



Yellow lupine extract modulates diabetes-induced extracellular matrix changes in ocular tissue

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Abstract

The relationship between diabetes and ocular extracellular matrix (ECM) turnover and the potential beneficial effects of yellow lupine extract (YLE) require further investigation. The main aim of this study was to investigate the involvement of diabetes in ocular ECM remodelling and the effect of YLE. Animal treatment included a diabetes group induced in rats by alloxan (250 mg/kg, ip) and normal and diabetic rats receiving 100 mg/kg YLE for 1 month. Collagen, fibronectin and laminin were measured in the samples using ELISA kits, and collagenase activity was measured using a fluorometric kit. Total gelatinase activity was measured in ocular homogenate samples. Ocular vessels were assessed by fundus angiography. Results showed that diabetes caused a significant increase in total collagen and fibronectin content by 83% ($p < 0.01$) and 88% ($p < 0.05$), respectively, compared to the control group. Treatment with YLE resulted in a decrease in total collagen and laminin content by 10% and 5%, respectively ($p < 0.05$). There was also a significant 50% increase in total gelatinase activity in the diabetic group compared to the control group ($p < 0.05$). These findings demonstrate that diabetes alters the composition of the ocular ECM and suggest that YLE may be a potential therapeutic agent for the prevention of diabetic retinopathy.

Keywords Extracellular matrix · MMP · Eyes · Diabetes · Yellow lupine

Introduction

Diabetes is a chronic metabolic disorder that is believed to be the most rapidly emerging health challenge (Akhter 2021). It is characterised by elevated blood glucose levels resulting from either the body's inability to produce insulin or insulin resistance. Prolonged hyperglycaemia can result in a range of complications, including damage to various organs, such as the eyes (Pont et al. 2020). Diabetic Retinopathy is a complication of diabetes that affects the blood vessels in the retina, the light-sensitive tissue at the back of the eye. The mechanism of DR is complex and involves multiple pathways, but can be broadly divided into two main

stages: the non-proliferative stage and the proliferative stage (Nian et al. 2021).

The underlying mechanisms that contribute to the development of DR include chronic hyperglycemia (high blood sugar), oxidative stress, inflammation, and dysfunction of the cells lining the blood vessels (endothelial cells) (Sahajpal et al. 2019; Kang and Yang 2020).

In the non-proliferative stage, diabetes causes damage to the small blood vessels (capillaries) that supply the retina with oxygen and nutrients. This damage can result in increased permeability of the blood vessels, which may subsequently lead to leakage of fluid and blood into the retina (Cunha-Vaz 2017; Mrugacz et al. 2021). This can result in retinal swelling and thickening, which may lead to visual impairment. Furthermore, the compromised blood vessels may also become obstructed, resulting in regions of the retina being deprived of oxygen (ischaemia) (Kusuhara et al. 2018).

The ECM is a complex network of proteins, glycoproteins, and proteoglycans that provides structural and functional support to tissues and organs. In the eye, the ECM plays a pivotal role in maintaining the structural integrity of various ocular tissues, including the cornea,

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sclera, retina, and lens (Qiu et al. 2020; Pouw et al. 2021). The cornea, the transparent outermost layer of the eye, is composed of several layers of specialized cells and a dense ECM composed of collagen fibrils, proteoglycans, and glycoproteins. The ECM provides the cornea with its mechanical strength and contributes to its transparency, which is essential for maintaining clear vision (Puri et al. 2020).

The sclera, the white outer layer of the eye, is composed of a layer of collagen fibrils embedded in a proteoglycan-rich ECM. The sclera provides the eye with structural support, thereby maintaining its shape (Boote et al. 2020). The ECM of the retina provide structural support and help maintain the integrity of the blood-retina barrier (Ebnetter and Zinkernagel 2016).

Herbal medicines have been described as having promising antioxidant, anti-inflammatory and hypoglycaemic properties. These plants contain bioactive compounds that help manage diabetes by reducing oxidative stress, inflammation and blood glucose levels, making them valuable alternatives in the treatment of diabetes (Byahut and Bag 2022; Ejiofor et al. 2023).

A number of plant extracts and compounds have been the subject of investigation with a view to determining their potential for the treatment of DR. For example, extracts from the plant *Salvia miltiorrhiza* have been demonstrated to possess anti-inflammatory and antioxidant properties, and have been investigated for their potential in the treatment of diabetes and DR (Jia et al. 2019; Gaonkar and Hullatti 2020). Furthermore, the flavonoids quercetin and resveratrol, which are present in numerous plant-based foods, have been demonstrated to exert beneficial effects on the microvascular complications of diabetes, including DR. (Rossino and Casini 2019).

