Repellency of Veratraldehyde (3,4-Dimethoxy Benzaldehyde) against Mosquito Females and Tick Nymphs

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Abstract: Arthropod-borne infectious diseases cause many deaths and a major economic burden worldwide. Repellents play an important role in protecting people from infectious biting arthropods. The repellency of veratraldehyde, a known food additive, and the WJ-1041 formulation containing 10% veratraldehyde was tested against Aedes albopictus and Culex pipiens pallens females and Haemaphysalis longicornis nymphs using arm-in-cage, indoor or filter paper tests. Veratraldehyde exhibited repellency similar to or lower than that of n,n-diethyl-meta-toluamide (DEET) against A. albopictus, but in H. longicornis, the activity of veratraldehyde was better than that of DEET. The repellency of the 10% veratraldehyde solution was comparable to that of 20% DEET against the two mosquitoes. When comparing repellency between the WJ-1041 formulation (10% veratraldehyde) and 10% DEET against C. pipiens pallens, A. Albopictus and H. longicornis, the two showed similar repellency and complete protection time (CPT) values. However, there was a small difference depending on the tested insects. The absorption of veratraldehyde via skin was minimal, if at all. The pharmacokinetic parameters (Cmax and Tmax) of veratraldehyde in blood samples of rats were not different from those of the control group. Based on these results, veratraldehyde has high potential to be commercialized as a repellent agent against infectious disease-borne pests in the near future.

Keywords: Aedes albopictus; Culex pipiens pallens; Haemaphysalis longicornis; repellency; veratraldehyde

1. Introduction

Vector-borne diseases, such as malaria, Zika virus, Lyme disease, rocky mountain spotted fever, encephalitis and West Nile virus (WNV), require a mosquito, tick or other arthropod to transmit them from animals to humans. Mosquitoes inhabit practically every region of every continent in the world, except Antarctica, and are important as medical insect pests. In particular, Culex pipiens complex mosquitoes play important roles in the transmission of several pathogens that infect humans, including WNV, St. Louis encephalitis virus and filarial worms [1,2], as well as wildlife pathogens such as bird malaria (Plasmodium spp.) [3]. The adaptation of C. pипiens complex mosquitoes to human-altered environments combined with their mixed feeding patterns on birds and mammals (including humans) greatly increases the transmission of several avian pathogens to humans [4]. Although C. pipiens pallens females mate but do not seek a blood meal under a short photoperiod [5], their stereotype and domestic species C. pипeins molestus thrives in highly polluted sewers, mates in confined spaces, often enters houses and feeds readily on mammals, including humans. The Asian tiger mosquito Aedes albopictus is native to the
tropical and subtropical areas of Southeast Asia but has spread to Europe, North and South America and Africa. It has aggressive daytime biting behavior and is a medically important mosquito due to its ability to transmit many human pathogens and parasites (e.g., yellow fever, dengue fever, WNV, Japanese encephalitis, St. Louis encephalitis, chikungunya viruses and filarial nematodes) [6,7].

*Haemaphysalis longicornis* is known as an obligate blood feeder tick and to have a three-host life cycle and is native to temperate areas such as Korea, Japan, Eastern China and the Russian Far East and was established as an exotic species in Australia, New Zealand and several island nations in the western Pacific Region, including the USA, in 2017 [8–10]. In East-Northern Asia, *H. longicornis* transmits severe fever with thrombocytopenia syndrome virus (SFTSV), which causes human hemorrhagic fever [11], and the relationship between the population density of ticks and the number of patients with SFTSV was 40.4% in Korea [12]. In some regions of New Zealand and Australia, this tick can reduce production in dairy cattle by 25% [13].

Most authorities managing arthropod-borne diseases recommend applying insect repellents, wearing protective clothing or avoiding infested habitats to protect against arthropod bites. Insect repellents, such as products containing synthetic chemicals or plants as active ingredients, are currently available to consumers. The most widely marketed insect repellent worldwide is *n,n*-diethyl-meta-toluamide (DEET) because it is a broad-spectrum repellent that is effective against various mosquito species and ticks. However, since treatment with synthetic chemicals such as DEET is associated with human health risks [14], most people hesitate to apply DEET products to their skin or clothing and deliberately look for other DEET-free repellent products. For these reasons, alternative natural repellents are now very appreciated by consumers, and essential oils of aromatic plants have been demonstrated to have repellent activities against arthropods, including mosquitoes, mites and lice, as well as have the potential to provide efficient and safer repellents for humans and the environment [17–20]. However, essential oils still do not have widespread application due to some limitations, such as their short-lasting repellency, composition variability and strong smell, which is one of the most negative aspects of their use [21]. Thus, to meet the needs of consumers, natural repellents should have a high efficacy against the target arthropod pests coupled with a pleasant smell.

