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Research Article

Influence of Maturity Stage and Sampling Time on the Metabolite Profiles of *Piper sarmentosum* Using Optimized NMR Parameters

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ABSTRACT

Optimization is a critical step in metabolomics workflows to ensure reliable data, particularly when handling large sample numbers. In this study, optimization was carried out for *Piper sarmentosum* Roxb., a medicinal herb of regional importance, to establish standardized procedures for NMR-based metabolomic analysis and sample collection. Extraction conditions were evaluated using different sample masses, with 75 mg of freeze-dried material providing the best signal intensity, particularly in the aromatic region. Several NOESY-based NMR parameter sets were then compared, and the settings adapted from Halabalaki et al. (2014) produced the highest spectral quality and were adopted for further analyses. Following optimization, the influence of leaf maturity and harvesting time on metabolite composition was assessed using 1D NOESY NMR spectroscopy. Partial least square discriminant analysis (PLS-DA) revealed clear metabolic distinctions between young and mature leaves, while samples collected in the morning and afternoon showed no notable differences, indicating that sampling time had minimal impact on metabolite composition. The optimized protocols established in this work minimize technical variability, enhance spectral reproducibility, and improve metabolite detectability. These outcomes provide a robust platform for large-scale metabolomics investigations of *P. sarmentosum* and other medicinal plants.

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1. INTRODUCTION

The genus *Piper* (Piperaceae) represents one of the most diverse and chemically rich groups of tropical plants, comprising more than 2,000 species distributed across Southeast Asia, South America, and the Pacific regions. These species range from aromatic shrubs to woody vines and are well known for their ethnomedicinal, culinary, and economic importance (Salleh et al., 2015). In many traditional healing systems, *Piper* plants have long been used to treat ailments such as inflammation, digestive disorders, pain, infections, and respiratory conditions, highlighting their deep cultural significance and broad therapeutic relevance (Salleh, 2020). Chemically, *Piper* species are renowned for producing complex secondary metabolites, including amides, alkaloids, flavonoids, lignans, terpenoids, and essential oils. These phytochemicals underpin a wide spectrum of bioactivities, including antibacterial, antifungal, antioxidant, cytotoxic, anti-inflammatory, anticholinesterase, and enzyme inhibition properties, making the genus a valuable target for ongoing natural product research (Salleh et al., 2019).

Piper sarmentosum Roxb. (Figure 1) is a well-recognized traditional medicinal plant in Southeast Asia and China (Mathew et al., 2004). Traditionally, *P. sarmentosum* has been used as a natural remedy with a wide range of claimed therapeutic properties, including anticancer, anti-osteoporotic, antibacterial, antioxidant, antihypertensive, anti-inflammatory, and antidiabetic effects (Azlina et al., 2019; Mohamad Asri et al., 2020; Suwannasom et al., 2022; Anjur et al., 2022; Ibrahim et al., 2020; Azmi et al., 2021 and Hematpoor et al., 2018). Its therapeutic potential has been linked to a diverse array of bioactive metabolites, including alkaloids, amides, flavonoids, and phenylpropanoids (Adib et al., 2023). The growing global demand for traditional medicines has raised concerns over product quality, safety, and authenticity, especially with issues of counterfeiting and inconsistency. Reliable analytical methods are needed to authenticate products and assess their chemical composition, as efficacy depends on trace levels of bioactive metabolites. Metabolomics offers a comprehensive approach for characterizing the chemical composition of plant species by profiling a broad range of metabolite. Advances in high-field NMR and mass spectrometry now enable precise metabolomic profiling, generating consistent chemical fingerprints that support standardization, batch reproducibility, and quality control in plant-based medicines (Lee et al, 2017; Xiao et al. 2022). Metabolic profiling of *P. sarmentosum* is therefore an important approach for characterizing its phytochemical composition and supporting standardization efforts in herbal research.

NMR spectroscopy provides several distinct advantages over other metabolomic platforms. It is non-destructive, inherently quantitative, and requires minimal sample preparation without chemical derivatization (Markley et al., 2017; Edison et al., 2021). In addition, NMR is highly reliable for the identification of novel compounds and offers excellent reproducibility and automation potential, making high-throughput metabolomic analyses more feasible compared to LC-MS or GC-MS. However, the quality of spectral data is highly dependent on the choice of acquisition parameters. Without optimization, sensitivity and resolution may be insufficient to capture relevant metabolites, particularly in studies with large sample sets. Metabolomics studies require carefully designed experiments to ensure reproducibility and reliability of the data. Factors such as plant growth conditions, randomization, replication, and analytical quality control must be considered, along with sample preparation variables including tissue type, maturity stage, time of harvest, and quenching methods (Ocampos et al., 2024). Because metabolite concentrations are highly dynamic and influenced by both developmental and environmental conditions, inconsistent sampling can introduce significant variability and confound data interpretation (Li et al., 2020). Standardization of harvesting conditions such as selecting uniform leaf maturity and collecting samples at the same time of day has been recommended to reduce bias and enhance comparability (Zhao et al., 2022).

