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Isolation of Khusimol from the Root of Vetiver (*Vetiveria zizanioides* L. Nash) Grown in Samarang – Garut and the Study of its Profile after Harvesting

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Abstract: Vetiver oil is a type of essential oil extracted from the vetiver plant's roots. Vetiver oil is commonly used as a major odor contributor in the fragrance and aromatherapy industry. This study aimed to isolation khusimol compound from vetiver oil, which will be used as a marker and determine khusimol content in various drying times after harvesting. The target compound was found in the combination of fraction 3 (GF-3). Furthermore, GF-3 was subfractionated by classical column chromatography. Structure elucidation of isolate X was performed by NMR. ^{1}H-NMR and ^{13}C-NMR data of isolate X as the same as ^{1}H-NMR and ^{13}C-NMR data of khusimol compound in literature. Molecular formula of khusimol $C_{15}H_{24}O$. Khusimol content in extract with various drying times after harvest time 0 hr, 6 hr, 12 hr, 24 hr, 36 hr and 48 hr were 1.95 ± 0.02; 2.15 ± 0.02; 2.30 ± 0.03; 2.81 ± 0.01; 1.72 ± 0.03 and 1.38 ± 0.02 mg/100 g. The odor contributor compound was khusimol ($C_{15}H_{24}O$). The highest khusimol content was revealed by vetiver extract, which drying for 24 hr after harvest. Drying vetiver more than 24 hr could reduce khusimol content.

Keywords: vetiver oil; Vetiveria zizanioides; Java vetiver oil; khusimol.

1. Introduction

The root of vetiver is one of the famous specialty commodities of Garut Region. The root of vetiver is found growing wild, semi-wild, and deliberately planted in various countries with tropical and subtropical climates. The root of vetiver is found in various countries globally with different geographies, and its components can vary because these geographies can influence them. The main producing countries of vetiver are Haiti, Java, China, India, and Brazil. Haiti is the largest major supplier in the world, and Java is the second-largest supplier after Haiti. The center for plant cultivation and vetiver oil production in Indonesia is in Samarang-Garut Region, West Java. Roots of vetiver plantations in the Garut area are spread across four sub-districts, namely Samarang, Bayongbong, Cilawu, and Leles Districts [1]. The root of vetiver is often known as Khus-Khus. The root of vetiver belongs to the Poaceae (Gramineae) family with the morphology of having smooth roots, pale yellow or gray to reddish, with long, thin, stiff leaves, and 1.5 meters high [2]. Each vetiver habitat has different

ecotypes with different physical and physiological characteristics such as different varieties, populations, genetic and geographic cultures [3]. Vetiver has many benefits, especially in agriculture, such as being used for erosion control, soil and water conservation, and preventing floods and landslides. In addition, it can increase soil fertility and phytoremediation to remove organic matter, nitrogen nitrate and treat petroleum-contaminated soil [4-7].

Vetiver oil is a type of essential oil that is extracted from the roots of vetiver plants. Extraction can be carried out by water distillation, steam distillation, or extraction with evaporated solvents. Extraction of vetiver root produced vetiver oils which are added to various products such as products in the cosmetic, fragrance, and food industries. The vetiver oil was added to the product used to mask unpleasant tastes and odors and make the product look more attractive [8,9]. Vetiver oil has a wide range of benefits, such as it is used in the manufacture of perfumes, cosmetic ingredients, scented soaps, insect repellents, and insecticides [10]. The essential oil of vetiver is a thick liquid with a variety of colors from pale yellow to dark brown and has a woody odor. Vetiver oil is commonly used as a major fragrance contributor in the fragrance and aromatherapy industry [11]. In the vetiver plant, which contains much oil is the root part, the oil is taken by distillation, while other parts such as leaves and stems do not contain oil [12]. The root of vetiver has many benefits in traditional medicine, commonly used to treat fever, anemia, hemoptysis, phthisis, edema, skin diseases, urinary disorders, jaundice, and flatulence [13]. In nutraceuticals applications, extracts or waste from vetiver are used as antioxidants for protection against oxidative stress. In commercial applications, the root of vetiver is used in agriculture, handicrafts, refrigeration, construction, and textiles, while its vetiver oil for fragrances and aromatherapy [14]. The economic value of the vetiver plant lies in its roots which can be distilled to produce oil containing sesquiterpenoid compounds, such as α-vetivone, β-vetivone, khusimone, isovalencenol, vetiselinenol [15]; khusinol [16]; khusimol, (+) – zizaene (syn. khusimene) [17]; δ-selinene, and β-vetivenene, valerenol, valerenal, β-cadinene [11]; nootaktone, nootkatol, bicyclo-vetivenol, epi-α-cadinol, and khusinol acetate [8]. The components of vetiver root that function as the main odor contributor are α-vetivon, β-vetivon, and khusimol, where α-vetivone, β-vetivone, and khusimol are in the fingerprint area [14]. Vetiver oil had activities as antimycobacterial [18]; antimicrobial [8,11]; antioxidant [19,20]; anti-inflammatory [21]; repellent [10,22]; antifungal [23].

