Pathophysiology 25 (2018) 57–62

Contents lists available at ScienceDirect

Pathophysiology

journal homepage: www.elsevier.com/locate/pathophys

Moringa oleifera phytochemicals protect the brain against experimental nicotine-induced neurobehavioral disturbances and cerebellar degeneration

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ARTICLE INFO

Article history:
Received 12 October 2016
Received in revised form
24 December 2017
Accepted 28 December 2017

Keywords:
Moringa oleifera
Nicotine
Cerebellum
Locomotion
Neurobehaviour

ABSTRACT

Nicotine is a neuro-stimulant that has been implicated in the pathophysiology of many brain diseases. The need to prevent or alleviate the resulting dysfunction is therefore paramount, which has also given way to the use of medicinal plants in the management of brain conditions. This study was designed to determine the histomorphological and neurobehavioural changes in the cerebellum of Wistar rats following nicotine insult and how such injuries respond to *Moringa* intervention. Twenty-four adult male Wistar rats were divided into 4 groups. Group A and B were orally treated with normal saline and *Moringa oleifera* respectively for twenty-eight days; Group C was treated with nicotine while group D was treated orally with *Moringa oleifera* and intraperitoneally with nicotine for twenty-eight days. Animals were subjected to the open field test on the last day of treatment. 24 h after last day treatment, the animals were anesthetized and perfusion fixation was carried out. The cerebellum was excised and post-fixed in 4% paraformaldehyde and thereafter put through routine histological procedures. Results revealed cytoarchitectural distortion and extreme chromatolysis in neuronal cells of the cerebellar cortical layers in the nicotine-treated group. The Purkinje cells of the cerebellum of animals in this group were degenerated. There were also reduced locomotor activities in the group. Moringa was able to prevent the chromatolysis, distortion of the cerebellar cortical cells and neurobehavioural deficit. Our result suggests that *Moringa oleifera* could prevent nicotine-induced cerebellar injury in Wistar rats, with the possibility of ameliorating the clinical features presented in associated cerebellar pathology.

1. Introduction

Nicotine is one of the major components of tobacco that is commonly consumed by ways of cigarette smoking, tobacco chewing or during nicotine replacement therapy [1]. Consumption of tobacco products has been associated with the development of many pathological conditions, including cardiovascular and cerebrovascular diseases, as well as defects in brain morphology and neurochemistry [2,3]. Nicotine is a major tobacco-specific alkaloid that is present in both first and second hand tobacco smoke exposure [4], and is responsible for the addictive effect of tobacco [3,5]. The risk for nicotine addiction depends on the ingested quantity and route of delivery, while addiction potential is directly proportional to rate of dose delivery and absorption [6]. Nicotine has been reported to cause transient nystagmus and dizziness thereby resulting into postural imbalance in smokers [7]. Prolonged nicotine treatment induces CYP2E1 expression in cortical pyramidal neurons and cerebellar Purkinje cells, while augmentation of CYP2E1 in the brain may contribute to oxidative stress [8]. Histological demonstration of the cerebellum in nicotine-treated rodents revealed significant loss of white matter, with possible predisposition to progressive impairment in the structural integrity and function of the cerebellum [9,10].

Moringa oleifera (MO) is a small-sized tree that is approximately 10–12 m high [11]. It is widely cultivated in most parts of the world for its nutritional and medicinal benefits. MO has been reported to have anti-inflammatory, antimicrobial, antioxidant and anticancer properties [12]. Phytochemicals present in the leaves of

