International Journal of Mosquito Research

ISSN: **2348-5906**
CODEN: **IJMRK2**
IJMR 2020; 7(4): 26-30
www.dipterajournal.com

Received: 14-05-2020
Accepted: 16-06-2020

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Evaluation of lavicidal efficacy of seed extract of *Azadirachta indica* in laboratory bioassay on *Anopheles* mosquitoes

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Abstract
Azadirachta indica extracts (ethanol and aqueous) were tested against the 2nd and 3rd instar larvae of *Anopheles* mosquitoes. *Anopheles* mosquito were collected from possible breeding sites such as the puddles, tires tracks, rice fields, gutters etc. around Mubi town. Probit analysis was used to determine the lethal concentration of the extracts at 50% and 95% i.e. LC_{50} and LC_{95}, respectively. The result of the laboratory bioassay revealed that *A. indica* extracts at 50, 100 and 200 mg/ml significantly (P>0.05) controlled larval *Anopheles* mosquitoes in Mubi. However, ethanol extract proved to be more potent than aqueous extract as 100% mortality was recorded after 24 hours in all its concentrations, compared to aqueous extract, where mortality was spread between 24 hours and 48 hours. The LC_{50} and LC_{95} further proved the superiority of ethanol extract over aqueous extract. LC_{50} (-12.309) and LC_{95} (3.589) calculated for ethanol extract was significantly lower compared to LC_{50} (2.24E+08) and LC_{95} (1.08E+09) recorded for aqueous extract. Therefore, *A. indica* is a promising phytochemical which can be used sustainably as an alternative for chemical insecticide in controlling mosquitoes.

Keywords: *Anopheles*, *Azadirachta indica*, bioassay, larvicidal, mortality, mosquito

Introduction
Mosquitoes of the genus *Anopheles* transmit malaria [1]. A single bite of *Plasmodium* infected female anopheline mosquito is sufficient to transmit malaria [2]. Malaria remains one of the major endemic diseases in the tropics due to high frequency of transmission of *Plasmodium* species by a large number of *Anopheles* mosquitoes [3]. It is the primary cause of mortality and morbidity in Nigeria [4, 5]. According to WHO [5], there are about 219 million clinical cases of malaria worldwide. Africa is the most hit among all the continents with 91% deaths in 2010, of which 80% were children under five years of age [6]. Nigeria has the highest malaria prevalence with 300,000 dying each year [6], hence its situation is regarded as quite burdensome with a serious consequence of underdevelopment [7]. Adamawa State in the northeastern part of Nigeria is malaria endemic with prevalence level as high as 80% in the southern part [8] and high childhood morbidity [9].

Unfortunately, inappropriate use of chemical insecticides has led to the development of resistant mosquito's population [10, 11], and this has become a major obstacle against malaria control. *Anopheles* mosquitoes resistance have been reported in West and East Africa [12]. The widespread advent of resistance to insecticides in *Anopheles* species is a crucial drawback and impediment to efficient vector management and malaria prevention. A clear cut understanding of the potency of plant products as biopesticides against malaria vectors in a particular location remains a desirable information that will assist in the formulation of workable interventions to encourage efficient vector management.

Researches have been geared towards search for a potential alternatives for mosquito control that are relatively less expensive, easily biodegradable to non toxic products, environmentally safe and user-friendly, non effect to non target organisms and are suitable for for use in mosquito control programs [13, 14, 15]. Many authors have identified and reported some biopesticides against insect pests, and have been used effectively in the control of larval mosquitoes [16, 17, 18].

Neem plant (*Azadirachta indica* A. Juss) belongs to the family Meliaceae and its derived products have shown insecticidal property [19]. Of the numerous biological active compounds

in neem, azadirachtin is the most active and potent [20]. A biologically active compounds in *A. indica* is an eco-friendly insecticide than synthetic insecticides that contribute in high cost and health effects [21, 22, 23]. Neem seed extracts have been reported to have larvicidal ability against vectors of diseases such as malaria, filaria, dengue, dengue haemorrhagic fever, and yellow fever [21]. Gianotti *et al.* [24] in their study evaluated neem seed powder on the breeding sites of *An. gambiae* at the rate of 10 gm/m2 of pool surface area and were found to effectively control larval mosquitoes. Therefore, this study was designed to determine the the larvicidal efficacy of neem seed extracts against larval *Anopheles* mosquitoes in Mubi, in a quest of finding a suitable alternative for malaria vector control.

