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Neuroprotective Role of Caffeic Acid in Lipopolysaccharide-induced Neurodegeneration in Mice

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This study aimed to investigate the anti-inflammatory role of Caffeic acid (CFA) on Lipopolysaccharide (LPS)-induced neurotoxicity by quantifying astrocyte and microglial activation. Mice were randomly divided into Groups A-D (n=8). Group A, the Control group, received water; B (Neurotoxicity model) received LPS for seven days and sacrificed; C (Neurotoxicity and anti-inflammatory model) received LPS and caffeic acid concurrently, D (Positive control) received caffeic acid. All administrations were through oral gavage once daily at 08:00 hours, and the experiment lasted 14 days. An open field test (OFT) was used to assess the effect of LPS and other treatments on the exploratory behavior of the animals. LPS-induced neurotoxicity reduced mice's motor activities, significant degeneration of cerebral neurons, and increased cerebral GFAP (glial fibrillary acidic protein) and Iba1 (ionized calcium-binding adapter molecule 1) immuno-reactivity. The study also establishes the ameliorating effect of CFA on LPS-induced neurotoxicity by restoring cerebral histoarchitecture, improving motor activities, and reducing cerebral GFAP and Iba1 expression.

Key words: Caffeic acid; Lipopolysaccharide; Neurodegeneration; Cerebral cortex; Mice

Introduction

Neurodegenerative disorders (ND) constitute a heterogeneous group of age-related disorders characterized by a slow but irreversible deterioration of brain functions [24]. Over two decades, evidence has implicated calcium-related homeostatic mechanisms, giving rise to the Ca^{2+} hypothesis of brain aging and cell death [28]. Neurodegenerative diseases have become a global problem affecting mostly older adults. Parkinson's disease (PD), a type of ND, is major motor disorder, and the second most common neurodegenerative age-related disorder [19]. This disease is of great clinical and

economic importance due to its effect on mobility in the elderly. The primary locus of PD, comprised of pigmented, dopaminergic neurons inside the substantia nigra pars compacta (SNpc) and attendant projections to the putamen, is characterized clinically by resting tremor, bradykinesia, rigidity, and postural instability. Even though the pathogenesis and differential diagnosis of PD is poorly understood, however, a multifactorial process appears to initiate dopaminergic neuron degeneration in PD. Moreover, several inflammatory responses have been suggested to play a key role in dopaminergic neuron degeneration and hence progression of the disease. The two most common inflammatory models of PD are those involving the use of polyinosinic polycytidylic acid (poly (I: C) and lipopolysaccharide (LPS), which activate toll-like receptors 3 and 4, respectively [21]. Toll-like receptors (TLR) recognize pathogen-associated molecular patterns, initiating an immune response and promoting the production of pro-inflammatory cytokines, chemokines, and oxidative factors [21]. The earliest duration of LPS-induced PD studied was 3 days, and it led to robust activation of substantia nigra (SN)-microglial cells. Between 1 and 2 weeks after starting LPS infusion, SN microglia became fully activated, exhibiting the characteristic amoeboid morphology [10].

Animal PD models have improved our knowledge of the disease and have played a critical role in developing neuroprotective drugs. Although much funding has been earmarked for the development of drugs or technology for the cure or treatment of ND, unfortunately, up till now, there is less effective management currently available for the amelioration of these elderly-related diseases. This has led to the advent of complementary means of control, including the use of Caffeic acid (CFA), a polyphenol produced through the secondary metabolism of vegetables [25], including olives, coffee beans, fruits, potatoes, carrots, and propolis, and constitutes the main hydroxycinnamic acid found in the diet of humans [27]. The phenolic acid constituents of coffee, such as CFA, have also been reported to possess antioxidants, anti-inflammatory, anti-apoptotic, and neuroprotective properties [27]. A study has disclosed that Caffeic acid can protect the blood-brain barrier (BBB) in a rodent model of traumatic brain injury [32], preventing neonatal hypoxic-ischemic brain injury. Another study also postulates that CFA attenuated dopaminergic neuronal loss in 6-OHDA Parkinson's model [3], and this could be due to its dihydroxy atom, which easily makes CFA a potent antioxidant molecule [26]. Furthermore, CFA has been proven to be a potent 5-lipooxygenase (LOX) inhibitor, and has subsequently demonstrated an ability to down-regulate NF-κB, IL-6, and IL-1β in inflammatory reactions [13]. These benefits of CFA have been captured in a large prospective epidemiological study which documented a reduced risk of developing PD with a relative risk ranging from 0.45 to 0.80 in coffee drinkers versus non-coffee drinkers [14]. Therefore, the index study was designed to evaluate the neuro-ameliorative role of caffeic acid on anxiety-like (anxiolytic or anxiogenic) behavior and how these dietary constituents modulate the general behavior, including the normal locomotion and depressive symptoms when presented with stressors like Lipopolysaccharides.

