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ANTIDIABETIC, ANTIOXIDANT, AND ANTI-INFLAMMATORY EFFECTS OF A NOVEL POLYHERBAL FORMULATION IN DIABETES MELLITUS

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Abstract
Diabetes mellitus is characterized by hyperglycemia, oxidative stress and chronic inflammation. Medicinal plants with complementary phytochemical profiles provide a promising multi-targeted strategy, but reproducible analytical standardization and integrated in vitro evaluations are often lacking. To prepare and standardize a polyherbal formulation (PHF) containing Withania somnifera, Tinospora cordifolia and Gymnema sylvestre and to evaluate its in vitro antioxidant, antidiabetic enzyme inhibition and anti-inflammatory properties using validated analytical and biochemical methods. Extracts of authenticated plant materials were prepared in 70% ethanol, concentrated and blended in a 2:2:1 (WS:TC:GS) ratio. Standardization included organoleptic assessment, pharmacopeial physicochemical parameters, total phenolic and flavonoid content, HPTLC fingerprinting and validated HPLC quantification of marker compounds. Antioxidant activity was measured by DPPH, ABTS, FRAP and nitric oxide scavenging assays. In vitro antidiabetic potential was assessed by α-amylase, α-glucosidase and DPP-4 inhibition assays. Anti-inflammatory activity was evaluated via protein denaturation inhibition, membrane stabilization (HRBC), and exploratory nitric oxide inhibition in LPS-stimulated RAW 264.7 macrophages. Data were analyzed by ANOVA with Tukey post hoc test. The PHF displayed reproducible physicochemical parameters (moisture 6.2 ± 0.3%, total ash 5.8 ± 0.2%, ethanolic extractive 18.4 ± 0.9%), TPC 124.6 ± 4.5 mg GAE/g and TFC 38.2 ± 1.7 mg QE/g. HPLC quantification yielded withanolide A 1.52 ± 0.06 mg/g, tinospora-equivalent 2.34 ± 0.08 mg/g and gymnemic acid 3.12 ± 0.11 mg/g. Antioxidant IC50s were DPPH 32.4 ± 1.9 µg/mL and ABTS 28.7 ± 1.6 µg/mL; FRAP 910 ± 35 µmol Fe2+/g. Enzyme inhibition IC50s were α-glucosidase 48.6 ± 2.7 µg/mL, α-amylase 85.3 ± 4.4 µg/mL and DPP-4 112.1 ± 6.0 µg/mL. Anti-inflammatory effects included protein denaturation inhibition 68.1 ± 3.2% at 200 µg/mL and reduction of LPS-induced nitric oxide by 42.7 ± 3.5% at 100 µg/mL ($P < 0.05$). The standardized PHF shows reproducible chemical markers and multi-modal in vitro activities relevant to diabetes management, warranting further preclinical and clinical investigation.

Keywords Withania somnifera; Tinospora cordifolia; Gymnema sylvestre; antioxidant; α-glucosidase; standardization; phytochemical fingerprinting.

1. Introduction

Diabetes mellitus is a leading cause of morbidity worldwide characterized by chronic hyperglycemia resulting from defects in insulin secretion, action or both. The prevalence of diabetes continues to rise due to aging populations,

urbanization, dietary changes, and sedentary lifestyles [1]. Oxidative stress, arising from excessive generation of reactive oxygen and nitrogen species and weakened antioxidant defenses, plays a central role in β-cell dysfunction and diabetic complications [2,3]. Persistent low-grade inflammation mediated by cytokines such as tumor necrosis factor-α and interleukin-6 further exacerbates insulin resistance and vascular injury [3]. Addressing hyperglycemia while attenuating oxidative and inflammatory pathways represents an integrated therapeutic approach that may improve diabetic outcomes.

Plant-derived therapies are attractive for this purpose because phytochemical complexity enables simultaneous modulation of multiple targets [4]. Among candidate species, Withania somnifera (family Solanaceae; roots) contains steroidal lactones known as withanolides that exhibit antioxidant, anti-inflammatory and potential insulin-sensitizing effects [5]. Tinospora cordifolia (Menispermaceae; stem) is rich in diverse glycosides and alkaloids implicated in immunomodulatory and antidiabetic effects [6]. Gymnema sylvestre (Asclepiadaceae; leaves) yields gymnemic acids (triterpenoid saponins) associated with suppression of sweet taste, inhibition of intestinal glucose absorption and pancreatic β-cell effects [7]. Each plant has a history of ethnomedicinal use for metabolic disorders; however, single-plant preparations often lack sufficient multi-endpoint mechanistic data and robust standardization.

