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Toxicity of white flesh *Citrus grandis* Osbeck fruit peel extracts against *Aedes aegypti* (Linnaeus) larvae and its effect on non-target organisms

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Abstract
The aim of the study is to evaluate the bio-efficacy of white flesh *Citrus grandis* fruit peel extracts, essential oil and isolated compounds against late third instar *Aedes aegypti* larvae. Furthermore, this study also investigates its effect on non-target organisms. Bioassay was conducted under laboratory condition to determine the LC_{50} and LC_{90} values at 24 and 48 hours post treatment. The most toxic extract was tested at sub-lethal dose against non-target organism namely, *Toxorhynchites splendens* and *Poecilia reticulata*. Results revealed that the hexane extract proved to be the most effective with lowest LC_{50} values at 24 hours (17.98 ppm) and 48 hours (10.19 ppm). The extract is proven safe when tested with *P. reticulata* but toxic towards younger instar of *Tx. splendens*. The finding indicates that white flesh *C. grandis* fruit peel has the potential to be used as alternative larvicides against *Ae. aegypti* larvae.

Keywords: *Citrus grandis*, *Aedes aegypti*, *Toxorhynchites splendens*, *Poecilia reticulata*.

1. Introduction
Current vector control programmes employ synthetic insecticides to control adult and larval mosquito populations [1]. However, synthetic insecticides have caused numerous ecological problems such as environmental contamination, harm to non-target insects, and development of resistance in target vectors [2]. Moreover, eradication of adult mosquitoes using adulticides has not been successful, as adults can easily escape the area being treated [3] and such treatments only result in a temporary reduction of the adult population [4]. Use of larvicides, on the other hand, is promising because it focuses on the larval stage within breeding sites [5]. When in the aquatic stage, larvae are confined within their aquatic habitats which are readily accessible, thus allowing larvicides to reach their target for effective control [6].
Plant based products offers better solution to evergrowing problem created by synthethic insecticides [7]. Unlike chemical larvicide which is based on a single active ingredient, botanical derived larvicide comprises of multiple active compounds which act concertedly. Therefore leaving little chance for the target insects to develop resistance [8].
Plants contain numerous chemicals, some of which are known for their medicinal and pesticidal properties [9]. Thus, in recent years, plant-based insecticides have become more and more popular. Unlike synthetic chemical insecticides, which have an adverse effects on the environment, phytochemical compounds are relatively safe [10]. There are more than 2000 plant species identified for producing secondary metabolites which is valuable in biological pest control and from this number, 344 plant species have been reported to display mosquitocidal activities [11].
In Malaysia, numerous studies on indigenous plants have been conducted to evaluate their mosquitocidal activities. For example, Rohani *et al.* [12] have studied the potential adulticidal activities of essential oils from *Litsea elliptica* (medang), *Polygonum minus* (pygmy smartweed), and *Acorus calamus* (sweet flag), and Jantan *et al.* [13] reported that essential oils from *L. elliptica*, *Piper aduncum* (spiked pepper), and *P. minus* exhibited larvicidal activities. Similarly, Hidayatulfathi *et al*. [14] found that hexane, chloroform, ethyl acetate and methanol extract of *A. calamus* displayed larvicidal effects against 4th instar *Ae. aegypti* larvae.

Yousaf and Zuharah [15] documented larvicidal acitivities of leaves and bark of *Melanochyla fasciculiflora*, *Gluta renghas* (rengas), *Anacardium occidentale* (cashew tree) and *Mangifera indica* (mango). Toxic effect of *Cyperus aromaticus* against *Aedes aegypti* and *Aedes albopictus* larvae was studied by Kamiabi *et al*. in 2015 [16]. Likewise, Ahbirahmi *et al*. [17] investigated bio-efficacy of leaf, flower and stem of *Ipomoea cairica* (railway creeper) against wild strain *Ae. aegypti* and *Ae. albopictus*

Citrus species which is well known for its economically important fruit has been identified for its larvicidal activities. The potential use of extracts from *Citrus* waste such as peel and seed as a mosquito larvicide has been studied previously. Essential oil of *Citrus aurantium* (sour orange), *Citrus grandis* (pomelo), *Citrus aurantifolia* (key lime) [18], *Citrus limon* (lemon), *Citrus sinensis* (red blood orange), *Citrus paradisi* (grapefruit), *Citrus reticulate* (mandarin orange) [19], *Citrus mitis* (calamondin), *Citrus jambhiri* (rough lemon) [20] and *Citrus limetta* (sweet lime) [21] waste were tested against dengue vectors. By using different extraction method and part of the plant, toxicity of *C. grandis* fruit peel [18, 20, 22, 23] and seed [19] has been tested against *Aedes aegypti* mosquitoes.