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https://doi.org/10.1016/j.pathophys.2017.12.003
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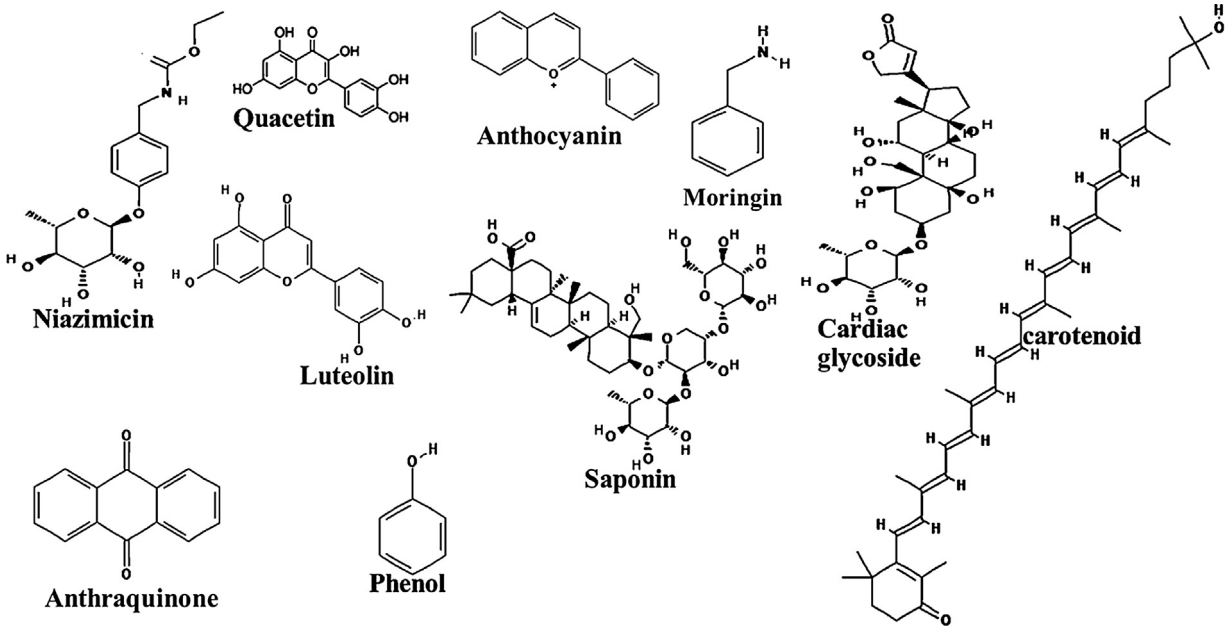

Fig. 1. structure of active phytochemicals of MO.

Table 1
Active phytochemical constituents of aqueous extract of *Moringa oleifera* leaves.

Phytochemicals	Abundance
Anthraquinone	11.68 g/100 g [14]
Terpenoids	4.84 g/100 g [14]
Cardiac glycoside	0.36 g/100 g [14]
Tannins	9.36 g/100 g [14]
Carotenoids	1.16 g/100 g [14]
Anthocyanin	0.06 g/100 g [14]
Saponins	1.46 g/100 g [14]
Quacetin	126 μg/g [15]
Luteolin	6.20 μg/g [15]
Phenols	118 mg/g [15]

MO include tannins, steroids, triterpenoids, flavonoids, saponins, anthraquinones, alkaloids, niazimicin, moringin and reducing sugars (Table 1, Fig. 1) [13,14]. The essential oil of MO has been found to contain flavonoids quercetin and luteolin [15]. The total phenolic component of MO leaf extract is 118 mg/g with a total antioxidant activity of 0.636 μmol Trolox/mg [16]. Recently, the attention of researchers has been focused on moringin (4-(α-L-rhamnosyloxy)benzyl isothiocyanate) because it has been shown to have anti-inflammatory as well as antioxidant effects, protecting against neurodegenerative disorders [17]. Chatchada et al. reported that *Moringa* leaf extract was neuroprotective against age-related dementia when administered to rats [18].

The cerebellum links the sensory and motor areas of the brain by means of output neurons that projects to brain structures regulating/coordinating general and fine movements. The aforementioned link forms the basis for precision and accuracy. In the present study, we sought to characterize behavioral functions and cerebellar morphology in rats following infusion of nicotine, while investigating the neuroprotective potentials of MO.

2. Materials and methods

2.1. Laboratory animals and care

A total of 24 adult male Wistar rats were used for this study. Ethical approval was sought and obtained from the ethical committee of the College of Health Sciences, University of Ilorin. Wistar rats were housed in a wire gauzed cage at the animal holding facility of the Faculty of Basic Medical Sciences, University of Ilorin. The animals were allowed to acclimatize for two weeks prior to commencement of study.

