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Phytochemical Profiling of In vitro Anticancer Active Fraction of *Physalis angulata* Using Mass-Based Dereplication

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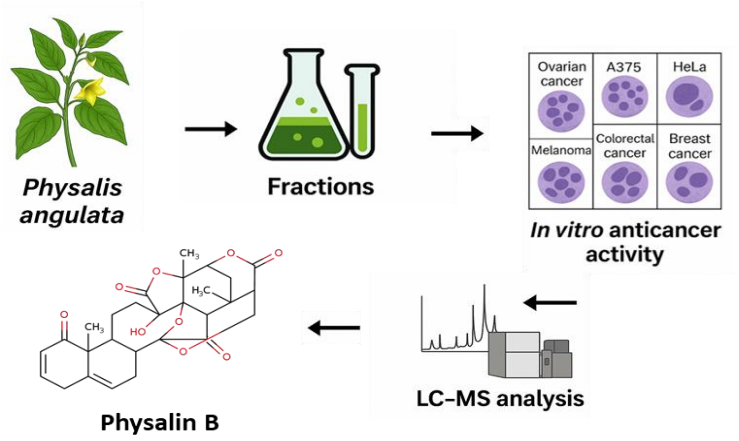
LC-MS
A375
HeLa
HT-29
MCF-7

ABSTRACT

Physalis angulata, a medicinal plant traditionally used for various ailments, was studied for its potential as a source of anticancer agents using a bioassay-guided fractionation method. The objective of the study was to assess the in vitro anticancer properties of fractions derived from the *P. angulata* whole plant and to identify the active constituents through liquid chromatography–mass spectrometry (LC-MS) analysis. Whole plant crude extracts were subjected to fractionation, and the obtained fractions were evaluated against a panel of six human cancer cell lines, which included ovarian cancer (A2780, SKOV3), melanoma (A375), cervical cancer (HeLa), colorectal cancer (HT-29), and breast cancer (MCF-7). Fraction 6.1 exhibited the highest level of cytotoxicity across all tested cell lines, suggesting its potential as a significant source of anticancer compounds. Following the LC-MS analysis of Fraction 6.1, a tentative identification of 19 chemical constituents was achieved through a dereplication process utilizing accessible mass spectral databases. Physalin B, a recognized bioactive steroidal lactone with established anticancer properties, was identified as the major component of the active fraction. The presence of additional physalins and withanolide-type compounds indicates a potential synergistic or additive effect contributing to the observed anticancer properties. The findings substantiate the ethnopharmacological application of *P. angulata* and highlight its potential as a candidate for additional phytochemical and pharmacological research. Future work will focus on the isolation of compounds, elucidation of their structures, and mechanistic studies to confirm the bioactivity of the identified compounds and evaluate their therapeutic significance in cancer treatment. This research establishes a scientific foundation for evaluating *P. angulata* as a potential natural source of anticancer compounds.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Cancer is a leading cause of mortality globally, necessitating the discovery of novel therapies to tackle challenges such as medication resistance and adverse effects. Natural products have consistently served as a significant source for the discovery of novel pharmaceuticals, particularly in cancer therapy [1]. *Physalis angulata*, commonly known as letup-letup, belongs to the Solanaceae family and is well-known in traditional medicine for its various pharmacological effects, including anti-inflammatory, antioxidant, and anticancer actions. Research has indicated the in vitro anticancer efficacy of *P. angulata* extracts against multiple cancer cell lines, suggesting the presence of bioactive metabolites that merit further investigation [2]. For example, withangulatin I, a withanolide extracted from *P. angulata*, has exhibited notable in vitro anticancer efficacy against human colorectal carcinoma (COLO 205) and gastric carcinoma (AGS) cell lines, with IC_{50} values of 1.8 µM and 65.4 µM, respectively [3]. Moreover, physalins B, D, and F, extracted from *P. angulata*, have demonstrated significant in vitro anticancer efficacy against various tumor cell lines, including KB (human nasopharyngeal carcinoma), A431 (human epithelial carcinoma), HCT-8 (intestinal adenocarcinoma), PC-3 (human prostate cancer), and ZR751 (human breast cancer), with EC_{50} values below 4 µg/mL [4]. These findings highlight the promise of *P. angulata* as a source of anticancer drugs and necessitate further exploration of its bioactive components. Bioassay-guided fractionation is an essential technique for isolating bioactive chemicals from intricate natural matrices, prioritizing fractions that demonstrate notable biological activity for subsequent study. This method optimizes the discovery process by concentrating on the most viable prospects. This study evaluated the crude extract and bioactive fractions derived from the entire plant of *P. angulata* for in vitro anticancer efficacy against six cancer cell lines: ovarian cancer (A2780, SKOV3), melanoma (A375), cervical cancer (HeLa), colorectal cancer (HT-29), and breast cancer (MCF-7). Numerous studies have proven the effectiveness of bioassay-guided fractionation in isolating in vitro anticancer compounds. The bioassay-guided fractionation of the ethanol extract from *Aquilaria sinensis* flowers resulted in the extraction of a novel cucurbitane-type triterpenoid, aquilarolide A, in addition to five recognized chemicals, all demonstrating in vitro anticancer activity against several cancer cell lines [5]. Likewise, bioassay-guided fractionation of methanolic extracts from *Pittosporum angustifolium* and *Terminalia ferdinandiana* led to the extraction of compounds with notable in vitro anticancer and antibacterial properties [6]. These examples highlight the efficacy of bioassay-guided fractionation in the identification and isolation of bioactive chemicals with prospective medicinal uses. To accelerate the identification of active metabolites in the most potent fraction, dereplication by LC-MS was utilized. Dereplication is an effective method that facilitates the swift recognition of recognized compounds inside intricate mixes, thus reducing redundancy and concentrating

efforts on novel bioactive compounds [7]. Prior research has shown the efficacy of LC-MS-based dereplication in the identification of anticancer agents from natural sources. A high-throughput approach for dereplication and evaluation of metabolite distribution in *Salvia* species utilizing LC-MS/MS has been established, enabling the identification of bioactive chemicals with potential anticancer effects [8]. Furthermore, LC–PDA–MS/MS-based dereplication has facilitated the isolation of novel optical isomers of 19,20-epoxycytochalasin-N, compounds demonstrating in vitro anticancer potential, highlighting the efficacy of this methodology in natural product research [9]. Previous studies underscore the effectiveness of LC-MS-based dereplication in facilitating the identification of new anticancer drugs from complex natural extracts. The aims of this study were to (i) identify anticancer fractions from *P. angulata* by bioassay-guided fractionation and (ii) dereplicate the active chemicals in these fractions utilizing LC-MS analysis. This comprehensive method highlights the potential of *P. angulata* as a viable source of anticancer drugs and facilitates further phytochemical and pharmacological research.

