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Research Article

NEUROPROTECTIVE EFFECT OF *CASSIA OCCIDENTALIS* AGAINST 3-NITROPROPIONIC ACID-INDUCED NEUROTOXICITY IN RATS

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Abstract: Huntington's disease (HD) is a neurodegenerative disorder characterized by motor impairment, cognitive decline and psychiatric symptoms. Systemic administration of 3-nitropropionic acid (3-NP) causes selective striatal degeneration similar to that seen in HD. Although the precise cause of neuronal cell death in HD is unknown, recent studies clearly demonstrate that increased oxidative stress is one of the major deleterious events in the 3-NP-induced neurodegenerative process. *Cassia occidentalis* (CO) has been studied for its antipyretic, anti-inflammatory and antioxidant properties. In this study was evaluated the neuroprotective activity of stalks and leaves extracts of CO against behavioral and biochemical parameters induced by intraperitoneal administration of 3-NP. The *in vitro* antioxidant activity was determined by the DPPH radical scavenging activity method. Behavior evaluations were performed using the models of open-field, rotarod and elevated plus maze. The *in vivo* antioxidant activity was evaluated in the striatal region after behavioral tests by the lipid peroxidation and the enzymatic activity of superoxide dismutase. Systemic administration of 3-NP (30 mg/kg, i.p. for 5 days) produced hypolocomotion, muscle incoordination and memory deficit. CO administration (400 and 800 mg/kg p.o.) improved 3-NP-induced dysfunctional behavior (locomotor, rotarod and memory retention). Biochemical analysis of the striatum revealed that systemic 3-NP administration significantly increased lipid peroxidation and decreased superoxide dismutase activity, which was attenuated by daily treatment with CO. These results suggest that the protective effects of CO against 3-NP-induced rat striatal degeneration are mediated through its antioxidant activity and suggest a potential therapeutic benefit of this plant in the treatment of HD.
Key words: antioxidant; *Cassia occidentalis*, Huntington, neuroprotection

1.0 INTRODUCTION

Huntington's disease (HD) is a progressive neurodegenerative disorder characterized by motor impairment, cognitive decline and psychiatric symptoms that worsen as the disease progress [1]. The most striking neuropathology in HD occurs within the striatum, in which gross atrophy of the caudate nucleus and putamen is accompanied by selective neuronal loss of the medium spiny GABAergic neurons and astrogliosis. Other regions, including the cerebral cortex, globus pallidus, thalamus, subthalamic nucleus, substantia nigra and cerebellum, show different levels of atrophy depending on the pathologic grade [2]. Despite the discovery of the genetic mutation of HD [3], the mechanisms of the pathogenesis of HD are still not understood. Oxidative stress, mitochondrial dysfunction and neuroinflammation [4,5] have been proposed as contributing factors for the appearance of motor alterations and cognitive decline [6]. A consistent dysfunction in the mitochondrial respiratory chain complex II–III has been found in the caudate nucleus of patients with HD. It has been proposed that a disruption in redox balance could lead to increased oxidative stress, neuronal dysfunction and HD [7, 8]. In this scenario, anti-oxidant therapy has been suggested as a potential method to prevent neurodegenerative disorder, such as HD. In this way, natural products from plants would be an important tool for treating these pathologies.

Cassia occidentalis is a bush from the Leguminosae family, found in many tropical areas of America, including the Amazon rainforest. In folk medicine, its roots, leaves and barks have been widely used as medicinal purposes, such as hepatoprotector, anti-inflammatory and antioxidant [9]. These pharmacological properties have been attributed to its chemical composition, which was enriched in anthraquinones, fatty oils, flavonoids, polysaccharides and tannins [10, 11, 12, 13]. In addition, it has demonstrated low level of toxicity by oral administration in rats [13, 14]. Thus, this study was undertaken to test the hypothesis that *Cassia occidentalis* oral intake may act by antioxidant mechanisms as neural protector against the development of HD in rats.