Mosquito larvae are normally found to co-exist with other organisms in nature. Insects such as psophora (*Toxorhynchites sp*.), cylcopoid copepods (*Mesocyclops*), odonate larvae (dragonfly and damselfly larvae) and larvivorous fishes (*Gambusia sp*. and *Poecilia sp*.) can be found to co-habit with mosquito larvae. These insects are indigenous predators which are also known as natural biological control agents [1, 24]. As efficient biological agents, this predators need to be protected from deleterious effects of larvicides [6].

With the growing environmental concern and regular dengue outbreak in Malaysia, the present study has been conducted to find a better option replacing synthethic larvicide. As the focus of other researchers are primarily on essential oil and on a selected extract of *C. grandis* fruit peel, this study was designed to investigate the toxicity of various extracts and fractions beside essential oil against late third instar larvae of *Aedes aegypti* (Linn.) under laboratory conditions. In addition, this study also evaluate the effect of the most potent extract on 2 non-target organism.

2. Materials and methods

2.1 Plant collection, extraction and isolation of compound

White flesh pomelo or *Citrus grandis* fruits were purchased from trusted vendor in Taiping, Perak. The fruits were brought to Herbal Processing Lab, Integrative Medicine Cluster, Advanced Medical and Dental Institute, USM for processing. A voucher of specimen (Referral number: MANO 2013-01) was deposited in the Herbarium Unit, Forest Research Institute Malaysia, Selangor, Malaysia. The fruit peels of white flesh pomelo has been processed, extracted (crude methanol extract, hexane fraction, ethyl acetate fraction, queous fraction and crude water and essential oil) and its phyto-compound isolated as described in Manorenjitha *et al*. [25].

2.2 Preparation of laboratory strain *Ae. aegypti* larvae

Eggs of *Aedes aegypti* were provided by the Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia. Eggs were vacuum hatched to ensure uniformity of larval age. Emerging larvae were reared at uniform density (300 larvae per enamel tray) in dechlorinated water. Larval food consisting of dog biscuit, beef liver, yeast, and milk powder in the ratio of 2:1:1:1 by weight was prepared as a fine powder and given to the *Ae. aegypti* larvae. The larvae were maintained under laboratory conditions (temperature: 26 ± 4º C, relative humidity: 70–80%, photoperiod cycles: 14 light: 10 dark). Late third instar larvae were used in this study.

2.3 Preparation of plant stock solution

Plant stock solutions was prepared by diluting one gram of crude methanol extract, ethyl acetate fraction and 0.2 g of hexane fraction in 10 ml of acetone while aqueous fraction and crude water extract was dissolved in 100 ml of distilled water. Similarly, 1 ml of essential oil was dissolved in 100 ml of acetone (stock solution). From the stock solution, serial dilution was prepared for each extract, fraction and oil.

2.4 Dose response study

Larvicidal activity (preliminary testing, dose response study and larval bioassay of naringin) was assessed following procedures described by WHO [26]. Six concentrations were tested against twenty late third instar *Aedes aegypti* larvae to determine the efficacies of each extract, and each test was replicated six times. Concentration tested: crude methanol extract (500, 750, 1000, 1250, 1500, 1750 ppm), hexane fraction (3.125, 6.25, 12.5, 25, 50, 75 ppm), ethyl acetate fraction (125, 250, 375, 500, 625, 750 ppm), aqueous fraction (500, 750, 1000, 1250, 1500, 1750 ppm), crude water extract (500, 750, 1000, 1250, 1500, 1750 ppm), essential oil (25, 30, 35, 40, 45, 50 ppm) and naringin, standard and isolated compound (10, 50, 100, 500, 1000, 1500 ppm). The control group was treated with 1 ml of acetone and distilled water. Bioassays were conducted under laboratory conditions to determine the LC_{50} and LC_{90} values at 24 and 48 h post-treatment. Numbers of dead larvae were counted at 24 h and 48 h post-treatment.

2.5 Toxicity of plant extract on non-target organisms

The effect of the hexane fraction of *Citrus grandis* fruit peel was tested against non-target mosquito predators, *Toxorhynchites splendens* (mosquito predator) and *Poecilia reticulata* (predatory fishes). *Toxorhynchites splendens* larvae were obtained from the Vector Control Research Unit, Universiti Sains Malaysia, whereas other predator species were purchased from an aquatic pet shop.