2. METHODOLOGY

2.1 Solvents, Chemicals, and Reagents

All analytical-grade solvents (purity ≥ 96%) for extractions and bioassay-guided fractionations (ethanol, methanol, hexane, dichloromethane, and ethyl acetate) were purchased from The Merck Group (Darmstadt, Germany). For HPLC and LC-MS analyses, HPLC-grade methanol, acetonitrile (purity ≥ 99.9%), and formic acid were used, all obtained from Merck (Darmstadt, Germany). Ultrapure water (UPW) with a resistivity of 18.2 MΩ, used for mobile phase preparation during fractionation and HPLC chromatography, was purified using an ELGA Ultrapure Water System (Göttingen, Germany).The chemicals and reagents used for the anticancer assays included standard cell culture media, fetal bovine serum (FBS), phosphate-buffered saline (PBS), and trypsin-EDTA solution, all of which were purchased from Gibco (Life Technologies Ltd., Paisley, UK) and Capricorn Scientific GmbH (Ebsdorfergrund, Germany). Dimethyl sulfoxide (DMSO) was used as the solvent for dissolving the fractions, and Sulforhodamine B (SRB) reagent was utilised for the cytotoxicity assay. All materials were of analytical grade and obtained from Sigma Aldrich (St. Louis, MO, USA).

2.2 Plant Material

The dried and ground Physalis angulata (4.66 kg) was purchased from Biofresh Wellness Trading (Johor Bahru, Johor) on 27 June 2023. The plant identity was confirmed by Ms. Tan Ai Lee (botanist). As the material was obtained directly from a commercial supplier, no voucher specimen was deposited in the Kepong Herbarium.

2.3 Extraction and Fractionation

Extraction was performed through cold maceration using methanol as the solvent. The plant material was immersed in methanol at room temperature for three days, repeated for three cycles to ensure complete extraction. The extract was filtered using Whatman No. 1 filter paper (Cytiva, Marlborough, MA, USA) and concentrated under reduced pressure, yielding 621.7 g of crude extract, corresponding to a 13.49% extraction yield. To remove chlorophyll and simultaneously fractionate the crude extract, the entire 621.7 g of extract was subjected to chromatography using Diaion HP20 (Mitsubishi Chemical Corp) columns. This process was performed using 11 individual columns to manage the large quantity of crude extract. The fractions collected from the 11 columns were analyzed using thin-layer chromatography (TLC) (Supelco, Bellefonte, PA, USA), and similar fractions were combined based on their TLC results, leading to six main fractions (F1–F6). All six fractions were tested for cytotoxic activity against six cancer cell lines, with Fraction F6 identified as the most active.

Further fractionation of Fraction F6 was conducted using flash chromatography with a silica universal column and a stepwise gradient of hexane and methanol as eluents. This process yielded six subfractions (F6.1–F6.6). Each subfraction was tested for anticancer activity, and Fraction F6.1 emerged as the most active subfraction.

2.4 Sulforhodamine B (SRB) Assay

The A2780 human ovarian carcinoma cell lines was purchased from European Collection of Cell Cultures (ECACC), whereas SKOV-3 ovarian cancer, MCF-7 breast cancer, HT-29 colorectal cancer, A375 melanoma and HeLa cervical cancer cell lines were purchased from American Type Culture Collections (ATCC), USA. These adherent cancer cell lines were sub cultured and maintained in Dulbecco's Modified Eagle's medium-high glucose (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.25% amphotericin B, 100 units penicillin, 100 µg/mL streptomycin and 0.01 mg/mL gentamicin at 37 C, 5% carbon dioxide in air (i.e. carbon dioxide incubator).

Each of the cancer cell line was seeded in the 96-well plate at 4000-5000 cells/well. The cells were incubated overnight and treated with seven fractions of *P. angulata* at five different concentrations in triplicate. The concentrations of the fractions used for the initial screening were 1, 10, 20, 50 and 100 µg/mL. Since the IC_{50} of F6.1, F56.2 and F6.3 falls below the lowest concentration tested which was 1 µg/mL, further screening was performed by treating the cell lines with lower ranged of concentrations of the fractions which were 0.032, 0.16, 0.8, 4 and 20 µg/mL. Non-treated cells were also included in the experiments.

The treated and non-treated cells were then incubated for the next 72 h in the carbon dioxide incubator and the reactions were stopped using Sulforhodamine-B (SRB) assay [10, 11].

The optical density (OD) of the treated and untreated cells per well was then measured at 492 nm using microplate reader (Tecan Infinite, Switzerland). The dose-response curves were plotted from the results of Percentage of Cells Viability versus concentrations (µg/mL) of the samples tested. The percentage of cells viability (%) was then calculated according to the formula:

Percentage of Cells Viability (%) = (OD_{492nm} treated/ OD_{492nm} non-treated) x 100

The IC_{50} values were obtained from percentage of cells viability versus concentrations of the samples tested. Three independent experiments were performed and the IC_{50} values were given as the mean ± SEM. Fraction that gave IC_{50} values below 20 µg/ mL was considered to be active [12, 13]. Statistical analysis was performed using Graphpad Prism version 5. One-way analysis of variance (ANOVA) was used to determine the statistical significance of differences among fractions with multiple cancer cell lines. When a statistically significant effect was observed ($p<0.05$), Tukey's post hoc test was applied to identify pairwise differences between groups.

2.5 LC-MS Analysis

2.5.1 Sample Preparation

Prior to LC-MS analysis, the methanol crude extract underwent pretreatment 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 LC-MS spectrum. The 300 mg/6 mL (Supelco, Bellefonte, PA, USA) 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.

