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Original article

Hibiscetin attenuates oxidative, nitrative stress and neuroinflammation via suppression of TNF-α signaling in rotenone induced parkinsonism in rats

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ABSTRACT

Parkinson's disease (PD) is the gradual and selective degradation of dopamine-releasing neurons in substantia nigra pars compacta (SNpc) and results in postural instability, stiffness, bradykinesia, and resting tremor. The goal of this research was to see how hibiscetin action on PD in rotenone-treated rats. Rats were administered orally with hibiscetin (10 mg/kg) after 1 h rotenone (0.5 mg/kg, s.c.). This therapy regimen was followed on a daily basis for 28 days.

Rats were tested for catalepsy and akinesia on day 29 after the last dosage of rotenone. Biochemical parameters were performed to measure reduced glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), malondialdehyde (MDA), nitrite, neuroinflammatory cytokines, and neurotransmitter and their metabolite levels such as dopamine (DA), norepinephrine (NE), serotonin (5-HT), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA). Rotenone-induced akinesia and catatonia in rats decreased endogenous antioxidant (GSH, CAT, and SOD) levels, increased MDA and nitrite levels, and changed neurotransmitter and metabolite levels. Hibiscetin effectively reduced rotenone-induced akinesia and catatonia, improved endogenous antioxidant (GSH, CAT and SOD) levels, and reduced oxidative and nitrative stress in the treated rats. Moreover, hibiscetin restored altered neurotransmitters and their metabolites to normal levels in rotenone-treated rats. The study results showed that hibiscetin has anti-Parkinson's activity against rotenone-induced PD in rats.

1. Introduction

Parkinson's disease (PD) is gradual and selective degradation of dopamine releasing neurons in substantia nigra pars compacta (SNpc) and results in postural instability, stiffness, bradykinesia and resting tremor (El-Ghaiesh et al., 2020, Wang et al., 2020). Previous research has found that genetic, stochastic and environmental factors play a role in the progression of PD (Zhang et al., 2017, Wang et al., 2020).

Reactive oxygen species (ROS) generation in mitochondria with subsequent DNA deletions defines PD and destroys dopaminergic neurons (El-Ghaiesh et al., 2020). In patients with PD, free

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radical-induced damage, oxidative stress, inflammatory alterations, and dysfunction of mitochondria have been seen in their brains (Zhang et al., 2017, Wang et al., 2020).

Immune dysregulation in brain leads to inflammatory cytokines production, create a chain of pro-inflammatory events that finally results in PD-related neurotoxicity (Tansey et al., 2022). The interferon gamma (IFNγ) and tumor necrosis factor- α (TNF-α) pathways are gaining more interest in research investigating PD pathogenesis (Tansey et al., 2022). TNF-α levels in the serum, cerebrospinal fluid (CSF) and brain of PD patients were shown to be elevated (Eidson et al., 2017, Tansey et al., 2022). Targeted neutralisation of soluble TNF-α signalling drastically reduces dopaminergic cell mortality in an experimental investigation, suggesting TNF-α function in nigral degeneration (Barnum et al., 2014, Tansey et al., 2022). Furthermore, increased neuroinflammatory cytokines have been observed in PD, and this correlates with impairment and disease severity (Brodacki et al., 2008, Reale et al., 2009, Tansey et al., 2022).

Although dopamine replacement therapy is increasingly used to treat PD, long-term treatment can cause dyskinesia and fluctuations in motor activities and free radical production, in some patients which can exacerbate neuronal degeneration (Garabadu and Agrawal 2020).

Several treatments have been studied for their ability to delay the onset of PD or to delay the destruction of dopaminergic neurons, but none have proven to be successful (Garabadu and Agrawal 2020). Currently used medications to treat PD include levodopa (associated with motor complications), catechol-O-methyl-transferase inhibitors (associated with hepatotoxicity), dopamine agonists (associated with peptic ulcer disease, erythromelalgia and pulmonary and retroperitoneal fibrosis), anticholinergics (associated with cognitive impairment, dry mouth and urinary symptoms) but side effects limit their use (Garabadu and Agrawal 2020). The natural products are being investigated in this context for the creation of a new medication that can prevent nigrostriatal system degeneration in adults.

Rotenone is a herbal pesticide which can interfere with mitochondrial complex-I activity and cause oxidative stress in neurons (Sonia Angeline et al., 2012). Rotenone has been used in numerous research to create PD-like symptoms in experimental animals (Sonia Angeline et al., 2012, von Wrangel et al., 2015, El-Ghaiesh et al., 2020). Because of its extremely lipophilic characteristics, rotenone easily penetrate blood–brain barrier and then dopaminergic neurons without the help of transporter (Sarbishegi and Charkhat Gorgich 2019). The rotenone-induced PD symptoms are linked to mitochondrial electron transport chain disruption, which results in the generation of excessive ROS, increased oxidative stress, depletion of ATP, neuronal death and motor impairments (Sarbishegi and Charkhat Gorgich 2019, Rodsiri et al., 2020).

