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Morin Hydrate Prevents Neurodegeneration in 3-Nitropropionic Acid Induced Huntington's Disease

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ABSTRACT

The present study was designed to investigate the neuroprotective effect of a flavonoid Morin Hydrate in 3-nitropropionic acid induced model of Huntington's disease.3-Nitropropionic acid was used to induce symptoms similar to that of Huntington's disease in male wistar rats. Rats were treated with Morin (50 mg/kg and 100 mg/kg) for 14 days. Body weight, brain weight, behavioural parameters, biochemical parameters were assessed and brain samples were sent for histopathology.3-nitropropionic acid administration for 14 days significantly induced symptoms such as reduced body weight, impaired gait, locomotor activity, muscle grip strength and also caused oxidative stress in striatum. The treatment with Morin 50 mg/kg was less effective as compared to that of Morin 100 mg/kg. Morin (100 mg/kg)showed slightly reduced oxidative stress as well as motor coordination deficits. Histopathology results also revealed reduced neuronal degeneration in Morin (100 mg/kg) samples as compared to Huntington control (HC) rats. This evidence suggest flavonoid Morin might have protective effect against 3-Np induced huntington's disease or disease like symptoms, and further studies on higher doses are needed to explore the usefulness of flavonoid Morin in HD.

Keywords: Huntington's disease, 3-Nitropropionic acid, Morin Hydrate, Antioxidant, Neuroprotective

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INTRODUCTION

Huntington's disease is a progressive, fatal, neurodegenerative disorder caused by an expanded CAG repeat in huntingtin gene that encodes for an abnormally long polyglutamine tract in a protein termed huntingtin (htt)[1]. It is associated with severe degeneration of basal ganglia neurons, especially the intrinsic neurons of the striatum and is characterized by progressive dementia and involuntary abnormal choreiform movements[2]. In addition, metabolic abnormalities such as wasting and altered energy expenditure are increasingly recognized as clinical hallmarks of the disease. Neuropathological analysis of HD brain reveals preferential and progressive neuronal loss of GABAergic medium- sized spiny neurons (MSNs) in the striatum[3]. One toxin that is gaining prominence for use in animal model of HD is 3-Nitropropionic acid. Mitochondrial toxin 3-Np is an irreversible inhibitor of succinate dehydrogenase that inhibits both the krebs cycle and complex II of the mitochondrial electron transport chain[4]. Administration of 3-Np to rats impairs energy metabolism and results in striatal lesions, probably through a glutamate-dependent excitotoxic mechanism[5]. Flavonoids are potent antioxidants with beneficial effect against oxidative stress-related disease such as HD, Alzheimer's disease, Diabetes, Cancer, and Parkinson's disease. Morin (2',3,4',5,7-pentahydroxyflavone) is a member of flavonoid family which consists of yellowish pigment. It has been reported to possess a variety of biological properties against oxidative stress-induced damage including protection of cardiovascular cells, glomerular mesangial cells, hepatocytes, oligodendrocytes and neurons damaged by oxidative stress[6]. It also produces different types of pharmacological benefits such as free radical scavenging activity, xanthine-oxidase inhibitor property, anti-inflammatory activity, protective effect of DNA from damage caused by free radical and anticancer activity[7]. Aim of the present study is to investigate the neuroprotective effect of Morin Hydrate against 3-Nitropropionic acid induced Huntington's disease.

MATERIALS AND METHOD

Morin was obtained from Sigma Aldrich.3-Nitropropionic acid, DTNB and NBT were procured from Research-lab fine chem industries (Mumbai, India). Malondialdehyde, reduced glutathion, griess reagent and superoxide dismutase was purchased from Sigma Aldrich (USA), obtained from Lobacheime, (Mumbai, India). All other reagents and chemicals were of analytical grade and purchased from local suppliers of Pune.

Experimental Animals

Male wistar rats (250- 310 g) were procured from National Institute of Biosciences, Pune. Rats were randomly placed separately in polypropylene cages with paddy husk as bedding. The animals

were maintained under standard laboratory conditions at temperature 23 ± 2ºC with relative humidity 55 ± 10 % under 12 h light and 12 h dark cycle throughout the experiment. Animals had free access to water and standard laboratory feed (Nutrivet Lab., Pune) *ad libitum*. All the experimental procedures and protocols used in this study were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of Sinhgad College of Pharmacy, Pune, constituted under Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA) by Ministry of Environment and Forests, Government of India, New Delhi, India. Ethical guidelines were strictly followed during all the experimental procedures.

