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Chemical Composition of the Essential Oil from Aerial Parts of Javanian *Pimpinella pruatjan* Molk. and Its Molecular Phylogeny

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Abstract: The species-rich and diverse genus *Pimpinella* is mainly distributed in Europe and Asia; a few species occur in Africa. Yet, the Javanian Pimpinella, *P. pruatjan*, which has been used as an aphrodisiac in Indonesian traditional medicine, was studied for the first time in the context of chemical composition, as well as phylogeny analysis and antimicrobial activity. We examined the chemical composition of the essential oil (EO) from aerial parts of *P. pruatjan* by gas liquid chromatography-mass spectrometry (GLC-MS). The main component of EO was (*Z*)-γ-bisabolene. Several oxygenated monoterpenes, oxygenated sesquiterpenes, and sesquiterpenes were also detected. The genetic relationship of *Pimpinella pruatjan* Molk. to other *Pimpinella* species was reconstructed using nucleotide sequences of the nuclear DNA marker ITS (Internal Transcribed Spacer). *P. pruatjan* clusters as a sister group to the African *Pimpinella* species. The EO did not exhibit an apparent antimicrobial activity.

Keywords: *Pimpinella pruatjan* Molk.; essential oil (EO); GLC-MS; ITS; phylogeny; antimicrobial

1. Introduction

Pimpinella is a member of the family Apiaceae and comprises approximately 200 species occurring in Europe, Asia, and some species in Africa [1–3]. *Pimpinella pruatjan* Molk. (formerly *Pimpinella alpina*) is an indigenous *Pimpinella* species to the Indonesian island Java [4,5]. The plant grows in restricted areas of the Dieng Plateau, Central Java [6,7], on the Pangrango mountain in West Java, and in the mountain area in East Java [8]. The root of *P. pruatjan* has been used as an aphrodisiac in Indonesia traditional medicine (Jamu). No medicinal uses have been reported from the aerial parts of this plant. In this study, we also examined the essential oils from the aerial part to investigate whether the oils possess antimicrobial activity as can be seen in other *Pimpinella* species.

The genus *Pimpinella* is known for its essential oils (EOs) [2,9–11]. The oils from *Pimpinella* species growing across Europe and Asia usually consist of monoterpenes (such as limonene, sabinene, α-pinene, β-pinene, γ-terpinene), norsesquiterpenes (geijerene, pregeijerene), sesquiterpenes

(such as himachalene, δ-elemene, β-bisabolone, caryophyllene, germacrene D), and a high content of phenylpropanoid derivatives with the 2-hydroxy-5-methoxy-1-(*E*)-propenylbenzene skeleton, known as pseudoisoeugenol [1–3,12–14]. The presence of pseudoisoeugenol is an important chemosystematic character in the genus of *Pimpinella* and most likely contributes to the antimicrobial activity of the EOs [2,15].

The EO from aerial parts of *P. pruatjan* has not been studied before nor its chemical composition and the phylogenetic position within the genus *Pimpinella*. We investigated the composition of the EO by GLC-MS and assessed its antimicrobial activity. The phylogeny of *P. pruatjan* was reconstructed using nucleotide sequences of the nuclear DNA marker *ITS* which is widely used in plants.

2. Experimental Section

2.1. The Plant Material and Isolation of the Oils

Plant material was collected from a mountain area in West Java, Indonesia, at an altitude of >1800 m above sea level, in April–May 2011. Botanical identification was carried out by Deden Girmansyah, M.S., at the Research Centre for Biology, Indonesian Institute of Sciences, Bogor, Indonesia. A voucher specimen was deposited at the Indonesian Spice and Medicinal Crops Research Institute, Indonesian Agency for Agricultural Research and Development (IAARD). For this study, the plant has been registered in the botanical collection of the Biology Department, Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, under the accession number P8259.

Dried aerial parts (1 kg) of *P. pruatjan* were crushed, followed by hydrodistillation for 3 h in a Clevenger-type apparatus to obtain the volatile oil. The EO was then dried over anhydrous sodium sulfate, evaporated in a Rotavapor, and stored in sealed vials at 4 °C for further analysis. The dried aerial parts contained about 0.23% of EO, calculated on dry weight base.