2.5.2 LC-MS Analysis Parameter

The LC-MS analysis of the active fractions was performed using a Thermo Scientific Vanquish Horizon UHPLC system coupled with a Thermo Scientific Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The chromatographic separation was achieved using an Accucore TM Vanquish C18+ column (2.1 × 100 mm, 1.5 µm) (Thermo Fisher Scientific, Bremen, Germany) maintained at 40°C. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). A gradient elution was employed, starting at 10% B at 0 minutes, increasing linearly to 100% B over 30 minutes, and maintained at 100% B for an additional 5 minutes. The flow rate was set at 0.2 mL/min, and the injection volume was 5 µL. UV detection was carried out at wavelengths of 210, 254, and 280 nm. Mass spectrometry was conducted using a heated electrospray ionization (H-ESI) source operating in both positive and negative ion modes. The spray voltage was set to 3500 V for positive mode and 2500 V for negative mode. The sheath, auxiliary, and sweep gas flow rates were set at 35, 7, and 1 Arb, respectively. The temperature of the ion transfer tube was 300°C, and the vaporizer temperature was 275°C.For MS^1 analysis, the Orbitrap detector operated at a resolution of 60,000, with a scan range of m/z 100–1000. The AGC target was set at 4.0e5 with a maximum injection time of 100 ms. MSn analysis was performed using a quadrupole isolation mode with an isolation window of 1.5 m/z. High-energy collision dissociation (HCD) was employed with stepped collision energies of 15%, 30%, 45%, and 60%. The Orbitrap resolution for MS^n was set at 15,000, with an AGC target of $5.0e^4$ and a maximum injection time of 22 ms. All data were collected in centroid mode.

2.6 Dereplication

The dereplication process was performed using MZmine software version 3.2.8 to mine the raw data obtained from UHPLC-MS/MS analysis. Raw data were first converted into mzML format using the MSConvert tool from ProteoWizard software. The data mining procedure included mass detection, ADAP chromatogram building, chromatogram resolving, and isotope filtering. For mass detection, the noise level threshold for MS1 was set to $1.5e^4$, and for MS^2, it was set to 0.0. ADAP chromatogram building used parameters such as a minimum group size of 4 scans, group intensity threshold of $1.5e^4$, minimum highest intensity of $3.5E^4$, scan-to-scan accuracy of 0.0015 m/z or 10 ppm, and retention time filters set between 0.0 and 25.0 minutes. Chromatogram resolving included parameters such as an S/N threshold of 10, a minimum feature height of $1.5e^4$, and a peak duration range of 0.0 to 1.0 minutes. The isotope filtering step applied an m/z tolerance of 0.001 m/z or 5 ppm and a retention time tolerance of 0.030. Monotonic shape filtering and a maximum charge set at 2 were used to enhance feature selection. After processing, data were exported in .mgf and .csv formats for further analysis. The .mgf file was analyzed using SIRIUS 5 software for in silico phytochemical annotation. Molecular formulas were predicted using CSI:FingerID and ranked with ZODIAC to enhance confidence. Structural annotations were linked to natural product databases, such as COCONUT and custom databases from the Physalis genus and *P. angulata*. Further classification was done using CANOPUS for ontology predictions. This workflow provided a comprehensive annotation of the phytochemicals in the active fractions.

3. RESULTS AND DISCUSSION

3.1 Bio-assay Guided Fractionation

The crude methanol extract obtained from the whole plant of *P. angulata* demonstrated notable cytotoxic activity across all six cancer cell lines tested. The IC_{50} values for the crude extract were 9.35 ± 0.22 µg/mL (A2780), 18.34 ± 0.42 µg/mL (SKOV3), 15.29 ± 0.21 µg/mL (A375), 21.90 ± 2.03 µg/mL (HeLa), 15.72 ± 0.84 µg/mL (HT-29), and 17.32 ± 1.21 µg/mL (MCF-7). Fractionation of the crude extract yielded six fractions (F1–F6), among which fractions F5 and F6 exhibited significant cytotoxic activity ($p<0.0001$, one-way ANOVA followed by Tukey's post hoc test). Fraction F5 displayed IC_{50} values of 7.21 ± 0.44 µg/mL (A2780), 10.90 ± 0.34 µg/mL (SKOV3), 12.73 ±

0.64 µg/mL (A375), 13.23 ± 0.55 µg/mL (HeLa), 11.18 ± 0.30 µg/mL (HT-29), and 11.67 ± 0.37 µg/mL (MCF-7). Fraction F6 was the most effective, showing IC_{50} values of 5.28 ± 0.13 µg/mL (A2780), 6.64 ± 0.017 µg/mL (SKOV3), 6.54 ± 0.11 µg/mL (A375), 6.92 ± 0.17 µg/mL (HeLa), 6.38 ± 0.079 µg/mL (HT-29), and 6.54 ± 0.054 µg/mL (MCF-7). Due to its superior activity, Fraction F6 was further fractionated into seven subfractions (F6.1–F6.7), which were subjected to cytotoxicity assays to identify the most active subfraction. Data for the subfractions are presented in **Table 1**.

Table 1 In vitro anticancer results (IC_{50} values) for sub-fractions (F6.1 to F6.7) of *P. angulata* against tested cancer cell lines.

Cancer cell/ Sub-fraction	A2780 Ovary (µg/mL)	SKOV-3 Ovary (µg/mL)	A375 Melanoma (µg/mL)	HeLa Cervical (µg/mL)	HT-29 Colorectal (µg/mL)	MCF-7 Breast (µg/mL)
F6.1	0.73 ± 0.028	0.92 ± 0.063	0.90 ± 0.040	1.36 ± 0.040	0.86 ± 0.061	0.90 ± 0.011
F6.2	0.79 ± 0.035	3.86 ± 0.380	3.68 ± 0.130	5.13 ± 0.170	2.84 ± 0.170	3.59 ± 0.270
F6.3	0.70 ± 0.028	3.58 ± 0.210	2.85 ± 0.14	4.49 ± 0.440	1.86 ± 0.170	2.74 ± 0.140
F6.4	6.93 ± 0.073	13.51 ± 0.250	13.04 ± 0.13	10.02 ± 0.092	12.34 ± 0.160	12.64 ± 0.19
F6.5	13.14 ± 0.350	27.06 ± 2.23	21.39 ± 1.09	14.33 ± 0.220	23.31 ± 0.470	23.38 ± 0.950
F6.6	12.20 ± 0.100	16.16 ± 0.44	14.41 ± 0.66	12.77 ± 0.330	15.65 ± 0.270	14.96 ± 0.300
F6.7	>100	>100	>100	>100	>100	>100

