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Original Article

EXTRACTION AND CHARACTERIZATION OF BLACK BETEL LEAF (*PIPER ACRE* BLUME.) ESSENTIAL OILS FROM EAST KALIMANTAN

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ABSTRACT

Objective: To determine the extraction technique and content of linalool, as the dominant compound, in black betel essential oil from East Kalimantan using a validated analytical method.

Methods: Extraction was carried out using steam distillation. Essential oils were assessed by observing the colour and solubility in ethanol. Using an Agilent GC-MS with a 5977B (GCMSD) detector, a DB-5MS column, and a helium gas carrier of 1 ml/minute. The level of dominant compounds was determined using a validated analytical method.

Results: The essential oil had characteristics that met SNI standards. Based on the results of GC-MS, the dominant compound in five samples was linalool. Validation of the analytical method was carried out with the following conditions established: injector temperature 250 °C, oven temperature 40 °C with a 2 min holding time, reaching 125 °C with a 10 min holding time, reaching 250 °C with a 2 min holding time, reaching 340 °C with a 10 min holding time. The resulted in a linearity of 0.999; a 1.637% relative standard deviation for precision; 102.27% recovery value for accuracy; 0.4% LOD; and 1.2% LOQ. The percentage linalool content of the samples was found in the sample code MADSH 4 (10.56% with a standard deviation of 0.6169).

Conclusion: The steam distillation extraction method can be used to obtain essential oil with characteristics that meet SNI standards. Based on the results of GC-MS, linalool is the dominant compound, with a concentration of 10.56% found in the MADSH4 sample.

Keywords: Extraction, Characteristics, Essential oil, Black betel

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INTRODUCTION

The industrial and food sectors utilise the constituent compounds of essential oils and their derivatives. Indonesia is one of the largest essential oil-producing countries [1]. The content of plant essential oils varies, even in the same family and within the same species. This can be influenced by environmental factors, which include plant genotype, environmental conditions, and harvest time and plant origin [2, 3]. Mann and Kaufman (2012) and Li *et al.* (2011) wrote that essential oils in the Zingiberaceae family vary in yield and composition depending on the species and varieties, even within the same species but in different geographical areas [4, 5].

Piper is one type of plant belonging to the family Piperaceae and has many species [6]. *Piper* species are also used as spice, but their secondary metabolites also exhibit biological effects on human health. This plant is rich in essential and non-essential oils, which can be found in the fruit, seeds, leaves and stems. Some *Piper* species, such as *Piper nigrum*, *Piper betle* and *Piper auritum*, contain various secondary metabolites, including alkaloids, flavonoids, saponins, tannins, phenols, triterpenoids and steroids [7].

Based on its identification, black betel (*Piper acre* Blume.) belongs to *Piper betle* L. *P. betle* from Nepal contains terpenoids such as caryophyllene and α-humulene, phenols such as chavibetol, and various other chemical compounds [8]. More detailed identification shows that black betel and *P. betle* L. have the same shape and characteristics. The difference is in the size of the black betel leaf, which is smaller with a darker or black leaf stalk. Differences in the morphology and varieties of species can cause differences in chemical content. The existence of morphological differences in the same species can result in differences in the chemical compounds contained in species that have these morphological differences [9].

Black betel has a very distinctive aroma. The leaves are reported to be used as mouth fresheners and in the treatment of infectious and non-infectious diseases, such as influenza, cough, asthma, constipation and others. This is because the plant contains essential oils, alkaloids, flavonoids, saponins and tannins [10].

Factors that influence the quality of essential oils include the extraction process, type, and quality of raw materials [11]. Methods used to obtain essential oils ranged from simple, fractional, vacuum and steam distillations and micro-wave assisted hydrodistillation. However, most of the essential oils are obtained by steam distillation. The essential oil produced by this method has high quality and the highest recovery [12].

