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Original Article

Novel approaches to improve systemic bioavailability of curcumin using probiotics for rotenone-induced Parkinson's disease in rodents

Arun Soni[1,2], Adarsh Patel[2], Sanjeev Acharya[2,3], Alkesh Patel[1], Umang Shah[1], Swayamprakash Patel[1], Nilay Solanki[1], Mehul Patel[1]

[1]Ramanbhai Patel College of Pharmacy, Charotar University of Science and Technology, CHARUSAT Campus, Changa, Anand, Gujarat 388421, India; [2]Department of Pharmacology, SSR College of Pharmacy, Sayli-Silvassa Road, Union Territory of Dadra and Nagar Haveli and Daman Diu, Sayli, Silvassa 396230, India; [3]Department of Pharmacognosy, Shree S. K. Patel College of Pharmaceutical Education and Research, Ganpat University, Ganpat Vidhyanagar, Mehsana-Gandhinagar Highway, Kherva, Gujarat 384012, India

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Abstract: Background and Aim: The prevalence of Parkinson's Disease (PD) is high, and treatment is not optimal to date. Curcumin possesses neuroprotective effects. Nevertheless, oral use is incommodious due to its poor bioavailability. Numerous attempts have been made to increase its systemic bioavailability. Among these, an effective way to increase the bioavailability of curcumin is by combining it with probiotics to target the glucuronidation reaction. The present study focuses on the bio-enhancement of curcumin using probiotics in animal models of PD by alleviating oxidative stress and ameliorating dopamine levels. Materials and Methods: Forty-two male rats were used for the study. Twelve animals were used for a bio-availability study in which curcumin was administered orally alone and concomitantly with a probiotic (*Lactobacillus Rhamnus,* 10^9 *cfu,* PO) to prove probiotics' ability to enhance curcumin's serum level by inhibition of β-glucuronidase activity. Serum curcumin level was estimated using the LC-MS/MS technique on 21 days of dosing. The remaining animals were used in an experimental study. PD was induced through 2.5 mg/kg rotenone (ROT). Subsequently, the animals were allocated to five groups and treated commensurately along with ROT. Three treatment groups were administered curcumin (alone, with 10^8 *cfu,* with 10^9 *cfu*). The standard control and disease control groups were supplied with sunflower oil. Effect on behavioral patterns, neurotransmitter and enzyme levels, and oxidative stress parameters were measured. Moreover, the brain was isolated for histopathology. Results and Conclusions: The bioavailability study revealed a significant (*P*-value <0.001) increase in the serum level of curcumin in concomitantly administered probiotics with curcumin-treated animals compared to curcumin-only treated animals. An experimental study showed improved behavioral parameters, brain dopamine level, acetylcholinesterase (AchE), and oxidative stress combined in the curcumin and *L. Rhamnosus* treated groups. A notable improvement in the histology of the brain was observed. These findings strongly indicate that combining *L. Rhamnosus* with curcumin may be an effective therapeutic solution.

Keywords: Parkinson's disease, β-glucuronidase activity, curcumin, rotenone, oxidative stress, neurotherapeutic

Introduction

The second most common disease after Alzheimer's disease, Parkinson's disease (PD), is a progressive neurological movement condition. PD is characterized by nigro-striatal dopaminergic neuron loss, which eventually causes striatal dopamine (DA) loss [1]. When nigro-striatal neurons die, common motor symptoms show up, including unstable posture, stiff muscles, resting tremors, and slow movement [2]. By 2030, it is expected that there will be almost 16.5 million PD patients in the most populated Asian nations [3]. Currently, in India, around 328 out of 100,000 people are suffering from PD [4], and the numbers are estimated to increase in the next few years.

