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Detemination of Kinship Among Orange Cultivars (*Citrus* Sp) Based on Flavonoid Profiles

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ABSTRACT

Orange (*Citrus* sp.) is a type of plant with a superior flavor and aroma of the fruit that satisfies many tastes in Indonesian society. As one of the exceptional fruit commodities, it has economic advantages and vast marketing. Increase public awareness of the need for a source of nutrition and the benefits generated by the orange as having antioxidant, antibacterial, antiviral, hypo-allergenic, and anticancer activity, as well as industrial raw materials such as jam, juice, syrup, and deodorizer. To measure the kinship of orange species based on flavonoid content. Exploration was carried out in a basic chemical laboratory; the parameters measured were the flavonoids by using the wavelength of maximum absorption of the UV-Vis spectrophotometry of citrus flavonoids. Furthermore, profile flavonoids with UV-Vis spectrophotometry include absorption bands of the type of flavonoid. Data analysis and results of chemical constituents in the form of a binary data matrix. After that, proceed with the analysis SIMQUAL (*Similarity of Qualitative Data*) to obtain a similarity matrix between phenetic cultivars of orange. The phenetic similarity matrix and the clustering analysis method *(Sequential Agglomerative Hierarchical and Nested),* the SAHN-UPGMA *(Unweighted Pair-Group Method Arithmetic Average)* program, and *NTSys-PC* version 2.0 were used to construct the phenetic family tree (dendrogram). The result showed that by using the UV-Vis spectrophotometry profile of flavonoids, a dendrogram is formed of two main clusters, namely cluster I, which consists of sweet orange cultivars, Kisar grapefruit, sour orange, lemon, suanggi orange, and pomelo. Cluster II consists of limau orange and lime cultivars. Furthermore, flavonoid profiles using UV-Vis spectrophotometry indicate that Kisar grapefruit and lemon tart have a coefficient of 0.88, which means close kinship with limau and lime, which have a coefficient of 0.88, meaning close kinship with cluster II. NaOH reagent 40%, $AlCl_3$ 5%, and NH_3 25% can produce color changes that indicate the presence of flavonoid content, while using distilled water does not. The kinship test consists of 2 clusters that have a difference of 0.52 (52%)

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INTRODUCTION

Orange (Citrus sp.) is one of the leading fruit crops in Indonesia, valued for its flavor and aroma, which appeal to the tastes of many Indonesians. Oranges exhibit high genetic diversity, as reflected by the numerous members of the *Citrus* genus, such as *C. nobilis* syn. *C. reticulata* (Siamese orange), *C. aurantifolia* (Lime), *C. limon* (Lemon), *C. reticulata* Blanco (Mandarin orange), *C. aurantium* (Sour orange), *C. grandis* (Bali orange), *C. maxima* (Pomelo), *C. hystrix* (Kaffir lime), *C. bergamia* (Bergamot), *C. nobilis* Lour. (Tangerine), *C. amblycarpa* (Sambal orange), and *C. medica* (Citron or Kates orange). Increasing public awareness of the importance of nutrition has drawn attention to the health benefits of citrus fruits, which include antioxidant, antibacterial, antiviral, anti-inflammatory, antiallergic, and anticancer properties. These biological effects are primarily attributed to flavonoids—bioactive compounds found abundantly in citrus fruit peels, seeds, and pulp. The main flavonoids present in citrus are naringin, narirutin, and hesperidin.

