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Pharmacognostic Standardization, Phytochemical Profiling, Antioxidant Activity, and Toxicological Evaluation of the Traditionally Used Medicinal Plant *Prunus wallichii* Steud.

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ABSTRACT

Introduction: Traditional medicinal plants play a crucial role in the management of several diseases and serve as a valuable source of bioactive compounds having significant therapeutic properties. *Prunus wallichii* Steud., a medicinal plant traditionally used by the Mizo tribe, remains unexplored despite its ethnomedicinal importance. **Objective:** The present study aimed to investigate the pharmacognostic characteristics, phytochemical profile, antioxidant potential, and safety evaluation of *P. wallichii*. **Methods:** The study investigates the pharmacognostic standardization, total phenolic and total flavonoid content, *in vitro* antioxidant activity, acute and sub-acute toxicity studies of extracts, and HPTLC qualitative analysis of methanolic extract. **Results:** Pharmacognostic standardization established the identity, purity, and quality of the crude drug. The methanolic extract exhibited higher total phenolic content (207.13 ± 2.041 mg GAE/g) and total flavonoid content (539.99 ± 25.23 mg QE/g) compared to the hydro-ethanolic extract (156.16 ± 6.55 mg GAE/g and 333.33 ± 24.035 mg QE/g, respectively). Both extracts demonstrated significant dose-dependent antioxidant activity in hydroxyl radical and hydrogen peroxide scavenging assays. Acute and sub-acute toxicity studies in rodents revealed the LD_{50} was greater than 2000 mg/kg showing no adverse toxic effects and mortality during the study period. Hematological, biochemical, and histopathological studies demonstrated the safety profile of the extracts. High-performance thin layer chromatography analysis confirmed the presence of two bioactive markers, scopoletin and taxifolin, which are known for their diverse pharmacological activities. **Conclusion:** The study findings provide scientific evidence supporting the traditional medicinal use of *P. wallichii* and highlight its potential for future therapeutic and pharmacological applications.

Key words: *Prunus wallichii* Steud.; Pharmacognostic standardization; Physicochemical; Acute toxicity; Sub-acute toxicity; HPTLC.

INTRODUCTION

Traditional medicinal plants are utilized worldwide as the primary healthcare needs due to their content of diverse secondary metabolites having therapeutic properties[1]. Scientific research studies are implemented on the traditional medicinal plants and discovery and development of novel therapeutic bioactive are still carried out due to their pharmacological effects, safety, and low costs. Billions of the world population consume medicinal plants for treatment of several ailments as an alternative to allopathic medicines. Due to this, proper documentation and investigation of traditional medicinal plants are necessary to determine their therapeutic benefits, bioactive components, as well as their adverse or toxic effects[2].

Prunus wallichii Steud., belonging to Rosaceae family is an evergreen shrub of approximately 12 m height, found in evergreen coniferous forests at an altitude of 500 to 3600 meters. It is native to the Southeast Asia, including Nepal, India, Bhutan, Bangladesh, Myanmar, Laos, Indonesia, Pakistan, China, Vietnam, and Thailand. In India, it is found in the northeastern region, such as Assam, Mizoram, Meghalaya, Manipur, and Sikkim. The indigenous people of Luangpawn village situated in Aizawl, Mizoram uses the leaf decoction to treat problems associated with diabetes to regulate the blood glucose level and inflammations, and is locally known as "thei-arlung." *Prunus* species are known to have profound pharmacological properties as several research studies and reviews have been extensively conducted where *P. armenica* L. is known to contain phenolics and flavonoids with significant antioxidant properties and other pharmacological properties[3,4]. The by-products of *P. persica* L. have notable polyphenols content, antioxidant activities, and showed no toxic effects on male Wistar rats[5,6,7,8]. The fruit extract of *P. laurocerasus* L. has shown significant antioxidant activity[9,10]. *P. spinosa* fruit extract contains phenolic and flavonoid compounds, exhibiting antioxidant, anticancer, and antimicrobial activities[11,12,13]. The by-products extract of *P. avium* L. also contains numerous phenolics and flavonoids, and exhibits significant antioxidant and other pharmacological activities[14,15]. Acute toxicity study and sub-acute oral toxicity study of *P. mume* fruit showed no significant toxic effects on mice[16]. The aqueous leaf extract of *P. africana* showed no significant toxic effects on studied animals[17]. Despite the ethnomedicinal claims and therapeutic potentials of *P. wallichii*, particularly by the Mizo tribe, scientific research study on this species remains unexplored, and there is a lack of systematic studies investigating the pharmacognostic as well as toxicological approaches.

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Therefore, the present study aims to standardize and evaluate the pharmacognostic parameters, phytochemical components, antioxidant potentials, and toxicological assessment of *P. wallichii* extracts. Pharmacognostic characterization was carried out on the crude drug by conducting organoleptic, microscopic, and physicochemical studies. Phenolic and flavonoid content and antioxidant properties of the methanolic and hydro-ethanolic extracts were evaluated by *in vitro* assays. Toxicological assessment was also conducted by acute toxicity and sub-acute toxicity studies to evaluate the safety profile of *P. wallichii* extracts. The methanolic extract fractions obtained from column chromatography were also carried out for HPTLC qualitative analysis using two standard reference scopoletin and taxifolin which are known to have wide ranges of therapeutic benefits. Therefore, our research findings could support and contribute in the development of evidence-based toxicological effects along with standardization, authentication, and quality control of the plant material.

MATERIALS AND METHODS

Plant Materials

An ethnobotanical survey was conducted at Luangpawn village located in Aizawl district, Mizoram, India, where the indigenous people of Luangpawn village consume the decoction of the leaf of *Prunus wallichii* Steud. in the management of diabetes, for regulating the blood glucose level and to relieve the disease-associated inflammation. An interview was conducted with Mr. Râlliana for documentation on the preparation and mode of administration. Guided by the information, collection of fresh leaves of *P. wallichii* was carried out in the forest region of Luangpawn during the months of October to December, 2020, providing a global positioning system (GPS) giving 23.856526° N (latitude) and 93.086295° E (longitude). The plant was deposited and taxonomically identified by Dr. N. Odyou, Scientist E and Head of Office, Botanical Survey of India, Eastern Regional Centre, Shillong, Meghalaya, with an identification number BSI/ERC/Tech/Identification/20/1061. The leaves were collected, washed, and dried under the shade in room temperature. Fresh leaves were used for the morphological studies and were also ground to coarse powder for extraction for further studies.

Organoleptic studies

The organoleptic studies of the leaf of *P wallichii* was carried out using standard protocol where the length, breadth, shape, colour, odour, taste, apex, margin, base, venation, and leaf arrangements were carefully determined[18,19].

Microscopic studies

Qualitative microscopic studies

Leaf microscopy

The leaf microscopic study was performed by peeling the upper and lower epidermal layer of fresh leaves where stomata was determined using projection microscope (ZEISS True Chrome II, T Capture Software) under 10x and 40x. The transverse section of the fresh leaf through the lamina and midrib was also cut in a thin section, mounted on a glass slide and several characteristics were studied using microscope at 10x[18,19].

Powder microscopy

A coarse powder of cleansed air-dried leaves was used to determine different microscopical characteristics. The powdered sample was placed on a glass slide, 1-2 drops of chloral hydrate solution was added, cover slip was placed on top carefully, and then observed for identification using microscope at 10x and 40x[18,19].

Quantitative microscopic studies

Stomatal number

Stomatal number refers to the mean number of stomata present per square millimetre of the leaf epidermis. The lower and upper epidermis of the fresh leaf was peeled off, placed on a glass slide, treated with 5% glycerine, and stained using dilute phloroglucinol hydrochloride. The number of stomata was counted under microscope at 10x for four different fields and the mean values were recorded[18,19].

Stomatal index

Stomatal index represents the percentage ratio of total stomata present to the total number of epidermal cells, where each stoma is counted as single cell. The lower and upper epidermis of the fresh leaf was carefully peeled off, placed carefully on a glass slide, treated with 5% glycerine, and stained using dilute phloroglucinol hydrochloride. Then, stomata and epidermal cells present were recorded using microscope at 10x for four different fields, and the mean values were recorded[18,19]. The stomatal index (S.I.) was calculated using the equation.

$$S.I. = \frac{S}{E+S} \times 100$$

Where, S is the number of stomata present per unit area and E is the number of epidermal cells present in the same unit area.

Vein islet and vein termination

For determination of vein islet and vein termination, the leaf was cut into square pieces excluding the midrib which was boiled in water bath with chloral hydrate solution until a clear transparent leaf was obtained. Then, the prepared sample was placed carefully on a glass slide and observed with a microscope at 10x and the number of vein islet and vein termination was counted for four different fields and mean values were recorded[18,19].

Physicochemical determination

Loss on drying

Using a glass-stoppered, shallow weighing bottle 5 gm of air-dried, coarsely powdered sample (in triplicate) was kept in hot-air oven (ELITE® Oven) without the stopper at 105 ± 2°C for 5 hr to obtain a stable weight. It was cooled at room temperature in a desiccator with the stopper, the weight for loss on drying was taken and mean values were recorded[18].

