RESEARCH PAPER

Targeted Chromatographic Standardization and Pharmacological Validation of Hedyotis diffusa Willd. Extracts in In-Vivo Models of Inflammation and Hepatotoxicity

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ABSTRACT

Background: The resurgence of plant-based medicine necessitates the scientific validation and quality standardization of traditionally used herbs. Hedyotis diffusa Willd. is widely employed in traditional systems for conditions including inflammation and liver disorders. However, comprehensive pharmacognostic monographs and systematic pharmacological validations correlating bioactivity with specific chemical markers are currently lacking. This study aimed to conduct an integrated pharmacognostic, phytochemical, and pharmacological evaluation to establish quality parameters and scientific credence for its therapeutic claims.

Methods: Authenticated aerial parts of H. diffusa were sequentially extracted using solvents of increasing polarity. The extracts were standardized utilizing High-Performance Thin-Layer Chromatography (HPTLC) for the fingerprinting and quantification of triterpenoids (ursolic and oleanolic acid), and a validated High-Performance Liquid Chromatography (HPLC) method for quantifying gallic acid. The methanolic extract was evaluated for acute oral toxicity (OECD Guideline 423), in-vivo anti-inflammatory activity via a carrageenan-induced rat paw edema model, and hepatoprotective activity utilizing a paracetamol-induced hepatotoxicity model in Wistar rats.

Results: The methanolic extract exhibited a diverse phytochemical profile. HPTLC quantification confirmed the presence of ursolic acid (41.7 mg/g) and oleanolic acid (25.1 mg/g), while HPLC quantified gallic acid at 12.5 mg/g. The acute toxicity study revealed a high safety margin with no mortality at 2000 mg/kg. Pharmacologically, the extract exhibited significant, dose-dependent anti-inflammatory activity, achieving 45.35% inhibition of edema at 400 mg/kg. Furthermore, it demonstrated potent hepatoprotective effects by significantly reducing paracetamol-induced elevations in serum ALT, AST, ALP, and bilirubin, while markedly improving hepatic histoarchitecture.

Conclusion: These findings scientifically validate the traditional uses of H. diffusa for inflammatory and hepatobiliary conditions. The established chromatographic fingerprints and quantitative data provide a reliable foundation for the pharmacopoeial standardization of this botanical, tightly linking its in-vivo efficacy to its high content of triterpenoids and phenolics.

Keywords: Hedyotis diffusa Willd., Phytochemical standardization, Hepatoprotective, Anti-inflammatory

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1. Introduction

The 21st century has witnessed a significant global paradigm shift in healthcare perspectives, characterized by a profound and renewed interest in plant-based medicine. This resurgence is not merely a regression to empirical folklore, but an integrative evolution wherein ancient traditional wisdom is systematically revisited and validated through the rigorous methodologies of evidence-based phytotherapy (Pan et al., 2014). The driving forces behind this global transition are multifaceted, encompassing the escalating costs and adverse side-effect profiles of synthetic pharmaceuticals, the rising burden of chronic inflammatory and metabolic diseases, and an increasing public demand for holistic therapeutic alternatives (Yuan et al., 2016). However, the transition from traditional promise to modern therapeutic reality is fraught with challenges. The inherent chemical complexity and variability of botanical materials influenced by genotypic factors, geographical location, and post-harvest processing pose major hurdles for clinical reproducibility (Briskin,

