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Original Article

Silymarin, Quercetin and Hesperidin Combination Ameliorates Learning and Memory Deficit in 3 Nitro Propionic Acid Induced Rat Model of Huntington's Disease

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ABSTRACT

Introduction: Huntington's disease is considered as the autosomal dominating with progression of neuro-degeneration with decreased cognitive function and behaviour changes. Bioflavonoids promote memory, learning, and cognitive function. The present study aims to summarize the synergistic effect of Silymarin, Quercetin and Hesperidin on ability to in 3-nitropropionic acid (3-NP) induced rat model of Huntington's disease (HD). **Materials and Methods:** Learning and memory deficits were induced in male Wistar rats through intraperitoneal administration of 3-NP. Bodyweight was taken on days 1st, 7th, 14th, and 21st post-treatment. Novel object recognition test (NORT) and elevated plus maze (EPM) were performed on days 21st and 22nd. Animals were sacrificed on day 22 for acetylcholinesterase (AChE) estimation, brain weight assessment, and histopathological study. **Results:** Administration of 3-NP at a dose of 10 mg/kg body weight for 21 days significantly induced learning and memory deficit similar to HD. It reduced body and brain weight, memory retention, and recognition index with enhanced function of acetyl cholinesterase in the brain striatum. Silymarin and Hesperidin as monotherapy significantly restored recognition index and memory loss induced by 3NP due to reduced neuronal damage and apoptosis in the brain striatum, Quercetin and Silymarin restored body weight and relative brain weight because of an increase in muscle weight and reduced brain atrophy. **Conclusion:** All bioflavonoids restored AChE activity, but their combination was better compared to individual drug effects. The current investigation proved that a combination of Silymarin, Quercetin, and Hesperidin is effective in the restoration of ability to learn and memory because of HD compared to monotherapy.

Keywords: Acetylcholinesterase, Bioflavonoids, Cognitive function, Monotherapy, Neurodegenerative disorder, Synergistic effect.

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INTRODUCTION

Huntington's disease is a very uncommon, neurological disease diagnosed by chorea (abnormal involuntary movements), behavioural manifestations, psychological behavioral symptoms, and cognitive impairment, which develop at the age of 30-50 years, and severity increases with age.[1-2] Worldwide prevalence ranges from 5.96 to 13.7 per 100,000.[3] The exact pathogenic mechanism underlying HD has not been explained yet but mitochondrial dysfunction, excitotoxicity, neuroinflammation, oxidative stress, neurochemical imbalance, and apoptosis are the most well-accepted mechanisms.[4-5] Tetrabenazine and Deutetrabenazine are the only drugs that have been approved by the US FDA for treatment of chorea related to HD but it has their limitations like drug interaction and side effects.[6-8]

3 nitro propionic acid (3-NP) induced rat model of HD is found to be the most suitable preclinical model.[9] It is a mycotoxin, at chronic administration (10 mg/kg/day, 3–6 weeks) induces some features similar to those displayed by HD patients. The 3-NP model can mimic and reproduce the hyperkinetic and hypokinetic symptoms of HD, depending on the time and dose administered, thus allowing the initial and late phases of HD to be evaluated.[10-12]

Bioflavonoids belong to a group of natural substances with variable phenolic structures and exhibit a variety of activities including anti-oxidant, anti-inflammatory, antiallergic, antiviral, and anticarcinogenic properties.[13-14] While there have only been a few studies on the potential beneficial effects of flavonoids in HD models, the published results suggest specific flavonoids could be of potential clinical use against HD.[15] Silymarin, a flavonolignan from the seeds of milk thistle (*Silybum marianum*), is a mixture of mainly three flavonolignans, which are silidianin, and silychristine, silybin and here silybin is considered to be more active.[16] Silymarin significantly inhibits the LPS (lipopolysaccharide)-induced activation of microglia and the production of inflammatory mediators, such as tumour necrosis factor-alpha and nitric oxide (NO), and reduced the damage to dopaminergic neurons and protects the neurons of SNC (substantia nigra pars compacta).[17-18] Quercetin is categorized as a flavonol, prominently found in variety of fruits and vegetables.[19] Quercetin reverses 3-NP induced inhibition of respiratory chain complexes, restores ATP level, attenuates mitochondrial oxidative stress in terms of lipid peroxidation, and prevents mitochondrial swelling. Long-term treatment with quercetin can safely and effectively improve selected elements of motor performance and increase muscle mass during initial phases of aging.[20] Hesperidin, a bioflavonoid, is an abundant and inexpensive by-product of 'Citrus cultivation' with abundant of pharmacological properties and medicinal uses.[21] Hesperidin inhibits elevation of TNF- α level, apoptosis, and excitotoxicity in a quinolinic acid-induced rat model of HD.[22] The role of hesperidin concentration of 100 mg/kg p.o. on reduction of MDA (Malondialdehyde) level, enhancement of CAT (Catalase) activity provides a strong sign that it has a beneficial role in the treatment of HD,

