PRUNETIN ATTENUATES D-GALACTOSE-INDUCED BRAIN AGING VIA INHIBITING AMYLOID-β AND TAU PROTEIN AGGREGATION

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ABSTRACT

The objective of this investigation was to assess the effect of prunetin in D-galactose-induced brain aging in rats and its regulating mechanisms. D-galactose (200 mg kg^{-1} body wt.) was given orally daily for 45 days to accelerate aging, and prunetin (10, 20,40, and 80 µg kg^{-1} body wt. respectively) was administered orally. The anti-oxidant and anti-brain aging activities of prunetin in serum were measured by the estimation of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) and malondialdehyde (MDA) levels and brain tissues were measured by western blot analysis and histopathological studies. Prunetin therapy decreases elevated levels of glucose, C-reactive protein, cholesterol, and triglyceride levels in the D-galactose-induced rats. D-galactose suppresses the levels of superoxide dismutase, glutathione peroxidase, catalase and total antioxidant capacity in rats; these levels were elevated by treating with prunetin. Malondialdehyde levels were elevated in D-galactose-induced rats. Prunetin significantly decreases the malondialdehyde levels in rat brain tissue. Prunetin decreases mitochondrial dysfunction induced by D-galactose, by improving the activities of Na^+K^+-ATPase and acetylcholinesterase enzyme activity. Western blot analysis results showed that the degree of brain tissue damage was significantly reduced by prunetin. The results of our study indicated that prunetin treatment reduced oxidative stress by exerting a protective effect against D-galactose-induced aging in rats, by significantly decreasing amyloid - β and tau protein levels in the brain tissue. Prunetin exhibits anti-oxidant activity by increasing anti-oxidant enzymes.

Keywords: Prunetin, D-galactose, acetylcholinesterase, mitochondrial dysfunction and amyloid-β

ABBREVIATIONS

AGE - Advanced glycation end product, SOD - Superoxide dismutase, GSH-Px - Glutathione peroxidase, CAT - Catalase, T-AOC - Total anti-oxidant capacity, MDA - Malondialdehyde, TNF - Tumour necrosis factor, IL-6 - Interleukin-6, CRP - C-reactive protein, ELISA - Enzyme linked immunoassay, SDS - sodium dodecyl sulfate, AChE - Acetylcholinesterase enzyme.

INTRODUCTION

Brain aging is a neurodegenerative disorder, outlined by a progressive decline in memory and cognition. Aging is mainly due to pathophysiological changes in the brain[1]. According to recent statistics, the number of neurodegeneration patients will exceed 45 million by 2025[2]. Different mechanisms are involved in the regulation of brain aging, with oxidative stress being one of the mechanisms that accelerates brain aging. Oxidative stress is not only a cause of brain aging, but it is also a major cause of other neurodegenerative disorders such as Alzheimer's and Parkinson's[3]. Excessive free radical generation harms cellular proteins, lipids, and DNA, causing aging and cell death[2,3]. Another cause for oxidative stress is the generation of advanced glycation end products (AGEs)[4]. At present, there is no effective treatment for aging and Alzheimer's disease. Cholinesterase inhibitors and NMDA receptor antagonists are used to slow down the progression of the disease[5].

D-Galactose induced model is widely used for examining brain aging, due to similar symptoms induced as natural aging. D-galactose is a monosaccharide molecule that induces aging by the development of

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advanced glycation end products (AGE), which in turn causes stimulation of the receptor for AGE. These glycation end products result in oxidative stress and cellular damage[6]. Oxidative stress accompanies impairment in cognition, and mitochondrial dysfunction, and diminishes the function of preventing the oxidation of cells. D-Galactose toxicity is mainly associated with mitochondrial dysfunction, a leading cause of aging and neurodegenerative diseases[7].

