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Secondary metabolite profile in mature and old leaves of four *piper* species: Forest Betel (*Piper aduncum* L.), Red Betel (*Piper crocatum* Ruiz & Pav.), Javanese Chili Betel (*Piper retrofractum* Vahl.), and Green Betel (*Piper betle* L.)

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Abstract

Piper species is a potential medicinal plant, empirically known for its effectiveness in curing various diseases, particularly in Indonesia. Therefore, this research aimed to evaluate the similarities and differences in the profile of secondary metabolite compounds in mature and old leaves of four *Piper* species using Gas Chromatography-Mass Spectrometry (GC-MS). The analysis was also carried out to identify the specific compounds found in each species at different leaf development stages. Samples used were mature and old leaves from four species of *Piper*, namely forest betel (*Piper aduncum* L.) (PA), red betel (*Piper crocatum* Ruiz & Pav.) (PC), Javanese chili betel (*Piper retrofractum* Vahl.) (PR), and green betel (*Piper betle* L.) (PB). Subsequently, samples were extracted using ethanol solvent and secondary metabolite profile was detected through GC-MS. A total of 40 secondary metabolite compounds were found in mature and old leaves of four species. The results showed that alkaloid content contributed 25% of the total compounds detected, while fatty acids yielded the largest portion (27.5%). Based on PCA score plot analysis, a significant grouping of secondary metabolite compounds was observed in all species, where PC was categorized separately on the right, and the other species were on the left. Several specific compounds were also found only in one species and not in others. Similar to mature and old leaves, some compounds were discovered in one of the developmental phases. Dillapiole and hexadecane were only found in PA, both in mature and old leaves; germacrene A, eugenol, and chavicol were found in mature and old leaves of PB, but beta-selinene, delta-guaiene, and linolelaidic acid were only found in mature leaves. In PC, there were specific compounds namely benzofuran, myrcene, and 1,6,10-dodecatrien that were found in mature and old leaves. Besides that, trans-ocimene and alpha-bisabolol were only found in old leaves of PC. In PR, only tetracosane was found in mature leaves, while piperidine, octanodecanoic acid, and benzylmalonic acid were found in old leaves.

Keywords

GC-MS; mature leaves; old leaves; *Piper*; profile; secondary metabolite

Introduction

The Piper plant is used for many different things all around the world. It can be used in herbal medicines, culinary applications, decorative displays, and

customary rites. It has substantial botanical diversity, with about 700 species recognized globally (1) and an estimated 1400–2000 variants arising from various nations. The Java Island in Indonesia alone is home to about 23 different species of Piper known to science. Most species are found to be able to survive at elevations between sea level and 2500 m, while very few can reach elevations over 3000 m (2). In particular, Indonesia uses the betel plant in traditional medicine. The well-known effects of Piper as an antidiabetic, immunomodulator, platelet inhibitor, antioxidant, and anticancer agent make it stand out (3).

Red betel (*Piper crocatum* Ruiz & Pav.) (PC) originated in Peru and has since spread to several countries, including Indonesia. The leaves are elliptical, acuminatus, sub-acute at the base with a tapered top, flat edges, shiny or hairless, with a length of 9–12 cm and width of 4–5 cm. Pinnatus leaf veins are found in the lower half, comprising 4–5 pairs, forming a bullulatus-lacunosa pattern. The top leaves display a dark green with silver markings along the veins, while the lower sections exhibit a purple coloration, having a slightly slimy texture and a bitter taste, along with a less distinct aroma (4). Red betel leaves are widely used to get rid of body odor, vaginal discharge, ulcers, fatigue, muscle aches, and to smoothen the skin, as well as for itching, red eye cleansers, and canker sores. Red betel leaves decoction is also believed to be able to eliminate bad breath in the mouth if used as a mouthwash (5).

The forest betel, also known as *Piper aduncum* L. (PA), is a shrub or small tree that can grow to a height of 7 m with a stem diameter of 10 cm or more. It has medium -hard, brittle wood, short silt roots, and aromatic foliage and twigs. The leaves are oval, distichous, and alternating, measuring 12 to 22 cm in length. *P. aduncum* contains an essential oil that is reportedly able to prevent bites of the *Aedes albopictus* mosquito (6).

