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Research

Phytochemical Profiles and Mosquito-Repellent Properties of Indigenous Plants from Pak Panang District, Nakhon Si Thammarat, Thailand

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ABSTRACT

This study investigated the mosquito-repelling ability of folkloric indigenous plants by phytochemical extraction using three distinct methods. The phytochemical composition and antioxidant activities of the extracts were evaluated using thin-layer chromatography. The average powder weights of *Citrus maxima* (CM), *Eleocharisdulcis plantaginea* (EP), and *Thespesia populnea* (TP) were 154.33, 156.67, and 153.78 mg/g dry weight, respectively, and are substantially different from the other ten species (p=0.05). However, this technique did not affect the extract yield. The region of interest (ROI) values for the antioxidant activity of these three species were 328.9, 924.0, and 735.0, respectively. The repellent activities of the CM, TP, and EP extracts were significantly lower than those of DEET; however, the repellent activities of the mixed extract did not differ (p=0.05). This study found that indigenous plants commonly used in the region have mosquito-repellant and antioxidant properties. Combining the three extracts would yield the same activity as DEET, an ingredient used in commercial mosquito repellents.

Key words: *Aedes*, antioxidant, herb, indigenous plant, repellent, TLC

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INTRODUCTION

Mosquitoes hold significant medical importance due to their role as disease vectors and their impact on human health, especially in light of the increasing incidence of dengue fever in the last three decades (Martini *et al*., 2020). According to a report by the World Health Organization, approximately 2.5 billion people in tropical regions are susceptible to contracting this disease (Romeo-Aznar *et al*., 2018). Consequently, efforts in mosquito management and surveillance have been focused on developing strategies to regulate various aspects of mosquito ecology, including egg-laying, growth, breeding sites, and the distribution of disease vectors (Marques-Toledo *et al*., 2019; Haddawy *et al*., 2019). Therefore, a comprehensive understanding of mosquito behaviors is essential for effective prevention. The primary activity of mosquitoes is their reproductive cycle, during which they lay eggs that develop into disease-transmitting adults (Nagao *et al*., 2012; Brown *et al*., 2021).

Disease-carrying mosquito control and eradication primarily involves monitoring mosquito breeding sites, such as stagnant water and water containers, to eliminate mosquito larvae (Higa, 2011; Sareein *et al*., 2019; Connelly *et al*., 2020; Brown *et al*., 2021). The application of mosquito repellent is a common strategy, with two main types in use. First, synthetic chemicals like DEET (*N, N*-diethyl-3-methylbenzamide) at a standard concentration of 20-25% have been traditionally employed to combat these insects and are prevalent in commercial mosquito repellent products. Permethrin, an insecticide derived from *Chrysanthemum cinerariifolium*, received approval as an insecticide and repellent in the United States in 1979 (Muangmoon *et al*., 2018; Khater *et al*., 2019).

The second category of repellents is botanical, where chemicals extracted from plants represent a novel innovation (Mint Mohamed Lemine *et al*., 2017). Folkloric plants are widely embraced as mosquito repellents due to their capacity to reduce reliance on pesticides and their eco-friendly nature (Halberstein, 2005; Wiwanitkit, 2011). Their significance extends to human and animal well-being, particularly in natural environments and plantations (Tanaka *et al*., 2008; Wiwanitkit, 2011; Teanpaisan *et al*., 2017). The efficacy of folkloric plants as repellents against disease vectors depends on the diversity of plant species and their phytochemical composition (Nair & Van Staden, 2014; Altemimi *et al*., 2017; Batool *et al*., 2019). Notably, alkaloids derived from basil (*Ocimum basilicum*) and citronella (*Cymbopogon nardus*) have proven to be effective insect repellents (Chansang *et al*., 2005; Phasomkusolsil & Soonwera, 2010; Lupi *et al*., 2013). Essential oils extracted from the bark of *Hazomalania voyronii* are utilized to formulate eco-friendly repellents and insecticides for managing mosquito vectors and agricultural pests (Benelli *et al*., 2020). Furthermore, *Carpesium abrotanoides*, containing key constituents like trans-nerolidol and caryophyllene, has shown the potential to serve as a mosquito repellent by completely inhibiting *Ae. aegypti* (Haris *et al*., 2022). With the decline in public Favor for synthetic repellent products, botanical-based repellents are gaining popularity as a safer and more environmentally friendly option. Natural products, having a long history of use, are both gentle on the skin and non-harmful to the environment, unlike their synthetic, non-biodegradable counterparts (Prakoso *et al*., 2018).