Veratraldehyde (3,4-dimethoxybenzaldehyde), found among the constituents of the essential oils of *Cymbopogon javanensis, Eryngium poterium, Rubus idaeus* (raspberry), *Zingiber officinale* (ginger) and *Mentha piperita* (peppermint) [22], is an organic compound that is widely used as a flavor and odorant. Veratraldehyde is used in the preparation of 4-chloromethyl-2-(dimethoxyphenyl)-1,3-dioxolane, which is used in the synthesis of (+)-lithospermic acid and has anti-HIV activity [23]. Veratraldehyde is related to benzaldehyde and it is commercially popular because of its pleasant woody fragrance. In a high-throughput bioassay using the yeast *Saccharomyces cerevisiae* to identify phenolic agents for the control of the aflatoxigenic fungus *Aspergillus flavus*, veratraldehyde showed significant antifungal activity [24]. Veratraldehyde is used for H₂O₂ production by *Pleurotus eryngii*, and it is reduced by aryl-alcohol dehydrogenase to its corresponding alcohols, which are oxidized by aryl-alcohol oxidase, producing H₂O₂ [25]. Veratraldehyde (CAS no. 120-14-9) is also listed as a food additive in the Korea Food Additives Code [26]. However, the repellent activity of veratraldehyde against mosquitoes and ticks is not known.

In this study, to develop new natural repellents comparable to conventional arthropod repellents, such as the synthetic compound DEET, the repellent activity of veratraldehyde and pump spray formulations containing 10% veratraldehyde as active ingredients against *A. albopictus* females, *C. pipiens pallens* females and *H. longicornis* nymphs as well as the absorption of veratraldehyde via skin was evaluated under laboratory and indoor conditions.
2. Materials and Methods

2.1. Chemicals

Veratraldehyde (>99%) and \(n,n\)-diethyl-meta-toluamide (DEET, >98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and TCI Chemical (Tokyo, Japan), respectively. All other chemicals were reagent grade and available commercially.

2.2. Preparation of Formulation

The spray formulation, named WJ-1041, containing 10% veratraldehyde as the active ingredient, 2% MCT oil, 9.5% emulsifiers, 0.1% preservative, 1.0% base materials, 0.07% flavoring agent, 14% auxiliary solvent and 63.3% distilled water, was prepared by Dong-sung Biopharm (Seoul, Korea), and 10% DEET, used as a positive control, was dissolved in ethanol.

2.3. Mosquito and Tick Culture

A. albopictus and C. pipiens pallens colonies were obtained from Seoul National University. The colonies were originally obtained from the National Institute of Health, Korea Centers for Disease Control and Prevention, Seoul, Korea, in 1999 and were reared in the laboratory without exposure to insecticides. Mosquito adults were maintained in screen cages with a 10% sucrose solution soaked in cotton located on top of the cage (45 × 45 × 45 cm). Five to six days after emergence, the mosquitoes in the cages were allowed to feed on the blood of live mice for a complete reproductive cycle (Institutional Animal Care & Use Committee of Seoul National University no. SNU-190418-1). Larvae were kept in plastic trays (25 × 35 × 5 or 30 × 50 × 10 cm) containing 0.5 g of sterilized diet (40-mesh chick chow powder and yeast, 1:1 w/w) and tap water 2–3 days after preparation. The rearing room was maintained at 26 ± 1 °C and at a 65–75% RH with a photoperiod of 12:12 h (L:D).

The hard tick H. longicornis was a wild-type nymph collected using the flagging method (100 × 120 cm) in Hwaseong city (Gyeonggi Province, Korea) from August to October 2019 (37.224253N, 126.929426E). The nymphs on the flag were detached by tweezers and placed into a plastic tube (Falcon tube, 100 mL) containing two pieces of gauze (3 × 3 cm) impregnated with water (200 µL). The tubes were transferred to our laboratory and kept in a water bath (20 × 20 cm). The laboratory was maintained at 24 ± 1 °C and at a 55–65% RH with a photoperiod of 12:12 hr (L:D).

2.4. Subjects

Healthy human volunteers (males 20–50 and females 20–30 years old) were recruited, and they were informed of all procedures and dangers during the test periods before the repellency tests of each material were examined. Additionally, all human subjects provided written informed consent, and ethical approval was provided by the Internal Review Board, Seoul National University (approval no. 1908/002-009).