Various factors, like excessive ROS levels, environmental neurotoxins, genetic factors, and endogenous toxins, may be responsible for potentiating the risk of PD [5]. Numerous ani-

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mal models can reproduce the pathological findings seen among PD patients. Among them, rotenone is a selective mitochondrial complex-I inhibitor that damages nigrostriatal dopaminergic neurons and models the induction of PD in rats [6]. It causes DA neuronal death, impaired function, and elevation in α-synuclein levels [7, 8]. Moreover, microglial activation strongly contributes to DA neuronal loss, as supported by various experimental studies [9]. Presently, few drugs inhibit the progression of PD, and the currently used therapeutic drugs often possess several side effects [10]. Thus, natural substances with significant neuroprotective properties, like curcumin, may be able to attenuate or reverse the neurodegenerative process in PD [11].

A polyphenolic substance called curcumin, or diferuloylmethane, is derived from the rhizomes of the *Curcuma longa* plant [12]. It also exhibits significant anti-inflammatory, free radical scavenging, mitochondrial protective, and antioxidant properties, making it a possible treatment for PD [11, 12]. However, despite its potency and wide range of medical applications, the therapeutic use of curcumin is highly restricted due to its low oral bioavailability. Much research on pharmacokinetics showed that the liver and intestines break down curcumin into glucuronide and sulfate metabolites, lowering blood levels and overall body bioavailability [13]. An effective natural way to enhance the bioavailability of curcumin is by targeting its metabolic pathway using probiotics. Beta-glucuronidase is an enzyme that hydrolyses the curcumin metabolite into its active form. It disrupts enzymatic metabolism, allowing curcumin to recirculate into the bloodstream and exert its effect. Probiotics can help modulate beta-glucuronidase activity, leading to increased levels of active curcumin in the body. This approach has shown promise for improving the therapeutic potential of curcumin as a natural remedy [14]. Bacterial sources like *L. Rhamnosus* synthesise the β-glucuronidase enzyme in the intestine, inhibiting the rapid metabolism and thus enhancing the systemic bioavailability of curcumin by several fold. Combining curcumin with probiotics may also help reduce gastrointestinal side effects often associated with high doses of curcumin supplementation. This synergistic approach could offer a more effective and tolerable way to harness the therapeutic benefits of curcumin [15].

The present investigation aims to determine the β-glucuronidase activity in *Lactobacillus Rhamnosus* species and evaluate the impact of curcumin combined with the probiotic *L. Rhamnosus* on rotenone-induced Parkinson's disease in SD rats. Additionally, the enhancement of the systemic bioavailability of curcumin by *L. Rhamnosus* was evaluated using the LC-MS/MS method. The results of this study may provide valuable insight into this combination for the treatment of neurodegenerative diseases like Parkinson's. Further research in this area may lead to the development of novel treatments that are both effective and well-tolerated.

Materials and methods

Chemicals and reagents

In India, *L. Rhamnosus* was procured from Unique Biotech Ltd. Standard curcuminoids (purity 95%) were purchased from Natbiome Specialty Healthcare Pvt. Ltd., Ahmedabad, India. Rotenone was procured from Tokyo Chemical Industries Co., Ltd. Substrate (Phenolphthalein-β-D-glucuronide) was procured from Sigma Aldrich. Analytical-grade chemicals were used for the study. All the chemical solutions and drugs were freshly prepared before use.

Experimental animals

Forty-two male Sprague Dawley rats from the Zydus Research Center in Ahmedabad were obtained for the experiment. Their weights ranged from 150 to 180 g. The Institutional Animal Ethics Committee of the SSR College of Pharmacy in Silvassa, Dadra, Nagar Haveli, Daman, and Diu approved the study (Protocol No. SSR/IAEC/2021/030). The rats were housed in standard laboratory conditions with a 12-hour light/dark cycle and had *ad libitum* access to food and water. All procedures were conducted according to the guidelines set by the Committee for Control and Supervision of Experiments on Animals (CPCSEA). All the animals in the study were euthanized with CO_2 asphyxiation technique.

Determination of viability and structure of L. Rhamnosus

A concentrated culture of *L. Rhamnosus* was prepared by suspending 0.1 g of bacteria in

MRS broth. The suspension was diluted 100 times and then added to test tubes. The suspension was mixed and incubated in a pre-calibrated incubator for 24 to 48 hours. The bacteria were then evaluated for morphologic appearance using the heat and fix method under an oil-immersion objective lens. The process was repeated multiple times to obtain the desired bacterial suspension.