Biodiversity studies, both at the national and international levels, have gained increasing attention. These studies encompass diversity both within and between species, as well as within and between populations. The study of inter-species relationships has advanced to include analyses of genome structure and evolution (Purwanto et al., 2002). To obtain accurate taxonomic data, phenetic methods grouping organisms based on observable similarities are now widely used. Phenetics involves organizing data based on shared characteristics, which is essential for plant classification. In addition to morphological features, chemical content can also serve as a reliable basis for classification. The use of chemical profiles to study relationships among species (inter-specific) or within species (intra-specific) is known as chemotaxonomy (Wahyu et al., 2005). Research on the kinship relationships among citrus cultivars based on flavonoid content is still limited.

diversity of citrus cultivars in Indonesia, especially in regions such as Ambon where various local types are widely cultivated, there is a pressing need to explore their biochemical characteristics as a complement to morphological studies. Understanding the flavonoid profiles of different citrus cultivars not only contributes to better classification and conservation efforts but also supports the development of functional foods and herbal-based health products. Moreover, accurate kinship analysis can aid breeding programs aimed at improving fruit quality, disease resistance, and adaptability to environmental stress (Purwanto et al., 2002). Chemotaxonomic approaches with modern analytical techniques such as UV-Vis spectrophotometry, researchers can uncover detailed flavonoid patterns that reflect evolutionary relationships and potentially uncover unique compounds with pharmacological value. Therefore, a study focusing on the chemotaxonomic relationship of citrus cultivars based on their flavonoid content is both timely and significant in advancing citrus biodiversity research and its practical applications.

However, such studies are necessary to complement morphological approaches and to enhance the understanding of citrus diversity. Given the extensive diversity of citrus cultivars found in Ambon, it is important to conduct research aimed at reorganizing and clarifying the classification of these cultivars. This can lead to improved taxonomic positioning and a better understanding of kinship relationships within the genus *Citrus*.

MATERIALS AND METHOD

Materials. The plants used in this study are citrus cultivars found in Ambon and Kisar which include *Citrus aurantium* (L.) (sour orange); Citrus *aurantifolia* (Cristm.) Swingle (lime); *Citrus medica* (L). (Suanggi orange); Citrus *maxima* (Burm) Merr (Big Orange); *Citrus sinensis* (L.) Osbeck (Sweet Orange), *Citrus limon* (L.) Burm.f. (Limon Orange), *Citrus hystrix* D.C. (Kaffir Lime) and *Citrus* sp. (Kisar Orange) were collected from Hutumuri (home yard and garden) and Kisar. Chemicals (reagents) used to identify flavonoids in the form of ammonia, NaOH 40%, and $_{AlCl3}$ 5%, to extract flavonoid content using chloroform and for the analysis of components and flavonoid groups in this study were silica gel $_{GF254}$ plates (E.Merck), mobile phase in the form of n-hexane: ethyl acetate (3: 1 v/v), general spray reagent (cerium (IV) sulfate) and ammonia solution.

Methods. The first step in flavonoid identification involves preparing all the necessary tools and materials. The citrus fruits selected for the study must be in good condition, free from any damage or deformities. Once selected, the fruits are thoroughly cleaned using running water to remove any dirt or debris and then allowed to drain. The cleaned fruit is then squeezed, and the juice is collected in an Erlenmeyer flask. Using a measuring pipette, 1 mL of the juice extract is measured and transferred into a labeled test tube, with each test tube corresponding to a specific citrus cultivar.

The identification process is then carried out using both reagent and non-reagent methods. For the non-reagent method, the extract is placed on a glass slide and mixed with five drops of distilled water. For the reagent method, the extract is similarly placed on a glass slide and treated with five drops of each identification reagent. The reagents used for flavonoid detection include ammonia solution, 40% NaOH, and 35% $AlCl_3$. After the reagents are added, any resulting color changes in the citrus extract are carefully observed, recorded on an observation sheet, and documented. These steps are repeated for each citrus sample with different reagents until all cultivars have been tested. The final step involves comparing the color changes before and after reagent application to determine the presence and reaction of flavonoid compounds in each sample. Extraction. Each fresh citrus specimen was peeled, and the fruit segments (grains) were separated and weighed to obtain approximately 100 grams of sample. The sample (simplisia) was then crushed and placed into an Erlenmeyer flask. To extract the flavonoid compounds, 100 mL of chloroform was added to the flask, which was then sealed tightly using plastic wrap. The maceration process was carried out for 24 hours. Afterward, the mixture was concentrated using a vacuum rotary evaporator set at 55°C until a thick extract was obtained.

