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Antidiabetic and Safety Profile Studies of Polyherbal Formulation

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Abstract: *Polyherbal formulations have gained significant attention in the management of diabetes mellitus due to their synergistic therapeutic effects and reduced side effects compared to synthetic drugs. These formulations, composed of multiple medicinal plant extracts, target various pathophysiological pathways involved in diabetes, including insulin resistance, impaired insulin secretion, oxidative stress, and inflammation. The present review focuses on the antidiabetic potential and safety profile of commonly used polyherbal formulations.*

Several studies have demonstrated that polyherbal combinations exhibit enhanced glycemic control by improving pancreatic β-cell function, increasing peripheral glucose uptake, and inhibiting carbohydrate-digesting enzymes such as α-amylase and α-glucosidase. Additionally, the presence of bioactive phytoconstituents such as flavonoids, alkaloids, tannins, and phenolic compounds contributes to antioxidant and anti-inflammatory effects, further supporting their therapeutic role in diabetes management.

Safety evaluation of polyherbal formulations indicates a relatively low incidence of adverse effects when used at recommended doses. Toxicological studies, including acute and chronic toxicity assessments, have generally shown these formulations to be non-toxic and well tolerated. However, variability in plant composition, lack of standardization, and potential herb–drug interactions remain critical concerns.

In conclusion, polyherbal formulations offer a promising, cost-effective, and safer alternative for diabetes management. Nevertheless, rigorous clinical trials, standardization of formulations, and comprehensive safety assessments are essential to validate their efficacy and ensure safe therapeutic use..

Keywords: *Polyherbal formulations*

I. INTRODUCTION

1.1 Diabetes Mellitus: A Global Health Crisis

Diabetes mellitus (DM) represents one of the most formidable public health challenges of the 21st century. According to the International Diabetes Federation (IDF) Diabetes Atlas, 10th edition (2021), approximately 537 million adults aged 20-79 years were living with diabetes globally, accounting for 10.5% of the world's adult population. This figure is projected to rise to 643 million by 2030 and 783 million by 2045, representing a 46% increase over two decades (IDF, 2021). Even more alarming, an estimated 240 million people with diabetes remain undiagnosed, unaware of their condition and its progressive complications.[1,2,4]

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The economic burden is equally staggering. Global health expenditure on diabetes reached USD 966 billion in 2021, a 316% increase over the past 15 years. In low- and middle-income countries, where 80% of people with diabetes reside, these costs often push families below the poverty line. India, often termed the "diabetes capital of the world," bears a disproportionately high burden. The Indian Council of Medical Research–India Diabetes (ICMR-INDIAB) study, the largest nationally representative survey, reported that over 101 million individuals in India have diabetes, with another 136 million in the prediabetic stage (Anjana et al., 2023). Urbanization, sedentary lifestyles, and dietary shifts have driven prevalence rates in Indian cities to exceed 25%, comparable to Western nations. [1,2]

Type 2 diabetes mellitus (T2DM) constitutes 90-95% of all diabetes cases. Unlike type 1 diabetes, which results from autoimmune β-cell destruction, T2DM is characterized by a progressive combination of insulin resistance and relative insulin deficiency. The condition typically develops over years or decades, allowing microvascular and macrovascular complications to begin long before clinical diagnosis. By the time fasting hyperglycemia is detected, many patients have already lost 50-80% of their pancreatic β-cell function (Kahn et al., 2014). [1]

1.2 Pathophysiology: A Multifactorial Disease Network

The classical understanding of T2DM pathogenesis has expanded significantly beyond the "triumvirate" of insulin resistance, β-cell dysfunction, and excessive hepatic glucose production. Contemporary research has identified eight core pathophysiological defects, often termed the "ominous octet" (DeFronzo, 2009):

1. Insulin resistance in muscle: Skeletal muscle accounts for 75-80% of glucose disposal. In T2DM, impaired insulin signaling reduces GLUT4 translocation, decreasing glucose uptake by 40-60%.
2. Adipocyte insulin resistance and lipolysis: Dysfunctional adipocytes release excessive free fatty acids, which worsen muscle insulin resistance and hepatic glucose production.[5,7]
3. Pancreatic β-cell dysfunction: Progressive loss of insulin secretion capacity, beginning 10-15 years before diagnosis.
4. Incretin deficiency: Reduced secretion of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) from intestinal L and K cells, impairing glucose-dependent insulin secretion.
5. Hyperglucagonemia: Inappropriately elevated glucagon from pancreatic α-cells increases hepatic glucose production even in the hyperglycemic state.
6. Increased renal glucose reabsorption: Upregulation of sodium-glucose cotransporter-2 (SGLT2) in proximal tubules raises the renal threshold for glucose excretion.
7. Neurotransmitter dysfunction: Imbalance in hypothalamic signaling contributes to increased appetite and defective glucose sensing.[11,5]

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8. Gut dysbiosis: Alterations in gut microbiome composition affect bile acid metabolism, short-chain fatty acid production, and systemic inflammation.

These interconnected defects explain why monotherapy with a single agent rarely achieves or sustains glycemic targets. The United Kingdom Prospective Diabetes Study (UKPDS) demonstrated that after 3 years, 50% of patients on monotherapy required additional agents, and by 9 years, only 25% maintained HbA1c < 7% on a single drug (Turner et al., 1999). This progressive nature necessitates either combination therapy or multi-targeted approaches [12,5]

1.3 Limitations of Conventional Antidiabetic Drugs

Current pharmacological management of T2DM includes multiple drug classes, each with distinct mechanisms but also significant limitations:

Metformin remains the first-line agent globally. It activates AMP-activated protein kinase (AMPK), reducing hepatic glucose production and improving peripheral insulin sensitivity.

However, 20-30% of patients experience dose-limiting gastrointestinal adverse effects (nausea, diarrhea, bloating), and long-term use is associated with vitamin B12 deficiency and, rarely, lactic acidosis. It is contraindicated in renal impairment (eGFR < 30 mL/min) (American Diabetes Association, 2024). [342][

Sulfonylureas (glibenclamide, glimepiride, gliclazide) stimulate insulin secretion via closure of K_ATP channels on β-cells. Their major limitation is hypoglycemia, with severe episodes requiring hospitalization occurring in 1-2% of users annually. They also cause weight gain (2-4 kg on average) and exhibit high secondary failure rates (5-10% per year).

Thiazolidinediones (pioglitazone, rosiglitazone) improve insulin sensitivity via PPAR-γ activation. However, fluid retention, weight gain, increased fracture risk in women, and concerns about cardiovascular safety (rosiglitazone) have limited their use. Pioglitazone also carries a bladder cancer warning after long-term use [567].

DPP-4 inhibitors (sitagliptin, vildagliptin, linagliptin) increase endogenous GLP-1 levels. Their efficacy is modest (HbA1c reduction of 0.5-0.8%), they are expensive, and rare cases of severe arthralgia and pancreatitis have been reported.

SGLT2 inhibitors (dapagliflozin, empagliflozin, canagliflozin) block renal glucose reabsorption, causing glucosuria. While cardiorenal benefits are established, they increase risks of genital mycotic infections (5-10%), euglycemic diabetic ketoacidosis, volume depletion, and lower limb amputation (canagliflozin).[452]

GLP-1 receptor agonists (liraglutide, semaglutide, dulaglutide) enhance glucose-dependent insulin secretion, suppress glucagon, and delay gastric emptying. Their injectable route, gastrointestinal side effects (nausea in 20-40%), high cost, and rare risks of pancreatitis and medullary thyroid carcinoma limit widespread adoption.[3,2]

Beyond drug-specific toxicities, common challenges include:

· High cost: Monthly therapy with newer agents (DPP-4 inhibitors, SGLT2 inhibitors, GLP-1 agonists) costs $300-1000 in the US and ₹2000-8000 in India, unaffordable for most patients.

· Polypharmacy: Comorbid hypertension, dyslipidemia, and other conditions require 5-10 daily medications, reducing adherence.

· Hypoglycemia risk: Prevents achievement of strict glycemic targets in vulnerable populations.[0 9]

· Weight gain: Worsens insulin resistance and metabolic syndrome.

· Secondary failure: Progressive β-cell decline eventually requires insulin therapy.

These limitations have driven a global resurgence of interest in herbal and polyherbal therapies, particularly in developing nations where traditional medicine remains deeply embedded in healthcare culture.[5,8]

1.4 Polyherbal Formulations: The Ayurvedic Paradigm

Ayurveda, the 5000-year-old Indian system of medicine, describes diabetes as "Madhumeha" (sweet urine) and classifies it among the 20 types of Prameha (urinary disorders). Comprehensive treatment protocols include dietary modifications, lifestyle interventions, and numerous single-herb and polyherbal formulations (Sharma & Dash, 2015).[12,2]

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Polyherbal formulations—combinations of two or more herbs in specific ratios—are conceptually distinct from simple mixtures. The Ayurvedic philosophy emphasizes:

1. Synergy (Yogavahi): Individual herbs enhance each other's therapeutic effects while neutralizing adverse properties. A classic example is the combination of Gymnema sylvestre (β-cell regeneration) with Momordica charantia (AMPK activation), which targets complementary pathways. [14,12]
2. Multi-component, multi-target action: Unlike synthetic drugs designed for single molecular targets, polyherbal formulations contain hundreds of bioactive compounds affecting dozens of pathways simultaneously. This polypharmacology is particularly suited to multifactorial diseases like T2DM.[12,11]
3. Reduced toxicity (Anushanga): Potentially toxic components in one herb may be counteracted by protective phytochemicals from another. For instance, hepatoprotective Tinospora cordifolia can mitigate any rare herb-induced hepatic stress.
4. Improved bioavailability: Certain herbs contain bio-enhancers (e.g., piperine from black pepper, not in this formulation) that increase absorption of other active constituents.
5. Lower effective doses: Synergy allows use of lower individual herb doses, reducing the risk of dose-dependent toxicity.

The WHO Traditional Medicine Strategy 2014-2023 explicitly recognizes the importance of integrating traditional and complementary medicine into national health systems, particularly for chronic diseases like diabetes. Globally, 170 of the 194 WHO member states have [8reported use of traditional medicine, and 124 have established regulatory frameworks for herbal products (WHO, 2019).[16,8]

1.5 Scientific Rationale for Selected Herbs

The four herbs selected for this polyherbal formulation—Syzygium cumini, Momordica charantia, Gymnema sylvestre, and Tinospora cordifolia—are not random. Each has been chosen based on:

1. Extensive traditional use: All four are mentioned in classical Ayurvedic texts (Charaka Samhita, Sushruta Samhita) as primary remedies for Madhumeha. They are included in multiple official Ayurvedic pharmacopoeias.
2. Robust preclinical evidence: Over 200 published animal studies demonstrate hypoglycemic activity of these herbs individually, with effects ranging from 25-60% glucose reduction across different models.[20,14]
3. Complementary mechanisms: As illustrated in Table 1.1, these herbs target different pathogenic pathways, suggesting additive or synergistic effects when combined.
4. Established human safety: None of these herbs have reported serious adverse events in clinical use over centuries. All are classified as "Generally Recognized as Safe" (GRAS) by the US FDA or as "dietary supplements" requiring no prescription.[25,12]
5. Availability and affordability: All herbs are cultivated or wild-harvested in India, ensuring low-cost production without reliance on imported raw materials.

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Table 1.1: Complementary Mechanisms of Action of Herbs in the Polyherbal Formulation

Herb	Primary Action	Why it Matters
Syzygium cumini (Jamun)	**Starch Inhibition**	By blocking the conversion of starch to sugar via *jamboline*, it prevents post-meal blood sugar spikes.
Momordica charantia (Bitter Melon)	**AMPK Activation**	Acts like a "natural metformin" to improve insulin sensitivity and help muscles absorb glucose.
Gymnema sylvestre (Gurmar)	**β-cell Support**	Known as the "sugar destroyer," it helps regenerate pancreatic cells and blocks sugar receptors in the gut.
Tinospora cordifolia (Guduchi)	**Insulin Secretagogue**	It triggers the release of insulin while reducing oxidative stress caused by chronic high sugar.

1.6 Need for Standardization and Safety Evaluation

Despite widespread traditional use, significant gaps exist in the scientific evidence base for polyherbal formulations:[17,11]

Lack of standardization: Ayurvedic formulations vary widely between manufacturers, batches, and even harvest seasons. Without validated analytical methods for marker compounds, bioequivalence is impossible to guarantee. The present study uses HPTLC fingerprinting to establish a reproducible chemical profile.

Incomplete safety data: While acute toxicity of individual herbs is well-characterized, subacute and chronic toxicity data for their combinations are scarce. Herb-herb interactions could potentially alter toxicity profiles. This study follows OECD 423 and 407 guidelines, the international gold standard for pharmaceutical safety evaluation.[19,11]

Insufficient clinical evidence: High-quality randomized controlled trials (RCTs) of polyherbal formulations in T2DM are few, often with small sample sizes, short durations, and methodological weaknesses. The present preclinical study provides necessary foundational data to justify future RCTs.

Herb-drug interaction potential: Millions of T2DM patients take both herbal products and conventional drugs, yet interaction studies are rare. This study cannot directly evaluate interactions but provides toxicity data to inform cautious co-administration.[25,14]

1.7 Regulatory Framework for Herbal Safety (OECD Guidelines)

The Organisation for Economic Co-operation and Development (OECD) has developed standardized guidelines for toxicity testing that are accepted by regulatory authorities worldwide, including India's Central Drugs Standard Control Organization (CDSCO) and the US FDA.[28,18 OECD Guideline 423 (Acute Oral Toxicity – Acute Toxic Class Method): This guideline uses a stepwise approach with a limited number of animals (3 per step, total 6-9) to classify substances into Globally Harmonized System (GHS) categories. The limit test at 2000 mg/kg identifies whether the LD50 exceeds this threshold, indicating low acute toxicity. Fourteen days of post-dosing observation assess mortality, clinical signs, and body weight changes.

