

UHPLC-Q Exactive Orbitrap HRMS-Based Metabolite Profiling and Determination of Total Phenolic and Flavonoid Contents in *Piper betle* L. Leaf Ethanolic Extract

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Keywords:	Abstract
<i>Piper betle</i> L.; UHPLC-Q Exactive Orbitrap HRMS; metabolite profiling; total phenolic content; total flavonoid content; phytochemical characterization	<i>Piper betle</i> L. leaves contain diverse phytochemical constituents; however, variations in extract composition highlight the need for complementary analytical characterization. This study aimed to characterize the metabolite profile of <i>Piper betle</i> L. leaf ethanolic extract and determine its total phenolic content (TPC) and total flavonoid content (TFC). Metabolite profiling was performed using ultra-high-performance liquid chromatography coupled with Q Exactive Orbitrap high-resolution mass spectrometry (UHPLC–HRMS) in positive and negative electrospray ionization (ESI) modes. Metabolites were tentatively annotated based on accurate mass measurements, tandem mass spectrometric fragmentation patterns, and database matching. TPC and TFC were determined using colorimetric assays with gallic acid and quercetin as reference standards, respectively, and all measurements were performed in triplicate. Thirteen metabolites were tentatively annotated, including phenylpropanoids, flavonoids, phenolic amides, and terpenoids. Hydrocinnamic acid exhibited the highest reported chromatographic signal intensity, followed by tetramethoxyflavanone, cinnamic acid, and coumaric acid derivatives. The TPC was 64.37 ± 4.01 mg gallic acid equivalents per gram of extract (mg GAE/g extract), whereas the TFC was 213.74 ± 1.93 mg quercetin equivalents per gram of extract (mg QE/g extract). These measurements provided complementary information regarding the phytochemical characteristics of the extract but could not be directly interpreted as the relative proportions of individual constituents. Overall, the integrated analytical approach provided a preliminary phytochemical profile of the investigated <i>Piper betle</i> L. leaf ethanolic extract, establishing a foundation for subsequent metabolite identification, targeted quantitative analysis, and comparative quality assessment. Further biological investigations are warranted to elucidate potential

INTRODUCTION

Medicinal plants remain valuable sources of bioactive compounds for pharmaceutical, nutraceutical, and functional food development. Advances in metabolomics and analytical chemistry have substantially improved the characterization of plant secondary metabolites, facilitating a more comprehensive understanding of the chemical constituents potentially responsible for their biological activities. Among the available analytical techniques, ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry (UHPLC–HRMS) has emerged as a powerful platform for metabolite profiling because of its high sensitivity, mass accuracy, and ability to detect a broad spectrum of phytochemicals within complex botanical matrices (Adeeyo et al., 2024; Wang et al., 2022).

Piper betle L. (Piperaceae), commonly known as betel leaf, is a medicinal plant widely distributed throughout South and Southeast Asia. It has traditionally been used to manage infections, inflammation, digestive disorders, and cardiovascular-related conditions (Panda & Panda, 2023; Singh et al., 2023). Previous studies have reported various biological activities associated with P. betle, including antioxidant, antimicrobial, anti-inflammatory, antidiabetic, and cytoprotective properties. These activities have been attributed, at least in part, to its diverse phytochemical constituents, including phenylpropanoids, phenolic acids, flavonoids, terpenoids, alkaloids, and other secondary metabolites (Lanting et al., 2026; Singh et al., 2023). Nevertheless, the phytochemical composition of P. betle may vary considerably depending on geographical origin, environmental conditions, cultivation practices, harvesting stage, and extraction procedures, potentially resulting in substantial differences in metabolite profiles across studies (Lanting et al., 2026).

A fundamental challenge in phytochemical investigations is that botanical identity alone does not guarantee chemical equivalence among plant extracts. (Narayanappa et al., 2024b) demonstrated compositional variation within P. betle by characterizing 20 sexually dimorphic genotypes using high-performance liquid chromatography with diode-array detection (HPLC–DAD). Their analysis identified several phenolic and organic acids, including caffeic acid, gallic acid, p-coumaric acid, and cinnamic acid, and revealed differences between male and female genotypes. These findings highlighted the influence of genetic variability on phytochemical composition and demonstrated the limitations of applying a universal chemical profile to all preparations of the species. Consequently, direct characterization of individual extracts is essential for establishing meaningful comparisons with previously investigated preparations and providing reliable chemical reference data for subsequent studies.

