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RESEARCH ARTICLE

Protective effect of *Calendula officinalis* Linn. flowers against 3-nitropropionic acid induced experimental Huntington's disease in rats

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Abstract

Oxidative stress (OS) and nitric oxide mechanisms have been recently proposed in 3-nitropropionic acid (3-NP)-induced neurotoxicity. The compounds, having antioxidant, anti-inflammatory and estrogenic effects, have been suggested for neuroprotection in different experimental models. *Calendula officinalis* Linn. flower extract (COE) is known for its potent antioxidant, anti-inflammatory, estrogenic and neuroprotective activities. Hence, the present study was designed to evaluate the neuroprotective effect of COE on 3-NP-induced neurotoxicity in rats by observing behavioral changes, OS and striatal damage in rat brain. Adult female Wistar rats were pretreated with vehicle or COE (100 and 200 mg/kg) for 7 days, followed by cotreatment with 3-NP (15 mg/kg, intraperitoneally) for the next 7 days. At the end of the treatment schedule, rats were evaluated for alterations in sensory motor functions and short-term memory. Animals were sacrificed and brain homogenates were used for the estimation of lipid peroxidation (LPO), glutathione, total thiols, glutathione *S*-transferase, catalase and nitrite. A set of brain slices was used for the evaluation of neuronal damage in the striatal region of the brain. 3-NP caused significant alterations in animal behavior, oxidative defense system evidenced by raised levels of LPO and nitrite concentration, and depletion of antioxidant levels. It also produced a loss of neuronal cells in the striatal region. Treatment with COE significantly attenuated behavioral alterations, oxidative damage and striatal neuronal loss in 3-NP-treated animals. The present study shows that COE is protective against 3-NP-induced neurotoxicity in rats. The antioxidant, anti-inflammatory and estrogenic properties of COE may be responsible for its neuroprotective action.

Keywords

Excitotoxicity, marigold, oxidative stress, neuroprotective, nitrative stress, TTC

History

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Introduction

Huntington's disease (HD) is a chronic progressive autosomal-dominant neurodegenerative disease, characterized by clinical triad movement disorder; dementia, and psychiatric disturbance resulting from striatal-specific neuronal degeneration (Leegwater-Kim & Cha, 2004). HD patients often exhibit deficits in executive tasks requiring planning, cognitive flexibility and problem solving. HD poses challenges for health- and social-care professionals because of its complexity and unpredictability of the disease (Kumar & Kumar, 2009a).

3-nitropropionic acid (3-NP), a metabolite of 3-nitropropanol, is a natural environmental toxin produced by various fungal species (*Aspergillus flavus*, *Astragalus* and *Arthrinium)* and is present in leguminous plants. 3-NP crosses the blood-brain barrier, and at the cellular level, it is an irreversible inhibitor of the electron transport enzyme, succinate dehydrogenase (SDH), a mitochondrial complex II enzyme responsible for the oxidation of succinate to fumarate in Krebs cycle. Subsequently, it blocks the transport of electrons in oxidative phosphorylation, causing decreased adenosine triphosphate (ATP) level in the brain. Neurons are metabolically highly active cells, hence processes that affect mitochondrial function invariably lead to neuronal death (Ahuja et al., 2008; Túnez et al., 2010). Hence, ingestion of 3-NP by humans or animals may lead to HD-like symptoms.

Calendula officinalis Linn. (Asteraceae), commonly known as marigold, is an herb of ancient medicinal repute in homeopathic and traditional medicine (Cetkovic et al., 2004). It has been reported to possess antibacterial, antifungal, antiviral, antimutagenic, hepatoprotective, renoprotective, free-radical– scavenging and anti-inflammatory actions and also possesses estrogenic and central nervous system properties (Cetkovic et al., 2004; Chandran & Kuttan, 2008; Duke, 1992; Korengath et al., 2009). Moreover, we recently reported protective action of *C. officinalis* flowers against monosodium-glutamate–induced oxidative stress (OS)

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and excitotoxic brain damage in rats (Shivasharan et al., 2012). The compounds, having antioxidant and -inflammatory properties, reportedly possess a beneficial effect in animal models of HD and other neurodegenerative disease (Kumar & Kumar, 2009b; Nagakannan et al., 2012; Thippeswamy et al., 2011). Hence, the present study has been undertaken with the aim of determining the neuroprotective effect of marigold flower extract against 3-NP-induced experimental HD in rats.

Methods

Chemicals

1-Chloro-2,4-dinitrobenzene (CDNB) and 3-NP were obtained from Sigma-Aldrich (St. Louis, MO). Reduced glutathione (GSH), 5,5′-dithiobis-2-nitrobenzoic acid, thiobarbituric acid, trichloroacetic acid (TCA) and 2,3,5 triphenyl tetrazolium chloride (TTC) were purchased from Hi-Media Laboratories Pvt. Ltd. (Mumbai, India). All other chemicals used were of analytical grade.

