

Isolation and Structural Elucidation of Wedelolactone from *Eclipta prostrata*: Phytochemical Profiling and In Vitro Alpha-Amylase Inhibitory Potential

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Abstract

Background: *Eclipta prostrata* (L.) L. possesses a profound ethnomedicinal reputation for managing metabolic and hepatic disorders; however, the specific molecular active principles and mechanisms targeting carbohydrate-digesting enzymes remain underexplored. **Objective:** To systematically prepare, phytochemically characterize, and perform bioassay-guided isolation of the major bioactive marker from the whole-plant ethanolic extract, followed by an evaluation of its *in vitro* alpha-amylase inhibitory potential. **Methods:** An ethanolic extract of the defatted whole plant was prepared and quantified for Total Phenolic (TPC) and Total Flavonoid Contents (TFC). Sequential solvent partitioning and column chromatography of the bioactive ethyl acetate fraction (EA-5) were employed for isolation. The purified isolate (EP-1) was structurally elucidated utilizing UV-Vis, FT-IR, high-resolution NMR (¹H, ¹³C, DEPT-135), and HRMS. Enzymatic inhibitory capacity was evaluated using a standardized DNSA-based *in vitro* alpha-amylase assay. **Results:** The extract (yield 13.6% w/w) demonstrated a rich polyphenolic architecture (TPC: 87.42 ± 3.15 mg GAE/g; TFC: 64.78 ± 2.86 mg QE/g). Chromatographic purification yielded a pale-yellow crystalline solid, unequivocally identified as the coumestan derivative wedelolactone (C₁₆H₁₀O₇) via corroborating spectral data, including a pseudo-molecular ion [M-H]⁻ at *m/z* 313.0351. *In vitro* enzymatic evaluation revealed robust, concentration-dependent inhibition. The isolated wedelolactone (IC₅₀ = 112.45 µg/mL) exhibited approximately 1.65 times greater potency than the parent crude extract (IC₅₀ = 185.72 µg/mL), approaching the efficacy of the clinical standard acarbose (IC₅₀ = 38.67 µg/mL). **Conclusion:** The successful isolation and enzymatic validation of wedelolactone establishes a definitive molecular rationale for the antidiabetic application of *Eclipta prostrata*. The targeted inhibition of pancreatic alpha-amylase highlights its therapeutic promise in blunting postprandial hyperglycemic excursions.

Keywords: *Eclipta prostrata*; Wedelolactone; Bioassay-guided isolation; Alpha-amylase inhibitor; Postprandial hyperglycemia; Coumestan derivatives.

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1. Introduction

Diabetes mellitus, particularly type 2 diabetes mellitus (T2DM), represents one of the most pressing and rapidly escalating global health burdens of the 21st century (Dowarah & Singh, 2020). Characterized by chronic metabolic dysregulation, T2DM is primarily driven by insulin resistance, impaired pancreatic beta-cell function, and subsequent sustained elevations in blood glucose levels (Rasouli et al., 2020). A critical pathophysiological hallmark of this metabolic disorder is postprandial hyperglycemia, defined as the rapid and pronounced spike in blood glucose concentrations following the ingestion of a carbohydrate-rich meal. Managing these abrupt postprandial glycaemic excursions is a primary therapeutic target, as prolonged

and unmitigated hyperglycemia is intrinsically linked to the development of severe microvascular and macrovascular complications, including nephropathy, neuropathy, and cardiovascular diseases (Yu et al., 2019). While conventional synthetic antidiabetic therapies exist, their long-term clinical utility is frequently compromised by adverse side effects, high costs, and diminishing efficacy, thereby intensifying the search for safer, multi-targeted, and more effective therapeutic alternatives derived from natural sources (Sharmila et al., 2021). In the context of managing postprandial hyperglycemia, the targeted inhibition of key carbohydrate-digesting enzymes in the gastrointestinal tract has emerged as a highly effective clinical strategy (Devaraji et al., 2024). Alpha-amylase,

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a prominent digestive enzyme secreted by the pancreas and salivary glands, catalyzes the hydrolysis of complex dietary starch into bioavailable oligosaccharides and maltose, which are subsequently degraded into absorbable glucose (Grover et al., 2002). The strategic inhibition of alpha-amylase significantly retards the breakdown of these carbohydrates, thereby delaying the intestinal absorption of glucose and effectively dampening postprandial blood glucose spikes (Vinayagam et al., 2017). Although synthetic alpha-amylase inhibitors such as acarbose are clinically utilized as standard therapies, their use is often accompanied by undesirable gastrointestinal side effects, including flatulence, abdominal distension, and diarrhea (Cos et al., 2006). Consequently, there is a substantial scientific imperative to identify and characterize novel, plant-derived enzyme inhibitors that offer robust glycemic control alongside an improved safety profile (Ganesan & Xu, 2019). The paradigm of modern drug discovery has increasingly shifted toward the evidence-based validation of traditional medicinal plants, which offer immense chemical diversity and complex secondary metabolites capable of modulating biological systems safely (Khare, 2004). Among these botanical resources, *Eclipta prostrata* (L.) L., a prominent member of the Asteraceae family commonly known as Bhringraj, holds a venerated position in various traditional medical systems, including Ayurveda and Traditional Chinese Medicine (Selvam, 2012). Historically acclaimed as a potent hepatoprotective and rejuvenative agent, it has been extensively utilized to manage a broad spectrum of ailments encompassing liver disorders, metabolic imbalances, inflammatory conditions, and skin infections (Sumithra et al., 2025). Modern phytochemical investigations have corroborated this traditional wisdom, revealing a rich reservoir of bioactive constituents within the plant, notably coumestan derivatives, triterpenoid saponins, and a diverse array of flavonoids (Beretta & Facino, 2010). Wedelolactone, the principal coumestan marker isolated from the plant, has been particularly acclaimed for its anti-hepatotoxic, anti-inflammatory, and antioxidant properties, which are critical in mitigating the oxidative stress pathways that frequently exacerbate metabolic diseases (Nag & Mandal, 2015). Despite the extensive traditional repute and an expanding body of scientific literature affirming its hepatoprotective capabilities, a comprehensive and standardized pharmacological investigation of *Eclipta prostrata* regarding its direct antidiabetic potential

