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RESEARCH ARTICLE

# Histomorphological and Neurobehavioral Study of Effect of *Spinacia Oleracea* (Spinach) Ethanolic Leaf Extract on Restraint Chronic Stress-Induced Neurodegeneration in Hippocampus of Experimental Rats

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## Abstract

**Background and objective**: Spinach (*Spinacia oleracea*) is nutritionally rich in antioxidants. This work aimed at evaluating the therapeutic potential of ethanolic leaf extract of *Spinacia oleracea* (SO) on restraint chronic stress (RCS)-induced neurodegeneration.

**Methods and study design:** Twenty-four male Wistar rats, weighing 180–200 grams, were divided into six different groups, namely: the normal control group (0.5ml of normal saline), the negative control (RCS and normal saline only), the low dose (200mg/kg), the medium dose (400mg/kg), the high dose (800 mg/kg) and fluoxetine (20mg/kg). Restraint chronic stress (RCS) were induced 2hours daily for 21 days followed by treatment with *Spinacia Oleracea* for another 14 days. Neurobehavioral assessments, including the Y-maze, novel object recognition, and elevated plus maze (EPM) tests, was done and video tapped to facilitate accurate

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scoring. After treatment is done, blood sample was collected for oxidative stress (SOD, MDA, GSH) and neuroinflammatory marker (IL-1β, TNF-α) assay. Histologically, using hematoxylin and eosin (H&E) stain, neuronal alterations were evaluated in the hippocampus sample.

**Results**: *S. Oleracea* (200mg/kg and 800mg/kg) treatment significantly cured RCS-induced memory impairment and cognition on Y-maze, and NORT respectively and time spent in the open arms of the EPM significantly raised ($p < 0.05$). Administration of SO reduced MDA level while increasing SOD and glutathione-S-transferase activity in the treatment groups and significantly reduced RCS-induced elevation of (TNF-α) and Interleukin-1 beta (IL-1β) ($p<0.05$) which are necrosis factor for tumor. Furthermore, SO enhanced histoarchitecture of hippocampus in the groups with treatment in compression to the negative control.

**Conclusion:** *S. Oleracea* is therapeutic against chronic stress-induced neurodegeneration in the hippocampus.

**Keywords:** Restraint Chronic stress, *Spinacia Oleracea,* Neurodegeneration, Neurobehavior.

## 1. Introduction

Stress is a prevalent experience that affects organisms' daily activities and well-being.[1] Chronic or prolong stress manifests itself in many different ways for individuals, including life and job stress, psychological stress, physical stress, and social stress. It is one of the significant variables influencing a significant portion of the nation's population. It puts physical and emotional endurance under stress, which is thought to be the primary cause of a number of psychiatric and physical problems.[2]

Prolonged periods of stress are a significant clinical and environmental increased risk for neurodegenerative illnesses, like depression, schizophrenia, and drug relapse.[3] It impairs cognitive function, and also has emotional and psychological effects.[4]

The frequency of neurodegenerative diseases has increased along with the average life expectancy worldwide, with global average of over 50million individuals, having a significant socioeconomic impact.[5] The biological processes leading to neurodegenerative diseases like Parkinson's disease (PD) and Alzheimer's disease (AD), which are connected to clinical presentation like anxiety, memory problem and movement difficulty, and an elevated level of pro-inflammatory and oxidative stress indicators in the blood and brain of those who have the disease, has been linked to neuroinflammation caused by stress.[3,4, 6]

The production of reactive oxygen species (ROS) is one the main mechanisms of stress.[7, 8] ROS are reduced by cellular antioxidant system, both enzymatic and non-enzymatic. Due to its widespread distribution among organisms, reduced glutathione (GSH) is a key marker for antioxidant activity that doesn't involve enzymes. Catalase and superoxide dismutase (SOD) are the most research focused enzymatic antioxidant.[8] The total antioxidant defense capacity (TAC), shows how well the body balances harmful ROS with its antioxidant capacity, it is frequently used to represent the overall redox state of an organism. When the production of reactive oxygen species (ROS) exceeds the antioxidant defense capacity, it results in lipid peroxidation (LPO), protein oxidation and DNA damage, all these contribute death of cell.[9] According to Lopez-López *et al*.[10], prolonged stress response leads to the release of catecholamine and glucocorticoid and into the circulation which may activate damage processes that, over time, result in metabolic changes linked to oxidative stress and inflammation.

Research has demonstrated that exposure to stressors such as immobilization, cold, and cold water immersion over a 21-day period leads to changes in antioxidant capacity, which are linked to oxidative stress in various organs of Westar rats.[11, 12]

Furthermore, prior research has demonstrated that those who experience prolong stress may be prone to decline memory, neurodegenerative diseases like Alzheimer's, worsening hippocampus damage, and depressive-like behavior.[13] Currently, it is believed that changes in dendritic and synaptic structure in stress-responsive brain areas, Areas like the medial prefrontal cortex, hippocampus, and the amygdala, influence fear and anxiety brought on by stressful life experiences.[14] In response to stress, the hippocampus glucocorticoid receptor is activated, which increases neuronal metabolism while also promoting dendritic atrophy, lowering cell survival and neurogenesis, and impairing long-term potentiation and cognition.[15, 16] It's interesting to note that dendritic atrophy is constantly brought on by immobilization or Persistent stress

due to limitations in the hippocampal CA3 region.[17] According to Ortiz and Conrad[17], the prolonged stress increase promotes neurotic death also inhibiting generation of new neurons in the hippocampus, which results in a decrease in the hippocampal volume. Therefore, alternative is needed to curb this chronic stress damages to hippocampus.