Based on its morphology, the Javanese chili betel (*Piper retrofractum* Vahl.) (PR) is a climbing plant, characterized by round leaves, lanceolate, and wide with green to dark green coloration. The stem is round and large, has a diameter of ± 5–7 mm, length of the main stem is 2.93–9.82 cm, with significantly varying color from black, and brown to blackish brown. The main chemical constituents that have been isolated and identified from *P. retrofractum* are amides, alkaloids, phenylpropanoids, alkyl glycosides, and lignans. This plant possesses antioxidant, hepatoprotective, cytotoxic, larvicidal, antiproliferative, antitubercular, antileishmanial, antiphotoaging, and antiobesity properties. *P. retrofractum* has the potential for the treatment of several diseases and disorders (7).

Green betel (*Piper betle* L.) (PB) is a perennial dioecious climber, with large leaves, 15–20 cm long, broadly ovate, slightly cordate, shortly acuminate, acute, entire, glabrous, yellowish or bright green, shining on both sides (8). Betel leaf has many properties such as anti-fungal, anti-septic, anti-microbial, anti-cancer, anti-diabetic, anti-allergic, anti-fertility, anti-filarial, wound healing and anti-dermatophytic. It also prevents gastrointestinal infections due to its immunomodulatory effect. It may be useful in the treatment of diabetes by maintaining blood sugar levels (9).

Piperaceae species are widely recognized for their rich content of essential oils, alkaloids, and phenols (10). Alkaloids and phenols, which are common components of the Piperaceae family, are significant physiological ingredients that aid in controlling plant growth and providing defense against diseases and insect pests (11). These compounds have also been extensively used as natural plant products, mainly applied in the pharmaceutical and food industries (12–14). Currently, the research on plant metabolomics has gained significant attention due to its potential for diagnosing metabolite changes in low molecular weight metabolite and the underlying biochemical mechanisms (15). This shows that the use of plant metabolomic research to investigate the distribution and profile of secondary metabolite in plant organs is an important step in identifying the medicinal properties of various plant parts.

The part of the Piper plant that is used in traditional medicine, especially in Indonesia, is the leaves. Based on previous investigations, there is no research that has explored and comprehensively compared secondary metabolite profile in mature and old leaves of four *Piper* species, namely forest betel (*P. aduncum* L.) (PA), red betel (*P. crocatum* Ruiz & Pav.) (PC), Javanese chili betel (*P. retrofractum* Vahl.) (PR), and green betel (*P. betle* L.) (PB). Consequently, this research aimed to evaluate the similarities and differences of secondary metabolite profile in mature and old leaves of four *Piper* species using GC-MS. The specific compounds found in each species at different ages of leaves were also explored because it is an important step in identifying medicinal properties of various plants in different stage of development.

Materials and Methods

Chemical and Plant Materials

Chemicals for extraction were carried out using Merck Sigma Aldrich's absolute ethanol and liquid nitrogen. Samples used were mature (M) and old leaves (O) from PA, PC, PR, and PB, with three replications for each age and species. Leaves samples were collected from Sleman Regency, Yogyakarta Special Province, Indonesia.

Metabolite Profile

Extraction of Secondary Metabolite

Sample extraction used the maceration method reported in previous research (16) with modifications. A sample of 20 g leaves was added with liquid nitrogen and crushed using a mortar to obtain a powder form. Subsequently, the powder was transferred to an Erlenmeyer flask, 15 mL ethanol was added, and stirred. Samples were incubated at room temperature for 72 hrs, filtered, and the extract was evaporated in a petri dish for analysis using GC-MS.

GC-MS Analysis

GC-MS analysis was conducted using Shimadzu GCMS-QP2010S equipped with an Agilent DB-5MS column. The column specifications included a length of 30 m, 0.25 mm ID, 0.25 um film, Helium carrier gas, and EI 70Ev ionizer. GC-2010 specifications were column oven temperature of 70.0°C, injection temperature of 300.00°C, splitless injection mode, sampling time of 1.00 min, flow control mode pressure, pressure 30.0 kPa, total flow 35.6 mL/min, column flow 0.65 mL/min, linear velocity 29.6 cm/sec, purge flow 3.0 mL/min, split ratio 49. Furthermore, the ion source temperature was 250°C, the interface temperature of 305°C, the solvent cut time was 5 min, and the detector gain relative mode. Total GC running time was 80 min and the relative percentage amount of each component was calculated by comparing its average peak area to the total areas.

