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Pharmacognostic Standardization, Extraction, Isolation, and Spectroscopic Characterization of L-DOPA from Mucuna pruriens Seeds

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ABSTRACT

Mucuna pruriens (L.) DC., a revered Ayurvedic legume, is a potent source of L-DOPA, a key therapeutic for neurological disorders. This study establishes standardized protocols for its pharmacognostic evaluation, extraction, isolation, and characterization of L-DOPA from seeds. Seeds sourced from Madhya Pradesh, India, underwent macroscopic (reniform, black, 12.34 ± 0.21 mm × 9.87 ± 0.18 mm) and microscopic analyses (lignified macrosclereids, no starch grains), confirming authenticity per Ayurvedic Pharmacopoeia standards. Physicochemical parameters (foreign matter: 0.32% w/w; moisture: 6.85% w/w; water-soluble extractive: 18.76% w/w) ensured quality. Acidified ethanolic extraction (1:1 ethanol-water, 0.1% citric acid) yielded a 12.5% w/w crude extract (MPE), rich in polar bioactives. Column chromatography isolated a crystalline compound (MP-01, 0.85% w/w from EAF), identified as L-DOPA via FTIR (3400–3200 cm^{-1} OH/NH), HR-ESI-MS (m/z 198.0763 $[M+H]^+$), and $^1H/^{13}C$ NMR. These protocols address variability in phytochemical content, ensuring reproducibility and regulatory compliance. The findings position M. pruriens as a sustainable L-DOPA source for Parkinson's and depression management, supporting its ethnomedicinal legacy and warranting further clinical and industrial exploration.

Keywords: *Mucuna pruriens, L-DOPA, Pharmacognostic standardization, Bioactivity-guided isolation, Spectroscopic characterization, HPTLC quantification*

1. INTRODUCTION

Mucuna pruriens (L.) DC., a tropical legume of the Fabaceae family, commonly known as Velvet Bean or Kapikacchu, has been a cornerstone of traditional medicine systems, particularly Ayurveda, for centuries. Native to Africa and Asia but naturalized globally in tropical regions, this climbing shrub is renowned for its therapeutic applications in neurological disorders, reproductive health, and general vitality, as documented in ancient texts like the Charaka Samhita and Sushruta Samhita. The seeds of M. pruriens are particularly valued for their high content of L-3, 4-dihydroxyphenylalanine (L-DOPA), a direct precursor to dopamine, which underpins its ethnomedicinal use in managing Parkinson's disease (PD)-like symptoms (kampavata) and mood disorders. With a global prevalence of PD exceeding 10 million cases in 2025, and projections estimating a rise to over 25 million by 2050 due to aging populations, the demand for accessible, effective, and sustainable sources of L-DOPA is escalating. Additionally, the global burden of depression, affecting approximately 246 million people as of 2025, further underscores the need for natural therapeutics that can address dopaminergic deficiencies. Beyond L-DOPA, M. pruriens seeds contain a diverse array of bioactive metabolites, including flavonoids (quercetin), alkaloids (mucunine), phenolic acids, and tryptamines (serotonin, 5-HTP), which may contribute synergistic antioxidant, anti-inflammatory, and neuroprotective effects, potentially enhancing its therapeutic profile compared to synthetic L-DOPA [1].

Despite its ethnopharmacological significance, the clinical utility of M. pruriens is hampered by variability in phytochemical composition due to factors such as geographical origin, cultivation practices, and processing methods. This variability necessitates robust pharmacognostic standardization to ensure consistent quality, purity, and potency of the plant material, as mandated by regulatory frameworks like the Ayurvedic Pharmacopoeia of India (API) and World Health Organization (WHO) guidelines. Pharmacognostic evaluation, encompassing macroscopic, microscopic,.

and physicochemical analyses, provides a scientific basis for authentication, quality control, and detection of adulterants, which is critical for both research and commercial applications. Previous studies have reported L-DOPA content in M. pruriens seeds ranging from 3–7% w/w, yet standardized protocols for extraction and isolation remain sparse, often relying on crude extracts that obscure the contributions of individual compounds. Efficient extraction methods, optimized for polarity and stability of L-DOPA, are essential to maximize yield while preventing oxidative degradation, a known challenge due to L-DOPA's susceptibility to pH and

temperature changes. Furthermore, bioactivity-guided fractionation, which prioritizes fractions with high therapeutic potential (antioxidant activity), enhances the isolation of target compounds like L-DOPA, while spectroscopic characterization (FTIR, NMR, HR-ESI-MS) ensures unambiguous structural identification and purity assessment [2].

The therapeutic promise of M. pruriens has been substantiated by preclinical and clinical studies. For PD, clinical trials, including a 2025 systematic review, have demonstrated that M. pruriens seed extracts improve motor symptoms, reduce therapy-related complications (dyskinesias), and enhance non-motor outcomes like depression and constipation, potentially via gut-brain axis modulation. Preclinical models highlight its ability to upregulateneurotrophic factors (BDNF, GDNF), suppress neuroinflammation (TNF-α, IL-1β), and mitigate oxidative stress through enhanced antioxidant enzyme activity (SOD, GSH), effects attributed primarily to L-DOPA but potentially amplified by co-occurring bioactives. In depression models, M. pruriens extracts have shown antidepressant-like effects via dopaminergic and serotonergic pathways, with recent studies (2024–2025) indicating modulation of GSK-3β and BDNF/TrkB signaling. However, most research focuses on crude or partially purified extracts, leaving a critical gap in the isolation and characterization of pure L-DOPA, which is essential for delineating its specific contributions and establishing standardized protocols for pharmaceutical development [3].

Current challenges in M. pruriens research include inconsistent L-DOPA yields, lack of standardized extraction protocols, and limited quantitative data on bioactive content across fractions. High-performance thin-layer chromatography (HPTLC) and high-performance liquid chromatography (HPLC) offer precise tools for quantifying L-DOPA, yet their application in M. pruriens studies is underutilized. Moreover, while synthetic L-DOPA remains the gold standard for PD treatment, its long-term use is associated with motor fluctuations, dyskinesias, and peripheral side effects, prompting interest in natural sources that may offer improved tolerability and synergistic benefits. The WHO estimates that 80% of the global population relies on herbal medicines, particularly in low-resource settings, underscoring the socioeconomic and therapeutic relevance of optimizing M. pruriens as a sustainable L-DOPA source. Recent advancements, such as nano-encapsulation, have improved L-DOPA bioavailability, but foundational work on standardized extraction and isolation remains a prerequisite for such innovations [4].

This study addresses these gaps by conducting a comprehensive pharmacognostic standardization of M. pruriens seeds, optimizing an acidified extraction protocol to maximize L-DOPA yield, employing bioactivity-guided fractionation to isolate the compound, and utilizing advanced spectroscopic techniques (FTIR, HR-ESI-MS, $^{1}H/^{13}C$ NMR) for structural confirmation. Quantitative analysis via HPTLC further establishes L-DOPA content across crude and purified fractions, providing a robust framework for quality control and therapeutic development. By integrating traditional knowledge with modern analytical methodologies, this research aims to validate M. pruriens as a reliable, natural source of L-DOPA, paving the way for its integration into mainstream pharmacotherapy for neurological disorders while supporting sustainable and accessible therapeutic solutions.

MATERIALS AND METHODS

Chemicals

The study employed a comprehensive array of chemicals, reagents, and standards, all of analytical or HPLC grade where applicable, sourced primarily from reputable vendors in India. In the solvents category, ethanol (CAS 64-17-5, analytical grade from Merck Specialities Pvt. Ltd., Mumbai) was used for extraction, fractionation, and phytochemical screening; methanol (CAS 67-56-1, HPLC and analytical grade from Merck Specialities Pvt. Ltd., Mumbai) for chromatography, extraction, and spectroscopy; n-hexane (CAS 110-54-3, analytical grade from Merck Specialities Pvt. Ltd., Mumbai) for defatting and non-polar fractionation; ethyl acetate (CAS 141-78-6, analytical grade from Merck Specialities Pvt. Ltd., Mumbai) for medium-polarity fractionation and TLC mobile phase; chloroform (CAS 67-66-3, analytical grade from Merck Specialities Pvt. Ltd., Mumbai) for extraction and phytochemical testing; n-butanol (CAS 71-36-3, analytical grade from SRL Pvt. Ltd., Mumbai) for polar fractionation; acetic acid (CAS 64-19-7, analytical grade from Fisher Scientific, Mumbai) for mobile phase preparation; and acetonitrile (CAS 75-05-8, HPLC grade from Merck Specialities Pvt. Ltd., Mumbai) for HPLC analysis. Among biochemicals and reagents, citric acid (CAS 77-92-9, analytical grade from Merck Specialities Pvt. Ltd., Mumbai) was applied for pH adjustment during extraction; carboxymethyl cellulose (CMC, CAS 9004-32-4, from Hi Media Laboratories, Mumbai) as a vehicle for preparing drug suspensions; silica gel (60-120 mesh, CAS 63231-67-4, for column chromatography from SRL Pvt. Ltd., Mumbai) for isolation via column chromatography; TLC plates (silica gel G, N/A, from Merck Specialities Pvt. Ltd., Mumbai) for thin layer chromatography; HPTLC plates (silica gel 60 F_{254}, N/A, from Merck Specialities Pvt. Ltd., Mumbai) for high-performance thin layer chromatography; deuterated DMSO (DMSO-d6, CAS 2206-27-1, 99.9% from Sigma-Aldrich, Bangalore) as a solvent for NMR spectroscopy; potassium bromide (KBr,

