

## Role of *Moringa Oleifera* Leaf Extract in alleviating cerebellar ataxia induced by monosodium glutamate in rodent model

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### Keywords

- Cerebellar ataxia
- *Moringa oleifera*
- Monosodium glutamate
- Oxidative stress
- Nrf2

### Abstract

**Objective:** Studying the antioxidant properties of *Moringa oleifera* (MO) leaf extract as a prevention and treatment therapy on monosodium glutamate (MSG)-induced cerebellar ataxia rat model and investigating the role of Nrf2-Keap1 pathway. **Methods:** Thirty male Sprague-Dawley rats were allocated into four groups. Control group: six rats were administered distilled water orally for ten days. MSG group: eight rats received 4 g/kg bw of MSG orally for ten days. MSG + MO prevention group: eight rats received oral MSG 4 g/kg bw for ten days and 400 mg/kg bw of MO leaf extract orally for 21 days. MSG + MO treatment group: eight rats received 4 g/kg bw MSG orally for ten days, then 400 mg/kg bw of MO leaf extract orally for 21 days. We conducted basal and motor behavioral assessments. We carried out a histopathological inspection and a biochemical analysis to measure oxidative stress markers MDA and GSH levels in the cerebellar tissue. We also conducted an immunohistochemical analysis to evaluate the nuclear expression of Nrf2. **Results:** MO prevention and treatment therapy increased some motor behavior parameters. Both increased Purkinje cell survival ( $P < 0.01$ ), GSH levels, and nuclear Nrf2 expression. MO prevention therapy decreased MDA levels compared to MSG  $P < 0.01$ . **Conclusions:** MO prevention and treatment therapy have a possible neuroprotective effect on MSG-induced cerebellar ataxia rat model through the Nrf2-Keap1 pathway. Still, MO prevention therapy may have the upper hand over treatment therapy.

## Introduction

Cerebellar ataxias (CAs) represent a category of diseases with different clinical manifestations showing a lack of coordination and balance in common (1). Hereditary ataxias commonly affect young adults and cause motor and cognitive impairment; therefore, it significantly affects their quality of life (2). Ataxia appears in 26 per 100,000 children and 50 per 100,000 people's lifetimes (3). In Al-Kharga District-New Valley, Egypt the prevalence rate is 38.34 per 100,000 (4).

Cerebellar neurons are predominantly vulnerable; therefore, many pathways may lead to neuronal death, including DNA/RNA deficits, toxic protein accumulation or a deficit in its clearance, oxidative stress, caspase activation, N-methyl-D-aspartate (NMDA)-mediated excitotoxicity, and apoptosis (5). These pathways may function individually or, more probably, interact with each other, causing cellular damage that finally results in neuronal death through several different events (6). Until now, symptomatic treatments have been provided for progressive ataxias patients, as we lack a particular treatment to stop the ataxias' course or reverse cerebellar atrophy (7). Consequently, it is medically necessary to develop more effective therapies for this category of debilitating illnesses (8). Several forms of primary CA and other neurodegenerative diseases have shown signs of oxidative stress. Oxidative stress is a recurrent pathway of cellular degeneration, which clarifies the interest in antioxidant therapy to improve effective drugs (2).

Monosodium glutamate (MSG) is a salt form of L-glutamic acid frequently utilized as a taste enhancer globally. It is known that a high dose of

MSG can damage cerebellar Purkinje cells (PCs) and affect motor coordination, which is why it is used to induce experimental CA in rodents (9).

*Moringa oleifera* (MO), a small tree commonly grown in most parts of the world, is known for its therapeutic and nutritional properties. Many studies showed that MO has antioxidant, antimicrobial, anti-inflammatory, anti-dyslipidemic, antihyperglycemic, antiproliferative, anti-ulcer, hepatoprotective, and anticancer effects. MO leaves contain a variety of beneficial phytochemicals, including flavonoids, tannins, moringin, steroids, triterpenoids, saponins, alkaloids, anthraquinones, niazimicin, and reducing sugar substances (10;11). MO leaf extract was observed to have a potential neuroprotective effect as it activates antioxidant enzymes and reduces oxidative stress (12).

Activation of nuclear factor-2 erythroid-related factor-2 (Nrf2) signaling is a principal antioxidant mechanism of MO, as MO contains  $\beta$ -carotene, which was found to promote nuclear translocation of Nrf2 and enhance target gene expression that decreases the expression of Keap1. Keap1 is a negative regulator that strongly modulates the activity of Nrf2 by binding to Nrf2 and keeping it in the cytoplasm. In case of oxidative stress, Nrf2 separates from Keap1, migrate to the nucleus to activate the expression of ARE-dependent gene. That results in antioxidant enzymes production as superoxide dismutase, heme oxygenase-1 and glutathione peroxidase, therefore cells become more resistant to oxidative stress (13; 14).

Does *Moringa Oleifera*'s role as an antioxidant acting through the Nrf2-Keap1 pathway and other neuroprotective mechanisms influence the pathophysiology of cerebellar ataxias? It remains an important question to answer.

## Material and method

### Experimental animals

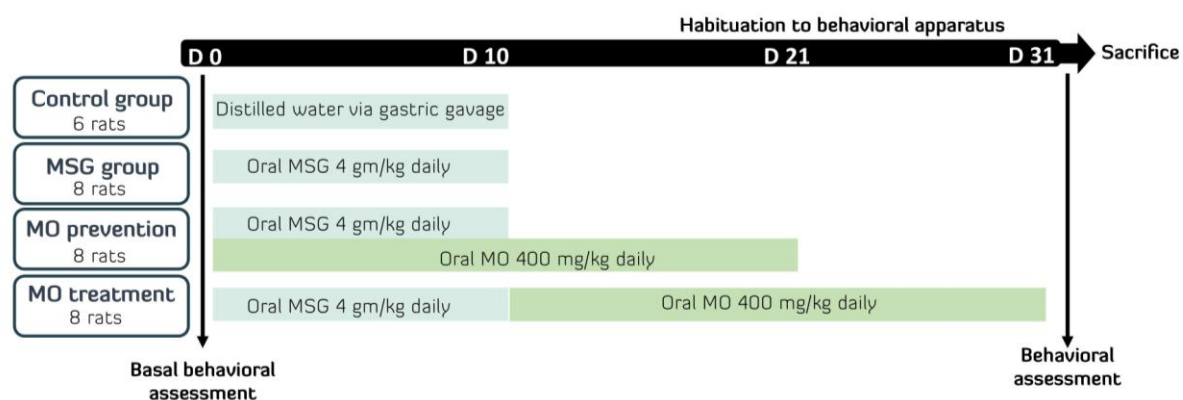
This study was done on thirty male Sprague Dawley rats, age (10-12 weeks), weight (250-350 gm). Rats were obtained from the Medical Experimental Research Centre (MERC), Mansoura faculty of Medicine. The housing circumstances were regulated, maintaining humidity levels between 40% and 70%, an inverted light-dark cycle of 12 hours each, and a temperature range of 20 to 22 °C, with food and drink available ad libitum. The IRB Committee approved this project (Code MS.22.04.1949). We used the "resource equation" method to determine the appropriate sample size (15).

### Chemicals

MO was acquired from the Egyptian Scientific Society of Moringa, National Research Centre, Dokki, Cairo, Egypt, and MSG was purchased from ALMASRIA for Chemicals, Egypt.

