Moringa oleifera and *Annona muricata* seed oil extracts as biopesticides against the second and fourth larval instar of *Aedes aegypti* L. (Diptera: Culicidae)

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ABSTRACT

Laboratory bioassay was conducted on the 2nd and 4th instar larvae of *Aedes aegypti* (Diptera: Culicidae) to investigate the efficacy of *Moringa oleifera* seed oil (MOSO) and *Annona muricata* seed oil (AMSO) (200, 100, 50, 25, 12.5 and 6.25 µL/mL) along with a control. Inhibition of emergence (IE) and larval mortality were monitored at 3-hours intervals for a period of 24 hrs. Results showed that MOSO at the highest concentration caused 94% and 70% mortality for both 2nd and 4th instar larvae respectively while AMSO gave 100% for both 2nd and 4th instar larvae. The level of IE was 100% for both toxicants after post-exposure culture for 10 days. Nevertheless, the 2nd instar larvae were more susceptible than the 4th instar. The LD_{50} values against the 2nd and 4th instar larvae were 18.19 µL/mL and 54.50 µL/mL for MOSO while AMSO were 9.49 µL/mL and 16.11 µL/mL respectively. AMSO was found to be more effective about twice at all stages than MOSO, therefore both oils show promise as biopesticides against *Ae. aegypti* mosquito larvae although further evaluations need to be carried out especially on the other mosquito genera and species.

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INTRODUCTION

Mosquitoes are important vectors of several tropical diseases including malaria, filariasis and numerous viral disease such as dengue, Japanese encephalitis and yellow fever (Glubler, 1998; WHO, 2002). Different measures targeted against these arthropod vectors have relied heavily on the use of synthetic pesticides resulting in several problems including environmental pollution, undesirable effect on non-target organisms, development of resistance, unacceptable levels of pesticide residue, escalating cost of production among others (Brown, 1986; Mazzari and Georghious, 1995). Hence it is imperative that alternatives measures are developed and put into general use as soon as possible. One potential alternative approach to the use of synthetic pesticides is the use of plant secondary metabolites like active compound and other volatile oils (Ogbonna *et al.*, 2014).

In the recent times, natural products from plants are widely under investigation against insects due to their excellent properties like cheap availability and renewable nature, environmentally friendly and presence of an array of characteristic such as insecticidal, antifeedant, ovicidal and larvicidal effects (Okonkwo *et al*., 2014; Ogbonna *et al.*, 2014). Various plants have been screened for pesticidal effects and other activities, for instance, Okonkwo *et al.* (2014) evaluated the toxicity effect of *Moringa oleifera* seed oil against the 1st and 4th instar larvae of *Aedes aegypti*. Larvicidal activity of *Phytolacca dodecandra* against *Aedes aegypti* was investigated by Akpa *et al.* (2003).

Aqueous extracts of leaves of *M. oleifera*, *Vernonia amygdalina* and *Annona muricata* were evaluated for the control of *Collectotrichum destructivum* on seeds of cowpea (*Vigna uniguculata*) (Akinbode and Ikotun, 2008). Badruddoza and Rahman (2008) reported the larvicidal action of *M. oliefera* root

together with other nineteen Indian plants against two mosquito species, *Ae. albopictus* and *Culex quinquefasciatus*. Donli and Dauda (2002) evaluated the aqueous Moringa seed extract as a seed treatment and bio-fungicide for groundnut in Nigeria. It is evident therefore, that *M. oleifera* has been applied into various uses for the management of pests and vectors of medical, veterinary and agricultural importance. *Annona muricata* on the other hand, is another plant that has shown potentials in pesticidal activities. The plant has shown insecticidal activities against the larva of *Ae. aegypti* (Nwankwo *et al.,* 2012). In India, the seed extracts of *Annona squamosa* showed high level of efficacy (70.8%) in the control of *Boophilus microphis* after 24hrs of treatment (Magadum *et al.,* 2009).

Moringa oleifera and *Annona muricata* have received considerable attention in recent times but more research efforts need to be carried out to explore all their potentials and applications especially in mosquito control. Against these back drops, investigations were conducted to evaluate the larvicidal activity of these two botanicals; *Moringa oleifera* and *Annona muricata* against the 2nd and 4th instar larvae of *Aedes aegypti* mosquito.

