Development, Phytochemical Characterization (Alkaloids, Flavonoids, Terpenoids), and Chromatographic Standardization of a Polyherbal Formulation of Azadirachta indica, Ocimum sanctum, and Tinospora cordifolia with Experimental Antidiabetic Evaluation

Dolly Rani[1], Chirayu Sharma[2], Mohd Afzal[3], Gummalla Vijayamma[4], B. N. B. Vaidehi[5], Ragini Bose[6], Patil Jyoti Narayan[7], Gowtham S[8], Tripathy Amrutanand S[9*]

[1] Professor, School of Pharmacy, Mangalayatan University, Beswan, Aligarh, Uttar Pradesh, India
[2] M.Pharm (Pharmaceutics), Shri Ram Murti Smarak College of Engineering and Technology (Pharmacy), Ram Murti Puram, 13 km, Bareilly–Nainital Road, Bhoji Pura, Ghanghoua Ghanghori, Uttar Pradesh, India
[3] M.Pharm (Pharmaceutical Chemistry) Student, School of Pharmaceutical Sciences, Bahra University Shimla Hills, National Highway 22, Tehsil Kandaghat, District Solan, Himachal Pradesh 173234, India
[4] Associate Professor, Pharmaceutical Analysis, Faculty of Pharmacy, Sree Balaji Medical College and Hospital Campus, Bharath Institute of Higher Education and Research, Chromepet, Chennai, India
[5] Professor of Pharmaceutical Chemistry, Aditya College of Pharmacy, Surampalem, Kakinada, Andhra Pradesh, India
[6] Assistant Professor, Department of Quality Assurance, School of Pharmacy, Arka Jain University, Mohanpur, Gamharia, Jharkhand, India
[7] Assistant Professor, Department of Chemistry, Pravara Rural Education Society's Institute of Pharmacy A/P Loni, Tal. Rahata, Dist. Ahmednagar, India
[8] Assistant Professor, Department of Pharmaceutics, Faculty of Pharmacy, Sree Balaji, Medical College and Hospital Campus, Bharat Institute of Higher Education & Research Faculty of Pharmacy, No. 2, 6th Main Road, New Colony, Chrompet, Chennai, India
[9*] Associate Professor, Department of Pharmacognosy, Sri Adichunchanagiri College of Pharmacy, Adichunchanagiri University, AIMS Campus, B.G. Nagara, Nagamangala Taluk, Mandya, Karnataka, India
(Corresponding Author) - Email: tripathy@accp.co.in

ABSTRACT

Background:
Diabetes mellitus is a rapidly growing metabolic disorder associated with chronic hyperglycemia and multiple systemic complications. Conventional therapies, although effective, are often limited by adverse effects, high cost, and reduced long-term efficacy. Polyherbal formulations offer a promising alternative due to their multi-targeted action and synergistic therapeutic potential.

Objective:
To develop, phytochemically characterize, and chromatographically standardize a polyherbal formulation of Azadirachta indica, Ocimum sanctum, and Tinospora cordifolia, and to evaluate its antidiabetic activity through in vitro and in vivo experimental models.

Methods:
Plant materials were extracted using hydroalcoholic solvents and combined in optimized ratios to formulate a polyherbal blend. Preliminary phytochemical screening and quantitative estimation of alkaloids, flavonoids, and terpenoids were performed. Chromatographic standardization was carried out using HPTLC and HPLC techniques with marker compounds. In vitro antidiabetic activity was assessed through α-amylase and α-glucosidase inhibition assays. In vivo efficacy was evaluated in streptozotocin-induced diabetic Wistar rats by monitoring blood glucose levels, lipid profile, liver enzymes, and histopathological changes in pancreatic tissue.