which may be because of the involvement of microglial pathway through inactivation of microglial cells.[23]

These findings suggest the bioflavonoids mentioned above may have a beneficial effect on Huntington's disease, that can be tested (induced) by using the 3NP. Therefore, this investigation aims to study the synergistic effect of combinatorial treatment of Silymarin, Quercetin, and Hesperidin on anxiety and depression in 3-NP-induced HD in Wistar rats.

MATERIALS AND METHODS

Drugs and Chemicals

Silymarin, Quercetin, Hesperidin, and 3NP were purchased from Sigma–Aldrich, St. Louis, MO, USA. Unless stated, all other chemicals and biochemical reagents of analytical grade employed (used) in this investigation were purchased from a local vendor in Pune, Maharashtra, India.

Experimental Animals

Male Wistar rats (200- 250 gm) were procured from Crystal biological solutions, Pune, (MS), India. All experimental rat models were kept under standard lab conditions at a temperature of 23 ± 2°C with a RH of 55 ± 10 % under 12 hr light and 12 hr dark cycles throughout the experiment. The Institutional Animals Ethics Committee (IAEC) reviewed and approved all the experimental procedures and protocols used in this study.

Preparation of Drugs and Chemicals

Silymarin (200 mg/kg B/W), quercetin (50 mg/kg B/W) and Hesperidin (50 mg/kg B/W) were administered via p.o. route up to 21 days as a suspension prepared in 0.5 % Carboxymethylcellulose (CMC) (w/v). 3NP (10 mg/kg B/W) was administered intraperitoneally, prepared freshly using normal saline, and administered for 21 days. 3NP was injected 90 min after the administration of test drugs.

Experimental Design

After 1 week of acclimatization, experimental animals were grouped into 8 categories and received treatment for 21 days. Group I (NC) - normal control received Normal saline (1 ml/kg i.p.) and 0.5% CMC (1 ml/100gm p.o.), Group II (HC) - Huntington control received 3-NP (10 mg/kg, i.p.) + 1 ml/gm 0.5% CMC p.o., Group III (ST), IV (QT) and V (HT) received silymarin (200 mg/kg; p.o.), Quercetin (50 mg/kg; p.o.) and Hesperidin (50 mg/kg; p.o.), respectively, with concomitant administration of 3NP. Group VI (S+Q+H T) received a combination of silymarin (200 mg/kg; p.o.), Quercetin (50 mg/kg; p.o.) and Hesperidin (50mg/kg; p.o.) whereas Group VII (S+Q T) received a combination of silymarin (200 mg/kg; p.o.) and Quercetin (50 mg/kg; p.o.), Group VIII (S+H T) received a combination of Silymarin (200 mg/kg; p.o.) and Hesperidin (50 mg/kg; p.o.) with concomitant administration of 3NP (10 mg/kg i.p.).

