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Vector control and Isolation of Larvicidal Compounds from *Melia azedarach* (A. Juss) Leaf using *Anopheles gambiae* Larva.

Olugbenga, A. Ojo [1*], Abidemi, E, Ojo[2]

[1]Department of Pharmacognosy, Madonna University, Elele, Rivers State, Nigeria
[2]Department of Microbiology, College of Biosciences, Federal University of Agriculture, Abeokuta, Nigeria.

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* *Corresponding Author:*
ojoolugbenga46@gmail.com
https://orcid.org/0000-0002-6104-6024
+2348060883753

ABSTRACT

Background: Despite ongoing efforts to control the disease, malaria remains a serious threat to the human race on earth. This disease is ubiquitous in Africa, especially those countries that are categorized as undeveloped. The present investigation aims to evaluate and isolate larvicidal principles in the leaves and stem bark of *Melia azedarach* from the Meliaceae family using the fourth instar larvae of *An. gambiae*.

Methods: Larvicidal activity was evaluated by exposing 4th instar larvae of *Anopheles gambiae* to serial concentrations (0.625-10 mg/mL) of extracts, fractions, and compounds of Melia azedarach. Larval mortality was recorded after 24h of exposure and 50% lethal concentration (LC_{50}) values were determined. L Results were compared to those of larvae exposed to N, N- diethyl-m-toluamid (DEET), the reference insecticides, and untreated groups.

Results: Larvicidal activity of the crude methanol extract of both leaves and stem bark of M. azedarach were 5.80 and 7.59 mg/mL respectively. The hexane soluble fraction of *M. azedarach* leaf displayed highly lethality at 0.625mg/mL with LC_{50} of 0.025 then the remaining fractions. Similarly, larvicidal activity of DEET was significantly low (LC_{50} = 1.09 mg/mL) than the hexane fractions ($p < 0.05$). Fractionation and purification of the active fractions led to the isolation of three compounds. Sub-fraction 35-49 (designated as MAZLFF) of *M. azedarach* showed larvicidal activity on larvae with LC_{50} of 0.19 mg/mL. Two compounds were isolated from MAZLFF using preparative thin layer chromatographic. Compounds 2 and 3 had Rf values of 0.75 and 0.63 respectively on silica gel G using CH_3Cl_3:CH_3OH (9.5: 0.5) and CH_3Cl_3: Acetone (9:1) respectively. Compounds 2 and 3 showed a positive test for the presence of triterpenoids after spraying with anisaldehyde/H_2SO_4. The presence of triterpenoids may be responsible for the insecticidal activity observed in the two compounds isolated from the leaves of *M. azedarach*.

Conclusion: The leaves of M. azedarach showed potent anti larvicidal activity. Compounds from this plant may serve as sources for the development of malaria vector control compounds.

1. Introduction

The increase in the occurrence of malaria in Africa is becoming alarming. Despite all efforts being put together to reduce these deadly diseases. Statistics from the World Health Organization (WHO) show sub – Saharan Africa was more affected. In 2006, there were 216 million cases of malaria, up from 211 million cases in 2015. The estimated number of malaria death stood at 445,000 in 2016, a similar number to the previous year, 446,000.[1] Among the cases infant, children, and pregnant women were prone to this infectious disease. Control of malaria is becoming increasingly difficult because of the development of resistance of mosquitoes to pesticides.[2] Hence, a marked increase in malaria infection has necessitated the need to control the vector which is a major delivery system that harbors the parasite, since parasites were resistant to malaria drugs. Control of mosquitoes is essential as many species of mosquitoes are vectors to prevalent and infectious diseases including malaria, filariasis, and many arboviral diseases and they constitute an intolerable biting nuisance.[3,4] Co-evolution has equipped plants with a