The thick extract was allowed to sit briefly to ensure complete evaporation, after which it was transferred to a clean Erlenmeyer flask. The flask was covered with filter paper and stored in a desiccator until the extract settled and formed a layer resembling citrus peel at the bottom of the flask. For the UV-Vis spectrophotometric analysis, the dried extract was dissolved in 30 mL of methanol and labeled accordingly in separate beakers. A 3 mL aliquot of each solution was placed into a cuvette and analyzed for its absorbance within the UV-Vis wavelength range of 180-780 nm.

Data Analysis

The analysis of flavonoid content in citrus was carried out using both microchemical color tests and UV-Vis spectrophotometry, followed by kinship analysis among citrus cultivars. In the microchemical identification of flavonoids, the data obtained were analyzed descriptively. Citrus extracts that reacted positively to flavonoids were indicated by a color change after the addition of specific reagents. A 25% ammonia solution produces a yellow coloration when reacting with flavone and flavonol groups, while a 35% $AlCl_3$ solution results in a pale yellow or orange hue when reacting with flavones. A 40% NaOH solution also produces noticeable color changes. To assist in identifying and differentiating these yellow and orange shades, the R Color Chart Sorted by Hue, Saturation, and Value was used as a visual reference.

The UV-Vis spectrophotometric profile of flavonoids was analyzed by observing the absorption wavelengths formed. Identification of the flavonoid types was based on their maximum absorbance wavelengths using the reference chart Maximum Absorption Wavelength UV-Vis Spectrophotometry to determine the kinship relationships among citrus cultivars, the absorbance bands obtained from UV-Vis analysis were used as distinguishing characters. These bands were tabulated into a binary matrix, where the presence of a specific absorbance band was given a value of 1, and its absence was marked as 0.

The resulting data were used to construct a dendrogram using numerical methods based on the association coefficient clustering approach. The degree of similarity was determined through cluster analysis, following the methodology of Sneath et al. (1975) and Purwanto et al. (2005). The phenetic relationships were analyzed through character coding, and the data were processed using the Numerical Taxonomy and Multivariate Analysis System (NTSys) version 2.0 (Gengler, 2002), resulting in a visual representation of the kinship among the citrus cultivars.

RESULTS AND DISCUSSION

Microchemical Identification of Flavonoids (Color Test)

Microchemical identification of flavonoids, also known as the color test, is a simple yet effective method used to detect the presence of flavonoid compounds in plant extracts based on characteristic color changes upon reaction with specific reagents. Each reagent interacts with particular flavonoid structures, resulting in visible color changes typically yellow to orange hues depending on the type of flavonoid present. For instance, ammonia reacts with flavones and flavonols to produce a yellow color, while $AlCl_3$ tends to yield a pale yellow or orange shade when reacting with certain flavonoid groups. This color based qualitative analysis provides preliminary evidence for the presence of flavonoid compounds before further quantitative or spectral analysis is conducted. The results of microchemical identification of flavonoids can be seen in **Table 1**.

Table 1. Results of Flavonoid Identification Microchemically (Color Test)

NO	Orange Extract	Reagents			
		Aquades	NaOH 40%	$AlCl_3$ 5%	NH_3 solution 25%
1.	Kaffir lime (*C. hystrix* D.C)	-	+	+	+
2.	Sweet orange (*C. sinensis* (L.) Osbeck)	-	+	+	+
3.	Sour orange (*C. aurantium* (L.))	-	+	+	+
4.	lime (*C. aurantifolia* (Cristm.) Swingle)	-	+	+	+
5.	Kisar orange (*Citrus* sp.)	-	+	+	+
6.	Suanggi orange (*C. medica* (L.)	-	+	+	+
7.	Lemon (*C. limon* (L.) Burm.f.)	-	+	+	+
8.	Big orange (*C. maxima* (Burm.) Merr)	-	+	+	+

