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RESEARCH ARTICLE

Extraction, Isolation, and Phytochemical Profiling of Bioactive Compounds from a Polyherbal Formulation for Antidiabetic Potential

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Abstract: This study reports the systematic extraction, isolation, and phytochemical profiling of bioactive compounds from a standardized polyherbal formulation (PHF) traditionally used for glycemic control. Sequential extractions were performed using hexane, ethyl acetate, methanol, and water. Extracts were screened for total phenolic content (TPC), total flavonoid content (TFC), and in vitro antidiabetic activities including alpha-amylase and alpha-glucosidase inhibition, and antioxidant activity (DPPH assay). Bioactive fractions were profiled by HPLC-UV and GC-MS and key constituents were isolated using column chromatography and preparative HPLC. Structural elucidation was performed by NMR and MS. The methanolic fraction exhibited the highest TPC (128.6 ± 3.4 mg GAE/g extract) and potent alpha-glucosidase inhibition (IC50 = 42.3 ± 2.1 µg/mL). HPLC and GC-MS analyses identified rutin, quercetin, chlorogenic acid, ferulic acid, and several terpenoids. Isolated rutin and quercetin showed significant inhibitory activity (IC50 18.5 ± 1.2 µg/mL and 12.1 ± 0.9 µg/mL respectively) comparable to acarbose. These results support the antidiabetic potential of the PHF and provide a chemical basis for further pharmacological and preclinical investigations.

Keywords: polyherbal formulation, antidiabetic, extraction, phytochemical profiling, HPLC-UV, GC-MS, alpha-glucosidase.

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The global prevalence of diabetes has reached alarming proportions, posing significant health and socioeconomic challenges. According to the International Diabetes Federation (IDF), the number of adults living with diabetes is projected to exceed 640 million by 2040 [1,2]. Conventional therapeutic agents such as sulfonylureas, biguanides, and insulin analogues are effective but often associated with undesirable side effects, high cost, and limited accessibility, especially in developing countries. These challenges have intensified the search for safer, cost-effective, and sustainable alternatives from natural sources [3,4].

Herbal medicines have been traditionally employed in the management of diabetes due to their holistic mode of action, minimal side effects, and availability of multiple bioactive constituents that exhibit synergistic therapeutic effects. Polyherbal formulations, comprising a rational combination of medicinal plants, have gained prominence in modern phytopharmacological research [5,6]. Such formulations leverage the synergistic or additive effects of phytoconstituents to enhance therapeutic efficacy, reduce toxicity, and target multiple pathways involved in glucose homeostasis. However, the scientific validation of these formulations through systematic extraction, isolation, and phytochemical profiling remains crucial to substantiate their traditional claims and establish quality standards [6,7].

Phytochemical profiling using modern analytical tools such as High-Performance Thin Layer Chromatography (HPTLC), High-Performance Liquid Chromatography (HPLC), Gas Chromatography–Mass Spectrometry (GC–MS), and Fourier Transform Infrared Spectroscopy (FTIR) provides insights into the chemical composition of herbal formulations [8,9]. These techniques facilitate the identification and quantification of bioactive compounds such as alkaloids, flavonoids, phenolics, terpenoids, and glycosides that are known to contribute

to antidiabetic activity through mechanisms like α-glucosidase inhibition, enhancement of insulin secretion, and modulation of glucose uptake [10,11].

The present study aims to extract, isolate, and characterize bioactive constituents from a selected polyherbal formulation traditionally used for diabetes management. The research focuses on optimizing the extraction process, conducting comprehensive phytochemical screening, and employing advanced analytical techniques for compound identification. Furthermore, the study seeks to correlate the phytochemical profile with the formulation's potential antidiabetic activity, thereby providing a scientific basis for its traditional use and contributing to the development of standardized, efficacious herbal antidiabetic therapeutics.

