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Quercetin or Rosemary extract mitigates manganese chloride-induced neurotoxicity through regulation of DNA methylation and histone acetylation and alleviation of apoptosis in rats

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ABSTRACT

Manganese (Mn) is a necessary trace mineral, but imbalanced levels in the body can lead to neurotoxicity. The neurotoxicity of manganese chloride ($MnCl_2$) is associated with dopaminergic neurodegeneration, oxidative damage and neuro-inflammation. This study was conducted to assess the neuroprotective effects of quercetin or rosemary extract on neurotoxicity induced by $MnCl_2$ in rats. Twenty-eight male albino rats were separated into four identical groups. G1(normal control): Rats were provided with purified water. G2 ($MnCl_2$): Rats were orally administered $MnCl_2$ at a dose of 1/25 LD_{50} (59.36 mg/kg b.wt) five times a week for six consecutive weeks. G3 ($MnCl_2$+ Quercetin): Rats were given $MnCl_2$ (59.36 mg/kg b.wt) along with Quercetin (50 mg/kg b.wt/day). G4 ($MnCl_2$+ Rosemary extract): Rats were given $MnCl_2$ (59.36 mg/kg b.wt) along with Rosemary extract (200 mg/kg b.wt/day). Results indicated that a significant upregulation of HAT1, HDAC1, and Phosphatidylinositol 3 kinase (PI3K) gene expression with Global DNA hyper-methylation were observed in brain of $MnCl_2$ exposed rats. Meanwhile, Quercetin or Rosemary extract co-treatment with $MnCl_2$ induce significant downregulation of HAT1, HDAC1 and PI3K expression with major Global DNA hypo-methylation in the brain of rats. Additionally, treating manganese-exposed rats with quercetin or Rosemary extract also resulted in the preservation of the brain's histological structure. These results suggest that quercetin and rosemary can modulate alterations in histone acetylation in rats brain cells when exposed to manganese through their antioxidant, anti-inflammatory, and anti-apoptotic properties.

Introduction

Manganese (Mn) is an essential trace element necessary for maintaining optimal brain health in humans. It plays a crucial role in the metabolism of proteins, fats, and carbohydrates, as well as serving as a cofactor for various antioxidant enzymes such as glutamine synthetase and superoxide dismutase. It is well recognized that prolonged manganese exposure can cause neurotoxicity (Takeda, 2003). Long-term manganese exposure is linked to neurotoxicity and the development of several neurological conditions. Oxidative stress-induced apoptosis is a recognized mechanism of cell death in manganese toxicity. The epigenetic mechanism controlling gene regulation could be altered by oxidative stress (Tarale *et al*., 2016)

Manganese can overexpress α-synuclein. The overexpressed α-synuclein enters the nucleus, attaches to histone protein (p300), and prevents HAT action, which results in DNA fragmentation and apoptosis (Jin *et al*., 2011). α-Synuclein regulates apoptosis through an epigenetically mediated interaction with histone proteins. Global DNA hypomethylation is also brought on by α-synuclein by cytoplasmically sequestering DNA methyltransferase (Tarale *et al*., 2016).

Exposure to neurotoxic metals can cause changes in DNA methylation, histone modification, and/or non-coding RNA, which are the mechanisms responsible for epigenetic alterations. Gene expression can be inhibited by DNA methylation through hypermethylation or activated through hypomethylation (Ijomone *et al*., 2020). An increasing amount of research has connected environmental contaminants to epigenetic variations, such as alterations to histone modifications, DNA methylation status, and other elements including miRNA incorporation and nucleosome remodeling. Histone regulatory enzymes, such as HATs, HDACs, histone methyltransferases (HMTs), and histone demethylases (HDMs), control two of the most researched histone modifications: acetylation and methylation (Ranjan and Sharma, 2016). Acute exposure to Mn can cause histone modification by upregulating the expression and activity of histone deacetylase (HDAC) and lowering that of histone acetyltransferase (HAT) in vitro (Streifel *et al*., 2013).

Flavonoids are secondary metabolites found in plants, and they are essential to their survival because they perform physiological functions and give them resistance against environmental stresses. Quercetin, found in a variety of plants such as onions, citrus fruits, and parsley, is a well-known bioflavonoid. It is classified as a flavone due to its chromen-4-one structural structure and five hydroxy groups (Rajesh and Dhanaraj, 2023). Quercetin is considered a highly effective chemoprotective substance in animal studies due to its various biological and pharmacological properties, such as anti-inflammatory, anti-carcinogenic, antiviral, and anti-apoptotic effects, making it promising in combating the harmful impacts of environmental pollutants (Murakami *et al*., 2008). Quercetin has the ability to regulate the toxicity caused by manganese in rats on the brain-pituitary-testicular axis by utilizing its antioxidant, anti-inflammatory, and anti-apoptotic characteristics (Adedara *et al*., 2017)

