

Essential oils from *Vitex trifolia* as an effective repellent for *Aedes aegypti*

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Manuscript received: 2 September 2020. Revision accepted: 29 September 2020.

Abstract. Arpiwi NL, Muksin IK, Kriswiyanti E. 2020. Essential oils from *Vitex trifolia* as an effective repellent for *Aedes aegypti*. *Biodiversitas* 21: 4536-4544. *Vitex trifolia* is a shrub or small tree grown wild from family Verbenaceae commonly found in the sandy shore habitat of the tropics and sub-tropical countries. This research aims to measure the essential oils yield of *V. trifolia* leaves, to investigate the secretory cells of the leaf, and to measure repellent activity of the essential oils against *Aedes aegypti*. Leaves and soil samples were taken from Panjer, Sanur, and Sidakarya Villages of Denpasar, Bali Indonesia. Oil was extracted from fresh leaf samples using steam distillation. The essential oils were analyzed for its constituents using Gas Chromatography-Mass Spectrometry. The leaf cross-sections were prepared using paraffin embedding method and stained with safranin. The essential oils of *V. trifolia* at different concentrations (0, 2, 3, 4, 5, 6% w/w) were formulated into lotions. The yield of essential oils was significantly different from the three locations. The main constituent of the oil was cis-ocimene. The essential oil yield was positively correlated with the sand content. The secretory structures of the leaf were idioblast and glandular trichome. Lotions formulated with the essential oils were soft, not greasy, and not sticky upon application to the skin and non-irritant. Lotions with 5 and 6% essential oils gave 100% protection for 3 hours from *Ae. aegypti*.

Keywords: Essential oils, idioblast, repellent, glandular trichome, *Vitex trifolia*

INTRODUCTION

The genus *Vitex* comprises more than 270 species distributed worldwide (Laxmikant 2012) contains many useful active constituents (Devika 2017). The genus is commonly used as traditional medicines for several ailments as well as complementary medicines by Asian folks. Among the genus, *Vitex agnus-castus* and *V. negundo* are well known for their ethnopharmacological properties, especially as an antibacterial. The two species grow in riverbanks and beaches in the Mediterranean region, Southern Europe, and in Central Asia (Rani and Sharma 2013). *Vitex trifolia* is a shrub or small tree grown wild from family Verbenaceae commonly found in sandy shore habitat of the tropics and sub-tropical countries including India, Sri Lanka, China, the Philippines, Indonesia, North Australia, New Caledonia, and French Polynesia (Laxmikant 2012).

The habitus of *V. trifolia* is low, wide branches dense shrub of fewer than 5 m in height. The leaf color is dark green with simple and trifoliate leaves (Thenmozhi et al. 2011). The leaflets are oblong or oblong-elliptical shapes with a length of 4-7 cm. The adaxial side of the leaf is smooth and shiny while the abaxial side is covered with sparse grey hairs. The aromatic soft leaves smell pungent after crushing (Rani and Sharma 2013). The flowers are white and violet is borne in terminal as an inflorescence with the length of 5-10 cm oblong panicles. The fruit is round shape (Chan et al. 2016; Laxmikant 2012).

There are numerous factors influencing the essential oil accumulation in the plants, such as soil types and soil texture, temperature, percentage of organic matter in the

soil, altitude (Yavaria et al. 2010), climates, geographic origin, and genetics. Sometimes it is difficult to determine a single factor that influences essential oils yield since the factors are interrelated (Dhifi et al. 2016). In *Vitex trifolia*, the yield of essential oils even varied between the seasons. Higher yield is obtained during spring and autumn as a response of plants to stress conditions and protection to abrupt changes in weather conditions. On the other hand the decrease in essential oils production during summer and winter due to alteration of biosynthesis pathway of the plant (Dehsheikh et al. 2019).

The main constituents of the essential oils from *V. trifolia* are monoterpenes and sesquiterpenes, namely 1R- α -pinene, ocimene, 3-carene, toluene, p-ethyl-, eucalyptol, and 3-methoxy-5-methylphenol (Devi and Singh 2014). In another study, caryophyllene and eucalyptol are the main constituents of the oil *V. trifolia*, which account for 38% and 26% respectively (Thomas et al. 2019). Constituents of the essential oils is also influenced by season of the year due to changes in the biosynthesis pathway. Environmental stresses, such as high temperature during summer and low temperature during winter have changed some constituents into the oxidases (Dehsheikh et al. 2019).

Essential oils from *V. trifolia* can repel mosquitos, therefore it can protect the human skin from landing or biting (Tawatsin et al. 2006). A mosquito is a vector of many infectious diseases such as malaria, chikungunya, dengue, and Japanese encephalitis. No vaccine has been found for these mosquito-transmitted diseases (Chandrasekaran et al. 2019), therefore, controlling the vector needs to be pursued to prevent transmission of diseases (Jing and Wang 2019). One attempt to do this is

by applying repellent onto the skin. Synthetic chemical repellent DEET (N-diethyl-3-methylbenzamide) is effective to control mosquito bites but it uses raises some health risks. Therefore, the use of plant-based insect repellent is a safe alternative (Diaz 2016).

The effectiveness of a natural anti-mosquito lotion depends on the concentration of the essential oils and of the type of species used (Uniyal et al. 2016; Arpiwi et al. 2020). The concentration of lotion containing 10% of essential oils from *V. trifolia* is more effective against three mosquito species, namely *Anopheles dirus*, *Culex quinquefasciatus*, and *Ae. albopictus* whereas it is less effective against *Ae. aegypti* (Tawatsin et al. 2006).