The NOESY (Nuclear Overhauser Effect Spectroscopy) experiment is commonly employed in NMR-based metabolomics for efficient water suppression (Nagana et al., 2021; Zhuoma et al, 2025). It exploits through-space dipolar interactions between protons, enabling selective attenuation of the water resonance while preserving metabolite signals (Kim et al., 2010). In this study, 1D NOESY NMR parameters were optimized to enhance data quality, and the effects of leaf maturity and sampling time on the metabolite profiles of *P. sarmentosum* were investigated. These optimizations ensured consistent sample quality, minimized technical variability, and improved data reliability. The methodology established here provides a solid foundation for subsequent large-scale profiling, where high throughput and reproducibility are essential.

(**A**) (**B**)

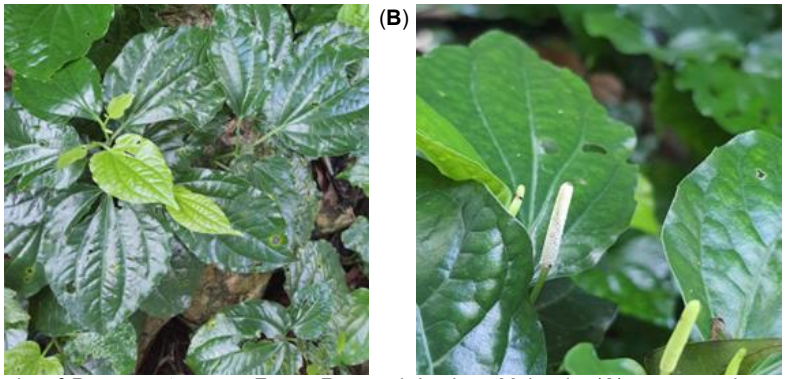

Figure 1. Photographs of *P. sarmentosum* at Forest Research Institute Malaysia; (**A**) mature and young leaves; (**B**) fruits

2. METHODOLOGY

2.1. Solvents and Chemicals

Deuterium oxide (D, 99.9%) and methanol-D4 (D, 99.8%) were purchased from Cambridge Isotope Laboratories, USA. Sodium deuteroxide solution (NaOD) (40wt, % in D_2O (D, 99.5%) and 3-(Trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt (TMSP) (D, 98.0 %) were purchased from Aldrich, USA. Meanwhile pottasium dihydrogen phosphate was from Merck, USA.

2.2. Plant Samples

P. sarmentosum samples were identified by a botanist at Forest Research Institute of Malaysia (FRIM). Leaf samples of *P. sarmentosum* were collected from the FRIM nursery in Kepong, consisting of both mature and young leaves at two time points (8:00 a.m. (shaded) and 1:30 p.m. (sunny)). Immediately after harvesting, samples were quenched in liquid nitrogen, ground using a cryo-mill, freeze-dried, and stored at -80 °C until further use (Kim et al., 2010). Herbarium samples were kept at FRIM.

2.3. Optimization of Sample Mass for NMR Analysis

An optimization was carried out using three different sample masses (25, 50, and 75 mg) of freeze-dried material to determine the appropriate amount for NMR analysis. The preparation method followed Kasim et al. (2022) with slight modification. Briefly, 75 mg of freeze-dried material were transferred into a microcentrifuge tube, and 1.0 mL of CH_3OH-d_4 was added. The mixture was vortexed for 2.0 min and sonicated for 20.0 min, followed by centrifugation at 13 000 rpm for 5.0 min at room temperature. The supernatant (500.0 µL) was transferred into a 2.0 mL microcentrifuge tube and mixed with 250.0 µL of KH_2PO_4 buffer (pH 6.0) containing 0.1% trimethylsilyl propionic acid sodium salt (w/v). The mixture was kept at 4.0 °C for 30.0 min, then centrifuged at 6 000 rpm for 5.0 min. The resulting supernatant (600.0 µL) was carefully transferred into a 5.0 mm NMR tube (DURAN® 178 × 4.95 mm) for analysis.