2. MATERIALS AND METHODS

2.1 Plant Material

The plant samples were collected in the city of Limoeiro, state of Pernambuco, Brazil. A voucher specimen was authenticated in the Department of Botany of the Federal University of Pernambuco (UFPE) and was deposited at Geraldo Mariz Herbarium (number 45936).

2.2 Extraction and phytochemical analysis

Extraction was performed by macerating air-dried, powdered *Cassia occidentalis* with 70% ethanol at room temperature for 7 days, and was occasionally shaken. In the laboratory, the ethanolic extract of *Cassia occidentalis* (CO)

was filtered, evaporated to dryness under reduced pressure and finally lyophilized (Tecnape, Technology in dehydration Ltda – Ribeirão Preto, Brazil) to yield approximately 15%. The dry residue was stored at 4 °C, and, at the time of use, was resuspended in distilled water. Phytochemical analysis was performed according methods of Harbone [15] and Wagner and Bladt [16]. As previously described by Silva et al. [13] CO extract showed the presence of flavonoids, anthraquinones, triterpenes, small amount of saponins and the absence of iridoids.

2.3 Animals

Male Wistar rats (*Rattus norvegicus* var. *albinus*) weighing between 250-300g were used for neuroprotective experiments. The animals were obtained from the Department of Physiology and Pharmacology from the UFPE. They were maintained under standard environmental conditions (12 h dark/light cycle) and temperature (22 ± 2 °C). Water and industrialized dry food (Labina®, Purina, Brazil) were available *ad libitum*. All experiments were carried out between 09:00 am and 3:00 pm. All protocols were approved by the Animal Experimentation Ethics Committee of the Biological Science Center - UFPE, under license n°. 23076.017121 in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.4 Experimental induction of Huntington's disease (HD) and experimental design

HD was induced by a neurotoxin 3-nitropropionic acid (3-NP), as previously described [17, 18]. It is known that systemic administration of this drug increases oxidative stress and produces neuronal death and bilateral lesion in striatum region of the rodents [17, 19]. In the present study, animals were divided randomly in four groups (n=8/group) according the treatment. The control group received vehicle (NaCl 0.9%, i.p.) for 5 consecutive days and water, by oral route, for 14 days (1 mL/kg, p.o.). The experimental group 1 received 3-NP (30 mg/kg, i.p.) for 5 days and after water (1 mL/kg, p.o.) for 14 days. The group 2 and 3, received 3-NP for 5 days and after *Cassia occidentalis* at dose of 400 mg/kg (3-NP + CO 400 mg/kg) or CO at dose of 800 mg/kg (3-NP + CO 800 mg/kg) for 14 days.

In order to assess the development of the HD in the groups, the animals were evaluated for body weight gain (percentage of change in relation of initial body weight), behavioral and biochemical parameters on days 1, 7 and 14 of treatment.

2.5 Evaluation of behavioral parameters

2.5.1 Locomotor activity

To quantify the general activity, rats were placed individually in the center of an open-field arena (a circular wooden box 100 cm in diameter and 40 cm high, with a floor divided into 19 regions). During 5 min, each animal was evaluated for: i) number of floor units entered by the animal with four paws (totl ambulatory activity); ii) number of times the animal stood on its hind legs (total rearing activity); and iii) time that the animal stood still without showing any movements (duration of immobility in seconds). After each evaluation, the device was cleaned with a 5% alcohol–water solution before placement of the animals to eliminate a possible bias effect due to odor clues left by previous rats.

2.5.2 Rotarod activity

All animals were evaluated for grip strength by using the rotarod. Each rat was given a prior training session before initialization of therapy to acclimatize them to a rotarod apparatus. The animal was placed on the rotating rod with a diameter of 7 cm (speed 25 rpm). Two separate trials were performed by each rat at 5 min intervals and a cut off time (180) was maintained throughout the experiment. The time when each animal fell off from the rod for the first time was recorded as fall off time (s) [20].

