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Synergistic Effect of Flavonoid-rich Ethanolic Extract of *Coccinia indica* and Hesperidin in Diabetes Induced Neuropathic Rat Model

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Degeneration of neuronal function and diminished sensation characterize diabetic neuropathy, the foremost prevalent complication connected with diabetes mellitus. This research focuses on investigating the combined impact of flavonoid rich ethanolic extract of *Coccinia indica* extract (CIE) and hesperidin in diabetes induced neuropathy. Male sprague dawley rats were instigated with

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diabetic neuropathy (DN), following 14 days of high fat diet treatment along with streptozotocin (35 mg/kg, *i.p*), then the animals were maintained for 4 weeks to allow the development of DN. In accordance to the previous research identifying increased lipid peroxidation and reduced enzymatic antioxidants (superoxide dismutase, catalase, and reduced glutathione) as hallmark indicators of diabetic neuropathy (DN), our study demonstrated a similar biochemical profile. Treatment with CIE (200 mg/kg) alone and in combination with hesperidin at doses (25, 50 mg/kg), orally were conducted for eight weeks, which produced substantial reduction in blood sugar and restored antioxidant levels. Models for neurological pain (Eddy's hot plate and Tail immersion) were utilized to assess the levels of antinociception. Post third week, the combined therapy of CIE and hesperidin exhibited significant (p<0.001) antinociception due to their ability to improve the insulin sensitivity (Increase in glucose tolerance) when compared with metformin. As CIE, hesperidin, and metformin are all known to activate AMP-activated protein kinase (AMPK), which subsequently leads to the inhibition of nuclear factor-kappa B (NF-κB) and other pertinent signaling pathways. This cascade ultimately results in the suppression of gluconeogenesis, inflammatory cytokines while enhancing insulin sensitivity and elevating antioxidant levels. This signifies that combination therapy with CIE+hesperidin is equivalent to metformin in delaying the progression of diabetes induced neuropathy. Histological examinations confirmed the absence of sciatic nerve damage. Combined therapy of CIE along with hesperidin yielded notable depletion in blood sugar concentration and prevented further development of diabetic neuropathy.

Keywords: Diabetic neuropathy; antioxidant; Coccinia indica leaf extract; nociception.

1. INTRODUCTION

Diabetes mellitus (DM) has existed from the prehistoric era, with Apollonius of Memphis presumably being the first to utilize the term *diabetes* (Isma'il and Isma'il, 2024). It is labeled as a metabolic dysfunction comprising of multiple illness. Chronic hyperglycemia involves disruption in the levels of lipids, saccharides and protein absorption which is possibly due to the insufficient insulin or its lack of action (Poznyak et al., 2020). Complications related to diabetes are inevitable at the later stages, and it is mostly classified into macrovascular and microvascular. The majority of health issues and fatality are linked with the latter one which encompasses retinopathy, nephropathy and neuropathy. Among the various co-morbidities diabetic neuropathy has emerged as one of the dominant complicacy affecting both type 1 and 2 diabetic patients (Tomic et al., 2020).

Diabetic neuropathy (DN) is a significant clinical condition marked by discomfort and considerable morbidity, mainly resulting from damage to the somatosensory nervous system. The typical clinical syndrome involves neuronal damage affecting the feet and soles, with symptoms worsening as they move from the extremities towards the centre of the body (Jaroslawska et al., 2020). The major markers for the prediction of diabetic neuropathy involves the prolongation of diabetes and Haemoglobin A1c (HbA1C) (Rahman et al.,2023). While HbA1c is linked with various metabolic conditions, genetics and inadequate blood sugar management, the precise mechanism by which diabetes mellitus affects the sensory nerves is yet to be ascertained. Nonetheless, a novel theory proposes that oxidative stress may be a major cause of diabetic neuropathy (Pang et al., 2020).

The disparity between the production of reactive oxygen species (ROS) and its antioxidant system arises due to oxidative injury. In diabetes, induction of oxidative stress is primarily due to the impairment in glucose metabolism, which redirects the excess glucose to other signal transduction pathways (Lin et al., 2020). This leads to the buildup of hazardous metabolites and overindulgence of nicotinic acid adenine dinucleotide phosphate (NADPH). NADPH contributes a significant impact in detoxification of ROS and depletion of it results in elevation of cellular oxidative stress, unusual alterations of protein and fats (Chandel, 2021). Excessive generation of ROS leads to damage of the mitochondrial energy units impairing their functions and further contributing to axonal damage. This, in turn, results in prolonged degenerative disorders such as diabetic neuropathy (Mallet et al., 2020). Mechanisms such as the polyol, advance glycated end products (AGE), hexosamine and the protein kinase C (PKC) pathway also contribute in the generation of free radicals causing oxidative stress (Paul et al.,2020).

