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Larvicidal efficacy screening of Anacardaciae crude extracts on the dengue hemorrhagic vector, *Aedes aegypti*

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Abstract. Vector-borne diseases are still rife because of the re-emergence of diseases transmitted by mosquitoes. The objective of this paper is to evaluate the larvicidal efficacy of crude leaf extract of *Mangifera indica*, *Gluta renghas*, and *Melanochyla fasciculiflora* against vector of dengue hemorrhagic fever, *Aedes aegypti*. These plant species are endemic species and widely distributed in Malaysian forests. Leaves of *Ma. indica*, *G. renghas and M. fascculiflora* were collected from Teluk Bahang National Park, Penang Malaysia. Fractions of leaves were segregated, air–dried, powdered and extracted using Soxhlet with methanol. The solvent was removed by using rotary evaporator to obtain the crude extract. Using WHO standard larval bioassay test method, third instar larvae of *Aedes aegypti* were exposed to concentration ranging from 200- 4500ppm of methanol extract for all plant species. Larval mortality was observed after 24 hours exposure. The highest susceptibility and toxicity was recorded by *Mangifera indica* with the lowest concentration at 800ppm followed by *M. fasciculiflora* and *G. renghas*. This indicates that crude plant extract is very effective in killing *Ae. aegypti* mosquitoes. This finding may lead to new low cost alternative, environmentally friendly method for mosquito control programs. To our knowledge, this is the first report on larvicidal bioefficacy from endemic Malaysian plants.

INTRODUCTION

Dengue is a mosquito-borne viral disease, becoming a huge public health problem in developing countries. Approximately two billion people live in the tropical and subtropical region (Rigau-Pérez *et al.*, 1998) around the world, and roughly around 120 million people travelling to these countries are at risk of being infected by dengue viruses (Gauzman & Kouri, 2002). Two billion people worldwide are at risk with annual infection rate of 2.5- 5.0%, and 2.5% of the fatal infection were mostly children (WHO, 2008a).

Since 1980, Malaysia with a population at approximately 27.7 million and a population density of 84 per sq. km, has consecutively recorded rising cases of dengue outbreaks (Lam, 1994). *Aedes aegypti* and *Aedes albopictus* are vectors for dengue fever and dengue hemorrhagic fever in Malaysia and has been reported since 1950s (Smith, 1956). The mosquitoes transmit dengue virus (DENV) to susceptible humans (Guha-Sapir & Schimmer, 2005). The increase in dengue cases is considered to be a reflection of the rampant development towards massive infrastructure and urbanization which is a favourable factor for breeding site of *Ae. aegypti* (Gubler, 2002; Muhammad Azami *et al.*, 2011).

To reduce the *Aedes* mosquito population, most of the mosquito control programs in the world usually apply chemical control methods. Prior to the discovery of

organochlorine and organophosphate insecticides in the late 1930s and early 1940s, insecticides were important products for pest management in industrialized countries (Isman, 2007). Public Health policies have adopted various control of Culicidae by using synthetic insecticides such as temephos, malathion and phenitrothion (Cavalva *et al.*, 2010). However, the rampant use of these insecticides inadvertently causes resistance evolution in pest, pest resurgence, environmental pollution, and contamination to human and other living things (Das *et al.*, 2007). Furthermore, the synthetic insecticides are more hazardous, leaving toxic residues in food products and are not easily biodegradable. The safest recommended dose of larvicide or pesticide in drinking water varies based on the WHO risk assessment to ensure accurate and reliable dose, which will not harm human populations. For example, methoprene should not exceed 1 mg/l (WHO, 2008b), while pyriproxyfen should not exceed 0.01mg/l (WHO, 2006a; 2006b).

One of the methods to overcome these problems is by using plant-derived bioproducts as replacements for synthetic insecticides or for use in integrated management programmes (Shaalan *et al.*, 2005). Unlike synthetics that kill both pests and non target organisms, natural insecticides are relatively inactive against the latter (Isman, 1997). Extracts of crude oils, which is of botanical origin, prove to have larvicidal activity against mosquito larvae. Plant based bioproducts are mostly non-toxic to humans and have a high degree of biodegradation (Rahuman *et al.*, 2009).

The family Anacardiaceae consists of 70 genera and 600 species of trees or climbers or shrubs. The bark is usually resinous and is found in warm temperate regions of Europe, eastern Asia and America. *Melanochyla fasciculiflora* and *Gluta renghas* are the endemic plants from the Anacardiaceae family that are usually found in the Malaysian forest. Comparatively, *Mangifera indica*, also from the Anacardiaceae family, is a common domestic plant because of its fruit and is usually found in residential areas. To the best of our knowledge, no information is available on larvicidal efficacy of endemic plant species from Malaysia (*M. fasciculiflora* and *G. renghas*). In this study, these three plant species were chosen due to their highly poisonous resin. The resin is known to cause irritation, allergy and nausea to humans (Goon & Goh, 2011). The resin is located in the inner fibrovascular system of the stems, roots and leaves. The bioactive constituent of Family Anacardiaceae could be from the poisonous resin either as a single or a mixture of different active ingredients.

