

Flavonoid Ameliorates Anxiety in High Fat High Fructose diet Induced Insulin Resistance in Rats

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ABSTRACT

Objectives: Literature review revealed that intake of high fat diet had a likelihood of causing metabolic syndrome and might be involved in causing anxiogenic symptoms. The current study was drafted to appraise hesperidin effect (bioflavonoid) in delaying the progression of anxiety-like symptoms caused by intake of High-Fat High-Fructose Diet (HFHFrD) in male albino Wistar rats. **Materials and Methods:** Rats were fed with HFHFrD for the induction of insulin resistance (Metabolic syndrome). The role of hesperidin (25 mg/kg p.o), metformin (100 mg/kg p.o) and exercise was assessed in insulin resistance mediated behavioural changes (anxiety). Behavioural parameters, lipid profile of brain/serum, antioxidant status and inflammatory cytokines were determined. **Results:** We observed elevated weight in body and brain in response to high-fat high-fructose diet and causing anxiogenic-like symptoms. Brain oxidative stress was confirmed by increased lipid peroxidation and neuroinflammation, as well as decline in brain antioxidant profile (SOD, CAT and GSH). Changes in neurobehavior and biochemical parameters significantly postponed the symptoms of anxiety due to prior treatment with hesperidin and metformin. Modest improvement with exercise. **Conclusion:** Hesperidin can be a potential therapy in the prevention of anxiety like disorders and can mitigate neuroinflammation and prevent it from neurodegeneration, behavioural changes and biochemical alteration linked with insulin resistance.

Keywords: Insulin Resistance, Neuroinflammation, Anxiety, Metformin, Hesperidin and Exercise.

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INTRODUCTION

Anxiety is a common form of mental disorder which are seen in both young and adult individuals characterized by persistent fear, high alert, motor tension and sympathetic hyper activity.¹ Sedentary lifestyle (westernized diet, physical inactivity) and obesity promote excess adipokine release, elevating Free Fatty Acids (FFAs) and Reactive Oxygen Species (ROS) that triggers Tumor Necrosis Factor-alpha (TNF- α) mediated low-grade inflammation and impair insulin signalling contributing to insulin resistance, promoting the release of pro-inflammatory cytokines (IL-6, IL-1 β and TNF- α) capable of crossing the Blood Brain Barrier (BBB), activating microglia and astrocytes in the CNS. This neuroinflammation, through NF- κ B, JNK and JAK-STAT pathways, disrupts neurotransmission and increases oxidative stress in hippocampus, prefrontal cortex, and amygdala,

contributing to anxiety-like behaviors.² Awareness for overweight and obesity-related issues promote the creation of numerous dietary supplements that can aid in the battle against the disease and also with anxiety-like symptoms linked with it. Under these circumstances we wanted to study the effect of Hesperidin a bioflavonoid found in citrus fruits like oranges, lemons, Tangerines, Mandarins etc., which has free radical scavenging property and shows promising antioxidant, anti-inflammatory and neuroprotective property that was studied in animal models. Hesperidin gets converted to hesperitin which is an aglycone form of hesperidin and has better absorption in the small intestine, this flavonoid supports neuron survival by activating the PI3K-Akt (Phosphoinositide 3-Kinase protein kinase-B) and MAPK (Mitogen-Activated Protein Kinase) signaling pathways and it also influences astrocyte function by promoting the recruitment of neuronal progenitor cells,³ hesperidin has also been shown to enhance insulin sensitivity in rat models, demonstrating that it has protective function against insulin resistance brought on by a high-fat diet by activation of the IR/PDK1 signaling pathway.⁴ There are many literatures available on its various pharmacological effects like antidiabetic, antihyperlipidemic, neuroprotective, nephroprotective, antithyroid, immunomodulatory, antiepileptic, anti-cancer, anti-ulcer effects etc. Some research also proves



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anxiolytic activity of hesperidin in type 2 diabetic rats.⁵ The current investigation was carried out to evaluate behavioural changes related with HFHFrD intake. We determined the biochemical effects in HFHFrD induced neuroinflammation and hesperidin anxiolytic, and neuroprotective effects.

MATERIALS AND METHODS

Reagents and Extract

EDTA (1 mM), phosphate buffer (100 mM, pH 7.4), Nitro blue tetrazolium (NBT), Sodium carbonate (Na₂CO₃), Hydroxylamine HCl (1 mM), Hydrogen peroxide (H₂O₂), Thio barbituric acid (TBA 0.6%), Phosphoric acid (H₃PO₄), n-Butanol, Sulfosalicylic acid (3%), NADPH₂, Glutathione reductase, GSH (Reduced glutathione), Dimethyl sulfoxide (DMSO), Glucose solution. Hesperidin was purchased from Otto Chemi. Pvt. Limited., India.

High-fat high-fructose diet preparation and induction of insulin resistance

The High Fat High Fructose Diet (HFHFrD) was prepared by blending 390 g regular rat chow with 40 g of sour starch and 110 g of vegetable fat, after dissolving in water at a concentration of 10 g per 100 mL in the form of pellets, while the fructose diet will be made by dissolving 30 g fructose in 100 ml water and shall be placed into their feeding bottle for administration excluding the normal control group which shall be fed with a normal pellet diet.⁶ HFHFrD was fed for 42 days for induction of IR and was confirmed by HOMA-IR

Assessment of OGTT (Oral glucose tolerance test)

A test for Oral Glucose Tolerance (OGTT) was conducted in overnight fasted rats that have access to water. Glucose solution (2 g/kg p.o.) was fed half an hour after the extracts were administered. The tail tip was used to draw blood at 0, 30, 60, 90, and 120 min of glucose administration.⁷ Glucose level was estimated by using a standard glucometer (Accucheck, India).

