Original Article

DOI: 10.5582/ddt.2022.01026

Effects of eugenol on the behavioral and pathological progression in the MPTP-induced Parkinson's disease mouse model

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SUMMARY Parkinson's disease (PD) is the world's second most common neurological disorder. Oxidative stress and neuroinflammation play a crucial role in the pathogenesis of PD. Eugenol is a phytochemical with potent antioxidant and anti-inflammatory activity. The present investigation is aimed to study the effect of eugenol in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced mouse model of PD and its relationship to antioxidant effect. The effects of seven days of oral pre-treatment and post-treatment with three doses of eugenol (25, 50 and 100 mg/kg/day) were investigated against the MPTP-induced PD mouse model. In addition to the assessment of behavioural parameters using various tests (actophotometer, beam walking test, catalepsy, rearing, rotarod), biochemical parameters including lipid peroxidation and reduced glutathione levels in brain tissues, were also estimated in this study. The binding mode of eugenol in the human myeloid differentiation factor-2 (*h*MD-2) was also studied. Results showed that MPTP administration in mice resulted in the development of motor dysfunction (impaired motor coordination and hypo locomotion) similar to that of PD in different behavioural studies. Pre-treatment with eugenol reversed motor dysfunction caused by MPTP administration while post-treatment with eugenol at a high dose aggravated the symptoms of akinesia associated with MPTP administration. MPTP resulted in increased lipid peroxidation while decreased reduced glutathione levels in the brains of mice. MPTP-induced increased lipid peroxidation and attenuated levels of reduced glutathione were found to be alleviated with eugenol pre-treatment while augmented with eugenol post-treatment. Eugenol showed a binding affinity of -6.897 kcal/mol against the MD2 coreceptor of toll-like receptor-4 (TLR4). Biochemical, as well as neurobehavioral studies, showed that eugenol is having a protective effect, but does not have a curative effect on PD.

Keywords Parkinson's disease, eugenol, MPTP, lipid peroxidation, reduced glutathione

1. Introduction

Parkinson's disease (PD) is the world's second most common neurodegenerative illness, with an estimated frequency of 0.1% to 0.2% (*1*). PD is affecting about 1% of adults older than sixty years of age (*2,3*). PD is mainly characterized by the depletion of dopaminergic neurons in the substantia nigra pars compacta (SNpc) region of the brain (*4,5*). Symptoms of PD involve both motor dysfunctions as well as non-motor symptoms. Motor dysfunctions include tremors, muscle rigidity, postural imbalance, bradykinesia and variable degree of cognitive impairment (*5*). The molecular mechanism for PD is still not clear but the aggregation of the presynaptic protein, α-synuclein leads to the formation of the Lewy body, which is a distinct pathological hallmark of PD (*6*). Apart from the deposition of the Lewy body, the generation of free radicals and oxidative stress plays a crucial role in the pathogenesis of PD (*7*). These generated free radicals can cause the formation of protein carbonyls, DNA damage and lipid peroxidation (*8*). Neural membranes consist of phospholipids with high content of polyunsaturated fatty acids. Lipid peroxidation results in the peroxidation of these polyunsaturated fatty acids and results in the formation of phospholipids and malondialdehyde (MDA). The earlier report shows increased lipid peroxidation activity in the PD patients (*9*).

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin that causes clinical manifestations that is quite similar to PD, and it is commonly used in experimental animals as a PD model (*10,11*). It has been reported that oxidation of MPTP produces the 1-methyl-4-phenylpyridinium (MPP+) ion,

which blocks the activity of mitochondrial complex I and thus results in the generation of reactive oxygen species (ROS) (*12*).

Current therapies for PD include levodopa, dopaminergic agonists and monoamine oxidase B inhibitors, and combinations of these medications. However, with time these methods become less effective (*13*). This emphasizes the requirement for the development of novel therapeutic strategies for the management and treatment of PD. Phytochemicals having anti-oxidant and anti-inflammatory activities have been presented as a possible treatment for PD (*14*). Eugenol is chemically 4-allyl-2-methoxyphenol and is the main bioactive compound present in herbs and spices like clove, cinnamon, and nutmeg, which has strong anti-oxidants and anti-inflammatory activities (*15*). World health organization (WHO) declared eugenol as a non-mutagenic and generally recognized as a safe (GRAS) substance. Since eugenol is hydrophobic, it may easily penetrate into the brain bypassing the blood-brain barrier when consumed orally. Several *in vivo* and *in vitro* experiments have demonstrated that eugenol exerts a neuroprotective effect in many CNS disorders (*16*). Eugenol is reported to have anti-stress properties (*17*). In previously reported animal studies, eugenol was found to be protective against neurotoxicity induced by acrylamide (*18*), aluminium (*19,20*), chlorpyrifos (*21*), scopolamine (*22*), as well as 6-hydroxydopamine (*23*). In gerbils, it has also been shown to protect against ischemia-induced brain toxicity (*24*). It also prevents amyloid protein aggregation (*25*) and thus prevents symptoms associated with Alzheimer's disease in insulin and Aβ-induced rat models (*26*). Eugenol also aided functional recovery after traumatic brain injury by reducing oxidative stress and neuronal death (*27*) and is also effective against spinal cord injury in rats (*28*). The neuroprotective benefits of eugenol can be attributed in part to its anti-oxidant and anti-inflammatory characteristics. Considering the therapeutic benefits of eugenol in various studies, the objective of this research is to investigate the effect of eugenol in MPTP-induced mouse model of PD and its link to antioxidant function.