Estimation of Morphological Parameters

% Change in Body Weight

Bodyweight was recorded on the 1st, 7th, 14th, and 21st day of the treatment, and the percentage change in body weight was calculated with the help of the following formula.[24]

$$\%\ \text{Change in body weight} = \left(\frac{\text{body weight on } 21^{st}\ \text{day}}{\text{body weight on } 1^{st}\ \text{day}}\right) \times 100$$

Brain Weight and Relative Brain Weight

Whole-brain weight was measured on 22nd day after completion of NORT and EPM and relative brain weight was calculated by using the following formula.[25]

$$\text{Relative brain weight} = \left(\frac{\text{Brain weight}}{\text{Body weight}}\right) \times 100$$

Evaluation of Behavioural Parameters

All behavioral procedures were carried out between 8:00 a.m. and 11:00 a.m. in a temperature and humidity-controlled room.

Novel Object Recognition Test (NORT)

NORT was done in the open field arena 72cm×72cm×36cm. At the very first trial (T1), at one corner of open area one object stimulus (O1) was kept while experimental rat was placed at opposite position of arena. Here the time required to explore the object was noted. The experiment was halted after animal has explored the object till 20 sec or maximum 10 min have been elapsed. While conducting the second trial (T2) which was taken after 30 min of T1, another object (O2) was placed in adjacent place with reference to object. The time taken by the experimental animals to explore the objects O1 (familiar) and O 2 (novel) was recorded. In the last trial (T3), performed after 24 hr of T1, O2 was exchanged with newer object (O3) and the time spent exploring the reference (O1) and novel (O3) objects were measured. The initial data received during the object recognition test was put into a Recognition Index, indicating the preference of the experimental test animals for the novel (O2) and the familiar object (O1).[26]

The closer this ratio to 1 more, the animal spent time explored the novel object

Recognition Index = $[t_{novel} / (t\ novel + t_{familiar})]$

Where $t_{familiar}$ is the time taken by animal to explore the familiar object and t_{novel} is the time for the new object.

Elevated Plus-maze (EPM)

Memory dysfunction was evaluated using EPM. The plus-maze has four arms (50 cm × 10 cm) with two open arms and two closed arms, closed with a 40 cm height wall connected with a central platform of 10 × 10 cm dimensions. Acquisition of memory was assessed on day 21st after scheduled treatment. A rat was 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 one of the closed arms was recorded as initial transfer latency (ITL). A rat could explore the maze for 30 sec after recording ITL and returned to its home cage. Retention transfer latency (RTL) was noted again on the 22nd day. The % memory retention was found out by using following formula.[27]

$$\%\ \text{Memory retention} = (\text{ITL} - \text{RTL})/\text{RTL} \times 100$$

Estimation of Biochemical Parameters in Brain Striatum

After completion of NORT and EPM on the 22nd day, all rats were sacrificed by cervical dislocation, and the brain was removed. The brain striatum was identified, separated, weighed, and used for all biochemical estimations and histopathology.

Preparation of Brain Homogenate

10% (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). The homogenate was subjected to centrifugation at 10,000 g up to 15 min. The supernatant aliquots was then separated and utilized for biochemical estimation.[28]

Acetylcholinesterase activity

To 0.4 ml of homogenate, 2.6 ml of phosphate buffer, and 100 µl of DTNB were added. The solution was mixed well; % absorbance was then estimated at 412 nm. After achieving a constant range of absorption, about 20 µl of acetyl-thiocholine was mixed and the fluctuation in absorption was recorded for 5 min with an interval of 1 min to detect the change in absorbance for 1min.[29]

The quantity of AChE was determined with the formula = 5.74×10−4 × change in absorbance per min /g wet weight of tissue.

Histopathology of Striatum

Three animals belonging to individual categories were used for histopathological studies. On day 22nd brain striatum was separated and stored in formalin (10% v/v) solution. After 24 hr are completed, the tissue was dehydrated with alcohol and defatted using xylene. These were fixed into the paraffin wax and sliced into 3-5 µm thick using a microtome. The hematoxylin and eosin (H&E) were the stains used to stain the slides. Slides were visualized using a compound microscope for neuronal degeneration, necrosis, and infiltration of the inflammatory cell along with vascular degeneration and glial cell infiltration with a gradation system from 0-3.

Histopathological Score = No Toxicity-0, Mild- 1, Moderate-2, Severe-3.

Scoring was carried out using the whole slide and the pictures show only some representative areas from the slide.

