Effect of Lupeol on the Expression of Neuro Inflammatory Signalling Molecules in Brain Tissues of High Fat Diet and Sucrose Fed Type-2 Diabetic Rats

Centre of Molecular Medicine and Diagnostics (COMManD), Department of Biochemistry,  
Saveetha Dental College & Hospitals, Saveetha Institute of Medical & Technical Sciences,  
Saveetha University, Chennai 600077, India

Abstract

Lupeol has anti-inflammatory, antimicrobial, anti-protozoal, anti-proliferative, anti-invasion, anti-angiogenic and cholesterol-lowering properties. However, the mechanisms underlying the effect of lupeol on neuroinflammatory signalling molecules have not yet been identified. The study was designed to study the effect of lupeol on the expression of inflammatory signalling molecules in brain tissue of high-fat diet and sucrose-fed type-2 diabetic rats. Adult male albino rats of Wistar 150–180 days old with 180–200 g body weight (b.wt) were divided into four groups of six rats each. Group I: Control (vehicle-treated); Group II: High fat diet-induced type-2 diabetic rats; Group III: Type-2 diabetic rats treated with lupeol (25 mg/kg b.wt/day) orally for 30 days and Group IV: Type-2 diabetic rats treated with metformin (50 mg/kg, b.wt/day orally for 30 days. After 30 days of treatment, the animals were anaesthetized, and brain tissue was dissected and used for the assessment of mRNA expression analysis. Type-2 diabetic animals showed a significant increase (p<0.05) in TNF-α and IL-6 mRNA levels in brain tissue in high-fat diet-induced type-2 diabetic animals. However, lupeol treatment, effectively reduced (p<0.05) the neuroinflammatory signaling molecules (TNF-α and IL-6 mRNA) showing that lupeol has significant role over the control of neuroinflammatory signaling. Our present findings clearly show that lupeol has a significant role in reducing neuroinflammation via the downregulation of TNF-α and IL-6 in brain tissues and hence, lupeol can be a potential natural drug for the treatment of diabetic neuropathy.

Keywords: Antidiabetic, Brain, High Fat Diet, Inflammation, Innovative Technique, IL-6, Lupeol, Novel Method, TNF-α.

Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by elevated blood glucose levels due to insulin deficiency, insulin resistance, or both. It represents a significant global health challenge, with its prevalence steadily increasing worldwide. According to recent statistics from the International Diabetes Federation (IDF), approximately 463 million adults aged 20-79 were living with diabetes in 2019, and this number is projected to rise to 700 million by 2045. The burden of diabetes extends beyond individual health, exerting substantial economic impacts on healthcare systems and society as a whole. Complications associated with diabetes, such as cardiovascular disease, kidney failure, blindness, and lower limb amputation, contribute to increased morbidity, mortality, and healthcare costs, highlighting the urgent need for effective management strategies [1-5].

Diabetic neuropathy, a common complication of diabetes mellitus, manifests as nerve damage leading to symptoms such as...
pain, numbness, tingling, and weakness, primarily affecting peripheral nerves. This condition significantly impacts patients’ quality of life and increases the risk of foot ulcers, infections, and lower limb amputations. Growing evidence suggests a strong association between diabetes and neuroinflammation, mediated by proinflammatory cytokines, which contribute to the pathogenesis of diabetic neuropathy and cognitive dysfunction. Proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β) are elevated in individuals with diabetes and are implicated in the activation of glial cells and the release of neurotoxic factors in the central nervous system. These cytokines disrupt neuronal function, promote oxidative stress, and induce apoptosis, ultimately leading to neuroinflammation and neuronal damage. Furthermore, neuroinflammation exacerbates insulin resistance in the brain, impairing insulin signaling pathways and exacerbating cognitive decline in individuals with diabetes. Understanding the role of proinflammatory cytokines in mediating neuroinflammation offers potential therapeutic targets for the prevention and treatment of diabetic neuropathy and cognitive impairment [6-10].

