Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Comparative Characterization of Four Medicinally Important Phyllanthus Species Using HPTLC, RP-HPLC, GC–MS/MS, ICP- OES, and Antioxidant Assays

Suman Mandal

sumanmandal785256@gmail.com

Central Ayurveda Research Institute

Lakshminarayana Misro

National Ayurveda Research Institute for Panchakarma

Puneet kumar Singh

Central Ayurveda Research Institute

Thirupataiah Boini

National Ayurveda Research Institute for Panchakarma

Biswajit Paul

Central Ayurveda Research Institute

Dhritismita Bayan

Central Ayurveda Research Institute

Anuja S Jeevan

National Ayurveda Research Institute for Panchakarma

Manajit Bora

Central Ayurveda Research Institute

Devanjal Bora

Central Ayurveda Research Institute

Prem Lal Bharti

Central Ayurveda Research Institute

Kousik Ghosh

Captain Srinivasa Murthy Central Ayurveda Research Institute

Vinodkumar Thallada

Captain Srinivasa Murthy Central Ayurveda Research Institute

Ajay Kumar Meena

Central Council for Research in Ayurvedic Science

Research Article

Keywords: Phyllanthus, HPTLC fingerprinting, RP-HPLC, GC–MS/MS, ICP-OES, Antioxidant activity

Posted Date: October 30th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11061113/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Comparative Characterization of Four Medicinally Important *Phyllanthus* Species Using HPTLC, RP-HPLC, GC–MS/MS, ICP-OES, and Antioxidant Assays

Suman Mandal[1*], Lakshminarayana Misro[2], Puneet Kumar Singh[1], Thirupataiah Boini[2], Biswajit Paul[1], Dhritismita Bayan[1], Anju S Jeevan[2], Manajit Bora[1], Devanjal Bora[1], Prem Lal Bharti1, Kousik Ghosh[3], Vinodkumar Thallada[3], and Ajay K. Meena[4]

*Correspondences

Suman Mandal

sumanmandal785256@gmail.com

[1]Department of Chemistry, Central Ayurveda Research Institute, Guwahati, Barsojai (Bhetapara), Beltola, Guwahati, Assam, India, Pin-781028

[2]Department of Chemistry, National Ayurveda Research Institute for Panchakarma, Cheruthuruthy, via Shoranur, Thrissur District, Kerala, India, Pin-679531

[3]Department of Chemistry, Captain Srinivasa Murthy Central Ayurveda Research Institute, 51, Anna Arch Rd, NSK Nagar, Anna Nagar, Chennai, Tamil Nadu, India, Pin- 600106

[4]Central Council for Research in Ayurvedic Sciences (CCRAS), 61-65, Opp. 'D' Block, Janakpuri Institutional Area, Janakpuri, New Delhi, Delhi, India-110058

Abstract

Phyllanthus species are widely recognized for their medicinal importance due to their wide range of therapeutic applications and diverse phytochemical composition, Nevertheless, there are still few comprehensive comparison studies that integrate elemental analy sis, metabolite profiling, chromatographic fingerprinting, and antioxidant evaluation across different Phyllanthus species. Four medicinally important *Phyllanthus* species (*P. sikkimensis, P. urinaria, P. amarus,* and *P. rheedei*) were comparatively evaluated using physicochemical and phytochemical analyses, HPTLC, RP-HPLC, GC–MS/MS, ICP-OES, TPC, TFC, and DPPH and ABTS antioxidant assays. Reproducible chromatographic fingerprints with both shared and species-specific bands were produced by HPTLC analysis, revealing unique phytochemical traits among the studied species. RP-HPLC analysis demonstrated that *P. sikkimensis* contained the highest concentrations of gallic acid (32.412 ± 0.002 mg/g) and ellagic acid (7.786 ± 0.005 mg/g), whereas *P. rheedei* exhibited the lowest concentrations of both phenolic markers among the four *Phyllanthus* species. GC–MS/MS detected 20, 30, 25, and 24 metabolites in *P. sikkimensis*, *P. urinaria*, *P. amarus*, and *P. rheedei*, respectively. All four species shared neophytadiene, phytol, squalene, 2-hexadecen-1-ol acetate, ascorbic acid 2,6-dihexadecanoate, linolenic acid and the chemotaxonomic markers lignans hypophyllanthin and niranthin. ICP-OES analysis demonstrated marked interspecific variation in mineral composition, with potassium being the predominant element. *P. sikkimensis* exhibited the strongest antioxidant activity and the highest phenolic and flavonoid concentrations among the taxa under investigation.

These findings offer thorough phytochemical, chemotaxonomic, and elemental evidence for disti nguishing pharmaceutically significant *Phyllanthus* species and establish solid quality control par ameters that will assist with their standardization, validity, and future pharmacological applicatio n.

Keywords: Phyllanthus; HPTLC fingerprinting; RP-HPLC; GC–MS/MS; ICP-OES; Antioxidant activity

1 Introduction

The genus *Phyllanthus* (family **Phyllanthaceae**), commonly known as "Bhuiamlki" in India, comprises more than 1000 species distributed worldwide (Webster, 1994; Unander et al., 1995). Species of this genus exhibit diverse growth forms, including trees, shrubs, and herbs (Sarin et al., 2014). Traditionally, species of *Phyllanthus* have been widely used in the management of liver, kidney, and bladder disorders, as well as intestinal parasitic infections and diabetes (Khatoon et al., 2006). Their broad therapeutic spectrum has been largely attributed to the presence of structurally diverse secondary metabolites, including lignans, flavonoids, tannins, phenolic acids, alkaloids, terpenoids, and hydrolysable tannins, many of which exhibit antioxidant, hepatoprotective, antiviral, antimicrobial, anti-inflammatory, immunomodulatory, and anticancer activities (Geethangili and Ding 2018; Mohanty et al., 2023).

Among the medicinally important members of the genus, *Phyllanthus amarus* and *P. urinaria* have been extensively investigated and are recognized as important sources of lignans such as phyllanthin, hypophyllanthin, and niranthin, together with phenolic constituents including gallic acid and ellagic acid (Shanker et al., 2011; Annamalai et al., 2017; Patel et al., 2021). *Phyllanthus sikkimensis*, commonly referred to as Sikkim leaf-flower, is a relatively underexplored species. It has been traditionally used for the treatment of fever, dysentery, wounds, ulcers, and gastrointestinal disorders. Methanolic leaf extracts of this plant have also been reported to exhibit analgesic activity (Al Masum et al., 2016). *Phyllanthus rheedei* has been used by the Muthuvan tribes of Kerala for the treatment of liver disorders. Previous studies have demonstrated the hepatoprotective potential of its ethanol extract against d-galactosamine-

induced liver damage in experimental models (Suresh and Asha, 2008). Comparative investigations involving these lesser-studied species are therefore essential to establish their phytochemical characteristics, authenticate botanical identity, and evaluate their medicinal potential.

Reliable quality control and authentication of medicinal plants require the integration of complementary analytical techniques capable of evaluating both chemical composition and biological activity. High-performance thin-layer chromatography (HPTLC) provides rapid and reproducible fingerprint profiles for species authentication, whereas reverse-phase high-performance liquid chromatography (RP-HPLC) enables accurate quantification of important phenolic marker compounds such as gallic acid and ellagic acid. Similarly, GC–MS/MS facilitates comprehensive metabolite profiling and identification of chemotaxonomic marker compounds, while ICP-OES offers precise determination of nutritionally important and potentially toxic mineral elements. Combined with measurements of total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity, these analytical approaches provide a comprehensive assessment of the quality, chemical diversity, and therapeutic potential of medicinal plants.

Although several studies have independently reported the phytochemistry or pharmacological properties of individual *Phyllanthus* species, comprehensive comparative investigations integrating chromatographic fingerprinting, quantitative marker analysis, metabolite profiling, elemental composition, and antioxidant evaluation across multiple medicinally important species remain scarce. To the best of our knowledge, no previous study has simultaneously compared *Phyllanthus sikkimensis*, *P. urinaria*, *P. amarus*, and *P. rheedei* using an integrated multi-platform analytical approach.

Therefore, the present study aimed to perform a comprehensive comparative evaluation of four medicinally important *Phyllanthus* species through physicochemical and preliminary phytochemical characterization, HPTLC fingerprint profiling, RP-HPLC quantification of gallic acid and ellagic acid, GC–MS/MS metabolite profiling, ICP-OES-based elemental analysis, and determination of total phenolic content, total flavonoid content, and antioxidant activities using DPPH and ABTS radical scavenging assays. The findings are expected to provide valuable chemotaxonomic information, establish reliable quality control parameters, and facilitate the standardization and future pharmacological utilization of these medicinally important *Phyllanthus* species.

2 Materials and Methods

2.1 Plant collection and authentication

All the plant materials *Phyllanthus amarus* Schumach. & Thonn., *Phyllanthus sikkimensis* Müll.Arg., *Phyllanthus urinaria* L., *Phyllanthus rheedei* Wight were collected from Rani Khamar, Kamrup district of Assam (25°51'00.4"N 91°21'43.5"E). The botanical identity of the species was formally authenticated by Dr. Devanjal Bora, Research Officer (Botany) and Mr. Biswajit Paul, Research Assistant (Botany), Central Ayurveda Research Institute, Barsojai (Bhetapara), Beltola, Guwahati, Assam, Pin-781028. and representative herbarium specimens were assigned the unique Voucher Number like *Phyllanthus amarus* Schumach. & Thonn. (NEHAR-8314), *Phyllanthus sikkimensis* Müll.Arg. (NEHAR-8315), *Phyllanthus urinaria* L. (NEHAR-8316), *Phyllanthus rheedei* Wight (NEHAR-8317) and has been deposited in the Index Herbarium NEHAR of the same institution for future reference. The leaves of the plants were used in this study.

2.2 Preparation of sample for HPTLC Analysis

1 g of the powdered plant material was sonicated with 10 mL of methanol for 15 min at 40°C. The extract was then filtered, and the filtrate was collected and stored at 5°C until further analysis. The sample was subsequently diluted to a concentration of 50 mg/mL for HPTLC analysis.

2.2.1 HPTLC Instrumentation

High-Performance Thin Layer Chromatography (HPTLC) analysis was carried out using a CAMAG HPTLC system, consisting of a Linomat V sample applicator, TLC Scanner IV, TLC Visualizer, and VisionCATS 3.2 software for data acquisition, processing, and quantification. Pre-coated silica gel 60 F_{254} plates (10 × 10 cm) were used as the stationary phase. Various mobile phase combinations were evaluated to achieve optimal separation. The best resolution and Rf values were obtained using a mobile phase composed of toluene:ethyl acetate:methanol:formic acid in the ratio of 6:4:0.5:0.5 (v/v/v/v).The sample extracts were applied onto the TLC plates using a 100 µL CAMAG syringe with the Linomat V applicator. Plate development was performed in a CAMAG Twin Trough Chamber (TTC), pre-saturated with the mobile phase for 20 minutes at 25 ± 2°C. After development, the plates were air-dried at room temperature for 5 minutes and subsequently derivatized using anisaldehyde sulphuric acid reagent (ASR). The plates were then heated at 105°C using a CAMAG plate heater until characteristic-coloured spots appeared. Following derivatization, the plates were re-visualized and scanned densitometrically at 545 nm using the TLC Scanner IV (Wagner and Bladt, 1996).

2.3 Preparation of Standard and Sample for HPLC Analysis

For sample preparation, 1 g of the finely powdered plant material was accurately weighed and transferred into a 25 mL solvent bottle. Subsequently, 10 mL of HPLC-grade methanol was added, and the mixture was sonicated for 15 minutes at 40°C. The resulting methanolic extract

was filtered through a 0.22 µm PTFE syringe filter. The filtrate was then diluted to a concentration of 10,000 ppm and transferred into HPLC vials for subsequent analysis and the standard solution was prepared by standard method (Misro et al., 2026).

