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Inhibition of Oxidative Stress and the NF-κB Pathway by a Vitamin E Derivative: Pharmacological Approach against Parkinson's Disease

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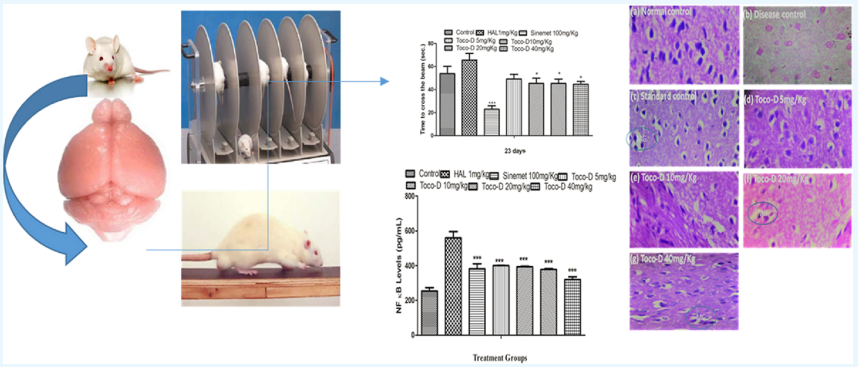

ABSTRACT: Parkinson's disease (PD) is a progressive neurodegenerative disorder. In this study, PD was induced via (ip) injection of haloperidol (1 mg/kg/day). Animals were divided into seven groups (n = 70). Group I received the vehicle carboxymethylcellulose (CMC; 0.5%), group II was treated with designated 1 mg/kg haloperidol, and group III received the standard drug Sinemet (100 mg/kg), while groups IV−VII received a tocopherol derivative (Toco-D) at dose levels of 5, 10, 20, and 40 mg/kg, respectively, via the oral route. All groups received haloperidol for 23 consecutive days after their treatments except the control group. The improvement in locomotor activity and motor coordination was evaluated by using behavioral tests. Oxidative stress markers, neurotransmitters, and monoamine oxidase B (MAO-B) as well as NF-κB levels in the whole brain were measured. mRNA expression analysis of α-synuclein was carried out using the PCR technique. Toco-D at 20 mg/kg showed the maximum improvement in locomotor activity. The levels of antioxidant enzymes and neurotransmitters were also increased by the treatment with Toco-D. Inflammatory cytokine levels and mRNA expression of α-synuclein were decreased by Toco-D in treated animals. This study concluded that Toco-D might be effective in the improvement of locomotor activity and motor coordination in haloperidol-induced PD.

INTRODUCTION

Parkinson's disease (PD) occurs due to the degeneration of dopaminergic neurons characterized by both motor and non-motor features due to accumulation of Lewy bodies, mitochondrial dysfunction, oxidative stress, neuroinflammation, genetics, and environmental toxins.[1] Reactive oxygen species (ROS) contribute to dopaminergic neuronal loss in the PD brain due to dopamine metabolism, hydrogen peroxide, biochemical pathways such as nitric oxide (NO) synthesis and arachidonic acid metabolism, and high concentrations of polyunsaturated fatty acids.[2] Previous studies showed that transcriptional-factor-like nuclear factor κB (NF-κB) is up-regulated during neuroinflammation in the brain, which leads to PD by destroying dopaminergic neurons through autophagy.[3] Mutations in α-synuclein also contributes to the progression of PD by enhancing inflammatory responses.[4]

Some studies revealed that foods rich in vitamin E showed a protective role against PD.[5] A previous study mainly focusing on the role of vitamins in neurodegenerative diseases reported that dietary food enriched with vitamin E decreased the risk factors of PD.[5b,6] Tocopherol (vitamin E) is an antioxidant substance that protects the cell membrane by binding with the cell membrane and chemically protects the body from oxygen radicals and also protects against oxidation of lipids, nucleic acids, and proteins in PD patients. Haloperidol-induced PD in

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experimental animals is a common method of chemically induced PD. Haloperidol is an antipsychotic drug and induces PD by inhibiting D2 receptors postsynaptically in the mesolimbic area of the brain. This inhibition leads to neurodegeneration and causes oxidative stress.[7]

Nitro-group-containing compounds react with ROS and attract particular attention as antioxidant agents.[8] The naphthalene nucleus attracts interest in the development of new drugs because of its flexible nature. Different drugs are available in the market that contain a naphthalene nucleus like rifampicin.[9] Previous studies showed that vitamin E and many naphthalene derivatives have therapeutic regenerating properties and are used in many neurodegenerative disorders. The main objective of this study was to evaluate any beneficial effect when vitamin E is combined with naphthalene and add up the therapeutic benefits of both compounds on PD. The main hypothesis of this study is to explore the therapeutic effect of a naphthalene-based vitamin E derivative (Figure 1) in PD.