2. Materials and Methods

2.1. Materials.

Freshly harvested vetiver roots and vetiver oil were obtained from the Samarang - Garut, West Java, Indonesia. The vetiver was determined at the Herbarium Bandungense, School of Biological Science and Technology, and identified as *Vetiveria zizanioides L. Nash*.

2.2. Methods.

2.2.1. Phytochemical screening.

Phytochemical screening was carried out on fresh crude drugs to determine the presence of secondary metabolites in the sample. The secondary metabolites included alkaloids, flavonoids, saponins, quinones, phenols, tannins, and steroids/triterpenoids.

2.2.2. Isolation of the main component of vetiver oil.

Vetiver oil was purchased from vetiver plantation, fractionated by classical column chromatography. Before doing classical column chromatography, vetiver oil was monitored by thin-layer chromatography (TLC). After finding the appropriate mobile phase, the fractionation of vetiver oil was carried out by classical column chromatography using gradient elution technic with n-hexane - ethyl acetate (95: 5, 90:10, 85:15, 80:20, and 75:25) as mobile phases. The fraction obtained was monitored by TLC using a stationary phase of silica gel GF_{254} and the mobile phase n-hexane - ethyl acetate (9:1), then sprayed with vanillin-sulfuric acid spots and heated at 105°C for 5-10 min. Fractions that have the same chromatogram pattern were combined. Subfractionation was conducted by classical column chromatography by isocratic elution technic using mobile phase ratio n-hexane - ethyl acetate (90:10). TLC monitored subfraction using a stationary phase of silica gel GF_{254} and n-hexane - ethyl acetate (9:1) as mobile phase, then sprayed with vanillin-sulfuric acid spots and heated at 105°C for 5-10 min. Subfractions that have the same chromatogram pattern were combined. The subfraction was tested for purity with a single development TLC with three different levels of polarity, namely n-hexane - ethyl acetate (9:1), toluene - acetone (9:1) methanol.

2.2.3. Structure elucidation of isolate using NMR spectroscopy.

The isolate obtained was identified using NMR spectroscopy.

2.2.4. Analysis of khusimol compound by TLC-densitometry.

TLC-densitometry analyzed Khusimol compound to see Rf, maximum wavelength, and Area Under Curve (AUC). Based on AUC data, it can be calculated the purity level of the compound.

2.2.5. Preparation of khusimol compound calibration curves.

Khusimol compound was weighed as much as 1.5 mg then dissolved with 1.5 ml (1500 µl) n-hexane to obtain a stock solution concentration of 1000 µg/ml. Furthermore, from the stock solution of 1000 µg/ml it was diluted to obtain a series solution of concentrations of 100, 200, 300, 400, 500, 600, 700, and 800 µg/ml. The concentration series solutions of 100, 200, 300, 400, 500, 600, 700, and 800 µg/ml were spotted using a micropipette with a volume of 5 µl on the TLC plate with silica gel GF_{254} as stationary phase and developed using n-hexane-ethyl acetate (9:1) as mobile phase. The AUC of each concentration solution was measured using a TLC-densitometer. A calibration curve was prepared between AUC and solution concentration to obtain a linear regression equation $y = A + Bx$, where x is the concentration (µg/ml), and y is AUC.

