Indonesian Journal of Applied Research
IJAR

CLUSTERIZATION OF RED BETEL LEAVES (*Piper crocatum*) FROM VARIOUS REGIONS IN INDONESIA BASED ON SECONDARY METABOLITE FINGERPRINT ANALYSIS AND CYTOTOXICITY VALUES

Eka Wahyu Purnama[1], Mega Safithri[1,2], Dimas Andrianto[1]

[1]Department of Biochemistry, Faculty of Mathematics and Natural Science, IPB University
[2]Tropical Biopharmaca Research Center, LPPM IPB University, Indonesia

Volume 4 Issue 2
(August 2023)
e-ISSN 2722-6395
doi: 10.30997/ijar.v4i2.293

ARTICLE INFO

Article history:
Received: 08-02-2023
Revised version received: 18-02-2023
Accepted: 31-07-2023
Available online: 10-08-2023

Keywords:
LC-MS/MS; LC_{50}; red betel

How to Cite:
Purnama, E. W., Safithri, M., & Andrianto, D. (2023). CLUSTERIZATION OF RED BETEL LEAVES (Piper crocatum) FROM VARIOUS REGIONS IN INDONESIA BASED ON SECONDARY METABOLITE FINGERPRINT ANALYSIS AND CYTOTOXICITY VALUES. *Indonesian Journal of Applied Research (IJAR)*, *4*(2), 170-182. https://doi.org/10.30997/ijar.v4i2.293

Corresponding Author:
Mega Safithri
safithri@apps.ipb.ac.id

ABSTRACT

The quality of the ethanol extract of red betel leaves as a medicinal plant is determined by secondary metabolites, which are influenced by geographical conditions and the plant growing environment. This study aims to classify red betel from various regions in Indonesia based on secondary metabolite fingerprint analysis and cytotoxicity values. Observation of the diversity of secondary metabolites of the ethanol extract of red betel leaves from seven different regions (Banda Aceh, Bandung, Bogor, Malang, Samarinda, Kendari, Jayapura) was carried out using a metabolomics approach using liquid chromatography-mass spectroscopy (LC-MS/MS) and determining cytotoxicity value using the Brine Shrimp Lethality Test (BSLT) method. Secondary metabolite fingerprinting analysis using cluster analysis with dendrogram yielded 12 compounds with 3 sample groups based on their region of origin, namely group 1 (Banda Aceh, Samarinda, Jayapura); group 2 (Bandung, Kendari, Malang); group 3 (Bogor). Group 1 samples identified eight compounds that had the highest relative abundance values. Group 2 samples identified 3 compounds that have the highest relative abundance values. Group 3 samples had 1 compound with the highest relative abundance value. Each compound has a different retention time. The cytotoxicity value (LC_{50}) of the ethanol extract of betel leaves was obtained from the Banda Aceh and Malang areas (2.64 µg/mL). The conclusions of this study based on the results of secondary metabolite fingerprinting analysis and cytotoxicity values identified 12 compounds with three clusters of secondary metabolite diversity based on their region of origin, namely group 1 (Banda Aceh, Jayapura, Samarinda); group 2 (Bandung et al.); group 3 (Bogor).

1. INTRODUCTION

Indonesia is a country with a high level of biodiversity. Biodiversity, which includes genes, species, and living things, contains plants and animals spread throughout Indonesia. Indonesia is estimated to have potential medicinal plants of 30,000 species, of which 940 have been declared medicinal. This number represents 90% of the medicinal plants found in the Asian region (Salim & Munadi, 2017). One of the medicinal plants that can be used is red betel. Red betel contains active compounds such as flavonoids, alkaloids, tannins, polyphenolics, and essential oils (Safithri et al., 2022). The active compounds contained in the red betel plant. This plant to have much potential to treat various diseases, including its potential as an antioxidant (Safithri et al., 2022), anti-inflammatory (Gong et al., 2021), antihyperglycemic (Hasibuan et al., 2016), anticancer by inhibiting the proliferation of cancer cells (Zulharini et al., 2018), anticancer colon in silico studies (Umar et al., 2023) antiviral (Diniatik, 2011), antiobesity (Apritya et al., 2020), antibacterial (Mangesa & Irsan, 2020) and also as an antidiabetic (Maha et al., 2020). Saraswati and Palupi (2019) show that red betel leaves collected from Kupang, East Nusa Tenggara contain 35 compounds with % quality > 90%, and are dominated by 5 compound components with the largest % area, namely β-Mirsen (13.80%), Linalool (3.29%), α-Thujen (1.52%), γ-Terpinen (1.36%), cis-β-Terpineol (1.15%). The red betel plant from North Sumatra is used as a herbal medicine to treat diabetes (Mindayani et al., 2020). The factors that influence the differences in the composition of the compounds from each sample in different areas are the height of the sample growing location, environment, climate, soil composition, and weather.

Research on the diversity of red betel plants in various regions has carried out research with the antioxidant test method DPPH obtained IC_{50} values successively from the smallest to the largest, namely Bogor, Bandung, Jayapura, Aceh, Jogjakarta, Samarinda, Malang, and Kendari with a value of 55.096 µg/mL (strong) very weak), and 479.286 µg/mL (very weak) and total phenolic where the total phenolic is highest, namely the Malang area with a value of 12.570 mg GAE/g and the lowest is the Yogyakarta area with a value of 6.389 mg GAE/g (Putri, 2022). The antioxidant test of the MDA-TBA method with the best results came from Malang, namely the ethyl acetate fraction with an IC_{50} value of 2.012 µg/mL, Yogyakarta, namely the n-hexane fraction of 3,810 µg/mL, Bogor, namely 70% ethanol extract of 0.432 µg/mL and Bandung, water fraction of 2.508 µg/mL (Chairunisa, 2022). Another research done by Umar et al. (2022) also supports the statement that the compounds in red betel leaves have antioxidant activity because the DPPH test result from red betel leaves extracts showed an IC_{50} score of 47.45 ppm and classified in a solid antioxidant class. One simple test that can be carried out is the toxicity test using the Brine Shrimp Lethality Test (BSLT) method to determine the bioactivity potential of red betel leaves. Previous studies have been carried out on boiled water of red betel leaves with an LC_{50} value of 544.82 ppm and 30% ethanol extract of red betel leaves with an LC_{50} value of 435.29 ppm, while methanol extract of red betel leaves has an LC_{50} value of 27.40 ppm (Safithri et al., 2012).