Extraction conditions represent another important source of phytochemical variability. (Nguyen, 2020) developed an HPLC method for the simultaneous quantification of hydroxychavicol, eugenol, and gallic acid in P. betle leaf extracts prepared using solvents of varying polarities. Under their experimental conditions, methanol extraction yielded the highest reported hydroxychavicol content (25.035 mg/g), whereas ethanol extraction produced the highest eugenol content (2.898 mg/g). Gallic acid recovery also varied substantially among extraction solvents. These findings demonstrated that solvent selection influenced not only extraction efficiency but also the relative abundance of individual phytochemical constituents.

Therefore, ethanolic *P. betle* leaf extracts require direct analytical characterization because their chemical profiles cannot be assumed to correspond to those obtained using aqueous, methanolic, or less polar extraction systems.

Although targeted chromatographic methods provide valuable quantitative information regarding selected phytochemical constituents, comprehensive metabolite profiling can reveal additional compounds within complex botanical matrices. Ultra-high-performance liquid chromatography coupled with high-resolution tandem mass spectrometry (UHPLC–HRMS/MS) enables the characterization of diverse metabolites through chromatographic separation, accurate mass measurements, and tandem mass spectrometric fragmentation patterns. (Li & Zhang, 2022) demonstrated the application of UHPLC–Q Exactive Orbitrap MS/MS to investigate chemical differences between the bulbs and flowers of *Fritillaria thunbergii*, integrating mass spectral information with database matching and molecular networking approaches. Although their investigation focused on a different medicinal plant, the findings provided methodological support for applying Orbitrap-based high-resolution mass spectrometry to characterize phytochemical diversity. The application of this analytical platform to *P. betle* may facilitate the detection and tentative annotation of compounds beyond predefined analytical markers while maintaining appropriate confidence levels in database-assisted metabolite identification.

In addition to metabolite profiling, total phenolic content (TPC) and total flavonoid content (TFC) analyses provide complementary information regarding the phytochemical characteristics of botanical extracts. These colorimetric assays quantify analytical responses relative to reference standards and facilitate comparisons among extracts when consistent experimental procedures, calibration methods, and reporting units are applied. However, their results require careful interpretation because different compounds may exhibit varying responses under identical assay conditions. Shraim et al. (2021) experimentally demonstrated limitations associated with the aluminum chloride colorimetric assay, particularly the assumption that structurally different flavonoids produce equivalent responses relative to a selected calibration standard. Accordingly, TFC values expressed as quercetin equivalents should be interpreted as method-dependent estimates rather than absolute measurements of all flavonoid constituents. Similarly, TPC values expressed as gallic acid equivalents reflect the response of the assay and do not necessarily represent the absolute concentration of individual phenolic compounds. Integrating these measurements with chromatographic metabolite profiling may therefore provide a more comprehensive characterization of plant extracts while recognizing that the analytical methods assess different chemical properties.

Previous investigations have established substantial knowledge regarding the phytochemical composition of *P. betle*, although several analytical considerations remain insufficiently addressed. Ferreres et al. (2014) characterized phenolic constituents in aqueous and ethanolic extracts, (Nguyen, 2020) quantified selected phytochemical compounds using different extraction solvents, and (Narayanappa et al., 2024b) investigated compositional variations among *P. betle* genotypes. Collectively, these studies demonstrated the chemical diversity of the species and the influence of extraction conditions and genetic characteristics on its phytochemical profile. However, the integration of broad-spectrum metabolite profiling using UHPLC–Q Exactive Orbitrap HRMS with parallel TPC and TFC measurements for the

specific ethanolic extract investigated in the present study remained an area requiring further examination. The research gap therefore concerned the combined application of complementary analytical approaches to characterize a particular botanical preparation rather than an absence of previous phytochemical investigations involving *P. betle*. Addressing this gap could provide a more integrated understanding of the chemical characteristics of the investigated extract and facilitate comparisons with previously reported phytochemical profiles.