In addition to improving cognitive function, medicinal herbs like spinach (*Spinacia oleracea*), a member of *Chenopodiaceae* family have been used to reduce other Alzheimer's disease symptoms.[18] Spinach a leafy cool-season vegetable, cultivated around the world, It is consumed fresh as in salad or boiled.[19, 20]

Beyond protecting against macromolecular oxidative damage and eliminating reactive oxygen species, phytochemicals and other bioactive components, Spinach has the ability to control the expression and activity of genes involved in inflammation, metabolism, proliferation, and antioxidant defense.[21] Spinach has an anti-inflammatory quality and a positive impacts on the central nervous system, thus this study aims to investigate how it can help in reducing prolonged stress-induced anxiety and depression such as behaviors by inhibiting the brain of rats with oxidative stress and inflammation of the neurons. The current investigation sought to determine whether stress or high glucocorticoid levels may stimulate neuronal metabolism in middle-aged, healthy rats towards the hippocampus and whether stressful life experiences could affect the onset or maintenance of about neurodegeneration. Due to its status as one of the earliest brain regions to exhibit neuropathology, we concentrated our research on the hippocampus.[22, 23] The findings suggest a potential mechanism via which stress and spinach influence emotional and cognitive behavior.

## 2.Materials and Methods

### 2.1 Collection and Identification of Plant Materials

The leaves of *Spinacia Oleracea* were gotten from the local market in Enugu state, then identified and verified at the Department of Plant Science and Biotechnology, Enugu State University of Science and Technology Agbani, Enugu state, Nigeria.

#### *2.1.1 Extract Preparation*

After being cleaned and allowed to air dry in the shade for a few days, spinach leaves were blended into a fine powder. Ethanolic extraction (EE) was prepared using fine powder (FP). Separately, 1500 ml of 99% ethanol and 500g of ground S. Oleracea were combined, and the combination was allowed to come to room temperature for 72 hours. After that, Whatman No. 1 filter paper was used to filter the solutions. To achieve the required concentration, the extract was then dissolved in distilled water, dried at 70°C, and then kept at 4°C.[24]

### 2.2 Experimental Animal

We bought 24 mature male Wistar rats, each weighing 180–200g, from a breeding facility in the Animal House of the University of Nigeria, Nsukka's Department of Pharmacology and Technology. In the Animal House of the Department of Anatomy at the University of Nigeria Enugu Campus, they were kept in an iron cage with adequate ventilation.Before being used in the experiment, the rats were kept in an area with regulated humidity and air pressure for two weeks. They were also given unrestricted access to clean water and fed normal livestock pellets (Guinea Feed Nigeria Limited).

### 2.3 Ethical Approval

All animals were handled under guidelines for animal research as detailed in the Guidelines for the care and use of laboratory animals. Ethical approval was obtained from the Faculty of Basic Medical Science research ethics committee of Enugu State University College of Medicine with the Ethical Right permission Number: ESUCOM/FBMS/ETR/2022/010 to carry out this research.

### 2.4 Experimental Design

For this investigation, twenty-four (n=4) mature male Wistar rats weighing between 180 and 200g were used. They were split up into six (6) groups of four rats each at random. Group 1 severed as normal control (no stress); the rats were fed with normal saline and feed only. Groups 2 through 6 were subjected to two hours of restraint every day for 21 days in a row in order to create chronic stress [25]. Group 2 severed as negative control; normal saline and feed. Rats in groups 3, 4, and 5 were experimental groups and administered 200, 400, and 800mg extract at different doses respectively. Group 6 were the positive control group (drug group); the rats were treated with 20mg of fluoxetine (Chart 1).

**Chart 1.** *Drug Administration Schedule*

| Group/Description | Treatment |
|---|---|
| 1 (Normal control) | Normal saline 2ml daily |
| 2 (Negative control) | Restraint stress + 2ml Normal saline |
| 3 (Low dose extract) | Restraint stress + 200mg of extract |
| 4 (Medium dose extract) | Restraint stress + 400mg of extract |
| 5 (High dose extract) | Restraint stress + 800mg of extract |
| 6 (Drug control) | Restraint +20mg of fluoxetine |

### *2.4.1 Restrain Method*

The rats' movements were restricted by placing them in a similar-sized perforated plastic tube for two hours daily for 21 days.[26] The purpose of the tube's perforations is to guarantee that the rats inside of them have adequate ventilation. Following the stress of restraint, behavioral evaluations were carried out post-treatment. Behavioral tests were performed in the following order: The Y-maze test, novel object recognition test (NOR), and elevated plus maze (EPM).