Identification of metabolites present in natural products can use several methods, namely the Fourier Transform InfraRed Spectroscopy (FTIR) method, High-Performance Liquid Chromatography (HPLC), Gas Chromatography-Mass Spectrometry (GC-MS), and Liquid Chromatography-Mass Spectrometry (LC-MS). Red betel samples were identified using LC-MS/MS from different growing locations. It is considered due to the possibility of differences in the compounds contained in each extract from different locations of the samples. LC-MS/MS are two tools combined into one by performing identification and quantitation, which reduces sample complexity and allows the separation of metabolites prior to detection. The LC-MS/MS analysis principle separates a compound mixture based on its polarity. The separate compounds are identified for their molecular weights, where the data obtained from the analysis results using LC-MS/MS are in the form of molecular weights with

the addition of some charge from the molecular weight of the solvent (Yuliana & Arianti, 2020).

The data obtained were processed by multivariate analysis, namely cluster or cluster analysis. Cluster analysis is a technique that classifies objects into relatively homogeneous groups called clusters. Cluster analysis, also called classification analysis or numerical taxonomy, deals with grouping procedures where each object is only included in one cluster. Cluster analysis aims to determine whether a data set contains subgroups or consists of one group, determining the number of clusters in the data and the extent to which cluster membership from sample observations can be classified accurately (Dalmaijer et al., 2022). Research has been carried out on the bioactive potential of red betel leaves in various regions. However, no one has identified secondary metabolites and red betel clustering based on the similarity of secondary metabolites from each region. Therefore, it is necessary to conduct this research to classify red betel from various regions in Indonesia based on the analysis of secondary metabolite fingerprints and cytotoxicity values. This research's benefit is knowing the cytotoxic activity and similarity of secondary metabolites from various regions based on their clustering.

2. METHODS

2.1. Tools and Materials

The tools used in this study were glassware, analytical balance, oven, micropipette, desiccator, multiwell plates, magnifying glass, aquarium, lamp, LC-MS/MS Thermo Scientific Vanquish Flex Ultra High-Performance Liquid Chromatography (UHPLC) tandem Q Exactive Plus Orbitrap High-Resolution Mass Spectrometer (Thermo Fisher Scientific, Waltham, United States) with the mobile phase used was 0.1% formic acid in water and 0.1% acetonitrile in water, the stationary phase used was an Accucore TM Phenyl Hexyl LC Column, 2.1 mm × 100 mm, 2.6 µm diameter (Thermo Fisher Scientific, Waltham, United States), a laptop set with some software, namely Thermo Scientific XCalibur 4.2 (Thermo Fisher Scientific Waltham, United States), Compound Discoverer 3.2 (Thermo Fisher Scientific Waltham, United States), and The Unscrambler X 10.14 (CAMO Analytics, Oslo, Norway).

The materials to be used in this study were red betel leaves from seven regions in Indonesia, namely Banda Aceh, Bandung, Bogor, Malang, Samarinda, Kendari, and Jayapura, distilled water, seawater, ethanol, shrimp larvae eggs (*Artemia Salina* Leach). The sample preparation process to analyze red betel leaf ethanol extract using LC-MS/MS at the LPPM IPB Excellence Research Laboratory.

2.2. Determination of Water Content and Extract Yield

Determination of the water content was carried out by preparing a porcelain cup that had been cleaned and dried in an oven at 105 ℃ for 15 minutes. Then, the cup is cooled in a desiccator and weighed as the empty weight of the cup. A total of 1 gram of red betel leaf simplicia was put into a cup and dried using an oven at 105 ℃ for 3 hours, then cooled in a desiccator for 15 minutes and weighed using an analytical balance.

The extract yield was carried out using dry test material as a powder, followed by an extraction process with ethanol. Dried red betel leaves were carried out by weighing 10 g and adding 40 mL of ethanol (1:4). The sample was homogenized until the solvent and sample were mixed evenly using a shaker for 24 hours. The extract mixture was filtered to obtain a filtrate; then, the filtrate was concentrated using a rotary evaporator at 50 ^{o}C to obtain a paste-shaped extract. Extraction was carried out three times.

2.3. Toxicity Test

Hatching of shrimp larvae. A total of 400 mL of seawater was filtered until clear. Seawater is poured into an Erlenmeyer flask. As much as 2 g of *A. salina* Leach eggs were put into an Erlenmeyer flask, irradiated with a lamp, and allowed to stand for 48 hours.

Preparation of stock and test solutions, 500 mL of seawater was filtered until clear. 30 mg of each sample was dissolved in 20 mL of seawater to make a stock solution of 4000 ppm. Dilutions were made for the test solutions with concentrations of 1000, 800, 600, 400, 200, 100, 50, and 10 ppm. Vortex the test solution until dissolved.

Toxicity test. 10 shrimp larvae in 1 mL of seawater were put into each well on the test plate. Controls were made with seawater containing 10 shrimp larvae without additional test solution. A total of 1 mL of the test solution was put into each well on the test plate. The test plates were left for 24 hours, and the number of still-alive and dead prawns was counted. The mortality of *A. salina* Leach larvae was analyzed using the Probit Minitab 19 for Windows analysis program to determine the LC_{50} value.