Experimental design

After one week of acclimatization, Wistar rats were randomly divided into four groups (n=6) and received treatment for 14 days as per the following schedule

1. **Control:** vehicle
2. **Huntington control:** 3-nitropropionic acid (10mg/kg i.p.)
3. **Low Dose:** 3-nitropropionic acid (10mg/kg i.p.)+ Morin (50mg/kg; p.o)
4. **High Dose:** 3-nitropropionic acid (10mg/kg i.p.)+ Morin(100mg/kg; p.o)

The observations were taken in between 9:00 A.M. to 12:00 P.M. throughout the study. At the end of the treatment animals were sacrificed by cervical dislocation, brain was isolated, weighed and used for various biochemical estimations.

Estimation of morphological parameters

Body weight and brain weight

Body weight was measured on 1st (before treatment) and 14th day post 3-nitropropionic acid administration, using electronic weighing balance (CONTECH, C7-6K1). Whole brain weight was measured at the end of the treatment.

Evaluation of behavioural parameters

a. Muscle grip strength

All animal were evaluated for motor ability and balance by using the rota rod apparatus (Techno, India). Rats were placed on the rotating rod with a diameter of 7 cm (speed 25 rpm). Each rat performed three separate trials after interval of 5 min. The average results were recorded as fall of time in seconds with cut off time of 180 s[8].

b. Locomotor activity

Animals were placed in Actophotometer where beam of light falls on photoelectric cells and basal activity score was recorded over the period of 5 min which is recorded as no. of beams cut during locomotion[9].

c. Narrow Beam Walking Test

This test was used to evaluate motor performance by progressively increasing the difficulty in the execution of the task. The animals were trained in crossing a 150 cm long wooden beam, from a platform at one end to the animal's home cage at the other end, placed horizontally 60 cm above the floor. The number of paw slips onto an under-hanging ledge and the time taken to traverse the beam was recorded. The maximum time allowed for the task was 2 min[1].

Estimation of biochemical parameters in brain striatum

Preparation of brain homogenate

After completion of behavioral evaluation animals were scarified by cervical dislocation, immediately for the biochemical analysis. Brain was dissected and striatum and hippocampus were identified, separated and weighed. 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 min and aliquots of supernatant was separated and used for biochemical estimation.

a. Estimation of lipid peroxidation (MDA)

Malondialdehyde (MDA), a product of lipid peroxidation was measured by method described earlier. Sample of 0.1 ml supernatant was taken and mixed with 0.2 ml 8.1% sodium dodecyl sulphate (SDS), 1.5 ml 20% glacial acetic acid and 1.5 ml of 0.8% thiobarbituric acid (TBA). Following these additions, tubes were mixed and heated at 95 °C for 1 h on a water bath and cooled under tap water before mixing 1ml of distilled water and 5ml mixture of n-butanol and pyridine (15:1). The mixture was centrifuged at 4000 rpm for 10 min. The amount of MDA formed was measured byabsorbance of upper organic layer at a wavelength of 532 nm using appropriate controls. A calibration curve was plotted using malondialdehyde bis-(dimethoxy acetyl) as a standard. The values were expressed as nmol/mg of wet tissue[10].

b. Estimation ofreduced glutathione (GSH)

Equal volumes of tissue homogenate (supernatant) and 20% trichloroacetic acid were mixed. The precipitated fraction was centrifuged and to 0.25ml of supernatant, 2ml of 0.6mM 5,5’-dithiobis (2-nitro benzoic acid) reagent was added. The final volume wasmade upto 3ml with phosphate buffer (0.2M, pH 8.0). The colour developed was read at 412nm against reagent blank. The values are expressed as µg/mg of wet tissue[11].

c. Estimation of nitric oxide (NO)

500 µl of Greiss reagent (1:1 solution of 1% sulphanilamide in 5% phosphoric acid and 0.1% napthaylamine-diaminedihydrochloric acid in water) was added to 0.1ml of supernatant and

absorbance was measured at 546 nm. Nitrite concentration was calculated using a standard curve for sodium nitrite and expressed as ng/g of wet tissue[12].

d. Estimation of Super oxide dismutase (SOD) activity

To 100µl serum, 1ml of sodium carbonate (1.06 g in 100 ml water), 0.4 ml of 24mM NBT and 0.2 ml of EDTA (37 mg in 100 ml water) was added and zero minute reading was taken at 560 nm. Reaction was initiated by addition of 0.4 ml of 1mM hydroxylamine hydrochloride, incubated at 25°C for 5 minutes and the reduction of NBT was measured at 560nm and expressed as µg/mg of wet tissue[13].

Histopathology

The Brain tissues as whole was removed from the animals under study and preserved in 10% neutral buffered formalin for 72 hrs. The brain section was sliced at centre horizontally and was rinsed in running water for removal of preservative. The tissue was further processed in ascending grades of alcohol for dehydration and was cleared in xylene and finally embedded in paraffin block. The tissue was then sectioned on automated microtome and about 5 µ sections were taken on glass slide. Cut sections were stained with haematoxylin and eosin protocol. Histopathology studies were performed at Precision Lab by Dr. Mote, MD (Pathology).