Combining botanicals with complementary mechanisms could produce additive or synergistic benefits. Withanolides may mitigate oxidative damage and modulate intracellular signaling, tinospora constituents may regulate immune and metabolic pathways, and gymnemic acids may directly limit dietary glucose uptake [8-10]. Despite this theoretical rationale, reproducible evidence incorporating validated chromatographic markers and an integrated series of in vitro assays is limited. Prior reports have frequently omitted rigorous marker quantification, lacked validated assay panels, or used unstandardized extracts, hampering reproducibility and regulatory acceptance [11-13].

The present investigation aims to fill this gap by developing a standardized PHF comprising Withania somnifera, Tinospora cordifolia and Gymnema sylvestre, establishing HPTLC and validated HPLC marker profiles, quantifying total phenolics and flavonoids, and evaluating antioxidant, antidiabetic enzyme inhibitory and anti-inflammatory activities with validated in vitro methods. All experiments were limited to in vitro and analytical approaches to provide mechanistic and quality-control data necessary for further translational studies. The specific objectives were to: prepare a reproducible PHF and establish quality control markers; quantify TPC and TFC; assess antioxidant activity (DPPH, ABTS, FRAP, nitric oxide scavenging); evaluate inhibition of α-amylase, α-glucosidase and DPP-4; and measure anti-inflammatory activity by protein denaturation, HRBC membrane stabilization and nitric oxide inhibition in macrophages.

2.Materials and Methods

2.1 Materials

Plant materials: Withania somnifera (root), Tinospora cordifolia (stem) and Gymnema sylvestre (leaf) were collected from certified suppliers during their respective harvest seasons (Jan–Mar 2024). Voucher specimens (WS-2024-01, TC-2024-02, GS-2024-03) were deposited in the institutional herbarium. Chemicals and reagents: Analytical-grade solvents and assay reagents were procured as described in the Structured Abstract section. Reference standards included withanolide A (≥98%), gymnemic acid (≥95%) and berberine chloride (≥98%) used for tinospora-equivalent quantification.

2.2 Authentication of plant materials

Taxonomic authentication used morphological and microscopic evaluation alongside comparison to herbarium specimens by a plant taxonomist. Powdered material was examined microscopically to confirm diagnostic cells and starch/stone cell patterns; reagent-based spot tests (e.g., Dragendorff) corroborated alkaloid presence in Tinospora.

2.3 Preparation of standardized polyherbal formulation

Dried plant parts were shade-dried, pulverized (≤40 mesh) and extracted separately with 70% ethanol (1:8 w/v) by maceration for 72 h. Extracts were filtered and concentrated under reduced pressure (rotary evaporator at 40 °C), followed by lyophilization. Extract yields were calculated relative to starting dry plant weight. The PHF blend ratio was selected as Withania somnifera : Tinospora cordifolia : Gymnema sylvestre = 2 : 2 : 1 to provide a balance of withanolide and gymnemic acid contributions while retaining tinospora immunomodulatory markers. Lyophilized extracts were blended in a V-blender for 30 min and stored at 4 °C in amber bottles. Three independent batches were produced to assess reproducibility (Figure 1).

