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Geraniol attenuates α-synuclein expression and neuromuscular impairment through increase dopamine content in MPTP intoxicated mice by dose dependent manner

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ABSTRACT

Parkinson's disease (PD) is characterized by progressive loss of dopamine (DA) neurons in the nigrostriatal system and by the presence of Lewy bodies (LB), proteinaceous inclusions mainly composed of filamentous α-synuclein (α-Syn) aggregates. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was adopted to generate PD models in C57BL/6 mice. In the present study, we investigated the effect of geraniol (GE) against α-Syn aggregation on MPTP induced mouse model of PD in dose dependant manner. When pretreatment of GE improved neuromuscular impairment, TH expressions and decreases α-Syn expressions in MPTP intoxicated PD mice by dose dependent manner. In addition, we confirmed that sub-chronic administration of MPTP in mice leads to permanent neuromuscular deficits and depletion of dopamine and its metabolites. Our results suggest that GE is beneficial for the treatment of PD associated with neuromuscular disability and LB aggregation.

1. Introduction

Parkinson's disease (PD) is a most common neurodegenerative and movement disorder. It is characterized by a progressive loss of neuromelanin containing dopaminergic (DA-ergic) neurons and its projecting from the substantia nigra (SN) to the striatum (ST) [1,2] leads to the loss of dopamine (DA) in the striatum manifests as motor disabilities that are characteristic of PD. The pathological changes in PD, as revealed by widespread presence of α-synuclein (α-Syn) positive inclusion bodies and α-Syn positive neuritis [3,4]. The recent studies highlighting the important roles for α-Syn in synaptic transmission and dopaminergic neuron physiology to add our understanding of PD etiology and provide a central link between the genetic findings and neurodegeneration observed in sporadic PD.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes damage to DA-ergic neurons in the nigrostriatal system, similar to that seen in PD patients [5]. After administration, MPTP is metabolized by an enzyme, monoamine oxidase B (MAO-B), in glial cells to form 1-methyl-4-phenylpiridinium (MPP^+) [6] and enters into DA-ergic neurons with a dopamine transporter (DAT). Inside the DA-ergic neurons, MPP^+ injures mitochondrial respiratory complex I [7], and subsequently generates oxidative free radicals that can lead to the oxidative nitration of α-Syn [8–10]. α-Syn oxidative nitration had been found in PD and is potentially important in the aggregation and toxicity of α-Syn [11]. The overexpression of α-Syn by itself can cause oxidative stress, increased inclusion formation, and mitochondrial structural abnormalities in cultured neurons [12].

Consumption of phytochemicals from natural products has been associated with reduced risk of neurodegenerative diseases [13–15]. To protect vulnerable targets, phytochemicals counteract the imbalance of the cellular redox homeostasis and the reactive oxygen species levels under the cytotoxic threshold. The phytochemical, Geraniol (GE), an acyclic monoterpene alcohol found in lemongrass and aromatic herb oils, proved to have cytoprotective and antioxidant potential in oxidative stress induced animal models [16]. We speculate that GE, through its antioxidant and anti-inflammatory properties, may exert the capacity to block the MPTP induced neurotoxicity. Consequently, in the present investigation, we demonstrate the neuroprotective effects of GE of MPTP induced neuromuscular deficits, dopamine depletion and action versus α-Syn aggregation in a mouse model of PD.

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2. Materials and methods

2.1. Animals and ethics statement

Adult male C57BL/6 mice (25–30 gm) purchased from the National Institute of Nutrition, Hyderabad, were used in the present study. The animals were kept under 12-h light/dark cycles, at 22 °C and 60% humidity with food and water *ad libitum*. The experimental protocols met with the National Guidelines on the Proper Care and Use of Animals in Laboratory Research (Indian National Science Academy, New Delhi, 2000) and were approved by the Animal Ethics Committee of the Institute (Reg. No. 160/1999/CPCSEA; Approval No: 969/2012) Raja Muthaiah Medical College, Annamalai University.