Lupinus luteus is an annual herbaceous plant that can attain a height of 30 to 50 cm (Parra-González et al. 2012). Lupine is characterised by the presence of high levels of indigestible carbohydrates and fibre. Lupine constituents have been employed in a multitude of applications, including the production of food (for example, as a substitute for animal protein), chemicals (such as in the manufacture of dyes, photographic plates and cinematographic films) and pharmaceuticals (for instance, in the production of peptone, albuminates, iron, iodine and lecithins) (van de Noort 2017).

The main objective of this research study is to investigate the relationship between diabetes and the ECM remodelling of the eyes. The objective of this study is twofold: firstly, to investigate the extent to which diabetes may be implicated in the remodelling of ECM components within the eye; secondly, to evaluate the potential benefits that may be derived from the use of *Lupinus luteus* extract, which is known for its rich antioxidant and anti-inflammatory properties.

Materials and methods

Preparation of yellow lupine extracts

Lupinus luteus seeds were purchased from a local herbal shop in Medenine (Tunisia) and identified by Dr Mouldi Gamoun, a botanist at the International Center for Agricultural Research in the Dry Areas (ICARDA). Extract was prepared as previously described (Sayari et al. 2022). Briefly, seeds were finely ground and extraction was carried out using 50% ethanol as solvent. A total of 15 g of the resulting powder was macerated in 100 ml of solvent under continuous stirring for 24 h at room temperature, protected from light. The mixture was then filtered to remove solid residues and the solvent was evaporated using a rotary evaporator. Finally, the extracts obtained were weighed to determine the extraction yield.

Animal treatment

Male Wistar rats were purchased from the Faculty of Sciences of Gabes, Tunisia and maintained at the same conditions of humidity (70%), temperature (23 °C) and 12-h light/dark cycle and fed commercial pellet diet and tap water ad libitum.

Rats were randomly assigned to specific protocol groups:

Diabetes mellitus (DM) Group (n = 7): diabetic rats that received intraperitoneal injection of saline solution (0.9%) for 1 month.

YLE Group (n = 7): normal rats that received intraperitoneal injection of YLE (100 mg/kg) diluted in saline solution (0.9%) for 1 month.

DM+YLE Group (n = 6): diabetic rats treated with YLE (100 mg/kg) diluted in saline solution (0.9%) for 1 month.

CTRL group: Normal rat group (n = 8) has received an intraperitoneal injection of saline solution (0.9%) for 1 month.

Diabetes was induced by a single dose of alloxan (250 mg/kg, ip) dissolved in saline solution (0.9% NaCl) as previously described (Sayari et al. 2022).

At the end of the study, rats were sacrificed by overdose of urethane in strict accordance with Institutional Animal Care and Use Committee guidelines. Urethane was chosen for its efficacy in producing a deep, long-lasting anaesthesia suitable for terminal procedures, ensuring a humane and painless sacrifice. The recommended dose of 1.5 g/kg IP, a dose known to be lethal, while maintaining stable anaesthesia without the need for supplementation. Adequate anaesthesia was confirmed by loss of muscle tone, palpebral reflex and absence of response to toe, tail or abdominal pinch before proceeding.



Blood samples were collected in serum vacutainers, centrifuged at 4200 g for 15 min at 4 °C and stored at – 80 °C until analysis. Eyes were removed, frozen and stored at – 80 °C.

Ocular angiography

The treated and control rats were examined by ocular angiography. This technique consists of taking images of the ocular fundus after injection of a contrast agent (sodium fluorescein 5% in saline (0.9% NaCl)). Angiography was performed at the Ophthalmology Department of the Habib Bourguiba University Hospital in Medenine.

Our experimental approach included:

- Application of 0.01% tropicamide eye drops to dilate the pupil and facilitate examination of the fundus.
- Intraperitoneal injection of 1 mL of sodium fluorescein 5% and examination of the fundus using a Canon CF-1 mydriatic fundus camera with a Canon 400 digital SLR camera.

Extraction of total proteins

Eyes from treated and control rats were homogenised in lysis buffer (0.25 M sucrose, 0.05 M Tris-HCL, 1 M EDTA, pH 7.4). After 24 h incubation at – 20 °C, samples were centrifuged at 12,000 rpm for 15 min. The supernatant was removed and stored at – 60 °C until use.

