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Toxicity of *Citrus aurantifolia* and *Citrus hystrix* against *Aedes albopictus* larvae

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Abstract

Dengue is a major threat for humans. Since 1970's dengue cases was found to be increasing in terms of frequency and intensity. Thus, in this study fruits from the *Citrus* species were used to evaluate the larvicidal activity against the 3rd instar of *Aedes albopictus*. Bioassay was carried out using the ethanolic extract of pulps of *C. aurantifolia* and *C. hystrix*. *C. aurantifolia* exhibits higher larvicidal activity. *C. aurantifolia* caused 66.70% of mortality at the concentration of 3.75mg/ml over 24 hours period of time whereas, *C. hystrix* caused the percentage of mortality of 56.70% at the concentration of 3.75mg/ml over 24 hours. Moreover, both the botanical extract exhibit similar outcomes as the synthetics at the concentration of 3.75mg/ml over 72 hours period. The median lethal concentration (LC_{50}) exhibited by *C. aurantifolia* was 2.95mg/ml and *C. hystrix* was 3.01mg/ml respectively. In nut shell, *C. aurantifolia* and *C. hystrix* was found to have larvicidal activity and thus, this fruits have potential to be develop as a larvicidal agent. Further study needed to identify the active compound that caused the toxicity and to do carry out experiment on field condition.

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Introduction

Dengue is an acute mosquito-borne viral infection that causes significant socioeconomic and disease burden on many tropical and subtropical regions all around the world (Gubler, 2011). The Dengue fever cases was found to increase every year in Malaysia and the government is spending millions of Ringgit for curing the patients, controlling the spreading via variety of measures and giving more attention to the research and development (Shepard *et al*., 2013). In Malaysia both *Aedes aegypti* and *Aedes albopictus* were widely distributed (Ishak 2014). Thus both Aedes play an important role of transmitting the infections to the communities.

This work was mainly done to determine the effectiveness of *Citrus aurantifolia* and *Citrus hystrix* individually and with combination towards the *Aedes albopictus* larvae by using ethanol as a solvent for extraction

Citrus aurantifolia also known as Key lime was believed from South East Asia around 4000 BC and its belongs to Indo-Malayan region (Enejoh *et al.,* 2015). *Citrus aurantifolia* have shown anticancer and radical scavenging activity (Poulose *et al*., 2005; Jayaprakasha *et al*., 2008), anti-microbial activity against *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella* spp (Oikeh *et al*., 2016). Besides that, *C. aurantifolia* leaf shows antifungal activity against *Aspergillusniger*, *Aspergillus fumigates*, *Mucor* spp and *Pencillium* (Pathan *et al*., 2012). *Citrus aurantifolia* were found to have insecticidal and repellant activity against *Camponotus nearcticus (*Mensah *et al*., 2014*). Citrus aurantifolia* shows repellant activity against mosquitoes (Effiom *et al*., 2012). *Citrus aurantifolia* were found to be effective against mosquito, cockroach and housefly in an aerosolic form (Ezeonu *et al*., 2001).

The leaf and Peel of *C. aurantifolia* were effective against the larvae of *Culex quinquefasciatus* and *Aedesalbopictus* (Nath *et al*., 2006).

Citrus hystrix commonly known as in the name of Limaupurut in Malay and Jerukpurut in Indonesia belongs to the family of Rutaceae family and this plant able to grow in the house garden (Nor, 1999). *Citrus hystrix* poses cardioprotective and hepatoprotective activity (Herwandhani *et al*., 2013; Abirami *et al*, 2015), anti-cancer properties (Manosroi *et al*., 2006), anti-helminthic activities (Onyilofe *et al*., 2014), anticholine esterase activity (Youkwan *et al*., 2010) and antioxidant activity (Aziman *et al*., 2012). *Citrus hystrix* were found to be effective as repellant agent against cockroaches (Thavara *et al*., 2007). Furthermore, the essential oil of the *C. hystrix* leaves were found to be effective against *Spodoptera litura* (Loh, *et al*., 2011). Moreover, *C. hystrix* were found to provide repellant activity against *Stitophilus oryzae* (Buatone and Indrapichate, 2011) and the leaves of *C. hystrix* were found to be effective against 3rd and 4th instar of *Aedes aegypti* larvae (Mya *et al*., 2015).

Materials and methods

Collection of fruits

Citrus aurantifolia and *Citrus hystrix* were purchased at Kuala Lumpur vegetable market. Both plants were sent for authentication at Institute of Bioscience, University Putra Malaysia Serdang. The plant authentication was done by Dr. Shamsul Khamis. The voucher number for *C. aurantifolia* is and *C. hystrix* is SK 2522/14 and is SK 2523/14 respectively.

Collection of larvae

Aedes albopictus larvae were collected from the Unit of Entomology, Institute of Medical Research (IMR), Malaysia and the larvae were maintained until the bioassay. The entire studies have been conducted in the Biomedicine laboratory, Asia Metropolitan University.