Nakhon Si Thammarat, a southern Thai province known for its tropical climate that fosters the growth of diverse flora and fauna (Ounlert & Sdoodee, 2015), is witnessing a growing prevalence of diseases transmitted by mosquitoes as vectors each year (Ferreira-de-Lima, 2018). In 2020, the province reported 4,283 cases of dengue with 12 fatalities (Department of Disease Control, Nakhon Si Thammarat Province, Ministry of Public Health, 2020), emphasizing the need for collaborative efforts to combat epidemics and address public health concerns associated with disease transmission via mosquito breeding sites (Martini *et al*., 2020). Exploring the development of mosquito repellents from herbal extracts derived from various parts of plants, including roots, leaves, flowers, stems, and seeds, in addition to combining plant species native to mosquito-endemic areas, offers a promising approach. This approach not only benefits people and the environment but also reduces the use of chemical agents and enhances income from herb harvesting. Thin-layer chromatography (TLC), a versatile and essential chromatographic technique, is primarily employed to separate non-volatile compounds from plant materials. Experiments involve the use of substrates such as glass slides, plastics, and aluminum plates coated with absorbent materials like silica gel G or GF, aluminum oxides, and cellulose, depending on the specific compounds of interest. The retention factor (R_F) on the TLC plate serves as a unique indicator of each active component's polarity, akin to a plant's fingerprint (Namir *et al*., 2019). TLC is predominantly used to analyze the phytochemical profiles of plants (Jesionek *et al*., 2015). Phytochemicals, bioactive compounds found in plant-based foods, offer additional health benefits and nutritional value (Reich & Widmer, 2009). Phytochemical screening is vital for the initial selection of pharmaceutical compounds for various applications. The study's objectives were based on two hypotheses. Firstly, to assess the phytochemical profile of indigenous plants and their efficacy as repellents against mosquito species prevalent in the region, assuming that community-utilized folkloric indigenous plants would be more effective as vector repellents. Secondly, to survey physical reservoirs of mosquito larvae, which directly impacts species abundance and diversity in the area. Understanding the preferences of vector mosquitoes for egg-laying served as a guideline for monitoring the spread of dengue-carrying mosquitoes.

MATERIALS AND METHODS

Sampling area

The sampling site was determined to be in the Pak Panang District, Nakhon Si Thammarat, with coordinates of 8°14'2" N latitude and 100°1'4" E longitude. Laem Talumphuk is a sandy geographical area approximately 3 km wide and 10 km long (Figure 1). The bay's shoreline was initially composed of tidal flats and mangrove trees, but much of it has since been transformed into shrimp farms.

Mosquito collection, identification, and rearing

Mosquito samples were collected using live mosquito traps in compliance with the World Health Organization (WHO) regulations (WHO, 2020). The trap equipment was left in situ for 4–5 hr, and all mosquitoes were caught inside and then released into a cage of 30 cm (width) × 30 cm (height) × 30 cm (length). The cage was then returned to the Specialized Research Unit on Insects and Herbs (SRUIH) at Nakhon Si Thammarat Rajabhat University’s Faculty of Science and Technology. Mosquitoes are initially separated based on their morphology (Rattanarithikul, 1982; Rueda, 2004; Becker *et al*., 2020) by examining them under a stereo microscope, and the species were verified by specialists at the Division of Vector-Borne Diseases, Department of Disease Control, Ministry of Public Health for confirmation. Next, the mosquitoes were raised in a 60 cm (width) × 60 cm (height) × 60 cm (length) cage in a facility with a relative humidity of 75±5% and a temperature of 29±5°C and fed water and honey until repellent activity testing.

