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Repellent Power of Kuit Lime Peel Ethanol Extract (*Citrus amblycarpa* L.) Against *Aedes aegypti* Mosquitoes

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ABSTRACT

This research investigated the capability of the kuit lime peel ethanol extract as a repellent against *Aedes aegypti* mosquitoes. This study used the post test only control group design approach and the experimental method recommended by the World Health Organization Pesticides Evaluation Scheme (WHOPES) with modification. This study used seven different experiment groups consisting of a negative control group using a mixture of Tween 20 and aquadest, a positive control group using commercial mosquito repellent (Autan®), and concentration groups using 5%, 15%, 30%, 45%, and 60% (m/v) of the kuit lime peel ethanol extract. Each experiment groups were applied on a volunteer's arms that are then inserted in a testing cage containing 25 female adult *Ae. aegypti* mosquitoes. Every mosquito that landed on the arm testing area will be calculated during the examination time (every 2 min for 30 min) to determine the repellent protection power percentage. Based on the result of the study using Two-Way Analysis of Variance and Duncan's Multiple Range Test, the kuit lime peel ethanol extract is confirmed to be the most effective as a repellent against *Ae. aegypti* mosquitoes at the 30% concentration and until the 29th minute post-application with a 100% protection power.

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INTRODUCTION

Commercial repellents containing chemically active substances such as *N, N-diethyl-meta-toluamide* (DEET) can cause local and systemic toxicity (Fitri *et al.*, 2022). The use of DEET on the skin often causes skin irritation (erythema and pruritus), whereas the use of DEET at high concentrations can cause more severe effects such as insomnia, muscle cramps, mood disturbances, and the formation of rashes. DEET is absorbed through the skin into the systemic circulation and 10-15% of each dose can be excreted in the urine, whereas DEET metabolites can persist in the skin and fatty tissue areas for 1-2 months (Futeri and Illahi, 2019). In addition, DEET repellents can pollute the environment, leave residues, and cause drug resistance (Aini *et al.*, 2016).

Based on the various damage caused by chemical repellents, it is necessary to develop alternative repellents that are effective and safe for both living organisms and the environment. One method is to utilize natural materials in the vicinity, such as local Indonesian plants. Kuit lime (*Citrus amblycarpa*) is a plant that has the potential to act as a repellent.

According to Irwan *et al.* (2017) and Irwan and Rosyidah (2019), kuit lime is a local and abundant citrus species in South Kalimantan, which is widely used as a typical kitchen spice, but its peel is disposed of as waste. Citrus peel contains valuable substances that can act as insecticides.

Phytochemical extracts from citrus peels have been shown to be effective mosquito repellents at certain concentrations (Effiom *et al.*, 2012). Kuit lime contains phytochemical compounds of secondary metabolites, such as alkaloids, saponins, steroids, triterpenoids, tannins, and flavonoids. These compounds have been shown to act as insecticidal larvicides (Ishak *et al*. 2021). Kuit lime peel also contains essential oils, such as limonene and linalool, which are repellents against arthropods (Nastiti, 2020). Gas chromatography-mass spectrometry (GC-MS) tests also showed that kuit lime peels contain bioactive compounds such as 2H-1-Benzopyran-2-one (Coumarin) and pentamethoxyflavone (Kasman *et al.*, 2019).

A previous study by Kasman *et al.* (2021) showed that the ethanol extract of kuit lime peel has potential as a larvicide against instar III larvae of *Ae. aegypti*, killing 96% of larvae after 24 h of measurement. Nastiti (2020) proved that ethanol extract of kuit lime peel with a concentration of 35% is effective as a repellent for *Culex* sp. mosquitoes with an average of 67% of the test mosquitoes did not land, while research on the repellent power of kuit lime peel ethanol extract against *Ae. aegypti* mosquitoes has never been carried out.

