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Hesperidin induced attenuation of oxidative damage and restoration of antioxidant potential in induced diabetic neuropathy model of mice

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Abstract

Oxidative stress is a major contributor to diabetes-associated neural injury, in which excessive reactive oxygen species (ROS) promote lipid peroxidation, protein oxidation and depletion of cellular reducing capacity. Hesperidin, a citrus-derived flavanone glycoside, has attracted considerable interest because of its antioxidant and neuroprotective properties. The present study investigated the effects of hesperidin on lipid peroxidation, oxidative protein modification, and ferric-reducing antioxidant capacity in the brain of alloxan-induced diabetic mice. Diabetes was induced by a single administration of alloxan (170 mg/kg b.w.). Alloxan-induced diabetes produced a pronounced oxidative imbalance in brain tissue. Compared with Group I, thiobarbituric acid reactive substances (TBARS) and protein carbonyl content (PCC) levels in Group II increased by 170.59% and 66.19%, respectively, indicating increased lipid peroxidation and oxidative protein modification. Hesperidin treatment in Group VI attenuated these alterations, with TBARS and PCC levels decreasing by 17.66% and 13.83%, respectively, relative to Group II. In addition, ferric reducing antioxidant power (FRAP) was 87.35% higher in Group VI than in Group II, indicating an enhancement of the ferric-reducing capacity of brain tissue following hesperidin treatment. Overall, hesperidin attenuated lipid and protein oxidative damage and enhanced reducing antioxidant capacity under diabetic conditions. These findings provide biochemical evidence supporting the potential protective role of hesperidin against diabetes-associated cerebral oxidative injury in experimental diabetic neuropathy (DN).

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by sustained hyperglycaemia resulting from inadequate insulin secretion, impaired insulin sensitivity, or dysfunction in both insulin secretion and action (Tripathy and Chavez, 2010; American Diabetes Association, 2013; Campbell and Newgard, 2021). Diabetes is a rapidly escalating global health burden, with its prevalence exceeding 500 million in 2024 and expected to approach 900 million by 2050 (Genitsaridi et al., 2026). DM is associated with a wide range of long-term metabolic and vascular (neuropathy, nephropathy, retinopathy, coronary artery disease, stroke, and peripheral arterial disease) complications (Cole and Florez, 2020). Among these, diabetic neuropathy (DN) is one of the most prevalent and debilitating manifestations, representing the leading cause of neuropathy worldwide (Dewanjee et al., 2018; Johannsen et al., 2001). Epidemiological

evidence indicates that approximately 30% of individuals with diabetes develop neuropathy, with lifetime prevalence rising to nearly 50% in individuals over the age of 50 (Starling, 2018). Clinically, DN is characterized by progressive nerve dysfunction, leading to sensory deficits, neuropathic pain, and functional impairment, thereby contributing substantially to morbidity and diminished quality of life (Feldman et al., 2019). Furthermore, it has been observed that the duration of diabetes and elevated glycated haemoglobin (HbA1c) levels are associated with increased neuropathy risk (Tesfaye et al., 2005).

The underlying pathogenesis of DN is complex and involves multiple interrelated mechanisms; however, various studies highlight that oxidative stress plays a crucial role in the onset and progression of the condition (Khan et al., 2025; Rusli et al., 2024). Oxidative stress results from an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defence system. Under diabetic conditions, chronic hyperglycaemia induces oxidative stress via the generation of ROS through activation of several interrelated biochemical mechanisms, including glucose auto-oxidation, nerve hypoxia/ischemia, excessive formation of advanced glycation end products (AGEs), activation of the polyol pathway mediated by aldose reductase, poly (ADP ribose) polymerase (PARP), hexosamine pathway, activation of protein kinase C pathway, elevation of pro-inflammatory cytokines namely interleukin-1β (IL-1β), interleukin (IL-6), tumour necrosis factor-α (TNF-α), and up-regulation of neurotrophin 3 resulting in cellular damage, neuronal dysfunction and degeneration, ultimately leading to development of DN (Dewanjee et al., 2018; Han et al., 2023).

Oxidative stress induces lipid peroxidation, leading to thiobarbituric acid reactive substances (TBARS) formation, and causes protein oxidation, resulting in the accumulation of protein carbonyls, which are stable markers of cellular injury (Kasznicki et al., 2012). To counteract oxidative damage, cells possess a highly coordinated antioxidant defence system comprising both enzymatic antioxidants, including glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT), and non-enzymatic antioxidants, such as glutathione (GSH).