2.2. Preparation of treatment solutions

Nicotine (95%) was obtained from the British Drug House (BDH) Chemical Ltd, Poole, England. Following titration procedure, it was observed that animals were able to tolerate a maximum dose of 13.76 mg/kg in 0.1 ml of vehicle. Dry *Moringa* leaves were weighed and then brewed in distilled water at 100 °C for 10 min. The solution was then evaporated and the extract/residue concentrated to 5 g of MO in 100 ml of distilled water.

2.3. Treatment of animals

The animals were randomly divided into four groups A–D. Group A received 1 ml of distilled water orally, Group B was administered 200 mg/kg aqueous extract of MO orally, Group C was administered 1.38 mg/kg nicotine bitartrate intraperitoneally (i.p), while Group D was concomitantly administered 200 mg/kg MO orally and 1.38 mg/kg nicotine bitartrate (i.p). All the groups were treated for 28 consecutive days.

2.4. Neurobehavioral study

Rats were tested in the open field apparatus following the last administration to assess locomotor and exploratory activities [19]. The open field apparatus was made from plywood measuring 100 cm × 100 cm with walls 50 cm high. The floor was divided into square grids each measuring 25 cm in length with a blue marker and a center square of the same length was drawn with a red marker. During the test, rats were picked by the tails and dropped at the center square and allowed to explore the open field for 10 min while a video camera located above the apparatus was used to record activities of rats for 10 min each. Three behaviors were scored from the recorded video by an independent observer who analyzed the number of lines crossed, rearing frequency and rearing duration. The rearing frequency and rearing duration were estimated as the

number of times the rat stood on its hind limbs, with both fore limbs raised, while the rearing duration is the total time spent while in the rearing position.

2.5. Tissue processing

Rats for histological and histochemical studies were euthanized by intramuscular injection of 25 mg/kg of ketamine and subjected to transcardial perfusion during which a flush of 50 ml of normal saline was followed by 500 ml of 4% paraformaldehyde (PFA). The brain tissues were thereafter excised, weighed and post-fixed in 4% paraformaldehyde (PFA) for 48 h. Rats for enzymatic studies were sacrificed by cervical dislocation (to eliminate interference of anesthetic agent with biochemical redox); the brains were then excised, weighed, rinsed in 0.25 M sucrose 3 times for 5 min each and stored in 30% sucrose at 4 °C. PFA-fixed tissue sections were stained using Haematoxylin and Eosin (H and E) and Cresyl Fast Violet (CFV) staining techniques.

2.6. Colorimetric assay for enzymatic studies

Enzymatic assay for MDA (malondialdehyde) and SOD (superoxide dismutase) activities were carried out in cautiously dissected cerebellar cortices of the rats using spectrophotometric techniques. MDA and SOD assay kits were procured from Abcam®, USA. Equal weighing brain tissues (0.085 g) were homogenized in 0.25 M sucrose using a homogenizer at 4 °C. The tissue homogenate was centrifuged for 15 min in a centrifuge at 5000 rpm to obtain supernatants containing organelle fragments and synaptosomes. The supernatants were aspirated into plain labeled glass cuvettes placed in ice. MDA and SOD activities were assayed according to manufacturer's instruction in the assay kit pack.

2.7. Data analysis

Data obtained from enzymatic assays and cerebellum/brain weight ratio (CBR) was analyzed using GraphPad Prism® software (version 6). Enzyme and CBR values were plotted in ANOVA using Tukey's multiple comparisons test. Data obtained were presented as mean ± standard error of mean, with significance level placed at p values less than 0.05, 0.01 or 0.005. The results obtained were represented in bar charts with error bars to show the mean and standard error of mean respectively.