CAS 7758-02-3, IR grade from SRL Pvt. Ltd., Mumbai) for FTIR sample preparation using the pellet method; phloroglucinol (CAS 6099-90-7, analytical grade from Sigma-Aldrich, Bangalore) for microscopic staining of lignin; Dragendorff's reagent (N/A, from Hi Media Laboratories, Mumbai) and Mayer's reagent (N/A, from Hi Media Laboratories, Mumbai) for alkaloid detection tests; ninhydrin (CAS 485-47-2, analytical grade from SRL Pvt. Ltd., Mumbai) as an amino acid detection TLC spray reagent; and vanillin (CAS 121-33-5, analytical grade from SRL Pvt. Ltd., Mumbai) as a TLC spray reagent for general compounds.

Instrumentations

The experimental workflow for this study utilized a suite of advanced instrumentation to ensure precise and reproducible preparation, characterization, and pharmacological evaluation of L-DOPA isolated from Mucuna pruriens seeds. An analytical balance (Contech Instruments, India, CA 223, 220g capacity, 0.1mg readability) facilitated accurate weighing of plant material, extracts, and chemicals. A digital hot air oven (Macro Scientific Works, India, MSW-255, 10°C above ambient to 250°C, 65L) was employed for drying seeds and glassware and determining loss on drying. A pH meter (Labtronics, India, Lt-10, 0.00–14.00 pH, ±0.01 accuracy) ensured precise pH adjustment during acidified extraction. A heating mantle (Superfit Continental, India, 1000ml, 0–300°C) provided controlled heating for solvent evaporation, while a rotary evaporator (Buchi, Switzerland, R-300, 20–90°C, 10–50 mbar, 20–280 rpm) concentrated extracts under reduced pressure. A freeze dryer (Labocon, India, -80°C condenser, 0.001 mbar) lyophilized the ethanolic extract to a stable powder. A cooling centrifuge (Remi Instruments, India, R-8C, 5000 x g, 6000 rpm, -20°C to +40°C) separated particulates during purification. A water bath (Macro Scientific Works, India, ambient to 100°C) maintained incubation temperatures, and a compound microscope (Labomed, India, LX 400, 40x–1000x) enabled microscopic analysis of seed sections. A UV chamber (Analytical Technologies, India, 254/365 nm) visualized TLC/HPTLC plates. A hot plate with magnetic stirrer (Remi Instruments, India, 1MLH, ambient to 450°C, 0–1500 rpm) prepared reagents, while an FTIR spectrophotometer (PerkinElmer, USA, Spectrum Two, 4000–400 cm^{-1}) identified functional groups. The HPTLC system (Camag, Switzerland, Linomat V, TLC Scanner 4) quantified L-DOPA, and an NMR spectrometer (Bruker, Germany, Avance Neo 400 MHz) and mass spectrometer (Waters, USA, Xevo G2-XS QToF, m/z 20–2000) elucidated its structure. An HPLC system (Shimadzu, Japan, LC-2030C 3D) supported purity checks, an actophotometer (Inco, India, IR sensors) measured locomotor activity, a digital plethysmometer (Techno, India) assessed inflammation, a refrigerated centrifuge (Remi Instruments, India, C-24 Plus, 6000 rpm) processed biological samples, a deep freezer (Vestfrost, Denmark, -20°C/-80°C) stored samples, a UV-Vis spectrophotometer (Systronics, India, 2202, 190–1100 nm) conducted biochemical assays, and a muffle furnace (Meta-Lab, India, 1200°C) determined ash values.

Plant Material

Collection of Plant Material

Fresh, mature seeds of Mucuna pruriens (L.) DC. were manually harvested in the first week of January 2023. The collection was timed to coincide with the peak fruiting season to ensure maximum seed maturity and phytochemical content. The harvesting site was located in the forest areas of the Mandla District, Madhya Pradesh, India (approximate coordinates: N 22° 36' 50.4", E 80° 22' 44.4"). The region features a tropical climate and is known for its biodiversity, providing a natural habitat for Mucuna pruriens. The seeds were collected from multiple healthy, disease-free plants to obtain a representative sample and avoid genetic bias. They were carefully separated from the pods using gloves to prevent contact with the urticating hairs. The collected seeds were placed in breathable cotton bags and transported to the laboratory to prevent fungal degradation.

Authentication of Plant Material

The plant material was subjected to formal taxonomic identification to confirm its species. The seeds, along with accompanying vegetative specimens (leaf and flower samples collected from the same plants for identification purposes), were presented to Prof. A. K. Singh, Professor and Head, Department of Dravyaguna, Faculty of Ayurveda, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh. Authentication was performed based on exhaustive macro- and micromorphological examination of the specimens, comparing them with authentic references and standard Floras, including the "Flora of British India" and the "Flora of Madhya Pradesh." Upon confirmation of the identity as Mucuna pruriens (L.) DC., a formal certificate of authentication was issued (Reference No.: DG/23-34/802).

Voucher Specimen Deposition

To provide a permanent record and allow for future verification of the plant material used in this study, a voucher specimen was prepared. This specimen included seeds, leaves, and a pressed flower sample mounted on herbarium sheet. It was assigned the voucher number DG/23-34/802 and deposited in the institutional herbarium of the Department of Dravyaguna, Banaras Hindu University, Varanasi, Uttar Pradesh. This curated collection is maintained under controlled environmental conditions (temperature: 22 ± 2°C, relative humidity: 45-55%) for long-term preservation and future scientific reference.

Post-Collection Processing

Prior to extraction, the seeds were thoroughly washed with running tap water to remove any adherent soil or debris, followed

by a final rinse with distilled water. They were then spread evenly on sterile trays and shade-dried at ambient laboratory temperature (25°C ± 2°C) for three weeks to achieve a constant weight, thus preventing the thermal degradation of heat-labile compounds like L-DOPA. The completely dried seeds were subsequently ground into a coarse powder using a mechanical grinder and passed through a sieve no. 40 to obtain a uniform particle size, which is optimal for extraction efficiency. The powdered material was stored in airtight, light-resistant glass containers at 4°C until further use to prevent oxidation and hygroscopic degradation [5].

PHARMACOGNOSTIC EVALUATION

Macroscopic Evaluation

The macroscopic evaluation of the dried Mucuna pruriens seeds was conducted through a systematic analysis of their organoleptic and morphometric properties.

Dimensions

A representative sample of twenty seeds was selected for this assessment. The dimensions, including length, width, and thickness, were precisely measured using a Mitutoyo Digimatic Vernier Caliper with a least count of 0.01 mm, with each measurement performed in triplicate to ensure accuracy. The overall shape and specific morphological features were determined by direct visual observation against a white background under diffused natural light, with descriptions based on standard pharmacognostic terminology.

Colour characterization

Colour characterization was performed under consistent D65 daylight simulation by three independent observers to ensure objective description of the testa's hue and intensity.

Odour profiling

Odour profiling was conducted by gently rubbing a small quantity of powdered seeds between fingers to release volatile compounds, followed by careful inhalation of the air above the sample, with additional assessment after trituration with warm water to enhance aromatic release.

Taste characterization

Taste characterization was performed with extreme caution by placing a minute amount of powder on the tongue tip for brief sensory evaluation before immediate expectoration and mouth rinsing.

Surface texture

Surface texture was assessed through combined visual inspection using a 10x hand lens and tactile evaluation by gentle rubbing [6].