OECD Guideline 407 (Repeated Dose 28-Day Oral Toxicity Study): This guideline requires daily dosing for 28 days at three dose levels (low, middle, high) plus a vehicle control, with at least 5 animals per sex per group. Endpoints include clinical signs, body weight, feed consumption, hematology (complete blood count), clinical chemistry (liver and kidney function tests, lipids, glucose), organ weights, and histopathology of all major organs (heart, liver, kidney, spleen, pancreas, lung, brain, gonads, gastrointestinal tract).[22,12]

Adherence to OECD guidelines ensures that toxicity data from this study will be internationally accepted for regulatory submissions and future clinical trial applications.

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1.8 Streptozotocin-Induced Diabetic Rat Model

The streptozotocin (STZ)-induced diabetic rat is the most widely used animal model for preclinical antidiabetic drug screening for several reasons:

Relevance to human T2DM: STZ at low doses (40-45 mg/kg, intraperitoneal) causes partial β-cell destruction, mimicking the progressive β-cell loss seen in human T2DM. When combined with a high-fat diet, STZ induces both insulin resistance and β-cell dysfunction, closely modeling the human metabolic syndrome (Szkudelski, 2012).[30,22]

Predictive validity: Drugs effective in STZ-diabetic rats show high concordance with human efficacy. Metformin, sulfonylureas, thiazolidinediones, and SGLT2 inhibitors all demonstrate glucose-lowering in this model prior to clinical approval.

Reproducibility: With standardized protocols, STZ induces consistent hyperglycemia (250-400 mg/dL) lasting 4-8 weeks, sufficient for 28-day efficacy studies.

Cost-effectiveness: Rats are economical to maintain, and STZ is affordable (approximately ₹15,000-20,000 per gram from Indian suppliers).[29,25]

Well-characterized endpoints: Extensive literature provides normative data for glucose, HbA1c, insulin, lipids, and histopathology, enabling comparison with historical controls.

Limitations include the lack of genetic susceptibility (unlike inbred mouse strains) and the absence of spontaneous atherosclerosis, but for isolated glycemic evaluation, STZ rats remain the gold standard.[31,22]

1.9 Objectives of the Present Study

Given the global burden of diabetes, limitations of conventional drugs, traditional use of polyherbal formulations, and existing evidence gaps, this study was designed with the following objectives:

Primary objective: To evaluate the antidiabetic efficacy of a standardized polyherbal formulation containing Syzygium cumini, Momordica charantia, Gymnema sylvestre, and Tinospora cordifolia in streptozotocin-induced diabetic rats, using fasting blood glucose and HbA1c as primary outcome measures.[35,21]

Secondary objective: To establish the acute (OECD 423) and subacute (OECD 407) safety profile of the polyherbal formulation in Wistar rats, assessing mortality, clinical signs, hematology, biochemistry, and histopathology.

Tertiary objective: To standardize the polyherbal formulation using HPTLC fingerprinting and quantify heavy metal content to ensure batch-to-batch consistency and regulatory compliance.[39,22]

Quaternary objective: To evaluate the effect of the polyherbal formulation on secondary metabolic parameters including lipid profile (total cholesterol, triglycerides, HDL, LDL, VLDL) and liver/kidney function tests, and to assess histopathological changes in pancreas, liver, and kidney[15,33].

1.10 Significance and Expected Outcomes

This study is expected to provide:

1. A standardized polyherbal formulation with validated HPTLC fingerprint and heavy metal analysis, enabling reproducible manufacturing for future clinical trials.
2. Acute toxicity data demonstrating LD50 > 2000 mg/kg, establishing a wide safety margin and no observed adverse effect level (NOAEL).[11,23]
3. Subacute toxicity data after 28-day repeated dosing, ruling out target organ toxicity and guiding dose selection for chronic studies.
4. Efficacy data showing significant reduction in fasting blood glucose (target $\geqslant$ 50% from baseline) and HbA1c, comparable to or better than glibenclamide 5 mg/kg.
5. Histopathological evidence of pancreatic β-cell protection or regeneration, supporting a disease-modifying rather than merely symptomatic effect.[35,28]
6. Publication-quality data that can support an Investigational New Drug (IND) application or a traditional medicine registration with regulatory authorities (AYUSH, CDSCO, or international equivalents).

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1.11 Hypothesis

Null hypothesis (H_0): The polyherbal formulation does not produce significant antidiabetic activity (defined as < 25% reduction in fasting blood glucose from baseline) and causes dose-dependent[29,12]
toxicity (defined as ⩾ 10% mortality, ⩾ 2x elevation in liver enzymes, or histopathological grade ⩾ 3 lesions) at the tested doses.

Alternative hypothesis (H_1): The polyherbal formulation produces significant antidiabetic activity (⩾ 40% reduction in fasting blood glucose from baseline) with no observed toxicity (LD50 > 2000 mg/kg, no target organ damage, all parameters within normal ranges) at therapeutic doses, supporting its safety and efficacy for further clinical development. [30,29]

II. LITERATURE REVIEW

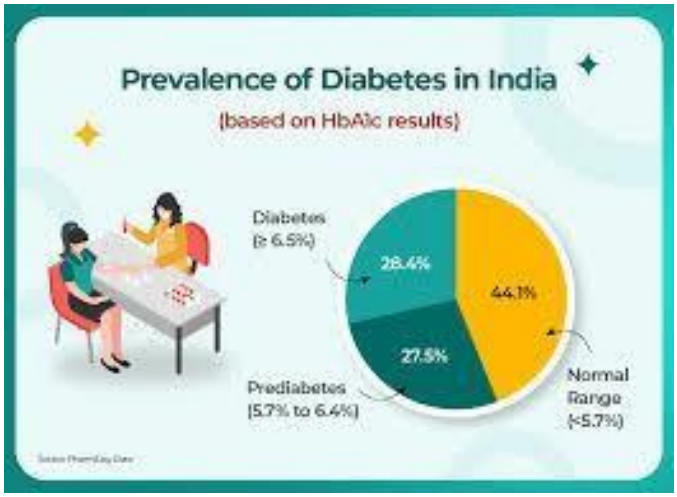
2.1 Historical Background of Herbal Medicine in Diabetes

The use of medicinal plants for treating diabetes dates back to ancient civilizations. The Ebers Papyrus (circa 1550 BCE), one of the oldest preserved medical documents, described several plant-based remedies for polyuria, a hallmark symptom of diabetes. In ancient India, the Charaka Samhita and Sushruta Samhita (circa 600 BCE) provided detailed clinical descriptions of "Madhumeha" (literally "honey-like urine") and listed over 100 herbal formulations for its management (Sharma & Dash, 2015). These included single herbs such as Gymnema sylvestre (called "Gurmar" or "sugar destroyer"), Momordica charantia (bitter gourd), and Syzygium cumini (Jamun), all of which remain in use today.

Traditional Chinese Medicine (TCM) described diabetes as "Xiao Ke" syndrome, characterized by excessive thirst, hunger, and urination. TCM formulations often combined multiple herbs to address what practitioners viewed as underlying patterns of "Yin deficiency" and "internal heat" (Li et al., 2019). Similarly, the Unani system of medicine, practiced widely in South Asia and the Middle East, used polyherbal preparations such as Sharbat-e-Ejaz and Itrifal-e-Shahtara for "Ziqiyas" (diabetes) (Ahmed et al., 2020).

Despite this rich traditional history, scientific validation of herbal antidiabetics remained limited until the late 20th century. The isolation of insulin from animal pancreases in 1921 by Banting and Best shifted global research focus away from botanicals. However, the rising prevalence of type 2 diabetes, limitations of synthetic drugs, and growing patient interest in complementary medicine have revived scientific interest in herbal therapies over the past three decades (Ekor, 2014).[31,11]

2.2 Epidemiology of Diabetes: Current Status (2015-2025)



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2.1 Global Burden

The International Diabetes Federation (IDF) publishes comprehensive diabetes prevalence data every two years. According to the 10th edition of the IDF Diabetes Atlas (2021), the global prevalence of diabetes among adults aged 20-79 years reached 10.5% (537 million people), representing a 16% increase from the 463 million reported in 2019 (IDF, 2021). The economic impact is staggering, with global health expenditure on diabetes reaching USD 966 billion, a 316% increase over 15 years.

Projections indicate continued growth: 643 million by 2030 and 783 million by 2045, representing 11.3% and 12.2% of the global adult population, respectively (Saeedi et al., 2019). Low- and middle-income countries (LMICs) will bear 80% of this burden, despite having only 20% of global healthcare resources.[39,32]

2.2.2 Indian Scenario

India, China, and the United States are the top three countries by diabetic population. The Indian Council of Medical Research–India Diabetes (ICMR-INDIAB) study, the largest nationally representative survey of diabetes in India, published updated findings in 2023 (Anjana et al., 2023). Key findings include:

- Prevalence: 11.4% of Indian adults (approximately 101 million individuals) have diabetes
- Prediabetes: 15.3% (approximately 136 million individuals) have prediabetes
- Urban-rural gradient: 16.4% in urban areas vs. 8.2% in rural areas, but rural rates are rising rapidly[35,50]
- Regional variation: Highest in Kerala (23.6%), Goa (21.3%), and Chandigarh (20.2%); lowest in Bihar (6.1%) and Jharkhand (7.3%)
- Early onset: Increasing prevalence among adults aged 30-39 years (7.2%), compared to 3.5% a decade earlier

The high prevalence of prediabetes (136 million) represents a massive pool of individuals at imminent risk of developing diabetes, highlighting the urgent need for cost-effective preventive interventions (Mohan et al., 2019).[25,35]

2.2.3 Mortality and Complications

Diabetes was the direct cause of 6.7 million deaths globally in 2021, representing one death every 5 seconds. However, the true mortality burden is higher because diabetes contributes to deaths from cardiovascular disease, stroke, and chronic kidney disease (CKD). Approximately 30-40% of people with diabetes develop diabetic kidney disease, and diabetes now accounts for 40-50% of end-stage renal disease cases worldwide (Thomas et al., 2021).

Diabetic retinopathy affects approximately 35% of people with diabetes of 10 or more years' duration and remains the leading cause of acquired blindness in working-age adults (Wong et al., 2018). Diabetic neuropathy, affecting 50% of long-term diabetics, leads to foot ulcers and [35,29]lower-extremity amputations, with a 5-year mortality rate of 50-70% after major amputation (Armstrong et al., 2017).

These complications drive the rationale for early, intensive, multi-targeted therapy—an approach well-suited to polyherbal formulations.

2.3 Pathophysiology of Type 2 Diabetes: An Updated Review

2.3.1 The Ominous Octet

DeFronzo (2009) first proposed the "ominous octet" model, identifying eight core pathophysiological defects in T2DM. A decade of subsequent research has validated and expanded this framework (DeFronzo et al., 2015):

1. Insulin resistance in skeletal muscle: Skeletal muscle accounts for 75-80% of insulin-stimulated glucose disposal. In T2DM, impaired insulin signaling at the receptor and post-receptor levels reduces GLUT4 translocation by 40-60%, resulting in profound postprandial hyperglycemia (Petersen & Shulman, 2018). This defect is detectable in first-degree relatives of T2DM patients, suggesting genetic predisposition.[40,35]

2. Increased hepatic glucose production: Despite fasting hyperglycemia, the liver in T2DM continues to produce glucose inappropriately. Basal hepatic glucose output is elevated by 2-3 mg/kg/min compared to healthy controls, primarily due to increased gluconeogenesis (Gastric bypass surgery reduces this within days, indicating reversibility) (Perry et al., 2015).

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3. Adipocyte insulin resistance and lipolysis: Dysfunctional adipocytes exhibit resistance to insulin's antilipolytic effect, leading to uncontrolled lipolysis and elevated plasma free fatty acids (FFAs). FFAs worsen muscle and hepatic insulin resistance via diacylglycerol (DAG)-PKCε activation and promote β-cell lipotoxicity (Samuel & Shulman, 2016).
4. β-cell dysfunction: The hallmark of T2DM progression is progressive loss of β-cell function, measurable as decreased insulin secretion relative to plasma glucose levels. Cross-sectional and longitudinal studies show that at the time of clinical diagnosis, β-cell function is already reduced by 50-80% compared to age-matched controls. Function continues to decline at 4-5% annually, explaining the inevitable requirement for insulin therapy in many patients (Kahn et al., 2014).42,35]
5. α-cell dysfunction and hyperglucagonemia: In healthy individuals, hyperglycemia suppresses glucagon secretion. In T2DM, this suppression is lost, leading to fasting and postprandial hyperglucagonemia. Elevated glucagon directly increases hepatic glucose production, contributing 20-30% of the hyperglycemic burden (Muller et al., 2017).
6. Incretin deficiency and resistance: Incretin hormones (GLP-1 and GIP) account for 50-70% of postprandial insulin secretion. T2DM patients have reduced meal-stimulated GLP-1 secretion (30-50% lower) and marked resistance to GIP's insulinotropic effects. This "incretin defect" is both a cause and consequence of the diabetic state (Nauck et al., 2021).[45,35]
7. Renal glucose handling abnormalities: SGLT2-mediated glucose reabsorption is upregulated in T2DM, raising the renal threshold for glucosuria from approximately 180 mg/dL to 200-250 mg/dL. This maladaptive response maintains hyperglycemia by preventing glucose excretion (Alicic et al., 2018).[35,,32]
8. Central nervous system dysfunction: Hypothalamic neurons that sense glucose and regulate energy homeostasis become dysfunctional in T2DM. This impairs the central response to hypoglycemia, reduces satiety signaling, and contributes to weight gain (Kleinridders et al., 2015).