### 2.5 Evaluation of Behavior

To avoid bias, behavioral protocols were carried out with the experimenter blinded to the testing groups, and all of the animals utilized in this study were included in behavioral studies without any exclusions. To minimize putting the animals under stress, more skilled workers were hired to ensure that the procedure was finished by midday. On the 22nd day after stress and the 15th day after treatment, the experimenter used testing rats from each experimental group to carry out testing methods on a rotating basis.

### *2.5.1 The Y-maze Test*

The Y-maze is composed of three arms, A, B, and C, each of which is slanted at a 120° angle and covered in black acrylic. The rats were put in the center and allowed to investigate every arm. The total number of entries was noted after each paw was on the arms' floor. Subsequent entries into each arm were measured when the rat entered the same arm in a different order. To assess the rat's cognitive function during disease control and therapy, the total number of entries was also tallied[6, 27]. **Formula: (**Total No of spontaneous Alteration / Total No of Arm entries -2) × 100

### *2.5.2 Novel object recognition test (NORT)*

To assess cognitive damage and restoration, the experiment was carried out using the rats' ability to discriminate between familiar and novel objects. The discrimination ratio was calculated by measuring the amount of time the rats spent investigating the new and old objects and different score for each group during the experiment, which was conducted following Lueptow's.[28] guidelines.

### *2.5.3 Elevated plus maze (EPM)*

Mehta *et al.,*'s Elevated Plus Maze (EPM)[29] was utilized to assess the anxiogenic and anxiolytic effects of spinach therapy and restraint chronic stress. The number of entries and the amount of time spent in the closed and open arms were used in this evaluation. The rat was given five minutes to explore the maze after being put in the center, across from the closed arm. The time spent on the open and closed arms was recorded by a camera that was attached to a computer.[30] After each animal was tested, 20% alcohol was used to clean the device.

### 2.6 Sacrifice and specimens collection

After the behavioral tests were finished on the sixteenth day following treatment, the animals were put to death. Ophthalmic arteries were used to draw blood for later analysis of various biochemical assays. The brain tissues were harvested and fixated in 10% formalin for Histological analysis.

### *2.6.1 Determination of MDA and Glutathione activity*

MDA, a marker of tissue lipid peroxidation, was quantitatively measured in the blood samples using a previously published method.[31] One milliliter of the sample supernatant was combined with a mixture of hydrochloric acid (HCl), thiobarbituric acid (TBA), and trichloroacetic acid (TCA). For 45 minutes, the mixture was incubated in boiling water. The entire solution was then centrifuged at 1000 g for 10 min after cooling. The following formula was used to determine the MDA concentration: C (M) = absorbance/ (1.56 × 105), where the absorbance of the supernatant was read at 535 nm. The activity of glutathione was estimated following the description by Baghcheghi *et al*.[31] 0.1 mL of the supernatant, the test sample, was diluted with 0.9 mL of phosphate (PO4)

buffer. 20% trichloroacetic acid (TCA) in a volume of 1 mL was added. For 20 minutes, this mixture was left alone. It was then centrifuged for 10 minutes at 10,000 rpm. The supernatant was collected, and 0.25 mL of it was added to 0.75 mL of phosphate buffer. 2mL of 2, 2-Dithiobis nitrobenzoic acid (DTNB) was added at 0.0006M, and the mixture was let to sit for 10 minutes. At 412 nm, absorbance was measured.

#### *2.6.2 Enzymatic Analysis*

The SOD activity was evaluated using Madesh and Balasubramanian's approach, which is based on the formation of SOD by auto-oxidation of pyrogallol (Merck) and as a result reduction of 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich) to colored formazan. It was noticed that Sigma-Aldrich's DMSO halts the process. Succinctly, the sample's supernatant was transferred onto a 96-well plate. The DMSO was added after five minutes, and a microplate reader set to the wavelength of 570nm was used to measure the solution absorbance. It was deduced that, one unit of SOD is the quantity of protein needed to prevent a 50% decrease in MTT.[32]

#### *2.6.3 Estimation of Corticosterone levels*

Levels of Corticosterone were tested using enzyme immunoassay kits.

#### *2.6.4 Tumor necrosis factor-alpha and interleukin-1β estimation*

ELISA kits were used to measure the levels of proinflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α).

#### *2.6.5 Histological analysis*

The rat brains were carefully dissected obtaining the hippocampus and processed through the stages of fixation in formalin. It was embedded in molten paraffin wax after being fixated and dehydrated in ascending grades of alcohol. After cutting and mounting coronal paraffin sections onto gelatin-coated slides, the sections were stained with Hematoxylin & Eosin (H&E) stain. After that, a digital light microscope was used to view the slides.

### 2.7 Statistical analysis

The data collected were recorded at M ± SED (standard error difference) and were analyzed using SPSS package version 23. To compare if the differences in the means of the various variables were significant, we used ANOVA, and a post-hoc analysis using tukey test were carried out.