In general, the compounds contained in essential oils are sterols, terpenes, sesquiterpenes, esters and aldehydes, including components such as limonene, mircene, noctanal, pinene, decanal, linanool, sabiene, neral, dodecanal, geranial and other minor compounds. Widyo *et al.* reported that the results of the GC-MS analysis of *P. betle* L. essential oil gave 10 dominant compounds, namely chavicol, eugenol, germacrene D, caryophyllene, eugenol acetate, 2-allylphenol, β-chamigrene, α-cadinen, terpineol and α-humulent [13]. *Piper crocatum* gave five dominant compounds, namely sabinene, β-mirsen, linalool, caryophyllene, and β-pinene, and *P. nigrum* gave five dominant compounds, namely β-pinene, limonene, linalool, caryophyllene, and germacrene B. *Piper cubeba* also gave five dominant compounds, namely β-pinene, linalool, caryophyllene, δ-cadinene, and cubebol [14]. The black betel, which taxonomically belongs to the *Piper* species. May have a different chemical content. Of these components, linalool is considered to be an active ingredient that can play a significant role compared to other minor compounds. Linalool is found in many essential oils spread throughout the world in more than 200 species of monocot and dicot plants [15, 16].

The determination of the components of black betel essential oil samples can be undertaken using a gas chromatography (GC) method based on the calibration curve method. Validated methods will help to assure that the analysis results can be trusted [17].

To develop traditional medicines that have been used for generations from the local area to a wider scope, it is necessary to characterise black betel essential oil and its dominant compounds [18]. Based on the description above, a research analysis and

characterisation of black betel essential oil was carried out using gas chromatography-mass spectroscopy (GC-MS), aiming to determine the characteristics of the essential oil, as well as the content of the dominant compound and its levels, using a method that has been validated using several samples of black betel essential oil from East Kalimantan as a basis for its use with black betel leaf. This research can also be considered as an attempt to develop essential oils through the analytical method used.

MATERIALS AND METHODS

Materials

The tools used in this research were a set of glassware, an analytical balance, tube clamp, test tube, volume pipette, a set of tools used for steam distillation and a GC-MS instrument (Agilent GC type 7890A (G3440A) with a detector type 5977B (GCMSD)).

Samples

Black betel leaf (*Piper acre* Blume.) was obtained from 5 different growing locations in East Kalimantan and was determined at LIPI Bogor.

Chemicals and reagents

Water (Milli-Q pure water from an Arium® Pro), chloroform and ethanol (grade pro analysis) from Merck, and standard linalool 99.0% (Sigma-Aldrich).

Methods

Sample preparation

The sampling of black betel leaf from East Kalimantan was carried out randomly and proportionally, with samples taken far apart to represent the area. Samples were therefore taken from five different locations, namely Berau, Kutai Kartanegara (Kukar), East Kutai (Kutim), Samarinda and Penajam. Each fresh sample was washed to remove existing impurities, and the leaves were then cut into smaller pieces ready for the extraction process.

Extraction of black betel leaf essential oil by steam-water distillation method

Each sample that is ready to be taken as much as 2 kg. Steam distillation was carried out for 5 h, starting from the first drop at a temperature of 50-70 °C until all the sample oil was taken, while being careful not to let the water boil. The results, separated between the oil and the condensate, include the yield, physical properties such as colour, solubility in ethanol and GC-MS analysis.

% Recovery of black betel leaf essential oil

The essential oil content contained in the black betel leaf was measured using a set of steam distillation apparatus, based on the equation below [19].

$$\% \text{ Recovery} = \frac{\text{Oil volume}}{\text{sample weight}} \text{ x } 100\%$$

Organoleptic identification

Up to 2 ml of the extracted black betel leaf essential oil was pipetted into a test tube, making sure that there were no air bubbles that interfered with it. The test tube containing the sample was propped up on white paper, and the colour was visually observed [20].

Solubility in ethanol

First of all, 2 ml of essential oil was placed in a test tube, 1 ml of 80% ethanol was added drop by drop, and the sample was then homogenised. The solubility of the essential oil in ethanol was then observed [20].

Essential oil analysis using GC-MS

Gas chromatography conditions

An Agilent GC brand gas chromatography type 7890A (G3440A) with a detector type 5977B (GCMSD) was used, with a DB-5MS column set by a manual injection system with a split method and an injector temperature of 250 °C. The initial temperature of the oven was 40 °C with a hold time of 2 min; then the temperature was increased to 125 °C with a hold time of 10 min, followed by a temperature increase to 250 °C with a hold time of 2 min, and a final temperature increase to 340 °C with a hold time of 10 min. The carrier gas flow rate was 1 ml/min with a split ratio of 20:1. Observations were made at a molecular weight of 40-700 m/z using the NIST 17 library.

