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Optimization of Polyphenol Extraction from Two *Ziziphus* Species via Central Composite Design-Response Surface Methodology: *In Vitro* Screening for Antioxidant, Antidiabetic, and Anti-Glycation Potentials

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ABSTRACT

Polyphenols are widely available in medicinal plants and show promising pharmacological activity for many chronic diseases like diabetes mellitus. This study is aimed at optimizing the polyphenols from *Ziziphus mauritiana* and *Ziziphus spina-christi* using central composite design–response surface methodology (CCD–RSM) and evaluating their antioxidant, antidiabetic, and anti-glycation potentials. The key extraction parameters used for the optimal yield and biological evaluation were ethanol concentration, extraction time, solid-to-liquid ratio, and temperature. Total phenolic content (TPC) was maximized at intermediate ethanol concentrations (50–70% v/v), moderate extraction times (20–40 min), solid-to-liquid ratios of 20–25 g/mL, and temperatures between 40.0 and 60.0°C, and TPC obtained with optimal conditions was 120.59 mg GAE/g DW for *Z. mauritiana* and 104.77 mg GAE/g DW for *Z. spina-christi*. For the optimized extracts, *Z. mauritiana* showed significantly higher DPPH and ABTS (131.73 ± 7.23 mg and 271.62 ± 6.23 mg VCEAC/g DE) activities than *Z. spina-christi* (DPPH: 111.29 ± 4.34 mg; ABTS: 236.71 ± 8.23 mg VCEAC/g DE), while *Z. spina-christi* exhibited greater FRAP activity compared to *Z. mauritiana* (149.17 ± 6.23 mg and 123.53 ± 5.23 mg VCEAC/g DE, respectively). *Z. spina-christi* also had higher flavonoid and tannin content. In vitro assays revealed moderate α-amylase inhibition (IC_{50} 112.30–176.20 µg/mL) and prominent anti-glycation effects (IC_{50} 118–146 µg/mL), suggesting potential for managing oxidative stress and diabetes-related complications. This study underscores the phytotherapeutic potential of *Ziziphus* species and highlights CCD–RSM as an effective tool for optimizing bioactive compound extraction.

Keywords: Polyphenol optimization, *Ziziphus* species, Response Surface Methodology, Antioxidant activity, Antidiabetic potential, Anti-glycation

Introduction

Polyphenols are a diverse group of plant-derived compounds recognized for their robust health benefits, including their capacity to mitigate diabetes, neutralize free radicals, and slow down glycation processes. Numerous bioactive polyphenols, such as ferulic acid, quercetin, resveratrol, epigallocatechin gallate, curcumin, ellagic acid, and chlorogenic acid, have been scientifically reported for their efficacy in reducing oxidative stress, regulating glucose metabolism, and inhibiting the formation of advanced glycation end-products (AGEs),[1-3] all of which are implicated in the pathogenesis of chronic diseases like neuropathy and cardiovascular disorders.[4] Interestingly, most of the aforementioned polyphenolic compounds have been evidenced in the plants we decided on for our study.[5] The increasing demand for natural therapeutics and functional substances has driven research into efficient extraction and analytical optimization of polyphenols from various plant sources, improving both yield and bioactivity.[6]

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This research is particularly relevant due to the growing market for plant-based nutraceuticals and the need for targeted extraction strategies.Among polyphenol-rich plants, species of the genus *Ziziphus* have emerged as valuable sources of bioactive compounds.[5] *Ziziphus mauritiana* (Indian jujube) and *Ziziphus spina-christi* (Christ's thorn jujube) are two prominent species traditionally consumed for their nutritional fruits and used in folk medicine for their leaves, bark, and roots.[5, 7] These species are widely distributed across arid and semi-arid regions of Asia, Africa, and the Middle East, with *Z. mauritiana* being more prevalent in South Asia (Indo-Malaysian region) and *Z. spina-christi* in the Middle East (e.g., Yemen).[8, 9] Both plants are valued for their edible fruits and their application in traditional remedies for diabetes, inflammation, and microbial infections.[10, 11] Notably, recent phytochemical analyses have demonstrated that *Ziziphus* species are rich in polyphenols, flavonoids, saponins, alkaloids, and other bioactive constituents, contributing to their ongoing relevance for both folk and evidence-based medicine.[10-12] For example, *Z. mauritiana* has demonstrated in vitro efficacy against breast cancer, further highlighting its therapeutic promise.[13]

A key novelty of this research lies in the systematic optimization of polyphenol extraction from *Z. mauritiana* and *Z. spina-christi* using advanced statistical approaches, which remains underexplored in current literature. The efficiency of polyphenol extraction is influenced by various factors, including the type and composition of the solvent, the extraction method and duration, temperature, and the solvent-to-solid ratio.[14, 15] Conventional extraction approaches, such as one-factor-at-a-time (OFAT), often fail to achieve optimal yields or

may compromise the quality of sensitive compounds.[16] However, statistical optimization methods, such as Central Composite Design–Response Surface Methodology (CCD–RSM), enable a more precise, multifactorial analysis to determine ideal conditions while preserving functional integrity.[17, 18] This study briefly contrasts both methods: while OFAT can offer baseline optimization, CCD-RSM accounts for interaction effects among parameters, thereby providing a holistic optimization technique strongly relevant to industrial and academic extraction processes. These approaches have become increasingly important in natural product research, as corroborated by recent extraction studies.