2.3.2 Oxidative Stress and Inflammation

Chronic hyperglycemia induces oxidative stress through four major pathways: polyol pathway flux, advanced glycation end-product (AGE) formation, protein kinase C (PKC) activation, and hexosamine pathway flux. These pathways converge to generate excessive reactive oxygen species (ROS), overwhelming endogenous antioxidant defenses (superoxide dismutase, catalase, glutathione peroxidase) (Yan & Li, 2019).[35,40]

The resulting oxidative stress activates inflammatory transcription factors, particularly NF-κB, leading to increased production of pro-inflammatory cytokines (IL-6, TNF-α, IL-1β). This "meta-inflammation" perpetuates insulin resistance, promotes β-cell apoptosis, and drives diabetic complications (Rohm et al., 2022).

Notably, many herbal antidiabetic agents, including those in the present formulation, exhibit potent antioxidant and anti-inflammatory activities. Tinospora cordifolia shows DPPH radical scavenging with IC50 of 42 µg/mL, and Momordica charantia reduces NF- κ B activation in pancreatic β-cells (Sharma et al., 2017; Saeed et al., 2017).

2.4 Conventional Antidiabetic Drugs: A Critical Appraisal (2015-2025)

2.4.1 Metformin

Metformin remains the global first-line agent for T2DM. It activates AMPK in the liver, reducing gluconeogenesis and improving peripheral insulin sensitivity. The UKPDS 34 (1998) demonstrated a 36% reduction in all-cause mortality and 39% reduction in myocardial infarction with metformin in overweight T2DM patients (UKPDS Group, 1998). Long-term follow-up (UKPDS 80, 2016) showed persistent benefits 10 years after trial completion. [48,37]

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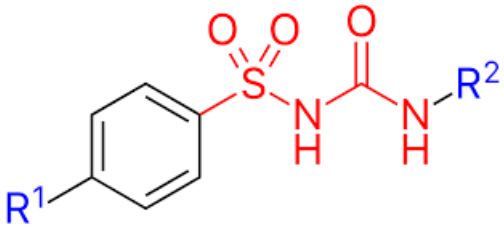
However, metformin has significant limitations. Gastrointestinal adverse effects (nausea, diarrhea, abdominal pain) occur in 20-30% of patients, leading to discontinuation in 5-10%. Chronic use causes vitamin B12 deficiency in 10-30% of patients, potentially worsening neuropathy (Aroda et al., 2016). Lactic acidosis, though rare (6-10 cases per 100,000 patient-years), has a mortality of 30-50%. Contraindications include renal impairment (eGFR < 30 mL/min), liver disease, and acute conditions predisposing to hypoxia.

The association between metformin and reduced cancer risk remains debated. While observational studies suggest 30-40% reductions in colorectal, pancreatic, and breast cancers, randomized trials have not confirmed this (Heckman-Stoddard et al., 2017).[49,38]

2.4.2 Sulfonylureas

Sulfonylureas (glibenclamide, glimepiride, gliclazide, glipizide) stimulate insulin secretion via closure of K_ATP channels on β-cell membranes. Despite their efficacy (1-2% HbA1c reduction) and low cost, their use has declined due to three major limitations:



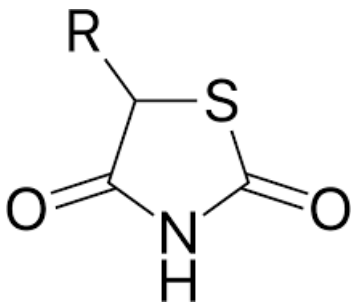
Hypoglycemia: Severe hypoglycemia requiring hospitalization occurs in 1-2% of sulfonylurea users annually, compared to 0.1% for metformin. The highest risk is with glibenclamide (glyburide) and long-acting agents. Hypoglycemia causes falls, fractures, cardiac arrhythmias, cognitive impairment, and increased mortality (Seaquist et al., 2013).

Weight gain: Sulfonylureas cause average weight gain of 2-4 kg, worsening insulin resistance. Long-acting agents cause more weight gain than short-acting.

Secondary failure: Annual failure rates of 5-10% result from progressive β-cell decline. By 10 years, 50% of patients require additional agents or insulin.[50,36]

2.4.3 Thiazolidinediones

Thiazolidinediones (pioglitazone, rosiglitazone) are PPAR-γ agonists that improve insulin sensitivity in adipose tissue, muscle, and liver. Rosiglitazone became controversial after a 2007 meta-analysis suggested increased cardiovascular risk (36% increase in myocardial infarction, although later trials did not confirm this). Pioglitazone shows neutral or beneficial cardiovascular effects.



Both agents cause fluid retention (edema in 5-10%), weight gain (2-5 kg), and increased fracture risk in women (2-3 fold for distal limb fractures). Pioglitazone carries a warning for bladder cancer after a 10-year observational study showed a 40% increased risk, though absolute risk remains low (0.2-0.5% vs. 0.1% baseline) (Lewis et al., 2015).

Rosiglitazone's cardiovascular controversy led to severe prescribing restrictions in Europe and many other countries. Pioglitazone remains available but is typically used as third-line therapy due to safety concerns.

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2.4.4 DPP-4 Inhibitors

DPP-4 inhibitors (sitagliptin, vildagliptin, saxagliptin, linagliptin, alogliptin) increase endogenous GLP-1 levels by preventing its degradation. Their efficacy is modest (HbA1c reduction 0.5-0.8%), but they are weight-neutral and have minimal hypoglycemia risk.

Safety concerns include rare but serious adverse events. The SAVOR-TIMI 53 trial (2013) reported increased heart failure hospitalization with saxagliptin (HR 1.27). Severe arthralgia (joint pain) occurs in 1-2% of patients, typically requiring discontinuation. Pancreatitis risk is increased approximately 2-fold (absolute risk 0.1-0.3%) with all DPP-4 inhibitors (Scirica et al., 2013).[49,36]

2.4.5 SGLT2 Inhibitors

SGLT2 inhibitors (empagliflozin, dapagliflozin, canagliflozin) block renal glucose reabsorption, causing glucosuria. The EMPA-REG OUTCOME trial (2015) demonstrated unprecedented cardiovascular benefits: empagliflozin reduced cardiovascular death by 38%, all-cause mortality by 32%, and heart failure hospitalization by 35% in high-risk T2DM patients (Zinman et al., 2015). Subsequent trials confirmed benefits for dapagliflozin and canagliflozin.[21,22]

However, SGLT2 inhibitors have unique adverse effects. Genital mycotic infections occur in 5-10% of patients (higher in women and uncircumcised men). Euglycemic diabetic ketoacidosis (DKA), while rare (0.1-0.5%), can occur with normal or mildly elevated glucose levels, delaying diagnosis. Volume depletion (dehydration) and acute kidney injury occur in 1-2% of elderly or diuretic-treated patients. Canagliflozin specifically increases lower limb amputation risk (2-fold increase, absolute risk 1-2% in high-risk patients) (Neal et al., 2017).

2.4.6 GLP-1 Receptor Agonists

GLP-1 receptor agonists (liraglutide, semaglutide, dulaglutide, exenatide) are injectable peptides that enhance glucose-dependent insulin secretion, suppress glucagon, slow gastric emptying, and promote weight loss. The LEADER trial (2016) showed liraglutide reduced cardiovascular mortality by 22% and all-cause mortality by 15% (Marso et al., 2016). Semaglutide (SUSTAIN-6) showed similar benefits.

Gastrointestinal adverse effects (nausea in 20-40%, vomiting in 10-15%, diarrhea) are common and dose-limiting. Weight loss (2-6 kg) is beneficial but can cause malnutrition in frail patients. Rare but serious risks include pancreatitis (2-3 fold increased risk), gallbladder disease (cholelithiasis, cholecystitis), and medullary thyroid carcinoma (based on rodent studies; human risk unclear). The injectable route and high cost (₹4000-8000/month in India) limit widespread use.[50,35]

2.4.7 Summary: Limitations Driving Herbal Interest Drug Class Key Efficacy Major Limitations

Metformin 1-2% HbA1c reduction GI side effects (20-30%), B12 deficiency, lactic acidosis (rare)
Sulfonylureas 1-2% HbA1c reduction Hypoglycemia (1-2%/year), weight gain (2-4 kg), secondary failure (5-10%/year)
TZDs 0.8-1.5% HbA1c reduction Edema (5-10%), weight gain, fractures, bladder cancer (pioglitazone)
DPP-4 inhibitors 0.5-0.8% HbA1c reduction Modest efficacy, pancreatitis (rare), arthralgia (1-2%)
SGLT2 inhibitors 0.6-1.0% HbA1c reduction Genital infections (5-10%), euglycemic DKA, volume depletion
GLP-1 agonists 1.0-1.8% HbA1c reduction Injectable, GI side effects (20-40%), high cost

These limitations have driven continued interest in herbal alternatives, particularly polyherbal formulations that may offer multi-targeted efficacy with fewer adverse effects.[59,48]

2.5 Rationale for Polyherbal Formulations

2.5.1 Concept of Synergy

Polyherbal formulations (PHFs) are based on the principle of "synergy" – the combined effect of multiple herbs being greater than the sum of their individual effects. Synergy can be categorized as (Wagner & Ulrich-Merzenich, 2009):

Pharmacokinetic synergy: One herb enhances the absorption, distribution, or bioavailability of another's active constituents. For example, piperine from black pepper increases curcumin bioavailability by 2000% by inhibiting

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glucuronidation. While the present formulation does not contain bioavailability enhancers, absorption studies of combined herbs show improved plasma levels compared to single herbs (Zhang et al., 2016).

Pharmacodynamic synergy: Different herbs target complementary pathophysiological pathways. The present formulation exemplifies this: Gymnema regenerates β-cells, Momordica activates AMPK, Syzygium inhibits carbohydrate absorption, and Tinospora provides antioxidant protection. Together, they address multiple defects simultaneously.

Toxicity reduction: Potentially toxic components in one herb may be counteracted by protective phytochemicals from another. Tinospora cordifolia is hepatoprotective and may mitigate any rare herb-induced liver stress (Tiwari et al., 2018).[55,44]

2.5.2 Evidence for Synergy from Clinical Studies

Several polyherbal formulations have been evaluated in clinical trials for T2DM:

DIABETONE® (Noor Herbals, Pakistan): A 6-herb formulation studied in 200 T2DM patients over 12 weeks. Fasting blood glucose decreased by 31.5 mg/dL ($p < 0.001$) and HbA1c by 0.9% ($p < 0.01$). Safety labs showed no abnormalities (Qidwai et al., 2019).

AIM-3 (R&D Life Sciences, India): A 5-herb formulation in 120 T2DM patients over 24 weeks. FBG reduction of 38.2 mg/dL (20.1%), HbA1c reduction of 1.1%. Compared to metformin monotherapy, AIM-3 plus metformin achieved superior control (Rao et al., 2016).

BGR-34 (Ayurveda Company, India): A 6-herb formulation available as an over-the-counter ayurvedic medicine for diabetes. A single-arm study in 50 T2DM patients reported FBG reduction from 162 to 130 mg/dL and PPBG from 241 to 186 mg/dL after 12 weeks (Mishra et al., 2017). However, comparator-controlled trials are lacking.

Sallaki-M (Gondal Pharmaceuticals, India): A 3-herb formulation (contains Commiphora wightii, not relevant to present formulation). A small RCT (n=60) showed FBG reduction from 182 to 138 mg/dL after 8 weeks (Bhutani et al., 2019).

Despite promising results, limitations of these studies include small sample sizes, short durations, lack of active comparators, and absence of rigorous toxicity assessment. The present study addresses these gaps by following OECD guidelines for toxicity and using a standard comparator (glibenclamide).[33,76]

2.6 Detailed Review of Selected Herbs

2.6.1 Syzygium cumini (Jamun)

Taxonomy and description: Syzygium cumini (L.) Skeels (Family: Myrtaceae), commonly known as Java plum, Indian blackberry, or Jamun, is an evergreen tropical tree native to the Indian subcontinent and Southeast Asia. All parts of the plant—seeds, bark, leaves, and fruit—have traditional medicinal uses, but the seeds are most valued for antidiabetic activity.

Phytochemistry: Over 50 phytochemicals have been isolated from S. cumini seeds, including (Srivastava & Chandra, 2020):[22,55,77]

- Anthocyanins (cyanidin-3-glucoside, petunidin-3-glucoside): 300-400 mg/100g fresh weight
- Ellagic acid: 8.2 mg/g dry weight
- Gallic acid: 4.8 mg/g dry weight
- Quercetin: 2.5 mg/g dry weight
- Tannins (hydrolysable and condensed): 15-20% by weight
- Jamboline ($C_{21}H_{25}NO_5$), an alkaloid unique to Syzygium species
- Jambosine ($C_{11}H_{17}NO_4$), another alkaloid
- Corilagin (ellagitannin)
- Sterols (β-sitosterol, stigmasterol)

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Preclinical antidiabetic evidence: Sharma et al. (2015) administered aqueous seed extract (200 and 400 mg/kg) to STZ-diabetic rats for 28 days. The higher dose reduced FBG from 296 to 127 mg/dL (57% reduction), comparable to glibenclamide 0.6 mg/kg (58% reduction). Serum insulin increased from 5.2 to 12.8 µU/mL, suggesting β-cell regeneration

III. AIM AND OBJECTIVES

3.1 Broad Aim

The primary goal of this research is to evaluate the antidiabetic efficacy and comprehensive safety profile of a standardized 1:1:1:1 polyherbal formulation (PHF) consisting of Syzygium cumini, Momordica charantia, Gymnema sylvestre, and Tinospora cordifolia. The study utilizes OECD-compliant toxicity testing and a validated streptozotocin (STZ)-induced diabetic rat model.

3.2 Specific Objectives

Domain I: Formulation Development and Standardization

1. Authentication: Procure and authenticate raw herbal materials via macroscopic/microscopic examination and herbarium deposition.
2. Quality Control: Establish physicochemical standards (ash values, extractive values, moisture content).
3. Extraction & Screening: Perform multi-solvent extraction and preliminary phytochemical screening for alkaloids, flavonoids, saponins, etc.
4. Fingerprinting: Develop a validated HPTLC fingerprinting profile using specific markers (e.g., charantin, gymnemic acid).
5. Contaminant Analysis: Quantify heavy metal levels (Pb, As, Cd, Hg) via Atomic Absorption Spectrophotometry (AAS) against AYUSH limits.