Plant material and extraction

Fresh flowers of *C. officinalis* L., were collected from the herbal garden of the University of Agricultural Sciences (Dharwad, India). The plant was identified and authenticated by Prof. K. Siddappa, Department of Botany, Sree Siddaganga College of Arts, Science and Commerce (Tumkur, India), and a voucher specimen of the plant was kept in the college herbarium.

Shade-dried flowers were extracted exhaustively with 70% methanol by a cold maceration process for 14 days. The obtained extract was concentrated to get a thick brown semisolid paste (yield, 18.66%).

Estimation of bioactive compounds in *C. officinalis* extract (COE) using the high-performance liquid chromatography (HPLC) method

For HPLC analysis, COE was dissolved in HPLC-grade methanol (1 mg/mL) and subjected to HPLC for analysis of chlorogenic acid, rutin and ferulic acid. The HPLC system (Shimadzu, Kyoto, Japan) was equipped with a dual-pump LC-20AD binary system, a photodiode array detector (SPD-M20A) and a Merck C18 reversed-phase column (I.D 4.6×250 mm) packed with 5-μm-diameter particles. Separation was achieved with a two-pump linear gradient program for pump A (acetonitrile) and pump B (water containing 0.1% formic acid), initially started with a gradient of 10% A changing to 70% in 20 minutes and followed by washing for 40 minutes. Flow rate and injection volume were 1.0 mL/min and 20 μL, respectively. Chromatographic peaks of analytes were confirmed by comparing their retention time and UV spectra with those of the reference standards. Results (mg/g dry weight) were obtained by comparison of peak areas (320 nm) of the samples with that of standards.

Animals and experimental design

Adult female Wistar rats (190–220 g) bred in the animal house of the Sree Siddaganga College of Pharmacy (Tumkur, India) were used. Animals were housed under standard laboratory conditions, maintained on a 12-hour light/dark cycle, and had free access to food and water. Experimental protocols were approved by the local institutional animal ethics committee (SSCPT/IAEC/73/2009-2010) and conducted according to Control and Supervision of Experiments on Animals (CPCSEA) guidelines, under Ministry of Animal Welfare Division, Government of India, New Delhi.

The weighed quantity of COE was suspended in distilled water using 1% Tween 80 (v/v) and administered orally to experimental animals at a constant volume of 5 mL/kg for 14 days. 3-NP was diluted with saline (pH 7.4) and administered intra-peritoneal at the dose of 15 mg/kg, for seven days to induce the neurotoxicity. Doses of 3-NP and COE were selected based on previous literature (Ahuja et al., 2008; Cetkovic et al., 2004). Dosages were adjusted daily according to body weight of animals.

Rats were randomly divided into five groups of 6 each and treated as below; Groups I and III, normal and 3-NP alone respectively, were treated with 1% Tween-80 (5 mL/kg/day). Groups II and V, COE alone and COE higher dose respectively, were treated with COE 200 mg/kg/day and Groups IV, COE low dose were treated with COE 100 mg/kg/day. All these treatments were given for 14 days orally. From days 8 to 14, after two h of the above treatments groups I and II received normal saline 2 mL/kg/day, i.p. and 3-NP (15 mg/kg/days i.p.) was administered to groups III to V. Body weights were measured on regular basis and percent changes in body weights from days 1 to 15 were calculated (Ahuja et al., 2008). On day 14 of the experiment, after 4 h of 3-NP administration, rats were evaluated for behavioral parameters. On day 15 after body weight measurements, rats were sacrificed and brains were isolated for biochemical estimations, and TTC staining.

Neurological scoring

A neurological score was determined for each animal in comparison to control animals. Score was as follows: 0, normal behavior; 1, general slowness of displacement resulting from mild hind limb impairment; 2, in coordination and marked gait abnormalities; 3, hind limb paralysis; 4, incapacity to move resulting from fore limb and hind limb impairment; and 5, recumbency (Ahuja et al., 2008).

Locomotor activity

At the end of the treatment schedule, spontaneous locomotor activity was recorded by using an actophotometer equipped with infrared-sensitive photocells. Before the locomotor task, animals were placed individually in the activity meter for 2 minutes for habituation. Thereafter, locomotor activity was recorded for a period of 5 minutes. Ambulatory activity was expressed in terms of total photo beam counts per 5 minutes (Kulkarni, 1999).

