LARVICIDAL EFFECTS OF MOMORDICA CHARANTIA METHANOL LEAF EXTRACT ON ANOPHELES TESSELLATUS

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ABSTRACT

Mosquito borne diseases contribute to the major disease burden around the world. The use of botanicals as an alternative to synthetic compounds have been explored due to its multifarious advantages. The present study investigated the larvicidal activity of Momordica charantia methanol leaf extracts against the second instar larvae of the laboratory reared, Anopheles tessellatus. Momordica charantia is a medical herb, belonging to the Cucurbitaceae family. The bioassay test was carried out by using the WHO procedure. The mean percentage mortality of Anopheles tessellatus was shown to increase with increasing concentration and increased time of exposure to the Momordica charantia methanol leaf extract. 100% larval mortality was observed at 48 hours following the exposure to plant extract at the highest concentration of 1000 ppm. The study revealed an LC50 value which decreased with time (251 ppm at 24 hours and 126 ppm at 48 hours). Additionally, behavioural changes during the larvicidal assay were observed in response to tactile stimuli. In order to identify histological changes in the cuticle and mid-gut upon exposure to Momordica charantia plant chemicals, the tissue was processed and sections were stained with Hematoxylin and Eosin. The most commonly observed characteristic changes in treated Anopheles tessellatus larvae include damage to cuticle and shrinkage of cells. Therefore, the present investigation revealed that Momordica charantia methanol extract demonstrated effective larvicidal properties against Anopheles tessellatus larvae which can be attributed to the phytochemicals present in the plant. Hence, the formulation of Momordica charantia methanol extracts may potentially be used as an effective and eco-friendly larvicide, which could be an alternative to malaria control.Keywords: Mosquito, Anopheles tessellatus, Momordica charantia, larvicidal effect, mortality

INTRODUCTION

Mosquitoes as vectors of diseases

Vector borne diseases are among the chief causes of illness and decease in many developing countries. Mosquitoes (Order: Diptera) are undoubtedly the most medicinally significant arthropod which are vectors for detrimental parasites and pathogens, including malaria, yellow fever, Japanese encephalitis, dengue, filariasis, chikungunya and Zika virus disease (Sharma et al., 2016). Malaria is a debilitating disease associated with high morbidity and mortality, caused by the Plasmodium parasite. Female mosquitoes depends on a blood meal of vertebrate hosts to initiate gametogenesis. These blood meals mediate the interaction between humans and the mosquito hosts in the parasite life cycle (Nyamoita et al., 2013).

According to the World Malaria Report, 214 million cases of malaria were reported globally in 2015 along with and 438 000 malaria deaths (World Malaria Report, 2016). In September 2016, Sri Lanka was declared as a Malaria free country, with less than <0.04% prevalence of the malaria vector and no reported incidences of Malaria since 2012. In order to maintain this status, vector control and surveillance measures have been implemented (Bulletin of the World Health Organization, 2017).

Methods of mosquito control

Mosquito control plays a fundamental role, in preventing the proliferation of mosquito borne diseases and in improving quality of public health. Due to the absence of an effective vaccine or antiviral therapy, vector control is the only currently available method to control mosquito borne diseases (El-Sayed and El-Bassiony, 2016).

Vector control can be achieved by biological, physical and chemical approaches. The strategy to combat these diseases primarily depends on disruption of the disease transmission cycle by either targeting the mosquito larvae at breeding sites through spraying of stagnant water or by killing/repelling the adult mosquitoes using insecticides (Bekele et al., 2014).

Control measures directed at the larval stages are more effective especially in areas where mosquito breeding sites are easily accessible. As the mosquito larvae are restricted within small aquatic habitats and due to their low mobility, it is unlikely to escape control measures (Mallick et al., 2016). Initially, mosquito control significantly relied on the application of broad-spectrum pesticides. Since the discovery of the insecticide Dichloro–diphenyl–trichloroethane (DDT), an organochlorine pesticide, mosquito control approach has been almost completely based on synthetic organic insecticides (Musau et al., 2016).

Nevertheless, the frequent use of chemical insecticides has led to undesirable side effects including, destabilization of the ecosystem, bioaccumulation, reduction in biodiversity and unfavourable biodegradability (Ollengo et al., 2016). Moreover, the extensive use of synthetic insecticides over the last five decades have resulted the development of physiological resistance in vector species (Ali et al., 2013). These factors have necessitated the urge for environmentally safe, cost-effective and target specific insecticides against mosquito species. Accordingly, a number of studies have emphasized the development of natural insecticides for controlling mosquitoes (Ileke et al., 2015; Singh et al., 2014; Varun et al., 2013).

Recent research have documented the use of natural plant products against mosquito vectors (Haq et al., 2016; Kweka et al., 2016; Elumalai et al., 2015; Govindarajan, 2011). Although insecticides of plant origin have been extensively used on agricultural pests, a very limited extent has been used against mosquito vectors of public health importance. Prospection for new larvicidal molecules based on plant derivatives are safer to use due to their innate biodegradability (Govindarajan et al., 2012; Kamaraj et al., 2010).

LITERATURE REVIEW

Plant based chemicals compounds

Sri Lanka is a varietal emporium of endemic and exotic flora enriched with phytochemicals having pharmacognostical and toxicological properties. The phytochemicals derived from plant sources can act as toxicant against the adult as well as larval stages of mosquitoes (Islam et al., 2011). Moreover, certain phytochemicals interfere with growth, reproduction or act as a repellent against various species of mosquitoes (Noutcha et al., 2016). These plant based botanicals have a variety of advantages including, multifarious control mechanisms against mosquitoes which decreases the likelihood of developing resistance and is target specific thereby non harmful to humans (Paul and Choudhury, 2016).