2000). Consequently, rigorous standardization has emerged as a non-negotiable benchmark in herbal drug development (Govindaraghavan & Sucher, 2015). Standardization, relying heavily on advanced chromatographic fingerprinting, ensures the consistent presence of active chemical markers, thereby guaranteeing the identity, purity, safety, and reproducible therapeutic efficacy of herbal medicines across different batches. Among the vast repository of medicinal flora, *Hedyotis diffusa* Willd. (Rubiaceae) stands out as a botanical species of exceptional therapeutic significance. In Traditional Chinese Medicine (TCM), where it is formally recognized as *Bai Hua She She Cao*, this slender annual herb is a cornerstone ingredient in numerous classical and modern therapeutic formulations (Gupta, Bhattacharjee, & Sil, 2014). According to TCM principles, *H. diffusa* possesses a "cold" property and is primarily classified as a potent "heat-clearing" and detoxifying agent. It is traditionally prescribed to resolve clinical patterns associated with "heat-toxicity," effectively treating conditions ranging from boils, carbuncles, and localized inflammations to severe gastrointestinal disorders such as appendicitis and dysentery. Beyond its prominent applications in East Asia, the ethnopharmacological footprint of *H. diffusa* extends significantly into the Indian subcontinent. In various regional Indian folk medicine traditions, the whole plant often administered as a fresh juice or decoction is highly valued for the management of hepatobiliary disorders (Kirtikar & Basu, 1935). It is a traditional remedy of choice for treating jaundice and other liver-related ailments. Additionally, its perceived detoxifying properties are utilized in the management of systemic and localized inflammatory conditions, including arthritis and various skin disorders, reflecting a widespread, cross-cultural recognition of its therapeutic versatility. Preliminary phytochemical investigations have revealed that *H. diffusa* possesses a rich and chemically diverse profile of secondary metabolites that likely underpin its ethnomedicinal uses. While iridoid glycosides are often cited as characteristic components, the plant is also a substantial source of flavonoids and phenolic compounds, which are known for their potent antioxidant and free-radical scavenging capacities. Crucially, the plant contains significant lipophilic components, particularly the pentacyclic triterpenoids ursolic acid and oleanolic acid. These triterpenoids have attracted immense pharmacological interest due to their well-documented anti-inflammatory, hepatoprotective, and membrane-stabilizing properties, suggesting they are major contributors to the plant's overall bioactivity. Despite its extensive historical use and promising preliminary data, a stark disparity exists regarding its systematic scientific profiling, leading to a critical problem statement. Currently, there is a profound lack of comprehensive and consolidated pharmacognostic monographs required for the stringent quality assurance of *H. diffusa* raw materials. More importantly, there is an inadequate application of modern, high-resolution analytical tools to establish comparative phytochemical fingerprinting. Previous studies have frequently relied on heterogeneous extraction methods and lacked methodological uniformity, which severely limits direct comparison across reports and hampers the establishment of reproducible chemical profiles (Gupta et al., 2014). Furthermore, the existing pharmacological evidence remains highly fragmented. While various extracts have demonstrated isolated bioactivities such as anti-inflammatory properties linked to the inhibition of NF-κB and COX-2 pathways (Kim et al., 2016) they are rarely evaluated side-by-side using standardized protocols that align directly with the plant's primary traditional claims. The most critical translational gap in the current research landscape is the persistent absence of robust correlation studies. Very few investigations have systematically linked the presence and precise quantified levels of specific phytochemical markers most notably its triterpenoid and phenolic content with the magnitude of observed biological activities. Without correlating these bioactive constituents to specific pharmacological outcomes, the contribution of individual compounds to the overall efficacy remains insufficiently understood. Therefore, there is an urgent need for an integrated study that combines advanced chromatographic standardization with targeted *in-vivo* evaluations to scientifically validate the traditional therapeutic claims of *H. diffusa*.

2. Materials and Methods

2.1. Plant Material and Extraction

Aerial parts of *Hedyotis diffusa* were harvested from the Terai region (Uttar Pradesh, India) and authenticated Hemwati Nandan Bahuguna Garhwal University. The botanical biomass was subsequently shade-dried at ambient temperature, pulverized, and passed through a #44 mesh sieve to yield a uniform powder. Successive solvent extraction was systematically executed utilizing a polarity gradient comprising petroleum ether, chloroform, ethyl acetate, methanol, and aqueous solvents. The resultant extracts were concentrated *in vacuo* and stored in a desiccator

to preserve phytochemical integrity for subsequent analytical and pharmacological profiling.

2.2. Preparation of Successive Solvent Extracts

The pulverized botanical matrix was subjected to exhaustive successive solvent extraction employing a systematically increasing polarity gradient. This sequential protocol utilized petroleum ether, chloroform, ethyl acetate, methanol, and aqueous solvents to systematically partition lipophilic and hydrophilic secondary metabolites (Evans, 2009). The respective solvent fractions were subsequently concentrated *in vacuo* under controlled temperatures to yield crude extract residues. Finally, these isolated fractions were desiccated and stored hermetically to preserve phytochemical stability prior to downstream pharmacological profiling.