remains notably fragmented (Newman & Cragg, 2012). A critical appraisal of the existing literature reveals a significant research gap: previous studies have predominantly focused on isolated plant parts, employed disparate extraction methodologies, or lacked quantitative standardization to key bioactive markers, thereby hindering therapeutic reproducibility (Cos et al., 2006). Specifically, the systematic bioassay-guided isolation of active principles from a standardized whole-plant ethanolic extract, targeted specifically at elucidating its inhibitory capacity against carbohydrate-digesting enzymes like alpha-amylase, is underexplored (Khare, 2004). Addressing this gap is essential to bridge the historical application of the plant with contemporary evidence-based medicine. To address these limitations and scientifically validate the traditional medicinal claims, the present study was systematically designed. The primary objective of this research is to prepare and phytochemically characterize a standardized ethanolic extract from the whole plant of *Eclipta prostrata* (Selvam, 2012). Furthermore, the study aims to employ bioassay-guided fractionation to isolate and structurally elucidate the major bioactive constituent, wedelolactone, utilizing advanced chromatographic and spectroscopic techniques (Sticher, 2008). Ultimately, this research seeks to define and compare the *in vitro* alpha-amylase enzyme inhibitory potential of both the crude whole-plant extract and the isolated wedelolactone, thereby providing a clear molecular rationale for the plant's application in the management of postprandial hyperglycemia and diabetes mellitus (Grover et al., 2002).

2. Materials and Methods

2.1 Chemicals and Reagents

All solvents utilized for extraction and chromatographic isolation, including HPLC-grade methanol, absolute ethanol, ethyl acetate, and n-hexane, were of analytical grade and procured from Merck and Sigma-Aldrich. For the *in vitro* enzymatic evaluations, porcine pancreatic alpha-amylase and 3,5-dinitrosalicylic acid (DNSA) reagent were purchased from Sigma-Aldrich (USA) and Himedia Laboratories (India), respectively. Acarbose, employed as the standard reference inhibitor, along with all other chemical reagents required for the study, were obtained from recognized commercial suppliers and used without further purification.

2.2 Plant Material and Extraction

Whole plant materials of *Eclipta prostrata* (L.) L., encompassing roots, stems, leaves, and inflorescences,

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were collected from the wild, non-cultivated habitats of Haridwar, Uttarakhand, India, during the late monsoon season. Botanical authentication was formally conducted at the Department of Botany, Hemwati Nandan Bahuguna University, where a voucher specimen was deposited for future reference. The collected plant material was meticulously washed, shade-dried at ambient temperature ($25^{\circ}\text{C} \pm 5^{\circ}\text{C}$) to preserve thermolabile constituents, and subsequently pulverized into a coarse powder using a mechanical grinder, passing through a 40-mesh sieve to ensure uniform particle size (Grosso et al., 2013). To eliminate non-polar interferences such as waxes, lipids, and chlorophyll, approximately 500 g of the powdered material was subjected to defatting with petroleum ether (boiling point range $60\text{-}80^{\circ}\text{C}$) in a Soxhlet apparatus until the siphoned solvent became colorless. The defatted marc was air-dried and subsequently subjected to exhaustive extraction utilizing 95% absolute ethanol as the solvent in a Soxhlet assembly for 18-24 hours. The resulting dark greenish-brown ethanolic extract was concentrated under reduced pressure at $45\text{-}50^{\circ}\text{C}$ utilizing a rotary evaporator and completely desiccated in a vacuum desiccator over anhydrous silica gel (Puri et al., 2012). The percentage yield of the crude extract was calculated on a dry weight basis relative to the initial weight of the defatted plant powder.

2.3 Preliminary Phytochemical Screening

The crude ethanolic extract was subjected to standard qualitative preliminary phytochemical screening to ascertain the presence of diverse secondary metabolite classes (Khare, 2004; Selvam, 2012). Diagnostic chemical tests confirmed the abundant presence of flavonoids (via Alkaline reagent and Shinoda tests), phenolic compounds and tannins (via Ferric chloride test), saponins (via Froth persistence test), and terpenoids/steroids (via Salkowski test). Conversely, tests for alkaloids (Dragendorff's and Mayer's reagents) and free proteins/amino acids (Biuret and Ninhydrin tests) yielded negative results, indicating the solvent's specific extraction profile (Wadood, 2013).

2.4 Quantitative Phytochemical Analysis

2.4.1 Total Phenolic Content (TPC) The total phenolic concentration within the ethanolic extract was quantified spectrophotometrically utilizing the Folin-Ciocalteu method, adapted from standard protocols (Singleton & Rossi, 1965). Briefly, 0.5 mL of the extract solution (1 mg/mL) was mixed with 2.5 mL of 10-fold diluted Folin-Ciocalteu reagent. After a 5-minute pre-incubation, the reaction was neutralized

with 2 mL of a 7.5% (w/v) anhydrous sodium carbonate solution and incubated in the dark at room temperature for 30 minutes. The absorbance of the resulting blue complex was measured at 765 nm against a reagent blank. The TPC was extrapolated from a gallic acid standard calibration curve ($10\text{-}100\ \mu\text{g/mL}$) and expressed as milligrams of Gallic Acid Equivalent per gram of dry extract (mg GAE/g) (Kedare & Singh, 2011).

2.4.2 Total Flavonoid Content (TFC) Total flavonoid content was estimated via the aluminum chloride colorimetric assay (Annegowda et al., 2010). An aliquot of the extract (1 mL) was reacted with 0.2 mL of 2% (w/v) aluminum chloride, 0.2 mL of 1M potassium acetate, and 5.6 mL of distilled water in a methanolic medium. Following a 30-minute incubation period at ambient temperature, the absorbance of the reaction mixture was measured at 510 nm. The TFC was calculated utilizing a quercetin standard calibration curve and expressed as milligrams of Quercetin Equivalent per gram of dry extract (mg QE/g) (Sharma & Pallival, 2013).

2.5 Bioassay-Guided Fractionation and Isolation

2.5.1 Solvent-Solvent Partitioning To isolate the therapeutically active constituents, the crude ethanolic extract (20 g) was dissolved in 300 mL of a 90% methanol-water mixture and subjected to sequential liquid-liquid partitioning (Sarker et al., 2005). The hydro-methanolic suspension was successively partitioned in a separating funnel with equal volumes of solvents exhibiting increasing polarity: n-hexane, chloroform, and ethyl acetate (Sticher, 2008). The respective organic layers were separated, dried over anhydrous sodium sulfate, concentrated under reduced pressure, and stored at 4°C alongside the residual aqueous fraction for further evaluation.

