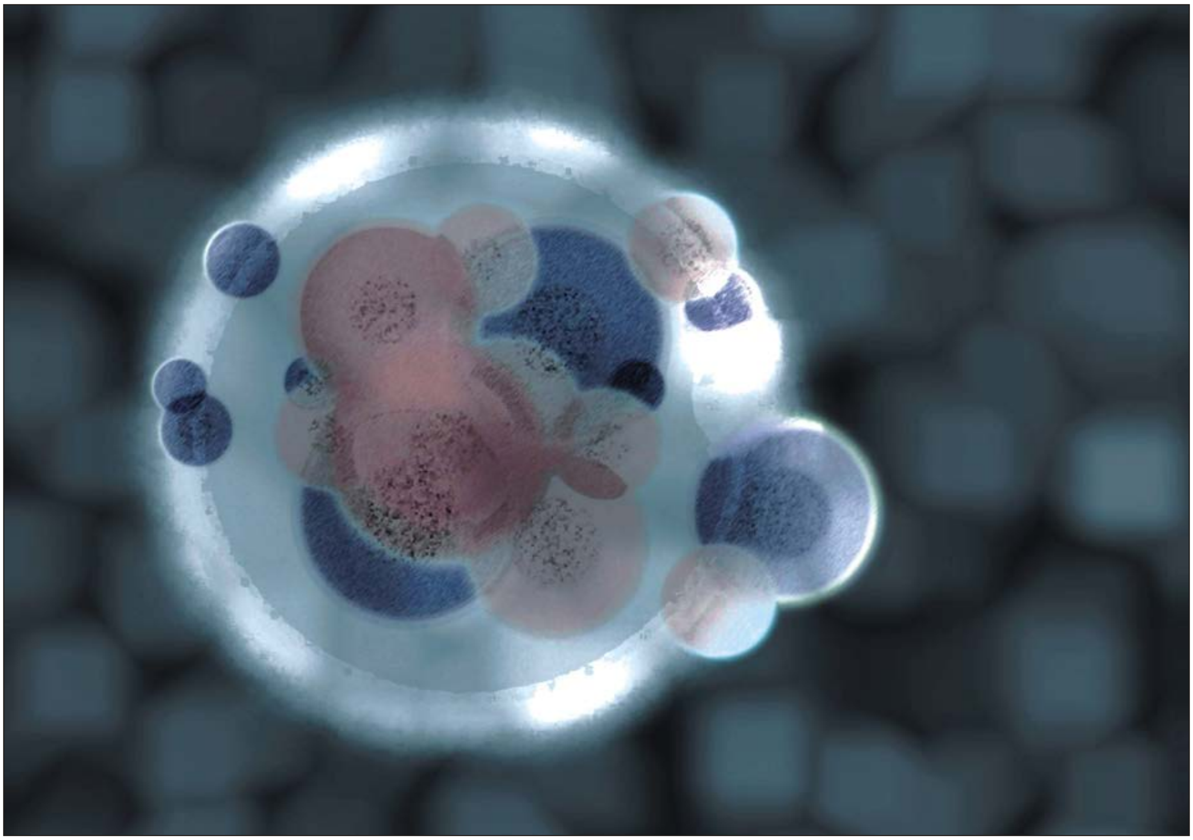

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# Research Article
# Secondary Metabolites Production in Clove (*Syzygium aromaticum*): Chemical Compounds

[1]Syahran Wael, [2]Tri Rini Nuringtyas, [2]Nastiti Wijayanti and [3]Pudji Astuti

[1]Department of Biology, Universitas Pattimura Maluku, Jl. Ir. M. Putuhena, Poka Ambon, Indonesia
[2]Department of Biology, Universitas Gadjah Mada Yogyakarta Indonesia, Indonesia
[3]Department of Veterinary Medicine, Universitas Gadjah Mada Yogyakarta Indonesia, Indonesia