However, in diabetic conditions, impairment of this endogenous defence mechanism leads to excessive accumulation of free radicals, which in

turn accelerates lipid peroxidation in neuronal cells (Bandeira et al., 2012) which are particularly vulnerable to such damage due to their high content of polyunsaturated fatty acids (PUFAs) (Yang et al., 2020). This process results in the generation of toxic by-products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which contribute to neuronal degeneration. Collectively, these oxidative and lipid peroxidative changes are closely associated with β-cell dysfunction, impaired glucose tolerance, insulin resistance, and the progression of neuropathic complications associated with diabetes (Villalón-García et al., 2023). Additionally, the total antioxidant capacity of a biological sample can be evaluated using the ferric reducing antioxidant power (FRAP) assay, which measures the overall reducing potential of plasma or tissue samples (Benzie and Strain, 1996). A decrease in FRAP levels in diabetic conditions indicates reduced antioxidant capacity and an increased sensitivity to oxidative stress-mediated cellular damage (Jiménez Morales and Cañizares-Macías, 2026).

In the present study, alloxan was administered to induce experimental diabetes in mice. Alloxan, a well-known diabetogenic compound, is the most commonly used agent for inducing diabetes in experimental animal models. Also, the similar structure of alloxan to that of glucose allows its selective uptake and accumulation in the pancreatic beta cells via the GLUT 2 glucose transporter and causes selective necrosis of pancreatic islets (Elsner et al., 2002; Etuk Etuk, 2010; Gorus et al., 1982; Lenzen, 2008; Shaw Dunn et al., 1943).

Given the central role of oxidative stress in the etiology of DN, therapeutic options that restore redox homeostasis and strengthen antioxidant defences via natural bioactive compounds, particularly flavonoids, have emerged as promising candidates for managing oxidative stress-related complications of diabetes. Several bioactive compounds, including resveratrol, curcumin, quercetin, berberine, sulforaphane and epigallocatechin-3-O-gallate, have demonstrated neuroprotective effects in experimental models of DN by attenuating oxidative stress, inflammation, mitochondrial dysfunction and neuropathic alterations (Kumar *et al*., 2007; Joshi *et al*., 2013; Zan *et* al., 2017; Moustafa *et* al., 2018; Abo-Salem *et* al., 2020; Zhang *et al*., 2021). These findings have encouraged increasing interest in naturally derived compounds as potential complementary approaches to target the

multifactorial mechanisms underlying DN. Hesperidin is one such bioactive flavonoid that has attracted considerable interest because of its broad pharmacological properties. Hesperidin is abundantly found in lemons, grapefruits, tangerines, and sweet oranges, and exhibits antioxidant, anti-inflammatory, neuroprotective, antidiabetic, antiulcer, sedative, antihyperglycemic, antidyslipidemic, analgesic, anticarcinogenic, anti-depressant, anti-fertility, antifungal, antibacterial, antiviral, antihypertensive, anti-allergic effect, immuno-modulatory, and diuretic effect, among several other reported effects (Akiyama et al., 2010; Antunes et al., 2014; Tamilselvam et al., 2013). Hesperidin has also demonstrated protective effects against pathological conditions, such as diabetic retinopathy, rheumatoid arthritis, and myocardial ischemia (Adefegha et al., 2021; Li et al., 2016; Shi et al., 2012). It attenuated cisplatin-induced neurotoxicity in the rat brain (Kamisli et al., 2015), 6-hydroxydopamine (6-OHDA)-induced neurotoxic effects in aged mice (Antunes et al., 2014), and increased xanthine oxidase levels in the brains of diabetic animal models (Ashafaq et al., 2014). Furthermore, in the past decade, several clinical trials have been conducted to evaluate the therapeutic potential of hesperidin. These findings highlight its growing clinical relevance and support its investigation in the present study as a promising candidate for mitigating oxidative stress-mediated neuronal damage.

The present study was done to evaluate the protective effect of hesperidin against oxidative stress in alloxan-induced DN in a mice model. Lipid peroxidation and protein oxidation were evaluated to measure oxidative damage. Additionally, the overall antioxidant capacity was also measured.