### Study design

Rats were randomly allocated into four groups. a) The control group: six rats were administered distilled water via oral gavage for ten days. B) MSG group: eight rats were administered 4 g/kg body weight of MSG daily for ten days; MSG was given via oral gavage after being dissolved in distilled water (16). To avoid crystallization, we prepared the dose daily. C) MSG + MO prevention group: eight rats were administered 4 g/kg of body weight of MSG orally for ten days and 400 mg/kg body weight/day of aqueous MO leaf extract for 21 days (12) MO intervention started with MSG protocol (10). D) MSG + MO treatment group: We administered oral MSG 4 g/kg body weight to eight rats for ten days, followed by a daily oral gavage of 400 mg/kg body weight of aqueous MO leaf extract for 21 days (10; 12); (Fig.1).



**Fig 1.** Study design showing timeline of the study groups

### Behavioral Baseline Assessment: spontaneous behavior in the open field

We conducted a behavioral baseline assessment on all the rats, following the Deacon protocol (17), before initiating any regimens. We used a 100cm×100cm×60cm (L ×W ×H) arena with dark walls and a divided base into 16 equal squares. We

left the rats in the room for 5-20 minutes before recording, then exposed them to the open field for the first time, and video-recorded their behavior for 3 minutes. We recorded the number of crossed squares, rearing frequency, fecal boli, and presence or absence of urination.

**Behavioral assessment after intervention: general locomotive activity and exploratory level using an open field test**

Each rat was video recorded for 10 minutes in the open field arena as a single trial. We conducted the automated analysis using the Fiji Image J software 2.9.0 version (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, Maryland, USA). Distance crossed, velocity, and immobility time were recorded. We manually recorded the frequency of urination, the number of fecal boli, and the presence or absence of urination (18).

**Motor coordination assessment by static rod test**

Two wooden rods 60 cm long, 5 cm, and 1.5 cm in diameter were fixed from one side on a shelf with the other protruding horizontally into space and elevated from the floor by 60 cm; both were marked 10 cm from the shelf. We first placed each rat on the broadest rod, placing its nose towards the rod's free end. Two measures were taken: orientation time, which is the duration needed to orient 180° from the initial place towards the shelf in seconds, and transit time, which is the duration in seconds required to travel after orientation to the mark 10 cm from the shelf. Both parameters have a maximum score of 120 seconds. If the rat failed to pass the first rod, a maximum score was spontaneously assigned to the small rod without testing. This protocol was modified from Deacon (19).

**Animal sacrifice and sample collection**

The day following the completion of the behavioral tests recording, the rats were deeply anesthetized and euthanized using a high dose of sodium thiopental (120 mg/kg) intraperitoneal injection. Next, we performed intracardiac

perfusion using 100 ml of saline (20). The brain was dissected, and the cerebellum was divided into two sections. One section was fixed in 10% formaldehyde at room temperature for one day until processing for histopathological and immunohistochemical assessment. The other section of the cerebellum was immediately weighed, then frozen, and kept at -80 °C till the biochemical assay of oxidative stress markers was done (21).

**Histopathological examination**

The fixed cerebellar tissue underwent the standard procedure for paraffin embedding, followed by the cutting of serial 20  $\mu$ m-thickness sections. Haematoxylin was applied to the sections for 15 minutes, followed by immersion in HCl alcohol solution for 35 seconds, then stained with eosin for 10 minutes and subsequently treated with 90% ethanol for 40 seconds (22). We used an Optika light microscope (OPTIKA Microscopes, Italy) to take five random photomicrographs of each specimen at 400 $\times$  magnification and do a histopathological evaluation of PC necrosis. For each photomicrograph, we calculated the PC linear density by dividing the counted PCs by the length of the PC layer in millimetres, obtained by drawing a line across the center of the PCs. Finally, we calculated the mean PC linear density for each rat (23).

**Biochemical study**

To prepare the cerebellar homogenate, we added 10 ml of ice-cold phosphate-buffered saline per gram of cerebellar tissue and used a Teflon-glass homogenizer. Next, we centrifuged the homogenate for 15 minutes at 5000 rpm and 4 °C (22). We then used the supernatant to measure the amounts of

oxidative stress markers reduced glutathione (GSH) and malondialdehyde(MDA) via a colorimetric assay, adhering to the manufacturer's guidelines. The kits were bought from Bio Diagnostic, Giza, Egypt (CAT. No. GR 25 11) and (CAT. No. MD 25 29) for GSH and MDA, respectively.

### **Immunohistochemical examination**

Tissue sections that had been deparaffinized were rehydrated, cleaned, submerged in 3% hydrogen peroxide, and digested with pepsin to extract antigens. After applying serum to prevent unspecific binding, we treated the sections with anti-Nrf2 polyclonal antibody (catalog # sc-13032 X; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. We generated a brown signal using a diaminobenzidine/peroxidase substrate. The section was cleaned, coated, dehydrated, and counterstained. The primary antibody was substituted with phosphate buffered solution (PBS), and adjacent sections functioned as a negative control (22). Five random photographs of serial sections of each specimen were taken using an Optika light microscope (OPTIKA Microscopes, Italy) at 400× magnification. Next, we conducted a qualitative and semi-quantitative analysis of Nrf2 expression. Qualitative analysis was done by counting the number of PCs showing positive nuclear Nrf2 expression in relation to the total number of PCs, represented as a percentage (24). Semi-quantitative determination of Nrf2 expression was done by using the open-source Fiji Image J software 2.9.0 version (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, Maryland, USA, <https://imagej.nih.gov/ij/>, 1997-2018.). Nrf2 expression was measured as mean

gray value % following Crowe and Yue protocol (25).

### **Statistical analysis**

IBM SPSS Statistics software (Version 26) was used to perform data analysis. To compare groups, the one-way ANOVA test with post hoc Bonferroni was used for parametric data. While Kruskal-wallis test was used to analyze non-parametric data, the bivariable comparison was done using repeated Mann-Whitney U test. The data were described as Mean  $\pm$  SD or median and interquartile range (IQR) or frequency according to its type. The results were considered statistically significant when p-value is lesser than 0.05. Correlation between different parameters was done using Pearson's or Spearman's correlation. The graphical representation was done using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, Inc., San Diego, CA).

### **Results**

#### **Animal survival**

Control group rats had 100% survival. Each of the other groups had 25% mortality, with a survival rate of 6 rats at the end of the study.

#### **Motor behavioral tests analysis**

Basal behavior assessment before any intervention showed no significant difference in all spontaneous open-field parameters among all study groups (Table 1).

After the intervention, the behavioral general locomotive activity and exploratory level using the open field test showed that the log distance crossed by the control, MO prevention, and MO treatment groups in the open field arena increased significantly compared to the MSG group by about 25-30%. The immobility time in the open field increased significantly in the MSG group

compared to the control, MO prevention, and MO treatment groups with  $P < 0.01$ ,  $P < 0.01$ , and  $P < 0.05$ , subsequently. There was no significant difference among the other open-field parameters

(Table ). Moreover, no significant variation was seen among the study groups regarding the static rods test (Table 3).

**Table 1** Parameters of spontaneous behavior in the open field before administration of MSG and MO among study groups.

	Control	MSG	MO prev.	MO treat.	P value
Squares (number)	36.2 ± 15.05	25.7 ± 14.7	38.7 ± 7.12	22 ± 19.2	0.179
Rearing (frequency)	9.5 ± 5.24	10 ± 7.24	15.2 ± 4.79	9.7 ± 4.46	0.256
Fecal boli (number)	2.5 (2 - 3)	3 (2 - 5)	2.5 (2 - 6)	2.5 (2 - 5)	0.802
Urination (number)	0/6	0/6	1/6	0/6	1

The number of squares crossed by rats in the open field and rearing are represented as mean ± SD, The One-way ANOVA test showed no significant difference among the studied groups. The number of fecal boli is expressed as median, min. to max, Kruskal Wallis test showed no significant difference among the studied groups. The presence or absence of urination is represented as frequency: number/total, Fischer exact  $\chi^2$  test showed no significant difference among the studied groups. n= 6 in each group.

