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Biochemical constituents and insecticidal activities of *Callistemon viminalis* essential oil against adults and eggs of *Pediculus humanus capitis* (Phthiraptera: Pediculidae)

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ABSTRACT

Background: Synthetic chemical pediculicides used for head-louse treatment do not kill louse eggs, can induce side effects in humans, and lead to genetic resistance in lice worldwide, including in Thailand. Use of phyto-constituents, particularly plant-derived essential oils, is alternatively recommended for head lice.
Purpose: To identify biochemical constituents of *Callistemon viminalis* essential oil (EO) and to assess the *ex vivo* effects of EO on head lice and their eggs.
Methods: The EO was extracted from fresh leaves *C. viminalis* by stream distillation, and the biochemical constituents were identified by gas chromatography-mass spectrometry (GC–MS). Louse samples were collected from schools in Khon Kaen Province, Thailand for *ex vivo* tests. Pediculicidal activity of EO was investigated using topical contact, and ovicidal activity was assessed using immersion and topical tests. Adult lice were observed for 8 h after treatment and eggs were observed for 3 weeks to assess pediculicidal and ovicidal effects, respectively. Mortality was noted and morphological change was recorded for head lice using scanning electron microscopy. Inhibition of egg hatching was evaluated.
Results: Forty-four phytochemical components were identified in EO obtained from fresh leaves of *C. viminalis*. The most abundant were 1,8-cineole (66.96%), α-pinene (18.74%) and o-cymene (7.02%). *Ex vivo, C. viminalis* EO at concentrations above 10% for 30 min caused 100% adult mortality using a topical bioassay. In a complete-immersion test, all head louse eggs failed to hatch after exposure to concentrations of *C. viminalis* EO of 10% or greater for 10 min. Eggs were less sensitive using a topical method. Permethrin 1%, coconut oil and distilled water exhibited low efficacy against both crawling and egg stages. Scanning electron micrographs indicated that *C. viminalis* EO caused obstruction in some louse spiracles but had no effect on the cuticle or sensory hairs.
Conclusion: The EO derived from *C. viminalis* had higher *ex vivo* efficacy against head lice and their eggs than 1% did permethrin. This EO may be a starting point for developing natural pediculicides to control head lice that are susceptible or resistant to permethrin.

Abbreviations

EO	Essential oil
GC-MS	Gas chromatography-mass spectrometry
MS	mass spectra
SEM	Scanning electron microscopy
LC50	Lethal concentration toxicity
RH	Relative humidity
EI	Electron ionization
RI	Retention indices

; mA, Milliampere.

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Introduction

Infestation with *Pediculus humanus capitis* De Geer (the head louse) is common on children, especially those aged 3 to 11 years (Coates et al., 2020). This ectoparasite causes distress to the affected hosts and leads to expenditure by public-health agencies. An estimated 6 to 12 million children worldwide are infested with this insect (Falagas et al., 2008). Head lice cannot jump or fly, but are easily spread by direct head-to-head contact or via indirect contact by sharing of personal items (Veracx and Raoult, 2012). The head louse is confined to the scalp and feeds exclusively on human blood (Bonilla et al., 2013). Intense itching is the most common symptom and is induced by the saliva of lice. Scaling of the scalp is associated with chronic lesions. Lice can also spread some pathogenic bacteria, but this is apparently not common (Coates et al., 2020).

Neurotoxic pediculicides (e.g., permethrin, malathion, benzene hexachloride, carbaryl and ivermectin) have long been used to control head louse infestations (Burgess, 2009a; Gunning et al., 2019). However, these substances do not kill eggs and can have various adverse effects on humans and the environment. Furthermore, it is well known that head louse populations develop resistance to pediculicides (Durand et al., 2012). To be effective in the control of pediculosis, treatments with pediculicides need to be repeated at about 2-week intervals to match the life cycle of the lice: eggs normally take 7 to 10 days to hatch and nymphs take 9 to 15 days to become adults (Cummings et al., 2018). Phytoconstituents, particularly plant-derived essential oils or non-neurotoxic substances, e.g., dimethicone or synthetic oils, serving as physical barriers, are recommended as novel alternatives to neurotoxic pediculicides (Mumcuoglu et al., 2020).

Phytochemicals with insecticidal and pediculicidal activities can be used singly or in various combinations (Candy et al., 2020; Di Campli et al., 2012). Plant-derived essential oils have been of particular interest following reports of their high pediculicidal and ovicidal abilities *ex vivo* (Candy et al., 2018b; Yones et al., 2016) and in clinical trials (Barker and Altman, 2011; Greive and Barnes, 2018). Plant-derived essential oils contain monoterpenes, sesquiterpenes and phenolic compounds (e.g., terpenoids, phenols, aldehydes, acids and hydrocarbons) as their main constituents (Bakkali et al., 2008). These essential oils often possess antibacterial, antiviral, antifungal, antiparasitic and insecticidal activities, while being safe for mammals, as evidenced by their frequent use in cosmetic products (Shaaban et al., 2012).

