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Research article

Phytochemical profiling and identification of chemical markers in four *Piper* species using liquid chromatography-high resolution mass spectrometry for authentication of herbal products

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Abstract

Importance of the work: Often, medicinal and pharmacological properties are determined based on phytochemical contents, emphasizing the need for phytochemical profiling of *Piper* spp.

Objectives: To analyze the phytochemical profiles of *Piper crocatum, Piper aduncum, Piper betle* and *Piper retrofractum* and to identify chemical markers specific to each species.

Materials and Methods: Leaves of the four *Piper* species were extracted using maceration, with three replications for each species. Each extract was analyzed using ultra high-performance liquid chromatography linear trap quadrupole-Orbitrap mass spectrometry and the MetaboAnalyst 6.0 software. Principal component analysis was used to determine sample groupings and similarities and differences of the compounds from the four *Piper* species.

Results: Each species had a specific phytochemical profile composed of different secondary metabolite compounds. There were substantial variations between the components of compounds of mature and old leaves. The qualitative and quantitative analysis of 83 secondary metabolite compounds successfully identified the presence of chemical markers in each species.

Main finding: The liquid chromatography-high resolution mass spectrometry method and multivariate data analysis showed potential to produce phytochemical profiles and to identify chemical markers in different *Piper* spp. for application in the development of modern drugs requiring authentication of the medicinal plant source.

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Introduction

The genus *Piper* has a geographical distribution in the tropical and subtropical regions of the world, covering Asia, Australia, Pacific Ocean Islands, Africa and South America Salehi et al., 2019). As a food and medicinal plant genus, *Piper* harbors many known species comprising a total of 2,834 (Plants of the World Online, 2025), with the highest diversity of 600 found in Southeast Asia. This genus has diverse habits ranging from herbs and lianas, to shrubs or small trees with various species, including *Piper nigrum*, *Piper cubeba*, *Piper retrofractum*, *Piper betle*, *Piper crocatum* and *Piper sarmentosum*, all predominantly being used as medicinal plants. Ethnobotanical studies in Asian countries have documented the traditional uses of *Piper* species. For example, a study by Chaveerach et al. (2006) reported that eight *Piper* species were the most popular in Thailand, with various uses such as spices and vegetables, as well as medicines, decorations and in traditional ceremonies. Hoi (2020) performed an ethnobotanical study in Vietnam and noted that *P. sarmentosum* was used traditionally to treat gout. Dapar et al. (2020) in an ethnobotanical study on the Manobo tribes of the Philippines noted three *Piper* species were used as medicinal plants to treat various health problems. Widowati et al. (2020) reported that *P. betle* was effective for women's health, particularly in postpartum treatment. The report of Kuncari (2020) identified seven species of *Piper* used as medicinal plants by the people of Southern Aceh from the Piperaceae in the Gunung Leuser National Park. Weking et al. (2023) studying the Banceuy indigenous people of West Java found that *P. betle, P. crocatum* and *P. sarmentosum* were used as traditional medicinal plants.

Other studies have investigated four *Piper* species, namely *P. betle, P. retrofractum, P. sarmentosum* and *P. aduncum* that are traditionally used as medicinal plant in Indonesia. These four species have various biological activities, including *P. betle* with pharmacological properties (antibacterial, antifungal, antioxidant, antiulcerogenic, analgesic, hepatoprotective), according to recent ethnopharmacological reviews (Biswas et al., 2022). *P. retrofractum* was found to have antitubercular, antiphotoaging, antioxidant, hepatoprotective and antiobesity properties (Salleh and Ahmad, 2020). *Piper sarmentosum* showed antibacterial, anti-inflammatory, anti-atherosclerotic, anti-osteoporosis and hypoglycemic properties (Sun et al., 2020). Taher et al., (2020) reported on *P. aduncum* and identified antimicrobial, anti-parasitic, antitumor and anticancer properties.

The phytochemical content and high diversity of secondary metabolites of *Piper* spp. play a major role in health benefits. Hartini et al. (2024) identified 12 groups of compounds extracted from leaves dominated by terpenes and terpenoid comprising the essential oils. These essential oils of *Piper* spp. consisted of terpenes, terpenoids, phenylpropanoids, benzenoids and non-terpenoid compounds (Salehi et al., 2019). Given that the phytochemical content determines the pharmacological and therapeutic properties, analysis of the phytochemical profile of *Piper* species is very important. Species belonging to the same genus share phytochemical profiles according to chemotaxanomic aspects but can be characterized by the specific composition of secondary metabolite compounds known as phytochemical markers. These markers influence the therapeutic effects of *Piper* spp. and determine their use as medicinal plants, showing the need to identify characteristic compounds.