Hibiscus sabdariffa (family: Malvaceae) is also known as "red sorrel" or "roselle". Its calyces are used as a flavouring agent in chocolates, ice cream, jam, puddings, cakes and beverages such as herbal drinks, fermented drinks, wine etc (Da-Costa-Rocha et al., 2014). It has been reported to possesses antioxidant (Subhaswaraj et al., 2017), antibacterial (Abdallah 2016), hepatoprotective (Yin et al., 2011), nephroprotective(Anwar Ibrahim and Noman Albadani 2014), diuretic (Alarcón-Alonso et al., 2012), antilipidemic (Hopkins et al., 2013), antidiabetic(Bule et al., 2020) and antihypertensive (Hopkins et al., 2013) properties. Flavonoids, anthocyanins, organic acids and polysaccharides are the primary elements of *H. sabdariffa* that are responsible for its pharmacological action (Da-Costa-Rocha et al., 2014). *H. sabdariffa* contains flavonoids including hibiscitrin (hibiscetin-3-glucoside) (Da-Costa-Rocha et al., 2014). The literature review revealed that the hibiscetin, aglycone part of hibiscitrin is not studied for its pharmacological activities including neuroprotective actions. Based on these findings, the current study was designed to assess the effect of hibiscetin on oxidative and nitrative stress and neuroinflammation in rotenone-Parkinson's disease in rats and possible role of TNF-α signalling pathway.

2. Material and methods

2.1. Chemicals and reagents

Griess reagent, rotenone and thiobarbituric acid (TBA) (Sigma Aldrich, St. Louis, U.S.A.) and reduced glutathione (GSH) (Hi-Media Laboratories Pvt., Ltd., Mumbai, India) were used. High grade hibiscetin (98.0% purity) was used in the study and obtained from TGS Pvt. Ltd. India. High-quality reagents and chemicals were used in the experiment.

2.2. Animals

Rats (Wistar 180–220 g) maintained under regular controlled circumstances following Institutional ethical committee recommendations (IAEC/ TRS/PT/022/010). The rats had unrestricted access to water and food pellets.

2.3. Experimental design

OECD ANNEX-423 standards were used to assess the acute oral toxicity of hibiscetin and as per the recent toxicity study, hibiscetin (10 mg/kg) was administered to rats orally (Gilani et al., 2022). Hibiscetin was administered orally to rats for 28 days after being diluted with a 0.5% sodium carboxymethyl cellulose (Na-CMC) solution. Rotenone was emulsified in sunflower oil and administered subcutaneously (0.5 mg/kg) to rats to induce neurotoxicity (Sharma and Bafna 2012).

Total 24 rats were divided into 4 groups and tread as shown below:

Group-1 (normal control): 1 ml 0.5% of Na-CMC solution (p.o.) + sunflower oil (s.c.).

Group-2 (rotenone control): 1 ml of 0.5% Na-CMC solution (p.o.) + 0.5 mg/kg of rotenone (s.c.).

Group-3 (hibiscetin per se): hibiscetin 10 mg/kg (p.o.) + sunflower oil (s.c.)Group-4 (test group): hibiscetin 10 mg/kg (p.o.) + 0.5 mg/kg of rotenone (s.c.).

This treatment schedule was followed daily for 28 days and there was 60 min gap between the above mentioned oral and subcutaneous treatment.

On day 29, rats were evaluated for catalepsy and akinesia. Then, they were euthanized, brain was removed for biochemical analysis such as endogenous antioxidants (reduced glutathione, catalase, superoxide dismutase), oxidative (malondialdehyde) and nitrative (nitrite) stress markers, neurotransmitters and their metabolites such as DA, 5-HT, NE, DOPAC, 5-HIAA and HVA and inflammatory mediators (IL-1β, IL-6 and TNF-α).

2.4. Behavioural parameters

2.4.1. Catalepsy

The catalepsy was assessed using a bar test according to the stated technique (Garabadu and Agrawal 2020). In this test, three trials were conducted to measure how long it took rats to remove one of their forelimbs from a bar (9 cm high) after being placed in the half-raising position and the cut-off time for test was 3 min.

2.4.2. Akinesia

Akinesia in the animals was measured by watching their latency (sec) while they moved all four limbs. Before the akinesia test, each rat was acclimatised on a hardwood raised (100 cm) platform. The time it took the animal to move all four limbs was timed using a stopwatch. In this test, for each animal 180 sec were given to complete the task (Venkateshgobi et al., 2018).

2.5. Biochemical examination of brain tissue

2.5.1. Brain tissue homogenate preparation

In an ice-cold phosphate buffer, the cleansed brain tissue was homogenised. The brain suspension was spun for 15 min at 10,000 g, and supernatant was biochemically analysed.