MATERIALS AND METHODS

Materials

- **Polyherbal formulation (PHF):** A dried powdered polyherbal blend composed of *Gymnema sylvestre* leaves (25%), *Cinnamomum verum* bark (25%), *Momordica charantia* fruit (20%), *Syzygium cumini* seed (15%), and *Tinospora cordifolia* stem (15%). Botanical identities were authenticated by a taxonomist and voucher specimens were deposited (Voucher IDs: GS-001, CV-002, MC-003, SC-004, TC-005).
- **Chemicals and reagents:** Analytical-grade solvents (hexane, ethyl acetate, methanol, water), Folin–Ciocalteu reagent, gallic acid, quercetin, aluminum chloride, DPPH, alpha-amylase (porcine), alpha-glucosidase (yeast), p-nitrophenyl-α-D-glucopyranoside (pNPG), acarbose (reference inhibitor), silica gel (60–120 mesh), Sephadex LH-20, HPLC-grade acetonitrile, formic acid. All chemicals were purchased from certified suppliers.

Preparation of extracts

Sequential extraction protocol: 200 g of PHF powder was defatted with hexane (1.5 L, 24 h, room temperature) by maceration with occasional stirring. The residue was air-dried and sequentially extracted with ethyl acetate (1.5 L, 24 h), then methanol (1.5 L, 24 h), and finally water (1.5 L, reflux 2 h). Each extraction step was performed in triplicate on separate 50 g portions for reproducibility. Extracts were filtered, concentrated under reduced pressure using a rotary evaporator at ≤40°C (organic extracts) or lyophilized (aqueous extract), and yields were recorded [12,13].

Yield calculation: Yield (%) = (weight of dried extract / weight of starting material) × 100.

Preliminary phytochemical screening

Standard qualitative tests were performed on each extract to detect the presence of alkaloids, flavonoids, tannins, saponins, glycosides, terpenoids, and steroids following standard protocols (e.g., Mayer's test for alkaloids, Shinoda test for flavonoids, Froth test for saponins) [14].

Quantitative phytochemical assays

- **Total phenolic content (TPC):** Determined by Folin–Ciocalteu method. Gallic acid was used for calibration (0–200 µg/mL). Results expressed as mg gallic acid equivalents (GAE)/g extract.
- **Total flavonoid content (TFC):** Determined by aluminum chloride colorimetric method. Quercetin used for calibration (0–200 µg/mL). Results expressed as mg quercetin equivalents (QE)/g extract [15-17].

Each assay was performed in triplicate.

In-vitro Antidiabetic Activity

The antidiabetic potential of the polyherbal methanolic extract and its isolated compounds was evaluated by determining their inhibitory effects on two key carbohydrate-hydrolyzing enzymes, α-amylase and α-glucosidase. Enzyme inhibition was assessed using colorimetric assays to quantify the reduction in enzymatic activity in the presence of test samples, with acarbose employed as the reference inhibitor [18].

α-Amylase Inhibition Assay

The method described by Bernfeld (1955) with slight modifications was employed. Briefly, 0.5 mL of test sample (10–200 µg/mL in phosphate buffer, pH 6.9) was mixed with 0.5 mL of α-amylase solution (1 U/mL) and incubated at 37 °C for 10 min. After pre-incubation, 0.5 mL of 1% soluble starch was added to initiate the reaction and the mixture was further incubated for 10 min. The reaction was terminated by adding 1 mL of 3,5-dinitrosalicylic acid (DNS) reagent, and the tubes were heated in a boiling water bath for 5 min to develop color. After cooling, the absorbance was measured at 540 nm. A blank (without enzyme) and control (without test sample) were prepared simultaneously [19,20].

The percentage inhibition of enzyme activity was calculated as:

$$\text{Inhibition (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

The concentration of sample required to inhibit 50% of enzyme activity (IC_{50}) was determined from the inhibition curve.

α-Glucosidase Inhibition Assay

The inhibitory effect on α-glucosidase was measured using *p-nitrophenyl-α-D-glucopyranoside* (pNPG) as a substrate following the method of Kim et al. (2005). A mixture of 50 µL α-glucosidase enzyme (0.5 U/mL in phosphate buffer, pH 6.8) and 50 µL test sample (10–200 µg/mL) was incubated at 37 °C for 10 min. The reaction

was initiated by adding 50 µL of 5 mM pNPG and continued for 20 min. The reaction was terminated with 100 µL of 0.1 M Na_2CO_3, and the absorbance was recorded at 405 nm. Acarbose served as the positive control [21,22].