## Abstract
**Background and Objective:** Clove (*Syzygium aromaticum* (L.) Merr. and Perry) used in folk medicinal and traditional plant to treat various diseases. This plant is widely used and has commercial value because the content of secondary metabolite compounds. Differences in the compound content of the plant have contributed to the taxonomic position of the plant. There are many varieties of cloves, i.e., zanzibar, sikotok, siputik and raja. This research was aimed to analyze compounds of cloves var. zanzibar, sikotok, siputik, raja and their phylogenetic relationship. **Materials and Methods:** The material used leaves of *Syzygium aromaticum* (Linn.) Var. zanzibar, sikotok, siputik and raja was taken from the plantation of Negeri Lima, Maluku, Indonesia. Extraction using maceration method with n-hexane pro-analysis solvent. Chemical compounds were determined by using GC-MS. GC-MS data from 4 varieties of clove plants were analyzed using SPSS version 20 statistical software using cluster analysis based on UPGMA method (unweighted paired group method with arithmetic mean). **Results:** Varieties zanzibar contained 5 compounds, i.e., eugenol (70.43%), β-caryophyllene (16.79%), α-humulene (3.05%), caryophyllene oxide (2.07%), tetratetracontane (3.12%). Var. raja compounds consisted of 77.24% eugenol, 16.15% β-caryophyllene, 1.52% α-humulene, 5.08% caryophyllene oxide. Var. sikotok consisted of 16.50% β-isoprophylideneglycerol, 48.33% eugenol, 28.80% β-caryophyllene, 2.97% α-humulene, 3.40% caryophyllene oxide. Var. siputik consisted of 80.15% eugenol, 13.44% β-caryophyllene, 1.50% α-humulene, 4.90% caryophyllene oxide. Similarity values are found in var. siputik and raja with a similarity index of 0.25 followed by zanzibar with siputik 11.9 and zanzibar with sikotok 14.34. **Conclusion:** The highest compound content is 80.15% eugenol found in var. siputik followed by 28.80% β-caryophyllene compound in var.sikotok, α-humulene compound 3.05% in var.zanzibar and 5.08% caryophyllene oxide compound in var.raja.

Key words: Clove, *Syzygium aromaticum*, chemical compound, metabolit sekunder, GC-MS, UPGMA

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Corresponding Author: Syahran Wael, Department of Biology, Universitas Pattimura Maluku, Jl. Ir. M. Putuhena, Poka Ambon Indonesia



Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Clove (*Syzygium aromaticum* (L.) Merr. and Perry) (synonym: *Eugenia caryopyllata*) is one of the plant that have secondary metabolites that are widely used in the world of health[1]. The main secondary metabolites of cloves consist of eugenol, β-caryophyllene and eugenol acetate belonging to the terpenoid group[2]. Clove are tropical medicinal plants[3] included in the Myrtaceae Family[4], that is native from the Maluku islands in east Indonesia[5]. The Maluku people recognized of cloves such as siputik, sikotok, zanzibar and raja and have been used at traditional medicinal herbs to cure various diseases. Clove would survive tens even up to hundreds of years and the results can be enjoyed for generations[6].

Maluku cloves contain different secondary metabolites with the highest content of compounds was found in var.zanzibar. Previous studies used gas chromatography method with methanol solvent reported that eugenol 88.58% in zanzibar, 76.10%, in sikotok and 70.97% in siputik[7]. The bioactive compounds are eugenol, β-caryophyllene and volatile compounds[8] and known to have pharmacological functions such as anesthetic, antimicrobial, antiseptic, stimulant, antioxidant, aphrodisiac, immunomodulator, anti-inflammatory and toothache[6,9].

The part of the clove plant contains different compounds ranging from flowers, leaves, stems and roots with the highest in flower[10]. It contains phenolic compounds[8] such as simple phenols, phenolic acids, phenyl acetic acids, cinnamic acids, coumarin, lignans, flavonoids, lignins, tannins, benzophenones, stilbene, quinones and betacyanin[11]. These compounds act as antioxidant[9],higher potential of free radical-resistance activity than vitamin C[2], inhibits lipid peroxide[10], maintains activity superoxide dismutase enzymes, catalase and glutathione peroxidase[12]. The highest content of clove compounds is eugenol found in flower of 87-96%, β-caryophyllene is highest in leaves 11-19%, the highest eugenol acetate is found in clove leaves[5] 8-21%.

Secondary metabolites of cloves in Maluku are not much known, in fact, it is a taxonomic character that can be used as markers to determine species or populations. Meanwhile, environmental factors can cause differences in morphological characters. Study on chemotaxonomy may involve secondary metabolites such as flavonoids (phenols), terpenes, alkaloids, lignans, sterols, fats and tannin[1]. Chromatography gas analysis of clove leaf compound found 23 compound that was dominated of eugenol (76.8%), β-caryophyllene (17.4%), α-humulene (2.1%), eugenol acetate (1.3%), limonene (0.1%), methyl salicylate (0.1%), methylchavicol (0.2%), chavicol (0.1%), α-famesen (0.1%), caryophyllene alcohol (0.1%), caryophyllene oxide (0.4%), α-humulene epoxide (0.1%) and cis-limonene oxide, trans-limonene oxide, methyleugenol, cis-isoeugenol, α-cloven, cis-methylisoeugenol, cis acetate and trans-isoeugenyl acetate[3].