2.2. Gas Liquid Chromatography—Mass Spectrometry (GLC-MS)

Hexane solutions of the volatile oils were examined using GLC-MS on an HP5890 Series II gas chromatograph equipped with an DB-5 capillary column (30 m; 0.25 mm inner diameter; 0.25 μm film thickness) and used mass range and scan-time of mass spectra analysis. The injector was operated at a 250 °C in split mode (1:50) at a head pressure of 15 kPa of Helium. The temperature program started with an isothermal step at 40 °C for 2 min. The temperature was then raised to 300 °C at a rate of 4 °C/min and held for 10 min. A mixture of n-alkanes (C_8–C_{28}) was co-chromatographed with the samples to get the Kovats retention index (RI) of the separated compounds. The GLC was coupled to a quadrupole mass spectrometry SSQ 7000 (Thermo-Finnigan). The mass spectra were recorded at 70 eV at a source temperature of 175 °C, with the Xcalibur vers.1.3 software. The peaks were identified by comparing their mass spectra and retention indexes (RI) relative to C_8–C_{28} n-alkanes, the values of our internal library, NIST and literature data [2,16,17].

2.3. DNA Barcoding of P. pruatjan

DNA was isolated from aerial parts of *P. pruatjan* using the standard CTAB method with slight modification [18]. The ITS region was amplified with the primer pairs ITS forward (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS reverse (5′-TCCTCCGCTTATTGATATGC-3′). DNA amplification buffer contained 5 μL of 10× high PCR buffer, 1.5 mL of dNTP mixture containing 2.5 mM each nucleotide, 1 μL of each primer from 10 pmoL/mL stock, 0.5 μL of 10 mg/mL BSA, 0.2 μL of Top-TAQDNA Polymerase (2.0 U/mL, BIORON), 1–3 μL of template DNA, and sterile water was used to bring to total volume of 50 μL. The amplification was performed using a thermocycler (Tprofessional Basic Gradient, Biometra, Analytik Jena, Goettingen, Germany) under the following conditions: initial denaturation at 94 °C for 1 min, followed by 38 cycles of denaturation of double-stranded template DNA at 94 °C for 50 s, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. After 38 cycles, DNA amplifications were followed by a final extension at 72 °C for 10 min. Four μL of PCR product

were analyzed by electrophoresis using 1% TAE agarose gels. The remaining PCR product was purified for direct sequencing (by company StarSEQ, Mainz, Germany).

Data analysis and reconstruction of phylogenetic tree were carried out as described before [19]. The aligned ITS sequence from *P. pruatjan* was compared with *ITS* sequences from 39 *Pimpinella* species retrieved from National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The sequence matrix was manually aligned using BioEditV 708. Genetic distances were determined using MEGA 7.0 using the Kimura 2-Parameter (K2P) model.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [20]. The tree with the highest log likelihood (-3088.5028) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 6.4064)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 43.3441% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 40 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. There were a total of 599 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [21].

2.4. Antimicrobial Activity

2.4.1. Microorganisms

The antimicrobial activity of the EO was examined using Gram-positive bacteria, including *Bacillus subtilis* ATCC 6051, *Staphylococcus aureus* ATCC BAA-977, *Streptococcus pyogenes* ATCC 12344, *Staphylococcus epidermis* ATCC 14990, *Enterococcus faecalis* ATCC 51299 VRE Neg, vancomycin resistant *Enterococcus faecalis* ATCC 51299 VanB, and methicillin-resistant *Staphylococcus aureus* NTCC 10442; Gram-negative bacteria included *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Klebsiella pneumoniae* ATCC 700603; and yeast, including *Candida albicans* ATCC 90028, *Candida parapsilosis* ATCC 22019, and *Candida glabrata* ATCC MYA-2950. All of the microorganism cultures were supplied by Medical Microbiology Laboratory, Hygiene Institute, Heidelberg University, Heidelberg, Germany.

2.4.2. Culture Preparation and Cultivation of Microorganism

Bacterial cultures were sub-cultured in Columbia medium with 5% sheep blood (Becton Dickinson, Heidelberg, Germany), Mueller-Hinton broth (MHB) (Fluka), and brain heart infusion (BHI) (Merck, Heidelberg, Germany), and incubated at 37 °C for 24 h, with the supply of CO_2 well-maintained in cultivation of *Streptococcus pyogenes*. Fungal cultures were sub-cultured in CHROMagar *Candida* (Becton Dickinson, Heidelberg, Germany) and incubated at 25 °C for 48 h.