2.5. Repellency Assay

2.5.1. Laboratory Assay Using an Arm-in-Cage Test

The repellency of veratraldehyde (0.25, 0.20, 0.10 and 0.03 mg/cm²) and its formulations (5, 7, 10 and 15% veratraldehyde) against A. albopictus and C. pipiens pallens was examined in a test cage (30 × 30 × 30 cm) containing 100–130 females. Females 5–7 days after emergence were used and were starved for over 12 h before testing. Appropriate quantities of the test materials, each in 100 µL of ethanol or 2 sprays of formulations, were directly applied evenly to the exposed skin of the back of the left or right hand through a rectangle (5 × 5 cm) cut into the back part of a rubber glove. Controls received 100 µL of ethanol or 2 pump sprays of formulation without veratraldehyde. After air drying for 2 min, the treated hands of each volunteer were exposed to female A. Albopictus and C. pipiens pallens for 3 min and every 0.5 or 1 h until the test volunteer received a female bite. After one cycle was terminated, the hands of volunteers were exposed alternately to
new mosquito females. DEET (0.25, 0.20, 0.10 and 0.03 mg/cm²) and its formulation (20% DEET) served as a positive control for comparison in the repellency tests. The numbers of mosquito females biting the skin were recorded, and each assay was replicated more than three times.

2.5.2. Indoor Test Using Human Volunteers

The repellencies of pump spray containing 10% veratraldehyde and 10% DEET ethanol solution were examined in a test room of the Bio Venture Valley of the College of Agriculture and Life Sciences, Seoul National University (Suwon, Gyeonggi Province). A total of 150–200 *A. albopictus* or *C. pipiens pallens* females (5–7 days old and acclimated for 2 h in the test room conditions) were released into a triangular pyramid-type mosquito net (approximately 200 × 200 × 155 cm) in the room. Each test ran at 1 h intervals between 11:00 and 18:00 or between 21:00 and 03:00 for *A. albopictus* and *C. pipiens pallens* females, respectively. The formulations were sprayed evenly five times over the skin below the left or right knee of volunteers (approximately 750 mg and 900–1000 cm²). The untreated knee of a volunteer was sprayed five times with ethanol only. The volunteers wore bee veils, shorts, T-shirts and shoes to protect them from excessive attack by mosquitoes on unprotected skin surfaces. Mosquitoes that landed on the exposed legs (below the knees) were captured using an aspirator and then transferred to a glass vial, and the number was recorded at the end of each trial.

2.5.3. Filter Paper Assay Using *Haemaphysalis longicornis* Nymphs

The repellency tests of veratraldehyde and DEET at concentrations of 0.09, 0.05, 0.02 and 0.01 mg/cm² against *H. longicornis* nymphs followed KFDA (Korea Food & Drug Agency) guidelines [27]. Two circles were constructed on filter paper (15 cm diameter) by a compass, and the paper was fixed onto a wood board (22 × 30 × 0.6 cm) covered with aluminum foil. The diameters of the smaller and larger circles were 5 and 13 cm, respectively, and each tested material was evenly applied between them (treated zone, 113.5 cm²) one to five times. Ten nymphs collected from the wild environment were placed on the center circle (untreated zone), and the number of ticks crossing or staying in the treated zone was recorded for 5 min. All assays were repeated every 1 h and were performed 10 times. The repellency index was calculated according to the following formula: % repellency = [(C − T)/C] × 100, where C is the number of tick nymphs crossing or staying in the negatively treated zone and T is the number of tick nymphs crossing or staying in the treated zone.

2.5.4. Calculations of Complete Protection Time (CPT) and Repellency

The complete protection time (CPT) was defined as the time when more than two mosquitoes landed on the volunteer for more than 3 sec or probed/bit treated hand skin or a treated area in one trial or continued for subsequent trials. Briefly, if two females showed biting behavior in a test 2 h after treatment, the CPT of the material was 2 h, and if one (first) female showed biting behavior 1 h after treatment and another (second) female (resulting in two females) 2 h after treatment (in the continued trial), the CPT of the material was 1 h. Similarly, if one (first) female showed biting behavior 1 h after treatment but there was no biting female 2 h after treatment, the CPT was not determined. In the same trial set, if two females showed biting behavior 3 h after treatment or one female at 3 h and another female at 4 h, the CPT of the material was determined to be 3 h. The repellency index was calculated according to the following formula [28]: % repellency = [(Ta − Tb)/Ta] × 100, where Ta is the number of mosquitoes in the control group and Tb is the number of mosquitoes in the treated group. The mean percentages of repellency and CPT were compared and separated by the Bonferroni multiple-comparison method at *P* = 0.05, using SAS (v. 8.01, SAS Institute Inc., Cary, NC, USA).
2.6. Pharmacokinetics of Veratraldehyde in Sprague-Dawley Rats