Potential metabolite clove is not well known to the public and to add value to its usefulness, some research on the search for active secondary metabolite compounds has been done government and institutions. The importance of research on the search for active substances of secondary metabolite compounds from cloves is caused as a source of raw materials of drugs that can be utilized for the benefit of industry and medical. Previous research on secondary metabolite of cloves is limited to one clove var. zanzibar, while others such as siputik, sikotok and raja have not been done. This study was used to compare the profile of chemical compounds in 4 varieties (zansibar, sikotok, siputik and raja) and their phylogenetic in the collection of clove plantation of Negeri Lima, Maluku, Indonesia.

## MATERIALS AND METHODS

**Collection of plant materials:** The material used in this research were clove leaves var. zanzibar, sikotok, siputik and raja, which were collected on January 25, 2017 from Negeri Lima plantation, Maluku, Indonesia. Each of clove leaves were taken from the 3rd to 7th leaf from the top. They were dried in sunless and powdered, respectively and stored in tight plastic containers for further use.

**Plant extract preparation:** The secondary metabolite compounds of clove leaf extract by maceration method using n-hexane pro-analysis solvent from April 6 to May 13, 2017 which was held at FALITMA Laboratory Faculty of Biology UGM. Materials have been dried grinded by blending. Total of 900 g of clove leaf powder was macerated with 1200 mL of solvent n-hexane pro-analysis for three days with occasional stirring. The extract obtained pasta was 23.19 g so that 2.57% of immersion.

**Method:** Evaluation of the chemical compound of clove leaf extract from 4 varieties of clove using GC-MS was carried out at the Organic Chemistry Laboratory of the Faculty of Mathematics and Natural Sciences UGM from June-August 2017. Gas chromatography spectrometry is a combination of two methods in the analysis of compounds namely gas chromatography to detect compounds and their intensity and mass spectrometry to determine the kinds of compounds.

Clove leaf extract was tested with GC-MS to separate the mixture of compounds contained in the extract in a fast and accurate time. Mass spectrometry will detect molecular components that have been separated on gas chromatography. The mass spectrometry is capable of producing the tested ion beam, choosing the ion to be a spectrum corresponding to the mass ratio to the charge and recording the relative abundance of each ion type present. The results of the combination of these two methods were processed by computer and presented in graphical form. The level of the compound was determined by the standard peak area compared to the peak area of the sample. The results of this combination of methods were processed by computer and presented in graphical form. The graph consists of peaks at certain retention times and was accompanied by spectroscopic results in the form of mass spectra and molecular weights.

The GC-MS analysis specification uses HP5 column (5% phenyl metyl siloxane) 30 m, with an initial column temperature of 100°C and a final temperature of 280°C, a baseline time of 5 min, an increase of 5°C $min^{-1}$. In this analysis used FID (Flame ionization detector) with temperature detector 300°C, injection temperature 280°C and gas carrier helium. Total flow 10 and split 60 with number of injection every 1 µL.

The extract was injected into 0.5 µL syringe of GC-MS QP2010S SHIMADZU, the temperature of the column was set to 120-310°C with a 10°C increase per minute, helium carrier gas was set to 13.7 kPa pressure, an electron impact ionizing detector. The spectrometer mass was used to detect the component molecules that have been separated on gas chromatography and then matched with GC-MS Data bank Library. Chromatography gas detects compound and determines was concentration on the sample of gas mixture. Trailers in the vapor form through the gas stream into the separation column and that it arrives at the detector. The detector produced a signal according to the number of compounds delivered by the carrier gas. Results from gas chromatography were visualized as chromatogram profiles with different time retention. Meanwhile, mass spectrometry was used to obtain molecular weight based on mass ratio with ion charge.

**Data analysis:** GC-MS data from 4 varieties of clove plants were analyzed using SPSS version 20 statistical software. The categorization of the four varieties was tested by cluster analysis based on the UPGMA method (Unweighted paired group method with arithmetic averages).

