

**PHYTOCHEMICAL SCREENING AND LARVICIDAL ACTIVITY OF
MURRAYA KOENIGII LEAVES ETHANOLIC EXTRACT AGAINST
*AEDES AEGYPTI***

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ABSTRACT

World Health Organization (WHO) estimated over 100 million dengue infections to happen annually worldwide involving more than 2.5 billion people. Temephos or abate is a larvicide that has been used in vector control to eradicate mosquito larvae. Though practically low risk, there had been resistance problem reported with continuous use. This study seeks to find an effective and safer alternative to abate by assessing the use of ethanolic extract of *Murraya koenigii* leaves as larvicidal agent against *Aedes aegypti*. *M.koenigii* leaves were macerated for 3 days with absolute ethanol and evaporated using rotary vapor to produce the crude extract. The crude extract was subjected to phytochemical screening using standard qualitative method. For bioassay, the crude extract underwent a serial dilution to produce 3 concentrations of 100 ppm (C₁), 50 ppm (C₂) and 10 ppm (C₃) with abate and absolute ethanol as negative and positive control respectively. Bioassay for larvicidal effect was conducted in accordance to WHO standard method. Phytochemical screening of ethanolic extract of *M. koenigii* leaves revealed the presence of alkaloid, steroid and saponin. The bioassay shows that after 24 hours, the mortality rate of C₁, C₂ and C₃ larvae were 100%, 38% and 0% and when further extended to 48 hours, the rate increased to 100% and 46% for C₂ and C₃ respectively. The LC₅₀ and LC₉₉ post 24 hours were 54.489 ppm and 93.961 ppm respectively whilst at post 48 hours, the LC₅₀ and LC₉₉ were 10.263 ppm and 16.176 ppm respectively. The results show that up to 48 hours duration of exposure, the mortality rate increase whilst the lethal concentration (LC₅₀ and LC₉₉) decreases. Upon examination on larvae deformities at post 24 and 48 hours, all test concentrations and negative control exhibit normal morphology. Positive control, however, exhibit deformities characterized by twisted and fragmented insides. When statistically analyzed, C₁ larvicidal activity was proven comparable with abate at 24 hours while C₂ needed 48 hours exposure to be on par. Based on the results, it could be argued that the ethanolic extract of *M.koenigii* leaves does hold promising value to be further developed as larvicidal.

KEYWORDS: Abate, *Ae. aegypti*, *M. koeginii*, LC₅₀, LC₉₉

INTRODUCTION

Vector-borne diseases accounted for 1 million mortalities each year and a large portion of casualties are contributed by dengue. Being the deadliest vector-borne disease in the world, dengue has affected more than half of the world populations. It has been noted as the world's fastest growing vector-borne disease with 30-fold increment of cases reported for the past 50 years. WHO estimated over 100 million dengue infections to happen annually worldwide involving more than 2.5 billion people (WHO 2015). Some factors contributing to the world's worsening situation of dengue include inadequate housing, poorly designed irrigation and water systems, deforestation and loss of biodiversity and poor waste disposal and water storage (WHO 2015). Insecticides and larvicides are the two most common control strategies in combating dengue. The usage of synthetic insecticides like pyrethroids and dichlorophenyltrichloroethane (DDT) are the two most common control strategies in combating dengue. The usage of synthetic insecticides like pyrethroids and dichlorophenyltrichloroethane (DDT) worked out well at first, but over time, continuous usage had raised resistance and environmental issues (Maharaj et al., 2012; Shankar et al., 2013).

Abate is one classic example of synthetic larvicide widely used in eradicating larval stages of mosquitoes. Though practically low risk, there had been resistance reporting problem upon continuous use (Grisales et al., 2013). Globally, efforts have been made by researchers to find safer and effective synthetic insecticides and larvicides. Bearing the same intention, this study has a few objectives namely to determine the phytochemical constituents of ethanolic extract of *M.koenigii* leaves, the larvicidal activity of ethanolic extract of *M.koenigii* against *Aedes aegypti*, the mortality rate of *Aedes aegypti* when exposed to ethanolic extract of *M.koenigii*, the LC₅₀ and LC₉₉, morphological deformities and to compare the larvicidal efficacy between test concentrations and control groups.