In Sri Lanka, variety of plant species including cinnamon, lemon grass, sweet flag, citronella, lemon, cardamom and ginger have been tested for mosquito larvicidal activity against several species of the subgenus Aedes, Culex and Anopheles (Ranaweera, 1996).

Momordica charantia and its phytochemical constituents

Momordica charantia Linn (Family: Cucurbitaceae), commonly known as bitter gourd/melon and balsam pear, is cultivated in tropical regions, including Asia, Caribbean, East Africa and South America (Das et al., 2015). Momordica charantia contains suborbicular leaves, with 5-7 deep blade palmate lobes and are variable in their size. Bitter gourd is an important functional food crop grown in low lands of Sri Lanka. This monoecious climber and scrambling harbaceous vine is locally known as, ‘Karawila’ in Sinhala and ‘Pavakka’ in Tamil (Giuliani et al., 2016).

Traditionally, the fruits, leaves, and roots of bitter melon were believed to have medicinal importance. These medicinal properties are attributed to the presence of secondary metabolites such as alkaloids, glycosides, tannins, triterpenes, protein, flavonoids, saponins, minerals and vitamins (Tan et al., 2014).

Over the past few decades collection of research have been carried out on bitter melon and these studies have identified, antidiabetic, antiviral, antitumor, antileukemic, antibacterial, anthelmintic, antimutagenic, antimycobacterial, antioxidant, antiulcer, anti-inflammatory, hypocholesterolemic, hypotriglyceridemic, hypotensive, immunostimulant, and insecticidal properties of Momordica charantia (Subramaniam et al., 2017; Saengsai et al., 2015; Nagappan and Gomathinayagam, 2014).

Momordica charantia as a potential larvicide

The first reported insecticidal activity of Momordica charantia was against the

mustard saw fly and mustard aphid, Lipaphis erysimi (Mishra et al., 2006; Kumar et al., 1979). More recently, the mosquito larvicidal property of Momordica charantia against three mosquito species of the subgenus Anopheles stephensi, Culex quinquefasciatus and Aedes aegypti have been reported (Kamaraj and Rahuman, 2010; Maurya et al., 2009; Rahuman and Venkatesan, 2008; Singh et al., 2006; Prabakar and Jebanesan, 2004).

The objective of this study was to assess the potential larvicidal effects of methanol extracts of Momordica charantia against the Anopheles tessellatus larvae. The aim of this study was to determine the mean percentage mortality of Anopheles tessellatus larvae following the exposure to methanol extract of Momordica charantia leaf. Furthermore, the focus of this research was to identify histological changes of the organism upon exposure to methanol extract of Momordica charantia leaf.

MATERIALS

Reagents

Methanol, Ethanol, Distilled water, De-chlorinated water, Xylene, 10% neutral buffered formalin, Hematoxylin, Eosin, Acid Alcohol, Canada Balsm, Paraffin wax, Dimethyl sulfoxide (DMSO).

Glassware and Consumables

Measuring cylinder (10ml and 100 ml), Beakers (50 ml and 500 ml), Micro-centrifuge tubes (1.5ml), Staining jars, Reagent bottles (50 ml), Aluminium foil, Whatman No.1 filter paper, Funnel, Petri dishes, Micropipettes (1000 µl), Steel spatula, Pipette tips, Pasteur pipette, Surgical forceps, Tissue paper, Air tight polythene sealer bags, Tissue Cassettes, Microscope slides, Aluminium ice box, Glass vials and Latex gloves.

Instrumentation

Analytical balance (SP 602 AM), Electrical grinder (LS750 W5), Forced Air Lab oven (DHG-9023A), Fume hood (FH1000), Inverted Microscope (TCM 400), Light Microscope (LX 300), Magnetic stirrer (2ML), Magnetic stirrer bar (Q-19-A), Paraffin Dispenser (TEC-2000), Rotary Microtome (F163851404), Slide Dryer (SD-2800), Vertical pressurized steam sterilizer (LS-B35L-I), Vortex Mixer (VM-300), Water bath (WB 2800).

METHODOLOGY

Collection of plant material

Mature leaves of Momordica charantia were collected from a cultivation in Colombo, Western Province, Sri Lanka (Figure 1). The geographical conditions of the region were 74 mm average precipitation, humidity 88% and mean annual temperature +27° C.

Figure 1. Map of Sri Lanka showing location of sample collection, Colombo Western Province. Palagama, GP coordinates 6.7645° N, 79.9654° E.

REARING OF MOSQUITO LARVAE

The eggs of Anopheles species mosquito were cultured and hatched to larvae at the Department of Parasitology, Faculty of Medicine, University of Colombo, Sri Lanka. These larvae were brought to the BMS laboratory and kept in the container for 2 hours. Thereafter, three larvae (n=3) were allocated into containers, each containing 10 ml of de-chlorinated water. Physiological parameters of the de-chlorinated water including temperature and pH were measured.

The containers containing mosquito larvae were covered by using a nylon net with small pore size and allowed to adapt to the environmental conditions for 24 hours, as shown in (Figure 2). During the acclimatization period, the larvae were fed with brewer's yeast. The humidity and light conditions were maintained in a homogenized manner.

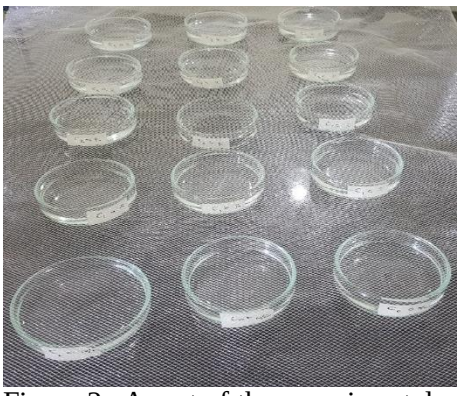


Figure 2. A part of the experimental set up

Morphological Species Identification - Larvae

Stage II and III larvae were placed individually in petri dishes and observed under an inverted microscope (Model-TCM 400) with an objective (×10 and x40). Using standard larval keys developed for Sri Lankan anophelines, the larvae were identified to the species level (Amerasinghe, 1992). Further, larval species identification was reconfirmed through adult identification.