2.4. Identification of Secondary Metabolites Using LC-MS/MS

Identification of metabolites refers to the method that has been carried out by Karomah (2019). Compound discoverer software processed data from LC-MS/MS in RAW format. The workflow is selected in the processing section for untargeted metabolomics with statistics detected unknown with ID using a local database. Then add files in RAW form in the workflow, and options appear, including selecting spectra, aligning retention time, detecting unknown compounds, grouping unknown compounds, predicting composition, searching mass lists, filling gaps, normalizing areas, and marking compound backgrounds. The detected unknown compound section uses an m/z tolerance of 5 ppm and a minimum peak intensity 2,000,000. In the search mass list, a manual database is used, then running is carried out to identify the compounds in the extract.

2.5. Data analysis

Data in a RAW format consisting of retention time and peak intensity were transposed on the data. After that, the range of the transposed data was defined. The defined range aims to create a sample group according to the solvent. Furthermore, the data before and after preprocessing are grouped using cluster analysis by creating a dendrogram. Data on shrimp larvae mortality from the BSLT test were analyzed using the Probit analysis method to find the lethality concentration at the 50% level (LC_{50}), assuming a Weibull distribution and a 95% confidence interval. Data processing uses the help of Minitab 19 for Windows software.

3. RESULTS AND DISCUSSION

3.1. Results

The water content of the simplicia obtained in this study varied from region to region (Table 1). The highest water content measurement results were in the Bandung area, with a value of 9.2%, and the lowest in Samarinda was 6%. The simplicia must be measured for its water content to determine its quality and shelf life. Extract yield calculation is calculated based on comparing the weight of the extract obtained with the simplicia used in the extraction process. The highest yield percentage was found in the Bandung area, with a value of 19.42%, and the lowest in the Malang area, with a value of 9.34%.

Table 1 Water content and yield of red betel leaf ethanol extract

Sample	Water Content (%)	Yield (%)
Banda Aceh	6.86 ± 0.07	15.90 ± 2.12[a,b]
Bandung	9.29 ± 0.37	19.42 ± 3.09[a]
Bogor	7.55 ± 0.33	17.38 ± 1.45[a,b]
Malang	8.05 ± 0.09	9.34 ± 0.50[c]
Samarinda	5.99 ± 0.09	15.78 ± 1.49[a,b]
Kendari	6.10 ± 0.07	17.90 ± 2.48[a,b]
Jayapura	7.03 ± 0.51	12.62 ± 0.54[b,c]

Toxicity was carried out using the Brine Shrimp Lethality Test (BSLT) method using *A. salina* Leach shrimp larvae as the object of observation. Red betel leaf extract from seven regions is classified as very toxic or can be categorized as an active extract where the lowest to highest LC50 values are respectively owned by red betel leaf extract from Malang (2.64 µg/mL), Banda Aceh (2.64 µg/mL), Samarinda (2.79 µg/mL), Bogor (4.65 µg/mL), Bandung (5 µg/mL), Jayapura (6.47 µg/mL), Kendari (7.49 µg/mL) (Figure 1). These results were supported by ANOVA statistical analysis, which showed that the seven red betel leaf extract regions were significantly different (p-value <0.05) based on their toxicity. It shows that differences in the region of origin of plant growth affect the differentiation of metabolites, which is reflected in their bioactivity. The results of the Tukey test ($\alpha = 0.05$) on the toxicity of the ethanol extract of red betel leaves from seven regions showed that there were 4 groups: (1) Kendari and Jayapura samples; (2) Jayapura and Bandung; (3) Bandung and Bogor; (4) Samarinda, Malang and Banda Aceh; which are grouped based on the closeness of the LC_{50} values they have (Figure 1).

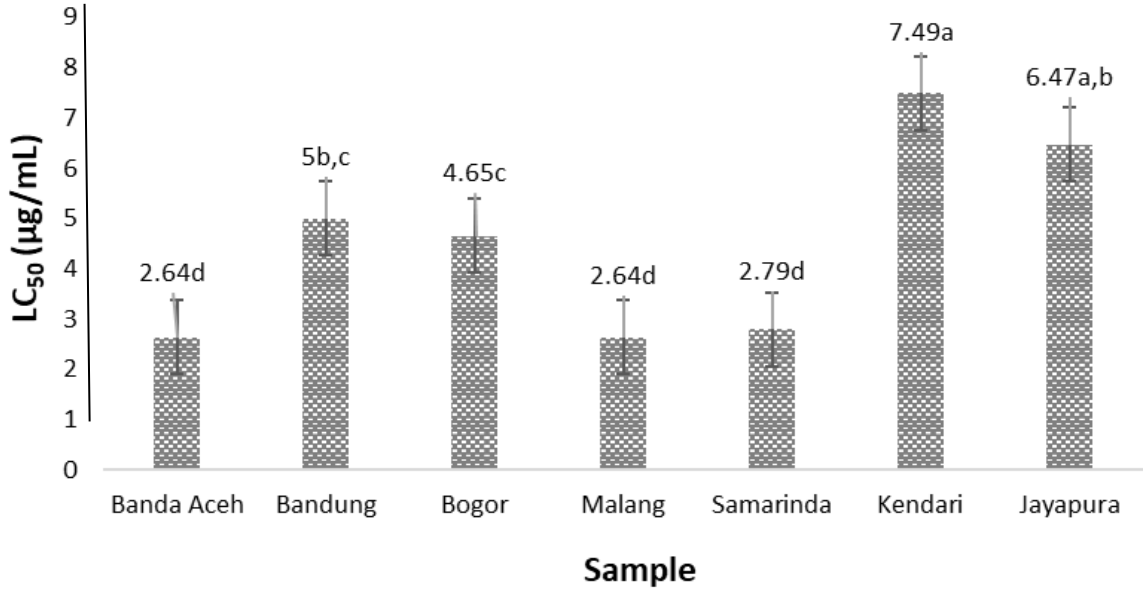

Figure 1 Lethal Concentration 50 (LC_{50}) of Red Betel Ethanol Extract Based on Toxicity Test (BSLT)

