

Protective effect of *Zizyphus spinachristi* on MPP⁺-induced oxidative stress

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ABSTRACT

Oxidative stress and mitochondrial dysfunction mediated neuro apoptosis is reported to play a major role in the pathology of Parkinson's disease. *Zizyphus spina-christi* fruits (ZSCF) are used as traditional medicines that are well-known for their high antioxidant properties. In the present study, we investigated the protective effects of ZSCF extract against 1-methyl-4-phenylpyridinium (MPP⁺) induced neurotoxicity in SH-SY5Y cell lines. The effect of ZSCF on MPP⁺ induced cell

viability (MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assay), membrane damage - (lactate dehydrogenase (LDH), oxidative stress (levels of ROS, nitric oxide and GSH and activities of SOD and catalase), mitochondrial membrane potential and apoptosis (activity of caspase 3 and protein expressions of cyto c, Bax and Bcl-2) were measured. Our results showed that ZSCF could be able to reduce the neurotoxicity of MPP⁺ and offer neuroprotection *in vitro*. This protective effect

of ZCF might be mediated by its potent antioxidant properties. However, further research is necessary to isolate active compounds and performing preclinical and clinical studies to confirm the neuro-protective effects of ZSCF in PD.

2. INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized clinically by symptoms of bradykinesia (slowness), postural instability (imbalance), rigidity, resting tremor, autonomic dysfunction and psychiatric manifestations (1,2). The progressive and selective degeneration of midbrain dopaminergic neurons of the substantia nigra (SN) pars compacta (SNpc) in the nigro-striatal pathway is the main pathological hallmark of PD. Although the etiology of PD remains unclear, numerous studies from our laboratory have provided substantial evidence for the involvement of oxidative stress and mitochondrial dysfunction (3,4). Various mitochondrial toxins that selectively inhibiting mitochondrial electron transport chain complex I activity, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine, or rotenone, are capable of producing neuronal cell death and have been widely used to produce experimental models of PD (5). MPTP and its active metabolite 1-methyl-4-phenylpyridinium (MPP⁺) are the most popular dopaminergic neurotoxins and established to induce *in vitro* and *in vivo* models of PD (6). MPP⁺ strongly inhibits mitochondrial complex I which increases the rate of mitochondrial reactive oxygen species (ROS) release, leading to neuronal cell death (7).

There is an increasing usage of plant derived natural compounds with high anti-oxidative and mitochondrial protective properties as an alternative sources of drugs or herbal extracts for therapeutic purpose (8). *Zizyphus spinachristi* fruits (ZSCF) belong to the family *Rhamnaceae* and are grown throughout the Middle Eastern region including Oman. It is commonly called as jujube or red dates in Oman and also known as Dom/Christ thorn in English. ZSCF can be consumed fresh or as an extract in dried form. It has been previously reported that zizyphus species are commonly used in treatment of various diseases including obesity, liver complaints, digestive disorders, anodyne, emollient, urinary troubles, skin infections, diabetes, loss of appetite, fever, bronchitis, anemia, diarrhea, and pharyngitis (9). Abdel Wahhab *et al.*, reported that the ZSCF extract having high antioxidants and offer protective effect against aflatoxicosis (10). Based on our literature survey, the effect of ZSCF on MPP⁺-induced toxicity has not been previously investigated. Therefore, herein we examined the effects of the ZSCF extract in SH-SY5Y neuroblastoma cells stressed with 1-methyl-4-phenylpyridinium (MPP⁺).

3. MATERIALS AND METHODS

3.1. Cell Culture and other chemicals

The SH-SY5Y cell line was obtained from the American Type Culture Collection (Manassas, VA). The SH-SY5Y human neuroblastoma cell line was cultured in Eagle's Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA) and antibiotics (100 µg/ml streptomycin, 100 U/ml penicillin; Gibco BRL) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The passage of the cells were used in the study were around 8 to 10. All other chemicals and reagents were from Sigma.

3.2. Preparation of sample

The freeze dried sample of ZSCF (60 g) was extracted with 150 ml of methanol: water (4:1, v/v), at room temperature (20 °C for 24 h using a magnetic stirrer). The extracts were then filtered and centrifuged at 6000 RCF, for 30 min at 3°C using Sanyo, Harrier MSE centrifuge, 18/80 Rotor No. AO9R8326 and the supernatant were concentrated under reduced pressure at 40°C for 3-4 h using a rotary evaporator to obtain the ZSCF methanolic crude extract. Total phenolics of ZSCF were measured by the Folin-Ciocalteu assay of Singleton and Rossi with slight modifications (9, 11). 250 µL of Folin-Ciocalteu reagent was mixed with 10 µL of ZSCF extract. After a short incubation of 5 min, 750 µL of sodium carbonate (1.9. M) was added and incubated for 2 h at 25 °C and the absorbance measured at 765nm and compared with gallic acid standards. The concentration of phenolics in ZSCF extracts was expressed as Gallic Acid Equivalents (GAE). All the measurements were taken in triplicate and the mean values were calculated. To find out the dose, different concentrations of ZSCF (10, 25, 50, 75, 100 µg GAE) were tested for cell viability and the best dose was used for further analysis (data not shown).

3.3. Cells Treatment

In Experiment I, cells were incubated with different concentrations of ZSCF (0, 20, 40, 50, 60, 80 and 100 µg GAE) for 24h, and MTT assay was performed to detect the toxicity of ZSCF. In Experiment II, cells were pretreated with different concentrations of ZSCF (0, 20, 40, 50, 60, 80 and 100 µg GAE) for 4? h and then incubated with MPP⁺ (1mM) for 2h. The effective dose of ZSCF was used to identify potential neuroprotective effects against MPP⁺ toxicity.