RESULTS

% Change in Body weight

Administration of 3-NP via intra-peritoneal route up to 21 days caused a significant reduction (p<0.0001) in the % body weight in the HC group when compared with NC rats. Silymarin treated group (200 mg/kg) and Quercetin treated group (50 mg/kg) showed a significant increase in % body weight (p<0.05) and (p<0.01) respectively but Hesperidin (50 mg/kg) treated group did not show any significant restoration in % change in total weight of body when compared to HC rats. Administration of Silymarin (200 mg/kg p.o.) with Hesperidin (50 mg/kg p.o.) produced a significant increased (p<0.05) in % body weight when compared with HC rats. Further, combination treatment of silymarin (200 mg/kg p.o.) and Quercetin (50 mg/kg p.o.) with and without Hesperidin (50 mg/kg p.o.) more significantly restored (p<0.001) body weight when compared to HC rats (Figure 1).

Results are expressed as mean ± SEM (n=6). Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. @@@@p<0.0001 when Huntington control compared with NC rats *p<0.05, **p<0.01, and ***p<0.001 when Treatment groups compared with Huntington control ST = Silymarin treated, QT = Quercetin Treated, HT = Hesperidin treated (Applicable for all Figures)

Relative Brain Weight

Administration of 3-NP via intra-peritoneal route up to 21 days produced a significant reduction (p<0.0001) in the relative brain weight in HC rats when compared with NC rats. Silymarin treated group (200 mg/kg) and Quercetin treated group (50 mg/kg) showed a significant increase in relative brain weight (p<0.01) and (p<0.05) respectively but Hesperidin (50 mg/kg) treated group did not restore relative brain weight as compared to HC rats. Administration of Silymarin (200 mg/kg p.o.) with Hesperidin (50 mg/kg p.o.) produced a significant increased (p<0.01) in % body weight when compared with HC rats. Further, combination treatment of Silymarin (200 mg/kg p.o.) and quercetin (50 mg/kg p.o. B/W) with and without Hesperidin (50 mg/kg p.o.) more significantly restored (p<0.001) relative brain weight when compared to HC rats (Figure 2).

Novel Object Recognition Test

Administration of 3-NP via intra-peritoneal route up to 21 days exhibited a significant reduction (p<0.0001) in recognition index in HC rats when compared with NC rats after 30 min and 24 hr. Silymarin treated group (200 mg/kg p.o.) exhibited remarkable hike in recognition index after 30 min (p<0.001) and after 24 hrs. (p<0.001) as compared to HC rats. Quercetin-treated group (50 mg/kg p.o.) does exhibit a significant increase in recognition index neither after 30 min nor after 24 hr. when compared with HC rats. Hesperidin treated group (50 mg/kg p.o.) produced a remarkable growth in recognition index (p<0.05) when performed after 24 hr. but did not produce any significant increase in recognition index when performed after 30 min. A combination of Silymarin (200 mg/kg p.o.) and Quercetin (50 mg/kg p.o.) restored the recognition index significantly when performed after 30 min (p<0.01) and after 24 hrs. (p<0.05) when compared with HC rats. A combination of silymarin (200 mg/kg p.o.) and Hesperidin (50 mg/kg p.o.) produced a significant increase in recognition index when performed after 30. min (p<0.01) and after 24 hrs. (p<0.01) as compared to HD rats. Further, a combination of Silymarin (200 mg/kg p.o.), Quercetin (50 mg/kg p.o.), and Hesperidin (50 mg/kg p.o.) more significantly restored the recognition index after 30 min (p<0.001) as well as after 24 hrs. (p<0.001) with reference to HC group. (Figure 3A and 3B)

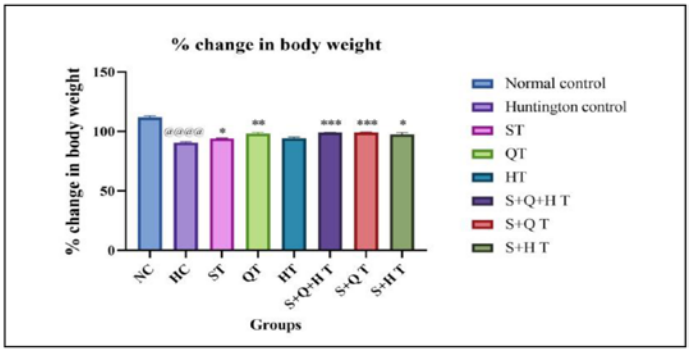


Figure 1: Effect of combination of silymarin, quercetin, and hesperidin on % change in body weight.

