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Original Article

Pinocembrin Isolated from Nigerian Propolis Prevents Elevation of Cytokines Implicated in the Aetiology of Diabetic Retinopathy in Rat Models of Diabetes Mellitus

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ABSTRACT

Propolis, a resin produced by bees-, contains the flavonoid compound pinocembrin, which shows promise for antioxidant and anti-inflammatory applications, though its therapeutic potential remains underexplored. Diabetic retinopathy, a common complication of diabetes, involves retinal inflammation and vascular damage. Previous research indicates that Nigerian propolis may have anti-hyperglycemic effects and the ability to lower glycosylated hemoglobin levels. This study evaluated the protective effects of pinocembrin, extracted from Nigerian propolis, against diabetic retinopathy in a streptozotocin-induced rat model. Diabetes was induced in male Sprague-Dawley rats through a single intraperitoneal injection of streptozotocin, resulting in sustained hyperglycemia. The diabetic rats were then administered oral pinocembrin at a dose of 50 mg/kg daily for 8 weeks. Pinocembrin effectively mitigated the elevation of inflammatory mediators, including Interleukin-1 (IL-1), Interleukin-8 (IL-8) and Tumor Necrosis Factor-alpha (TNF-α), within the retinal tissues of the treated diabetic rats. Furthermore, pinocembrin enhanced levels of the antioxidant enzymes such as Superoxide Dismutase (SOD) and Glutathione Peroxidase (GSH-Px), and also improved glycemic control and glycosylated hemoglobin levels. The results indicate that pinocembrin possesses significant therapeutic potential for preventing or mitigating diabetic retinopathy. Its capacity to regulate inflammatory processes and strengthen antioxidant defenses underscores its potential as a treatment strategy for managing this vision-threatening complication associated with diabetes mellitus.

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1. Introduction

Propolis, a resinous material gathered by honeybees from diverse plant sources, exemplifies nature's remarkable medicinal capacity. Produced by honeybees, propolis is created as bees collect resins (1), waxes (2), and other botanical exudates from various plant sources (3), blending them with enzymes and beeswax. This complex mixture serves essential roles in the hive, such as acting as a sealant to protect against drafts (4), maintaining hive hygiene (5), and defending against invading pathogens .Beyond its structural and protective functions within the hive, propolis has been recognized for centuries for its potential health benefits (6). Traditional medicine systems worldwide have employed propolis for its purported wound healing, antimicrobial, and anti-inflammatory properties (7). Modern scientific investigations have begun to unravel the complex chemical composition of propolis, revealing a rich source of bioactive compounds, including flavonoids, phenolic acids, terpenes, and other phytochemicals (8). The diverse constituents of propolis gives it a wide range of pharmacological properties, making it a promising source for the development of novel therapeutic agents (9). One particularly intriguing component of propolis is the flavonoid compound pinocembrin (10). While the composition of propolis varies depending on geographical origin (11) and plant sources, its consistent presence in beehives worldwide highlights its essential role in bee health and its potential for unlocking valuable therapeutic applications for human health.

Diabetic retinopathy (DR), a common microvascular complication associated with diabetes mellitus, significantly impairs the quality of life for millions around the world. Diabetic retinopathy (DR) is characterized by the gradual deterioration of retinal blood vessels, which can result in visual impairment and potentially lead to blindness if not properly managed (12). The development of DR is multifaceted, with persistent hyperglycaemia serving as a primary driver that initiates a cascade of pathological processes, including inflammation, oxidative stress, and increased vascular permeability. Increased levels of pro-inflammatory cytokines, including Interleukin-1, Interleukin-8, and Tumor Necrosis Factor-alpha, have been associated with the development and advancement of DR (13). These pro-inflammatory cytokines contribute to vascular endothelial dysfunction, increased vascular permeability and abnormal retinal angiogenesis, ultimately resulting in retinal damage and visual impairment (14).

