LARVICIDAL EFFECTS OF *CURCUMA LONGA* ETHANOL RHIZOME EXTRACT ON *ANOPHELES TESSELLATUS*

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ABSTRACT

Adult Anopheles tessellatus is a vector of Malaria, which is responsible for increased morbidity and mortality in many tropical and subtropical countries. During epidemics, the emphasis is given to the use of insecticides as a control measure against mosquitoes. As an alternative to synthetic insecticides, the use of bio-degradable phytochemicals against mosquito larvae is considered to be one of the safest approaches to control mosquito-borne diseases. This project was carried out to monitor the effect of ethanolic rhizome extract of Curcuma longa on second larval instar of Anopheles tessellatus. Bio-assay was performed to determine the percentage mortality of Anopheline larvae following exposure to different concentrations of plant extract. The study indicated that the mortality rate following exposure increases in a time and dose-dependent manner. The plant demonstrated its strongest larvicidal activity as it caused 100% mortality by its rhizome extract at 500 ppm following 48 hours of exposure. Anopheles tessellatus larvae were subjected to histological analysis after 48 hours of exposure to 300 ppm of the extract. The histology revealed a disruption in the cuticle and mid-gut epithelium of the larvae. Additionally, a DNA fragmentation analysis was also performed to detect possible induction of apoptosis and the results indicated no significant DNA degradation. The results of this study suggest a potential utilization of the rhizome extract of Curcuma longa for the control of Anopheles tessellatus larvae. *Keywords: Anopheles tessellatus, Larvae, Curcuma longa, Rhizome, Ethanol*

INTRODUCTION

Prevalence of vector-borne diseases

Vector-borne diseases are one of the major causes of morbidity and mortality in many developing countries. Over 350 mosquito species are vectors of pathogens that cause life-threatening diseases including malaria, Japanese encephalitis, lymphatic filariasis, yellow fever and dengue (Mohankumar, Shivanna & Achuttan, 2016; Bekele *et al.*, 2014).

Malaria is caused by *Plasmodium* sp. parasites that are transmitted to humans through the bites of infected female Anopheline mosquitoes (World Health Organization, 2017). *Anopheles culicifacies* is regarded as the main vector of malaria in Sri Lanka. In addition to that, other species that have consistently been incriminated as malarial vectors are *Anopheles subpictus, Anopheles annularis, Anopheles varuna*, and *Anopheles tessellatus* (Gunathilaka *et al.*, 2015).

The global burden of Malaria

According to the latest estimates of World Health Organization (WHO), there were 216 million cases of malaria and 445 000 deaths reported in 2016 (Figure 1) (World Health Organization, 2017). Malaria was endemic in Sri Lanka for centuries and was a significant public health issue associated with great economic loss and social disruption. However, Sri Lanka has achieved a remarkable success by eliminating malaria where the last indigenous case was reported in October 2012 (Karunaweera, Galappaththy & Wirth, 2014). Although indigenous transmission of malaria has been controlled, still there is a risk for the occurrence of imported malaria cases mainly from India and African countries. Therefore, reducing the risk of imported malaria as well as controlling the vector is important to maintain the achieved success (Sri Lanka. Ministry of Health, 2016).

Different approaches have been developed to lessen the global prevalence of malaria. Among these strategies, the use of indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs) which are mainly directed against the adult mosquito have contributed to a significant reduction of malaria incidences over the past decade (World Health Organization, 2017; Samuel *et al.*, 2016).

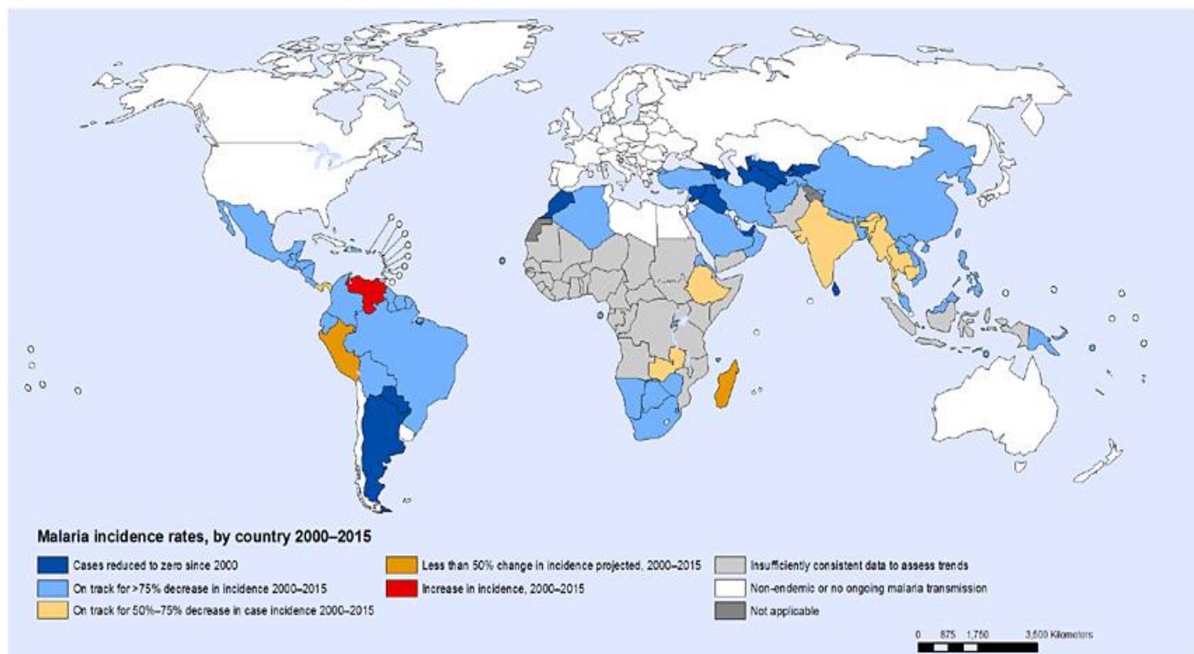


Figure 1. Global malaria incidence rates, by country, 2000-2015 (World Health Organization, 2017)

Conversely, control measures that target the immature stages of mosquitoes such as the larval stage, are useful for the control of malaria in regions where the breeding sites of mosquitoes are reachable and restricted in number and size. In contrast to the highly mobile adult mosquitoes, the immature stages such as larvae are limited within comparatively small aquatic habitats and incapable of escaping the control methods. Therefore, larval source management (LSM) plays a progressively significant role in vector control (Tomass *et al.*, 2011).