## 3. Results

### 3.1 Behavioral Test

#### *3.1.1 Effect of spinach on the impaired spatial memory activity by restraint chronic stress administration*

This test was performed to check the spatial memory function of stress-induced rats, treatment with spinach. The stress-induced rats showed a marked decline (7.8±1.3%) in the % spontaneous alterations as compared to the controls (10.5 ±5.3) while the spinach-treated rats demonstrated a rise in 200mg/kg ($p < 0.743$, 61.2±11.8%) and 800mg/kg (69.9 ±15.1%) in the activity as compared with the stress-induced and control groups. The total number of entries into each arm by the stress-induced groups decreased compared with the control group. However, treatment with spinach showed an increase in the number of entries as compared with the stressed group (Table 1a and b)

**Table 1a.** *Effect of restraint chronic stress on the Y-maze test*

| Group/Description | Mean No. of Entries | Mean No. of Possible triads | Mean No. of Alternations | Mean Percentage Alternation |
|---|---|---|---|---|
| 1 (Normal control) | 12.5 ± 5.2 | 6.8 ± 3.8 | 62.3 ± 20.9 | 10.5 ± 5.3 |
| 2 (Negative control) | 9.8 ± 1.2 | 4.8 ± 1.7 | 61.1 ± 16.9 | 7.8 ± 1.3 |
| 3 (Low dose extract) | 8.0 ± 2.6 | 4.0 ± 1.4 | 72.5 ± 25.0 | 6.0 ± 2.6 |
| 4 (Medium dose extract) | 10.3 ± 2.9 | 5.5 ± 2.8 | 64.5 ± 21.9 | 8.3 ± 2.9 |
| 5 (High dose extract) | 8.5 ± 5.0 | 5.5 ± 3.7 | 90.7 ± 11.4 | 6.5 ± 5.0 |
| 6 (Drug control) | 6.3 ± 2.6 | 2.5 ± 2.0 | 51.0 ± 34.4 | 3.8 ± 3.3 |
| P. Value | 0.116 | 0.380 | 0.276 | 0.220 |

*All the values are presented as the mean ± SEM. Correlation among the groups was done using Tukey's test by one-way ANOVA. p value = < 0.05 is significant.*

**Table 1b.** *Effect of Spinacia Oleracea on the Y-maze test*

| Group/Description | Mean No. of Entries | Mean No. of Possible triads | Mean No. of Alternations | Mean Percentage Alternation |
|---|---|---|---|---|
| 1 (Normal control) | 9.8 ± 3.8 | 5.3 ± 1.7 | 7.8 ± 3.8 | 74.1 ± 18.1 |
| 2 (Negative control) | 7.3 ± 2.9 | 2.8 ± 1.5 | 5.3 ± 2.9 | 61.8 ± 27.8 |
| 3 (Low dose extract) | 10.5 ± 1.3 | 5.3 ± 1.5 | 8.5 ± 1.3 | 61.2 ± 11.8 |
| 4 (Medium dose extract) | 8.8 ± 3.4 | 4.3 ± 3.1 | 6.3 ± 4.3 | 50.5 ± 34.5 |
| 5 (High dose extract) | 10.0 ± 2.3 | 5.5 ± 1.7 | 8.0 ± 2.3 | 69.9 ± 15.1 |
| 6 (Drug control) | 5.3 ± 2.5 | 2.5 ± 2.4 | 3.3 ± 2.5 | 70.2 ± 24.6 |
| P. Value | 0.124 | 0.195 | 0.173 | 0.743 |

*All the values are presented as the mean ± SEM. Correlation among the groups was done using Tukey's test by one-way ANOVA. p value = < 0.05 is significant.*

### *3.1.2 Spinach ameliorated non-spatial memory impairment as induced by chronic stress*

The discrimination ratio (DR) and difference score (DS) was calculated as mentioned in the methodology and a positive score indicated more time spent in the novel object, whereas a negative score indicated more time spent with the familiar object. While analysis reveal difference score and discrimination ratio in each group (Table 2a), it revealed stress effects on each group's ability to recognize familiar and novel objects. One-way ANOVA showed that there was a decline in memory and recognition in stressed groups compared to control animals ($P = 0.077$). In group 2-6 animals, DR and DS were negative respectively (Table 2a). Collectively, these data indicate that stressed rats spent more time with a familiar object than the novel object. Also, these data indicate that rats treated with spinach spent significantly more time with the novel object compared to control animals except in group 4 (Table 2b).

**Table 2a.** *Effect of restraint chronic stress on the NOR test.*

| Group/Description | Difference Score | Discrimination Ratio |
|---|---|---|
| 1 (Normal control) | 54.0 ± 92.7 | 0.8 ± 0.2 |
| 2 (Negative control) | -28.0 ± 41.8 | 0.2 ± 0.1 |
| 3 (Low dose extract) | 7.8 ± 32.7 | 0.3 ± 0.2 |
| 4 (Medium dose extract) | -19.5 ± 41.6 | 0.7 ± 0.3 |
| 5 (High dose extract) | 46.0 ± 78.0 | 0.4 ± 0.3 |
| 6 (Drug control) | -0.8 ± 43.6 | 0.2 ± 0.3 |
| P. Value | 0.336 | 0.074 |

*All the values are presented as the mean ± SEM. Correlation among the groups was done using Tukey's test by one-way ANOVA. p value = < 0.05 is significant.*