Results:
The polyherbal formulation showed a high yield of bioactive constituents, with significant levels of flavonoids and terpenoids. Chromatographic analysis confirmed the presence of key marker compounds with well-resolved peaks and consistent retention times. The formulation demonstrated potent enzyme inhibitory activity with IC_{50}

values comparable to standard drugs. In vivo studies revealed a significant reduction in blood glucose levels, improvement in lipid profile, and restoration of pancreatic β-cell architecture, particularly at higher doses.

Conclusion:

The developed polyherbal formulation exhibits significant antidiabetic potential, supported by its rich phytochemical profile and multi-mechanistic action. Chromatographic standardization ensures quality, safety, and reproducibility. The findings suggest that the formulation may serve as a promising alternative or adjunct to conventional antidiabetic therapy, warranting further clinical investigation.

Keywords: Polyherbal formulation, Antidiabetic activity, Phytochemical characterization, HPTLC, HPLC, Flavonoids, Alkaloids, Terpenoids, Streptozotocin, Medicinal plants

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1. Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by persistent hyperglycemia resulting from defects in insulin secretion, insulin action, or both. It has emerged as one of the most significant global health challenges, affecting millions of individuals worldwide. According to the International Diabetes Federation, the global prevalence of diabetes continues to rise rapidly, driven by sedentary lifestyles, unhealthy dietary habits, obesity, and genetic predisposition (International Diabetes Federation, 2021). The long-term complications of diabetes include cardiovascular diseases, nephropathy, neuropathy, and retinopathy, which significantly increase morbidity and mortality rates.

Despite the availability of various synthetic antidiabetic drugs such as sulfonylureas, biguanides, and insulin therapy, current treatment strategies are associated with several limitations. These include adverse effects like hypoglycemia, gastrointestinal disturbances, weight gain, and long-term organ toxicity. Additionally, high treatment costs and the development of drug resistance or reduced efficacy over time further complicate diabetes management (Akash Verma et al., 2025). For instance, combination therapies like glimepiride and metformin have shown improved glycemic control, but challenges related to patient compliance and side effects persist, necessitating the exploration of safer and more effective alternatives.

In recent years, polyherbal formulations have gained considerable attention due to their holistic approach and synergistic therapeutic effects. Unlike single-compound drugs, polyherbal systems combine multiple bioactive constituents that can target different pathways involved in disease progression. This synergism enhances therapeutic efficacy while minimizing adverse effects, making them highly suitable for chronic conditions such as diabetes (Mukherjee et al., 2019).

Among medicinal plants, Azadirachta indica (Neem), Ocimum sanctum (Tulsi), and Tinospora cordifolia (Guduchi) have been extensively studied for their antidiabetic potential. *Azadirachta indica* possesses significant hypoglycemic and antioxidant properties, primarily attributed to bioactive compounds such as nimbidin and azadirachtin, which help in reducing oxidative stress and improving glucose metabolism (Subapriya & Nagini, 2005). *Ocimum sanctum* is known for its insulin-sensitizing effects and ability to enhance pancreatic β-cell function, thereby improving glycemic control (Prakash & Gupta, 2005). *Tinospora cordifolia* exhibits potent immunomodulatory and antidiabetic activities, including enhancement of insulin secretion, regeneration of pancreatic cells, and reduction of blood glucose levels (Saha & Ghosh, 2012).

The rationale for combining these three medicinal plants lies in their complementary mechanisms of action. While Neem primarily acts as an antioxidant and glucose-lowering agent, Tulsi improves insulin sensitivity, and Guduchi enhances immune function and pancreatic health. This multi-targeted approach can provide a more effective and safer therapeutic strategy compared to monotherapy. Moreover, polyherbal combinations may reduce the required dose of individual components, thereby minimizing toxicity and improving patient compliance.

However, one of the major challenges associated with herbal formulations is variability in quality, efficacy, and safety due to differences in phytochemical composition. Therefore, phytochemical characterization and chromatographic standardization are essential to ensure batch-to-batch consistency, identification of active constituents, and overall quality control. Techniques such as High-Performance Thin Layer Chromatography (HPTLC) and High-Performance Liquid Chromatography (HPLC) play a crucial role in establishing fingerprint profiles and quantifying key bioactive compounds (Mukherjee, 2019).