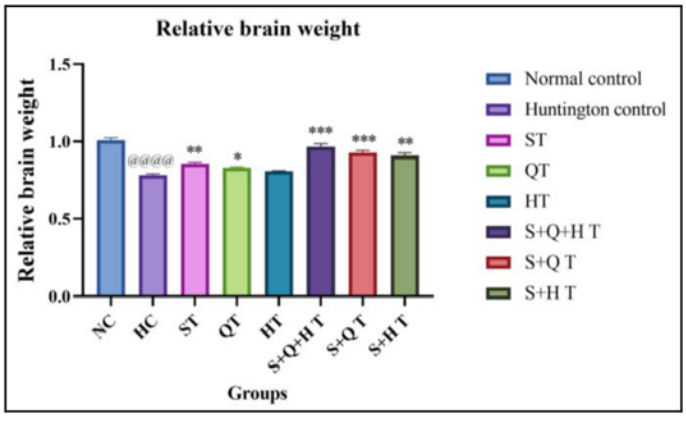


Figure 2: Effect of combination of silymarin, quercetin, and hesperidin on relative brain weight.

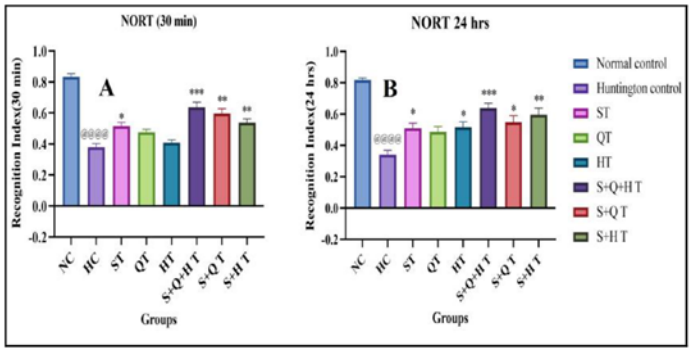


Figure 3: Effect of combination of silymarin, quercetin, and hesperidin submitted to novel object recognition test after 30 min (A) and after 24 hrs (B).

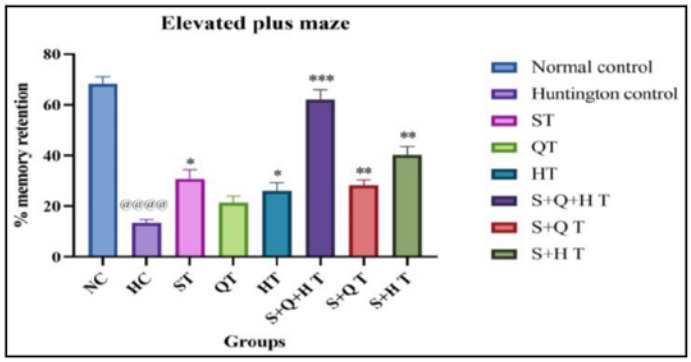


Figure 4: Effect of combination of silymarin, quercetin, and hesperidin submitted to the elevated plus-maze.

Elevated Plus-maze

Administration of 3-NP via intra-peritoneal route up to 21 days exhibited a significant reduction (p<0.0001) in memory retention in HC rats when compared with NC rats. Silymarin treated group (200 mg/kg p.o.), Hesperidin treated group (50 mg/kg p.o.) significantly increased (p<0.05) memory retention but Quercetin treated group (50 mg/kg p.o.) did not produce any significant increase in memory retention when compared to HC rats. Administration of silymarin (200 mg/kg p.o.) with either quercetin (50 mg/kg p.o.) or Hesperidin (50 mg/kg p.o.) significantly restored (p<0.01) memory retention when compared with HC rats. Further, combination treatment of Silymarin (200 mg/kg p.o.) with quercetin (50 mg/kg p.o.) and Hesperidin (50 mg/kg p.o.) more significantly restored (p<0.001) the memory retention when compared to HC rats (Figure 4).