Data Processing and Statistics

The data from GC-MS analysis was summarized based on the Similarity Index (SI) value > 80%. Compound names, chemical formula, and retention time values were tabulated and analyzed with the MetaboAnalyst program (https://www.metaboanalyst.ca/MetaboAnalyst/). The distribution and sample grouping were visualized using principal component analysis (PCA), an unsupervised approach that lowered the dimension of the data sets. The threshold for identifying possible outliers in the dataset was set at a 95% confidence interval in the PCA score plot.

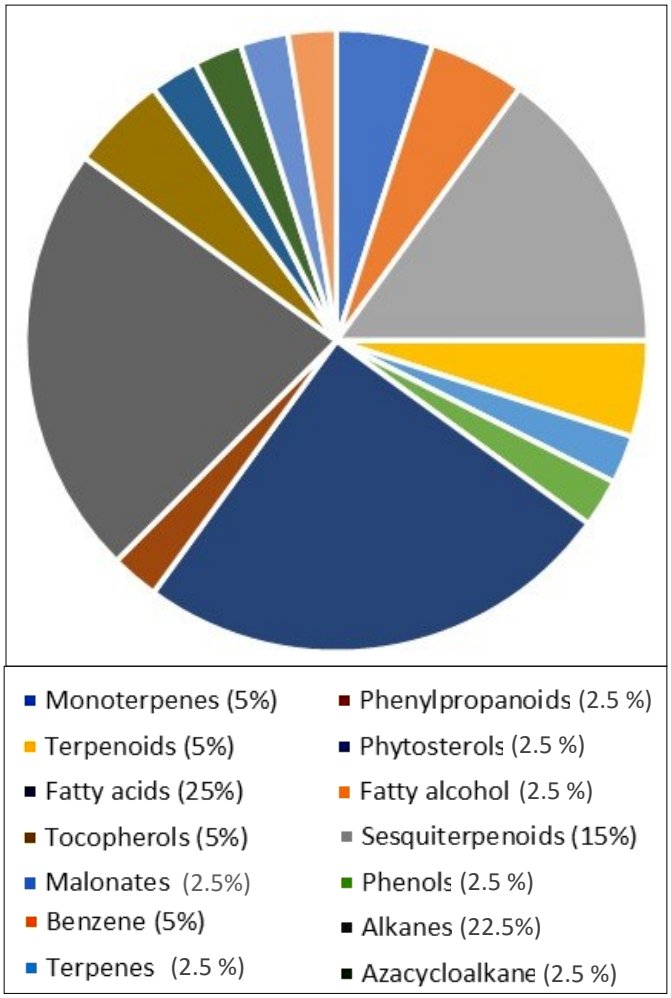

Fig. 1. Classification of 40 secondary metabolite compounds in mature and old leaves of forest betel (*Piper aduncum* L.), red betel (*Piper crocatum* Ruiz & Pav.), Javanese chili betel (*Piper retrofractum* Vahl.), and green betel (*Piper betle* L.).

Results and Discussion

Metabolomic Profile of Samples

The results of GC-MS analysis showed that 40 secondary metabolite compounds were detected in mature and old leaves of all *Piper* species. These compounds were divided into 12 groups, namely monoterpenes (5%), sesquiterpenoids (15%), terpenes (2.5%), fatty acids (25%), alkanes (22.5%), phytosterols (2.5%), benzene (5%), terpenoids (5%), phenols (2.5%), phenylpropanoids (2.5%), tocopherols (5%), and azacycloalkalene (2.5%), as presented in Fig. 1. *Piper* species has been widely explored and the phytochemical investigations globally led to the isolation of several physiologically active compounds, including alkaloids, amides, propenyl phenols, lignanes, neolignanes, terpenes, steroids, kawapyrones, piperloids, chalcons, dihydrochalcones, and flavones. Biological activities of different species have special emphasis, indicating a wide spectrum of pharmacological activities (17). Furthermore, the 40 compounds detected were analyzed with MetaboAnalyst to determine the grouping of specific compounds in each species with different ages of leaves.

Grouping Compounds

In order to make it easier to see the similarities and differences across the data sets, PCA was utilized to categorize metabolite phenotypes and find the differential metabolite. Every point of a PCA score represented a single sample, and the score plot displayed the distribution of samples. This demonstrated that whilst distinct metabolomic activities were distributed, similar metabolomic processes were clustered (18). As seen in Fig. 2, PCA was used to find variations in metabolite profiles across eight datasets that included two leaf development stages and four *Piper* species.