Callistemon viminalis (Sol. ex Gaertn.) G.Don (weeping bottlebrush, Myrtaceae family) was reported to possess fumigant toxicity against parasites, mainly due to the presence of 1,8-cineole, alpha-terpineol and alpha-pinene (Ahmad and Athar, 2017). Despite its known insecticidal action, there has been no previous report on the effects of essential oils of *C. viminalis* on head lice, either on the crawling stage or on eggs. Commercial pediculicidal products containing pyrethroids or permethrin are generally used for head louse treatment in Thailand. Frequent exposure of lice in Thailand to pediculicides has led to some resistance developing and genetic differnce (Brownell et al., 2020; Yingklang et al., 2021). Essential oils may provide the basis for further development of phytomedicines for controlling lice, whether susceptible or resistant to pediculicide, and limit the spread of resistance alleles in the landscape. Therefore, in this study, we aimed to identify biochemical constituents of *C. viminalis* essential oil (EO) and assessed the *ex vivo* effects of EO on head lice and their eggs.

Materials and methods

Human ethical statement and identification of head lice

This study was conducted between August and December 2019. It was approved by the human ethics committee of Khon Kaen University (HE621476) following the principles of the Declaration of Helsinki. In total, 212 children (6–12 years of age) were enrolled from two primary schools in Khon Kaen Province, Thailand. In both schools, there had been previous pediculicidal treatments (conducted by the Department of Parasitology, Khon Kaen University) using 1% permethrin. Before collection of head louse samples, permission was obtained from the directors of the two schools. In addition, all parents gave written, informed consent and the children's assent was obtained prior to collection of samples. All children were examined for head lice infestation by our staff using a fine-tooth comb. Head louse eggs were detected by the naked eye or by use of a magnifying glass. Eggs were usually attached to the hair shaft less than 1 cm from the scalp, especially behind the ears and on the back of the neck. Of the 212 children examined, 120 (56.6%) were found to be infested (presence of motile lice and/or live eggs).

Collection of head-louse samples

To collect head lice, the fine-tooth comb was passed through the hair of the volunteers from the scalp to the ends of the hair. Live lice were thus combed onto a sheet of white paper and transferred to a small plastic box. A magnifying glass was used to assist visual observation of the white or yellow eggs of head lice attached to each black or brown hair shaft of the volunteers. The hair shaft with attached eggs was then cut, 0.2 cm from the scalp. All head louse samples (crawling and egg) were kept in the plastic containers (one container per volunteer) and immediately transported to the laboratory for testing within 30 min. The total time elapsing between sample collection and laboratory work was 2.5 h. Head louse samples (crawling and egg) were collected three times from each school at intervals of one to two weeks (based on the time required for head louse development). At least 200 eggs and 400 crawling specimens were collected each time.

Plant material and extraction

Fresh leaves of *C. viminalis* (Sol. ex Gaertn.) G.Don were collected from the herbal garden of the Faculty of Medicine, Khon Kaen University, Thailand (In August and September 2019). The plant identification was confirmed by Prof. Arunrat Chaveerach, and a voucher specimen was kept in the herbarium at the Department of Biology, Faculty of Science, Khon Kaen University. Thirty kilograms of fresh leaves of *C. viminalis* were cleaned with water and half dried at 28–29 °C (room temperature) for 1 h. A subsample of 10 kg was used for each extraction. This quantity, along with 6 L of distilled water, was loaded into the basket chamber of an essential-oil distiller (from our establishment). Crude essential oil was extracted using stream distillation for 3 h. The crude EO was then dissolved with sodium sulfate anhydrous (Sigma-Aldrich, Darmstadt, Germany) in a separate funnel flask and gently shaken to remove water from the oil. The EO which was collected was stored in brown bottles at 4 °C until used.

Phytochemical analysis

The phytochemical components of *C. viminalis* EO were analyzed using GC–MS series QP2010 Shimadzu (Tokyo, Japan). Separation was carried out using a capillary column Rtx-5MS (5% diphenyl and 95% dimethyl polysiloxane, 30 m length, 0.25 mm internal diameter, 0.25 μm film thickness (Restek, Bellefonte, PA, USA). Helium carrier gas was used at a flow rate of 1 mL/min. The sample was diluted at 1:100 v/v with hexane and 1 μL of the diluted sample was injected into the capillary column with a split ratio of 50:1. The injection port temperature was set at 250 °C. The analytical column temperature was initially set at 50 °C for 5 min, then gradually increased to 100 °C at 3 °C/min and maintained for 5 min, followed by an increment rate at 5 °C/min to 250 °C and retained for 3 min, and finally increased to 280 °C at a rate of 5 °C/min and held for 5 min. The ion-source temperature and interface temperature were 230 °C and 280 °C, respectively. The mass spectrometer using an electron ionization (EI) system was set at the ionization energy of 70 eV. The mass acquisition range was scanned from 40 to

550 *m/z* (Chen et al., 2014). Total run time was 71 min with no identified peaks observed after 41 min under the preset conditions. Compounds were identified based on their mass spectra (MS) and computer matching was accomplished with the National Institute of Standards Technology (NIST 14) libraries. The retention indices (RI) were calculated using a homologous series of n-alkanes (C7-C30) (Sigma-Aldrich, USA).