The adaxial surface of *V. trifolia* leaf is smooth, even with thick wall epidermis and thin cuticle layer. While the abaxial surface is uneven, with narrow cylindrical epidermal cells and covered by dense non-glandular trichomes (Thenmozhi et al. 2011). Glandular trichomes are also present in *V. trifolia* and trichome is one of morphological leaf characters of Verbenaceae (Munsif 2007).

Glandular trichomes are epidermal hairs that modified into structure for synthesis and secretion of plant secondary metabolites including terpenoid essential oils which are frequently found in leaves and stems (Lange et al. 2013). There are two types of glandular trichomes, namely peltate and capitate, which differ from each other in the number of basal, stalk, and apical cells (Guo et al. 2013). Peltate

glandular trichome is characterized by a short stalk (usually one cell) and large secretory apical cells, whereas capitate glandular trichomes have variable sizes and generally have longer stalk and smaller secretory apical cells (Huchelmann et al. 2017).

The aims of the present study were to measure the essential oils yield of *V. trifolia* leaves, to investigate the secretory cells of the leaf, to measure repellent activity of the lotions containing essential oils from *V. trifolia* against *Ae. aegypti*.

MATERIALS AND METHODS

Oil extraction

Oil was extracted from fresh leave samples using steam distillation according to the method by Arpiwi et al. (2020). Leaf samples were taken from 3 locations/villages in Denpasar, Bali, Indonesia, namely Sidakarya, Sanur, and Panjer Villages (Figure 1). Briefly, 5 kg of fresh leaves was placed in the sample tank and closed tightly. The boiler was filled with a half-full with water and the condenser was equipped with water inlet and outlet. Extraction was conducted for 4 hours at water boiling temperature. The mixture of essential oils and hydrosol was collected in the separator. The essential oils were filtered using a filter paper.

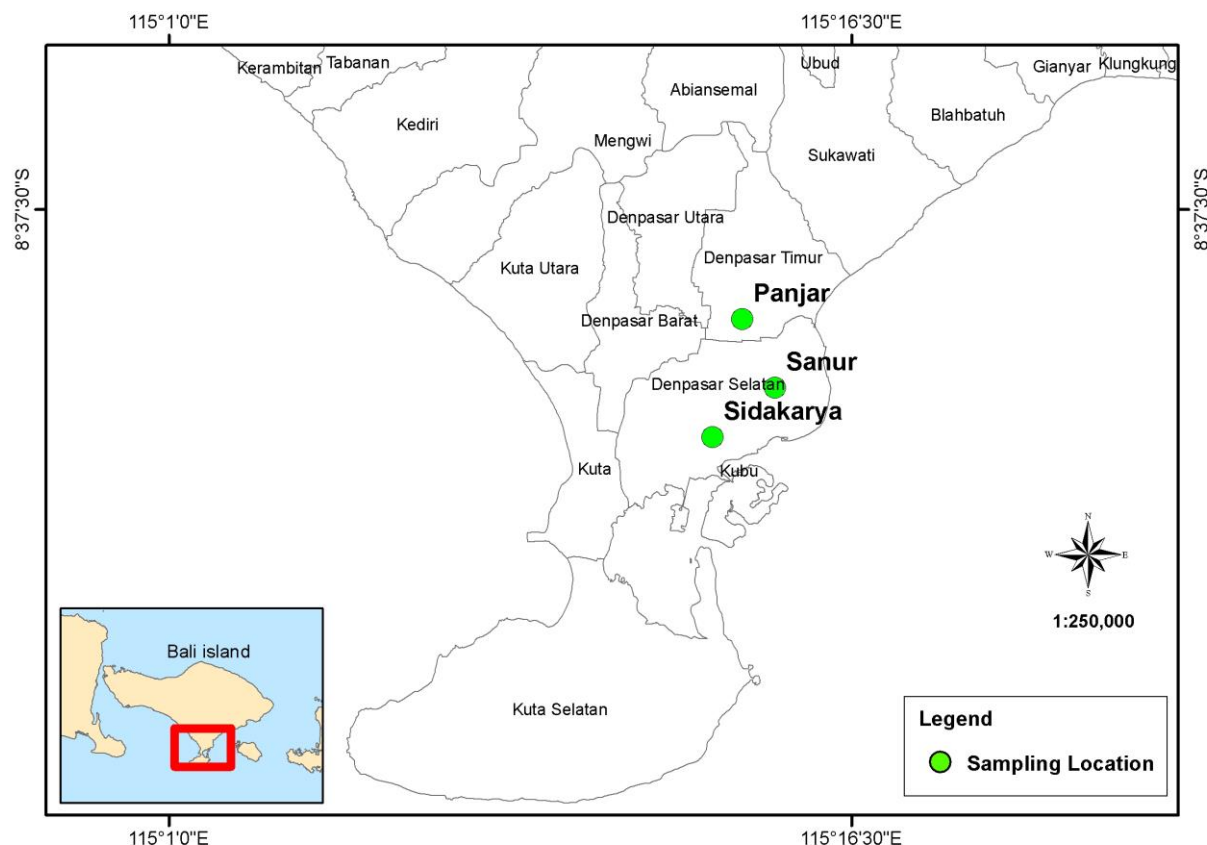


Figure 1. Study area for leaves and soil samplings from Panjer, Sidakarya, and Sanur Villages of Denpasar, Bali, Indonesia

Analysis of chemical constituents of the essential oils

The essential oils were analyzed for its constituents using Gas Chromatography-Mass Spectrometry (GC-MS, Shimadzu-QP2010 Ultra) assay. Oil sample (20 µL) was diluted using 5 mL absolute ethanol and then filtered using Whatman no 1 filter paper. An aliquot of 1 µL was injected into the column (DB-5 MS 122-0132) with a length of 30 meters and a diameter of 0.22 µm. Injector temperature was 250°C and helium was used as a carrier gas with flow rate 3 mL/minute. The chemical constituents of the essential oils were identified by comparing peaks with the library.