The ethanol extract of red betel leaves from Banda Aceh has 5 compounds with the highest relative abundance compared to 6 other regions (Table 2). Among the red betel leaves tested, red betel leaves from Banda Aceh have the highest potential to isolate compounds. Red betel leaves ethanol extracts from Bandung, Samarinda, and Kendari also have many compounds that can be isolated. Aside from the Banda Aceh sample, which contains 5 compounds with the highest relative abundances, the Samarinda and Bandung samples contain only 1 compound with the highest relative abundance. The sample from Kendari identified many compounds but did not have a compound that had a dominant relative abundance. Whereas the ethanol extract samples from Bogor, Malang, and Jayapura had fewer identified compounds, the samples from Bogor identified 9 compounds, with 1 compound having the highest relative abundance. Samples from Malang identified 11 compounds, with 2 compounds having the highest relative abundance. Samples from Jayapura identified 11 compounds, with 2 compounds having the highest relative abundance (Table 2).

The dendrogram describes the grouping of each sample based on measurement variables, namely secondary metabolites identified by secondary metabolite fingerprinting analysis. The cluster analysis results classified red betel from seven regions into three clusters based on the region of origin (Figure 2). Red betel from Banda Aceh has the same secondary metabolite as Samarinda and Jayapura. Red betel from Bandung has the same secondary metabolites as Kendari and Malang. Red betel from Bogor is clustered independently because it has different secondary metabolites from other regions.

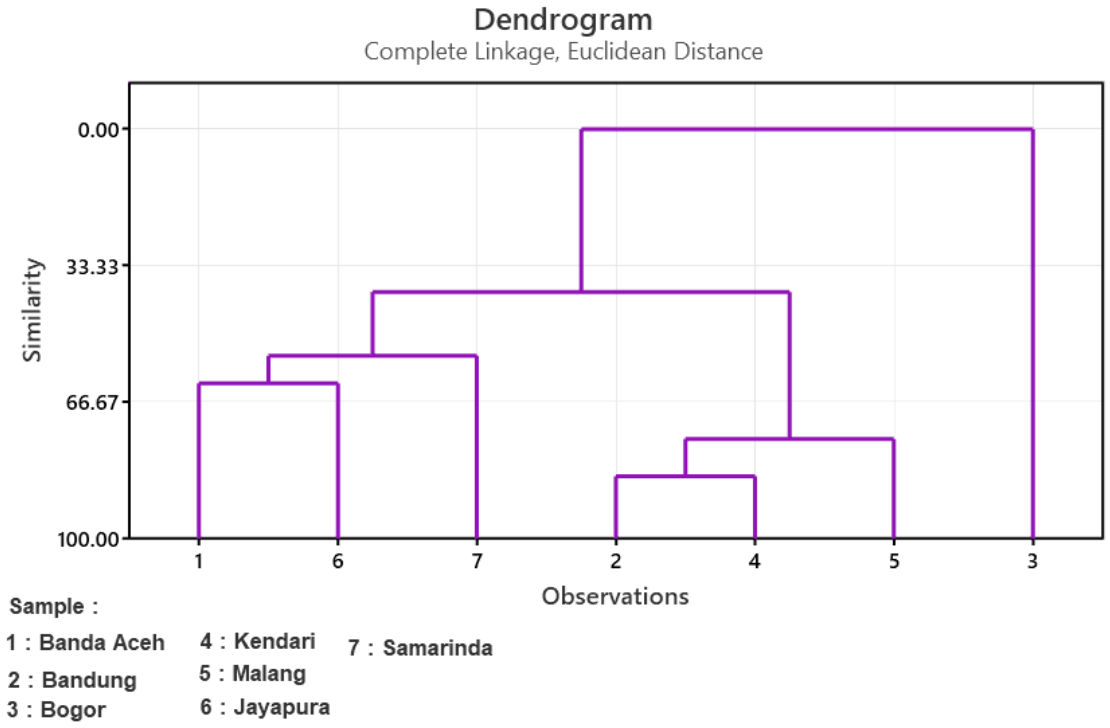

Figure 2 Dendrogram of Similarity of Secondary Metabolites of Red Betel Leaves from Seven Regions in Indonesia

Table 2 List of secondary metabolites of red betel ethanol extract in each sample

Compound name	RT (min)	Relative abundance (%)						
		Banda Aceh	Bandung	Bogor	Malang	Samarinda	Kendari	Jayapura
(2S)-4-Methyl-2-({[(3S,4S,5R)-2,3,4-trihydroxy-5-(hydroxymethyl)tetrahydro-2-furanyl]methyl}amino) pentanoic acid	1.523	1.90	1.97	3.19	0.71	1.30	1.89	0.59
5-Hydroxytryptophol	2.015	1.04	0.42	-	0.85	0.86	1.04	3.67
DL-Tryptophan	4.946	1.15	1.19	-	0.57	1.54	0.61	1.37
2-(4-Allyl-2,6-dimethoxyphenoxy)-1-(3,4,5-trimethoxyphenyl)-1-propanol	14.18	4.46	15.08	8.08	14.21	2.95	13.92	7.06
Tetrahydrocurcumin	15.33	0.95	0.93	0.62	1.35	0.38	0.76	1.22
Piperine	16.98	7.94	10.71	7.50	14.63	2.82	12.85	8.72
Hernanol	16.111	1.30	0.98	1.01	-	0.69	1.05	2.51
Piperanine	16.954	2.99	1.66	1.02	1.31	1.36	1.31	-
Schisandrin C	18.124	3.23	1.15	-	2.37	0.97	1.45	3.03
Xanthohumol	18.214	12.92	3.15	2.44	4.47	5.75	3.05	8.06
Fusarin C	18.225	4.35	1.03	0.51	0.62	2.10	1.00	2.31
Garcinone C	18.252	3.02	0.73	0.64	1.64	0.93	1.16	1.14