Total protein was assayed by the Bradford method by mixing 20 µl of each sample with 3 ml of Bradford reagent. The tubes were gently vortexed and incubated for 5 min at room temperature. The absorbance is measured at 595 nm.

Assay of total collagen, fibronectin and laminin

ELISA kits from BioVision (K218-100), Abcam (ab108850) and LifeSpan (LS-F6465) were used to quantify collagen, fibronectin and laminin, respectively, according to the manufacturers' protocols. For collagen, tissue samples were homogenized in ddH₂O (100 µl per 10 mg tissue), hydrolyzed with an equal volume of 12 M HCl at 120 °C for 3 h, clarified with activated charcoal and centrifuged at 10,000×g for 3 min. Hydrolyzed samples (2–30 µl) were evaporated to dryness at 70 °C prior to ELISA analysis. For fibronectin, tissue samples were extracted in 0.1 M phosphate-buffered saline (pH 7.4) with 1% Triton X-100, centrifuged at 14,000×g for 20 min, the supernatant was collected, the protein concentration measured and samples diluted as required. For laminin, reagents and samples were equilibrated to room temperature and 100 µL of each sample was incubated at 37 °C for 1 h, followed by washing, ELISA detection and measurement at 450 nm.

Assay of matrix metalloproteinase activity

The measurement of collagenase activity was carried out using a Biovision kit as per the manufacturer's guidelines. In brief, 2 µl of total protein extract (100 µg/µl) was added to 98 µl of collagenase buffer, followed by the addition of 40 µl of FALGPA and 60 µl of collagenase buffer. The reaction mixture was shaken, and the absorbance at 345 nm was measured between 5 and 15 min.

For gelatinase activity, the Gelatinase (Fluorometric) Assay Kit (Bio Vision, Cat. No. K444-100) and as previously reported (Chu et al. 2019) and according to the manufacturer's instructions. 50 µg of supernatant and 50 µl of purified enzyme were added to a 96-well plate. Additionally, a positive control was prepared by adding 2 µl of Enzyme Positive Control to 18 µl of Gelatinase Assay Buffer.

Statistical analysis

Statistical analyses were conducted using the Statistica software (StatSoft France). A one-way ANOVA was performed to compare group means, followed by Tukey's post hoc test to identify specific group differences. Normality of the data was verified using the Shapiro–Wilk test, and homogeneity of variance was confirmed with Levene's test. Results are presented as mean values with standard error of the mean ($\pm$ SEM). Differences were considered statistically significant at $p < 0.05$.

Results

Evolution of ECM level

ECM proteins, including total collagen, laminin, and fibronectin, were quantified in eye homogenate samples to investigate the effect of diabetes and yellow lupin extract treatment on ECM composition. Our results showed a significant increase in the content of total collagen and fibronectin in the diabetic group compared to the control group, by 83% and 88%, respectively ($p < 0.01$ for both). In contrast, treatment with yellow lupin extract resulted in a decrease in total collagen and laminin content of 10% and 5%, respectively, in comparison to the diabetic group (Fig. 1).

Evolution MMP activities

The activity of collagenases and gelatinases, including MMP-1, MMP-8, and MMP-13, was evaluated in eye homogenate samples to investigate the effect of diabetes and YLE treatment on matrix metalloproteinase (MMP) activity. The findings demonstrated a notable elevation in total gelatinase activity within the diabetic group, by 50% ($p < 0.05$).