2. Materials and Methods

2.1. Chemicals

MPTP was procured by Sterling biologicals, Ahmedabad while Hank's balanced salt solution (HBSS) was obtained from Invitrogen (Thermo Fisher Scientific), Bangalore. Eugenol, 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) (Ellman's Reagent) and thiobarbituric acid (TBA) were purchased from Sigma Aldrich (USA). Sodium dodecyl sulphate (SDS), 1,1,3,3-tetraethoxypropane (TEP), trichloroacetic acid, pyridine, n-butanol, glacial acetic acid and all additional solvents and reagents were acquired by HiMedia Laboratories Pvt. Ltd., Mumbai. Eugenol was suspended in 40% propylene glycol in phosphate buffer with pH 6.8 as it is hydrophobic. MPTP is water-soluble so it was dissolved in sterile saline.

2.2. Animals

In the present study, swiss male albino mice were used weighing 30-35 gm. This study was undertaken at the central animal house, Institute of Pharmacy, Nirma University, Ahmedabad, India. All the studies were conducted as per the national institute of health "Guide for the care and use of laboratory animals". All the experimental procedures were approved by the institutional animal ethics committee (IP/PCOL/MPH/25/2019/012).

2.3. Experimental design

This study includes the evaluation of pre-and post-treatment with eugenol (25, 50 and 100 mg/kg) against the MPTP-induced mouse model of PD. A schematic representation of the experimental design is shown in Figure 1. Eight groups of six animals each (n = 6) were formed by randomly assigning the animals to the groups. The list of groups and details of each group is mentioned below: Group I: Control (saline + no MPTP administration); Group II: MPTP; (MPTP (3 mg/kg, *i.p.*) consecutively for five days); Group III: Eug-25 + MPTP (seven days of treatment with eugenol (25 mg/kg, *p.o.*) followed by co-administration of MPTP (3 mg/kg, *i.p.*) for five days from the third day of eugenol treatment); Group IV: Eug-50 + MPTP (seven days of treatment with eugenol (50 mg/kg, *p.o.*) followed by co-administration of MPTP (3 mg/Kg, *i.p.*) for five days from the third day of eugenol treatment); Group V: Eug-100 + MPTP (seven days of treatment with eugenol (100 mg/kg, *p.o.*) followed by co-administration of MPTP (3 mg/kg, *i.p.*) for five days from the third day of eugenol treatment); Group VI: MPTP + Eug-25 (five days of treatment with MPTP (3 mg/kg, *i.p.*) followed by seven days treatment with eugenol (25 mg/kg, *p.o.*)); Group VII: MPTP + Eug-50 (five days of treatment with MPTP (3 mg/kg, *i.p.*) followed by seven days treatment with eugenol (50 mg/kg, *p.o.*)); Group VIII: MPTP + Eug-100 (five days of treatment with MPTP (3 mg/kg, *i.p.*) followed by seven days treatment with eugenol (100 mg/kg, *p.o.*)).

Lastly, behavioural parameters using various tests (actophotometer, narrow beam walking test, catalepsy bar test, cylinder test and rotarod) were estimated and then animals were sacrificed and their brain was isolated, collected and stored in -20°C cold storage. Other biochemical parameters like oxidative stress *i.e.*, lipid peroxidation and endogenous antioxidant i.e., reduced glutathione were estimated.