2.2. Experimental design

The dose dependent study was conducted with three different doses of GE (50, 100 and 200 mg/kg) to determine the effect of GE in MPTP induced PD mice. The mice were randomized and divided into six groups of fifteen animals each. The group I: normal mice treated with vehicle (saline) were served as control. Group II: mice injected with MPTP (30 mg/kg/day b.w dissolved in saline, i.p.) alone, at 24 h intervals for 7 days to produce the sustained model of PD [17]. Group III: GE (50 mg/kg dissolved in saline; (orally) (1 h prior to each MPTP injection) [17] administered on a 7 days schedule with an interval 24 h between consecutive doses + MPTP (injected same as group II). Group IV and Group V of mice were received MPTP same as above mentioned manner prior to GE was given (100 and 200 mg/kg b.w orally) as different dose. Group VI: GE (100 mg/kg b.w) was dissolved in saline and administered for 7 days by oral gavage [18]. Mice were sacrificed two weeks after the first injection of MPTP or saline. The time point chosen for scarifying animals were based on previous study [19] and was to investigate dopamine depletion and the alterations of α-Syn expression.

2.3. Hang test

Mice were placed on a horizontal grid and were supported until they held the grid. Then, the grid was inverted so that the mice were allowed to hang upside down. Staying time was measured as described previously [20].

2.4. Striatal Monoamine Oxidase-B (MAO-B) activity

MAO-B activity was performed by commercially available kit (Amplex Red Monoamine Oxidase Kit, Molecular Probes, Invitrogen, UK). Briefly, brain samples were sonicated in ice cold 20 mM phosphate buffered saline (pH 7.4) and centrifuged at 13,000*g* for 10 min at 4 °C. Protein amount in the supernatant was determined by the method of Lowry et al. [21] and a volume corresponding to 250 μg was diluted in assay buffer to a final volume of 500 μL. To facilitate discrimination between MAO-A and MAO-B, samples were pre incubated 30 min at room temperature with clorgyline (1 μM) as a specific MAO-A inhibitor. The fluorimetric assay started when 500 μL of a reaction mixture containing Amplex Red reagent (400 μM), horseradish peroxidase (2 U/mL) and benzylamine (2 mM) as a specific substrate for MAO-B was added. The assay was conducted in cuvette at room temperature for 45 min. At the end of the incubation time, specific fluorescence of resurofin, the oxidation product of Amplex Red reagent, was measured using a Bioteck Kontron cuvette fluorimeter (560 nm excitation and 590 nm emission). The amount of resurofin in the samples was determined using a resurofin standard curve, and one unit of MAO-B was defined as the amount of enzyme generating 1 μmol of resurofin per minute.

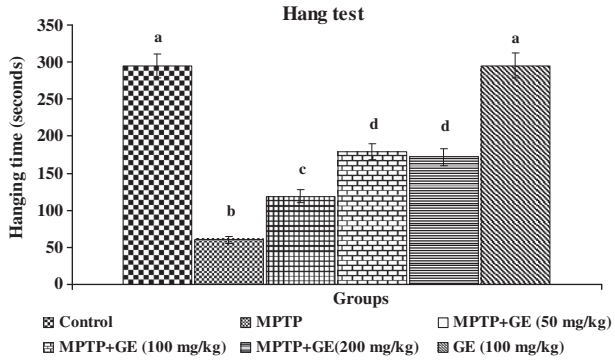

Fig. 1. Effect of GE on hanging performance. Variation in the neuromuscular strength measured in terms of hang time in the control and experimental mice. The values are expressed as mean ± SD. b $P < 0.05$, compared with a. c, d, d $P < 0.05$, compared with the b.

Table 1
Effects of GE on the activity of striatal monoamine oxidase B (MAO-B).

Groups/variables	MAO-B (mU/mg protein)
Control	3.250 ± 0.24[a]
MPTP	5.961 ± 0.45[b]
MPTP + GE (50 mg/kg)	5.8917 ± 0.44[b]
MPTP + GE (100 mg/kg)	4.950 ± 0.31[c]
MPTP + GE (200 mg/kg)	4.9160 ± 0.30[c]
GE	3.240 ± 0.24[a]

Values are given as mean ± SD ($n = 6$), values not sharing common superscript are significant with each other $p < 0.05$, ANOVA followed by DMRT. b $p < 0.05$, compared with a. c, $p < 0.05$, compared with the b.

