Metabolite Profiles of Agarwood *Gyrinops versteegii* (Gilg) Domke Leaves collected from Different Locations

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Abstract
The leaves of Gyrinops versteegii, an agarwood-producing plant, are reported to have many benefits, including antioxidant, anti-malaria and an anti-proliferation activity against several cancer cell lines. The plants originate from the Eastern part of Indonesia and are now cultivated for resin production in several parts of Indonesia. Secondary metabolites are known to be highly influenced by environmental factors. This study was conducted to evaluate the effect of different planting locations on the leave metabolite profiles of G. versteegii. Leaf samples were collected from two different locations, Bogor Botanical Gardens, West Java and Research and Development Institute of Technology Non-Timber Forest Product Mataram, Lombok Island. Samples were extracted by maceration overnight with non-polar to polar solvents of chloroform, ethyl acetate and ethanol at a ratio of 1:13 (w/v). The extracts were air-dried and then analyzed using GC-MS. The chromatograms were further processed for confirmation of the metabolites content. The metabolite profiles were compared using multivariate analysis in SIMCA P software and significant differences were confirmed with a t-test. The Bogor sample's metabolite profile was different from the Mataram sample, specifically fifteen compounds including fatty acids, monoterpenes, sesquiterpenes and triterpenes.

This difference may be due to the different soil composition between the two locations. The soil in Bogor had higher concentrations of K and Mn, but lower N and Ca compared to the Mataram soil. The different location was confirmed by the metabolite profiles of agarwood leaves and the G. versteegii leaves from Bogor are different from those from Mataram.

Keywords: *Gyrinops versteegii*, secondary metabolites, chemometrics, SIMCA P, GC-MS.

Introduction

Indonesia is a country with abundant natural resources because of its tropical climate[6] and has many tropical rainforests. The tropical rainforest generates resources such as wood and non-wood products. One example of a non-wood product from Indonesian tropical rainforests is agarwood[7]. Agarwood is widely used as traditional medicine, especially in traditional Chinese medicine (TCM) and traditional Indian medicine (TIM). In TCM, agarwood is utilized as cold medicine for stomachaches, vomiting, hiccups, chest and abdominal pain, diarrhea, hypertension and fatigue. In contrast, the agarwood in TIM is believed to be a sedative, cardiotonic (strengthening heart performance), anti-inflammatory, anti-leprosy, depurative, carminative and detoxifying[24].

Gyrinops versteegii is an agarwood-producing tree identified in 1932 in Indonesia[19] and is especially abundant in the Eastern part of Indonesia. *Gyrinops versteegii* has many benefits including bioactivity as an antioxidant[19,22], an immunomodulator, antiviral against dengue serotype three virus[18] and cytotoxic activity against cancer cell lines[19]. *G. versteegii*'s leaves are used as herbal tea. People consume herbal tea for supporting health and wellness, attributed to the presence of secondary metabolites in leaves[24]. The metabolites profiles of *G. versteegii* leaves have been reported using GC-MS[31].

However, environmental factors influence the content and composition of secondary metabolites in the area where the plants grow. A previous study in our group showed that *G. versteegii* leaves from Bogor have antioxidant activity and cytotoxicity to HeLa cell lines[19]. Due to the high commercial value of agarwood's resin, *G. versteegii* is currently cultivated in many places across Indonesia. Hence, agarwood research is of the interest of the Research and Development Institute of Technology Non-Timber Forest Product Mataram, West Nusa Tenggara.

The different environments of Bogor, West Java and West Nusa Tenggara might be attributed to by the differences in elevation and that Bogor is far from the beach while Mataram is near. Bogor has an average temperature around 20 – 30 ºC, a humidity of 70 % and average rainfall 3.500 – 4000 mm/year[23], but Mataram has an average temperature around 23 – 32 ºC, with humidity around 80 – 88 % and average rainfall around 2.843 mm/year[5].

In this research, we compared the metabolite profile of *G. versteegii* leaves from Bogor to those from Mataram. We also analyzed the soil composition of the two locations to understand the difference.

Materials and Methods

The materials used in this study were *G. versteegii* leaves. The *G. versteegii* leaves were collected from the Bogor Botanical Gardens, West Java and the Research and Development Institute of Technology Non-Timber Forest

Product Mataram, West Nusa Tenggara. Only physiologically matured leaves were used in this study. Solvents for extraction were chloroform (Merck), ethyl acetate (Merck) and ethanol (Merck). All solutions were in p.a. grade.