Morphological Species Identification – Adult Mosquito

The adult mosquito was preserved in 70% ethanol and stored in the refrigerator overnight. Thereafter, the mosquito was dissected by using surgical forceps and the parts were mounted by using Canada Balsm. Prepared permanent slides were observed and identified under the light microscope (Model-LX 300). The mosquito was identified to species level by using standard illustrated adult mosquito keys (Gunathilaka, 2017).

Preparation of plant extract

Plant leaves (Figure 3) were initially washed with tap water and allowed to air dry for five days in a shaded environment at room temperature.

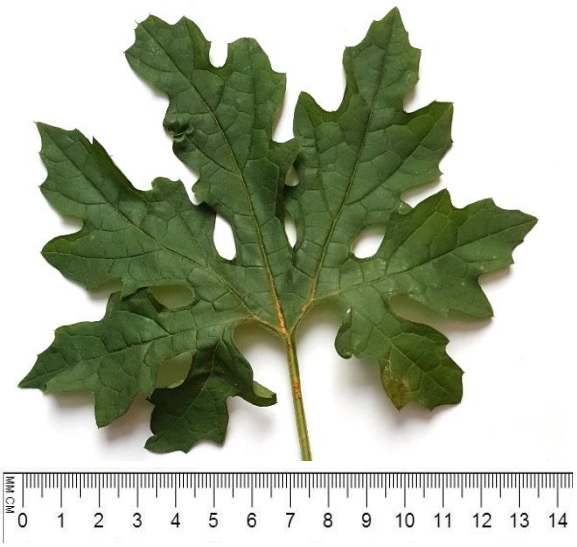


Figure 3. Leaf of Momordica charantia

The dried leaves were powdered mechanically using an electric grinder (Model- LS750 W5). The powdered product was sealed in an air tight polythene bag wrapped in aluminium foil and stored in the refrigerator at +4°C prior to extraction.

The methanol extract of the plant was prepared by dissolving 40g of dried powder in 100 ml of solvent. The mixture was completely covered in aluminium foil left in the dark at room temperature for 24 hours with shaking at regular intervals. Thereafter, the mixture was subjected stirring at 500 rpm for 20 minutes in a magnetic stirrer (Model-2ML). The solution was filtered using a glass funnel and Whatman no. 1 filter paper. Filtrates were dried in the fume hood. The evaporated plant extract was transferred into an airtight container covered in aluminium foil and was refrigerated at +4o C until further use.

A 10 mg/ml (1%) stock solution was prepared by dissolving 100 mg of Momordica charantia methanol extract in 100 µl of DMSO. Once the residue was completely mixed, it was dissolved by adding de-chlorinated water. The stock solution was vigorously vortexed to obtain a homogenized mixture.

Larvicidal Bioassay test

Stage II Instar larvae were sorted and chosen for the assay. Three sets of larval triplicates (n=3) were transferred into the containers and treated with different concentrations of the Momordica charantia methanol extract (Table 1). The bioassay for the larvicidal activity was carried out by using the WHO procedure with slight modifications.

The control set was prepared by adding 0.1% DMSO. The exposed larvae were kept in a cool, low light area in the lab for 24 hours in an unfed state. All containers covered with a net to reduce evaporation of the de-chlorinated water and to facilitate oxygen dissolving. The mortality were recorded following 24 hours and 48 hours of exposure. Moreover, to analyze the behavioral changes, the response to tactile stimuli were assessed.

Table 1. Group and treatment for larvicidal bioassay

Treatment	**Concentration of Momordica charantia (ppm)**
C0 [control]	0
C1	25
C2	50
C3	80
C4	100

Histological Studies

For histological studies, live larvae were obtained following 48 hours of exposure to Momordica charantia methanol leaf extract. The larvae were fixed in 5 ml of 10% neutral buffered formalin and refrigerated. Following the dehydration in a graded ethanol series of 70%, 80%, 90%, and two washes in 100% ethanol, the larvae were bathed in two washes in xylene (Refer appendix). Thereafter, the larvae were subjected to two paraffin wax baths at 58-60o C, before infiltration. By using a wax dispenser (Model-TEC-2000), the larvae was embedded in paraffin. Once solidified, the paraffin block was kept at +4ºC until use. The paraffin block was sectioned using rotary microtome (Model-F163851404) at 5 µm. Sections were mounted on to glass slides which were homogenizedly coated with egg albumin. The slides were placed on the slide dryer (Model-SD-2800) at 38o C for 30 minutes. Thereafter, the slides were observed under the light microscope (Model-LX300) at objective 40x to ensure the presence of larval tissue.

The larval tissues were stained using Hematoxylin and Eosin staining procedure (Refer appendix) and observed under the light microscope (Model-LX300). The histopathological effect on mid-gut epithelium of larvae and morphological aberration induced by the methanol extracts of Momordica charantia leaves were examined.

Statistical Analysis

The experimental data were expressed as mean % mortality ± SE of three replicates at each concentration. The data were statistically analyzed using multiple factor ANOVA (Analysis of Variance) using the statistical software SPSS, in which the significance was considered at a 95% confidence level ($P<0.05$). Furthermore, individual mean significant difference was calculated by using post hoc Tukey test by using SPSS software. Additionally, using Microsoft Excel, the data were subjected to log–probit regression analysis to calculate the median lethal concentrations, LC50 at 95% confidence intervals.

