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Cyperus rotundus; Rhodiola rosea; Zostera marina; polyherbal mixture; streptozotocin; Wistar rats; fasting blood glucose; HbA1c; lipid profile; metformin

Antidiabetic Efficacy of Ethanolic Extracts of Cyperus Rotundus Rhizome, Rhodiola Rosea Root and Zostera Marina Leaf and Their 1:1:1 Polyherbal Mixture in Streptozotocin-Induced Diabetic Wistar Rats

Polyherbal Antidiabetic Evaluation in Streptozotocin-Diabetic Rats

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ABSTRACT:

Purpose: The main objective of the current research work was to evaluate the antidiabetic efficacy of 70% ethanolic extracts of *Cyperus rotundus* rhizome (CR), *Rhodiola rosea* root (RR) and *Zostera marina* leaf (ZM), and of a 1:1:1 polyherbal mixture of the three extracts (MIX), in streptozotocin (STZ)-induced diabetic Wistar rats. The single extracts and the mixture were compared at the same total oral dose (250 mg/kg/day) against metformin (50 mg/kg/day). To our knowledge, this combination of a terrestrial sedge, an alpine adaptogen and a marine angiosperm has not been tested in an STZ model earlier.

Methods: Thirty-five male Wistar rats were allocated by computer-generated randomisation to seven groups of five (normal control, diabetic control, metformin, CR, RR, ZM and MIX). Diabetes was induced in 30 rats by a single intraperitoneal dose of STZ (55 mg/kg in citrate buffer, pH 4.5), and rats with fasting glucose of 300 mg/dL or more at 72 h were included. Treatments were given by oral gavage once daily for 28 days. Fasting blood glucose and body weight were recorded weekly, and HbA1c, serum lipid profile, ALT, AST and creatinine were measured at termination. Treated groups were compared with diabetic control by Dunnett's test and with each other by Tukey's test.

Results: All 30 STZ rats were included (72 h glucose 339 to 535 mg/dL) and no animal died. At week 4, fasting glucose was 94 ± 9 mg/dL in normal control and 439 ± 48 mg/dL in diabetic control, and it was lowered to 264 ± 57 mg/dL by metformin (40%), 243 ± 70 mg/dL by CR (45%), 272 ± 48 mg/dL by RR (38%), 300 ± 67 mg/dL by ZM (32%) and 235 ± 50 mg/dL by MIX (46%) (Dunnett, all $p < 0.01$). HbA1c fell from 8.8 ± 0.5% in diabetic control to 6.9 to 7.7% in the treated groups ($p < 0.05$), and total cholesterol, triglycerides, LDL-C, HDL-C, ALT, AST and creatinine moved towards normal in all five treated groups. MIX carried one-third of the single-extract dose of each plant and gave the lowest mean glucose, total cholesterol, triglycerides, LDL-C and creatinine, but no two treated groups differed significantly (Tukey, smallest $p = 0.18$).

Conclusion: It can be concluded that the three ethanolic extracts and their 1:1:1 mixture lowered fasting glucose, HbA1c and the atherogenic lipids in STZ-diabetic rats

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to about the same extent as metformin, and that the mixture did so at one-third of the dose of each constituent. Dose-response arms for each extract and a factorial design are the next steps for the mixture.

1. INTRODUCTION

Diabetes affected about 537 million adults aged 20–79 years in 2021, and the projection for 2045 is 783 million (Saeedi et al., 2019; Sun et al., 2021). Most of this burden is type 2 diabetes, which develops through insulin resistance in muscle, liver and adipose tissue together with a progressive loss of pancreatic β-cell function (DeFronzo et al., 2015; Stumvoll & Goldstein, 2005). Obesity and ectopic lipid accumulation drive much of the resistance (Kahn et al., 2006; Samuel & Shulman, 2012), and as eight separate organ-level defects have been described behind the hyperglycaemia, agents with different targets are combined in treatment (DeFronzo, 2009). In the STZ model the hyperglycaemia is accompanied by dyslipidaemia, raised liver enzymes and higher creatinine, so a candidate is judged on all of these end points and not on glucose alone (Ramesh et al., 2023; Wen et al., 2019). Streptozotocin (STZ) destroys the pancreatic islet β-cells and it is the usual chemical route to insulin-deficient diabetes in mice and rats, both for studying the consequences of diabetes and for screening candidate therapies (Furman, 2015). The model is easy to run and hard to reproduce. Preparation and stability of the STZ solution, dose, route, diet, species, age, body weight, sex and the glucose threshold used to define hyperglycaemia all change the outcome, and badly chosen conditions raise mortality and animal suffering (Goyal et al., 2015). Reports of STZ dose, time to onset and severity are often limited and inconsistent, which makes a new study difficult to design (Deeds et al., 2011). Hence each of these factors was fixed in advance in the current study and is reported in Section 2.

Cyperus rotundus L. (Cyperaceae) rhizomes were first tested for antidiabetic activity as a hydro-ethanolic extract in alloxan-diabetic rats, where 500 mg/kg given for seven days lowered blood glucose (Raut & Gaikwad, 2006). An ethanolic rhizome extract at 250 and 500 mg/kg for three weeks improved body weight and lowered blood glucose, SGPT, SGOT, cholesterol and triglycerides in STZ-diabetic mice (Singh et al., 2015). Work on the mechanism points in several directions. An ethyl acetate extract raised GLUT4 and GLUT1 expression and membrane translocation in L6 myotubes and limited lipid accumulation in 3T3-L1 adipocytes through the AMPK and ACC pathways (Pichetkun et al., 2024), and stilbenoid oligomers captured from the extract were strong α-glucosidase inhibitors (Deng et al., 2019). The 70% ethanol and water extracts of the plant were among those screened for α-glucosidase and lipase inhibition (Elbashir et al., 2018), and the hexane fraction reduced hepatic lipogenic gene expression in mice fed a high-sucrose diet through the LXRα–SREBP-1c axis (Oh et al., 2015). A hydroalcoholic extract (100 to 400 mg/kg/day for 21 days) reversed olanzapine-induced weight gain and metabolic change in rats (Kanagali et al., 2021). α-Cyperone, one of its active constituents, improved renal function in diabetic rats with renal ischaemia–reperfusion injury (Daude et al., 2024), and zinc oxide nanoparticles made with the plant lowered retinal inflammation in STZ-diabetic rats (Zhang et al., 2020).

For *Rhodiola rosea* L. (Crassulaceae), a water extract lowered plasma glucose dose-dependently in STZ-diabetic rats, an effect that depended on opioid μ-receptors and adrenal β-endorphin, and three days of dosing raised GLUT4 expression in skeletal muscle (Niu et al., 2014). Salidroside, the main phenylethanoid glycoside of the root, lowered fasting glucose and protected the kidney of STZ-diabetic Wistar rats at 50 mg/kg/day for eight weeks (Pei et al., 2022), and it is reported to improve insulin resistance through AMPK, PI3K, Akt and GSK-3β signalling (Chaitanya et al., 2025; Zheng et al., 2015). Rhodiosin from *R. crenulata* reduced postprandial glucose in normal and diabetic mice by inhibiting both the activity and the production of α-glucosidase (Wang et al., 2024), and an ethanolic root extract of *R. heterodonta* at 100 mg/kg lowered glucose in glucose-, alloxan- and adrenaline-induced diabetic rats (Zaripova et al., 2024). Protective effects of salidroside or *Rhodiola* preparations have since been described in diabetic kidney, heart, hippocampus, retina and skeletal muscle (Feng et al., 2024; Li et al., 2025; Ren et al., 2024; Wang et al., 2025; Wang et al., 2023; Zhou et al., 2026), and in the gut microbiota and iron metabolism of diabetic db/db mice (Shi et al., 2022).

