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Therapeutic and Neuroprotective potentials of *Spinacia oleracea* (Spinach) Extract on Stress-induced Neurodegeneration in the Hippocampus of adult Wistar Rats

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Abstract

Background of the study: Exposure to chronic stress is a risk factor for developing memory deficits. This work evaluated the therapeutic potential of ethanolic leaf extract of *Spinacia oleracea* (SO) on chronic restraint stress-induced neurodegeneration.

Material and Method: Twenty-four adult male Wistar rats (180-200g) were assigned into six groups, namely: the normal control group (0.5ml of normal saline), the stress control (2ml of normal saline), the low dose (stress + 200mg/kg extract), medium dose (stress + 400mg/kg extract), high dose (stress + 800 mg/kg extract) and positive control (stress + 20mg/kg of Fluoxetine). Chronic restraint stress was induced 2 hours daily for 21 days, followed by post-treatment with *Spinacia Oleracea* for another 14 days. Neurobehavioral (Y-maze, novel object recognition and elevated plus maze) tests were videotaped for proper scoring. At the end of the treatment, the blood sample was collected for oxidative stress and neuroinflammatory markers assay. The neuronal alteration was assessed histologically using Haematoxylin and Eosin staini on the hippocampus sample. The data collected were analyzed using the IBM SPSS package version 23 with p-value < 0.05.

Result: *S. oleracea* (200mg/kg and 800mg/kg) treatment significantly cures stress-induced memory impairment and cognition on Y-maze ^and novel object recognition test, respectively and increases time spent in the open arm in the elevated plus maze test. Administration of *S. oleracea* decreased lipid peroxidation level while increasing superoxide dismutase and glutathione-S-transferase activity in the treatment groups and significantly reduced stress-induced elevation of tumor necrosis factor (TNF-α) and

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Interleukin-1 (IL-1β). Furthermore, *S. oleracea* enhanced the histoarchitecture of the hippocampus in the treatment groups compared to the stress control.
Conclusion: *S. Oleracea* demonstrated therapeutic potential against chronic stress-induced neurodegeneration in the hippocampus.

Keywords: Chronic stress, *Spinacia oleracea,* neurodegeneration, memory impairme

INTRODUCTION

Stress is a prevalent condition that impacts an organism's everyday functioning and overall health [1]. Individuals experience chronic or persistent stress in a variety of ways, such as social, physical, psychological, and occupational stress, as well as stress related to life and work. It is among the important factors affecting a sizable section of the populace of the country. It stresses both physical and emotional endurance, which is assumed to be the main factor behind a number of mental and medical issues [2].

Neurodegenerative diseases like depression, schizophrenia, and drug relapse have a substantial clinical and environmental risk factor in chronic stress[3]. It affects emotional and psychological states in addition to impairing cognitive function[4]. Global average life expectancy has increased with the prevalence of neurological disorders, with over 50 million people globally having a major socioeconomic impact [5]. Stress-induced neuroinflammation has been linked to the pathogenesis of neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD). These diseases are characterized by symptoms such as anxiety, memory loss, and motor impairments, as well as an increase in proinflammatory and oxidative stress markers in the blood and brain of those affected [3,4,6].

The production of reactive oxygen species (ROS) is one of the key mechanisms of stress [7,8]. ROS are decreased by both enzymatic and non-enzymatic antioxidant defense systems in cells. Glutathione reduction (GSH), which is widely distributed throughout organisms, is an essential signal for the non-enzymatic antioxidant activities. Catalase (CAT) and superoxide dismutase (SOD) are the two enzymatic antioxidant systems that have been studied the most [8]. The general redox state of an organism is commonly represented by the total antioxidant capacity (TAC), which indicates a balance between the creation of ROS and antioxidant capacity. Lipid peroxidation (LPO), protein oxidation, and DNA damage occur when ROS generation surpasses antioxidant capability and can lead to cellular death [9]. [10] claim that long-term damage mechanisms that lead to oxidative stress and inflammation-related metabolic alterations may be triggered by the persistent release of catecholamines and glucocorticoids into the bloodstream during the stress response.

Research has demonstrated that oxidative stress in several organs is linked to changes in antioxidant capacity in stress caused by immobility, cold, and immersion in cold water for 21 days in Wistar rats [11,12]. Chronic stress also increases the likelihood of memory loss, neurodegenerative disorders like Alzheimer's, progressive hippocampal damage, and depressive-like behavior, according to earlier study [13].According to [14] fear and anxiety resulting from stressful life experiences are thought to be influenced by alterations in dendritic and synaptic structure in stress-responsive brain regions like the

hippocampus, the medial prefrontal cortex, and the amygdala. The glucocorticoid receptor in the hippocampal region is activated in response to stress, which raises neuronal metabolism at the expense of dendritic atrophy, reduces cell survival and neurogenesis, and affects cognition and long-term potentiation [15,16]. Interestingly, immobilization or prolonged restraining stress in the CA3 area of the hippocampus is a consistent cause of dendritic atrophy. [17] suggest that prolonged periods of stress exacerbate neuronal death and suppress neurogenesis in the hippocampus, hence reducing the volume of the hippocampus. Consequently, a different approach is required to stop the hippocampal damage caused by ongoing stress. Medicinal plants have been utilized to improve cognitive performance and reduce other symptoms linked with Alzheimer's disease, such as spinach (*Spinacia oleracea*) [18]. In the family Chenopodiaceae is spinach. This leafy, cool-season vegetable is grown all over the world and is typically eaten raw in salads or after boiling [19,20]. The leaves of the spinach plant include phytochemicals such as tannin, flavonoids, phenol, and carbohydrates [21]. Phytochemicals and other bioactive components found in spinach not only scavenge reactive oxygen species but also inhibit macromolecular oxidative damage and regulate gene expression and activity associated with metabolism, inflammation, proliferation, and antioxidant defense [22]. Due to the anti-inflammatory effects of spinach and its positive effects on the central nervous system, the present study investigated the therapeutic and neuroprotective effect of Spinacia oleracea (spinach) ethanolic leaf extract on chronic restraint stress-induced neurodegeneration in the hippocampus of experimental rats.

