Larvicidal against mosquito vectors and brine shrimp activities of extracts from the flowers of *Moringa oleifera* Lam

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ABSTRACT

This paper reports the larvicidal and cytotoxicity activity of *Moringa oleifera* flowers extracts against laboratory reared late third instars larvae of mosquito vectors (*Anopheles gambiae* Giles s.s, *Culex quinquefasciatus* Say and *Aedes aegypti*) and brine shrimps respectively. The larvicidal assay was according to WHO protocols. The petroleum (MOFP), ethyl acetate (MOFE) and methanolic (MOFM) demonstrated larvicidal activity with LC_{50} ranges of 9.220-859 µg/mL, 15.82-500 µg/mL and 175.47-513.156 µg/mL against *A. gambiae*, *A. aegypti* and *C. quinquefasciatus* respectively. The Petroleum ether extract exhibited higher activity compared to other extracts with LC_{50} values of 9.22 µg/mL and 15.82 µg/mL against *A. Gambiae* and *A. aegypti*, respectively. The activity against *C. Quinquefasciatus* was low to all extracts to the extent that there was no mortality observed after 48 hours with the exception of MOFE which had LC_{50} value of 367.068 µg/mL. All extracts exhibited low toxicity to brine shrimps with LC_{50} value > 100 µg/mL. The *M. oleifera* flowers are therefore potential source of botanical larvicidal agents.

KEY WORDS: *Moringa oleifera; Anopheles Gambiae*; *Culex quinquefasciatus; Aedes aegypti;* larvicidal

{Citation: John Wilson Nkya, Paul Erasto, Musa Chacha. Larvicidal against mosquito vectors and brine shrimp activities of extracts from the flowers of *Moringa oleifera* Lam. American Journal of Research Communication, 2014, 2(8):15-29} www.usa-journals.com, ISSN: 2325-4076.

INTRODUCTION

Mosquitoes are insect vectors responsible for the transmission of parasitic and viral infections to millions of people worldwide with substantial morbidity and mortality (Petrić, 2010). It is believed that, mosquitoes transmit more diseases than any other group of arthropods to both human and livestock throughout the world (Roger and Randolph, 2000; Nyamoita *et al.*, 2013). The most common mosquito vectors responsible for transmission of infectious diseases are *Anopheles gambiae, Aedes aegypti* and *Culex quinquefasciatus* Say which transmits malaria, yellow fever and lymphatic filariasis respectively (Arivoli and Samuel, 2011). The vector-borne diseases caused by different species of mosquitoes constitute an unsurpassed health problem all over the world especially sub-Sahara Africa (Craig *et al.,* 1999). For instance, malaria alone, is one of the most important causes of direct and indirect infant, child and adult mortality with approximately two to three million new cases arising every year in Tanzania (Sachs and Malaney, 2002; Olsen *et al.,* 2002).

According to current estimate, around 2.5 billion people are at now risk of dengue, which has become the most rapidly spreading mosquito-borne viral disease (Lalchhandama, 2011). There have been recurrent outbreaks of dengue fever in Tanzania April 2014 associated with rapid increase and spread of *A. Aegypti* in different part of the country, Dar-es salaam been the most affected province, the total of 757 cases with 460 confirmed have been reported including 3 deaths (WHO, 2014). Similarly, lymphatic filariasis and a number of arboviruses including St. Louis encephalitis virus and West Nile virus (Kamaraj *et al.,* 2008), are among the tropical diseases with around 120 million people infected in the world (Anees, 2008) and approximately 44 million people having common chronic manifestation (Rahuman *et al.,* 2009). This calls for the immediate action to safeguard the life of people and consequently the economy of the developing countries affected with diseases transmitted with mosquitoes.

The use of different parts of plants as mosquito repellents has been well documented (Cavalcanti *et al.,* 2004; Markout *et al.,* 2000; Prabakar and Jebanesan, 2004; Thomas *et al.,* 2004). From these plants, secondary metabolites with insect growth regulator property, repellent activity and ovipositor attractant activity have been reported (Patil *et al.,* 2010; Das *et al.,* 2007). Thus screening for indigenous plants for possible larvicidal activity offers a promising endeavour. It is

in this vein that *Moringa oleifera* (Moringaceae) flower petroleum ether, ethyl acetate and methanolic extracts were screened for possible larvicidal and cytotoxicity activity and the findings are reported in this paper.

