

# Manganese Induces Oxidative Stress, Redox State Unbalance and Disrupts Membrane Bound ATPases on Murine Neuroblastoma Cells In Vitro: Protective Role of Silymarin

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**Abstract** Manganese (Mn) is an essential trace element required for ubiquitous enzymatic reactions. Chronic over-exposure to this metal may promote potent neurotoxic effects. The mechanism of Mn toxicity is not well established, but several studies indicate that oxidative stress play major roles in the Mn-induced neurodegenerative processes. Silymarin (SIL) has antioxidant properties and stabilizes intracellular antioxidant defense systems. The aim of this study was to evaluate the toxic effects of MnCl<sub>2</sub> on the mouse neuroblastoma cell lines (Neuro-2A), to characterize the toxic mechanism associated with Mn exposure and to investigate whether SIL could efficiently protect against neurotoxicity induced by Mn. A significant increase in LDH release activity was observed in Neuro-2A cells associated with a significant decrease in cellular viability upon 24 h exposure to MnCl<sub>2</sub> at concentrations of 200 and 800  $\mu$ M ( $P < 0.05$ ) when compared with control unexposed cells. In addition, exposure cells to MnCl<sub>2</sub> (200 and 800  $\mu$ M), increases oxidant biomarkers and alters enzymatic and non enzymatic antioxidant systems. SIL treatment significantly

reduced the levels of LDH, nitric oxide, reactive oxygen species and the oxidants/antioxidants balance in Neuro-2A cells as compared to Mn-exposed cells. These results suggested that silymarin is a powerful antioxidant through a mechanism related to its antioxidant activity, able to interfere with radical-mediated cell death. SIL may be useful in diseases known to be aggravated by reactive oxygen species and in the development of novel treatments for neurodegenerative disorders such as Alzheimer or Parkinson diseases.

**Keywords** Manganese · Oxidative stress · Neuro-2A cells · Silymarin · Glutathione

## Introduction

Chronic environmental or occupational exposure to manganese (Mn) in mammals is identified as a dystonic neurodegenerative movement disorders that results from excessive accumulation of Mn in multiple brain regions and presents with neurologic symptoms resembling feature of Parkinson diseases [1]. Mn exposure can cause motor deficits in mammals that are characterized by extrapyramidal motor abnormalities, such as rigidity, hypokinesia, tremor, and behavioural disturbances [2]. Although the pathological changes and symptoms of Mn exposure are well known, the primary mechanisms of neurotoxicity have yet to be established. Several mechanisms of Mn neurotoxicity have been proposed, including disruption of mitochondrial metabolism [3], disturbance of neurotransmitter metabolism [4], alteration of iron homeostasis [5] and induction of oxidative stress via direct or indirect formation of free radicals in both neurons and astrocytes [6–8]. Upon entering cells via facilitated diffusion and receptor-gated cation channels [9], Mn avidly accumulates

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in mitochondria [10] and inhibits the complexes of the electron transport chain [11], which impairs oxidative phosphorylation and ATP production [12]. The resulting decreased energy production and alters mitochondrial permeability transition, leading to excessive  $\text{Ca}^{2+}$  influx and massive reactive oxygen species (ROS) production [13]. Moreover, superoxide, produced in the mitochondrial electron transport chain (ETC.) [14], may catalyze the transition shift of  $\text{Mn}^{2+}$ – $\text{Mn}^{3+}$  through a set of reactions similar to those mediated by superoxide dismutase and thus lead to the increased oxidant capacity of this metal [15].

Knowing that oxidative stress plays a central role in Mn-induced neurotoxicity, several authors have investigated the potential neuroprotective effect of antioxidants against Mn-induced toxicity. In mitochondrial preparations, N-acetylcysteine (NAC), glutathione, and vitamin C prevent ROS production caused by high concentrations of Mn [16]. In cultured astrocytes, Chen and Liao [17] observe that NAC attenuates the pro-oxidant effects of Mn. Ex vivo, Hazell et al. [18] report that NAC blocks the astrocytosis caused by acute exposure to Manganese.

Though several synthetic antioxidants are available, a growing trend has been targeted toward the use of natural products (polyphenols, flavonoids, vitamins, carotenes and lycopenes) as antioxidants in view of toxicity condemn [19]. Bioflavonoids are the ubiquitous group of polyphenolic substances that are present in most plants [20]. Silymarin is a flavonoid isolated from the fruits and seeds of the milk thistle (*S. marianum*). It is classified as a benzopyranone [21] consisting of a mixture of polyphenolic flavonoid like silibin, isosilybin, silydianin and silychristin. It has been reported to possess multiple pharmacological activities and considered as an anti-hepatotoxic agent for the treatment of various diseases [22]. In addition several recent studies have shown the potential hepatopreventive and therapeutic efficacy of silymarin in different animal models and cell culture systems [23, 24]. Its positive effects have been ascribed to the putative anti-oxidant, anti-inflammatory and anti-proliferative properties based on the modulation of specific signalling pathway, transcription factor and gene expression [25]. But its potential protection for central nervous system (CNS) and the mechanisms of action are still unclear.

In the current study, we established the mechanism of SIL-mediated attenuation of reactive oxygen species (ROS) generation and the possible involvement of its neuroprotection against Mn toxicity. The preventive effect of silymarin against oxidative stress promoted by Mn was studied by assessing biochemical parameters in Neuro-2A cell, in order to evaluate enzymatic antioxidant defenses, hydrogen peroxide content, glutathione redox, lipid peroxidation and protein carbonyl content. We further addressed whether the neuroprotective effects of SIL against Mn toxicity were mediated via their antioxidant properties.

## Materials and Methods

### Materials

Lactate deshydrogenase (LDH) kit was purchased from Biomaghreb ref. 20012 (Ariana, Tunis, Tunisia). Minimum Essential Medium (MEM) was purchased from Invitrogen (Glasgow, UK), foetal bovine serum (FBS) was provided by Gibco (Invitrogen, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 5,50-dithiobis-(2-nitrobenzoic acid) (DTNB), Silymarin and all other chemical products used in this study were obtained from Sigma Chemical Co. (St. Louis, France). Culture plates and flasks were purchased from IWAKI (Japan).