Table 1 presents the in vitro anticancer results (IC_{50} values) for sub-fractions (F6.1 to F6.7) of *P. angulata* against tested cancer cell lines. Fraction F6.1 showed the strongest anticancer effects on most cell lines, with IC_{50} values between 0.73 ± 0.028 µg/mL for A2780 ovarian cells and 0.86 ± 0.061 µg/mL for HT-29 colorectal cells. These results suggested that F6.1 contains bioactive compounds that effectively inhibit cancer cell growth. Fraction F6.2 also displayed excellent anticancer activity, with IC_{50} values ranging from 3.86 ± 0.38 µg/mL for SKOV-3 ovarian cells to 2.84 ± 0.17 µg/mL for HT-29 colorectal cells, but with a lower potency than F6.1, followed by fractions F6.3 to F6.6 with IC_{50} values as high as 13.14 ± 0.35 µg/mL for F6.4 in SKOV-3 cells and 12.20 ± 0.100 µg/mL for F6.6 in A2780 cells. Fraction F6.7 was found to be inactive against all the tested cancer cell lines with IC_{50} values greater than 100 µg/mL. Hence, the three most active subfractions (F6.1, F6.2, and F6.3), along with the crude methanol extract, were then subjected to LC-MS profiling to identify the compounds present in these subfractions.

3.2 LC-MS Profiling and Dereplication

The LC-MS analysis was conducted on the crude methanol extract and the three most active subfractions: F6.1, F6.2, and F6.3, focusing solely on the positive ion mode due to its greater diversity of detected metabolites. While LC–MS data were acquired in both positive and negative ionization modes, the results presented herein focus on the positive ion mode, which produced a broader range and higher abundance of annotated metabolites. The negative ion mode yielded substantially fewer features and did not reveal additional unique compounds beyond those detected in positive ion mode. The resulting chemical profiles were presented in **Figure 1**. Fraction F6.1 exhibited the most pronounced peaks in the LC-MS profile, particularly around 15.74 and 16.39 minutes, indicating the concentration of specific bioactive compounds. The cytotoxicity data further supports the idea that F6.1 contains the most potent anticancer compounds, with IC_{50} values ranging from 0.73 ± 0.028 µg/mL for A2780 ovarian cells to 0.86 ± 0.061 µg/mL for HT-29 colorectal cells. These findings indicate that F6.1 has strong activity across multiple cancer cell lines, making it the most promising fraction for further investigation. The intense peaks observed in the LC-MS profile of F6.1 correlate with the bioactivity data, suggesting that the compounds responsible for the observed cytotoxicity are concentrated in this fraction. Therefore, the comprehensive profiling of phytochemicals in the most active sub-fraction (F6.1) was conducted using a

dereplication approach based on high-resolution tandem liquid chromatography-mass spectrometry (LC-MS) data.

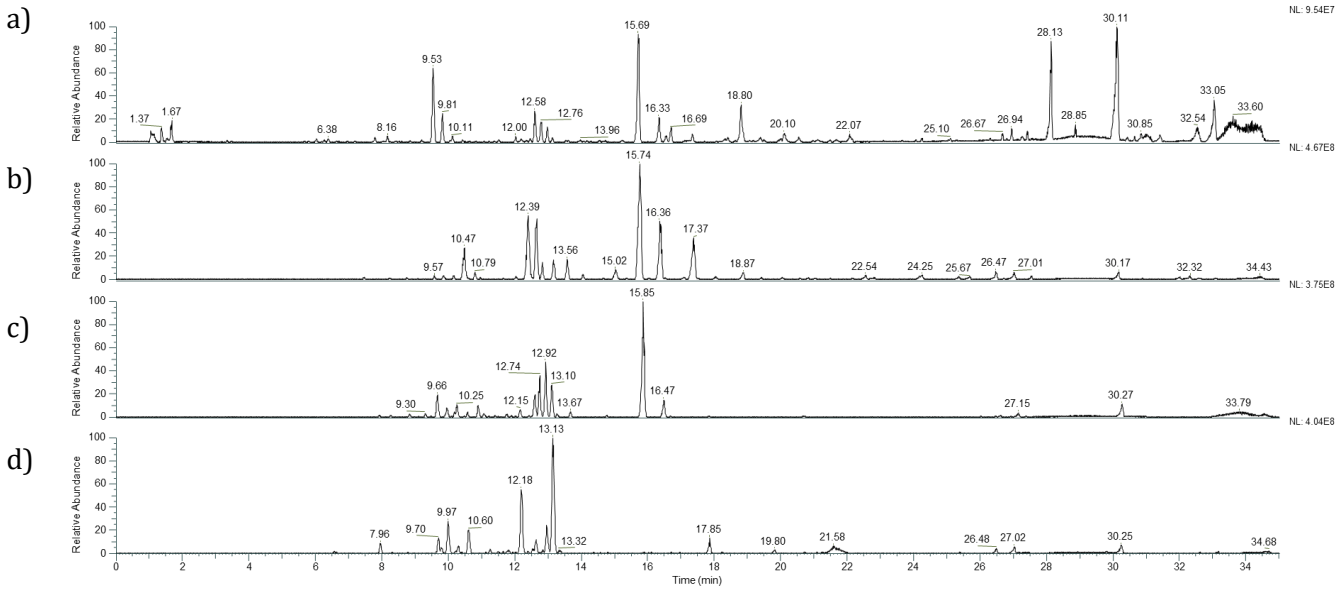


Figure 1 LC-MS profile of (a) methanol crude extract; (b) fraction F6.1; (c) fraction F6.2; and (d) fraction F6.3 from *P. angulata* whole plant in positive mode.