Extraction of *Citrus aurantifolia*

C. aurantifolia was thoroughly washed with running tap water and the outer skin is removed by using the knife.

The fruits were put into the commercially available juice extractor machine to get the fresh juice and dried for 3 weeks in room temperature.

A paste like consistency has been formed after 3 weeks of drying. 20g of the paste were mixed with 160ml of 60% of ethanol. The mixture was then filtered after 48 hours and the filtrate was dried in hot air oven at 50°C ± 2°C for 3 days. Similar method was used for the extraction of *C. hystrix* pulp (Arivoli and Tennyson, 2012).

Toxicity bioassay

The assay was conducted with a slight modification from Subramaniam *et al.*, (2012). Plastic cup was filled with 190ml of distilled water and 10 ml of the *C. aurantifolia* pulp extract. The concentration that was used in this study was 1.25mg/ml, 2.50mg/ml and 3.75mg/ml. Ten larvae of 3rd instar of *Aedes albopictus* were pipetted from the collecting jar and introduced into the plastic cups. The test was replicated 3 times. The same method was used for the other concentration. Synthetics; Abate and 1,8-cineole was used as positive controls and ethanol was used as negative control. The mortality was recorded for 24, 48 and 72 hours.

The corrected percentage of mortality was calculated by using Abbott's formula (1925), as shown below. Similar method was used for *C. hystrix* pulp extract.

$$\text{Percentage of corrected mortality} = \frac{\%\text{ of mortality in treated} - \%\text{ of mortality in control}}{100 - \%\text{ of mortality in control}} \times 100$$

Statistical analysis

Paired "*T* test" were performed by using SPSS version 16.0 to obtain the significant values. Probit analysis was done to calculate LC_{50} for the first 24 hours by using the method used by Randhawa (2009).

Results

The mean percentage of mortality of *Aedes albopictus* larvae against the ethanolic extract of *C. aurantifolia* pulp on 24, 48 and 72 hours of observation were summarized in Table 1.After 24 hours of test, the concentration of 1.25mg/ml provide mean mortality of 3.00 ± 0.00 with a percentage of mortality of 30%. The concentration at 2.50mg/ml exhibit larvicidal activity with mean of 3.33 ± 0.58 and with the percentage of mortality of 33.33%, in which it shows an increment of 0.33% as compared to the previous concentration of 1.25mg/ml.

Table 1. Mean mortality and percentage of mortality of *Aedes albopictus* larvae against ethanolic extract of *Citrus aurantifolia* pulp extract.

Concentration	24 h		48 h		72 h	
	Mean mortality	Percentage of mortality	Mean mortality	Percentage of mortality	Mean mortality	Percentage of mortality
1.25 mg/ml	3.00 ± 0.00^{a}	30.00^{a}	4.67±1.15^{a}	46.70^{a}	6.67± 0.58^{a}	66.70^{a}
2.50 mg/ml	3.33 ± 0.58^{a}	33.33^{a}	6.33±0.58^{b}	63.33^{b}	6.67±0.58^{a}	66.70^{a}
3.75 mg/ml	6.67 ± 0.58^{b}	66.70^{b}	8.33 ± 0.58^{c}	83.33^{c}	9.00 ± 1.00^{b}	90.00^{b}
Abate (0.5mg/ml)	10.00 ± 0.00^{c}	100.00^{c}	10.00 ± 0.00^{d}	100.00^{d}	10.00 ± 0.00^{b}	100.00^{b}
1,8-cineole (0.5mg/ml)	10.00 ± 0.00^{c}	100.00^{c}	10.00 ± 0.00^{d}	100.00^{d}	10.00 ± 0.00^{b}	100.00^{b}

Means within the same column followed by the same letter are not significantly different by *t*-test ($P<0.05$).

The maximum concentration that was used in these studies was 3.75mg/ml which shows the mean mortality 6.67 ± 0.58, with the percentage 66.70% and it shows an increment of 33.37% compared to 2.50mg/ml. Even though the synthetics; abate and 1,8- cineole is potent by causing 100% of mortality at the concentration 0.5mg/ml, similar effects can be interpreted from the extract at 3.75mg/ml on the third day (72 hour) with the percentage of mortality of 90%.

The mean and percentage of mortality of *Aedes albopictus* larvae against ethanolic of extract *C. hystrix*pulpextract were summarized in Table 2. At 24 hours the concentration of 1.25 mg/ml shows larvicidal activity with the mean mortality of 2.33 ± 0.58 with the percentage of mortality was 23.33%. Whereas, at the concentration of 2.50mg/ml the mean mortality was 5.00 ± 1.00 and the percentage of mortality were 50.00%, in which it shows an increment of 26.67% compared to 1.25mg/ml. The concentration of 3.75mg/ml shows mean mortality of 5.67 ± 0.58 and percentage of mortality of 56.70% which shows an increment of 6.70% compared to 2.50mg/ml. At 72 hours similar effect to the synthetics can be elucidate at 3.75mg/ml with percentage of mortality 96.70%.

