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OPTIMIZATION OF *CITRUS LIMON* PEEL EXTRACTION, DETERMINATION OF MAIN CHEMICAL COMPONENTS AND EFFECTIVENESS IN REPELLENCY AGAINST *AEDES* MOSQUITO DENGUE FEVER VECTOR

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SUMMARY

Currently, scientific publications are focusing on the pharmacological actions of *Citrus limon*'s extract and essential oil. Its essential oil is rich in bioactive monoterpenoids, such as D-limonene, β-pinene, γ-terpinene. *Citrus limon* essential oils have been recognized as the potential source of natural insecticides because of their selectivity, ability to be biologically degraded into non-toxic compounds, low impacts on biodiversity and the environment. Many previous studies have reported anti-bacterial, anti-fungal, anti-inflammatory, anti-cancer, hepato-regenerative, and cardio-protective activities of *Citrus limon* essential oil. In this study, we collected the discarded *Citrus limon* peel source in Gia Lai province to utilize as a material to build an optimized extraction process with the following criteria: extraction solvent, solvent/sample ratio, extraction temperature, and extraction time. The results showed a stable extraction process with a maximum extraction efficiency of 4.02%, at 40°C, for 3 hours, with two extraction times using 95% ethanol for solvent. Using GC/MS method, the determined limonene content accounted for 12.2% of the extract. The *Citrus limon* peel extract exhibited potency against *Aedes aegypti* (arbovirus vector) at a concentration of 0.01 mL, with protection time of 70 minutes and biting percentage of 0.9%, compared to negative control with statistically significant ($P < 0.05$). The above results correspond with the most recent publications about the effects of mosquito repellence of certain plant-based essential oils. This study has proven that *Citrus limon* peel in this locality signifies a promising candidature for future studies regarding its main active compound, limonene, in the control of dengue-transmitting vectors. Therefore, *Citrus limon* peel extract brings hope to develop new mosquito repellency products in the future.

Keywords: *Citrus limon* peel extraction, Extraction process optimization, Limonene, Mosquito repellence, Protection time

INTRODUCTION

Due to the complication of the current epidemic, notably the dengue fever and Zika transmitted by *Aedes aegypti*, anti-mosquito measurement using the synthetic chemical is currently the most effective and popular approach. Nevertheless, the pesticide-resistant ability of the insect remains to be challenging. The trend of finding alternative preventive products with low resistance ability has pointed to plant extracts. Common synthetic insect repellents, like DEET (*N,N*-Diethyl-3-methylbenzamide), acts on blocking the insect odor-sensory receptor (olfactory receptor, ORx) and suppressing the detection of carbon dioxide as the insect is attracted to 1-octen-3-ol in the human breath and sweat (Swale *et al.,* 2014). Similar odor receptors responding to DEET have been demonstrated to occur in the mosquito *Culex quinquefasciatus* (Syed *et al.,* 2008). For *Anopheles gambiae,* DEET OR83b receptors are stimulated by citronellal and modulated by cation channel TRPA1 (Kwon *et al.*, 2010). However, the use of DEET has raised concerns about potential risks to environmental and human health, especially in children (Khanikor *et al.*, 2013). Thereby, plant-based essential oils with repellent properties and low toxicity to both environment and health have been regarded as alternatives for conventional synthetic pesticides (Tong *et al.*, 2013).

Vietnamese lime (*Citrus aurantifolia*) belongs to the *Rutaceae* family and is a small herbal species. The lime peel appears green and becomes yellow when riped. The flesh divides into several sections. The juice has a sour taste. Fresh fruits are harvested throughout the year for juice drinking to improve the body's immune response. Therapeutic benefits of the lime essential oil include anti-inflammatory, disinfectant, anti-cancer and anti-parasitic effects. The lime essential oil is rich in biologically active monoterpenoids such as D-limonene (70.37%), β-pinene (3.24%), γ-terpinene (0.90%) (Russo *et al*., Jing *et al*., 2015). These compounds, particularly limonene, are highly oxygenated secondary metabolites, primarily found in the seeds, pulp and bark of citrus fruits, including lemons (Gualdani *et al*., 2016). Many studies have shown that the concentration of compounds depends on fruit development and maturation stages, and stays high in unripe fruits compared to ripe ones (Huang *et al*., 2019). Limonene has fragrance and several effects, especially mosquito repellence (Klimek *et al*., 2020). In the meantime, the lime peel generally gets discarded after taking the juice. The folk has used the lime peel to deter the mosquito in the past. Accordingly, our study is to optimize the process for lime peel extraction from which its extract was evaluated for actions on *Aedes aegypti* in order to create a novel bioproduct for repelling harmful insects.