3. Results

3.1. Moringa attenuates deficits in locomotor activities in nicotine-treated rats

Open field tests reveal locomotor and exploratory activities in rodents, as well as levels of anxiety. On subjecting animals from the different experimental groups to the tests, it was observed that there was no significant difference in the number of lines crossed and the rearing frequency in the MO-treated groups compared to control. High frequency of lines crossed and rearing indicates low level of anxiety in rats. On the other hand, we observed significant reduction in number of lines crossed as well as frequency and duration of rearing in the nicotine-treated group when compared to rats that received MO ($p < 0.005$) and control group ($p < 0.05$) (Figs. 2–4). It was observed that rats in the nicotine-treated group remained inactive for a larger percentage of time spent in the open field, leading to reduction in the locomotor activities which indicates increased anxiety. Oral infusion of MO prevented this degenerative changes in the group that was treated both nicotine then MO, as it was observed that the number of lines crossed with frequency and duration of rearing were significantly higher than those of the nicotine-treated group ($p < 0.005$).

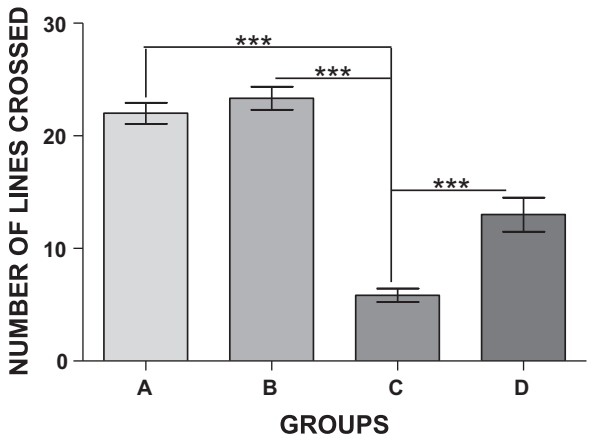

Fig. 2. Number of lines crossed by animals in the open field test. A = control, B = *Moringa oleifera*, C = nicotine and D = nicotine and *Moringa*. *** is the significant levels of comparison at $p < 0.005$.

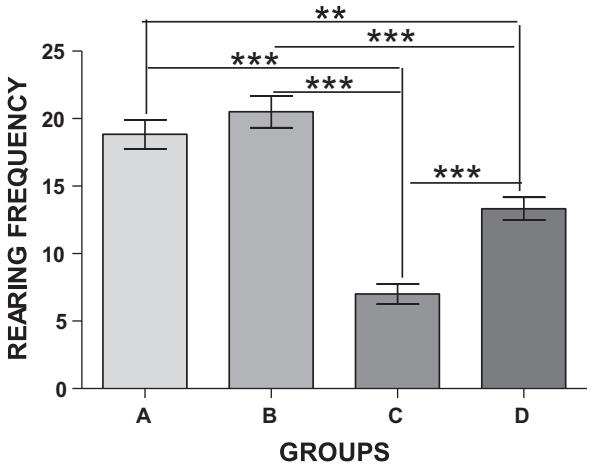

Fig. 3. Rearing frequency of animals in the open field test. A = control, B = *Moringa oleifera*, C = nicotine and D = nicotine and *Moringa*. ** and *** are significant levels of difference at $p < 0.01$ and 0.005 respectively.

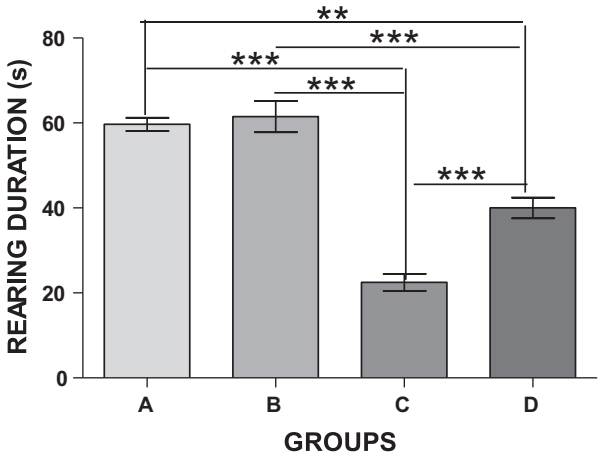

Fig. 4. Rearing duration of animals in the open field test. A = control, B = *Moringa oleifera*, C = nicotine and D = nicotine and *Moringa*. *** is significant levels of difference at $p < 0.005$.