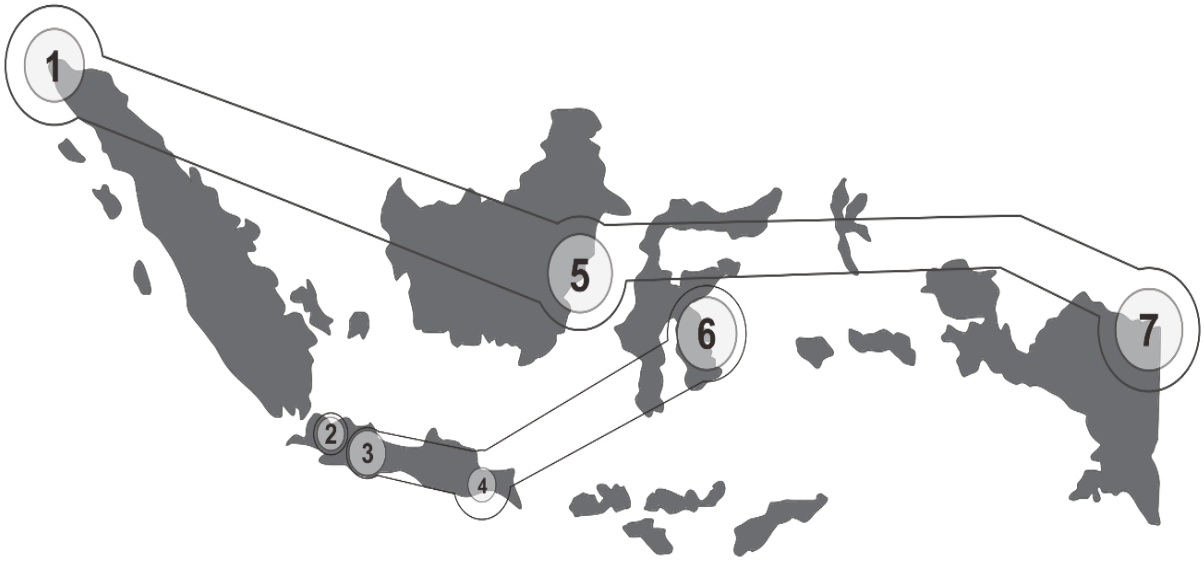

Figure 3 Map of the Red Betel, Clusters in Indonesia: (1) Banda Aceh, Aceh; (2) Bogor, West Java; (3) Bandung, West Java; (4) Malang, East Java; (5) Samarinda, East Kalimantan; (6) Kendari, Southeast Sulawesi; (7) Jayapura, Papua.

3.2. Discussion

The results showed that the water content of the simplicia obtained varied from region to region (Table 1). The highest water content measurement results were in the Bandung area, with a value of 9.2%, and the lowest in Samarinda was 6%. The simplicia must be measured for its water content to determine its quality and shelf life. According to BPOM (2014), the water content of simplicia is said to be good if it is below 10%. Water content above 10% or too high can cause decay and damage to the simplicia more quickly than the water contained in the simplicia, so measuring the water content is very important (Sulistiawati et al., 2016). The yield of the extracts produced in this study varied for each region (Table 1). The highest yield was obtained from the Bandung area at 19.4% and the lowest from Malang at 9.3%. Extract yield can be affected by the length of maceration, pH, temperature, light, and the type of solvent used (Wahyuni & Widjanarko, 2015). The mortality of the shrimp larvae obtained is closely related to the toxicity of the test extract (Raharjo & Ningsih, 2015).

The bioactivity test of the ethanol extract of red betel leaves using the BSLT method aims to determine the diversity of the composition of red betel secondary metabolites from seven different regions based on their toxicity. The toxicity of the extract is expressed by LC_{50} (lethal concentration), the concentration of the tested extract that can kill 50% of the population after a 24-hour incubation period. The lower the LC_{50}, the higher the toxicity of the extract or compound. According to Meyer et al. (1982), the crude extract was said to be highly toxic if it had an LC_{50} value <100 µg/mL and was categorized as non-toxic if the LC_{50} value was > 1000 µg/mL. Meanwhile, according to Albuntana et al. (2011), an extract can have the potential as an anticancer if it has an LC_{50} value <30 µg/mL. The results show that the ethanol extract of red betel leaves has an LC_{50} value below 30 µg/mL, which is included in the very toxic category and reflected in its bioactive potential.

Twelve metabolites were identified from the ethanol extract of red betel leaves with different relative abundance percentages (Table 2). Geographical and environmental characteristics in each area will make differences in genetics and bioactive compounds. It is proven by the research of Sukandar and Widayat (2017) that plants from different places contain different compounds. One of the differences in the percentage of relative abundance can be influenced by the altitude factor, namely Banda Aceh, Aceh with a height of 2 meters above sea level (05°16'15"-05°36'16" North Latitude, 95°16'15"-95° 22'35" East); Bandung, West Java with an altitude of 768 meters above sea level (107° 36' E, 6° 55' S); Bogor, West Java with an altitude of 330 meters above sea level (106°43'30" E - 106°51'00" E, 30'30" South Latitude - 6°41'00" South Latitude); Malang, East Java with an altitude of 506 meters above sea level (7.06° - 8.02° South Latitude, 112.06° - 112.07° East Longitude); Samarinda, East Kalimantan with an altitude of 25 meters above sea level (0°21'81"- 1°09'16" S, 116°15'16"-117°24'16" E); Kendari, Southeast Sulawesi with a height of 2 meters above sea level (3°54'40" - 4°5'5" S, 122°26'33" - 122°39'14" E); Jayapura, Papua with an altitude of 287 meters above sea level (137°27' - 141°41' E and 1°27' - 3°49' S).