MATERIALS AND MEDHODS

Chemicals and organisms tested

Ethanol (absolute), petroleum ether and ethyl acetate were bought from Fluka Chemie GmbH (Sigma-Aldrich®, Zwijndrecht, Netherlands). Dimethyl sulfoxide (DMSO) was purchased from Sigma® (Poole, Dorset, UK). Cyclophosphamide was purchase from Khandelwal laboratories pvt ltd, 79/87 D.LADPATH, Mumbai 400033 India. The Brine Shrimps eggs were purchased from Aquaculture innovations (Grahamstown 6140, South Africa) and sea salt was prepared locally by evaporating sea water collected from the Indian Ocean, along the Dar es Salaam Coast. *Culex quinquefasciatus, Anophelles gambiae* and *Aedes aegypti* larvae were obtained and reared at the Tropical Pesticides Research Institute (TPRI) Arusha, Tanzania.

Collection and preparation of plant materials

Health flowers of *M. oleifera* were detached from the inflorescence rachis at the joint in the pedicel, from domesticated *M. oleifera* tree farms at Lushoto District, Tanga Region, Tanzania. The plant was identified by Mr. Haji Seleman, a botanist from the Department of botany, University of Dar es Salaam and the voucher specimen (MOLT 2143) was kept at Nelson Mandela African Institute of Science and Technology, Arusha. Flowers were dried under room temperature and pulverized at the Institute of Traditional Medicine, Muhimbili Health and Allied Sciences (MUHAS).

Extraction process

The pulverised flower of *M. oleifera* (1000 g) was sequentially macerated using petroleum ether, ethyl acetate and ethanol for 48h twice for each solvent. The respective extracts were filtered through muslin cloth on a plug of glass wool in a glass column and solvents were evaporated in *vacuo* using a rotary evaporator and stored in refrigerator at -20°C.

Larvicidal activity assay

The larvicidal test was performed according to World Health Organization (WHO) protocol (WHO, 1996) with minor modification. In this protocol, mosquito larvae were reared at a fixed density of 100 larvae per tray and fed with tetramine® for *A. gambiae* and dog biscuits for *C. quinquefasciatus* and *A. aegypti* during the whole time of experiment (Kasap and Demirhan, 1992). The reason of targeting mosquito at the larval stage is that, they cannot escape from their breeding sites until the adult emerges (Rajkumar and Jebanesan, 2009). 100 mg of the plant extracts were dissolved in 1 mL of DMSO making up a stock solution of 100mg/mL. From the stock solution, different concentration levels of 50, 100, 250, 500, and 1000 µg/mL were prepared by serial dilution. Ten late third instars laboratory reared *C. quinquefasciatus, A. gambiae and A. aegypti* mosquito larvae were then introduced in the test solution and mortality was observed after 24h, 48h and 72h. Negative control tests contained mosquito larvae, DMSO (0.5%) and water only. All tests were carried out in triplicate for statistically significant results under controlled temperature (25 ± 2°C) and relative humidity of 75-85%. The larvae were considered dead if they were immobile and unable to reach the water surface when probing them by the Pasteur pipette (Andemo *et al.,* 2014). The mean results of the percentage mortality were plotted against the logarithms of concentrations using the Fig P computer program (Biosoft Inc, USA). The concentrations killing fifty percent of the larvae (LC_{50}), confidence interval (C.I) and regression coefficient (R^2) were calculated from the regression equations obtained from the graphs.

Brine shrimp test (BST)

The brine shrimp lethality test (BST) as described by Meyer *et al.*, (1982) was adopted with minor modification. In this assay, brine shrimp eggs were placed in sea water (3.8 g/L of distilled water) and incubated. Eggs were hatched within 48 h, providing a large number of brine shrimp larvae (nauplii). 40 mg of the plant extracts were dissolved in 1 mL of DMSO making up a stock solution of 40mg/mL. From the stock solution, different concentration levels were prepared by serial dilution and 10 nauplii were introduced into 10 ml vials. The volume was then adjusted to 5ml with artificial sea water (3.8% w/v sea salt in distilled water). Each concentration was tested in triplicates with two negative controls running simultaneously containing 10 nauplii, sea water and 5% DMSO only for comparison, Cyclophosphamide was used as a positive control. The

vials were incubated under light for 24 h. The average number of dead larvae in each triplicate was recorded after 24h.