Description
(+) = contains flavonoids
(-) = no contains flavonoids

Description of Flavonoid Compound Profile by Spectrophotometry

The results of flavonoid profiling by spectrophotometry (wavelength, absorbance, band, and flavonoid type isolates) can be seen in Table 2. These results provide detailed information about the unique spectral characteristics of each citrus cultivar, including specific absorbance peaks corresponding to different types of flavonoids. By comparing the maximum absorption wavelengths and intensity levels, it is possible to identify the presence and relative abundance of compounds such as naringin, hesperidin, and narirutin. This spectrophotometric data serves as the basis for determining similarities and differences in flavonoid composition among cultivars, which is essential for subsequent chemotaxonomic and kinship analyses.

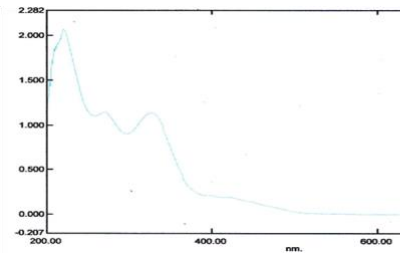
Table 2. Wavelength, Absorbance, Band, and Flavonoid Type Data of*Citrus* Cultivar Isolates (*Citrus* sp.)

No	Orange Extract	wavelength (nm)	Abs.	Ribbon	Types of flavonoids
1.	Kaffir lime (C. hystrix D.C)	420.50	0.191	+	Auron
		413.50	0.191	+	
		326.50	1.139	+	Flavonon, Flavon
		298.00	0.903	+	Bilavonil, Auron, Antosianidin
		270.00	1.145	+	
		258.50	1.103	+	Flavanone Glycoside (Rutin)
		220.50	2.075	+	Flavanone (Naringenin), Flavanone Glycoside (Hesperidin)
2.	Lime (*C. aurantifolia* (Cristm.) Swingle)	483.50	0.008	+	Antosianidin
		334.50	2.962	+	Flavonol
		324.50	2.856	+	Flavon
		316.00	3.286	+	Flavon

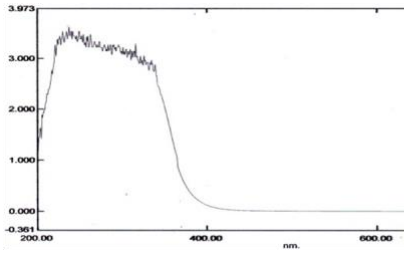
		310.50	2.962	+	Flavon
		295.50	3.263	+	Flavonol (Quercetin)
		288.00	3.068	+	Flavanone (Naringenin)
		279.00	3.263	+	Flavonon
		277.00	3.135	+	Flavonon
		267.50	3.325	+	Flavone (Luteolin)
		264.50	3.215	+	Flavanone Glycoside (Rutin)
		262.50	3.462	+	Flavone (Luteolin) Flavanone Glycoside (Rutin)
		257.50	3.215	+	Flavanone Glycoside (Rutin)
		249.00	3.524	+	Kalkon, Auron, Flavanone Glycoside (Naringin)
		247.00	3.325	+	Kalkon, Auron, and Flavanone Glycoside (Naringin)
		240.00	3.524	+	Kalkon, Auron and Flavanone Glycoside (Naringin)
		231.50	3.311	+	Kalkon dan Auron
		230.00	3.524	+	Kalkon, Auron and Flavanone Glycoside (Naringin)
3.	Kisar orange (*Citrus* sp.)	511.50	0.002	+	Antosianidin
		327.00	0.582	+	Flavon
		293.00	0.430	+	Flavanon dan Bilavonil
		220.50	1.294	+	Flavanone Glycoside (Hesperedin) and Flavanone (Narigenin)
4.	Sour orange(*C. aurantium* (L.))	513.00	0.007	+	Antosianidin
		326.50	0.993	+	Flavon dan Flavonon
		298.00	0.809	+	Isoflavon
		221.50	2.389	+	Flavanon (Naringenin) and Flavanone Glycoside (Hesperidin)
5.	Lemon (C. limon (L.) Burm.f.)	470.00	0.005	+	Antosianidin;
		316.00	1.005	+	Flavon
		299.50	0.918	+	Flavonol (Myricetin) dan Isoflavon
		272.50	0.971	+	Antosianidin dan Bilavonil
		264.50	0.969	+	Flavanone Glycoside (Rutin) and Flavone (Luteolin)
		222.50	2.914	+	Flavanone (Narigenin) and Flavanone Glycoside (Hesperidin)
6.	suanggi orange (C.medica (L.)	500.00	0.007	+	Antosianidin
		338.00	2.960	+	Flavonol dan Flavon
		336.00	2.874	+	Flavonol
		300.50	3.436	+	Flavonon dan Bilavonil
		298.50	3.135	+	Isoflavon
		290.50	3.612	+	Flavonol (Quercetin)
		288.50	3.175	+	Flavanone (Naringenin)
		286.00	3.263	+	Flavonoan and Bilavonil
		278.00	3.175	+	Flavonon