Addressing this analytical gap is particularly relevant because the continued investigation and potential development of botanical preparations require reliable and interpretable chemical characterization. Variations in plant material, environmental conditions, and extraction procedures may complicate comparisons across studies and hinder the identification of suitable chemical markers for quality assessment. An integrated analytical approach can provide information regarding tentatively detectable metabolites and the aggregate responses of an extract in commonly employed phytochemical assays. For *P. betle*, such information may support subsequent compound confirmation, targeted quantitative analysis, and comparative evaluation of independently prepared extracts. Nevertheless, phytochemical characterization alone cannot establish biological efficacy, clinical safety, or batch-to-batch consistency. These outcomes require additional experimental validation, including biological activity assessments, toxicological investigations, and standardized analytical procedures.

The novelty of this study lies in the complementary application of high-resolution metabolite profiling and colorimetric phytochemical assays to the same ethanolic *P. betle* leaf extract. Specifically, UHPLC–Q Exactive Orbitrap HRMS analysis employing positive and negative electrospray ionization (ESI) modes was combined with TPC and TFC determinations to characterize tentatively annotated metabolites and evaluate aggregate assay responses. This integrated approach enabled the simultaneous consideration of individual metabolite annotations and overall phenolic- and flavonoid-related analytical responses without assuming that chromatographic signal intensities represented absolute compound concentrations. Furthermore, gallic acid equivalents and quercetin equivalents were interpreted as distinct, assay-dependent measurements rather than directly interchangeable indicators of phytochemical abundance. The principal contribution of this approach was therefore to establish an integrated chemical profile of the investigated preparation and identify potential analytical markers requiring further structural confirmation and targeted quantification.

Accordingly, this study aimed to characterize the metabolite profile of *P. betle* leaf ethanolic extract using UHPLC–Q Exactive Orbitrap HRMS and determine its total phenolic and flavonoid contents. Specifically, the study sought to tentatively annotate detectable metabolites based on accurate mass measurements, tandem mass spectrometric (MS/MS) fragmentation patterns, and database matching; classify the identified metabolites according to their chemical groups; and quantify TPC and TFC using gallic acid and quercetin as reference standards, respectively. The findings were expected to expand the available phytochemical evidence concerning *P. betle* ethanolic extracts and provide a foundation for subsequent analytical marker confirmation, comparative extract characterization, and quality assessment. Ultimately, the study aimed to provide researchers and botanical product developers with a

documented chemical reference for future standardization efforts, targeted phytochemical investigations, and biological activity evaluations.

METHODS

Materials

The ethanolic extract of *Piper betle* L. leaves was used as the study material. LC–MS-grade methanol and acetonitrile, formic acid, aluminum chloride (AlCl₃), sodium nitrite (NaNO₂), sodium hydroxide (NaOH), sodium carbonate (Na₂CO₃), and Folin–Ciocalteu reagent were obtained from Merck KGaA (Darmstadt, Germany). Quercetin and gallic acid reference standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Ultra-high-performance liquid chromatography–high-resolution mass spectrometry (UHPLC–HRMS) analysis was performed using a Vanquish UHPLC system coupled to a Q Exactive Plus Orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was achieved using an Accucore C18 column (100 × 2.1 mm, 1.5 µm particle size; Thermo Fisher Scientific, Waltham, MA, USA).

Determination of Total Flavonoid Content

The total flavonoid content (TFC) of the *P. betle* leaf ethanolic extract was determined using the aluminum chloride (AlCl₃) colorimetric method according to Chang et al. (2002), with minor modifications based on the Indonesian Herbal Pharmacopoeia, Second Edition (Ministry of Health of the Republic of Indonesia, 2017). Quercetin was used as the reference standard, and the results were expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g extract) (Chang et al., 2002; Kemenkes RI, 2017).

Quercetin standard solutions (10–50 µg/mL) were prepared from a 1000 µg/mL stock solution in methanol. The sample solution was prepared by dissolving 1 mg of extract in 1 mL of methanol. For the assay, 250 µL of the standard or sample solution was mixed sequentially with distilled water, 5% NaNO₂, 10% AlCl₃, and 1 M NaOH, followed by incubation at each step. The absorbance was measured at 510 nm using a UV–Visible spectrophotometer. All analyses were performed in triplicate.

The TFC was calculated from the quercetin calibration curve and expressed as mg QE/g extract using the equation:

$$TFC \text{ (mg QE/g extract)} = \frac{C \times V \times DF}{m}$$

where *C* is the flavonoid concentration obtained from the calibration curve (mg/mL), *V* is the total volume of the sample solution (mL), *DF* is the dilution factor, and *m* is the mass of extract used (g). Results were expressed as mean ± standard deviation (SD) of three independent determinations.