MATERIALS AND METHODOLOGY

Collection and Preparation of Extract

Spinacia oleracea leaves were purchased from a Shoprite supermarket in Polo Park, Enugu State of Nigeria, between October 2021 and November 2021. The leaves were cleaned and air-dried in the shade for a few days before being ground to a powder in a blender. Separately, 500g of pulverized S. oleracea was mixed with 1500 ml of 99% ethanol, and the mixture was normalized to room temperature for 72 hours. Then, the solutions were filtered through the Whatman No. 1 filter paper. Next, the extract was dried at 70ºC, stored at 4ºC, and dissolved in distilled water to obtain the desired concentration [23].

Experimental Animal

Twenty-four adult male Wistar rats weighing 180-200g were purchased from a breeding house in the Animal House of the Department of Pharmacology and Technology, University of Nigeria, Nsukka. They were housed in a well-ventilated iron cage in the Animal House of the Department of Anatomy, University of Nigeria Enugu Campus. The rats were maintained under controlled atmospheric pressure and humidity and acclimatized to the environment for two weeks before experimental use. They were also allowed free access to clean water and standard livestock pellets (Guinea Feed Nigeria Limited).

Ethical Approval

All animals were handled under guidelines for animal research as detailed in the Guidelines for the Care and Use of Laboratory Animals [24]. 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.

Experimental Design

Twenty-four adult male Wistar rats of 180-200g were used for this study. They were randomly divided into six (6) groups of 4 rats each. Group 1 (the normal control) received normal saline (2ml/kg, daily). Chronic stress was induced in groups 2 to 6 by placing them in a restrainer for 2 hours per day for 21. Group 2 (stress control) received normal saline (2ml/kg b.w, daily). Rats in groups 3, 4, and 5 were treated daily through oral gavage of 200, 400, and 800mg/kg extract of spinach at different concentrations, respectively for 14 days commencing from days 22-35. Also, Group 6 (positive control) received Fluoxetine (20mg/kg) daily for 14 days from days 22-35, [25] (Chart 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 restraint stress, behavioural evaluations were carried out post-treatment. The Y-maze test, novel object recognition test (NOR), and elevated plus maze (EPM) test were the order in which behavioural tests were conducted.

Behavioural assessment

All animals used in this study were subjected to behavioural experiments with no exclusion, and behavioural protocols were done with the experimenter blinded to the testing groups to prevent bias. 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, testing protocols were carried out on a rotating basis by the experimenter using testing rats from each experimental group.

Y-maze Test

Three arms—A, B, and C—each slanted at 120° and covered in black acrylic—make up the Y-maze. The rats were placed in the middle and free to explore the arms. The total number of entries was recorded when every paw was on the floor of the arms. When the rat entered the same arm in a different order, subsequent entries into each arm were measured. The total entries were counted to evaluate the rat's cognitive performance under disease control and treatment [6,27]. Formula: **(**Total No of spontaneous Alteration / Total No of Arm entries -2) × 100

Novel Objection Recognition Test (NOR)

The experiment was conducted to evaluate cognitive impairment and restoration based on the rats' capacity to distinguish between familiar and novel objects. The time spent exploring the new and old objects was used to determine the discrimination ratio and difference score for each group during the experiment, which was conducted following guidelines of [28].

Elevated plus maze (EPM)

The Elevated Plus Maze (EPM), described by [29], was used to measure the anxiolytic and anxiogenic effects of restraint chronic stress and spinach treatment. Time spent in the closed and open arms and the numbers of entries were employed in this assessment. The rat was placed in the central area opposite the closed arm and allowed to explore the maze for 5 min. 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, the device was cleaned using 20% alcohol.

Sacrifice and collection of specimens

Immediately after the behavioural test, animals were anaesthetized under light ether anesthesia to prevent marked chemical alteration. Thereafter, 5 mL of the blood of each animal was collected via cardiac puncture into separate lithium-heparinized tubes. Samples were centrifuged at 4000 revolutions per minute for 15 min at – 4 °C, using a cold centrifuge (Centurium Scientific, Model 8881) and supernatant serum was separated from the clot as soon as possible and stored at 80 °C before the biochemical assays period. Animal's brains were exercised immediately, cleaned and fixated in 10% formalin for Histological analysis [31].

Determination of MDA and Glutathione activity

Using a previously reported technique, Malondialdehyde (MDA), a marker of tissue lipid peroxidation, was quantified in the blood samples [32]. A mixed solution containing hydrochloric acid (HCl), thiobarbituric acid (TBA), and trichloroacetic acid was added to 1 ml of the sample supernatant. The mixture was incubated in boiling water for 45 min. The entire solution was centrifuged at 1000g for 10 min after cooling. The concentration of MDA was calculated using the following formula: C (M) = absorbance/(1.56 × 105), reading the supernatant's absorbance at 535 nm.
Glutathione activity was estimated as described by [32]. The test sample, 0.1 mL of supernatant, was diluted in 0.9 ml of phosphate (PO4) buffer. 20% trichloroacetic acid (TCA) in 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 was added to 0.75 mL phosphate buffer. After adding 2 mL of 2 2-Dithiobis nitrobenzoic acid (DTNB) at 0.0006M, the mixture was let to sit for 10 minutes. At 412 nm, absorbance was measured.

Enzymatic analysis

The SOD activity was measured using method of [33], which is based on the generation of SOD through 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. DMSO (Sigma-Aldrich) stops the reaction. Briefly, the supernatant of the sample was poured on a 96-well plate. After 5 min, the DMSO was added, and the solution absorbance was read using a microplate reader at a wavelength of 570 nm. One unit of SOD was described as the amount of protein required to inhibit a 50% reduction in MTT.

Corticosterone levels

Enzyme immunoassay kits measured corticosterone levels.

Estimation of tumor necrosis factor-alpha and interleukin-1β

Proinflammatory cytokines of tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β) were estimated using ELISA kits.