		275.50	3.374	+	Flavonon
		267.00	3.141	+	Flavonol (Kaempferol)
		253.00	3.612	+	Flavonol (Myricetin);
		251.00	3.374	+	Flavonol, Flavon, and Isoflavon
		244.00	3.913	+	Kalkon, Auron dan Flavanone Glycoside (Naringin)
		241.50	3.436	+	Kalkon, Auron, dan Flavanone Glycoside (Naringin)
		235.00	3.612	+	
		227.00	3.311	+	Flavanone (Naringenin) and Flavanone Glycoside (Naringin)
		225.00	3.524	+	Flavanone (Naringenin) Flavanone Glycoside (Hesperidin)
7.	Kaffir lime (C. hystrix D.C)(C. hystrix D.C)	486.00	0.007	+	Antosianidin
		360.00	2.872	+	
		357.00	2.752	+	Kalkon
		355.00	2.985	+	
		349.50	2.791	+	
		338.50	3.039	+	
		333.00	2.800	+	Flavonol
		323.50	3.101	+	Flavon
		314.00	2.914	+	
		304.00	3.215	+	Bilavonil and Flavonon
		299.50	3.073	+	Isoflavon
		294.50	3.414	+	Bilavonil dan
		292.50	3.073	+	Flavonon
		287.00	3.325	+	Flavanone (Naringenin)
		280.00	3.135	+	Antosianidin
		271.00	3.263	+	Antosianidin and Bilavonil
		268.00	3.135	+	Flavone (Luteolin)
		259.50	3.263	+	Flavonol (Quercetin) and Flavanone Glycoside (Rutin)
		259.00	3.215	+	Flavanone Glycoside (Rutin)
		254.00	3.524	+	Flavone (Luteolin)
		240.00	3.325	+	Kalkon, Auron
		238.00	3.612	+	dan Flavanone Glycoside (Naringin)
		230.00	3.215	+	
		227.00	3.436	+	Flavanone (Naringenin) dan Flavanone Glycoside (Naringin)
8.	Big orange (C.maxima (Burm.) Merr)	513.00	0.005	+	Antosianidin
		312.00	2.389	+	Flavon

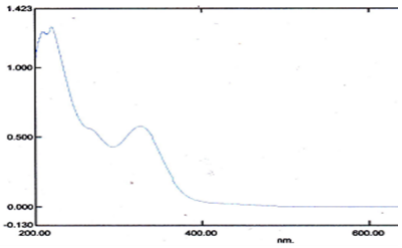
279.50	1.526	+	Flavonon
245.00	3.436	+	Kalkon, Auron,
236.00	3.263	+	Flavanone Glycoside (Naringin)
234.00	3.436	+	
229.00	3.215	+	Flavanone (Hesperetin)
224.00	3.524	+	Flavanone (Narigenin) Flavanone Glycoside (Hesperetin)