Extractive values

For determination of extractive values, 5 gm of air-dried, coarsely powdered sample was separately macerated with 100 mL of chloroform-water and ethanol (in triplicate) in a closed flask for 24 hr. The mixtures were shaken frequently during the initial 6 hr and allowed to stand undisturbed for the rest 18 hr. The extracts obtained was rapidly filtered using Whatman No.1 filter paper and 25 mL of each filtrate was evaporated to dryness on petri dish at 105°C. The final weight was taken and percentage of solvent soluble extractive was calculated with reference to the air-dried powdered sample[18].

Determination of total ash

The silica crucible was heated in hot-air oven to remove moisture for 30 min and kept cooled in a desiccator and the weight was recorded. 2 gm of the air-dried powdered samples was placed in the crucible, incinerated in muffle furnace (Jupiter Scientific Co., Tamil Nadu, India) at a temperature 105°C for 1 hr and increased to 600 ± 25°C until samples become carbon-free ash. Then, samples were allowed to

cool and weight of the ash was taken and percentage of the ash was calculated with respect to the air-dried powdered sample[18].

Determination of acid-insoluble ash

The ash obtained was boiled with 25 mL of 2 M hydrochloric acid for 5 min where the insoluble matter was collected on ashless filter paper. The collected residue was washed with hot water, ignited for 15 min, and cooled in a desiccator. The weight was recorded and percentage of acid insoluble ash was calculated with respect to the air-dried powdered sample.[18]

Determination of water-soluble ash

The ash obtained was boiled with 25 mL of water for 5 min where insoluble matter was collected using ashless filter paper. The collected residue was wash washed with hot water, ignited a temperature not exceeding 450°C for 15 min at and allowed to cool in a desiccator. The weight of the insoluble matter was deducted from the weight of total ash, and differences in the result weight indicates the water-soluble ash and percentage of water-soluble ash was calculated with respect to the air-dried powdered sample[18].

Extraction

Approximately 1000 gm of the coarsely ground leaves was utilised for extraction by hot-continuous extraction using Soxhlet apparatus with methanol, and using cold maceration with hydro-ethanol (50% ethanol). The extracts obtained was filtered with Whatman filter paper No. 1, concentrated by Rotavapor (Buchi) at 20-50°C, and evaporated to dryness by lyophilisation (Scanvac Coolsafe, Labogene) at -110°C, 120 lbs which was stored in air-tight container at 4°C for further studies.

Estimation of total phenolic content and total flavonoid content

Total phenolic content of methanolic and hydro-ethanolic extracts were determined by Folin-Ciocalteu's method[20]. In this assay, 1 mL of the sample extract at 50mg/mL concentration was mixed with 5 mL of Folin-Ciocalteu's reagent and was kept for 3 min; 4 mL of 0.7 M sodium carbonate solution was added and kept for 1 hr in room temperature and absorbance was measured at 765 nm (Specord 50 Plus, Analytik Jena), using the solvent as blank. Standard gallic acid was used for calibration curve preparation and the assay was performed in triplicate. The amount of phenolic content was determined and expressed as gallic acid equivalent in milligrams per gram (mg GAE/g) of the dried extract.

Total flavonoid content was determined by aluminium chloride colorimetric method[21]. In this assay, 1 mL of sample extract at 50 mg/mL concentration was mixed with 2 mL of distilled water and kept for 5 min; 3 mL of 5% sodium nitrite and 0.3 mL of 10% aluminium chloride solution was added and kept for 6 min; 2 mL of 1 M sodium hydroxide solution was added; volume was made up to 10 mL with distilled water, kept for 1 hr in room temperature and absorbance was measured at 510 nm, using solvent as blank. Standard quercetin was used for calibration curve preparation and the assay was performed in triplicate. Results were expressed as quercetin equivalents in milligrams per gram (mg QE/g) of the dried extract.

In vitro antioxidant studies

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging assay of methanolic and hydro-ethanolic extracts was performed as described in Anupong et al., 2023 with slight modification[22]. To a mixture solution of 0.45 mL of sodium phosphate buffer (pH 7.0, 200 mM), 0.15 mL of deoxyribose (10 mM), 0.15 mL of ferrous chloride (500 mM), 0.1 mL of EDTA (1 mM), 0.15 mL of hydrogen peroxide (10 mM), and 0.525 mL of distilled water, different concentration of the extracts (50, 100, 200, 400, and 800 µg/mL) were added, incubated for 1 hr at 37°C. Then, 1 mL of thiobarbituric acid (1% w/v) prepared in sodium hydroxide solution (0.025 M) along with 1 mL of ice cold trichloroacetic acid (2.8% w/v) were added, heated in boiling water bath for 10 min. The reaction mixture was cooled in room temperature and absorbance was measured at 520 nm. Ascorbic acid was used as standard drug and the percentage inhibition of hydroxyl radical scavenging activity was calculated using the equation.

$$Percentage\ inhibition\ (\%) = \frac{(A_0 - A_1)}{A_0} \times 100$$

Where, A_0 = absorbance of control and A_1 = absorbance in the presence of extract/standard.

Hydrogen-peroxide radical scavenging assay

Hydrogen peroxide radical scavenging assay of the extract samples were determined as per the method described in Shanmuganathan et al., 2023[23]. In this assay, to 1 mL of different extract solution (100, 200, 300. 400, and 500 µg/mL), 0.6 mL of hydrogen peroxide (43 mM) prepared in phosphate buffer (pH 7.4, 0.1 M) was added, and 3.4 mL of phosphate buffer was added and kept for 10 min. The absorbance was measured at 230 nm, gallic acid was used as standard, phosphate buffer was used as blank, and the percentage inhibition was calculated by using the equation.

$$Percentage\ inhibition\ (\%) = \frac{(A_0 - A_1)}{A_0} \times 100$$

Where, A_0 = absorbance of control and A_1 = absorbance in the presence of extract/standard.

Toxicological assessment

Animal procurement and ethical approval

Healthy adult *Mus musculus*, Swiss albino mice (35-40 gm), male and female (nulliparous and non-pregnant) were obtained from Saha Enterprise, Kolkata, India (CCSEA registered). Animals were acclimatized for 7 days with access to a standard pellet diet and water were provided *ad libitum*. Animals were housed in groups (n=5, same sex) in a controlled room temperature of 25 ± 2°C, with relative humidity of 55-60%, with artificial lighting cycle of 12 hr light and 12 hr dark. They were fasted for 12 hr before dosing and after dosing for 3-4 hr, but were provided with free access to water. During the study period, CPCSEA guidelines were strictly followed to ensure the ethical care and proper handling of laboratory animals, thereby minimizing pain and stress[24]. The ethical approval was obtained from the Institutional Animal Ethics Committee (IAEC) of the Department of Pharmaceutical Sciences, Dibrugarh University (Regd. No. 1576/GO/ERe/S/11/CPCSEA, dated 30/10/2018), with an approval number IAEC/DU/200, dated 19/05/2021.

Acute oral toxicity study

Acute oral toxicity of methanolic and hydro-ethanolic extracts of *P. wallichii* was conducted as per the Organisation for Economic Cooperation and Development (OECD) Guidelines 425 (Up-and-down procedure, limit test at 2000 mg/kg b.w.)[25]. Female Swiss albino mice were divided in three groups (n=6), group I serves as control group receiving the vehicle- distilled water, group II receives 2000 mg/kg b.w. of methanolic extract (PWM), and group III receives 2000 mg/kg b.w. of hydro-ethanolic extract (PWH). Animals were fasted overnight, but given access to water before dosing and after dosing

for 3-4 hr, and their weight were recorded. The first mouse of each group was orally fed with the samples and were carefully observed for mortality and toxicity signs, with special attention for the first 4 hr (for short-term toxicity), and periodically for 48 hr. If the animals survive, the remaining five mice from both the groups were orally fed with the samples, sequentially at 48 hr interval. Then, all the animals were carefully observed daily for 14 days (for long-term toxicity). Their body weight was recorded on the 1st day and 14th day and clinical toxicity signs including changes in weight, skin, fur, eyes, and mucous membrane, diarrhoea, tremors and convulsions, salivation, lethargy, behavioural changes, sleeping disorder, and mortality were monitored during the study period[26].

Sub-acute toxicity study

Sub-acute toxicity assessment was performed as per the OECD Guidelines 407 (Repeated dose 28-day oral toxicity in rodents, limit test at 1000 mg/kg b.w.)[27]. Both the sexes of Swiss albino mice were used for the study, divided into three groups (n=6, 3 males and 3 females), where group I serves as control receiving vehicle- distilled water, group II receives 1000 mg/kg b.w./day of PWM, and group III receives 1000 mg/kg b.w./day of PWH. All the treatment were given by oral route at the volume of 1mL/100 gm b.w./day during 28 days. Animals' health condition and clinical toxicity signs were observed daily, food and water consumption were checked weekly, and weight changes were recorded on the 1st, 14th, and 28th days. On the 29th day, animals were anesthetized, humanely killed and the blood and vital organs were collected for checking the relative organ weight, haematological, biochemical, and histopathological studies[28].

