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Demethoxycurcumin ameliorates rotenone-induced toxicity in rats

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1. ABSTRACT

Rotenone, an environmental toxin, is used to induce neurodegeneration in both the cellular and animal model of Parkinson's disease. Demethoxycurcumin (DMC), derivative of curcumin has been reported to have antioxidant and anti-inflammatory characteristics in *in vitro* and *in vivo* PD conditions. The present study was aimed to evaluate the efficacy of DMC in the management of neurodegeneration in PD. Male Wistar rats were radomized and divided into control, rotenone, DMC +rotenone and rotenone alone treated animals. Pre-treatment with DMC one hour prior to the rotenone injection, attenuated the motor and non-motor deficits. Western blot analysis indicated that the administration of DMC to PD rats eased the protein expression of dopaminergic and apoptotic indices. These findings showed that DMC effects on ameliorating the PD symptoms induced by rotenone might be associated with the neuroprotective and antioxidant effects of this compound.

2. INTRODUCTION

Neurodegenerative diseases (NDDs) are expected to surpass cancer as the second most cause of death by 2040 worldwide (1). Parkinson's disease (PD) is one of the most common NDDs that mainly affect the movement of aged population. It is characterized clinically by resting tremor, bradykinesia, rigidity and postural instability that primarily arises due to the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) that synthesize, dopamine (DA), a chemical messenger responsible for transmitting signals to produce smooth and focused muscle activity. Depletion of DA causes the striatal nerve cells to fire out of control, leaving patients unable to direct their normal movements (2). As a result, the amount of DA needed for neurotransmission in the ST is lowered. Parkinsonian signs appear when dopaminergic neuronal death surpasses 70-80% (3). Generally, dopaminergic impairment is assessed by measuring DA level and the expressions of dopaminergic neuronal markers such as tyrosine hydroxylase (TH), a rate-limiting enzyme involved in the DA synthesis (4).

The factors that enhance the risk of PD were pesticide exposure, prior head injury, β-blocker use, agricultural occupation, rural living, well-water drinking. The incidence of PD is more in farmers in rural areas, which could be due to the enhanced pesticides/ herbicides exposure as compared to the general population (5). Rotenone belongs to cytotoxic retinoid family extracted from some plants of Leguminosae family like *Derris mallaccensis*, *Derris elliptica*, *Lonchocarpus utilis* and *Lonchocarpus urucu* (6). It is widely used as pesticide and insecticide. Like MPTP, it is highly lipophilic and can easily cross the BBB followed by accumulation within organelles such as mitochondria, where it inhibits complex I of the ETC (7). Rotenone is reported to cause ROS generation, ATP depletion and cell death in neurons due to its inhibitory action on mitochondrial complex I. Rotenone toxicity mimics many pathological hallmarks of PD, including loss of dopaminergic neurons in SN and formation of Lewy bodies which is presumably due to oxidative damage, mitochondrial dysfunction and disruption of axonal transport (7).

Various pharmacological agents, including MAO-B inhibitors (8), DA agonists, calcium antagonists, NMDA antagonists, glutamate release inhibitors (9), immunosuppressants, nitric oxide syntheses inhibitors, dimethyl thiourea (10), sulfhydryl drugs and other antioxidants (11) along with L-DOPA combination has been shown to protect experimental animals against various PD toxins. Current pharmacological therapies for the PD are inadequate; these are only able to provide symptomatic relief and after long use produce stern side effects and even worsen the condition.

Curcuma longa (turmeric) is used for medical purpose in the history of folk and Ayurvedic medicine. The neuroprotective effect of turmeric attributed due to the presence of curcumin by its antioxidant (12), mitochondrial protective, signal modulating (13), anti-inflammatory (14) and anti-apoptotic functions (15). The ratio of curcumin compounds present in commercially available preparations of curcumin are curcumin: demethoxycurcumin (DMC): bisdemethoxycurcumin (BDMC) in the ratio of about 66:23:11. Both the DMC and BDMC are also used for domestic cooking, food industry and folk medicines (16). Previous studies from our lab demonstrated the neuroprotective role of DMC against rotenone induced motor deficits, neurochemical alteration, oxidative stress and the expression of inflammatory markers in rats (17, 18). However the role of DMC in rotenone induced motor and non-motor symptoms, dopaminergic and apoptosis markers were not investigated.

3. MATERIALS AND METHODS

3.1. Chemicals

Rotenone, DMC, was purchased from Sigma Chemical Company, Bangalore, India. All other reagents used were of analytical grade and were procured locally.Anti-Bcl-2, anti-Bax, Caspase-3, Caspase-6, Caspase- 8, Caspase-9 and TH antibodies were obtained from Cell Signalling (USA) and b-actin antibodies were purchased from Santa Cruz Biotechnology, Inc, (USA). Anti rabbit HRP conjugated secondary antibody (Sigma chemical, USA). All other chemicals were of analytical grade.