### Cell Culture

Neuro-2A mouse neuroblastoma cell line, obtained from the ATCC (CCL-131), was routinely grown in 75 cm<sup>2</sup> flasks and maintained in MEM supplemented with 10% FBS, 3,500 units/ml polymyxin B sulfate, 1,400 units/ml neomycine sulfate and 7.5 mg/ml streptomycin base. Cultures were maintained in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. Cell dissociation was achieved with 0.05% trypsin-0.02% verséne (triplex III). Briefly, cells were seeded on 96, 24 and 6-well culture plates in medium at an approximate density of  $1 \times 10^5$  cells/ml. After 24 h stabilization, neuronal cells were co-cultured with medium containing various concentrations of  $\text{MnCl}_2$  (200 and 800  $\mu\text{M}$ ) and/or silymarin (10, 50, and 100  $\mu\text{M}$ ) for 24 h. The concentration of  $\text{MnCl}_2$  was selected based on previously reported cytotoxic levels in cultured cells [26]. For stock solution,  $\text{MnCl}_2$  was dissolved in MilliQ Plus sterilized water at the concentration of 800 mM. Silymarin was dissolved in dimethylsulfoxide (DMSO) to obtain 100 mM. The experimental concentrations were freshly prepared in the basal medium with a final DMSO concentration 0.1%.

### Cell Viability Assay and Morphological Assessment in Neuro-2A Cells

Cell viability was determined colorimetrically using the MTT reduction assay. Neuro-2A cells were seeded in 96-well plate ( $1 \times 10^5$  cells/well) for 24 h and were exposed following the above cell treatment protocol. Briefly, at the end of treatment, 10  $\mu\text{l}$  of MTT [5 mg/ml in phosphate buffered saline (PBS)] solution were added to each well and the plates were incubated at 37°C for additional 4 h. Then, the medium containing MTT was removed and 200  $\mu\text{l}$  of DMSO was added to each well to achieve solubilization of the formazan crystal formed in viable cells. The absorbance was read at 570 nm with DMSO used as the blank. Cell viability was expressed as a

percentage of the control culture. Changes on the cell morphology were observed with an Olympus model (CK2, Japan) inverted phase-contrast microscope fitted with a digital camera to capture images.

### LDH Assay

Membrane integrity was evaluated by measuring the LDH activity in the culture medium of control and treated cells. After 24 h treatment, the culture medium was removed and the LDH activity was measured following the manufacturer's protocol (Biomagreb, ref. 20012 Ariana, Tunis, Tunisia). The percent of LDH activity released was determined as percent of enzyme activity in the incubation medium to the total LDH activity. The absence of the viable cells corresponds to 100% of LDH activity in the incubation medium.

### Measurement of Intracellular ROS

The production of intracellular ROS was assessed by 2',7'-dichlorofluorescein diacetate (DCFDA) oxidation. Twenty-four hours after treatment with SIL and/or Mn, cells were incubated with 20  $\mu$ M DCFH-DA at 37°C for 1 h and washed twice with PBS. The fluorescence intensity of DCF was measured in a microplate-reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Results were expressed as percentage of controls.

### Measurement of Cellular H<sub>2</sub>O<sub>2</sub>

The H<sub>2</sub>O<sub>2</sub> concentration was measured in Neuro-2A by a modification of the ferrous oxidation-xylenol orange (FOX1 reagent) assay adapted to microtiter plates [27, 28]. 50  $\mu$ l of Neuro-2A cells ( $1.0 \times 10^6$  cells/ml) were added to 950  $\mu$ l of FOX 1 reagent and incubated for 30 min at room temperature. This assay is based on the ability of H<sub>2</sub>O<sub>2</sub> to oxidize the ferrous Fe<sup>2+</sup>-ions to the ferric Fe<sup>3+</sup>-ions which react with xylenol orange to a coloured complex. The absorbance of the samples was read at 570 nm and the concentration of H<sub>2</sub>O<sub>2</sub> was determined using standard peroxide solutions in the same microtiter plate.

### Determination of Stress Markers and Antioxidant Enzymes

#### *Preparation of the Cellular Extracts*

After the different treatments, Neuro-2A cells were trypsinized and harvested. The pellets obtained after centrifugation (2 min, 1,500 rpm, 4°C) were resuspended and washed twice in isotonic Tris-HCl buffer (20 mM, pH 7.4). Cells suspension was lysed by 5 freeze-thaw cycles (liquid

nitrogen  $-37^\circ\text{C}$ ). After centrifugation of the lysates at  $8,000 \times g$  for 10 min at 4°C they were stored at  $-80^\circ\text{C}$ , until analysis.

#### *Assay of Antioxidant Enzymes*

Catalase (CAT) activity was assayed by the decomposition of hydrogen peroxide according to the method of Aebi [29]. A decrease in absorbance due to H<sub>2</sub>O<sub>2</sub> degradation was monitored at 240 nm for 1 min and the enzyme activity was expressed as  $\mu\text{mol H}_2\text{O}_2$  consumed/min/mg protein.

Superoxide dismutase SOD (MnSOD and Cu/ZnSOD) activities were evaluated by measuring the inhibition of pyrogallol activity as described by Marklund and Marklund [30]. Based on the competition between pyrogallol oxidation by superoxide radicals and superoxide dismutation by SOD. The specific Cu/Zn-SOD inhibition by potassium cyanide allowed the Mn-SOD determination in the same conditions. Assays were monitored by spectrophotometry at 420 nm. One unit (U) corresponded to the enzyme activity required to inhibit the half of pyrogallol oxidation. SOD activity was expressed as U/mg protein.

Glutathione peroxidase (GPx) activity was measured according to Flohe and Gunzler [31]. The enzyme activity was expressed as nmoles of GSH oxidized/min/mg protein.

#### *Assays of Oxidant Markers*

*Thiol, Disulfide (SH/SS Redox State) and GSH Assays* Thiols (SH) and disulfides (SS) were determined according to Zahler and Cleland [32]. The method is based on the reaction of DTNB with the samples producing a yellow product, thionitrobenzoic acid (TNB). The disulfide content was calculated through the difference between the two determinations. The SH/S-S ratio was also calculated. Results were expressed as nmol TNB/mg protein.

Total Cellular Glutathione (GSH) levels were determined as described previously by Fairbanks and Klee [33]. Distilled water was used as blank and standards were prepared from GSH. The slope of the reaction rate was used to calculate the GSH content.