In this study, we tested the larvicidal efficacy from crude extracts of Family Anacardiaceae namely *M. fasciculiflora*, *G. renghas*, and *Ma. indica.* Crude extracts were tested against *Ae. aegypti* in a search for an effective and affordable natural new alternative to mosquito control program.

MATERIALS AND METHODS

Plant Collection and Identification

Leaves of three plants from Anacardaciae family: *M. fasciculiflora*, *G. renghas* and *Ma. indica* were collected from Teluk Bahang National Park, Penang, Malaysia (5°27'38.56"N, 100°12'18.69"E). Taxonomic authentication was done by the Botanical Laboratory staff, School of Biological Sciences.

Preparation and extraction of plant extracts

Leaves were left to dry for 7-14 days in the laboratory until all water has evaporated and the weight of leaves become stable. The dried leaves were blended using commercial Panasonic stainless steel blender. A total of 40gm powdered ground leaves were extracted with acetone (2000ml, Qualigens) in a Soxhlet apparatus. Soxhlet apparatus was set at boiling point of methanol at 66°C for 3 hours until the solvent colour became translucent to ensure all of the possible plants contents were extracted. This procedure was repeated for 3 times in order to have enough crude extracts for larval bioassay experiments. In order to remove excess solvent from the crude extract, we then evaporated the crude extracts using rotary

vacuum evaporator. The water bath was set up at boiling point of acetone (66°C) with 100 rpm speed. Then, the crude extracts were placed in the oven at 37°C for 24 h to remove excess solvent. Crude extracts were kept in the refrigerator at 4°C for storage.

Preparation of stock and test concentrations

One gram of crude extract was dissolved in 100ml of methanol to prepare the stock solution at 10,000 ppm. From the stock solution, subsequent serial concentrations were prepared with distilled water. Serial concentration ranged between 150-4500 ppm.

Mosquito culture

Aedes aegypti from Vector Control Research Unit (VCRU), Universiti Sains Malaysia strain was used in this experiment. Eggs were hatched a week prior to experiment and maintained at temperature of 28±2°C, 70%-85% relative humidity (RH), with a photo period of 14 h light, 10 h dark. Larvae were fed with a mixture of dog biscuit, beef liver, yeast, and milk powder with ratio of 2:1:1:1 by weight and prepared as a fine powder. Late 3rd instar and early 4th instar larvae which have the strongest and stable metabolism phase were used in this experiment.

Larval Bioassays

A total of 25 late 3rd and early 4th instar larvae of *Aedes aegypti* were used in the experiment, using the WHO standard procedure for larvicidal bioassay (WHO, 2005). These larvae were placed in 250ml paper cup and introduced to different serial concentration of extracts prepared using distilled water. 1 ml of 10% methanol was also added into test cups to minimize the effects of solvent on the larval mortality. Whereas, only 1ml of 10% methanol in 249ml of distilled water was used as control. Larval mortality was observed 24 h post treatment. Dead larvae are those that are insensitive and motionless to probing with a needle. Moribund larvae are those incapable of rising to the surface for respiration (Macedo *et al.*, 1997; WHO, 2005). The experiment was replicated three times for each concentration and conducted at laboratory conditions with temperature of 28 ± 3°C with relative humidity of 80 ± 10%.

Statistical analysis

To test the effects of different crude extracts on larval mortality, we ran a two-way ANOVA by using SPSS program version 19. The percentage of larval mortality is considered as dependent variable whereas concentration and plant species were considered as fixed factors. Larval mortality was expressed as percentage and log-transformed prior to the analysis to satisfy the assumption of ANOVA. The LC_{50} and LC_{95} values were calculated using Probit analysis in SPSS 20.0 (IBM SPSS, 2011). The level of significance for the statistic analyses were set at $P<0.05$.

RESULTS

Among all three plants tested, data revealed that 100% of *Ae. aegypti* larvae mortality from the lowest concentration (800ppm) was observed for *Ma. indica* crude extract (Fig. 1). At the same concentration, *M. fasciculiflora* only gave 10% mortality and 0% mortality for *G. renghas* crude extract. There are significant differences on larval mortality between all these three plant crude extracts ($F= 138.51$, $df=12$, $p=0.00$; Table 1). Result shows that *Ma. indica* showed the highest toxicity with the lowest concentration followed by *M. fasciculiflora* and *G. renghas*. No mortality was recorded for control experiment.