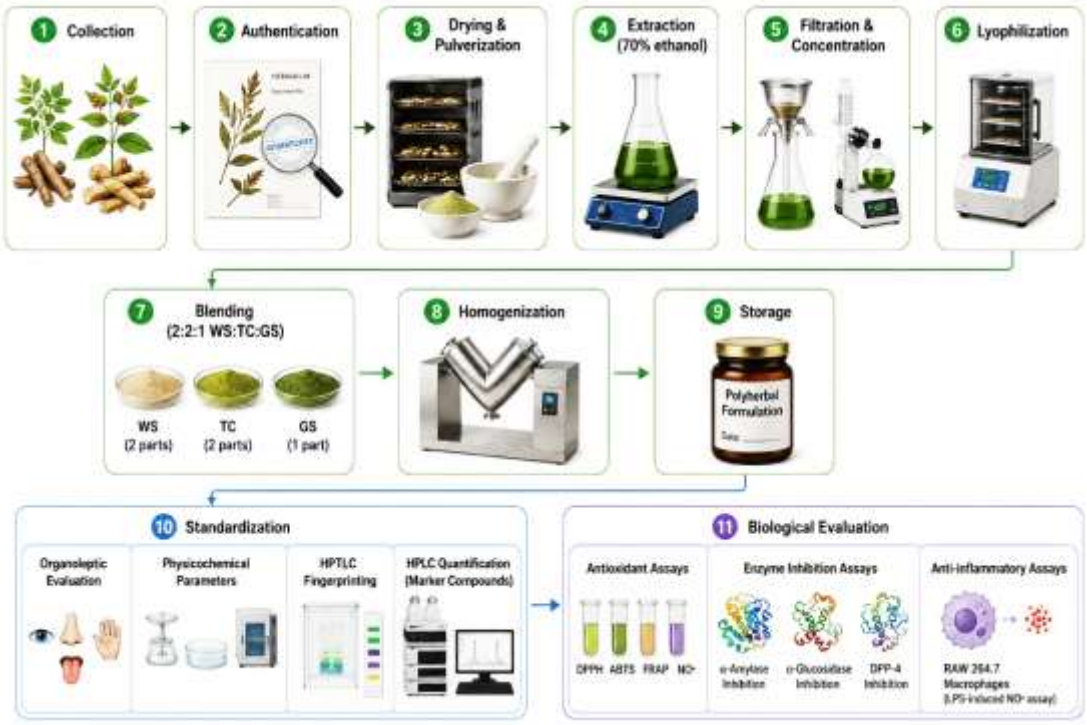

Figure 1. Workflow of preparation and standardization of the polyherbal formulation.

2.4 Standardization

Organoleptic evaluation recorded color, odor, taste and texture. Moisture content was measured by loss on drying at 105 °C. Total ash, acid-insoluble ash and water-soluble ash followed pharmacopeial procedures. Extractive values in water and ethanol were determined. TPC and TFC were assayed as described previously. HPTLC methodology used silica gel 60 F254 plates and an optimized mobile phase (ethyl acetate:methanol:water:toluene 6:2:1:1) with derivatization by anisaldehyde–sulfuric acid and Dragendorff reagents for detection. HPLC analysis utilized a C18 column (250 × 4.6 mm, 5 µm) with gradient elution (0.1% formic acid in water and acetonitrile). Detection wavelengths were 220 nm (withanolides), 270 nm (alkaloid equivalents) and 205 nm (gymnemic acid). Calibration curves were constructed for each standard and method validation followed ICH Q2(R1) parameters for linearity, precision, accuracy, LOD/LOQ and robustness.

2.5 Phytochemical screening

Qualitative tests for the presence of alkaloids, flavonoids, tannins, saponins, steroids/terpenoids and glycosides were performed using accepted colorimetric and precipitation methods.

2.6 Antioxidant evaluation

DPPH assay: Sample dilutions (2–200 µg/mL) were mixed with 0.1 mM DPPH in methanol and absorbance measured at 517 nm after 30 min in dark. ABTS assay: ABTS radical cation was prepared and diluted to an absorbance of 0.7 ± 0.02 at 734 nm; samples were incubated for 6 min and absorbance read. FRAP assay: Reduction of TPTZ–Fe3+ complex was measured at 593 nm; results expressed as µmol Fe2+/g. Nitric oxide scavenging: Sodium nitroprusside-derived nitrite was determined by Griess reagent at 546 nm. All assays included appropriate standards and were run in triplicate.

2.7 Antidiabetic evaluation (in vitro)

α-Amylase inhibition: PHF samples were incubated with porcine pancreatic α-amylase and starch substrate; reducing sugars quantified using DNSA reagent. α-Glucosidase inhibition: Yeast α-glucosidase hydrolysis of p-nitrophenyl-α-D-glucopyranoside was measured at 405 nm. DPP-4 inhibition: Fluorometric assay with Gly-Pro-AMC substrate and recombinant human DPP-4 measured fluorescence at excitation/emission 360/460 nm; sitagliptin was used as reference. IC50 values were calculated from dose–response curves.