For the past few years, numerous plant based substances and bioactive compounds with oxidant scavenging and inflammation lowering properties has been identified as a potent therapeutic option. Furthermore drug discovery efforts targeting these natural compounds have yielded great potency with minimal complications (Gupta et al., 2021). *Coccinia indica* a natural compound is often regarded as a natural alternative for insulin due to its effectiveness in managing hyperglycemia (Pioglitazone et al., 2020). The diverse phytoconstituents found in the leaves and stems of *coccinia indica* has gained recognition for their effectiveness in the treatment for various conditions including diabetes, inflammation, hyperlipidemia, nociceptive pain, microbial infections and pyrexia (Suresh et al., 2024, Hossain et al., 2021). Similarly, alongside *coccinia indica*, another natural compound hesperidin a significant polyphenolic compound is well-known for its diverse therapeutic effects against oxidation and inflammation (Li et al., 2023). It has reported to show valuable impact in the management of neurodegenerative complications. Moreover it also activates the cellular defense mechanism, which serves an essential role in protecting cells from oxidative damage, making it beneficial for diabetic neuropathy (Eid et al., 2023),(Kaur et al., 2024).

Hesperidin and *coccinia indica*, have each demonstrated significant potential in managing various complications individually; however, there is currently no evidence on the efficacy of combining these two natural compounds. Hence, the objective of this research is to determine if CIE solely or with an amalgamation of hesperidin can mitigate the increasing neuronal damage induced by type 2 diabetes in rodents.

2. MATERIALS AND METHODS

2.1 Materials

Streptozotocin (STZ) has been procured from Sigma-Aldrich chemical company, St. Louis, MO, USA. The *coccinia indica* leaves (ethanolic) yield utilized in the research was generously provided as a gift sample by Green Chem Laboratories, located in Bangalore, India.

2.2 Preparation of Extract (Pioglitazone et al., 2020)

The *coccinia indica* leaves were desiccated and extracted using ethanol. The extraction process was done by warming the plant material for 5-6hrs in a compact system that recirculates the extract onto the initial plant bed. Under minimal tension and low thermal state the extract was fused and intensified, after repeating the procedure for two times. The concentrated extract were then transferred into the drying cell, where it was processed into a powdered state. The powder were progressively refined to minute consistency using grinder mill and thoroughly mixed to ensure a consistent even batch.

2.3 Research Animals

Male (sprague-dawley) rats carrying weight about 240 and 280g was housed at the animal facility, Krupanidhi College of Pharmacy for research objectives. These rats were maintained under a regulated environment with temperatures ranging within 27° ± 2°C, humidity between 55% and 60%, and 12-hour light-dark rhythm. The investigative protocol was approved by the designated Ethics Control (Reference: KCP/IAEC/PCOL/130/AUG2023). The rats were accomodated in adherence to directives founded by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Prior to the manipulation in diet, all rodents had unrestricted access to regular pellet food and water.

2.4 Induction of Type 2 Diabetes

2.4.1 Preparation of fructose diet (Said et al., 2023)

A fresh fructose diet was carefully prepared each day, which included high fructose, protein and fat (660:100:80g). Along with it zinc spar 0.04 grams, essential nutrients, and lastly 150g of commercial-grade cellulose.

2.4.2 Development of type 2 diabetes in rats (Srinivasan et al., 2005, Hassan et al., 2020)

Experimental rodents were given high-fat diet (HFD) once daily for two weeks. The animals were then fasted throughout the night and was followed by an intraperitoneal infusion of streptozotocin (35mg/kg), mixed with 1M// citrate based buffer. Experimental rodents exhibiting non fasted blood glucose concentrations, exceeding 250 mg/dl is classified as diabetic. In the end, samples were obtained through the tail vein, and sugar levels were determined with the help of a glucometer (Accucheck, India).

2.5 Experimental Groups

The rats were segregated across 7 groups consisting of 6 animals per group. Usual diet was fed to group 1. Group 2-7 were given fat rich diets for 14 days, with subsequent administration of streptozotocin (35mg/kg *i.p*). After which, Group 2 was served as diabetic control (receiving no treatment) and the remaining groups 3-7 were treated orally with their assigned medication/extract for 56 days following the onset of insulin resistance. Carboxy methyl cellulose (0.5% w/v) was utilized as suspension for all compounds. Administration of hesperidin, (high dose) *coccinia indica* solely and in conjunction with hesperidin (low dose), metformin was done accordingly to groups (Tomic et al., 2022), (Jaroslawska et al., 2021), (Rahman et al., 2023), (Pang te al., 2020), (Lin et al.,2023).

2.6 Test Studies

2.6.1 Prior and subsequent therapy glucose estimation

Following glucose intake orally, alteration in blood sugar concentration overtime is influenced through the proportion of glucose getting absorbed in gut and metabolism mediated by insulin. Diabetic rats were fasted throughout the night for the screening of glucose tolerance. At the beginning of the therapy a glucose tolerance test was conducted in groups 1,3,5 and 7 (negative control, *Coccinia indica* leaf extract, hesperidin and metformin treatment) so, as to ensure the non-hypoglycemic effect of these drugs. After the 8 week treatment period groups 1-7 (namely negative control, positive control, *Coccinia indica* extract, hesperidin (high dose), combination of *Coccinia indica* with hesperidin (low dose) and metformin) underwent through glucose tolerance test. Thirty minutes after the dose administration, glucose (2 g/kg) is given orally, following which blood samples was obtained through tail (vein) at an interval of 0,30,60 and 120 minutes post-glucose ingestion. The glucose concentrations were assessed using AccuCheck testing strips.

2.7 Models of Neuropathic Pain

The following diabetic neuropathic animal models were used in investigating the antinociceptive activity.