Relative organs weight

The relative weight of the collected organs such as liver, heart, and kidneys was calculated using the equation[29].

$$Relative\ organ\ weight = \frac{Organ\ weight\ (gm)}{Body\ weight\ (gm)} x1000$$

Hematological and biochemical analysis

The blood samples collected were stored in anticoagulant EDTA tubes for the evaluation of hematological parameters using Automated Hematology Analyzer, Sysmex XN-1000™, Kobe, Japan. The red blood cell count (RBC), white blood cell count (WBC), hemoglobin (HGB), hematocrit (HCT), mean cell volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelet count (PLT) were measured. The collected blood samples were also centrifuged (REMI) at 4000 rpm for 15 min to separate the plasma for biochemical analysis. The collected plasma layer was transferred to clean vials for determination of aspartate aminotransferase (AST), alanine amino-transferase (ALT), alkaline phosphatase (ALKP), total protein, albumin, bilirubin, urea, and creatinine using analyzer (VITROS® 5600, QuidelOrtho™, San Diego, USA)[30].

Histopathological study

For histopathological study, the liver, heart, and kidneys of the animals were collected, washed with normal saline, 70% ethanol, and preserved in 10% formalin. The organs were embedded in paraffin wax, sectioning was performed using microtome (Leica RM2125 RTS microtome, Leica Biosystems, Mumbai, India) with 4-6 µm thickness for each organ, and stained using eosin and hematoxylin dyes on the glass slides. The slides were observed under projection microscope and photomicrographs were taken for comparison and examination of histopathological alterations for the control and treated groups[28].

High Performance Thin Layer Chromatographic (HPTLC) studies

For HPTLC analysis, methanolic extract was first carried out for column chromatography and two fractions were selected for carrying out the qualitative study using Camag HPTLC System using pre-coated silica gel 60 F_{254} HPTLC plates (50 × 100 mm; Merck, Darmstadt, Germany) as the stationary phase. Two standard markers scopoletin and taxifolin having a wide range of therapeutic properties were acquired as standard markers for comparison of the sample fractions. Samples were prepared in ethanol and applied to the plate in varying volumes (5.0, 6.0, 8.0, and 9.0 µL) using a CAMAG Linomat 5 automatic applicator (Serial No. 100632, CAMAG, Muttenz, Switzerland). Sample bands were applied 8.0 mm from the lower edge of the plate, with a band length of 8.0 mm and an inter-track distance of 10.4 mm. The dosage speed was set at 100 nL/s, and a pre-dosage volume of 0.20 µL was used to ensure accurate sample delivery. Chromatographic development was performed in a twin-trough glass chamber (20 × 10 cm) pre-saturated for 20 minutes with the respective mobile phase for each sample. Saturation was carried out using appropriate filter paper liners to ensure consistent vapour phase conditions. Plates were developed up to a solvent front distance of 70 mm, followed by drying in ambient room temperature for 5 minutes. Densitometric evaluation of the plates was conducted using a CAMAG TLC Scanner 3 (Serial No. 140507), operated under visionCATS software version 3.2.23095.1 (CAMAG, Switzerland). Scanning was performed in absorbance mode at multiple wavelengths of 254 and 366 nm using deuterium and tungsten lamps. The scanner was configured with automatic detector mode at a scanning speed of 20 mm/s, a resolution of 100 µm/step, and a slit dimension of 5 × 0.45 mm (micro). Data acquisition and processing included Savitzky-Golay smoothing (window size 7), baseline correction using the lowest slope method, and peak detection using a Gaussian legacy algorithm with sensitivity set to 0.1, peak separation of 1, and a threshold of 0.1. Integration was carried out over an Rf range of 0.00 to 1.00. Each sample volume yielded consistent peak patterns with reproducible Rf values, supporting the robustness of the method. All operations were executed in accordance with CAMAG standard practices and analytical guidelines for HPTLC[31,32,33].

Statistical analysis

The results data were statistically analyzed and calculated using GraphPad Prism Software version 8.0.1, GraphPad Software, San Diego, CA.

RESULTS

Organoleptic studies

The organoleptic or macroscopical analysis of the leaf of *P. wallichii* was carried out to determine the morphological structure and the observation results as given in Table 1.

Table 1. Organoleptic observation of the leaf of *P. wallichii*.

Parameter	Observation (Mean ± SEM)
Length	16.06 ± 0.517 cm
Breadth	5.83 ± 0.120 cm
Shape	Aristate
Colour	Green
Odour	Grassy smell
Taste	Slightly bitter
Apex	Caudate
Margin	Entire
Base	Rounded
Venation	Cross-venulate
Leaf arrangement	Alternate

Microscopical studies

Qualitative microscopic studies

Leaf microscopy

The microscopic analysis of the leaf of *P. wallichii* revealed the presence of anomocytic stomata, defined by four subsidiary cells surrounding the guard cells (Figure 1A). The upper and lower epidermal cells were also observed which is crucial for the gaseous exchange and transpiration (Figure 1B and 1C). The vein islet and vein termination were observed showing a distinctive pattern of the species (Figure 1D), and the transverse section of the midrib showed the presence of vascular bundles consisting of xylem and phloem, upper and lower epidermis, palisade tissues, collenchyma cells, and parenchyma cells (Figure 1E). The observation of these anatomical features of *P. wallichii* by microscopic studies supports the identification and pharmacognostic standardization.

Powder microscopy

The powder microscopic analysis disclosed the essential diagnostic features of *P. wallichii*. The observation of epidermal cells, trichomes, starch grains, and calcium oxalate crystal (Figure 2A and 2E) confirms the presence of plant tissues that functions in moisture supply, protection, secretory function, energy storage, and helps in the diagnostic characterization of the plant. Simple trichome and labiacious trichome (Figure 2B), vessel (Figure 2C), simple fiber (Figure 2D) identified confirms the structural features that functions in the mechanical support, water, and minerals transport, as well as for defense mechanism purpose.

Quantitative microscopic studies

The quantitative microscopic studies were performed to evaluate the stomatal number, stomatal index, and number of vein islet and vein termination within a defined area of 1000 μm^2 across four different fields using projection microscope. Detailed results were presented in Table 2.

Physicochemical determination

Physicochemical studies were conducted to evaluate the quality control of the crude drug which is crucial for pharmacognostic standardization. Loss on drying is determined to control, minimize, and prevent decomposition of the crude drug. The study was performed in triplicate and the findings were given as percentage of loss on drying as shown in Table 3.

Extractive values were determined by maceration using chloroform-water and ethanol as solvents since most of the active constituents such as glycosides, tannins, plant acids, resins, etc., gets solubilized. The study was performed in triplicate and the percentage of water-soluble extractive and ethanol-soluble extractive values were shown in Table 3.

Total ash, acid-insoluble ash, and water-soluble ash values were also evaluated to measure the total amount of residual contents which remains after ignition, mainly to determine the identity, purity, and adulterants present in the crude drug. The studies were carried out in triplicate and the percentage values were shown in Table 3.

Total phenolic content and total flavonoid content

The total phenolic content and total flavonoid content were evaluated using the calibration curves of gallic acid (y = 0.0099x + 0.0019, R^2

Table 2. Quantitative microscopic parameters of the leaf of *P. wallichii*.

Microscopic parameters	Upper epidermis (Mean ± SEM)	Lower epidermis (Mean ± SEM)	Vein numbers (Mean ± SEM)
Stomatal number	0.75 ± 0.47	103.25 ± 8.92	-
Stomatal index	0.17 ± 0.09	44.47 ± 5.74	-
Vein islet	-	-	2.00 ± 0
Vein termination	-	-	2.75 ± 0.25

Values were expressed as mean ± SEM (n = 4).

Table 3. Physicochemical parameter results of *P. wallichii*.

Physicochemical parameters	Constant values in % w/w (Mean ± SEM)
Loss on drying	12.43 ± 0.51
Water-soluble extractive	4.74 ± 0.15
Ethanol-soluble extractive	2.20 ± 0.12
Total Ash	5.50 ± 0.50
Acid-insoluble ash	1.00 ± 0.50
Water-soluble ash	2.00 ± 0.50

Values were expressed as mean ± SEM (n = 3).

Table 4. Effect of 28 days oral administration on PWM and PWH (at 1000 mg/kg) on body weight (g) of male and female mice in sub-acute toxicity study.

Days	Body weight (g)		
	Normal control	PWM	PWH
Male			
Day 1	33.556 ± 0.382	31.783 ± 0.464	29.65 ± 0.41
Day 14	33.98 ± 0.446	32.103 ± 0.396*	29.80 ± 0.457*
Day 28	34.033 ± 0.429	32.356 ± 0.367*	30.026 ± 0.493*
Female			
Day 1	32.366 ± 0.371	30.40 ± 1.184	30.80 ± 1.209
Day 14	32.65 ± 0.375	30.606 ± 1.146*	31.296 ± 1.162*
Day 28	32.853 ± 0.305	30.733 ± 1.176*	31.34 ± 1.127*

Values are expressed as mean ± SEM (n = 6), PWM = methanolic extract of *P. wallichii*, PWH = hydro-ethanolic extract of *P. wallichii*. Statistical analysis was performed by using one-way ANOVA followed by Dunnett's multiple comparisons test, where the mean of normal control group was compared with the mean of tested groups, *$p < 0.05$.

