RESEARCH PAPER

Neuroprotective effect of Carnosic acid in Rotenone induced Parkinson's disease in mice.

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ABSTRACT

Background/Introduction: Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by dopaminergic neuronal loss, oxidative stress and mitochondrial dysfunction. Current therapies mainly provide symptomatic relief without preventing disease progression. Carnosic acid (CA), a natural phenolic diterpene from Rosmarinus officinalis, possesses antioxidant and anti-inflammatory properties with potential neuroprotective effects. This study evaluated the neuroprotective effects of CA, alone and combined with levodopa, in a rotenone-induced mouse model of Parkinson's disease.

Methodology: Forty-two adult male Swiss albino mice were randomly assigned to seven groups (n=6/group): control, rotenone, CA 20 mg/kg, CA 40 mg/kg, CA 20 mg/kg + levodopa, CA 40 mg/kg + levodopa and levodopa alone. Parkinsonism was induced with rotenone (2.5 mg/kg, intraperitoneally) for 21 days. Serum triiodothyronine, nitric oxide, lipoxygenase, total antioxidant capacity, and superoxide dismutase were estimated. Anxiety-like behavior was assessed using the Elevated Plus Maze and Open Field Test. Data were analyzed using one-way ANOVA with Bonferroni post-hoc test.

Results: Rotenone reduced serum T3, TAC, and SOD while increasing NO and LPX levels, indicating oxidative stress. It also induced anxiety-like behaviour. CA treatment dose-dependently improved biochemical and behavioural changes, with CA 40 mg/kg +levodopa producing near-normal antioxidant status, reduced oxidative stress markers, restored T3 levels, and the greatest improvement in behavioral performance.

Conclusion: Carnosic acid exerted significant neuroprotective effects against rotenone-induced Parkinsonism by restoring antioxidant defenses, reducing oxidative stress, normalizing thyroid hormone levels, and improving anxiety behaviour. Combined therapy with levodopa showed superior benefits, suggesting CA as a promising adjunctive treatment targeting multiple pathogenic mechanisms in Parkinson's disease.

Keywords: *Carnosic acid, Levodopa, Neuroprotection, Oxidative stress, Parkinson's disease, Rotenone-induced Parkinsonism,*

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INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disorder marked by the selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), resulting in striatal dopamine deficiency and cardinal motor symptoms include bradykinesia, rigidity, resting tremor, postural instability along with non-motor features such as cognitive impairment, sleep disorders and autonomic dysfunction. It affects upto 2% of the people aged 60 years and above. The neuropathological hallmarks include neuronal loss in SNpc and formation of Lewy bodies rich in α-synuclein aggregates. [1-3]

According to the Global Burden of Disease Study 2021, the global prevalence of PD was approximately 11.77 million individuals, with age-standardized rates of incidence (15.63/100,000), prevalence (138.63/100,000) and disability-adjusted life years (DALYs) (89.59/100,000), all showing increasing trends over the past three decades. Projections indicate that by 2050, the number PD cases worldwide could rise to 25 million, reflecting a substantial increase in disease burden due to factors such as population aging and improved life expectancy. [4,5] In India, the estimated number of people living with PD in 2019 was about 7,71,000 with 45,000 deaths, reflecting a significant and growing public health burden.[6]

Although the precise etiology of remains unclear, mitochondrial dysfunction, oxidative stress, neuroinflammation and apoptotic/necrotic cell death are consistently implicated in both human disease and experimental models. One widely used model is rotenone-induced PD in rodents: rotenone is a lipophilic pesticide that inhibits mitochondrial complex I, leading to overproduction of reactive oxygen species (ROS), impaired ATP generation, activation of apoptotic pathways, α-synuclein aggregation, and dopaminergic neuronal loss in SNpc, with behavioral deficits reflective of PD. [7-9]

