J Herbmed Pharmacol. 2024; 13(3): 390-398.

http://www.herbmedpharmacol.com doi: 10.34172/jhp.2024.44768

Journal of Herbmed Pharmacology

Neuroprotective effect of *Launaea taraxacifolia* against neuroinflammation, memory loss and neurobehavioral deficit in a rat model of hypertension: biochemical and immunohistochemical approaches

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ARTICLE INFO

Article Type:
Original Article

Article History:
Received: 12 December 2022
Accepted: 4 April 2024

Keywords:
Dementia
Astrogliosis
Microgliosis
Oxidative stress
Neuro-inflammation
Neuroprotection

ABSTRACT

Introduction: Alterations of antioxidant defense, neuroinflammation, and neurodegeneration are common pathological occurrences associated with neurodegenerative diseases. This study evaluated the neuroprotective effect of *Launaea taraxacifolia* (LT), popularly known as African Wild lettuce, against neuroinflammation, memory loss, and neurobehavioral deficit.

Methods: Adult Wistar rats were used following random assignment into groups 1 to 5. Group one was the normal control. Groups four to five received 40 mg/kg N^{ω}-nitro-l-arginine methyl ester (L-NAME). In addition to L-NAME exposure, groups three and four received 100 and 200 mg/kg LT, whereas group five received 10 mg/kg lisinopril. The experiment lasted for five weeks. Markers of oxidative stress, neurobehavioural studies, histology, and immunohistochemistry of glial fibrillary acidic protein (GFAP), ionised calcium-binding adaptor molecule 1 (Iba-1), as well as anti-calbindin for staining astrocytes, microglia, and Purkinje cells were determined.

Results: Malondialdehyde (MDA) and protein carbonyl in the L-NAME alone group were heightened compared to those treated with LT. However, treatment with LT significantly reduced neuronal oxidative stress, neuroinflammation, and neurobehavioural changes. Quantitative analysis of immunohistochemical staining revealed heightened glial fibrillary acidic protein (GFAP), ionised calcium-binding adaptor molecule 1 (Iba-1), as well as anti-calbindin as indicated by astrogliosis, microgliosis, and Purkinje cell degeneration in untreated rats. Moreover, the observed ultrastructural anarchy induced by L-NAME was restored in rats treated with LT ($P<0.05$).

Conclusion: Together, the leaf extract of LT can be effective as a neuroprotective drug candidate.

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Implication for health policy/practice/research/medical education:
Co-treatment with *Launaea taraxacifolia* significantly improved astrogliosis, microgliosis, Purkinje cell degeneration, and depletion of neuronal antioxidant defense status. The results highlighted neuroprotective properties associated with *L. taraxacifolia* administration in experimental hypertension. Hence, *L. taraxacifolia* is a viable drug candidate for the treatment of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases.
Please cite this paper as: Oyagbemi AA, Hassan FO, Adebiyi OE, Adigun KO, Folarin OR, Ajibade TO, et al. Neuroprotective effect of *Launaea taraxacifolia* against neuroinflammation, memory loss and neurobehavioral deficit in a rat model of hypertension: biochemical and immunohistochemical approaches. J Herbmed Pharmacol. 2024;13(3):390-398. doi: 10.34172/jhp.2024.44768.

Introduction

N^{ω}-nitro-l-arginine methyl ester (L-NAME) selectively inhibits biosynthesis of the endogenous vasodilator, nitric oxide (NO), with consequent mediation of oxidative stress-induced organ damage in rats (1,2). In the brain, neurobehavioral deficit has been associated with L-NAME administration in rats following experimental hypertension (3). Moreover, alterations in NO production disrupt normal nervous system functioning due to neuro-inflammation and oxidative stress (4,5).

Launeae taraxacifolia (LT) is a widely used leaf crop in Africa, where it is commonly gathered from the wild for local use due to its high nutritional and medicinal values (6,7). Traditionally, LT, due to its very potent antioxidant properties, is widely used for the management of varying ailments such as hypertension, anemia, diabetes, and hepatic diseases (8). The ethanolic extract of LT mitigated oxidative stress and ameliorated histological alterations of different anatomical portions of the brain in rats following mercuric chloride exposure (9). The evaluation of neuroprotective potency associated with LT administration on neuroinflammation, alterations of cognitive functioning, and behavioural deficits in L-NAME exposed rats was the aim of this study.