2.5.2. Endogenous antioxidants

The Ellman method was used to calculate the amount of reduced glutathione (GSH) (Ellman 1959). SOD was estimated using the Misra and Frodvich method (Misra and Fridovich 1972), the supernatant of brain homogenate (0.2 ml) was mixed with 0.8 ml 50 mM glycine buffer (pH 10.4). By adding 0.02 ml of epinephrine in it initiated the reaction. After 5 min the absorbance was measured at 480 nm. To estimate catalase activity, 0.1 ml of supernatant was added to 1.9 ml phosphate buffer (pH 7.0, 50 mM) in the cuvette. 1.0 ml of freshly prepared H_2O_2 (30 mM) was added to initiate the reaction. The catalase activity (CAT) was represented as $\mu M/H_2O_2$ decomposed/min (Aebi et al., 1974).

2.5.3. Malondialdehyde (MDA) and nitrite

MDA was calculated using the Wills method (Wills 1966) and represented as nmol/mg tissue. For nitrite estimation, the Griess method was utilised and expressed as μg/mg tissue (Green et al., 1982, Nagakannan et al., 2012).

2.5.4. Neurotransmitters and their metabolites

The neurotransmitter and their metabolites such as dopamine (DA), norepinephrine (NE), serotonin (5-HT), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA) were estimated using analytical kits.

2.5.5. Cytokines

The TNF-α, IL-6 and IL-1β levels were measured by immunoassay kit and expressed in pg/ml of sample.

2.6. Histopathology

In order to analyze the brains from all groups, they were separated, rinsed with cold saline, and stored in formalin buffer. Fixing the tissues and slicing them into 3–4 μm slices were followed by staining with haematoxylin and eosin. Histological changes were examined by a pathologist using a light microscope (10x lens).

2.7. Statistical analysis

The results were reported as mean ± S.E.M. One way ANOVA followed by Tukey's test with limits of $P < 0.05$.

3. Results

During acute oral toxicity testing, hibiscetin was found to be safe in rodents with no mortality or adverse effects. The dose of hibiscetin selected was 10 mg/kg based on safety data.

3.1. Behavioural parameters

3.1.1. Catalepsy

In the catalepsy test, normal control rats retracted their limb from the elevated bar (3.0 ± 0.34 sec) and corrected their posture quickly. The rotenone control rats were not able to retract their limb from the elevated bar and correct their posture quickly. Rotenone treated rats required significantly (17.38 ± 1.40 sec, $P < 0.001$) more time to correct their posture when compared to normal control. Hibiscetin (10 mg/kg) improved muscular rigidity in treated rats and they took significant reduced (5.76 ± 0.37 sec, $P < 0.001$) time to remove limb from the elevated surface. No changes were observed in hibiscetin alone treated rats compared to the normal. Catalepsy test results are shown in Fig. 1.

3.1.2. Akinesia

Normal control animals initiated a normal movement when they moved all four limbs on a hardwood raised platform. Rotenone-treated rats took considerably longer time ($P < 0.001$) to remove all four paws, showing difficulty in movement initiation. The administration of hibiscetin improved (1.60 ± 0.12 sec) the rotenone-induced difficulties in movement start when compared to the rotenone control group. The results were statistically significant ($P < 0.001$) Vs rotenone control rats. The hibiscetin alone therapy had no effects on akinesia in rats. Fig. 2 depicts the outcome of influence of hibiscetin on rotenone-induced akinesia.

3.2. Biochemical examination of brain tissue

3.2.1. Endogenous antioxidants

Rotenone reduced endogenous antioxidant levels in the rat brain, with lowered levels of GSH (6.38 ± 0.41, $P < 0.001$), SOD (1.15 ± 0.10, $P < 0.001$), and CAT (2.75 ± 0.22, $P < 0.001$) reported in rotenone-treated rats in comparison with normal control rats.

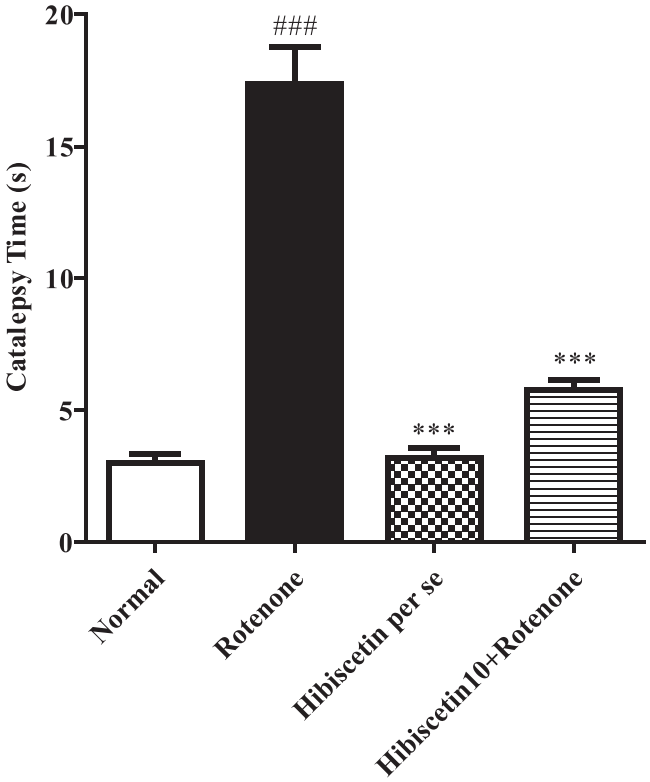

Fig. 1. Effect of hibiscetin on catalepsy in rotenone treated rats. Mean ± S.E.M. (n = 6). $^{###}P < 0.001$ vs normal control and $^{***}P < 0.001$ vs rotenone control.