3.2. Animals

Male Wistar rats (225-250 g) were procured from the Biogen Laboratory, Bangalore India. They were kept under the ambient conditions and fed with standard pellet and water *ad libitum*. All 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 (IAEC/KMPC/230/2015-2016).

3.3. Experiment

Twenty-four animals were randomised and distributed into four groups (n=6): control (0.5. ml of sunflower oil *i.p.* for 45 days), rotenone (2.5. mg/kg/ day *i.p.* in sunflower oil for 45 days) (19), rotenone as group II + DMC (10 mg/kg b.w. *p.o.*for 45 days) and DMC (10 mg/kg) alone treated. After the end of the experimental period, behaviour tests (open field, akinesia and catalepsy test) were carried out. Then the animals were sacrificed by cervical dislocation and the SN was dissected, rinsed in ice-cold saline

and stored at -80°C for protein expression studies of dopaminergic and apoptotic markers.

3.4. Behavioral analysis

3.4.1. Open field test

The floor of the wooden rectangular open field apparatus (100 x 100 x 40 cm) was covered by rexin cloth with drawn lines that are dividing them into 25 equal squares (20 x 20 cm). Animals were placed individually in the corner of the apparatus and its behavior was observed for 5 min; peripheral locomotor activity- the number of lines crossed in the outer 16 squares with two fore paws, central locomotor activity the number of lines crossed in the inner 9 squares; rearing activity the number of the time rat standing on its fore legs with its hind legs on the ground and grooming activity the number of times the rat licking the fur or washing face or scratching. Between each session the apparatus was thoroughly cleaned with alcohol and dried (20).

3.4.2. Catalepsy

The term implies the inability of an animal to correct an externally imposed posture. The animal is lifted by its tail and is allowed to place its forepaws on a horizontal wooden bar (diameter: 1.2.5 cm; height: 10 cm), which was just above and parallel from the base. Catalepsy was measured as the time elapsing before it climbed down from the bar was recorded. The duration taken for the first movement of paws was measured as cataleptic time. The maximum descent latency for at least 30 s was said to be cataleptic and given one point and maximum time was fixed at 180 s (21).

3.4.3. Akinesia

This tests replicates the difficulty in initiating movement in PD. Akinesia was performed by noting the latency in seconds (s) of the animals to move all four limbs with the test finished within 180 s time frame. Before carrying out each akinesia test rats were acclimatized for 5 min on a wooden elevated (100 cm) platform (100 x 150 cm). Using a stopwatch, the time taken by the animal to move all the four limbs was recorded (22).

3.4.4. Forced Swim Test

In FST, developing an immobile posture, when rodents are exposed to an inescapable situation, resembles depression in humans. The test was conducted in two sessions. First, in the training session, the rats were allowed to swim in water tank (20 x 20 x 40 cm) containing water in room temperature at a depth of 15 cm for 15 min. Twenty-four hours after the training session, the rats were subjected to the forced swimming test for 5 min, for subsequent quantification of immobility time (time required to attain lack of motion of the whole body with only of the necessary movements to keep the animal's head above the water). The water was changed after each animal to avoid the influence of smell (23).

3.4.5. Sucrose preference test

Sucrose intake test is used to measure anhedonia, a decreased ability to experience pleasure, is a core symptom of human depression. The animals were transferred into single housing cages with free access to food. Each rat was provided with two bottles of water, pre-weighed, on the extreme sides of the cage during the 24 h training phase to adapt the rats to drink from two bottles. After training, one bottle was randomly switched to contain 1 % sucrose solution for 1 h, as described previously (24), and 24 h later, the bottles were reversed, and provided for 1 h, to avoid perseveration effects. The sum of water consumption and sucrose solution consumption was defined as the total intake. The percentage of sucrose intake was calculated by using the following equation (% sucrose preference = sucrose intake x 100/total intake). The test was carried out between 9:00 and 11:00 a.m., beginning 1 week prior to the rotenone exposure (to provide baseline values). After the sucrose preference test, all the rats received free access to food and water.

3.4.6. Dissection and homogenization

Animals were sacrificed by decapitation immediately after behavioral assessment and the brain was harvested quickly to procure ST and SN for protein expression studies.