Susceptibility of *Tx. splendens* larvae to the *C. grandis* fruit peel fraction was conducted following the methods by Lek-Uthai *et al*. [27] with some modifications. The first and fourth instar larvae of *Tx. splendens* were used in this study. Individual mosquito larva of the selected instar was introduced into the paper cup and acclimatized for a few hours before introducing the fruit peel fraction. After calculation, the amount of hexane fraction which gave test solutions for LC_{50} and LC_{90} were introduced into the cups to a final volume of 100 ml. Control cups were prepared using acetone as the final concentration. The test was conducted in triplicate and mortality was observed at 24 and 48 hours post-exposure.

The method using *Poecilia reticulata* as non-target organism was adopted from Patil *et al*., [28]. *P. reticulata* was acclimatized to the laboratory condition for about 5 days. Assessment of toxicity was carried out at LC_{50} and LC_{90}

value. Thirty healthy *P. reticulata* fish were placed in a rectangular, glass aquarium containing 400 ml of dechlorinated water and fruit peel hexane fraction in 3 replicates. At the same time a control group consisting of 30 fish in dechlorinated water with acetone was also set up. Mortality was recorded at 24 and 48 hours post-exposure.

All experiments were conducted in a controlled laboratory condition (temperature: 27 ± 3ºC and relative humidity: 75 ± 10 %) without aeration or renewal of water. The predators were observed for mortality and other abnormalities such as sluggishness and reduced swimming activity after 24 hours of exposure. The exposed predators were also observed continuously for ten days to understand the post treatment effects of this extract on survival and swimming activity.

2.6 Statistical analysis

The extract concentration at which 50% of the population (LC_{50}) and 90% of the population (LC_{90}) was killed, the regression equation, the 95% upper confidence limit (UCL) and lower confidence limit (LCL), and chi square values were calculated using computer software programme called SPSS analysis (Statistical Package for the Social Sciences) version 20.0. Lethal concentration (LC) values were considered to be significantly different from each other at $P < 0.05$ if confidence intervals did not overlap.

For non-target organism, the results was expressed as mean ± S.E (Standard Error). The data obtained was analysed for normality test. When appeared not to be normally distributed, the data was subjected to a non-parametric analysis using SPSS version 20.

3. Results and Discussion

3.1 Larval bioassay of *C. grandis* fruit peel extracts tested against *Ae. aegypti* larvae

In the present study, organic solvent extract and essential oil was tested to determine the efficacy of *C. grandis* fruit peel against the dengue vector. Among the six extracts tested, crude methanol extract, ethyl acetate fraction, hexane fraction and essential oil exhibited larvicidal activity against late third instar *Ae. aegypti* larvae. However, larvicidal activity of hexane fraction was found to be superior followed closely by essential oil.

The larvicidal effects of *C. grandis* peel extracts against *Ae. aegypti* larvae depended on the concentration of the extract. Tables 1 and 2 show the larvicidal activity of the *C. grandis* extracts at 24 h and 48 h post-treatment, respectively. The hexane fraction, which caused 50% larval mortality at 17.98 ppm, was the most effective among the extracts tested. This was followed closely by the essential oil extract, which induced 50% larval mortality at 29.43 ppm. The ethyl acetate fraction and crude methanol extract were the least effective, with LC_{50} values of 203.87 and 981.06 ppm, respectively. No larvicidal effect was detected at 1000 ppm for the aqueous and water extracts.

This is the third available report that documents the potency of *C. grandis* fruit peel as a larvicide. The first available document on larvicidal activity of *C. grandis* fruit peel was by Vu *et al*. in 1993 [29]. They found that the essential oil of the fruit peel was effective against *Culex quinquefasciatus*, *Culex tritaeniorhynchus* and *Ae. aegypti* at an LC_{50} value of 0.02 ml/L. In a recent study, Torres *et al*. [23] reported that crude hexane extract of *C. grandis* fruit peel from Davao, Philippines has the lowest lethal concentration (LC_{50} = 1.11 ppm) compared to fruit peel from other localities when tested against 3rd and 4th instar *Ae. aegypti* larvae. This result is noticeably lower than the present study. In some studies, the toxicity of *C. grandis* fruit peel was found to be inferior when compared to other citrus species. For example, Akram *et al*. [20] reported that essential oils of *Citrus jambhiri* (rough lemon) and *Citrus limon* (lemon) were most effective against the fourth instar larvae of *Aedes albopictus* at a lethal concentration (LC_{50}) of 119.993 ppm and 137.258 ppm, respectively. In contrast, essential oil *C. grandis* gave a slightly higher LC_{50} value (334.874 ppm). Similarly, Din *et al*. [19] found that essential oil of *C. limon* was the most toxic among all citrus fruit peels and seeds. At 24 hours of exposure, the LC_{50} value of the essential oil of *C. limon* was 468.69 ppm for fruit peel and 395.59 ppm for seeds while *C. grandis* fruit peel (605.62 ppm) and seeds (446.24 ppm) registered higher LC_{50} values when tested against fourth instar larvae of *Ae. albopictus*. In addition, Astarini *et al*. [18] reported that the essential oil from the *C. grandis* fruit peel was less effective with an LC_{50} value of 955.64 ppm compared to *C. aurantium* with an LC_{50} value of 299.95 ppm when tested against third instar *Ae. aegypti* larvae.