2.3.1 RP-HPLC Instrumentation

A Reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed for the quantitative determination of gallic acid and ellagic acid in the different *Phyllanthus* species. The analytical technique was developed using Shimadzu RP-HPLC, model: i – series LC- 2050C fitted with a C18 column (250 mm × 4.6 mm, 5 µm particle size). The efficient chromatographic separation was achieved by the isocratic low pressure gradient elution with mobile phase comprising of 0.3 % orthophosphoric acid in water and methanol in the ratio (30 - 70 v/v). Both 0.3 % orthophosphoric acid in water and methanol were degassed for 30 minutes using ultrasonic bath sonicator. The injection volume was 10 µL for each injection. The flow rate was set at 1 ml/min. The column oven temperature was maintained at 35^0C. PDA (photo diode array) detector was used for the detection and detection was carried out at 272 nm and 367nm (GA & EA). The runtime for the analysis was 30 min. Detailed conditions are provided in Table 1.

Table 1 RP-HPLC Instrumentation data

S. No.	Parameters	Unit	Value
1.	Injection Volume	µL	10
2.	Flow Rate	mL/ min	1
3.	Column Temperature	ºC	35
4.	Run Time	Min	30
5.	Wavelength	nm	272 (GA), 367 (EA)

6.	Retention time (R_t)	Min	GA – 2.682, EA – 3.600
7.	Detector	-	Photo Diode Array Detector (PDA)
8.	Column	mm	C_{18} 5 µm particle size, and diameter× length 4.6 × 250
9.	Pressure	Kgf/cm^2	300
10.	Mode	-	Low-pressure gradient, Isocratic mode
11.	Mobile Phase	-	Methanol: 0.3 % orthophosphoric acid in water (70: 30)

2.4 Preparation of Sample for GC-MS/MS Analysis

Approximately 1.0 g of the powdered sample was extracted with 10 mL of HPLC-grade ethyl acetate by sonication for 30 min at room temperature. The extract was filtered through Whatman No. 1 filter paper, and then again, the solution was passed through a 0.22 µm PTFE syringe filter to remove suspended particles. An aliquot of the filtered extract was transferred to a 2 mL amber GC vial fitted with a PTFE/silicone septum, and 1 µL of the sample was injected into the GC–MS/MS system for analysis.

2.4.1 GC–MS Instrumentation

GC–MS analysis of *Phyllanthus* species extracts was performed using a Shimadzu GC-MS-TQ8050 NX System equipped with an SH-I-5Sil MS capillary column and helium as carrier gas. Compounds were identified based on retention time and mass spectra by comparison with the National Institute of Standards and Technology (NIST) library (National Institute of Standards and Technology, 2020); detailed conditions are provided in Table 2.

Table 2 GC–MS/MS Instrumentation data

S. No.	Parameters	Unit	Values
1.	Column	-	H-I-5Sil MS capillary column (30 m × 0.25 mm I.D. ×

			0.25 µm film thickness)
2.	Carrier Gas	-	Helium (99.999% purity)
3.	Carrier Gas Flow Rate	mL/min	1.0
4.	Injection Volume	µL	1.0
5.	Injection Mode	-	Splitless
6.	Injector Temperature	°C	250
7.	GC Oven Program	-	60°C (2 min), ramp at 10°C/min to 280°C, hold for 10 min
8.	Ionization Mode	-	Electron Ionization (EI)
9.	Electron Energy	eV	70
10.	Ion Source Temperature	°C	230
11.	Interface Temperature	°C	280
12.	Solvent Cut Time	Min	3.0
13.	Acquisition Mode	-	Electron Ionization (EI)
14.	Mass Scan Range	-	*m/z* 40–600
15.	Detector	-	Triple Quadrupole Mass Analyzer
16.	Identification	-	NIST Mass Spectral Library

2.5 Sample preparation for ICP-OES analysis and Instrumentation

A simple two-step microwave digestion method was carried out to prepare each sample. Samples weighing accurately 200 mg were put in polytetrafluoroethylene-tetrafluoromethane (PTFE-TFM) containers. 8 mL of NIST traceable 67–70% concentrated nitric acid (HNO_3) was then added to each vessel. Following that, the medium pressure vessels were tightly sealed and

subjected to a microwave using a rotor-equipped Anton Paar microwave digestion system. In this process, A two-step microwave digestion program was employed to set the temperatures to 100, and 180 °C. The samples were completely digested at 180 °C. As a result, we carried out the procedure for each sample, fixing the end temperature to 180 °C. Digested samples were properly filtered through Whatman No. 41 filter paper once the samples had reached room temperature. The filtered samples were then stored as a stock sample solution after being diluted 50 mL with pure Milli-Q water. The NIST traceable multi-element standard (1000 ppm) in high purity for each specified element was employed to make the standard solution. Solutions with different concentrations, including 0.1, 0.5, 2, 5, 10, 20, and 50 ppm, were prepared from this stock standard. For every element, linear calibration curves with correlation coefficients of 0.9998 were obtained. Table 3 illustrates the microwave digesting system's various operational parameters.

Table 3 Different operational parameters of the Microwave Digestion system

S. No.	Parameters	Unit	Value
1.	Power	W	1030–1800
2.	Step 1: Maximum Temperature	°C	100
3.	Step 1: Ramp Time	Min	10
4.	Step 1: Hold Time	Min	15
5.	Step 2: Maximum Temperature	°C	180
6.	Step 2: Ramp Time	Min	10
7.	Step 2: Hold Time	Min	30

2. 5.1 ICP-OES Instrumentation

Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) with an Agilent SPS4 Autosampler (Agilent 5800) was utilized for elemental analysis. A double-pass cyclonic spray chamber, the Sea Spray glass concentric nebulizer, and the Easy-fit semi-demountable VDV torch with an inner diameter of 1.8 mm are all included. The ICP Expert Pro software program was utilized to establish, refine, and administer all of the variable parameters linked to the ICP-OES method throughout the experiment. Table 4 provides a comprehensive overview of the various operational parameters employed in the ICP-OES instrument, along with their individual values and settings.

Table 4 Various operational parameters of the ICP-OES

S. No.	Parameters	Unit	Value
1.	Sample Uptake time	*Sec*	*30*
2.	Stabilization time	*Sec*	*15*
3.	Rinse Time	*Sec*	*30*
4.	Pump Speed	*Rpm*	*12*
5.	RF Power	*KW*	*1.25*
6.	Auxiliary Flow	*L/min*	*1.0*
7.	Plasma Flow	*L/min*	*12.5*
8.	Nebulizer Flow	*L/min*	*0.7*
9.	Viewing Height	*mm*	*16*
10.	Makeup Flow	*L/min*	*0.0*
11.	Beam selector Position	-	*Axial*
12.	Peltier	°C	*-40*
13.	Polychromator	°C	*35*

2.6 Preparation of Sample Extract for Total Phenolic, Total Flavonoid, and Antioxidant Studies

Exactly 2 g of dried *Phyllanthus* powder was extracted with 100 mL of methanol and subjected to continuous agitatation for full day using a mechanical shaker. The resulting mixture was filtered through Whatman No. 41 filter paper and filtrate was used for study.

2.6.1 DPPH Free Radical Scavenging Activity

5 mL of the methanolic extract of *Phyllanthus* species at different concentrations (2.5, 5, 10, 15, 20, and 25 µg/mL) was mixed with 5 mL of the prepared DPPH solution (Concentration 40 µg/mL). The absorbance was then measured at 517 nm using a UV–Visible spectrophotometer. The percentage of DPPH radical scavenging activity was calculated using the following formula: [Ac - (As - Ao)] x 100 / Ac. Here, Ac stands for absorbance of control DPPH solution, As for absorbance of sample or standard + DPPH, and Ao for absorbance of sample without DPPH interaction (Mandal et al., 2025). Ascorbic acid was used as reference standard.

2.6.2 ABTS Free Radical Scavenging Assay

5 mL of *Phyllanthus* extract at different concentrations (8, 16, 32, 48, 64, and 80 µg/mL) was mixed with 5 mL of the prepared ABTS solution (Concentration1000 µM). The reaction mixtures were thoroughly shaken and allowed to stand for 3 minutes at room temperature. The absorbance was then measured at 734 nm using a UV–Visible spectrophotometer. The percentage of ABTS radical scavenging activity was calculated using the following formula used for DPPH radical scavenging (Mandal et al., 2025). Trolox was used as reference standard.

2.6.3 Total Phenolic Content

The total phenolic content (TPC) of methanolic extract *Phyllanthus* species was determined using the Folin–Ciocalteu reagent (FCR) method. The absorbance of the resulting blue-coloured

complex was measured at 765 nm using a UV–Visible spectrophotometer. The total phenolic content of the extracts was calculated from the calibration curve constructed using gallic acid and expressed as µg gallic acid equivalents (GAE) per mg of dry extract (Mandal et al., 2025). Experimental details are provided in the Supplementary Information **[S1]**.

2.6.4 Total Flavonoid Content

The total flavonoid content (TFC) of methanolic extract *Phyllanthus* species was determined using the aluminium chloride ($AlCl_3$) colorimetric assay. The absorbance was measured at 436.5 nm using a Shimadzu UV–Visible spectrophotometer. The total flavonoid content was calculated from the calibration curves and expressed as µg Quercetin equivalents (QE)/mg and µg Rutin equivalents (RE)/mg of dry sample (Mandal et al., 2025). Experimental details are provided in the Supplementary Information **[S2]**.

2.7 Physico-Chemical and Phytochemical Analysis

All physico-chemical and phytochemical analyses were carried out in accordance with standard pharmacopoeial procedures (World Health Organization, 1998).

2.8 Statistical Analysis

All experiments were performed in triplicate, unless otherwise stated. The results were presented as mean ± SD (standard deviation). Pearson's correlation analysis was performed to evaluate the relationships among antioxidant activity, total phenolic content (TPC), total flavonoid content (TFC), gallic acid, and ellagic acid.

3 Results and discussion

3.1 HPTLC Analysis

Comparative HPTLC analysis of methanolic extract of four *Phyllanthus* species (*P. urinaria*, *P. amarus*, *P. rheedei*, and *P. sikkimensis*) revealed distinct and reproducible fingerprint profiles under UV (254 nm and 366 nm) and post-derivatization conditions.

In HPTLC fingerprint profiling, at 254 nm, all species exhibited multiple well-defined bands, indicating the presence of UV-absorbing phytoconstituents such as phenolics and lignans. Under UV light at 254 nm, the chromatograms exhibited 4, 7, 6, and 3 prominent dark bands, respectively, each with characteristic Rf values. A common Rf range of approximately 0.50–0.55 was observed across all species, suggesting the presence of shared bioactive compounds within the genus. However, variations in band number and distribution—particularly the higher number of bands observed in *P. amarus* compared to *P. sikkimensis*—indicate interspecific phytochemical diversity.

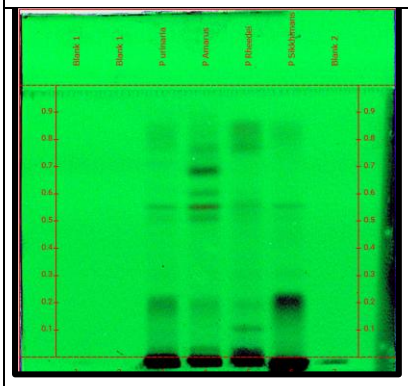
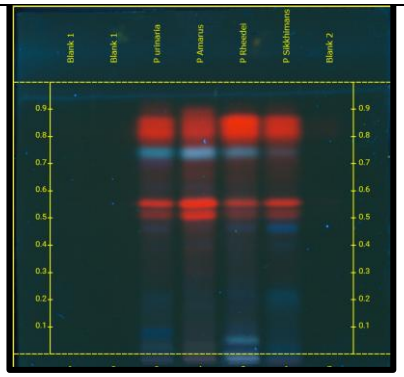
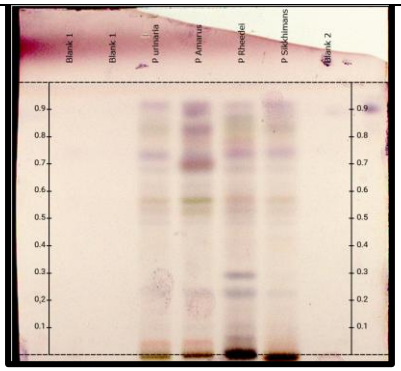
At 366 nm, enhanced fluorescence enabled improved visualization of secondary metabolites. The presence of blue, red, and cyan fluorescent bands suggests the occurrence of flavonoids, coumarins, and related conjugated compounds. Upon observation at 366 nm, fluorescence detection revealed 7, 9, 8, and 8 bands, respectively, indicating the presence of diverse fluorescent phytoconstituents. A consistent band around Rf ~0.73 was observed in all species, indicating conserved metabolites, while additional fluorescent bands in *P. amarus* and *P. rheedei* may serve as distinguishing chemical markers.