## RESULTS AND DISCUSSION

**Identification of clove leaf extract compounds with GC-MS:** Chromatography is a technique of separating the compound based on the difference of affinity between the mobile phase and the stationary phase of the compound to be separated[13]. Gas chromatography is used to analyze volatile chemical compounds such as cloves. The four varieties of clove studied have compounds belonging to the terpenoid group comprising the secondary metabolite class with the most members groups found sesquiterpene compound group in 4 varieties that is compound β-caryophyllene, β-isoprophylideneglycerol, caryophyllene oxide and α-humulene[14]. Groups of sesquiterpenes found in all four varieties are composed of isocaryophyllene compounds such as caryophyllene, α-caryophyllene, β-caryophyllene and caryophyllene oxide[1]. Eugenol is a major essential oil component in the clove and that is useful as anti-bacterial, anti-inflammatory, anti-tumor, pesticides and so on[15].

The results showed that there are difference in the amount of compounds content of clove leaf extract. Eugenol, β-caryophyllene, α-humulene and caryophyllene oxide found in 4 varieties with different values. GC-MS analysis in Fig. 1, shows that var. zanzibar contained 5 compounds consisted of peak (1) 70.43% eugenol ($C_{10}H_{12}O_2$) with molecular weight 164, peak (2) 16.79% β- caryophyllene ($C_{15}H_{24}$) with molecular weight 204, peak (3) 3.05% α-humulene ($C_{15}H_{24}$) with molecular weight of 204, peak (4) 2,07% with caryophyllene oxide ($C_{15}H_{24}$) molecular weight of 220, peak (5) 3.12% tetratetracontane ($C_{44}H_{90}$) molecular weight 618.

GC-MS analysis of var. raja in Fig. 2, containing 4 compounds consisted of peak (1) 77.24% eugenol ($C_{10}H_{12}O_2$) with molecular weight 164, peak (2) 16,15% β-caryophyllene ($C_{15}H_{24}$) with molecular weight 204, peak (3) 1.52% α-humulene $C_{15}H_{24}$) with molecular weight of 204, peak (4) caryophyllene oxide ($C_{15}H_{24}$) 5.08% by weight of molecule 220.

The result of GC-MS analysis of var.sikotok in Fig. 3, contained 5 compounds consisted of peak (1) 16,50% β-isoprophylideneglycerol ($C_6H_{12}O_3$) with molecular weight 132, peak (2) 48.33% eugenol ($C_{10}H_{12}O_2$) with molecular weight 164, peak (3) 28.80% β-caryophyllene ($C_{15}H_{24}$) with molecular weight 204, peak (4) 2.97% α-humulene ($C_{15}H_{24}$) with molecular weight 204, peak (5) caryophyllene oxide ($C_{15}H_{24}$) 3.40% molecular weight 220.

The results of GC-MS analysis of var. siputik in Fig. 4, contained 4 compounds consisted of peak (1) 80,15% eugenol ($C_{10}H_{12}O_2$) with molecular weight 164, peak (2) 13,44%