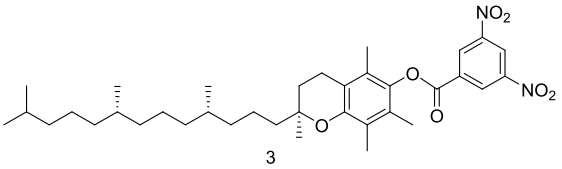

Figure 1. 2,5,7,8-Tetramethyl-2-((4*S*,8*S*)-4,8,12-trimethyltridecyl)-chroman-6-yl 3,5-dinitrobenzoate.

RESULTS

Behavioral Test. *Effect of Toco-D Treatment on Catalepsy by Using the Block Method.* The catalepsy time of the disease groups increased from the 7th to 21st days of treatment in comparison to that of the control. The catalepsy time of the tocopherol derivative (Toco-D) treatment groups significantly ($P < 0.001$) decreased on the 14th and 21st days of treatment when compared with that of the disease group (Figure 2A,B).

Effect of Toco-D Treatment on Catalepsy by Using a Triple Horizontal Bar Apparatus. At the 4 and 6 mm bars, Toco-D at a dose of 40 mg/kg (***$P < 0.001$) scored higher as compared to lower doses but comparatively lower than the standard group (Figure 3A).

Effect of Toco-D Treatment on Motor Coordination by Using a Rotarod Apparatus. The haloperidol (HAL) group showed a reduction in falloff time when compared with the control. Sinemet and Toco-D at all doses showed a significant increase ($P < 0.001$) in endurance time when compared with the disease group (Figure 3B).

Effect of Toco-D Treatment on Locomotor Activity Using an Actophotometer. Toco-D at a dose of 20 mg/kg raised the locomotor activity, and this increase was significant ($P < 0.001$) when compared to that of the disease group. The effect of Toco-D decreased when moving from 20 to 5 mg/kg dose levels (Table 1).

Effect of Toco-D Treatment on Motor Coordination Activity Using the Balance Beam Test. The disease group showed a significant decrease in locomotion when compared with the control. Toco-D at a dose of 40 mg/kg raised the locomotion activity, and this increase was significant ($P < 0.05$) when compared to that of the disease group (Figure 4).

Effect of Toco-D at Different Dose Levels on Biochemical Assays. Toco-D at a dose level of 20 mg/kg (↑32%) increased the superoxide dismutase (SOD) level, and this increase was significant as compared to that of the HAL-treated group as shown in Table 2. Toco-D at all dose levels decreased the malondialdehyde (MDA) level; this decrease was significant compared to that of the HAL-treated group. The level of protein is reduced in the disease group in comparison with that of the control group. Toco-D showed a dose-dependent increase in brain protein level.

Effect of Toco-D on Neurotransmitters and MAO-B in Mice's Brains. Animals treated with Toco-D at all dose levels showed a significant increase in the dopamine and serotonin levels as compared to the HAL-treated group (Figure 5) as decrease in the level of dopamine in the HAL group. Toco-D treatment at all dose levels significantly ($P < 0.001$) decreased the enzyme level as compared to that of the disease group.

ELISA Analysis. NF-κB levels were significantly ($P < 0.001$) decreased in the treated animals with both Toco-D and Sinemet (Figure 6).

RT-PCR Analysis. The mRNA expression of the PD marker (α-synuclein) was significantly decreased by Toco-D when compared with that of the disease group (Figure 7), which was significantly raised in the HAL group. The reduction in the mRNA expression of α-synuclein demonstrated the reduction in the severity of PD in animals treated with HAL.