2.4. Optimization of 1D-NOESY NMR Acquisition Parameters for Metabolomic Analysis

For NMR-based metabolomics, NOESY 1D-NMR spectra were recorded at 300.0 K (26.85 °C) on a Bruker AVANCE 600 MHz spectrometer equipped with a CryoProbe™ operating at a frequency of 600.21 MHz. CH_3OH-d_4 was used as the internal lock. Four acquisition parameter sets previously applied in plant metabolomics studies were tested to determine suitable conditions. The parameters varied during optimization were the number of scans (NS), relaxation delay, and acquisition time, while the spectral width (SW) and mixing time were kept constant:

(i) NS= 64, relaxation delay= 1.0 s, acquisition time= 3.666 s, mixing time= 0.01 s, SW = 20 ppm (Abdul Hamid et al., 2017)
(ii) (NS= 64, relaxation delay= 2.0 s, acquisition time= 2.045 s, mixing time= 0.01 s, SW= 20 ppm (Abd Ghafar et al., 2020)
(iii) NS= 64, relaxation delay= 4.0 s, acquisition time= 3.98 s, mixing time= 0.01 s, SW= 20 ppm (Halabalaki et al., 2014)

(iv) NS= 128, relaxation delay= 4.0 s, acquisition time= 3.98 s, mixing time= 0.01 s, SW = 20 ppm (Halabalaki et al., 2014)

A presaturation sequence was used to suppress the residual H_2O frequency during the recycle delay. Free induction decays (FIDs) were Fourier transformed with a line broadening of 0.3 Hz and zero-filled to 32.0 K points. The resulting spectra were manually phased, baseline-corrected, and calibrated to TSP at 0.0 ppm using TopSpin (version 4.3.0, Bruker). Subsequently, to study the effects of developmental stage and sampling time on metabolite composition, the following parameters were applied: NS= 128, relaxation delay= 4.0 s, acquisition time= 3.98 s, mixing time= 0.01 s, and spectral width (SW)= 20 ppm (Halabalaki et al., 2014).

2.5. NMR Data Processing for Metabolomics Analysis

The FID data of NOESY 1D-NMR experiment were converted into ASCII file using NMR Assure Bruker software (v. 1.0, Swiss). Spectral intensities were scaled to the TSP signal and reduced to integrated regions of 0.01 ppm width, yielding 829 bins per NMR spectrum. The region from 3.15 to 4.1 ppm was excluded to eliminate interference from residual water and solvents. The averaged binned integral of the NOESY 1D-NMR data were then subjected to multivariate data analysis. Partial Least Squares Discriminant Analysis (PLS-DA) was performed with the SIMCA-P software (v. 13.0.3, Umetrics, Umea, Sweden). A hierarchical clustering heatmap was developed using MetaboAnalyst (version 6.0) to visualize variations in metabolite profiles according to leaf maturity and sampling time.

3. RESULTS AND DISCUSSION

3.1. Optimization of Sample Mass

In metabolomics, extraction is a crucial step that determines the quality and representativeness of the metabolic profile obtained. An efficient extraction must maximize metabolite recovery while minimizing degradation or selective loss of compounds. Therefore, careful optimization of the extraction procedure is essential to ensure reliable, reproducible, and comprehensive metabolite coverage (Kaiser et al., 2009; Martin et al., 2015). Methanol is widely preferred due to its intermediate polarity, enabling extraction of both polar and semi-polar metabolites for a comprehensive profile. Chloroform, though effective for nonpolar compounds such as lipids and terpenoids, often co-extracts excessive lipids that can mask signals in NMR. Meanwhile, water is suitable for hydrophilic metabolites like sugars, organic acids, and amino acids, but fails to extract semi-polar and nonpolar metabolites, excluding key groups such as alkaloids and flavonoids that are important in *P. sarmentosum* (Adib et al., 2024). Methanol thus provides a balanced extraction, recovering sugars, amino acids, organic acids, phenolics, alkaloids, and flavonoids, making it suitable for NMR profiling of *P. sarmentosum* in this study.