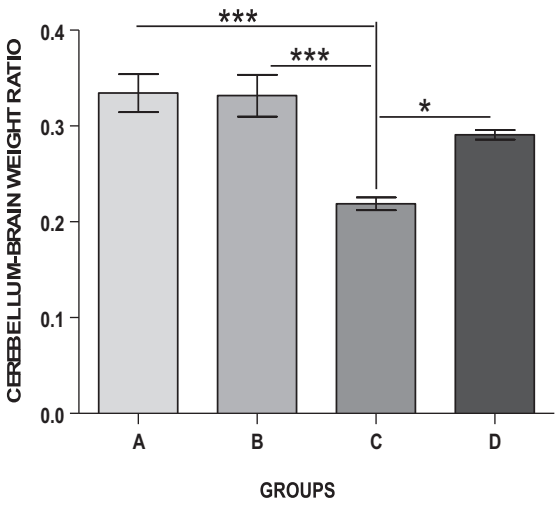

Fig. 5. Cerebellum to brain weight ratios. A = control, B = *Moringa oleifera*, C = nicotine and D = nicotine and *Moringa*. * and *** are significant levels of difference at $p < 0.05$ and 0.005 respectively.

3.2. Cerebellum to brain weight ratio (CBR)

Following sacrifice, rats whole brain weights were taken, before the cerebelli were excised and weighed to contrast the ratio of both. The CBR of rats in MO-treated group was noted to be similar to control. As expected, nicotine-treated rats had significantly lower CBR when compared to control (Fig. 4). Interestingly, when compared with control and MO groups, the MO plus nicotine group had reduced CBR but this observed reduction is not significant. The CBR of the nicotine group was significantly lower than that of MO plus nicotine group, implying that MO mitigated nicotine-induced cerebellar weight reduction (Fig. 5).

3.3. Moringa oleifera counterbalances nicotine-induced oxidative stress

In the present study, cerebellar SOD and MDA activities were assessed. SOD level in MO-treated group was similar to control. There was significant ($p < 0.005$) reduction in the level of SOD in nicotine-treated rats when compared to both control and MO-treated groups. Rats that received a combined treatment of MO and nicotine had a significantly ($p < 0.01$) higher level of SOD when compared to the nicotine-treated group, but a lower SOD level relative to the control and MO-treated groups ($p < 0.05$). The observed higher SOD expression in rats that received a combined treatment of MO and nicotine showed the protective effects of MO against nicotine-induced degenerative changes (Fig. 6).

Nicotine administration resulted in increased lipid peroxidation in this study, as it was observed that the nicotine-treated group has higher ($p < 0.005$) MDA expression when compared to other treatment groups. Observed nicotine-induced neural lipid peroxidation was prevented by MO treatment in the group that received combined treatment with nicotine, as evidenced by a significantly ($p < 0.05$) higher level of MDA relative to the control and MO-treatment. This suggests that MO may have the capacity to prevent excessive lipid peroxidation induced by nicotine (Fig. 7).

3.4. Histological and histochemical observations

The general morphological presentation of cerebellar layers in control rats and those treated with MO were unaltered as shown by H and E staining (Fig. 7a). In these two groups, the arrangement of cells within the cerebellar cortex are highly organized from the molecular layer to the granular layer-which surrounds the less populated inner medullary layer of white matter. In addition, cellular density within the groups appears normal across all cortical layers, although the Purkinje cell layers are not clearly demonstrated. On the other hand, cerebellum of nicotine-treated rats is characterized by distorted cerebellar histoarchitecture, with less dense granular layer compared to other groups (at low-power magnification). Rats that received a combined treatment of MO and nicotine displayed normal cerebellar architecture in contrast to cerebellar morphology in group C.