Determination of Total Phenolic Content

The total phenolic content (TPC) was determined using the Folin–Ciocalteu colorimetric method according to the Indonesian Herbal Pharmacopoeia, Second Edition (Ministry of Health of the Republic of Indonesia, 2017). Gallic acid was used as the reference standard, and the results were expressed as milligrams of gallic acid equivalents per gram of extract (mg GAE/g extract) (Kemenkes RI, 2017).

Gallic acid standard solutions (5–20 µg/mL) were prepared from a 1000 µg/mL stock solution in methanol. The sample solution was prepared by dissolving 4 mg of extract in 4 mL of methanol. For the assay, 250 µL of the standard or sample solution was mixed with distilled water, Folin–Ciocalteu reagent, and 20% Na₂CO₃ solution, followed by incubation at room temperature in the dark. The absorbance was measured at 765 nm using a UV–Visible spectrophotometer. All measurements were performed in triplicate.

The TPC was calculated from the gallic acid calibration curve using the following equation:

$$TFC \text{ (mg GAE/g extract)} = \frac{C \times V \times DF}{m}$$

where *C* is the phenolic concentration obtained from the calibration curve (mg/mL), *V* is the total volume of the sample solution (mL), *DF* is the dilution factor, and *m* is the mass of extract used (g). Results were expressed as mean ± standard deviation (SD) of three independent determinations.

UHPLC–Q Exactive Orbitrap HRMS Analysis

The metabolite profile of *Piper betle* L. leaf ethanolic extract was analyzed using an Ultra-High Performance Liquid Chromatography–High Resolution Mass Spectrometry (UHPLC–HRMS) system coupled with a Q Exactive Plus Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA, USA). Briefly, 5 mg of extract was dissolved in 1 mL of methanol, centrifuged, filtered through a 0.2 µm nylon membrane, and subjected to UHPLC–HRMS analysis.

Chromatographic separation was performed on a Vanquish UHPLC system equipped with an Accucore™ C18 column (100 × 2.1 mm, 1.5 µm). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) under gradient elution. The flow rate was 0.2 mL/min, injection volume was 2 µL, and the column and autosampler temperatures were maintained at 30°C and 10°C, respectively.

Mass spectrometric detection was conducted using an electrospray ionization (ESI) source in both positive and negative ionization modes over an *m/z* range of 100–1500. Raw data were processed using Compound Discoverer™ version 3.2, and tentative metabolite identification was performed using the mzCloud and ChemSpider databases. Metabolites were annotated based on accurate mass measurements, MS/MS fragmentation patterns, retention times, and spectral library matching.

RESULTS AND DISCUSSION

Phytochemical Characterization of *Piper betle* L. Leaf Ethanolic Extract

The metabolite profile of *Piper betle* L. leaf ethanolic extract was characterized using UHPLC–Q Exactive Orbitrap HRMS. The representative base peak chromatograms obtained in positive and negative ionization modes are shown in Figure 1. Based on accurate mass measurements, retention times, MS/MS fragmentation patterns, and database matching, a total of 13 metabolites were tentatively identified (Table 1). These compounds were classified into several groups of secondary metabolites, including phenolic acids, phenylpropanoids, flavonoids, flavanones, phenolic amides, and terpenoids.

Among the identified metabolites, hydrocinnamic acid was the most abundant compound, followed by 5,6,7,4'-tetramethoxyflavanone, cinnamic acid, and *p*-coumaric acid,

suggesting that phenylpropanoids constitute a major fraction of the phytochemical composition of *P. betle* leaf ethanolic extract. The occurrence of cinnamic acid, *p*-coumaric acid, hydrocinnamic acid, and 4-methoxycinnamic acid is consistent with previous reports identifying phenylpropanoids as characteristic constituents of *P. betle* leaves (Narayanappa et al., 2024; Ferreres et al., 2014). These findings further support the role of phenylpropanoids as one of the dominant phytochemical groups in this species.