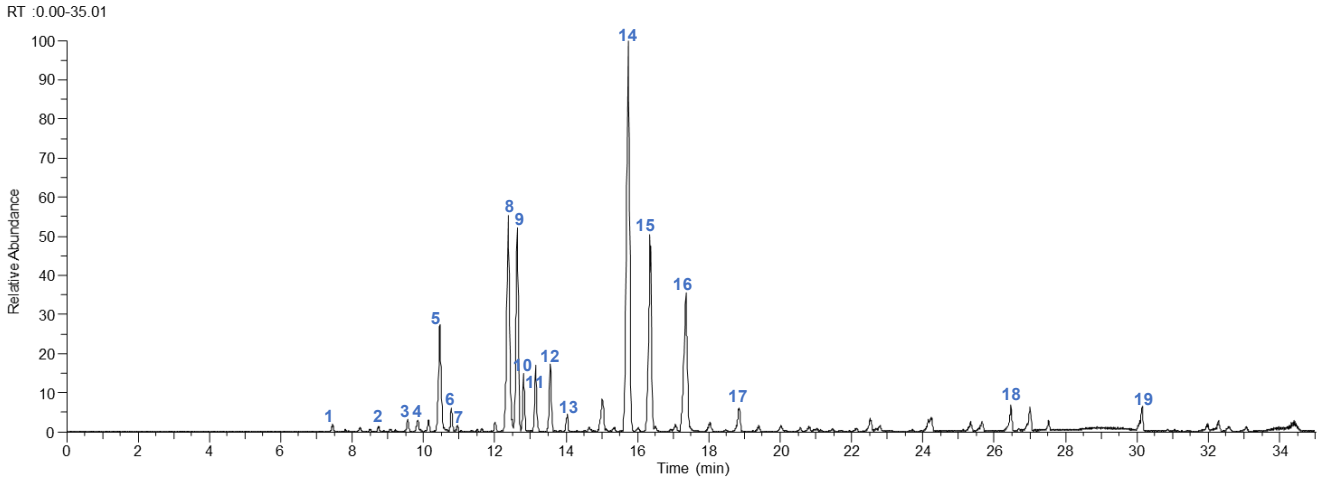


Figure 2 LC-MS profile of fraction F6.1 in positive mode.

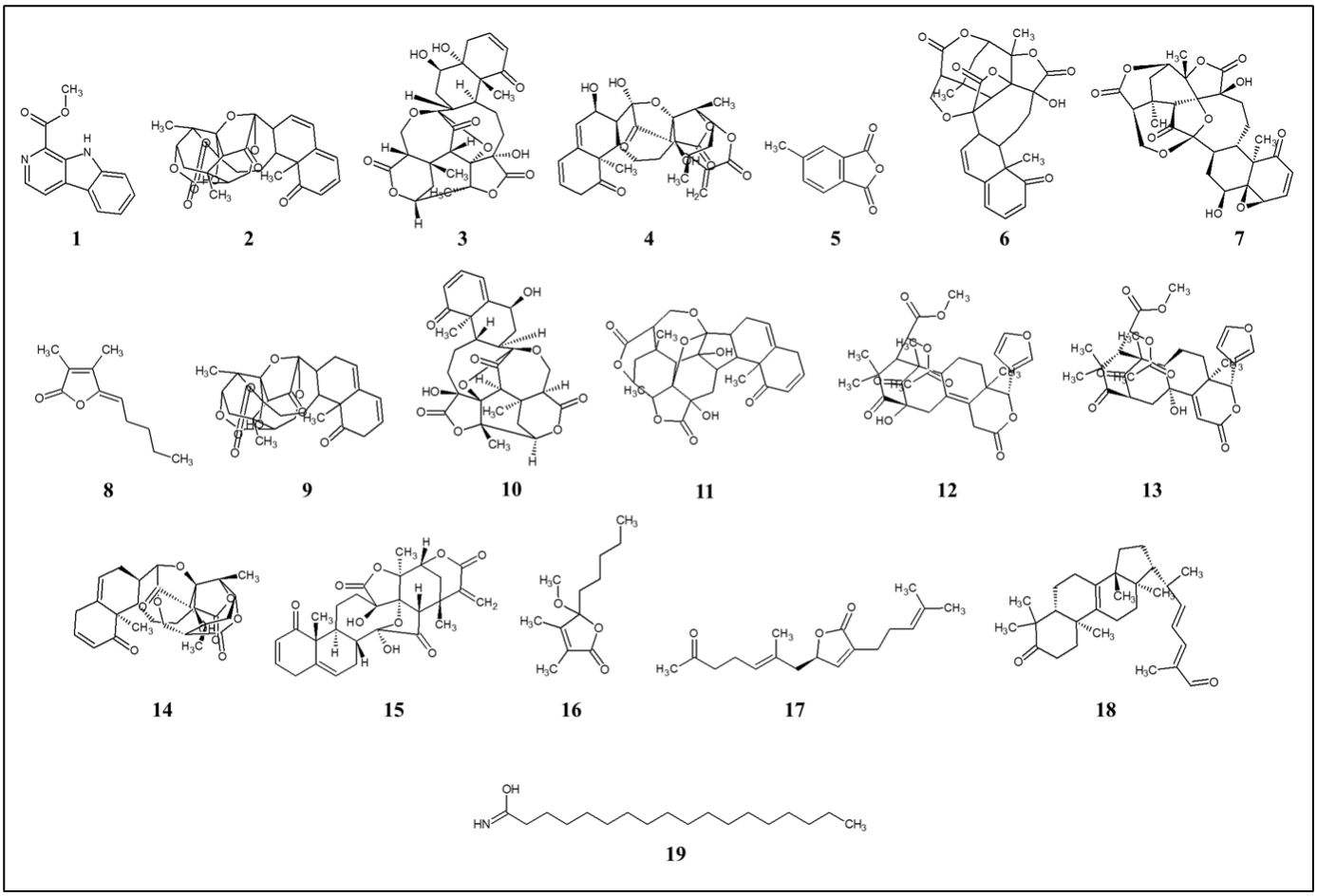


Figure 3 Chemical structures of the identified compounds from this the most active fraction (F6.1).

