

Pharmacognostic Standardization and in Vitro Antidiabetic Evaluation of *Cyperus Rotundus* Rhizome, *Rhodiola Rosea* Root and *Zostera Marina* Leaf Extracts and Their 1:1:1 Polyherbal Combination in Insulin-Resistant 3T3-L1 Adipocytes

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Abstract

Purpose: The main objective of the current research work was to standardise three plant materials from different ecological habitats, *Cyperus rotundus* rhizome (tropical sedge), *Rhodiola rosea* root (high-altitude adaptogen) and *Zostera marina* leaf (marine seagrass), and to evaluate their methanolic extracts individually and as a 1:1:1 combined polyherbal extract (CPE) for in vitro antidiabetic activity. To our knowledge, this combination of a terrestrial sedge, an alpine adaptogen and a marine angiosperm has not been evaluated earlier.

Methods: The plant materials were authenticated by macroscopy, microscopy, UV-Visible spectroscopy and high-performance thin-layer chromatography (HPTLC), and physicochemical parameters were determined as per IP/WHO guidelines. Cytotoxicity was determined by MTT assay in 3T3-L1 cells (7.8125–1000 µg/mL, 24 h), and glucose uptake was measured by 2-NBDG in insulin-resistant differentiated 3T3-L1 adipocytes at 62.5, 125 and 250 µg/mL with insulin (100 nM) as standard. α -Amylase and α -glucosidase inhibition were determined against acarbose (15.625–500 µg/mL) and IC₅₀ values were obtained by logistic non-linear regression.

Results: All parameters were within the specified limits, and HPTLC confirmed salidroside (R_f 0.43) and rosin (R_f 0.60) in *R. rosea*, an α -cyperone-type sesquiterpenoid band (R_f 0.45) in *C. rotundus* and rosmarinic acid (R_f 0.71) in *Z. marina*. Viability remained $\geq$ 80% up to 250 µg/mL for all four materials (CR 86.69%, RR 90.41%, ZM 81.28% and CPE 86.00%). CPE at 250 µg/mL restored glucose uptake to 89.36% of normal control against 46.95% in the insulin-resistant control (Dunnett, $p < 0.001$), which was close to insulin (92.31%). CPE showed the lowest IC₅₀ for α -amylase (86.28 µg/mL) and α -glucosidase (68.25 µg/mL) among the extracts, followed by CR, RR and ZM, while acarbose gave 58.26 and 44.63 µg/mL.

Conclusion: It can be concluded that the 1:1:1 combination of *C. rotundus*, *R. rosea* and *Z. marina* acts on peripheral glucose uptake and on both carbohydrate-hydrolysing enzymes, and the combined extract was more active than each single extract even though it carried one-third the dose of each component.

Keywords: *Cyperus rotundus*; *Rhodiola rosea*; *Zostera marina*; polyherbal formulation; 3T3-L1 adipocytes; 2-NBDG glucose uptake; α -amylase; α -glucosidase; HPTLC fingerprinting; pharmacognostic standardization.

1. Introduction

In 2021 about 537 million adults aged 20–79 years were living with diabetes, and the number is projected to reach 783 million by 2045 (Saeedi et al., 2019; Sun et al., 2021). Most of these cases are type 2 diabetes, in which insulin resistance in muscle, liver and adipose tissue runs alongside a progressive loss of pancreatic β -cell function (DeFronzo et al., 2015; Stumvoll et al., 2005). Obesity and ectopic lipid accumulation drive much of this resistance (Kahn et al., 2006; Samuel & Shulman, 2012), and eight separate organ-level defects have been described behind the hyperglycaemia, which is the main argument for combining agents with different targets (DeFronzo, 2009).

Plant-derived agents can act at two of these points. The first point is the digestion of dietary starch by pancreatic α -amylase and intestinal α -glucosidase, which decides the size of the postprandial glucose rise. Acarbose acts at this step and it was shown to reduce the conversion of impaired glucose tolerance to type 2 diabetes, but gastrointestinal side effects limit its acceptance (Chiasson et al., 2002; van de Laar et al., 2005). Plant polyphenols such as flavonoids, phenolic acids and their glycosides inhibit both enzymes and they have been proposed as milder alternatives (Hanhineva et al., 2010; Kumar et al., 2011; Papoutsis et al., 2020; Proença et al., 2017; Tadera et al., 2006; Tundis et al., 2010). The second point is peripheral glucose disposal through insulin-stimulated translocation of the GLUT4 transporter in adipocytes and skeletal muscle (Huang & Czech, 2007; Leto & Saltiel, 2012). Polyphenols are also reported to improve insulin signalling and glucose uptake at this level (Shahwan et al., 2022).

Ayurveda and other traditional systems mostly use plants in combination, on the reasoning that constituents of different plants act on different targets and may give additive or synergistic effects (Wagner & Ulrich-Merzenich, 2009; Williamson, 2001). Network pharmacology describes the same multi-target idea in drug-

discovery terms (Hopkins, 2008), and recent polyherbal antidiabetic formulations have been evaluated with glucose uptake and enzyme inhibition models (Das et al., 2025; Wang et al., 2023). *Cyperus rotundus* L. (Cyperaceae) is a constituent of such formulations and its rhizome is rich in sesquiterpenes such as α -cyperone, cyperene and cyperotundone along with flavonoids (Park et al., 2018; Thebtaranonth et al., 1995; Wang et al., 2022). Its extracts were reported to inhibit α -amylase and α -glucosidase, to improve glucose handling in diabetic rats and to modify glucose uptake in adipocytes and myotubes (Abdella et al., 2024; Mohamed et al., 2021; Pichetkun et al., 2024), and anti-obesity effects were also described (Joshi et al., 2026; Majeed et al., 2022). *Rhodiola rosea* L. (Crassulaceae) is a high-altitude adaptogen whose roots contain salidroside and the cinnamyl alcohol glycosides rosavin, rosarin and rosin (Panossian et al., 2010). *Rhodiola* extracts inhibit α -glucosidase and salidroside activates AMP-activated protein kinase (AMPK) and improves insulin sensitivity (Kwon et al., 2006; Li et al., 2008; Zheng et al., 2015; Zheng et al., 2019).

Zostera marina L. (eelgrass) is a marine angiosperm and its phenolic chemistry is dominated by rosmarinic acid, caffeic acid derivatives and the sulphated phenolic zosteric acid (p-(sulphooxy)cinnamic acid) (Grignon-Dubois & Rezzonico, 2023; Kurth et al., 2015; Li et al., 2022; Zhang et al., 2023; Zhu et al., 2026). Seagrass extracts have shown antioxidant activity and traditional use against diabetes, skin disease and wounds (Ameen et al., 2024; Custódio et al., 2015; Vasarri et al., 2021), and rosmarinic acid itself increases glucose uptake and reverses insulin resistance in muscle cells (Den Hartogh et al., 2023; Vlavcheski et al., 2017). However, *Z. marina* has not been evaluated for antidiabetic activity in an adipocyte model and it has not been combined with terrestrial antidiabetic plants.

The three plants come from a tropical lowland, the Himalayan alpine zone and the coastal sea floor, and they carry different chemical classes, namely sesquiterpenoids and flavonoids, phenylethanoid and phenylpropanoid glycosides, and phenolic acids. Hence the current research was aimed to standardise the three plant materials as per pharmacopoeial guidelines, to fingerprint them by UV-Visible spectroscopy and HPTLC, and to evaluate their methanolic extracts and a 1:1:1 combined polyherbal extract (CPE) for cytotoxicity, glucose uptake in insulin-resistant 3T3-L1 adipocytes, and α -amylase and α -glucosidase inhibition.

2. Materials and methods

2.1. Plant material, chemicals and reagents

Dried rhizomes of *C. rotundus* were obtained from Authentic Herbal Suppliers, Neemuch, Madhya Pradesh (post-monsoon harvest, October 2024, about 480 m), dried root slices of *R. rosea* were obtained from Himalayan Bio-Organics Pvt. Ltd., Leh (late autumn harvest, September 2024, about 3,420 m) and dried leaves of *Z. marina* were obtained from Coastal Flora Traders, Jamnagar, Gujarat (early summer harvest, May 2024, sea level). Voucher specimens (CR/2024/07A, RR/2024/07B and ZM/2024/07C) were deposited in the departmental herbarium (Table 1, Figure 1). Salidroside ($\geq 98\%$) was obtained from Sigma-Aldrich and rosavin ($\geq 98\%$) was obtained from ChromaDex, pre-coated silica gel 60 F₂₅₄ aluminium plates were obtained from Merck, and the remaining chemicals and reagents used were of analytical grade.

Table 1: Source documentation and collection details of the plant materials

Parameter	<i>Cyperus rotundus</i>	<i>Rhodiola rosea</i>	<i>Zostera marina</i>
Voucher specimen no.	CR/2024/07A	RR/2024/07B	ZM/2024/07C
Part used	Rhizome (dried)	Root (dried, sliced)	Leaf (dried)
Habitat	Tropical sedge	High-altitude adaptogen	Marine seagrass
Altitude	~480 m	~3,420 m	Sea level
Season of harvest	Post-monsoon (Oct 2024)	Late autumn (Sep 2024)	Early summer (May 2024)
Supplier	Authentic Herbal Suppliers, Neemuch, MP	Himalayan Bio-Organics Pvt. Ltd., Leh	Coastal Flora Traders, Jamnagar, GJ



Figure 1: Plant materials used in the study: *Cyperus rotundus* growing plant (A) and harvested rhizomes (B), *Rhodiola rosea* flowering plant (C) and roots (D), and *Zostera marina* meadow (E) and dried leaves (F)

2.2. Macroscopic and microscopic evaluation

Organoleptic characters (appearance, colour, odour, taste, texture and size) of the dried materials were recorded as per standard pharmacognostic procedures (Khandelwal, 2015; Trease & Evans, 2020). Free-hand transverse sections (TS) were taken, stained and observed under a compound microscope, and powder microscopy was done on the 40-mesh powders to record the diagnostic elements.

