

OLIVE LEAF EXTRACT AMELIORATES HYPERGLYCAEMIA, OXIDATIVE STRESS AND PANCREATIC AND HEPATIC INJURY IN STREPTOZOTOCIN-INDUCED DIABETIC RATS: A DOSE-DEPENDENT COMPARISON WITH METFORMIN

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Abstract

We evaluated whether a standardised olive leaf extract (OLE; *Olea europaea* L., 94.7 mg/g oleuropein) lowers fasting blood glucose in a dose-dependent manner and restores pancreatic and hepatic antioxidant capacity, using metformin as a reference. Fifty-eight male Wistar rats were used; forty-eight of them were allocated to six groups (n = 8): Normal, Normal + OLE 400, Diabetic, Diabetic + OLE 200 or 400 mg/kg, and Diabetic + metformin 200 mg/kg; streptozotocin (55 mg/kg, i.p.) induced diabetes. After four weeks, we assessed glycaemic indices, oral glucose tolerance, and pancreatic and hepatic oxidative markers (malondialdehyde, MDA; reduced glutathione, GSH; superoxide dismutase, SOD; catalase, CAT) with blinded histopathology. Streptozotocin produced marked hyperglycaemia, elevated HbA1c, hypoinsulinaemia and tripled MDA with antioxidant depletion. OLE produced a dose-dependent recovery; at 400 mg/kg, it lowered fasting glucose by 59% (Cohen's d = 4.85, 95% CI 2.81-6.90). It restored antioxidant enzymes to values not significantly different from metformin. Histopathology showed attenuation of pancreatic and hepatic lesions, with no overt adverse change in non-diabetic animals. Standardised OLE exerts dose-dependent anti-hyperglycaemic and antioxidant effects and protects pancreatic and hepatic tissue.

Rezumat

Studiul a evaluat efectul unui extract standardizat de frunze de măsline (OLE; *Olea europaea* L., 94,7 mg/g oleuropeină) asupra glicemiei bazale într-o manieră doză-dependentă, precum și a capacității antioxidante la nivel pancreatic și hepatic, metformina fiind utilizată ca referință. Au fost incluși 58 de șobolani Wistar masculi, dintre care 48 au fost repartizați în șase loturi (n = 8): martor normal, normal + OLE 400, diabetic, diabetic + OLE 200 sau 400 mg/kg și diabetic + metformină 200 mg/kg; diabetul a fost indus cu streptozotocină (55 mg/kg, i.p.). După patru săptămâni, au fost evaluați indicii glicemici, toleranța orală la glucoză și markerii de stres oxidativ pancreatici și hepatici (malondialdehidă, MDA; glutation redus, GSH; superoxid dismutază, SOD; catalază, CAT), precum și examenul histopatologic efectuat în orb. Streptozotocina a determinat hiperglicemie marcată, valori crescute ale HbA1c, hipoinsulinemie și triplarea MDA, cu depleția sistemelor antioxidante. OLE a produs o revenire doză-dependentă: la doza de 400 mg/kg, a redus glicemia bazală cu 59% și a readus activitatea enzimelor antioxidante la valori care nu au diferit semnificativ de cele obținute sub metformină. Examenul histopatologic a evidențiat atenuarea leziunilor pancreatice și hepatice, fără modificări evidente la animalele nediabete. Extractul standardizat de frunze de măsline exercită efecte antihiperglicemice și antioxidante doză-dependente și protejează țesutul pancreatic și hepatic.

Keywords: *Olea europaea*, oleuropein, diabetes mellitus, oxidative stress

Introduction

Diabetes mellitus is among the most prevalent non-communicable metabolic disorders and imposes a growing clinical and economic burden worldwide. It is characterised by chronic hyperglycaemia arising from defective insulin secretion, impaired insulin action, or both, and is associated with progressive damage to the pancreas, liver, kidney, retina, and vasculature (1).

Oxidative stress is a central mechanism linking hyperglycaemia to tissue injury. Sustained hyperglycaemia accelerates reactive oxygen species generation through glucose auto-oxidation, advanced glycation

end-product formation, and mitochondrial overload, overwhelming endogenous antioxidant defences and promoting lipid peroxidation and cellular damage (2, 3). Because conventional therapies have recognised limitations and side effects, there is sustained interest in plant-derived adjuvants that combine hypoglycaemic and antioxidant properties (4).

Olive (*Olea europaea* L.) leaves are an abundant agricultural by-product rich in polyphenols, particularly oleuropein, hydroxytyrosol, tyrosol, and flavonoids. These constituents have demonstrated antioxidant, anti-inflammatory, hypolipidaemic, and hypoglycaemic activities in experimental models (5-12), and recent reviews have consolidated the therapeutic

potential of olive-leaf polyphenols (13, 14). Despite this evidence, integrated studies that combine biochemical, functional, and histopathological endpoints and directly compare graded doses of olive leaf extract (OLE) with metformin within a single controlled design remain limited.

We therefore evaluated the dose-dependent effect of OLE (200 and 400 mg/kg) on glycaemic control, insulin status, oral glucose tolerance, pancreatic and hepatic oxidative stress, and the histopathology of the pancreas and liver in a streptozotocin (STZ)-induced diabetic rat model, with metformin as the reference drug. Because a single high-dose STZ injection produces a predominantly insulin-deficient, type 1-like model, we included metformin as a clinically established anti-hyperglycaemic comparator, and interpret the findings within that context. We tested the hypothesis that OLE lowers week-4 fasting blood glucose in a dose-dependent manner and restores pancreatic and hepatic antioxidant capacity to a degree approaching that of metformin, while producing no overt adverse effect in non-diabetic animals.

Materials and Methods

Plant material and preparation of OLE

Fresh olive leaves (*Olea europaea* L., *Oleaceae*) were collected from Makkah Province, Saudi Arabia (21.62° N, 39.89° E) in March 2025 and authenticated by a plant taxonomist at the Department of Biology, Umm Al-Qura University, Saudi Arabia. A voucher specimen was deposited at the Herbarium of the Department of Biology, Umm Al-Qura University, Saudi Arabia, under accession number UQU-OE-2025-017.

Dried, powdered leaves were extracted with 70% aqueous ethanol at a plant-to-solvent ratio of 1:10 (w/v) at 60°C for 2 h under continuous magnetic stirring. The suspension was filtered through Whatman No. 1 paper, concentrated under reduced pressure at 40°C, and freeze-dried to a dry OLE (yield 23.4 ± 0.8% w/w), stored at -20°C and freshly suspended in distilled water before administration.

The extract was standardised to its oleuropein content by reversed-phase HPLC-UV (Shimadzu LC-20AD; LabSolutions v5.97) against an authentic oleuropein reference standard (Sigma-Aldrich, O9755; ≥ 98% purity). Separation used a Phenomenex Luna C18 column (250 mm × 4.6 mm i.d., 5 µm) at 30°C with 0.1% aqueous formic acid (A) and acetonitrile (B) in gradient mode (0 - 5 min, 5% B; 5 - 20 min, 5 - 40% B; 20 - 25 min, 40% B; 25 - 26 min, 40 - 5% B; 26 - 30 min, 5% B) at 1.0 mL/min, a 20 µL injection, and detection at 254 nm; oleuropein eluted at 14.3 ± 0.2 min. The extract was dissolved in methanol (1 mg/mL), sonicated for 10 min, and filtered through a 0.22 µm PTFE membrane before injection. Oleuropein content was 94.7 ± 2.3 mg/g of dry extract. Calibration was linear over 1 - 200 µg/mL ($R^2 = 0.9997$), with a limit of detection of 0.8 µg/mL, a limit of quantification of 2.5 µg/mL, and intra- and inter-day precision of 0.9% and 1.6% RSD, respectively. Supplementary Figure S1 shows a representative chromatogram. Accordingly, the 200 and 400 mg/kg OLE doses delivered oleuropein-equivalent doses of 18.94 and 37.88 mg/kg, respectively.

Chemicals and assay kits

We obtained STZ (Sigma-Aldrich, S0130) and metformin hydrochloride (Sigma-Aldrich, PHR1084) from Merck KGaA (Germany). We used ketamine (Narkomed, Germany) and xylazine (Bayer, Rompun, Germany) for anaesthesia. All other reagents were of analytical grade; catalogue and lot numbers are listed in the Supplementary Source Data.

We measured all biochemical endpoints with the commercial kits listed in Table I, used strictly according to the manufacturer's instructions, and read them on a microplate spectrophotometer. The classical publications cited alongside each oxidative-stress marker are given as the source of the assay principle on which the corresponding kit is based, or, where the kit chemistry differs, as the classical assay for the same analyte; they are not separate manual procedures, and no manual assay was performed in parallel.