Dietary flavonoids and micronutrients are natural antioxidants mostly present in fruits and vegetables. Recent studies mainly concentrate on the development of natural immunity and antioxidant activity, which can prevent oxidative stress[8]. Holistic medicine attracts them due to its safety, potential, availability and affordability. Antioxidants' free radical scavenging activity reduces oxidative damage and slows down aging[9]. The natural product is a promising alternative therapy for aging-related disorders. Prunetin is an O-methylated isoflavone, one group of flavonoids. First, it was extracted from Oregon cherry, and later on, it was extracted from many plant sources, mainly from cabbage, pea, etc.,[10]. Prunetin exhibits antioxidant, antihyperlipidemic, anti-inflammatory and proteolytic activity[11]. It is a potent inhibitor of aldehyde dehydrogenase in the human liver which is responsible for antihyperlipidemic activity[12,13]. Proteolytic activity of prunetin in article II chondrocytes helps treat osteo-arthritis[14]. Antiproliferative effect of prunetin *in vitro* and in silico was quoted by Preeti Vetrivel, Seong min Kim et al, in 2020[15]. Prunetin exhibits cytotoxic action in human stomach cancer cell lines, according to the researchers. In the present study, Wistar rats induced brain aging with D-galactose, and prunetin is used to treat brain aging by showing its effect on improving cognitive function.

MATERIALS AND METHODS

Reagents and drugs

D-Galactose was purchased from Clear Synth Labs Research Center, Mallapur, Hyderabad. Prunetin was purchased from Merck, Hyderabad. Commercial kits were used for the determination of total antioxidant capacity (T-AOC), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), malondialdehyde (MDA), and monoamine oxidase. Kits were purchased from Sigma Aldrich (St. Louis, USA). Donepezil HCl was purchased from Vasudha Pharma Chem. Ltd., Parawada, Visakhapatnam. Antibodies used for western blot were purchased from Rockland immunochemical, Inc. Limerick, USA.

Animals

Wistar rats weighing about 200 to 220 g of both sexes (male & female) were grouped and housed separately in polyacrylic cages in an animal house maintained under standard laboratory conditions at Maharajah's College of Pharmacy, Andhra University, Vizianagaram, Andhra Pradesh, India. The rats were housed under a 12-h light and 12-h dark cycle at 25 – 28 °C and 50 ± 5 % humidity, with a standard pellet diet and water *ad libitium*. CPCSEA guidelines were followed and were approved by the IAEC (Institutional Animal Ethical Committee) Reg. No. IAEC/GIP-1287/GSS-F/4/2020-2021, GITAM Deemed to be University, Visakhapatnam, Andhra Pradesh, India.

For 6 days, both animals were acclimatized to laboratory settings. The rats were randomized into 6 groups (n= 6/ group). Rats in Group I was injected with normal saline (Control group). Group -II was administered with D- galactose at a dose of 200 mg kg^{-1} body wt. orally once daily for 45 days, to induce brain aging. Group - III was orally administered with D-galactose and donepezil at a dose of 3 mg kg^{-1} body wt. Group IV, V, VI, and VII were orally administered with D-galactose and prunetin at a dose of 10, 20, 40, and 80 µg kg^{-1}, respectively. Prunetin was given along with D-galactose on the same day in respective groups. The total period of study was 45 days.

The weight of the rats was recorded at the start of the experiment, at weekly intervals, and at the end of the experimental studies. Behavioural studies were performed at regular intervals. The animals were sacrificed at the end of the trial following an overnight fasting period of 45 days, on the 46th day.

Behavioural studies

Morris water maze test

The MWM test was performed to examine spatial memory and learning[16,17]. The round pool about 6 feet in diameter and 3 feet in depth, was filled with water that was turned opaque with non-toxic tempera paint. The pool was divided into 4 quadrants. An escape platform was built in the centre of one of the pool's quadrants. The platform was hidden at 2cm underwater. During training, four trial sessions were given to rats per day for five days. If the rat found the hidden platform within 60 secs, the rat could rest on the platform for 15 secs. If the rat failed to find the platform within 90 seconds, a rat was placed on the platform for 15 secs. The latency to locate the platform and the swimming speed of each animal was monitored and recorded. The procedure was repeated every 6 days up to the 45th day. On the 45th day, rats were placed in a pool and permitted to swim freely for 60 seconds. The

time was noted down, how much time the rat spent on the hidden platform, the number of times the rat crossed the hidden platform, and the swimming speed.

Object recognition test (ORT)

The Novel object recognition test is another name for it[18]. It is used to evaluate an animal's cognitive ability. It is a useful tool for assessing various stages of learning and memory. The test was performed in three phases: - Habituation phase, Training phase and Test phase. In the habituation session, the animals were habituated in the square box which consists of 2 transparent glass beakers filled with white cement or all-purpose flour. In a training session, the rats were exposed to 2 identical objects, while in the test session one of the previously exposed objects was replaced with a novel object. In the test phase, the sniffing time for the novel and familiar objects was measured. If the rat has a memory deficit, there is no difference in exploration for 2 objects at the test phase.