2.5.3 Elevated plus maze test for spatial memory

This protocol was performed according to previous description by Kumar et al. [21]. Briefly, the elevated plus maze consists of two opposite open arms (50×10 cm), crossed with two closed arms of the same dimensions with 40 cm high walls. The arms are connected with a central square (10×10 cm). After 5 days of 3-NP treatment, memory acquisition was assessed on days 1, 7 and 14. The animals were placed individually at one end of an open arm facing away from the central square and the time taken by the animal to move from the open arm and enter one of the closed arms was recorded as initial transfer latency (ITL). The rat was allowed to explore the maze for 30s after recording the initial acquisition latency and returned to its home cage. Retention latency was noted again on days 2, 8 and 15, after 5 days of 3-NP treatment.

Percentage memory retention was calculated by the formula: Transfer latency (first analysis − second analysis) × 100/Transfer latency (first analysis).

2.6 Biochemical parameters

2.6.1 Striatum samples

At days 2, 8 and 15, the animals were sacrificed by decapitation immediately after the behavioral assessment, for the biochemical analysis. Brains were removed, and the forebrains were dissected out and the cerebellum discarded. Samples were kept cold and the striatum were separated. A tissue homogenate (10% w/v) was prepared in PBS 1X, to which was added butylated hydroxytoluene (0.004% w/v) for preventing oxidation of the samples. The homogenate was centrifuged at 10.000 *g* for 15 min and an aliquot of supernatant was separated. Protein content of the samples was assessed by the Bradford method [21] using bovine serum albumin as a standard.

2.6.2. Measurement of lipid peroxidation

The quantitative measurement of lipid peroxidation in the striatum samples was performed according to the method described by Buege and Aust [23]. The amount of malondialdehyde (MDA) was used as an indirect measure of lipid peroxidation and was determined by reaction with thiobarbituric acid. Briefly, aliquots (500 µL) of supernatant were placed in test tubes and added to 1 mL of tiobarbituric acid (TBA) reagent: TBA 0.38% (w.w.), 250 mL of 1N hydrochloride acid (HCl), trichloroacetic acid (TCA 15%) and 20 mL of ethanoic BHT (2%). The solution was shaken and heated to 100 °C for 15 minutes, followed by cooling in an ice bath. N-butanol (1.5 mL) was added, shaken and centrifuged to 3000 *g*. After centrifugation, the upper layer was collected and assessed with a spectrophotometer at 532 nm. All

determinations were made in triplicate. For calculations, a standard curve was constructed with 1,1,3,3, tetra methoxy propane (TMP). Results were expressed as nmol MDA/mg protein.

2.6.2 Superoxide dismutase (SOD) activity

SOD activity was determined according to the method described by Misra and Fridovich [24]. One hundred microliters of supernatant was added to 880 µL (0.05 M, pH 10.2, 0.1 mM EDTA) of carbonate buffer. Twenty microliters of epinephrine (30 mM in 0.05% acetic acid) was added to the mixture at 480 nm for 4 min on spectrophotometer. The enzyme activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equal to 1 unit.

2.7.1. Evaluation of DPPH radical scavenging activity of Cassia occidentalis extract

DPPH (2.2-diphenyl-1-picrylhydrazyl) radicals scavenging activity of *Cassia occidentalis* extract was evaluated as described by Brand-Williams, Cuvelier and Berset [25]. One mL of DPPH solution (30 mM, in 95% ethanol) was incubated with 2.5 mL of different concentrations of *Cassia occidentalis* extract (2.5-250 µg/mL). The reaction mixture was well shaken and incubated for 30 min at room temperature, and the absorbance of the resulting solution was analyzed by spectrophotometry at 515 nm. The synthetic antioxidant butylated hydroxytoluene (BHT) was included in experiments as a positive control. The IC_{50} value for each sample, defined as the concentration of the test sample leading to 50% reduction of the initial DPPH concentration, was calculated against the mean percentage of the radical scavenging activity.

