data are expressed as mean ± SD, n = 5. $^{###}p < 0.001$ L-glu group vs. control group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. L-glu group. One-way ANOVA followed by Tukey multiple comparison tests. L-glu: L-glutamic acid, Vit C: Vitamin C.

in *C. edulis*-treated mice with doses of 314 and 625 mg/kg, respectively compared to the L-glutamic group. The treatments with the extract show results comparable with those of the reference substance vitamin C.

4.7. Histopathological analysis

Histopathological changes in the hippocampus of mice examined by cresyl violet staining are shown in Fig. 6A. Sections of the CA3 region of the hippocampus in the control group showed a regular histological architecture, with pyramidal cells uniform in size and regularly arranged without any visible histopathological changes. The photomicrograph showed that administration of L-glutamic acid-induced damage in the hippocampus CA3 region. This is characterized by affected pyramidal cells with massive disruption, irregular shape, and

Table 1
Effects of *Carissa edulis* aqueous extract on antioxidant enzymes activities, malondialdehyde content and total proteins after L-glutamic acid administration.

Treatments	Doses (mg/kg)	MDA (nmol/g tissue)	CAT (µM H_2O_2/ min/mg tissue)	GSH (µmol/g tissue)
Control	–	202.56 ± 10.44	62.58 ± 4.54	1.57 ± 0.16
L-glu	2 g/kg	389.82 ± 10.73$^{###}$	42.16 ± 5.88$^{##}$	0.85 ± 0.14$^{###}$
C. edulis	62.8	370.34 ± 18.5	39.60 ± 6.10	0.88 ± 0.11
	157	344.52 ± 11.58*	51.60 ± 4.33	1.28 ± 0.12*
	314	292.99 ± 13.67***	62.05 ± 3.58*	1.40 ± 0.09**
	628	221.36 ± 15.22***	66.05 ± 4.98**	1.52 ± 0.11***
Vit C	250	214.35 ± 12.67***	62.49 ± 9.8**	1.47 ± 0.14***

Data are expressed as mean ± SD, n = 5. $^{##}p < 0.01$, $^{###}p < 0.001$ L-glu group vs. control group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. L-glu group. One-way ANOVA followed by Tukey multiple comparison tests. L-glu: L-glutamic acid, Vit C: Vitamin C, MDA: Malondialdehyde, CAT: Catalase, GSH: Reduced glutathione

eosinophilic changes. Most neurons were shrunken, with hyperchromatic nuclei and vacuolated cytoplasm. The hippocampus CA3 region of mice treated with the doses of 314 and 628 mg/kg of *C. edulis* showed a marked improvement in the pyramidal neurons similar to the normal architecture of the control group. The dose of 62.8 mg/kg did not show any protection. The pyramidal cells were destroyed and their cytoplasm remained dense, small, and dark. Moreover, in the hippocampus of mice treated with vitamin C, there were no significant histopathological changes compared to the control group.

The hippocampal survival neuronal density was 105 ± 7 cells/mm^2 in the control group and only 35 ± 5 cells/mm^2 in the L-glu group ($p < 0.001$). As shown in Fig. 6B, administration of *C. edulis* at doses of 314 and 628 mg/kg showed an evident increase in neuronal densities of

