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Neuroprotective effect of pet ether extract *of Ficus religiosa* (L.) leaves in 3-nitropropionic acid induced Huntington disease

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Abstract: Huntington's disease (HD) is a neurodegenerative disease that leads to progressive motor impairment, cognitive dysfunction and abnormal body movements. Systemic intraperitoneal administration of 3 nitropropionic acid (3 NP) inhibit oxidative phosphorylation and cause striatum neuronal degeneration as seen in HD. Nowadays modernization of lifestyle and none of the suitable drug treatment available for the management of HD, there is clear need to investigate alternative. Based on above, present study has been designed to explore the possible role of pet ether extract of *Ficus religiosa* (PEFRE) against 3 NP induced neurotoxicity. 14 days administration of 3 NP caused significant decreased motor in coordination (locomotor and rotarod performance) and significant cognitive impairment (elevated plus maze performance) as compared to vehicle treated animals. Biochemical analysis showed significant increase in lipid peroxidation and acetylcholineterase enzyme level; and depleted superoxide dismutase, catalase and reduced glutathione level. Daily administration of pet ether extract of *F. religiosa* (400 mg/kg) significantly improved motor and cognitive performance. *F. religiosa* significantly attenuated oxidative damage. Lower dose of PEFRE did not show significant activity. These finding suggest that neuroprotective action of *F. religiosa* could be used as an effective therapeutic agent in the management of Huntington's disease.
Keywords: Huntington's disease, Oxidative stress, *Ficus religiosa*, 3 Nitropropionic acid.

1. Introduction

A chronic progressively neurodegenerative Huntington's disease (HD) is characterized by progressive cognitive motor impairment, psychiatric disturbances and weight loss. Neuropathophysiology of HD involves the generation of excitotoxic events, the alteration in energy metabolism, mitochondrial dysfunction and oxidative stress. Several experiments have been made to develop HD in rodents (1). Neurotoxins such as kainic, quinolinic and ibotenic acid have been injected into specific region of brain to develop HD.

One such toxin, 3 nitropropionic acid (3 NP), a naturally occurring plant mycotoxin, produced by the fungus *Arthrinium spp*. 3 NP is a suicidal irreversible inhibitor of respiratory chain and kreb's cycle enzyme, succinate dehydrogenase. 3 NP interfers with ATP synthesis and causes striatal neuropathy similar to these seen in HD patients (2, 3). In spite of several attempts and extensive research this disease remains incurable. The current available drug treatments for HD possess various side effects. Therefore, herbal therapies should be considered as alternative/complementary medicines for therapeutic approach.

Ficus religiosa Linn (Moraceae) commonly known as 'Pimpala' or 'Pipal' tree is a large widely deciduous tree, heart-shaped without aerial roots from the branches, with spreading branches and grey bark (4-6). *F. religiosa* has been used in traditional medicine for a wide range of ailments. Its bark, fruits, leaves, roots, latex and seeds are medicinally used in different forms, sometimes in combination with other herbs (7). The whole parts of the plant exhibit wide spectrum of activities such as anticancer, antioxidant, antidiabetic, antimicrobial, anticonvulsant, anthelmintic, antiulcer, antiasthmatic, antiamnesic etc. Bark of the plant have been used for astringent, cooling, aphrodisiac, antibacterial against *Staphylococcus aureus* and *Escherichia coli*, gonorrhoea, diarrhoea, dysentery, haemorrhoids and gastrohelcosis, anti-inflammatory and burns. The leaves of the plant have been used for haemoptysis, epistaxis, haematuria, menorrhagia, blood dysentery and skin diseases. Leaf juice has been used for asthma, cough, sexual disorders, diarrhea, haematuria, toothache, migraine, eye troubles, gastric problems and scabies. Fruits of the plant were used in asthma, laxative and digestive. Seeds of the plant used in Refrigerant, laxative and Latex used in Neuralgia, inflammations and haemorrhages (8). As *F. religiosa* has been used traditionally in the treatment of neurodegenerative disorders (including Huntington's disease) and has also been reported to possess antioxidant activity; this plant may prove to be effective in the remedy of HD. Hence *F. religiosa* was evaluated for its neuroprotective effect using neurotoxin i.e. 3 NP induced Huntington's disease model in rats.

2. Materials and Methods

2.1. Collection of plant material.

Fresh leaves of *Ficus religiosa* were collected from local area of Ahmedabad district, Gujarat, India during July-September. This plant was identified and authenticated by Dr. A. Benniamin, Scientist D, Botanical Survey of India, Pune. Voucher specimens no. (BSI/WC/Tech./2015/JOB-1) have been kept in Botanical Survey of India, Pune, Maharashtra, India.

2.2. Animals.

Adult male Wistar rats, weighing 180-220 g and albino mice of either sex weighing 25-30 g were used and acclimatized to laboratory condition for one week. All animals were housed in well ventilated polypropylene cages at 12:12 h light/dark schedule at 25±2 ºC and 55-65 % RH. The rats were fed with commercial pelleted rats chow and water *ad libitum* as a standard diet. Institutional Animal Ethics Committee approved the experimental protocol in accordance with the committee for the purpose of control and supervision of experiments on animals (CPCSEA).