RESULTS

Species Identification

Morphological Species Identification - Larvae

The water dwelling larvae were observed to lie parallel to the water surface, which is a characteristic feature of Anopheles genus. Three body regions, head, thorax and abdomen were distinct as shown in Figure 4

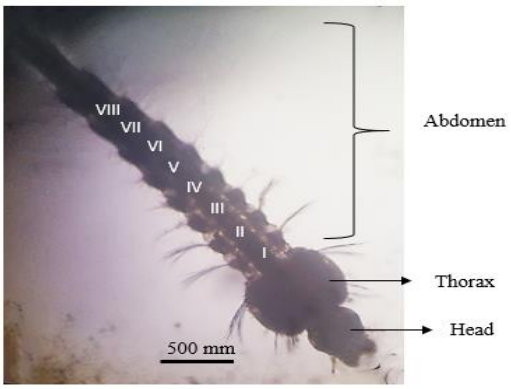


Figure 4. *Light Microscopic view of Anopheles stage II instar larvae (100x).*

(A) ***(B)***

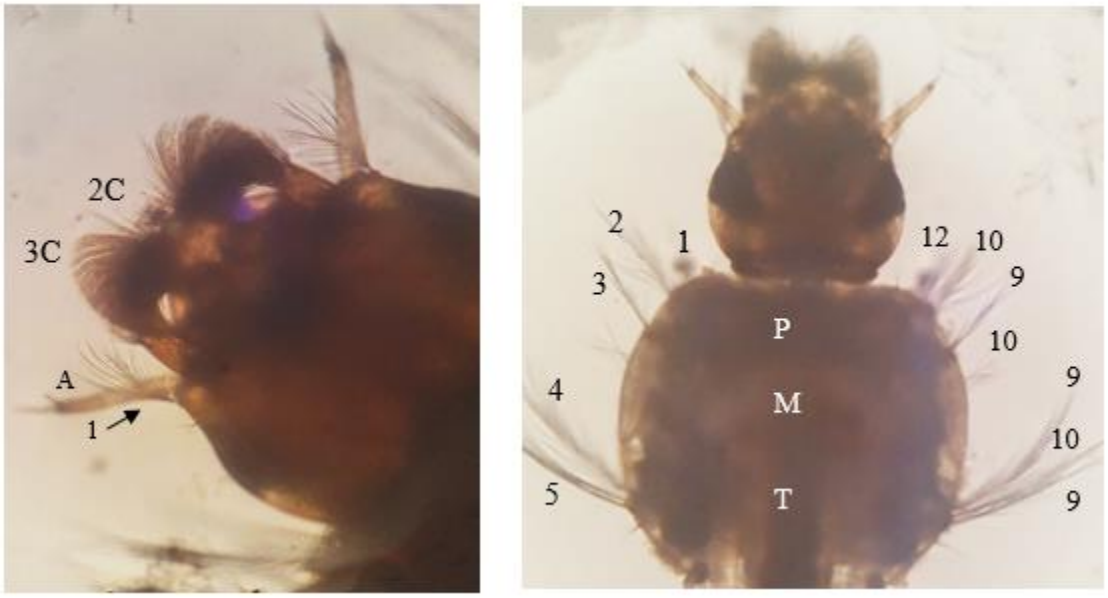


Figure 5. (A) Dorsal view of head (400x) (B) Ventral view of prothorax and thorax (400x)

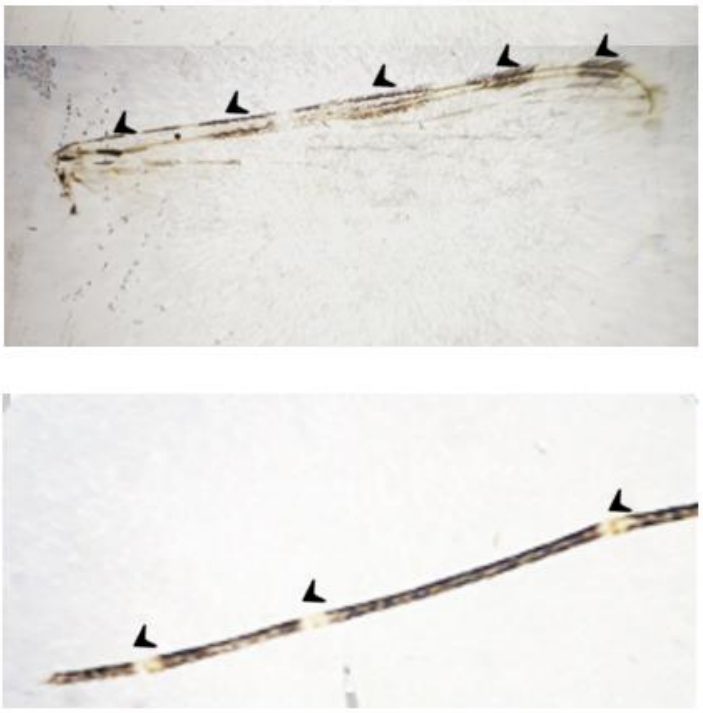
Morphological features including, Seta 1-A simple; 2-C inserted at least as far apart as the distance between 2-C and 3-C on one side, in the dosal view of head as shown in (Figure 5A) were observed. Thus confirming the subgenus Cellia. (Amerasinghe, 1992).

In the ventral view of the thorax, the following morphological features were observed, long thoracic pleural setae 9, 10, 12-P, 9, 10-M and 9, 10-T simple as shown in (Figure 5B). Hence the taxonomic series was identified as Neomyzomyia. Anopheles tessellatus and Anopheles elegans have been reported under Neomyzomyia series in Sri Lanka (Amerasinghe, 2014).

