Chrysin exerts anti-oxidant properties and restores motor function in MPTP induced mouse model of Parkinson disease

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ABSTRACT
Parkinson’s disease (PD) is a neurodegenerative disorder. It is characterized by a loss in substantianigra and striatum of dopaminergic neurons. The present study is to measure the antioxidant activity of chrysin and to assess behaviour in MPTP mice model of Parkinson’s disease. Male C57BL/6J mice divided into six groups (n=9). Different concentration (50, 100, 200 mg/kg) of chrysin was administered for five consecutive days, MPTP administered 80 mg/kg (2 X 40 mg/kg 16 h interval) via intraperitoneal. After 48 hours of MPTP injection, a behavioural assessment was performed. After behavioural analysis, animals were sacrificed, and brains were collected. Collected brains were subjected for the analysis of Molecular studies, Biochemistry and Histopathology. Chrysin increased Glutathione peroxidase and Nitric oxide activity compared to MPTP induced mice. Similarly, when compared with the MPTP treated group, TRX 1 also increased in chrysin treated (100 and 200mg/kg) groups. It shows chrysin’s anti-oxidants scavenging activity, and these modifications lead to major behavioural changes in mice treated with chrysin, these results being comparable with standard drug L-Dopa. All the groups were compared with the control groups.

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INTRODUCTION
Parkinson’s disease is the second most common neurodegenerative disorder characterized by loss of dopaminergic neurons in the substantia nigra pars compacta and dopamine depletion in Striatum regions in the midbrain. Dopaminergic neurons are responsible for regulating voluntary movement, and degeneration of these neurons leads to postural instability, bradykininesia, resting tremors and rigidity (Brichta et al., 2013). The cause of PD remains unknown, and currently, available treatment is symptomatic management. Defects in energy metabolism and oxidative stress were the main reason for dopaminergic neurodegeneration (Jenner and Olanow, 1996; Fahn and Cohen, 1992). The investigations for novel treatment either to modify the disease ailments or to provide a definitive cure. It remains a challenging task between clinical research and basic science that aims to understand its disabling disorder. Dopamine agonists, Glutamate antagonists, monoamine oxidase-B (MAO-B) inhibitors, catechol-O-methyltransferase (COMT) inhibitors, anticholinergics, β-blockers are currently used for the treatment. Studies provide a strong indication to use levodopa and dopamine agonists for Parkinson’s motor symptoms...
stages (Connolly and Lang, 2014). L-Dopa tops the existing multiple symptomatic PD treatments. Despite the short plasma life of L-Dopa (1-2 hours), frequent intake results in fluctuated plasma profiles. Further, patients with PD exhibit the fluctuations of motor symptoms in the disease progression. Thus, the development of neuroprotective drugs against neurodegeneration is necessary. So, there is a need for developing neuroprotective drugs against neurodegeneration. The present research shows the neuroprotective properties of Chrysin, MPTP is a neurotoxin affecting the dopaminergic system and produces pathological neurodegenerative changes similar to Parkinson’s disease. It also increases ROS activity in SNPC and Striatum. MPTP models of Parkinson’s disease in mice revealed behavioural changes similar to that of humans. Stride length test also showed relevance to the damage of striatum caused due to MPTP. Chrysin is a member of the flavonoid family, abundantly present in plant extracts, propolis and honey. Chrysin is a bioactive compound with many pharmacological properties like antihypertensive, anticancer and anti-diabetic properties (Hwang et al, 2018; Lukačínová et al, 2008). Antioxidant scavenging activity and stride length neurobehavioral activity of chrysin was evaluated in the present study against MPTP intoxicated mice.

MATERIALS AND METHODS

Reagents

L – DOPA, TRIzol reagent, MPTP and Chrysin chemicals were obtained from Sigma Aldrich, USA. DMSO was procured from Himedia, India. The natural animal feed procured from Provimi Animal Nutrition India Pvt. Ltd, Bengaluru, India.