Pediculicidal testing

Callistemon viminalis EO was prepared in coconut oil and adjusted to 1, 5, 10, 20, 50 and 100% v/v. Ten motile adult lice (either male or female) in each experimental group were used. These were examined under a stereomicroscope to confirm morphological integrity (complete organs and active movement) before testing. Ten microliters of each concentration of *C. viminalis* EO were directly dropped over the body of each individual louse on a petri dish (90 × 15 mm). Coconut oil, distilled water and permethrin 1% w/w cream (Hexin Lice Killer®, Bangkok, Thailand) were simultaneously used as dilution solution, negative control and positive control, respectively. Head louse samples were exposed to each solution for 5, 10, 20 and 30 min. At the end of the exposure period, the treated lice were transferred to a new petri dish, then rinsed with 10% diluted non-pediculicidal shampoo (Sunsilk®, Thailand) and distilled water twice for 1–2 min. This was done to make sure that the reagent used in each test did not persist on the lice, potentially distorting the results. Louse samples were dried on a filter paper (Whatman® No.1) and their mortality rate was determined by observation at 5, 15, 20, 30, 60, 120 and 480 min (8 h) under a light stereomicroscope. Lice were deemed to have died if they did not present any movement of an external structure (antennae and legs) or peristalsis of internal organs (gut), even when pricked with a needle (Carpinella et al., 2007; Heukelbach et al., 2008). All experimental tests were performed in triplicate. Lice from the 30 min exposure from each experimental group were preserved in Kanofsky fixative solution for investigation of morphological change using scanning electron microscopy (SEM).

Ovicidal testing

Using a compound light microscope, head louse eggs were identified as early (presence of a yoke cell) or late stages (presence of eye spots, nymph visible inside and/or movement) before conducting tests. Ten late-stage eggs per experimental group were used for tests. These were exposed to *C. viminalis* EO, distilled water, coconut oil or 1% permethrin for 10 min using topical contact (10 μL) or for 20 min using a dipping method (immersion test) (100 μL). These time-periods were chosen based on the results of the pediculicidal screening tests. Following exposure, the eggs were maintained at 29–30 °C; 69% relative humidity, corresponding to the ambient conditions during August to December in our area, for 2–3 weeks (Cueto et al., 2006; Sonnberg et al., 2010). The eggs in petri dishes were daily sprayed with water to control the relative humidity. Eggs were examined every day for 3 weeks under compound and stereomicroscopes and numbers of nymphs hatching were recorded. The rate of hatching was calculated on days 7, 14 and 21. Eggs were deemed to have died if they did not hatch or the nymph was dead inside the egg and/or the egg disintegrated within 21 days. Experiments on eggs were performed in duplicate.

Scanning electron microscopy

Preserved lice (adults) were washed with phosphate-buffered saline and dehydrated through a series of alcohol concentrations (50, 70, 80, 90, 95 and 100% for 10 min each). They were then placed in amyl acetate solution and dried with liquid CO_2 using a critical-point drier K850 (ASHFORD, Kent, United Kingdom) for 30 min. The samples were mounted on aluminum stubs, then coated with gold at 30 mA for 2 min and visualized using a SEM machine (JSM-840A, JEOL, München, Germany).

Table 1
Biochemical components of *C. viminalis* essential oil detected by GC–MS analysis.

RT (min)	Peak Name	Molecular class	% Area	Retention index
8.3	Isobutyl isobutyrate	Carboxylic acid ester	0.03	917
8.7	α-Thujene	Monoterpene	1.57	926
9.0	α-Pinene	Monoterpene	18.74	932
9.6	Camphene	Monoterpene	0.04	946
10.9	β-Pinene	Monoterpene	1.21	974
11.8	β-Myrcene	Monoterpene	0.15	993
12.1	2-Carene	Monoterpene	0.10	1000
12.3	α-Phellandrene	Monoterpene	1.48	1004
12.6	3-Carene	Monoterpene	0.18	1009
12.9	Isopentyl 2-methylpropanoate	Carboxylic acid ester	0.17	1016
13.1	2-Methylbutyl isobutyrate	Carboxylic acid ester	0.04	1019
13.4	o-cymene	Monoterpene	7.02	1024
13.7	1,8-Cineole (synonym Eucalyptol)	Monoterpene	66.96	1031
14.7	β-Ocimene	Monoterpene	0.13	1052
15.1	γ-Terpinene	Monoterpene	1.09	1059
15.3	1,3-Dimethylbutyl isobutyrate	Carboxylic acid ester	0.07	1063
16.6	Terpinolene	Monoterpene	0.31	1089
17.4	Linalool	Monoterpene	0.18	1105
17.8	Fenchol	Monoterpene	0.03	1114
19.1	trans-Pinocarveol	Monoterpene	0.08	1139
20.4	(-)-Borneol	Monoterpene	0.02	1167
21.0	Terpinen-4-ol	Monoterpene	0.64	1179
21.7	α-Terpineol	Monoterpene	1.00	1194
31.3	2-Isopropyl-5-methyl-7-azabicyclo[4.1.0]heptane	Monoterpene	0.02	1365
31.5	exo-2-Hydroxycineole	Monoterpene	0.01	1370
31.6	2,2,4,4-tetramethylcyclohexane-1,3,5-trione	Monoterpene	0.03	1372
31.7	Bioallethrin	Sesquiterpenes	0.01	1373
33.6	Caryophyllene	Sesquiterpenes	0.30	1419
34.3	Aromandendrene	Sesquiterpenes	0.06	1441
34.8	α-Humulene	Sesquiterpenes	0.06	1456
35.1	Alloaromadendrene	Sesquiterpenes	0.04	1463
35.2	5-Hydroxy-2,2,6,6-tetramethyl-4-propionylcyclohex-4-ene-1,3-dione	monoterpene	0.04	1466
36.0	Taylorione	Sesquiterpenes	0.04	1491
36.4	2-Hydroxycineole, isobutyrate	Sesquiterpenes	0.05	1501
37.0	Cinerolone	monoterpene	0.07	1526
37.7	6-Isobutyryl-2,2,4,4-tetramethylcyclohexane-1,3,5-trione	monoterpene	0.77	1549
38.4	Alloaromadendrane-4β,10α-diol	Sesquiterpenes	0.05	1574
38.7	Spathulenol	Sesquiterpenes	0.57	1584
38.8	Caryophyllene epoxide	Sesquiterpenes	0.66	1588
39.5	Humulene epoxide II	Sesquiterpenes	0.06	1616
39.7	2,2,4,4-Tetramethyl-6-(2-methylbutanoyl) cyclohexane-1,3,5-trione	Sesquiterpenes	0.26	1625
39.9	Isoleptospermone	Sesquiterpenes	1.61	1634
40.3	Spathulenol	Sesquiterpenes	0.08	1647
40.4	2-methyl-4-Octanone	Sesquiterpenes	0.07	1652
Total			100.00	