2. Materials and Methods

2.1 Chemicals

Alloxan (2244-11-3), hesperidin (520-26-3), and glutathione reductase (9001-48-3) were procured from the Sigma-Aldrich Chemicals Company, USA. 2-thiobarbituric acid (TBA) (504-17-6), ethylenediaminetetraacetic acid (EDTA) (6381-92-6), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) (69-78-3), 2,4-dinitrophenylhydrazine (DNPH) (119-26-6), NADPH (reduced nicotinamide adenine dinucleotide phosphate) (53-57-6), 2,4,6-Tri(2-pyridyl)-*s*-triazine (TPTZ) (3682-35-7), methionine (63-68-3) and riboflavin (83-88-5) were procured from Himedia, India. All other reagents and chemicals used were of analytical grade.

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2.2 Experimental Animals

The experiments were conducted on male Swiss albino mice procured from Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar, Haryana, India (Reg. No. 1669/Go/Re/Bi-Bt-S/Re-L/12/CPCSEA). Healthy, 6-8 weeks old Albino mice (weighing 22 ± 2 g) were acclimatized and maintained in the Animal House, Department of Zoology, University of Rajasthan, Jaipur (Reg. No. 1678/GO/Re/S/2012/CPCSEA). The mice were housed in polypropylene cages containing sterile husk as bedding and were fed on a standard pelleted diet and water *ad libitum* throughout the experiment. They were weighed daily as body mass is an indicator of metabolic change. The maintenance of mice was as per the guidelines of the Committee for Control and Supervision of Experiments on Animals (CCSEA), Government of India, and was approved by the Institutional Animal Ethics Committee (IAEC), Department of Zoology, University of Rajasthan, Jaipur under the approved protocol No. UDZ/IAEC/2023-I/20 dated 11.08.2023.

2.3 Experimental design

For the study, mice were divided into six groups (six animals in each group). Group I was a control group that received normal saline (0.85% NaCl). Mice in group II were administered a single dose of alloxan (170 mg/kg body weight) to induce diabetes based on a previous study (Kashyap and Gupta, 2019). Group III was provided with metformin (180 mg/kg body weight). Group IV and VI were given alloxan (170 mg/kg b.w.) on the first day. After confirmation of diabetes within 24 hours, group IV was administered metformin (180 mg/kg b.w.), while group VI was administered hesperidin (8 mg/kg b.w.). Mice in group V were given hesperidin only. The duration of all treatments except alloxan was 40 days. Exposure to alloxan, metformin, and hesperidin was administered intraperitoneally (i.p.) (Table 1).

Table 1: Experimental design

Groups	Treatment	Dose
Group I	Control	Normal saline (0.85%)
Group II	Alloxan treated	170 mg/kg b.w.
Group III	Metformin treated	180 mg/kg b.w.
Group IV	Alloxan + Metformin	170 mg/kg b.w. + 180 mg/kg b.w.

Group V	Hesperidin	8 mg/kg
Group VI	Alloxan + Hesperidin	170 mg/kg b.w. + 8 mg/kg b.w.

2.4 Induction of Diabetes and its Validation

Diabetes was induced in mice through a single intraperitoneal injection of alloxan monohydrate solution (170 mg/kg body weight) following the previously reported protocol of Kashyap and Gupta (2019). Before its administration, mice were fasted overnight but given water *ad libitum*. The induction of diabetes was validated 24 hours after alloxan administration by measuring blood glucose levels. A small blood sample was collected from the tail tip using the tail-snip method, and glucose concentration was determined using a glucometer (Dr. Morphen, India). Mice with glucose levels exceeding 200 mg/dL were considered diabetic and included in the diabetic groups. Following confirmation of diabetes, the respective treatments were administered for a 40-day treatment period.

2.5 Biochemical Studies

At the end of the treatment period, the mice were sacrificed by cervical dislocation, and the brains were immediately removed. Brain homogenates (10%) were prepared in 0.1 M phosphate buffer and further used to assess the oxidative responses of the brains of differently treated mice.

2.5.1 TBARS

TBARS levels were measured as an indicator of oxidative stress by Ohkawa *et al.* (1979) method. LPO is a physiological indicator of oxidative stress. MDA is formed as a result of peroxidation of lipids, which reacts with 2-thiobarbituric acid (TBA) and forms an adduct that gives a pink-coloured complex and is observed spectrophotometrically at 532nm.