Determination of fecal β-glucuronidase activity

The study used the phenolphthalein method to assess enzyme activity in a color reaction. Deionized water was used to mix the enzyme supernatant, potassium phosphate buffer, bovine serum albumin, and phenolphthalein glucuronide substrate solution to carry out the reaction. The amount of phenolphthalein released was calculated using a standard curve, and the fecal protein concentration was determined using the Biuret method [16-19].

Bioavailability investigation of curcumin by LC-MS/MS

LC-MS/MS analysis was done to investigate the bioavailability of curcumin. A total of 12 animals were divided into four categories, each having three animals. Group I (Control group) received saline. Group II (LR) was administered *L. Rhamnus* (10^9 CFU), Group III (CUR) was administered curcumin (1000 mg/kg), and Group IV (CUR + LR) was administered curcumin (1000 mg/kg) in conjunction with *L. Rhamnus* (10^9 CFU). After the 11th day of administration, blood samples were collected and centrifuged at 3000 g for 5 minutes. LC-MS/MS assessed the serum curcumin level in the separated serum following centrifugation [20].

Experimental design

A total of thirty rats were used for the study. Every group except the control group received rotenone every day for 21 days at a dose of 2.5 mg/kg SC, dissolved in sunflower oil [21]. The animals were divided into five groups: Group I (C) received only sunflower oil through IP; Group II (Ro) received rotenone; Group III (Cu) received rotenone along with curcumin (500 mg/kg); Group IV (CuLR-L) received rotenone along with curcumin (500 mg/kg) and LR (0.1×10^9 CFU); and Group V (CuLR-H) received rotenone along with curcumin (500 mg/kg) and LR (1×10^9 CFU).

Physical findings

Behavioral findings: Various tests, such as the hanging wire test [22], akinesia [23, 24], and catalepsy [25, 26], were performed to check neurological changes during and after the induction of PD and curcumin treatment.

Neurochemical findings: All neurochemical measurements, like dopamine level [27] and acetylcholinesterase activity [28], were performed using brain homogenate at 10% w/v. It was produced in 0.1 M phosphate buffer (pH 7.4). For an estimation by a neuro-biochemical assay, the homogenate was centrifuged at 15,000 rpm for 10 to 15 minutes [26]. Additionally, anti-oxidants like superoxide dismutase (SOD) [29], catalase [30], reduced glutathione (GSH) [29, 30], and lipid peroxidation (LPO) [29-32] were measured.

Histopathologic examination: The removed brains were cleaned in a 10% formaldehyde solution and stored in a cold phosphate buffer. Hematoxylin and eosin staining was used to evaluate the histopathology of brain sections.

Statistical analysis

Graph Pad Prism version 5.0 was used to analyze the data, expressed as mean ± SEM. A two-way analysis of variance was used to estimate behavior measurements at various time points, followed by Bonferroni's comparison test, and a one-way analysis of variance was used to assess neurochemical measurements.

Results

Evaluation of β-glucuronidase activity in L. Rhamnosus

Animals in group II that were given *L. Rhamnosus* had significantly ($P<0.05$) higher levels of phenolphthalein released than animals in the control group. This shows that *L. Rhamnosus* has β-glucuronidase activity (**Figure 1**). Compared to the control group, the amount of phenolphthalein released with CUR alone or combined with LR was not significantly elevated ($P<0.05$).