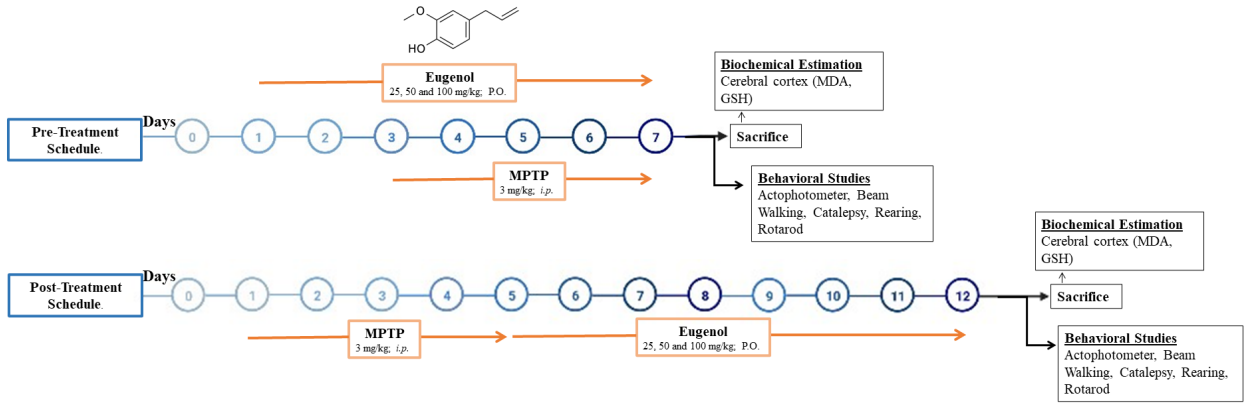

Figure 1. An illustration showing the treatment schedule and design of the study. P.O.: per oral; GSH: reduced glutathione; MDA: malondialdehyde.

2.4. Behavioral parameters

2.4.1. Actophotometer

Actophotometer is mainly used to check the locomotor activity of the rodent. Actophotometer consists of photoelectric cells coupled in a circuit with a counter. When a beam of light strikes the photoelectric cell, the circuit is complete. The animal was placed in the metal box and when it walked through it, the lights of the photocell were being cut off and then a count was recorded. All the animals were initially placed in the apparatus for five minutes for acclimatization. In the main test, mice were kept for five minutes in the apparatus and then the readings were recorded as activity scores (*29*).

2.4.2. Narrow beam-walking test

A narrow beam-walking test is a useful tool for assessing the balance and motor coordination of animals. The main objective of this technique was to monitor the functional recovery of locomotor capacity in rodents following sensorimotor cortex damage (*30,31*). The width, length and thickness of the beam were 12 mm × 50mm × 6mm. For this neurobehavioral test, the mice were placed on one end of the thin beam where they were trained to walk across the elevated beam to reach the platform on the other side of the arm. The time taken in seconds to cross the beam was noted for each animal (*32*).

2.4.3. Catalepsy bar test

Catalepsy is a condition in which an animal maintains an aberrant posture for an extended period. Catalepsy bar test is often performed in laboratory animals to investigate the effects of medications on extrapyramidal symptoms associated with PD (*33*). The catalepsy was measured using a bar test. The front paws of animals were alternately placed on a horizontal bar of 3 cm and 9 cm above and parallel to the base in this bar test. The time was recorded at which the animal removes its paw from the bar. The severity of catatonic response was observed and recorded as follows: Stage I: When a mouse is placed on a table, it moves normally, scoring 0. Stage II. The mouse starts moving only when pushed or touched, scoring 0.5. Stage III. When the front paws of the mouse are alternatively put on a 3 cm high block and the animal fails to straighten its posture after 10 seconds, give it a 0.5 for each paw and a total scoring of 1 for this level. Stage IV. When the front paws of the mouse are alternately put on a 9 cm block, the mouse fails to remove it; give it a 1 for each paw, with a total scoring of 2 for this level. Thus, the highest possible scoring for a single mouse would be 3.5, indicating complete catatonia.

2.4.4. Spontaneous forelimb elevation (cylinder) test

This test was conducted to observe the exploratory behaviour (rearing behaviour) in the subject animal. The animal was placed in a cylindrical jar in such a way that both the hind paws should be in the upright position against the wall of the cylinder. The number of rearing behaviour was observed for each animal for five minutes (*34*).

2.4.5. Rotarod

Rotarod is an apparatus that is used to check motor coordination and balance. In this test, the mice were placed on the horizontal rod that rotates about its long axis. The time taken by each of the mice to fall off was recorded. The normal mouse should remained on the rod for a long time while the mouse treated with MPTP and that showed the Parkinson's-like symptoms were fallen off from the rod as they were not able to maintain their motor coordination and balance (*35*).

