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Research Article
Miracle Tree (*Moringa oleifera*) Attuned GFAP and Synaptophysin Levels, Oxidative Stress and Biomarkers in Cerebellar Fluorosis of Pregnant Rats

[1]Amira El-Helaly, [1]Amoura M. Abou-El-Naga, [2]Khulud M. Alshehri and [1]Mai Alaa El-Dein

[1]Department of Zoology, Faculty of Science, Mansoura University, Mansoura 35561, Egypt
[2]Department of Biology, Al-Baha University, Al Bahah 23334, Saudi Arabia

Abstract

Background and Objective: Cerebellar fluorosis is a health issue associated with excessive exposure to fluoride (F) either in direct or indirect ways as pesticides, drinking water and caries preventing prescriptions. It is characterized by elevation in oxidative stress, inflammation, demyelination and Purkinje cell loss. *Moringa oleifera* (M), is a widely cultivated plant used as a health-booster agent in modulating various disorders because of its high content of vitamins and minerals. The beneficial effect of moringa against fluoride-induced cerebellar toxicity in pregnant rats was investigated in this study. **Materials and Methods:** Twenty pregnant rats were administered daily 300 mg kg^{-1} *M. oleifera* aqueous extract incorporated with 10 mg kg^{-1} of F intoxication from the 1st day of gestation until the 20th day. Following the termination of the trial, sera were collected and cerebellar tissue was removed for further examinations, along with the assessment of maternity. **Results:** The *M. oleifera* significantly normalized serum FSH, LH, progesterone, dopamine and serotonin levels of F-intoxicated mothers. Additionally, *M. oleifera* markedly prevented the lipid peroxidation and DNA fragmentation indicated by the tail length and moment in comet assay (-34.4 and -75.3%, respectively, when compared to the fluoride intoxicated group), while sustaining the levels of SOD and CAT revealing its antioxidant activity. The *M. oleifera* regressed the cerebellar α-amylase (-25.4%) and acetylcholinesterase activity (-40.6%), also attenuated GFAP (-73.4%, $p<0.0001$), synaptophysin level (216.6%, $p<0.0001$) and IL-6 expression (-91.2%) comparing to fluoride only treated mothers. **Conclusion:** Histological and ultrastructural examinations confirmed the recuperating effects of *M. oleifera* on mothers' cerebellar tissue intoxicated with fluoride indicated by intact folia and restored Purkinje cells number and architecture. The maternal study emphasized the anti-abortifacient activity of moringa against fluoride induced-fetotoxicity.

Key words: Cerebellar fluorosis, GFAP, synaptophysin, folia, Purkinje cells, fetotoxicity

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Corresponding Author: Mai Alaa El-Dein, Department of Zoology, Faculty of Science, University of Mansoura, Mansoura 35516, Egypt Tel: 00201111886616

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In homes, fields, on crops and in shipping containers, fluorinated pesticides as sodium fluoride and sulfuryl fluoride (SO_2F_2) are frequently employed as a fumigant pesticide and strong greenhouse gas despite their serious risk to humans[1]. Fluorine, the 13th most abundant element in the earth's crust, is widely released into the ecosystem naturally via volcanic emissions or industrially via minerals and fertilizers manufacturing, fungicides and rodenticides. That is why it is widely recognized that fluoride is an important natural and industrial ecological pollutant. Despite low levels of fluoride triggers healthy bone growth and plays an essential role in dental health by preventing dental caries and tooth decay[2].

Fluoride is present in foods, plants, tea, toothpaste and other dental and pharmaceutical items used by humans. Consuming tainted water is the main route of exposure to fluoride[3]. High levels of fluoride exposure are detrimental to human health because they can cause fluorosis, a condition that is poisonous and debilitating[4]. The concentration, duration and tissue susceptibility of an environmental contaminant's harmful effects depends on the exposure to that pollutant[5].

The common toxic mechanism of all toxic metals is through oxidative stress that leads to the production of lipid peroxidation (LPO), secondary to provoking of free radicals in the tissues. So, reactive oxygen species (ROS), which are beneficial in adequate amounts, result in damage to various biomolecules like DNA, proteins and membrane lipids, enzymes, in addition to simultaneously impairing the antioxidant defense system[6]. Fluoride may have a deleterious impact on cytokines because they are crucial in controlling immune response and cell proliferation. Recent studies showed that they play a role in the inflammatory response as well as the development and progression of tumors through proliferation, invasion, matrix destruction and ultimately apoptosis[7,8]. Moreover, fluoride can activate c-Jun N-terminal kinase (JNK) pathway by upregulating the P-$MEK_{4/7}$, JNK mRNA as well as protein expression level[9]. The JNK pathway is responsible for regulating the synthesis of proinflammatory cytokines, including IL-6, IL-2 and TNF-α[10]. Furthermore, fluoride can activate both normal and necrotic cell death via triggering Fas Ligand (FasL)/Fas signaling pathway in addition to Tumor Necrosis Factor-α and its Receptor (TNF-R1)[11].

Maternal fluoride exposure threatens fetal advancement[5], during embryonic development, the F has a very sensitive influence on the central nervous system due to its inadequate protective mechanisms[12]. Fluoride causes abnormal menstruation and elevates pregnancy complications, teratogenicity and frequency of miscarriages[13]. Neurodegeneration takes place either due to pathological conditions or ecological toxins that have impacts on the nervous tissue constituents and they are typically debilitating and incurable and often lead to ataxia, dementia or any other cognitive incapacity[14]. The neurotoxicological consequences of F in the brain of rats encompass oxidative stress, DNA harm, disruption of protein and neurotransmitter levels, as well as modifications in the activities of specific enzymes[15,16].

Traditional medicine (herbal medicine) is used nowadays as an alternative remedy as the use of plants and plant extracts improves human health. A wide array of botanical species has been employed in the treatment of cognitive disorders, encompassing neurodegenerative diseases and various neuropathological conditions. Therefore, herbal medicine has gained great attention and is recommended as a biological alternative with limited adverse effects to maintain one's health[17].

Moringa oleifera (M) which belongs to the Moringaceae family, is considered one of the most well-known among approximately 13 different species of moringa[18]. It has been experienced as antioxidant, cardiovascular enhancer, anti-cancer, antimicrobial, anti-inflammatory, anti-hepatotoxic, anti-urinary[19] and anti-neurotoxic properties[20]. The neuroprotective potential of *M. oleifera* may be due to its ability to eliminate oxidative stress and enhance the cholinergic function[21]. The *M. oleifera* functions as a free radical scavenger within the central nervous system, hence enhancing the endogenous antioxidant system[22]. Additionally, it contains numerous elements that are essential for life and play a part in immune system development, oxygen transport, enzyme activation and stimulation of nerve impulses. The *M. oleifera* is renowned for being a strong source of protein and abundant minerals, particularly iron and zinc[23]. The *M. oleifera* with other antioxidants can diminish reactive oxygen species.

This investigation was done to detect the ameliorative influence of moringa on mothers' cerebellar fluorosis biologically, inflammatory, hormonally, enzymatically, pathologically, immunohistochemically, ultra-structurally and maternally.

MATERIALS AND METHODS

Study area: This study was conducted at the Animal House and Laboratory Facilities of the Faculty of Science, Mansoura University, Mansoura, Egypt during the period from March, 2022 to May, 2023.

Chemicals: Sodium fluoride was obtained from Sigma (Sigma- Aldrich Co., St. Louis, Missouri, USA).

Moringa leaves aqueous extract preparation: Early in the morning, fresh leaves of *M. oleifera* were picked from gardens in the Faculty of Agriculture, Mansoura University, Egypt. Using distilled water, *M. oleifera* leaves were meticulously separated from the stem, thoroughly cleansed and rid of any sand or other impurities. Using an electric kitchen blender, the powder was created from the fresh leaves. A 250 mL conical flask was used for mixing 100 mL of distilled water with 5 g of finely pulverized *M. oleifera* leaves. The quantity of plant leaves extracted is 320 g per 5.6 L of distilled water. The flask contents were located on a shaker at 70°C for 20 min. Whatman filter paper No. 1 (125 mm, Cat. No. 1001 125, Germany) was used to filter the mixture after it had been extensively homogenized and sieved with a large size of Whatman filter paper (24 cm). Following extraction in the oven at 70°C, the extracted components were concentrated to a predetermined volume. Until it was used, the filtrate that resulted was kept in the freezer (-20°C)[24].

Experimental animals: Rats used in this study were obtained from The Egyptian Vaccine Company (VACSERA, Giza, Egypt), which were housed in the Mansoura University, Faculty of Science, experimental animal center. Animals were placed in metallic containers containing shavings as bedding material and exposed to alternating 12 hrs periods of light and darkness, while maintaining a somewhat stable temperature (23±2°C) and humidity (50%) supplied with enough food and drink. In this study, ten male rats and twenty virgins, sexually mature female rats weighing between 150 and 180 g each were employed.

Experimental design: After an acclimatization period, both female and male rats were housed together overnight for mating, each three virgin female rats with one fertile male in separate cages with (3:1) ratio. Next day morning, the presence of vaginal plug or sperms in the vaginal smears was considered the first day of pregnancy, as shown in Fig. 1a afterward, pregnant rats were randomly divided into 4 groups of 5 animals as follows:

- **Control group (C):** Pregnant rats were fed on a standard diet and water and kept for 20 days of gestation
- **Moringa extract treated group (M):** Pregnant rats were orally supplemented with moringa extract in a dose of 300 mg kg^{-1} daily starting from the first day till the 20th day of gestation
- **Fluoride treated group (F):** Pregnant rats received daily oral doses of sodium fluoride (10 mg kg^{-1}) starting from the first day till the 20th day of gestation
- **Combined group (M+F):** Pregnant rats treated daily with both sodium fluoride (10 mg kg^{-1}) combined with moringa leaf extract at a dose of 300 mg kg^{-1} from the first day till the 20th day of gestation

Samples collection: Rats were restrained overnight on their 20th gestational day and sacrificed 24 hrs after the last treatment. Clean centrifuge tubes were used to collect blood samples, which were then allowed to clot before being centrifuged at 1500 rpm for 15 min at 4°C to extract sera. Subsequently, the sera were promptly frozen at a temperature of -20°C to facilitate subsequent biochemical investigation. While this was going on, mothers' cerebellar tissues were divided, weighed and a portion of them were homogenized in a glass-Teflon homogenizer with ice-cold 0.01 M Tris-HCl buffer (pH 7.4). After centrifuging the homogenates at 1500 rpm, the supernatant was stored at -20°C until biochemical tests. Other areas of the cerebellum, however, were retained for future investigations and fixed in neutral formalin (10%) or glutaraldehyde (4%) for histological and ultrastructural analyses.