Statistical analysis

Evaluation of the lethal concentration (LC50) was analyzed using Probit analysis. Mortality was described by mean number and percentage of nonviable lice. Egg hatching was determined by the numbers of non-viable eggs cannot hatch. Relative efficacy of the experimental

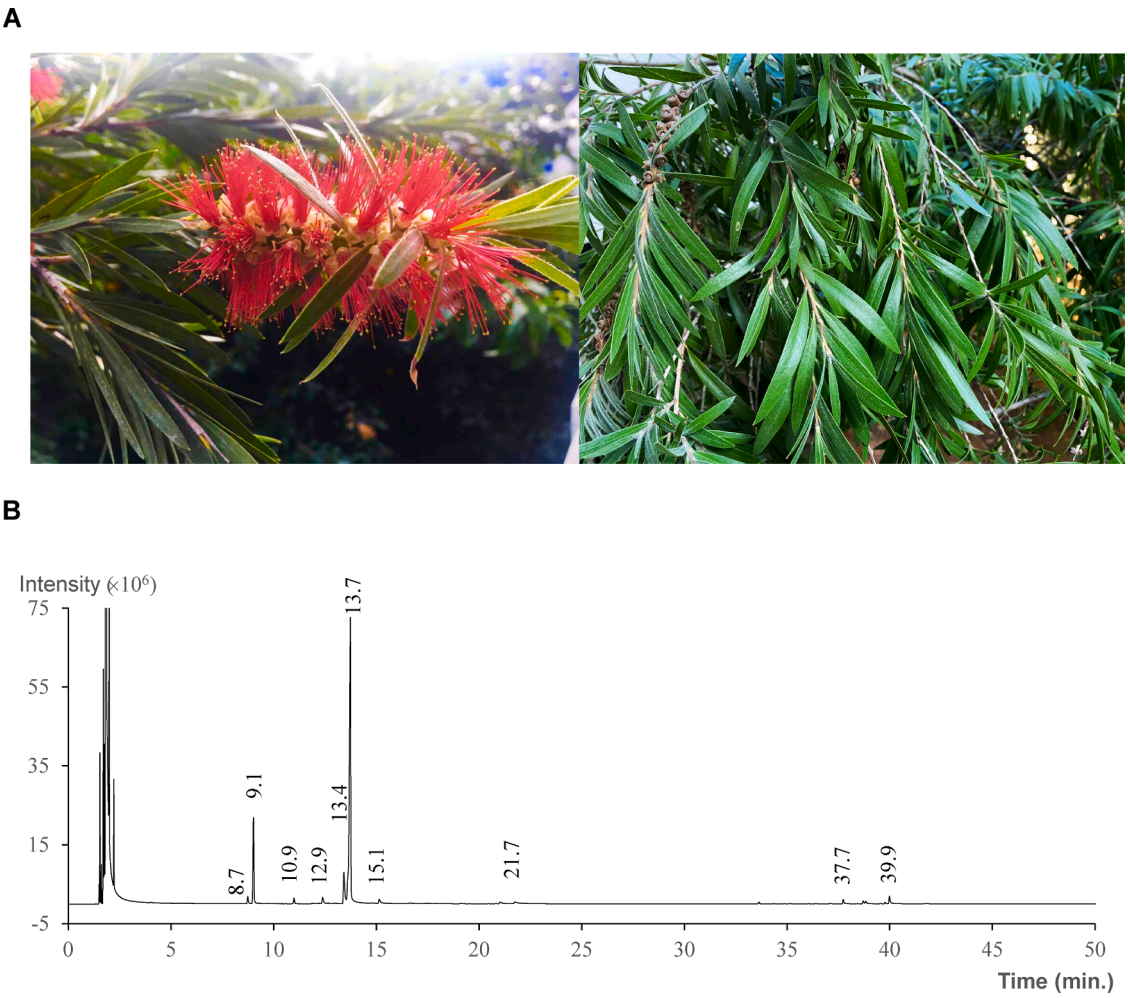

Fig. 1. *C. viminalis* picture and chromatogram profile of peak retention of *C. viminalis* essential oil compounds using GC–MS. A: *C. viminalis* flower and leaves. B: Chromatogram profile of *C. viminalis* essential oil biochemical constituents.

treatments was analyzed using Kruskal–Wallis and Dunn's tests as implemented in STATA package version 10.1 (StataCorp LLC, College Station, TX, USA). Any *p*-value less than 0.05 was accepted as statistically significant.

Results

A total of six milliliters of *C. viminalis* EO was obtained from thirty kilograms of fresh leaves (yield = 0.02% (v/w)). The GC–MS analysis revealed forty-four biochemical constituents of fresh leaves *C. viminalis* EO (Table 1 & Fig. 1), the most abundant being monoterpenes such as 1,8-cineole (66.96%), α-pinene (18.74%) and o-cymene (7.02%).