MATERIALS AND METHODS

Materials

The adult mosquitoes were captured from rearing *Aedes aegypti* mosquito larvae in water containers in Chu Puh district, Gia Lai province. Fresh lime peels were obtained in Gia Lai province.

The lime peel was collected from localities in Gia Lai province in July 2020. The discarded materials included rotten peels and ones contaminated with mold. The selected lime peel was ground into powder before extraction.

Optimizing the *Citrus limon* peel extraction process

Investigation of solvents: lime peel powder (50 g) in a 1-liter flask was added *n*-hexane or solvents (ethyl acetate, acetone, ethanol (EtOH)), warmed at 40°C for 3 hours, and filtered; the combined solvent was concentrated to obtain the corresponding extract; experiments were performed in triplicate.

Extraction temperature: lime peel powder (50 g) added to EtOH (500 mL) and the temperature adjusted at four different temperatures, ranging from 30ºC, 40°C, 50°C, and 60°C. Filter and distillation of solvent afforded the respective extract; each procedure was repeated 3 times.

Investigation of extraction time: lime peel powder sample (50 grams) added to the solvent (EtOH), warmed to 40°C for timepoints (2 hours, 3 hours, and 4 hours), filtered, and distilled the solvent to obtain the corresponding extracts to each extraction time; each experiment was triplicate.

Quantification of limonene in *Citrus limon* peel extract by GC/ MS analysis

The limonene content of lime peel extracts was quantified by GC Thermo Scientific Trace 1310 connected to MS Thermo Scientific ITQ 900, USA. Chromatographic column was TG-5MS column (30 m x 0.25 mm, 0.25 µm) using limonene as the standard reference. Parameter settings for LC/MS systems: use TG-5MS as the column; the probe temperature was 200°C; the flow rate was 0.5 mL/min; the injection volume was 5 µL; the analysis time was 30 minutes; the column temperature was 25°C.

Sample preparation, calibration curve plotting: reference sample was dissolved in *n*-hexane to a concentration of 20 mg/mL, then diluted into a series of different concentrations (10; 5; 2; 1; 0.5; 0.1 mg/mL) to establish standard calibration curve; the lime peel extracts were mixed evenly and measured for specific weights; the solvent was then used to dissolve the samples to obtain an analytical sample that has a concentration of 392 mg/mL; the reference solution and the analytical passed through a 0.45 µm filter before injecting into the GC/MS system.

Establishing the limonene's quantitative standard curve: the equation y = a.x + b was applied to describe the relationship between the selected UV peak area (y) and the corresponding concentration of the reference (x); the quantitative standard curves had high linearity with a correlation coefficient of $R^2 \geq 0.999$ by the quantitative methods using DAD.

Analysis of signals on the GC/MS system: ionization chromatogram selected at the molecular mass response (KLPT) of 136; the limonene's selected signal peak was stable on the GC/MS system at the retention time Rt of 11.66 minutes regarding the reference samples on the quantitative scale; the computed calibration curve constructed using Chemstation software based on fragment ion peak molecular weight of 136 at retention time Rt of 11.66 minutes.

Testing effect of the extract as repellent against *Aedes aegypti*

The test was carried out by the method of Phasomkusolsil *et al.* (2010) with appropriate changes. Mosquito cages with a cage size (30 cm × 30 cm × 30 cm) contained 200 female mosquitoes *Aedes aegypti* (5 to 7 days old). Each cage of 2 cells had a drop net with an outside circulation having a size of 10 cm × 10 cm, where the arms have been in contact with the sample.