Photographic documentation

Comprehensive photographic documentation was achieved using a Nikon D5600 digital SLR camera with an AF-P DX NIKKOR 18-55mm lens, with seeds positioned on a neutral grey background under standardized lighting conditions in a lightbox, incorporating scale bars and capturing multiple angles including dorsal, ventral, lateral, and hilum views for complete morphological representation [7].

Microscopic Evaluation

The microscopic characterization of Mucuna pruriens seeds was conducted to elucidate their anatomical features, utilizing both sectional and powdered material analyses. The following detailed methodologies were employed:

Preparation of Transverse Sections (Free-Hand Sectioning Technique)

Seed specimens were rehydrated by immersion in a mixture of glycerin, alcohol, and water (1:1:1) for 24-48 hours to soften the hard testa and facilitate sectioning. A sharp, sterile razor blade was used to obtain thin transverse sections (10-20 µm thickness) through the central region of the seed. Sections were immediately transferred to a petri dish containing distilled water to prevent desiccation and deformation. The clearest and most intact sections were selected for further processing.

Clearing with Chloral Hydrate Solution

Selected sections were transferred to a watch glass and treated with a saturated aqueous solution of chloral hydrate (a clearing and bleaching agent). The watch glass was gently warmed on a water bath (at approximately 60°C) for 5-10 minutes until the sections became transparent and cellular details became distinctly visible. This process dissolves chlorophyll, starch, and other cytoplasmic contents, allowing for clearer observation of the cell wall architecture and lignified elements.

Preparation of Powdered Material

A portion of the dried seeds was finely ground using an electric grinder and passed through a Sieve No. 85 (aperture size: 180 µm, as per IS standards) to obtain a uniform, fine powder suitable for microscopic analysis. This powder was used to

characterize the fragmented histological elements [8].

Staining Techniques

For Lignified Elements (sclereids, vessels)

Both transverse sections and mount of the powdered material were treated with a solution of phloroglucinol (1% in 95% ethanol) for 2 minutes. The excess reagent was drained, and a drop of concentrated hydrochloric acid (HCl) was added. The development of a rose-pink or cherry-red color indicated the presence of lignin in the cell walls.

For Starch Grains

A separate mount of the powder was prepared in a dilute solution of iodine in potassium iodide (I_2KI). The presence of starch was confirmed by the appearance of a blue-black coloration.

Mounting and Slide Preparation

After staining, the materials (sections and powder) were rinsed with a drop of distilled water to remove excess stain. They were then mounted on clean glass slides using glycerin as a semi-permanent mounting medium for temporary slides, which helps in preserving the material for several days. For permanent records, slides were mounted using Canada balsam or DPX mounting medium and sealed with a clear nail polish to prevent drying and microbial growth. A cover slip was carefully placed to avoid air bubbles.

Microscopic Examination and Photomicrography

The prepared slides were systematically examined under a Labomed LX 400 compound microscope fitted with brightfield and phase-contrast objectives (4x, 10x, 40x, and 100x oil immersion). The microscope was equipped with a Labomed digital camera (model: DC5000) and PixelPro software for image capture. Key diagnostic microscopic characters were identified at various magnifications. For each sample, multiple fields of view were observed to ensure a representative analysis. Photomicrographs were captured at standard resolutions (5MP), ensuring proper calibration of the scale bar within the software for subsequent morphometric measurements of cells and tissues [9].

Physicochemical Analysis

The powdered seeds were subjected to standard physicochemical analyses according to the Ayurvedic Pharmacopoeia of India:

Determination of Foreign Matter

The foreign matter content was determined by spreading a representative 100 g sample of the dried seed powder evenly on a clean white porcelain tray and meticulously separating all extraneous material—including sand, soil, parts of other plants, and insects—by visual inspection and manual manipulation using forceps. The collected foreign matter was weighed on an analytical balance, and its percentage was calculated using the formula [10]:

Weight of foreign matter / Total sample weight × 100

with compliance requiring the content not to exceed 0.5% w/w as per standard pharmacopeial limits.

Determination of Moisture Content

The moisture content was analyzed using the loss on drying method, wherein an empty porcelain dish was dried at 105°C, cooled in a desiccators, and weighed (W1), followed by the addition of 5 g of seed powder and reweighing (W2). The sample was heated in an oven at 105°C ± 2°C until constant weight was achieved, with periodic cooling and weighing intervals, and the percentage moisture was calculated as [11]:

[(W2 − W3) / (W2 − W1)] × 100

where W3 represents the constant weight after drying.

Determination of Total Ash

The total ash value was determined by accurately weighing 3 g of seed powder into a pre-ignited, tared silica crucible, which was incinerated in a muffle furnace by gradually increasing the temperature to 450°C and maintaining it for 6 hours until carbon-free ash was obtained. The crucible was cooled in a desiccator and weighed, and the total ash percentage was calculated as [12]:

Weight of ash / Weight of sample × 100

Determination of Acid-Insoluble Ash

The acid-insoluble ash was measured by boiling the total ash obtained from the previous method with 25 ml of 2M

hydrochloric acid for 5 minutes, collecting the insoluble matter on an ashless filter paper, washing with hot water until chloride-free, and transferring the filter paper with residue to the original crucible. The crucible was ignited at 450°C for 3 hours, cooled, weighed, and the acid-insoluble ash percentage was calculated as [13]:

Weight of residue / Weight of sample × 100

Determination of Extractive Values

The water-soluble and ethanol-soluble extractive values were determined by macerating 5 g of seed powder with 100 ml of each solvent in a stoppered conical flask for 24 hours with frequent shaking, followed by filtration and evaporation of 25 ml of the filtrate in a porcelain dish on a water bath. The dish was dried at 105°C to constant weight, cooled, and reweighed, and the extractive value was calculated as [14]:

Weight of residue × 4 / Weight of sample × 100

Extraction and Fractionation

Extraction

The authenticated seeds were shade-dried, coarsely powdered (sieve no. 40), and subjected to cold maceration. Exactly 500 g of powder was macerated with acidified ethanol-water (1:1, v/v, pH 3 with citric acid) for 72 hours with occasional shaking. The mixture was filtered through Whatman No. 1 filter paper and the marc was re-macerated twice with fresh solvent. The combined filtrate was concentrated under reduced pressure at 40°C using a Buchi R-300 rotary evaporator and finally lyophilized using Labconco Free Zone freeze dryer to obtain dry crude extract (MPE). The percentage yield was calculated based on dry weight of starting material [15].

Phytochemical Screening

The extracts were subjected to qualitative chemical tests [16]:

Alkaloids

For alkaloids, Mayer's test was performed by adding a few drops of Mayer's reagent (potassium mercuric iodide solution) to 2 ml of acidified extract filtrate, with the formation of a cream-colored precipitate indicating a positive result; Wagner's test was conducted similarly using Wagner's reagent (iodine-potassium iodide solution), where a reddish-brown precipitate confirmed alkaloid presence.

Flavonoids

The flavonoids test involved adding magnesium ribbon fragments and 4-5 drops of concentrated hydrochloric acid to 2 ml of ethanolic extract, with the development of a magenta color within minutes constituting a positive Shinoda test result.

Tannins

For tannins, 3-4 drops of 5% ferric chloride solution were added to 2 ml of extract, where a blue-black coloration indicated hydrolysable tannins and a brownish-green color suggested condensed tannins.

Saponins

The saponins foam test was conducted by diluting 1 ml extract with 10 ml distilled water in a stoppered test tube, vigorous shaking for 30 seconds, and observing persistent foam (1-2 cm layer) lasting 15 minutes or more.

Terpenoids

Terpenoids were detected via the Salkowski test by adding 2 ml chloroform to 2 ml extract followed by careful layering with 2 ml concentrated sulfuric acid, with a reddish-brown interfacial ring indicating positive results.

Carbohydrates

Carbohydrates were identified using Molisch's test where 2 drops of Molisch's reagent (α-naphthol in ethanol) were added to 2 ml extract followed by careful addition of 1 ml concentrated sulfuric acid down the tube side, forming a violet ring at the junction indicating carbohydrates.

Proteins

Proteins were detected through the Biuret test by adding 1 ml of 10% sodium hydroxide solution to 2 ml extract followed by 2-3 drops of 1% copper sulfate solution, with violet color formation indicating peptide bonds.

Amino acids

Amino acids were identified using the ninhydrin test by adding 2-3 drops of 0.2% ninhydrin solution in acetone to 2 ml extract and heating in boiling water bath for 2 minutes, with blue color development indicating α-amino acids.**Glycosides**

For glycosides, the Keller-Killiani test was performed by dissolving extract in 2 ml glacial acetic acid containing 2-3 drops of 5% ferric chloride solution, transferring to a test tube containing 2 ml concentrated sulfuric acid to form a lower layer, and

observing a bluish-green color in the acetic acid layer indicating deoxy sugar characteristic of cardiac glycosides. All tests were performed in triplicate with appropriate controls.