Table 5. Effect of oral administration of PWM and PWH on the average organs weight and relative organs weight (g) of male and female mice.

	Organs (g)	Normal control	PWM	PWH
Average organs weight				
Male	Heart	0.159 ± 0.001	0.155 ± 0.002	0.154 ± 0.001
	Liver	0.872 ± 0.001	0.868 ± 0.001	0.885 ± 0.003
	Kidneys	0.337 ± 0.001	0.334 ± 0.001	0.335 ± 0.0006
Female	Heart	0.159 ± 0.001	0.152 ± 0.0003	0.157 ± 0.0006
	Liver	0.872 ± 0.0006	0.867 ± 0.001	0.886 ± 0.002
	Kidneys	0.337 ± 0.0003	0.334 ± 0.001	0.336 ± 0.0006
Relative organs weight				
Male	Heart	4.673 ± 0.086	4.797 ± 0.042	5.131 ± 0.109
	Liver	25.639 ± 0.298	26.831 ± 0.27	29.514 ± 0.563
	Kidneys	9.914 ± 0.100	10.334 ± 0.112	11.162 ± 0.164
Female	Heart	4.861 ± 0.071	4.971 ± 0.195	5.023 ± 0.190
	Liver	26.545 ± 0.238	27.981 ± 0.857	28.371 ± 1.107
	Kidneys	10.279 ± 0.104	10.922 ± 0.438	10.750 ± 0.414

Values are expressed as mean ± SEM (n = 6), PWM = methanolic extract of *P. wallichii*, PWH = hydro-ethanolic extract of *P. wallichii*. Statistical analysis was performed by using one-way ANOVA followed by Dunnett's multiple comparisons test, where the mean of normal control group was compared with the mean of tested groups, and values were found to be non-significant.

Table 6. Effect of oral administration of PWM and PWH on the hematological parameters in sub-acute toxicity study in male and female mice.

	Parameters	Normal control	PWM	PWH
Male	WBC (10^3/μL)	4.79 ± 0.050	10.233 ± 0.173*	13.206 ± 0.173*
	RBC (10^6/μL)	5.393 ± 0.049	7.226 ± 0.302*	7.79 ± 0.021*
	Haemoglobin (g/dL)	11.166 ± 0.185	11.233 ± 0.233	12.866 ± 0.033*
	Haematocrit (%)	22.533 ± 0.033	30.233 ± 0.970*	31.766 ± 0.088*
	MCV (fL)	39.50 ± 0.723	47.766 ± 0.837*	40.133 ± 0.290
	MCH (pg)	19.30 ± 0.057	18.40 ± 0.404*	16.633 ± 0.145*
	MCHC (g/dL)	46.566 ± 0.185	39.333 ± 0.466*	43.033 ± 0.348*
	Platelet count (10^3/μL)	444.66 ± 0.882	243 ± 1.732*	270.66 ± 1.856*
Female	WBC (10^3/μL)	4.766 ± 0.053	10.623 ± 0.642*	13.51 ± 0.121*
	RBC (10^6/μL)	5.516 ± 0.150	7.416 ± 0.334*	7.843 ± 0.046*
	Haemoglobin (g/dL)	11.00 ± 0.472	11.066 ± 0.328	12.533 ± 0.120*
	Haematocrit (%)	22.433 ± 0.203	29.633 ± 1.992*	31.866 ± 0.120*
	MCV (fL)	40.10 ± 1.401	47.533 ± 0.545*	39.433 ± 0.491
	MCH (pg)	19.633 ± 0.145	18.20 ± 0.208*	16.50 ± 0.173*
	MCHC (g/dL)	46.566 ± 0.088	39.50 ± 0.529*	41.866 ± 0.920*
	Platelet count (10^3/μL)	442.33 ± 1.453	234.66 ± 2.403*	266.33 ± 0.882*

Values are expressed as mean ± SEM (n = 6), PWM = methanolic extract of *P. wallichii*, PWH = hydro-ethanolic extract of *P. wallichii*, WBC = Leukocytes/white blood cell count, RBC = Erythrocytes/red blood cell count, MCV = mean cell volume, MCH = mean corpuscular haemoglobin, MCHC = mean corpuscular haemoglobin concentration. Statistical analysis was performed by using one-way ANOVA followed by Dunnett's multiple comparisons test, where the mean of normal control group was compared with the mean of tested groups, *$p < 0.05$.

= 1) (Figure 3 A) and quercetin (y = 0.0003x + 0.0005, R^2 = 0.9952), respectively (Figure 3 B). The total phenolic content of PWM and PWH were shown to be 207.13 ± 2.041 mg GAE/g and 156.16 ± 6.55 mg GAE/g, respectively. The total flavonoid content of PWM and PWH were shown to be 539.99 ± 25.23 mg QE/g and 333.33 ± 24.035 mg QE/g, respectively. The results indicate that PWM had higher phenolic and flavonoid content when compared to PWH.

In vitro antioxidant studies

The antioxidant activity of PWM and PWH were evaluated by *in vitro* methods. In hydroxyl radical scavenging assay, the standard ascorbic acid and samples showed concentration-dependent activity, where standard was found to be more potent than the samples, giving an IC_{50} values of 147.366 ± 0.284 μg/mL for ascorbic acid, compared to 249.033 ± 0.809 μg/mL and 183.40 ± 0.173 μg/mL for PWM and PWH, respectively.

Similarly in hydrogen peroxide radical scavenging assay, standard gallic acid and samples also showed concentration-dependent activity, giving an IC_{50} values of 83.06 ± 0.385 μg/mL for gallic acid, 96.103 ± 0.276 μg/mL, and 98.39 ± 0.280 μg/mL for PWM and PWH, respectively.

Graphical representations of antioxidant activity were depicted in Figure 4. From the assays, it was found that both PWM and PWH extracts possess antioxidant activity when compared with the standards.

Data are expressed as mean ± SEM (n = 3). Nonlinear regression analysis (log [inhibitor] vs. response—variable slope, four-parameter model) was performed using GraphPad Prism 8.0.1 to determine the IC_{50} values, and the log IC_{50} values were shown in the graphs. For curve fitting, bottom and top parameters were constrained to 0 and 100, respectively, to reflect the normalized percentage inhibition values. (A) Percentage inhibition vs. log concentration graph for hydroxyl radical scavenging activity of PWM and PWH. (B) Percentage inhibition vs. log concentration graph for hydrogen peroxide radical scavenging activity of PWM and PWH. All the assays were repeated independently at least thrice with consistent results, ascorbic acid and gallic acid were used as a positive control in the assays.

Table 7. Effect of oral administration of PWM and PWH on the biochemical parameters in sub-acute toxicity study in male and female mice.

	Parameters	Normal control	PWM	PWH
Male	AST (IU/L)	141 ± 1.527	154 ± 3.055	150 ± 0.577
	ALT (IU/L)	109.33 ± 0.666	116 ± 1.527*	111 ± 0.577
	ALKP (IU/L)	178.66 ± 0.882	180 ± 1.154	168.66 ± 1.454
	Bilirubin (g/dL)	2.10 ± 0.208	2.273 ± 0.023	2.066 ± 0.008
	Total Protein (g/dL)	8.01 ± 0.006	7.57 ± 0.145	7.936 ± 0.053
	Albumin (g/dL)	3.456 ± 0.008	3.896 ± 0.211	3.866 ± 0.138
	Urea (mg/dL)	25.226 ± 0.075	25.003 ± 0.187	26.086 ± 0.129
	Creatinine (mg/dL)	0.48 ± 0.006	0.45 ± 0.005*	0.50 ± 0.005*
Female	AST (IU/L)	139 ± 0.577	142.33 ± 1.453	145.66 ± 1.453
	ALT (IU/L)	108 ± 0.577	116 ± 3.605*	108 ± 0.577
	ALKP (IU/L)	170.33 ± 3.179	177 ± 1.527	162.66 ± 1.453
	Bilirubin (g/dL)	2.066 ± 0.009	2.096 ± 0.049	2.05 ± 0.021
	Total Protein (g/dL)	8.023 ± 0.012	7.58 ± 0.235	7.60 ± 0.134
	Albumin (g/dL)	3.44 ± 0.005	3.286 ± 0.143	3.543 ± 0.014
	Urea (mg/dL)	23.703 ± 0.104	23.253 ± 0.579	24.923 ± 0.255
	Creatinine (mg/dL)	0.47 ± 0.005	0.45 ± 0.011*	0.493 ± 0.009*

Values are expressed as mean ± SEM (n = 6), PWM = methanolic extract of *P. wallichii*, PWH = hydro-ethanolic extract of *P. wallichii*, AST = Aspartate Aminotransferase, ALT = Alanine Aminotransferase, ALKP = Alkaline phosphatase. Statistical analysis was performed by using one-way ANOVA followed by Dunnett's multiple comparisons test, where the mean of normal control group was compared with the mean of tested groups, *$p < 0.05$.

Table 8. Rf values of standards and samples of HPTLC study at 254 nm.