Current treatments focus on symptom management and glycemic control; however, recent research suggests phytotherapy, utilizing plant-derived compounds, may offer therapeutic benefits. Phytochemicals like flavonoids, alkaloids, and polyphenols found in medicinal plants exhibit neuroprotective effects by reducing oxidative stress, inflammation, and neuronal apoptosis. Herbal extracts and botanical preparations have shown promise in preclinical and clinical studies for alleviating neuropathic pain and improving nerve function in diabetic patients, presenting a potential adjunctive or alternative approach to conventional therapies [11-13].

Phytotherapy offers a promising avenue for managing diabetic neuropathy, providing alternative treatments that address limitations of conventional therapies and improve outcomes. Research continues to elucidate mechanisms of action and optimize efficacy and safety of phytotherapeutic interventions. Further exploration of plant-derived compounds and their potential synergistic effects may lead to novel therapeutic strategies for diabetic neuropathy, enhancing patient care and quality of life [14-20].

Amidst the growing prevalence and burden of diabetes, there is increasing interest in exploring alternative and complementary approaches to conventional management. Natural medicine, including herbal remedies, dietary supplements, and traditional practices, holds potential as adjunctive or standalone therapies for diabetes control. Various natural compounds, such as berberine, curcumin, and resveratrol, have demonstrated promising antidiabetic effects through mechanisms such as improving insulin sensitivity, reducing inflammation, and regulating glucose metabolism. Additionally, mind-body practices like yoga and meditation have been shown to help manage stress and improve glycemic control in individuals with diabetes. Integrating alternative medicine into diabetes care offers a holistic approach that may complement conventional treatments, enhance overall well-being, and potentially mitigate the burden of the disease.

Lupeol, a natural pentacyclic triterpene widely distributed in plants, exhibits diverse medicinal properties, making it a promising candidate for therapeutic applications. Its antioxidant activity has been demonstrated in numerous studies. Lupeol scavenges free radicals, reduces lipid peroxidation, and enhances antioxidant enzyme activity, protecting cells from oxidative stress-induced damage. This antioxidant potential contributes to its cytoprotective effects and its potential role in preventing various diseases linked to oxidative stress, including diabetes and cancer [20-24]. In addition to its antioxidant...
properties, lupeol shows promise as an antidiabetic agent. Studies have highlighted its ability to improve insulin sensitivity, stimulate glucose uptake in cells, and modulate key enzymes involved in glucose metabolism. Lupeol supplementation has been associated with reduced blood glucose levels and improved lipid profile in diabetic animal models, suggesting its potential as a therapeutic agent for managing diabetes mellitus (Salehi et al., 2019; Salehi et al., 2020). However, the mechanism underlying the effects of lupeol on proinflammatory signalling molecules in high-fat diet-induced-type-2 diabetic rats is obscure. Here, we have shown the possible effects of lupeol in attenuating the expression of proinflammatory molecules towards the consideration of therapeutic drug [20-24].

**Materials and Methods**

**Chemicals**

The chemicals and reagents used in this study were purchased from Sigma Chemical Company St. Louis, MO, USA; Invitrogen, USA; Eurofins Genomics India Pvt Ltd, Bangalore, India; New England Biolabs (NEB), USA; Promega, USA. Lupeol was procured from Sigma Chemical Company St. Louis, MO, USA; Total RNA isolation reagent (TRIR) was purchased from Invitrogen, USA. The reverse-transcriptase enzyme (MMuLv) was purchased from Genet Bio, South Korea purchased from Promega, USA. Interleukin-6 (IL-6), Tumor Necrosis factor-α and β-actin primers were purchased from Eurofins Genomics India Pvt Ltd, Bangalore, India.

**Animals**

Animals were maintained as per the National Guidelines and Protocols approved by the Institutional Animal Ethics Committee (IAEC no: 006/2016). Healthy adult male Wistar albino rats 150–180 days old weighing 180–200 g were used in this study and maintained in clean polypropylene cages at the Central Animal House Facility, Meenakshi Medical College and Research Institute (Meenakshi Academy of Higher Education and Research) under specific humidity (65±5%) and temperature (21±2°) with constant 12 h light and 12 h dark schedule.