Histological analysis

The rat brains were carefully dissected obtaining the hippocampus and fixated in 10% formalin; fixed specimens were dehydrated in an ascending grade of alcohol (ethanol), cleared in xylene and embedded in paraffin wax. Serial sections of 10um thick were obtained using a rotatory microtome. The sections were deparaffinized and stained using haematoxylin and eosin. Thereafter, slides were viewed with a digital light microscope and a photomicrograph was captured.

Statistical analysis

The data collected were recorded at M ± SED (standard error difference) and analyzed using the SPSS package version 23. ANOVA was used to compare the differences in the means of the various variables where significant, and a post-hoc analysis using the Tukey test was carried out. Differences were considered statistically significant at $p<0.05$.

RESULTS

Effect of spinach administration on impaired spatial memory activity by chronic restraint stress

This test was performed to check the spatial memory function of stress-induced rats treated with spinach. The stress-induced rats showed a marked decline (61.1±16.9) in the percentage of spontaneous alterations as compared to the controls (62.3 ±20.9). In contrast, the spinach-treated rats demonstrated a rise in 200mg/kg (61.2±11.8%) and 800mg/kg (69.9 ±15.1%) in the activity compared to the stress-induced and control groups with p-value >0,05 across 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 and possible triads as compared with the stressed group (Table 1a and b).

Spinach ameliorated non-spatial memory impairment induced by chronic stress.

The discrimination ratio and difference score were calculated as mentioned in the methodology, and a positive score indicated more time spent on the novel object. In contrast, a negative score indicated more time spent with the familiar object. While analysis revealed a different score and discrimination ratio in each group (Fig. 1), it revealed stress effects on each group's ability to recognize familiar and novel objects. One-way ANOVA showed a decline in memory and recognition in stressed groups compared to control animals ($p>0.05$). In groups 2-6 animals, discrimination ratio I and difference score I declined with groups 5 and 6 having negative scores respectively. These data indicate that stressed rats spent more time with a familiar object than a novel object. Also, these data indicate that rats treated with spinach

spent significantly more time with the novel object compared to control animals as shown in discrimination ratio II and difference score II with p>0.05 (Fig. 1).

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 with p >0.05. The time spent in the closed arm by the animals of groups 2 to 6 was longer than the control group (245.0 ± 33.6) (Table 2a). Animals in the spinach-treated (193.8 ± 92.9), (244.3 ± 84), and (234.3 ± 38.3) groups had a shorter time in the closed arms than the stressed group (Table 2b). The results of the one-way ANOVA revealed that the time in the open arm was affected by stress (p > 0.05). Also, the stressed rats spent less time in the open arm than the control group (58.3 ± 29.8) (Table 2b). The spinach extract administration increased the time spent in the open arm compared to the stress group (p> 0.05; Table 2b).

Effect of *Spinacia oleracea* on chronic restraint stress-induced Histopathological Alterations in the hippocampus

Microscopically, the hippocampus of normal control rats revealed a normal histological structure (Fig. 5a). On the 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), and group 6, treated with Fluoxetine, a standard drug, showed normal histoarchitecture (Fig. f).

Effect of *Spinacia oleracea* on Oxidative Stress Markers level in Chronic Restraint Stress-induced Neurodegeneration in Wistar Rats.

Chronic restraint stress-induced neurodegeneration resulted in a significant increment in lipid peroxidation marker (MDA) in the brain (p < 0.006). In contrast, SOD and GST significantly declined (p < 0.043) and (p < 0.002), respectively, as compared to control rats (Fig. 2). On the other hand, treatment 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. Comparison across groups shows a statistically significant difference between the spinach-treated groups and stress, positive control and normal control groups. The results demonstrated the anti-oxidative potential exerted by spinach against chronic stress-induced oxidative stress (Fig. 2).

Effect of *Spinacia oleracea* on Cortisol level in chronic restraint stress-induced Neurodegeneration in Wistar Rats

One-way ANOVA performed for serum cortisol showed a significant interaction between the effects of stress and spinach. The stress group showed higher serum cortisol levels than the control groups (p <

0.05). The spinach extract groups presented lower serum cortisol levels than the stress group ($p < 0.05$). Also, there was a statistically significant difference between the stress control, low-dose treatment and standard drug groups (p-value 0.04) (Fig. 3).

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

The level of inflammatory markernt t (TNF-α) in the stress group increased (279 ± 51.3) compared with the control group (113.0 ± 0.4). On the other hand, spinach at a low dose (200 mg/kg) and high dose (800mg/kg) showed statistically significant differences (158.6 ± 34.4, 164.3 ± 14.1), respectively, in the TNF-α in comparison to the stress control and normal control group ($p < 0.005$). The IL-1β levels increased in the stress control group (79.3 ± 4.2) compared to the normal group (30.5 ± 4.2). When treated with a low dose (200 mg/kg) of spinach, it decreased 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 ($p<0.001$) (Fig. 4).

DISCUSSION

The current research revealed that treatment of adult rats with spinach reduced neuroinflammation, anxiety, and depression-like behaviours, cancelled learning, and memory impairment induced by chronic restraint stress. These results suggest that chronic stress effects could be a result of neurodegeneration. It has been proposed that chronic restraint stress (CRS) can imitate socioenvironmental stress [34,35]. Research on both humans and rodents has shown that stress has a variety of detrimental effects, including an increase in the prevalence of psychopathologies. According to [4], restraint stress is known to negatively impact the physiological, psychological, and reproductive axis in rats. Male rats were restrained for 2 hours straight every day for 21 days and spinach extract was administered following the stress induction for 14 days at different concentrations.

In the current study, we evaluated anxiety, depression, memory, and cognition using three behavioural test models, including the EPM, Y-maze, and NORT. Finally, treatment with spinach improved cognitive impairment, depressive-like behaviours, and anxiety in Wistar rats. CRS produces memory loss, locomotor impairments, and anxiogenic behaviour due to the inflammatory processes. These behavioural deficiencies are also linked to several severe neurodegenerative disorders. We used a Y-maze behavioural test to evaluate the rats' spatial memory. Rats who had previously visited an arm were watched to determine the amount of spontaneous activity. According to our findings, the stress group's spontaneous alteration significantly decreased compared to the control group, indicating behavioural toxicity and correlating with studies of [36] and [13]. The time and travelled paths were improved in the spinach-treated group. Treatment with spinach increased the percentage alteration, thereby improving cognition. These improvements were seen mostly in groups 3 and 4 which received 200 and 400mg concentration of the extract.