The evidence for *Zostera marina* L. (Zosteraceae, eelgrass) is thinner and mostly indirect. Among two seagrasses from southern Portugal, *Z. marina* had the highest total phenolic content and radical-scavenging activity, with rosmarinic acid as its main identified compound (Custódio et al., 2015). Apigenin-7-O-glucoside, chrysoeriol and luteolin were isolated from the leaves (Kim et al., 2004). Rosmarinic acid, luteolin and its sulfated derivatives, the main constituents of the *Zostera* polyphenol complex, have been reviewed for effects on carbohydrate and lipid metabolism in diabetes and hyperlipidaemia models

(Styshova et al., 2017). Mixtures of polar lipids from sea macrophytes including *Z. marina*, with the *Z. marina* polyphenol complex and echinochrome, were tested in rats with experimental atherosclerosis and diabetes (Krivoshapko et al., 2012), and the earlier work was reviewed (Krivoshapko & Popov, 2011). Recent reviews cover the antioxidant and anti-inflammatory properties of Mediterranean seagrasses (Vasarri et al., 2025) and list antidiabetic qualities among those reported for marine plants (Ribas-Taberner et al., 2025). Combining plants that act on different targets is the reasoning behind polyherbal practice (Wagner & Ulrich-Merzenich, 2009; Williamson, 2001), and network pharmacology describes the same multi-target idea in drug-discovery terms (Hopkins, 2008). GB5, a cold-percolated ethanolic extract of five plants including *C. rotundus* in equal proportions, was built on this basis and characterised in vitro, in yeast and in 3T3-L1 adipocytes (Das et al., 2025), and Trimad, which combines *C. rotundus* tubers with two other plants in a 1:1:1 ratio, was tested at 50, 100 and 200 mg/kg in high-fat-fed Wistar rats (Joshi et al., 2026). The studies cited above used different extraction solvents, rodent species, doses, durations and end points, and hence they cannot be ranked against each other. In the preceding in vitro study from this laboratory, a 1:1:1 combination of the methanolic extracts of the three plants restored glucose uptake in insulin-resistant 3T3-L1 adipocytes to 89.36% of normal control at 250 µg/mL and gave lower IC50 values for α-amylase (86.28 µg/mL) and α-glucosidase (68.25 µg/mL) than each single extract. A mixture of the three extracts has not been tested against the single extracts at the same total dose in an STZ model. Hence the current research was aimed to evaluate 70% ethanolic extracts of *C. rotundus* rhizome, *R. rosea* root and *Z. marina* leaf, and their 1:1:1 mixture, head to head in one standardised STZ-diabetic Wistar rat model at an identical total oral dose of 250 mg/kg/day, with metformin (50 mg/kg/day) as the reference drug, on fasting blood glucose, HbA1c, serum lipid profile, body weight and hepatorenal markers.

2. MATERIALS AND METHODS

2.1. Plant material, chemicals and reagents

Rhizomes of *C. rotundus* L. (Cyperaceae), roots and rhizomes of *R. rosea* L. (Crassulaceae) and leaves and shoots of *Z. marina* L. (Zosteraceae) were obtained from certified botanical suppliers (Table 1). Each lot was authenticated by a botanist from macroscopic and microscopic characters and a voucher specimen was lodged in the herbarium. Identity of each extract was also confirmed by thin-layer chromatography (TLC) against a reference marker, which was α-cyperone for *C. rotundus* (Daude et al., 2024), salidroside for *R. rosea* (Chaitanya et al., 2025) and rosmarinic acid for *Z. marina* (Custódio et al., 2015). The material was washed in running water, shade-dried, powdered to 60 mesh and stored in airtight containers at room temperature, protected from light. Loss on drying was determined gravimetrically at 105 °C to constant weight. STZ (analytical grade, ≥ 98% purity) was obtained from Sigma-Aldrich, metformin hydrochloride was of USP/IP reference grade, sodium carboxymethylcellulose (CMC, medium viscosity) was obtained from SRL Diagnostics, citric acid monohydrate, trisodium citrate dihydrate and anhydrous D-glucose were obtained from HiMedia/Merck and the remaining chemicals and reagents used were of analytical grade.

Table 1: Source documentation, extraction parameters and yield of the plant materials

Parameter	***Cyperus rotundus***	***Rhodiola rosea***	***Zostera marina***
Part used	Rhizomes (tubers)	Roots and rhizomes	Leaves and shoots
Source and batch	Certified Botanical Supplier, CR-2026-01	Certified Botanical Supplier, RR-2026-02	Certified Marine Botanical Supplier, ZM-2026-03
Herbarium voucher no.	ARS-HERB-CR-014	ARS-HERB-RR-015	ARS-HERB-ZM-016
Authenticated by	Taxonomy & Pharmacognosy Division	Taxonomy & Pharmacognosy Division	Taxonomy & Pharmacognosy Division
Loss on drying (%)	7.8	6.3	8.0
Powdered material extracted (g)	300.0	300.0	300.0
Dry extract obtained (g)	38.1	52.8	30.0
Extractive yield (% w/w)	12.7	17.6	10.0
TLC reference marker	α-Cyperone	Salidroside	Rosmarinic acid

Extraction in 70% (v/v) ethanol; all extracts were concentrated at ≤ 45 °C under vacuum.

2.2. Preparation of extracts

For each plant, 300.0 g of shade-dried 60-mesh powder was extracted by triple maceration in 70% (v/v) aqueous ethanol at room temperature. The first extraction used 3.0 L for 72 h with periodic agitation, and it was followed by two successive 24 h extractions of the marc with 2.0 L of fresh solvent each (7.0 L in total). The pooled liquid extracts were filtered through Whatman No. 1 paper, concentrated under reduced pressure in a rotary evaporator at 45 °C and dried to constant mass in a vacuum desiccator. The dry extracts were weighed to calculate the extractive yield (Table 1) and stored in amber glass containers at 4 °C under desiccation until use (Figure 1).

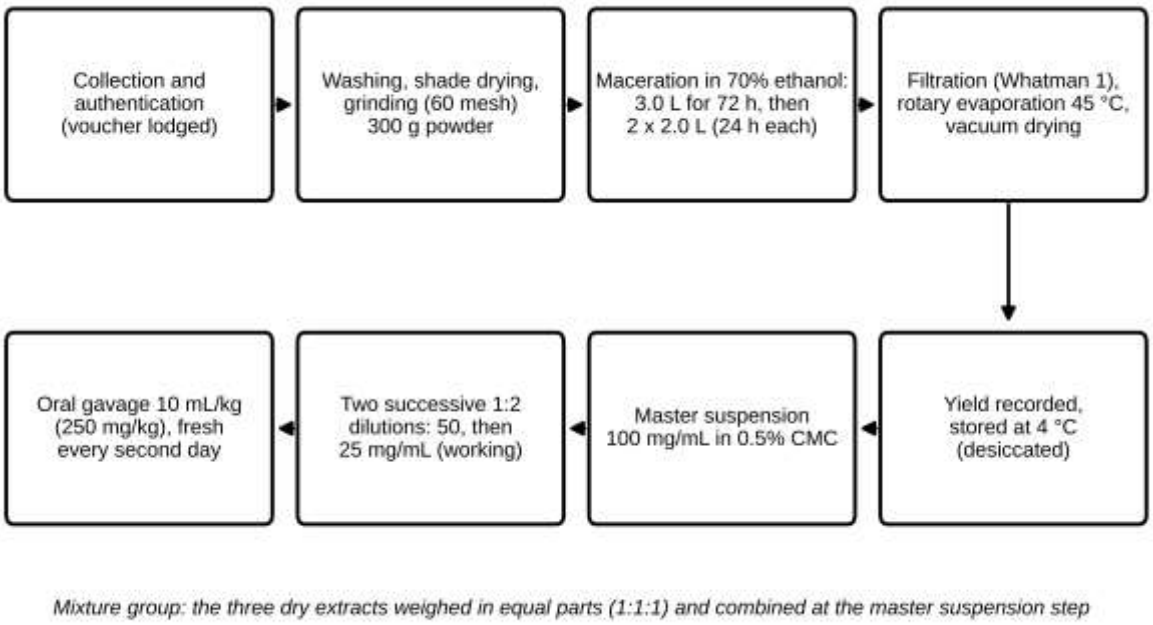


Figure 1: Preparation chain from plant material to oral dosing suspension. Each extract followed the same route, and the mixture group received equal weights of the three dry extracts

2.3. Dosing formulations

The vehicle for all oral preparations was 0.5% (w/v) CMC in distilled water. Extract and metformin suspensions were prepared fresh every second day from dry material weighed once a week, kept at 4 °C between doses and mixed by vortex before each dose. Each extract master suspension was made at 100 mg/mL (0.50 g triturated with a small volume of vehicle and made up to 5.0 mL) and diluted twice in 1:2 steps to the 25 mg/mL working concentration, so that a dose of 250 mg/kg was delivered in 10 mL/kg. A 200 g rat therefore received 2.0 mL containing 50 mg of extract. The mixture was prepared in the same way from equal weights of the three dry extracts (16.7 mg of each in the 2.0 mL dose), and metformin was prepared at 5 mg/mL for 50 mg/kg in the same volume. Volumes were recalculated weekly from the most recent body weight, and across the study they ranged from 1.70 to 2.26 mL per rat. Over 28 days the five rats in each extract group consumed 6.99 g (CR), 6.99 g (RR) and 6.78 g (ZM) of extract, the mixture group 6.66 g of mixture and the metformin group 1.37 g of drug.