Morphological characteristics observed in the ventral view of prothorax, including Seta 1-P weak, 2-5 branched; 1, 2-P arising from separate basal tubercles and only tubercle of 2-P prominent and sclerotized, as shown in (Figure 5B). Based on these features, the subgenus was identified to be Anopheles Tessellatus. Species identification was further confirmed through adult identification (Amerasinghe, 2014).

Morphological Species Identification – Adult Mosquito

(A)



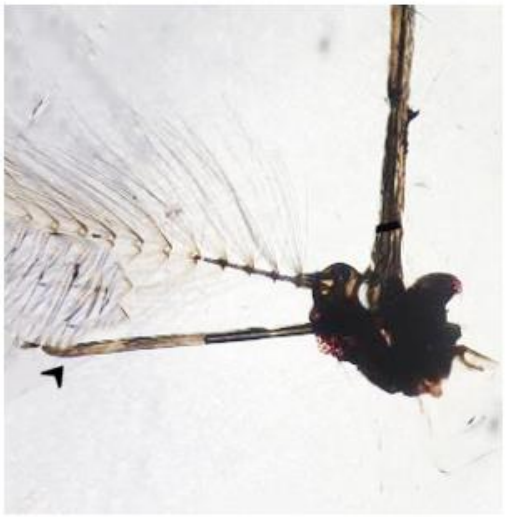
(B)

Figure 6. (A). Mosquito Wing (200x) (B) Femur and tibia speckled (400x)

The morphological characteristics of the mosquito wing including the presence of four or more darks marks involving both costa and veins R-R1 accessory sector pale (ASP) spot present on costa and/or subcostal, as shown in (Figure 6A) These features aided in distinguishing subgenus Cellia from those of subgenus Anopheles (Gunathilaka et al., 2014).

Four taxonomical series of Sri Lankan anophelines belong to the subgenus Celia. Among which Anopheles elegans and Anopheles tessellatus, members of the Neomyzomyia series, Anopheles Maculatus and Jamesii group, belonging to Neocellia series exhibit speckled femur and tibia as shown in (Figure 6B).

(A)



(B)

Figure 7. (A) (B) Apical half of the proboscis pale scaled (400x)

Based on the characteristics of apical half of the proboscis pale scaled, as shown in figure 7, the species was confirmed to be Anopheles tessellatus (Gunathilaka, 2017).

Larvicidal Bioassay

The larval mortality of Anopheles tessellatus after treatment with methanol leaf extract of Momordica charantia was observed. Table 2 shows the larval mortality of Anopheles tessellatus (II instar) after treatment at different concentrations (250 – 1000 ppm).

Table 2. Larval mortality of the methanol extract of Momordica charantia leaf against Anopheles tessellatus

Treatment	***Momordica charantia* methanol extract** **Concentration (ppm)**	**Mean % Mortality ± SE**	
		24 hrs	**48 hrs**
N/C	0	0 ± 0	0 ± 0
C1	250	11.11 ± 11.00	55.56 ± 11.33
C2	500	22.22 ± 11.00	66.67 ± 19.34
C3	800	77.78 ± 22.33	100 ± 0
C4	1000	100 ± 0	100 ± 0

The larval mortality was recorded at 24 hour and 48 hours after exposure to methanol extract of *Momordica charantia* leaf. The results are presented in the graph in (Figure 8).