METHODS

Plant material

M. koenigii leaves were harvested in three batches from Felda Bukit Sagu 3, Kuantan (3°57'15.2"N 103°12'30.4"E). The leaves were authenticated by Dr Shansul Khamis, a plant taxonomist from University Putra Malaysia. The specimen was deposited at herbarium of Kulliyah of Pharmacy, International Islamic University Malaysia (Voucher no: PIIUM 0258).

Plant extraction

2.95 kg of *M.koeginii* leaves were blended using blender and subjected to 3 days maceration using absolute ethanol at room temperature (Suganya et al., 2013). Macerated *M.koeginii* leaves were sifted using plastic mesh strainer first before filtered using filter paper. Filtered extract was then subjected to rotary evaporation with 250 mbar pressure and water bath of 60°C. After

evaporating, crude extract was spooned out from the flask using spatula. Crude extracts were kept in falcon tubes in fume hood. Falcon tubes were wrapped with aluminium foil and few holes were made on top to let excess water evaporate. Once all water evaporated, crude extracts were weighed and kept at 4°C until use.

Phytochemical screening

All chemical tests (Mayer's, Libermann Burchard's, Shinoda, Froth and Ferric Chloride Test) for phytochemical screening were conducted according to the standard described by Sofowora (1993), Trease and Evans (1989) and Harborne (1973).

Larvicidal bioassay

Healthy third instar larvae were transferred into test cup containing 150 ml distilled water, 1.5 mL test concentrations (C_1 ; 100 ppm, C_2 ; 50 ppm, C_3 ; 10 ppm) and 1.5 mL absolute ethanol. 25 larvae were transferred using loop strainer into respective test cups. For positive control, 1 mL of abate solution was added into 249 mL of distilled water and stirred for homogeneity. From the stock solution, only 150 mL was used and 25 larvae were added using loop strainer into each positive control cup. Each test concentrations and control groups were done in 4 replicates. Bioassay was run at 27°C and relative humidity of 61 with photoperiod of 12 hours light followed by 12 hours dark, extending from 24 hours to 48 hours. No food was provided to the larvae during the experiment.

Mortality assessment and morphological examination

At the end of 24 hours, larvae were considered dead if there is no sign of swimming or movement when probed with a disposable pipette in the siphon. Moribund larvae that were incapable of rising to the surface when water was disturbed were also considered dead. Dead larvae were transferred from the test solution into universal bottle containing 70% ethanol for preservation purpose. The bioassay was continued until 48 hours before it was terminated. Larvae that were still alive were killed by pouring hot water. All data were recorded and the mortality rate after each exposure was calculated. Observation of deformities on preserved dead larvae post 24 and 48 hours were done using light microscope under magnification power 40x, focusing on the head, thorax, abdomen and end parts.

Data analysis

LC_{50} and LC_{99} of ethanolic extract of *M. kongenii* leaves against *Ae. aegypti* were determined with probit regression and comparison between treatment and control groups were analyzed using one way analysis of variance (ANOVA). The data was analyzed using IBM SPSS Statistics 20 software. Statistical significance was considered at $p < 0.05$.

RESULTS AND DISCUSSION

Phytochemical screening

Phytochemical screening of ethanolic extract of *M. koeginii* leaves (Table I) shows the presence of alkaloid, steroid and saponin. Contrarily, a similar phytochemical study in India revealed the presence of alkaloid, phenol, terpenoid and flavonoid (Saravanan et al., 2014). Another study conducted in India shows that the ethanolic extract of *M. koeginii* roots possesses chemical constituents like sterols, flavonoids, carbohydrate and amino acid (Vats et al., 2011). Differences in phytochemical screening results could be contributed by geographical differences in cultivation sites (Khattak and Rahman 2015). Additionally, humidity, age of plant, soil type, climate change, temperature and presence of different microorganism could also contribute to the variation in phytochemical compounds (Chansang et al., 2005).