Treatment of head lice for 5 min with concentrations of *C. viminalis* EO of 50–100% caused 100% mortality by the end of the 480 min observation period (LC50 = 9.4% v/v; 95%CI: 5.72, 15.42). Treatment with *C. viminalis* EO concentrations of 20% or greater caused 100% mortality at 10 min (LC50 = 7.9% v/v; 95%CI: 4.66, 13.61), and at 20 min exposure (LC50 = 2.2% v/v; 95%CI: 1.91, 4.06). A 30 min treatment with *C. viminalis* EO at concentrations of 10% or greater caused 100% mortality (LC50 = 0.8% v/v; 95%CI: 0.29, 2.25) (Fig. 2). In contrast, low pediculicidal activity was observed in treatments with 1% permethrin and coconut oil, while distilled water did not have any effect at any application times. Adult mortality caused by application of 10–100% *C. viminalis* EO for 5 min, ≥10% EO for 10 min, ≥1% EO for 20 min, and ≥5% EO for 30 min was significantly greater than mortality in the 1% permethrin groups at each of these exposure times ($p < 0.05$) (Fig. 2).

The SEM demonstrated that 100% *C. viminalis* EO obstructed the spiracles of head lice, while there was no loss of sensory hairs or cuticle when compared with the negative controls (Fig. 3).

Hatching data for eggs are shown in Tables 2 and 3. In the immersion test, no eggs hatched by day 21 following 10 min exposure to ≥10% of *C. viminalis* EO (Table 2). In contrast, only concentrations of 50% and 100% of *C. viminalis* EO killed all eggs using the topical method (treatment for 20 min) (Table 3). In the negative (distilled water and coconut oil) and positive control (1% permethrin) groups, 90–100% of eggs hatched in both immersion and topical tests by 7–14 days.

Discussion

Several methods (e.g., fumigant, topical, ingestion, dipping or immersion tests) have been used to investigate the *ex vivo* insecticidal activities of a range of substances, but results varied according to the type of insect and of insecticide investigated (Candy et al., 2020). In this study, we used topical direct contact to assess pediculicidal activity of *C. viminalis* EO against head lice. This method more closely resembles the real-world application of treatment on the scalp of children (Candy et al., 2018a). We killed 100% of lice by application of 10% v/v *C. viminalis* EO for 30 min. Distilled water had the lowest efficiency against adult lice, followed by coconut oil and 1% permethrin after 30 min application. Permethrin 1% had little effect on the lice after exposure for 5, 10 or 20 min, but the louse mortality was increased to 33.3%