2.8.1 Drugs

3-Nitropropionic acid (3-NP), butylated hydroxytoluene (BHT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), malondialdehyde (MDA), thiobarbituric acid (TBA), epinephrine and 1,1,3,3-tetra methoxy propane (TMP) were purchased from Sigma (St. Louis, MO, USA).

2.9.1 Statistical analysis

Data were expressed as means ± standard error of mean (SEM). Differences between the experimental groups were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Tukey’s multiple comparison test. The comparisons were carried out by GraphPad Prism software (GraphPad Software, version 5.0) and differences were considered significant at $p<0.05$.

3 RESULTS

3.1 Effect of *Cassia occidentalis* on the ponderal gain in HD rats

Figure 1 shows the effects of the 3-NP treatment on the body weight gain of the rats. After 3-NP treatment, all animals of the experimental group decreased significantly the body weight in relation to control group (Fig. 1, 1st day). Starting from the 7th day of treatment with CO (400 and 800 mg/kg), the animals had body weight gain similar to control group, while 3-NP group remained with lower values of body weight gain during whole treatment.

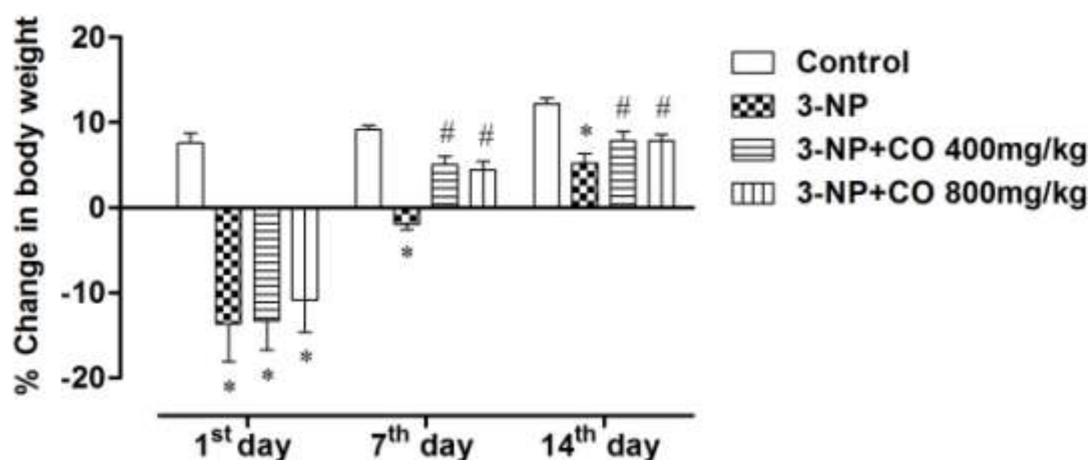

Figure.1. Effect of *Cassia occidentalis* (CO 400 and 800 mg/kg, p.o.) on body weight of 3-nitropropionic acid treated rats (3-NP; 30 mg/kg, i.p). Each value represents the mean±S.E.M. *$p<0.05$ when compared to the vehicle-treated control group; #$p<0.05$ when compared to 3-nitropropionic acid treated group (3-NP) (One-way ANOVA followed by Tukey's test).

3.2 Effect of *Cassia occidentalis* on the locomotor activity in HD rats

In figure 2 was presented the effects of the *Cassia occidentalis* on the locomotor activity in HD rats. Administration of 3-NP for 5 days significantly reduced the total ambulatory and total rearing activities (Fig. 2 A and Fig. 2 B, 1st day) and increased the duration of immobility of the animals (Fig. 2 C, 1st day). Treatment with CO extract (400 and 800 mg/kg, p.o.) for 7 and 14 days significantly improved locomotor activity in 3-NP treated rats, producing increased ambulatory and rearing activities and decreased duration of immobility (Fig. 2 A, B and C, 7th and 14th days).