The discrimination index, the difference in exploration time of familiar and novel objects, was calculated to evaluate cognitive performance.

Preparation of tissue samples / Brain tissue processing

Animals were anesthetized and sacrificed by decapitation after behavioural testing on the 46th day. Brains were quickly removed and washed with ice-cold 0.9 % saline solution. The brain was completely crushed by using a syringe plunger. 10 % homogenate was prepared by adding 10 mL of ice cold 0.1M Tris HCl buffer to the crushed brain. The homogenate was centrifuged at 10,000 rpm for 20 minutes and the supernatant was used for biochemical assay. A part of the brain tissue was stored at -80 °C for further studies i.e., western blot analysis.

Biochemical analysis

Serum samples were isolated by centrifugation at 4°C and 13000 rpm for 10 minutes. The brain tissue homogenate was centrifuged at 10,000 rpm at 4 °C for 20 minutes, and the supernatant was collected. Serum triglycerides (TG's), blood glucose, insulin, and CRP were measured by using a biochemical analyzer (Cora Lab Touch, Tulip, India). Antioxidant assay of SOD, GSH-Px, MDA, CAT, T-AOC, and Na^+ / K^+- ATPase contents in the brain tissue were determined using commercially available kits purchased from Sigma - Aldrich (St. Louis, USA). The levels of TNF-α and IL-6 in brain tissue and serum were measured using commercial ELISA kits. All procedures were performed according to the instructions of the manufacturers.

Histopathological studies

The brain tissue was isolated and immobilized in 4 % paraformaldehyde for 24 h. After that, it was embedded in paraffin and sliced into 5 µm thick sections. Then, the sections were stained with hematoxylin and eosin. Each slide was examined by a light microscope to observe the histopathological changes (as indicated in Fig. 7).

Determination of acetylcholinesterase and Na^+/ K^+ - ATPase activities

The AChE and Na^+/K^+ - ATPase activities in brain tissue were measured by using commercially available kits (Sigma Aldrich, St. Louis, USA). The activities of the enzymes were expressed as units per gram (U g^{-1}) of protein.

Western blotting analysis

Western blot technique is used to separate and identify proteins[19]. Brain tissue was homogenized with ice-cold lysis buffer by using an electric homogenizer, then constant agitation maintained for 2 h at 4 °C. Minimum samples loaded onto gels was 0.1 mg mL^{-1} and the optimal concentration was 1- 5 mg mL^{-1}. After centrifugation for 20 min at 12000 rpm at 4 °C in a microcentrifuge, the supernatant was collected and kept in ice.

The protein concentrations were determined by using bicinchoninic acid (BCA) assay (Info bio kits, Delhi, India). Equal amounts of proteins were subjected to sodium dodecyl sulfate (SDS). SDS proteins bind to proteins specifically in a mass ratio of 1:4:1. The membranes were incubated with indicated antibodies at 4 °C overnight, followed by incubation with secondary antibodies at 25 °C for 2 h. The expressions of proteins were measured by using an Electrochemiluminescence kit (ECL) (Infobio kits, Delhi, India).

Statistical analysis

The data represented were expressed as mean ± standard deviation. Statistical evaluation was performed by using one-way analysis of variance (ANOVA) with Graph Pad instant demo version and a P-value of < 0.05 was considered statistically significant.

RESULTS

Morris water maze test

Prunetin alleviates learning and memory impairment of D-galactose-induced rats.

Morris water maze test was conducted to evaluate spatial learning and memory functions of D-galactose-induced aging rats. During the training period, the time that rat spent to find out the hidden platform from the 1st day to the 5th day was decreased. Gradually the latency period was shortened to locate the hidden platform (as indicated in Table I). D-Galactose-induced rats spent more time reaching the hidden platform even after the training period when compared with the control group, indicating that long-term exposure to D-galactose reduced the learning and memory abilities of a rat. Our results express that prunetin 10 µg kg^{-1} day^{-1} and prunetin 20 µg kg^{-1} day^{-1} (Fig. 1 indicates $P<0.05$ and $P< 0.01$) prevent the D-galactose-induced neurological impairment and improve learning and memory abilities of prunetin treated rats. When compared to the 7 groups of rats, the swimming speed of rats indicated that D-galactose + prunetin (10 µg kg^{-1} day^{-1}) and D-galactose + prunetin (20 µg kg^{-1} day^{-1}) group rats exhibit no damage to the motor area ($P<0.05$, $P<0.01$), as indicated in Fig. 1.