**Table 2** General locomotive and exploratory parameters in the open field test after administration of MSG and MO among the different study groups.

	Control	MSG	MO prev.	MO treat.	P value
Log (Distance cm)	2.39 ± 0.29*	1.85 ± 0.21	2.41 ± 0.24*	2.52 ± 0.36**	0.003 F= 6.62
Velocity (cm/sec)	351.7 (114.2-842.9)	223.8 (27.7-497.1)	315.4 (123-728.4)	344.5 (128.3-709.7)	0.456
Immobility time (sec)	182.6** (88.5-551.3)	729.2 (563.3-747.1)	507.9** (175-548.1)	402.8* (200.5-669.4)	0.002
Rearing frequency	2.5 (0 - 19)	0 (0 - 3)	2.5 (1 - 8)	3 (1 - 13)	0.225
Fecal boli number	3 (1 - 4)	1.5 (0 - 2)	2.5 (0 - 4)	2.5 (0 - 4)	0.253
Urination number	1/6	0/6	0/6	2/6	0.573

The log of the distance crossed in cm by rats is represented as mean ± SD, A One-way ANOVA test with post hoc Bonferroni test was used. Velocity (cm/sec), Immobility time (in seconds), fecal boli, and rearing are represented as median, min. to max, Kruskal Wallis test was used then a bivariable comparison using the Mann-Whitney test was done. The presence or absence of urination is represented as frequency: number/total, Fischer exact  $\chi^2$  test was used. \* Significantly different from the MSG group, P value <0.05. \*\* Significantly different from the MSG group, P value <0.01. n= 6 in each group.

**Table 3.** Comparison between orientation time and transit time on the static rods among study groups after administration of MO in MSG-induced cerebellar ataxia.

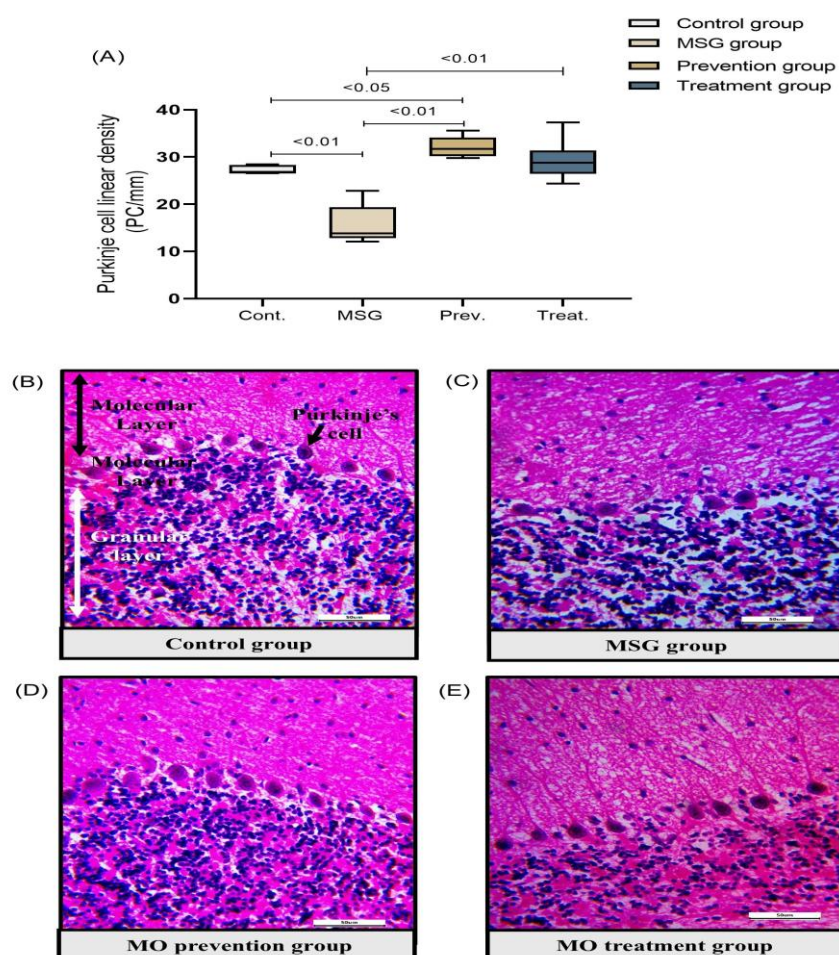
	Control	MSG	MO prev.	MO treat.	P value
Orientation t.1 (sec.)	5 (3 - 14)	6 (2 - 120)	3.5 (3 - 14)	6 (3 - 120)	0.719
Transit t. 1 (sec.)	64 (3 - 120)	120 (11 - 120)	63 (2 - 120)	120 (11 - 120)	0.492
Orientation t. 2 (sec.)	120 (2 - 120)	120 (5 - 120)	64 (3 - 120)	120 (4 - 120)	0.963
Transit t. 2 (sec.)	120 (2 - 120)	120 (9 - 120)	120 (3 - 120)	120 (120 - 120)	0.395

Orientation time and transit time on two different diameters of the static rods expressed as median, min. to max. Kruskal Wallis test showed no significant difference among the studied groups. n= 6 in each group.

### Histopathological analysis of Purkinje cell survival

Comparison analysis of the Purkinje cell linear density among study groups showed that the MSG group (134 PCs per 8601.786  $\mu\text{m}$  total layer length in 30 slices from 6 rats) was significantly reduced than the control group (260 PCs per 9614.076  $\mu\text{m}$  total layer length in 30 slices from 6 rats) with  $P < 0.01$ . Interestingly, the MO prevention group (314 PCs per 9847.602  $\mu\text{m}$  total layer length in 30 slices from 6 rats) revealed a significant increase compared to the MSG group and the control group with  $P < 0.01$  and  $P < 0.05$ , subsequently. Also, the MO treatment group (291 PCs per 10123.634  $\mu\text{m}$

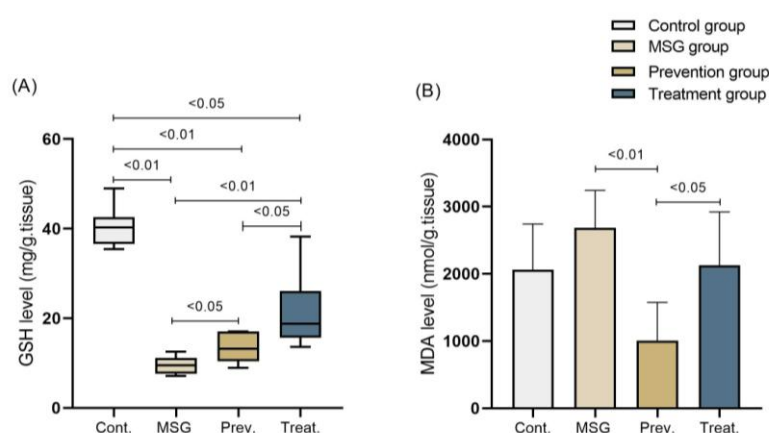
total layer length in 30 slices from 6 rats) showed a significant increase relative to the MSG rats,  $P < 0.01$  with no significant difference to the control group (**Fig. 2A**). Haematoxylin and eosin-stained sections examination under the microscope showed uniform alignment of a row of PCs between molecular and granular layers in the control group (**Fig. 2B**). The MSG group sections revealed disturbed alignment in the PCs layer with scattered, deformed, and irregular cells (**Fig. 2C**). Yet MO prevention (**Fig. 2D**) and MO treatment groups (**Fig. 2E**) exhibited regular and ordinary morphology of uniformly aligned PCs more like the control group.