Preparation and extraction of *Gyrinops versteegii* leaves: Fresh leaves were cleaned using tap water to remove dirt and then air-dried to remove the water completely. The leave samples were weighed and then dried at room temperature for several days. The leaves were then placed in a 40 °C oven until the sample reached a constant weight. The dried samples were weighed, then pulverized into a powder with an electric blender.

Extraction of *Gyrinops versteegii* leaves: The extraction of agarwood leaves was carried out by the maceration method with chloroform, ethyl acetate and ethanol. An amount of 5 g of a dried sample was added to 65 mL of solvent in a beaker glass for a ratio of 1:13 (w/v). The maceration process was conducted for 24 h by mixing regularly. At the end of the extraction process, the mixtures were filtered and the solvent was collected. The solvent was dried until it reached a constant weight and was then transferred to a 10 mL falcon tube coated with aluminum foil. Extracts were stored in the fridge for the next experiment.

Metabolite profiles analysis using GC-MS: Analysis of the metabolite profiles was carried out using GC-MS at the Organic Chemistry Laboratory of the Faculty of Mathematics and Natural Sciences, Universitas Gadjah Mada, Indonesia. Each extract was dissolved in the respective solvent and analyzed using GC-MS (Shimadzu GC 2010). The GC-MS condition was as follows: temperature of 70 – 300 °C with 5 °C increment every 1 min with a total of running 80 min. The GC columns were HP 5 or Rtx 5 MS semipolar type with 30 m length, 0.25mm diameter and 0.25 µm thickness. Helium gas was the carrier gas with a flow rate of 28 $mL \cdot min^{-1}$. An average of 3 µL of the sample was injected in the split-less mode. The GC-MS detector was a 70 electron volt ionization electron detector (EI 70 Ev). Each sample was analyzed in triplicate using GC-MS.

Soil element analysis: Soil was collected from the habitat area of the agarwood, the Bogor Botanical Gardens, West Java and Mataram, West Nusa Tenggara. Both soils were analyzed for the mineral content, especially for nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), sodium (Na), magnesium (Mg), iron (Fe), manganese (Mn), copper (Cu) and zinc (Zn). The soil analysis was conducted using Atomic Absorption Spectrometer (AAS) at the Soil Science Laboratory, Faculty of Agriculture, Universitas Gadjah Mada, Indonesia.

Data analysis: The chromatogram obtained from GC-MS was cleaned to remove possible signals contaminants. The chemical structure analysis and metabolite functions were confirmed through cross-validation in the PubChem database (https://pubchem.ncbi.nlm.nih.gov). A comparison of the two locations' metabolite profile was performed using multivariate data analysis of SIMCA P, version 14. The possible metabolites corresponding to the difference were analyzed using a t-test to identify statistical significance between the two locations.

Results and Discussion

The extraction process follows the like-dissolve-like principal. Each solvent has a different capability to extract metabolite compounds according to their polarity. Different mass of extracts was observed among different solvents and different location. The ethanol extract of Mataram has the highest extract mass followed by Bogor's ethanol extract. The lowest extract mass was observed on the ethyl acetate extract of Mataram (Table 1). This may indicate the differences in profile metabolites between *G. versteegii* leaves from Mataram and Bogor. The high semipolar metabolites content of G. *versteegii* leaves was also reported by Fadhilah[10].

In general, the groups of compounds extracted using ethanol are tannins, polyphenols, polyacetylenes, flavonols, terpenoids, sterols and alkaloids[30] while compounds extracted using chloroform are terpenoids, flavonoids[30], phenolic compounds and tannins. The phytochemicals extracted using ethyl acetate are alkaloids, steroids, flavonoids, saponins, tannins[8] and phenolic[32].

Table 1
The results of maceration extraction from gaharu leaves *Gyrinops versteegii*

Region	Solvent	Weight (g)		Yield
		Dried sample	Extract mass	(%)
Mataram	Ethanol	5	0.48	9.56
	Chloroform	5	0.27	5.47
	Ethyl acetate	5	0.28	5.62
Bogor	Ethanol	5	0.38	7.60
	Chloroform	5	0.27	5.30
	Ethyl acetate	5	0.18	3.52

The ethanol extract of *G. versteegii* leaves from Bogor contains 19 compounds. The chloroform extract contains 15 compounds, while the ethyl acetate extract contained 9 compounds (Table 2). The ethanol extract contains more compounds from fatty acid and sesquiterpene. The chloroform and ethyl acetate extracts contain more compounds from the fatty acids and alkanes.