Estimation of FSH, LH and progesterone serum level: The quantitative determination of serum hormone was performed by ELISA Kit using the Double Antibody Sandwich technique with FSH, Cat. No. MBS263261, while LH, with Cat. No. MBS764675 and Progesterone, with Cat. No: MBS762170 was determined by Competitive-ELISA detection method.

Determination brain dopamine and serotonin: The levels of dopamine (DA) were assessed by employing a rat Enzyme-Linked Immunosorbent Assay (ELISA) kit obtained from CUSABIO TECHNOLOGY LLC, Houston, USA. Additionally, the quantification of serotonin levels was conducted using a kit identified by the code E-EI-0033.

Oxidative stress assessment: Using assays purchased from Biodiagnostic, Egypt, the levels of the antioxidant enzymes catalase (Catalogue # CA 2517) and superoxide dismutase (SOD with Catalogue # SD 2521) and the lipid peroxidation product malondialdehyde (MDA with Catalogue # MD 2529) were measured in the sera.

Neurodegeneration markers evaluations: The brain α-amylase (CSBEL001689RA) and acetylcholinesterase with catalog number (CSB-E11304r) were measured. The Rat Enzyme-Linked Immunosorbent Assay (ELISA) Kit, manufactured by CUSABIO TECHNOLOGY LLC in Houston, USA, was utilized.

Immunohistochemistry: The immunohistochemical investigation was assessed to identify the expression of GFAP and synaptophysin antigens as described earlier within cerebellar tissue. After being deparaffinized in xylene and rehydrated in falling ethanol concentrations. Tissue slices were antigen-retrieved in sodium citrate buffer for 10 min in a microwave at 100°C. By incubating with 3% hydrogen peroxide for 10 min at room temperature, endogenous peroxidase was suppressed. The tissue slices were treated with primary anti-synaptophysin or anti-GFAP (Thermo Fisher Scientific, Fremont, California, USA) at 4°C overnight after being blocked in 10% normal goat serum for 30 min. Rat monoclonal anti-GFAP anti-body (1:2000; Abcam; Cambridge, UK) and anti-synaptophysin anti-body (1:1000; catalogue number 701503; Millipore; Temecula, California, USA) were the main antibodies used. The main antibody, biotinylated secondary antibody and streptavidin-HRP (1:1000) were all incubated on the tissue slices for 1 hr each. The tissue slices were then exposed to streptavidin-conjugated horseradish peroxidase for 30 min at 37°C (ExtrAvidin Kit, Sigma, St. Louis, Missouri). The sections were initially subjected to hematoxylin staining, followed by the application of a diaminobenzidine (DAB, Sigma) substrate for 5 min at ambient temperature to facilitate the development of the staining.

Quantitative PCR: By the guidelines provided by the manufacturer, the Trizol reagent (Takara, Maebashi, Japan) was employed to extract the entirety of RNA from the cerebellum of a pregnant female rat. Using a NanoDrop2000 (Thermo Scientific), the quantity of RNA was calculated based on the A260/A280 ratio. The cDNA synthesis kit (Bio-Rad, Hercules, California, USA) was used to create first-strand cDNA from total RNA by the manufacturer's instructions. For PCR amplification, Ultra SYBR Mixture (2 L of cDNA in 25 L) was utilized. As 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec were performed on the PCR mixture after it had been denatured at 94°C for 2 min. The following primer sequences were employed in this study.

The IL 6 (NM_012589.2) forward: 5'-GCCCTTCAGGAA CAGCTATGA and reverse: 3'-TGTCAACAACATCAGTCCCAAGA. The following is how the relative gene expression was calculated: The 2-Ct technique was used to normalize gene expression or fold change to the housekeeping gene (β-actin) expression or fold change in the target gene[25].

Detection of DNA damage: As previously described by Giovannelli *et al.*[26], a single-cell gel electrophoresis assay (also known as a comet assay) was evaluated. Approximately 100,000 cells were immersed within a 0.65% low melting agarose matrix, which was subsequently applied onto entirely frosted microscope slides pre-coated with a layer of 0.75% normal agarose. The normal agarose was prepared using a Mg^{2+} and Ca^{2+} free phosphate-buffered saline (PBS) solution. A final layer consisting of low-melting agarose with a concentration of 0.65% was carefully added to the uppermost surface. Then put the slides in cold lysate solution containing (10% DMSO+1% Triton X-100+89% of 10 mmol L^{-1} Tris+1% sodium lauryl sarcosine+2.5 mol L^{-1} NaCl+100 mmol L^{-1} Na_2 EDTA, pH 10) for 1-2 hrs at 5°C. Afterward, incubated the slides for 15 min in an ice-cold electrophoresis chamber (model GNA-200, Pharmacia, Milan, Italy) containing (300 mmol L^{-1} NaOH+1 mmol L^{-1} Na_2EDTA, pH 12) and exposed to 25 V/300 mA for 20 min. Pre-incubation and electrophoresis were made in an ice bath. After soaking the slides for 3 to 5 min in 0.4 M Tris, pH 7.5, the DNA was stained by adding fifty microliters of ethidium bromide (20 μg mL^{-1}) to each slide. After 5 min of staining, slides were washed in distilled water and covered for microscopic examination. Under a fluorescent microscope (Olympus B-60F5) fitted with an excitation filter of 549 and 590 nm barrier filter and linked to a CCD camera (Kodak, USA) and analyzed 1000× magnification images. A total of one hundred cells were randomly chosen for analysis on each slide. The assessment of DNA damage in these cells was conducted by determining the ratio of tail DNA content to the overall cellular DNA content.

Histopathological examination: Maternal cerebellar tissue samples were fixed in 10% neutral buffered formalin (PH 7.2) for histological investigations. Sections underwent a rinsing process using a 70% alcohol solution, followed by dehydration using escalating concentrations of alcohol and were further cleaned using xylene. The specimen was sliced into sections with a microtome, resulting in a thickness of 5 m. These sections were subsequently treated with paraffin wax immersion and stained with Hematoxylin and Eosin (H&E). The field regions for each slice were then chosen and they were evaluated under a light microscope at 100 and 400× for histopathological alterations.

Ultrastructural examination: Segments of maternal cerebellar tissue from control and other treatment groups, measuring approximately 1 cubic millimeter each, were immersed in a buffered glutaraldehyde solution (pH 7.4) and fixed at a temperature of 5°C for a duration of 4 hrs. Next, the specimens underwent a triple wash using phosphate buffer, with two changes. Following this, the specimens were refixed in a 1% aqueous buffered solution of osmium tetroxide (OsO_4) at room temperature for a duration of 2 hrs. Subsequently,

the samples underwent a buffer rinse, followed by dehydration using progressively higher concentrations of alcohol. Finally, the samples were implanted at the apex of an inverted polythene beam capsule that was filled with liquid resin. The sections were sliced using an ultramicrotome into semithin sections with a thickness of 0.5 µm, as well as ultrathin sections. Toluidine blue was used to stain the semithin sections. The ultrathin sections, ranging from 50 to 90 nm in thickness, were subjected to staining using uranyl acetate and lead citrate. Subsequently, these sections were analyzed using a Transmission Electron Microscope (TEM) of the model JEOL 100 CX.

Ethical approval and permission to participate: All procedures involving the care and use of the animals in this study followed the protocol established by the Institutional Animal Ethics Committee (IAEC) of Mansoura University (SCI-Z-M-2021-68) and in compliance with the Guiding Principles for the Use of Animals in Toxicology.

Statistical evaluation: The standard error of the mean (SEM) was used to express the results (n = 5). One-way ANOVA was used to analyze the statistical differences and Tukey's *post hoc* test was used to confirm the results. When the p-value was less than 0.05, a significant difference was taken into consideration. GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, California, USA) was used for all statistical analyses.

RESULTS

Changes in body weight (BW) and organs coefficients of pregnant rats: Changes in the final BW and vital organs efficiency were examined at day 20 of gestation as shown in (Fig. 1b). All animal groups recorded comparable initial BW, at the end of the experiment, oral intake of *M. oleifera* extract insignificantly decreased the final BW (202.4±15.7 g, -5.5%) compared to the normal untreated mothers (214.2±8.6 g). Meanwhile, fluoride exposure showed a remarkable decrease in the final BW (166.4±9.1 g) with -22.3% compared to the normal group. Co-treatment with both moringa extract and fluoride salts depicted a non-marketable elevation in the final BW (207.4±8.5 g) with 24.6% compared to the fluoride-only treated group.

Changes in the vital organ weight of pregnant rats were also assessed. A nonsignificant regression in brain, cerebellum, kidney, liver and spleen weights (Fig. 1c-g, respectively) was noticed by oral dosing of moringa aqueous extract respecting to normal pregnant mothers (-11.1, -10.5, -9.1, -17.3 and -3.9%, respectively). Oral injection of fluoride salts to pregnant animals resulted in a markable depletion in weights of brain (-27.8%, $p<0.05$), cerebellum (-36.8%), kidney (-15.8%), liver (-38.2%, $p<0.01$), while elevated spleen weight (57.7%) comparing to normal group. Introducing moringa extract to fluoride treatment elevated the weight of brains, cerebella, kidneys and livers (25.4, 33.3, 12.9 and 41.7%, respectively), meantime decreased spleens weight (-31.7%) respecting to fluoride treated mothers.

Moringa normalized FSH, LH and progesterone (P) concentrations in mothers' sera: The administration of moringa extract to normal pregnant rats unremarkably depleted FSH level (-8.7%), P level (-6.78%), while elevated LH level (+0.4%), for normal untreated pregnant rats. A significant regression in serum levels of FSH, LH and P (-26.1%, $p<0.05$; -25.3%, $p<0.01$ and -29.4, $p<0.0001$, respectively) was noticed in F treated group concerning C group. Meanwhile, supplementation of *M. oleifera* extract together with F intoxication recorded a marked elevation in FSH level (21.8%) and a high significant upgrading in LH level ($p<0.0001$, 48.7%) and progesterone level ($p<0.0001$, 45.6%) comparable to F-treated groups (Fig. 2a-c).

Moringa attenuated serum dopamine and serotonin levels: Interestingly, treatment with *M. oleifera* aqueous extract remarkably elevated serum dopamine (111.5%) and serotonin levels (39.2%) as compared to control untreated pregnant rats. But oral administration of F salt significantly regressed serum levels of both dopamine ($p<0.05$, -186.7%) and serotonin ($p<0.01$, -184.3%) respecting to *M. oleifera* extract treated animals and (-26.1 and -51%), respectively comparing to control animals. On the other hand, rats supplemented with moringa extract together with F exhibit a non-significant increase in dopamine and serotonin serum levels (77.1 and 91.1%), respectively when compared with F alone treated animals (Fig. 2d-e).

