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Metabolite profiles and biomarkers of three *Selaginella* (Selaginellaceae) medicinal plant species in Java Island, Indonesia

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Abstract. *Khoirunisa A, Chikmawati T, Nugroho G, Miftahudin. 2025. Metabolite profiles and biomarkers of three* Selaginella *(Selaginellaceae) medicinal plant species in Java Island, Indonesia. Biodiversitas 26: 434-443.* Metabolite content in plants is an important taxonomic marker that facilitates the realistic delimitation of species. Substantial improvement is needed for the metabolomic data of several fern species, including *Selaginella*, which is widely used as a medicinal plant on Java Island. Therefore, this research aimed to profile metabolite compounds in *Selaginella ornata*, *S. plana*, and *S. willdenowii*, and identify biomarkers for species differentiation. Metabolite content and data were determined with Liquid Chromatography-Mass Spectrometry (LC-MS) and MZmine 3.1.0 beta software, respectively. Meanwhile, metabolite profiling, heatmap clusters, and cluster analysis were carried out using MetaboAnalyst 5.0. A total of 113 metabolites were detected in three *Selaginella* species observed. Based on metabolite characteristics, cluster analysis categorized all individuals into three groups, showing that individuals from the same species were more similar than others, with *S. ornata* metabolites appearing more similar to *S. willdenowii* than to *S. plana*. Three species had similarities in the compounds 1,3,5-benzenetricarbonitrile, 2-hydroxyisocaproic acid, 3-furoic acid, 3-methyl-2-oxovaleric acid, amentoflavone, avobenzone, ibuprofen, kojic acid, and skyrin. Metabolites only possessed by each species of *S. plana*, *S. ornata*, and *S. willdenowii* included 4-vinylphenol, velutin, and axahine B, respectively. This research reported for the first time several low-weight secondary metabolites with potential application as biomarkers to differentiate three species.

Keywords: Biomarkers, cluster analysis, heatmap, LC-MS/MS, low-weight secondary metabolites

INTRODUCTION

Biochemical characters have been used as taxonomic evidence for several hundred years. The chemical compounds are used extensively in plant systematics to analyze infraspecific variation, understand the evolutionary relationships of various taxa categories, and solve taxonomic problems. Leading chemical compounds that are systematically useful include secondary metabolites, such as alkaloid, phenolic, betalain, anthocyanin, terpenoid, and flavonoid (Singh 2016). Moreover, several metabolomics research have analyzed plant diversity with low molecular weight compounds, including secondary metabolites obtained from living organisms (Shen et al. 2023; Quiros-Guerrero et al. 2024). In recent years, a key technology for metabolomics research is Liquid Chromatography-Mass Spectrometry (LC-MS), which combines physical separation capabilities with mass analysis capabilities. LC-MS often produces larger data volumes and is superior in sensitivity, speed, and efficiency, compared to Gas Chromatography-Mass Spectrometry (GC-MS) (Wu et al. 2021). In this context, the profile data obtained is used to group plant species based on similarities and differences in the content of metabolite compounds, while each species has a particular structure, product, and metabolite biosynthesis pathway (Arbona et al. 2015; Singh 2016). The resulting grouping will be consistent with previously known plant evolutionary relationships (Liu et al. 2017; Freitas et al. 2021). Previous research reported that metabolite-based classification successfully differentiated *Citrus* spp. and determined the species-specific metabolites (Peng et al. 2021). Using untargeted metabolomics data on mosses has promoted a more realistic delimitation of species (Peters et al. 2023).

In addition to taxonomic purposes, low-weight secondary metabolite data can be used to estimate the potential future application of plants as a source of herbal-based cosmetic ingredients, herbal nutritional supplements, or medicinal ingredients (Salem et al. 2020). Plants have been used in treating health problems for thousands of years by many ethnic groups, including Indonesia, and are considered a source of various modern medicinal ingredients. Approximately 30% of drugs sold worldwide are reported to contain compounds derived from plant materials (Calixto 2019; Salmerón-Manzano et al. 2020). Many people use herbal drugs because of the belief that medicinal plants have advantages over drugs derived from chemicals, including lower side effects, relatively affordable prices, easy accessibility, safety, and effectiveness (Nimesh et al. 2020). Currently, herbal drugs are being used by various populations worldwide, both in developing and industrialized countries (Tangkiatkumjai et al. 2020). Approximately 50,000-80,000 species have been used as medicinal plants in the world (Pimm et al. 2014), and around 6000 in Indonesia are known to contain bioactive compounds, with 16.7% of the species being used as traditional medicinal plants (Elfahmi et al. 2014). However, the potential of

various plant ingredients has yet to be well documented for development into drugs.

Selaginella is a genus of pteridophytes used to produce drugs, which belongs to the Selaginellaceae family with high species diversity (Zhou et al. 2016). The number of *Selaginella* in the world is around 800 species spread across all continents except Antarctica, with the highest diversity in tropical and subtropical regions (Zhou and Zhang 2015). Between 1994-2014, 39 species were found distributed across nine islands in Indonesia, with Java Island containing the most significant number (22) (Wijayanto 2014). Furthermore, *Selaginella* is easily distinguished from other pteridophytes by several characteristics, such as small leaves (microphylls) arranged opposite each other in four rows on the stem, with two of these rows consisting of larger leaves arranged laterally and the remaining two comprising smaller median leaves on branches facing forward. This species is heterosporous with mega- and microsporangia surrounded by sporophylls and arranged in strobili at the tips of branches (Valdespino et al. 2015; Weststrand and Korall 2016).