2.3. Preparation of extracts

The dried materials were powdered and passed through a 40-mesh sieve. Each powder was macerated in methanol (1:10 w/v) for 72 h at room temperature with intermittent shaking, and the filtrate was concentrated in a rotary evaporator at 40 °C to give the methanolic extracts of *C. rotundus* (CR), *R. rosea* (RR) and *Z. marina* (ZM). The combined polyherbal extract (CPE) was prepared by mixing CR, RR and ZM in a 1:1:1

(w/w) ratio on a dry-extract basis. For CPE the concentrations are expressed as total extract concentration, so that 250 µg/mL CPE contains 83.33 µg/mL each of CR, RR and ZM (Table 2).

Table 2: Component contribution of CR, RR and ZM in the combined polyherbal extract (CPE, 1:1:1 w/w)

CPE total concentration (µg/mL)	CR (µg/mL)	RR (µg/mL)	ZM (µg/mL)
1000	333.33	333.33	333.33
500	166.67	166.67	166.67
250	83.33	83.33	83.33
125	41.67	41.67	41.67
62.5	20.83	20.83	20.83
31.25	10.42	10.42	10.42
15.625	5.21	5.21	5.21
7.8125	2.60	2.60	2.60

2.4. UV-Visible spectrophotometric analysis

Methanolic extracts (1 mg/mL) were filtered through 0.45 µm PTFE membrane and scanned from 200 to 400 nm in a double-beam UV-Visible spectrophotometer (Shimadzu UV-1800) against methanol as blank. Absorbance at the characteristic wavelengths was recorded for three independent replicates, and each determination was performed in triplicate and the average and standard deviations were calculated.

2.5. Physicochemical parameters

Loss on drying, total ash, acid-insoluble ash, water-soluble ash, swelling index and water-soluble and alcohol-soluble extractive values were determined on the 40-mesh powders as per the Indian Pharmacopoeia and WHO guidelines (Indian Pharmacopoeia Commission, 2022; Khandelwal, 2015; World Health Organization, 2007). For swelling index, 1.0 g of powder was placed in 25 mL of distilled water in a stoppered graduated cylinder, macerated for 24 h with intermittent shaking and the volume of the swollen sediment was measured. For water-soluble and alcohol-soluble extractives, 5 g of powder was macerated with 100 mL of distilled water or ethanol (95% v/v) for 24 h, filtered, and an aliquot of the filtrate was evaporated and dried at 105 °C. For successive extractive values, 20 g of powder was macerated successively with petroleum ether (60–80 °C), chloroform, ethyl acetate, methanol and water (6 h per solvent). Each determination was performed in triplicate and the average and standard deviations were calculated.

2.6. HPTLC fingerprinting

HPTLC was performed on silica gel 60 F₂₅₄ aluminium plates (10 × 10 cm, 0.2 mm). Methanolic extracts (10 mg/mL, 5 µL) were applied as 8 mm bands, 10 mm from the lower edge and 15 mm apart, with a Linomat 5 applicator (CAMAG). Plates were developed to 80 mm in a twin-trough chamber pre-saturated with mobile phase for 20 min and scanned with a CAMAG TLC Scanner 4 (winCATS software) at 254 nm and 366 nm (Reich & Widmer, 2008; Xie et al., 2006). For *C. rotundus*, toluene : ethyl acetate : formic acid (7 : 2.5 : 0.5, v/v/v) was used as mobile phase, and the plate was derivatised with anisaldehyde–sulphuric acid reagent and heated at 105 °C for 10 min. For *R. rosea*, ethyl acetate : methanol : water : formic acid (77 : 13 : 10 : 1, v/v/v/v) was used without derivatisation and salidroside and rosavin standards were co-chromatographed on the same plate. For *Z. marina*, chloroform : ethyl acetate : formic acid (5 : 4 : 1, v/v/v) was used and the plate was derivatised with natural products–polyethylene glycol (NP/PEG) reagent and observed at 366 nm. Peak area was expressed as percentage of total densitometric area.

2.7. Cell culture and adipocyte differentiation

3T3-L1 pre-adipocytes were maintained in DMEM high glucose supplemented with 10% foetal bovine serum and 1% penicillin–streptomycin at 37 °C under 5% CO₂ and seeded in 96-well black clear-bottom plates. Two days after confluence (day 0), differentiation was induced with growth medium containing 0.5 mM 3-isobutyl-1-methylxanthine, 1 µM dexamethasone and 10 µg/mL insulin for 48 h. From day 2 to day 4 the cells were kept in growth medium with 10 µg/mL insulin, and thereafter in growth medium alone, changed every 48 h, until day 8, when more than 90% of the cells showed lipid droplets (Green & Kehinde, 1975). Insulin resistance was induced in the mature adipocytes by exposure to 1 µM dexamethasone for 48 h (Luan et al., 2019), and the insulin-resistant (IR) control received the same treatment without extract (Rotter et al., 2003). The normal control was kept in growth medium without dexamethasone. The extracts were dissolved in dimethyl sulfoxide and diluted in assay medium so that the final vehicle concentration (≤ 0.5% v/v) was kept constant in all wells.

2.8. MTT cytotoxicity assay

Cells were seeded at 1 × 10⁴ cells/well in 96-well flat-bottom plates (100 µL) and allowed to attach for 24 h. Cells were then exposed to CR, RR, ZM and CPE (7.8125–1000 µg/mL, two-fold serial dilution) for 24 h. MTT

(0.5 mg/mL in PBS, 20 µL/well) was added and the plates were incubated for 4 h in the dark, the formazan was dissolved in 100 µL DMSO and absorbance was read at 570 nm with 630 nm as reference in a microplate reader (BR-580, BR Biochem Life Sciences) (Mosmann, 1983; van Meerloo et al., 2011). Vehicle control was taken as 100% viability and 0.1% Triton X-100 was used as positive cytotoxic control. Each OD value was corrected against its corresponding extract blank, because coloured plant extracts and antioxidants can reduce or absorb at the MTT wavelength (Berridge et al., 2005; Bruggisser et al., 2002; Ghasemi et al., 2021). Cell viability was calculated by using the following equation:

$$\text{Cell viability (\%)} = [(OD_{\text{sample}} - OD_{\text{sample blank}}) / (OD_{\text{vehicle control}} - OD_{\text{media blank}})] \times 100$$

The highest concentration retaining $\geq 80\%$ viability was used to select the non-cytotoxic working doses for the functional assay. Representative phase-contrast images were captured at each concentration.

2.9. 2-NBDG glucose uptake assay

Glucose uptake was measured in insulin-resistant differentiated 3T3-L1 adipocytes using the fluorescent glucose analogue 2-NBDG (Yamada et al., 2007; Zou et al., 2005). After induction of insulin resistance, the cells were serum-starved for 2 h in low-glucose DMEM and treated for 24 h with CR, RR, ZM and CPE at 62.5, 125 and 250 µg/mL, and insulin (100 nM, 30 min) was used as standard. Normal control, IR control and vehicle control were run in parallel. The cells were washed with Krebs–Ringer phosphate HEPES (KRPH) buffer and incubated with 100 µM 2-NBDG in glucose-free KRPH buffer for 30 min at 37 °C, washed three times with ice-cold PBS to stop the uptake, and fluorescence was read at 485 nm excitation and 535 nm emission on a fluorescence microplate reader and recorded as relative fluorescence units (RFU). Wells without cells carried through the same steps served as blank. Relative glucose uptake and the increase over the IR control were calculated by using the following equations:

$$\text{Relative glucose uptake (\%)} = [(RFU_{\text{sample}} - RFU_{\text{blank}}) / (RFU_{\text{normal control}} - RFU_{\text{blank}})] \times 100$$

$$\text{Increase vs IR control (\%)} = [(Uptake_{\text{treatment}} - Uptake_{\text{IR control}}) / Uptake_{\text{IR control}}] \times 100$$

2.10. α -Amylase inhibition assay

α -Amylase inhibitory activity was determined with starch as substrate and 3,5-dinitrosalicylic acid reagent, and absorbance was recorded at 540 nm. Extracts and acarbose (reference standard) were tested at 15.625–500 µg/mL. Each reaction OD was corrected against the corresponding reagent or sample blank and the inhibition was calculated by using the following equation:

$$\text{Inhibition (\%)} = [(Corrected OD_{\text{control}} - Corrected OD_{\text{sample}}) / Corrected OD_{\text{control}}] \times 100$$

2.11. α -Glucosidase inhibition assay

α -Glucosidase inhibitory activity was determined with p-nitrophenyl- α -D-glucopyranoside as substrate and the released p-nitrophenol was recorded at 405 nm. Extracts and acarbose were tested at 15.625–500 µg/mL, OD values were blank-corrected and inhibition was calculated by using the same equation as for the α -amylase assay.