In addition to phenylpropanoids, several flavonoids and flavanones, including rhoifolin, 5,6,7,4'-tetramethoxyflavanone, tangeretin, and 8-prenylnaringenin, were tentatively identified. The flavonoid profile identified in the present study differs from earlier reports, which predominantly detected catechin, rutin, luteolin, apigenin, kaempferol, and naringenin as major flavonoid constituents of *P. betle* leaves (Ferreres et al., 2014; Narayanappa et al., 2024). Such variations may be attributed to differences in genotype, geographical origin, environmental conditions, extraction procedures, and analytical platforms. The high sensitivity and mass accuracy of Orbitrap HRMS also facilitated the detection of structurally diverse metabolites, including phenolic amides, oxygenated phenolics, and terpenoids that may be overlooked using conventional analytical techniques.

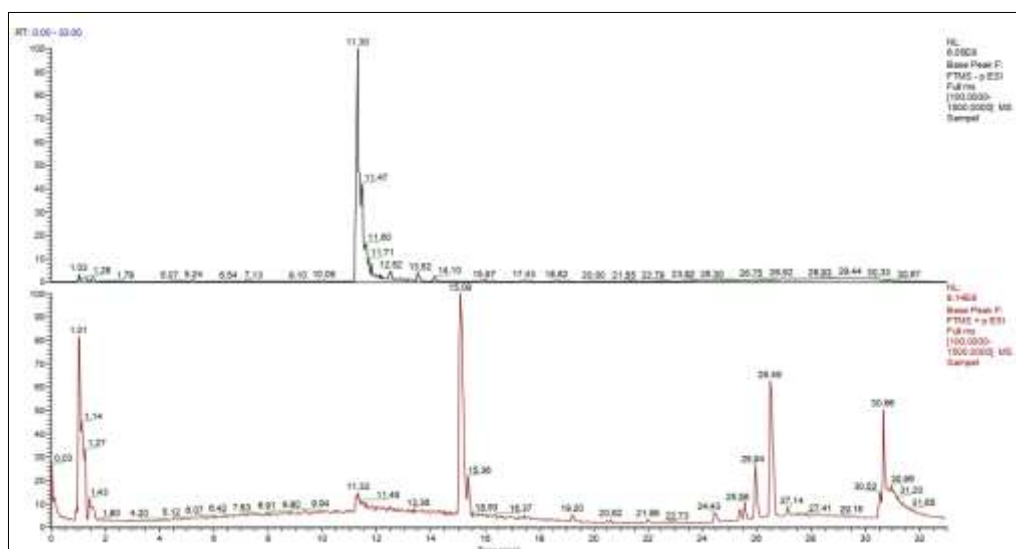


Figure 1. Base peak chromatograms (BPCs) of *Piper betle* L. leaf ethanolic extract obtained by UHPLC-Q Exactive Orbitrap HRMS analysis in (a) negative electrospray ionization (ESI⁻) mode and (b) positive electrospray ionization (ESI⁺) mode.

Table 1. Metabolite Profile of the Ethanolic Extract of *Piper betle* L. Leaves Identified by UHPLC Vanquish Tandem Q Exactive Plus Orbitrap HRMS

No.	Retention Time (min)	Compound Name	Molecular Formula	Molecular Weight (Da)	Ion Mode	Observed <i>m/z</i>	ΔMass (ppm)	Peak Area
1	1.08	3-(4-Hydroxy-3,5-dimethoxyphenyl)-1-(2,4,6-trihydroxy-3-methoxyphenyl)-1,2-propanedione	C ₁₈ H ₁₈ O ₉	3.780.934	[M-H] ⁻	377.09	-4.54	6.11 × 10 ⁸
2	8.28	Rhoifolin	C ₂₇ H ₃₀ O ₁₄	5.781.636	[M+H] ⁺	579.17	0.12	2.39 × 10 ⁸