3.4.7. Western Blotting

Tissue samples were homogenized in RIPA buffer and centrifuged at 10,000 rpm for 30 min to isolate the supernatant. Protein amount was estimated according to method of Lowry *et al.* (25), and the sample containing 50 µg protein was loaded onto the polyacrylamide gels. The gel was then transferred onto a nitrocellulose membrane (PALL Corporation, Biotrace). 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 blots were probed with various antibodies: TH, Caspases-3, -6, - 8, -9, Bax, Bcl-2 and β-actin (1: 1000) with gentle shaking overnight at 4ºC. After this, membranes were incubated with their corresponding secondary antibodies (anti-rabbit IgG conjugated to HRP) for 2 h at room temperature. The membrane was washed thrice with TBST for 30 min. Immunoreactive protein was visualized by the chemiluminescence protocol (GenScript ECL kit, USA). Densitometry analysis was performed with a computer using a gel

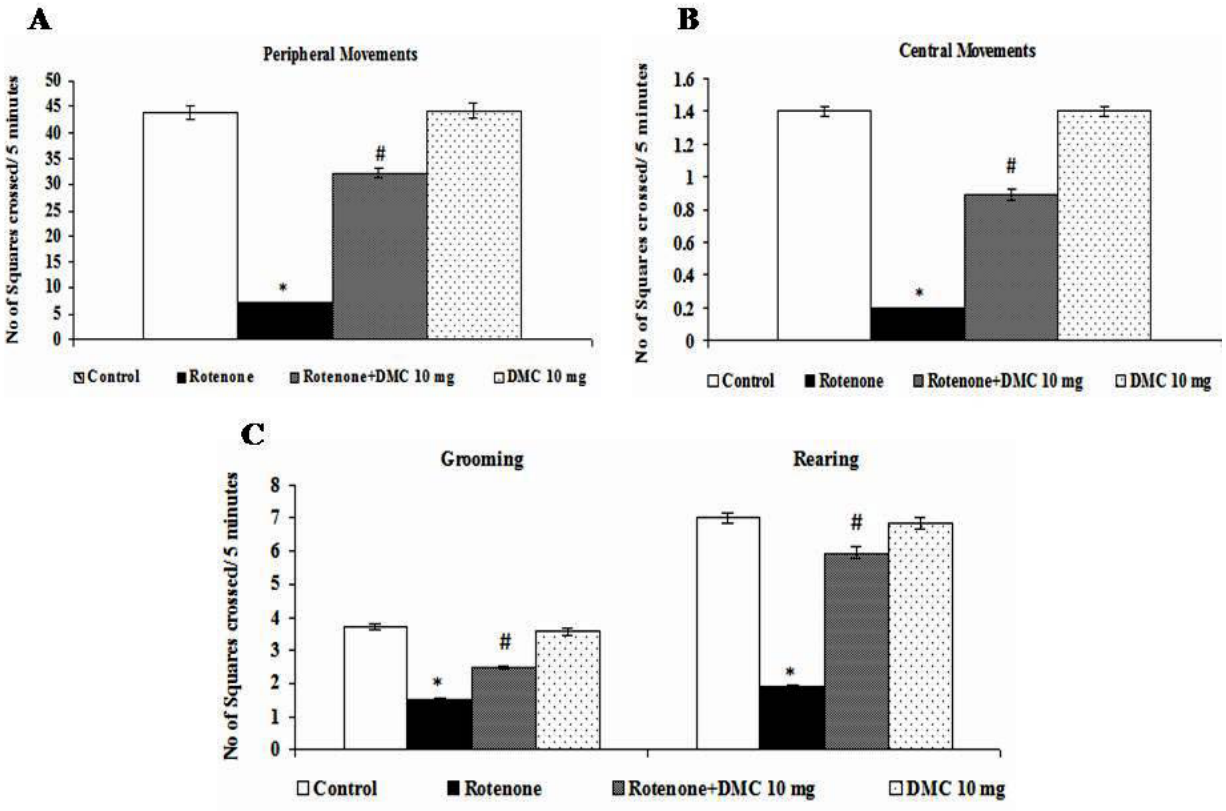

Figure 1. Open field behavior of control and experimental rats: Movements (Peripheral (a) and Central (b)) and activities (Grooming and Rearing (c)) were sign ificantly reduced by rotenone treatment when compared with the control group. However, this reduction in motor activity was attenuated by pre treatment with DMC. Values are given as mean ± SD. #p<0.05 compared to the control, *p<0.05 compared to the rotenone group.

image analysis program. The data were then corrected by background subtraction and normalized against β-actin as an internal control.

3.4.8. Statistical analysis

Statistical analysis was performed by one-way analysis of variance followed by Duncan's multiple range test (DMRT) using Statistical Package for the Social Science (SPSS) software package version 15.0. All data are expressed as mean ± SD for six rats in each group. Results were considered statistically significant at p<0.0.5.

4. RESULT

4.1. Effects of DMC on rotenone induced locomotion and exploratory activity

Rotenone injection led to a significant reduction in the peripheral and central movements (Figure 1, A, B) along with diminished rearing and grooming activities (Figure 1, C). Co-administration of DMC (10 mg/kg b.w) to rotenone treated rats showed a significant increase in the locomotion and non-locomotion activities as compared to rotenone alone intoxicated rats. However, there was no significant changes were observed in DMC (10 mg/kg b.w) alone treated rats as compared to saline treated control rats.