The combined application of SIRIUS and GNPS successfully identified 38 compounds in the methanol crude extract, of which 19 were detected in fraction F6.1. Notably, 7 of these 19 annotated compounds have been previously reported as isolates from *P. angulata*. 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 [15]. Therefore, the annotated compounds were validated using available known isolated compounds from *P. angulata*. In this study, the annotated compounds were classified at levels 1 and 2 according to the Metabolite Identification Confidence (MIC) levels proposed by Schrimpe-Rutledge et al., 2016 [15]. 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 from methanolic extract is presented in **Table 2**, and the list of annotated compounds in the active fraction F6.1 is presented in Table 3. The total ion chromatography (TIC) of the active fraction F6.1, with the assigned annotated compounds, is shown in **Figure 2**. The chemical structures of the annotated compounds from the most active fraction (F6.1) are illustrated in **Figure 3**. The dereplication analysis of F6.1 reveals several metabolites, with physalin B being the most intense and prominently featured compound across different fractions (in **Figure 1**). It appears at a retention time of 15.74 minutes and is identified as the major peak in fraction F6.1, with a molecular formula of $C_{28}H_{29}O_8$ and an experimental mass of 509.1814 m/z. Physalin B was the most abundant compound in F6.1, with a relative peak area (TIC integration) approximately twice that observed in F6.2 (**Figure 1**). These values are semi-quantitative, reflecting relative signal intensities in positive ion mode LC–MS, and

indicate a higher abundance of physalin B in F6.1 compared to F6.2. This metabolite is also detected in fraction F6.2 and the crude extract, indicating that physalin B is a significant component of the plant's chemical profile. Its intense presence in the LC-MS profile, along with its molecular characteristics, suggests it may play a key role in the observed anticancer activity, particularly in the active F6.1 fraction. Physalin B has been previously reported for its various biological activities, including anticancer, anti-inflammatory, and immunomodulatory effects [16]. Studies have shown that physalin B exhibits potent cytotoxicity against various cancer cell lines, including those derived from ovarian, melanoma, and cervical cancers. The activity of physalin B in *P. angulata* is consistent with the strong anticancer effects observed in this study, particularly in fractions where it is enriched, such as F6.1. The concentration of physalin B in both the crude extract and the fractions likely contributes significantly to the anticancer activity, as reflected by its correlation with the anticancer data. The fact that physalin B is present in F6.2, although it has a slightly reduced intensity compared to F6.1, suggests that it may also contribute to the moderate activity observed in this fraction. While other metabolites, such as 4,7-didehydrophysalin B and isophysalin B, were detected, they appeared with lesser intensity and may contribute less to the overall bioactivity compared to physalin B. In summary, the dereplication data supports the hypothesis that physalin B is a major bioactive compound in *P. angulata*, contributing significantly to its anticancer properties. The strong presence of physalin B across different fractions, including F6.1, F6.2, and the crude extract, correlates with its previously reported anticancer activity and suggests that it may be a key compound responsible for the therapeutic potential of *P. angulata*. Further isolation and structural elucidation of physalin B, alongside the study of its mechanisms of action, could provide helpful information regarding its potential as a therapeutic agent in cancer treatment.

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Table 2 Annotated Compounds from Methanolic Extract of *P. angulata*

No	RT (min)	Identification	Molecular formula	Experimental mass $[M+H]^+$	Calculated mass $[M+H]^+$	Mass error (ppm)	MS/MS Fragmentation	Database
1	6.38	Loliolide	$C_{11}H_{16}O_3$	197.1175	197.1173	-1.0	197.1064, 161.0957, 135.1164, 133.1007, 107.0851, 95.0488, 93.0695, 91.0539, 43.0177	COCONUT
2	7.44	1-Methoxycarbonyl-beta-carboline*	$C_{13}H_{10}N_2O_2$	227.0818	227.0818	0.0	213.0657, 195.0549, 185.0706, 167.0600, 85.6843	COCONUT
3	8.16	Physalin E	$C_{28}H_{32}O_{11}$	545.2026	545.2013	-2.4	509.1810, 491.1708, 463.1754, 445.1651, 371.1629, 173.4332, 129.1254	LR_FA, LR_GF, COCONUT
4	8.73	4,7-Didehydrophysalin B*	$C_{28}H_{28}O_9$	509.1814	509.1803	-2.1	491.1710, 465.1542, 373.1286, 329.1384, 217.0851, 147.0793	LR_GF, COCONUT
5	9.53	Physalin D*	$C_{28}H_{32}O_{11}$	545.2029	545.2013	-2.9	509.1811, 491.1706, 473.1599, 445.1650, 159.0799	LR_FA, LR_GF, COCONUT
6	9.81	Isophysalin A*	$C_{28}H_{30}O_{10}$	527.1921	527.1908	-2.5	509.1819, 491.1698, 463.1754, 289.1074, 217.0858, 175.0750, 147.0801, 135.0438, 121.0643, 85.0280	LR_FA, LR_GF, COCONUT
7	10.11	Physalin N	$C_{28}H_{30}O_{10}$	527.1923	527.1908	-2.8	509.1819, 491.1698, 463.1754, 289.1074, 217.0858, 175.0750, 147.0801, 135.0438, 121.0643, 85.0280	LR_FA, LR_GF, COCONUT
8	10.43	4-Methylphthalic anhydride*	$C_9H_6O_3$	163.0391	163.0393	1.2	135.0441, 105.0444, 95.0489, 92.0255, 77.0384, 51.0228	COCONUT
9	10.46	Ganbajunin C	$C_{34}H_{26}O_8$	563.1685	563.1698	2.3	545.1575, 527.1467, 491.1702, 473.1533, 445.1656, 399.1588, 265.0621, 171.0798, 123.0433	COCONUT
10	10.75	4,7-Didehydroneophysalin B*	$C_{28}H_{28}O_9$	509.1814	509.1803	-2.2	491.1708, 463.1756, 445.1652, 329.1547, 239.0702, 211.0754, 171.0802, 147.0800, 121.0643	LR_GF, COCONUT
11	10.96	(1R,2R,4S,5R,7R,11R,12S,15S,18S,19R,20S,21S,23R,26S)-4,15-	$C_{28}H_{30}O_{11}$	543.1872	543.1857	-2.8	525.1752, 507.1643, 479.1702, 461.1599, 289.1064, 217.0850, 175.0749, 161.0592, 137.0589	LR_GF, COCONUT