Larval and pupal stages of mosquito can be controlled with the application of synthetic insecticidal chemicals such as fenthion, temephos, malathion, diflubenzuron, and methoprene. However, similar to the adult vector, these life stages of anopheline mosquitoes also have been reported to develop resistance against synthetic insecticides (Liu *et al.*, 2014; Tomass *et al.*, 2011).

Phytochemicals as natural insecticides

The use of synthetic insecticides in the aquatic sources causes many risks to the humans and environment. In present, application of many of the synthetic insecticides in mosquito control programs has been restricted due to many reasons including the concern for environmental sustainability, risks on human health, and their non-biodegradable nature (Ghosh, Chowdhury & Chandra, 2012).

Natural products from botanical sources with insecticidal properties have been studied in the recent past for the control of diverse vectors (Parte *et al.*, 2015). Many researchers have stated the effectiveness of plant extracts against mosquito larvae (Kumar *et al.*, 2012). Phytochemicals

derived from plant sources have been reported as potential repellents, larvicides, oviposition attractants, and insect growth regulators. These phytochemicals can play a significant role in mosquito control and thus can interrupt the transmission of mosquito-borne diseases at both individual and community level (Srinivasan *et al.*, 2014).

The effectiveness of phytochemicals against mosquito larvae may present significant variations depending on the species of plant, plant parts used, maturity of the plant, as well as upon the presented vector species (Ghosh, Chowdhury & Chandra, 2012). Hence, there is a necessity to identify target-specific, easy to use, eco-friendly and low-cost alternative insecticidal constituents from plant resources. Many previous studies have discovered the presence of insecticidal plants belonging to diverse families with a wide geographical distribution across the world. The crude solvent extracts of many plant materials have been identified to have different levels of bioactivity against the larval stages of mosquitoes (Tomass *et al.*, 2011).

Biological properties of *Curcuma longa*

Curcuma longa plant (common name, turmeric) (Figure 2) is a perennial herb belonging to the ginger family which is extensively cultivated in the south and southeast tropical Asia (Himesh *et al.*, 2010). The rhizome of turmeric is highly beneficial and widely been used for medicinal and culinary purposes. The most active constituent of *Curcuma longa* is curcumin, which is about 2 to 5% of the species. Turmeric consists of an extensive variety of phytochemicals including curcumin, demethoxycurcumin, bisdemethoxycuicumin, curcumenol, curcumol, zingiberene, eugenol, triethylcurcumin, tetrahydrocurcumin, turmerin, turmeronols and turmerones. The characteristic yellow color of *Curcuma longa* is due to the presence of curcumin, which comprises curcumin I (94%), curcumin II (6%) and curcumin III (0.3%). It has a hydrophobic nature and freely soluble in ethanol, acetic acid, chloroform and ketone (Himesh *et al.*, 2010).

Figure 2. The Curcuma longa plant

For the past few decades, extensive work has been undertaken to evaluate the pharmacological activities and biological actions of Curcuma longa and its extracts. Curcumin (diferuloylmethane), the chief bioactive constituent of the plant has been found to have a broad-spectrum of biological actions. These include its antioxidant, anti-carcinogenic, anti-inflammatory, anti-mutagenic, anticoagulant, anti-diabetic, antifertility, antibacterial, antiviral, antifungal, anti-fibrotic, anti-venom, antiulcer, hypotensive and

hypocholesteremic properties (Krup, Prakash & Harini, 2013; Prashar et al., 2011; Chattopadhyay et al., 2004).

In view of a cumulative interest in developing insecticides from plant origins as an alternative to chemical insecticide, the current study was performed to evaluate the larvicidal potential of the ethanolic ribosome extracts of Curcuma longa against the Anopheles tessellatus larvae.

METHODOLOGY

Plant material

The fresh rhizomes of Curcuma longa were collected from a field in Neelanmahara area of Maharagama, Sri Lanka (annual temperature of 32^{0}C and rainfall of 4 mm) (Figure 3). The rhizomes were thoroughly washed under running tap water, sliced into smaller pieces and dried in a hot-air oven at 37°C for 3 days.

Figure 3. The map of Sri Lanka showing Neelanmahara area (latitude 6.8376267, longitude 79.9263141 and 25 m above sea level)

Rearing mosquito larvae

The eggs of *Anopheles tessellatus* were allowed to hatch into larvae and procured from Department of Parasitology, Faculty of Medicine at the University of Colombo. Three larvae (n=3) were allocated into containers, each containing 10.00ml of de-chlorinated water. Physiological parameters of the de-chlorinated water including temperature and pH were measured. Larval forms were fed on an appropriate food mixture. Containers were covered by using nylon net with small pore size and allowed to adapt to the environmental conditions for 24hrs.

Larval identification

Stage II and III larvae were placed individually in petri dishes and observed under an inverted light microscope (TCM 400) with an objective (x10 and x40). Using standard larval keys developed for Sri Lankan anopheline (Amarasinghe, 1992), they were identified to the species level. Further, larval species identification was reconfirmed through adult identification.