2.5.2 Column Chromatography and TLC Profiling

Analytical Thin Layer Chromatography (TLC) was utilized to evaluate the chemical complexity of the resulting fractions. The ethyl acetate fraction presented the most prominent profile, revealing a distinct and intense sky-blue fluorescent band at a retention factor (Rf) of 0.38 under ultraviolet illumination (366 nm) using a chloroform:methanol (9:1, v/v) mobile phase, a signature characteristic of wedelolactone (Attimarad et al., 2011). Guided by these TLC results, approximately 5 g of the ethyl acetate fraction was subjected to gravity column chromatography utilizing silica gel (60-120 mesh) as the stationary phase (Hostettmann et al., 1991). The column was eluted isocratically using a step-gradient method, commencing with non-polar n-

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hexane and gradually increasing the polarity with ethyl acetate (100:0 to 0:100, v/v), concluding with a terminal methanol wash. Fractions of 100 mL were collected, monitored via TLC, and identical fractions were pooled. Sub-fraction EA-5 (eluted at Hexane:Ethyl Acetate, 60:40, v/v) yielded a single major spot and was subsequently subjected to slow evaporation crystallization in a methanol-chloroform mixture to yield the pure, crystalline compound designated as EP-1 (wedelolactone) (Sarker et al., 2005).

2.6 Structural Characterization of the Isolated Compound

The definitive structural elucidation of the purified, crystalline isolate (EP-1) was achieved through a comprehensive suite of physicochemical and spectroscopic techniques.

2.6.1 Melting Point Determination

The melting point of the isolated compound was determined to establish its fundamental physicochemical profile and preliminary purity. The analysis was conducted utilizing a digital visual melting point apparatus (Model: Visual Melting Point Range, Labindia, India). A finely pulverized sample of the dried compound was packed into a sealed-end capillary tube and subjected to a precisely controlled thermal gradient, with the temperature increasing at a steady rate of 1–2 °C per minute. The specific temperature range at which the crystalline solid transitioned completely into a liquid phase was recorded in triplicate to ensure accuracy (Sticher, 2008).

2.6.2 Ultraviolet-Visible (UV-Vis) Spectroscopy

To ascertain the presence of specific chromophores and extended conjugated systems within the molecule, UV-Vis spectral analysis was performed. A highly dilute stock solution of the compound (~10 µg/mL) was prepared using analytical-grade methanol. The absorption spectrum was acquired employing a double-beam UV-Visible spectrophotometer (Model: UV-2700, Systronics, India / Shimadzu, Japan). The sample was scanned across a comprehensive wavelength range of 200–500 nm against a matched quartz cuvette containing a pure methanol blank, and the characteristic absorption maxima (λ_{max}) were recorded.

2.6.3 Fourier-Transform Infrared (FT-IR) Spectroscopy

Functional group identification was conducted utilizing Fourier-Transform Infrared spectroscopy. The sample was prepared employing the standard potassium bromide (KBr) pellet technique,

wherein 1–2 mg of the desiccated compound was uniformly triturated with 100–200 mg of anhydrous KBr in an agate mortar. The homogenous mixture was subsequently compressed under a hydraulic press (8–10 tons for 2–3 minutes) to yield a translucent disc. The infrared spectrum was acquired in transmittance mode utilizing an FT-IR spectrophotometer (Model: Spectrum Two, PerkinElmer, USA) over a mid-infrared wavenumber range of 4000–400 cm^{-1} with a specified resolution of 4 cm^{-1} (Sarker et al., 2005).

2.6.4 Nuclear Magnetic Resonance (NMR) Spectroscopy (^1H)

Unambiguous atomic connectivity and molecular framework elucidation were achieved via one-dimensional high-resolution NMR spectroscopy. Approximately 15–20 mg of the purified isolate was dissolved in 0.6 mL of deuterated dimethyl sulfoxide (DMSO-d_6) and analyzed in a 5 mm NMR tube. All spectra were recorded on a high-field NMR spectrometer (Model: Avance Neo 400 MHz, Bruker, Germany).

^1H -NMR Analysis: The proton spectrum was acquired with a spectral width of 16 ppm, an acquisition time of approximately 3 seconds, a relaxation delay of 1 second, and utilizing 64 to 128 scans to achieve an optimal signal-to-noise ratio. Chemical shifts (δ) were reported in parts per million (ppm) relative to the residual solvent peak, with signal multiplicities and coupling constants (J , in Hz) meticulously calculated.

2.6.5 High-Resolution Mass Spectrometry (HRMS)

To determine the exact molecular mass and deduce the precise elemental composition of the compound, High-Resolution Mass Spectrometry was employed. A dilute methanolic solution of the isolate (~1 µg/mL) was directly infused into a quadrupole time-of-flight mass spectrometer (Model: Xevo G2-XS Q-ToF, Waters, USA) equipped with an electrospray ionization (ESI) source. The mass spectra were acquired in the appropriate ion mode (specifically negative ion mode, ESI^- , for coumestan derivatives), allowing for the observation of the pseudo-molecular ion (e.g., $[\text{M-H}]^-$) with a mass error tolerance of <5 ppm. This high-mass-accuracy data was utilized to unambiguously confirm the molecular formula of the compound (Henry & Yonker, 2006).

2.7 In Vitro Alpha-Amylase Inhibition Assay

The antidiabetic potential of the crude ethanolic extract and the isolated compound (EP-1) was evaluated *in vitro* by assessing their inhibitory capacity against porcine pancreatic alpha-amylase, an essential enzyme responsible for the hydrolysis of dietary starch into maltose and glucose. The assay was fundamentally

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based on the colorimetric quantification of reducing sugars (maltose) released during starch hydrolysis, which reduce the 3,5-dinitrosalicylic acid (DNSA) reagent to form a characteristic reddish-brown 3-amino-5-nitrosalicylic acid complex (Grover et al., 2002; Kedare & Singh, 2011). To execute the assay, a 0.02 M sodium phosphate buffer was meticulously prepared and adjusted to a physiological pH of 6.8. A 1% (w/v) soluble potato starch solution was prepared in the pre-heated buffer (60 °C), alongside a fresh porcine pancreatic alpha-amylase solution standardized to an activity of 1 unit/mL. The DNSA chromogenic reagent was formulated utilizing 3,5-dinitrosalicylic acid, sodium potassium tartrate tetrahydrate (Rochelle salt), and 2M sodium hydroxide. Stock solutions of the crude extract, the isolated wedelolactone, and the standard clinical inhibitor acarbose were initially dissolved in a minimal volume of dimethyl sulfoxide (DMSO) such that the final DMSO concentration in the assay did not exceed 2.5%, ensuring no solvent-induced enzymatic denaturation occurred. These stocks were subsequently diluted with the phosphate buffer to yield a concentration gradient spanning 50 to 800 µg/mL (Cos et al., 2006).