Table I

Assay kits, quantification principle and readout for each biochemical parameter

Parameter	Kit/instrument (manufacturer, catalogue no.)	Quantification principle	Read-out
Fasting blood glucose	GOD-POD kit (BioSystems S.A., Spain; BT 1000)	GOD-POD colourimetric method	Absorbance at 505 nm
HbA1c	HbA1c kit (Crystal Chem Inc., USA; 80310)	Boronate-affinity assay; glycated haemoglobin as % of total Hb	Microplate reader
Serum insulin	Rat insulin ELISA (Mercodia AB, Sweden; 10-1250-01)	Solid-phase two-site sandwich ELISA; detection limit ≤ 0.15 µg/L (manufacturer's specification)	Absorbance at 450 nm
MDA (pancreas, liver)	TBARS kit (Cayman Chemical, USA; 10009055)	MDA-thiobarbituric acid adduct (principle of Ohkawa <i>et al.</i>)	Absorbance at 532 nm
GSH (pancreas, liver)	GSH kit (Cayman Chemical, USA; 703002)	DTNB (Ellman's reagent) reduction to TNB	Absorbance at 412 nm

Parameter	Kit/instrument (manufacturer, catalogue no.)	Quantification principle	Read-out
SOD (pancreas, liver)	SOD kit (Cayman Chemical, USA; 706002)	Tetrazolium salt detection of xanthine-oxidase-generated superoxide; the pyrogallol-oxidation method of Marklund and Marklund is the classical assay for the same analyte	Absorbance at 450 nm
CAT (pancreas, liver)	CAT kit (Cayman Chemical, USA; 707002)	Reaction of catalase with methanol in the presence of H ₂ O ₂ ; formaldehyde detection (principle of Aebi)	Absorbance at 540 nm
ALT, AST	Mindray BS-380 reagents	IFCC-standardised kinetic method	Automated analyser
ALP	Mindray BS-380 reagents	p-Nitrophenyl phosphate kinetic method	Automated analyser
Creatinine	Mindray BS-380 reagents	Kinetic Jaffe method	Automated analyser
Total protein (normalisation)	Bradford reagent, BSA standard	Coomassie dye-binding	Absorbance at 595 nm

GOD-POD, glucose oxidase-peroxidase; TBARS, thiobarbituric acid reactive substances; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); BSA, bovine serum albumin; MDA, malondialdehyde; GSH, reduced glutathione; SOD, superoxide dismutase; CAT, catalase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase

Animals and ethical approval

Adult male Wistar rats (180 - 220 g) were obtained from the institutional central animal house and housed under standard conditions (22 ± 2°C, 12 h light/dark cycle) with free access to a standard pellet diet and water, and acclimatised for one week. We studied only males to limit oestrous-cycle variability in glycaemic and oxidative-stress endpoints; we acknowledge the resulting constraint on generalisability to females in the Discussion. All procedures were approved by the Biomedical Research Ethics Committee, Umm Al-Qura University, Saudi Arabia (Approval No. HAPO-02-K-012-2025-02-3449; 3 February 2025) and conducted in accordance with the ARRIVE 2.0 guidelines (15) and Directive 2010/63/EU (16).

Induction of diabetes and animal flow

After an overnight fast, diabetes was induced by a single intraperitoneal injection of STZ (55 mg/kg) freshly dissolved in cold 0.1 M citrate buffer (pH 4.5) and protected from light; control animals received an equivalent volume of citrate buffer. This regimen produces a predominantly insulin-deficient, type 1-like model through selective β -cell ablation (17, 18). Seventy-two hours later, fasting blood glucose was measured, and rats with FBG $\geq$ 250 mg/dL were considered diabetic and included. Treatment began immediately after this confirmation, *i.e.* 72 h after the STZ injection.

Animal flow (ARRIVE item 2). Fifty-eight rats were used in total. We allocated 48 rats to six experimental groups (8 *per* group): 32 STZ-injected animals to the four diabetic groups and 16 non-injected animals to the two non-diabetic groups. We maintained an additional ten rats in parallel as a reserve cohort under identical housing and handling conditions; two were injected with STZ and confirmed diabetic alongside the main cohort and followed the same vehicle schedule from day 0, and the remaining eight were not injected and were never entered into the study. Thirty-four animals therefore received STZ in total, and all thirty-four reached the inclusion criterion of fasting blood

glucose (FBG) $\geq$ 250 mg/dL at the 72 h assessment; there were no non-responders, and none were excluded on that ground. We state this explicitly, as it is a more complete response rate than is usually reported for this regimen, and we can offer no explanation for it beyond the STZ batch and the strict overnight fast before injection. Two animals in the untreated diabetic group died during the four-week treatment period and were replaced by the two reserve diabetic animals, which had therefore completed the full four weeks of the protocol rather than a shortened one. Mortality was 2 of 34 STZ-injected animals, confined to the untreated diabetic group, and we report it here as an outcome in its own right rather than only as an attrition figure. No animal was excluded on any other ground after allocation, and no data point was removed from any analysis other than those of the two animals that died, so that all six groups were analysed at *n* = 8. Supplementary Figure S2 shows the flow.

Experimental design and sample-size calculation

Confirmed diabetic animals were randomly allocated to treatment groups using a computer-generated randomisation sequence, with the Normal and Normal + OLE groups drawn from non-injected animals, giving six groups of eight rats each (Table II). Treatments (OLE 200 or 400 mg/kg, or metformin 200 mg/kg) were administered once daily by oral gavage for four consecutive weeks; control and untreated diabetic animals received the vehicle. Metformin (200 mg/kg/day) served as an anti-hyperglycaemic reference. Doses between 100 and 500 mg/kg/day are used in STZ-diabetic rodent studies; the comparator study we cite used 100 mg/kg in diet-STZ Sprague-Dawley rats (19), and we selected the higher dose to avoid under-dosing the reference arm in a severely hyperglycaemic, insulin-deficient model. The 200 and 400 mg/kg OLE doses were chosen to bracket the 100 - 200 mg/kg range in which OLE has shown antioxidant and anti-hyperglycaemic activity in rats (6, 20); no dose-ranging study was performed by us,

and this is acknowledged as a limitation. Samples for the biochemical assays and all histological slides were coded by a third party, so that the analyst and the histopathological scorer were unaware of group identity. We do not claim blinding of the glycaemic measurements themselves: a tail-vein reading of 440 *versus*

90 mg/dL discloses allocation, and the gavage operator necessarily knew which treatment was being administered. We recorded body weight at baseline and weekly, and recorded daily food and water intake for each animal individually throughout the study.

Table II

Experimental groups and treatment protocol

Group	Treatment	Dose	Route	Duration
Normal	Vehicle (distilled water)	not applicable	Oral	4 weeks
Normal + OLE	Non-diabetic + OLE	400 mg/kg/day	Oral	4 weeks
Diabetic	STZ induction, then untreated (vehicle)	STZ 55 mg/kg (single dose)	STZ i.p.; vehicle by oral gavage	STZ once (day 0); vehicle daily, 4 weeks
OLE 200	Diabetic + OLE	200 mg/kg/day	Oral	4 weeks
OLE 400	Diabetic + OLE	400 mg/kg/day	Oral	4 weeks
Metformin	Diabetic + metformin	200 mg/kg/day	Oral	4 weeks

STZ, streptozotocin; OLE, olive leaf extract; i.p., intraperitoneal

We estimated the sample size *a priori* for the primary endpoint, week-4 fasting blood glucose. Assuming a between-group difference of 120 mg/dL, a pooled standard deviation of 75 mg/dL, $\alpha = 0.05$, and 80% power, we required a minimum of seven animals *per* group; we used eight *per* group to allow for attrition.

Anaesthesia, euthanasia and sample collection

At the end of the treatment period, rats were fasted overnight and deeply anaesthetised with ketamine/xylazine (80/10 mg/kg, i.p.). Blood was collected under deep anaesthesia for the determination of FBG, HbA1c, and serum insulin; animals were then euthanised by exsanguination under deep anaesthesia, followed by cervical dislocation as a confirmatory (adjunctive) step. Exsanguination under general anaesthesia and physical confirmation of death are both accepted for rats under the AVMA Guidelines for the Euthanasia of Animals (21) and Annex IV of Directive 2010/63/EU (16); cervical dislocation was used only as a secondary confirmatory measure on an already unconscious, exsanguinated animal and never as the primary method. The pancreas and liver were rapidly excised, rinsed in ice-cold saline, and divided: one portion was homogenised for oxidative-stress markers, and another was fixed in 10% neutral-buffered formalin for histopathology.

Tissue homogenisation and total protein determination

Approximately 100 mg of pancreatic or hepatic tissue was blotted dry and homogenised in ice-cold 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA at a tissue-to-buffer ratio of 1:10 (w/v), using a Teflon-glass homogeniser held on ice (10 strokes, 30 s intervals). The homogenate was centrifuged at $10,000 \times g$ for 15 min at 4°C; the clear supernatant was aliquoted, snap-frozen and stored at -80°C, and each aliquot was thawed only once before assay. Pancreatic tissue was processed within 5 min of excision to limit autolysis by endogenous proteases. Total soluble protein in every supernatant was

determined by the Bradford dye-binding method against a bovine serum albumin (BSA) standard curve (22), and all four oxidative-stress markers were expressed *per* mg of soluble protein on this basis.

Biochemical assays

Fasting blood glucose was measured by the glucose oxidase-peroxidase (GOD-POD) colourimetric method at 505 nm; HbA1c by a boronate-affinity assay expressed as a percentage of total haemoglobin; and serum insulin by a sandwich ELISA read at 450 nm. The rat insulin ELISA (Mercodia 10-1250-01) is a solid-phase two-site sandwich enzyme immunoassay read at 450 nm; 10 μ L of each undiluted sample was assayed in duplicate, and all samples from all six groups were measured on a single plate of one lot. We did not determine assay precision in-house for this run. The manufacturer's specification for this kit states a measuring range of 0.15 - 5.5 μ g/L and, in Directions for Use version 7.0, an intra-assay coefficient of variation of 5.1%, 3.1% and 2.8% at mean concentrations of 0.47, 0.96 and 2.8 μ g/L respectively, an inter-assay coefficient of variation of 10%, 4.4% and 3.2% at the same three concentrations, and a detection limit of 0.15 μ g/L or lower; these are the manufacturer's figures and not measurements made in this laboratory, and we report them as such. These manufacturer's figures are stated in the mass units used in the Directions for Use (μ g/L). The calibrators supplied with the kit and used to construct the standard curve in this study are expressed in μ IU/mL; serum insulin values, and the HOMA indices derived from them, are therefore reported in μ IU/mL throughout, and no unit conversion was applied to the measured concentrations. We left the manufacturer's precision and range figures in the units they report and did not restate them in μ IU/mL. Because every sample was measured on one plate, between-plate variation cannot contribute to the between-group comparisons reported here. In both pancreatic and hepatic homogenates, we assessed lipid peroxidation as MDA

and the antioxidant defence system as GSH, SOD, and CAT; the quantification principle and detection wavelength of each assay are given in Table I. For MDA, GSH, and CAT, the kit principle follows the classical method cited alongside it – Ohkawa *et al.* (23), Ellman (24), and Aebi (25), respectively. For SOD, the kit chemistry differs from the method cited, and we distinguish the two here. We measured SOD activity with the Cayman Chemical kit 706002 (Table I), in which superoxide radicals generated by xanthine oxidase reduce a tetrazolium salt; we quantify enzyme activity by the sample's inhibition of that reduction, read at 450 nm. The classical assay of Marklund and Marklund (26) measures the same enzyme by a different chemistry, the inhibition of pyrogallol autoxidation. Reference (26) is therefore cited as the established method for this analyte, and not as the source of the assay chemistry used in the present study. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and creatinine were measured on a Mindray BS-380 automated biochemistry analyser using the manufacturer's reagents.