plethora of chemical defenses against insect predators. According to Feinstein [5], more than 2,000 species of plants representing 170 families are said to have insecticide properties. The Meliaceae plant family is known to contain a variety of compounds that show insecticidal, antifeedant, growth-regulating, and development-modifying properties. [6, 7] As a result of allelochemicals embedded in Meliaceae plants affect the biochemical and physiological processes of the insect system and nullify the resistance mechanism and also increase the pathogenicity of microbial pesticides. *Melia azedarach* commonly known as Chinaberry or Persian lilac tree is a deciduous tree that is native to northwestern India and has long been recognized for its insecticidal properties. Fruit extracts of *M. azedarach* and *Azadirachta indica* elicit a variety of effects in insects such as antifeedant, growth retardation, reduced fecundity, molting disorder, morphogenetic defects, and changes of behavior.[8, 9] The leaves are commonly laid between the pages of books and in folded woolen clotting as protection against insect attack.[10] The bark, flowers, and leaves of *M. azedarach* are toxic, but they are less toxic than fruits.[11] The present study aimed to isolate the vector control compounds from the leaf and stem bark of *Melia azedarach*.

2. Materials and methods

2.1 Plant material

The leaves and stem barks of *M. azedarach* were collected from the Botanical Garden of University Ibadan, Nigerian, in July 2010. Plants materials were authenticated by Mr. Oluwaseun Osiyemi of the Forest Research Institute of Nigeria (FRIN), Ibadan, and voucher specimens were deposited under FHI 108966, in FRIN.

2.2 Preparation of Plant Extract

The dried powdered leaf and stem bark of *M. azedarach* were2.3 exhaustively extracted with methanol by cold maceration for 72 h at room temperature (RT). Extracts were filtered and the solvents evaporated to dryness using a rotary evaporator at 40°C. The resulting extracts were stored in the refrigerator at 4°C until needed for analysis. The most active crude extract was partitioned into hexane, chloroform, and ethyl acetate in a separating funnel to obtain fractions that were equally tested. The active fraction was subjected to chromatographic techniques including column and thin-layer chromatography

2.3 Isolation of compounds

The hexane soluble fraction of *M. azedarach* (10 g) was chromatographed on silica gel using column chromatographic technique and eluted with Hex: $CHCl_3$, $CHCl_3$: EtOAc, and then EtOAc: CH_3OH mixture of increasing polarity (ranging from 100% Hex to 100% $CHCl_3$ and then 100% EtOAc to 20% CH_3OH). 100Ml fractions were collected and monitored by analytical TLC. Fifty sub-fractions were obtained and fractions with similar R_f values on analytical TLC (Silica gel GF_{256}) were pooled to give nine main sub-fractions. The nine sub-fractions (designated as $MAZLF_{A-I}$) were all evaporated using a rotator evaporator and each was subjected to larvicidal assay. Fraction twenty-one (21) of the fifty fractions that were eluted with 100% $CHCl_3$ crystallized to obtain compound 1. This compound was compared with standard β-sitosterol by co-spotting on TLC plate and R_f values were recorded as follows: fraction 21, R_f = 0.4; standard β-sitosterol, R_f = 0.4; silica gel, 100% $CHCl_3$. It was found that the compound has similar R_f with standard reference of β-sitosterol. The chromatogram was sprayed with vanillin/sulphuric acid spraying reagent and heated until the maximum colour of deep brown colour developed

Sub-fraction ($MAZLF_F$) was the most active of all the pooled sub-fractions and was subjected to purification on a column and eluted with Hex: $CHCl_3$ with a stepwise increase in chloroform. Fifty-nine fractions obtained were monitored with TLC and pooled into 3 different groups viz: Sub-fraction 21-27 ($MAZLF_{F1}$), 28-47 ($MAZLF_{F2}$), and 48-59 ($MAZLF_{F3}$). Sub-Fraction ($MAZLF_{F3}$) was further separated using preparative TLC into two compounds. Compounds 2 and 3 had R_f values of 0.75 and 0.63 respectively on silica gel G using CH_3Cl_3-CH_3OH (9.5: 0.5) and CH_3Cl_3-Acetone (9:1) respectively.