2.12. Statistical analysis

All data are expressed as mean $\pm$ standard deviation (SD) of three replicates ($n = 3$). Cell viability was analysed by two-way ANOVA (extract $\times$ concentration), and comparisons at a single concentration were analysed by one-way ANOVA with Tukey's honestly significant difference (HSD) post hoc test. In the glucose uptake assay every group was compared with the IR control by Dunnett's test. IC₅₀ values were obtained by logistic non-linear regression of percentage inhibition against log₁₀ concentration, with the lower asymptote constrained to 0% and the upper asymptote and slope fitted on all replicate points; 95% confidence intervals were derived from the regression and goodness of fit was expressed as R^2 (Gadagkar & Call, 2014; Ritz et al., 2015; Sebaugh, 2011). A value of $p < 0.05$ was taken as significant. All computations were performed in Python (SciPy and statsmodels).

3. Results

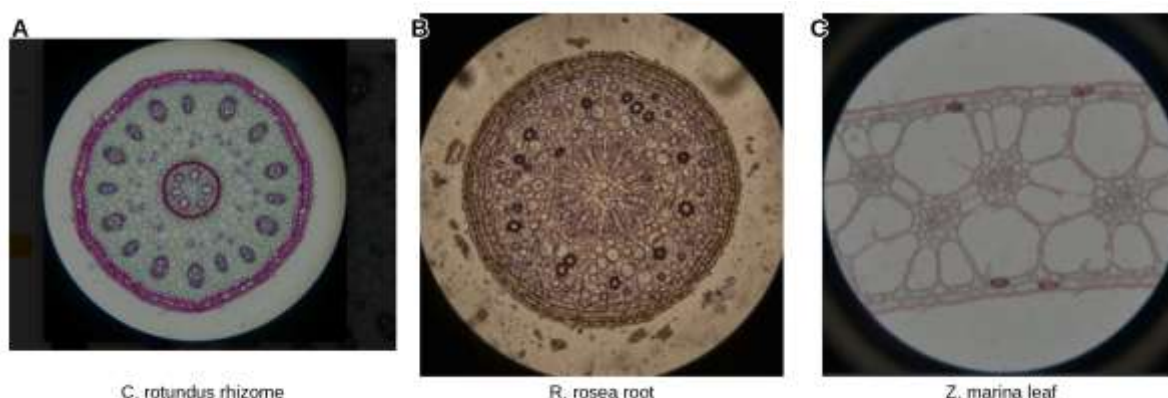
3.1. Macroscopic and microscopic characters

The organoleptic characters of the three materials are given in Table 3. *C. rotundus* rhizomes were ovoid to fusiform, dark reddish-brown externally and cream internally, with an aromatic camphoraceous odour and a bitter, then pungent taste. *R. rosea* slices were brownish-yellow with a rose-gold sheen on fracture and a distinct rose-like odour, and *Z. marina* leaves were ribbon-like, dull green, with a saline marine odour and mucilaginous taste. These observations are consistent with the standard pharmacognostic descriptions (Khandelwal, 2015; Trease & Evans, 2020).

Table 3: Macroscopic and organoleptic characteristics of the dried plant materials

Feature	<i>C. rotundus</i> (rhizome)	<i>R. rosea</i> (root)	<i>Z. marina</i> (leaf)
Appearance	Whole, dried, ovoid to fusiform	Transversely cut slices, uneven	Fragmented, ribbon-like strips
Colour	Externally dark reddish-brown to blackish; internally cream to pale brown	Brownish-yellow with rose-gold sheen on fracture	Dull green to brownish-green
Odour	Aromatic, camphoraceous, slightly pungent	Distinctly rose-like, fragrant	Faintly marine, saline, seaweed-like
Taste	Initially bitter, followed by aromatic, pungent aftertaste	Slightly bitter, astringent	Saline, mucilaginous
Texture	Hard, woody; fibrous fracture	Hard, rough; granular fracture	Brittle, papery; parallel venation prominent
Size range	1.4–3.7 cm length; 0.7–2.1 cm diameter	1.8–5.3 cm diameter (slices variable)	18–52 cm length; 0.3–0.9 cm width

In TS of *C. rotundus* rhizome, a thick-walled epidermis with thin cuticle and a parenchymatous cortex with scattered conjoint, collateral, closed vascular bundles were observed. An endodermis with prominent Casparian strips surrounded the pericycle and stele, and the parenchyma contained simple starch grains (8–18 μm) and oil globules, while cork cells were sparse. In TS of *R. rosea* root, a cork layer of thin-walled tangentially elongated cells, a cortex with stone cells and concentrically striated starch grains (15–25 μm), and radially arranged vascular bundles with spiral and reticulate xylem vessels were observed. The *Z. marina* leaf showed a thin astomatous epidermis with mucilage-secreting cells, a mesophyll of extensive aerenchyma without differentiated palisade or spongy layers, small vascular bundles in a parenchymatous sheath and occasional silica deposits near the epidermis (Figure 2). Powder microscopy confirmed the same diagnostic elements for each material.

**Figure 2: Transverse sections of *C. rotundus* rhizome (A), *R. rosea* root (B) and *Z. marina* leaf (C) showing the diagnostic histological features**

3.2. UV-Visible spectral characteristics

The UV-Visible spectra of the three methanolic extracts are shown in Figure 3 and the absorbance at the characteristic wavelengths is given in Table 4. It was observed that *C. rotundus* showed maxima at 276 and 321 nm, *R. rosea* at 252 and 278 nm and *Z. marina* at 328 nm. The relative standard deviation of the replicates was below 3% at every wavelength. The bands at 276–278 nm correspond to phenolic and phenylethanoid chromophores such as salidroside (Panossian et al., 2010), and the 328 nm maximum of *Z. marina* is in agreement with the caffeoyl chromophore of rosmarinic acid and caffeic acid derivatives reported in eelgrass (Grignon-Dubois & Rezzonico, 2023; Harborne, 1998; Li et al., 2022).

Table 4: Absorbance of the methanolic extracts at characteristic wavelengths

Wavelength (nm)	R1	R2	R3	Mean $\pm$ SD	RSD (%)
<i>C. rotundus</i> (methanolic extract, 1 mg/mL)					
216	1.1420	1.1280	1.1570	1.1423 $\pm$ 0.0145	1.27
254	0.7140	0.7030	0.7290	0.7153 $\pm$ 0.0131	1.82

Wavelength (nm)	R1	R2	R3	Mean $\pm$ SD	RSD (%)
276 ^a	0.8470	0.8310	0.8620	0.8467 $\pm$ 0.0155	1.83
305	0.5830	0.5720	0.5980	0.5843 $\pm$ 0.0131	2.23
321 ^a	0.6120	0.6270	0.5960	0.6117 $\pm$ 0.0155	2.53
R. rosea (methanolic extract, 1 mg/mL)					
219	1.1240	1.1380	1.1070	1.1230 $\pm$ 0.0155	1.38
252 ^a	0.9230	0.9410	0.9160	0.9267 $\pm$ 0.0129	1.39
278 ^a	0.7680	0.7590	0.7820	0.7697 $\pm$ 0.0116	1.51
310	0.4310	0.4470	0.4230	0.4337 $\pm$ 0.0122	2.82
Z. marina (methanolic extract, 1 mg/mL)					
254	0.4870	0.5020	0.4740	0.4877 $\pm$ 0.0140	2.87
292	0.5340	0.5210	0.5490	0.5347 $\pm$ 0.0140	2.62
310	0.6120	0.5980	0.6270	0.6123 $\pm$ 0.0145	2.37
328 ^a	0.8910	0.8740	0.9070	0.8907 $\pm$ 0.0165	1.85

^aCharacteristic absorption maximum ($\lambda_{\max}$). *Each value represents Mean $\pm$ standard deviation (SD) of 3 observations

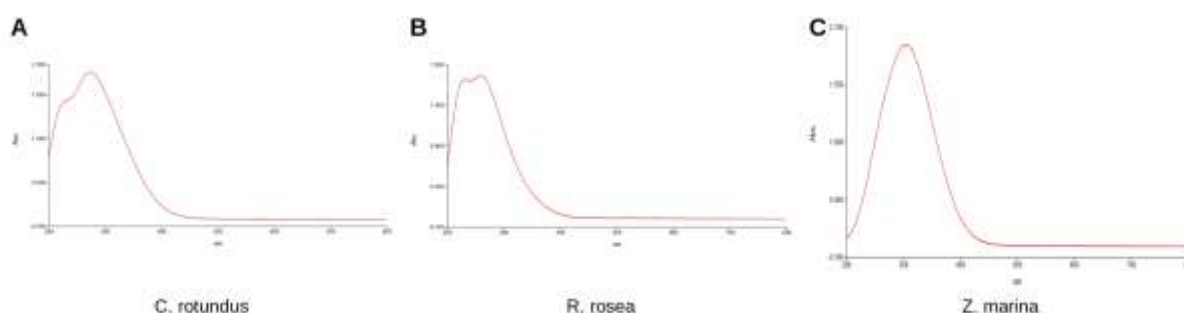


Figure 3: UV-Visible absorption spectra (200–800 nm) of the methanolic extracts of *C. rotundus* (A), *R. rosea* (B) and *Z. marina* (C)

3.3. Physicochemical parameters

The results obtained are given in Table 5. It was found that loss on drying was $7.81 \pm 0.13\%$ for *C. rotundus*, $9.03 \pm 0.12\%$ for *R. rosea* and $11.47 \pm 0.24\%$ for *Z. marina*, which were within the limit of not more than 10–12%. Total ash ($5.16 \pm 0.10\%$ and $3.53 \pm 0.08\%$) and acid-insoluble ash ($1.24 \pm 0.06\%$ and $0.51 \pm 0.04\%$) of *C. rotundus* and *R. rosea* were within the pharmacopoeial limits, confirming low inorganic contamination. *Z. marina* showed high total ash ($19.16 \pm 0.22\%$) and water-soluble ash ($12.57 \pm 0.18\%$), and this is characteristic of marine angiosperms, which accumulate mineral salts and silicates from sea water (Ameen et al., 2024; Vasarri et al., 2021). Its acid-insoluble ash ($4.15 \pm 0.14\%$) was within the limit of not more than 5% applied for the marine leaf, so siliceous contamination was not significant.