Oral glucose tolerance test (OGTT)

The OGTT was performed at the end of the four-week treatment period and 12 - 24 h after the last treatment dose, so that the acute post-gavage response did not confound the curve; the animals were then returned to food, and terminal anaesthesia and tissue collection were performed on the following day after a second overnight fast, so that the fasting glucose, insulin and HOMA values reported here were not measured in the hours following a glucose load. This means that the animals were fasted twice within approximately 36 h, which we note because it is a non-trivial burden in severely catabolic diabetic rats. After an overnight fast of 8 - 12 h, glucose (2 g/kg) was administered by oral gavage and blood was sampled from the lateral tail vein at 0, 30, 60, 90 and 120 min. Plasma was separated immediately at each time point, and glucose was quantified with the same GOD-POD kit used for fasting glucose (BioSystems BT 1000; absorbance at 505 nm). No handheld glucometer was used at any stage of this study; every glucose value reported, whether fasting or within the tolerance test, derives from this single colourimetric method, so the two are directly comparable. We calculated the total area under the glucose-time curve (AUC) using the trapezoidal rule.

Histopathological examination

Formalin-fixed pancreatic and hepatic specimens were dehydrated, cleared, embedded in paraffin, sectioned at 4 - 5 μ m, and stained with haematoxylin and eosin (H&E). Sections were examined independently by two observers blinded to treatment; lesions were graded semi-quantitatively on a 0 - 3 scale (0 = none, 1 = mild, 2 = moderate, 3 = severe), and inter-observer agreement was assessed by weighted kappa (kappa =

0.86), with discordant scores resolved by consensus. Pancreatic and hepatic sections were scored independently on separately coded slides, so that the assessment of one organ could not influence that of the other. Pancreatic injury was scored for islet shrinkage/degeneration, acinar degeneration and vacuolation, interstitial oedema, inflammatory infiltrate, and congestion/haemorrhage; hepatic injury was scored for hepatocyte degeneration/ballooning, sinusoidal dilatation, sinusoidal and central-vein congestion, inflammatory infiltrate, and necrosis. Each organ was scored on five categories, giving a summed score of 0 - 15 *per organ per animal*.

Islet sampling

Three non-consecutive sections at least 100 μ m apart were examined *per* pancreas, and ten non-overlapping fields *per* animal were captured across those sections. Every islet falling within those fields contributed to the islet component of the score, and no islet was selected, excluded or preferentially sought on the basis of size, location or appearance. A separate count of islets examined *per* animal was not recorded during scoring, so the pancreatic score is reported *per* animal rather than *per* islet, and we do not present an islet-level denominator.

We quantified hepatic sinusoidal congestion/haemorrhage area by colour-threshold image analysis (ImageJ v1.54, NIH, USA) and expressed it as a percentage of total tissue area; the animal was the unit of analysis throughout. We acquired images with a 40 $\times$ objective under identical illumination and white-balance settings. We performed thresholding in the HSB colour space with the ImageJ Colour Threshold plugin, using the plug-in's default automatic thresholding method; we left the hue, saturation, and brightness limits at their defaults and did not adjust them manually for any image. We applied the same default procedure to every image in every group, with the operator blinded to group identity, so no per-image human judgement entered the measurement. We note for transparency, since the point bears on reproducibility, that this means the limits were determined algorithmically for each image rather than pinned to a single fixed set of values across the whole series; the trade-off is that the procedure is operator-independent and carries no risk of a threshold being tuned, consciously or otherwise, to a known group.

Statistical analysis

Quantitative data are expressed as mean $\pm$ standard error of the mean (SEM) for $n = 8$ *per* group. We assessed normality and homogeneity of variance using the Shapiro-Wilk and Levene tests. Levene's test was significant for week-4 fasting blood glucose ($P = 0.040$) and for HOMA- β ($P = 0.032$), and the Shapiro-Wilk test was significant in isolated groups for fasting glucose, GSH and hepatic congestion area (values for these endpoints are given in the Supplementary Source Data, sheet Statistical_Annex); for these

endpoints, Welch's ANOVA, reported below, is the confirmatory analysis. We compared normally distributed variables using one-way ANOVA followed by Tukey's post-hoc test; we analysed OGTT glucose-time data using repeated-measures (mixed) ANOVA with group and time as factors. Ordinal histopathology scores are presented as median (Q1 - Q3) and compared by the Kruskal-Wallis test with Dunn's post-hoc test and Bonferroni adjustment. As a sensitivity analysis, we performed Welch's ANOVA (robust to unequal variances) for the continuous endpoints, which yielded the same conclusions (Supplementary Source Data). Analyses were performed in GraphPad Prism 9.5, and we considered $P < 0.05$ significant.

Effect sizes are reported for every endpoint, not only for selected contrasts: partial η^2 with its 95% confidence interval for every one-way omnibus model, Cohen's d with its 95% confidence interval for every pairwise contrast of those models, and the rank-biserial correlation for the ordinal histopathology comparisons. For the repeated-measures OGTT model, we report partial η^2 without a confidence interval, because the interval for a within-subject design is not comparable with those above, and we prefer not to present the two as though they were. The complete set is tabulated in the Supplementary Source Data (sheets Omnibus_ANOVA and PostHoc_Tukey), with the computational method for each quantity documented in the sheet Statistical_Methods_Note; the contrasts that carry the argument are quoted in the text. Confidence intervals on Cohen's are unadjusted for multiplicity, whereas the accompanying P values are family-adjusted; therefore, the two are not interchangeable, and a contrast may show a confidence interval excluding zero alongside a non-significant adjusted P .

HOMA indices were computed for each animal from its own week-4 fasting values as:

$$HOMA - IR = \frac{\text{Fasting glucose (mmol/L)} \times \text{Fasting insulin (}\mu\text{IU/mL)}}{22.5};$$

$$HOMA - \beta = \frac{20 \times \text{Fasting insulin (}\mu\text{IU/mL)}}{\text{Fasting glucose (mmol/L)} - 3.5}; \quad (27).$$

Because both are non-linear functions whose denominators approach zero in severely hyperglycaemic, hypoinsulinaemic animals, the group mean is not a meaningful summary; HOMA values are therefore reported as median (range) and were compared descriptively only. More fundamentally, HOMA is a steady-state model calibrated in humans with intact β -cell function, and at the glucose and insulin concentrations reached in this model (approximately 24 mmol/L and 6 $\mu\text{IU/mL}$ in untreated diabetic rats) the equations operate far outside the range over which they were derived; even in rodent studies where HOMA-IR has been formally validated, that validation applies to diet-induced insulin resistance rather than to β -cell-ablated animals (28). Accordingly, HOMA is

presented only as a descriptive index and is not used to support any inference about insulin resistance or β -cell function in this study.

State two further limits of the analysis where the reader encounters the results, not only in the Discussion. First, no equivalence test was performed, and no equivalence margin was pre-specified; where a contrast between OLE and metformin is reported as non-significant, that is an absence of evidence for a difference and not evidence of equivalence, and at $n = 8$ per group this design cannot exclude differences of the order of one standard deviation. Second, we controlled multiplicity within each endpoint family using Tukey for the continuous endpoints and Bonferroni-adjusted Dunn for the ordinal ones, but not across the twenty-five endpoint families examined; therefore, read the individual contrasts as descriptive rather than as confirmatory hypothesis tests.

Results and Discussion

OLE lowers fasting blood glucose dose-dependently

At baseline, all STZ-injected groups were comparably hyperglycaemic relative to normal controls ($P < 0.001$), confirming successful induction. After four weeks, untreated diabetic rats remained severely hyperglycaemic, whereas OLE lowered FBG dose-dependently (one-way ANOVA $F(5,42) = 113.04$, $P < 0.001$, partial $\eta^2 = 0.931$, 95% CI 0.874 - 0.946; Figure 1). Relative to untreated diabetic animals, FBG fell by 43% with OLE 200 mg/kg ($d = 3.49$, 95% CI 1.87 - 5.11) and by 59% with OLE 400 mg/kg ($d = 4.85$, 95% CI 2.81 - 6.90); the reduction with metformin was 55% ($d = 4.69$, 95% CI 2.69 - 6.68). OLE 400 mg/kg and metformin did not differ ($d = 0.79$, 95% CI - 0.24 to 1.81; $P = 0.918$), whereas OLE 400 mg/kg was superior to OLE 200 mg/kg ($d = 2.60$, 95% CI 1.23 - 3.98; $P = 0.003$), establishing the dose-response. The Normal + OLE group remained normoglycaemic, indicating that FBG was not reduced in non-diabetic OLE-treated animals.