2.5. Dopamine and its metabolites

Determination of dopamine, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in SN of mice were measured by high performance liquid chromatography (HPLC) with electrochemical detection as reported previously [22].

2.6. Immunohistochemistry analysis

TH (tyrosine hydroxylase) immunohistochemistry was done according to previously described protocol [23]. We used the following primary antibody (at the following dilution): TH antimouse antibody (1:1000, Sigma–Aldrich). The intensity of TH immunoreactivity in the ST was quantified by optical density (OD) measurements using the Micro Computer Imaging Device (MCID) software; data were presented as a percent of the control group values.

Table 2
Changes in the levels of DA, DOPAC and HVA in substantia niagra.

Groups/variables	DA (ng/mg of tissue weight)	DOPAC (ng/mg of tissue weight)	HVA (ng/mg of tissue weight)
Control	5.683 ± 0.43[a]	1.961 ± 0.15[a]	0.785 ± 0.05[a]
MPTP	2.250 ± 0.13[b]	0.543 ± 0.04[b]	0.430 ± 0.03[b]
MPTP + GE (50 mg/kg)	2.380 ± 0.17[b]	0.877 ± 0.06[b]	0.548 ± 0.06[b]
MPTP + GE (100 mg/kg)	4.020 ± 0.28[c]	1.636 ± 0.12[c]	0.640 ± 0.04[c]
MPTP + GE (200 mg/kg)	4.080 ± 0.26[c]	1.644 ± 0.12[c]	0.671 ± 0.04[c]
GE (100 mg/kg)	5.511 ± 0.36[a]	1.921 ± 0.14[a]	0.762 ± 0.05[a]

Results present the dopamine and its metabolite levels measured in control and experimental mice substantia niagra in dosing regimenin. Values are represented as mean ± SD ($n = 6$), values not sharing common superscript are significant with each other $p < 0.05$, ANOVA followed by DMRT. b $p < 0.05$, compared with a. c, $p < 0.05$, compared with the b.

2.7. Extraction of total mRNA

Total mRNA was isolated from the SN and ST using mRNA extraction kit (Genei Bangalore, India), following the manufacturer's instructions. The mRNA integrity was determined by agarose gel electrophoresis and the concentration and purity were measured spectrophotometrically [24].

Total mRNA was converted to single stranded cDNA using 2 μg of total mRNA as a template. Oligo (dT) 12–18 primer (Invitrogen Life Technologies) and Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Invitrogen Life Technologies) were used as per manufacturer's instruction. The following primers were used for the mRNA expression: α-syn: forward primer 5′-GGAGTGACAACAGTGGCTGA-3′, reverse primer 5′-GCTCCCTCCACTGTCTTCTG-3′; β-actin: forward primer 5′-GCGAGAAGATGACC CAGATC-3′, reverse primer 5′-CCAGTGGTACGGCCAGAGG-3′ [25]. The thermocycling conditions were initiated at 95 °C for 10 min, followed by 40 PCR cycles of denaturation at 95 °C for 15 s, and anneal/extension at 60 °C for 1 min. Melting (dissociation stage) was performed by the end of each cycle to ascertain the specificity of the primers and the purity of the final PCR product. The amplified products were electrophoresed with 1.5% agarose gel analyzed changes by Image J software.

2.8. Western blotting

The nigrostriatal tissues were collected from the each group of mice as described previously [23]. Samples fractions containing equal amounts of protein (50 μg) were separated in 10% SDS–polyacrylamide gel electrophoresis. The membranes were incubated with the blocking buffer containing 5% non-fat dry milk powder or BSA for 2 h to reduce non-specific binding sites and then incubated in with anti-α-Syn (mouse monoclonal, 1:1000) with gentle shaking for overnight at 4 °C. After this, membranes were incubated with their corresponding secondary antibodies (anti-mouse or anti rabbit IgG conjugated to HRP) for 2 h at room temperature. The membrane was washed thrice with TBST for 30 min. Protein bands were visualized by an enhanced chemiluminescence's method using ECL-kit (GenScript ECL kit, USA). Bands were scanned using a scanner and quantitated by Image J, a public domain Java