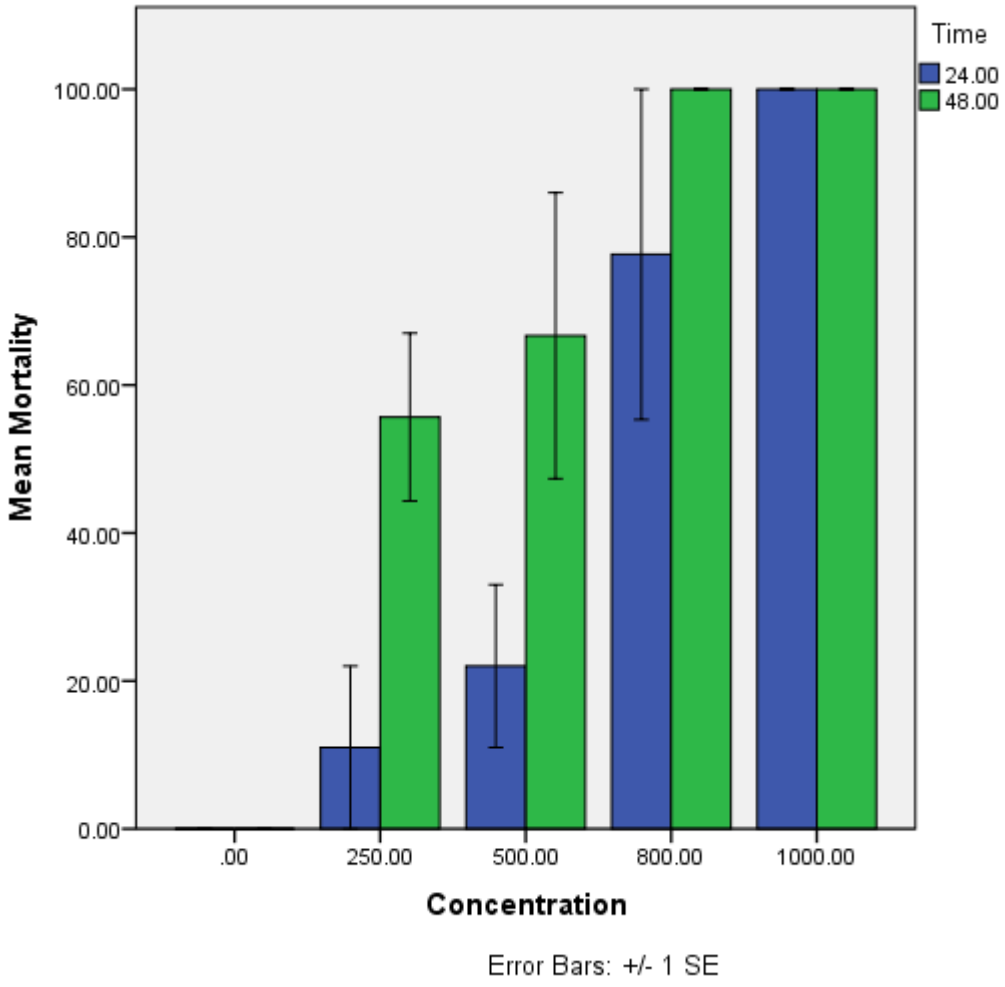


Figure 8. *Graphical representation of the mean percentage mortality of Anopheles tessellatus after 24 hr and 48 hr of exposure between five different concentrations of Momordica charantia methanol leaf extract. Mean % mortality at each exposure time at each concentration ± SE is presented.*

The graph indicated that the percentage mortality of Anopheles tessellatus increased with increasing concentration and increased time of exposure to the plant extract ($P<0.05$, Tukey's pairwise tests after one-way ANOVA). The replicates with the highest concentration of the extract, 1000 ppm, showed maximum % mortality. The lowest concentration of 250 ppm showed lowest % mortality while the control larvae showed 100% survival. At the highest concentration (1000 ppm), the

% mortality of the larvae was significantly different with other concentrations including control (p=0.000), 250 ppm (p=0.000) and 500 ppm (p=0.001) (P<0.05, Tukey's pairwise tests after one-way ANOVA).

Moreover, statistical data clearly indicated (Table 3) a significant increase in % mortality with exposure time period (P<0.05, Tukey's pairwise tests after one-way ANOVA). The highest % mortality was detected in every concentration apart from control in 48 hours of exposure.

Table 3. Summary of the two-way ANOVA balanced design between the Momordica charantia methanol leaf extract concentrations and the time of exposure upon the percentage mortality of Anopheles tessellatus larvae.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Time	3740.833	1	3740.833	10.029	.005
Concentration	40580.800	4	10145.200	27.199	.000
Time * Concentration	2992.667	4	748.167	2.006	.132
Error	7460.000	20	373.000		
Total	140001.000	30			
Corrected Total	54774.300	29			

Median Lethal Concentration (LC50)

Table 4. Log-Probit regression

Time (hrs)	LC_{50}	Regression Equation	R^2
24	251 ppm	y = 2.1769 x -0.1733	0.94
48	126 ppm	y = 2.3398 x – 0.1475	0.95

Behavioral changes in larvae

Physiological parameters of the de-chlorinated water including, temperature and pH were recorded as 28 ^{0}C and 6.6, respectively. Following 24 hours of exposure, the control larvae were rapidly responding to tactile stimuli and were alternating between the surface and the bottom of the container. At 250, 500 and 800 ppm the larvae were lethargic and moderate response to stimuli were observed. At 1000 ppm, 100 % mortality and immobility were observed and thus larvae remained at the bottom of the container and were unresponsive to stimuli.

Following 48 hours of exposure, the control larvae rapidly responded to tactile stimuli and were alternating between the surface and the bottom of the container. At 250 and 500 ppm, the larvae were moribund, incapable of rising to the surface and not showing characteristic diving action when the water was disturbed. At 800 and 1000 ppm, 100 % mortality and immobility were observed. The larvae did not induce movement when probed in the abdomen region.

Histological Effects

The histological effects of *Anopheles tessellatus* larvae treated with *Momordica charantia* methanol extract were compared with untreated larvae (Figure 9 and 10).

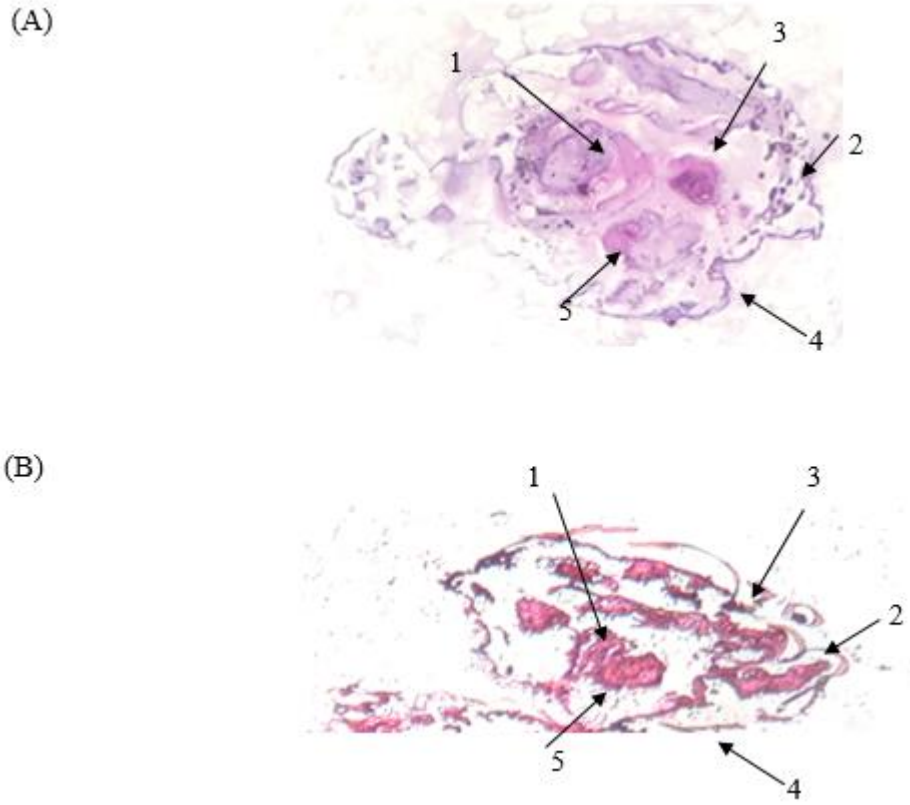
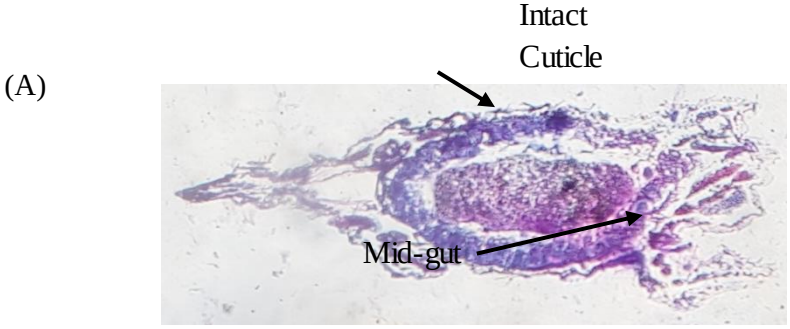


