

## Research Article

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# Embelin and levodopa combination therapy for improved Parkinson's disease treatment

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**Abstract:** Parkinson's disease (PD), a progressive neurodegenerative disorder, affects dopaminergic neurons. Oxidative stress and gut damage play critical roles in PD pathogenesis. Inhibition of oxidative stress and gut damage can prevent neuronal death and delay PD progression. The objective of this study was to evaluate the therapeutic effect of embelin or the combination with levodopa (LD) in a rotenone-induced PD mouse model. At the end of experimentation, the mice were sacrificed and the midbrain was used to evaluate various biochemical parameters, such as nitric oxide, peroxynitrite, urea, and lipid peroxidation. In the substantia nigra (midbrain), tyrosine hydroxylase (TH) expression was examined by immunohistochemistry, and Nurr1 expression was evaluated by western blotting. Gut histopathology was evaluated on tissue sections stained with hematoxylin and eosin. *In silico* molecular docking studies of embelin and  $\alpha$ -synuclein ( $\alpha$ -syn) fibrils were also performed. Embelin

alone or in combination with LD ameliorated oxidative stress and gut damage. TH and Nurr1 protein levels were also significantly restored. Docking studies confirmed the affinity of embelin toward  $\alpha$ -syn. Taken together, embelin could be a promising drug for the treatment of PD, especially when combined with LD.

**Keywords:** gut pathology, Nurr1, oxidative stress, tyrosine hydroxylase, *in silico* docking studies

## 1 Introduction

Parkinson's disease (PD) is the second most common progressive, multifactorial neurodegenerative disorder after Alzheimer's disease. It has a complex etiology and mostly affects elderly people [1]. Pathophysiologically, PD is characterized by the degeneration of midbrain dopaminergic neurons in the substantia nigra (SN) and is associated with motor dysfunctions. The neurotransmitter dopamine (DA) has a major role in movement, motivation, memory, reinforcement, motor skills, and other functions. Decreased or altered neurotransmission as a result of degeneration of dopaminergic neurons leads to cardinal features of PD, such as tremors at rest, rigidity, bradykinesia (akinesia), and postural instability [2]. PD is often associated with neuropsychiatric problems, as well as abnormalities in the olfactory, visual, somatosensory, and autonomic systems, although it is primarily a motor disorder [3]. The exact mechanism of dopaminergic neurodegeneration is not clear. Mitochondrial damage, oxidative stress, excitotoxicity, misfolding and aggregation of proteins, and impairment of protein clearance pathways may be responsible for the onset and progression of PD [4–7]. These factors are closely linked to autophagy, a highly conserved cellular catabolic process that involves elimination of cellular dysfunctional proteins and organelles. Growing evidence indicates the importance of autophagy and dysregulation in the process of neurodegeneration [8].

Dysfunctional mitochondria show a reduction in energy production and utilization. The effect of environmental

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toxins such as herbicides, pesticides, fungicides, and insecticides on health is a major concern [9,10]. Many studies suggest a close relationship between exposure of these toxic chemicals and the development of PD [11–14]. By products of oxidative phosphorylation, such as hydrogen peroxide and superoxide radicals, are scavenged by antioxidants in cells under normal condition. However, reactive oxygen species (ROS) levels are significantly increased in pathological conditions associated with mitochondrial dysfunction [15–19]. Despite brain energy dysfunction, an increase in brain urea has been unraveled in other neurodegenerative disorders, such as Alzheimer's and Huntington diseases [20–23]. An animal model of PD also shows this increase in brain urea [24]. The transcription factor nuclear receptor related-1 (Nurr1) is critical for the homeostasis of dopaminergic neurons, because it plays a vital role in synthesis, survival, and functional integrity and also exerts protective effects against oxidative stress. Nurr1 downregulation is associated with the progressive neuropathology of dopaminergic neurons and PD pathogenesis [25–27]. Nurr1 plays a role in maintaining the homeostasis of nigral dopaminergic neurons as well as in situations of stress and cell damage. It is important to explore its functions in PD. Researchers have reported that the level of Nurr1 in midbrain dopaminergic neurons decreases in the elderly [28] as well as in PD [29]. One hypothesis is that hypoxia-inducible factor-1 alpha (HIF-1 $\alpha$ ) and Nurr1 are crucial for the development and survival of dopaminergic neurons, and PD pathogenesis is aggravated due to deficiencies in these proteins [30,31]. Restoring Nurr1 activity could be a useful therapeutic approach for PD [30].

The enteric nervous system (ENS), also referred to as the “second brain,” is a bidirectional integrated system that communicates extensively with the central nervous system (CNS) [32–34]. Recent studies on the gastrointestinal (GI) tract have emphasized the role of the ENS in the development of PD [35,36]. Researchers have reported that  $\alpha$ -synuclein ( $\alpha$ -syn) migrates from the GI tract to the brain [37]. Epidemiological studies have also shown a strong link between exposure to noxious environmental factors, such as heavy metals, insecticides, herbicides, and pesticides, and the evolution of neurodegenerative processes in PD by causing oxidative stress and genetic changes [38–40]. Generation of a massive amount of ROS as a result of oxidative stress alters proteins and lipids and disrupts homeostasis [41,42].

The current treatments available for PD are focused on symptomatic improvement. It is critical to find a potential candidate drug that targets the molecular mechanisms of PD pathogenesis to delay neurodegeneration. Natural products and their derivatives from plants are vital for novel

drug development [43]. One promising plant is *Embelia ribes* Burm. f. (Family: Myrsinaceae). *E. ribes* berry extract has been extensively investigated for its various pharmacological properties, including antioxidant, anxiolytic, antidepressant, anticonvulsant, antidiabetic, and antimicrobial effects [44]. It has also tested in animal models of Alzheimer's [45] and Huntington [46] diseases, with promising findings. The main active principal isolated from *E. ribes* berries is embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone). This compound can cross the blood–brain barrier to promote beneficial effects on the CNS [47]. Toxicity studies of embelin in mice (50 or 100 mg kg<sup>-1</sup>) was found to be safer and did not change body weight [48]. Mice carrying xenograft pancreatic tumors were fed embelin (75 mg kg<sup>-1</sup>) for 6 weeks. It was well tolerated and showed no toxic effects [49].

The standard drug levodopa (LD) is the only approved symptomatic treatment for PD but shows several problems: “dopa-resistant” motor symptoms (postural abnormalities, freezing episodes, and speech impairment) and non-motor symptoms, such as autonomic dysfunction, mood and cognitive impairment, and/or drug-related side effects (especially psychosis, motor fluctuations, and dyskinesias) [50,51]. It would be useful to identify a treatment that could complement LD, allowing for the reduction of the LD dose while preventing the various motor and non-motor symptoms that sometimes accompany this reduction. The mechanisms concerning neurodegeneration seem to involve aggregation of the presynaptic molecule  $\alpha$ -syn into toxic oligomers and fibrils. Hence, it would be useful to identify a treatment that could bind to  $\alpha$ -syn, breaking up toxic aggregates or preventing aggregates from forming. This ability could ameliorate symptoms in patients with PD. Overall, the currently available PD therapy does not adequately treat the disease. The present work employed a mouse model with rotenone-induced PD symptoms to evaluate the antioxidant potential of embelin alone or in combination with LD. Furthermore, *in silico* docking studies were used to characterize how embelin binds to  $\alpha$ -syn.

## 2 Materials and methods

### 2.1 Chemicals and reagents

Rabbit polyclonal  $\beta$ -actin and rabbit polyclonal Nurr1 primary antibodies were procured from Invitrogen, Thermo Fisher Scientific (Waltham, MA, USA). The main chemicals used in this study were procured from the following

sources: rotenone (TCI Chemicals Pvt. Ltd., Chennai, India); the Abcam Urea Assay Kit ab83362 (Boston, MA, USA); thiobarbituric acid (TCI Chemicals Pvt. Ltd., Tamil Nadu, India); olive oil, Thuruthel Drug Lines (ERUMBA Pharmacy, Payyannur, Kerala, India); L-DOPA (product code: 77201; Sisco Research Laboratories Pvt. Ltd., Maharashtra, India); dimethyl sulfoxide (DMSO) (Pure Chemicals Co., Chennai, India); and hydroxypropyl cellulose (HiMedia, Mumbai, India). An authenticated sample of *E. ribes* (berries) was provided by Dr. V. Chelladurai, Botanist, Thirunelveli, Tamil Nadu, India. All other chemicals and reagents used were of analytical grade.