Additionally, Herbal medicines have also been considered as safe, alternate therapy for toxicities. Rosemary (*Rosmarinus officinalis*), is one common household plant, has several phytochemicals, such as rosmarinic acid, betulinic acid, ursolic acid, camphor, caffeic acid, and antioxidants including carnosic acid (Akela *et al*., 2018). Extracts from rosemary leaves display various biological activities in Vitro, including anti-inflammatory, antithrombotic, antiviral, antinociceptive, antioxidant, antibacterial, anti-tumor, antiulcerogenic, antidiuretic, and neuroprotective effects (Tousson *et al*., 2019). The molecular mechanisms that produce Mn-induced neurotoxicity are unknown. The purpose of this study was to explore the possible protective benefits of Quercetin and Rosemary leaf extract, nat-

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ural products, against neurotoxicity and epigenetic alterations induced by manganese chloride in rats through the evaluation of HAT, HDAC, Global DNA methylation, and histopathological examination of brain tissue.

Materials and methods

Experimental Animals

Twenty-eight male albino rats, weighing from 150 to 200 grams and aged four to five weeks, were acquired from the Laboratory Animals Research Center at Faculty of Veterinary Medicine, Benha University, Egypt. The animals were housed in stainless steel cages with a 12-hour light and dark cycle, maintained at a temperature of 23±2°C, and with a relative humidity ranging from 50 to 70%. Water and food were available at all times. The rats were housed for a period of fifteen days before the experiment began. The Experimental protocol was conducted following the guidelines of the Institutional Animals Care and Use Committee, approved by the Research Ethics Committee of the Faculty of Veterinary Medicine at Benha University (BUFVTM 07-02-23).

Chemicals and natural agents

The chemicals and natural antioxidants used in this study were:
Manganese chloride (99%): Manganese chloride tetrahydrate (MnC_{l2}. $4H_2O$) was Manufactured by Advent Chembio Pvt. Ltd, Navi Mumbai, India. Rats were given manganese orally for a period of six weeks at a dose of 1/25 LD50 (59.36 mg/kg b.wt) diluted in distilled water and administered five times per week (Vez´er *et al*., 2005).
Quercetin: Dihydrate of 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one and dehydrate of 3,3',4',5,7-pentahydroxyflavone. Quercetin was obtained from Aktin Chemicals, Inc., a company in Chengdu, China that specializes in promoting natural health products.

Preparation of Quercetin stock solution

About 200 mg of Quercetin was mixed with 1 ml of dimethyl sulfoxide (DMSO). After that, 0.5 ml of the Quercetin DMSO solution was combined with 1 ml of Tween 80 and then diluted with normal saline until the total volume reached 7 ml. Each rat received 1 ml of this solution orally Rats were administered a daily dose of (50 mg/kg body weight) quercetin for six weeks. The quercetin was dissolved in DMSO, Tween 80, and normal saline solution (Indap *et al*., 2006).
Rosemary: Rosemary was purchased from Al-Harraz Co., for Agriculture Seeds, Herbs and Medicinal plants, Cairo, Egypt.

Preparation of Rosemary leaves extract

To prevent the decomposition of the chemical contents, nearly 250 grams of dried rosemary leaves were finely crushed into powder. The plant powder was then mixed with ethanol (70% ethanol and 30% water) in a sealed container, and the mixture was left at room temperature for a minimum of three days. Then, the mixture was filtered to create a liquid extract, which was then concentrated at 50°C and low pressure using a rotary evaporator. This procedure was repeated a minimum of three times. Finally, the extract was measured and kept at 20°C until required (Abdel-Gawad *et al*., 2021).

Design of experimental work

The rats were separated into four groups, with each group consisting of seven rats.
G1: 1 (Normal control): The rats were provided with distilled water.
G2: (Manganese chloride): Rats were administered Manganese chloride at a dose of 1/25 LD_{50} (59.36 mg/kg body weight) orally, five days a week for a duration of 6 weeks.
G3: (Manganese chloride + Quercetin): Rats were given quercetin orally at a dose of (50 mg/kg b.wt per day) for six weeks, along with five weekly doses of manganese chloride at a dose of 59.36 mg/kg b.wt.
G4: (Manganese chloride + Rosemary extract): Rats were administered Rosemary extract daily for six consecutive weeks at a dose of 200 mg/kg body weight orally (Al-Attar and Shawush, 2014), along with five weekly doses of Manganese chloride at a dose of 59.36 mg/kg body weight orally.