Table I: Latency time (in a sec) of training rats on MWM Test

Group	Latency time (in sec)
Vehicle group	2.5
Disease control	8.1
Standard group	2.4
Test 1	3
Test 2	2.89
Test 3	2.78
Test 4	2.7

Object recognition test

In this test, the D- galactose-induced rats showed decreased exploration time of new objects compared to the control. Test and standard groups show a significant increase in exploration time compared to disease control. An increase in exploration time shows an effect on recognition memory. Exploration time indicates the recognition of a new object. In this ORT prunetin, 20 µg kg^{-1} day^{-1} (indicated as Fig. 2) and Prunetin 40 µg kg^{-1} day^{-1} (indicated as Table II) treated rats showed recognition of new object by exploration. Here $P<0.05$, was exhibited by Group-V and VI when compared to the control group.

Table II: Exploration time (in sec) of training rats on ORT

Group	Exploration time (in sec)
Vehicle group	11.2
Disease control	6.4
Standard group	10.8
Test 1	9.8
Test 2	10.2
Test 3	10.6
Test 4	10.6

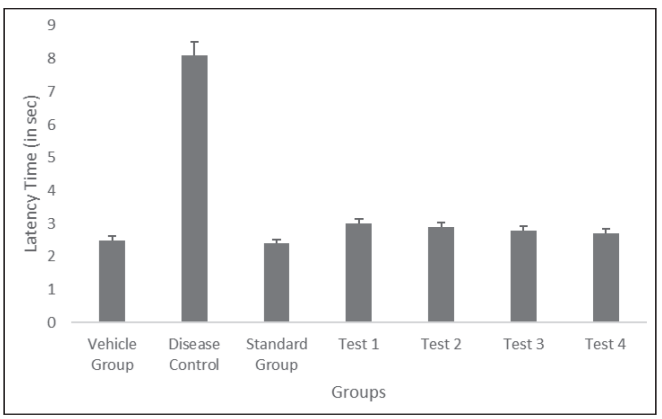

Fig. 1: Effect of prunetin on the spatial learning and memory impairments on the MWM

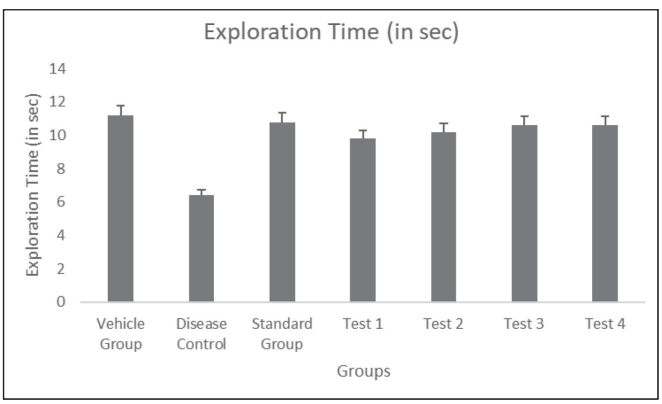

Fig. 2: Effect of prunetin on the recognition of newer objects on ORT

Biochemical analysis

There is a significant difference ($P<0.05$) in the levels of glucose, insulin, and CRP observed among the seven groups of animals (shown in Table III). Levels of glucose, insulin, and CRP are higher in disease-control rats than in vehicle control. Prunetin significantly controls the level of glucose and CRP levels at a dose of 40 µg kg^{-1} day^{-1}.

Serum triglycerides were significantly (P<0.05) higher in D-galactose-induced rats than in vehicle control rats. Levels of cholesterol and triglycerides were significantly decreased in prunetin-treated rats at a dose of 20 µg kg^{-1} and 40 µg kg^{-1} body wt. Prunetin treated rats at a dose of 20 µg kg^{-1} and 40 µg kg^{-1} body wt. showed results similar to that of standard drug-treated rats. Depending upon the results, we observed that a dose of 40 µg kg^{-1} was effective in reducing triglycerides, glucose, insulin, and CRP levels (as indicated in Fig. 3).