Standard	Volume	Rf values	Samples	Volume	Rf values
Scopoletin 1	1.0 µL	0.717	PT78	5.0 µL	0.708
Scopoletin 2	2.0 µL	0.713	RC256	5.0 µL	0.706
Scopoletin 3	4.0 µL	0.710			
Taxifolin 1	2.0 µL	0.642	PT78	5.0 µL	0.613, 0.647
Taxifolin 2	4.0 µL	0.615, 0.652	RC256	5.0 µL	0.613
Taxifolin 3	6.0 µL	0.607, 0.644			

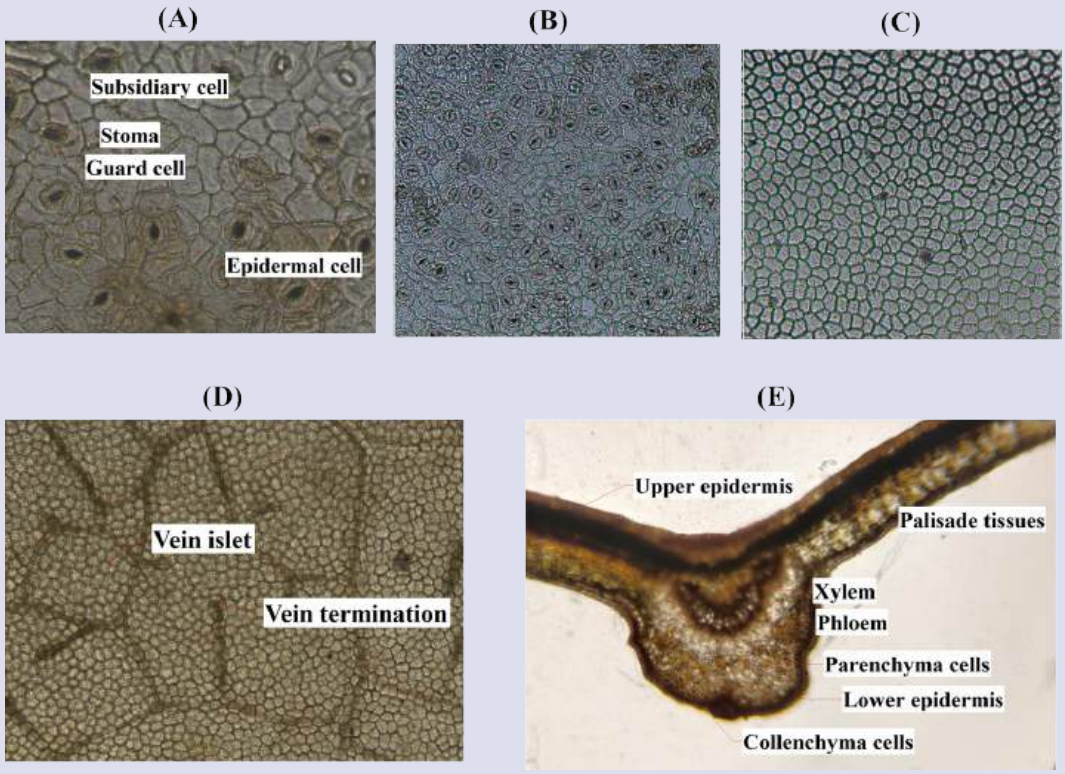

Figure 1. Microscopic studies of *P. wallichii* leaf showing (A) anomocytic stomata observed at 40x, (B) upper epidermis with numerous stomata at 10x, (C) lower epidermis at 10x, (D) vein islet and vein termination at 10x, and (E) transverse section of the leaf at 10x.

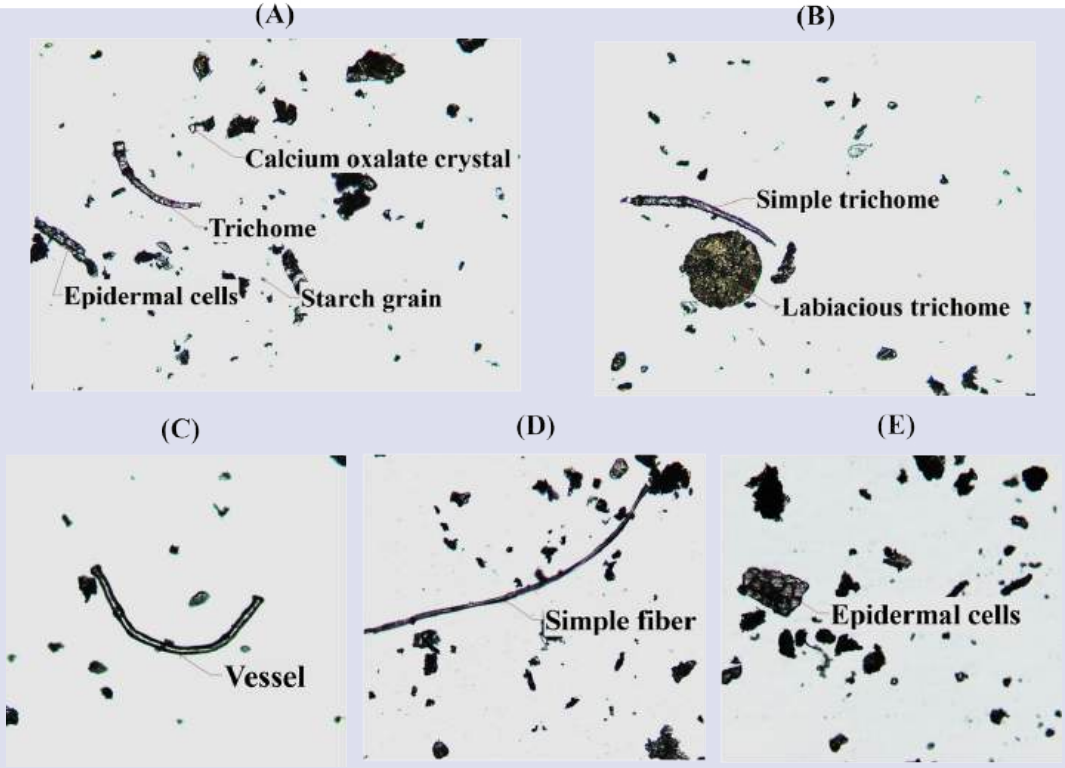

Figure 2. Powder microscopy of *P. wallichii* at 10x magnification, showing diagnostic anatomical features for species identification.

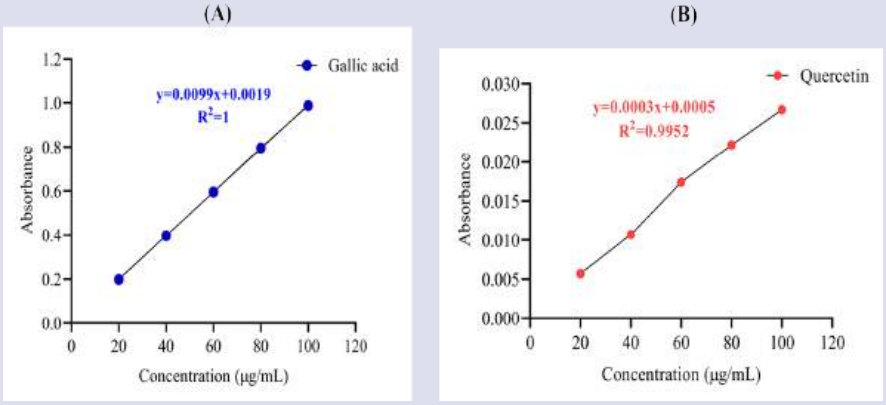

Figure 3. Calibration curve for estimation of (A) total phenolic content, and (B) total flavonoid content.

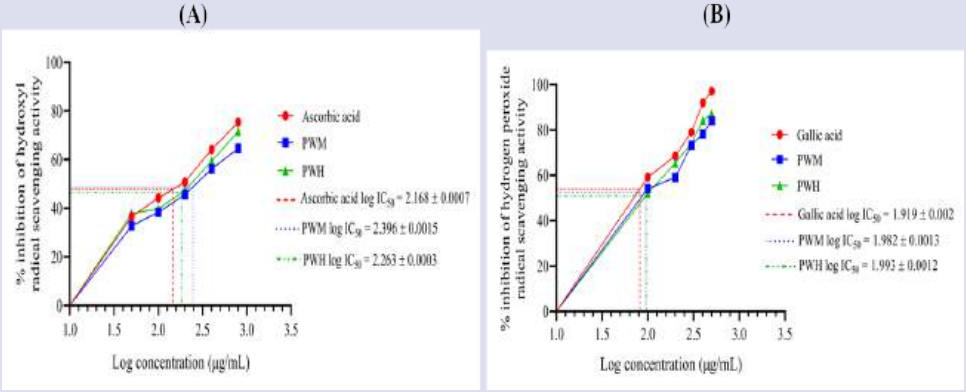

Figure 4. Antioxidant activity of *P. wallichii* methanolic extract (PWM) and hydro-ethanolic extract (PWH).

Acute toxicity study

Acute toxicity study was determined as per the OECD guideline 425, using the limit test at the dose of 2000 mg/kg body weight on female Swiss albino mice. The extract samples PWM and PWH were administered orally to evaluate their toxicity potentials and observations showed that there were no clinical toxicity signs, mortality, and no significant body weight variation on the tested animals during the 14 days study period. Therefore, the samples were found to be safe at the dose of 2000 mg/kg, and the LD_{50} was found to be greater than 2000 mg/kg.