4.2. Effects of DMC on rotenone induced movement impairments

Impaired coordination in movement was observed by catalepsy test. Chronic administration of rotenone caused impairment in correction of an externally imposed posture (catalepsy) as compared to control rats. Co-administration of DMC to rotenone treated rats significantly attenuated rotenone induced catalepsy. No significant changes were observed between control and DMC alone treated rats (Figure 2).

4.3. Effects of DMC on rotenone induced initial movement impairment

Impairment in initiation of movement was measured by akinesia test. Chronic administration of rotenone caused impaired ability to initiate movement (akinesia) as compared to control rats (p<0.0.5). Oral administration of DMC significantly attenuated rotenone induced akinetic movement however it did not restored completely as that of normal animals (Figure 3). Moreover no significant variations were observed between control and DMC alone treated rats.

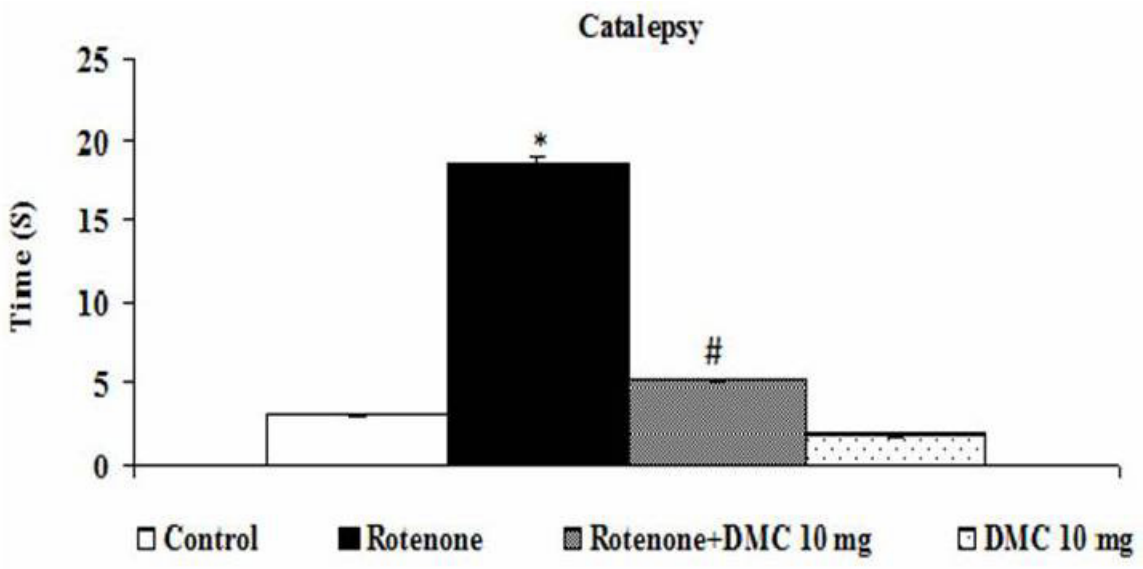

Figure 2. Effect of DMC on rotenone induced control and experimental rats. Rotenone administration caused significantly more latency to move all the four limbs or to correct an externally imposed posture (catalepsy) as compared to the control group. The animals treated with the DMC alone did not show any effect in catalepsy. Data are expressed as mean ± SD. #P <0.05, compared with the control group. *P<0.05, compared with the rotenone group.