Evaluation of Insulin Resistance (HOMA-IR Method)

The animals were subjected to fasting for 12 hr with access to water. Fasting glucose levels was estimated using a Glucometer (accucheck, india) and the insulin levels during fasting were measured using a commercial kit for radioimmunoassay following the instructions provided by the manufacturer (Linco Research, United States, St. Charles, MO). The assessment of the homeostasis model (HOMA) index of insulin resistance was calculated using the equation:

$$\text{HOMA} = [\text{glucose (mM)} \times \text{insulin } (\mu\text{U/mL})] / 22.5$$

As previously reported by Pikavance *et al.*⁸

Experimental Animals and Treatment Protocol

After reviewing and approving the experimental protocol, the Institutional Animal Ethics Committee (Krupanidhi College of Pharmacy, Bengaluru, Karnataka, India) bearing Approval No. KCP/IAEC/PCOL/157/July-2024. The study involves the use of male albino wistar rats that weigh 200 to 250 g. All animals permitted to adjust to standard laboratory circumstances prior to the start of experiment. Briefly, the experimental rodents were maintained in a controlled environment with temperatures ranging from 25 to 29°C, relative humidity (55-60%), and regulated 12-hr dark/light photoperiod. The animals were offered unlimited provision of water and maintained a standard diet prior to their dietary changes. Prior to initiating the experimental investigation, we standardized the induction of the Insulin Resistance (IR) model in rats based on protocol described by Melo *et al.*, The animals were divided into six groups at random. Eight rats in each group ($n=8$). Group I was maintained on a standard diet throughout the study and served as normal control. Group II to VI were fed a High-Fat High-Fructose Diet (HFHFrD) for 6-weeks to induce insulin resistance and was continued till end of the study. This dietary model involved administering a high-fat diet as feed, accompanied by 30% fructose dissolved in drinking water and fed via water bottles. Treatments began simultaneously with HFHFrD administration, Group II (positive control) feed with only HFHFrD, group III was administered with hesperidin (25 mg/kg, p.o.), group IV undergoing exercise (5 days/week for 14 weeks), group V received metformin (100 mg/kg, p.o), group VI being administered hesperidin alone (25 mg/kg, p.o) without HFHFrD. Treatment was carried out from day one of HFHFrD administration for hesperidin, metformin and exercise. Diazepam was treated after induction of IR. Animals body weight was tracked weekly. Behavioural tests were assessed after the induction of IR.

Assessment of behavioural Parameter

Elevated Plus Maze (EPM)

EPM was employed to evaluate anxiety-related behaviour in rats. EPM apparatus comprised of four arms arranged in a plus-shaped configuration, raised 50 cm above the floor. Two arms were open (30 × 5 cm), the other two were enclosed by opaque walls (30 × 5 × 20 cm). All arms extending from a 5 × 5-centimetre center platform. At beginning of the test, each rat was gently positioned at the center zone fronting an open arm and behavior was monitored for 5 min. How much time is spent in the open versus closed arms was recorded by two independent observers. Greater occupancy of the arms enclosed was considered signs of anxiousness, whereas increased entry and time spent with arms wide open reflected anxiolytic activity.^{9,10}

Light Dark Model

To assess anxiety-related behavior, a Light/Dark Box was employed. The apparatus contained two connected chambers: one brightly illuminated and the other kept dark. The dark section (27 × 18 × 29 cm) was dimly lit using red light, while the light section (27 × 27 × 29 cm) was exposed to a 40-W white bulb. Both Sections were divided by a wall with a 7 × 7 cm doorway that permitted free passage between the two zones. At the beginning of each trial, rats were put in the light chamber facing the entrance to dark side and behavior was recorded for a standard observation period. The key variables noted were the time spent in the illuminated compartment, the number of complete entries in light side (four paws inside), how long it takes to first enter dark area and overall locomotor activity in both sections. After each test, the chambers were cleansed with 10% ethanol to get rid of odor traces.¹¹

Open Field Test (OFT)

Open field test was conducted following procedure originally Walsh and Cummins' description (1976),¹² with modifications as outlined by Sethi and others. Test was conducted in a square-shaped field measuring 70 × 70 × 60 cm, the floor separated into 49 equal squares (each 10 × 10 cm). Rats were positioned in the middle of the arena, and testing commenced 10 sec later. Behavioral assessments were carried out over three 5 min sessions. The parameters recorded included: (A) time spent in the open field's center, (B) distance travelled in total, (C) duration of freezing behavior. Freezing was defined as the period during which the rat remained still either lying or sitting with minimal movements, such as slow head shifts or breathing-related motion. All behaviors were analyzed offline after testing.

Rotarod Test

The rotarod apparatus consisted of a horizontal rod (wooden or metallic) coated with rubber to enhance grip, with a diameter of approximately 3 cm. The rod was connected to a motor that allowed adjustment of the rotation speed. It was positioned at a height of about 50 cm above the tabletop to prevent animals from voluntarily jumping off. Beneath each section, individual cages were placed to safely contain the animals after they fell from the rotating rod and to minimize further movement. The time for which the animal falls, will be measured.¹³

Swimming exercise

Exercise intervention was provided in the form of swimming. Training was conducted in a glass tank (100 × 40 × 60 cm) water-filled and kept at 32°C. Level of water was adjusted to allow the rats to swim freely without support. On the first day, animals swam for 15 min and the duration was increased by 5 min each day until a maximum of 30 min was reached for 3-16 weeks. The exercise group performed daily swimming sessions of 30 min, five days per week.¹⁴

Assessment of Biochemical Parameters in Brain Tissue

After the treatment period, animals were subjected to overnight fasting and then sacrificed. Brain was quickly removed, and the hippocampus was dissected out. Tissue homogenization was performed in KH₂PO₄/K₂HPO₄ phosphate buffer saline (50 mM, pH 7.4) using a Elvehjem-Potter homogenizer. The homogenates were centrifuged for 10 min at 10,000 g at 4°C and resulting supernatant was utilized for estimation of protein content, reactive oxygen species, reduced glutathione, antioxidant enzyme activity, Malondialdehyde (MDA) and lipid profile. A separate portion of the hippocampus was processed following the method described by Gupta and Kaur for cytokine measurement.¹⁵

Serum lipid content

Free Fatty Acids (FFA), Triglycerides (TG), High-Density Lipoprotein cholesterol (HDL), and serum Total Cholesterol (TC) and concentrations were estimated using Semiauto analyser-Erba chem 5-plus (Erba Diagnostics, Germany) with standard enzymatic colorimetric kits following manufacturer's protocols.

