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Comprehensive Phytochemical Profiling of Malaysian *Piper sarmentosum* Leaves using Mass-Based Dereplication and Molecular Networking

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A comprehensive phytochemical analysis of the methanolic extract of Malaysian *Piper sarmentosum* was carried out utilizing a mass-based dereplication strategy coupled with a molecular networking approach. The dereplication analysis using SIRIUS software in conjunction with the GNPS platform yielded 62 compounds in the extract, including 30 amide alkaloids, 7 phenylpropanoids, 6 lignans, 6 monoacylglycerols, 9 flavonoids, 2 coumarins, 1 terpene and 1 benzofuran. The LCMS phytochemical profile was verified using seven isolated compounds, namely γ-asarone **1**, *trans*-asarone **2**, cepharanone B **3**, sarmentosine **4**, N-[3-(4-methoxyphenyl)propanoyl]pyrrole **5**, N-(3-phenylpropanoyl)pyrrole **6**, and andamanicin **7**. Pyrrole alkaloids **5** and **6** are reported for the first time as constituents of the Malaysian species. These were selected as marker compounds for quantification analysis. The plant extract contained 13.30 µg/mg of **5** and 66.80 µg/mg of **6** through a validated HPLC assay.

Keywords: *Piper sarmentosum*; dereplication; molecular networking; quantification

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P. sarmentosum is an herbaceous plant belonging to the *Piperaceae* family, widely distributed across Southeast Asia, Northeast India, and South China. The plant features stalks with heart-shaped leaf blades and obovoid berry fruits. Its leaves are arranged in an alternating pattern and have a glossy, dark green color. Commonly known as wild betel, this plant has a wide range of applications in both culinary practices and traditional medicine. Due to their spicy taste and intense aroma, the leaves are often used as flavorings in traditional cuisine. Additionally, the plant is believed to treat various ailments, including cough, dysentery, flu, lumbago, malaria, pleurisy, toothache, muscle weakness, and bone pain [1, 2].

A comprehensive review on the traditional uses, metabolites, and biological properties of *P. sarmentosum* was recently published [3]. The plant has been reported to contain a wide range of compounds, including amide alkaloids [4-7], flavonoids [8-10], steroids [4, 10-11], phenylpropanoids [5, 10, 12], benzenoids [11], alkenylphenols [13], terpenes [10-11], lignans [10], and chalcones [11]. The diverse phytochemical composition of this plant contributes to its various pharmacological activities, such as antibacterial [14, 15], anti-inflammatory [16-18], anticancer [19], antioxidant [20], antihypertensive [21], and antidiabetic properties [22]. These attributes have made this plant highly valued, leading to its inclusion in the Malaysian Herbal Monograph 2015.

However, from a regulatory perspective, concerns about authenticity and inconsistency in composition of an herbal plant use as traditional medicine remains a critical issue in developing quality control of herbal medicines. In this regard a plant's fingerprint which refers to unique pattern indicating the presence of multiple markers is a powerful and well accepted technique for the quality control of herbal medicinal products. Preempting the inclusion of *P. sarmentosum* in the Malaysian Herbal Pharmacopeia, which is currently being developed, we herein report the establishment of *P. sarmentosum* leaves chemical fingerprint based on LCMS molecular networking analysis.

EXPERIMENTAL

In this study, we conducted a thorough phytochemical profiling of Malaysian *P. sarmentosum*, utilizing a mass-based dereplication method along with molecular network analysis. The dereplication study was

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Figure 1: *P. sarmentosum.*

implemented using SIRIUS software and Feature-Based Molecular Networking (FBMN) analysis from the Global Natural Products Social Molecular Networking (GNPS) platform. The generated molecular network, visualized with Cytoscape software, improved the depiction of the chemical space of the plant extract. The dereplication analysis was verified using isolated compounds to increase the confidence level of the phytochemical annotation. Additionally, this study performed an HPLC validation method to quantify two isolated compounds derived from this plant, which act as marker class compound for this species.

Chemical Solvents and Reagents

Analytical-grade solvents such as acetone, acetonitrile, ethyl acetate, chloroform, hexane, isopropanol, and methanol were used for extraction, fractionation, and isolation purposes. Acetonitrile HPLC-grade solvent was used for HPLC analysis, and deuterated chloroform ($CDCl_3$) NMR-grade solvent was used for NMR analysis.

Plant Materials and Extraction

The leaves of *P. sarmentosum* were identified by ethnobotanist Tan Ai Lee from the Forest Research Institute Malaysia (FRIM). The plants were collected in June 2018 from Maran, Pahang, Malaysia. A voucher specimen of the collection, labelled K(PS)-PHG-001, was deposited at the herbarium in FRIM. The dried, ground leaves of *P. sarmentosum* (2.5 kg) were extracted by percolation at room temperature using methanol. The extracts were then concentrated with a rotary evaporator, yielding 117.73 g of methanol extract. Figure 1 shows a picture of the *P. sarmentosum* plant.

Pre-Treatment using Solid Phase Extraction (SPE)

Prior to conducting UHPLC-Orbitrap analysis, the methanol leaves extract underwent pre-treatment via the Solid Phase Extraction (SPE) technique. This process was essential for eliminating pigments and sample interferences, thereby facilitating the acquisition of a high-quality LCMS spectrum. The C18 cartridge was first activated with 100% ultrapure water, then flushed with 100% methanol, and conditioned with 95% methanol. A sample concentration of 30 mg/mL was loaded onto the sorbent and eluted with 95% methanol. The resulting filtrate was then dried for subsequent analysis.

Phytochemical Profile by High-Resolution Tandem Liquid Chromatography Mass Spectrometry

The mass data of the pre-treated extract was recorded by Thermo Scientific™ Orbitrap Fusion™ Tribrid™ Mass Spectrometer. This system was equipped with Thermo Scientific Vanquish Horizon UHPLC and connected with the system of degasser, quaternary pump, diode array detector, and auto-sampler system, where FreeStyle Version 1.6 software was used as interface mass data analysis. The sample was eluted onto Accucore™ Vanquish™ C18 UHPLC column (2.1 x 100mm, 1.5 µm, Thermo Scientific, USA) at 40 °C with the injection volume of 1 µL under gradient elution system of deionized water (A): acetonitrile (B) with additional 0.1% formic acid. The solvent was eluted at a flow rate of 0.2 mL/min with the gradient system of 25-100% B in 25 min, 100% B in 5 min, 100-25% B in 2 min, and 25% B in 5 min. Full MS scan was acquired in positive and negative ion modes using Dual Electrospray Ionization (ESI) at capillary voltage 3500 V, sheath

gas 35 arb, auxiliary gas 7 arb, ion transfer tube temperature 300 °C, and vaporized temperature 275 °C. The total ion chromatography (TIC) was recorded from 150 to 1500 mass units with mass resolution 60 000. Positive ionization mode yielded richer signals and thus the sample was further ionized for MS2 level using assisted collision energy mode at 20, 35, 50, and 60 with an orbitrap resolution of 15 000.