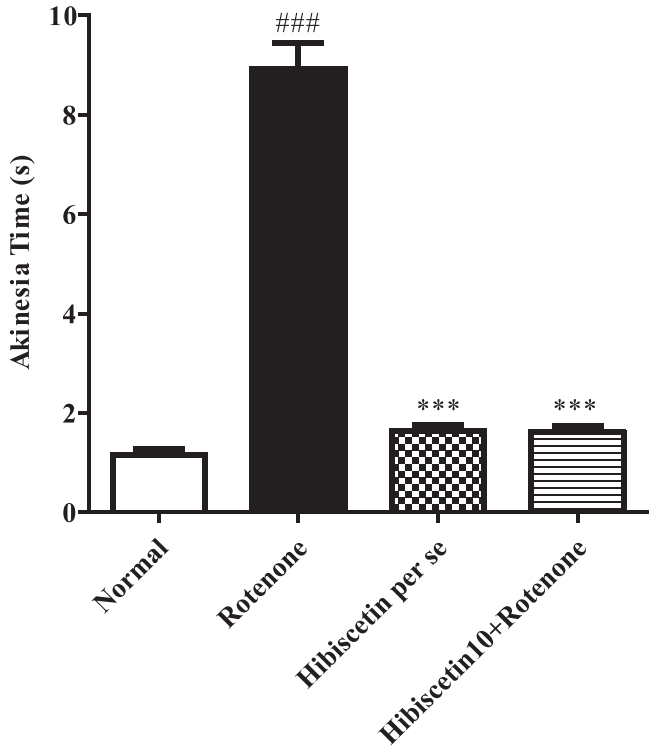

Fig. 2. Effect of hibiscetin on akinesia in rotenone treated rats. Mean ± S.E.M. (n = 6). $^{###}P < 0.001$ vs normal control and $^{***}P < 0.001$ vs rotenone control.

Compared to rotenone control rats, hibiscetin (10 mg/kg) recovered the levels of GSH (12.10 ± 1.20, $P < 0.05$), SOD (3.06 ± 0.18, $P < 0.001$), and CAT (6.11 ± 0.39, $P < 0.05$). The administration of hibiscetin to normal animals produced no difference compared to the normal control. Fig. 3 depicts the endogenous antioxidant levels.

3.2.2. MDA and nitrite

Rotenone increased MDA (10.55 ± 1.22, $P < 0.001$) and nitrate (249.4 ± 8.52, $P < 0.001$) levels in animals in comparison with normal control rats. Hibiscetin reduced MDA levels (3.30 ± 0.30, $P < 0.001$) and nitrite levels (149.9 ± 8.67, $P < 0.001$) when compared to the rotenone control group. These results were statistically significant as compared to rotenone control rats. Hibiscetin therapy alone had no effect on MDA or nitrite levels Vs control. Fig. 4 depicts the MDA and nitrite results.

3.2.3. Neurotransmitters and their metabolites

Rotenone administration lowered neurotransmitter levels in rats, including DA (0.80 ± 0.10, $P < 0.05$), NE (22.08 ± 2.14, $P < 0.001$), and 5-HT (12.85 ± 0.97, $P < 0.001$). Rotenone, on the other hand, boosted DOPAC (352.7 ± 15.29, $P < 0.001$) and HVA (420.0 ± 16.35, $P < 0.001$) levels in the treated animals while decreasing 5-HIAA (50.37 ± 4.18, $P < 0.001$). The levels of neurotransmitters and their metabolites in rotenone control mice were significantly higher ($P < 0.001$) than in normal control animals. After rotenone injection, hibiscetin restored DA (1.75 ± 0.13, $P < 0.05$), NE (33.03 ± 3.03, $P < 0.05$), 5-HT (28.88 ± 3.69, $P < 0.01$), DOPAC (134.3 ± 11.98, $P < 0.001$), HVA (207.6 ± 11.41, $P < 0.001$), and 5-HIAA (80.20 ± 4.58, $P < 0.01$) to normal. Hibiscetin alone did not cause any substantial changes in neurotransmitters or their metabolites. Fig. 5 depicts the outcomes of these estimations.

3.2.4. Neuroinflammatory cytokines

The rotenone control animals had considerably higher levels of IL-6 (109.5 ± 6.48, $P < 0.001$), IL-1β (110.6 ± 6.22, $P < 0.001$), and TNF-α (5.23 ± 0.28, $P < 0.001$). Hibiscetin administration to rotenone-injected rats reduced IL-6 (69.52 ± 3.11, $P < 0.001$), IL-1β (59.13 ± 3.48, $P < 0.001$), and TNF-α (2.41 ± 0.19, $P < 0.001$) in comparison to normal rotenone control rats. Hibiscetin alone had no effect on neuroinflammatory indicators Vs normal control rats. Fig. 6 depicts the neuroinflammatory cytokine results.