Brain specimens

Rats were euthanized in accordance with Animal Ethics Committees after the 6-week experiment, then the skull was carefully opened and the brains were immediately removed. After being dissected, the brain tissue specimens were split into two parts. The first part was rinsed with sterile saline solution to remove any blood cells and clot, then transferred to Eppendorf tubes, rapidly kept in liquid nitrogen, and stored at -80°C until RNA extraction for gene expression analysis using RT-PCR. The remaining part of the brain was fixed in 10% formalin for histopathological analysis.

Molecular analysis

The levels of mRNA expression for Histone acetyltransferases (HAT1), Histone deacetylase (HDAC1), and Phosphatidylinositol 3 kinase (PI3K) genes in the rat brain were quantified using real-time quantitative polymerase chain reaction analysis (RT-qPCR) (Table 1). Pure RNA was extracted from brain tissues using a complete RNA purification kit (Thermo Scientific, Fermentas, #K0731) following the manufacturer's instructions. To generate cDNA samples, reverse transcription was carried out using the Revert Aid TM First Strand CDNA synthesis kit (#EP0451, Thermo Scientific, Fermentas, USA). Real-time PCR with SYBR Green and gene-specific primers, following the manufacturer's protocol (Thermo Scientific, USA, # K0221), was performed to assess gene expression levels. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used to normalize the target gene expression levels, using the 2-ΔΔCt method.

Moreover, assessment of global DNA methylation was conducted using the Colorimetric Base Catalog # P-1030 method. The level of global DNA methylation was determined using the MethylFlashTM Global DNA Methylation (5-mC) ELISA Easy Kit from EpiGentek in Farmingdale, NY, USA, following the protocol by Li *et al*. (2018).

Histopathological examination

Liver tissue specimens were examined using histopathology after being preserved in a solution of 10% neutral buffered formalin, as described by Bancroft and Gamble (2008). After being properly fixed, the specimens

Table 1. Sequences of forward and reverse primers for genes used in RT- qPCR.

Gene	Forward primer (/5 ------ /3)	Reverse primer (/5 ------ /3)
HAT1	CGACTGCTGGTGACTGACAT	TGCTCGTGCTGCACACTTAT
HDAC1	GCGAGCAAGATGGCGCAGACT	GTGAGGCTTCATTGGGTGCCCT
PI3K	AACACAGAAGACCAATACTC	TTCGCCATCTACCACTAC
GAPDH	CAACTCCCTCAAGATTGTCAGCAA	GGCATGGACTGTGGTCATGA

were dehydrated using different concentrations of ethanol, then cleared with xylene, embedded in paraffin, and carefully blocked. The specimens were cut into sections measuring 5 µm for analysis under a microscope, and then stained with hematoxylin and eosin (H&E).

Statistical analysis

The data was presented using means±SEM. An analysis of variance (ANOVA) was conducted using SPSS 18.0 software version in 2011 to determine the statistical significance, with Duncan's multiple range test (DMRT) utilized for individual comparisons. A p-value of ≤ 0.05 was considered statistically significant.

Results

The results of the qPCR analysis presented in Table 2 showed a significant upregulation in the expression of Histone acetyltransferases (HAT1), Histone deacetylase (HDAC1), and Phosphatidylinositol 3 kinase (PI3K) genes in the brains of rats exposed to manganese chloride compared to the control group. However, the gene expression levels of HAT1, HDAC1, and PI3K were significantly downregulated in rats treated with quercetin or rosemary extract after manganese intoxication when compared to untreated rats.

The results presented in Table 3 demonstrated a significant global DNA hypermethylation in rats exposed to Manganese chloride compared to the control group. In contrast, treatment with Quercetin or Rosemary extract in Manganese chloride-intoxicated rats resulted in significant global DNA hypomethylation when compared to manganese chloride untreated group.

Table 2. Protective impact of Quercetin or Rosemary extract on the relative expression of HAT1, HDAC1 and PI3K genes in the brain of rats intoxicated with Manganese chloride.

Parameters Animal groups	HAT1 Fold change	HDAC1 Fold change	PI3K Fold change
G1: Normal control	1.00^{d}±0.00	1.00^{d}±0.00	1.00^{d}±0.00
G2: Manganese chloride	5.39^{a}±0.22	7.26^{a}±0.34	6.50^{a}±0.27
G3: Manganese chloride + Quercetin	3.25^{b}±0.14	3.25^{b}±0.12	3.68^{b}±0.15
G4: Manganese chloride + Rosemary extract	1.95^{c}±0.10	2.04^{c}±0.09	1.87^{c}±0.11

Data is displayed as Mean±Standard Error of the Mean (SEM). Means within the same column carrying different superscript letters are significantly different (P≤ 0.05).

Table 3. Effects of Quercetin or Rosemary extract administration on the Global DNA methylation level in the brains of rats exposed to Manganese chloride.