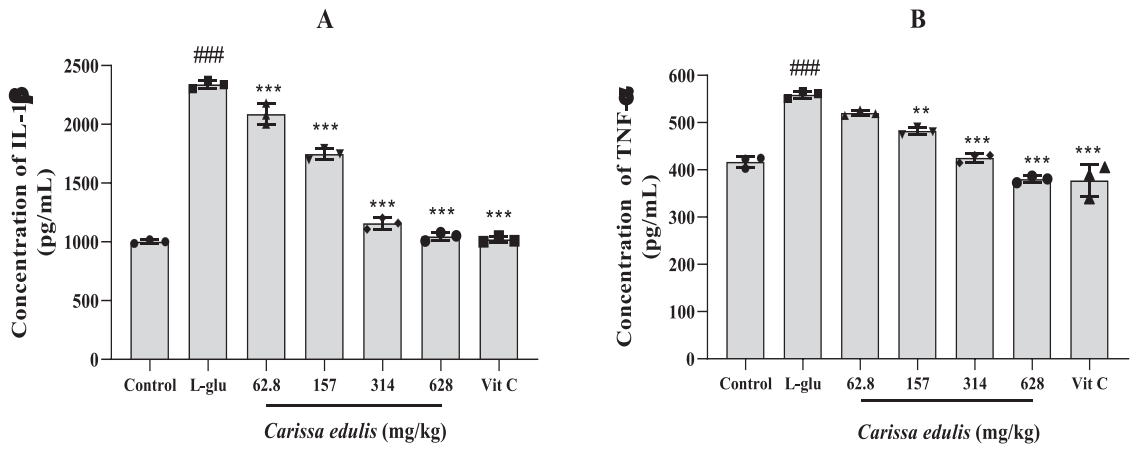

Fig. 5. Effect of *C. edulis* aqueous extract on brain cytokines levels of L-glutamic-treated mice. A) Concentration of IL-1β, B) Concentration of TNF-α. Data are expressed as mean ± SD, n = 5. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ L-glu group vs. control group. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. L-glu group. One-way ANOVA followed by Tukey multiple comparison tests. L-glu: L-glutamic acid, Vit C: Vitamin C.

80 ± 6 and 94 ± 5 cells/mm2, respectively ($p < 0.001$) when compared to the L-glu group. The neuronal density in the hippocampus of the vitamin C group was 100 ± 5 cells/mm^2 ($p < 0.001$) compared to the L-glu group respectively.

5Discussion

Glutamic acid, or glutamate, is considered the most important excitatory neurotransmitter in the brain. In high concentrations, it induces excitotoxicity, leading to the death of nerve cells with significant long-term damage to many brain areas (Waggas, 2009). The over-activation of NMDA receptors with glutamate disrupts calcium homeostasis, which is involved in neuronal damage (Thonda et al., 2014). This prolonged activation leads to a Ca2 + ion influx resulting in a cascade of membrane disruption and cytoplasmic and nuclear events, these being the pathological hallmark for mitochondrial dysfunction and generation of free radicals resulting in neuronal death (Maya et al., 2018). This entire phenomenon is called excitotoxicity, which is a key factor in numerous neurodegenerative conditions. The present study was designed to investigate the therapeutic effects of *Carissa edulis* aqueous extract against L-glutamic acid-induced neurotoxicity by regulating oxidative stress and inflammation in mice. The results of the study revealed that the injection of glutamic acid-induced memory impairment, locomotion and muscle strength impairment, oxidative stress, neuroinflammation, and cell degeneration in the hippocampus. These results are consistent with Thonda et al. (2014) and Hazzaa et al., (2020), who showed that glutamate induces neurotoxic impairment in mice.

Glutamate plays a vital role in various brain functions including cognitive and motor functions, particularly through its influence on the basal ganglia circuitry (Jenner and Caccia, 2019, Pagonabarraga et al., 2021). This neurotransmitter is crucial for synaptic plasticity, which underlies learning and memory, and it is involved in regulating movement by modulating the activity of neural circuits. Glutamate is known to impair locomotor activity by causing damage to neurons in the basal ganglia by generating free radicals. Moreover, glutamatergic overstimulation of the basal ganglia output nuclei and substantia nigra pars compacta lead to motor complications (movement and locomotion impairment) as the basal ganglia receive and process sensory and motor information to coordinate voluntary motor activity (Swamy et al., 2013; Takakusaki, 2017; Pagonabarraga et al., 2021). The neurological scoring test demonstrated that the locomotor activity of the L-glutamic acid-treated group was notably impaired. This finding aligns with existing literature, which also indicates that L-glutamic acid induces disturbances in locomotor activity (Wang et al., 2020). The Study of Thonda et al. (2014) and Swamy et al. (2013) have shown that the administration of monosodium glutamate in rats induced locomotion and muscle impairment and also oxidative stress. In this study, the locomotor impairment may be due to oxidative stress in the basal ganglia. In vitro studies have demonstrated that L-Glu induces oxidative stress by increasing intracellular reactive oxygen species (ROS), reducing levels and function of the antioxidant defence system, and impairing mitochondrial function, ultimately leading to neuronal damage and loss. Treatment with aqueous extract of *C. edulis* as well as the standard drug significantly reversed the L-glutamic acid-induced decrease in locomotion. Treatment with the aqueous extract of *C. edulis* inhibits the deleterious effect of glutamate suggested by its antioxidant properties thus protecting the neurons of mice against oxidative stress due to glutamatergic overstimulation. The hanging wire test, which assesses the neuromuscular disturbance of muscle strength in mice based on their grip strength, also yielded insightful results. Upon administration of L-glutamic acid, the suspension time of mice was reduced likely due to excessive stimulation of the glutamate pathway and inhibition of GABA release (Swamy et al., 2013). However, treatment with *C. edulis* and vitamin C notably increased this suspension time. This suggests that the reversal effect of L-glutamic acid induced by treatment with *C. edulis* can be attributed to its various beneficial properties.