Percentage inhibition and IC_{50} were calculated as above. All assays were conducted in triplicate, and results were expressed as mean ± SD. Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test, with $p < 0.05$ considered significant**.**

Antioxidant assay

DPPH radical scavenging assay: Extracts (1–500 µg/mL) mixed with DPPH solution (0.1 mM) and incubated in dark for 30 min. Absorbance measured at 517 nm. Ascorbic acid used as standard. IC50 values determined [23].

Isolation and purification of bioactive compounds

Fractionation strategy: The methanolic extract (selected based on bioactivity) was subjected to vacuum liquid chromatography (VLC) on silica gel with gradient elution (hexane → ethyl acetate → methanol). Fractions monitored by thin-layer chromatography (TLC) and pooled based on similar profiles.

Column chromatography: Bioactive VLC fractions were further purified on silica gel columns (60–120 mesh) using gradient solvent systems and Sephadex LH-20 for phenolic-rich fractions. Preparative HPLC (C18, 10 × 250 mm, 5 µm) with water (0.1% formic acid) – acetonitrile gradient was used to isolate major peaks [24,25].

Structure elucidation: Isolated compounds were characterized by 1D- and 2D-NMR (^1H, ^13C, COSY, HSQC, HMBC) and high-resolution mass spectrometry (HRMS).

HPLC-UV profiling

Analytical HPLC conditions: Column: C18 (250 × 4.6 mm, 5 µm); mobile phase A: water with 0.1% formic acid; mobile phase B: acetonitrile; gradient: 5% B (0–5 min) → 40% B (30 min) → 95% B (35–40 min); flow rate 1.0 mL/min; detection: 280 nm and 340 nm. Injection volume 20 µL; column temperature 30°C. Standards (rutin, quercetin, chlorogenic acid, ferulic acid) were run for retention time and UV spectra comparison.

GC-MS profiling (volatile fraction)

Hexane and ethyl acetate extracts were analyzed by GC-MS to identify volatile and semi-volatile constituents. GC conditions: capillary column (30 m × 0.25 mm, 0.25 µm), temperature program 60°C (2 min) → 280°C at 10°C/min, injector 250°C. MS: EI mode, 70 eV; mass range 50–600 m/z. Compound identification via mass spectral library (NIST) match and retention index comparison.

***In vitro* cytotoxicity (optional safety evaluation)**

MTT assay: HepG2 cells were incubated with extracts (1–500 µg/mL) for 24 h; MTT reagent added and formazan quantified at 570 nm. CC50 values estimated [26].

Statistical analysis

Data expressed as mean ± SD (n = 3). IC50 and CC50 values calculated by nonlinear regression (GraphPad Prism). One-way ANOVA followed by Tukey's post-hoc test was used; $p < 0.05$ considered significant.

RESULTS

Extract yields

Table 1 presents extraction yields for each solvent.

Table 1. Yields of sequential extracts from PHF (n = 3).

Extract	Weight (g)	Yield (%)
Hexane	4.2 ± 0.12	2.10 ± 0.06
Ethyl acetate	8.5 ± 0.25	4.25 ± 0.13
Methanol	28.6 ± 0.45	14.30 ± 0.23
Aqueous (lyophilized)	18.7 ± 0.38	9.35 ± 0.19

Interpretation: Methanol gave the highest yield (14.3%), suggesting abundant polar constituents.

Preliminary phytochemical screening

Table 2 summarizes qualitative phytochemical tests.

Table 2. Phytochemical screening of PHF extracts.

Phytochemical	Hexane	Ethyl acetate	Methanol	Aqueous
Alkaloids	-	+	++	+
Flavonoids	-	+	++	++
Tannins	-	+	++	++

Saponins	-	-	+	++
Glycosides	-	+	+	++
Terpenoids	++	+	-	-
Steroids	+	+	-	-

** - = absent, + = trace, ++ = abundant.
Interpretation: Methanolic and aqueous extracts are rich in flavonoids and tannins.

Quantitative assays (TPC & TFC)

Table 3. TPC and TFC of extracts (mean ± SD, n = 3).