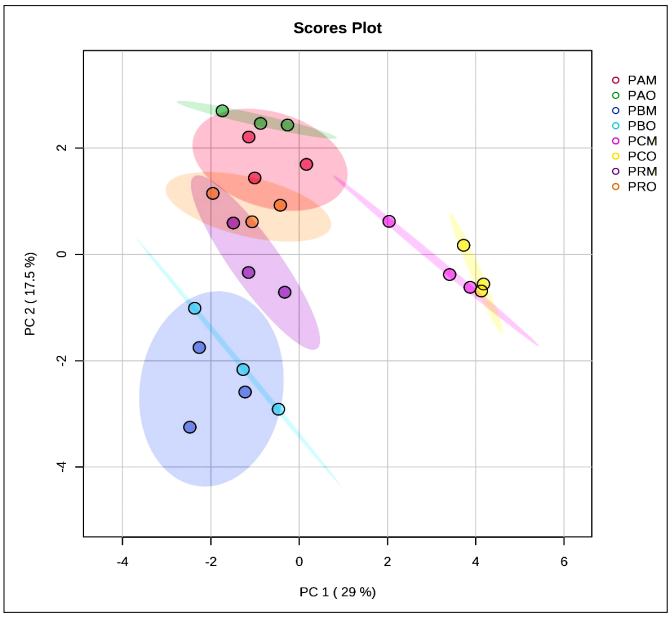

Fig. 2. PCA score plot of forest betel (*Piper aduncum* L.) (**PA**), red betel (*Piper crocatum* Ruiz & Pav.) (**PC**), Javanese chili betel (*Piper retrofractum* Vahl.) (**PR**), and green betel (*Piper betle* L.) (**PB**) in mature (**M**) and old (**O**) leaves.

In this research, PCA was carried out on 40 secondary metabolite compounds resulting from GC-MS analysis. The score plot showed that samples from the same *Piper* species were closely clustered, while those from different species were separated from each other. PCA results of all detected compounds identified four groups, where secondary metabolite compounds in PC were grouped separately on the right. Meanwhile, PA, PR, and PB were categorized on the left, as presented in Fig. 2. PC1 and PC2 accounted for 29% and 17.5% of the variance in the data, respectively, showing specific secondary metabolite compounds found in each *Piper* species. However, there were no differences in the grouping of compounds in mature and old leaves of each species.

The results of PCA analysis showed that PC had a different group of compounds compared to other species. Morphologically, PC had the most different characters compared to others, namely in the morphological characters of leaves. The upper leaves are dark green with a silvery appearance around the veins, while the lower parts are purple (4). These leaves are slimy and have a bitter taste with a less specific odor compared to other species. PC leaves are found to include flavonoids, polyphenolic chemicals, tannins, and essential oils, according to chromatography analysis (19).

Heatmap analysis was carried out on the 40 detected compounds to determine the grouping and profile of specific compounds in each sample, as shown in Fig. 3. Based on heatmap analysis, the profile of secondary metabolite-specific compounds for each species was identified at different ages. In PC, specific compounds were found in mature leaves (PCM), namely Benzofuran, 2,3-dihydro (benzene), Myrcene (Monoterpenes), and 1,6,10-Dodecatrien (Terpenoids), while old leaves (PCO) contained Trans-Ocimene (Monoterpenes), and alpha Bisabolol (sesquiterpenes). The extract from PC leaves contained alkaloids, carbohydrates, water, tannins, phenols, flavonoids, and essential oils, as per an earlier study (19). According to the results of (20), certain compounds were found in PC leaves, such as flavonoids with groups like quercetin and aurone, and essential oils with monoterpene components like α-thujene, α-pinene, sabinene, ß-myrcene, α-terpinene, ß-phellandrene, γ-terpinene, α-terpineol, terpinolene, and copaene. Neo-lignans, including 1-allyl-3,5-dimethoxy7-methyl-oxo-6-(3,4,5-trimethoxyphenyl) bicyclo[3,2,1]oct-2-en-8-yl acetate, and sesquiterpenes including caryophyllene, α-caryophyllene, and germacrene D, were also found. Additionally, compounds such as alkaloids, tannins-polyphenols, steroids-terpenoids, and saponins were also