Materials and Methods

Experimental design

Adult Wistar rats were used following random assignment into groups 1 to 5. Group one was the normal control. Groups four to five received 40 mg/kg L-NAME. In addition to L-NAME exposure, groups three and four received 100 and 200 mg/kg LT, whereas group five received 10 mg/kg lisinopril. The experiment lasted for five weeks. Neurobehavioral studies were conducted on the last five days of the experiment before the sacrifice of the animals. On the last day of dosing, various samples and organs were carefully removed from anesthetized rats following euthanasia for biochemical analysis and immunohistochemistry procedures.

Plant collection, processing, and extraction

The fresh leaves of LT were prepared and authenticated at the Department of Botany, University of Ibadan herbarium with the deposition of a specimen with herbarium number UIH-22760. Thereafter, the dried pulverized leaves were defatted using n-hexane, extracted with methanol, filtered, and subsequently subjected to evaporation to dryness at 40°C. The resulting extract was used for this study.

Chemicals

Acetylcholine iodide, thiobarbituric acid, ammonium ferrous sulphate, Biuret's reagent, Greiss reagent, N-(1-naphthyl)ethylenediamine, sulphanilamide, phosphoric acid, sodium hydroxide, creatinine reagent, copper sulphate, trichloroacetic acid, and reduced glutathione (GSH) were used in this experiment.

Hanging wire grip test

The hanging wire grip test was performed to evaluate the grasping ability of the forelimb and coordination of movement. The rats were placed with their forelimbs suspending their bodies from a wire, 0.3 cm in diameter, 40 cm in length, and 45 cm above soft ground (10). The time spent holding the wire was recorded during 2 daily trials for each rat. A maximum cut-off latency time of 120 seconds was recorded.

Open field test

Locomotion in an open field box was measured once during the animal's light cycle. Each rat was placed individually in the center of an open field box (72 × 72 cm, 36 cm high). One of the sides of the box was covered with a plexiglass so that the rats could be visible in the apparatus. Black lines were drawn from the floor with a marker and each rat could explore the open field arena for five minutes. Line crossing, stretched-attend posture, rearing, grooming, center square, and freezing duration were recorded as described by (11).

Morris water maze test

Spatial learning and reference memory in the experimental animals were determined with the Morris water maze (MWM) test (12). A submerged escape platform was placed in the Northeast quadrant of a black circular tank (diameter of 150 cm and depth of 50 cm) filled with water. The water was made opaque with powdered non-fat milk. During the spatial learning trial, each rat was tested four times per day for three consecutive days. Learning by using distal cues to navigate to the hidden platform when starting from four different quadrants (North, East, West,

and South) was assessed in the animals. At first, the rats were placed on the platform in the maze for 20 seconds before lowering them gently from the start positions, facing the wall. Each subject was given 120 seconds to find the escape platform. In case the subject failed to locate the platform within the set time it was gently guided towards the platform (13). Each animal was allowed 15 minutes of inter-trial rest. The latency to locate the escape platform was recorded with a stopwatch.

A single spatial probe trial was conducted the day after the last acquisition testing; the pool was set up as before except the hidden platform was removed. The time spent within the quadrant where the escape platform was previously located was documented.