Following derivatization with anisaldehyde–sulphuric acid reagent (ASR), the chromatographic plates were examined under white light, where 8, 7, 11, and 8 distinct colored bands were observed for *P. urinaria*, *P. amarus*, *P. rheedei*, and *P. sikkimensis*, respectively, each possessing characteristic *R*f values. The appearance of violet, pink, green, and brown bands reflect the presence of diverse chemical classes such as phenolics, terpenoids, and sugars. Among the

studied species, *P. rheedei* exhibited the highest number of derivatized bands, suggesting a comparatively richer phytochemical profile, whereas *P. sikkimensis* showed fewer but distinct bands useful for species identification. The developed chromatographic profiles and corresponding Rf values are presented in Table 5 and Fig. 1.

Table 5 Rf values and colour of spots

Sl. No.	**Sample**	**Developed plate**		**Derivatized plate**
		@ 254 nm	**@ 366 nm**	**@ White light**
1	*P. urinaria*	0.21, 0.55, 0.71,0.78	0.09(blue), 0.16(blue), 0.46(light brown), 0.51(light red), 0.55(red), 0.73(cyan blue), 0.80(red)	0.05(brown), 0.22(violet), 0.49(light pink), 0.56(brown), 0.67(light green), 0.73(violet), 0.82(light green), 0.91(violet)
2	*P. amarus*	0.15, 0.50, 0.55, 0.60, 0.67, 0.76, 0.82	0.16(light blue), 0.31(brown), 0.40(brown), 0.49(red), 0.53(red), 0.65(brown), 0.71(cyan blue), 0.80(red), 0.88(red)	0.22(violet), 0.51(violet), 0.56(green), 0.67(violet), 0.73(pink), 0.81(pink), 0.87(violet)
3	*P. rheedei*	0.09, 0.18, 0.52, 0.54, 0.75, 0.85	0.05(cyan blue), 0.20(blue), 0.46(cyan blue), 0.50(light brown), 0.55(red), 0.73(cyan blue), 0.78(red), 0.84(red)	0.06(blue), 0.11(pink), 0.21(violet), 0.28(violet), 0.48(pink), 0.52(light pink), 0.56(brown), 0.68(light blue), 0.74(violet), 0.83(green), 0.90(pink)
4	*P. sikkimans*	0.17, 0.54, 0.77	0.16(cyan blue), 0.39(brown), 0.45(cyan blue), 0.50(red), 0.55(red), 0.73(cyan blue), 0.79(red), 0.84(red)	0.08(pink), 0.21(violet), 0.47(pink), 0.56(brown), 0.68(pink), 0.74(violet), 0.81(green), 0.89(violet)

At UV 254 nm	At UV 366 nm	Post-derivatization under white light
		
1 2 3 4	1 2 3 4	1 2 3 4

Track 1: *P. Urinaria*; **Track 2:** *P. Amarus*; **Track 3:** *P. Rheedei*; **Track 4:** *P. Sikkimensis*

Fig. 1 HPTLC fingerprint profiling of *Phyllanthus* species

3.2 RP-HPLC Analysis

Quantitative estimation of gallic acid (GA) and ellagic acid (EA) in four *Phyllanthus* species (*Phyllanthus urinaria*, *P. amarus*, *P. rheedei*, and *P. sikkimensis*) was carried out using reverse-phase high-performance liquid chromatography (RP-HPLC).

Among the investigated taxa, *P. sikkimensis* showed the highest concentration of both EA (**7.786 ± 0.005 mg/g**) and GA (**32.412 ± 0.002 mg/g**), suggesting a comparatively greater abundance of hydrolysable tannin-derived phenolic constituents. In contrast, *P. rheedei* contained the lowest concentration of both EA (**0.162 ± 0.001 mg/g**) and GA (**2.737 ± 0.002 mg/g**), indicating a comparatively reduced accumulation of these marker constituents. Intermediate concentrations were recorded in *P. urinaria* and *P. amarus*. The concentration pattern observed across species

showed that GA was consistently present at higher levels than EA. The quantitative data and chromatograms are presented in Table 6 and Fig. 2.

Table 6 Concentration (mg/g) of EA and GA

Plant name	**Concentration (mg/g)**	
	EA	**GA**
P. urinaria	1.994±0.004	25.213±0.002
P. amarus	0.682±0.001	7.909±0.005
P. rheedei	0.162±0.001	2.737±0.002
P. sikkimensis	**7.786±0.005**	**32.412±0.002**

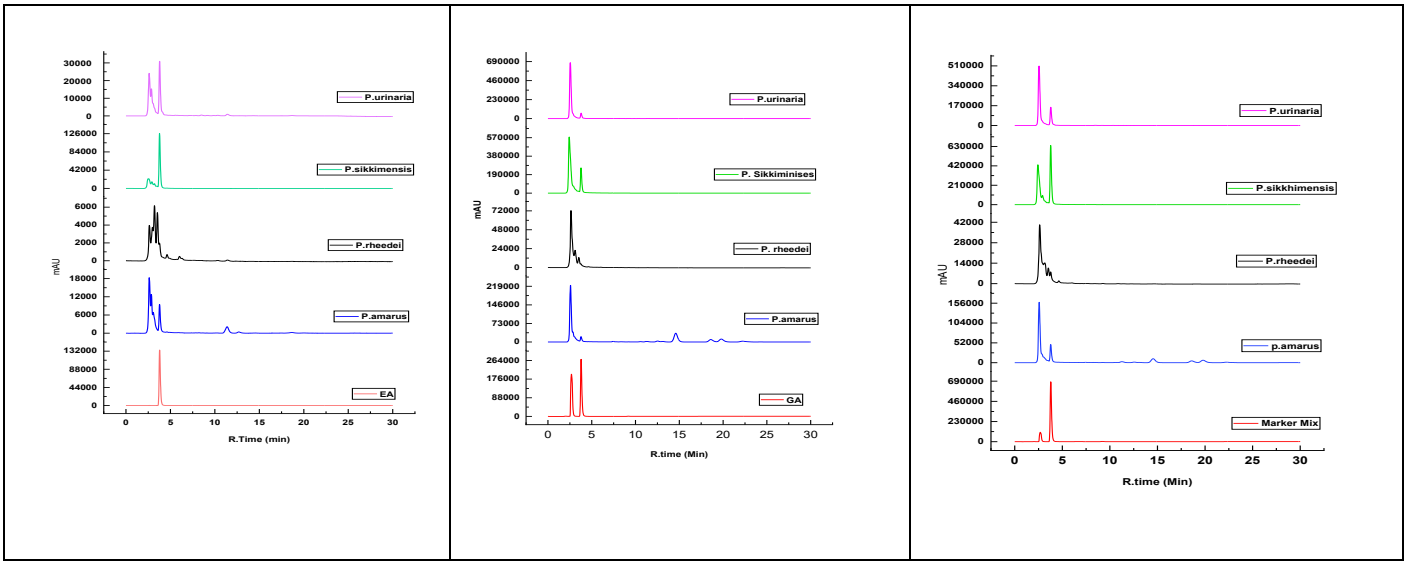


Fig. 2 HPLC chromatogram of *Phyllanthus* species and marker compounds

A common pattern of peaks was consistently observed across all *Phyllanthus* species analyzed, with the exception of *P. amarus*. These shared peaks exhibited retention times around 11.12, 11.46, and 11.89 minutes, suggesting the presence of structurally related or identical compounds across *P. sikkimensis*, *P. rheedei*, and *P. urinaria*. The absence of these peaks in *P. amarus*

indicates a potential chemotypic variation within the genus, which may be attributed to species-specific biosynthetic pathways or ecological adaptations **[S3].**

One of the earlier quantitative chromatographic studies reported the simultaneous estimation of phyllanthin, hypophyllanthin, gallic acid, and ellagic acid in *Phyllanthus amarus* (Dhalwal et al., 2006). The reported concentrations of phyllanthin, hypophyllanthin, gallic acid, and ellagic acid were 0.37% (w/w), 1.16% (w/w), 0.36% (w/w), and 0.17% (w/w), respectively. However, in the present study, the concentrations of gallic acid and ellagic acid detected in *P. amarus* were considerably higher than those reported previously.

3.3 GC–MS/MS profiling

GC–MS/MS analysis was performed to characterize the bioactive constituents present in the four *Phyllanthus* species. A total of 20, 30, 25, and 24 compounds were identified in ethyl acetate extracts of *P. sikkimensis*, *P. urinaria*, *P. amarus*, and *P. rheedei*, respectively. Comparative analysis revealed 6 compounds that were consistently detected across all four species, including neophytadiene, phytol, squalene, 2-Hexadecen-1-ol acetate, Ascorbic acid 2,6-dihexadecanoate, Linolenic acid. The widespread occurrence of these compounds suggests that they represent characteristic phytochemical constituents of the investigated *Phyllanthus* species. A Venn diagram was constructed to illustrate the distribution of GC–MS-identified compounds among the four *Phyllanthus* species, facilitating a comparative analysis of shared and species-specific phytoconstituents which is presented in Fig.3.

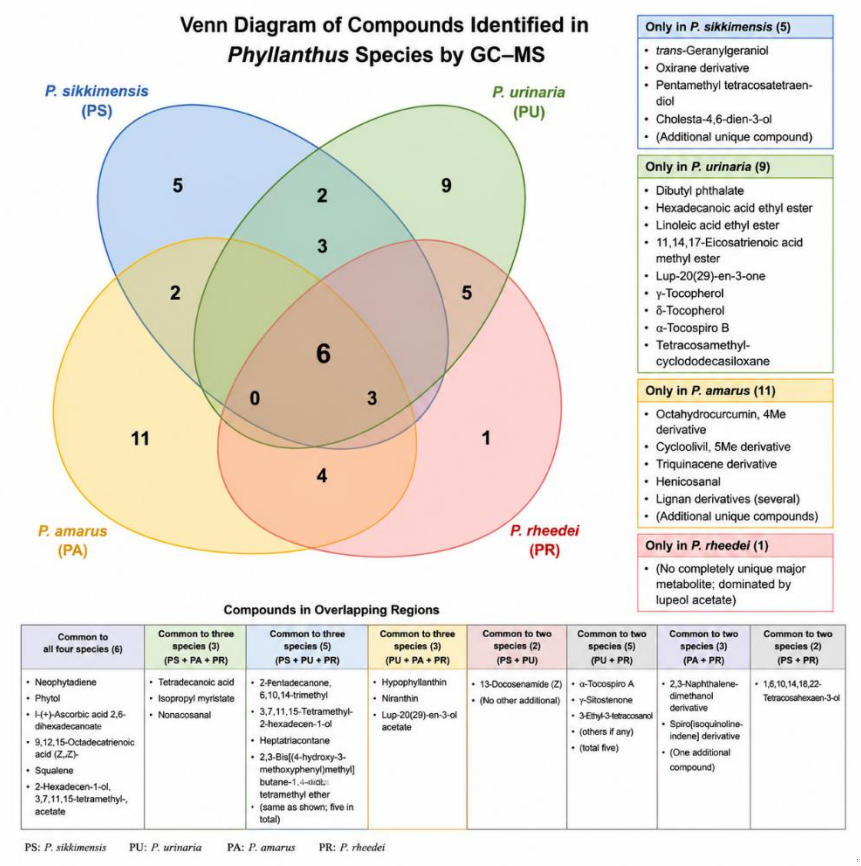


Fig.3 Venn diagram of compounds identified in *Phyllanthus* species

Among the identified metabolites, hypophyllanthin and niranthin, which are well-recognized chemotaxonomic marker lignans of the genus *Phyllanthus*, were detected in all four species. GC–MS/MS analysis revealed the presence of the lignans hypophyllanthin and niranthin in the ethyl acetate extracts of *P. urinaria*, *P. amarus*, and *P. rheedei*. In contrast, these two compounds were detected in the hexane extract of *P. sikkimensis* **[S4]**. However, their relative abundance was considerably lower in *P. sikkimensis*, *P. urinaria*, and *P. rheedei* than in *P. amarus*, where both compounds were present at comparatively higher concentrations. The GC–MS/MS total ion chromatograms (TICs) of the four *Phyllanthus* species are presented in Fig. 4, Fig. 5, Fig. 6, and Fig. 7.