RESULTS AND DISCUSSION

Effect of Morin hydrate on body weight

Administration of Morin 50mg/kg did not increase the weight significantly, but Morin 100mg/kg showed significant increase in weight as compared to Huntington Control (HC) group as shown in Table 1 and Figure 1.

Table 1: Effect of Morin hydrate on body weight

Sr. No.	Groups	Body Weight (Day 1)	Body Weight (Day 14)
1.	Normal Control	298.7 ± 4.96	296.3 ± 7.10[a]
2.	Huntington Control	296.8 ± 6.063	153.2 ± 5.55*
3.	Morin Hydrate 50 mg/kg	294.5 ± 6.048	193.3 ± 6.64[b]
4.	Morin Hydrate 100 mg/kg	299.0 ± 3.786	249.3 ± 8.88[a$]

Table 2: Effect of Morin hydrate on brain weight

Sr. No.	Groups	Brain Weight
1.	Normal Control	2.505 ± 0.09[a]
2.	Huntington Control	1.440 ± 0.057*
3.	Morin 50 mg/kg	1.840 ± 0.052[b]
4.	Morin 100 mg/kg	2.16 ± 0.079[a%]

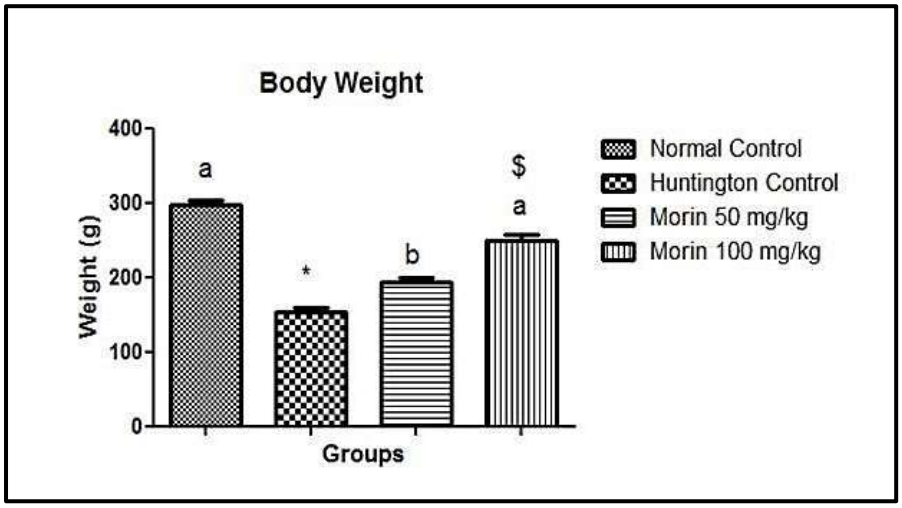

Figure 1: - **Effect of Morin hydrate on body weight**

Each value represents mean ± S.E.M. n=6.

[a]P< 0.001 compared with HC rats, [$]P< 0.001compared with M 50mg/kg, [b]P< 0.01 compared with HC

HC= *Huntington control, M = Morin Hydrate*

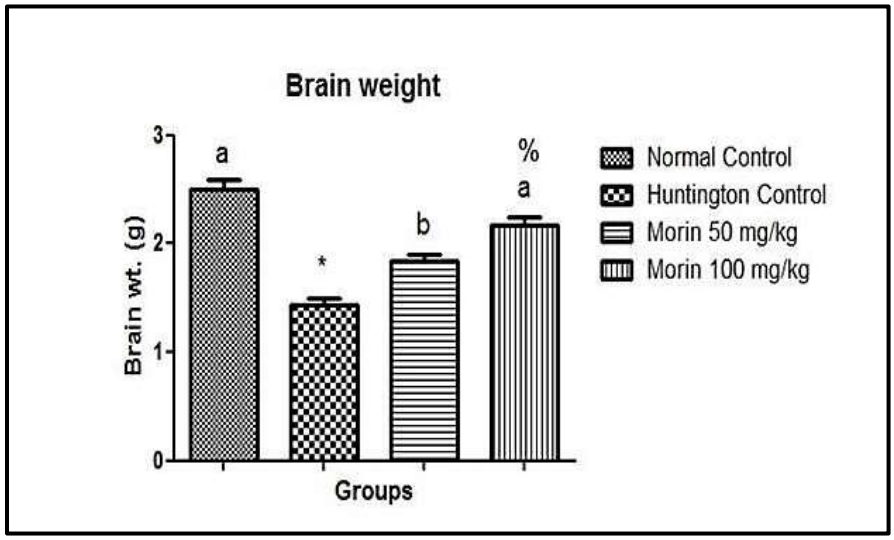

Figure 2: - Effect of Morin hydrate on brain weight

Each value represents mean ± S.E.M. n=6.