Table 1 Phytochemical constituents of ethanolic extract of *M. koeginii* leaves

Chemical constituents	Presence (+) / Absence (-)
Alkaloid	+
Terpenoid	-
Steroid	+
Flavanoid	-
Saponin	+
Phenol	-

Mortality of larvae is most likely related to the phytochemical constituents in the *M. koeginii* leaves. Alkaloids, for instance, could disrupt membrane integrity and impair microtubules function. They could also cause impairment in digestive system by inhibiting hydrolytic enzyme (Mahajan et al., 2010). Saponins are toxic to some cold-blooded animal and insects. They are bitter and able to induce toxicity (Foerster 2009). Apart from the specific role of certain compound, synergistic effect of closely related compounds in plants could also contribute to plant natural defense (Isman 1997).

Larvicidal bioassay

Mortality rate

Figure 1 shows the percentage larvae mortality rate post 24 and 48 hours of exposure. Based on the results, the larvae mortality rate was highest in C1 (100 ppm) yielding 100% and lowest at C3 (10 ppm) yielding 1% post 24 hours. Meanwhile, for C2 (50 ppm), longer exposure (48 hours) was needed to yield 100% mortality rate. This proven that the mortality rate in this study was concentration dependent. Similarly, another study also presented a concentration dependent result whereby 17.6% mortality of larvae was induced at lowest concentration (50 ppm) and

64.4% mortality at highest concentration (650 ppm) (Suganya et al., 2013). However, this study produced a more promising result as the concentration used is much lower and yet yield higher mortality rate.

In another study, hexane extraction of *M. koeginii* leaves produced 100% and 59.2% larvae mortality rate at 100 ppm and 50 ppm respectively at 24 hours (Arivoli et al., 2015). At 100 ppm, the data by Arivoli et al. (2015) and this study presented similar mortality rate. However, at 50 ppm, Arivoli et al. (2015) presented better result as it causes higher larvae mortality as compared to this study (38%). Solvent choice for extraction is crucial as different chemicals yield different phytochemical constituents. Polarity of solvents will determine the constituents to be extracted out. Polar solvent would extract polar molecules and nonpolar solvent would extract nonpolar molecules. Polar solvent like water can extract large polar molecules like protein which is non-contributory to larvicidal activity (Ghosh et al., 2012). In contrast, nonpolar molecules like hexane could extract useful constituents like essential oils and alkaloid which contribute to larvae mortality due to their toxic properties (Arivoli et al., 2015).