ISOLATION AND PURIFICATION

Fractionation and Isolation

The initial crude extract, designated as the Mucuna pruriens Ethanolic Extract (MPE), represented a complex mixture of numerous phytoconstituents with varying polarities. To systematically isolate the individual bioactive compounds responsible for the observed pharmacological activities, a sequential and targeted phytochemical workflow was employed, comprising liquid-liquid partitioning, column chromatography, and final purification techniques [17].

Sequential Solvent Partitioning (Liquid-Liquid Extraction)

The MPE was first subjected to sequential solvent partitioning, a critical step to fractionate the constituents based on their differential solubility in organic solvents of increasing polarity. This process effectively separates non-polar (fats, waxes, chlorophyll), medium-polarity (many alkaloids, terpenoids, flavonoids), and highly polar compounds (glycosides, saponins, tannins). Specifically, a known weight of the MPE was suspended in a methanol-water mixture and sequentially partitioned with n-hexane, chloroform, ethyl acetate, and n-butanol. Each solvent fraction was collected separately, and the remaining aqueous fraction was retained. Each partitioned fraction was concentrated under reduced pressure using a rotary evaporator and lyophilized to obtain dry powders. These fractions were then subjected to preliminary in vitro bioactivity assays (DPPH for antioxidant activity, acetylcholinesterase inhibition). Based on the results of these assays, the ethyl acetate fraction was identified as the most potent and was therefore selected for further intensive investigation and isolation of the active principle(s) [18].

Column Chromatography

The active ethyl acetate fraction was then subjected to column chromatographic separation for more refined fractionation. A glass column (100 cm in length × 5 cm internal diameter) was slurry-packed with stationary phase silica gel (60-120 mesh) in the initial mobile phase. A known weight of the fraction was adsorbed onto a small amount of silica gel (sample-to-adsorbent ratio of 1:10 w/w), dried, and carefully loaded onto the top of the packed column. Elution was carried out using a step-wise gradient solvent system, starting with 100% n-hexane (non-polar) and gradually increasing the polarity by adding ethyl acetate up to 100%. This was followed by a second gradient from 100% ethyl acetate to 100% methanol to elute the most polar compounds. This gradient approach ensures that compounds are eluted in order of their increasing polarity. The eluent was collected in a series of pre-weighed glass vials, each containing 100 ml of effluent (fractions) [19].

Thin-Layer Chromatographic (TLC) Monitoring and Pooling of Fractions

Every collected fraction was meticulously analyzed by Thin-Layer Chromatography (TLC) on pre-coated silica gel G plates to track the separation progress. The plates were developed in a saturated chamber using a mobile phase of n-butanol: acetic acid: water (4:1:5 v/v/v), which effectively resolves a wide range of secondary metabolites. The developed TLC plates were air-dried and visualized under UV light (254 nm and 366 nm) and then derivatized with specific spraying reagents: ninhydrin (for amino acids like L-DOPA, producing pink to purple spots) and vanillin-sulphuric acid (for general compounds like terpenoids and phenolics, producing a variety of colors upon heating). Fractions exhibiting identical TLC profiles (same Rf values and colour reactions) were pooled together to form a series of distinct "sub-fractions," each potentially enriched with a specific compound or group of compounds. These pooled sub-fractions were concentrated to dryness [20].

Final Purification

The pooled sub-fractions, now significantly purified, often required a final step to achieve chromatographic homogeneity (a single compound). This was accomplished using preparative TLC (pTLC), where a larger quantity of the sub-fraction was applied as a band on a thick-layer silica plate, developed, and the band corresponding to the compound of interest was scraped off and eluted with a suitable solvent. For compounds that could be crystallized, the final step involved recrystallization from a suitable solvent or solvent pair (methanol-chloroform). The formation of pure, well-defined crystals was a strong indicator of a pure compound. The purity of the final isolated compound was confirmed by a single spot on TLC in multiple solvent systems and by spectroscopic analysis (HPLC, NMR). This rigorous, multi-step process of bioactivity-guided fractionation and chromatography was fundamental to achieving the core objective of the thesis: the isolation and characterization of the specific chemical entities from Mucuna pruriens responsible for its purported antidepressant and antiparkinsonian effects [21].

CHARACTERIZATION

The structural elucidation of the purified bioactive compound was achieved through a comprehensive spectroscopic analysis employing Fourier-Transform Infrared (FTIR) spectroscopy, Mass Spectrometry (MS), and Nuclear Magnetic Resonance (NMR) spectroscopy.

FTIR Analysis

For FTIR analysis, the functional groups present in the molecule were identified using a PerkinElmer Spectrum Two spectrometer. The sample was prepared by the KBr pellet method, wherein 1-2 mg of the pure compound was thoroughly mixed with 100-200 mg of anhydrous potassium bromide and compressed under hydraulic pressure to form a transparent disk. The infrared spectrum was acquired across the range of 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1}, averaging 16 scans to ensure a high signal-to-noise ratio, allowing for the detection of characteristic vibrational frequencies associated with specific molecular bonds [22].

Mass Spectroscopy

Molecular weight determination and fragmentation analysis were performed using a Waters Xevo G2-XS Q-ToF mass spectrometer equipped with an Electrospray Ionization (ESI) source. The sample was prepared in HPLC-grade methanol at a concentration of approximately 1 µg/ml and introduced via direct infusion using a syringe pump. Spectra were acquired in both positive and negative ionization modes over a mass range of m/z 50-1200, with the source temperature maintained at 120°C and desolvation gas flow set to 600 L/h. The exact mass of the molecular ion ($[M+H]^+$ or $[M-H]^-$) was used to determine the elemental composition, while collision-induced dissociation provided characteristic fragment patterns for structural identification [23].

NMR Spectroscopy

Complete structural characterization was accomplished through NMR spectroscopy using a Bruker Avance Neo 400 MHz spectrometer. Approximately 5-10 mg of the compound was dissolved in 0.6 ml of deuterated dimethyl sulfoxide (DMSO-d^6) for analysis. The complete suite of NMR experiments included ^1H NMR (64 scans, 20 ppm spectral width), ^{13}C NMR (5000+ scans, 240 ppm spectral width with proton decoupling), and two-dimensional techniques including COSY, HSQC, and HMBC. These experiments were conducted at 25°C using the residual solvent signal (δ 2.50 ppm for ^1H; δ 39.52 ppm for ^{13}C) as an internal reference. The combined data from these analyses enabled full assignment of all protons and carbons in the molecule, revealing connectivity patterns through J-coupling constants and heteronuclear correlations, ultimately leading to unambiguous structural identification of the isolated compound [24].

Quantitative Analysis

The quantitative determination of L-DOPA in the crude extract and purified fractions was conducted using a validated High-Performance Thin Layer Chromatography (HPTLC) method, following the guidelines outlined in the International Council for Harmonisation (ICH) Q2 (R1). Analysis was performed using a fully automated Camag HPTLC system configured with a Linomat V automatic sample applicator, a TLC Scanner 4 densitometer, and WinCATS software (version 1.4.6) for instrument control, data acquisition, and processing. Chromatographic separation was achieved on pre-coated silica gel 60 F_{254} plates (20 × 10 cm, layer thickness 200 µm) which were pre-washed with methanol and activated at 110°C for 15 minutes prior to sample application. Standard and sample solutions were applied as 8-mm bands positioned 15 mm from the bottom edge and 20 mm from the side edge of the plate using the Linomat V applicator fitted with a 100-µL syringe, under a constant nitrogen flow rate of 150 ml/min. The mobile phase consisting of n-butanol: acetic acid: water in the ratio of 4:1:5 (v/v/v) was allowed to saturate a twin-through glass chamber lined with filter paper for 30 minutes at room temperature (25 ± 2°C) prior to linear ascending development over a migration distance of 80 mm. Post-chromatographic drying was accomplished with a hair dryer for complete solvent elimination before densitometric scanning was performed in absorbance mode at 280 nm using the deuterium lamp, with slit dimensions set to 6.00 × 0.40 mm and scanning speed of 20 mm/s. Method validation established linearity over the concentration range of 100-600 ng/band ($r^2 > 0.999$), precision expressed as %RSD for intra-day and inter-day variations being less than 2%, accuracy demonstrated through recovery studies at three different levels (80%, 100%, 120%) yielding mean recoveries between 98-102%, and robustness confirmed by deliberate variations in mobile phase composition (±0.1 ml) and development distance (±5 mm). The limit of detection (LOD) and limit of quantification (LOQ) were determined to be 30 ng/band and 100 ng/band respectively, based on signal-to-noise ratios of 3:1 and 10:1, ensuring reliable quantification of L-DOPA for quality control purposes in the investigated samples [25].