Adult mosquito identification

The adult mosquito immersed in 70% ethanol and refrigerated overnight. Thereafter, the mosquito was dissected and parts were mounted by using Canada balsam. The prepared permanent slides were observed and identified under the light microscope (LABOMED) using standard adult mosquito keys developed for

Sri Lankan anopheline (Gunathilaka, 2017).

Preparation of plant extract

The dried turmeric was ground to fine powder form with an electric grinder and stored at 4°C in air-tight plastic bags covered with aluminum foils until use (Figure 4). The ethanol extract of *Curcuma longa* was prepared by dissolving 50.0g of dried powder in 100.0ml of absolute alcohol. The mixture was left in the dark at room temperature for 24 hours and shaken at regular intervals. Thereafter, the mixture was subjected to stirring at 500 rpm for 20 minutes in a magnetic stirrer. The solution was filtered using a glass funnel and Whatman number 1 filter papers. Filtrates were dried in the fume hood. The evaporated plant extract was collected into air-tight containers, covered with aluminum foils and refrigerated at 4°C until further use.

Figure 4. The rhizomes (left) and the power (right) of turmeric

Preparation of stock solution

A 5.00 mg/ml of stock solution was prepared by dissolving 0.05g of plant extract in 100.00µl of DMSO. Once the residue was completely dissolved, it was further diluted by 9.90ml of de-chlorinated water (The ultimate concentration of DMSO in the stock solution was 0.1%). The stock solution was vigorously vortexed to obtain a homogenized mixture.

Larvicidal Bioassay test

Three set of second instar larvae triplicates (n=3) with more or less similar body length were sorted out (Figure 5) and treated with different concentration of plant extract (Table 3). The larvicidal activity of the turmeric extract was assessed by the WHO standard larvicidal bioassay test (WHO, 2005). The control set was prepared by adding 0.1% DMSO. The larval behavior at regular time intervals and the mortality were recorded following 2, 4, 24 and 48 hours of exposure. Larvae were kept in a cool and low light area in the lab for 48 hours in the unfed state. The containers were covered with the net in order to reduce evaporation and to facilitate dissolve oxygen level.

Table 3. The different concentrations of turmeric extract

Group	Treatment
N/C	0.1% of DMSO
C1	25 ppm
C2	50 ppm
C3	100 ppm
C4	300 ppm
C5	500 ppm

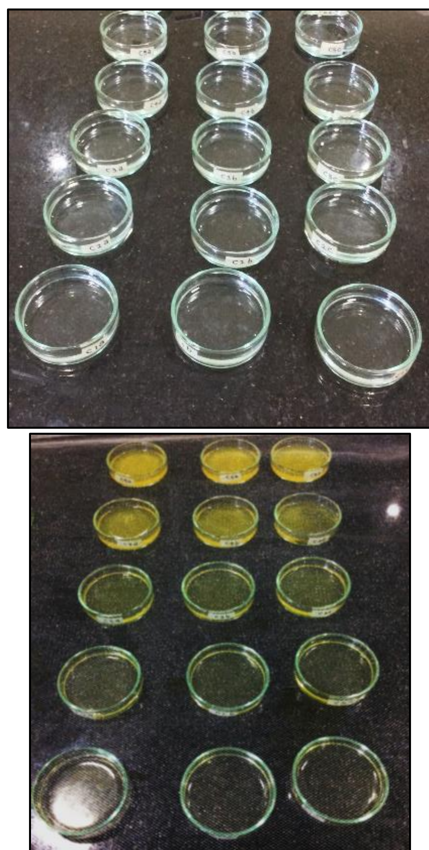


Figure 5. The bioassay setup before (left) and after (right) the exposure

Histological Procedure

For histological studies, the live C4 larvae (300ppm) after 48 hours exposure to the plant extract were fixed in 5 ml of 10% neutral buffered formalin and refrigerated. After dehydration in a graded ethanol series, the larvae were subjected to two washes in xylene. Thereafter, larvae were subjected to two paraffin wax baths at 58-60°C before infiltration by using a wax dispenser (TPC 2000). Once solidified, paraffin blocks were kept at 4°C until use. The paraffin block was sectioned using rotary microtome (SHANDON FINESSE 325) at 5-7 µm. The sections were mounted onto glass slides which were coated with egg albumin. The slides were placed on the slide dryer (SD 2800) at 38°C for 30 minutes. The slides were observed under the light microscope (LABOMED) at 100x to ensure the presence of larval tissue.

The larval tissues were stained using Hematoxylin and Eosin staining procedure (Appendix 1) and observed under the light microscope at 200x to compare the histological changes with the control larvae.

DNA fragmentation analysis:

The C2 (100ppm) larvae obtained 24 hours post-exposure was fixed in 5 ml of ice-cold PBS. For the DNA extraction procedure, PBS buffer was removed from the micro-centrifuge tube. Thereafter, the larvae were homogenized in Liffton's buffer and kept for 30 minutes. Subsequently, Proteinase K was added to the homogenized sample and vortexed. The mixture was incubated for 1 hour at 55°C.

This was followed by the addition of phenol: chloroform: isoamyl alcohol to the sample. The sample was vortexed and centrifuged at 12000 rpm for 15 minutes. The aqueous layer of the sample was transferred to a new micro-centrifuge tube and an equal volume of ice-cold Isopropanol was added. The extract was stored in -20°C for 30 minutes. Thereafter, the extract was centrifuged at 12000 rpm for 15 minutes. The supernatant was removed from the micro-centrifuge tube and the pellet was dried. Subsequently, nuclease-free water was added to micro-centrifuge tube. The extracted sample was run on 1% agarose gel at 50V for 10 minutes and at 100V for 1 hour.