Data Mining using MZmine Software

MZmine software version 3.2.8 was used for data mining of the raw data obtained from the UHPLC-MS/MS analysis. Before the data mining process, the raw data were converted into mzML format using the MSConvert tool from Proteo Wizard software. The data mining procedure consisted of four steps: mass detection, ADAP chromatogram building, chromatogram resolving, and isotope filtering.

For mass detection, the noise level threshold for MS1 was set to 1.5E4 and to 0.0 for MS2. The ADAP chromatogram builder, one of the LCMS feature detection steps, used the following parameters: minimum group size in number of scans = 4, group intensity threshold = 1.5E4, minimum highest intensity = 3.5E4, scan-to-scan accuracy = 0.0015 m/z or 10 ppm, and filters with retention time set from 0.0 to 25.0 min, MS level = 1, polarity = positive, and spectrum type = centroided.

The parameters for the chromatogram resolving step were set as follows: the original feature list was kept, MS/MS scan pairing was enabled, S/N threshold = 10, minimum feature height = 1.5E4, coefficient/area threshold = 50, peak duration range = 0.0 to 1.0 min, and RT wavelet range = 0.0 to 0.1 min.

Finally, the isotope filtering step was performed using these parameters: m/z tolerance = 0.001 m/z or 5 ppm, retention time tolerance = 0.030, monotonic shape was enabled, maximum charge = 2, representative isotope = most intense, "never remove feature with MS2" was enabled, and the original feature list was kept.

The data mining procedure yielded two types of data files: .mgf and .csv formats, which were exported to SIRIUS software and the GNPS platform, respectively, for phytochemical annotation analysis. The .mgf format file was generated using the following parameters: select spectra to merge = across samples, m/z merge mode = weighted average (remove outliers), intensity merge mode = sum intensities, expected mass deviation = 0.001 m/z or 10 ppm, cosine threshold (%) = 70.0, signal count threshold (%) = 20.0, isolation window offset (m/z) = 0.0, isolation window width (m/z) = 1.0, merge MS/MS = true, and m/z tolerance = 0.002 m/z or 5 ppm.

***In silico* Phytochemical Annotation using SIRIUS 5 Software**

The .mgf file containing spectral features derived from the data mining procedure was exported to the open-source, Java-based SIRIUS 5 software framework [23] for the in-silico annotation of phytochemicals. SIRIUS 5 software provides a combination of tools, including CSI: FingerID, COSMIC CANOPUS, and ZODIAC, which help annotate chemical compounds using a fast computational approach. The phytochemical annotation process began with molecular formula identification, with the following parameters set: adduct = $[M+H]^+$, instrument = Orbitrap, filter by isotope pattern = true, MS2 mass accuracy (ppm) = 15, MS/MS isotope scorer = score, candidates stored = 10, minimum candidates per ion stored = 1, and elements allowed in the molecular formula = carbon, hydrogen, oxygen, and nitrogen.

To improve molecular formula ranking, the ZODIAC tool was used. It re-ranks molecular formula candidates based on compound similarities in the dataset using fragmentation trees as input [24]. Next, the CSI: FingerID tool [25, 26] was utilized to predict and search molecular fingerprints in structure databases. In this step, the molecular structure was narrowed down to be identified only in the selected natural products database, such as COCONUT. Custom databases of isolated compounds from the *Piper* genus and *P. sarmentosum* species, generated from the Dictionary of Natural Products (DNP) database, were also used to increase the confidence of phytochemical annotation. The CSI: FingerID tool parameters were set as follows: fallback adducts = $[M+H]^+$ and score threshold = true. COSMIC represents a systematic approach that enables the assignment of confidence levels to structural annotations. For each structure annotated by CSI:FingerID, COSMIC offers a confidence score (a value ranging from 0 to 1) that indicates the probability of the accuracy of this annotation [26]. It operates without any parameters. CANOPUS is an abbreviation for class assignment and ontology prediction utilizing mass spectrometry. This free parameter tool was used to predict compound classes based on the molecular formula generated by CSI:FingerID [27-29].

Visualization of Feature-Based Molecular Networking

The feature-based molecular network (FBMN) of methanol extracts of *P. sarmentosum* was generated by uploading the .csv and mzML format files of the LCMS spectral data to the Global Natural Product Social Molecular Networking (GNPS) website (https://gnps.ucsd.edu) using an FTP server. The parameters used to generate the molecular network were as follows: the quantification table source was MZmine, the precursor ion mass tolerance (PIMT) was set to 0.02 Da, and the fragment ion mass tolerance (FIMT) was also set to 0.02 Da. The minimum cosine score for a pair of consensus MS/MS

spectra was 0.7, the network topK was 10, and the minimum matched fragment ions (Min Matched Peaks) was 6. Additionally, the maximum shift between precursors was set to 500 Da, and the maximum connected component size was 100. The library search required a minimum of 6 matched peaks, and the score threshold for an advanced spectral library search was 0.7. The settings for searching analogs were set to "don't search," with a maximum analog search mass difference of 100 Da, and the top results to report per query were limited to 1.

Fractionation and Isolation of Chemical Constituents of *Piper sarmentosum* Leaves

The 50 g of methanol extract was fractionated using the column chromatography (CC) method with an ion exchange resin (DIAIONTM, Mitsubishi Chemical Corporation) as the stationary phase. The CC method utilized a mobile phase consisting of a mixture of water and methanol, which was subsequently flushed with ethyl acetate and then acetone. This fractionation technique yielded four fractions, named F1, F2, F3, and F4.

The F4 (10 g) was subjected to medium-pressure liquid chromatography (MPLC) using a reverse-phase system with a mobile phase of water and methanol to isolate promising compounds, producing five subfractions (F4M1–F4M5). Three subfractions (F4M2, F4M3, and F4M4) were further purified using a WATERS preparative high-performance liquid chromatography system (Prep-HPLC; Agilent Eclipse XDB-C18 column: 21.2 × 250 mm, 7 µm; mobile phase: water and acetonitrile) and recycling preparative high-performance liquid chromatography (Recycling HPLC; JAI; C18 [JAIGEL-ODS-AP, SP-120-15, 20 × 250 mm or JAIGEL-ODS-AP-30, SP-120-15, 30 × 250 mm]; mobile phase: water and methanol).

The purification process resulted in the isolation of γ-asarone **1** (12 mg) and *trans*-asarone **2** (11 mg) from the F4M2 fraction. The F4M3 fraction yielded four amide alkaloids: cepharanone B **3** (1.7 mg), sarmentosine **4** (1.2 mg), N-[3-(4-methoxyphenyl) propanoyl]pyrrole **5** (7 mg), and N-(3-phenylpropanoyl) pyrrole **6** (6 mg). Lastly, the F4M4 fraction produced andamanicin **7** (7 mg).

Method Validation of HPLC

The HPLC method validation was performed following the International Conference on Harmonization (ICH) guidelines [30]. The method was validated by considering several analytical parameters, including specificity, linearity and range, limit of detection (LOD), limit of quantitation (LOQ), precision, and accuracy. Compounds **5** and **6** were used as standard compounds and quantified in this work.