		dihydroxy-11,18,21-trimethyl-6,17,24,28,29-pentaoxanonacyclo[17.9.1.11,20.02,12.05,7.05,11.015,19.018,23.021,26]triacont-8-ene-10,16,25,30-tetrone*						
12	11.08	Physalin K	$C_{28}H_{30}O_{12}$	559.1818	559.1806	-2.1	541.1751, 525.1757, 495.1654, 467.1705, 449.1594, 187.0752, 159.0800, 135.0436, 95.0126	LR_GF, COCONUT
13	11.60	Atalantin; 6-Deoxo,19-Oxo(Lactone),11-Acetoxy,7-Ketone	$C_{29}H_{32}O_{11}$	557.2027	557.2013	-2.5	525.1755, 507.1649, 479.1698, 461.1595, 433.1636, 173.4305, 127.0385, 95.0124	COCONUT
14	12.03	Physalin U	$C_{29}H_{34}O_{11}$	559.2182	559.2169	-2.3	528.1958, 527.1912, 509.1802, 499.1960, 481.1852, 429.1545, 173.4303	COCONUT
15	12.39	Bovolide*	$C_{11}H_{16}O_2$	181.1225	181.1224	-0.55	163.1123, 145.1014, 135.1170, 107.0854, 93.0698, 79.0541	COCONUT
16	12.45	Khayanolide E	$C_{29}H34O_{11}$	559.2177	559.2169	1.4	541.2071, 499.1965, 481.1859, 429.1548, 241.0874, 213.0913, 171.0807	COCONUT
17	12.59	Isophysalin B*	$C_{28}H_{30}O_9$	511.1965	511.1959	-1.2	493.1857, 475.1747, 431.1853, 243.1014, 187.0753, 145.0648, 121.0646	LR_FA, LR_GF, COCONUT
18	12.78	Physalin G*	$C_{28}H_{30}O_{10}$	527.1915	527.1908	-1.3	509.1806, 491.1703, 465.1546, 213.0905, 187.0747, 171.0798, 121.0639	LR_FA, LR_GF, COCONUT
19	12.97	Ganbajunin E	$C_{34}H_{26}O_8$	563.1685	563.1698	-2.3	545.1575, 527.1467, 509.1795, 491.1703, 473.1595, 463.1754, 417.1697	COCONUT
20	13.12	15,28-dihydroxy-9,12,19-trimethyl-2,6,13,29-tetraoxanonacyclo[15.10.1.1^{1,11}.0^{4,9}.0^{7,12}.0^{10,28}.0^{11,15}.0^{18,27}.0^{19,24}]nonacosa-21,24-diene-5,14,20-trione*	$C_{28}H_{30}O_9$	511.1965	511.1959	-1.2	493.1857, 475.1747, 431.1853, 385.1803, 243.1015, 213.0913, 187.0753, 145.0648, 121.0646	LR_FA, LR_GF, COCONUT
21	13.19	[7-(3-furyl)-1,8,12,16,16-pentamethyl-5,10,15-	$C_{28}H_{32}O_8$	497.2177	497.2166	-2.2	451.2122, 433.2017, 415.1901, 309.1492, 235.1113, 197.0958, 187.1115, 169.1011, 155.0850, 125.0592, 97.0644	COCONUT

		trioxo-3,6-dioxapentacyclo[9.8.0.0^{2,4}.0^{2,8}.0^{12,17}]nonadec-13-en-19-yl] acetate						
22	13.56	6-Acetoxy-2-Hydroxymexicanolide*	$C_{29}H_{34}O_{10}$	543.223	543.222	-1.8	525.2114, 493.1856, 475.1749, 431.1849, 413.1745, 385.1799, 243.1008, 187.0748, 171.0798, 145.0641, 121.0641	COCONUT
23	14.01	Angolensin C*	$C_{29}H_{34}O_{10}$	511.197	543.222	-1.8	525.2114, 493.1856, 475.1749, 431.1849, 413.1745, 385.1799, 243.1008, 187.0748, 171.0799, 145.0641, 121.0641	COCONUT
24	14.62	6-Dehydroxykhayanolide E	$C_{29}H_{34}O_{10}$	543.2233	543.222	-1.8	525.2110, 511.1958, 493.1855, 483.2012, 465.1906, 451.1750, 296.8709, 215.1075	COCONUT
25	14.97	Pyrazine-2-carboxylic acid [1-(1-ethylaminooxalyl-propylcarbamoyl)-3-methyl-butyl]-amide	$C_{18}H_{27}N_5O_4$	378.2126	378.2135	2.4	361.1859, 347.1698, 201.1121, 143.0700, 111.0444	PubChem
26	15.71	Physalin B*	$C_{28}H_{30}O_9$	511.1964	511.1959	-1.0	493.1858, 475.1749, 451.1753, 429.1698, 387.1593, 341.1538, 253.0860, 171.0805, 131.0853	LR_FA, LR_GF, COCONUT
27	16.33	Physalin C*	$C_{28}H_{30}O_9$	511.1969	511.1959	-2.0	493.1856, 475.1749, 447.1802, 429.1697, 383.1645, 171.0805, 155.0854, 131.0855, 123.0803	LR_GF, COCONUT
28	17.33	5-methoxy-3,4-dimethyl-5-pentylfuran-2-one*	$C_{12}H_{20}O_3$	213.1488	213.1485	-1.4	195.1380, 181.1220, 163.1115, 135.1166, 111.0437, 85.0645	COCONUT
29	17.70	Embelin	$C_{17}H_{26}O_4$	295.1908	295.1902	-2.0	277.1797, 231.1741, 203.1794, 175.1114, 161.0958, 137.0594, 111.0438, 81.0696	COCONUT
30	18.82	Tuberatolide A*	$C_{18}H_{26}O_3$	291.1956	291.1953	-1.0	249.1845, 233.1533, 203.1065, 161.0958, 133.100, 107.0853, 57.0696	COCONUT
31	19.64	13-hydroxyoctadeca-9,11,15-trienoic acid	$C_{18}H_{30}O_3$	295.2268	295.2265	-1.0	277.2160, 259.2047, 249.2207, 231.2098, 173.4301, 151.1113, 135.1162, 107.0852, 95.0853, 81.0696, 67.0540	COCONUT
32	22.55	cis-Parinaric acid methyl ester	$C_{19}H_{30}O_2$	291.2321	291.2316	-1.7	259.2054, 149.1321, 135.1165, 121.1007, 93.0696, 81.0696, 79.0539, 67.0540, 55.0540	COCONUT