Table III: Levels of glucose, CRP, cholesterol, and triglycerides in the serum

Group	Glucose (mmol L^{-1})	CRP (mg dl^{-1})	Cholesterol (mmol L^{-1})	Triglycerides (mmol L^{-1})
Vehicle group	4.1	1.36	2.01	2.23
Disease control	4.93	3.25	2.7	3
Standard group	3.92	1.4	2.13	2.31
Test 1	3.89	1.8	2.1	2.26
Test 2	4	1.5	1.83	2.22
Test 3	4.3	1.39	1.72	2.01
Test 4	3.98	1.84	1.61	1.83

Table IV: Activities of superoxide dismutase, glutathione peroxidase, malondialdehyde, catalase and total antioxidant capacity in the brain tissue

Group	SOD (U mg^{-1} Protein)	GSH-Px (U mg^{-1} Protein)	MDA (nmol mg^{-1} Protein)	Catalase (U mg^{-1} Protein)	T-AOC Activity
Vehicle group	66.73	16.28	3.64	4.62	0.5
Disease control	42.06	7.52	5.09	1.86	0.22
Standard group	64.68	18.83	3.83	4.3	0.32
Test 1	59.31	16.89	3.43	4	0.26
Test 2	62.21	18.52	3.56	4.13	0.25
Test 3	63.02	19.72	3.92	4.35	0.24
Test 4	59.89	17.53	3.96	4.21	0.24

Activities of SOD, GSH-Px, MDA, CAT, T-AOC levels in the brain

To determine whether prunetin attenuated oxidative damage in the brain tissue of D- galactose-treated rats, we measured the activities of SOD, GSH-Px, MDA, and

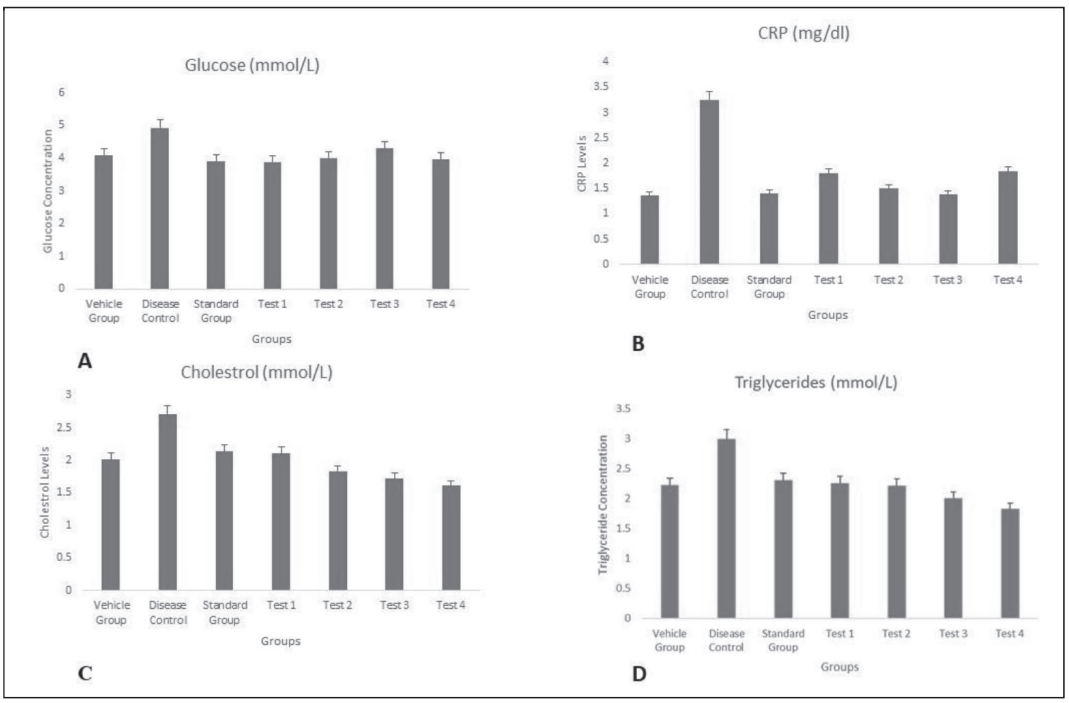

Fig. 3: Effect of prunetin on (A) glucose (B) CRP (C) cholesterol (D) triglycerides. All data are expressed as mean ± SEM. P<0.05 versus normal, P<0.05 versus disease control, P<0.01 versus mode