For the STZ-injected groups, the bar labelled Week 0 is the confirmatory measurement taken 72 h after the STZ injection, at the point of inclusion, and not a pre-injection value; for the two non-diabetic groups, it is the pre-treatment baseline. STZ-diabetic rats are markedly hyperglycaemic; OLE lowers FBG dose-dependently, with 400 mg/kg approaching metformin. N + OLE, non-diabetic rats treated with OLE 400 mg/kg.

Effects on HbA1c, insulin and glucose tolerance

HbA1c and serum insulin followed the same dose-ordered pattern (Figure 2A and Figure 2B). Relative to untreated diabetic animals, HbA1c fell by 27% with OLE 200 mg/kg ($d = 2.11$, 95% CI 0.86 - 3.36), by 41% with OLE 400 mg/kg ($d = 4.10$, 95% CI 2.29 - 5.91) and by 37% with metformin ($d = 3.70$, 95% CI

2.01 - 5.38; omnibus $F(5,42) = 31.83$, $P < 0.001$, partial $\eta^2 = 0.791$, 95% CI 0.631 - 0.838). Serum insulin rose by 77%, 134% and 108% for OLE 200 mg/kg, OLE 400 mg/kg and metformin respectively (OLE 400 vs diabetic $d = -6.40$, 95% CI - 8.96 to - 3.83;

$F(5,42) = 71.76$, $P < 0.001$, partial $\eta^2 = 0.895$, 95% CI 0.810 - 0.919). Neither HbA1c nor insulin differed significantly between OLE 400 mg/kg and metformin (both adjusted $P \geq 0.378$).

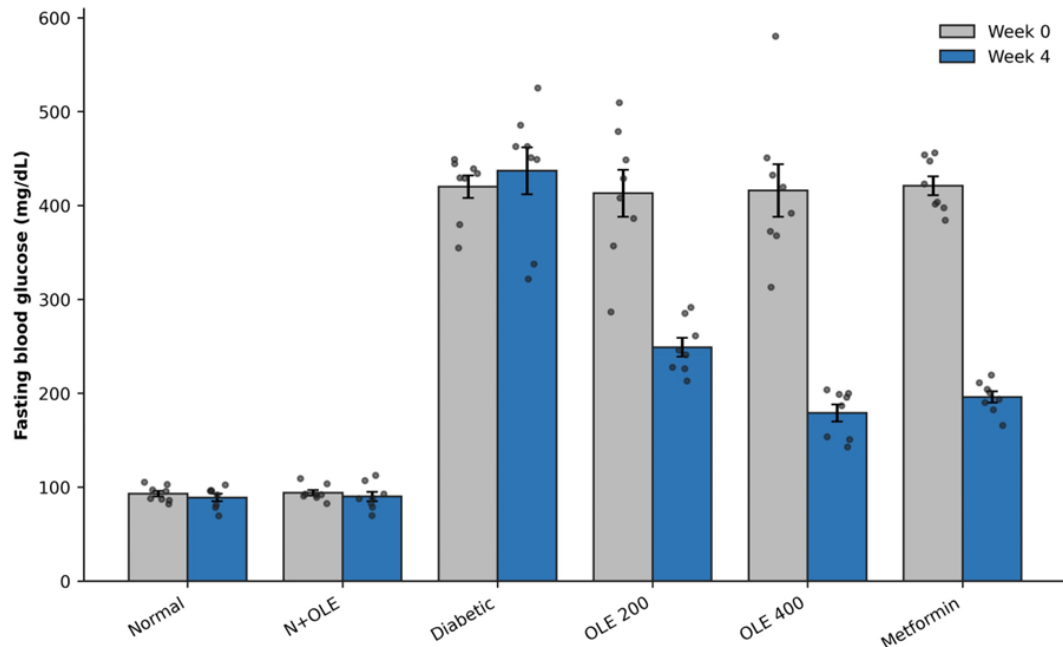


Figure 1.

Fasting blood glucose (mg/dL) at baseline and at week 4 across the six groups (mean $\pm$ SEM, $n = 8$; individual values shown; baseline, grey bars; Week 4, coloured bars)

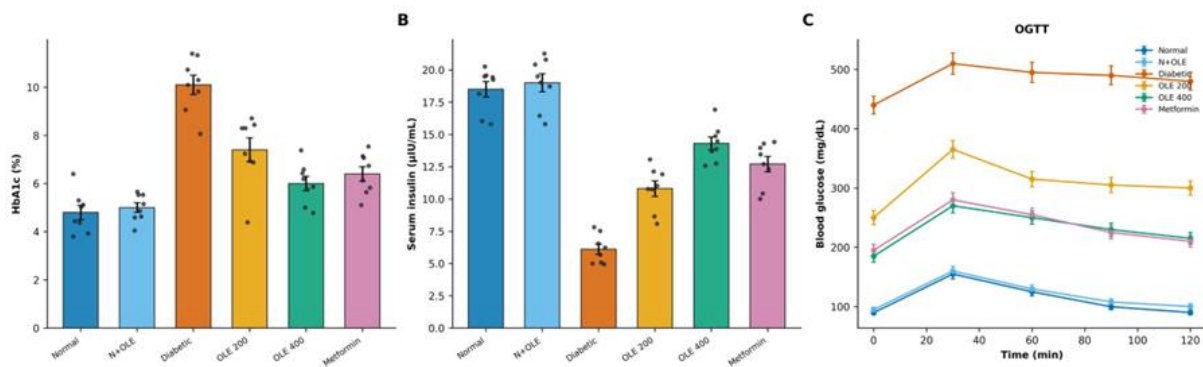


Figure 2.

OLE dose-dependently lowers HbA1c, raises insulin, and improves glucose tolerance (A) HbA1c, (B) serum insulin and (C) OGTT, oral glucose tolerance test (mean $\pm$ SEM, $n = 8$; individual values shown in A and B)

The HbA1c result requires an explicit caveat. The boronate-affinity assay used here is calibrated for human haemoglobin. It has no established rat reference interval, and the four-week treatment window covers well under one full erythrocyte lifespan in the Wistar rat, which is approximately 60 days (29). A diabetic HbA1c of 10.1% therefore does not correspond to the mean glucose that would be inferred from human calibration, and the internal discrepancy between HbA1c and the concurrently measured fasting glucose is expected rather than anomalous. HbA1c is

therefore interpreted only as a relative, within-study change between concurrently processed groups, not as an absolute index of mean glycaemia; the fasting glucose and OGTT data carry the glycaemic argument.

In the OGTT, repeated-measures ANOVA showed significant main effects of group ($F(5,42) = 956.55$, $P < 0.001$, partial $\eta^2 = 0.991$) and time ($F(4,168) = 43.32$, $P < 0.001$) with a non-significant group $\times$ time interaction ($F(20,168) = 0.83$, $P = 0.670$). The dose-dependent improvement is therefore expressed in the

height of the curve rather than in its shape: relative to untreated diabetic animals, the glucose AUC fell by 36% with OLE 200 mg/kg ($d = 8.74$, 95% CI 5.36 -

12.12), by 51% with OLE 400 mg/kg ($d = 12.29$, 95% CI 7.63 - 16.95) and by 51% with metformin ($d = 12.16$, 95% CI 7.55 - 16.77; Figure 2C and Table III).

Table III

Oral glucose tolerance and homeostasis model indices ($n = 8$ per group)

Group	OGTT AUC (mg·min/dL), mean $\pm$ SEM	HOMA-IR, median (range)	HOMA- β , median (range)
Normal	14.096 $\pm$ 267	4.09 (2.72 - 4.93)	215.2 (177.8 - 861.8)
Normal + OLE	14.856 $\pm$ 211	4.23 (3.36 - 5.39)	248.1 (119.4 - 1,029.3)
Diabetic	58.646 $\pm$ 1,107	5.99 (4.03 - 9.37)	6.0 (4.5 - 8.6)
OLE 200	37.809 $\pm$ 442	7.03 (4.51 - 7.77)	20.6 (15.7 - 26.5)
OLE 400	28.500 $\pm$ 527	6.19 (5.20 - 8.51)	41.8 (33.5 - 67.1)
Metformin	28.875 $\pm$ 523	6.10 (4.10 - 7.75)	34.5 (26.6 - 38.3)

OGTT AUC, area under the oral glucose tolerance curve (trapezoidal rule), mean $\pm$ SEM. Fasting glucose, HbA1c and insulin are plotted in Figure 1 and Figure 2 and are not repeated here