Specificity

Specificity refers to a validation procedure that should confirm the ability of an analytical method to assess unmistakably the analyte in the presence of other components or a complex mixture. The specificity test was performed by comparing the retention time of **5** and **6** in *P. sarmentosum* extract with that of standard compounds.

Linearity and Range

The following standard solutions were prepared: Compound **5** at concentrations of 10, 20, 30, 40, 50, and 60 µg/mL, and Compound **6** at concentrations of 20, 40, 60, 80, 100, and 120 µg/mL. The prepared standard solutions were filtered through a 0.22 µm membrane filter and analyzed in triplicate to create a calibration curve for each of the two standard compounds independently. The calibration curves were constructed by plotting the standard solution concentrations (x, µg/mL) against peak areas (y, mAU). Regression coefficients (R^2), slopes, intercepts, and standard deviations were calculated from the calibration curves. The calculated R^2 was used to verify the linearity of the relationship between the standard solution concentrations and peak areas.

Limit of Detection (LOD) and Limit of Quantification (LOQ) Analysis

The limit of detection (LOD) is the lowest concentration of an analyte in a sample that can be detected. The limit of quantification (LOQ) is the lowest concentration of an analyte in a sample that can be quantified with admissible accuracy and precision under stated operational conditions. The LOD and LOQ values were calculated based on the standard deviation of the response and the slope of the calibration curve obtained from the linearity test. The following equations were used for the calculation of the LOD and LOQ values.

The LOD can be expressed as: $\mathrm{LOD} = \frac{3.3\sigma}{S}$

The LOQ can be expressed as: $\mathrm{LOQ} = \frac{10\sigma}{S}$

Where σ = the standard deviation of the response, S = the slope of the calibration curve.

Precision

Precision is a measure of the reproducibility of a result. It involves repeated measurements under the same operating conditions over a short interval of time, which should yield consistent results. The precision of the analytical method was evaluated by considering both intra-day and inter-day tests. Intra-day precision was assessed by analyzing the standard solutions at three different concentrations (10, 30, and 60 µg/mL for **5**,

and 20, 60, and 120 µg/mL for **6**) three times for each concentration within a single day. Inter-day precision was determined by injecting the standard solutions at the same three concentrations (10, 30, and 60 µg/mL for **5**, and 20, 60, and 120 µg/mL for **6**) in triplicate each day, over three consecutive days. The results of the precision tests were expressed as relative standard deviation (% RSD). The formula used to calculate % RSD is: % RSD = (standard deviation / mean measured amount) x 100.

Accuracy

Accuracy is the degree of agreement between the measured value and the true value. The accuracy analysis was performed by spiking a known amount of standard solution of **5** and **6** at three different levels (low, medium, and high) into 1000 µg/ml of *P. sarmentosum* extract. All the spiked samples were analyzed in triplicate. The results of the recovery analysis were calculated based on the measured concentration of the un-spiked sample, the spiked sample, and the amount of spiked standard compounds. The recovery (%) was calculated based on the following equation:

$$Recovery\ (\%) = \frac{(concentration\ of\ spiked\ sample - concentration\ of\ un-spiked\ sample)}{amount\ of\ spiked\ standard} \times 100$$

RESULTS AND DISCUSSION

Phytochemical Annotation using High-Resolution Tandem Liquid Chromatography-Mass Spectrometry

The comprehensive profiling of phytochemicals in the methanolic extract of *P. sarmentosum* leaves was conducted using a dereplication approach based on high-resolution tandem liquid chromatography-mass spectrometry (LCMS) data, including molecular networking analysis. The plant extract was analyzed in positive ion mode using an Orbitrap Fusion™ Tribrid™ mass spectrometry system. The spectrum data of the plant extract, covering a mass range from m/z 150 to 1500, was processed using Mzmine software before proceeding with phytochemical annotation. The pre-processed LCMS data was then imported into the SIRIUS software application [23] for in-silico metabolite annotation. Subsequently, the spectral LC-MS data was uploaded to the Global Natural Products Social Molecular Networking (GNPS) platform (https://gnps.ucsd.edu/) for spectral library searches and advanced molecular networking through Feature-Based Molecular Networking (FBMN) analysis. This process enhanced the confidence in phytochemical annotation and improved visualization of the plant extract's chemical composition.

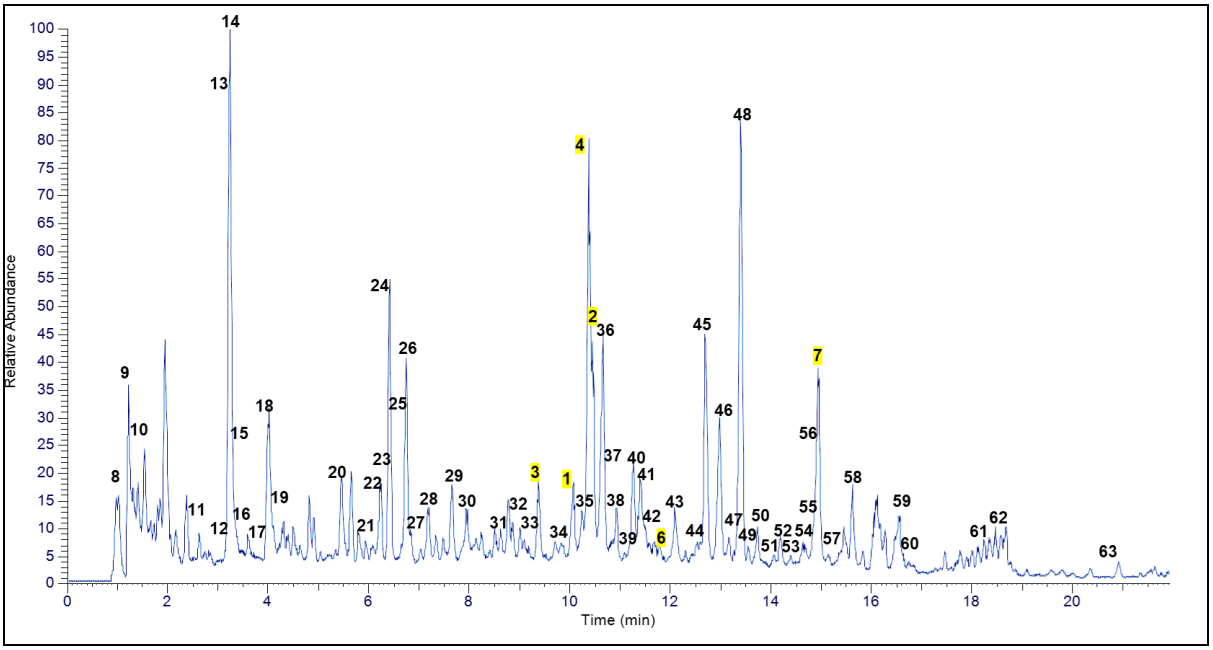

Figure 2. Total Ion Chromatogram (TIC) of the Methanol Extract of *P. sarmentosum* Leaves with Annotated Compounds.
Note: The highlighted numbers are the isolated compounds.