Method validation

Linearity and calibration curve

Linearity was carried out by preparing seven standard solutions of linalool in chloroform with a concentration range of 0.1% to 6.4%. Then, 1l was injected into the GC system and linear regression analysis was performed. A calibration curve was made using the concentration of the standard linalool solution and the area depicted by the graph. Linearity can be seen from the obtained R^2 value.

Precision

The precision of the method was assessed by testing the linalool compound in the black betel leaf essential oil sample six times, diluting using chloroform. Then, 1 l was injected into the GC system to ensure sufficient data to represent the data population for statistical testing. The sample concentration was calculated based on the peak area plotted against the calibration curve, and the relative standard deviation (RSD) was calculated and compared with Horwitz CV (%).

Accuracy

The external standard method was used, where the calibration curve obtained previously states the relationship between the peak area of the chromatogram and the concentration of the standard substance. For the assays using concentrations of 0.8% and 6.4%, three repetitions were used and results were determined by establishing the area of the chromatogram peak for each concentration and then substituting this into the calibration curve regression equation.

LOD/IOQ

The limit of detection (LOD) and limit of quantification (LOQ) are calculated based on the standard deviation (SD) of the response and the slope of the standard linearity.

Determination of linalool content

Each sample of black betel leaf essential oil was 0.1 ml in 10 ml chloroform, and 1 l was injected into the validated GC-MS system. Three replicates of each sample were used.

RESULTS AND DISCUSSION

Sample extraction was carried out using the steam-water distillation method. The essential oil produced from each sample is shown in table 1. The organoleptic results of black betel leaf before and after drying are presented in table 2. The colour and appearance of the black betel leaf after washing and after drying are depicted in fig. 1.

Table 1: Recovery results for black betel leaf essential oil

Sample code	Fresh sample weight (g)	Essential oil weight (g)	Recovery (%)
DSH 1	2000	0.096	0.0048
DSH 2	2000	0.071	0.0035
DSH 3	2000	0.082	0.0041
DSH 4	2000	0.092	0.0046
DSH 5	2000	0.065	0.0032
Mean		0.0812	0.00404
SD		0.01325	0.00068

Table 2: Results of organoleptic analysis of black betel leaf

Sample code	Scent		Color	
	After washing	After drying	After washing	After drying
MADSH 1	Weak characteristic	Strong distinctive	Fresh light green	Blackish green
MADSH 2	Weak characteristic	Strong distinctive	Fresh green	Blackish green
MADSH 3	Weak characteristic	Strong distinctive	Fresh dark green	Blackish green
MADSH 4	Weak characteristic	Strong distinctive	Fresh dark green	Blackish green
MADSH 5	Weak characteristic	Strong distinctive	Fresh green	Blackish green

Fig. 1: (a) Black betel leaf after washing (b) Black betel leaf after drying

Table 3: Characteristics of black betel leaf essential oil

Sample code	Colour		Solubility in ethanol	
	Extraction results	SNI reference	Extraction results	SNI reference
MADSH 1	Pale yellow	Pale yellow to brown	2:1	2:1
MADSH 2	Pale yellow	Pale yellow to brown	2:1	2:1
MADSH 3	Pale yellow	Pale yellow to brown	2:1	2:1
MADSH 4	Pale yellow	Pale yellow to brown	2:1	2:1
MADSH 5	Pale yellow	Pale yellow to brown	2:1	2:1

Fig. 2 showed a chromatogram displaying the five samples of black betel leaf essential oil from East Kalimantan.