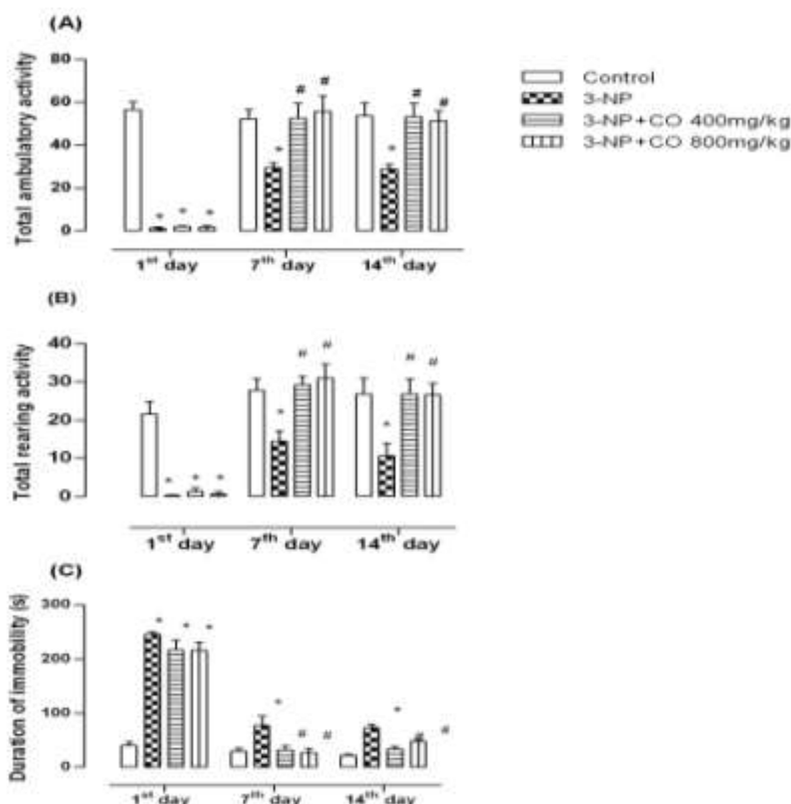

Figure. 2. Effect of *Cassia occidentalis* (CO 400 and 800 mg/kg, p.o.) on the total ambulatory activity (A), total rearing activity (B) and duration of immobolity (C) of 3-nitropropionic acid treated rats (3-NP; 30 mg/kg, i.p.) by the open field evaluation. Each value represents the mean±S.E.M. *p<0.05 when compared to the the vehicle-treated control group; $^{\#}$p<0.05 when compared to the 3-nitropropionic acid-treated group (One-way ANOVA followed by Tukey's test).

3.3 Effect of *Cassia occidentalis* on rotarod activity in HD rats

In figure 3 was presented the effects of *Cassia occidentalis* on rotarod activity in HD rats. Treatment with 3-NP significantly decreased the fall-off time when compared to the control animals. Daily treatment with CO extract (CO 400 and 800 mg/kg, p.o.) for 7 and 14 days increased the fall-off time when compared to 3-NP group.