2.3. Preparation of leaf extract.

The leaves were collected and dried in shade and ground. Coarsely powdered plant material (1000 g) was weighed and extracted with 5 lit of solvents like petroleum ether (60–80 ºC), ethyl acetate and ethanol by successive extraction in a soxhlet apparatus for 72 h. After each extraction the solvent was distilled off and concentrated extract was transferred to previously weighed petri dish and evaporated to dryness at room temperature (45-50 ºC) to obtain dried extracts. The dried extract was weighed and the percentage yield of the extracts was calculated as follow:

$$\%\ \text{of extractive yield}\ \left(\frac{w}{w}\right) = \frac{\text{Weight of dried extract}}{\text{Weight of dried leaves powder}} \times 100$$

The yield of petroleum ether, ethyl acetate and ethanol extract were found to be 18.2, 10.6 and 26.8 % (w/w) respectively.

2.4. Preliminary phytochemical studies.

Preliminary qualitative phytochemical screening were done for the presence of different group of chemicals i.e. alkaloids, flavonoids, saponins, tannins, sterols, carbohydrates, and glycosides as described by Harborne (9).

2.4.1 Test for tannins and phenols. 5 ml of extract was added to 2 ml of 5 % of alcoholic ferric chloride solution. Blue-Black precipitate indicated the presence of tannins and phenols.

2.4.2 Test for Alkaloids. To the dry extract (10-20 mg) dilute hydrochloric acid (1-2 ml) was added, shaken well and filtered. With filtrate following tests were performed.

2.4.2.1 Mayer's test. To 2-3 ml of filtrate, 2-3 drops of Mayer's reagent was added. Appearance of precipitate indicated presence of alkaloids.

2.4.2.2 Wagner's test. To 2-3 ml of filtrate, Wagner's (3-5 drops) reagent was added. Appearance of reddish brown precipitate indicated presence of alkaloids.

2.4.2.3. Hager's test. To 2-3 ml of filtrate, 4-5 drops of Hager's reagent was added. Appearance of yellow precipitate indicated presence of alkaloids.

2.4.2.4 Dragendorff's test. To 2-3 ml of filtrate, 4-5 drops of Dragendorff's reagent was added. Appearance of orange brown precipitate indicated presence of alkaloids.

2.4.3 Test for saponins. About 1 g of dried powdered sample was boiled with 10 ml of distilled water. Frothing persistence indicated the presence of saponins.

2.4.4 Test for terpenoids. 5 ml of extract was mixed with 2 ml of chloroform and few drops of concentrated sulphuric acid was carefully added to form a layer. Red ring indicated the presence of terpenoids.

2.4.5 Test for steroids (Liebermann Burchard reaction). 5 ml of extract was mixed with 10 ml of chloroform and 1 ml acetic anhydride and few drops of concentrated sulphuric acid were added. Green ring indicated the presence of steroids.

2.4.6 Test for flavonoids (Shinoda test). To dry extract (10-20 mg), 5 ml of ethanol (95 %), 2-3 drops of hydrochloric acid and 0.5 g magnesium turnings were added. Change of color of solution to pink indicated presence of flavonoids.

2.4.7 Test for Carbohydrates (Molish's test). Few drops (2-3) of α-naphthol solution in alcohol was added to 2-3 ml of solution of extract, shaken for few min and then 0.5 ml of concentrated sulphuric acid was added from the side of test tube. The formation of violet ring at the junction of two solutions indicated presence of carbohydrates.

2.4.8 Test for glycosides.

2.4.8.1 Legal's test. To the extract, 1 ml of pyridine and 1 ml of sodium nitroprusside were added. Change in color to pink or red indicated presence of cardiac glycosides.

2.4.8.2 Keller-Killiani test. Glacial acetic acid (3-5 drops), one drop of 5 % of ferric chloride and concentrated sulphuric acid was added to the test tube containing 2 ml of solution of extract. Appearance of reddish brown color at the junction of two layers and bluish green in the upper layer indicated presence of cardiac glycosides.

2.4.8.3 Borntrager's test. Dilute sulphuric acid was added to 2 ml of solution of extract, boiled for few min and filtered. To the filtrate 2 ml of benzene or chloroform was added and shaken well. Separated the organic layer and ammonia was added. The change in color of ammonial layer to pink red indicated presence of anthraquinone glycosides.

2.4.9 Test for phlobatannins. About 2 ml of extract was boiled with 2 ml of 1 % hydrochloric acid. Deposition of red color indicated the presence of phlobatannins.

2.4.10 Test for amino acid (Ninhydrin test). 5 to 6 drops of Ninhydrin reagent were added in 5 ml of extract and heated over boiling water bath for 5 min. Purple coloration indicated the presence of amino acid.

2.4.11 Test for proteins (Biuret test). 5-6 drops of 5 % sodium hydroxide and 5-7 drops of 1 % of copper sulfate were added in 2 ml of extract. Violet color indicated the presence of protein.

2.5. Acute oral toxicity of the extract.

The mice were divided into 5 groups of 10 animals each. The mice were fasted for 6 h and had access to only water *ad libitum* before experimental study. Group I received only vehicle (distilled water). Groups II, III, IV and V received different doses of pet. ether extract of *F. religiosa* (PEFRE) i.e. 1000, 2000, 3000 and 4000 mg/kg respectively. All the doses and vehicle were administered orally. The animals were observed for 72 h for mortality (10).