Brain lipid content

Triglyceride (TG), High-Density Lipoprotein (HDL), and Total Cholesterol (TC) contents in the brain were assessed using commercial diagnostic kits provided by Randox Laboratories (Ardmore, Northern Ireland, UK).¹⁶

Assessment of Antioxidant Status

Protein concentration was quantified using the Hartree method,¹⁷ a modified version of the classical Lowry technique. Lipid peroxidation, expressed as Malondialdehyde (MDA), was estimated by the thiobarbituric acid reaction method.¹⁸ Reduced Glutathione (GSH) levels were measured following the procedure of Sedlak and Lindsay,¹⁹ while total sulfhydryl groups were analyzed using Ellman's method.²⁰ Glutathione Peroxidase (GPx) activity in brain tissue was assessed according to the protocol described by Flohé and Günzler.²¹ Activity of the enzyme Superoxide Dismutase (SOD) was measured using Fridovich and Misra method,²² and Catalase (CAT) activity was evaluated following the method of Aebi.²³

Assessment of ROS Levels: Reactive Oxygen Species and inflammatory mediators

Levels of H₂O₂ (hydrogen peroxide) in the hippocampus were measured according to the procedure of Dineon *et al.*,²⁴ In this method, H₂O₂ interacts with 4-aminoantipyrine and p-hydroxybenzoic acid when peroxidase is present, producing a pink complex of quinoneimine, which is quantified spectrophotometrically at 505 nm. The hydroxyl radical generation was assessed following the protocol of Payá *et al.*,²⁵ Briefly, hydroxyl radicals were produced via EDTA H₂O, the Fe³⁺ ascorbate system, which oxidizes deoxyribose. The hippocampal

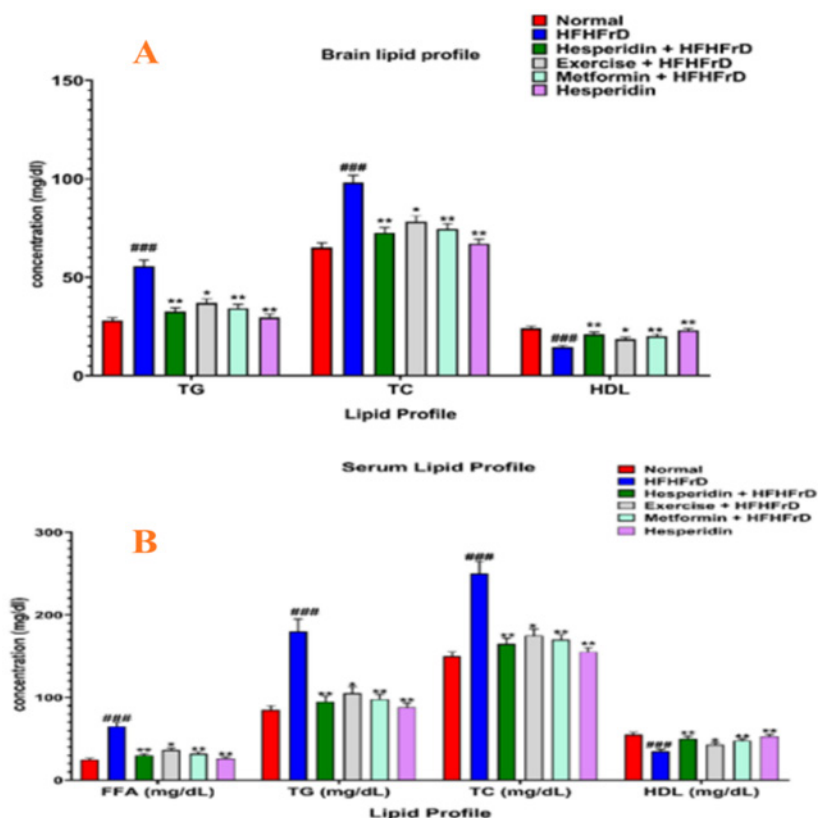


Figure 1: Lipid Profile: A) Brain lipid profile B) Serum lipid profile. Data expressed here are in Mean \pm SD ($n=8$), ** $p<0.01$ vs HFHFrD, * $p<0.05$ vs. HFHFrD, ### $p<0.001$ vs. Normal.

homogenate was incubated with the reaction mixture for 1 hr at 37°C, after which reaction was halted through addition of (2.8%) trichloroacetic acid and (1%) thiobarbituric acid. The samples were then boiled for 20 min at 100°C min, and absorbance was measured at 532 nm in comparison to a blank that contained only buffer and deoxyribose. Radical superoxide levels were determined based on the method of Marklund and Marklund,²⁶ through minor modifications. Pyrogallol was added after homogenates were incubated in Tris-HCl buffer to initiate reaction. After a 4-min incubation at 25°C, the response was terminated with Hydrogen Chloride (HCl). At 420 nm, absorbance was measured in comparison to the blank.

Statistical evaluation

Data are presented as Mean \pm Standard Error of the Mean (SEM). Statistical comparisons between groups were carried out between groups, using the unpaired Student's *t*-test or One-Way Analysis of Variance (ANOVA), after confirmation of data normality distribution across all measured parameters. *post hoc* multiple comparisons were performed via Tukey's test. p value ≤ 0.05 was regarded as statistically significant. Analyses were carried out using GraphPad Prism (version 8.0).

RESULTS

Influence of Hesperidin and HFHFrD on body, brain and abdominal fat weights

Rats fed with HFHFrD demonstrated notable increases in abdominal, brain and body fat weight in contrast to those who fed a regular diet. Supplementation with hesperidin significantly reduced these elevations and metformin produced similar protective effects (Table 1).

Effects on Lipid profile

Brain Lipid profile

The Brain lipid profile revealed that HFHFrD feeding resulted in notable increase in triglycerides, total cholesterol and alongside a decline in HDL cholesterol level in contrast to rats with normal diet. Hesperidin administration ameliorated these disturbances, significantly lowering cholesterol and triglyceride levels, while partially restoring HDL in the brain. Metformin produced a similar normalization of lipid parameters (Figure 1A).

Serum lipid profile

The serum lipid profile revealed that HFHFrD feeding resulted in notable increase in free fatty acids, total cholesterol, triglycerides and alongside a decline in HDL cholesterol level in contrast to rats with normal diet. Hesperidin administration ameliorated these disturbances, significantly lowering cholesterol and triglyceride

levels while partially restoring HDL. Metformin produced a similar normalization of lipid parameters (Figure 1B).

Impact on Oral Glucose Tolerance (OGTT) and insulin resistance

Oral Glucose Tolerance Testing (OGTT) showed progressive impairment in glucose clearance among HFHFrD-fed rats. At baseline, no intergroup differences were evident; however, by week 6, HFHFrD animals displayed significantly higher blood glucose at multiple time points, which persisted and worsened through weeks 10 and 14. Hesperidin improved glucose tolerance, beginning at week 8 with greater improvements at weeks 10 and 14. Metformin produced comparable glucose-lowering effects throughout the study. The HOMA-IR analysis further confirmed severe IR in HFHFrD rats as compared to control. Both hesperidin and metformin significantly reduced HOMA-IR values, indicating enhanced insulin sensitivity and improved glycemic regulation (Figures 2A-2E).