**Fig 2.** Histopathological changes (A) difference in the median, min. to max of Purkinje cell linear density (PC/mm). Kruskal Wallis test was done and a bivariable comparison using the repeated Mann-Whitney U test. Significant differences are shown on the graph.  $n = 6$  in each group. The morphological changes in Purkinje cells stained by hematoxylin and eosin in the control group (B) showed regular and ordinary morphology with normal density of Purkinje cells. (C) with MSG showed scattered Purkinje cells with disturbed alignment, irregular appearance, and notably decreased density of Purkinje cells. (D), (E) with MO as a prevention and treatment respectively showed morphology more like the normal control group. The scale bars represent 50  $\mu\text{m}$ , at 400 $\times$  magnification.

### Biochemical analysis

The control group had a significant increase in the level of antioxidant marker GSH in the cerebellar tissue compared to MSG, MO prevention, and MO treatment groups. Also, MO prevention and MO treatment groups had a significant elevation in the level of GSH in cerebellar tissue compared to the MSG group, with  $P < 0.05$  and  $P < 0.01$ , respectively. Interestingly, the MO treatment group had a significantly high GSH level ( $P < 0.05$ ) compared to the MO prevention group (Fig. 3A). However,



**Fig 3.** Effect of MSG and MO administration on (A) GSH level is represented as median, min. to max, Kruskal Wallis test was done with a bivariable comparison using the repeated Mann-Whitney U test. (B) MDA level is represented as mean  $\pm$  SEM, A One-way ANOVA test with post hoc Bonferroni test was used. P-value  $< 0.05$  is considered significant. Significant differences are shown in the graph.  $n = 6$  in each group. GSH: reduced glutathione and MDA: malondialdehyde.

### Immunohistochemical analysis: MO increased nuclear Nrf2 expression

The percentage of PCs' nuclei with Nrf2 expression was significantly elevated in the control rats compared to the MSG rats. Interestingly, the MO prevention and MO treatment groups increased the percentage of stained nuclei by more than two and a half times compared to the MSG group ( $P < 0.001$ ). Image J software supported these results by revealing a significantly elevated mean gray value % in the control rats compared to the MSG rats. Also, MO prevention and MO treatment groups had significantly higher mean gray value % than the MSG group,  $P < 0.01$  and  $< 0.05$ , respectively. Yet, we found no significant

on comparing the level of MDA to the other research groups, we found no significant difference. Yet, MDA level was significantly reduced in the MO prevention rats relative to the MSG rats with  $P < 0.01$ . Interestingly, the MO prevention group also had a significantly reduced MDA level than the MO treatment group ( $P < 0.05$ ). However, no significant change was observed when comparing MDA levels between the MO therapy and MSG group (Fig. 3B).

difference among the control, MO prevention, and MO treatment rats (Fig. 4).

### Correlation between study variables

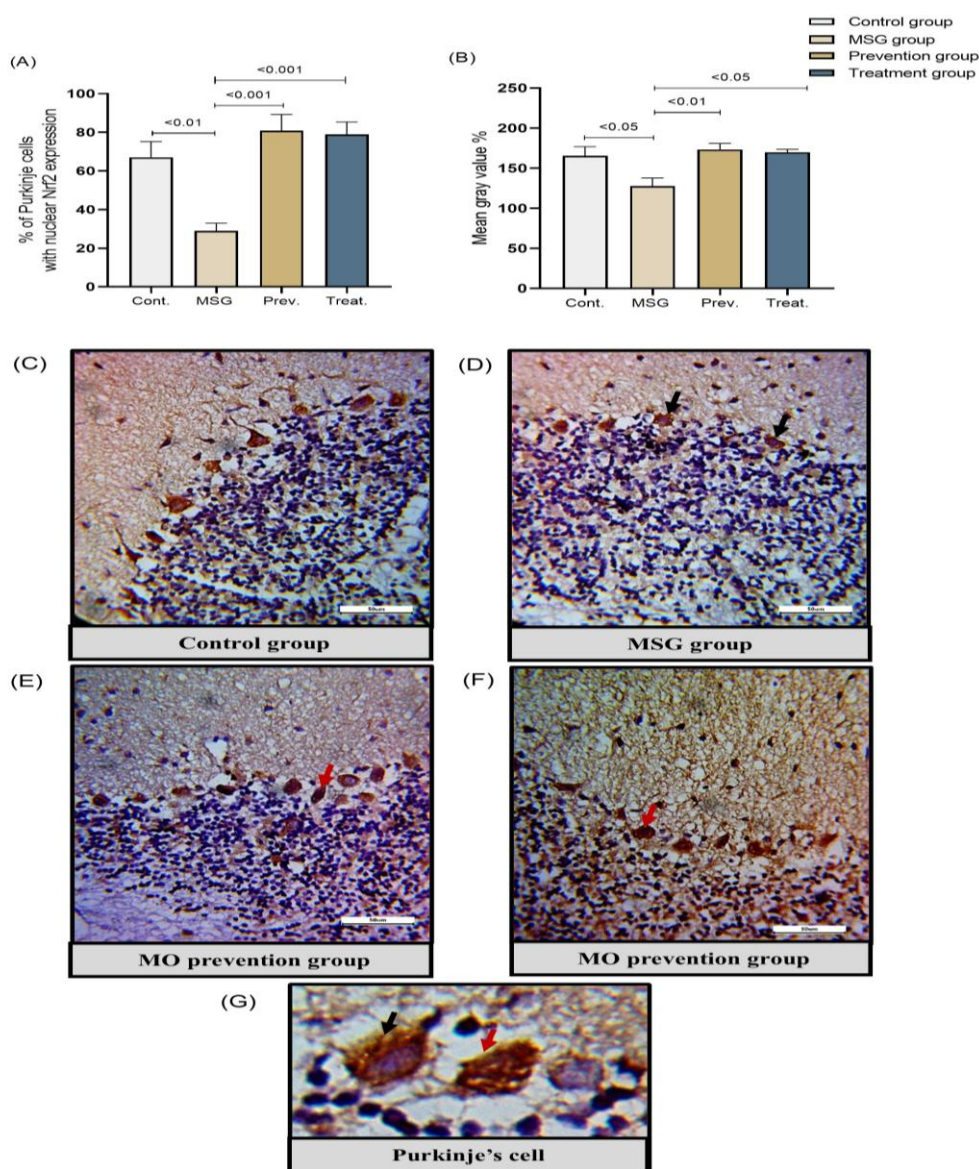
Spearman's rho correlation analysis indicated a significant negative correlation between immobility time and log distance crossed in the open field arena (Fig. 5A) and a significant positive correlation between GSH level and log distance (Fig. 5C). While Pearson's correlation analysis indicated a significant positive correlation between the percentage of PC nuclei expressing Nrf2 and log-distance (Fig. 5B).