Materials and methods

Study area

The study area is Mubi. It is located between latitude 10º12'N and longitude 13º10'E, and has a tropical climate and is found within the Sudan savanna [25]. Average temperature is about 32^0C, with a minimum of 15.2ºC, usually in December and January period [25]. The area has an average relative humidity from 28% to 45% and annual rainfall of about 1050 mm. The rainy season is between May and October, while the dry season between November and April [25, 26].

Collection of plant materials

The seed of *Azadirachta indica* was collected from various plantations around Adamawa State University Mubi, between September and October, 2019. The seed of *A. indica* was decorticated, then cupboard-dried at room temperature for 2 to 3 weeks, before grinding into fine powder, using electric blender. The powdered sample was stored in a dark bottle with screw cap top.

Preparation of ethanol and aqueous extracts of *Azadirachta indica* seed powder

Ethanol and aqueous extracts were prepared using maceration method as performed by Dahchar *et al*. [27]. 100g of each powder sample was soaked in 200 ml of ethanol (80% v/v). This was allowed to stand for 72 hours in a dark cupboard under room temperature. The content was shaken at a regular interval to ensure proper mixture. Thereafter, the mixture content was filtered through Whatman's Filter Paper (No. 42). After the filtrate was obtained, the ethanol content of the mixture was removed using a water bath at 60 to 65°C. the stock solution obtained was however, used for the preparation of different concentrations of the treatments used in the experiment. For aqueous extract, distilled water was used in place of ethanol and of the same quantity.

Collection of Mosquito Larvae and Maintenance

Anopheles mosquito were collected from possible breeding sites such as the puddles, tires tracks, rice fields, gutters etc. around Mubi town. The larvae were identified as described by WHO [28] procedure base on their resting position on water lying horizontally on the surface of the water. *Anopheles* larvae collected were transferred to the Zoological laboratory of Adamawa State University, Mubi, and were maintain on biscuits and yeast feed in a ratio of 1:10 respectively.

Larvicidal bioassay

The Larvicidal Bioassay was carried out using WHO Standard test procedure [29] on second and third instar larvae i.e. L2 and L3, respectively. 20 to 25 healthy L2 and L3 instar larvae were introduced into the treatment beaker (250ml) using a rubber pipette. The control was set up where 20 to 25 instar larvae were introduced into 250 ml beaker, and no treatment was added. Both the treatment and the control experiment were replicated four times. Treatment beakers were covered with muslin cloth to avoid entry of any foreign material and for proper aeration. The larval mortality was observed for 24hrs, 48hrs and 72hrs. During the bioassay, the larvae were not fed [30]. Mortality was regarded when there is no sign of any movement or even after mild touch with glass rod [31], and dead larvae were counted. If 30% mortality was recorded in the control, the experiment was discarded or the mortality was corrected using Abbott's formula [32], as follows:

$$\text{Corrected Mortality} = \frac{\text{Mortality in Test Bottle (\%)} - \text{Mortality in Control Bottle (\%)}}{\text{100\%} - \text{Mortality in Control Bottle(\%)}} \times 100$$

Data analysis

The average larval mortality data obtained was subjected to probit analysis for calculating LC_{50} and LC_{95} (lethal concentrations) values, and their 95% confidence limits was estimated using a probit regression model to observe the relationship between percentage mortality of larvae and logarithmic concentration of the *A. indica*. Separate probit models were used for each extract [33]. The analysis was carried out using the SPSS (statistical package for social science) version 19.0.

Results

Mortality effect of *A. indica* in extract on *Anopheles* mosquito larvae

All the extracts tested exhibited larvicidal potential against the test organism and proved to be toxic; although there was a remarkable difference in the concentrations and timing of their activity.

Aqueous seed extract

The lethality pattern of the aqueous seed extract of *A. indica* on *Anopheles* larvae showed that, although the aqueous seed extract exhibited a certain degree of larvicidal effect on the test organism, its effect was minimal as mortality recorded in all the treatment concentration was spread across the period of exporsure, and 100% mortality was recorded at 72hrs of exposure as shown in Table 1.