Effects on Antioxidant Profile of Brain

HFHFrD feeding triggered a marked oxidative imbalance in brain tissue. Compared with normal controls, HFHFrD rats exhibited significantly elevated Malondialdehyde (MDA) levels, reflecting increased lipid peroxidation. Antioxidant defences were also compromised as evidenced by reduced Catalase, Glutathione Peroxidase (GPx) and Superoxide Dismutase (SOD), along with depleted Glutathione (GSH) levels. Hesperidin treatment significantly countered these alterations, restoring GPx, CAT and SOD activities, while elevating GSH and lowering MDA. Metformin and exercise interventions displayed similar antioxidant-protective actions (Figures 3A-3E).

Effects on Behavioral Parameters

Effect on open field test

HFHFrD-fed animals showed clear anxiety-like behaviors, including reduced central zone exploration, shorter distance travel, and increased freezing episodes relative to controls. Hesperidin treatment significantly improved exploratory activity and reduced immobility. Metformin demonstrated comparable benefits, while exercise produced moderate anxiolytic effects. Diazepam, as expected, showed strong anxiolytic efficacy across all behavioral indices (Tables 2-4).

Effect on elevated plus maze

Rats receiving HFHFrD spent significantly shorter time in open arms and longer period of time in closed arm compared to controls, reflecting elevated anxiety. Hesperidin administration increased open-arm time and diminished closed-arm preference at the end of treatment compared with HFHFrD controls, suggesting an anxiolytic-like action. Metformin and exercise also improved EPM performance with similar trends (Tables 5 and 6).

Effects on light and dark test

HFHFrD feeding reduced duration of stay in light zone, increased dark zone period compared to normal, consistent with anxiety-like behavior. Hesperidin reversed these alterations, enhancing light-zone exploration and reducing dark-zone preference. Comparable effects were observed with metformin and exercise (Tables 7 and 8).

Effects on brain Reactive Oxygen Species (ROS) and inflammatory mediators.

HFHFrD fed rats showed excessive ROS generation in hippocampal tissues, A) Hydrogen peroxide and B) hydroxyl

Table 1: Fat weight. Data expressed here are in Mean±SD (n=8), * p<0.05 vs HFHFrD, ## p<0.01 vs normal.

Parameter (g)	Normal	HFHFrD	Hesperidin+HFHFrD	Metformin+HFHFrD
Brain weight	1.42±0.02	1.65±0.04 ##	1.50±0.02 *	1.47±0.02*
Body weight	240.46±2.67	294.70± 14.00 ##	252.84±5.90 *	247.96±4.75*
Abdominal weight	8.21±1.99	19.23±6.30 ##	12.65±3.90 *	10.92±2.72*

Table 2: Open field test- center time (s). Data expressed here are in Mean±SD (n=8), * p<0.001 vs HFHFrD, ** p<0.01 vs HFHFrD, * p<0.05 vs HFHFrD, ### p<0.001 vs normal, ## p<0.01 vs normal.**

Group	Week 8	Week 10	Week 12	Week 14
Normal	42.0±2.0	42.0±2.0	42.0±2.0	42.0±2.0
HFHFrD	28.0±1.5 ##	23.0±1.2 ###	21.0±1.3 ###	19.0±1.2 ###
Hesperidin+HFHFrD	33.0±1.6 *	38.0±1.5 **	40.0±1.6 ***	41.0±1.4 ***
Metformin+HFHFrD	32.0±1.7 *	34.2±1.5 *	36.5±1.4 **	38.0±1.3 **
Exercise+HFHFrD	27.0±1.4	29.0±1.3	32.0±1.3 *	37.5±1.2 **
Hesperidin	40.5±1.5 ***	41.0±1.3 ***	41.5±1.2 ***	42.0±1.1 ***

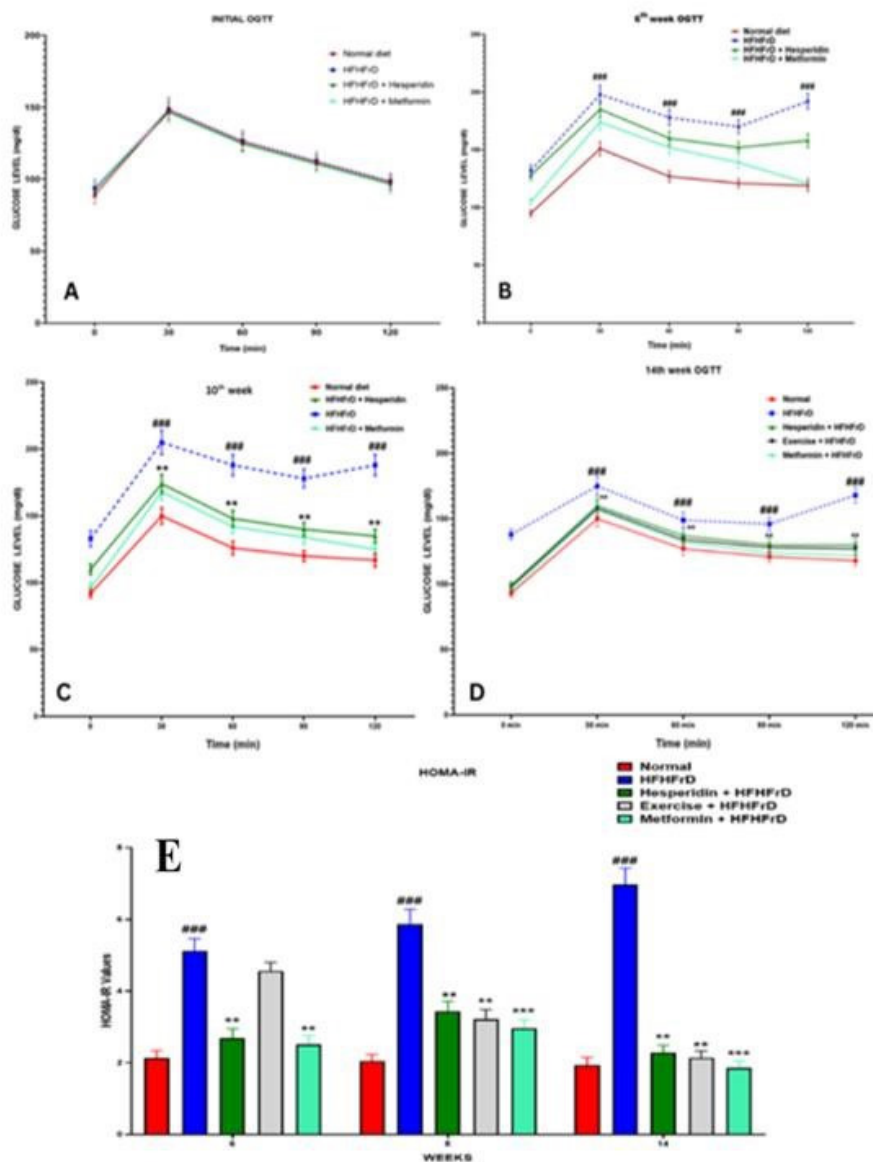


Figure 2: Oral glucose tolerance test and HOMA-IR. A) Initial B) 6th week C) 10th week D) 14th week E) HOMA-IR. Data expressed here are in Mean±SD (n=8), ** $p > 0.01$ vs HFHFrD, * $p > 0.05$ vs HFHFrD, *** $p > 0.001$ vs HFHFrD, ### $p > 0.001$ vs Normal.