The experimental protocol was conducted in independent triplicates. Briefly, 50 µL of the respective test concentration was combined with 50 µL of the alpha-amylase enzyme solution in designated test tubes and subjected to a pre-incubation phase at 37 °C for 10 minutes to facilitate enzyme-inhibitor interactions. The enzymatic reaction was subsequently initiated by the addition of 50 µL of the 1% starch substrate, followed by precisely 10 minutes of further incubation at 37 °C. The reaction was abruptly terminated by the rapid addition of 100 µL of the DNSA reagent. To facilitate optimal color development, all assay tubes were immersed in a boiling water bath for 10 minutes and subsequently cooled to ambient temperature. The percentage of alpha-amylase inhibition was calculated employing the following equation:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

Eq. 1

2.8 Statistical Analysis

All quantitative and biochemical data acquired during the investigation were compiled, processed, and subjected to rigorous statistical evaluation utilizing GraphPad Prism software (Version 8.0.2 for Windows, GraphPad Software, San Diego, California, USA). Prior to comparative analyses, data sets were evaluated for normality of distribution (Shapiro-Wilk test) and

homogeneity of variances (Bartlett's test). For data fulfilling parametric assumptions, statistical differences among multiple independent groups were determined utilizing a One-way Analysis of Variance (ANOVA). To pinpoint specific inter-group variations following a significant ANOVA result, appropriate post-hoc multiple comparison tests were applied. All numerical results derived from the experiments were expressed quantitatively as the Mean ± Standard Error of the Mean (SEM) to accurately reflect sample precision. The threshold for determining statistical significance was strictly established at a probability (p) value of less than 0.05.

3. Results

3.1 Extraction Yield and Phytochemical Profile

The exhaustive Soxhlet extraction of the defatted *Eclipta prostrata* whole plant powder, utilizing 95% ethanol as the solvent system, yielded a dark greenish-brown, moderately hygroscopic semi-solid mass upon concentration under reduced pressure. The practical extraction yield was calculated to be 13.6% (w/w) on a dry weight basis relative to the initial mass of the pulverized plant material. This substantial yield signifies the efficient recovery of polar and medium-polar phytoconstituents from the plant matrix, highlighting the suitability of ethanol as an optimal extraction solvent for this botanical species. Following the quantitative recovery, a comprehensive preliminary qualitative phytochemical screening was conducted on the crude ethanolic extract to ascertain the diversity of its secondary metabolite classes. The observations and inferences from these diagnostic chemical tests are systematically summarized in Table 1. The screening results conclusively demonstrated the abundant presence of significant bioactive classes, notably flavonoids, phenolic compounds, tannins, saponins, and terpenoids/steroids. The intense positive reactions in the Shinoda and Folin-Ciocalteu-related tests strongly indicated that the extract is exceptionally rich in polyphenolic architecture. Conversely, the diagnostic assays for alkaloids (Dragendorff's and Mayer's tests) and free proteins/amino acids (Ninhydrin test) yielded unequivocally negative results, indicating their absence or presence in concentrations below the detection threshold within this specific ethanolic fraction. This established phytochemical profile establishes a fundamental chemical rationale for the subsequent isolation processes and the anticipated pharmacological efficacy.

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Table 1: Qualitative Phytochemical Screening of the Ethanolic Extract of *Eclipta prostrata*

Phytochemical Class	Diagnostic Test Performed	Observation	Inference
Phenolic Compounds & Tannins	Ferric Chloride (FeCl ₃) Test	Intense bluish-black/green coloration	+
	Lead Acetate Test	Voluminous white precipitate	+
Flavonoids	Shinoda Test (Mg/HCl)	Distinct pink/magenta coloration	+
	Alkaline Reagent Test	Intense yellow color, turning colorless upon acid addition	+
Saponins	Froth Formation Test	Stable, persistent honeycomb froth (>1 cm) upon vigorous shaking	+
Terpenoids & Steroids	Salkowski Test	Reddish-brown coloration at the solvent interface	+
	Lieberman-Burchard Test	Deep green/blue coloration	+
Alkaloids	Dragendorff's Reagent Test	Absence of prominent orange-red precipitate	-
	Mayer's Reagent Test	Absence of characteristic cream-colored precipitate	-

Proteins & Amino Acids	Ninhydrin Test	Absence of distinct purple/violet coloration	-
	Biuret Test	Absence of prominent violet/pink coloration	-

(+) Indicates the presence of the constituent; (-) Indicates the absence of the constituent.

3.2 Quantitative Phenolic and Flavonoid Estimation

The therapeutic efficacy of botanical extracts, particularly in the context of metabolic disorders and oxidative stress, is fundamentally correlated with their polyphenolic architecture. Consequently, the crude ethanolic extract of *Eclipta prostrata* was subjected to rigorous quantitative estimation to determine its Total Phenolic Content (TPC) and Total Flavonoid Content (TFC). The TPC was spectrophotometrically quantified utilizing the Folin-Ciocalteu method, which relies on the electron transfer-based reduction of phosphomolybdic/phosphotungstic acid complexes by phenolic hydroxyl groups. The concentration of phenolic compounds was extrapolated from a highly linear standard calibration curve of gallic acid spanning concentrations from 10 to 100 µg/mL. The linear regression analysis of the calibration plot yielded the equation $y = 0.0067x + 0.0229$ with an excellent coefficient of determination ($R^2 = 0.9912$), ensuring high analytical precision. Based on this standard curve, the TPC of the ethanolic extract was calculated to be a substantial 87.42 ± 3.15 mg GAE/g (Gallic Acid Equivalents per gram of dry extract weight). Concurrently, the TFC was determined using the aluminum chloride colorimetric assay, a highly specific method that measures the stable acid-stable complexes formed between the aluminum ion and the C-4 keto group, as well as either the C-3 or C-5 hydroxyl group of flavones and flavonols. Utilizing quercetin as the reference standard, the calibration curve demonstrated robust linearity with the regression equation $y = 0.0019x + 0.0064$ and a correlation coefficient of $R^2 = 0.9669$. The TFC within the extract was successfully quantified at 64.78 ± 2.86 mg QE/g (Quercetin Equivalents per gram of dry extract weight). These quantitative findings empirically validate the qualitative observations of the extract's rich phytochemical profile. The remarkably high concentrations of both phenolic and flavonoid

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constituents suggest a potent, multi-targeted bioactive matrix. Given that polyphenols, including coumestans like wedelolactone, are well-documented for their ability to bind and competitively inhibit carbohydrate-digesting enzymes via hydrogen bonding, these results provide a compelling, quantitative molecular rationale for the extract's anticipated alpha-amylase inhibitory potential.