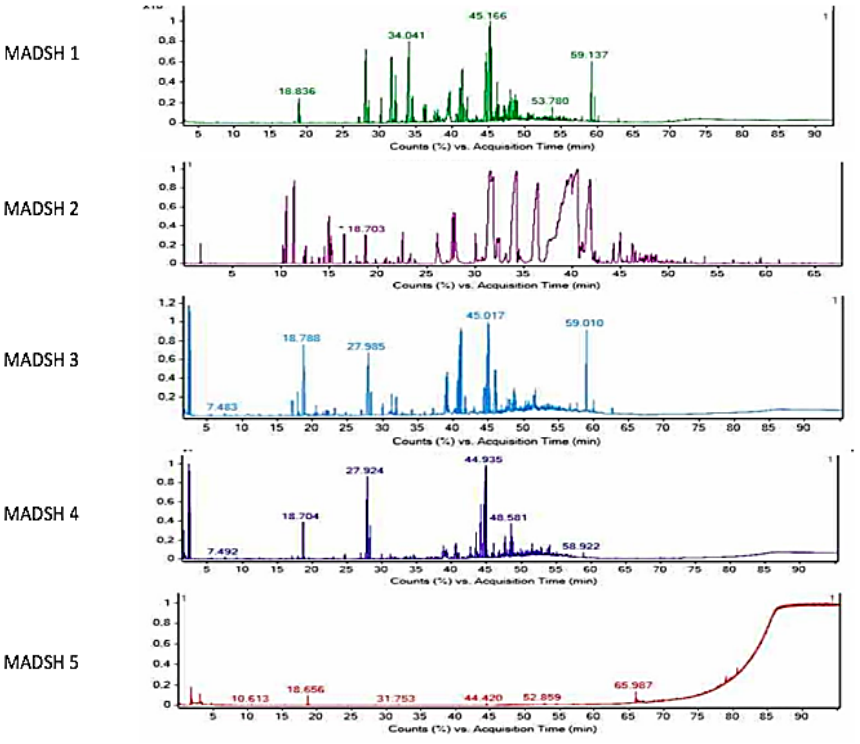

Fig. 2: Black betel leaf essential oil chromatogram

Table 4: Group of compounds from the five samples of black betel leaf essential oil

Sample code	RT	Area	m/z	Compound name	Similarity (%)
MADSH 1	18.836	5110049859	154.25	Linalool	95.36
	34.041	7961307602	204.35	Germacrene D	90.5
	45.166	725612033	204.35	Caryophyllene	93.64
MADSH 2	11.350	13031237524	136.23	Camphene	93.48
	18.703	1059381509	154.25	Linalool	94.11
	30.038	1890013663	204.35	⟨-cubebene	96.96
MADSH 3	7.483	678554229	72.1057	2-butanone	92.62
	18.788	2047572787	154.25	Linalool	91.66
	27.985	1803187668	170.29	2-undecanone	96.16
MADSH 4	7.492	361496367	72.11	2-butanone	92.45
	18.704	1023589611	154.25	Linalool	96.48
	27.924	4025491594	170.29	2-undecanone	93.06
MADSH 5	10.613	2744479	138.14	Methanesulfonyl acetic acid	81.59
	18.656	20099454	154.25	Linalool	90.45
	31.753	31949067	86.14	2-propanone	86.88

MS fragment and the structure of the linalool compound can be seen in fig. 3.