3.3.1. MTT reduction assay for cell viability

Cellular viability was measured in a 96-well plate by quantitative colorimetric assay with

MTT, which represents an appropriate indicator for mitochondrial activity of living cells (12). After the treatment schedule, medium was removed and the cells were incubated with 0.2.5 mg/ml MTT for 4 h at 37°C. The reaction was stopped by adding DMSO. The amount of MTT formazan product was determined by measuring absorbance in a microplate reader at a test wavelength of 570 nm and a reference wavelength of 630 nm.

3.3.2. Lactate dehydrogenase (LDH) assay for cell membrane damage

The amount of LDH released into the medium is an index of cell membrane damage because of the enzyme's high intra cellular localization. The plasma membrane damage was evaluated by measuring extracellular LDH activity in the medium. SH-SY5Y cells were pretreated with concentrations of the ZSCF extract (50µg GAE), and then exposed to 1mM MPP⁺ for 24 h. After the incubation, 50 µl of culture supernatants were collected from each well. The LDH activity was determined with a colorimetric LDH assay kit. Total cellular LDH activity was determined by solubilizing the cell with 0.2.% Triton X-100. The release of intracellular LDH to the extracellular medium was expressed as a percentage of total cellular LDH activity.

3.3.3. Effect of ZSCF extract on generation of free radicals

The levels of intracellular reactive oxygen species (ROS) were determined by the change in fluorescence resulting from the oxidation of the fluorescent probe DCFH-DA (13). When applied to intact cells, DCFH-DA readily diffuses through the cell membrane and is hydrolyzed enzymatically by intracellular esterases to non-fluorescent DCFH (14). In the presence of ROS, DCFH is oxidized to highly fluorescent DCF (13). DCF fluorescent intensity is proportional to the amount of ROS formed intracellular level. In this assay, SH-SY5Y cells were pretreated with ZSCF extract, and then exposed to 1mM MPP⁺ for 24 h. After the medium was removed, the cells were incubated with 20 µM DCFH-DA for 30 min, and the cells were washed to remove the extracellular DCFH-DA. The cells were then suspended in PBS. The fluorescence intensity (relative fluorescence unit) was determined using a Cyto Fluor 4000 fluorescence plate reader at the excitation wavelength of 485 nm and emission wave length of 535 nm.

3.3.4. Determination of extracellular nitric oxide (NO)

The accumulated level of nitrite (an indicator of NO) in the culture supernatants was measured using the colorimetric reaction with the Griess reagent. The supernatants (100 ml) were transferred to a

separate plate, and were reacted with 100ml of Griess reagent in the dark for 10 min at room temperature. Absorbance at 550 nm was measured. For each experiment, freshly prepared NaNO₂ that had been serially diluted was used as a standard, in parallel with culture supernatants.

3.3.5. Measurement of anti-oxidant enzyme activity

SOD activity was measured using assay kits purchased from Dojindo (Kumamoto, Japan). The catalase activity assay was performed as described previously (15).

3.3.6. Estimation of GSH

Determination of GSH was performed as described previously (16). The reaction mixture contained 0.1. M sodium phosphate buffer (pH8.0.), 5.0. mM EDTA, 20 µl o-phthaldehyde (1.0. mg/ml), and 20 µl of the above-noted sample. After incubation for 15 min at room temperature, fluorescence at emission 420 nm and excitation at 350 nm was recorded.

3.3.7. Assessment of mitochondrial membrane potential

The mitochondrial membrane potential was measured using a fluorescent dye, JC-1 reagent. SH-SY5Y cells were pretreated with ZSCF extract, and then exposed to 1mM MPP⁺ for 24 h. The treated cells were incubated with 1X JC-1 reagent solution at 37°C for 15 min. The red and green fluorescence were measured using a fluorescence microplate reader, with excitation at 585 and 510 nm and emission at 590 and 527 nm, respectively. The ratio of red to green fluorescence was quantified from the cells of interest.

3.3.8. Caspase activity

In SH-SY5Y cells were pretreated with ZSCF extract, and then exposed to 1mM MPP⁺ for 24 h, Caspase activity was determined by using acetyl-Asp-Glu-Val-Asp-aldehyde-AFC (Clontech USA), a pseudo substrate used to measure caspase activity (mainly for caspase-3). In the presence of caspase, this substrate is cleaved to the fluorochrome 7-amino-4-trifluoromethyl coumarin (AFC), which was quantified by measuring fluorescence intensity with a F-4500 HITACHI fluorescence spectrophotometer (400 nm excitation and 505 nm emission).

3.4. Western blot analysis

Briefly, cells in 6 well plates were harvested and washed with PBS. Cells were lysed in 100µl lysis buffer (20 mM Tris-HCl, pH 7.4., 150 mM NaCl, 1 mM EDTA, 30µg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) followed

by centrifugation at 1,000g for 5 min at 4°C. The supernatants (cytosolic fractions) were saved and the pellets solubilized in the same volume of mitochondrial lysis buffer (50 mM Tris pH 7.4., 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2 % Triton X-100, 0.3 % NP-40, 100mM PMSF, 10µg/ml leupeptin, 2µg/ml aprotinin), kept on ice and vortex for 20 min followed by pelleting at 10,000g for 10 min at 4°C and subjected to 12.5 % poly acrylamide gel electrophoresis lane (19). A total volume of 40 µg of protein was loaded per lane. The separated proteins were blotted onto a PVDF membrane by semi-dry transfer (BIORAD). After blocking with 5 % nonfat milk in TBS, the membranes were then incubated with various antibodies: Bax, Bcl-2, cytochrome c and β -actin. The following dilutions were used for Bax, Bcl-2 and cytochrome c (1:1,000), and β -actin (1:2,000). After primary antibody incubation, the membranes were incubated with secondary antibody at a concentration of 1:2,000. Then the membranes were washed with Tris-buffered saline and 0.05 % Tween-20 thrice for 10 min interval, after extensive washes in TBST, the bands was visualized by treating the membranes with 3, 3' - diaminobenzidine tetrahydrochloride (Western blot detection reagent, Sigma, USA). Densitometry was done using 'Image J' analysis software.

