Borneo Journal of Resource Science and Technology (2025), 15(1): 146-155
DOI: https:doi.org/10.33736/bjrst.6982.2025

Essential Oil from *Citrus medica* Waste and Its Repellent Activity Against Mosquitoes (Diptera: Culicidae)

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Received: 19 April 2024 Accepted: 28 August 2024 Published: 30 June 2025

ABSTRACT

Citrus medica is enriched with beneficial antioxidant agents and has promising potential as a mosquito repellent. Most commercial mosquito repellents contain N,N,-diethyl-3-methybenzamide (DEET), damaging the synthetic fabric and plastic, thus producing toxic reactions. This study was conducted to identify the application of *C. medica* peels as new mosquito repellents formulated using essential oil of *C. medica* peels. Methodologically, the essential oil of *C. medica* peels was extracted via hydro-distillation method and analysed by gas chromatography-mass spectrometry. The insect repellent of *C. medica* essential oil nanoemulsion (EON) spray was formulated. This EON was further characterized and assessed for its stability as well as mosquito repellency properties. Major chemical constituents were successfully identified in *C. medica* peels, in which D-Limonene constituted almost 64.57%. The formulated EON was found to be slightly turbid, bluish-white, and isotropic. The pH of EON was 5.45, which was skin-friendly, with 0.8896 ± 0.0016 cP viscosity at 27 °C, which was lower than water (0.8539 cP). The conductivity readings (- 234V) used to establish the oil-in-water nanoemulsion were substantiated by spherical and homogenous shapes with no aggregation seen on a scanning electron microscope. From the repellency test, EON showed good potential with more than 70% mosquito repellency. In conclusion, mosquito repellents formulated from *C. medica* peel essential oil showed good mosquito repellency that effectively reduces vector-borne diseases, which significantly threaten many lives.

Keywords: Citrus, essential oil, food waste, mosquito repellency, nanoemulsion spray

INTRODUCTION

Citrus medica is a member of the Rutaceae family and is referred to as 'limau susu' locally (Mahdi *et al*., 2020). The *C. medica* is a short tree with an oblong fruit of 8 cm to 12 cm in length (Onyeyirichi *et al*., 2014). This species abundantly grows in Malaysia, India, China, Japan, and other tropical and subtropical areas (Langgut, 2015; Onyeyirichi *et al*., 2014). The fruit has long been known to contain many secondary metabolites such as ascorbic acid (vitamin C), retinol (vitamin A), niacin, and thiamine, which are recognized as good antioxidant agents (Bhuiyan *et al*., 2009). These metabolites were also essential nutrients for humans. While the fruit is consumed worldwide as fresh fruit and juice, the peel is often discarded as food waste (Muhd Rodhi *et al*., 2020). It is estimated that 48 million tons of *C. medica* wastes (seeds and peels) are produced globally (Badalamenti *et al*., 2022). Until recently, this circumstance prompted a fresh shift in direction through the enhancement and utilization of previously disregarded by-products, which were once seen as having limited commercial worth and were otherwise left unused (Boccia *et al*., 2021). The essential oil extracted from *C. medica* peel is well-known for its pleasant odor and flavor intensity (Li *et al*., 2019). In contemporary times, there exists a significant need for this vital oil across various sectors such as food production, pharmaceuticals, cosmetics, and perfumery industries (Zamakshshari *et al*., 2023). Its established properties encompass pain relief, anxiety reduction, neuroprotective effects, and demonstrating antibacterial and antifungal effectiveness (Yen & Failloux, 2020).

The vector-borne diseases (malaria, dengue, Chagas disease, yellow fever, Japanese encephalitis, and onchocerciasis) affect millions globally through mosquitoes transmitting infectious pathogens from animals to humans. The World Health Organization (WHO) reports that more than 700,000 people die annually from vector-borne diseases (WHO, 2020). Finding a safe, effective, and affordable tool to fight these diseases is urgent. Mosquito repellent is one of the tools that can be used to tackle this problem. The mechanism of mosquito repellent is to minimise contact with mosquitoes and avoid the transmission of infectious pathogens (Yen & Failloux, 2020). A synthetic repellent of N, N, -diethyl-3-methybenzamide (DEET) is used commercially as a mosquito repellent (Tan *et al*., 2019). However, DEET application will lead to toxic reactions and cause damage to plastic and synthesis fabric (Gillij *et al*., 2008). A wonderful and potent protein target for the mosquito would be the odorant binding protein (OBP) as this protein, initially identified from moths, could be used as molecular targets for the creation of mosquito and moth repellent (Leite *et al*., 2009).

