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Original Article

ACTIVITY ASSAY AND IDENTIFICATION OF LARVICIDAL AND REPELLENT COMPOUND GROUPS OF MARIGOLD FLOWER (*TAGETES ERECTA* L.)

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ABSTRACT

Objective: The mosquito *Aedes aegypti* is the primary vector for spreading the dengue virus, which causes Dengue Hemorrhagic Fever (DHF). Marigold (*Tagetes erecta* L.) is a plant with potential as a biopesticide. This study aims to determine marigold's larvicidal and repellent activities and identify the chemical groups responsible.

Methods: Extraction was performed using the maceration method with 96% ethanol. Fractionation was carried out using the Liquid-Liquid Extraction (LLE) method with three solvents: n-hexane, ethyl acetate, and water. Fraction separation utilized Vacuum Liquid Chromatography (VLC), followed by classical column chromatography, and compound groups were identified through Thin Layer Chromatography (TLC) monitoring. Larvicidal tests were conducted using samples of the Mariold Flowers Extract (MFE), n-Hexane Fraction (nHF), Ethyl Acetate Fraction (EAF), and Water Fraction (W. F.). Repellent testing was conducted using samples of the MFE and nHF at 1% and 2% concentrations.

Results: Phytochemical screening identified flavonoids, polyphenols, steroids/triterpenoids, monoterpenoids, and sesquiterpenoids in MFE. The nHF showed the highest larvicidal efficacy against *A. aegypti* larvae, with an LC_{50} value of 18.80 µg/ml. The nHF exhibited the highest repellent activity, achieving 88.07% repellency at 2% concentration after 3 h. Terpenoids, detected through TLC with an R_f value of 0.58, were further analyzed using Gas chromatography–mass spectrometry (GC-MS), revealing o-cymene, a monoterpene derivative with potential larvicidal and repellent properties.

Conclusion: Marigold flowers have the potential for development as a natural larvicide against *A. aegypti* larvae and as a mosquito repellent.

Keywords: *Tagetes erecta* L., Repellent, Larvicide, n-hexane fraction, Monoterpene

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INTRODUCTION

The *Aedes aegypti* mosquito is the primary vector for transmitting the dengue virus, which causes Dengue Hemorrhagic Fever (DHF). According to a report by the World Health Organization (WHO) in 2022, approximately 3.5 billion people are at risk of contracting dengue, with 1.3 billion of them living in endemic DHF areas, except for Korea [1]. The use of synthetic pesticides has begun to be abandoned and switched to a bio-based pesticide that is easily available, and safe for humans and the environment [2]. Chemicals such as N, N-diethyl-m-toluamide (DEET) are often used as repellents, with the mechanism of action inhibiting the chemical receptors of lactic acid and carbon dioxide in mosquitoes. Temophos (Abate) is a type of insecticide that kills insects at the larval stage. Abate works by binding and destroying the cholinesterase enzyme in the larvae resulting in continuous muscle contractions, convulsions, and eventually, the larvae will die. However, the use of chemicals causes resistance in mosquitoes and has negative effects on humans. Therefore, the use of plants containing active compounds as an alternative to repellents is considered safer and more effective to avoid its adverse effects [3, 4].

Due to their remarkable larvicidal, pupicidal, and adulticidal properties, phytochemicals with mosquito potential are now recognized as powerful alternative insecticides to replace synthetic insecticides in mosquito control programs [5]. One plant that has potency as a biopesticide is a the marigold flower (M. F.) (fig. 1). The marigold plant is native to Mexico and other warm regions of the Americas. This plant belongs to the Asteraceae family [6]. The main components in the essential oil of M. F. are hydrocarbon monoterpenes (ocimene, limonene, terpinene, and others), acyclic monoterpene ketones (tagetone, dihydrotagetone, and tagetenone) and lower amounts of sesquiterpenes and oxygenated compounds [7]. The secondary metabolites of this plant have natural insecticidal effects that have been widely demonstrated in MF by various methods as larvicides and repellents. Because these metabolites, especially for mosquitoes, can cause contact toxicity, respiratory and digestive disorders [8]. The study presents a method to track groups of compounds based on the activity of M. F., which have the potential to be larvicides and insect repellents, utilizing ethanol solvent extraction. Compound analysis of Vacuum Liquid Chromatography (VLC) sub-fraction results using Gas Chromatography-Mass Spectrometry (GC-MS).

MATERIALS AND METHODS

Instrument and materials

The instruments used in this study are GC-MC. Gas chromatography instruments (Perkin Elmer Clarus 500, USA) and mass spectrometer (Perkin Elmer Clarus SQ 8S). Yellow M. F. are collected from Manoko Experimental Garden, Lembang, which is the main material. The chemicals used include ethanol 96% (Merck Jakarta, Indonesia), n-hexane (Merck Jakarta, Indonesia), ethyl acetate (Merck Jakarta, Indonesia), chloroform, methanol, toluene, silica gel GF_{254} plates (Merck Jakarta, Indonesia), silica gel H60. The experimental animals used in this study were *A. aegypti* instar III/IV larvae collected from ITB Bandung and adult female *A. aegypti* mosquitoes aged 3-5 days from LOKA Pangandaran Health Laboratory, West Java.