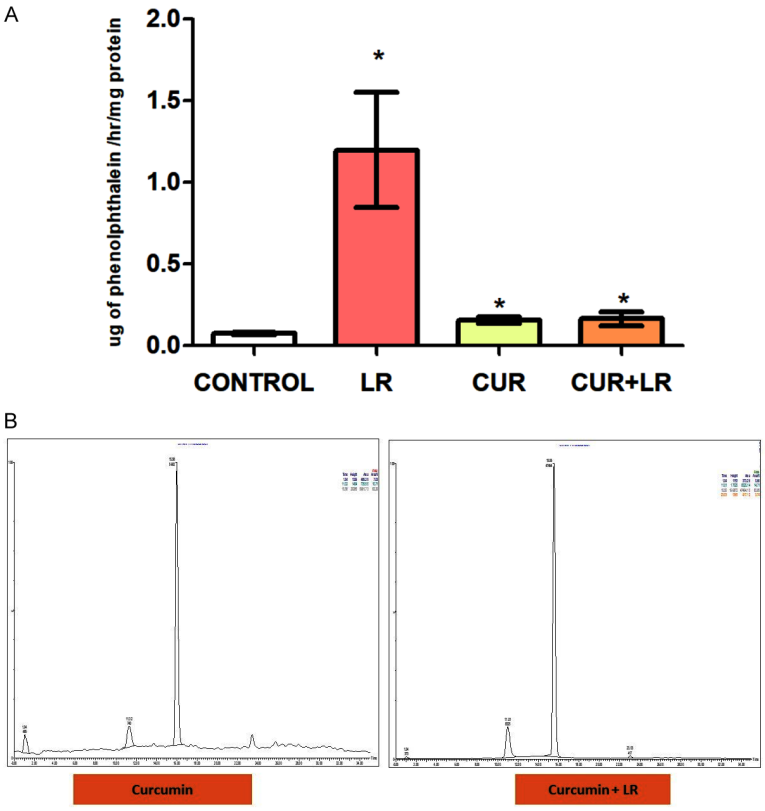

Figure 1. A. β-glucuronidase activity in rat fecal samples (The data is the mean ± SEM (n = 3). One-way ANOVA followed by Tuckey's test. * denotes P≤0.05 when compared with the CUR and CUR + LR groups); B. LCMS chromatograms of serum curcumin levels.

Determination of curcumin bioavailability using LCMS-MS

Data were used to construct pharmacokinetic curves of the curcumin concentration vs. time. The total bioavailability of curcumin using the LCMS-MS technique indicates that the animals who received Curcumin with L. *Rhamnosus* had more area (47464.10) than curcumin alone (5681.73). This reflects a 7-fold increase, depicting that *L. Rhamnus* enhances the bioavailability of curcumin (**Figure 1**).

Evaluation of behavioral tests

Hanging wire test: The hanging time in the ROT-induced group was significantly lower than that of the normal control group (**Figure 2**). In the treated groups, such as CUR, CUR + LR (LD), and CUR + LR (HD), the hanging time significantly increased compared to the ROT group.

Akinesia: The akinetic time in the ROT-induced group was considerably higher than of the normal control group. In the treated groups, such as CUR, CUR + LR (LD), and CUR + LR (HD), the akinetic time was significantly decreased compared to the ROT group (**Figure 2**).

Catalepsy: The cataleptic time in the ROT-induced group was considerably longer than of the standard control group. However, it was significantly shorter in the treatment groups, such as CUR, CUR + LR (LD), and CUR + LR (HD), compared to the ROT group (**Figure 2**).

Evaluation of neurochemical measurements

Dopamine level: Compared to the standard control group of rats, the level of dopamine was considerably ($P<0.05$) reduced after administration of Rotenone. The levels of dopamine in the CUR alone, CUR plus LR (LD) ($P<0.05$), and CUR plus LR (HD) ($P<0.05$) groups were much higher than those of the ROT group after therapy (**Figure 3**).

Effect on acetylcholinesterase (AChE) activity: Comparing the ROT group to the control group, the AChE levels were significantly higher ($P<0.05$) in the ROT group. When compared to the ROT group, AChE levels went down in the CUR treatment ($P<0.05$), CUR + LR (LD) ($P<0.05$), and CUR + LR (HD) ($P<0.05$) groups (**Figure 4**).

Evaluation of anti-oxidant parameters

SOD level: When compared to the control group, administration of ROT significantly ($P<0.05$) reduced the amount of SOD in the ROT group. In comparison to the ROT group of rats, the treatments with CUR ($P<0.05$), CUR + LR (LD) ($P<0.05$), or CUR + LR (HD) ($P<0.05$) raised the SOD levels (**Figure 5**).