2.2.6. Determination of khusimol content in extracts with various drying times.

Freshly harvested vetiver roots were cleaned, washed, and cut into small pieces, then crushed and weighed 50 g. Then the fresh roots were left for drying times of 0 hr, 6 hr, 12 hr, 24 hr, 36 hr, and 48 hr. After being allowed to dry at various times, each sample was weighed again. This was performed to determine whether the sample weight is reduced or not when left at room temperature. After that, each sample was macerated with 500 ml of methanol. After being macerated, then filtered, then monitored by TLC. The vetiver and isolate methanol

extract was placed on the TLC plate of silica gel GF254 using a micropipette with a volume of 5 µl, with a mobile phase of n-hexane ethyl acetate (9:1). Then the AUC of each sample was measured using a TLC scanner.

3. Results and Discussion

In this study, the ingredients used were fresh vetiver roots and vetiver oil. This study aims to isolate the marker compound that function as aroma compounds from vetiver oil and to determine the content of marker compounds from the extract with various drying times after harvest, namely 0 hr, 6 hr, 12 24 hr, 36 hr, and 48 hr.

3.1. Phytochemical screening.

Phytochemical screening was carried out on freshly harvested crude drugs to see secondary metabolites such as alkaloids, flavonoids, saponins, quinones, phenols, tannins, and steroids/terpenoids.

Table 1. Phytochemical screening of crude drug.

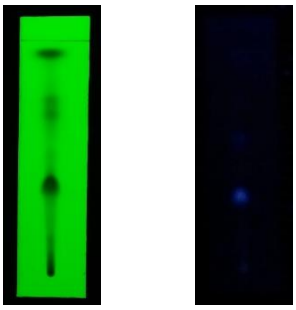
Secondary metabolite	Results
Alkaloid	-
Flavonoid	+
Saponin	-
Kuinon	-
Fenol	-
Tannin	-
Steroid/Triterpenoid	+

Description: + = detected, - = not detected

In Table 1, the results of phytochemical screening show that qualitatively the freshly harvested crude drug contained flavonoids and steroids/triterpenoids.

3.2. Isolation of the main component of vetiver oil.

Vetiver oil was purchased from vetiver plantations then fractionated using classical column chromatography. TLC monitoring was performed to optimize the appropriate mobile phase in classical column chromatography.



(a) (b)

Figure 1. Thin layer chromatogram of vetiver oil. Note: stationary phase: silica gel GF_{254}, mobile phase: n-hexane - ethyl acetate (9:1), (**a**) under UV λ 254 nm; (**b**) under UV λ 366 nm.

The purpose of mobile phase optimization was to find an eluent system or mobile phase that provided the best separation that will be used for the separation process using classical column chromatography—optimization of mobile phase with silica gel GF_{254}, using various

comparisons of n-hexane-ethyl acetate starting with the comparison of n-hexane-ethyl acetate 9:1, 8:2, 7:3, 6:4 and 5:5. The optimization results showed that the mobile phase of n-hexane-ethyl acetate (9:1) presented a better chromatogram pattern.

The monitoring results showed a unique compound at Rf 0.37, which then became the target compound for isolation (Figure 1). After finding the appropriate mobile phase, then the vetiver oil fractionation was carried out using classical column chromatography. Classical column chromatography was used to separate the components present in vetiver oil into subfractions. The mobile phase of n-hexane-ethyl acetate (9:1) exposed better separation. Therefore the selection of the mobile phase in classical column chromatography refers to the mobile phase. The elution technique used was the gradient elution technique, which uses several eluents as the mobile phase. The mobile phase with gradient elution prepared, namely the ratio of n-hexane-ethyl acetate 95:5; 90:10; 85:15; 80:20, and 75:25. Fractionation by classical column chromatography produced 250 vials and was monitored by TLC using silica gel GF_{254} as stationary phase and n-hexane - ethyl acetate (9:1) mobile phase, then sprayed with vanillin-sulfuric acid and heated with oven at 105˚C for 5-10 min. After being sprayed with vanillin-sulfuric acid showed red to purplish-red spots, indicated that vetiver oil contained terpenoid groups. The utilization of vanillin-sulfuric acid was followed by heating at 105°C to visualize terpenoid groups. In general, terpenes derivative compounds still have at least one double bond, a nucleophile group that reacts with an electrophile such as an aldehyde group from vanillin, a coupling compound so that it will appear in visible light [24-25]. Then the fractions that had the same chromatogram pattern were combined, and 5 combined fractions were obtained. Then the combination of fraction 3 (GF-3) was chosen because the target compound was found in GF-3, and there was no other spot at the top, so that the GF-3 was subfractionated by classical column chromatography, using the isocratic elution technique.