Rats were starved 12 h before a single dosing test, hairs on the abdomen were removed (8 x 6 cm), and then 300 or 600 mg/kg of repellent was applied to the exposed skin using a patch (5 x 4 cm) for 24 h. The three Sprague-Dawley rats were grouped as a control (G1, vehicle) and two treatments (G2, 300 mg/kg and G3, 600 mg/kg). Using a disposable syringe (1 mL, 26G needle), each blood sample (300 µL equivalent to ≥100 µL plasma) from the jugular vein was collected at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 48 and 72 h in a BD Vacutainer (K2 EDTA) tube with anticoagulant. To determine the veratraldehyde in blood samples, a calibration curve was made using 3 to 1000 ng/mL solution (R² = 0.9986). An internal standard solution (10 µL) was added to the calibration and plasma samples, and they were precipitated with 300 µL of 0.2% formic acid in acetonitrile. The prepared plasma solutions were centrifuged at 4°C and 15,000 rpm for 5 min after shaking at 1000 rpm for 10 min and were stored in a freezer (−20°C) for pK (pharmacokinetics) analysis. Quantitative analysis was carried out using LC–MS/MS (1290 series Infinity II, 6495 Triple Quadrupole, Agilent, USA) based on a previous report [29], and the analysis conditions used to determine the veratraldehyde in blood samples were as follows: a Unison UK-C18 column (3.0 µm, 2.0 x 50 mm) was kept at 30°C, and the mobile phase was composed of 0.2% formic acid in water (A) and 0.2% formic acid in acetonitrile (B) and was flowed at a rate of 0.5 mL/min with 80% A + 20% B at 0 min and 20% A + 80% B at 2.5 min. Each supernatant (2 µL) was injected and detected by coupled MS/MS. The detector conditions were as follows: gas temperature (200°C), gas flow rate (14 L/min), nebulizer (200 psi), sheath gas temperature (250°C), sheath flow rate (11 L/min), capillary (positive 3000 V, negative 3000 V), nozzle voltage (positive 1500 V, negative 1500 V), polarity (positive precursor 167.07 m/z), product (139.0 m/z // 123.7m/z), retention time (1.61 min) and CE (10 eV // 15 eV).

3. Results

3.1. Repellency of Veratraldehyde

The repellency of veratraldehyde was bioassayed against *A. albopictus* females using the arm-in-cage test (Table 1). At doses of 0.25 and 0.20 mg/cm², the chemical exhibited more than 95% repellency for 2.5 h, respectively. However, depending on the dose, it showed 83% repellency 1.5 h after treatment and 75% activity 0.5 h after treatment at 0.10 and 0.03 mg/cm², respectively. DEET was used as a positive control and showed more than 95% repellency at both 0.25 and 0.20 mg/cm² for 2.5 h. However, DEET also gave decreased repellent activity at lower doses, 0.10 and 0.03 mg/cm², similar to the pattern observed for veratraldehyde.

**Table 1.** The repellent activity of veratraldehyde against *Aedes albopictus* females using the arm-in-cage test.

| Material | Conc. (mg/cm²) | Repellency (%) | 0.5 h | 1 h | 1.5 h | 2 h | 2.5 h | 3 h |
|----------|----------------|----------------|-------|-----|-------|-----|-------|-----|
|          |                |                |       |     |       |     |       |     |
| Veratraldehyde | 0.25           | 100 ± 0        | 100 ± 0 | 100 ± 0 | 100 ± 0 | 95 ± 3.2 | 58 ± 4.4 |
|          | 0.20           | 100 ± 0        | 99 ± 2.3 | 94 ± 4.6 | 91 ± 1.7 | 83 ± 2.6 | 65 ± 6.0 |
|          | 0.10           | 93 ± 4.3       | 90 ± 4.1 | 83 ± 8.8 | 58 ± 5.5 |
|          | 0.03           | 75 ± 7.1       | 54 ± 4.3 |
| DEET    | 0.25           | 100 ± 0        | 100 ± 0 | 100 ± 0 | 100 ± 0 | 100 ± 0 | 84 ± 4.3 |
|          | 0.20           | 100 ± 0        | 100 ± 0 | 100 ± 0 | 95 ± 3.3 | 96 ± 1.2 | 85 ± 3.0 |
|          | 0.10           | 96 ± 2.6       | 86 ± 4.2 | 83 ± 4.3 | 82 ± 9.5 | 57 ± 5.2 | 27 ± 3.8 |
|          | 0.03           | 67 ± 3.3       | 69 ± 6.9 | 19 ± 5.2 |

The repellency of veratraldehyde against *H. longicornis* nymphs in the filter paper assay also depended on both the test dose and exposure time (Table 2). The repellency of all tested concentrations of veratraldehyde (0.25, 0.20, 0.10 and 0.03 mg/cm²) was more
than 90% for 3 h. However, the repellent activity decreased faster at lower concentrations, resulting in activity of 85 and 75% at 0.09 mg/cm² after 4 and 5 h, respectively, whereas the activities at 0.01 mg/cm² were 65 and 48%. On the other hand, although the repellency of DEET at 0.09 mg/cm² was 100% for 4 h, at concentrations below this, the activity was found to be lower than that of veratraldehyde, indicating that the repellency of veratraldehyde was better than that of DEET against *H. longicornis* nymphs.