Elevated plus maze test

Memory dysfunction is evaluated using the elevated plus maze test, which consists of two opposite open arms (50×10 cm), crossed with two closed arms of the same dimensions with a 40-cm-high wall. The arms are connected with a central square (10×10 cm). Acquisition of memory was assessed on day 14 after initiating COE treatment. Rats were placed individually

at one end of an open arm facing away from the central square. The time taken by the animal to move from the open arm and enter into one of the closed arms was recorded as initial transfer latency (ITL). Rats were allowed to explore the maze for 30 seconds after recording ITL and returned to its home cage. Retention transfer latency (RTL) was noted again on day 15 (Kumar & Kumar, 2009a). Percent retention of memory was calculated by the following formula:

$$\frac{(\mathrm{ITL}-\mathrm{RTL})}{\mathrm{RTL}}\times 100$$

Beam walking test

The beam walking task was used to assess the motor coordination of animals, where the ability of rats to traverse a horizontal narrow beam (2.3×120 cm) suspended 50 cm above a foam-padded cushion was measured. During testing, rats were given 2 minutes to traverse the beam. If they did not complete the task or if they fell off the beam, the trial was ended, and rats were placed back into their home cages. For successful performers, latency to cross the beam was recorded (Song et al., 2006; Yan et al., 2007).

Limb withdrawal test

In this test, the animal was placed on a 20-cm-high, 30×30 cm platform containing two holes of 5 cm in diameter for hind limbs and two holes with a diameter of 4 cm for forelimbs. The rat was placed on the platform by positioning first the hind limb and then the forelimbs into the holes. The time taken by the animal to retract its first hind limb and the contralateral hind limb were recorded. The difference between the retraction times of both hind limbs was determined (Tariq et al., 2005).

Hanging wire test

The rat was allowed to hold, with the forepaws, a steel wire (2 mm in diameter and 80 cm in length), placed at a height of 50 cm over a cushion support. The length of time the rat was able to hold the wire was recorded. This latency to grip loss is considered as an indirect measure of grip strength (Hunter et al., 2000), and the cut-off time was taken as 90 seconds.

Biochemical estimations

After behavioral tests, animals were sacrificed under deep ether anesthesia and perfused transcardially with an ice-cold saline. Brains were homogenized in cold phosphate-buffered saline (PBS; 10%, w/v), and the suspension was centrifuged at $12{,}000\times g$ (Remi Motors Ltd., Mumbai, India) for 15 minutes at 4 °C. The supernatant was used for the following biochemical analysis.

Total protein content of the brain homogenate was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. LPO was measured by estimating the amount of malondialdehyde (MDA) formed, as described by Gelvan & Saltman (1990,) and the results were expressed as nmol MDA/mg protein. Reduced glutathione (GSH) was measured according to the method of Ellman (1959), and values were expressed as nmol/mg protein. Total thiol (TT) was determined by the method described by Sedlak & Lindsay (1968), and the results were expressed as nmol/mg protein. Glutathione *S*-transferase (GST) activity was measured as described by Habig et al. (1974), and the results were expressed as nmol of CDNB conjugate formed/min/mg protein. Catalase (CAT) activity was measured as described by Claiborne (1985) and expressed as units/mg protein. Nitrite content in brain homogenates was determined by the method described by Green et al. (1982), and the results were expressed as nmol/g tissue.

TTC staining

Animals were deeply anesthetized and transcardially perfused with ice-cold normal saline. Brains were removed, sliced coronally into 2-mm-thick sections, and incubated in PBS (pH 7.4) containing 2% of TTC for 30 minutes at 37 °C in the dark, followed by fixing in 10% neutral-buffered formalin overnight. Images of stained sections were acquired by using a high-resolution scanner, and the lesion area was compared visually (Bederson et al., 1986)

Statistical analysis

Values were expressed as mean ± standard error of the mean (SEM). Statistical analysis was carried out by one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. p-values <0.05 were considered as significant.

Results

Estimation of bioactive compounds in COE using HPLC

Different compositions of acetonitrile and water containing 0.1% formic acid were tried to resolve different components in COE. Gradient composition (acetonitrile/water containing 0.1% formic acid, 10:90 to 70:30) was run up to 20 minutes to give the resolution of chlorogenic acid [running time (RT) = 10.31], rutin (RT = 13.63), and ferulic acid (RT = 15.14) in the presence of other compounds in COE (Figure 1). Presence of chlorogenic acid, rutin, and ferulic acid in COE was confirmed by comparing the UV spectrum of respective standard compounds. The amount of chlorogenic acid, rutin, and ferulic acid in COE was found to be 0.31, 0.02, and 0.1 mg/g of extract, respectively, as quantified by the proposed HPLC method (Figure 1).

Body-weight change

Administration of 3-NP (15 mg/kg i.p. for 7 days) resulted in a significant ($p<0.001$) change in body weight, when compared to normal rats. In the case of 3-NP-alone–treated rats, body weight was drastically decreased and was found to be $85.60\pm 1.06\%$ of initial body weight, whereas normal control rats showed an increased body weight and was $107.10\pm 1.18\%$ of initial body weight. Treatment with COE (100 and 200 mg/kg, p.o.) significantly ($p<0.001$ and $p<0.05$, respectively) attenuated the 3-NP-induced decrease in body weight, and the percentage change in body weights were found to be $98.84\pm 1.40\%$ and $92.19\pm 2.50\%$ of initial body weight, respectively (Table 1).