Subfractionation was carried out using classical column chromatography with n-hexane-ethyl acetate (90:10) as the mobile phase. The subfraction obtained is accommodated 3-4 ml using the vial. Then the vial is aerated so that the solvent evaporates. The subfractionation resulted in 100 vials and was monitored by TLC using silica gel GF_{254} as stationary phase, n-hexane-ethyl acetate (9:1) as mobile phase, then sprayed with vanillin-sulfuric acid and heated at 105˚C for 5-10 min. After spraying vanillin-sulfuric acid in subfraction monitoring, subfractions with the same chromatogram pattern were combined, and 4 combined subfractions were obtained. Then GS-3 was chosen to proceed to the purity test because the target compound was present in GS-3 and there was no other spot on the top. Purity test of GS-3 was performed by single development TLC with 3 mobile phases with different polarities, namely n-hexane - ethyl acetate (9:1), toluene - acetone (9:1), and methanol. The results showed that one spot was obtained on each TLC plate, indicating that GS-3 was a pure isolate, referred to as isolate X.

3.3. Characterization and structure identification of isolate X by NMR.

The next step was to structure elucidation of isolate X using the Nuclear Magnetic Resonance Spectroscopy (NMR) method consisting of ^{1}H-NMR and ^{13}C-NMR.

Based on ^{1}H-NMR spectrum data of isolate X (Figure 2), it showed the presence of two tertiary methyl groups at 1.05 ppm (s, 3H) and 1.08 ppm (s, 3H), the CH_2OH methylene group has two non-equivalent protons at 3.47 ppm (lH, dd, J = 7.7 and 10.3 Hz) and at 3.74 ppm (lH, dd, J = 6.3 and 10.3 Hz), and the vinyl methylene group CH_2=C at 4.60 ppm (1H, s, J = 1.5 to 1.8 Hz) and at 4.73 ppm (1H, s, J = 1.5 to 1.8Hz).

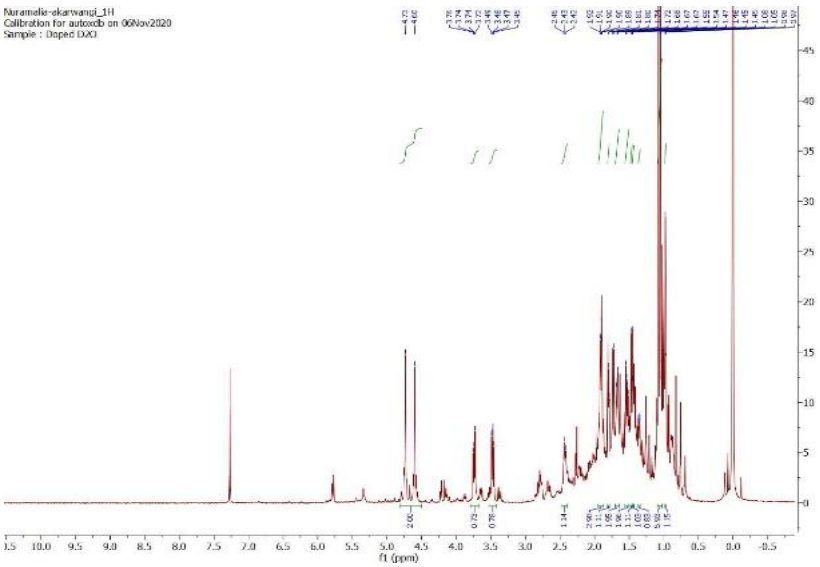


Figure 2. ^{1}H-NMR spectrum of isolate X.