Sub-acute toxicity study

Sub-acute toxicity study was conducted on the extract samples as per the OECD guideline 407, where all the treated group animals with the samples (1000 mg/kg b.w.) and normal control group survived throughout the 28 days study period. There were no clinical toxicity signs and mortality observed among the sample group when compared with the normal control group.

Measurement of changes in body weight and relative organ weight

There were no significant changes in the mean body weight of animals after oral administration of the samples (1000 mg/kg b.w.) during the 28 days study period when comparing the sample group with the normal control group. The results indicate that PWM and PWH had no effect on the growth of the animals (Table 4).

The average organs weight and relative organs weight of the animals including the heart, liver, and kidneys was recorded and calculated, and there were no significant changes when comparing the treated group against the normal control group (Table 5). The results indicated that the vital organs of the treated animals were not adversely affected by the samples throughout the study period.

Hematological and biochemical parameters

Hematological parameters and biochemical parameters were found to be within the normal ranges when compared with the normal control group (Table 6 and Table 7).

Histopathological study

The histopathological studies of vital organs such as heart (Figure 5 A-C), liver (Figure 5 D-F), and kidneys (Figure 5 G-I) were compared between the extract-treated groups and vehicle-treated normal control group. There were no remarkable changes observed and the treated groups were found to be similar with the observation in the normal control group.

High performance thin layer chromatographic (HPTLC) analysis

HPTLC analysis of two methanolic fractions of *P. wallichii* (PT78 and RC256) obtained from column chromatography was carried out for qualitative study by using hexane: ethyl acetate: formic acid (7:3:0.2 v/v/v) as mobile phase by comparing with scopoletin and taxifolin as standard markers. The developed chromatograms observed under 254 and 366 nm along with the post-derivatization showed a distinct band corresponding to the standards. The Rf values of the bands were found to be 0.708 ± 0.0 in PT78 and 0.706 ± 0.0 in RC256 which matched closely with scopoletin- 0.713 ± 0.002; 0.63 ± 0.017 in PT78 and 0.613 in RC256 which matched closely with taxifolin- 0.632 ± 0.0088,

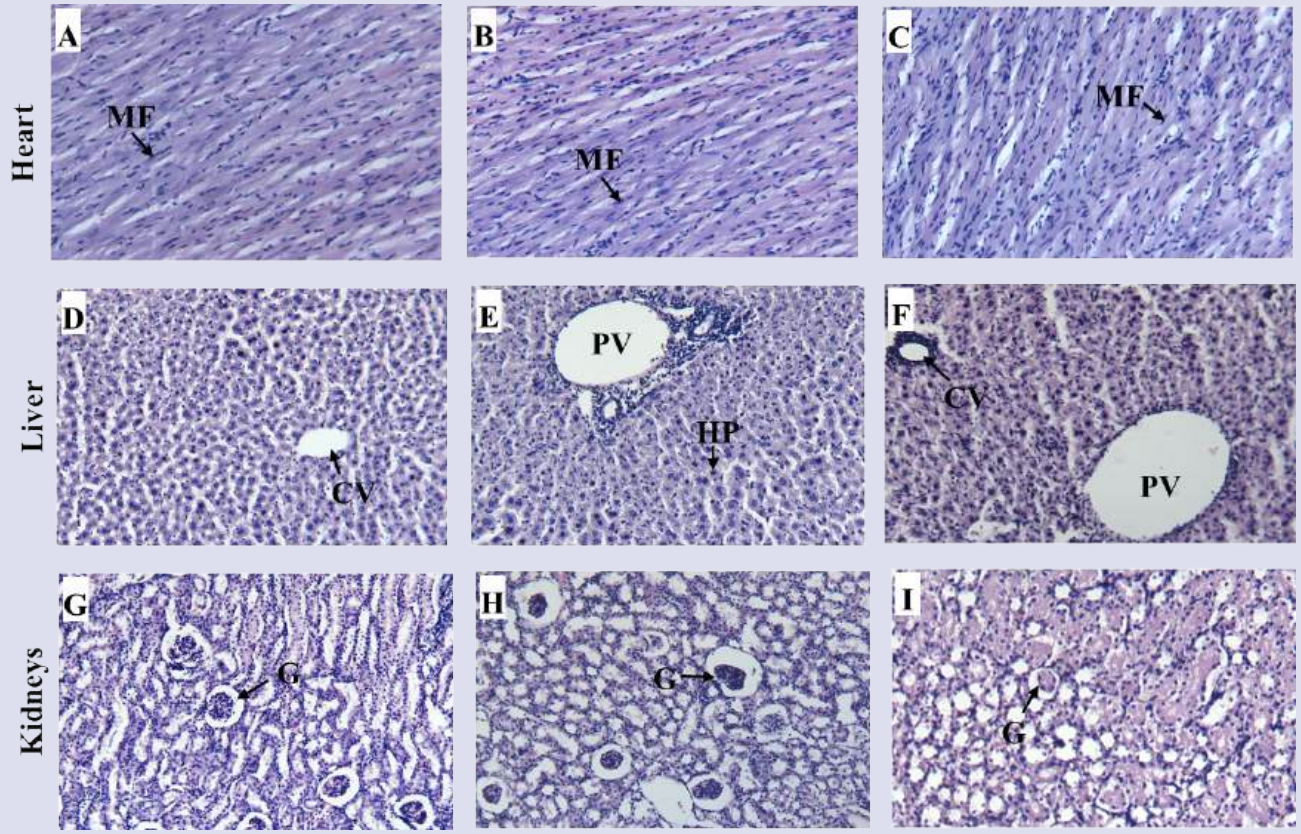

Figure 5. Histological sections of the sub-acute toxicity (observed at 10X) of heart (A-C), liver (D-F), and kidneys (G-I) of vehicle-treated normal control, PWM (1000 mg/kg), and PWH (1000 mg/kg) treated groups respectively. MF = Myocardial fibers, CV = Central vein, PV = Portal vein, HP = Hepatocyte plates, G = Glomerulus.

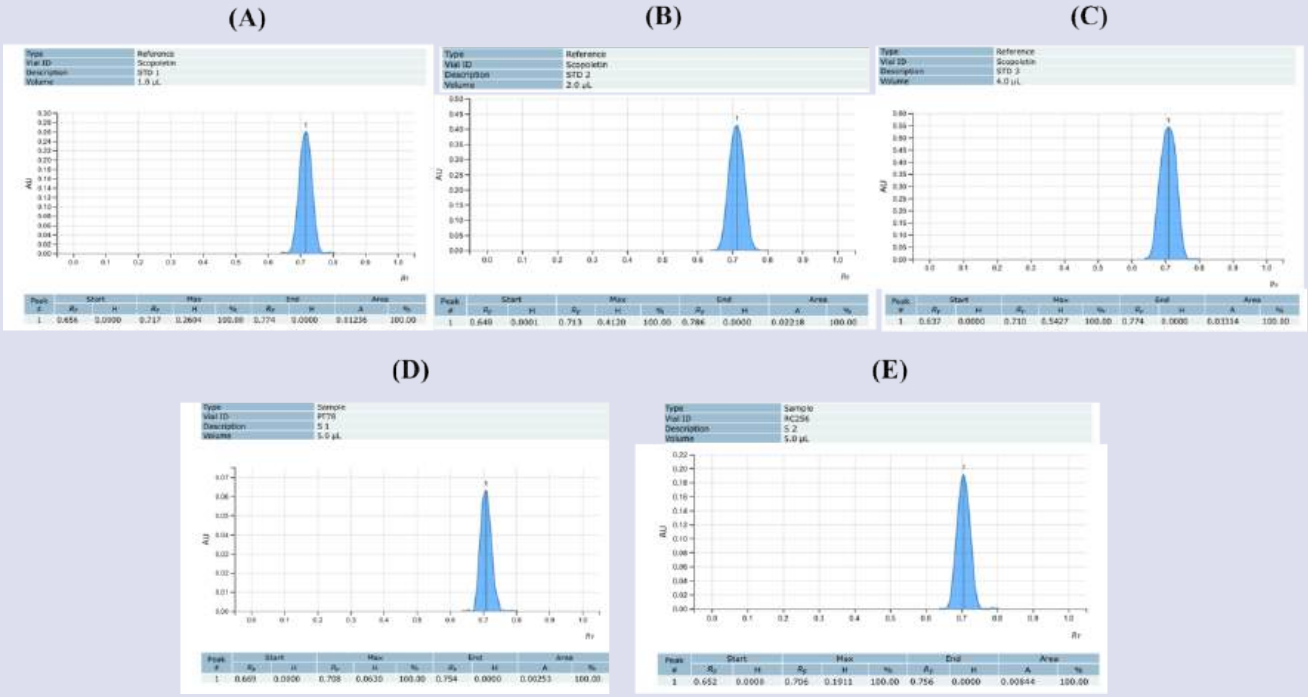

Figure 6. HPTLC chromatograms of [A] Scopoletin at 1.0 µL, [B] Scopoletin at 2.0 µL, [C] Scopoletin at 4.0 µL, [D] PT78 at 5.0 µL, and [E] RC256 at 5.0 µL using visionCATS software.