## 2.2 Preparation of rotenone

Fifty milligrams of rotenone was dissolved in 1 mL of DMSO. From this solution, 0.2 mL was mixed with 19.8 mL olive oil to prepare the stock solution.

## 2.3 Preparation of embelin

Coarsely powdered *E. ribes* berries (1.5 kg) were extracted three times with 2 L of *n*-hexane using the cold extraction method (24 h for each extraction). The solvent was decanted, pooled, and distilled in a boiling water bath. The extract was concentrated *in vacuo* and subjected to column chromatography over silica gel (100–200 mesh). Benzene was used to elute embelin from the column. This procedure yielded an orange powder that, when crystallized with diethyl ether, afforded orange plates of embelin (yield: 2 g) [52]. The structural characterization of embelin was confirmed in earlier work by Fourier transform infrared (FT-IR) spectroscopy [53].

## 2.4 Animal grouping and experimental design

Male Swiss albino mice (25–30 g) were procured from M/s Biogen Laboratory (Bengaluru, India). Animals were housed in ventilated polypropylene cages and acclimatized for 7 days to laboratory conditions before starting experiments. They were kept under ambient conditions with a natural light/dark cycle at  $25 \pm 2^\circ\text{C}$  and 40–60% relative humidity and fed with standard pellet diet and water *ad libitum*.

The mice were randomly divided into seven groups (10 per group). The treatments were as follows: group I, vehicle control, namely olive oil ( $2\text{ mL kg}^{-1}$  orally [p.o.]);

groups II–VII, rotenone ( $2.5\text{ mg kg}^{-1}$ , intraperitoneally); group III, embelin ( $20\text{ mg kg}^{-1}$ , p.o.); group IV, embelin ( $40\text{ mg kg}^{-1}$  p.o.); group V, embelin ( $20\text{ mg kg}^{-1}$  p.o.) and LD ( $7.5\text{ mg kg}^{-1}$  p.o.); group VI, embelin ( $40\text{ mg kg}^{-1}$  p.o.) and LD ( $7.5\text{ mg kg}^{-1}$  p.o.); and group VII, LD ( $7.5\text{ mg kg}^{-1}$  p.o.). The treatment protocol in mice as indicated above was for a period of 21 days.

**Ethical approval:** The research related to animals' use has been complied with all the relevant national regulations and institutional policies for the care and use of animals. All animal experimental procedures were performed according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India, and were approved by Institutional Animal Ethics Committee (reference number: SU/CLAR/RD/003/2019).

## 2.5 Preparation of the PD mouse model

Previous work has shown that chronic daily intraperitoneal injection of rotenone that is prepared using a natural oil causes behavioral (locomotor) deficits and neurochemical abnormalities characteristic of PD [54,55]. Therefore, in the present work, rotenone was prepared in a mixture of olive oil and DMSO (99:1) and administered intraperitoneally to Swiss mice for 21 days.

## 2.6 Cardiac perfusion and brain homogenate preparation

At the end of the experiment, the animals were anesthetized using isoflurane and subjected to cardiac perfusion using normal saline (0.9% NaCl) to eliminate the blood. The skull of the mice was opened and the midbrain carefully isolated and washed with ice-cold phosphate-buffered saline (PBS), pH 7.4. The whole midbrain tissue isolated from each mouse was kept on ice and homogenized with 0.1 M PBS (pH 7.0) using a Potter–Elvehjem PTFE-coated pestle and glass tube (PRO Scientific Inc., Oxford, CT, USA).

## 2.7 Isolation of the postmitochondrial fraction

The midbrains were immediately immersed in 0.7 mL of ice-cold isolation buffer (IB) composed of 225 mM mannitol, 75 mM sucrose, 1 mM ethylene glycol-bis( $\beta$ -aminoethyl

ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 5 mM HEPES-KOH (pH 7.2), and 1 mg mL<sup>-1</sup> of essential fatty acid-free bovine serum albumin (BSA) in a 5 mL plastic tube. The tissue was homogenized at 25,300g using a tissue homogenizer (REMI Lab Homogeniser RQ-127A/D, Maharashtra, India) and transferred into a 1.5 mL microcentrifuge tube. The brain homogenate was centrifuged at 1,100g for 2 min in a refrigerated centrifuge (Eppendorf, Model 5425R, Hamburg, Germany). The supernatant was placed aside and the pellet was resuspended in 0.2 mL of the buffer and centrifuged again at 1,100g for 2 min. The pellet was discarded and the supernatant was combined with that from the first centrifugation. Next, 0.5 mL of combined supernatants was mixed with 0.07 mL of 80 vol% Percoll solution, carefully layered on top of 0.7 mL of 10% Percoll solution, and centrifuged at 18,500g for 10 min. Then, 80% Percoll was diluted in buffer to prepare the 10% solution. The mitochondria-enriched fraction was collected at the bottom of the tube and resuspended in 0.7 mL of washing buffer composed of 250 mM sucrose, 5 mM HEPES-KOH (pH 7.2), 0.1 mM EGTA, and 1 mg mL<sup>-1</sup> BSA. The suspension was centrifuged at 10,000g for 5 min. The final mitochondrial pellet was resuspended in 0.07 mL of washing buffer and stored on ice. To isolate mitochondria, the same procedure was employed as described above, except that IB was supplemented with 0.01% digitonin at the tissue homogenization step, to release mitochondria. The mitochondrial protein content was measured with a Lowry assay kit (Bio-Rad, Haryana, India) according to the manufacturer's instructions. After removing the pellet, the postmitochondrial supernatant fraction was used to estimate the levels of nitric oxide (NO), urea, peroxynitrite, and malondialdehyde (MDA), a measure of lipid peroxidation (LPO).

## 2.8 Measurement of LPO

The MDA content was determined by the thiobarbituric acid (TBA) reaction as described previously [56]. To 0.5 mL of homogenate, 1.5 mL of 20% acetic acid, 0.2 mL of sodium dodecyl sulfate (SDS), and 1.5 mL of TBA were added. The mixture was adjusted to 4.0 mL with distilled water and then heated for 60 min at 95°C using a glass ball as a condenser. After cooling, 4.0 mL of a butanol-pyridine mixture was added and the mixture was shaken well. After centrifugation at 1,792g for 10 min, the organic layer was removed and its absorbance was read at 532 nm. The standards and blanks were treated in a similar manner. The MDA concentration, an indicator of LPO, is expressed as nmol mL<sup>-1</sup>.

## 2.9 Measurement of NO

Nitrite (NO<sub>2</sub><sup>-</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>) are stable final products of NO metabolism and may be used as indirect markers for the presence of NO. The total NO concentration is commonly determined as the sum of the NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> concentrations. In the analyzed samples, NO<sub>3</sub><sup>-</sup> was reduced to NO<sub>2</sub><sup>-</sup> in the presence of cadmium and then converted to nitric acid; this process used Griess's reagent to produce a colored product (Sigma-Aldrich, Stainheim, Germany). The NO<sub>2</sub><sup>-</sup> concentration was determined by spectrophotometric analysis at 540 nm [57].