Selaginella ornata (Hook & Grev.) Spring, *S. plana* (Desv. ex Poir.) Hieron., and *S. willdenowii* (Desv.) Baker abundant on Java Island have been widely used as medicinal plants by residents in several areas in West Java Province, mainly for post-natal care and broken bone treatment (Chikmawati et al. 2009). Miftahudin et al. (2019) have also reported that these three species have antioxidant activity. This ability is probably due to the three species containing alkaloids, flavonoids, saponins, tannins, and steroids (Chikmawati et al. 2012). Limited information is available regarding the primary chemical compounds in the species, particularly *S. ornata* and *S. plana*, leading to a need for more investigation that can contribute significantly to the fields of taxonomy, pharmacy, and health. Therefore, this research aimed to analyze non-targeted metabolite compounds in extracts of *S. ornata*, *S. plana*, and *S. willdenowii* with medicinal potential and determine the differentiating compounds between species using LC-MS/MS-based metabolomics method.

MATERIALS AND METHODS

Sample preparation and extraction

Plant samples for *S. plana* and *S. willdenowii* were collected from IPB Univesity green area (-6.55500, 106.72159), Dramaga Campus, Bogor, while *S. ornata* was obtained from Curug Nangka area (-6.66886, 106.72641), Bogor, Indonesia (Supplementary Files-1). The samples were collected in June 2022 from forested areas or cliffs near irrigation canals that have high humidity. Rhizome drying and extraction were performed at the Plant Resources and Ecology Laboratory, Department of Biology, IPB University, and the Analytical Chemistry Research Laboratory, Department of Chemistry, IPB University. Untargeted metabolites were analyzed at the Advance Research Laboratory of IPB University.

The collected *Selaginella* samples were washed using tap water, drained, and dried in the oven at 40-50°C for 72 h, then the dried samples (simplisia) were ground using a blender. Sample extraction followed the procedure by Gayathri et al. (2005), which was modified on the duration of maceration, and conducted in three replications for each species. An amount of 5 g of simplisia from each *Selaginella* species was extracted using 100 mL of 70% ethanol or with the simplisia to solvent ratio of 1:20 (w/v) and stirred for 5 hours. After incubation at room temperature for 24 hours, the filtrate was separated using Whatman No.1 filter paper. A total of 50 mL filtrate was concentrated on a rotary evaporator at 50°C to form a paste.

Metabolite analysis

Separation and identification of metabolite components of *Selaginella* extract were performed under the following conditions: An amount of 2 mg paste sample was analyzed. The extracts were filtered using a 0.2 µm PFTE filter, and 2.5 µL of sample was injected. The analysis was performed using the UHPLC Vanquish Tandem Q Exactive Plus Orbitrap HRMS (ThermoScientific, USA). The column used was Accucore C18 (1.5µm 2.1×100 mm) with temperature condition at 30°C. The mobile phase consisted of water + 0.1% Formic Acid (A) and Acetonitrile + 0.1% Formic Acid (B), with a flow rate of 0.2 mL/min over 30-minute gradient. The gradient program was as follows, i.e. 0-1 min (95% A + 5% B), 1-25 min (5-95% B), 25-28 min (95% B), and 28-30 min (95% A + 5% B). The mass spectrometry was performed using electrospray ionization in positive and negative mode, with a scan range of 100-1500 m/z. The system was set with the following conditions, i.e. capillary temperature at 320°C, sheath gas flow at 15 µL/min, Aux gas flow at 3 L/min, spray voltage at 3.8 kV and S-lens RF level at 50.

LC-MS/MS data analysis

The data obtained in a complex raw format from LC-MS/MS instruments were processed using MZmine 3.1.0 beta software to identify metabolite compounds (Schmid et al. 2023). The identification step consisted of mass detection, ADAP chromatogram builder, chromatogram deconvolution, isotopic grouper, alignment, and identification.

Mass detection

The raw data method and features detection menu were selected after entering the raw data into MZmine software. The mass detection was set with the parameters, including scan number (1), Retention Time (RT) (0.00-30.00 min), MS level (1), scan types (all scan types), mass detector (centroid), and noise level (1.0E5). In the next stage, peak detection was used to identify and measure the signal intensity corresponding to the sample molecules.

ADAP chromatogram builder

ADAP chromatogram was built with the following parameters, RT (0.00-30.00 min), MS level (1), min group size in # of scans (5), group intensity threshold (3.0E0), min highest intensity (1.0E5), scan to scan accuracy (0.002 m/z or 10 ppm), and suffix (chromatogram). This stage

aimed to produce a chromatogram from the extracted ions and detected chromatogram peaks.

Chromatogram deconvolution

Chromatogram deconvolution was carried out using parameters, including suffix (resolved), original feature list (remove), dimension (RT), chromatographic threshold (50%), min search range RT/mobility (0.05), min relative height (0.1%), min absolute height (1.0E5), min ratio of peak top/edge (2), peak duration range (0-5), and min # of data points (5). This stage targeted to combine chromatograms near each other to form a definite m/z value.