Glutamate is particularly concentrated in brain regions essential for cognitive processes, especially in the cerebral cortex, hippocampus, striatum, and dentate gyrus (Abass and El-Haleem, 2011). The learning and memory abilities were performed using the elevated plus maze test. The EPM is the most widely used behavioural test for the assessment of anxiety response in rats (Pellow et al., 1985) and mice (Lister, 1987). It has been used later to evaluate learning and memory in rats and mice (Sharma and Kulkarni, 1992). This EPM model is considered a reliable apparatus for the drug's effect evaluation on learning and memory performances (Dhingra and Kumar, 2012, Pahaye et al., 2017). L-glutamic administration significantly increased the latency time of entrance into the enclosed arm, while the treatment with *C. edulis* aqueous extracts decreased this latency time in a dose-dependent manner. The longer the latency time, poor is the retention memory, and the shorter the latency time, the greater the retention memory (Morales-Delgado et al., 2018). Thus, a reduction in latency time between the acquisition and the retention trials indicates an improvement in the memory performance of mice (Yarube et al., 2016). In our previous study, it was found that *C. edulis* improved the learning ability and memory performance of mice in the T-maze and object recognition task. Also, it had the capacity to inhibit acetylcholinesterase enzyme (AChE), suggesting a potential capacity to improve cognitive functions (Yadang et al., 2020). This capacity is explained by the polyphenols it contains (Yadang et al.,