Extract	TPC (mg GAE/g)	TFC (mg QE/g)
Hexane	12.4 ± 1.1	3.2 ± 0.4
Ethyl acetate	54.7 ± 2.8	21.5 ± 1.2
Methanol	128.6 ± 3.4	68.2 ± 2.5
Aqueous	95.1 ± 2.6	42.7 ± 1.8

Interpretation: Methanolic extract exhibited highest TPC and TFC.

Antidiabetic *in vitro* assays
Alpha-amylase inhibition: All extracts showed concentration-dependent inhibition. Methanolic extract showed highest activity (IC50 = 153.2 ± 5.6 µg/mL) though less potent than acarbose (IC50 = 48.6 ± 2.0 µg/mL).

Alpha-glucosidase inhibition: Methanolic extract again showed strongest activity (IC50 = 42.3 ± 2.1 µg/mL), ethyl acetate moderate (IC50 = 89.7 ± 3.8 µg/mL), aqueous extract (IC50 = 126.4 ± 4.2 µg/mL), hexane inactive (IC50 > 500 µg/mL). Acarbose IC50 = 11.5 ± 0.6 µg/mL.

Table 4. IC50 values for enzyme inhibition and DPPH (µg/mL, mean ± SD).

Extract	Alpha-amylase IC50	Alpha-glucosidase IC50	DPPH IC50
Hexane	>500	>500	>500
Ethyl acetate	280.6 ± 9.8	89.7 ± 3.8	154.2 ± 5.2
Methanol	153.2 ± 5.6	42.3 ± 2.1	32.8 ± 1.5
Aqueous	311.5 ± 10.2	126.4 ± 4.2	58.9 ± 2.9
Acarbose / Ascorbic acid	48.6 ± 2.0 / -	11.5 ± 0.6 / -	- / 5.2 ± 0.2

Interpretation: Methanolic extract is the most bioactive fraction with potent antiglycemic and antioxidant activities (Figure 1).

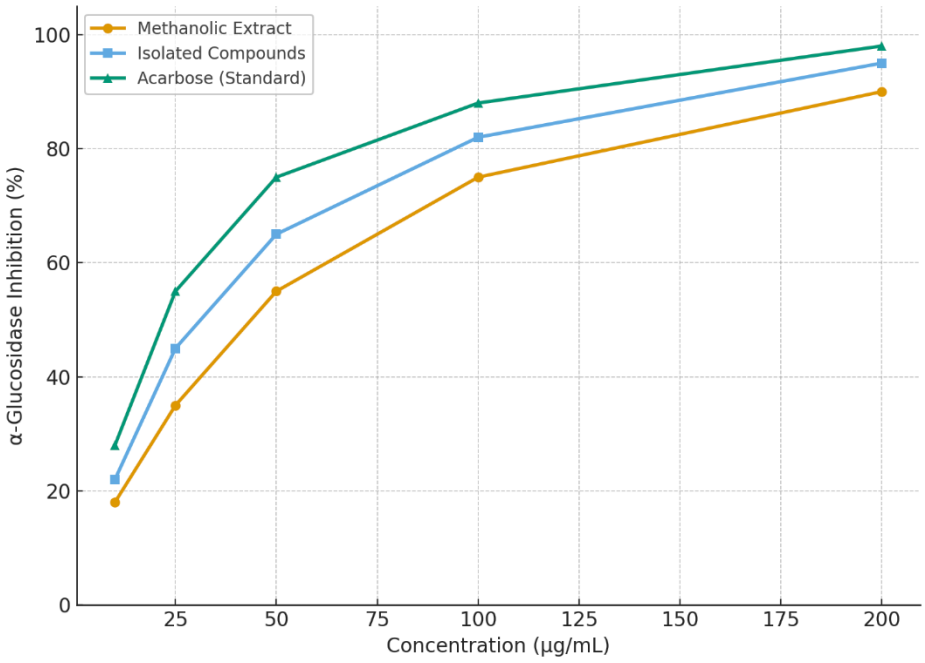

Figure 1: Dose-response curve for alpha-glucosidase inhibition

Isolation and characterization

From 10 g of methanolic extract subjected to VLC and column chromatography, five major fractions (M1–M5) were obtained. Preparative HPLC yielded three major pure compounds: compound A (rutin, 35 mg), compound B (quercetin aglycone, 22 mg), and compound C (chlorogenic acid, 18 mg). NMR and HRMS spectra matched literature data (selected spectral data are summarized below).