The combined application of SIRIUS and GNPS led to the identification of 62 compounds in the methanolic extract of *P. sarmentosum* leaves. The phytochemical annotation revealed a diverse range of compounds, including 30 amide alkaloids, 9 flavonoids, 7 phenylpropanoids, 6 lignans, 6 monoacylglycerols, 2 coumarins, 1 benzofuran, and 1 terpene. Notably, 31 out of the 62 annotated compounds were previously isolated from *P. sarmentosum*. The mass error for these annotated compounds was under 5 ppm. Although in-silico metabolite databases provide valuable annotation guidance, validating retention time and MS/MS fragmentation data with reference standards is essential for achieving high confidence in metabolite identification [34]. Therefore, the annotated compounds were verified using seven known isolated compounds from *P. sarmentosum*: **1** [12], **2** [12], **3** [31], **4** [32], **5**, **6** [32] and **7** [33].

In this study, the annotated compounds were classified at levels 1 and 2 according to the Metabolite Identification Confidence (MIC) levels proposed by [34]. Level 1 indicates compounds identified by comparing MS/MS fragmentation patterns with reference standards, while level 2 involves comparison of MS/MS fragmentation patterns only. The list of annotated compounds is presented in Table 1, and the total ion chromatography (TIC) of the plant extract, with the assigned annotated compounds, is shown in Figure 2. The chemical structures of the isolated compounds from this plant extract are illustrated in Figure 3. Figure 4 displays the molecular network generated from FBMN analysis, visualizing the network of identified phytochemicals using Cytoscape software.

Table 1. Annotated Compounds from Methanolic Extract of *P. sarmentosum* Leaves.

No.	t_R (min)	Compounds	Molecular Formula	Experimental $[M+H]^+$	Calculated $[M+H]^+$	δ_{ppm}	MS/MS	SIRIUS/GNPS
8	1.03	hexagol	$C_{12}H_{26}O_7$	283.1753	283.1757	1.41	239.1481, 195.1200, 177.0900, 151.1000, 133.0854	Both
9	1.27	4'-*O*-glucosylvitexin.	$C_{27}H_{30}O_{15}$	595.1663	595.1663	0.00	433.1140, 415.1030, 379.0820, 313.0710, 271.0600	Both
10	1.35	vitexin-2"-*O*-rhamnoside.	$C_{27}H_{30}O_{14}$	579.1714	579.1714	0.00	433.1119, 415.1015, 379.0804, 313.0698, 283.0594	Both
11	2.62	loliolide	$C_{11}H_{16}O_3$	197.1174	197.1177	-1.52	179.1061, 161.0956, 135.1162, 133.1006, 107.0849, 93.0693	Both
12	3.23	vanillin	$C_8H_8O_3$	153.0547	153.0551	2.61	125.0590, 111.0434, 97.0641, 93.0328	Both
13	3.24	2,6-dimethoxy-4-(prop-2-en-1-yl)phenol	$C_{11}H_{14}O_3$	195.1017	195.1021	-2.05	167.0697, 165.0905, 153.0541, 133.0642, 133.0642, 105.0693, 79.0537	SIRIUS
14	3.38	paprazine	$C_{17}H_{17}NO_3$	284.1284	284.1286	0.70	164.0697, 147.0432, 121.0640, 119.0484, 105.0327, 95.0484	Both
15	3.52	scoparone	$C_{11}H_{10}O_4$	207.0655	207.0657	0.97	163.0383, 151.0747, 146.0365, 135.0433, 107.0484, 79.0536	Both
16	3.59	moupinamide	$C_{18}H_{19}NO_4$	314.1391	314.1392	0.32	177.0547, 145.0283, 121.0644, 117.0331, 103.0538	Both
17	3.87	sinapaldehyde	$C_{11}H_{12}O_4$	209.0809	209.0813	-1.91	177.0541, 149.0589, 131.0482, 121.0640, 103.0534, 95.0454, 79.0535	SIRIUS
18	4.02	gazarin	$C_{10}H_{12}O_4$	197.081	197.0814	2.03	182.0567, 169.0853, 154.0618, 138.0675, 123.0441	Both
19	4.1	genipin	$C_{11}H_{14}O_5$	227.0915	227.0919	1.76	209.0802, 177.0546, 149.0597, 121.0648, 69.0335	Both
20	5.46	piperlotine D	$C_{16}H_{21}NO_4$	292.1547	292.1549	0.68	221.0802, 178.0617, 167.0695, 124.0751, 98.0593, 70.0645	SIRIUS
21	6.19	3-(2*H*-1,3-benzodioxol-5-yl)-1-(piperidin-1-yl)prop-2-en-1-one	$C_{15}H_{17}NO_3$	260.1284	260.1286	0.77	218.1173, 200.1069, 131.0485, 103.0537	SIRIUS
22	6.24	(*E*)-1-cinnamoylpyrrolidine	$C_{13}H_{15}NO$	202.1228	202.1232	1.98	131.0487, 103.0537, 98.0595, 72.0802	SIRIUS
23	6.38	isokaempferide	$C_{16}H_{12}0_6$	301.0709	301.0711	0.66	258.0518, 240.0399, 229.0487, 184.0514, 136.0147, 121.0276	Both