There are three compounds observed in all three extracts of *G. versteegii* leaves from Bogor including isopropyl myristate, methyl stearate and squalene (Figure 1).

Isopropyl myristate is a fatty acid ester compound that has antimicrobial activity[13]. Methyl stearate is a fatty acid methyl ester compound that acts as a metabolite, has activities as an anti-inflammatory, lipid metabolism regulator, gastrin inhibitor, anthelmintic and antinociceptive[1]. Squalene is a terpenoid compound and acts as a precursor in cholesterol biosynthesis[25]. Squalene has anticancer, antioxidant and detoxifier activity[15].

The ethanol extract of the Bogor sample has two specific compounds, neophytadiene and phytol. Neophytadiene is a sesquiterpene compound that has analgesic, antipyretic, anti-inflammatory, antimicrobial and antioxidant activity[28]. Phytol is generally used as a precursor in vitamin E biosynthesis and has anti-inflammatory, antimicrobial and antitumor activity[16]. The specific compound in the chloroform extract is 9-eicosyne, which has antimicrobial and cytotoxic activity[17]. The ethyl acetate extract did not have a particular compound but has a high level of 1-octadecyne and squalene (Figure 1). 1-Octadecyne is a long chain hydrocarbon compound with antimicrobial, antioxidant and anticancer activity[3].

The ethanol extract of the *G. versteegii* leaves from Mataram contained 16 compounds. The chloroform extract detected 15 compounds and the ethyl acetate extract contained 19 compounds (Table 3). This study observed that ethanol and chloroform extracts contain more compounds from fatty acids and alkane. In comparison, the ethyl acetate extract contains more compounds from sesquiterpene and alkane.

Table 2
Compounds in ethanol. chloroform and ethyl acetate extract of *Gyrinops versteegii* leaves from Bogor by GC-MS

S.N.	Name of Compound	Class of Compound	Area (%)		
			Ethanol	Chloroform	Ethyl acetate
1	1-Octadecyne	Alkane	—	18.55	20.97
2	Dotriacontane	Alkane	—	0.82	—
3	Heneicosane	Alkane	1.22	—	—
4	Heptacosane	Alkane	—	5.56	11.16
5	Heptadecane	Alkane	—	0.58	0.53
6	Hexadecane	Alkane	1.73	0.47	—
7	Hexatriacontane	Alkane	—	10.41	9.93
8	Nonacosane	Alkane	1.75	—	—
9	9-Eicosyne	Alkyne	—	3.00	—
10	Phytol	Diterpenoid	8.51	—	—
11	Dodecanoic acid	Fatty acid	2.59	—	—
12	Ethyl linoleate	Fatty acid	1.15	—	—
13	Ethyl myristate	Fatty acid	6.30	0.95	—
14	Ethyl pentadecanoate	Fatty acid	14.16	—	—
15	Ethyl stearate	Fatty acid	—	1.11	—
16	Isopropyl myristate	Fatty acid	2.07	5.99	7.62
17	Methyl oleate	Fatty acid	1.80	6.57	—
18	Methyl palmitate	Fatty acid	0.76	11.52	—
19	Methyl ricinoleate	Fatty acid	3.35	—	—
20	Methyl stearate	Fatty acid	0.76	0.60	4.63
21	Methyl vaccenate	Fatty acid	—	5.82	6.93
22	Octadecanal	Fatty aldehyde	0.25	—	—
23	alpha.-Springene	Sesquiterpene	1.55	—	0.88
24	Hexahydrofarnesyl acetone	Sesquiterpene	1.68	—	—
25	Neophytadiene	Sesquiterpene	23.24	—	—
26	Patchouli alcohol	Sesquiterpene	6.75	—	—
27	Squalene	Triterpenoid	13.18	26.94	37.34
— symbol means that compound wasn't detected in the extract					