Animal husbandry

The adult Male mice (C57BL/6j) were procured from biogen laboratory animal facility, Attibele, Bangalore ( CPCSEA Reg. No. 971/bc/06/CPCSEA). The animals (body weight : 18-22 g) were grouped into 6 and they were housed in a ventilated room (air cycles: 15/min, recycle ratio: 70:30) under 22 ± 3°C ambient temperature, 12-h light/dark photoperiod, 40 - 60% relative humidity and they are fed with rodent feed and purified water ad libitum. Initially, male mice acclimatized for seven days before the start of the experiment for adapting the laboratory conditions. This study strictly followed (Institute of Laboratory Animal Resources, National Academic Press, 1996; NIH publication number #85-23, revised 1996) “Guide for the Care and Use of Laboratory Animals”. IAEC, Sri Ramachandra Medical College and Research Institute approved the current study (IAEC/XLIII/SRU/429/2015).

Experimental design

The mice were separated into six groups and each group contains nine animals (n=9). Group 1 animals received 2 % of DMSO with saline served as a control (n=9), Group 2 animals received MPTP + 2% DMSO, Group 3, 4 & 5 animals received different concentration of chrysin (50, 100 and 200 mg/kg) along with MPTP. Group 6 animals received L- DOPA (100 mg/kg) and administrated two different doses + MPTP. Chrysin or Vehicle was administered for five consecutive days. The intraperitoneal administration of MPTP (80 mg/kg) divided into two doses (2 X 40 mg/kg 16 h interval) on 3rd and 4th days. All animals were sacrificed after behavioural (motor function) analysis and Collected brain samples were for biochemical analysis.

Nitrous Oxide (NO) estimation

Nitrous oxide interacts with oxygen to create nitrite which was evaluated by griess reagent. In order to obtain protein precipitation, about 0.2 ml of 10 % homogenised tissue samples (Kept in 10% ice cold potassium chloride) were thoroughly mixed with 1.8 ml of saline and 0.4 ml. The supernatant was collected after centrifugation at 4000 rpm for 10 min. To 1ml of the supernatant, 2 ml Griess reagent was added, mixed well and allowed to stand for 20 minutes under dark condition. The UV spectrophotometer is used to measure the chromogen intensity at 540 nm. The expressed values given in μM/mg protein.

Estimation of Glutathione Peroxidase (GPx)

The GPx levels in brain tissue homogenate was measured as previously described (Yamamoto and Takahashi, 1993). Concisely, the mixture of assay contains 100 μL of 1M Tris-HCl (pH-8.0) 5mM of EDTA, 100μL of glutathione reductase solution (10U/mL), 20 μL of 0.1MGSH, 100 μL of 2mM NADPH, 650 μL of dH2O, 10 μL of 7mM butyl hydroperoxides, and 10 μL homogenate brain samples. Spectrometrically NADPH oxidation was observed at 340 nm. The amount of GPx required to oxidize 1 μmol of NADPH per min.

Stride length

The stride length testwas performed according to Fernagut et al. (2002) method. A black wooden box 6.5 (h) × 14.5 × 20 (w) (l) consisted of a runway 9.5 (h) × 40 (l) × 4.5 (w), placed at one end of the runway. Two halogen lamps provided 50 W each in the runway, mice was placed towards the box. The box had 45 mm in diameter a hole facing the runway. The hind - paws and
Figure 1: Effect of Chrysin on NO. The comparison test ## indicates p value 0.01 Vs group I ** indicates p value <0.01 respectively Vs group II. The values are expressed in Mean ± SEM (n=3)

Figure 2: The Effect of Chrysin on GPx. The comparison test ## indicates p value < 0.01 Vs group I , *,** indicates p value 0.05 <0.01 respectively Vs group II. The values are expressed in Mean ± SEM (n=3)

fore - paws of the mice wetted with colour ink red and green (non-toxic) and other end covered white paper. The time taken by the mice to cross the runway the stride length of fore limbs and hind limbs were measured (3 consecutive readings).