Immunohistochemistry

The immunolocalization of cerebrum and cerebellum was described as earlier reported (14) to determine the expression of glial fibrillary acidic protein (GFAP) for astrocytes, ionized calcium-binding adaptor molecule 1 (Iba1) for microglia, and cerebellar Ca^{2+} binding protein calbindin D-28k (CB) in the brain (Purkinje neurons). Brain samples were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned at a thickness of 5 µm. Antigen retrieval was done in 10 mM citrate buffer (pH = 6.0) for 25 minutes, with subsequent peroxidase quenching in 3% H_2O_2/methanol. The sections were blocked in 2% milk for 1 hour and probed with the following antibodies overnight: anti-GFAP Rabbit Polyclonal (1:1000; Dako, Denmark), anti-Iba-1 (1:800; Wako, USA) and anticalbindin rabbit monoclonal antibody D-28k (1:12 000; Swant, Switzerland) for 16 hours at 4 °C. After washing, the sections were incubated for 2 hours at room temperature in the appropriate biotinylated secondary antibodies (1:500; Vector Labs). The sections were then reacted in avidin-biotin-peroxidase solution (ABC kit, Vectastain, Vector Labs, USA) using 3, 30-diaminobenzidine (DAB) as chromogen, according to the manufacturer's protocol. Sections were observed with light microscope (Leica LAS-EZ®) using the Leica software application suite version 3.4 equipped with a digital camera. Immunoreactivity was quantified using ImageJ software.

Biochemical analysis

The markers of oxidative stress were analysed in the post mitochondria fraction (PMF) of brain homogenates using standard methods previously described by Woff (15) for hydrogen peroxide (H_2O_2), by Varshney and Kale (16) for malondialdehyde (MDA), and by Reznick and Packer (17) for protein carbonyl. Similarly, the markers of the antioxidant defence system were analysed with the methods previously described by Rotruck et al (18) for glutathione peroxidase, by Habig et al (19) for glutathione S-transferase, by Beutler et al (20) for reduced glutathione, and by Misra and Fridovich (21) for superoxide dismutase. Furthermore, the methods of Olaleye et al (22) and of Xia and Zweier (23) were used for the determination of NO and myeloperoxidase serum levels, whereas protein contents were estimated using the method of Gornal et al (24).

Statistical analysis

Statistical analysis was carried out on the data from this study with One-way ANOVA with Dunnett's post-test at a 95% confidence limit. All values were expressed as mean ± SD. The test of significance between the two means was also estimated by Student's t test.

Results

Neurobehavioural studies

Open field test

The results of the open feed test revealed that the grooming frequency, freezing, stretch attend posture, and rearing between the tests and the control group of rats were similar (Figure 1). However, the escape latency (Figure 1) across all the groups decreased with increasing trial day in the MWM test suggesting that learning was not impaired in the treated and control rats over time. Likewise, the probe trial results on the last day (Day 4) showed that the times spent in the target quadrant by rats administered with L-NAME only decreased significantly relative to LT-treated rats (Figure 2). The hanging latency was similar in all experimental groups in the hanging-wire test

Biochemical analyses

The neuronal MDA, H_2O_2, protein carbonyl, and NO were significantly ($P<0.05$) elevated in rats administered L-NAME alone compared with these oxidative stress markers in the LT-treated rats (Table 1). Also, LT significantly ($P<0.05$) increased superoxide dismutase, glutathione S-transferase, glutathione peroxidase, vitamin C, and protein and non-thiols in comparison with the rats treated with L-NAME alone (Table 2). In addition, acetylcholinesterase (AchE) activity increased significantly, but the myeloperoxidase activity decreased in LT administered rats compared with the rats exposed to L-NAME without LT treatment (Table 3).

Immunohistochemistry

Distortion in Purkinje cellular morphology was observed following L-NAME exposure in the absence of LT treatment; however, LT administration significantly mitigated this morphological distortion (Figure 3). Microglial and astrocytic cellular infiltrations in L-NAME-treated rats were heightened relative to LT-treated rats (Figures 4 and 5). Treatment of rats with LT significantly ($P<0.05$) attenuated L-NAME-induced reduction in Purkinje cell counts and distortion in Purkinje cellular morphology, astrocytosis, and microgliosis.