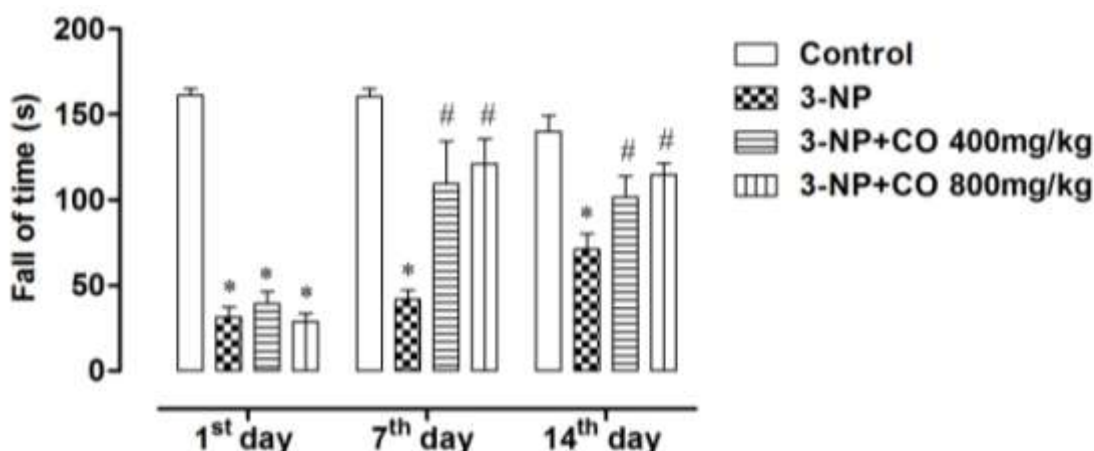

Figure. 3. Effect of *Cassia occidentalis* (CO 400 and 800 mg/kg, p.o.) on the fall off time in 3-nitropropionic acid treated rats (3-NP; 30 mg/kg, i.p.) by the Rotarod performance. Each value represents the mean±S.E.M. *p<0.05 when compared to the vehicle-treated control group; $^{\#}$p<0.05 when compared to the 3-nitropropionic acid-treated group (One-way ANOVA followed by Tukey's test).

3.4 Effect of *Cassia occidentalis* on spatial memory performance in elevated plus maze test

Intraperitoneal administration of 3-NP caused a marked memory loss as shown by a decrease in the % retention of memory in rats when compared to the control animals (Fig. 4). Daily treatment with extract of CO (400 and 800 mg/kg, p.o.), increased the % retention of memory when compared to 3-NP group.

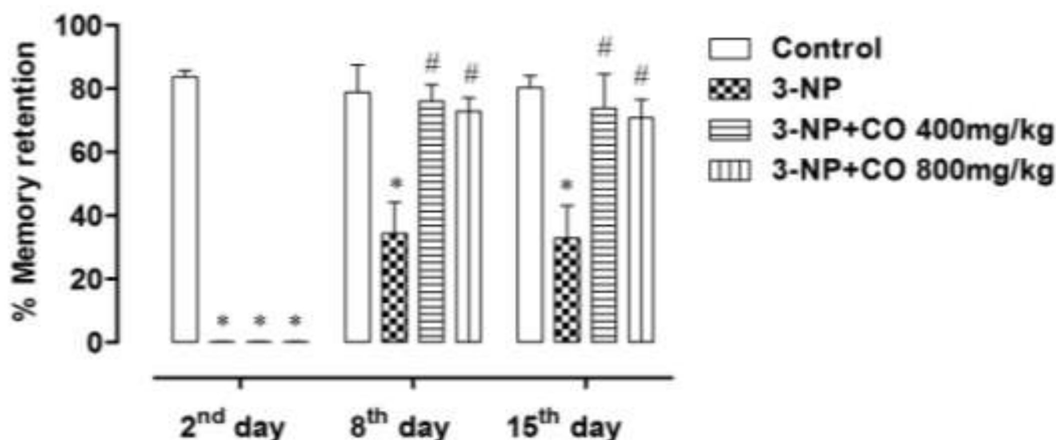

Figure. 4. Effect of *Cassia occidentalis* (CO 400 and 800 mg/kg, p.o.) on the % of memory retention of 3-nitropropionic acid treated rats (30 mg/kg, i.p.) by the elevated plus maze task. Each value represents the mean±S.E.M. *p<0.05 when compared to the vehicle treated control group; $^{\#}$p<0.05 when compared to the 3-nitropropionic acid treated group (One-way ANOVA followed by Tukey's test).