According to Parfati and Windono (2017), red betel is classified into the kingdom Plantae, Magnoliophyta division, class Magnoliopsida, family Piperales, Order Piperaceae, Genus Piper, species *Piper crocatum* Ruiz & Pav. Red betel leaf extract contains various phytochemical compounds, including essential oils, alkaloids, saponins, tannins, and flavonoids. In addition, there are other compounds such as hydroxychavicol, cavicol, cavibetol, carvacol, eugenol, p-synthes, cineol, caryophyllene, estragol kadimen, terpenene, and phenyl (Novilia et al., 2018). Twelve secondary metabolites were identified from seven regions, namely Banda Aceh, Bandung, Bogor, Malang, Samarinda, Kendari, and Jayapura, which were analyzed using LC-MS/MS. Table 2 shows that all samples have specific typical metabolites. The results showed that Indonesian red betel leaves, based on the similarity of secondary metabolites, were divided into three main groups based on cluster analysis in the form of dendrograms. Dendrograms can be reconstructed based on morphological similarity indices and their compound components with close relationships between regions based on metabolite content expressed by the Euclidean distance, starting from 10-100 with the equation of the number of groups with the results of the dendrogram allegedly occurring due to the character of the diversity of each plant phenotype (Purnomo & Khotimah, 2019).

These results indicate that red betel leaves from Banda Aceh have similarities with Samarinda and Jayapura based on secondary metabolite profiles. The same environmental conditions can cause the similarity of secondary metabolites. According to Husnul Khatimah in the book Tugu Khatulistiwa (2018), the provinces of Sumatra, Kalimantan, and Papua are provinces that are traversed by zero degrees latitude or which are traversed by the equator which will affect the condition of environmental factors, namely temperature. Following the research of Moghaddam and Pirbalouti (2017), the metabolite content in plants is highly dependent on the environmental conditions of growth. Environmental conditions can make a significant contribution to variations in the content of metabolites in plants. Biogeographic conditions could be one of the reasons Bandung, Malang, and Kendari become one cluster. Climatic conditions and land topography in this area have similarities. Malang is an area that has topography with a height of 506 meters above sea level. The soil types in this area are reddish-brown latosol, blackish-gray alluvial, Mediterranean, brown andosol. Likewise, the Bandung area is 768 meters above sea level with andosol and alluvial soil types. The Kendari area has alluvial, glisol, litosol, and podzolic soil types. The same thing was also reported by Purwanto and Rispriandari (2020): the metabolic pathway for the formation of secondary

metabolites can be influenced by several external factors such as light, pH, altitude, temperature, humidity, harvest time, and nutrition. Soil types, pests/diseases, and physiological conditions of plants will affect the level of secondary metabolite production in plants (Zhang et al., 2021).

Bogor is clustered independently because it does not have the same secondary metabolites as other regions. The rainfall condition with an average annual rate of 3500-4000 mm (BPS 2021) is relatively high, and humidity reaches 70%. Following Maulana and Herlina (2020) that rainfall intensity has a strong effect on water reserves in the soil; this is because the availability of water in the soil drives the rate of decomposition of organic matter and the formation of soil structure so that root penetration can get nutrients deeper, which will affect the components of secondary metabolites. According to Jains et al. (2017), plants' lack of light intensity will affect secondary metabolites. Secondary metabolites synthesized as a plant defense mechanism are produced in response to changes in temperature, drought, humidity, salinity, UV radiation, and pathogens that function as detoxifying agents, allelopathic compounds, and signaling molecules (Idris et al**.,** 2018).

The dendrogram describes the grouping of each sample based on measurement variables, namely secondary metabolites identified by secondary metabolite fingerprinting analysis. The cluster analysis results classified red betel from seven regions into three clusters based on the region of origin (Figure 2). The clustering results of the three red betel clusters may also occur due to climate and soil topography, as in the study of (Andrianto et al., 2019). Green betel from Sumber Baba and Demta are included in the same cluster because based on climate and soil topography where Sumber Baba and Demta have the same climate and soil. Suliantari et al. (2018) stated that the origin of the betel influences the active compounds in betel leaves.

4. CONCLUSION

Secondary metabolite profile analysis of ethanol extract of red betel leaves (*Piper crocatum* Ruiz & Pav) using LC-MS/MS and integration of multivariate statistical cluster analysis can be used to cluster the diversity of secondary metabolites based on their region of origin. The quality of secondary metabolites of red betel leaves is influenced by various environmental factors where the plant grows. The results of identifying secondary metabolites of the ethanol extract of red betel leaves in seven regions showed twelve compounds detected. Cluster analysis with a dendrogram yielded three clusters of secondary metabolite diversity based on their region of origin, namely group 1 (Banda Aceh, Jayapura, Samarinda); group 2 (Bandung, Kendari, Malang); group 3 (Bogor). This is supported by the results of the BSLT toxicity test through the Tukey statistical test, which classifies based on its bioactive potential with the most active extracts from the Banda Aceh and Malang regions.

ACKNOWLEDGMENT

The authors would like to thank Ms. Mega Safithri as supervisor 1 with Research Contract Number 3881/IT3.L1/PT.01.03/P/B/2022 and Mr. Dimas Andrianto as supervisor 2 who have supported the research process. We are also very grateful to the reviewers for suggestions to improve the quality of this article.