Bacterial or fungal colonies from 24 h or 48 h cultures were suspended to a 0.5 McFarland turbidity $\approx 1 \times 10^8$ colony forming unit per mL (CFU/mL). For the antimicrobial assay, the turbidity of bacterial or fungal solution was then adjusted to the concentration of 1×10^5 (CFU/mL).

2.4.3. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Microbicidal Concentration (MMC)

MIC was determined by the micro dilution method according to NCCLS (2006) [22]. Samples were diluted in DMSO to give a stock solution concentration of 200 mg/mL. Serial concentrations were made to obtain a final concentration of 10 to 8×10^{-2} mg/mL and placed in a 96-well plate (Greiner Bio-one, Frickenhausen, Germany). A suspension of bacterial or fungal cells with a concentration of 1×10^5 (CFU/mL) was then added and 96-well plates were incubated at 37 °C for 24 h for bacteria and at 25 °C for 48 h for yeast. MIC was defined as the lowest concentration that showed no visible turbidity. The observation was directly visualized without coloring reagent such as tetrazolium or

Alamar blue and directly compared to the negative growth control containing no bacteria and positive growth control containing bacteria. MMC was determined by inoculating 3 µL from each clear well and incubated at 37 °C for 24 h for bacteria and at 25 °C for 48 h for yeast. The MMC value was defined as the lowest concentration of samples that showed no growth of bacteria or yeast after incubation. Streptomycin and vancomycin (Applichem, purity > 93%) with the highest tested concentration of 50 µg/mL were used as a positive control against bacteria and nystatin (Cellpharm, purity > 90.6%, concentration 50 µg/mL) against yeast.

3. Results and Discussion

3.1. Relationships between P. pruatjan and Other Pimpinella Species

ITS sequences of *P. pruatjan* were used for DNA barcoding and to investigate the genetic relationship of *P. pruatjan* with other *Pimpinella* species distributed in Europe, Asia, and Africa. The phylogenetic analyses were based on a representative sampling of the genus *Pimpinella*, which includes 16 accessions from Africa and Madagascar and 26 from Eurasia. Another Apiaceae, *Foeniculum vulgare* served as an outgroup for the phylogeny reconstruction.

The phylogeny reconstruction reveals four major clades, supported with high bootstrap values (Figure 1). *P. pruatjan* clusters in a clade of Eurasian taxa as a sister group to the African samples with high bootstrap support (Figure 1). This finding could suggest that *P. pruatjan* might share phytochemical characteristics with the African *Pimpinella* species. European and Asian *Pimpinella* species have significant biological activities, such as antifungal [23], antiviral [24], and anticonvulsant effects in mice [25]. Unfortunately, no report could be found so far about the biological activity of African *Pimpinella* species.