Collectively, this work is novel in its combined application of CCD-RSM and rigorous in vitro bioactivity screening (antioxidant, antidiabetic, and anti-glycation assays) for *Z. mauritiana* and *Z. spina-christi* polyphenols. To our knowledge, no prior research has systematically optimized extraction parameters in these species with subsequent pharmacological evaluations, thus filling a crucial gap in both the analytical and therapeutic fields. The findings are anticipated to promote the sustainable utilization of *Ziziphus* species in health-related fields and provide valuable insights regarding the development of innovative nutraceuticals.

Materials and Methods

Collection and authentication of plants

Two plants of the same genus but different species were used for the study. Leaves of *Ziziphus mauritiana* were collected in India from Mysore city in Boghadi village at estimated GPS No. 12.3048997, 76.5673809, while the other species, i.e., *Ziziphus spina-christi*, was collected from the Republic of Yemen, Al-Mahwit governorate, Alwasitah village at estimated GPS No. 15.4576598, 43.3625020. Both plants were authenticated in India by the Foundation for Revitalization of Local Health Traditions (FRLHT), Bangalore, with accession numbers FRLH-123440 for *Z. mauritiana* and FRLH-123441 for *Z. spina christi*.

Chemicals

Standard analytical-grade chemicals and solvents were locally acquired from reputable vendors to ensure consistency. From Sigma-Aldrich (USA), materials including bovine serum albumin (BSA), glucose, acarbose, and aminoguanidine were obtained. From SRL and HiMedia (India), Folin-Ciocalteu reagent, DPPH, TPTZ, dinitro salicylic acid, sodium carbonate, sodium azide, vanillin, soluble starch, sulfuric acid, and standards (gallic acid, ascorbic acid, quercetin, and ellagic acid) were obtained. The enzymes used in the inhibition tests, which included α-amylase, were supplied by Sigma-Aldrich and Cayman (Germany).

Extraction of plant material for the one-factor-at-a-time

For each experiment, 1 g of the plant material was placed in a 25 mL beaker. Different volumes of variable ratios of hydro-alcohol were added and then subjected to sonication for selected durations. Table 1 presents the variables. To assess the OFAT, the extracted samples were spun in a centrifuge, concentrated, and dried at the set temperature, and the total amount of phenols was measured using the Folin-Ciocalteu (FC) colour assay for polyphenols. The result was shown as mg gallic acid equivalents per gram dry weight (mg GAE/g DW).[19, 20]

Extraction of plant material by optimized method

About 500 grams of *Z. mauritiana* and *Z. spina christi* leaves were extracted by an optimized sonication process, and the obtained menstruum was then concentrated using a rotary evaporator at a suitable temperature, followed by drying in an electric water bath. The prepared extracts were stored in an airtight container at 4°C.

Central composite design for optimizing sonication assisted extraction

RSM-based CCD was designed using Minitab statistical software 22 and the optimal ranges of experimental variables from the OFAT, with the aim of optimizing sonication extraction to enhance polyphenols in *Z. mauritiana* and *Z. spina christi* extracts. A three-level full factorial face-centered CCD was designed utilizing three continuous numerical factors and 20 base runs that included 8 cube points, 6 center points in the cube, and 6 axial points. A similar design was developed for *Z. mauritiana* and *Z. spina christi*, which included three independent experimental variables: ethanol concentration (A, % v/v), extraction duration (B, minutes), and solid-to-liquid ratios (C, g/mL). Table 2 shows the coded and non-coded values used in the model development of *Z. mauritiana* and *Z. spina christi*. The polynomial equation for generating the optimal TPC from the two plant materials will be expressed in the following equation 1:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum\sum_{i<j} \beta_{ij} X_i X_{ij} \ldots\ldots\ldots \text{(Equation 1)}$$

Where X_i and X_j stands for the levels of the numerical variables, k is the number of numerical variables that need to be modelled. The coefficients consist of β_0, which is constant.

Determination of total phenolic content (TPC)

The TPC of OFAT/CCD-designed menstruum (2.5–10 µL) and phenolic-rich extracts (2.5–17.5 µg) was determined using the FC method with modifications.[19, 20] Briefly, 400 µL of sample or gallic acid standard (HiMedia, India; ≥98% purity; 2.5–17.5 µg/400 µL) was mixed with 2 mL of 10% (v/v) Folin-Ciocalteu reagent (HiMedia, India; ≥98% purity, analytical grade) in a 5 mL test tube. After 5 min incubation at 25 ± 1°C, 1.6 mL of 7% (w/v) sodium carbonate solution (SRL, India; ≥99% purity, analytical grade) was added, followed by dark incubation for 1 h. Absorbance was measured at 765 nm (Shimadzu UV-1800 spectrophotometer). TPC was calculated as mean ± SD (n=3) mg GAE/g DW or dry extract (mg GAE/g DE) using a gallic acid standard curve.

DPPH radical scavenging activity

The antioxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay following Brand-Williams et al. with modifications.[21, 22] A 0.1 mM DPPH solution was prepared in HPLC-grade methanol (SRL, India; 99.9% purity) using DPPH radicals (HiMedia, India; ≥97% purity). In a 5 mL test tube, 1 mL of sample or vitamin C standard (L-ascorbic acid, HiMedia, India; ≥99% purity; 2.5–17.5 µg) was mixed with 1 mL of the DPPH solution. After incubation in the dark at 25 ± 1°C for 30 min, absorbance was measured at 517 nm (Shimadzu UV-1800 spectrophotometer). The decrease in absorbance relative to methanol controls was expressed as vitamin C equivalents antioxidant capacity (mg VCEAC/g DE).