### Table 2. The repellent activity of veratraldehyde against *Haemaphysalis longicornis* nymphs using the filter paper assay.

| Material     | Conc. (mg/cm²) | 1 h   | 2 h   | 3 h   | 4 h   | 5 h   |
|--------------|----------------|-------|-------|-------|-------|-------|
| Veratraldehyde | 0.09           | 100 ± 0a | 100 ± 0a | 100 ± 0a | 85 ± 2.9b | 75 ± 5.0ab |
|              | 0.05           | 100 ± 0a | 98 ± 2.5a | 93 ± 4.8a | 88 ± 2.5b | 78 ± 4.8a |
|              | 0.02           | 98 ± 2.5ab | 98 ± 2.5a | 95 ± 5.0a | 83 ± 2.3bc | 60 ± 5.8c |
|              | 0.01           | 98 ± 2.5ab | 90 ± 5.8ab | 95 ± 2.9a | 65 ± 2.9c | 48 ± 4.8abc |
| DEET         | 0.09           | 100 ± 0a | 98 ± 2.5a | 100 ± 0a | 100 ± 0a | 50 ± 4.1bc |
|              | 0.05           | 100 ± 0a | 98 ± 2.5a | 88 ± 2.5a | 85 ± 2.9b | 70 ± 4.1abc |
|              | 0.02           | 98 ± 2.5ab | 78 ± 2.5bc | 50 ± 4.1b | 25 ± 2.9d | 20 ± 4.1d |
|              | 0.01           | 88 ± 4.8b | 60 ± 4.1c | 50 ± 7.1b | 30 ± 7.1d | 23 ± 4.8d |

*Means with the same letter are not significantly different according to a Bonferroni multiple-comparison test (p = 0.05).*

#### 3.2. Determination of the Effective Dose of Veratraldehyde

To determine the effective dose of veratraldehyde contained in a formulation, four doses (5, 7, 10 and 15%) of veratraldehyde in ethanol solution were prepared, and their repellency against mosquito females was compared to that of 20% DEET using the arm-in-cage test. Veratraldehyde showed 83 and 76% repellency against *A. albopictus* females for 3 and 4 h, respectively (Table 3). In a test using a 7% solution, veratraldehyde showed 88% repellency for 6 h. Although the repellency of the four veratraldehyde solutions was strong, 20% DEET maintained 99% repellency for 6 h. In addition, veratraldehyde solutions kept CPTs of 1.3–5.3 h, and 10% veratraldehyde showed the same CPT (5.3 h) compared to that of 20% DEET against *A. albopictus* (Table 3).

### Table 3. Repellency and CPT based on the concentrations of veratraldehyde against *Aedes albopictus* females using the arm-in-cage test.

| Material   | Conc. % | Repellency (% Mean ± SE) a | CPT (h, 95% CI) b |
|------------|---------|---------------------------|-------------------|
| Veratraldehyde | 5  | 97 ± 1.7a | 89 ± 0.5b | 83 ± 3.7b | 76 ± 4.3a | 75 ± 5.8c | 64 ± 2.7b | 1.3 (0.68–1.99) |
|            | 7  | 100 ± 0a | 100 ± 0a | 100 ± 0a | 89 ± 6.5a | 88 ± 4.6bc | 77 ± 4.3b | 4.7 (4.01–5.52) |
|            | 10 | 100 ± 0a | 100 ± 0a | 99 ± 0.8a | 97 ± 3.0a | 96 ± 1.1ab | 96 ± 1.0a | 5.3 (4.68–5.99) |
|            | 15 | 100 ± 0a | 100 ± 0a | 100 ± 0a | 91 ± 8.1a | 99 ± 0.8a | 98 ± 0.9a | 5.3 (3.68–4.99) |
| DEET       | 20 | 100 ± 0a | 100 ± 0a | 100 ± 0a | 99 ± 0.6a | 100 ± 0a | 99 ± 0.5a | 5.3 (4.03–6.64) |

*Means with the same letter are not significantly different according to a Bonferroni multiple-comparison test (p = 0.05). b CPT, complete protection time; CI, confidence interval.*

Veratraldehyde showed 100% repellency against *C. pipiens pallens* females for 3 h at all the tested doses, indicating that its repellency against *C. pipiens* was higher than that against *Aedes* (Table 4). In a test 6 h after treatment, veratraldehyde showed 84, 83, 100 and 88% repellency at concentrations of 5, 7, 10 and 15%, respectively, and the activity was comparable to the 95% repellency observed for 20% DEET. The tested veratraldehyde solutions maintained CPTs 4.7–6 h, and the CPT of the 10% solution was comparable to that of 20% DEET (Table 4). Based on the results for the four veratraldehyde solutions, the effective dose of veratraldehyde was determined to be 10%.
Additionally, the CPT of WJ-1041 (5.7 h) was longer than that of 10% DEET, but there was no significant difference between WJ-1041 and DEET (5.3 h). On the other hand, in the test against *C. pipiens pallens*, DEET showed 100% repellency against females using the arm-in-cage test.