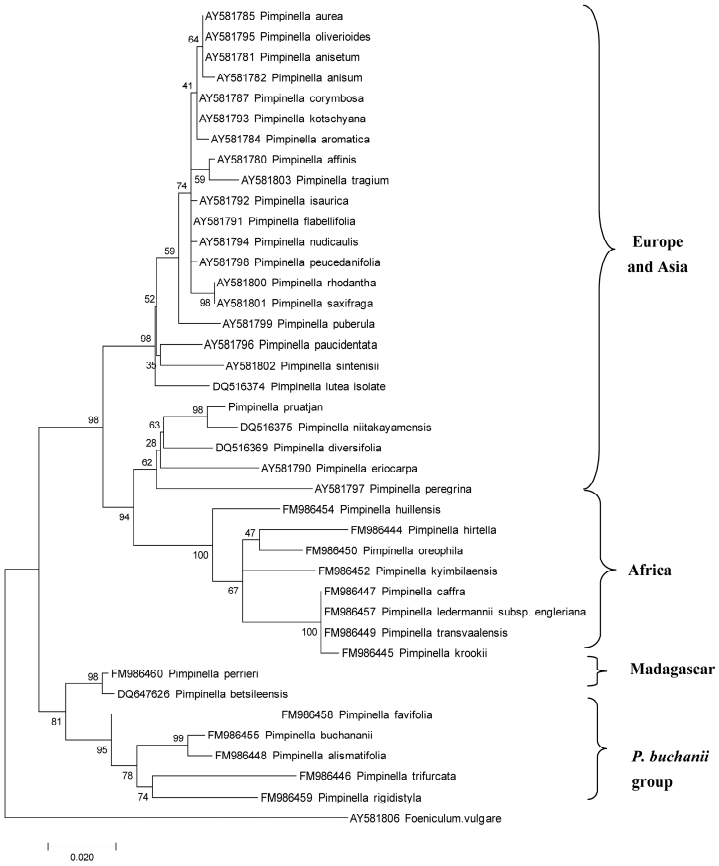

Figure 1. Molecular Phylogenetic analysis of *Pimpinella pruatjan* and the connection to the African species by Maximum Likelihood method. Numbers at the nodes are Bootstrap values from 500 replications.

3.2. Chemical Composition of the Essential Oil

Table 1 summarizes the components of the volatile oil from the aerial parts of *P. pruatjan*, their retention indices, and their relative abundances. The EO consists of oxygenated monoterpenes, sesquiterpenes, oxygenated sesquiterpenes, and arylpropanoids (derivatives of pseudoisoeugenol). Twenty secondary metabolites could not be identified; they could be primary or secondary metabolites. The main component was (*Z*)-γ-bisabolene (25.28%). Several oxygenated monoterpenes, such as linalool oxide, linalool, verbenol, borneol, p-cymen-8-ol, α-terpineol, trans-caren-2-ol, thymol, carvacrol, and *p*-vinyl guaiacol were detected at concentrations ranging from 0.1% to 1.2%. Three monoterpenes were detected, namely *p*-cymene, γ-terpinene, and p-cymenene. Sesquiterpenes, which are normally shared among the genus *Pimpinella*, were also discovered, such as α-cubebene, α-copaene, β-cubebene, β-elemene, and ar-curcumene. A number of oxygenated sesquiterpenes, such as dictamnol, caryophyllene oxide, humulene epoxide II, α-cadinol, and amorpha-4,9-dien-2-ol were also identified at concentrations ranging from 0.1% to 2.8%. The important chemosystematic characters in the genus *Pimpinella*, geijerene and the pseudoisoeugenol could also be detected albeit in low quantities (Table 1).

Table 1. Chemical composition of aerial part essential oils(Eos) from *P. pruatjan*.

No.	Identified Compounds	RI *	RI [15] and Ref.	Abundance (%) *	Identification
1	2-Hexanol	800	803 [26]	0.36	MS
2	3-Methylcyclopentanol	835	836 [27]	0.25	MS
3	Heptanal	891	902	0.12	MS, RI
4	Unidentified I	899		0.66	
5	Nonane (n)	902	900	3.70	MS, RI
6	Unidentified II	914		0.12	
7	2,2-Dimethylpentanal	920	826 [28]	4.53	MS
8	Unidentified III	936		0.21	
9	Unidentified IV	940		0.20	
10	Unidentified V	943		1.93	
11	Unidentified VI	955		2.76	
12	Isopropyl tiglate	968	976	6.92	MS, RI
13	Unidentified VII	994		0.38	
14	Unidentified VIII	997		0.50	
15	Octanal	1003	998	0.25	MS, RI
16	Unidentified IX	1005		0.24	
17	*p*-Cymene	1023	1024	0.60	MS, RI
18	Benzene acetaldehyde	1042	1042	0.33	MS, RI
19	Unidentified X	1043		0.23	
20	γ-Terpinene	1059	1059	0.44	MS, RI
21	trans-Linalool oxide	1072	1072	0.11	MS, RI
22	*p*-Cymenene	1089	1091	0.18	MS, RI
23	2-Nonanone	1093	1090	0.10	MS, RI
24	Linalool	1101	1096	0.20	MS, RI
25	Nonanal	1105	1100	0.12	MS, RI
26	Geijerene	1141	1143	Tr	MS, RI
27	*trans*-Verbenol	1149	1144	0.18	MS, RI
28	(*E*)-2-Nonenal	1160	1161	0.25	MS, RI
29	Borneol	1165	1169	0.12	MS, RI
30	2-Methoxy-3-(1-methylpropyl) pyrazine	1172	1172	Tr	MS, RI
31	Terpinen-4-ol	1176	1177	0.54	MS, RI
32	Octanoic acid	1179	1171	0.16	MS, RI
33	*p*-Cymen-8-ol	1184	1183	0.74	MS
34	α-Terpineol	1190	1188	0.14	MS, RI
35	Unidentified XI	1195		0.11	
36	*trans*-3-Caren-2-ol	1223		0.31	MS
37	2-Isopropenyl-5-methyl-1,4-benzenediol	1232		7.20	MS
38	Thymol methyl ether	1237	1235	7.80	MS, RI
39	Carvacrol methyl ether	1246	1244	0.13	MS, RI
40	Geraniol	1256	1252	0.21	MS, RI
41	Bornyl acetate	1289	1288	0.34	MS, RI
42	6-Hydroxy-m-anisaldehyde	1295		0.28	MS
43	Thymol	1298	1290	0.13	MS, RI
44	Carvacrol	1304	1299	0.37	MS, RI
45	4-Ethenyl-2-methoxyphenol	1315	1309	0.20	MS, RI
46	Unidentified XII	1343		0.14	
47	α-Cubebene	1353	1348	0.20	MS, RI
48	γ-Nonalactone	1363	1361	0.14	MS, RI
49	1-(4-Hydroxybenzylidine)acetone	1367		0.12	MS
50	α-Copaene	1379	1376	0.56	MS, RI