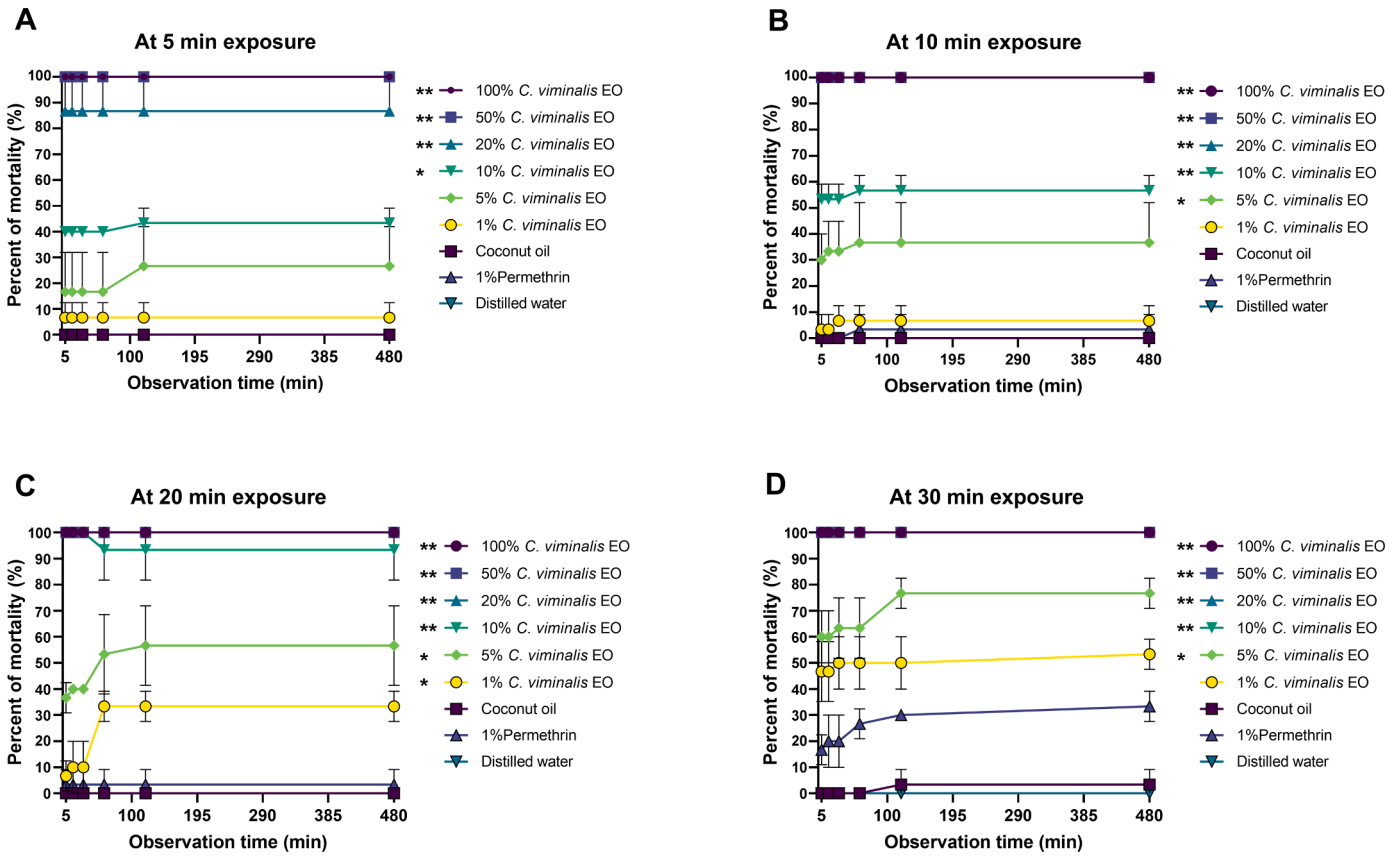

Fig. 2. Percent of head lice mortality (adult stage) at 5 to 480 min (8 h) observation time after applications of *C. viminalis* EO; coconut oil, 1% permethrin; and distilled water. The color represents of each test. A: lice exposure with 50 and 100% of *C. viminalis* EO at 5 min are superimposed line of 100% mortality; B: lice exposure with 20–100% EO at 10 min showed 100%; C: lice exposure with 10–100% EO at 20 min showed 95–100%; and D: lice exposure with 10–100% EO at 30-min showed 100%. Permethrin 1% is a positive control and distilled water is negative control. The experiments were conducted in triplicate (n = 10 adults per experimental group). * p <0.05; ** p <0.001 based on Kruskal–Wallis and Dunn's test (compared mean number of adult lice with positive control group: 1% Permethrin).

after exposure for 30 min. The most interesting finding is that *C. viminalis* EO yielded better results than did 1% permethrin, suggesting that *C. viminalis* EO might be used in areas where permethrin has low efficacy.

Based on findings of previous workers (Candy et al., 2018a; Heukelbach et al., 2008), we observed adult lice for 8 h after treatment. We found that head lice stopped movement without peristalsis of any organs more rapidly after exposure to water than to *C. viminalis* EO or any other solution tested. However, these individuals fully recovered and could walk again after completing an 8 h exposure, agreeing with a previous study (Burgess, 2009b). This behavior might be a form of "playing dead" in response to stressful and threatening conditions (Rogers and Simpson, 2014). Our results and previous reports suggest that investigation of louse mortality using only the criterion of immobility can be misleading and that a long observation time (several hours) is required.

Whereas synthetic insecticides against insect species, including head lice, have known neurotoxicity (Sparks and Nauen, 2015), the mechanisms by which essential oils affect insects are largely unclear. It is generally claimed that essential oils (plant or vegetable oils) cause suffocation of insects (Richling and Bockeler, 2008). We noted that lice exposed to coconut oil, water and 1% permethrin for 30 min stopped moving but became active again during the 8 h observation period. This finding indicates that a short topical application of these solutions is not adequate to induce asphyxiation. Indeed, coconut oils may need to be applied for more than 12 h to kill a significant number of lice (Mumcuoglu, 1999). Increased application time of 1% permethrin might improve its efficacy, but is not recommended by the manufacturers and the side effects on human skin might be increased. Head lice can also survive in water for several hours because of their protective layer of cuticular wax (Barnett et al., 2012). However, it is still unclear whether head lice are able to close their spiracles to prevent penetration of water and other fluids though the tracheal system or whether spiracles are also involved in water or waste excretion (Burgess, 2009b).

Our SEM observations of head louse adults after exposure for 30 min to either low or high concentrations of *C. viminalis* EO or other solutions revealed no loss of sensory hairs or spiracle swelling. This was contrary to a previous study by Akkad et al. that found loss of sensory hairs and swelling of spiracles after treatment with Tea-Tree Head Lice Gel product (containing 5% diluted tea-tree oil and ethanol 20%) for 1 h (Akkad et al., 2016). The different physical effects on louse morphology may be due to the different methods used, exposure time, and dilution of EO. However, we noticed that lice treated with high concentrations of *C. viminalis* EO for 30 min exhibited partial obstruction of some spiracles, suggesting some physical effects (Fig. 3). In addition, constituents of EO are of low molecular-weight and might be able to disrupt the integrity of insect cuticle lipids, hence increasing permeability of the cuticle (Di Campli et al., 2012), or vapor from volatile constituents might act via the louse respiratory system as suggested by Yang et al. (Yang et al., 2004).