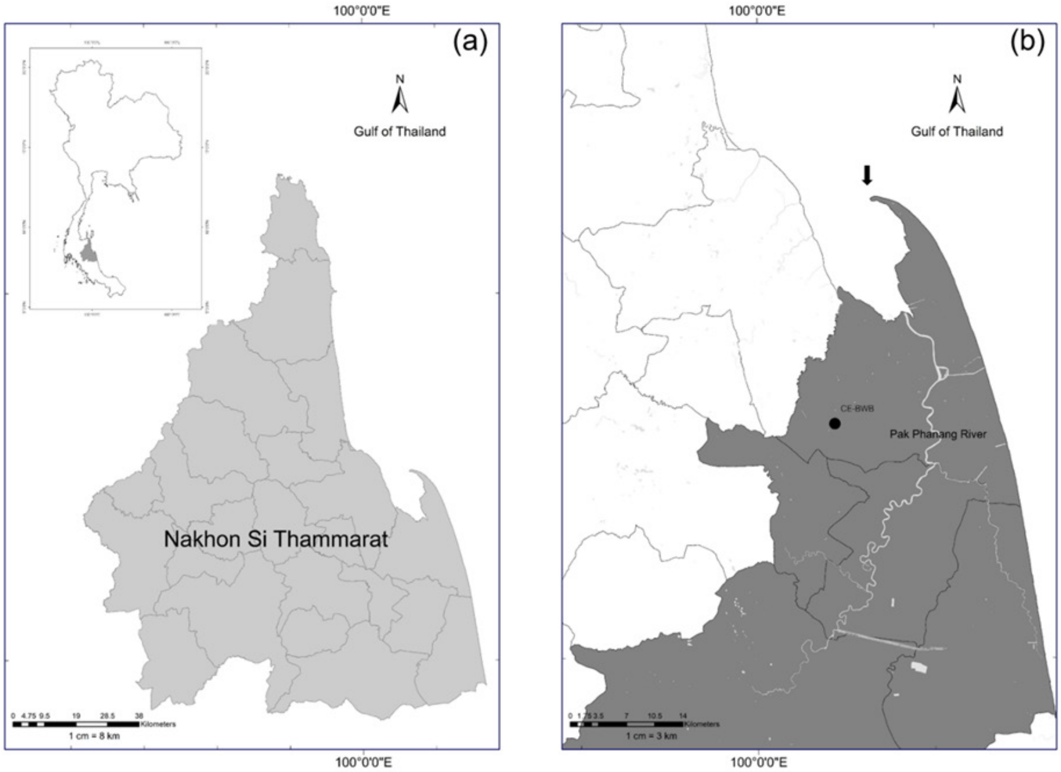

Fig. 1. The map of Thailand represents the Nakhon Si Thammarat, located in the south (a), and the sampling site for plant and mosquito collection and survey is magnified (b).

The larvae distribution and identification

Mosquito larvae were selected at random from nearby water reservoirs within the province during the rainy season, spanning from May to October 2022. Initially, comprehensive data was recorded, including water levels, color, composition, and the number of larvae or pupae inhabiting the reservoir. Subsequently, the larvae and pupae obtained from the reservoir were carefully placed into a 50 mL falcon tube containing a 70% alcohol solution and transported to the SRUIH. There, a morphology screening was conducted under the guidance of morphology criteria (Becker *et al.*, 2020) using a stereomicroscope. Following this taxonomic assessment, the species identification was further validated by a specialist from the Division of Vector-Borne Diseases, Department of Disease Control, Ministry of Public Health.