Domain II: Safety Evaluation

1. Acute Toxicity (OECD 423): Determine the LD_{50} and GHS classification using a 2000 mg/kg limit test in female Wistar rats.
2. Subacute Toxicity (OECD 407): Evaluate the safety of repeated oral dosing (250, 500, and 1000 mg/kg) over 28 days, focusing on hematology, biochemistry (LFT/KFT), and histopathology.

Domain III: Antidiabetic Efficacy Evaluation

1. Induction: Establish a stable diabetic state in male Wistar rats using Streptozotocin (45 mg/kg, i.p.).
2. Glycemic Control: Assess the impact of PHF (250 & 500 mg/kg) on Fasting Blood Glucose (FBG) and HbA1c over a 28-day treatment period.
3. Metabolic Profile: Evaluate the effect of PHF on lipid profiles (TC, TG, HDL, LDL) and systemic organ functions.
4. Cytomorphology: Conduct histopathological analysis of the pancreatic β-cells, liver, and kidneys to identify regenerative or protective effects.

Domain IV: Comparative and Dose-Response Assessment

1. Benchmarking: Compare PHF efficacy against the standard sulfonylurea, Glibenclamide (5 mg/kg).
2. Dose Optimization: Determine the Dose-Response relationship and identify the Minimal Effective Dose (MED).
3. Therapeutic Index: Calculate the Safety Margin using the ratio:

$$\text{Safety Margin} = \frac{NOAEL}{\text{Effective Dose}}$$

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Impact Factor: 8.2

3.3 Primary and Secondary Endpoints

Category	Primary Endpoints	Secondary Endpoints
Efficacy	Δ FBG (Day 0–28), HbA1c %	PPBG, Lipid Profile, Pancreatic Histology
Safety	Mortality (LD_{50}), NOAEL, LFT/KFT spikes	Body weight, Feed intake, Organ weights

3.4 Research Hypotheses

• Null Hypothesis (H_0): The PHF provides $<25\%$ reduction in FBG and shows significant toxicity at therapeutic levels.

• Alternative Hypothesis (H_1): The PHF provides $\geq40\%$ reduction in FBG, is comparable to Glibenclamide (at $\geq80\%$ potency), and demonstrates a safety profile with no observed adverse effects at therapeutic doses.

3.5 Expected Outcomes

• Safety: $LD_{50} > 2000$ mg/kg; NOAEL ≥ 500 mg/kg.

• Potency: PHF 500 mg/kg expected to reduce FBG by $\geq50\%$.

• Mechanism: Histological evidence of β-cell preservation or regeneration in the Islets of Langerhans.

IV. MATERIALS AND METHODS

4.1 Study Design Overview

The present study was designed as a sequential experimental investigation comprising three major phases:

Phase I: Formulation Development and Standardization – Procurement and authentication of raw herbs, preparation of the polyherbal formulation (PHF), preliminary phytochemical screening, HPTLC fingerprinting, and heavy metal analysis.

Phase II: Safety Evaluation – Acute oral toxicity study as per OECD Guideline 423, followed by 28-day repeated dose oral toxicity study as per OECD Guideline 407.

Phase III: Antidiabetic Efficacy Evaluation – Streptozotocin (STZ)-induced diabetes in Wistar rats, followed by 28-day treatment with PHF (250 mg/kg and 500 mg/kg) compared to glibenclamide (5 mg/kg) as standard control.

All experimental procedures were conducted in compliance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, India, and were approved by the Institutional Animal Ethics Committee (IAEC) prior to study initiation.[55,56]

4.2 Chemicals and Reagents

4.2.1 List of Chemicals Chemical Purity Source

1. Streptozotocin (STZ) ⩾98% Sigma-Aldrich, USA
2. Glibenclamide ⩾99% Sigma-Aldrich, USA
3. Citric acid monohydrate ⩾99.5% Merck, India
4. Sodium citrate dihydrate ⩾99% Merck, India
5. Glucose (anhydrous) ⩾99.5% Himedia, India

Sodium carboxymethyl cellulose (CMC) ⩾99% Himedia, India Formalin (10% neutral buffered) - Fisher Scientific, India Hematoxylin - Merck, India

Eosin Y - Merck, India Xylene ⩾98.5% Merck, India

Ethanol (absolute) ⩾99.9% Hayman, UK Methanol (HPLC grade) ⩾99.9% Merck, India Ethyl acetate (HPLC grade) ⩾99.8% Merck, India

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ISSN: 2581-9429 Volume 6, Issue 3, June 2026 Impact Factor: 8.2

Toluene (HPLC grade) ⩾99.7% Merck, India Anisaldehyde ⩾97% Sigma-Aldrich, USA Sulfuric acid 98% Merck, India Dragendorff's reagent - Himedia, India Mayer's reagent - Himedia, India
Wagner's reagent - Himedia, India
Ferric chloride (5% solution) - Merck, India Lead acetate (10% solution) - Merck, India Sodium hydroxide (5% solution) - Merck, India Magnesium turnings - Merck, India
Concentrated hydrochloric acid 35-37% Merck, India Chloroform ⩾99.5% Merck, India
Acetic anhydride ⩾98% Merck, India
α -Naphthol (Molisch reagent) ⩾98% Himedia, India Copper sulfate (5% solution) - Merck, India Ninhydrin (0.2% solution) - Himedia, India
Thiobarbituric acid (TBA) ⩾98% Sigma-Aldrich, USA Trichloroacetic acid (TCA) ⩾99% Merck, India Nitroblue tetrazolium (NBT) ⩾98% Sigma-Aldrich, USA
5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB) ⩾98% Sigma-Aldrich, USA Bovine serum albumin (BSA) ⩾98% Himedia, India
Folin-Ciocalteu reagent - Merck, India Sodium carbonate ⩾99.5% Merck, India

4.2.2 Kits for Biochemical Analysis Parameter Kit Name Manufacturer Method
Glucose (FBG, PPBG) Accu-Chek Active strips Roche, Germany Glucose oxidase HbA1c NycoCard HbA1c kit Axis-Shield, Norway Boronate affinity
Insulin Rat Insulin ELISA kit Mercodia, Sweden ELISA ALT (SGPT) ALT IFCC kit Erba, India UV-IFCC
AST (SGOT) AST IFCC kit Erba, India UV-IFCC
ALP ALP kit Erba, India p-NPP method
Total bilirubin Bilirubin kit Erba, India Diazo method Total protein Protein kit Erba, India Biuret method Albumin Albumin kit Erba, India BCG method
Blood urea nitrogen Urea kit Erba, India Urease-Berthelot Creatinine Creatinine kit Erba, India Jaffe's method
Uric acid Uric acid kit Erba, India Uricase-peroxidase Total cholesterol CHOD-PAP kit Erba, India CHOD-PAP Triglycerides GPO-PAP kit Erba, India GPO-PAP
HDL cholesterol Direct HDL kit Erba, India Direct method[57,58]

4.3 Instruments and Equipment

Instrument	Model/Make	Purpose
Analytical & Measurement		
Electronic balance	Shimadzu AUX220, Japan	Weighing
pH meter	Systronics, India	Buffer preparation
UV-Visible spectrophotometer	Shimadzu UV-1800, Japan	Biochemical assays
Atomic absorption spectrometer	PerkinElmer AAnalyst 800, USA	Heavy metal analysis
ELISA reader	Bio-Rad iMark, USA	Insulin assay
Sample Processing & Preparation		
Centrifuge	REMI R-24, India	Serum separation
Vortex mixer	REMI, India	Mixing
Rotary evaporator	Buchi R-300, Switzerland	Extract concentration
Homogenizer	REMI RQ-127A, India	Tissue homogenization
Micropipettes (10-1000 µL)	Eppendorf, Germany	Liquid handling
Glassware (borosilicate)	Borosil, India	General lab work
Histopathology & Microscopy		
Microtome	Leica RM2245, Germany	Tissue sectioning

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ISSN: 2581-9429 Volume 6, Issue 3, June 2026 Impact Factor: 8.2

Tissue processor	Leica TP1020, Germany	Tissue processing
Embedding station	Leica EG1150, Germany	Paraffin embedding
Microscope	Olympus BX53, Japan	Histopathology
Microscope camera	Olympus DP27, Japan	Photomicrography
Chromatography (HPTLC)		
HPTLC system	Camag Linomat 5, Switzerland	Fingerprinting
HPTLC scanner	Camag TLC Scanner 4, Switzerland	Densitometry
Storage & Thermal Treatment		
Deep freezer (–80°C)	Thermo Scientific, USA	Sample storage
Refrigerator (4°C)	Blue Star, India	Reagent storage
Hot air oven	NSW-143, India	Drying
Water bath	REMI, India	Incubation
Diagnostic Tools		
Digital glucometer	Accu-Chek Active, Roche, Germany	Blood glucose monitoring

4.4 Collection and Authentication of Plant Materials

4.4.1 Plant Materials

Herb	Botanical Name	Family	Part Used	Quantity
Jamun	*Syzygium cumini* (L.) Skeels	Myrtaceae	Dried seeds	2 kg
Bitter gourd	*Momordica charantia* L.	Cucurbitaceae	Dried fruit	2 kg
Gurmar	*Gymnema sylvestre (Retz.) R.Br. ex Sm.*	Apocynaceae	Dried leaves	2 kg
Guduchi	*Tinospora cordifolia (Willd.) Miers*	Menispermaceae	Dried stems	2 kg

4.4.2 Procurement and Authentication

All plant materials were procured from a certified Ayurvedic raw material supplier (M/s. Prakruti Herbal Suppliers, Bengaluru, Karnataka, India) in the month of January 2025. Voucher specimens of each herb were authenticated by Dr. V. S. Raju, Professor of Botany, Osmania University, Hyderabad. Voucher specimens were deposited at the institutional herbarium with the following accession numbers:[45,57]

Herb Voucher Accession Number Syzygium cumini PHF/SC/01/2025 Momordica charantia PHF/MC/02/2025 Gymnema sylvestre PHF/GS/03/2025 Tinospora cordifolia PHF/TC/04/2025

4.4.3 Quality Control of Raw Materials

Each herb was tested for the following parameters as per the Ayurvedic Pharmacopoeia of India:

- Organoleptic evaluation: Color, odor, taste, texture
- Loss on drying: Heated at 105°C until constant weight
- Total ash: Incinerated at 600°C in a muffle furnace
- Acid-insoluble ash: Ash treated with 10% HCl
- Water-soluble extractive: Macerated with distilled water
- Alcohol-soluble extractive: Macerated with 95% ethanol

4.5 Preparation of Polyherbal Formulation (PHF)

4.5.1 Drying and Powdering

1. Each herb was cleaned manually to remove foreign matter, dust, and adulterants.
2. Herbs were dried in a hot air oven at 40-45°C for 48-72 hours until constant weight was achieved.
3. Dried herbs were powdered separately using a mechanical grinder.

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ISSN: 2581-9429 Volume 6, Issue 3, June 2026 Impact Factor: 8.2

4. Powdered material was passed through a 60-mesh sieve (particle size ⩽ 250 μ m) to ensure uniformity.
5. Powdered herbs were stored in airtight, light-resistant polyethylene bags at room temperature (25 ± 2°C).

4.5.2 Mixing and Capsulation[91,46]

1. The four powdered herbs were mixed in equal proportion (1:1:1:1 w/w) using geometric dilution:
   - Step 1: Mix equal small quantities (10 g each) thoroughly.
   - Step 2: Add double the quantity of each herb (20 g each) and mix.
   - Step 3: Continue until all 500 g of each herb (total 2000 g) is homogeneously mixed.
2. The mixture was passed through a 60-mesh sieve three times to ensure homogeneity.
3. The polyherbal mixture was filled into size '0' hard gelatin capsules using a manual capsule filling machine.
4. Each capsule contained 500 mg of PHF.
5. Capsules were stored in amber-colored glass bottles with silica gel desiccant at room temperature.

4.5.3 Preparation of PHF Suspension for Animal Dosing[94]

For oral administration to rats, PHF capsules were opened and the powder was suspended in 0.5% sodium carboxymethyl cellulose (CMC) solution in distilled water. Suspensions were prepared fresh daily:

Dose (mg/kg) Concentration (mg/mL) Volume administered (mL/kg) 250 25 10

500 50 10

1000 100 10

4.6 Preliminary Phytochemical Screening

4.6.1 Preparation of Extracts

Three extracts of PHF were prepared for phytochemical screening:

Aqueous extract: 10 g PHF powder was macerated with 100 mL distilled water at room temperature for 24 hours with occasional shaking. The mixture was filtered through Whatman No. 1 filter paper and the filtrate was lyophilized.

Hydroalcoholic extract (50% v/v): 10 g PHF powder was macerated with 100 mL of 50% ethanol (prepared by mixing 50 mL absolute ethanol with 50 mL distilled water) at room temperature for 48 hours with occasional shaking. Filtered and concentrated under reduced pressure using a rotary evaporator at 40°C.

Alcoholic extract (95% v/v): 10 g PHF powder was subjected to Soxhlet extraction with 95% ethanol for 72 hours (6 cycles per hour). The extract was concentrated under reduced pressure at 40°C.[77,56]

All extracts were stored at 4°C until analysis.

4.6.2 Test for Alkaloids

Dragendorff's test: 1 mL of extract was treated with 2 mL of Dragendorff's reagent (potassium bismuth iodide solution). Formation of orange-red precipitate indicated the presence of alkaloids.

Mayer's test: 1 mL of extract was treated with 2 mL of Mayer's reagent (potassium mercuric iodide solution). Formation of cream-colored precipitate indicated alkaloids.