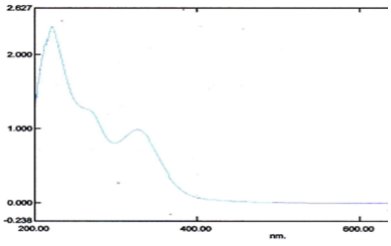
Sweet orange (*C. sinensis* (L.) Osbeck



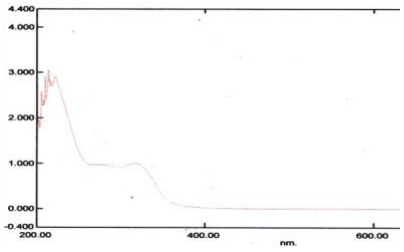
Lime (*C. aurantifolia* Cristm. Swingle



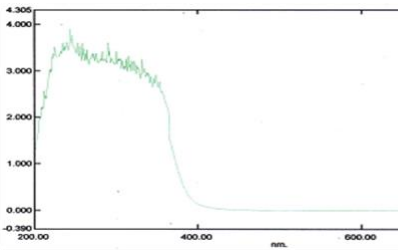
Kisar orange (*citrus* sp)



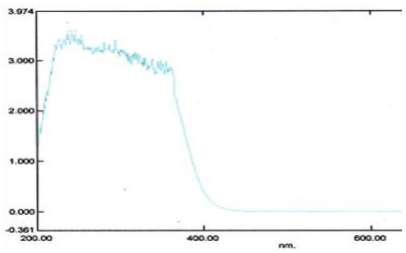
Sour orange (*C. aurantium* (L.))



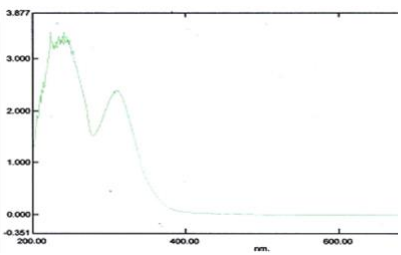
Lemon (*C. limon* (L.) Burm.f.)



Suanggi orange (*C. medica* (L.))



Kaffir lime (*C. hystrix* D.C)



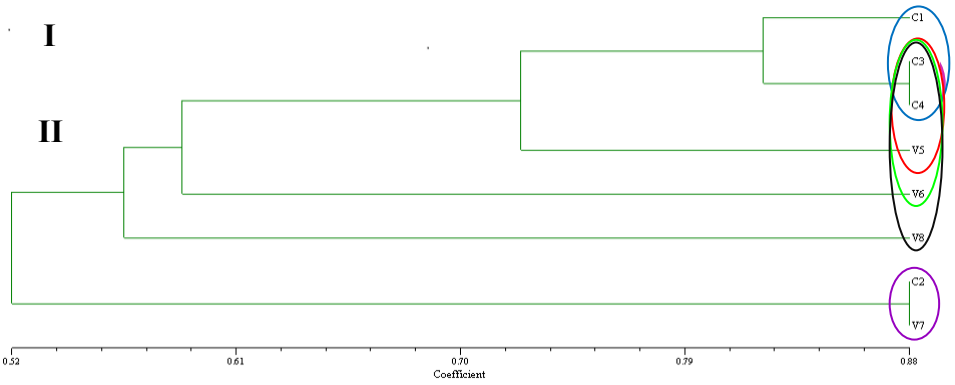
Big orange (*C.maxima* Burm. Merr)

UV-Vis Spectrophotometer Results of Citrus CultivarIsolates

(*Original documentation*)

Kinship Relationship of 8 orange Cultivars Based on Band Profiles

The results of the analysis of citrus kinship based on banding produced a dendrogram that divides 8 citrus cultivars into 2 large clusters that can be seen.