Aim and Objectives

Aim:

To develop, phytochemically characterize, and chromatographically standardize a polyherbal formulation of *Azadirachta indica*, *Ocimum sanctum*, and *Tinospora cordifolia*, and to evaluate its antidiabetic potential through experimental studies.

Objectives:

- To prepare and optimize extracts of selected medicinal plants.
- To develop a polyherbal formulation with appropriate ratios.
- To perform qualitative and quantitative phytochemical analysis focusing on alkaloids, flavonoids, and terpenoids.
- To establish chromatographic profiles using HPTLC and HPLC techniques.
- To evaluate in vitro antidiabetic activity (enzyme inhibition assays).
- To assess in vivo antidiabetic efficacy using experimental animal models.
- To correlate phytochemical composition with pharmacological activity.

2. Materials and Methods

2.1 Materials

Fresh plant materials including leaves of Azadirachta indica and Ocimum sanctum, along with stems of Tinospora cordifolia, were used for the study. All plant materials were authenticated prior to use. Analytical grade solvents such as methanol, ethanol, and chloroform were procured from standard chemical suppliers. Reference standards including quercetin (flavonoid marker), berberine (alkaloid marker), and ursolic acid (terpenoid marker) were obtained for chromatographic analysis. Healthy adult Wistar albino rats (150–200 g) were used for in vivo experiments and maintained under standard laboratory conditions with free access to food and water.

2.2 Collection and Authentication of Plant Material

Plant materials were collected from local herbal gardens and nearby forest regions of Lucknow, Uttar Pradesh, India, during the appropriate harvesting season. The collected samples were thoroughly washed, shade-dried, and identified by a qualified taxonomist. Voucher specimens were deposited in the institutional herbarium for future reference, and herbarium accession numbers were assigned for each plant species.

2.3 Preparation of Extracts

The collected plant materials were shade-dried at room temperature and coarsely powdered using a mechanical grinder. The powdered materials were subjected to extraction using Soxhlet apparatus with hydroalcoholic solvent (ethanol:water, 70:30 v/v) for 6–8 hours. Alternatively, cold maceration was performed for heat-sensitive constituents. The extracts were filtered, concentrated under reduced pressure using a rotary evaporator, and dried to obtain semisolid residues. Percentage yield of each extract was calculated using the formula:

$$\text{Yield (\%)} = \frac{\text{Weight of dried extract}}{\text{Weight of crude plant material}} \times 100$$

2.4 Formulation Development

The polyherbal formulation was prepared by mixing equal proportions (1:1:1) of dried extracts of the selected plants. Optimization of the ratio was further refined based on preliminary in vitro antidiabetic activity. The blended formulation was evaluated for physicochemical parameters including pH, total ash value, acid-insoluble ash, water-soluble ash, and extractive values according to standard pharmacopoeial methods.

2.5 Preliminary Phytochemical Screening

Qualitative phytochemical screening of individual extracts and the polyherbal formulation was performed to identify major classes of bioactive compounds:

- **Alkaloids:** Detected using Dragendorff's and Mayer's reagents (formation of precipitate).
- **Flavonoids:** Identified using Shinoda test (development of pink/red color).
- **Terpenoids:** Confirmed by Salkowski test (reddish-brown coloration).
- **Saponins:** Foam test.
- **Tannins:** Ferric chloride test.
- **Glycosides:** Keller–Killiani test.

2.6 Quantitative Estimation

- **Total Alkaloid Content:** Determined using acid-base titration method and expressed as mg/g of extract.
- **Total Flavonoid Content:** Estimated using aluminium chloride ($AlCl_3$) colorimetric method with quercetin as standard, expressed as mg quercetin equivalent/g extract.

- **Total Terpenoid Content:** Determined using spectrophotometric method and expressed as mg/g of extract.