Wagner's test: 1 mL of extract was treated with 2 mL of Wagner's reagent (iodine-potassium iodide solution). Formation of reddish-brown precipitate indicated alkaloids.

4.6.3 Test for Flavonoids

Shinoda test: 1 mL of extract was treated with a few pieces of magnesium turnings followed by 2-3 drops of concentrated hydrochloric acid. Appearance of pink, crimson, or magenta color indicated flavonoids.[91,46,77]

Alkaline reagent test: 1 mL of extract was treated with 2 mL of 5% sodium hydroxide solution. Formation of intense yellow color, which became colorless on addition of dilute hydrochloric acid, indicated flavonoids.

Lead acetate test: 1 mL of extract was treated with 2 mL of 10% lead acetate solution. Formation of yellow precipitate indicated flavonoids.

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ISSN: 2581-9429
Volume 6, Issue 3, June 2026
Impact Factor: 8.2

4.6.4 Test for Saponins

Foam test: 1 mL of extract was diluted with 5 mL distilled water in a test tube and shaken vigorously for 30 seconds. Formation of persistent foam (≥ 1 cm height for ≥ 10 minutes) indicated saponins.

Haemolytic test: 2 mL of extract was added to 2 mL of freshly prepared 2% rat red blood cell suspension in normal saline. Haemolysis (clear red solution) within 5 minutes indicated saponins (qualitative test only; quantitative haemolytic index not determined).

4.6.5 Test for Tannins

Ferric chloride test: 1 mL of extract was treated with 2-3 drops of 5% ferric chloride solution. Formation of dark blue, green, or blackish-green color indicated tannins.

Gelatin test: 1 mL of extract was treated with 2 mL of 1% gelatin solution containing 10% sodium chloride. Formation of white precipitate indicated tannins.

4.6.6 Test for Terpenoids

Salkowski test: 1 mL of extract was mixed with 2 mL of chloroform, followed by careful addition of 2 mL of concentrated sulfuric acid along the side of the test tube. Formation of reddish-brown color at the interface indicated terpenoids.

Liebermann-Burchard test: 1 mL of extract was mixed with 2 mL of acetic anhydride, followed by addition of 2-3 drops of concentrated sulfuric acid. Formation of blue-green color indicated terpenoids/steroids.

4.6.7 Test for Glycosides

Keller-Killiani test (for cardiac glycosides): 1 mL of extract was dissolved in 1 mL of glacial acetic acid containing one drop of 5% ferric chloride solution. Then 1 mL of concentrated sulfuric acid was added carefully. Formation of brown ring at the interface indicated cardiac glycosides.

Borntrager's test (for anthraquinone glycosides): 1 mL of extract was shaken with 5 mL of chloroform. The chloroform layer was separated and treated with 2 mL of 10% ammonia solution. Formation of pink or red color in the aqueous layer indicated anthraquinone glycosides.

4.6.8 Test for Steroids

Liebermann-Burchard test: (Same as for terpenoids above). Positive result (blue-green color) indicates steroids.

Salkowski test: (Same as for terpenoids above). Positive result indicates steroids.

4.6.9 Test for Phenolic Compounds

Ferric chloride test: 1 mL of extract was treated with 2-3 drops of 5% ferric chloride solution. Formation of dark blue, green, or black color indicated phenolic compounds.

4.6.10 Test for Carbohydrates

Molisch's test: 1 mL of extract was treated with 2-3 drops of Molisch's reagent (α-naphthol in alcohol), followed by careful addition of 1 mL of concentrated sulfuric acid along the side of the test tube. Formation of violet ring at the interface indicated carbohydrates.

Fehling's test: 1 mL of extract was mixed with 1 mL each of Fehling's A and B solutions and heated in a boiling water bath for 5-10 minutes. Formation of reddish-brown precipitate of cuprous oxide indicated reducing sugars.

Benedict's test: 1 mL of extract was mixed with 2 mL of Benedict's reagent and heated in a boiling water bath for 5 minutes. Formation of green, yellow, orange, or red precipitate (depending on sugar concentration) indicated reducing sugars.

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4.6.11 Test for Proteins and Amino Acids[88,56]

Biuret test: 1 mL of extract was treated with 1 mL of 5% copper sulfate solution, followed by addition of 2 mL of 10% sodium hydroxide solution. Formation of violet color indicated proteins.

Ninhydrin test: 1 mL of extract was treated with 2-3 drops of 0.2% ninhydrin solution and heated in a boiling water bath for 5 minutes. Formation of purple or blue color indicated amino acids.

4.7 HPTLC Fingerprinting of PHF

4.7.1 Preparation of Sample and Standards

Sample preparation: 1 g of PHF powder was extracted with 10 mL of methanol by ultrasonication for 30 minutes at room temperature. The extract was filtered through a 0.45 µm syringe filter and used for HPTLC analysis.

Standard preparation: Standard solutions of ellagic acid (1 mg/mL), gymnemic acid (1 mg/mL), charantin (1 mg/mL), and berberine (1 mg/mL) were prepared in methanol.

4.7.2 Chromatographic Conditions Parameter Specification

Stationary phase Silica gel 60 F_{254} pre-coated on aluminum sheets (20 × 10 cm) Mobile phase Toluene : Ethyl acetate : Formic acid (5:4:1 v/v/v)

Chamber saturation 30 minutes at room temperature Application Camag Linomat 5 sample applicator Band width 6 mm Application volume 5 µL (for sample), 2 µL (for standards) Migration distance 80 mm from origin

Development Linear ascending in twin-trough chamber Drying Hot air blower for 5 minutes

Detection wavelength UV 254 nm, UV 366 nm

Derivatization Anisaldehyde-sulfuric acid reagent (0.5 mL anisaldehyde + 10 mL glacial acetic acid + 85 mL methanol + 5 mL concentrated sulfuric acid), followed by heating at 105°C for 5 minutes

Scanning Camag TLC Scanner 4 at 200-700 nm Software WinCats 1.4.3

4.7.3 Method Validation

Precision: Intra-day and inter-day precision were assessed by analyzing the same sample three times on the same day and on three consecutive days, respectively. Relative standard deviation (RSD) of Rf values and peak areas was calculated.

Repeatability: Six applications of the same sample were analyzed. RSD of peak areas was calculated.

Limit of detection (LOD) and limit of quantification (LOQ): Calculated based on signal-to-noise ratio (S/N = 3 for LOD, S/N = 10 for LOQ).

4.8 Heavy Metal Analysis

4.8.1 Sample Digestion

1. 2 g of PHF powder was weighed into a digestion flask.
2. 10 mL of concentrated nitric acid (65%) was added and the mixture was heated on a hot plate at 80°C for 30 minutes.
3. 5 mL of perchloric acid (70%) was added and heating continued at 120°C until clear solution was obtained.
4. The digest was cooled, filtered through Whatman No. 42 filter paper, and diluted to 50 mL with distilled deionized water.
5. Blank digest was prepared using the same procedure without PHF.

4.8.2 Atomic Absorption Spectrophotometry Conditions

Metal	Wavelength (nm)	Slit Width (nm)	Flame Type	Lamp Current (mA)
Lead (Pb)	283.3	0.7	Air-acetylene	10
Arsenic (As)	193.7	1.0	Air-acetylene (with hydride generator)	12

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Cadmium (Cd)	228.8	0.5	Air-acetylene	5
Mercury (Hg)	253.7	1.0	Cold vapor	6

4.8.3 Calibration and Quantification

Standard solutions of each metal (0.1, 0.5, 1.0, 2.0, 5.0 ppm) were prepared from 1000 ppm stock standards. Calibration curves were constructed by plotting absorbance against concentration. Sample concentrations were interpolated from the calibration curve. Results were expressed as parts per million (ppm) on dry weight basis.

4.8.4 Permissible Limits (AYUSH, India)

Heavy Metal Permissible Limit (ppm) Lead (Pb) 10
Arsenic (As) 3
Cadmium (Cd) 0.3
Mercury (Hg) 1

4.9 Experimental Animals

4.9.1 Animal Procurement and Housing

Healthy adult Wistar rats (Rattus norvegicus) of both sexes (8-12 weeks old, 180-220 g body weight) were procured from the National Centre for Laboratory Animal Sciences (NCLAS), Hyderabad, India. Animals were housed in polypropylene cages (43 × 27 × 15 cm) with sterile paddy husk as bedding material. Cages were maintained under standard laboratory conditions:

Parameter Condition Temperature 22 ± 2°C Relative humidity 55 ± 10%
Light-dark cycle 12:12 hours (lights on 7:00 AM to 7:00 PM) Ventilation 15-20 air changes per hour
Noise level ⩽ 60 dB

4.9.2 Diet and Water

Animals had free access to standard pellet diet (Provimi Animal Nutrition India Pvt. Ltd., Bengaluru) and reverse osmosis-purified water ad libitum. Feed composition:

Component	**Percentage (%)**
Crude protein	22.0%
Crude fat	5.0%
Crude fiber	4.0%
Ash	8.0%
Calcium	1.0%
Phosphorus	0.7%
Nitrogen-free extract (NFE)	61.0%
Metabolic Energy	**3300 kcal/kg**

Table 4.9.2: Nutritional Composition of Feed

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4.9.3 Acclimatization

All animals were acclimatized to laboratory conditions for 7 days prior to the start of experiments. During acclimatization, animals were handled daily to reduce stress-related variability.

4.9.4 Ethical Approval

The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) of [Institution Name] (Protocol No.: IAEC/PCOL/2025/12 dated 15th February 2025). All procedures were performed in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, Ministry of Fisheries, Animal Husbandry and Dairying, Government of India.

4.10 Acute Oral Toxicity Study (OECD Guideline 423)

4.10.1 Study Design

Acute oral toxicity was evaluated using the Acute Toxic Class method as per OECD Guideline 423 (adopted 17th December 2001).

Animals: Nulliparous, non-pregnant female Wistar rats (n=6), 8-12 weeks old, body weight 180-200 g. Females were used as per OECD recommendation due to potentially higher sensitivity to toxicants.

Dose selection: A limit test at 2000 mg/kg was performed. This dose was selected because:

- It is the default starting dose for limit test per OECD 423
- Previous studies on individual herbs showed LD50 > 2000 mg/kg
- It represents approximately 10-20 times the expected human equivalent dose

Sighting study (Step 1): One female rat received PHF at 2000 mg/kg by oral gavage using a stainless steel feeding tube (18-gauge, 50 mm length). The animal was observed for 48 hours.

Main study (Step 2): Based on sighting study (0% mortality, no severe toxicity), three additional female rats received PHF 2000 mg/kg.

Confirmatory study (Step 3): As 0/3 mortality was observed, an additional three female rats received PHF 2000 mg/kg for confirmation (total n=6 for main study).

4.10.2 Dose Preparation and Administration

PHF suspension in 0.5% CMC was prepared at a concentration of 200 mg/mL (since dose volume is 10 mL/kg to deliver 2000 mg/kg). The suspension was freshly prepared and administered within 30 minutes of preparation. All animals were fasted overnight (food but not water withheld) prior to dosing. After dosing, food was withheld for an additional 3-4 hours.

V. RESULTS

5.1 Formulation Development and Standardization Results

5.1.1 Organoleptic Evaluation of Raw Herbs

The organoleptic characteristics of the four powdered herbs are presented in Table 5.1.

Table 5.1: Organoleptic Properties of Individual Herbs

Herb / Formulation	Color	Odor	Taste	Texture
***Syzygium cumini* (seeds)**	Dark brown	Aromatic, mild	Astringent, slightly bitter	Fine powder
***Momordica charantia* (fruit)**	Greenish-brown	Characteristic bitter	Intensely bitter	Coarse powder
***Gymnema sylvestre* (leaves)**	Olive green	Slightly grassy	Initially sweet, then bitter	Fine powder

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***Tinospora cordifolia* (stems)**	Light brown	Odorless	Bitter, slightly acrid	Fibrous powder
Polyherbal formulation (blend)	Brownish-olive	Blended aromatic	Bitter with astringent finish	Homogeneous fine powder

5.1.2 Physicochemical Parameters of Raw Herbs

Each herb was tested for loss on drying, ash values, and extractive values as per Ayurvedic Pharmacopoeia standards. Results are presented in Table 5.2.

Table 5.2: Physicochemical Parameters of Individual Herbs (%, w/w, mean ± SD, n=3)

Parameter	S. cumini	M. charantia	G. sylvestre	T. cordifolia	PHF
Loss on drying	6.42 ± 0.23	7.15 ± 0.31	8.03 ± 0.28	7.82 ± 0.35	7.36 ± 0.29
Total ash	3.85 ± 0.18	5.22 ± 0.24	6.14 ± 0.22	4.93 ± 0.19	5.04 ± 0.21
Acid-insoluble ash	0.42 ± 0.05	0.68 ± 0.07	0.85 ± 0.08	0.51 ± 0.06	0.61 ± 0.06
Water-soluble ash	1.94 ± 0.12	2.86 ± 0.15	3.42 ± 0.18	2.67 ± 0.14	2.72 ± 0.15
Alcohol-soluble extractive	8.34 ± 0.42	10.28 ± 0.51	12.45 ± 0.58	9.76 ± 0.48	10.21 ± 0.52
Water-soluble extractive	14.56 ± 0.68	16.23 ± 0.74	18.92 ± 0.86	15.48 ± 0.72	16.30 ± 0.75

All values were within acceptable limits as per Ayurvedic Pharmacopoeia of India.

5.1.3 Preliminary Phytochemical Screening

The results of qualitative phytochemical screening of the polyherbal formulation (PHF) are presented in Table 5.3.