ABTS radical cation decolorization assay

The $ABTS^+$ [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] radical scavenging activity was determined according to Re et al. with modifications.[23, 22] The $ABTS^+$ radical cation was generated by reacting 7 mM ABTS (HiMedia, India; ≥98% purity) with 2.45 mM potassium persulfate (SRL, India; ≥99% purity) in the dark for 16 h at 25 ± 1°C. The solution was then diluted with ethanol (SRL, India; analytical grade) to an absorbance of 0.70 ± 0.02 at 734 nm. For the assay, 0.2 mL of sample (2.5-17.5 µg) was combined with 1.8 mL of diluted $ABTS^+$ solution in a 5 mL test tube and incubated for 6 min in the dark. Absorbance was measured at 734 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800). Results were expressed as mg VCEAC/g DE relative to the control.

Total tannin content (TTC)

The TTC was determined using the vanillin-HCl method according to Price et al.,[24] with modifications by Bhat et al.[25] Briefly, 500 µL of sample (2.5–17.5 µg) was mixed with 1.25 mL of 15% (w/v) vanillin solution (prepared in methanol using vanillin from HiMedia, India; ≥99% purity) and 1.25 mL of 32% (v/v) HCl (SRL, India; analytical grade). The reaction mixture was incubated at 25 ± 1°C for 15 min, and absorbance was measured at 500 nm using a Shimadzu UV-1800 spectrophotometer. Catechin (HiMedia, India; ≥98% purity) served as the standard, with results expressed as mg catechin equivalents per gram of dry extract (mg CAE/g DE).

Ferric reducing antioxidant power (FRAP) assay

The reducing power was evaluated using the FRAP method according to Benzie and Strain.[26] The FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-

tripyridyl-s-triazine; HiMedia, India; ≥98% purity) in 40 mM HCl (SRL, India; analytical grade), and 20 mM $FeCl_3 \cdot 6H_2O$ (SRL, India; analytical grade) in a 10:1:1 ratio (v/v/v). For the assay, 500 µL of sample (2.5-17.5 µg) was combined with 2 mL of FRAP reagent in a 5 mL test tube and incubated at 37°C for 10 min. Absorbance was measured at 593 nm using a Shimadzu UV-1800 spectrophotometer. Results were quantified against a mg VCEAC/g DE.

Determination of total flavonoid content (TFC)

The TFC was determined using a modified colorimetric assay with aluminum chloride.[27] Briefly, 250 µL of sample or quercetin standard (2.5-17.5 µg; HiMedia, India; ≥98% purity) was mixed with 2.750 mL of 2% aluminum chloride solution (prepared from $AlCl_3$; SRL, India; ≥99% purity) in methanol (SRL, India; HPLC grade) using 5 mL test tubes. After 30 min incubation at 25 ± 1°C, absorbance was measured at 510 nm using a Shimadzu UV-1800 spectrophotometer. The TFC was calculated from a quercetin standard calibration curve and expressed as mg quercetin equivalents per gram of dry extract (mg QE/g DE), with all measurements performed in triplicate (n=3).

In Vitro α-Amylase Inhibition Assay

The α-amylase inhibitory activity was determined spectrophotometrically using a modified DNSA (3,5-dinitrosalicylic acid) method.[28] The reaction system contained 1 mL of test sample (100-800 µg/mL) or acarbose standard (0.1–10 µg/mL) (Sigma-Aldrich, USA; ≥98% purity) at varying concentrations, which was pre-incubated with 1 mL of porcine pancreatic α-amylase solution (EC 3.2.1.1; Sigma-Aldrich, Germany; ≥95% purity; 1.5 U/mL in 20 mM phosphate buffer, pH 6.9) at 37°C for 10 min. Subsequently, 1 mL of 1% soluble starch substrate (SLR, India; analytical grade) in phosphate buffer was added and incubated for an additional 30 min at 37°C. The reaction was terminated by adding 1 mL DNSA reagent (Sigma-Aldrich, USA; analytical grade), followed by heating at 90°C for 5 min in a water bath. After cooling to room temperature, the reaction mixture was diluted to 10 mL with distilled water, and absorbance was measured at 540 nm using a Shimadzu UV-1800 spectrophotometer. The percentage inhibition was calculated relative to control reactions, and the IC_{50} values were determined from dose-response curves, with all experiments performed in triplicate (n=3).

In vitro BSA glycation and AGEs inhibition assay

The anti-glycation activity was evaluated by measuring inhibition of advanced glycation end-product (AGE) formation using a bovine serum albumin (BSA)-glucose model system.[29] The reaction mixture contained 300 µL of test sample (100-800 µg/mL) or aminoguanidine standard 1 mg/mL (Sigma-Aldrich, USA; ≥98% purity), 50 µL of BSA solution (10 mg/mL; Sigma-Aldrich, USA; ≥98% purity), and 50 µL of 0.5 M glucose (Sigma-Aldrich, USA; analytical grade) in phosphate buffer (pH 7.4; SRL, India; analytical grade) containing 0.02% sodium azide (SRL, India; ≥99% purity) as preservative. The mixture was incubated in 96-well black microplates at 37°C for 7 days under dark conditions. AGE formation was quantified by measuring fluorescence intensity at excitation 370 nm and emission 440 nm using a BioTek Synergy H1 microplate reader (BioTek Instruments, USA). The percentage inhibition was calculated relative to glycation control wells, and IC_{50} values were determined from dose-response curves, with all experiments performed in triplicate (n=3). Method validation followed established protocols as previously described.