Soil analysis

Soil samples (1 kg each) were taken from the same locations as leaf sampling, namely from Sidakarya, Sanur, Panjer of Denpasar, Bali, Indonesia (Figure 1). Soils were analyzed for pH, electric conductivity, the content of C organic, total nitrogen (N), available phosphorus (P), available potassium (K), content of water, sand, silt, and clay. Soil was suspended in water with soil to water ratio of 1 : 2.5 and pH of the suspension were measured using a pH meter. Electric conductivity (EC) was measured at 25°C. Carbon (C) organic content was measured using Walkley & Black method (Walkley and Black 1964) and total nitrogen was by Kjeldhall methods. Available P and K were determined by Bray 1 method (Bray and Kurtz 1945). Water content was measured based on field capacity using gravimetry method. Soil texture was determined by pipette method.

Microscopic evaluation of the leaf

Fully developed fresh leaf samples were harvested at the 5th node from a shoot tip with triplicate from each location. The leaf was cross-sectioned and fixed using 10% Neutral Buffer Formalin (NBF) solution for 24 hours. Tissues then dehydrated using a tissue processor (Tissue Tek II, Model RX-II B, Sakura Seiki Co, Ltd). Dehydration began with 10% NBF solution for 2 hours and then alcohol 70% for 2 hours, absolute alcohol 3 times for 2 hours each, followed by two times in toluol for 3 hours each and finally in liquid paraffin for 3 hours. Leaf tissue was embedded in liquid paraffin and then sliced in the lamina and midrib using a microtome with thickness of 4 µm. The sectioned tissue was placed into an object-glass and then stained with 1% aqueous safranin. The slide was closed with a cover glass and sealed with Canada balsam. The slice was cleaned with absolute alcohol and then xylol three times each. The leaf structure of the lamina and midrib was observed under a microscope and photographs were taken using an Optilab camera. The thickness of leaf tissues was measured using Image Raster 3.

Preparation, sensorial and quality analysis of the lotions

Lotions were prepared based on a formula in Table 1 following a method by Arpiwi et al. (2020) with modification in the ingredients used. Lotions contained oil and aqueous phases and united by emulsifiers. The oil and aqueous phases were heated at 70°C in different containers. The oil phase consisted of virgin coconut oil (VCO), cetyl alcohol, stearic acid and dimethicone while aqueous phase

consisted of glycerin, triethanolamine, nipagin, and distilled water. The aqueous phase was added to the oil phase slowly while stirring to mix thoroughly to form a lotion base. The temperature of the lotion base was down to 40°C and then essential oils from *V. trifolia* was added at 0, 2, 3, 4, 5, and 6%. The lotion was mix gently to homogenous mixture.

Sensorial analysis of the lotions was assessed for color, odor, sensitivity and consistency according to the method by Yadav et al. (2014). The color of the lotion was observed visually and the odor was smelled. Sensitivity was assessed to 20 human volunteers (10 males and 10 females) aged from 20 to 40 years and body weight from 50 to 70 kg. Each volunteer was given 6 lotions (F0, F1, F2, F3, F4, and F5) and applied once each day. Lotion (1 gram) was applied behind the ears and elbow crease before night sleeping for 6 hours. Observation was conducted the next morning for any feeling of itchiness, signs of swelling, and skin rash. Consistency of the lotion was measured on softness, greasiness, and stickiness. The lotion must be easily spread and soften upon application which indicates uniform consistency. The quality of the lotion including pH, viscosity, and homogeneity were measured according to Arpiwi et al. (2020) and it was compared to the Indonesian National Standard (SNI 16-4399-1996).

Repellent activity of the lotions

The repellent activity of the lotions was measured under laboratory conditions (Phasomkusolsil and Soonwera 2010). Twenty-five females starve mosquitos were placed in 20 x 20 x 20 cm cage covered with perforated cloth. The treatments were control + (a commercial anti-mosquito lotion), control-(lotion base) and lotion with different concentrations of *V. trifolia* essential oils (F1 = 2%, F2 = 3%, F3 = 4%, F4 = 5% and F5 = 6%). Lotion (1 mL) was applied to the human hand where the right hand as a control and the left hand as treatments. The right hand was applied with basis lotion and left hand with anti-mosquito lotion. Right hand was inserted into the cage for 5 minutes every hour for a total period of six hours. The left hand was inserted into the cage soon after right hand. The number of mosquito landing or biting the hand was counted. Repellent activity was measured using the following formula by Wathoni et al. (2018).

$$\text{Protection ability (\%)} = \frac{\Sigma(C - T)}{\Sigma C} \times 100$$

Where; C: mosquito perch on control hand, T: mosquito perch on treated hand.