The varying larvicidal activities of *C. grandis* fruit peel extracts between the present study and those by the other authors [18-20, 23, 29] may be due to the types of soil [9], geographic and seasonal variation, method of extraction [30, 31], age of the plant [9, 31] plant parts used as well as the solvent used during extraction which influenced the concentration of plant bioactive components [9].

Several researchers reported that different solvent extracts derived from several plants parts exhibited larvicidal activity [32]. Lija-Escaline *et al*. [33] reported that exposure to *Piper nigrum* (black pepper) caused more than 90% death in fourth instar larvae of *Ae. aegypti* with an LC_{50} value of 32.23 ppm. Similarly, Liu *et al*. [34] found that early fourth instar *Aedes albopictus* larvae were susceptible to essential oil of *Tetradium glabrifolium* (bee bee tree) fruits at an LC_{50} values of 8.2 µg/ml. Recently, Mallick *et al*. [32] found that acetone extract of *Annona reticulata* (custard apple) leaves proved its larvicidal activity when tested against different larval stages (1st, 2nd, 3rd and 4th instar larvae) of *Anopheles stephensi* (LC_{50} values between: 3.56-8.57 ppm), *Culex quinquefasciatus* (LC_{50} values between: 1.42 – 4.94 ppm) and *Ae. aegypti* (LC_{50} values between: 5.89 – 16.69 ppm) after 24 hours exposure. Likewise, the toxicity of *Blumea mollis* (nari pachha), *Chloroxylon swietenia* (Ceylon satinwood), *Clausena anisata* (kattu-veppilai), *Feronia limnonia* (wood apple), *Lantana camara* (big-sage), *Plectranthus amboinicus* (Indian mint) and *Tagetes erecta* (Mexican marigold) were evaluated by Jayaraman *et al*. [35] against *Cx. quinquefasciatus*, *Ae. aegypti* and *Anopheles stephensi* larvae. They reported that ethyl acetate extract of *Chloroxylon swietenia* displayed remarkable larvicidal activity against 4th instar larvae of *Cx. quinquefasciatus* ($LC_{50:}$ 12 hrs= 94.12 ppm, 24 hrs= 194.22 ppm), *Ae. aegypti* ($LC_{50:}$ 12 hrs= 80.58 ppm, 24 hrs= 173.04 ppm) and *An. stephensi* ($LC_{50:}$ 12 hrs= 76.24 ppm, 24 hrs= 167.28 ppm) compared to other plant species. In another study, Sharma *et al*. [36] investigated the larvicidal activity of leaf and stem of 5 plant species namely, *Achyranthes aspera* (prickly chaff flower), *Cassia occidentalis* (coffee weed), *Catharanthus roseus* (periwinkle), *Lantana camara*,

Xanthium stumarium (common cocklebur) against early 4th instar *Ae. aegypti* larvae. Hexane extract of leaves and stems of the five plant species were showed potent larvicidal properties, and hexane exract of *A. aspera* stem was found to be the most potent with an LC_{50} value 68.133 ppm after 24 hours exposure. Mallick *et al*. [21] observed larvicidal activity of *C. limetta* fruit peel extracts extracted using different organic solvent and tested against 3rd larval instar of *Cx. quinquefasciatus*. They documented the lowest LC_{50} value for n-hexane extract (661.27 ppm), while methanol (939.43 ppm) and ethyl acetate (1268.67 ppm) extracts exhibited higher lethal concentration. Toxicity effect was also evaluated by Musau *et al*. [37] for six plant species (Tagetus minuta (wild marigold), *Adansonia digitata* (baobab tree), *Ocimum suave* (clove basil), *Plecthranthus barbatus* (Indian coleus), *Azadirachta indica* (neem) and *Lantana camara*) obtained from the district of Msambweni, Kenya. They concluded that aqueous, acetone and hexane extracts of the six plants (with test concentration range: 1, 0.5, 0.25 and 0.125%) were toxic against 4th instar larvae of *Ae. aegypti*. On top of that, acetone extract of *T. minuta* and hexane extract of *O. suave* were the most toxic as they produced 100% larval mortality in all test concentrations.