Statistical analysis

Data are expressed as mean ± SD (n=3). Statistical design and optimization were performed using Response Surface Methodology Central Composite Design (CCD-RSM) in Minitab® 22 (Minitab LLC, Version 22.3, Release Year: 2025, USA). Model significance was assessed by ANOVA ($p < 0.05$). Group comparisons were performed by independent t-test and one-way ANOVA followed by Tukey's HSD post hoc test using GraphPad Prism version 10.4.2. $P < 0.05$ was considered statistically significant.

Results and Discussion

Polyphenols for One-Factor-at-a-Time

The OFAT analysis was done to carefully study how four important extraction factors: ethanol concentration, extraction time, solid-to-liquid ratio, and temperature affected the TPC extracted from *Z. mauritiana and Z. spina-christi*. As shown in Figure 1, the darker red shading in the heatmaps, TPC was maximized at intermediate ethanol concentrations (50-70% v/v), moderate extraction times (20-40 min), solid-to-liquid ratios of 20-25 g/mL, and temperatures between 40 and 60°C. At higher ethanol concentrations (≥90% v/v) and temperatures (≥80°C), TPC declined sharply. *Z. spina-christi* consistently yielded higher TPC than *Z. mauritiana* under optimal conditions.

The results indicate that TPC extraction was highly dependent on the ethanol concentration. Maximum TPC was observed at intermediate ethanol concentrations (around 50–70% v/v), with a decline at lower and higher concentrations. This pattern matches earlier studies on other plants, like *Nephelium lappaceum* L., where a 54% ethanol concentration was best for extracting polyphenols, resulting in more polyphenols and better antioxidant activity.[30] The lesser efficacy at higher ethanol concentrations may be due to the reduced solubility of more polar phenolic compounds, whereas lower concentrations may not effectively damage plant cell walls and release fewer polar phenolics. This statement may also apply to other medicinal plants, where a more polar solvent, such as distilled water, may be the best solvent for polyphenol extraction, as is the case for *Euphorbia resinifera* O. Berg.[31]

TPC increased with extraction time up to a certain threshold, after which prolonged extraction did not yield further significant gains and, in some cases, resulted in decreased TPC. This trend aligns with literature reports indicating that optimal extraction times are usually brief (e.g., 10–30 minutes for ultrasound or microwave-assisted extraction), after which degradation or oxidation of phenolics may take place.[32, 33] An increased solid-to-liquid ratio generally improved TPC up to an optimal point, after which further increases did not enhance extraction and sometimes reduced efficiency. This is likely due to the saturation of solvent with solutes and decreased mass transfer efficiency at higher ratios. Comparable findings were reported in *Quercus cerris* and *Sideritis raeseri* extractions, where a 1:10 to 1:40 (g/mL) ratio was optimal for polyphenol recovery.[34, 35] Too low a ratio limits solute availability, while too high a ratio leads to inefficient solvent use. Temperature had a significant effect, with TPC increasing as temperature rose to moderate levels (typically 40–60°C) but declining at higher temperatures (above 70°C). Elevated temperatures can enhance the solubility and diffusion of phenolic compounds, but excessive heat may promote degradation or polymerization of sensitive polyphenols, as documented in several extraction studies.[36]

These findings are consistent with previous reports on *Ziziphus* species, where polyphenol extraction efficiency was shown to depend strongly on solvent polarity and extraction conditions. For instance, studies on *Z. mauritiana* have demonstrated that ethanolic extracts are particularly rich in polyphenols and exhibit notable antioxidant and anti-inflammatory activities, supporting the use of ethanol-water mixtures for optimal extraction.[13, 37, 38] Similarly, *Z. spina-christi* leaves have been shown to yield high TPC and strong antioxidant activity when extracted with hydro-alcoholic solvents, as measured by the FC method.[39] The observed decline in TPC at higher temperatures and ethanol concentrations is likely due to thermal degradation and reduced solubility of certain phenolics.

Moreover, the literature highlights the significant pharmacological potential of polyphenol-rich extracts and other secondary metabolites from both species, including free radical scavenging, anti-inflammatory, anti- nociceptive, anxiolytic and antimicrobial effects.[13, 37-41] These bioactivities are closely linked to the extraction efficiency and the optimization of process variables, reinforcing the importance of the present findings for both research and application in natural product development.

Fitting and validation of CCD:

Following OFAT screening, RSM-CCD was used to enhance polyphenol extraction from *Z. mauritiana* and *Z. spina-christi*, maximizing phenolic content removal from the plants. This

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methodology is based on determining the link between the response variable (TPC) and the extraction variables. Table 3 discusses the extraction variables and response values throughout all 20 experimental trials. The estimated TPC was entered into the model, which was then assessed using ANOVA. The program suggested that the entire quadratic model was an optimal fit, with ANOVA displaying significant p-values and an insignificant lack of fit. Table 4 summarizes the mean square values, as well as the model's P and F values. Following the software's recommendation, a second-degree polynomial equation was chosen, which provided the greatest fit for all three independent variables and answers. Equations 2-3 show the relationship between the

Table 1: One-factor-at-a-time experimental design for polyphenol extraction from plant material.