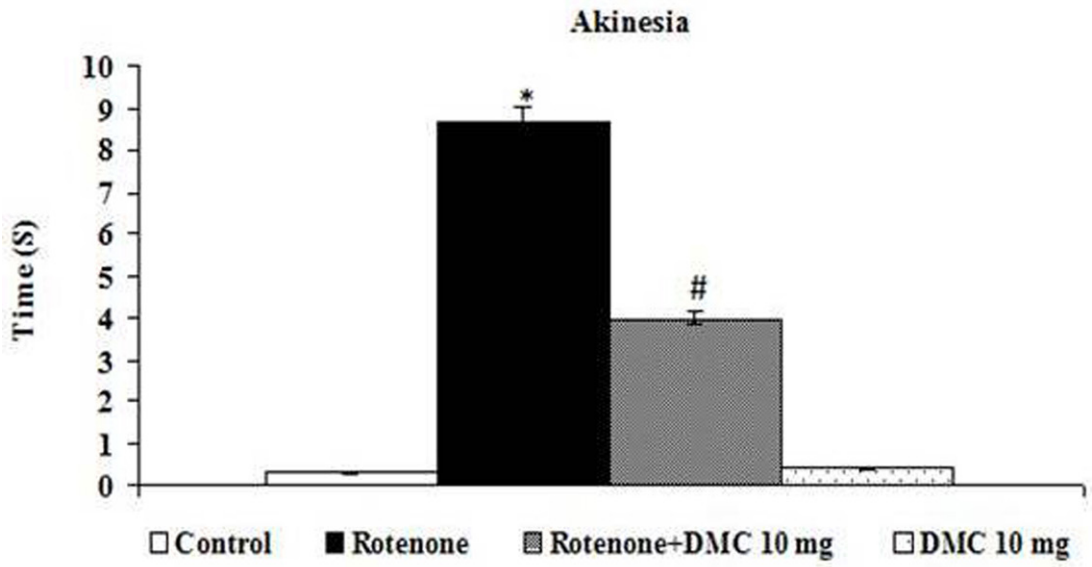

Figure 3. Effect of DMC on rotenone induced control and experimental rats. Rotenone administration caused significantly more latency to move all the four limbs or to correct an externally imposed posture (catalepsy) as compared to the control group. The animals treated with the DMC alone did not show any effect in akinesia. Data are expressed as mean ± SD. #P <0.05, compared with the control group. *P<0.05, compared with the rotenone group.

4.4. Effects of DMC on rotenone induced forced swim test

The forced swim test is one of the most commonly used animal model for assessing antidepressant like behaviour. Rotenone administered rats showed more immobility time in forced swim test as compared to control rats (Figure 4). Rotenone and DMC co-administrated animals had significantly reduced immobility time as compared to rotenone alone treated animals. No significant differences were observed between control and DMC alone treated animals ($p < 0.0.5$).

4.5. Effect of DMC on rotenone induced Sucrose preference test

Depressive like behaviour was indicated by sugar intake test to investigate anhedonia. Rotenone induced animals showed significant decrease in pleasure to drink sweet water (sucrose intake test) as compared to control rats (Figure 5). Co-administration

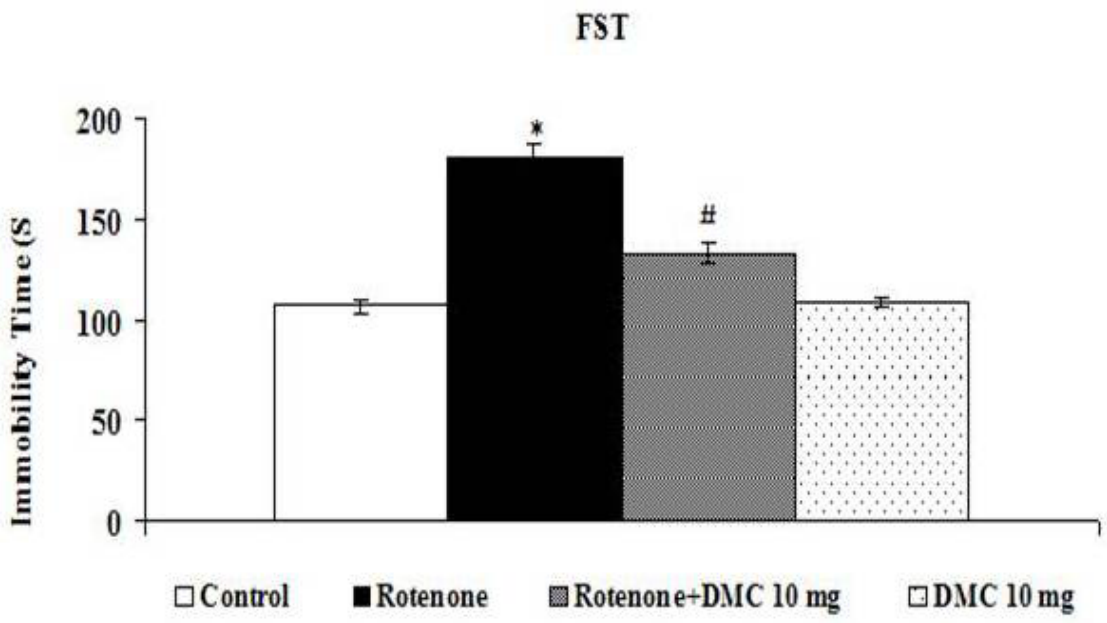

Figure 4. Preventive effect of DMC in rotenone induced swim disability in control and experimental rats. After 3 min, the time spent in immobility was measured, and a difference between the rotenone treated groups and pretreated DMC to rotenone treated rats and no significant change observed in saline treated control group and alone DMC treated group. Data are expressed as mean ± SD. (p0.05). #P <0.05, compared with the control group. *P<0.05, compared with the rotenone group.