The application of plant-based repellents has become popular because they are free of toxicity and adverse reactions (Ojewumi *et al*., 2021). The essential oil isolated from plants has shown excellent repellent activity in several phytophagous insects and hematophagous such as mosquitoes. Studies showed that essential oil nanoemulsion (EON) repellent from *Eucalyptus globulus* and *Mentha piperita* is efficient compared to synthetic repellent, with lower protection times against mosquitoes (Mohammadi *et al*., 2019; Navayan *et al*., 2017). The essential oil contains secondary metabolites such as limonene, citronella, 1,8-cineole, geraniol, and eugenol and these compounds have a good insect repellent activity as the compounds pose unbearable odour for the insects (da Silva & Ricci-Júnior, 2020). To date, the EONs from *C. medica* have been developed by researchers for food and beverage applications. The development is due to their biological activities, such as antibacterial, anti-inflammatory, antioxidant, antibiofilm, followed by human gut protective activity (Li *et al*., 2018; Liu *et al*., 2021; Lou *et al*., 2017). However, no EON formulation of *C. medica* has been reported specifically for mosquito repellent application (Diptera: Culicidae). Therefore, this study aimed to identify the application of *C. medica* waste in developing new mosquito repellents.

MATERIALS AND METHODS

Chemicals

Each chemical substance was procured from Merck (Darmstadt, Germany) and Sigma-Aldrich (Chemie, Steinheim, Germany). Additionally, all solvents employed held the chromatographic or analytical grade quality standard.

Sample Collection and Preparation

Citrus medica fruits were purchased from the local market, Kota Samarahan, Malaysia, in January to March 2023. The fruit underwent multiple rinses with distilled water to eliminate surface impurities such as dirt, dust, microorganisms and any lingering pesticide traces. Subsequently, the peels were sliced into small fragments using a stainless-steel knife.

Extraction of Essential Oil from *Citrus medica*

The methodology of hydro-distillation via the Clevenger-type apparatus was employed in this work according to Zamakshshari *et al*. (2023). About 2.198 kg of freshly cut *C. medica* peel was placed in a 5 L round bottom flask with the addition of ultrapure water. The sample was heated for 4 h. The resulting steam, carrying the oil, was channelled through a condenser, and collected within the Clevenger-type apparatus. The condensate (including oil) in the conical flask was collected. For separation, liquid-liquid extraction was performed using 150 mL of ethyl acetate. The resulting supernatant was collected, and subsequent removal of any residual water molecules was achieved by introducing anhydrous sodium sulfate. Further purification involved the removal of ethyl acetate through a rotary evaporator (Buchi, Germany) to yield the essential oil. The procedure was repeated several times, and the essential oil was stored in a small vial covered with aluminum foil before gas chromatography-mass spectrometry (GC-MS) analysis (Zamakshshari *et al*., 2023). The essential oil's percentage yield was determined using the formula provided accordingly; Eq. (1):

Essential oil percentage yield (w/w) % = Mass of essential oil (g) / Mass of sample (g) x 100 Eq.(1)

Chemical Profiling Using Gas Chromatography-Mass Spectrometry (GC-MS)

The essential oil of *C. medica* peels was analysed using GC-MS (Agilent 7890B) equipped with capillary column HP-5 MS (30 m × 250 µm × 0.25 µm) coupled with a quadrupole mass spectrometer (Agilent 5977) as reported by Mohammed *et al*. (2022) with slight modifications. An aliquot of 1 µL of the sample was injected in splitless mode. Then, the temperature of the column, initially 50 °C (1 min hold), was increased to 280 °C at the rate of 5 °C min^{-1} and maintained at this temperature for 10 min, injection temperature of 280 °C, detector temperature of 300 °C. Helium was the carrier gas with a constant flow rate at 1 mL min^{-1} and the total analysis time was 57 min. Electron ionization at 70 eV determined the mass spectra in the m/z range of 50-500 Da. The chromatogram percentage area was measured and identified using the MassHunter Qualitative Analysis software. The matching percentage was assigned to greater than 79% by comparing their mass ionization spectra with the NIST17 library (National Institute of Standards and Technology, 2017) without any correction factors (Dob *et al*., 2005).