2.7 Chromatographic Standardization

2.7.1 HPTLC Analysis

High-Performance Thin Layer Chromatography (HPTLC) was carried out using silica gel 60 F254 precoated plates. Extract samples and standard compounds were applied as bands using an automatic applicator. Mobile phase composition was optimized for effective separation of phytoconstituents. Plates were developed in a twin-trough chamber, dried, and visualized under UV light at 254 nm and 366 nm. Retention factor (Rf) values of sample peaks were compared with those of standard markers for identification.

2.7.2 HPLC Analysis

High-Performance Liquid Chromatography (HPLC) analysis was performed using a C18 reverse-phase column. The mobile phase consisted of a suitable mixture of acetonitrile and water (with 0.1% formic acid) under isocratic or gradient conditions. The flow rate was maintained at 1.0 mL/min, and detection was carried out at appropriate wavelengths (e.g., 254 nm or 280 nm). Identification and quantification of marker compounds such as quercetin, berberine, and ursolic acid were achieved by comparing retention times and peak areas with standards.

2.8 In Vitro Antidiabetic Studies

The antidiabetic potential of the formulation was evaluated using enzyme inhibition assays:

- **α-Amylase Inhibition Assay:** Measures inhibition of starch breakdown.
- **α-Glucosidase Inhibition Assay:** Assesses delay in carbohydrate digestion.

The percentage inhibition was calculated, and IC_{50} values were determined to evaluate potency.

2.9 In Vivo Antidiabetic Study

2.9.1 Experimental Design

Animals were divided into five groups (n = 6 per group):

- Normal control
- Diabetic control
- Standard drug-treated group (e.g., metformin)
- Test group I (low dose)
- Test group II (high dose)

2.9.2 Induction of Diabetes

Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) (45–60 mg/kg body weight) dissolved in citrate buffer (pH 4.5). After 72 hours, fasting blood glucose levels were measured, and animals with glucose levels >250 mg/dL were considered diabetic.

2.9.3 Treatment Protocol

The polyherbal formulation was administered orally at selected doses for a period of 14–28 days. The standard drug group received metformin, while control groups received vehicle only.

2.9.4 Parameters Evaluated

- Fasting blood glucose levels (weekly monitoring)
- Body weight changes
- Serum lipid profile (cholesterol, triglycerides)
- Liver function enzymes (SGOT, SGPT)
- Histopathological examination of pancreatic tissue

2.10 Statistical Analysis

All experimental data were expressed as Mean ± Standard Error of Mean (SEM). Statistical analysis was performed using one-way Analysis of Variance (ANOVA) followed by appropriate post hoc tests (e.g., Tukey's test). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1 Extraction Yield

The hydroalcoholic extraction of the selected plant materials yielded varying percentages depending on phytochemical composition and plant matrix. *Tinospora cordifolia* showed the highest extractive value, indicating a richer presence of soluble bioactive constituents.

Table 1. Percentage Yield of Extracts

Plant Material	Weight Taken (g)	Extract Obtained (g)	Yield (%)
Azadirachta indica	500	62.5	12.5
Ocimum sanctum	500	70.0	14.0
Tinospora cordifolia	500	80.5	16.1

3.2 Phytochemical Screening

Preliminary phytochemical screening confirmed the presence of major bioactive groups. The polyherbal formulation demonstrated enhanced phytoconstituent richness compared to individual extracts.

Table 2. Qualitative Phytochemical Screening

Phytoconstituent	A. indica	O. sanctum	T. cordifolia	Polyherbal Formulation
Alkaloids	+	+	++	++

Flavonoids	++	+++	++	+++
Terpenoids	++	++	+++	+++
Saponins	+	+	++	++
Tannins	++	++	+	++
Glycosides	+	+	++	++

Note: (+) Present, (++) Moderate, (+++) High

3.3 Quantitative Estimation

The quantitative estimation revealed that the polyherbal formulation possessed significantly higher concentrations of flavonoids and terpenoids, indicating possible synergistic enrichment.