## 2.10 Measurement of peroxynitrite

Peroxynitrite levels were measured according to the method described by Beckman *et al.* [58] Peroxynitrite-mediated nitration of phenol was measured spectrophotometrically at 412 nm. In brief, 100 µL of homogenate was placed in a glass test tube, to which 5 mM phenol in 5 M sodium phosphate buffer (pH 7.4) was added to a final volume of 2 mL. After mixing, the solution was incubated for 2 h at room temperature and then 15 µL of 0.1 M sodium hydroxide was added and the absorbance was read at 412 nm. The method is based on the oxidation of *o*-phenylenediamine, a colorless substance, by peroxynitrite to yield a colored product. The absorbance increase of this chemical reaction is linearly related to the concentration of peroxynitrite in the range of  $4.4 \times 10^{-7}$  to  $8.0 \times 10^{-6}$  mol L<sup>-1</sup>, with a detection limit of  $1.7 \times 10^{-7}$  mol L<sup>-1</sup> (3σ).

## 2.11 Measurement of urea

Elevated brain urea levels in neurodegenerative Huntington's disease patients were reported as a major metabolic defect [59]; hence, brain urea levels were measured. In a 96-well plate, a 50 µL reaction mix containing urea assay buffer (42 µL), oxiRed probe (2 µL), enzyme mix (2 µL), developer (2 µL), and converter enzyme (2 µL) was added to each well containing 50 µL of urea standards, test samples, or blank control. The reaction mixture was mixed well and incubated for 60 min at 37°C (protected from light). The absorbance at 570 nm was measured using spectrophotometer (Elico, B-200, Hyderabad, India).

## 2.12 Western blot analysis

The SN tissue homogenate was prepared in lysis buffer containing 1% protease inhibitor (HiMedia Laboratories)

for 1 h at 4°C. Samples were then centrifuged at 10,000g for 15 min at 4°C. Supernatants were collected and the protein concentration was determined using a Bradford protein assay kit. Equal amounts of protein (40 µg) were separated on 10% acrylamide gels using SDS–polyacrylamide gel electrophoresis and electrotransferred onto a polyvinylidene difluoride membrane. Membranes were incubated with 5% nonfat milk for 1 h at room temperature (to block non-specific protein binding) prior to incubation overnight at 4°C in a solution of rabbit polyclonal anti-β-actin (1:1,000) and rabbit polyclonal anti-Nurr1 (1:1,000). The next day, membranes were washed three times with Tris-buffered saline with 0.5% Tween 20 (TBST), incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5,000) for 1 h at room temperature, and then washed three times with TBST. The target proteins were visualized using ECL reagent and exposed to a piece of X-ray film. The densities of specific protein bands were determined using Adobe Photoshop CS6 software (version 12.0). The Nurr1 (66 kDa) protein level was normalized to the β-actin (42 kDa) protein level (loading control).

### 2.13 Gut histopathology and brain immunohistochemical staining

Gut tissue was fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin, and then sectioned at a thickness of 5 µm and stained with hematoxylin and eosin (H&E) for histopathological investigation. For histomorphometry analysis, the gut tissue samples were well-oriented with longitudinally cut crypts to precisely assess alterations in the overall tissue architecture. Regarding gut tissue samples, at least 4–5 consecutive villi well distended from the base to tip were rated. The number of goblet cells per villus was counted in 10 well-oriented and adjacent crypt villi on each section stained with H&E. All measurements were made at 100× magnification. On each slide, one section that represented the best view of the villus and crypts was selected for analysis. The slides were examined under an Olympus light microscope and photomicrographs were taken with a Sony camera.

For brain tissue, mice in each group were anesthetized with isoflurane and then transcardially perfused with 0.9% normal saline to clear the blood from the brain. The brain from each mouse was immersed in 10% neutral buffered formalin (the fixative solution) for 4 h. The tissues were cryoprotected in 30% sucrose, embedded in tissue-freezing medium with liquid nitrogen, and sectioned at 3–5 µm using a cryostat. Sections were stored under anti-

freeze buffer. Parallel free-floating sections were subjected to endogenous peroxidase quenching with 1% hydrogen peroxide in PBS, followed by treatment with blocking buffer (5% normal chicken serum and 0.3% Triton X-100 in PBS overnight at 4°C) to block nonspecific protein binding. Sections were incubated overnight at 4°C with mouse monoclonal anti-tyrosine hydroxylase (TH; diluted 1:3,000; Thermo Fisher Scientific, Invitrogen). After washing with PBS, tissues were incubated for 2 h at room temperature with affinity-purified rabbit anti-mouse IgG antibody (diluted 1:50 dilution; Sigma-Aldrich). Then, the sections were exposed to an avidin–biotin peroxidase complex for 2 h. The peroxidase activity was visualized using a stable diaminobenzidine solution. All immunoreactions were observed using a compound light microscope and these results were quantified using the ImageJ 1.46 (National Institutes of Health, Bethesda, MD, USA). For quantification, fragmentation (F) reflects that TH expression (brown patches in the image) that appeared as either single, homogeneous, and continuous or complex, heterogeneous, and discontinuous patches. Based on this protein expression classification,  $F$  was calculated as:  $F = N/A$ , where  $N$  is the number of protein expression patches in the image and  $A$  is the total area (in pixels) of the protein signal.

### 2.14 Molecular docking studies

Molecular docking studies were executed with Autodock4.2 software [60] using the solid-state nuclear magnetic resonance of α-syn fibrils retrieved from the RCSB Protein Data Bank (PDB code 2N0A). This structure holds 10 units of the peptide (140 amino acids), which are arranged in parallel by presenting features that commonly stabilize amyloid folds (such as intermolecular salt bridges, a glutamine ladder, steric zippers involving hydrophobic residues, and in-register parallel-β-sheet hydrogen-bonding with a Greek key motif) [61]. The embelin structure was downloaded from the PubChem database. Before performing docking, all water molecules were removed and polar hydrogen was added to α-syn fibrils by using the hydrogen module in AutoDock Tools, followed by assigning Kollman united atom partial charges. The grid maps representing the proteins in the actual docking process were calculated with AutoGrid. The grids were chosen to be sufficiently large to include the active site. The dimensions of the grids were thus 126 Å × 126 Å × 126 Å, with the spacing of 0.375 Å between the grid points. The *in silico* bonding mode of ligand with the receptor was carried out using the empirical free energy function and the Lamarckian genetic algorithm,

applying the following standard protocol with an initial population of 150 randomly placed individuals, a maximum of  $2.5 \times 10^7$  energy evaluations, GA crossover mode = two points. A genetic algorithm of 100 independent docking cycles was carried out for the ligand. A genetic algorithm of 100 independent docking conformers was carried out for the compound, and the best conformer was taken based on their lowest binding energy for further analysis. PyMOL (<https://pymol.org/2/>) and LigPlot 2.2 tools were used to visualize the docking results [62]. The entire structure of embelin bound to the target protein was examined for binding energy and also for specific and nonspecific interacting residues.

## 2.15 Statistical analysis

The data are presented as the mean  $\pm$  standard error of the mean. Differences among the groups were compared with a one-way analysis of variance (ANOVA), followed by the Bonferroni test for multiple comparisons. The *F* and *P* values for the one-way ANOVA are shown in each panel of each figure. Significant differences between groups are denoted by asterisks, with details indicated in each figure legend. SigmaPlot 14.5 (Systat, USA) was used for statistical analysis.

## 3 Results

### 3.1 Changes in brain MDA, NO, peroxynitrite, and urea levels

The mean MDA level in rotenone-treated mice was more than two times higher than in the control mice ( $11.36 \pm 0.40$  and  $5.48 \pm 0.17$  nmol mL<sup>-1</sup>, respectively; Figure 1a). The mice exposed to rotenone plus embelin and/or LD showed markedly reduced MDA compared with rotenone treatment alone (Figure 1a;  $P < 0.001$ ). Notably, the combination of 40 mg kg<sup>-1</sup> embelin and 7.5 mg kg<sup>-1</sup> LD rendered even better protection against LPO. The mean peroxynitrite level in the rotenone-treated mice (group II) was markedly higher than the control mice ( $0.19 \pm 0.001$  and  $0.13 \pm 0.007$  nmol mL<sup>-1</sup>, respectively,  $P < 0.001$ ; Figure 1b). The addition of embelin and/or LD to rotenone-treated mice significantly reduced the peroxynitrite level compared with the rotenone-treated mice ( $P < 0.001$ ). Rotenone-treated mice that received 40 mg kg<sup>-1</sup>

embelin or 40 mg kg<sup>-1</sup> embelin plus 7.5 mg kg<sup>-1</sup> LD showed almost similar peroxynitrite levels to the control mice (Figure 1b).