MATERIALS AND METHODS

Kuit limes were collected from kuit limes plantations at coordinates (-3.32692, 114.97567), Astambul sub-district, Banjar district, South Kalimantan. The limes were identified and determined at the Plant Physiology Laboratory, Faculty of Science and Technology, Universitas Airlangga Surabaya. Kuit limes were skinned, sorted, and then cleaned of dirt by washing with clean water, draining, and drying the newspaper evenly. The peels were aerated for approximately seven days by placing them in a place protected from sunlight, and then placed in an oven at 50 °C for 10 hours. After the orange peel was dry, it was mashed using a blender and sieved to form a fine simplicia (Kasman *et al.*, 2021).

Preparation of kuit lime peel (KLP) ethanol extract was prepared at the Pharmacology Laboratory, Faculty of Veterinary Medicine, Universitas Airlangga Surabaya. Fine simplicia of KLP was weighed as much as one kilogram and macerated with technical ethyl alcohol solvent for three days in a tightly closed container and stirred occasionally every day so that it was well mixed. The filtrate was then filtered through a sieve and evaporated using a rotary evaporator at 40 °C to obtain a concentrated extract with a concentration of 100% (m/m) (Kasman *et al.*, 2021). The extract was then diluted to obtain a liquid extract with the emulsifying agent Tween 20 and distilled water solvent to obtain various concentrations of KLP (5% (P1), 15% (P2), 30% (P3), 45% (P4), and 60% (P5)) (m/v). The concentration of KLP ethanol extract was determined by the dilution formula with the note that V_1M_1 and V_2M_2 have the same density:

$$V_1 \times M_1 = V_2 \times M_2$$

Description:
V_1 = Volume of initial solution (ml)
M_1 = Initial concentration (%)
V_2 = Volume of solution after diluted (ml)
M_2 = Concentration after dilution (%)
(Nastiti, 2020).

Samples of *Ae. aegypti* mosquitoes were obtained from the Institute of Tropical Disease (ITD) Laboratory of the Universitas Airlangga, Surabaya. The sample size criteria for the *Ae. aegypti* mosquitoes based on the WHOPES guidelines (2009) were 25 adult female mosquitoes aged 5-7 days, actively seeking hosts, fasted for 12 h before the study, and sampled randomly per test cage.

Repellent testing was conducted according to WHOPES guidelines (2009). A mixture of emulsifying agent (Tween 20 1%) and distilled water solvent was applied evenly with a brush to the test arm as a negative control, allowed to dry at room temperature, and then the test arm was inserted into the mosquito test cage. The number of mosquitoes that landed on the test arm within the 1-minute observation period was counted and recorded. After 1 min, the test arm was carefully removed from the test cage and allowed to rest for 1 min. The test arm was reinserted into the test cage for 1 min to observe and count the number of mosquito landing events. After 1 minute, the test arm was carefully removed from the cage. The test procedure was repeated for each treatment with KLP ethanol extract at various concentrations (5%, 15%, 30%, 45%, and 60%) (m/v) and positive control (Autan® repellent containing 15% DEET), respectively. The test was conducted in four replicates on different days, but at the same test time.

The number of mosquito landings on the test arm was then converted into a formula for the protection power percentage as follows:

$$\text{Protection Power Percentage (\%)} = \left(\frac{\Sigma C - \Sigma T}{\Sigma C}\right) \times 100\%$$

Description:
C = the total of mosquitoes in contact with the control arm
T = the total of mosquitoes in contact with the treatment arm/test group

Data on the protection power percentage from various test groups were statistically analyzed using the SPSS version 28 application based on WHOPES (2009) and modifications from Sudiarti *et al.* (2021) with the Two-Way Analysis of Variance (ANOVA) test, followed by Duncan's Multiple Range test.