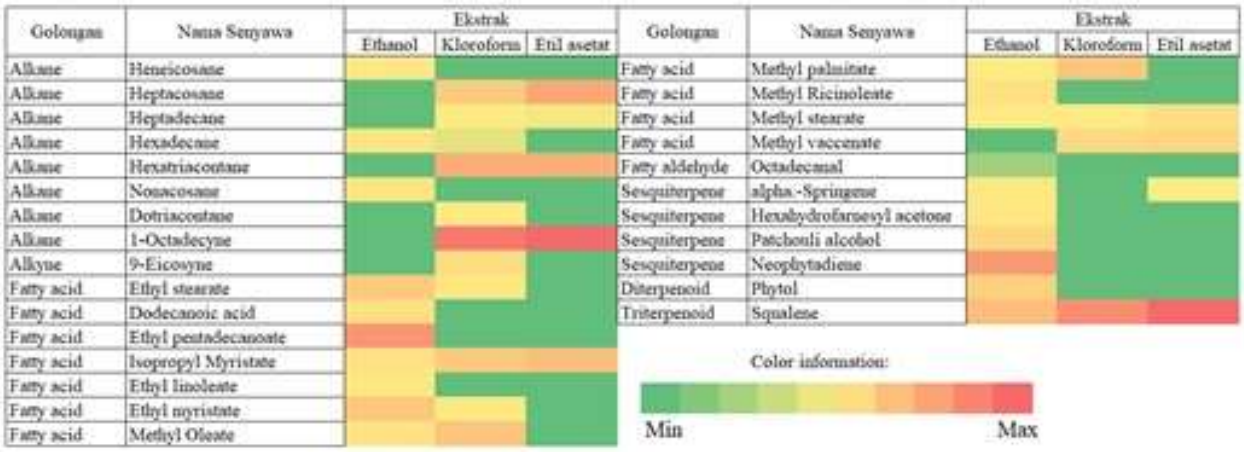


Figure 1: Heatmap of *Gyrinops versteegii* leaves from Bogor.

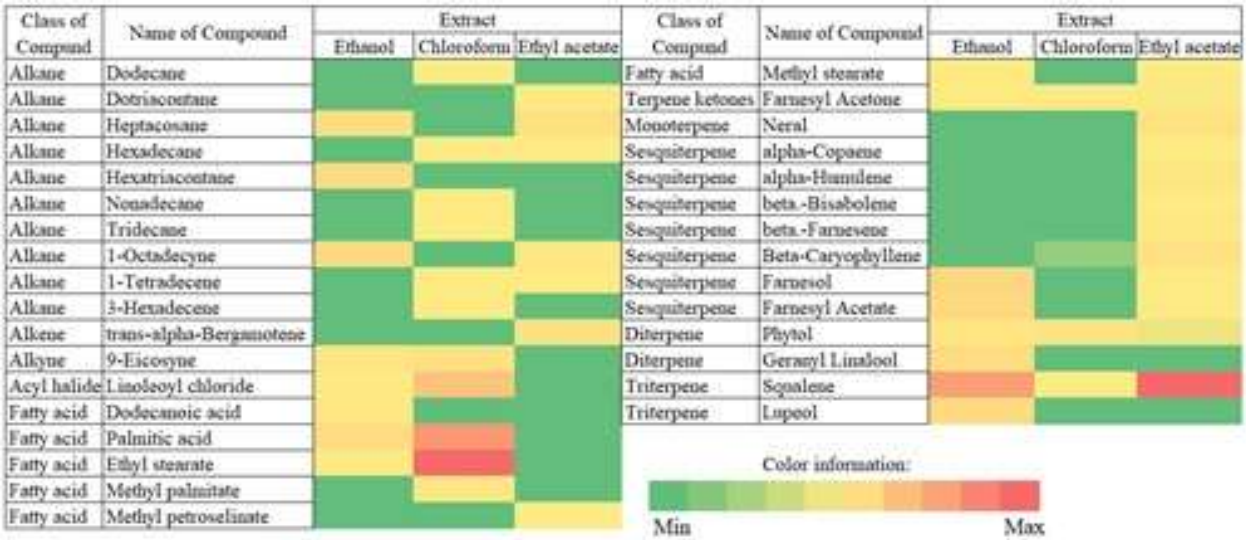


Figure 2: Heatmap of *Gyrinops versteegii* leaves from Mataram

There were three compounds found in all three solvents of the extract of *G. versteegii* leaves from Mataram; farnesyl acetone, phytol and squalene (Figure 2). Farnesyl acetone is a terpene ketone compound that is a hormone and metabolite. The ethanol extract contained two specific compounds, geranyl linalool and lupeol. Lupeol is a pentacyclic triterpene with bioactivities as an anticancer and chemopreventive agent[26].