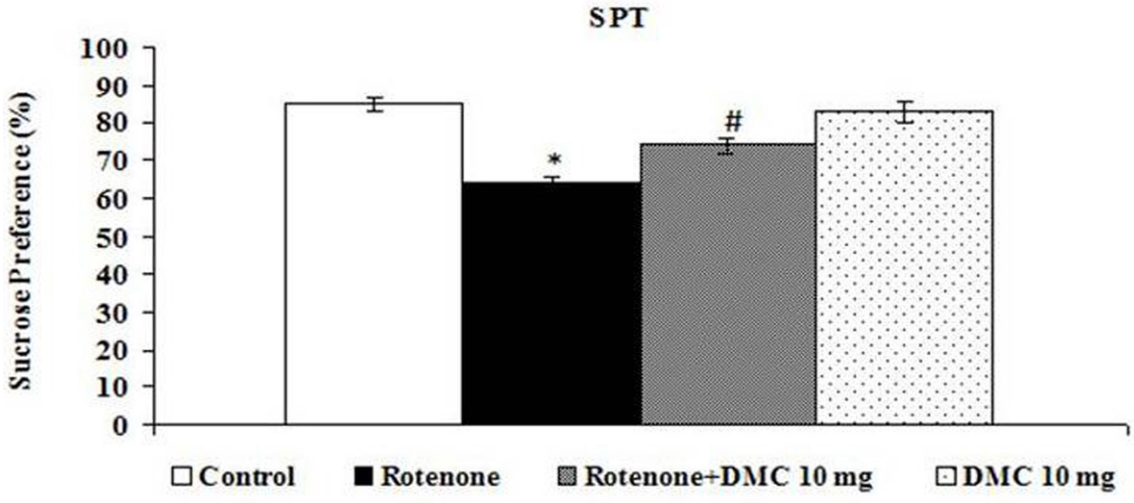

Figure 5. Effect of DMC on rotenone induced depressive like behavior that is indicated by sugar intake in sucrose intake test and immobility time in forced swim test. Data are shown as mean ± SD. #p<0.05 compared to the control rats *p<0.05 compared to the rotenone treated rats.

of DMC significantly improved the sucrose intake. Moreover no significant variations were observed between control and DMC alone treated rats ($p < 0.0.5$).

4.6. Effect of DMC on rotenone induced expression of TH in SN and ST

Rotenone treatment significantly reduced TH expression as compared to control. However, oral administration of DMC (10 mg/kg b.w) significantly enhanced TH expression as compared to rotenone injected rats (Figure 6). Quantification of TH expression showed significant protection in SN and ST by DMC from rotenone induced loss ($p < 0.0.5$). DMC alone treated rats showed no significant changes in the TH expression as compared to control rats ($p < 0.0.5$).

4.7. DMC suppressed rotenone induced apoptosis

As shown in Figure. 7, rotenone significantly enhanced the expression of pro-apoptotic (Bax, Caspase-3, -6, -8 and -9 markers and diminished

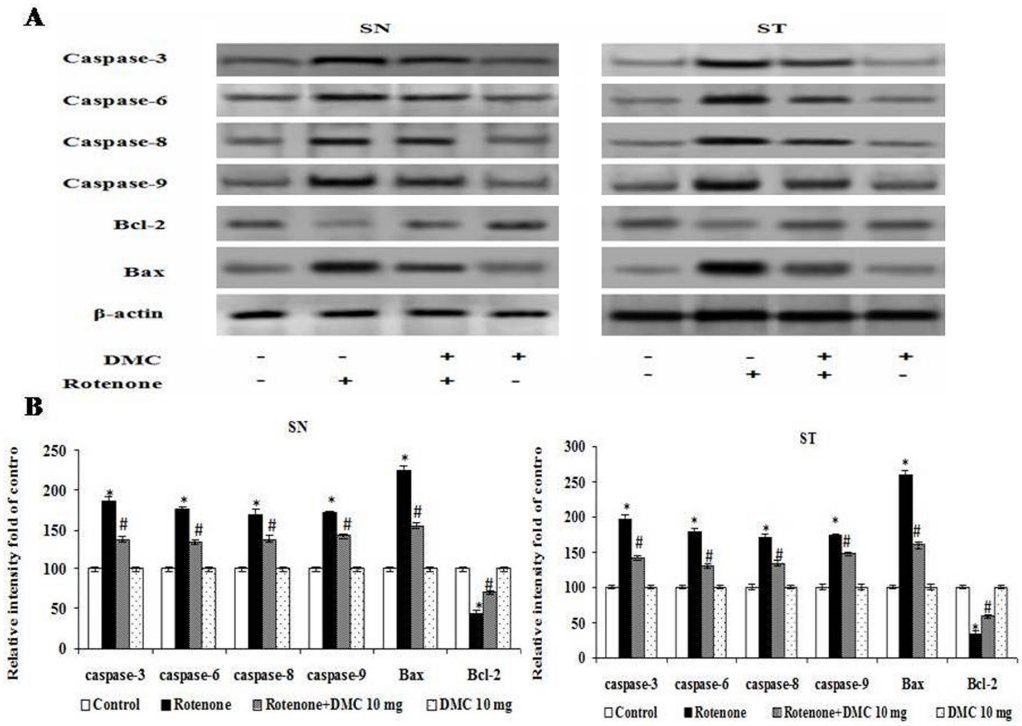

Figure 6. Effect of DMC on expressions of the TH proteins in SN and ST of control and experimental rats. Figure a represents autoradiogram of these protein expressions by using β-actin as an internal control. Figure show the band density was quantified by scanning densitometry. Data are shown as mean ± SD. #p<0.05 compared to the control rats, *p< 0.05 compared to the rotenone treated rats.