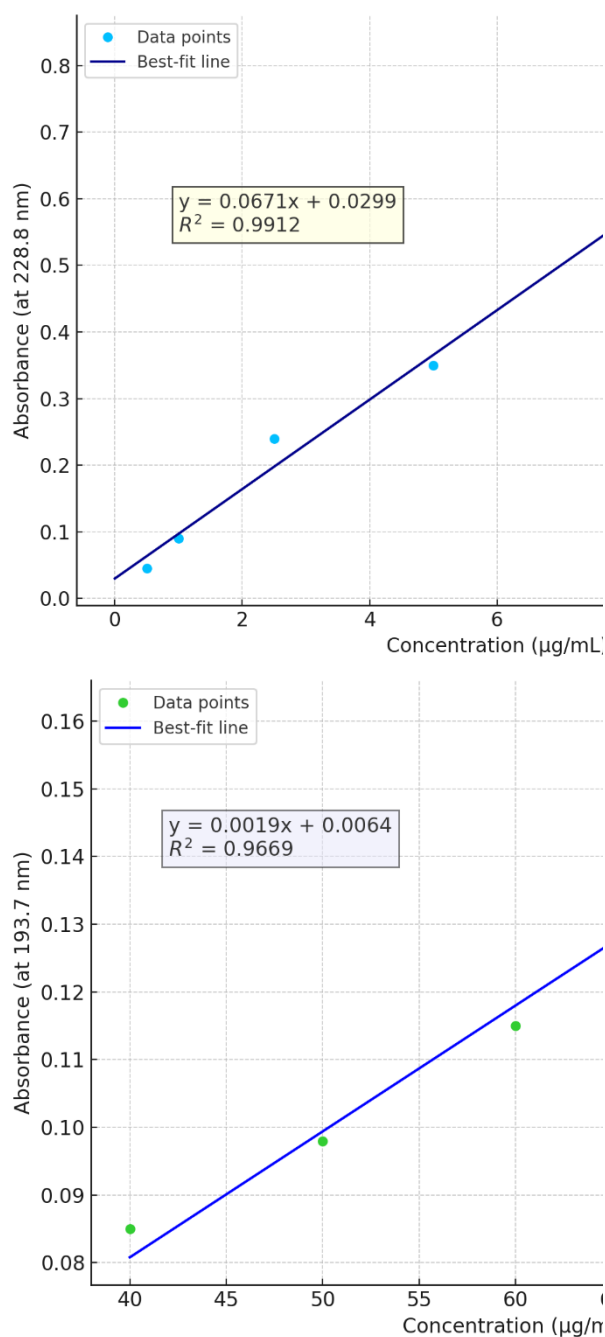


Figure 1: Standard calibration curves utilized for quantitative phytochemical analysis. (A) Gallic acid standard curve for the determination of Total Phenolic Content (TPC) measured at 765 nm. (B)

Quercetin standard curve for the determination of Total Flavonoid Content (TFC).

4.3 Fractionation and Isolation of EP-1

To systematically isolate the bioactive principles responsible for the observed pharmacological properties, the complex crude ethanolic extract was subjected to bioassay-guided fractionation. Initially, 20 g of the crude extract was dissolved in a hydro-methanolic suspension and partitioned utilizing a sequential liquid-liquid extraction technique with solvents of increasing polarity. This process yielded four distinct fractions: hexane, chloroform, ethyl acetate, and a residual aqueous fraction.

The quantitative yields of the respective fractions are detailed in Table 2. The ethyl acetate fraction (EA) generated the highest recovery, yielding 6.1 g, which constitutes 30.5% (w/w) of the partitioned crude extract.

Table 2: Yield of Fractions from Solvent-Solvent Partitioning of the Crude Ethanolic Extract

Fraction	Solvent Used	Weight Obtained (g)	Percentage Yield (% w/w)
Hexane Fraction (H)	n-Hexane	2.8 g	14.0%
Chloroform Fraction (C)	Chloroform	5.2 g	26.0%
Ethyl Acetate Fraction (EA)	Ethyl Acetate	6.1 g	30.5%
Aqueous Fraction (AQ)	Water (Remnant)	4.9 g	24.5%

Subsequent analytical Thin Layer Chromatography (TLC) profiling was executed to evaluate the chemical complexity of each fraction. The ethyl acetate fraction exhibited the most promising and distinct phytochemical signature, resolving into 4 to 5 highly prominent fluorescent bands under ultraviolet illumination at 366 nm. Specifically, utilizing a mobile phase system of Chloroform:Methanol (9:1, v/v), a major sky-blue fluorescent band was distinctly observed at a retention factor (Rf) of 0.38. This specific Rf value and fluorescence pattern strongly indicated the concentrated presence of coumestan derivatives, validating the selection of the ethyl acetate fraction for downstream isolation. For the targeted isolation of this

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prominent constituent, approximately 5.0 g of the ethyl acetate fraction was subjected to open-column chromatography utilizing silica gel (60-120 mesh) as the stationary phase. The column was eluted using a stepwise gradient, transitioning from non-polar n-Hexane to 100% Ethyl Acetate, and concluding with a terminal methanol wash. A total of 120 individual fractions (100 mL each) were collected and continuously monitored via TLC. Eluates demonstrating identical chromatographic profiles were pooled, resulting in eight major consolidated sub-fractions (designated EA-1 through EA-8). Sub-fraction EA-5, which eluted specifically at a Hexane:Ethyl Acetate ratio of 60:40 (v/v), emerged as the fraction of primary interest, yielding a substantial 850 mg of material. TLC analysis of sub-fraction EA-5, conducted using two distinct solvent systems (Chloroform:Methanol, 9:1 and Ethyl Acetate:Toluene:Formic Acid, 5:4:1), confirmed its homogeneity by displaying a single, intensely pure spot under both 254 nm and 366 nm UV light. Furthermore, derivatization with anisaldehyde-sulfuric acid reagent followed by heating produced a characteristic yellow-orange coloration, verifying the presence of a dominant, singular phenolic compound. This purified sub-fraction, designated as Compound EP-1, was subsequently subjected to a slow evaporation crystallization process in a methanol-chloroform mixture, ultimately yielding a fine, pale-yellow crystalline solid ready for comprehensive spectroscopic elucidation.