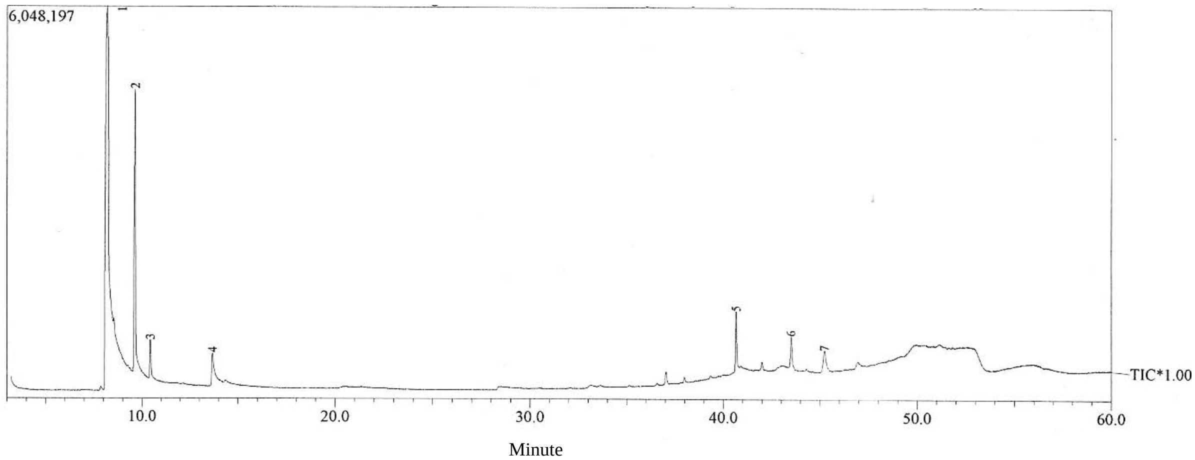


Fig. 1: Histogram clove leaf extract of var.zanzibar (Peak 1 Eugenol, peak 2 β-caryophyllene, peak 3 α-humulene, peak 4 caryophyllene oksida, peak 5 tetratetracontane)

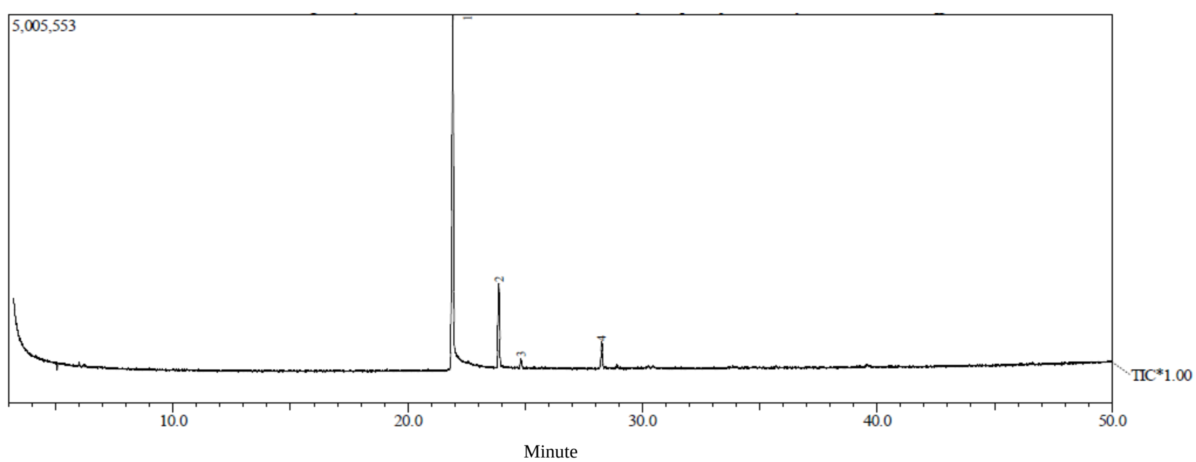


Fig. 2: Histogram clove leaf extract of var.raja (Peak 1 Eugenol, peak 2 β-caryophyllene, peak 3 α-humulene, peak 4 caryophyllene oksida)

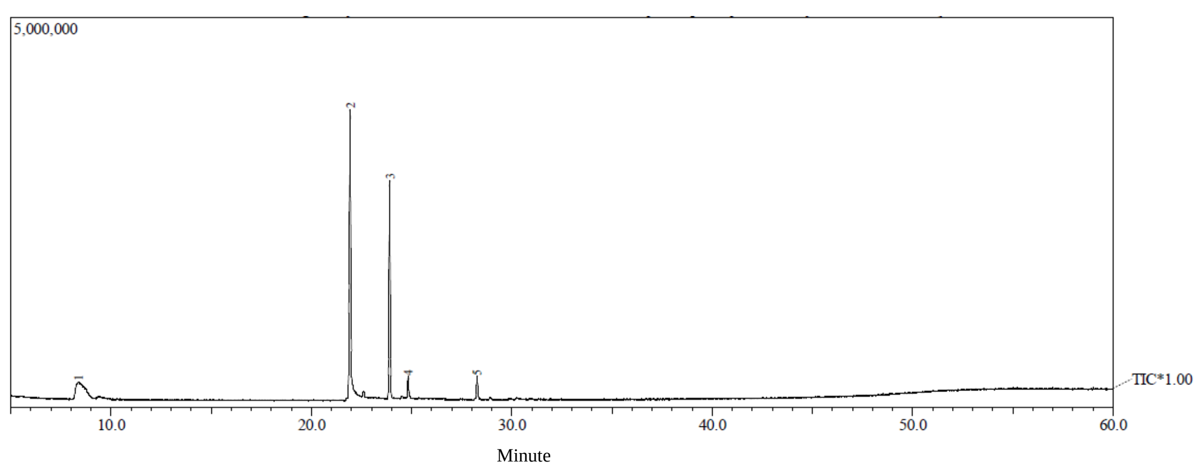


Fig. 3: Histogram clove leaf extract of var.sikotok (Peak 1 β-isoprophylideneglycerol peak 2 Eugenol, peak 3 β-caryophyllene, peak 4 α-humulene, peak 5 caryophyllene oksida)