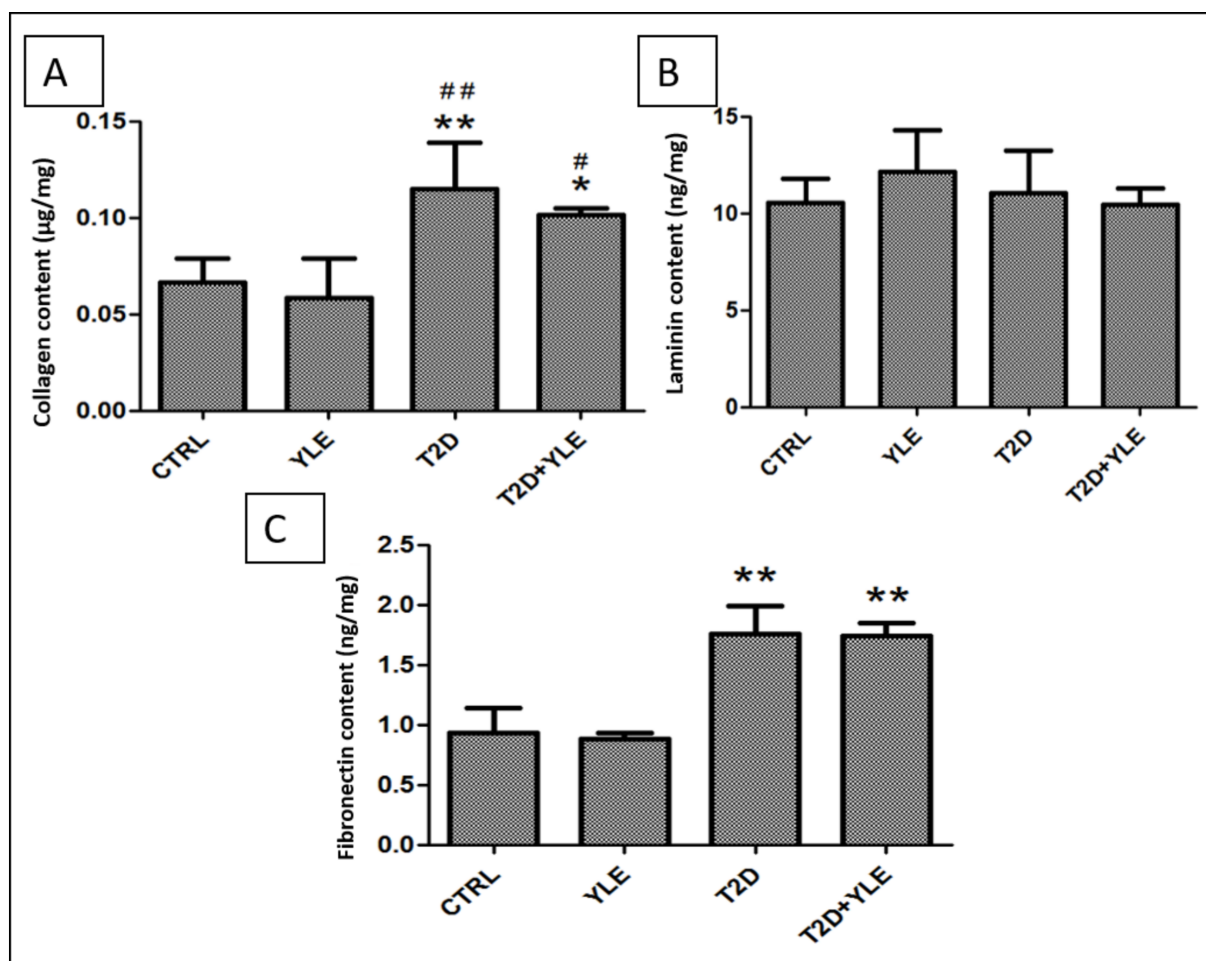


Fig. 1 Variation of the content of total collagen (µg/ml) (A), laminin (ng/mg) (B) and fibronectin (ng/mg) in the eyes of different groups of control and treated rats. The results are expressed as mean $\pm$ standard deviation. * $p < 0.05$, ** $p < 0.01$ vs. CTRL group; # $p < 0.05$, # $p < 0.01$

vs. YLE group. CTRL: control rats; YLE: control rats treated with 100 mg/kg yellow lupine extract; DM: diabetic rats by injection of alloxan 250 mg/kg, ip; DM+YLE: diabetic rats treated with 100 mg/kg ethanol–water extract (50:50) of yellow lupine for 1 month

However, treatment with YLE led to a slight increase of 9% in total gelatinase activity compared to the diabetic group (Fig. 2). In contrast, no significant difference in collagenase activity was observed among the groups of rats.

Ocular angiography

A fundus examination by ocular angiography was performed in control and diabetic rats to investigate the effect of diabetes on retinal blood vessels and perfusion. In control animals, the fundus blood vessels appeared straight and non-dilated, with no signs of ischaemia or perivascular hyperfluorescence following contrast agent injection. In contrast, diabetic rats exhibited significant vascular changes, including dilation of veins and areas of perivascular hyperfluorescence due to the diffusion of fluorescein into the surrounding tissue. Furthermore, signs of ischaemia and poor perfusion were

evident, particularly around the optic nerve, as dark areas on the angiographic images following contrast infusion (Fig. 3).