Current treatments for Parkinson's disease, such as levodopa and dopamine agonists, mainly address symptoms and do not halt neuronal degeneration. Therefore, there is a need for neuroprotective agents that can halt disease progression. Natural compounds like Carnosic acid (CA), a phenolic diterpene found in rosemary (Rosmarinus officinalis) and sage, show potential due to their antioxidant, anti-inflammatory, and mitochondrial-protective effects. CA is activated under oxidative stress, interacts with Keap 1 to stabilize and translocate Nrf2

(Nuclear factor erythroid 2-related factor 2), which then induces antioxidant and detoxifying genes (e.g., HO-1, NQO1), restoring redox homeostasis and potentially protecting dopaminergic neurons. [10-12]

CA exhibits neuroprotective effects by activating pathways, preserving mitochondrial function, reducing oxidative stress and protecting neurons from injury. While CA has shown benefits in PD models, such as 6-hydroxydopamine-induced neurotoxicity,[13] studies in rotenone-induced models, which closely mimic sporadic PD, are limited. This study aims to evaluate the effects of CA on behavioral and biochemical parameters in a rotenone-induced PD mouse model.

MATERIALS AND METHODS

Experimental Animals: Adult male Swiss albino mice, weighing 30-50 grams and aged 8-12 weeks, were obtained from Biogen animal facility in Bengaluru, India. The mice were housed individually in polypropylene cages and maintained under standard laboratory conditions, at a temperature of 25±5ºC and relative humidity of 45- 55% with a natural light/dark cycle and provided unrestricted access to food and water. Prior to the start of the experiments, the animals were acclimatized to the laboratory environment for seven days.

Ethical Approval: The experimental protocol involving animals was approved by the Institutional Animal Ethics Committee (Approval No. SU/CLAR/RD/003/2019), and all procedures were conducted in accordance with the guidelines of the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Chemicals: Rotenone was purchased from TCI (Plau, USA), while olive oil was obtained from Thuruthel Drug Lines, ERUMBA Pharmacy, Payyannur, Kerala, India. Levodopa was procured from SR Laboratories, Maharashtra, India; dimethyl sulfoxide (DMSO) from Pure Chemicals; and hydroxypropyl cellulose (HPC) from HIMEDIA, India. Carnosic acid was isolated as per the procedure in the Central Research laboratory (CRL) facility of Department of Research and Development, Saveetha Institute of Medical and Technical Sciences, Chennai, India. All other chemicals and reagents used in the study were of analytical grade.

Preparation of Rotenone stock solution: 50 mg rotenone was weighed and dissolved in 1 mL of DMSO. From this, an aliquot of 0.2 mL was taken and diluted with 19.8 mL of olive oil to prepare a stock solution.

Preparation of Carnosic acid and Levodopa treatment drugs: 100 mg of Carnosic acid was dissolved in 1 mL of DMSO. From this, an aliquot of 1 mL was taken and diluted with 9 mL of olive oil to prepare the stock solution. Levodopa was weighed 15mg and dissolved in 20mL of 10% of hydroxypropyl cellulose (HPC).

Experimental Design: The mice were divided randomly into seven groups with six animals in each group. The grouping was done one day prior to the start of experiments. The duration of the experiment (injection and oral dosing) was 21 days. The tail of each mouse was marked with non-toxic permanent marker pen for identification. All the animals were weighed individually and the animal body weights were recorded. Group-1 (Control) received olive oil (2mL/kg) by oral administration (p.o.) each day, Group-2 received 2.5 mg/kg rotenone by intraperitoneal route (i.p.) each day, Group-3 received Carnosic acid 20mg/kg p.o. along with 2.5 mg/kg rotenone everyday by i.p., Group-4 received Carnosic acid- 40mg/kg by p.o. along with 2.5 mg/kg rotenone everyday by i.p., Group-5 received Carnosic acid-20mg/kg and levodopa 7.5 mg/kg p.o. along with 2.5 mg/kg rotenone everyday by i.p., Group 6 received Carnosic acid-40mg/kg and levodopa 7.5mg/kg p.o. along with 2.5 mg/kg rotenone everyday by i.p. Group-7 received 7.5 mg/kg levodopa p.o. along with 2.5 mg/kg b.w (body weight) rotenone everyday by i.p. On day 22, animals were anesthetized with isoflurane and 0.25 mL of blood was collected retro-orbitally for serum analysis of triiodothyronine (T3), Nitric Oxide (NO), Lipoxygenase (LPX), total antioxidant capacity (TAC) and superoxide dismutase (SOD).