Cerebellar α-amylase and acetylcholinesterase (AChE) levels: Figure 2f-g depicted that treatment with *M. oleifera* extract unremarkably depleted α-amylase level, while elevated AChE level in mothers' cerebellar tissue (-12.96 and 47.9%), respectively when compared to normal mothers). At the same time oral administration of F salt insignificantly elevated tissue α-amylase level respecting to C group (17.7%) and all other experimental groups, meanwhile significantly elevated AChE concentration (127.8%, $p<0.05$ respecting the normal mothers). Combined injection of moringa extract together with fluoride salt decreased both α-amylase (-25.4%) and AChE tissue level (-40.6%) compared to fluoride treated group.

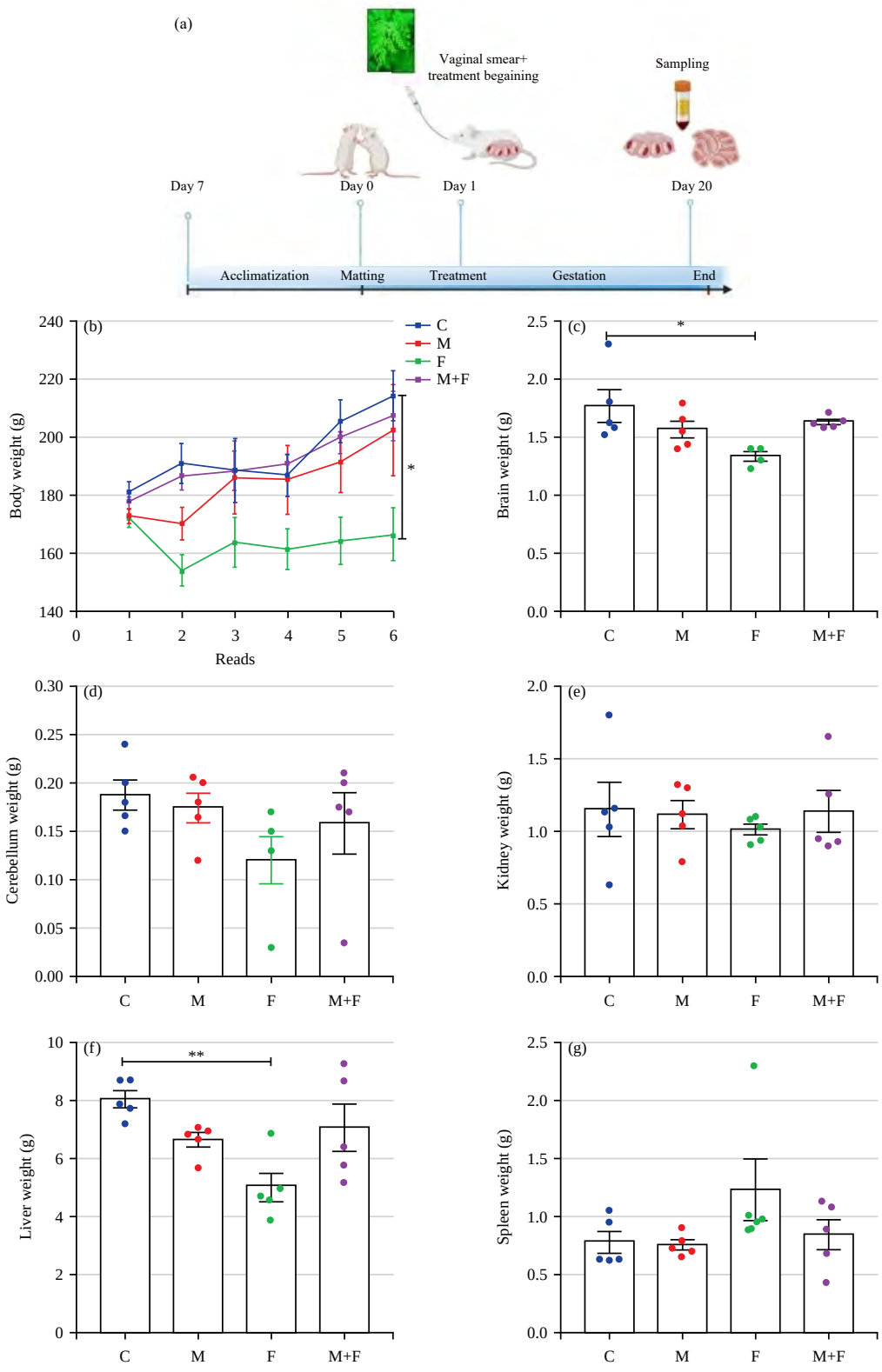

Fig. 1(a-g): Ischemic illustration of experimental timeline, (a) Effect of fluoride and\or moringa on BW of mothers in different groups, (b) Effect of fluoride and\or moringa on vital organs weight of mothers in experimental groups; (c) Brain, (d) Cerebellum, (e) kidney, (f) Liver and (g) Spleen

*p<0.05 and **p<0.01 with respect to control (n = 5)

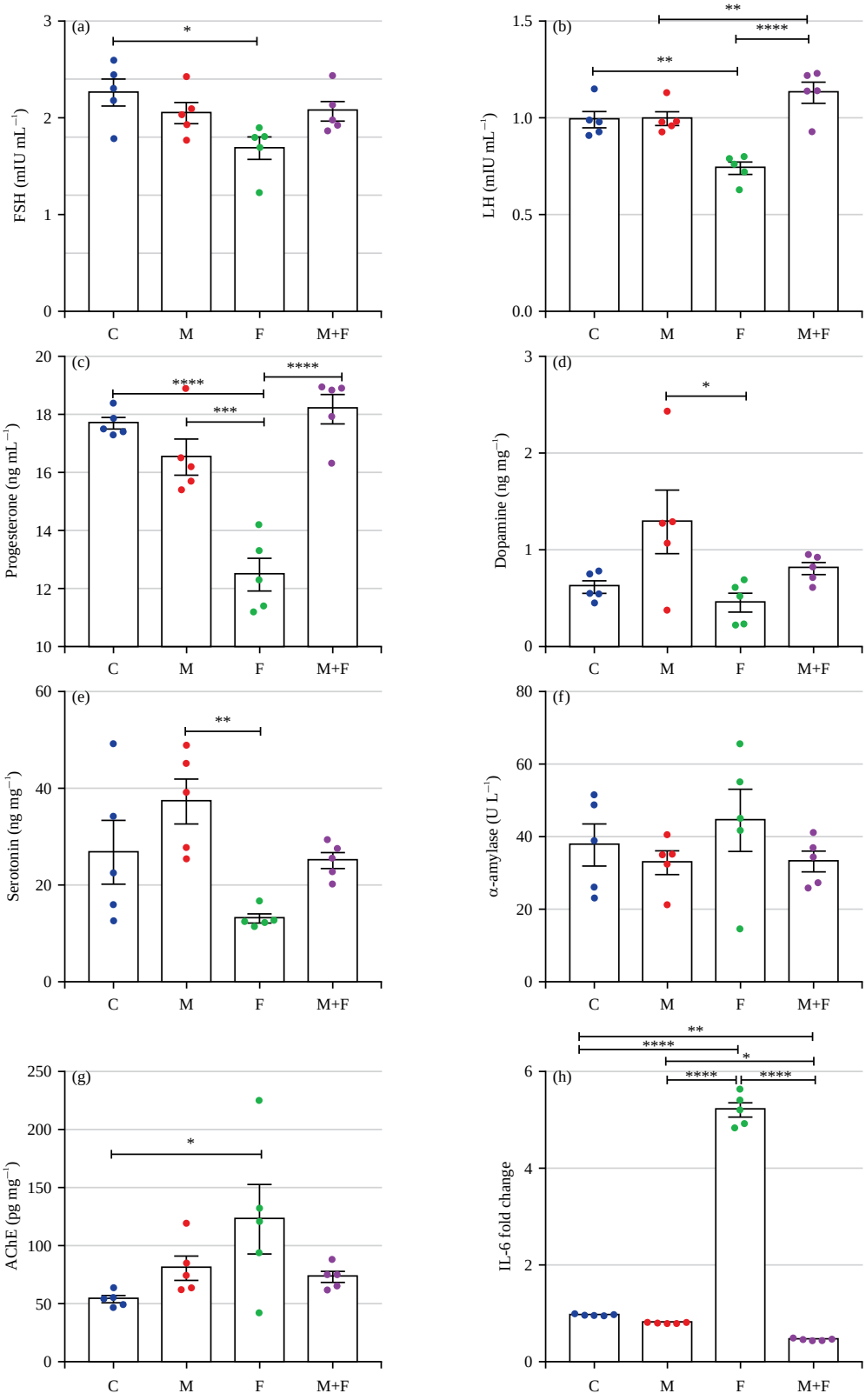

Fig. 2(a-h): Effect of F and\or M on; (a) FSH serum level, (b) LH serum level, (c) Progesterone serum level of mothers, (d) Dopamine serum level, (e) Serotonin serum level, (f) Acetylcholinesterase (AChE) cerebellar level, (g) α-amylase cerebellar level and (h) IL-6 fold change

*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 with respect to control (n = 5)