Naturally-derived plant compounds have received significant interest as potential therapeutic agents for various disease, including DR. Propolis, a sticky substance gathered by bees from a variety of plant sources, has been widely recognized for its extensive pharmacological properties (7), including anti-inflammatory, antioxidant, and anti-diabetic effects. Pinocembrin, a major flavonoid compound in propolis (15), has shown promising therapeutic potential in studies for various conditions, including neurological disorders and cardiovascular diseases.

This study aimed to investigate the protective effects of pinocembrin, isolated from Nigerian propolis, on diabetic retinopathy in a streptozotocin-induced diabetic rat model. The study focused on evaluating the impact of pinocembrin treatment on the retinal concentrations of critical inflammatory cytokines in the diabetic rat model.

2. Materials and Methods

2.1 Propolis Extract Preparation

Nigerian propolis samples were collected from Federal University of Abeokuta, Abeokuta (7.1475° N, 3.3619° E) in southern Nigeria, and subjected to a solvent extraction procedure. The propolis samples were first ground into a fine powder using a mechanical grinder. The powdered propolis was then extracted with ethanol under reflux conditions for 4 hours. The crude propolis extract was obtained by filtering the extract and subsequently removing the solvent under reduced pressure.

2.2 Chemicals and Reagents

Streptozotocin (STZ, > 98% purity) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and used to induce experimental diabetes in the animal models. Pinocembrin (> 98% purity) was isolated from Nigerian propolis through a reverse-phase high-performance liquid chromatography purification (HPLC) method. Enzyme-linked immunosorbent assay (ELISA) kits for the quantification of inflammatory cytokines, including Interleukin-1 (IL-1), Interleukin-8 (IL-8), and Tumour Necrosis Factor-alpha (TNF-α) were sourced from Bio-Rad Laboratories and Cayman Chemical Company. All other standard laboratory reagents and consumables were of high analytical quality and obtained from reputable commercial sources and reputable suppliers. Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) assay kits were purchased from Sigma-Aldrich. Blood

glucose levels were assessed using On-Call Plus glucometer from Acon Laboratories, Inc. Glycosylated haemoglobin (HbA1c) concentrations were measured using assay kits from Bio-Rad Laboratories.

2.3 Experimental Animals

The study utilized male Sprague-Dawley rats as the experimental subjects. The animals were maintained in a controlled environment with a 12-hour light/dark cycle, and the temperature and humidity were maintained consistently . The animals were housed under controlled environmental conditions, with free access to standard rodent feed and water. The rats were randomly assigned into four experimental groups: non-diabetic control, diabetic control, diabetic rats treated with pinocembrin, and diabetic rats administered metformin as a positive control.

2.4 Experimental Design

Diabetes was induced in the appropriate animal groups through a one-time intraperitoneal injection of streptozotocin at a dose of 55 mg per kilogram of body weight. Animals with fasting plasma glucose levels greater than 250 mg/dL were considered diabetic and included in the study. The purified pinocembrin fraction was administered orally to the treatment group at a dose of 50 mg/kg daily for 8 weeks, while the non-diabetic and diabetic control groups received vehicle treatment. The metformin group received oral metformin at a dose of 300 mg/kg daily. The metformin treatment group was included as a positive control to assess the effectiveness of pinocembrin in mitigating the progression of diabetic retinopathy. Following the 8-week treatment period, the animals were humanely euthanized, and their retinal tissues were collected for analysis to quantify the levels of inflammatory cytokines.

Retinal tissue samples were homogenized in lysis buffer using a tissue homogenizer. Specifically, 500 µL of lysis buffer was added per 100 mg of tissue along with a 5-mm stainless steel bead. The samples were then placed in the tissue homogenizer and processed at 25 Hz for 1 minute. Following homogenization, the samples were centrifuged at 16,000 × g for 10 minutes at 4°C, and the supernatant was collected for further analysis.

The study evaluated oxidative stress markers, including superoxide dismutase and glutathione peroxidase, in the retinal tissue samples. Additionally, the study protocol was reviewed and approved by the Ahmadu Bello University Zaria animal ethics committee, and all experiments were conducted in compliance with the guidelines for the care and use of laboratory animals.