Isotopic grouping

The grouping stage helped to arrange monoisotopic peaks with corresponding isotope and chromatogram peaks possessing similar isotope patterns. This stape applied parameters, including name suffix (deisotoped), m/z tolerance (0.01 m/z or 5 ppm), RT tolerance (0.03), monotonic shape (selected), maximum charge (2), and representative isotope (most intense).

Alignment

The alignment stage combined and compared metabolites between samples according to m/z and RT. The data were aligned using several parameters, including features list name (aligned features list), m/z tolerance (0.001 m/z or 5 ppm), RT tolerance (0.01), RT tolerance after correction (1), min number of points (25%), and threshold value (2).

Identification

The identification stage compared the detected m/z values with online data sources or data present in literature reviews (Höcker et al. 2021). The following parameters were chosen to annotate the data, namely MS level (2), precursor m/z tolerance (0.001 m/z or 5 ppm), CCS tolerance (5%), min ion intensity (0), crop spectra to m/z overlap (√), spectral m/z tolerance (0.0015 m/z or 10 ppm), and min matched signals (4). Compounds in *Selaginella* extract were identified by comparing the precise mass values of the detected peaks with the accurate mass values of metabolites in PubChem library (https://pubchem.ncbi.nlm.nih.gov/) and the spectrum library in MassBank of North America (MONA) database (https://mona.fiehnlab.ucdavis.edu), GNPS (https://gnps.ucsd.edu), dan HMDB (https://hmdb.ca/). The identification stage compared the detected m/z values with online data sources or data in literature reviews (Höcker et al. 2021). The matching parameters used was min cosine similarities = 0.8 (80%) with confidence level of 2 (putative metabolites).

Heatmap and cluster analyses

MetaboAnalyst 5.0 software was used for statistical analysis to construct a clustered heatmap and a dendrogram. This applied the complete grouping method with the Euclidean dissimilarity index (Pang et al. 2021).

RESULTS AND DISCUSSION

Profile of *Selaginella* metabolite compounds

The profile of metabolite compounds was described by metabolomics analysis, which aimed to collect various compounds in cells and tissues (Van Dam and Bouwmeester 2016; Wuolikainen et al. 2016). Estimating metabolites in *Selaginella* extracts was performed by matching m/z values with the compound patterns in the MONA database and online data sources in PubChem. The results obtained from several stages of analysis were in the form of a table containing compound identity, RT, mass of detected peaks, chromatogram image with normalized peaks, and other information.

Chromatograms produced by the ethanol extracts of three *Selaginella* species required an elution time of approximately 30 minutes. They generated nearly the same peaks for each replicate but different peaks between species (Supplementary Files-2). The Chromatogram profile of *S. plana* extract comprised more peaks than *S. ornata* and *S. willdenowii*. These results showed that the number and type of compounds detected in individuals of one species are almost the same, but different in individuals from various species. *Selaginella plana* contained more metabolites than the other two species. The metabolite components identified from the extracts of three *Selaginella* species amounted to 113 essential compounds. The number of compounds in each species replication was calculated based on the type of compound detected with different m/z values and times. The number of metabolites found in each replication varied in the three species investigated. The variation in the number of metabolites per replication in *S. plana* was higher than in the other two species (Figure 1). The distribution of metabolite compounds identified in three *Selaginella* species showed that *S. plana* had the highest metabolite content (Figure 2). This result is based on the chromatogram description of *S. plana*, which has the highest number of peaks. According to the results of metabolomic analysis, the metabolite compound groups of three *Selaginella* species consisted of amino acid, fatty acid, carboxylic acid, phenols, flavonoid, alkaloid, terpenoid, and other compound groups (Figure 3). The most significant number of compounds identified was the group of amino acids from the total identified metabolite compounds.

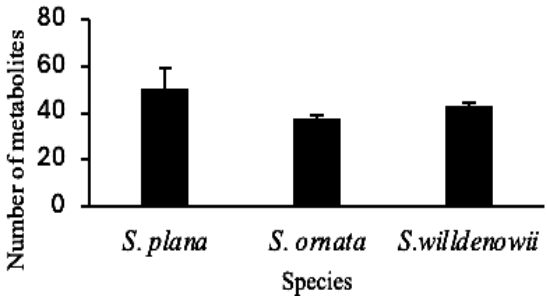

Figure 1. Number of metabolites in three *Selaginella* species

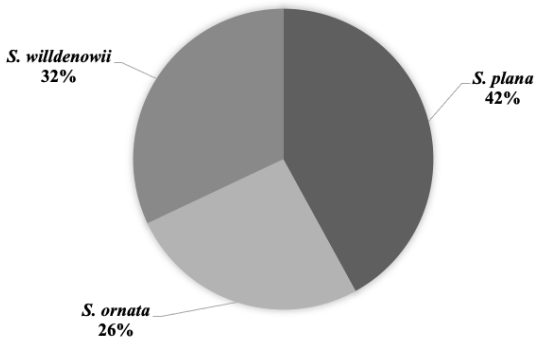

Figure 2. The proportion of total metabolites detected in *S. plana*, *S. ornata*, and *S. willdenowii* plants