A 1.0% agarose gel was placed on the electrophoresis chamber in a horizontal position and TBE buffer was added to the chamber until it covers the gel. Then 8.0µL ethidium bromide was added to the

chamber. Subsequently, 10 µL of DNA extract was mixed with 5µL of gel loading buffer and loaded into the wells. The lid of the chamber was closed and the gel was allowed to run at 100V for 1 hour. Then the gel was placed in the UV Trans-illuminator and bands were observed using the software and the monitor.

Statistical analysis

Statistical analysis was performed using SPSS version 21.0 statistical software and Microsoft Excel 2013. Results were evaluated for its normality. Two-way ANOVA was performed for normally distributed data with the significance of $p<0.05$. The significant differences further estimated using Tukey's pairwise test. The mean mortality percentages of larvae were subjected to probit analysis in order to calculate LC_{50} values following 24 and 48 hours of exposure along with the regression equation.

DATA ANALYSIS

Species identification

Larval identification

Anopheline larvae can be distinguished in the field based on their resting positions in the water. Due to the absence of siphon, the larvae belonging to the genus *Anopheles* generally position parallel to the water surface (Figure 6) (Centre of Disease Control and Prevention, 2017).

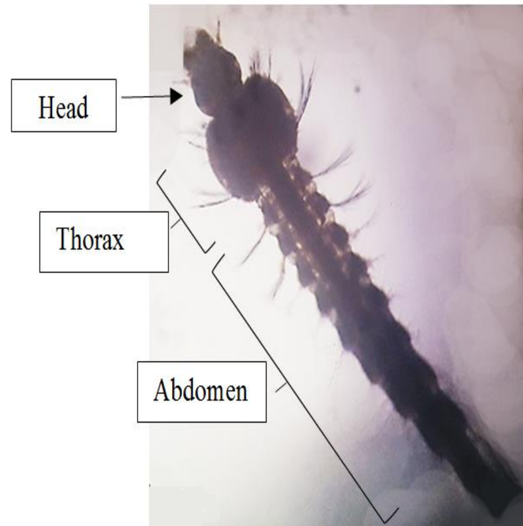


Figure 6. Light microscope view of the Anopheles 4th instar larvae (100x)

Three distinct body regions; head (antennal hairs, inner clypeal hairs, outer clypeal hairs, frontal hairs and sutural hairs), thorax (thoracic palmate hairs, shoulder hairs, pro, meso and meta thoracic hairs), abdomen (abdominal tergal plates, palmate hairs in abdominal segments, lateral hairs in the abdominal segments) were noted.