Acetylcholinesterase Activity

Administration of 3-NP via intra-peritoneal route up to 21 days exhibited a significant increase (p<0.0001) in AChE activity in HC rats when compared with NC rats. Silymarin treated group (200 mg/kg p.o.), Hesperidin treated group (50 mg/kg p.o.) showed a remarkable fall in (p<0.01) AChE activity whereas Quercetin treated group (50 mg/kg p.o.) produced a noticeable decrease in (p<0.05) action of AChE in relation to HC rats. Administration of Silymarin (200 mg/kg p.o.) in combination with either Quercetin (50 mg/kg p.o.) or hesperidin (50 mg/kg p.o.) produced a significant decrease in (p<0.001) AChE activity when compared with HC rats Further, combination treatment of Silymarin (200 mg/kg p.o.) with quercetin (50 mg/kg p.o.) and Hesperidin (50 mg/kg p.o.) more significantly restored (p<0.0001) AChE activity when compared to HC rats (Figure 5).

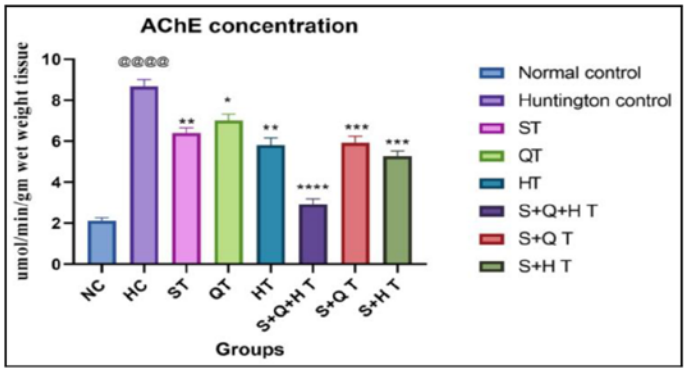


Figure 5: Effect of combination of silymarin, quercetin, and hesperidin submitted acetylcholinesterase concentration.

Histopathology of Brain Striatum

Huntington's control group showed pathological degenerative changes, necrosis, and infiltration of the inflammatory cell along with vascular degeneration and glial cell infiltration in the brain when compared to the control group. Silymarin-treated groups and other combination groups showed promising results in preventing neuronal degeneration when compared with Huntington's control group. Hesperidin proved effective to prevent pathological changes as compared to Huntington's control group. The Quercetin-treated group was found less significant in preventing neurodegeneration when compared with Huntington's control group (Table 1 and 2).