2.6. Induction of Huntington's disease and Experimental design.

HD was induced by a neurotoxin i.e. 3-nitropropionic acid (3 NP) (11, 12). It is known that systemic intraperitoneal administration of 3 NP increases oxidative stress leads to neuronal death and bilateral lesion in striatum region of the rodents. In the present investigation, animals were divided into Six groups of 8 rats in each. Group I served as vehicle control, received normal saline (10 ml/kg, p.o.). Group II to VI were induced with 3 NP (30 mg/kg, i.p.) daily for 14 days and treatment schedule was as follows: Group II served as a 3 NP control, received normal saline (10 ml/kg, p.o.), Group III served as a standard, received Rivastigmine (1 mg/kg, p.o.), Group IV to VI served as a test drug, received PEFRE (100, 200 and 400 mg/kg, p.o. respectively). Rivastigmine and PEFRE were given 1 hour prior to 3 NP administration for 14 days.

2.7. Behavioral Assessment

2.7.1. Locomotor activity.

The spontaneous locomotor activity was monitored using digital actophotometer (Hicon instrument, India) equipped with infrared sensitive photocells. The apparatus was placed in a darkened, light and sound attenuated and ventilated testing room. Each interruption of a beam on the X or Y axis generated an electric impulse, which was presented on a digital counter. Each animal was observed over a period of 5 min on days 1, 3, 7, 10 and 14 after 3 NP administration and values expressed as counts per 5 min (13).

2.7.2. Rotarod activity.

All animals were evaluated for grip strength by using the rotarod. The rotarod test is widely used in rodents to assess their "minimal neurological defeit" such as motor function and coordination. Each rat was given a prior training session before initialization of therapy to acclimatize them on a rotarod apparatus (EIE instrument, India). Animal was placed on the rotating rod with a diameter of 7 cm (speed 25 rpm). Each rat was subjected to three separate trials at 5 min interval on days 1, 3, 7, 10 and 14 after 3 NP administration and cut off time (180 s) was maintained throughout the experiment. The average results were recorded as fall of time (14).

2.7.3. Elevated plus maze test.

Memory dysfunction was evaluated using elevated plus maze, which consists of two opposite black open arms (50 × 10 cm), crossed with two closed walls of the same dimensions with 40 cm high walls. The arms are connected with a central square of dimensions 10 × 10 cm. The entire maze was elevated to a height of 50 cm from the floor. Memory dysfunction was assessed 60 min after administration of the vehicle or test drug on days 1, 3, 7, 10 and 14. The animals were placed individually at one end of an open arm facing away from the central square. During a 5 min test session, number of entries into open arm, number of entries into closed arm, time spent in the open arm and time spent in the closed arm were noted (15-17).

2.10. Dissection and Homogenization.

After the treatment period, animals were sacrified by decapitation immediately after behavioral assessment. The brains were immediately removed, forebrain was dissected out, and cerebellum was discarded. Brains were put on ice and rinsed in ice-cold isotonic saline to remove blood. A 10 % (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). The homogenate was centrifuged at 10,000 g for 15 minutes and aliquots of supernatant obtained was used for biochemical estimation.

2.11. Biochemical Estimation

2.11.1. Malondialdehyde (MDA) level.

The amount of malondialdehyde was used as an indirect measure of lipid peroxidation and was determined by reaction with thiobarbituric acid (TBA) (18). Briefly, 1 ml of aliquots of supernatant were placed in test tubes and added to 3 ml of TBA reagent: TBA 0.38 % (w/w), 0.25 M hydrochloric acid (HCl), trichloroacetic acid (TCA 15 %). The solution was shaken and placed for 15 min, followed by cooling in an ice bath. After cooling, solution was centrifuged to 3500 g for 10 min. The upper layer was collected and assessed with a spectrophotometer at 532 nm. All determinations were made in triplicate. Results were expressed as nanomoles per mg of protein. The concentration of MDA was calculated using the formula:

$$\text{Conc. of MDA} = \frac{\text{Abs}_{532} \times 100 \times V_T}{(1.56 \times 10^5) \times W_T \times V_U}$$

Where Abs_{532} is absorbance, V_T is total volume of mixture (4 ml), 1.56×10^5 is molar extinction coefficient, W_T is weight of dissected brain (1 g) and V_U is aliquote volume (1 ml)

2.11.2. Superoxide Dismutase (SOD) level.

SOD activity was determined according to the method described by Beyer and Fridovich 1987 (19). 0.1 ml of supernatant was mixed with 0.1 ml of EDTA ($1x10^{-4}$ M), 0.5 ml of carbonate buffer (pH 9.7) and 1 ml of Epinephrine (1 mM). The optical density of formed adrenochrome was read at 480 nm for 3 min on spectrophotometer. The enzyme activity was expressed in terms of U/min/mg. One unit of enzyme activity is defined as the concentration required for the inhibition of the chromogen production by 50 % in one minute under the defined assay conditions.