Specific compounds functioning as phytochemical markers can be determined using several methods. Recently, liquid chromatography-high resolution mass spectrometry (LC-HRMS) has been widely used as to determine phytochemical profiles and identify marker compounds; as this method has separation ability and the high sensitivity of liquid chromatography and when combined with the sensitivity of HRMS, it produces high specificity (Xu et al., 2022), which is suitable for phytochemical analysis. Several studies have used LC-HRMS for phytochemical profiling of medicinal plants, including Nunez et al. (2020), who applied the method for characterization and authentication of *Curcuma longa* from *C. zedoaria* in curry samples based on curcuminoid and polyphenolic profiles. Ma et al. (2022) reported on another application of LC-HRMS for phytochemical profiling in the quality control and authentication of herbal components in traditional Chinese medicine. Rafi et al. (2023) used this method to evaluate the phytochemical profiles and antioxidant properties of *Cosmos caudatus*, a medicinal plant species commonly consumed as vegetable either raw or cooked. Furthermore, Luca et al. (2021) identified 42 piperamide compounds from *P. nigrum, P. longum, P. retrofractum, P. borbonese, P. guineense and P. cubeba*.

Characteristic compounds in medicinal plants must be identified for authentication of medicinal raw materials. Authentication of plant species as a procedure for quality control of herbal materials can be carried out based on morphological, phytochemical and molecular characterization (Weil et al., 2021; Pauzi et al., 2022). Among these three methods, phytochemical characterization is directly related

to the therapeutic effect through the identification of species-specific compounds. Authentication of quality control of medicinal plant based on phytochemical profiling has been reported in many species. For example, Rashid et al. (2019) conducted phytochemical characterization of *Lallemantia royleana* seeds using the spectrophotometric method for authentication of herbal medicine. Li et al. (2023) used the UPLC-LTQ/Orbitrap MS method for metabolomics analysis to produce 67 chemical components extracted from corn silk of different varieties, as plant products used in beverages, food and herbal medicine. Recently, comprehensive phytochemical profiling of *Withania somnifera* was reported by Sahoo et al. (2024), who used thin-layer chromatography, high-performance thin-layer chromatography, spectrometry and nuclear magnetic resonance analysis.

Considering the importance of *Piper* as a medicinal plant with several therapeutic properties, this study aimed to evaluate the phytochemical profiles and to identify characteristic compounds in *P. betle, P. retrofractum, P. sarmentosum* and *P. aduncum*. The results should provide an important contribution as a scientific basis for the development of authentication methods for medicinal plants in modern herbal products.

Materials and Methods

Plant materials

Samples of the *Piper* species were collected from Merapi Farma Herbal, a pharmaceutical company in Sleman, Yogyakarta, Indonesia. Verification of species identity was performed at the Laboratory of Plant Systematics, Faculty of Biology, Universitas Gadjah Mada, Indonesia.

Preparation of plant extract

Dirt was removed from fresh leaf samples of *P. betle, P. retrofractum, P. sarmentosum* and *P. aduncum* using running tap water. Then, the samples were placed between two layers of absorbent paper to eliminate water on the leaf surface. Next, the leaves were sun-dried for 3 d, followed by rapid drying using an electric oven at 60ºC to reduce the water content in the dried leaves to 10%. For each plant sample, 20 g of dried leaves were powdered manually using a mortar and pestle with the addition of liquid nitrogen. Then, each powder sample (15 g) was soaked in 150 mL 96% ethanol PA and incubated at room temperature in a rotary shaker at 150 revolutions per minute for 24 hr. The mixture was passed through Whatman filter paper and the extract was collected in an Erlenmeyer flask, which was subsequently placed in rotary evaporator (Buchi R-210) at 50ºC for 15 mins. The resulting paste was transferred into a 500 µL tube and kept in a refrigerator.