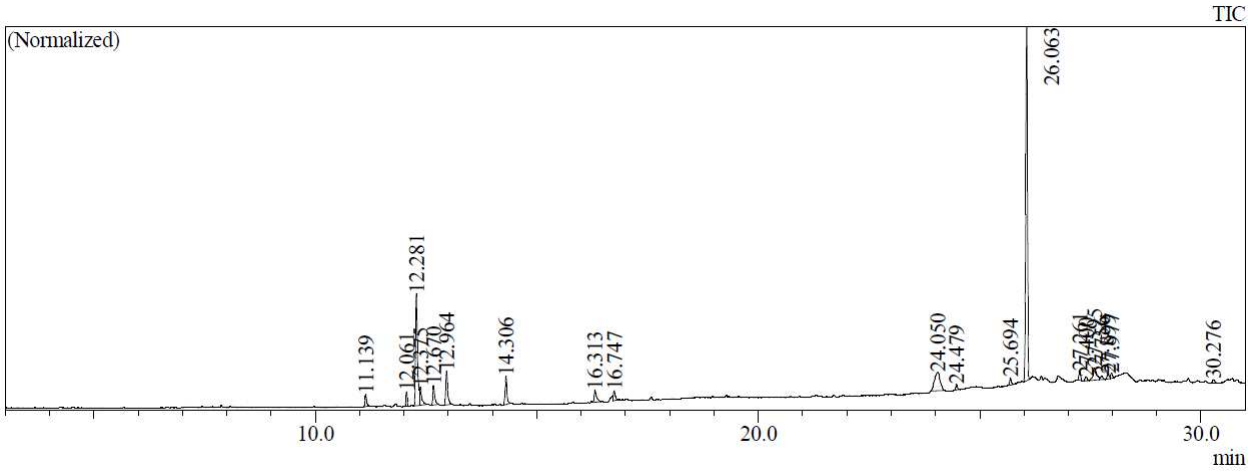


Fig. 4. GC-MS/MS Total Ion Chromatogram (TIC) of *P. sikkimensis*

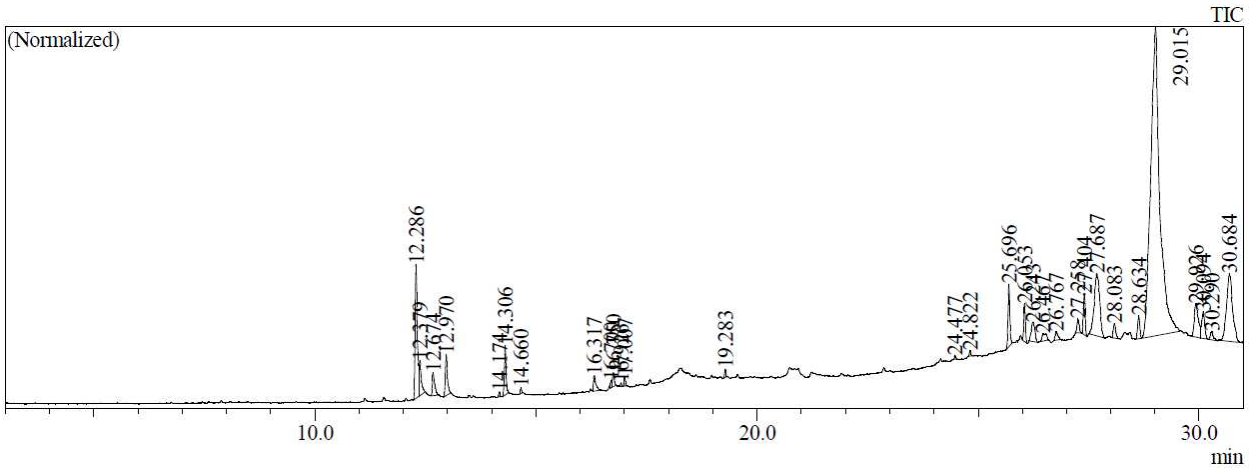


Fig. 5. GC-MS/MS Total Ion Chromatogram (TIC) of *P. urinaria*

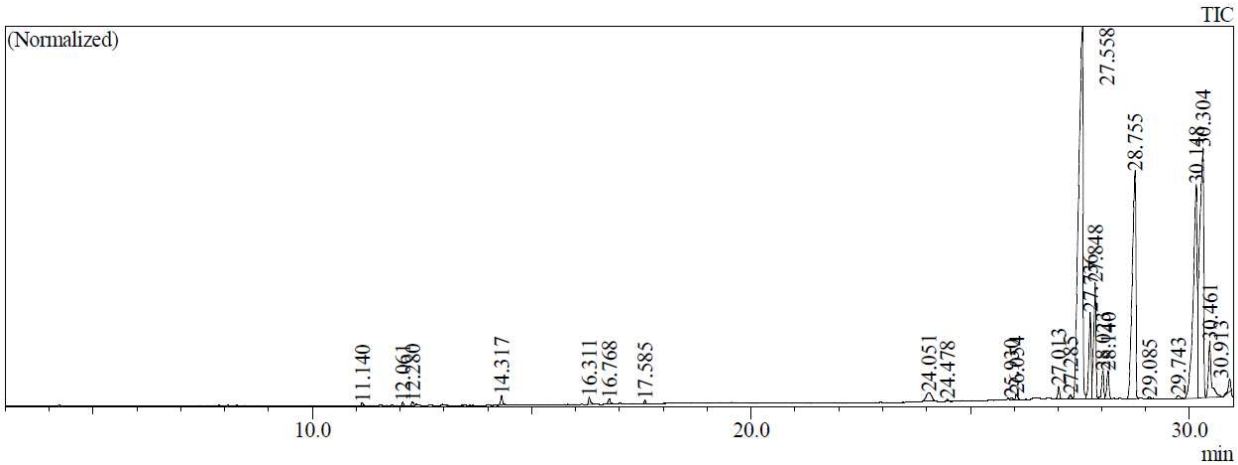


Fig. 6. GC-MS/MS Total Ion Chromatogram (TIC) of *P. amarus*

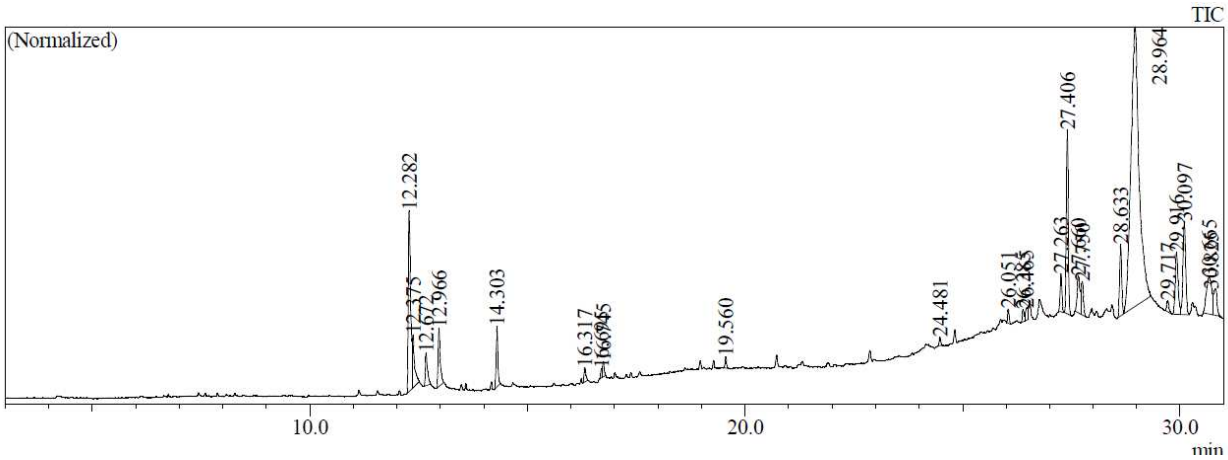


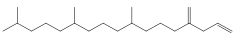
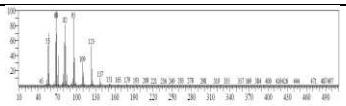
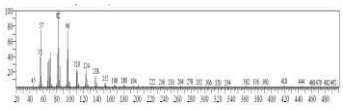
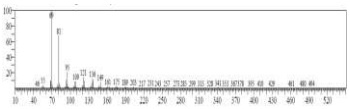
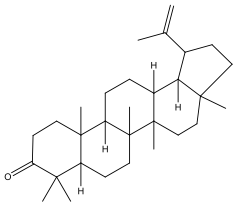
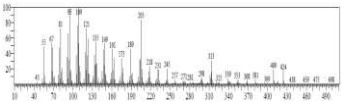
Fig. 7. GC-MS/MS Total Ion Chromatogram (TIC) of *P. rheedei*

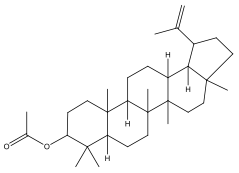
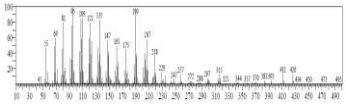
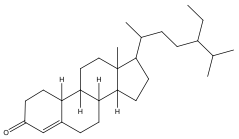
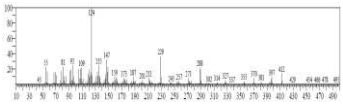
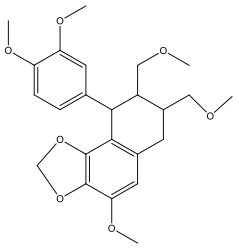
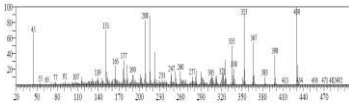
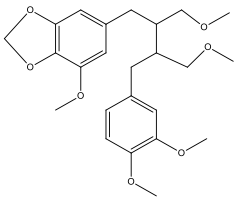
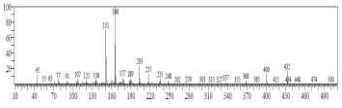
The predominant constituents of *P. sikkimensis* were squalene (45.05%), neophytadiene (16.38%), and nonacosanal (8.02%). In *P. urinaria*, the major compounds identified were lup-20(29)-en-3-ol, acetate (3β)- (56.33%), γ-sitostenone (8.22%), lup-20(29)-en-3-one (6.87%), and neophytadiene (5.76%). The principal constituents of *P. amarus* included Secoisolariciresinol tetramethyl ether, tetramethyl ether (31.15%), niranthin (19%); and hypophyllanthin (15.47 %). In contrast, the major constituents detected in *P. rheedei* were lup-20(29)-en-3-ol, acetate (3β)- (47.84%), neophytadiene (8.66%), 2,3-bis[(4-hydroxy-3-methoxyphenyl)methyl]butane-1,4-diol, tetramethyl ether (7.31%), niranthin (5.33%), γ-sitostenone (4.24%) and hypophyllanthin (3.72%).

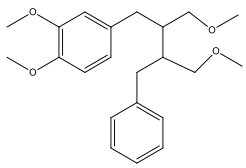
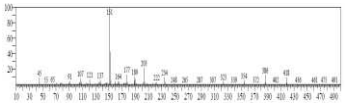
The chemical structures, characteristic mass fragmentation patterns, molecular formula, mass peak and reported biological activities of the major bioactive compounds are summarized in Table 7.