As shown in Table 2, there is a positive correlation between plant crude extracts and larvae mortality. Among the three methanolic crude extracts tested, *Ma. indica* showed the strongest larvicidal effects with the lowest lethal concentration among all with LC_{50} at 630.39 ppm and LC_{95} at 779.08 ppm. *Melanochyla fasciculiflora* and *G. renghas* showed weak larvicidal effects of more than 2000 ppm [*M. fasciculiflora* LC_{50} 2337.89 ppm; *G. renghas* LC_{50} 2854.07 ppm]. However, the lethal concentration that kills 95% of *Aedes* larvae was lower for *G. renghas* (LC_{95} 3979.86 ppm) and almost doubled for *M. fasciculiflora* (LC_{95} 6481.04 ppm).

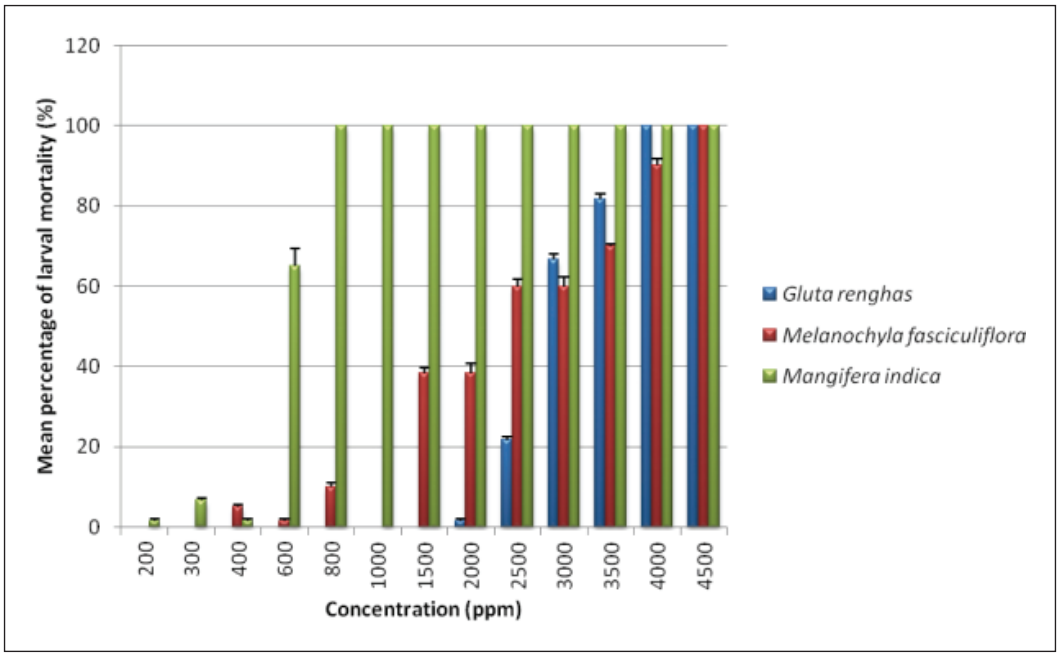

Figure 1. Percentage mortality of *Aedes aegypti* larvae due to different concentration of three plant crude extracts; *Gluta renghas*, *Melanochyla fasciculiflora* and *Mangifera indica*

Table 1. Two-way analysis of variance assessing the effects of different concentrations and plant species on *Aedes aegypti* larvae mortality after 24 h exposure

Source	df	MS	F-ratio	*P*
Concentration	12	11.04	138.51	0.00*
Plant species	2	18.04	226.26	0.00*
Concentration x plant species	24	1.67	21	0.00*

df = degree of freedom, MS = mean squared values. *Significant values are in bold at 5% significant level

Table 2. Mean LC_{50} and LC_{95} (in ppm) of larval efficacy on *Aedes aegypti* for three plant species, *Gluta renghas*, *Melanochyla fasciculiflora* and *Mangifera indica*

Plant crude extract	LC_{50} (95% confidence limit)	LC_{95} (95% confidence limit)	Regression Equation
Gluta renghas	2854.07 (2752.46–2940.52)	3979.86 (3762.09–4332.25)	Y = -39.36 + 11.39X
Melanochyla fasciculiflora	2337.89 (1957.51–2983.31)	6481.04 (4596.53–11694.48)	Y = 112.51 + 3.71X
Mangifera indica	630.39 (523.92–769.15)	779.08 (672.26–1379.84)	Y = -5.65 + 0.009X