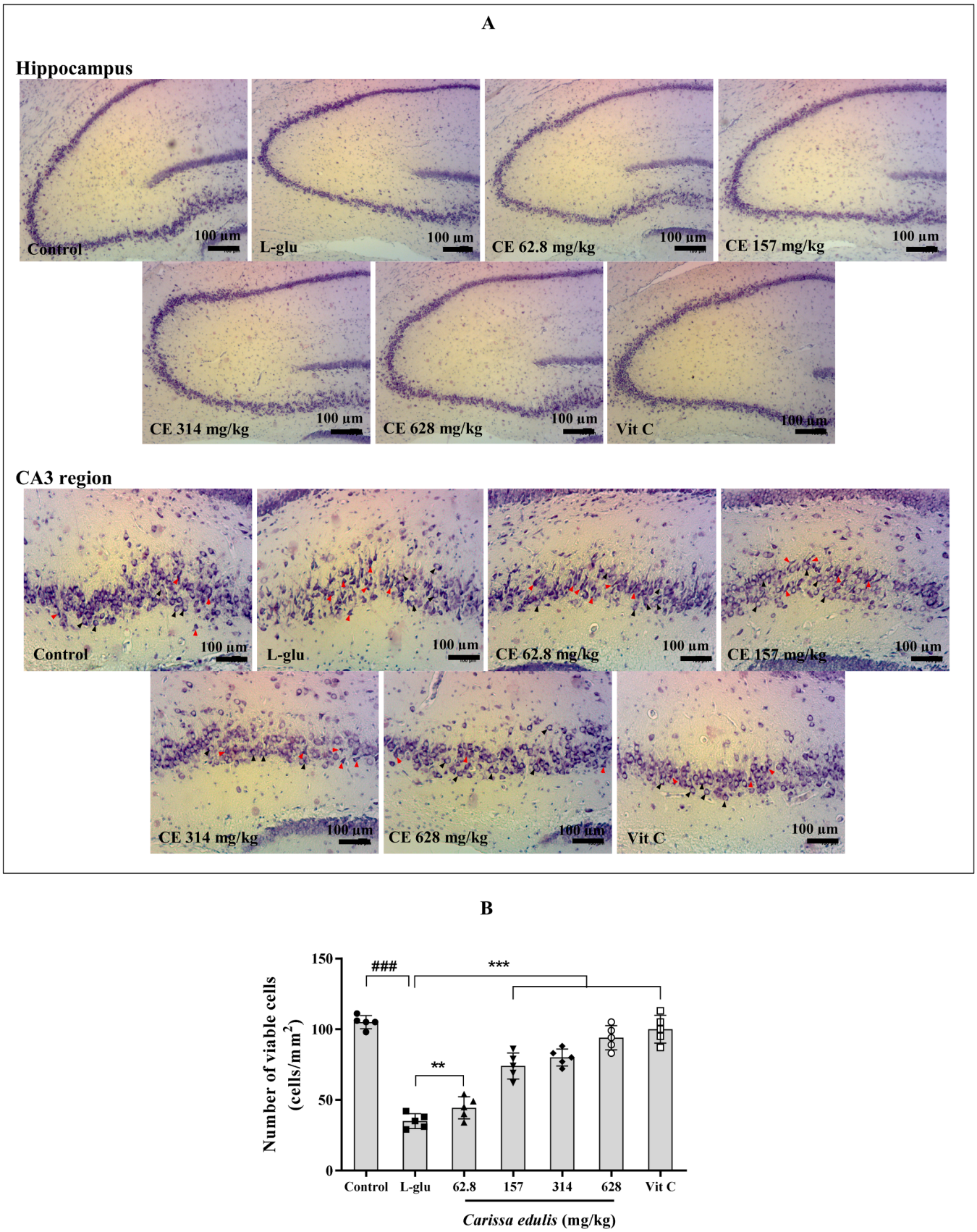

Fig. 6. Effects of *C. edulis* aqueous extract on histopathological changes in hippocampus examined by cresyl violet staining in L-glutamic acid-treated mice. A) Histopathological changes in hippocampus and CA3 region, Scale bar = 100 µm. B) Hippocampal neuron survival density and cells count using ImageJ software. Data are expressed as mean ± SD, n = 5. $^{###}p < 0.001$ vs. control group; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. L-glu group. One-way ANOVA followed by Tukey multiple comparison tests. Red arrows represent degenerated cells and black arrows represent normal cells. L-glu: L-glutamic acid, CE: *Carissa edulis*.

2019), as one property of polyphenols includes the improvement of memory (Vauzour, 2012).