Table 7 Major Phytoconstituents Identified in the *Phyllanthus* Species

Phytochemical compounds	Mol. formula	Structure	Mass peak	Mass fragmentation patterns	Biological activity

Neophytadiene	$C_{18}H_{38}$		266		Anti-inflammatory, Antioxidant, Antimicrobial (Rajeswaran et al., 2025)
Nonacosanal	$C_{29}H_{58}O$		296		Antimicrobial (Soares et al., 2017)
Squalene	$C_{30}H_{50}$		347		Antioxidant (Huang et al., 2009), Anti-inflammatory (Popa et al., 2015), Anticancer (Smith, 2000), Cardioprotective (Huang et al., 2009)
Lup-20(29)-en-3-one	$C_{30}H_{48}O$		370		Anticancer (Shanmugam et al., 2014), Anti-obesity (Siddique and Saleem, 2020), Antioxidant (Dzubak et al., 2006)

Lup-20(29)-en-3-ol, acetate, (3.beta.)-	$C_{32}H_{52}O_2$		378		Anticancer (Shanmugam et al., 2014), Anti-obesity (Siddique and Saleem, 2020), Antioxidant (Dzubak et al., 2006)
.gamma.-Sitostenone	$C_{29}H_{48}O$		376		Antidiabetic (Kumar et al., 2021),
Hypophyllanthin	$C_{24}H_{30}O_7$		259		Immunomodulatory (Harikrishnan et al., 2018), anti-inflammatory (Suresh and Asha, 2008), Anti-allergic (Abd Rani NZ et al., 2021)
Niranthin	$C_{24}H_{32}O_7$		298		Anti-hepatitis B (HBV) (Liu et al., Antiviral (Nikule et 2024) and omodulatory (Jantan 019)

Secoisolariciresinol tetramethyl ether	$C_{24}H_{34}O_6$		350		Antioxidant (Pan et al, 2009), and Phytoestrogenic (Adlercreutz, 2007)

3.4 Mineral analysis

Essential elements such as potassium, iron, copper, manganese, and zinc play important roles in enzyme activation, antioxidant defence, photosynthesis, and various metabolic processes. Therefore, the differences in mineral composition among these *Phyllanthus* species may contribute to their nutritional value and therapeutic potential and could partly explain variations in their pharmacological activities. ICP–OES analysis revealed considerable variation in the mineral composition among the four *Phyllanthus* species. Potassium (K) was the most abundant element in all species, with concentrations ranging from 4609.70 to 5678.04 mg/kg, indicating that all four species are rich sources of this essential macronutrient. *P. sikkimensis* exhibited the highest potassium content (5678.04 mg/kg), closely followed by *P. rheedei* (5659.50 mg/kg), whereas *P. amarus* contained the lowest level (4609.70 mg/kg).

Among the micronutrients, *P. rheedei* showed the highest concentrations of aluminium (985.45 mg/g), cobalt (0.23 mg/kg), copper (4.92 mg/kg), iron (675.20 mg/kg), nickel (1.64 mg/kg), and zinc (28.48 mg/kg), suggesting its greater capacity for mineral accumulation. In contrast, *P. urinaria* contained the second-highest levels of aluminium (761.25 mg/kg) and iron (581.20 mg/kg), while also exhibiting the highest sodium concentration (154.11 mg/kg). *P. sikkimensis* was characterized by the highest manganese content (85.42 mg/kg) but relatively lower concentrations of most trace elements compared with *P. rheedei*. *P. amarus* showed the highest

sodium (195.52 mg/kg) and comparatively high zinc (24.78 mg/kg) but contained the lowest levels of potassium, manganese, and nickel. The quantitative data are presented in Table 8.

Table 8 Mineral composition in *Phyllanthus* species

Plant name	Concentration (mg/kg)								
	Al	Co	Cu	Fe	K	Na	Ni	Mn	Zn
P.sikkimensis	144.71	0.04	2.16	117.93	5678.04	79.96	0.91	85.42	19.24
P.urinaria	761.25	0.18	4.12	581.2	5092.46	154.11	0.94	44.81	17.48
P.amarus	204.24	0.13	4.05	285.6	4609.7	195.52	0.54	22.62	24.78
P.rheedei	985.45	0.23	4.92	675.2	5659.5	82.48	1.64	47.06	28.48

3.5 Physico-Chemical Analysis

Physico-chemical evaluation is an important component of pharmacognostic standardization because it provides information on the purity, quality, stability, and extraction efficiency of plant materials. In the present study, notable variation was observed among the four investigated *Phyllanthus* species with respect to moisture content, ash values, extractive values, and pH. *P. sikkimensis* showed the highest LOD (**6.694 ± 0.02 %**), whereas *P. urinaria* exhibited the lowest value (**3.489 ± 0.027 %**). Among the evaluated species, *P. urinaria* recorded the highest total ash value (**11.781 ± 0.006 %**), while *P. rheedei* exhibited the lowest (**6.283 ± 0.006 %**). *P. urinaria* showed the highest acid insoluble ash (**2.823 ± 0.006 %**), whereas *P. rheedei* displayed the lowest value (**1.483 ± 0.006 %**). Alcohol soluble extractive values varied considerably, with *P. rheedei* showing the highest extraction yield (**16.623 ± 0.006 %**) and *P. sikkimensis* the lowest (**7.529 ± 0.014 %**). The higher alcohol soluble extractive value of *P. rheedei* suggests greater abundance of alcohol-soluble constituents. Similarly, *P. rheedei* again exhibited the highest

water-soluble extractive value (**24.477 ± 0.015 %**), followed by *P. urinaria* (**21.847 ± 0.014 %**), indicating predominance of polar constituents. The pH of the 10% suspension ranged from **3.746 to 5.059**, demonstrating that all species were mildly acidic. The details physico-chemical data was presented in Table 9.

Table 9 Physico-chemical results of *Phyllanthus* species

Parameters	**Plant name**			
	P. Urinaria	*P. Amarus*	*P. Rheedei*	*P. sikkimensis*
Loss on drying (%w/w)	3.489±0.027	6.216±0.008	4.415±0.008	6.694±0.02
Total Ash (%w/w)	11.781±0.006	8.66±0.007	6.283±0.006	8.177±0.005
Acid insoluble Ash (%w/w)	2.823±0.006	1.943±0.006	1.483±0.006	1.815±0.008
Alcohol soluble extractive (%w/w)	12.946±0.008	10.533±0.004	16.623±0.006	7.529±0.014
Water soluble extractive (%w/w)	21.847±0.014	17.956±0.01	24.477±0.015	19.379±0.016
pH (10% suspension)	4.413±0.026	4.856±0.038	3.746±0.053	5.059±0.045

3.6 Phytochemical analysis

Preliminary phytochemical screening of methanolic extracts demonstrated that all investigated *Phyllanthus* species possess diverse classes of secondary metabolites. The preliminary phytochemical screening of methanolic extracts of *Phyllanthus amarus*, *P. rheedei*, *P. urinaria*, and *P. sikkimensis* is summarized in Table 7. The phytochemical analysis revealed the presence of proteins, phenolics, alkaloids, tannins, and flavonoids in all four species. The abundance of phenolics and tannins is consistent with the well-documented antioxidant, antimicrobial, and anti-inflammatory properties of *Phyllanthus* species. Glycosides were detected in *P. sikkimensis*, *P. urinaria*, and *P. amarus* but were absent in *P. rheedei*. Carbohydrate analysis showed positive

Fehling's and Molisch's reactions in *P. sikkimensis*, *P. rheedei*, and *P. urinaria*, whereas *P. amarus* showed negative results while Saponins were observed exclusively in *P. rheedei*. These phytochemical differences may contribute to the variations observed in their biological activities and chemical fingerprints obtained through chromatographic and spectroscopic analyses. The phytochemical analysis results are presented in Table 10.

Table 10 Phytochemical results of methanol extract of *Phyllanthus* species

Sl. No.	Test parameters		Name of Sample			
			P. sikkimensis	*P. rheedei*	*P. urinaria*	*P. amarus*
1.	Glycosides	Salkowski's test	+	-	+	+
2.	Protein	Xanthoproteic test	++	+	++	++
3.	Phenols	Ferric chloride test	+++	+++	+++	+++
		Lead acetate test	+++	+++	+++	+++
4.	Carbohydrates	Fehling's test	++	+	+++	-
		Molisch's test	+++	+++	+++	-
5.	Alkaloids	Mayer's test	+	++	+	+
		Wagner's test	+	++	+	+
		Hager's test	+	+	+	+
6.	Tannins	Braymer's test	+++	+++	+++	+++
7.	Saponins	Foam test	-	+	-	-
8.	Flavonoids	Shinoda test	-	-	-	-
		Alkaline reagent test	+	-	++	+
		Lead acetate test	+++	+++	+++	+++

Note: +++ Very good, ++ Good, + Moderate, - Absent

3.7 Antioxidant Assay and Total Phenolic and Total Flavonoid Content

The antioxidant potential and phytochemical composition of the investigated *Phyllanthus* species were evaluated using DPPH and ABTS radical scavenging assays together with estimation of total phenolic content (TPC) and total flavonoid content (TFC). All extracts exhibited a concentration-dependent increase in radical scavenging activity, indicating a direct relationship between extract concentration and antioxidant capacity as shown in Fig. 8.

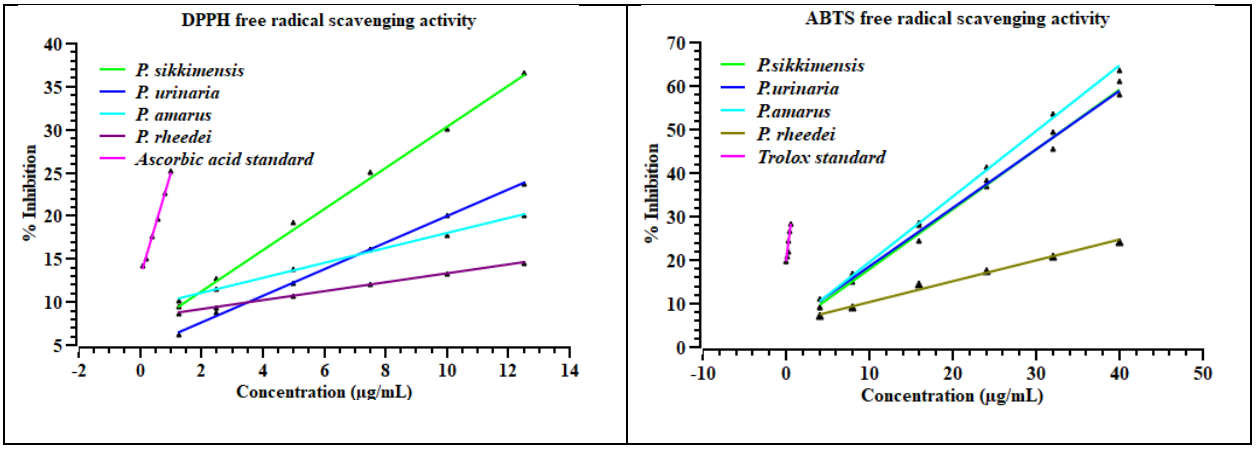


Fig. 8. DPPH and ABTS free radical scavenging activity of methanolic leaf extracts of *Phyllanthus* species.

In the DPPH assay, antioxidant activity varied markedly among species, as reflected by differences in IC_{50} values. Since lower IC_{50} values correspond to stronger free radical scavenging activity, *P. sikkimensis* exhibited the highest DPPH scavenging potential among the tested extracts with an IC_{50} value of **18.212 ± 0.159 µg/mL** and the weakest activity was observed in *P. rheedei* (**77.803 ± 2.148 µg/mL**). A similar trend was observed in the ABTS assay, although some variation in species ranking was evident. *P. amarus* showed the strongest ABTS scavenging activity among the plant extracts with an IC_{50} value of **30.226 ± 0.15 µg/mL**, whereas *P. rheedei* exhibited the lowest activity **92.883 ± 0.395 µg/mL**. The variation between

DPPH and ABTS results may be attributed to differences in reaction mechanisms, radical characteristics, and the differential response of individual phytochemical classes toward each assay system.