Neurological scoring

Intraperitoneal administration of 3-NP resulted in motor abnormalities. Out of 6 rats, 3 showed uncoordination and

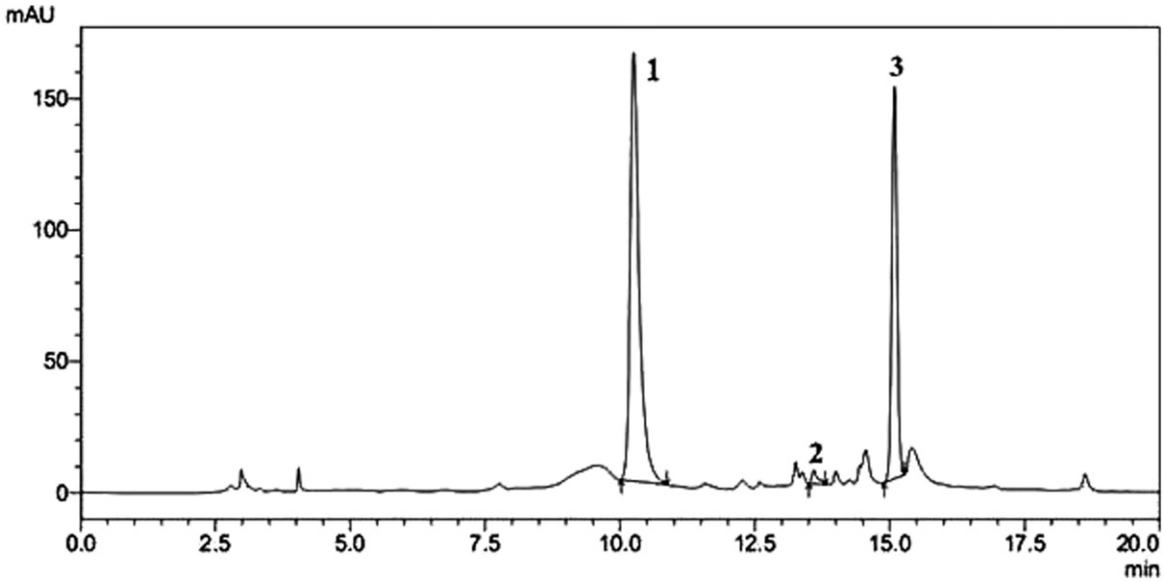

Figure 1. Reversed-phase HPLC chromatogram of hydroalcoholic extract of *C. officinalis* Linn. flowers (COE). Where 1 = chlorogenic acid, 2 = rutin and 3 = ferulic acid.

Table 1. Effect of COE on body weight and behavioral parameters in 3-NP treated rats.

Treatment	% Body weight changes	Neurological scoring	Locomotor activity (Counts/5 min)	Balance beam test (s)	Limb withdrawal test (s)	Hanging wire test (s)
Normal	107.10 ± 1.18^{c}	0.0^{c}	218.80 ± 21.55^{c}	4.61 ± 0.44^{c}	2.82 ± 0.79^{c}	75.64 ± 9.1^{c}
COE alone	103.10 ± 0.95	0.0	191.60 ± 1.69	4.21 ± 0.27	3.2 ± 0.74	$83.22 \pm .78$
3-NP alone	85.60 ± 1.06	2.83 ± 0.47	84.75 ± 15.34	95.13 ± 16.4	46.3 ± 11.4	17.16 ± 4.07
COE 100	98.84 ± 1.40^{c}	0.60 ± 0.40^{c}	183.80 ± 9.19^{b}	7.83 ± 3.20^{c}	6.55 ± 1.42^{c}	60.63 ± 8.50^{b}
COE 200	92.19 ± 2.50^{a}	1.40 ± 0.40^{a}	146.40 ± 8.67^{a}	56.64 ± 8.23^{a}	19.16 ± 5.07^{a}	30.55 ± 1.43

Each value are expressed as mean ± SEM ($n = 6$), $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$ when compared to 3-NP-alone–treated rats. One-way ANOVA followed by Tukeys post-hoc test.

marked gait abnormalities, 2 showed hind limb paralysis, and 1 was unable to move resulting from hind limb and forelimb impairment and gained a significantly high ($p < 0.001$) neurological score (2.83 ± 0.47), when compared to normal control rats. Pretreatment with COE (100 and 200 mg/kg) to 3-NP-treated rats showed significant ($p < 0.001$ and $p < 0.05$) improvement in behavioral changes, when compared to 3-NP-alone–treated animals, and the neurological score was found to be 0.60 ± 0.40 and 1.40 ± 0.40, respectively (Table 1).