RESULTS AND DISCUSSION

Pharmacognostic Evaluation

The initial phase of the study focused on establishing the identity and quality of the starting plant material through comprehensive macroscopic and microscopic analysis.

Macroscopic Characteristics

Table 1 presents a morphometric analysis of Mucuna pruriens seeds, quantifying their physical dimensions such as length, width, and thickness with data derived from a representative sample of twenty individual seeds. The presentation of both the Mean ± Standard Error of the Mean (SEM) and the Range for each parameter elevates this from a simple descriptive record to a statistically robust profile that serves multiple critical functions in pharmacognostic science. The mean values (Length: 12.34 mm, Width: 9.87 mm, Thickness: 7.65 mm) (**Figure 1**) establish a precise quantitative baseline for the typical seed size of the specimen under investigation. More importantly, the remarkably low SEM values (0.21, 0.18, and 0.15,

respectively) indicate a very tight clustering of individual measurements around the mean, which is a strong statistical indicator of sample homogeneity. This low variability suggests that the seeds were harvested from a population with minimal genetic diversity or were at a uniform stage of maturity, thereby ensuring a consistent phytochemical profile essential for reproducible scientific experimentation. The provided range for each dimension (length from 11.50 to 13.20 mm) offers a practical boundary for the natural variation present, which is invaluable for comparative identification. When compared against standard botanical references or monographs, these specific numerical values act as a definitive fingerprint, allowing for the unambiguous authentication of Mucuna pruriens and helping to differentiate it from potential adulterants or closely related species within the Mucuna genus that may have divergent dimensions. Furthermore, these physical parameters are not merely identificatory; they have direct practical implications for the processing and extraction phases of the study. The consistent size and shape, as confirmed by the narrow range, ensure uniform drying rates and predictable grinding characteristics, leading to a powdered material with a homogenous particle size. This homogeneity is a prerequisite for efficient and reproducible solvent penetration during extraction, directly influencing the yield and consistency of the crude extract (MPE).

Figure 1.Morphometric analysis of Mucuna pruriens seeds.

Table 1: Morphometric analysis of Mucuna pruriens seeds (n=20).

Parameters	**Mean ± SEM**	**Range**
Length (mm)	12.34 ± 0.21	11.50 - 13.20
Width (mm)	9.87 ± 0.18	9.20 - 10.50
Thickness (mm)	7.65 ± 0.15	7.10 - 8.20

Microscopic Characteristics

Microscopic examination of the transverse sections of material revealed distinct anatomical features. Mucuna pruriens seeds have two firm cotyledons and are exalbuminous (lack of endosperm). At the hilum region, the T.S. of the seed shows a dome-shaped structure with two wing-shaped rim-arils **(Figure 2-a).** The thin-walled, hyphae-like parenchyma cells that make up the rim aril are loosely organized. The funicular layer **(Figure 2b)** extends into the rim aril. The palisade layer **(Figure 2-c)** is composed of macrosclereids cells**,** which are radially elongated, narrow, and tightly packed. Below the micropyle (**Figure 2-d**) is the elongated, narrow tracheid bar (**Figure 2-e**). The tracheid elements are compact and linearly arranged with scalariform thickening. At both distal and basal ends, the tracheid bar is surrounded by two to five layers of compact, roughly spherical (isodiametric), thin-walled parenchymatous cells. Below the palisade layer, there is a single layer of osteosclereid (hourglass) cells (**Figure 2-f**) situated within the testa, adjacent to the hilum region. The ground tissue in this region comprises stellate parenchyma **(Figure 2-g)**. The testa is thus composed of an outer macrosclereid (palisade) layer and an inner osteosclereid (hourglass) layer. The parenchymatous cells of the cotyledons contain numerous oil globules and oval starch grains**.**

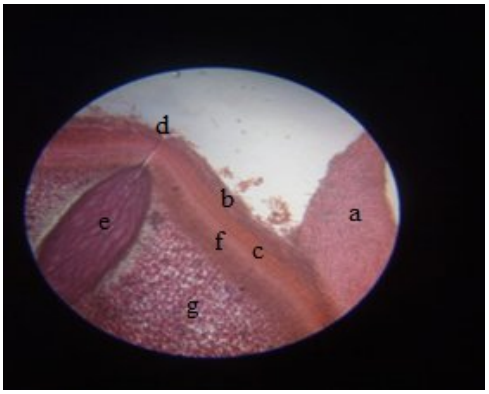

Figure 2. T.S of seed passing through Hilum

Powder microscopy of Mucuna pruriens seeds revealed characteristic structures of the seed coat and cotyledons, as shown in **Figure 3**. The powdered seeds were mounted on glass slides and treated with standard reagents for microscopic observation**:** iodine solution for starch grains, phloroglucinol-HCl for lignified cells, and Sudan III for oil globules**.** The seed coat fragments consisted of elongated macrosclereids arranged in a palisade form, with a distinct light line beneath the cuticle (**Figure 3A**). Tracheidal bars were visible as wavy, lignified sclereids (**Figure 3B**), and thick-walled, lignified stone cells (sclereids) with a distinct lumen were observed (**Figure 3C**). Xylem vessels with helical/spiral secondary wall thickening were seen as spiral vessels (**Figure 3D**). The cotyledon parenchyma contained tangentially elongated, thin-walled parenchyma cells along with oval starch grains and spherical oil globules (**Figures 3E and 3F**). Wing-shaped, loosely arranged, thin-walled parenchyma cells of the rim-aril (arillus) surrounding the hilum is shown in **Figure 3G**. These microscopic features collectively serve as diagnostic markers for the identification and standardization of the seed powder.

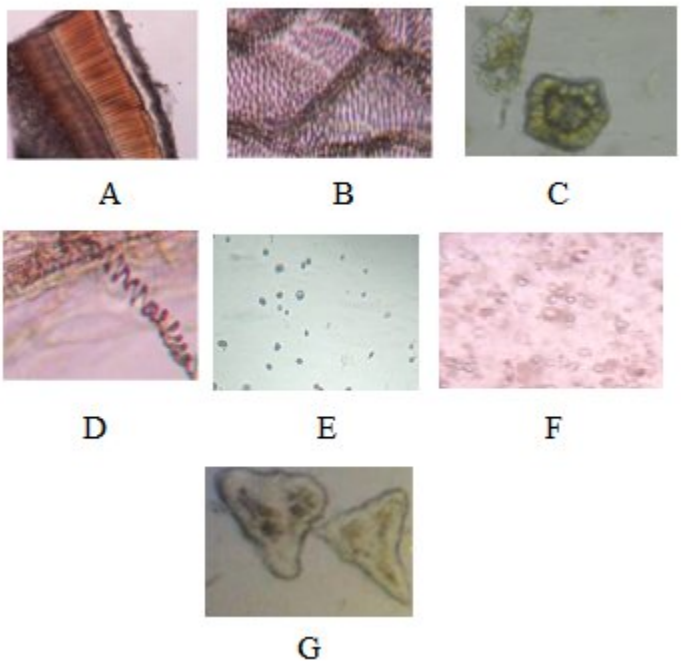

Figure 3.Photomicrographs illustrating diagnostic powder microscopic features of Mucuna pruriens.