3.3. Histopathological changes

In the rotenone control rats, histopathological examination revealed many pyknotic neurons characterized by dark cytoplasm, shrinking, and vacuolation. A greater percentage of degenerated neurons were found in the rotenone group. Compared to rotenone control rats, hibiscetin (10 mg/kg) to rats ameliorated percent of

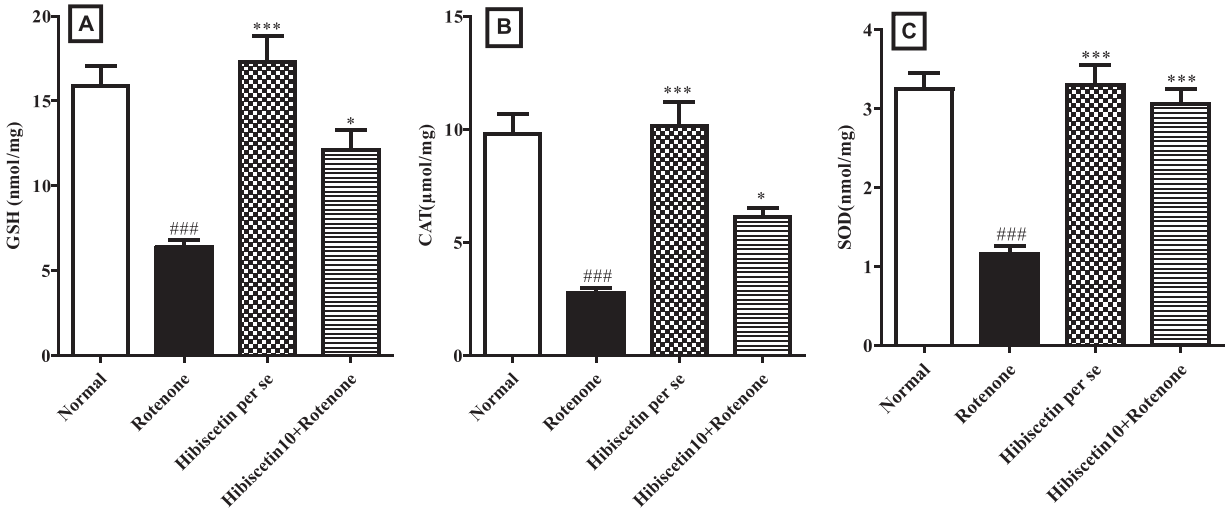

Fig. 3. Effect of hibiscetin on A] GSH, B] catalase and C] SOD activities in rotenone treated rats. Mean ± S.E.M. (n = 6). $^{###}P < 0.001$ vs normal control and $^{*}P < 0.05$ and $^{***}P < 0.001$ vs rotenone control.

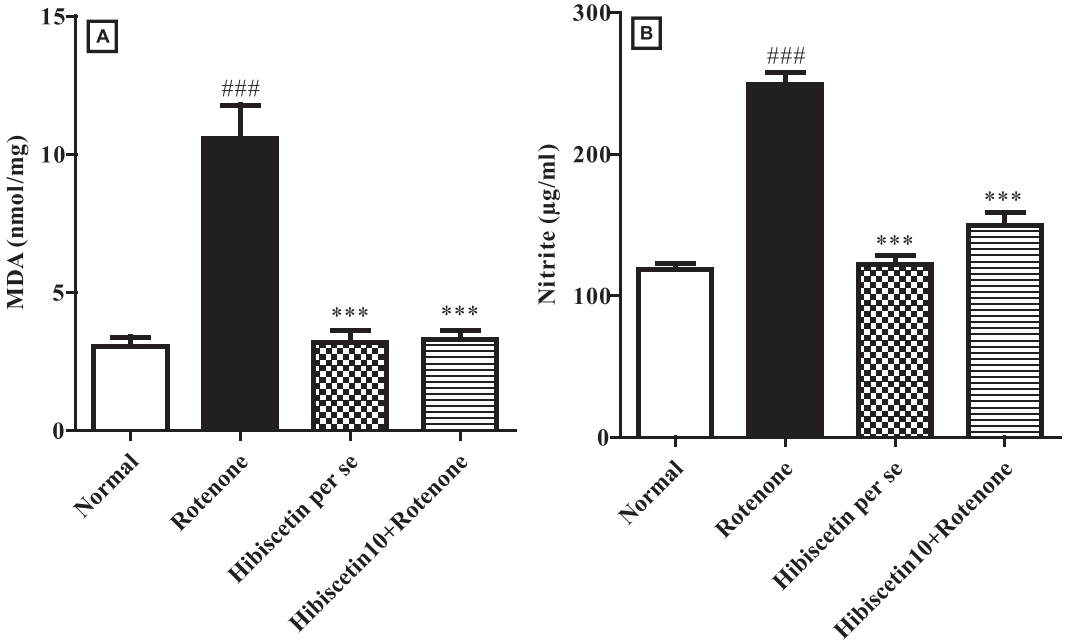

Fig. 4. Effect of hibiscetin on A] MDA and B] Nitrite in rotenone treated rats. Mean ± S.E.M. (n = 6). $^{###}P < 0.001$ vs normal control and $^{***}P < 0.001$ vs rotenone control.