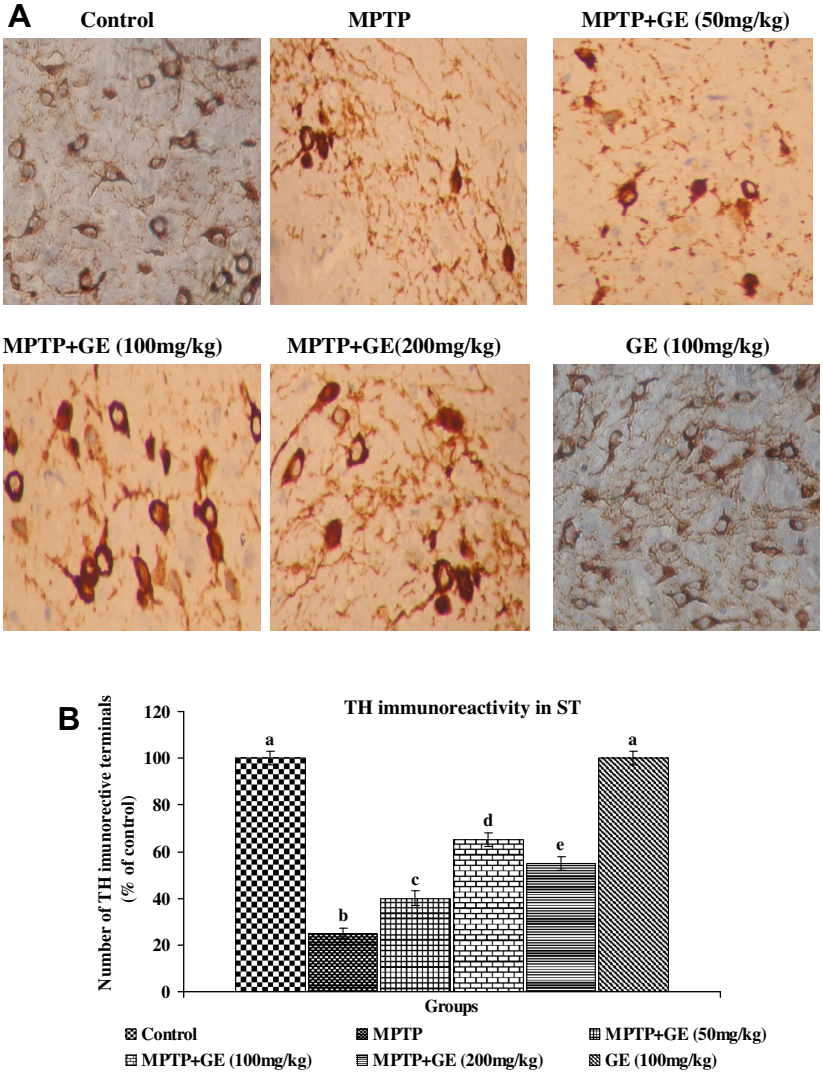

Fig. 2. Immunohistochemical analysis of TH in ST of control and experimental mice: (A) (40× magnification) illustrates TH immunoreactivity was significantly reduced following MPTP administration, an effect that was attenuated by pre-treatment with GE. (B) Quantification of TH positive terminals in the ST. Pre-treatment with GE significantly rescued TH positive terminals in ST by dose dependent manner from death induced by MPTP. The mean value for DAT-IR was determined for each group, and was expressed as a percentage of that matched control mice. Values are presented as mean ± SD of three mice per group.

image processing software, which of control was set to 1.

2.9. Statistical analysis

All the data were expressed as mean ± SD of a number of experiments ($n = 6$). Statistical significance was evaluated by one way analysis of variance (ANOVA) using SPSS version 15.0 software and individual comparisons were obtained using Duncan's Multiple Range Test (DMRT). Values were considered statistically significant if $p < 0.05$.

3. Results

3.1. Effect of GE on hang test

Neuromuscular strength was observed by hang test as shown in Fig. 1. The average hanging time of MPTP administered mice were lowered as compared to control mice Moreover, significantly increased hanging time were observed in the GE Pretreated group as compared to the MPTP alone treated group ($p < 0.05$). No significant changes observed between GE alone treated mice and saline treated control mice ($p < 0.05$).