radical levels were significantly elevated compared to controls. Hesperidin alone these elevations, lowering both hydrogen peroxide and hydroxyl radical levels. Likewise, C) IL-6 and D) IL-1 β , a pro-inflammatory cytokine was markedly elevated in the HFHFrD group but were significantly attenuated following hesperidin administration. Comparable antioxidant and anti-inflammatory effects were also observed with metformin and exercise interventions (Figures 4A-4D).

Effect on Rotarod

The rotarod test revealed that rats receiving HFHFrD diet showed progressive motor coordination decline, with significant deficits emerging by week 8, worsening by week 14 compared to controls. Hesperidin treatment (alone and combined), metformin and exercise interventions all provided significant protection against HFHFrD-induced motor impairment from week 8 onward,

with hesperidin alone showing the most robust neuroprotective effects. These findings demonstrate that all tested interventions effectively counteracted the progressive motor deficits induced by chronic HFHFrD consumption (Figure 5).

DISCUSSION

The current research highlights deleterious effects of a HFHFrD on metabolic, neurochemical and behavioural parameters and provides compelling evidence that hesperidin and metformin treatment effectively reversed these impairments, particularly in the context of neuroinflammation and delay in anxiety-like behaviours. Prolonged HFHFrD intake led to Insulin Resistance (IR) in our animal model as shown by elevated insulin levels which were confirmed by HOMA-IR and impaired glucose regulation. These outcomes are in line with existing literature,⁶

which attributes these metabolic disturbances to the high energy density, low satiety and high palatability of fat-rich diets that drive overconsumption and adiposity accumulation.²⁷⁻³⁰ Our findings validate these results, showing that six weeks of HFHFrD significantly altered body composition and glucose metabolism. Importantly, hesperidin and metformin interventions successfully reduced weight gain and nearly normalized glucose and insulin levels, supporting their potential in improving insulin sensitivity and regulating systemic metabolism. Results also indicated a pattern of anxiety-like behaviour, which progressively worsened in HFHFrD-fed animals, becoming statistically significant by the 8th week as confirmed through behavioural assessments including EPM, OFT, and LDT. Hesperidin-treated animals exhibited a delayed onset of significant behavioural changes, showing only mild anxiety like effects at week 8 but anti-anxiety significance. Exercise showed a similar trend, with modest improvement during the early phase (week 8 and 10) and more robust effects by week 12 onward. Metformin showed a gradual trend toward improvement, although without reaching statistical significance compared to HFHFrD, which aligns for the anxiolytic property

that has been previously seen in type-2 diabetic rats.⁵ In addition to metabolic disruptions, HFHFrD profoundly affected the brain's lipid profile, increasing total cholesterol levels and triglycerides while HDL remained low, such lipid dysregulation in the brain is particularly detrimental given the limited capacity for self-regulation of lipid substrates in neural tissue. Chronic accumulation of lipids contributes to systemic and organ-specific damage, including in the brain.³¹⁻³⁴ Our findings align with these observations and further demonstrate that hesperidin mitigates brain hypercholesterolemia, potentially by restoring lipid homeostasis and preventing cerebral lipid overload. A major mechanism linking HFHFrD to neurological dysfunction is oxidative stress, develops when Reactive Oxygen Species (ROS) production surpasses the available capacity of the antioxidant protection system. In our study, this was evident from elevated Malondialdehyde (MDA), an index of lipid peroxidation, along with decreased activity of key antioxidants, including CAT, SOD and GPx, and non-enzymatic Glutathione (GSH) antioxidants in HFHFrD fed rats. These outcomes are in line with research findings that have shown oxidative damage in brains of rats with

Table 3: Open field test- distance travelled. Distance travelled (m). All data are represented are in Mean±SD (n=8), * p<0.001 vs HFHFrD, ** p<0.01 vs HFHFrD, * p<0.05 vs HFHFrD. ### p<0.01 vs normal, ### p<0.001 vs normal.**

Group	Week 8	Week 10	Week 12	Week 14
Normal	5.38±0.39	5.36±0.37	5.39±0.41	5.41±0.42
HFHFrD	3.45±0.38 ##	3.00±0.34 ###	2.80±0.36 ###	2.65±0.34 ###
Hesperidin+HFHFrD	4.15±0.33 *	4.00±0.32 **	4.45±0.31 ***	4.85±0.30 ***
Metformin+HFHFrD	4.25±0.35 *	4.08±0.34 *	4.51±0.32 **	4.90±0.29 ***
Exercise+HFHFrD	3.90±0.34	3.80±0.33	4.00±0.31 *	4.20±0.30 **
Hesperidin	5.18±0.31 ***	5.40±0.28 ***	5.53±0.27 ***	5.60±0.26 ***

Table 4: Open field test- Freezing time (s). Data expressed here are in Mean±SD (n=8), * p<0.05 vs HFHFrD, * p<0.001 vs HFHFrD, ** p<0.01 vs HFHFrD, ### p<0.001 vs normal, ## p<0.01 vs normal.**

Group	Week 8	Week 10	Week 12	Week 14
Normal	48.0±2.5	50.0±2.0	48.5±2.0	47.5±2.0
HFHFrD	68.0±2.5 ##	78.0±2.8 ###	82.0±2.5 ###	88.0±2.5 ###
Hesperidin+HFHFrD	60.0±2.0 *	59.0±2.0 *	55.0±1.8 **	53.0±1.8 ***
Metformin+HFHFrD	59.0±2.0 *	60.0±2.0 *	58.0±1.8 **	55.0±1.8 ***
Exercise+HFHFrD	65.0±2.3 *	63.0±2.0 *	60.0±1.9 **	56.0±1.9 **
Hesperidin	48.5±2.0 **	49.0±2.0 **	48.0±1.8 ***	47.0±1.7 ***