RESULTS AND DISCUSSION

The five treatments of KLP ethanol extract had different percentages of protective power based on concentration. P1 provided an average protection

power of 86.86%, P2 provided an average protection power of 94.8%, P3 provided an average protection power of 97.26%, P4 provided an average protection power of 99.46%, and P5 provided an average protection power of 100%. These results showed that the higher the concentration of the extract, the higher the protection power and the content of active ingredients in the extract (Figure 1). The active ingredients emit a pungent odor that repels mosquitoes (Qadafi *et al.*, 2021). The results of this study are in accordance with those of Wahyuni and Adiwanto (2019), who tested the lime peel extract (*Citrus aurantifolia*) against *Ae. aegypti.*

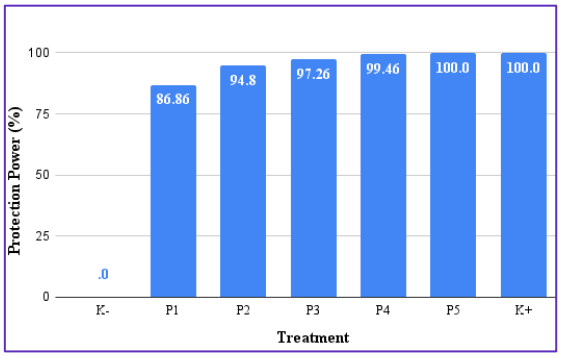

Figure 1. Mean protection power percentage (%) of kuit lime peel ethanol extract against *Ae. aegypti* based on the extract concentration.

The protection power provided by KLP ethanol extract is presumed to be because it contains several active ingredients that have been proven as repellents against mosquitoes, such as secondary metabolite group compounds (alkaloids, saponins, steroids, triterpenoids, tannins, and flavonoids), according to the method described by Irwan *et al.* (2017), which was validated with the Thin Layer Chromatography (TLC) test by Kasman *et al.* (2019). KLP also contains limonene and linalool essential oils (Nastiti, 2020). GC-MS test results show that KLP also contains the main active essential oil compounds that have potential as bioinsecticides, such as the compound 2H-1-Benzopyran-2-one (Coumarin) and the flavonoid group pentamethoxyflavone (Kasman *et al.*, 2019).

According to Effiom *et al.* (2012), the mode of action of the active ingredients is that when applied to the skin, they have an inhibitory effect on the mosquito's lactic acid receptor cells, namely by closing or changing lactic acid, which normally works as an attractant that mosquitoes can smell. The active ingredients within the extract are also volatile, along with CO_2 from the host, and change to resemble a plant's CO_2. This confuses or distracts the mosquito, as it cannot identify the host as its food source, preventing blood-sucking contact.

The way essential oils within an extract work according to Husna *et al.* (2015) and Aini *et al.* (2016) is when the extract is applied evenly on the test arm of volunteers, it seeps into the pores of the skin. As a result of body heat, essential oils evaporate into the air and emit an aroma that is detected by chemoreceptors or olfactory receptors on the antennae of mosquitoes. The receptor converts it into an impulse that is passed on by the axon of the sensory nerve to the nerve center (brain). The aroma of essential oil that is not favored by mosquitoes is then translated into the brain so that mosquitoes will avoid the source of the aroma, and thus volunteers will avoid being bitten by vector mosquitoes.

Treatment	Mean (%) ± SD
K(-)	$0.00^{d} \pm 0.00$
P1 (5%)	$86.86^{c} \pm 6.34$
P2 (15%)	$94.8^{b} \pm 4.81$
P3 (30%)	$97.26^{a} \pm 3.36$
P4 (45%)	$99.46^{a} \pm 0.83$
P5 (60%)	$100.00^{a} \pm 0.00$
K(+)	$100.00^{a} \pm 0.00$

Table 1. Mean and standard deviation of protection power percentage of KLP ethanol extract as repellent against *Ae. aegypti* mosquitoes
Note: Different superscripts indicate significant differences in each group ($p<0.05$)

Based on the results of the statistical tests in Table 1, the significance value is $p = 0.000$ ($p < 0.05$), which means there is a significant difference between treatment groups based on the extract concentrations. Statistical analysis revealed that there was no significant difference between P3 and the treatments with higher concentrations (P4 and P5) or K(+). However, P3 was found to be significantly different from P1, P2, and K(-). This confirms that KLP ethanol extract at a concentration of 30% (m/v) (P3) is significantly superior as a repellent against *Ae. aegypti* mosquitoes because it did not show a significant difference compared to the highest concentration (60% concentration) (b/v) (P5). Statistically, this result is consistent with the results of Pitarani *et al.* (2019). This was also supported by Alfiah *et al.* (2015), who stated that the effective concentration is the minimum concentration that can provide a very strong inhibition response.