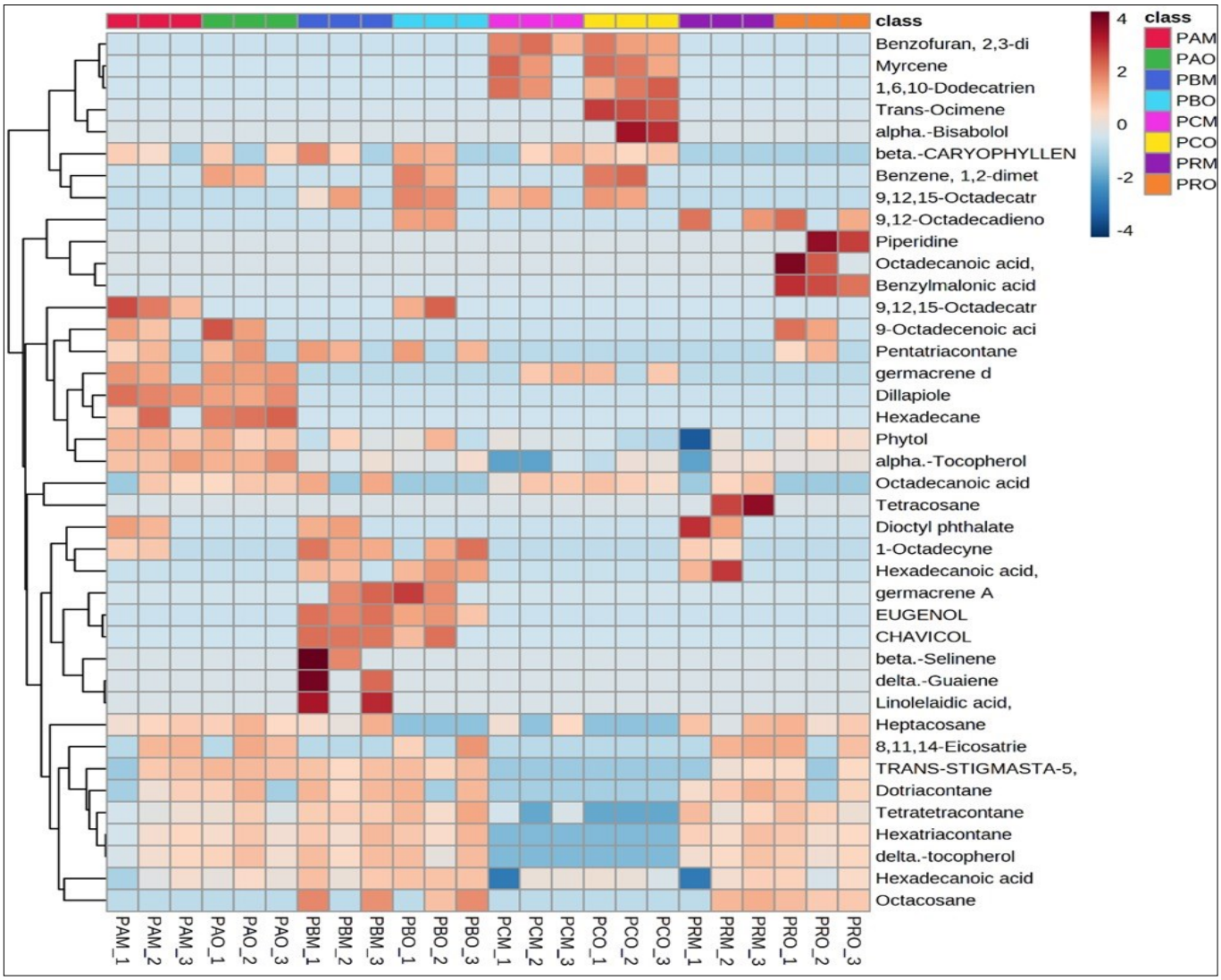

Fig. 3. Heatmap clustering among forest betel (*Piper aduncum* L.) (**PA**), red betel (*Piper crocatum* Ruiz & Pav.) (**PC**), Javanese chili betel (*Piper retrofractum* Vahl.) (**PR**), and green betel (*Piper betle* L.) (**PB**) in mature (**M**) and old (**O**) leaves.

discovered. PC leaves have been shown to have anti-inflammatory, antibacterial, antifungal, antihyperglycemic, and anti-proliferative qualities in a number of investigations into their pharmacological characteristics.