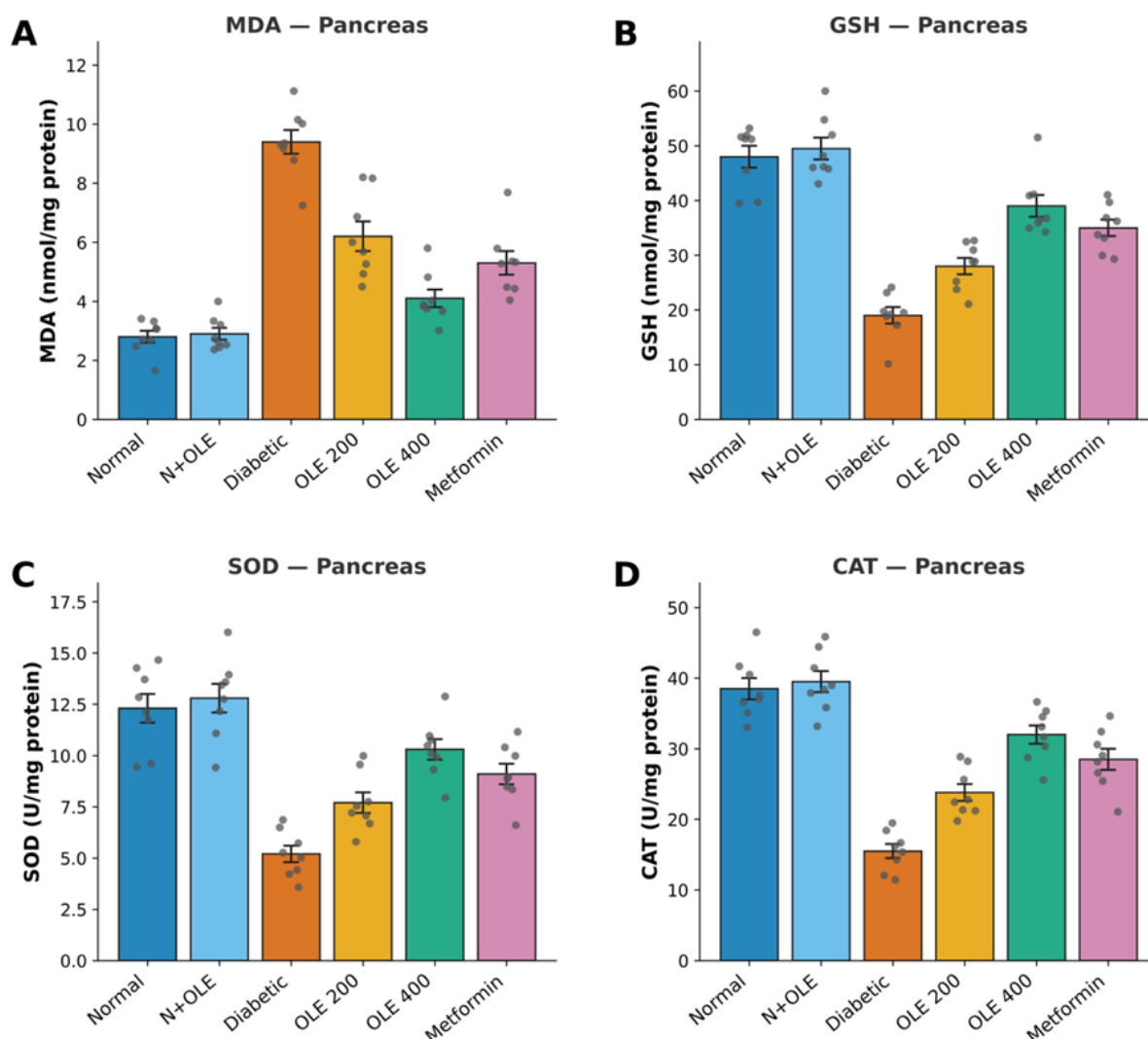


Figure 3.

OLE dose-dependently lowers lipid peroxidation and restores antioxidant defences in pancreatic tissue.

Pancreatic oxidative stress markers: (A) MDA, (B) GSH, (C) SOD and (D) CAT

(mean $\pm$ SEM, $n = 8$; individual values shown)

OLE restores the tissue antioxidant balance

Diabetes produced a pronounced oxidative imbalance in both organs, with a more than three-fold rise in MDA and depletion of GSH, SOD and CAT (one-way ANOVA *per* marker and tissue, all $P < 0.001$; full

omnibus statistics, effect sizes and post-hoc comparisons in the Supplementary Source Data). OLE reversed these changes dose-dependently. In the pancreas, OLE 400 mg/kg lowered MDA by 56% relative to untreated diabetic animals ($d = 5.30$, 95% CI 3.11 - 7.50;

Figure 3A) and raised GSH, SOD and CAT by 105%, 98% and 106% ($d = -4.00$, 95% CI - 5.78 to - 2.22, Figure 3B; $d = -3.98$, 95% CI - 5.75 to - 2.21, Figure 3C; and $d = -5.03$, 95% CI - 7.14 to - 2.93, Figure 3D). The hepatic response was of the same direction and larger magnitude: MDA fell by 64% ($d = 9.71$, 95% CI 5.98 - 13.44; Figure 4A) and GSH, SOD and CAT rose by 187%, 190% and 156% ($d = -6.89$, 95% CI - 9.62 to - 4.16, Figure 4B; $d = -8.74$, 95% CI - 12.13 to - 5.36, Figure 4C; and $d = -7.74$, 95%

CI - 10.77 to - 4.71, Figure 4D). For every marker in both tissues, OLE 400 mg/kg was superior to OLE 200 mg/kg (all adjusted $P \leq 0.025$) and did not differ significantly from metformin (all adjusted $P \geq 0.175$), with point estimates lying closer to the normal control values; no equivalence test was performed, and these non-significant contrasts should not be read as demonstrating equivalence. The Normal + OLE group did not differ from controls for any marker in either tissue (all adjusted $P \geq 0.465$).

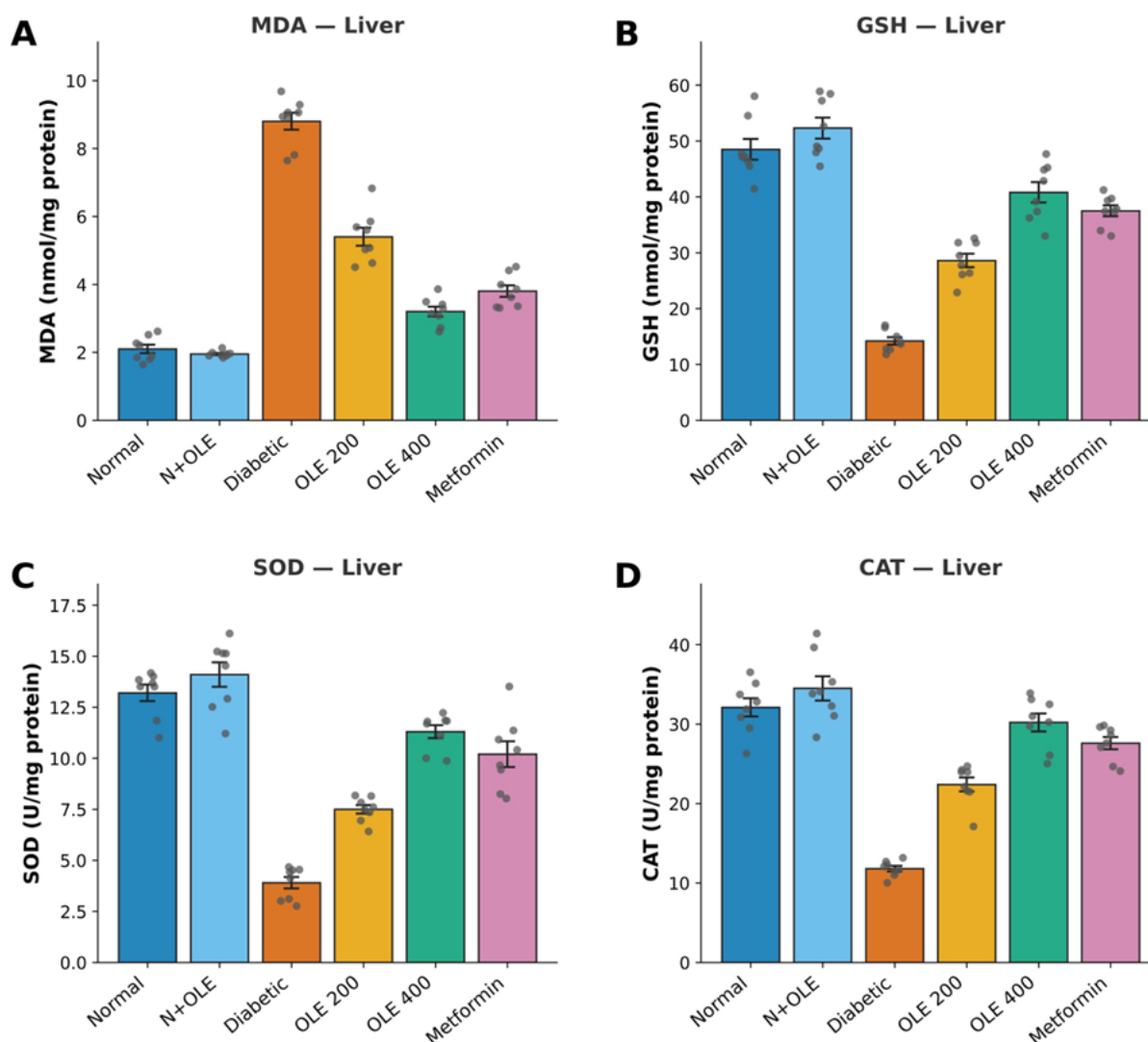


Figure 4. Hepatic oxidative-stress markers: (A) MDA, (B) GSH, (C) SOD and (D) CAT (mean $\pm$ SEM, $n = 8$; individual values shown)

The hepatic response parallels the pancreatic one and is of larger magnitude.

Histopathology of the pancreas

Pancreatic histology mirrored the biochemical findings (Figure 5). Normal controls and the Normal + OLE group showed intact, compactly arranged acini with normal islets and preserved lobular architecture. Untreated diabetic rats exhibited the most severe

changes: widely separated, degenerated acini, marked interstitial oedema, cytoplasmic vacuolation, inflammatory infiltration, and shrunken islets. OLE 200 mg/kg produced partial recovery with residual acinar separation, whereas OLE 400 mg/kg restored a near-normal acinar architecture with well-defined islets; the metformin group showed similar preservation.