**Table 2b.** *Effect of Spinacia Oleracea on the NOR test*

| Group/Description | Difference Score | Discrimination Ratio |
|---|---|---|
| 1 (Normal control) | 25.5 ± 41.5 | 0.7 ± 0.3 |
| 2 (Negative control) | -97.5 ± 127.9 | -0.3 ± 0.3 |
| 3 (Low dose extract) | 15.3 ± 27.8 | 0.6 ± 0.2 |
| 4 (Medium dose extract) | 5.3 ± 16.2 | 0.7 ± 0.4 |
| 5 (High dose extract) | 8.8 ± 12.6 | 0.5 ± 0.3 |
| 6 (Drug control) | 2.3 ± 12.1 | 0.5 ± 0.4 |
| P. Value | 0.160 | 0.443 |

*All the values are presented as the mean ± SEM. Correlation among the groups was done using Tukey's test by one-way ANOVA. p value = < 0.05 is significant.*

### *3.1.3 Spinach ameliorated chronic stress-induced anxiolytic activity*

The results of the one-way ANOVA revealed that the time spent in the close arm was affected by stress P < 0.750 for post stress test. The time spent in the closed arm by the animals of groups 2 to 6 was longer than the control group (55 ± 33.6) (Table 3a). Animals in the spinach treated (58.3 ± 29.8), (55.8 ± 84) and (65.8 ± 38.3) groups had a shorter time in the closed arms than the stressed group (Table 3b). The results of the one-way ANOVA revealed that the time in the open arm was affected by stress (P < 0.869 for post-stress). Also, the stressed rats spent less time in the open arm compared to the control group (245 ± 35.6) (Table 3a). The spinach extract increased the time spent on the open arm compared to the stress group (P < 0.783; Table 3b).

**Table 3a.** *Effect of restraint chronic stress on the EPM test.*

| Group/Description | No. of Entries in open arm | Duration in open arm | No. of entries in closed arm | Duration in closed arm |
|---|---|---|---|---|
| 1 (Normal control) | 5.8 ± 2.9 | 245.0 ± 35.6 | 5.3 ± 3.3 | 55.0 ± 33.6 |
| 2 (Negative control) | 5.3 ± 2.9 | 84.0 ± 78.5 | 6.0 ± 1.0 | 237.0 ± 76.7 |
| 3 (Low dose extract) | 5.3 ± 2.6 | 79.8 ± 34.2 | 5.3 ± 2.6 | 220.3 ± 34.2 |
| 4 (Medium dose extract) | 4.8 ± 2.9 | 75.8 ± 67.4 | 5.0 ± 3.5 | 224.3 ± 67.4 |
| 5 (High dose extract) | 4.0 ± 3.2 | 79.8 ± 63.1 | 4.3 ± 2.7 | 235.2 ± 39.9 |
| 6 (Drug control) | 4.8 ± 0.5 | 91.3 ± 42.0 | 4.8 ± 0.5 | 208.8 ± 42.0 |
| P. Value | 0.949 | 0.869 | 0.977 | 0.750 |

*All the values are presented as the mean ± SEM. Correlation among the groups was done using Tukey's test by one-way ANOVA. p value = < 0.05 is significant.*

**Table 3b.** *Effect of Spinacia Oleracea on the EPM test*

| Group/Description | No. of Entries in open arm | Duration in open arm | No. of entries in closed arm | Duration in closed arm |
|---|---|---|---|---|
| 1 (Normal control) | 5.5 ± 1.3 | 252.3 ± 23.9 | 5.3 ± 0.9 | 47.8 ± 23.9 |
| 2 (Negative control) | 5.5 ± 3.5 | 106.3 ± 92.9 | 5.3 ± 3.8 | 193.8 ± 92.9 |
| 3 (Low dose extract) | 4.5 ± 2.6 | 241.8 ± 29.8 | 4.3 ± 2.6 | 58.3 ± 29.8 |
| 4 (Medium dose extract) | 2.5 ± 2.4 | 244.3 ± 84.0 | 2.5 ± 2.4 | 55.8 ± 84.0 |
| 5 (High dose extract) | 4.0 ± 1.8 | 234.3 ± 38.3 | 4.3 ± 1.7 | 65.8 ± 38.3 |
| 6 (Drug control) | 3.3 ± 1.5 | 239.8 ± 51.6 | 3.5 ± 1.9 | 60.3 ± 51.6 |
| P. Value | 0.401 | 0.783 | 0.577 | 0.783 |

*All the values are presented as the mean ± SEM. Correlation among the groups was done using Tukey's test by one-way ANOVA. p value = < 0.05 is significant.*

### 3.1.4 Effect of *Spinacia Oleracea* on restraint chronic stress-induced Histopathological Alterations in the hippocampus

Microscopically as shown in Figure 1, the hippocampus of normal control rats revealed a normal histological structure (Fig. a). On contrary, the hippocampus of stress-rats (group 2) exhibited severe neuropathic alterations which included necrosis, shrunken, and pyknosis of neurons, neuronophagia of necrotic neurons (Fig. b,) as well as the formation of neuronal tissue vacuolation (Fig. b), neurogenesis (proliferation of glia cells) (Fig. c). However, the hippocampus of stress rats co-treated with spinach in group 4 showed histopathological alterations (Fig. d). Meanwhile, at the high dose of spinach extract the hippocampus histoarchitecture appears normal (Fig. e) as well as in group 6 treated with fluoxetine a standard drug showed normal histoarchitecture (Fig. f).