Total phenolic content (TPC) analysis revealed significant variation among the four *Phyllanthus* species. *P. sikkimensis* exhibited the highest TPC **(31.406 ± 1.792 µg/mg GAE)**, followed by *P. urinaria*, *P. amarus*, and *P. rheedei*. A similar trend was observed for total flavonoid content (TFC), with *P. sikkimensis* recording the highest values **(458.581 ± 16.093 µg/mg RE and 47.916 ± 2.077 µg/mg QE)**, followed by *P. amarus*, *P. urinaria*, and *P. rheedei*. These findings indicate that *P. sikkimensis* is comparatively richer in phenolic and flavonoid constituents, suggesting its potential as a valuable source of natural antioxidant phytochemicals. The detailed quantitative values of IC_{50} for DPPH assay and ABTS assay, TPC and TFC are presented in Table 11.

Table 11 IC_{50} values, total phenolic and total flavonoids result of methanol extract of *Phyllanthus* species

Plant names	DPPH assays IC_{50} values	ABTS assays IC_{50} values	TPC (µg/mg, GAE)	TFC (µg/mg, RE)	TFC (µg/mg, QE)
P. sikkimensis	18.212±0.129	33.399±0.375	31.406±1.792	458.581±16.093	47.916±2.077
P. urinaria	28.718±0.58	33.352±0.254	26.261±1.792	217.053±7.07	26.181±1.627
P. amarus	46.723±0.134	30.226±0.15	18.234±1.461	315.801±6.043	33.106±2.49
P. rheedei	77.803±2.148	92.883±0.395	8.524±0.571	193.429±6.548	25.331±2.482
Ascorbic acid	3.375±0.248				
Trolox		1.932±0.026			

3.8 Pearson correlation analysis

Pearson correlation analysis was performed to investigate the relationships among the quantified marker compounds, total phenolic content (TPC), total flavonoid content (expressed as rutin equivalents, TFC-RE, and quercetin equivalents, TFC-QE), and antioxidant activities determined by the ABTS and DPPH radical scavenging assays. The correlation matrix revealed strong associations among the phytochemical constituents and antioxidant parameters, demonstrating that the phenolic and flavonoid composition plays a major role in determining the antioxidant potential of the investigated *Phyllanthus* species.

Gallic acid showed an exceptionally strong positive correlation with TPC ($r = 0.944$), indicating that gallic acid is one of the principal phenolic constituents contributing to the total phenolic content. The antioxidant assays exhibited strong inverse relationships with the phytochemical parameters. DPPH IC_{50} values showed very strong negative correlations with TPC ($r = -0.971$), gallic acid ($r = -0.936$), and ellagic acid ($r = -0.771$). Similarly, ABTS IC_{50} values were negatively correlated with TPC ($r = -0.793$), gallic acid ($r = -0.645$), ellagic acid ($r = -0.445$), TFC-RE ($r = -0.560$), and TFC-QE ($r = -0.472$). Because lower IC_{50} values indicate stronger free-radical scavenging activity, these negative correlations clearly demonstrate that increasing concentrations of phenolic compounds and flavonoids are associated with enhanced antioxidant capacity. Overall, the Pearson correlation analysis demonstrates that gallic acid, ellagic acid, total phenolic content, and total flavonoid content are closely associated with the antioxidant activity of the investigated *Phyllanthus* species. Among these variables, total phenolic content exhibited the strongest negative correlation with DPPH IC_{50}, suggesting that phenolic constituents are the major contributors to antioxidant activity. The Pearson correlation data was presented in Table 12.

Table 12 Pearson Correlation Matrix of EA, GA, TPC, TFC (RE), TFC (QE), ABTS (IC_{50}) and DPPH (IC_{50})

Variable	EA	GA	TPC	TFC (RE)	TFC (QE)	ABTS (IC_{50})	DPPH (IC_{50})
EA	1.000	0.861	0.805	0.867	0.883	-0.445	-0.771
GA	0.861	1.000	0.944	0.616	0.621	-0.645	-0.936
TPC	0.805	0.944	1.000	0.691	0.615	-0.793	-0.971
TFC (RE)	0.867	0.616	0.691	1.000	0.951	-0.560	-0.675
TFC (QE)	0.883	0.621	0.615	0.951	1.000	-0.472	-0.636
ABTS IC_{50}	-0.445	-0.645	-0.793	-0.560	-0.472	1.000	0.870
DPPH IC_{50}	-0.771	-0.936	-0.971	-0.675	-0.636	0.870	1.000

4 Conclusion

Using an integrated multi-platform analytical approach, the present study provides one of the first comprehensive comparative phytochemical, chromatographic, elemental, and antioxidant evaluations of four medicinally relevant *Phyllanthus* species (*P. sikkimensis, P. urinaria, P. amarus, and P. rheedei*). Reproducible chromatographic profiles with both common and species-specific bands were obtained by comparative HPTLC fingerprinting, demonstrating their value for chemotaxonomic discrimination, quality assessment, and authentication. Gallic acid and ellagic acid concentrations varied significantly between species, RP-HPLC analysis demonstrated that P. *sikkimensis* had the highest quantities of both phenolic markers. Numerous bioactive substances were identified by GC–MS/MS metabolite profiling, including six compounds common to all four species. The presence of the lignans niranthin and hypophyllanthin further demonstrated their importance as unique chemotaxonomic identifiers of

the genus. The elemental composition of the examined species differed significantly, according to ICP-OES analysis, with potassium being the most common mineral in every sample, highlighting their nutritional importance. Furthermore, *P. sikkimensis* was consistently demonstrated to possess the strongest antioxidant potential by total phenolic content, total flavonoid content, and antioxidant assays. This was verified by Pearson correlation analysis, which revealed strong correlations between phenolic constituents, especially gallic acid, and free radical scavenging activity. These findings indicate that phenolic compounds have an important function in the antioxidant properties of the Phyllanthus species under investigation. This work's comprehensive analytical approach provides a strong framework for distinguishing closely related species and encourages their evidence-based application in herbal therapy. To confirm its therapeutic effectiveness and aid in its development into standardized herbal formulations, more metabolomic research, bioactivity-guided isolation of active constituents, molecular authentication, and in vivo pharmacological tests are necessary.

Acknowledgements

The authors are very grateful to CCRAS, New Delhi, India, the Director-General CCRAS, Ministry of AYUSH, New Delhi, for providing encouragement and facilities for carrying out this work.

Author Contributions

Suman Mandal and Lakshminarayana Misro: Methodology, investigation, formal analysis, data curation, visualization, and writing—original draft. Puneet Kumar Singh and Thirupataiah Boini: Conceptualization, supervision, methodology, and critical revision of the manuscript. Biswajit Paul, Dhritismita Bayan, and Anju S. Jeevan: Investigation, experimental work, and data curation. Manajit Bora, Devanjal Bora, Kousik Ghosh, P.L. Bharti, Vinodkumar Thallada, and Ajay K. Meena: Supervision, project administration, and manuscript review.

Financial support and sponsorship

This research received no external funding.

Data availability

All data generated or analyzed during this study are available in this article and supplementary information files.

Declarations

Ethics approval and consent to participate

The required permissions/licences for collection of the plant specimens were obtained from the appropriate authority, where applicable.This study involves comparative phytochemical profiling, HPTLC fingerprinting, quantification of selected phenolic markers, and in vitro antioxidant evaluation. No human participants or vertebrate animal subjects were involved in this research; therefore, formal ethical approval from an institutional review board was not required. Not applicable. This study did not involve human participants.

Consent for publication

Authors give consent for the publication of identifiable details in this manuscript to be published at your journal.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

Abd Rani NZ, Lam KW, Jalil J, Mohamad HF, Mat Ali MS, Husain K. Mechanistic studies of the antiallergic activity of Phyllanthus amarus Schum. & Thonn. and its compounds. Molecules. 2021;26(3):695. doi:10.3390/molecules26030695.

Adlercreutz H. Lignans and human health. Crit Rev Clin Lab Sci. 2007;44(5-6):483-525. doi:10.1080/10408360701612942.

Al Masum MA, Kabir MSH, Shoibe M, Hasan M, Chakrabarty N, Islam MJ, Basak M, Hasan MN, Chowdhury MHM, Sayeed MA. Analgesic activities of methanol extract of Phyllanthus sikkimensis Muell. Arg. leaves. World J Pharm Res. 2016;5(7):1717-1725. doi:10.20959/wjpr20167-6628.

Annamalai M, Sanjay ER, Nagendra Prasad HN, Radhakrishna Rao M, Joshi MB, Rai DPS, Kapaettu S. Quantitative analysis of Phyllanthus amarus and Phyllanthus urinaria for bioactive molecules using high-pressure liquid chromatography and liquid chromatography–mass spectrometry. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences. 2017. DOI: 10.1007/s40011-017-0839

Dhalwal K, Biradar YS, Rajani M. High-performance thin-layer chromatography densitometric method for simultaneous quantitation of phyllanthin, hypophyllanthin, gallic acid, and ellagic acid in Phyllanthus amarus. J AOAC Int. 2006;89(3):619- 623. doi:10.1093/jaoac/89.3.619.

Dzubak P, Hajduch M, Vydra D, Hustova A, Kvasnica M, Biedermann D, Markova L, Urban M, Sarek J. Pharmacological activities of natural triterpenoids and their therapeutic implications. Nat Prod Rep. 2006;23(3):394-411. doi:10.1039/B515312N.

Geethangili M, Ding ST. A review of the phytochemistry and pharmacology of Phyllanthus urinaria L. Front Pharmacol. 2018;9:1109. doi:10.3389/fphar.2018.01109.

Harikrishnan H, Jantan I, Haque MA, Kumolosasi E. Anti-inflammatory effects of hypophyllanthin and niranthin through downregulation of NF-κB/MAPKs/PI3K-Akt signaling pathways. Inflammation. 2018;41(3):984-995. doi:10.1007/s10753-018-0752-4.

Huang ZR, Lin YK, Fang JY. Biological and pharmacological activities of squalene and related compounds: potential uses in cosmetic dermatology. Molecules. 2009;14(1):540-554. doi:10.3390/molecules14010540.

Jantan I, Haque MA, Ilangkovan M, Arshad L. An insight into the modulatory effects and mechanisms of action of Phyllanthus species and their bioactive metabolites on the immune system. Front Pharmacol. 2019;10:878. doi:10.3389/fphar.2019.00878.

Khatoon S, Rai V, Rawat AKS, Mehrotra S. Comparative pharmacognostic studies of three Phyllanthus species. J Ethnopharmacol. 2006;104(1-2):79-86. doi:10.1016/j.jep.2005.08.048.

Kumar KJS, Lin C, Tseng YH, Wang SY. Fruits of Rosa laevigata and its bio-active principal sitostenone facilitate glucose uptake and insulin sensitivity in hepatic cells via AMPK/PPAR-γ activation. Phytomed Plus. 2021;1(4):100109. doi:10.1016/j.phyplu.2021.100109.

Liu S, Wei W, Shi K, Cao X, Zhou M, Liu Z. In vitro and in vivo anti-hepatitis B virus activities of the lignan niranthin isolated from Phyllanthus niruri L. J Ethnopharmacol. 2014;155(2):1061-1067. doi:10.1016/j.jep.2014.05.064.

Mandal S, Mandal AK, Sundharamoorthy S, Narayanan SKK, Bayan D, Bora D, Bora M, Baruah D, Singh PK, Ramachandran S. Some pharmacopoeial tests for a folklore herb Cordyline fruticosa (L.) A. Chev. and monographic standards with antioxidant assay thereof. Indian J Nat Prod Resour. 2025;16(2):325-334. doi:10.56042/ijnpr.v16i2.13614.