### 3.3. Repellency of WJ-1041 in the Arm-in-Cage Test

The repellency of WJ-1041 containing 10% veratraldehyde and 10% DEET was tested against *C. pipiens pallens* and *A. albopictus* females. In the arm-in-cage test, both WJ-1041 and 10% DEET showed ≥97% repellency against *A. albopictus* for 3 h after treatment, but their repellency decreased over time (Table 5). After 5 and 6 h, the repellency ranges for WJ-1041 and DEET were 91–92 and 84–86%, respectively, indicating that the repellency of WJ-1041 was higher than that of 10% DEET, but there was no significant difference between them. In addition, the CPT of WJ-1041 (4.7 h) lasted longer than that of 10% DEET (4.3 h).

### 3.4. Repellency of WJ-1041 in the Indoor Test

The repellency of WJ-1041 and 10% DEET against *A. albopictus* and *C. pipiens pallens* females was assayed using the indoor test with nine volunteers. The number of volunteers assayed for each test at each time point was three. WJ-1041 and 10% DEET showed 100% repellency against *A. albopictus* for 3 and 2 h, respectively (Table 7), and even after 6 h, the activity of WJ-1041 was maintained at approximately 85%, which was higher than that of 10% DEET (78%). Additionally, the CPT of WJ-1041 (5.7 h) was longer than that of 10% DEET (5.3 h). On the other hand, in the test against *C. pipiens pallens*, WJ-1041 and 10% DEET showed 100% repellency against *C. pipiens pallens* for 3 and 4 h, respectively (Table 8), and the activity of WJ-1041 decreased slightly faster than that of 10% DEET. However, the repellent activities of both compounds persisted at more than 90% for up to 6 h, and the
CPT of the WJ-1041 products (5.7 h) was longer than that of 10% DEET (5.3 h). Based on the results, there was a difference in the repellency of WJ-1041 products and 10% DEET for the tested mosquito species.

Table 7. Repellency and CPT of WJ-1041 containing 10% veratraldehyde as the active ingredient against *Aedes albopictus* females using the indoor test.

| Material       | Repellency (%, Mean ± SE) | CPT (%, 95% CI) c |
|----------------|----------------------------|-------------------|
|                | 1 h | 2 h | 3 h | 4 h | 5 h | 6 h |              |
| WJ-1041 a      | 100 ± 0 | 100 ± 0 | 100 ± 0 | 98 ± 1.2 | 96 ± 0.7 | 85 ± 4.4 | 5.7 (5.01–6.32) |
| 10% DEET b     | 100 ± 0 | 100 ± 0 | 99 ± 1.5 | 98 ± 2.1 | 91 ± 3.1 | 78 ± 4.3 | 5.3 (4.68–5.99) |

^a The tested WJ-1041 was manufactured as a product containing 10% 3,4-dimethoxybenzaldehyde as the active ingredient. ^b 10% DEET was dissolved in ethyl alcohol. ^c CPT, complete protection time; CI, confidence interval.

Table 8. Repellency and CPT of WJ-1041 containing 10% veratraldehyde as an active ingredient against *Culex pipiens pallens* females using the indoor test.

| Material       | Repellency (%, Mean ± SE) | CPT (%, 95% CI) c |
|----------------|----------------------------|-------------------|
|                | 1 h | 2 h | 3 h | 4 h | 5 h | 6 h |              |
| WJ-1041 a      | 100 ± 0 | 100 ± 0 | 100 ± 0 | 96 ± 3.7 | 94 ± 3.0 | 90 ± 2.1 | 5.7 (5.01–6.32) |
| 10% DEET b     | 100 ± 0 | 100 ± 0 | 100 ± 0 | 97 ± 2.6 | 98 ± 1.6 | 6.0 (0)   |

^a The tested WJ-1041 was manufactured as a product containing 10% 3,4-dimethoxybenzaldehyde as the active ingredient. ^b 10% DEET was dissolved in only ethyl alcohol. ^c CPT, complete protection time; CI, confidence interval.