DISCUSSION

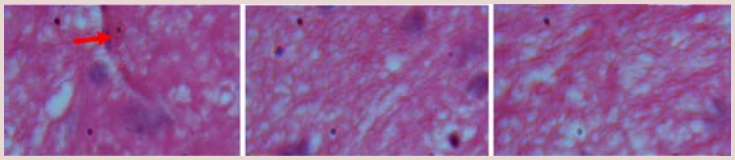
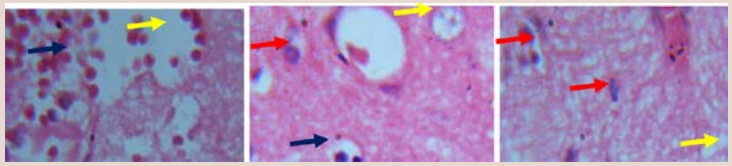
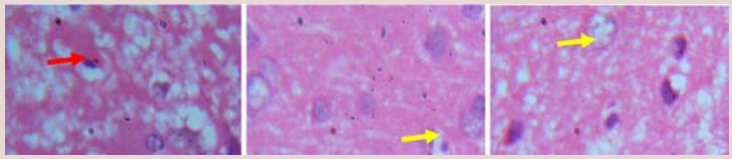
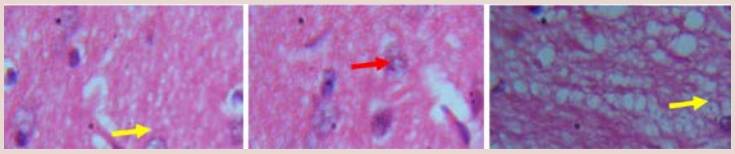
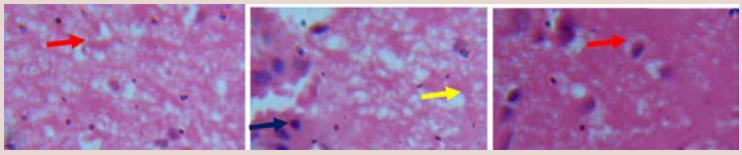
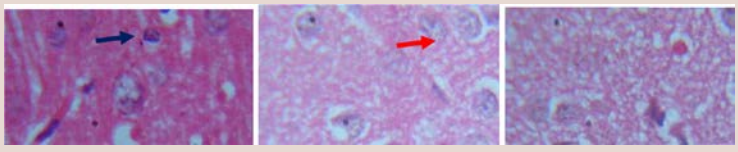
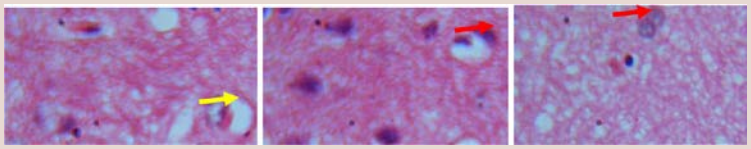
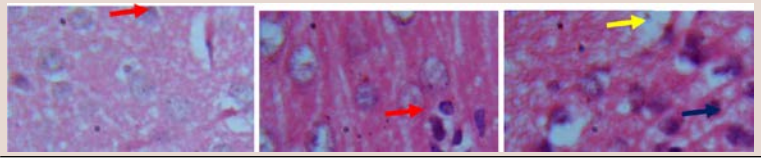
HD is considered as a disorder of the nerve cells in selected areas of the brain like the striatum and hippocampus degenerate, which causes movement, and psychiatric and cognitive difficulties.[30] Memory disorders are frequently discussed in disease, and can be confounded with or exacerbated by attention disorders. There have been several therapeutic advances while treating the disease such as fetal neural transplantation, RNA interference, and transglutaminase inhibitor, but there is no cure for neurodegenerative diseases.[31]

Modern research has showed that the protective effects of flavonoids includes the defending neurons with respect to injury resulting from neurotoxins, a property to lower inflammation of neurons, and the ability to enhance memory, learning, and cognitive function.[32] The mechanisms of flavonoids are performed via preventing of cholinesterase including AChE, butyrylcholinesterase, β-secretase, free radicals, and modulation of signaling pathways that are implicated in cognitive and neuroprotective functions. As per the previous study, Silymarin was found to be a putative neuroprotective agent against many neurologic diseases which may be owing to its tendency to inhibit oxidative stress in the brain and by influencing pathways such as b-amyloid aggregation, inflammatory mechanisms, and cellular apoptotic machinery.[33] Available data shows that silibinin which is the major active component of silymarin ameliorates the impairment of ability to learn and memorize, possibly because of the activation of the ROS-BDNF-TrkB pathway in the hippocampus and the suppression of inflammatory response.[34] Recently Kolhori *et al.* proved that silymarin probably because of to its antioxidant effects causes improvement of memory function and disorder of learning.[35] As per the data available, quercetin shows improvement in learning ability and memory by increasing the GSH level and decreasing the OH– content.[36] Quercetin hinders action of acetylcholinesterase and decreases MDA levels.[37] Previous data suggests, Hesperidin exerts its neuroprotective effect owing to its antioxidant, maintenance of mitochondrial function,

Table 1: Histopathological Score = No Toxicity-0, Mild- 1, Moderate-2, Severe-3. Vascular degeneration (VD), neuronal degeneration (ND), Glial cell Infiltration (GCI).