Parameter Animal groups	Global DNA methylation 5-mC (%)
G1: Normal control	0.77^{d}±0.04
G2: Manganese chloride	2.65^{a}±0.11
G3: Manganese chloride + Quercetin	1.80^{b}±0.08
G4: Manganese chloride + Rosemary extract	1.32^{c}±0.06

The data is displayed as Mean±Standard Error of the Mean (SEM). Means within the same column carrying different superscript letters are significantly different (P≤ 0.05).

Histopathological findings

The brain of control group displayed a typical histological appearance of the cerebellar cortex, with the molecular layer being the outermost, a dense neuropil network with many axonal and dendritic processes. The layer of Purkinje cells is located at the border of the deeper interior granular cell layer and the molecular layer (Fig. 1A, B). Meanwhile, the brain of toxic group treated with manganese chloride showed degenerating neurons in the Purkinje cell layer characterized by darkly pigmented pyknotic nuclei and shrinking cell bodies and bright eosinophilic cytoplasm. Few cells became significantly distorted, with an indistinct in both form and content in the form of vacuolated cytoplasm as well as the absence of their nuclei. The granule cells were arranged together in large gaps, seemed smaller in size, and had pyknotic darkly pigmented nuclei (Fig. 1C). Furthermore, the overlying molecular layer showed irregular neuropil vacuolation including shrunken eosinophilic neurons with pyknotic nuclei (Fig. 1D).

Microscopic analysis of the brains of rats given quercetin and manganese chloride exhibited mild improvement in the brain histological appearance in comparable with that of the manganese chloride treated group. The brain revealed nearly normal structure of cerebellum with mild vacuolation characterized by presence of large, clear, round vacuoles in the Purkinje cell and molecular cell layer. Additionally, a few necrotic neurons were also noticed (Fig. 2A, B). Meanwhile, the examined brain of rats in manganese chloride and rosemary group demonstrated considerable restoration of the normal cerebellar architecture as compared to intoxicated rats (Fig. 2C, D).

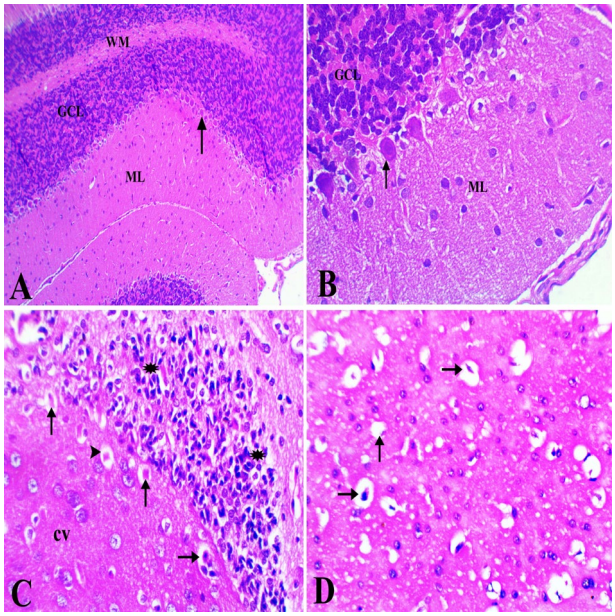

Fig. 1. Brain sections of control (A, B), and toxic (C, D) groups, H&E. Control group showing normal histological appearance of cerebellum contains a sparsely cellular molecular layer (ML), a Purkinje cell layer (arrow), a granular cell layer (GCL), and white matter (WM) (A, x100& B, x400). (C) Toxic group showing degenerating Purkinje neurons (arrow) with darkly stained pyknotic nuclei and bright eosinophilic cytoplasm. Also notes, complete degeneration of few Purkinje cells (arrowhead) with vacuolated cytoplasm and disappearance of its nucleus. The granule cells appear shrunken with pyknotic darkly stained nuclei and clumped in groups (asterisk) with wide spaces in between (x400), (D) The molecular layer showing irregular neuropil vacuolation (arrow) with shrunken eosinophilic neurons having pyknotic nuclei (x400).