The result also revealed that there was no significant difference $p \leq 0.005$ between the treatment concentration in the mortality recorded after 24 hrs and 48hrs but differs significantly $p \leq 0.05$ when compared with the control (untreated expirement) (Table 1).

Ethanol seed extract

The lethality pattern of ethanol seed extract of *A. indica* on *Anopheles* larvae revealed that the ethanol seed extract had an excellent larvicidal potential against the test organism. 100% mortality was recorded in all the treatment concentrations at just 24hrs of exporsure as shown in Table 1. The remaining treatment concentrations, just as the aqueous extract did not differ significantly in the mortality recorded after 24hrs.

Lethal concentration of *A. indica* extracts against anopheles larvae

Table 2, shows the 50 and 95% lethal concentrations (LC_{50} and LC_{95}) of the aqueous and ethanol seed extract of *A. indica*, against *Anopheles* mosquito lavae. The result showed that aqueous extract had 2.24E+08 mg/ml as LC_{50} and 1.08E+09 mg/ml as LC_{95} which is significantly higher than the values recorded in ethanol extract; where -12.309 mg/ml and 3.589 mg/ml were recorded for LC_{50} and LC_{95} respectively (Table 2). This is further evident in Figure 1, where log concentration was plotted against probit of the treatment extracts, and aqueous extract appeared to have the highest value.

Table 1: Percentage mortality of neem seed extracts against *Anopheles* mosquito larvae at 24hr, 48hr and 72hr of treatments

Extract	Trt. Conc. (mg/ml)	% Mortality after 24 Hours Mean±SD	% Mortality after 48 Hours Mean±SD	% Mortality after 72 Hours Mean±SD
Aqueous	Control	0.00±0.00^a	0.00±0.00^a	0.00±0.00^a
	50	31.50±12.77^b	211.25±84.22^b	0.00±0.00^a
	100	35.00±12.33^b	233.50±84.26^b	0.00±0.00^a
	200	31.50±12.77^b	211.25±84.22^b	0.00±0.00^a
Ethanol	Control	0.00±0.00^a	0.00±0.00^a	0.00±0.00^a
	50	100.00±0.00^a	0.00±0.00^a	0.00±0.00^a
	100	100.00±0.00^a	0.00±0.00^a	0.00±0.00^a
	200	100.00±0.00^a	0.00±0.00^a	0.00±0.00^a

The mean values having the same superscript are not significantly different ($P \leq 0.05$).

Table 2: Lethal Concentrations of neem treatment extracts against *Anopheles* mosquito larvae.

Extract	Exposure Time (Hrs)	Percent mortality (%)			Lethal Concentration	
		50 ppm	100 ppm	200 ppm	LC_{50} (95%CL)	LC_{95} (95%CL)
Aqueous	24	50	60	80	2.24E+08	1.08E+09
	48	50	40	20		
	72	-	-	-		
Ethanol	24	100	100	100	-12.309	3.589
	48	-	-	-		
	72	-	-	-		