2.4 Larval Toxicity Assay

Larvae (*Anopheles gambiae*) were collected at the Ojoo area, Ibadan, Oyo State, Nigeria, from tyre-print breeding sites and reared in plastic bowels containing distilled water at room temperature (between 28°c to 30°c). They were fed with dog biscuit (tetramin^R fish baby)

Stock solutions of each extract were prepared at 20 mg/mL with ethanol. Test solutions of concentrations of 10, 5, 2.5, 1.25, and 0.625 mg/mL were prepared by serial dilution of the stock solution with ethanol. Sterile disposable cups (250 mL) were used for the study. Dilution of extracts consisting of 1mL was transferred into a cup containing 99 mL of well water (99 mL). Twenty fourth-instar larvae were released into each cup containing 100 mL solution of each test concentration. After 24 h, the number of dead larvae in each cup was counted (assessed by the inability of larva to get to

the surface when agitated). The experiment was extended to 48 h. Control experiments carried out with DEET and with 1% ethanol were run in parallel. Experiments were done in duplicates.

2.5 Statistical Analysis

Results were expressed as mean ± SEM of two independent experiments. Larval toxicities were reported as LC_{50} obtained from GraphPad Prism statistical software, analysis.

3. Results

Two extracts from *M. azedarach* plant belonging to Meliaceae family were screened. Table 1 summarizes the mean percentage mortalities exhibited by each extract. In the assay, M. azedarach leaves extract was most toxic displaying highest toxic against larvae while activity displayed by *M. azedarach* stem bark extract showed some toxicity but not enough to kill larva. As a result of this *M. azedarach* leaves was subjected to partition process. The results of fractions obtained by partitioning the crude methanol leaf extracts into hexane, chloroform and ethyl acetate showed that the non polar fractions were the most active. The hexane soluble of fraction of *M. azedarach* leaf exhibited the highest toxicity among the fractions although chloroform and ethyl acetate also displaced toxicity but not as active as hexane fraction. The hexane leaf fraction of *M. azedarach* produced 100% mortality at 2.5 mg/ml (fig. 1), although chloroform, ethyl acetate produced 94.7%, 96.5% at 5mg/ml respectively. Table 2 shown the larvicidal activity of hexane, chloroform and ethyl acetate fractions of *M. azedarach* leaf while table 3 shown LC_{50} value of crude methanol extract of *M. azedarach* leaf and stem bark.

Table 1: Showing percentage mortalities of crude extracts

Concentration (mg/mL)	MAL	Mean % Mortality (±SEM)	
		MAS	DEET
10	**100**	100 ± 25.0	100
5	**100**	87.5 ± 12.5	45.0 ± 1.0
2.5	**77.5** ± 2.5	25.0 ± 12.1	37.5 ± 2.0
1.25	**82.5** ± 2.5	25.0 ± 0.0	30.0 ± 2.5
0.625	**75.0** ± 5.0	12.5 ± 12.5	20.0 ± 0.5

MAL: *M. azedarach* leaf; MAL- *M. azedarach* stem bark

Table 2: larvicidal activity of hexane, chloroform, and ethyl acetate fraction of *M. azedarach* leaf on *An. Gambiae*

Concentration (mg/mL)	Hexane	Mean % Mortality (±SEM)		
		Chloroform	Ethyl acetate	DEET
10	100 ± 0.0	100 ± 0.0	97.5 ± 2.5	100± 0.0
5	100 ± 0.0	94.7 ± 0.0	96.5 ± 0.0	45.0± 1.0
2.5	100 ± 0.0	86.8 ± 2.6	95.0 ± 5.0	37.5± 2.0
1.25	100 ± 0.0	76.3 ± 2.6	95.0 ± 5.0	30.0± 2.5
0.625	96.0 ± 0.0	71.1 ± 2.6	95.0 ± 5.0	20.0± 0.5
1% Ethanol	5.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Water 100%	0.00			

24 hrs exposure to 1 % ethanol resulted in 5% mortality, 100% water resulted in 100% survival