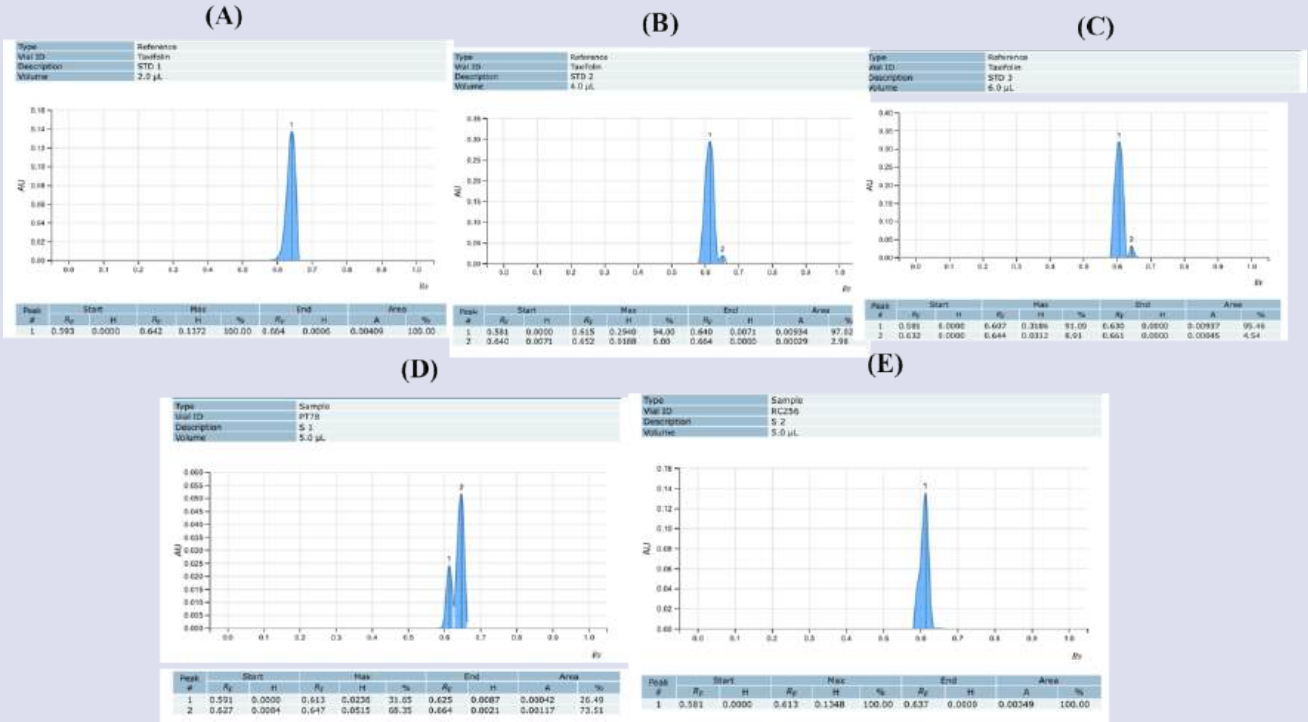

Figure 7. HPTLC chromatograms of [A] Taxifolin at 2.0 µL, [B] Taxifolin at 4.0 µL, [C] Taxifolin at 6.0 µL, [D] PT78 at 5.0 µL, and [E] RC256 at 5.0 µL using visionCATS software.

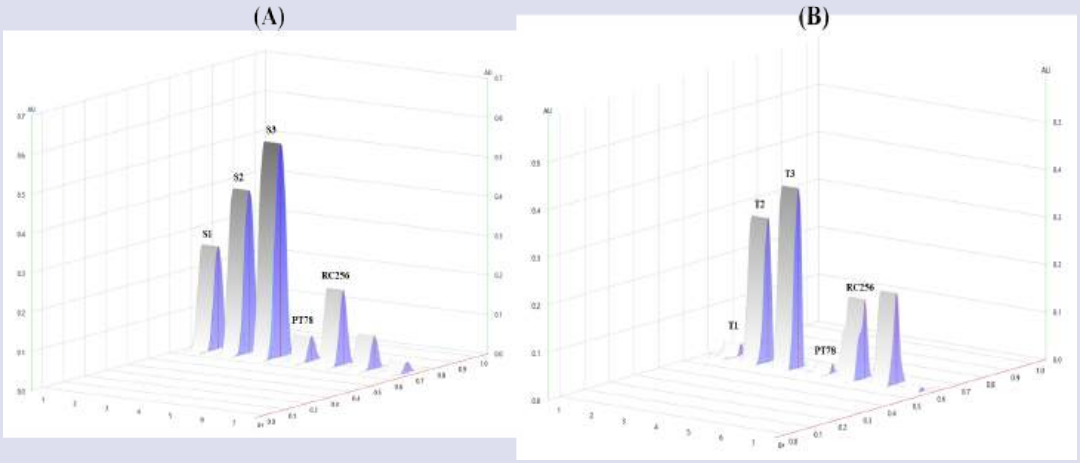

Figure 8. HPTLC densitometric peaks of [A] Scopoletin (S1, S2, S3) and samples PT78 and RC256; [B] Taxifolin (T1, T2, T3) and samples PT78 and RC256 using visionCATS software.

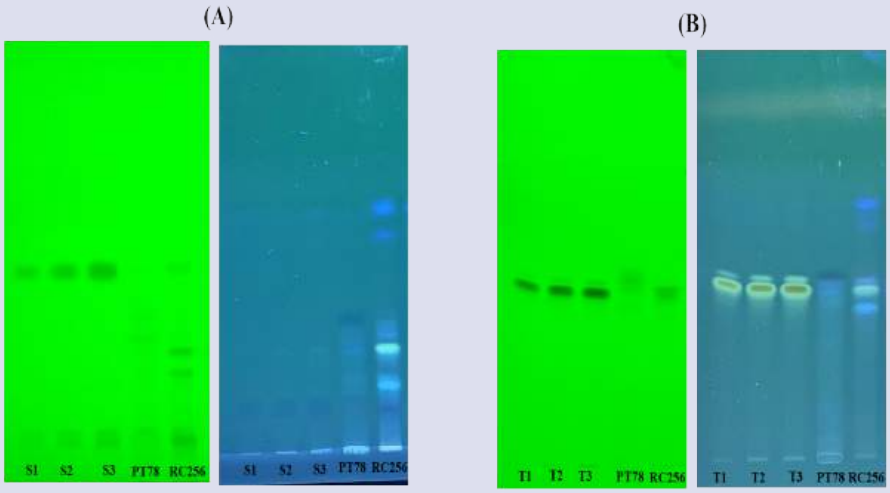

Figure 9. HPTLC plates of [A] Scopoletin (S1, S2, S3) and samples PT78 and RC256 under 254 and 366 nm; [B] Taxifolin (T1, T2, T3) and samples PT78 and RC256 under 254 and 366 nm using CAMAG Twin Trough Chamber.

revealing a similar chemical profile between the standard markers and the fractions, demonstrating a successful fractionation of the bioactive constituents during column chromatography (Table 8). The Rf values comparison of standard markers and samples were depicted in Table 8 along with chromatograms and TLC plates given in Figure 6 to Figure 9.

DISCUSSION

Traditional medicinal plants have been used as remedies to cure and prevent several diseases due to their low costs and significant therapeutic properties. These medicinal plants are known to be highly complex and research studies have been carried out extensively to understand their complexity from systemic evaluations like identification, standardization, quality control, bioactive components, therapeutic efficacies, along with the toxicological effects[34]. Despite the significant utilization as a natural source of remedy, the preliminary evaluations including pharmacognostic standardization, toxicological assessment, and phytochemical components have not been explored on *P. wallichii*. Pharmacognostic studies were conducted on the leaf of the plant to understand various parameters including the organoleptic, qualitative microscopic, and quantitative microscopic characteristics. Organoleptic evaluation demonstrated that the leaf had aristate shape, caudate apex, entire margin, rounded base, cross venation, and alternate leaf arrangement, which is green in colour, having grassy smell, tasteless, and the length and breadth of the leaf was found to be 16.06 ± 0.517 cm and 5.83 ± 0.120 cm respectively (Table 1). The qualitative microscopic study of the leaf showcases the detection of anomocytic type of stomata, revealing the guard cells, subsidiary cells, and epidermal cells (Figure 1A-C). The vein islet and vein termination (Figure 1D), along with the transverse section highlighting the vascular bundles, epidermis, palisade tissues, collenchyma cells, and parenchyma cells (Figure 1E) were also observed which plays a crucial role for gaseous exchange, transpiration, barrier protection, minerals, and sugar transportation for the plant.[19] Powder microscopic determination showcases the presence of epidermal cells, trichomes, starch grains, calcium oxalate crystals, fibers, and vessels (Figure 2A-E) which functions as crucial roles for the plant. The qualitative microscopic studies including stomatal number (upper- 0.75 ± 0.47 and lower- 103.25 ± 8.92 epidermis), stomatal index (upper- 0.17 ± 0.09 and lower- 44.47 ± 5.74 epidermis), vein islet (2.00 ± 0.0), and vein termination numbers (2.75 ± 0.25) quantify the presence of each parameter observed within 1000 μm^2 for four different fields (Table 2). Here, lower epidermis was found to contain more stomatal number

and stomatal index when compared to upper epidermis due to less exposure of direct sunlight and wind, and photosynthesis occurred on the shaded side of the plant leaf to balance carbon-dioxide uptake and prevention of excess water loss due to transpiration[19]. These microscopic features collectively support the qualitative evaluation for the identification and pharmacognostic standardization of *P. wallichii*.