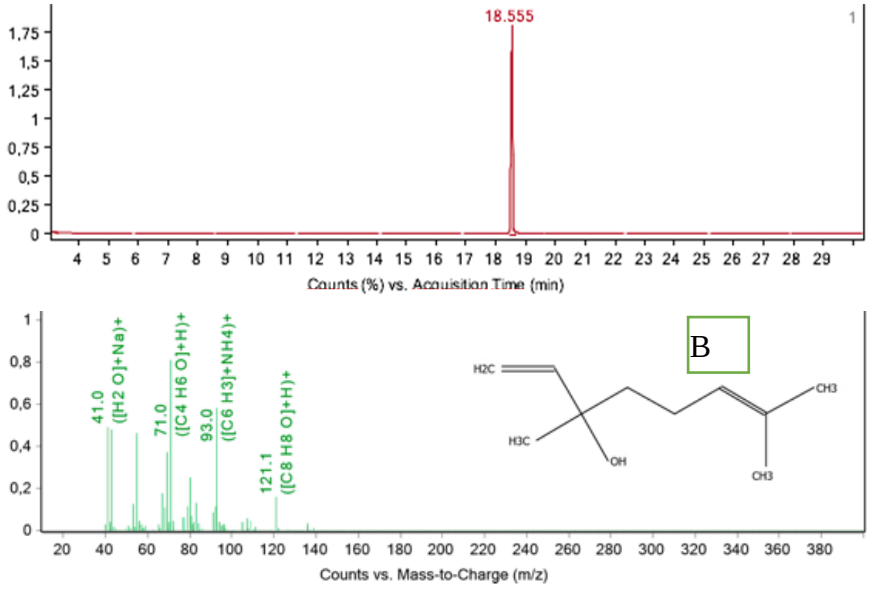

Fig. 3: (a) Standard chromatogram of linalool (b) MS section and structure of linalool

A calibration curve based on the concentration of the linalool standard solution and the area depicted by the graph in the fig. 4. The precision data can be seen in table 5. The percentage recovery results are shown in table 6. The LOD and LOQ results are shown in table 7. The levels of linalool contained in the samples the results are shown in table 8.

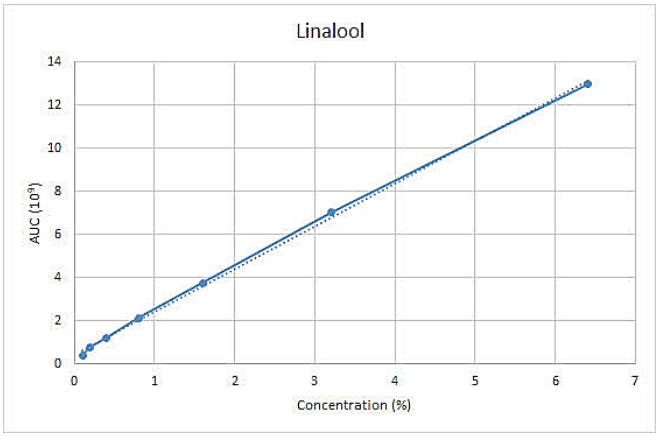

Fig. 4: Linearity of linalool standard solution

Table 5: Precision results

Preparation	Concentration (%)
1	0.878
2	0.840
3	0.858
4	0.841
5	0.851
6	0.850
Mean	0.853
SD	0.0139
%RSD	1.637

Table 6: Accuracy determination results

Concentration (%)	Area	Substitution concentration (%)	% Recovery
0.8	2120895375	0.840	105.05
0.8	2156795839	0.858	107.31
0.8	2121575651	0.841	105.09
6.4	13056635370	6.352	99.26
6.4	13000000000	6.324	98.82
6.4	12908931518	6.278	98.10

Table 7: LOD and LOQ nilai values

Parameter	Value
Slope	1983862950
Deviation Standard (SD)	239669431.90
LOD	0.40
LOQ	1.21

Table 8: Linalool content in black betel sample

Sample code	Content (%)	Mean	SD
MADSH 1	0.1423	0.1369	0.0060
	0.1382		
	0.1304		
MADSH 2	0.1180	0.1197	0.0016
	0.1200		
	0.1212		
MADSH 3	0.5811	0.5671	0.0264
	0.5837		
	0.5366		
MADSH 4	10.919	10.560	0.6169
	9.848		
	10.914		
MADSH 5	0.0906	0.1032	0.0158
	0.1211		
	0.0981		

DISCUSSION

The essential oil produced in this study is extracted from betel leaf. Prior to the essential oil extraction process, pre-treatment was carried out in the form of washing, which aims to remove impurities attached to the black betel leaf, followed by simple drying out of direct sunlight until the leaves are no longer wet and the condition of the leaves is not too dry. This simple drying process is done to reduce the water content in the betel leaf and involves the evaporation of water from the material. This release of water causes the breakdown of the oil cells, making it easier for the oil to be taken up during distillation [21].