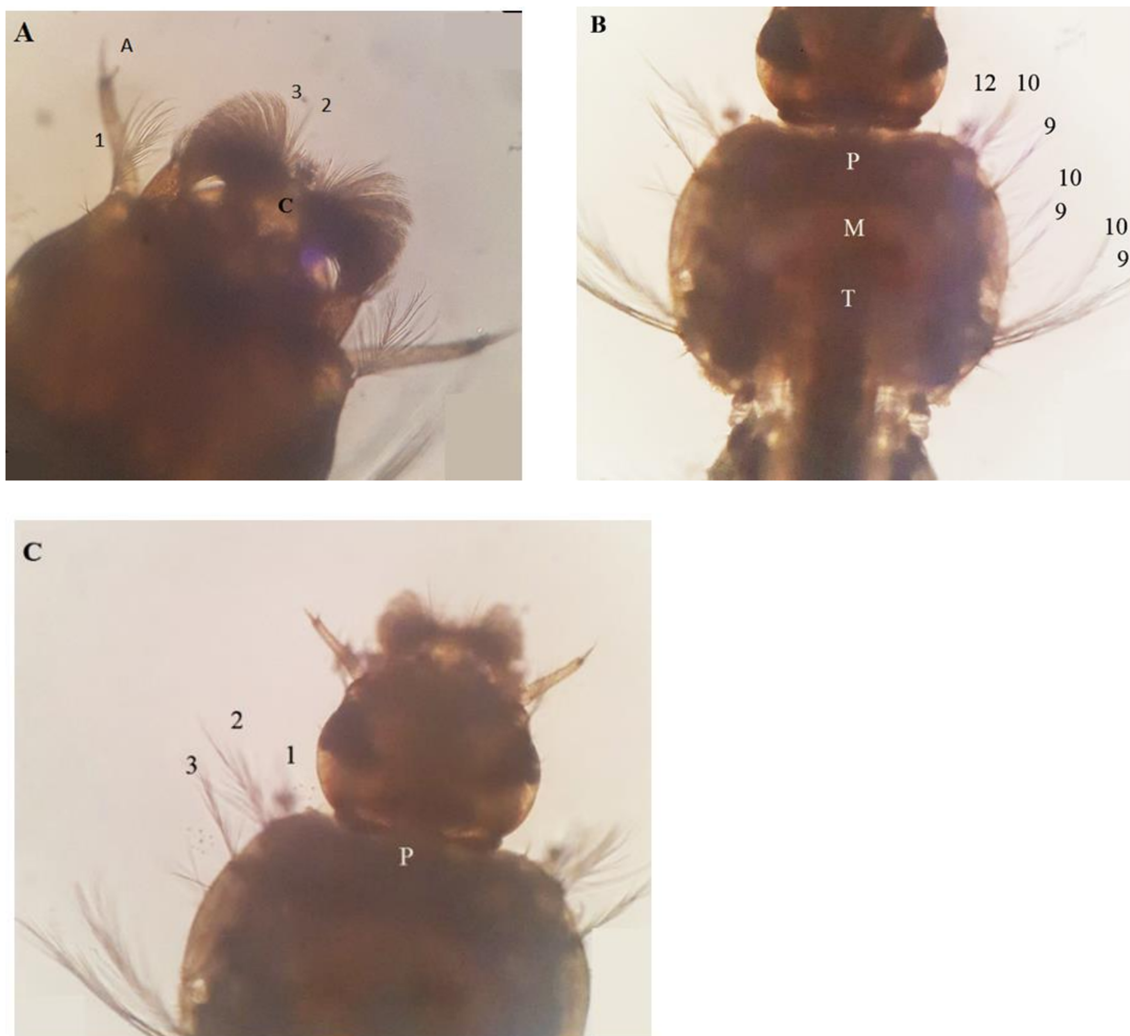


Figure 7: The morphological view of head and the thorax of Anopheles larvae (400x) (A) Seta 1-A simple; 2-C inserted at least as far apart as the distance between 2-C and 3-C on one side (B) Long thoracic pleural setae 9, 10, 12-P, 9, 10-M and 9, 10-T simple (C) Seta 1-P weak, 2-5 branched; 1, 2-P arising from separate basal tubercles, only tubercle of 2-P prominent and sclerotized

Based on the morphological characteristics of the dorsal view of head (Figure 7A), the subgenus *Cellia* was distinguished from the subgenus *Anopheles* (Amarasinghe, 1992). The Sri Lankan anophelines of subgenus *Cellia* are categorized into four taxonomic series: *Pyretophorous, Myzomyia, Neomyzomyia* and *Neocellia* series. The observed ventral view of thorax (Figure 7B) confirmed the taxonomic series as *Neomyzomyia*. Two species, *Anopheles tessellatus*, and *Anopheles elegans* have been reported under *Neomyzomyia* series in Sri Lanka. Based on the features observed in the dorsal view of prothorax (Figure 7C), the species was distinguished as *Anopheles Tessellatus* from *Anopheles elegans* (Gunathilaka, 2017). The species identification was further confirmed by observing the morphological features of the adult mosquito.

Anopheline adult mosquito identification

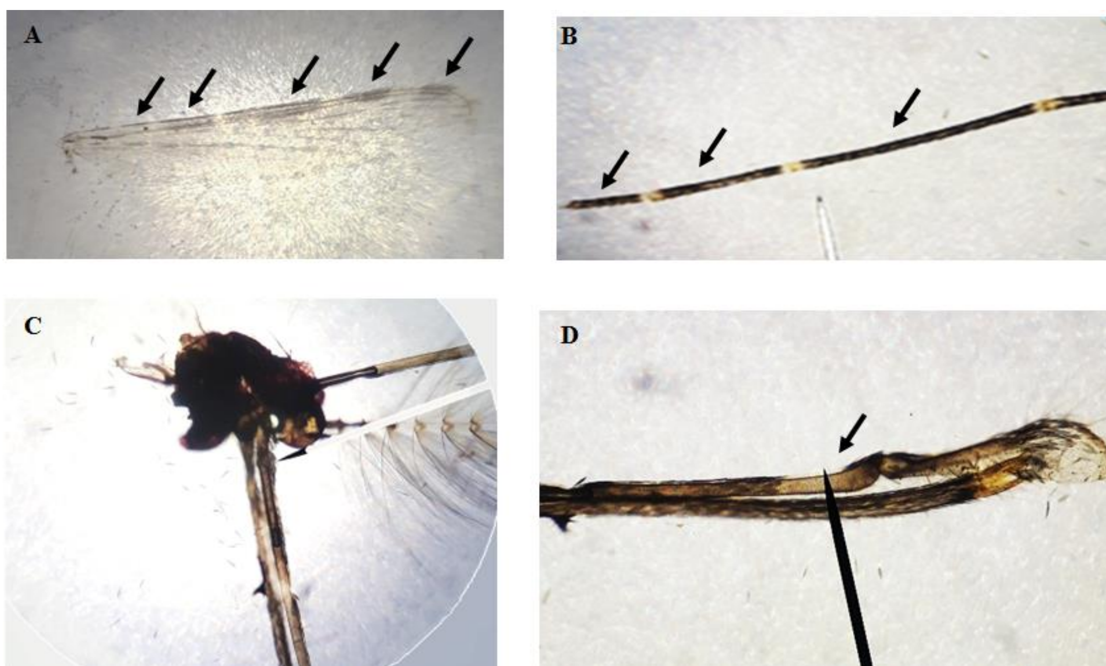


Figure 8. Light microscopic view of adult Anopheline mosquito (400X)(A) Wing with 4 or more darks marks involving both costa and veins R-R1 accessory sector pale (ASP) spot present on costa and/or subcostal, (B) Femur and tibia speckled (C) The head of the mosquito with a part of proboscis (D) Apical half of the proboscis pale scaled

The observed morphological features of the wing of Anopheline (Figure 8A) distinguished the subgenus *Cellia* from the subgenus *Anopheles.* Among the species belongs to subgenus *Cellia*, *A. elegans* and *A. tessellatus,* which belong to *Neomyzomyia* series and *A. Maculatus* and *jamesii* group which belong to *Neocellia* series feature speckled femur and tibia (Figure 8B). Based on the characteristics observed on the proboscis (Figure 8D) the species was confirmed as *Anopheles tessellatus* (Gunathilaka, 2017).

Larvicidal bioassay

Physical characteristics of water used for the study, such as median temperature 28 ± 0.2^{0}C and pH 6.6 ± 0.2 were within the permissible limits throughout the study period. The results of *Anopheles tessellatus* larval mortality tested against *Curcuma longa* crude ethanolic extract are presented in Table 4. The results of the present study revealed the high larvicidal activity of turmeric rhizome extract against *Anopheles tessellatus*.