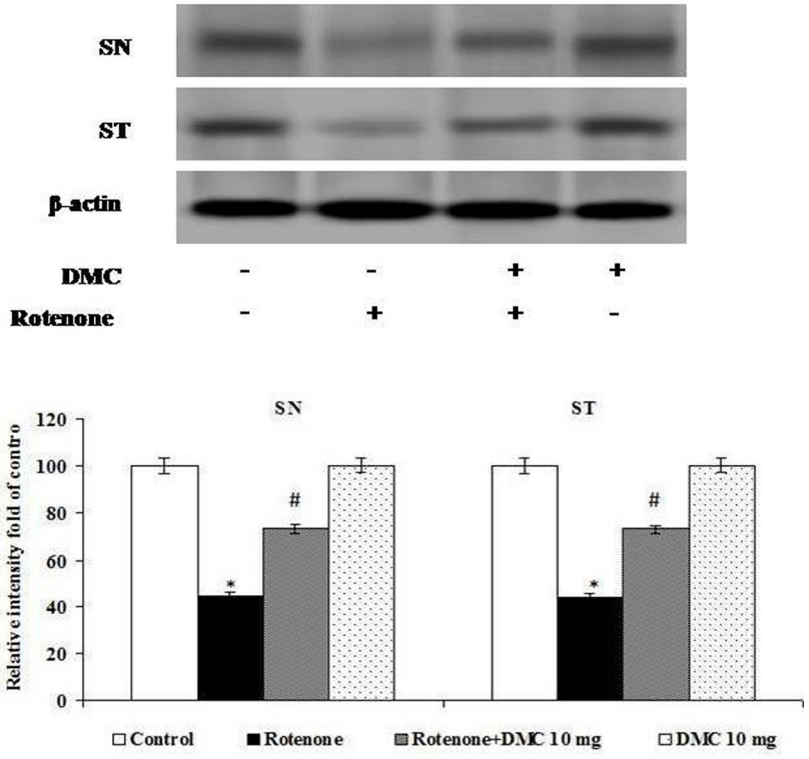

Figure 7. Effect of DMC on rotenone induced changes in the expressions of apoptotic protein markers (Bax, caspase 3, 6, 8 and 9 in control and experimental animals. Figure a represents autoradiogram of these protein expressions by using β-actin as an internal control. Figure A and B show the band density was quantified by scanning densitometry. Data are shown as mean ± SD. #p<0.05 compared to the control rats, *p< 0.05 compared to the rotenone treated rats.

the expression of anti-apoptotic protein Bcl-2 when compared to control ($p < 0.0.5$). Meanwhile, treatment with DMC significantly prevented the expression of pro-apoptotic markers when compared to rotenone group by restoring the expression of Bcl-2 ($p < 0.0.5$).

5. DISCUSSION

Motor function is normally measured by performing a number of behavioural analysis specifically, open field test, hang test, narrow beam walking (4), rotarod performance, stride length (26), swim test, akinesia, catalepsy, pole test (27). Behavior analysis is reported as more sensitive technique to detect functional impairments in PD rodent models and to quantify the therapeutic efficacy that restores dopaminergic function. Baydar *et al.,* (28) reported that the analyses of behavioural changes are more sensitive than neurochemical alterations during neurotoxin exposures. Various behaviour test were performed to measure depression, anxiety and memory impairment (29). As rotenone model mimics both the motor and NMS of PD (30), the neuroprotective effect of DMC against rotenone induced motor and non-motor impairments were assessed in this study.

DA levels are closely associated with the open field activity. In the open field test, peripheral square crossing indicates the general motor performance and acclimatization attempt, whereas central square crossing indicates the exploratory behaviour. Rearing and grooming activities are also indicators of stress. Rearing is known to be highly sensitive to ST or SN lesions (31). Reduced performance of square crossing and activities in rotenone induced animals could be associated with dopaminergic loss (32). In the present study, administration of rotenone exhibited impaired ability to initiate movement (akinesia) and rigidity or inability to correct an externally forced posture (catalepsy). Behavioural assessment of akinesia in rodent models of PD resembles limb akinesia and gait problems of PD patients (33). Rotenone destroyed the dopaminergic neurons selectively and resulting in impaired motor function (34). Depletion of brain DA levels in rotenone treated rats (18) caused behavioural abnormalities as seen in PD patients, whereas enhancement of striatal DA (18) and its regulators including TH in this study, by DMC clearly indicated the neuroprotective efficiency of DMC in protecting dopaminergic neurons and thereby normalizing the behaviour (35).