No.	t_R (min)	Compounds	Molecular Formula	Experimental $[M+H]^+$	Calculated $[M+H]^+$	δ_{ppm}	MS/MS	SIRIUS/GNPS
24	6.4	pralina	$C_{10}H_8O_2$	161.0598	161.0602	-2.48	133,0640, 118.0406, 105.0692, 95.0483, 90.0458, 79.0536	SIRIUS
25	6.41	piperlotine A	$C_{14}H_{17}NO_3$	232.1334	232.1337	1.29	232.1334, 161.0590, 133.0640	SIRIUS
26	6.74	piplartine	$C_{17}H_{19}NO_5$	318.134	318.1341	0.31	276.1228, 131.0484, 128.0700, 103.0537	SIRIUS
27	6.82	piperolactam A	$C_{16}H_{11}NO_3$	266.0815	266.0817	0.75	223.0622, 196.0513, 194.0595	Both
28	7.18	3-(3,5-dimethoxyphenyl)-1-(pyrrolidin-1-yl)prop-2-en-1-one	$C_{15}H_{19}NO_3$	262.1441	262.1443	0.76	191.0696, 176.0462, 163.0747, 148.0512, 135.0800	SIRIUS
29	7.64	piperolactam B	$C_{17}H_{13}NO_4$	296.092	296.0924	1.35	263.0572, 238.0493, 235.0621, 209.0595, 207.0671, 181.0642	SIRIUS
30	7.99	4-methylcoumarin	$C_{10}H_8O_2$	161.0598	161.0602	2.48	133.0641, 118.0406, 103.0535, 95.0484, 79.0536	Both
31	8.5	piperylin	$C_{16}H_{17}NO_3$	272.1283	272.1286	1.10	201.0546, 171.0439, 159.0439, 137.0831, 135.0437, 115.0537, 98.0595	Both
32	9.04	chrysin	$C_{15}H_{10}O_4$	255.0654	255.0657	1.18	153.0175, 152.0615, 129.0329, 103.0536, 68.9965	Both
33	9.26	acacetin	$C_{16}H_{12}O_5$	285.076	285.0763	1.05	213.0540, 185.0595, 168.0562, 133.0641, 124.0152	Both
3	9.35	cepharanone B	$C_{17}H_{13}NO_3$	280.0971	280.0973	0.71	219.0673, 209.0591, 206.0594, 181.0641, 180.0801, 152.0614	SIRIUS
34	9.82	luteolin 3',4'-dimethyl ether	$C_{17}H_{14}O_6$	315.0866	315.0868	0.63	272.0675, 243.0643, 201.0538	Both
1	10.07	γ-asarone	$C_{12}H_{16}O_3$	209.117	209.1177	3.34	177.0905, 165.0905, 149.0591, 121.0641, 107.0485, 91.0536	SIRIUS
35	10.24	yangambin	$C_{24}H_{30}O_8$	447.2018	447.2019	0.22	383.1491, 279.1221, 247.0959, 232.1088, 187.0746, 181.0852	SIRIUS
4	10.37	sarmentosine	$C_{18}H_{21}NO_3$	300.1598	300.1599	0.33	201.0905, 187.0746, 161.0591, 131.0484, 98.0593, 72.0802	SIRIUS
2	10.44	*trans*-asarone	$C_{12}H_{16}O_3$	209.1174	209.1177	1.43	177.0905, 165.0905, 149.0591, 121.0641, 107.0485, 91.0536	SIRIUS
36	10.66	7,7'-epoxy-3,3'-dimethoxy-4,5:4',5'-bis(methylenedioxy)lignan	$C_{22}H_{24}O_7$	401.1601	401.16	-0.25	352.1303, 249.1116, 207.1009, 168.0774, 153.0540	SIRIUS
37	10.74	piperolactam C	$C_{18}H_{15}NO_4$	310.1076	310.1079	-0.98	280.0601, 277.0729, 249.0781, 221.0820, 193.0888	SIRIUS

No.	t_R (min)	Compounds	Molecular Formula	Experimental $[M+H]^+$	Calculated $[M+H]^+$	δ_{ppm}	MS/MS	SIRIUS/GNPS
38	10.79	1-piperettylpyrrolidine; (*E*,*E*,*E*)-form	$C_{18}H_{19}NO_3$	298.1441	298.1443	0.67	227.0698, 199.0749, 169.0642, 161.0591, 141.0691, 98.0594, 72.0802	SIRIUS
39	11.1	piperamide-C7:1 (6*E*)	$C_{18}H_{23}NO_3$	302.1754	302.1756	0.66	213.0903, 173.0954, 135.0433, 129.0692, 98.0593, 72.0801	SIRIUS
40	11.27	5-methoxy-3,4:3',4'-bis(methylenedioxy)lignan-9,9'-olide	$C_{21}H_{20}O_7$	385.1287	385.1287	0.00	353.1397, 237.4124, 189.0910, 175.0755, 121.0642	SIRIUS
41	11.46	monolinolenin (9c,12c,15c)	$C_{21}H_{36}O_4$	353.2691	353.2692	0.28	335.2583, 279.2315, 261.2208, 233.2259, 109.1005	Both
42	11.88	1-[(2E,4E)-7-(3,4-methylenedioxyphenyl)-2,4-heptadienoyl]pyrrolidine	$C_{18}H_{21}NO_3$	300.1598	300.1599	-0.33	227.0104, 201.0905, 187.0746, 161.0591, 131.0484, 98.0593, 72.0802	SIRIUS
6	11.94	*N*-(3-phenylpropanoyl)pyrrole	$C_{13}H_{13}NO$	200.1071	200.1075	1.99	146.0962, 133.0645, 105.0694, 91.0537, 68.0490	SIRIUS
43	12.15	7-(2*H*-1,3-benzodioxol-5-yl)-1-(piperidin-1-yl)hepta-2,6-dien-1-one	$C_{19}H_{23}NO_3$	314.1754	314.175	1.27	201.0901, 161.0590, 138.0903, 135.0438, 131.0484, 112.0752, 186.0960	SIRIUS
44	12.53	3',4',5'-trimethoxycinnamyl alcohol	$C_{12}H_{16}O_4$	225.1123	225.1127	1.78	207.1012, 193.0854, 179.1060, 163.0750, 151.0748, 105.0694	SIRIUS
45	12.71	sarmentine	$C_{14}H_{23}NO$	222.1854	222.1858	1.80	150.0907, 124.0750, 110.0957, 98.0593, 81.0329, 72.0802	SIRIUS
46	13.15	piperamide-C9:3 (2*E*,4*E*,8*E*)	$C_{20}H_{23}NO_3$	326.1755	326.1756	0.30	227.1061, 211.0747, 161.0590, 135.0433, 98.0593, 72.0802	SIRIUS
47	13.37	pellitorine	$C_{14}H_{25}NO$	224.2011	224.2014	1.33	196.2045, 168.1376, 151.1110, 123.1161, 69.0693	Both
48	13.4	brachyamide B	$C_{20}H_{25}NO_3$	328.1913	328.1912	-0.30	227.1059, 213.0912, 161.0591, 135.0434, 98.0593, 72.0802	SIRIUS
49	13.66	tectochrysin	$C_{16}H_{12}O_4$	269.081	269.0814	1.49	226.0624, 225.0541, 197.0595, 152.0615, 124.0149	Both
50	13.77	apigenin 7,4'-dimethyl ether	$C_{17}H_{14}O_5$	299.0917	299.0919	0.67	256.0724, 241.0489, 227.0697, 213.0539, 167.0332, 124.0147	Both
51	13.81	glyceryl Palmitate	$C_{19}H_{38}O_4$	331.2846	331.2848	0.60	123.1162, 109.1005, 95.0848, 85.1005, 71.0855	Both
52	14.19	piperamide-C9:1 (8E)	$C_{20}H_{27}NO_3$	330.2067	330.2069	0.61	175.0748, 161.0592, 147.0799, 135.0619, 72.0801	SIRIUS