Misro L, Jeevan AS, Maurya R, Radhakrishnan T, Boini T, Rajeesh VR, Rohit KS, Kumar V, Meena AK, Srikanth N, Acharya R. Impact of regional variation on the

bioactive composition and antioxidant potential of Piper longum Linn. Chem Biodivers. 2026;23(1):e02460. doi:10.1002/cbdv.202502460.

Nikule HA, Nikam TD, Borde MY, Pawar SD, Shelke DB, Nitnaware KM. Phytochemical and pharmacological insights into Salacia chinensis L. (Saptarangi): an underexplored important medicinal plant. Discover Plants. 2024;1:67. doi: 10.1007/s44372-024-00067-2

National Institute of Standards and Technology. NIST/EPA/NIH Mass Spectral Library. Gaithersburg (MD): National Institute of Standards and Technology; 2020.

Pan JY, Chen SL, Yang MH, Wu J, Sinkkonen J, Zou K. An update on lignans: natural products and synthesis. Nat Prod Rep. 2009;26(10):1251-1292. doi:10.1039/B910940D.

Patel J, Nagar PS, Pal K, Singh R, Dhanani T, Patel V, Srivastava S, Kumar S. Comparative profiling of four lignans (phyllanthin, hypophyllanthin, nirtetralin, and niranthin) in nine Phyllanthus species from India using a validated reversed phase HPLC-PDA detection method. J AOAC Int. 2021;104(2):485-497. doi:10.1093/jaoacint/qsaa133.

Popa O, Băbeanu NE, Popa I, Niță S, Dinu-Pârvu CE. Methods for obtaining and determination of squalene from natural sources. Biomed Res Int. 2015;2015:367202. doi:10.1155/2015/367202.

Rajeswaran S, Rajan DK. Neophytadiene: biological activities and drug development prospects. Phytomedicine. 2025;143:156872. doi:10.1016/j.phymed.2025.156872.

Sarin B, Verma N, Martín JP, Mohanty A. An overview of important ethnomedicinal herbs of Phyllanthus species: present status and future prospects. ScientificWorldJournal. 2014;2014:839172. doi:10.1155/2014/839172.

Shanker K, Singh M, Srivastava V, Verma RK, Gupta AK, Gupta MM. Simultaneous analysis of six bioactive lignans in Phyllanthus species by reversed phase hyphenated high performance liquid chromatographic technique. Acta Chromatogr. 2011;23(2):321-337. doi:10.1556/AChrom.23.2011.2.10.

Shanmugam MK, Dai X, Kumar AP, Tan BKH, Sethi G, Bishayee A. Oleanolic acid and its synthetic derivatives for the prevention and therapy of cancer: preclinical and clinical evidence. Cancer Lett. 2014;346(2):206-216. doi:10.1016/j.canlet.2014.01.016.

Siddique HR, Saleem M. Beneficial health effects of lupeol triterpene: a review of preclinical studies. Life Sci. 2011;88(7-8):285-293. doi:10.1016/j.lfs.2010.11.020.

Smith TJ. Squalene: potential chemopreventive agent. Expert Opin Investig Drugs. 2000;9(8):1841-1848. doi:10.1517/13543784.9.8.1841.

Soares DB, Duarte LP, Cavalcanti AD, Silva FC, Braga AD, Lopes MT, Takahashi JA, Vieira-Filho SA. Psychotria viridis: Chemical constituents from leaves and biological properties. Anais da Academia Brasileira de Ciências. 2017;89(02):927-38.

Suresh V, Asha VV. Preventive effect of ethanol extract of Phyllanthus rheedii Wight. on D-galactosamine induced hepatic damage in Wistar rats. J Ethnopharmacol. 2008;116(3):447-453. doi:10.1016/j.jep.2007.12.011.

Unander DW, Webster GL, Blumberg BS. Usage and bioassays in Phyllanthus (Euphorbiaceae). IV. Clustering of antiviral uses and other effects. J Ethnopharmacol. 1995;45(1):1-18. doi:10.1016/0378-8741(94)01189-7.

Wagner H, Bladt S. Plant drug analysis: a thin layer chromatography atlas. 2nd ed. Berlin, Heidelberg: Springer-Verlag; 1996.

Webster GL. Synopsis of the genera and suprageneric taxa of Euphorbiaceae. Ann Mo Bot Gard. 1994;81(1):33-144. doi:10.2307/2399909.

World Health Organization. Quality control methods for medicinal plant materials. Geneva: World Health Organization; 1998.

Supplementary data

S1. Total Phenolic Content

The total phenolic content (TPC) of different *Phyllanthus* species (*Phyllanthus amarus*, *P. rheedei*, *P. urinaria*, and *P. sikkimensis*) was determined using the Folin–Ciocalteu reagent (FCR) method.

Briefly, 1 mL of methanolic extract (1250 µg/mL for all samples except *P. rheedei*, which was prepared at 500 µg/mL) was mixed with 2 mL of Folin–Ciocalteu reagent (10% v/v in deionized water) and 2 mL of sodium carbonate solution (7% w/v in deionized water). The reaction mixture was then made up to a final volume of 10 mL with deionized water, thoroughly mixed, and incubated for 30 minutes at room temperature. For the calibration curve, gallic acid (GA) was used as the reference standard. Standard solutions of gallic acid at concentrations of 1, 2, 3, 4, and 5 µg/mL were prepared and treated in the same manner as the samples, along with a reagent blank.

The absorbance of the resulting blue-coloured complex was measured at 765 nm using a UV–Visible spectrophotometer. The total phenolic content of the extracts was calculated from the calibration curve constructed using gallic acid and expressed as µg gallic acid equivalents (GAE) per mg of dry sample.

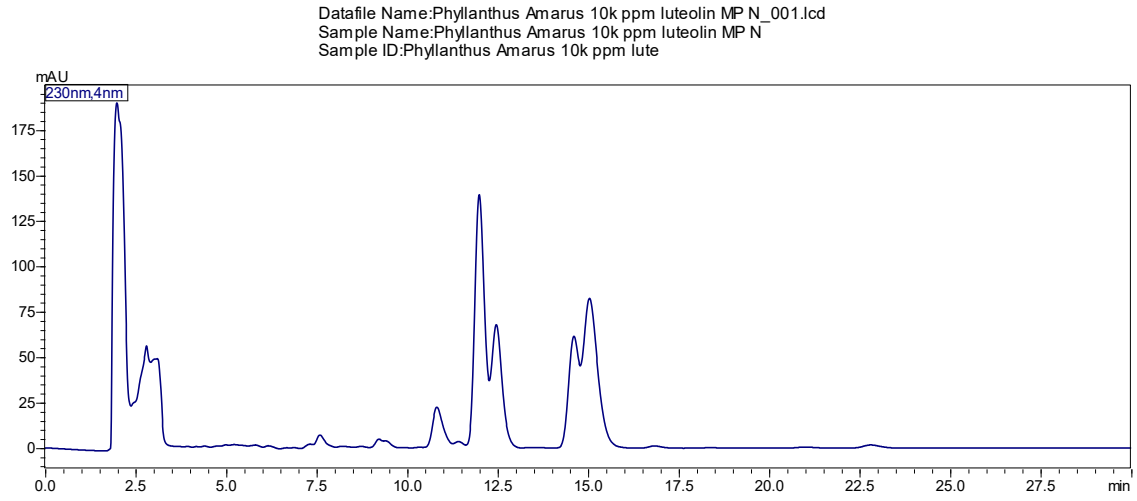
S2. Total Flavonoid Content

The total flavonoid content (TFC) of different *Phyllanthus* species (*Phyllanthus amarus*, *P. rheedei*, *P. urinaria*, and *P. sikkimensis*) was determined using the aluminium chloride ($AlCl_3$) colorimetric assay. The details Quercetin and rutin were used as reference standards for the estimation of total flavonoid content. Calibration curves were constructed using quercetin at concentrations of 5, 10, 20, 32, and 100 µg/mL, and rutin at concentrations of 10, 20, 40, 64, and

80 µg/mL. For sample analysis, 4 mL of methanolic extract (5000 µg/mL) was transferred into a 10 mL volumetric flask containing 0.2 mL of saturated potassium acetate solution (1 M). After a few minutes, 0.2 mL of aluminium chloride solution (10% w/v in deionized water) was added, and the volume was made up to 10 mL with distilled water. The reaction mixtures were mixed thoroughly and incubated in the dark for 30–60 minutes at room temperature. A similar procedure was followed for the preparation of standard and blank solutions. The absorbance was measured at 436.5 nm using a Shimadzu UV–Visible spectrophotometer. The total flavonoid content was calculated from the calibration curves and expressed as µg quercetin equivalents (QE)/mg and µg rutin equivalents (RE)/mg of dry sample.

S3. Details HPLC data

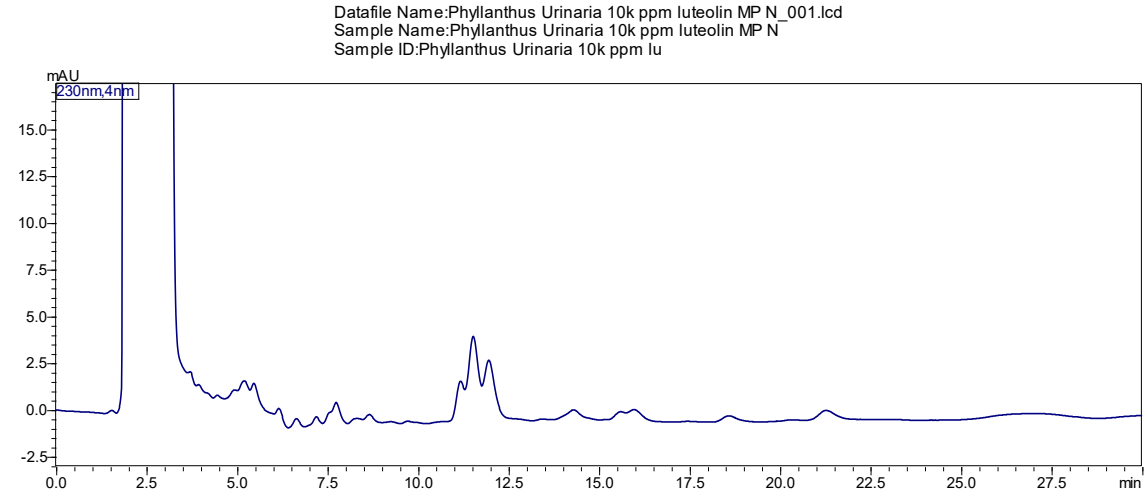
P. amarus HPLC chromatogram



Peak#	Ret. Time	Area	Height	Area%	Height%	A/H	
1	**1.986**	**350831 6**	**182807**	**41.192**	**36.764**	**19.191**	**Gallic acid**
2	**2.808**	**153301**	**13771**	**1.8**	**2.77**	**11.132**	**Ellagic acid**
3	**3.103**	**281496**	**22941**	**3.305**	**4.614**	**12.27**	
4	3.942	2130	325	0.025	0.065	6.556	
5	4.178	1734	284	0.02	0.057	6.099	

6	4.409	4380	518	0.051	0.104	8.448	
7	4.819	2371	98	0.028	0.02	24.267	
8	4.994	2671	391	0.031	0.079	6.825	
9	5.22	6891	451	0.081	0.091	15.294	
10	5.819	9706	828	0.114	0.166	11.724	
11	6.16	14105	1010	0.166	0.203	13.963	
12	6.675	2638	278	0.031	0.056	9.493	
13	6.882	1768	256	0.021	0.051	6.917	
14	7.326	9932	758	0.117	0.152	13.108	
15	7.592	79957	5675	0.939	1.141	14.089	
16	8.189	8912	581	0.105	0.117	15.33	
17	8.733	6960	603	0.082	0.121	11.545	
18	9.223	24411	2329	0.287	0.468	10.481	
19	9.41	8065	731	0.095	0.147	11.033	
20	10.828	402454	21228	4.725	4.269	18.959	
21	11.426	17406	1574	0.204	0.317	11.057	
22	**11.996**	**184581 6**	**117607**	**21.672**	**23.652**	**15.695**	**may be phyllanthin**
23	**12.468**	**620727**	**41564**	**7.288**	**8.359**	**14.934**	**may be Hypophyllanthin**
24	**14.61**	**471896**	**31174**	**5.541**	**6.269**	**15.138**	**may be Nirtetralin**
25	**15.041**	**102903 4**	**49460**	**12.082**	**9.947**	**20.805**	**may be Niranthin**