Collection and preparation of simplicia

Yellow marigold plants obtained from the Manoko Experimental Garden Lembang were determined at Herbarium Jatinangoriense, Biosystematics and Molecular Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Padjadjaran University, Bandung to determine the clarity of the plant species used in the study. The Yellow M. F. are collected in the morning and then washed with water to remove dust and other foreign objects, then the petals are separated from the flower crowns and then the flower crowns are spread on a tray to be dried in an oven at 40 °C. After drying, it is then blended until it becomes a fine powder. MF simplicia powder is sieved using a 60 mesh sieve until a fine powder of homogeneous size is obtained and weighed as much as 500 g for maceration.

Standardization of simplicia

To keep the quality of traditional medicines produced maintained, it is necessary to standardize herbal medicinal raw materials. Standardization of simplicia using non-specific methods, including drying loss, moisture content, total ash content, acid-insoluble ash content, water-soluble essence content and ethanol soluble-essence content are specific parameters [9]. The equations to determine the percentage of standardization are as below:

$$\%\text{Total ash content} = \frac{\text{Total ash weight}}{\text{Weight of simplicia}} \text{ x } 100\%$$

$$\%\text{Acid-Insoluble Ash} = \frac{\text{Acid soluble ash weight}}{\text{Weight of simplicia}} \text{ x } 100\%$$

$$\%\text{Water-Soluble Ash} = \frac{\text{Weight of water insoluble ash}}{\text{Weight of simplicia}} \text{ x } 100\%$$

$$\%\text{ Moisture Content} = \frac{\text{Final volume obtained}}{\text{Weight of simplicia}} \text{ x } 100\%$$

$$\%\textit{Drying loss} = \frac{\textit{Weight of vaporizer cup and simplicia-Weight of empty vaporizer cup}}{\textit{Weight of simplisia before heating}} \textit{ x } 100\%$$

$$\%\text{Water-Soluble Essence Content} = \frac{\text{Extract weight}}{\text{Weight of simplicia}} \text{ x } \frac{\text{solvent volume}}{\text{volume of the filtrate taken}} 100\%$$

$$\%\text{Ethanol-Soluble Essence Content} = \frac{\text{Extract weight}}{\text{Weight of simplicia}} \text{ x } \frac{\text{solvent volume}}{\text{volume of the filtrate taken}} 100\%$$

Preparation of extract

The dried M. F. simplification powder (500 g) for 2x24 h was extracted by maceration using 1 l of 96% ethanol solvent, the extraction process was repeated 3 times. The obtained macerate was concentrated using a rotary evaporator at 50 ℃ and vaporized on a water bath to obtain a concentrated extract. The concentrated extract is measured, and the percentage yield is calculated using the equation below:

$$\%\text{ Yield: } \frac{\text{Extract weight}}{\text{Simplicia weight}} \text{ x } 100\%$$

Phytochemical screening

Phytochemical screening procedures for simplicia and marigold flower extracts (MFE) based on the Indonesian Herbal Pharmacopoeia of 2017, which includes flavonoids, alkaloids, tannins, polyphenols, saponins, steroids/triterpenoids, monoterpenoids, and sesquiterpenoids [10].

Fractionation

A certain amount of MFE dissolved in 100 ml of water. The water extract was put into a separatory funnel and n-hexan was added in a ratio of 1: 1. After that, it was shaken and allowed to stand until the two solvents separated, this process was carried out 3 times. The water layer was put back into the separating funnel and then ethyl acetate (1:1) was shaken and allowed to separate, repeated up to 3 times. The water fraction (W. F.), ethyl acetate fraction (EAF), and n-hexane fraction (nHF) were evaporated using a rotary evaporator to obtain a concentrated fraction. The three obtained fractions are then evaporated using a water bath to obtain concentrated fractions, and the yield percentage is calculated using the equation below:

$$\%\text{ Yield: } \frac{\text{Fraction weight}}{\text{Extract weight}} \text{ x } 100\%$$

Phytochemical profile monitoring using thin layer chromatography (TLC) method

MFE, WF, EAF, and nHF were subjected to TLC monitoring. The TLC elution process was carried out using a chromatography chamber containing mobile phases toluene: ethyl acetate: formic acid (4:3:1), chloroform: acetone: formic acid (10:2:1), chloroform: methanol (9:1) and n-hexane: ethyl acetate (7:3). With stationary phase using silica gel GF_{254} plate with size 4x7 cm. The GF_{254} silica gel plate was spaced 1 cm at the bottom and 0.5 cm at the top as the elution limit. After the elution process was completed, it was observed under UV 254 nm and UV 366 nm. The TLC plate was then sprayed with H_2SO_4 5% and Liebermann Burchard (LB) spotting reagents. Then heated briefly which was then observed again under UV 254 nm and UV 366 nm.

Preliminary larvicide assay

Preliminary assays were carried out to ensure the concentration range by using various concentration variations to determine the estimated toxicity. The test samples used were MFE and fractions. MFE and all fractions were made 10,000 µg/ml mains solution obtained by weighing 500 mg and then dissolved in 50 ml of distilled water. Then a concentration range was made, which is 10 µg/ml, 100 µg/ml, and 1000 µg/ml by using the dilution of the main solution. Each concentration was made in 100 ml of distilled water and put into a plastic cup. A total of 10 *A. aegypti* larvae that have reached instar III or IV were each put into a plastic cup containing the test sample. The negative control was 100 ml of distilled water, while the positive control was temephos 1% (Abate). The test was conducted three times and was conducted at room temperature 25-28 ℃. After 24 h of testing, the number of dead mosquito larvae was counted. The results obtained were recorded in the mortality table. From the % mortality data of *A. aegypti* larvae, the concentration that causes 50% larval mortality will be determined. The results obtained were recorded in the mortality table of the treatment groups were corrected using the equation:

$$\%\text{ Mortality: } \frac{\text{Total deaths of larvae}}{\text{Total larvae}} \text{ x } 100\%$$