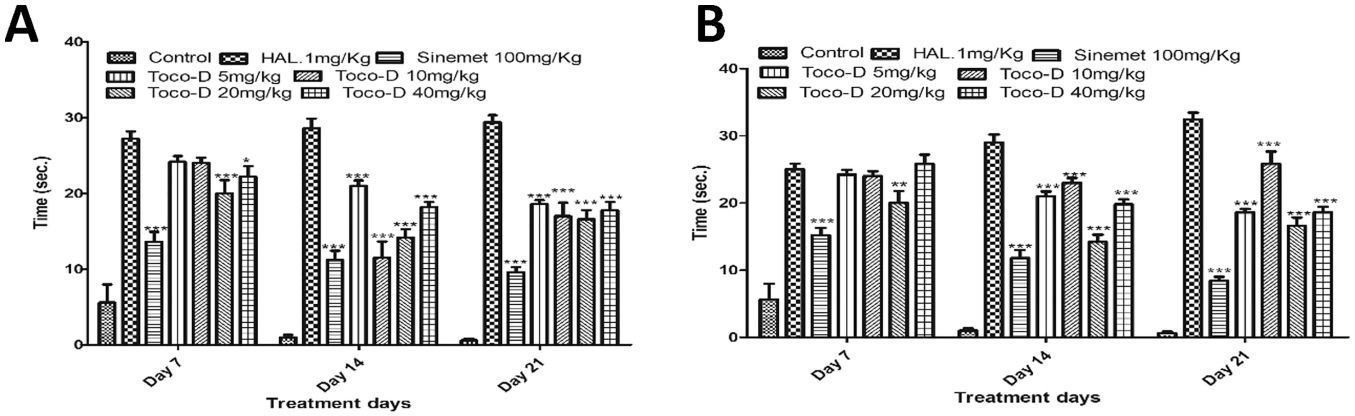

Figure 2. Effect of Toco-D treatments at different dose levels on the catalepsy time using the block method: (A) 3 cm and (B) 5 cm. Data are expressed as mean ± SEM, $n = 10$. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ when compared with the disease group.

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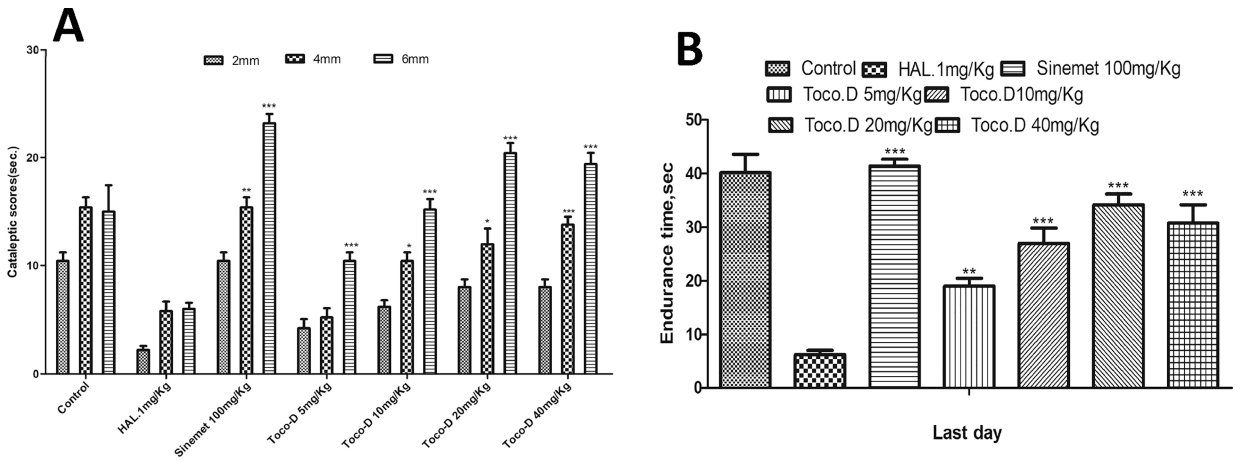

Figure 3. (A) Effect of Toco-D treatment at different dose levels in the catalepsy test by using a triple horizontal apparatus. (B) Endurance time using the rotarod method. Data are expressed as mean ± SEM, $n = 10$. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ was given 2 mm block vs 4 and 6 mm block (A); $^{**}P < 0.01$ and $^{***}P < 0.001$ in comparison to the disease group (B).

Table 1. Effect of Toco-D Treatment on Locomotor Activity Using an Actophotometer[a]

treatment groups	dose (mg/kg)	locomotor activity (number of movements)
control (0.5% CMC)	1	62 ± 2.05
HAL	1	25 ± 1.67[nsb] (↓59.6%)
Sinemet	100	55 ± 2.78*** (↑120%)
Toco-D	5	30 ± 1.8* (↑20%)
Toco-D	10	40 ± 4.0*** (↑60%)
Toco-D	20	46 ± 1.7*** (↑84%)
Toco-D	40	42 ± 2.5*** (↑68%)

[a]Data are expressed as mean ± SEM, $n = 10$. $^{*}P < 0.05$ and $^{***}P < 0.001$ when compared with the disease group. [b]ns = $P > 0.05$. Non-significance.