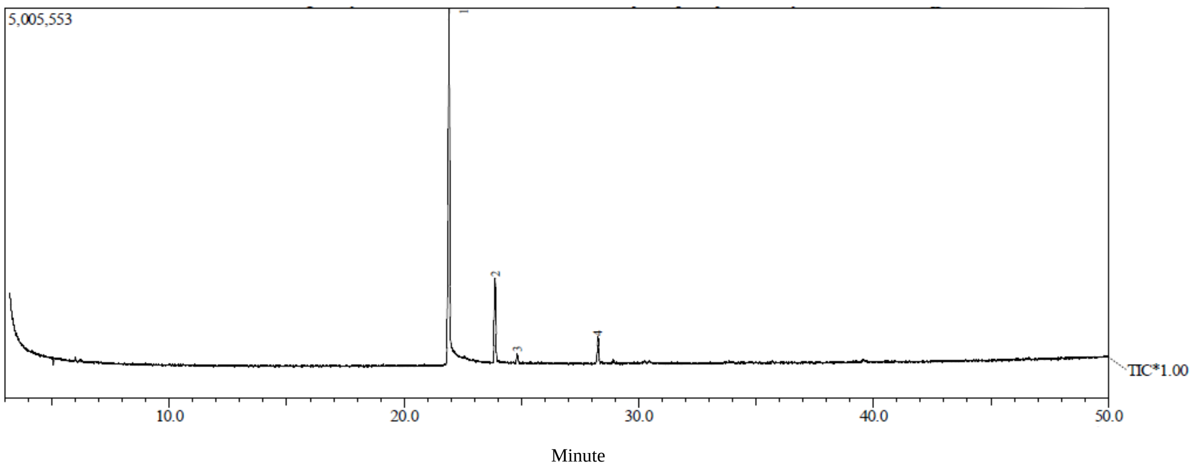


Fig. 4: Histogram clove leaf extract of var.siputik (Peak 1 Eugenol, peak 2 β-caryophyllene, peak 3 α-humulene, peak 4 caryophyllene oksida)

β-caryophyllene ($C_{15}H_{24}$) with molecular weight 204, peak (3) 1,50% α-humulene $C_{15}H_{24}$) with molecular weight of 204, peak (4) 4,90% caryophyllene oxide ($C_{15}H_{24}$) molecular weight 220.

The eugenol compound ($C_{10}H_{12}O_2$)was found to be highest in all four varieties compared to other compounds. Eugenol is a derivative of the allyl-substituted guaiacol, including a group of phenol compounds[16]. Groups of phenol compounds are widely found as secondary metabolite products in the plant[1]. The content of eugenol compound in cloves consists of var.zanzibar of 70.43%, var.raja 77.24%, var.sikotok 48.33% and var.siputik 80.15%.

The eugenol compound is an essential oil component derived from phenylpropene[1]. This compound is known for its pharmacological properties, such as analgesics, local anesthetics, anti-inflammatory, antimicrobial, aphrodisiac, antioxidant and antitumor[9]. In addition eugenol and often used as a food preservative. Some eugenol derivatives such as methyleugenol and eugenol acetate are available as perfume components, food taste additives and attractant compound were used for observation of mating behavior in male animals[17]. The tetratetracontane compound is only identified in the var.zanzibar and the β-isoprophylideneglycerol compound in the var.sikotok. The β-isoprophylideneglycerol compound is a phenylpropene compound whose structure is composed of a benzene ring with substituted hydroxy and profenil groups[2]. This compound is widely used as a perfume.