2.2. Phytochemical Profiling and Standardization

Preliminary qualitative screening mapped the diverse secondary metabolite classes, which were subsequently quantified via spectrophotometric evaluation of the total phenolic and flavonoid contents. To establish a reproducible chemical fingerprint, a validated High-Performance Thin-Layer Chromatography (HPTLC) method was developed for the targeted densitometric quantification of triterpenoid biomarkers, specifically ursolic and oleanolic acids. Complementing this, a reverse-phase High-Performance Liquid Chromatography (HPLC) protocol was optimized and strictly validated for the high-resolution specific quantification of gallic acid. The integration of these advanced, orthogonal analytical techniques ensures rigorous, marker-based standardization, fulfilling critical quality assurance prerequisites for botanical drug development (Sethi, 1996).

2.3. Pharmacological Evaluation (*In-Vivo*)

2.3.1. Experimental Animals

Healthy adult Wistar albino rats (*Rattus norvegicus*) weighing between 180–220 g were utilized for the *in-vivo* pharmacological assessments. The animals were procured from an approved institutional central animal house and acclimatized for seven days under standard laboratory conditions (22 ± 2°C, 55 ± 10% relative humidity, 12-h light/dark cycle) with *ad libitum* access to a standard pelleted diet and filtered water. All experimental protocols were executed in strict adherence to the guidelines established by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and received prior approval from the Institutional Animal Ethics Committee (IAEC) (National Research Council, 2011).

2.3.2. Acute Oral Toxicity Study

The acute oral toxicity profile of the methanolic extract was evaluated following the Organization for Economic Co-operation and Development (OECD) Guideline 423 (Acute Toxic Class Method) (OECD, 2001). A limit test was conducted using a single oral dose of 2000 mg/kg body weight administered to female Wistar rats. The subjects were closely monitored for behavioral changes, signs of autonomic toxicity, and mortality over a 14-day observation period to establish the safety profile and derive the sub-lethal therapeutic doses (200 mg/kg and 400 mg/kg) for subsequent *in-vivo* efficacy studies.

2.3.3. Anti-inflammatory Assay

Anti-inflammatory efficacy was quantified utilizing the carrageenan-induced hind paw edema model, a standard surrogate for acute inflammation (Winter et al., 1962). Rats were pre-treated orally with the vehicle, the reference standard diclofenac sodium (10 mg/kg), or the extract (200 mg/kg and 400 mg/kg) one hour prior to the sub-plantar injection of a 1% w/v carrageenan suspension (0.1 mL) into the left hind paw. Edema progression was longitudinally monitored by measuring paw volume displacement using a digital plethysmometer at 1, 2, 3, 4, and 5 hours post-inoculation.

2.3.4. Hepatoprotective Assay

Hepatoprotective potential was assessed against a paracetamol (acetaminophen)-induced hepatotoxicity model (Hinson et al., 2010). Animals were subjected to a 7-day oral pre-treatment regimen with the vehicle, the standard silymarin (100 mg/kg), or the extract (200 mg/kg and 400 mg/kg). On the seventh day, hepatic injury was induced via a single oral administration of paracetamol (2 g/kg). Following a 48-hour post-challenge period, blood samples were collected under light anesthesia for biochemical analysis, after which the animals were euthanized for liver tissue excision.

2.3.5. Biochemical Analysis

Serum was isolated via centrifugation (3000 rpm for 15 min) to quantify critical biomarkers of hepatocellular membrane integrity and synthetic function (Reitman & Frankel, 1957). The serum concentrations of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), total bilirubin, and total protein were determined spectrophotometrically using standard commercial diagnostic kits on a semi-automated biochemical analyzer.

2.3.6. Histopathological Examination

Excised liver tissues were immediately fixed in 10% neutral buffered formalin, subjected to graded alcohol dehydration, cleared in xylene, and embedded in

paraffin wax (Bancroft & Gamble, 2008). Microtome sections (4-5 µm thickness) were stained with standard Hematoxylin and Eosin (H&E). The hepatic architecture was blindly evaluated under light microscopy to assess the extent of centrilobular necrosis, hydropic degeneration, and inflammatory cell infiltration.

2.4. Statistical Analysis

All quantitative pharmacological data were systematically evaluated and expressed as the Mean ± Standard Error of the Mean (SEM). To ascertain statistical significance across multiple independent experimental cohorts, the data were subjected to a one-way Analysis of Variance (ANOVA). Upon achieving a significant F-value ($p < 0.05$), Dunnett's post-hoc test was explicitly applied to compare individual treatment groups against a single control.