**Induction of Type-2 Diabetes**

Rats were subjected to 60 days of a high-fat diet containing cholesterol 3%, cholic acid 1%, coconut oil 30%, standard rat feed 66%, and 30% sucrose through drinking water. On the 58th day of treatment, after fasting, blood glucose level was checked and the rats that had blood glucose above 120 mg/dL were chosen as type-2 diabetic rats. Sucrose feeding through drinking water with a high-fat diet was continued until the end of the study.

**Experimental Design**

Adult male albino rats of Wistar 150–180 days old with 180–200 g body weight (b.wt) were randomly divided into five groups of six rats each. Group I – control (vehicle-treated); Group II – type-2 diabetic rats; Group III – type-2 diabetic rats treated with lupeol (25 mg/kg b.wt/day) orally for 30 days; Group IV – type-2 diabetic rats treated with metformin (50 mg/kg, b.wt/day orally for 30 days; Two days before sacrifice, control and experimental animals were subjected to oral glucose tolerance (OGT) test and insulin tolerance test. At the end of the treatment, animals were anaesthetized with sodium thiopentone (40 mg/kg b.wt), blood was collected through the cardiac puncture, and sera were separated and stored at −80°C. Brain tissues from control and experimental animals were immediately dissected and used for assessing the various parameters.

**Assessment of Fasting Blood Glucose (FBG)**

The blood glucose was estimated using On-Call Plus blood glucose test strips (ACON Laboratories Inc., USA). From the rat tail tip, the blood was collected and results were expressed as mg/dl.
Oral Glucose Tolerance Test (OGTT)

For oral glucose tolerance tests, animals were fasted overnight. After giving the oral glucose load (10 ml/kg; 50% w/v), blood glucose level was estimated at various time periods (60, 120, and 180 min) by using On-Call Plus blood glucose test strips. Before giving glucose load, the value of blood glucose is considered as 0 min value. Results were marked as mg/dl.

Isolation of Total RNA

Total RNA was isolated from control and experimental samples using TRIR (total RNA isolation reagent) kit. Briefly, 100 mg fresh tissue was homogenized with 1 ml TRIR and the homogenate was transferred immediately to a microfuge tube and kept at -80°C for 60 min to permit the complete dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform was added, vortexed for 1 min and placed on ice at 4°C for 5 min. The homogenate was centrifuged at 12,000 x g for 15 min at 4°C. The aqueous phase was carefully transferred to a fresh microfuge tube and an equal volume of isopropanol was added, vortexed for 15 sec and placed on ice at 4°C for 10 min. The samples were centrifuged for 10 min at 4°C. The supernatant was discarded and the RNA pellet was washed with 1 ml of 75% ethanol by vortexing and subsequent centrifugation for 5 min at 7,500 x g (4°C). The supernatant was removed and RNA pellets were mixed with 50 μl of autoclaved Milli-Q water and dissolved by heating in a water bath for 10 min at 60°C.

Quantification of RNA

Diluted RNA samples were quantified spectrophotometrically by measuring the absorbance (A) at 260/280 nm. A ratio of absorbance at 260/280 nm > 1.8 is generally considered as good quality RNA (Fourney et al., 1988). The purity of RNA obtained was 1.8.

Conversion of cDNA from Total RNA

RT-PCR is an approach for converting and amplifying a single-stranded RNA template to yield abundant double-stranded DNA products. 1. First strand reaction: Complementary DNA (cDNA) is made from the mRNA template using Oligo dT, dNTPs & reverse transcriptase. 2. Second strand reaction: After the reverse transcriptase reaction is complete, standard PCR (called the “second strand reaction”) is initiated. Principle RT-PCR is a method used to amplify cDNA copies of RNA. It is the conversion of mRNA into a single cDNA template. A specific oligodeoxynucleotide primer is hybridized to the mRNA and is then extended by an RNA-dependent DNA polymerase to create a cDNA copy. First-strand DNA synthesis The RT kit was purchased from Eurogentec (Seraing, Belgium). Reagents 1. 10X RT buffer: One vial containing 1.4 ml of 10X RT buffer. 2. EuroScript reverse transcriptase: One tube containing 75 μl of Moloney Murine leukemia virus reverse transcriptase (3750 U at 50 U/μl).