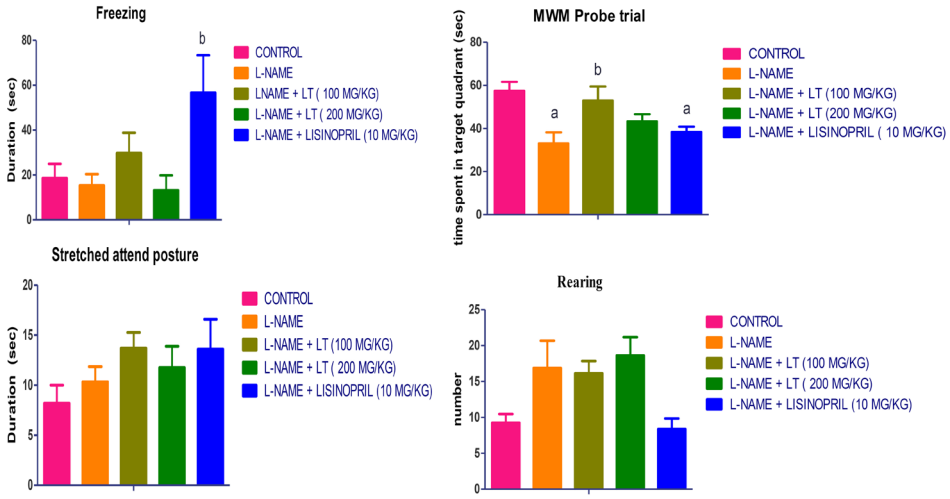

Figure 1. Ameliorative effect of *Launaea taraxacifolia* on neurobehavioural responses associated with *N*$^{\omega}$-nitro-l-arginine methyl ester (L-NAME)-induced neurotoxicity on Wistar rats (Open field tests 1). Group 1 (control group), group 2 (L-NAME 40 mg/kg) orally, group 3 (100 mg/kg of LT plus 40 mg/kg L-NAME, group 4 (200 mg/kg of LT plus 40 mg/kg L-NAME, group 5 (10 mg/kg of LT plus 40 mg/kg L-NAME). Different letters on columns indicate significantly different ($P < 0.05$).

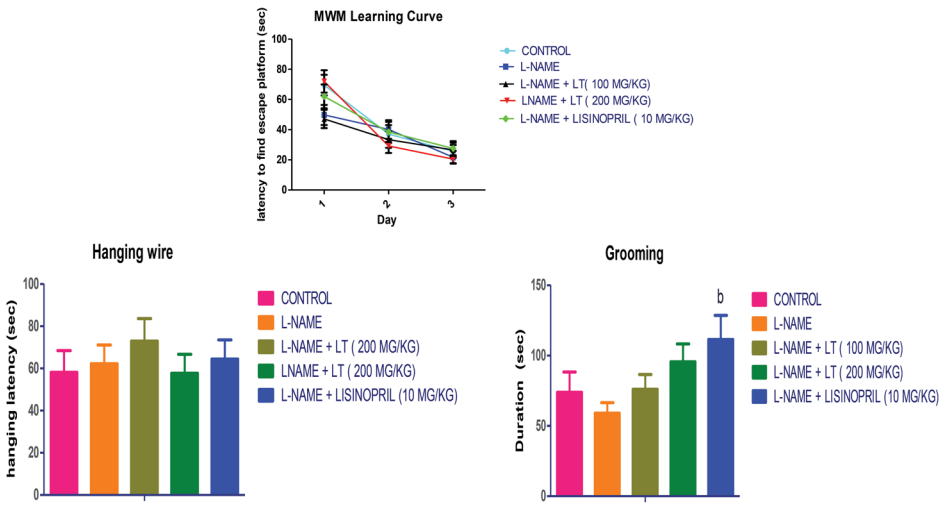

Figure 2. Ameliorative effect of *Launaea taraxacifolia* on neurobehavioural responses associated with N(ω)-nitro-L-arginine methyl ester (L-NAME)-induced neurotoxicity on Wistar rats (Open field tests 2). Group 1 (control group), group 2 (L-NAME 40 mg/kg) orally, group 3 (100 mg/kg of LT plus 40 mg/kg L-NAME, group 4 (200 mg/kg of LT plus 40 mg/kg L-NAME, group 5 (10 mg/kg of LT plus 40 mg/kg L-NAME). Different letters on columns indicate significantly different ($P < 0.05$).

Discussion

In this study, the administration of L-NAME alone significantly increased neuronal NO, H_2O_2, MDA, and protein carbonyl contents relative to the control and LT-treated groups. It is probable that the exaggerated production of reactive oxygen species and free radicals might be associated with the uncoupling of the NOS with the concomitant production of superoxide anion radicals and enhanced generation of neuronal NO (25,26).