Materials and Methods

Experimental Animals

Thirty-two (32) young male Swiss mice weighing 20 g and 30 g were obtained from the Animal House of the University of Medical Sciences, Ondo City. They were kept and housed in plastic cages at room temperature and 12:12 h light-dark cycle, and fed with a balanced rodent pellet diet and water ad libitum. Mice were acclimatized for fourteen days before the commencement of experiments. The NIH Guideline on experimental procedures for the Care and Use of Laboratory Animals for research was strictly adhered during our experiment.

Drugs and Chemicals

Caffeic acid, lipopolysaccharide – LPS (*Escherichia coli* serotype, 055: B5), acetylthiocholine, Ellman Reagent [5′, 5′-Dithiobis- (2-nitrobenzoate) DTNB] and thiobarbituric acid (TBA) were obtained from Sigma-Aldrich, St. Louis, USA. Trichloroacetic acid (TCA) was obtained from Burgoyne Burbidge & Co., Mumbai, India. Primary antibodies (Anti-Iba 1, GFAP), Polymer Anti-mouse igG Reagent, DAB peroxidase (HRP) Substrate kit (Vector®) were purchased from Vector Labs, Burlingame, CA, USA, Elite Vectastain ABC kit), visualization was with diaminobenzidine (DAB) (Vector Labs, peroxidase substrate kit, SK-4100).

Experimental Procedure

The animals were randomly assigned into four experimental Groups (A-D), n=8. Group A serves as the Control group and received water and vital pellet feed only for 14 days; Group B (LPS) received water, vital pellet feed, and 5 mg/kg of LPS for seven days to induce neurodegeneration [4, 12]; Group C (CFA + LPS) received water, Vital pellet feed, 5 mg/kg of LPS, and 40 mg/kg of caffeic acid for 14 days to determine the protective potential of caffeic acid; Group D (CFA) received water, Vital pellet feed and caffeic acid only for 14 days. All administrations were done via oral gavage using an improvised oral cannula once a day at 08:00 hours, and the whole experiment lasted for 14 days.

Behavioral paradigms

Open field test

An open field test (OFT) was used to assess the effect of LPS and other treatment groups on the exploratory behavior of the animals. Locomotor activity (LMA) was assessed in mice individually placed into a clean, novel glass arena (30×30×60 cm) that was divided into nine virtual quadrants (10×10 cm each). Locomotor activity was measured by counting the number of crossings, the number of rearing, centre square entries and time in the centre, and grooming over a 5 min period. Between the experiments, the apparatus was cleaned with 70% ethanol [29].

Animal Sacrifice and Tissue Excision

The mice were sacrificed 24 hours after the last administration. Animals were sacrificed via cervical dislocation, carried out by a skilled personnel. The brain was exposed by a sagittal incision of the skull, and the brain harvested and fixed in 10% neutral buffered formalin before processing for histology. The recommended procedure of Drury and Wallington was adopted [6].

Histological and Immunohistochemical Procedure

The brain samples were processed for routine histological processing for hematoxylin and eosin (H&E) staining technique. Brain sections for immunohistochemistry were stained for astrocyte and microglia with GFAP and IBA, as described by Gray and Hand [9].

Photomicrography and Image Analysis

The histological and Immunohistochemical slides were viewed under a Digital Light microscope, and an attached camera took digital photomicrographs at ×400, ×100, and x40 magnifications using OMAX software. The cell counter plug-in, NIH-sponsored Image J software, was used to digitally analyze photomicrographs with a resultant quantification of protein expression [11].

Data Analysis

One-way ANOVA was carried out to analyze data from behavioral tests, GFAP, and Iba1 immunoreactivity, followed by the Tukey test for multiple comparisons. A GraphPad Prism 8 was utilized for statistical analysis. A significant difference was set at $p < 0.05$.