2.5 HPLC UV-VIS Analysis of Pinocembrin Content in Nigerian Propolis

Pinocembrin was isolated from Nigerian propolis using a reverse-phase high-performance liquid chromatography (RP-HPLC) method.

The mobile phase for HPLC analysis consisted of HPLC-grade methanol and deionized water. HPLC-grade formic acid was incorporated as a modifier in the mobile phase. The propolis extract was introduced into the HPLC system and separated on a C18 column using a binary mobile phase composed of methanol and water. The mobile phase was pumped at a constant rate of 1 mL/min, with sample injections of 10 µL, and the column temperature was maintained at 30°C to optimize the separation performance. Pinocembrin was eluted at a retention time of 17.8 minutes and exhibited a peak absorbance at 290 nm, which was monitored using a UV-Vis detector. The peak area, rather than solely the peak height, was utilized to calculate the concentration of the eluted compounds based on standards (Figure 1 and Table 1).

2.6 Quantification of Cytokines

Levels of inflammatory cytokines, including Interleukin-1, Interleukin-8, and Tumor Necrosis Factor-alpha, were measured in the retinal samples using enzyme-linked immunosorbent assay (ELISA) techniques. The kits used had a minimum detectable concentration below 5 pg/mL for the cytokines and were providedby commercial manufacturers Bio-Rad Laboratories and Cayman Chemical Company.

2.7 Glucose and Glycosylated Haemoglobin (HbA1c) measurement

Measurement of the fasting blood glucose levels employed the glucose oxidase method using the On-Call Plus glucometer from Acon Laboratories, Inc. while glycosylated haemoglobin (HbA1c) concentrations were determined using the ELISA method with assay kits from Bio-Rad Laboratories.

2.8 Assessment of Oxidative Stress

Retinal tissues were analyzed for the activities of antioxidant enzymes, including superoxide dismutase and glutathione peroxidase, using commercial assay kits from Cayman Chemical Company.

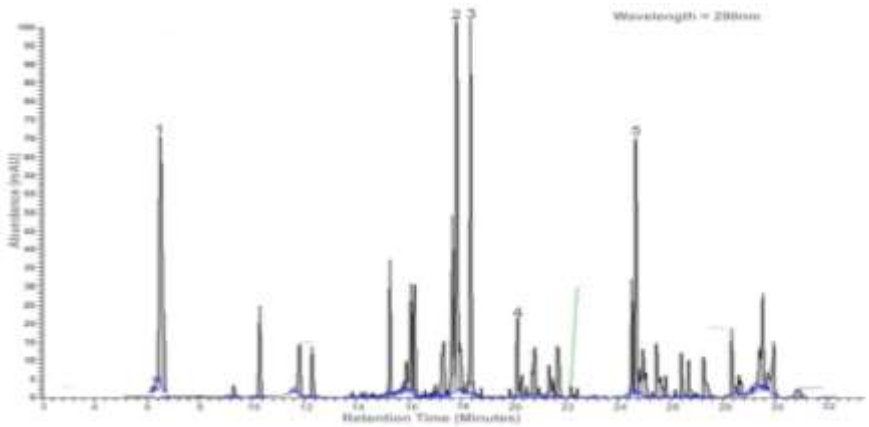

Figure 1. Chromatogram of the isolation of some components of Nigerian propolis showing different peaks. Pinocembrin is the peak numbered 2. Its elution properties are shown in the table below.

Table 1. Showing properties of the constituent compounds isolated from Nigerian propolis using HPLC, and with peaks labelled 1 to 5 in the chromatogram shown in Plate 1 above. Pinocembrin is numbered 3.

	Peak	Height (mAU)	Retention Time	Area	Molecular Formula	Class
1	Gallic acid	71.12	6.5	53154	$C_7H_6O_5$	Phenolic acid
2	Pinocembrin	99.96	17.8	109036	$C_{15}H_{12}O_4$	Flavonoid
3	Chrysin	99.94	18.5	86500	$C_{15}H_{10}O_4$	Flavonoid
4	Piperine	23.08	20.3	30007	$C_{17}H_{19}NO_3$	Alkaloid
5	Glycyrrhizin	73.23	24.6	39012	$C_{42}H_{62}O_{16}$	Saponin

2.9 Statistical Analysis

The data were presented as the mean ± standard error of the mean (SEM). Intergroup comparisons were conducted using one-way and two-way analysis of variance, followed by Tukey's post-hoc test. A statistical significance threshold of $p < 0.05$ was established.