2.11.3. Catalase (CAT) level.

The catalase activity was assessed by the method Aebi 1974 (20). The assay mixture consists of 0.05 ml of supernatant of tissue homogenate (10 %) and 1.95 ml of 50 mM phosphate buffer (pH 7.0) in 3 ml cuvette. 1 ml of 30 mM hydrogen peroxide (H_2O_2) was added and changes in absorbance were followed for 30 s at 240 nm at 15 s intervals. The catalase activity was calculated using the millimolar extinction coefficient of H_2O_2 (0.071 mmol cm^{-1}) and the activity was expressed as micromoles of H_2O_2 oxidized per minute per milligram protein.

$$\text{CAT activity} = \frac{\delta \text{ O.D.}}{E \times \text{Vol. of Sample (ml)} \times \text{mg of protein}}$$

Where δ O.D. is change in absorbance/minute; E is extinction coefficient of hydrogen peroxide (0.071 mmol cm^{-1})

2.11.4. GSH Level (Reduced Glutathione).

For the estimation of reduced glutathione, 1 ml of tissue homogenate was precipitated with 1 ml of 10 % TCA. To an aliquot of the supernatant, 4 ml of phosphate solution and 0.5 ml of 5, 5'-dithio-bis- (2-nitrobenzoic acid) (DTNB) reagent were added and absorbance was taken at 412 nm (21). The values were expressed as nM of reduced glutathione per mg of protein.

$$\text{GSH level} = \frac{Y - 0.00314}{0}.0314 \times \frac{D_F}{B_T \times V_U}$$

Where Y is Abs_{412} of tissue homogenate, D_F is dilution factor (1), B_T is brain tissue homogenate (1 ml) and V_U is aliquote volume (1 ml).

2.11.5. Acetyl Cholinesterase (AChE) Activity.

AChE is a marker of loss of cholinergic neurons in the forebrain. The AChE activity was assessed by Ellman method (22). The assay mixture contained 0.05 ml of supernatant, 3 ml of 0.01 M sodium phosphate buffer (pH 8), 0.1 ml of acetylthiocholine iodide, and 0.1 ml of DTNB (Ellman reagent). The change in absorbance was measured for 2 min at 30 s interval at 412 nm using UV VISIBLE spectrophotometer. The rate of the reaction was calculated using following formula:

$$R = \frac{\delta A}{Co} \times 5.74 \times 10^{-4}$$

Where, R is rate in moles of substrate hydrolyzed / minute / g tissue; δA is change in absorbance / min; CO is original concentration of the tissue (mg/ml).

2.12 Histopathological studies.

A section of the rat brains were fixed in 10 % formalin prior to embedding in paraffin wax. The tissue was then processed and sectioned (5 µm thick) using a rotary microtome. The sections were stained with haematoxyline and eosin (H and E stain) dye for histopathological observations. The slides were the mounted with DPX (mixture of 10 g of sistyrene, 80.5 ml of dibutyl phthalate and 35 ml of xylene) and observed under the light microscope for morphological changes.

2.13 Statistical analysis.

All the values were expressed as Mean ± SEM. Statistical evaluation of the data was done by one-way ANOVA (between control and drug treatments) followed by Dunnett's t-test for multiple comparisons and two-way ANOVA followed by Bonferroni's multiple comparison test, with the level of significance chosen at $P < 0.001$ using Graph-Pad Prism 5, San Diego, CA software.

3. Results

3.1. Phytochemical Screening.

Table 1 showed the phytochemical screening of the different extract of *F. religiosa.*

Table 1: Phytochemical investigation of *F. religiosa* Linn leaves

Sr. No.	Name of Test	Pet. Ether	Ethyl Acetate	Ethanol
1	Tannins and Phenols	-ve	-ve	+ve
2	Alkaloids	+ve	+ve	+ve
3	Saponins	-ve	-ve	-ve
4	Terpenoids	-ve	+ve	+ve
5	Steroids	-ve	-ve	-ve
6	Flavonoids	-ve	+ve	+ve
7	Carbohydrates	-ve	-ve	-ve
8	Glycosides	-ve	-ve	+ve
9	Phlobotannins	-ve	-ve	-ve
10	Amino acid	-ve	-ve	-ve
11	Protein	-ve	-ve	-ve

3.2. Acute Toxicity.

The PEFRE was found to be safe at all the doses used and there was no mortality found up to the dose of 4000 mg/kg of PEFRE when administered orally. Therefore, we have taken 400 mg/kg as the therapeutic dose and made variations by taking 100 mg/kg as lower dose and 400 mg/kg as higher dose.

3.3.1. The effects of PEFRE on 3 NP induced Huntington's disease in the Locomotor activity.

Neurotoxin i.e. 3 NP treated group showed significant ($p<0.001$) reduction in locomotor activity as compared to vehicle treated group. Chronic oral administration of PEFRE of higher doses (200 and 400 mg/kg) showed significant ($p<0.001$) increase in the locomotor activity on day 3, 7, 10 and 14 as compared to 3 NP treated control animals. However, administration of PEFRE (100 mg/kg) did not show significant activity. Rivastigmine (1 mg/kg) significantly ($p<0.001$) increased locomotor activity (Figure 1).