MATERIALS AND METHODS

Mosquito collection and rearing

Aedes aegypti eggs were collected and identified at the Federal Ministry of Health, Department of Public Health, National Arbovirus and Vectors Research Center, 33 Park Avenue G.R.A Enugu State Nigeria. They were reared in the laboratory at 29±2^{O}C, 87±5% RH. The eggs were placed in plastic containers containing 500 mL of distilled water. The eggs were allowed to hatch to both 2nd and 4th instar larvae. The larvae were feed with Yale Fortune cabin sweetened biscuit after hatching (Nwankwo *et al*., 2012; Okonkwo *et al.,* 2014).

Processing of moringa and annona seeds

The *M. oleifera* seeds were collected from the moringa plantation in Enugu State, Nigeria while *A. muricata* seeds were obtained from fruits bought from Eke-Awka in Anambra State. The moringa and annona seeds were dried under shade and decorticated to obtain a white and a light-brown kernel respectively. The dried seeds were later pulverized using electric blender to obtain a fine powder.

Extraction of the moringa and annona seed oil

The extraction of the pulverized samples (90 g each) was done using Soxhlet extractor for three hours using petroleum ether (250 mL). This system was heated about 5 cm above a hot electric plate while cold water was allowed to flow in and out the condenser compartment to cool the system. After many refluxes for about three hours, the petroleum ether was gradually evaporated.

Formulation of the oils

Serial dilutions of the oil extract of both the *M. oleifera* and *A. muricata* seed were prepared in acetone. The extracts were taken as 100% concentration which was then diluted serially to 20%, 10%, 5%, 1.25%, and 0.625% by adding 4 mL, 2 mL, 1 mL, 0.5 mL, 0.25 mL and 0.125 mL of the oils respectively using 20 mL syringe yielding 200 µL/mL, 100 µL/mL, 50 µL/mL, 25µL/ml, 12.5 µL/mL and 6.25 µL/mL respectively.

Laboratory *in vitro* larvae bioassay

The different concentrations of the technical material obtained from diluting in acetone were used. Appropriate aliquots of 1 mL dosage in mL/mL of the formula were added in plastic cups containing 2000 mL of distilled water for both Annona and Moringa treatments. Twenty (2nd and 4th) instar larvae of *Ae. aegypti* mosquito were used for the bioassay. Each treatment and control was replicated four times and each bioassay repeated. The bioassay was carried out at laboratory temperature of 29±2^{0}C, 87±5% relative humidity and photoperiod 12:12 light and dark period. Assessments were made at every 3-hour interval; the dead larvae were counted until all test organisms died or survivals observed to emergence (Mulla, 1986; WHO, 1981).

Data analysis

The data obtained were analyzed using GenStat package 9.2 (9th edition). Mortality data obtained were corrected by Abbot Formula (1925) and Log-probit analysis was carried out (Finney, 1971) for determining LD_{50}. The percentage inhibition of emergence (IE%) was based on the initial number of larvae used. Analysis of variance (ANOVA) was also performed on the mortality data and means separated using least significant different (LSD).

RESULTS AND DISCUSSION

The results in table 1 below showed that increase in concentration and time of exposure led to an

increase in mortality of the *Ae. aegypti* larvae. The highest concentration of *M. oliefera* seed oil caused 94% and 70% mortality to the 2nd and 4th instar larvae of *Ae. aegypti* mosquito after 12 hours of exposure, respectively while the least concentration of 6.25 µL/mL gave 40% and 20% mortality to the 2nd and 4th instar larvae, respectively (Tables 1). The LD_{50} (IE_{50}) of MOSO against 2nd instar larvae of *Ae. aegypti* mosquito was obtained as 18.19 µL/mL while that of the 4th instar larvae was obtained as 54.5 µL/mL, respectively.

The Analysis of variance (ANOVA) of the effect of *M. oliefera* against the 2nd instar larvae showed that there was a significant difference (P<0.05, P=0.049) in the mortality obtained by the different concentrations. The mean separation compared to the LSD value showed that the mean mortality of 10.3 obtained at the highest concentration of 200 µL/mL was only significantly different with the least concentration of 6.25 µL/mL (mean mortality 2.2) and the control (P<0.05, P<0.001) (Table 1). The mean mortality of 11.9 obtained by the highest timing of 12 hours was significantly different with mean mortality of 1.4 obtained at the least timing of 3 hours (P>0.05, P=0.085) (Table 1). The separation of the means of the mortality at different time interval showed that the mortality obtained after 9 hours was significantly lower (P<0.05, P<0.001) compared to that obtained after 12 hours (Table 1)

Table 1. The percentage mortality effects of different concentrations of *Moringa oliefera* seed oil on 2nd and 4th instar larvae of *Aedes aegypti* mosquito.