[a]P< 0.001and [b]P< 0.01when compared with HC, [%]P< 0.05 when compared with M 50 mg/kg.

HC= *Huntington control, M = Morin Hydrate*

Effect of Morin Hydrate on brain weight

Rats treated with Morin 100 mg showed significant increase in brain weight as compared to HC rats. While Morin 50mg/kg showed slight effect on brain weight as compared to HC rats as shown in Table 2 and Figure 2.

Effect of Morin hydrate on muscle grip strength

Rats treated with Morin 100 mg/kg did show improvement in grip strength and increased fall time as compared to HC rats and Morin 50 mg/kg group as shown in Table 3 and Figure 3.

Table 3: Effect of Morin hydrate on muscle grip strength

Sr. No.	Groups	Fall off Time (Sec)
1.	Normal Control	65.67 ± 1.56[a]
2.	Huntington Control	15.33 ± 1.62*
3.	Morin 50 mg/kg	24.67 ± 1.70[c]
4.	Morin 100 mg/kg	38.67 ± 2.55[a$]

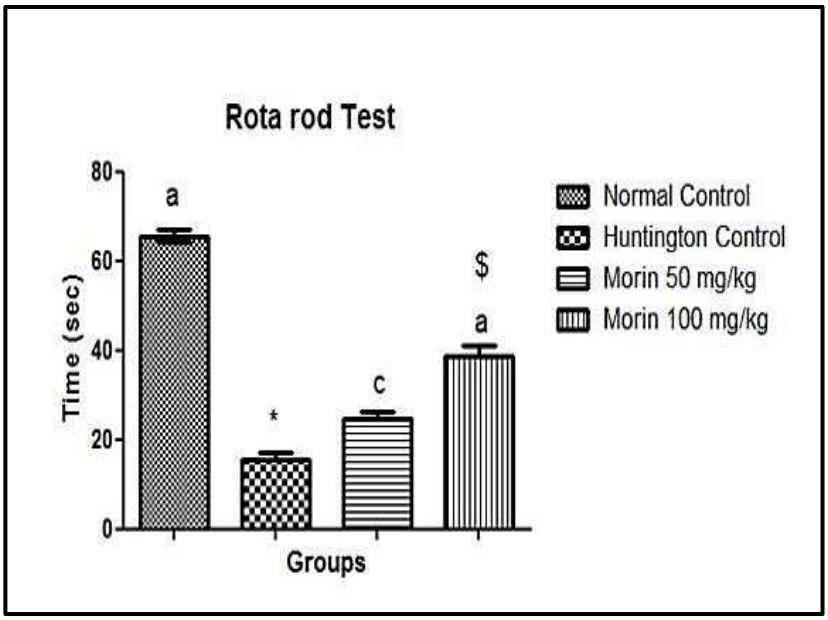

Figure 3: -Effect of Morin hydrate on muscle grip strength

Each value represents mean ± S.E.M. n=6.

[a]P< 0.001 compared with HC, [c]P< 0.05 compared with HC, [$]P< 0.001compared M 50 mg/kg.

HC= *Huntington control, M = Morin Hydrate*

Effect of Morin hydrate on Locomotor activity

Rats treated with Morin 100 mg/kg did show significant increase in the No. of locomotions as compared to HC and Morin 50 mg/kg treated rats as shown in Table 4 and Figure 4.

Table 4: Effect of Morin hydrate on Locomotor activity

Sr. No.	Groups	No. of Locomotions
1.	Normal Control	334.7 ± 13.33[a]
2.	Huntington Control	118.5 ± 7.88*
3.	Morin 50 mg/kg	162.3 ± 9.32[c]
4.	Morin 100 mg/kg	242.8 ± 9.69[a$]

Table 5: Effect of Morin Hydrate on Gait using Narrow Beam Walking Test

Sr. No.	Groups	No. of Paw slips	Time (Sec) to transverse the beam
1.	Normal Control	4.00 ± 0.730[a]	14.33 ± 2.57[a]
2.	Huntington Control	9.16 ± 1.35*	106.0 ± 4.09*
3.	Morin 50 mg/kg	9.00 ± 0.966	82.33 ± 4.44[b]
4.	Morin 100 mg/kg	7.16 ± 0.477	40.33 ± 3.63[a$]