Estimation of Serum T3 levels: Blood collected in clot activator vacutainer tube was allowed to clot for 20 min at room temperature, then centrifuged at 3000 rpm for 10 min at 4 °C to obtain serum. About 150 µL of serum was used to measure T3 levels using BIOBASE ELISA kits on the IMMULITE 2000 immunoassay system (Siemens Healthcare Diagnostics, Chennai, India). [14]

Super oxide dismutase (SOD) assay: Tubes containing 0.5 mL of sample was brought to a volume of 2.5mL by adding 0.5 mL of 0.6mM EDTA and 0.5 mL of 300 mM carbonate buffer. The reaction was initiated by adding 0.4 mL of 1.8 mM adrenaline, and the increase in absorbance at 480 nm was recorded using a UV spectrophotometer. A control tube without the enzyme was used to measure 50% autoxidation of adrenaline to adrenochrome. Enzyme activity was expressed as units/minute/mg protein.[15]

Estimation of total antioxidant capacity (TAC): Serum TAC was estimated using the method of Benzie and Strain. The FRAP working solution was prepared by mixing 10 mM of 2,4,6-Tripyridyl-S-triazine (TPTZ) in 40 M HCL, 0.02 M $FeCl_3$, and acetate buffer pH 3.6. To this, 8 µL of serum and 240 µL of FRAP reagent were added and incubated for 10 min at room temperature. Absorbance was then measured at 593 nm and TAC was expressed as mmol/L. [16]

Estimation of Nitric Oxide (NO): Nitric oxide levels were estimated by the Griess reagent method.[17] Brain tissue homogenate (10% w/v in PBS, pH 7.4) was centrifuged at 10,000 rpm for 15 min at 4°C. To 100 µL of the supernatant, 100 µL of Griess reagent (1% sulfanilamide and 0.1% NED in 2.5% phosphoric acid) was added and incubated at room temperature for 10 min. Absorbance was measured at 540 nm, and nitrite concentration was calculated using a sodium nitrite standard curve and expressed as µmol/mg protein.

Estimation of Lipoxygenase (LOX): LOX activity was assayed as per Axelrod et al. [18] The reaction mixture contained 2.9 mL phosphate buffer (0.2 M, pH 6.5) and 0.1 mL linoleic acid (2.5 mM). The reaction was initiated by adding 0.1 mL enzyme extract, and the increase in absorbance was read at 234 nm for 3 min at 25°C. One unit of enzyme activity was defined as an increase in absorbance of 0.001 per min and expressed as units/mg protein.

Elevated Plus Maze (EPM): The EPM test is employed to evaluate the behaviour of rodents and has been accepted to measure anti-anxiety properties. It measures the time spent in open and closed arms, reflecting a conflict between their preference for safe, enclosed areas and innate motivation to explore novel environments. The test relies on the rodent's natural fear of heights, open spaces and preferences for dark, enclosed areas. The apparatus comprised of two open arms (51× 12 cm) and two closed arms (51 × 12 × 40.6 cm) extending from a central platform (10 × 10 cm), elevated 72 cm above the floor. Each animal was placed on the central

platform facing an open arm and allowed to explore freely for five minutes. The apparatus was cleaned with ethanol after each trial. Behaviour was video-recorded, and anxiety levels were assessed based on the number of entries into open and closed arms. The proportion of time spent in the closed arm was taken as an indicator of anxiety-like behaviour.