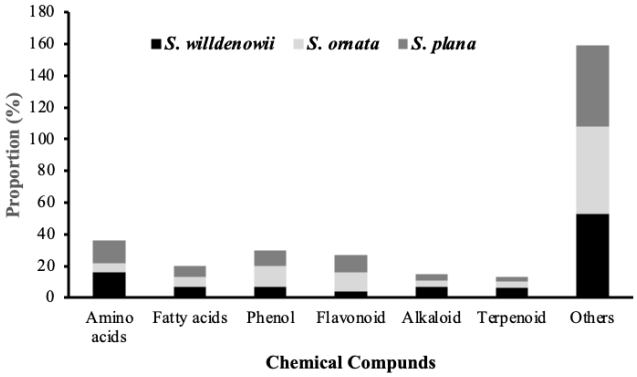

Figure 3. The proportion of each group of chemical compounds detected in *S. plana*, *S.ornata*, and *S. willdenowii*

Amino acid

Amino acid is an essential organic compound acting as a building block for protein, leading to an extensive investigation for use in medicine (Parthasarathy et al. 2021). The group of amino acid compounds in *Selaginella* varies between species, 14% in *S. plana*, 6% in *S. ornata*, and 16% in *S. willdenowii* (Figure 3). Amino acid groups detected in *S. plana* included gabapentin, with m/z value of 187.10 at 1.52 minutes (Table 1). The gabapentin compound can be used as a seizure reliever for people living with epilepsy and tends to relieve nerve pain, leading to the inclusion in the class of anti-seizure drugs (Rocha et al. 2019). *S. plana* and *S. willdenowii* have the same 4 amino acid compounds, namely deferrioxamine e, diprotin b, l-2-aminoadipic acid, l-pyroglutamic acid, and n-acetyl-l-leucine. The amino acid compound only detected in *S. ornata* was aspartic acid, while axahine B was found in *S. willdenowii*. Aspartic acid is the key compound in the amino acid metabolism, serving as a precursor for basic amino acids formation and enhancing heat stress tolerance (Lei et al. 2022). Aspartic acid compounds exist in L- and D-isoforms (L-Asp and D-Asp), with L-Asp playing a role in the pathogenesis of psychiatric and neurological disorders and changes in BCAA levels in diabetes and hyperammonemia. D-Asp has a role in brain development and hypothalamic regulation (Holeček 2023).

Kapahine B, isolated from the marine sponge Cribrochalina olemda (Nakao et al. 1995), showed anticancer activity against P-388 murine leukemia cells with an IC50 value of 5.0 µg/mL (Gul and Hamann 2005). Based on the pharmacological properties of the phytochemical compounds, *S. willdenowii* leaf extract is reportedly used as an anticancer, antioxidant, anti-inflammatory, antitumor, and antimicrobial drug (Susilo and Wardhani 2023). The extract of *Selaginella* also contains L-tyrosine that plays crucial role as antioxidant, attractants, and plant defense to environmental stress (Schenck and Maeda 2018).

Fatty acid

Fatty acid compounds detected in three *Selaginella* species were nearly the same amount, 7%, 6%, and 7% in *S. plana*, *S. ornata*, and *S. willdenowii*, respectively (Figure 3). A fatty acid found in three *Selaginella* species was gamma-linolenic acid with m/z value of 277.22 at 24.2 minutes (Table 2). This compound is an unsaturated fatty acid that can act as an anti-inflammatory drug (Sergeant et al. 2016). Palmitic acid is a saturated fatty acid compound detected in *S. plana* with m/z value of 255.23 at 19.26 minutes. Palmitic acid can interact with DNA topoisomerase to induce apoptosis in MOLT-4 leukemia cancer cells (Kwan et al. 2014). Palmitic acid could inhibit plant pathogens in soil and have a positive effect on rhizosphere conditions (Ma et al. 2021).

Table 1. Amino acid metabolite compounds detected in three *Selaginella* species

Compounds	Formula	m/z	RT (minute)	Species
Aspartic acid	$C_4H_7NO_4$	134,05	1.3	*S. ornata*
Deferrioxamine E	$C_{27}H_{48}N_6O_9$	639,31	9.62	*S. plana, S. willdenowii*
Diprotin B	$C_{16}H_{29}N_3O_4$	327,22	11.45	*S. plana, S. willdenowii*
Gabapentin	$C_9H_{17}NO_2$	171,21	1.52	*S. plana*
Kapakahine B	$C_{49}H_{52}N_8O_6$	849,41	15	*S. willdenowii*
L-2-Aminoadipic acid	$C_6H_{11}NO_4$	160,06	1.43	*S. plana, S. willdenowii*
L-glutamine	$C_5H_{10}N_2O_3$	130,05	1.59	*S. plana*
L-Pyroglutamic acid	$C_5H_7NO_3$	128,04	2.27	*S. plana, S. willdenowii*
L-Tyrosine	$C_9H_{11}NO_3$	180,07	1.54	*S. plana*
N-Acetyl-L-Leucine	$C_8H_{15}NO_3$	172,10	6.75	*S. plana, S. willdenowii*