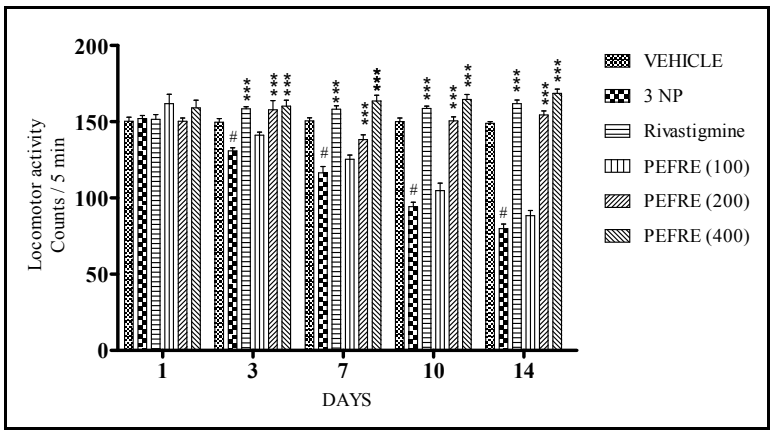

Figure 1: The effects of PEFRE on *3 NP induced Huntington's disease* in the Locomotor activity

Group I: Vehicle control group; Group II: 3 NP treated group; Group III: Rivastigmine + 3 NP treated group; Group IV: PEFRE (100 mg/kg) + 3 NP treated group; Group V: PEFRE (200 mg/kg) + 3 NP treated group; Group VI: PEFRE (400 mg/kg) + 3 NP treated group. ***$p<0.001$ as compared to 3 NP treated negative control group (Group II). # $p<0.001$ 3 NP treated negative control group (Group II) compared to vehicle treated control group (Group I)

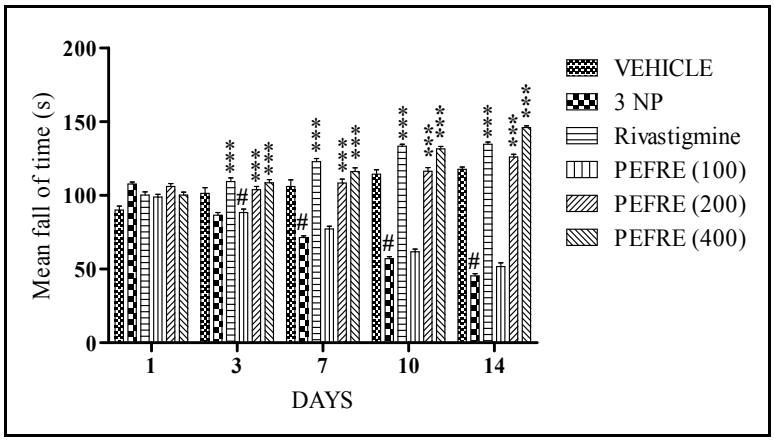

Figure 2: The effects of PEFRE on *3 NP induced Huntington's disease* in the Rotarod performance

Group I: Vehicle control group; Group II: 3 NP treated group; Group III: Rivastigmine + 3 NP treated group; Group IV: PEFRE (100 mg/kg) + 3 NP treated group; Group V: PEFRE (200 mg/kg) + 3 NP treated group; Group VI: PEFRE (400 mg/kg) + 3 NP treated group. ***$p<0.001$ as compared to 3 NP treated negative control group (Group II). # $p<0.001$ 3 NP treated negative control group (Group II) compared to vehicle treated control group (Group I)

3.3.2. The effects of PEFRE on 3 NP induced Huntington's disease in the Rotarod performance.

Treatment with 3 NP significantly decreased the mean fall of time when compared to the vehicle control animals. Chronic oral administration of Rivastigmine (1 mg/kg) and PEFRE (200 and 400 mg/kg) significantly ($p<0.001$) increased the mean fall of time when compared to 3 NP group on day 3, 7, 10 and 14. Administration of PEFRE (100 mg/kg) did not show significant activity (Figure 2).

3.3.3 The effects of PEFRE on 3 NP induced Huntington's disease in the Elevated plus maze performance.

In elevated plus maze test, administration of 3 NP significantly decreased number of entries and time spent in open arm and significantly increased number of entries and time spent in closed arm as compared to vehicle treated group. In contrast, chronic oral administration of Rivastigmine (1 mg/kg) and PEFRE (400 mg/kg) significantly (P<0.001) increased number of entries and time spent in open arm and significantly decreased the number of entries and time spent in closed arm as compared to 3 NP treated group. However, PEFRE (100 and 200 mg/kg) did not show any significant activity (Figure 3).