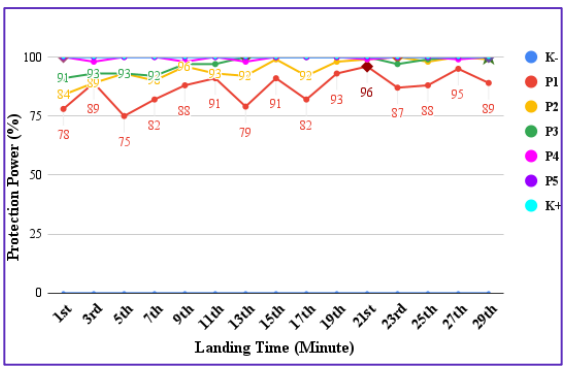

Figure 2. Protection power percentage (%) of KLP ethanol extract against *Ae. aegypti* based on landing

The five treatments of KLP ethanol extract had different percentages of protective power based

on each *Ae. aegypti* mosquitoes landing time on the test arm which can be viewed in Figure 2. At the 1st minute of treatment, P1 and P2 provided >50% protection against *Ae. aegypti* mosquitoes. In P3, the protection power was >90%, whereas P4 and P5 had 100% protection power. P1 reached a maximum protection power of 96% at the 21st minute of treatment. P2 achieved 100% protection power at the 23rd minute of treatment, P3 at the 13th minute of treatment, and P4 and P5 from the 1st minute of treatment. Based on these findings, it was revealed that the longer the observation period, the higher the percentage of protective power. This result contradicts the research of Wasistha *et al.* (2022), who showed that the longer the observation period, the lower the percentage of repellency. This is thought to be due to the observation time in this study, which is less than that of Wasistha *et al.* (2022), who tested ethanol extracts of lime leaves (*Citrus hystrix* DC) for up to 60 min and 120 min, as well as the WHOPES standard (2009), which recommends testing for up to 8 h. A longer observation period is required to determine the time required by the KLP ethanol extract when its performance decreases.

After reaching the maximum protection power, the percentage of protection power for the five treatments at each landing time fluctuated. However, the overall percentages remained >50% and >90% until the last landing time (29th minute). At the 29th minute of treatment, P1 provided 89% protection power, P2 provided 99% protection power, and P3, P4, and P5 provided 100% protection power. The same finding was also reported by Mufidah *et al*. (2020) and Hidayah *et al.* (2018). The increase and decrease in protective power are caused by the evaporation of chemical compounds that occur during the test (Mufidah *et al*., 2020). The evaporation phenomenon was stated as the crude repellent extract's true method of work, according to Mukandiwa *et al.* (2016). The crude repellent extract applied to the test arm evaporated in the test cage, affecting mosquitoes in two ways: through air contact with volatile active compounds within the extract and inhibition contact when mosquitoes land on the test arm. Both contact methods are very important for World Health Organization (WHO)-approved repellents. Crude repellent extracts are also superior to repellents made from pure fractions because they do not evaporate quickly and do not reduce the repelling effect against mosquitoes when repeated applications are made (Santya and Hendri, 2013)

Statistical tests proved otherwise that there was no significant difference in the protection power between treatments, with a significance value of $p = 0.445$ ($p > 0.05$). This result is consistent with the findings of Mufidah *et al*. (2020). Statistical tests also showed that the most effective landing time (17th minute) was not significantly different from the last landing time (29th minute). The last landing time was also not significantly different from other observation times. The observed repellent activity of the *Ae. aegypti* by KLP ethanol extract was sustained for up to 29 minutes.