Notes: m/z: mass-to-charge ratio value; RT: Retention Time

Table 2. Fatty acid metabolite compounds detected in three *Selaginella* species

Compounds	Formula	m/z	*RT* (minute)	Species
3,5-Dihydroxydecanoic acid	$C_{10}H_{20}O_4$	227.13	13.3	*S. ornata*
Dihydrojasmonic acid	$C_{12}H_{20}O_3$	213.15	13.37	*S. willdenowii*
Gamma-Linolenic acid	$C_{18}H_{30}O_2$	277.22	24.2	*S. ornata, S. plana, S. willdenowii*
Isopalmitic acid	$C_{16}H_{32}O_2$	295.23	20.03	*S. plana, S. willdenowii*
Linoleic acid	$C_{18}H_{32}O_2$	279.23	25.74	*S. willdenowii*
Lipoic acid	$C_8H_{16}O_2S_2$	207.05	1.49	*S. plana*
Palmitic acid	$C_{16}H_{32}O_2$	255.23	19.26	*S. plana*

Notes: m/z: mass-to-charge ratio value; RT: Retention Time

In addition, the lipoic acid compound (m/z value 207.05 and RT 1.49 minutes) was detected in *S. plana*, which could help reduce skin aging due to its antioxidant properties (Kim et al. 2021). Compounds only detected in *S. willdenowii* included dihydrojasmonic acid and linoleic acid (or omega-6), often found in skincare ingredients. This compound is among the essential fatty acids needed to work optimally, but the human body cannot produce it. Linoleic acid is required as an ingredient for ceramide, which protects and maintains skin moisture. A body that lacks linoleic acid can experience dry skin, hair loss easily, slower wound healing, and decreased cell regeneration. Conjugated linolenic acid, an omega-5 fatty acid, comprises antioxidant and anti-inflammatory properties and has been shown to stimulate keratinocyte proliferation and epidermal regeneration. Conjugated linolenic acid can shorten recovery time after fractionated ablative laser resurfacing of the face (Wu and Goldman 2017). Dihydrojasmonic acid compound, also known as dihydrojasmonate, possesses antioxidant properties similar to the isopalmitic acid detected in *S. plana* and *S. willdenowii*.

Phenol

Phenolic compounds are secondary metabolites of phenol groups containing hydroxyl on one or more aromatic benzene rings (de M. Castro and Demarco 2008). According to Figure 3, most of these compounds (13%) were detected in *S. ornata* extracts, such as catechol, 2-hydroxybenzaldehyde, and aspalathin (Table 3). Catechol was found in *S. ornata* with an m/z value of 109.03 at RT of 1.22 minutes and reported by Lim et al. (2016) to be capable of inhibiting lung cancer growth. Catechol has also been reported to induce root elongation, but inhibited the root hair elongation (Wang et al. 2016). 2-hydroxycinnamic acid was detected in three species at 8.05 minutes with an m/z value of 165.05. This compound can be used as an ingredient in cosmetic products due to its antioxidant and anti-aging potential (Taofiq et al. 2017). 4-vinylphenol and 3,4-dimethoxycinnamic acid were only detected in *S. plana*, with 4-vinylphenol showing potential as an anti-breast cancer agent due to inhibiting metastasis and stemness of CSC-enriched breast cancer cells (Leung et al. 2018). Based on the compounds, three *Selaginella* species have the potential to be antioxidant, anti-aging agents, and anticancer agents. Phenylacetic acid is an auxin compound, regulates plant growth through cell expansion and key compound in plant interaction with soil-microbial (Cook 2019).

Flavonoid

Approximately 11%, 12%, and 4% of flavonoid secondary metabolites were respectively identified in *S. plana*, *S. ornata*, and *S. willdenowii* (Figure 3). These include amentoflavone detected in three species at 13.85 minutes with an m/z value of 539.10 and is a biflavonoid derivative with many health benefits (Table 4). The result is based on research by Chikmawati et al. (2012), which identified an amentoflavone compound in *S. willdenowii* from Java Island. Leaf extract of *S. willdenowii* contains three known biflavones, namely 4′,7″-di-O-methyl-amentoflavone, isocryptomerin, and 7″-O-methylrobustaflavone, reported to effectively inhibit tumor cell growth (Silva et al. 1995). Additionally, amentoflavone can reduce inflammatory activity in brain microglial cells, serve as an anti-inflammatory drug, and induce the apoptotic activity of cervical cancer cells (Lee et al. 2011). In Chinese medicine, *S. willdenowii* is used for cardiovascular diseases and the treatment of nose, liver, throat, and lung cancer (Shumon and Ashrafuzzaman 2021).

Other flavonoid compounds include naringin, with an m/z value of 579.18 identified at 9.32 minutes, and can be used as anti-diabetic type 2 (Ahmed et al. 2012). Rhoifoilin detected at 10.38 minutes had an m/z value of 577.15 and antioxidant, anti-inflammatory, antimicrobial, and anticancer benefits (Refaat et al. 2015). In the plant, rhoifolin plays scavenging reactive oxygene species in plant cell (Peng et al. 2020). Aloeresin A was detected at 9.32 minutes with an m/z value of 541.18 and anti-inflammatory properties (Mwale and Masika 2010). Compounds found in *S. ornata* only were gardenin A and velutin, while those observed in *S. plana* were isoschaftoside, isovitexin 2"-O-arabinoside, kaempferol-7-neohesperidoside, and vitexin. Isoschaftoside is a C-glycosyl flavonoid identified at 7.16 minutes with an m/z value of 565.16 and has neuroprotective effects by reducing oxidative stress (Guan et al. 2022). Isovitexin 2"-O-arabinoside also possesses antioxidant activity with an m/z value of 564.49 and an RT of 7 minutes (Shao et al. 2019; Li et al. 2021). Recent research showed that bioactive compounds from *S. plana* could inhibit the growth of a fungal cell causing candidiasis (*Candida albicans*), through the disruption of ergosterol biosynthesis (Warella et al. 2023).