*Lipid Peroxidation End Products and Protein Carbonyl Assays* The extent of lipid peroxidation in Neuro-2A cell homogenates, was determined by measuring the release of thiobarbituric acid reactive substance (TBARS) in terms of malondialdehyde (MDA) formation according to Draper and Hadley method [34]. Results were expressed as nmol of MDA/mg of cellular protein.

The protein carbonyl PCO content of Neuro-2A homogenates was determined according to the method of Evans et al. [35]. Results were expressed as nmol DNPH conjugated/mg protein.

### Assay of $\text{Na}^+\text{-K}^+\text{-ATPase}$ and $\text{Ca}^{2+}\text{-ATPase}$ Activities

$\text{Na}^+\text{-K}^+\text{-ATPase}$  and  $\text{Ca}^{2+}\text{-ATPase}$  were measured as previously described [16, 36]. The inorganic phosphate–molybdate complex was quantitated by spectrophotometer at 690 nm. The  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity was calculated as the difference between the total ATPase activity and the ATPase activity inhibited by ouabain, the specific-inhibitor of  $\text{Na}^+\text{-K}^+\text{-ATPase}$ . In the presence of  $\text{Ca}^{2+}$ ,  $\text{Ca}^{2+}\text{-ATPase}$  activity appeared and was measured in the presence of  $\text{Ca}^{2+}$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$  ions and ouabain. The ATPase activity was expressed as  $\mu\text{mol Pi/hour/mg}$  of protein.

### Measurement of Nitrite, Tumor Necrosis Factor $\alpha$ (TNF $\alpha$ ) and Interleukin 6 (IL-6)

Neuro-2A cells were seeded on 24-well plates. After being treated with SIL and/or Mn, the supernatants were collected and stored at  $-80^\circ\text{C}$  until assays. As an indicator of nitric oxide production, the amount of nitrite accumulated in culture supernatant was determined with a colorimetric assay using Griess reagent as previously described [37]. The concentration of nitrite in samples was determined using a sodium nitrite standard curve.

TNF $\alpha$  and IL-6 levels were detected by mouse TNF $\alpha$  and IL-6 enzyme-linked immunosorbent assay sandwich method (ELISA) kits according to the procedures provided by the manufacturers. Microtiter (96-well) plates were coated with anti-mouse IL-6 and TNF- $\alpha$  (BD Pharmingen) in coating buffer at  $4^\circ\text{C}$  overnight. After the plates were blocked and washed, 100  $\mu\text{l}$  supernatant was added, and plates were incubated for 1 h at room temperature. The plates were then washed and treated with biotinylated antibodies against IL-6 and TNF- $\alpha$ , then Extravidin—peroxidase conjugate (Sigma). A standard curve generated by using purified recombinant IL-6, and TNF- $\alpha$  was used to determine cytokine concentration.

### Protein Assay

The protein concentration was determined using the BCA<sup>TM</sup> Bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA) with bovine serum albumin as a standard.

### Statistical Analysis

Results were expressed as mean  $\pm$  standard deviation (mean  $\pm$  SD). All analysis was carried out with GraphPad Prism 4.02 for Windows (GraphPad Software, San Diego, CA). Significant differences between treatment effects were determined by 1-way ANOVA, followed by Tukey's

post-hoc test for multiple comparisons with statistical significance of  $P < 0.05$ .

## Results

### Protective Effect of SIL on Mn-Induced Toxicity in Neuro-2A Cells

To determine the toxic effect of Mn, the cell viability and cell membrane damage were analysed by MTT and LDH release assay, respectively. Cytotoxicity was proportionally enhanced with increased Mn concentrations (200 and 800  $\mu\text{M}$ ). As illustrated in Fig. 1a, the viability of cells treated with 200 and 800  $\mu\text{M}$   $\text{MnCl}_2$  for 24 h showed a significant dose-dependent decrease ( $P < 0.05$ ) compared to control group. As shown in Fig. 1b, cells treated with the same doses induced significant increase ( $P < 0.05$ ) of the LDH level in the culture medium indicative of over cytotoxicity.