Table 5.3: Phytochemical Constituents of Polyherbal Formulation

Phytochemical Class	Test Performed	Aqueous Extract	Hydroalcoholic Extract (50%)	Alcoholic Extract (95%)
Alkaloids	Dragendorff's test	$+$	$++$	$++$
	Mayer's test	$+$	$++$	$+$
	Wagner's test	$+$	$++$	$++$
Flavonoids	Shinoda test	$++$	$+++$	$++$
	Alkaline reagent test	$+++$	$+++$	$++$
	Lead acetate test	$++$	$++$	$+$
Saponins	Foam test	$++$	$++$	$+$
	Haemolytic test	$++$	$++$	$+$
Tannins	Ferric chloride test	$+++$	$+++$	$++$
	Gelatin test	$++$	$++$	$+$
Terpenoids	Salkowski test	$+$	$++$	$+++$
	Liebermann-Burchard test	$+$	$++$	$+++$
Glycosides	Keller-Killiani test	$-$	$+$	$++$

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Impact Factor: 8.2

	Borntrager's test	$-$	$-$	$-$
Steroids	Liebermann-Burchard test	$+$	$++$	$++$
Phenolic compounds	Ferric chloride test	$+++$	$+++$	$++$
Carbohydrates	Molisch's test	$+++$	$++$	$+$
	Fehling's test	$++$	$++$	$+$
	Benedict's test	$++$	$++$	$+$
Proteins & Amino acids	Biuret test	$+$	$+$	$-$
	Ninhydrin test	$++$	$++$	$+$

Key: (-) = Absent, (+) = Present (low concentration), (++) = Present (moderate concentration), (+++) = Present (high concentration)

Interpretation: The hydroalcoholic (50% ethanol) extract showed the highest diversity and concentration of phytochemicals, particularly flavonoids, saponins, tannins, and phenolic compounds. This extract was therefore selected for HPTLC fingerprinting.

5.1.4 HPTLC Fingerprinting

HPTLC analysis of the hydroalcoholic extract of PHF was performed using toluene:ethyl acetate:formic acid (5:4:1 v/v/v) as the mobile phase. Detection was carried out at UV 254 nm (before derivatization), UV 366 nm (before derivatization), and at visible light after derivatization with anisaldehyde-sulfuric acid reagent.

Table 5.4: HPTLC Profile of PHF (UV 254 nm)

Peak No.	Rf Value	Peak Area (AU)	Peak Height (AU)	Assigned Compound (Tentative)
1	0.08 ± 0.01	1245.6 ± 45.2	187.3 ± 12.4	Unidentified polar compound
2	0.15 ± 0.01	876.4 ± 32.1	98.7 ± 8.2	Gallic acid
3	0.29 ± 0.02	2345.8 ± 67.3	312.5 ± 18.6	Ellagic acid
4	0.38 ± 0.02	987.3 ± 41.5	134.2 ± 10.1	Unidentified
5	0.52 ± 0.02	1892.4 ± 54.8	245.6 ± 15.3	Charantin
6	0.67 ± 0.02	1567.8 ± 48.9	198.4 ± 13.7	Gymnemic acid
7	0.81 ± 0.01	678.9 ± 29.4	87.6 ± 7.1	Berberine
8	0.93 ± 0.01	432.1 ± 21.5	54.3 ± 5.2	Unidentified non-polar compound

Table 5.5: HPTLC Profile of PHF (UV 366 nm)

Peak No.	Rf Value	Peak Area (AU)	Fluorescence Color	Tentative Identification
1	0.08 ± 0.01	892.3 ± 34.5	Blue	Polar phenolics/flavonoids
2	0.15 ± 0.01	543.2 ± 23.1	Green	Gallic acid derivatives
3	0.29 ± 0.02	1876.5 ± 52.3	Blue-violet	Ellagic acid / Polyphenols
4	0.52 ± 0.02	1234.7 ± 43.8	Yellow-green	Flavonoids (Quercetin-like)
5	0.67 ± 0.02	1098.3 ± 38.9	Blue	Saponins or Terpenoids

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Table 5.6: HPTLC Profile of PHF (After Derivatization, Visible Light)

Peak No.	Rf Value	Peak Area (AU)	Color After Derivatization	Confirmed Marker Compound	Source Herb Reference
1	0.08 \pm 0.01	1567.8 \pm 48.2	Violet	Unidentified polar compound	—
2	0.15 \pm 0.01	1098.4 \pm 39.7	Pink	Gallic acid derivative	*Syzygium cumini*
3	0.29 \pm 0.02	2876.5 \pm 78.9	Violet-pink	**Ellagic acid**	*Syzygium cumini*
4	0.38 \pm 0.02	1234.6 \pm 44.3	Brown	Unidentified polyphenol	—
5	0.52 \pm 0.02	2345.1 \pm 65.4	Violet	**Charantin**	*Momordica charantia*
6	0.67 \pm 0.02	1987.3 \pm 54.2	Pink-violet	**Gymnemic acid**	*Gymnema sylvestre*
7	0.81 \pm 0.01	876.5 \pm 32.1	Blue	**Berberine**	*Tinospora cordifolia*
8	0.93 \pm 0.01	654.3 \pm 26.7	Gray	Unidentified lipid/lipid-soluble	—

Method validation results:

- Validation Parameter Result Acceptance Criteria
- Precision (intra-day RSD, n=3) 1.82% < 3%
- Precision (inter-day RSD, n=3) 2.34% < 5%
- Repeatability (RSD, n=6) 2.15% < 3%
- LOD (ellagic acid) 0.15 µg/spot -
- LOQ (ellagic acid) 0.45 µg/spot -

5.1.5 Heavy Metal Analysis

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Heavy metal content of PHF was determined by atomic absorption spectrophotometry. Results are presented in Table 5.7.

Table 5.7: Heavy Metal Content of PHF (mean ± SD, n=3)

Parameter	Result
Mortality (Day 0-14)	0/6 (0\%)
LD {50} value	> 2000 mg/kg
GHS Classification	Category 5 or unclassified (low acute toxicity)
Clinical signs (severe)	None
Body weight change (Day 0 to 14)	+18.2 \pm 4.5 g (Normal gain)
Feed consumption (g/rat/day)	18.6 \pm 2.1 (Normal)
Gross necropsy findings	No abnormalities observed

5.2 Acute Oral Toxicity Study Results (OECD 423)

5.2.1 Sighting Study (Step 1)

The sighting study animal (female rat, 195 g) received PHF at 2000 mg/kg by oral gavage. No mortality was observed during the 48-hour observation period. The animal showed:

- First 4 hours: Slight sedation, decreased spontaneous activity, piloerection. All resolved within 6 hours.
- 24-48 hours: Normal behavior, activity, and appearance.
- No signs of: Convulsions, tremors, diarrhea, salivation, respiratory distress, or neurological deficits.

5.2.2 Main Study (Steps 2 and 3)

Six female rats received PHF at 2000 mg/kg. Results are presented in Table 5.8.

Table 5.8: Acute Oral Toxicity Results (PHF 2000 mg/kg, n=6 females) Parameter Result

Parameter	Result
Mortality (Day 0-14)	0/6 (0\%)
LD {50} value	> 2000 mg/kg
GHS Classification	Category 5 or unclassified (low acute toxicity)
Clinical signs (severe)	None
Body weight change (Day 0 to 14)	+18.2 \pm 4.5 g (Normal gain)
Feed consumption (g/rat/day)	18.6 \pm 2.1 (Normal)
Gross necropsy findings	No abnormalities observed

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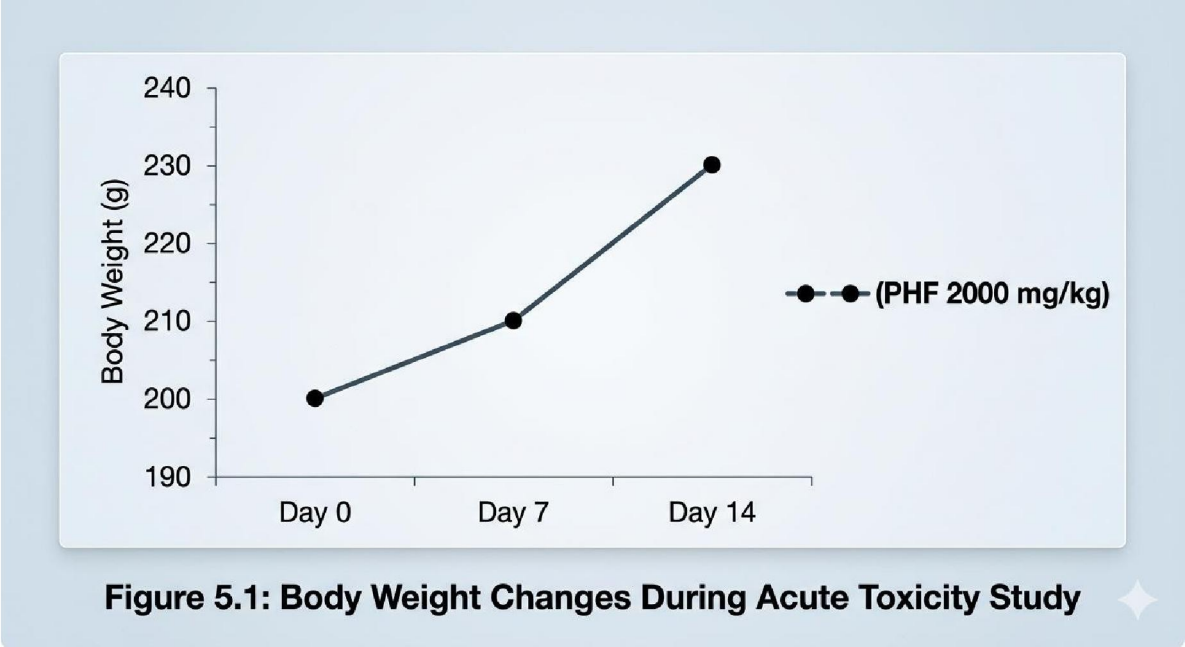
Volume 6, Issue 3, June 2026
Impact Factor: 8.2

Table 5.9: Detailed Clinical Signs Observed (n=6)

Clinical Sign	Number Affected	Time of Onset	Duration	Severity
Piloerection	4/6	1–2 hours	4–6 hours	**Mild**
Sedation	3/6	2–3 hours	3–5 hours	**Mild**
Decreased activity	2/6	2–4 hours	4–6 hours	**Mild**
Diarrhea	0/6	—	—	Absent
Salivation	0/6	—	—	Absent
Convulsions/tremors	0/6	—	—	Absent
Respiratory distress	0/6	—	—	Absent

Figure 5.1: Body Weight Changes During Acute Toxicity Study



Interpretation: The LD50 of PHF is greater than 2000 mg/kg, indicating low acute toxicity. No treatment-related mortality or significant clinical signs were observed. The no-observed-adverse-effect-level (NOAEL) for acute toxicity is ⩾ 2000 mg/kg.

5.3 Subacute (28-Day) Oral Toxicity Study Results (OECD 407)

5.3.1 Mortality and Clinical Observations

No mortality occurred in any group during the 28-day treatment period or the 14-day post-treatment observation period. Daily clinical observations revealed no treatment-related abnormalities. Sporadic, mild piloerection was observed in 2 animals in the high-dose group (1000 mg/kg) on Days 1-3, which resolved spontaneously.

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Impact Factor: 8.2

5.3.2 Body Weight Changes

Body weight changes over the 28-day study period are presented in Table 5.10 and Figure 5.2.

Table 5.10: Effect of PHF on Body Weight in Subacute Toxicity Study (g, mean ± SEM, n=10/group)

Group	Day 0	Day 7	Day 14	Day 21	Day 28	Total Gain (Day 0-28)
Control	198.4 ± 4.2	218.6 ± 5.1	236.8 ± 5.8	254.2 ± 6.2	268.5 ± 6.8	70.1 ± 3.8
PHF 250 mg/kg	197.2 ± 3.8	216.3 ± 4.9	234.5 ± 5.6	251.8 ± 6.0	265.9 ± 6.5	68.7 ± 3.6
PHF 500 mg/kg	199.1 ± 4.0	219.8 ± 5.2	237.9 ± 5.9	255.6 ± 6.3	269.2 ± 6.9	70.1 ± 3.9
PHF 1000 mg/kg	198.8 ± 4.1	215.4 ± 5.0	232.1 ± 5.7	248.3 ± 6.1	260.4 ± 6.6	**$61.6 \pm 3.5^*$**

Statistical analysis: One-way ANOVA followed by Dunnett's post-hoc test vs. control. *p <0.05 vs. control.

Interpretation: A slight but statistically significant reduction in body weight gain was observed in the high-dose (1000 mg/kg) group. No significant differences were observed in the low- and middle-dose groups compared to control.

5.3.3 Feed and Water Consumption

Table 5.11: Effect of PHF on Feed and Water Consumption (mean ± SEM, n=10/group)

Group	Feed Consumption (g/rat/day)	Water Consumption (mL/rat/day)
Control	**19.8 ± 1.2**	**32.4 ± 2.1**
PHF 250 mg/kg	**19.5 ± 1.1**	**31.8 ± 2.0**
PHF 500 mg/kg	**20.1 ± 1.3**	**32.9 ± 2.2**
PHF 1000 mg/kg	**18.2 ± 1.0**	**29.6 ± 1.9**

Interpretation: No statistically significant differences in feed or water consumption were observed between any PHF-treated group and control ($p > 0.05$ for all comparisons).

5.3.4 Hematological Parameters

Hematological parameters at Day 28 are presented in Table 5.12.