Conc. (µL/mL)	Log Dose	Time interval								Mean ± Se.		Probit		% Mortality	
		3hours		6hours		9hours		12hours							
		2nd	4th	2nd	4th	2nd	4th	2nd	4th	2nd	4th	2nd	4th	2nd	4th
200	2.3	3.3	2.3	6.3	5.0	12.8	8.0	18.8	14.0	10.3±3.5	7.3±2.5	6.6	5.5	94.0	70.0
100	2.0	2.8	1.3	5.3	3.8	10.8	6.8	16.5	11.3	8.9±3.0	5.8±2.2	5.9	5.2	82.5	56.5
50	1.7	1.0	0.8	3.8	2.5	7.3	5.0	11.8	9.8	6.0±2.3	4.5±2.0	5.8	5.0	79.0	49.0
25	1.4	0.5	0.5	2.8	2.3	5.3	4.8	10.0	7.3	4.7±2.0	3.7±1.7	5.0	4.7	50.0	36.5
12.5	1.1	0.5	0.3	1.8	1.3	3.8	2.3	8.5	6.3	3.7±1.8	2.6±1.3	4.8	4.5	42.5	31.5
6.25	0.8	0.3	0.0	0.5	0.8	2.5	1.8	5.5	3.8	2.2±1.2	1.6±0.8	4.4	4.1	27.5	19.0
Mean±s.e		1.4±0.5	0.9±0.3	3.4±0.9	2.6 ±0.6	7.1±1.6	4.8±1.0	11.9±2.0	8.8±1.5						
Control		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0						

2nd instar larvae--Means of four replicates (±s.e), LSD for concentration=6.610 and LSD for time=3.672

4thinstar larvae-- LSD for time=2.528

Toxicity of Moringa seed

Also the result in table 2 showed that increase in the concentrations and exposure time of AMSO against *Ae. aegypti* mosquito larvae led to increased mortality. The highest concentration of 200 µL/mL resulted in 100% mortality for both the 2nd and 4th instar larvae but it was observed that the 100% mortality was recorded at the 6th hour of exposure for that of the 2nd instar larvae while that of the 4th instar larvae occurred at the 12th hour of exposure. Also, at 100 µL/mL concentrations, AMSO equally recorded 100% mortality to the 2nd instar larvae while at the same concentration 90% mortality was observed for that of the 4th instar larvae. The least concentration of 6.25 µL/mL gave 34% mortality to the 2nd instar larvae while a lower mortality of 16.5% was recorded for that of the 4th instar larvae. The LD_{50} (IE_{50}) of AMSO against 2nd instar larvae of *Ae. aegypti* mosquito was obtained as 9.49 µL/mL while that of the 4th instar larvae was obtained as 16.11 µL/mL, respectively.

The ANOVA showed that the mortality recorded for the concentrations of AMSO for both the 2nd and 4th instar larvae were significantly different (P<0.05, P<0.001) compared to the control and also the mortality recorded for the highest exposure time of 12 hours was significantly (P<0.05, P=0.003) higher than the least exposure time of 3 hours for both the second and fourth instar larvae of *Ae. aegypti*.

Table 2. The mean mortality effects of different concentrations of *Annona muricata* seed oil on 2nd and 4th instar larvae of *Aedes aegypti* mosquito.

Conc. (µL/mL)	Log Dose	Time interval								Mean ± Se.		Probit		% Mortality	
		3hours		6hours		9hours		12hours							
		2nd	4th	2nd	4th	2nd	4th	2nd	4th	2nd	4th	2nd	4th	2nd	4th
200	2.3	19.0	18.3	20.0	19.0	20.0	19.8	20.0	20.0	19.8±0.3	19.3±0.4	8.9	8.9	100.0	100.0
100	2.0	16.8	15.5	18.8	17.0	20.0	17.8	20.0	18.0	18.9±0.3	17.1±0.6	8.9	6.3	100.0	90.0
50	1.7	14.3	13.3	17.3	15.0	18.3	16.3	19.0	17.3	17.2±1.0	15.5±0.9	6.6	6.1	95.0	86.5
25	1.4	11.8	10.5	14.3	12.8	16.8	13.0	17.8	15.8	15.2±1.3	13.0±1.1	6.2	5.8	89.0	79.0
12.5	1.1	8.8	3.3	10.8	6.8	11.5	7.3	13.3	8.0	11.1±0.9	6.4±1.3	5.4	4.7	66.5	40.0
6.25	0.8	2.3	1.0	3.0	2.3	5.3	2.8	6.8	3.3	4.4±1.0	2.4±0.5	4.6	4.0	34.0	16.5
Mean±s.e				14.0±2.6	12.2±2.6	15.3±2.4	12.8.0±2.8	16.2±2.1	13.7±2.7						
Control		12.2±2.5 0.0	10.3±2.8 0.0	0.0	0.0	0.0	0.0	0.0	0.0						