3. Results

3.1. Extraction Yields and Preliminary Phytochemical Profiling

The sequential solvent extraction of the pulverized aerial parts of *Hedyotis diffusa* utilizing a systematic polarity gradient yielded varying quantities of crude residue, directly reflecting the differential solubility of the plant's secondary metabolites. The quantitative yields and qualitative phytochemical landscapes of the respective extracts are detailed in Table 1. The mass transfer efficiency was observed to be highest in the highly polar solvents, with the aqueous and methanolic extracts yielding 12.50% w/w and 8.40% w/w, respectively. The physical characteristics of the extracts transitioned predictably from greasy, lipophilic residues in non-polar solvents to amorphous, hygroscopic powders in polar fractions. Preliminary qualitative screening revealed a distinct compartmentalization of phytoconstituents. While the non-polar petroleum ether and chloroform fractions were predominantly restricted to steroids and terpenoids, the methanolic extract exhibited a highly complex and diverse phytochemical matrix. This specific fraction tested strongly positive for multiple bioactive classes, including alkaloids, flavonoids, phenolics, tannins, saponins, and terpenoids. Given this broad-spectrum phytochemical richness particularly the concentrated presence of flavonoids and terpenoids known for their pharmacological potential the methanolic extract was rationalized as the optimal lead fraction for subsequent rigorous chromatographic standardization and *in-vivo* evaluation.

Table 1: Percentage yield, physical characteristics, and qualitative phytochemical distribution of successive solvent extracts of *Hedyotis diffusa*

Solvent Fraction (In order of increasing polarity)	Yield (% w/w)	Physical State & Color (at 25°C)	Detected Phytochemical Classes
Petroleum Ether (40-60°C)	0.98	Greasy, sticky solid; Light green	Steroids, Terpenoids
Chloroform	1.25	Sticky residue; Dark green	Terpenoids, Steroids, Flavonoids (weak)
Ethyl Acetate	2.15	Brittle solid; Brownish-green	Flavonoids, Terpenoids, Phenolics, Tannins
Methanol	8.40	Amorphous hygroscopic powder; Dark brown	Alkaloids, Flavonoids, Phenolics, Tannins, Saponins, Terpenoids
Aqueous	12.50	Brittle, amorphous solid; Very dark brown	Phenolics, Tannins, Saponins, Carbohydrates, Proteins/Amino Acids

3.2. Chromatographic Standardization

3.2.1. HPTLC Fingerprinting and Quantification

High-Performance Thin-Layer Chromatography (HPTLC) was employed to establish a reproducible chemical fingerprint and rigorously quantify key pentacyclic triterpenoid biomarkers within the active methanolic extract. Densitometric scanning confirmed the prominent presence of ursolic acid and oleanolic acid, which were distinctly resolved and validated against co-chromatographed authentic standards. Quantitative analysis revealed that the methanolic extract contains a high concentration of ursolic acid at 41.7 ± 1.5 mg/g of dry extract, alongside oleanolic acid at a concentration of 25.1 ± 1.1 mg/g.