Figure 9. (A) Cross section of untreated Anopheles tessellatus larvae (200x) (B) Cross section of Anopheles tessellatus larvae treated with 500 ppm methanol extract of Momordica charantia leaf, showing the effect after 48 hours of exposure (200x). 1. Midgut and peritrophic matrix; 2. Wing imaginal disc; 3. Gastric caeca; 4. Leg imaginal disc; 5. Thoracic ganglion

The results of the histological studies confirmed that Momordica charantia methanol extract caused distinguished damaged to the cuticle leading to completely destroyed and shrunken cells. When treated with Momordica charantia, the midgut epithelium and caeca of the larval tissue was affected, as indicated in (Figure 10).



(B)

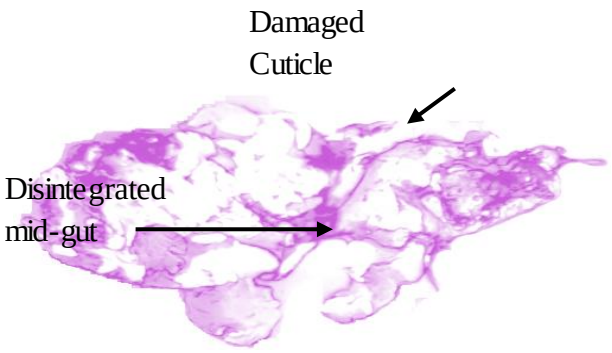


Figure 10. (A) Longitudinal section of untreated Anopheles tessellatus larvae showing intact thick cuticle layer (200x) (B) Longitudinal section of Anopheles tessellatus larvae treated with 500 ppm of Momordica charantia methanol plant extract (200x) , showing the effect after 48 hours of exposure.

DISCUSSION

In the emerging field of research, in mosquito control programmes, botanical phytochemicals have been recognized as potential alternatives to synthetic insecticides, due to their larvicidal properties (Zhao et al., 2017). The present study was undertaken to assess the larvicidal potential of the methanol extracts of Momordica charantia leaf against Anopheles tessellatus. Targeting the stationary larvae phase with plant-derived larvicides is desirable as this will supress the fecundity of mosquitoes.

A number of compounds, such as flavonoid, glycosides, alkaloid, and saponin have been isolated from Momordica charantia and have reported to exhibit larvicidal activity (Mala and Tulika, 2014). Additionally, momordicin I and momordicin II, have exhibited anti-feedant and larvicidal activity in insects (Ling et al., 2008; Li et al., 2015). Moreover, terpenoid compounds including, momordicside-L and momordicoside-K, have demonstrated significant mortality in Armyworm larvae (Wardhani, Abadi, and Himawan, 2015). Furthermore, bioactive compounds of Momordica charantia including, apigenin and tannins have established potential anthelmintic effect (Vedamurthy et al., 2015).

The phytochemical components in Momordica charantia could contain polar and non-polar compounds, which have to be dissolved in solvents in order to release its chemical constituents (Ghosh, Chowdhury, and Chandra, 2012). Methanol was selected as the solvent of choice, based on the polarity and the nature of phytochemicals present in Momordica charantia. Since methanol has a polarity of 5.1, it is capable of extracting various polar and certain non-polar components. Therefore, the methanol extract of Momordica charantia dissolved in DMSO stock solution, revealed a significant difference ($p<0.05$) between percentage mortality and concentration. Hence, the bioassay showed potent larvicidal activity (Edriss, Satti and Alabjar, 2013).

The LC50 value of the present study is nearly similar to literature values. Rahuman and Venkatesan have reported that the methanol extract of Momordica charantia were highly effective against the larvae of A. aegypti (LC50= 199.14±13.13 ppm), at 24 hours of exposure Similarly, the larvicidal efficacies of methanol extracts of Momordica charantia, tested with LC50 values were 207.61±13.50 ppm, against the late third larval age group of C. quinquefasciatus (Rahuman and Venkatesan, 2008). Thus, a significant number of studies demonstrate that the leaf extracts of Momordica charantia are toxic to larval species. Momordica charantia may, therefore, act as an effective bio-larvicide against mosquitoes in the future (Singh, Dhiman and Mittal, 2006).

According to the results of the probit analysis at 95% confidence interval, the LC50 had decreased over time. Hence an increase in toxicity of the Momordica charantia methanol extract was observed at longer exposure period compared to short exposure period. This implies that the chemical constituents in the Momordica charantia methanol extract are released at a steady rate over a period of time (Ramalingum and Mahomoodally, 2014). As a consequence of exposure to the plant extract, induction of apoptosis in larval tissue could occur during long exposure periods (Dusfour et al., 2015). A significant mean percentage mortality was observed at the concentration with close proximity to the LC50, hence the larvae were evaluated for possible histological changes.