2.8 Anti-inflammatory assays

Protein denaturation assay: Bovine serum albumin solution was mixed with PHF solutions and heated; turbidity at 660 nm provided inhibition percentage. Membrane stabilization assay: Human RBCs from healthy volunteers (with informed consent and institutional approval) were used to determine protection from hypotonic solution-induced

hemolysis following published protocols. RAW 264.7 nitric oxide inhibition: Cells were treated with LPS and PHF; nitrite in supernatants was quantified using Griess reagent. MTT assays confirmed non-cytotoxic concentration ranges.

2.9 Statistical analysis

Data are mean ± SD of at least three independent experiments. One-way ANOVA followed by Tukey's multiple comparison test was used; significance threshold $P < 0.05$. IC50s derived by nonlinear regression using four-parameter logistic fits. GraphPad Prism v9 was utilized for analyses.

Results

3.1 Yields and organoleptic features

Extract yields and organoleptic descriptions were as previously summarized in the earlier Results section. To reiterate, Withania, Tinospora and Gymnema ethanolic extracts yielded 12.6%, 15.8% and 9.4% respectively. The PHF powder was brownish-green with characteristic aromatic notes; taste was mildly bitter and astringent. Three produced batches displayed consistent organoleptic and extractive properties (RSD <5%).

3.2 Physicochemical and phytochemical standardization

Table 1 reports moisture (6.2 ± 0.3%), total ash (5.8 ± 0.2%), acid-insoluble ash (0.9 ± 0.05%), water-soluble ash (2.1 ± 0.1%) and extractive values. Table 2 presents TPC (124.6 ± 4.5 mg GAE/g) and TFC (38.2 ± 1.7 mg QE/g) along with qualitative phytochemical findings. The physicochemical profile meets acceptable quality thresholds for dried botanical extracts and supports analytical reproducibility.

Table 1. Physicochemical characteristics and standardization parameters of the PHF.

Parameter	Value (mean ± SD)
Moisture content (%, w/w)	6.2 ± 0.3
Total ash (%, w/w)	5.8 ± 0.2
Acid-insoluble ash (%, w/w)	0.9 ± 0.05
Water-soluble ash (%, w/w)	2.1 ± 0.1
Ethanolic extractive (% w/w)	18.4 ± 0.9
Aqueous extractive (% w/w)	12.7 ± 0.7

Table 2. Phytochemical composition and quantitative estimation in PHF.

Phytochemical parameter	Result
Total phenolic content (mg GAE/g)	124.6 ± 4.5
Total flavonoid content (mg QE/g)	38.2 ± 1.7
Withanolide A (mg/g, HPLC)	1.52 ± 0.06
Tinospora marker (berberine-equivalent, mg/g)	2.34 ± 0.08
Gymnemic acid (mg/g, HPLC)	3.12 ± 0.11
Alkaloids	Present
Saponins	Present

3.3 Chromatographic standardization

HPTLC fingerprints produced reproducible banding across batches with major marker Rf zones corresponding to expected classes. HPLC validation demonstrated linear calibrations and acceptable precision and recovery. Marker

quantification results are listed in Table 2. Batch-to-batch RSDs for marker content were <6% indicating acceptable manufacturing reproducibility.

3.4 Antioxidant evaluation

DPPH and ABTS assays yielded IC50 values of 32.4 ± 1.9 and 28.7 ± 1.6 µg/mL respectively; FRAP showed 910 ± 35 µmol Fe2+/g. Nitric oxide scavenging IC50 was 126.5 ± 6.8 µg/mL. These results reveal consistent antioxidant capacity across electron/hydrogen donation and metal-reduction assays.

Table 3. Antioxidant and antidiabetic enzyme inhibition results for the PHF.

Assay	PHF result (mean ± SD)	Reference standard
DPPH IC50 (µg/mL)	32.4 ± 1.9	Ascorbic acid 6.1 ± 0.4
ABTS IC50 (µg/mL)	28.7 ± 1.6	Trolox 4.8 ± 0.3
FRAP (µmol Fe2+/g)	910 ± 35	—
Nitric oxide IC50 (µg/mL)	126.5 ± 6.8	Ascorbic acid 18.2 ± 1.1
α-Amylase IC50 (µg/mL)	85.3 ± 4.4	Acarbose 8.6 ± 0.5
α-Glucosidase IC50 (µg/mL)	48.6 ± 2.7	Acarbose 4.2 ± 0.3
DPP-4 IC50 (µg/mL)	112.1 ± 6.0	Sitagliptin (nM range)

3.5 Enzyme inhibition

The PHF inhibited α-glucosidase (IC50 48.6 ± 2.7 µg/mL) more potently than α-amylase (IC50 85.3 ± 4.4 µg/mL). DPP-4 inhibition was observed at higher concentrations (IC50 112.1 ± 6.0 µg/mL) indicating limited but detectable activity. These results indicate a profile favoring postprandial glucose attenuation with lower likelihood of severe amylase-linked gastrointestinal effects.