Compound identification using liquid chromatography-high resolution mass spectrometry

Each paste sample (5 mg) was dissolved in 1 mL of MeOH, filtered through a 0.2 µm nylon membrane and the filtrate was collected in a vial tube. Metabolite profiling was performed using an UHPLC Vanquish Tandem Q Exative Plus Orbitrap HRMS (Thermo Scientific), with an Accucore C18, 100 mm × 2.1 mm, 1.5 µm diameter column (Thermo Scientific) and a gas flow rate of 0.2 mL/min. The column temperature was maintained at 30ºC. The sample injection volume was 2 µL, with a mobile phase of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The program for gradient elution with the total run time was 33 min, comprising 0–1 min (5% B), 1–25 min (5–95% B), 25–28 min (95% B) and 28–33 min (5% B). The MS full-scan range was 100–1,500 m/z in the negative mode of ionization. This mode was applied since the compounds analyzed were polar compounds which are more easily ionized using negative ionization mode rather than positive mode (Luca et al., 2021; Ma et al., 2022).

Data analysis

The peak value area data obtained from UHPLC-Q-Orbitrap HRMS were analyzed using the MetaboAnalyst 6.0 software (https://www.metaboanalyst.ca/). Principal component analysis (PCA) was performed to determine sample grouping, similarities and differences of the isolated compounds from the four *Piper* species. Subsequently, a two-dimensional PCA scatterplot was generated featuring the grouping of plant samples according to their phytochemical profiles. A heatmap of the hierarchical cluster analysis was constructed to show the grouping patterns of the identified compounds and their corresponding clustering among the *Piper* species.

Results and Discussion

Based on the results of the phytochemical profiling on *P. aduncum, P. betle, P. crocatum* and *P. retrofractum* using LC-HRMS, 83 secondary metabolite compounds were identified.

The dominant groups of compounds were: organic acids, fatty acids, flavonoids, terpenes, piperidine and phenylpropanoids. Each *Piper* species had a different compound composition, as shown in the heatmap presented in Fig. 1. Based on the results of the LC-HRMS analysis, 432 compounds were detected from the samples. Next, these 432 compounds were screened based on the occurrence of each compound in three repetitions as a standard analysis method for ensuring their identity. The first screening resulted in 276 compounds which were allocated to a compound group using the relevant database search. The final screening resulted in the recognition of 83 compounds being reliably identified as in the four *Piper* species. Then, these 83 compounds were subjected to multivariate analysis using the Metaboanalyst 6.0 software.