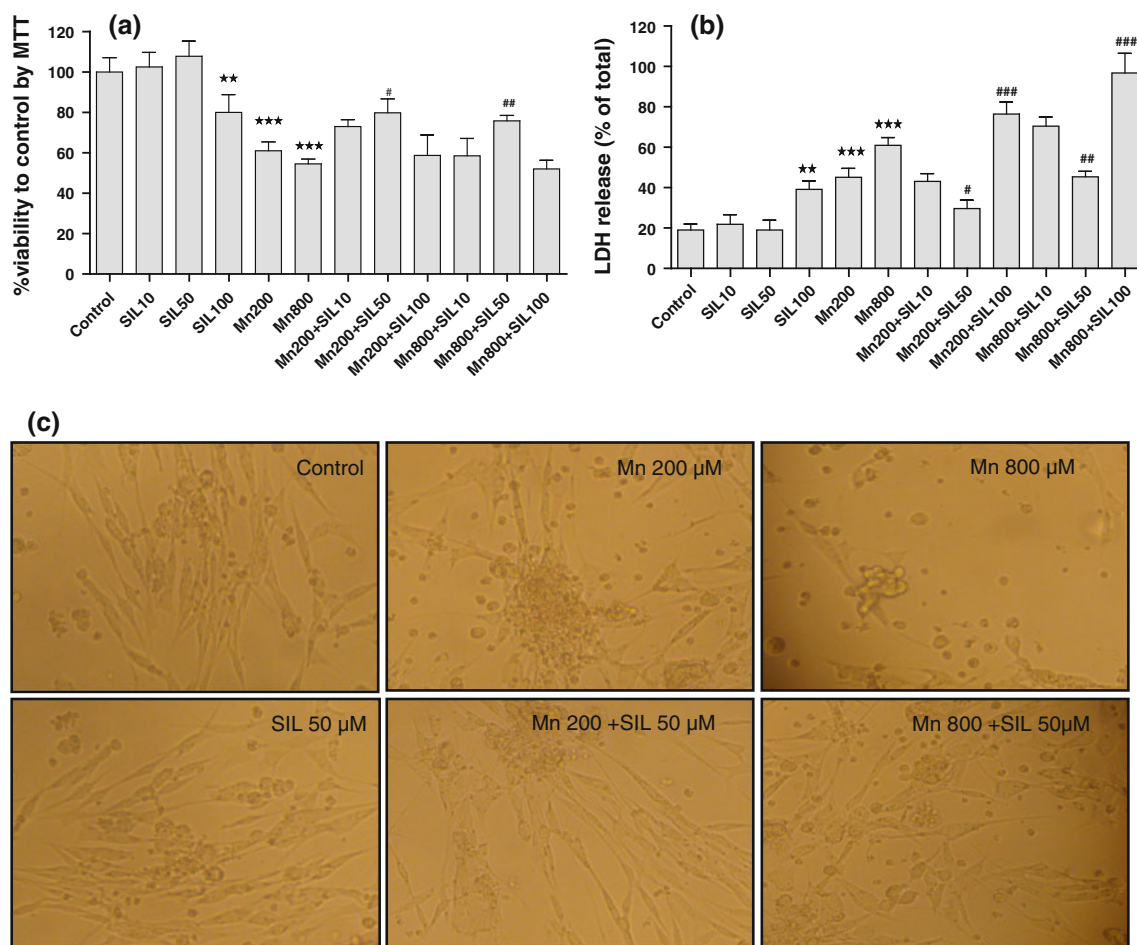
The effect of SIL on Mn-induced toxicity was investigated by testing three concentrations of the flavonoid (10, 50 and 100  $\mu\text{M}$ ). The treatment of Neuro-2A cells with SIL alone (10 and 50  $\mu\text{M}$ ) showed no toxicity after 24 h of exposure. However, the highest concentration of SIL (100  $\mu\text{M}$ ) did not provide significantly greater antioxidant activity than 50  $\mu\text{M}$ , and the culture began to exhibit signs of toxicity with significant reduction ( $P < 0.05$ ) in cell viability compared to that of control. These data showed a dose-dependent effect of SIL with 50  $\mu\text{M}$  evoking the greatest protection against Mn toxicity. Based on those results, this concentration was used in subsequent studies to determine the neuroprotective effects against Mn toxicity.

In addition, we observed the effect of SIL on morphology in Neuro-2A cells treated with Mn. As shown in Fig. 1c, under phase contrast microscopy, untreated Neuro-2A cells were long fusiform in shape with slender processes. After treatment of Mn, cells lost their normal spindle-like morphology, shrank or became round. They were detached and some even floated into the medium. Co-treatment with SIL (50  $\mu\text{M}$ ) markedly ameliorated Mn-induced morphological alterations. Cells treated with SIL alone displayed normal morphology.

### Inhibitory Effect of SIL on ROS Production in Mn-Induced Neuro-2A Cells

Figure 2 presented the inhibitory effect of SIL on ROS production in Mn-induced Neuro-2A cells. Compared to control, treatment of Neuro-2A cells with Mn at 200 and 800  $\mu\text{M}$  for 24 h increased ROS by 30 and 40% ( $P < 0.05$ ) and cellular  $\text{H}_2\text{O}_2$  by 140 and 200%, respectively. However, co-treatment with 50  $\mu\text{M}$  of SIL significantly





**Fig. 1** The effect of silymarin on Mn-induced toxicity in Neuro-2A cells. **(a)** Dose-dependent effects on cell viability was assessed using MTT assay. **(b)** Cell death was quantified by the release of LDH, from Neuro-2A cells into the medium treated with Mn and/or various concentrations of Silymarin exposure for 24 h. **(c)** The morphology

of the treated cells was examined by phase contrast photomicrography using olympus inverted microscope. Data represent the group mean  $\pm$  SD of 6 independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus vehicle control group. # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  versus Mn-treated alone group

reduced the levels of Mn-induced ROS and  $H_2O_2$  production ( $P < 0.05$ ) compared to Mn treated cells. There were no changes in ROS and  $H_2O_2$  levels after treatment with 50  $\mu$ M of SIL alone (Fig. 2).

#### Effect of SIL on Antioxidant Enzyme Activities in Mn-Induced Neuro-2A Cells

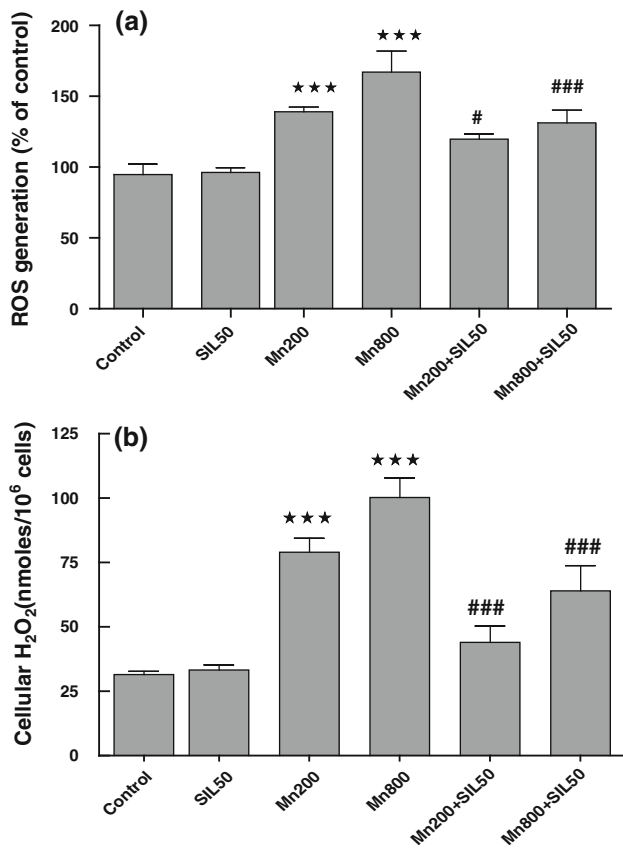
Intracellular levels of antioxidant enzymes such as Mn-SOD, Cu-Zn SOD, CAT and GPx in Neuro-2A cells following exposure to  $MnCl_2$  and SIL for 24 h were measured by spectrophotometric analysis (Table 1). Neuro-2A cells exposed to  $MnCl_2$  showed a significant decrease in the specific activities of Mn-SOD, Cu-Zn SOD, CAT and GPx when compared to cells treated with a vehicle control ( $P < 0.05$ ). Whereas, their activity was increased significantly ( $P < 0.05$ ) when Mn-treated cells were co-cultured with SIL as compared to Mn-treated cells.