It was observed that *Z. marina* showed the highest swelling index (8.27 ± 0.21 mL/g), which reflects its mucilage and hydrocolloid content. *C. rotundus* showed moderate swelling (3.13 ± 0.15 mL/g) due to its starch content, while *R. rosea* showed minimal swelling (2.50 ± 0.10 mL/g), consistent with the absence of mucilaginous constituents in the root (Panossian et al., 2010). *Z. marina* yielded considerably higher water-soluble ($29.04 \pm 0.26\%$) than alcohol-soluble extractive ($15.84 \pm 0.18\%$), reflecting its polysaccharide and salt content, whereas *R. rosea* gave the highest alcohol-soluble extractive ($19.06 \pm 0.15\%$), consistent with its glycoside-rich profile. *C. rotundus* gave moderate values in both solvents ($14.95 \pm 0.23\%$ and $11.72 \pm 0.17\%$), which is in agreement with its terpenoid–phenolic composition (Thebtaranonth et al., 1995; Wang et al., 2022).

Table 5: Physicochemical parameters of the powdered plant materials

Parameter	<i>C. rotundus</i>	<i>R. rosea</i>	<i>Z. marina</i>	Limit
Loss on drying (% w/w)	7.81 ± 0.13	9.03 ± 0.12	11.47 ± 0.24	NMT 10–12%
Total ash (% w/w)	5.16 ± 0.10	3.53 ± 0.08	19.16 ± 0.22	NMT 8% (CR), 5% (RR), 25% (ZM)
Acid-insoluble ash (% w/w)	1.24 ± 0.06	0.51 ± 0.04	4.15 ± 0.14	NMT 3% (CR), 1% (RR), 5% (ZM)

Parameter	<i>C. rotundus</i>	<i>R. rosea</i>	<i>Z. marina</i>	Limit
Water-soluble ash (% w/w)	2.56 ± 0.07	2.01 ± 0.04	12.57 ± 0.18	NLT 2%
Swelling index (mL/g)	3.13 ± 0.15	2.50 ± 0.10	8.27 ± 0.21	—
Water-soluble extractive (% w/w)	14.95 ± 0.23	24.12 ± 0.22	29.04 ± 0.26	—
Alcohol-soluble extractive (% w/w)	11.72 ± 0.17	19.06 ± 0.15	15.84 ± 0.18	—

NMT, not more than; NLT, not less than. Limits as per the Ayurvedic Pharmacopoeia of India (*C. rotundus*), WHO guidelines for herbal drugs and the limits applied for marine plant material (*Z. marina*). *Each value represents Mean ± standard deviation (SD) of 3 observations

The successive extractive values are given in Table 6. For *C. rotundus*, the non-polar fractions gave 4.15% (petroleum ether) and 3.55% (chloroform), and together with the ethyl acetate fraction (5.87 ± 0.08%) this confirms the sesquiterpenoid essential oil (α -cyperone, cyperene) character of the rhizome (Park et al., 2018; Wang et al., 2022). *R. rosea* gave high polar yields in methanol (16.01 ± 0.17%) and water (19.10 ± 0.22%), in line with its polar glycosides salidroside, rosavin and rosarin (Panossian et al., 2010; Zhang et al., 2021). *Z. marina* gave the highest aqueous yield (22.59 ± 0.19%) with a low lipid fraction (< 1%), which reflects marine polysaccharides and inorganic salts.

Table 6: Successive extractive values of the plant materials (% w/w)

Solvent	<i>C. rotundus</i>	<i>R. rosea</i>	<i>Z. marina</i>
Ethyl acetate	5.87 ± 0.08	4.66 ± 0.13	3.15 ± 0.12
Methanol	9.17 ± 0.15	16.01 ± 0.17	11.57 ± 0.21
Aqueous (residual)	10.60 ± 0.16	19.10 ± 0.22	22.59 ± 0.19

Successive maceration: petroleum ether (60–80 °C) → chloroform → ethyl acetate → methanol → water, 6 h per solvent. *Each value represents Mean ± standard deviation (SD) of 3 observations

3.4. HPTLC fingerprinting

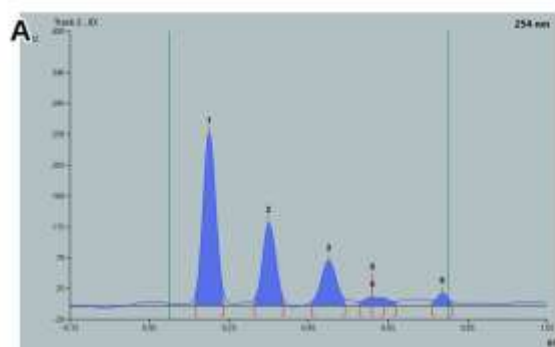
The HPTLC densitograms and plate photographs are shown in Figure 4 and the peak profiles are given in Table 7. For *C. rotundus*, it was found that the dominant peak at R_f 0.45 (34.20% of total area at 254 nm) gave a reddish-brown colour after anisaldehyde–sulphuric acid derivatisation, consistent with the α -cyperone-type sesquiterpenoid fraction which is the principal marker of the rhizome (Park et al., 2018; Thebtaranonth et al., 1995). The band at R_f 0.28 (21.80%) gave a bluish-purple colour consistent with quercetin/kaempferol glycosides reported in this species (Abdella et al., 2024; Wang et al., 2022), the band at R_f 0.67 (18.50%) corresponded to a caffeic or ferulic acid derivative and the non-polar band at R_f 0.82 (12.30%) corresponded to sesquiterpene hydrocarbons such as cyperene.

For *R. rosea*, the sample peaks at R_f 0.43 and 0.60 superimposed on the co-chromatographed salidroside (R_f 0.44) and rosavin (R_f 0.61) standards, confirming their identity. Salidroside was the dominant marker (28.70% of the profile at 254 nm) followed by rosavin (19.30%), and a band at R_f 0.74 (12.10%) was assigned to rosarin/rosin on the basis of R_f and literature. The presence of the full rosavin–rosarin–rosin group, and not salidroside alone, distinguishes authentic *R. rosea* from other *Rhodiola* species and from adulterated commercial material (Booker et al., 2015; Booker et al., 2016). For *Z. marina*, the intense blue-fluorescent band at R_f 0.71 (38.90%) was assigned to rosmarinic acid, which matches the UV maximum at 328 nm, and the orange-fluorescent band at R_f 0.55 (14.20%) was tentatively assigned to zosteric acid, the sulphated phenolic of *Zostera* species studied for its anti-fouling activity (Jendresen & Nielsen, 2019; Kurth et al., 2015; Zhu et al., 2026). A green fluorescent band at R_f 0.82 (11.50%) corresponded to a caffeic acid derivative.

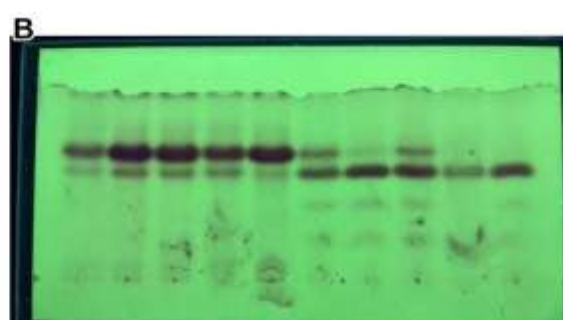
Table 7: HPTLC fingerprint profiles of the methanolic extracts

Peak	R_f	Detection response	Assignment	Peak area (% of total)
<i>C. rotundus</i> — toluene : ethyl acetate : formic acid (7 : 2.5 : 0.5); anisaldehyde–H₂SO₄; 254 nm				
1	0.28	Dark quenching / bluish-purple	Flavonoid glycoside (R_f consistent with quercetin-3-glucoside)	21.80
2	0.45	Moderate quenching / reddish-brown	Sesquiterpenoid (α -cyperone type)	34.20
3	0.67	Faint quenching / brownish-red	Phenolic acid (caffeic/ferulic acid derivative)	18.50