Table 4. Mortality rates of Anopheles tessellatus larvae following exposure to ethanolic extract of Curcuma longa

Sample	Concentration of the extract (ppm)	Mortality Rate (%)	
		24hrs	48hrs
N/C	0	00.00 ± 00.00	00.00 ± 00.00
C1	25	00.00 ± 00.00	00.00 ± 00.00
C2	50	00.00 ± 00.00	11.11 ± 11.11
C3	100	11.11 ± 11.11	33.33 ± 00.00
C4	300	33.33 ± 19.25	66.67 ± 19.25
C5	500	88.89 ± 11.11	100 ± 00.00

An 88.89% and 100% of larvae mortality were observed at the highest dose rate of 500ppm following 24 and 48 hours of exposure (Figure 9). Further, the effect of larval mortality was dependent on the concentration of rhizome extract and the exposure time duration.

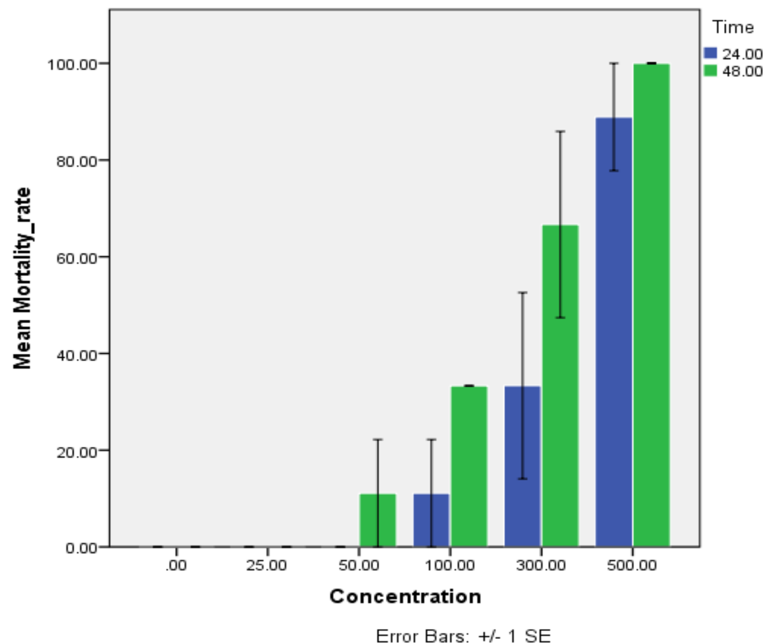


Figure 9. Percentage of larvae mortality at different hours following treatment with different concentrations of ethanolic extract of turmeric.

The highest rate of mortality in larvae was observed where the highest concentration of extract (500 ppm) was exposed. Significant differences were observed between the mortality rates caused by different concentrations of the plant extract against Anopheles tessellatus larvae of laboratory strains (MS = 8401.388, df = 5, P = 0.000) (Table 5). In addition to that, the time or exposure period was also found to have a statistically significant effect on the mortality rates (MS = 1512.173, df = 1, P = 0.028). Therefore, P< 0.05, Tukey's pairwise tests were performed following one way ANOVA. The highest concentration (500 ppm) was found to have a significantly difference from other tested concentrations (N/C, P = 0.000; 25 ppm, P = 0.000; 50 ppm, P = 0.000; 100 ppm, P = 0.000, 300 ppm = P = 0.001).

Table 5. Two-way ANOVA measuring the effects of different concentrations of Curcuma longa extract on Anopheles tessellatus larvae mortality after 24 h and 48 h of exposure

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Concentration	42006.938	5	8401.388	30.245	.000
Time	1512.173	1	1512.173	5.444	.028
Concentration * Time	1265.383	5	253.077	.911	.490
Error	6666.667	24	277.778		
Total	81110.889	36			
Corrected Total	51451.161	35			

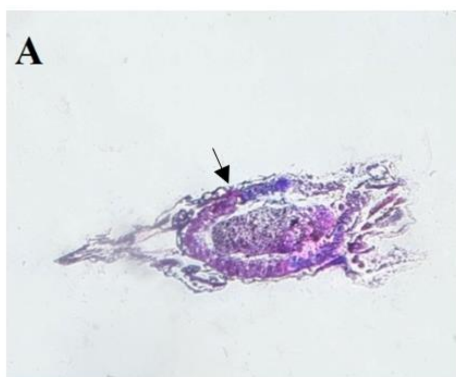
The result of log-probit analysis (at 95% confidence level) revealed a gradual decrease in the LC_{50} value with the increasing exposure period. The rhizome extract of *Curcuma longa* presented a high larvicidal activity with LC_{50} values of 467.74 ppm and 151.36 ppm after 24 and 48 hours of exposure respectively. The concentration-dependent mortality was detected to be clear, as the mortality rate was positively correlated with the concentration. However, the low amount of data and the variance may have an impact on the observed regression coefficient value (Table 6).

Table 6. Probit analysis of larvicidal efficacy of turmeric rhizome extracts against Anopheles tessellatus

	LC_{50} (ppm)	Regression equation	R squared value
24 hours	467.74	y = 2.4399x - 1.5209	0.635302178
48 hours	151.36	y = 2.8696x - 1.2699	0.763292029

Significant behavioral changes were observed in mosquito larvae exposed to turmeric extract within 30 minutes of exposure. The most apparent sign of behavioral changes observed following 24 hours of exposure in Anopheles tessellatus larvae was the inability to come on the surface. The larvae also presented restlessness, loss of equilibrium and eventually death. Histological experiments

Histology of *Anopheles tessellatus* larvae in the control group presented the normal form of the body regions. The cuticle layer of the body was intact with well-developed mid-gut epithelium exhibiting the brush border (Figure 10A).