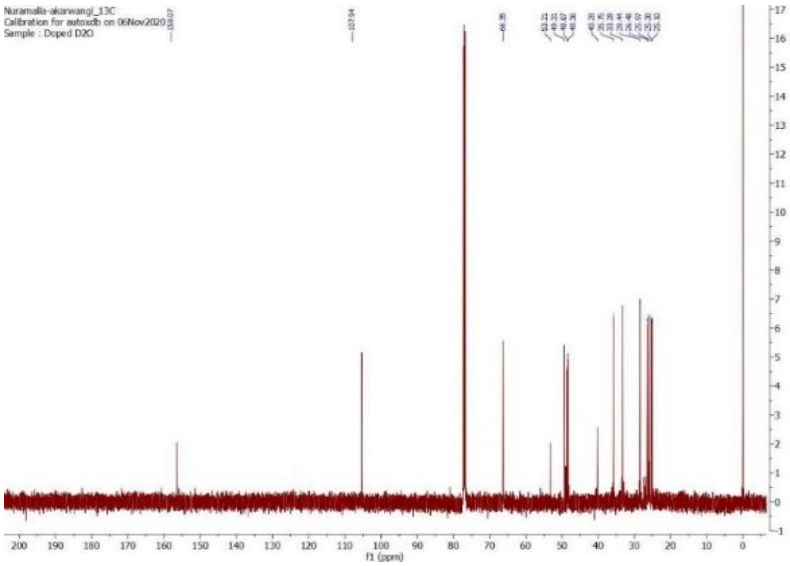


Figure 3. ^{13}C-NMR spectrum of isolate X.

Whereas the ^{13}C-NMR spectrum of isolate X presented the presence of three quarternary carbon groups (C), two methyls (CH_3), seven methylene (CH_2), and three methine groups (CH) in the molecule (Figure 3).

Table 2. Comparison of ^{1}H-NMR and ^{13}C-NMR of isolate X and literature

No.	Isolate X		Khusimol	
	^{13}C NMR (ppm)	**^{1}H NMR (mult., *J* in Hz)**	**^{13}C NMR (ppm)**	**1H NMR (mult., *J* in Hz)**
1	53.2	-	53.2	-
2	48.4	1.91 (*m*, 1H)	48.2	1.92 (*m*, 1H)
3	26.5		26.5	1.89 (*m*, 1H) 1.42 (*m*, 1H)
4	25.1	1.68 (*m*, 1H) 1.52 (*m*, 1H)	25.1	1.68 (*m*, 1H) 1.51 (*m*, 1H)
5	48.7	2.43 (*t*, 1H)	48.7	2.43 (*ddd*, 1H)
6	158.1	-	156.5	-
7	40.3	-	40.3	-
8	49.3	1.80 (*m*, 1H)	49.3	1.80 (*m*, 1H))
9	25.4	1.46 (*m*, 1H) 1.35 (*m*, 1H)	25.4	1.46 (*m*, 1H) 1.34 (*m*, 1H)
10	35.8	1.87 (*t*, 1H) 1.54 (*t*, 1H)	35.8	1.86 (*t*, 1H) 1.54 (*t*, 1H)
11	33.3	1.47 (*d*, 1H)	33.3	1.47 (*d*, 1H)

No.	Isolate X		Khusimol	
	^{13}C NMR (ppm)	^{1}H NMR (mult., *J* in Hz)	^{13}C NMR (ppm)	1H NMR (mult., *J* in Hz)
		0.98 (*d*, 1H)		0.98 (*d*, 1H)
12	66.4	3.74 (*dd*,1H) 3.47 (*dd*,1H)	66.4	3.73 (*d*, 1H) 3.48 (*d*, 1H)
13	26.0	1.05 (*s*, 3H)	26.0	1.05 (*s*, 3H)
14	28.4	1.08 (*s*, 3H)	28.5	1.07 (*s*, 3H)
15	107.9	4.73 (*s*, 1H) 4.60 (*s*, 1H)	105.3	4.73 (*d*, 1H) 4.60 (*d*, 1H)

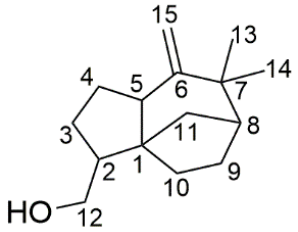


Figure 4. Molecular structure of khusimol.

In Table 2, it can be suggested that the ^{1}H-NMR and ^{13}C-NMR data of isolate X was similar to the khusimol compounds isolated by Rao and Gal (1994) [26]. Based on the results of the comparison of data isolate X with literature, isolate X was khusimol compound with the molecular formula $C_{15}H_{24}O$ [27].