Insect Repellent of Oily-Based Nanoemulsion Spray Formulation

The spray was formulated using the emulsion phase inversion method of Ostertag *et al*. (2012) with slight modifications. The essential oil was immersed with Tween 80 to activate the surfactant, followed by the co-surfactant glycerin. The EON was formulated according to a ratio of 1:1.5:10 of essential oil, surfactant and co-surfactant, respectively. The nanoemulsion was stirred using a magnetic stirrer for 30 min. About 1.25 g of the nanoemulsion was diluted to 100X with a 100 Mm acetate buffer and 0.5 g of tocopherol as the final EON spray product.

Characterisation and Stability Test On Nanoemulsion Spray

pH

EON spray's pH was determined at room temperature by a pH meter (MW 102 pH, Milwaukee Instruments, Hungary, Budapest). The pH measurements were taken in triplicate for the sample tested. The pH meter was calibrated using a buffer solution with pH 10.01, 7.01 and 4.01 as quality control before measurements were made.

Conductivity

The conductivity of the EON spray was measured at ambient temperature using a digital conductivity measuring device (Jenway 4510 Conductivity/TDS Meter). This test determined the emulsion's continuous phase, whether it was o/w emulsions (conductive) or w/o emulsions (unconducive) (Azmi *et al*., 2019). The conductivity test was carried out in triplicate for the sample tested. Before any measurements were taken, the meter was calibrated using deionised water.

Thermodynamic and Shelf Stability

The stability studies were based on Shafiq-un-Nabi *et al*. (2007) with slight modifications. The thermodynamic stability test can be divided into three parts: (i) centrifugal separation, in which the EON was centrifuged at 1350 rpm for 5 min, (ii) temperature cycling, involving six cycles, alternating between refrigerated conditions at 4 ℃ and elevated temperature at 40 ℃, were conducted (the samples were stored at each temperature for a minimum of 24 h) and (iii) freeze-thaw cycling, in which the formulations were subjected to three freeze-thaw cycles, alternating between temperatures of -21 ℃ and 25 ℃ (the samples were stored at each temperature for a minimum of 24 h). The thermodynamically stable EON formulation was subjected to shelf-life stability; EON was assessed for cracking, creaming or phase separation via macroscopy for 84 days.

Viscosity Measurement and Scanning Electron Microscope (SEM)

A U-tube viscometer was utilised to determine the viscosity of the EON spray at 27 °C. Morphology of the nanostructure of the EON was characterised using SEM (JEOL JSM-6390LA, Japan). The EON was subjected to serial dilution using ethanol. A sample drop was smeared onto a zinc plate, air-dried, and subjected to platinum coating before the SEM imaging.

Mosquito Repellent Assay

The excito chamber method was used to observe the mosquito behavior change in the form according to Anuar and Yusof (2016) with slight modifications. The excito chamber is shown in Figure 1. Both chambers A and B were constructed with transparent plastic, with each side measurement of 30 cm × 30 cm × 30 cm, forming a cube. A small entry opening (5 cm × 5 cm) equipped with a short netting sleeve on the outside was made at chamber A to lead mosquitos into the chamber. A transparent plastic cylinder was placed as a bridge connecting chamber A and B. A fabric (4 cm × 4 cm) was treated with insect repellent spray or cream. Prior to the test, the mosquitoes were fasted overnight or for a minimum duration of 4 h. The behaviour of the mosquitoes was monitored in relation to the count of mosquitoes that managed to move to a different area and the mosquitoes that remained within the enclosure containing the treated substance. This observation was documented after a 30 min exposure period. The experiments were carried out during daylight hours in triplicates. The percentage of mosquito repellency was computed using the subsequent formula, Eq.(2):

Mosquito repellency (%):

$$(NME + NMD)/(N_{total}) \times 100 \qquad \text{Eq.(2)}$$

Whereby, NME = the count of mosquitoes that managed to escape, NMD = number of deceased mosquitoes, and Ntotal = number of mosquitoes that were exposed.