Locomotor activity

A significant ($p < 0.001$) decrease in locomotor counts (measured as counts/5 minutes) were observed in 3-NP-alone–treated animals (84.75 ± 15.34), when compared to normal control animals (218.8 ± 21.55). Pretreatment with COE (100 and 200 mg/kg) to 3-NP-treated rats significantly ($p < 0.001$ and $p < 0.05$) increased locomotor counts, when compared to 3-NP-alone–treated animals and was found to be 183.8 ± 9.19 and 146.4 ± 8.67, respectively (Table 1).

Elevated plus maze test

In the present experiment, mean ITL on day 14 was relatively stable in all animals within the group. 3-NP-alone–administered rats showed a significant ($p < 0.001$) increase in mean RTL, compared to normal control animals. Normal control animals entered the closed arm quickly and mean RTL was shorter, when compared with its own ITL ($49.02 \pm 13.70\%$). In contrast, 3-NP-treated rats performed poorly and showed an increased mean RTL ($136.0 \pm 48.11\%$), compared with its own ITL. This indicates there is cognitive dysfunction in 3-NP-treated animals. Chronic pretreatment with COE (100 and 200 mg/kg, p.o.) to 3-NP-treated rats showed significant ($p < 0.01$ and $p < 0.05$) improvement in memory performance, when compared to 3-NP-alone–treated rats. Mean RTL was reduced ($22.03 \pm 9.49\%$ and $21.87 \pm 8.27\%$, respectively), when compared with its ITL (Figure 2).

Balance beam test

Motor coordination and body balance were significantly ($p < 0.001$) affected in 3-NP-administered rats, when compared to normal control rats. Normal control animals traversed the beam in 4.61 ± 0.44 seconds, whereas 3-NP-alone–treated rats showed 195.99% increase in latency to cross the beam (95.13 ± 16.47 seconds). Pretreatment with COE (100 and 200 mg/kg) significantly ($p < 0.001$ and $p < 0.05$) improved motor coordination and body balance in 3-NP-treated rats and showed a decrease (7.83 ± 3.20 and 56.64 ± 8.23 seconds, respectively) in latency to traverse the beam (Table 1).

Limb withdrawal test

Reaction time difference between the hind limbs was significantly ($p<0.001$) higher in 3-NP-alone–treated rats, when compared to normal control rats; this indicates the functional abnormalities in the hind limbs in 3-NP-treated rats. Normal control rats were able to quickly retract both their hind limbs (2.82 ± 0.79 seconds) and was increased by 93.89% in 3-NP-alone–treated rats (46.31 ± 11.42 seconds), demonstrating the extent of 3-NP-induced striatal degeneration. Pretreatment with COE (100 and 200 mg/kg) significantly ($p<0.001$ and $p<0.05$) improved hind limb functions (6.55 ± 1.42 and 19.16 ± 5.07 seconds) in 3-NP-treated rats, when compared to 3-NP-alone–treated rats (Table 1).

Hanging wire test

Animals treated with 3-NP significantly ($p<0.001$) lost grip strength, when compared to normal control animals. 3-NP-alone–treated rats showed 77.31% decreased fall latency (17.16 ± 4.07), when compared to normal control animals (75.64 ± 9.11). Pretreatment with COE (100 and 200 mg/kg) improved grip strength (60.63 ± 8.5 and 30.55 ± 1.43 s) in 3-NP-treated rats. COE-alone (200 mg/kg)-treated rats showed better grip strength (83.22 ± 6.78 seconds), when compared to normal control animals (Table 1).

Biochemical estimations

LPO The levels of LPO (measured as nmol of MDA/mg protein) where found to be significantly ($p<0.001$) higher in 3-NP-treated rats, when compared to normal control animals. 3-NP-alone–treated rats showed 121.03% higher levels of LPO (4.42 ± 0.23) than normal control animals (2.00 ± 0.31). Pretreatment with COE (100 and 200 mg/kg) decreased the level of LPO significantly ($p<0.01$ and $p<0.05$) in 3-NP-treated rats, when compared to 3-NP-alone–treated animals (2.86 ± 0.39 and 2.90 ± 0.26, respectively) (Table 2).

GSH There was significant ($p<0.001$) depletion of GSH in 3-NP-alone–treated rats, as compared to control rats (measured as nmol/mg protein). Normal control rats showed a 4.90 ± 0.44 level, whereas 3-NP-alone–treated rats showed an 80.28% reduced level of GSH (0.97 ± 0.20). Both the tested doses of COE pretreatment significantly ($p<0.05$ and $p<0.001$) protected animals against 3-NP-induced GSH depletion, and GSH levels in these animals were found to be 3.29 ± 0.31 and 4.37 ± 0.86, respectively (Table 2).