2.4. Acute oral toxicity and dose selection

The acute oral toxicity of each extract was assessed earlier in a separate cohort as per OECD Test Guideline 423, the acute toxic class method (OECD., 2002). Nulliparous, non-pregnant female Wistar rats (three per step) were dosed at 2000 mg/kg as a 100 mg/mL suspension in 0.5% CMC at 20 mL/kg after overnight fasting, and they were observed closely for the first 4 h and then daily for 14 days. No animal died and none showed signs of toxicity, and body weight gain was normal for all three extracts. The therapeutic dose of 250 mg/kg is one-eighth of this limit dose. MIX was dosed at the same 250 mg/kg in

total, so each extract in it was given at one-third of its single-extract dose and the single extracts and the mixture were compared at equal total exposure; equal parts were also used in the polyherbal formulations of Das et al. (2025) and Joshi et al. (2026). The single-extract dose lies at the lower end of the 250 to 500 mg/kg range used for the ethanolic *C. rotundus* extract in STZ-diabetic mice (Singh et al., 2015), and within the 100 to 300 mg/kg range of the *R. heterodonta* root extract (Zaripova et al., 2024) and an ethanolic flower extract in STZ-diabetic rats (Kotb El-Sayed et al., 2020). One dose level per extract was used because the cohort was fixed at 35 animals (five per group). Metformin at 50 mg/kg matches the dose used in STZ-diabetic Wistar rats by Adams and Yakubu (2019) and is close to the 55 mg/kg used by Arabloei Sani et al. (2022).

2.5. Experimental animals, husbandry and ethics

Thirty-five healthy male Wistar rats, 8 to 10 weeks old and weighing 185 to 219 g on Day −1, were procured from a CPCSEA-registered breeding facility. Male animals were selected because sex is a known biological variable that alters susceptibility to STZ-induced β-cell ablation (Goyal et al., 2015). Rats were housed five per cage in autoclaved polypropylene cages with dust-free paddy-husk bedding under controlled conditions (22 ± 2 °C, 55 ± 10% relative humidity, 12 h light and 12 h dark cycle), with standard laboratory rodent pellet diet and reverse-osmosis water ad libitum, and they were acclimatised for seven days before the experiment. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC Protocol No. ARS/IAEC/2026/014) and conducted in compliance with CPCSEA guidelines, and the reporting follows the ARRIVE 2.0 guidelines (Percie du Sert et al., 2020). Humane endpoints were set before the start: body weight loss of more than 20% of the Day 0 weight, a glucometer reading above the 600 mg/dL ceiling with clinical distress, or inability to eat or drink. No animal reached any humane endpoint.

2.6. Randomisation, allocation and blinding

Animals were numbered R01 to R35 on arrival and marked on the fur with picric acid. On Day −1 a computer-generated random order assigned them to the seven groups, five per group, and mean body weight on Day −1 did not differ among groups (one-way ANOVA, $F(6, 28) = 0.90$, $p = 0.51$). Each group occupied one cage, and the cage positions on the two-shelf rack were drawn at random and moved one position clockwise each week so that no group stayed in one location. Dosing, glucose readings and weighing were done in a fixed cage order that was itself rotated weekly. Staff measuring HbA1c and serum biochemistry, and the analyst, worked from cage and animal codes and did not know group identity until the database was locked. The person giving the doses could not be blinded to the colour of the suspensions, which differed between extracts. Each animal was the experimental unit (Festing & Altman, 2002).

2.7. Induction of diabetes

Rats were fasted for 12 h with free access to water. The 30 rats in groups 2 to 7 received STZ at 55 mg/kg intraperitoneally (20 mg/mL, 10.2 to 12.0 mg per rat), dissolved in cold 0.1 M citrate buffer (pH 4.5) immediately before use and injected within 15 min, while the normal control rats received the same volume of buffer. This dose and buffer were used by Arabloei Sani et al. (2022) in Wistar rats. Plain water was replaced by 5% glucose solution for the next 24 h to prevent early hypoglycaemia. At 72 h (Day 3), after a 12 h fast, a drop of blood from the tail vein was read on the glucometer, and rats with fasting glucose of 300 mg/dL or more were included, the same threshold that Arabloei Sani et al. (2022) applied at 48 h. This reading became the week 0 baseline.

2.8. Treatment and sampling schedule

The seven groups are given in Table 2. Treatment started on Day 4 and continued for 28 days (28 doses, Day 4 to Day 31), once daily at 09:00 by oral gavage with a ball-tipped stainless steel cannula. Fasting glucose (12 h fast, tail vein, before that day's dose) and body weight were recorded on Day 3 (week 0) and on Days 11, 18, 25 and 32 (weeks 1 to 4), as shown in Figure 2. The duration lies between the 21 days used by Singh et al. (2015) in mice and the 42 days used by Wen et al. (2019), and it equals the four weeks of metformin dosing of Arabloei Sani et al. (2022). On Day 32 the rats were anaesthetised with isoflurane, blood was collected by cardiac puncture for HbA1c (EDTA) and serum, and the animals were euthanised by exsanguination under deep anaesthesia.

Table 2: Experimental groups and oral dosing regimen

Group	Code	n	STZ 55 mg/kg i.p.	Oral treatment (once daily, 28 days)	Dose (mg/kg)	Conc. (mg/mL)
1	NC	5	No (citrate buffer)	Vehicle, 0.5% CMC	–	–
2	DC	5	Yes	Vehicle, 0.5% CMC	–	–
3	MET	5	Yes	Metformin	50	5
4	CR	5	Yes	*C. rotundus* ethanolic extract	250	25
5	RR	5	Yes	*R. rosea* ethanolic extract	250	25
6	ZM	5	Yes	*Z. marina* ethanolic extract	250	25
7	MIX	5	Yes	Mixture of the three extracts, 1:1:1 by weight	250 (total)	25 (total)

CMC, sodium carboxymethylcellulose. All oral doses were given at 10 mL/kg. MIX supplied about 83.3 mg/kg of each extract.