REFERENCES

[BPOM RI]. Badan Pengawas Obat dan Makanan Republik Indonesia. (2014). Peraturan Kepala Badan Pengawas Obat dan Makanan Nomor 12 Tahun 2014 tentang Persyaratan Mutu Obat Tradisional. Jakarta (ID): BPOM RI

[BPS] Badan Pusat Statistik. (2017). Jumlah Curah Hujan dan Jumlah Hari Hujan di Stasiun Pengamatan BMKG. Jakarta: Badan Pusat Statistik

Albuntana, A., Yasman, Wardhana, W. (2011). Uji toksisitas ekstrak empat jenis teripang suku Holothuriidae dari Kepulauan Panjalirin Timus, Kepulauan Seribu, Jakarta menggunakan Brine Shrimp Lethality Test (BSLT). J Iltek Keltrop, 3, 65-72. https://doi.org/10.29244/jitkt.v3i1.7835

Andrianto, D., Husnawati, Hermita, S., & Haryanti, S. (2019). The Classification of betel leaves (Piper betle) from 15 ethnics in eastern Indonesia based on phytochemicals fingerprint analysis. *Biodiversitas Journal of Biological Diversity*, *21*(1). https://doi.org/10.13057/biodiv/d210133

Apritya, D., Sigit, M., Yunani, R., & Lestari, F. (2020). Pemanfaatan infusa daun sirih merah (Piper crocatum) sebagai anti-obesitas pada mencit (Mus musculus). *VITEK: Bidang Kedokteran Hewan*, *10*, 50–57. https://doi.org/10.30742/jv.v10i0.49

Chairunisa, F. (2022.). *Penambatan molekuler senyawa bioaktif sirih merah (Piper crocatum) terhadap lipoksigenase dan penghambatan malondialdehid in vitro*.

Dalmaijer, E. S., Nord, C. L., & Astle, D. E. (2022). Statistical power for cluster analysis. *BMC Bioinformatics*, *23*(1), 205. https://doi.org/10.1186/s12859-022-04675-1

Diniatik, K. (n.d.). AM, & Purwaningrum, O.(2011). UJI AKTIVITAS ANTIVIRUS EKSRAK ETANOL DAUN SIRIH MERAH (Piper crocatum Ruitz & Pav) TERHADAP VIRUS NEWCASTLE DISEASE (ND) DAN PROFIL KROMATOGRAFI LAPIS TIPISNYA. *Pharmaceutical Journal of Indonesia*, *8*(1).

Gong, Y., Li, H. X., Guo, R. H., Widowati, W., Kim, Y. H., Yang, S. Y., & Kim, Y. R. (2021). Anti-allergic Inflammatory Components from the Leaves of Piper crocatum Ruiz & Pav. *Biological and Pharmaceutical Bulletin*, *44*(2), 245–250. https://doi.org/10.1248/bpb.b20-00726

Hasibuan, M. S., Yasni, S., Bintang, M., & Ranti, A. S. (2016). Antihyperglycemic activity of Piper crocatum leaves and Cinnamomum burmannii bark mixture extract in streptozotocin-induced diabetic rats. *J. Math. Fund. Sci*, *48*(2), 178–191. https://doi.org/10.5614/j.math.fund.sci.2016.48.2.8

Idris, A., Linatoc, A. C., Muhammad, S. M., Aliyu, A. M., & Bakar, M. F. A. (2018). Effect of light intensity on the total flavonoid and total phenolic contents of Mikania micrantha and Tridax procumbens. *Journal of Science and Technology*, 10(4). https://doi.org/10.30880/jst.2018.10.04.001

Jain, C., Khatana, S., & Vijayvergia, R. (2019). Bioactivity of secondary metabolites of various plants: a review. *Int. J. Pharm. Sci. Res*, 10(2), 494-504.

Khatimah, H. (2018). Tugu khatulistiwa. Jakarta Timur: Badan Pengembangan dan Pembina Bahasa.

Karomah, A. H. (2019). Pemprofilan Metabolit Ekstrak Daun dan Batang Sambiloto (Andrographis paniculata) Menggunakan Kromatografi Cair-Spektroskopi Massa. IPB University. Bogor.

Maha, A., Elmiyati, E., & Nola, S. (2020). PENGARUH PEMBERIAN AIR REBUSAN SIRIH MERAH (Piper crocatium Ruiz &Pav) TERHADAP PENURUNAN GLUKOSA DARAH PADA MENCIT. *Jurnal Aceh Medika*, *4*(2), 15–23.

Mangesa, R., & Irsan, I. (2020). Efektifitas Fraksi Aktif Metanol Daun Sirih Merah (Piper Crocatum) Yang Berpotensi Sebagai Antibakteri Salmonellas Typhi:(the Effectiveness of Methanol Active Fraction of Red Better Leaves [Piper Crucatum] That Potential as

an Antibacterial Salmonellas Typhi). *Uniqbu Journal of Exact Sciences*, *1*(2), 40–45. https://doi.org/10.24042/biosfer.v10i1.4230

Maulana, A. R., & Herlina, N. (2020). Hubungan Unsur Iklim Terhadap Produktivitas Tanaman Ubi Kayu (Manihot esculenta Crantz) di Kabupaten Malang. *PLANTROPICA: Journal of Agricultural Science*, *5*(2), 118–128. https://doi.org/10.21776/ub.jpt.2020.005.2.3

Meyer, B. N., Ferrigni, N. R., Putnam, J. E., Jacobsen, L. B., Nichols, D. E. J., & McLaughlin, J. L. (1982). Brine shrimp: a convenient general bioassay for active plant constituents. *Planta medica*, *45*(05), 31-34. https://doi.org/10.1055/s-2007-971236

Mindayani, S., Susanti, W., Agustin, N., & Tina, J. (2020). Efektivitas rebusan daun sirih merah (Piper crocatum) terhadap penurunan kadar gula darah penderita diabetes mellitus. *Jurnal Riset Hesti Medan Akper Kesdam I/BB Medan*, *4*(2), 119–125. https://doi.org/10.34008/jurhesti.v4i2.145