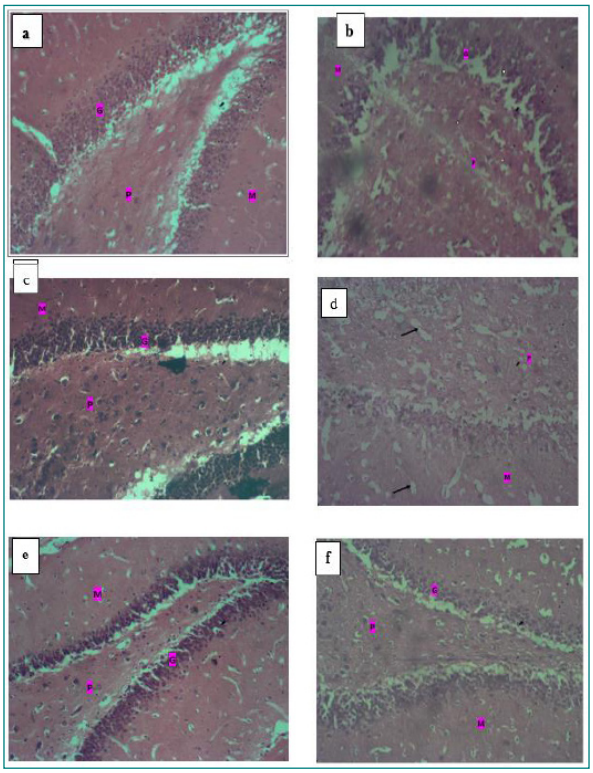


**Figure 1.** *Representative Photomicrographs of H&E-stained sections of the hippocampus: a normal control brain showing the normal histological architecture. b-f restraint chronic stress-exposed brains b necrosis, shrunken, and pyknosis of neurons as well as neuronophagia; c normal histoarchitecture; d neuronophagia and formation of neuronal vacuolation (black arrow); e high dose spinach treated brain showing no histopathological alterations. f Fluoxetine + stress co-treated brain showing normal histoarchitecture. Molecular (M), granular (G), and pyramidal (P) cell layers. H&E X200.*

### 3.1.5 Effect of *Spinacia Oleracea* on Oxidative Stress markers in restraint chronic stress-induced Neurodegeneration in Wistar rats

Restraint chronic stress-induced neurodegeneration resulted in a significant increment in lipid peroxidation marker (MDA) in the brain (5.4 ± 0.2, $p < 0.006$), whereas SOD and GST were significantly declined (8.5 ± 2.1, $p < 0.043$) and (9.7 ± 0.2, $p < 0.002$) respectively, as compared to control rats (Table. 4a). On the other hand, treatment of stress-group with spinach resulted in a statistically significant reduction in MDA and a significant elevation in SOD and GST respectively, as compared to stress only and control groups. A statistically significant difference was detected in MDA, GST, and SOD contents in healthy treatment rats that received spinach, as compared to control rats. The results demonstrated the anti-oxidative potential exerted by spinach against chronic stress-induced oxidative stress.

**Table 4a.** *Effect of Spinacia Oleracea on oxidative stress in restraint chronic stress-induced neurodegeneration.*

| Group/Description | MDA | SOD | GST |
|---|---|---|---|
| 1 (Normal control) | 1.3 ± 0.5 | 15.6 ± 2.7 | 18.4 ± 1.7 |
| 2 (Negative control) | 5.4 ± 0.2[b] | 8.5 ± 2.1[b] | 9.7 ± 0.2[b] |
| 3 (Low dose extract) | 3.3 ± 0.9 | 9.8 ± 1.8 | 11.5 ± 1.5[c] |
| 4 (Medium dose extract) | 3.4 ± 0.3 | 11.5 ± 0.3 | 10.4 ± 0.1[d] |
| 5 (High dose extract) | 4.3 ± 0.8[e] | 9.9 ± 0.1 | 14.1 ± 1.2 |
| 6 (Drug control) | 2.2 ± 0.8[f] | 11.7 ± 0.7 | 15.2 ± 0.9[f] |
| P. Value | 0.006 | 0.043 | 0.002 |

*SOD: Superoxide dismutase; MDA, Malondialdehyde; GST, Glutathione-S-Transferase*

*Values expressed as mean ±SD, 4 animals/group. [b]$p < 0.05$ vs control group and group 6, [c]$p < 0.05$ vs control group, [d]$p < 0.05$ vs control group and group 6, [f]$p < 0.05$ vs negative control and group 4.*

### *3.1.6 Effect of Spinacia Oleracea on corticosterone in restraint chronic stress-induced Neurodegeneration in Wistar rats*

One-way ANOVA performed for serum corticosterone showed a significant interaction between the stress and spinach effects. The stress group showed higher serum corticosterone compared to the control groups ($p < 0.05$ Table 4b). The stress + spinach groups presented lower serum corticosterone compared to the stress group only ($p < 0.05$). However, there was a statistically significant difference between the negative control, low-dose treatment and standard drug groups.