3.2. Effects of GE on the Activity of Monoamine Oxidase B (MAO-B)

Table 1 depicts that MPTP treatment induced a marked increase in MAO-B activity as compared to control mice. Meanwhile, pre-treatment with GE the activity of MAO-B showed no significant decrease as compared to MPTP control mice ($p < 0.05$). No significant changes observed between the saline treated control and GE alone treated mice respectively.

3.3. Changes in DA, DOPAC and HVA in SN and ST

DA, DOPAC and HVA levels were significantly decreased in SN (Table 2) of MPTP-treated mice as compared to control mice. Pretreatment with GE following MPTP exposure significantly improved the levels of DA and its metabolites. However, treatment with GE alone control mice did not alter the levels of DA and its metabolites as compared to control mice.

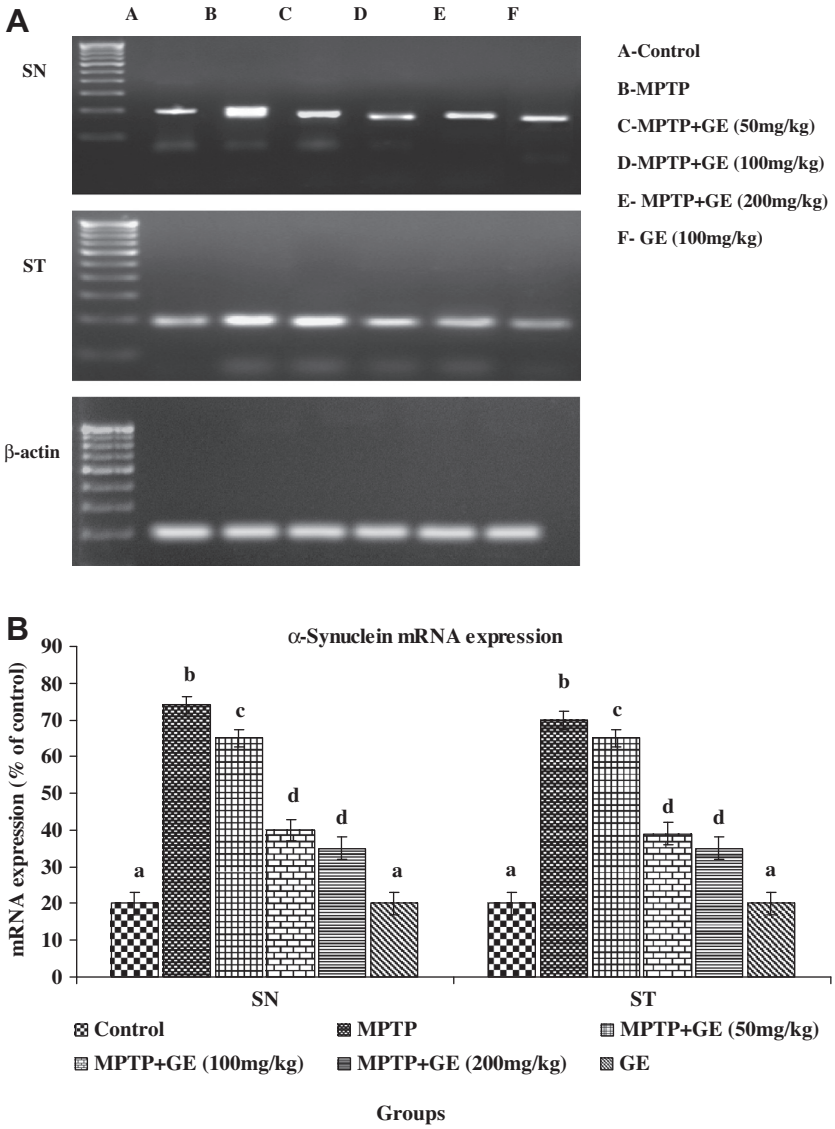

Fig. 3. (A) Dose dependent effect of GE on MPTP induced nigrostriatal α-synuclein mRNA expression in control and experimental mice. β-actin mRNA were used as housekeeping gene for the normalization of mRNA expressions. (B) Quantification graphs Values are expressed as mean ± SD of three mice per group. b $P < 0.05$, compared with a. c, d, d $P < 0.05$, compared with the b.