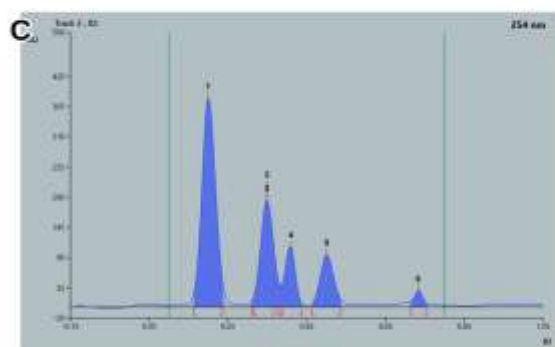
Peak	R _f	Detection response	Assignment	Peak area (% of total)
4	0.82	No quenching / violet	Sesquiterpene hydrocarbon (cyperene/ β -selinene)	12.30
—	—	—	Minor peaks / baseline	13.20
R. rosea — ethyl acetate : methanol : water : formic acid (77 : 13 : 10 : 1); no derivatisation; 254 nm				
1	0.22	Faint quenching	Polar constituent (no matching standard)	8.40
2	0.43	Strong quenching	Salidroside (standard R _f 0.44)	28.70
3	0.60	Moderate quenching	Rosavin (standard R _f 0.61)	19.30
4	0.74	Faint quenching	Rosarin/rosin (R _f and literature)	12.10
—	—	—	Minor peaks / baseline	31.50
Z. marina — chloroform : ethyl acetate : formic acid (5 : 4 : 1); NP/PEG; 366 nm				
1	0.39	Faint blue fluorescence	Phenolic constituent	6.80
2	0.55	Orange fluorescence	Zosteric acid (tentative)	14.20
3	0.71	Intense blue fluorescence	Rosmarinic acid	38.90
4	0.82	Green fluorescence	Caffeic acid derivative	11.50
—	—	—	Minor peaks / baseline	28.60



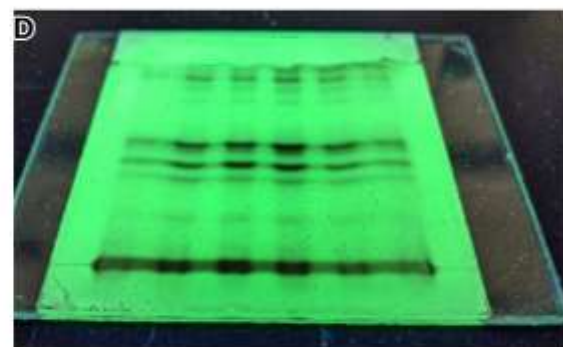
C. rotundus, densitogram 254 nm



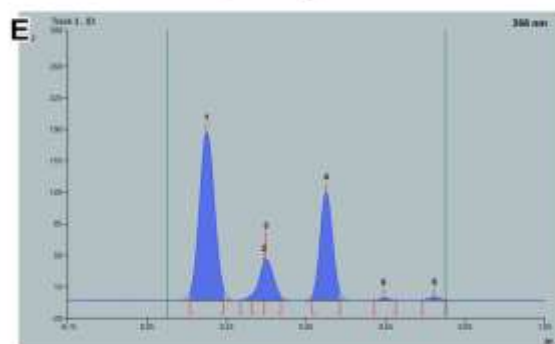
C. rotundus, plate 254 nm



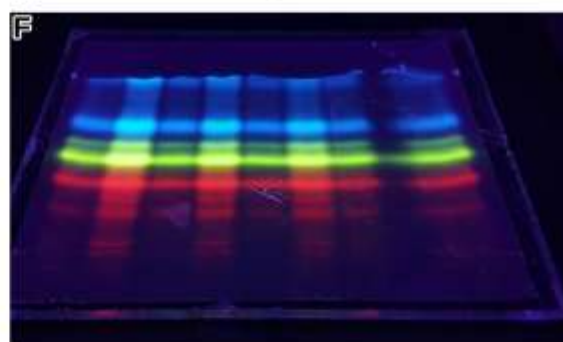
R. rosea, densitogram 254 nm



R. rosea, plate 254 nm



Z. marina, densitogram 366 nm



Z. marina, plate 366 nm (NP/PEG)

Figure 4: HPTLC densitograms and plate photographs of *C. rotundus* at 254 nm (A, B), *R. rosea* at 254 nm (C, D) and *Z. marina* at 366 nm after NP/PEG derivatisation (E, F)

3.5. Cytotoxicity in 3T3-L1 cells

The control OD values are given in Table 8. Vehicle control (0.9477 ± 0.0065) did not differ from the normal control (101.12% viability), and 0.1% Triton X-100 reduced the viability to 12.30%. A graph was plotted by taking % cell viability on Y-axis and concentration on X-axis, which is shown in Figure 5, and the viability values are given in Table 9. It was found that all four materials reduced viability in a concentration-dependent manner. Two-way ANOVA showed significant effects of extract ($F = 348.04$), concentration ($F = 3933.39$) and their interaction ($F = 21.80$; all $p < 0.001$).

It was observed that viability remained $\geq 80\%$ up to $250 \mu\text{g/mL}$ for every material, with $86.69 \pm 0.23\%$ for CR, $90.41 \pm 1.41\%$ for RR, $81.28 \pm 0.18\%$ for ZM and $86.00 \pm 0.51\%$ for CPE. At $1000 \mu\text{g/mL}$ the viability was 71.49% for RR, 66.19% for CR, 63.79% for CPE and 58.41% for ZM, and all pairwise differences at this concentration were significant (one-way ANOVA $F = 141.09$, $p < 0.001$; Tukey $p \leq 0.025$). The phase-contrast images (Figure 6) showed the spindle-shaped fibroblast-like morphology of 3T3-L1 cells up to $250 \mu\text{g/mL}$, and rounding and loss of cell density were seen only at the higher concentrations and with Triton X-100. Hence 62.5, 125 and $250 \mu\text{g/mL}$ were finally selected as working concentrations for all four materials, which kept the comparison uniform while remaining within the non-cytotoxic range.

Table 8: Control OD₅₇₀ values of the MTT assay on 3T3-L1 cells

Control	R1	R2	R3	Mean OD $\pm$ SD	Cell viability (%)
Blank	0.0850	0.0870	0.0860	0.0860 ± 0.0010	—
Normal control	0.9510	0.9630	0.9580	0.9573 ± 0.0060	101.12
Vehicle control	0.9410	0.9540	0.9480	0.9477 ± 0.0065	100.00
Triton X-100 (0.1%)	0.1890	0.1900	0.1970	0.1920 ± 0.0044	12.30

*Each value represents Mean $\pm$ standard deviation (SD) of 3 observations

Table 9: Effect of CR, RR, ZM and CPE on OD₅₇₀ and cell viability of 3T3-L1 cells after 24 h

Concentration ($\mu\text{g/mL}$)	CR	RR	ZM	CPE
1000	0.6563 ± 0.0093 (66.19 $\pm$ 1.08)	0.7020 ± 0.0052 (71.49 $\pm$ 0.60)	0.5893 ± 0.0035 (58.41 $\pm$ 0.41)	0.6357 ± 0.0078 (63.79 $\pm$ 0.90)
500	0.7617 ± 0.0153 (78.41 $\pm$ 1.78)	0.8030 ± 0.0044 (83.21 $\pm$ 0.51)	0.6943 ± 0.0100 (70.60 $\pm$ 1.16)	0.7450 ± 0.0060 (76.48 $\pm$ 0.70)
250	0.8330 ± 0.0020 (86.69 $\pm$ 0.23)	0.8650 ± 0.0121 (90.41 $\pm$ 1.41)	0.7863 ± 0.0015 (81.28 $\pm$ 0.18)	0.8270 ± 0.0044 (86.00 $\pm$ 0.51)
125	0.8820 ± 0.0078 (92.38 $\pm$ 0.91)	0.9107 ± 0.0078 (95.71 $\pm$ 0.90)	0.8583 ± 0.0100 (89.63 $\pm$ 1.16)	0.8883 ± 0.0012 (93.11 $\pm$ 0.13)
62.5	0.9133 ± 0.0035 (96.02 $\pm$ 0.41)	0.9393 ± 0.0023 (99.03 $\pm$ 0.27)	0.8973 ± 0.0057 (94.16 $\pm$ 0.66)	0.9237 ± 0.0050 (97.21 $\pm$ 0.58)
31.25	0.9357 ± 0.0046 (98.61 $\pm$ 0.54)	0.9553 ± 0.0006 (100.89 $\pm$ 0.07)	0.9260 ± 0.0010 (97.49 $\pm$ 0.12)	0.9427 ± 0.0015 (99.42 $\pm$ 0.18)
15.625	0.9503 ± 0.0032 (100.31 $\pm$ 0.37)	0.9633 ± 0.0029 (101.82 $\pm$ 0.34)	0.9400 ± 0.0044 (99.11 $\pm$ 0.51)	0.9520 ± 0.0036 (100.50 $\pm$ 0.42)
7.8125	0.9563 ± 0.0061 (101.01 $\pm$ 0.71)	0.9583 ± 0.0023 (101.24 $\pm$ 0.27)	0.9477 ± 0.0038 (100.00 $\pm$ 0.44)	0.9570 ± 0.0053 (101.08 $\pm$ 0.61)

Values are mean OD₅₇₀ $\pm$ SD with cell viability (% of vehicle control, mean $\pm$ SD) in parentheses. *Each value represents Mean $\pm$ standard deviation (SD) of 3 observations