2.7.1 Eddy's Hot plate method (Surwase and Patil, 2022,Aburas et al., 2023)

The intensity in pain response observed over thermal plate was believed to arise due to the union of neuronal system. Experimental rodents was individually positioned over the top of thermal analgesiometer fixed at 50±1°C. Duration until initial response, either by paw withdrawal or an attempt of jump, were recorded as an indicator of nociceptive resistance. A maximum cutoff limit of fifteen seconds was imposed to prevent potential paw injury due to prolonged exposure to the discomfort stimulus. Documentation of reactions was done seven days post administration of streptozotocin.

2.7.2 Tail immersion method (Kumar et al., 2009)

Nociception was evaluated using the tail immersion test. After one week of STZ injection, the subject's tail was submerged inside warm water (45° ± 1°C). The duration of tail flick response was noted with a 15-second time limit.

2.8 Assessment of Antioxidants

2.8.1 Superoxide estimation (Gao et al., 2024, Owira et al., 2022)

The estimation of Superoxide Dismutase (SOD) relies on detecting oxygen (O_2) through the auto-oxidation of hydroxylamine hydrochloride, resulting in generation of nitrite, subsequently quantified colorimetrically at 560 nm. SOD value is determined with measurements specified for quantity which hinders partially with decrease in nitroblue tetrazolium (NBT). NBT undergoes reduction via hydroxylamine auto-oxidation, resulting in the formation of nitrite, along with the inclusion of EDTA, detectable through colorimetric means. In a test tube 100 µl tissue homogenate was added along with 0.25M phosphate buffer, combined with 1 ml sodium carbonate mixture, 0.4ml NBT, and 0.2ml EDTA for baseline measurement at 560nm. Subsequently, 0.4 ml 1mM hydroxylamine hydrochloride initiated the reaction. After 5 minute incubation at 25°C, NBT reduction was measured at 560 nm.

2.8.2 Estimation of catalase (Jing et al., 2020,Manesh et al., 2023)

Catalase serves by decomposing hydrogen peroxide into harmless components namely water and molecular oxygen, thereby acting as a protective agent against oxidative damage. Many pathogens utilize catalase to protect themselves from the host's immune system, which often employs hydrogen peroxide as a defense

mechanism alongside oxidative stress. 100 microlitres tissue extract prepared with 0.15M potassium chloride solution were combined with 0.25M buffer (neutral pH), optical density were recorded at 240nm. Subsequently, the reaction mixture were introduced to H_2O_2 and incubated for a minute. Absorbance was recorded again, with phosphate buffer used as the reference sample.

2.8.3 Estimation of reduced glutathione

Reduced glutathione (GSH) were quantified following Ellman's method (Jitca et al., 2021). An equal volume of homogenized sciatic nerve was combined with 10% (trichloroacetic acid) and centrifuged to precipitate the proteins. Subsequently, (0.01 ml) of the resulting extract was mixed with the buffer (phosphate), 5,5-dithiobis(2-nitrobenzoic acid) and distilled water. The solution was subsequently subjected to thorough centrifugation, and optical density measurements were taken at 412 nm over a 15-min period. The concentration of GSH were quantified and expressed as *µ*g/mg of protein.

2.8.4 Lipid estimation (Chen and Zhang, 2016, Aguilar et al., 2020)

Lipid peroxidation levels were evaluated through the interaction between malondialdehyde (MDA) and thiobarbituric acid (TBA), forming a complex. The standard protocol utilized for estimating Thiobarbituric acid reactive substances (TBARS) in blood serum. Upon reaction of TBA alongside MDA, a hue of crimson colour emerged, which were quantified at 532 nm. 500µl of the sample was mixed with hydrochloric acid (300µl), trichloroacetic acid , and TBA, then it underwent heating at 95°C lasting 15mins. Once cooled, the solutions were subjected for centrifugation. Thereafter resulting supernatants were gathered, followed by the measurement of absorbance at 532nm in comparison to a blank solution.

2.9 Histopathological Analysis (Kumar et al.,2009, Bayir et al., 2023)

Sciatic nerve samples underwent immersion at 10% formalin fixative solution and sliced into 4µm thick sections. Staining was performed using haematoxylin along with eosin. The portion of nerve tissue was examined using an optical microscope at 320x magnification to assess axonal degeneration and vascular abnormalities.

2.10 Statistical Analysis

Data are reported as mean ± standard error of the mean (SEM). The antinociceptive data were evaluated using two-way ANOVA, while the biochemical parameters were evaluated using one-way. Tukey's significance range test were employed in both analyses to make post-hoc comparisons (Chen et al., 2022).

A *p-value* of less than 0.05 was considered to indicate statistical significance.

3. RESULTS

3.1 Pre and Post Therapy Glucose Measurement

3.1.1 OGTT- prior to the initiation of therapy

Therapeutic effectiveness of hesperidin, *coccinia indica*, metformin is shown in Fig. 1. It was discovered that glucose levels peaked one hour post administration, before returning back to its normal fasting levels two hours later. Comparable outcomes were noted in *coccinia indica* leaf extract, hesperidin and metformin administered groups. The increase in blood sugar levels observed at half an hour plus one hour post glucose loading, followed by the return to baseline at two hours, was not considered significant when compared to negative control rodents and within treatment groups as well. This suggests that *coccinia indica* extract, hesperidin, and metformin do not induce hypoglycemia in normal rats.