No.	t_R (min)	Compounds	Molecular Formula	Experimental $[M+H]^+$	Calculated $[M+H]^+$	δ_{ppm}	MS/MS	SIRIUS/GNPS
53	14.37	7,7'-epoxy-8,8'-lignan-3,3',4,4',5,5'-hexol; (7R*,7'R*,8R*,8'R*)-form, 3,4-methylene, 3',4',5,5'-tetra-Me ether	$C_{23}H_{28}O_7$	417.1911	417.1913	0.48	294.1246, 249.1116, 235.1322, 195.0644, 181.0852, 151.0747	SIRIUS
54	14.61	linolenate	$C_{18}H_{30}O_2$	279.2319	279.2324	1.79	137.1315, 123.1162, 109.1004, 95.0847, 81.0691	Both
55	14.69	5-hydroxy-3,7-dimethoxy-2-(4-methoxyphenyl)chromen-4-one	$C_{18}H_{16}O_6$	329.1022	329.1025	0.91	313.0700, 271.0594, 243.0645, 215.0698, 135.0432	Both
56	14.85	N-(2-methylpropyl)undeca-2,4-dienamide	$C_{15}H_{27}NO$	238.2166	238.2171	-2.10	168.1379, 151.1112, 133.1007, 123.1162, 95.0485, 81.0692, 69.0693	SIRIUS
7	14.93	Andamanicin	$C_{24}H_{32}O_6$	417.2274	417.2277	0.72	235.1315, 209.1166, 181.0853, 169.0851, 153.0901	SIRIUS
57	15.17	2S)-2,3-dihydroxypropyl (9Z,12Z)-octadeca-9,12-dienoate	$C_{21}H_{38}O_4$	355.2846	355.2849	0.84	337.2734, 263.2365, 245.2259, 123.1162, 95.0849, 81.0693	Both
58	15.63	1-[11-(3,4-Methylenedioxyphenyl)-2,4,10-undecatrienoyl]pyrrolidine; (2E,4E,10E)-form	$C_{22}H_{27}NO_3$	354.2068	354.2069	0.28	255.1375, 206.1532, 161.0590, 135.0433, 96.0593, 72.0802	SIRIUS
59	16.63	1-[(2E,4E)-2,4-Dodecadienoyl]Pyrrolidine	$C_{16}H_{27}NO$	250.2168	250.2164	1.60	150.0913, 124.0755, 110.0962, 98.0598, 95.0489, 72.0805	SIRIUS
60	16.86	N-Isobutyl-(2E,4E)-Dodecadienamide	$C_{16}H_{29}NO$	252.2323	252.2325	-0.79	196.1693, 179.1426, 154.1222, 135.1165, 95.0848, 81.0329, 69.0693	SIRIUS
61	18.67	Guineesine	$C_{24}H_{33}NO_3$	384.2539	384.2538	-0.26	201.0908, 187.0750, 175.0746, 161.0590, 149.0592, 135.0433	Both
62	18.82	Bollex	$C_{19}H_{32}O_2$	293.2478	293.248	0.68	261.2206, 243.2103, 161.1317, 137.1316, 95.0847	SIRIUS
63	20.71	Tetradeca-2E,4E-Dienoic Acid Pyrrolidide	$C_{18}H_{31}NO$	278.2481	278.2485	-1.43	150.0909, 124.0750, 110.0957, 98.0594, 95.0484, 72.0802	SIRIUS

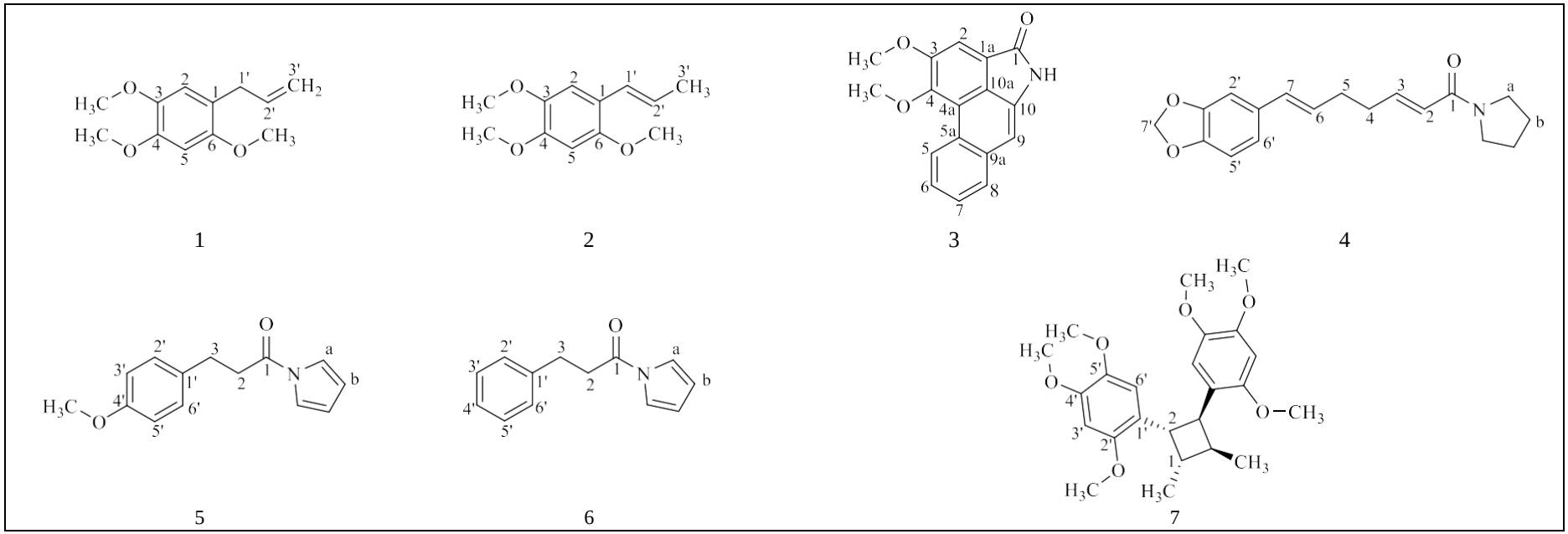

Figure 3. Isolated Compounds of *P. sarmentosum* Leaves.