Data analysis

Essential oils content, mosquito repellent activity, quality of lotions, and soil properties were analyzed using One Way Anova with SPSS at significance level 5%. The presence of any significant differences followed by Post Hoc analysis using Duncan. The correlation between essential oils yield and soil properties was analyzed using Pearson at 0.05 and 0.01 levels of significances.

RESULTS AND DISCUSSION

Essential oils yield

The yield of essential oils and climate data from each location is presented in Table 1. The yield of essential oils varied significantly ($P < 0.05$) among growth locations of the plants. The highest oil yield was obtained from Sidakarya (0.0627% w/w) while the lowest was from Panjer (0.0147 % w/w).

The yield of essential oils from fresh leaf samples in the present study was lower than another study in Iran with 0.10 to 0.22% oil yield varies seasonally (Dahsheikh et al. 2019). Growth location of the plant influenced the essential oil yield. The climate data of each location showed that during 2019 the mean of minimal and maximal temperatures ranged from 25.82 to 27.34°C, mean of minimal and maximal humidity ranged from 81.11 to 83.14%, and mean of rainfall ranged from 3.08 to 3.14 mm/year. Since temperature, relative humidity, and rainfall were quite similar among locations it was likely soil properties of each location might contribute to different yields of essential oils.

Chemical constituents of the essential oils from *Vitex trifolia*

The constituents of essential oils from *V. trifolia* were identified using GC-MS (Figure 2). There were 5 peaks in the chromatogram with very close retention time from one another, which ranged from 4.09 to 5.107 minutes. The 5 peaks were identified as constituents of the essential oils

with different retention times and percent area (Table 2). The main constituents were cis-ocimene (44.57%), this was followed by alpha Thujene (25.63%), cyclopentene,3-isopropenyl-5,5-dimethyl (18.19%), α -pinene (6.38%), and β -pinene (5.22%).

There were 5 chemical constituents in the essential oils from *V. trifolia* and these were much lower than other studies. For example, Thomas et al. (2019) find 16 compounds where caryophyllene and α -Eucalyptol are the major which constitutes from 38.36% and 25.72% respectively. Chandrasekaran et al. (2018) find 89 compounds in the essential oils from *V. trifolia* with eucalyptol, sabinene, and caryophyllene are the main constituent represents for 16.35%, 9.44%, and 8.91% respectively. The difference in the essential oil constituents probably due to internal, external factors and their interactions. For example, the maturity of plant concern, genetics, soil type, climate, and geographic origin (Figueiredo et al. 2008; Dhifi et al. 2016).

Table 2. Chemical constituents of essential oils from *Vitex trifolia*

Compounds	Retention time (min.)	Area (%)
α -Pinene	4.09	6.38
α -Thujene	4.261	25.63
cis-ocimene	4.383	44.57
Cyclopentene,3-isopropenyl-5,5-dimethyl	4.711	18.19
β -Pinene	5.107	5.22

Table 1. Detail locations of leaf and soil samplings, essential oils yield, and climate data during 2019

Location	Latitude	Longitude	Oil yield	Mean of minimal and maximal temperatures (°C)	Mean of minimal and maximal humidity (%)	Rainfall (mm/year)
Panjer	8°39'59"S	115°14'01"E	0.0147±0.001a	25.89-27.28	81.86-82.89	3.13
Sidakarya	8°42'39"S	115°13'21"E	0.0627±0.010c	25.82-27.34	82.10-83.13	3.14
Sanur	8°41'32"S	115°14'46"E	0.0473±0.004b	25.88-27.28	81.11-83.14	3.08

Values of oil yields were mean \pm standard deviation (SD) from triplicates. Values in the same column followed by different letters were significantly different at $P < 0.05$.

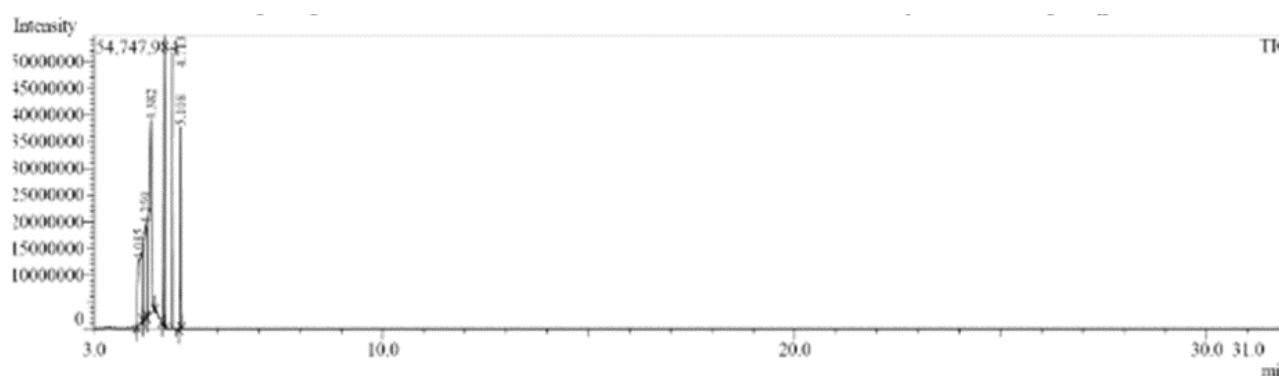


Figure 2. Gas Chromatography-Mass Spectrometry (GC-MS) profile of the essential oils from *Vitex trifolia*

Soil properties

Soil properties from each sampling location are presented in Table 3. All locations had similar pH, electric conductivity, and available potassium. The content of C organic in Panjer and Sanur was 2.99 and 2.56% respectively, and in Sidakarya was 1.6%. Total nitrogen content was 0.13% both in Panjer and Sanur and 0.06% in Sidakarya. The available phosphorus was very high both in Panjer and Sanur and high in Sidakarya. Water content (field capacity) was 37.02% in Panjer, 26.01% in Sanur, and 27.62% in Sidakarya. Soil texture in Panjer was loam, while in Sanur and Sidakarya were loamy sand respectively.