3.1.2 OGTT- post therapy

Following 8 weeks' duration of intervention with several predetermined regimens on diabetic rodents a glucose challenge test was conducted. Sugar levels (mg/dl) at the fasting state for the negative and positive control were 74.83 ± 3.24, 295.73 ± 12.34, respectively. In diabetic rodents administration of *coccinia indica*, hesperidin (high dosage), *coccinia indica* plus hesperidin (low dose), metformin determined blood glucose levels as 130.32 ± 2.78, 141.56 ± 3.36, 128.46 ± 2.54, 113.23 ± 3.43, 110.13 ± 2.45 respectively. In Fig. 2, the data for group 1 rats indicated that the maximum glucose level was achieved (1 hr) after glucose loading and then returned close to the basal line two hours later. The non-treated diabetic rodents had a slight rise in sugar concentration after twohour period. Rodents undergone treatment significantly displayed low peaks in blood glucose levels, post glucose administration (2 hrs) determining a potent antihyperglycemic effect. In comparison to individual treatment the combination therapy of *coccinia indica* and hesperidin (low dose) had a stronger antihyperglycemic impact.

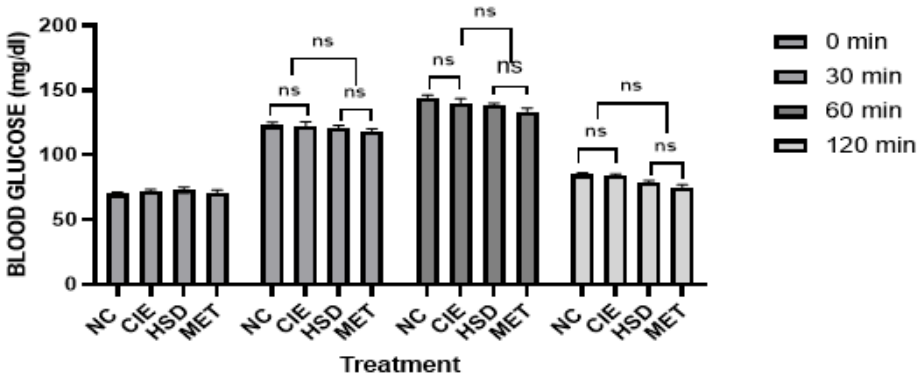

Fig. 1. Impact of *coccinia indica*, hesperidin, metformin on glucose challenge test in negative control rodents (pre therapy) assessed at four time points: baseline, half an hour, one hour, two hours

Data's are expressed as mean ± SEM (n = 6). (NS) denoting non-significance, upon comparing with both negative control and among various experimental groups

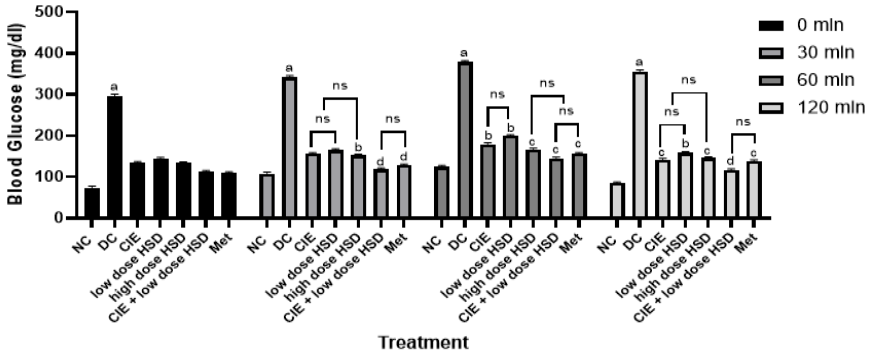

Fig. 2. Impact of hesperidin (low/high dose), *coccinia indica* , co-administration of *coccinia indica* alongside hesperidin, metformin on glucose challenge test in positive rodents, following an 8- week treatment regime

Data's are expressed as mean ± SEM (n = 6), [a] p < 0.0001, [b] p < 0.001, [c] p < 0.01 [d] p < 0.05 upon comparison with negative control as well as between the groups

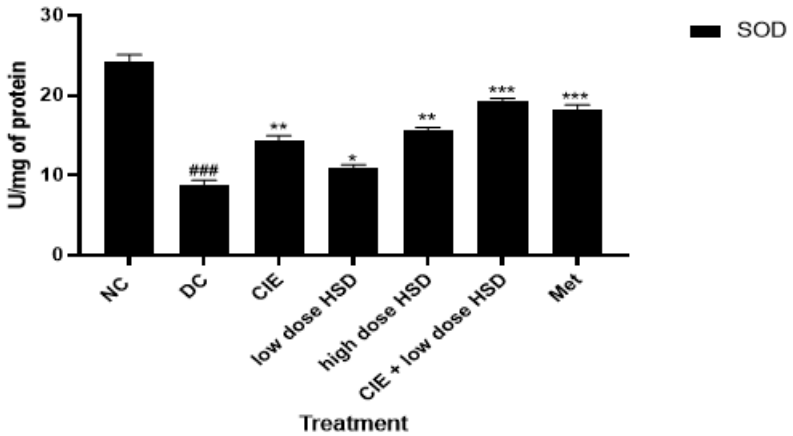

Fig. 3. Data are represented as Mean ± SEM, (n = 6 rats per group). ###*p*< 0.001 upon comparison with normal control. ***p<0.001,p<0.01,*p<0.05 when compared with disease control**