Effect on catalase activity: Compared to the standard control group of animals, the level of

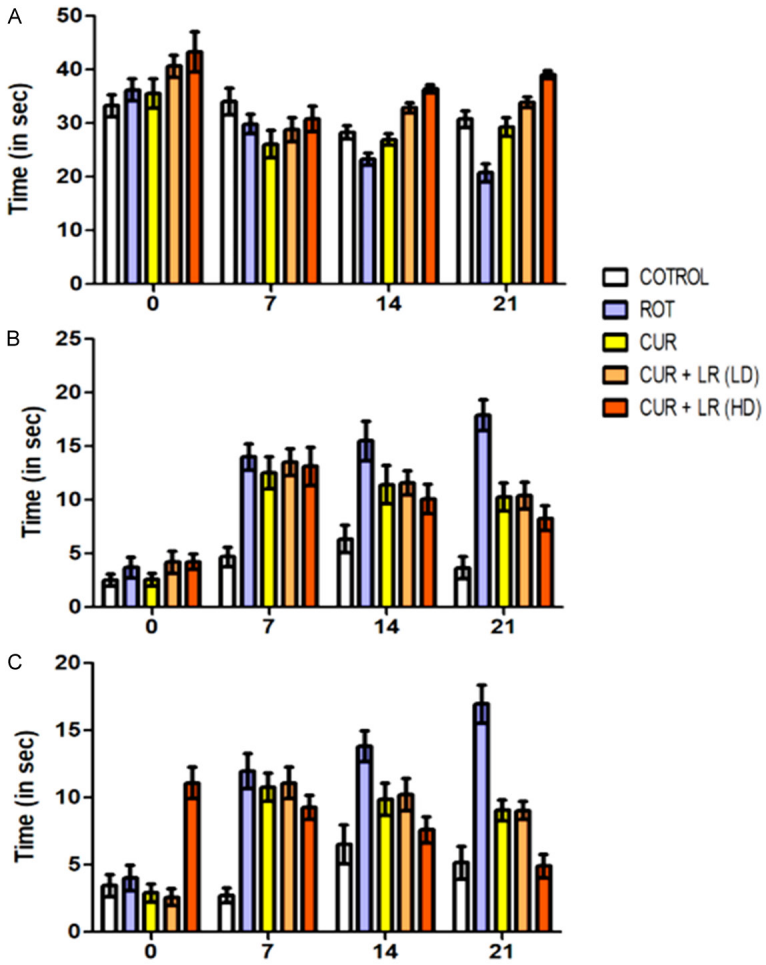

Figure 2. Behavioral tests. A. Hanging wire test; B. Akinesia; C. Catalepsy. Data are presented as mean ± SEM (n = 6). A two-way ANOVA followed by Bonferroni's test was applied to check statistical difference.

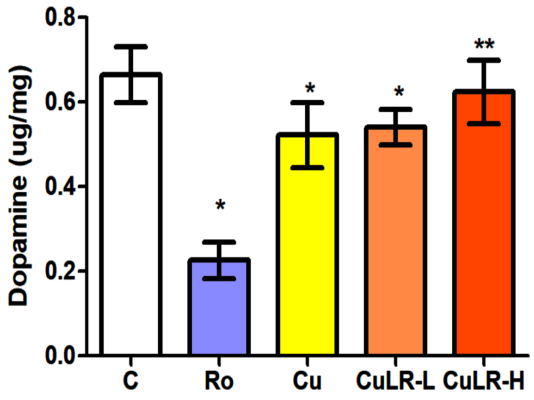

Figure 3. Brain dopamine level. Data are presented as mean ± SEM (n = 6). A one-way ANOVA followed by Tukey's test was applied. *, ** denote P≤0.05 and P≤0.01, respectively, when compared with the ROT group.

CAT was considerably (P< 0.05) lowered after receiving ROT. CAT levels were much higher in the CUR alone, CUR plus LR (LD) (P<0.05), and CUR plus LR (HD) (P<0.05) groups than in the ROT group after treatment (**Figure 5**).