Before application of the repellents, arms of two volunteers washed and cleaned thoroughly with distilled water. Both arms were covered with rubber sleeves with a window area of 3 cm × 10 cm. On the ventral part of forearm, the left arm was for treatment and the right arm was for control. A total of 0.01 mL of the samples (including the extract from *Citrus limon* peel, limonene mixed with coconut oil, insect repellent lotion, and coconut oil) was applied to the treatment area of the left forearm of each volunteer and used the coconut oil as a negative control and insect repellent lotion (containing DEET 25.63%) as a positive control. After applying the test repellent, the volunteer was instructed not to rub, touch, or wet the treated forearm. The right forearm, which acted as a negative control, was not treated, and was exposed for up to 30 seconds to the mosquito cage contained female mosquitoes. If having at least two mosquitoes landed on or bit the arm, the repellency test kept performing. The test continued until at least two bites occurred in a three-minute period. If no mosquitoes bit or landed during the three-minute period, the arm was withdrawn from the cage. The repellency test period was carried out every 30 minutes until fewer than 2 mosquitoes bit or landed during the three minutes, at which period, the repellency test stopped. The time between applications of the repellents was recorded as the protection time. For comparison, a percentage of mosquito biting was calculated for each test using the following formula:

Biting % = 100 × C/200

Where: C is the total number of biting by the end of the test. The test was carried out 3 times per sample.

The test to determine the repellent effect of the *Citrus limon* peel extraction was stopped when C < 1% for 3 minutes. In the testing and control lots, the laboratory conditions are verified at 27 ± 2°C.

RESULTS AND DISCUSSION

Optimizing extraction process of *Citrus limon* peel

Many studies have demonstrated that *Citrus limon* peel contains chemical compositions possessing different polarities, including limonene, pinene, terpinene and other compounds, also a high content of essential oils being soluble in the non-polar solvents, such as *n*-hexane, and polar solvents, such as dichloromethane, ethyl acetate, methanol, and ethanol (Jing *et al.*, 2015). We herein examined solvents with increasing polarity, including *n*-hexane, ethyl acetate, acetone, ethanol.

Figure 1 showed that, when using *n*-hexane, acetone and ethyl acetate, the total extract content varied within a range of 1.0 to 1.1%. For polar solvent ethanol (EtOH), the total extract efficiency has doubled (4%). From the results, we chose 95% EtOH as the extracting solvent.

In the extraction of active ingredients in the *Citrus limon* peel, the amount of solvent greatly affects the extraction efficiency. We set up extraction conditions in which the reaction time was 3 hours/extraction at the reaction temperature of 40°C. For each of 50 g of sample, 95% EtOH at 400 mL, 500 mL, and 650 mL were used to assess the necessary amount for extraction efficiency optimized. The results in Figure 2 showed that the extraction efficiency lifted gradually to the ratio of solvent to sample being 10:1 when increasing the extraction solvent EtOH from 400 mL to 500 mL. The amount of solvent continued to rise with an insignificant change in the extraction efficiency. For one time of extraction, the efficiency was only 1.8%. For two times of extraction, the efficiency was about 4%. No substantial increase was identified for three times of extraction. Therefore, by extracting with EtOH twice, the ratio of solvent to sample being 10:1 is the most effective.

During the extraction process, temperature plays a crucial role in extraction efficiency. The examination was carried out at rising temperatures: 30°C, 40°C, 50°C, and 60°C.