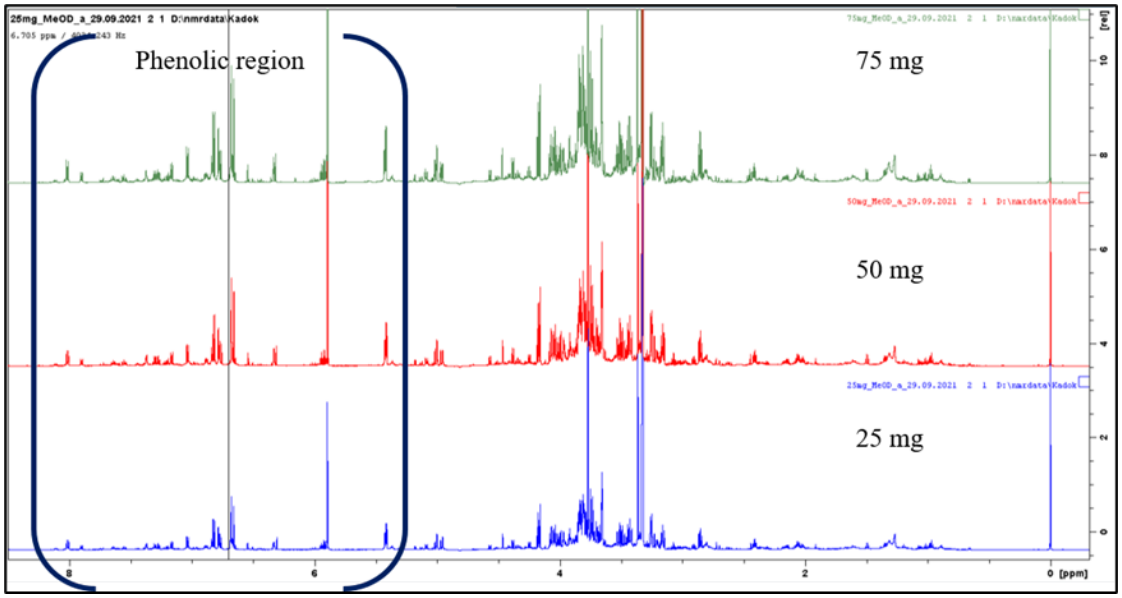

Figure 2. 1D NOESY NMR spectra of *P. sarmentosum* leaves extracts using different sample weights

Optimization of sample mass was first carried out using three amounts: 25 mg, 50 mg, and 75 mg of freeze-dried material dissolved in MeOD. This step was performed to determine the optimal sample quantity that would yield sufficient signal intensity for reliable metabolite detection. The resulting NMR spectra showed that 75 mg produced the highest signal intensity, particularly in the 5-8 ppm region where many aromatics and olefinic metabolites resonate (Figure 2). These metabolites are often critical in metabolomics studies, and the higher sample mass ensured greater metabolite concentration in the NMR tube, resulting in improved signal-to-noise ratios, enhanced detection of low-abundance compounds, and more reliable spectral interpretation.

3.2. Optimization of NMR Parameters for Metabolic Analysis

In addition to sample mass, experimental parameters for the 1D NOESY experiment were optimized to achieve consistent spectral quality across all samples. Key acquisition parameters such as number of scans (NS), relaxation delay, acquisition time, mixing time, and spectral width are known to strongly influence sensitivity and reproducibility (Wu et al., 2014). Four parameter sets previously applied in plant metabolomics studies were tested (Figure 3): (i) NS= 64, relaxation delay= 1.0 s, acquisition time= 3.666 s, mixing time= 0.01 s, SW= 20 ppm (Abdul Hamid et al., 2017); (ii) NS= 64, relaxation delay= 2.0 s, acquisition time= 2.045 s, mixing time= 0.01 s, SW= 20 ppm (Abd Ghafar et al., 2020); (iii) NS= 64, relaxation delay= 4.0 s, acquisition time= 3.98 s, mixing time= 0.01 s, SW= 20 ppm (Halabalaki et al., 2014) and (iv) NS= 128, relaxation dela = 4.0 s, acquisition time= 3.98 s, mixing time= 0.01 s, SW= 20 ppm (Halabalaki et al., 2014). Among these, the parameters adapted from Halabalaki et al. (2014) produced the strongest signals in the phenolic region. The region between 5-8 ppm often includes signals from aromatic and olefinic compounds, which are critical for metabolomics analysis. The higher mass ensures a greater concentration of metabolites in the NMR tube, enhancing signal-to-noise ratios and improving the detectability of metabolites, thereby enabling more reliable spectral interpretation and metabolite identification.