RT PCR analysis
The brain regions of ST and SNpc was used for RNA extraction using the TRIzol method. The tissue samples were homogenised and incubated for 10 min at room temperature. The ice-cold Chloroform was added and allowed to incubate for 5 min at 37°C, centrifuged at 12,000 rpm/15 minutes. Then, ice-cold isopropyl alcohol added and samples were incubated for 15 mins to precipitate total RNA and centrifuged for 12,000 rpm/15 min., supernatant was decanted and pellet was washed three times with ethanol, centrifuged at 10,000 rpm for 10 minutes. The pellet was resuspended after the ethanol wash with RNase free water, stored -80°C until use. The isolated RNA was subjected to gene expression and polymerization reaction to obtain cDNA using PCR master cycler qiagen. The gene expression level was analyzed using CT values.

β-actin: sense, 5'-CTTCTATGCCAACAC AGT GC -3';
antisense, 5'-GTACTCCTGCTTGCTGATCC-3',

Histopathology
The tissue Samples were dissected for histopathological analysis. The 5 mm thick paraffin sections through matched coronal levels of the SNPC and ST were stained with cresyl violet. The stained histopathological samples observed under the light
Figure 3: Effect of Chrysin on Stride length behavior analysis. The comparison test ## indicates p value 0.01Vs group I, ** indicates p value 0.01Vs group II. The values are expressed in Mean ± SEM (n=9) PCR

Figure 4: Effect of Chrysin on TRX 1. The comparison test ## indicates p value < 0.01 Vs group I, *, ** indicates p value 0.05 < 0.01 respectively Vs group II. The values are expressed in Mean ± SEM (n=3)
**Data analysis**

Data were expressed as Mean ± Standard error of the mean (SEM). Mean differences between groups were analysed by one way ANOVA followed by Tukey's multiple comparison as post hoc test using Graph pad Prism 5.0 (San Diego, USA) software.

**RESULTS**

**Effect of Chrysin on NO**

A significant increase in NO content (F(5,12) = 61.82, p<0.01 and F(5,12) = 48.51, p<0.01 respectively) were observed in SNpc and ST regions of MPTP mice when compared to vehicle-treated normal mice.

The administration (Oral) of Chrysin 50, 100, 200 mg/kg significantly decreased NO content (p<0.05 and 0.01) in ST and SNpc regions when compared to MPTP mice. Figure 1

**Effect of Chrysin on GPx**

A significant decrease in GPx content (F(5,12) = 4.547, p<0.01 and F(5,12) = 3.248, p<0.01 respectively) were observed in SNpc and ST regions of MPTP treated mice when compared to control group. The treatment of chrysin 200 mg/kg significantly increased the GPx content (p<0.01; p<0.05 and 0.01) in ST and SNpc, when compared to MPTP treated groups shown in Figure 2.

**Chrysin attenuated MPTP induced abnormal stride length behaviour**

The MPTP treated group taken long time cross the

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**Figure 5: Effect of Chrysin on Histopathological changes in SNpc and ST region. (n=3)**
Peroxynitrite is a strong oxidant provoked using induced parkinsonism. Treatment reversed the suppression against MPTP. In our present study, MPTP intoxicated mice groups show the protection of neurons with MPTP treated group shown in Figure 4.

Histopathological Changes in SNPC and ST
Coronal sections of SNPC and ST regions examined under the light microscope where the MPTP treated groups show the shrinkage neurons when compared to the control groups. Chrysin treated (100 and 200 mg/kg) groups show the protection of neurons with MPTP treated group shown in Figure 5.