2.5.2 PCC

Protein carbonyl content (PCC) was determined as an indicator of oxidative modification of proteins by measuring protein carbonyl groups following the method of Levine *et al.* (1990). The carbonyl groups react with 2,4-dinitrophenylhydrazine (DNPH) to form corresponding dinitrophenylhydrazones. The resulting absorbance was measured spectrophotometrically at 370 nm.

2.5.3 FRAP

FRAP measurements were made in accordance with the method outlined by Benzie and Strain (1996). The FRAP assay is used to determine the antioxidant activity of a substance in a reaction medium. It is based on the reduction of the ferric (Fe^{3+})–TPTZ complex to its ferrous (Fe^{2+}) form at low pH, producing a blue-coloured

complex with an absorption maximum at 593 nm. The increase in absorbance is directly proportional to the reducing power of the sample.

3. Statistical evaluation

Results are expressed as mean ± SEM. Significance was determined using one-way ANOVA followed by Tukey's post hoc test ($p<0.05$ considered significant).

4. Results

4.1 Thiobarbituric Acid Reactive Substances (TBARS)

TBARS levels were significantly elevated in the alloxan-treated group (Group II) compared with the control group ($p<0.0001$). This increase in TBARS levels indicates enhanced lipid peroxidation following alloxan administration. In metformin-treated mice (Group III), TBARS levels were significantly reduced compared with Group II ($p<0.0001$), although the levels remained higher than those of Group I ($p<0.01$). Similarly, diabetic mice treated with metformin (Group IV) showed a significant reduction in TBARS levels relative to Group II ($p<0.01$); however, the levels remained significantly higher than those in Group I ($p<0.0001$) and Group III ($p<0.05$). Hesperidin-treated non-diabetic mice (Group V) also showed significantly lower TBARS levels compared with Group II ($p<0.0001$). In the group VI in which diabetic mice received hesperidin (Group VI), TBARS levels were significantly reduced relative to group II ($p<0.0001$) (Fig. 1).

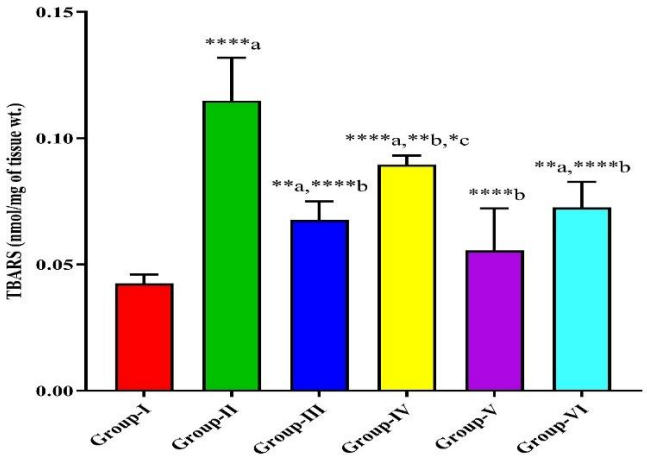


Fig 1. TBARS level in brains of differently treated mice. Results are expressed in nanomoles of MDA formed per milligram of tissue weight. *$p<0.05$, **$p<0.01$, ****$p<0.0001$; a = compared to control; b = compared to group II; c = compared to group III

4.2 Protein Carbonyl Content (PCC)

PCC levels were markedly increased in the alloxan-treated group (Group II) compared with the control group (Group I; $p<0.0001$), indicating enhanced protein oxidation following alloxan administration. Metformin-treated mice (Group III) showed significantly lower PCC levels than Group II ($p<0.0001$), although the levels remained higher than those of Group I ($p<0.05$). In diabetic mice treated with metformin (Group IV), PCC levels were significantly reduced compared with Group II ($p<0.01$), but remained significantly higher than those in Group I ($p<0.0001$) and Group III ($p<0.05$).

Hesperidin-treated non-diabetic mice (Group V) exhibited significantly lower PCC levels than Group II ($p<0.0001$). In diabetic mice receiving hesperidin (Group VI), PCC levels were also significantly reduced compared with Group II ($p<0.01$), although they remained significantly higher than those of Group I ($p<0.0001$) and Group III ($p<0.05$).