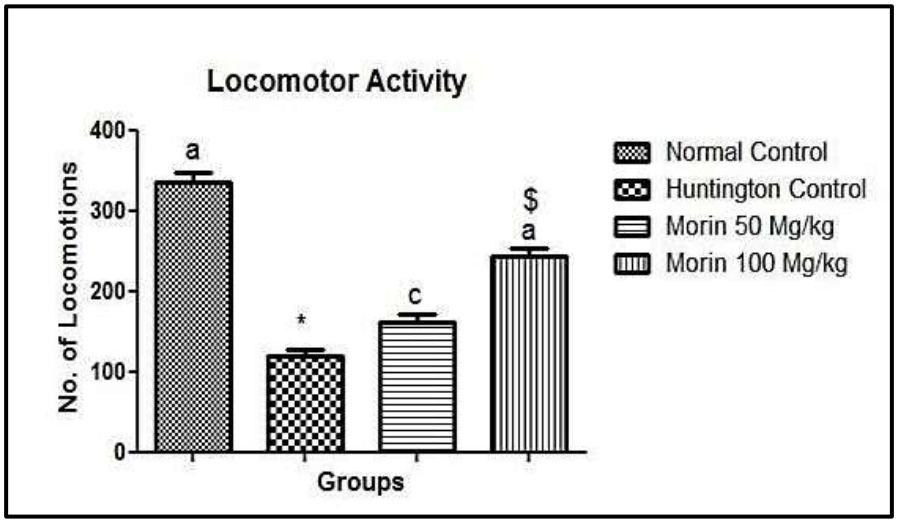

Figure 4: Effect of Morin hydrate on Locomotor activity

Each value represents mean ± S.E.M. n=6.

[a]P< 0.001, [c]P< 0.05 compared with HC, [$]P<0.001compared with M 50 mg/kg.

HC= *Huntington control, M = Morin Hydrate*

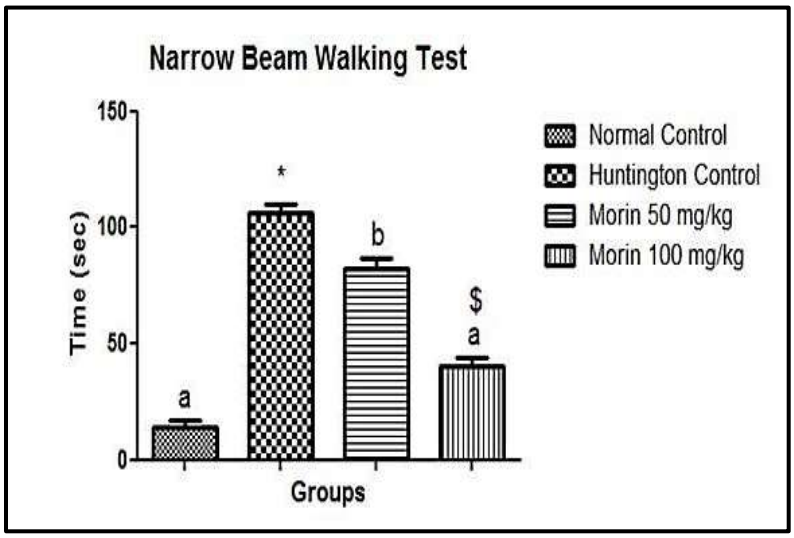

Figure 5: Effect of Morin hydrate on Gait (Time taken to transverse the Beam)

Each value represents mean ± S.E.M. n=6.

[a]P< 0.001, [b]P< 0.01compared with HC, [$]P<0.001compared with M 50 mg/kg.

HC= *Huntington control, M = Morin Hydrate*

Effect of Morin Hydrate on Gait

Rats treated with Morin 100 mg/kg showed significantly improved gait, reduced time to cross the beam but had no significant effect on no. of paw slips as compared to HC rats and Morin 50 mg/kg treated rats as shown in Table 5 and Figure 5, 6.

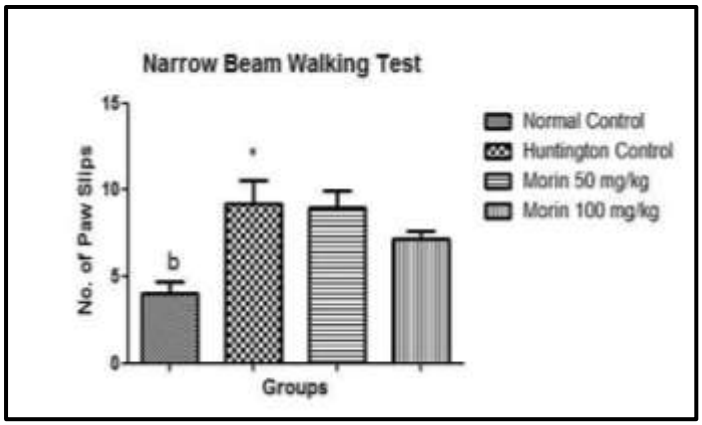

Figure 6: Effect of Morin hydrate on Gait (No. of Paw slips)

Each value represents mean ± S.E.M. n=6.

[b]P< 0.01compared with HC, M 50 mg/kg and M 100 mg/kg are not significant as compared to HC.