At higher magnification, cerebellar morphology in groups A and B were characterized by Purkinje cells with noticeable cell bodies and dendrites that were deeply projecting into the molecular layers. The granular layer in these groups consisted of small granule neurons which were densely disposed. Purkinje cell layers also showed conspicuous dendritic spines. The Purkinje cell layer of the nicotine-treated group shows several signs of degeneration as both the cell bodies and dendrites were sparsely visible when compared to the other groups at higher power magnification. Granule cells in this group were also sparsely distributed within the cerebellar granule cell layer (Fig. 8a).

Histochemical demonstration of the cerebellar cortex of *Moringa*-treated rats showed appropriately stained Purkinje and granule cells, in similarity to what was observed from rats in the control group. Conversely, nicotine-treated rats presented with extreme peripheral and mild central chromatolysis as shown by

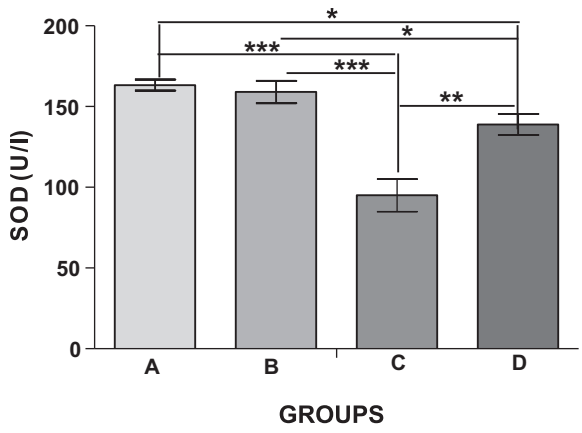

Fig. 6. Cerebellar levels of superoxide dismutase. A = control, B = *Moringa oleifera*, C = nicotine and D = nicotine and *Moringa*. *, ** and *** are significant levels of difference at $p < 0.05$, $p < 0.01$ and $p < 0.005$ respectively.

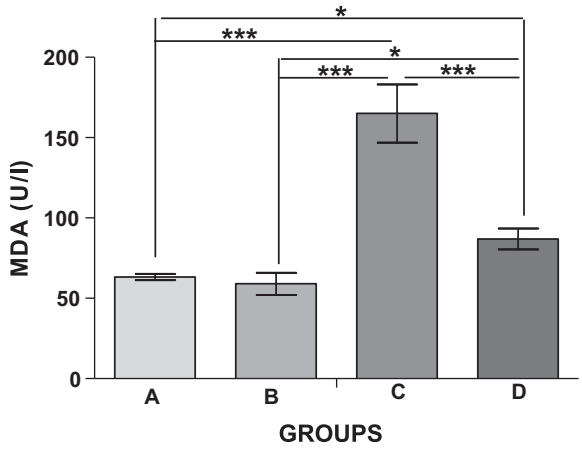

Fig. 7. Cerebellar levels of malondialdehyde. A = control, B = *Moringa oleifera*, C = nicotine and D = nicotine and *Moringa*. * and *** are significant levels of difference at $p < 0.05$ and $p < 0.005$ respectively.