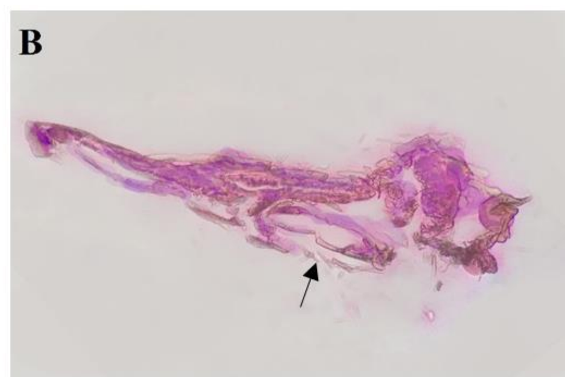


Figure 10. Histological analysis A- Histology of A. tessellatus larvae of control group showing intact cutical layer of the body (indicated by arrows), B- Histology of the A. tessellatus larvae treated with 300ppm of turmeric extract for 48 hours showing the disrupted cuticle layer (indicated by arrows).

After 48 h exposure to Curcuma longa extract, the cuticle was observed to be disrupted with partially degraded epithelial cells began through the dilated basal membrane (Figure 10 B).

DNA fragmentation analysis

To examine the effects of the plant extract on the larval DNA, the larvae which were exposed to 100ppm of turmeric extract were analyzed to detect intact DNA on 1% agarose gel electrophoresis. No apparent deviations of the larval DNA were detected on the gel image suggesting that the exposure to the plant extract does not affect the larval structure in molecular level (Figure 11).

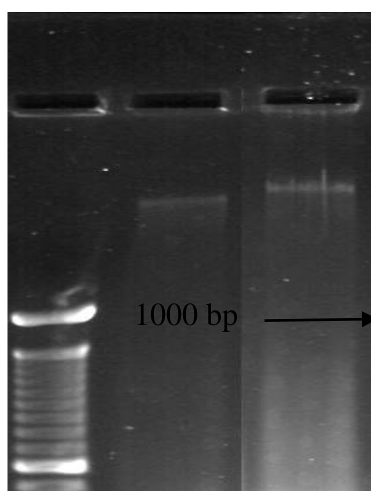


Figure 11. The gel image of DNA analysis. Lane M: molecular ladder; Lane 1: negative control; Lane 2: DNA sample of the exposed larva analysis. Lane M: molecular ladder; Lane 1: negative control; Lane 2: DNA sample of the exposed larva

DISCUSSION

Vector control is crucial to prevent the proliferation of mosquito-borne diseases and to enhance the quality of public health (Ghosh, Chowdhury & Chandra, 2012). The extensive usage of synthetic organic insecticides in mosquito control operation during the last five decades has caused the development of physiological resistance in target vector population and accumulation of toxic substances in the environment. This has necessitated the need of an alternative which is bio-degradable, environmentally safe, and easy to use as a vector control measure (Samuel *et al.*, 2016; Kumar *et al.*, 2012).

Numerous researchers in the field of vector control have discovered the effectiveness of different phytochemicals of botanical origin against different mosquito species (Rawani *et al.*, 2017). Among these botanical sources, turmeric has been long found to exhibit numerous properties along with potential larvicidal activity (Krup, Prakash & Harini, 2013; Prashar *et al.*, 2011; Chattopadhyay *et al.*, 2004).

The 24 hours bioassay is a major tool which is applied to evaluate the toxicity of phytotoxins, and many researchers have been using this method to measure the toxic effects of different plant extractions against mosquitoes (Viji & Nethaji, 2015). The current study discovered the toxic nature of ethanolic rhizome extract of *Curcuma longa* that demonstrated its larvicidal activity against the malaria vector, *Anopheles tessellatus*. The mortality percentage of larvae was detected to be increased with the increasing concentration of extract and the exposure duration. The result of log-probit analysis (at 95% confidence level) revealed a significant reduction in LC_{50} value following 48 hours of exposure (LC_{50} = 151.36 ppm) in comparison to LC_{50} value at 24 hours of exposure (LC_{50} = 467.74 ppm). This may suggest that the efficacy of the plant compounds which are responsible for the larval mortality readily increases with the increasing time of exposure. Conversely, a reduction in LC_{50} values with the increasing exposure period was also reported in case of aqueous extract of

Curcuma longa tested against *Anopheles stephensi*. However, the reported activity is more effective compared to the activity in the present report (Singha & Chandra, 2011).

The ethanolic extract of *Curcuma longa* is reported to contain 22.6% curcumin, 6.1% desmethoxycurcumin, and 15.4% ar-turmerone with 6.6% bisdesmethoxycurcumin, which may responsible for its larvicidal activity (Ali, Wang & Khan, 2015). In contrast to the crude extract, the essential oil of *Curcuma longa* has been reported to possess much higher potential as a natural larvicide. Studies suggest that the larvicidal effects of essential oil of *Curcuma longa* may be due to the greater amount of Phytol (18.89%) (Albert *et al*., 2014). Curcumin is presently been used in many clinical trials and interestingly, it is found to be non-toxic to humans. The non-toxic nature makes it a very attractive applicant for consequent field experiments with expected insignificant non-specific species toxicity and environmental inferences (Sagnoua *et al*., 2012).

Other members of the genus *Curcuma* have also been reported for their larvicidal activity towards mosquito larvae. For instance, Sukari *et al*., (2010) have observed the larvicidal action *Curcuma heyneana, Curcuma xanthorrhiza*, and *Curcuma mangga*, against the dengue mosquito larvae of *Aedes aegpyti*. The authors further reported that a high toxicity against larvae of *Aedes aegypti* was observed in the cases of hexane extract of *C. xanthorrhiza* with a LC_{50} value of 26.4 μg/ml, and the petroleum ether extract of *C. heyneana* with a LC_{50} value of 34.9 μg/ml. Correspondingly, The LC_{50} value of *Curcuma aromatica* on Japanese encephalitis vector *Culex vishnui* larvae was reported to be 17.25 ppm following 72 hours of exposure (Mallick, Bhattacharya & Chandra, 2014).