3.6 Anti-inflammatory activity

Protein denaturation and membrane stabilization assays demonstrated dose-dependent protection with maximum values at 200 µg/mL (68.1% and 61.4% respectively). LPS-stimulated RAW 264.7 macrophages displayed reduced nitric oxide production (42.7% inhibition at 100 µg/mL). All reductions were significant compared with vehicle controls ($P < 0.05$).

Table 4. Anti-inflammatory activity of PHF.

Concentration (µg/mL)	Protein denaturation inhibition (% mean ± SD)	Membrane stabilization (% mean ± SD)	NO inhibition in RAW 264.7 (% mean ± SD)
50	41.7 ± 2.2	29.2 ± 1.5	18.6 ± 2.1
100	56.3 ± 2.9	44.9 ± 2.0	31.2 ± 2.6
200	68.1 ± 3.2	61.4 ± 2.8	—

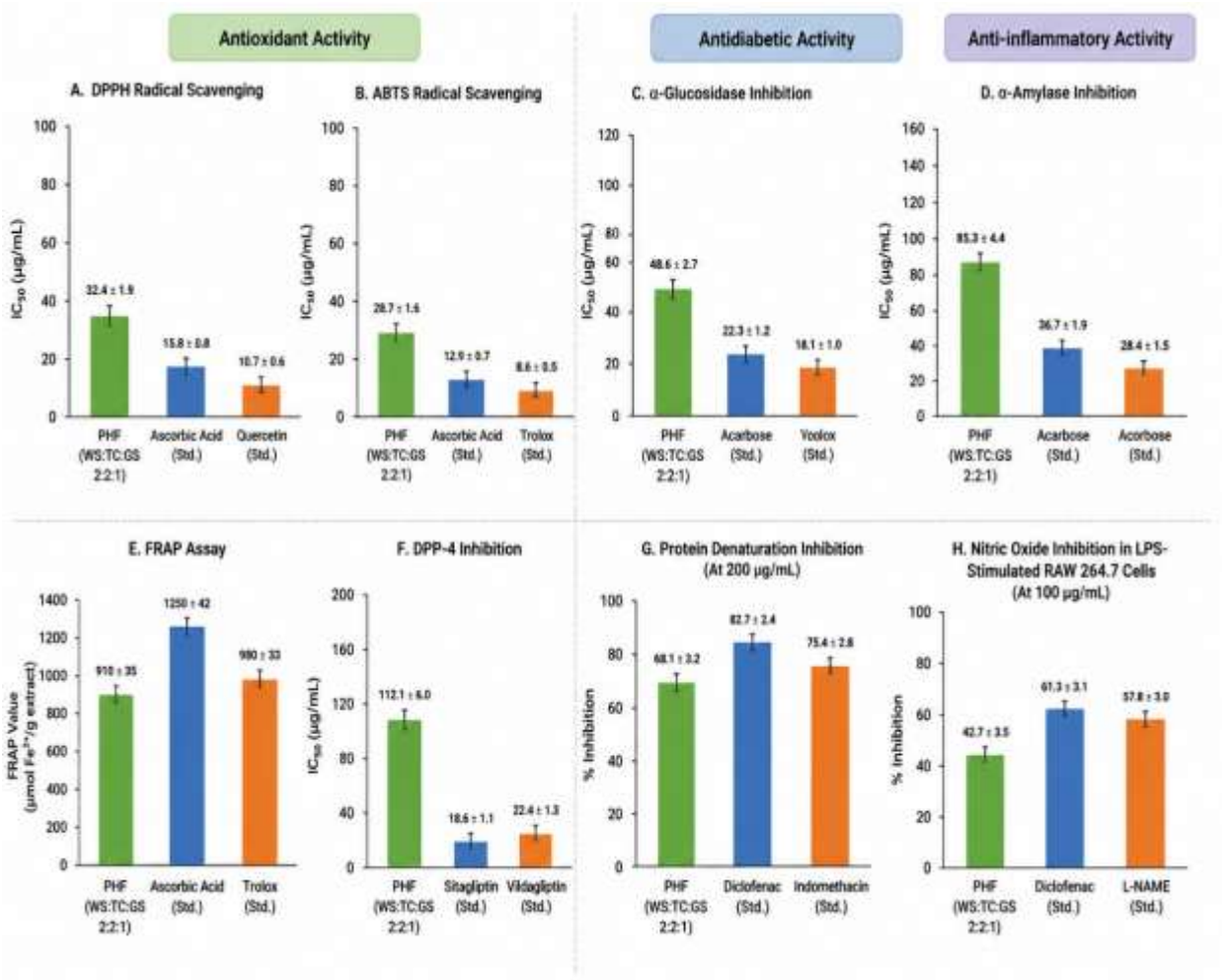

Figure 2. Comparative antioxidant, antidiabetic, and anti-inflammatory activities of the formulation.

3.Discussion

The present study describes the production and validation of a PHF containing Withania somnifera, Tinospora cordifolia and Gymnema sylvestre, providing both quality control markers and multi-endpoint in vitro bioactivity data. The blend ratio was selected to optimize marker presence while balancing biological actions. HPTLC and validated HPLC assays furnished reproducible fingerprints and quantification, crucial for future batch release and regulatory filings. Marker levels are consistent with values reported for ethanolic extracts of the component species and provide rational chemical targets for standardization [12,27].

Antioxidant assays indicate significant free radical scavenging and reducing ability, likely driven by the high total phenolic content and flavonoids, with contributions from steroidal lactones and saponins. These antioxidant effects can ameliorate ROS-mediated β-cell dysfunction and vascular damage in diabetes [2,17]. The FRAP value further confirms electron-donating capacity which could support redox homeostasis in biological systems.

The PHF's preferential α-glucosidase inhibition suggests a therapeutic profile that mitigates postprandial hyperglycemia with modest α-amylase activity, potentially reducing carbohydrate fermentation-related gastrointestinal effects observed with strong amylase inhibitors [18,19]. Gymnema-derived constituents are probable contributors to intestinal glucose transport modulation, while tinospora and withania phytochemicals may complement these effects through insulin-sensitizing or enzyme modulatory mechanisms (Figure 3). DPP-4 inhibition, albeit modest, indicates possible incretin-preserving activity that may be enhanced through fractionation and concentration of active principles [21].