In an earlier study, GC-MS analysis of crude methanol extract, ethyl acetate fraction, hexane fraction and essential oil of *C. grandis* fruit peel revealed the presence of hundreds of phtyochemical compounds. The presence of several compounds which is known for its larvicidal activity such as hexadecanoic acid (or palmitic acid) and 9,12-octadecadienoic acid (Z,Z) (or linoleic acid) was observed in hexane fraction, ethyl acetate and essential oil, respectively. GC-MS analysis of the hexane fraction that caused the highest mortality in test larvae population revealed the presence of hexadecanoic acid, which was found to be a major bioactive compound in the fraction [25].

This finding is in agreement with a few authors, who reported the larvicidal potential of hexadecanoic and linoleic acid acid against vector mosquitoes. Isolated fatty acids from fruit of *Solanum lycocarpum* (wolf apple) were found to display larvicidal activity against 3rd and 4th instar *Cx. Quinquefasciatus* larvae [38]. Unripe fruit with large amount of hexadecanoic acid (41.5%) and ripe fruit with linoleic acid (75.5%) were found to be the most potent larvicide with the lowest LC_{50} value (between 0.70-1.33 ppm). Similarly, the GC-MS study on ethanolic leaf extract of *Heliotropium indicum* (scorpion weed) detected the presence of 9,12-octadecadienoic acid (Z,Z) while in *Mukia maderaspatana* (bristly bryony) confirmed the presence of 9,12-octadecadienoic acid (Z,Z) and hexadecanoic acid. Both extracts were found to be highly toxic towards 4th instar *Ae. aegypti* larvae [39]. In another study, isolated compound of palmitic acid from *Feronia limonia* (wood apple) leaves extract were found to be effective larvicide against *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* with LC_{50} values of 57.23, 79.58 and 129.24 ppm, respectively [40]. Likewise, Naik *et al*. [41] reported that synthesized silver nanoparticles of *Pongamia pinnata* (pongam oiltree) leaves showed 2 prominent compounds namely 9-octadecadienoic acid (Z) and n-hexadecanoic acid. They assumed that both compounds are responsible for the larvicidal activity of *P. pinnata* against *Ae. aegypti* larvae with LC_{50} values of 0.25 ppm for synthesized nanoparticles. Ravaomanarivo *et al*. [42] also discovered presence of various fatty acids including palmitic acid and linoleic acid in the oil extract of *Annona squamosa* (sweetsops) and *Annona muricata* (soursop) seeds. However, *A. muricata* revealed more fatty acids compounds compared to *A. squamosa* and the differences in number of compounds is reflected on their larvicidal performance. Extracts (aqueous, ethanol and dichloromethane) of *A. muricata* was found to induce high mortality rate in *Ae. aegypti* and *Cx. quinquefasciatus* larvae compared to *A. squamosa* at all concentrations tested (concentration range: 0.5%, 1% and 2%).

The second most potent extract in the present study was the essential oil. Plant secondary metabolites found in essential oil of *C. grandis* fruit peel namely limonene was the major bioactive compound [24]. This was expected as limonene was reportedly found in abundance in citrus fruit peels [18, 43-45]. Several studies have demonstrated the potential of limonene against vector mosquitoes. Larvicidal activity of pure constituents extracted from *Mentha spicata* or spear mint (carvone, cis-carveol and limonene) was recorded for *Anopheles stephensi* (LC_{50} values: 19.33, 28.50, 8.83 ppm), *Aedes aegypti* (LC*50* values: 23.69, 32.88, 12.01 ppm) and *Culex quinquefasciatus* (LC_{50} values: 25.47, 35.20, 14.07 ppm) larvae by Govindarajan *et al*. [46]. They noted that limonene appeared to be most potent against the three vector mosquitoes. Sedaghat *et al*. [47] reported the larvicidal potency of *Cupressus arizonica* (Arizona cypress) leaves against *An. stephensi* at LC50 of 79.30 ppm. They also reported that limonene was the major component (14.44%) of the essential oil. Rocha *et al*. [48] documented toxicity effect of essential oil from two different cultivar of *Foeniculum vulgare* (fennel) against 3rd instar *Ae. aegypti* larvae with LC_{50} values of 23.3 µL/L (Cape Verde cultivar) and 28.2 µL/L (Portugal cultivar). They assumed that the presence of limonene, trans-anethole and estragole in the essential oil of the aerial parts exhibited strong larvicidal activity against the dengue vector larvae. In another study, essential oils of *C. aurantium* and *C. paradisi* fruit peels with 94.81% of limonene as the main constituent, displayed larvicidal effect against *An. stephensi* at LC_{50} values 31.20 ppm and 35.71 ppm, respectively [49].