3. Results

3.1. Effect of Pinocembrin on Glycaemic Control

Induction of diabetes through streptozotocin administration resulted in a significant elevation in fasting blood glucose levels and glycosylated haemoglobin (HbA1c) concentrations in the DR untreated group compared to the non-diabetic control. Treatment with pinocembrin significantly reduced both fasting blood glucose and HbA1c levels compared to the DR untreated group (Figure 2). The effects of pinocembrin on glycemic parameters were comparable to those observed in the metformin-treated group.

3.2 Effect of Pinocembrin on Proinflammatory Cytokines in Diabetic Retinopathy

In Figure 3, Retinal tissue analysis revealed significantly elevated levels of inflammatory cytokines, including IL-1, IL-8, and TNF-α, to levels indicative of diabetic retinopathy (DR) in the diabetic (untreated) control group compared to non-diabetic controls. Treatment with pinocembrin significantly reduced the levels of these proinflammatory cytokines in the diabetic rats. The metformin-treated group showed a greater reduction in the levels of IL-1 and TNF-α but a lesser reduction of IL-8 than the pinocembrin-treated group.

3.3 Effect of Pinocembrin on Oxidative Stress in Diabetic Retinopathy

The retinal tissues of diabetic control rats exhibited significantly diminished activities of the antioxidant enzymes superoxide dismutase and glutathione peroxidase compared to non-diabetic controls, suggesting elevated oxidative stress. Administration of Pinocembrin effectively restored the activities of these antioxidant enzymes, thereby mitigating oxidative stress in the retina (Figure 4).

4. Discussion

Studies have demonstrated the therapeutic properties of propolis samples from around the world, highlighting the need to standardize these samples by isolating their active constituents. Our previous studies have shown the anti-hyperglycaemic and antioxidative effects of crude extract of Nigerian propolis (16). GC-MS analysis of Nigerian propolis revealed that it contained flavonoids, alkaloids,

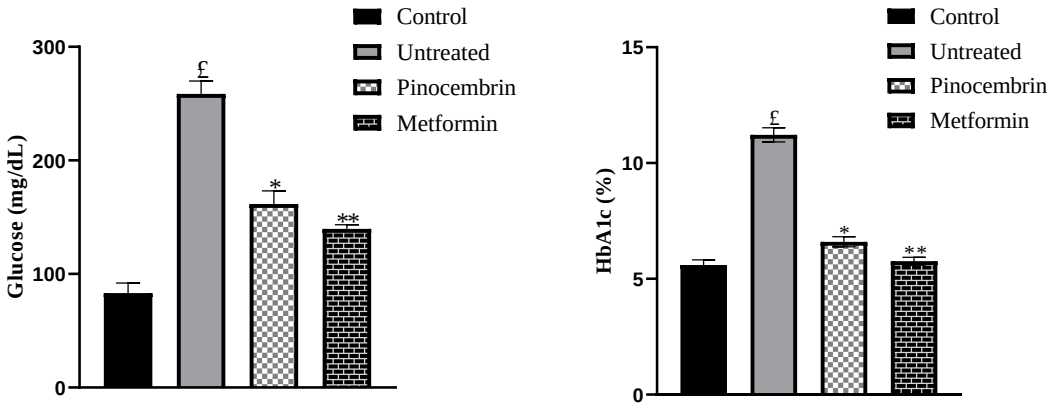

Figure 2. Effect of Pinocembrin on Glycaemic Control. The data were presented as the mean ± SEM. Intergroup comparisons were conducted using one-way analysis of variance, followed by Tukey's post-hoc test with $p < 0.05$ taken as the level of statistical significance. $^{(£)}$ $p < 0.001$ compared with the Control; $^{(*)}$ $p < 0.05$ compared with the DR Untreated; $^{(**)}$ $p < 0.001$ compared with the DR Untreated.