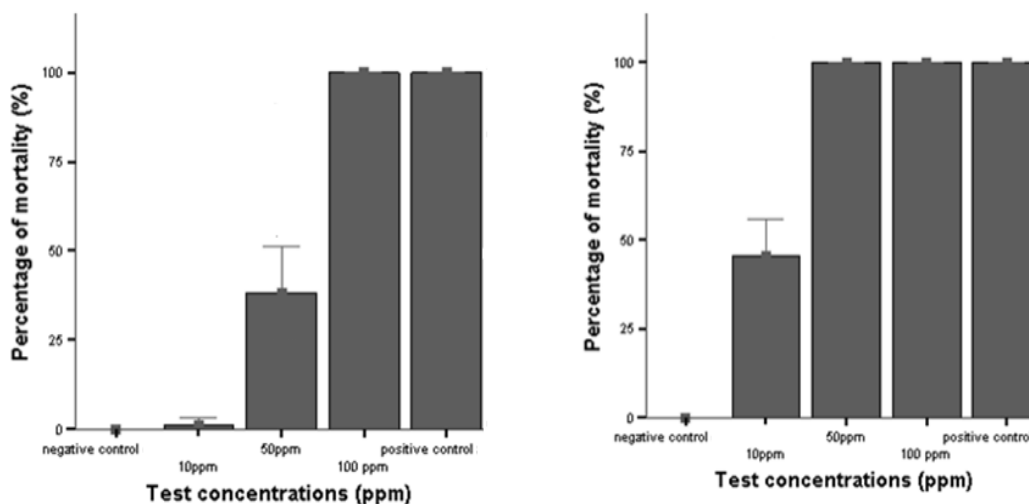


Figure 1 Percentage of larvae mortality post 24 and 48 hours exposure

Morphological deformities

Figure 2, 3 and 4 shows the morphology of larvae, focusing on 3 parts namely the head and thorax, abdomen and end parts. The figures show that all larvae exposed to test concentrations and negative control exhibit normal morphology with the exception of larvae exposed to positive control whereby the larvae exhibit twisted and fragmented insides. Abnormal larvae tend to possess characteristics like blackish and twisted insides and deformed abdomen (Khater and Shalaby 2008).

Head and Thorax

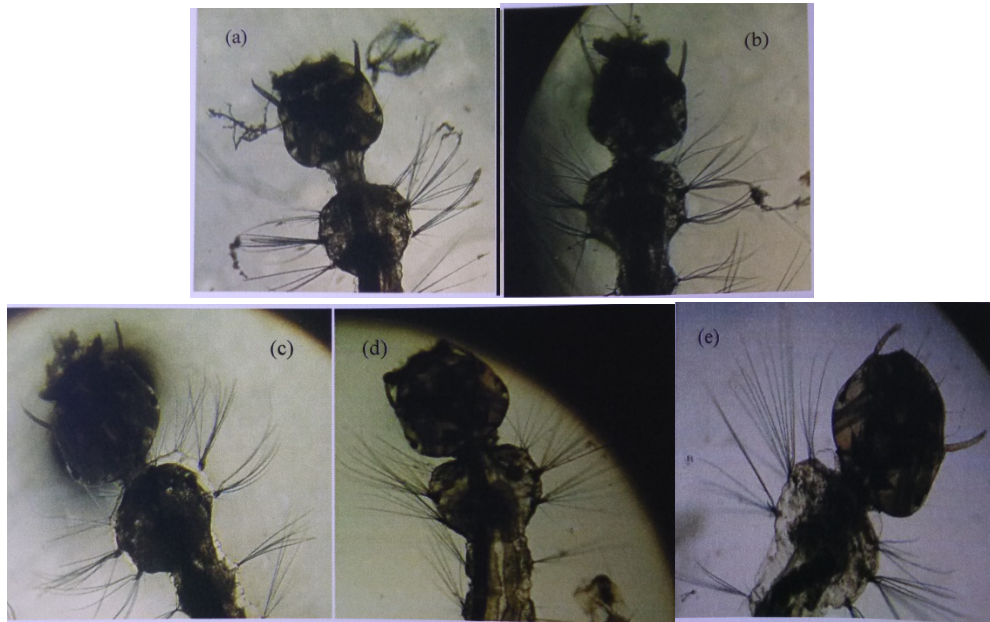


Figure 2 Head and thorax part of larvae at (a) C1, (b) C2, (c) C3, (d) Negative control and (e) Positive control