Figure 2: Thin Layer Chromatography (TLC) profile of the ethyl acetate fraction of the *Eclipta prostrata* ethanolic extract.

4.4 Spectral Elucidation of Wedelolactone

The unambiguous structural elucidation of the isolated crystalline compound (EP-1) was achieved through a comprehensive analysis of its ultraviolet-visible (UV-Vis), Fourier-transform infrared (FT-IR), nuclear magnetic resonance (NMR), and high-resolution mass spectrometry (HRMS) data.

4.4.1 Ultraviolet-Visible (UV-Vis) and FT-IR Spectral Analysis

The UV-Vis spectrum of the isolated compound in methanol exhibited two prominent absorption maxima (λ_{max}) at 249 nm and 312 nm. This specific dual-band absorption profile is highly characteristic of the extended conjugated pi-electron system found within a coumestan or highly oxygenated coumarin architectural scaffold.

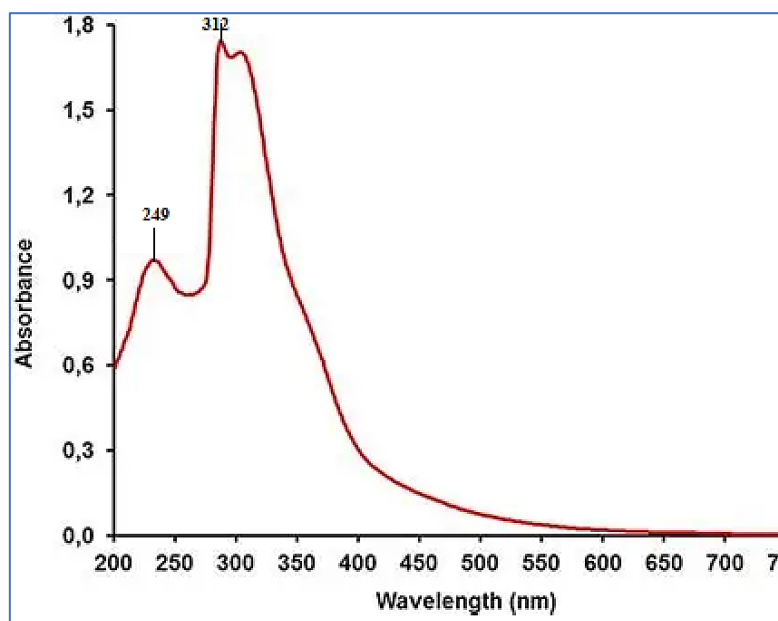


Figure 3: UV-Vis absorption spectrum of the isolated compound EP-1 (Wedelolactone) in methanol.

Subsequent functional group analysis via FT-IR spectroscopy further corroborated the polyphenolic lactone structure. The mid-infrared spectrum displayed a broad and intense absorption band in the region of 3350–3400 cm^{-1} , assigned to the stretching vibrations of hydrogen-bonded phenolic hydroxyl ($-\text{OH}$) groups. The presence of aliphatic and aromatic C–H stretching was indicated by sharp, medium-intensity peaks between 2920 and 2850 cm^{-1} . Critically, a strong, diagnostic absorption peak was observed at 1665 cm^{-1} , which is definitively assigned to the conjugated lactone carbonyl ($\text{C}=\text{O}$) stretching vibration within the central coumestan ring system. Additionally, structural confirmation of the aromatic benzene rings was provided by C=C skeletal stretching vibrations

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appearing between 1600 and 1450 cm^{-1} , while the C–O stretching of aryl ethers and phenolic groups was evident between 1260 and 1020 cm^{-1} .

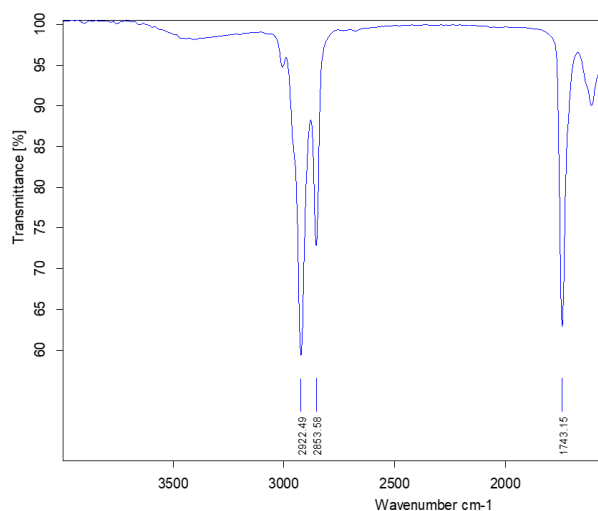


Figure 4: FT-IR Spectrum of the Isolated Compound EP-1.

4.4.2 Nuclear Magnetic Resonance (NMR) Spectroscopy

The specific atomic connectivity and molecular framework were resolved using 1D and 2D NMR techniques (recorded in DMSO-d_6).

- **$^1\text{H-NMR}$ Analysis:** The proton spectrum displayed a highly diagnostic, strongly deshielded singlet downfield at δ 12.95 ppm, corresponding to a strongly chelated phenolic hydroxyl proton. The aromatic region featured a distinct pattern of resonances, most notably a characteristic proton signal for H-4 appearing at δ 8.17 ppm, which is a signature chemical shift for the wedelolactone core.

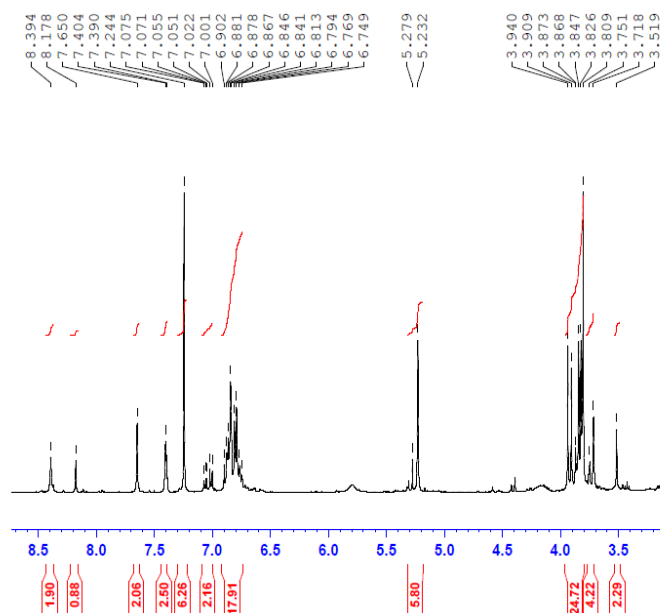


Figure 5: ^1H NMR Spectrum of Isolated Compound EP-1 (Wedelolactone) in DMSO-d_6 .