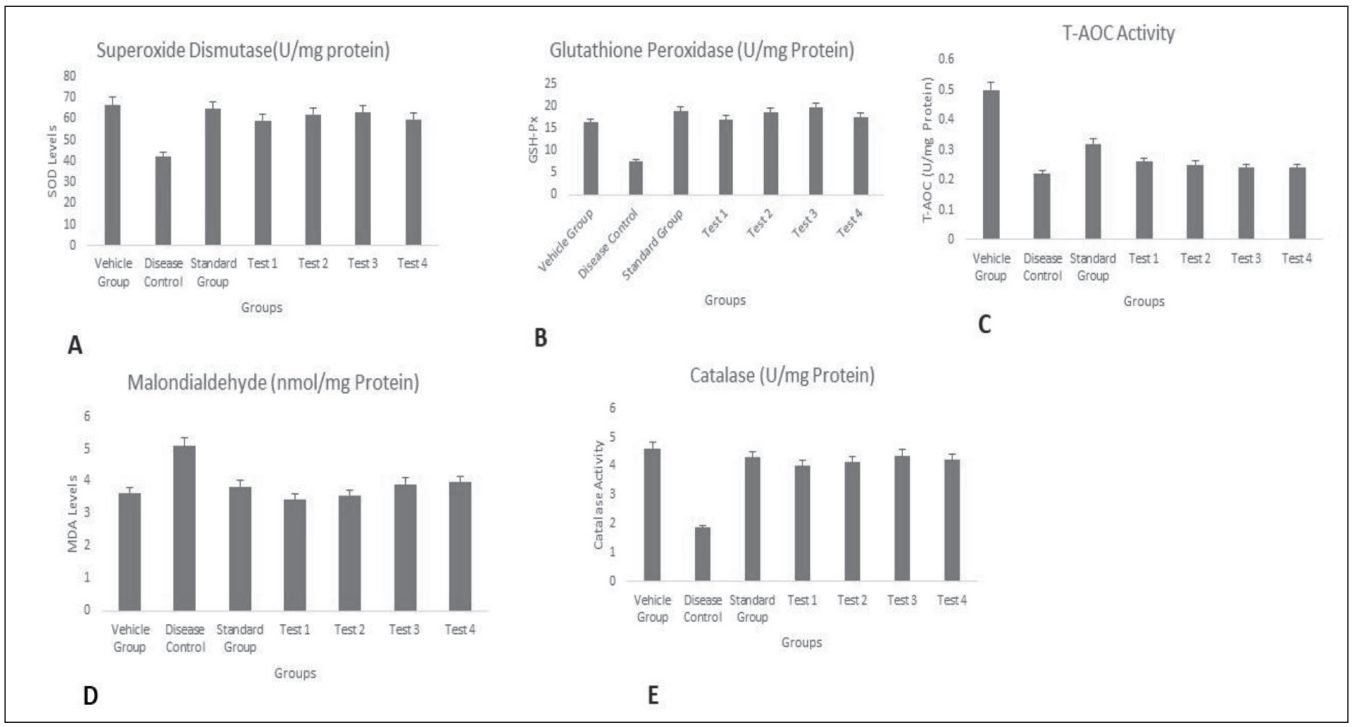

Fig. 4: Effect of prunetin on antioxidant enzymes and lipid peroxidation. (A) SOD activity (B) GSH-Px activity (C) T-AOC activity (D) MDA Content (E) CAT activity

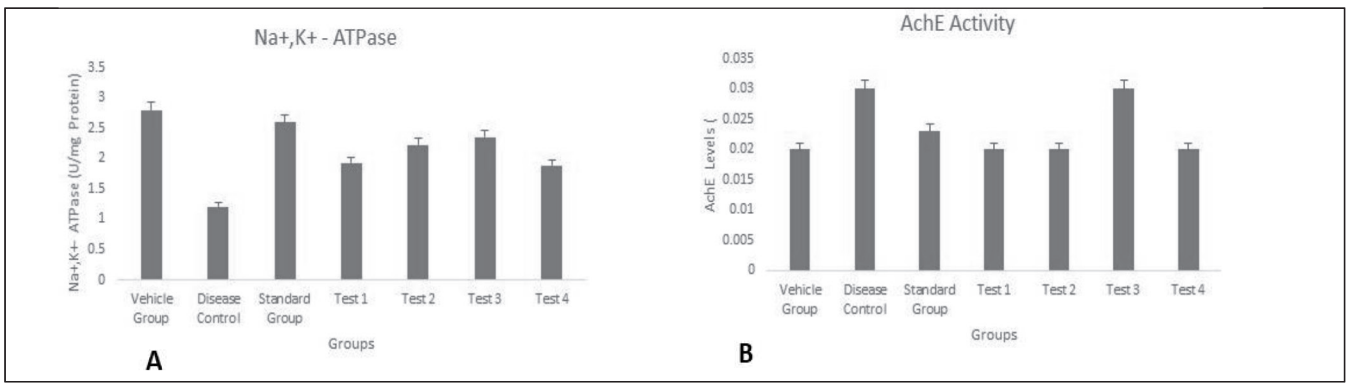

Fig. 5: Activities of (A) Na^+/K^+ - ATPase (B) Acetylcholinesterase enzyme in brain tissue