mRNA Expression Analysis

Procedure

Procedure Real-Time PCR was carried out on CFX 96 Real-Time system (Bio-Rad). The reaction mix (10 μl) was prepared by adding 5 μl of 2X reaction buffer, 0.1 μl of sense and antisense primer, 1 μl of cDNA and 3.8 μl of sterile water. The thermal cycler protocol was as follows: Initial denaturation at 95°C for 3 min, followed by 40 cycles of PCR, denaturation at 95°C for 10 sec, annealing at 60°C for 20 sec and extension at 72°C for 20 sec. All reactions were performed in triplicate along with no template control (NTC). Melt curve analysis was performed using the thermal cycling programmed at 50-95°C for each
sample to determine the presence of multiple amplicons, non-specific products and contaminants. The results were analysed using CFX 96 Real Time system software (Bio-Rad). As an invariant control, the present study used rat β-actin. The following primers were used in this study: Rat IL-6- FW-5’-GAAATGAGAAAAGAGTTGTGC -3’; RW-5’-GGAAGTTGGGTAGGAGGAC; Rat TNF-α- FW -5’-ACGCTTCTCTGCTACTG-3’; RW-5’GGATGACACGCCCAGTCG-3’; Rat β-actin- FW – 5’- TACAGCTTCACCACCACAGC - 3’; RW– 5’- TCTCCAGGGAGGAGGAT - 3’

**Statistical Analysis**

GraphPad Prism 8.0 was used for statistical calculations. Mean values with SEM were presented. One-way ANOVA was used in statistical studies, and then the Newman-Keuls assessment for numerous comparisons. Statistics were deemed significant at \( p < 0.05 \).

**Results**

**Effect of Lupeol on TNF-α in the Brain Tissue of Type-2 Diabetic Rats**

![Amplification Graph](image)

TNF-alpha (tumour necrosis factor-alpha) has been implicated in the pathogenesis of diabetic neuropathy, a common complication of diabetes mellitus. Elevated levels of TNF-alpha are associated with neuroinflammation, nerve damage, and the development of neuropathic pain in diabetic individuals. Targeting TNF-alpha signalling pathways may offer therapeutic potential for the management of diabetic neuropathy by mitigating inflammation and preserving nerve function. Additionally, TNF-alpha contributes to the development of diabetic complications by promoting endothelial dysfunction, impairing insulin signalling pathways, and inducing oxidative stress, highlighting its role as a potential therapeutic target in T2DM management. Fig. 1. shows the level of TNF-α in control and experimental rats. A significant increase (\( p<0.05 \)) in TNF-α mRNA levels in brain tissue was observed in type-2 diabetic animals, whereas lupeol treatment decreased the TNF-α mRNA levels in the type-2 diabetic animals. Lupeol treatment to control rats did not show any significant change in the TNF-α mRNA levels.
Figure 1. Shows the Effect of Lupeol on mRNA Expression of TNF-α Type-2 Diabetic Rats. The mRNA Expression of TNF-α was Assessed by Real Time-PCR

Effect of Lupeol on IL-6 in the Brain Tissue

Interleukin-6 (IL-6) has emerged as a key player in the development and progression of diabetic neuropathy, a debilitating complication of diabetes mellitus. Elevated levels of IL-6 have been observed in diabetic patients with neuropathy, correlating with the severity of nerve damage and neuropathic pain. IL-6 contributes to neuroinflammation, peripheral nerve damage, and sensitization of pain pathways, suggesting its involvement in the pathophysiology of diabetic neuropathy and potential as a therapeutic target. Fig. 2. shows the level of IL-6 in control and experimental rats. A significant increase (p<0.05) in IL-6 mRNA levels in brain tissue was observed in type-2 diabetic animals, whereas lupeol treatment decreased the TNF-α mRNA levels in the type-2 diabetic animals. Lupeol treatment to control rats did not show any significant change in the TNF-α mRNA levels.
**Discussion**