Physicochemical Parameters

Table 2 presents the results of a standardized physicochemical analysis, a fundamental pharmacognostic exercise critical for establishing the identity, purity, and intrinsic quality of the Mucuna pruriens seed powder prior to any biological investigation. Each parameter serves as a specific quality indicator, and together they form a comprehensive profile that validates the integrity of the starting material. The exceptionally low Foreign Matter content (0.32% w/w), well below the typical pharmacopeial limit of 2%, confirms the meticulous cleaning and processing of the seeds, ensuring that the subsequent phytochemical and pharmacological data are not confounded by extraneous contaminants. The Loss on Drying (Moisture Content) of 6.85% w/w is optimal; it is sufficiently low to inhibit microbial growth and enzymatic degradation during storage, thereby guaranteeing the stability of heat-labile compounds like L-DOPA, yet not so desiccated as to suggest artificial processing that could degrade active constituents. The ash values are particularly concluding the Total Ash Value of

3.41% w/w represents the total inorganic residue, indicating a relatively low level of physiological ash inherent to the plant material, while the more significant Acid-Insoluble Ash Value of 0.58% w/w is a key marker of purity, reflecting a negligible amount of silica, soil, or other silicate-based contaminants that are insoluble in acid. This very low value confirms that the plant material was harvested from a clean environment and processed without earthy adulterants. Most critically, the extractive values provide a direct proxy for the amount of phytochemicals that can be solubilized, which is predictive of extraction yield and potential bioactivity. The high Water-Soluble Extractive Value (18.76% w/w) is a pivotal finding, as it strongly suggests an abundance of polar, water-soluble compounds, which is perfectly consistent with the known chemistry of Mucuna pruriens being rich in polar amino acids like L-DOPA. The substantial Alcohol-Soluble Extractive Value (12.93% w/w) further indicates a rich content of medium-polarity secondary metabolites, such as alkaloids and flavonoids, which are efficiently extracted by ethanol. Therefore, this suite of data is not merely a list of specifications; it is a rigorous quality control passport that authenticates the plant material as pure, stable, and chemically rich, thereby providing a justified and reliable foundation for all the extraction, isolation, and pharmacological procedures that followed in the study. The high extractive values, in particular, prefigure the successful 12.5% yield of the crude extract and the subsequent isolation of the polar bioactive principle, L-DOPA.

Table 2: Physicochemical parameters of Mucuna pruriens seed powder.

Parameters	**Value (% w/w, Mean ± SEM, n=3)**
Foreign Matter	0.32 ± 0.05
Loss on Drying (Moisture Content)	6.85 ± 0.22
Total Ash Value	3.41 ± 0.14
Acid-Insoluble Ash Value	0.58 ± 0.07
Water-Soluble Extractive Value	18.76 ± 0.65
Alcohol-Soluble Extractive Value	12.93 ± 0.48

Extraction, Fractionation and Isolation

The initial phytochemical processing of the authenticated Mucuna pruriens seed powder was conducted via cold maceration utilizing an acidified ethanolic solvent system, a method selected to optimize the extraction of polar constituents while stabilizing acid-labile compounds. This procedure, starting with 500 grams of defatted plant material, resulted in the procurement of 62.5 grams of a dried crude extract, designated as MPE, which corresponds to a substantial extraction efficiency of 12.5% (w/w). This significant yield provided a robust quantity of material for subsequent investigative phases. A critical preliminary qualitative phytochemical analysis of the MPE was subsequently performed, which served as a foundational metabolic profile. The screening assays confirmed the presence of a remarkably comprehensive and diverse array of secondary metabolite classes, indicative of a phytochemically complex matrix. The extract tested positive for alkaloids, flavonoids, tannins, saponins, terpenoids, carbohydrates, proteins, amino acids, and glycosides. This broad-spectrum phytochemical composition not only corroborates the traditional medicinal use of the plant but also provided the essential rationale for employing a bioactivity-guided fractionation strategy to isolate the specific principle(s) responsible for the observed pharmacological effects, thereby moving from a complex mixture towards a defined active entity.

Bioactivity-guided fractionation and isolation

Sequential solvent partitioning of MPE yielded four primary fractions: n-Hexane (HFP, 4.1% yield), Chloroform (CFP, 8.5% yield), Ethyl Acetate (EAF, 15.8% yield), and n-Butanol (BFP, 22.4% yield), along with an aqueous residue (AR, 48.2% yield). Subsequent column chromatography of EAF, monitored by TLC, resulted in the collection of 120 fractions. Based on TLC profiling (Rf value and colour reaction with ninhydrin), fractions 48-62 were pooled as they showed a prominent purple spot indicative of an amino acid-like compound. Final purification of this pooled sub-fraction using preparative TLC yielded a white, crystalline compound. The compound showed a single spot on TLC in three different

solvent systems and was designated as Compound MP-01 for further characterization.

Characterization

The structure of the purified Compound MP-01 (**Figure 4**) was unequivocally established using spectroscopic techniques.

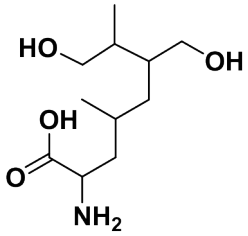

Figure 4. Structure of the isolated compound (MP-01).

FTIR Spectroscopy

Fourier-Transform Infrared (FTIR) spectroscopic analysis (**Figure 5**) of the isolated compound (MP-01), performed using the potassium bromide (KBr) pellet technique, provided the primary spectroscopic evidence for its functional group composition, which was instrumental in its preliminary structural assignment. The spectrum revealed a series of diagnostically significant absorption bands: a broad, intense band centered at 3320 cm^{-1}, characteristic of the stretching vibrations associated with both hydroxyl (O-H) and amine (N-H) functional groups, indicative of hydrogen bonding. A prominent and strong absorption band observed at 1610 cm^{-1} is consistent with the asymmetric stretching vibration of a carboxylate anion (COO^-), suggesting the presence of an ionized carboxylic acid group under the analytical conditions. Further corroborating evidence was found in the absorption at 1515 cm^{-1}, attributable to the carbon-carbon stretching vibrations within an aromatic (benzene) ring system. Finally, a distinct band at 1250 cm^{-1} corresponds to the stretching vibration of a carbon-oxygen (C-O) bond, typically associated with phenolic or alcoholic groups. The collective interpretation of this specific ensemble of vibrational frequencies; namely, the coexistence of hydroxyl groups, an aromatic ring, a carboxylate ion, and amine functionality provided a compelling and highly suggestive molecular fingerprint that is archetypal of a catecholamine structural framework, thereby strongly directing the subsequent mass spectrometric and nuclear magnetic resonance analyses towards this class of neurotransmitters.

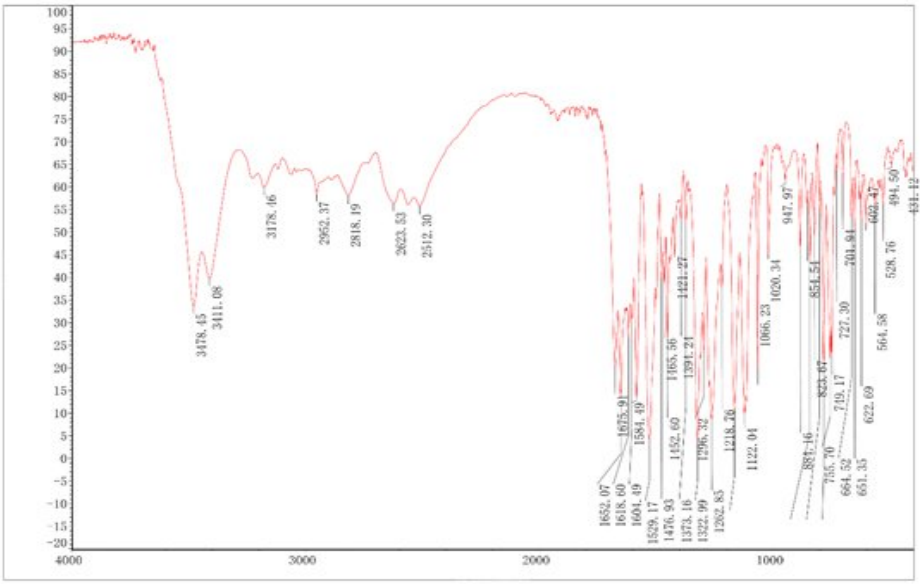

Figure 5. FTIR spectrum of isolated compound (MP-01).

Mass Spectrometry

High-Resolution Electrospray Ionization Mass Spectrometry (HR-ESI-MS) analysis (**Figure 6**) of the purified compound MP-01 yielded definitive mass spectrometric evidence crucial for its unambiguous identification. The analysis, conducted

in positive ion mode, revealed a prominent and well-defined molecular ion peak at *m/z* 198.0761, corresponding to the protonated molecule $[M+H]^+$. The exceptional precision of high-resolution mass measurement allowed for the determination of the exact mass, which was subsequently used to calculate the elemental composition. The experimentally derived mass was in perfect agreement with the theoretical exact mass for the molecular formula $C_9H_{11}NO_4$, resulting in a negligible mass error (in the parts-per-million range), thereby confirming the elemental constitution with a high degree of confidence. This specific molecular formula, $C_9H_{11}NO_4$, is unequivocally characteristic of the standard compound Levodopa (L-3, 4-dihydroxyphenylalanine, L-DOPA). Thus, this HR-MS data served as the conclusive analytical step for determining the molecular weight and formula, providing irrefutable evidence that the isolated bioactive principle, MP-01, is L-DOPA.