Histopathological Studies. In the disease group, neuronal cell damage and microgliosis were seen. Toco-D-treated animals at all dose levels had reduced microgliosis and cellular damage. Overall brain health was intact in Toco-D-treated animals (Figure 8).

DISCUSSION

PD is a recognized type of neurodegenerative disease characterized by bradykinesia, tremors at rest, rigidity of muscles, and a shuffling gait.[10] Vitamin E is a lipophilic antioxidant that protects membranes from peroxidation and induces a protective mechanism against oxidative stress.[11] Toco-D at all doses levels showed a significant reduction in catalepsy time in the block method and a significant increase in grip strength in the horizontal bar method. Mice with dopamine depletion have demonstrated impaired motor skills in some cases, as dopamine degeneration impairs spatial memory tasks.[12] All doses of Toco-D demonstrated improved endurance time and bradykinesia on the rotarod and balance beam tests.

An actophotometer was used to check the locomotor activity of the mice. Increased vertical and horizontal movements within the cage depicted increase mental alertness. A decreased activity score was taken as an attribute of CNS depression.[13] An increase in locomotor activity (↑84%) was seen at a dose of 20 mg/kg, but this was less than that of the standard group.

Antioxidants are compounds that have the capability to delay, prevent, or even treat the damage by different diseases, including neurodegenerative diseases. Different endogenous antioxidants, either enzymatic or non-enzymatic, play an

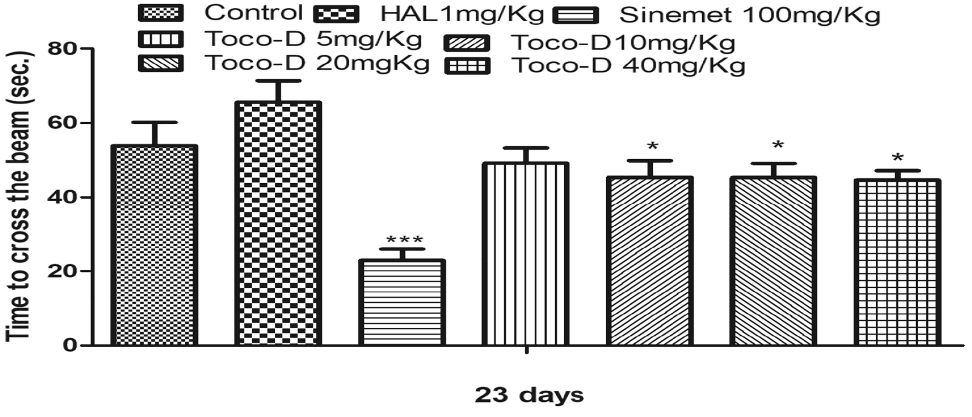

Figure 4. Effect of Toco-D treatment at different dose levels on motor coordination activity using the balance beam test. Data are expressed as mean ± SEM, $n = 10$. $^{*}P < 0.05$ and $^{***}P < 0.001$ when compared with the disease group.

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Table 2. Effect of Toco-D Treatment at Different Dose Levels on Biochemical Assays in the Brain[a]

treatment groups	dose (mg/kg)	GSH (μg/mg of protein)	CAT (μmol/min/mg of protein)	SOD (μmol/mg of protein)	MDA (μmol/mg of protein)	nitrite (μg/mg)	protein (μg/mg)
control (0.5% CMC)	1	7.72 ± 0.15	64 ± 3.39	29.06 ± 1.8	1.11 ± 0.05	1.92 ± 0.09	6.88 ± 0.52
HAL	1	6.7 ± 0.16***	52.55 ± 2.54	22.22 ± 0.88**	1.35 ± 0.17***	2.11 ± 0.36***	5.57 ± 0.37**
Sinemet	100	8.2 ± 0.23	80.07 ± 2.44	31.80 ± 0.78	1.11 ± 0.24	1.42 ± 0.16	8.90 ± 0.24
Toco-D	5	7.49 ± 0.31	55.70 ± 2.6***	24.64 ± 1.18*	1.21 ± 0.05	1.73 ± 0.21	6.90 ± 0.12
Toco-D	10	7.56 ± 0.29[ns]	71.33 ± 0.22***	26.68 ± 1.5	1.72 ± 0.18***	2.09 ± 0.14***	7.13 ± 0.05***
Toco-D	20	7.71 ± 0.17***	69.70 ± 0.20*	28.61 ± 0.16**	1.09 ± 0.10***	1.88 ± 0.11***	7.21 ± 0.11***
Toco-D	40	7.35 ± 0.20***	60.55 ± 2.49	27.35 ± 1.39**	1.39 ± 0.22	1.51 ± 0.19***	6.79 ± 0.48***

[a]Data are expressed as mean ± SEM, $n = 10$. [ns] *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ when compared with the disease group.