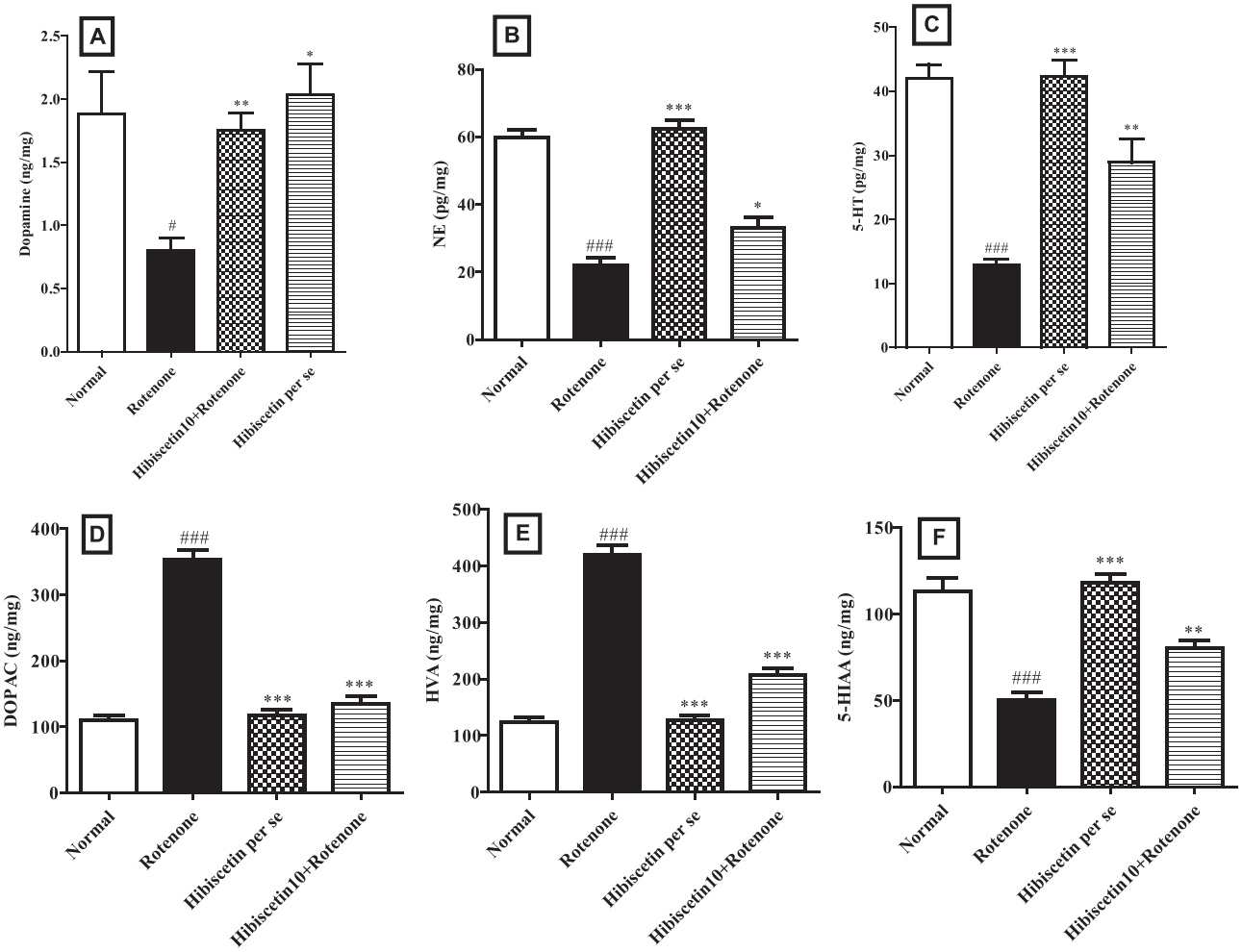

Fig. 5. Effect of hibiscetin on A] Dopamine, B] NE, C] 5-HT, D] DOPAC, E] HVA and F] 5-HIAA in rotenone treated rats. Mean ± S.E.M. (n = 6). $^{###}P < 0.001$ vs normal control and $^{**}P < 0.01$ and $^{***}P < 0.001$ vs rotenone control.