In this study, the observed behavioral changes of mosquito larvae following exposure to plant extract were compatible with other studies. Viji and Nethaji (2015) reported similar behavioral effects on larvae of *A. aegpyti* following exposure to crude ethanolic extract of *C. longa*. Conversely, Remia and Logaswamy (2010) also reported these behavioral effects in the cases of *Catharanthus roseus* and *Lantana camara* extracts where the effect of *Catharanthus roseus* was found to be more pronounced after exposure. The authors further stated that these effects may be due the presence of neurotoxic compounds in the plant materials.

Besides causing death to mosquito larvae, the effect of intoxication also manifests through the aberration of structures. It is evident as *Aedes aegypti* larvae treated with extracts of natural compounds such as dried fruits of peppercorns and red seaweed *Laurencia dendroidea* has been reported to have darkening and shrunken cuticle of anal papillae after the exposure (Bianco *et al*., 2013; Kumar, Warikoo & Wahab, 2010). In this study, the observed histological variations of *Anopheles tessellatus* following the exposure to turmeric extract presented high structural impairments including the cuticle and the mid-gut.

As the main absorption area in mosquito gut, midgut owns fine microvilli in the apex of cells (Alves, Serrão & Melo, 2010). In the present study, histological variations were observed in the mid-gut of *Anopheles tessellatus* including partial degradation of the epithelial cells and disruption of the

microvilli and the basal membrane with the loss their normal appearance. Al-Mehmadi & Al-Khalaf (2010) have reported that such impairments may lead to larval mortality due to the interaction of the gut contents with the hemolymph. These alternations were in covenant with previous studies. The mid-gut of *Culex quinquefasciatus* larvae that exposed to *Murraya paniculata* leaf extract was presented with separated epithelial cells from the basal membrane, separated elongations protruded into the lumen, and disrupted the appearance of microvilli (Kjanijou *et al*., 2012). Similar features including epithelial cell separation from the basal membrane have been observed in *Culex quinquefasciatus* following exposure to *Melia azedarach* extract (Al-Mehmadi & Al-Khalaf, 2010).

Active compounds of *Curcuma longa* may cause disruption of the midgut epithelium, where its constituents are been absorbed. Irrespective of the type of constituents used, the correspondence of detrimental changes observed in the different species indicates that these variations are a mutual response to cellular intoxication (Kjanijou *et al*., 2012).

Larvicidal substances are also been reported to cause protein and DNA destruction where a reduction of total body protein and DNA content were observed in the red cotton bug *Dysdercus cingulatus* larvae treated with *P. pavonica* extract (Sahayaraj & Kalidas, 2011). Such reduction can result in prolongation of larval duration and reduction of adult emergence (Yu *et al*., 2015). Moreover, a study by Magalhães *et al*., (2009) on Schistosoma mansoni have reported that the increasing concentration curcumin can lead to an increased larval mortality, decrease worm viability and reduced egg production which may associate with transcriptional repression (Morais *et al*., 2013). However, the observed result in DNA fragmentation analysis in the current study did not present any significant changes in larval DNA after exposure to *Curcuma longa* extract.

The present study evaluated the efficacy of ethanolic extract of rhizome of *Curcuma longa* against the second instar larvae of *Anopheles tessellatus*. Although apparent histological alternations were observed in larvae following exposure to *Curcuma longa*, no significant DNA degradations were observed at the molecular level. Thus, during the present study, the observed disruption of the cuticle and the mid-gut of the larvae as well as the behavioral changes including reduced mobility, may be accountable for larval mortality in this bioassay experiment.

FUTURE WORK

The results of this study which revealed the efficacy of *Curcuma longa* ethanolic extract as a potential larvicidal agent against *Anopheles tessellatus* can be decoded to affect diverse aspects of mosquito control agent development. As curcumin is the major bioactive compound in turmeric which has been found to be responsible for it larvicidal activity, isolation of curcumin from *C. longa* may offer a high efficacy against the target vector population.

Preparation of solvent extracts initially from non-polar to polar chemicals is essential to determine the most effective solvent for the extraction process. Moreover, the application of evaporation methods with increase precision in order to obtain the solid residue from the liquid solvent may lead to the determination of

lethal dose with a higher accuracy. The column chromatography and thin layer chromatography can be used to purify the phytochemicals of turmeric and thereby analyze its larvicidal potentiality. Furthermore, a field evaluation of its larvicidal activity can be performed against other instar stages of Anopheline larvae as well as on different Anopheles species.

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APPENDIX 1: Hematoxylin and Eosin staining procedure (H & E Staining)

The mounted larval tissue slides were stained with using following H & E Staining Procedure.

- Xylene I - 5 minutes
- Xylene II - 5 minutes
- 100% Ethanol I - 3 minutes
- 100% Ethanol II - 3 minutes
- 95% Ethanol - 3 minutes
- 70% Ethanol -3 minutes
- Distilled Water - 30 Seconds
- Hematoxylin - 45 Seconds
- Tap water – 2 to 3 minutes
- Distilled water – 1 to 2 minutes
- Acid Alcohol - 15 Seconds
- 70% Ethanol -3 minutes
- Eosin – 5 to10 Seconds
- 95% Ethanol - 30 Seconds
- 100% Ethanol I - 3 minutes
- 100% Ethanol II - 3 minutes
- Xylene I - 3 minutes
- Xylene II -3 minutes

After the staining procedure, a drop of Canada balsam was put on the section and placed a cover slip, press to remove air bubbles. Let it dry for 5-10 minutes.