3.5. Statistical Analysis

Data are expressed as mean \pm SD for each group (n = 3). The statistical significance of changes in different groups was evaluated by one way-ANOVA and Duncan's multiple range test (DMRT) using Graph Pad Prism 6.0. software. The statistical significance at P<0.05 was considered as significant and mentioned as *,* # in the figures.

4. RESULTS

4.1. Effect of ZSCF on MPP⁺-induced cell death and oxidative stress

In high concentration (100 µg GAE), ZSCF alone treatment to SH-SY5Y cells induced exhibited slight cytotoxicity (Figure 1, "A"). ZSCF protected against cell death induced by 1mM MPP⁺ (Fig.1 A), significantly at the concentration of 50 µg GAE (Figure 1, "B"). Pretreatment with ZSCF (50 µg GAE) significantly diminished the release of LDH induced by MPP⁺ (Figure 2). (Figure 3, 4, 5, "A, B" and "C") shows the levels of ROS, NO and GSH and activities of SOD and catalase in MPP⁺ treated SH-SY5Y cells incubated with or without ZSCF. Compared with untreated cells, MPP⁺ treatment (1mM) increased the levels of ROS and NO and decreased the levels of GSH significantly in SH-SY5Y cells. Pre-treatment with ZSCF to MPP⁺, decreased levels of ROS and NO and enhanced GSH significantly, compared to MPP⁺

alone treated group. Compared with untreated cells, MPP⁺ (1 mM) treatment decreased SOD and catalase activities in SH-SY5Y cells. Pre-treatment with ZSCF to MPP⁺ treated cells enhanced the activities of SOD and catalase significantly, compared to MPP⁺ alone treated group.

4.2. Effect of ZSCF on MPP⁺-induced mitochondrial dysfunction

Figure 6, "A" shows the alterations in mitochondrial membrane potential ($\Delta\Psi_m$) measured by red/green fluorescence ratio after the treatment with rhodamine-123. MPP⁺ treated cells are depolarised and show significant dissipation of ($\Delta\Psi_m$)s with highly green colour fluorescence as compared with control. ZSCF pre-treatment to MPP⁺ treated cells displayed red/green fluorescence indicating a polarized state of mitochondrial membrane as compared to MPP⁺ treated cells.

4.3. Effect of ZSCF on MPP⁺-Induced caspase activity

Administration of 1 mM MPP⁺ for 24 h induced the enhanced activities of caspase 3, observed by fluorescence spectrophotometer. ZSCF pre-treatment prevented the enhancement in the activity of caspase observed in neuroblastoma cells treated with MPP⁺ indicating that ZSCF prevented the apoptosis induced by MPP⁺ in SH-SY5Y cells (Figure 7; Table 1).

To further characterize the mechanism by which ZSCF prevents MPP⁺ induced apoptosis, the expression of cyt. c and caspase 3 and 9 were studied by Western blot analysis (Figure.8 A and B; Table 1). The protein expressions of cytosol cyt. c and caspase 3 and 9 were increased significantly and mitochondrial cyt. c was decreased significantly in the MPP⁺ treated cells compared to the control cells. ZSCF, which had no effect in control cells receiving vehicle, prevented the increase in the expression of cytosol cyt. c and caspase 3 and 9 and the decrease in mitochondrial cyt. c observed in the MPP⁺ treated cells.

5. DISCUSSION

In this study, we showed the neuroprotective effect of ZSCF extract against MPP⁺-induced neurotoxicity in SH-SY5Y cells (Figure 1A and B). Cytotoxic effect of MPP⁺ was measured by MTT and LDH assays (Figure 2). Results of the MTT assay indicated that the MPP⁺ (1mM) treatment for 24 h destroyed SH-SY5Y cells and caused approximately half-maximal inhibition of cell viability (~54%). Similar type of results was obtained from LDH assay also. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells. Lactate dehydrogenase (LDH),