Moghaddam, M., & Pirbalouti, A. G. (2017). Agro-morphological and phytochemical diversity of Iranian Cuminum cyminum accessions. *Industrial Crops and Products*, *99*, 205–213. https://doi.org/10.1016/j.indcrop.2017.02.003

Novilia, L., Harahap, U., & Hasibuan, P. A. Z. (2018). EVALUATION OF HEPATOPROTECTIVE EFFECT OF ETHANOLIC EXTRACT FROM RED BETEL (PIPER CROCATUM RUIZ AND PAV.) LEAVES. *Asian Journal of Pharmaceutical and Clinical Research*, *11*(7), 248. https://doi.org/10.22159/ajpcr.2018.v11i7.25542

Parfati, N., & Windono, T. (2016). Sirih merah (Piper crocatum Ruiz & Pav.) kajian pustaka aspek botani, kandungan kimia, dan aktivitas farmakologi. *Media Pharinaceutica lndonesiana*, *1*(2), 106-115. https://doi.org/10.24123/mpi.v1i2.193

Purnomo, P., & Khotimah, N. (2019). Variations and Phenetic Analysis of Peanut Cultivars (Arachis hypogaea L.) Based on Morphological Characteristics. *Journal of Tropical Biodiversity and Biotechnology*, *4*(1), 24. https://doi.org/10.22146/jtbb.39390

Purwanto, U. M. S., & Rispriandari, A. A. (2020). *Perbedaan Bagian Tanaman Krokot (Portulaca grandiflora Hook.) Terhadap Kandungan Total Fenolik dan Flavonoid serta Aktivitas Antioksidan*. https://doi.org/10.29244/cb.7.1.10

Putri, R. S. (2022). *Kajian In Silico Aktivator Enzim Cu/Zn-SOD dan Aktivitas Antioksidan In Vitro Sirih Merah (Piper crocatum Ruiz & Pav) Asal Berbagai Daerah di Indonesia*.

Raharjo, S. J., & Ningsih, R. W. (2015). *CYTOTOXIC ACTIVITIES OF ETHYL ACETATE FRACTIONS FROM PETROLEUM ETHER EXCTRACT AND METHANOL EXTRACT OF PISTIAE LEAVES*.

Safithri, M., Fahma, F., & Marlina, P. W. N. (2012). Analisis proksimat dan toksisitas akut ekstrak daun sirih merah yang berpotensi sebagai antidiabetes. *Jurnal Gizi Dan Pangan*, *7*(1), 43–49. https://doi.org/10.25182/jgp.2012.7.1.43-49

Safithri, M., Nur Faridah, D., Ramadani, F., & Pratama, R. (2022). Antioxidant activity of ethanol extract and fractions of Piper crocatum with Rancimat and cuprac methods. *Turkish Journal of Biochemistry*, *47*(6), 795–801. https://doi.org/10.1515/tjb-2021-0300

Salim, Z., & Munadi, E. (2017). Info komoditi tanaman obat. *Jakarta: Badan Pengkajian Dan Pengembangan Perdagangan Kementerian Perdagangan Republik Indonesia*, 1–2.

Saraswati, A., & Palupi, S. (2019). Analisis Kualitatif Dan Kuantitatif Minyak Atsiri Daun Sirih Hijau (Piper betle L.) Dan daun sirih Merah (Piper crocatum Ruiz & Pav.) Berasal Dari Kupang, NTT. *CALYPTRA*, *7*(2), 1640–1659.

Sukandar, P. B., & Widayat, T. (2017). Eksplorasi Pengetahuan Lokal Etnomedisin dan Tumbuhan Obat Berbasis Komunitas di Indonesia Provinsi Kalimantan Timur, Kalimantan Utara (Laporan Penelitian).

Suliantari., Jenie, B. S. L., Suhartono, M.T., Apriyantono, A. (2018). Antibacterial activity of green sirih (*Piper betle* L) extract towards food pathogens. *Jurnal teknologi dan industri pangan,* 19(1), 1-7. https://doi.org/10.6066/jtip.2012.23.2.217

Sulistiawati, E., Santosa, I., Aps, Y. R., & Saka, A. A. (2016). Pengaruh Suhu pada Pengeringan Tepung Kimpul (Xanthosoma sagittifolium). *CHEMICA: Jurnal Teknik Kimia*, *2*(2), 57. https://doi.org/10.26555/chemica.v2i2.4568

Umar, M., Safithri, M., & Pratama, R. (2023). In Silico Study of Anticancer Activity of Red Betel Leaves Bioactive Compounds against Colon Cancer Marker Proteins. *HAYATI Journal of Biosciences*, *30*(1), 113–121. https://doi.org/10.4308/hjb.30.1.113-121

Wahyuni, D. T., & Widjanarko, S. B. (2015). *PENGARUH JENIS PELARUT DAN LAMA EKSTRAKSI TERHADAP EKSTRAK KAROTENOID LABU KUNING DENGAN METODE GELOMBANG ULTRASONIK*. *3*(2).

Yuliana, A., & Arianti, W. (2020). *MEASURMENT OF MONASCUS PUPUREUS USING LC-MS*. *20*.

Zhang, S., Zhang, L., Zou, H., Qiu, L., Zheng, Y., Yang, D., & Wang, Y. (2021). Effects of Light on Secondary Metabolite Biosynthesis in Medicinal Plants. *Frontiers in Plant Science*, *12*, 781236. https://doi.org/10.3389/fpls.2021.781236

Zulharini, M., Sutejo, I. R., Fadliyah, H., & Jenie, R. I. (2018). Methanolic extract of red betel leaves (Piper crocatum Ruiz & Pav) perform cytotoxic effect and antimigration activity toward metastatic breast cancer. *Indonesian Journal of Cancer Chemoprevention*, *8*(3), 94–100. https://doi.org/10.14499/indonesianjcanchemoprev8iss3pp94-100