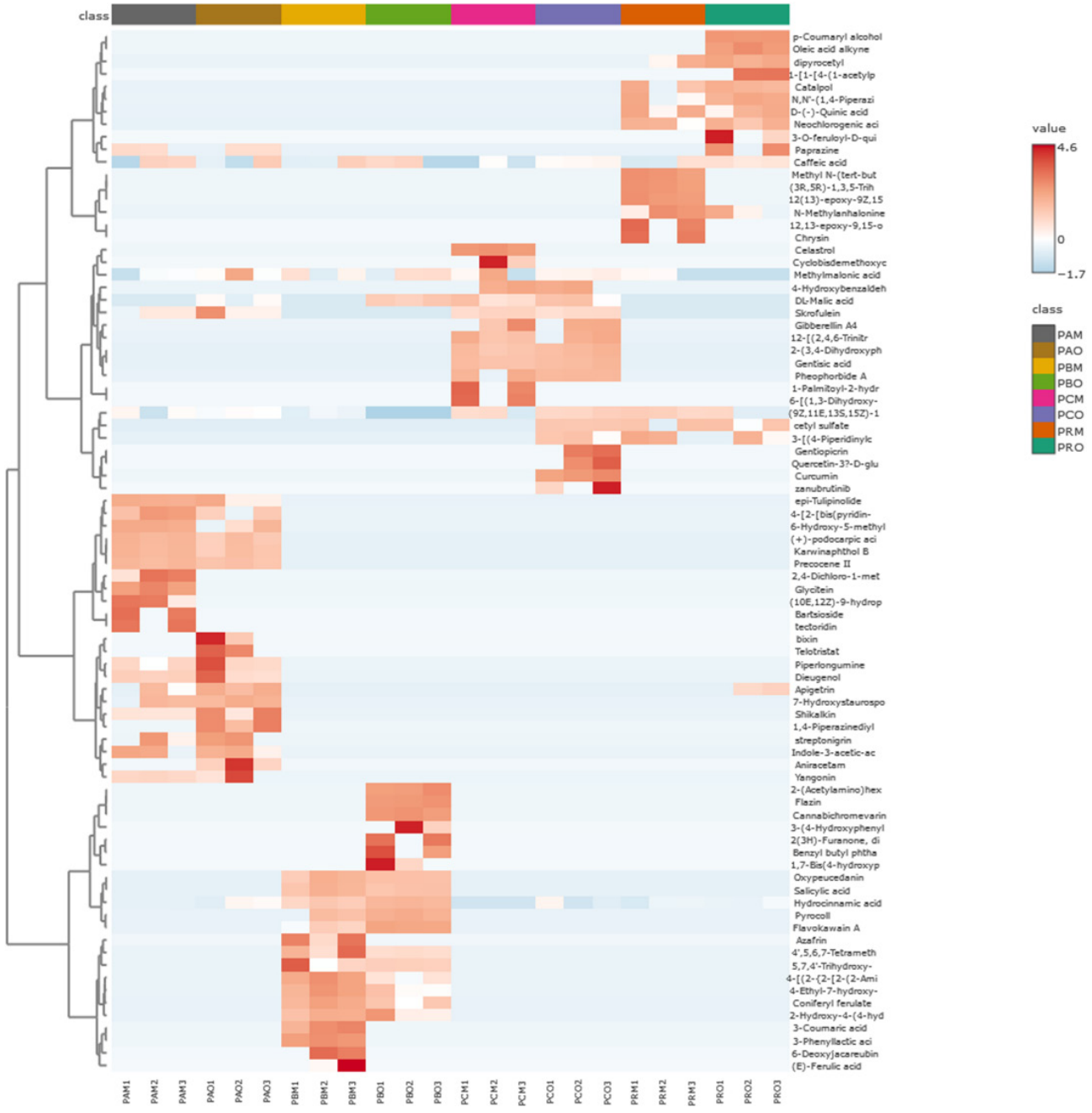

Fig. 1 Heatmap of four *Piper* species based on secondary metabolite profiles.

PAM (*Piper aduncum*, mature leaves)
PAO (*Piper aduncum*, old leaves)
PBM (*Piper betle*, mature leaves)
PBO (*Piper betle*, old leaves)
PCM (*Piper crocatum*, mature leaves)
PCO (*Piper crocatum*, old leaves)
PRM (*Piper retrofractum*, mature leaves)
PRO (*Piper retrofractum*, old leaves)

Volatile and non-volatile compounds in various *Piper* species have had reportedly different compositions. For example, Salehi (2019) conducted *Piper* spp. phytochemistry and noted that each species had a different essential oil composition. In addition, phytochemical profiling has been applied based on spectrophotometric and chromatographic methods, with Weil et al. (2021) reporting there were differences in the essential oil and piperine contents among *P. nigrum*, *P. borbonense*, *P. guineense* and *P. capense*. Kuncari (2020) found differences in the qualitative phytochemical profiles in an ethnopharmacological study of seven *Piper* species traditionally used as medicinal plants by communities around Gunung Leuser National Park, Aceh, Indonesia.

Based on their results of chemical profiling of the genus *Piper*, Luca et al. (2021) concluded that LC-HRMS was a sensitive analysis method, as based on the composition of piperamide compounds, LC-HRMS identified similarities and differences between species; for example, *P. nigrum* had a piperamide profile identical to *P. longum*, but different from *P. cubeba*, *P. guineense* and *P. retrofractum*. In addition, Ma et al. (2022) emphasized the advantages of using LC-HRMS as an analytical method for the separation and identification of complex compounds in medicinal plants. In the current study, LC-HRMS was considered an appropriate analysis method for the characterization, authentication and detection of illegal practices of mixing herbal medicinal ingredients.

As shown in Fig. 1, each *Piper* species had several specific compounds that could be categorized as indicative chemical markers. Identification of these compounds was supported by the results of the PCA, based on the character loading values on the first and second principal components. The compounds analyzed for identification of chemical markers were isolated in the ethanolic extracts of both mature and old leaves in the four *Piper* species, as shown in Table 1. The characteristic compounds identified were mostly not classified as essential oil components because of the drying treatment. In this study, treatment was carried out to make leaves closely resemble the conditions of herbal materials used in the pharmaceutical industry.