The essential oils yield was negatively correlated with electric conductivity, C organic, available P and K, water content, content of silt, and clay. This means that an increase in essential oils yield is accompanied by a decrease in those soil properties. In other plant species, such as *Ziziphora clinopodioides*, the essential oil yield is negatively correlated with EC and pH (Amiri et al. 2019). The only highly positive correlation on oil yield was with sand content with Pearson correlation values of 0.927. This indicated that the yield of essential oils is greatly influenced by the sand content of the soil. The soil sample from Sidakarya contained 78.74% sand and *V. trifolia* from this location contained the highest oil (0.0627% w/w). Similar result is also reported in other plant species of *Thymus migricus* where the essential oil yield is positively correlated with sand content in the soil, whereas clay and silt contents are negatively correlated (Yavaria et al. 2010).

Microscopic evaluation of leaf

The cross-section of lamina is presented in Figure 3. The leaf thickness ranged from $160.18 \pm 1.11 \mu\text{m}$ to $225.97 \pm 1.27 \mu\text{m}$. The adaxial epidermis was 16.9 ± 0.91 - $25.59 \pm 0.85 \mu\text{m}$ thick, layered with thin cuticle and had sparse single-celled non-glandular trichomes. A double layer hypodermis of 51.46 ± 1.53 - $56.92 \pm 1.21 \mu\text{m}$ thick was beneath the epidermis, where the second layer was interrupted in places by 4-18 palisade cells. The mesophyll consisted of 4-5 layers cylindrical palisade cells. Idioblasts were observed in the mesophyll which was large and isodiametric. Spongy parenchyma was found beneath the palisade. The abaxial epidermis was thinner than the adaxial and had dense glandular and non-glandular trichomes. These refer to glandular and non-glandular hairs (Dogan et al. 2008). Idioblast is an internal secretory cell which is bigger and differ in shape from neighboring cells and contains terpenes in *Lantana camara* of the Verbenaceae family (Zabele et al. 2005; Passos et al. 2009). In terms of cell size, it was not the case of idioblast in the present study where it was hardly distinguished from the variable sizes of neighboring cells. Some neighboring cells were smaller, some bigger and some nearly the same sized. According to Muliya et al. (2018), idioblast can be distinguished by the cell content since idioblast is morphologically similar to neighboring cells. In the present study, idioblast stained red color more intense than neighboring cells, possibly indicating the cell content.

Table 3. Soil properties at different locations

Location	pH	EC (mmhos/cm)	C organic	N total	Available P	Available K	Water content (%)	Content of (%)			Texture
								Sand	Silt	Clay	
Panjer	7.7	0.68	2.99	0.13	107.95	216.03	37.02	42.67	46.49	10.84	Loam
Sidakarya	7.7	0.54	1.6	0.06	26.48	171.54	27.62	78.74	15.74	5.52	Loamy sand
Sanur	7.8	0.52	2.56	0.13	48.38	202.92	26.01	77.73	20.36	1.96	Loamy sand

Note: The correlation between essential oils yield and soil properties is presented in Table 4. Some soil parameters negatively and positively correlated with oil yield at 0.05 and 0.01 levels of significances

Table 4. Correlation between essential oils yield and soil properties

	Oil yield (% w/w)	pH	EC (mmhos/cm)	C organic (%)	N total (%)	Available P (ppm)	Available K (%)	Water content (%)	Sand (%)	Silt (%)	Clay (%)
Oil yield (% w/w)	1	0.276	-0.881**	-0.890**	-0.751*	-0.963**	-0.883**	-0.901**	0.927**	-0.956**	-0.695
pH	0.276	1	-0.346	-0.700	0.162	-0.271	-0.063	-0.410	0.361	-0.307	-0.503
EC mmhos/cm	-0.881**	-0.346	1	0.659	0.485	0.923**	0.658	0.960**	-0.974**	0.952**	0.915**
C organic (%)	-0.890**	-0.07	0.659	1	0.943**	0.888**	0.998**	0.688*	-0.760*	0.834**	0.387
N total (%)	-0.751*	0.162	0.485	0.943**	1	0.736*	0.940**	0.465	-0.569	0.661	0.157
Available P (ppm)	-0.963**	-0.271	0.923**	0.888**	0.736*	1	0.884**	0.937**	-0.937**	0.993**	0.764*
Available K (%)	-0.883**	-0.063	0.658	0.998**	0.943**	0.884**	1	0.679*	-0.754*	0.830**	0.376
Water content (%)	-0.901**	-0.41	0.96**	0.688*	0.465	0.937**	0.679*	1	-0.987**	0.966**	0.923**
Sand (%)	0.927**	0.361	-0.974**	-0.760*	-0.569	-0.973**	-0.754*	-0.987**	1	-0.992**	-0.888**
Silt (%)	-0.956**	-0.307	0.952**	0.834**	0.661	0.993**	0.830**	0.966**	-0.992**	1	0.822**
Clay (%)	-0.695*	-0.503	0.915**	0.387	0.157	0.764*	0.376	0.923**	-0.888**	-0.822**	1