Discussion

Human neuronal physiology and cognition are linked to changes in Mn homeostasis, and neurological dysfunctions can result from either overexposure or, less frequently, deficiency (Balachandran *et al.*, 2020). There are several hypothesized pathways for Mn neurotoxicity, including oxidative stress, mitochondrial damage, and functional alterations in neurotransmission (Aschner and Erikson, 2017). High Mn levels cause oxidative stress because they promote excessive ROS formation that goes be

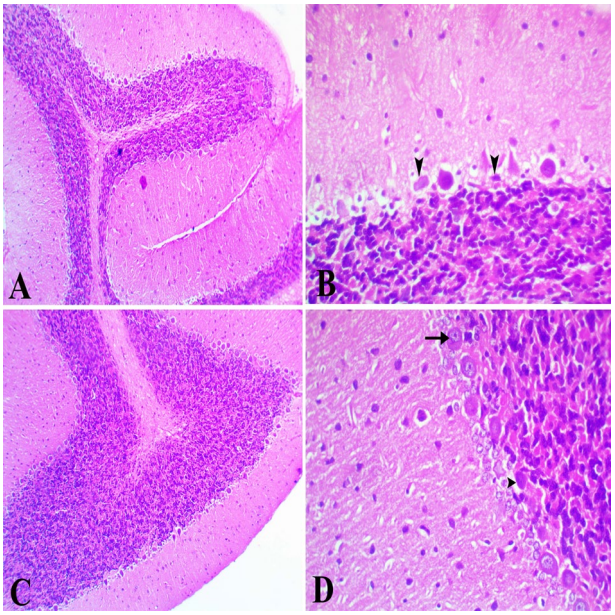

Fig. 2. Brain sections of treated groups, (A, B) manganese chloride+ quercetin, and (C, D) manganese chloride+ rosemary, H&E. (A) nearly normal layers of cerebellum (x100), (B) few neurons appear shrunken with deeply stained pyknotic nuclei (arrowhead) (x400). (C) normal cerebellar architecture (x100), (D) normal Purkinje cells with pale vesicular nucleus and prominent deeply stained marinating nucleolus (arrow). Only a few cells are seen shrunken with deeply stained pyknotic nucleus (arrowhead) (x400).

yond physiological limits (Owumi and Dim, 2019). Specific cell signaling pathways, such as mitogen-activated protein kinases (MAPK), caspases, and protein kinase C (PKC), are triggered when reactive oxygen species (ROS) have a significant impact on neuronal cell death. The disruption of Mn antioxidant activity makes the brain more susceptible to Mn-induced oxidative stress. This is especially true in synaptic clefts and dopaminergic-rich areas of the brain (Harischandra *et al*., 2019). The results obtained showed a considerable upregulation of Histone acetyltransferases (HAT1), Histone deacetylase (HDAC1) and Phosphatidylinositol 3 kinase (PI3K) gene expression gene expressions in $MnCl_2$ exposed rats. By raising HDAC activity and lowering HAT activity, manganese chloride might prevent the histones acetylation of H3 and H4, which ultimately results in cell damage and apoptosis in neural cells. $MnCl_2$ effect on histone deacetylase (HDAC) and histone acetyltransferase (HAT) was investigated by Guoa *et al*. (2018). who demonstrated that in PC12 cells, $MnCl_2$ enhanced HDAC activity while decreasing HAT activity. In neuronal cells, $MnCl_2$ induced histone hypoacetylation by increasing the activity and protein levels of HDAC while decreasing the activity and protein levels of HAT.

$MnCl_2$ was found to block the acetylation of core histones in cell culture models of Parkinson's disease (PD). Additionally, trichostatin A (TSA) inhibition of HDAC activity has shown to be protective against cell death caused by manganese, indicating that histone acetylation could play a crucial role in manganese-induced damage to dopaminergic neurons. These results raised the possibility that manganese-induced dopaminergic neuron injury may be mediated through blocking histone acetylation. Histone alteration has also been shown to occur after Mn exposure. During the hypo-acetylation process in neurons, $MnCl_2$ is responsible for increasing the activity and protein expression of HDAC; and decreasing the activity and protein expression of HAT (Guo *et al*., 2018). Zhang *et al*. (2017) found that enhanced histone acetylation could play a role in higher levels of reactive oxygen species (ROS) and lower levels of glutathione in neurons. This may be due to the fact that when histone acetylation is present. This could be because when histone acetylation is present brain cells exposed to Mn exhibit higher levels of Nrf2/HO-1 pathway expression. Magnesium is necessary for many physiological activities because it is an enzyme cofactor in different processes such as energy production, reproduction, and protection against cellular stress through antioxidants (Tuschl *et al*., 2013). Hence, our suggestion was that $MnCl_2$ might inhibit HAT activity and histone acetylation in the same way. The catalytic role of HDACs and other deacetylases is dependent on a single transition metal ion. Studies have demonstrated that apart from the Zn2+ binding site of HDAC enzymes, the catalytic metal ion binding site in histone deacetylases is similar to the Mn2+ binding site. This indicates that the use of $MnCl_2$ may enhance the activity of HDAC enzymes (Patrick *et al*., 2011).