Chronic restraint stress is known to impair spatial memory, a different test—the Novel Object Recognition Test—was also conducted to assess the ill rats' ability to recognize objects and their memories. Similar to earlier research [37], the findings showed a decline in object identification following stress induction compared to controls, with a negative difference score and discrimination. However, the animal's ability to discriminate between familiar and novel objects in the stress-induced rats' dose-dependently increased memory acquisition (Fig. 2), and their capacity for spatial learning, all indicated an improvement in cognitive functioning, suggesting spinach. Spinach extract shows therapeutic potential in this study by improving learning and memory recognition.

Moreover, stressed rats were less likely to enter and stay in the open arm to assess anxiety in the EPM test. Compared to normal control rats, rats that experienced prolonged restraint stress spent more time in the closed arm. This behaviour may be seen as maladaptive because the animal restricts its exploration and opportunity to gather resources without a direct threat. Anxiety-like behaviour resulted from prolonged immobility. Animals under constant stress may establish an adaptive defence against a reasonably immediate threat [25]. Taking low, medium, and high concentrations 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 stress control group, and this is in line with the studies of [23]. Administration spinach in this present study shows its potential to reduce anxiolytic behaviours and locomotive disorders.

Furthermore, histological staining with H and E was done on the hippocampus (Fig. 5). From the results of the H and E staining of the hippocampus, it was observed that chronic stress caused poor structural arrangement, neuronal vacuolation, shrunken, pyknosis of neurons, and neuronophagia, confirming the susceptibility of these affected brain regions to chronic restraint stress-induced neurodegeneration. The hippocampus reportedly involves learning, memory, and accompanying motor responses toward goal-oriented behaviour and exploratory activity. The hippocampus has also been implicated in anxiety with input from the amygdala [38]. The photomicrograph sections of the Fluoxetine and spinach-treated groups improved in a dose-dependent manner, with the group receiving 800 mg/kg bw significantly reversing the disruption seen in the chronic stress-treated group. This demonstrated that persistent stress damages neurons, in line with the behaviour measured, and treatment with spinach at 800 mg reduced the effects of chronic stress on the neurons and enhanced behaviour by promoting neuronal cell integrity and organization of neurons in the affected hippocampal regions of the brain. This analysis also supports the anti-anxiolytic, memory deficit and learning improvement potential of spinach extract and can be linked to its antioxidant and phytochemical constituent.

The brain is highly vulnerable to the generation of ROS since it has a low antioxidant capacity compared to 20% of the metabolized oxygen [10]. CRS accelerates metabolism and produces more ROS [39]. Oxidative stress results from high amounts of ROS damaging cells and tissues [9,40] and this is evidenced in the photomicrograph presented in this study where shrunken, neuronal vacuolation and structural disintegration were seen following chronic stress induction. In the present study, CRS 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 defence

system of the animal. CRS creates free radicals and contributes to oxidative stress in the brain by lowering antioxidant defenses and glutathione levels, increasing lipid peroxidation, and changing SOD activity. According to [13], stressed rats had lower SOD and GST activity and higher MDA levels in the hippocampus. The results we obtained are in line (Fig. 3). These findings show that CRS causes anxiety and cognitive decline in rats by causing oxidative stress in the brain. CRS-related oxidative damage can cause mental health issues like anxiety and cognitive loss [7,41]. In this current research, the treatment of spinach improved both GST and SOD activity while decreasing MDA levels in reference to the antioxidant potential of spinach. Administration of spinach extract at different concentrations restored the antioxidant defence system of the animal. This is evident in groups 3 to 5 and comparable to standard drug-fluoxetine and normal control groups. This study is comparable to another one where the administration of spinach to mice enhanced SOD and GST levels while decreasing 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 levels in the hippocampus region during cognitive decline and anxiety have not been mainly studied. Additionally, we discovered that after spinach treatment, TNF-α and IL-1β levels were significantly reduced in all treatment groups compared to the stress group, which had higher levels of these cytokines. These findings demonstrated that spinach can protect the brain from the damaging effects of elevated cytokines during neuroinflammatory conditions [45]. However, the stress control had higher cortisol levels. Another study that came to a similar conclusion found that modest chronic stress raised the level of cortisol in the blood. The behavioural disorders brought on by CRS may be the result of cortisol instability, which plays a significant role in the anxiety-like behaviour observed in this study [46]. Additionally, we discovered that rats given spinach treatment had reduced cortisol levels following our analysis; this can be linked to its phytochemical constituents like tannin, phenol and flavonoid.

The findings of this study provide substantial evidence that spinach extract effectively minimizes cognitive impairment, recognition deficits, and anxiety disorders induced by chronic restraint stress (CRS) in a dose-dependent manner. The antioxidant and anti-inflammatory properties of spinach, attributed primarily to its phytochemical constituents, particularly flavonoids and tannins [21], play a central role in its neuroprotective effects. These bioactive compounds have demonstrated therapeutic potential by reducing oxidative stress, restoring antioxidant enzyme activity (GST, SOD), and lowering inflammation through the suppression of cytokines like IL-1β and TNF-α. Additionally, spinach extract reduced elevated cortisol levels, further alleviating stress-related behavioural and physiological disturbances. Also, the mechanism of action is linked to the spinach extract's ability to combat CRS-induced oxidative stress and neuroinflammation, thereby restoring normal cognitive and behavioural functions. These findings suggest that spinach extract offers a promising natural intervention for stress-related neurodegenerative disorders, highlighting its therapeutic potential as a neuroprotective agent.