Table 1 List of indicative chemical markers in four investigated *Piper* species

No	Plant species	Compound name	Molecular weight	Retention time	Sample area (unit)
1	*Piper aduncum*	Piperlongumine	317.1	13.8	112947691.5
		Dieugenol	326.2	21.2	141356661.9
		Karwinaphthol B	288.1	21.0	371613098.3
		Precocene II	220.1	16.8	637343013.4
		Shikalkin	288.1	15.0	1014504639
		7-Hydroxystaurosporine	482.2	18.2	115270354.4
		epi-Tulipinolide	290.2	16.3	102829232.6
		(+)-Podocarpic acid	274.2	18.0	652498595
		6-Hydroxy-5-methyl-4,11-Dioxoundecanoic acid	244.1	10.6	255852338.5
2	*Piper betle*	Flavokawain a	314.1	14.5	168056753.7
		Salicylic acid	138.0	10.1	134010807.1
		Hydrocinnamic acid	150.1	11.5	28587579719
		Pyrocoll	186.0	11.5	140978868.8
		Oxypeucedanin	286.1	13.9	146290788,2
		4',5,6,7-Tetramethoxyflavanone	344.1	13.6	1000895017
		5,7,4'-Trihydroxy-6-prenylflavanone	340.1	17.9	135288630,8
		Coniferyl ferulate	356.1	17.1	164809960,3
		4-Ethyl-7-hydroxy-3-(p-methoxyphenyl)coumarin	296.1	17.1	238439953,4
		2-Hydroxy-4-(4-hydroxyphenyl)butanoic acid	196.1	11.5	599263742,8
3	*Piper crocatum*	Gentisic acid	154.0	4.0	68821129,91
		12-[(2,4,6-trinitrophenyl)amino]dodecanoic acid	426.2	6.3	57254224,8
		Skrofulein	314.1	16.6	242835528
		Pheophorbide a	592.3	26.8	52871509,99
		2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4h-chromen-3-yl 6-o-β-d-xylopyranosyl-β-d-glucopyranoside	596.1	8.4	51579604,52
4	*Piper retrofractum*	Catalpol	362.1	4.0	49381754,71
		Quinic acid	192.1	1.1	142978877,4
		n,n'-(1,4-Piperazinediyldi-3,1-propanediyl) bis[2-(3,4,5-trimethoxyphenoxy)acetamide]	648.3	18.0	145497335,6
		Neochlorogenic acid	354.1	6.5	153099047
		Dipyrocetyl	238.0	9.4	53079865,46

Piperidine, an alkaloid compound, was identified as the dominant component of the four *Piper* species in the current study. Xiang et al. (2016) carried out a phytochemical profile study of the Piperaceae and reported that alkaloids were the dominant secondary metabolite compound, along with some other groups such as flavonoids, chalcones and kava-pyrones. In the current study, flavonoids were another class of compound detected with relatively high diversity. Salleh et al. (2020) in a study of *P. abbreviatum* successfully identified five methoxylated flavonoids with potential as chemical markers, while Salleh and Ahmad (2020) reviewed the phytochemistry of *P. retrofractum* and reported that alkaloids, amides, phenylpropanoids, alkyl glycosides and lignan were the dominant groups of compounds characterizing the species. Morais et al. (2023) in an analysis of essential oils found that the main compound in *P. aduncum* was dillapiole, an arylpropanoid compound, while Ware et al. (2024) carried out a phytochemical analysis using LC-HRMS and reported that the dominant compound groups in *P. sarmentosum* leaf extract were flavonoids, lignans and phenyl propanoids.

As presented in Fig. 2, the 3D scatterplot plot generated from the PCA clearly separated the four *Piper* species, with the spatial placement based on the composition of compounds identified in the ethanolic extracts of their leaves. In the current study, each species was made up of samples from two groups—those representing mature and those representing old leaves. On the scatterplot, samples from the mature and old leaves were grouped in close proximity, indicating that there were only slight differences in the composition of secondary metabolite compounds between the two groups of samples.

The markers in *Piper* species come from the essential oil group, including various types of chemical compounds. Each *Piper* species is characterized by dominant and specific compounds, which are essential for chemical characterization. Based on a review of various chemotypes of *P. betle*, Mondal (2021) summarized volatile markers including chavicol, chavibetol, eugenol, isoeugenol, eugenol acetate, germacrene-D, safrole, anethole, alpha-cadinol. The compositions of these chemical markers vary depending on the geographical location and the variety of *P. betle*, showing the influence of environmental and genetic factors on chemical characteristics.