Linalool is a monoterpene commonly found in plants that has activity as an anticancer and anti-inflammatory agent[20]. The specific compound in the chloroform extract is methyl palmitate. The ethyl acetate extract has a unique pattern because it has more compound that is otherwise absent in the two other solvents (Figure 2).

The ethyl acetate extract contains neral and alpha-humulene. Neral has cytotoxic activity against cancer cells, specifically HepG2 and HeLa and is not typically toxic to normal cells[4]. Alpha-humulene is a monocyclic sesquiterpene compound. Alpha-humulene and beta-caryophyllene have anticancer activity against MCF-7 (breast cancer) and DLD-1 (colon cancer)[2].

The GC-MS data were then analyzed with multivariate analysis (MVA) to differentiate between the leaves derived from Bogor and Mataram. The scatter plot of partial least squares discriminant analysis (PLS-DA) was colored by location and solvents. The scatter plots colored based on differences in planting locations, leaves extracts from Mataram clearly separated from the Bogor samples by the PC1 (Figure 3A). The R2 and Q2 values indicate a good model of PLS-DA. R2 is used to show how good the model is, while Q2 indicates the model's predictability or predictive power. The Q2 value was a good model if it was higher than 0.521. The R2 and Q2 values of this model were 0.89 and 0.81 respectively.

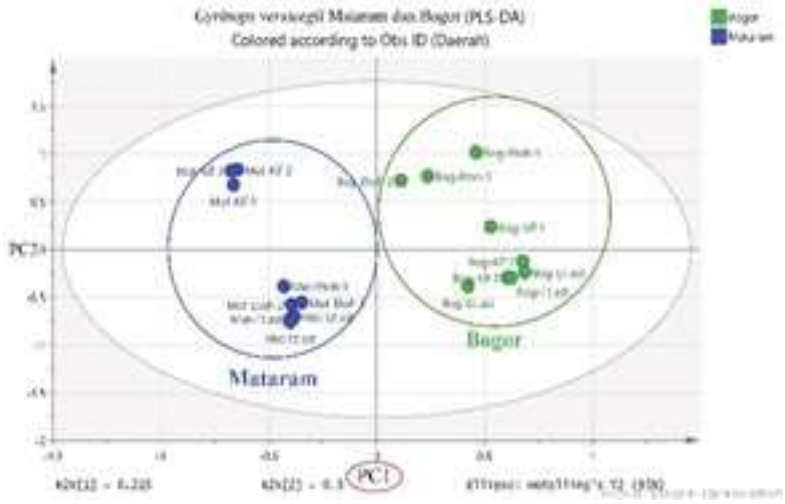
Therefore, the model predicted data well. Loading plots served to determine the metabolites responsible for separation among Mataram and Bogor samples (Figure 3B). Fifteen compounds were detected different between Mataram and Bogor. These include trans-α-were, neral, lupeol, isopropyl myristate, heptacosane, geranyl linalool, farnesyl acetone andfarnesyl acetate, farnesol, beta-caryophyllene, beta-farnesene, beta-bisabolene, alpha-humulene, alpha-copaene and 1-octadecyne (Figure 4).

The metabolite profile of *G. versteegii* from Bogor was characterized by a high level of several compounds, 1-octadecyne, heptacosane and isopropyl myristate. This profile differed from Mataram's metabolite profile with more diverse compounds that distributed almost in comparable concentration. This significant difference in metabolite profile can be caused by differences in the biotic environment in the growing area of *G. versteegii* itself. The *G. versteegii* Mataram grows with other agarwood plants (monoculture), whereas in Bogor, *G. versteegii* grows with other plants (polyculture). Thus, allelopathic interactions may occur.