OFAT for	Ethanol concentration	Extraction time	Solid to liquid ratio	Temperature
Ethanol concentration	0 to100 % V/V	30 Minutes	20 g/mL	60 ° C
Extraction time	50% V/V	0 - 60 Minutes	20 g/mL	60 ° C
Temperature	50 % V/V	30 Minutes	20 g/mL	40-80 ° C
Solid to liquid ratio	50 % V/V	30 Minutes	10 - 30 g/mL	60° C

Fixed conditions for non-varied factors: 1 gm plant material in 25 mL solvent, sonicated, centrifuged, and analysed via FC assay. The underlined values represent the testing ranges for each selected parameter in the OFAT design.

Table 2: Coded and un-coded experimental variables employed for developing CCD for *Z. mauritiana and Z. spina Christi*

Variables	Units	Coded and uncoded level of variables					
		Z. mauritiana			*Z. spina christi*		
		-1	0	+1	-1	0	+1
Ethanol concentration	% (v/v)	50	60	70	30	40	50
Extraction time	Minutes	30	35	40	25	30	35
Solid to liquid ratios	g/mL	20	25	30	15	20	25

Table 3: TPC levels obtained for the designed models as per CCD matrix.

Run	Block	*Ziziphus mauritiana*				Block	*Ziziphus spina Christi*			
		A	B	C	TPC in mg GAE/g DM		A	B	C	TPC in mg GAE/g DM
1	1	1	-1	1	101.82	2	0	-1	0	74.58
2	1	0	0	0	100.82	2	0	0	1	57.98
3	1	0	0	0	82.75	2	1	0	0	102.03
4	1	0	0	0	73.61	2	0	0	0	106.15
5	1	1	1	-1	77.16	2	0	0	-1	69.41
6	1	1	1	1	70.65	2	0	1	0	87.3
7	1	-1	1	-1	72.24	2	0	0	0	102.17
8	1	-1	-1	1	101.98	2	-1	0	0	80.63
9	1	-1	1	1	119.77	1	-1	-1	-1	104.47
10	1	1	-1	-1	103.14	1	-1	1	1	101.08
11	1	0	0	0	117.77	1	0	0	0	103.22
12	1	-1	-1	-1	121.21	1	1	1	-1	75.42
13	2	0	0	1	124.76	1	0	0	0	74.46
14	2	0	-1	0	112.59	1	1	-1	-1	107.69
15	2	0	0	0	107.83	1	0	0	0	61.1
16	2	1	0	0	123.12	1	-1	1	-1	92.86
17	2	0	1	0	76.18	1	0	0	0	67.27
18	2	0	0	0	78.24	1	1	1	1	60.09
19	2	0	0	-1	79.13	1	-1	-1	1	90.86

20	2	-1	0	0	119.77	1	1	-1	1	56.97	

Table 4: ANOVA for the second order quadratic model with regression coefficients for TPC

Source	*Z. mauritiana*			*Z. spina Christi*		
	Mean square values	**F-Value**	**P-Value**	**Mean square values**	**F-Value**	**P-Value**
Model	827.53	179.75	< 0.0001^S	679.76	103.86	< 0.0001^S
Ethanol concentration	1.12	0.24	0.632	67.88	10.37	0.009^S
Extraction time	6.56	1.42	0.260	25.36	3.87	0.077
Solid to liquid ratio	49.53	10.76	0.008^S	216.66	33.10	< 0.0001^S
Ethanol concentration * Ethanol concentration	901.66	195.85	< 0.0001^S	1438.53	219.80	< 0.0001^S
Extraction time * Extraction time	797.31	173.18	< 0.0001^S	383.98	58.67	< 0.0001^S
Solid to liquid ratio * Solid to liquid ratio	237.71	51.63	< 0.0001^S	31.45	4.81	0.053
Ethanol concentration * Extraction time	1.61	0.35	0.567	33.59	5.13	0.047^S
Ethanol concentration * Solid to liquid ratio	43.73	9.50	0.012^S	1.98	0.30	0.594
Extraction time * Solid to liquid ratio	14.16	3.08	0.110	76.16	11.64	0.007^S
Lack-of-Fit	2.79	0.43	0.809	6.80	1.08	0.467
R^2	0.9938			0.9894		
Adjusted R^2	0.9883			0.9799		

'S' indicates significant values with $p < 0.05$, where '*' indicates "Factor * Factor" terms represent the quadratic (nonlinear) effects of that factor in the regression model.

Table 5: Predicted and experimental values of TPC under optimized variables.