The mean NO level in rotenone-treated mice was significantly higher than in the control mice ( $19.0 \pm 1.1$  and  $11.2 \pm 1.9$   $\mu$ mol L<sup>-1</sup>, respectively,  $P < 0.001$ ; Figure 1c). Administering embelin and/or LD to rotenone-treated mice markedly reduced NO levels compared with mice that only received rotenone ( $P < 0.001$ ). In particular, the NO levels in the rotenone- and drug-treated mice (40 mg kg<sup>-1</sup> embelin plus 7.5 mg kg<sup>-1</sup> LD) were not different from the control group.

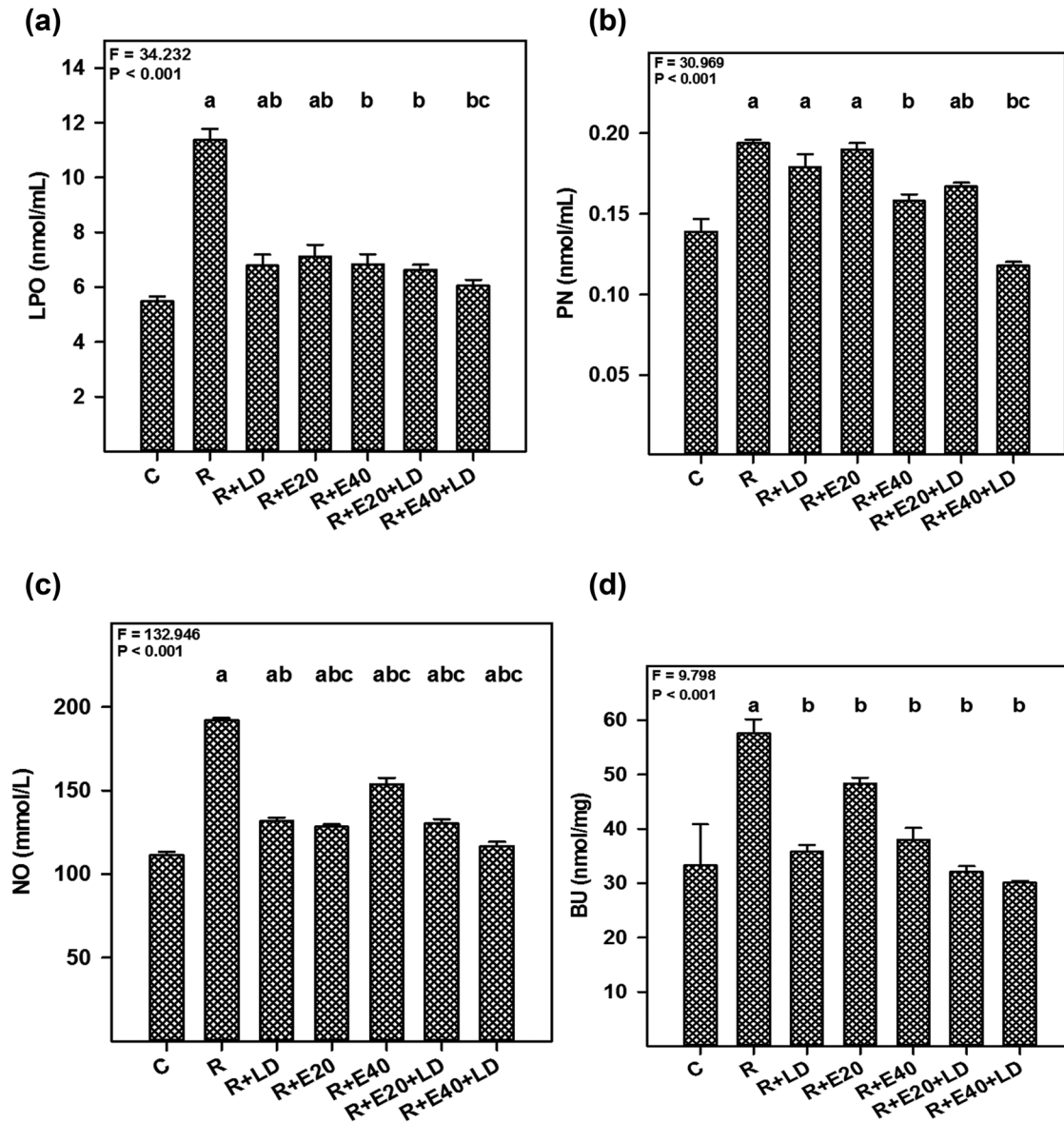
The mean brain urea in the rotenone-treated mice was nearly 75% higher than the control mice ( $57.60 \pm 2.59$  and  $33.30 \pm 7.51$  nmol mg<sup>-1</sup>, respectively,  $P < 0.001$ ; Figure 1d). The addition of embelin and/or LD to rotenone-treated mice markedly reduced the brain urea levels compared with mice that only received rotenone ( $P < 0.001$ ). Indeed, treatment returned the urea level to near what was found in control mice. Based on the above findings, treatment with embelin alone or embelin plus LD reversed the biochemical alterations caused by rotenone administration.

### 3.2 Nurr1 protein changes in the SN

Figure 2 shows the western blot analysis of Nurr1 (66 kDa) protein in the SN. Nurr1 expression in the rotenone-treated mice was significantly decreased ( $P < 0.05$ ) compared with the control group. Nurr1 expression in the rotenone-treated mice administered LD standard therapy (7.5 mg kg<sup>-1</sup>) was restored to the control level. Embelin (40 mg kg<sup>-1</sup>) plus LD (7.5 mg kg<sup>-1</sup>) combination therapy in rotenone-treated mice also reverted the Nurr1 protein expression to almost the control level. However, the effect of embelin alone (40 mg kg<sup>-1</sup>) in restoring the Nurr1 protein level was comparatively less than the embelin plus LD combination therapy. Therefore, embelin plus LD combination therapy has a better protective effect on the brain dopaminergic system.

### 3.3 Gut histopathological changes and histomorphometry

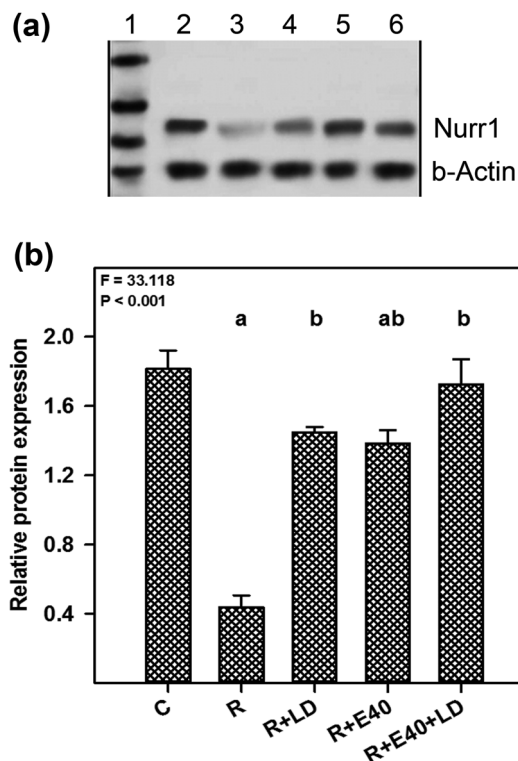
Micrographs of H&E-stained gut sections are shown in Figure 3. The control mice showed normal architecture of all three mucosa levels (Figure 3a, circle), with normal crypts and no inflammatory cell infiltration. In the rotenone-treated mice (Figure 3b), there was remarkable