3.5 Biochemical parameters

Administration of 3-NP resulted in significant changes on biochemical parameters when compared to the control animals. The inoculation of 3-NP for 5 days induced oxidative stress, as indicated by an increase in the striatum MDA levels, and a decrease of SOD activity when compared to the control group. The treatment with extract of CO (400 and 800 mg/kg, p.o.) for 7 and 14 days, after 3-NP administration, attenuated the increase in lipid peroxidation in the striatum as shown by a significant decrease in MDA levels (Table 1). Similarly, daily treatment with CO for 7 and 14 days attenuated the decrease in SOD activity due to 3-NP treatment (Table 2).

Table 1. Effect of *Cassia occidentalis* (CO) on malondialdehyde levels in the striatum of rat brain from animals treated with 3-nitropropionic acid (3-NP).

	Malondialdehyde levels(nmol/mg protein)					
Treatments	1st day		7th day		14th day	
Control	0.076	±0.002	0.096	±0.003	0.080	±0.004
3-NP	0.155	±0.016*	0.178	±0.003*	0.155	±0.002*
3-NP+ CO 400mg/kg	0.167	±0.006*	0.076	±0.003$^{\#}$	0.088	±0.001$^{\#}$
3-NP+ CO 800mg/kg	0.156	±0.021*	0.068	±0.,009$^{\#}$	0.083	±0.007$^{\#}$

Each value represents the mean±S.E.M. *p<0.05 as compared to vehicle treated control group; $^{\#}$p<0.05 as compared to 3-nitropropionic acid treated group (One-way ANOVA followed by Tukey's test).

Table 2. Effect of *Cassia occidentalis* (CO) on superoxide dismutase activity in the striatum of rat brain from animals treated with 3-nitropropionic acid (3-NP).

	Superoxide dismutase (nmol/mg protein)					
Treatment	1st day		7th day		14th day	
Control	0.077	±0.007	0.087	±0.004	0.080	±0.007
3-NP	0.050	±0.001*	0.051	±0.001*	0.049	±0.002*
3-NP+ CO 400mg/kg	0.053	±0.002*	0.070	±0.001#	0.067	±0.002#
3-NP+ CO 800mg/kg	0.056	±0.003*	0.079	±0.002#	0.070	±0.002#

Each value represents the mean±S.E.M. *p<0.05 as compared to vehicle treated control group; $^{\#}$p<0.05 as compared to 3-nitropropionic acid (3-NP) treated group (One-way ANOVA followed by Tukey's test).

3.6 DPPH radical scavenging activity

All concentrations of CO were able to promote the capture of the DPPH radical. The IC_{50} of CO extract was 53.7 µg/mL, while the synthetic antioxidant BHT showed an IC_{50} = 73.8 µg/mL.

4 DISCUSSION

The main findings of the present study were: i) 3-NP systemic administration in rats produces neurobehavioral and biochemical changes mimicking those observed in HD; ii) the treatment of those rats with CO produced a neuroprotection and was able to prevent the systems of HD progression due to its antioxidant activity, revealing its pharmacological potential for treating HD.

It is well known that increased oxidative stress contribute significantly for HD progression [26]. Thus, pharmacological tools, as 3-NP, have been employed to produced HD in animal models. Systemic administration of 3-NP induces important increase in free radicals formation, leading to degenerative process in striatal region and HD development [7, 8].

In the present study, the treatment of rats with 3-NP produced motor and behavioral abnormalities, including bradykinesia, weakness and rigidity of muscles and considerable reduction in body weight. These findings are in agreement with earlier reports who also observed a variety of neurobehavioral and motor abnormalities in rats after 3-NP administration [26]. The observed weight loss could be related to the metabolic impairment induced by 3-NP, in other words, energy metabolism injury, mobilization of energy reserves and lipid peroxidation, which constitute peripheral effects of inoculation. However, it cannot be ignored that the striatal lesion and bradykinesia can also act as important factors on weight loss [17, 27]. The treatment with CO (400 and 800 mg/kg, p.o. for 7 and 14 days) significantly improved the weight loss when compared with 3-NP treated group.