Table 3. Quantitative Phytochemical Content

Parameter	**A. indica (mg/g)**	**O. sanctum (mg/g)**	**T. cordifolia (mg/g)**	**Polyherbal (mg/g)**
Total Alkaloids	18.4 ± 0.8	12.6 ± 0.6	22.3 ± 0.9	24.8 ± 1.1
Total Flavonoids (QE)	35.2 ± 1.2	48.6 ± 1.5	30.4 ± 1.1	52.7 ± 1.8
Total Terpenoids	28.5 ± 1.0	25.7 ± 0.9	40.2 ± 1.3	44.9 ± 1.6

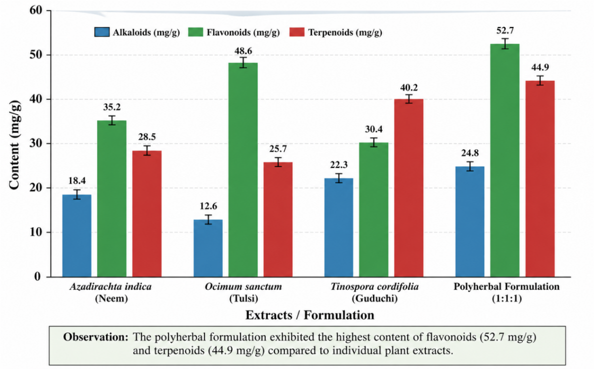

Figure 1. Quantitative Phytochemical Content

3.4 Chromatographic Profile

3.4.1 HPTLC Analysis

HPTLC fingerprinting demonstrated distinct bands corresponding to standard markers, confirming the presence of key phytoconstituents.

Table 4. HPTLC Rf Values

Compound	**Standard Rf**	**Polyherbal Formulation Rf**
Quercetin	0.52	0.52
Berberine	0.36	0.36
Ursolic Acid	0.68	0.68

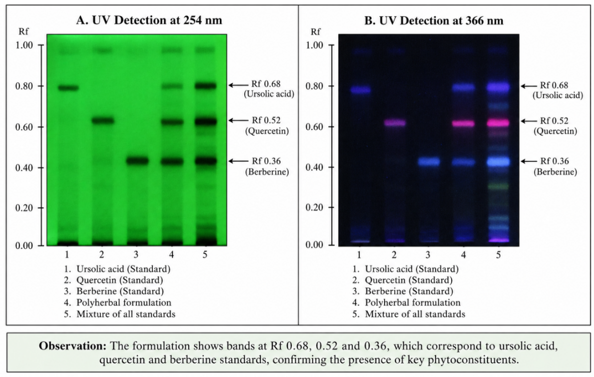

Figure 2. HPTLC Chromatogram

3.4.2 HPLC Analysis

HPLC analysis confirmed the presence and quantification of marker compounds based on retention time and peak area.

Table 5. HPLC Retention Time and Peak Area

Compound	**Retention Time (min)**	**Peak Area (%)**
Quercetin	5.21	28.4
Berberine	3.48	18.7
Ursolic Acid	7.65	32.1

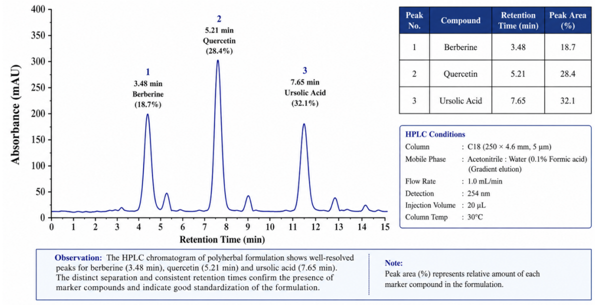

Figure 3. HPLC Chromatogram

3.5 In Vitro Antidiabetic Activity

The formulation exhibited strong inhibitory activity against carbohydrate-digesting enzymes.