Table 5: Elevated plus maze- time spent in open arm (s). Data expressed here are in Mean±SD (n=8), * p<0.05 vs HFHFrD, ** p<0.01 vs normal, * p<0.001 vs HFHFrD. ### p<0.001 vs normal, ## p<0.01 vs normal.**

Group	Week 8	Week 10	Week 12	Week 14
Normal	131.6±5.2	132.8±4.9	134.1±5.0	133.9±5.1
HFHFrD	99.0±4.5 ##	87.0±4.1 ###	74.0±3.9 ###	66.5±3.8 ###
Hesperidin+HFHFrD	108.0±4.3 *	112.5±4.0 **	127.5±4.2 ***	130.0±3.8 ***
Metformin+HFHFrD	100.0±4.4	104.0±4.1 *	118.0±4.4 **	129.0±3.7 ***
Exercise+HFHFrD	102.0±4.1	98.5±4.0	105.0±4.3 *	112.0±3.9 **
Hesperidin	125.5±4.6 **	128.0±4.8 ***	130.0±5.0 ***	131.0±4.9 ***

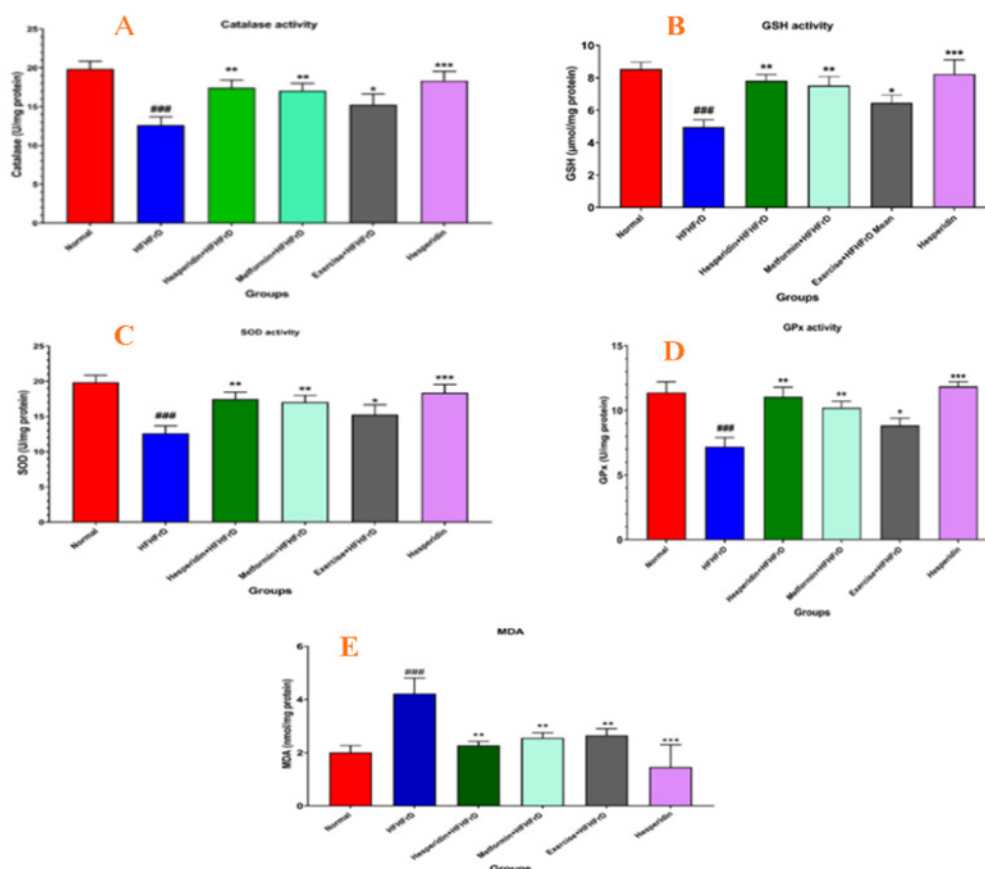


Figure 3: Anti-oxidant status of brain. A) CAT B) GSH C) SOD D) GPx E) MDA. Data expressed here are in Mean±SD (n=8), *** p<0.001 vs HFHFrD, ** p<0.01 vs HFHFrD, * p<0.05 vs HFHFrD, ### p<0.001 vs normal.

Table 6: Elevated plus maze- time spent in closed arm (s) Data showed are in Mean±SD (n=8), * p<0.05 vs HFHFrD, ** p<0.01 vs HFHFrD, *** p<0.001 vs HFHFrD. ## p<0.01 vs normal, ### p<0.001 vs normal.

Group	Week 8	Week 10	Week 12	Week 14
Normal	78.4±3.9	77.1±3.6	75.3±3.7	76.1±3.8
HFHFrD	102.8±4.1 ##	115.5±4.3 ###	127.5±4.2 ###	134.5±4.0 ###
Hesperidin+HFHFrD	92.0±3.8 *	87.5±3.9 **	81.0±4.0 ***	77.0±4.0 ***
Metformin+HFHFrD	93.0±3.9 *	89.0±4.0 *	82.5±4.1 **	77.5±4.2 ***
Exercise+HFHFrD	98.5±4.2	102.0±4.1	95.0±4.4 *	88.0±4.1 **
Hesperidin	81.5±4.0 **	78.5±4.2 ***	76.7±4.1 ***	74.3±4.0 ***

high fat and high-sugar diets.³⁵ Batandier and others documented elevated oxidative stress in the frontal cortex and reduced Nrf2 expression in HFHFr fed rats.³⁶ In our study, treatment with hesperidin and metformin effectively reversed these redox disturbances, as evidenced by elevated levels of GPx, CAT, SOD and GSH levels with decreased levels of MDA and there by restoration of this antioxidant enzyme activity to normal levels, confirms its powerful antioxidant role.