Alkaloid

Alkaloid is a secondary metabolite produced from the biosynthesis of shikimic acid in tryptophan and is included

in the class of compounds formed from molecules containing amine groups. This metabolite protects plants from disease, and the toxic agent components prevent herbivore attacks (Matsuura and Fett-Neto 2015). Ergonovine maleate is a group of alkaloid compounds detected in *S. willdenowii* with an m/z value of 343.21 at 11.85 minutes, which functions as a drug to help reduce bleeding after child delivery (McEvoy 2014) (Table 5). Additionally, xanthosine was identified with an m/z value of 285.08, RT of 1.1 minutes, and the ability to increase milk production and the number of mammary gland stem cells in cows (Choudhary et al. 2018).

Terpenoid

Terpenoid is a secondary metabolite produced through the mevalonic acid biosynthesis pathway, with functions including growth regulation and stimulation, as well as the protection of plants from microbes and insects (Tholl 2015). Tryptophenolide was detected from the terpenoid group at 12.91 minutes in three *Selaginella* species with an m/z value of 311.17 (Table 6). He et al. (2016) reported that this compound could act as an anti-androgen, inhibiting prostate cancer cell growth. Additionally, geranic acid detected at 12 minutes with an m/z value of 169.12 often produces a distinctive odor, facilitating the application as a perfume ingredient (Jaworska et al. 2015).

Clustering *Selaginella* species based on metabolite compound content

Chemotaxonomy includes using differences in the content of metabolite compounds as taxonomic evidence to distinguish species. This method is used to differentiate between plant species that have high morphological similarities (Singh 2016). An example of chemotaxonomy is the differentiation of several *Rhodiola* species with various classes of phenolic and flavonoid compounds due to their similar morphological characteristics (Liu et al. 2013). The analysis results of metabolite compounds showed a clear group division between three *Selaginella* species observed and presented on the heatmap combined with hierarchical clusters (Figure 4).

Table 3. Phenol group metabolite compounds detected in three *Selaginella* species

Compounds	Formula	m/z	RT (minute)	Species
2-Hydroxybenzaldehyde	$C_7H_6O_2$	121.03	21.73	*S. ornata*
2-Hydroxycinnamic acid	$C_9H_8O_3$	165.05	8.05	*S. ornata, S. plana, S. willdenowii*
2-Hydroxyphenylacetic acid	$C_8H_8O_3$	151.04	1.41	*S. ornata, S. willdenowii*
3,4-Dimethoxycinnamic acid	$C_{11}H_{12}O_4$	209.08	7.17	*S. plana*
4-Vinylphenol	C_8H_8O	119.05	7.39	*S. plana*
Aspalathin	$C_{21}H_{24}O_{11}$	451.12	1.11	*S. ornata*
Catechol	$C_6H_6O_2$	109.03	1.22	*S. ornata*
Phenylacetic acid	$C_8H_8O_2$	135.04	6.42	*S. plana, S. ornata*

Notes: m/z: mass-to-charge ratio value; RT: Retention Time

Table 4. Flavonoid metabolite compounds detected in three *Selaginella* species

Compounds	Formula	m/z	RT (minute)	Species
Amentoflavone	$C_{30}H_{18}O_{10}$	539.10	13.85	*S. ornata, S. plana, S. willdenowii*
Aloeresin A	$C_{28}H_{28}O_{11}$	541.18	9.32	*S. ornata, S. plana*
Gardenin A	$C_{21}H_{22}O_9$	313.07	8.26	*S. ornata*
Isoschaftoside	$C_{26}H_{28}O_{14}$	565.16	7.16	*S. plana*
Isovitexin 2"-O-arabinoside	$C_{26}H_{28}O_{14}$	564.49	7	*S. plana*
Kaempferol-7-neohesperidoside	$C_{27}H_{30}O_{15}$	593.15	6.01	*S. plana*
Naringin	$C_{27}H_{32}O_{14}$	579.18	9.32	*S. ornata, S. plana*
Rhoifolin	$C_{27}H_{30}O_{14}$	577.15	10.38	*S. ornata, S. plana*
Velutin	$C_{17}H_{14}O_6$	313.07	8.25	*S. ornata*
Vitexin	$C_{21}H_{20}O_{10}$	433.12	9.53	*S. plana*

Notes: m/z: mass-to-charge ratio value; RT: Retention Time

Table 5. Alkaloid metabolite compounds detected in three *Selaginella* species

Compounds	Formula	m/z	RT (minute)	Species
Ergonovine maleate	$C_{19}H_{23}N_3O_2$	343.21	11.85	*S. willdenowii*
Thalsimine	$C_{38}H_{40}N_2O_7$	637.29	9.42	*S. plana, S. willdenowii*
Thymine	$C_5H_6N_2O_2$	127.05	2.73	*S. plana*
Xanthosine	$C_{10}H_{12}N_4O_6$	285.08	1.11	*S. willdenowii*