Data analysis

The mean results of the percentage mortality were plotted against the logarithms of concentrations using the Fig P computer program. Regression equations obtained from the graphs were used to obtain LC_{16}, LC_{50}, LC_{84} and the 95% CI values (Litchfield and Wilcoxon, 1949). For the brine shrimp assay, extracts with LC_{50} value greater than 100 µg/mL were considered non toxic (Gupta et al., 1996). For larvicidal assay, extracts with LC_{50} greater than 750 µg/mL were considered non-toxic, weakly effective when the LC_{50} ranges from 200 to 750 µg/mL, moderate when the LC_{50} ranges from 50 to 100 µg/mL and high when the LC_{50} is less than 50 µg/mL (Komalamisra *et al.* 2005).

RESULTS

Larvicidal activity of *Moringa oleifera* flower extracts.

The larvicidal nature of flower extracts of *M. oleifera* showed greater impacts on the development and survival of third instars larvae of *A. gambiae, A. aegypti* and *C. quinquefasciatus*. Severe lethal effects were observed showing an increasing trend towards higher concentration in dose and time dependent manner as shown in Table 1, 2 and 3. *Moringa oleifera* flower petroleum ether extract (MOFP) exhibited maximum larvicidal activity against *A. gambiae,* and *A. aegypti* larvae with LC_{50} values of 9.22 µg/mL and 15.82 µg/mL respectively (Table 1). *C. quinquefasciatus* larvae were the most resistant (Table 3) against the tested extracts as compared to *A. gambiae, A. aegypti*. The larvicidal activity extracts against *C. Quinquefasciatus* was generally low to the extent that there was no mortality observed after 48 hours with the exception of MOFE which had LC_{50} value of 367.068 µg/mL. Even after 72 hours, MOFE was relatively the most active (LC_{50} value of 175 µg/mL) followed by MOFM (LC_{50} of 366.385 µg/mL) and MOFP was the least with LC_{50} value of 513.156 µg/mL.

The negative control cohorts showed no mortality during the time of experiment, the larvae developed into pupae and then adults within 48–72h. Despite the fact that *C. Quinquefasciatus* was resistant against the tested extracts, *A. Gambiae* and *A. Aegypti* were strongly inhibited by the tested extracts.

Brine shrimp activity of *Moringa oleifera* flower extracts

Moringa oleifera flower petroleum, ethyl acetate and methanolic extracts exhibited lethality activity against the brine shrimps with the LC_{50} values of 697.18, 350.57 and 1027 µg/mL (Table 4). Since the LC_{50} values observed were higher than 100 µg/mL, *Moringa oleifera* flower extracts were considered non-toxic to brine shrimp larvae. The upper limit for extracts to be considered toxic against brine shrimp larvae is 100 µg/mL (Meyer *et al.,* 1982).

Table 1: Larvicidal activity of *Moringa oleifera* extracts against *Anopheles gambiae*

Extract code	Time of Observation	LC_{50} µg/ml	95% (UCL – LCL)	R^2	Regression equation
MOFP	24h	489.900	220.450-1086.470	0.9853	Y=27.187logx-23.124
	48h	24.178	11.754 -47.840	0.9846	Y=31.771logx +6.0476
	72h	9.220	4.8079-17.680	0.9456	Y=25.816logx+25.095
MOFEA	24h	278.350	213.310-383.030	0.9371	Y=70.294logx-121.84
	48h	152.810	111.500-216.050	0.9371	Y=68.561logx-99.748
	72h	87.140	66.85-110.730	0.9747	Y=72.454logx-90.575
MOFM	24h	859.085	460.63-1602.190	0.9796	Y=34.792logx-52,081
	48h	25.461	13.997-46.314	0.9295	Y=36.249logx-0.9613
	72h	15.178	7.696-29.934	0.9450	Y=31.933logx+12.28
Control (-VE)	NM	-	-	-	-

KEY; CI = confidence interval, R^2= regression coefficient; Y=Regression equation, NM= No mortality at all levels of concentration tested; LC_{50} = lethal concentration (concentration to kill 50% of test organisms); UCL = Upper Confidence limit; LCL = Lower Confidence limit; MOFP =*M. oleifera* flower, petroleum ether extract; MOFEA=*M. oleifera* flower, Ethyl Acetate extract; MOFM=*M. oleifera* flowers, methanolic extract.