Spearman's rho correlation analysis demonstrated a significant negative correlation between immobility time and Purkinje cell linear density, as

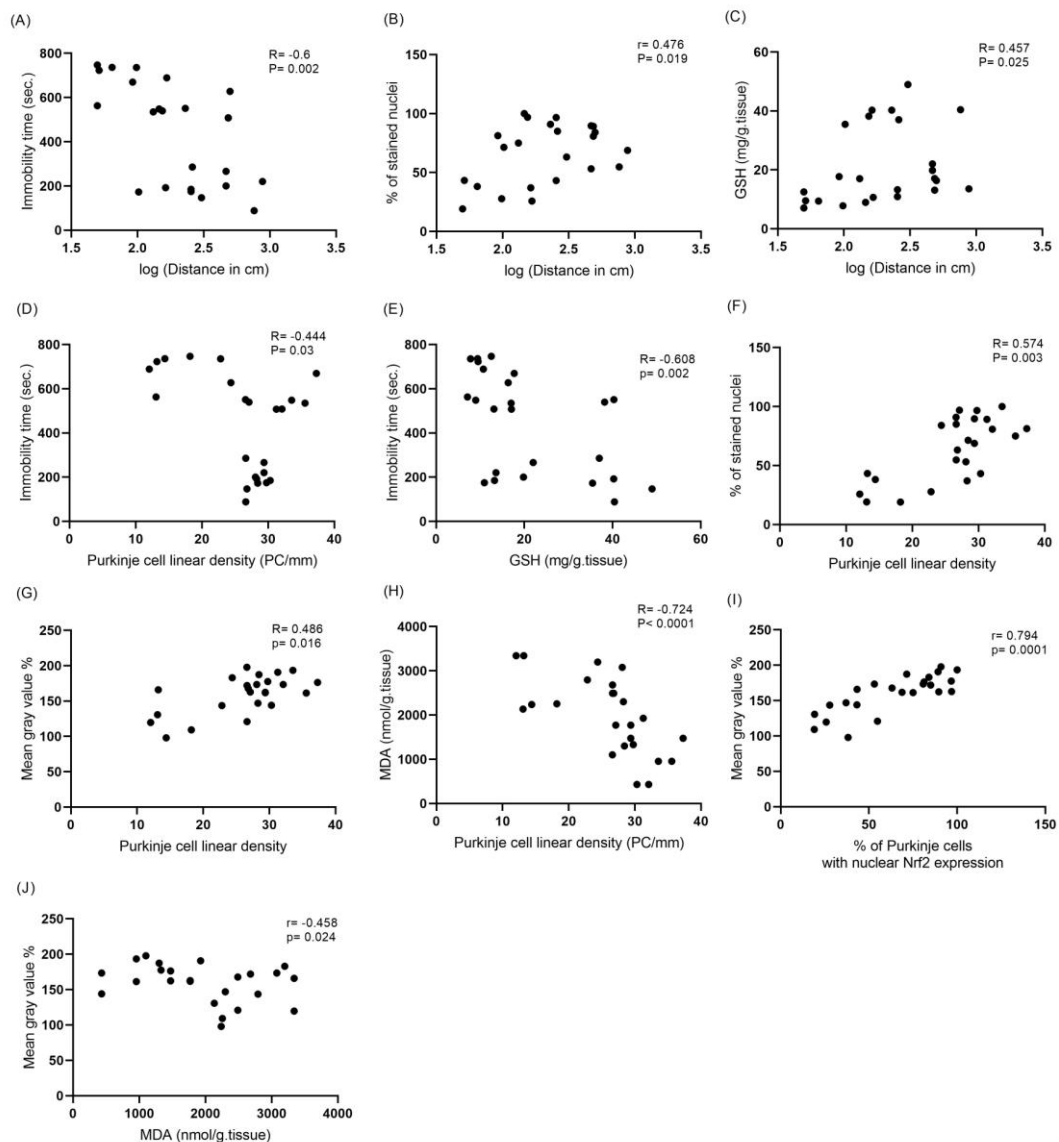


well as immobility time and GSH levels (**Fig. 5D; E**). Furthermore, it showed a significant positive correlation between Purkinje cell linear density, the percentage of stained nuclei expressing Nrf2 (**Fig. 5F**), and the mean gray value % of Nrf2 expression (**Fig. 5G**). However, it had a highly significant negative correlation with the oxidative stress marker MDA level among all study groups (**Fig. 5H**).

On the other hand, Pearson's correlation analysis revealed a highly significant positive correlation between the mean gray value % of Nrf2 expression and the percentage of PCs with nuclear Nrf2 expression (**Fig. 5I**). Moreover, it had a significant negative correlation with cerebellar MDA levels among study groups (**Fig. 5J**). We found no correlation between other study parameters among study groups.



**Fig 4.** Effect of MSG and MO administration on the nuclear Nrf2 expression. (A) Qualitative analysis of nuclear Nrf2 expression, (B) quantitative analysis of nuclear Nrf2 expression. The percentage of nuclear Nrf2 expression and mean gray value % are represented as mean  $\pm$  SEM, One-way ANOVA with post hoc Bonferroni test was used. P-value  $< 0.05$  is considered significant. Significant differences are shown in the graph.  $n = 6$  in each group. Examination of the Purkinje cell layer under the microscope showed in (C) the normal control group shows the normal expression of nuclear Nrf2, (D) MSG-induced (MSG 4 g/kg) cerebellar ataxia group shows a decreased expression of nuclear Nrf2, (E), (F) administration of MO (400 mg/kg) as prevention and treatment showed a significant increase in nuclear Nrf2 expression compared to MSG only group. The scale bars represent 50  $\mu$ m at 400 $\times$  magnification. (G) Black arrow: Purkinje's cell without nuclear Nrf2 expression. Red arrow: Purkinje's cell with nuclear Nrf2 expression.



**Fig5.** Correlations between different study parameters. r: Pearson’s correlation coefficient, R: Spearman’s rho correlation coefficient, and P = probability significance.

**Discussion**

We found that MO, either as a prevention or a treatment therapy, alleviated some of the motor behavioral disturbances and increased PC survival in the cerebellar cortex. These findings were supported by the increased level of the antioxidant marker GSH in the cerebellar tissue of the rats administered MO prevention and treatment therapy. Additionally, there was a decrease in the level of the oxidative marker MDA in the cerebellar tissue of rats administered MO as a

prevention therapy. Nrf2- Keap1 signaling pathway may mediate this neuroprotective effect of MO, as both MO prevention and treatment therapy increased the overall expression of Nrf2 and its nuclear translocation. Although both MO prevention and treatment therapy showed a possible neuroprotective role of MO on cerebellar ataxia, our results suggest that MO prevention therapy may have the upper hand as it could inhibit the damaging cascade from the start rather than solely reversing the damage.

In the present study, a 4 g/kg body weight MSG for ten days was used to induce the cerebellar ataxia model in rats to evaluate the hypothesis of the neuroprotective benefits of *Moringa oleifera* leaf extract in CA. Before the induction of cerebellar ataxia, baseline levels of behavior were assessed by spontaneous behavior in the open field test, and we found that all groups possessed comparable motor function behavior with no significant change between different groups. After MSG and MO intervention, all rats were subjected to behavioral assessment utilizing the open field and static rod tests.

Statistical analysis using the Shapiro-Wilk test showed the distance data in the open field was not normally distributed; therefore, we used log distance instead. Statistical analysis revealed a significant decrease in the log distance crossed by MSG group rats compared to the control group, indicating a motor deficit in the general locomotive action of these rats. After MO prevention and treatment, we found significant improvement in this motor deficit as the log distance crossed increased significantly in these rats compared to the MSG group rats. We noted that the log distance was more significant in the MO treatment group.

We also found a significant elevation in the immobility time in the MSG rats relative to the control rats, indicating a motor deficit after CA induction. These results aligned with a previous study by Att et al. who found that MSG induced poor mobility in the open field test relative to controls regarding the number of crossed squares and mobility duration (26). There was also a significant reduction in the immobility time in MO prevention and treatment rats compared to the

MSG group, which demonstrated alleviation in motor deficit after MO intervention. This decrease in immobility time was more remarkable in the MO prevention group. Furthermore, there was no significant variation in the number of fecal boli and urination across the various groups, suggesting the absence of anxiety.