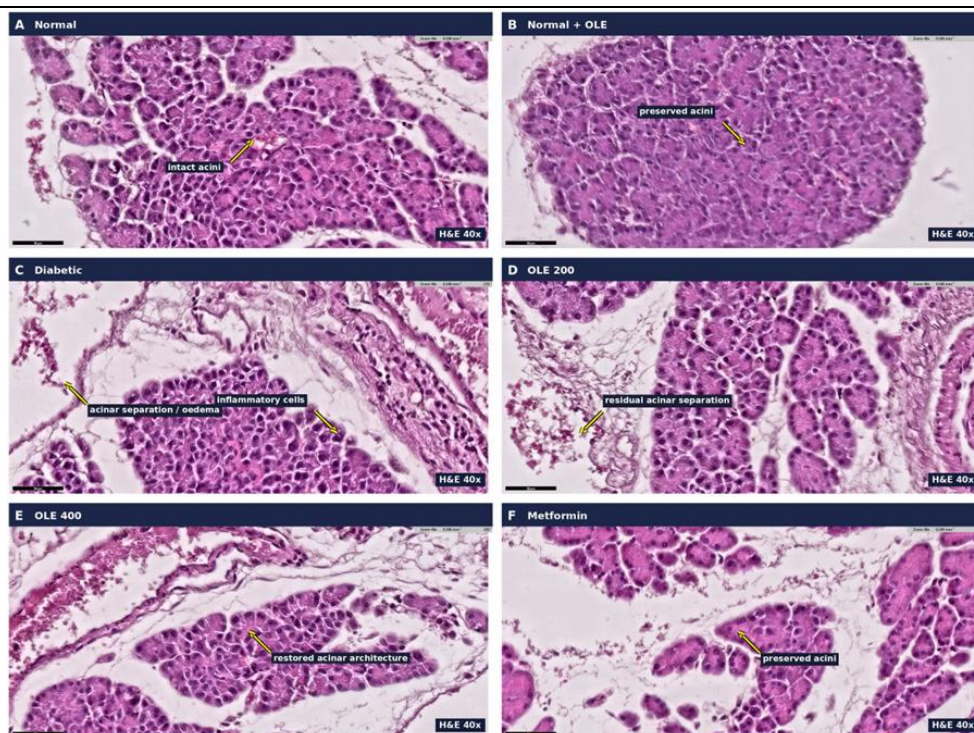


Figure 5.

Representative photomicrographs of pancreatic tissue (H&E, 40× objective; scale bar = 50 μm). (A) Normal; (B) Normal + OLE; (C) Diabetic; (D) OLE 200; (E) OLE 400; (F) Metformin. Arrows indicate representative lesions, including acinar separation/interstitial oedema and inflammatory infiltration in the diabetic pancreas. The grey annotation in the upper right of each panel is the acquisition software's own overlay, recording the objective and the field area

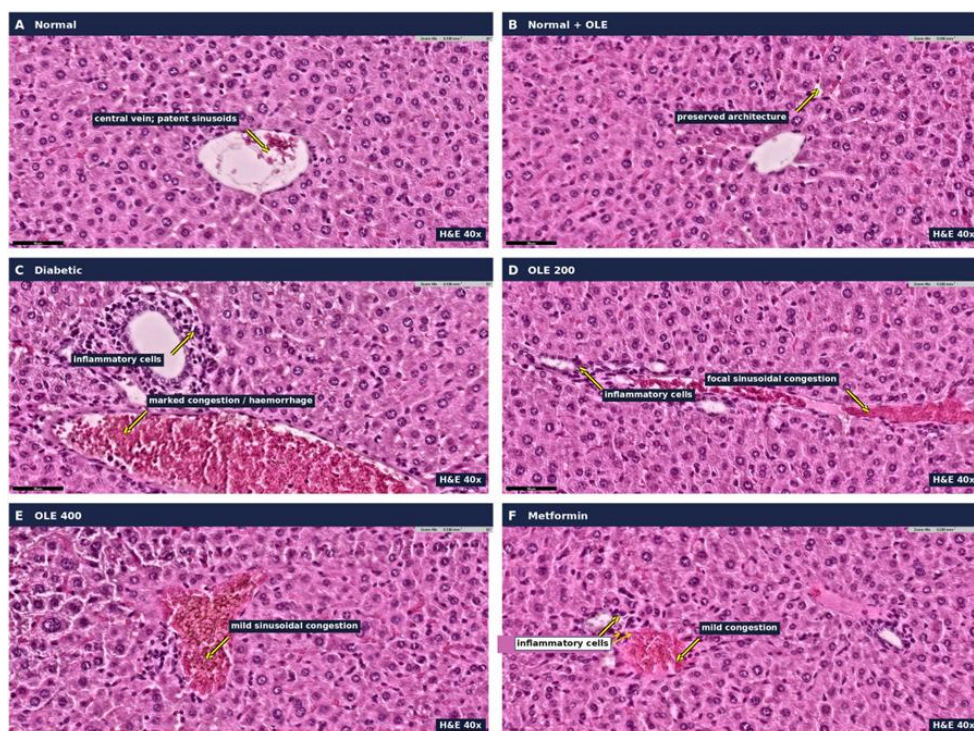


Figure 6.

Representative photomicrographs of hepatic tissue (H&E, 40× objective; scale bar = 50 μm). (A) Normal; (B) Normal + OLE; (C) Diabetic; (D) OLE 200; (E) OLE 400; (F) Metformin. Arrows indicate representative lesions, including sinusoidal congestion/haemorrhage and inflammatory infiltration. The grey annotation in the upper right of each panel is the acquisition software's own overlay, recording the objective and the field area

Histopathology of the liver

Hepatic sections showed a parallel pattern (Figure 6). Control and Normal + OLE livers displayed normal architecture with radiating hepatocyte cords and patent sinusoids. Diabetic livers showed the most pronounced injury, with marked sinusoidal and central-vein congestion, focal haemorrhage, hepatocyte degeneration, and portal inflammation. OLE 200 mg/kg showed partial improvement with a residual focal inflammatory infiltrate, whereas OLE 400 mg/kg restored an almost normal lobular architecture with only mild sinusoidal congestion; the metformin group showed only mild focal mononuclear infiltration.

Quantitative histopathology

Blinded semi-quantitative scoring reproduced the qualitative observations (Kruskal-Wallis: pancreas $H = 42.49$, liver $H = 39.64$, both $P < 0.001$; Figure

7A and Figure 7B, and Table IV). After Dunn's test with Bonferroni adjustment, only the OLE 400 mg/kg contrast differed significantly from the untreated diabetic group in both organs; neither metformin nor OLE 200 mg/kg reached significance (all adjusted P values in Table IV). This is a limitation of the summed ordinal score, whose ceiling and small rank spread give the Dunn procedure low power at $n = 8$, rather than evidence that metformin failed to protect tissue; the continuous morphometric endpoint below separates all treated groups from untreated diabetic animals unambiguously.

Group values for panel C (mean $\pm$ SEM, $n = 8$) are: Normal 0.31 ± 0.04 ; Normal + OLE 0.33 ± 0.05 ; Diabetic 4.24 ± 0.22 ; OLE 200 2.08 ± 0.15 ; OLE 400 0.89 ± 0.08 ; Metformin 0.44 ± 0.05 .

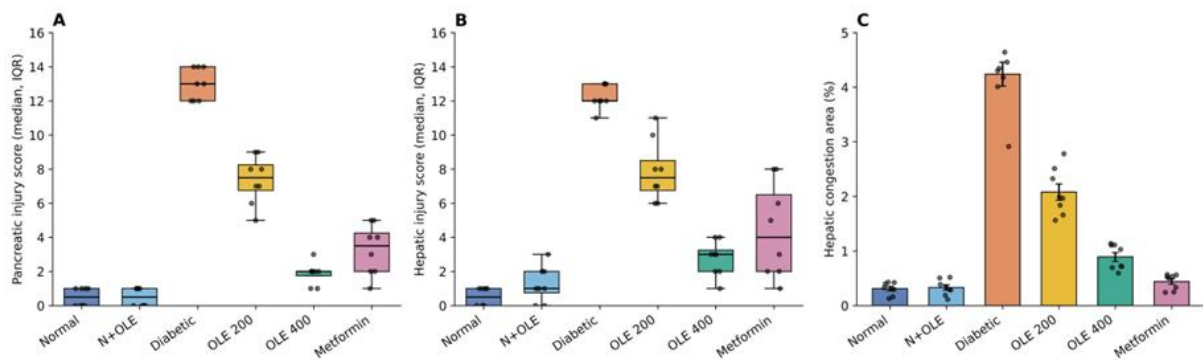


Figure 7.

Quantitative histopathology. (A) Pancreatic injury and (B) hepatic injury scores (median and interquartile range). (C) Hepatic sinusoidal congestion/haemorrhage area (% of tissue) by colour-threshold image analysis (mean $\pm$ SEM; one-way ANOVA $F(5,42) = 172.2$, $P < 0.001$)

Table IV

Semi-quantitative histopathological injury grades and Dunn post-hoc comparisons *versus* the untreated diabetic group ($n = 8$ per group)

Group	Pancreas: descriptive grade	Pancreas: P vs Diabetic (Dunn, Bonferroni)	Liver: descriptive grade	Liver: P vs Diabetic (Dunn, Bonferroni)
Normal	Normal	< 0.001	Normal	< 0.001
Normal + OLE	Normal	< 0.001	Minimal	< 0.001
Diabetic	Severe	n.a.	Severe	n.a.
OLE 200	Moderate	1.000	Moderate	1.000
OLE 400	Mild	0.012	Mild	0.018
Metformin	Mild	0.130	Mild	0.102

Kruskal-Wallis: pancreas $H = 42.49$, liver $H = 39.64$, both $P < 0.001$. P values are Bonferroni-adjusted Dunn comparisons against the Diabetic group; the summed numerical scores themselves are plotted in Figure 7A-7B and are not repeated here. n.a., not applicable (reference group)

Objective colour-threshold image analysis of hepatic sections showed that the sinusoidal congestion/haemorrhage area rose more than thirteen-fold in untreated diabetic rats relative to controls and fell progressively with treatment (one-way ANOVA $F(5,42) = 172.2$, $P < 0.001$; group values in the legend to Figure 7C). Relative to untreated diabetic animals, the congested area was 51% lower with OLE 200 mg/kg ($d = 4.06$, 95% CI 2.26 - 5.85), 79% lower with OLE 400 mg/kg ($d = 7.16$, 95% CI 4.33 - 9.98) and 90% lower with

metformin ($d = 8.42$, 95% CI 5.15 - 11.69), closely mirroring the biochemical findings.