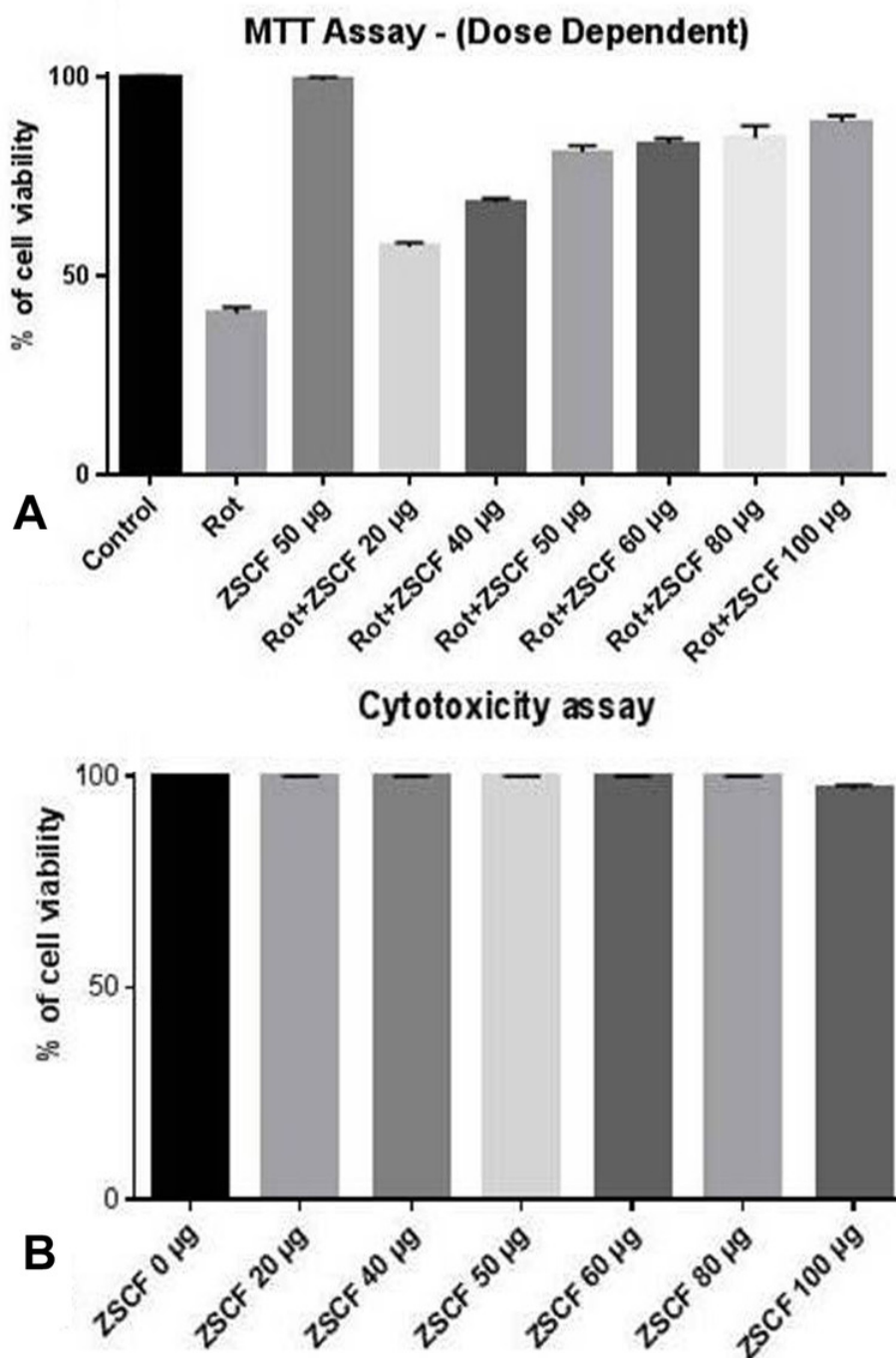


Figure 1. A: Effect of ZSCF extract on MPP⁺ induced cell death in dose dependent manner. Cell viability was assessed by MTT reduction assay. Cytotoxicity of different doses also tested. B. Effect of ZSCF extract on MPP⁺ induced cell death. Cell viability was assessed by MTT reduction assay. * $p < 0.05$; MPP⁺ compared to control and ** $p < 0.05$; ZSCF + MPP⁺ compared to MPP⁺ alone; One way ANOVA.

which is a soluble cytosolic enzyme present in most eukaryotic cells, releases into culture medium upon cell death due to damage of plasma membrane. In rodents and primates, dopamine transporter (DAT) transports MPP⁺ ions into dopaminergic neurons, whereas nor-epinephrine transporter expressed by SH-SY5Y cells is known to transport MPP⁺ at a slower rate than

DAT and induced neuronal degeneration (17). This scenario is likely to be comparable to the situation in surviving neurons of SNc in parkinsonian patients after initial diagnosis of PD (18). In the present study, both MTT and LDH assays indicated that the pretreatment of ZSCF extract significantly enhanced cell viability in a dose-dependent manner.

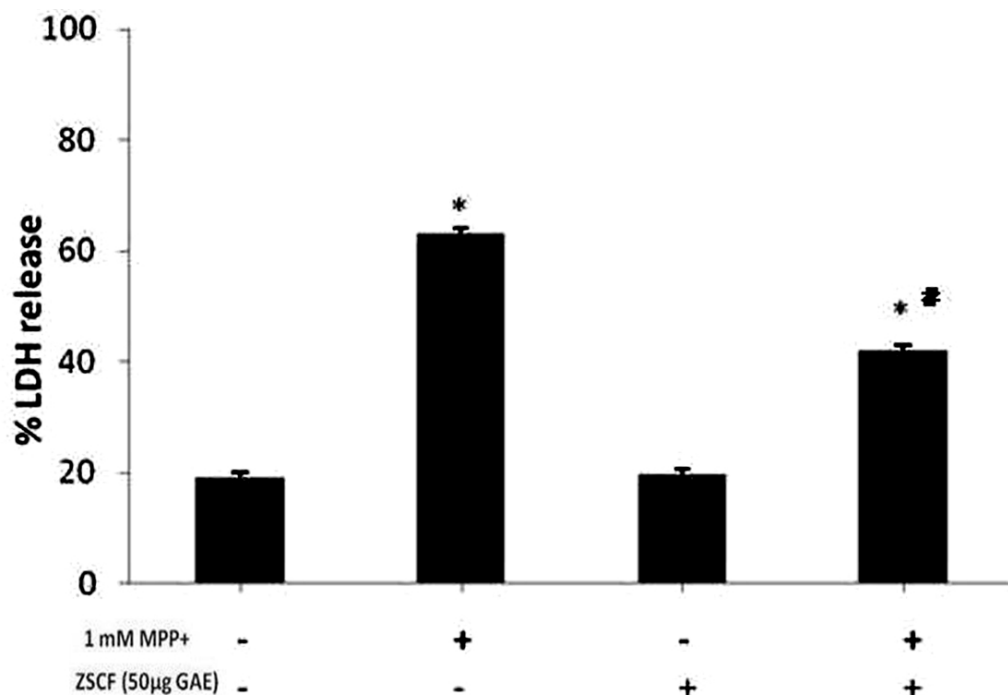


Figure 2. Effect of ZSCF extract on MPP⁺ induced cell membrane damage, assessed by LDH release. **p* < 0.0.5; MPP⁺ compared to control and ***p* < 0.0.5; ZSCF + MPP⁺ compared to MPP⁺ alone; One way ANOVA.