DISCUSSION

All plants tested showed toxicity towards *Ae. aegypti* larvae. Crude extracts from Anacardaciae family indicated that the highest toxicity on *Ae. aegypti* larvae is from by *Ma. indica* followed by *M. fasciculiflora*, and *G. renghas*. At the lowest concentration of 800 ppm extracted using methanol solvent, *Ma. Indica* crude extract can kill 100% *Aedes* larvae after 24 h exposure. The bioactive compound in plants that induced larvicidal or adulticidal response might be from various compounds including phenolics, terpenoids, flavanoids, and alkaloids as single compound or joint compounds (Elumalai *et al*., 2012). *Mangifera indica* leaves also contain different compounds such as alkaloids, anthracenosides, coumarins, avonones, reducing sugars, tannins, saponins, steroids and triterpenoids (Godfrey *et al*., 2007). These compounds are found in many plants and were reported to have antimicrobial activity on a variety of microorganisms such as bacteria, fungi, viruses and many others (Marjorie, 1999; Bbosa *et al*., 2007; Doughari *et al*., 2008).

The effectiveness of *Ma. indica* crude extracts against *Culex quinquefasciatus* has been studied previously. At 1000 ppm, only eight *Cx. quinquefasciatus* larvae died after 24 h exposure (Rahuman *et al*., 2008). However, our results indicated that *Ma. indica* crude extract is more effective on *Ae. aegypti* larvae with 25 larvae killed at 800ppm after 24 h exposure. This indicates that *Ma. indica* plant extract have a strong larvicidal effect and worked better on *Ae. aegypti*. We suggested that *Ma. indica* plant extract is an effective new source of larvicidal agent.

The other two crude leaf extracts of Anacardaciae that were chosen were *G. renghas* and *M. fasciculiflora*. These two plants were included in the study based on the records of their poisonous resin. Both plants are also endemic plant species of the Malaysian forest. We discovered that both of these plants gave medium larvicidal efficacy with more than 2000 ppm to kill 50% of *Ae. aegypti*. However, both plants are still viable as a potential botanical insecticide. It is worthwhile to identify the active components that cause larval mortality and explore its remarkable larvicidal properties as an alternative to chemical larvicides. We believe both of these plants have potential as biocontrol agents and can possibly cause sub-lethal effects on oviposition deterrence and effects on next *Ae. aegypti* progeny.

Larvicidal efficacy against a particular mosquito species may be due to the synergistic effects from combination of phytochemicals in plant extracts (Tawatsin *et al*., 2006b). The bioactivity of plant based insecticides against mosquito larvae varies significantly according to solvent used in extraction and the mosquito species tested (Shaalan *et al*., 2005). Activity of chemical compound also varies based on solvent polarity and plant parts (Oliveira *et al*., 2010). Polar solvents such as acetone and methanol mainly extract steroids and alkaloids. In this study, we discovered that methanol extract enhanced the bioactive compound in *Ma. indica*, *G. renghas and M. fasciculiflora*.

The quality of essential oils and plant extracts varies based on plant species (variety), cultivating conditions, maturation of plants, plant storage, plant preparation and method of extraction (Tawatsin *et al*., 2006a). In this study, all the leaves was harvested in the morning (0900) from single parent tree of more than two years of age. The leaves were brought to the lab and immediately dried under room temperature and extracted within a week. Crude extracts were stored in refrigerator at 4°C to keep it fresh before the experiment. The precise and swift procedure is important to ensure all the possible active chemical compounds in the crude extracts are preserved in large quantities.

Aedes aegypti is usually associated with cosmopolitan/urban areas with dense human population where the mosquitoes bite indoors. Therefore, it is important to create a safe natural and nontoxic product in controlling mosquito populations. Based on the potential source of bioactive chemical and free from harmful effects, plant extracts can be considered as one of the alternative source for mosquito killing agent (Das *et al*., 2007).

In this study, we focused on finding a new safe natural botanical product derived from natural forest using low-tech control, low cost, easy to handle and can be integrated into current vector control programs. Control program using conventional insecticide are very expensive (Kudom *et al.*, 2011). Studies have shown that crude extracts can be extracted at low cost compared to purified compounds and it is effective against mosquitoes (Cavalcanti *et al.*, 2004; Jenson *et al.*, 2006). *Mangifera indica*, *G. renghas*, *M. fasciculiflora* trees are easily available in the Malaysian forest throughout the year and easy to be harvested. We suggest that these three plant species with significant larvicidal potential are feasible to be implemented into the current mosquito control program especially *Ma. indica*.

The result from this study has revealed new potential larvicidal agents produced from natural forest. Further investigations of effectiveness of crude extract should be made in understanding its potential in controlling mosquitoes and further research is needed to improve the plant formulation in enhancing the bioactive activity. We hope that the plants will be acceptable to replace the use of conventional insecticide in mosquito control program.

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