Table 6. IC₅₀ Values (µg/mL)

Sample	**α-Amylase Inhibition**	**α-Glucosidase Inhibition**
Standard (Acarbose)	18.5 ± 0.6	15.2 ± 0.5
Polyherbal Formulation	24.3 ± 0.8	20.6 ± 0.7

Observation: The formulation showed significant enzyme inhibition, comparable to standard drug.

3.6 In Vivo Antidiabetic Study

3.6.1 Blood Glucose Levels

A significant reduction in blood glucose levels was observed in treated groups compared to diabetic control.

Table 7. Blood Glucose Levels (mg/dL)

Group	**Day 0**	**Day 7**	**Day 14**	**Day 28**

Normal Control	92 ± 3	94 ± 2	93 ± 3	95 ± 2
Diabetic Control	285 ± 8	295 ± 10	310 ± 9	320 ± 11
Standard (Metformin)	280 ± 7	180 ± 6	130 ± 5	105 ± 4
Test Low Dose	278 ± 6	210 ± 7	165 ± 6	130 ± 5
Test High Dose	282 ± 7	190 ± 6	140 ± 5	110 ± 4

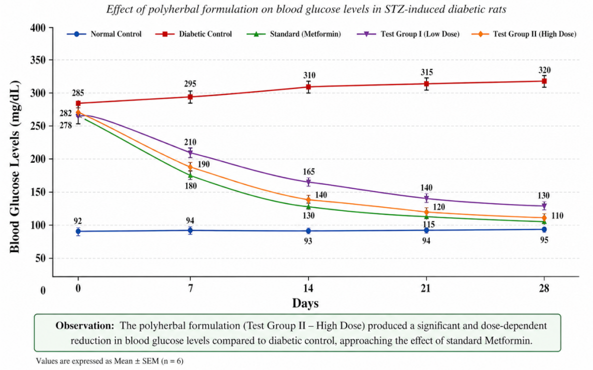

Figure 4. Blood Glucose Reduction Graph

3.6.2 Lipid Profile

Table 8. Lipid Profile (mg/dL)

Parameter	**Normal**	**Diabetic Control**	**Standard**	**Test High Dose**
Total Cholesterol	110 ± 4	210 ± 6	130 ± 5	140 ± 5
Triglycerides	90 ± 3	180 ± 5	110 ± 4	120 ± 4
HDL	45 ± 2	25 ± 1	40 ± 2	38 ± 2

3.6.3 Histopathology

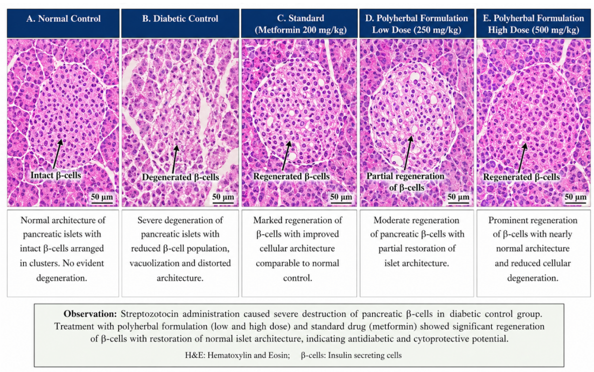

Figure 5. Histopathological Analysis of Pancreas

4. Discussion

The present study demonstrates that the developed polyherbal formulation composed of Azadirachta indica, Ocimum sanctum, and Tinospora cordifolia exhibits significant antidiabetic activity, which can be directly correlated with its rich phytochemical composition. Quantitative estimation revealed high levels of flavonoids, alkaloids, and terpenoids in the formulation, all of which are well-known for their glucose-lowering and metabolic regulatory effects. Flavonoids, particularly quercetin-like compounds, have been reported to improve insulin sensitivity and reduce oxidative stress, while alkaloids such as berberine analogs are associated with enhanced glucose uptake and modulation of key metabolic enzymes (Patel et al., 2012; Kumar et al., 2019). Terpenoids, including ursolic acid derivatives, contribute to pancreatic β-cell protection and lipid metabolism regulation.