3.2 Biochemical Indices

3.2.1 Effect of CIE, hesperidin along with its conjunction on the levels of superoxide dismutase and catalase in peripheral nerve (sciatic)

Diabetic animals exhibited remarkable decline in SOD concentration against non-treated rodents. However eight weeks treatment with *CIE*, hesperidin and their combination displayed significant increase in SOD levels, upon comparing with the diabetic control (Fig. 3). Subsequently, diabetic control animals showed decrease in sciatic nerve catalase activity upon comparing with negative control. Post eight week treatment period, *CIE* plus hesperidin treated group demonstrated noteworthy (*P*<0.001) growth in catalase activity against positive control. Likewise, remaining treated rodents exhibited crucial (*P*<0.01) rise in catalase concentration (Fig. 4).

3.2.2 Effect of CIE, hesperidin along with its conjunction on peripheral nerve (sciatic) lipid peroxidation and glutathione

Malondialdehyde (MDA) levels in the sciatic nerve were notably higher in positive control rats compared to non-diabetic rats. A notable decrease in the levels of reduced glutathione was also observed. Eight weeks of therapy with CIE, hesperidin and their combination remarkably (*P* < 0.05) reduced MDA content and moreover increased concentration of reduced glutathione (Fig. 5).

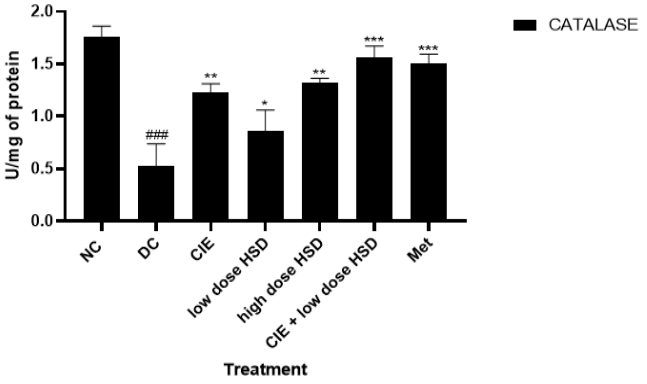

Fig. 4. Data are represented as Mean ± SEM, (n = 6 rats in each group). ###*p*< 0.001 upon comparison with negative control. ***p<0.001,p<0.01,*p<0.05 when evaluated against disease control**

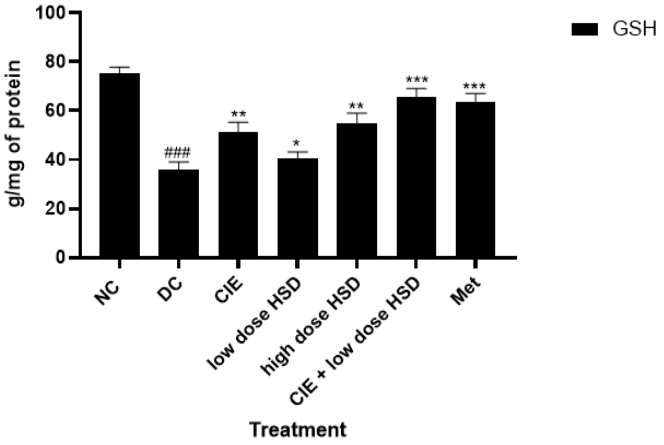

Fig. 5. Values are represented as Mean ± SEM, (n = 6 rodents per group). ###*p*< 0.001 upon comparison with negative control.*p<0.001,**p<0.01,*p<0.05 when compared to disease control**

3.3 Evaluation of Antinociceptive Activity

The nociceptive sensitivity showed a notable decrease in positive control rats, in comparison to the baseline values assessed in both the tail-immersion and thermal hyperalgesia. CIE, hesperidin and their combination therapy for eight weeks notably increased the latency threshold and sustained it throughout the therapy. The positive rodents fed with CIE, hesperidin and their amalgamation exhibited a concentration alongside time influenced rise over pain sensitivity upon comparing it with the non-treated diabetic rats (Tables 1 and 2).

3.4 Impact of CIE and Hesperidin on Sciatic Nerve Histopathology

Diabetic rodents displayed decreased fiber density in multiple neuronal sites, with fiber loss being chronic and non-uniform in the positive control. Moreover, endoneurial vessels marked thickening and hyalinization upon comparing it with negative control. Although, CIE + hesperidin (low dose) remedy was found to preserve fiber density.

4. DISCUSSION

As the number of instances for non-insulin dependent diabetes mellitus rises, so do complexities mediated through diabetes. Health conditions including obese, elevated triglycerides, high blood sugar status altogether characterize the metabolic disorder. The initial onset of neuropathy is evidenced by a study over a group of individuals with impairment in glucose tolerance, who exhibited decrease in their neuronal transmission (Smith et al., 2022). The peripheral neuronal injury in hyperglycemic individuals is marked by the gradual loss of nerve fibres. Individuals with impairment in glucose tolerance frequently experience deprivation of distal nerve fibres in the lower legs (34. Prem Kumar et al., 2009). The appropriate management of blood sugar levels may help in preventing the onset and the advancement of diabetes induced neurodegeneration. Reactive oxygen species generated through diabetes related oxidative stress contribute to vascular injury in the sciatic nerves of diabetic rodents (Mallet et al., 2020). Dietary regime rich with free radical scavengers have enhanced vascular resilience in diabetic rats (Salomi and Parimala, 2023).