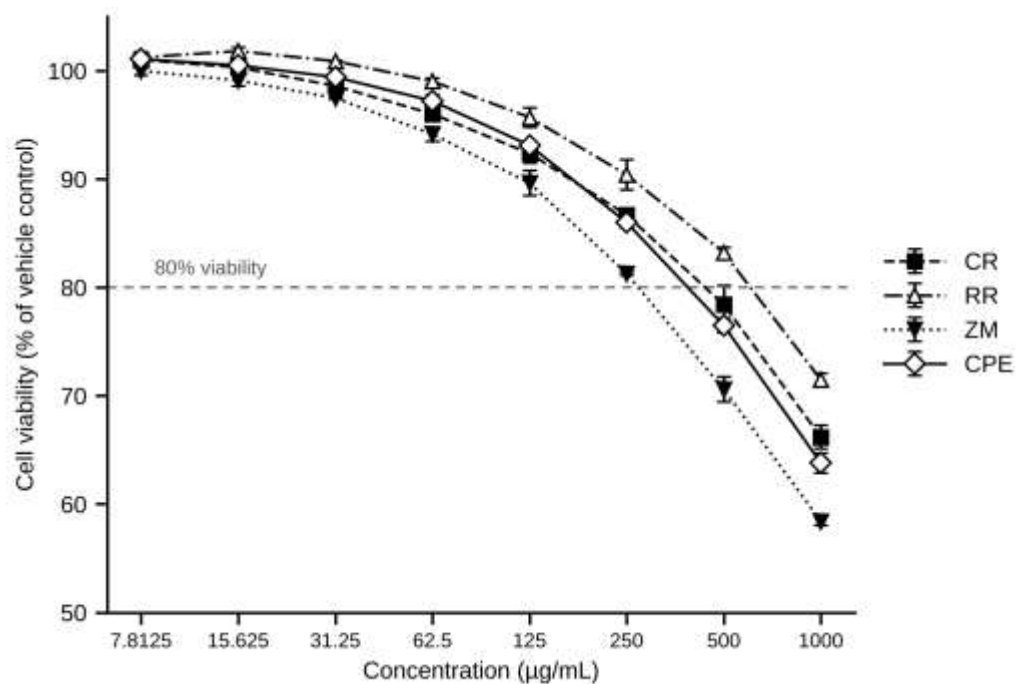


Figure 5: Concentration-dependent effect of CR, RR, ZM and CPE on the viability of 3T3-L1 cells after 24 h exposure (MTT assay). Data are mean $\pm$ SD (n = 3); the dashed line marks 80% viability

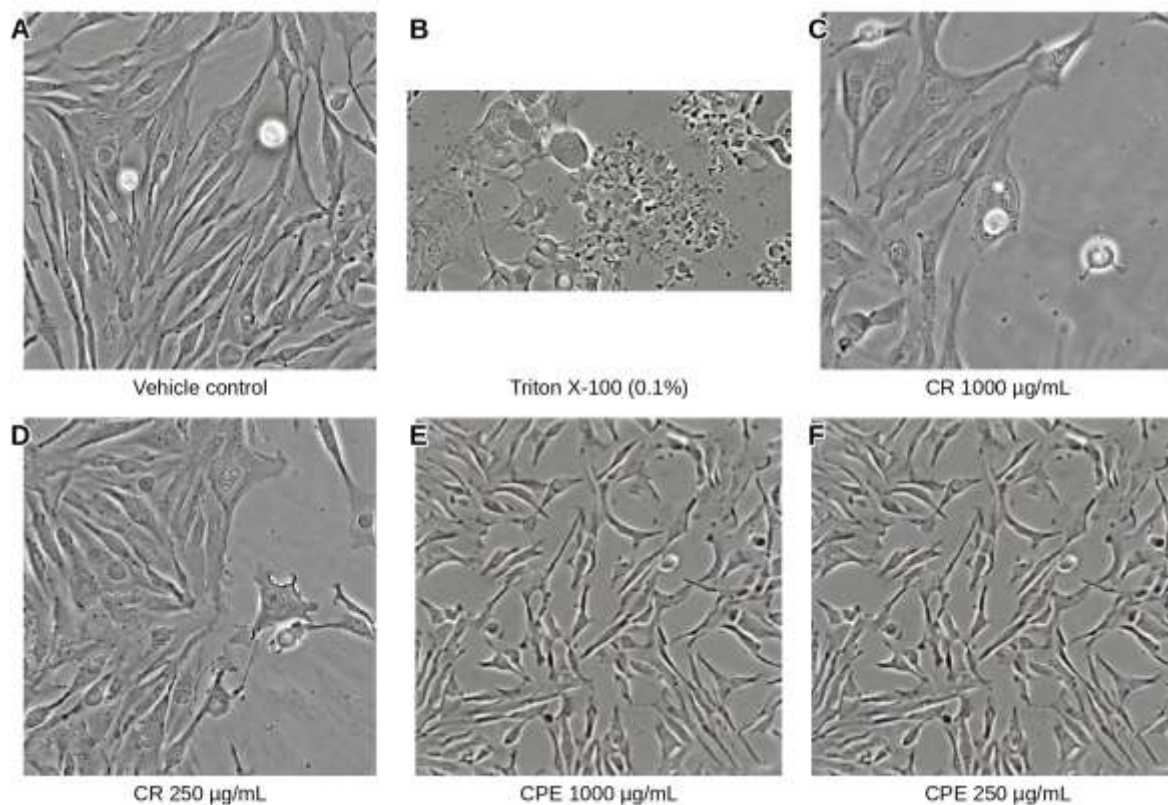


Figure 6: Representative phase-contrast images of 3T3-L1 cells: vehicle control (A), 0.1% Triton X-100 (B), CR at 1000 μ g/mL (C) and 250 μ g/mL (D), and CPE at 1000 μ g/mL (E) and 250 μ g/mL (F)

3.6. Glucose uptake in insulin-resistant adipocytes

The results obtained are given in Table 10 and shown in bar graph, which is shown in Figure 7. It was observed that the IR control showed reduced glucose uptake of 46.95% of normal control, and the vehicle control (46.61%) did not differ from it (Dunnett p = 1.000). Insulin (100 nM) restored the uptake to 92.31%. One-way

ANOVA across all groups was significant ($F = 765.25$, $p < 0.001$), and every extract and CPE treatment increased glucose uptake over the IR control in a dose-dependent manner (Dunnett, all $p < 0.001$).

It was found that among the single extracts CR has shown the highest uptake at 250 $\mu\text{g/mL}$ (80.43%), followed by RR (77.69%) and ZM (71.75%), which corresponds to increases of 71.33%, 65.49% and 52.83% over the IR control. CPE has shown the strongest extract-based response at every dose, reaching 89.36% relative uptake at 250 $\mu\text{g/mL}$ (90.35% increase over IR control). At 250 $\mu\text{g/mL}$, CPE was significantly higher than CR (mean difference 8.93%), RR (11.67%) and ZM (17.62%) (Tukey, all $p < 0.001$), and it was only 2.95% below the insulin standard ($p = 0.021$). CR was also higher than RR (2.74%, $p = 0.033$) and RR than ZM (5.94%, $p < 0.001$) (Table 13).

Table 10: Effect of CR, RR, ZM and CPE on 2-NBDG glucose uptake in insulin-resistant 3T3-L1 adipocytes

Group	Conc. ($\mu\text{g/mL}$)	RFU (mean $\pm$ SD)	Relative uptake (%)	Increase vs IR control (%)	p vs IR control
Normal control	—	912.67 $\pm$ 5.69	100.00 $\pm$ 0.71	113.01	< 0.001
IR control	—	487.00 $\pm$ 6.00	46.95 $\pm$ 0.75	—	Reference
Vehicle control	—	484.33 $\pm$ 5.51	46.61 $\pm$ 0.69	-0.71	1.000 (ns)
Insulin (100 nM)	—	851.00 $\pm$ 7.55	92.31 $\pm$ 0.94	96.64	< 0.001
CR	62.5	605.00 $\pm$ 7.55	61.65 $\pm$ 0.94	31.33	< 0.001
CR	125	688.67 $\pm$ 6.51	72.08 $\pm$ 0.81	53.54	< 0.001
CR	250	755.67 $\pm$ 7.09	80.43 $\pm$ 0.88	71.33	< 0.001
RR	62.5	598.33 $\pm$ 8.02	60.82 $\pm$ 1.00	29.56	< 0.001
RR	125	670.00 $\pm$ 9.00	69.75 $\pm$ 1.12	48.58	< 0.001
RR	250	733.67 $\pm$ 7.51	77.69 $\pm$ 0.94	65.49	< 0.001
ZM	62.5	565.00 $\pm$ 8.00	56.67 $\pm$ 1.00	20.71	< 0.001
ZM	125	626.00 $\pm$ 9.00	64.27 $\pm$ 1.12	36.90	< 0.001
ZM	250	686.00 $\pm$ 8.00	71.75 $\pm$ 1.00	52.83	< 0.001
CPE	62.5	649.33 $\pm$ 8.50	67.18 $\pm$ 1.06	43.10	< 0.001
CPE	125	745.67 $\pm$ 8.50	79.19 $\pm$ 1.06	68.67	< 0.001
CPE	250	827.33 $\pm$ 8.50	89.36 $\pm$ 1.06	90.35	< 0.001

Blank RFU 110.33 $\pm$ 3.06. p values by Dunnett's test against the IR control; ns, not significant. *Each value represents Mean $\pm$ standard deviation (SD) of 3 observations