2nd instar….Means of four replicates (±S.E), LSD (P for concentrations=2.596, LSD for time=6.241; 4th instar. LSD for concentration=2.149, LSD for time=7.03.

Toxicity of Annona seed oil

The oil extracts of *M. oleifera* and *A. muricata* seed exhibited various levels of toxicity against the 2nd and 4th instar larvae of *Ae. aegypti* mosquito, which suggest the presence of some toxic substances found in some plants. This is in agreement with the work recently done by Okonkwo *et al.* (2014), where *M. oleifera* seed oil was shown to be toxic to the 1st and 4th instar larvae of *Ae. aegypti* mosquito and also the work done by Ogbonna *et al.* (2014) where the oil of *Zingiber officinale* rhizome (ginger) was equally effective against adult *Prostephanus truncatus*. Also the effectiveness of plant oil in inhibiting growth of many insect and fungal development has been reported previously (Nwankwo *et al*., 2009; Manas *et al*., 2005; Sharma and Sexena, 1994; Mwangi and Mukiama, 1988). Nwankwo *et al.* (2012) also evaluated the larvicidal properties of *Annona Muricata* seed oil on *Ae. aegypti* and it was found to be effective. The result showed that increase in concentrations of *M. oleifera* and *A. muricata* seed oil extracts led to an increase in the mortality and the rate of inhibition of emergence of both the 2nd and the 4thinstar larvae of *Ae. aegypti* mosquito and this also suggests that higher concentrations contain higher level of the active substance. This is consistent with studies by Okonkwo *et al.* (2014) and Ogbonna *et al*. (2014).

It was observed from the results that, at the same concentration of the oils, the 2nd instar larvae recorded a higher mortality than the 4th instar larvae, this shows that the 2nd instar larvae was more susceptible than the 4th instar larvae as it suggests to be due to the tender nature of the chitin in the early instar of mosquitoes. Similar studies by Enam (2001) suggest that plant essential oils block the spiracles of more susceptible insects when added in water. The observation by Akpa *et al.* (2003) that 3rd instar was more susceptible than 4th instar with a plant, *Phytolaccado decandra* verifies this finding.

Alkaloids isolated from *Annona squamosa* have shown larvicidal growth regulating activities and mortality against *Anopheles stenphensi* at concentrations of 50 – 200 ppm (Saxena *et al.*, 1993) as this might equally be responsible for the increased growth regulatory inhibition and mortality recorded in the present studies. This is also consistent with the study by Pushpalatha and Muthukrishnam (1995) where it was reported that leaf extracts of *Vitex negundo* at very low concentration had larvicidal activity against *Culex quinquefasciatus* and *An. stenphensi* and also extended the duration of larval instar pupation.

Also at the same concentration it was observed that the seed oil extract of *A. muricata* gave a higher mortality compared to that *M. oleifera*, this shows that the seed of *A. muricata* at the same concentrations has a higher level of active substance compared to that of *M. oleifera*. The LD_{50} values goes further to confirm that the seed oil of *A. muricata* is more potent than that of *M. oleifera* since the LD_{50} value of *A. muricata* is lower than that of *M. oleifera*. The present study has shown that both *M. oleifera* and *A. muricata* seed oil extracts could be used locally as larvicide in the control of *Ae. aegypti* mosquitoes in order to curtail the outbreak of yellow fever and this will equally help to reduce the impact of convectional insecticide in

the environment. Therefore *Moringa oleifera* seed oil and *Annona muricata* seed oil should be further exploited and developed for mosquito and other insect pests control and be made commercially available especially in the tropics and subtropics where these insect pests are mostly abundant.

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