Table 1. Effect of *coccinia indica,* hesperidin along with its conjunction in thermal analgesiometer

	0	2	4	6	8
Control	8.93±0.45	8.60±0.42	8.71±0.43	8.83±0.46	8.21±0.40
DC	8.57±0.46	8.38±0.41	7.61±0.44	7.24±0.44	7.03±0.41
CIE	8.66±0.43	8.45±0.45	8.23±0.43^{c}	8.41±0.47^{b}	8.04±0.43^{b}
Hes(low)	8.62±0.41	8.42±0.44	8.15±0.42^{c}	8.21±0.41^{c}	7.91±0.45^{c}
Hes(high)	8.71±0.45	8.53±0.46	8.27±0.41^{c}	8.59±0.43^{b}	8.1±0.42^{b}
CIE+Hes	8.89±0.42	8.65±0.44	8.57±0.44^{b}	8.79 ±0.45^{a}	8.57±0.42^{a}
Met	8.81±0.45	8.59±0.42	8.48 ±0.43^{b}	8.71±0.42^{a}	8.48±0.40^{a}

Data are represented as mean ± SEM, n=6, $^{a}p<0.001$, $^{b}p<0.01$, $^{c}p<0.05$ upon evaluating it against positive control

Table 2. Impact of *coccinia indica,* hesperidin along with its conjunction in tail immersion test

	0	2	4	6	8
Control	8.2± 0.3	8.47±0.55^{a}	8.61±0.43^{a}	8.57±0.44^{a}	8.51± 0.37^{a}
DC	8.05±0.40	7.92±0.42	7.71±0.41	7.66±0.41	7.54± 0.38
CIE	8.08±0.42^{c}	8.03±0.40^{a}	7.9±0.41^{a}	8.14±0.42^{c}	7.8±0.40
Hes(low)	8.06±0.41	8.01±0.44^{a}	7.7±0.42^{a}	8.12±0.41^{c}	7.6±0.45
Hes(high)	8.09±0.41^{c}	8.04±0.46^{a}	7.9±0.44^{a}	8.15±0.43^{c}	**7**.9±0.42
CIE+HSD	8.01±0.40 ^{a}	8.32±0.42^{b}	8.40±0.42^{c}	8.21±0.41^{c}	8.27±0.43^{c}
Met	8.20±0.42^{a}	7.96±0.41	7.78±0.40 ^{a}	7.89±0.41^{c}	7.71±0.40

Data are represented as mean ± SEM, n=6, $^{a}p<0.001$, $^{b}p<0.01$, $^{c}p<0.05$ upon evaluating it against positive control