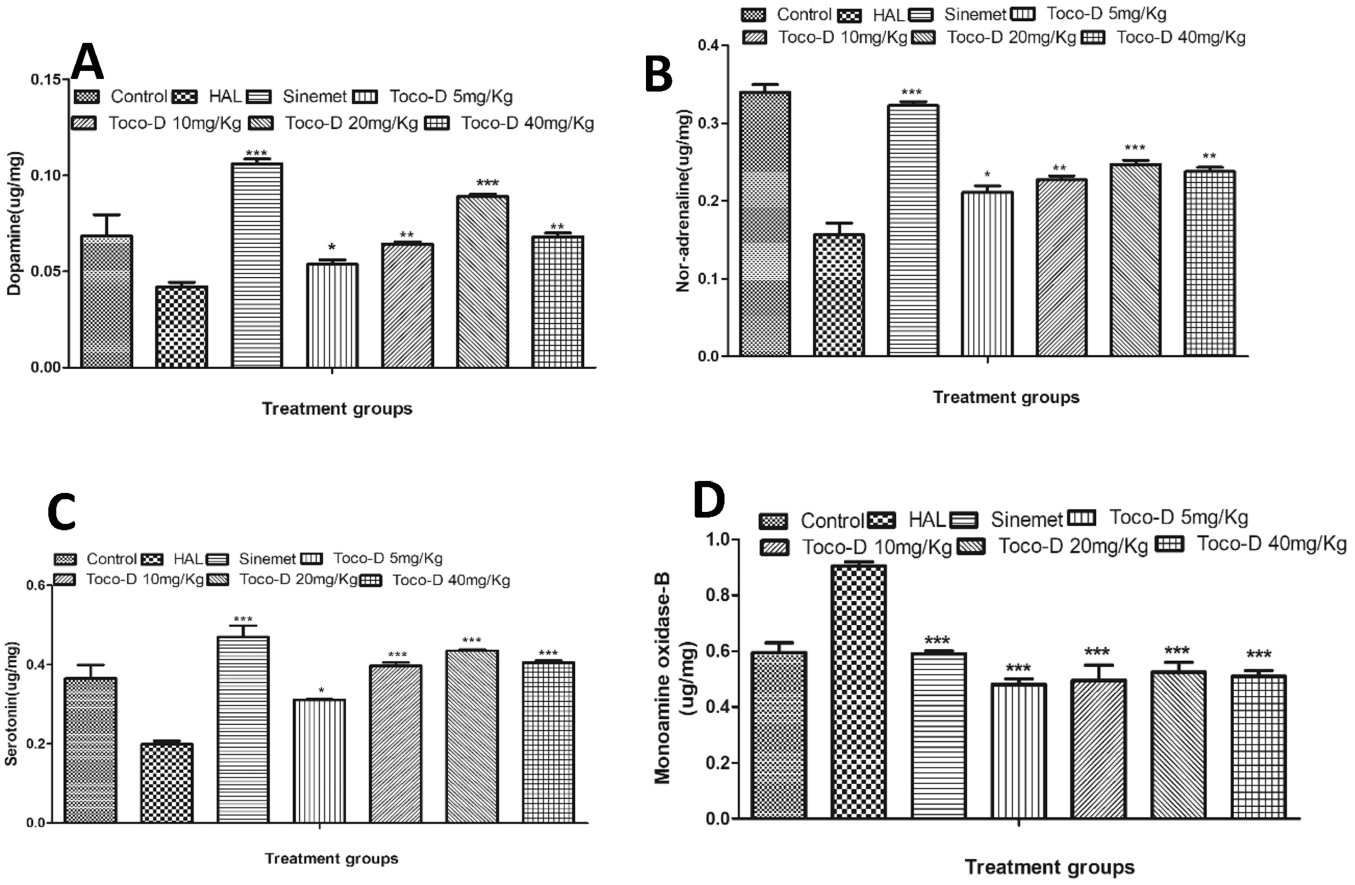

Figure 5. Effect of Toco-D treatment on (A) dopamine, (B) noradrenaline, (C) serotonin, and (D) monoamine oxidase in HAL-induced PD model. Data are expressed as mean ± SEM, $n = 3$. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ when compared with the disease group.