Larvicidal assay

The concentration range used in the larvicide test refers to the results of the preliminary test by making a master solution and then diluting it according to the dilution rules with each concentration made in 100 ml of distilled water. The negative control used was only 100 ml of distilled water while for the negative control, Temephos 1% (Abate) was used. The amount of *A. aegypti* larvae used was 25. The test was conducted three times and was conducted at room temperature 25-28 ℃. After 24 h of testing, the number of dead mosquito larvae from each plastic cup was counted with the toxicity category according to Martiningsih 2013 (table 1). The results obtained were recorded in the mortality table of the treatment groups were corrected using the equation [11]:

$$\%\text{ Mortality: } \frac{\text{Total deaths of larvae}}{\text{Total larvae}} \text{ x } 100\%$$

Table 1: Toxicity category based on LC_{50} value [12]

No	LC_{50} (µg/ml)	Category
1	<30	Highly toxic
2	30-1000	Toxic
3	>1000	Non-toxic

Larvicide assay data analysis

Data analysis for the results of larvicide testing using statistical analysis Krusikal Wallis test by comparing the effect of each sample with the control. Then carried out probit analysis. Probit analysis is used in biological testing to determine the response of the subject under study by the presence of larvicidal stimuli to determine the mortality response. The Lethal Concentration 50 (LC_{50}) value was obtained by calculating using the probit analysis method. LC_{50} is calculated by converting the percent (%) mortality into probit values and making a linear regression against the logarithm of concentration.

Repellent assay

The Health Research Ethics Committee of the Faculty of Medicine, Jenderal Achmad Yani University, has approved the repellent test procedure (protocol number M3.2408.025, letter number 033/UM3.08/2024). The samples used for the repellent assay were MFE and the active fraction from the larvicidal assay was made into a cream dosage form while the control cream base was used only. *A. aegypti* mosquitoes were obtained from the Pangandaran Health

Laboratory Institute. A total of 25 female *A. aegypti* mosquitoes that were fasted 12 h before to testing were prepared for each treatment and then introduced into each test cage. The mosquito repellency test used the volunteer's arm as bait. Previously, the volunteer's arm should be washed with running water and dried with a tissue. Volunteers were advised not to consume cigarettes for 12 h before the test and during the test [13]. The left and right arms of the volunteers before examination were first cleaned with running water and then dried. Next, the left hand was applied with base cream as a control and immediately put into a cage containing mosquitoes. Then the number of mosquitoes that landed for 10 seconds with 10 repetitions was counted. Completed testing on the control. Next, the right arm was applied to the test sample and immediately inserted into the mosquito cage counted the number of mosquitoes that landed in 10 seconds, and 10 repetitions were made. This test was carried out from the 0th hour to the 6th hour with the same testing process. The repellent testing process was carried out twice for each concentration. After the test, the percentage of rejection power was calculated using the equation:

$$\% \text{ Repellency: } \frac{C-T}{C} \text{ x } 100\% \text{ [14]}$$

Description:

C: Total mosquitoes that landed on the control group

T: Total mosquitoes that landed on the test sample group

Repellent assay data analysis

The data was then analyzed using the ANOVA test with the SPSS 26.0 application. The research data was previously checked first with a normality test because it is a requirement before testing with the ANOVA method. Normality test by looking at the results of the Shapiro-Wilk table calculation, all research variables have a significance value smaller than 0.05, which means that the research data is not normally distributed and cannot be tested using ANOVA because it does not meet the requirements. The Kruskal Wallis test was chosen as an alternative test for the ANOVA test if it did not meet the requirements, for example on normality. If the results are significant differences, the Mann-Whitney U test analysis is performed [15].

Separation by VLC

Separation using VLC is a technique for separating compounds based on their polarity. A total of 70 g of silica gel 60 H was compacted in a column using a vacuum. A 7 g sample was mixed with 10 g of silica, stirred until homogeneous, and placed into the VLC column. After leveling and adding filter paper, a gradient eluent (starting with n-hexane, then ethyl acetate, and finally methanol) was applied. After elution, the column was washed with ethanol. Sub-fractions from VLC were analyzed using TLC on silica gel 60 F_{254} plates, followed by U. V. light observation at 254 nm and 365 nm [9].

GC-MS analysis

GC-MS analysis was analyzed at Biotek Rekayasa Indonesia Bogor to determine the compound components contained in the combined sub-fractions of M. F. VLC results with the instrument method which can be seen in table 2.

Table 2: The instrument method GC-MS

Components	Conditions
Column	Perkin Elmer, Elite-5ms Capillary Column-30 m x 0.25 mm I. D. x 0.25 µm
Electron ionization	70 eV
MS Scan	40 to 450 Da
Gas Carrier	Helium
Split mode	10:1
Injector Temperature	250 °C
Injection Volume	1 µl
Solvent delay	2 min

Separation using column chromatography (CC)

A cotton filter was placed at the bottom of the column, followed by 3 g of silica gel 60 powder dissolved in approximately 20 ml of solvent. The sample was dissolved in the same solvent and added dropwise to the column using a dropper, with the flow rate set to one drop per second. Droplets were collected in vials (1-5 ml each), replacing the vials when the droplet color changed. This process was repeated with the introduction of new eluent until the droplets became clear. Sub-fractions were monitored using TLC, and their R_f values were calculated to identify potential larvicidal and repellent compounds from M. F.