Discussion

This study aimed to explore the effects of diabetes and YLE treatment on ocular health, specifically on the composition of the ECM and MMP, as well as retinal blood vessels. Our main results showed that the diabetic group exhibited a significant increase in total collagen and fibronectin levels, as well as total gelatinase activity, compared to the control group. However, treatment with YLE resulted in a decrease in total collagen and laminin levels and a slight increase in total gelatinase activity compared to the diabetic group. In other hand, ocular angiography revealed significant vascular changes and signs of ischaemia and poor perfusion, especially around the optic nerve, in diabetic rats.



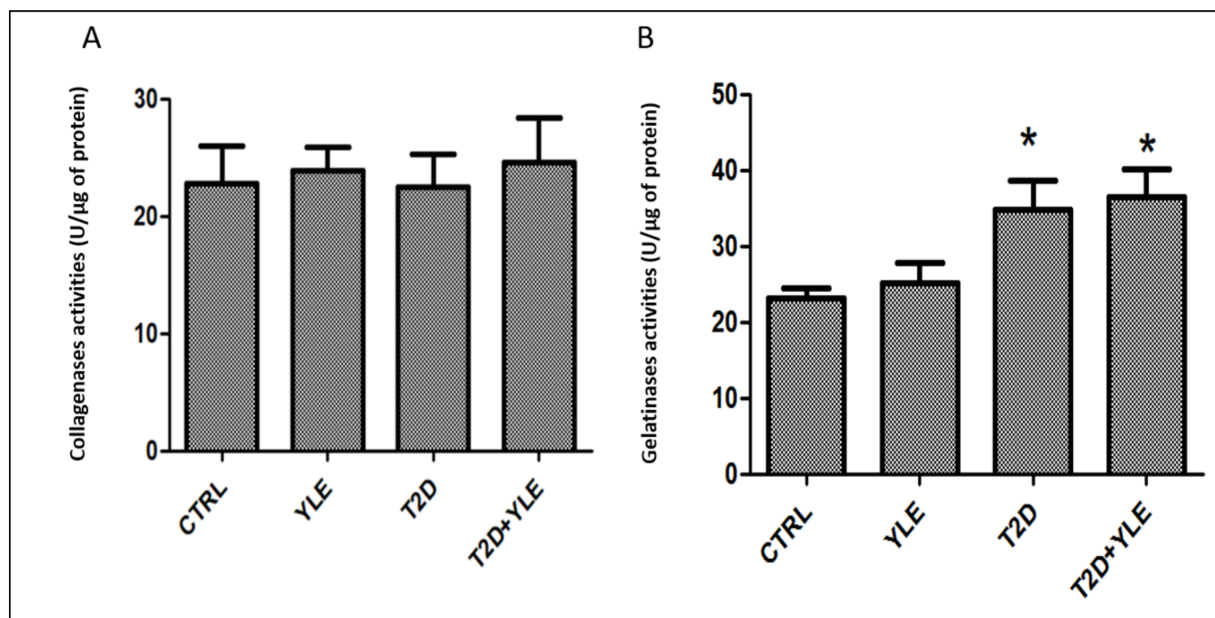


Fig. 2 Variation of the activity of collagenases (MMP-1, MMP-8, MMP-13) in U/μg (A) and the activity of gelatinases (MMP-2 and MMP-9) (B) at the level of the eye from the different treated and control rats. Results are expressed as mean $\pm$ standard deviation. CTRL:

control rats; YLE: control rats treated with 100 mg/kg yellow lupine extract; DM: diabetic rats by injection of alloxan 250 mg/kg, ip; DM+YLE: diabetic rats treated with 100 mg/kg ethanol–water extract (50:50) of yellow lupine for 1 month

The dose of YLE (100 mg/kg) was chosen based on evidence from previous studies showing significant biological activity at this concentration (Farghaly and Hassan 2012). This dose has been widely used in similar experimental models, demonstrating efficacy without inducing toxicity. Furthermore, our own previous studies in liver and kidney tissues (Sayari et al. 2022) confirmed that the 100 mg/kg dose produced relevant and promising results, further justifying its application in the present study. These findings reinforce the appropriateness of this dose level for assessing the potential effects of YLE under the current experimental conditions.

DR is one of the most common complications of diabetes mellitus and a leading cause of blindness worldwide. DR is characterized by changes in the retinal vasculature, which ultimately leads to impaired blood flow, hypoxia, and ischemia, resulting in visual loss (Trott et al. 2022; Kropp et al. 2023).