Recently, many findings supported that the elevation of reactive oxygen species leading to oxidative stress correlated with elevated inflammatory markers is associated with low cognitive performance in age-related disorders (Liguori et al., 2018). Furthermore, the increased level of intracellular ROS is highly implicated in the pathogenesis of glutamate-induced neurotoxicity leading to necrosis and apoptosis of neurons (Pavlovic et al., 2009; Ezza and Khadrawyb, 2014; Xin et al., 2019). The overstimulation of N-methyl-D-aspartate (NMDA) receptors by L-glutamate results in increased intracellular calcium levels, which subsequently activates various pathways that generate ROS, including mitochondrial dysfunction and activation of nitric oxide synthase (NOS) (Aschner et al., 2007, Shah et al., 2016). Moreover, the influx of Ca2 + into mitochondria can disrupt their function, leading to increased production of ROS as a byproduct of altered oxidative phosphorylation processes. This mitochondrial ROS can further exacerbate oxidative stress and contribute to cellular damage (Liu et al., 2023). During this study, the oxidative stress status was evaluated. The results obtained revealed that the level of the lipid peroxidation end-product namely MDA increased in the brain of mice treated with L-glutamic acid while the level of glutathione and catalase activity decreased. The significant imbalance in the brain is considered as an indication of oxidative stress and neuronal damage (Lee et al., 2020). This increased level of MDA may result in the disruption of neuronal membrane structure with the non-specific permeability to ions leading to the loss in function and integrity of the neurons (Swamy et al., 2013). The treatment with the aqueous extract of *Carissa edulis* dose-dependently the MDA level compared to the L-glutamic acid-treated group, which suggests a protective effect of *Carissa edulis* against the oxidative stress induced by L-glutamic acid. There is a significant decrease in the glutathione level for the L-glutamic model group compared to normal animals. Glutathione is the major intracellular antioxidant that plays a role in the non-enzymatic antioxidant defense system. It is considered an antioxidant scavenging free radicals and other ROS and an inhibitor of lipid peroxidation mediated (Swamy et al., 2013). The depletion of glutathione level induced by the excitotoxicity is due to the inhibition of cysteine transport blocking the formation of glutathione in mouse brain (Thonda et al., 2014) and thus induces the reduction in the level of glutathione as observed in the present study. *Carissa edulis* aqueous extract administration increased the glutathione level compared to the model group, indicating the protection of neurons against the neurotoxicity induced by L-glutamic acid. The result of the catalase activity showed a significant decrease in the catalase activity for the group treated with the L-glutamic group compared to the normal group. Catalase is an antioxidant enzyme produced naturally by the body which reacts with hydrogen peroxide to form water and oxygen (Lee et al., 2020). Catalase protects cells against free radical damage by removing the H_2O_2 formed with the conversion of O^{2-} radical into H_2O_2 by superoxide dismutase (Swamy et al., 2013; Lee et al., 2020). Decrease, inhibition, or malfunction in catalase activity results in the enhancement of excitotoxicity and increased level of ROS, indicating the important role of catalase enzyme in maintaining the oxidative balance (Terlecky et al., 2006, Lee et al., 2020). In the present study, oxidative stress induced by L-glutamic acid administration significantly reduced the catalase activity. However, treatment with *C. edulis* aqueous extract significantly increases this catalase activity. Previous studies have shown that *C. edulis* aqueous extract possesses antioxidant activity, which has decreased the MDA level, increasing catalase activity and glutathione level in the brain (Yadang et al., 2020). Also, the study has shown that *C. edulis* contains a high level of bioactive compounds such as polyphenols, flavonoids, and tannins which are the major contributors to its antioxidant activity. These compounds are known to be good radical scavengers (Yadang et al., 2019). Thus, the antioxidant property of *C. edulis* may protect the mice's brains against the neuronal damage induced by L-glutamic acid.