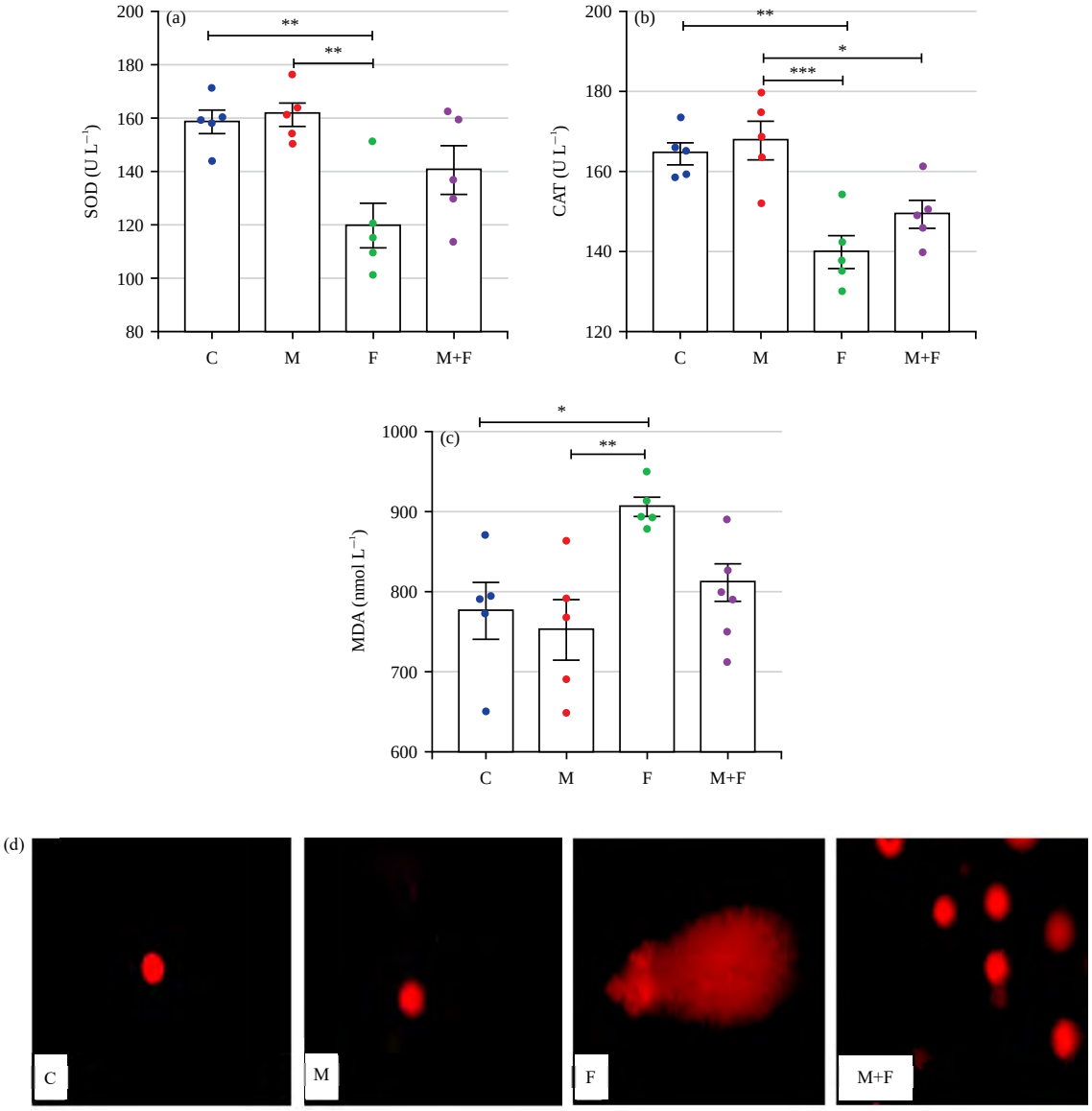

Fig. 3(a-d): Effect of fluoride and\or moringa on (a) Serum SOD (U L^{-1}), (b) Serum CAT (U L^{-1}), (c) Serum MDA (nmol L^{-1}) of mothers in different groups and (d) Representative photomicrographs demonstrating DNA damage in mother's cerebellar tissues using comet assay

C: Control group, M: Moringa aqueous extract group, F: Fluoride group, M+F: Moringa together with fluoride group, *p<0.05, **p<0.01 and ***p<0.001 with respect to control (n = 5)

Moringa ameliorated cerebellar IL-6 level: Fluoride salt administration significantly elevated IL-6 level (p<0.0001) respecting all other different groups. Meanwhile, supplementation of *M. oleifera* extract significantly regressed IL-6 level (-84.6%) concerning fluoride intoxicated animals. At the same time, combined treatment with both moringa and fluoride significantly diminished IL-6 level (-91.2%) respecting to fluoride treated group (Fig. 2h).

Moringa extract ameliorated oxidative stress and DNA damage: The level of SOD and CAT in serum was significantly reduced after administering moringa (p<0.01) compared to the control group. While administration of moringa extract accompanied with fluoride markedly improved serum SOD and CAT levels (17.4 and 6.7%), respectively compared to fluoride only receiving group as shown in Fig. 3a-b. Serum MDA level exhibits a remarkable increase in the F group

Table 1: Comet assay parameters obtained by image analysis in mother's cerebellar cells of the different groups

Group	Control	Moringa	Fluoride	Moringa+Fluoride
DNA in tail (%)	2.9±0.47	3.56±0.41	15.29±0.76^{A,B}	5.86±0.52^{a,C,D}
Tail length (μm)	1.132±0.09	1.31±0.13	3.197±0.16^{A,B}	2.1±0.18 ^{A,b,D}
Tail moment (Unit)	2.94±0.49	4.26±0.56	47.02±2.96^{A,B}	11.63±1.43 ^{b,C,D}

Each value represents the Mean±SE of 50 nuclei in each group, ^{A,a}Significantly different from the control group at 0.0001 and 0.001 level, respectively, ^{B,b,C}Significantly different from the M group at 0.0001, 0.001 and 0.05 level, respectively and ^{D} Significantly different from the F group at 0.0001 level

compared to both the control and moringa-treated group (14.4%, p<0.05; 16.9%, p<0.01, respectively). At the same time, a combined approach of both moringa and fluoride insignificantly minimized MDA level (-10.5%) respecting to fluoride treated group (Fig. 3c). No statistical difference was recorded between the control and the moringa treated group.

The extent of DNA damage in cerebellar cells of the different groups was assessed using single-cell gel electrophoresis Fig. 3d and Table 1. The cerebellar cells of mothers exposed to fluoride salt showed a significant increase in DNA fragmentation as reflected by the measured tail length and tail moment, compared to the control group. This damage represents a 182.4 and 1500% increase, respectively of the control group. Conversely, cerebellar tissue of pregnant rats treated with moringa aqueous extract together with fluoride showed a less elevation in tail length and tail moment by only 81.36 and 295.7%, respectively, versus the control group and -34.4 and -75.3%, respectively versus fluoride intoxicated group.

Histopathological examination: Histopathological examination of maternal cerebellar tissue excised from the normal untreated group revealed common nerve fibers, dispersed stellate and basket cells make up the majority of the superficial portion of the cerebellar cortex's molecular layer, which is compact and weakly pigmented. Then, a typical, single layer of big pyriform Purkinje cells (cell bodies with a greater diameter) extends deep into the cerebellar cortex. Then, adjacent to the white matter of the cerebellum and deep to the Purkinje cell layer, there were densely packed granular cells (small neurons with heterochromatic darkly pigmented nuclei) (Fig. 4C and C1). Similarly, mothers who took moringa extract showed intact cerebellar architecture (Fig. 4M and M1). Otherwise, daily supplementation with fluoride salt markedly degenerated the cerebral folial layers. Thin regretted granular layers with either basophilic apoptotic or eosinophilic necrotic neurons were depicted (Fig. 4F), which is further emphasized with ultrastructure investigations. Reactive gemistocytic astrocytes can be noticed here and there in the molecular layer (Fig. 4F2 and F3). A great loss in the pyriform cell layer was reported at low focus, accompanied with karyolysed deeply acidophilic Purkinje cells at higher magnification (Fig. 4F and F1). Elongated irregular glial cells were activated due to the toxic insult of fluoride salt intake (Fig. 4F1). In another focus fluoride ions deposition could be detected in the neuronal tissue (Fig. 4F2). Generally, a combined approach of fluoride salts and moringa extract significantly recuperate cerebral folia, but still, some granule cells of the cerebellar internal granule cell layer have this pyknotic basophilic feature (Fig. 4M+F). Many Purkinje cells retain a normal appearance despite the regional granule cell necrosis together with multiple active gemistocytic astrocytes and dilatation in blood capillary in the outermost molecular layer (Fig. 4M+F1 and 2). Intoxication with fluoride significantly reduced the approximate Purkinje cells number (p<0.001) compared to all other experimental groups, while cotreatment with moringa and fluoride markedly improved this number (Fig. 4I).

Immunohistochemical investigations

GFAP protein expression: Immunohistochemical observation of the cerebellar tissues from control and M-only treated animals showed mild expression in GFAP protein (Fig. 5C, C1, M and M1). While fluoride salt administration significantly elevated GFAP expression respecting the normal group (p<0.0001) and showed a remarkable protein expression in the tissue (Fig. 5F and F1). Same time, oral dosing of moringa extract together with fluoride salt significantly regressed GFAP level (-73.42%, p<0.0001) compared to fluoride-treated mothers and normalized its tissue expression (Fig. 5M+F and M+F1). The GFAP labelling index was recorded (Fig. 5I).

Synaptophysin (SYN) protein expression: Immunohistochemical examination of mother's cerebellar tissue for SYN protein exhibited intense expression in both the control and M-treated group (Fig. 6C, C1, M and M1), by contrary, oral administration of fluoride salt significantly regressed SYN level (p<0.000) with -68.70% respecting to the control group accompanied with scant tissue expression (Fig. 6F and F1). On the other hand, mothers supplemented with moringa extract together with fluoride exhibited a significant elevation of SYN level (p<0.0001) with 216.64% when compared with fluoride-treated animals and almost restored its dense expression (Fig. 6M+F and M+F1). The SYN labelling index was recorded (Fig. 6I).