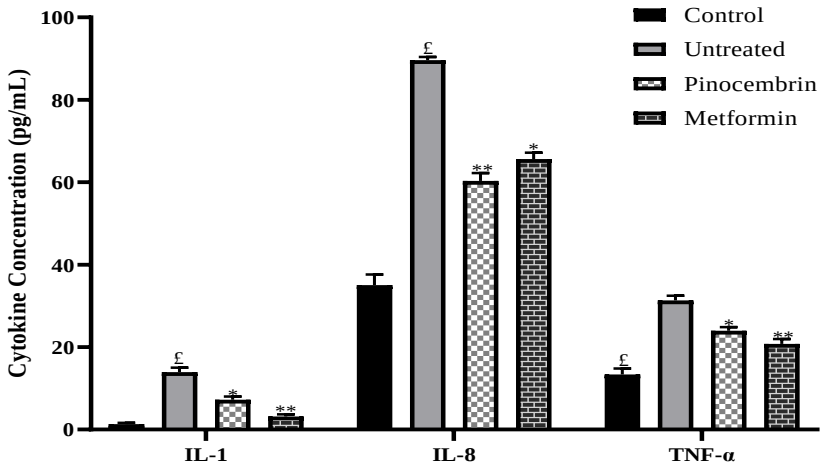

Figure 3. Effect of Pinocembrin on Proinflammatory Cytokines in Diabetic Retinopathy. The data were presented as the mean ± SEM. Intergroup comparisons were conducted using two-way analysis of variance, followed by Tukey's post-hoc test with $p < 0.05$ taken as the level of statistical significance. $^{(£)}$ $p < 0.001$ compared with the Control; $^{(*)}$ $p < 0.05$ compared with the untreated; $^{(**)}$ $p < 0.001$ compared with the untreated.

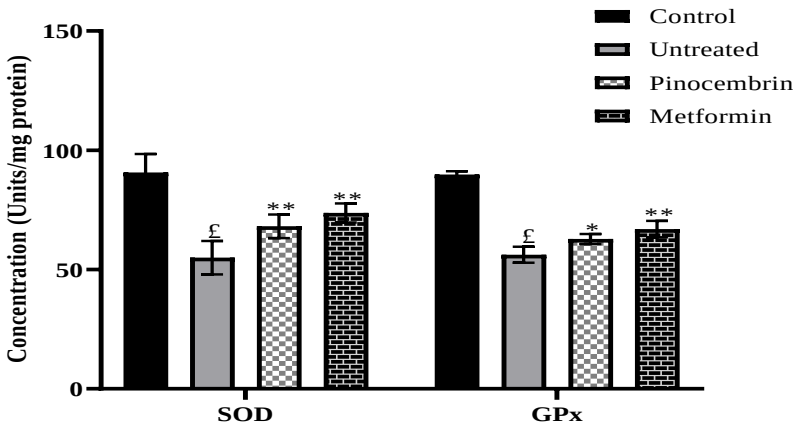

Figure 4. Effect of Pinocembrin on Oxidative Stress in Diabetic Retinopathy. The data were presented as the mean ± SEM. Intergroup comparisons were conducted using one-way analysis of variance, followed by Tukey's post-hoc test with $p < 0.05$ taken as the level of statistical significance. $^{(£)}$ $p < 0.001$ compared with the Control; $^{(*)}$ $p < 0.05$ compared with the untreated; $^{(**)}$ $p < 0.001$ compared with the untreated.

steroids, glycosides, saponins, tannins, phlobatannins and phenolic compounds (16). Hence, in the present study, pinocembrin was isolated from the Nigerian propolis and found to inhibit the progression of diabetic retinopathy in a rat model of diabetes mellitus.