The antioxidant effects of hesperidin are well documented elsewhere by Khan and coworkers and demonstrated its ability to lower ROS and lipid peroxidation simultaneously enhancing the antioxidant defence system in the brain.^{37,38} Notably, in our findings it is observed that hesperidin and metformin significantly decreased brain ROS levels as compared to HFHFrD

feed groups near to normal levels. More interestingly our data showed hesperidin and metformin treatment abolished proinflammatory cytokines in rat's brain. Alongside oxidative stress, neuroinflammation was a prominent feature in our HFHFrD induced insulin resistance mediated anxiety model marked by heightened production of proinflammatory cytokines like IL-6 and TNF-α. These inflammatory mediators are known to activate glial cells, particularly microglia and astrocytes, resulting in neuroinflammatory cascades and gliosis.³⁹ Our results resonate with previous findings where HFHFrD-induced elevations in Free Fatty Acids (FFAs) activate microglia, disrupt hypothalamic signaling and trigger anxiety-like behavior.⁴⁰ Consistent with these studies, we observed elevated FFAs and increased neuroinflammatory markers in HFHFrD-fed rats

brain, that were significantly reduced following hesperidin and metformin treatment. The behavioural effects of HFHFrD were also consistent with previous research as follows. Rats exhibited classic anxiety-like actions, such as spending more time in the elevated plus maze's closed arms, reduced centre time in OFT and higher selection of the dark section in the dark/light box. Such behavioural changes are commonly reported following high-fat diet consumption and are believed to disrupt neurotransmitter function, impaired synaptic plasticity, and neuroinflammation.⁴¹⁻⁴³ Our findings not only confirm these anxiogenic effects but also demonstrate that hesperidin and metformin significantly reversed these behaviour parameters, restoring exploratory activity and reducing anxiety-like responses. Emerging evidence suggests that hesperidin may modulate other neurotransmitter systems,

including serotonin, dopamine, and GABA and also influence the axis of the Hypothalamus, Pituitary and Adrenal (HPA) axis.^{44,45} These pathways are critical in anxiety regulation, and although our study did not directly assess neurotransmitter levels, the reversal of anxiety-like behaviour implies possible involvement of these systems, warranting further investigation. According to earlier research, diazepam lowers anxiety via increasing the brain's GABA-A receptor activation, where diazepam showed definite anti-anxiety benefits via this GABA route when evaluated in diabetic stressed rats utilizing the elevated plus maze.⁴⁶ Our findings also reflect the many-sided therapeutic potential of hesperidin along with reflecting its potential in delaying the progression of insulin resistance mediated anxiety symptoms. Hesperidin crosses the blood-brain barrier and

Table 7: Light and Dark test- time spent in light zone (s). Data showed are in Mean±SD (n=8), * $p < 0.05$ vs HFHFrD, ** $p < 0.01$ vs HFHFrD, * $p < 0.001$ vs HFHFrD, ### $p < 0.001$ vs normal, ## $p < 0.01$ vs normal.**

Group	Week 8	Week 10	Week 12	Week 14
Normal	132.6±7.2	131.8±6.8	130.9±7.0	130.5±6.6
HFHFrD	91.0±5.9 ##	78.0±6.1 ###	65.0±5.3 ###	57.0±4.9 ###
Hesperidin+HFHFrD	102.0±6.0 *	108.0±5.8 **	118.0±5.6 ***	126.0±5.2 ***
Metformin+HFHFrD	104.0±5.7 *	110.0±6.0 *	119.0±5.5 **	127.0±5.3 ***
Exercise+HFHFrD	96.0±6.1 *	101.0±6.4*	110.0±6.0 *	115.0±5.8 **
Hesperidin	125.5±7.0 ***	128.0±6.9 ***	130.0±6.8 ***	132.0±6.6 ***

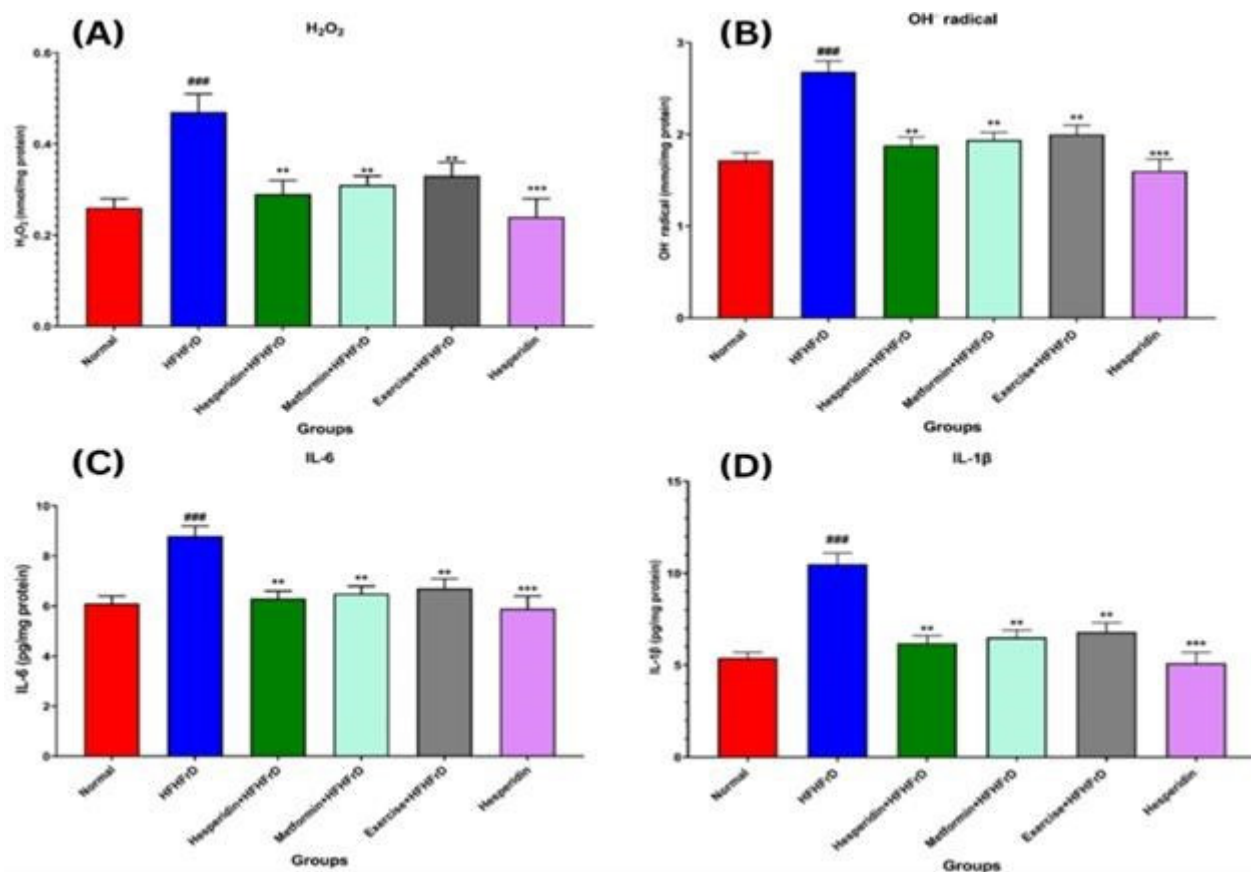
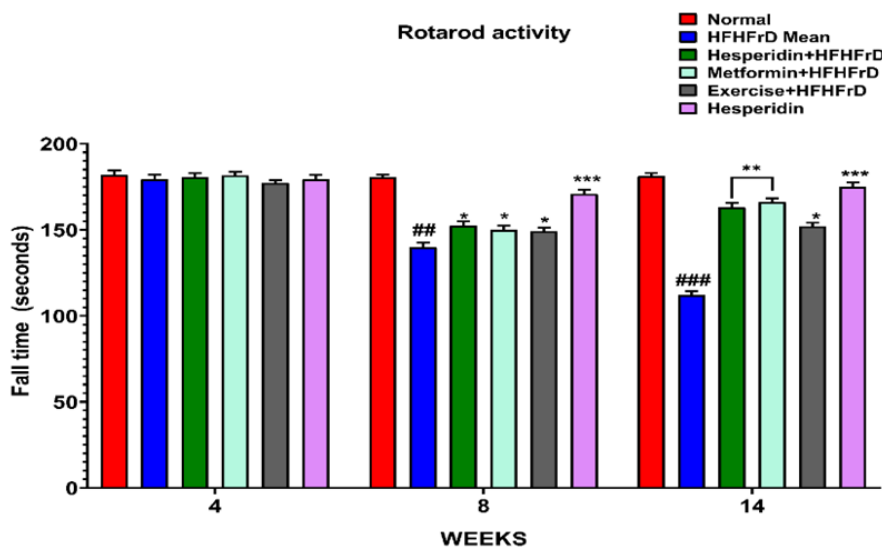