Reactive oxygen species and cytokines are closely related in many cellular processes and the relationship between both is complex and bidirectional. An excessive production of ROS can stimulate the production of pro-inflammatory cytokines, such as IL-1β and TNF-α through various signalling pathways, including the mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways (Padgett et al., 2013). Cytokines, in turn, can lead to the generation of more ROS (Ranneh et al., 2017, Petrie et al., 2018). Cytokines like IL-1β can upregulate NADPH oxidase activity in cells, leading to increased ROS generation (Lambeth, 2004). Also, Cytokines can down-regulate the expression and activity of enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase, which are responsible for ROS detoxification (Jena et al., 2023). Microglia, the principal immune cell of the CNS, when activated produces several inflammatory cytokines, including IL-1β, IL-6, and TNF-α, highly expressed during neurodegenerative diseases (Ishijima, Nakajima, 2021). Many studies have shown the presence of cytokines, particularly IL-1β in the brain of patients with Alzheimer's disease (Hoozemans et al., 2011). Moreover, treatment with a high concentration of glutamate-induced an increase in the production of Tumor necrosis factor-α in rat's brains (Abass and El-Haleem, 2011). Glutamate toxicity has been reported in many cases of neurodegenerative disorders (Xin et al., 2019). Several research papers have shown the relation between glutamate, ROS and cytokines (Neveen and Iman, 2010, Xin et al., 2019, Shah et al., 2016, Yang et al., 2017, Qiang et al., 2020). Moreover, Yang et al. (2017) have shown in their study that glutamate-induced elevation of lipid peroxidation, tumour necrosis factor-α, interferon-gamma (IFN-γ), interleukin-1β, nitric oxide, reactive oxygen species, NADPH oxidase, caspase-3, calpain activity, and Bax. Studies have shown correlations between cognitive decline and plasma levels of some cytokines in the early and moderate stages of Alzheimer's disease, suggesting an interaction between lymphocytes and glial cells (Paccalin et al., 2009). The present study showed that the administration of L-glutamic acid resulted in the production and up-regulation expression of cytokines, thus, showing the installation of an underlying inflammatory process. *Carissa edulis* aqueous extract significantly inhibited the TNF-α and down-regulated the expression of IL-β. This activity could be due to active compounds such as tannins which have anti-inflammatory properties (Fraga-Corral et al., 2021; Ambreen and Mirza, 2020). Also, the group that received vitamin C, an antioxidant, has seen their brain protected from neuroinflammation. Vitamin C deficiency in glial cells triggers pro-inflammatory microglia activation and neuroinflammation (De Nuccio et al., 2021). The study of Nualart has reported the antioxidant and neuroprotective effects of vitamin C against glutamate excitotoxicity in the brain and cerebrospinal fluid (Nualart et al., 2014). Treatment with vitamin C showed the ability to reduce microglial activation in a rat model of ethanol-induced neuroinflammation (Ahmad et al., 2016). Similarly, the study of Zhang et al. showed that treatment with vitamin C reduced the microglial activation and the proinflammatory cytokine production in a lipopolysaccharide mice model (Zhang et al., 2018). Antioxidants, both endogenous and exogenous, can modulate cytokines production and activities, reducing the inflammatory response and ROS generation. Antioxidant intake is then necessary and beneficial to protect the brain against oxidative stress and underlying neuroinflammation. That is why *Carissa edulis*, which has both antioxidant and anti-inflammatory properties, is a suitable candidate for a palliative treatment or alternative drug in the fight against neurodegenerative diseases like Alzheimer's disease. These results demonstrate the therapeutic activity of *Carissa edulis* via its anti-inflammatory properties.

Oxidative stress and inflammation can occur in different regions of the brain and are involved in the pathophysiology of various brain diseases. Therefore, evaluating damage in the hippocampus, the brain region most associated with learning and memory functions (Anand and Dhikav, 2012), is crucial. Moreover, the CA3 region has attracted major attention for its specific role in memory processes, susceptibility to

seizures, and neurodegeneration. It is important for rapid encoding of memory and the architecture of CA3 circuits seems to be well adapted for the rapid storage and retrieval of memories (Cherubini and Miles, 2015; Rebola et al., 2017). The hippocampus is characterized by high neuronal network activity and plasticity, making it particularly vulnerable to disruptive events such as degenerative processes. Furthermore, neurons in the hippocampus are especially susceptible to oxidative stress due to their high metabolic activity and low levels of antioxidant defenses (Wang and Michaelis, 2010). Elevated levels of ROS can damage cellular components like lipids, proteins, and DNA, ultimately leading to neuronal damage (Mohan Kumar et al., 2023). Moreover, ROS are generated within mitochondria following calcium influx triggered by glutamate receptor stimulation. The presence of these ROS can activate signalling pathways related to inflammation and cell damage. Pro-inflammatory cytokines such as IL-1β and IL-6 are released from activated microglia and astrocytes in response to oxidative stress (Padgett et al., 2013). High levels of ROS are associated with synaptic dysfunction and a loss of plasticity in hippocampal neurons, which are essential for cognitive functions (MohanKumar et al., 2023).