The ECM provides a scaffold for the structural organization of tissues and plays a crucial role in cell signalling and tissue homeostasis. Changes in ECM composition have been reported in various diseases, including DR (Resnikoff et al. 2022). In our study, we observed a significant increase in total collagen and fibronectin content in the diabetic group compared to the control group. These findings are consistent with previous studies reporting increased collagen deposition in the retina of diabetic animals (Hachana et al. 2020). Fibronectin is a glycoprotein that plays a role

in cell adhesion, migration, and differentiation. Increased fibronectin expression has been reported in diabetic retinas, and its accumulation has been associated with the development of retinal vascular dysfunction (Zhang and Kong 2020). Similarly, previous studies have reported an increase in total collagen content in the retinas of diabetic rats, which was associated with the development of basement membrane thickening and capillary occlusion (Roy and Kim 2021).

In contrast, treatment with yellow lupin extract resulted in a decrease in total collagen and laminin content compared to the diabetic group. Laminin is a glycoprotein that plays a role in the formation and maintenance of the basement membrane. Its dysregulation has been implicated in the pathogenesis of DR (Resnikoff et al. 2022). Our findings suggest that YLE may have a beneficial effect on ECM composition in the diabetic retina. In this context, previously we demonstrate that lupine extract can prevent collagenases activities in diabetes rats and provide an anti-hyperglycemic effect and improve lipid parameters level in kidney and liver of diabetic rats (Sayari et al. 2022).

Plant consumption has been associated with numerous health benefits, including the potential to improve eye health. Many plant-based foods contain antioxidants, anti-inflammatory compounds, and other bioactive molecules that may help protect the eye from damage and reduce the risk of certain eye diseases (Sharma et al. 2022; Mulpuri et al. 2023).

Previous investigations reported the increasing interest in natural products for pharmacotherapy due to their