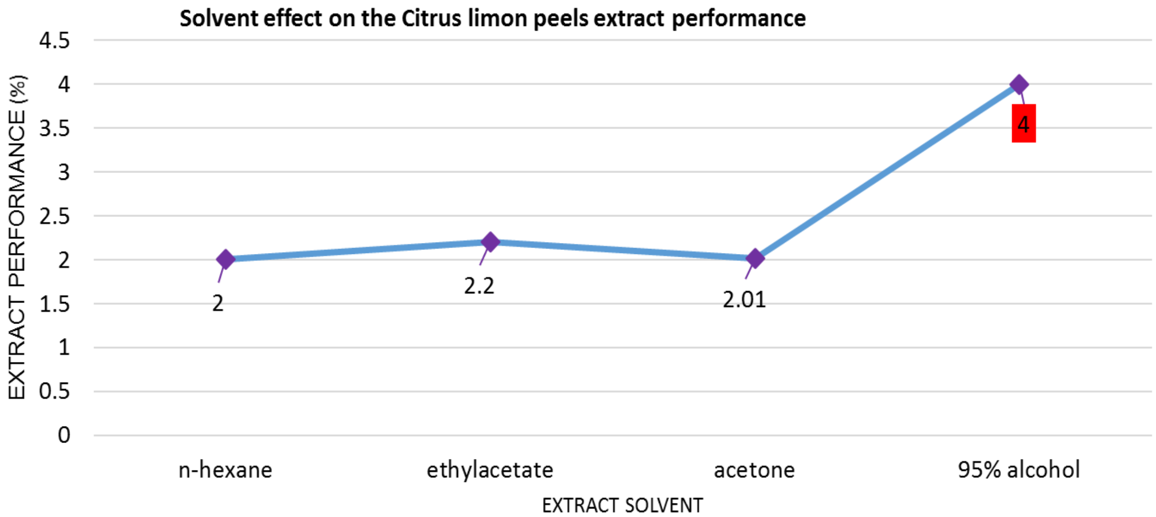

Figure 1. Effect of solvents on the performance of *Citrus limon* peel extraction

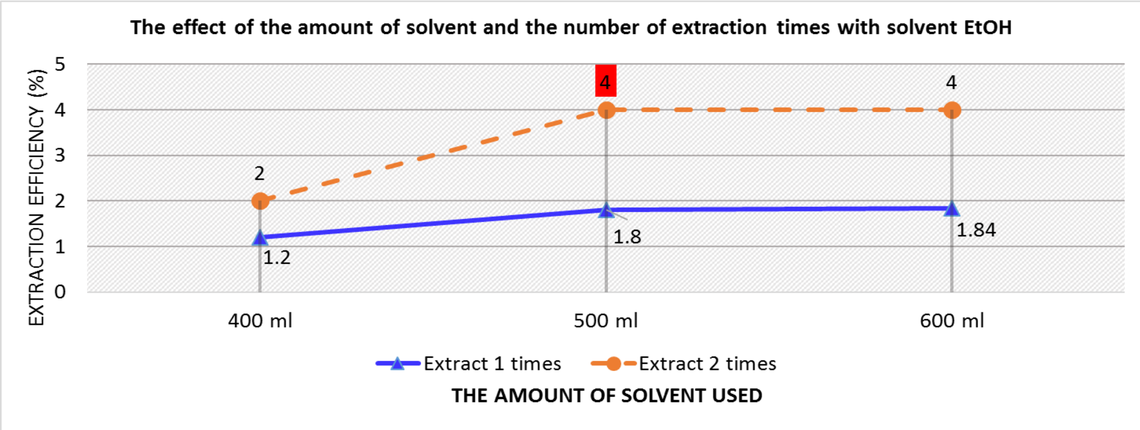

Figure 2. Effect of amount of solvent and number of repeated extractions on *Citrus limon* peel.

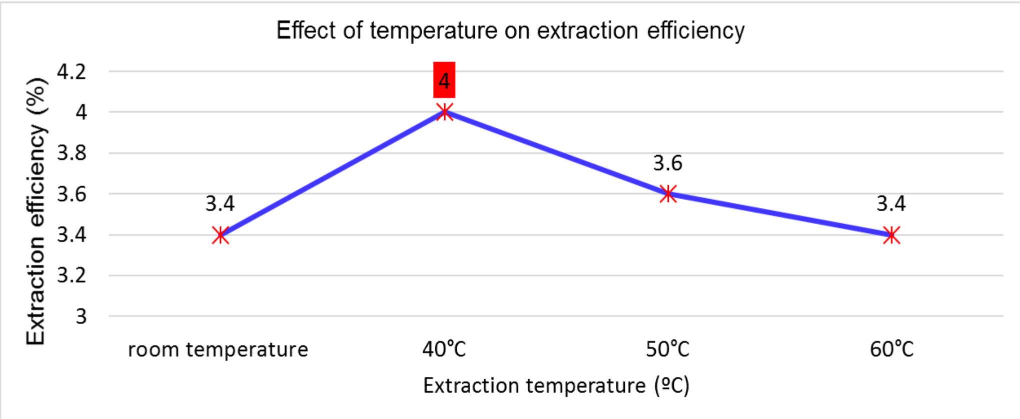

Figure 3. Effect of temperature on extraction efficiency of *Citrus limon* peel.