Results

Open Field Test

Behavioral activity data from the LPS mice group, control mice group, LPS + CFA group, and caffeic only group were collected on the 3rd, 5th, 7th, 10th, 12th, and 14th day of the experiment. The days chosen for the test were for convenience and to allow for enough time for drug actions. However, the data recorded for day 5, 7, and 12 were used for general presentation of our data analysis (**Fig. 1**).

Total Distance Traveled

The mice' total distance travelled on the open field test over the three days was calculated as a graphical representation shown in **Fig. 1** (**chart 1**). The control group mice had a mean total distance travelled of 449.00 ± 14.43 (cm), which was significantly reduced to 126.90 ± 5.17 ($P < 0.0001$) in the LPS-only group. LPS + Caffeic acid increased the mean total distance travelled to 307.90 ± 6.14 ($P = 0.0001$) compared to LPS-group mice. Similarly, caffeic acid-group mice increased the mean distance travelled to 424.30 ± 15.53 ($P = 0.0001$) across the 5th, 7th, and 12th day of the open field experiment.

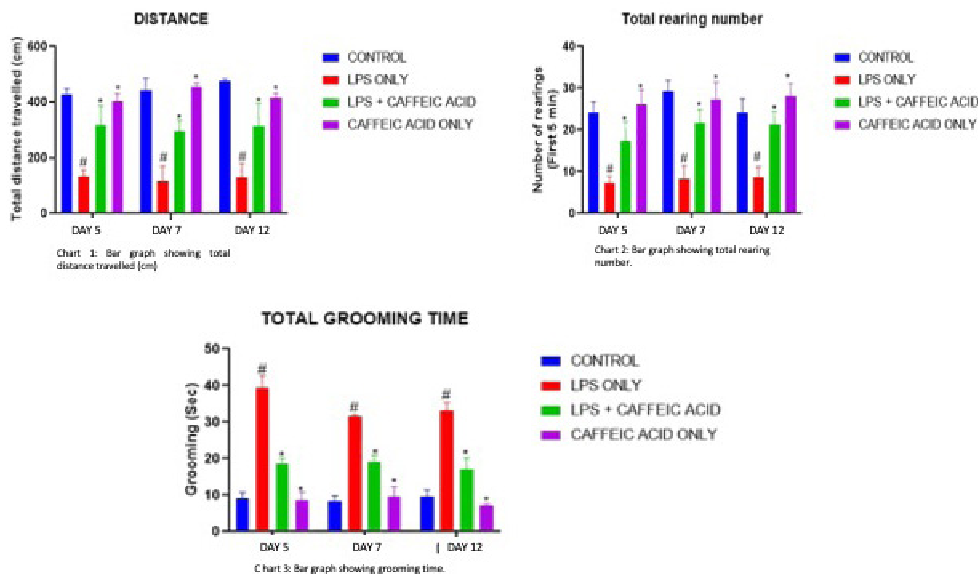

Fig. 1. Behavioral assessment using Open Field Test. Chart 1. Total distance of mice travelled on the open field test; Chart 2. Total rearing number; Chart 3. Grooming time in the open field test.

Total Rearing Number

The mean total rearing number in the control mice was 26.33 ± 3.48, 25.33 ± 1.20, and 25.67 ± 2.03 on the 5th, 7th, and 12th day, respectively (**Fig. 1, chart 2**). LPS-group significantly reduced the mean total rearing number on the 5th, 7th, and 12th day to 7.33 ± 1.86 ($P < 0.0001$), 9.00 ± 0.00 ($P < 0.0001$), 8.00 ± 1.53 ($P < 0.0001$) compared to the control group. LPS + CFA-group increased the mean total rearing number to 21.67 ± 2.33 ($P = 0.0022$) 16.33 ± 1.667 ($P < 0.0001$) and 22.33 ± 0.33 ($P = 0.0002$) compared to LPS-group. CFA-group also increased the mean total rearing number to 27.33 ± 1.20, 25.67 ± 0.67, 28.33 ± 3.18 ($P < 0.0001$).