Effect on reduced GSH activity: Compared to the standard control group of animals, the level of GSH was considerably (P<0.05) lower after receiving ROT. However, levels of GSH were much higher in the CUR alone, CUR plus LR (LD) (P<0.05), and CUR plus LR (HD) (P<0.05) groups than in the ROT group. This is shown in **Figure 5**.

Effect on lipid peroxidation activity (TBARS): The level of TBARS was significantly (P< 0.05) higher in the animals given ROT compared to the control group. When compared to the ROT group, the treatment with CUR alone (P<0.05), together with LR (LD) (P<0.05), but not CUR + LR (HD) (P<0.05), considerably lowered the level of MDA toward normal (**Figure 5**).

Histopathologic study

The histopathological images were recorded under 400× (40× with a scale bar of 10 μm). According to **Figure 6**, the control group (A) had regular histological features. The rotenone-treated animals (B) displayed the presence of dark and well-differentiated eosinophilic lesions in the midbrain of rats. The number of eosinophilic lesions in the CUR-treated group (C), CUR + LR (LD)-treated group (D), and CUR + LR (HD)-treated group (E) was much lower as compared to the ROT-treated group, as shown in **Figure 6**. The CUR + LR (HD)-treated group showed maximum protection against rotenone-induced eosinophilic lesions. This group displayed lighter, smaller, fewer, and more dispersed lesions than the rotenone-treated

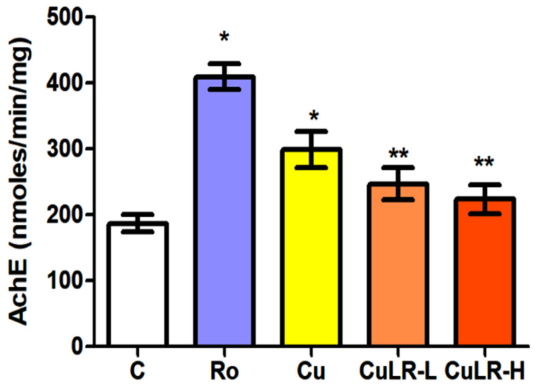

Figure 4. Brain AchE activity. Data are presented as mean ± SEM (n = 6). A one-way ANOVA followed by Tukey's test was applied. *, ** denote P≤0.05 and P≤0.01, respectively, when compared with the ROT group.

group. Moreover, eosinophilic lesions are less with the treatment when compared to the ROT-treated group. Eosinophilic lesions are shown as arrows in the histology of the brain.

Discussion

Parkinson's disease (PD) is a widespread neurodegenerative motor condition that affects a considerable proportion of the world's population. The nigrostriatal region's dopaminergic neurons degenerate, decreasing motor and behavioral function, which is the cause of the condition. This study shows that curcumin and the probiotic *L. Rhamnosus* can protect neurons in rats that have been given rotenone, which causes PD. Despite its diverse therapeutic activity, curcumin's clinical use is highly restricted owing to its poor oral bioavailability due to rapid liver and intestinal metabolism [33]. The main enzyme, UDP-glucuronosyl transferase, breaks down curcumin into its byproducts, which makes it less available in the body [34]. To compensate, a curcumin adjuvant such as *L. Rhamnosus* was used, which can synthesize and generate the β-glucuronidase enzyme, reverting curcumin to its active form [35, 36]. So, an organized *in vitro* study was done to find out and confirm the β-glucuronidase enzyme activity in rat feces to support the above idea. The enzyme β-glucuronidase was thought to break down the substrate, phenolphthalein-β-D-glucuronide, and release phenolphthalein. This would support the notion that β-glucuronidase would also break down the curcumin-glucuronide conjugates made after metabolism. The existing study revealed that the phenolphthalein released was highest in the *L. Rhamnosus*-treated group compared to other groups. This shows that the probiotic plays a significant role in making the β-glucuronidase enzyme that reduces curcumin breakdown, which makes it more bioavailable when taken by mouth, as was already reported [37]. To determine how *L. Rhamnosus* enhanced the systemic bioavailability of curcumin, an LC-MS/MS investigation was also carried out. The study found that when curcumin was mixed with L. *Rhamnosus*, the area from the curve was more significant than when curcumin was used by itself. This meant that curcumin was up to seven times more bioavailable overall. This displayed the role of *L. Rhamnosus* in increasing the blood levels of curcumin. The reported method was used for the induction of PD [38].