The observable enhanced production of neuronal MDA was not surprising because the brain has very high content of lipids. Hence, peroxidation of polyunsaturated fatty acid in the brain expectedly will generate high MDA. This observation suggests that L-NAME has the capacity to induce neuroinflammation and neuronal oxidative stress. Interestingly, co-administration of L-NAME with LT showed better adaptation to oxidative stress. High protein carbonyl contents in the rats administered L-NAME alone, observed in this study, is indicative of oxidation of proteins with critical roles in the central nervous system such as neurofibrillary tangles, beta-amyloid protein, and tau. The oxidation, misfolding, and subsequent precipitation of these proteins are hallmarks of Alzheimer's disease (27,28). Furthermore, LT administration abates the L-NAME-induced abnormally elevated MDA, protein carbonyl, NO, and H_2O_2 generation. Also, the

Table 1. Effect of *Launaea taraxacifolia* (LT) on neuronal markers of oxidative stress

Studied parameters	Control	L-NAME 40 mg/kg	100 mg/kg of LT plus 40 mg/kg L-NAME	200 mg/kg of LT plus 40 mg/kg L-NAME	10 mg/kg of lisinopril and 40 mg/kg L-NAME
H_2O_2 generation (µmole/min/mg protein)	0.65±0.02	0.71±0.15[a]	0.79±0.04[b]	0.84±0.11[b]	0.89±0.04[b]
Protein carbonyl (nmole /mg protein)	74.11±5.87	80.36±5.39[a]	75.61±7.57[a]	72.33±9.00[a]	83.94±1.77[a, b]
Malondialdehyde (µmole MDA formed/mg protein)	7.00±1.00	8.00±1.00[a]	7.00±1.00[b]	7.00±1.00[b]	5.00±0.10[b]

Values are presented as mean ± standard deviation (n = 10). Superscript ([a]) indicates a significant difference at $P < 0.05$ compared with control (group 1), while superscript ([b]) indicates a significant difference at $P < 0.05$ compared with group 2 in each row. (One-way analysis of variance plus student's t-test was used).

Table 2. Effect of *Launaea taraxacifolia* (LT) on neuronal non-enzymic antioxidant defence system

Studied parameters	Control	L-NAME 40 mg/kg	100 mg/kg of LT plus 40 mg/kg L-NAME	200 mg/kg of LT plus 40 mg/kg L-NAME	10 mg/kg of lisinopril and 40 mg/kg L-NAME
Vitamin C (µmole/mg protein)	1.55±0.21	1.49±0.08	1.66±0.22	1.72±0.54	1.57±0.15
Non-protein thiol (µmole/mg protein)	129.23±24.8	88.63±4.29[a]	96.66±5.3[a]	99.79±6.28[a]	76.02±4.52[a]
Protein thiol (µmole/mg protein)	49.89±9.77	44.8±4.22	47.09±7.21	48.01±3.34	45.81±8.93
Reduced glutathione (µmole/mg protein)	84.09±9.9	64.52±7.34[a]	74.29±5.99	83.36±9.26[b]	91.34±4.45[a, b]

Values are presented as mean ± standard deviation (n = 10). Superscript ([a]) indicates a significant difference at $P < 0.05$ compared with control (group 1), while superscript ([b]) indicates a significant difference at $P < 0.05$ compared with group 2 in each row. (One-way analysis of variance plus student's t-test was used).

Table 3. Effect of *Launaea taraxacifolia* (LT) on neuronal enzymic antioxidant defence system and neuroinflammatory markers

Studied parameters	Control	L-NAME 40 mg/kg	100 mg/kg of LT plus 40 mg/kg L-NAME	200 mg/kg of LT plus 40 mg/kg L-NAME	10 mg/kg of lisinopril and 40 mg/kg L-NAME
GST (mmol CDNB formed/mg protein)	58.87±8.64	43.95±3.74[a]	53.65±8.06[b]	42.72±6.03[b]	43.59±8.76[b]
SOD (Units/mg protein)	17.38±0.99	13.79±0.64[a]	15.55±0.69[a]	14.67±1.15[a, b]	11.58±1.36[a, b]
GPx (Units/ mg protein)	187.11±2.23	169.96±8.21[a]	206.03±11.81[a, b]	178.39±13.28[a]	168.23±3.83[a]
AChE (nmole/mg protein)	410.18±56.51	327.58±20.65[a]	439.78±51.94[b]	313.26±45.49[a]	368.61±55.68[b]