**Figure 1:** Antioxidant potential of embelin (E) and embelin plus LD therapies in mice treated with rotenone, based on the measurement of brain (a) LPO, denoted by MDA, (b) NO, (c) peroxynitrite (PN), and (d) brain urea (BU) levels. The values represent the mean  $\pm$  standard error ( $n = 6$ ). For the embelin groups, the number indicates the dose (in  $\text{mg kg}^{-1}$  body weight). The  $F$  and  $P$  values are based on the one-way analysis of variance. The level of significance was found to be  $P < 0.001$ . The superscript letters indicate the results of the Bonferroni multiple comparison test. <sup>a</sup>Significantly different from the control group. <sup>b</sup>Significantly different from the rotenone group. <sup>c</sup>Significantly different from the rotenone + LD group.

destruction of the mucosa and surface epithelia (arrow mark). Moreover, inflammatory cell infiltration (arrow head) was noted in the lamina propria. In the group treated with  $10 \text{ mg kg}^{-1}$  embelin, there were epithelial destruction, mucosal edema (small arrow), intense inflammatory cell infiltration, and crypt distortion (Figure 3c), whereas in the group treated with  $20 \text{ mg kg}^{-1}$  embelin, there was inflammation in the mucosal layer but no damage to the epithelial layer (Figure 3d). Reduced crypt damage, less edema, and

less inflammation were seen in this group. In the group treated with  $10 \text{ mg kg}^{-1}$  embelin plus LD (Figure 3e), there were epithelial destruction and moderate infiltration; normal arrangement of all the three mucosal layers is visible. However, in the groups treated with  $20 \text{ mg kg}^{-1}$  embelin plus LD (Figure 3f) or LD alone (Figure 3g), there were less damage in gut tissues, no edema, proper arrangement of mucosal layers, and less inflammatory cell infiltration.



**Figure 2:** (a) Western blot analysis showing Nurr1 protein changes in the SN. Lane 1, molecular weight ladder; lane 2, control; lane 3, rotenone (R); lane 4, R + embelin (E, 40 mg kg<sup>-1</sup>); lane 5, R + E (40 mg kg<sup>-1</sup>) + LD (LD, 7.5 mg kg<sup>-1</sup>); lane 6, R + LD (7.5 mg kg<sup>-1</sup>). (b) The bar graph shows the relative expression of Nurr1 in various groups. The values present the mean ± standard error ( $n = 4$ ). The  $F$  and  $P$  values are based on a one-way analysis of variance. The level of significance was found to be  $P < 0.001$ . The letters indicate the results of the Bonferroni multiple comparison test. <sup>a</sup>Significantly different from the control group. <sup>b</sup>Significantly different from the rotenone group. <sup>c</sup>Significantly different from the rotenone + LD group.

Based on a scoring system established by Okayasu *et al.* [63], inflammatory cell infiltrates, epithelial changes, and the mucosal architecture were scored based on specific criteria. The changes in the gut histomorphometry were analyzed and the overall scores for each group are presented in Table 1. Similar to the histopathological changes, gut from mice treated with 20 mg kg<sup>-1</sup> embelin plus LD showed substantial improvement compared with the other treated groups.

### 3.4 Effect of embelin and embelin plus LD treatments on the rotenone-induced reduction of the TH-positive cells in the SN

TH catalyzes the rate-limiting step in DA biosynthesis in dopaminergic neurons. The number of TH-positive cells

was evaluated in the SN (Figure 4). Compared with the control group (Figure 4a), there was a marked reduction in TH-positive cells in the rotenone-treated mice (Figure 4b). By contrast, in the drug-treated groups (Figure 4c–g), there was protection of TH-positive cells. Figure 4h shows the percentage of TH-positive cells.

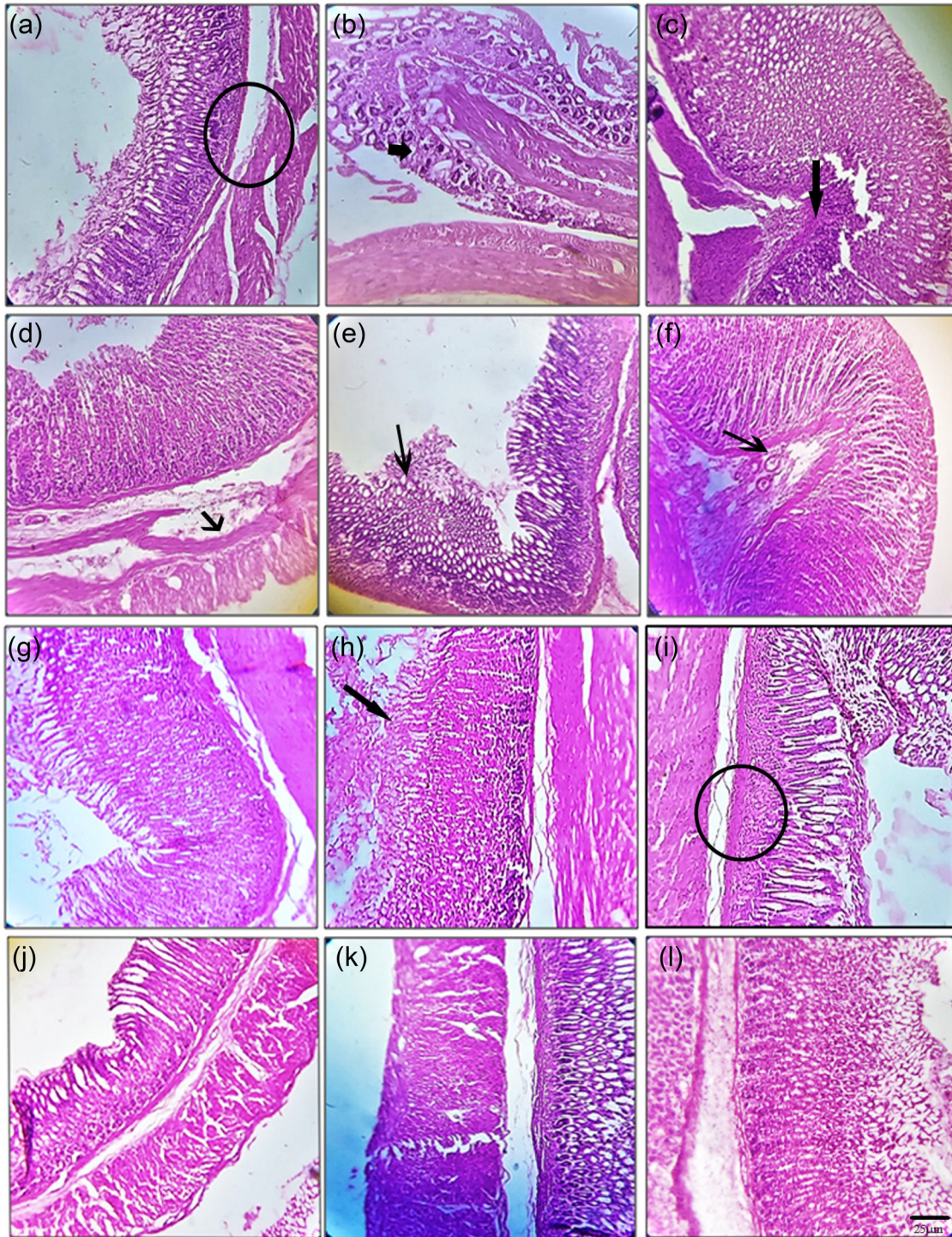
### 3.5 *In silico* studies revealed the binding affinity of embelin for $\alpha$ -syn fibrils

The binding mode of embelin with  $\alpha$ -syn fibrils was analyzed using the Autodock 4.2 suite. Thirteen putative binding sites were identified (Figure 5a). Site 2 (with residues Y39, S42, and T44), site 9 (with residues G86, F94, and K96), and site 3/13 (with residues L43, L45, V48, and H50) showed a high probability of interaction [64]. The embelin scoring energy ( $-4.18$  kcal mol<sup>-1</sup>) is most favorable for interaction with site 3/13. For this interaction, two hydrogen bonds are formed. The first hydrogen bond is between the second oxygen atom of embelin with the ND1 group of the polar residue His50(D), with a bond distance of 3.0 Å (Figure 5b and c). The second hydrogen bond is between the fourth oxygen atom of embelin and the NZ group of positively charged residue Lys45(D), with a bond distance of 2.9 Å (Figure 5b and c). In addition, residues Val48(C), Val48(D), Val48(F), His50(G), and His50(F) are involved in hydrophobic interactions to sustain the stability of the embelin– $\alpha$ -syn complex. Furthermore, van der Waals interactions occur with the residues Lys45(C), Val48(E), Val49(C), Val49(D), Val49(E), Val49(F), His50(C), and His50(E) (Figure 5b and c).