No.	Retention Time (min)	Compound Name	Molecular Formula	Molecular Weight (Da)	Ion Mode	Observed <i>m/z</i>	Δ Mass (ppm)	Peak Area
3	10.81	4-Methoxycinnamic acid	C ₁₀ H ₁₀ O ₃	1.780.629	[M+H] ⁺	179.07	−0.37	4.56 × 10 ⁸
4	11.29	1-(2,4-Dihydroxy-6-methoxy-3,5-dimethylphenyl)-3-phenylpropan-1-one	C ₁₈ H ₂₀ O ₄	3.001.363	[M−H] [−]	299.13	0.49	8.45 × 10 ⁸
5	11.30	Hydrocinnamic acid	C ₉ H ₁₀ O ₂	1.500.673	[M−H] [−]	149.06	−5.37	7.56 × 10 ¹⁰
6	11.38	<i>p</i> -Coumaric acid	C ₉ H ₈ O ₃	1.640.467	[M−H] [−]	163.04	−3.99	1.28 × 10 ⁹
7	11.39	Cinnamic acid	C ₉ H ₈ O ₂	1.480.525	[M+H] ⁺	149.06	0.42	1.31 × 10 ⁹
8	13.51	(2E)-N-[2-(Dihydroxymethyl)-4-hydroxy-5-methoxyphenyl]-3-(4-hydroxy-3-methoxyphenyl)acrylamide	C ₁₈ H ₁₉ NO ₇	3.611.165	[M−H] [−]	360.11	0.96	3.96 × 10 ⁸
9	13.51	5,6,7,4'-Tetramethoxyflavanone	C ₁₉ H ₂₀ O ₆	3.441.263	[M−H] [−]	343.12	−0.80	2.19 × 10 ⁹
10	16.40	Tangeretin	C ₂₀ H ₂₀ O ₇	3.721.204	[M+H] ⁺	373.13	−1.33	2.29 × 10 ⁸
11	17.44	8-Prenylnaringenin	C ₂₀ H ₂₀ O ₅	3.401.315	[M−H] [−]	339.12	1.11	3.44 × 10 ⁸
12	18.77	Quinoprene	C ₁₈ H ₂₈ O ₂	2.762.087	[M+H] ⁺	277.22	−0.96	2.49 × 10 ⁸
13	22.00	Curcumene	C ₁₅ H ₂₂	2.021.721	[M+H] ⁺	203.18	−0.30	3.58 × 10 ⁸

Overall, the metabolite profile revealed that *P. betle* leaf ethanolic extract possesses a chemically diverse composition dominated by phenylpropanoids and flavonoids, accompanied by phenolic amides and terpenoid compounds. These results are in agreement with previous HRMS- and GC-MS-based studies demonstrating the phytochemical complexity of *P. betle* leaves (Osman et al., 2021; Nugroho et al., 2025).

Quantification of Total Phenolic and Flavonoid Contents

The calibration curves of gallic acid and quercetin exhibited excellent linearity, with correlation coefficients (R^2) greater than 0.99 (Figures 2), indicating the suitability of the analytical methods for the quantification of total phenolic and flavonoid contents.

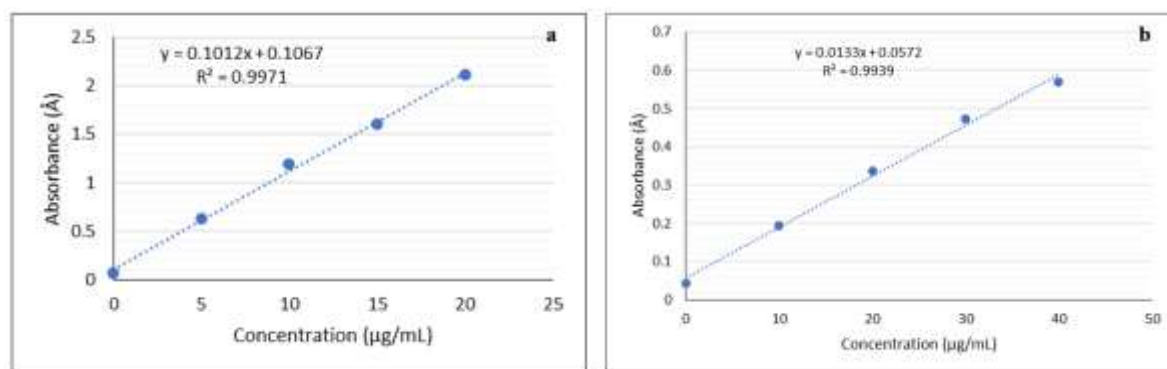


Figure 2. Calibration curves used for the quantification of total phenolic and flavonoid contents in *P. betle* L. leaf ethanolic extract: (a) gallic acid standard curve and (b) quercetin standard curve.

The total phenolic content (TPC) and total flavonoid content (TFC) of the *Piper betle* L. leaf ethanolic extract are presented in Tables 2 and 3. The extract contained 64.37 ± 4.01 mg gallic acid equivalents (GAE)/g extract and 213.74 ± 1.93 mg quercetin equivalents (QE)/g extract for TPC and TFC, respectively. The relatively low standard deviation values indicate good repeatability of the analytical measurements.