Body weight, food and water intake

STZ-diabetic rats showed the classic metabolic signs of uncontrolled diabetes: untreated diabetic rats lost weight over four weeks whereas normal controls gained, and the diabetic group developed polyphagia and polydipsia (all $P < 0.001$). Treatment attenuated these changes dose-dependently, and 400 mg/kg OLE converted the net four-week change from loss to gain,

with food and water intakes comparable to metformin; OLE 200 mg/kg was intermediate. Group values are given in Table V and are not repeated here.

Serum liver and kidney function markers

Serum transaminases and the cholestatic marker ALP paralleled the hepatic histology: ALT, AST and ALP were markedly raised in untreated diabetic rats and fell dose-dependently with OLE, reaching values

comparable to metformin at 400 mg/kg. Serum creatinine, elevated in diabetic rats, was likewise attenuated by OLE 400 mg/kg, with no evidence of worsening hepatic or renal function (Table VI). Creatinine at OLE 400 mg/kg nevertheless remained above the control value, and since creatinine was the only renal endpoint measured, we do not claim renal protection.

Table V

Body weight, food and water intake (mean $\pm$ SEM, $n = 8$ per group)

Group	BW wk0 (g)	BW wk4 (g)	Δ BW (%)	Food (g/day)	Water (mL/day)
Normal	210.0 $\pm$ 4.44	268.0 $\pm$ 5.28	27.9 $\pm$ 3.03	18.2 $\pm$ 0.41	32.5 $\pm$ 1.73
Normal + OLE	211.0 $\pm$ 1.12	271.0 $\pm$ 5.20	28.4 $\pm$ 2.12	17.8 $\pm$ 0.45	31.8 $\pm$ 2.00
Diabetic	209.0 $\pm$ 3.21	168.0 $\pm$ 5.16	- 19.6 $\pm$ 2.32	36.4 $\pm$ 2.69	98.6 $\pm$ 3.45
OLE 200	210.0 $\pm$ 5.21	205.0 $\pm$ 5.84	- 2.1 $\pm$ 3.35	28.7 $\pm$ 0.98	68.2 $\pm$ 3.51
OLE 400	210.0 $\pm$ 3.95	238.0 $\pm$ 6.65	13.7 $\pm$ 4.16	22.4 $\pm$ 0.57	48.3 $\pm$ 2.58
Metformin	209.0 $\pm$ 4.16	232.0 $\pm$ 3.96	11.2 $\pm$ 2.26	23.1 $\pm$ 1.40	52.7 $\pm$ 2.18

BW, body weight. One-way ANOVA: BW wk4 $F(5,42) = 52.35$; Δ BW% $F(5,42) = 38.57$; food intake $F(5,42) = 27.62$; water intake $F(5,42) = 89.26$; all $P < 0.001$. Partial η^2 with 95% confidence intervals and all pairwise effect sizes for these endpoints are given in the Supplementary Source Data

Table VI

Serum liver and kidney function markers (mean $\pm$ SEM, $n = 8$ per group)

Group	ALT (U/L)	AST (U/L)	ALP (U/L)	Creatinine (mg/dL)
Normal	28.4 $\pm$ 1.3	62.1 $\pm$ 2.4	78.5 $\pm$ 2.1	0.62 $\pm$ 0.03
Normal + OLE	27.8 $\pm$ 0.3	60.5 $\pm$ 2.1	76.2 $\pm$ 2.7	0.60 $\pm$ 0.04
Diabetic	89.6 $\pm$ 3.0	124.3 $\pm$ 4.8	182.4 $\pm$ 11.0	1.48 $\pm$ 0.05
OLE 200	54.2 $\pm$ 3.4	88.7 $\pm$ 3.9	128.6 $\pm$ 3.8	1.02 $\pm$ 0.05
OLE 400	38.1 $\pm$ 1.7	70.4 $\pm$ 3.5	96.3 $\pm$ 2.6	0.78 $\pm$ 0.04
Metformin	41.5 $\pm$ 1.9	74.8 $\pm$ 2.2	102.7 $\pm$ 6.3	0.82 $\pm$ 0.03

One-way ANOVA: ALT $F(5,42) = 112.05$; AST $F(5,42) = 52.17$; ALP $F(5,42) = 49.26$; creatinine $F(5,42) = 70.37$; all $P < 0.001$. Partial η^2 with 95% confidence intervals and all pairwise effect sizes for these endpoints are given in the Supplementary Source Data

Integrated interpretation

Taken together, these data show that a standardised OLE exerts a dose-dependent anti-hyperglycaemic and antioxidant effect and protects both pancreatic and hepatic tissue in STZ-induced diabetic rats. The value of the design lies in the convergence of several classes of evidence measured in the same animals: a functional glycaemic class (FBG and OGTT), a hormonal endpoint (serum insulin), a biochemical class (MDA, GSH, SOD and CAT in two organs), and a histological class comprising an ordinal blinded score and a continuous morphometric measurement. We describe these as convergent rather than independent, since FBG and the OGTT area under the curve are arithmetically related once the group $\times$ time interaction is null, and the four oxidative markers are strongly intercorrelated. They move in the same direction and in the same dose order, and the higher OLE dose did not differ significantly from metformin on the glycaemic and oxidative endpoints, with the qualification set out below.

STZ selectively destroys pancreatic β -cells, producing insulin deficiency, hyperglycaemia and a well-characterised state of oxidative stress (17, 18). In our model, this was reflected by very high FBG and HbA1c, low serum insulin, impaired glucose tolerance, and a

striking rise in MDA with depletion of GSH, SOD and CAT, accompanied by the expected histological correlates.

Mechanistic basis of the antioxidant effect

The magnitude of the antioxidant response – a 56–64% fall in MDA with a simultaneous doubling to near-tripling of GSH, SOD and CAT – is larger than would be expected from direct radical scavenging by the ingested polyphenol alone, and points instead to an inducible, transcriptionally mediated response. The most plausible candidate is the Keap1-Nrf2-ARE axis. OLE at doses closely comparable to those used here (100 – 200 mg/kg orally in rats) has been shown to up-regulate Nrf2/antioxidant-response-element/haem oxygenase-1 signalling and to restore SOD, CAT and GSH while suppressing lipid peroxidation in cyclophosphamide-injured rat kidney (20), which is precisely the pattern we observe in both pancreas and liver. If this is the operative route, the enzyme activities we measured would be induced rather than merely spared; however, we did not measure enzyme protein, transcript, or turnover, so our data cannot distinguish induction from reduced consumption, and this remains a hypothesis rather than an observation. A second, complementary route is AMPK activation. Oleuropein activates AMPK and improves insulin

sensitivity in C2C12 myotubes (7), and in a mouse model of gestational diabetes it activates AMPK signalling while lowering blood glucose, insulin and hepatic oxidative-stress markers (30). AMPK activation and Nrf2 induction need not be competing explanations, and a single upstream polyphenolic stimulus could, in principle, drive both the metabolic and antioxidant arms of the response we recorded. We emphasise that we did not measure either pathway in this study; we infer both from the literature, and we list direct confirmation as a priority for future work.

Evidence bearing on a β -cell action, and its limits

The partial recovery of serum insulin (134% at OLE 400 mg/kg versus untreated diabetic animals) in a model whose defining lesion is β -cell ablation is the most interesting observation in this dataset, and also the one most easily over-interpreted. We therefore state the limits of the inference explicitly. HOMA- β rises in dose order across the treated groups, but we do not treat this as evidence: as set out in the Statistical analysis, HOMA- β is a hyperbolic function whose denominator approaches zero in these animals; its group mean is not interpretable, and the model is calibrated for humans with intact β -cells (27, 28). The argument for a β -cell-directed effect therefore rests on one measured observation, the restoration of circulating insulin, supported by a qualitative one, the better-preserved islet appearance recorded by the blinded scorer within the islet category of the pancreatic score. We note that the representative photomicrographs in Figure 5 annotate acinar and inflammatory features rather than islets, so the figure illustrates the general histological picture and is not offered as evidence for this particular point. We are explicit that the second is not a quantified endpoint: the pancreatic score is a single summed ordinal across five lesion categories, and the islet component was not analysed; we did not analyse the islet component separately, so no islet-specific statistic exists in this study. Neither leg is a HOMA index.

Even so interpreted, the finding remains correlative. An increase in circulating insulin with improved islet morphology is compatible with protection of surviving β -cells from ongoing oxidative injury, reduced β -cell apoptosis, or reduced glucotoxic stress secondary to lower ambient glucose, and the present data cannot distinguish these. OLE has been reported to preserve islet integrity and to normalise insulin and glycation markers in a rat model of gestational diabetes (31), and oleuropein lowers glycaemia and restores antioxidant status in alloxan models (5, 8) – although those two studies measured neither islet nor β -cell endpoints, so they establish the antioxidant and anti-hyperglycaemic context rather than a β -cell action. The interpretation is therefore plausible but not established. Insulin and cleaved caspase-3 immunohistochemistry with islet-level morphometry would be

required before a causal β -cell-protective mechanism could be asserted, and we do not assert one here.