Values are presented as mean ± standard deviation (n = 10). Superscript ([a]) indicates a significant difference at $P < 0.05$ compared with control (group 1), while superscript ([b]) indicates a significant difference at $P < 0.05$ compared with group 2 in each row. (One-way analysis of variance plus student's t-test was used).

results obtained from the MWM test showed that the rats administered L-NAME alone displayed memory deficit and impaired neurobehavioural changes. However, the locomotor activity and muscular strength were not significantly altered as expected. However, co-treatment with methanol leaf extract of LT significantly improved memory loss, locomotor activity, and muscular strength. Based on these results, it can be inferred that LT might contain cocktails of phytochemicals that are essential drug candidates for the prevention and treatment of neurodegenerative disease conditions.

Under experimental conditions, the observation of elevated NO in the brain was reported to be linked with cognitive deficit (29). In this study, the administration of L-NAME caused a significant increase in the level of NO in the brains of rats. This observation may suggest the induction of neuronal inflammation and damage in the L-NAME-administered rats. This was observed to decrease following treatment with LT. Surprisingly, the activity of AChE reduced significantly in the L-NAME administered group in comparison with the LT group. Several neuronal pathologies associated with AChE have been extensively reported in other studies (30,31). Furthermore, the connection between AChE inhibition and autonomic nervous dysfunctions has also been reported (32). Furthermore, the induction of oxidative stress, neuroinflammation, and modulation of AchE has been documented as part of the pathophysiology of AD culmination in memory loss and cognitive aberration (33). The inhibition of AChE by L-NAME could also contribute to neuronal loss, memory deficit, cognitive function, and impaired neuronal signaling. However, LT and Lisinopril treatment reversed the inhibited AChE except for the highest dosage of LT.

Several mechanisms have been proposed as crosstalk between neuroinflammation and neuronal oxidative stress (34). The product of lipid peroxidation and protein oxidation, known as MDA and protein carbonyl, respectively were significantly lowered in LT-administered rats suggesting a mitigation of oxidative stress and a lower

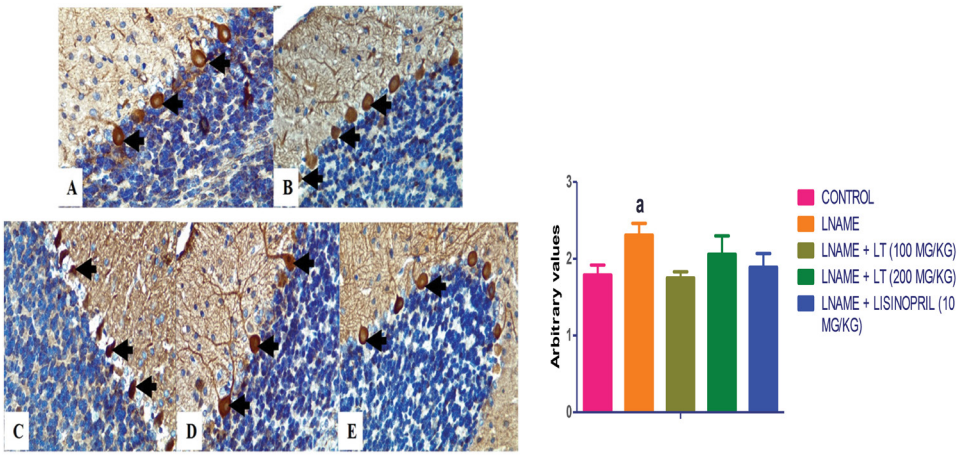

Figure 3. The immunohistochemistry of anti-calbindin in the Purkinje layer of the cerebellum. A: control group; B: L-NAME 40 mg/kg orally; C: 100 mg/kg of *Launaea taraxacifolia* (LT) plus 40 mg/kg L-NAME; D: 200 mg/kg of LT plus 40 mg/kg L-NAME); E: 10 mg/kg of LT plus 40 mg/kg L-NAME. The arrows represent immunohistochemical expressions of anti-calbindin in the Purkinje layer of the cerebellum. Different letters on columns indicate significantly different ($P < 0.05$).