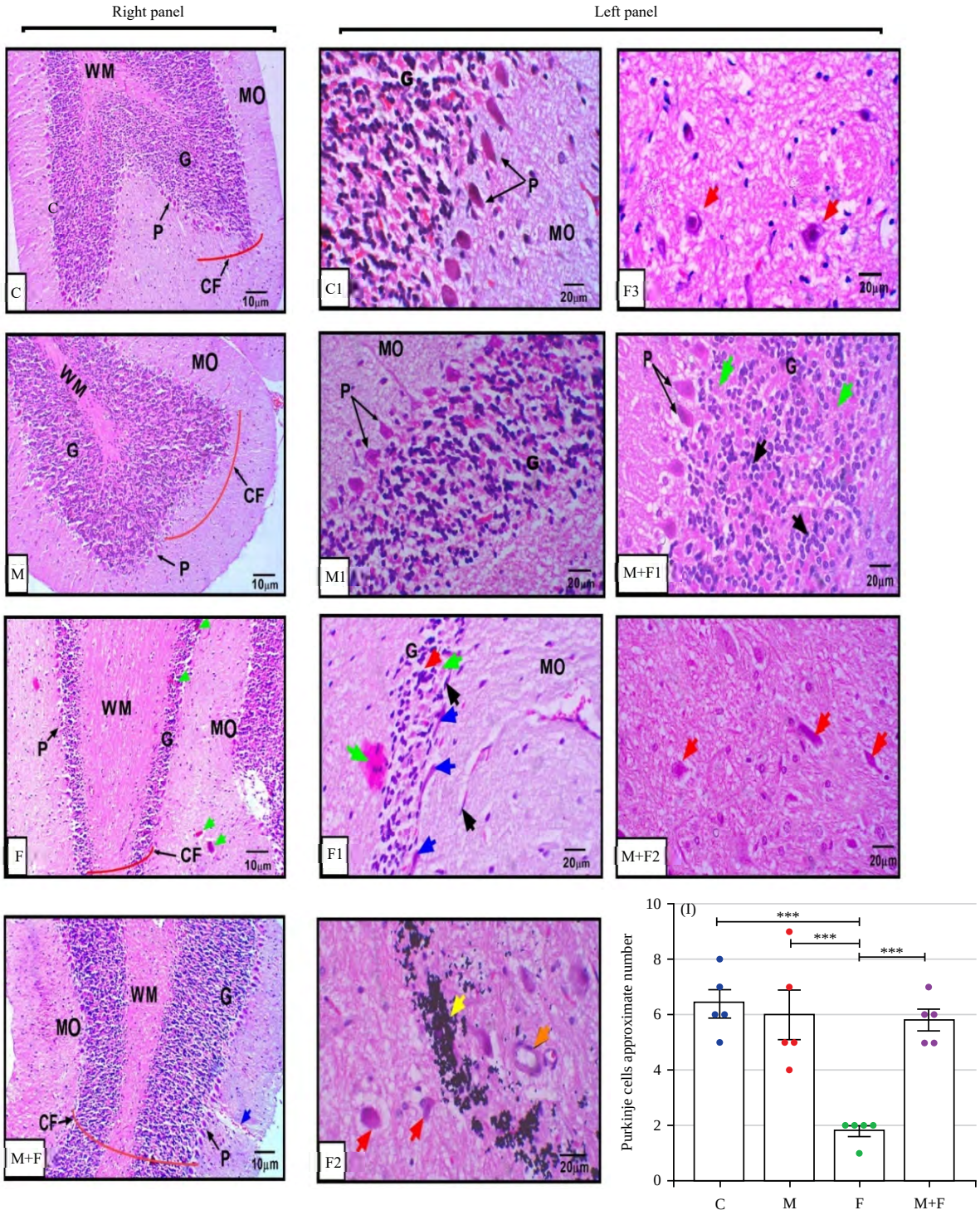

Fig. 4: Photomicrographs of H&E of cerebellar cortex sections from all groups, (C, C1) Control , (M, M1) Moringa extract-treated group, (F, F1, F2 and F3) Fluoride treated group, (M+F, M+F1 and M+F2) Combined treatment and (I) Approximate Purkinje cells number in different experimental groups

Sections (C, C1, M and M1) depict normal cerebral folium (CF), cortical outer molecular layer (MO), intact densely stained Purkinje cell layer (P), followed by multicellular granular layer (G), finally white matter (WM). While sections (F, F1, F2, and F3) shows multi-focal necrosis (green arrows) in cerebral folium (CF), molecular layer (MO), Purkinje cell layer (P), degenerated granular layer (G) and white matter (WM), gemistocytic astrocyte (red arrows) and reactive glial cells (black arrows), endothelial hypertrophy (orange arrow), and fluoride ion deposition (yellow arrows). Sections (M+F, M+F1 and M+F2) show intact piriform Purkinje cells (P) and molecular layer (MO). Black arrowhead identifies a pyknotic nucleus amid numerous normal granule cells, granular layer (G) still suffering from multifocal necrosis (green arrow). Many reactive astrocytes here and there (red arrow). Right panel 100×, left panel 400× and ***p<0.001

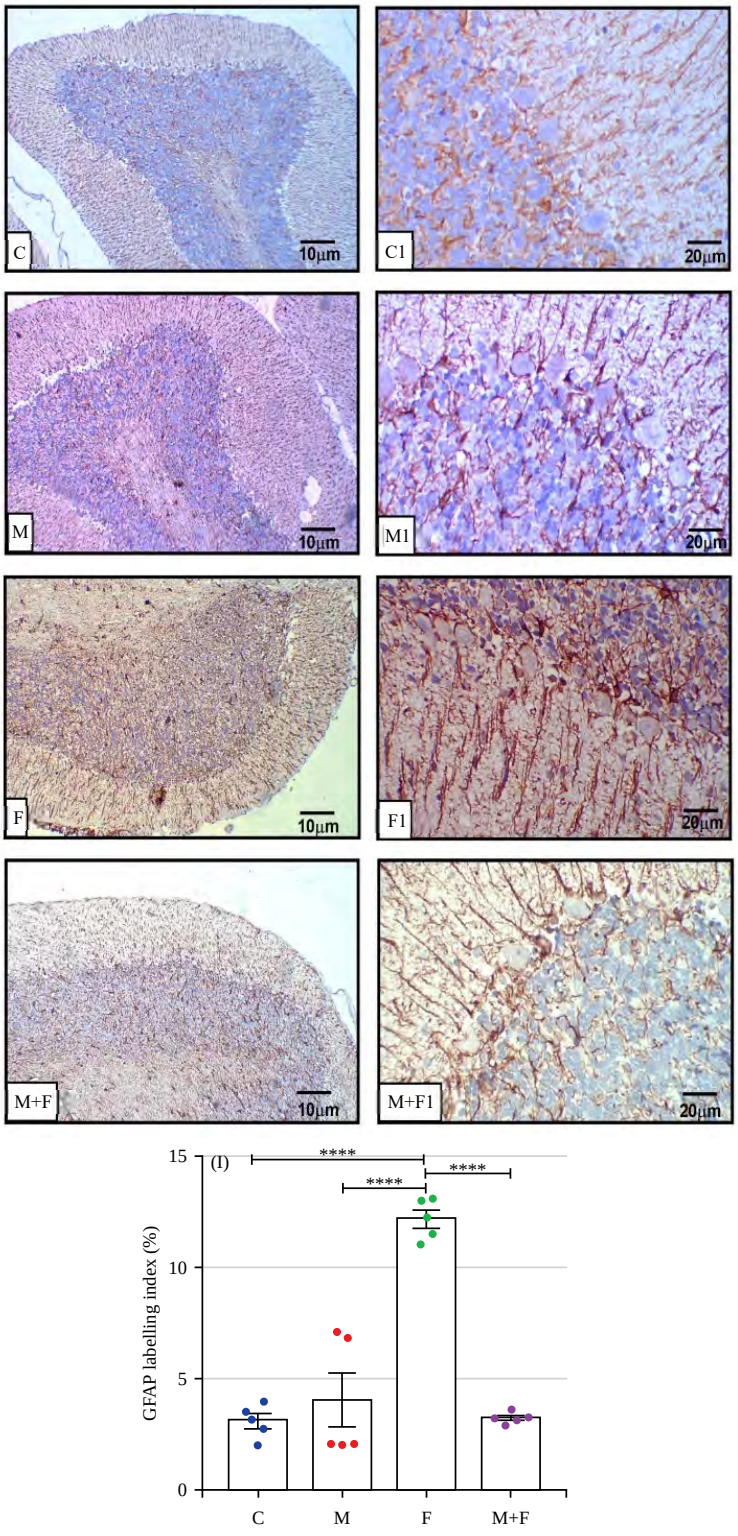

Fig. 5: Expression levels of GFAP protein in cerebellar tissue of different groups, (C and C1) Control, (M and M1) Moringa extract-treated group shows a mild protein expression in tissue, while (F, F1) Fluoride treated group showed a marked GFAP expression (M+F and M+F1) Combined treatment depicts week protein expression in the cerebellar tissue and (I) GFAP labelling index

Right panel 100×, left panel 400×, the values are expressed as the Mean±SEM of 5 microscopic fields/tissue samples of GFAP immune-expression and ****p<0.0001

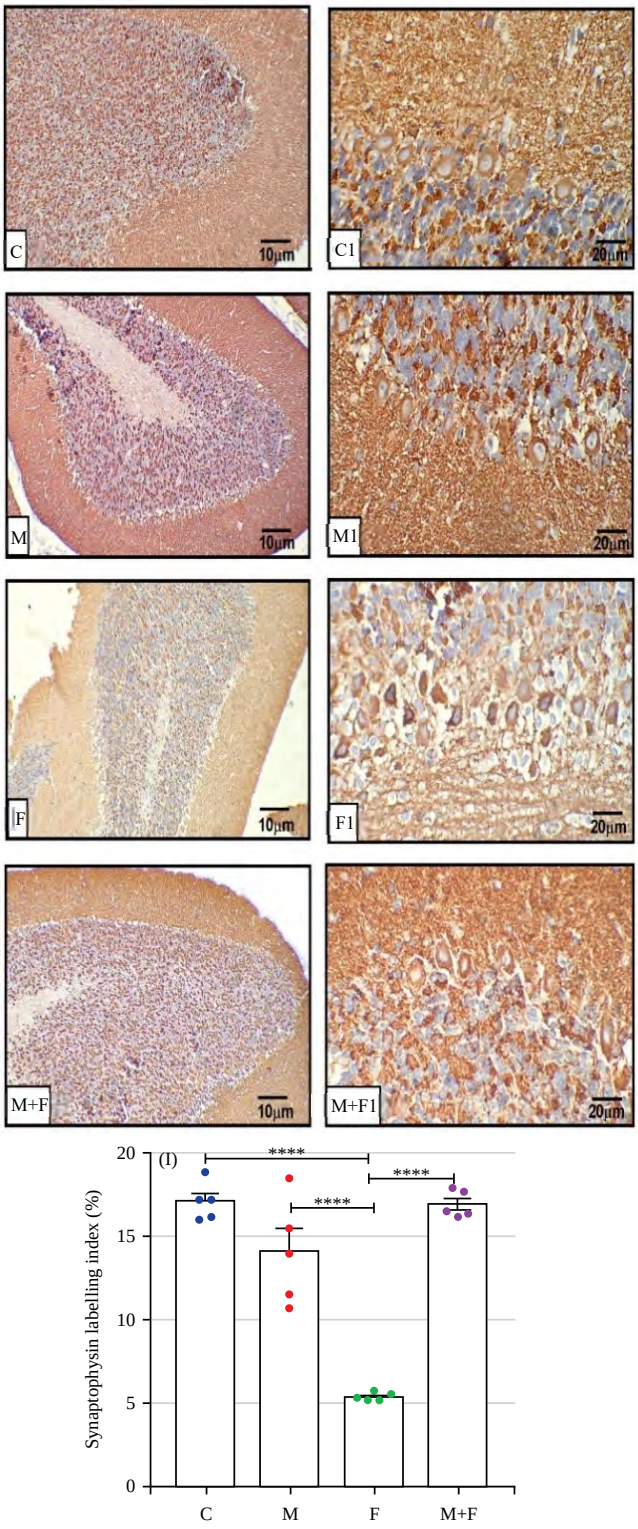

Fig. 6: Expression levels of SYN protein in cerebellar cortex of different groups, (C and C1) Control, (M and M1) Moringa extract-treated group displays a dense protein expression in tissue, while (F, F1) Fluoride treated group showed a little SYN expression (M+F and M+F1) Combined treatment depicts increased protein expression in the cerebellar tissue and (I) SYN labelling index