CONCLUSION

The current study investigated the effects of spinach extract on chronic restraint stress-induced neurodegeneration in adult Wistar rats. The results showed that CRS-induced anxiety, memory loss, and recognition impairments, evident in the behavioural test are linked to oxidative stress, inflammation, and increased cortisol levels. However, histological analysis of the hippocampus revealed that spinach treatment reversed the neuronal damage caused by CRS, supporting its neuroprotective effects. Spinach also restored antioxidant enzyme activity (GST, SOD) and decreased oxidative damage (MDA levels), as well as reduced inflammatory cytokines -IL-1β and TNF-α, which are critical in neuroinflammatory conditions. Also, spinach treatment significantly improved cognitive function reduced anxiety and depression-like behaviours, and restored antioxidant defence systems, particularly with 200, 400, and 800 mg/kg concentration.

CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest.

DATA AVAILABILITY

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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LIST OF TABLES

Chart 1: Drug Administration Schedule

Group/Description	Treatment
1 (Normal control)	Normal saline 2ml daily
2 (Stress 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 (Positive control)	Restraint +20mg of Fluoxetine

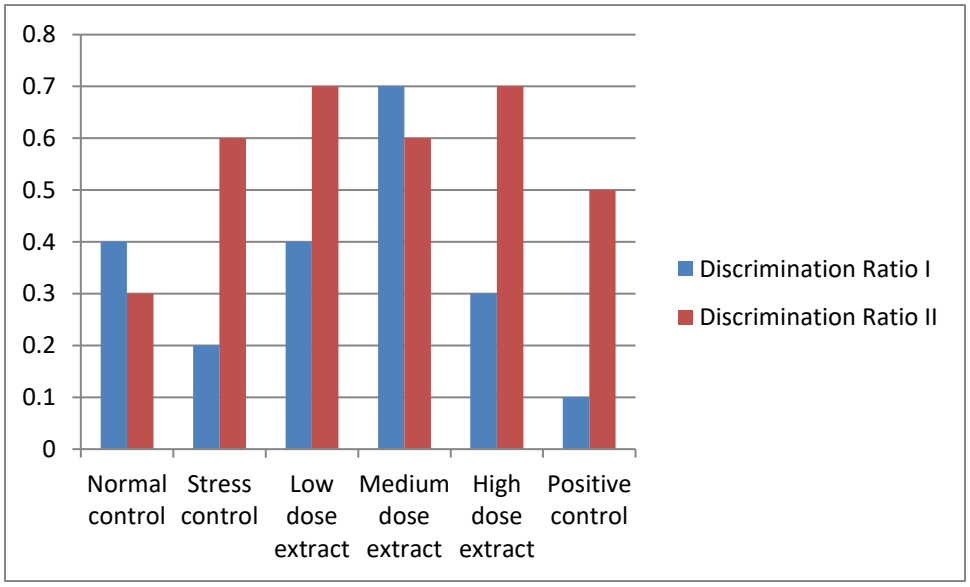
Table 1a: Effect of chronic restraint stress on the Y-maze test

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

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

Group/Description	Mean No. of Entries	Mean No. of Spontaneous Alteration	Mean No. of Possible Triads	Mean Percentage Alternation
1 (Normal control)	7.3 ± 2.9	2.8 ± 1.5	5.3 ± 2.9	61.8 ± 27.8
2 (Stress control)	10.5 ± 1.3	5.3 ± 1.5	8.5 ± 1.3	61.2 ± 11.8
3 (Low dose extract)	9.8 ± 3.8	5.3 ± 1.7	7.8 ± 3.8	74.1 ± 18.1
4 (Medium dose extract)	10.0 ± 2.3	5.5 ± 1.7	8.0 ± 2.3	69.9 ± 15.1
5 (High dose extract)	8.8 ± 3.4	4.3 ± 3.1	6.3 ± 4.3	50.5 ± 34.5
6 (Positive control)	5.3 ± 2.5	2.5 ± 2.4	3.3 ± 2.5	70.2 ± 24.6

All the values are presented as the mean ± SD. Correlation among the groups was done using one-way ANOVA with p-value >0.05.



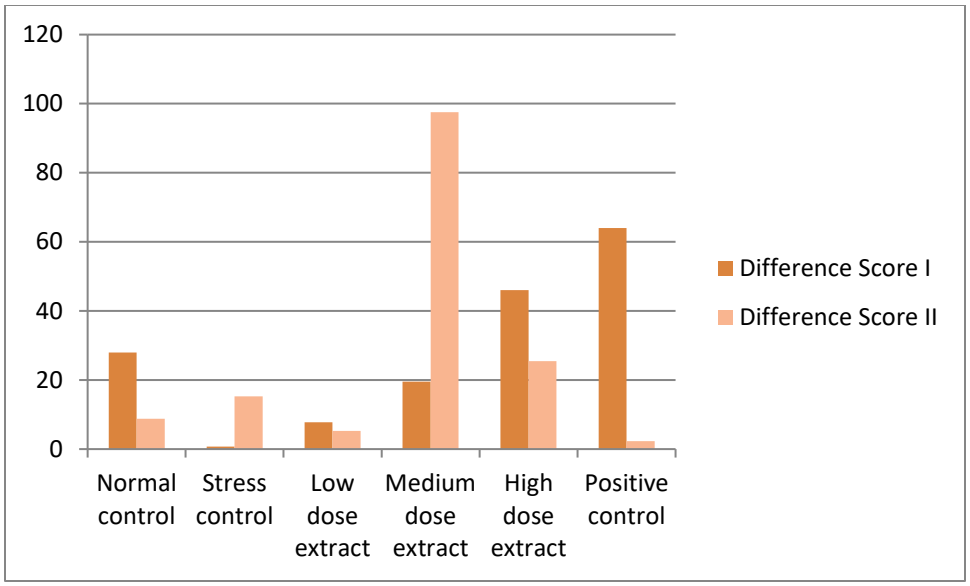


Figure 1: Effect of *Spinacia oleracea* on Novel Object Recognition test following chronic restraint stress-induced neurodegeneration in rats. I; after stress, II; after treatment