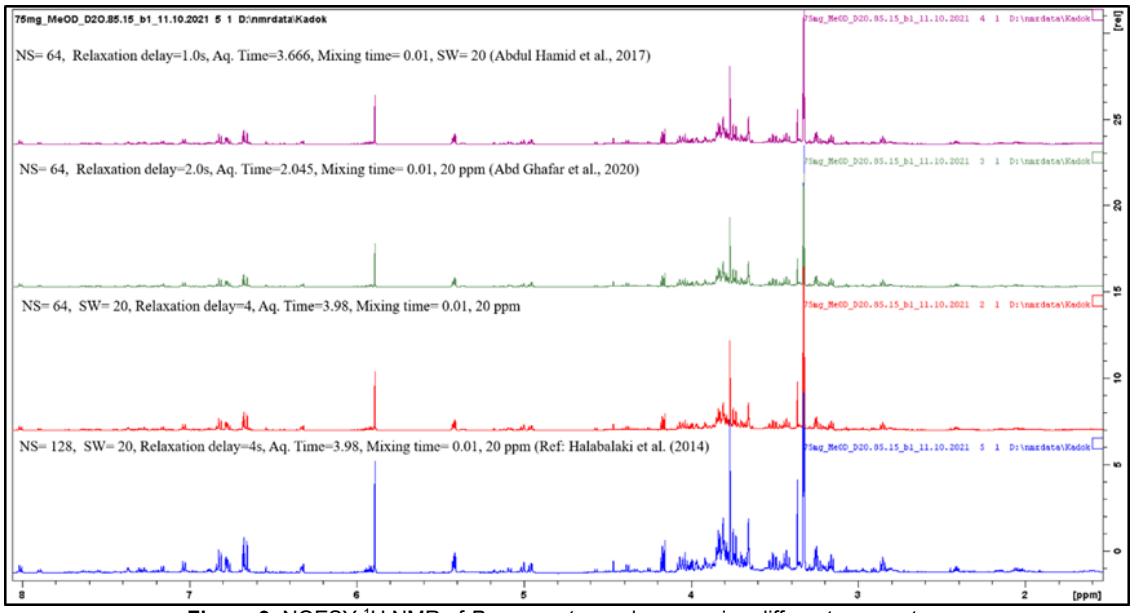

Figure 3. NOESY ^{1}H NMR of *P. sarmentosum* leaves using different parameters

3.3. Effect of Developmental Stage and Sampling Time on NMR-Based Metabolite Composition

Plant metabolomics analysis involves several critical steps, including experimental design, plant growth management, harvesting, sample preparation, metabolite extraction, instrumental analysis, data preprocessing, and statistical evaluation (Ocampos et al., 2024). Careful planning of each stage is essential to ensure reproducibility, accuracy, and meaningful interpretation of data. In particular, harvesting is a crucial step, as variations in tissue type, developmental stage, and time of collection can affect metabolite profiles. In this study, the NMR profiles of mature and young *P. sarmentosum* leaves showed clear differences (Figure 4).

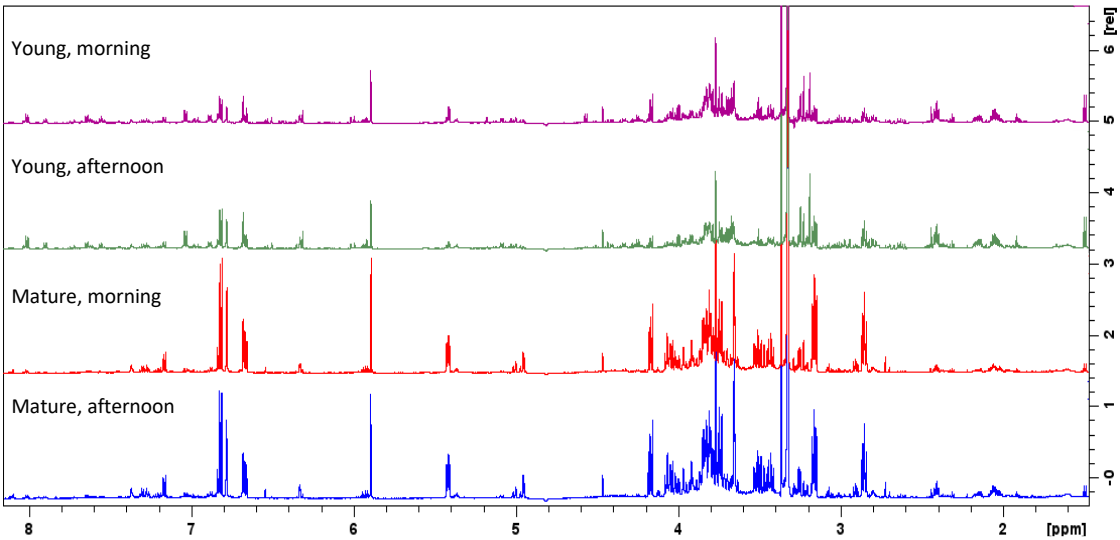

Figure 4. Representative ^{1}H NMR spectra of *Piper sarmentosum* mature and young leaves sampled at different time, showing clear differences in metabolite profiles, with mature leaves exhibiting higher signal intensities compared to young leaves