Preparation of plant samples and extraction

Plants were cleaned and cut into small pieces 1–5 cm in size and then dried at 50 °C for 48 hr before ground into powder. Three extraction procedures were used to compare the contents and efficacy of the extracts. First, the maceration method: Herbal extraction was carried out by weighing 500 g of herbs and extracting them with 1,000 mL of methanol for three days. Sonication method: Finely powdered plant (250 g) was extracted with 500 mL of MeOH for 30 min at 70 °C. Soxhlet method: The same weight-to-solvent ratio used for sonication was extracted for 1 h at the solvent melting point using SyncorePlus (BUCHI). The residue was filtered using Whatman No.1 filter paper. The solution was then vacuum-dried at Interface I-100 (BUCHI) to determine the powder weight.

Thin layer chromatography

Ten microliters of each extract (1 mg/mL in MeOH) were spotted on a 10 × 20 cm TLC Silica gel 60 F254 aluminum plate using the semi-automatic CAMAG® Linomat 5 control by the VisionCATS ver.2. The spotted plate was then developed in a saturated chamber (CAMAG® Automatic Developing Chamber 2 (ADC 2)) containing the mobile phase in the following proportions: toluene: acetonitrile: ethyl acetate: acetic acid (35: 15: 10: 0.15). When the mobile phase reached the solvent front, approximately 80.00 mm from the starting point, the plate was removed, and the capillary reaction was terminated by blowing dry. The phytochemical profile was viewed and documented using a CAMAG® TLC Visualizer 2 under white light and 254 and 366 nm UV-transillumination.

Phytochemical profile detection

The phytochemical profile in the plate was then detected by spraying reagents to identify significant secondary metabolites. First, terpenoids were detected by spraying the anisaldehyde-sulfuric acid reagent over the fingerprint on the plate and heating it for 10 min on a TLC hot plate at 110 °C. Next, the organic components were identified by iodine fuming, which turned purple (Merck, 1980). The plate was then sprayed with the vanillin-sulfuric-acid reagent to detect phenolic compounds before heating for 10 min on a TLC hot plate at 110 °C. Finally, alkaloids were detected by spraying the plate with Dragendorff's reagent.

Antioxidant activity

The phytochemicals on the TLC plates were derivatized with 2, 2-diphenyl-1-picrylhydrazyl (DPPH; Sigma-Aldrich) to evaluate the antioxidant activity of the compounds. The CAMAG® TLC Visualizer2 was used to visualize the phytochemical profile by reacting with DPPH and illuminating it under white light. The color area size was determined after spraying 0.5% DPPH in MeOH and incubating without light for 15 min as the region of interest (ROI) using the ImageJ software (Rueden *et al*., 2017).

Repellent activity assessment

The arm-in-cage (AIC) test was used to assess the repellent activity following the WHO guidelines. The test was conducted by inserting the arm in the cage while wearing a pair of long black gloves that reached the elbow to protect the hand from mosquito bites. Six participants were involved in the study, and informed consent was obtained before the study. All the study participants are asked to avoid alcohol intake and products such as perfumes or lotion. In addition, any specific measures to be followed before or during the experiment could be added. The groove cage had a hole on the top to connect a clear plastic sheet 10 × 10 cm in size and 1 mm holes to smell the body, and the treated forearm was inserted in a cage sized 30 cm (width) × 30 cm (height) × 30 cm (length) at regular intervals. The cage contained 250 female mosquitoes that did not feed blood for 2–3 days. The protection against the number of mosquitoes in the control and extract groups was determined. The activity was assessed in triplicate for 3 min, followed by a 30-min rest period. Distilled-H_2O was used as a negative control, *N, N*-diethyl-m-toluamide (DEET) was used as a positive control, and 1 mL of 1 mg/mL extract in distilled-H_2O was the test compound. The effectiveness of the repellent activity was evaluated based on the first mosquito bite.

Data analysis

All measurements are expressed as mean ± standard error (SE). The antioxidant activity on the TLC plate and the repellent activity were analyzed in R (version 4.2.0 for Windows) using one-way Analysis of Variance (ANOVA) to compute statistical differences and Tukey's multiple comparisons (Gentlemen *et al*., 2006). The Statistical Package for the Social Sciences (SPSS) for Windows was used to analyze and handle the mosquito larvae distribution. The *chi-square* test was used to test the research's hypothesis that the physical characteristics of the reservoir including the water level, the material made of the reservoir, and the color of the stagnant water container affect mosquito larvae distribution and the relationship between the dependence (No. of larvae) variable and independence (the physical features) variable was tested by Pearson's correlation (Masuadi *et al*., 2021). In all analyses, the significant statistical difference was set with a 95% confidence interval.