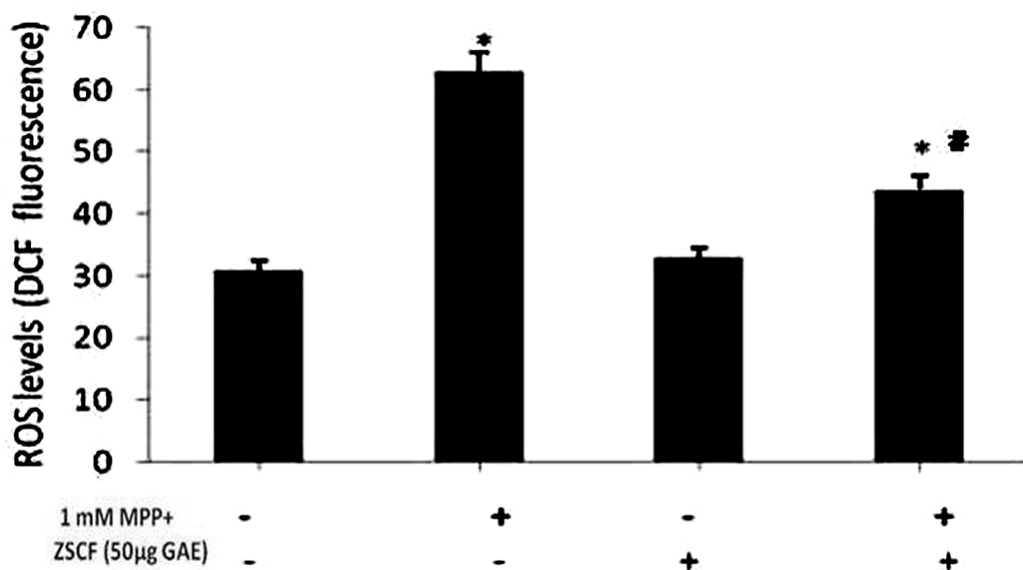


Figure 3. Effect of ZSCF extract on MPP⁺ induced free radical (ROS) generation in SH-SY5Y cells. ROS levels were measured by DCF fluorescence assay. **p* < 0.0.5; MPP⁺ compared to control and ***p* < 0.0.5; ZSCF + MPP⁺ compared to MPP⁺ alone; One way ANOVA.

Mitochondria are the main site of oxygen metabolism, accounting for approximately 85–90% of the oxygen consumed by the cell (19). In normal conditions, due to leakage of electrons at complex I and complex III, it has been estimated that 0.2.% to 2.0.% of O₂ consumed by mitochondria generates

superoxide anion (O₂⁻) (20). Then superoxide anion undergoes spontaneous or SOD-catalyzed dismutation to form H₂O₂. Catalase and glutathione peroxidase catalyzes the decomposition of H₂O₂ to H₂O and O₂. Complex I could be a critical site of mitochondrial ROS production and relatively small

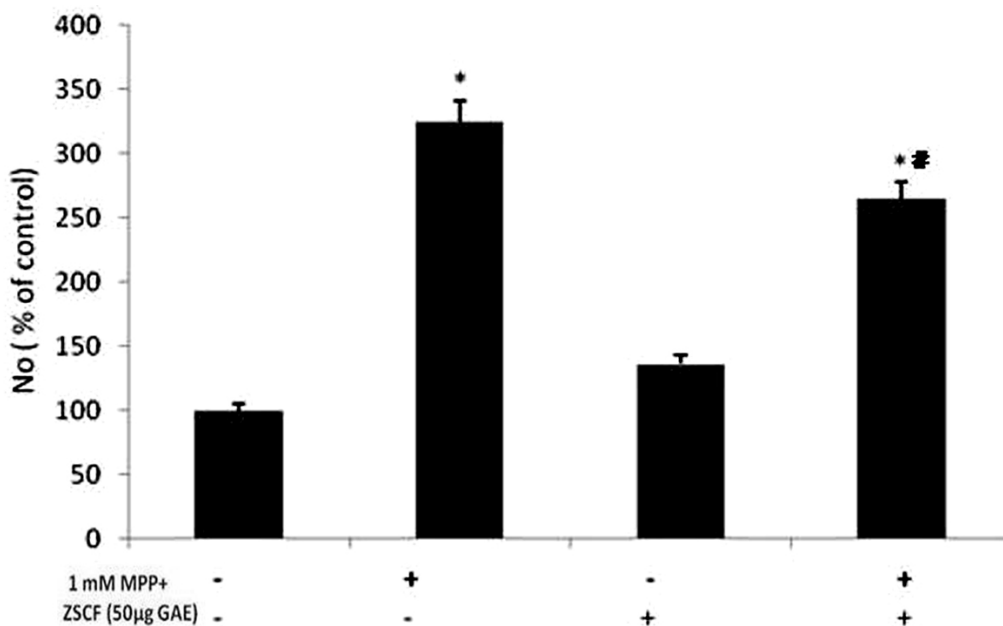


Figure 4. Effect of ZSCF extract on MPP⁺ induced nitric oxide generation in SH-SY5Y cells. **p* < 0.05; MPP⁺ compared to control and ***p* < 0.05; ZSCF + MPP⁺ compared to MPP⁺ alone; One way ANOVA.

level of inhibition is sufficient to increase ROS generation (O₂⁻, H₂O₂ etc) (21).