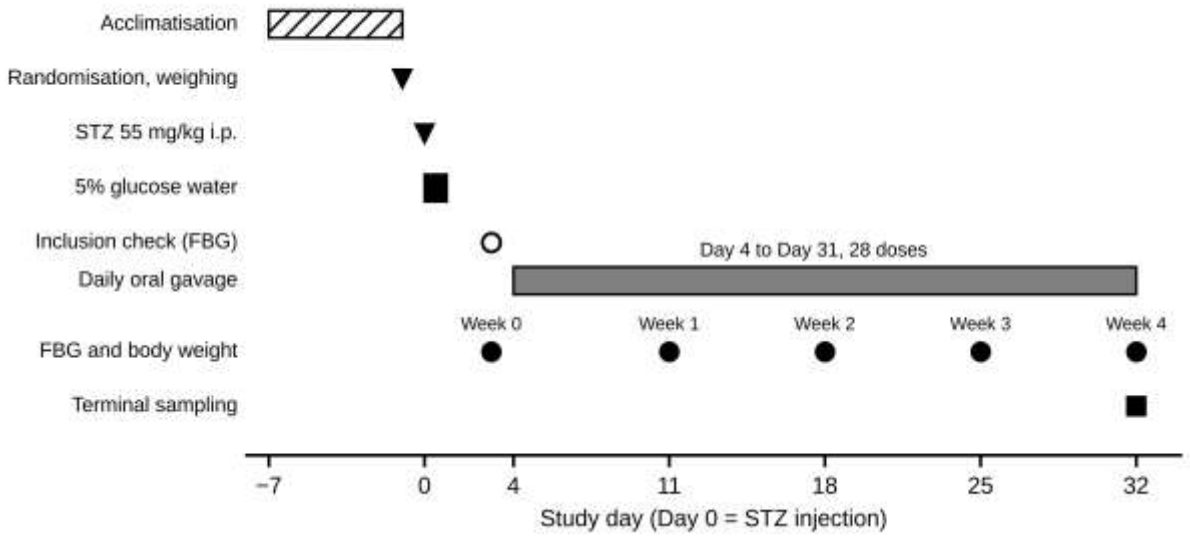


Figure 2: Schedule of study procedures. Day 0 was the day of STZ injection, and fasting glucose and body weight were recorded on Days 3, 11, 18, 25 and 32

2.9. Biochemical and clinical pathology measurements

Fasting glucose was measured on tail-vein blood with a calibrated glucose-oxidase glucometer (Accu-Chek Active, Roche Diagnostics; upper reading limit 600 mg/dL), and body weight was recorded to 1 g. At termination HbA1c (%) was measured in EDTA whole blood by an immunoturbidimetric assay, and serum was separated at 3000 rpm for 15 min. Total cholesterol was measured by the cholesterol oxidase–peroxidase method, triglycerides by the glycerol phosphate oxidase–peroxidase method and HDL-C after precipitation of apolipoprotein B-containing lipoproteins. VLDL-C was calculated as triglycerides/5 and LDL-C by the Friedewald equation (total cholesterol − HDL-C − VLDL-C), and triglycerides were below 400 mg/dL in every sample. ALT and AST were measured by kinetic assay and creatinine by the kinetic Jaffé method. The serum panel was analysed on a clinical biochemistry analyser (Erba Chem 7 / EM200, Transasia Bio-Medicals) with validated commercial kits and quality control sera, and all assays were run in duplicate and the mean was used.

2.10. Study validity and acceptance criteria

Criteria for accepting the run were set before dosing from published values for STZ-diabetic Wistar rats and from the variability attainable with five animals per group (Table 3). A run was accepted if every criterion was met.

2.11. Statistical analysis

Each rat was the experimental unit (n = 5 per group) and the data are expressed as mean ± standard deviation (SD). Fasting glucose at each week, the area under the glucose–time curve (AUC, trapezoidal rule, mg·week/dL), body weight, HbA1c, lipids and the safety markers were each analysed by one-way ANOVA. Normality of residuals was checked by the Shapiro–Wilk test and equality of variances by the Brown–Forsythe test, and both assumptions held for all end points reported ($p > 0.05$). Treated groups and normal control were compared with diabetic control by Dunnett's test, and all pairwise comparisons were made by Tukey's honestly significant difference (HSD) test. A value of $p < 0.05$ (two-sided) was taken as significant. All computations were performed in Python 3 with SciPy, and with 35 animals in 7 groups the one-way design leaves 28 error degrees of freedom.

3. RESULTS

3.1. Induction of diabetes, baseline comparability and study acceptance

All 30 STZ-injected rats were hyperglycaemic at 72 h, with fasting glucose of 339 to 535 mg/dL, and all were included. No rat died and none was withdrawn. Normal control fasting glucose at week 0 was 92 ± 4 mg/dL. Baseline glucose did not differ among the six STZ groups (one-way ANOVA, $F(5, 24) = 0.73$, $p = 0.61$), nor did body weight at week 0 ($F(5, 24) = 1.11$, $p = 0.38$). STZ-injected rats showed polyuria and polydipsia from Day 2. Dosing was tolerated without abnormal behaviour, diarrhoea or local injury, and no group differed from another in clinical observations apart from the signs of diabetes. The results obtained against the pre-specified criteria are given in Table 3, and every criterion was met.

Table 3: Pre-specified study acceptance criteria and observed values

Criterion	Acceptable range	Observed	Met
Fasting glucose at 72 h, every STZ-injected rat	≥ 300 mg/dL	Minimum 339 mg/dL	Yes
Mortality or withdrawal	None	0 of 35	Yes
Normal control, glucose at week 4 (group mean)	86 to 99 mg/dL	94 mg/dL	Yes
Diabetic control, glucose at week 4 (group mean)	410 to 497 mg/dL	439 mg/dL	Yes
Normal control, HbA1c (group mean)	3.9 to 4.4%	4.3%	Yes
Diabetic control, HbA1c (group mean)	8.4 to 9.5%	8.8%	Yes
Diabetic control, total cholesterol (group mean)	123 to 143 mg/dL	127 mg/dL	Yes
Diabetic control, triglycerides (group mean)	172 to 214 mg/dL	196 mg/dL	Yes
Diabetic control, HDL-C (group mean)	23 to 27 mg/dL	25 mg/dL	Yes
Diabetic control, body weight change, week 0 to 4 (group mean)	−22 to −7 g	−10 g	Yes
Metformin, glucose at week 4 (group mean)	192 to 296 mg/dL	264 mg/dL	Yes
Mixture, glucose at week 4 (group mean)	187 to 309 mg/dL	235 mg/dL	Yes
Metformin versus diabetic control, glucose at week 4	Dunnett $p < 0.05$	$p < 0.001$	Yes

Ranges are the interval within which a group mean was accepted.

3.2. Fasting blood glucose and glucose AUC

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A graph was plotted by taking fasting blood glucose on Y-axis and week of treatment on X-axis, which is shown in Figure 3, and the values obtained are given in Table 4. It was found that fasting glucose in diabetic control stayed between 412 and 439 mg/dL (weekly means) and was 439 ± 48 mg/dL at week 4, which is 365% above normal control (94 ± 9 mg/dL). Week 4 glucose differed among groups ($F(6, 28) = 18.18$, $p < 0.0001$). It was observed that metformin lowered it to 264 ± 57 mg/dL (40% below diabetic control, Dunnett $p < 0.001$), CR to 243 ± 70 mg/dL (45%, $p < 0.0001$), RR to 272 ± 48 mg/dL (38%, $p < 0.001$), ZM to 300 ± 67 mg/dL (32%, $p < 0.01$) and MIX to 235 ± 50 mg/dL (46%, $p < 0.0001$). No two treated groups differed at week 4 (Tukey, smallest $p = 0.48$), and no group came back into the normal range.

Metformin, CR, RR and MIX were below diabetic control from week 1 onwards ($p < 0.01$ at every week), whereas ZM separated from diabetic control at week 3 (week 1 $p = 0.076$, week 2 $p = 0.13$, week 3 $p < 0.01$). The AUC over the four weeks differed among groups ($F(6, 28) = 23.83$, $p < 0.0001$) and it was lower than in diabetic control with metformin, CR, RR and MIX ($p < 0.01$ for each) and with ZM ($p < 0.05$). The glucose of every rat is shown in Figure 4, and at week 4 all 5 rats of each treated group were below their own week 0 value.