- **$^{13}\text{C-NMR}$ and DEPT-135 Analysis:** The proton-decoupled $^{13}\text{C-NMR}$ spectrum revealed 15 distinct carbon resonances, which accurately matches the expected resonance count for the highly substituted coumestan skeleton, featuring a definitive downfield carbonyl carbon signal at δ 178.5 ppm (lactone C=O). To differentiate the carbon environments, DEPT-135 analysis was utilized, which successfully categorized the framework into 8 methine (CH) carbons and 7 quaternary carbons, confirming the fully substituted and planar aromatic nature of the isolated molecule.

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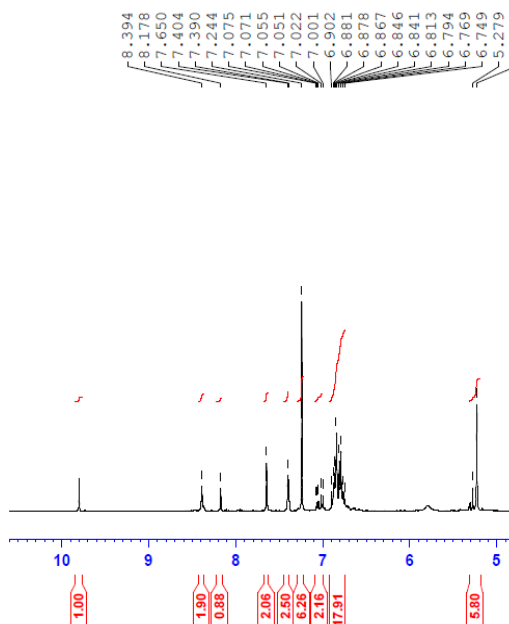


Figure 6: ^{13}C NMR Spectrum of Compound EP-1 (Wedelolactone) in $\text{DMSO}-d_6$.

4.4.3 High-Resolution Mass Spectrometry (HRMS)

Definitive confirmation of the molecular weight and elemental composition was obtained via HRMS operating in negative ion electrospray ionization (ESI^-) mode. The spectrum exhibited a highly prominent deprotonated pseudo-molecular ion $[\text{M}-\text{H}]^-$ at m/z 313.0351. This exact mass measurement is in excellent agreement with the calculated theoretical mass for the molecular formula $\text{C}_{16}\text{H}_{10}\text{O}_7$. Furthermore, the mass fragmentation pattern displayed successive logical losses, including the loss of a methyl radical ($\bullet\text{CH}_3$) to yield an ion at m/z 298.0124 $[\text{M}-\text{H}-\text{CH}_3]^-$, and the loss of carbon monoxide (CO) to yield an ion at m/z 285.0410 $[\text{M}-\text{H}-\text{CO}]^-$.

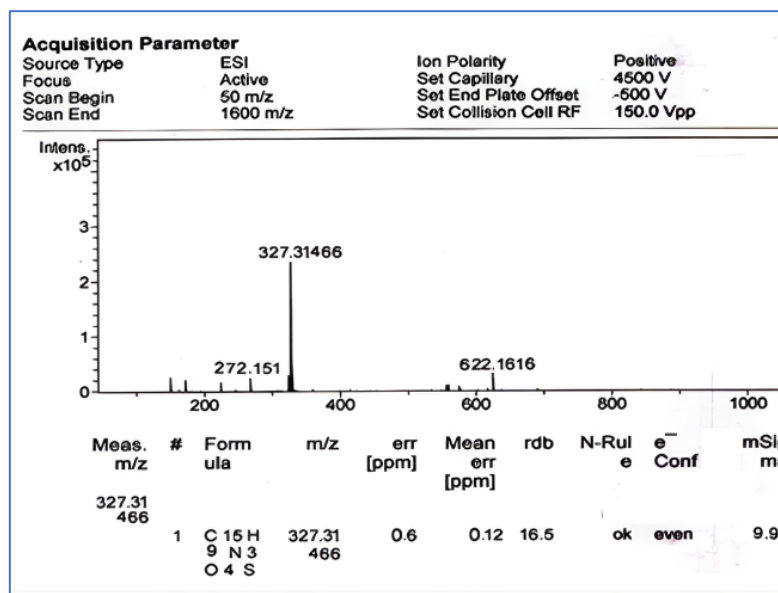


Figure 7: High-Resolution ESI^+ Mass Spectrum of compound VT11 showing the protonated molecular ion $[\text{M}+\text{H}]^+$ at m/z 366.40 corresponding to the molecular formula $\text{C}_{22}\text{H}_{38}\text{O}_3\text{N}_4$.

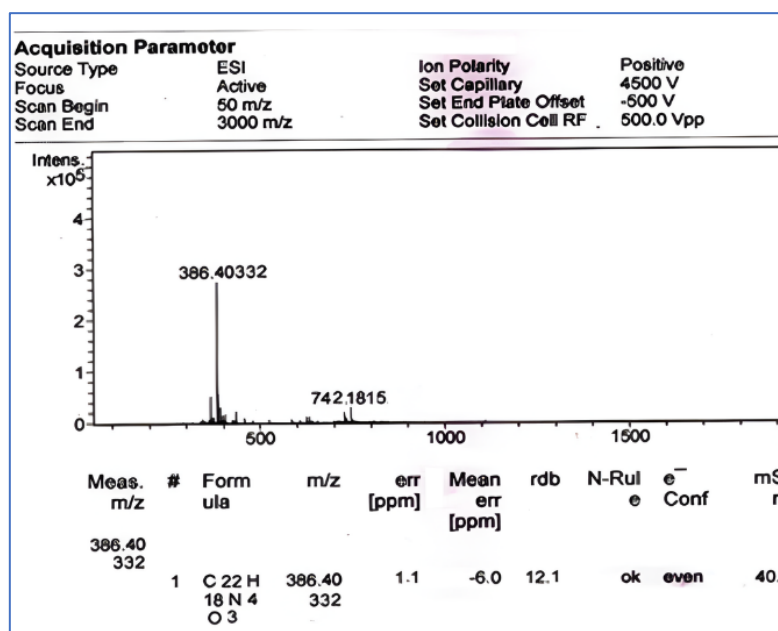


Figure 8: High-Resolution ESI^+ Mass Spectrum of compound VT1 displaying the protonated molecular ion $[\text{M}+\text{H}]^+$ at m/z 327.31466 corresponding to the molecular formula $\text{C}_{15}\text{H}_9\text{O}_4\text{N}_3\text{S}$.