Open Field Test (OFT): The OFT measures and assesses general activity and exploratory behavior in both rats and mice. OFT measures both quality and quantity activity in animals. The apparatus, typically circular or square or rectangular enclosure, prevents escape and allows evaluation of locomotor and behavioral responses to various compounds. In this study, mice were placed at the center of a square arena (43 × 43 cm) and allowed to explore for five minutes. Time spent in resting, number of rearing's, and ambulatory movements were recorded. The arena was cleaned with ethanol after each trial.

STATISTICAL ANALYSIS

Results were expressed as mean ± standard error of mean (SEM). Group comparisons were performed using one-way analysis of variance (ANOVA), followed by Bonferroni multiple comparison post-hoc test to determine differences between treatment groups. A p-value <0.05 was considered statistically significant.

RESULTS

Table 1. Protective effect of Carnosic acid on rotenone induced changes in tri-iodothyronine (T3), Nitric oxide (NO) and lipoxygenase (LPX) Values are mean + SE (n = 6 each).

Group	Tri-iodothyronine (ng/dL)	Nitric oxide (µ mol)	Lipoxygenase (LPX) (units/mg)
Control	156.8 ± 6.14	15.95 ± 2.08	1.24 ± 0.11
Rotenone	82.61 ± 7.42	19.82 ± 1.44	3.12 ± 0.22
Rotenone+ Carnosic acid 20mg/kg	93.89 ± 4.49	21.39 ± 1.13	2.34 ± 0.07
Rotenone+ Carnosic acid 40mg/kg	104.52 ± 4.86	18.05 ± 1.48	2.12 ± 0.10
Rotenone+ Carnosic acid 20mg/kg + Levodopa	120.11 ± 5.95	13.25 ± 1.05	1.72 ± 0.08
Rotenone+ Carnosic acid 40mg/kg+ Levodopa	138.62 ± 7.17	11.93 ± 1.23	1.34 ± 0.08
Rotenone+ Levodopa	114.18 ± 2.29	14.39 ±3.38	1.85 ± 0.10

ANALYSIS OF SERUM TRIIODOTHYRONINE (T3), NITRIC OXIDE (NO) AND LIPOXYGENASE (LPX)

The reference range for total T3 in normal mouse is 0.08-0.18 µg/dL. The mean and SE of T3 (ng/dL) for 1 to 7 groups were 156.8 ± 6.14, 82.61 ± 7.42, 93.89 ± 4.49, 104.52 ± 4.86, 120.11 ± 5.95, 138.62 ± 7.17 and 114.18 ± 2.29 respectively. The mean and SE of Nitric oxide (µ mol) for the 1 to 7 groups were 15.95 ± 2.08, 19.82 ± 1.44, 21.39 ± 1.13, 18.05 ± 1.48, 13.25 ± 1.05, 11.93 ± 1.23 and 14.39 ±3.38. The mean and SE of Lipoxygenase (LPX) (units/mg) for the groups were 1.24 ± 0.11, 3.12 ± 0.22, 2.34 ± 0.07, 2.12 ± 0.10, 1.72 ± 0.08, 1.34 ± 0.08 and 1.85 ± 0.10 (Table 1). In the present study, serum T3, nitric oxide (NO) and Lipoxygenase (LPX) levels showed significant alterations following rotenone administration. Rotenone treated mice (group 2) showed a marked reduction in T3 levels compared to the control group, representing a 1.9-fold decrease ($p<0.05$). Treatment with Carnosic acid alone (groups 3 and 4) showed partial restoration of T3 levels, while combination therapy with Carnosic acid + Levodopa (groups 5 and 6) demonstrated a pronounced improvement. Group 6 exhibited only a 1.1-fold decrease relative to control, indicating substantial recovery.