**Table 4b.** *Effect of Spinacia Oleracea on Serum cortisol levels of restraint chronic stress-induced neurodegeneration in rats*

| Group/Description | CORTISOL |
|---|---|
| 1 (Normal control) | 177.9 ± 6.4 |
| 2 (Negative control) | 385.9 ± 7.5 |
| 3 (Low dose extract) | 273.1 ± 64.8 |
| 4 (Medium dose extract) | 228.1 ± 22.9[d] |
| 5 (High dose extract) | 235.2 ± 10.9[e] |
| 6 (Drug control) | 266.6 ± 6.3 |
| P. Value | 0.004 |

*Values expressed as mean ±SD, 4 animals/group. dp < 0.05 vs negative control, group 3 and group 6, ep < 0.05 vs negative control, and group 6.*

### *3.1.7 Effect of Spinacia Oleracea on neuroinflammatory parameters in restraint chronic stress-induced Neurodegeneration in Wistar rats*

The level of inflammatory markers (TNF-α) in the restraint chronic stress group increased (279 ± 51.3) compared with the control group. On the other hand, spinach at a low dose (200 mg/kg) and high dose (800mg/kg) showed statistically significantly different (158.6 ± 34.4, 164.3 ± 14.1) respectively in the TNF-α in comparison to the negative control and normal control group ($p < 0.005$). The IL-1β levels increased (79.3 ± 4.2) in the negative control group as compared with the normal group. When treated with a low dose (200 mg/kg) of spinach, it displayed a decline in the IL-1β (46.6 ± 3.9). When treated with a higher dose (800 mg/kg) of spinach, a significant decrease was observed in IL-1β as compared with the negative control group (Table 4c)

**Table 4c.** *Tumor Necrosis Factor-α (TNF-α) and Interleukin-1β (IL-1β)*

| Group/Description | TNF-α | IL-1β |
|---|---|---|
| 1 (Normal control) | 113.0 ± 0.4 | 30.5 ± 4.2 |
| 2 (Negative control) | 279.0 ± 51.3 | 79.3 ± 4.2 |
| 3 (Low dose extract) | 158.6 ± 34.4[e] | 46.6 ± 3.9 |
| 4 (Medium dose extract) | 209.0 ± 6.1 | 51.1 ± 1.3 |
| 5 (High dose extract) | 164.3 ± 14.1[e] | 41.3 ± 0.4 |
| 6 (Drug control) | 114.0 ± 1.4 | 53.9 ± 0.8 |
| P. Value | 0.005 | 0.001 |

*Values expressed as mean ±SD, 4 animals/group. cp < 0.05 vs negative control and normal control group, ep < 0.05 vs negative control and normal control group.*

## 4. Discussion

From the recent research, we found out that administering spinach extract on adult rats reduced neuroinflammation, anxiety, and behaviors associated with depression, canceled learning, and cognitive impairment due to restraint chronic stress. These results suggest that chronic stress effects could be a result of neurodegeneration.

It has been proposed that restraint chronic stress (RCS) can imitate socioenvironmental stress.[33, 34] Research

on both humans and rodents has shown that there are a lot of negative effects associated with stress, of which increased prevalence of psychopathologies is inclusive. Restraint stress is known to negatively impact the physiological, psychological, and reproductive axis in rats, according to Lee and MacKenzie.[4] Over the course of 21 days, male rats were restrained for only 2 hours each day.

In this current study, we evaluated anxiety, depression, memory, and cognition using three behavioral test models, including the EPM, Y-maze, and NORT. Finally, treatment with spinach improved cognitive impairment, depressive-like behaviors, and anxiety in Wistar rats. RCS is reported to produce memory loss, locomotor impairments, and anxiogenic behavior as a result of the inflammatory processes. These behavioral deficiencies are also linked to several serious neurodegenerative disorders. To evaluate the rats' spatial memory, we used a Y-maze behavioral test. Rats who have previously visited an arm were watched to determine the amount of spontaneous activity. According to our findings, there was a significant decrease in the stress group's spontaneous alteration when compared to the control group, indicating behavioral toxicity and it correlates with studies of Fang *et al*.[35] and Zhang *et al.*[13] Time and traveled paths were improved in the stress-treated group. Increase in the % spontaneous alteration, which however improved cognition was noted when spinach was used for treatment.

Since chronic restraint is known to impair spatial memory, a different test, the Novel Object Recognition Test was also carried out, assessing the ill rats' object recognition ability and their memories. Similar to earlier research, the findings showed a decline in object identification in the stressed group compared to controls, with a negative difference score and discrimination <0.5.[36] However, The animals' capacity to distinguish between known and unfamiliar objects, the rats' dose-dependently enhanced memory acquisition and their spatial learning abilities all indicated an improvement in cognitive functioning, suggesting spinach.