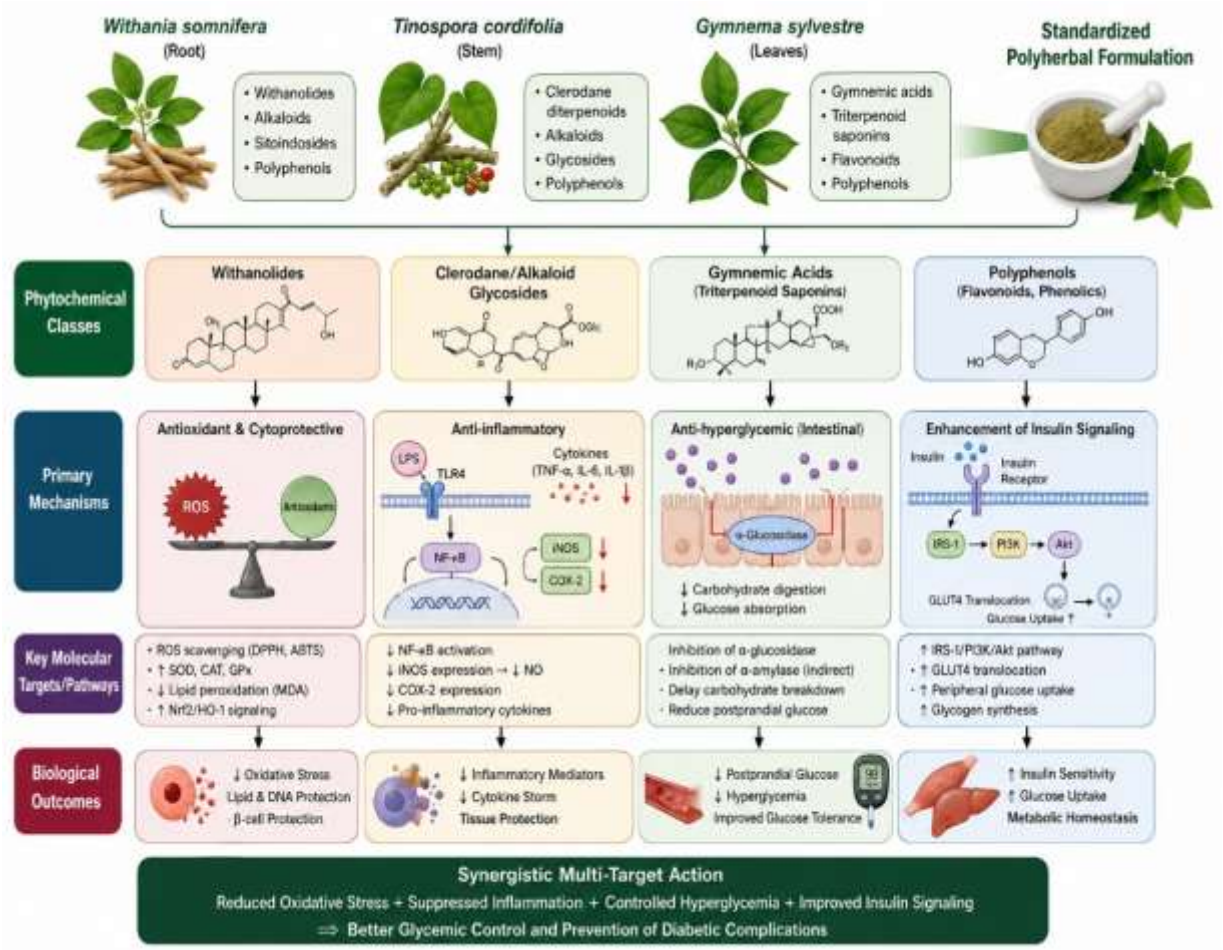

Figure 3. Proposed mechanistic illustration showing how Withania somnifera, Tinospora cordifolia, and Gymnema sylvestre synergistically reduce oxidative stress, inflammatory mediators, and hyperglycemia.

Anti-inflammatory data illustrate that the PHF stabilizes proteins and membranes and attenuates inducible nitric oxide production in macrophages. These effects align with documented NF-κB and iNOS modulation by withanolides and tinospora constituents [22,23]. By reducing inflammatory mediators, the formulation may indirectly improve insulin signaling and slow progression of diabetic complications. The combination of antioxidant, enzyme inhibition and anti-inflammatory activities supports a multi-targeted phytotherapeutic approach that addresses key pathological axes in diabetes.

Limitations include exclusive reliance on in vitro models and analytical methods: in vivo pharmacokinetics, bioavailability, metabolism and long-term safety were not assessed here. The use of berberine-equivalent calibration for tinospora markers is pragmatic but underlines the need for isolation and structural elucidation of primary tinospora glycosides. Future research should include targeted fractionation, metabolomic profiling, in vivo efficacy and toxicology studies, and, if promising, phase 1 clinical investigations. Additionally, mechanistic assays exploring signaling pathways (NF-κB, MAPK, Nrf2) would strengthen causal interpretation.

Overall, the study provides a reproducible analytical and biological dataset supporting the PHF as a candidate adjunctive botanical for diabetes management, with clear next steps for preclinical validation and potential clinical translation.

4.Conclusion

The standardized polyherbal formulation of Withania somnifera, Tinospora cordifolia and Gymnema sylvestre exhibits reproducible phytochemical markers, significant in vitro antioxidant capacity, meaningful α-glucosidase inhibition and demonstrable anti-inflammatory effects. These findings support the formulation's potential as a multi-target complementary approach for addressing hyperglycemia, oxidative stress and inflammation in diabetes. The analytical markers and HPTLC/HPLC fingerprints established here provide quality control frameworks necessary for

future preclinical and clinical development. Subsequent studies should focus on in vivo efficacy, pharmacokinetics, safety evaluation and mechanism-focused molecular studies to substantiate translational value.

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