RESULTS AND DISCUSSION

Mosquito collection and the larvae distribution in the physical reservoir

Ae. albopictus, more commonly captured than the other species, was collected and tested in a laboratory. Adult mosquitoes were collected using the method devised by WHO (2009) and reared in the laboratory for repellent testing. The distribution of larvae was surveyed, and the larvae of *Ae. aegypti*, *Ae. albopictus*, *Culex* spp., and other species, including pupae, were identified based on their physical characteristics. Mosquito larvae were placed in 12 of 82 reservoirs made of various materials, including plastic, natural materials, aluminum, tire, cement, and foam. The reservoir was white, black, gray, blue, brown, light blue, or transparent. The container index was determined by calculating the percentage of containers with Aedes larvae, resulting in a value of 14.63. This value significantly surpasses the CI figures observed in Southeast Asia, which range from 1.94% to 3.43% (Matini *et al*., 2020; Triana *et al*., 2021). In contrast, Mexico reported a CI of 14.00% (Morales-Pérez *et al*., 2020), indicating a high level of risk as per the CI criteria established by the Division of Vector-Borne Diseases in Thailand (2020), where a CI exceeding 10% is considered indicative of high risk. The water level in the reservoir ranged from 0.50 to 110 cm and contained 31 *Ae. aegypti* larvae, 83 *Ae. albopictus* larvae and 15 *Culex* spp. larvae. There were 45 unidentified species and 21 pupae. The presence or absence of larvae in waterlogged vessels was moderately linear (contingency coefficient = 0.567) and significantly correlated at the 0.05 level (p=0.046) using the *Chi-square* test. Then, a regression model was developed to explain and predict the relationship between physical characteristics and larval number. The multiple linear regression was conducted based on the general formula as $Y = \alpha + \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_k X_k + \varepsilon$, where the regression coefficient of physical features variables of X_1 to X_k and ε was the error term. On the other hand, the number of mosquito larvae and the water level was significantly higher (p=0.01; Pearson's correlation = 0.781, df = 80, F = 124.884) as determined by the linear relationship model using the following equation:

No. of larvae = – 2.065+0.683 (water level in the reservoir) – Equation 1

This equation can predict the number of larvae when the reservoir's water level is known.

The relationship between the presence of mosquito larvae and the color of the reservoir and the material was significantly correlated with the contingency coefficient, which was 0.716 (n=196, df = 24, *Chi-square* = 201.5) (Figure 2a) and 0.738 (n=196, df = 20, *Chi-square* = 234.7), respectively (Figure 2b). The reservoir color and water level were linked to mosquito larval encounters.