Serum NO levels were significantly elevated in the PD group (group 2) compared to control ($p<0.05$), confirming oxidative/nitrosative stress induced by rotenone. Carnosic acid at 20 mg/kg slightly increased NO, while the 40 mg/kg dose moderately reduced it. Notably, combined Carnosic acid + Levodopa (groups 5 and 6) significantly lowered NO levels compared to the PD group, approaching near-normal values. LPX levels also increased sharply in the PD group compared to control ($p<0.001$), indicating lipid peroxidation. Carnosic acid alone reduced LPX activity, while combination of Carnosic acid with Levodopa resulted in the most significant improvement, with group 6 showing near-normal LPX values. Levodopa alone also significantly improved all parameters compared to PD. Overall, rotenone induced significant alterations in T3, NO and LPX levels, while treatment with Carnosic acid especially in combination with Levodopa effectively reversed these biochemical disruptions, demonstrating strong neuroprotective and antioxidant effects.

Table 2. Protective effect of Carnosic acid on rotenone induced changes in total anti-oxidant Capacity (TAC) and superoxide dismutase (SOD). Values are mean + SE (n = 6 each).

Group	Total anti-oxidant capacity (mmol/L)	Superoxide dismutase (µmol/mL)
Control	2.12 ± 0.29	5.24 ± 0.63
Rotenone	0.92 ± 0.10	1.28 ± 0.63
Rotenone+ Carnosic acid 20mg/kg	1.24 ± 0.01	4.32 ± 0.96
Rotenone+ Carnosic acid 40mg/kg	1.52 ±0.05	4.48 ± 0.53
Rotenone+ Carnosic acid 20mg/kg + Levodopa	1.82 ± 0.001	4.75 ± 0.70
Rotenone+ Carnosic acid 40mg/kg+ Levodopa	2.02 ± 0.16	5.15 ± 0.88
Rotenone+ Levodopa	1.75 ± 0.02	4.62 ± 0.38

ANALYSIS OF SERUM TOTAL ANTIOXIDANT CAPACITY (TAC) AND SUPEROXIDE DISMUTASE (SOD) LEVELS

The mean ± SE of Total Antioxidant Capacity (mmol/L) for 1 to 7 groups were 2.12 ± 0.29, 0.92 ± 0.10, 1.24 ± 0.01, 1.52 ±0.05, 1.82 ± 0.001, 2.02 ± 0.16 and 1.75 ± 0.02 respectively. The mean ± SE of Superoxide Dismutase (SOD) (µmol/mL) for 1 to 7 groups were 5.24 ± 0.63, 1.28 ± 0.63, 4.32 ± 0.96, 4.48 ± 0.53, 4.75 ± 0.70, 5.15 ± 0.88 and 4.62 ± 0.38. One-way ANOVA showed a significant difference in TAC and SOD levels between the groups ($p < 0.05$). Rotenone-treated mice (Group 2) demonstrated a 56.6% reduction in TAC and a 75.6% reduction in SOD levels compared to the control group, indicating marked oxidative stress.

Treatment with Carnosic acid alone (Group 3 and 4) significantly increased TAC and SOD levels compared to Group 2 ($p<0.05$), with group 4 (40mg/kg) showing greater restoration. The combination therapies (Group 5 and 6) showed even more pronounced improvements. Group 6 (Carnosic acid 40 mg/kg + Levodopa) showed TAC and SOD levels nearly comparable to control, with only 4.7% decrease in TAC and 1.7% decrease in SOD relative to Group 1. Thus, combined Carnosic acid and Levodopa treatment markedly attenuated rotenone-induced oxidative damage, showing statistically significant restoration of both TAC and SOD levels.

Table 3. Protective effect of Carnosic acid and its combination with L-DOPA on rotenone- induced anxiety behavioural changes in mice using EPM. Values are mean + SE (n = 6 each).