important role in scavenging oxidative free radicals in the normal homeostasis process. Exogenous antioxidants like phenols, flavonoids, carotenoids, and vitamin E are mostly present in food.[14] Various antioxidants from plants (*Mucuna pruriens*) are used for the management of PD like ursolic acid and chlorogenic acid, which supports that antioxidants have the potential to reverse the disease induced by MTPT.[15,16] Endogenous antioxidants like glutathione, catalase, and SOD have a scavenging activity against the accumulation of oxygen species, and a decrease of the levels of these endogenous antioxidants in the brain has been attributed to oxidative stress.[17] The brain homogenate of the animals treated with Toco-D exhibited significant increases in glutathione (GSH), catalase (CAT), SOD, and protein levels as shown in Table 2.

PD is a neurodegenerative disease due to functional deficits of dopamine, noradrenaline, serotonin, and MAO-B.[18] The starting factor is the degeneration of dopaminergic neurons in the striatum.[19,20] In our study, Toco-D treatment elevated the levels of neurotransmitters in the brain, which helps in restoring the motor and non-motor functions of the brain.

Different products that produce inflammation by increasing inflammatory cytokines like TNF-α damage cells by activating the inflammatory response through the activation of the NF-κB pathway.[21] In chronic inflammatory conditions like asthma, Alzheimer's disease, and PD, NF-κB activation is the principal event.[22] The activation of NF-κB further regulates the various pro-inflammatory mediators (COX-II, TNF-α) that play an important role in the pathophysiology of various diseases like Alzheimer's disease.[23] Studies showed that pioglitazone, curcumin,[24] and salmeterol[25] regenerated dopaminergic neurons by inhibiting the activation of NF-κB. In our study,

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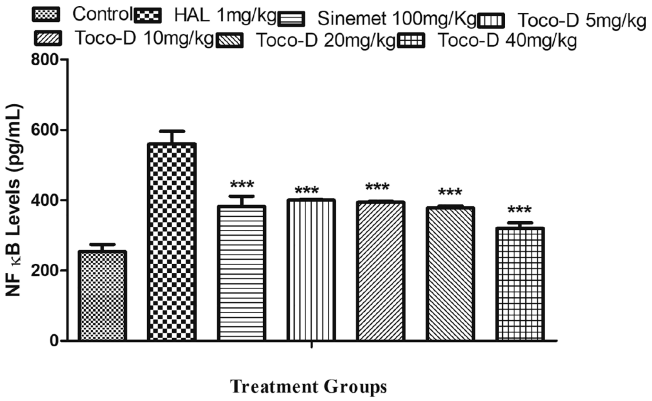

Figure 6. Effect of Toco-D treatment on NF-κB levels in the brain in HAL-induced PD. Data are expressed as mean ± SEM, $n = 3$. ***$P < 0.001$ when compared with the disease group.

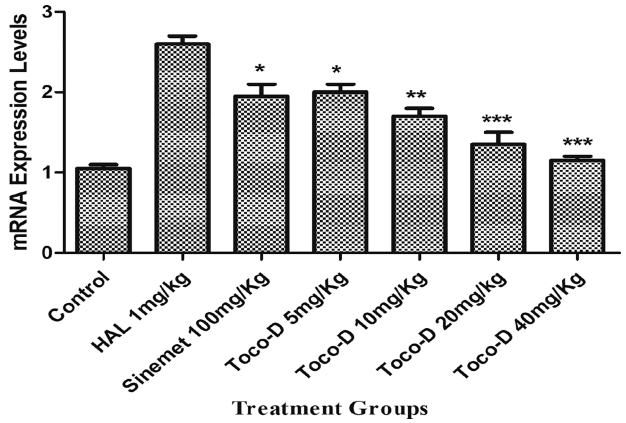

Figure 7. Effect of Toco-D treatment on mRNA expression of α-synuclein in HAL-induced PD. Data are expressed as mean ± SEM, $n = 3$. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ when compared with the disease group.