## 4 Discussion

Chronic intraperitoneal administration of rotenone induces a gradual movement disorder that recapitulates PD symptoms [54]. The rotenone-treated mice in this study developed similar symptoms, and the herbal drug embelin ameliorated oxidative stress and associated complications in these mice. Importantly, embelin plus LD therapy rendered better protective effects against PD-related dysfunction compared with embelin treatment alone. Of several molecular mechanisms proposed in the etiology and pathogenesis of PD, oxidative stress and ROS generation play key roles in certain regions of the brain of people with PD by triggering mitochondrial dysfunction [65–67]. The embelin treatment doses in the present study were slightly different





**Figure 3:** Representative micrographs of H&E staining of gut sections ( $n = 4$ ). The control group (a) shows normal architecture of all three mucosal layers (circle), with normal crypts and no inflammatory cell infiltration. In the rotenone-treated group (c), there is destruction of the mucosa and surface epithelia (arrow mark). Moreover, inflammatory cell infiltration (small and thick arrow) is apparent in the lamina propria (b). In the rotenone + embelin ( $20 \text{ mg kg}^{-1}$ ) group (d), there is mucosal edema (small and thin arrow), with intense inflammatory infiltration and crypt distortion (e). In the rotenone + embelin ( $40 \text{ mg kg}^{-1}$ ) group, there were epithelial destruction and moderate inflammatory cell infiltration (f) apart from normal arrangement of all the three mucosal layers (g). In the rotenone + embelin ( $20 \text{ mg kg}^{-1}$ ) + LD ( $7.5 \text{ mg kg}^{-1}$ ) group, all three mucosal layers showed a normal arrangement (i, circle), although there were epithelial destruction and moderate inflammatory cell infiltration (h). In the rotenone + embelin ( $40 \text{ mg kg}^{-1}$ ) + LD ( $7.5 \text{ mg kg}^{-1}$ ) group, there were little damage, no edema, proper arrangement of mucosal layers, and limited inflammatory cell infiltration (j and k). The rotenone + LD ( $15 \text{ mg kg}^{-1}$ ) group showed similar features (l). The scale bar is  $25 \mu\text{m}$ .

**Table 1:** Scoring pattern for gut histomorphometry

Number	Groups	Histomorphometry	Mean score
1	C	Intact surface epithelium without inflammatory cells	0
2	R	Infiltration of inflammatory cells, mucosal ulceration, irregular crypts, hyperplasia, goblet cell loss, cryptitis, and erosion	3.8
3	R + LD	Infiltration of inflammatory cells, hyperplasia, goblet cell loss, cryptitis, and erosion	2.6
4	R + E20	Infiltration of inflammatory cells extended to the submucosa, hyperplasia, marked epithelial changes, goblet cell loss, irregular crypts, and erosion	3.7
5	R + E40	Infiltration of inflammatory cells, goblet cell loss, crypt loss, and erosion	2.5
6	R + E20 + LD	Infiltration of inflammatory cells, goblet cell loss, crypt loss, and erosion	1.7
7	R + E40 + LD	Minimal infiltration of inflammatory cells, goblet cell loss and crypt loss with granular tissue repair, and new capillary formation	1.0

C, control; R, rotenone; LD, levodopa; E, embelin (20 or 40 mg kg<sup>-1</sup>).

Scoring – no effect = 0; minimal = 1; mild = 2; moderate = 3; severe = 4.

The mean score is an average of three animals in each group.

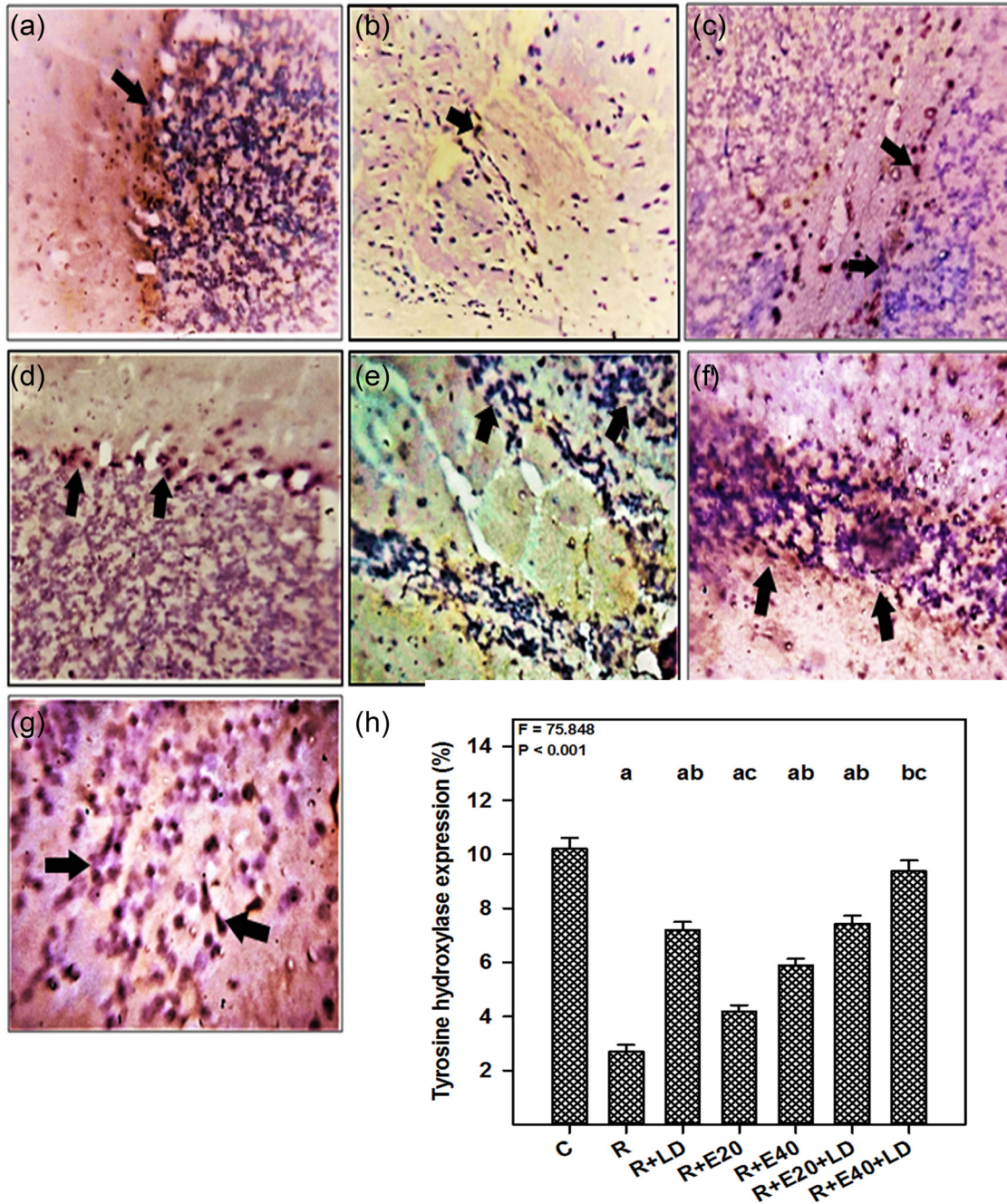
from earlier works [68,69]; they were chosen by taking into consideration the ability of embelin to protect the brain against oxidative stress and inflammation. In the present work, although a 50% reduced dose of LD (7.5 mg kg<sup>-1</sup>) was administered to the rotenone-treated mice [70], a previous study supported this dose as a therapy for an animal model of PD [71].