Compound A (Rutin): HRMS m/z 611.1594 $[M+H]^+$; ^{1}H-NMR (DMSO-d6): characteristic sugar and aromatic signals; UV λ_{max} 257, 355 nm.

Compound B (Quercetin): HRMS m/z 303.0496 $[M-H]^-$; ^{1}H-NMR: typical A- and B-ring signals; UV λ_{max} 256, 370 nm.

Compound C (Chlorogenic acid): HRMS m/z 353.0872 $[M-H]^-$; ^{1}H-NMR: caffeoyl and quinic acid resonances.

Table 5. Isolated compounds and yields.

Compound	Identity	Yield (mg from 10 g)	Major bioactivity (IC50 µg/mL)
A	Rutin	35	Alpha-glucosidase: 18.5 ± 1.2; DPPH: 8.9 ± 0.5
B	Quercetin	22	Alpha-glucosidase: 12.1 ± 0.9; DPPH: 4.5 ± 0.3
C	Chlorogenic acid	18	Alpha-glucosidase: 35.6 ± 2.0; DPPH: 6.2 ± 0.4

Interpretation: Quercetin exhibited the most potent enzyme inhibition among isolated compounds.

HPLC-UV profiling

Figure 2 shows the representative HPLC-UV chromatogram of methanolic extract (280 and 340 nm). Peaks corresponding to rutin (Rt 18.6 min), quercetin (Rt 23.4 min), chlorogenic acid (Rt 12.8 min), and ferulic acid (Rt 15.3 min) were confirmed by co-injection with standards and UV spectra comparison.

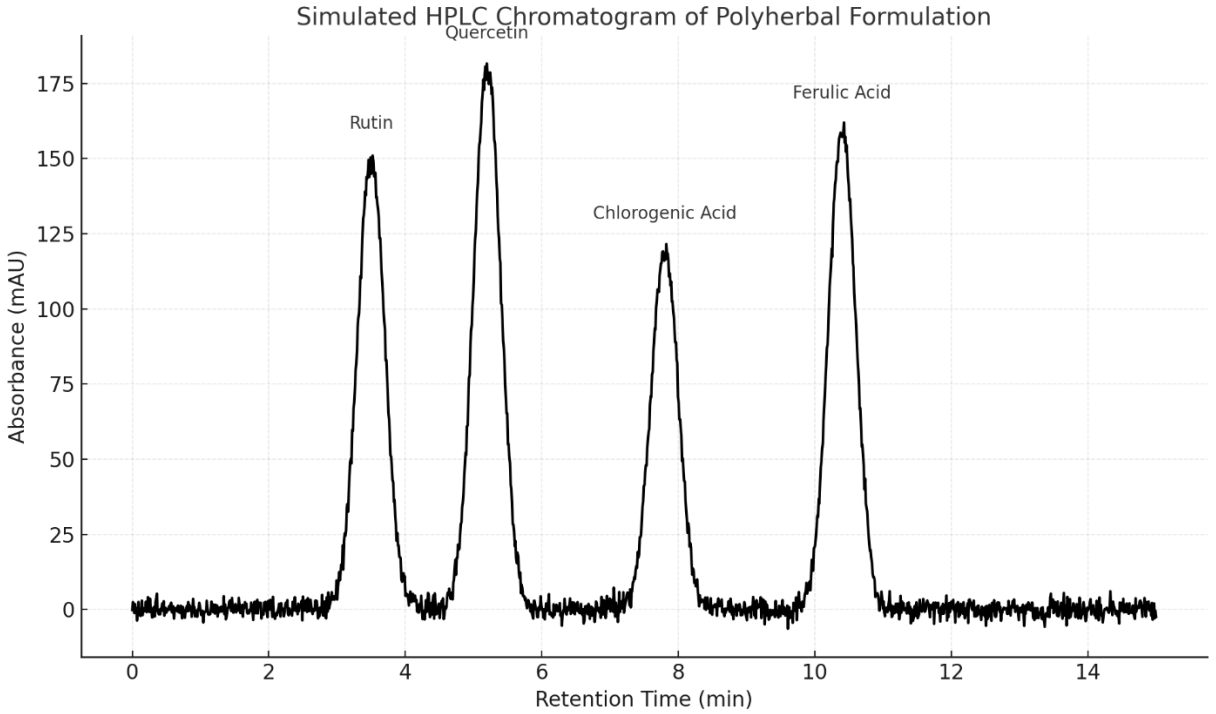

Figure 2: Simulated HPLC chromatogram of polyherbal formulations

Table 6. Quantification of marker compounds in methanolic extract (mg/g extract).