The current study has certain limitations in that we observed changes in the velocity, rearing frequency in the open field, and a change in motor behavior using a static rod test; however, these changes were not statistically significant. After searching the literature, we found varied results. Some studies found that MSG administration reduced rearing frequency in the open field test (27;28). Other studies were consistent with our findings, as Hassaan et al. found no change in the rearing frequency and motor activity on the static rod test after MSG induction (29). It was also observed in the 3-acetylpyridine-induced CA model as researchers observed a difference in motor behaviors, with different impairment severity between various tasks and studies (30).

Some possible explanations could be that the dose and the route of administration of MSG may influence motor symptoms, and a higher dose of MSG may be needed to notice these changes. We have already ruled out the option of using a larger dosage of MSG in this study, as it was lethal for the rats. Another possibility is that the rats may have had a compensatory strategy to cope with the altered motor function that allowed them to maintain their velocity in the open field (31). It may also be because static rod tests might require more significant deficits to produce measurable differences in performance, which needs further research. This highlights the necessity of using

several tasks to characterize rodent behavioral impairments (30). That's why we performed more than one behavioral test to detect motor dysfunction in the first place.

In the histopathological study, we noticed that the PCs had normal morphology, and there was a regular arrangement in the PC layer of the cerebellar cortex in MO prevention, treatment and the control group. However, MSG group rats had abnormal, distorted and shrunken PCs with distorted alignment in the cerebellar cortex. These observations were confirmed by measuring the Purkinje cell linear density; we found that ataxic rats had a significant reduction in the Purkinje cell linear density relative to the control group, indicating that MSG exerted its excitotoxic effect on PCs, resulting in neuronal death and disturbed alignment of PC layer in the cerebellar cortex. Since PCs are the only outputs from the cerebellar cortex and significantly contribute to the pathophysiology of CA, any disruption to PC function or structure can significantly impact cerebellar output (32). This observation was consistent with the findings of other studies (26; 16; 9).

Surprisingly, the PC linear density increased significantly in the MO prevention rats compared to the ataxic and control rats, indicating the possible neuroprotective role of MO on PCs in improving their survival. Moreover, the MO treatment group had a significant increase in the Purkinje cell linear density compared to the ataxic rats, which supported the presence of a neuroprotective effect by MO. This finding aligned with the study by Omotoso et al. (33) who observed a protective impact of MO on PCs

compared to rats with cuprizone-induced cerebellar damage.

To further study the neuroprotective effect of MO and investigate its antioxidant effect on the cerebellar PCs. We measured GSH and MDA levels in the cerebellar tissue of the different study groups. We found a significant elevation in GSH levels in the cerebellar tissue of the control, MO prevention, and treatment rats relative to ataxic rats. We also found a significant elevation in the MDA level in the cerebellar tissue of the MSG rats relative to the MO prevention rats. However, we didn't find a significant increase in MDA level relative to the control group. MSG excitotoxicity was reported to cause  $Ca^{2+}$  overload in the mitochondria, increasing ROS production and impairing the mitochondrial antioxidant role, making the cell more vulnerable to apoptosis (34). This explains the decreased level of GSH in the MSG group compared to the other groups and the increased level of MDA in the MSG group relative to the MO prevention group. This aligned with a previous study, which found that MSG significantly decreased GSH and increased MDA levels in the brain tissue (35). Despite the absence of significant difference in MDA levels compared to the control in this study, it is essential to consider the broader context of MSG-induced neurotoxicity. Other potential mechanisms like glutamate excitotoxicity or neuroinflammation could still be involved.

Several components of MO can pass through the blood-brain barrier, with  $\beta$ -carotene as a major component. Thus, they positively affect the neuronal system (36; 13). MO is known for its antioxidant properties; it prevents the generation of ROS and blocks the produced free radicals. MO

can work enzymatically and non-enzymatically. MO activates an enzymatic defense system of antioxidants, increasing the activity of glutathione peroxidase, catalase, and superoxide dismutase. The non-enzymatic effect of MO is due to the presence of reduced thiol, lipo-, and hydro-soluble metabolic compounds. Additionally,  $\beta$ -carotene removes singlet oxygen and enhances its antioxidant properties (13;37). Also, through the Nrf2-Keap1 pathway, Nrf2 migrates to the nucleus and initiates ARE-dependent gene expression. Which lead to antioxidant enzymes production, including those involved in synthesizing and conjugating glutathione (13), thus ultimately increasing GSH level.

GSH is a small molecular weight antioxidant, a category of compounds that hinder chain reactions propagation and biomolecules oxidation by directly reacting with the free radicals (38). Uncontrolled oxidative stress causes direct damage to lipids by a process known as lipid peroxidation, which produces different oxidation products; one of them is MDA, which has a cytotoxic effect and promotes cell death (39).

The current study revealed that MO prevention and treatment groups significantly increased GSH level compared to the MSG group, indicating that MO played a role through the antioxidant GSH. This observation aligns with the study by Karimah et al. (12) who found that MO increased GSH levels in PCs in mice administered with methylmercury. Interestingly, we found that the increase in GSH level was more significant in MO treatment rats, and there was also a significant increase in GSH level in MO treatment relative to the prevention therapy. This finding could imply that MO acts more effectively to restore reduced GSH levels

after MSG has initiated oxidative stress, which needs further investigation.

We also found that MO prevention therapy significantly reduced MDA level relative to the MSG group, indicating the effect of MO prevention therapy in reducing lipid peroxidation and protecting the cell from the cytotoxic effect of MDA. This result is consistent with Omotoso et al.'s observation that MO administration reduced MDA levels in the nicotine-induced cerebellar degeneration model (10).

Interestingly, we found that MDA levels were significantly reduced in the MO prevention rats compared to the MO treatment rats. It highlights the potential effect of MO prevention therapy in alleviating oxidative damage before it occurs. However, we didn't observe a significant change in the MDA level in the MO treatment group compared to the MSG group. A previous study noted that MO decreased MDA levels the pre- and post-treatment as well. Yet, they reported that pre-treatment with MO showed better outcomes than post-treatment in reducing oxidative stress in the testicular tissue of cadmium-exposed rats (40).

To further investigate MO's possible underlying neuroprotective mechanism, we performed an immunohistochemical analysis of Nrf2 expression. Our data demonstrated a significant reduction in the mean gray value% of Nrf2 expression and the percentage of PCs with nuclear Nrf2 expression in ataxic rats compared to the control. We also observed a significant elevation in the mean gray value% of Nrf2 expression and the percentage of PCs with nuclear Nrf2 expression in MO prevention and treatment groups compared to the MSG group. Notably, the elevation in the mean

gray value% of Nrf2 expression was more significant in the MO prevention group.

Khan et al. (13) reported that one of the mechanisms of the neuroprotective role of MO is its influence on the Nrf2/ Keap1 pathway. MO affects the Nrf2/ Keap1 pathway in two ways; MO was found to potentiate Nrf2 dissociation from Keap1 and its nuclear translocation to activate ARE-dependent gene expression. MO was also reported to reduce Keap1 expression, negatively regulating Nrf2 activity through ubiquitination, which results in the accumulation of newly formed Nrf2 and its activation as well (41).