DISCUSSION
The lipophilic compound of MPTP quickly penetrates the BBB and reported to produce degeneration of dopaminergic neurons in the midbrain and leads to PD-like neurochemical and histopathological changes in nonhuman primates and mice (Betarbet et al., 2002; Liu et al., 2008). MPTP has been used in our study to stimulate PD in the mouse. In this study, the chrysin exhibits the neuroprotection against MPTP intoxicated Parkinson's mouse model. Chrysin administration repressed NO activity and up-regulated GPx and thioredoxin reductase activity. Additionally, animals treated with chrysin showed better Stride length activity.

Parkinson's disease caused by various biochemical changes caused by oxidative stress and mitochondrial dysfunction, and PD etiology is unknown, the factors that cause neurodegeneration (Schapira, 2008)

GPx is a naturally occurring antioxidant enzyme that catalyzes the removal of glutathione-dependent hydrogen per oxide (Bensadoun et al., 1998). The GPX family proved that total GPx activity plays a critical role in PD. In our present study, MPTP intoxication suppressed the GPx activity, and chrysin treatment reversed the suppression against MPTP induced parkinsonism.

Peroxynitrite is strong oxidant provoked using the interpolated response of NO and superoxide plays a critical role in MPTP induced Parkinson's model (Aoyama et al., 2008). Microglial originated NO and O2 are designated as the mediators that connect inflammation and abnormal SYN in PD (Gao et al., 2011). The Chrysin group animals showed decreased NO levels which may be expected to the obstruction. This strongly evidences the antioxidant potential of chrysin against MPTP induced neurodegeneration.

Thioredoxin reductase is a redox-active selenoenzyme, possesses a selenocysteine residue in its active site. It performs its action in association with thioredoxin to form a vital cellular disulfide reductase system. It reduces the number of other protein disulfides and a huge number of oxidized low molecular weight compounds. Consequently, thioredoxin reductase plays a vital role in conserving redox balance inside cells. Liu et al. (2013) showed that thioredoxin reductase mRNA levels were markedly lowered in midbrains of Parkinson's disease mice which donate to oxidative stress and thereby it was concluded that diminished thioredoxin enzyme provoke reactive oxygen species aggregation and promote a harmful shift in the redox balance within cells (Chen et al., 2015). The present study reveals that chrysin pretreatment exhibited neuroprotection by enhancing thioredoxin reductase activity MPTP prompted in mice SHNPC and ST. In contrast, the thioredoxin reductase decreased in the MPTP treated group.

Fernagut et al. (2002) stated that Parkinsonian gait was characterized by a reduced stride length and it was used as an index of basal ganglia dysfunction (Fernagut et al., 2002). Reduced stride length significantly correlated with the magnitude of cell loss, either in substantia nigra or in the lateral midstriatum (Yang et al., 2007). In this study, stride length reduced by MPTP and stride length behaviour was reversed significantly by Chrysin against MPTP. The protective effect of chrysin prevents the MPTP induced neurotoxicity in PD mice, coronal sections of SNPC stained by CV staining. The appearance of shrinkage neurons in the SNPC region of MPTP intoxicated mice reflects neural inflammation and damage. Chrysin treatment protected against MPTP intoxication.

CONCLUSION
Current therapies for Parkinson's disease significantly improved the quality of life among patients suffering from this neurodegenerative disease, yet none of the new therapies has shown to be gentle or to prevent disease development. This reveals
the neuroprotective properties of chrysin in reversing the antioxidant scavenging abilities and also improvement in Stride length behaviour against MPTP neuro toxicity preclinically. Therapeutic supplementation of this compound may prove to be a neuroprotective agent for the management of Parkinson’s disease and may be taken over for human clinical trials.

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Conflict of interest
The author declares that there is no conflict of interest.

ABBREVIATIONS

PD, Parkinson’s disease; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NO, Nitric oxide; GPx, Glutathione peroxidase; ANOVA, Analysis of variance; RNA, Ribonucleic acid; NADPH, Nicotinamide adenine diphosphate; EDTA, Ethylene diamine tetra acetic acid; ROS, Reactive oxygen species.

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