HC= *Huntington control, M = Morin Hydrate*

Effect of Morin hydrate on MDA level in Brain

Rats treated with Morin 100 mg/kg showed significantly low levels of MDA as compared to HC rats as shown in Table 6 and Figure 7.

Table 6: Effect of Morin hydrate on MDA level in brain

Sr. No.	Groups	MDA level
1.	Normal Control	10.97 ± 1.17[a]
2.	Huntington Control	32.17 ± 1.55*
3.	Morin 50 mg/kg	24.21 ± 1.16[a]
4.	Morin 100 mg/kg	17.05 ± 0.857[a@]

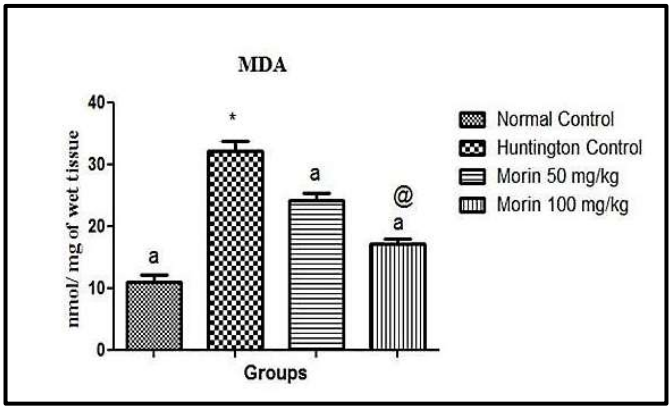

Figure 7: Effect of Morin hydrate on MDA Level

Each value represents mean ± S.E.M. n=6.

[a]P< 0.001 compared with HC, [@]P<0.01 compared with M 50 mg/kg.

HC= *Huntington control, M = Morin Hydrate*

Effect of Morin hydrate on GSH activity

Rats treated with Morin 100 mg/kg showed significant increase in GSH activity in striatum as compared to HC rats as well as Morin 50mg/kg treated rats as shown in Table 7 and Figure 8.

Table 7: Effect of Morin hydrateon GSH activity

Sr. No.	Groups	GSH Level
1.	Normal Control	111.5 ± 4.206[a]
2.	Huntington Control	49.66 ± 4.614*
3.	Morin 50 mg/kg	69.67 ± 3.340[c]
4.	Morin 100 mg/kg	88.54 ± 4.256[a%]

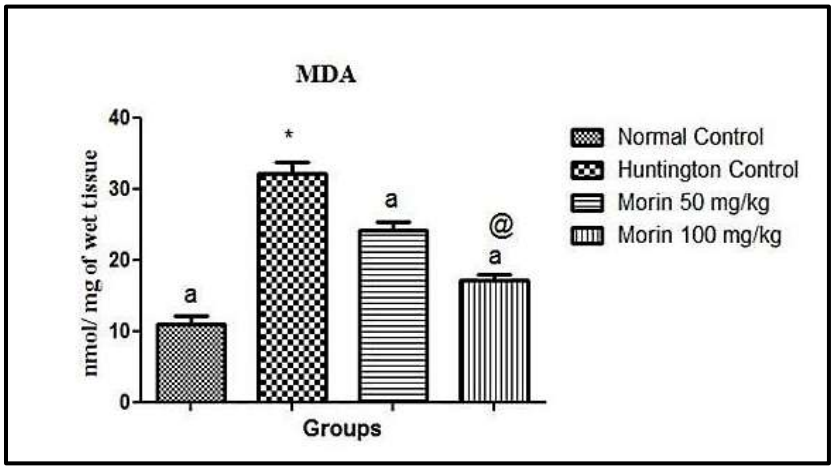

Figure 7: Effect of Morin hydrate on MDA Level

Each value represents mean ± S.E.M. n=6.

[a]P< 0.001 compared with HC, [@]P<0.01 compared with M 50 mg/kg.

HC= *Huntington control, M = Morin Hydrate*

Effect of Morin hydrate on NO activity

Rats treated with Morin 50 mg/kg did not have significant effect on NO levels. But rats treated with Morin 100 mg/kg did show significant decrease in NO levels as compared to HC rats as shown in Table 8 and Figure 9.

Table 8: Effect of Morin hydrate on NO activity

Sr. No.	Groups	NO level
1.	Normal Control	118.4 ± 6.37[a]
2.	Huntington Control	263.1 ± 10.06*
3.	Morin 50 mg/kg	225.2 ± 5.26[b]
4.	Morin 100 mg/kg	117.1 ± 5.87[a$]