Rotenone is a mitochondrial-complex-I inhibitor that causes Parkinson's disease (PD) symptoms upon prolonged exposure. Its chemical name is [(2R,6aS,12aS)-1,2,6,6a,12,12a-hexahydro-2-isopropenyl-8,9-dimethoxychromeno[3,4-b] fluro (2,3-h) chromen-6-one]. Due to the delay in gastric emptying that occurs during rotenone intoxication and other GIT-related problems, as stated by Sharma and Nehru, 2013, this model casued a considerable loss in body weight of the ROT-treated group as compared to other groups of animals [39]. The body weight loss was greater in the other group than in the ROT group. The motor and behavioral impairment caused by rotenone exacerbates PD symptoms. Rats' motor and behavioral abilities were evaluated using physical tests. Except for the CUR-treated group, which showed less hanging time, as reported in earlier research conducted by Ramkumar et al., 2018, the ROT group's hanging time was considerably reduced compared to other treatment groups [40]. Other tests, such as akinesia and catalepsy, also showed significant results. The treatment groups' akinetic and catalytic times were much shorter than the ROT-treated group. Therefore, combining probiotics and curcumin significantly reduces motor and behavioral symptoms.

The balance of dopamine and acetylcholine in the striatum affects how motor and cognitive action sequences are learned. This is because

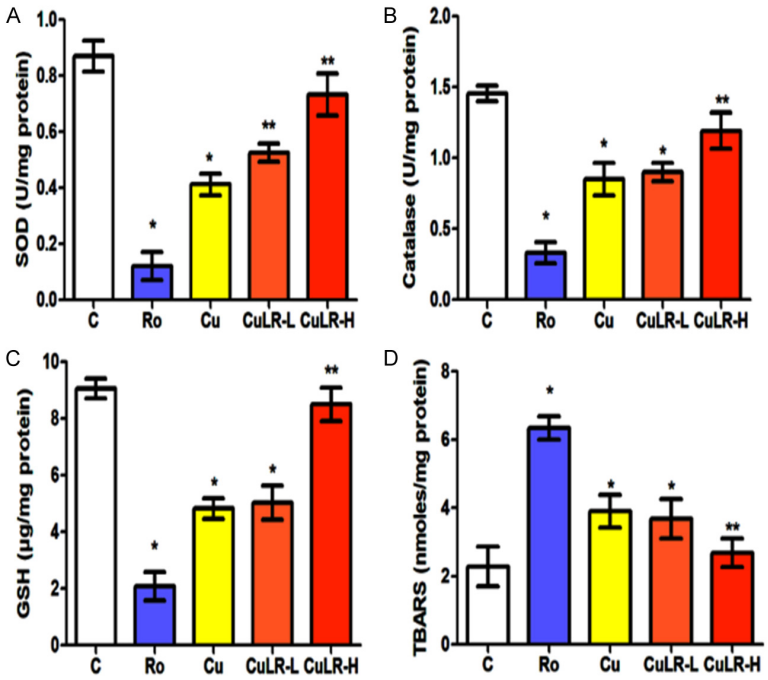

Figure 5. Brain antioxidant levels. A. SOD; B. Catalase; C. GSH; D. MDA. Data are presented as mean ± SEM (n = 6). A one-way ANOVA followed by Tukey's test was applied. *, ** denote P≤0.05 and P≤0.01, respectively, when compared with the ROT group.

dopamine and acetylcholine strongly interact at many presynaptic and postsynaptic sites in the striatum, which is the central input station of the basal ganglia [41]. This shows that the dopamine and acetylcholine balance in the nigrostriatal region is essential for regulating motor and behavioral functions. While using facts, the levels of dopamine and AChE in the rat brain were calculated. Except for the CUR alone and CUR + LR (LD) groups, which showed just a slight increase, the dopamine level in the brain was significantly increased in the CUR + LR (HD) group compared to the ROT-treated group. Earlier studies also showed an increase in the dopamine level of curcumin in the rat brain [42]. Rotenone changes the balance of dopamine and acetylcholine, which raises AChE levels. This means that the significantly higher AChE levels in the ROT-treated group support what was described previously [43].