2.5. Biochemical parameters

2.5.1. Evaluation of lipid peroxidation

MDA is a byproduct of lipid peroxidation

(polyunsaturated fatty acids) in cells. As a result, MDA level is a good indicator of lipid peroxidation. The amount of MDA in brain tissue was assessed as described before by Draper and Hadley (*36*) with minor modifications. The cortical area of brain tissue was weighed (100 mg). Weighted tissue was homogenized with HBSS buffer (5 mL) at 3,000 rpm in three cycles of 30 seconds each with a 30-second interval in a tube. Centrifugation of the homogenized tissue was done at 3,000 rpm for 10 minutes at 25°C. The supernatant was discarded, and the cell pellet was recovered and then mixed in tubes with 1.5 mL of acetic acid, 1.5 mL of TBA, 0.2 mL of SDS and 0.7 mL of MilliQ water. The control tubes were filled with HBSS (0.1 mL) at the place of homogenate for reference solution. For 1 hour, the tubes were immersed in boiling water. 1 mL Milli Q was added to each tube after it had boiled. Each tube received 5 mL of butanol: pyridine (15:1) solution and then vortexed for 5 minutes. The top organic layer was collected following centrifugation at 3,000 rpm for 10 minutes at 25°C. The amount of MDA generated was determined by measuring the absorbance at 532 nm of the top organic layer. MDA concentration was calculated in µM/mg of brain tissue using standard curves created by TEP.

2.5.2. Assessment of reduced glutathione

The levels of reduced glutathione were measured using Ellman (*37*) method with minor modifications. 100 mg of tissue from the cortical region of the brain was homogenized in ice-cold phosphate buffer (5 mL). The tissue homogenate was treated with 0.1 mL of trichloroacetic acid, followed by a 10 minutes centrifugation at 3,900 g at 25°C. One millilitre of supernatant was collected and then combined with one millilitre each of DTNB and phosphate buffer, vortexed for one minute, and then incubated for five minutes at room temperature. As a blank, 1 mL of DTNB was added to 2 mL of phosphate buffer. At 412 nm, both test samples and blanks were evaluated for absorbance. A standard curve was used to calculate the reduced glutathione levels and expressed as µM/mg of brain tissue.

2.6. *In silico* docking studies

Lipopolysaccharide (LPS) is activating the toll-like receptor 4 (TLR4)/myeloid differentiation factor 2 (MD-2) complex, which then activates an innate immune response. A docking study was carried out using Autodock 4.2 software (*38*). Crystal structures of *human* MD-2 (*h*MD-2) complex with antiendotoxic lipid IVa (PDB ID: 2E59) (*39*) solved at 2.21 Å resolution were downloaded from the protein data bank (PDB). Auto-dock tools (ADT) were used to prepare eugenol and *h*MD-2, all water molecules were deleted, polar hydrogen atoms were added and Kollman United Atoms charges were loaded to perform docking calculations. Co-crystalized lipid IVa was removed from the *h*MD-2 structure. A grid box was constructed at 0.375 Å spacing with grid points in xyz dimensions of 40, 44, and 35, respectively. The Lamarckian genetic algorithm was selected as the search algorithm.

2.7. Statistical analysis

Graph Pad Prism 9 was utilized for the statistical analysis. The data were analyzed by using the one-way ANOVA method followed by Tukey's multiple comparison test. Each bar in the graph represents a mean ± SEM, with each group containing 6 animals. It is statistically significant if the significance value (*p*-value) is less than 0.05.

3. Results

3.1. Eugenol improved the MPTP-induced lowered activity score of the mice in actophotometer

Figure 2 illustrates the effect of pre-and post-treatment with eugenol on the locomotor activity of mice treated with MPTP using an actophotometer. MPTP administration for five consecutive days significantly ($p < 0.05$) reduced the activity score of mice in the actophotometer. Pre-treatment with eugenol (100 mg/kg) significantly ($p < 0.05$) mitigated the MPTP-induced deterioration in the performance of mice in the actophotometer while 25 and 50 mg/kg doses of eugenol were found ineffective. On the contrary, post-treatments with 25 and 50 mg/kg doses of eugenol were not showing any significant protective effect against MPTP-induced deterioration in the locomotor activity of mice in the actophotometer. However, post-treatment

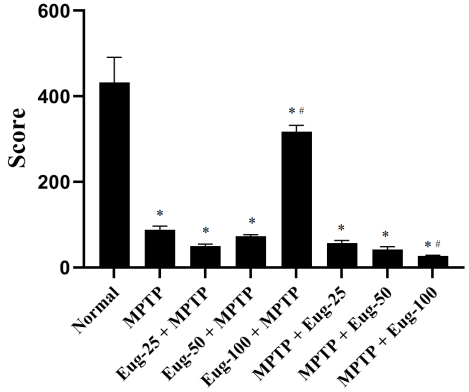

Figure 2. Effect of seven days pre- and post-treatment with eugenol on mean activity score in actophotometer apparatus against mouse model of MPTP-induced PD. Each bar represents data in mean ± SEM ($n = 6$). $^{*}p < 0.05$ compared to control and $^{\#}p < 0.05$ compared to MPTP using one-way ANOVA followed by Tukey's multiple comparisons test.

with eugenol at 100 mg/kg resulted in aggravating significantly ($p < 0.05$) the MPTP-induced deterioration in the locomotor activity of mice in the actophotometer.