Table 4: Fasting blood glucose (mg/dL) by week of treatment and glucose AUC (mg·week/dL)

Group	Week 0	Week 1	Week 2	Week 3	Week 4	AUC
Normal control	92 ± 4	95 ± 6	93 ± 6	92 ± 9	94 ± 9	373 ± 24
Diabetic control	412 ± 18	439 ± 30	432 ± 59	429 ± 11	439 ± 48†	1726 ± 113†
Metformin	446 ± 53	349 ± 58	294 ± 51	272 ± 61	264 ± 57***	1269 ± 207**
C. rotundus	405 ± 22	340 ± 47	298 ± 71	258 ± 53	243 ± 70****	1220 ± 210**
R. rosea	411 ± 53	316 ± 35	303 ± 57	290 ± 61	272 ± 48***	1250 ± 191**
Z. marina	428 ± 66	375 ± 50	347 ± 87	300 ± 89	300 ± 67**	1386 ± 286*
Mixture (1:1:1)	446 ± 52	335 ± 30	296 ± 50	262 ± 46	235 ± 50****	1234 ± 162**

Each value represents Mean ± standard deviation (SD) of 5 observations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ against diabetic control (Dunnett's test); † $p < 0.0001$ for diabetic control against normal control. Week 0 is Day 3.

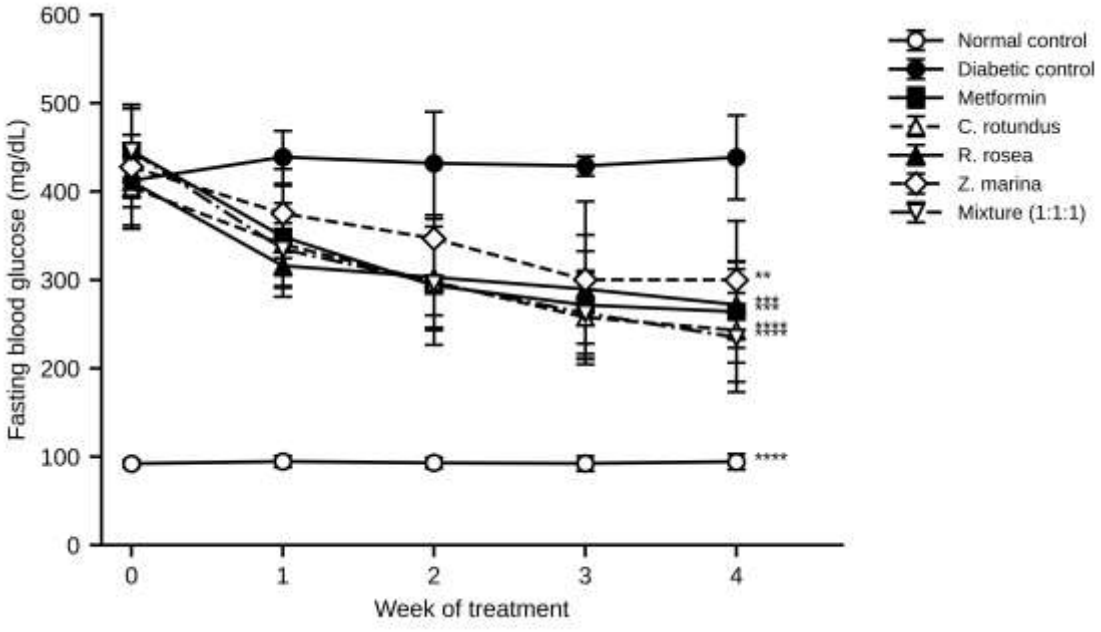


Figure 3: Fasting blood glucose during treatment. Data are mean ± SD (n = 5). Asterisks at week 4 show Dunnett's test against diabetic control: ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (normal control is compared with diabetic control)

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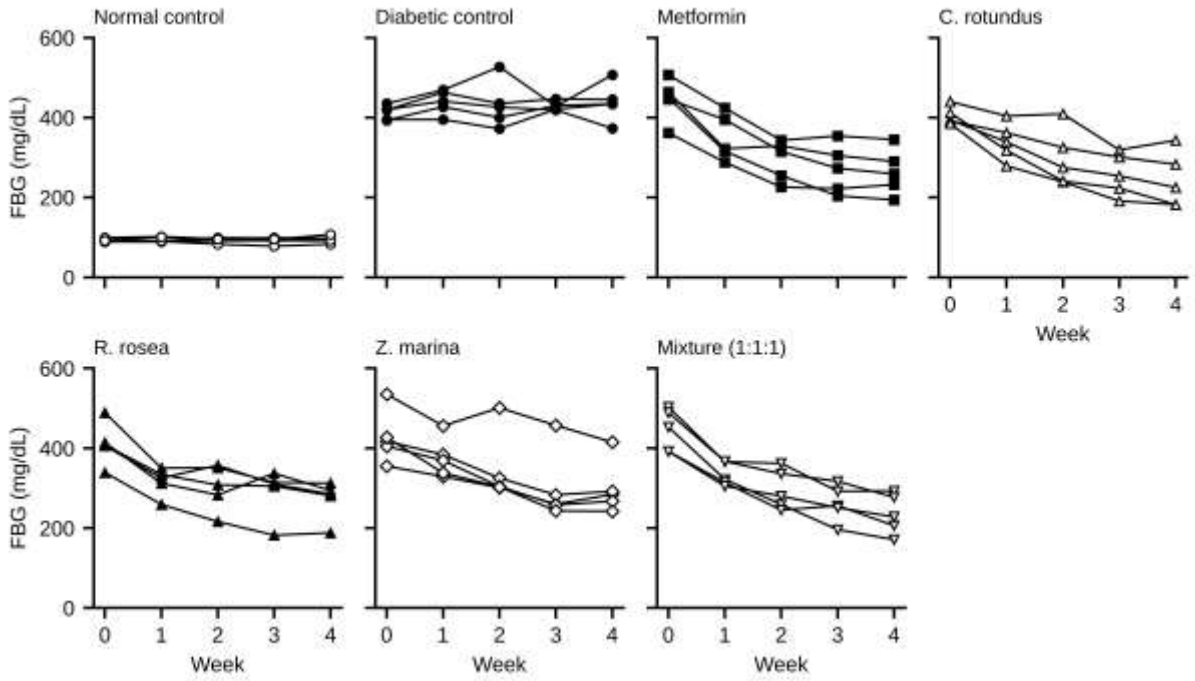


Figure 4: Fasting blood glucose of each rat over the four weeks, one line per animal, by group

3.3. Body weight

The results obtained are given in Table 5 and shown in Figure 5. It was found that normal control rats gained 37 ± 4 g between week 0 and week 4 and diabetic control rats lost 10 ± 6 g ($F(6, 28) = 14.34$, $p < 0.0001$). Weight change was better than in diabetic control with metformin (+10 ± 7 g), CR (+9 ± 11 g), RR (+10 ± 11 g) and MIX (+13 ± 10 g) ($p < 0.01$ for each), whereas with ZM (+1 ± 7 g) the difference was not significant ($p = 0.19$). Final body weight did not differ from diabetic control in any treated group ($p = 0.11$ to 0.70), and no treated group regained the weight of normal control.

Table 5: Body weight (g) from baseline to week 4

Group	Day −1	Week 0	Week 4	Change, week 0 to 4
Normal control	201 ± 4	204 ± 4	241 ± 1	37 ± 4
Diabetic control	206 ± 8	196 ± 8	187 ± 8	−10 ± 6†
Metformin	202 ± 6	192 ± 5	202 ± 7	10 ± 7**
C. rotundus	203 ± 11	197 ± 11	206 ± 21	9 ± 11**
R. rosea	206 ± 6	197 ± 8	206 ± 13	10 ± 11**
Z. marina	204 ± 10	195 ± 10	196 ± 15	1 ± 7
Mixture (1:1:1)	196 ± 7	186 ± 9	199 ± 15	13 ± 10**

Each value represents Mean ± standard deviation (SD) of 5 observations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ against diabetic control (Dunnett's test); † $p < 0.0001$ for diabetic control against normal control.