RESULTS

The general research process starting from the determination to the identification of MF compounds can be seen in fig. 1. The determination of marigold plants at Herbarium Jatinangoriense, Biosystematics and Molecular Laboratory, Department of Biology, FMIPA UNPAD No. 381/lBM/IT/III/2024 explained that the type of plant under study has the scientific name *Tagetes erecta* L., synonym *Tagetes major* Gaertn, local name marigold flower/tahi ayam and family Asteraceae.

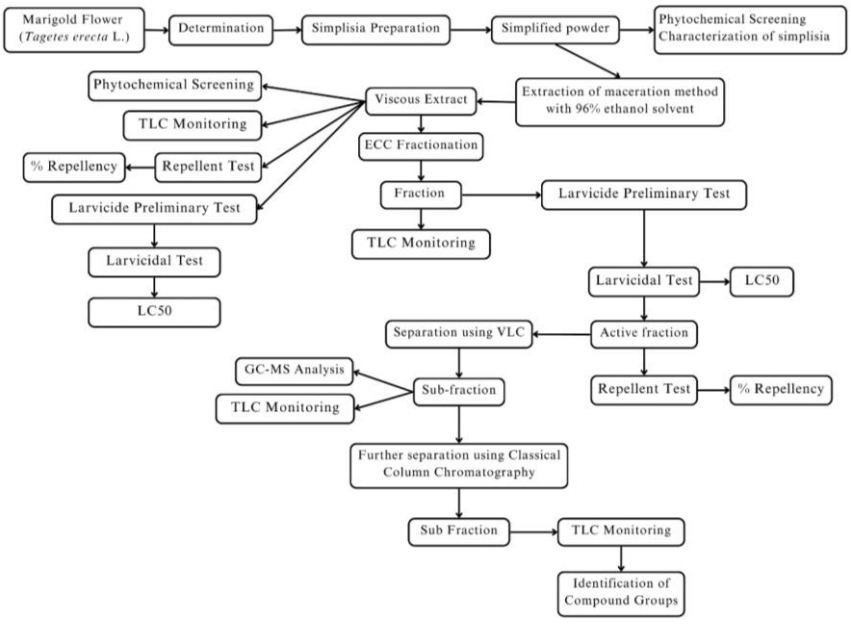


Fig. 1: Overview of research methods

The standardization of simplicia was performed three times to ensure the reliability and validity of the obtained percentage results and the results can be seen in the table 3. Extraction was carried out by weighing 500 g of M. F. then macerating them in 96% ethanol solvent obtained a yield of 23.13% (115.64 g) (table 4).

Fig. 2: Marigold plant (*Tagetes erecta* L.)

Phytochemical screening was performed to identify secondary metabolites present in the simplicia and extract of M. F. The results of M. F. simplisia contain flavonoids, saponins, tannins, polyphenols, steroids/triterpenoids, monoterpenoids, and sesquiterpenoids. While the MFE contains flavonoids, polyphenols, steroids/triterpenoids, monoterpenoids, and sesquiterpenoids (table 5).

Table 3: Standardization of M. F. Simplicia

Standardization type	Result
Total ash content	5.82±0.41 % w/w
Acid-Insoluble Ash	0.26±0.06 % w/w
Water-Soluble Ash	3.75±0.22 % w/w
Water-Soluble Essence Content	24.17±4.08 % w/w
Ethanol-Soluble Essence Content	31.01±2.71 % w/w
Moisture Content	6.33±1.40 % v/w
Drying loss	4.55±0.18 % w/w

Description: weight/weight (w/w); volume/weight (v/w)

Table 4: MFE weight and yield

Sample	Weight	Yield
MFE	115.64 g	23.13%

Table 5: Phytochemical screening results of M. F. simplicia and MFE

Compound group	Result	
	M. F. Simplicia	MFE
Flavonoids	+	+
Alkaloids	-	-
Saponin	+	-
Tannins	+	-
Polyphenols	+	+
Steroids/triterpenoids	+	+
Monoterpenes and sesquiterpenes	+	+

Description: (+) Detected, (-) Not detected

50 g of MFE was fractionated using n-hexane, ethyl acetate, water solvents, and obtained EAF had the largest yield of 37.44% (18.72 g), followed by W. F. at 32.26% (16.13 g), and nHF at 27.24% (13.62 g) (table 6). TLC monitoring was performed on MFE, nHF, EAF, and W. F. samples. The mobile phases were used toluene: ethyl acetate: formic acid (4:3:1), observation under 365 nm UV lamp after spraying with LB spotting agent and heating showed the component detected in lanes 1 (MFE) and 2 (nHF) (fig. 8).

Table 6: Weight and yield of nHF, EAF and W. F.