Right panel 100×, left panel 400×, the values are expressed as the Means±SEM of 5 microscopic fields/tissue samples of SYN immune-staining and ****p<0.0001

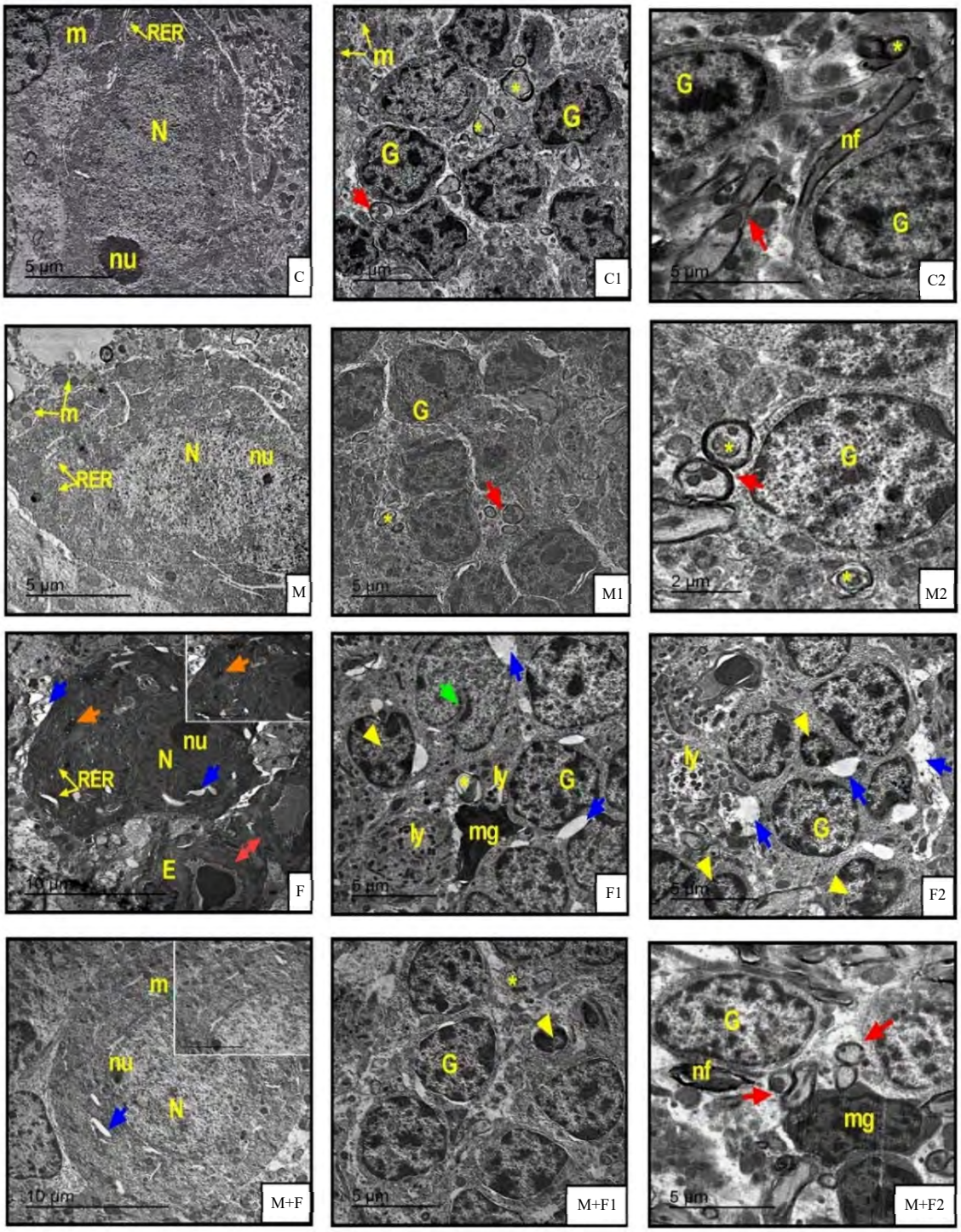

Fig. 7: Electron micrographs of cerebellar cortex sections from all groups, (C, C1 and C2) Control (M, M1 and M2) Moringa extract-treated group, (F, F1 and F2) Fluoride treated group and (M+F, M+F1 and M+F2) Combined treatment

Sections (C, C1, C2, M, M1 and M2) depict normal Purkinje cells containing normal eukaryotic nucleus (N) and noticeable nucleolus (nu). The cytoplasm contains many RER and granulocytes (G) and is rich in mitochondria (m) and granular layer shows many myelinated nerve fibers (nf), neural synapsis (red arrow) and also myelinated axons (*). While sections (F, F1 and F2) reveal purkinje cells with atrophied pyknotic nucleus (yellow head arrow), dilation in RER and dark patches (orange arrow), thickening in endothelial wall (red double headed arrow) and granular layer also depicts destructed granulocytes (G) with pyknotic nuclei and invaginated nuclear envelope (green arrow), many dark lysosomes (ly), neuropil vacuolation (blue arrow) and microglial cells (mg). (M+F, M+F1 and M+F2) showed quite intact Purkinje cells with normal nucleus (N), nucleolus (nu), dilated rough endoplasmic reticulum (blue arrow), granulocytes (G), normal neurofilaments (nf), neural axons (*) and pre- and post-synapsis (red arrow). But still suffering from apoptotic cells (yellow head arrow) and active microglial cells (mg).

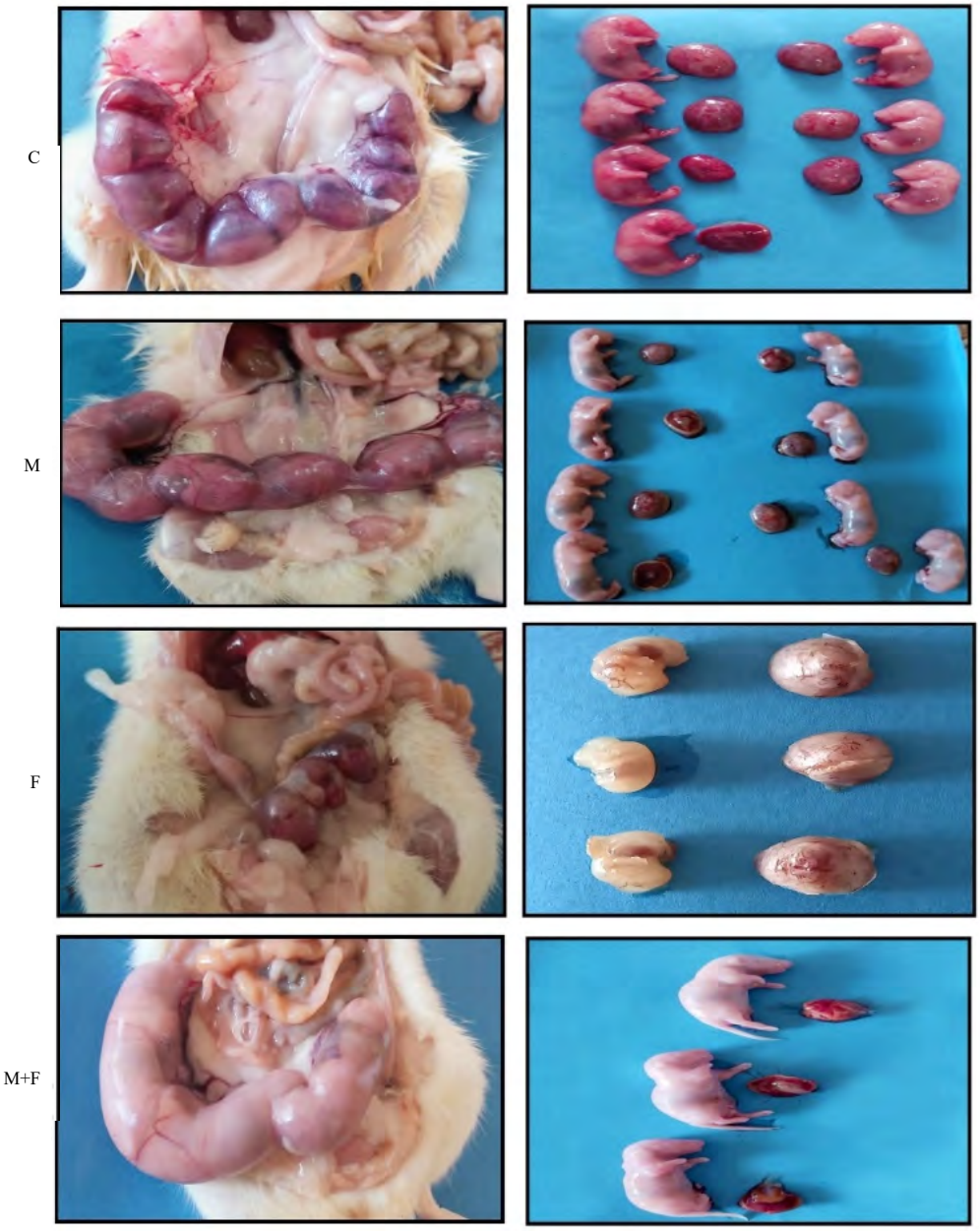

Fig. 8: Photographs of mothers at day 20 of gestation showing uterus of pregnant rats and their fetuses from all groups, (C) Control, (M) Moringa extract-treated group, (F) Fluoride treated group and (M+F) Combined treatment
Control and moringa extract-treated group reveals normal uterus and uterine horns, normal fetuses and no abortion or resorption. Fluoride treated group depicts presence of one uterine horn, mortality, many abortions and protrusion

Ultrastructural examination: Ultrastructure examination of the mothers' cerebellar cortex of control depicts an intact pyriform cell with a well-defined eukaryotic nucleus and marked electron-dense nucleolus, its cytoplasm is rich in RER and secretory granules and both rounded and elongated mitochondria. The granular layer showed normal granulocytes with mostly peripheral and little central euchromatin, centric heterochromatin and distinct nucleoli. Fairly sheathed myelinated axons, nerve fibers and both pre and post-synapsis filled with neurotransmitter vesicles could be seen here and there in the neuropil. A patent mossy rosette is noticed. Administration of moringa to normal pregnant rats revealed quite normal Purkinje cells with a euchromatic nucleus and rich RER, golgi apparatus and different-sized mitochondria. The granular layer showed normal organization as that of the control (Fig. 7C, C1 and C2).