Prior research used leaf clove from India with GC-MS method of acetone solvent showed that eugenol compound 80.19%, eugenyl acetate 7.91%, caryophyllene 3.79%, tetrahydro 3 methyl 2.26% and methylhydrazine 1.54%[9]. Clove compound tested in three different countries namely Madagascar, Indonesia and Zanzibar used gas chromatography, in Madagascar found eugenol 91.81-96.65%, β-caryophyllene 1.66-4.48%, α-humulene 0.22-0.79%, eugenyl acetate 0.37-2.53%, caryophyllene oxide 0.14-0.6%, in Indonesia found eugenol 88.76-89.28%, β-caryophyllene 7.40-7.75%, α-humulene 0.93-1.31%, eugenyl acetate 0.07-0.17%, caryophyllene oxide 0.20-0.26%, in Zanzibar found eugenol 87.52-89.47%, β-caryophyllene 7.19-9.70%, α-humulene 0.75-1.08%, eugenyl acetate 0.55-0.88%, caryophyllene oxide 0.25-0.68%[5], from Bangladesh compound of eugenol 74.28%, eucalyptol 5.78%, caryophyllene 3.85%, α-cardinol 2.43%, limonal 2.08% while clove shoots eugenol 49.7%, caryophyllene 18.9%[4].

This study found secondary metabolites from four varieties of clove with different compositions. In var.raja, obtained eugenol 77.24%, β-caryophyllene 16.15%, α-humulene 1.52%, caryophyllene oxide 5.08%, var.siputik eugenol 80.15%, β-caryophyllene 13.44%, α-humulene 1.50%, caryophyllene oxide 4.90%, var.sikotok eugenol 48.33%, β-caryophyllene 28.80%, α-humulene 2.97%, caryophyllene oxide 3.40%, var.zanzibar eugenol 70.43%, β-caryophyllene 16.79%, α-humulene 3.05%, caryophyllene oxide 2.07%. The results of this study revealed that secondary metabolites of clove found were not much different from cloves from Bangladesh, Madagascar and India, although their research did not mention secondary metabolites of these types of cloves. Composition secondary metabolites of clove in this study is higher in compounds compared to regions from Lombok, Toli-Toli, Minahasa and Bali. Clove from Lombok obtained eugenol 67.27%, β-caryophyllene 9.43%,


Fig. 5(a-d): Morphology of leaf and flower of four varieties *S. aromaticum* in plantation Negeri Lima, Maluku, Indonesia (a) Siputik, (b) Sikotok, (c) Zanzibar and (d) Raja

Table 1: Matrix similarity clove on phytochemical

| | Zanzibar | Siputik | Sikotok | Raja |
|---|---|---|---|---|
| Zanzibar | 0.000 | | | |
| Siputik | 11.909 | 0.000 | | |
| Sikotok | 14.348 | 17.955 | 0.000 | |
| Raja | 11.890 | 0.215 | 15.682 | 0.000 |

α-humulene 1.24%, caryophyllene oxide 2.22%[18], from Toli-Toli obtained compounds eugenol 66.37%, β-caryophyllene 15.38%, α-humulene 1.97%, eugenol acetate 12.99%, chavicol 0.18% and caryophyllene oxide 0.47%, from Bali obtained compounds of eugenol 72.34%, β-caryophyllene 12.51%, α-humulene 2.34%, eugenol acetate 5.33%, chavicol 0.25% and caryophyllene oxide 0.18%[19].

Currently, the types of clove in Indonesia consist of var.zanzibar, var.sikotok, var.raja and var.siputik. Evaluation of eugenol compound in these three clove showed different content, eugenol on var. zanzibar 88.58 mg $g^{-1}$, var.sikotok 76,10 mg $g^{-1}$ and var.siputik 70,97 mg $g^{-1}$ [7]. This indicated that morphological contrast is different. Clove plant in Maluku more var.sikotok whilst var.siputik mostly in Sumatera[20].

The morphology of clove var.siputik in Fig. 5 would identified from yellow leaf buds to light green, yellowish green stalks, old green leaves and large, almost shiny, shady trees, yellow flowers, large, less than 15 flowers[20]. The sikotok have small leaf blades, the leaves are easily reddish, the petioles and the branches are easily yellowish green, the old leaves are small green and slightly shiny, the trees are very shady, dense, the flowers of the bunches exceed 15 flowers and are yellow while the base slightly red[21]. Varieties zanzibar have long leaf strands, red to pink leaf buds, leaflets and young branches of red, dark green leafy leaves, small shiny leaves, very shady trees, number of flowers more than 15 flowers and colored red. Varieties raja of the leaves are rather large, the leaves are dark green, the flowers are larger, the number of each tanden is not much, the tree is not shady[20]. Clove plant in Maluku more var.sikotok and raja. Varieties raja grow wild in the forest and flowering twice a year. while zanzibar, sikotok and siputik flowering once year.