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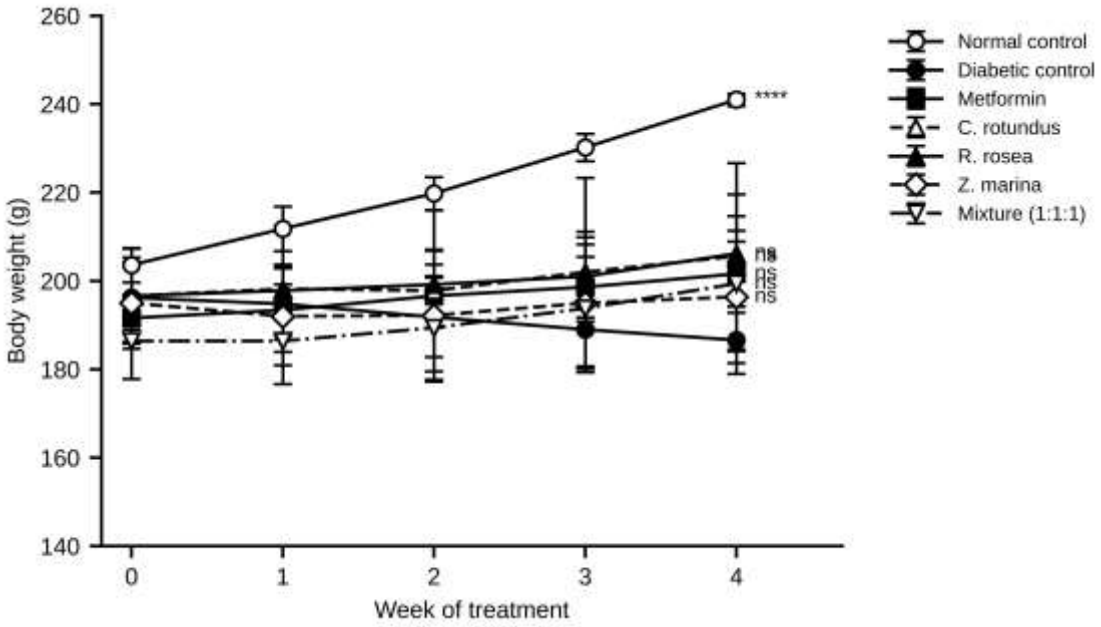


Figure 5: Body weight during treatment. Data are mean ± SD (n = 5). Symbols at week 4 show Dunnett's test of final body weight against diabetic control: **** $p < 0.0001$ for normal control; ns, not significant

3.4. Glycated haemoglobin (HbA1c)

The results obtained are given in Table 6 and shown in Figure 6. HbA1c was 4.3 ± 0.1% in normal control and 8.8 ± 0.5% in diabetic control, a rise of 107% ($F(6, 28) = 24.30$, $p < 0.0001$). It was observed that metformin lowered it to 7.0 ± 0.8% ($p < 0.001$), CR to 6.9 ± 0.5% ($p < 0.001$), RR to 7.1 ± 0.6% ($p < 0.01$), ZM to 7.7 ± 1.0% ($p < 0.05$) and MIX to 7.1 ± 0.6% ($p < 0.001$). No group returned to the normal control value. HbA1c tracked the mean glucose of each rat over the study: across the 30 STZ rats the correlation between HbA1c and mean weekly glucose was $r = 0.96$.

3.5. Serum lipid profile

Diabetic control rats had higher total cholesterol (127 ± 10 mg/dL), triglycerides (196 ± 17 mg/dL) and LDL-C (64 ± 9 mg/dL), and lower HDL-C (25 ± 2 mg/dL), than normal control (69 ± 4, 71 ± 5, 15 ± 6 and 40 ± 3 mg/dL; all $p < 0.0001$). All five treatments lowered total cholesterol, triglycerides and LDL-C and raised HDL-C against diabetic control (Table 6, Figure 7): $p < 0.05$ or smaller in every case, $p < 0.01$ for metformin on all four, and $p < 0.001$ for CR, RR and MIX on all four. Total cholesterol fell by 19% with CR, 21% with RR, 17% with metformin and 24% with MIX, and triglycerides fell by 31%, 31%, 29% and 37% in the same order. Lipid values moved with glycaemia, as across the 30 STZ rats mean weekly glucose correlated with triglycerides ($r = 0.81$) and total cholesterol ($r = 0.82$).

Table 6: Terminal HbA1c (%) and serum lipid profile (mg/dL) at Day 32

Group	HbA1c (%)	TC	TG	HDL-C	LDL-C	VLDL-C
Normal control	4.3 ± 0.1	69 ± 4	71 ± 5	40 ± 3	14.9 ± 5.7	14.3 ± 1.0
Diabetic control	8.8 ± 0.5†	127 ± 10†	196 ± 17†	25 ± 2†	63.5 ± 9.3†	39.3 ± 3.4†
Metformin	7.0 ± 0.8***	105 ± 12**	139 ± 31***	30 ± 3**	47.3 ± 8.4**	27.9 ± 6.1***
C. rotundus	6.9 ± 0.5***	103 ± 6***	136 ± 18***	33 ± 2****	42.8 ± 4.5***	27.2 ± 3.5***
R. rosea	7.1 ± 0.6**	101 ± 8***	135 ± 17****	33 ± 2****	40.6 ± 6.2***	27.0 ± 3.4****
Z. marina	7.7 ± 1.0*	110 ± 10*	145 ± 20***	30 ± 2**	50.5 ± 8.8*	28.9 ± 4.0***
Mixture (1:1:1)	7.1 ± 0.6***	97 ± 8****	125 ± 16****	34 ± 3****	38.9 ± 7.3****	24.9 ± 3.2****

Each value represents Mean ± standard deviation (SD) of 5 observations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ against diabetic control (Dunnett's test); † $p < 0.0001$ for diabetic control against normal control. TC, total cholesterol; TG, triglycerides. LDL-C and VLDL-C were calculated (Friedewald).

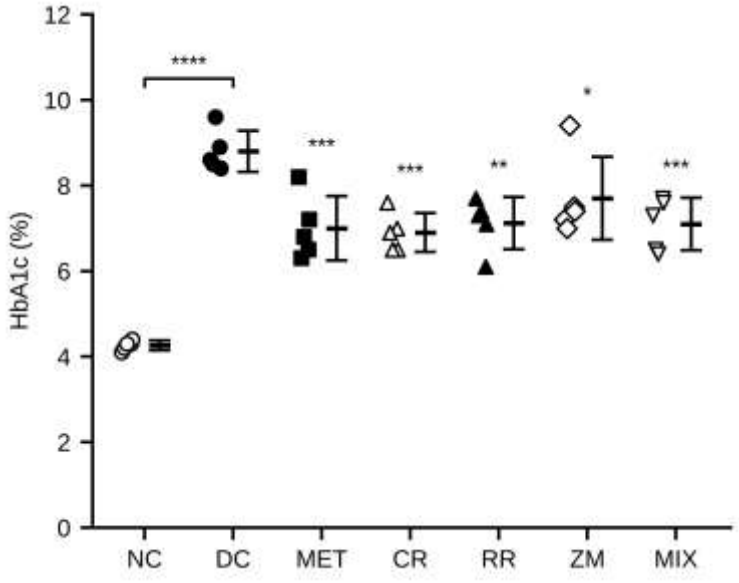


Figure 6: HbA1c at termination. Each symbol is one rat, and the bar and error bars to the right of each group are mean ± SD. Dunnett's test against diabetic control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; bracket, normal control against diabetic control

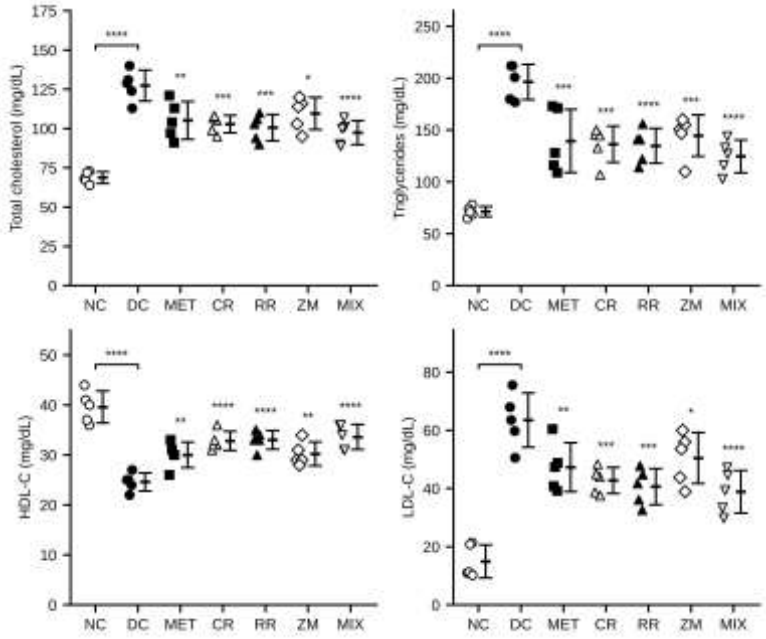


Figure 7: Serum lipid profile at termination (total cholesterol, triglycerides, HDL-C and LDL-C). Symbols and statistics as in Figure 6