Chronic Mn exposure activates Phosphatidylinositol 3 kinase (PI3K) / Serine-threonine protein kinase (Akt) {PI3K/Akt}signaling pathway in hippocampus of rats. PI3K is an intracellular kinase that regulates cell development, differentiation, and apoptosis and is linked to oxidative stress (Vanhaesebroeck *et al*., 2016). A critical mediator of cell survival signaling initiated by PI3K is the serine-threonine protein kinase Akt. It shows anti-apoptotic activity by preventing the release of cytochrome C from mitochondria and inhibiting Forkhead box (FOX) transcription factors (TFs) (Song *et al*., 2005). Furthermore, FoxO3a, BAD, caspase-9, NF-kappa B, and other downstream target proteins are among the proteins that Akt can either activate or inhibit (Exil *et al*., 2014). Cheng *et al*. (2019) demonstrated that rats' hippocampal PI3K/Akt signaling pathway is activated by prolonged exposure to Mn. The researchers noticed a decrease in the levels of Akt-1 and FoxO3a in mRNA and an increase in Akt phosphorylation to p-Akt. This suggested that long-term exposure to Mn could activate PI3K/Akt signaling through p-Akt, thereby preventing the transcriptional activation of genes related to apoptosis. Moreover, Xiaofei *et al*. (2022) investigated the role of the PTEN/PI3K/AKT Signaling Pathway in inducing liver cell apoptosis in chickens exposed to manganese toxicity. The signaling pathway known as PTEN/PI3K/AKT plays a crucial role in regulating both cell division and programmed cell death. The Mn-induced apoptotic damage is controlled by the signaling pathway PTEN/PI3K/AKT. Who stated that the expression of the anti-apoptotic gene Bcl-2 decreased after exposure to Mn, while the expression of eleven pro-apoptotic genes (RIP1, RIP3, MLKL, Bax, Caspase-3, FADD, Cyt-C, ERK, JNK, Caspase-8, and P38) increased after exposure to Mn. This indicates that exposure to Mn caused apoptosis in the liver cells of chicks and that the molecular mechanism of apoptosis caused by an excess of Mn included the PTEN/PI3K/AKT signaling pathway. In addition, Cheng *et al*. (2019) investigated the impact of prolonged exposure to manganese sulfate (MnSO4) on the regulation of the PI3K/Akt signaling pathway in rats. Who revealed that prolonged exposure to manganese sulfate can lead to the programmed cell death of central nervous system cells, trigger the PI3K/Akt signaling pathway in the hippocampal region of rats, and increase the transcription and translation of Hsp70.

The existing finding showed significant hyper methylation of global DNA was observed in Manganese chloride exposed rats. Exposure to manganese results in the generation of reactive oxygen species (ROS) through redox cycling, which ultimately causes oxidative stress. The oxidative stress induced by manganese is responsible for the death of dopaminergic cells and malfunction of mitochondria (Farina *et al*., 2013). Studies have indicated that oxidative stress triggers DNA abnormalities such as changes in chromosome structure, modifications in DNA bases, and deletions. This may impact the ability of DNA methyltransferase to effectively utilize DNA as a substrate, consequently disrupting overall or gene-specific methylation patterns (Donkena *et al*., 2010). The global DNA methylation microarray profiling of the hippocampal dentate gyrus after manganese exposure showed increased methylation of multiple genes related to differentiation processes (Ijomone *et al*., 2020). These results imply that disruptions in epigenetic mechanisms may play a role in influencing hippocampal neurogenesis following manganese exposure. Furthermore, DNA methylation does not occur randomly but rather as a response to various environmental factors like exposure to heavy metals (Jordan *et al*., 2017). Following manganese exposure, there was global DNA methylation. The microarray profiling of the hippocampal dentate gyrus showed that several genes linked to developmental processes were hypermethylated (Ijomone *et al*., 2020). This indicates that disturbances in epigenetic processes could impact the response of hippocampal neurogenesis to manganese exposure. Furthermore, exposure to heavy metals is one of several environmental triggers that cause DNA methylation, which is not a spontaneous process (Jordan *et al*., 2017). Demethylation of DNA has also been discovered lately, as shown by the closure of the covalent bond that once bound the methyl group and the 5′ carbon atom of a cytosine ring. Shen *et al*. (2014) indicated that there are two potential reasons for this demethylation process: either DNMT1 is absent during recurrent replication, or enzymes are actively involved in removing or altering methyl groups from cytosine, resulting in the formation of cytosine without any methyl modifications.