Notes: m/z: mass to charge ratio value; RT: Retention Time

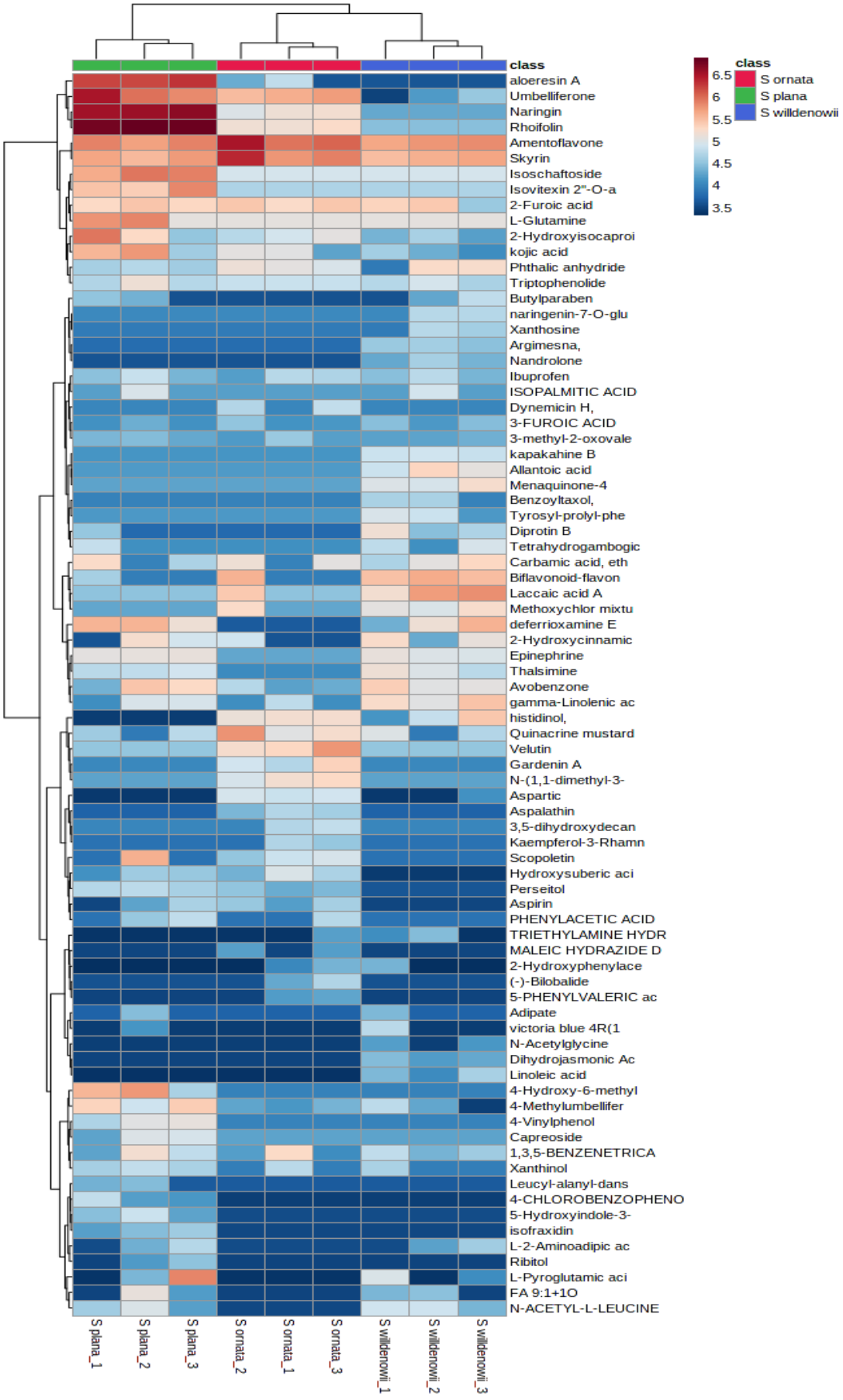

Figure 4. Heatmap clusters based on metabolite compounds contained in *Selaginella plana*, *S. ornata*, and *S. willdenowii*

Table 6. Terpenoid metabolite compounds detected in three *Selaginella* species

Compounds	Formula	m/z	RT (minute)	Species
Benzoyltaxol	$C_{54}H_{55}NO_{15}$	957.36	15.69	*S. willdenowii*
Geranic acid	$C_{10}H_{16}O_2$	169.12	12	*S. willdenowii*
Murolic acid	$C_{21}H_{36}O_5$	401.29	25.5	*S. plana*
Triptophenolide	$C_{20}H_{24}O_3$	311.17	12.91	*S. ornata*, *S. plana*, *S. willdenowii*

Notes: m/z: mass-to-charge ratio value; RT: Retention Time

Table 7. Candidate characteristic compounds for *Selaginella ornata*, *S. plana*, and *S. willdenowii*

Species	Biomarker candidates
S. plana	Aloeresin A, Naringin, Rhoifolin, Isoschaftoside, Isovitexin 2"-O-arabinoside, 4-vinylphenol, and Isofraxidin.
S. ornata	Velutin, Aspalathin, Gardenin A, and N-(1,1-dimethyl-3-oxobutyl)acrylamide.
S. willdenowii	Kapakahine B, Dihydrojasmonic Acid, Linoleic Acid, Alantoic Acid, Menaquinone, and Nandrolone.