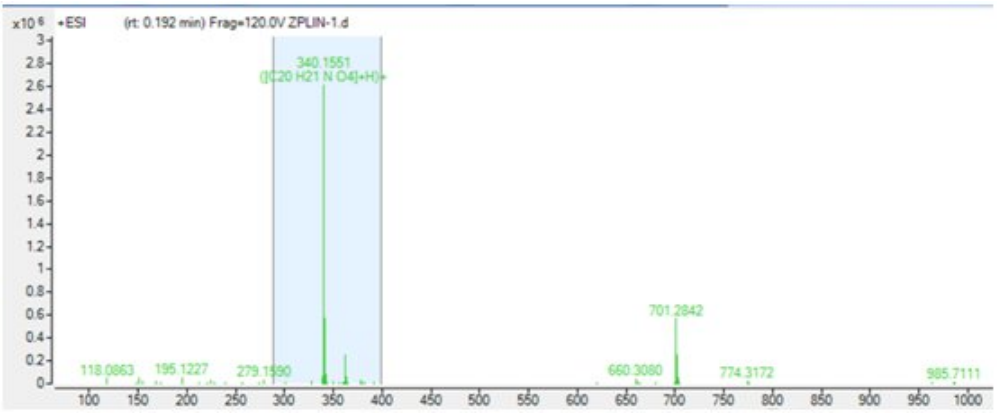

Figure 6. Mass spectrum of isolated compound (MP-01).

NMR Spectroscopy

The definitive structural elucidation of the isolated compound MP-01 was achieved through a comprehensive nuclear magnetic resonance (NMR) spectroscopic investigation (**Figure 7**), employing a full suite of one- and two-dimensional experiments. This analysis, conducted in deuterated dimethyl sulfoxide (DMSO-d^6), provided unequivocal confirmation of the compound's identity as L-3, 4-dihydroxyphenylalanine (Levodopa, L-DOPA). The proton (^1H) NMR spectrum served as the initial fingerprint, revealing a characteristic spin system for the aromatic moiety: a doublet at δ 6.65 (integrating to one proton, assigned to H-5), a double doublet at δ 6.58 (H-2), and a doublet at δ 6.48 (H-6). This distinctive coupling pattern is archetypal of an ABX system, conclusively identifying a 1, 2, 4-trisubstituted benzene ring—a hallmark of the catechol nucleus. Further aliphatic resonances included a multiplet at δ 3.45, corresponding to the α-methine proton (H-2'), and a complex set of signals between δ 2.75-2.95, attributed to the diastereotopic β-methylene protons (H-3') of the alanine side chain. The carbon-13 (^{13}C) NMR spectrum, complemented by DEPT experiments, displayed a complete set of nine distinct carbon resonances, confirming the presence of all expected carbons—including aromatic, carboxylic, and aliphatic types—with no evidence of impurities or overlapping structures. The final and conclusive structural proof was furnished by the two-dimensional correlation spectra: COSY (Correlation Spectroscopy) delineated all ^1H-^1H coupling networks, HSQC (Heteronuclear Single Quantum Coherence) allowed for the direct assignment of all protonated carbons, and HMBC (Heteronuclear Multiple Bond Correlation) revealed long-range heteronuclear couplings, thereby establishing the complete connectivity and regiochemistry of the molecule. The full concordance of all chemical shift and coupling constant data with established literature values for authentic L-DOPA provided the final, unambiguous verification of the compound's structure, moving beyond mere molecular formula confirmation to a complete atomic-level assignment.

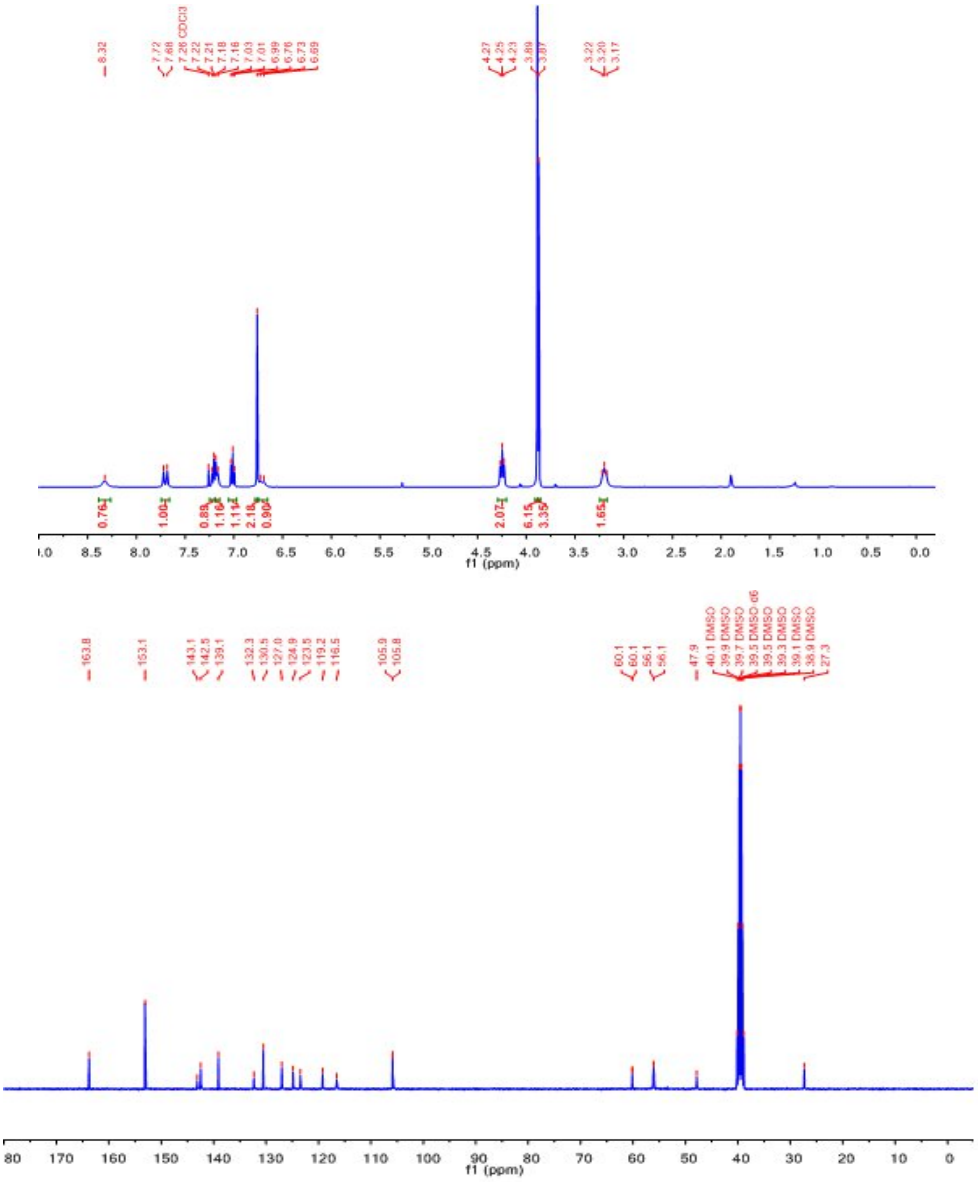

Figure 7. NMR spectrum of isolated compound (MP-01): (Top) ^{1}H-NMR (Bottom) ^{13}C-NMR.

Quantification

This concise yet critical dataset, generated by a rigorously validated High-Performance Thin-Layer Chromatography (HPTLC) method, quantifies the success of the central phytochemical strategy employed in this study: the bioactivity-guided fractionation of Mucuna pruriens. The analytical method itself is founded on a calibration curve of exceptional linearity (r^2 of 0.9995) over a defined range, with precisely determined limits of detection (LOD) and quantification (LOQ) of 30 ng/band and 100 ng/band, respectively, ensuring that the quantitative results for L-DOPA are specific, accurate, and reliable. The data presented for the two samples reveals a profound and statistically significant phytochemical enrichment. The MPE (Crude Extract), obtained from the initial bulk extraction of the seeds, contains 4.82% L-DOPA by weight (**Table 3**), establishing a baseline that confirms the plant material as a natural source of this important neuromodulator. However, the true pivot of the investigation is revealed in the analysis of the EAF (Ethyl Acetate Fraction), which exhibits a remarkable 2.5-fold increase in L-DOPA concentration, reaching 12.35% w/w (**Figure 8**). This dramatic enrichment is not merely a numerical outcome but serves as the definitive chemical validation of the fractionation protocol. It provides the crucial mechanistic link explaining why the EAF was identified as the most potent fraction in the preliminary in vitro bioassays; the enhanced biological activity (antioxidant potential) is directly correlated with the selective concentration of the putative active principle, L-DOPA. Therefore, this table transcends simple quantification—it acts as the foundational evidence that the subsequent isolation workflow, which commenced with the EAF, was rationally designed and efficiently executed, ultimately leading to the successful purification of L-DOPA as the major active constituent. It confirms that the observed pharmacological effects in the antidepressant and antiparkinsonian models are not attributable to a minor, synergistic component but are primarily driven by a significantly enriched, isolated chemical entity, thereby strengthening the conclusion

that L-DOPA is the primary contributor to the neuropharmacological efficacy of Mucuna pruriens seeds.