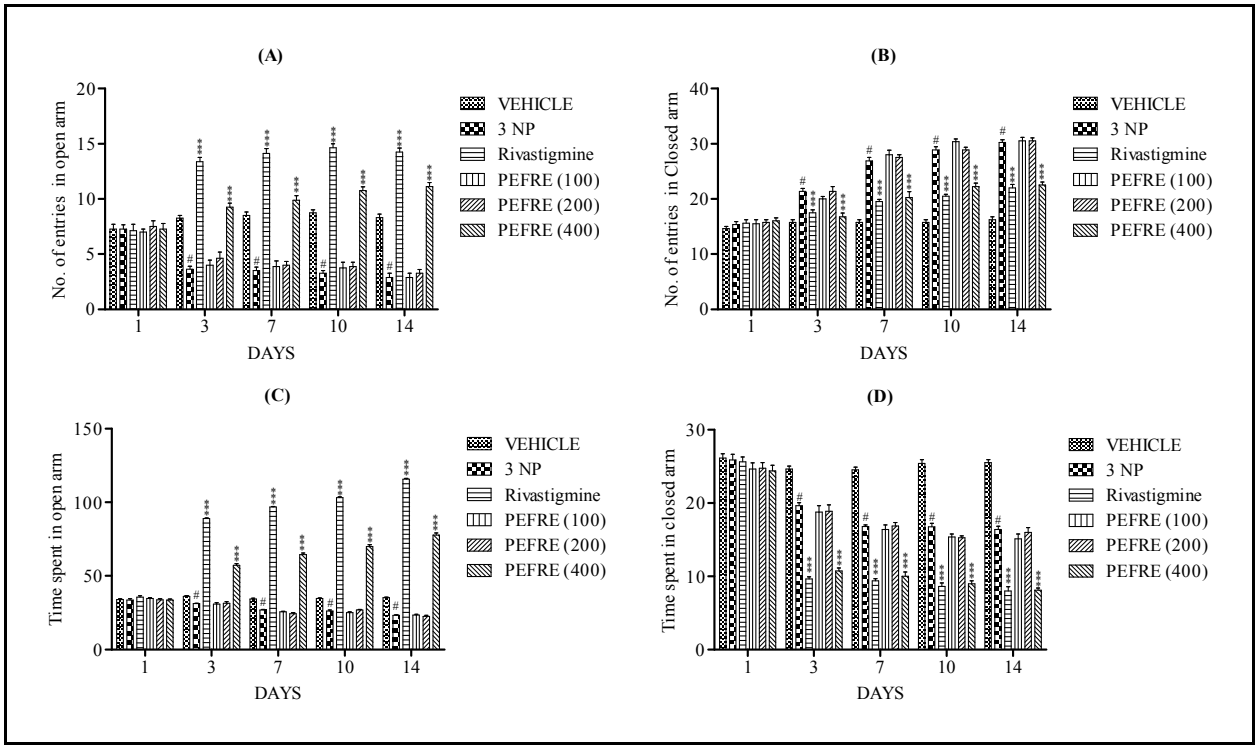

Figure 3: The effects of PEFRE on *3 NP induced Huntington's disease* in the Elevated plus maze performance ((A) Number of entries in open arm; (B) Number of entries in closed arm; (C) Time spent in open arm; (D) Time spent in closed arm)

Group I: Vehicle control group; Group II: 3 NP treated group; Group III: Rivastigmine + 3 NP treated group; Group IV: PEFRE (100 mg/kg) + 3 NP treated group; Group V: PEFRE (200 mg/kg) + 3 NP treated group; Group VI: PEFRE (400 mg/kg) + 3 NP treated group. ***p<0.001 as compared to 3 NP treated negative control group (Group II). # p<0.001 3 NP treated negative control group (Group II) compared to vehicle treated control group (Group I)

3.3.4. The effects of PEFRE on 3 NP induced Huntington's disease in MDA, CAT, SOD, GSH and AChE level.

The inoculation of 3 NP induced oxidative stress, as indicated by decreased in the CAT, SOD and GSH levels when compared to vehicle control animals in brain levels. Daily administration of PEFRE (400 mg/kg) showed significant (P<0.001) increase in CAT, SOD and GSH levels in the brain with 3 NP treated group. Similarly, treatment with pet ether extract of FRE (400 mg/kg, p.o.) attenuated the increase in lipid peroxidation and acetylcholinesterase enzyme level as shown by a significant (P<0.001) decreased in MDA and AChE enzyme levels (Table 2).

Table 2: Effect of PEFRE on the levels of lipid peroxidation (MDA), catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH) and Acetylcholinesterase enzyme (AChE) levels in the brain of 3 NP treated animals.

Group	MDA (nM/mg of protein)	CAT (µmoles of H_2O_2 used/min/mg protein)	SOD (Units/mg protein)	GSH (nM/mg of protein)	AChE (µmoles/min/g of tissue)
Vehicle control	1.37±0.06	4.02±0.05	5.11±0.09	5.63±0.09	10.14±0.18
3 NP control	2.72±0.31#	2.61±0.41#	2.70±0.43#	3.90±0.02#	11.65±0.31#
Rivastigmine	1.50±0.06***	5.26±0.16***	8.85±0.54***	8.92±0.33***	7.408±0.29***
PEFRE (100)	2.57±0.17	3.28±0.02	2.99±0.64	4.50±0.01	11.19±0.18
PEFRE (200)	2.09±0.18	3.28±0.02	3.58±0.34	5.14±0.08	10.52±0.32
PEFRE (400)	1.44±0.06***	4.42±0.10***	6.60±0.35***	9.19±0.76***	8.938±0.097***

Values are expressed as Mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ as compared to 3 NP treated control group (Group II) [Groups III to VI were compared with Group II], # $p<0.001$ as compared to vehicle treated group (Group I) [Groups II was compared with Group I]