Rotenone treatment exhibited cognitive impairment such as depression and anxiety as evidenced by sucrose intake and forced swim test. Sucrose preference is frequently used as a measure of anhedonia, another form of depression in rodents. Damages to dopaminergic, serotonergic and noradrenergic systems have been postulated to the prevalence of depression in PD (35). Forced swim test is based on the observation of rodents that were placed in an in escapable and stressful situations develop an immobile posture, after exerting initial escape oriental movements. In a consequent exposure, the commencement of the immobility is faster and more marked. This phenomenon is called "behaviour despair" and is arised due to the animals response to the development of depression process (36). Depletion of monoamine neurotransmitters by rotenone leads to depressive behaviour (37), whereas DMC offers antidepressive action as evidenced by behaviour analysis.

Administration of rotenone induced the loss of TH-immunopositive neurons in SN with significant motor defects. Experimental studies suggested that the decrease in the TH activity or its levels have been due to the underlying pathogenesis of PD (38). In the present study, rotenone treated rats exhibited significant decrease in the expression of TH in SN and ST demonstrated the possible degeneration of dopaminergic neurons. Oxidative stress induces post-translational modification of TH and diminished its catalytic function (38). Furthermore, S-glutathionylation of TH enzyme has been accelerated by ROS (39). It was reported that antioxidants exert a protective effect on TH expression (40). Oral treatment of DMC may enhance TH expression in ST and SN mainly due to its antioxidant property (17,18).

There are several evidences implicating apoptosis and caspase activation in patients as well as in *in vivo* models of NDDs. Mitochondrial dysfunction mediated oxidative damage could further initiate apoptotic neuronal cell death in PD (41). Loss of MMP leads to release of cyt-c into the cytoplasm where it can initiate the activation of caspase cascade by the activation of caspase-9, which leads to the activation of capsase-3, resulting in the morphologic alterations associated with apoptosis (41). On the other hand, activation of FADD leads to the activation of caspase-8, which translocates to mitochondria which, induces cyt-c release and ultimately to caspase-3 activation. Caspase-3, 8, 9 expressions were significantly enhanced in rotenone treated animals as compared to control animals. DMC attenuated rotenone induced apoptosis by inhibiting the release of cyt-c and reducing the expression of caspases (17). These results indicated that DMC treatment had a clear neuroprotective effect against rotenone toxicity. Neurotoxins such as MPTP, rotenone, paraquat and maneb induced cell death by the activation of members of the B cell lymphoma 2 family of proteins (Bcl-2) (42) and Bcl-2 plays an important role in the regulation of mitochondrial mediated apoptosis. Bcl-2 proteins are classified into three groups: those inhibit apoptosis (Bcl-xL, Bcl-2, Bcl-w, Mcl-1, Bcl-10 and Bcl-2 related protein A1); those promote apoptosis (Bax, BAK, Bcl-

rambo, Bcl-xs, BOK/Mtd); and the pro-apoptotic BH3 proteins (Bad, BID, Bik/Nbk, BIM, BLK, Bmf, Hrk/DP5) that regulate the action of anti-apoptotic Bcl-2 proteins. Thus, the balance between Bcl-2 family members performs a vital role in determining cell survival or death. In our study, rotenone administration significantly elevated the expressions of pro-apoptotic Bax and reduced the anti-apoptotic Bcl-2, indicating that rotenone toxicity could be in favour of apoptosis. Bcl-2 act as a frustrating force to reduce apoptotic damage by diminishing lipid peroxidation reactions triggered by cytotoxic agents such as ROS (43). Bcl-2 was also found to prevent the release of cyt-c. In contrast, Bax enhances apoptosis by (i) dimerizing with anti-apoptotic Bcl-2 proteins, (ii) enhancing cyto-c release and subsequent activation of caspase-3 that finally leads to cell death (Ethell and Fei, 2009). However, DMC treatment prevented rotenone induced apoptosis by reducing the expression of Bax, caspases and enhancing the expression of Bcl-2. The multi pharmacological effect of DMC may be responsible for its therapeutic potential and in future DMC may be used alone or alone with present drugs for the treatment of PD. However clinical studies are warranted to support these findings.

6. CONCLUSION

The findings obtained from this and our previous studies showed that DMC offers neuroprotective effect by ameliorating the PD symptoms induced by rotenone might be associated with its antioxidant, mitochondrial protective and anti-inflammatory effects.

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