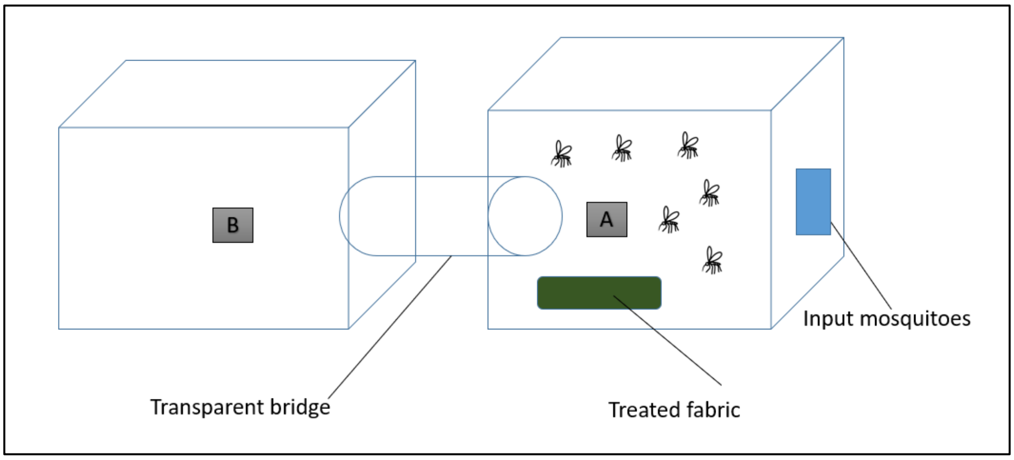

Figure 1. Excito chamber

Statistical Analysis

The data on the extraction of essential oil, stability test, and mosquito repellent assays were represented as mean ± standard deviation and were conducted in three replicates analyses.

RESULTS AND DISCUSSION

Yield of Essential Oil Extract and Qualitative Analysis of Essential Oil Using GC-MS

The *C. medica* peel essential oil was extracted using the hydro-distillation process for 4 h, yielding 0.23% (w/w) (Table 1). The essential oil obtained was pale yellow. Similarly, Venturini *et al*. (2014) reported that 0.40 - 0.72% (w/w) essential oil was extracted from *C. medica* peels using the same method. However, the percentage yield of essential oil obtained in this study is slightly different from previously reported extractions (Amelia *et al*., 2017). This suggests that factors might be due to the *C. medica* origin, sample preparation technique before hydro-distillation, duration of hydro-distillation, diversity of various *C. medica* genetics, and environmental factors such as terrestrial factors (drought, water, soil), climate factors (temperature, light, stressors), and human factors (fertilizers, pollution, deforestation) (Upadhyay *et al*., 2022).

The library score matched with a percentage greater than 80% was selected by comparing their mass ionisation spectra with the NIST17 library (National Institute of Standards and

Technology, 2017) without any correction factors (Dob *et al*., 2005). A total of 11 compounds comprising 99.99% of the essential oil content were identified (Figure 2). Monoterpenes were the major constituent in essential oil (76.50%), followed by oxygenated monoterpenes (5.28%). Specifically, three major constituents abundantly found in essential oil were D-limonene (64.57%), γ-terpinene (10.58%), and linalool (6.08%). Other chemical constituents present in the essential oil were (S)-(+)-5-methyl-1-heptanol (1.36%), 1,3-cyclohexadiene, 1-methyl-4-(1-methylethyl)- (1.35%), terpinen-4-ol (1.17%), α-terpineol (2.50%), decanal (1.18%), 6-oct-1-ol,3,7-dimethyl-, formate (1.70%), 1-cyclohexene-1-carboxaldehyde, 4-(1-methylethenyl)- (0.43%) and dodecanal (0.41%), as shown in Table 2. A value of less than 0.1% was denoted as traces (Dob *et al*., 2005). In *C. medica*, D-limonene is known to have high biological activity; thus, to improve the high biological activity of the extract it needs to contain high D-limonene. According to Santiago *et al*. (2020), the hydro distillation method can result in high levels of D-limonene in the extract, which is a compound with potential biological activity (Amelia *et al*., 2017). Figure 3 shows the chemical structure of chemical constituents that are present in essential oil.