Table 1. *Cont.*

No.	Identified Compounds	RI *	RI [15] and Ref.	Abundance (%) *	Identification
51	8-epi-Dictamnol	1382	1380	0.53	MS, RI
52	β-Cubebene	1387	1388	0.58	MS, RI
53	β-Elemene	1394	1390	0.40	MS, RI
54	Methyl eugenol	1405	1403	0.52	MS, RI
55	α-Cedrene	1418	1411	0.22	MS, RI
56	(*E*)-Caryophyllene	1423	1419	1.12	MS, RI
57	2,5-Dimethoxy-p-cymene	1427	1426	4.39	MS, RI
58	Dictamnol	1432	1429	1.92	MS, RI
59	6,9-Guaiadiene	1440	1444	0.16	MS, RI
60	α-Himachalene	1448	1451	Tr	MS, RI
61	allo-Aromadendrene	1454	1460	Tr	MS, RI
62	α-Humulene	1458	1454	0.26	MS, RI
63	Sesquisabinene	1461	1459	0.22	MS, RI
64	cis-Cadina-1(6)-4-diene	1466	1463	2.86	MS, RI
65	Unidentified XIII	1472		0.14	
66	γ-Muurolene	1482	1479	0.26	MS, RI
67	ar-Curcumene	1488	1480	2.96	MS, RI
68	(*E*)-Methyl isoeugenol	1492	1492	0.38	MS, RI
69	Viridiflorene	1501	1496	0.36	MS, RI
70	*trans*-β-Guaiene	1505	1502	0.47	MS, RI
71	*cis*-γ-Bisabolene	1518	1515	25.28	MS, RI
72	δ-Cadinene	1530	1523	1.10	MS, RI
73	Spathulenol	1582	1578	0.13	MS, RI
74	Caryophyllene oxide	1589	1583	2.80	MS, RI
75	Unidentified XIV	1611		0.23	
76	Humulene epoxide II	1614	1608	0.96	MS, RI
77	Unidentified XV	1625		0.51	
78	3-Thujopsanone	1651	1654	0.14	MS, RI
79	α-Cadinol	1660	1654	0.54	MS, RI
80	Unidentified XVI	1674		0.22	
81	14-Hydroxy-9-epi-(*E*)-caryophyllene	1677	1669	0.24	MS, RI
82	Unidentified XVII	1682		0.12	
83	Amorpha-4,9-dien-2-ol	1692	1700	0.46	MS, RI
84	Unidentified XVIII	1714		0.10	
85	Unidentified XIX	1728		0.13	
86	Unidentified XX	1732		0.11	
87	*trans*-Pseudoisoeugenol isobutyrate	1747	1742	0.13	MS, RI
88	Epoxypseudoisoeugenyl isobutyrate	1797	1793	0.18	MS, RI
89	*trans*-Pseudoisoeugenyl-2-metylbutyrate	1842	1842	1.38	MS, RI
90	Epoxypseudoisoeugenyl 2-methylbutyrate	1901	1895	0.14	MS, RI
91	Hexadecanoic acid	1963	1960	0.35	MS, RI
92	*cis*-Falcarinol	2038	2036	0.51	MS, RI
93	Nezukol	2141	2133	0.18	MS, RI

Abbreviations: RI (retention indices); MS (mass spectrometry); Tr (traces); RI *: measured linear retention indices on a capillary column with an active phase DB-5. * total peak area was set 100%.