After entering neurons, rotenone blocks mitochondrial complex I activity, increases ROS production, and inhibits proteasome activity, generating proteolytic stress. Rotenone decreases DA and reduced glutathione (GSH) levels and increases LPO in dopaminergic neurons, resulting in oxidative damage [72,73]. The significantly elevated levels of brain lipid peroxides, NO, peroxynitrite, and urea in the rotenone-treated mice suggest the effect of rotenone treatment on oxidative stress-related damage, implicating free radicals and defective mitochondrial respiration [24,74–76]. Therefore, increased generation of reactive oxygen metabolites and the ensuing oxidative stress are considered important pathogenic mechanisms that underlie rotenone-induced cell death. By contrast, embelin and embelin plus LD combination therapies in rotenone-treated mice exerted an antioxidative effect by reversing the changes in brain levels of LPO, peroxynitrite, NO, and urea. These effects might have contributed to the neuroprotective and therapeutic benefits exerted by the compounds. Hence, the antioxidant and neuroprotective properties of embelin [44–46,77–79] and the ability of LD to improve the brain DA availability and nerve conduction should have ameliorated the rotenone-induced symptoms in the mice.

Increased NO generation is also an important contributor to the mechanisms by which rotenone mediates neuronal damage [80]. Rotenone increases the expression of inducible nitric oxide synthase (iNOS) in the

striatum and SN and increases the level of NO in rodent brains [81]. Increased NO generation via neuronal nitric oxide synthase could also be involved in rotenone-mediated neurotoxicity. This increase in the NOS activity and 3-nitrotyrosine and nigrostriatal damage in rats treated with rotenone was reduced by administration of the neuronal NOS inhibitor 7-nitroindazole [80]. Evidence implicates increased NO formation for a prolonged time during inflammation and toxicity as a cause of neuronal death. The reaction of NO with molecular oxygen yields reactive oxides of nitrogen, including nitrogen dioxide (NO<sub>2</sub>) and dinitrogen trioxide (N<sub>2</sub>O<sub>3</sub>). The reaction of NO with the superoxide anion (O<sub>2</sub><sup>-</sup>) forms reactive peroxynitrite; this species leads to oxidation, nitrosylation of thiols in proteins or GSH, and nitration of tyrosine residues in proteins [82,83]. Oxidative/nitrosative stress and inactivation of several mitochondrial electron transport complexes, including complex I, cause mitochondrial dysfunction, inhibition of mitochondrial respiration, depletion of cellular energy, and, ultimately, neuronal cell death [84]. In agreement with previous reports [45,46,77], the neuroprotective effect of embelin against PD observed in the present study could be attributed to its antioxidant and free radical scavenging capacity. The amelioration of brain urea levels in PD brains by embelin and embelin plus LD combination therapies implicates their intricate role in the restoration of brain energy functions. Notably, treatment with 40 mg kg<sup>-1</sup> embelin or 40 mg kg<sup>-1</sup> embelin plus LD provided the best effects.

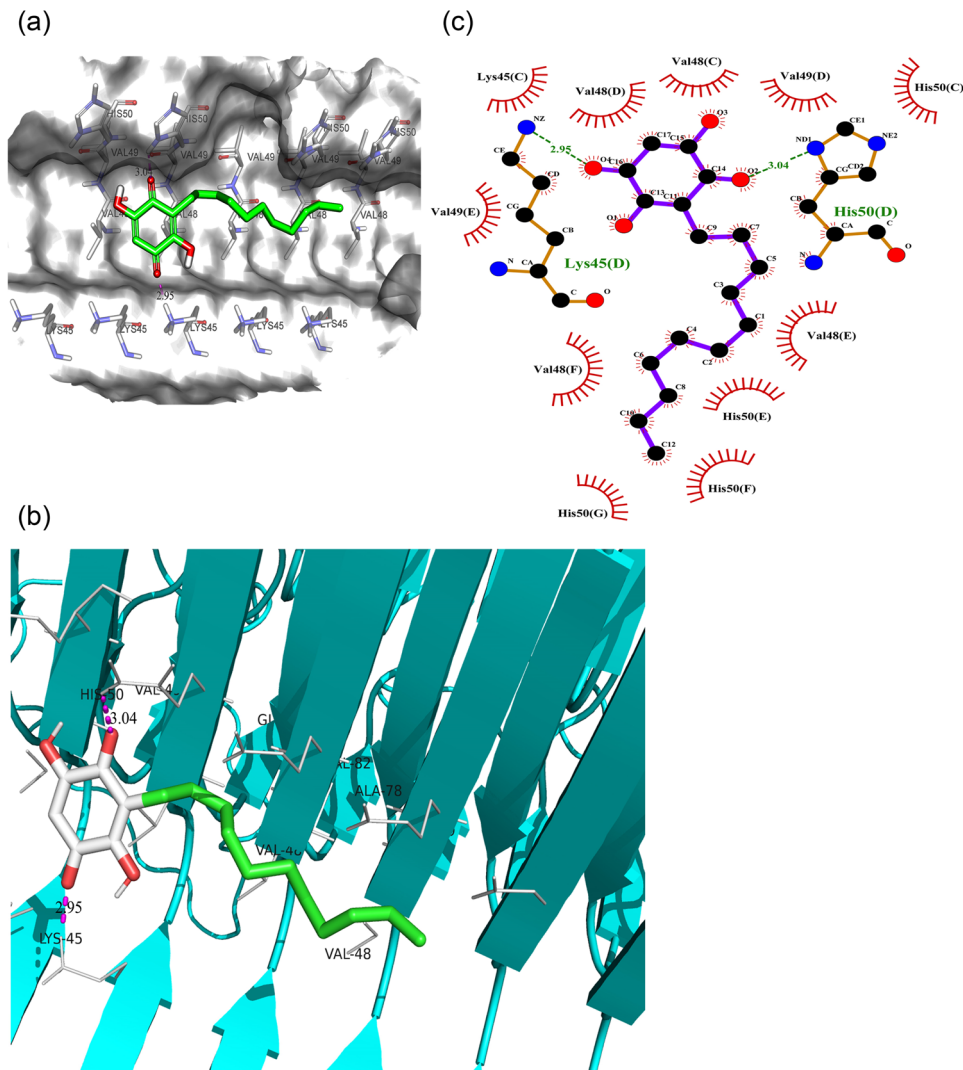
The nuclear receptor transcription factor Nurr1, through its interaction with several key factors, is crucial for mesencephalic dopaminergic neuron survival. Specifically, this transcription factor contributes to regulate the expression of TH, DA transporter, vesicular monoamine transporter 2, and aromatic L-amino acid decarboxylase and to support



**Figure 4:** The effect of embelin and embelin + LD therapies on the TH protein expression level. TH immunostaining in the SN from the (a) control, (b) rotenone, (c) rotenone + embelin ( $20 \text{ mg kg}^{-1}$ ), (d) rotenone + embelin ( $40 \text{ mg kg}^{-1}$ ), (e) rotenone + embelin ( $20 \text{ mg kg}^{-1}$ ) + LD ( $7.5 \text{ mg kg}^{-1}$ ), (f) rotenone + embelin  $40 \text{ mg kg}^{-1}$  + LD  $7.5 \text{ mg kg}^{-1}$ , and (g) rotenone + LD ( $7.5 \text{ mg kg}^{-1}$ ) groups. The arrows denote the TH-positive cells. (h) Quantitative analysis of TH protein expression. The results are expressed as the mean + standard error of the mean ( $n = 4$ ). The  $F$  and  $P$  values are based on the one-way analysis of variance with Bonferroni multiple comparison test. The level of significance was found to be  $P < 0.001$ . <sup>a</sup>Significantly different from the control group. <sup>b</sup>Significantly different from the rotenone group. <sup>c</sup>Significantly different from the rotenone + LD group.