The results of the study in Table 2 show the percentage of protection power of the KLP ethanol extract as a repellent based on the concentration of the extract and the landing time of *Ae. aegypti* mosquitoes. Analysis of the statistical results showed that the two variables contributed to each other in influencing the percentage of protection power, with a significance value of $p = 0.000$ ($p < 0.05$). The interaction can be seen at P3 and the 29th minute of treatment, when the KLP ethanol extract reached the maximum percentage of protection power (100%). From these results, it can be concluded that the KLP ethanol extract works most effectively at a concentration of 30% (m/v) and is still effective for up to 29 minutes with 100% protective power.

Table 2. Protection power percentage (%) of KLP ethanol extract against *Ae. aegypti* based on the concentration and landing time.

	Minute	Treatment						
		K(-)	P1	P2	P3	P4	P5	K(+)
Protection Power (%)	1st	0^{af}	78^{ae}	84^{ad}	91^{ac}	100^{ac}	100^{ac}	100^{ac}
	3rd	0^{af}	89^{ae}	89^{ad}	93^{ac}	98^{ac}	100^{ac}	100^{ac}
	5th	0^{abf}	75^{abe}	93^{abd}	93^{abc}	100^{abc}	100^{abc}	100^{abc}
	7th	0^{abf}	82^{abe}	90^{abd}	92^{abc}	100^{abc}	100^{abc}	100^{abc}
	9th	0^{abf}	88^{abe}	96^{abd}	97^{abc}	98^{abc}	100^{abc}	100^{abc}
	11th	0^{abf}	91^{abe}	93^{abd}	97^{abc}	100^{abc}	100^{abc}	100^{abc}
	13th	0^{abf}	79^{abe}	92^{abd}	100^{abc}	98^{abc}	100^{abc}	100^{abc}
	15th	0^{abf}	91^{abe}	99^{abd}	100^{abc}	100^{abc}	100^{abc}	100^{abc}
	17th	0^{bf}	82^{be}	92^{bd}	100^{bc}	100^{bc}	100^{bc}	100^{bc}
	19th	0^{abf}	93^{abe}	98^{abd}	100^{abc}	100^{abc}	100^{abc}	100^{abc}
	21st	0^{abf}	96^{abe}	99^{abd}	100^{abc}	99^{abc}	100^{abc}	100^{abc}
	23rd	0^{abf}	87^{abe}	100^{abd}	97^{abc}	100^{abc}	100^{abc}	100^{abc}
	25th	0^{abf}	88^{abe}	98^{abd}	99^{abc}	100^{abc}	100^{abc}	100^{abc}
	27th	0^{abf}	95^{abe}	100^{abd}	100^{abc}	99^{abc}	100^{abc}	100^{abc}
	29th	0^{abf}	89^{abe}	99^{abd}	100^{abc}	100^{abc}	100^{abc}	100^{abc}

Note: Different superscripts indicate significant differences in each group ($p<0.05$)

CONCLUSION

Kuit lime peel (KLP) ethanol extract (*Citrus amblycarpa*) is effective as a repellent against *Ae. aegypti* mosquitoes at a concentration of 30% (m/v) with 100% protective power. The repellent effect of the KLP ethanol extract against *Ae. aegypti* mosquitoes still last for up to 29 minutes. KLP ethanol extract worked most effectively as a repellent against *Ae. aegypti* mosquitoes at a concentration of 30% (m/v) and a duration of 29 minutes.

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AUTHORS' CONTRIBUTIONS

FNA designed and performed the experiment, data collection, data analysis, and writing of the manuscript. PH conceptualized, developed, and supervised the research and proofreading of the manuscript. ML was involved in developing the research methodology and proofreading the manuscript. RK, ES, and AS guided data analysis, data interpretation, and literature evaluation of the manuscript. All authors have read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ETHICAL APPROVAL

This research procedure has obtained a Certificate of Ethical Appropriateness by the Research Ethics Commission of the Faculty of Public Health, Universitas Airlangga through letter number 193/EA/KEPK/2023.

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