Intoxication with fluoride induced cerebellar microstructural alterations in pregnant rats, as Purkinje cells were shown apoptotic with a dark atrophied pyknotic nucleus and dimpled envelope. Its cytoplasm revealed vacuolation, dilatation in RER and dark patches here and there. A noticeable thickening in the endothelial wall with the dark indented nucleus. Some nerve fibers were noticed, disrupted or split in myelin sheaths in the granular layer. Additionally, many neuronal granulocytes appeared destructed with pyknotic nuclei with invaginated nuclear envelope in another focus. Many dark lysosomes with multiple-sized showed scattered in the granulocyte cytoplasm (Fig. 7F and F1). Neuropil displayed unequivocal vacuolation indicating neuronal loss and disruption (Fig. F2). Furthermore, a granular microglial cell was observed.

Combined modality of moringa and fluoride attenuated pyriform cell microstructure, its nucleus was seen intact with abundant euchromatin and peripheral nucleolus enclosed with a well-distinguished envelope. Its cytoplasm was filled with numerous mitochondria and secretory granules, but still suffered from vacuolation (Fig. 7M+F). Typical granulocytes with undamaged nuclei and well-defined nucleoli, euo- and heterochromatin and numerous dark active mitochondria were exhibited. Neuropil was shown to be relatively recuperated having multi mitochondria, secretory granules, pre- and post-synapsis, neurofilaments and myelinated axons (Fig. 7M+F1 and 2).

Maternity study: Strikingly, administration of *M. oleifera* extract to normal untreated pregnant rats markedly showed normal uterine horns with no abortion or resorption, also fetuses appeared normal without malformations (Fig. 8M) these results were comparable to normal untreated uterine horns and fetuses (Fig. 8C). On the other hand, oral administration of fluoride revealed a high rate of abortion, resorbed fetuses, only one uterine horn was observed and the other is replaced by residual bodies, fetuses were dead with abnormal bending of the fetal body (protrusion) (Fig. 8F). Pregnant rats supplemented with moringa together with fluoride depicted the ameliorated effect of moringa against fluoride as it revealed no abortion, normal uteri and normal fetuses without any malformation (Fig. 8M+F).

DISCUSSION

Fluorinated compounds have been demonstrated to have serious impacts on the brain whether used in medical prescriptions field pesticides or as a food fumigant on post-harvest food. After being absorbed by the digestive system, fluoride reaches the circulation[27,28]. Toxic mechanisms of fluoride are involved in many biological processes like neuroinflammation, generation of free radicals[29], disruption of metal homeostasis[30], protein inhibition[31] and tissue damage[32]. The placental and blood-brain barriers may both be crossed by fluoride. According to previous studies, it has been linked to a delay in brain development during the prenatal and neonatal periods[12,33].

The use of herbal plants besides pharmaceutical drugs is an alternative method for enhancing our health. *Moringa oleifera* has many therapeutic potentials for many diseases like immune regulation, hyperglycemia, hypertension, hyperlipidemia, liver disease, inflammation and cancer[34]. Previous investigations reported that the phytochemicals flavonoids and phenolics are what give moringa its biological action[35]. Moreover, it has inherent natural antioxidants that can diminish the generation of free radicals, restrict the magnitude of lipid peroxidation and enhance the efficacy of antioxidant enzymes[36]. Additionally, moringa leaves have many benefits to overcoming nutritional and health implications in pregnant women and also prevent adverse pregnancy outcomes[37]. The protective effects of *Moringa oleifera* leaves on neurons and glia in lead poisoning have been shown[38,39]. Additionally, these leaves have been found to combat oxidative stress in rat models of Alzheimer's disease[21,22,40].

The objective of the present study was to elucidate the underlying mechanism via which the extract of *Moringa oleifera* leaves might mitigate the adverse effects of fluoride poisoning in pregnant rats. Body weight is crucial since it offers a broad perspective on the animals' condition of health. Generally, a key symptom of toxicity may be an abnormal rise or fall in body weight and the weight of other organs. The measurement of organ weight in toxicity is thus a crucial factor. Concerning body weight and vital organ weight, rats intoxicated with fluoride showed a significant regression in the body weight, uterus and placenta. These results were consistent with preclinical studies that discovered that fluoride-treated rats' body weight dramatically declined by Djouadi and Derouiche[44]. Similarly, another study provided that fluoride causes significant regression in uterine weight and also causes alterations in the uterine coefficient[40]. On the other hand, administration of moringa prevented regression in body weight. Also, co-treatment of both moringa and fluoride elevated body weight alleviated the reduction followed by fluoride exposure, significantly increased uterine and placental weights and narrowed the differences between the control and fluoride treated groups. Similar observation reported an elevation in body weight of rats treated with moringa, indicating that moringa may enhance anabolic processes resulting in the accumulation of adipose tissues of rats[41]. This result was in the same line with earlier results found that moringa causes elevation in body weight at a low dose[42].

Furthermore, mothers treated with fluoride alone showed a marked regression in vital organs weight (kidney, liver, brain and cerebellum) and in contrast elevated spleen weight. These outcomes, were consistent with earlier findings that revealed that fluoride causes an elevation in the relative weight of the spleen[43]. On the other hand, moringa treatment improves the reduction in vital organ weight, confirming with other studies demonstrated that moringa improves kidney weight and keeps it near to the normal weight[44]. This result was in parallel with previous results reported that moringa regressed the changes in organ weight and narrowed the differences between the control and cypermethrin treatment[45].

Considering to hormonal level, the anterior pituitary glandular gonadotroph cells secrete FSH and LH[46]. Gonadotropin-releasing hormones from the brain control how much of them are secreted. FSH's primary role is to stimulate ovarian expansion and to encourage the formation of follicles and estradiol. The LH is essential for follicular maturation, ovulation, corpus luteum formation and steroid hormone production[47]. The adverse impact of fluoride on the reproductive performance of females was seen to result in reduced levels of Follicle-Stimulating Hormone (FSH), Luteinizing Hormone (LH) and progesterone, along with a reduction in uterine weight and the number of follicles in the ovaries[48]. The results of this experiment showed that the injection of fluoride caused a considerable regression in the levels of the hormones progesterone, LH and FSH in the serum. Present study results were nearly similar to that reported in another study, explaining that intaking of fluoride resulted in a decline in FSH and LH in rats intoxicated with fluoride[49]. Furthermore, the regression of progesterone level is associated with a decrease in the number of healthy follicles[50]. However, in contrast to the fluoride-treated group, current study results showed that oral administration of moringa extract along with fluoride elicited an increase in FSH, LH and progesterone hormones. Similar observations were made, reported that moringa leaves increase female reproductive hormones, particularly FSH, LH and progesterone as well as increase the number of implantations[51]. This indicated that moringa may enhance the reproductive performance in females by increasing the secretion of hormones.

Furthermore, the elevated rate of free radicals like H_2O_2 generation may be the cause of the considerable changes in the brain catecholamine neurotransmitters (dopamine and serotonin) in inebriated rats. Previous findings demonstrated that these radicals could lead to the oxidation of dopamine and serotonin, which can result in neurodegenerative illnesses including Parkinson's and Alzheimer's[52].

The current study revealed that rats intoxicated with fluoride exhibit a significant regression in serum levels of both dopamine and serotonin. These findings were nearly similar to the preliminary results reported that fluoride causes depletion in both dopamine and serotonin levels[53]. The regression in serotonin level may be attributed to the conversion of serotonin to melatonin to inhibit the oxidative stress caused by fluoride exposure as melatonin is considered a powerful antioxidant and serotonin and melatonin exist in proportion to each other[54]. Moreover, administration of moringa causes elevation in serum levels of both dopamine and serotonin as compared to fluoride alone treated rats. Our findings were in agreement with another study that found that administration of moringa significantly elevated both dopamine and serotonin in male rats intoxicated with cobalt chloride[55].

At the biochemical level, α-amylase hydrolyzes carbohydrates into shorter molecules like maltose and saccharides predominantly before glucose synthesis. This enzyme is essential for the breakdown of starch and glycogen. It is mainly produced in the salivary gland and pancreas however, it is also found in other different organs[56]. In the brain, α-amylase can be released by astrocytes and is particularly linked to glycogen degradation involved in cell reactivity[57]. The present study showed an elevation in tissue α-amylase level followed by oral administration of fluoride. Our finding was in agreement with a previous study that demonstrated that the activity of amylase after rinsing with NaF was higher than after rinsing with distilled water[58].

Moreover, administration of moringa extract lowered α-amylase tissue level. Also, co-treatment with both fluoride and moringa regressed α-amylase levels indicating protective effect of moringa against fluoride toxicity. The present discovery was consistent with the prior research by Ademiluyi *et al.*[59], which demonstrated that the extract derived from moringa leaves effectively inhibits the activity of α-amylase. This finding aligns with another study that has shown the antioxidative and antidiabetic properties of tannins found in moringa. These properties are believed to arise from the tannins' capacity to bind with carbohydrates and proteins, leading to the suppression of digestive enzymes such as α-amylase[60].

According to a recent study, acetylcholinesterase (AChE), is an enzyme that breaks down acetylcholine at synaptic junctions, controlling how nerve signals and impulses are transmitted[61]. This may eventually result in the oxidation of serotonin and dopamine, which may cause neurodegenerative diseases including Parkinson's and Alzheimer's disease[52]. Administration of fluoride causes a significant elevation in tissue AChE level as compared to the control. This finding was in the same line with the earlier results found that fluoride causes a significant elevation in AChE in fluoride exposed animals[62]. This increment may be due to the interconnection between fluoride and two or more hydrogen donors needed to stimulate the enzyme[63].

Preliminary findings demonstrated that supplementation with antioxidants like whey protein extract regresses serum AChE levels in elderly rat brain, indicating that supplementation with antioxidants may enhance the antioxidant potential[64]. The present study finding revealed that the administration of moringa extract causes a reduction in AChE level. This finding shows that supplementation with moringa, as it has many antioxidants, may effectively prevent the effect of fluoride on some enzymes and maintain them near to the normal level.

Regarding inflammation, it is well-known that both people and animals may benefit from inflammation[65]. Numerous factors, including microorganisms, damaged cells and toxic substances, can cause it[66]. According to preclinical research, fluoride poisoning is linked to an inflammatory response in mouse testicles[67]. Moreover, the group that received fluoride treatment had a substantial increase in the expression of the pro-inflammatory marker IL-6. The obtained result was consistent with other findings which indicated that fluoride has the potential to induce the production of the pro-inflammatory marker IL 6 in the brain of rats[68]. Moreover, preclinical studies have demonstrated that neuroinflammation and release of pro-inflammatory cytokines are accompanied by cognitive impairment like Alzheimer disease[69]. The production of these cytokines is predominantly attributed to the activation of monocytes, macrophages and T cells and they play a crucial role in the control of the inflammatory response[70]. The release of these cytokines is initiated by activated microglial cells, potentially resulting in tissue injury and brain cell destruction[71]. On the other hand, *Moringa oleifera* leaves, which are considered a natural product, have many antioxidants that succeeded in the reduction of inflammatory effect and regression of IL 6 pro-inflammatory marker[72]. Moreover, bioactive extract of moringa might effectively suppress iNOS and COX-2 protein expression as well as inhibit the production of Por-inflammatory cytokines like IL-6, IL 1β, TNF-α[73], this was in agreement with the preceding outcomes. These findings were in parallel with our results that found a significant regression in pro-inflammatory marker IL6 in moringa treated groups.