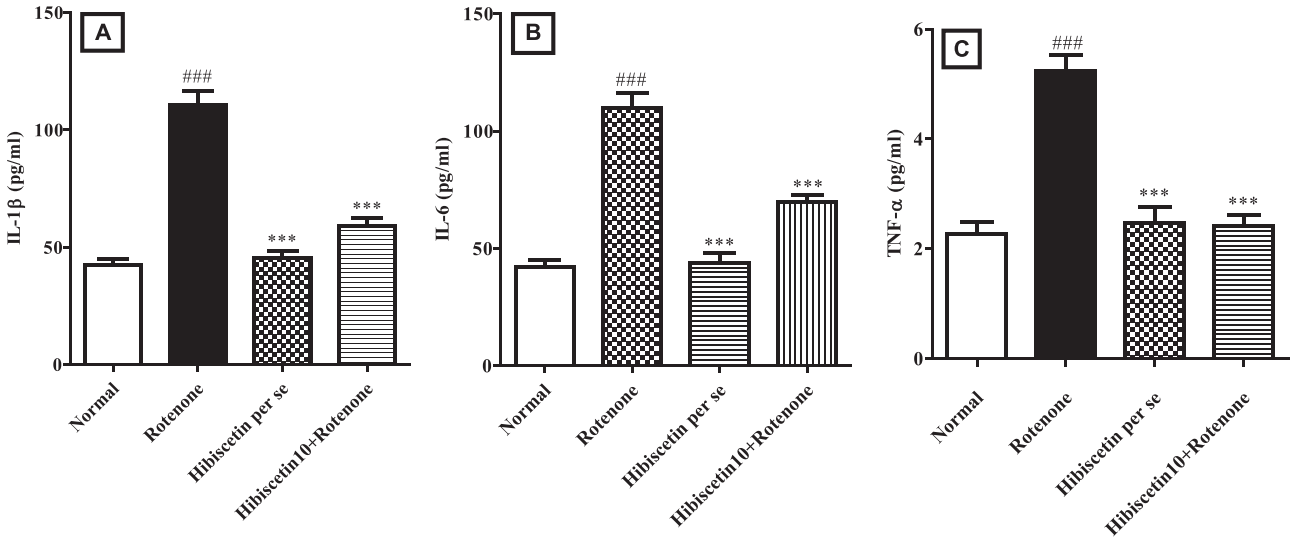

Fig. 6. Effect of hibiscetin on A] IL-1β, B] IL-6, and C] TNF-α in rotenone treated rats. Mean ± S.E.M. (n = 6). $^{###}P < 0.001$ vs normal control and $^{***}P < 0.001$ vs rotenone control.

neurons and showing pyknosis versus the rotenone control group (Fig. 7).

4. Discussion

Rotenone-induced PD in rodents is one of the commonly used preclinical model to study anti-Parkinson's effects of drugs (Lawana and Cannon 2020). Rotenone-induced PD mimics the symptoms of Parkinson's patient. Rotenone inhibits NADH oxidation and produces neurotoxicity by suppressing the mitochondrial respiratory chain complex-I enzyme (Lawana and Cannon 2020). Because dopaminergic neurons are more susceptible to oxidative stress, exposing them to rotenone selectively kills these neurons and causes PD-like symptoms (Lawana and Cannon 2020).

Neuronal degeneration in the movement-controlling area of the brain happens in PD, resulting in early symptoms such as muscle rigidity, a shuffling step, difficulty starting movement(Geibl et al., 2019, Klein et al., 2019). The current study results substantially support the previously reported findings of rotenone-induced muscular rigidity, loss of muscle control, and restricted body mobility in rotenone-treated rats, as demonstrated by severe cata-