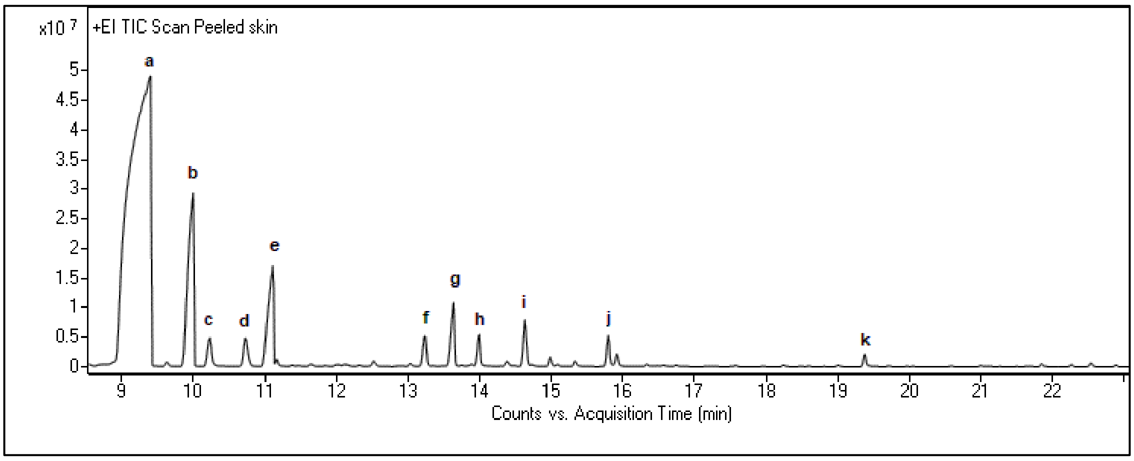

Figure 2. Chromatogram of *Citrus medica* peels

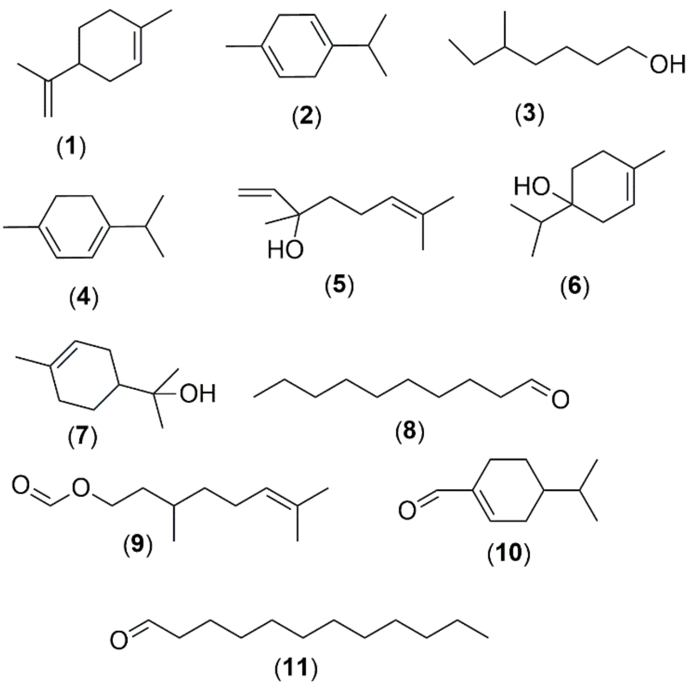

Figure 3. Chemical structures of the compounds obtained from *Citrus medica* essential oil from hydro-distillation method

Table 1. Percentage yield of *Citrus medica* peels essential oil

Sample	Sample mass (kg)	Weight of essential oil (g)	Percentage yield (w/w) %	Colour of essential oil
Citrus medica peels	2.198	4.52	0.23	Pale yellow