Figure 4: Brain ROS and inflammatory mediators. A) Hydrogen peroxide (H₂O₂), B) Hydroxyl radical (OH⁻), C) Interleukin-6 (IL-6), D) Interleukin-1 beta (IL-1β). Data showed are in Mean±SD (n=8), ** $p < 0.01$, * $p < 0.001$ vs HFHFrD, ### $p < 0.001$ vs normal, ## $p < 0.01$ vs normal.**

Table 8: Light and Dark test- time spent in dark zone (s). Data showed are in Mean±SD (n=8), * $p < 0.05$ vs HFHFrD, ** $p < 0.01$ vs HFHFrD, *** $p < 0.001$ vs HFHFrD), ### $p < 0.001$ vs normal, ## $p < 0.01$ vs normal.

Group	Week 8	Week 10	Week 12	Week 14
Normal	67.3±4.8	68.1±4.6	69.0±4.4	69.4±4.7
HFHFrD	108.5±5.7 ##	121.0±5.6 ###	134.5±5.9 ###	142.0±6.2 ###
Hesperidin+HFHFrD	96.0±5.2 *	85.0±5.7 **	75.0±5.4 ***	74.0±5.1 ***
Metformin+HFHFrD	98.0±5.0 *	90.0±5.6 *	88.0±5.5 **	76.0±5.2 ***
Exercise+HFHFrD	106.0±5.3	118.0±5.9	99.0±6.2 *	85.0±5.9 **
Hesperidin	73.5±4.4 ***	70.9±4.2 ***	68.6±4.0 ***	67.9±3.8 ***

**Figure 5:** Rotarod activity- Rotarod fall time (seconds). Data showed are in Mean±SD (n=8), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs HFHFrD. ### $p < 0.001$ vs normal, ## $p < 0.01$ vs normal.

acts on several signaling pathways, including MAPK and PI3K-Akt, downregulates cytokines that promote inflammation (IL-1 β , IL-6, and TNF- α) and also reduces iNOS expression in microglia.⁴⁷ Continued research is in progress to elucidate the underlying mechanism of the hesperidin's anxiolytic effect in insulin resistance mediated anxiety like behaviour.

CONCLUSION

The present study demonstrates a close association between insulin resistance, neuroinflammation and anxiety-like behaviour, with species of reactive oxygen playing important part in the detrimental effects of HFHFrD. Rats fed HFHFrD for 6 weeks developed insulin resistance and glucose intolerance, accompanied by anxiety-like symptoms. These metabolic changes were linked to decreased antioxidant enzyme activity and increased lipid peroxidation (GPx, SOD, CAT), depletion of glutathione, excessive ROS generation (H₂O₂ and OH \cdot), increased cytokines that promote inflammation (IL-1 β and IL-6). Hesperidin treatment from day one effectively attenuated these changes and showed delayed onset of anxiety-like behaviour's, with significant protection evidence from week 8 onwards. Hesperidin prevented progression of insulin resistance, preserved

antioxidant defences, reduced neuroinflammation, and improved behavioural outcomes, highlighting its preventive and anxiolytic role. Overall, these findings confirm that insulin resistance and anxiety are interconnected through oxidative and inflammatory pathways and suggest hesperidin as a promising preventive strategy to delay or counteract metabolic and neuropsychiatric complications induced by HFHFrD in rats.

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ABBREVIATIONS

HFHFrD: High-Fat High-Fructose Diet; **IR:** Insulin Resistance; **HOMA-IR:** Homeostasis Model Assessment-Insulin Resistance; **OGTT:** Oral Glucose Tolerance Test; **EPM:** Elevated Plus Maze; **OFT:** Open Field Test; **ROS:** Reactive Oxygen Species; **SOD:** Superoxide Dismutase; **CAT:** Catalase; **GPx:** Glutathione Peroxidase; **GSH:** Reduced Glutathione; **MDA:** Malondialdehyde; **IL-6:** Interleukin-6; **IL-1 β :** Interleukin-1 beta; **TNF- α :** Tumor Necrosis Factor-Alpha; **CNS:** Central Nervous System; **p.o.:** Per os (Oral Administration).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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SUMMARY

This study investigated the preventive potential of hesperidin, a citrus bioflavonoid, against high-fat high-fructose diet induced insulin resistance and associated anxiety-like behaviors in male Wistar rats. High-fat high-fructose diet feeding induced significant insulin resistance confirmed by elevated insulin resistance markers, along with anxiety-like behaviors assessed through behavioral testing methods. Hesperidin treatment from day one effectively prevented metabolic disturbances, restored antioxidant enzyme activities, reduced neuroinflammation markers, and significantly delayed the onset of anxiety-like symptoms with protective effects becoming evident during the study period. The study demonstrates hesperidin's multi-target preventive approach against metabolic syndrome-related neuropsychiatric complications through antioxidant protection, anti-inflammatory action and neuroprotective mechanisms.

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