Table 2: Larvicidal activity of *Moringa oleifera* extracts against *Aedes aegypti*

Extract Code	Time of Observation	LC_{50}µg/mL	95% (UCL – LCL)	R^2	Regression equation
MOFP	24h	737.340	456.560-1190.800	0.9804	Y=45.234logx-79.716
	48h	25.163	15.068-42.022	0.8497	Y=42.284logx-9.2302
	72h	15.820	8.690-28.760	0.7856	Y=36.24logx+6.5387
MOFEA	24h	500.993	315.686-795.076	0.9958	Y=46.967logx-76.803
	48h	369.022	245.035-555.747	0.9967	Y=52.921logx-85.851
	72h	219.444	148.978-229.010	0.9885	Y=56.032logx-81.189
MOFM	24h	462.922	313.208-684.199	0.9144	Y=55.499ogx-97.93
	48h	171.920	127.730-231.400	09846	Y=73.023logx-113.23
	72h	107.010	81.750-139.650	0.9843	Y=81.088logx-114.56
Control	NM	-	-	-	-

KEY; LC_{50} = lethal concentration (concentration to kill 50% of test organisms); UCL = Upper Confidence limit; LCL = Lower Confidence limit; R^2=regression coefficient; Y=Regression equation, NM= No mortality at all levels of concentration tested; MOFP =*M. oleifera* flowers, petroleum ether extract; MOFEA=*M. oleifera* flowers, Ethyl Acetate extract; MOFM=*M. oleifera* flowers, methanolic extract.

Table 3: Larvicidal activity of *Moringa oleifera* extracts against *Culex quinquefasciatus*

Extract Code	Time of observation	LC_{50}µg/mL	95% (UCL –LCL)	R^2	Regression equation
MOFP	24h	NM	-	-	-
	48h	NM	-	-	-
	72h	513.156	356.358-737.41	0.8743	Y=59.81logx-112.1
MOFEA	24h	NM	-	-	-
	48h	367.068	271.700-495.909	0.9241	Y=72.01logx-134.69
	72h	175.470	135.08-227.94	0.8896	Y=82.85logx-135.93
MOFM	24h	NM	-	-	-
	48h	NM	-	-	-
	72h	366.385	272.079-492.421	0.838	Y=73.27logx-137.87
CONTROL(-VE)	NM	-	-	-	-

KEY; LC_{50}= Lethal concentration (concentration to kill 50% of test organisms); UCL= Upper Confidence limit; LCL= Lower Confidence limit; R^2= regression coefficient; Y= Regression equation; NM= No mortality at all levels of concentration tested; MOFP= *M. oleifera* flowers petroleum ether extract; MOFEA= *M. oleifera* flowers, Ethyl Acetate extract; MOFM=*M. oleifera* flowers, methanolic extract.

Table 4: Lethality assay of *Moringa oleifera* flower extracts against brine shrimp larvae

Extract Code	LC_{50}µg/mL	95%(UCL-LCL)	R^2	Regression equestion	Toxicity Class
MOFP	697.180	381.180-1275.140	0.9365	Y=35.92logx-52.133	NT
MOFEA	350.570	203.810-602.960	0.957	Y=39.98logx-51.714	NT
MOFM	1027.250	473.610-2228.110	0.9126	Y=28.00logx-34.336	NT
Control(-Ve)	NM	-	-	-	NT
Control(+Ve)	16.255	12.006 - 22.305	0.9949	Y = 69.9logx -34.936	T

KEY; LC_{50}= Lethal concentration (concentration to kill 50% of test organisms); UCL= Upper Confidence limit; LCL= Lower Confidence limit; R^2= regression coefficient; Y=Regression equation; NM=No mortality at all levels of concentration tested; NT=Non Toxic; T=Toxic; MOFP=*M. oleifera* flowers; petroleum ether extract; MOFEA=*M. oleifera* flowers, Ethyl Acetate extract; MOFM=*M. oleifera* flowers, methanolic extract.