33	24.25	9,11,13-Octadecatrienoic acid, methyl ester	$C_{19}H_{30}O_2$	293.2477	293.2472	-1.7	261.2209, 173.1323, 123.1164, 109.1009, 95.0852, 81.0696, 67.0540, 55.0540	COCONUT
34	26.32	Hexadecanamide	$C_{16}H_{33}NO$	256.2638	256.2632	-2.3	214.2151, 173.4279, 130.1223, 116.1066, 102.0909, 88.0753, 74.0597, 57.0696	COCONUT
35	26.42	Astraeusin A*	$C_{30}H_{44}O_2$	437.3416	437.3408	-1.8	409.3470, 391.3364, 281.2265, 213.1636, 173.1322, 109.1008, 69.0695	COCONUT
36	26.99	Ganoderal A	$C_{30}H_{44}O_2$	437.3416	437.3408	-1.8	409.3470, 391.3364, 281.2265, 213.1636, 173.1322, 109.1008, 95.0851, 69.0695	COCONUT
37	28.13	3-[18-(1-hydroxy-3-methoxy-3-oxo-propyl)-3,7,12,17-tetramethyl-8,13-divinyl-22,24-dihydroporphyrin-2-yl]propanoic acid	$C_{35}H_{36}N_4O_5$	593.2771	593.2755	-2.7	3-[18-(1-hydroxy-3-methoxy-3-oxo-propyl)-3,7,12,17-tetramethyl-8,13-divinyl-22,24-dihydroporphyrin-2-yl]propanoic acid	COCONUT
38	30.14	Octadecanamide*	$C_{18}H_{37}NO$	284.2952	284.2944	-2.8	130.1223, 116.1066, 102.0908, 72.0440, 57.0696, 46.0649	COCONUT

*Compounds detected in F6.1.

Table 3 Annotated Compounds from Active Fraction F6.1

No	RT (min)	Identification	[M+H]⁺	Molecular formula
1	7.46	1-Methoxycarbonyl-beta-carboline	227.0818	$C_{13}H_{10}N_2O_2$
2	8.75	4,7-Didehydrophysalin B	509.1814	$C_{28}H_{28}O_9$
3	9.56	Physalin D	545.2029	$C_{28}H_{32}O_{11}$
4	9.85	Isophysalin A	527.1918	$C_{28}H_{30}O_{10}$
5	10.47	4-Methylphthalic anhydride	163.0391	$C_9H_6O_3$
6	10.79	4,7-Didehydroneophysalin B	509.1814	$C_{28}H_{28}O_9$
7	10.95	(1R,2R,4S,5R,7R,11R,12S,15S,18S,19R,20S,21S,23R,26S)-4,15-dihydroxy-11,18,21-trimethyl-6,17,24,28,29-pentaoxanonacyclo[17.9.1.11,20.02,12.05,7.05,11.015,19.018,23.021,26]triacont-8-ene-10,16,25,30-tetrone	543.1872	$C_{28}H_{30}O_{11}$
8	12.39	Bovolide	181.1225	$C_{11}H_{16}O_2$
9	12.64	Isophysalin B	493.1861	$C_{28}H_{30}O_9$
10	12.81	Physalin G	527.1915	$C_{28}H_{30}O_{10}$
11	13.15	15,28-dihydroxy-9,12,19-trimethyl-2,6,13,29-tetraoxanonacyclo[15.10.1.1^{1,11}.0^{4,9}.0^{7,12}.0^{10,28}.0^{11,15}.0^{18,27}.0^{19,24}]nonacosa-21,24-diene-5,14,20-trione	511.1965	$C_{28}H_{30}O_9$
12	13.56	6-Acetoxy-2-hydroxymexicanolide	543.223	$C_{29}H_{34}O_{10}$
13	14.04	Angolensin C	543.223	$C_{29}H_{34}O_{10}$
14	15.74	Physalin B	511.197	$C_{28}H_{30}O_9$
15	16.36	Physalin C	511.169	$C_{28}H_{30}O_9$
16	17.37	5-methoxy-3,4-dimethyl-5-pentylfuran-2-one	213.1488	$C_{12}H_{20}O_3$
17	18.87	Tuberatolide A	291.1956	$C_{18}H_{26}O_3$
18	26.47	Astraeusin A	437.3416	$C_{30}H_{44}O_2$
19	30.14	Octadecanamide	284.2952	$C_{18}H_{37}NO$

3 CONCLUSION

This study achieved its objectives of identifying anticancer fractions from *P. angulata* through bioassay-guided fractionation and dereplicating the active constituents in these fractions using LC–MS analysis. Fraction F6.1 was identified as the most active, exhibiting significant cytotoxicity across multiple cancer cell lines, and physalin B was determined to be its primary bioactive component. These findings indicate that Fraction F6.1, which is enriched in physalin B, warrants further investigation for its potential incorporation as an active ingredient in herbal preparations for cancer treatment. Given the promising efficacy of this fraction, additional studies are necessary to explore its potential in preclinical and clinical settings, assessing the therapeutic effectiveness of the fraction in its entirety rather than isolating individual compounds. The results of this study provide a robust foundation for advancing the development of *P. angulata* as a natural source of anticancer agents, focusing on its active fractions for future therapeutic applications.

Author contributions

Fauziah Abdullah: Conceptualization, methodology, investigation, writing—original draft, writing—review and editing. **Nurhanan Murni Yunos**: Conceptualization, methodology, investigation, writing—reviewing and editing, validation, project administration, and supervision. **Nursabrina Najwa Salmin**: Data curation, formal analysis, investigation, LC-MS analysis, visualization, and writing—review and editing. **Nor Jannah Sallehudin**: Investigation, formal Analysis, validation, and in vitro assay execution. All authors have read and approved the final version of the manuscript.

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Conflict of Interest

The authors declare that there are no financial or commercial conflicts of interest in connection with this research.

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