3.2. Eugenol improved MPTP-induced deteriorated performance of mice on narrow beam walking test

The effect of pre-and post-treatment with eugenol on the performance of mice treated with MPTP in the narrow beam walking test is shown in Figure 3. MPTP administration for five consecutive days significantly ($p < 0.05$) increase the time taken by mice to cross the narrow beam. The oral pre-treatment with eugenol at all the doses (25, 50 and 100 mg/kg) showed a significant improvement in MPTP-induced deteriorated performance of mice in the beam-walking test. On the contrary, post-treatment with eugenol at all the doses resulted in aggravating significantly ($p < 0.05$) the MPTP-induced deterioration in the performance of mice in the beam walking test.

3.3. Effect of eugenol on motor behaviour of mice in catalepsy bar test

The effect of pre-and post-treatment with eugenol on the performance of mice treated with MPTP in the catalepsy bar test is illustrated in Figure 4. This test was mainly assessed the immobility or muscle rigidity in mice after MPTP administration for five consecutive days. MPTP administration for five consecutive days in mice significantly ($p < 0.05$) increased the catalepsy score. Pretreatment with eugenol at two doses (50 and 100 mg/kg) showed significant alleviation of MPTP-induced augmented catalepsy score while eugenol at the dose of 25 mg/kg was found ineffective. Post treatments with all the doses of eugenol (25, 50 and 100 mg/kg) were not showing any significant protective effect against MPTP-induced increase in catalepsy score in mice.

3.4. Eugenol improved MPTP-induced decreased rearing behaviour in mice

The effect of pre-and post-treatment with eugenol on the rearing behaviour of mice treated with MPTP is shown in Figure 5. This test was mainly performed to evaluate the exploratory behaviour of mice. In this study, MPTP administration for five consecutive days in mice significantly ($p < 0.05$) decreased the rearing behaviour of mice. Pre-treatment with eugenol (50 and 100 mg/kg) showed a significant increase in the MPTP-induced decrease in rearing behaviour while pre-treatment with eugenol at the dose of 25 mg/kg was not showing any improvement. Post treatments with all the doses of eugenol (25, 50 and 100 mg/kg) were not showing any significant alteration in the MPTP-induced decreased rearing behaviour in mice.

3.5. Eugenol alleviates motor incoordination of mice administered with MPTP in rotarod apparatus

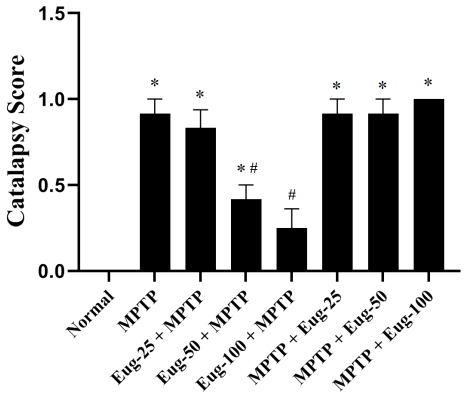

Figure 4. Effect of seven days pre and post treatment with eugenol on catalepsy behaviour against mouse model of MPTP-induced PD. Each bar represents data in mean ± SEM ($n = 6$). $^{*}p < 0.05$ compared to control and $^{\#}p < 0.05$ compared to MPTP using one-way ANOVA followed by Tukey's multiple comparisons test.

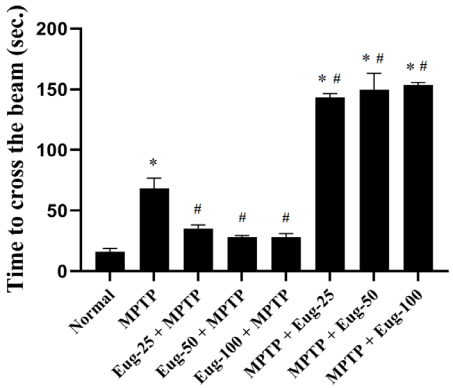

Figure 3. Effect of seven days pre- and post-treatment with eugenol on performance in beam walking test against mouse model of MPTP-induced PD. Each bar represents data in mean ± SEM ($n = 6$). $^{*}p < 0.05$ compared to control and $^{\#}p < 0.05$ compared to MPTP using one-way ANOVA followed by Tukey's multiple comparisons test.

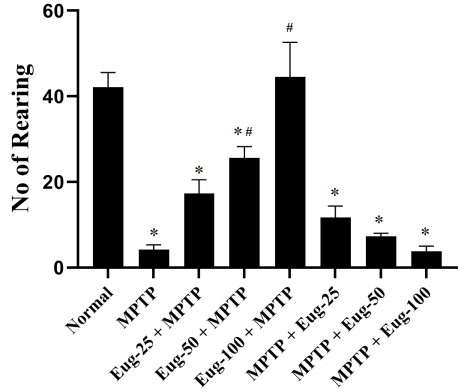

Figure 5. Effect of seven days pre and post treatment with eugenol on rearing activity against mouse model of MPTP-induced PD. Each bar represents data in mean ± SEM ($n = 6$). $^{*}p < 0.05$ compared to control and $^{\#}p < 0.05$ compared to MPTP using one way ANOVA followed by Tukey's multiple comparisons test.