TT A significantly ($p<0.05$) reduced level (measured as nmol/mg protein) of TT was observed in 3-NP-alone–treated animals (18.48 ± 0.28), when compared to normal control animals (25.78 ± 2.70). Pretreatment with COE (100 and 200 mg/kg) elevated TT content (23.64 ± 0.70 and 21.36 ± 1.44, respectively) in 3-NP-treated rats, when compared to 3-NP-alone–treated rats, but the difference was statistically insignificant (Table 2).

GST Activity of GST (measured as nmole CDNB conjugate formed/min/mg protein) was significantly ($p<0.001$) reduced in 3-NP-treated rats (7.21 ± 1.01), when compared to normal control animals (15.19 ± 1.01). Pretreatment with COE (100 and 200 mg/kg) significantly ($p<0.01$ and $p<0.05$) prevented decline in GST activity (12.07 ± 0.70 and 9.14 ± 1.14, respectively) in 3-NP-treated rats, when compared to 3-NP-alone–treated rats (Table 2).

CAT Administration of 3-NP (15 mg/kg for 7 days) resulted in a significant ($p<0.001$) decrease in CAT activity (measured as U/mg protein), as compared to control animals. CAT activity in normal control animal showed a level of 21.52 ± 2.40, and administration of 3-NP alone caused a depletion of 70.80% of CAT activity (6.28 ± 0.67). Both the tested doses of COE pretreatment to 3-NP-treated rats showed significantly ($p<0.01$) increased CAT level (15.38 ± 1.26 and 12.22 ± 1.27, respectively) (Table 2).

Nitrite Tissue nitrite content (measured as nmol/g tissue) was significantly ($p<0.01$) elevated in 3-NP-treated rats (37.13 ± 2.59), when compared to normal control rats (25.42 ± 1.19). Pretreatment with COE (100 and 200 mg/kg) significantly ($p<0.001$ and $p<0.01$) reduced nitrite content (22.21 ± 2.52 and 22.86 ± 1.45, respectively) in 3-NP-treated rats, when compared to 3-NP-alone–treated rats (Table 2).

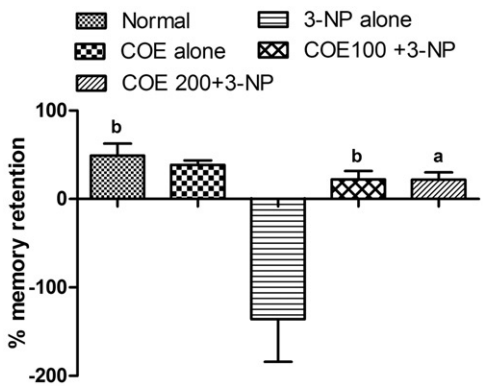

Figure 2. Effect of COE on elevated plus maze test in 3-NP-treated rats. Each bar represents mean ± SEM ($n=6$); $^{a}p<0.05$ and $^{b}p<0.01$ when compared to 3-NP-alone–treated rats. One-way ANOVA followed by Tukey's *post-hoc* test.

Table 2. Effect of COE on biochemical estimations in 3-NP treated rats.

Treatment	LPO (nmol MDA/mg protein)	GSH (nmol/mg protein)	TT (nmol/mg protein)	GST (nmol CDNB conjugate formed/min/mg protein)	CAT (U/mg protein)	Nitrite (nmol/g tissue)
Normal	2.00 ± 0.32^{c}	4.90 ± 0.44^{c}	25.78 ± 2.70^{a}	15.19 ± 1.01^{c}	21.52 ± 2.40^{c}	25.42 ± 1.19^{b}
COE alone	2.02 ± 0.21	4.63 ± 0.75	23.75 ± 1.60	12.05 ± 0.94	20.76 ± 1.56	25.83 ± 2.10
3-NP alone	4.43 ± 0.24	0.97 ± 0.20	18.48 ± 0.28	7.21 ± 1.01	6.28 ± 0.67	37.13 ± 2.59
COE 100	2.86 ± 0.39^{b}	3.29 ± 0.31^{a}	23.64 ± 0.70	12.07 ± 0.70^{b}	15.38 ± 1.26^{b}	22.21 ± 2.52^{c}
COE 200	2.90 ± 0.26^{a}	4.37 ± 0.86^{b}	21.36 ± 1.44	9.14 ± 1.14	12.22 ± 1.27^{b}	22.86 ± 1.44^{b}

Each value are expressed as mean ± SEM ($n=6$), $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$when compared to 3-NP-alone–treated rats. One-way ANOVA followed by Tukeys *post-hoc* test.