Improved glucose tolerance and carbohydrate digestion

The OGTT result is informative about mechanism precisely because of its shape. The group $\times$ time interaction was not significant, so treatment shifted the whole curve downwards rather than blunting the post-load excursion, as would be expected if the dominant effect were on basal glycaemia rather than on handling an oral glucose bolus. Nevertheless, two absorptive mechanisms plausibly contribute to the 51% reduction in AUC. Hydroxytyrosol is a non-competitive inhibitor of α -glucosidase and oleuropein an uncompetitive one, and hydroxytyrosol additionally shows mild inhibition of α -amylase (32), which would slow the appearance of glucose from the gavage load; and an olive-derived agonist of the bile-acid receptor TGR5 improves glucose tolerance and lowers serum glucose and insulin in mice (11). Because we administered the load orally rather than intravenously, the design cannot separate these gut-level contributions from systemic insulin-mediated disposal – an intra-peritoneal glucose tolerance test or an incretin measurement would be needed for that.

Hepatic protection

The hepatic response deserves separate comment because it was consistently larger than the pancreatic response for every oxidative marker, and because it was corroborated by two independent read-outs – serum ALT, AST, and ALP, and an operator-independent morphometric measurement of sinusoidal congestion. The liver is both the principal site of first-pass exposure to orally administered polyphenols and the organ in which AMPK- and PPAR α -dependent effects of oleuropein have been most clearly demonstrated: dietary oleuropein reverses the diet-induced hepatic lipid accumulation and dysfunction through PPAR α /FGF21 signalling (33), and hepatic AMPK activation by oleuropein has been shown directly *in vivo* (30). A larger hepatic than pancreatic effect would therefore be expected on first-pass grounds; we did not measure plasma or hepatic exposure, so we offer this as a plausible account rather than a pharmacokinetic finding.

Bioavailability and the identity of the active species

An important qualification applies to the mechanistic discussion as a whole. Oleuropein undergoes extensive pre-systemic hydrolysis, and hydroxytyrosol rather than intact oleuropein is the dominant circulating species after oral administration in both animals and humans (34). The 18.94 and 37.88 mg/kg oleuropein-equivalent doses delivered here should therefore not be read as systemic oleuropein exposures, and the effects we observed may be attributable to hydroxytyrosol, to other olive-leaf polyphenols present in the standardised extract, or to a luminal action of unabsorbed oleuropein on carbohydrate digestion, in any combination. We did not measure plasma

oleuropein or hydroxytyrosol, and this is a genuine limitation of the present work rather than a technicality.

Comparison with the metformin reference

OLE 400 mg/kg did not differ significantly from metformin on any glycaemic or oxidative endpoint, and the point estimates favoured OLE on several of the antioxidant markers. Two qualifications must be attached before that is read as equivalence. First, we performed no equivalence test and pre-specified no equivalence margin, so a non-significant contrast at $n = 8$ reflects an absence of evidence for a difference and nothing more. The second is that the comparison is not uniform across endpoints: on the objective morphometric endpoint, hepatic sinusoidal congestion area, metformin was numerically better than OLE 400 mg/kg by a margin whose confidence interval on the standardised difference excludes zero ($d = 2.38$, 95% CI 1.07 - 3.70; group values in the legend to Figure 7), even though the family-adjusted P did not reach significance. We record this rather than let the summary claim absorb it. Metformin acts principally by inhibiting hepatic gluconeogenesis and improving peripheral insulin sensitivity through AMPK-dependent and AMPK-independent routes (35), mechanisms that are least favourable in a predominantly insulin-deficient, type 1-like model; the comparator is therefore working at a disadvantage in this design, and equivalence here should not be extrapolated to type 2 diabetes. At the same time, metformin is not devoid of antioxidant activity – partial inhibition of mitochondrial complex I reduces reactive oxygen species generation, and anti-inflammatory effects are well documented (36) – so the improvement it produced in MDA and in the antioxidant enzymes is expected rather than anomalous, and the numerical margin favouring OLE should not be over-read given that the contrasts were not statistically significant.

Relation to human evidence

OLE has been evaluated in humans, and the direction of effect is consistent with ours. However, the human evidence is weaker and less consistent than our animal data, and we present it as such. A 2024 systematic review and meta-analysis of randomised controlled trials reported a favourable association between OLE and glucose metabolism in its narrative synthesis, but its pooled estimate for fasting glycaemia in the low-dose studies significantly favoured the control group (37); the pooled human evidence therefore does not yet support a glucose-lowering effect. An earlier controlled study reported reduced HbA1c and fasting plasma insulin in type 2 diabetic patients (12), and the extract has an established human safety and tolerability record from hypertension trials at comparable exposure (38). Effect sizes in humans are, unsurprisingly, an order of magnitude smaller than those recorded here in a severe, chemically induced rodent model, and the human trials studied type 2 diabetes rather than

an insulin-deficient phenotype. A rodent bridge between the two settings already exists: in a high-fat-diet plus low-dose STZ model, which reproduces the insulin-resistant type 2 phenotype rather than the β -cell-ablated one studied here, olive leaf treatment likewise improved glycaemic control (39) – suggesting that the effect is not confined to the insulin-deficient state, although we did not test that model. The convergence is therefore encouraging as to direction but says nothing about the transferability of the magnitudes we report.

Safety in non-diabetic animals

OLE administration to non-diabetic animals produced no glycaemic or oxidative deterioration: the Normal + OLE group remained normoglycaemic, did not differ from controls for any oxidative marker in either organ, and showed serum ALT, AST, ALP and creatinine within the control range. The only departure was a minimal upward shift in the ordinal liver score, which did not separate from controls on Dunn testing ($P = 1.000$; Supplementary Source Data, sheet PostHoc_Tukey) and was not accompanied by any change in the continuous morphometric congestion area (Figure 7C) or in serum transaminases (Table VI). This safety signal is nonetheless preliminary: it derives from a single dose level over four weeks in eight animals, with no dedicated toxicological endpoints, and should be interpreted as an absence of overt toxicity rather than as a demonstration of safety.

Limitations

Several constraints bound these conclusions. First, we used a single, type 1-like STZ model in male rats over four weeks, so the findings should not be generalised to type 2 diabetes or, without further work, to females. Second, as set out above, HbA1c cannot be read as an absolute index of mean glycaemia in this setting, and HOMA supports no inference about insulin resistance or β -cell function. Third, the β -cell-protective interpretation is supported by measured insulin and H&E morphology but not by insulin or apoptosis immunohistochemistry. Fourth, the molecular pathways (Nrf2, AMPK and PPAR α) were inferred from the literature rather than measured. Fifth, we did not determine plasma exposure to oleuropein and hydroxytyrosol, so we cannot separate systemic from gut-level contributions. Sixth, the summed ordinal histopathology score has limited power at $n = 8$ after multiplicity adjustment, which is why the continuous morphometric endpoint was included; that endpoint is hepatic only, so the pancreatic ordinal result stands on its own, and neither OLE 200 mg/kg nor metformin separated from untreated diabetic pancreas on it. Seventh, no equivalence test was performed, so none of the OLE-*versus*-metformin comparisons should be read as demonstrating equivalence; on hepatic congestion area, the point estimate favoured metformin. Eighth, the two animals that died were in the untreated diabetic group and were replaced

from the reserve cohort; because the animals lost were plausibly the most severely affected in the comparator group, the dispersion of that group may be narrower than it would otherwise have been, and the standardised effect sizes computed against it may be correspondingly generous. Future work should include longer durations and both sexes, insulin and caspase-3 immunohistochemistry with islet-level quantification, direct assessment of the proposed pathways, and pharmacokinetic characterisation of the standardised extract.

Conclusions

Standardised OLE produces a dose-dependent improvement in glycaemic control, insulin status and tissue oxidative balance, and protects pancreatic and hepatic architecture in a type 1-like STZ diabetic rat model. At 400 mg/kg, its effect did not differ significantly from metformin on the glycaemic and oxidative endpoints, with large effect sizes and consistent dose ordering across convergent classes of evidence, and without overt adverse glycaemic, biochemical or histological changes in non-diabetic animals. No equivalence test was performed, and on the one continuous morphometric endpoint the point estimate favoured metformin, so this should be read as a failure to detect a difference rather than as a demonstration of equivalence. The improvement in circulating insulin, together with better-preserved islet morphology, is compatible with protection of surviving β -cells, but the present data are correlative on this point, and the homeostasis model indices do not support the inference; immunohistochemical confirmation is required. Within the limits of a single preclinical model of insulin-deficient diabetes, these findings support the potential of standardised OLE as a natural adjuvant and justify further mechanistic, dose-ranging and ultimately clinical investigation before any extrapolation to human diabetes.

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Not applicable.

Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article. The complete animal-level dataset (individual biochemical values, OGTT time-course readings, histopathology scores and morphometric congestion-area measurements) is provided as Supplementary Source Data and is openly available in Zenodo under the concept DOI 10.5281/zenodo.20687524 (<https://doi.org/10.5281/zenodo.20687524>; file Supplementary_Source_Data.

xlsx, licensed CC BY 4.0). This concept DOI is version-independent: it is registered and resolves to the current version of the deposit, so it will continue to direct readers to the most recent version of the dataset.

Ethics approval and consent to participate

All experimental procedures were approved by the Biomedical Research Ethics Committee, Umm Al-Qura University (approval No. HAPO-02-K-012-2025-02-3449; 3 February 2025), and were conducted in accordance with Directive 2010/63/EU and the ARRIVE 2.0 guidelines. The study did not involve human participants.

AI use disclosure

During the preparation of this manuscript, the author(s) used Claude (Anthropic) for editing assistance and language improvement, specifically to improve spelling, grammar, word choice, readability, and clarity. The author(s) reviewed and revised all outputs from these tools and take full responsibility for the final content of the work. The authors generated and verified all scientific content, experimental data, statistical analyses, and interpretations.

Conflict of interest

The authors declare no conflict of interest.

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