Table 1: Dose response of *Ae. aegypti* larvae against six *C. grandis* extracts, fraction and oil at 24 hrs post treatment

Extracts/ Fractions/ Oil	LC_{50} (ppm) (95% Confidence limit)	LC_{90} (ppm) (95% Confidence limit)	Slope ± SE	Chi square (X^2)
Hexane	17.98[a] (13.17-26.17)	87.16 (50.80-246.27)	1.87 ± 0.15	5.656
Ethyl Acetate	203.87[b] (175.45-229.88)	713.13 (601.03-903.90)	2.36 ± 0.23	3.772
Methanol	981.06[c] (465.40-2466.05)	1776.56 (1211.92-2674273.46)	4.97 ± 0.38	42.654
Aqueous*	-	-		
Water*	-	-		
Essential Oil	29.43[a] (26.40-31.71)	43.75 (40.07-50.59)	7.45 ± 0.58	8.895

LC_{50} values will be considered to be significantly different ($P \leq 0.05$) from each other if the confidence intervals did not overlap. n=6.
*Testing were discontinued as no mortality were recorded at 1000 ppm

Table 2: Dose response of *Ae. aegypti* larvae against six *C. grandis* extracts, fraction and oil at 48 hrs post treatment

Extracts/ Fractions/ Oil	LC_{50} (ppm) (95% Confidence limit)	LC_{90} (ppm) (95% Confidence limit)	Slope ± SE	Chi square (X^2)
Hexane	10.19[a] (8.84-11.84)	45.50 (34.08-68.70)	1.97 ± 0.19	1.719
Ethyl Acetate	120.13[b] (0.38-197.69)	478.64 (292.77-122211.93)	2.14 ± 0.29	4.598
Methanol	774.20[c] (530.36-971.57)	1445.23 (1114.56-3249.99)	4.73 ± 0.37	17.730
Aqueous*	-	-		
Water*	-	-		
Essential Oil	25.23[a] (10.20-29.09)	36.91 (31.95-94.89)	7.75 ± 0.92	5.191

LC_{50} values will be considered to be significantly different ($P \leq 0.05$) from each other if the confidence intervals did not overlap. n=6.
*Testing were discontinued as no mortality were recorded at 1000 ppm

3.2 Larval bioassay of naringin tested against *Ae. aegypti* larvae

Another focus of the present study was the isolated compound from ethyl acetate fraction. Naringin, isolated from the *C.grandis* fruit peels [25] and standard compound purchased from SIGMA displayed toxic effect against 4th instar *Ae. aegypti* larvae. However, the isolated compound was found to be more potent towards *Ae. aegypti* larvae compared to the standard compound (Table 3). To the best of my knowledge, only one larvicidal activity of naringin (isolated compound) has been studied against vector mosquitoes. The only available document evaluated the toxic effect of flavonoid compound extracted from *Poncirus trifoliate* (Japanese or Chinese bitter orange) such as naringin, poncirin, rhoifolin and marmesin [50]. The report stated that naringin was able to control 50% of the 4th instar *Ae. aegypti* larvae at 0.122 ppm after 24 hours exposure. However, it is considered least potent when compared to other flavonoid compounds.

Based on the current observation, the larvicidal performance of plant extract depends on the extraction method and solvent used. The extraction method used in this study (serial exhaustive extraction) allowed the phytochemical compounds present within the plant material to be solubilised. This method ensured that the solvents diffused into the plant material and solubilised compounds according to the polarity of the solvent used [51]. Different solvent types can significantly affect the potency of extracted plant compounds [52] because of the polarity range [53].