Figure 6 illustrates the effect of pre-and post-treatment with eugenol on the performance of mice treated with MPTP in rotarod apparatus. This test was mainly performed to assess the motor coordination activity. MPTP treatment in mice resulted in a decrease in the fall of time in comparison to control. Thus, MPTP administration in mice caused deterioration in motor coordination. The pre-treatment with a 100 mg/kg of dose of eugenol showed a significant improvement in the motor coordination of mice in rotarod apparatus while the other two doses of eugenol (25 and 50 mg/kg) were found ineffective. On the contrary, post-treatments with the two doses of eugenol (25 and 50 mg/kg) were not showing any significant protective effect against MPTP-induced deterioration in the motor coordination activity of mice in rotarod apparatus. However, post-treatment with eugenol at 100 mg/kg resulted in aggravating significantly ($p < 0.05$) the MPTP-induced deterioration in the motor coordination of mice in rotarod apparatus.

3.6. Eugenol attenuated the MPTP-induced increased lipid peroxidation in the brain of mice

Figure 7 depicts the effect of pre-and post-treatment with eugenol on the lipid peroxidation in the brain of mice treated with MPTP. MDA is the marker of lipid peroxidation. In this study, it was observed that when compared with the control group, MPTP administration in mice augmented the MDA levels in their brain. The pre-treatment with eugenol at all the doses (25, 50 and 100 mg/kg) followed by MPTP administration reduces the MDA level when compared with the MPTP group, which indicates the neuroprotective effect of eugenol. The post-treatment group of eugenol at the dose of 100 mg/kg further increased the MDA levels in the brain of mice treated with MPTP while the two doses of post-treatment of eugenol were not showing any significant alteration.

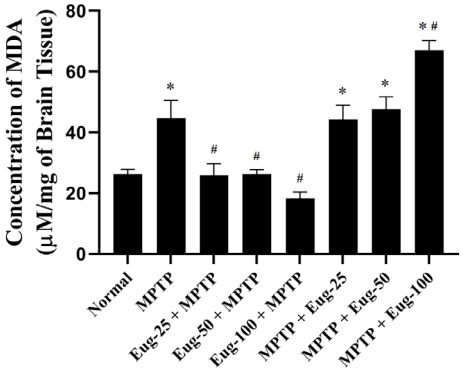

Figure 7. Effect of seven days pre and post treatment with eugenol on MDA levels against mouse model of MPTP-induced PD. Each bar represents data in mean ± SEM ($n = 6$). $^{*}p < 0.05$ compared to control and $^{\#}p < 0.05$ compared to MPTP, using one-way ANOVA followed by Tukey's multiple comparisons test.

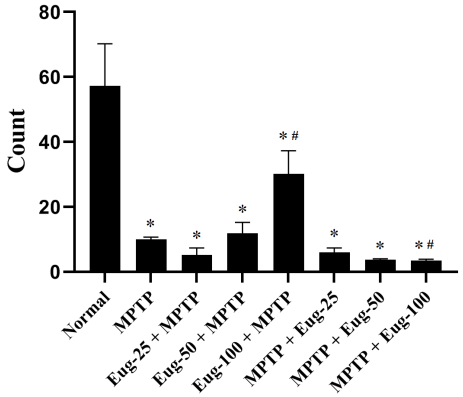

Figure 6. Effect of seven days pre and post treatment with eugenol on motor coordination in rotarod apparatus against mouse model of MPTP-induced PD. Each bar represents data in mean ± SEM ($n = 6$). $^{*}p < 0.05$ compared to control and $^{\#}p < 0.05$ compared to MPTP using one way ANOVA followed by Tukey's multiple comparisons test.

3.7. Eugenol mitigated the MPTP-induced decreased reduced glutathione in the brain of mice

The effect of pre-and post-treatment with eugenol on the reduced glutathione levels in mice treated with MPTP is shown in Figure 8. Reduced glutathione is an endogenous antioxidant. In this study, it was observed that when compared with the control group, MPTP administration in mice decreased the reduced glutathione levels in the brain. The pre-treatment with eugenol (50 and 100 mg/kg) significantly alleviated the MPTP-induced attenuated levels of reduced glutathione in the brain of mice while the dose of 25 mg/kg was found to be ineffective. The post-treatment with all the doses of eugenol were not showing any significant alteration in the MPTP-induced decreased reduced glutathione levels in the brain of mice.