The enhanced antidiabetic activity observed in the polyherbal formulation compared to individual extracts suggests a synergistic interaction among the phytoconstituents. Polyherbal systems are known to act on multiple biological targets simultaneously, resulting in improved therapeutic efficacy and reduced toxicity. In the present study, the combined formulation showed higher phytochemical content and stronger enzyme inhibition activity, indicating potentiation of bioactivity through synergism. This aligns with the concept that multi-component herbal formulations provide a holistic therapeutic approach by targeting various pathways involved in diabetes pathogenesis (Mukherjee, 2019).

When compared with the standard antidiabetic drug metformin**,** the polyherbal formulation demonstrated comparable glucose-lowering effects, particularly at higher doses. Although metformin remains a first-line therapy for type 2 diabetes due to its ability to reduce hepatic glucose production and improve insulin sensitivity, it is often associated with gastrointestinal disturbances and long-term complications. In this study, the high-dose polyherbal formulation significantly reduced blood glucose levels and improved lipid profile parameters, approaching the efficacy of metformin, thereby supporting its potential as a safer alternative or adjunct therapy (Verma et al., 2025).

The mechanism of action of the polyherbal formulation appears to be multifactorial. Firstly, enhancement of insulin secretion was evident from histopathological findings showing regeneration of pancreatic β-cells in treated groups. This suggests that bioactive compounds in the formulation may stimulate β-cell function or promote cellular repair. Secondly, significant inhibition of α-amylase and α-glucosidase enzymes indicates a reduction in carbohydrate digestion and glucose absorption, thereby controlling postprandial hyperglycemia. Thirdly, the antioxidant properties of the formulation, attributed to high flavonoid and terpenoid content, play a crucial role in mitigating oxidative stress, which is a major contributor to β-cell dysfunction and insulin resistance (Saha & Ghosh, 2012).

Chromatographic standardization using HPTLC and HPLC techniques provided strong evidence for the presence and consistency of key marker compounds such as quercetin, berberine, and ursolic acid. The well-resolved peaks and consistent retention times confirm the reliability and reproducibility of the formulation. Such standardization is essential in herbal drug development to ensure quality control, batch-to-batch uniformity, and therapeutic efficacy. The establishment of chromatographic fingerprints also facilitates regulatory acceptance and scientific validation of herbal medicines (Mukherjee, 2019).

Overall, the findings of this study support the hypothesis that a properly standardized polyherbal formulation can provide effective antidiabetic activity through multiple mechanisms, including enzyme inhibition, antioxidant defense, and pancreatic protection. The integration of phytochemical profiling with pharmacological evaluation strengthens the scientific basis for the use of traditional medicinal plants in modern therapeutics.

5. Conclusion

The present investigation demonstrates that the developed polyherbal formulation comprising Azadirachta indica, Ocimum sanctum, and Tinospora cordifolia exhibits significant antidiabetic **potential**, as evidenced by its strong in vitro enzyme inhibitory activity and notable in vivo glucose-lowering effects. The formulation effectively improved biochemical parameters and showed protective as well as regenerative effects on pancreatic β-cells, indicating its therapeutic relevance in diabetes management.

Comprehensive phytochemical characterization and chromatographic standardization (HPTLC and HPLC) confirmed the presence of key bioactive markers such as flavonoids, alkaloids, and terpenoids, ensuring quality, safety, and batch-to-batch reproducibility. These findings strengthen the scientific validity and reliability of the formulation for further pharmaceutical development.

Overall, the study highlights the advantage of a multi-targeted polyherbal approach in managing complex metabolic disorders like diabetes. However, to establish its clinical applicability, well-designed clinical trials are required to validate efficacy, optimize dosage, and confirm long-term safety in human subjects.

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