The phytochemical character of 6 accessions of clove plants species has the composite components and the intensity of the compounds. The phytochemical character of the leaves is dominated of terpenoid compounds. Different components of the compounds can be influenced by two factors, namely genetic and environmental. Secondary metabolites function in plant defense mechanisms in the environment where the environment is a response to environmental influences in the form of pests and diseases or drought[22]. In this study the four varieties clove were taken from the clove plantation of Negeri Lima, Maluku so it was considered homogeneous, thus it can be concluded that the secondary metabolite is influenced by genetic factors.

Based on quantitative data of GC-MS profile, the calculation of the similarity index in all clove was followed by analysis of UPGMA cluster (Unweighted paired group method with arithmetic averages). Cluster analysis is a multivariate analysis used in analyzing phytochemicals in taxonomy. In the cluster analysis of GC-MS data, the calculation of the similarity index on 4 varieties leaf was tested. The calculation of the similarity index is done based on the correlation coefficient. The result of the calculation is a similarity matrix. The similarity matrix is then further analyzed of UPGMA clustering algorithm or average linkage to obtain the result of dendrogram. The similarity matrix and clonal analysis dendrogram of the four varieties can be seen in the Table 1.

Based on Table 1 it can be seen that the closest similarity values are found in siputik and raja varieties with a similarity index of 0.25 followed by zanzibar with siputik 11.9 and zanzibar with sikotok 14.34 and in Fig. 6, dendrogram the four varieties of clove clustered at a low similarity index value. The grouping of these four accessions is based on the

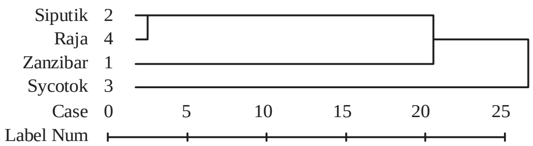


Fig. 6: Dendrogram cluster analysis (UPGMA) phytochemical data of clove

phytochemical character of the clove leaf compound. The cluster analysis found that the four clove accessions were clustered into 2 clusters, namely cluster 1 consisting of siputik, raja, zanzibar and cluster 2 consisting of sikotok. The chemical compounds of each species are characteristic of the adaptation of the species to the environment. The character of the plant chemical compound becomes the differentiat or that can be used as evidence in the classification. Research on the nature of chemical compounds is useful in explaining the evolutionary relationships of each clove.

The other research on phylogenetic of 28 accession of 15 year old clove plant in Sukapura experiment garden based on var.zanzibar morphology characteristic 19 accession, 5 accession siputik, 2 accession and 2 accession sikotok, each accession was observed 10 adults leaf and 5 flowers shows a wide genetic diversity, except for leaf length and diameter of petals with a diversity coefficient of more than 20%, observation of stalk and flower characters have a coefficient of diversity[22] smaller than 20%. The results of clove plant exploration in Maluku are rich in germplasm. This plant spreads almost all over the Maluku islands. The difference in secondary metabolites in clove plants showed the diversity of genetic factors to the expressed trait. Genetically controlled phenotype traits will be inherited to the next generation although environmental factors also affect the expression.

## CONCLUSION

Secondary metabolites of clove from four varieties (zanzibar, siputik, sikotok and raja) with the highest compound content is 80.15% eugenol found in var.siputik followed by 28.80% β-caryophyllene compound in var.sikotok, α-humulene compound 3.05% in var.zanzibar and caryophyllene oxide 5.08% in var.raja. Results of UPGMA cluster (Unweighted paired group method with arithmetic averages) the similarity matrix and dendrogram of the four varieties of clove showed diversity with the closest similarity values in siputik and raja varieties with a similarity index of 0.25 followed by zanzibar with siputik 11.9 and zanzibar with sikotok 14.34.

## SIGNIFICANCE STATEMENT

This study discovers the possible different secondary metabolites in four varieties of clove that can be beneficial for synthesize eugenol, β-caryophyllene, α-humulene caryophyllene oxide. This study will help the research to uncover the critical areas of synthesizing natural compounds in which many researchers were not able to explore. Thus, a new theory about the different composition of secondary metabolites in four varieties of clove and the possibility of other secondary metabolites can be obtained.

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