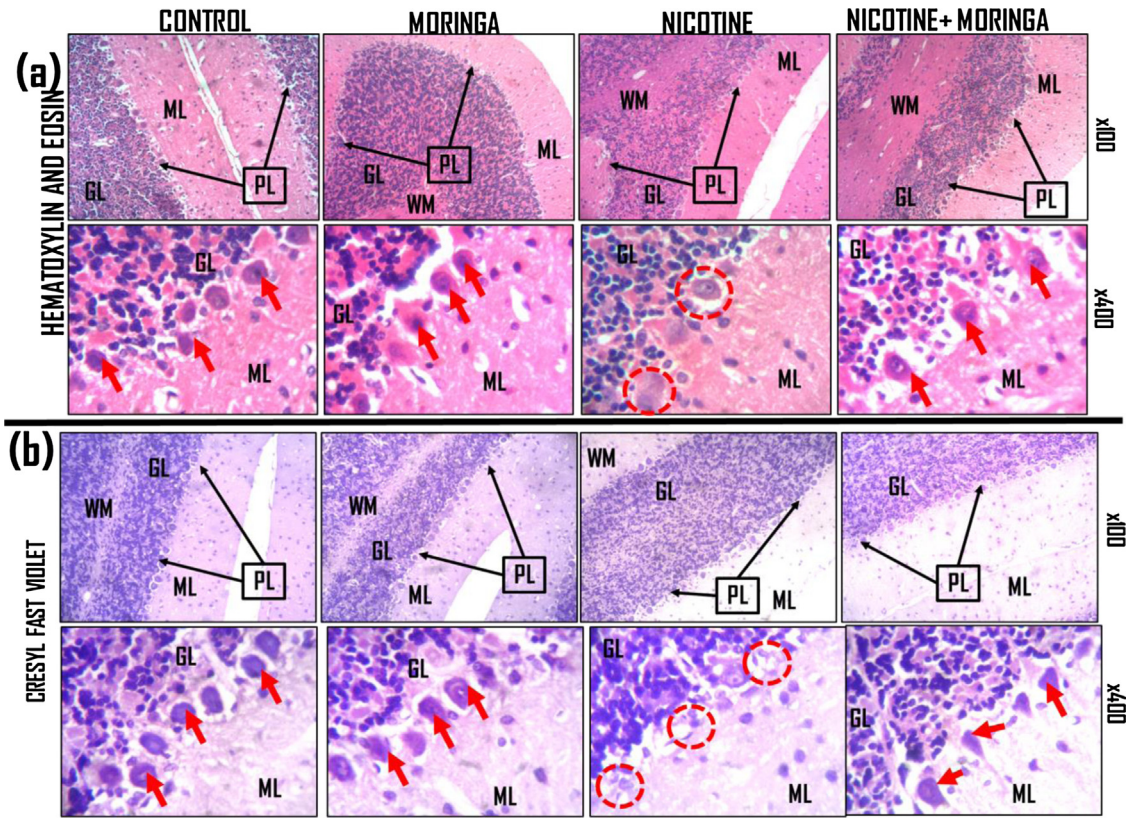

Fig. 8. a and b: Representative photomicrographs showing the histomorphology and Nissl profile of the cerebellum of Wistar rats. ML = molecular layer, GL = granular layer, PL = Purkinje cell layer and WM = white mater. Fig. 8a shows panoramic view and high power magnification of the general histology cerebellum of treated rats. Deeply stained and characteristically normal cellular layering of the granule cell layers with well-outlined Purkinje cell layers can be seen in control, Moringa and nicotine + Moringa groups. The Purkinje cell layer in the nicotine group is distorted and poorly outlined in the lower magnification. At a higher magnification (×400), control, Moringa and nicotine + Moringa group show large Purkinje cell (red arrows) with axons jetting into the molecular layer, whereas the Purkinje cells in the Purkinje layer in the nicotine group appear degenerated as they are laconically expressed (dotted red circles). Fig. 8b shows the Nissl profile of the cerebellum of Wistar rats stained by Cresyl fast violet stain. At a panoramic view (×100), all the micrographs appear to have the same staining intensity and cellular density within the granular layer. Meanwhile, at a higher power magnification (×400), the Purkinje cells of the control moringa and nicotine + moringa are deeply stained and well expressed (red arrow). Nicotine group present with chromatolytic Purkinje cell (dotted red circles). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the poorly stained Nissl proteins within Purkinje cells. The observed intense chromatolytic change in the Purkinje cells was prevented by the actions of *Moringa* in the group that received a combined treatment of Nicotine and MO (Fig. 8b).