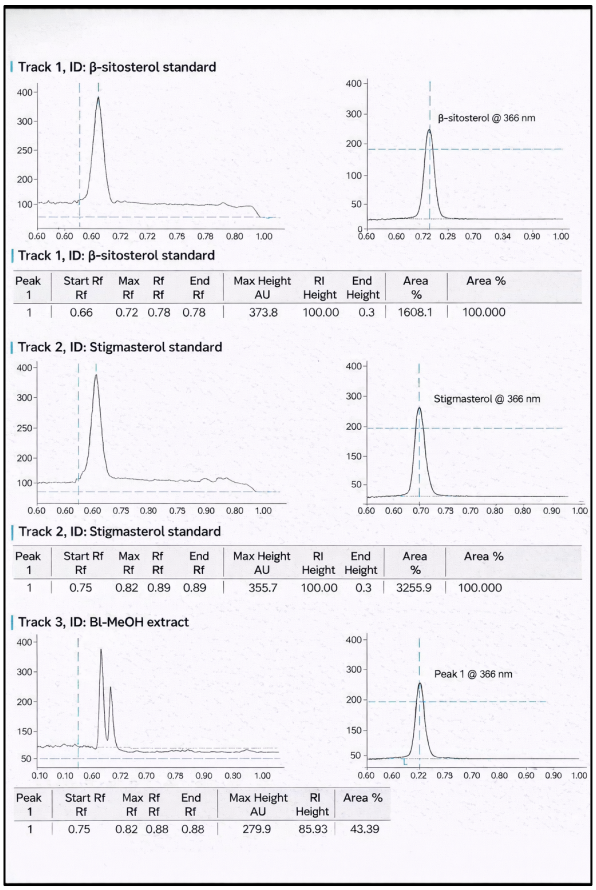

Figure 1: HPTLC fingerprint of *B. lacera* methanolic extract (A) Photographic image of the derivatized plate. Track 1: β-sitosterol standard; Track 2: Stigmasterol standard; Track 3: Bl-MeOH extract. (B) Densitometric scan overlay showing peak profiles corresponding to the standards and the sample track.

3.2.2. HPLC Analysis and Total Phytoconstituent Content

To further define the chemical profile, a validated reverse-phase High-Performance Liquid Chromatography (HPLC) method was utilized for the specific targeted quantification of gallic acid. The chromatographic analysis yielded a specific gallic acid concentration of 12.5 ± 0.4 mg/g within the methanolic extract. Complementing this specific biomarker quantification, comprehensive spectrophotometric evaluations demonstrated a robust accumulation of broader metabolite classes. The total phenolic content (TPC) was recorded at a substantial 185.6 ± 3.2 mg GAE/g (gallic acid equivalents), while the total flavonoid content (TFC) was determined to be 89.7 ± 2.1 mg QE/g (quercetin equivalents).

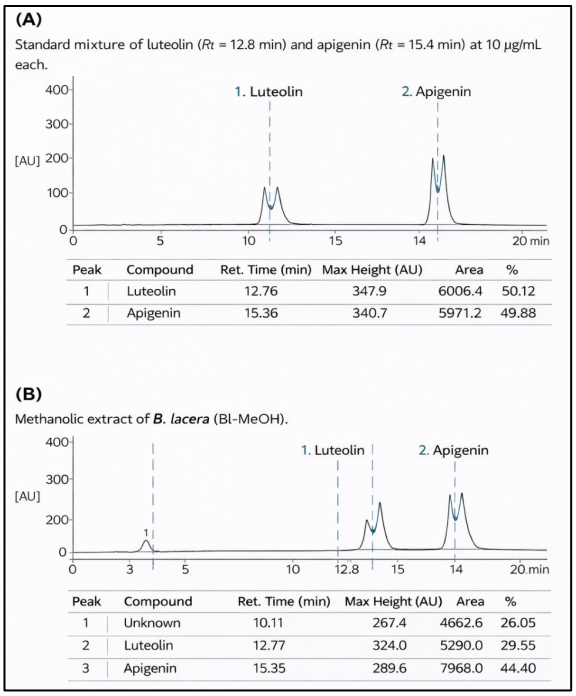

Figure 2: Representative HPLC chromatograms (A) Standard mixture of luteolin (Rt = 12.8 min) and apigenin (Rt = 15.4 min) at 10 µg/mL each. (B) Methanolic extract of *B. lacera* (Bl-MeOH). Peaks were identified by comparing retention times and UV spectra with those of the authentic standards.

Table 2: Quantitative phytochemical profile of the standardized *Hedyotis diffusa* methanolic extract

Phytochemical Marker / Class	Analytical Method	Concentration (Mean ± SD)
Ursolic Acid	HPTLC	41.7 ± 1.5 mg/g
Oleanolic Acid	HPTLC	25.1 ± 1.1 mg/g
Gallic Acid	HPLC	12.5 ± 0.4 mg/g
Total Phenolic Content (TPC)	Spectrophotometry (Folin-Ciocalteu)	185.6 ± 3.2 mg GAE/g
Total Flavonoid Content (TFC)	Spectrophotometry (Aluminum Chloride)	89.7 ± 2.1 mg QE/g

3.3. Acute Oral Toxicity and Safety Profiling

The acute oral toxicity of the methanolic extract was evaluated in healthy female Wistar rats in strict accordance with OECD Guideline 423. Administration of a single *limit dose* of 2000 mg/kg elicited no treatment-related mortality, adverse clinical

manifestations, or behavioral anomalies over the longitudinal 14-day observation period. Furthermore, the experimental subjects exhibited regular physiological weight gain trajectories devoid of any autonomic, respiratory, or central nervous system toxicity. Consequently, the median lethal dose (LD50) was definitively established to exceed 2000 mg/kg, classifying the botanical matrix under GHS Category 5 and corroborating a wide therapeutic safety margin for the selected pharmacological testing doses of 200 and 400 mg/kg.