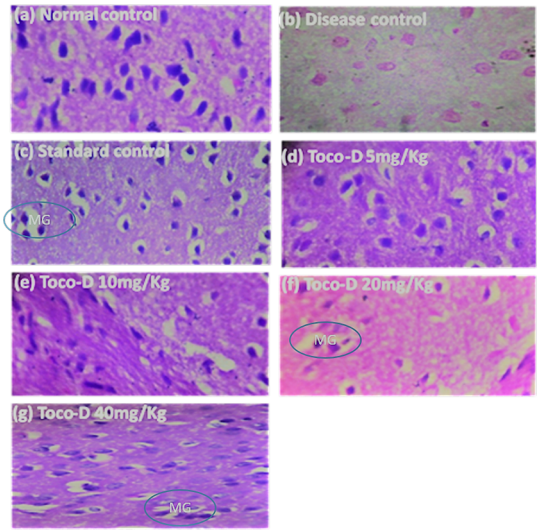

Figure 8. (a–g) Effect of Toco-D treatments on brain histology against HAL-induced toxicity in mice.

Toco-D at all dose levels significantly reduces the levels of NF-κB in the brain, which might be the reason for alleviating PD.

It is well evident that the α-synuclein protein inhibited the synthesis of dopamine by down-regulating the activity of the tyrosine hydrolase enzyme, a rate-limiting step in the synthesis of dopamine.[26] The destruction of dopamine neurons and intra-neuronal deposition of α-synuclein proteins (Lewy bodies) are the major hallmarks of PD.[18,27] Studies reported that SNCA genes are replicated, triplicated, and mutated during PD.[28] At present, the main mechanism behind this is not clear yet. PCR analysis showed a reduction of the mRNA expression of α-synuclein by the treatment of Toco-D. According to histopathological studies, the normal control group showed intact neuronal cells and no phenomenon of apoptosis appeared there.

CONCLUSIONS

This study concluded that Toco-D improved catalepsy and locomotion due to the increasing concentration of dopamine in the substantia nigra. Toco-D may improve the symptoms by reducing oxidative stress levels and enhancing neurotransmission. Down-regulation of NF-κB and α-synuclein further supported our study. The basic limitation of the study is not finding out the exact mechanism of the action of the vitamin E derivative and any of its toxic effects on animals. Further molecular and cellular level studies will also be conducted to evaluate the mechanism of this compound.

MATERIALS AND METHODS

Experimental Animals. Swiss albino mice ranging from 3 to 4 months old, male, and weighing 25–40 g were obtained from the Riphah Institute of Pharmaceutical Sciences (RIPS) animal house. All procedures described were reviewed and approved by the research ethical committee of Riphah International University, Lahore, with an authorized number of REC/RIPS-LHR/2017 043.

Experimental Design. Animals were categorized into seven groups ($n = 10$). Groups II–VII received HAL (1 mg/kg/day) intraperitoneally for 23 days along with other treatments except for the control. Groups IV–VII were administered Toco-D orally at 5, 10, 20, and 40 mg/kg doses, respectively.

Behavioral Tests. A block test was performed using by using 3 and 5 cm blocks on the 7th, 14th, and 21st days by measuring the forepaw removal time with 300 s as the cutoff time for catalepsy measurement.[29] The triple horizontal bar method was performed with placement of mouse front limbs on the 49 cm high bar, holding the bar (2 mm) from the floor. If the mouse stayed on the first bar (2 mm) for 30 s, it was then subjected to thicker bars (4 and 6 mm).[30] The rotarod test was performed on pre-trained mice subjected to trials for three consecutive days at a fixed speed (5 rpm) for 5 min.[31] The time at which animals fall from the rod was noted. The actophotometer test was performed on the 22nd day of dosing, and the total locomotor activity was recorded.[32] In the balance beam test, the time it took pretrained animals to complete a walk on the apparatus was recorded; the maximum time allowed per mouse was 180 s.[33]

Estimation of Biochemical Markers. *Preparation of Tissue Homogenates.* Tissue homogenates were prepared by adding sodium phosphate buffer (pH 7.4) in the excised brain after washing it with cold saline at a ratio of 1:10 (w/v) in a tissue homogenizer. Homogenates were then centrifuged at 4000 rpm for 10 min at 4 °C, and the supernatant was used for further studies.[34]