Group	Closed arm Time in Seconds	Open arm Time in Seconds	Center Time in Seconds	Standing Time in Seconds
Control	31.3 ± 5.45	55.5 ± 5.28	33.1 ± 8.20	2.5 ± 0.22
Rotenone	101 ± 3.13	12.6 ± 2.6	4.6 ± 1.2	0.9 ± 0.08
Rotenone+ Carnosic acid 20mg/kg	91 ± 2.2	19.3 ± 2	9.6 ± 1.4	1.1 ± 0.16
Rotenone+ Carnosic acid 40mg/kg	88.3 ± 2.02	19.3 ± 1.5	12.1 ± 1.7	1.3 ± 0.2
Rotenone+ Carnosic acid 20mg/kg + Levodopa	83.6 ± 1.89	21 ± 0.8	15.3 ± 2.4	1.1 ± 0.16
Rotenone+ Carnosic acid 40mg/kg+ Levodopa	49.6 ± 2.9	46.3 ± 1.6	24 ± 4	2 ± 0.2
Rotenone+ Levodopa	84.6 ± 1.9	27.3 ± 1.7	8 ± 1.4	1.1 ± 0.16

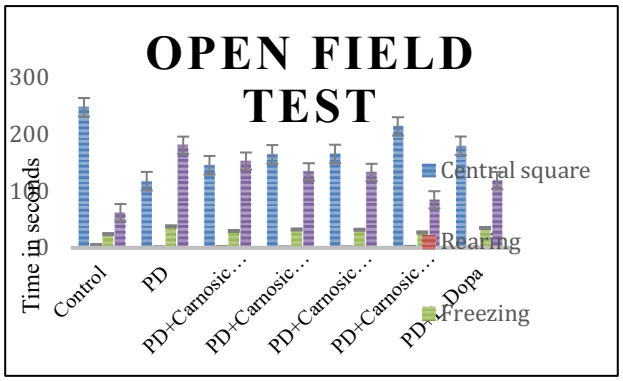
ANXIETY ASSESSMENT VIA ELEVATED PLUS MAZE (EPM)

There was a significant increase ($p<0.001$) in anxiety behaviour in the rotenone-treated PD mice compared to the control group, as evidenced by the marked in closed-arm time from 31.3 ± 5.45 seconds in controls to 101 ± 3.13 seconds in the PD group, representing a 3.22-fold increase. There was a pronounced decrease in open-arm time from 55.5 ± 5.28 seconds to 12.6 ± 2.6 seconds corresponding to a 4.40-fold reduction. Following the treatment with the

combination of Carnosic acid 40 mg/kg + Levodopa showed the most notable improvement, with closed-arm time decreasing to 49.6 ± 2.9 seconds and open-arm time increasing to 46.3 ± 1.6 seconds, which was statistically significant compared to the PD group ($p<0.001$). Compared to the control group, this treatment group showed only 1.58-fold increase in closed-arm time and a 1.20-fold decrease in open-arm time, indicating substantial reversal of anxiety-like behaviour compared to untreated PD mice. These findings confirm that the combination of Carnosic acid 40 mg/kg and Levodopa produced the most effective anxiolytic response among the treated groups (Table 3 and Figure 3).

Table 4. Protective effect of Carnosic acid and its combination with L-DOPA on rotenone- induced anxiety behavioural changes in mice using OFT. Values are mean + SE (n = 6 each).

Group	Central square duration in Seconds	Rearing in Seconds	Freezing in Seconds	Peripheral square duration in Seconds
Control	249 ± 8.4	5.8 ± 1	24.3 ± 2.5	62.3 ± 8.38
Rotenone	118.1 ± 5.1	2.1 ± 0.4	38.1 ± 4.4	181.8 ± 5.1
Rotenone+ Carnosic acid 20mg/kg	146.3 ± 6	2.3 ± 0.4	29.8 ± 3.2	153.6 ± 6
Rotenone+ Carnosic acid 40mg/kg	165.6 ± 11	2 ± 0.3	32.5 ± 2.3	134.6 ± 11.1
Rotenone+ Carnosic acid 20mg/kg + Levodopa	166.3 ± 4.6	2 ± 0.3	32 ± 1.8	133.6 ± 4.6
Rotenone+ Carnosic acid 40mg/kg+ Levodopa	215 ± 14.6	2.8 ± 0.4	27.3 ± 2.9	85 ± 14.6
Rotenone+ Levodopa	180.8 ± 8.7	1.8 ± 0.3	35.1 ± 1.9	119.1 ± 8.7