Table 3: Dose response of *Ae. aegypti* larvae against naringin, standard and isolated compound of *C. grandis* extracts at 24 and 48 hrs post treatment

Naringin	LC_{50} (ppm) (95% Confidence limit)	LC_{90} (ppm) (95% Confidence limit)	Slope ± SE	Chi Square (X^2)
24 hours				
Standard Compound	691.07[b] (493.99-1099.50)	5070.17 (2594.05-16432.99)	1.48 ± 0.22	1.384
Isolated compound	115.87[a] (31.29-392.59)	2056.08 (534.41-358421.54)	1.03 ± 0.13	7.382
48 hours				
Standard Compound	263.74[b] (48.38-2599.22)	1618.62 (499.99-445619873.4)	1.63 ± 0.20	5.146
Isolated compound	74.71[a] (49.53-114.02)	1125.34 (539.17-4140.29)	1.09 ± 0.17	0.951

LC_{50} values will be considered to be significantly different ($P \leq 0.05$) from each other if the confidence intervals did not overlap. n=6.

3.3 Non-target organism

The bio-efficacy of hexane fraction of *Citrus grandis* fruit peel on *Ae. aegypti* larvae was re-evaluated on 2 non-target insects which was commonly found co-habiting with the dengue vector. The result is shown in Table 4. It was found that among the 2 target species, first instar *Tx splendens* seemed to be the most susceptible with 100% mortality for both LC_{50} and LC_{90} dose. The fourth instar *Tx splendens* showed resistance at LC_{90} with average mortaliy of 20.0% at 24 hours and 26.7% at 48 hours post exposure. *Poecilia reticulata* showed tolerance at LC_{50} concentration while at the higher dose (LC_{90}) average mortality of 73.3% and 75.6% was recorded at 24 and 48 hours post exposure, respectively.

In recent years, concern on adverse effects of larvicides on non-target organisms that breed in the same habitat as vector mosquitoes have initiated extensive investigation [27, 54, 55]. Moderate information is available about low dosages of larvicides that may have impact the aquatic organisms that share the same niche of mosquitoes such as their natural enemies [55].

In this study, sub-lethal dose of hexane fraction of *Citrus grandis* fruit peel proved to be safer to *Poecilia reticulata* as it showed no toxic effects at LC_{50}. However, an average death of more than 70% was recorded for *P. reticulata* when exposed to higher dose (LC_{90}). As for *Toxorhynchites* splendens larvae, the hexane fraction of *C. grandis* fruit peel appeared to be toxic to the first instar larvae compared to the fourth instar larvae. The first instar *Tx.splendens* larvae registered 100% mortality for sub-lethal concentration (LC_{50}) at 24 hours exposure while the fourth instar larvae treated with sub-lethal dose registered less than 50% mortality at 24 hours and 48 hours. It showed that the fourth instar *Tx. splendens* larvae were tolerant to the toxicity of the hexane fraction of the fruit peel.

Several studies have reported the safe use of botanically derived larvicide on larvivorous fish, *P. reticulata* (guppy fish). For instance, out of 14 plant extract studied by Promsiri *et al.* [56] only five plant extracts (*Anethum graveolens* (dill weed), *Cinnamomum porrectum* (safrol laurel), *Phyllanthus pulcher* (phyllanthus weed), *Anacardium occidentale* (cashew nut tree) and *Annona muricata* (soursop)) were recorded as having no adverse effect on *P. reticulata* at LC_{50} value of 12.5 µg/ml, 50.0 µg/ml, 12.5 µg/ml, 6.3 µg/ml and 50.0 µg/ml, respectively. On the other hand, *Mammea siamensis* (sarapi, a Thai medicinal plant) displayed slight toxicity at the sub-lethal dose of 3.2 µg/ml with 10 % mortality recorded for *P. reticulata*. Likewise, a study on methanolic extract of *Atlantia monophylla* (wild lime) found that the extract is non-toxic against non-target organism such as *P. reticulata*, *Gambusia affinis* and *Diplnychus indicus* at maximum concentration of 5 mg/l [57]. In 2012, Patil *et al.* [58] also found no toxic effect induced by silver nanoparticles synthesized using *Pergularia*

daemia (trellis-vine) latex at concentration value obtained from larval bioassay against fourth instar of *Ae. aegypti* (LC_{50}= 6.18 ppm; LC_{90}= 12.95 ppm) and *An. stephensi* (LC_{50}= 6.47 ppm; LC_{90}= 14.08) when tested on *P. reticulata*. Hence, they suggested the use of *P. daemia* plant latex extract as well as synthesized silver nanoparticles of the latex as part of an integrated vector control against *Aedes aegypti* and *Anopheles stephensi* in the presence of *P. reticulata*. Similarly, Anogwih *et al.* [6] reported that Spinosad, a product derived from the fermentation of soil bacterium (*Saccharopolyspora spinose*) is compatible with *P. reticulate* at concentration not greater than 49µgL^{-1}. In a recent study by Alshebly *et al.* [59], *P. reticulata* was found to be tolerant towards sublethal dose of the essential oil of *Hedychium larsenii* (white garland lily) and its isolated compound (ar-curcumene and epi-β-bisabolol) with LC_{50} values higher than 1500 ppm.