Sl. no	Group	Animal 1			Animal 2			Animal 3			Total Score	Average score
		VD	ND	GCI	VD	ND	GCI	VD	ND	GCI		Total
1	Normal Control	1	0	0	0	0	0	0	1	0	2	0.22
2	Huntington Control	3	2	2	3	2	2	2	3	3	21	2.33
3	Silymarin treated	1	1	1	1	1	1	1	1	2	10	1.11
4	Quercetin treated	2	1	2	2	1	2	2	2	2	16	1.77
5	Hesperidin treated	2	1	1	1	1	1	1	2	2	12	1.33
6	S+Q+H treated	0	1	1	0	1	1	1	1	0	06	0.66
7	S+Q treated	0	2	1	1	1	1	2	1	1	10	1.11
8	S+H treated	1	1	1	1	1	1	1	1	1	9	1.00

Table 2: Effect of Silymarin, Quercetin, and Hesperidin along with their combinations on vascular degeneration (VD), neuronal degeneration (ND), and Glial cell Infiltration.

Group name	Histopathological image (100x) hematoxylin and eosin (H&E) stain
Normal Control	
Huntington Control:	
Silymarin treated	
Quercetin treated	
Hesperidin treated	
S+Q+H treated	
S+Q treated	
S+H Treated	

Blue arrow- Glial cell Infiltration, Red arrow- neuronal degeneration, yellow arrow- Vascular Degeneration

and antiapoptotic properties in a neuroblastoma cell line.[38] It increases the action of antioxidant enzymes (SOD, glutathione GPx, GRx, and CAT) and GSH levels and decreases MDA in the hippocampal area, and shows improvement in memory retrieval and recognition memory consolidation.

Bodyweight reduction and brain atrophy are fundamental symptoms of HD. As the disorder spreads, swallowing become more difficult, leading to decreased intake of calories, contributing to weight loss. Brain hypotrophy leads to brain weight loss. In our study, Silymarin and Quercetin individually increase body weight, but Hesperidin alone does not produce any significant change. Similarly, Quercetin administration showed significant restoration of brain weight reduction, but Silymarin and its combination with Hesperidin are proven more significant than Quercetin in regaining of brain weight. A combination of Silymarin and Quercetin is the best combination any other, but the addition of Hesperidin does not cause a remarkable gain in brain weight.

Here, we employed EPM and NORT as a behavioral model to screen ability to learn and memory in rats. The action can be investigated via transfer latency (TL) as the criteria for acquisition and restoration of memory process on EPM both in rats and mice.[40] In our study, Silymarin and Hesperidin proved effective in the improvement of % memory retention but Quercetin is not effective as single-drug therapy. A combination of all three bioflavonoids was more prominent when compared to a combination of any one drug with Silymarin for improvement of % memory retention.

The NOR test evaluate the rodent's capacity to identify a already explored object. The NOR particularly relies upon the rodent's innate choice to explore novel over familiar stimuli. Here, Silymarin exhibited remarkable increase in recognition index whether perform after 30 min or 24 hr. but Quercetin could not improve the recognition index. A combine effect of Silymarin with either Quercetin or Hesperidin was better than single-drug therapy but the combination of all three was best effective in improving the recognition index. A previous study says that Ach functions as neurotransmitter that neurons use to communicate with each other. An increased level of enzyme Acetylcholinesterase worsens the symptoms of HD.[41] With acetylcholinesterase activity, all three drugs are significantly effective as monotherapy, but Silymarin was proven best as compared with other drugs. Further, the combination of all three drugs was found to be the best combination for the reduction of acetylcholinesterase activity.

ACh is highly enriched in the striatum and vitally important for normal function. HD striatal damage and increased level of AChE decreases the normal level of ACh which contributes to many neurodegenerative state disease states.[41] In our investigation, we found that Silymarin and Hesperidin along with their combinations can prevent striatal disruption and neuro-inflammation. In conclusion, the present investigation proves that a combination of Silymarin, Quercetin, and Hesperidin significantly improves learning and memory deficit induced by 3 nitro propionic acid in a experimental animal rat model of HD. The aforementioned findings can be useful to extend future study.

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