Source	Extraction variable			TPC in mg GAE/g DW		% Difference (CV)
	A	**B**	**C**	**Experimental**	**Predicted**	
Z. mauritiana	60	25.5	25.59	122.34 ± 2.72	120.59	1.43
Z. spina christi	40.50	25.85	23.78	106.32 ± 3.18	104.771	1.46

Table 6: Optimized extract antioxidant activity of *Z. mauritiana* and *Z. spina christi*

Extracts	DPPH (mg VCEAC/g DE)	ABTS (mg VCEAC/g DE)	FRAP (mg VCEAC/g DE)
Z. mauritiana	131.73 ± 7.23^a	271.62 ± 6.23^a	123.53 ± 5.23^b
Z. spina christi	111.29 ± 4.34^b	236.71 ± 8.23^b	149.17 ± 6.23^a

Values are expressed as mean ± SD (n = 3). Different superscript letters (a, b) within the same column indicate statistically significant differences ($p < 0.05$) determined by independent samples t-test

Table 7: Optimized extract phytochemical content in *Z. mauritiana* and *Z. spina christi*

Extracts	TPC GAE/g DE	TFC QE/g DE	TTC TAE/g DE
Z. mauritiana	163.15 ± 8.41^a	15.94 ± 2.32^b	36.23 ± 2.35^b
Z. spina christi	134.63 ± 4.75^b	23.47 ± 1.678^a	45.35 ± 3.42^a

Values are expressed as mean ± SD (n = 3). Different superscript letters (a, b) within the same column indicate statistically significant differences ($p < 0.05$) determined by independent samples t-test.

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Table 8: Optimized extract α-amylase inhibition and Antiglycation of *Z. mauritiana* and *Z. spina christi*

Extracts	α-amylase inhibition in IC_{50} (µg/mL)	Antiglycation in IC_{50} µg/mL)
Z. mauritiana	112.32 ± 8.21^{b}	118 ± 4.23^{b}
Z. spina christi	176.2 ± 7.34^{c}	146 ± 6.62^{c}
Acarbose	0.5 ± 0.1^{a}	---------
Aminoguanidine	------	87 ± 3.3^{a}

Values are expressed as mean ± SD (n = 3). Different superscript letters (a, b, c) within the same column indicate statistically significant differences ($p < 0.05$) determined by one-way ANOVA followed by Tukey's HSD post-hoc test. Acarbose and aminoguanidine were used as positive controls for α-amylase inhibition and antiglycation, respectively.

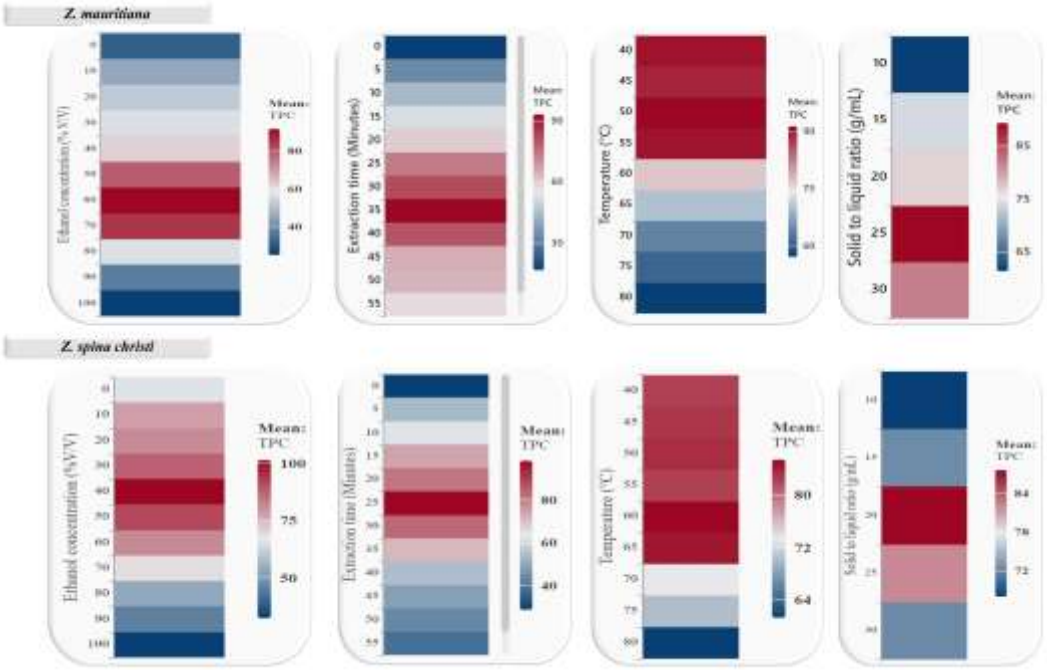

Figure 1: One factor analysis for *Z. mauritiana* and *Z. spina christi*

TPC and the experimental variables for *Z. mauritiana* (*Y1*) and *Z. spina christi* (*Y1*), respectively.

$Y_1 = -1559.3 + 20.21\ A + 48.31\ B + 18.10\ C - 0.1811\ A^2 - 0.6811\ B^2 - 0.3719\ C^2 + 0.0089\ AB + 0.0468\ AC - 0.0532\ BC$……(Equation 2)

$Y_2 = -636.9 + 19.78\ A + 23.12\ B + 3.65\ C - 0.2287\ A^2 - 0.4727\ B^2 - 0.1353\ C^2 - 0.0410\ AB - 0.0100\ AC + 0.1234\ BC$……(Equation 3)

Upon analysing the model using ANOVA, it was found the model showed significant *P* values and *F* values followed by insignificant lack of fit along with the R^2 values for the two models, which are 0.9938 and 0.9894 for *Z. mauritiana and Z. spina christi*, respectively, indicating the well-fitted model, which can be used to establish a more established relationship between the experimental variables and the response. The software was also used to analyse the contour and the RSM, which provides a detailed understanding of the relationship between the independent variable and the response variables.