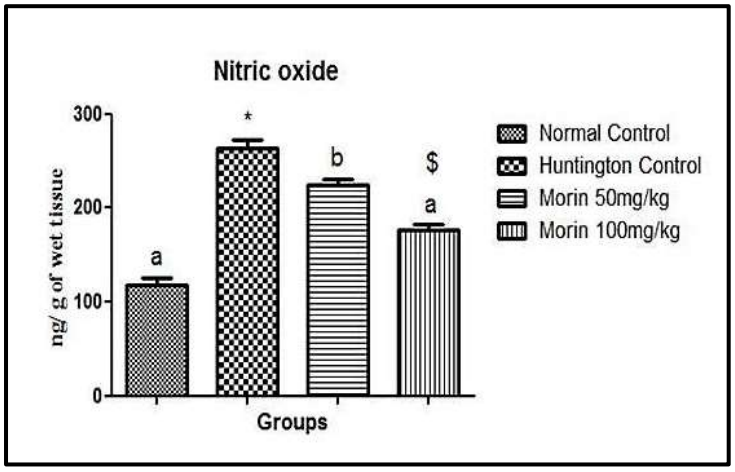

Figure 9: Effect of Morin hydrate on NO activity

Each value represents mean ± S.E.M. n=6.

[a]P< 0.001, [b]P< 0.01compared with HC, [$]P<0.001compared to M 50 mg/kg.

HC= *Huntington control. M= Morin Hydrate*

Effect of Morin hydrate on SOD activity in brain

Rats treated with Morin 100 mg/kg showed significant increase in SOD levels in striatum as compared to Huntington Control rats as shown in Table 9 and Figure 10.

Table 9: Effect of Morin Hydrate on SOD activity in brain

Sr. No.	Groups	SOD level
1.	Normal Control	57.30 ± 3.75[a]
2.	Huntington Control	20.42 ± 1.53*
3.	Morin 50 mg/kg	32.08 ± 2.11[c]
4.	Morin 100 mg/kg	43.02 ± 2.25[a%]

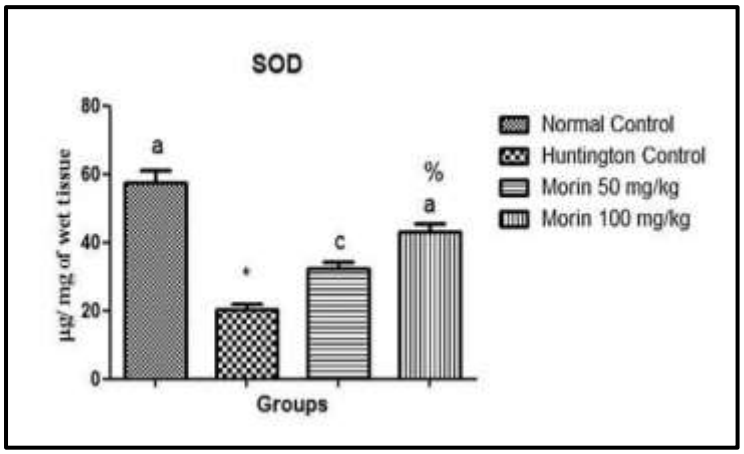

Figure 10: Effect of Morin hydrate on SOD activity

Each value represents mean ± S.E.M. n=6.

[a]P< 0.001, [c]P< 0.05 compared with HC, [%]P<0.05 compared to M 50 mg/kg.

HC=*Huntington control, M= Morin Hydrate*

Histopathology

Histopathological evaluation showed less neurodegeneration in Morin 100 mg/kg as compared to Huntington Control as shown in Figure 11.

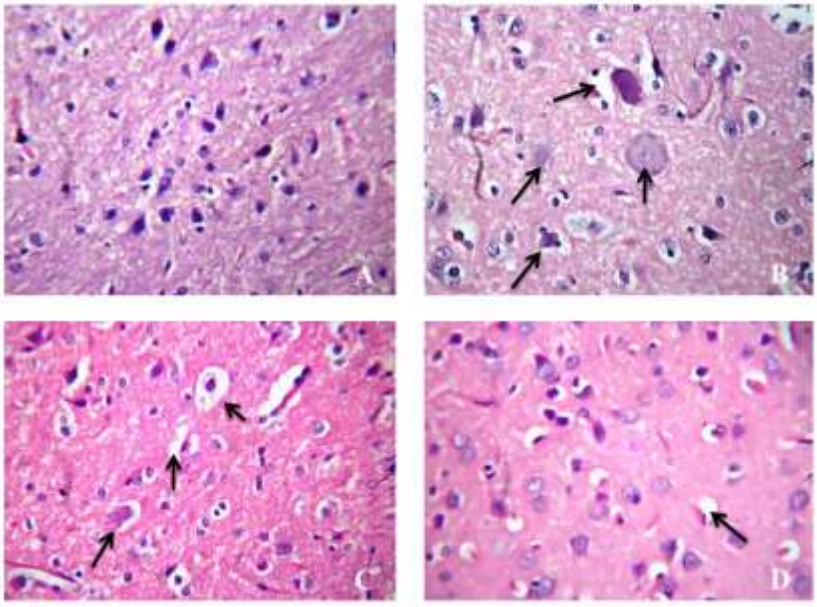

Figure 11: Histopathology (Hematoxylin and Eosin stain)