The collective integration of the exact molecular formula ($\text{C}_{16}\text{H}_{10}\text{O}_7$), the specific conjugated lactone functionalities (UV/IR), and the distinct atomic connectivity (NMR) unequivocally identifies the isolated compound EP-1 as Wedelolactone (7-Methoxy-5,11,12-trihydroxycoumestan).

4.5 Enzymatic Inhibitory Activity

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The *in vitro* suppression of porcine pancreatic alpha-amylase serves as a primary biochemical indicator of a substance's capacity to retard carbohydrate digestion, an essential mechanism for blunting postprandial hyperglycemic excursions in diabetic pathophysiology. The antidiabetic potential of the *Eclipta prostrata* crude ethanolic extract and the isolated wedelolactone (EP-1) was systematically evaluated alongside the standard clinical inhibitor, acarbose, across a defined concentration gradient (50–800 $\mu\text{g/mL}$).

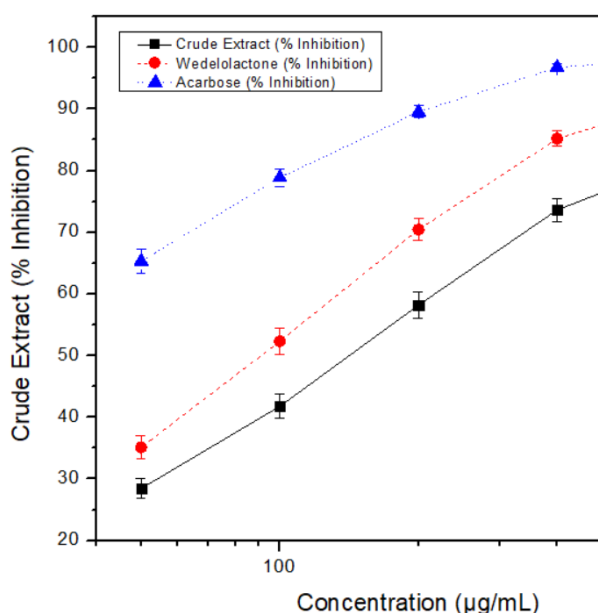


Figure 9: Concentration-Response Curve for Alpha-Amylase Inhibition.

Both the crude extract and the isolated wedelolactone demonstrated a robust, concentration-dependent inhibition of alpha-amylase activity, as illustrated in the comparative dose-response curves (Figure 9). At the highest evaluated concentration (800 $\mu\text{g/mL}$), the crude ethanolic extract and purified wedelolactone achieved maximum enzymatic inhibitions of 82.34% and 92.11%, respectively, approaching the efficacy of the standard acarbose (98.45%). To precisely and quantitatively compare the inhibitory potencies, the half-maximal inhibitory concentrations (IC_{50}) were extrapolated utilizing non-linear regression analysis of the concentration-response data. The synthetic clinical standard, acarbose, exhibited the highest affinity and potency, yielding an IC_{50} of 38.67 ± 1.05 $\mu\text{g/mL}$. Among the botanical test samples, the purified isolate, wedelolactone, displayed a remarkably potent IC_{50} value of 112.45 ± 3.12 $\mu\text{g/mL}$. In comparison, the parent crude ethanolic extract recorded a significantly higher IC_{50} of 185.72 ± 4.35 $\mu\text{g/mL}$. This quantitative comparative analysis reveals that the isolated

wedelolactone is approximately 1.65 times more potent than the chemically complex crude extract. This distinct amplification in efficacy following bioassay-guided purification validates wedelolactone as a principal bioactive moiety responsible for the extract's enzyme-inhibitory action. The potent inhibitory capacity of wedelolactone is structurally justified by its dense polyphenolic architecture. It is hypothesized that the rigid, planar coumestan core, coupled with its multiple free phenolic hydroxyl groups, facilitates robust intermolecular hydrogen bonding and pi-pi stacking interactions within the catalytic domain or allosteric sites of the alpha-amylase enzyme, thereby sterically hindering substrate binding and subsequent starch hydrolysis. Collectively, these *in vitro* enzymatic findings establish a definitive, target-specific molecular rationale for the utilization of *Eclipta prostrata* in managing postprandial hyperglycemia and provide a robust foundation for the observed *in vivo* antihyperglycemic efficacy.

5. Conclusion

The present systematic investigation provides a definitive molecular rationale for the traditional utilization of *Eclipta prostrata* in the management of metabolic disorders. Through rigorous bioassay-guided fractionation of the standardized whole-plant ethanolic extract, this study successfully isolated and unequivocally characterized wedelolactone as the principal bioactive coumestan derivative. The comprehensive spectroscopic elucidation integrating UV-Vis, FT-IR, high-resolution ^1H and ^{13}C NMR, and HRMS confirms the structural integrity of the isolated marker. Crucially, the *in vitro* enzymatic evaluation establishes, for the first time in a standardized comparative model, that both the chemically complex crude extract and the purified wedelolactone exert robust, concentration-dependent inhibitory effects against pancreatic alpha-amylase. The superior inhibitory potency of isolated wedelolactone ($\text{IC}_{50} = 112.45$ $\mu\text{g/mL}$) compared to the parent extract ($\text{IC}_{50} = 185.72$ $\mu\text{g/mL}$) pinpoints its critical role in retarding carbohydrate hydrolysis. Ultimately, these findings substantiate that wedelolactone serves as a highly promising, natural multi-target scaffold capable of mitigating postprandial hyperglycemic excursions. This research not only bridges ethnobotanical knowledge with empirical molecular evidence but also establishes a critical foundation for the future development of standardized, *Eclipta prostrata*-derived phytotherapeutics for the clinical management of type 2 diabetes mellitus.

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