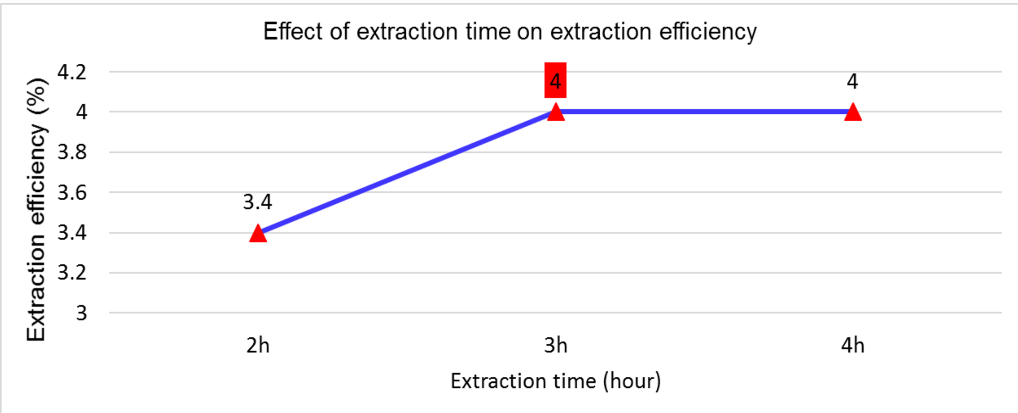

Figure 4. Effect of extraction time on *Citrus limon* peel.

The results showed that extraction at 40°C gave the highest extract amount, approximately 4% in comparison to the original material, while at room temperature or temperature rising at

50°C or 60°C, the amount of extract was only reached 3.4% - 3.6% (Figure 3). Hence, we choose the extraction temperature at 40°C for the effective extraction.

As presented in Figure 4, the extraction efficiency approached 3.4% and 4% after 2 hours and 3 hours, respectively. When the extraction time increased to 4 hours, the extraction efficiency did not increase (Figure 4). For economic benefit, 3 hours was optimal for the *Citrus limon* peel extraction process.

Thus, in laboratory settings, 50 g of freshly ground *C. limon* peel powder sample was extracted with 250 mL of solvent and EtOH at 40°C for 3 hours with stirring. After filtering the extract, the residue was re-extracted using 250 mL of solvent with EtOH and distilled in a vacuum at 50°C. The obtained extracts were 2 g (4%).

The results in Figure 5 showed that the extraction efficiency achieved stability (4%) as examining at the scale of 1 kg, 2 kg, 3 kg and 5 kg. Phung *et al*. (2019) reported that the *Citrus limon* peel extracted with ethanol and water in the ratio of 5:2:1 for 5 hours at 60°C demonstrated the extraction efficiency at 2.7% (Phung Thi Kim Hue *et al*., 2019). Another study using a microwave-assisted steam distillation for 45 minutes to extract the lime oil achieved an optimum yield of 1.15% (Shakir *et al*., 2015). Thus, our study has discovered the optimal process to get extracts from the *Citrus limon* peel with an efficiency of 4.0%.