The most affected tissue was the mid gut epithelium when compared with the untreated mid gut. The untreated mid gut consists of a unicellular epithelium layer resting upon a basement membrane. Histopathological alteration were seen in the mid-gut of the treated larvae, including separation of the epithelial cells from the basement membrane. However, in the treated mid-gut the cells were not pronounced and could not been identified in some areas at the base of the epithelial cells due to the sever destruction of the epithelium. Epithelial cells of the treated larvae were destroyed, large vacuoles were present in the cells, irregular and disappear cell boundaries were observed. Additionally, the cells appeared dislodged, sloughed and detached from each other. The control larvae revealed an intact cuticle whereas the cuticle in treated larvae appear to be discontinuous (Farnesi et al., 2012). This is consistent with the histological changes observed when mosquito larvae were exposed to plant chemicals (Sarra et al., 2017; Yu et al., 2015). Several reports suggested that the larvicide substances lead to morphological damage in epithelial cells of the mid-gut which are likely where these substances are absorbed (Perumalsamy et al; 2013; Al-Mehmadi, and Al-Khalaf, 2010).

The behavioral changes observed, are suggestive that the exposure to Momordica charantia methanol extract impaired the spontaneous motor activity of the larvae and provoked a change in propulsive movement, which was assessed by the response to sensory stimuli. Hence, it is suggestive that at high concentrations of the Momordica charantia methanol extract, the larvae demonstrates neuro-behavioral effects, including restlessness, loss of equilibrium and death (Bailey, Oliveri, and Levin, 2013).

A study conducted by Liu, evaluated the possible mechanism of action of compounds that demonstrate larvicidal

properties. Alkaloids are nitrogenous compounds present in Momordica charantia, which are active molecules against mosquito larvae. The most common effect Liu and colleagues observed was paralysis of the larval musculature, as demonstrated by the lack of mobility following exposure to Momordica charantia methanol extract. This is possibly due to inhibition of neuro-muscular transmission or disruption of enzymatic activity involved in energy synthesis (Veerakumari, 2015). Larvae that suffered a spastic paralysis assumed a bent or twisted posture, while in flaccid paralysis, the larvae were straight and relaxed (Islam and Bloomquist, 2015). Another proposed mode of action is the acetylcholinestrase (AChE) or sodium channels, as inhibition of acetylcholinesterase activity is responsible for terminating the nerve impulse transmission through synaptic pathway. Alkaloids function by depressing autonomic nervous system activity hence facilitating the plant extracts effectiveness in killing mosquito larvae (Liu et al., 2012). Generally the active toxic ingredients of plant extracts are secondary metabolites. The mosquito larvae feed on these secondary metabolites potentially encountering toxic substances with a wide range of molecular targets (Nagarani, Abirami, and Siddhuraju, 2014). These in turn, affects the larval physiology in various ways at numerous receptor sites, the principal of which is abnormality in the nervous system (Ebadollahi et al., 2014).

The purpose of this study was successfully achieved and Momordica charantia methanol leaf extracts have shown a potential larvicidal activity against Anopheles tessellatus in laboratory experiments. Toxicological studies have revealed that Momordica charantia is safe for human health (Joseph and Jini, 2013; Rawani et al., 2017). The larvicidal property of Momordica charantia can be attributed to the phytochemicals present in the plant. Hence, the formulation of Momordica charantia methanol extracts may potentially be used as an effective and eco-friendly larvicide, which could be an alternative to malaria control.

Several other aspects of this research can be further studied. These include determination of the larvicidal activity of different solvent extracts of Momordica charantia plants on Anopheles species to identify the most efficient solvent for extraction. Furthermore, isolation and purification of compounds present in Momordica charantia methanol extract using column chromatography could be performed in order to confirm the bioactive compounds responsible for the larvicidal property. Moreover, the larvicidal activity of the methanol extract of Momordica charantia could be exploited for use in potable waters against mosquito larvae. Implementation of field trials could be performed to assess the efficacy and cost-effectiveness of this method of mosquito control.

In conclusion, an attempt was made to evaluate the role of plant extracts in mosquito larvicidal activity. The results of the present study may encourage further researches on using simple and inexpensive application methods for controlling mosquitoes in their breeding sites (Soleimani-Ahmadi et al., 2017).

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APPENDIX

Dehydration procedure for Histological studies of larval tissues

Alive larvae were fixed in 5 ml of 10% neutral buffered formalin and refrigerated (2-24 hours). The dehydration procedure was carried out in a graded ethanol series, 70% Ethanol for 40 minutes, 80% Ethanol for 30 minutes, 90% Ethanol for 30 minutes, 100% Ethanol for 30 minutes and 100% Ethanol for 1 hour. Thereafter, the larvae were subjected to two washes in Xylene, for 7 and 15 minutes, respectively.

Hematoxylin and Eosin staining procedure (H & E Staining)

In order to remove paraffin, slides containing larvae sections were bathed in the following series of solutions: xylene (2 washes of 5 minutes each), 100% ethanol (two washes of 3 minutes each), 95% ethanol (3 minutes), 70% ethanol (3 minutes) and distilled water (30 seconds). The HE staining was initiated with a bath in hematoxylin solution (45 seconds), followed by tap water, and distilled water for 2 minutes. To remove excess hematoxylin the slides were bathed in 1% acid alcohol for 1 minute and 30 seconds, followed by 70% ethanol (3 minutes), eosin solution (6 seconds), 95% ethanol (30 seconds), 100% ethanol (2 washes of 3 minutes each) and xylene (2 washes of 3 minutes each). After the xylene bath, slides were mounted with cover slips, using Canada Balsm.


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