Moreover, stressed rats were less likely to enter and stay in the open arm to assess anxiety in the EPM test. When compared to normal control rats, rats that experienced prolonged restraint stress spent noticeably more time in the closed arm. Animals that are under constant stress may be establishing an adaptive defense against a reasonably immediate threat. Alternately, their behavior might be seen as maladaptive because the animal restricts its exploration and opportunity to gather resources in the absence of a direct threat.[25] Anxiety-like behavior resulted from prolonged immobility. Taking low, medium, and high dosages of the extract significantly increased the percentages of open-arm entry and time spent in open-arm during the EPM test when compared to the negative control (stress only) group, which is consistent with Tarasi and Asle[24] studies.

Histological staining with H and E was done on the hippocampus. According to the findings of the hippocampal H and E staining, long-term stress led to poor structural organization, neuronal vacuolation, constricted, pyknosis of neurons, plus neuronophagia, verifying the vulnerability of these impacted brain areas to restraint chronic stress-induced neurodegeneration, as documented by Ortiz and Conrad[17] and Hei *et al*.,[30] Learning, memory, and the accompanying motor responses to goal-oriented behavior and exploratory activity are all said to be mediated by the hippocampus. Also, the hippocampus has been linked to anxiety with contribution from the amygdala.[37] The photomicrograph sections of the fluoxetine and spinach-treated groups improved in such a way that is dose-dependent, with the group receiving 800 mg/kg bw significantly reversing the disturbance seen in group treated with chronic stress. It demonstrated that persistent stress damages neurons, in line with the behavior measured, and treatment with spinach at 800 mg lessened the impact of chronic stress on the neurons and improved neuronal cell integrity and neuronal architecture in the impacted hippocampus regions of the brain,

Because the brain has a poor antioxidant capacity compared to 20% of the metabolized oxygen, it is particularly susceptible to the production of ROS.[10] RCS accelerates metabolism and produces more ROS.[38] Oxidative stress is a result of high amounts of ROS damaging cells and tissues.[9, 39] In the present study, RCS increased MDA levels and decreased GST and SOD function, causing oxidative damage to the brain. The oxidant-antioxidative systems' stability is changed by this damage, which also affects the antioxidant defense system. Free radicals are created by RCS and contribute to the brain's oxidative stress by lowering the levels of glutathione and the defenses of antioxidants, increasing the peroxidation of lipid, and changing the activity of SOD.[31, 38, 40] This study discovered that RCS reduced GST and SOD function while raising MDA levels, causing oxidative damage

to the brain. According to Zhang et al.[13], stressed rats had lower SOD and GST activity and higher MDA levels in the hippocampus. The results we obtained are in line. These findings show that RCS may cause anxiety and cognitive decline in rats by causing oxidative stress in the brain. RCS-related oxidative damage can cause mental health issues like anxiety and cognitive loss.[7, 41] In this current research, treatment of spinach improved both GST and SOD activity while decreasing MDA levels. This study is comparable to another one where the administration of spinach to mice enhanced SOD and GST levels while decreased MDA levels.[42, 43] The mechanism behind these can be linked to the flavonoid constituent of the extract.

Furthermore, CRS promotes inflammatory cytokines such as IL-1β and TNF-α, which have been proposed to be critical players in the pathophysiology[44]. That accords with what we discovered. To our knowledge, the effects of spinach on IL-1β and TNF-α in the hippocampus region during cognitive decline and anxiety have not been particularly studied. Furthermore, we discovered that after spinach treatment, the leveld of TNF-α and IL-1β were drastically reduced across all treatment groups when compared to the stress group, that higher cytokines levels was seen. Our findings demonstrated the ability of spinach in protecting the brain from the damaging impacts of cytokines that are elevated during neuroinflammatory conditions.[45]

However, we discovered that there was higher levels of corticosterone on the stress-group (negative control). Another study that came to a similar conclusion found that modest chronic stress raised the level of corticosterone in the blood. The behavioral disorders brought on by RCS may be the result of corticosterone instability, which plays a major role in anxiety.[46] Additionally, we discovered that rats given spinach treatment had reduced corticosterone levels. It can be hypothesized that spinach in our study minimizes cognitive impairment and anxiety in rats given.

## 5. Conclusion

The current study proved that S. oleracea inhibits proinflammatory cytokine expression to prevent neurodegeneration induced by chronic stress. Additionally, preventing oxidative stress damage, decreasing corticosterone levels, and improving neuronal viability in many brain regions associated with cognition and anxiety assist this activity.

### Author Contributions

KEN and AGE: Investigation, Resources, Supervision, Funding acquisition, Writing—review & editing. KEN and AGE: Methodology, Data curation, Visualization, Formal analysis, Writing—original draft. KEN and AGE, CKC, JCI, CNC, OIS, OJO, PTA, ITA, SCO, GO, VCO, ONU, OJN, CCV, SCE, SEO, CEN and IKU: Writing—review & editing, Sample collection. F.S.: Sample collection. KEN and AGE: Supervision. KEN and AGE: Resources. KEN and AGE: Funding acquisition, Writing—review & editing. All authors have read and agreed to the published version of the manuscript.

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### Author Disclosures

The authors declare no conflicts of interest regarding this manuscript.

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