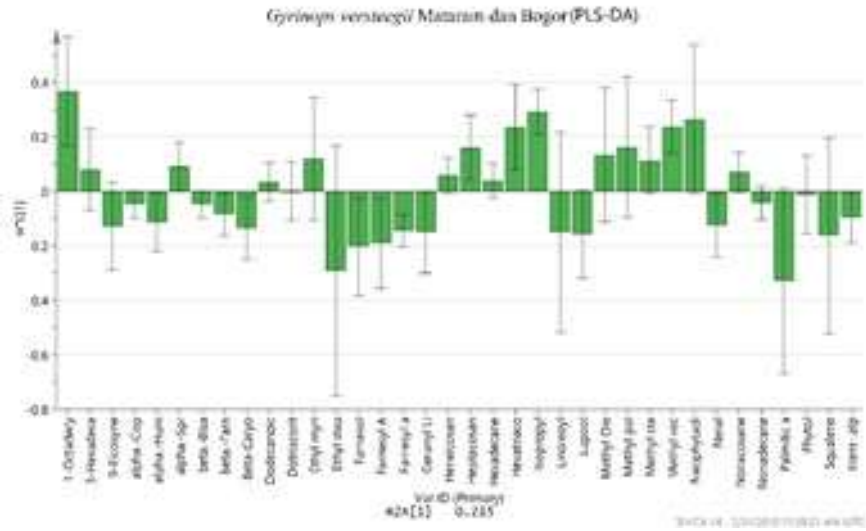
There are four soil element differences between Mataram and Bogor (Table 4): Ca, K, N and Mn. Calcium plays a role in plant metabolic pathways, especially in the phenylpropanoid pathway, which plays a role in synthesizing phenolic compounds such as lignin. At high concentrations, calcium directly affects the metabolism of the phenolic compounds, where excess calcium will reduce the activity of phenylalanine ammonia-lyase (PAL), which is an enzyme to synthesize phenolic compounds. Declining levels of phenolic compounds can indicate decreased activity of PAL enzymes on the phenylpropanoid pathway caused by Ca.[29]

Table 3
Compounds in ethanol, chloroform and ethyl acetate extract of *Gyrinops versteegii* leaves from Mataram by GC-MS

S.N.	Name of Compound	Class of Compound	Area (%)		
			Ethanol	Chloroform	Ethyl acetate
1	1-Octadecyne	Alkane	5.24	—	1.02
2	1-Tetradecene	Alkane	—	0.19	0.41
3	3-Hexadecene	Alkane	—	0.15	—
4	Dodecane	Alkane	—	0.17	—
5	Dotriacontane	Alkane	—	—	0.84
6	Heptacosane	Alkane	4.05	—	3.36
7	Hexadecane	Alkane	—	0.82	0.41
8	Hexatriacontane	Alkane	4.94	—	—
9	Nonadecane	Alkane	—	0.65	—
10	Tridecane	Alkane	—	0.59	—
11	trans-.alpha.-Bergamotene	Alkene	—	—	3.10
12	9-Eicosyne	Alkyne	1.76	3.53	—
13	Linoleoyl chloride	Asil halida	0.64	12.16	—
14	Geranyl Linalool	Diterpenoid	7.28	—	—
15	Phytol	Diterpenoid	3.42	5.16	0.64
16	Dodecanoic acid	Fatty acid	1.45	—	—
17	Ethyl stearate	Fatty acid	0.82	39.67	—
18	Methyl palmitate	Fatty acid	—	1.34	—
19	Methyl petroselinate	Fatty acid	—	—	0.48
20	Methyl stearate	Fatty acid	0.73	—	1.05
21	Palmitic acid	Fatty acid	4.68	25.58	—
22	Neral	Monoterpenoid	—	—	5.03
23	alpha.-Copaene	Sesquiterpene	—	—	0.81
24	alpha.-Humulene	Sesquiterpene	—	—	4.32
25	beta.-Bisabolene	Sesquiterpene	—	—	0.79
26	beta.-Farnesene	Sesquiterpene	—	—	2.32
27	Beta-Caryophyllene	Sesquiterpene	—	0.24	5.38
28	Farnesol	Sesquiterpene	10.10	—	1.46
29	Farnesyl acetate	Sesquiterpene	8.67	—	1.35
30	Farnesyl acetone	Terpene ketones	1.30	1.70	0.70
31	Lupeol	Triterpenoid	8.21	—	—
32	Squalene	Triterpenoid	36.18	1.16	61.33
— symbol means that compound wasn't detected in the extract					



(a)



(b)

Figure 3: Partial Least Square Discriminant Analysis of leaves extract of *Gyrinops versteegii* collected from Bogor and Mataram. A. Scatter plot colored base on region. B Loading plot of *Gyrinops versteegii* Compounds between Mataram and Bogor