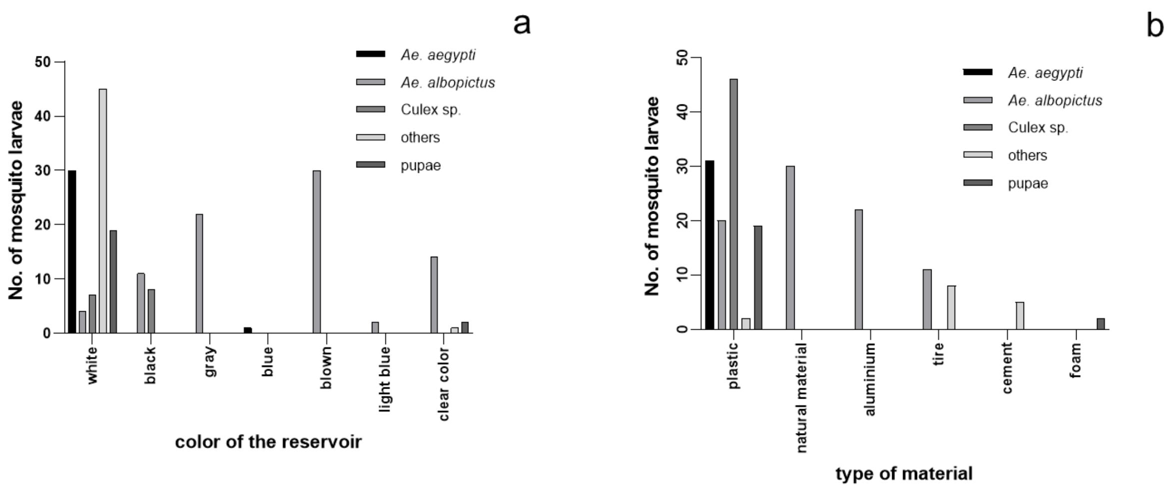

Fig. 2. The correlation between the number of mosquito larvae, the reservoir color (a), and the type of material (b).

Plant collection

Plant species were sampled based on local sage interviews for their use as insect repellants, as well as plant parts. There were 10 orders and 13 families of indigenous species in the plant collection, including *Citrus maxima* Merr. (CM), *Thespesia populnea* L. Sol. Ex Correa. (TP), *Garcinia mangostana* L. (GM), *Paederia foetida* L. (PF), *Vitex rotundifolia* L.f. (VR), *Piper retrofractum* Vahl. (PR), *Litsea petiolata* Hook. f. (LP), *Stachytarpheta jamaicensis* (L.) Vahl (SJ), *Cleome viscosa* Linn. (CV), *Andrographis paniculata* (Burm.f.) Nees (AP), *Bouea oppositifolia* (Roxb.) Meisn. (BO), *Eleocharisdulcis plantaginea* (EP), and *Chromolaena odoratum* (L.) (CO). Among the plants collected, the Tum Tim Siam variation of CM (the variety well recognized as a specific species in Pak Phanang District) had a distinct taste and appearance. The EP is a sedge from which locals harvest subterranean fruit for food. The TP, also known as the Indian tulip tree, is an indigenous plant that grows in the sand along the coast, similar to VR. The LP is a small leaf plant commonly used to prepare chili paste and regional cuisines. Mangosteen (GM) is a vital commercial tropical fruit throughout the province. The remaining plants, PF, PR, BO, and CV, are consumed as fresh vegetables with spicy meals by the province's residents, while CO and AP are used to treat various ailments according to the village sage in the province.

Plant extraction

The average dry weight of the extract ranged from 123.44 mg/g dry weight (SJ) to 154.56 mg/g dry weight (EP). The preliminary ANOVA yielded an F-test statistic for the species with a value of

12.372 and a *p*-value less than 0.05. The average dry weight varied significantly from the mean weight per species for each pair when compared by Tukey's method. The extraction process did not affect the dry weight of the extract. None of the three extraction methods affected the mean dry weight, where maceration, reflux, and sonication yielded mean dry weights of 135.87 mg/g, 132.51 mg/g, and 134.72 mg/g, respectively. ANOVA was performed, and the F-test statistic for extraction was 0.374, with a *p-value* of 0.689. The crude extracts from each species were combined and reconstituted in methanol (data shown in Supplementary Table 1 & Table 2).

The phytochemical profile

The fingerprints of the plant compounds on TLC were examined at 254 and 366 nm UV-Transilluminescent wavelengths (Figure 3a & 3b). The intensity of the bands varied, indicating the concentration of the chemical ingredients. In addition, the fingerprint of each track was linked to the reagents used for flavonoids, alkaloids, and tannins (data shown in Supplementary Table 3).