Dendrogram of Orange Cultivars Grouping by Bands

Dendrogram above, 2 main clusters were formed consisting of cluster I and II separated by a coefficient value of 0.52. Cluster I is cultivars C1 (*C. sinensis*), C3 (*C.*sp), C4 (*C. aurantium*), V5 (*C. limon*), V6 (*C. medica*), V8 (*C. maxima*') with a coefficient of 0.57. Cluster II consists of cultivars C2 (*C. aurantifolia*') and V7 (*C. hystrix*) with a coefficient value of 0.88. In cluster I, between cultivars C3 (*C.* sp*)* and C4 (*C. aurantium*), both have a close kinship relationship with a coefficient of 0.88 then between cultivars C3 (*C.* sp*)* C4 (*C. aurantium*), and C1(*C. sinensis*), all three have a distant kinship relationship, the coefficient value is 0.825. Furthermore, between cultivars C3 (*C.* sp*)* C4 (*C. aurantium*), C1 (*C. sinensis*) and V5 (*C. limon*), have a distant kinship relationship, the coefficient value is 0.728. Between cultivars C3 (*C.* sp*)* C4 (*C. aurantium*), C1 (*C. sinensis*) V5 (*C. limon*), and V6 (*C. medica*), the relationship is distant, the coefficient value is 0.597. Then cultivars C3 (*C.* sp*)* C4 (C. *aurantium*), C1(*C. sinensis*) V5 (*C.limon*), V6 (*C. medica*) and V8 (*C. maxima*), have a distant kinship, the coefficient value is 0.57. In cluster II, which consists of two cultivars, C2 (*C. aurantifolia*) and V7 (*C.hystrix*) with a coefficient of 0.88, has a close relationship with cluster II, namely cultivars C3 (*C.* sp*)* and C4 (*C. aurantium*).

Experimental taxonomy is very important in identification efforts, testing classifications that have been made based on morphological traits, knowing kinship relationships, and knowing the effect of the environment on populations. The similarity of chemical content in plants can also be used as a means to classify plants (Purwanto, et al., 2002). Chemotaxonomy is based on Linnaeus concept that plants that have similar morphological characteristics generally also have similar chemical content. Chemotaxonomy is very important, because plant chemical compounds are valuable emperic properties, and can often be used to determine the identity of a plant with precision (Purwanto, et al., 2013). This property does not require that specimens are intact and well stored, even those that have been crushed can be analyzed for chemical content with very satisfactory results and correctly determined taxonomic status, as long as there is no contamination that interferes with the purity of the material (Listyawati, et al., 2001).

Kinship Relationship of 8 orange Cultivars Based on Band Profiles

The phenetic kinship of citrus cultivars is a kinship based on the similarity of characteristics possessed by the citrus cultivar. The higher the percentage of similarity, the closer the kinship of a plant and the lower the percentage of similarity, the further the kinship of a plant. The size of the similarity percentage will be influenced by the extent of variability (Winarti, 2004. Based on the results of the study, there were 17 flavonoid characteristics of the bands to see the phenetic kinship relationship (Table 4) so that a dendrogram of close and distant kinship relationships was obtained, which divided the 8 citrus cultivars into 2 main clusters (Figure 3). Similarity values >85% are classified in groups of plants that have close kinship while similarity values <85% are classified in groups of plants that have distant kinship (Singh, 1999).

CONCLUSION

Based on the results of research conducted on 8 citrus cultivars to identify the flavonoid content of each citrus cultivar, it can be concluded that the microchemical color test using distilled water solvent does not produce color changes that indicate there is no flavonoid content, on the contrary by using the reagent NaOh 40%, $AlCl3_{5\%}$, $_{NH3}$ 25% there is flavonoid content, but the results of this color test do not determine the kinship relationship. Meanwhile, the kinship test of 8 citrus cultivars based on flavonoid bands consisting of 2 clusters where clusters I and II are separated with a coefficient of 0.52 (52%) meaning that they have a distant kinship, because there are 2 types of flavonoids, namely 1 (flavone) and 7 (anthocyanidin).

AUTHORS CONTRIBUTION

Watuguly, T. W., Kakisina, A.G., Lesbatta, K.J designed and conducted the research, analysed and interpretation the data. Thenu wrote the draft of manuscript.

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