Table 2. Mean mortality and percentage of mortality of *Aedes albopictus* larvae against ethanolic extract of *Citrus hystrix* pulp extract.

Concentration	24 h		48 h		72 h	
	Mean mortality	Percentage of mortality (%)	Mean mortality	Percentage of mortality (%)	Mean mortality	Percentage of mortality (%)
1.25 mg/ml	2.33 ± 0.58^{a}	23.33^{a}	3.67±0.58^{a}	36.70^{a}	7.33±0.58^{a}	73.33^{a}
2.50 mg/ml	5.00±1.00^{b}	50.00^{b}	7.33±0.58^{b}	73.33^{b}	8.67±0.58^{b}	86.70^{b}
3.75 mg/ml	5.67±0.58^{b}	56.70^{b}	8.67±1.53^{b,c}	86.70^{b,c}	9.67±0.58^{c}	96.70^{c}
Abate (0.5mg/ml)	10.00±0.00^{c}	100.00^{c}	10.00±0.00^{c}	100.00^{c}	10.00±0.00^{c}	100.00^{c}
1,8-cineole (0.5mg/ml)	10.00±0.00^{c}	100.00^{c}	10.00±0.00^{c}	100.00^{c}	10.00±0.00^{c}	100.00^{c}

Means within the same column followed by the same letter are not significantly different by *t*-test($P<0.05$).

Citrus aurantifolia pulp extract were found to be more susceptible compared to *Citrus hystrix* pulp extract.

The effective concentration of *C. aurantifolia* extract that kills 50% population of *Aedes albopictus* larvae was 2.95mg/ml (Table 3). On the other hand the LC_{50} for *C. hystrix* was 3.01mg/ml.

Discussion

The results reveal that, ethanolic extract of *C. aurantifolia* pulp has larvicidal activity against *Aedes albopictus,* with a significant *P* value < 0.05. The bioassay shows that at the concentration of 3.75mg/ml the percentage of mortality was 66.70%. In similar research studies the essential oil from the *C. aurantifolia* shows larvicidal activity against 3rd instar of *Culex quinquefasciatus* with a 96% of mortality at the concentration of 1mg/ml (Manimaran *et al.*, 2012).

Table 3. Determination of Median Lethal Concentration LC_{50} of *Citrus aurantifolia* and *Citrus hystrix*.

Plant	LC_{50}	Regression equation	R^2	95% of confidence interval (lower to upper)
C. aurantifolia pulp extract	2.95mg/ml	Y=0.38x + 3.8767	R^2 = 0.8204	1.77-4.13
C. hystrix pulp extract	3.01mg/ml	Y= 0.36x + 3.9133	R^2 = 0.8857	1.77-4.25

The *Cytrixhystrix* peel extract shows percentage of mortality of 56.70% at the concentration of 3.75mg/ml and in a similar study the ethanolic extract of *C. hystrix*leaves shows that at the concentration of 12mg/ml the mortality of *Aedes aegypti* larvae was 2% on 3rd and 4th instars (Mya *et al.*, 2015).

From our study, the LC_{50} of both fruits was 2.95mg/ml for *C. aurantifolia* and 3.01mg/ml for *C. hystrix*. Furthermore, the LC_{50} of the essential oil extracted from the seed of *Citrus aurantium* against *Aedes albopictus* larvae of 4th instar was 0.905mg/ml (905.96 ppm) and *Citrus lemon* shows LC_{50} of 0.137

mg/ml (137.258ppm) at 24 hours (Akram *et al.*, 2010). The essential oil from the seed of *Citrus sinensis* (Valencia late) and *Citrus reticulate* (Feutrall early). Valencia late shows LC_{50} of 0.36mg/ml (368ppm) and Feutrall early shows LC_{50} of 0.5647 mg/ml (564.7ppm) at 24 hours against the 3rd instar of *Aedes albopictus* (Bilal *et al.*,2012). Upon comparing our study with similar studies above, it can be elucidate that the essential oil from the seed probably have the major active constituent that responsible for the lethal activity toward the larvae. From the study, it shows that *C. aurantifolia* pulp extract is more effective than the *C. hystrix* pulp extract. This result probably may be due to the presence high percentage of limonin and ethanol has the ability to extract the terpenoids from the botanical extract (Tiwari *et al.*, 2011). The result obtained, also indirectly indicates that, if the concentration is increased, probably the extracts effects might be similar to the synthetics.

Conclusion

Citrus aurantifolia and *Citrus hystrix* were found to have a larvicidal potential against *Aedes albopictus*. Thus, upon determining the bioactive compounds, the concentration that required to kill or retard the growth of the mosquitoes larvae without killing the non-targeted organism should be taken into an account and formulation of the bioactive components should be emphasized for proper delivery of the compounds.

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