CAT along with T- AOC levels in the brain tissue (shown in Table IV and Fig. 4). SOD, GSH-Px, CAT as well as T-AOC were significantly lower in positive control rats than in-vehicle control rats. D-galactose treatment reduced the level of MDA. Supplementation with prunetin effectively treated the brain damage caused by D-galactose.

Activities of AChE and Na^+/K^+ - ATPase

The effect of prunetin on the AChE enzyme activity showed that prunetin potentially inhibited the AChE activity in the brain tissue in the rat in a dose-dependent manner. Our results showed that the disease control group remarkably increased AChE activity in the brain tissue, whereas these effects were reversed significantly by prunetin 20 µg kg^{-1} and prunetin 40 µg kg^{-1} supplementation.

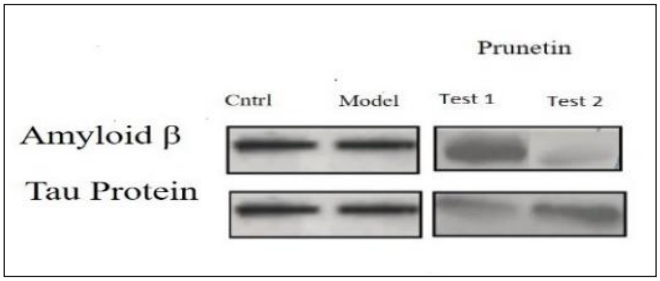

Fig. 6: Effect of prunetin on Amyloid-β and Tau protein expression in brain

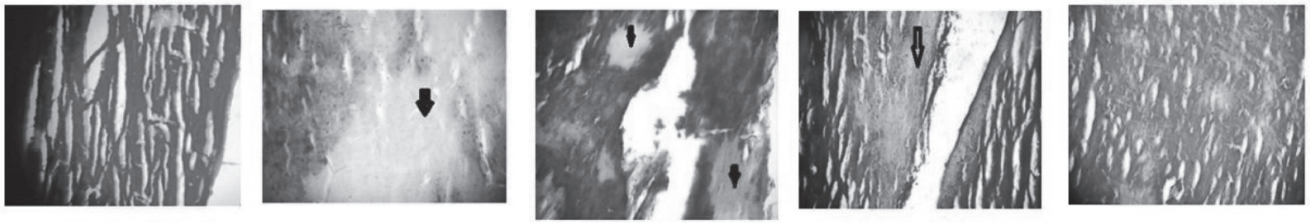

Fig: A Normal | Fig: B Disease Control | Fig: C Standard | Fig: D Test 1 | Fig: E Test 2

Fig. 7: Histopathological slides of brain tissue (A) Control (B) Disease Control (C) Standard (D) Test 1 and (E) Test 2

The effect of prunetin on the Na^+/K^+ -ATPase activity was significantly lower in D- galactose-induced rats than in-vehicle control rats. The Na^+/K^+-ATPase activity was significantly increased in prunetin-treated rats compared with those in the positive control group (as indicated in Table V and Fig. 5).

Western blot analysis

To know the mechanisms intricated in the decrease in amyloid -β and tau protein aggregation by prunetin, we investigate the expression of Aβ and tau protein in the brain. We found that prunetin at 40 µg kg^{-1} exerts a more protective effect on the rats in the Western blot assay. The expression of Aβ and tau proteins was found to be significantly more in the diseased control group than that in normal rats. Prunetin administration significantly inhibited the Aβ and tau expressions compared to the control group (Fig. 6).