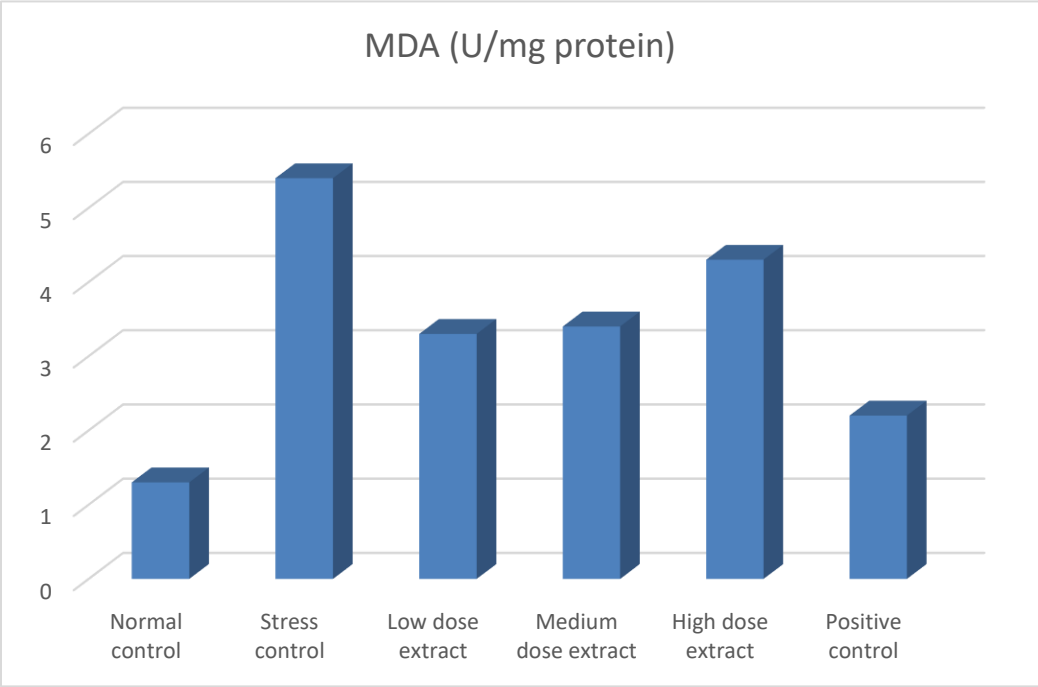
Table 2a: Effect of chronic restraint stress on the EPM test.

Group/Description	No. of Entries in Close arm	Duration in Close-arm	No. of entries in Open-arm	Duration in Open-arm	
1 (Normal control)	5.8 ± 2.9	245.0 ± 33.6	5.3 ± 3.3	55.0 ± 33.6	
2 (Stress control)	5.3 ± 2.6	220.3 ± 34.2	5.3 ± 2.6	79.8 ± 34.2	
3 (Low dose extract)	5.3 ± 2.9	237.0 ± 76.6	6.0 ± 1.0	84.0 ± 78.5	
4 (Medium dose extract)	5.0 ± 2.9	195.8 ± 67.4	4.8 ± 3.5	104.3 ± 67.4	
5 (High dose extract)	4.3 ± 3.0	235.3 ± 39.9	4.0 ± 3.2	79.8 ± 63.1	
6 (Positive control)	4.8 ± 1.0	208.8 ± 42.0	4.8 ± 0.5	91.3 ± 42.0	

Table 2b: Effect of *Spinacia Oleracea* on the EPM test

Group/Description	No. of Entries in Close arm	Duration in Close-arm	No. of entries in Open-arm	Duration in Open-arm
1 (Normal control)	4.5 ± 2.6	241.8 ± 29.8	4.3 ± 2.6	58.3 ± 29.8
2 (Negative control)	5.5 ± 1.3	252.3 ± 23.9	5.3 ± 0.9	47.8 ± 23.9
3 (Low dose extract)	5.5 ± 3.5	193.8 ± 92.9	5.3 ± 3.8	106 ± 93.0
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

All the values are presented as the mean ± SD. Correlation among the groups was done using one-way ANOVA with p-value >0.05.