3.6. Hepatic and renal markers

Diabetes raised ALT (113 ± 13 against 43 ± 5 U/L), AST (231 ± 27 against 116 ± 13 U/L) and creatinine (0.92 ± 0.09 against 0.49 ± 0.04 mg/dL; all $p < 0.0001$). It was found that all five treated groups were lower than diabetic control for ALT

and creatinine ($p < 0.001$ for each) and for AST ($p < 0.001$ for metformin, CR, RR and MIX, and $p < 0.01$ for ZM), and no extract raised any marker above the diabetic control mean (Table 7, Figure 8).

Table 7: Terminal serum ALT, AST and creatinine at Day 32

Group	ALT (U/L)	AST (U/L)	Creatinine (mg/dL)
Normal control	43 ± 5	116 ± 13	0.49 ± 0.04
Diabetic control	113 ± 13†	231 ± 27†	0.92 ± 0.09†
Metformin	81 ± 12***	161 ± 36***	0.62 ± 0.07****
C. rotundus	65 ± 13****	155 ± 15***	0.62 ± 0.05****
R. rosea	68 ± 10****	159 ± 29***	0.64 ± 0.10****
Z. marina	76 ± 6****	175 ± 18**	0.69 ± 0.10***
Mixture (1:1:1)	65 ± 8****	155 ± 21***	0.57 ± 0.04****

Each value represents Mean ± standard deviation (SD) of 5 observations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ against diabetic control (Dunnett's test); † $p < 0.0001$ for diabetic control against normal control.

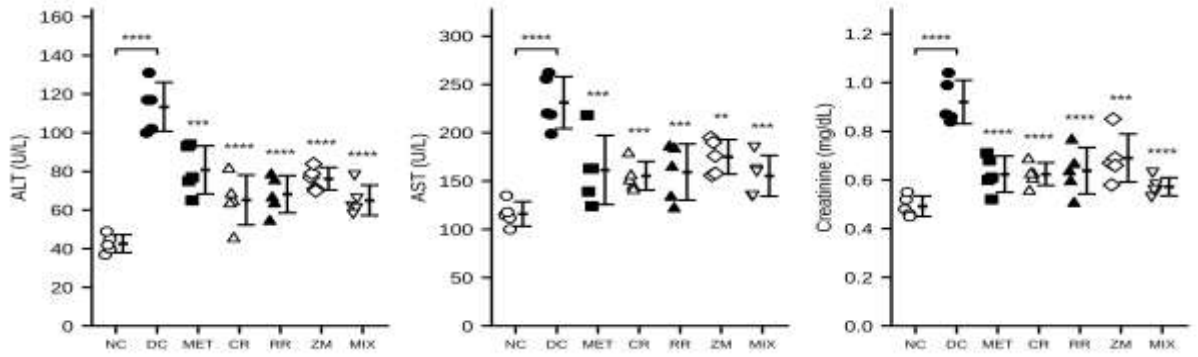


Figure 8: Serum ALT, AST and creatinine at termination. Symbols and statistics as in Figure 6

3.7. Comparison among the treated groups

The exact p-values of the Tukey comparisons among the five treated groups are given in Table 8. It was found that none of the 10 pairs differed significantly for fasting glucose at week 4, HbA1c, total cholesterol, triglycerides or weight change ($p \geq 0.31$), and across all 12 end points analysed the smallest p-value among treated groups was 0.18. By mean week 4 glucose the order was MIX (235 mg/dL), CR (243), metformin (264), RR (272) and ZM (300).

Table 8: Tukey HSD pairwise comparisons among the treated groups (exact p-values)

Comparison	Fasting glucose, week 4	HbA1c	Total cholesterol	Triglycerides	Weight change
Metformin vs CR	0.995	1.000	0.999	1.000	1.000
Metformin vs RR	1.000	1.000	0.978	1.000	1.000
Metformin vs ZM	0.932	0.571	0.983	0.999	0.674
Metformin vs MIX	0.975	1.000	0.784	0.871	0.997
CR vs RR	0.975	0.997	1.000	1.000	1.000
CR vs ZM	0.626	0.417	0.871	0.991	0.782

Comparison	Fasting glucose, week 4	HbA1c	Total cholesterol	Triglycerides	Weight change
CR vs MIX	1.000	0.999	0.953	0.955	0.988
RR vs ZM	0.979	0.756	0.656	0.980	0.719
RR vs MIX	0.923	1.000	0.997	0.976	0.995
ZM vs MIX	0.479	0.727	0.312	0.634	0.338

Tukey's test over all seven groups (28 error degrees of freedom). CR, *C. rotundus*; RR, *R. rosea*; ZM, *Z. marina*; MIX, 1:1:1 mixture.

4. DISCUSSION

The STZ protocol produced stable hyperglycaemia in every injected rat, without deaths. At week 4 the glucose of diabetic control was about 4.6 times that of normal control and HbA1c was 107% higher, body weight fell, and lipids, liver enzymes and creatinine all moved in the direction seen in insulin-deficient diabetes. The dose and buffer match those of Arabloei Sani et al. (2022), who used 55 mg/kg in 0.1 M citrate buffer (pH 4.5) in Wistar rats with a threshold above 300 mg/dL. The 72 h inclusion check after a 12 h fast and the glucose drink after the injection were chosen with the variation in STZ preparation, dose and mortality in mind (Deeds et al., 2011; Goyal et al., 2015). Glucose in diabetic control did not fall over the four weeks, which shows that spontaneous recovery of β-cells was not a confounder at this dose, and every pre-specified acceptance criterion was met (Table 3). Metformin at 50 mg/kg lowered fasting glucose by 40% and HbA1c by 1.8 percentage points, improved weight change and lipids, and lowered ALT, AST and creatinine, and it did not normalise glucose. It was the reference arm at 50 mg/kg for 14 days in STZ-diabetic Wistar rats (Adams & Yakubu, 2019) and at 55 mg/kg for four weeks (Arabloei Sani et al., 2022).

C. rotundus gave the lowest glucose of the three single extracts, 45% below diabetic control, with the difference already significant at week 1, and the lowest HbA1c of any group (6.9 ± 0.5%). Total cholesterol fell by 19% and triglycerides by 31%, against 17% and 29% with metformin and 21% and 31% with *R. rosea*. The lipid response agrees with the lipid-directed actions described for the plant, namely reduced LXRα–SREBP-1c-driven lipogenesis by the hexane fraction (Oh et al., 2015), AMPK and ACC signalling in adipocytes (Pichetkun et al., 2024) and reversal of olanzapine-induced weight gain and lipid change in rats (Kanagali et al., 2021). The outcome agrees in direction with the three-week study of Singh et al. (2015), in which an ethanolic rhizome extract improved body weight and lowered glucose, SGPT, SGOT, cholesterol and triglycerides in STZ-diabetic mice. Inhibition of α-glucosidase by stilbenoid oligomers (Deng et al., 2019) might contribute to the glycaemic effect alongside the glucose-uptake mechanism, and the fall in creatinine is compatible with the renal effect of α-cyperone in diabetic rats (Daude et al., 2024).