The principal target of MPP⁺ is the mitochondria, where it inhibits Complex I in the mitochondrial respiratory chain, with the consequent cessation of oxidative phosphorylation (22). It is known to bind to complex I of the electron transport chain, preventing the transfer of electrons from iron sulfur clusters to ubiquinone (23), reducing oxidative phosphorylation, and generating more and more reactive oxygen species (ROS) (24). Several studies have shown the involvement of radical oxygen species (ROS) in MPP⁺ -induced neurotoxicity (25-27). MPP⁺ treatment increased the levels of ROS (Figure 3) and nitric oxide (Figure 4) and decreased the levels of GSH significantly, whereas pre-treatment with ZSCF to MPP⁺ decreased the levels of ROS and NO and enhanced GSH significantly. Excessive NO accumulation, however, causes an imbalance in redox reactions and is highly toxic to dopaminergic neurons (28). Multiple studies show that NO is involved in MPP⁺ -induced DNA damage, PARP-1 activation, and cell death (29, 30). In SH-SY5Y cells, MPP⁺ treatment decreased the activities of SOD and catalase, whereas pre-treatment with ZSCF to MPP⁺ attenuated the activities of enzymatic antioxidants. Oxidative stress, characterized by increased levels of reactive oxygen species (ROS) and diminished levels and activities of antioxidants, also take a place in the death of dopaminergic cells (31). Decreased levels of GSH and activities of SOD and catalase in rotenone alone exposed SH-SY5Y cells which were

probably due to a response inclined towards increased concentration of ROS, which corroborates with our previous studies (Figure 5 A, B and C). It is well documented that ZSC extract is enriched in flavonoid compounds. These flavonoids compounds were found to have the ability to reduce the production of reactive oxygen species (ROS), the inhibition of protein and DNA synthesis and the apoptosis caused by aflatoxin and showed good scavenging power, in accordance with the observed inhibition of NO production (32) and suggesting that the extract attenuated the aflatoxin-mediated decrease in the activities of SOD, which corroborates with our study. Moreover ZSC significantly reduced AOM-induced colonic aberrant crypt foci development and azoxymethane -induced oxidative stress as indicated by restoration of endogenous glutathione depletion and abrogating the impairment of total antioxidant capacity (33), which is also consistent with our results.

It is generally accepted that low MPP⁺ doses lead to apoptosis, whereas high MPP⁺ doses is acutely toxic, leading to necrotic degeneration (34). Neuronal cell death due to MPP⁺ is mediated by opening of the mitochondrial permeability transition pore, releases of Ca²⁺ and cytochrome c, and activation of caspases (35). In our study, MPP⁺ treated cells showed significant dissipation of ΔΨ (Figure 6; Table 1) with enhanced cyt. c release and caspase 3 activity (Figure 7; Table 1), whereas pre-treatment with ZSCF to MPP⁺ attenuated these changes. ROS overproduction mediated by MPP⁺ can severely disrupt the mitochondrial membrane potential, by opening

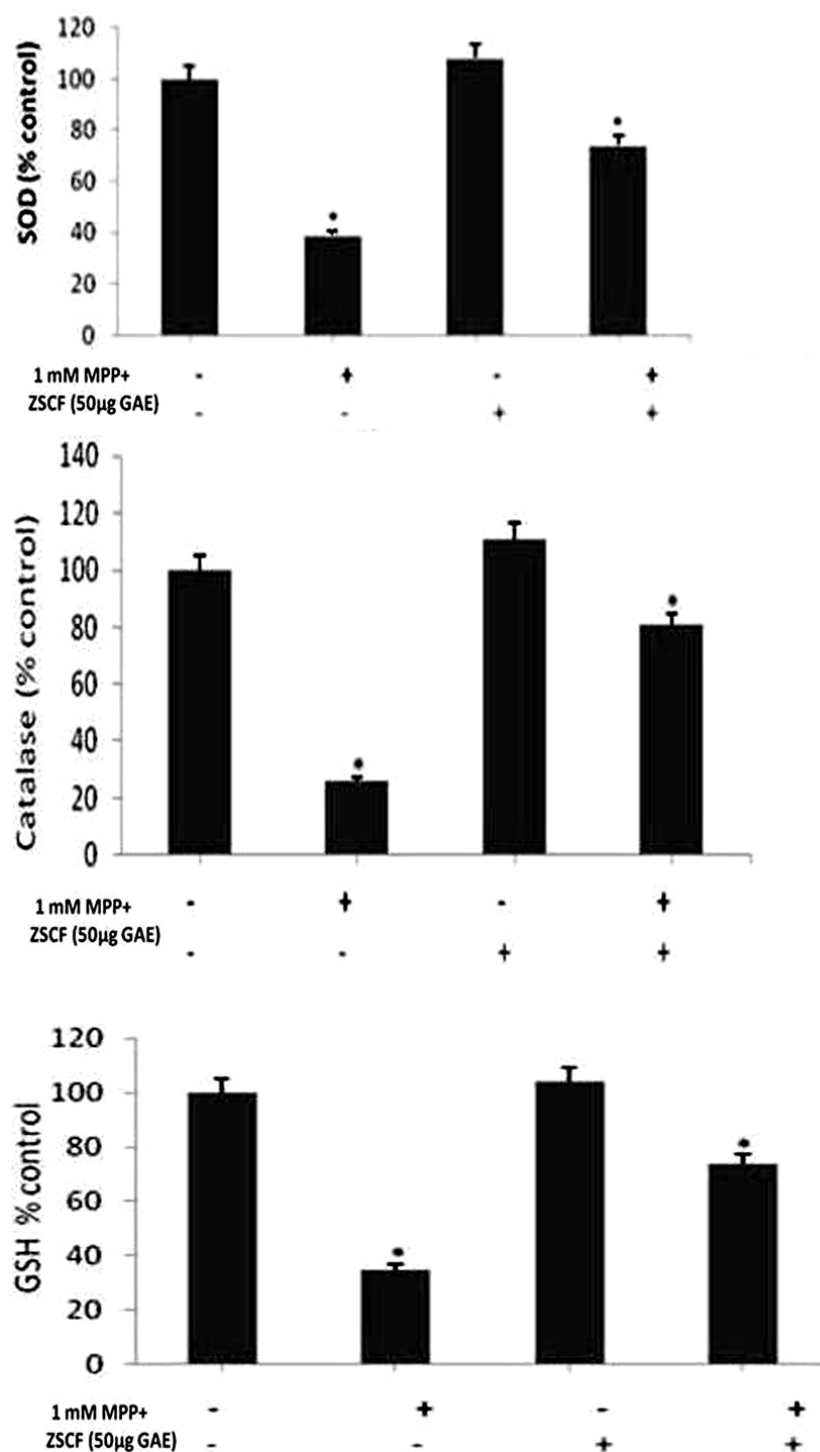


Figure 5. Effect of ZSCF extract on MPP⁺ induced (A) SOD, (B) catalase activities, and (C) GSH levels in SH-SY5Y cells. *p < 0.05; MPP⁺ compared to control and **p < 0.05; ZSCF + MPP⁺ compared to MPP⁺ alone; One way ANOVA.