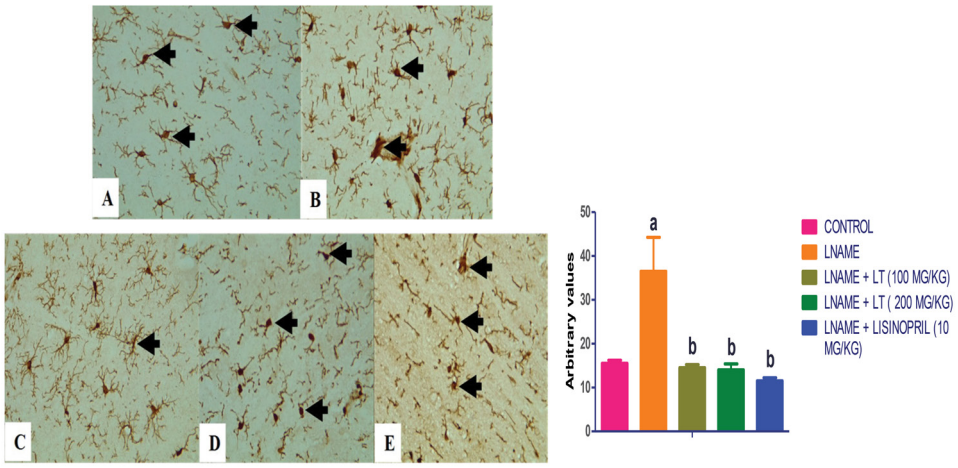

Figure 4. The immunohistochemistry of ionized calcium-binding adapter molecule 1 (Iba 1) in the cerebellum. A: control group; B: L-NAME 40 mg/kg orally; C: 100 mg/kg of *Launaea taraxacifolia* (LT) plus 40 mg/kg L-NAME; D: 200 mg/kg of LT plus 40 mg/kg L-NAME); E: 10 mg/kg of LT plus 40 mg/kg L-NAME. The arrows represent immunohistochemical expressions of Iba 1 in the cerebellum. Different letters on columns indicate significantly different ($P < 0.05$).

probability of the development of neurodegenerative diseases. Therefore, the leaf extract of LT might be of great value for the prevention of chronic neurodegenerative disease conditions as earlier reported (35).

Furthermore, all enzymic neuronal antioxidant defense systems were significantly elevated by LT treatment in comparison with L-NAME-exposed rats. The observed disruptions in the antioxidant defense pathways induced by L-NAME worked in tandem to facilitate the pathogenesis of neuroinflammation and oxidative stress following the administration of L-NAME. Depletion of serum NO bioavailability is usually associated with hypertension as seen in the mechanism action of L-NAME involving the inhibition of NO synthase. Therefore, we hypothesize that memory loss may occur in hypertensive patients with no locomotor dysfunction Previously, memory impairment and cognitive dysfunction have been reported in hypertensive states (36,37). Therefore, our study confirmed the involvement of NOS inhibitors such as L-NAME as an inducer of memory impairment and cognitive dysfunction, which are complications that might arise from hypertension. More so that LT might serve as a good drug candidate against hypertension, its ameliorative effect of hypertensive complication is an added advantage as a medicinal plant.

Higher expressions of Glial fibrillary acidic protein (GFAP) were observed in the L-NAME alone treated groups, which were significantly reduced in the LT treated group. This indicates the infiltration of damaged cells due to on-going inflammatory conditions in the brain. The observable astrogliosis was attenuated in groups co-administered with either LT or Lisinopril, a standard antihypertensive agent. The astrogliosis precipitated by L-NAME could be a pathological response from