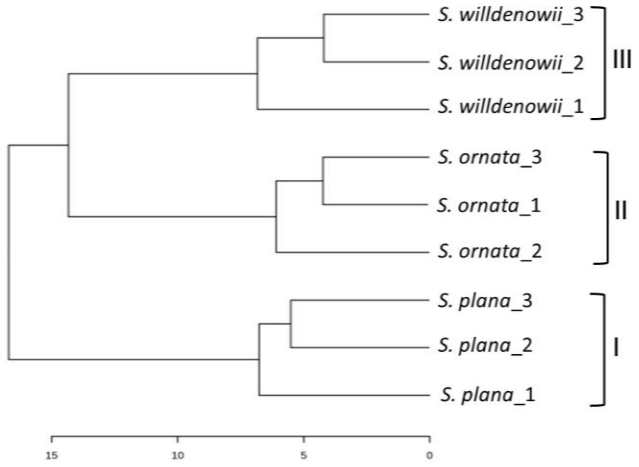

Figure 5. Dendrogram of three *Selaginella* species based on metabolite content analyzed using the complete grouping method with the Euclidean dissimilarity index

Heatmap data visualization is widely used in metabolomics research because it can describe several metabolite data and the differences in sample groups. The color rows on the heatmap represent the various metabolite compounds, while the columns are the species analyzed. Based on the heatmap formed from 80 chemical compounds, metabolites with a deep red color possess higher concentrations. Amentoflavone and skyrin had high concentrations in all individuals of three species and were highest in *S. ornata* replicate 2. The concentrations of Aloeresin A, Umbelliferone, Naringin, Rhoifolin, Isoschaftoside, and Isovitexin 2”-O-A were the highest in *S. plana*.

Metabolite profiles in plants can be used as candidate compound markers (biomarkers) of a species. Candidate characteristic compounds are determined from compounds only detected in one species and not found in others belonging to the same genus or group (Kim et al. 2017). These include 4-vinylphenol, velutin, and axahine B identified in *S. plana*, *S. ornata*, and *S. willdenowii*, respectively. All candidate characteristic compounds with the highest concentrations selected for each species group based on the heatmap are shown in Table 7.

Grouping between species was conducted through cluster analysis in the form of a dendrogram based on the level of Euclidean dissimilarity using the MetaboAnalyst 5.0 program. The results based on metabolite characteristics showed that *Selaginella* species in this research formed three groups (Figure 5).

Each group consists of individuals from the same species showing higher similarities in metabolite compounds than those from different species. However, all species have similar chemical compounds that facilitate the categorization into the same genus. The similarities in the compound content of the three species include 1,3,5-benzenetricarbonitrile, 2-Hydroxyisocaproic acid, 3-furoic acid, 3-methyl-2-oxovaleric acid, amentoflavone, avobenzone, ibuprofen, kojic acid, and skyrin. Groups 2 and 3 have a higher level of similarity due to containing 20 identical metabolite compounds, namely 2-hydroxyphenylacetic acid, laccaic acid a, histidinol, triethylamine hydro-chloride, methoxychlor, menaquinone-4, taurodehydro-cholic acid, oxytetracycline, maleic hydrazide diethanolamine, allantoic acid, azo-mustard, biflavonoid-flavone base + 3o, cephalochromin, cis-aconitate, mesaconic acid, bilobalide, cladribine, guanosine, fibracillin, and pretilachlor. Group 1 is on a different branch because it does not comprise some of the compounds present in the other two groups. The difference between Group 1 and the other groups depends on 4-chlorobenzophenone, 4-hydroxy-6-methyl-2-pyrone, 4-vinylphenol, and 5-hydroxyindole-3-acetic acid.

Three *Selaginella* species investigated vary in types and concentrations of low-density chemical compounds. *S. plana* has the most significant types and several compounds with higher concentrations than the other two species. Individuals from the same species possess higher metabolite similarity than those from different species. These results show that different *Selaginella* originating from the same location comprise various metabolite contents; hence, the types of metabolites possessed can be used as biochemical markers to differentiate between species. All *Selaginella* species contained metabolite compounds with antioxidant, anti-tumor, anti-cancer, anti-seizure, anti-inflammatory, anti-microbial, anti-aging, and anti-androgen activities. Therefore, three species have prospects to be

developed as ingredients for drugs, cosmetics, and perfumes in manufacturing.

In conclusion, this research showed that metabolite profiling of three *Selaginella* species through an untargeted metabolomics method using LC-MS/MS identified 113 essential metabolite compounds. Furthermore, amino acids, flavonoids, phenol, fatty acids, carboxylic acid, alkaloids, and terpenoids were detected in high quantities. Metabolites found in three species observed were 1,3,5-benzenetricarbonitrile, 2-hydroxyisocaproic acid, 3-furoic acid, 3-methyl-2-oxovaleric acid, amentoflavone, avobenzone, ibuprofen, kojic acid, and skyrin. Metabolites only possessed by each of *S. plana, S. ornata,* and *S. willdenowii* were 4-vinylphenol, velutin, and axahine B, respectively. Heatmap and dendrogram analyses showed that three species could be grouped based on metabolite characteristics, with *S. ornata* and *S. willdenowii* comprising high metabolite content similarity. According to the results, individuals from the same species had higher similarities in chemical compound characteristics than those from different species, signifying the potential to use metabolomics data as taxonomic markers in *Selaginella*.

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