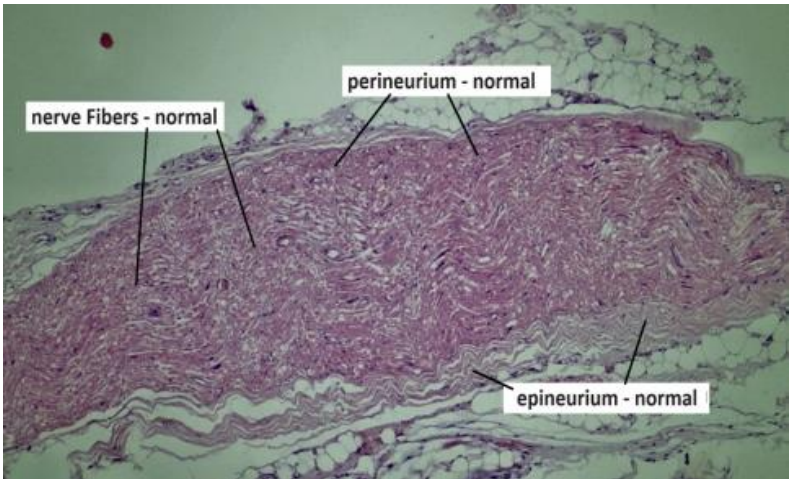

Fig. 6. Negative control

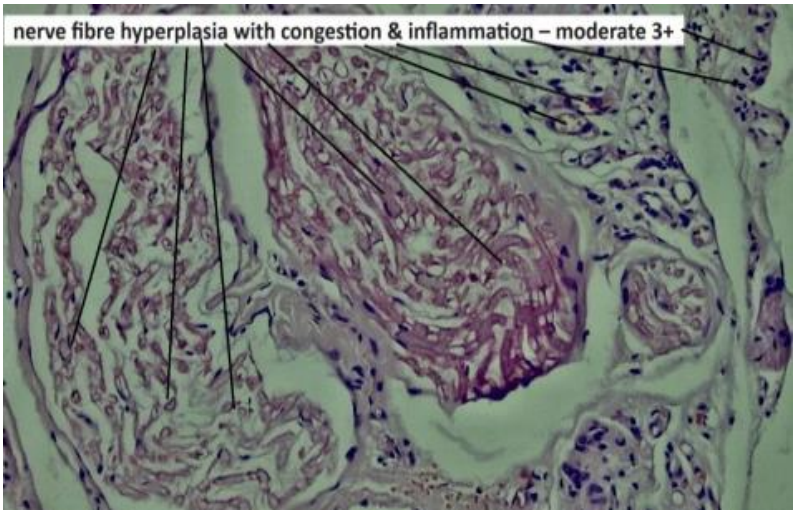

Fig. 7. Positive control

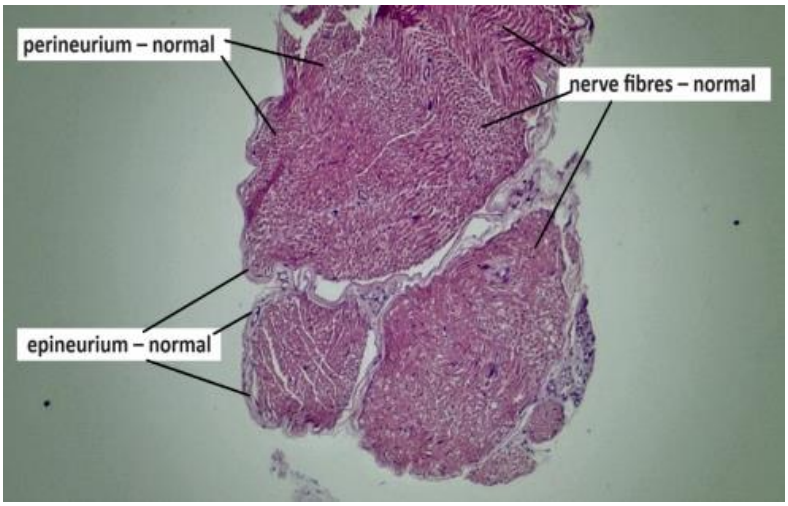

Fig. 8. CIE + hesperidin, showing normal and preserved outer layers

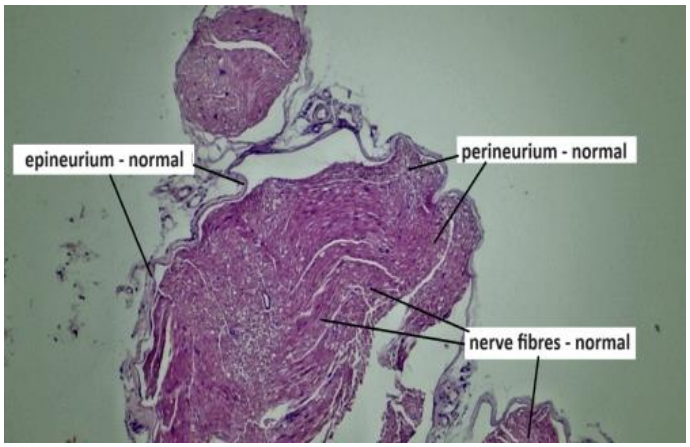

Fig. 9. Metformin Normal and preserved outer connective layers

The study offers valuable insights into the therapeutic potential of flavonoid combinations for diabetic neuropathy. It presents a scientifically rigorous approach by integrating biochemical, physiological, and histological analyses to assess treatment efficacy. The use of suitable animal models, along with the evaluation of oxidative stress markers and neuropathic pain symptoms, further underscores the study's technical robustness and comprehensive design. A fructose enriched dietary regime was implemented for the induction of insulin resistance, given the well-established role of fructose in exacerbating diabetic disorders (Srinivasan et al., 2005). Additionally streptozotocin was injected so as to induce a diabetic condition equivalent to the insulin resistance in a person. Experiments involving diabetes undergo significant alterations in enzymatic metabolism of glucose and fats. One key driver for such metabolic changes is the chronic hyperglycemic condition, which eventually contributes in the development of diabetes induced neurodegenerative disorders (Wu et al.,2023). In our current investigation both CIE and hesperidin has lowered the glucose levels close to the normal glycemic range, which serves as a crucial catalyst for restoring physiological equilibrium. Potential pathway through which CIE exerts its glucose lowering effect may involve enhancing the insulin activity in plasma, either through activation of insulin release by pancreatic beta cells or by promoting secretion through stored sites (Siddiqua et al., 2023). A possible mechanism for hesperidin could be due to the enhancement of glucokinase pathway while reducing the levels of D-glucose-6-phosphate (G6P) and phosphoenolpyruvate carboxykinase (PEPCK). Glucokinase one of the most responsive enzyme for glycolysis in hyperglycemia, when stimulated it promotes its activity of utilizing blood glucose for deposition of glycogen in the liver. In comparison to CIE treatment solely, the combined therapy of CIE and hesperidin (low dose) has demonstrated greater potency in reducing the blood glucose (Fig. 2). Decrease in sugar concentration could be ascribed for its capacity in stimulating insulin release as well as glucose utilization (Siddiqua et al.,2021, Osama et al.,2023). This strongly indicates that CIE along with its combination of hesperidin (low dose) has more pronounced effect on insulin sensitivity.