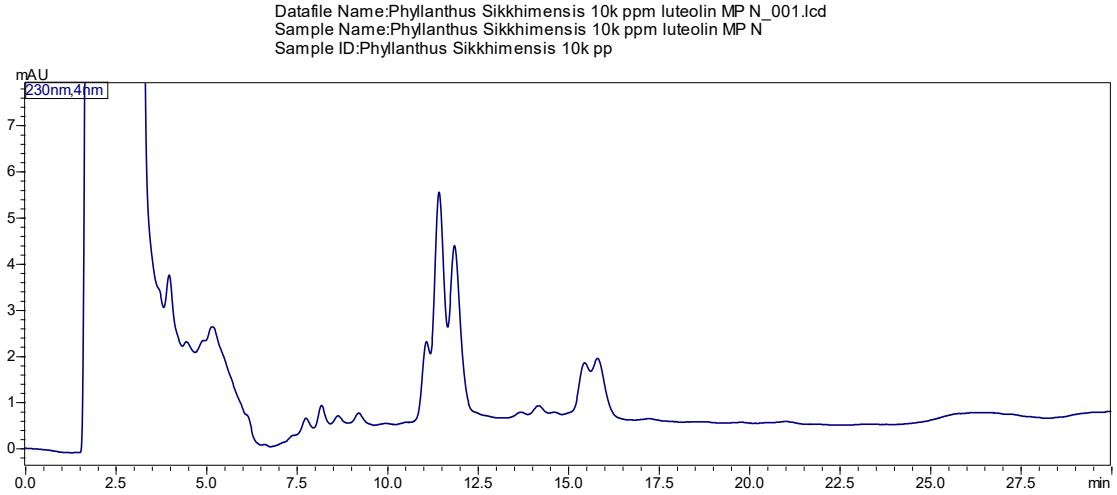
P. urenaria HPLC chromatogram



1	**1.99**	**1097759**	**570018**	**95.564**	**94.341**	**19.258**	**Gallic acid**

			4				
2	**2.813**	**362172**	**23567**	**3.153**	**3.901**	**15.368**	**Ellagic acid**
3	**3.703**	**2234**	**311**	**0.019**	**0.051**	**7.191**	
4	5.201	4629	438	0.04	0.072	10.578	
5	5.466	5281	596	0.046	0.099	8.867	
6	6.151	4776	508	0.042	0.084	9.401	
7	6.639	4589	424	0.04	0.07	10.826	
8	7.19	4151	407	0.036	0.067	10.187	
9	7.739	8117	735	0.071	0.122	11.044	
10	8.655	3185	284	0.028	0.047	11.209	
11	**11.173**	**12113**	**1023**	**0.105**	**0.169**	**11.84**	
12	**11.526**	**36023**	**2713**	**0.314**	**0.449**	**13.278**	
13	**11.957**	**26138**	**1801**	**0.228**	**0.298**	**14.513**	
14	14.298	8130	398	0.071	0.066	20.448	
15	15.845	2082	-8	0.018	-0.001	-246.19	
16	15.962	3049	192	0.027	0.032	15.881	
17	18.583	7896	308	0.069	0.051	25.603	
18	21.27	15005	494	0.131	0.082	30.402	

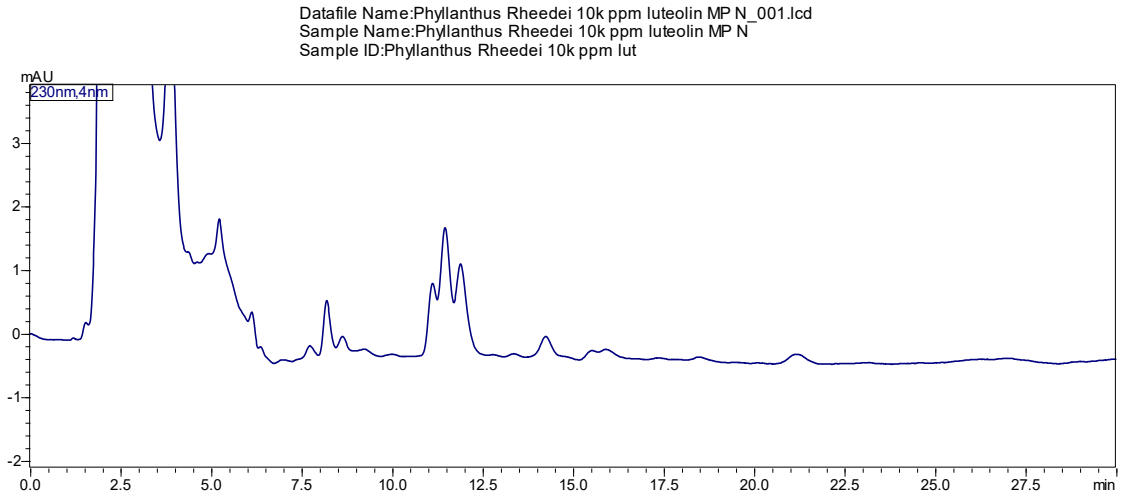
P. sikkiminises HPLC chromatogram



		1790332					
1	**2.09**	**3**	**916283**	**95.035**	**90.894**	**19.539**	**Gallic acid**
2	**2.809**	**460725**	**42634**	**2.446**	**4.229**	**10.807**	**Ellagic acid**
3	**3.122**	**331820**	**39138**	**1.761**	**3.882**	**8.478**	

4	3.983	9779	965	0.052	0.096	10.138
5	5.164	12441	521	0.066	0.052	23.893
6	7.76	3615	278	0.019	0.028	12.997
7	8.189	5075	432	0.027	0.043	11.753
8	8.649	1564	138	0.008	0.014	11.307
9	9.213	3236	221	0.017	0.022	14.664
10	**11.09**	**7927**	**685**	**0.042**	**0.068**	**11.567**
11	**11.44**	**43250**	**3189**	**0.23**	**0.316**	**13.561**
12	**11.867**	**34913**	**2296**	**0.185**	**0.228**	**15.209**
13	13.705	1091	72	0.006	0.007	15.077
14	14.193	2826	173	0.015	0.017	16.354
15	14.623	316	30	0.002	0.003	10.399
16	15.46	6764	449	0.036	0.045	15.06
17	15.818	9960	573	0.053	0.057	17.378

P. Rheedi HPLC chromatogram



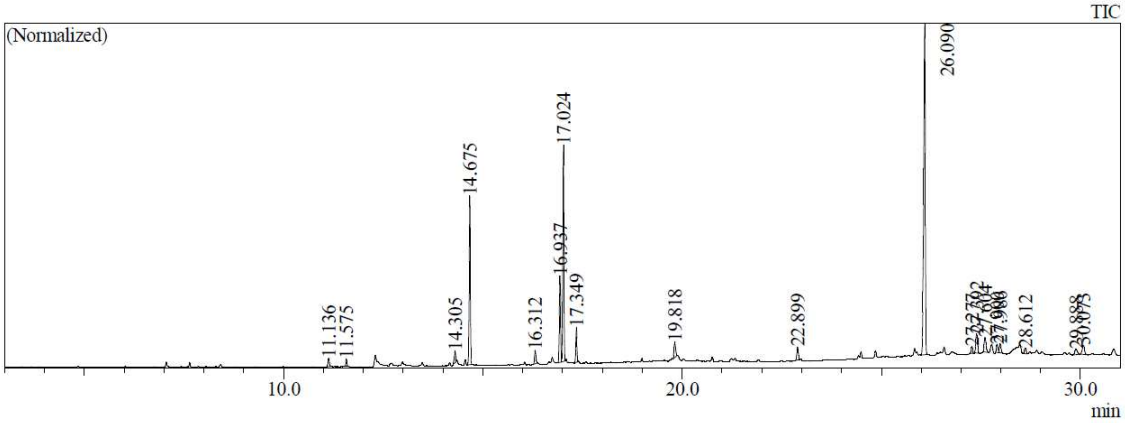
1	**2.117**	**1517883**	**78167**	**75.032**	**64.911**	**19.419**	**Gallic acid**
2	**2.814**	**124201**	**11166**	**6.14**	**9.272**	**11.123**	**Ellagic acid**

3	**3.117**	**263339**	**23036**	**13.017**	**19.13**	**11.431**
4	3.884	43352	2656	2.143	2.205	16.324
5	4.283	283	-1	0.014	-0.001	-236.827
6	5.072	326	-11	0.016	-0.009	-31.003
7	5.227	5156	555	0.255	0.461	9.294
8	6.124	2326	299	0.115	0.248	7.774
9	6.375	362	61	0.018	0.051	5.914
10	6.976	610	41	0.03	0.034	14.818
11	7.727	2138	164	0.106	0.137	13.009
12	8.195	9697	792	0.479	0.658	12.244
13	8.629	2246	190	0.111	0.158	11.805
14	9.236	1152	65	0.057	0.054	17.858
15	10.003	628	36	0.031	0.03	17.264
16	**11.12**	**5812**	**506**	**0.287**	**0.42**	**11.483**
17	**11.467**	**15027**	**1152**	**0.743**	**0.957**	**13.044**
18	**11.896**	**12519**	**831**	**0.619**	**0.69**	**15.068**
19	12.496	-2	-1	0	-0.001	2.257
20	13.369	732	47	0.036	0.039	15.504
21	14.243	6265	307	0.31	0.255	20.422
22	15.519	1057	69	0.052	0.057	15.388
23	15.903	1328	69	0.066	0.057	19.252
24	17.366	438	23	0.022	0.019	18.896
25	18.506	1130	53	0.056	0.044	21.245
26	21.178	4965	149	0.245	0.124	33.27

S4: GC-MS/MS report of hexane extract of P.sikkimensis

Peak Report TIC

Peak#	Name	R.Time	Area%	Height%	Similarity
1	Tetradecanoic acid	11.136	0.89	0.86	95
2	Tetradecanoic acid, ethyl ester	11.575	0.60	0.75	93
3	l-(+)-Ascorbic acid 2,6-dihexadecanoate	14.305	1.17	1.21	90
4	Hexadecanoic acid, ethyl ester	14.675	14.25	17.12	95
5	Phytol	16.312	1.41	1.36	97
6	Linoleic acid ethyl ester	16.937	7.74	8.76	95
7	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	17.024	18.16	21.95	93
8	Octadecanoic acid, ethyl ester	17.349	2.82	3.55	94
9	Thunbergol	19.818	1.86	1.57	78
10	Docosanoic acid, ethyl ester	22.899	1.38	1.32	75
11	Squalene	26.090	39.32	33.62	95
12	Tetrapentacontane	27.277	0.77	0.71	95
13	is[(4-hydroxy-3-methoxyphenyl)methyl]butane-1,4-diol, tetramethyl	27.392	2.32	1.93	89
14	14,18,22-Tetracosahexaen-3-ol, 2,6,10,15,19,23-hexamethyl-, (all-E)	27.604	1.91	1.46	91
15	2,6,10,15,19,23-Pentamethyl-2,6,18,22-tetracosatetraen-10,15-diol	27.900	1.11	0.82	92
16	Hexadeca-2,6,10,14-tetraen-1-ol, 3,7,11,16-tetramethyl-	27.986	1.00	0.93	91
17	Hypophyllanthin	28.612	0.73	0.57	75
18	Hypophyllanthin	29.888	1.01	0.57	74
19	Niranthin	30.073	1.57	0.94	93
			100.00	100.00	


TIC
(Normalized)
11.136
11.575
14.305
14.675
16.312
16.937
17.024
17.349
19.818
22.899
26.090
28.612
10.0
20.0
30.0
min

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Suplimentarydata.docx