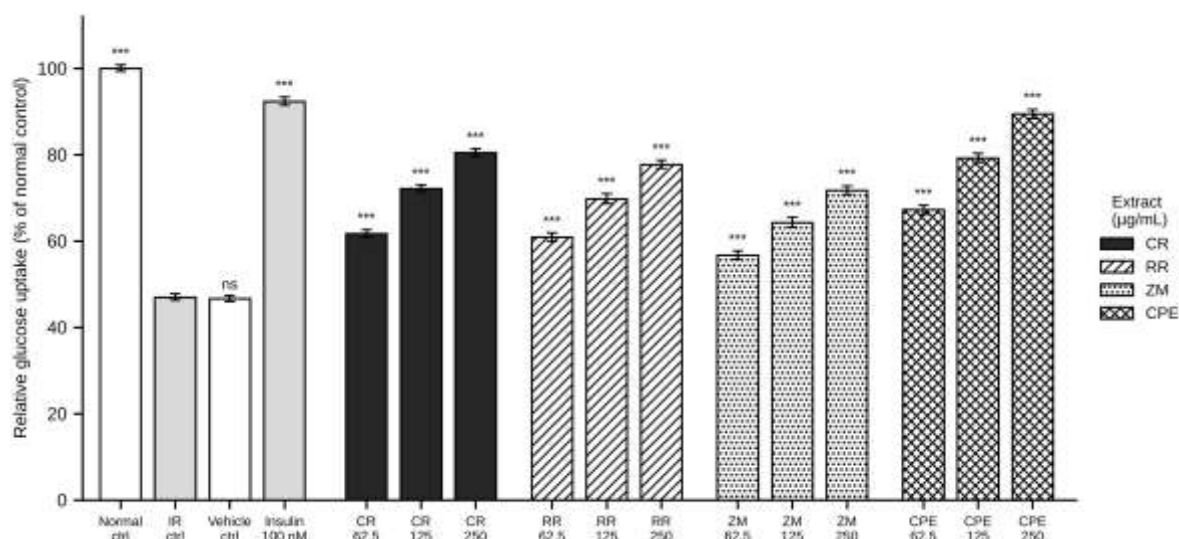


Figure 7: Effect of CR, RR, ZM and CPE on 2-NBDG glucose uptake in insulin-resistant 3T3-L1 adipocytes. Data are mean $\pm$ SD (n = 3). * $p < 0.001$ versus IR control (Dunnett's test); ns, not significant**

3.7. α -Amylase and α -glucosidase inhibition

A graph was plotted by taking % inhibition on Y-axis and concentration on X-axis, which is shown in Figure 8, and the inhibition values are given in Table 11. It was found that the inhibition of both enzymes increased in a concentration-dependent manner for every material. At 500 $\mu\text{g/mL}$, acarbose inhibited α -amylase by 87.03 $\pm$

0.93% and α -glucosidase by $91.37 \pm 0.75\%$, and CPE inhibited the two enzymes by $77.43 \pm 1.08\%$ and $83.26 \pm 0.97\%$. Among the single extracts, CR has shown higher inhibition than RR and ZM at every concentration. The logistic fits were good for every material ($R^2 \geq 0.9956$) and the IC_{50} values are given in Table 12. It was observed that CPE has shown the lowest IC_{50} among the extracts for α -amylase ($86.28 \mu\text{g/mL}$) and α -glucosidase ($68.25 \mu\text{g/mL}$), followed by CR (128.68 and $108.37 \mu\text{g/mL}$), RR (148.00 and $112.21 \mu\text{g/mL}$) and ZM (165.97 and $127.04 \mu\text{g/mL}$), while acarbose gave 58.26 and $44.63 \mu\text{g/mL}$. One-way ANOVA at $250 \mu\text{g/mL}$ was significant for both enzymes ($F = 298.68$ and 300.22 , $p < 0.001$), and Tukey's test separated every pair, including acarbose versus CPE ($p < 0.001$), CR versus RR ($p = 0.004$ and 0.005) and RR versus ZM ($p < 0.001$ and $p = 0.002$) (Table 13). Hence the ranking acarbose > CPE > CR > RR > ZM was identical for both enzymes, and it matched the ranking of the glucose uptake assay.

Table 11: Percentage inhibition of α -amylase and α -glucosidase by the extracts, CPE and acarbose

Conc. ($\mu\text{g/mL}$)	Acarbose	CPE	CR	RR	ZM
α-Amylase (OD_{540})					
15.625	22.37 ± 1.28	17.52 ± 1.12	12.84 ± 0.93	10.57 ± 0.81	6.72 ± 0.69
31.25	36.64 ± 1.51	29.84 ± 1.36	21.62 ± 1.21	18.76 ± 1.03	12.47 ± 0.86
62.5	51.46 ± 1.73	41.95 ± 1.61	32.90 ± 1.46	28.94 ± 1.30	21.83 ± 1.16
125	65.52 ± 1.41	54.21 ± 1.48	45.30 ± 1.67	39.42 ± 1.58	32.46 ± 1.43
250	78.16 ± 1.06	66.36 ± 1.26	57.11 ± 1.37	51.84 ± 1.34	44.27 ± 1.52
500	87.03 ± 0.93	77.43 ± 1.08	69.48 ± 1.16	63.27 ± 1.18	55.83 ± 1.30
α-Glucosidase (OD_{405})					
15.625	26.58 ± 1.18	22.18 ± 1.08	15.46 ± 0.97	12.92 ± 0.89	9.36 ± 0.74
31.25	42.83 ± 1.43	35.92 ± 1.28	25.62 ± 1.16	23.15 ± 1.08	18.55 ± 0.93
62.5	58.19 ± 1.57	48.35 ± 1.47	37.82 ± 1.41	34.59 ± 1.33	29.12 ± 1.18
125	72.47 ± 1.22	61.08 ± 1.37	50.74 ± 1.61	46.29 ± 1.50	40.66 ± 1.37
250	84.52 ± 0.98	73.12 ± 1.19	63.41 ± 1.31	58.46 ± 1.27	52.94 ± 1.46
500	91.37 ± 0.75	83.26 ± 0.97	75.27 ± 1.09	70.12 ± 1.12	64.59 ± 1.22

Reagent blank OD_{540} 0.0407 ± 0.0015 and blank-corrected assay control OD_{540} 0.8120 ± 0.0090 (α -amylase); reagent blank OD_{405} 0.0330 ± 0.0010 and blank-corrected assay control OD_{405} 0.7350 ± 0.0080 (α -glucosidase).

*Each value represents Mean $\pm$ standard deviation (SD) of 3 observations

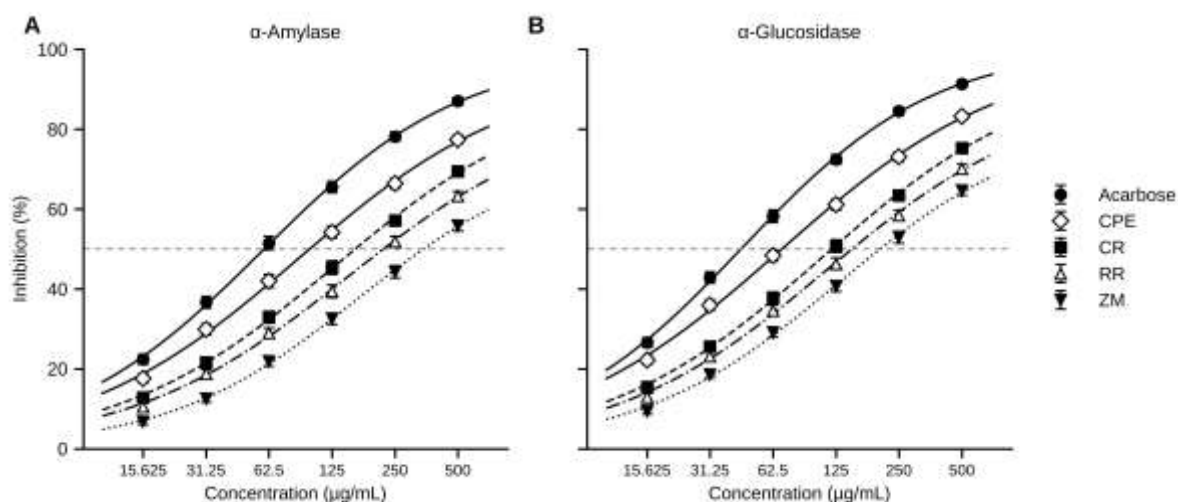


Figure 8: Concentration–response curves for inhibition of α -amylase (A) and α -glucosidase (B) by acarbose, CPE, CR, RR and ZM. Symbols are mean $\pm$ SD ($n = 3$); lines are logistic fits; the dashed line marks 50% inhibition

Table 12: IC_{50} values of the extracts, CPE and acarbose for α -amylase and α -glucosidase inhibition

Sample	α -Amylase IC_{50} ($\mu\text{g/mL}$) (95% CI)	R^2	α -Glucosidase IC_{50} ($\mu\text{g/mL}$) (95% CI)	R^2	Rank
Acarbose	58.26 (42.61–73.92)	0.9972	44.63 (30.29–58.97)	0.9975	Standard
CPE	86.28 (62.39–110.17)	0.9956	68.25 (46.67–89.83)	0.9963	1
CR	128.68 (84.59–172.76)	0.9963	108.37 (80.96–135.79)	0.9970	2

Sample	α -Amylase IC ₅₀ (μ g/mL) (95% CI)	R ²	α -Glucosidase IC ₅₀ (μ g/mL) (95% CI)	R ²	Rank
RR	148.00 (85.51–210.50)	0.9960	112.21 (79.84–144.58)	0.9960	3
ZM	165.97 (105.55–226.39)	0.9963	127.04 (89.52–164.56)	0.9960	4

IC₅₀ from logistic non-linear regression of inhibition against log₁₀ concentration (lower asymptote 0%), fitted on all replicate points; lower IC₅₀ indicates stronger inhibition.