Our results from GC–MS analysis revealed forty-four biochemical constituents of EO from *C. viminalis* grown in Thailand. The most abundant were 1,8-cineole (66.96%), α-pinene (18.74%) and o-cymene (7.02%). In South Africa, the major components of *C. viminalis* were 1,8-cineole 83.2%, α-pinene 6.4%, and α-terpineol 4.9%: less represented components included β-pinene, α-terpinene, terpinen-4-ol, and myrcene (Oyedeji et al., 2009). Constituents of *C. viminalis* EO vary according to

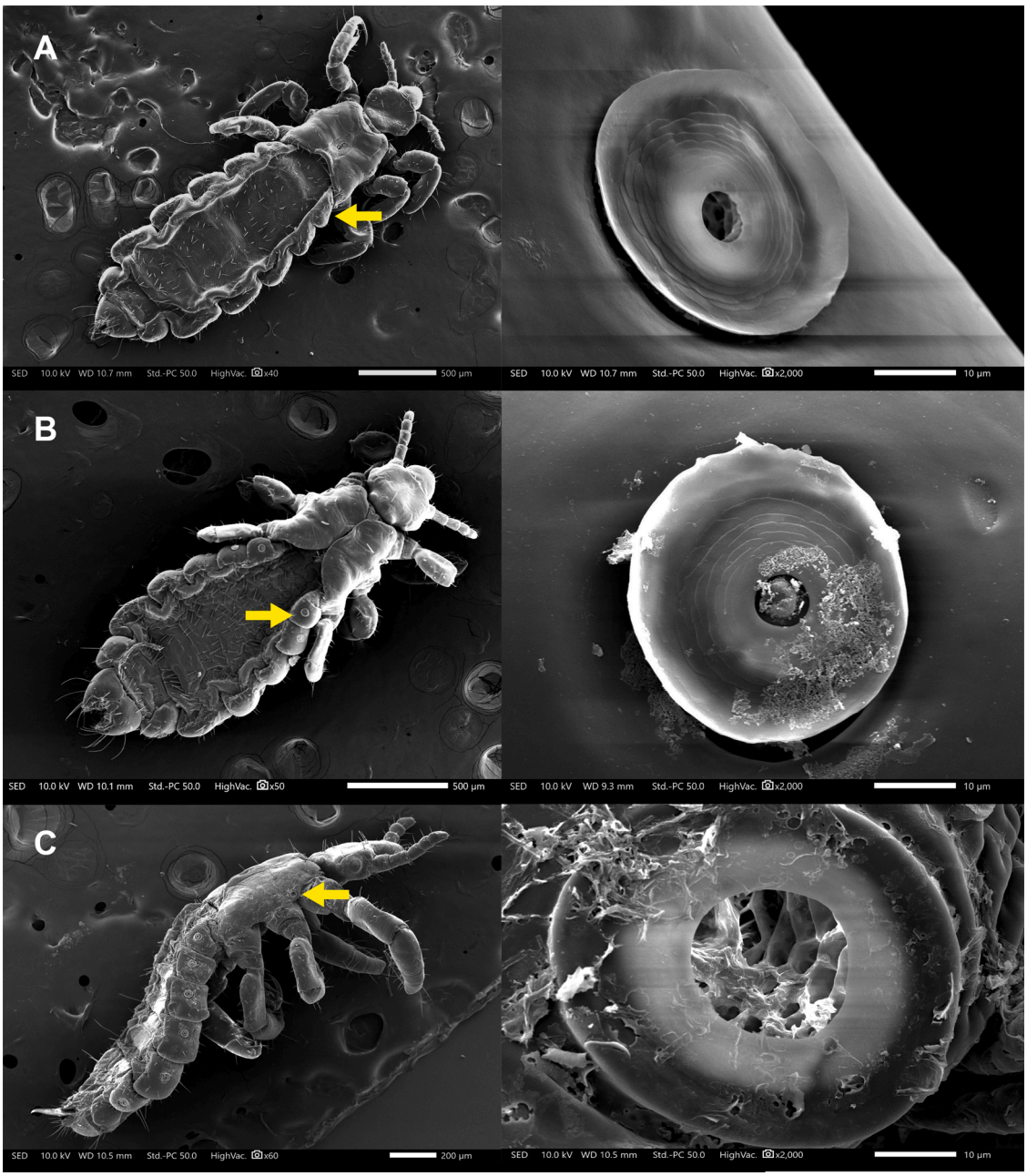

Fig. 3. Scanning electron micrograms of head lice morphological change after expose with various solutions at 30 min using SEM. A: Head lice body surface (HighVac 40x) and some spiracle (HighVac 2,000x) after exposure with water; B: Head lice body surface (HighVac 50x) and some spiracle obstruction (HighVac 2,000x) after exposure with 100% *C. viminalis* EO; C: Head lice body surface (HighVac 60x) and some spiracle (HighVac 2,000x) after exposure with commercial 1% Permethrin.

the location where plant materials are grown, age, season, environment and extraction method (Gad et al., 2019).

Coconut oil was used to prepare various dilutions of *C. viminalis* EO. Since coconut oil by itself had no effect on mortality of head lice or their eggs, we can discount any effect it might have had as a diluent: all observed effects were due to the constituents of *C. viminalis* EO alone. The rapid action of some components of essential oils against insect species might be indicative of a neurotoxic mode of action (Jankowska et al., 2017). The main component of *C. viminalis* EO, 1,8-cineole, has been reported to inhibit the activity of acetylcholinesterase in hematophagous insects (mosquitoes and flies) (Jankowska et al., 2017). This compound is a monoterpene abundant in several aromatic and medicinal plants (e.g., *Eucalyptus globulus, Melaleuca alternifolia* and *Zingiber officinale*). Extracts from these plants have often shown *ex vivo* activity to kill head lice (Candy et al., 2018b; Greive and Barnes, 2018; Toloza et al., 2010). However, the *C. viminalis* EO is a complex mixture of phytochemical components. Monoterpenes (o-cymene, α-pinene, 3-carene and alpha-phellandrene) and sesquiterpenes also have efficient and highly synergistic effects against head lice and other insects (Candy et al., 2020; Langsi et al., 2020). Further physiological study of the effects of *C. viminalis* EO on inhibition of acetylcholinesterase, or other neurotoxic actions against head-lice will be of value.