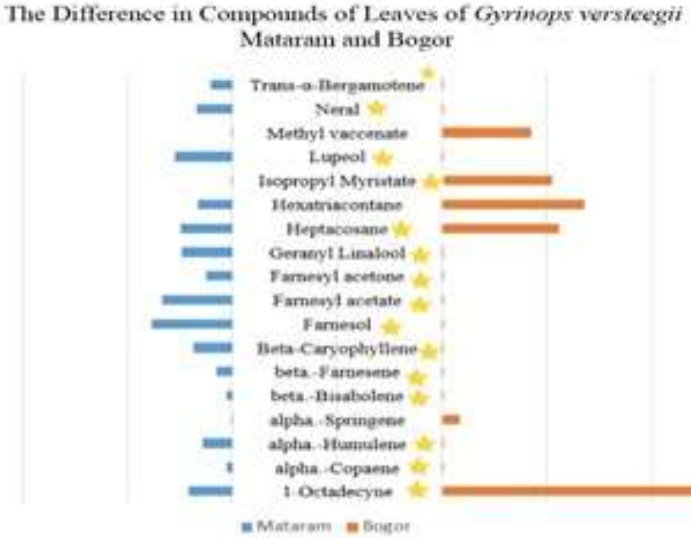


Figure 4: The Compounds Difference of *Gyrinops versteegii* Bogor and Mataram ★ indicating the significant difference between Mataram and Bogor on that compound based on Independent Sample T-Test with alpha= 0.05.

Table 4
Result of soil elements analysis

Daerah	N	P	K	Ca	Na	Mg	Fe	Mn	Cu	Zn
	Total	**Total**	**Total**	**Total**	**Total**	**Total**	**Total**	**Total**	**Total**	**Total**
	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
Bogor	**0.42**	0.05	**0.44**	**0.02**	0.08	0.05	0.74	**0.09**	0.01	0.01
Mataram	**0.60**	0.06	**0.19**	**0.14**	0.11	0.06	0.74	**0.03**	0.01	0.01
* Bold numbers indicates a significant difference between soil elements in Mataram and Bogor analyzed using t-test $p<0.05$										

Nitrogen plays a role in the metabolic synthesis of phenolic compounds through the phenylpropanoid pathway. The lack of nitrogen causes a decrease in the PAL activity, thereby reducing the phenolic compounds' levels in plants[12]. This is different from the Ca, where the excess decreases PAL activity, while N plays a role in increasing PAL activity. High PAL activity will cause higher levels of phenolic compounds.

Potassium (K) plays a role in protein synthesis, glycolytic enzymes, photosynthesis, coenzyme and inactivating the enzyme in the metabolic pathway. According to Ibrahim et al[14], there was an increase in the total production of phenolic compounds and flavonoids with an increase in K levels because it stimulates photosynthesis, where the results of the photosynthesis in the form of carbohydrates can be used as precursors to form flavonoids and phenolics. In addition to its role in stimulating photosynthesis, K also acts as a cofactor of the PAL enzyme, so that with more K, the PAL activity will increase and will increase the biosynthesis of secondary metabolites, mainly phenolic and flavonoids compounds.

Manganese (Mn) is an element that functions in the synthesis of chlorophyll, photosynthesis, electron transport and the synthesis of riboflavin, ascorbic acid and carotenoids. Manganese plays a direct role in the biosynthetic pathway of shikimic acid and phenylpropanoid so that Mn can control the biosynthesis of lignin and suberin[9]. Based on Ghannadnia et al[11], the addition of Mn can increase levels of the terpenoid compounds in white cumin. Biosynthesis of secondary metabolites in plants is influenced by many factors, one of which is nutrients. Manganese plays an essential role in plant metabolism, including the synthesis of chlorophyll, photosynthesis and acting as a cofactor for several enzymes of monoterpene synthase[11] such that increased levels of Mn can cause an increase in terpenoid compounds in a plant.

Conclusion

The metabolite profile of *Gyrinops versteegii* from Mataram and Bogor was significantly different. There were fifteen compounds responsible for these differences: trans-α-bergamotene, neral, lupeol, isopropyl myristate, heptacosane, geranyl linalool, farnesyl acetaone, farnesyl acetate farnesol, beta-caryophyllene, beta-farnesene, beta-bisabolene, alpha-humulene, alpha-copaene and1-octadecyne.

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