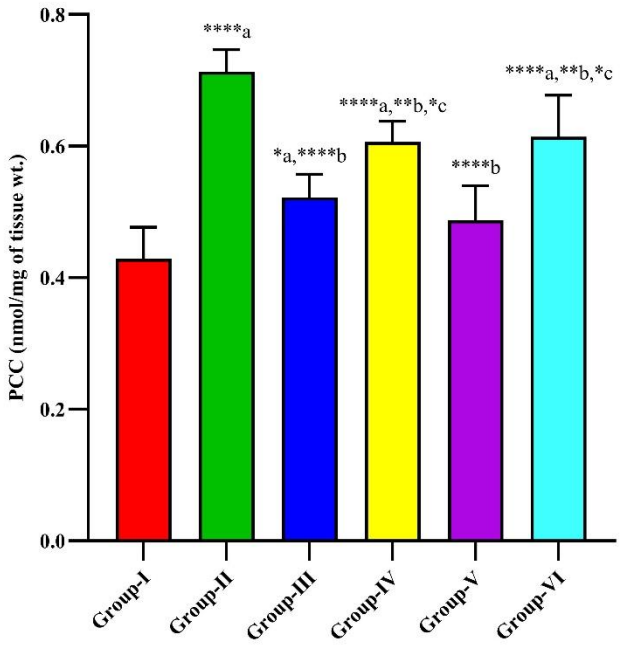


Fig 2. PCC level in brains of differently treated mice. Results are expressed in nanomoles of carbonyl content formed per milligram of tissue weight. *$p<0.05$, **$p<0.01$, ****$p<0.0001$; a = compared to control; b = compared to group II; c = compared to group III.

4.3 Ferric Reducing Antioxidant Power (FRAP)

FRAP levels were significantly increased in the metformin-treated group (Group III) compared with the control

group (Group I; $p<0.05$). A further increase was observed in the alloxan + metformin group (Group IV), which showed significantly higher FRAP levels than Group I ($p<0.01$). Hesperidin treatment alone (Group V) produced an increase in FRAP levels compared with the control, although the difference was not statistically significant ($p>0.05$). In diabetic mice treated with hesperidin (Group VI), FRAP levels were significantly higher than those of the control ($p<0.05$). No statistically significant difference was observed between the alloxan-treated group II and the control group ($p>0.05$).

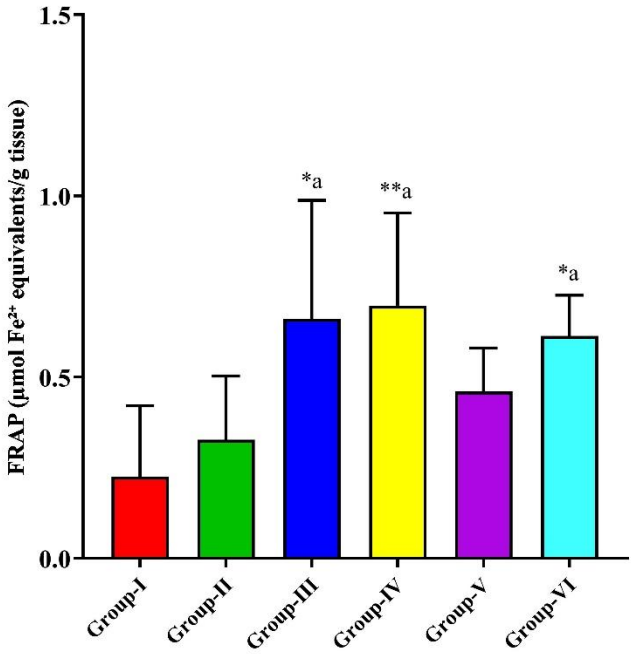


Fig 3. FRAP levels in brains of differently treated mice. Results are expressed as µmol Fe^{2+} equivalents per gram of brain tissue. *$p<0.05$, **$p<0.01$; a = compared to control

Discussion

The present study demonstrates that alloxan-induced diabetes was associated with a pronounced disturbance in cerebral oxidative balance. The alloxan-treated group (Group II) showed a substantial increase in TBARS and PCC along with a reduction in FRAP compared with the control group (Group I). These changes indicate that diabetes was associated with increased oxidative damage to membrane lipids and proteins, accompanied by a weakened reducing environment in the brain. Oxidative stress is considered an important contributor to the development of DN complications, in which persistent metabolic stress promotes excessive ROS production and disrupts the balance

between oxidative damage and antioxidant protection (Wright *et al*., 2006; Lin *et al*., 2023; Chen *et al*., 2025).