Conversely, treatment with Quercetin or Rosemary extract to manganese exposed rats showed significant downregulation in HAT1, HDAC1, and PI3K gene expression with global DNA hypo methylation in brain as compared with manganese non-treated group. Certain natural phytochemicals with chemopreventive capacities may be able to act as epigenetic regulators. Quercetin, a dietary supplement derived from natural flavonoid glycosides, has been associated with various health benefits such as anti-inflammatory, anticancer, antiapoptotic, antihypertensive, and neuroprotective effects. Quercetin is capable of influencing both conventional biochemical signaling pathways and epigenetic networks. Curcumin has been shown to reduce the activity of histone acetyltransferase as well as potentially inhibit DNA methyltransferase and histone deacetylase (HDAC) activity (Zhu *et al*., 2023). According to a study by Kanwal *et al*. (2016), it was discovered that quercetin has the potential to influence both liver cell metabolism and DNMT activity, as shown in both in vivo and in vitro experiments. In a study conducted by Liu *et al*. (2015), it was shown that quercetin can impact the Nrf2/HO-1 and p38/STAT1/

NF-κB pathway, consequently leading to the inhibition of inflammation and DNA methylation caused by nickel. Furthermore, quercetin seems to be a strong protective agent for the liver in response to inflammation caused by nickel exposure, with its effectiveness related to its ability to control the Nrf2 nuclear translocation and the activity of HO-1. In addition, quercetin has been shown to prevent global hypermethylation, including reducing DNA hypomethylation of CpG islands at the promoter of Nrf2 in the livers of mice (Liu *et al*., 2015). Also, quercetin treatment of human cervical cancer cells for 24 and 48 hours at 25 and 50 μM decreased the activity of HDACs via interacting with the catalytic domains of DNMTs, leading to overall levels of DNA hypomethylation. Quercetin and sodium butyrate have complementary effects on the growth and metastasis of esophageal cancer tumors. This synergy is linked to the downregulation of HDAC1, DNMT1, NF-κBp65, and cyclin D1, which act as histone deacetylase inhibitors through the HDAC-NF-κB pathway (Zheng *et al*., 2014).

Moreover, the administration of quercetin resulted in a decrease in the levels of DNMT1 and DNMT3a in both mRNA and protein levels in vitro and in vivo, as shown in xenograft models (Alvarez *et al*., 2018). Kedhari Sundaram *et al*. (2019) also found that treating U937 and HL60 cell lines with quercetin at 50 μmol for 48 hours led to a decrease in the expression of DNMT1 and DNMT3a. When human cervical cancer cells were treated with quercetin at 25 and 50 μM for 24 and 48 hours, there was a reduction in HDACs activity due to quercetin's interaction with the catalytic domains of DNMTs, resulting in DNA hypomethylation. The combination of quercetin and sodium butyrate has been shown to have synergistic effects on the growth and metastasis of esophageal cancer by downregulating HDAC1, DNMT1, NF-κBp65, and cyclin D1 through the HDAC-NF-κB pathway, acting as histone deacetylase inhibitors (Zheng *et al*., 2014).

Additionally, Quercetin is capable of crossing the blood-brain barrier and acting as a neuroprotectant, reducing the rate of brain cell death following focal cerebral ischemia (Paula *et al*., 2019). The neuroprotective effects of quercetin are associated with its ability to activate Akt signaling (Lei *et al*., 2015), which inhibits apoptosis (Yao *et al*., 2012), oxidative stress (Ishisaka *et al*., 2011), and neuro-inflammation (Rinwa and Kumar, 2013). Quercetin has been demonstrated in several disease models to initiate HAT and reduce HDACs via regulation of gene expression through epigenetic mechanisms. Quercetin has been proposed to exert a neuroprotective effect in the brain and hippocampus of ovariectomy rats via inhibiting HDACs and activating ERK. Moreover, quercetin helps to maintain the levels of HAT and HDAC and can reverse the activation of genes associated with neuroplasticity. In general, quercetin was found to mitigate the cognitive decline induced by ovariectomy by controlling the balance of histone acetylation (Aggarwal *et al*., 2020).

Rosmarinic acid (RA), the primary phenolic compound found in rosemary, is utilized in various products such as tea, oil, and medicine for its proven anti-inflammatory, antioxidant, and anti-cancer properties. Rosmanol prevented p38 MAPK, PI3K, and ERK1/2 from being activated in response to lipopolysaccharide (LPS) (Lai *et al*., 2009). Gene expression is controlled through epigenetic processes involving histone methyltransferases (HMTs) and DNA methyltransferases (DNMTs), which methylate both histone proteins and DNA (Kanwal *et al*., 2016). They suggest that dietary flavones can affect the activities of DNMT, HMT, histone protein methylation, and DNA methylation, all of which regulate epigenetic changes. Their potential anticancer effects could be attributed to their ability to modify the epigenetic processes associated with cancer development. Similarly, S-adenosyl-L-methionine (SAM) is utilized by certain DNA methyltransferases (DNMTs) as a source of methyl groups to catalyze the DNA methylation process. Tumor suppressor genes such BRCA1, CDKN2A/p16, RASSF1A, and GSTP1 are repressed by this mechanism (Baxter *et al*., 2014). Similar to this, histone methyltransferases (HMTs) catalyze the histones methylation at the lysine amino acid residues of H3 and H4 by using the methyl group donor SAM (Momparler *et al*., 2014). HDACs are enzymes that eliminate acetyl groups from histones, which is essential for gene expression (Jang *et al*., 2018). HATs and HDACs, two types of enzymes, work in balance to carefully regulate protein acetylation in a cell to maintain cellular homeostasis (Gujral *et al*., 2020). HATs are involved in the genesis of various diseases and govern basic cellular activities within the cell. Particularly, advancement is associated with dysregulated HAT activity (Cheng *et al*., 2019).