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Biochemical Marker Estimations. SOD was estimated by adding 2.8 mL of phosphate buffer at pH 7.4 along with 0.1 mL of the tissue homogenate and 0.1 mL of a pyrogallol solution, and the absorbance was measured at 325 nm. The tissue homogenate (1 mL) and 1 mL of 10% trichloroacetic acid were mixed and precipitated. To the supernatant, a phosphate solution (4 mL) and 0.5 mL of the dithiobis-nitrobenzoic acid (DTBN) reagent were added, and the absorbance was measured at 412 nm to measure the level of reduced GSH.[35] The tissue homogenate (0.05 mL), 1.95 mL of PO_4 buffer at pH 7.0 (50 mM), and 1 mL of hydrogen peroxide (30 mM) were added. The absorbance was measured at 240 nm for determination of CAT activity. The thiobarbituric acid (TBA) reagent (3 mL) was added to 1 mL aliquots of the brain homogenate. The mixture was centrifuged at 3500*g* for 10 min. The absorbance was taken at 532 nm for estimation of MDA. Protein estimation was done using the Lowry method at 660 nm. The Griess reagent method was used for the estimation of nitrite levels.[34]

Estimation of Neurotransmitters. *Preparation of the Aqueous Phase.* The tissue homogenate was prepared in 5 mL of HCl−butanol. The mixture was centrifuged at 2000 rpm for 10 min. An aliquot of the supernatant (1 mL), 2.5 mL of heptane, and 0.31 mL of HCl (0.1 M) were added and centrifuged for 10 min at 2000 rpm for separation of the two phases. The aqueous phase was removed and used for estimation of noradrenaline, serotonin, and dopamine.

Estimation of Dopamine Serotonin and Noradrenaline Levels. In a 0.2 mL portion of the aqueous phase, 0.05 mL of (0.4 M) HCl and 0.1 of mL EDTA/sodium acetate (pH 6.9) were added. An iodine solution (0.1 mL, 0.1 M) was added for the oxidation process. This was stopped by the addition of Na_2SO_3 (0.1 mL), and 0.1 M acetic acid was added to it after one and a half minute and the mixture was heated at 100 °C for 6 min. The absorbance values of dopamine and noradrenaline were taken at 350 and 450 nm, respectively. For the estimation of the serotonin level, 0.2 mL of the aqueous phase was added to 0.25 mL of *o*-phthaldialdehyde (OPA) and the mixture was heated to 100 °C for about 10 min; the absorbance was taken at 440 nm using 0.25 mL of HCl as a blank.[34]

Estimation of MAO-B. The tissue homogenate (250 μL) was mixed in 250 μL of serotonin and 250 μL of the buffer at 37 °C for 20 min. A 200 μL portion of 1 M HCl was added to arrest the reaction. The mixture was extracted with 5 mL of cyclohexane. The absorbance of the organic phase was taken at 242 nm with blank samples of 1 M HCl (20 μL). The MAO-B activity is expressed in moles per milligram of protein.[36]

ELISA Analysis. An NF-κB ELISA kit (cat. no. 85-86081-11) was used for the analysis of NF-κB levels by complexing it with the horseradish peroxidase (HRP) enzyme for conjugate formation. The TBM solution was added as stop solution, and the absorbance was measured at 450 nm. Regression lines of the standard were made.[37]

RT-PCR Analysis. RNA was extracted from the homogenate of the brain tissue in TRIzol using a Polytron device. cDNA was prepared from RNA by using a reverse transcription kit. PCR was used for replication at 40 cycles at 90 °C for 5 min. The primer sequences of α-synuclein and GAPDH used were forward TCGAAGCCTGTGCATCCATC and reverse CTCCCTCCTTGGCCTTTGAA and forward GGAGTCCC-CATCCCAACTCA and reverse GCCCATAACCCCCACAA-CAC, respectively.

Histopathological Studies. The brains of mice from the control and treatment groups were collected after scarification and preserved in 10% formalin. They were fixed in paraffin wax and were H&E stained for histopathological observations.

Statistical Analysis. All results were expressed as mean ± SEM. Data was evaluated using the GraphPad Prism version 5.01 software. Analyses were performed using one-way ANOVA (Dunnett's *t*-test) and two-way ANOVA by Bonferroni's multiple comparison tests. $P < 0.05$ was considered significant.

CONSENT FOR PUBLICATION

All authors gave their consent for publication.

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Author Contributions

A.I., T.I., S.S.K., A.K., and F.A. did all the related research work, studied and inspected the results, and critically examined the important data. Designing the research protocol was conducted by F.A. and B.A. The reliability of work was investigated by U.S. and M.G. along with proper guidance for publication.

Notes

The authors declare no competing financial interest.

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