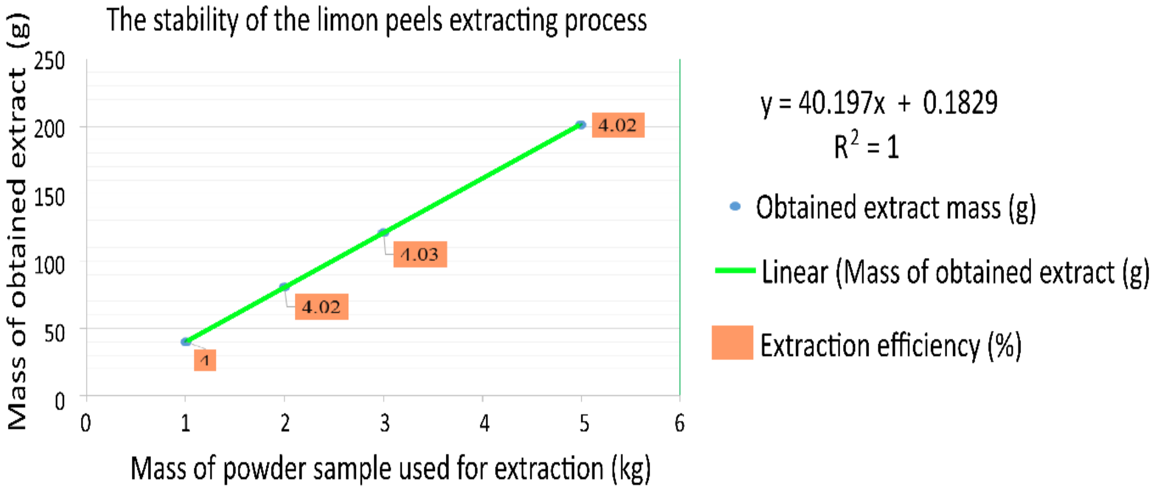

Figure 5. Examination of the stability of the *C. limon* peel extraction process.

Quantification of limonene from *Citrus limon* peel extract by GC/MS

The determining organic matter components in the *Citrus limon* peel extract were investigated by gas chromatography and mass spectrometry (GC/MS). The obtained results (Figure 6) showed that the limonene content in the extract was 12.2%. According to available studies, the limonene is high in the *Citrus limon* peel (Jing *et al*., 2015; Shakir *et al*., 2015) and has anti-mosquito activity (Soleimani *et al*., 2017). Limonene is also a common constituent of many plant extracts having a repellent feature (Russo *et al*., 2017).

Thereby, the peel of limes has been referred a byproduct having low commercial importance. Most of them are discarded that increases the risk of environmental pollution without being recognized the highly applicable potentials as shown above. Exploiting them is not only for economic values, but also to build closed agriculture and sustainable development.