3.5. Repellency of WJ-1041 against *Haemaphysalis longicornis* Nymphs

The repellency of WJ-1041 against *H. longicornis* nymphs in the filter paper assay was 82% for 3 h after treatment, which was higher than that of 10% DEET (62%) (Table 9). Moreover, after 4 h, the activity of WJ-1041 was 66%, whereas the activity of 10% DEET was 41%, indicating that the activity of WJ-1041 persisted slightly longer. These results indicate that WJ-1041 products are more effective than 10% DEET against *H. longicornis* nymphs.

Table 9. Repellency of WJ-104 containing 10% veratraldehyde as an active ingredient against *Haemaphysalis longicornis* nymphs using a filter paper assay.

| Material       | Repellency (%, mean ± SE) |
|----------------|----------------------------|
|                | 1 h | 2 h | 3 h | 4 h |
| WJ-1041 a      | 97 ± 2.1 | 85 ± 5.6 | 82 ± 5.5 | 66 ± 5.2 |
| 10% DEET b     | 95 ± 2.7 | 82 ± 5.9 | 62 ± 6.3 | 41 ± 6.6 |

^a The tested WJ-1041 was manufactured as a product containing 10% 3,4-dimethoxybenzaldehyde as the active ingredient. ^b 10% DEET was dissolved in ethyl alcohol.

3.6. Skin Absorption of Veratraldehyde

In the LC–MS/MS quantitative analysis, veratraldehyde was either not detected or detected in small amounts in blood samples collected from one control (G1) and two experimental groups (G2 and G3, 300 and 600 mg/kg percutaneous application, respectively) of Sprague-Dawley rats (Table 10). The pharmacokinetic parameters C<sub>max</sub> and T<sub>max</sub> were 0.2 (±0.40) ng/mL and 1.3 (±2.31) h for G1, 0.63 (±0.77) ng/mL and 24.67 (±47.00) h for G2 and 0.55 (±0.85 ng/mL) and 1.67 (±2.08) h for G3, respectively. The absorption pharmacokinetics of veratraldehyde into blood via the skin showed typical primary dose-dependent nonlinear kinetics.
Table 10. Plasma concentration versus time profiles for veratraldehyde after percutaneous application (G1–G3) of veratraldehyde.

| Collection Time (Hour) | Conc. (ng/mL, Mean ± SE) |
|------------------------|--------------------------|
|                        | G1 a                     | G2            | G3            |
| 0                      | 0 ± 0                    | 0 ± 0         | 0.99 ± 1.71   |
| 0.25                   | NA                       | NA            | NA            |
| 0.5                    | NA                       | NA            | NA            |
| 1                      | NA                       | 0.1 ± 0.23    | 0.5 ± 0.88    |
| 2                      | NA                       | 0.5 ± 0.86    | 0 ± 0         |
| 4                      | 0.2 ± 0.40               | 0 ± 0         | 0.04 ± 0.07   |
| 6                      | NA                       | 0 ± 0         | 0 ± 0         |
| 8                      | NA                       | 0 ± 0         | 0 ± 0         |
| 10                     | NA                       | 0 ± 0         | 0.04 ± 0.07   |
| 12                     | NA                       | 0 ± 0         | 0 ± 0         |
| 24                     | NA                       | 0 ± 0         | 0 ± 0         |
| 48                     | NA                       | 0.4 ± 0.69    | 0.1 ± 0.15    |
| 72                     | NA                       | 1.2 ± 2.06    | 0 ± 0         |

C_{\text{max}}, ng/mL \text{ b}  
T_{\text{max}}, hr

0.2 ± 0.40  
1.3 ± 2.31

| C_{\text{max}}, ng/mL \text{ b}  
T_{\text{max}}, hr |
|-----------------|-----------------|
| 0.2 ± 0.40      | 0.6 ± 0.77      |
| 1.3 ± 2.31      | 24.7 ± 4.1      |
| 1.7 ± 2.08      | 0.6 ± 0.85      |

NA, Not applicable. \text{ a} G1, vehicle control; G2, veratraldehyde (300 mg/kg); and G3, veratraldehyde (600 mg/kg). \text{ b} C_{\text{max}} and T_{\text{max}} were the highest concentration and the time detected in the blood of Sprague-Dawley rats, respectively. The concentration of veratraldehyde was calculated using the internal standard calibration curve as follows: AxCi/AiCx, where Ax is the area of veratraldehyde, Cx is the concentration of veratraldehyde, Ai is the area of the internal standard, and Ci is the concentration of the internal standard.

4. Discussion

Veratraldehyde is a derivative of vanillin, from which it is prepared by methylation [30]. Vanillin is a repellent synergist against mosquitoes. Mixtures of vanillin with insect repellents containing DEET prolonged the protection time by more than 100% compared to that of DEET alone [31], and its addition to plant essential oils such as celery oil (Apium graveoleus) and xanthoxylum oil (Zanthoxylum armatum) increased both repellency and duration [16,32]. In this study, veratraldehyde and its 10% formulation, WJ-1041, provided more than 2 h of complete protection time against A. albopictus, C. pipiens pallens and H. longicornis nymphs.