the mitochondrial permeability transition (MPT) pore through which, intermembrane proteins were released out of the mitochondria, which in turn activated a downstream executive caspase-3 and cell death (36). The interplay between pro- and anti-apoptotic

Bcl-2 family members may determine the fate of cells by regulating the permeability of mitochondrial membrane and controlling the release of cytochrome c from mitochondria (37). Our results showed that treatment of cells with MPP⁺ induced an increase in

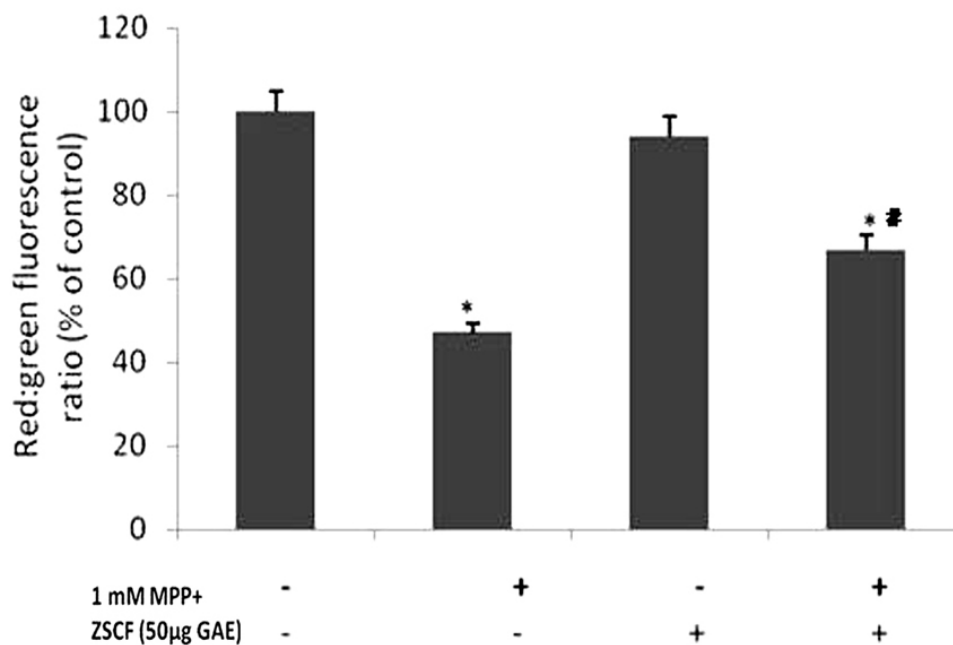


Figure 6. Effect of ZSCF extract on MPP⁺ induced mitochondrial membrane depolarization in SH-SY5Y cells. * $p < 0.05$; MPP⁺ compared to control and ** $p < 0.05$; ZSCF + MPP⁺ compared to MPP⁺ alone; One way ANOVA.

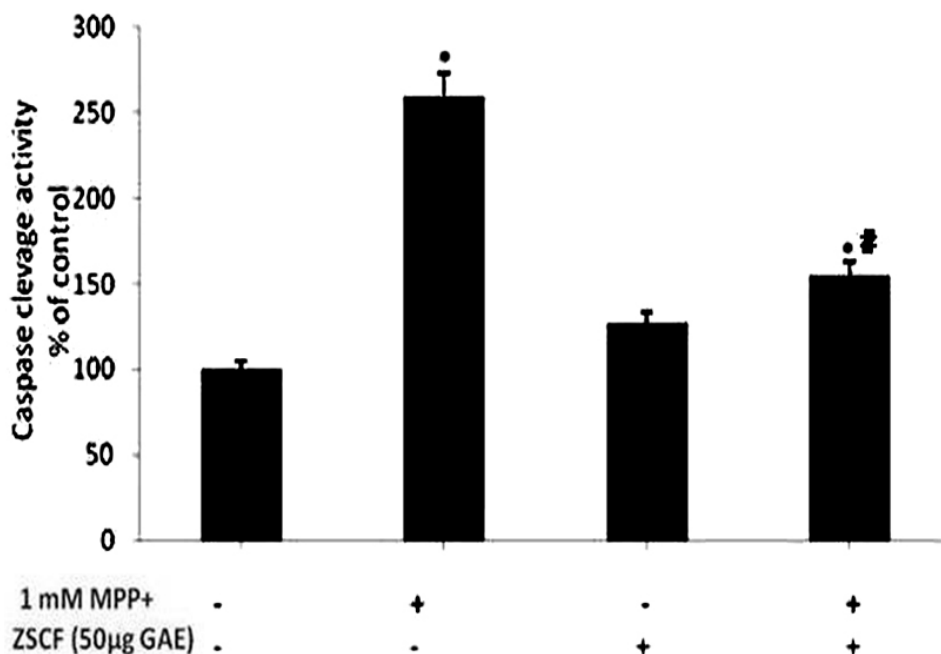


Figure 7. Effect of ZSCF extract on MPP⁺ induced caspase cleavage activity in SH-SY5Y cells. * $p < 0.05$; MPP⁺ compared to control and ** $p < 0.05$; ZSCF + MPP⁺ compared to MPP⁺ alone; One way ANOVA.

the expression of Bax and decrease in the expression of Bcl-2 (Figure 8 A and B; Table 1), whereas ZSCF treatment ameliorates the MPP⁺-induced changes. Bax, a pro-apoptotic protein is thought to be upstream of cytochrome c release in the mitochondria-mediated

apoptosis pathway and Bcl-2, a anti-apoptotic protein is reported to inhibit the release of cyt. C. There are two major pathways through which apoptosis were induced; one involves death receptors and is exemplified by Fas mediated caspase-8 activation,