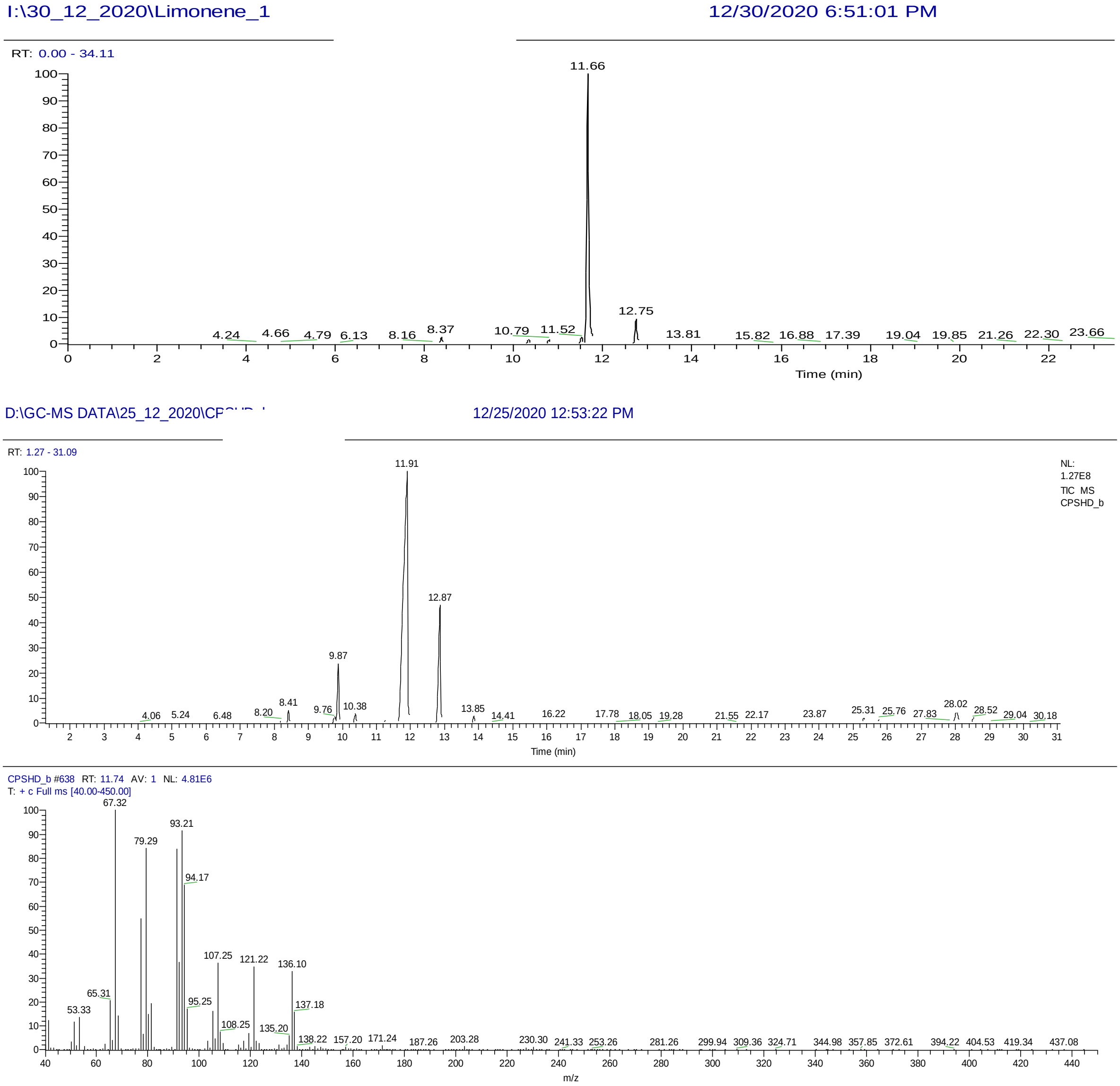

Figure 6. Determining limonene content in *C. limon* peel extract by GC/MS analysis.

Aedes aegypti mosquito repellent effect of limonene and the *Citrus limon* peel extraction

To evaluate the mosquito repellent effect of the active ingredient extracted from the peel of *C. limon*, we exposed 0.01 mL of the sample to the adult *Ae. aegypti* after the extraction and determination of the main active ingredient as described in the method section. The results (Table 1, Figure 7) determined that the *Citrus limon* peel extract showed effectiveness against *Ae. aegypti* (arbovirus vector) at the concentration of 0.01 mL, protection time of 70 minutes, biting percentage of 0.9%. The limonene had a better protective effect against the bite of *Ae. aegypti* (protection time was 90.0 ± 3.00 and biting percentage was 0.7 ± 1.48, compared to negative control which was statistically significant (P < 0.05)), when compared with the positive control (mosquito repellent product containing DEET 25.63%) the repellent effect of limonene and *C. limon* peel extract was worse. The result was consistent with the latest publications about effects of repelling mosquitoes of certain aromatic essential oils. Soleimani has reported that lemongrass oil had a high protection time of 98.66 and 98.00 minutes for *Ae. aegypti* (Soleimani *et al*., 2017). The oil of *C. odorata* exhibited high potency against *Ae. aegypti* with a biting percentage of 0.93% (Muturi *et al*., 2017). Sritabutra and Soonwera applied limonene-containing orange peel extract mixed with coconut oil on the skin that showed the protection time of 54 minutes and the biting percentage of 0.94% (Sritabutra and Soonwera, 2013).