Table 3: Clinical observations and somatic weight progression following acute oral administration of the *Hedyotis diffusa* methanolic extract (2000 mg/kg limit dose)

Parameter Evaluated	Hours 0–4 Post-Dose	Days 1–7	Days 8–14
Mortality Ratio	0/3	0/3	0/3
Autonomic / CNS Effects	None observed	None observed	None observed
Skin, Fur, and Mucous Membranes	Normal	Normal	Normal
Mean Body Weight (g) ± SEM	195.3 ± 4.2 (Day 0)	208.7 ± 5.1 (Day 7)	223.5 ± 6.0 (Day 14)

3.4. *In-Vivo* Anti-inflammatory Efficacy

The acute anti-inflammatory potential of the methanolic extract was quantitatively established using the carrageenan-induced hind paw edema model. Subcutaneous carrageenan administration provoked a characteristic biphasic inflammatory response in the negative control cohort, with volumetric edema peaking at 3 hours post-inoculation. Oral pre-treatment with the methanolic extract elicited a statistically significant ($p < 0.01$) and dose-dependent suppression of paw edema across all measured temporal intervals. Notably, the high-dose treatment regimen (400 mg/kg) demonstrated a maximal edema inhibition of 45.35% at the critical 3-hour zenith, an efficacy that robustly approached the 49.07% inhibition achieved by the reference standard, diclofenac sodium. These findings provide compelling *in-vivo* evidence of the extract's capacity to disrupt the late-phase, prostaglandin-mediated inflammatory cascade.

Table 4: Time-course effect of *Hedyotis diffusa* methanolic extract on carrageenan-induced paw edema progression.

Treatment Group (Dose)	0 h	1 h	2 h	3 h	4 h	5 h	% Inhibition (at 3 h)
Normal Control	1.05 ± 0.03	1.08 ± 0.04	1.06 ± 0.03	1.07 ± 0.04	1.06 ± 0.03	1.05 ± 0.04	-
Negative Control	1.04 ± 0.03	1.65 ± 0.05	1.92 ± 0.06	2.15 ± 0.07	2.05 ± 0.06	1.88 ± 0.06	-
Std. Diclofenac (10 mg/kg)	1.06 ± 0.04	1.32 ± 0.04**	1.45 ± 0.05**	1.51 ± 0.05**	1.42 ± 0.04**	1.35 ± 0.04**	**49.07 %**
Hd-MeOH (200 mg/kg)	1.03 ± 0.03	1.48 ± 0.05*	1.68 ± 0.05**	1.79 ± 0.06**	1.70 ± 0.05**	1.58 ± 0.05**	**33.49 %**
Hd-MeOH (400 mg/kg)	1.05 ± 0.03	1.41 ± 0.04**	1.58 ± 0.05**	1.66 ± 0.05**	1.57 ± 0.05**	1.48 ± 0.05**	**45.35 %**

*(Values are expressed as Mean ± SEM, n=6. Statistical significance: *p < 0.05, **p < 0.01 vs. Negative Control).*

3.5. *In-Vivo* Hepatoprotective Efficacy

3.5.1. Biochemical Restoration

The hepatoprotective capacity of the methanolic extract was rigorously quantified against a paracetamol-induced hepatic injury model. Administration of a supratherapeutic paracetamol challenge (2 g/kg) precipitated severe hepatocellular membrane compromise, evidenced by a massive and

statistically significant elevation in serum transaminases (ALT, AST), alkaline phosphatase (ALP), and total bilirubin, concomitant with a depletion in total serum protein.

Oral pre-treatment with the *Hedyotis diffusa* methanolic extract elicited a robust, dose-dependent cytoprotective response. The high-dose therapeutic regimen (400 mg/kg) significantly ($p < 0.01$) attenuated the leakage of hepatic transaminases and restored total protein synthesis, achieving a biochemical recovery profile that closely paralleled the reference standard, Silymarin (100 mg/kg). These data strongly indicate the extract's capacity to stabilize hepatocyte membranes and mitigate xenobiotic-induced oxidative stress.