R. rosea lowered glucose by 38%, close to metformin (40%), and HbA1c, weight change, lipids, ALT, AST and creatinine all improved. The separation from diabetic control was significant at week 1, and an early effect is plausible given the report that a water extract lowered plasma glucose through adrenal β-endorphin and opioid μ-receptors, with higher skeletal muscle GLUT4 and lower hepatic PEPCK expression after three days of dosing (Niu et al., 2014). Salidroside given for eight weeks lowered fasting glucose in STZ-diabetic Wistar rats from 26.0 to 20.5 mmol/L (about 468 to 369 mg/dL) and improved renal markers (Pei et al., 2022), and the fall in creatinine seen here with the whole extract points the same way. An ethanolic *R. heterodonta* root extract at 100 mg/kg lowered glucose in glucose-, alloxan- and adrenaline-induced diabetic rats (Zaripova et al., 2024), and the dose used here is 2.5 times that. Inhibition of intestinal α-glucosidase by rhodiosin (Wang et al., 2024) acts on postprandial rather than fasting glucose, so it is unlikely to explain a fasting effect. AMPK, PI3K/Akt and GSK-3β signalling (Chaitanya et al., 2025; Zheng et al., 2019) remain candidate mechanisms for the fasting and HbA1c responses.

Z. marina was the least effective single extract. Glucose fell by 32% ($p < 0.01$ at week 4), the separation from diabetic control came at week 3 and not at week 1, and the weight change did not differ from diabetic control ($p = 0.19$). HbA1c, total cholesterol, triglycerides, LDL-C, ALT, AST and creatinine were lower than in diabetic control, but the changes were smaller than with the other treatments. The plant is rich in phenolics, mainly rosmarinic acid (Custódio et al., 2015) and luteolin and its glycosides (Kim et al., 2004), and the polyphenol complex of *Zostera* species and its constituents were reported to act on

carbohydrate and lipid disturbances in animal models (Krivoshapko & Popov, 2011; Krivoshapko et al., 2012; Styshova et al., 2017). The phenolic content of eelgrass varies with season and geography (Grignon-Dubois & Rezzonico, 2023; Li et al., 2022), and this might be one reason for a modest response from a single lot. A recent review of marine plants calls the antidiabetic evidence promising and in need of further work (Ribas-Taberner et al., 2025). At 250 mg/kg the extract produced a modest effect, and a higher dose would be the obvious test of whether the response is dose-limited. MIX was given at 250 mg/kg in total, so each rat received about 83 mg/kg of each extract, one-third of the single-extract dose. It had the lowest mean week 4 glucose of any group (235 ± 50 mg/dL, 46% below diabetic control), the lowest total cholesterol, triglycerides, LDL-C and creatinine, and the highest HDL-C and weight change among the treated groups. None of these differences from the single extracts or from metformin was significant (Tukey, smallest $p = 0.18$ across all end points, Table 8), so the result is that the mixture matched them at one-third of each constituent's dose and did not exceed them. If each extract acted in proportion to its dose and independently of the others, the mixture would fall near the average of the three single-extract responses, and that average was 38% against 46% observed. A response above the average fits either a dose-response curve that flattens towards 250 mg/kg, so that a third of the dose gives more than a third of the effect, or an interaction between constituents. Separating the two needs a dose-response arm for each extract and a factorial design, which the present study did not include, and the interaction can be quantified by the combination index method (Chou, 2006; Chou, 2010; Chou & Talalay, 1984). Equal-part combinations containing *C. rotundus* have been built before, namely GB5, a five-plant ethanolic extract characterised in vitro, in yeast and in 3T3-L1 adipocytes (Das et al., 2025), and Trimad, a 1:1:1 combination of *C. rotundus* tubers, *Embelia ribes* fruits and *Plumbago zeylanica* roots tested in high-fat-fed rats (Joshi et al., 2026). Neither used the plants studied here or an STZ model, so they do not give a direct comparison. The lipid and organ-marker responses followed glycaemia. *Z. marina*, which had the smallest glucose effect, also had the highest HbA1c, total cholesterol, triglycerides, LDL-C, AST and creatinine of the treated groups, MIX had the lowest total cholesterol, triglycerides, LDL-C and creatinine, and across the STZ rats mean glucose and triglycerides were strongly correlated ($r = 0.81$). A parallel is seen in other STZ studies where plant extracts lowered glucose together with total cholesterol, triglycerides and LDL and raised HDL, for example with an ethanolic seed extract of *Annona reticulata* at 50 and 100 mg/kg over 42 days (Wen et al., 2019), with p-cymene given for four weeks (Arabloei Sani et al., 2022) and with an ethanolic extract of *Plicosepalus acaciae* flowers at 150 and 300 mg/kg for 15 days (Kotb El-Sayed et al., 2020). In high-fat-fed rats a 200 mg/kg methanolic extract of *Securigera securidaca* lowered total cholesterol, triglycerides, LDL-C and VLDL-C in groups of five (Aldal'in et al., 2023). Raised liver enzymes and renal markers in diabetic animals, and their fall with treatment, match the pattern in a 21-day study of a *Rivina humilis* extract that measured liver and kidney function and HbA1c (Ramesh et al., 2023) and in STZ-diabetic rats given a *Spilanthes filicaulis* leaf extract at 150 and 300 mg/kg for 15 days, where ALT, AST, lipids, glucose and HbA1c improved (Ojo et al., 2024). For tolerability, none of the three extracts or the mixture raised a marker above the diabetic control level at a dose one-eighth of the acute limit dose. The preceding in vitro study from this laboratory evaluated methanolic extracts of the same three plants, and their 1:1:1 combination, for glucose uptake in insulin-resistant 3T3-L1 adipocytes and for inhibition of α-amylase and α-glucosidase. The ranking in all three assays was the combination, then *C. rotundus*, *R. rosea* and *Z. marina*, and the in vivo ranking by mean week 4 glucose was MIX (46% reduction), *C. rotundus* (45%), *R. rosea* (38%) and *Z. marina* (32%), which is the same order of the four test materials, although the differences among them were not significant in the rat model. The extracts here were ethanolic and those in vitro were methanolic, so the two sets of results are compared for direction and not for magnitude. Fasting glucose and HbA1c in an insulin-deficient STZ model reflect basal hepatic glucose output and peripheral tissue glucose disposal, whereas the intestinal enzyme inhibition seen in vitro acts mainly on the postprandial excursion, which a 12 h fasting sample does not capture. Hence the glucose-uptake finding is the one that the present data can be set beside, and the enzyme findings need an oral glucose tolerance test (OGTT). An OGTT, terminal serum insulin and C-peptide, pancreatic islet histopathology, dose-response arms for each single extract and a factorial design for the mixture are the logical next steps.

5. CONCLUSION

In STZ-diabetic Wistar rats, ethanolic extracts of *C. rotundus*, *R. rosea* and *Z. marina* at 250 mg/kg for 28 days lowered fasting blood glucose by 45%, 38% and 32% relative to untreated diabetic rats, and a 1:1:1 mixture of the three at 250 mg/kg in total lowered it by 46%, against 40% for metformin at 50 mg/kg. *C. rotundus*, *R. rosea* and the mixture lowered HbA1c, total cholesterol, triglycerides and LDL-C to about the same extent as metformin, and *Z. marina* had smaller effects in the same direction and did not improve weight change significantly. All three extracts and the mixture lowered ALT, AST and creatinine relative to diabetic control. By mean week 4 glucose the order was the mixture, *C. rotundus*, metformin, *R. rosea* and

Z. marina, with no significant difference among the treated groups. It can be concluded that the three plants have antidiabetic activity in an STZ model, that the mixture matched the single extracts and metformin at one-third of the dose of each constituent, and that dose-response work on each extract and a factorial test of the mixture are the next steps.

Ethical issues

The study protocol was approved by the Institutional Animal Ethics Committee (IAEC Protocol No. ARS/IAEC/2026/014) and was conducted in compliance with CPCSEA guidelines. The reporting follows the ARRIVE 2.0 guidelines.

CONFLICT OF INTEREST

It is hereby declared that the authors do not have any declaration of interest.

DATA AVAILABILITY

The animal-level data (glucose, body weight, HbA1c, lipid profile and organ-marker values of each rat), the cage allocation log and the analysis script supporting the findings of this study are available from the corresponding author on reasonable request.

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