Table 2. Chemical composition of *Citrus medica* peels

Peak no.	Retention time	Compound name	Molecular formula	Percentage area (%)	Library score
a	9.39	D-Limonene (**1**)	$C_{10}H_{16}$	64.57	91.35
b	9.97	γ-Terpinene (**2**)	$C_{10}H_{16}$	10.58	90.01
c	10.23	(S)-(+)-5-methyl-1-heptanol (**3**)	$C_8H_{18}O$	1.36	90.41
d	10.73	1,3-cyclohexadiene, 1-methyl-4-(1-methylethyl) (**4**)	$C_{10}H_{16}$	1.35	90.41
e	11.10	Linalool (**5**)	$C_{10}H_{18}O$	6.08	84.45
f	13.23	Terpinene-4-ol (**6**)	$C_{10}H_{18}O$	1.17	87.61
g	13.63	α-Terpineol (**7**)	$C_{10}H_{18}O$	2.50	83.35
h	13.99	Decanal (**8**)	$C_{10}H_{20}O$	1.18	85.49
i	14.63	6-Oct-1-ol,3,7-dimethyl-, formate (**9**)	$C_{11}H_{20}O_2$	1.70	85.49
j	15.92	1-Cyclohexene-1-carboxaldehyde, 4-(1-methylethenyl) (**10**)	$C_{10}H_{14}O$	0.43	91.64
k	19.38	Dodecanal (**11**)	$C_{12}H_{24}O$	0.41	85.00
Total					99.99

Formulation and Characterisation of EON Spray

The essential oil of *C. medica* is insoluble in water; thus, limiting its usage. To overcome this situation Tween 80 and glycerol were used as surfactant and cosurfactant (Saberi *et al*., 2013). The final EON formulation was obtained in the ratio of 1:1.5:10 of essential oil, surfactant and co-surfactant in 100 mL of acetate buffer (pH 5.45) as the aqueous phase. The formulation was chosen in the specific ratio because studies have proven that 1000 mg L^{-1} or 0.1 mL of this similar essential oil is effective as a mosquito repellent (Choochote *et al*., 2007; Nascimento *et al*., 2017). The visual observation of the EON spray showed as a slightly turbid, bluish-white, and isotropic solution which are characteristics of good nanoemulsion formation (Shafiq *et al*., 2007). The extensive freeze-thaw, cooling, and heating centrifugation test were also performed to determine the thermodynamic stability. The SEM results (Figure 4) confirmed that the EON formulated has high thermodynamic stability as there was no visual evidence of creaming, cracking, coalescence, phase separation and inversion in the finished product, indicating that the preliminary stability of the EON is acceptable. The EON remained stable for two months at room temperature as none of the above-mentioned physical properties were observed. The essential oil degradation led to some colour fading of the EON after six months, but the strong citric aroma of the spray remained unchanged.

The additional characterisation of the formulation was evaluated, including pH, viscosity and conductivity, as exhibited in Table 3. The formulated EON exhibited a skin-friendly pH of which is 5.45, as the general optimal pH value of human skin is around pH 4 to 6 (Lukić *et al*., 2021). The viscosity of the formulation was detected as 0.8896 ± 0.0016 cP, slightly thicker than water at 0.8539 cP at 27 ℃, as this is a phenomenon caused by emulsification whereby it thickens the liquid (Geetanjali *et al*., 2021). A slightly higher viscosity than water is favourable as the EON is not too thickened, which would affect its transmittance as a repellent. This is proven by Okpalaku *et al*. (2022) as the lemon nanoemulsion had the lowest viscosity and the highest transmittance percentage when compared to turmeric and coconut nanoemulsion.

Besides that, the SEM results also showed that EON's supportive oil-in-water (o/w) nanoemulsion character with bright droplets appearing in the dark background (Figure 4).

The EON exhibited spherical and homogenous shapes, which was also a justification for the conductivity measurements (-234 ± 5V) for o/w nanoemulsion confirmation. The results exhibited no evidence of the emulsion droplets coalescing with each other and aggregating into larger clusters, suggesting the oil droplets are stably dispersed (Chuesiang *et al*., 2018; Geetanjali *et al*., 2021; Okpalaku *et al*., 2022). The conductivity of o/w nanoemulsions is higher than that of w/o nanoemulsions (Okpalaku *et al*., 2022). Therefore, EON must be of the o/w type, as their high conductivity values indicate. Nanoemulsion components, such as oil, water and surfactants, each impact their viscosity to varying degrees.