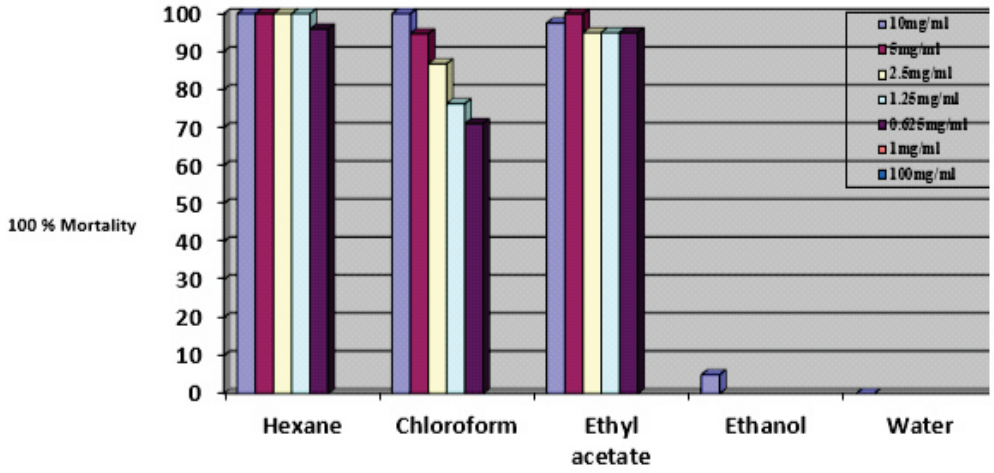

Fig 1 Larvicidal activity of fractions obtained from leaf of *M. azedarach*

Based on TLC analysis, 50 Sub-fractions collected were pooled together and grouped into 9 groups as a result of similar R_f they have in common. The 9 groups ($MAZLF_{A-I}$) are shown in Table 4, were subjected to larvicidal activity. Out of these groups, sub-fraction ($MAZLF_F$) displayed the highest activity at 2mg/ml against third instar larvae of *Anopheles gambiae*, and continue to inhibit metamorphosis of larvae to pupa even at 0.5mg/ml. Comparatively, the fraction ($MAZLF_H$) was the least active of the fractions at LC_{50} of 703.5mg/ml compared to LC_{50} of the highest active fraction at 0.19mg/ml. Fraction ($MAZLF_C$), ($MAZLF_D$) and ($MAZLF_G$) displayed moderate LC_{50} of 1.12, 0.49 and 0.2mg/ml respectively. Thin-layer chromatographic analysis of the fraction $MAZLF_F$ revealed the presence of three compounds which were identified by visualizing the plate under UV light at 254nm. This fraction was re again subjected to a purification column chromatography in which 59 fractions were collected and analyzed on Tlc plates as a result of pooling them together. The three groups that resulted from pooling viz: $MAZLF_{F1}$, $MAZLF_{F2}$, and $MAZLF_{F3}$ were tested on *Anopheles gambiae* mosquitoes larvae and LC_{50} of the fractions were 5.023, 1.218, and 507.14 mg/ml respectively. Sub-fraction, $MAZLF_{F2}$ was the most active and on the Tlc plate, two compounds were revealed which were identified by visualizing the plate under UV light at 254nm.

Preparative thin-layer chromatography was finally used to separate the compounds and two separate bands were revealed which were prominent under UV light 254nm. Each band was spotted on a separate Tlc plate and each of the Tlc plates revealed the presence of one spot under UV light at 254nm. The spot produced was spraying with anisaldehyde/H_2SO_4 and gave dark brown color in daylight and indicated that the compounds belong to triterpenoids. This result is consistent with earlier works by [12], that both leaves and seed extracts of *Melia azedarach* revealed the presence of triterpenoids and steroids, and both also presented alkaloids and condensed tannins. Compounds present in leaves are different from those in seeds since the former inhibits mainly egg hatching and the later, larval development.