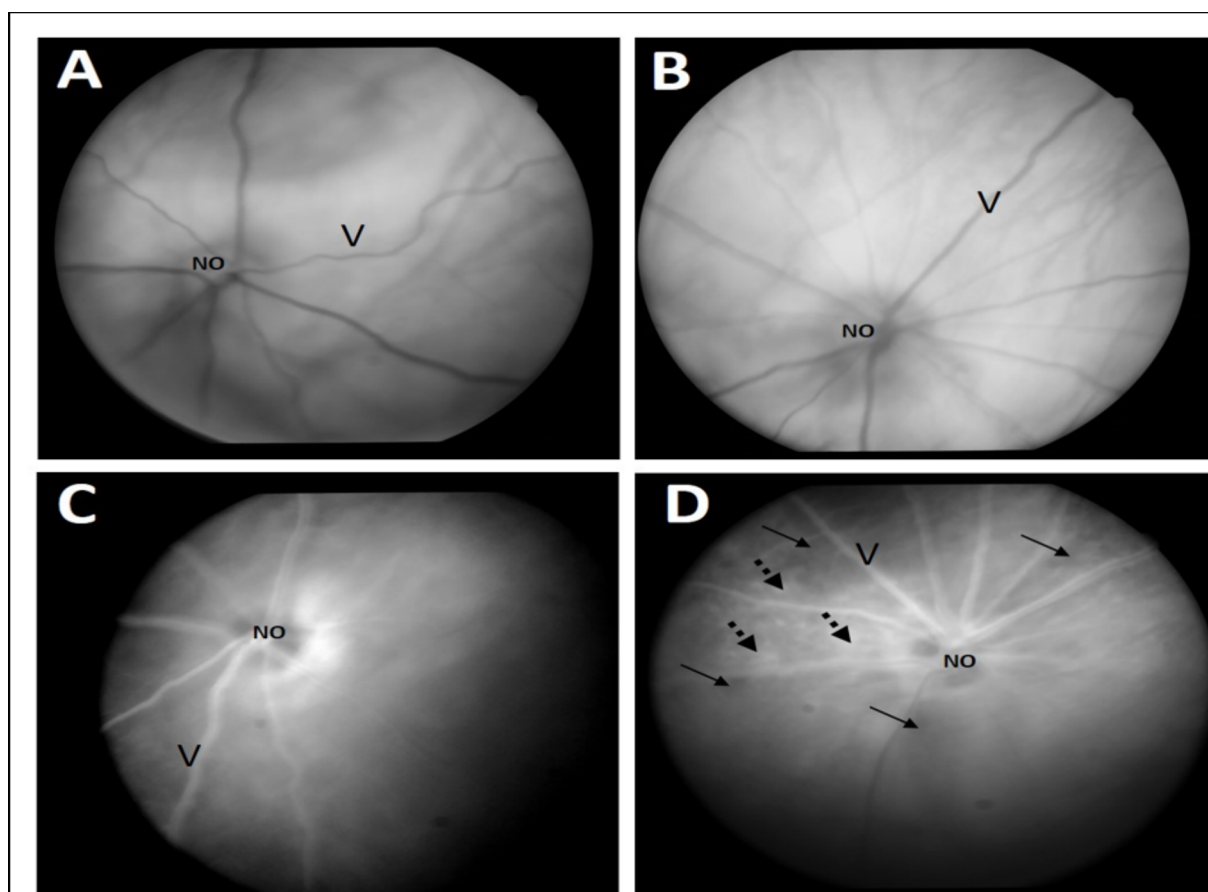


Fig. 3 Fundus fluorescein angiogram of control rats (**A**), rats treated with 100 mg/kg yellow lupine extract (**B**), diabetic rats by injection of alloxan 250 mg/kg (**C**) and diabetic rats treated with 100 mg/kg etha-

nol–water extract (50:50) of yellow lupine for 1 month (**D**). NO: optic nerve, V: vein, solid arrow: areas of ischemia, dashed arrow: perivascular diffusion of the contrast product. $n=4$ for each group

chemical diversity and highlights the potential of traditional herbs, such as *Pinus pinaster*, *Azadirachta indica*, *Anisodus tanguticus*, *Salvia miltiorrhiza*, *Stephania tetrandra*, and *Gymnema sylvestre*, for managing and potentially reversing DR-related pathogenesis (Singh et al. 2020). One class of bioactive compounds found in many plant-based foods is carotenoids. These are pigments that give fruits and vegetables their vibrant colors, and they have been shown to have a protective effect on eye health. For example, lutein and zeaxanthine, two carotenoids found in high concentrations in leafy green vegetables like spinach and kale, have been associated with a reduced risk of age-related macular degeneration (AMD) and cataracts (Mrowicka et al. 2022). In this context, we demonstrate in previous study that LC–MS analysis of ethanol 50% extract of yellow lupine contains phenolic acids such as gallic acid, quinic acid and ferulic acid in addition to flavonoids such as apigenin, quercetin, naringin, apigenin and acacetin (Sayari et al. 2022). Moreover, plant-based compounds, such as polyphenols, have been found to have potential benefits for eye health. Flavonoids and anthocyanins are two types of polyphenols that possess

antioxidant and anti-inflammatory properties which improve night vision and prevent eye fatigue (Detaram et al. 2021).

Our study found a significant increase in total gelatinase activity in the diabetic group compared to the control group, consistent with previous reports of increased MMP activity in DR (Kowluru and Mishra 2017). Gelatinase activity is known to play a role in the breakdown of the blood-retina barrier and the development of retinal neovascularization (Opdenakker and Abu El-Asrar 2019). Interestingly, treatment with YLE resulted in a slight increase in total gelatinase activity compared to the diabetic group. While this finding could suggest a potential pro-angiogenic effect of YLE on the retina, it may also indicate a remodeling process that supports retinal repair, as gelatinases are involved in extracellular matrix turnover and tissue regeneration. However, given the limited studies on gelatinase activity in the context of YLE and retinal health, further research is necessary to confirm whether this increase reflects a beneficial remodeling process or a pro-angiogenic effect, as well as to explore the underlying molecular mechanisms. In contrast, we did not observe any significant difference in collagenase

activity among the groups of rats. Previously it has been reported that patients with proliferative DR had higher levels of MMP-14, VEGF, and MMP-9 compared to non-diabetic controls and that MMP-14 expression was observed in various cells of the epiretinal membranes and was found to be associated with increased blood vessel formation in rats with induced diabetes (Opdenakker and Abu El-Asrar 2019).