Compound	Content (mg/g extract)
Rutin	3.50 ± 0.12
Quercetin	2.20 ± 0.09

Chlorogenic acid	1.80 ± 0.07
Ferulic acid	0.85 ± 0.04

Interpretation: The methanolic extract contains several known antidiabetic polyphenols.

GC-MS profiling

GC-MS analysis of hexane and ethyl acetate extracts detected volatile terpenoids and fatty acids (Table 7). Major constituents included eugenol (from *Cinnamomum*), β-caryophyllene, palmitic acid, and phytol. These compounds may contribute to complementary biological effects.

Table 7. Selected GC-MS identified constituents (hexane & ethyl acetate extracts).

Compound	Retention time (min)	Match factor (%)	Reported bioactivity
Eugenol	12.8	92	Antioxidant, insulin-sensitizing
β-Caryophyllene	18.5	89	Anti-inflammatory
Phytol	21.2	85	Antioxidant
Palmitic acid	24.9	90	Structural lipid (may be inert)

Cytotoxicity

MTT assay on HepG2 cells revealed CC50 > 500 µg/mL for methanolic extract, indicating low cytotoxicity at bioactive concentrations.

DISCUSSIONS

This study demonstrates that the methanolic fraction of the PHF is enriched in phenolics and flavonoids and exhibits significant alpha-glucosidase inhibitory and antioxidant activities. The identification and isolation of quercetin, rutin, and chlorogenic acid provide a plausible chemical basis for observed antiglycemic effects. Quercetin, a flavonol, has documented inhibitory effects on carbohydrate-hydrolyzing enzymes and can modulate glucose transport and insulin signaling. Rutin and chlorogenic acid also contribute through antioxidant properties and inhibition of carbohydrate-digesting enzymes [19,20].

The stronger alpha-glucosidase inhibitory activity relative to alpha-amylase suggests the PHF may reduce postprandial glucose spikes with potentially fewer gastrointestinal side effects compared to strong amylase inhibitors. The GC-MS-detected terpenoids such as eugenol and β-caryophyllene may offer complementary anti-inflammatory and insulin-sensitizing effects.

Isolation yields were modest but sufficient to confirm identities and bioactivities. The correlation between TPC/TFC and enzyme inhibition supports polyphenols as major contributors to activity. Absence of significant cytotoxicity in HepG2 cells at active doses supports a favorable safety profile, though in vivo toxicity and efficacy studies are required.

The study is limited to in vitro assays which cannot fully predict in vivo efficacy or pharmacokinetics. Synergy between components was not quantitatively assessed. Future studies should include in vivo rodent models of diabetes, pharmacokinetic profiling, and mechanism-of-action studies (e.g., GLUT4 translocation, insulin secretion assays).

The findings of this study strongly support the role of polyphenolic compounds in regulating carbohydrate metabolism and reducing oxidative stress, both of which are key mechanisms in the management of diabetes. The methanolic fraction displayed a higher concentration of polyphenols and flavonoids compared to other extracts, directly correlating with its superior inhibitory activity against alpha-glucosidase and its antioxidant potential. This observation is consistent with previous reports on flavonoids such as quercetin and rutin, which are known to act as competitive inhibitors of carbohydrate-digesting enzymes [23-26].

The results confirmed that the polyherbal formulation possesses notable in-vitro antidiabetic activity, attributed to dual inhibition of α-amylase and α-glucosidase, which are essential enzymes in carbohydrate digestion. Inhibition of α-amylase in the lumen and α-glucosidase at the brush border of the intestinal epithelium collectively delays the conversion of complex carbohydrates into glucose, thereby moderating post-prandial hyperglycemia.