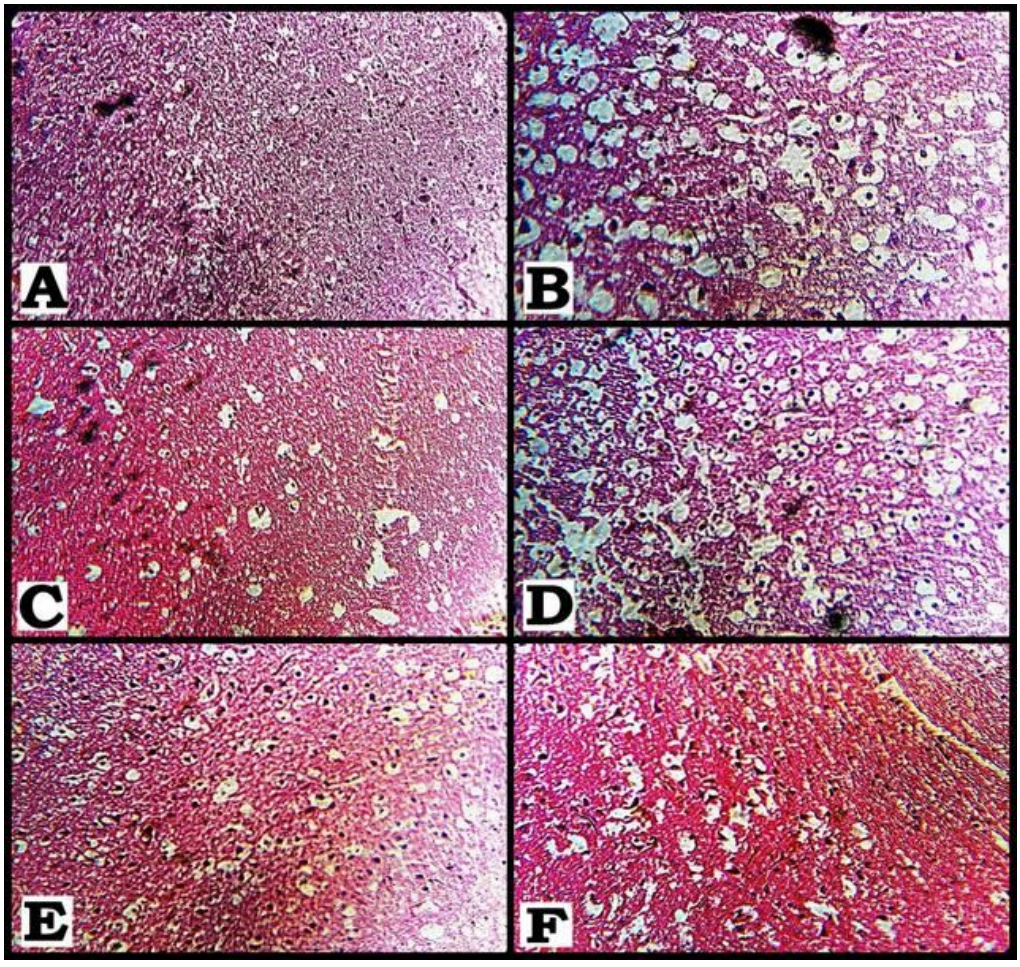

Figure 4: Effect of PEFRE on histopathological changes in the brain of normal and 3 NP treated animals (H & E staining; original magnification, 40 X)

(A) Normal control showing normal brain architecture; (B) Rats treated with 3 NP showing degeneration of neurons, hypertrophy of glial cells, neuroinflammation, necrosis; (C) Rats treated with 3 NP and Rivastigmine (1 mg/kg) showing minimal changes in neuronal cell integrity and architecture; (D) Rats treated with 3 NP and PEFRE (100 mg/kg) and (E) PEFRE (200 mg/kg) showing mild decrease in neurons and cellular hypertrophy; (F) PEFRE (400 mg/kg) treated rats showing minimal changes in neuronal cell populations.

3.3.5. Effect of PEFRE on histopathological changes in the brain of normal and 3 NP treated animals.

The histopathological study showed that neurotoxins i.e. 3 NP caused degeneration of neurons, hypertrophy of glial cells, neuroinflammation in form of swelling, decreased density of cells, alterations of architecture, and necrosis. (Fig. 4B) compared with normal vehicle treated rats (Fig. 4A). Rivastigmine (1 mg/kg) (Fig. 4C) and PEFRE (400 mg/kg) (Fig. 4F) significantly reverse the neuronal damage or neuronal alterations compared to 3 NP treated rats. Treatment with PEFRE (100 mg/kg) (Fig. 4D) and PEFRE (200 mg/kg) (Fig. 4E) did not show marked significant recovery of neuronal damage.

4. Discussion

Huntington's disease is an autosomal dominant inherited chronic neurodegenerative disorder characterized by progressive decline in motor and cognitive functions (23). The exact etiopathogenesis of HD is not clear yet, but literature suggest that oxidative stress, mitochondrial dysfunction, neuroinflammation, the alteration in energy metabolism play a crucial role in the pathogenesis of HD (24).