the production and storage of DA, a critical brain neurotransmitter [85]. Reduced Nurr1 expression that is observed in PD might not only result in dopaminergic neuron loss and motor impairments but also impair mitochondrial

function and oxidative phosphorylation [86]. Consistent with the fact that Nurr1 is vital for the development and survival of midbrain dopaminergic neurons, studies have reported that overexpression of  $\alpha$ -syn (WT or an A53T



**Figure 5:** (a) An illustration of embelin (green structure) in the substrate-binding pocket of  $\alpha$ -syn. The crucial residues of  $\alpha$ -syn are shown as atom stick figures. (b) The secondary structure view of the embelin- $\alpha$ -syn complex. Hydrogen bonds are represented by the magenta dashed line. (c) A LigPlot view of the embelin- $\alpha$ -syn complex. The Lys45(D) and His50(D) residues of  $\alpha$ -syn are highlighted. The image shows the hydrogen bonds and hydrophobic and van der Waals interactions that mediate embelin- $\alpha$ -syn binding.

mutant) in mice caused a striking reduction in Nurr1 protein, inhibited nuclear factor kappa B (NF- $\kappa$ B) expression and transcriptional activity, and decreased binding of NF- $\kappa$ B to the Nurr1 promoter [86]. Moreover, Nurr1 expression in microglia and astrocytes might help protect dopaminergic neurons from inflammation-associated death. Therefore, the anti-inflammatory role of Nurr1 seems important in the suppression of PD pathology [87,88]. Because Nurr1 helps regulate dopaminergic neuron functions via neurotrophic signaling by the glial-derived growth factor (GDNF) receptor Ret, the GDNF-Ret-Nurr1 pathway may play a major role in protecting dopaminergic neurons and their functions. Moreover, interaction between Nurr1 and Foxa2 is critical for the protection of midbrain dopaminergic neurons

from toxic insults [89]. As a regulator of neuroprotective genes, Nurr1 drives the cAMP response element-binding protein-mediated neuroprotective response in neurons following oxidative stress and excitotoxic insults [90].

While LD has provided great benefit to patients with PD, its chronic use is associated with issues. For example, patients can develop non-motor behavioral disorders, including impulse control disorders, psychosis, hallucinations, and (hypo)mania [91]. Moreover, chronic LD treatment can lead to oxidative stress [92,93]. The ability to add a natural compound could allow reducing the LD dose administered to patients while preventing the negative consequences that could result from this dose reduction. This potential requires additional studies,

including the examination of behavioral changes in animal models of PD.

The survival of dopaminergic neurons relies on two key proteins, namely HIF-1 $\alpha$  and Nurr1. Therefore, researchers have proposed that PD pathogenesis could be due to the deficiencies in these genes [30]. Therapeutic agents that increase the expression of these two genes ameliorated PD by restoring striatal DA content and motor functions and mitigated the PD-induced histological changes in SN [30]. As embelin and the embelin plus LD combination considerably increased the expression of Nurr1 protein in the midbrain region of rotenone-treated mice, the findings collectively indicate that Nurr1 could be a promising therapeutic target [86] by which degenerating midbrain dopaminergic neurons could be protected in a bidirectional mode by promoting intracellular survival pathways in midbrain dopaminergic neurons as well as by modulating the toxic environment surrounding these neurons. The binding affinity of embelin toward the active site of  $\alpha$ -syn was predicted by *in silico* molecular docking. The findings emphasize the ability of embelin to bind to and dissolve  $\alpha$ -syn fibrils. These fibrils are 1,000-fold more toxic than the oligomers or other precursors with regard to motor impairment, dopaminergic neuronal loss, and synaptic damage [94,95]. Therefore, the docking results in this study implicate that embelin can likely bind to  $\alpha$ -syn fibrils and break them up, an eventuality that could protect the organism from  $\alpha$ -syn-mediated toxicity. Taken altogether, embelin in combination with LD could be a viable treatment for PD.

Several works have substantiated gut pathology and damage in patients with PD as well as PD animal models. Some authors have speculated that PD originates outside of the CNS through some involvement with the ENS [96,97]. Indeed, Lewy bodies are found at every layer of the GI tract in patients with PD [98]. In support of this hypothesis, several studies have demonstrated that the gut microbiota may contribute to PD [99–101], and these effects could increase  $\alpha$ -syn expression in the gut. While there is growing evidence that emphasizes the importance of both brain and gut pathological changes in PD, there is no definitive proof as to which organ is affected first. The gut histopathological alterations caused by systemic rotenone administration suggest colonic inflammation [100]. Hence, intestinal dysfunction-related problems could be involved in PD progression [102]. Moreover, the increase in the cellularity of the lamina propria in the colon provides evidence for the occurrence of irritable bowel syndrome that affects the colonic mucosa [103]. There is plausible proof of a link between gut bacteria

and neurodegenerative diseases [104,105] that affects the ENS. Thus, the gut microbiota is implicated in motor dysfunction, neuronal inflammation, and  $\alpha$ -syn pathology.

Several studies indicate that patients with inflammatory bowel disease, which is known to increase intestinal permeability, have an increased risk of developing PD. This eventuality suggests a role for GI inflammation in the development of PD [106]. PD is also closely associated with inflammation caused by specific microbial cell structures and pattern recognition receptor signaling pathways. Inflammation in the ENS could be due to increased accumulation of *Escherichia coli* [93]. Evidence supports that gut dysfunction might aggravate oxidative stress and mucosal inflammation in PD apart from inducing  $\alpha$ -syn accumulation in the ENS [107]. Both inflammation and oxidative stress play a synergistic key role in gut dysbiosis-mediated changes in gut DA production as well as secretion of DA in the SN through activation of microglia NLRP3 inflammasome and iNOS expression [108]. The restoration of gut damage in rotenone-treated mice by embelin and embelin plus LD therapies implicates the antimicrobial, [109], antioxidant [76,110,111], and anti-inflammatory functions of embelin [78,112,113] and the antioxidant capacity [114,115] and gut epithelium protection rendered by LD [116]. Overall, our earlier investigation [53] and the present findings have substantiated embelin's therapeutic potential.

We used the well-established rotenone model of PD, which was first reported in 2000 [117]. Since this report, there has been a debate as to how well this model recapitulates the PD phenotype. There have been efforts to improve the administration of rotenone to achieve a consistent phenotype, and it serves as useful model to test neuroprotective strategies [118]. Moreover, mice treated with rotenone develop two of the hallmarks of PD: degradation of dopaminergic neurons in the SN and Lewy body formation in the surviving dopaminergic neurons [119]. Another issue is that rotenone induces an acute lesion – dopaminergic cell death and DA deficiency – whereas PD is a chronic, degenerative disease. However, we still feel that the rotenone model is appropriate to test potentially neuroprotective compounds. Our findings underscore the benefit of this compound to ameliorate hallmarks of PD, and this compound could be tested in other models of PD, including those generated by altering the genes that have been implicated in familial PD [120]. Like embelin, other natural products have also proven their efficacies when tested in both *in vitro* and *in vivo* models of PD [121,122], thereby implicating the importance of herbal treatment in PD.

## 5 Conclusion

The findings from this study demonstrate that embelin could be a potential drug for PD treatment, either alone or in combination with LD. Embelin attenuates rotenone toxicity to protect the brain through antioxidant mechanisms as well as by the upregulation of Nurr1 and TH proteins. Notably, *in silico* binding studies revealed that embelin can bind to  $\alpha$ -syn fibrils and break up these fibrils. This could ameliorate the deficits caused by  $\alpha$ -syn aggregation. Overall, embelin is a promising molecule for the treatment of PD.

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**Author contributions:** SS and ES designed the research. VHR, SS, and AK performed experiments. SA performed and analyzed the *in silico* molecular docking studies. SS and RV analyzed and interpreted the data. VHR and SS wrote the article. MDH critically edited and revised the manuscript. VHR, SS, AK, SA, MDH, ES, and RV approved the article.

**Conflict of interest:** Authors state no conflict of interest.

**Data availability statement:** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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