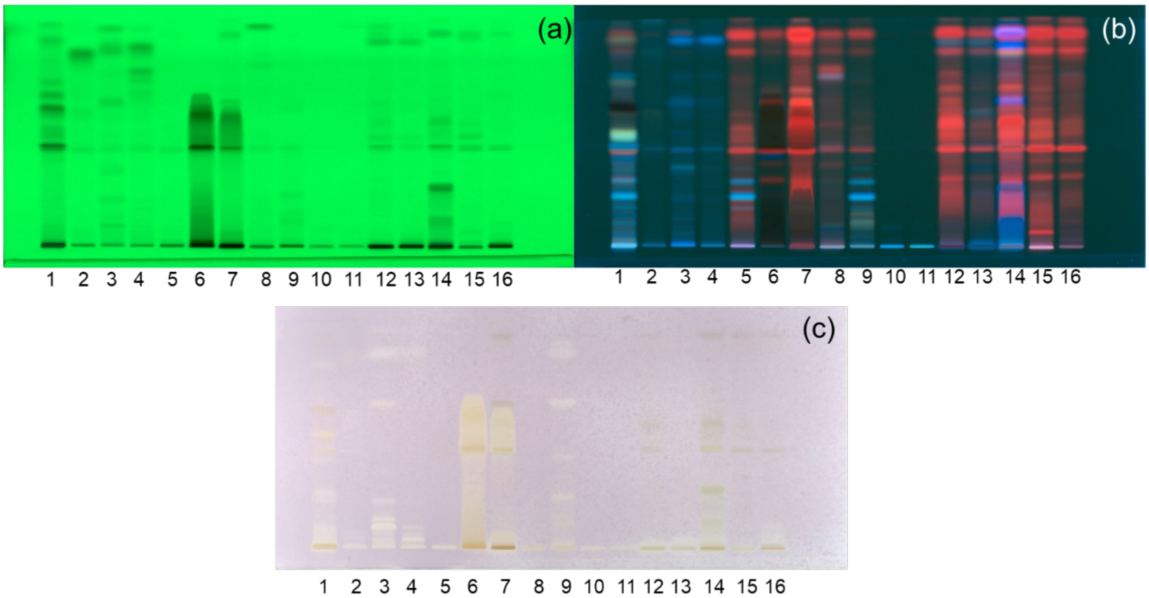

Fig. 3. The TLC chromatogram depicts the phytochemical profiles of 13 indigenous plants. The reaction of phytochemicals to DPPH on a TLC plate (a). A hue represents the reaction response on a TLC plate (b). Antioxidant comparison chart (c); Track 1: CM peels; Track 2: BO leaves; Track 3 and Track 4: GM pericarps and leaves, respectively; Track 5: SJ leaves; Track 6 and 7: TP fruits and leaves, respectively, Track 8 and 9: EP roots and leaves, respectively; Track 10: PF leaves; Track 11: VR leaves; Track 12: PR fruits; Track 13: CO leaves; Track 14: LP leaves; Track 15: CV leaves; and Track 16: AP leaves.

Antioxidant activity

The phytochemical profile on the TLC plate may be utilized to trace the response to DPPH and the antioxidant activity at track 1 for CM peels, tracks 3 and 4 for GM pericarps and leaves, tracks 6 and 7 for TP leaves and flowers, and track 9 for EP leaves as a white area in the presence of compounds (Figure 3c). The average region of interest (ROI) of antioxidant activity on the TLC plate was the highest in TP leaves and flowers, followed by CM, GM, and EP, with statistically significant differences from the rest at 328.92±25.60, 182.41±15.00, 308.53±15.40, 230.55±0.64, 924.02±25.20, 735.03±44.70, and 328.51±0.02 (*n*=3). The mean ROI of each crude extract was statistically different when compared using ANOVA at F = 0.22 and *p-value* = 0.05 for at least one pair, followed by Tukey's multiple comparisons, which revealed differences in the ROI, as presented by boxplot and the normal distribution statistic (Supplementary Figure 1). The ROI on the TLC plate represented the interaction between a compound in the crude extract and DPPH, showing antioxidant activity. Therefore, a high ROI indicated increased DPPH or antioxidant activity. The activity accuracy was validated in a standardized manner. As a result, the DPPH reaction with the crude extracts from CM, GM, TP, and EP shown on TLC plates is an intriguing choice for this study as it initially includes antioxidants when evaluated and will add additional value in the case of the generated products. However, this study tested CM, TP, and EP crude extracts for their mosquito-repellent activity. This was because all three plants were dispersed throughout the region. They were considered weeds, but the government encouraged their use for local benefits such as food and medicine.