Note: Water content was measured based on the field capacity. C: carbon, EC: electric conductivity, K: potassium, N: nitrogen, P: phosphorous, *: Correlation is significant at the 0.05 level, **: Correlation is significant at the 0.01 level

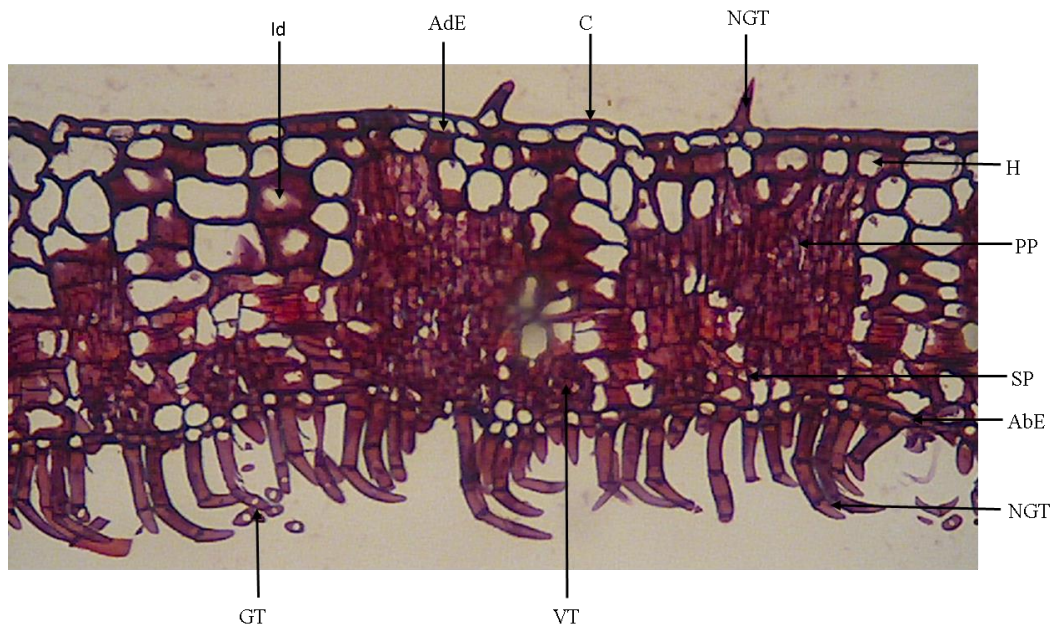


Figure 3. Cross-section of *Vitex trifolia* leaf via lamina. AbE: abaxial epidermis, AdE: adaxial epidermis, C: cuticle, GT: glandular trichome, H: hypodermis, I: idioblast, PP: palisade parenchyma, SP: spongy parenchyma, NGT: non-glandular trichome, VT: vascular tissue

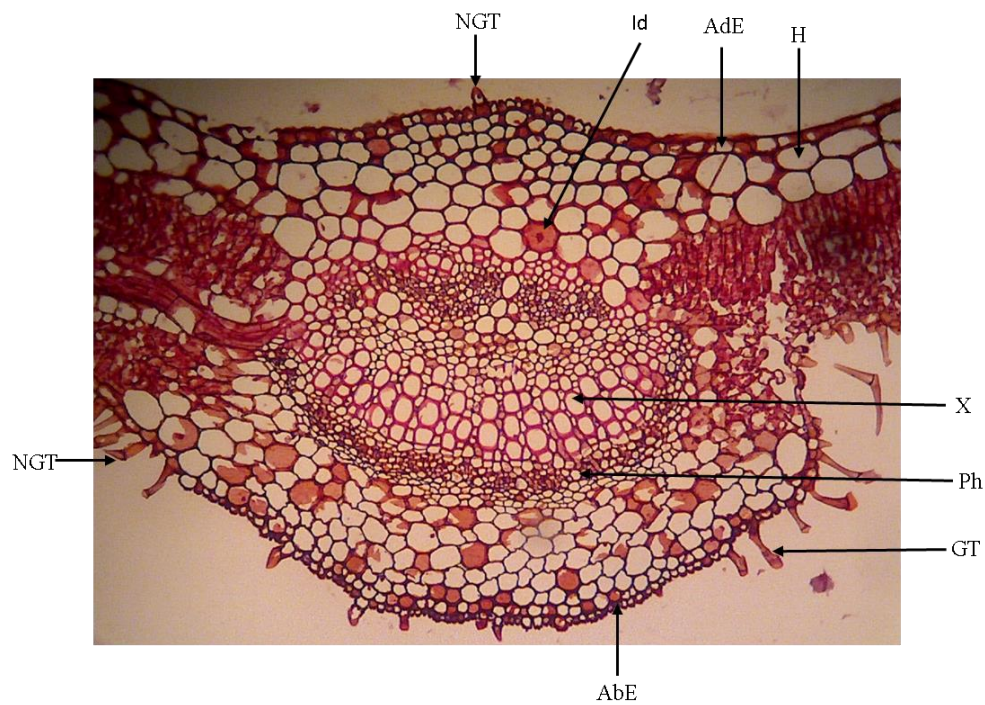


Figure 4. Cross section of *Vitex trifolia* leaf via midrib. AbE = abaxial epidermis, AdE = adaxial epidermis, C = cuticle, GT = glandular trichome, H = hypodermis, I = idioblast, Ph = phloem, PP = palisade parenchyma, SP = spongy parenchyma, NGT = non-glandular trichome, VT = vascular tissue, X = xylem