In the present study we evaluated the neurodegenerative effect of pet ether extract of *F. religiosa* in neurotoxins (3 NP) induced Huntington's disease in experimental animals.

Chronic intraperitoneal administration of 3 NP, a mitochondrial toxin produced progressive metabolic impairment and behavioral abnormalities. 3 NP administration have been reported to cause selective damage to the striatum and induce motor deficits (25). In this experiment, we induce HD by 3 NP (30 mg/kg for 14 days, i.p.) in rats, showing neurobehavioral and biochemical changes in rats similar to HD patients. 3 NP treated animals showed a significant decreased body weight and induced movement abnormalities in rats. Loss of body weight and movement abnormalities could be due to ATP loss. It has been reported that neuronal loss is responsible for reducing food intake and appetite (26). Pretreatment with pet ether extract of *F. religiosa* caused significant improvement in body weight and movement activity in 3 NP treated animals.

Typically, 3 NP cause HD begins with involuntary movements. In the present study, chronic administration of 3 NP caused significant decreased in locomotor activity and muscle activity. Lack of locomotion and motor coordination have been reported in HD condition (27). This could be due to decreased energy metabolism. Pretreatment with PEFRE (200 and 400 mg/kg) showed significant increase in locomotion and muscle activity. In the cognitive task, daily administration of 3 NP showed significant decreased the number of entries and time spent in open arm and increased the number of entries and time spent in closed arm in elevated plus maze. This could happened due to changes in striatum neuronal degeneration, a region functionally connected by motor cortex afferents. Pretreatment with PEFRE at a dose of 400 mg/kg showed significant increased the number of entries and time spent in open arm and decreased the number of entries and time spent in closed arm.

3 NP administration cause excitotoxicity that leads to depolarization of neuronal membrane potential, followed by release of free radicals and blockage of NMDA receptor ion channel. Increased levels of glutamate leads to excitotoxicity and calcium influx results in mitochondrial dysfunction and increased oxidative stress (28, 29).

Oxidative stress, a pathological condition leading to cell death, is generated as a result mitochondrial dysfunction play a important role in HD (30). GSH is an antioxidant that plays a vital role in reduction of ROS in brain by interacting directly to detoxify certain ROS. A decrease in the availability of reduced glutathione would impair the capacity of neurons to detoxify hydrogen peroxide and increase the risk of free radical formation and lipid peroxidation. Lipid peroxidation, a sensitive marker of oxidative stress, was estimated by measuring the levels of thiobarbituric acid. Lipid peroxidation occurs due to attack by radicals on double bond of unsaturated fatty acid and arachidonic acid which generate lipid peroxyl radicals and that initiate a chain reaction of further attacks on other unsaturated fatty acid. Lipid peroxidation leads to the loss of cell function in conditions like oxidative stress in brain and in neurodegenerative disorders (31). Reaction of O_2 with Nitric oxide results in the production of ONOO- which is highly cytotoxic and induces hydroxyl radical formation that makes it critical in the pathogenesis of neurodegenerative disorders (32). Catalase is an antioxidant enzyme that is essential for protection against oxidative damage to the cell (20) Superoxide dismutase (known as SOD) is an enzyme which acts as a catalyst in the process of dismutation of superoxide into nonreactive oxygen species and

hydrogen peroxide. It is therefore a critical antioxidant defense which is present in nearly all cells which are exposed to oxygen (33-34). In the present study, 14 days administration of 3 NP increased oxidative stress in the brain as observed by significant increased levels of lipid peroxidation and depletion of endogenous antioxidants enzyme such as catalase, superoxide dismutase and reduced glutathione. Treatment with higher dose of petroleum ether extract of *F. religiosa* (400 mg/kg) resulted in a decreased level of MDA and increased levels of SOD, catalase and GSH, indicating its antioxidant effect in the brain of 3 NP treated animals.

Reduced levels of acetylcholine in the brain are one of primary biochemical alterations seen in HD leading to cognitive defects. Acetylcholinesterase (AChE) is an enzyme that terminate synaptic transmission by catalysing hydrolysis of acetylcholine (35). 14 days administration of 3 NP increased AChE levels, whereas daily oral administration of PEFRE (400 mg/kg) administration restored the levels of AChE to normal. Neuroprotective effect of *F. religiosa* was further determined by histopathological examinations. Histopathological findings showed that pet. ether extract of *Ficus religiosa* treated animals had decreased degeneration of neurons, hypertrophy of glial cells, neuroinflammation, and increased density of cells, regained normal architecture and moderate necrosis in striatum region of brain.

Some constituents in the *Ficus religiosa* leaves have been identified such as campestrol, stigmasterol, isofucosterol, α-amyrin, lupeol, tannic acid, arginine, serine, aspartic acid, glycine, threonine, alanine, proline, tryptophan, tryosine, methionine, valine, isoleucine, leucine, n-nonacosane, n-hentricontanen, hexa-cosanol and n-octacosan (36-37). The neuroprotective activity of *F. religiosa* may be due to any one or more of the constituents in the extract for example alkaloids.

5. Conclusion

The results from this study indicated that *Ficus religiosa* plant could showed antihuntington's activity. The use of this plant for Huntington's disease treatment is promising but we appreciate further detailed precise molecular studies of this plant.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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