The daily administration with extract of CO for 7 and 14 days attenuated significantly the hypolocomotion (open-field performance), motor incoordination (rotarod performance) and cognitive impairment (elevated plus maze performance) caused by injection of 3-NP. According to Seaman [28], motor and cognitive impairment produced by 3-NP could be related to energy levels reduction and, consequently, changes on neural processing. The motor alterations observed following the 3-NP administration are assigned mainly to the striate neuronal degeneration, a region functionally connected by motor cortex afferents [29, 30]. The striatal neurodegeneration after 3-NP injection may be associated with free radicals production, due to mitochondrial dysfunction and to the selective vulnerability of dopaminergic motor neurons [18, 31].

It is well established that free radicals capture by antioxidants is related to their hydrogen donation ability [32]. Studies reported that treatment with 3-NP significantly decreases the antioxidant enzyme levels (SOD, catalase) and increases the oxidant enzyme levels (lipid peroxidation, nitrate level), suggesting the role of oxidative stress in neurodegenerative process [33]. Earlier reports have demonstrated clearly that increased oxidative stress may be one of the mainly deleterious events in HD [26, 27].

After 3-NP administration, there was an increase in MDA (lipid peroxidation pointer due to free radicals) and a decrease on superoxide dismutase antioxidant enzyme at striatal region, suggesting an enhancement of central oxidative stress. These effects were attenuated by the treatment with CO, for 7 and 14 days, indicating the antioxidant action of the extract. Furthermore, the antioxidant activity of CO also was demonstrated *in vitro*, by the DPPH radical assay, which showed that CO could decrease free radical levels.

Works have reported that the etiology of HD may be related to dysfunction in the mitochondrial energy production and/or the increase in reactive species of oxygen, which can lead to neuronal excitoxicity [34]. There have been numerous reports suggesting that generation of free radicals may be underlying the neurotoxic effects of succinate dehydrogenase inhibitors as 3-NP and methylmalonic acid [10]. The systemic administration of 3-NP leads to cellular depletion of ATP and induces striatal-specific lesions that are similar to HD [11]. The oxidative stress, in general, is prevented by endogenous antioxidant pathways and free radicals scavengers. Therefore, the use of several antioxidant agents have demonstrated to improve oxidative dysfunction and lesions produced by 3-NP [8, 31, 35].

CO is a species largely used in folk medicine due to its antibacterial, antifugical, laxative, diuretic, analgesic, anti-inflammatory and antioxidant properties [9, 36, 37]. In regard to its chemical composition, this species is well studied, where were found senosideos [38], anthraquinones [10], flavonoids [11], polysaccharides and tannins [12] which are connected with many properties of this species. The flavonoids, a large group of polyphenolic compounds found in fruits and vegetables, are capable to neutralize the neuronal lesion, delaying the progress of neurodegenerative diseases. These benefits are usually attributed to their antioxidant properties [39] that makes them potential targets for use in treatment of many pathologies where the free radicals are involved. Studies demonstrated that phenolic compounds in the CO were the main responsible for antioxidant properties, by hydroxyl radical scavenging activity, metal chelating activity and nitric oxide scavenging activity [40]. Altogether these data suggest that the preventive effects of CO against HD progression may be attributed to antioxidant properties of phenolic compounds in CO and that treatment for up to 14 days could prevent the development of HD in rats. In this way, the present study highlights the prophylactic effect of CO in neurodegenerative conditions, as HD.

5 CONCLUSION

In conclusion, the results of this study confirm that the administration of 3-NP in rats induces neurobehavioral and biochemical changes mimicking those observed in HD. In addition, the treatment of rats with CO for up to 14 days by oral route produced significant neuroprotection and decrease in clinical symptoms of HD in rats, probably mediated through its antioxidant activity. Thus, the present work brought out new insights for the treatment of neurodegenerative diseases, as HD, and highlighted the pharmacological properties of CO, suggesting its effective use in clinical conditions in human. However, the clinical relevance of CO for the treatment of HD warrants further studies.

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