Maintaining the normal GSH to oxidized glutathione ratio depends upon the availability of NADPH. In hyperglycemic conditions there is significant loss of NADPH which eventually leads in reduction of GSH and elevation of lipid oxylation (Mahmoud et al.,2012). Reduction in essential cellular antioxidants greatly increases the exposure to oxidative stress. Experimental rats (diabetic) showed a notable rise in MDA levels, an indicator of lipid peroxidation, accompanied by lowering of enzymatic anti-radicals, which correlates with the data that has already been established in previous research (Faheem et al., 2022). All of these markers returned to its normal levels following treatment with CIE and hesperidin which in turn proves the radical scavenging potential of these compounds. Research indicates that hesperidin may confer its beneficial effects through the modulation of nitric oxide levels. Furthermore, hesperidin functions as an effective scavenger of peroxynitrite ($ONOO^-$) and enhances cellular protective responses against diseases associated with $ONOO^-$ (Mahmoud et al.,2012). CIE and hesperidin contain antioxidants such as vitamin C, which are known to be beneficial in

diabetes (Hossain et al.,2021). Moreover both these compounds effectively scavenges superoxide and also prevents its production (Meenatchi et al.,2017, Parhiz et al., 2015), which can be evidenced by the increase in SOD and catalase levels in the current study. CIE and their combination with hesperidin caused increased tail flick response time in the tail immersion assay and increased paw retraction latency in Eddy's thermal test, which determines its potential in preventing the further degeneration of neurons in diabetic neuropathy.

Research has shown that NF-κB (nuclear factor-kappa B) plays a critical role in the induction of oxidative stress, primarily by upregulating pro-inflammatory cytokines such as IL-2, IL-6, and TNF-α, while also promoting pro-apoptotic pathways through caspase-3 and caspase-9 activation. This cascade contributes to neurodegenerative processes (Nanang and Nurrochmad, 2023). The combined therapeutic approach using CIE and hesperidin mitigates oxidative stress by activating adenosine 5′-monophosphate-activated protein kinase (AMPK). This activation leads to the phosphorylation of NF-κB, facilitating its translocation and inducing the expression of antioxidant enzymes, including heme oxygenase 1 (HO-1) (Weitong et al., 2020). It also facilitates the glucose transport and inhibits the mTOR (mammalian target of rapamycin), effectively suppressing protein synthesis and gluconeogenesis (Arpita et al.,2022). The combination of CIE and hesperidin also inhibits the increase in inflammatory cytokines preventing further neuroinflammation.

Subsequently, the synergistic threapy also initiates the enhancement of peroxisome proliferator-activated receptors (PPARs), which are integral in the regulation of essential metabolic and immunological pathways. Activation of PPARγ improves insulin sensitivity, reduces glucose and lipid levels (Germoush et al., 2020). In addition to their significant impact on metabolism, *coccinia indica* and hesperidin facilitate the activation of PPARγ, resulting in low sugar concentration and enhanced activity of anti-radical enzymes (Faheem et al., 2020, Elshazly et al., 2018).

These effects are essential in slowing the progression of hyperglycemia-related neurodegeneration which is made evident by the histopathological reports of sciatic nerve in our study (sec 3.4). Thus through our research it can be hypothesized that the synergistic therapy of *coccinia indica* (leaf extract) along with hesperidin can effectively impede the advancement of neuronal degeneration, primarily by the activation of AMPK and simultaneous enhancement of PPARγ which mediates its anti-hyperglycemic as well as radical scavenging property. The neuroprotective effects of CIE combined with hesperidin (low dose), as demonstrated in our research, were more effective than those in rats treated with metformin in alleviating diabetic neuropathy.

This manuscript holds significance for the scientific community as it investigates the potential of flavonoid-rich "*Coccinia indica*" extract and hesperidin in addressing diabetic neuropathy, a common complication of diabetes. By demonstrating synergistic interactions between these natural compounds, the study paves the way for more effective therapeutic strategies. Its focus on a natural product-based approach to an urgent medical need highlights the possibility of developing safer, more accessible treatments. Positive outcomes from this research could inform future therapeutic interventions, influence public health policies, and encourage further interdisciplinary exploration of natural compounds in treating neurodegenerative complications of diabetes.

5. CONCLUSION

Diabetes mellitus and its complications stem from hyperglycemia and oxidative stress. Although monotherapy has shown promising results in rodent studies, there is a growing trend towards using combination therapy to alleviate painful diabetic neuropathy. Our findings indicate that the progression of neurodegeneration was markedly delayed following the synergistic administration of "*Coccinia indica*" and low-dose hesperidin. This is evidenced by the significant elevation in antioxidant markers, including superoxide dismutase, catalase, and reduced glutathione, with subsequent reduction in the lipid peroxidation, alongside a reduction in blood glucose levels when compared to the positive control group. The increase in tail flick and pain threshold levels in eddy's thermal analgesiometer indicates the effectiveness of combination therapy by serving as an anti-nociceptive agent owing to its anti-hyperglycemic and radical scavenging attributes. The future of combination therapy in diabetic neuropathy holds significant potential for transforming treatment. A key advantage is enhanced therapeutic efficacy,

achieved by using agents with complementary mechanisms, such as *"Coccinia indica*" and low-dose hesperidin, which simultaneously mitigates hyperglycemia and oxidative stress. Moreover, prolong therapy with metformin has significant adverse effects but this approach can optimize outcomes in patients with variable responses to standard treatments, reducing adverse effects while maximizing therapeutic benefits. In addition, dietary modifications, lifestyle adjustments, and regular exercise can be strategically employed to effectively delay the progression of diabetic neuropathy and withdraw metformin.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

ETHICAL APPROVAL

The investigative protocol was approved by the designated Ethics Control (Reference: KCP/IAEC/PCOL/130/AUG2023). The rats were accomodated in adherence to directives founded by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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