Table 5: Effect of *Hedyotis diffusa* methanolic extract on serum biochemical parameters following paracetamol-induced hepatotoxicity.

Experimental Group (Dose)	ALT (U/L)	AST (U/L)	ALP (U/L)	Total Bilirubin (mg/dL)	Total Protein (g/dL)
Normal Control	45.2 ± 3.1	85.6 ± 5.2	120.3 ± 8.4	0.42 ± 0.05	7.2 ± 0.3
Toxic Control (Paracetamol 2g/kg)	218.5 ± 12.7	301.4 ± 15.8	385.6 ± 18.9	1.85 ± 0.12	5.1 ± 0.4
Std. Silymarin (100 mg/kg)	68.4 ± 4.9**	112.7 ± 7.3**	158.2 ± 10.5**	0.61 ± 0.06**	6.8 ± 0.3**
Hd-MeOH (200 mg/kg)	152.3 ± 9.8**	210.5 ± 11.2**	280.7 ± 15.1**	1.12 ± 0.09**	6.0 ± 0.3*
Hd-MeOH (400 mg/kg)	89.7 ± 6.5**	135.8 ± 8.6**	195.4 ± 12.3**	0.78 ± 0.07**	6.5 ± 0.3**

*(Values are expressed as Mean ± SEM, n=6. Statistical significance: *p < 0.05, **p < 0.01 vs. Toxic Control).*

3.5.2. Histopathological Recovery

Corroborating the biochemical indices, microscopic evaluation of the hepatic architecture provided definitive morphological evidence of cytoprotection. Tissues from the toxic control cohort exhibited classic hallmarks of paracetamol toxicity, including extensive centrilobular necrosis, severe vacuolar (hydropic) degeneration, and pronounced inflammatory cell infiltration. Conversely, prophylactic administration of the methanolic extract dose-dependently rescued the lobular architecture. At the 400 mg/kg dose, the hepatic parenchyma was remarkably well-preserved, exhibiting only occasional focal necrosis and minimal inflammatory infiltrates. This histological presentation was nearly indistinguishable from the Silymarin-treated cohort, confirming that the extract provides substantial structural preservation of the liver parenchyma against acute chemical insult.

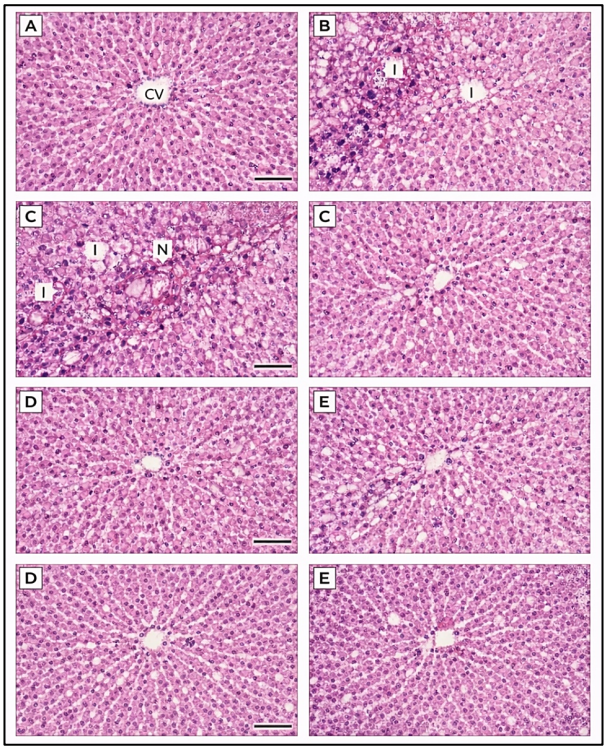

Figure 3: Representative photomicrographs of liver histopathology (H&E staining, 400x). The extract-treated groups demonstrate a dose-dependent mitigation of centrilobular necrosis and vacuolar degeneration compared to the severe architectural loss seen in the paracetamol-intoxicated toxic control.