Total Grooming Time

The grooming time in the open field test of control-group mice was 8.33 ±2.33, 8.00 ±0.58, 10.67 ± 0.88 *sec* on days 5, 7, and 12 (**Fig. 1, chart 3**). LPS-group significantly increased mean grooming time to 35.67 ± 5.24 ($P < 0.0001$) on the 5th day, 34.67 ± 1.45 ($P < 0.0001$) on the 7th day and 33.67 ± 1.76 ($P < 0.0001$) on the 12th day. Treatment of LPS-group mice with caffeic acid consequently significantly reduced this means grooming time to 18.33 ±1.86 ($P = 0.0022$), 18.33 ± 3.712 ($P < 0.0001$), 18.00 ± 0.58 ($P=0.0002$) compared to LPS-group. For the CFA-group, there was no significant difference in the mean grooming time [7.33 ± 0.33 ($P> 0.05$), 8.67 ± 2.186 ($P > 0.05$), and 9.33 ± 2.85 ($P > 0.05$)] when compared to the control-group, suggesting no major adverse effect of CFA in the mice.

Demonstration of histologic features with hematoxylin and eosin staining

The hematoxylin and eosin staining of the cerebrum shows distinct pyramidal neurons and oligodendrocytes (**Fig. 2**). Control (**Fig. 2A**) and CFA (**Fig. 2D**) groups show normal cerebral histology. LPS-group (**Fig. 2B**) shows obvious degenerative features in neurons characterized by cellular shrinking, loss of nuclear constituent, and cytoplasmic vacuolations. Treatment with CFA helped restore cerebral histology in the LPS+CFA-group (**Fig. 2C**). However, some visible cytoplasmic vacuolations are still present compared to control and CFA-group.

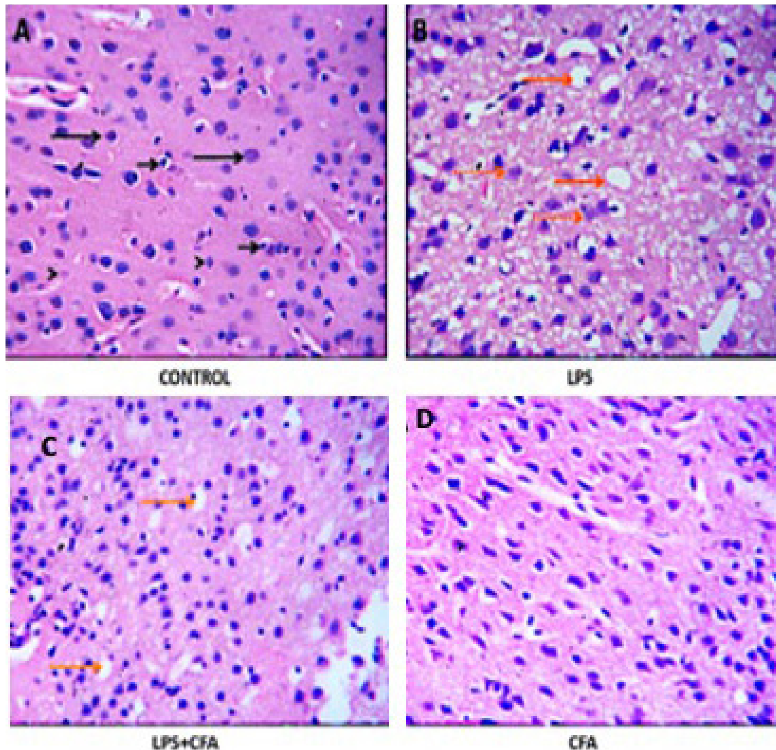

Fig. 2. Photomicrographs of H&E staining of the cerebrum of control, LPS, CFA, and LPS+CFA groups (H&E, ×400). Black arrows – pyramidal neurons; Short arrows – oligodendrocytes; Arrowhead – astrocytes; Brown arrows – cytoplasmic vacuolations; Dashed arrows – degenerating neurons

Quantification of immuno-expressed GFAP

The present study shows increased GFAP immunoreactivity in the LPS-group (15.00±1.77) compared to the control-group (1.00 ± 0.21), CFA-group (4.00 ± 0.77), and LPS + CFA group (6.25 ± 1.61). The CFA group (4.00 ± 0.77) and LPS + CFA group (6.25 ± 1.61) showed no significant difference in immunoreactivity when compared to the control group (1.00 ± 0.21) (**Fig. 3**).