#### Effect of SIL on Lipid Peroxidation Product and Protein Carbonylation in Mn-Treated Neuro-2A Cells

We tested next the effect of SIL on the lipid peroxidation product (MDA) and protein carbonyls (PCO) in Mn-treated cells as presented in Fig. 3. A significant increase ( $P < 0.05$ ) in MDA and PCO levels was observed in Mn-treated cells compared to a vehicle control. Co-administration of Silymarin at 50  $\mu$ M significantly decreased ( $P < 0.01$ ) the levels of these parameters. Further, there were no changes in the MDA and protein carbonyl levels after silymarin treatment as compared to control cells.

#### Effect of SIL on ATPase Activity in Mn-Induced Neuro-2A Cells

Table 2 illustrated  $Na^+K^+$ -ATPase and  $Ca^{2+}$  activities in Neuro-2A cells. Mn exposure caused a significant



**Fig. 2** The effects of silymarin on ROS generation were measured by increased fluorescence with the DCFH-DA assay (a) as well as on cellular H<sub>2</sub>O<sub>2</sub> accumulation (b) induced by manganese in Neuro-2A cells. Cells were co-cultured with Mn (200 and 800 μM) and SIL (50 μM) for 24 h. The values are mean ± SD of 6 independent experiments. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 versus vehicle control group. #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 versus Mn-treated alone group

inhibition (*P* < 0.05) in the Na<sup>+</sup>-K<sup>+</sup>-ATPase and Ca<sup>2+</sup> activities in Neuro-2A cells compared to those of control group. Co-incubation cells with SIL at 50 μM significantly (*P* < 0.05) ameliorated the decreasing activities of these enzymes obtained in the Mn-treated cells.

**Table 1** The effects of the Mn exposure (200 and 800 μM) and/or SIL 50 μM on antioxidant enzymes activities in Neuro-2A lysates

	Groups					
	Control	SIL50	Mn200	Mn800	Mn200 + SIL50	Mn800 + SIL50
Mn-SOD	1.43 ± 0.11	1.34 ± 0.10	0.92 ± 0.17***	0.77 ± 0.05***	1.16 ± 0.07 <sup>#</sup>	1.02 ± 0.09 <sup>#</sup>
Cu/Zn-SOD	2.23 ± 0.18	2.45 ± 0.17	1.48 ± 0.21***	1.05 ± 0.12***	1.8 ± 0.16 <sup>#</sup>	1.56 ± 0.14 <sup>##</sup>
CAT	12.75 ± 0.5	13.25 ± 0.44	6.8 ± 0.3***	6.35 ± 0.7***	10.1 ± 0.24 <sup>###</sup>	9.69 ± 0.68 <sup>###</sup>
GPx	8.84 ± 0.7	8.65 ± 0.91	7.25 ± 0.54**	4.62 ± 0.26***	6.05 ± 0.3 <sup>#</sup>	6.00 ± 0.6 <sup>#</sup>

Data are expressed as Mn-SOD and Cu-Zn SOD (unit/mg protein), CAT (μmole H<sub>2</sub>O<sub>2</sub>consumed/min/mg protein) and GPx (nmoles GSH oxidized/min/mg protein)

Results represent mean ± SD (*n* = 6)

\* *P* < 0.05; \*\* *P* < 0.01; \*\*\* *P* < 0.001 versus vehicle control group

# *P* < 0.05, ## *P* < 0.01, ### *P* < 0.001 versus Mn-treated alone group

### Effect of SIL on Thiol (SH), Disulfide (SS) Contents, as Well as on SH/SS Ratio and GSH in Mn-Treated Neuro-2A Cells

The in vitro effect of SIL on SH/SS redox status in Mn-treated cells was studied by measuring thiol and disulfide contents. Table 3 showed that Mn significantly decreased the total thiol content and increased disulfide levels in a dose-dependent manner (*P* < 0.01). The SH/SS ratio was significantly reduced. These effects were significantly mitigated by 50 μM of silymarin compared to those of Mn-treated cells. These biochemical parameters did not differ between control and silymarin treated cells.

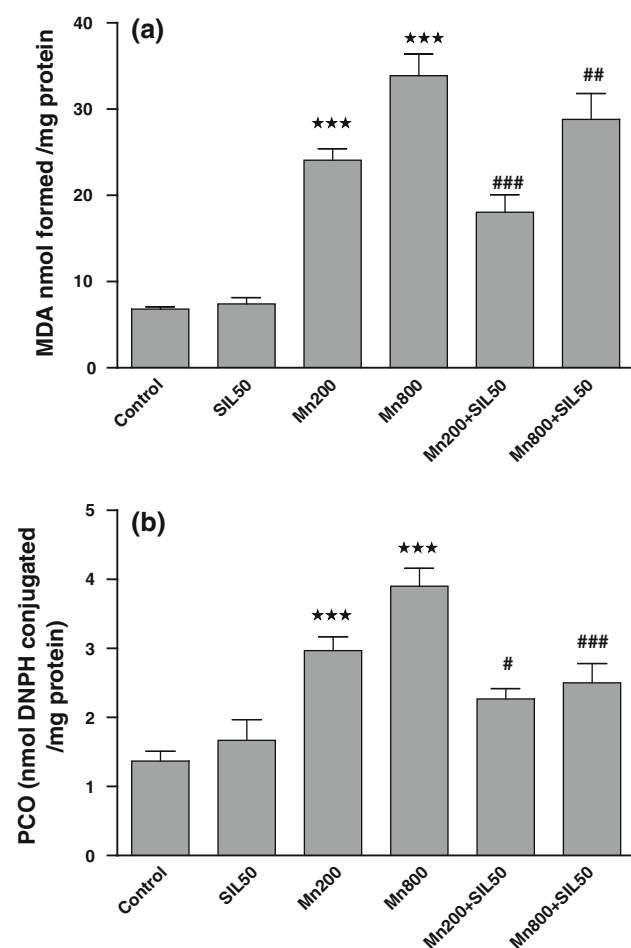
The effects of varying concentrations of MnCl<sub>2</sub> on intracellular GSH were also evaluated and presented in Table 3. Mn treatment led to a significant decrease in GSH level (*P* < 0.05) compared to that of control group. Co-treatment with SIL significantly improved GSH levels in the cell lysates when Neuro-2A cells were treated with Mn only.