Optimization of CCD design to maximize polyphenols

The main objective of the study was to prepare a polyphenolic-rich extract using leaves of *Z. mauritiana and Z. spina christi.* After successful validation and fitting of the model, the response optimizer tool was used to maximize the TPC from the plants, maintaining the experimental variables in range. The final optimized extraction variable of ethanol concentration at 60.00% (v/v), with an extraction time of 25.5 minutes by maintaining solid-liquid ratios of 25.59 g/mL, will yield a maximum TPC of 120.59 mg GAE/g DW for *Z. mauritiana*, and for *Z. spina christi*, *a* maximum phenolic content of 104.77 mg GAE/g DW can be achieved by maintaining 40.50% (v/v), 25.85 minutes, and 23.78 g/mL. Both the optimized models showed desirability of 0.922 and 0.942, respectively (Figure 2). The optimized parameters were used to extract the plant materials, and the TPC values were found to be in accordance with that of the predicted TPC values with the coefficient of variance of 1.43% and 1.46%, respectively, as discussed in Table 5. The close match between the predicted and experimental TPC values shows that the optimization and model are strong. These results are in line with what other studies have found: that moderate levels of ethanol and extraction durations give the best results for polyphenol output in *Ziziphus* species and other medicinal plants.[42, 43]

Antioxidant activity of optimized extracts

The antioxidant activities of the optimized extracts were statistically compared using DPPH, ABTS, and FRAP assays (Table 6). Statistical analysis revealed that *Z. mauritiana* exhibited significantly higher DPPH (131.73 ± 7.23 mg VCEAC/g DE) and ABTS (271.62 ± 6.23 mg VCEAC/g DE) activities compared to *Z. spina-christi* (DPPH: 111.29 ± 4.34 mg; ABTS: 236.71 ± 8.23 mg VCEAC/g DE), with $p < 0.05$. Conversely, *Z. spina-christi* displayed significantly greater FRAP activity (149.17 ± 6.23 mg VCEAC/g DE) than *Z. mauritiana* (123.53 ± 5.23 mg VCEAC/g DE), as confirmed by independent samples t-test analysis. These significant differences emphasize the impact of plant species on antioxidant potentials when subjected to optimized extraction protocols.

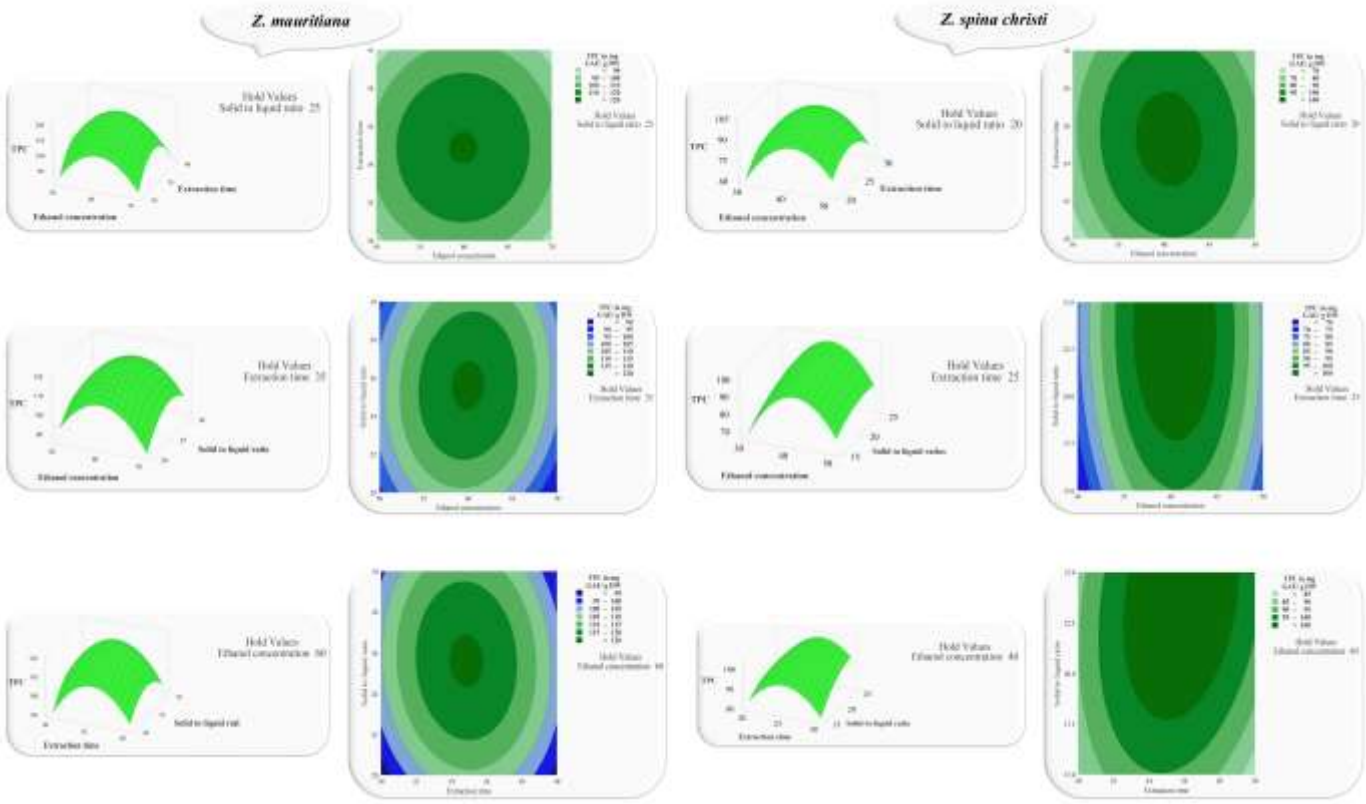

Figure 2: RSM and contour plots showing the effects of extraction variables TPC of *ziziphus mauritiana* and *Ziziphus spina-christi* extracts