Table V: Activities of Na^+/K^+ - ATPase and AChE in brain tissue

Group	Na^+/K^+ - ATPase	AChE Activity
Vehicle group	2.8	0.02
Disease control	1.21	0.03
Standard group	2.6	0.023
Test 1	1.93	0.02
Test 2	2.23	0.02
Test 3	2.35	0.03
Test 4	1.89	0.02

DISCUSSION

In this study, we observed that prunetin supplementation led to reversing the declined learning and memory in D-galactose-induced brain aging rats. Our results showed that 8 weeks of administration of prunetin attenuates the D-galactose-induced cognitive decline in rats.

Oxidative stress plays a major role in neurodegeneration. When the activity of anti-oxidants decreases and the number of reactive oxygen species will increase due to increased production of ROS, it will damage the brain, mainly the hippocampus which is responsible for learning and memory[20]. Na^+/K^+ ion concentration is necessary for impulse propagation and release of neurotransmitters. Na^+/K^+ ATPase enzyme is important for maintaining Na^+/K^+ - levels. Oxidative stress also decreases the activity of Na^+/K^+ - ATPase, which leads to learning and cognitive decline[21,22].

Superoxide dismutase, glutathione peroxidase and catalase are the major enzymes that exhibit anti-oxidant activity by inhibiting free radical production[23]. During oxidative stress, these anti-oxidant enzyme levels are decreased and the production of ROS increases[24].

In our study, 45 days of exposure to a high dose of D-galactose leads to aging and affects the spatial learning and memory capabilities of rats. Our data showed that prunetin 20 µg kg^{-1} and prunetin 40 µg kg^{-1} treatment remarkably decreases the amyloid-β and tau protein levels in the brain. Prunetin at a dose of 10 µg kg^{-1} attenuated oxidative damage, whereas prunetin at doses of 20 µg kg^{-1} and 40 µg kg^{-1} significantly reduced oxidative stress induced by D-galactose.

Superoxide dismutase, GSH-Px and catalase are major enzymes that exhibit anti-oxidant activity by inhibiting free radical production. During oxidative stress, these anti-oxidants enzyme levels are decreased and the production of ROS increases.

The levels of oxidative damage can be determined by assessing the antioxidant enzyme activity such as CAT, SOD and MDS. Anti-oxidant enzyme activities including SOD, CAT, GSH-Px and MDA levels

were decreased. MDA is one of the markers of lipid peroxidation. The key indicator of oxidative damage. CAT, SOD, and GSH-Px levels were decreased and MDA levels were increased in the serum of rats exposed to D- galactose.

Our results expressed that the CAT, SOD, and GPx activity was increased and the MDA levels were decreased in the serum of rats treated with prunetin. Prunetin opposes the oxidative damage caused by D-galactose, even at a dose of 10 μg kg^{-1}.

T-AOC determination facilitates the quantification of non-enzymatic anti-oxidant potential. We observed that in prunetin-treated rats, T-AOC increased in the brain, which indicates the anti-oxidant defense system in D-galactose-treated rats.

Neuroinflammation is another cause of learning and memory impairment. CRP is released in response to inflammatory cytokines. CRP levels were elevated in the serum of D- galactose induced rats. In prunetin-treated rats' serum, the CRP levels were suppressed.

Recent researchers demonstrated that cholinergic neurodegeneration is one of the common causes of Alzheimer's disease and other neurodegenerative disorders[25]. Acetylcholinesterase enzyme degrades the acetylcholine into acetate and choline. AChE inhibition had a role in the treatment of the neurodegenerative disorder. Prunetin inhibits AChE activity and elevates the acetylcholine neurotransmitter, which plays an important role in treating neurodegeneration.

Neurodegeneration due to Aβ and tau plaque formation in brain tissue is the common cause of brain aging and Alzheimer's disease. In Western blot analysis reports, prunetin exhibits a protective effect in the brain by decreasing the production of Aβ and tau proteins (indicated in prunetin treated group). In the Western blot image (control group), the thickness of the band indicates the formation of the protein plaque.

In the behavioral parameters, Morris water maze test and object recognition test, prunetin exerted positive effects to protect rats from D-galactose-induced brain aging. Thus, prunetin improves the cognitive and memory ability of D-galactose-induced rats.

CONCLUSION

In conclusion, the findings of our investigation revealed that prunetin administration considerably lowers Amyloid- β and tau protein levels in the brain. It increases the antioxidant enzyme activity and decreases lipid peroxidation. Myricetin at a dose of 10 μg kg^{-1} also exerts a protective effect. Based on reports, prunetin could be developed into a promising drug for the treatment of neurodegenerative disorders like brain aging and Alzheimer's disease, by preventing oxidative damage.

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