### Effect of SIL on MnCl<sub>2</sub> Generated of Proinflammatory Cytokines and Nitrite in Neuro-2A Cells Culture

As shown in Fig. 4, and compared to control cultures, no significant release of either TNFα or IL-6 was detected in the medium of Neuro-2A cells exposed to Mn. Since, the elevated accumulation of nitrite (*P* < 0.05), in a concentration-dependent manner, was observed compared to a vehicle control. Co-incubation of Mn treated cells with SIL significantly attenuated NO levels without reaching control values.

## Discussion

Manganese, 1 of the essential elements for the living body, is required for lipid, protein and carbohydrate metabolism and also in the function and the development of the brain



**Fig. 3** The effects of the Mn exposure (200 and 800  $\mu$ M) and/or SIL (50  $\mu$ M) on MDA and protein carbonyl levels in Neuro-2A lysates. MDA are expressed as nmol formed/mg protein and protein carbonyl (nmol DNPH conjugated/mg protein). Results represent mean  $\pm$  SD ( $n = 6$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus vehicle control group. # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  versus Mn-treated alone group

[38]. Its excessive amounts cause parkinsonian symptoms and which were implicated in the pathogenesis of Parkinson's disease (PD) via several pathways. In vitro models represent an effective system for determining the

mechanisms involved in neurodegenerative effects of Mn. The present study focused on characterising the mode of cell death in Neuro-2A cells treated with Mn and the possible mechanisms in neuroprotective effects of silymarin. To our knowledge, this is the first work which showed that silymarin protected Neuro-2A cells against Mn-induced ROS generation and glutathione depletion.

Neuronal injury was assessed by measuring MTT and LDH released activity. The MTT assay has been used to study neuronal injury, which quantifies mitochondrial activity by measuring the formation of a dark blue formazan product formed by the reduction of the tetrazolium ring of MTT [39]. LDH is an indicator of the integrity of cell membrane, normally existing in the cell was released into the medium from damaged neurons. In our study, Mn used at 200 and 800  $\mu$ M for 24 h induced toxicity of Neuro-2A cells as evidenced by an increase of Lactate dehydrogenase (LDH) release and a decrease of cellular viability (MTT assay) which was supported by morphological examinations. These results are consistent with earlier observations in other cell types [40–42] corroborating that impairment of mitochondria function is one of the key mechanisms in Mn cytotoxicity. However, Gunter et al. [43] demonstrate that  $Mn^{2+}$ , preferentially accumulated in mitochondria, can disrupt oxidative phosphorylation and enhances the generation of reactive oxygen species (ROS). The increase of ROS, as demonstrated by both DCFH-DA and FOX detection of cellular  $H_2O_2$ , support these findings. In our present study, the co-incubation of Neuro-2A cells with silymarin protects them against cytotoxicity induced by Mn. These observations indicate that the neuroprotective effect of silymarin, at least partially, is attributed to its antioxidant and free radical scavenging properties. Similar results report that silymarin decreases lipid peroxidation, a sensitive marker of oxidative stress, in liver microsome and isolated hepatocytes [44]. Furthermore, it has been demonstrated that the antioxidative activity of silymarin is related to the scavenging of free radicals [45, 46].

Oxidative stress occurs when the antioxidant defence system is overwhelmed by the production of reactive

**Table 2** The effects of the Mn exposure (200 and 800  $\mu$ M) and/or SIL 50  $\mu$ M on  $Na^+K^+$ -ATPase and  $Ca^{2+}$ -ATPase activities measured in Neuro-2A cells' homogenates

	Groups					
	Control	SIL50	Mn200	Mn800	Mn200 + SIL50	Mn800 + SIL50
$Na^+K^+$ -ATPase	7.76 $\pm$ 0.17	7.26 $\pm$ 0.84	4.40 $\pm$ 0.39***	3.33 $\pm$ 0.49***	5.83 $\pm$ 0.45#	4.62 $\pm$ 0.39#
$Ca^{2+}$ -ATPase	3.48 $\pm$ 0.35	3.72 $\pm$ 0.15	2.04 $\pm$ 0.12***	1.10 $\pm$ 0.10***	2.58 $\pm$ 0.13#	1.89 $\pm$ 0.8##

ATPase activities are expressed as  $\mu$ mol Pi/mg protein/h

Results represents mean  $\pm$  SD ( $n = 6$ )

\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$  versus vehicle control group

#  $P < 0.05$ , ##  $P < 0.01$ , ###  $P < 0.001$  versus Mn-treated alone group

**Table 3** The effects of the Mn exposure (200 and 800  $\mu\text{M}$ ) and/or SIL 50  $\mu\text{M}$  on intracellular GSH concentration, thiol (SH) and disulfide (SS) contents, and SH/SS ratio measured in Neuro-2A cells homogenates

	Groups					
	Control	SIL50	Mn200	Mn800	M 200 + SIL50	Mn800 + SIL50
GSH	46.44 $\pm$ 4.26	45.07 $\pm$ 2.13	30.53 $\pm$ 3.34***	22.26 $\pm$ 4.37***	34.92 $\pm$ 3.50####	33.64 $\pm$ 3.93#
SH content	25.38 $\pm$ 3.25	25.05 $\pm$ 3.17	19.90 $\pm$ 0.15***	16.5 $\pm$ 0.49***	22.40 $\pm$ 0.70#	20.51 $\pm$ 0.35####
SS content	5.43 $\pm$ 0.36	5.91 $\pm$ 0.35	10.45 $\pm$ 0.17***	14.90 $\pm$ 1.25***	8.25 $\pm$ 0.93##	10.45 $\pm$ 0.65####
SH/SS ratio	4.96 $\pm$ 0.29	5.05 $\pm$ 0.25	2.00 $\pm$ 0.18***	1.25 $\pm$ 0.40***	2.48 $\pm$ 0.13#	2.25 $\pm$ 0.11####