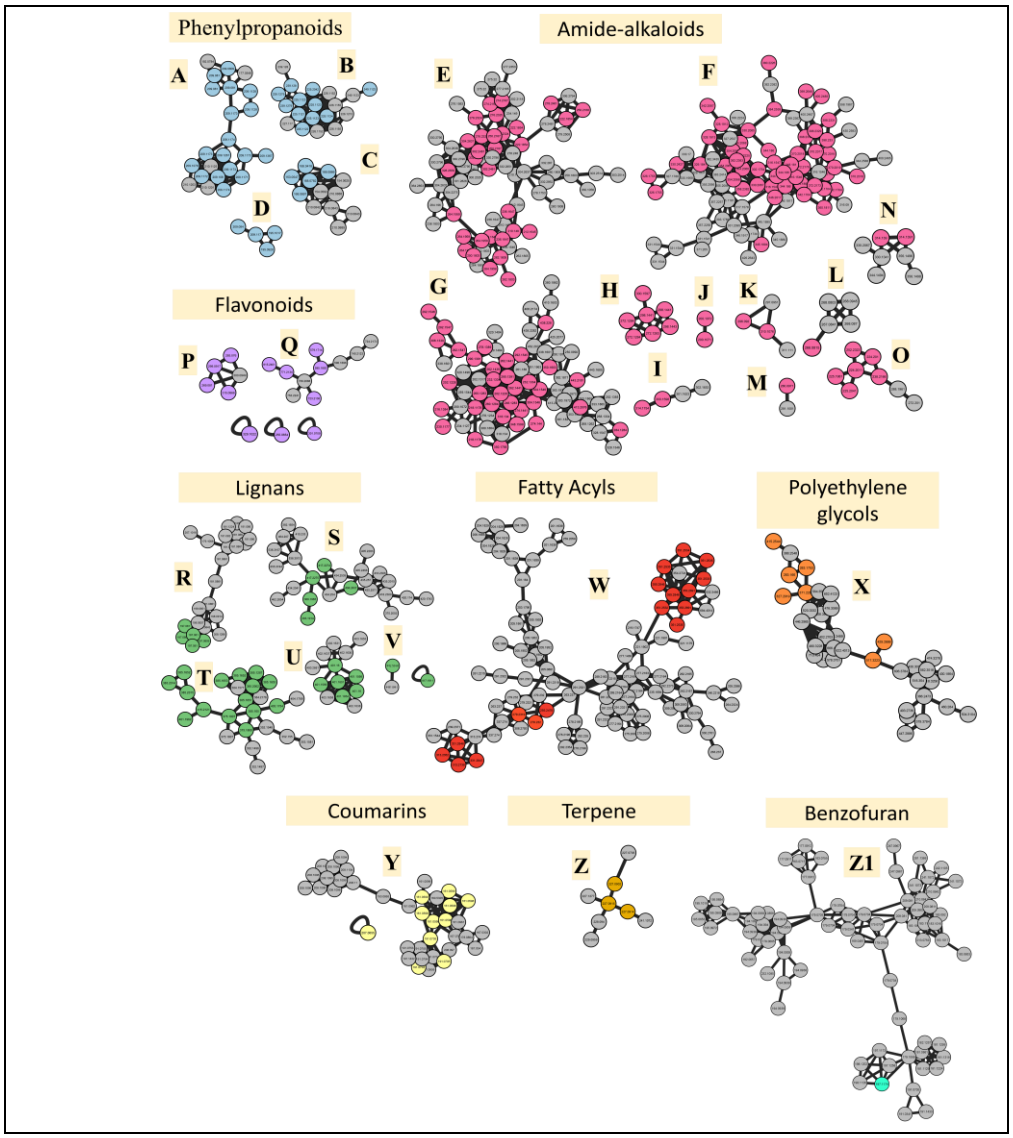

Figure 4. Molecular Networks of Annotated Phytochemicals of *P. sarmentosum* Leaves.

Visualization of the Molecular Network of Annotated Compounds

The visualization of the generated molecular network (Figure 4) consists of nodes and edges. The nodes represent each spectrum labeled as precursor ion mass (m/z), while the edges connected between the nodes represent the alignment from one spectrum to another. The FBMN analysis resulted in the classification of 28 clusters (Cluster A-Z1), which refers to the molecular networks of the 62 identified compounds. In this study, a variety of amide-alkaloids were identified in the methanolic plant extract, including pyrrolidines (16; Cluster E-I), pyrroles (2; Cluster G and J), piperidine (2; Cluster G and I), aphorphine (4; Cluster K-M), and N-acyl amines (6; Cluster F, G, N, and O). Additionally, this work has successfully annotated constituents from other classes of compounds, including phenylpropanoids (6; Cluster A-D), flavonoids (9; Cluster P and Q), lignans (6; Cluster R and S), fatty acyls (5; Cluster W), polyethylene glycols (1; Cluster X), coumarins (3; Cluster Y), terpenes (1; Cluster Z), and benzofurans (1; Cluster Z1).

The phytochemical profiling of *P. sarmentosum* through LCMS analysis has yielded a comprehensive overview of the chemical composition of its methanolic leaf extract. The resulting data offer valuable chemical "fingerprints" that can be used for authenticity testing and quality control in the production of herbal medicines. Moreover, the molecular networking analysis that has been carried out has further enriched the understanding of the phytochemistry of this plant by revealing the relationships between related chemical compounds based on their fragmentation patterns of LCMS data. Together, these findings not only establish a detailed profile of the plant's phytochemicals but also lay the groundwork for future research, potentially guiding the development of therapeutic applications and enhancing the quality standards in the herbal medicine industry.

Validation HPLC Method for Quantification 5 and 6 in Leaves of *P. sarmentosum*

A validated HPLC method was established utilizing the two isolated pyrrole alkaloids, **5** and **6** as the reference standards. This work is the first to report a validated method for HPLC analysis of *P. sarmentosum* leave extract, which is important as a base for analytical quality control tools for this plant. The choice of **5** and **6** as standards in the validated method is based on the fact the pyrrole alkaloids characteristics of *P. sarmentosum*. In our work, pyrrole alkaloids **5** and **6** are isolated for the first time from Malaysian *P. sarmentosum* making them available to be used for the establishment of the validated method. Pyrrole and pyrrolidine classes of compounds exhibit a wide range of therapeutic applications, including agents of antibiotics, anti-inflammatory, anti-tubercular, antitumor, and cholesterol-reducing drugs. This class of compounds has garnered significant attention due to their biological and pharmacological significance [35].

The HPLC method was validated using several analytical parameters including specificity, linearity, range, limit of detection (LOD), limit of quantitation (LOQ), precision, and accuracy. The HPLC method validation was conducted by following the guidelines set by the International Conference on Harmonization (ICH). The subsections below discuss the detailed results of the HPLC method validation.

Specificity

The specificity test was verified by analyzing the HPLC chromatograms of **5** and **6**, as well as the methanolic *P. sarmentosum* leaves extract (Figure 5). The retention times of **5** and **6** in the extract were compared to those of the standard compounds. The specificity test identified **5** and **6** in the plant extract at 14.29 and 14.72 minutes, respectively.

Linearity, Range, Limit of Detection (LOD), and Limit of Quantification (LOQ)

The linearity analysis has generated calibration curves for both standard compounds (Figure 6). The linear regression equation obtained for **5** is y=16.2700x+ 7.0469, while for **6**, it is 7.0317x-20.2280. The regression coefficients (R^2) obtained for both standards were more than 0.9900 with a concentration range of 10–60 µg/ml for **5** and 20–120 µg/ml for **6**, indicating good linearity within the proposed range. The LOD values obtained for **5** and **6** are 0.2591 µg/ml and 6.2301µg/ml, respectively. The LOQ values obtained for **5** and **6** are 1.6644 µg/ml and 10.0081µg/ml, respectively.