There are important differences in main constituents of the EO among the members of the genus *Pimpinella*. For instance, *P. aurea* DC, growing in Iran, contains limonene in the EO of aerial parts, epoxy pseudoisoeugenyl-2-methylbutyrate in the EO of roots, and β-bisabolone in the EO of fruits [2,3,29]. Trans-anethole is the main component in EO of *P. anisum* and *P. anisetum*, but it cannot be found in other *Pimpinella* species, such as in *P. peregrina* L., *P. tragium* Vill ssp. *polyclada* (Boiss. & Heldr.) Tutin, and *P. affinis* Ledeb [16]. (*Z*)-γ-Bisabolene, the main constituent in the aerial part EO of *P. pruatjan*, has not been found as a major compound in other EO of *Pimpinella*.

We detected a C_{17}-polyacetylene, falcarinol (Figure 2) in the EO of *P. pruatjan* for the first time in the genus *Pimpinella*. Other polyacetylenes have been reported to be present in some *Pimpinella* species, such as those of pentadeca-2,4,6,8-tetraene, 2,8-decadiene-4,6-diene-1-al, 2,8-tridecadiene-4,6-diene-10-ol in *P. major* L., 2,8,10-tridecatriene-4,6-diene in *P. major* L. and *P. anisum* L., 2-tridecaene-4,6-diene-8-ol-10-on in *P. major* L. and *P. saxifraga* L. [30]. The pathway leading to polyacetylene is common to this genus and the occurrence of falcarinol is perhaps due to the modification of the polyacetylene biosynthesis or species specific of their enzymatic machinery.

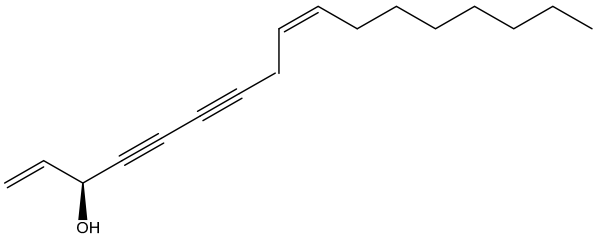

Figure 2. Falcarinol.

3.3. Antimicrobial Activity

The antimicrobial activity of the EO was examined against three *Candida* species and some pathogenic bacteria, including those with multidrug-resistance as representative for pathogenic bacteria causing human infection diseases. The EO exhibited no antibacterial or antifungal activity (at concentrations below 0.5 mg/mL) against pathogenic bacteria, including multidrug resistant bacteria, MRSA and VRE. The known high and moderate antimicrobial activities in the genus *Pimpinella* is mainly due to the presence of epoxypseudoisoeugenol and their derivatives; in particular, species which contain high epoxypseudoisoeugenol concentrations showed high antimicrobial activity [2,16]. These secondary metabolites carry epoxide groups which are powerful electrophiles. They can react with nucleophilic groups (e.g., NH_2, NH, and SH groups of amino acid) in proteins and thus affect the biological system in microorganisms [2,31,32]. In the EO of *P. pruatjan* epoxypseudoisoeugenols are present in low amounts which would explain the absence of antibacterial activity as compared to some other *Pimpinella* species [2,16]. Polyacetylene, including falcarinol showed profound antimicrobial activity against several bacteria, such as *Staphylococcus aureus*, *S. epidermidis*, *Micrococcus luteus* and *Enterococcus faecalis* and multidrug resistant *Staphylococcus aureus* [33,34]. The presence of falcarinol in *P. pruatjan* in a low amount barely affects the antimicrobial activity.

4. Conclusions

In conclusion, this is the first study suggesting that, differing from most Eurasian species, Javanian species possess (*Z*)-γ-bisabolene as the main component in the essential oil and the presence of known falcarinol, which is not common in the Eurasian species. Javanian *Pimpinella* cluster as a sister to the African species together with a few Eurasian species. Further investigations of the phytochemistry pharmacology of African *Pimpinella* are needed to understand the evolutionary background of *Pimpinella* species across the world.

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Conflicts of Interest: The authors declare no conflict of interest.

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