Table 13: Tukey HSD pairwise comparison at 250 μ g/mL across the three functional assays

Comparison	Glucose uptake mean difference (%) (p)	α -Amylase inhibition mean difference (%) (p)	α -Glucosidase inhibition mean difference (%) (p)
Insulin / acarbose vs CPE	+2.95 (0.021)	+11.81 (< 0.001)	+11.40 (< 0.001)
CPE vs CR	+8.93 (< 0.001)	+9.25 (< 0.001)	+9.70 (< 0.001)
CPE vs RR	+11.67 (< 0.001)	+14.52 (< 0.001)	+14.65 (< 0.001)
CPE vs ZM	+17.62 (< 0.001)	+22.08 (< 0.001)	+20.18 (< 0.001)
CR vs RR	+2.74 (0.033)	+5.27 (0.004)	+4.95 (0.005)
CR vs ZM	+8.68 (< 0.001)	+12.83 (< 0.001)	+10.47 (< 0.001)
RR vs ZM	+5.94 (< 0.001)	+7.56 (< 0.001)	+5.52 (0.002)

Mean difference = first group – second group. Reference standard: insulin (100 nM) for glucose uptake and acarbose (250 μ g/mL) for the enzyme assays. One-way ANOVA: glucose uptake F = 765.25; α -amylase F = 298.68; α -glucosidase F = 300.22 (all p < 0.001).

4. Discussion

The chemical composition of a herbal material changes with source, season and processing (World Health Organization, 2007; Xie et al., 2006), so the identity and purity of each batch were fixed before any bioassay was run. Identity was established by macroscopy, microscopy, UV-Visible spectroscopy and HPTLC, and purity by moisture, ash and extractive values. HPTLC was chosen because one run shows the whole chemical pattern of a sample and places it beside reference standards on the same plate (Ahmad et al., 2014; Reich & Widmer, 2008; Verma & Gupta, 2014). Salidroside and rosavin were co-chromatographed for *R. rosea* because about one fifth of commercial *R. rosea* products were found to lack rosavin, and 30% of market samples were adulterated with other *Rhodiola* species that carry salidroside but little rosavin (Booker et al., 2015; Booker et al., 2016). The high ash of *Z. marina* was expected for a marine angiosperm and it did not reflect contamination, because the acid-insoluble fraction remained low.

Plant extracts and antioxidants can reduce MTT without any cells present, or absorb at the reading wavelength, and either effect inflates the apparent viability (Bruggisser et al., 2002; Ghasemi et al., 2021; Mosmann, 1983). Each OD in this study was therefore corrected against its own extract blank. All four materials kept $\geq 80\%$ viability up to 250 μ g/mL (van Meerloo et al., 2011), so the glucose uptake and enzyme results at 62.5–250 μ g/mL were not a by-product of cell loss. *Z. marina* was the most cytotoxic material at 1000 μ g/mL, and this might be due to its high salt and mucilage load at that concentration.

3T3-L1 cells have served as a white-adipocyte model since their differentiation in culture was described (Green & Kehinde, 1975), and insulin resistance can be induced in the mature cells by inflammatory cytokines or glucocorticoids (Luan et al., 2019; Rotter et al., 2003), and treated adipocytes respond with increased glucose consumption (Yuan et al., 2019). 2-NBDG gives a direct fluorescent measure of glucose entry into the cell (Yamada et al., 2007; Zou et al., 2005). In this study uptake fell to 46.95% in the IR control and insulin brought it back to 92.31%, so the model responded as designed. Uptake in adipocytes depends mainly on GLUT4 translocation, which is controlled by the insulin–PI3K–Akt pathway and by AMPK (Huang & Czech, 2007; Leto & Saltiel, 2012). Salidroside stimulates glucose uptake in muscle cells by AMPK activation and it improves insulin resistance through a mitochondria-associated AMPK/PI3K/Akt/GSK3 β pathway (Li et al., 2008; Zheng et al., 2015; Zheng et al., 2019). Quercetin and kaempferol, which are among the flavonoids of *C. rotundus*, also increase glucose uptake through AMPK and JAK2 signalling (Dhanya et al., 2017; Kitakaze et al., 2020), and rosmarinic acid of *Z. marina* increases muscle glucose uptake and attenuates insulin resistance (Den Hartogh et al., 2023; Jayanthi et al., 2017; Vlaveciski et al., 2017). *C. rotundus* extract itself was shown to modify glucose uptake in myotubes and adipocytes (Pichetkun et al., 2024), and an equal-proportion polyherbal containing *C. rotundus* increased glucose uptake in 3T3-L1 adipocytes by 32.3% at 2.0 mg/mL (Das et al., 2025). CPE raised uptake by 90.35% over the IR control at 250 μ g/mL, one-eighth of that concentration.

Inhibition of α -amylase and α -glucosidase delays the release of glucose from dietary starch and reduces the postprandial peak, which is the basis of acarbose therapy (Chiasson et al., 2002; van de Laar et al., 2005). For flavonoids, the inhibition of both enzymes depends on the number and position of hydroxyl groups (Proença et al., 2017; Tadera et al., 2006; Tundis et al., 2010). *C. rotundus* extracts were reported to inhibit α -amylase with an IC_{50} of about 67 μ g/mL (Abdella et al., 2024) and α -glucosidase with an IC_{50} of 383.75 μ g/mL for an aqueous extract (Mohamed et al., 2021), and the CR values in the present study (128.68 and 108.37 μ g/mL) lie within this range. *R. rosea* extracts were shown to inhibit α -glucosidase and α -amylase in relation to their phenolic content (Kwon et al., 2006), and rhodiosin from *Rhodiola crenulata* reduced postprandial hyperglycaemia by α -glucosidase inhibition (Wang et al., 2024). Salidroside has a wider pharmacological profile including antidiabetic effects (Zhang et al., 2021), and *Rhodiola* extract also inhibits adipogenesis (Lee et al., 2011). The weaker but clear activity of ZM is in line with the antidiabetic and insulin-sensitising actions reported for rosmarinic acid and caffeic acid (Ganguly et al., 2023; Leong et al., 2025), and with the phenolic richness of eelgrass, which varies with season and geography (Elkattan et al., 2024; Grignon-Dubois & Rezzonico, 2023; Li et al., 2022).

At any total concentration, CPE carried only one-third the dose of each extract, yet at 250 μ g/mL it gave higher glucose uptake than 250 μ g/mL of any single extract, and its IC_{50} values were lower than those of each extract for both enzymes. If the three extracts were simply dose-additive, the mixture would be expected to respond within the range of the single extracts at the same total concentration, whereas CPE exceeded even the most active single extract in every assay. This points to at least an additive and possibly synergistic interaction among the sesquiterpenoid–flavonoid fraction of CR, the phenylethanoid and cinnamyl glycosides of RR and the phenolic acids of ZM. Herb–herb interaction of this kind is the reasoning behind polyherbal practice (Wagner & Ulrich-Merzenich, 2009; Williamson, 2001), and here it might be attributed to two mechanisms working in one preparation, enzyme inhibition at the gut level and GLUT4-mediated uptake at the tissue level (Das et al., 2025; Hopkins, 2008). The 1:1:1 ratio is also used in traditional formulations of *C. rotundus* such as Trimad (Joshi et al., 2026). The degree of interaction can be quantified by the combination index method of Chou and Talalay, which requires full dose–response curves for each component and the mixture at fixed ratios (Chou, 2006; Chou, 2010; Chou & Talalay, 1984). α -Cyperone, the principal sesquiterpenoid marker identified in CR, has also protected the kidney of diabetic rats against ischaemic injury at 50 mg/kg (Daude et al., 2024).

5. Conclusion

All pharmacognostic and physicochemical values of *C. rotundus* rhizome, *R. rosea* root and *Z. marina* leaf were within the specified limits. HPTLC confirmed salidroside and rosmarinic acid in *R. rosea*, an α -cyperone-type sesquiterpenoid in *C. rotundus* and rosmarinic acid in *Z. marina*. All extracts and CPE were non-cytotoxic to 3T3-L1 cells up to 250 μ g/mL. It was found that CPE at 250 μ g/mL restored glucose uptake in insulin-resistant adipocytes to 89.36%, close to insulin (92.31%), and it has shown the lowest IC_{50} among the extracts for α -amylase (86.28 μ g/mL) and α -glucosidase (68.25 μ g/mL). The ranking CPE > CR > RR > ZM was identical in all three functional assays. It can be concluded that the 1:1:1 combination is more active than each single extract, even at one-third the dose of each component, and that it works at two points, adipocyte glucose uptake and starch digestion through α -amylase and α -glucosidase. Combination-index analysis and in vivo evaluation are the next steps for CPE.

Ethical issues

Not applicable. The study was performed on an established cell line and did not involve human participants or animals.

Conflict of interest

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

Data availability

The raw replicate data, instrument printouts and analysis scripts supporting the findings of this study are available from the corresponding author on reasonable request.

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