Abdomen

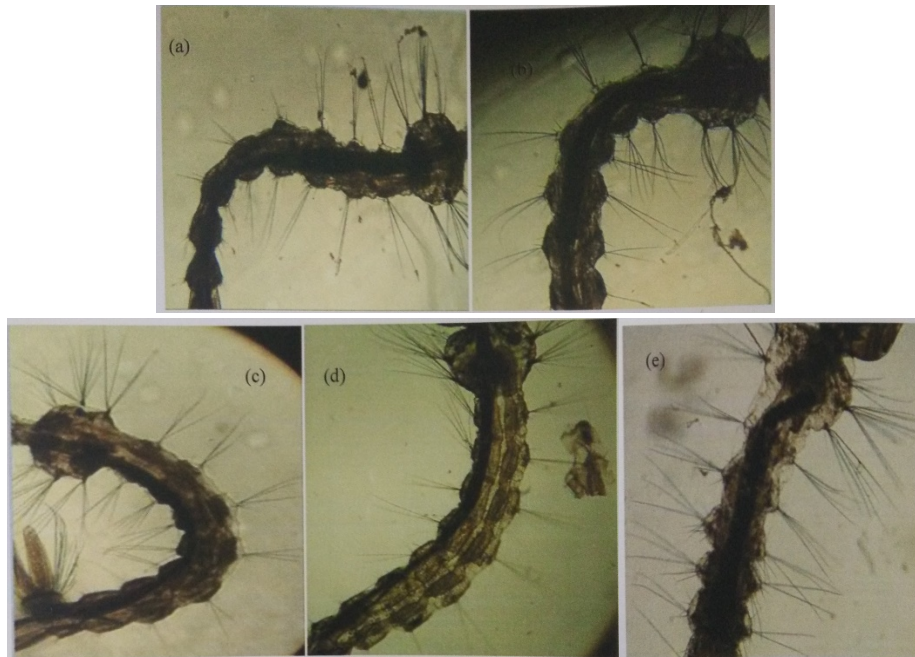


Figure 3 Abdomen part of larvae at (a) C1, (b) C2, (c) C3, (d) Negative control and (e) Positive control

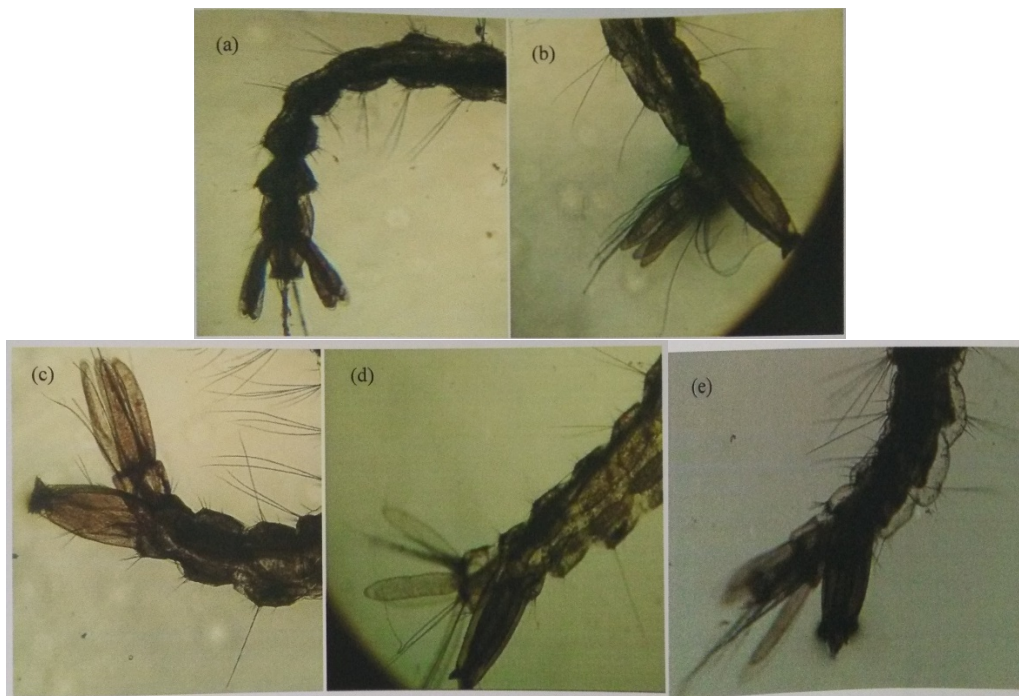
End parts

Figure 4 End parts of larvae at (a) C1, (b) C2, (c) C3, (d) Negative control and (e) Positive control