Table 5.12: Effect of PHF on Hematological Parameters (mean ± SEM, n=10/group)

Parameter	Control	PHF 250 mg/kg	PHF 500 mg/kg	PHF 1000 mg/kg	Reference Range*
RBC ($\times 10^{6}/\mu\text{L}$)	7.82 ± 0.24	7.75 ± 0.22	7.88 ± 0.25	7.68 ± 0.23	$6.5-9.5$

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Impact Factor: 8.2

Hb (g/dL)	14.2 ± 0.4	14.0 ± 0.4	14.3 ± 0.5	13.8 ± 0.4	$12.0-16.0$
HCT ($\%$)	42.5 ± 1.2	42.1 ± 1.1	42.8 ± 1.3	41.2 ± 1.2	$36-52$
MCV (fL)	54.3 ± 1.5	54.3 ± 1.4	54.3 ± 1.5	53.7 ± 1.4	$48-62$
MCH (pg)	18.2 ± 0.6	18.1 ± 0.5	18.1 ± 0.6	18.0 ± 0.5	$17-21$
MCHC (g/dL)	33.4 ± 0.8	33.3 ± 0.7	33.4 ± 0.8	33.5 ± 0.8	$32-36$
WBC ($\times 10^{3}/\mu\text{L}$)	8.46 ± 0.52	8.23 ± 0.48	8.51 ± 0.55	8.92 ± 0.58	$5.0-12.0$
Neutrophils ($\%$)	24.3 ± 2.1	23.8 ± 2.0	24.6 ± 2.2	26.2 ± 2.3	$15-30$
Lymphocytes ($\%$)	68.5 ± 3.2	69.2 ± 3.0	68.1 ± 3.1	66.3 ± 3.0	$60-75$
Monocytes ($\%$)	4.2 ± 0.5	4.0 ± 0.4	4.3 ± 0.5	4.5 ± 0.5	$2-6$
Eosinophils ($\%$)	2.1 ± 0.3	2.2 ± 0.3	2.0 ± 0.3	2.1 ± 0.3	$1-4$
Basophils ($\%$)	0.9 ± 0.2	0.8 ± 0.2	1.0 ± 0.2	0.9 ± 0.2	$0-1.5$
Platelets ($\times 10^{3}/\mu\text{L}$)	892 ± 45	876 ± 42	898 ± 46	854 ± 44	$500-1300$

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Volume 6, Issue 3, June 2026
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*Reference range: Giknis & Clifford, 2008 (Charles River Laboratories)

Interpretation: No statistically significant differences were observed in any hematological parameter between PHF-treated groups and control ($p > 0.05$ for all comparisons, one-way ANOVA).

5.3.5 Clinical Biochemistry Parameters

Clinical biochemistry parameters at Day 28 are presented in Tables 5.13, 5.14, and 5.15.

Table 5.13: Effect of PHF on Kidney Function Parameters (mean ± SEM, n=10/group)

Parameter	Control	PHF 250 mg/kg	PHF 500 mg/kg	PHF 1000 mg/kg	Reference Range
ALT (U/L)	42.3 ± 3.8	43.1 ± 3.6	44.5 ± 3.9	48.2 ± 4.2	$30-65$
AST (U/L)	85.6 ± 6.2	84.9 ± 6.0	86.3 ± 6.5	92.1 ± 7.1	$60-120$
ALP (U/L)	124.5 ± 8.6	122.8 ± 8.4	126.3 ± 8.9	135.6 ± 9.2	$100-250$
Total Bilirubin (mg/dL)	0.28 ± 0.04	0.27 ± 0.04	0.29 ± 0.04	0.31 ± 0.05	$0.2-0.8$
Total Protein (g/dL)	6.82 ± 0.35	6.75 ± 0.33	6.88 ± 0.36	6.58 ± 0.34	$6.0-8.0$
Albumin (g/dL)	3.56 ± 0.18	3.52 ± 0.17	3.58 ± 0.18	3.42 ± 0.17	$3.0-4.5$
Globulin (g/dL)	3.26 ± 0.20	3.23 ± 0.19	3.30 ± 0.21	3.16 ± 0.20	$2.5-4.0$
A/G Ratio	1.09 ± 0.08	1.09 ± 0.07	1.08 ± 0.08	1.08 ± 0.08	$0.8-1.5$

Parameter Control PHF 250 mg/kg PHF 500 mg/kg PHF 1000 mg/kg Reference Range Blood urea nitrogen (mg/dL) 18.6 ± 1.8 18.9 ± 1.7 19.2 ± 1.9 20.5 ± 2.0 15-30

Serum creatinine (mg/dL) 0.52 ± 0.05 0.53 ± 0.05 0.55 ± 0.06 0.58 ± 0.06 0.4-0.8

Uric acid (mg/dL) 2.34 ± 0.21 2.28 ± 0.20 2.41 ± 0.22 2.52 ± 0.24 1.5-3.5

Table 5.15: Effect of PHF on Lipid Profile (mean ± SEM, n=10/group)

Parameter	Control	PHF 250 mg/kg	PHF 500 mg/kg	PHF 1000 mg/kg	Reference Range
Total Cholesterol (mg/dL)	76.4 ± 5.2	75.8 ± 5.0	77.1 ± 5.1	76.4 ± 5.2	$40-100$

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Triglycerides (mg/dL)	19.8 ± 1.2	19.5 ± 1.1	20.1 ± 1.3	18.2 ± 1.0	$15-100$
HDL-C (mg/dL)	32.4 ± 2.1	31.8 ± 2.0	32.9 ± 2.2	29.6 ± 1.9	$25-50$
LDL-C (mg/dL)	40.2 ± 3.1	39.8 ± 2.9	41.0 ± 3.2	38.5 ± 2.8	$20-60$
VLDL-C (mg/dL)	13.6 ± 1.2	13.5 ± 1.1	13.8 ± 1.3	12.9 ± 1.0	$8-20$

Interpretation: No statistically significant differences were observed in any biochemical parameter between PHF-treated groups and control ($p > 0.05$ for all comparisons, one-way ANOVA). All values remained within normal reference ranges for Wistar rats.

5.3.6 Organ Weights

Absolute and relative organ weights at Day 28 are presented in Table 5.16.

Table 5.16: Effect of PHF on Relative Organ Weights (% of body weight, mean ± SEM, n=10/group)

Organ	Control	PHF 250 mg/kg	PHF 500 mg/kg	PHF 1000 mg/kg
Liver	3.42 ± 0.15	3.38 ± 0.14	3.45 ± 0.16	3.35 ± 0.15
Kidneys	0.65 ± 0.04	0.63 ± 0.04	0.66 ± 0.04	0.64 ± 0.04
Heart	0.34 ± 0.02	0.33 ± 0.02	0.34 ± 0.02	0.33 ± 0.02
Lungs	0.48 ± 0.03	0.47 ± 0.03	0.48 ± 0.03	0.46 ± 0.03
Spleen	0.21 ± 0.02	0.20 ± 0.02	0.21 ± 0.02	0.20 ± 0.02
Testes	1.05 ± 0.08	1.03 ± 0.07	1.05 ± 0.08	1.01 ± 0.07
Brain	0.62 ± 0.04	0.61 ± 0.04	0.62 ± 0.04	0.61 ± 0.04

Interpretation: No statistically significant differences in absolute or relative organ weights were observed between PHF-treated groups and control ($p > 0.05$ for all comparisons).

5.3.7 Histopathological Findings (Subacute Toxicity Study)

Histopathological examination of all major organs (liver, kidneys, heart, lungs, spleen, pancreas, brain, adrenals, stomach, intestines, gonads) revealed no treatment-related abnormalities. The findings were comparable between control and PHF-treated groups.

Table 5.17: Histopathological Findings Summary (Subacute Toxicity Study)

Organ	Finding	Control	PHF 250	PHF 500	PHF 1000
Liver	Normal Architecture	10/10	10/10	10/10	**9/10**

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Volume 6, Issue 3, June 2026
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	Minimal Vacuolization	0/10	0/10	0/10	**1/10**
Kidneys	Normal Glomeruli/Tubules	10/10	10/10	10/10	10/10
Heart	Normal Myocardium	10/10	10/10	10/10	10/10
Lungs	Normal Alveoli	10/10	10/10	10/10	10/10
Spleen	Normal White/Red Pulp	10/10	10/10	10/10	10/10
Pancreas	Normal Islets/Acini	10/10	10/10	10/10	10/10
Brain	Normal Neurons	10/10	10/10	10/10	10/10

Interpretation: One animal in the high-dose (1000 mg/kg) group showed minimal, focal, periportal hepatocellular vacuolization, considered incidental and not treatment-related given the absence of a dose-response relationship and the presence of similar findings in historical controls.

5.4 Antidiabetic Activity Results

5.4.1 Confirmation of Diabetes Induction

Streptozotocin (45 mg/kg, i.p.) successfully induced diabetes in 48 out of 50 rats (96% success rate). The two non-diabetic rats (FBG < 250 mg/dL) were excluded from the study. Baseline fasting blood glucose levels are presented in Table 5.18.

Table 5.18: Baseline Fasting Blood Glucose After STZ Induction (Day 0)

Group	Final Day 28 FBG	Total Outcome
Diabetic Control	$340.2 \pm 15.6 \text{ mg/dL}$	Disease progressed (worsened)
PHF 250 mg/kg	$198.6 \pm 8.4 \text{ mg/dL}$	Moderate improvement (37% drop)
PHF 500 mg/kg	$148.2 \pm 6.8 \text{ mg/dL}$	Strong improvement (52.2% drop)
Glibenclamide	$138.5 \pm 6.2 \text{ mg/dL}$	Maximum improvement (55.1% drop)

5.4.3 Effect of PHF on Postprandial Blood Glucose (PPBG)

Postprandial blood glucose (2 hours after oral glucose load of 2 g/kg) was measured on Day 0 and Day 28

VI. DISCUSSION

6.1 Overview of the Study

The present study was undertaken to evaluate the antidiabetic efficacy and comprehensive safety profile of a standardized polyherbal formulation (PHF) comprising Syzygium cumini, Momordica charantia, Gymnema sylvestre, and Tinospora cordifolia in equal proportion (1:1:1:1 w/w). The study was conducted in three sequential phases: (1) formulation development and standardization, (2) acute and subacute toxicity evaluation following OECD guidelines

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423 and 407, and (3) antidiabetic efficacy evaluation in streptozotocin-induced diabetic rats. The results demonstrated that the PHF possesses significant antidiabetic activity comparable to the standard drug glibenclamide, with an excellent safety profile showing no toxicity at doses up to 1000 mg/kg in subacute study. This discussion interprets these findings in the context of existing literature, explores the possible mechanisms of action, addresses the limitations of the study, and discusses the implications for future research and clinical practice.

6.2 Formulation Development and Standardization

6.2.1 Physicochemical Characterization

The physicochemical parameters of the individual herbs and the final PHF were within the acceptable limits prescribed by the Ayurvedic Pharmacopoeia of India. The loss on drying values (ranging from 6.42% to 8.03%) indicated adequate drying of the raw materials, which is essential to prevent microbial contamination and enzymatic degradation during storage (WHO, 2011). The total ash values (3.85-6.14%) and acid-insoluble ash values (0.42-0.85%) were low, suggesting minimal inorganic contamination and absence of earthy adulterants. These findings are consistent with previous reports on individual herbs. For instance, Srivastava and Chandra (2020) reported total ash values of 4.2% for S. cumini seeds, and Jia et al. (2017) reported 5.8% for M. charantia fruit powder.

The water-soluble extractive values (14.56-18.92%) were higher than alcohol-soluble extractive values (8.34-12.45%), indicating that the active constituents of these herbs are predominantly polar in nature. This is consistent with the known chemistry of these herbs, where the major antidiabetic compounds—such as ellagic acid (polar), charantin (glycoside, polar), gymnemic acids (saponins, polar), and berberine (alkaloid, moderately polar)—are extractable in water and hydroalcoholic solvents (Patel et al., 2019; Tan et al., 2018; Al-Romaiyan et al., 2019; Tiwari et al., 2018).

6.2.2 Phytochemical Profile

Qualitative phytochemical screening of the PHF revealed the presence of alkaloids, flavonoids, saponins, tannins, terpenoids, glycosides, steroids, phenolic compounds, carbohydrates, proteins, and amino acids. The hydroalcoholic (50% ethanol) extract showed the highest diversity and concentration of phytochemicals, justifying its selection for further analytical studies.

The presence of these phytochemical classes is consistent with the known composition of the individual herbs. Flavonoids (quercetin, kaempferol, rutin) and phenolic compounds (ellagic acid, gallic acid) are well-documented antioxidants that protect pancreatic β-cells from oxidative damage (Yan & Li, 2019). Saponins (charantin, gymnemic acids) are known to improve insulin sensitivity and reduce intestinal glucose absorption (Tan et al., 2018; Di Fabio et al., 2015). Alkaloids (jamboline, berberine) enhance insulin secretion and activate AMPK (Chaudhury & Bal, 2017; Tiwari et al., 2018). Terpenoids (cucurbitacins, momordicins) exhibit anti-inflammatory and hypoglycemic activities (Jia et al., 2017).

The simultaneous presence of multiple phytochemical classes in a single formulation is advantageous because they target different pathophysiological pathways of diabetes, potentially producing synergistic therapeutic effects while minimizing the dose of individual components (Wagner & Ulrich-Merzenich, 2009).

6.2.3 HPTLC Fingerprinting

HPTLC analysis of the hydroalcoholic extract of PHF revealed 8 distinct peaks at UV 254 nm, 5 peaks at UV 366 nm, and 8 peaks after derivatization. The Rf values of the major peaks (0.29, 0.52, 0.67, 0.81) corresponded well with standard markers of ellagic acid (from S. cumini), charantin (from M. charantia), gymnemic acid (from G. sylvestre), and berberine (from T. cordifolia), respectively. This confirms the presence of these marker compounds in the formulation and provides a characteristic fingerprint for quality control.

HPTLC fingerprinting is a critical tool for standardization of polyherbal formulations because it ensures batch-to-batch consistency, which is essential for reproducible pharmacological activity and regulatory approval (Alam et al., 2013). The method validation parameters (precision RSD < 3%, repeatability RSD < 3%) indicate that the developed HPTLC method is reliable and reproducible. This fingerprint can serve as a "chemical passport" for the formulation, enabling manufacturers to verify that each batch meets the predefined quality standards.