ASSESSMENT OF ANXIETY BEHAVIOUR USING THE OPEN FIELD TEST (OFT)

The OFT was used to assess exploratory activity, locomotion, and anxiety-like behaviour in rodents. Rotenone-treated PD mice showed a notable reduction in locomotion and enhanced anxiety compared to control. These mice exhibited a 2.1-fold decrease in central square duration (249 s to 118.1 s), indicating heightened anxiety. The group receiving Carnosic acid 40 mg/kg + Levodopa showed only a 1.16-fold decrease (215 s) reflecting a protective effect. Similarly, peripheral square duration increased 2.92-fold in the rotenone group (62.3 s to 181.8 s), demonstrating strong anxiety behaviour, whereas the same treatment group showed only 1.36-fold increase (85 s). Freezing time was also elevated in rotenone-treated mice, showing a 1.57-fold increase (24.3 s to 38.1 s), while the combination treatment showed only a 1.12-fold increase (27.3 s), indicating reduced anxiety. Overall, treatment groups, particularly those receiving Carnosic acid with Levodopa, demonstrated improved behavioral outcomes compared to rotenone group. It highlights their potential anxiolytic and neuroprotective effects and all results were statistically significant ($p<0.001$) as determined by one-way ANOVA with Bonferroni post-hoc analysis.

DISCUSSION

The present study demonstrates that Carnosic acid (CA) provides strong neuroprotective effects against rotenone induced PD in mice. Rotenone produced clear biochemical and behavioural features of PD, which is consistent with established literature describing its ability to inhibit mitochondrial complex-I, increase reactive oxygen species, impair OTP production, trigger α-synuclein aggregation and inhibit dopaminergic neurodegeneration.[7] CA treatment, alone and more profoundly in combination with levodopa, effectively reduced these harmful effects. These findings are consistent with earlier research showing that CA's antioxidant, mitochondrial-protective and Nrf2-activating effects in experimental models of neurodegeneration. [11,12]

A notable finding in this study was the decrease in serum T3 levels caused by rotenone and their restoration after treatment with CA and CA + levodopa combination. Although the

relationship between thyroid hormones and dopaminergic function in PD have been inconsistently reported, some clinical studies have showed reduced T3 levels in PD patients, particularly in those with akinetic-rigid symptoms. [19] The present results also consistent with the study by Koppal A et al. [20], which mention altered thyroid hormone patterns in PD and their correction with effective neuroprotective therapies. Since mitochondrial dysfunction affects overall metabolic processes, the restoration of T3 levels with CA treatment may indicate a broader systemic benefit through improved mitochondrial function and reduced oxidative stress. This is consistent with the previous literature highlighting CA's mitochondrial-protective functions. The greater improvement with CA + levodopa suggests that dopaminergic activity may stabilize hypothalamic-pituitary-thyroid axis regulation. [11,21]

Rotenone-exposed mice in the present study showed elevated nitric oxide (NO) and lipoxygenase (LPX) levels, indicating nitrosative stress and lipid peroxidation. Elevated NO is commonly seen in PD models due to microglial activation and iNOS induction [9], while increased LPX reflects excessive oxidation of polyunsaturated fatty acids in degenerating dopaminergic neurons [22,23]. The significant decrease in NO and LPX levels after CA and CA + Levodopa treatment highlights CA's ability to inhibit pro-oxidant enzymes and restore membrane integrity. This antioxidant effect is consistent with CA's known modulation of Nrf2-regulated cytoprotective genes.[10] These results also match the findings from the study by Koppal A et al. [20], where antioxidant treatments restore oxidative stress markers, reinforcing that phytochemicals with mitochondrial benefits offer broader neuroprotection in PD.