Trichome is a hair-like epidermal derivate present in aerial parts of plants. There are two types of trichomes, glandular and non-glandular. Glandular trichome is a site of essential oils secretion and accumulation in many plant species (Huchelmann et al. 2017) including member of the Verbenaceae family (Combrinck et al. 2007). Non-glandular trichome in *V. trifolia* was the single-celled

conical shape of the adaxial surface, and long multiple celled in the abaxial surface of the leaf. Both glandular and non-glandular trichomes play important roles in plant protection and defense against biotic and abiotic stresses, such as UV and oxidative stress, water deficit, and herbivores (Glas et al. 2012; Karabourniotis et al. 2020).

The cross-section of midrib is presented in Figure 4. The midrib ranged from 650.29 ± 17.27 to 930.29 ± 4.46 μm thick and predominantly to the abaxial side of the leaf. It consists of adaxial epidermis with sparse non-glandular trichomes. Beneath the adaxial epidermis was two hypodermal layers with large rectangular, square, and isodiametric cells. The vascular system was in the median arch of midrib consists of several parallel rows of xylems and surrounded by several layers of phloem elements. Isodiametric idioblast presented in the ground tissue stained red. Abaxial epidermis covered with glandular and non-glandular trichomes with smaller and shorter than those in the lamina.

The secretory structure of *V. trifolia* leaves was internal secretory cells idioblast and external secretory cells, glandular trichome. These were found in both lamina and midrib. In other species of the Verbenaceae, such as *Lantana camara*, those secretory structures also exist (Passos et al. 2009; Moura et al. 2005). The secretory structures are site of essential oils biosynthesis, accumulation, and storage (Lee and Ding 2016).

Sensorial analysis of the lotions

Sensorial analysis of the lotions including color, odor, sensitivity, and consistency is presented in Table 5. The color of lotion base (F0) and lotion with 2% essential oils was white, while the color of F2, F3, F4, and F5 was yellowish-white due to the addition of essential oils from *V. trifolia*. The odor of lotion ranged from weak, mild to strong as the percentage of essential oils added increases. The lotion base had no odor since it was not added with essential oils. All lotions did not cause rash, itchy, and swell to the human volunteers, meaning that the lotion did not cause sensitivity. The lotions were soft, not greasy, and not sticky upon application to the skin indicating acceptable consistency.

Sensorial analysis of the lotions revealed that the lotion has weak, mild to strong odors of essential oils of *V. trifolia*. Lotion with 5% essential oils had a mild odor while lotion with 6 % essential oils had strong odor and both had similar protection abilities. This might be of the customer preferences who might like mild or strong odors. The lotions were non-irritant and this confirmed safe for human skins. The lotions were not sticky or greasy and these give comfort to the skins. Safety, effectiveness, and pleasant during uses are prerequisites of plant-based insect repellent (Diaz et al. 2016). These prerequisites were fulfilled by the lotions formulated with essential oils from *V. trifolia*. In addition, the quality of formulated lotions met the requirement of National Indonesian Standard (SNI 16-4399-1996) for pH, viscosity, and homogeneity. Composition of the lotions along with mixing techniques greatly influenced consistency and homogeneity of the lotions (Yadav et al. 2014).

The quality of the lotions including pH, viscosity, and homogeneity were compared to the Indonesian National Standard (SNI 16-4399-1996) is presented in Table 6. The pH of lotions ranged from 6.41 in F0 to 6.87 in F5. The viscosity of the lotions ranged from 3016.66 in F0 to 2694.00 in F5 and all lotions were homogenous. These indicated that the formulated lotions fulfilled the requirements of the standard.

Repellent activity of the lotions

The repellent activity of the lotions containing essential oils from *V. trifolia* was measured as percentage of protection ability during the 6-hour experiment. Protection ability (%) of the lotions is presented in Table 7. There was significantly different ($P < 0.05$) among formulated lotions in their protection ability.

Table 5. Sensorial analysis of the lotions

Lotion	Color	Odor	Sensitivity	Consistency
F0	White	No odor	Nil	Acceptable
F1	White	Weak <i>V. Trifolia</i> oil	Nil	Acceptable
F2	Yellowish white	Mild <i>V. Trifolia</i> oil	Nil	Acceptable
F3	Yellowish white	Mild <i>V. Trifolia</i> oil	Nil	Acceptable
F4	Yellowish white	Mild <i>V. Trifolia</i> oil	Nil	Acceptable
F5	Yellowish white	Strong <i>V. Trifolia</i> oil	Nil	Acceptable

Note: F0: lotion base, F1: lotion with 2% essential oils, F2: lotion with 3% essential oils, F3: lotion with 4% essential oils, F4: lotion with 5% essential oils, F5: lotion with 6% essential oils