In general, the ^{1}H-NMR spectra of plant extracts can be categorized into three regions: the organic acid and amino acid region (0.00-3.00 ppm), the carbohydrate/glucose region (3.01-5.00 ppm), and the aromatic region (6.00-8.00 ppm) (Kim et al., 2010). These ^{1}H-NMR spectra are considered fingerprint spectra because there are no samples with identical ^{1}H-NMR spectra. Mature leaves exhibited higher signal intensities and more prominent peaks, reflecting greater accumulation of secondary metabolites such as flavonoids, amides, and phenylpropanoids. In contrast, young leaves displayed weaker spectra, consistent with their prioritization of primary metabolism to support growth. These differences highlight the strong influence of developmental stage on metabolite composition and emphasize the need to standardize leaf maturity for metabolomics investigations. Meanwhile, the samples of the same maturity but collected at different times showed no observable differences in their NMR spectra.

Direct visual inspection of spectra is often limited by overlapping peaks and subtle intensity differences across samples, making it difficult to identify systematic variation by simple observation. To further evaluate the effects of leaves maturity and sampling time on the metabolic profiles of *P.sarmentosum*, Partial Least Squares Discriminant Analysis (PLS-DA) was applied to the NMR dataset (Figure 5A). Multivariate data analysis such as PLS-DA enables the extraction of meaningful patterns from large spectral datasets by reducing dimensionality and emphasizing group-related variance. As a supervised multivariate method, PLS-DA uses prior class information to maximize separation spectral variables that contribute most to discrimination. This approach helps to uncover relationships between samples and highlight metabolites responsible for group differentiation (Kalivodová et al., 2015). The score plots showed that both maturity stage significantly influenced metabolite profiles. Mature leaves clustered on the positive side of PC1, while young leaves were separated on the negative side, reflecting clear metabolic differences between developmental stages. The PLS-DA model yielded values of $R^2X = 0.746$, $R^2Y = 0.783$, and $Q^2 = 0.508$, with PC1 and PC2 together explaining 71.2% of the total variation in the dataset. R^2X represents the proportion of variance in the X-matrix (NMR spectral data) that is explained by the model, while R^2Y reflects how well the model explains the separation between predefined sample groups. Q^2 indicates the predictive reliability of the model as determined by cross-validation. Collectively, these values suggest that the model provides a good description of the data with moderate predictive power.

The loading line plot from the PLS-DA model indicated distinct NMR chemical shift regions that contributed to the separation between mature and young *P. sarmentosum* samples (Figure 5B). Peaks located further from the baseline reflected stronger discriminatory power, and when combined with the variable importance in projection (VIP) scores, several chemical shifts were highlighted as significant contributors (VIP > 1.0) (Kasim et al., 2022). The most prominent signals included δ 1.52 (VIP 5.51), δ 5.44 (VIP 4.58), δ 2.88 (VIP 3.65), δ 4.20 (VIP 3.63), δ 4.16 (VIP 2.87), δ 6.84 (VIP 2.71), δ 6.80 (VIP 2.58), δ 6.68 (VIP 2.35), and δ 5.92 (VIP 2.16). Additional discriminant peaks were observed at δ 4.96 (VIP 1.70), δ 1.36 (VIP 1.92), δ 1.32 (VIP 1.75), δ 4.60 (VIP 1.50), and δ 5.20 (VIP 1.39). These values indicate that both high- and low-field regions of the spectrum contributed to class separation, suggesting that multiple metabolite classes underlie the observed maturity differences. The PLS-DA column loading plot (Figure 5C) illustrates metabolites with higher loading values exhibited stronger

influence on group separation, with positive loadings corresponding to compounds more abundant in mature leaves and negative loadings representing those enriched in young leaves. This suggests that mature leaves contained a higher abundance of metabolites responsible for group differentiation.

At this stage, the exact metabolites corresponding to these discriminant peaks could not yet be identified. However, the chemical shift regions already provide a preliminary picture of the metabolite groups involved. Signals between δ 6.6-7.0 ppm (δ 6.84, 6.80, 6.72, and 6.68) correspond to aromatic protons, due to phenolic, alkaloids or flavonoid derivatives. Peaks in the δ 4.0-5.5 ppm region (δ 5.44, 4.96, 4.20, 4.16, and 5.20) are commonly assigned to carbohydrate or glycosidic protons, suggesting the involvement of sugars or glycosylated secondary metabolites. The strong signal at δ 2.88 ppm may reflect methylene groups adjacent to electronegative atoms, while the aliphatic signals at δ 1.52, 1.36, and 1.32 ppm could be related to fatty acids, terpenoids, or other aliphatic components. Similar stage-dependent patterns have been reported in other medicinal plants, including *Andrographis paniculata*, where young and mature leaves showed distinct metabolite profiles (Tajidin et al., 2019). Comparable variations were also observed across growth stages in *Peganum harmala* (Li et al., 2018) and *Cynomorium songaricum* (Xue et al., 2020). Together, these studies reinforce the present findings that leaf maturity strongly affects metabolite composition, highlighting the importance of standardizing developmental stage in metabolomics investigations.