Oxidative stress arises from the disparity between the generation of reactive oxygen species (ROS) and the efficacy of antioxidant enzymes. Superoxide dismutase (SOD) and catalase (CAT) are the primary antioxidant enzymes in biological systems[74]. Polyunsaturated fatty acids eventually produce malondialdehyde (MDA) as an end product and it is the most accurate ROS[75] indicator. Furthermore, it has been shown that heightened levels of oxidative stress have the potential to trigger the apoptotic cell death pathway, leading to the occurrence of neurodegeneration[76]. Preclinical studies demonstrated that fluoride intake may cause oxidative stress and release free radicals in various biological systems[77]. Activities of superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) activities serve as indicators of the oxidative state present in both tissue and blood. The findings of this research indicated that the levels of antioxidant enzymes SOD and CAT in the blood of rats exposed to fluoride were lowered, resulting in a diminished capacity to scavenge reactive oxygen species (ROS). Moreover, the heightened concentration of MDA signifies a significant production of free radicals. Additionally, the increased level of MDA indicates a high level of free radicals' generation. These results were in parallel with previous studies, which explained that fluoride caused regression in the activity of antioxidant enzymes SOD and CAT in addition to increased production of MDA in rat brain[78]. Another study demonstrated that maternal exposure to fluoride during pregnancy and lactation regressed the activity of CAT enzyme in the prefrontal cortex, the striatum and the hippocampus[79].

Furthermore, moringa has powerful antioxidant activities, according to the results of the current investigation, treating rats with moringa reverses the negative effects of fluoride intoxication on the activity of antioxidant enzymes and lipid peroxidation by considerably raising blood levels of SOD and CAT while lowering levels of MDA. These findings corroborated earlier research that showed moringa reduced the lipid peroxidation biomarker MDA while increasing the activity of the antioxidant enzymes SOD and CAT[80]. According to preclinical research reported that the phytoconstituents in leaves extract, such as flavonoids, polyphenols, ellagic acid, quercetin, apigenin and tannins, can inhibit oxidases, scavenge free radicals and increase the activity of antioxidant enzymes[81].

Moreover, DNA fragmentation may be brought on by excessive ROS generation and an imbalance between the antioxidant defense system's ability to combat free radicals[82]. Oxidative stress causes DNA damage to the cells via directly and indirect ways inducing breaks in the double strands of DNA. It was stated that fluoride may induce DNA fragmentation of hepatic cells[83]. Current study findings demonstrated the ability of fluoride to cause cerebellum DNA damage as shown in comet assay results, these results go in the same hand with other studies indicating the oxidative damage of DNA in the brain of mice intoxicated with fluoride[84]. On the other hand, co-administration of both fluoride and moringa showed a marked reduction in cerebellum DNA damage which was parallel to a previous report by Omotoso *et al.*[85]. This may be due to the antioxidative properties of moringa that restore cerebellum DNA integrity.

In the current study, cerebellar damage was found in pregnant rats intoxicated with fluoride. Exposure of cerebellum to fluoride causes multifocal necrosis in cerebral folium, degenerated granular layer, reactive glial cells, necrotized Purkinje cells, necrotic and pyknotic granular cells as well as fluoride ion deposition. This finding was consistent with other studies that showed fluoride's ability to produce considerable abnormalities in the cerebellum, as seen by the neurons' multifocal shrinkage, eosinophilia and pyknotic changes[41]. In addition, it showed degeneration in the Purkinje cells, neuronal injury appeared along with gliosis as well as degeneration of neurons in the Purkinje layer. Preclinical studies demonstrated that fluoride could accumulate in the brain, increasing free radicals and peroxidation levels as well as causing oxidative stress that leads to neurotoxicity and resulting in neuronal damage[86].

However, treatment with moringa leaves improves cerebellar structure in the form of reduction of pyknotic basophilic appearance of granule cells and normal appearance of Purkinje cells. These findings were in collaboration with preclinical outcomes[87] reported that *Moringa oleifera* administration prevented acute apoptosis and chromatolysis in cerebellar cortex of rats treated with nicotine as well as alleviated locomotive deficits observed in nicotine treated rats. Similar observations revealed the capability of moringa to prevent neuronal cell damage through enhancing the antioxidant capacity of neuronal cells[88]. These findings explained that *Moringa oleifera* aqueous extract can ameliorate the histopathological changes induced by fluoride as well as improving cerebellar bioactivities by co-treatment of both moringa and fluoride indicating the synergistic effect of moringa against fluoride toxicity.

In the current study, immunohistochemical staining of GFAP showed significant elevation in glial fibrillary acidic protein expression in fluoride treated rats. The GFAP is regarded as a type III intermediate filament protein that preserves astrocyte mechanical strength. The increment in GFAP expression has been reported as a neurotoxicity marker[89]. This result was in parallel with with previous study which demonstrated the rise of GFAP-positive cells caused by fluoride. Moreover, GFAP was known as a specific marker of mature astrocytes in the Central Nervous System (CNS)[27].

The GFAP expression is crucial for healthy white matter architecture and blood-brain barrier integrity. As astrocytes respond quickly to any neurotoxic insult by manufacturing numerous neurotoxic chemicals and more GFAP, which was described as a marker protein for astrogliosis, the rise in GFAP expression indicates the harmful effect on the CNS[90,91].

On the contrary, fluoride causes a significant reduction in synaptophysin immunoreactivity. This result was also confirmed by the morphometric analysis, as there was a significant regression in synaptophysin expression in fluoride-treated group. Synaptophysin, which is considered a specific biomarker for synapses in mammals[92], is affected by fluoride. These results were in agreement with the previous report which explained that fluoride causes a reduction in the expression of synaptophysin[93].

Previous research has shown that a reduction in synaptophysin expression is linked to impaired learning and memory in animals[94]. This finding was consistent with another study that found a decrease in pre-synaptic proteins, including synaptophysin, following exposure to sodium fluoride. This decline in synaptophysin levels may adversely affect synaptic function, which is believed to play a crucial role in the formation of Central Nervous System (CNS) lesions following fluoride exposure. Preliminary findings stated that faint synaptophysin reactivity indicates abnormal axon transmission and impaired synaptic function leading to cognitive deficits, the diminished expression of synaptophysin may be due to the possible inhibition of synaptophysin protein biosynthesis via DNA fragmentation[95]. Furthermore, the administration of moringa exhibits a significant regression in GFAP expression, indicating the antioxidant capacity of moringa against fluoride neurotoxicity. Strikingly, moringa treatment showed diminished expression of immunohistochemical GFAP in rats intoxicated with aluminum[96].

According to preliminary research, *Moringa oleifera* extract acts as a neuroprotective support and normalizes GFAP[97]. Moreover, these findings suggested that moringa could suppress the expression of GFAP via the antioxidant capability present in it. On the other hand, moringa treatment causes a significant elevation in synaptophysin immunostaining as well as increased the area of synaptophysin expression in moringa-only treated rats or along with fluoride. This result was in the same line with the earlier results stated that *Moringa oleifera* has a recovery effect on synaptic proteins like synaptophysin and this could be attributed to the anti-inflammatory, antioxidative as well as neuroprotective properties[98]. Accordingly, the antioxidant properties of moringa are reflected in the immunohistochemical staining of rat cerebellar cortex as it may prevent the adverse effects induced by fluoride toxicity.

In the present study, structural alterations in the cerebellum of rats treated with fluoride were assessed using a transmission electron microscope. The cerebellar cortex of fluoride-treated group revealed features of neurodegeneration as the Purkinje layer in fluoride-treated group shows apoptotic Purkinje cells with an atrophied pyknotic nucleus and dimpled envelope. Furthermore, cytoplasmic ultrastructure exhibits changes in cytoplasm, dilation in RER and many dark lysosomes with multiple sizes. Alrafiah[27] established the connection between fluoride and oxidative stress by demonstrating how fluoride causes the nuclear membrane to fold inward and other cytoplasmic alterations. Moreover, the appearance of disrupted nerve fibers, destructed neuronal granulocytes and neuropil vacuolation indicate neuronal loss. These findings were in agreement with previous results and explained that fluoride causes disruption of myelination in rat cerebellar cortex as well as neuronal injury[99]. According to another research, this may be explained by changes in the myelin basic protein that result from membrane damage and axonal degeneration following exposure to hazardous substances[100].

Furthermore, microglial cells appeared in fluoride treated rats as well and endothelial wall was thickened, confirming another study conducted with[27]. In addition to their primary role as scavenger cells, activated microglial cells can perform a variety of additional tasks, including immune response induction, neuronal degeneration and tissue healing. Microglial activation may sustain chronic inflammation leading to neuronal dysfunction and cell death[101].

In this study, the group treated with moringa extract reduced the severity of cerebellar alterations seen in the fluoride treated group. This agreed with a previous study that pointed out that treatment with *Moringa oleifera* reversed most of the pathological changes induced by lead toxicity, indicating that moringa acts as a neuroprotective agent due to its capability to inhibit NO and MDA production[102]. Interestingly, the combined approach of moringa extract together with fluoride showed a remarkable improvement in the ultrastructure of the cerebellum after being destroyed by the administration with fluoride alone.

CONCLUSION

The aqueous extract of moringa has been found to have a substantial beneficial impact on enzymatic levels, hormonal balance, oxidative inflammatory stress, GFAP and synaptophysin levels in cerebellar tissue of pregnant mothers after fluoride intoxication. This improvement was also reflected in the histoarchitecture and ultrastructure of the tissue. Additionally, the extract has shown to enhance maternity evaluation. According to these findings, it is strongly advised to consume herbal remedies, particularly moringa extract, to minimize daily fluoride intoxication, especially in pregnant women.

SIGNIFICANCE STATEMENT

In this study, the adverse effect of fluorinated compounds on the cerebellum of pregnant mothers was investigated and the beneficial role of moringa extract at the end of the gestation period: Biochemically, pathologically, immunohistochemically, ultrastructurally and teratologically. Our findings give hope for pregnant mothers to overcome the toxicological effect of fluoride during the gestation period on their cerebellar tissue and fetuses.

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