Table 6. Quality of the anti-mosquito lotions

Characteristics	SNI 16-4399-1996	Lotion					
		F0	F1	F2	F3	F4	F5
pH	4.5-8.0	$6.41 \pm 0.59a$	$6.53 \pm 0.38b$	$6.63 \pm 0.38c$	$6.69 \pm 0.20c$	$6.78 \pm 0.01d$	$6.87 \pm 0.01e$
Viscosity	2000-5000	$3016.66 \pm 7.37f$	$2969.00 \pm 15.39e$	$2865.33 \pm 8.62d$	$2783.33 \pm 14.84c$	$2659.66 \pm 14.84a$	$2694.00 \pm 3.60b$
Homogeneity	Homogenous	Homogenous	Homogenous	Homogenous	Homogenous	Homogenous	Homogenous

Note: F0: lotion base, F1: lotion with 2% essential oils, F2: lotion with 3% essential oils, F3: lotion with 4% essential oils, F4: lotion with 5% essential oils, F5: lotion with 6% essential oils. Values of pH and viscosity are means of raw three measurements. Mean of pH and viscosity in the same followed by different letters are significantly different at $P < 0.05$

Table 7. Repellent activity of anti-mosquito lotion with different concentrations of essential oils from *Vitex. trifolia* at different time (hour) of observation

Lotion	Protection ability (%) at different time of observation (hour)					
	0	1	2	3	4	5
Control-	40.00±0.49a	31.21±0.98a	29.00±0.95a	27.23±1.19a	24.88±1.20a	20.51±1.86a
Control +	95.78±0.52d	92.14±1.05d	85.31±1.45d	62.77±1.80c	51.62±0.87c	44.86±1.87b
F1	61.48±1.15b	56.20±0.65b	52.22±1.30b	50.41±0.69b	48.85±0.82b	46.15±1.75b
F2	69.83±0.68c	68.79±0.84c	65.52±0.96c	61.6±0.62c	59.95±1.09d	57.47±0.89c
F3	100.00±0.00e	100.00±0.00e	100.00±0.00e	99.74±0.51d	92.15±1.18e	85.74±0.74d
F4	100.00±0.00e	100.00±0.00e	100.00±0.00e	100.00±0.00d	95.55±0.92f	90.62±0.75e
F5	100.00±0.00e	100.00±0.00e	100.00±0.00e	100.00±0.00d	96.97±0.79f	85.79±0.75d

Note: Control-: lotion base, control +: commercial anti-mosquito lotion, F1-F5: lotion with essential oils from *V. trifolia* at 2, 3, 4, 5, and 6% respectively. Values are mean ± standard deviation (SD) with 4 replications. Mean of protection ability in the same column followed by different letters are significantly different at $P < 0.05$

The highest protection ability was achieved by lotion with 5% and 6% essential oils while the lowest was by control-(lotion base). Lotion base had 40% protection ability at 0 h and it decreased gradually over time and finally was 18.71% at 6 h. Commercial anti-mosquito lotion (control +) had 95.78% protection at 0 h and it decreased every hour and at 6 hours, it was 35.18%. Lotions with 5 and 6% essential oils gave 100% protection for 3 hours from *Ae. aegypti*. The protection remained high which was 80.77% to 85.16 % during six hours of experiment.

The protection ability of formulated lotions was much higher and longer compared to commercial anti-mosquito lotion contains DEET (Tabel 6). These results indicated that essential oils from *V. trifolia* at low concentration (5-6%) incorporated into lotions were an effective repellent for *Ae. aegypti*. The repellent activity is due to the synergy effect of chemical constituents presented in the essential oils (Nerio et al. 2010). In other research, repellent activity is measured as protection time, which is the time from application of essential oils until two successive bites observe. By using this method the protection time of lotion with 10% essential oils from *V. trifolia* is 1.8 hours against *Ae. aegypti*, 8 hours against both *Ae. albopictus* and *Anopheles dirus*, and 7.5 hours against *An. quinquefasciatus*. *Ae. aegypti* is more aggressive than other species tested, therefore the species has shorter protection time than others (Tawatsin et al., 2006). Therefore, the protection ability of lotions containing essential oils from *V. trifolia* needs to be tested using other diseases-vector mosquito species, such as *An. dirus* and *An. quinquefasciatus*. Nonetheless, essential oils from *V. trifolia* possessed good repellent activity against *Ae. aegypti*, where at concentrations of 5-6% in lotions gave 100% protection for 3 hours and it remained high (81.17% and 85.16%) at 6 h.

Chemical constituents of essential oils that have strong repellent activity are monoterpenes and sesquiterpenes (Nerio et al. 2010). The essential oils components in *V. trifolia* were α -Pinene, α -Thujene, cis-ocimene, and β -Pinene and according to Tan et al. (2015), these compounds belong to monoterpenes group.

In conclusion, the yield of essential oils from *V. trifolia* ranged from 0.0147±0.001 to 0.0627±0.010 % w/w. The

constituents of the oil were cis-ocimene, α -Thujene, Cyclopentene,3-isopropenyl-5,5-dimethyl, α -Pinene, and β -Pinene. The oil yield was positively correlated with the sand content of the soil. The secretory structure of the leaf was idioblast and glandular trichome. Lotions formulated with essential oils from *V. trifolia* were soft, not greasy and not sticky upon application and non-irritant. Lotions with 5 and 6% essential oils gave 100% protection for 3 hours from *Ae. aegypti* and the protection remained high during six hours of experiment.

ACKNOWLEDGEMENTS

We would like to thank Ministry Education and Culture Republic of Indonesia for funding this research via DIPA PNPB 2020, Udayana University, Bali, Indonesia with research scheme *Penelitian Unggulan Udayana*.

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