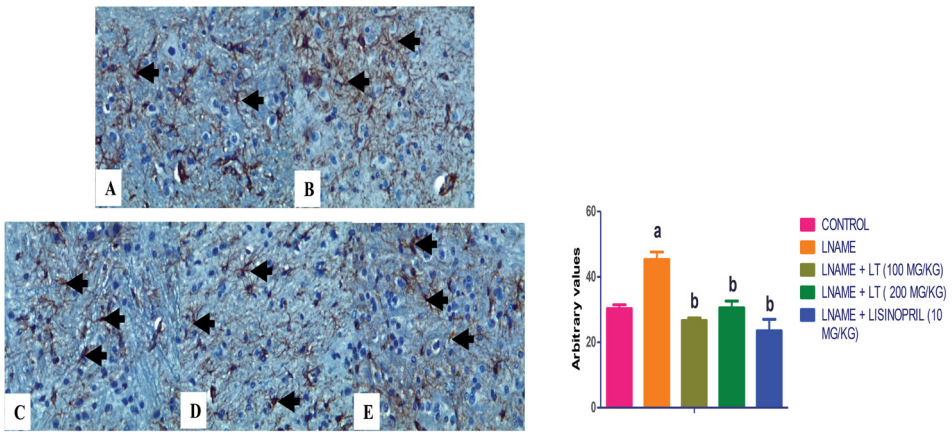

Figure 5. The immunohistochemistry of Glial fibrillary acidic protein (GFAP) in the cerebellum. A: control group; B: L-NAME 40 mg/kg orally; C: 100 mg/kg of *Launaea taraxacifolia* (LT) plus 40 mg/kg L-NAME; D: 200 mg/kg of LT plus 40 mg/kg L-NAME); E: 10 mg/kg of LT plus 40 mg/kg L-NAME. The arrows represent immunohistochemical expressions of GFAP in the cerebellum. Different letters on columns indicate significantly different ($P < 0.05$).

neuroinflammation and coupled with nitrosative stress (38,39). This could primarily contribute to impaired memory and neurobehavioural abnormalities thereby linking hypertension and neurobehavioural deficit. Research reports have documented the involvement of gliopathy giving rise to cognitive deterioration, memory, and attention loss (40,41). The level found in the higher dose of LT has almost similar effect as seen in the Lisinopril-treated group indicating high activity of the higher dose of LT.

The microglia exert a protective function because they phagocytize damaged neuronal cells and remove aggregates of pathological proteins from the brain (42). Our results show heightened microgliosis, which may be a response to acute inflammation and cellular apoptosis resulting from L-NAME induced neurotoxicity. It has been well established that both astrocytes and microglia become reactive in response to injury or disease in the brain, thereby serving as neuronal housekeepers (43). Also from our results, LT was able to mitigate the on-going neuronal pathology as indicated by astrogliosis and microgliosis. Purkinje cells degeneration have been previously reported by Takahashi et al (44) in cerebellum of valproic acid rat model of autism. The results of our study on the morphology of Purkinje cells suggest the deleterious effect on L-NAME on neuronal cells and loss of functions. However, the significant amelioration of this pathology in rats treated with LT is suggestive of the neuroprotective effect of LT. This finding was also supported by the fact that spatial memory and learning was not altered in the treatment groups that received LT. Another finding worthy of note is that Purkinje cells of LT rats were not affected morphologically suggesting a preservation of their structural and functional integrity.

Conclusio

Co-treatment of L-NAME with LT significantly ameliorated astrogliosis, microgliosis, Purkinje cells degeneration, oxidative stress, and improved memory and neurobehavioural deficit. Hence, the antioxidant property of *Launeae taraxacifolia* could be immensely beneficial for the management of neurobehavioral and cognitive deficit in hypertensive subjects.

Acknowledgments

Professor J.O. Olopade acknowledges the funding from Tertiary Education Trust Fund (TETFUND) of Nigeria. We acknowledge the Neuroscience laboratory, Department of Veterinary Anatomy, University of Ibadan for their technical assistance and provision of antibodies.

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Conflict of interests

All authors declared no conflict of interest.

Ethical considerations

The study was approved by the University of Ibadan Animal Care and Use Research Ethical Committee (ACUREC), with the ethical code UI-ACUREC/18/0135 assigned.

Funding/Support

The authors thank Professor O.O. Oguntibeju for providing financial assistance.

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