3.8. *In silico* docking interactions of eugenol with TLR4

TLR4/MD-2 heterodimer complex formed *via* transport

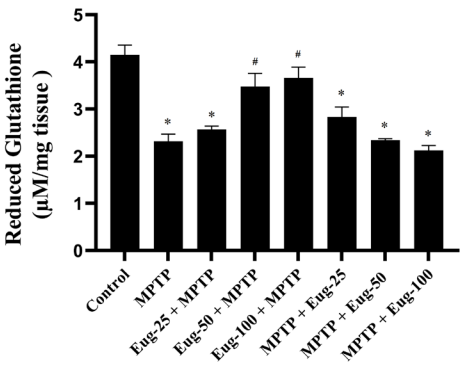

Figure 8. Effect of seven days pre and post treatment with eugenol on reduced glutathione levels against mouse model of MPTP-induced PD. Each bar represents data in mean ± SEM ($n = 6$). $^{*}p < 0.05$ compared to control and $^{\#}p < 0.05$ compared to MPTP, using one-way ANOVA followed by Tukey's multiple comparisons test.

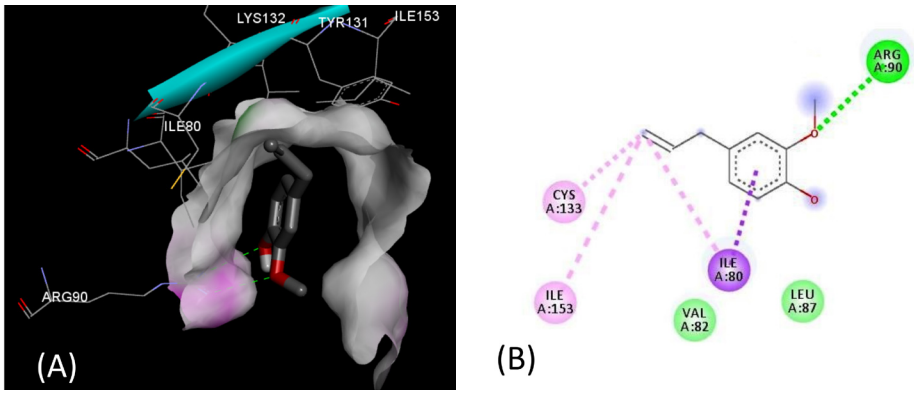

Figure 9. Binding mode of eugenol in the *h*MD-2 proposed by docking studies using AutoDock 4.2. Eugenol is in stick model with colour by atoms and labelled protein residues are capped in wireframe with colour by atom. Hydrogen bonds are shown as green dotted lines **(A)**. 2D view of interacting amino acid residues in the *h*MD-2 with eugenol **(B)**.

of LPS at MD-2 site, which activates TLR4 and its co-receptor MD-2 that will lead to activation of the intracellular signaling. The formation and activation of this complex can be prevented through the binding of an antagonist ligand to the extracellular domain, which will block further intracellular signaling events. In the absence of the X-ray structure of the *h*TLR4, we selected the crystal structure of *h*MD-2 (PDB-ID: 2E59) to study the binding of eugenol in the *h*MD-2. Eugenol showed a binding free energy value (ΔG) of -6.897 kcal/mol. Aromatic ring in eugenol showed pi-sigma interaction with Ile80, One oxygen atom of methoxy group and another oxygen atom of hydroxyl group formed conventional hydrogen bonds with Arg90, which is an important residue for binding of lipid IVa. The methylene group also showed pi-alkyl interaction with Ile153 and van dar Wall interaction with Tyr131. The overall orientation of eugenol in the binding site of *h*MD-2 is shown in Figure 9.

4. Discussion

PD is one of the most idiopathic diseases of neurodegenerative disorders, which is characterized clinically by the presence of tremor, rigidity, postural inability and bradykinesia (*40,41*). MPTP is a neurotoxin that causes clinical manifestations that is quite similar to PD, and it is commonly used in experimental animals as a PD model (*10,11*). The current investigation also reveals that MPTP administration in mice caused the neuromotor impairment in five battery tests (actophotometer, narrow beam walking test, catalepsy bar test, cylinder test and rotarod test). Pre-treatment with eugenol at 50 mg/kg and 100 mg/kg showed a significant reduction in most of the behavioural abnormalities that were observed in the MPTP-induced mouse model of PD. On the contrary, post-treatments with any of the doses of eugenol were not showing any significant protective effect against MPTP-induced Parkinson's symptoms. Moreover, post-treatment with eugenol at 100 mg/kg resulted in aggravating the Parkinson's-like symptoms (Figure 2-6).