Fraction type	Weight	Yield
nHF	13.62 g	27.24%
EAF	18.72 g	37.44%
WF	16.13 g	32.26%

MFE, nHF, EAF and W. F. were conducted preliminary larvicidal tests agains *A. aegypti* larvae with three repetitions. The results show that the average percentage of each sample that causes 50% mortality of *A. aegypti* larvae is MFE 100 µg/ml, nHF 10 µg/ml, EAF 100 µg/ml and W. F. 1000 µg/ml (fig. 3). The results of larvicidal testing at all concentrations of W. F., EAF, nHF, and MFE showed an increasing percentage of mortality as the concentration increased. Then the concentration of each sample that showed statistically significant differences compared to the negative control using the Kruskal-Wallis test ($p<0.05$) were MFE 200 µg/ml ($p=0.033$), MFE 250 µg/ml ($p=0.009$) and MFE 300 µg/ml ($p=0.004$). nHF 30 µg/ml ($p=0.025$), nHF 40 µg/ml (0.012), and nHF 50 µg/ml (0.004). EAF 100 µg/ml ($p=0.032$), EAF 150 µg/ml ($p=0.006$), and EAF 200 µg/ml ($p=0.003$). W. F. 1100 µg/ml ($p=0.038$), W. F. 1200 µg/ml ($p=0.009$), and W. F. 1300 µg/ml ($p=0.004$) (fig. 4). The LC_{50} value of each sample obtained from the analysis using linear regression (probit) in fig. 5 shows that nHF is more effective in killing *A. aegypti* larvae with an LC_{50} value of 18.80 µg/ml with a highly toxic category compared to other samples (table 7).

The repellent test protocol has obtained ethical approval from the Health Research Ethics Commission of the Faculty of Medicine, Jenderal Achmad Yani University, Cimahi No. 033/UM3.08/2024. The samples used are MFE and nHF. Where nHF gave effective results in the larvicide test and the concentrations used were 1% and 2%, respectively. In nHF with a concentration of 2%, the best repellency was obtained at 88.07% at 3 h after application (table 8). Repelen data were analyzed first using the normality test before ANOVA analysis. Where the results showed the data were not normally distributed ($p>0.05$). Then continued with the Kruskal Wallis analysis and the results showed significant differences ($p<0.05$) between control with 1% MFE, control with 2% MFE, control with 1% nHF and control with 2% nHF, each of which gave $p=0.000$ (fig. 7).

nHF gave effective results from the larvicide and repellent tests, the separation was continued using VLC and obtained 17 sub-fractions. Furthermore, TLC profile was monitored using toluene mobile phase: ethyl acetate: formic acid (4:3:1). The same spot appeared after being sprayed with H_2SO_4 spot expositor under UV 365 nm at numbers 6 to 9 (fig. 8) which were then combined because they had the same TLC profile. GC-MS analysis was carried out for the combination to find out what compounds were thought to have potential as insecticides. The MS results showed the presence of an ion fragmentation peak with a mass to charge ratio (m/z) of 119, which is a characteristic fragment of the o-cymene compound and confirmed with the spectrum library database (MAINLIB), showing a high match with the compound (fig. 6). Subsequently, the separation was carried out again using CC and TLC profile monitoring was carried out using the same mobile phase in TLC monitoring of VLC results. Spots appeared after being sprayed with LB spot evaluator under UV 365 nm at numbers 1 to 5 with R_f values of 0.54; 0.54; 0.46; 0.49 and 0.51, respectively (fig. 8). Subsequently, the spots that had the same TLC profile were recombined and further monitoring was carried out using TLC with the same treatment as before. A clearer spot appeared after being sprayed with LB spot evaluator under UV 365 nm which was bluish green in color with an R_f value of 0.56 (fig. 8). The results of this TLC profile are suspected to be terpenoid compounds which are one of the monoterpene.

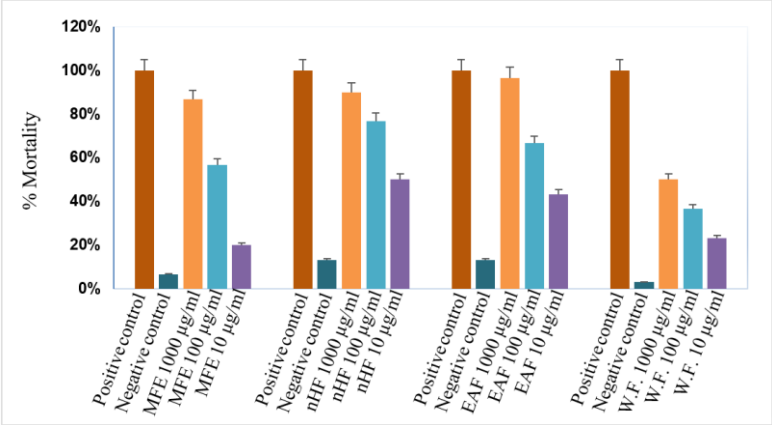


Fig. 3: The results of the average percent mortality of the preliminary larvicide test with three concentrations (n=3)