A – Normal Brain, B - Huntington Control: Neuronal Degenerative changes with Loss of nucleus of neurons, Dark Neurons, Neuronal swelling observed, **C - Morin Hydrate 50 mg/kg Body Weight:** Brain, Perivascular cuffing. MNC aggregation around blood vessel, degenerative change in supporting matrix of brain, congested blood vessel wasobserved, **D – Morin Hydrate 100 mg/kg Body Weight:** Mildbrain perivascular cuffing and MNC aggregation around blood vessels was observed as compared to HC and Morin 50 mg/kg.

→ = Indicates Neurodegeneration (Vacuolization, Neuronal Swelling).

The results of current study revealed that: (i) 3-Nitropropionic acid (i.p.) for 14 days induced neuronal oxidative stress and other Huntington like symptoms; (ii) Morin Hydrate at dose of 50 mg/kg did not produce significant effect as compared to Huntington control rats; (iii) Morin Hydrate at a dose of 100 mg/kg reduced the oxidative stress caused by 3-Np and significantly improved behavioural as well as morphological parameters. Huntington disease is a disorder in which nerve cells in certain parts of the brain waste away, or degenerate. HD occurs due to CAG repeat which occurs many more times than it is supposed to. HD causes movement; psychiatric and cognitive difficulties. Cognitive decline is more pronounced and can interfere with day-to-day

functioning like planning, organizing[14]. In accordance with earlier reports, as disease progresses, there is reduction of body weight. This may be due to difficulty in swallowing which results into less calorie intake. The striatal lesions, neuronal loss and bradykinesia are partly responsible for reduced appetite and weight loss[15]. In the present investigation it was found that administration of 3-nitropropionic acid for 14 days significantly reduced body weight when compared with control rats. Administration of Morin 100mg/kg body weight restores body weight significantly as compared to Huntington control group. 3-NP causes neuronal loss through mitochondrial dysfunction and decrease brain weight. 3-NP also induces an increased membrane permeability which leads to apoptotic cell death. Earlier reports on Morin suggest that it is anti-apoptotic agent which modulates mitochondrial membrane potential and maintains the levels of apoptotic and anti-apoptotic proteins[16]. In the present study administration of Morin significantly prevented reduction in brain weight as compared to Huntington control group. Muscle weakness is one of the major sign of HD. In HD there is muscle atrophy due to impaired energy uptake[8]. In the present study administration of 3-NP (i.p.) induced muscle atrophy that led to muscle weakness and decrease in muscle grip strength. Treatment with Morin prevented impairment of muscle grip strength and therefore decrease fall off time significantly as compared to Huntington Control. Gross motor disability is a hallmark of Huntington's disease. Narrow Beam Walking Test was performed to assess the motor coordination of the animals, number paw slips and time taken by the animal to transverse the beam was measured[1]. In this study administration of 3-NP caused significant postural defect, increased number of paw slips as well as time taken to transverse the beam. Morin Hydrate significantly reduced the total time taken to transverse the beam but did not have any significant effect on paw slips as compared to Huntington Control group. Neurobehavioral impairment is a characteristic symbol of striatal cell loss. Increased oxidative stress and mitochondrial dysfunction following 3-NP administration can result in poor cognitive and motor functions[1]. 3-NP administration causes muscular atrophy and leads to decrease in locomotion counts as compared to control group. In current investigation administration of 3-NP resulted in reduced number of locomotion. Treatment with Morin increased number of locomotion significantly compared to Huntington control group. The molecular mechanisms mediating neuronal death in HD seem to be related to oxidative stress, excite toxicity and misbalance in energetic metabolism[17]. In this study we evaluated the oxidative stress parameters like MDA, SOD, GSH and NO. Administration of 3-NP in Huntington Control group showed increased oxidative stress when compared with control group. 3-NP increased MDA and NO levels and decreases GSH, SOD activity. Treatment with Morin Hydrate at a dose of 100mg/kg significantly

reduced the oxidative stress by decreasing the level of MDA and NO & increasing the level of SOD and GSH through its antioxidant activity. To sum up, Morin hydrate showed significant effect in the management of HD. In this study we observed Morin 50mg/kg was less effective as compared to Morin 100mg/kg (p.o.). High dose of Morin produced significant effect as compared to low dose.

CONCLUSION

In conclusion, Morin Hydrate decreased the oxidative stress, prevented progression of Motor disability and further neuronal degeneration. Thus, Morin Hydrate acts as a potential antioxidant which can be engaged in the management of Huntington disease and other neurodegenerative disorders. Detailed studies are needed to substantiate the use of Morin Hydrate in prevention of 3-NP induced neurodegeneration.

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