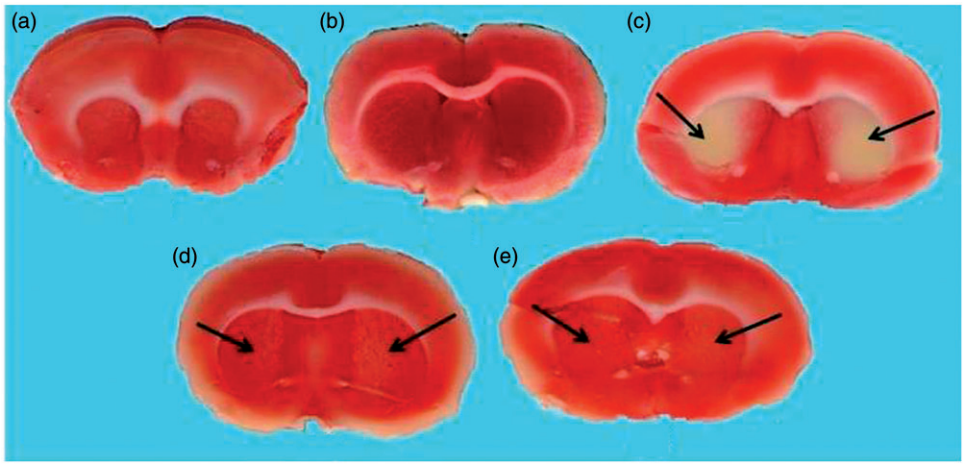

Figure 3. A representative photograph of rat brain coronal sections after staining with TTC in experimental rats. (a) Normal control; (b) COE alone; (c) 3-NP alone; (d) 3-NP + COE 100 mg/kg; (e) 3-NP + COE 200 mg/kg. Arrow indicates area of brain damage.

TTC staining

Extent of depletion of SDH was determined by TTC staining, which produces deep red color on reaction with this enzyme. The 3-NP-alone–treated group exhibited a large lesion in the striatal region, indicating a massive depletion of SDH. Pretreatment with COE markedly attenuated the depletion of this enzyme in 3-NP-treated animals. Brain sections of COE-treated animals showed a decreased disappearance of red color in the striatal region, indicating the antagonistic effect against 3-NP-induced striatal degeneration (Figure 3).

Discussion

Neurons demand a high-energy source to maintain ion gradients across the plasma membrane that is critical for the generation of action potentials. This intense energy requirement is continuous; even brief periods of oxygen or glucose deprivation can result in neuronal cell death (James et al., 2005)

Mitochondrial toxin 3-NP is an irreversible inhibitor of SDH (respiratory chain complex II), and the TCA cycle inhibits energy metabolism and induces HD-like symptoms in the caudate putamen. Mitochondrial membrane potential ($\Delta\Psi m$) collapse is also well documented in 3-NP toxicity (Ahuja et al., 2008; James et al., 2005), $\Delta\psi m$ is directly related to ATP production, and reduced $\Delta\Psi m$ likely contributes to energy failure (Leegwater-Kim & Cha, 2004). Hence, $\Delta\Psi m$ is a critical event in the life-death decision of neurons. Moreover, mitochondria are cytosolic organelles essential for generating the energy that fuels normal cellular function and, at the same time, are the major intracellular source of cytotoxic free radicals and the primary determinants of cell death (James et al., 2005)

In the present study, subchronic administration of 3-NP (15 mg/kg, i.p., for 7 days) produced a significant decrease in body weight, motor and cognition related behaviors, and antioxidant status in the rat brain; these observations exactly mimic the symptoms of HD patients (Leegwater-Kim & Cha, 2004).

The reduction of body weight in 3-NP-treated rats could be due to 3-NP-induced metabolic impairment (i.e. mobilization of energy stores and impairment in energy metabolism). However, bradykinesia and striatal lesions may be contributing factors for weight loss (Beal et al., 1993a; Fontaine et al., 2000). Both the tested doses of COE pretreatment prevented the body-weight reduction produced by 3-NP, indicating mitochondrial protection ability of COE.

Administration of 3-NP is associated with both hypo- and hyperactivity, depending on time and frequency of treatment (Ahuja et al., 2008). Both the tested doses of COE pretreatment improved the hypoactivity and rigidity produced by 3-NP, as evident by behavioral investigations, such as movement analysis and locomotor activity. Rigidity and movement disorders are related to basal ganglia lesions (Guyot et al., 1997). Hence, the beneficial effect of COE pretreatment on hypoactivity and rigidity depicts its protection against 3-NP-induced striatal lessons.

Although 3-NP-induced lesions are mostly striatal specific, many reports suggest that the neurons located in the hippocampus, thalamus and brain cortex are also affected (Túnez et al., 2010). 3-NP causes lesions in hippocampal CA1 and CA3 pyramidal neurons – the brain area associated with cognitive performance (Patocka et al., 2000). Chronic systemic administration of 3-NP causes memory dysfunction, where cognitive dysfunction is one of the characteristic features of HD patients (Brandt & Strauss, 1986; Butters et al., 1978). In the present study, COE treatment for 2 weeks significantly prevented the decline in cognitive performance and showed improved memory retention in 3-NP-treated rats.