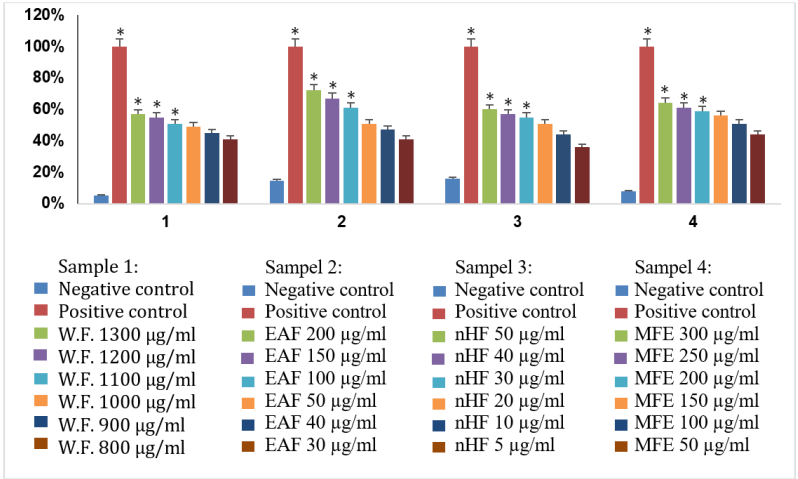
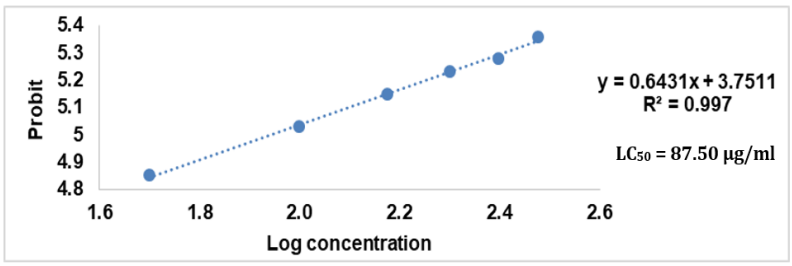
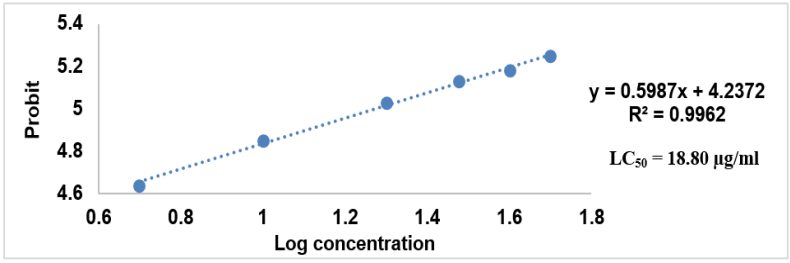


Fig. 4: The average percentage of larvicidal test results against *A. aegypti* larvae of 1) W. F.; 2) EAF; 3) nHF; 4) MFE. The x-axis is samples; the y-axis is % Mortality. *) significantly different compared to the negative control $p<0.05$. analysis using the kruskal wallis test



A



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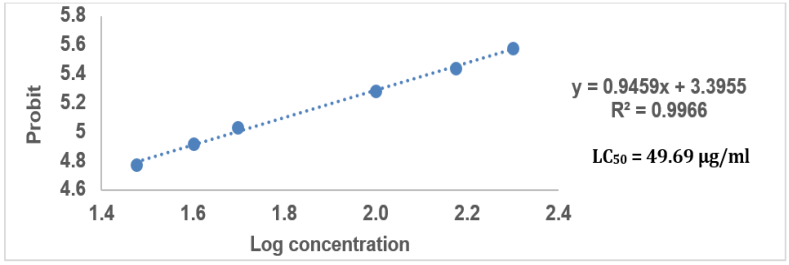

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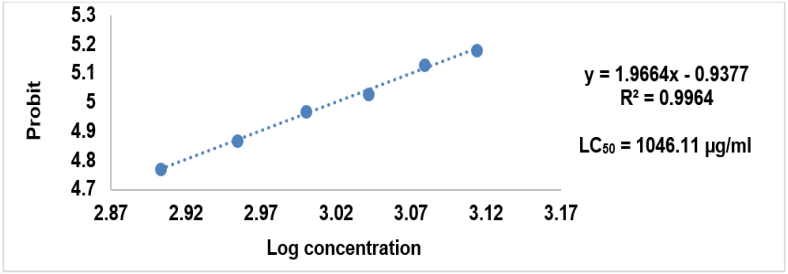

D

Fig. 5: Linear regression curve of probit value of *A. aegypti* larvae against Log C Concentration of A) MFE; B) nHF; C) EAF; D) W. F. The x-axis is the log concentration in µg/ml; the y-axis is probit value

Table 7: LC_{50} Value of MFE and fractions against larvae of *A. aegypti* mosquitoes

LC_{50} (µg/ml)			
MFE	**nHF**	**EAF**	**W. F.**
87.498	18.80	49.69	1046.11
Toxic	Highly toxic	Toxic	Non toxic

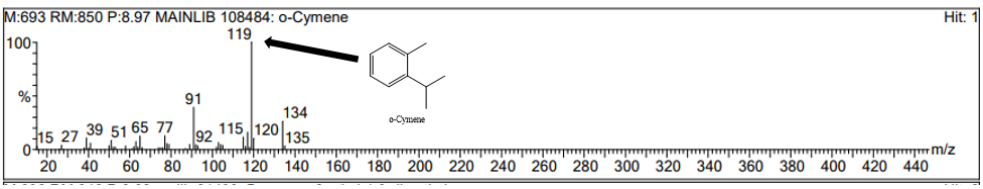

Fig. 6: The results of MS analysis of o-cymene compounds from the combination of sub-fractions 6-9 Shown with the highest peak of fragment 119

Table 8: Average results of repellent percentage of each sample against *A. aegypti* mosquitoes (n=2)