3.4. GE ameliorates against MPTP induced striatal degeneration

Immunohistochemistry for TH was applied on paraffin embedded sections of control and experimental mice ST, to investigate the loss of TH positive terminals. A drastic decrease of TH immunoreactivity was detected in MPTP treated mice ST compared with saline treated control (Fig. 2A, B). Prior administration of GE to MPTP drastically improves TH positive terminals as compared with MPTP alone treated mice by dose dependent manner ($p < 0.05$). When treated with saline and GE alone treatment didn't affect TH positive terminals in ST.

3.5. Effects of GE on the expression of α-Syn

To study the effects of α-Syn expression on via DA-ergic neurons, the MPTP treated mice increased levels of mRNA gene (Fig. 3A, B) and protein (Fig. 4A, B) expression levels in SN and ST were observed as compared to control mice. However, pretreatment with GE showed a significant decrease in the expression of α-Syn in both SN and ST as compared to MPTP alone treated animals ($p < 0.05$).

4. Discussion

Nigrostriatal pathway is a neural pathway that connects the SN with the striatum via DA-ergic neurons. The primary function of SN appears to be the control of motor function an important factor in the appearance of Parkinsonian signs is the degree of striatal DA depletion [26]. Thus, when studying behavioral consequence of MPTP induced nigrostriatal damage in the mouse, a considerable depletion is required to obtain longer lasting functional deficits [27]. In the present study, the neuromuscular strength assessed by hang test performance. The MPTP treated mice showed a significant reduction in neuromuscular strength as compared with control [20]. We found that pretreatment with GE showed marked improvement in neuromuscular strength in dose manner in comparison with MPTP alone treated group.

The search for new compounds to ameliorate existing deficits is the major goal for the treatment of PD. The therapeutic use of antioxidants may be beneficial in motor disorders [28]. They have the ability to penetrate the blood–brain barrier [29] and reported to have neuroprotective effect against various neurotoxins. The phytochemical GE has cytoprotective and antioxidant property against oxidative stress, the protective effect was also evident by the restoration of mitochondrial membrane potential which is an indicator of a protective effect for proper mitochondrial functioning [16].

The neurotoxicity of MPTP not only depends on the levels of the astrocytic MAO-B, but also the cerebrocapillary endothelium plays an important role in the neurotoxic pathway, mainly because the endothelial cells in the microvasculature of the blood brain barrior also contain MAO-B, which converts MPTP to MPP^+ at this site [30,31]. The importance of MAO-B inhibition to alleviate symptoms in patients with idiopathic PD has been previously postulated [32,33]. Furthermore, we found that administration of GE failure to inhibit the effect of MPTP induced MAO-B, which suggests that the neuroprotective effect of GE was not owing to the MAO-B inhibition. Epigallocatechin-3-gallate [13], kaempferol [14] and fenofibrate [15] prevented MPTP induced neurodegeneration by inhibiting oxidative stress and other mechanisms but not by inhibiting MAO-B activity, which were corroborated with our results.

Decreases in dopamine and its metabolites are associated with the progressive degeneration of DA-nergic neurons in the SN of PD [34,35]. Thus DA, DOPAC, and HVA levels reflect the activity of DA-nergic neurons [36]. The alteration occurs in the nigrostriatal DA level leads to impaired motor functions [37]. Drugs that are able to ameliorate MPTP induced neuronal damage are considered to be neuroprotective. HPLC analysis was conducted to monitor the changes in the levels of DA, DOPAC and HVA. We identified effects of pretreatment with GE against the neurotoxicity induced by subchronic MPTP treatment in mice. Reduction in the level of DA and