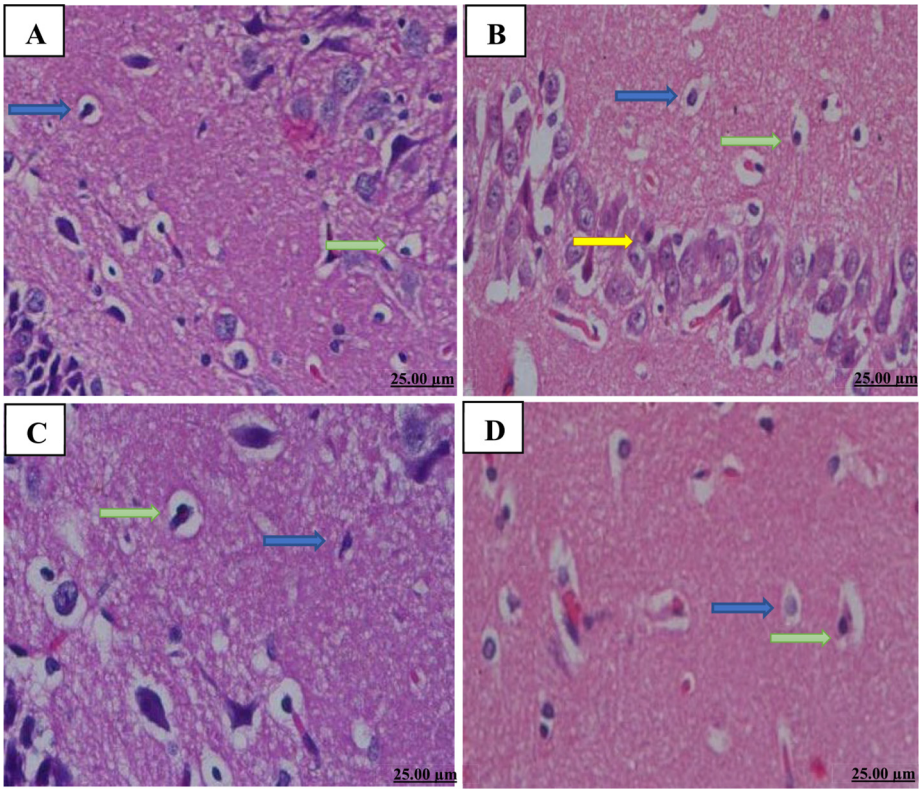

Fig. 7. Effect of hibiscetin on histological changes in all the groups. A. Normal group, B. Rotenone induced group, C. Hibiscetin per se (10 mg/kg), D. Hibiscetin (10 kg/kg) + Rotenone. The arrows indicate **Blue-** normal neuronal cell **Green-** Pyknotic cells **Yellow-** degenerated neurons.

tonia and akinesia. Hibiscetin treatment of rotenone-treated rats reduced catatonic behaviour and increased the moments of animals. These findings supported positive effects of hibiscetin on rotenone-induced muscle rigidity and, as a result, reduced body mobility in the rats.

Rotenone reduced endogenous antioxidants while increasing MDA and nitrate levels in the rat brain. Hibiscetin reduced rotenone-induced depletion of endogenous antioxidant levels as well as oxynitrative stress in rats, suggesting its anti-rotenone antioxidant activity. Dysfunction of mitochondria and nitroxidative stress are two interrelated mechanisms implicated in PD neurodegeneration (Nunes and Laranjinha 2021).

Dopamine is metabolised by the enzyme monoamine oxidase (MAO) to DOPAC, which is then transformed to HVA by catechol-O-methyl transferase (COMT) (Wallace and Traeger 2012, Sharma et al., 2020). Dopamine is metabolised to 3-MT by the enzyme COMT, which is then turned to HVA by the enzyme MAO (Wallace and Traeger 2012, Sharma et al., 2020). The third metabolic end-product of dopamine is norepinephrine (Gnegy et al., 2012). DOPAC degrades into hazardous metabolites in existence of H_2O_2, causing damage to dopamine storage vesicles. This could contribute to the failure of levodopa therapy for PD. In patients with PD, an MAO-B inhibitor can help with these issues. In the current work, rotenone treatment causes dopamine and norepinephrine depletion while increasing DOPAC and HVA levels in rat brains. In contrast, treatment of hibiscetin to rotenone-treated rats increased dopamine and norepinephrine levels while decreasing DOPAC and HVA levels, indicating that hibiscetin has an MAO-B inhibitor-like activity.

The serotonergic system is involved in the aetiology of PD non-motor symptoms (Miguelez et al., 2014, Tong et al., 2015). As a result, 5-HT and its metabolite 5-HIAA may be used as indicators for PD (Miguelez et al., 2014, Tong et al., 2015). Rotenone administration drastically reduced 5-HT and, ultimately, 5-HIAA in rats, indicating depression in the rodents. Hibiscetin reduced rotenone-induced 5-HT and 5-HIAA depletion in rats. This finding suggests that hibiscetin may be useful in rotenone-induced non-motor symptoms in rats.

Pathogenesis of PD is complicated, and inflammation plays a role in neurodegeneration (Caggiu et al., 2019). PD patients have higher amounts of inflammatory cytokines (Caggiu et al., 2019). In present findings, rotenone-induced inflammatory markers, largely validate prior findings. The administration of hibiscetin reduced the rotenone-induced TNF-α, IL-1β, and IL-6 levels. This demonstrates hibiscetin's anti-inflammatory properties in rats with rotenone-induced neuroinflammation.

Histomorphological observations confirmed the effectiveness of hibiscetin against rotenone-lesioned rats. According to previous studies, rotenone groups showed neuronal degeneration (Ameen et al., 2017, Abdel-Salam et al., 2018). Hibiscetin (10 mg/kg) improved neuronal structure and attenuated pyknosis in rats.

These findings revealed that hibiscetin protects the prominent effects on behavioural activity, endogenous biomarkers and inhibited inflammation via enhancement of mitochondrial function. By ameliorating the toxic effects of rotenone, hibiscetin might alter neuronal properties to provide neuroprotection. Furthermore, hibiscetin per se treatment had no effects on behavioural activity and endogenous biomarkers and pathways which is still unexplainable at the moment.

5. Conclusion

The current study demonstrates that hibiscetin lowers rotenone-induced catalepsy, akinesia, and metabolic abnormalities in rats by lowering the inflammatory response and free radical damage. The observed favourable effect could be attributable to neuroinflammatory cytokine inhibitory actions of hibiscetin.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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