Hours	MFE 1%	MFE 2%	nHF 1%	nHF 2%
0	0.25±39.68%	0.15±77.34%	0.33±69.58%	0.01±79.48%
1	0.34±34.42%	0.15±60.72%	0.03±62%	0.04±69.45%
2	0.06±68.37%	0.08±72.62%	0.06±74.59%	0.03±70.58%
3	0.22±31.23%	0.03±77.28%	0.16±69.23%	0.02±88.07%
4	0.04±46.75%	0.13±52.78%	0.13±70.03%	0.11±70.45%
5	0.02±29.49%	0.18±40.39%	0.11±63.84%	0.03±55.36%
6	0.14±44.12%	0.13±49.65%	0.05±43.53%	0.04±50.74%

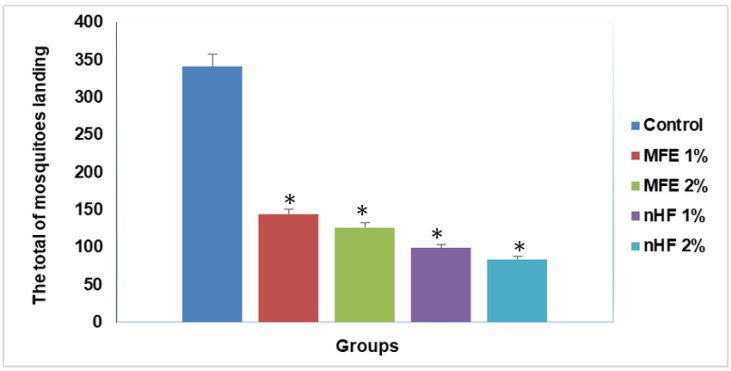

Fig. 7: Results of the total number of mosquitoes that landed on each sample with a test time of 7 h (n=2). *Significantly different compared to the control p<0.05, analysis using the kruskal wallis test

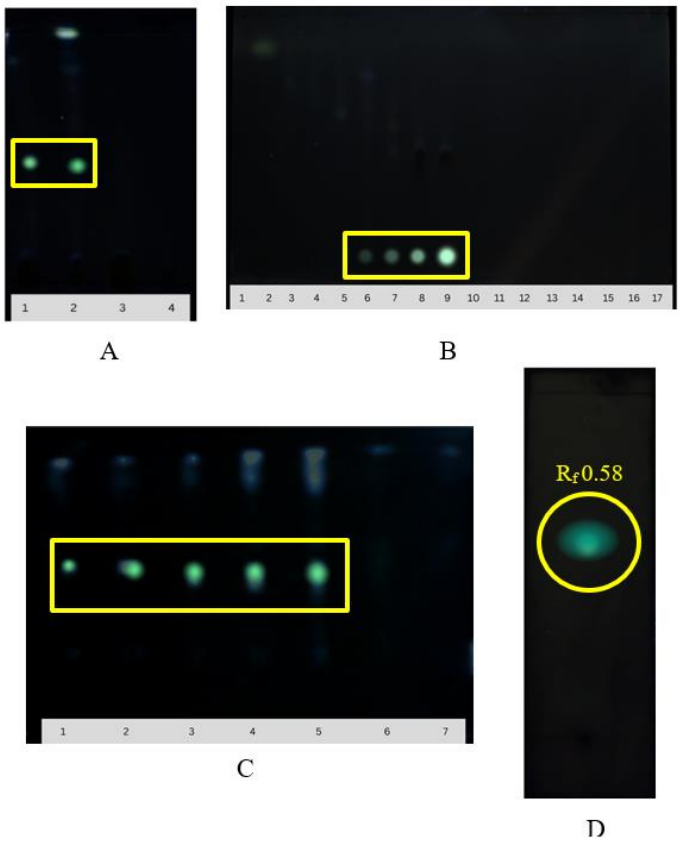

Fig. 8: A) TLC profiles of MFE and fractionated fractions; B) TLC profile of sub-fractions from separation using VLC; C) TLC profile of combined sub-fractions 6-9 from CC separation D) TLC profile of combined sub fractions 1-5

DISCUSSION

The type of plant used in this study is the marigold plant (*Tagetes erecta* L.) based on the results of determination. Table 3 is the result of the standardization of dried M. F. showing the quality level of the materials used. Ethanol-soluble content showed that the amount of ethanol-soluble material (31.01±2.71% w/w) included various types of secondary metabolites such as alkaloids, flavonoids, and

terpenoids. The high value indicates that the sample is rich in ethanol-soluble components and this becomes one of the main factors that determine the solvent used for extraction and ethanol is a good solvent to dissolve secondary metabolites [16]. The total moisture content in dried M. F. was 6.33±1.40 % v/w indicating the sample had better storage stability. However, the result of drying loss (4.55±0.18 % w/w) has a smaller value than the moisture content. This is because loss on drying measures not only the water content but also other substances that can evaporate during drying, such as residual solvents or essential oils. If the sample contains other volatile substances, differences in loss on drying results may occur despite similar water content [17].

Drying aims to decrease the moisture content, ensuring the material remains stable in storage, preventing fungal growth, and avoiding the occurrence of enzymatic reactions or processes that can decrease the quality of the ingredients [18].

Next, dried M. F. were extracted using maceration. Maceration was chosen because it can preserve secondary metabolites and high yields. The maceration principle is the slow transport of a solute to the solution until a balance is reached [16]. Table 4 shows that the weight of the extract obtained is 115.64 g with a yield of 23.13%. This result is smaller than the standardized ethanol-soluble extract content, which is due to evaporation or loss during drying the loss of active compounds during evaporation or drying can reduce the extract yield [19]. But when compared to the results of the research of Rombot *et al.* in 2020, in marigold flowers, a yield of 5.42 g was obtained from the weight of the extracted simplisia weighing 50 g with a solvent amount of 200 ml [20], this result has a higher yield (23.13%) and shows higher efficiency in isolating active compounds from simplicia.