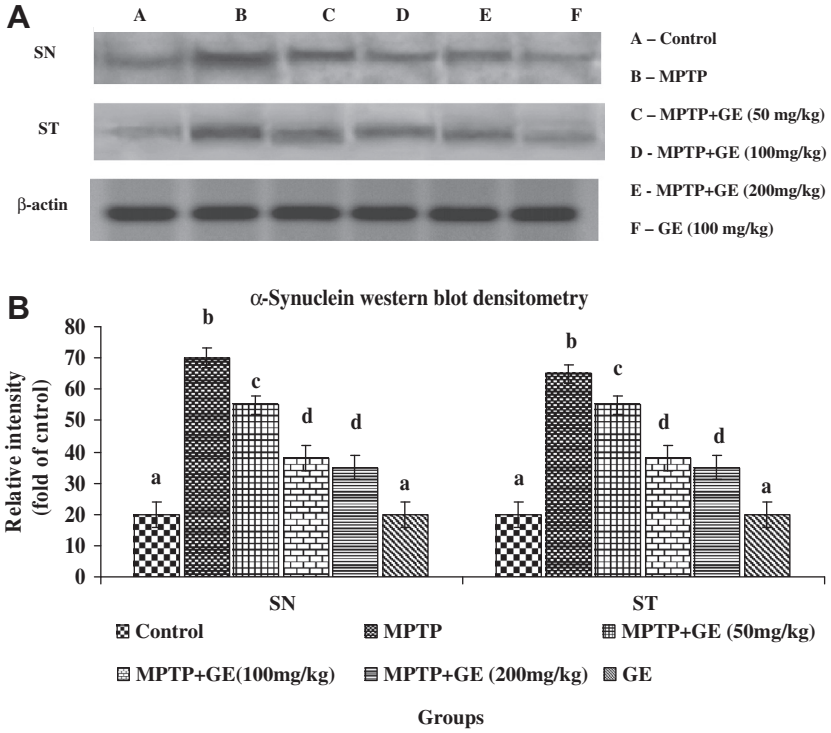

Fig. 4. (A) Dose dependent effect of GE on MPTP induced nigrostriatal α-synuclein protein expression levels in control and experimental mice. β-Actin was shown as an internal standard for the normalization of protein expression. (B) Quantification graphs Values are expressed as mean ± SD of three mice per group. b $P < 0.05$, compared with a. c, d, d $P < 0.05$, compared with the b.

its metabolites was observed in SN after MPTP treatment. Our results also supported previous reports [22]. The results from this study showed that GE significantly improved DA, DOPAC and HVA levels when compared to the control.

TH is the rate-limiting enzyme for the formation of DA. Its expression was used to identify neurotic processes, dopaminergic cell bodies of surviving [38]. TH immunoreactivity describes the number of survival neurons. In PD patients showed a decrease in the activity of TH which is pronounced especially in the nigrostriatal system [39] and the dramatic drop in TH have been suggested to be of underlying importance in the pathogenesis of PD. Our results also supported to previous findings. The immunohistochemical localization of TH in ST region further strengthens and support the protective action of GE against MPTP induced PD, as reported in the present study.

The MPTP intoxicated mice expressing high levels of α-Syn demonstrates that one of the major pathological feature of PD. The roles of α-Syn in normal cell function and in neurodegeneration have not been fully elucidated, but its potential roles in synaptic plasticity [40], neuronal differentiation [41], the up regulation of dopamine release [42], and mitochondrial dysfunctions [43,44] have been reported previously. Recently, the dual roles of α-Syn in neuroprotection and neurotoxicity were described [45–47]. Phytochemicals can inhibit formation and fibrillation of α-Syn, and prevent Aβ-peptide fibrillation and oligomerization as well [48,49]. In a mouse model, it has well reported that phytochemicals able to neuroprotective effects against MPTP induced neurotoxicity by decreasing the fibrillation and aggregation of α-Syn [48,49]. In this study, we observed that MPTP treated animals increased α-Syn expressions in the SN and ST. Pretreatment with GE on MPTP injection showed a decreased the expression of α-Syn inconsistent with previous reports [50–52].

In conclusion, our results show a protective role of bioactive GE against MPTP mice, suggesting that it could represent a potential treatment in early phases of PD. The GE pretreatment improves neuromuscular strength and reduced the α-Syn inclusion by reducing the mRNA expression. The attenuated effect against neurotoxicity by GE could be the basis for their purported action as neuroprotectant and this hypothesis is worthwhile of testing in future studies using animal models of PD, involving MPTP or other neurotoxins. In this regard, it has been reported that GE is a good neuroprotective agent against α-Syn inclusion.

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