These results align with recent studies reporting a positive correlation between TPC and antioxidant efficacy in *Ziziphus* species.[44] For instance, Jain et al. found ethanol extracts of *Z. mauritiana* leaves demonstrated high DPPH scavenging ability (IC_{50}: 8–12 µg/mL), a finding consistent with the strong antioxidant capacity observed in the current study.[42] The elevated FRAP in *Z. spina-christi* likely stems from its higher concentration of tannins and flavonoids, compounds known to contribute to reducing power.[45] Our findings reinforce the potent antioxidant capacity observed across diverse natural products, a shared biochemical strength that positions them as promising candidates for mitigating oxidative stress-linked disorders.[46] Like nature's own defense network, these compounds exhibit a triple therapeutic potential: combating glycation-driven complications, regulating metabolic imbalances in hypoglycemia,[47] and shielding against lipid peroxidation-induced cellular damage.[48]

Phytochemical content

Quantitative phytochemical analysis showed significant interspecies differences with respect to flavonoid and tannin content (Table 7). *Z. spina-christi* contained significantly higher TFC (23.47 ± 1.68 mg QE/g DE) and TTC (45.35 ± 3.42 mg TAE/g DE) than *Z. mauritiana* (TFC: 15.94 ± 2.32 mg QE/g DE; TTC: 36.23 ± 2.35 mg TAE/g DE), based on t-test results ($p < 0.05$). However, *Z. mauritiana* had a statistically higher TPC (163.15 ± 8.4 mg GAE/g DE) compared to *Z. spina-christi* (134.63 ± 4.75 mg GAE/g DE).

These findings are consistent with recent studies highlighting the phytochemical richness and health-promoting properties of *Ziziphus* species.[49] Notably, the TPC values reported for both species in this study surpass those documented for *Z. mauritiana* fruit juice (16.9 mg GAE/g DW), demonstrating the advantage of optimized extraction from leaves over fruits.[50] The enhanced polyphenol, flavonoid, and tannin concentrations position these extracts as promising candidates for further functional food and nutraceutical development.

α-Amylase inhibition and antiglycation activity

α-Amylase inhibition results (Table 8) demonstrated that *Z. mauritiana* extract (IC_{50} = 112.32 ± 8.21 µg/mL) was statistically more potent than *Z. spina-christi* (IC_{50} = 176.2 ± 7.34 µg/mL, $p < 0.05$), but both were much less active than the reference drug acarbose (IC_{50} = 0.5 ± 0.1 µg/mL). These data are statistically supported by one-way ANOVA followed by Tukey's HSD test. Recent comparative studies echo these moderate inhibitory activities, suggesting *Ziziphus* extracts,[51] while less potent than pharmaceuticals, still hold promise for complementary glycaemic management strategies.[5]

This study is the first to report statistically significant antiglycation activity for *Z. mauritiana* and *Z. spina-christi* leaf extracts (Table 8). Optimized extracts exhibited notable AGE inhibition (IC_{50}: 118 ± 4.23 µg/mL for *Z. mauritiana*, 146 ± 6.62 µg/mL for *Z. spina-christi*), although less potent than aminoguanidine (IC_{50} = 87 ± 3.3 µg/mL). The antiglycation effects are likely attributed to high polyphenol, flavonoid, and tannin content, which are well-established glycation inhibitors in recent literature.[2, 52] Similar antiglycation actions have been observed in *Ziziphus oxyphylla* extracts,[53] though this report is the first for these two species. These results underscore the extracts' utility as functional, natural antiglycation agents, warranting further compound isolation studies.

In summary, the significant, statistically validated differences in antioxidant, phytochemical, and enzyme-inhibitory activities between

Z. mauritiana and *Z. spina-christi* indicate species-specific strengths and suggest targeted uses for each. Both demonstrated moderate α-amylase and antiglycation effects, the highest TPC in *Z. mauritiana*, and the highest TFC/TTC/FRAP in *Z. spina-christi*, all backed by up-to-date references. These findings reinforce the ongoing relevance of Ziziphus species as sources of natural bioactive compounds and support their integration as functional ingredients in foods and nutraceuticals, rather than as replacements for conventional drugs.

Conclusion

The optimized extracts of *Ziziphus mauritiana* and *Ziziphus spina-christi* demonstrate robust antioxidant and moderate antidiabetic activities, with phytochemical profiles that support their traditional uses and potential applications in health promotion. These results are well-aligned with current literature, confirming the value of optimized extraction in maximizing the bioactive potential of *Ziziphus* species. Looking ahead, further research should focus on isolating and characterizing the key bioactive compounds responsible for the observed activities, as well as evaluating the efficacy and safety of these extracts in in vivo models and clinical settings. Exploration of formulation strategies for functional foods, nutraceuticals, and adjunct therapies may help realize the full health potential of *Ziziphus* species. Additionally, integration with sustainable cultivation and extraction practices could promote the wider adoption of these plants in evidence-based traditional and modern healthcare.

Conflict of Interest

The author`s declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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