Data are expressed as GSH ( $\mu\text{mol}/10^6$  cells), SH and SS (nmol TNB/mg protein)

Results represent mean  $\pm$  SD ( $n = 6$ )

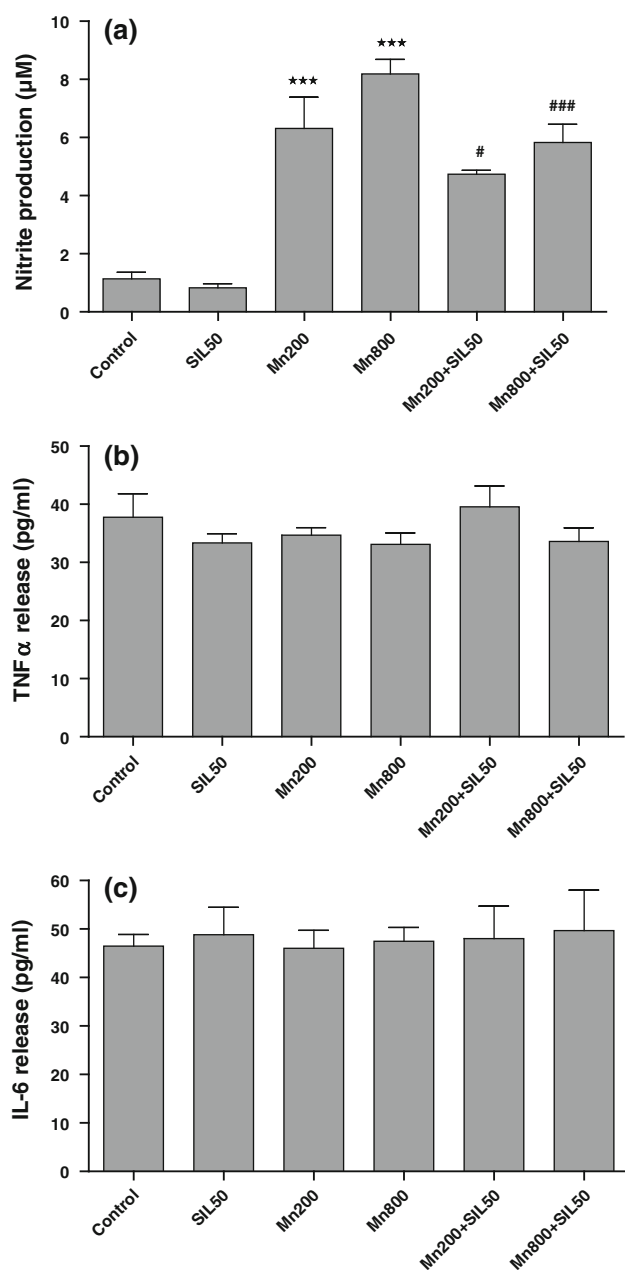
\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$  versus vehicle control group

#  $P < 0.05$ , ##  $P < 0.01$ , ####  $P < 0.001$  versus Mn-treated alone group

oxygen species. The latter can induce lipid peroxidation and protein oxidation, hence potentially causing cell death [47, 48]. Therefore, increasing the antioxidant capacity of neurons will provide a potential strategy to protect neurons from Mn-induced oxidative damage. In this study, cells treated with Mn increased significantly MDA and protein carbonyl levels with a significant depressed level in the activity of the antioxidant enzymes, including isoforms of SOD, CAT and GPx in Neuro-2A cells. These results indicated that the cytotoxic actions of Mn in Neuro-2A cells were mediated, at least in part, by an oxyradical mechanism involving overproduction of ROS and down regulation of certain key antioxidant enzymes. Both events would lead to oxidative stress in Mn-exposed cells. Treatment with silymarin improved MDA, protein carbonyl levels and antioxidant enzyme activities. Based on these findings, it is possible that silymarin might be able to down regulate Mn-induced oxidative damages through the inhibition of ROS generation and indirectly by activation of antioxidative system including GSH biosynthesis. One of the defense mechanisms that protect the cell from oxidative damage is the dismutation of superoxide anion radical to hydrogen peroxide by SOD which is further converted to water by GPx, using GSH as a substrate. The generated hydrogen peroxide can be converted in spontaneous reaction catalyzed by metals such as iron (Fenton reaction) to the highly reactive hydroxyl radical, reacting with various biological macromolecules including polyunsaturated fatty acids by which the process of lipid peroxidation is initiated [49]. In our study, a clear-cut enhancement of GPx activity caused by SIL might conceal an expected increase in GSH levels. As a result, this facilitated defense mechanism shifts the conversion of hydrogen peroxide to water thereby reducing the formation of highly reactive hydroxyl radical, and eventually leading to a lipid peroxidation decrease and hydrogen peroxide accumulation.

Previous studies demonstrate that hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) can provoke cellular injuries, including the oxidation of redox sensitive cysteinyl thiols in proteins [50, 51]. In oxidative conditions, S-nitrosation is a reversible modification able to protect protein cysteinyl thiols against unwanted and possibly irreversible oxidation into sulfenic, sulfinic and sulfonic acids and intra/intermolecular disulfide bonds [52, 53]. As an abundant cellular antioxidant, GSH, like its precursors, has a protective action in oxidative stress conditions. In cells, GSH is oxidized to the GSSG disulfide by the action of GSH-peroxidase through a mechanism that consumes  $\text{H}_2\text{O}_2$  [54]. In its S-nitrosated form, S-nitroso-glutathione (GSNO), GSH could act as a transporter and a stable reservoir of NO [55], as does Cysteine (Cys) [56]. Redox sensitive Cysteine residues of cellular proteins ( $-\text{SH}$ ) become S-oxidized ( $-\text{SOx}$ ) providing an important non-enzymatic cellular means to eliminate  $\text{H}_2\text{O}_2$  [50]. Excessive oxidation can however, severely damage proteins by the formation of irreversible S-S bonds and the conversion of SH groups into sulfinic and sulfonic acids. These attacks on sulfhydryls have been detected in Mn-treated cells. GSH was reported to provide cell protection possibly by consuming  $\text{H}_2\text{O}_2$ , generating extracellular GSSG and protein S-glutathionylated adducts ( $-\text{SSG}$ ) [57] and thus decreasing the amount of oxidant that can attack sulfhydryls. Considering that the total thiol content was significantly reduced by Mn while disulfide levels were enhanced, a significant reduction in SH/SS ratio was observed, suggesting that Mn was able to disturb the thiol redox status in Neuro-2A cells lysates. Given the numerous processes that are dependent upon cellular redox state, alteration of SH/SS ratio is considered as an important parameter indicative of oxidative stress. GSH is used to detoxify ROS and itself is converted to the oxidized form, GSSG. Our results are in accordance with previous observation which demonstrates the reduction of total GSH in the SK-N-SH (neuron-like) cells induced by manganese