In PR, specific compounds were detected in mature (PRM) and old leaves (PRO), including Piperidine (azacycloalkane), Octadecanoid acid (fatty acids), and Benzylmalonic acid (malonates). Additionally, high concentrations of tetracosane compounds (alkalenes) were detected in PRM. The main chemical constituents that were isolated and identified from PR included amides, alkaloids, phenylpropanoids, alkyl glycosides, and lignans (4), while Piperidine was found in leaves (19). Tetracosane is a straight-chain alkane containing 24 carbon atoms, playing a role as a plant metabolite and a volatile oil component. Tetracosane is a natural product found in *Cryptotermes brevis, Erucaria microcarpa*, and other organisms (20, 21), which has been explored in the pharmaceutical field. According to previous research (22), tetracosane is a potential bioactive compound, providing a rationale for its traditional use in peptic ulcer treatment.

Piper betle L. has the highest number of secondary metabolite compounds compared to other species, with 23 in total. Specific compounds had been found in PB, both in mature (PBM) and old leaves (PBO) (Table 1), including germacrene A (sesquiterpenoids), eugenol (terpenoids), and chaviol (phenols). These compounds were responsible for the distinctive aroma of PB, (23) which occurred due to the presence of oils, including terpenes and phenols. In this research, it was also discovered that the compounds beta-selinene (sesquiterpenoids), delta-Guaiene (sesquiterpenoids), and Linolelaidic acid (fatty acids) were only found in PBM at high concentrations, producing more essential oil compared to PBO. The main constituent of leaves is oil with a chemical composition depending on the location observed, namely asbetle oil. Leaves produce compounds such as hydroxychavicol acetate, allylpyrocatechol, chavibetol, piperbetol, methylpiperbetol, piperol A and B. Hydrocydroxychavicol and eugenol, including phenolic compounds, consist of monocyclic fragrant ring with an alcoholic, aldehydic, or carboxylic group, which are essential in PB leaves, contributing to several bioactivities. Chavibetol is the main com-

Table 1. Secondary metabolite compound in mature (**M**) and old (**O**) leaves of four *Piper* species: *Piper aduncum* L. (**PA**), *Piper crocatum* Ruiz & Pav. (**PC**), *Piper retrofractum* Vahl. (**PR**), and *Piper betle* L. (**PB**).

No.	Compound Name	PCM	PCO	PBM	PBO	PAM	PAO	PRM	PRO
1	Myrcene	✓	✓		✓				
2	Trans-Ocimene		✓		✓				
3	Benzofuran, 2,3-Dihydro-	✓	✓		✓				
4	Benzene, 1,2-Dimethyl-		✓		✓		✓		
5	Beta.-Caryophyllene	✓	✓	✓	✓	✓	✓		
6	Germacrene D	✓	✓		✓	✓	✓		
7	Germacrene A			✓	✓				
8	Alpha.-Bisabolol		✓		✓				
9	Beta.-Selinene			✓	✓				
10	Delta.-Guaiene			✓					
11	1,6,10-Dodecatrien-3-Ol, 3,7,11-Trimethyl-, S-(Z) -	✓	✓						
12	Phytol	✓	✓	✓	✓	✓	✓	✓	✓
13	Eugenol			✓	✓				
14	Chavicol			✓	✓				
15	1-Octadecyne			✓	✓	✓		✓	
16	Octadecanoic Acid, Methyl Ester								✓
17	Octadecanoic Acid	✓	✓	✓		✓	✓	✓	
18	Hexadecanoic Acid	✓	✓	✓	✓	✓	✓	✓	✓
19	9,12,15-Octadecatrienoic Acid, Methyl Ester, (Z,Z,Z)-	✓	✓	✓	✓				
20	8,11,14-Eicosatrienoic Acid, (Z,Z,Z)-				✓	✓	✓	✓	✓
21	9-Octadecenoic Acid (Z)-, Methyl Ester					✓	✓		✓
22	Hexadecanoic Acid, Ethyl Ester			✓	✓			✓	
23	Linolelaidic Acid, Methyl Ester								
24	9,12-Octadecadienoic Acid, Methyl Ester, (E,E)-				✓			✓	✓
25	9,12,15-Octadecatrien-1-Ol				✓	✓			
26	Dioctyl Phthalate			✓		✓		✓	