Loss on drying was evaluated to determine the loss of weight of the sample by determining the amount of water and volatile content as excessive moisture content can degrade the quality of the sample which can lead to chemical changes and microbial contamination[35,36]. The percentage for loss on drying exhibit 12.43 ± 0.51% for the leaf of *P. wallichii* (Table 3). Extractive values were evaluated for the crude drug and the findings revealed that percentage for water-soluble extractive exhibit 2.20 ± 0.12%, and percentage for ethanol-soluble extractive exhibit 4.74 ± 0.15% (Table 3). This study is crucial for estimation of the presence of phytochemical constituents like tannins, glycosides, plant acids, resins, etc. in the crude drug which further helps in assessing specific constituents soluble in the specific solvents[36,37]. Determination of ash values was conducted to determine the identity and purity as well as the presence of adulterants in the crude drug[37]. There are two types of ash, physiological ash which derives from plant tissues, and non-physiological ash that consists of residues like sand, soil, and other extraneous matter present in the sample. Total ash consists of mainly oxides, phosphates, carbonates, silicates, and silica. The acid-insoluble ash evaluates the amount of silica content, determining the dirt and sand, and water-soluble ash evaluate the content of inorganic elements present in the sample[19,36]. Our study findings revealed that percentage of total ash exhibit 5.50 ± 0.50%, percentage of acid-insoluble ash exhibit 1.00 ± 0.50%, and percentage of water-soluble ash exhibit 2.00 ± 0.50% (Table 3). Estimation of the above physicochemical characteristics were important for quality control and pharmacognostic standardization, which is reported for the first time for *P. wallichii*.

Plants consist of several secondary metabolites having beneficial therapeutic effects that are mostly found in the extracts of the crude drug. Secondary metabolites like flavonoids and phenolic acids are known to be present abundantly in medicinal plants. They are also known to have strong antioxidant property and noted to play crucial role in the physiology and controlling of several diseases[38]. In our study, polar solvents like methanol and hydro-ethanol were utilised for extracting the bioactive components present in *P. wallichii*. The results of quantitative estimation of total phenolic content and total flavonoid content strongly suggests that phenolics and flavonoids present in the extracts attributes to its therapeutic potential of the plant. The methanolic extract (PWM) was found to have better phenolic and flavonoid contents, when compared with hydro-ethanolic extract (PWH). The total phenolic content of PWM and PWH were found to be 207.13 ± 2.041 mg GAE/g and 156.16 ± 6.55 mg GAE/g, and total flavonoid content of PWM and PWH were found to be 539.99 ± 25.23 mg QE/g and 333.33 ± 24.035 mg QE/g respectively.

The antioxidant activity of the extracts was evaluated by carrying out hydroxyl radical and hydrogen peroxide radical scavenging assays. Hydroxyl radicals and hydrogen peroxide radicals are the common reactive oxygen species (ROS) which damages and attack the DNA, proteins, and lipids at cellular level if present excessively within the biological system, and can lead to pathology of several diseases[39]. The potent phytochemicals present in medicinal plants can reduce and neutralize these ROS, due to which evaluation of the activity of plant extracts or bioactive compounds becomes crucial using *in vitro* assays for preliminary assess of the potency of the plant extracts[40]. In this assay, PWM and PWH were determined for their potential to neutralize the hydroxyl radicals, and samples were found to have significant concentration-dependent activity showing an IC_{50} values of 249.033 ± 0.809 µg/mL and 183.40 ± 0.173 µg/mL for PWM and PWH, respectively and standard ascorbic acid gave an IC_{50} values 147.366 ± 0.284 µg/mL (Figure 4A). Likewise, redundancy of hydrogen peroxide radicals also causes oxidative stress, damaging the biomolecules within the biological system[41], and determination of hydrogen peroxide radical scavenging assay of PWM and PWH also gave concentration-dependent activity, with an IC_{50} values of 96.103 ± 0.276 µg/mL and 98.39 ± 0.280 µg/mL, and standard gallic acid gave an IC_{50} values 83.06 ± 0.385 µg/mL (Figure 4B).

The safety profile assessment of medicinal plants is necessary to describe the range of doses and to prevent the toxicity risks that can arise prior to development of the product[42]. There is either scientific research nor evidence on the safety and efficacy of the studied plant, as this can produce limitation on its therapeutic applications. Therefore, acute oral toxicity (14 days) and sub-acute toxicity (28 days) studies were performed to assess the safety profile of *P. wallichii*. After oral administration of the extracts PWM and PWH to the experimental animals in acute toxicity study, there were no signs of toxicity, mortality, and weight changes observed, since these factors are mainly used to determine the onset of drug toxicity. The LD_{50} of both the extracts were more than 2000 mg/kg, which was found to be nontoxic[25]. Then, in sub-acute toxicity study, no clinical toxicity signs and mortality were observed during the study period at the dose of 1000 mg/kg. Significant changes in animals' body weight associates with toxicity of the tested drug, and as per our findings there were few significant changes ($^*p < 0.05$) in the body weight of experimental animals when comparing normal control group with the tested groups (Table 4). Significant changes in the organs weight is also an indicator of toxicity of the drug[43]. The average and relative weights of vital organs like heart, liver, and kidneys were found to be normal when compared with normal control group (Table 5), indicating there was no toxic effects of the samples on the organs of animals, and was found to be statistically non-significant. Checking of the blood profile by haematological parameters provides information whether the sample group has drug-associated toxicity in the blood system when compared with the normal control group, and there was some statistical significance ($^*p < 0.05$) by comparing sample treated groups with normal control group (Table 6). The biochemical parameters provide information on the hepatic and renal profile; observations remained within the normal physiological ranges with no adverse effects on the hepatic and renal functions. Our findings showed some statistical significance ($^*p < 0.05$) by comparing treatment group with normal control group (Table 7). The histopathological studies of vital organs such as heart, liver, and kidneys (Figure 5) showed no morphological changes or damages of the organs by comparing the standard and sample treated groups with the normal control group.

HPTLC analysis was carried out for identification, separation, visualization, and standardization of the phytocomponents present in the samples which is an essential step in authentication and standardization of medicinal plants. HPTLC is considered as a reliable indicator for compound identity in the research study of traditional medicinal plants due to the sensitivity and specificity of HPTLC[44]. The qualitative HPTLC analysis of two selected fractions PT78 and RC256 were performed to identify the presence of the standard markers scopoletin and taxifolin, where the fractions displayed distinct bands that matched the Rf values of the standards. Both the fractions exhibit bands corresponding to the scopoletin and taxifolin confirming their presence giving an Rf values of 0.708 ± 0.0 in PT78 and 0.706 ± 0.0 in RC256 which matched closely with scopoletin Rf values of 0.713 ± 0.002; and Rf values of 0.63 ± 0.017 in PT78 and 0.613 in RC256 which matched closely with taxifolin Rf values of 0.632 ± 0.0088 (Table 8). Thus, our HPTLC analysis validated the presence of coumarin (scopoletin) and flavonoid (taxifolin) supporting the therapeutic potential of the studied plant extract which serve as an effective approach for authentication, quality assessment, and standardization of the plant extract. Therefore,

assessments of these parameters provide information and supports the safety profile of *P. wallichii* for its therapeutic usage in pharmacological activity studies as well as its extensive use as a traditional medicine.

CONCLUSION

The present study provides a comprehensive investigation of the pharmacognostic standardization, phytochemical composition, and toxicological evaluation supporting the traditional medicinal use of *P. wallichii*. Organoleptic studies, quantitative and qualitative microscopic studies, and physicochemical parameters determine the identity, purity, and quality which is required in pharmacognostic standardization of the sample crude drug. The quantitative estimation of phenolic content and flavonoid content, along with the *in vitro* antioxidant assays revealed the presence and efficacy of significant bioactive composition in the methanolic and hydro-ethanolic extracts. Acute toxicity and sub-acute toxicity studies revealed that the extracts are safe upon oral administration and animals showed no signs of adverse effects and death throughout the study period. The hematological, biochemical, and histopathological findings also showed no significant changes which further demonstrates the safety of the studied samples. The HPTLC study findings confirms the presence of two important phytoconstituents scopoletin (coumarin) and taxifolin (flavonoid) by qualitative analysis of the fractions obtained from methanolic extract. For advancing this research findings, *in vitro* and *in vivo* pharmacological studies and in-depth study of the bioactive constituents are crucial to fully achieve the therapeutic potential of *P. wallichii* which will encourage and provides the link between traditional knowledge and modern pharmacological science for the therapeutic benefits.

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