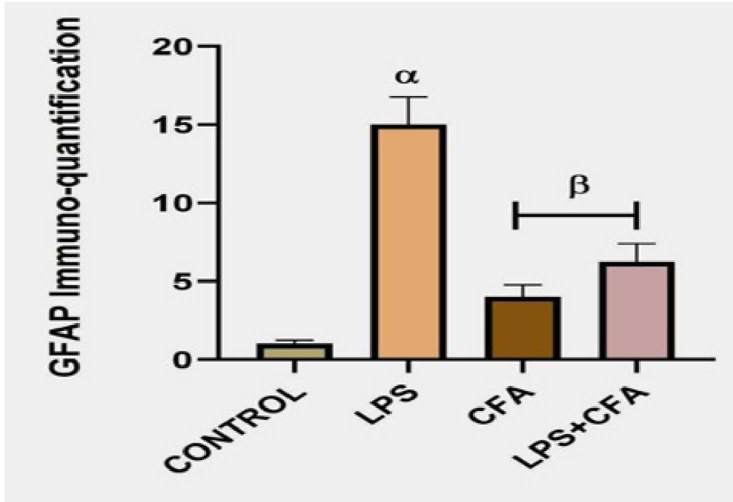

Fig 3: The bar chart depicts the number of GFAP immunoreactivity in control and treated groups, Bars are Mean ± SE. α denotes significant increase ($p<0.05$) compared to control group. β denotes significant decrease ($p<0.05$) compared to LPS only treated group. One-way ANOVA followed by Tukey test .

Quantification of immuno-expressed IBA1

The present study shows increased Iba1 immunoreactivity in the LPS-group (7.17 ± 0.18) compared to the control group (3.09 ± 0.59), CFA-group (4.00 ± 0.47), and LPS + CFA-group (4.14 ± 0.39). The CFA-group (4.00 ± 0.47) and LPS + CFA-group (4.14 ± 0.39) showed no significant difference in immunoreactivity when compared to the control-group (3.09 ± 0.59) (**Fig. 4**).

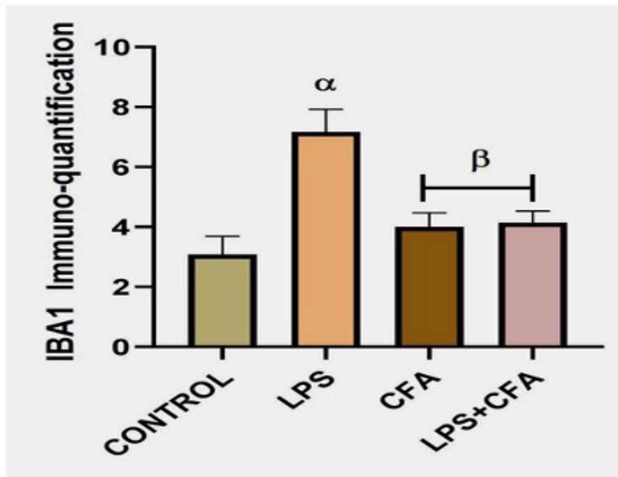

Figure 4: The bar chart depicts the number of IBA1 immunoreactivity in experimental groups, Bars are Mean ± SE. α denotes significant increase ($p<0.05$) compared to control group. β denotes significant decrease ($p<0.05$) compared to LPS only treated group. One-way ANOVA followed by Tukey test.

Discussion

The current study examines the anti-inflammatory effect of caffeic acid (CFA) on LPS-induced cerebral neuroinflammation by quantifying astrocyte and microglial cell activation. Lipopolysaccharide (LPS) is the polysaccharide component of the gram-negative bacterial cell wall. Once recognized by the immune system, LPS elicits a proinflammatory response [31] and has thus become extensively used in research to induce neuroinflammation [16]. Neuroinflammation plays an important role in the etiology and progression of neurodegenerative diseases, including Parkinson's disease [8]. However, CFA possesses unique biological characteristics, including antioxidant, anti-inflammation, and immune regulation [20]. It also exerts strong antioxidant effects by blocking reactive oxygen species [5]. CFA's anti-inflammatory and antioxidant activity have been demonstrated in many cells [7]. In addition, CFA was shown to exert direct neuronal protection through up-regulation of endogenous antioxidants and modulation of inflammatory homeostasis [17]. While several studies have used osmotin [2] and dihydrotestosterone [30], among other anti-inflammatory agents, to mitigate the neuroinflammatory effect of LPS, none have reported the anti-inflammatory activity of CFA in LPS-induced cerebral neuroinflammation. Therefore, this study aims to demonstrate that CFA can attenuate cerebral neuroinflammation following LPS-induced neurotoxicity in experimental mice.