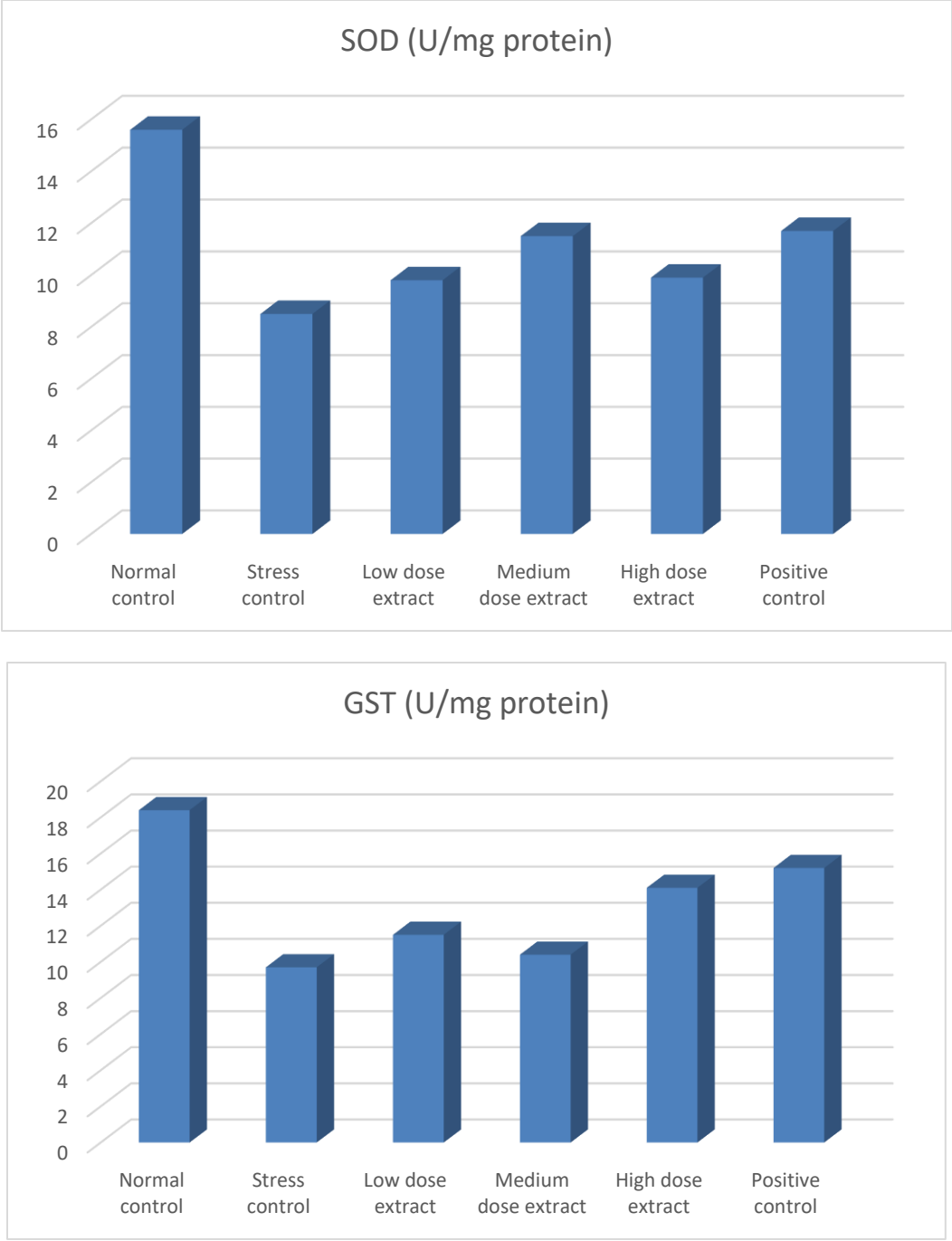


Figure 2: Effect of *Spinacia oleracea* on oxidative stress markers following chronic restraint stress-induced neurodegeneration in rats

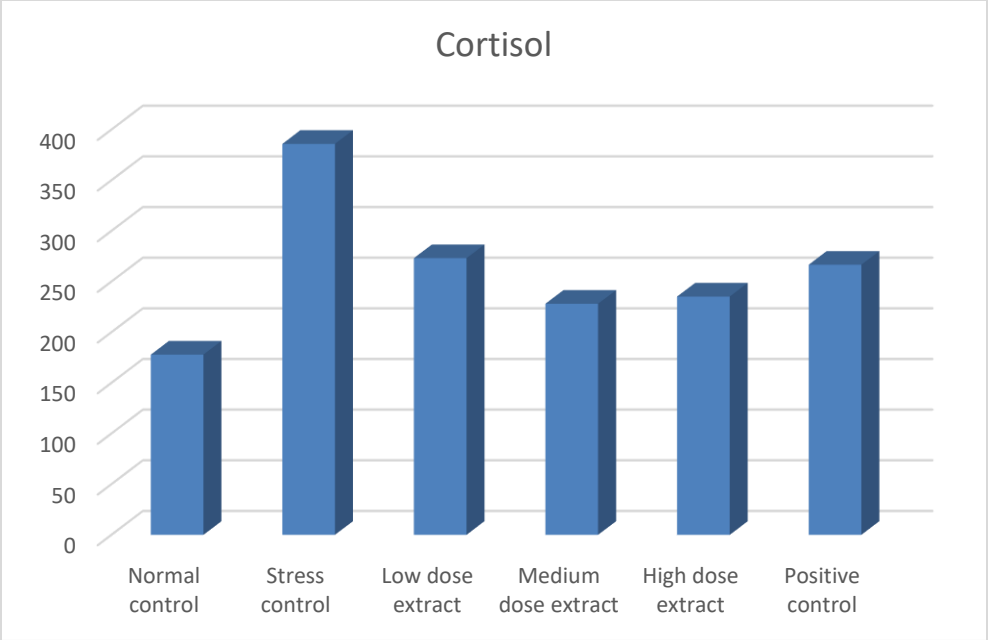
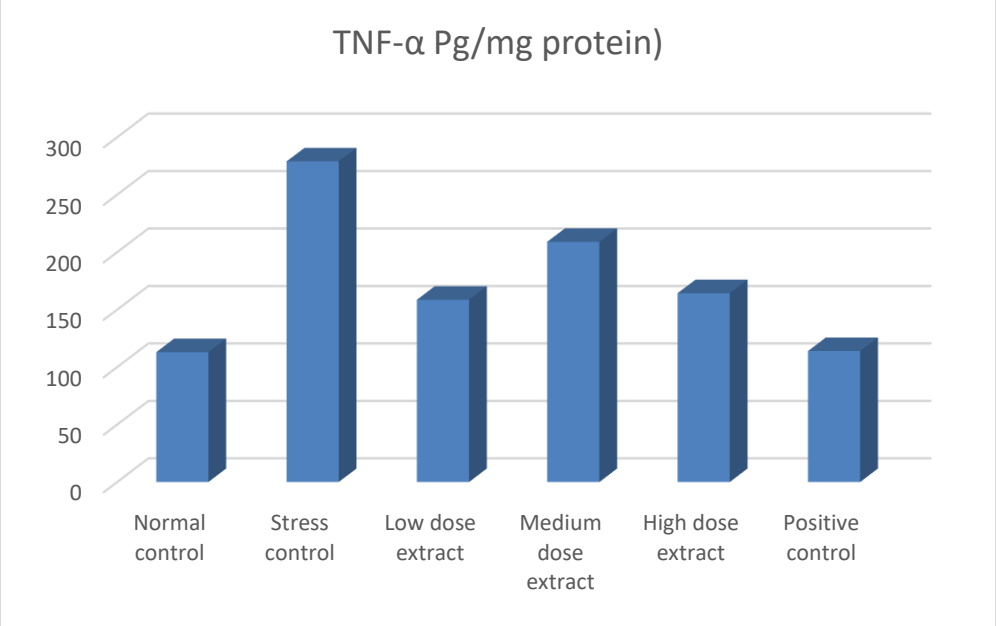


Figure 3: Effect of *Spinacia Oleracea on* Serum cortisol levels of chronic restraint stress-induced neurodegeneration in rats