3.4. Analysis of khusimol compounds by TLC-Densitometry.

Khusimol compound was analyzed by TLC-densitometry to see Rf, maximum wavelength, and AUC. The results of the TLC analysis with the silica gel GF_{254} stationary phase and the mobile phase n-hexane-ethyl acetate (9:1) specifically gave an Rf value of 0.36, and a maximum wavelength of 248 nm. Based on the AUC of isolate X and AUC total, the level of purity of khusimol compound 92.05%.

3.5. Preparation of khusimol calibration curve.

The calibration curve of khusimol was prepared using several series concentration solutions of 100, 200, 300, 400, 500, 600, 700, and 800 µg/ml. Then AUC will be obtained at each concentration. A calibration curve can be presented (Figure 5). The linear regression equation of khusimol was y = 6.8722x – 15.092, with R^2 0.9851.

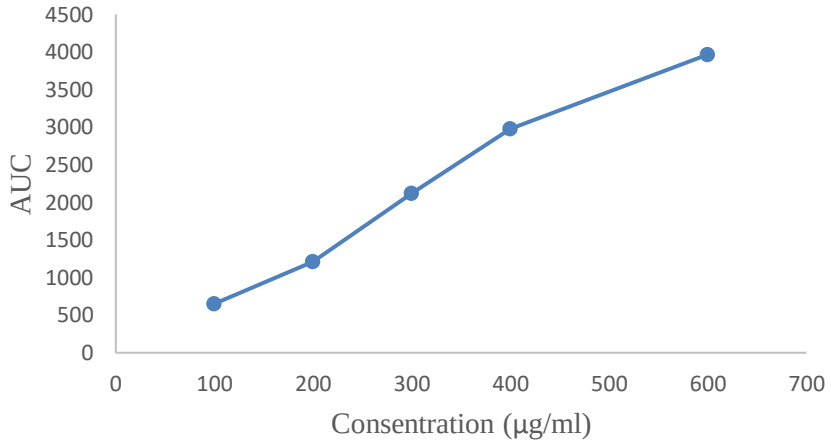


Figure 5. Khusimol calibration curve.

3.6. Determination of khusimol content in various drying times.

The vetiver's freshly harvested roots were cleaned, washed, and cut into small pieces, then crushed and weighed 50 g to be macerated. The extraction method chosen was maceration. The maceration method is the simplest extraction method, low cost, simple equipment, and does not require heat treatment, so it is the right choice for extracting thermolabile compounds [28]. After being left for various times at room temperature, each sample was weighed again to determine whether the sample weight was reduced or not (Table 3). All samples have an initial weight of 50 g; they are left at various times at room temperature before being macerated.

Table 3. The sample weights before and after being left at room temperature.

Times	Start weights	Weight after being left at room temperature
0 hr	50 g	-
6 hr	50 g	47 ± 0.50
12 hr	50 g	44 ± 0.71
24 hr	50 g	40 ± 0.58
36 hr	50 g	35 ± 0.60
48 hr	50 g	31 ± 0.71

In Table 3, it can be seen that after being left at room temperature, water and volatile compounds were evaporated. After being left for various times (0 hr, 6 hr, 12 hr, 24 hr, 36 hr, and 48 hr) at room temperature, then maceration was carried out with 500 ml of methanol. Maceration was carried out for 1 x 24 hr using methanol. Methanol solvent is chosen in the extraction process because methanol is a polar solvent. It will attract more components in the roots because anatomically, vetiver oil is found in the oil sacs in the cortex and endodermis the vetiver plant roots. The solvent will penetrate the oil stored under the subcutaneous oils' surface layer to pull the active compound out [12]. After being macerated with methanol then filtered and the filtrate is added to 500 ml. Furthermore, each sample of methanol extract of vetiver and isolate was placed 5 µL on the TLC plate of silica gel GF_{254} using a micropipette and was developed using the mobile phase n-hexane-ethyl acetate (9:1). Then the plates were dried, and the AUC was measured using a TLC scanner. The concentration of khusimol in the extract at various drying times can be seen in Table 4.

Table 4. Khusimol content in various drying times.