It's also critical to note that histopathology results further supported all of the earlier biochemical molecular findings. Histopathological investigation of the cerebellar cortex of the brain tissue in manganese chloride-intoxicated rats showed numerous histological alterations, involving degenerating Purkinje neurons with darkly stained pyknotic nuclei. Also notes, complete degeneration of few Purkinje cells with vacuolated cytoplasm and disappearance of its nucleus. These results were parallel to those of Opoola and Ajibade (2023), who revealed loss and degeneration of neurons, especially Purkinje cells, in the cortical layers of the groups treated with manganese as compared with normal cerebella histoarchitecture in the control rats. According to them, wistar rats given manganese chloride showed neurodegenerative alterations in the cortical layers of the cerebellum, which may have an adverse effect on certain cerebellar functions. Also, Aboutaleb *et al*. (2021) and Abu-Elfotuh *et al*. (2022) reported that, manganese chloride induced nuclear pyknosis and highly degeneration in the neurons of the cerebral cortex and the fascia dentate. Additionally, $MnCl_2$ induced neuronal degeneration in the caudate nucleus, putamen, globus pallidus, and substantia nigra, as well as morphological changes in neurons in the frontal cortex, hippocampus, midbrain and pons (Yamada *et al*., 1986). Experiments conducted in vitro and in vivo have indicated that neuroinflammation and oxidative stress are significant mediators of Mn-caused neurodegeneration (Milatovic *et al*., 2009; Sriram *et al*., 2010).

Conversely, microscopic examination of the brains of rats given quercetin and manganese chloride displayed mild improvement in the brain histological appearance in comparable with that of the manganese chloride treated group. The brain revealed a nearly normal structure of the cerebellum with mild vacuolation characterized by the presence of large, clear, round vacuoles in the Purkinje cell and molecular cell layer, indicating that these natural substances could ameliorate the structural changes induced by $MnCl_2$. Similar results were reported by Aboutaleb *et al*. (2021), also, Mehany *et al*. (2022) noted that, administering quercetin during environmental changes (high altitude, low oxygen, and low pressure) led to a mild improvement in the histological picture of the brain in rats. The rat cerebellar cortex's histological structure, where the majority of Purkinje cells have maintained their normal characteristics, reflects the antioxidant and anti-inflammatory impact of quercetin (Alsemeh *et al*., 2019). Antioxidants are elements that prevent and interrupt oxidation of substrates, even though they are present in low amounts. In addition to scavenging free radicals, nutritional antioxidants reduce peroxide levels, repair oxide membranes, and reduce reactive oxygen species (Flora, 2009). Also, there has been evidence that antioxidants control gene expression and regulate signal pathways, preventing cell death (Young and Woodside 2001). Moreover, a number of anti-inflammatory agents have been shown to reduce the neurotoxicity of Mn (Santos *et al*., 2012). This may be connected to the finding that the pathophysiology of Mn-induced toxicity involves inflammatory mechanisms (Milatovic *et al*., 2009; Sriram *et al*., 2010). Additionally, the investigated brain of rats in manganese chloride and rosemary group demonstrated considerable restoration of the normal cerebellar architecture as compared to manganese intoxicated rats, which is in accordance with findings performed by (Lahouel *et al*., 2020; Al-Safo and Al-Duliami 2022). Rosemary leaf extract has also been shown to heal tissue damage in brain tissues, due to its high concentration of phenolic compounds, the brain is a best place to start (Cui *et al*., 2018).

Conclusion

The results of this study suggest that exposure to $MnCl_2$ could be harmful to neurons and lead to alterations in intracellular PI3K, DNA methylation, and histone acetylation. However, Quercetin and rosemary extract have been shown to lower HDAC activity and protect against brain cell apoptosis caused by exposure to manganese. This indicates that histone acetylation plays a significant role in the development of manganese-induced neurotoxicity. Further research is needed to investigate the relationship between manganese-induced neurotoxicity and epigenetic alterations, which will help in developing accurate prognosis and treatment strategies.

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Conflict of interest

The authors declare that they have no conflict of interest.

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