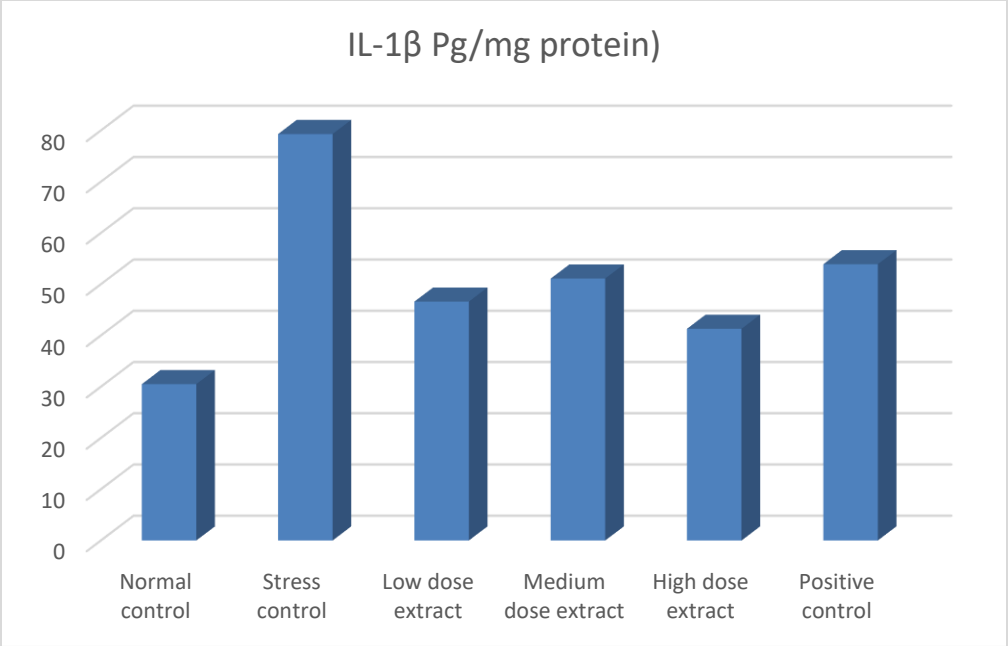
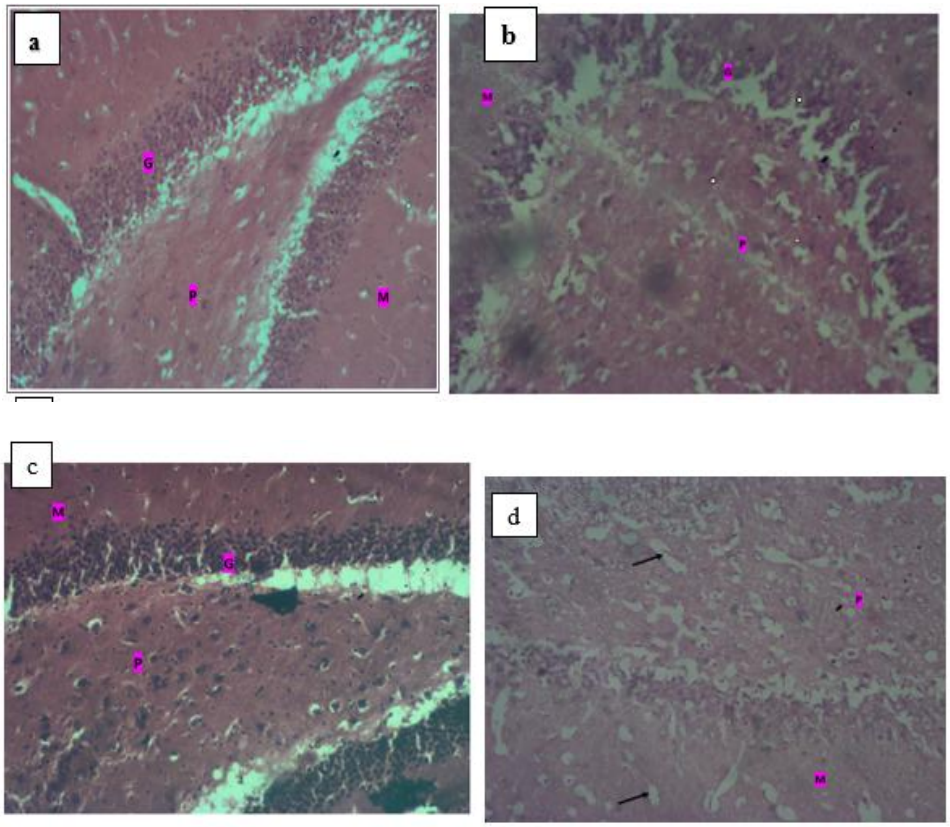


Figure 4: Effect of *Spinacia oleracea on* inflammatory marker level of chronic restraint stress-induced neurodegeneration in rats



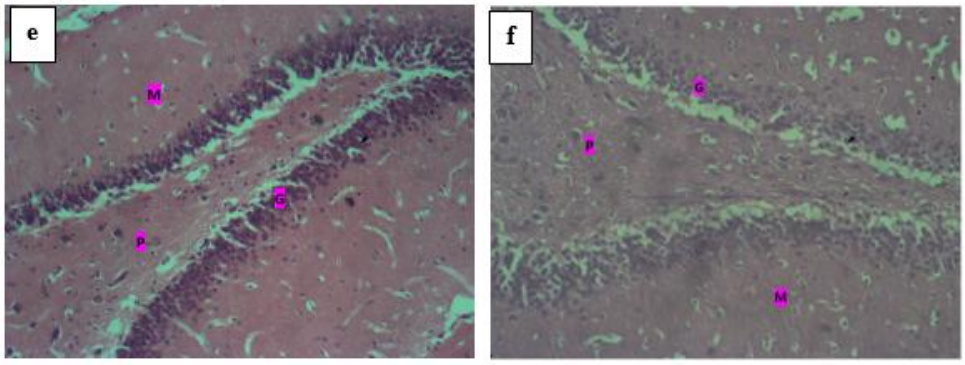


Figure 5: Representative Photomicrographs of H&E-stained hippocampus sections: a normal control brain showing the normal histological architecture. b-f restraint chronic stress-exposed brains b necrosis, shrunken, and pyknosis of neurons as well as neuronophagia; c normal histoarchitecture; d neuronophagia 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.