Drying times	Khusimol content (mg/100 g)
0 hr	1,95 ± 0.02
6 hr	2,15 ± 0.02
12 hr	2,30 ± 0.03
24 hr	2.81 ± 0.01
36 hr	1.72 ± 0.03
48 hr	1.38 ± 0.02

In Table 4, the results of the drying time of the roots at various times showed that the highest concentration of khusimol was given by the extract whose roots left for 24 hr after harvesting, namely 2.81 ± 0.01 mg/100 g. The yield of this khusimol content was associated with the drying period of the roots at room temperature. The khusimol content decreased after more than 24 hours of root drying to 1.72 ± 0.03 mg/100 g at 36 hours and 1.38 ± 0.02 mg/100 g at 48 hours after harvest. This may be due to the loss of more volatile compounds during drying and storage and possibly to chemical degradation. Sanford & Heinz (1971) reported that

the storage of nutmeg (*Myristica fragrans*) for a long time resulted in changes in the composition of volatile compounds [29]. Abdi *et al*. (2019) reported that the composition and accumulation of essential oils in peppermint with changes in plant growth stages and giving mild to moderate water stress treatment could change the content of essential oils. The essential oil content of plants that experience mild water stress increases, while plants with moderate water stress treatment cause a significant decrease in essential oil content at various stages of plant growth [30]. Drying vetiver at room temperature for more than 24 hr can reduce the content of khusimol. Harborne (1996) analyzed the essential oil from peppermint leaves showing a quantitative change in the essential oil content according to time travel in both leaf and fruit tissue [24]. Research by Kabede *et al*. (2020) also revealed potential markers of change of volatile components during the accelerated shelf life of pasteurized apple juice which was monitored at temperatures of 20, 30, and 40°C. The change in volatile components is related to the aroma of grassy, green, and fresh apple, especially from hexanal and trans-2-hexenal compounds, which decrease during storage and cause a change in the aroma of apple juice [AUTHOR: This sentence is unclear 31]. Yan *et al*. (2020) studied changes in volatile components and the quality of blueberries during postharvest storage, which were stored at 0°C for 0 days, 15 days, and 60 days, and stored at 25°C for 8 days to test the quality of blueberries. The results showed that the volatile compounds decreased during storage caused by fluctuations in ethyl acetate and decreased terpenoids [32]. Research by Ludlow *et al*. (2021) revealed the effect of storage time and temperature on volatile organic compounds, alliinase activity, and quality of garlic after harvest, which was stored for 9 months at -1.5°C and 22°C. The results exposed that garlic storage caused a decrease in the volatile organic compound (VOC) of organosulfur and a decrease in alliinase activity in garlic stored for 6 months at -1.5°C while garlic stored at 22°C experienced decay after 12 months of storage [33]. The other research reported that the effect of various duration and storage conditions on lavender essential oil which was stored at various temperatures, namely laboratory temperature (24°C), refrigerator temperature (4°C), and freezer temperature (-18°C) for 100 and 200 days and to determine the essential oil components during storage analyzed using GC/FID-MS. The results demonstrated that during storage, evaporation, and conversion processes have occurred so that most of the essential oil components decreased, especially components such as α-pinene, camphene, β-myrcene, γ-terpinene, α-terpinolene, trans-sabinene hydrate. Meanwhile, D-carvone disappeared during the oxidation process because the light could damage the lavender essential oil component [34]. Important compounds that give vetiver's distinctive fragrance are α-vetivone and β-vetivone, khusimol, khusimone, khusimene, vetiselinenol, isovalencenol, β-vetivenene, δ-selinene, prezizaene, eudesmol. The chemical composition of vetiver essential oil from nine countries (Brazil, China, Haiti, India, Java, Madagascar, Mexico, Reunion and Salvador) analyzed using GC/MS showed about 110 compounds. The multivariate statistical analysis data showed no significant differences in the samples from nine geographies. The main components of vetiver oil are khusimol (3.4-13.7%), α-vetivone (2.5-6.3%), β-vetispirene (1.6-4.5%), and vetiselinenol (1.3-7.8%). The chemical composition of vetiver oil is influenced by various factors, such as the environment and location [35].

4. Conclusions

The isolated compound from vetiver oil was khusimol ($C_{15}H_{24}O$). The content of khusimol given by vetiver extract left for 24 hr after harvest was 2.81 ± 0.01 mg/100 g. Drying

vetiver for more than 24 hours could reduce the content of khusimol, because drying and storage resulted in a loss of volatile compounds or chemical degradation.

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Conflicts of Interest

The authors declare no conflict of interest.

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