Consistent with previous reports, rotenone administration caused severe oxidative stress, reflected by a significant reduction in Total antioxidant capacity (TAC) and superoxide dismutase (SOD) levels. This pattern is well-documented, as rotenone and similar mitochondrial toxins markedly impairs endogenous antioxidant defenses, contributing to dopaminergic vulnerability.[8] These results also match with findings described by Rao SP et al. [24], where MPTP- induced oxidative stress lowered TAC and SOD levels in PD before they were treated with embelin. CA effectively restored both TAC and SOD levels, with 40 mg/kg dose showing stronger protective effect, suggesting robust free-radical scavenging and helping maintain normal mitochondrial activity. This is consistent with Wu et al. [13], who reported that CA increases SOD and catalase activities while reducing oxidative damage in 6-OHDA-induced Parkinson's disease. The enhanced improvement with CA + Levodopa combination may be due to improved dopaminergic transmission with CA's mitochondrial protection, reflecting the advantages described in other combination-therapy studies. [25,26]

Behaviorally, rotenone induced clear anxiety-like symptoms in both the Elevated Plus Maze (EPM) and Open Field Test (OFT), consistent with previous reports showing that chronic rotenone exposure impairs locomotor and emotional pathways and increases oxidative damage in brain regions involved in anxiety and mood regulation. [1] Treatment with CA significantly improved and corrected these behavioural abnormalities. The combination of CA 40 mg/kg with levodopa produced responses close to normal, such as increased open-arm exploration, reduced freezing time and greater central-zone exploration. These observations are similar to findings from other phytochemical-based neuroprotection studies, where antioxidant compounds reduced anxiety by restoring neurotransmitter balance and lowering oxidative stress. [27-29] The present results also align with previous findings where CA enhances synaptic function, reduces neuroinflammation and improves both motor and emotional behaviors by activating the Nrf2 pathway and suppressing pro-inflammatory cytokines. [12]

Mechanistically, the overall benefits of CA in this study can be attributed to its multi-target actions. Under oxidative stress, CA is converted into electrophilic quinone derivatives that interact with Keap1, allowing Nef2 to stabilize and translocate to the nucleus, where it activates detoxifying enzymes such as HO-1 and NQO1, thereby strengthening cellular antioxidant defenses. [10] CA also helps to maintain mitochondrial membrane potential, supports mitochondrial biogenesis and reduces microglia-driven inflammation, all of which are essential in counteracting rotenone-induced neurotoxicity. These mechanisms are consistent with earlier studies on CA and other plant-based antioxidants, which highlight the value of phytochemicals in targeting underlying degenerative pathways rather than only replacing dopamine. [10,11,30]

The improved outcomes seen with CA + levodopa indicate a pharmacological synergy. While levodopa restores dopamine, its long-term use can increase oxidative stress; CA counteracts this by strengthening antioxidant defenses and supporting mitochondrial function. This action explains the near-normal biochemical and behavioural results in the combination groups.

CONCLUSION

The present work illustrates that Carnosic acid provides neuroprotective effects against rotenone-induced Parkinsonism by restoring antioxidant balance, reducing nitrosative and lipid peroxidation damage, normalizing thyroid hormone disturbances and improving anxiety-related behavioural deficits. The combination of Carnosic acid with Levodopa showed more pronounced biochemical and behavioral improvements, suggesting a strong synergistic effect. Overall, Carnosic acid seems to be a promising adjuvant therapeutic approach that can target multiple pathogenic processes in Parkinson's disease, thereby offering a broader protective effect compared to dopaminergic therapy alone.

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