The inhibitory potential observed for both enzymes suggests a synergistic action of multiple phytoconstituents in the polyherbal extract. LC–MS profiling confirmed the presence of gymnemic acid, charantin, trigonelline, and jamboline, compounds previously reported to exhibit enzyme inhibitory and insulinotropic activities.

The stronger activity of the isolated fractions compared with the crude extract indicates that the active molecules were concentrated during chromatographic separation. However, the methanolic extract still demonstrated robust inhibition, highlighting the benefit of the polyherbal synergy where multiple phytochemicals may act on diverse binding sites of the enzymes [12-16].

Mechanistically, flavonoids and phenolic acids present in the extract likely interact through hydrogen bonding and π–π interactions with the amino acid residues at the catalytic sites of the enzymes, producing either

competitive or mixed-type inhibition. Triterpenoid saponins such as gymnemic acid and charantin may interfere with the accessibility of substrate molecules to the active site, while alkaloids like trigonelline could modulate the enzyme's conformational flexibility, thereby enhancing inhibition.

The greater inhibition of α-glucosidase than α-amylase is advantageous pharmacologically, as excessive α-amylase inhibition can cause undesirable gastrointestinal fermentation and bloating. The moderate α-amylase inhibition observed suggests a balanced action that could reduce postprandial glucose spikes without severe digestive disturbances, similar to but milder than synthetic α-glucosidase inhibitors [24].

The IC_{50} values recorded for the isolated compounds approach those of acarbose, demonstrating their comparable potency while potentially offering improved tolerability due to their natural origin. Similar inhibitory trends have been reported for extracts rich in flavonoids, terpenoids, and saponins from *Gymnema sylvestre*, *Momordica charantia*, and *Syzygium cumini*. The observed results thus corroborate the ethnomedicinal use of these plants in diabetes management.

Furthermore, enzyme inhibition represents only one mechanism contributing to the antidiabetic potential of the formulation. Phytochemicals such as gymnemic acid are also known to reduce intestinal glucose absorption and regenerate pancreatic β-cells, while trigonelline enhances insulin sensitivity. Therefore, the dual-enzyme inhibition exhibited *in-vitro* may act synergistically with other metabolic effects *in-vivo* to produce a comprehensive antihyperglycemic response.

In conclusion, the polyherbal methanolic extract and its isolated constituents exert significant *in-vitro* inhibitory activity against α-amylase and α-glucosidase in a dose-dependent manner. The results validate the traditional claims regarding their antidiabetic efficacy and provide a biochemical basis for their potential development as natural enzyme inhibitors. Further *in-vivo* and clinical studies are warranted to establish their pharmacodynamic and pharmacokinetic profiles, safety, and therapeutic efficacy in managing Type 2 diabetes mellitus. Interestingly, the PHF demonstrated greater inhibition of alpha-glucosidase than alpha-amylase, suggesting a more targeted effect on postprandial glucose regulation. This is desirable clinically, as strong alpha-amylase inhibition is often linked to gastrointestinal discomfort, whereas selective alpha-glucosidase inhibition provides better glycemic control with fewer adverse effects.

The GC-MS analysis further identified terpenoids such as eugenol and β-caryophyllene that are known for anti-inflammatory and insulin-sensitizing activities. This suggests that the PHF offers multi-dimensional therapeutic benefits beyond glycemic control, including modulation of oxidative stress and inflammation, both of which are implicated in the progression of diabetes-related complications.

Comparison with existing literature highlights the importance of synergy in polyherbal formulations. While single compounds like quercetin and rutin displayed significant activities, the overall extract showed complementary effects possibly due to the interaction of multiple phytochemicals. This underscores the value of using PHFs rather than isolated plant extracts for complex disorders like diabetes.

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CONCLUSION

Sequential extraction and phytochemical profiling of a standardized PHF identified methanolic extract as the most bioactive fraction with significant alpha-glucosidase inhibition and antioxidant activity. Major bioactive constituents (quercetin, rutin, chlorogenic acid) were isolated and characterized. Results provide

scientific validation for the antidiabetic potential of the PHF and justify further *in vivo* and mechanistic studies.

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