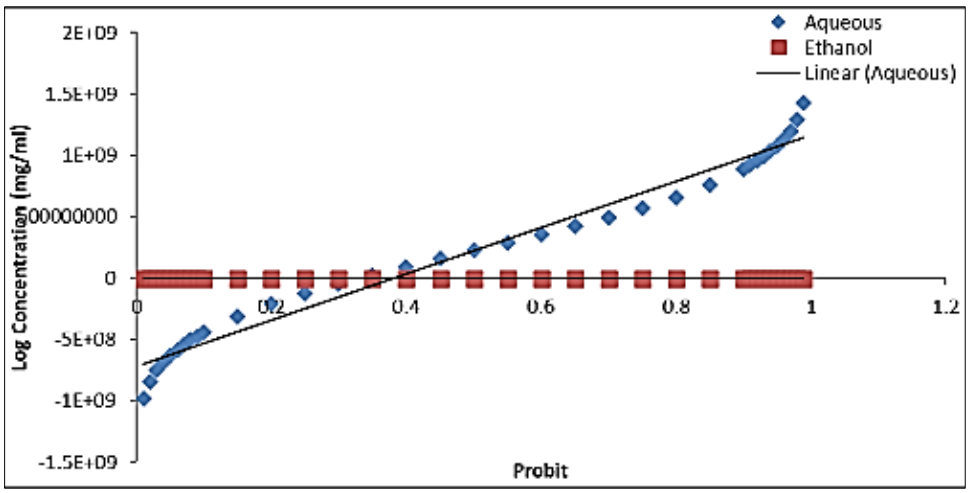

Fig 1: A plot showing the log conc. (ppm) against probit of the neem seed extracts to estimate the LC_{50} and LC_{95} against mosquito larvae.

Discussion

Plant products may be quite useful as biopesticides because they produce a large variety of compounds that increase potency against insect pests [34, 35]. Bio-pesticides with plant origins have been used effectively against several insect species especially disease-transmitted vectors, based on the fact that they are safe, as they are devoid of phytotoxic properties and friendly to the environment [35, 36]. In this study, neem seed extracts were effective against larvae *Anopheles* mosquitoes in Mubi. When compared with the control experiment, neem seed extracts significantly controlled larval *Anopheles* mosquitoes. The result of the study revealed that *Azadirachta indica* may be effective alternative to the conventional synthetic insecticides for the control of larval *Anopheles* mosquitoes. This however, uplifts hope of reducing the use of the much needed chemical insec ticides in our environment, which sre biodegradable and costly. This is also a confirmation to the fact that plant derived toxicants are valuable sources of larvicides in insect control [37].

In this study, larvicidal activity of aqueous and ethanol extracts of *A. indica* seed was evaluated on 2nd and 3rd instar larvae of the malaria vector, *Anopheles* mosquito. The result revealed that neem seed extracts are effective larvicide against *Anopheles* mosquito larvae, because of its high toxicity to *Anopheles* mosquito larvae. The high rates of larval mortality observed within 72 in all the concentrations indicated its high toxicity to mosquito larvae. Although, there was a remarkable difference in the impact of the concentrations and timing of

their activity. Ethanol extract exhibited superiority in controlling *Anopheles* larvae as 100% mortality was recorded within 24 hours of exposure. The result agrees with the findings of Okumu *et al*. [38] and Ayinde *et al*. [39], who both reported that neem oil formulation is a highly effective larvicide for anopheline mosquito vector control in Nairobi, Kenya and Ibadan, Nigeria, respectively. In the two studies, neem oil was an effective larvicide against *An. gambiae* larvae and inhibited the development of pupae. *A. indica* oil has also been reported to show an excellent larvicidal potential against different mosquito genera, including *Aedes*, *Anopheles* and *Culex* under field conditions [21, 40].

The result also showed a strong time-dependent correlation between the treatment concentrations and the mortality rate of the *Anopheles* larvae. This was evident in the trend of mortality recorded, where, the longer time of exposure to treatments, the more mortality was recorded in the larval population at the same concentration of extracts as shown in Table 1. This finding corresponds with that of Mgbemena [41] where his comparative evaluation of larvicidal potentials of ethanol extracts of *Azadirachta indica* on *Ae. aegypti* larvae showed similar characteristics.

Similar trend was observed when the lethal concentration at 50 and 95% for aqueous and ethanol neem seed extracts were calculated. The neem extracts used in the experiment were toxic to *Anopheles* larvae with LC_{50} of 2.24E+08 mg/ml and -12.309 mg/ml for aqueous and ethanol extracts, respectively. Similar trend was recorded for LC_{95}, and its evident in Figure 1, where log concentrations of aqueous and ethanol extracts was ploted against probit of the extracts. This was also observed in the study conducted by Dua *et al*. [21] where larvicidal activity of neem oil formulation was evaluated against mosquitoes.

Conclusion

In conclusion, neem seed extracts were found to be effective in controlling *Anopheles* mosquito larvae under laboratory conditions. The fact that neem seed extracts are relative less toxic to humans and the environment, active against few number of insect species (Alkofahi *et al*., 1989), less expensive and biodegradable, makes neem suitable for use in mosquito control programs. This therefore, could also be used as an alternative to other pesticides for control of malaria vector.

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