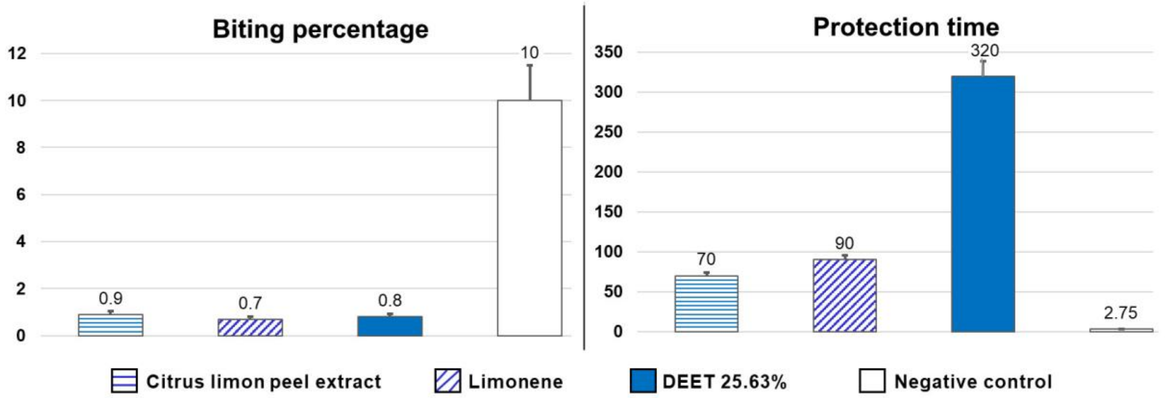

Figure 7. The protection time and biting percentage of *Aedes aegypti* after treatment.

Table 1. Solvent effect on the *Citrus limon* peel extract performance. **P value ≤ 0.05 means statistically significant*

Sample	Biting percentage	Protection time
Citrus limon peel extract	0.9 ± 0.10	70 ± 18*
Limonene	0.7 ± 1.48*	90 ± 3*
Negative control	10 ± 0.70	2.75 ± 0.06

In this study, the results showed that both *Citrus limon* peel extract and mixture of limonene and coconut oil exerted significant activity against *Ae. aegypti*. The main active ingredient that causes insect evasion in *C. limon* peel extract is likely limonene. Many studies have proven that limonene extracted from citrus peel was a fragrance agent (Xiao *et al*., 2017), limonene both created fragrance and repelled mosquitoes (Klimek *et al*., 2020; Gualdani *et al*., 2016). Insect odor and taste receptors are highly sensitive detectors of food (Hallem *et al*., 2006). Limonene may be a volatile substance, and its molecules created an odor. Limonene was the key odorant for the overall aroma of *C. limon* peel extract (Xiao *et al*., 2017). Odors bound to receptor proteins located on the hairs of specialized odor-receptor neurons exposed to the outside environment (Ditzen *et al*., 2008). It is also possible that limonene is a plant-based volatile active ingredient that is highly toxic to the insects (Gershenzon *et al*., 2007) making the insects eluding odors. TRPA1 (mutation affecting the TRPA1) is required for the activation of a BK channel to modulate odors-evoked action potentials, and for aversion to odors of the insects (Kwon *et al*., 2010). Currently, DEET is popular. DEET masks host odor by inhibiting subsets of heteromeric insect odorant receptors that require the OR83b coreceptor (Khanikor *et al.*, 2013). However, the use of synthetic chemicals to control insects and arthropods raises several concerns on the environment and human health and the ability to repel insects through essential oils of plants is favored (Lee *et al*., 2018, Tong *et al*., 2013).

The wide use of synthetic repellents against the *Aedes* mosquito has raised some issues on safety and health risks to the human and the environment. Thereby, our results may contribute to finding alternative options for the use of mosquito-repellent synthetic chemicals and support potential usages of natural product-based repellents in vector control. At present, the further development of environment-friendly bioproducts based on this product with more effective and long-lasting protection is a necessity.

CONCLUSION

In this study, we optimized extract process of discharged *Citrus limon* peel collected in Gia Lai, at the temperature of 40°C with 3 hours for each extraction time (repeated twice) using 95% ethanol as solvent. The obtained results also demonstrated the stability of the extract process with a maximum extraction efficiency of 4.0%. With GC/MS method, we determined that the content of limonene in the extract was 12.2%. *Citrus limon* peel extraction showed effectiveness against *Aedes aegypti* larvae (arbovirus vector) at the concentration of 0.05 mL, protection time of 90 minutes, biting percentage of 0.9%. The results also determined that *Citrus limon* peel extraction had high potency to control the species of vector mosquitoes. Further studies on the identification of active compounds, toxicity and field trials are needed to recommend the active fraction of these plant extracts for the eco-friendly development for control insect vectors.

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