Sample extraction was carried out using the steam-water distillation method. The characteristic of this method is that the steam is always in a saturated wet state, not too hot, and the sample being distilled is only in contact with the steam and is not exposed to heat. The principle is the same as in steaming rice. The sample material is in contact with steam. A total of 2 kg of black betel leaf, cut into pieces, was extracted by steam distillation for 5 h. The resulting essential oil was then added to anhydrous Na_2SO_4, which serves to remove the water content. The essential oil produced from each sample was 0.096 g, 0.071 g, 0.082 g, 0.092 g and 0.065 g, respectively, with a SD of 0.01325 and the % recovery was 0.0048%, 0.0035%, 0.0041%, 0.0046% and 0.0032%, respectively, with a SD of 0.00068.

According to the results, the essential oil obtained is very small compared to the quality requirements of ISO 959-1. According to these requirements, the essential oil content produced is at least 2%. In this study, the low total oil recovery can also be influenced by the growth of the plant itself. It is suspected that not in the appropriate growing environment or not in the ideal growing environment. A'yun *et al.* (2020) stated that the recovery percentage results would be different if plants were in different locations; plants need a certain level of sunlight to achieve maximum recovery [22].

Organoleptic testing also has an important role as an early detection method for assessing quality and identifying deviations and changes in the product. The implementation of organoleptic tests can be carried out directly, and this assessment can sometimes provide results that are considered good (SNI, 2006).

Dried black betel leaf gives off a stronger aroma than fresh leaves that have been washed. From this, the purpose of drying the raw materials can be said to decompose substances that do not have a characteristic odour into substances that have a distinctive smell. This difference in smell can also be influenced by the sensitivity of the sense of smell [23]. Organoleptic analysis of the black betel leaf essential oil also showed the suitability of observing the colour and distinctive odour of the essential oil produced.

The results of the extraction of the black betel leaf essential oil by steam distillation were analysed for characteristics including organoleptic properties and solubility in 80% alcohol. Because there is no SNI standard relating to black betel leaf essential oil, the characteristics were observed using the quality standard of green betel leaf essential oil, based on SNI 06-3953-1995.

Based on the data table 3 above, the black betel leaf essential oil met the range of essential oil quality standards based on SNI 06-3953-1995. The results of the solubility in ethanol 80% also show that it meets the specified standards.

There is a slight difference in the black betel leaf essential oil produced from some of the samples; however, all of the distinctive colours and odours produced based on organoleptic observations show conformity based on SNI 06-3953-1995. The differences may be due to differences in the components and/or the number of constituent components of the essential oil produced in each sample. The alcohol solubility test gives an idea of whether or not an oil is easily soluble; the more soluble the oil in alcohol, the more polar compounds are in the oil. Alcohol solubility is an important factor in essential oil testing because it can determine the quality of the essential oil [12].

The identification of components within the black betel leaf essential oil was carried out using GC-MS. The resulting output is in the form of molecular weight, with the system set at a molecular weight range of 40-700 m/z. Fig. 2 shows a chromatogram displaying the five samples of black betel leaf essential oil from East Kalimantan.

Based on the GC-MS results in table 4, the most dominant compound in some samples of black betel leaf essential oil was linalool. These results are in line with the research of Aprotosoaie *et al.*, who highlighted that linalool was one of the components contained in essential oils [15]. The linalool standard chromatogram with the same instrument conditions, as well as the MS fragment and the structure of the linalool compound, can be seen in fig. 3 below.

In this study, linalool was the dominant compound in the five samples of black betel leaf essential oil that were analysed using the same method and treatment. Linalool ($C_{10}H_{18}O$) is a monoterpenoid found in plant essential oils. It has a molecular weight of 154.25 and has properties that are less soluble in water because of its nonpolar structure but very soluble in organic solvents [24]. Linalool is a volatile compound found in many plants parts, such as flowers and leaves. It is widely used in various industries, including the food flavouring industry, cosmetics and pharmaceuticals [17].

The specialty of linalool is that it has a pleasant aroma, derived from monoterpenoids (C_{10}), which can be useful, playing a role in anti-anxiety (relaxation) effects [25]. Linalool can be used in the cosmetic field (in perfume, shampoo, soap and others) and in food additives as an aroma and flavouring [15]. Based on the literature, linalool has antioxidant, antibacterial, antimicrobial and anticancer activity [26]. It has also been stated that linalool is one of the compounds that has strong antileukemic activity against U937 lymphoma cells (IC_{50} = 3.51 g/ml) [19]. Toxicity data has demonstrated that it is safe, and it is considered as a valuable fragrant compound with significant therapeutic potential [15].