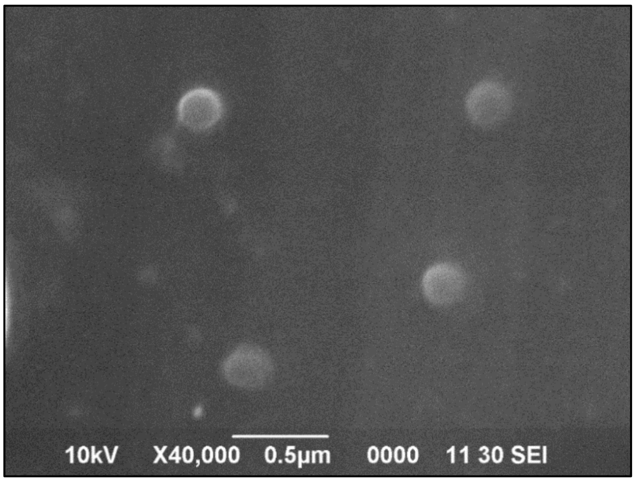

Figure 4. Scanning electron microscopic image of essential oil nanoemulsion (EON)

Table 3. Physicochemical characterization and shelf stability of essential oil nanoemulsion (EON)

Physicochemical Properties	Results
pH	5.45
Conductivity	-234 ± 5 V
Viscosity	0.8896 ± 0.0016 cP
Centrifugation	Pass
Heating cooling cycle	Pass
Freeze-thaw cycle	Pass
Shelf Stability (days)	
1	Pass
7	Pass
14	Pass
28	Pass
56	Pass
84	Pass

Mosquito Repellent Activity

The formulated oily-based nanoemulsion spray from *C. medica* peel essential oil was tested for its mosquito repellent activity using the excito chamber (Nararak *et al*., 2019). Ten mosquitoes were used for each set of experiments. The first set of the experiment gave 70% mosquito repellence, with four deaths and three mosquitoes escaping to the next box. Meanwhile, the second set of experiments gives 90% mosquito repellence, with five deaths and four mosquitoes escaping to the next box. The third trial gives 80% mosquito repellence, with six deaths and two mosquitoes escaping to another box. All sets of experiments showed that the EON spray has above 50% mosquito repellent activity, suggesting good mosquito repellence properties. The repellent activity is believed to be due to the presence of D-limonene, α-terpineol, 6-oct-1-ol,3,7-dimethyl-, formate, γ-terpinene and 1,3-cyclohexadiene, 1-methyl-4-(1-methylethyl)- as a constituent in the essential oil of *C. medica* peels. Additionally, D-Limonene was used in the organic agriculture industry as pest control due to its good repellent activity against two insect pests: *Tribolium castaneum* and *Lasioderma serricorne* (Pang *et* *al*., 2021). Meanwhile, the γ-terpinene, also found in wild carrot essential oil, shows excellent cytotoxic activity against three types of mosquito larva: *Aedes aegypti* L., *Culex pipiens* L. and *Culex restuans* Theobald larvae (Muturi *et al*., 2019). All sets of experiments showed a high mortality rate of mosquitoes. α-Terpineol is an aromatic agent widely used in the food industry and exhibits bio properties including antioxidative, growth-inhibiting, antimicrobial, anti-inflammatory, and pain-relieving properties as pharmaceutic (Sales *et al*., 2020).

CONCLUSION

In conclusion, this study successfully demonstrated the potential of *C. mdeica* peel waste as an effective natural mosquito repellent. The hydro-distillation method efficiently extracted essential oil, predominantly containing D-limonene, which was identified as a major active component. The formulated EON spray exhibited promising mosquito repellent properties, achieving over 70% repellency. Furthermore, the EON spray was characterised by skin-friendly pH, suitable viscosity and stable thermodynamic properties, making it a viable alternative to synthetic repellent like DEET. These findings underscore the dual benefits of utilising food waste for sustainable repellent production and offering a safer solution to mitigate mosquito-borne diseases. Future research should focus on large-scale production and comprehensive field testing to further validate the efficacy and safety of *C. medica* essential oil-based repellents.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Universiti Malaysia Sarawak (UNIMAS) for the PILOT Research Grant Scheme (UNI/F07/PILOT/85369/2022) and the Sarawak Biodiversity Centre (SBC) for granting the permission to conduct this research.

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