Except for the CUR-treated group, which reported less significant outcomes, the treatment groups all showed a substantial reduction in AChE levels, which improved motor symptoms. Oxidative stress is the primary pathologic marker responsible for the nigrostriatal neurodegeneration in PD. Curcumin possesses remarkable antioxidant properties due to its chemical structure. Curcumin has three active sites: methoxy and phenolic groups on benzene rings and the -diketone moiety, which are oxidized *via* electron transfer and hydrogen abstraction to provide antioxidant activity [44, 45]. In rats with rotenone-induced PD, the treatment groups had considerably lower amount of TBARS and the antioxidant activity of SOD, GSH, and CAT, as shown in earlier studies [43, 46]. Histopathologic analysis revealed that chronic rotenone exposure caused significant damage to the midbrain region of rats. In the ROT-treated group, many eosinophilic inclusions were detected (**Figure 6**) that resembled the pale bodies (Lewy bodies) found in the substantia nigra of PD animals. The other treatment groups prevented the development of these well-scattered, smaller-in-size, and fewer-in-number bodies compared to the ROT group.

Conclusion

The current work significantly reveals the function of curcumin in combination with *L. Rhamnosus* in rotenone-induced PD rats by reducing motor and behavioral symptoms, restoring dopamine levels, maintaining AChE levels, and enhancing antioxidant capacity. Additionally, these results provide a rationale for curcumin and *L. Rhamnosus* co-administration, which may slow the progress of PD. Clinical pharmacokinetic investigations are needed to address a possible role of probiotic L. *Rhamnosus* in enhancing curcumin's bioavailability.

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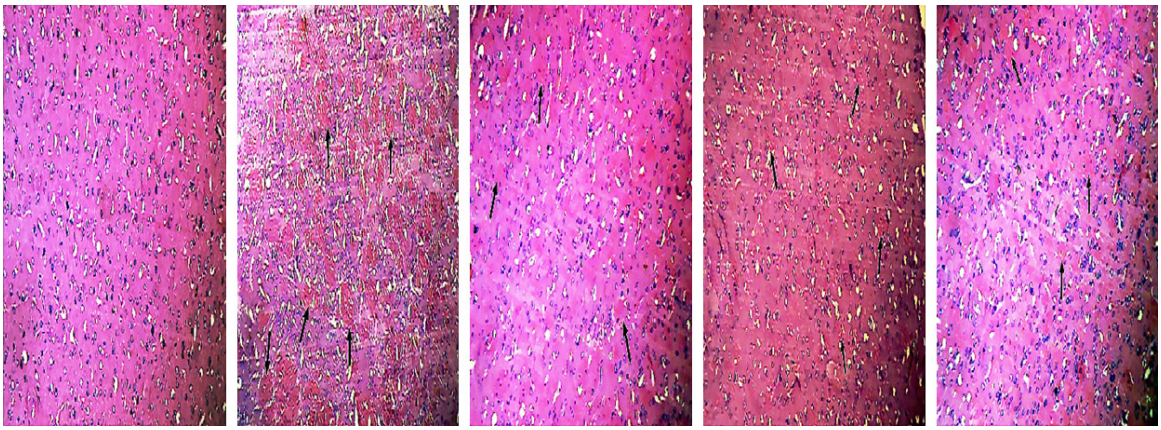

Figure 6. Brain histology (Histopathology was performed under 40× objective with 10× ocular).

Disclosure of conflict of interest

None.

Address correspondence to: Arun Soni, Ramanbhai Patel College of Pharmacy, Charotar University of Science and Technology, CHARUSAT Campus, Changa, Anand, Gujarat 388421, India. Tel: +91-9106186196; E-mail: arunsoni.ph@charusat.ac.in

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