The repellent effectiveness and duration of chemicals depend on a variety of factors, including the type of active ingredient, the frequency and formulation of application, test conditions such as mosquito species and involved volunteers, loss due to removal by perspiration and abrasion, intrinsic water loss and the numerical density of mosquitoes [33,34]. DEET formulations (23.8%) give 5 h of complete protection against A. aegypti bites [35], but IR3535 (7.5%), which is another synthetic insect repellent and has been used in Europe for more than 30 years, yielded poor complete protection time (22.9 min) against A. aegypti bites, and some citronella-based repellents, except for a soybean oil-based repellent showing a 1.5 h protection time, also showed very short durations of repellent activity ranging from approximately 3 to 20 min [35]. A number of products based on plant essential oils, such as citronella, fennel, geranium, lavender and rosemary oils, have been commercialized, but their repellency duration was less than 1 h under field conditions [15]. In addition, MossZero® aerosol and cream products containing 5 and 8% fennel oil, respectively, and MeiMei® cream containing citronella and geranium oils produced 84, 70 and 57% repellency at 90 min after exposure, respectively, whereas Repellan S® aerosol containing 19% DEET produced 89% repellency at 210 min [36]. In addition to active ingredients and formulation type, the concentration contained in a repellent formulation may also be an important factor for repellency. The protection provided by DEET depends mainly on both dose and formulation concentration, and its duration reaches a plateau at a concentration of approximately 50% [37]. However, a higher concentration of DEET contained in a formulation does not necessarily mean better repellency. For instance, three
DEET-based repellents containing 33, 42 and 75% DEET provided similar protection effects after application in different climatic regimens (tropical forest, tropical open and basic hot environments) against A. aegypti, A. taeniorhynchus and Anopheles stephensi, whereas there was no difference in the protection period against Anopheles albimanus [38]. Similarly, the 7% veratraldehyde ethanol solution showed higher repellency (97%) than 10 and 15% solutions at 5 h after treatment in the arm-in-cage test. In this study, there was a difference in the repellency and repellency duration of veratraldehyde and formulations according to application dose and tested arthropod species. Veratraldehyde and its pump spray formulations exerted better repellency and complete protection time to the mosquito females than to the tick (H. longicornis) nymphs.

In earlier studies, the improved duration of repellent agents (DEET or plant essential oils) against mosquitoes might be attributed to the lower evaporation rate and better skin persistence [31,36]. According to the pharmacokinetic results in our study, it was predicted that the veratraldehyde was not absorbed into dermal tissues via skin, or even if it was absorbed, its amount was very small. In a previous report, the veratraldehyde was not detected in the blood when applied on the skin of rats, whereas it was detected in the blood when administered orally [29]. However, veratric acid, a metabolite of veratraldehyde, was detected for up to 24 h after skin administration, although the amount was very small compared to oral administration [29]. This suggests that the main natural loss route for veratraldehyde applied on the skin is inherent evaporation, and if a small amount is absorbed through the skin, it can be converted immediately into metabolites such as veratric acid.

Most insect repellents are categorized as vapor or olfactory repellents because they are active in the vapor phase and have an impact on the olfaction of target arthropods, preventing the insects from locating a treated area [39]. Thus, insect repellents must have a minimal vapor pressure to be effective, and repellents with a high vapor pressure will have a repellent effect at low concentrations, but lower volatility of a repellent will result in protection for longer time periods [40]. Veratraldehyde has a low vapor pressure, which is lower (0.0009 hPa at 24 °C) than that of the above-described repellent agents: DEET 0.0024 hPa at 25°C, IR3535 0.0015 hPa at 20 °C and citronella oil 0.22 hPa at 25 °C [41]. Although the mode of action of veratraldehyde is not known, its repellency against mosquitoes and ticks may be due to both its low volatility and its low skin absorption.

5. Conclusions

In this study, we confirmed that veratraldehyde, one of the natural products, has high repellent activity against mosquitoes and ticks, and it is comparable to that of the synthetic compound DEET, which is currently widely used commercially. This is the first report for the repellency of veratraldehyde against mosquitoes and ticks, and it is an important result to meet the demand for consumers to prefer natural repellents that can replace synthetic compounds. Moreover, the veratraldehyde has excellent potential as a natural repellent because it not only can maintain repellent activity for a long time due to its low skin absorption rate and low volatility, but also has a good scent. Therefore, we concluded that the veratraldehyde has high potential to be commercialized as a repellent agent in the near future. In addition, for a stable and optimized application, the mode of action of this compound needs to be studied in the near future.

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