Existing research has highlighted the critical involvement of inflammatory factors, including the cytokines evaluated in this investigation, in the development and progression of diabetic retinopathy (17). Elevated levels of these cytokines promote vascular endothelial dysfunction, heightened permeability, retinal neovascularization, and culminating in retinal injury and vision impairment (18). This is particularly true for Interlukin-8 (18), which was found in this study to be greatly elevated in diabetes. Though previous studies have demonstrated that pinocembrin from various sources can modulate multiple inflammatory pathways, including the inhibition of nuclear factor-kappa B signalling and the suppression of proinflammatory cytokine production, which may contribute to its diverse therapeutic potential (19) and its versatility in therapeutic properties (20), the present study investigated the effect of pinocembrin isolated from Nigerian propolis on inflammation-induced retinal damage in a diabetic rat model. The results of the study demonstrated that pinocembrin treatment effectively mitigated the elevated levels of key inflammatory cytokines, such as Tumour Necrosis Factor-alpha, Interleukin-1 and more drastically Interleukin-8 in the retinal tissues of the diabetic rats. Interestingly, the levels of these inflammatory mediators were almost equally low in the pinocembrin-treated group, and even lower for Interleukin-8, compared to the positive control (metformin-treated) group. These findings suggest that pinocembrin, a flavonoid compound isolated from Nigerian propolis, possesses potent anti-inflammatory properties that may have the potential to attenuate the development and progression of diabetic retinopathy, a vision-threatening complication of diabetes mellitus.

Additionally, pinocembrin significantly lowered the blood glucose and glycated haemoglobin (HbA1c) levels in the diabetic rats, either through enhancement of insulin secretion or increased glucose uptake in peripheral tissues, which may further contribute to its retinal protective effects (20). Although its anti-inflammatory effect in the retina may also be independent of its general anti-hyperglycaemic effect (21), since the metformin-treated group had lower blood glucose levels than the pinocembrin-treated group but showed a somewhat equal effect on the inflammatory cytokine levels.Furthermore, this study revealed that pinocembrin treatment effectively restored the activities of crucial antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase, within the retinal tissues of the diabetic rats. This suggests that pinocembrin derived from Nigerian propolis also possesses the capacity to alleviate oxidative stress, similar to crude Brazillian propolis, Poplar type propolis, and Red propolis type (22). Thus, pinocembrin obtained from the Nigerian propolis mitigates oxidative stress, which is another major driver of the pathological processes underlying diabetic retinopathy by enhancing the body's natural antioxidant defences.

The findings of this study are further supported by the growing body of evidence on the pharmacological effects of propolis and its bioactive constituents (23). Propolis, a sticky material gathered by bees from various plant sources, has long been recognized for its diverse medicinal properties (7), including anti-inflammatory, antioxidant, and anti-diabetic activities (24). The identification of pinocembrin as a potent compound within Nigerian propolis underscores the importance of continued exploration of natural products as potential sources of novel therapeutic agents for the management of complex, multifactorial diseases like diabetic retinopathy.

The study demonstrates the therapeutic potential of pinocembrin, a compound from Nigerian propolis, in mitigating diabetic retinopathy. Pinocembrin reduced key inflammatory cytokines in the retinas of diabetic rats, suggesting its potent anti-inflammatory properties. These findings, along with the known benefits of propolis, highlight the importance of continued research into natural compounds as treatments for complex diseases like diabetic retinopathy. Further studies are needed to clarify more mechanisms and evaluate the clinical applications of pinocembrin.

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Authors' Contribution

Study concept and design: MI. O.

Acquisition of data: MI. O, J. L.

Analysis and interpretation of data: MI. O.
Drafting of the manuscript: J. L.
Critical revision of the manuscript for important intellectual content: J. L, Y. T.
Statistical analysis: MI. O, Y. T, J. L.
Administrative, technical, and material support: Y. T.

Ethics

On behalf of all the co-authors, I hereby confirm that I have reviewed and complied with the relevant instructions to Authors, the Ethics in Publishing policy, and the Conflicts of Interest disclosure.

Conflict of Interest

No conflict of interest declared.

Data Availability

All data are available on demand.

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