DISCUSSION

Mosquito borne diseases are one of the most public health problems in the developing countries (Mosley and Chen, 1984). Mosquito can be controlled by preventing mosquito bite using repellent, causing larval mortality and killing mosquitoes (Kumar and Maneemegalai, 2008). In the present study, the findings confirm the presence active ingredients which are responsible for larvicidal characteristics of *M. oleifera* flower petroleum ether, ethyl acetate and methanol extracts. The larvae of *A.gambiae* and *A. aegypti* were relatively more susceptible with good to moderate effect than *C. quinquefasciatus* Say after 24 h, 48 h and 72 h of exposure.

The highest mosquito larvae mortality exhibited with petroleum extracts suggests that non-polar secondary contained in the flowers posses larvicidal activity against *A. gambiae* and *A. aegypti*. Petroleum ether extract had weak larvicidal activity against *C. quinquefasciatus* suggesting that non-polar compounds lacks affinity to *C. quinquefasciatus* receptor sites. It has been established that pyrethrins, pyrethroids, dichlorodiphenyltrichloroethane (DDT) and DDT derivatives induces neurotic effects to insects when they penetrate the cuticle and reach the nervous system (Jukes, 1971). Despite the fact that pyrethrins and DDT analogs have different structural features, they have similar mode of action (Schleier III and Peterson, 2011). It is also possible that the larvicidal compounds contained in the *M. oleifera* flower petroleum ether extract might share the same mechanism of action. Pyrethrins are extracted with a light petrol-based solvent like kerosene from flower heads of *Chrysanthemum cinerariaefolium* (Ginsburg, 1930) and kerosene has almost the same polarity as petroleum ether because both are non polar solvents (Wasewar and Keshav, 2010).

Moringa oleifera flower ethyl acetate demonstrated relatively high activity against *C. quinquefasciatus* as compared to petroleum ether and methanolic extracts. The activity displayed by ethyl acetate extract might be due to the presence of β-sitosterol which has been reported from the flower of *M. oleifera* (Pace-Asciak *et al.,* 1995; Nikkon *et al.,* 2003) and was reported to exhibit larvicidal activity with LC_{50} value of 11.49, 3.58 and 26.67 ppm against *Aedes aegypti* L, *Anopheles stephensi* Liston and *C. quinquefasciatus* Say respectively (Rahuman et al., 2008). *Beta* amyrin, an isolate from the flowers of *M. oleifera* (Pace-Asciak *et al.,* 1995; Nikkon *et al.,* 2003) and exhibited larvicidal activity against malaria vector *A. stephensi* (Kuppusamy *et al.,* 2009) might have contributed to larvicidal activity displayed by *M. oleifera* flower ethyl acetate extract. Also methanolic extract had relatively high activity against *A. gambiae* at 72h, this might be contributed by the presence of 9- Octadecenoic acid which are found in *M. oleifera* flower methanolic extract (Vijay and Samrot, 2010) with larvicidal activities (Paripooranaselvi and Meenakshi, 2012).

Moringa oleifera flower petroleum ether, ethyl acetate and methanol extracts were evaluated for the potential to induce toxicity to humans. Brine shrimp larvae were employed in this assay. Extracts exhibited LC_{50} values higher than 100 µg/mL and are therefore considered to be non-

toxic against brine shrimp larvae. This suggests that secondary metabolites in *M. oleifera* flowers extracts have low toxicity to man and are therefore good candidates for the development of eco-friendly mosquito repellents. Similar results were obtained by Nyamoita *et al.* (2013) where it was reported that, *Vitex schiliebenii* leaves and stem bark exhibit low toxicity to brine shrimp but high larvicidal activity. These results prove that the flower extracts of *M. oleifera* can be used as mosquito repellent without causing side effects to humans.

CONCLUSION

The results from the present study suggest that *M. oleifera* flower petroleum ether and ethyl acetate extracts are potential mosquito repellents. Thus, compounds contained in these extracts might contribute to fight against mosquito borne diseases such as malaria, dengue, yellow fever and lymphatic filariasis.

ACKNOWLEDGEMENT

The authors would like to acknowledge the Commission for Science and Technology (COSTECH) through The Nelson Mandela African Institution of Science and Technology for funding this study. Appreciations are to Mr Abdul Kidukuli of the Institute of Traditional Medicine, Muhimbili University of Health and Allied Sciences, Dar es Salaam for technical support.

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