We can say that MO possibly had a neuroprotective effect on PCs by increasing Nrf2 translocation to the nucleus, which is confirmed by the increased percentage of PCs with nuclear Nrf2 expression in MO prevention and treatment rats compared to the ataxic rats. MO also possibly decreased the negative regulation of Nrf2 by Keap1, which led to the accumulation of newly synthesized Nrf2; this is suggested by the increase of the overall expression of Nrf2 indicated by increased mean gray value% of Nrf2 expression in MO prevention and treatment groups.

The increase in the mean gray value% of Nrf2 expression was more noticeable in the MO prevention group, suggesting that the prevention therapy had more effect on decreasing the negative regulation and ubiquitination of Nrf2 by Keap1; this interesting observation needs further research.

The neuroprotective effect of MO was documented in previous studies (42), yet to our knowledge, its positive impact on Nrf2 expression in PCs of the cerebellar cortex is a novel finding.

To dig deeper into the impacts of MO, we carried out a correlation analysis. We noticed a significant

positive correlation between the log distance crossed in the open field and the percentage of PCs with nuclear Nrf2 expression and between the log distance and GSH level. Furthermore, we found a significant negative correlation between the immobility time in the open field and the PC linear density, as well as between immobility time and GSH level. Suggesting the possible role of increased survival of PCs, increased Nrf2 expression in the nuclei of PCs, and increased level of the antioxidant GSH in cerebellar tissue in alleviating motor behavioral disturbances in the open field.

Additionally, the correlation analysis revealed a significant positive correlation between the PC linear density, the percentage of PCs with nuclear Nrf2 expression, and the mean gray value % of Nrf2 expression. This indicates that Nrf2 activation may have played a role in preventing PCs death.

Furthermore, we found a significant negative correlation between MDA level in cerebellar tissue and PC linear density as well as the percentage of PCs with nuclear Nrf2 expression. This highlights the possible protective role of MO. As the decreased MDA level in the MO prevention group was associated with the highest survival rate in PCs, even more than in the control group. It also suggests the possible involvement of Nrf2 activation in the mechanisms of reducing the oxidative marker MDA.

Therapeutic options for CA remain restricted, and no cure has been detected (43). Even if the etiology of CAs varies, several common paths may exist in disordered cellular activities, creating opportunities for novel therapies (44). Thus, targeting several pathways at once may be

therapeutically necessary to protect neuronal integrity and stop neurodegeneration (6).

During the preceding decade, growing evidence indicates using medicinal plants and their natural compounds as antioxidant treatments (37). Moreover, the global economic collapse, especially in African countries, will probably encourage more individuals to adopt herbal medicine (45). Delivering antioxidant therapies to the site of free radical production is one of the challenging parts in developing neuroprotective therapies. A previous study stated that several components of MO can cross the blood-brain barrier. Moreover, many studies have reported the neuroprotective effects of MO through different mechanisms, including its potent antioxidant effect (36).

Our findings may open a door for developing a treatment or a prevention therapy for CA using the active constituents of MO. Still, our study has some limitations as we didn't study other possible protective pathways of MO, such as the ubiquitin-proteasome pathway or the potent anti-inflammatory activity of MO by inhibiting NF- $\kappa$ B and PI3K/Akt pathways. We hope we can cover these points in future studies.

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#### **Conflict of interest**

All authors affirm the absence of any conflict of interest in this study.

#### **Ethics approval**

The Mansoura Faculty of Medicine IRB Committee approved this study (Code MS.22.04.1949).

#### **References**

1. **Manto M, Gandini J, Feil K, Strupp M.** Cerebellar ataxias: An update. *Curr Opin Neurol.* 2020;33(1):150–60.
2. **Barca E, Emmanuele V, DiMauro S, Toscano A, Quinzii CM.** Antioxidant Drugs: Novelties and Clinical Implications in Cerebellar Ataxias. *Curr Neuropharmacol.* 2018;17(1):21–32.
3. **Balaei MR, Ghiyamihoor F, Rad AA, Ashtari N, Toback M, Bergen H, et al.** Contemporary Clinical Neuroscience Development of the Cerebellum from Molecular Aspects to Diseases Second Edition [Internet]. 2023 [cited 2024 May 3]. Available from:[https://link.springer.com/chapter/10.1007/978-3-031-23104-9\\_2](https://link.springer.com/chapter/10.1007/978-3-031-23104-9_2)
4. **Farghaly WM, El-Tallawy HN, Rageh TA, Shehata GA, Metwally NA, Abo-Elfetoh NM.** Epidemiology of Cerebellar Ataxia in Al-Kharga district-new Valley (Egypt). *Egyptian Journal of Neurology, Psychiatry and Neurosurgery.* 2010;47(3):527–32.
5. **Manto M, Marmolino D.** Cerebellar ataxias. *Curr Opin Neurol.* 2009;22(4):419–29.
6. **Dueñas AM, Goold R, Giunti P.** Molecular pathogenesis of spinocerebellar ataxias. *Brain.* 2006;129(6):1357–70.

7. **Chien HF, Zonta MB, Chen J, Diaferia G, Viana CF, Teive HAG, et al.** Rehabilitation in patients with cerebellar ataxias. Vol. 80, Arquivos de Neuro-Psiquiatria. Associacao Arquivos de Neuro-Psiquiatria; 2022. p. 306–15.
8. **Marmolino D, Manto M.** Past, Present and Future Therapeutics for Cerebellar Ataxias. *Curr Neuropharmacol.* 2010;8(1):41–61.
9. **Prastiwi D, Djunaidi A, Partadiredja G.** High dosage of monosodium glutamate causes deficits of the motor coordination and the number of cerebellar Purkinje cells of rats. *Hum Exp Toxicol.* 2015;34(11):1171–9.
10. **Omotoso GO, Gbadamosi IT, Olajide OJ, Dada-Habeeb SO, Arogundade TT, Yawson EO. Moringa oleifera** phytochemicals protect the brain against experimental nicotine-induced neurobehavioral disturbances and cerebellar degeneration. *Pathophysiology [Internet].* 2018;25(1):57–62. Available from: <http://dx.doi.org/10.1016/j.pathophys.2017.12.003>
11. **Saini RK, Sivanesan I, Keum YS.** Phytochemicals of *Moringa oleifera*: a review of their nutritional, therapeutic and industrial significance. *3 Biotech.* 2016;6(2).
12. **Karimah A, Dyah N, Lastuti R, Kuncorojakti S.** Effect of *Moringa oleifera* Leaf Extract on the Percentage of Necrotic Purkinje Cells in Mice ( *Mus musculus* ) Induced by Methylmercury. 2017;(November 2016).
13. **Khan MF, Yadav S, Banerjee S.** Review Article on Effects of *Moringa* on Central Nervous System. *Journal of Young Pharmacists.* 2021;13(4):315–9.
14. **El-Hefnawy MA, Yehia A, Nashar EM El, Saad S, Obydah W, Alghamdi MA, et al.** Effect of vanillic acid on pentylenetetrazole-kindled rats: Nrf2/HO-1, IGF-1 signaling pathways cross talk. *J Integr Neurosci.* 2022;21(1):015.
15. **Charan J, Kantharia N.** How to calculate sample size in animal studies? Vol. 4, *Journal of Pharmacology and Pharmacotherapeutics.* 2013. p. 303–6.
16. **Aidaros AE mawla E sayed, Ibrahim AAS, Mohammed HO, Hassan NH.** Effect of monosodium glutamate on the cerebellar cortex of male albino rat and protective role of vitamin C. *Zagazig University Medical Journal [Internet].* 2019 Mar;25(2):250–60. Available from: [www.zumj.journals.ekb.eg](http://www.zumj.journals.ekb.eg)
17. **Deacon RMJ.** Housing, husbandry and handling of rodents for behavioral experiments. *Nat Protoc.* 2006;1(2):936–46.
18. **Mann A, Chesselet MF.** Techniques for Motor Assessment in Rodents [Internet]. Second Edi. *Movement Disorders: Genetics and Models: Second Edition.* Elsevier Inc.; 2015. 139–157 p. Available from: <http://dx.doi.org/10.1016/B978-0-12-405195-9.00008-1>
19. **Deacon RMJ.** Measuring motor coordination in mice. *J Vis Exp.* 2013;(75):1–8.
20. **Hussein AM, Adel M, El-Mesery M, Abbas KM, Ali AN, Abulseoud OA.** L-carnitine modulates epileptic seizures in pentylenetetrazole-kindled rats via suppression of apoptosis and Autophagy and