LC₅₀ and LC₉₉

Table 2 shows that post 24 hours, 50% larvae population was killed at 54.489 ppm whilst 99% larvae population was killed at 93.961 ppm. Upon exposure extension (48 hours), 50% larvae population was killed at 10.263 ppm and 99% larvae population was killed at 16.176 ppm. This indicate that longer exposure lower the LC₅₀ and LC₉₉ values. Similar to morality rate, the LC₅₀ and LC₉₉ values differ when different solvents were used. This can be seen in a study conducted by Sukari et al. (2013) which presented the decrease in larvicidal efficacy beginning with hexane with LC₅₀ of 1.88 ppm, followed by acetone with LC₅₀ of 24.21 ppm and lastly diethyl ether with LC₅₀ of 511.12 ppm. Additionally, when compared, the LC₅₀ in this study is higher than the previous study indicating the need of higher concentration to deplete half of larvae population (Suganya et al., 2013). Difference in LC₅₀ value using the same solvent and plant may be contributed by geograprahical distribution of the plant (Tiwary et al., 2007).

Table 2 Regression parameters of probit analysis for mortality of *Ae. aegypti*

Observations (hours)	LC ₅₀ (ppm)	LC ₉₉ (ppm)	Chi Square (X ²)
24	54.489	93.961	13.678
48	10.263	16.176	3.386

Comparison between treatment and control groups

Table III shows comparison of controls with test concentration post 24 and 48-hour exposure. After 24 hours of exposure, all test groups display significant difference ($p = 0.000$) when compared to negative control (ethanol), except for C₃ that showed no significant difference ($p = 1.000$). This indicates that C₃ was basically as weak as negative control in its larvicidal efficiency. However, when the duration is extended, C₃ showed significant difference ($p = 0.000$) when compared to negative control. This practically means that only after 48 hours of exposure, C₃ shows larvicidal efficiency. In another comparison, p value of C₁ is not significant ($p = 1.000$) when compared with positive control after 24 hours of exposure. This shows that C₁ is as much effective as positive control in its larvicidal efficiency. In contrast, C₂ and C₃ showed significant difference ($p = 0.000$) indicating they are not comparable and as effective as positive control. But, after 48 hours of exposure, C₂ showed no significant difference when compared with positive control ($p = 1.000$), thus proving it to be as effective as positive control and C₁.

Table 3 Comparison of controls with test concentrations post 24 and 48 hours exposure

Groups		p -value (post 24 hours exposure)	p -value (post 48 hours exposure)
Negative control	100.00 (C1)	0.000*	0.000*
	50.00 (C2)	0.000*	0.000*
	10.00 (C3)	1.000	0.000*
	31.25(Positive ctrl)	0.000*	0.000*
Positive control	100.00 (C1)	1.000	1.000
	50.00 (C2)	0.000*	1.000
	10.00 (C3)	0.000*	0.000*
	0.00 (Negative ctrl)	0.000*	0.000*

*Represents statistically significant at $p < 0.05$

CONCLUSION

The study concluded that the ethanolic extract of *M. koeginii* leaves were able to induce 100% mortality rate to larvae in just 24 hours at 100 ppm concentration. Lower concentration (50 ppm) with longer exposure (48 hours) could also induce 100% mortality rate to larvae population. Larvae mortality could be strongly reasoned with the revelation of potent constituents like saponin and alkaloid. Morphological examination, however, revealed no deformities to all larvae exposed with ethanolic extract of *M. koeginii* leaves. LC₅₀ of this study was also lower and more relevant as compared to previous study which used the same extraction solvent and plant. When compared with commercially available larvicide, ethanolic extract of *M. koeginii* was shown to be equally effective and on par. Thus, it is hoped that positive outcome of this study could be further developed in a more intricate manner and at a larger scale.

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