Table 2. Total Phenolic Content of *P. betle* L. Leaf Ethanolic Extract

Replicate	Absorbance (Å)	Gallic Acid Equivalent (µg/mL)	TPC (mg GAE/g extract)
1	1.6180	14.93	59.74
2	1.7901	16.63	66.54
3	1.7974	16.71	66.83
Mean ± SD	-	-	64.37 ± 4.01

Table 3. Total Flavonoid Content of *P. betle* L. Leaf Ethanolic Extract

Replicate	Absorbance (Å)	Quercetin Equivalent (µg/mL)	TFC (mg QE/g extract)
1	0.7608	52.90	211.61
2	0.7696	53.56	214.26
3	0.7733	53.84	215.37
Mean ± SD	-	-	213.74 ± 1.93

The TFC value was approximately three times higher than the TPC value, suggesting that flavonoid-related constituents represent a major proportion of the polyphenolic compounds present in the extract. This observation is consistent with the UHPLC-Q Exactive Orbitrap HRMS analysis, which tentatively identified several flavonoid and flavanone derivatives, including rhoifolin, 5,6,7,4'-tetramethoxyflavanone, tangeretin, and 8-prenylnaringenin. In addition, phenylpropanoid compounds such as cinnamic acid, *p*-coumaric acid, hydrocinnamic acid, and 4-methoxycinnamic acid were also detected, indicating a chemically diverse polyphenolic composition (Poudel et al., 2024; Sikdar, 2025; Singh et al., 2025; Sonphakdi et al., 2024).

Previous phytochemical studies have reported that *P. betle* leaves are rich in phenolic and flavonoid compounds, although their concentrations may vary depending on genotype, geographical origin, environmental conditions, extraction solvent, and analytical methodology (Narayanappa et al., 2024a; Nguyen et al., 2020). The relatively high TPC and TFC values obtained in the present study are in agreement with the metabolite profiling results and further support the abundance of polyphenolic constituents in the ethanolic extract.

The combination of UHPLC-Q Exactive Orbitrap HRMS profiling with TPC and TFC determination provides a comprehensive phytochemical characterization of *P. betle* leaf ethanolic extract. While HRMS analysis enables the tentative identification of individual metabolites, TPC and TFC measurements offer quantitative information regarding the abundance of major polyphenolic constituents. These findings contribute to the establishment of a phytochemical fingerprint that may serve as a useful reference for future quality assessment and standardization of *P. betle* leaf extracts.

CONCLUSION

This study characterized the phytochemical composition of Piper betle L. leaf ethanolic extract using ultra-high-performance liquid chromatography coupled with Q Exactive Orbitrap high-resolution mass spectrometry (UHPLC–HRMS), complemented by total phenolic content (TPC) and total flavonoid content (TFC) determinations. Thirteen metabolites were tentatively annotated, representing various chemical classes, including phenylpropanoids, flavonoids, phenolic amides, and terpenoids. The TPC was 64.37 ± 4.01 mg gallic acid equivalents per gram of extract (mg GAE/g extract), whereas the TFC was 213.74 ± 1.93 mg quercetin equivalents per gram of extract (mg QE/g extract). These measurements reflected assay-specific responses relative to different reference standards and could not be directly interpreted as the relative proportions of individual phytochemical constituents. Collectively, the findings provided complementary chemical information regarding the investigated extract and established a preliminary foundation for subsequent metabolite characterization, analytical standardization, and comparative quality assessment.

Future research should prioritize confirming the tentative metabolite annotations using authentic reference standards, targeted tandem mass spectrometry (MS/MS), and validated analytical methods for quantifying selected phytochemical constituents. Comparative investigations involving independently prepared extracts from different geographical origins, harvesting periods, and extraction conditions are recommended to assess phytochemical variability and identify reproducible chemical markers. Further evaluation of assay selectivity, potential matrix effects, and analytical interference would improve the reliability and interpretation of TPC and TFC measurements. Additionally, biological activity and toxicological studies are needed to investigate potential relationships between the characterized phytochemical constituents and their biological effects, as well as to evaluate the extract's safety and suitability for future pharmaceutical, nutraceutical, and functional food applications.

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