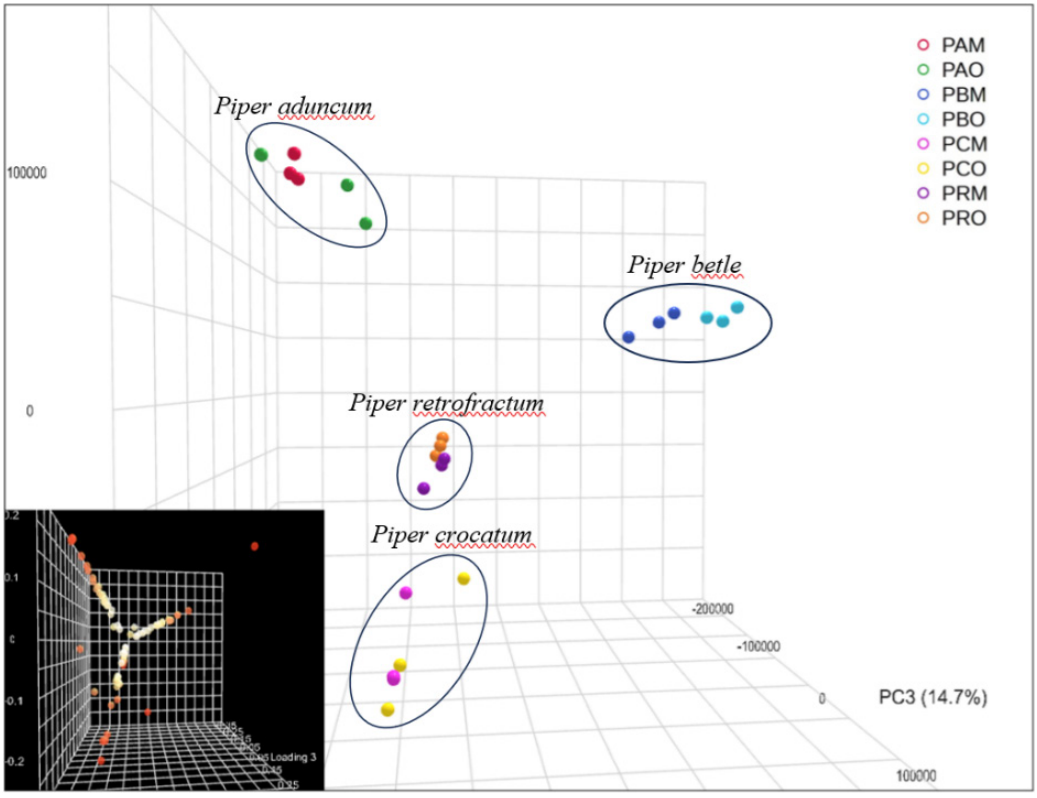

Fig. 2 Three-dimensional scatterplot of four *Piper* species based on secondary metabolite profiling. PC = principal component; PAM = *Piper aduncum*, mature leaves; PAO = *Piper aduncum*, old leaves; PBM = *Piper betle*, mature leaves; PBO = *Piper betle*, old leaves; PCM = *Piper crocatum*, mature leaves; PCO = *Piper crocatum*, old leaves; PRM = *Piper retrofractum*, mature leaves; PRO = *Piper retrofractum*, old leaves .

Amides have been reported to be chemical markers of *Piper* species, while piperolactam C and piperlongumine were found in *P. retrofractum* and *P. nigrum* (Luca et al., 2021). Fan et al. (2023) identified lignan in various *Piper* species reporting that lignans had high compound diversity with varying levels in each species. Identification of chemical markers for *Piper* authentication by Maulidya et al. (2023) showed that piperenamide A was a specific compound found only in the *Piper acre*. This compound was not detected in two other species (*Piper betle* and *P. crocatum*).

Identification of marker compounds is essential for the authentication of medicinal plants. Commonly, the authentication of herbal medicinal ingredients is carried out based on chemical fingerprinting as a quality control parameter (Reddy et al. 2021), because the efficacy of medicinal herbs is highly dependent on their composition and phytochemical content (Rafi et al., 2023). Ma et al. (2022) stated that authentication could be used to ensure the effectiveness and safety of medicinal herbal. Similarly, Mück et al. (2024) explored the use of traditional Chinese medicine and found that phytochemical profiling generated from chromatographic analysis could serve as a valuable method in authenticating herbal ingredients.

Conclusions

Phytochemical profiling based on the analysis of the ethanol leaf extract from *Piper* species using LC-HRMS was able to identify several compounds categorized as indicative chemical markers for *P. aduncum, P. betle, P. crocatum* and P. *retrofractum*. These compounds should be useful for developing species-specific chemical markers in the authentication and standardization of herbal products derived from the four *Piper* species analyzed in this study.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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