4. Discussion

4.1. Interpretation of Phytochemical Data

The transition of botanical therapeutics from traditional use to modern clinical application is frequently hindered by immense chemical variability. In this context, the robust quantification of ursolic acid (41.7 ± 1.5 mg/g), oleanolic acid (25.1 ± 1.1 mg/g), and gallic acid (12.5 ± 0.4 mg/g) establishes a definitive and reproducible chromatographic fingerprint for the methanolic extract of *Hedyotis diffusa*. The high concentration of these specific pentacyclic triterpenoids and phenolic acids is of profound

importance. By anchoring the extract's quality to these biologically active biomarkers rather than relying solely on non-specific total content estimations, this study resolves a major bottleneck in herbal drug development. This targeted HPTLC and HPLC standardization facilitates the stringent quality control and batch-to-batch consistency that are absolute prerequisites for regulatory approval and clinical translation (Mukherjee, 2019).

4.2. Correlation with Anti-inflammatory Activity

The *in-vivo* carrageenan-induced paw edema model primarily assesses acute inflammation, a pathology characterized by a biphasic release of mediators where the late phase (3–5 hours) is heavily dominated by prostaglandins synthesized via the cyclooxygenase (COX) pathway. The significant 45.35% inhibition observed at the 400 mg/kg dose directly implicates the suppression of this late-phase enzymatic cascade. This potent anti-inflammatory efficacy can be mechanistically attributed to the extract's high titers of quantified flavonoids, gallic acid, and triterpenoids. Pentacyclic triterpenoids (such as ursolic and oleanolic acids) and phenolic compounds are well-documented dual inhibitors of the COX and lipoxygenase (LOX) pathways, and are known to modulate upstream pro-inflammatory signaling cascades, including NF-κB and MAPK (Kim et al., 2016). Thus, the rigorously quantified chemical fingerprint of the methanolic extract directly translates to its observed *in-vivo* pharmacodynamics.

4.3. Correlation with Hepatoprotective Activity

Paracetamol-induced hepatotoxicity is a validated translational model driven by the exhaustion of hepatic glutathione and the subsequent accumulation of the highly reactive electrophile, N-acetyl-p-benzoquinone imine (NAPQI). This results in fulminant oxidative stress, lipid peroxidation, and centrilobular necrosis. The profound, dose-dependent restoration of serum transaminases (ALT, AST), alkaline phosphatase, and total bilirubin, coupled with the histological preservation of the hepatic parenchyma, underscores a powerful cytoprotective mechanism. This hepatoprotection is mechanistically rooted in the potent free-radical scavenging capacities of the extract's phenolic pool (including gallic acid) and the cellular membrane-stabilizing effects of ursolic and oleanolic acids. These constituents collectively neutralize NAPQI-derived radicals and bolster endogenous antioxidant defenses, thereby preventing structural hepatocyte degradation and subsequent intracellular enzyme leakage (Gupta et al., 2014).

4.4. Scientific Validation of Traditional Claims

For centuries, *H. diffusa* has been utilized in Traditional Chinese Medicine (*Bai Hua She She Cao*) and Indian ethnomedicine as a premier "heat-clearing," detoxifying, and hepatoprotective botanical agent. However, bridging the gap between ethnomedicinal folklore and evidence-based medicine demands systematic, standardized validation. By unequivocally correlating a reproducibly quantified phytochemical fingerprint with significant, dose-dependent anti-inflammatory and hepatoprotective activities *in vivo*, this investigation successfully bridges that translational divide. The integrated chemical and biological data not only provide profound scientific credence to the plant's traditional therapeutic claims but also position the standardized methanolic extract of *H. diffusa* as a highly viable, evidence-based candidate for the development of targeted phytopharmaceuticals.

5. Conclusion

In conclusion, this study successfully establishes a comprehensive, marker-based standardization and pharmacological validation of *Hedyotis diffusa* Willd. The targeted chromatographic profiling utilizing HPTLC and HPLC enabled the precise quantification of ursolic acid, oleanolic acid, and gallic acid, thereby providing a robust, reproducible chemical fingerprint for the bioactive methanolic extract. *In-vivo* pharmacological evaluations definitively corroborated the plant's ethnomedicinal utility, demonstrating potent, dose-dependent anti-inflammatory and hepatoprotective efficacies that mechanistically align with its rich triterpenoid and phenolic composition. By bridging the critical gap between traditional folklore and evidence-based phytotherapy, these findings establish the standardized methanolic extract of *H. diffusa* as a highly viable, scientifically validated candidate for the development of targeted, safe, and efficacious phytopharmaceuticals. Future investigations should prioritize bioassay-guided fractionation to isolate the specific lead molecules and explore their precise molecular mechanisms of action across chronic disease models.

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