Neuronal loss in the cortex is closely related to cognitive and behavioral dysfunction. Accumulating evidence indicates that systemic LPS injection in mice induces cognitive deficits, including spatial learning and memory impairment, as measured by the open field test (OFT), which is the most popular test of cerebral-dependent cognitive functions. The current study utilizes OFT to measure locomotive activity in experimental mice. The reported data indicate that LPS treatment decreased distance travelled, total rearing number, and increased total grooming time in mice, suggesting decreased locomotor activity, a clinical symptom of Parkinson's disease [8]. Previous studies have shown that LPS administration in mice affects locomotors and motor activity [1]. Herein, our results showed that locomotors' activity was improved by CFA, as indicated by increased distance travelled and total rearing time, and reduced total grooming time in the LPS + CFA-group. Previous studies indicate that the cerebral cortex undergoes neurodegenerative changes following LPS induction [1]. In the present study, the light microscopic examination of H&E-stained cerebral sections of the LPS-treated group revealed marked neurodegenerative changes in the pyramidal neurons of the cerebral cortex. The pyramidal cells were undergoing karyolysis and had no visible nuclei. Also, there are several visible cytoplasmic vacuoles. Due to the anti-inflammatory role of CFA in previous works, this study hypothesizes that treatment with CFA could help rescue the neurodegenerative changes observed in the cerebral cortex following LPS exposure. This study demonstrated restorative changes in the histological features of LPS-treated mice following CFA administration.

Chronic neuroinflammation mediates neuronal damage and apoptosis in the pathogenesis of neurodegenerative diseases, including Parkinson's disease [15]. Aberrant glial activation and neuroinflammation play a prominent role in the pathogenesis of neurodegenerative diseases [30]. This study presents data that supports neuroinflammation in the cerebral cortex following LPS-induced neurotoxicity. To quantify the extent of neuroinflammatory changes in the cerebral cortex, GFAP and Iba1 was demonstrated via immunohistochemistry [24].

Astrocytes participate in the generation and control of inflammatory mediators. The activation states of astrocytes are determined based on increased GFAP immunolabeling [24]. GFAP is a key component of the astrocyte's cytoskeleton that maintains cell integrity and resilience. There was a significant increase in GFAP expression following LPS exposure in mice cortex, consistent with Khan et al. findings [12]. CFA attenuated cerebral inflammation by down-regulating GFAP activation. As resident macrophages in the brain, microglia, typically marked by Iba1, can be activated and trigger the innate immune response by sensing exogenous neurotoxic substances, such as LPS and proinflammatory stimuli. Rapid microglial activation and associated inflammatory reactions are responses to combat the effect of insults and contribute to immune defense and tissue repair in the central nervous system (CNS) [22]. This acute activation is considered protective. By contrast, persistent microglial activation will ultimately result in the vast production of proinflammatory mediators, chemokines, and the recruitment of peripheral immune cells [18] to the brain that characterizes chronic neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease [23]. In this study, over-expression of cerebral Iba1 was established following LPS-induced neurotoxicity, consistent with other findings [12, 24]. In addition, there was a significant reduction in Iba1 expression in treatment with CFA. Therefore, CFA-induced down-regulation of microglia and astrocytes, as evident by reduced Iba1 and GFAP levels, respectively, shows that CFA could be a potential therapeutic agent to mitigate cerebral neurotoxicity.

Conclusion

In conclusion, this study presents novel evidence that CFA can mitigate LPS-induced neuroinflammation and neurodegeneration in the cerebral cortex of experimental mice via reduced astrocyte and microglia activation, consequently rescuing LPS-induced locomotive impairment. In summary, the study results provide evidence that the anti-inflammatory property of CFA can enable it to exert neuroprotection on the cerebral cortex. Therefore, CFA should be extensively studied as it may be a therapeutic agent against neuroinflammation and neurodegenerative diseases, such as Parkinson's.

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