This study provides new insights into the importance of developing precise and accurate analytical methods for the quantitative determination of linalool in black betel leaf essential oil. The method used in this research is a development method and has not yet become a standard method, and it is therefore necessary to validate the method. Method validation is a process to prove that an analytical method can be used according to certain parameters. Validated methods help to provide assurance that the analytical results can be trusted [16]. Therefore, in this study, validation of the method was carried out by GC. The validation parameters used as a reference included linearity and determination of standard curves, precision, accuracy and LOD/lOQ.

This determination is based on the calibration curve method, comparing a substance in a sample whose concentration is not known to a standard sample whose concentration is known. The concentrations of the linalool standard solutions with known concentration were 0.1%, 0.2%, 0.4%, 0.8%, 1.6%, 3.2% and 6.4%. The standard solutions were injected into the GC system and the results were obtained in the form of data on the area of each concentration of standard solution. The data obtained was used to create a calibration curve based on the concentration of the linalool standard solution and the area depicted by the graph in the fig. 4.

The linalool standard calibration curve shows the relationship between area and concentration. This means that the greater the concentration value of the standard solution, the greater the area value. The linear regression equation obtained is y = 1983862950x+453721520, with a coefficient of determination R^2 = 0.9986, which indicates that it is in accordance with the standard because it exceeds the minimum value of 0.995 [27]. This method also provides very good linearity, as seen from the R^2 value of more than 0.995, and the slope shows a proportional response between the input and output.

The precision was determined using the repeatability test method, measured as the SD or the RSD (coefficient of variation). The precision of the method is expressed as the RSD of six repetitions of the black betel leaf essential oil sample. The RSD value obtained for the linalool compound was 1.637%, which is greater than the %CV Horwitz (1.086%). This shows that the method has very good precision [28].

The accuracy of this method was determined using an external standard method, using the previously obtained calibration curve. The difference in the yield value between the substitution results in the calibration curve regression equation was compared with the actual concentration. The results obtained in table 6 indicate that the accuracy of the method is good because it is in the specified range, with % recovery values in the range of 80–110% [29].

The LOD and LOQ were obtained from the linear calibration curve by calculating the value of the linalool standard solution based on the SD of the response and the slope of the standard linearity.

The GC chromatogram showed linalool as the dominant component of some samples of black betel leaf essential oil. The quantity of linalool in the black betel leaf essential oil samples was 0.14%, 0.11%, 0.12%, 10.92% and 0.09%, respectively. There were variations in the compound components and linalool content of the black betel leaf essential oils, which were influenced by the origin of the clove leaf producing area, climate, season, location, geography and geology.

Based on the validation results obtained from each parameter, it can be said that the GC method is suitable for the determination of the linalool levels in samples of black betel leaf essential oil from East Kalimantan, because the results of all validation parameters out meet the requirements.

The linalool content in the black betel essential oil sample was determined by injecting the sample into the validated GC system. The data obtained shows that the highest linalool content was found in the black betel leaf essential oil sample with the code MADSH4. The average amount of linalool content in this sample is 10.56%, with an SD of 0.6169. Some *Piper* species have linalool as the main essential oil component in the leaves and seeds, such as *Piper jacquemontianum* leaves (14.5%) [29], *Piper claussenianum* leaves (2-5%) [30], *P. crocatum* leaf essential oil (3.29%), *P. nigrum* seeds (0.12%) and *P. cubeba* (0.98%) [15]. In this study, the average linalool content in black betel leaf essential oil from East Kalimantan (10.56%) was in the range of these linalool levels. Many *Piper* species contain lower amounts of linalool [31].

CONCLUSION

It can be concluded that the extraction and characteristics of black betel leaf essential oil meet the SNI standard of the Piperaceae

family based on organoleptic identification. Based on the GC-MS results, the most dominant compound in some samples of black betel leaf essential oil from East Kalimantan was linalool. The linalool content in black betel leaf essential oil was highest in the sample code MADSH 4, which was 10.56% with a SD of 0.6169.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

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