Table 1. The band analysis by Image J software

	Control	MPP ⁺	ZSCF	MPP ⁺ +ZSCF
Bax	100±7.6.4	160±13.6.2*	100±9.1.6	125±9.1.6 [#]
Bcl2	100±6.8.1	70±4.9.3*	100±8.3.4	86±5.7.6 [#]
Cyt. c (cytosol)	100±5.7.6	156±14.0.9*	100±6.8.1	122±10.6.9 [#]
Cyt. c (mito)	100±9.8.7	72±5.7.6*	100±7.6.4	86±6.2.6 [#]

Values are presented as mean ± SD. *p< 0.0.5; compared to control and # p< 0.0.5 compared to MPP⁺ alone; One way ANOVA

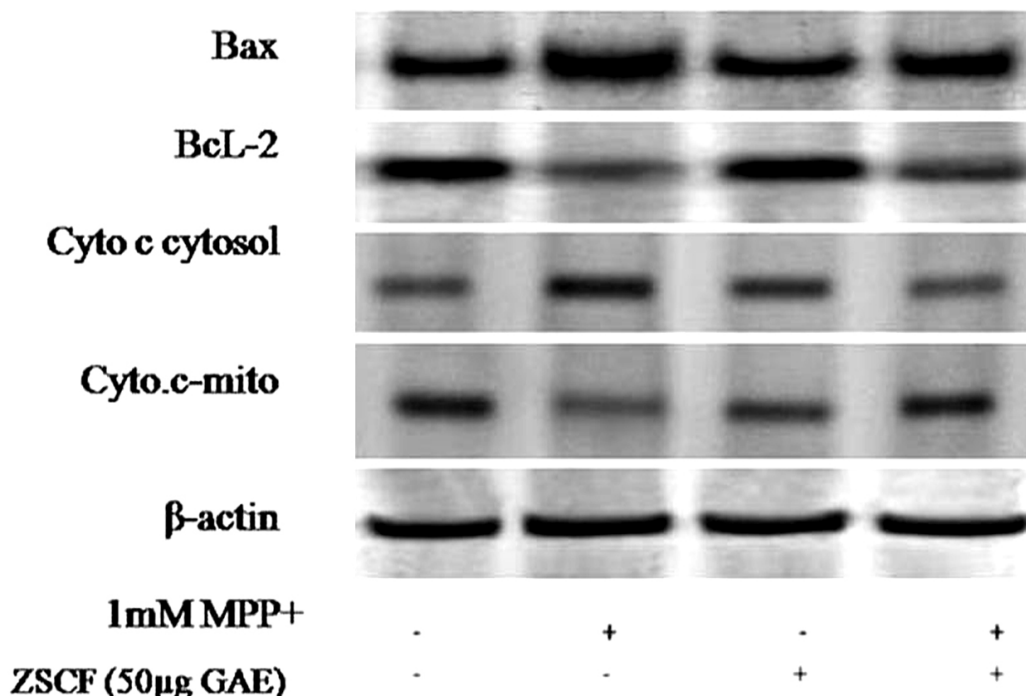


Figure 8. A: Effect of ZSCF extract on MPP⁺ induced alteration in the expressions of apoptotic markers in SH-SY5Y cells. *p< 0.0.5; MPP⁺ compared to control and **p< 0.0.5; ZSCF + MPP⁺ compared to MPP⁺ alone; One way ANOVA. B.

and the other is the stress-mediated or mitochondria-mediated caspase-9 activation pathway. Both pathways converge on caspase-3 activation, resulting in nuclear degradation and cellular morphological change (38).

The main drawback of this study is the usage of undifferentiated cells and lack of positive controls. Despite their tumoral origin, SH-SY5Y neuroblastoma cell lines have been used frequently to model PD, either in an undifferentiated state (39,40), or in a neuron-like differentiated state after induction with all-trans-retinoic acid (RA) (41,42). Undifferentiated cell line shows biochemical properties of immature catecholaminergic neurons and express only immature neuronal markers and lack mature neuronal markers (43,44). Differentiation of neuroblastoma cells with retinoic acid induced resistance to drug therapy (45) and to apoptosis triggered by chemotherapeutic agents

(46,47). So if retinoic acid differentiated cells are used, then more concentrations of toxin is needed to induce PD and high concentration of ZSCF is needed for the treatment.

Previously we have reported natural products could offer benefits to Alzheimer's disease, Parkinson's disease and traumatic brain injury (48-50), which also support our findings in this study. In line with our study, pinocembrin, the most abundant flavonoid in propolis (51), *Chrysanthemum indicum* Linn, oriental medicine (52), a fully acetylated EGCG (53), lycopene, a member of the carotenoid family of phytochemicals (54), theaflavins (55) and astaxanthin, a potent antioxidant (56) inhibited apoptosis in *in vitro* models of PD partially by lowering the ROS levels, enhancing mitochondrial membrane potential, decreasing Bcl-2/Bax ratio and preventing the release of cytochrome c.

Here, ZSCF extract blocks MPP⁺-induced SH-SY5Y cell death; ameliorates ROS generation; diminishes intracellular oxidative stress; prevents MMP reduction and the release of cytochrome C from damaged mitochondria and attenuates apoptosis in SH-SY5Y cells. However, further research is necessary to isolate active compounds and performing preclinical and clinical studies is of the mandate to confirm the neuro-protective effects of ZSCF in PD.

6. CONCLUSION

In conclusion, the results of this study suggest that ZSCF might be able to offer protection to MPP⁺ induced neuronal damage by normalizing oxidative stress, mitochondrial dysfunction by reducing apoptosis and improving antioxidant status. However further extensive research needed to find out the active components and pre-clinical/clinical trials to validate ZSCF as a new therapeutic agent for PD related conditions.

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