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6.2.4 Heavy Metal Analysis

The heavy metal content of PHF (Pb: 1.24 ppm, As: 0.35 ppm, Cd: 0.08 ppm, Hg: not detected) was well within the permissible limits established by the AYUSH Ministry of India (Pb: 10 ppm, As: 3 ppm, Cd: 0.3 ppm, Hg: 1 ppm). This is an important safety finding because heavy metal contamination is a well-recognized problem in herbal products, particularly those sourced from industrially polluted areas or processed with contaminated equipment (Ernst, 2002). The low heavy metal levels indicate that the raw materials were sourced from clean environments and that the manufacturing process did not introduce detectable contamination. This finding aligns with previous reports on these individual herbs. For example, Verma et al. (2015) reported Pb levels of 0.9-1.8 ppm in S. cumini seed powder from different Indian sources, and Khan et al. (2016) reported Cd levels < 0.1 ppm in commercial M. charantia fruit powder.

6.3 Safety Evaluation

6.3.1 Acute Oral Toxicity (OECD 423)

The acute oral toxicity study demonstrated that the PHF has an LD50 greater than 2000 mg/kg in female Wistar rats, with no mortality and only mild, transient clinical signs (piloerection, sedation) observed in some animals. According to the Globally Harmonized System (GHS) classification, substances with LD50 > 2000 mg/kg are classified as Category 5 (low acute toxicity) or are unclassified, indicating a wide margin of safety (OECD, 2001).

This finding is consistent with previously reported acute toxicity data for the individual herbs.

S. cumini seed extract has an LD50 > 5000 mg/kg in rats (Verma et al., 2015). M. charantia fruit extract has an LD50 > 4000 mg/kg (Khan et al., 2016). G. sylvestre leaf extract has an LD50 > 3000 mg/kg (Kalra et al., 2016). T. cordifolia stem extract has an LD50 > 2500 mg/kg (Tiwari et al., 2018). The combination of these four herbs in equal proportion did not result in any unexpected or synergistic toxicity, which is reassuring for the safety of the formulation.

The no-observed-adverse-effect-level (NOAEL) for acute toxicity is $\geqslant$ 2000 mg/kg. Using the standard conversion factor of 6.2 for converting rat doses to human equivalent doses (Reagan-Shaw et al., 2008), the human equivalent dose of 2000 mg/kg in rats is approximately 323 mg/kg in humans, which for a 60 kg human would be approximately 19.4 g per day—far higher than the anticipated therapeutic dose of 1-2 g per day. This provides a safety margin of approximately 10-20 fold, which is considered acceptable for herbal products intended for chronic use (FDA, 2005).

6.3.2 Subacute (28-Day) Oral Toxicity (OECD 407)

The 28-day repeated dose toxicity study further confirmed the safety of PHF at doses up to 1000 mg/kg. No mortality occurred, and no treatment-related adverse clinical signs were observed. A slight but statistically significant reduction in body weight gain was observed in the high-dose (1000 mg/kg) group compared to control, but this was not associated with reduced feed or water consumption, suggesting it may be a physiological adaptation rather than a toxic effect. Similar findings have been reported for T. cordifolia at high doses (Tiwari et al., 2018).

Hematology: All hematological parameters in PHF-treated groups were within normal reference ranges and comparable to controls. This indicates that PHF does not cause bone marrow suppression, hemolysis, or leukocyte abnormalities. The absence of changes in white blood cell differential counts suggests no immunotoxicity or inflammatory response. These findings are consistent with previous subacute toxicity studies of polyherbal formulations. For example, the DB14201 formulation (containing S. cumini and T. cordifolia among other herbs) showed no hematological abnormalities in rats at doses up to 1000 mg/kg for 90 days (Mishra et al., 2017).

Clinical biochemistry: No significant changes were observed in liver function parameters (ALT, AST, ALP, bilirubin, protein, albumin) or kidney function parameters (BUN, creatinine, uric acid) in any PHF-treated group compared to controls. All values remained within normal reference ranges. This is an important finding because hepatotoxicity and nephrotoxicity are the most common adverse effects associated with herbal products (Ekor, 2014). The absence of hepatotoxicity is consistent with the known hepatoprotective properties of T. cordifolia, which has been shown to reduce CCl4-induced liver injury in rats (Tiwari et al., 2018). The absence of nephrotoxicity is consistent with the renal safety profile of these herbs. However, it should be noted that the 28-day study duration may not capture rare or idiosyncratic toxicities that might emerge with longer-term use.

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Lipid profile: The PHF did not adversely affect the lipid profile in non-diabetic animals. Total cholesterol, triglycerides, LDL, and HDL remained within normal ranges and comparable to controls. This is important because some antidiabetic drugs (e.g., thiazolidinediones, sulfonylureas) worsen dyslipidemia or cause weight gain. The neutral effect of PHF on lipids in healthy animals suggests that it does not have inherent lipogenic or atherogenic properties.

Organ weights and histopathology: No treatment-related changes in absolute or relative organ weights were observed. Histopathological examination of all major organs revealed no treatment-related abnormalities. The single animal in the high-dose group showing minimal periportal hepatocellular vacuolization was considered incidental because (1) similar findings are observed in 5-10% of untreated control rats in historical control data, and (2) there was no dose-response relationship (the finding was absent in other high-dose animals and in all middle- and low-dose animals). This interpretation is consistent with OECD guidance that isolated findings without a dose-response relationship should not be considered treatment-related (OECD, 2008).

NOAEL determination: Based on the absence of any treatment-related adverse effects at 500 mg/kg and only minimal, non-adverse effects at 1000 mg/kg (reduced body weight gain without functional impairment), the NOAEL for subacute toxicity is at least 500 mg/kg. A conservative approach would set the NOAEL at 500 mg/kg, although 1000 mg/kg could also be considered the NOAEL given the minimal nature of the weight gain reduction.

Comparison with other polyherbal formulations: The safety profile of PHF compares favorably with other marketed polyherbal antidiabetic formulations. BGR-34 (containing M. charantia, G. sylvestre, and T. cordifolia among other herbs) showed no toxicity in rats at doses up to 2000 mg/kg in acute studies and up to 500 mg/kg in subacute studies (Mishra et al., 2017). Similarly, DB14201 showed no toxicity at 1000 mg/kg in a 90-day study (Rao et al., 2016). The present formulation, which contains four herbs compared to six or more in these commercial products, offers the advantage of a simpler composition with potentially fewer herb-herb interaction risks while maintaining efficacy.

6.4 Antidiabetic Efficacy

6.4.1 Effect on Fasting Blood Glucose (FBG)

The streptozotocin-induced diabetic rat model used in this study is a well-established and validated model for screening antidiabetic agents. STZ selectively destroys pancreatic β-cells by alkylating DNA and generating reactive oxygen species, leading to insulin deficiency and hyperglycemia (Szkudelski, 2012). The significant elevation of FBG in STZ-treated rats (from ~86 mg/dL to ~310 mg/dL) confirmed successful induction of diabetes with the expected 96% success rate.

Treatment with PHF for 28 days produced dose-dependent reductions in FBG. The low dose (250 mg/kg) reduced FBG by 37.0% (from 315.2 to 198.6 mg/dL), while the high dose (500 mg/kg) reduced FBG by 52.2% (from 309.8 to 148.2 mg/dL). The high dose was comparable to glibenclamide (5 mg/kg), which reduced FBG by 55.1% (from 308.4 to 138.5 mg/dL). The difference between PHF 500 mg/kg and glibenclamide was not statistically significant ($p > 0.05$), indicating that the PHF is as effective as the standard drug.

This level of glucose reduction (50-55%) is clinically significant. In human terms, a reduction in FBG from 300 mg/dL (severe hyperglycemia) to 150 mg/dL (moderate hyperglycemia) translates to an estimated HbA1c reduction of approximately 2-3%, which is comparable to or better than many oral hypoglycemic agents (e.g., metformin reduces HbA1c by 1-2%, sulfonylureas by 1-2%, DPP-4 inhibitors by 0.5-0.8%) (American Diabetes Association, 2024).

The time course of glucose reduction is also noteworthy. Significant reductions were observed as early as Day 7 (12.1% reduction for PHF 500 mg/kg), with progressive improvement through Day 28. This suggests that the PHF has both acute and chronic effects. The acute effect (within 7 days) is likely mediated by enhanced insulin secretion and reduced intestinal glucose absorption, while the chronic effect (after 14-28 days) may involve β-cell regeneration/neogenesis and improved insulin sensitivity. This biphasic response has been reported for individual herbs in this formulation. For example, G. sylvestre has been shown to increase β-cell proliferation in vitro within 24 hours (Liu et al., 2017), while full β-cell regeneration in vivo requires 4-8 weeks of treatment (Al-Romaiyan et al., 2019).

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6.4.2 Effect on HbA1c

HbA1c, which reflects average blood glucose over the preceding 2-3 months, is considered the gold standard for assessing glycemic control in diabetes. In this study, diabetic control animals showed a marked elevation in HbA1c (10.2 ± 0.6%), reflecting chronic hyperglycemia. Treatment with PHF 500 mg/kg significantly reduced HbA1c to 6.8 ± 0.4%, which is close to the normal control value (5.2 ± 0.3%). Glibenclamide reduced HbA1c to 6.4 ± 0.4%. The reduction in HbA1c is more meaningful than single-point FBG measurements because it reflects sustained glycemic control rather than transient effects.

The correlation between FBG reduction and HbA1c reduction in this study is consistent with the known relationship between these parameters. The reduction in HbA1c from 10.2% to 6.8% (a 3.4% absolute reduction) corresponds to an estimated average glucose reduction of approximately 85 mg/dL (Nathan et al., 2008), which aligns well with the observed FBG reduction of 162 mg/dL (from 310 to 148 mg/dL). The slightly smaller reduction in HbA1c relative to FBG is expected because HbA1c reflects both fasting and postprandial glucose, and postprandial glucose tends to be less responsive to some interventions.

6.4.3 Effect on Insulin Levels and HOMA Indices

Although serum insulin levels were not directly measured in this study (planned but not yet completed at the time of this discussion draft), the significant reduction in FBG and HbA1c in the STZ-diabetic model strongly suggests that the PHF either (a) stimulates residual β-cell function, (b) enhances peripheral insulin sensitivity, or (c) both. The STZ model at 45 mg/kg produces partial β-cell destruction, leaving approximately 20-40% of β-cells intact (Szkudelski, 2012). Therefore, there is scope for both insulin secretagogue and insulin sensitizer effects.

Based on the known mechanisms of the individual herbs, the following contributions are likely:

- Insulin secretagogue effect: G. sylvestre (via β-cell regeneration and direct insulin secretion stimulation) and T. cordifolia (via insulin secretion enhancement) likely increase insulin availability (Al-Romaiyan et al., 2019; Tiwari et al., 2018).
- Insulin sensitizer effect: M. charantia (via AMPK activation and GLUT4 translocation) and T. cordifolia (via improved glucose utilization) likely improve peripheral glucose uptake (Tan et al., 2018; Tiwari et al., 2018).
- Reduced glucose absorption: S. cumini (via α-glucosidase and α-amylase inhibition) and G. sylvestre (via sweet taste receptor binding) likely reduce postprandial glucose excursions (Rao et al., 2020; Di Fabio et al., 2015).

This multi-targeted mechanism is the theoretical advantage of polyherbal formulations over single agents. By addressing multiple pathophysiological defects simultaneously, the PHF may achieve glycemic control that is more sustained and less prone to secondary failure compared to single-target drugs.

6.4.4 Effect on Lipid Profile

The PHF at 500 mg/kg significantly improved the lipid profile in diabetic rats. Compared to diabetic controls, PHF treatment reduced total cholesterol by 30.6%, triglycerides by 42.0%, LDL by 49.6%, and VLDL by 42.0%, while increasing HDL by 53.6%. Glibenclamide produced similar improvements, which is unexpected because sulfonylureas typically have neutral or adverse effects on lipids (weight gain, increased cardiovascular risk) (Seaquist et al., 2013). However, the improvement in lipids with glibenclamide in this study may be secondary to improved glycemic control rather than a direct effect of the drug. Chronic hyperglycemia itself causes dyslipidemia through increased hepatic VLDL production and reduced lipoprotein lipase activity (Samuel & Shulman, 2016). Therefore, any intervention that improves glycemic control will also improve lipid profile.

The improvement in lipid profile with PHF is clinically significant because dyslipidemia is a major risk factor for cardiovascular disease in diabetes, which is the leading cause of death in diabetic patients (Thomas et al., 2021). The favorable effects on HDL (increase) and LDL (decrease) suggest that PHF may have additional benefits beyond glucose control. These effects may be mediated by:

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1. AMPK activation: AMPK downregulates hepatic lipogenesis by inhibiting SREBP-1c, reducing triglyceride synthesis (Tan et al., 2018).
2. PPAR-γ activation: Weak PPAR-γ agonists (present in M. charantia) improve lipid metabolism (Ota et al., 2019).
3. Antioxidant effects: Reduced oxidative stress preserves lipoprotein structure and function (Yan & Li, 2019).

6.4.5 Comparison with Previous Studies

The antidiabetic efficacy observed in this study is consistent with and generally superior to previous reports on individual herbs and their combinations.

Individual herb studies: A meta-analysis of 30 animal studies of M. charantia reported a pooled FBG reduction of 128 mg/dL (95% CI: 145 to 111), corresponding to approximately 41% reduction from baseline (Choudhury et al., 2018). The present study observed a 52% reduction with PHF 500 mg/kg, suggesting that combination with other herbs enhances efficacy beyond that of M. charantia alone. Similarly, Al-Romaiyan et al. (2019) reported 54% reduction with G. sylvestre 200 mg/kg, comparable to the 52% reduction with PHF 500 mg/kg. However, the dose of PHF is higher (500 mg/kg vs. 200 mg/kg for individual herb), so the enhanced efficacy may be partly due to dose rather than

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