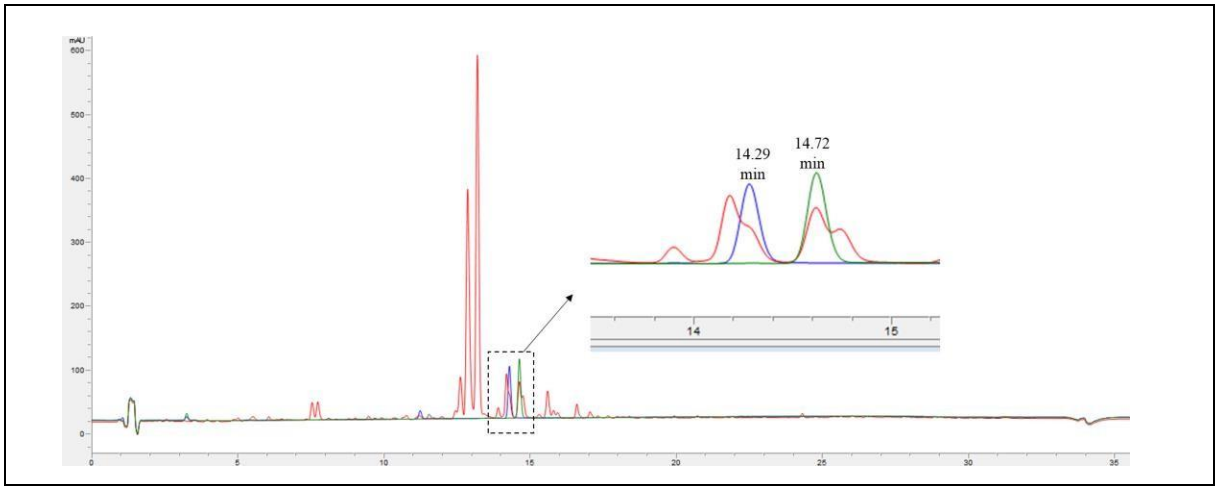

Figure 5. Overlay Chromatograms of Methanolic *P. sarmentosum* Leaves Extract and Standard Compounds (**5** and **6**)
Notes: Red: Methanolic *P. sarmentosum* Leaves Extract, Blue: **5**, Green: **6**

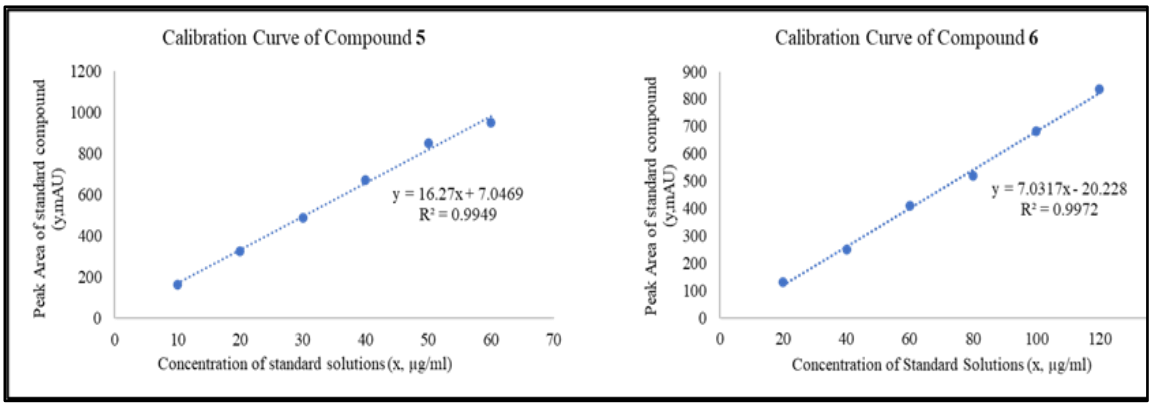

Figure 6. Calibration curve of **5** and **6**.

Table 2. Precision Test.

Compounds	Replication	Concentration (µg/mL)	Intra-day Test Result			Inter-day Test Result		
			Measured (µg/ml)	SD	RSD (%)	Measured (µg/ml)	SD	RSD (%)
5	1	10.00	9.60	0.07	0.72	9.47	0.05	0.52
	2		9.73			9.55		
	3		9.65			9.56		
	1	30.00	29.57	0.08	0.27	29.31	0.12	0.41
	2		29.52			29.08		
	3		29.68			29.12		
	1	60.00	57.94	0.08	0.14	57.78	0.14	0.24
	2		58.08			57.51		
	3		58.07			57.62		
6	1	20.00	21.62	0.05	0.23	21.06	0.28	1.35
	2		21.58			20.74		
	3		21.67			20.51		
	1	60.00	61.13	0.10	0.16	58.95	0.36	0.61
	2		61.19			59.16		
	3		61.33			58.45		
	1	120.00	121.55	0.3	0.25	116.95	0.78	0.66
	2		121.89			117.11		
	3		122.03			118.37		

Table 3. Accuracy Test.

Compounds	Concentration (µg/ml)	Spiked Standard (µg/ml)	Spiked Sample (µg/ml)	Recovery (%)	SD	RSD (%)
5	13.30	11.00	23.80	95.45	0.47	1.97
		13.00	26.30	100.00	0.5	1.90
		16.00	31.00	110.63	0.6	1.64
6	66.80	53.00	115.8	92.45	1.47	1.27
		67.00	132.7	98.36	1.99	1.50
		80.00	139.7	91.13	1.44	1.03

Precision

The precision of the analytical method was evaluated through intra-day and inter-day tests. Three different concentrations of the standard solution of **5** (10, 30, and 60 µg/ml) and **6** (20, 60, and 120 µg/ml) were analysed in triplicate within a day for intra-day testing. The inter-day test was performed by analysing the same concentrations of standard solution as in the intra-day test but conducted on three consecutive days. The relative standard deviation (RSD) values for both tests were calculated based on the measured concentration of the standard solutions. The obtained (RSD) values of the intra-day and inter-day precision were in the range of 0.14–0.72% and 0.24-1.25%, respectively, as shown in Table 2 The achieved RSD% values fell within acceptable limits (<2%), showing a high level of precision in the proposed method.

Accuracy

The accuracy of the proposed analytical method was validated by performing a recovery test. The test was performed by spiking the *P. sarmentosum* extract samples with standard solutions of **5** and **6** at three different levels of concentration. The test for both standards exhibited recoveries within the range of 91.13% to 110.63%, with relative standard deviation (RSD) values below 2.0%, indicating that the suggested analytical approach possesses high accuracy. Table 3 shows the results obtained from the accuracy test.

Quantification of 5 and 6 in *P. sarmentosum* Leaves

The validated analytical HPLC method was applied to quantify **5** and **6** in the methanolic *P. sarmentosum* leaves extract. The identification of the compounds was performed by comparing their retention time with the plant extract under the same HPLC conditions. Based on the quantification analysis, the plant extract was found to contain 13.30 µg/mg and 66.80 µg/mg of **5** and **6**, respectively.

CONCLUSION

The comprehensive study of the phytochemicals in the methanolic leaf extract of *P. sarmentosum* using a dereplication approach based on High-Resolution Tandem Liquid Chromatography Mass Spectrometry (LCMS) data, including molecular networking analysis, resulted in the annotation of 62 compounds, with alkaloids being the predominant class. The extract was found to contain 13.30 µg/mg of N-[3-(4-methoxyphenyl)propanoyl]pyrrole **5** and 66.80 µg/mg of N-(3-phenylpropanoyl)pyrrole **6**, as determined through a quantitative analysis by HPLC. The identification of pure isolated compounds and the detailed LCMS profile of this plant extract provide valuable information for further studies. These findings support the development of consistent and high-quality herbal products by providing more scientific evidence on the phytochemical composition of *P. sarmentosum*.

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