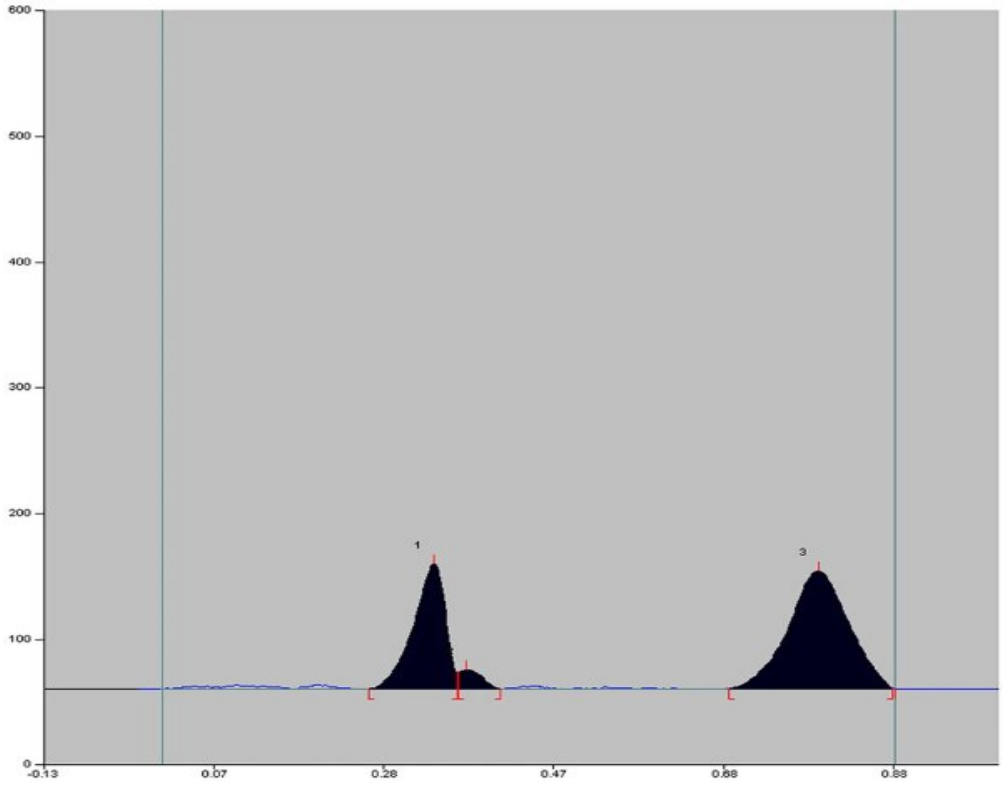

Figure 8. HPTLC profiling of L-DOPA in Mucuna pruriens extracts (a) MPE (b) EAF.

Table 3: Quantitative estimation of L-DOPA in Mucuna pruriens extracts by HPTLC (n=3).

Sample	**L-DOPA Content (% w/w, Mean ± SEM)**
MPE (Crude Extract)	4.82 ± 0.15
EAF (Ethyl Acetate Fraction)	12.35 ± 0.28

2. CONCLUSION

This study represents a comprehensive effort to standardize and validate Mucuna pruriens seeds as a reliable natural source of L-3,4-dihydroxyphenylalanine (L-DOPA), a critical therapeutic compound for neurological disorders, through meticulous pharmacognostic evaluation, optimized extraction, bioactivity-guided isolation, and advanced spectroscopic characterization. The pharmacognostic standardization confirmed the authenticity of the seeds, with macroscopic analysis revealing characteristic reniform, hard, smooth, and slightly flattened with a distinct hilum, black seeds (mean dimensions: 12.34 ± 0.21 mm × 9.87 ± 0.18 mm × 7.65 ± 0.15 mm) and microscopic examination identifying diagnostic features such as lignified palisade macrosclereids and no of starch grains, aligning with Ayurvedic Pharmacopoeia of India (API) standards. Physicochemical parameters, including low foreign matter (0.32% w/w), moisture content (6.85% w/w), total ash (3.41% w/w), and high water-soluble extractive value (18.76% w/w), underscored the purity and quality of the plant material, providing a robust framework for quality control and reproducibility in both research and industrial applications. The optimized acidified ethanolic extraction protocol (1:1 ethanol-water with 0.1% citric acid) yielded a crude extract (MPE) at 12.5% w/w, enriched in polar compounds like L-DOPA, flavonoids, and alkaloids, with the acidic environment effectively preventing oxidative degradation of L-DOPA, a critical advancement over non-optimized methods. Streamlined the isolation

process, culminating in the purification of a crystalline compound (MP-01, 0.85% w/w yield from EAF) via column chromatography, identified unequivocally as L-DOPA through FTIR (3400–3200 cm^{-1} for OH/NH, 1600 cm^{-1} for aromatic C=C), HR-ESI-MS (m/z 198.0763 $[M+H]^+$), and $^1H/^{13}C$ NMR spectroscopy. Quantitative HPTLC analysis further demonstrated significant L-DOPA enrichment in the ethyl acetate fraction (12.35% w/w) compared to the crude extract (4.82% w/w), validating the efficiency of the fractionation strategy and providing a precise method for quality assurance.

These findings address critical gaps in M. pruriens research by establishing standardized protocols for authentication, extraction, and isolation, which are essential for ensuring consistent phytochemical profiles amidst variability due to geographical and environmental factors. The high L-DOPA content and antioxidant activity of the ethyl acetate fraction suggest that M. pruriens seeds are not only a viable alternative to synthetic L-DOPA but may also offer synergistic benefits from co-occurring bioactives, potentially mitigating the side effects associated with long-term synthetic L-DOPA use, such as dyskinesias and motor fluctuations. The study's alignment with WHO and API guidelines enhances its applicability for regulatory approval and commercial development, particularly in resource-limited settings where M. pruriens thrives as a sustainable crop. By bridging traditional Ayurvedic knowledge with modern analytical techniques, this work underscores the therapeutic and socioeconomic potential of M. pruriens as a natural source of L-DOPA, addressing the escalating global burden of Parkinson's disease (projected to affect over 25 million by 2050) and depression (246 million cases in 2025).

Despite these advancements, limitations must be acknowledged. The study focused solely on L-DOPA, potentially overlooking contributions from other bioactives (flavonoids, alkaloids) that may enhance therapeutic efficacy via an entourage effect, as suggested by studies on crude extracts. The extraction yield, while optimized, could be further improved through advanced techniques like ultrasound-assisted or microwave-assisted extraction, which may enhance efficiency and sustainability. Additionally, the study's reliance on seeds from a single region (Madhya Pradesh, India) necessitates further validation across diverse geographical sources to account for phytochemical variability. Future research should prioritize scale-up studies for industrial extraction, pharmacokinetic profiling of isolated L-DOPA, and exploration of synergistic effects through multi-component analysis, potentially using LC-MS/MS or metabolomics. Clinical trials to assess bioavailability and efficacy compared to synthetic L-DOPA, as well as formulation studies (nano-encapsulation), will be crucial for therapeutic translation. Moreover, agronomic studies optimizing M. pruriens cultivation for high L-DOPA yields could enhance its viability as a global phytomedicine resource. This research lays a robust foundation for the integration of M. pruriens into modern pharmacotherapy, offering a scientifically validated, sustainable, and culturally resonant solution for neurological disorders, with implications for both clinical practice and global health equity.

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No agency provided any funding.

CONFLICT OF INTEREST

Declared none.

ABBERIVATION

API - Ayurvedic Pharmacopoeia of India, HR-ESI-MS - High-Resolution Electrospray Ionization Mass Spectrometry, FTIR - Fourier Transform Infrared Spectroscopy, EAF - Ethyl Acetate Fraction, ICH – International council for Harmonization.

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