In the present study, the increase in TBARS observed in G-II, following alloxan-induced diabetes, provides evidence of enhanced lipid peroxidation in the brain. This elevation suggests enhanced oxidative damage under diabetic conditions, which is consistent with previous findings by Kumari *et al.* (2025), who reported that increased TBARS levels were associated with oxidative stress and neuronal degeneration. Studies suggest that chronic diabetes or hyperglycaemia can promote ROS generation through mitochondrial dysfunction, activation of NADPH oxidases, glucose auto-oxidation and several glucose-dependent metabolic pathways (Rekatsina *et al*., 2020). The resulting ROS can generate secondary reactive aldehydes, which can further modify proteins and disrupt membrane integrity, and mitochondrial function. Experimental DN has similarly been associated with increased lipid-peroxidation products and reduced antioxidant protection (Wang *et al*., 2022). Hesperidin treatment in Group VI measurably improved the oxidative profile of diabetic animals. Hesperidin markedly reduced TBARS levels compared with G-II, indicating that hesperidin attenuated diabetes-associated lipid oxidative damage. This reduction in TBARS is consistent with previous experimental evidence showing that hesperidin attenuated brain lipid peroxidation and restored antioxidant parameters in rats exposed to acrylonitrile-induced oxidative injury (El-Sayed *et al*., 2008). The effect observed in G-VI may therefore reflect the ability of hesperidin to reduce the oxidative burden generated during diabetes.

PCC levels increased in Group II compared with Group I, indicating enhanced oxidative modification of proteins following alloxan administration. Protein oxidation can alter protein structure, enzyme activity, cellular transport, and energy metabolism, thereby amplifying functional consequences of oxidative stress (Vincent *et al*., 2004). Thus, lowering protein carbonylation may help preserve neuronal biochemical functions under diabetic conditions. Hesperidin treatment also attenuated this protein oxidative damage in diabetic brain tissue. PCC levels decreased in hesperidin-treated Group VI relative to Group II. This parallel reduction in protein and lipid oxidation demonstrates that the protective effect of hesperidin extended beyond membrane lipids to oxidative protein damage. Particularly relevant to the present findings, hesperidin has been shown to suppress both

lipid peroxidation and protein carbonyl formation under high-glucose conditions while simultaneously improving endogenous antioxidant defences (Liu *et al*., 2017).

In contrast to the increases in oxidative damage markers, FRAP was markedly reduced in G-II, indicating a reduction in ferric-reducing capacity. This reduction in FRAP in diabetic mice indicates an overall decline in the capacity of brain tissue to provide reducing equivalents against oxidative challenge; therefore, it complements measurements of oxidative damage. Hesperidin treatment in Group VI showed improvement in FRAP relative to the diabetic group, indicating that hesperidin restored the reducing environment of diabetic brain tissue. The restoration of FRAP following hesperidin treatment suggests an improved overall antioxidant system. Similar enhancements in FRAP and antioxidant enzymes have been reported following flavonoid treatment in experimental diabetes-associated oxidative damage (Ashafaq *et al*., 2014). The concurrent reduction in TBARS and PCC, together with increased FRAP levels, indicates that hesperidin treatment was associated with reduced lipid and protein oxidation and improved antioxidant reducing capacity.

The improvement observed following hesperidin treatment is consistent with previous evidence indicating its involvement in the modulation of oxidative and inflammatory pathways in DN. Its antioxidant effects may be mediated, at least in part, through modulation of endogenous redox-regulatory mechanisms. Metformin treatment also improved the oxidative profile compared with the diabetic group, as reflected by favourable changes in the oxidative stress parameters.

Taken together, the present findings demonstrate that alloxan-induced diabetes induced a marked cerebral oxidative imbalance, characterized by increased lipid peroxidation and protein oxidation together with reduced ferric-reducing antioxidant capacity. Hesperidin treatment attenuated these alterations, as evidenced by the significant reductions in TBARS and PCC and the significant increase in FRAP in Group VI compared with the diabetic group. These findings support the potential antioxidant role of hesperidin in protecting against oxidative alterations associated with DN.

CONCLUSION

Overall, the data suggest that hesperidin has a protective effect against diabetes-associated cerebral oxidative damage. Importantly, the simultaneous

improvement of TBARS, PCC and FRAP indicates that its effect was not confined to a single oxidative endpoint but involved several interconnected aspects of cerebral redox homeostasis. These findings provide a biochemical basis for further investigation of hesperidin as a potential adjunctive strategy for limiting oxidative damage associated with DN.

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Conflict of Interest

Authors have no conflict of interest.

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