In astrocytes, the innocuous MPTP is bio-activated to the dangerous metabolite 1-methyl-4-phenyl-pyridinium ion MPP+ by the synthesis of a dihydropyridinium intermediate by monoamine oxidase (*42*). The dihydropyridinium intermediate passes the cellular membrane easily and performs autoxidation in the extracellular area to produce superoxide anion in addition to MPP+ (*42*). MPP+ stimulates the production of ROS (*43-45*). As a result, oxidative stress is important in the pathogenesis of MPTP-induced PD, as well as the establishment and development of neurobehavioral deficits. The outcomes obtained in this study presented that MPTP administration in mice led to the augmented MDA and attenuated reduced glutathione levels in the brains. MDA is an indicator of lipid peroxidation and oxidative stress, while reduced glutathione is an endogenous antioxidant. Pre-treatment with eugenol at 50 mg/kg and 100 mg/kg significantly alleviated the MPTP-induced altered levels of MDA and reduced glutathione. Thus, pre-treatment with eugenol decreased lipid peroxidation and increased endogenous antioxidants in mice treated with MPTP. However, post-treatment with eugenol with any of the doses did not show any significant effect against MPTP induced augmented levels of MDA, as well as attenuated levels of reduced glutathione in the brain while, post-treatment with eugenol at 100 mg/kg, resulted in aggravating the MPTP-induced augmented levels of MDA in the brain. Similar types of results were depicted in the previously published work (*23,46*). Eugenol has been shown to have direct free radical scavenging properties (*47*). Hence, it appears that the beneficial impact of eugenol pre-treatment on MPTP-induced lipid peroxidation in mice brains was mediated, at least in part, by its free radical scavenging and antioxidant properties (Figure 7). Healthy mice can develop antioxidant systems such as reduced glutathione in response to the oxidative stress induced by MPTP administration. Therefore, pretreatment with eugenol can prevent MPTP-induced neurotoxicity. Damaged mice, on the other hand, have a lower tolerance for oxidative stress and are unable to build up adequate antioxidant systems.

This might make MPTP-induced oxidative stress worsened. These effects are called hormesis (*48*). Taking this statement of result into an account, it shows that the pretreatment of eugenol in a dose-dependent manner shows antioxidant activity and exerts a neuroprotective effect that will ultimately inhibit the oxidative stress while post-treatment at higher dose results in aggravating neurodegeneration. This hormesis is also seen with other phytochemicals such as Zingerone (*46,49*). These findings suggest that following the development of PD symptoms, we should avoid swallowing these spice constituents. However, for further confirmation *in vivo* work needs to be done.

Previous studies indicated that TLR4 is upregulated in the brain of MPTP-treated mice (*50,51*). Several recent pieces of literature report the TLRs as novel disease-modifying therapeutic targets in PD patients (*52-54*). Hence, targeting TLR4 for the management of neurodegenerative diseases including PD has gained a lot of attention around the world. For a better insight into the mechanism behind the anti-inflammatory activity we have docked the eugenol against the MD2 co-receptor of TLR4. Results of the present study elucidated that eugenol had a binding potential against the active site of the MD2 with a binding affinity of -6.897 kcal/mol. The important binding interactions include pi-sigma interaction between the aromatic ring of eugenol with Ile80, conventional hydrogen bonds between one oxygen atom of methoxy group and another oxygen atom of the hydroxyl group with Arg90, which is an important residue for binding of lipid IVa. The methylene group also showed pi-alkyl interaction with Ile153 and van dar Wall interaction with Tyr131 (Figure 9). A previous report showed that eugenol significantly inhibited the gene expression of TLR4 markers (*55*). Eugenol suppressed the expression of LPS-induced pro-inflammatory mediators in human macrophages (*56*) where LPS is the natural agonist of TLR4. Thus, the anti-inflammatory activity of eugenol might be partial because of its antagonistic activity at TLR4. However, this is a very preliminary finding and further *in-vitro*, as well as *in-vivo* studies, are required for confirmation.

5. Conclusion

It can be concluded from all the above-obtained results, that herbal therapy for the approach of various disease treatments can be a novel and most feasible approach. Pre-treatment of eugenol can be proved a novel herbal drug for the treatment of PD. Our *in vivo* study demonstrated that pre-treatment of eugenol can reduce behavioural impairments and improve the oxidative stress parameters as well as antioxidant mechanism. From all these findings, it is revealed that the inclusion of eugenol in the diet reduces the chances of developing Parkinson's symptoms in an elderly individual. These discoveries may encourage the food and pharmaceutical industries to develop safe and effective medicinal goods. However, still further preclinical as well as clinical studies are required to understand its molecular mechanism in detail.

Funding: This work was financially supported by Institute of Pharmacy, Nirma University, Ahmedabad.

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received March 26, 2022; Revised July 1, 2022; Accepted August 13, 2022.

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Released online in J-STAGE as advance publication August 24, 2022.