Treatment of rats with 3-NP produced muscle weakness and rigidity similar to HD patients (Túnez et al., 2010) and was evident by beam walking task and grip strength test observations of these animals. Pretreatment with COE (100 and 200 mg/kg) to 3-NP-treated rats showed decreased latency to cross the beam, indicating improved motor coordination and decreased muscle rigidity. Further, there is increased hanging wire latency by these animals, which reflects its increased grip and muscle strength.

3-NP-induced striatal neuronal degeneration produces functional abnormalities in the hind limbs (Tariq et al., 2005). In the present study, increased reaction time between the hind limbs in 3-NP-treated rats well supports the above-described finding. Pretreatment with COE (100 and 200 mg/kg) normalized hind limb functions in 3-NP-treated

rats, which indicate the protection of COE against striatal neuronal degeneration.

Oxidative damage and involvement of glutamate-related excitotoxicity play an important role in 3-NP-induced neurotoxicity (Beal et al., 1993b; Schulz et al., 1996). Increased influx of Ca^{++} resulting from increased glutamate levels may lead to activation of Ca^{++}-dependent nitric oxide synthase, causing an increase in OS and nitrative stress (Reynolds & Hastings, 1995). In the present study, a significantly elevated level of nitrite and MDA and a decreased level of antioxidants (GSH, TT, GST and CAT) in 3-NP-treated rat brains well supports the above-described findings. Treatment with COE (100 and 200 mg/kg) in 3-NP-treated rats significantly prevented the depletion of antioxidant enzymes and decreased OS and nitrative stress. COE has very good antioxidant potential (Cetkovic et al., 2004) and can form relatively stable phenol-derived free radicals that are stabilized as a result of resonance and steric hindrance possessed by the molecule. These findings are further confirmed by TTC staining, where COE prevented the changes produced by 3-NP and showed protection against 3-NP-induced HD-like symptoms.

There are enough reports stating that 3-NP-induced neurotoxicity involves inflammatory response (Ahuja et al., 2008; Fontaine et al., 2000), and *C. officinalis* flowers reportedly possess an anti-inflammatory property (Korengath et al., 2009) that may contribute to its neuroprotective action. In addition to antioxidant and -inflammatory activity, *C. officinalis* flowers are well known for their estrogenic property (Duke, 1992; Naguib et al., 2005). Accumulating reports indicates that estrogen protects neurons from degeneration in different experimental models of neurotoxicity. Hence, in addition to antioxidant and -inflammatory activities, the estrogenic effect of COE may play an important role in protecting animals from 3-NP-induced neurotoxicity.

Estrogen exerts neuroprotective action through its high antioxidant capacity, increased blood supply to the brain, hepatic coagulation protein expression, decreasing apoptotic and increasing antiapoptotic gene expression, counteracting release of cytochrome c from mitochondria, and inhibiting inflammation through downregulation of tumor necrosis factor alpha, especially in the stroke condition (Strom et al., 2009).

Concentration of estrogen in blood circulation plays an important role in its neuroprotective action (Brann et al., 2007). Estrogen prevents the production of reactive oxygen species and apoptosis only at low concentrations (Wang et al., 2001). The antioxidant effects of estrogens may prevail at physiologic concentrations, whereas redox cycling at higher estrogen concentrations may lead to increased radical generation and damage to neurons (Carswell et al., 2004; Kirsty et al., 2005; Strom et al., 2009). This may be the reason for which, in the present study, a higher dose of COE showed a less-beneficial effect, compared with its lower dose.

The flowers of *C. officinalis* Linn. contains flavonoids, such as quercetin, isorhamnetin, isoquercetin, calendoflaside, calendoflavoside, calendoflavobioside and rutin. The flowers are also rich in carotenoids, such as α-carotene and β-carotene, and quinones, such as plastoquinone, phylloquinone and α-tocopherol (Muley et al., 2009). Flavonoids, carotenoids and quinones are well documented for their beneficial effect in protecting organs against various toxic insults.

Even though the literature suggested the presence of various flavonoids in *C. officinalis*, in this study, we are reporting the presence of chlorogenic acid, rutin and ferulic acid in the title plant. The amount of chlorogenic acid, rutin and ferulic acid in COE was found to be 0.31, 0.02 and 0.1 mg/g of extract, respectively, as quantified by the proposed HPLC method (Figure 1). The observed beneficial effects of *C. officinalis* in 3-NP-induced neurotoxicity may be attributed to the identified bioactive compounds (chlorogenic acid, rutin and ferulic acid), in addition to its earlier reported constituents. Further, it could also result from the synergizing action of a combination of several components acting by modulating the multiple factors involved in 3-NP toxicity.

Conclusion

Conclusively, the hydroalcoholic extract of *C. officinalis* Linn. flower has potential protective effect against 3-NP-induced neurotoxicity in the rats. The observed protective effects may be the result of antioxidant, anti-inflammatory and estrogenic properties of COE. However, further research is required to elucidate the specific mode of action.

Declaration of interest

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