The phytochemical screening results show a decrease of saponin and tannin components in the MFE (table 5). This could be caused by of tannins' propensity to form complexes with proteins or other molecules, therefore changing their characteristics during the extraction process. Tannins that create insoluble complexes in the solvent will not be found in the extract [21], and saponins can interact with other compounds in the simplicia to produce insoluble or precipitating complexes during the extraction process. This can cause saponins to be lost from the extract solution, even if they are present in the simplicia [22].

Fractionation was carried out, resulting in three fractions: nHF, AEF, and W. F. MFe and each fraction were monitored using TLC. Several mobile phases were used, such as toluene: ethyl acetate: formic acid (4:3:1), observation under 365 nm UV lamp after spraying with LB spotting agent and heating showed the component detected in lanes 1 (MFE) and 2 (nHF), suggesting that this compound may be non-polar to semi-polar, such as terpenoids [23]. This indicates the presence of compounds with lower polarity, which were detected in nHF and MFE. This observation indicates that non-polar to semi-polar components are more prevalent in the marigold flower fractions.

The results of the larvicide testing indicate that nHF is very effective in killing the larvae of *A. aegypti* compared to the other test samples (table 7). Non-polar extracts from plants, such as those isolated using n-hexane solvent, often contain active compounds like terpenoids. These compounds are lipophilic, which facilitates their penetration into the insect body, making them effective in disrupting the central nervous system and causing death in the larval stage of insects [24]. Fig. 5 shows a very high linear relationship indicating that the treatment used (log concentration) has a significant and measurable effect on the larval response (probit value) [25] so the concentration can be said to have a significant effect on mortality or measured changes in *A. aegypti* larvae.

Research on nHF (88.07%) at low concentrations (2%) showed longer protection, although effectiveness decreased after 3-4 h (table 8). In comparison, research with tahi kotok flower using much higher concentrations, such as 10% and 20%, resulting in high protection of 79.2% [26] and 85.86% [27]. However, the duration of the protection was relatively shorter, lasting only about 2 h. Thus, while tahi kotok flower provides strong protection at higher concentrations, MFE and nHF offer a longer duration of protection even at lower concentrations. Other studies using higher extract concentrations, such as marigold 10% [3] and citronella 97.91% [28], provided higher protection, between 79.2%-97.91%. This indicates that nHF has potential as a repellent agent with a good duration of protection even when used in low concentrations and provides an advantage in active ingredient use efficiency compared to previous studies. However, optimization is required to maintain its effectiveness after 3-4 h of duration.

7 g of nHF was loaded onto the chromatographic column, and 21 subfractions were obtained via VLC. 17 subfractions were further analyzed using TLC with toluene: ethyl acetate: formic acid as mobile phase (fig. 8). The TLC results showed that lanes 6 to 9 had the same spot after spraying with H_2SO_4, indicating the presence of the same compound. These subfractions were combined (0.752 g) for further separation. The results of GC-MS analysis conducted at Biotek Rekayasa Indonesia with number 228-08/BIOTEK/VIII/2024. Analysis of the mass spectrum of o-cymene showed that the fragment $C_9H_{11}^+$ appeared as the dominant peak, indicating that this ion is the main product of the fragmentation process. this fragment (m/z 119) shows high stability due to electron delocalization in its structure. This reflects the aromatic nature of the compound, where delocalization of electrons in the benzene ring provides additional stability to the resulting ion (fig. 6) [29], a monoterpene derivative, which was suspected to be a potential larvicide and repellent The results of research by Akeumbiwo Tchumkam *et al.* in 2023, Essential oils from the leaves of plants of the Asteraceae family containing o-cymene as one of the main components with a concentration of 10% showed significant repellent activity against *Anopheles coluzzii* mosquitoes, with repulsion levels comparable to positive controls such as *Cymbopogon citratus* essential oil [30]. These compounds cause larval death through disruption of their respiratory and metabolic systems [31].

The results of the separation obtained 27 vials. Only vial numbers 1 to 6 showed a clear spot after being given the reagents H_2SO_4 and LB. Then the merger of the number range was carried out and TLC monitoring was carried out again with the same mobile phase (fig. 10) the results showed a spot with an R_f of 0.58 with a specific reagent vanillin sulfate 10%. Based on the TLC profile, it is suspected that the group of compounds identified is terpenoids, which is reinforced by the results of GC-MS analysis, there is one compound that belongs to the monoterpene (terpenoid) group, namely o-cymene. The results of research from Akeumbiwo on plants with the Asteraceae family identified the compound o-cymene at 10.3% which has the potential as a repellent against *Anopheles colluzi* mosquitoes [30]. Vanillin sulfate spotting agent is a universal detector for terpenoid compounds that give red and blue colors [32].

CONCLUSION

This study provides valuable insights into the potential of marigold flower extracts as repellents and larvicides for *A. aegypti* mosquitoes.

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AUTHORS CONTRIBUTIONS

Fahrauk Faramayuda (Supervisor), Uwan Purnama (Researcher), and Soraya Riyanti (Cosupervisor) experimented and wrote the manuscript.

CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare. We certify that the submission is original work and is not under review at any other publication.

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