Obesity and overweight are the fifth leading cause of death worldwide [25]. Diet-induced obesity is a key player in the pathogenesis of type 2 diabetes mellitus, responsible for up to 85% of the physiological effects of insulin on blood glucose clearance [26]. Therefore, several studies have focused on the mechanisms that control the intake of glucose as a target of antihyperglycemic therapies [27,28]. Due to their extraordinary ability in terms of pharmacodynamics and pharmacokinetic activities, plant-derived medicines are considered an alternative to currently available medicines [27]. In addition, Lupeol is the bioactive compound present in Cassia fistula. C. fistula belongs to the *Caesalpiniaceae* family commonly known as Amaltas, Indian laburnum, and golden showers. It is widely used in different traditional medicines such as Ayurveda, Unani, and Chinese [29-31]. The high fat and high sucrose diet-induced rats treated with lupeol in the current study demonstrate the major changes and expression in the brain compared to control rats showing the antidiabetic activity of lupeol in the diet-induced model.

Nearly all human diseases are connected with local or systemic inflammatory responses. Epidemiological and experimental reports showed that individuals with preexisting inflammatory conditions are more prone to the harmful effects of environmental harm [32]. Different anti-inflammatory drugs including metformin, thiazolidinediones, etc., are widely used to control inflammation and thus insulin resistance in type 2 diabetic patients [33-39]; Several lines of evidence suggest that diet-induced diabetes leads to the production of inflammatory markers. We investigated whether lupeol had any impact on the expression of inflammatory markers. This study reported short-term exposure to the high-sucrose or high-fat diet increased the inflammatory marker TNF-alpha in vivo with alterations in its mRNA expressions in diabetic rats as compared to normal rats. Alternatively, lupeol administration to the HFD-induced diabetic rats altered the TNF- mRNA expression to the near normal when compared to the control. These findings indicate that lupeol has been found to reduce TNF levels by inhibiting its expression. Lupeol has been reported to suppress the IL-6 which may be

![Figure 2. Shows the Effect of Lupeol on mRNA Expression of IL-6 Type-2 Diabetic Rats. The mRNA Expression of IL-6 was Assessed by Real Time-PCR](image-url)
imputable to better glycaemic rule [40-41].

These results were in line with another study that, Lupeol treatment (10–100 μM) is also shown to decrease the generation of pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and Interleukin β (ILβ) in lipopolysaccharide-treated macrophages [33](28). Lupeol treatment showed a decrease in the generation of pro-inflammatory cytokines such as IL-beta in lipopolysaccharide-treated macrophages [42-44].

Increased inflammatory marker concentrations signal an increased complication of diabetes, either through increased development or through decreased destruction. Lupeol has been reported to inhibit the IL-6 which may be attributable to better glycemic regulation and antioxidant status. Similarly, Lupeol treatment was observed to cause a reduction in inflammation by decreasing levels of type II cytokines IL-4, IL-5 and IL-13 in a bronchial asthma mouse model.[45-47].

Taken together, these compelling evidence suggest that the therapeutic usefulness of Lupeol for inflammatory conditions is attractive and warrants further investigation.

**Conclusion**

Increased fat intake and Western diets have been linked to insulin resistance to be associated with a high risk of diabetes mellitus and related metabolic diseases. Our present findings clearly show that lupeol has a significant role in reducing neuroinflammation via the downregulation of proinflammatory cytokines in brain tissues and hence, lupeol can be a potential natural drug for the treatment of diabetic neuropathy. Further studies on the role of lupeol on further signalling molecules involved in neuroinflammation and insulin signalling in brain tissues need to be analyzed to ascertain its potential mechanisms of action.

**Conflicts of Interest**

The authors declare that there are no conflicts of interest in the present study.

**Acknowledgement**

The authors would like to thank all the participants for their valuable support and Saveetha Dental College for their support in conducting the study.

**References**


study on hexavalent chromium [Cr(VI)] remediation using iron oxide nanoparticles based beads. *Environmental Nanotechnology, Monitoring & Management*, 1, 14, 100333.