For comparison of sampling times, samples were collected only in the morning and afternoon to represent two contrasting environmental conditions. The morning period reflected cooler, shaded conditions, while the afternoon represented warmer, high-light exposure without overextending the sampling schedule. These two time points were selected to capture potential diurnal variation in metabolite composition while maintaining consistency in post-harvest processing. Since each sample required immediate handling steps such as surface cleaning, cutting, cryo-milling, and storage under controlled conditions, increasing the number of collection times would have prolonged the processing period and introduced unwanted variability. Limiting sampling to two key time points ensured experimental consistency.

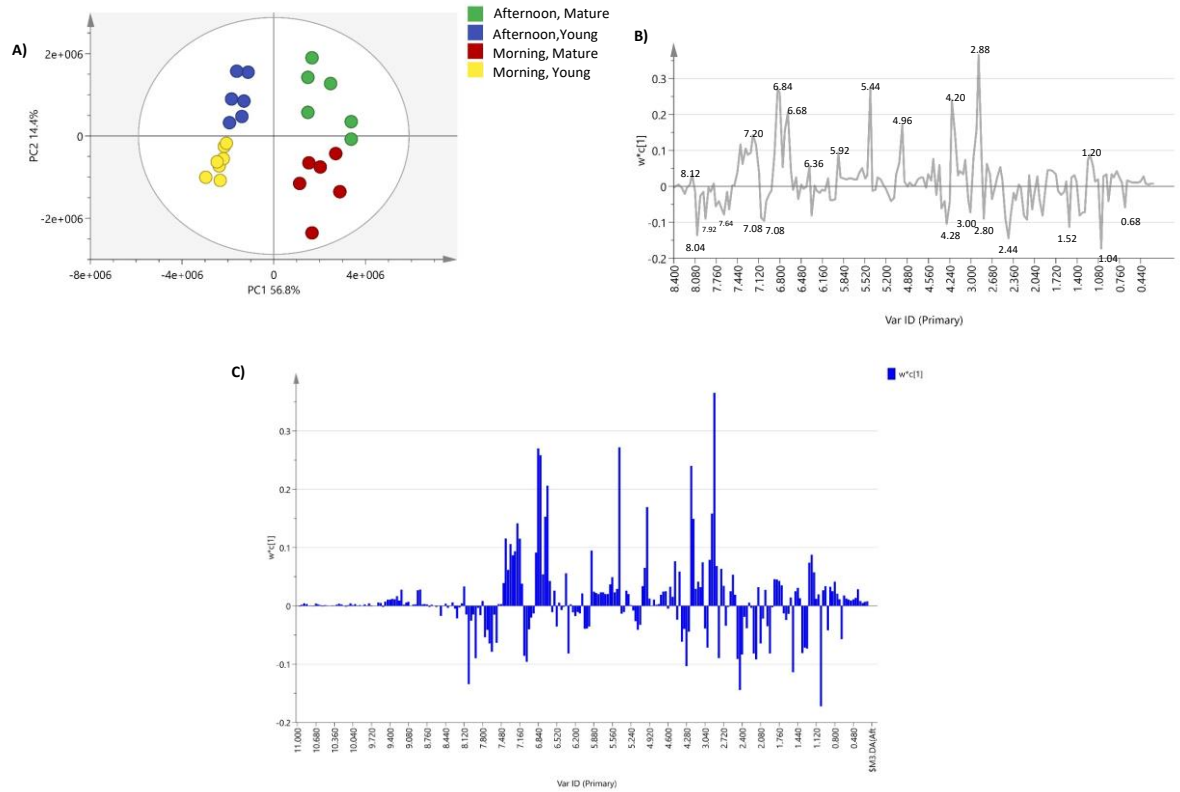

Figure 5. (**A**) PLS-DA score plot showing the effects of maturity stage and sampling time on *P. sarmentosum* metabolite profiles. (**B**) Loading line plot and (**C**) Column loading plot indicating metabolites contributing to the separation between mature and young samples

A hierarchical clustering heatmap was constructed to visualise the variation in metabolite profiles according to leaf maturity and harvesting time (Figure 6). Samples collected in the morning (8:00 a.m.; 26-27 °C, shaded conditions) and those harvested in the afternoon (1:30 p.m.; 30-31 °C, sunny conditions) showed no clear separation within the same maturity group. The clustering pattern

revealed a distinct separation between mature and young leaves, indicating that maturity was the major factor influencing the metabolite composition, while harvesting time had minimal effect. The colour gradient from red to blue represents relatively higher and lower metabolite intensities, respectively. Overall, these findings indicate that samples can be collected at either time of day, as the sampling time had minimal influence on metabolite composition compared to leaf maturity.