The repellent activity

The efficacy of the extract as a mosquito-repellent was evaluated and the following extracts were found to be the most effective: CM, TP, Mix, and EP (Table 1). Although the use of crude extract conditions, both single and combined, was effective in repelling adult *Ae. albopictus*, the study did not include a mixed test. Therefore, four varieties of CM pericarps were extracted and used as *Ae. aegypti* repellants. In addition, Jordanian TP leaf extract was tested for efficacy against *Ae. aegypti* and *Ae. albopictus* repellant. EP extract has not been reported to be insect or mosquito-repellent. The average number of mosquitoes resting in the testing area was 4.33±0.33, 7.33±0.12, 9.33±0.33, 9.00±0.00, and 5.00±1.73 for DEET, CM, TP, EP, and the mixture of the three extracts, respectively (Table 1). The preliminary ANOVA yielded an F-test statistic for the species with a value of 7.20 and a *p*-value of less than 0.05. The number of the average mosquitoes varied significantly from the mean mosquito per species extract of each pair when compared by Tukey's comparison.

Table 1. The mosquito-repellent activity of the plant extract (n=3)

Number of Mosquitoes (Mean ±SE)					
H_2O	DEET	CM Extract	TP Extract	EP extract	CM+TP+EP
11.00±0.12[a]	4.33±0.33[c]	7.33±0.12[ac]	9.33±0.33[ab]	9.00±0.00[ab]	5.00±1.73[bc]

* a, b, and c are different values that differ statistically significantly (p<0.05).

Table 1 represents the average count of *Ae. albopictus* in the test region treated with crude extract. Among the native plant extracts tested, three demonstrated better mosquito repellent properties compared to water, used as a negative control, albeit they were not as potent as DEET, a readily available commercial mosquito repellent. Specifically, the native plant *Citrus maxima* (CM) exhibited mosquito-repelling qualities similar to *Citrus* sp. extracts previously examined for their impact on mosquito larvae. However, it's important to note that the CM used in this study displayed regional variability (Murugan *et al*., 2012; Castillo *et al*., 2017; Visakh *et al*., 2022). On the other hand, *Thespesia populnea* (TP), an indigenous plant to the area, had not previously been reported for its insect-repellent properties. Surprisingly, when assessed in this study, TP showed a more significant mosquito-repelling effect than the negative control. *Eleocharisdulcis plantaginea* (EP), which belongs to the sedge family and is locally consumed by villagers, also exhibited noteworthy mosquito-repellent efficacy. Notably, EP was as effective as TP, with each of the three plants emitting a distinct odor. Interestingly, when all three extracts were combined and tested, their combined effectiveness was comparable to that of DEET, a commonly used insect repellent.

CONCLUSION

This research unveiled a significant correlation between the Container Index (CI) and Thailand's dengue surveillance control criteria, indicating a substantial risk of dengue endemicity in the area, with CI being a crucial factor at 14.63%. The presence and distribution of mosquito larvae were found to be closely associated with the water level in the reservoir. These findings have practical implications for monitoring the spread of dengue fever through vector surveillance. Furthermore, among the thirteen endemic indigenous plant species studied, three were identified as potential vector repellents, offering a safer alternative to chemical repellents for both human health and the environment. These three plants exhibited high levels of phytochemical compounds with strong antioxidant properties, which were found throughout the area. Notably, the effectiveness of these repellents against *Aedes albopictus* was comparable to that of DEET.

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ETHICAL STATEMENT

The Human Research Ethics Committee Certificate COA No.060/65 Nakhon Si Thammarat Rajabhat University supported research initiatives that adhered to globally standardized human research ethical norms. The Animal protocol was approved by the Walailak University Institutional Animal Care and Use Committee (WU-IACUC) No. WU-ACUC-65078.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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