27	Heptacosane	✓			✓		✓	✓	✓	✓
28	Tetratetracontane	✓			✓	✓	✓	✓	✓	✓
29	Hexatriacontane				✓	✓	✓	✓	✓	✓
30	Dotriacontane				✓	✓	✓	✓	✓	✓
31	Octacosane				✓	✓			✓	✓
32	Pentatriacontane				✓		✓	✓		✓
33	Dillapiole						✓	✓		
34	Hexadecane						✓	✓		
35	Tetracosane								✓	
36	Delta.-Tocopherol				✓	✓	✓	✓	✓	✓
37	Alpha.-Tocopherol			✓	✓	✓	✓	✓	✓	✓
38	Trans-Stigmasta-5,22-Dien-3.Beta.-Ol				✓	✓	✓	✓	✓	✓
39	Piperidine									✓
40	Benzylmalonic Acid									✓

ponent of the essential oil, characterized by a highly spiced odor. Hydroxychavicol has also shown beneficial bioactivities like anticarcinogenic and antimutagenic activities (23).

In PA, specific compounds were detected, namely Dilapiole and Hexadecane, which belong to the alkanes group. According to previous research (24), PA leaves and fruit contains 0.30% and 0.33% of essential oil, respectively. Apiol was the most abundant chemical compound obtained in the essential oil of leaves and fruit, with concentrations of 57.10% and 66.31%, respectively. This essential oil successfully inhibited the growth of *Aspergillus niger* and *Cladosporium* sp. but was unable to inhibit *Fusarium oxysporum* and *Fusarium solani*. In this research, Germacene D, a class of sesquiterpenes compounds, was also detected. Similarly, it was discovered (25) that based on gas chromatography analysis, 17.16% of Germacrene D was detected in leaves. This compound also suppressed the growth of lung cancer and leukemia cells *in vitro*.

Apart from specific compounds that are found in one species, 8,11,14-Eicosatrie, Trans-stigmasta-5, Dotriacontane, Tetratetracontane, Hexatriacontane, Delta-tochopherol, and Hexadecanoic acid, were also found in all species (Table 1). These compounds were only found in PA, PC, and PB, due to a separate grouping in PR compared to the other three *Piper* species.

Conclusion

In conclusion, based on the GC-MS results, a total of 40 secondary metabolite compounds were detected in four *Piper* species. Alkaloid content contributed 25% of the total compounds detected, while fatty acids had the largest portion of 27.5%. PCA score plot analysis showed that there was a significant grouping of the compounds, where PC was grouped separately on the right, while other species were on the left. Furthermore, there were several specific compounds found in one species and not in others. Similar to mature and old leaves, some compounds were only found in one of these developmental phases. Dillapiole and hexadecane were only found in *Piper aduncum* L., both in mature and old leaves; germacrene A, eugenol, and chavicol were found in mature and old leaves of *Piper betle* L., but beta-selinene, delta-guaiene, and linolelaidic acid were only found in mature leaves. In *Piper crocatum* Ruiz & Pav., there were specific compounds namely benzofuran, myrcene, and 1,6,10-dodecatrien that were found in mature and old leaves. Besides that, trans-ocimene and alpha-bisabolol were only found in old leaves of *P. crocatum* Ruiz & Pav. In *Piper retrofractum* Vahl., only tetracosane was found in mature leaves, while piperidine, octanodecanoic acid, and benzylmalonic acid were found in old leaves. The isolation and identification of active molecules with pharmacological properties should be the main focus of future research. In addition, most pharmacological research has been done on leaves; nevertheless, further research is required to look at the bioactivity of other plant parts.

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Authors' contributions

YSH contributed to the design of the research, wrote the manuscript, and coordinated. U participated in PCA analysis and wrote the manuscript. JJ was involved in collecting materials and compiling data. CP was involved in collecting the references, samples, and extraction. LHN participated in GC-MS analysis, final review of manuscript and coordination. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

Supplementary data

Supplementary Fig. 1. Results of GC-MS analysis.

Supplementary Fig. 2. Determination results for each *Piper* species.

Supplementary Fig. 3. Secondary metabolite data that was submitted in MetaboAnalyst.

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