**Fig. 4** The effects of the Mn exposure (200 and 800  $\mu\text{M}$ ) and/or SIL (50  $\mu\text{M}$ ) on the production of nitrite (a), TNF $\alpha$  (b), and IL-6 (c). The data are expressed as mean  $\pm$  SD from triplicate assays. \*\*\* $P < 0.001$  versus vehicle control group. # $P < 0.05$ , ### $P < 0.001$  versus Mn-treated alone group

[58]. Several explanations by which silymarin regulates Mn-induced oxidative stress remain possible: (1) As a polyphenolic flavonoid, silymarin has a strong free radical-scavenging activity [46]. It reacts with a damaging free radical and forms a flavonoid radical, with a great stability and breaks the free radical chain reaction [59]. SIL, having potential antioxidative properties is known for its anti-lipid peroxidative capability against a variety of oxidative stress and thus can suppress oxidative damage [60–62]. (2) The

neurotoxicity of Mn is mediated by forming  $\text{H}_2\text{O}_2$ , which generates lipid peroxidation adducts and protein carbonyl modifications in various cellular compartments. Incubation with the  $\text{Fe}^{3+}$  chelator desferrioxamine has been shown to decrease the toxicity of Mn. It is possible that silymarin attenuates the Mn-induced oxidative stress by chelating metal ions [63, 64]. (3) Oxidative stress occurs when the antioxidative defenses are broken down by the overproduction of reactive oxygen species. Expanding the antioxidant capacity of neuronal cells will provide a potential strategy to protect neurones from oxidative damage [65, 66]. Silymarin is known to induce the expression of antioxidative enzymes [67, 68]. Therefore, it is possible that silymarin alleviates oxidative damage indirectly by activation of antioxidative system including GSH biosynthesis.

Free radicals can attack polyunsaturated fatty acids (PUFA) in the biomembrane and initiate lipid peroxide chain reactions, leading to membrane dysfunction thereby destroying his special arrangement and impairing local enzyme activities.  $\text{Na}^+\text{-K}^+\text{-ATPase}$ , a membrane-bound enzyme responsible for maintaining  $\text{Na}^+$  and  $\text{K}^+$  gradients across the plasma cell membranes [69], was reported to be inhibited by ROS in primary cultured neurons [70]. ATP-driven plasma membrane calcium-pump ( $\text{Ca}^{2+}\text{-ATPase}$ ), of crucial importance in maintaining a low resting intraneuronal  $\text{Ca}^{2+}$  particularly in excitable cells such as neurons, is involved in the final common pathway leading to cell death [71]. The decrease in the activities of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  and  $\text{Ca}^{2+}\text{-ATPase}$  observed in Neuro-2A cells supports these findings. Previous studies have found that  $\text{Mn}^{2+}$  could alter the balance between the simultaneous uptake and release of  $\text{Ca}^{2+}$  across the mitochondrial inner membrane [72]. In fact, it has been shown that oxidative stress increases intracellular  $\text{Ca}^{2+}$  concentrations leading to activation of endonucleases which degrade DNA and, ultimately, contribute to cell death. Furthermore, it has been demonstrated that ROS overproduction may inhibit  $\text{Ca}^{2+}\text{-ATPases}$  and lead to alter regulation of  $\text{Ca}^{2+}$  levels and cell death. In the current study, we found that the co-incubation of Neuro-2A cells with silymarin effectively alleviated the decline of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  and  $\text{Ca}^{2+}\text{-ATPase}$  activities caused by Mn and subsequently stabilized transmembrane protein.

Upon activation, Neuro-2A cells secrete numerous bioactive factors such as interleukins, TNF- $\alpha$  and nitric oxide (NO). The nature of the released factors appears to be dependent on the nature of the causative agents. For example, lipopolysaccharide (LPS) was reported to increase the release of IL-6, TNF- $\alpha$  and NO [73]. In this study, NO was released but no significant release of cytokines TNF $\alpha$  and IL-6 was detected in Neuro-2A cells cultures treated with  $\text{MnCl}_2$  (200, 800  $\mu\text{M}$ ). This result is consistent with findings reported by others who

demonstrate that in the mouse N9 microglial cells where no release of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 was detected after treatment with MnCl<sub>2</sub> at doses lower than 500  $\mu$ M [73]. Moreover, a significant accumulation of nitrite was observed in the Neuro-2A cells cultures following treatment with MnCl<sub>2</sub> at two doses used (200, 800  $\mu$ M). Similar results were observed in primary mouse astroglia treated for 8 h with 10  $\mu$ M MnCl<sub>2</sub> [74] or C6 glioma cells treated for 24 h with 100  $\mu$ M MnCl<sub>2</sub> [75]. Although the NO synthesized by neuronal nitric oxide synthase (NOS) facilitates the formation of memories under physiological conditions [76], NO production from inducible nitric oxide synthase (iNOS) is deleterious under pathological conditions. Because of its independence of elevated intracellular Ca<sup>2+</sup> levels [77], iNOS catalyzes a high-output pathway of NO production [78] that is able to cause neuronal peroxynitrite-mediated damage and dysfunction [79]. Silymarin significantly inhibited the increase of extra-cellular nitrite (NO) as reported by us and suppressed the expression or activation of iNOS in several tissues including a glial cell line as it has been reported by others [49, 80].

The results from the present study confirm, for the first time, that silymarin could alleviate the toxicity induced by Mn in Neuro-2A cells. The beneficial effect may be attributed to the prevention of oxidative damage, measured in terms of the amount of peroxidized lipid and the level of GSH. Currently, the exact mechanism of SIL-induced neuroprotection and its potential therapeutic applications in neurodegenerative disorders are still unclear and warrant further investigations.

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