Research indicates that oxidative stress and inflammation are significant contributors to hippocampal neuronal damage in various neurological conditions, including Alzheimer's disease, traumatic brain injury, and cerebral ischemia (Wang and Michaelis, 2010). For instance, increased ROS levels and elevated expression of pro-inflammatory cytokines have been observed in the hippocampus of animal models with neurodegenerative diseases (Hamadi et al., 2016, Talebi et al., 2022). Loss of hippocampal structure was noted in mice treated with L-glutamic acid resulting in memory impairment and a decrease in the percentage of memory retention in the elevated plus maze test. The results of this study clearly show that L-glutamic acid administration to mice causes neuronal loss in the hippocampus with a decrease in pyramidal cell number. The cell death in the hippocampus induced by the administration of L-glutamic acid may be attributed to oxidative damage that leads to mitochondrial dysfunction with the release of cytochrome C and activation of caspases (Thonda et al., 2014; Hazzaa et al., 2020). Therefore, the use of antioxidants is a vital process in inhibiting neuronal damage. Treatment with *Carissa edulis* showed normal pyramidal cells with no histopathological changes. This neuroprotective activity of *Carissa edulis* to protect cells against degeneration may be attributed to high polyphenols content which confers the ability to modulate redox activity and to reduce oxidative stress. These polyphenol compounds are thought to be powerful antioxidants (Yadang et al., 2019, Yadang et al., 2020), thus providing neuronal protection and preventing neuronal damage by L-glutamic acid-induced excitotoxicity. The neurotoxicity of L-glu is not only specific to the hippocampus. While the hippocampus is particularly vulnerable to excitotoxicity due to its high density of glutamate receptors and its role in learning and memory, other brain regions also exhibit sensitivity including the cerebral cortex, striatum, basal ganglia, and amygdala (Wang and Michaelis, 2010, Ganesan et al., 2013). Thus, the hippocampus, vulnerable brain regions to glutamate-mediated excitotoxicity, plays an important role in spatial learning and memory (Hajihasani et al., 2020). Protecting this brain region from degeneration may increase the learning ability of mice, thus improving memory.

6Conclusion

The present study evaluated the protective effect of *Carissa edulis* on L-glutamic acid-induced neurotoxicity via the regulation of oxidative stress and neuroinflammation. Taken together, the aqueous extract of *Carissa edulis* improved the locomotor activity and increased the hanging latency time of mice in the muscle strength test. It improves learning and memory in the elevated plus maze by decreasing the latency time to enter into the closed arms. The antioxidant activity evaluated showed that *Carissa edulis* aqueous extract decreases the level of MDA and enhances the activity of catalase and the level of glutathione, thus conferring protection to neurons against neurotoxicity and eventual death. In addition, increases in proinflammatory markers IL-1β and TNF-α were inhibited by *Carissa edulis* aqueous extract treatment. *Carissa edulis* aqueous extract, therefore, protects the brain of mice against the installation of neuroinflammation. Also, *Carissa edulis* protects against pyramidal cell loss and neuronal degeneration. Thereby, *Carissa edulis* ameliorated L-glutamic acid-induced neurotoxicity and neurodegeneration in brain mice by regulating oxidative stress and inflammation. Also, vitamin C protects against the neurotoxicity induced by L-glutamic acid. Vitamin C is a potent antioxidant that has been shown to mitigate oxidative stress and damage induced by L-glutamate. However, plant extracts can offer additional benefits that go beyond what Vitamin C alone can provide. These plant-based options including *Carissa edulis* are often more available and more accessible to the local population. Furthermore, they offer various advantages including synergistic effects, anti-inflammatory and neuroprotective properties, as well as nutritional benefits. This study demonstrates that *Carissa edulis* extract has a direct effect on inhibiting oxidative stress and inflammatory cytokines. This effect is attributed to its various bioactive components, such as polyphenols, flavonoids, and tannins, which are the primary contributors to its antioxidant and anti-inflammatory properties. The findings suggest that *Carissa edulis* is a potent natural agent against neurotoxicity induced by L-glutamic acid. However, compounds will be isolated and further research conducted to understand and clarify the molecular mechanisms of *Carissa edulis* neuroprotection against neurotoxicity caused by L-glutamic acid.

Compliance with ethical standards

Animal handling and the experimental procedure were carried out in accordance with international guidelines for the care and use of laboratory animals from the National Institutes of Health (NIH Publications No. 8023, revised 1978). The study was approved by the COMSATS Institute of Information Technology Ethics Committee, Abbottabad, Pakistan (Reg. No. 73 Phm. Eth/FA17-CS-M10/17–010–71).

There is no human subject involved in this study and informed consent is not applicable.

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CRediT authorship contribution statement

Agbor Gabriel Agbor: Writing – review & editing, Supervision. **Sotoing Taiwe Germain:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Ngo Bum Elisabeth:** Writing – review & editing, Validation, Supervision, Conceptualization. **Fanta Yadang Sabine Adeline:** Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Nguezeye Yvette:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

None.

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