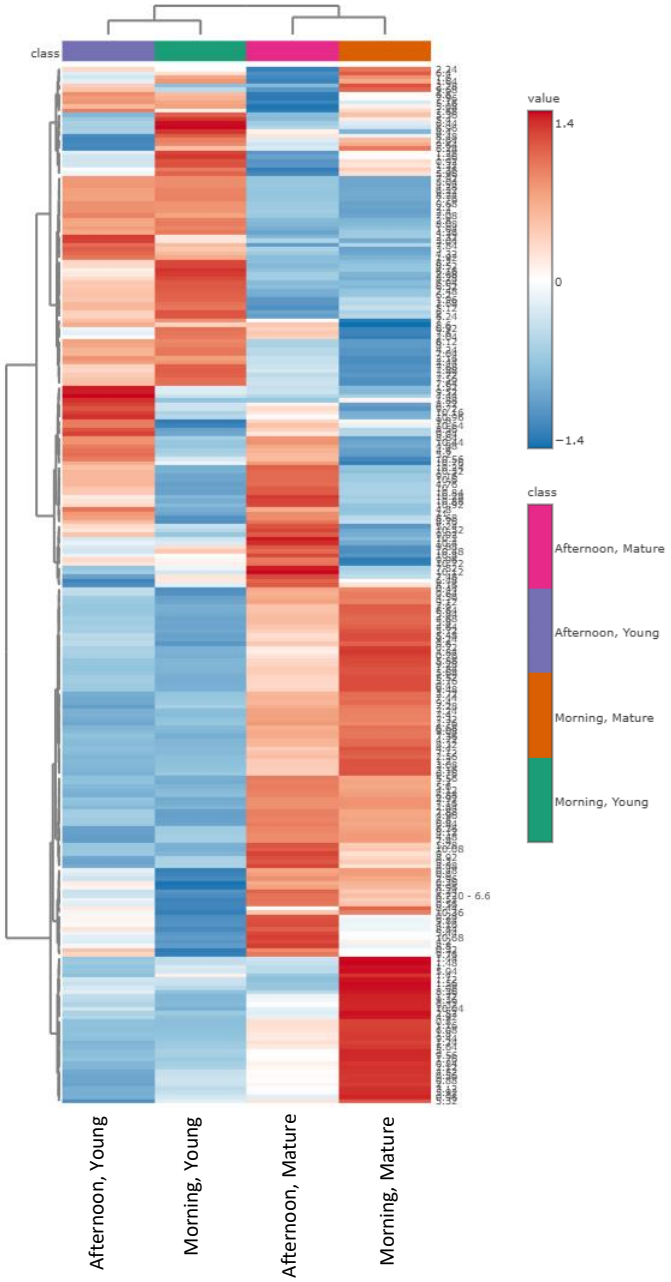

Figure 6. Heatmap visualization of NMR-derived metabolite variation between maturity stages and sampling time

4. CONCLUSION

Optimization of sample mass and NMR acquisition parameters enhanced spectral quality and ensured reliable metabolite detection. This study also demonstrated that while leaf maturity significantly influenced the metabolite profile of *Piper sarmentosum*, sampling time had minimal effect. Therefore, samples can be collected either in the morning or afternoon without substantial impact on metabolite composition. Together, these findings establish a framework for standardized NMR-based metabolomic analysis of *Piper sarmentosum*, providing greater consistency and reproducibility for future studies.

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CONFLICT OF INTEREST
It should disclose any financial or non-financial interests such as political, personal, or professional relationships that may be interpreted as having influenced the manuscript. The phrase "The authors declare no conflicts of interest" should be included if there is no conflict of interest.

AUTHOR CONTRIBUTION
Adiana Mohamed Adib: Conceptualization, methodology, data curation and writing original draft; Ling Sui Kiong: Funding acquisition, resources, investigation and supervision; Nurunajah Ab Ghani: Writing, review and editing; Nor Hadiani Ismail: Investigation and supervision; Noraini Kasim: Conceptualization, review, editing and corresponding author.

DATA AVAILABILITY
The authors confirm that the data supporting the findings of this study are available within the article.

DECLARATION OF GENERATIVE AI
Not applicable.

ETHICS
Not applicable.

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