In this study, we also tested the effects of *C. viminalis* EO against head louse eggs, which are not killed by synthetic chemical pediculicides (Burgess, 2009a). We explored the activity of various concentrations of *C. viminalis* EO on egg hatching during a follow-up observation period of 7–21 days. The effect of our EO on head louse eggs depended on the volume of the solution, length of exposure and method used. Complete

Table 2
Percentage of late-stage eggs hatching at 7, 14 and 21 days after 10 min exposure to each solution based on the immersion test.

Tests	Observation time (Culture) Mean number of eggs hatching/ total ((%) ± SE)	
	7 days	14- 21 days
100% *C. viminalis* EO	0/10 (0 ± 0.00)*	0/10 (0 ± 0.00)*
50% *C. viminalis* EO	0/10 (0 ± 0.00)*	0/10 (0 ± 0.00)*
20% *C. viminalis* EO	0/10 (0 ± 0.00)*	0/10 (0 ± 0.00)*
10% *C. viminalis* EO	0/10 (0 ± 0.00)*	0/10 (0 ± 0.00)*
5% *C. viminalis* EO	1.5/10 (15 ± 0.71)*	1.5/10 (15± 0.71)*
1% *C. viminalis* EO	5.0/10 (50± 1.41)*	5.0/10 (50± 1.41)*
Coconut oil	9.0/10 (90± 1.41)	9.5/10 (95± 0.71)
1% permethrin	9.5/10 (95± 0.71)	10/10 (100± 0.00)
Distilled water	10/10 (100± 0.00)	10/10 (100± 0.00)

The experiments were conducted in duplicate (n = 10 eggs per experimental group).
* $p<0.001$ based on Kruskal–Wallis and Dunn's tests (compared mean number of eggs hatching with positive control group: 1% permethrin).

Table 3
Percentage of late-stage eggs hatching at 7, 14 and 21 days after 20 min exposure to each solution based on the topical test.

Tests	Observation time (Culture) Mean number of eggs hatching/ total ((%) ± SE)	
	7 days	14- 21 days
100% *C. viminalis* EO	0/10 (0 ± 0.00)*	0/10 (0 ± 0.00)*
50% *C. viminalis* EO	0/10 (0 ± 0.00) *	0/10 (0 ± 0.00)*
20% *C. viminalis* EO	1.0/10 (10± 1.41) *	1.0/10 (10± 1.41)*
10% *C. viminalis* EO	4.0/10 (40± 1.41)*	5.0/10 (50± 0.00)*
5% *C. viminalis* EO	3.5/10 (35± 2.12)*	5.5/10 (55± 2.12)*
1% *C. viminalis* EO	5.0/10 (50± 1.41)*	7.0/10 (70± 1.41)*
Coconut oil	9.5/10 (95± 0.71)	9.5/10 (95± 0.71)
1% permethrin	10/10 (100± 0.00)	10/10 (100± 0.00)
Distilled water	10/10 (100± 0.00)	10/10 (100± 0.00)

The experiments were conducted in duplicate (n = 10 eggs per experimental group).
* $p<0.001$ based on Kruskal–Wallis and Dunn's tests (compared mean number of eggs hatching with positive control group: 1% permethrin).

immersion of eggs in EO was very effective, which is not surprising. Using topical contact only, *C. viminalis* EO was less effective. Conditions of exposure to *C. viminalis* EO that killed crawling louse stages were less effective against eggs. This might be due to the structure of the louse egg shell, which consists of a complex of proteins (Burkhart and Burkhart, 2005). In addition, because of low viscosity of *C. viminalis* EO, it may more easily penetrate through the large spiracle of the adult stage than through the small and complicated aeropyle on the operculum of the egg stage (Burkhart et al., 1999).

One limitation of the present study is the small sample size of head lice. Louse samples had to be pooled from several children because of the low intensity of infestation. It may have been better to collect samples from individual children without pooling them. Low effectiveness of 1% permethrin to kill head lice in this study may be a consequence of resistance arising locally following previous treatment with permethrin. In this study, we only observed adult lice for 8 h following treatment to check for signs of life. It is unclear whether this is long enough to confirm their death. *Ex vivo* tests reported here provide the first step in determining conditions required to kill lice before conducting any clinical trials. The EO of *C. viminalis* had higher efficacy against head lice than did 1% permethrin. Use of pure chemical components of *C. viminalis* EO should be evaluated.

Conclusion

We identified the main biochemical components of *C. viminalis* EO and demonstrated that it has higher *ex vivo* insecticidal activities against head lice and their eggs than does 1% permethrin. The EO obtained from *C. viminalis* is a potential source of pediculicidal and ovicidal compounds for controlling head louse infestations, especially in strains of lice that are resistant to synthetic insecticides. Further studies of the acute and chronic toxicities of *C. viminalis* EO in children are necessary.

Contribution authors

Conceptualization: M.Y., P.P., S.P.; Data curation: M.Y., A.P., N.N., B. S., P.P.; Formal analysis: M.Y., A.P., N.N., B.S., P.P., S.P.; Funding acquisition: M.Y., S.P.; Methodology: M.Y., A.P., N.N., B.S., P.P.; Project administration: S.P.; Supervision: S.P.; Validation: S.P., A.P.; Writing –original draft: M.Y.; Writing – review & editing: M.Y., A.P., N.N., B.S., P.P., S.P. All authors have read and agreed to the published version of the manuscript.

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Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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