Behavioural alteration was observed in the 1st instar *Tx. splendens* larvae treated with sub-lethal dose of hexane extract of *C. grandis* fruit peel. Larvae exposed to the extract increased its swimming activity and movements to the water surface. Changes in colour or darkening of *Tx. splendens* were visibly noted after the introduction of the extract in to the testing cup. However, minimal behavioural changes were observed in the 4th instar *Tx. splendens* larvae. The sensitivity of the test invertebrates was found to differ according to the life stage and sex [60].

The present study is in agreement with a study by Xue *et al.* [60]. They noted that 1st and 4th instar of *Taxorhynchites amboinensis* produced mixed results. They found that, the 1st instar larvae of *Tx. amboinensis* sensitive towards 3 types of mosquito repellents in liquid form at concentration ranging from 0.1%, 0.05%, 0.01%, 0.005% and 0.001% compared to the 4th instar larvae. They believed that differences in the findings were probably due to the smaller size of the 1st instar *Tx. splendens* larvae compared to the 4th instar larvae. In contrast to the present study, Anjali *et al.* [61] reported that crude and chloroform-methanol extracts of *Polianthes tuberosa* (tuberose) bud can be applied in aquatic body inhabited by *Culex quinquefasciatus* as it is safe to *Toxorhynchites* larvae which co-habitat the breeding habitat. Interestingly, the same observation was also reported for *Toxorhynchites* larvae after exposed to 1.26 ppm (sub-lethal concentration, LC_{50}) of synthesized silver nanoparticles of *Solanum nigrum* (green berry) fresh leaves which have exhibited 50% mortality to *Cx. quinquefasciatus* larvae [62]. Similarly, sub-lethal concentration (LC_{50}) of crude extract (0.13%) and ethyl acetate extract (77.03 ppm) of *Drypetes roxburghii* (lucky bean tree) mature fruits which were found to be toxic against 3rd instar *Cx. quinquefasciatus* larvae was reported non-toxic towards *Tx. splendens* larvae [63]. Likewise, lethal concentration of aqueous extract (321.75 ppm) and silver nanoparticles (9.65 ppm) of *Berberis tinctoria* (nilgiri barberry) leaf which killed 50% of 4th instar *Aedes albopictus* larvae were found to be safe against *Tx. splendens* larvae and *Mesocyclops thermocyclopoides* (copepod) larvae [64].

Table 4: Effect of LC_{50} and LC_{90} hexane fraction of *C. grandis* fruit peel against *P. reticulata* and *Tx. splendens*

Species	Control	Average mortality ± SE (%)	
		LC_{50}	LC_{90}
24 hours			
P. reticulata Tx. splendens	0	0d	73.3 ± 1.2c
(First instar)	0	100a	100a
(Fourth instar)	0	20.0 ± 3.7c	100a
48 hours			
P. reticulata Tx. splendens	0	0d	75.6 ± 0.9b
(First instar)	0	100a	100a
(Fourth instar)	0	26.7 ± 2.1b	100a

Mean percentage of mortality followed by the same letters within the same columns are not significantly different (P>0.05, non parametric Mann-Whitney U Test). n=3.

4. Conclusion

In conclusion, the present study has demonstrated the larvicidal activity of *C. grandis* fruit peel extracts against the late third instar *Ae. aegypti* larvae. It seems that the presence of high amount of hexadecanoic acid in hexane extract and limonene in essential oil of white flesh *C. grandis* fruit peel provides the best toxic effect on *Ae. aegypti* larvae. Futhernore, the present study indicated that hexane extract of *Citrus grandis* fruit peel extract can be applied at concentration lower than 17.98 ppm in aquatic body infested with *Aedes aegypti* larvae and co-inhabited by *Poecilia reticulata* and *Toxorhynchites splendens* larvae. At a lower concentration the *C. grandis* fruit peel may be applied as part of an integrated vector control programme for an efficient control of dengue vector.

5. Acknowledegment

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