- Upregulation of Hsp70. *Brain Sci.* 2018 Mar 1;8(3).
- 21. Shan chong pit, Khairuddin S, Chung Kwan Tse A, Fhung Hiew L, Lok Lau C, Lim Tipoe G, et al.** Hericium erinaceus potentially rescues behavioural motor deficits through ERK-CREB-PSD95 neuroprotective mechanisms in rat model of 3-acetylpyridine-induced cerebellar ataxia. 2020;10:14945. Available from: <https://doi.org/10.1038/s41598-020-71966-z>
- 22. El Nashar EM, Obydah W, Alghamdi MA, Saad S, Yehia A, Maryoud A, et al.** Effects of Stevia rebaudiana Bertoni extracts in the rat model of epilepsy induced by pentylenetetrazol: Sirt-1, at the crossroads between inflammation and apoptosis. *J Integr Neurosci.* 2022 Jan 1;21(1).
- 23. Chong PS, Khairuddin S, Tse ACK, Hiew LF, Lau CL, Tipoe GL, et al.** Hericium erinaceus potentially rescues behavioural motor deficits through ERK-CREB-PSD95 neuroprotective mechanisms in rat model of 3-acetylpyridine-induced cerebellar ataxia. *Sci Rep* [Internet]. 2020;10(1):1–18. Available from: <https://doi.org/10.1038/s41598-020-71966-z>
- 24. Moussa AT, Singh B, Al-Dissi AN.** Immunohistochemical expression of nuclear factor erythroid-2-related factor 2 and heme oxygenase 1 in normal bovine lung and bovine lung infected with *Mannheimia haemolytica*. *Canadian Journal of Veterinary Research.* 2015;79(2):81–6.
- 25. Crowe A, Yue W.** Semi-quantitative Determination of Protein Expression Using Immunohistochemistry Staining and Analysis: An Integrated Protocol. *Bio Protoc.* 2019;9(24).
- 26. Att R, Ameen A, Korayem H, Abogresha N, El-Wazir Y.** Adipose tissue-derived mesenchymal stem cells have better restorative capacity than bone marrow-derived cells in a cerebellar ataxic rat model. *Archives of Medical Science.* 2020 Nov 13;
- 27. Onaolapo OJ, Aremu OS, Onaolapo AY.** Monosodium glutamate-associated alterations in open field, anxiety-related and conditioned place preference behaviours in mice. *Naunyn-Schmiedeberg's Arch Pharmacol.* 2017;390(7):677–89.
- 28. Kardeşler AÇ, Başkale E.** Investigation of the behavioral and neurochemical effects of monosodium glutamate on neonatal rats. *Turk J Med Sci.* 2017;47(3):1002–11.
- 29. Hassaan PS, Dief AE, Zeitoun TM, Baraka AM, Deacon RMJ, Elshorbagy A.** Cortical tau burden and behavioural dysfunctions in mice exposed to monosodium glutamate in early life. *PLoS One.* 2019;14(8):1–14.
- 30. Wecker L, Engberg ME, Philpot RM, Lambert CS, Kang CW, Antilla JC, et al.** Neuronal nicotinic receptor agonists improve gait and balance in olivocerebellar ataxia. *Neuropharmacology.* 2013;73:75–86.
- 31. Jones TA.** Motor compensation and its effects on neural reorganization after stroke. Vol. 18, *Nature Reviews Neuroscience.* Nature Publishing Group; 2017. p. 267–80.
- 32. Cook AA, Fields E, Watt AJ.** Losing the Beat: Contribution of Purkinje Cell Firing Dysfunction to Disease, and Its Reversal.

- Vol. 462, Neuroscience. Elsevier Ltd; 2021. p. 247–61.
- 33. Omotoso GO, Gbadamosi IT, Afolabi TT, Abdulwahab AB, Akinlolu AA.** Ameliorative effects of Moringa on cuprizone-induced memory decline in rat model of multiple sclerosis. *Anat Cell Biol.* 2018;51(2):119–27.
- 34. Farhat F, Nofal S, Raafat EM, Ali A, Ahmed E.** MONOSODIUM GLUTAMATE SAFETY, NEUROTOXICITY AND SOME RECENT STUDIES. Vol. 64, *J. Pharm Sci.* 2021.
- 35. Umukoro S, Oluwole GO, Olamijowon HE, Omogbiya AI, Eduviere AT.** Effect of Monosodium Glutamate on Behavioral Phenotypes, Biomarkers of Oxidative Stress in Brain Tissues and Liver Enzymes in Mice. *World J Neurosci.* 2015;05(05):339–49.
- 36. Srivastava G, Ganjewala D.** An update on the emerging neuroprotective potential of Moringa oleifera and its prospects in complimentary neurotherapy. Vol. 4, *Phytomedicine Plus.* Elsevier BV; 2024.
- 37. Enogieru AB, Momodu OI.** The Developing Cerebellum as a Target for Toxic Substances: Protective Role of Antioxidants. *The Cerebellum* [Internet]. 2021;20:614–30. Available from: <https://doi.org/10.1007/s12311-021-01231-0>
- 38. Turrens JF.** Superoxide Dismutase and Catalase. *Comprehensive Toxicology*, Second Edition. 2010 Aug 12;4:219–27.
- 39. Ayala A, Muñoz MF, Argüelles S.** Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Vol. 2014, *Oxidative Medicine and Cellular Longevity.* Landes Bioscience; 2014.
- 40. Chatterjee PK, Anantharaya VNM, Shiva RK, Kumar NA, Shetty SB, Budihal SV, et al.** Pre and post-Treatment effects: Estimation of serum testosterone and lipid peroxidation levels on Moringa olifera extract induced cadmium exposed rats. *Pharmacognosy Journal.* 2014;9(6):846–9.
- 41. Bellezza I, Giambanco I, Minellia A, Donato R.** Nrf2-Keap1 signaling in oxidative and reductive stress. *Biochim Biophys Acta Mol Cell Res* [Internet]. 2018;1865(5):721–33. Available from: <https://doi.org/10.1016/j.bbamcr.2018.02.010>
- 42. Chen P, Li L, Gao Y, Xie Z, Zhang Y, Pan Z, et al.**  $\beta$ -carotene provides neuroprotection after experimental traumatic brain injury via the Nrf2-ARE pathway. *J Integr Neurosci.* 2019 Jun 1;18(2):153–61.
- 43. Genki H.** Oxidative stress :pathomechanism and biomarker in Friedreich' s ataxia [DISSERTATION]. [Irvine]: University of California; 2009.
- 44. Mitoma H, Manto M.** The Era of Cerebellar Therapy. *Curr Neuropharmacol.* 2018;17(1):3–6.
- 45. Igado OO, Olopade JO.** A review on the possible neuroprotective effects of Moringa oleifera leaf extract. *Nigerian Journal of Physiological Sciences.* 2016;31(2):183–7.