4. Discussion

Exposure to nicotine caused various degrees of alterations in the cytoarchitechture of the cerebellar cortex, including severe cellular chromatolysis. The chromatolytic and apoptotic changes in the cerebellar cortices of rats treated with only nicotine was more pronounced in the Purkinje cell layer, where apparent reduction in cell population was observed. Earlier studies reported that nicotine exposure adversely affects the development of Purkinje cells of the cerebellum, with reduced number of cells [20,21]. One of the mechanisms by which nicotine causes damage to neuronal cells is by inducing oxidative stress; nicotine causes an increase in the level of free radicals such as O2− which scavenge electrons from lipids around neuronal membrane [22,23]. Observed changes in neural enzymatic expressions in this study further substantiate the hypothesis that induction of oxidative stress underlies the primary mechanism through which nicotine induced cerebellar cytotoxicity. Nicotine was shown to increase generation of superoxide, judging from the observed reduction in the level of SOD in the cerebellum of nicotine-treated rats. The excessive superoxide probably resulted into lipid peroxidation in neurons of the cerebellum which was evident by the observed increase in MDA level. Consequences of nicotine-induced neurochemical alterations in cerebellum of treated rats are spotlighted by the observed morphological degeneration. Interactions between nicotine and nicotinic acetylcholine receptors on the Purkinje cells may successively activate the apoptotic process that leads to the reduced population of Purkinje cells. A previous report showed that the activation of nicotinic receptors by nicotine resulted in apoptotic cell death in primary hippocampal progenitor cells [24], validating the capacity of nicotine in facilitating apoptosis.

It is important to note that the end-point of afferent pathways to the cerebellar cortex is the characteristic Purkinje cell [25]. The modulation of cerebellar output also occurs at the level of the Purkinje cells which may be responsible for the motor learning aspect of cerebellar functions [25,26]. Therefore, affectation of Purkinje neurons by nicotine ingestion may result into circuit degeneration within the cerebellum of rats, which may thus account for the altered motor activities observed in behavioral characterization.

Neurons in the pontine nuclei collect a projection from the cerebral cortex and then convey the information to the contralateral cerebellar cortex. The axons from the pontine nuclei and other sources called mossy fibers synapse on granule cells in the granule cell layer of the cerebellar cortex. The cerebellar granule cells rise to specialized axons called parallel fibers that ascend to the molecular layer of the cerebellar cortex where they bifurcate to form T-shaped branches that relay information via excitatory synapses onto the dendritic spines of the Purkinje cells. Correspondingly, rats treated with nicotine manifested deficits in the behavioral tests carried

out in this study. The open field test for locomotor activities [26] showed a significant reduction in the number of lines crossed, rearing frequency and rearing duration when compared to the control and MO treated groups. Meanwhile, in line with findings of this study, an earlier study also found that nicotine ingestion causes postural imbalance in smokers [7].

Furthermore, the current study showed that concomitant administration of MO with nicotine prevented severe chromatolysis and apoptosis in the cerebellar cortex, and as well inhibited locomotive deficits observed in rats treated with nicotine only. This might be due to the anti-oxidant properties of *Moringa*, as earlier studies suggested that the protective effects of MO extract could be attributed to the presence of phytoconstituents that scavenge free radicals and activate endogenous antioxidant enzymes [27,28]. Similarly, at the appropriate dose, it has been shown that *Moringa* prevents possible nicotine-induced neuronal cell damage by boosting the antioxidant status of neuronal cells [29].

The antioxidant capacity manifested by MO in the present study is due to the presence of various types of antioxidant compounds such as ascorbic acid, flavonoids, phenolics and carotenoids [30,31]. Quercetin is of particular interest. It contains phenolic hydroxy groups with antioxidant action with documented therapeutic uses [32]. In fact, studies have shown that quercetin like other flavonoids strongly inhibit the production of both reactive oxygen and nitrogen species [33]. Luteolin present in MO extract also has a strong antioxidant activity and exhibits a protective capability on DNA [34]. Luteolin has free radical scavenging and anti-inflammatory capacities [35].

5. Conclusion

In conclusion, our study showed that MO possesses potent phytoactive constituents that can suppress morphological changes in nicotine-induced cerebellar toxicity. A major highlight of the cytoprotective potentials of MO is seen in its ability to boost the antioxidant status of neuronal cells, thereby counteracting nicotine neurotoxicity and maintaining the integrity of the cerebellar neurons.

Conflict of interest

No conflict of interest declared.

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