Several studies have reported a positive correlation between oxidative stress, cytokines, and MMP expression in DR. For instance, previous study found that high glucose levels can increase oxidative stress and upregulate the expression of MMP-9 and TNF- α in retinal endothelial cells, which can lead to endothelial dysfunction and retinal inflammation (Zhu et al. 2017). Moreover, various MMPs are upregulated, including MMP-14 and MMP-9, which can further promote inflammation and angiogenesis in the retina (Ünal et al. 2022). Moreover, recent studies have shown that mitochondrial damage and the production of inflammatory mediators are early indicators of DR, preceding histological changes in the retina (Opdenakker and Abu El-Asrar 2019). In the context of diabetes, increased oxidative stress initiates the NF- κ B/MMP-9 signalling pathway, which is critical for the development and progression of DR. Oxidative stress-induced MMP-9 has been shown to contribute to retinal vascular dysfunction, including breakdown of the blood-retinal barrier and degradation of tight junction proteins, leading to increased vascular permeability and neovascularization (Caban et al. 2022). Interestingly, bioflavonoids, which may have similar properties to YLE, have been shown to inhibit MMP-9 activity and suppress NF- κ B-mediated inflammation, suggesting a potential therapeutic avenue for the treatment of DR. Furthermore, MMP-1 and MMP-3 have been reported to be highly inducible by oxidative stress in the retinal pigment epithelium, a critical cell type in retinal health (Sorrentino et al. 2018). These MMPs play an important role in ECM turnover and their dysregulated activity may contribute to retinal damage and progression of DR. It is conceivable that YLE may act through similar oxidative stress and inflammation-mediated pathways to influence MMP activity, ultimately affecting ECM remodelling and retinal health (Alge-Priglinger et al. 2009).

Angiography is a valuable tool for evaluating retinal vascular changes in diabetic retinopathy (DR). In our study, we performed fundus examination by ocular angiography to investigate the effect of diabetes on retinal blood vessels and perfusion. We observed significant vascular changes in the diabetic group, including dilation of veins and areas of perivascular hyperfluorescence, which were indicative of fluid leakage into the surrounding tissue due to the disruption of the endothelial barrier. This phenomenon of hyperfluorescence is often associated with inflammation-induced leakage, where vascular permeability is increased, allowing fluorescein to diffuse beyond the vessels and into the

surrounding tissues. Furthermore, signs of ischemia and poor perfusion were evident, particularly around the optic nerve, as dark areas on the angiographic images following contrast infusion. These findings are consistent with previous studies reporting alterations in retinal blood flow and vascular permeability in diabetic animals (Rudraraju et al. 2020). Moreover, our findings demonstrated that diabetes induced significant changes in retinal vasculature, including dilation of veins and perivascular hyperfluorescence. These results are in line with previous studies, which have reported that diabetes causes retinal vascular dysfunction and morphological changes, leading to retinopathy (Sorrentino et al. 2018). This vascular leakage is supported by recent insights into the mechanisms of endothelial dysfunction, where an increase in endothelial permeability plays a central role in vascular leakage. Despite substantial advances in understanding these mechanisms, effective pharmacological treatments to counteract vascular leakage in diabetic retinopathy remain limited (Shiels et al. 2000). The complexity of endothelial permeability regulation, with multiple pathways contributing to vascular leakage, underscores the need for targeted therapies that address the underlying mechanisms, such as the modulation of intracellular calcium in endothelial cells (van Nieuw Amerongen and van Hinsbergh 2002).

However, treatment with yellow lupin extract did not result in significant improvement in retinal vascular changes.

The observed dilation of veins in diabetic rats in this study is in agreement with other studies that have found increased venous diameter in DR which can be due to the accumulation of ECM proteins, such as collagen and fibronectin, which lead to thickening of the vascular basement membrane and subsequent vascular remodelling (Sankaramoorthy and Roy 2021). Treatment with YLE resulted in a reduction in retinal vascular changes induced by diabetes, as evidenced by a decrease in perivascular hyperfluorescence. This suggests that YLE may have a protective effect on the retinal vasculature in DR.

The findings of this study suggest that YLE may be a promising therapeutic option for DR by modulating ECM composition. Further studies are needed to investigate the underlying mechanisms of this effect and to determine whether the observed changes in ECM composition translate into improvements in retinal function and structure.

Conclusion

Our study demonstrated that diabetes causes significant changes in ECM composition, including an increase in total collagen and fibronectin levels, as well as total gelatinase activity, in the retina of rats. These changes were associated with retinal vascular dysfunction and signs of ischemia and poor perfusion. Treatment with YLE resulted in a decrease

in total collagen and laminin content and a slight increase in total gelatinase activity compared to the diabetic group, suggesting a potential beneficial effect on ECM composition in the diabetic retina. Plant-based compounds, including carotenoids, polyphenols, and bioactive molecules found in YLE, have been associated with improved eye health and may have therapeutic potential in the management and prevention of DR-related pathogenesis. However, further studies are needed to investigate the underlying molecular mechanisms of these effects.

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Data availability Not available.

Declarations

Conflict of interest The authors have not disclosed any competing interests..

Ethical approval The animal protocols used for this study were approved by the University Animal Care and Use Committee of the University of Gabes, Tunisia and were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

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