Table 3: LC_{50} value of crude methanol extract of *M. azedarach* leaf and stem bark

Plant extracts	LC_{50}
***M. azedarach* leaf**	5.808
***M. azedarach* stem bark**	7.594

Table 4: LC_{50} of Sub-fractions of *Melia azedarach* n-hexane leaf fraction obtained from the column chromatography

Sub-Fractions	LC_{50} (mg/mL)
MAZLF $_A$	5.89
MAZLF $_B$	25.01
MAZLF $_C$	1.12
MAZLF $_D$	0.49
MAZLF $_E$	8.24
MAZLF $_F$	0.19
MAZLF$_G$	0.28
MAZLF$_H$	703.7
MAZLF $_I$	0.82
DEET	1.09

MAZLF – *Melia azedarach* hexane leaf fraction; DEET- N, N - diethyl - m – toluamide (Reference insecticides)

4. Discussion

Going by the facts and figures, malaria remains one of most infectious diseases, infects about half a billion people resulting in the death of 1.5 and 2.7 persons, annually with a high mortality rate among children.[13] Control of such diseases is becoming increasingly difficult because of increasing resistance of mosquitoes to pesticides.[14] A marked increase in the malaria has necessitated for the needs to control the vector, which is a major delivery system that harbor the parasite, since parasites were resistant to malaria drugs. Plant-derived preparations have been traditionally used as insecticides and have the potential to be the best pest control strategy as part of the overall agronomic pest control strategy including integrated pest management. In addition to this natural plant product which serve as general toxicant against mosquito larvae, botanical insecticides also have potential uses as growth and reproduction inhibitors, repellents, growth regulation, fecundity suppression, male sterility, larvicidal, ovicidal and oviposition activity mostly as deterrence [15]. Studies on plant extracts against insect larvae have been reported globally. The methanol extract of *Melia dubia* showed growth inhibitory and toxic effect against the larvae of *Helicoverpa armigera*. [16] This work demonstrates the potency of *M. azedarach* leaf and stem bark as an effective larvicidal against *An. gambiae* larvae. *Melia azedarach* leaf extract was highly toxic to mosquito larvae. The high rates of larval mortality observed at 2.5 to 5 mg/mL within 24 hr with LC_{50} value 5.808 mg/mL (Table 3)

indicate the high toxicity of the extract. Previous studies have shown that *M. azedarach* extracts possessed significant larvicidal activity and it has been reported that ethanolic leaf extract of *M. azedarach* is a strong larvicide on *Aedes aegypti*, and all tested larvae died before pupation, and significantly delayed development time, in addition to its inhibition ability of oviposition by the vector females.[17] The activity displayed by *M. azedarach* was better than activity displayed by DEET, the positive control, included in the study at value of 1.09 mg/mL. Triterpenoids are known to possess insect antifeedant and growth regulation activity against a variety of agriculture pests. [18] Many triterpenoids present in plants of the Meliaceae family are described as showing insecticidal activity. Bohnenstengel and co-workers [19] isolated three meliacarpins derivatives from the leave of *Melia azedarach* which was incorporated into the artificial diet of larvae of the polyphagous pest of *Spodoptera littoralis* is a chronic feeding bioassay. Quassinoids, which are modified triterpenoids, can thus stand a good chance of being used as insecticides. The result from this study will go a long way in reducing the spread of malarial disease in our environment since the principal host has been eliminated or inhibited by the allelochemicals produced by this fraction. The result is consistent with earlier studies from this plant on other mosquitoes excluding *An. gambiae* has proven to be an inhibitor of the emergence of larvae to pupa. The newest research work by Coria [17], proved that ethanolic leaf extract of *M. azedarach* is a strong larvicide on *A. aegypti*, and all tested larvae died before pupation, and significantly delayed development time, in addition to its inhibition ability of oviposition by the vector females. In comparison with leaf extract, the fruit extract showed much weaker effects.

5. Conclusion

The present studies revealed that methanol leaf extract may be used directly as larvicidal agents in volume in aquatic habitats or breeding sites. This agreed with the findings of Cabral *et al*., [19], revealed that the viability of the larval stage of *Musca domstica* was reduced by all the tested samples of *M. azedarach* mainly by methanol fraction of the seed of *M. azedarach* that induced a reduction of 31% when compared with those of control group. The mode of action of this leaf extract on mosquito larvae are not known, but previous studies demonstrated that phytochemical interfered with the proper functioning of mitochondria more specifically at the proton transferring sites [20] and other studies by David *et al*. [21], found that phytochemicals primarily affect the midgut epithelium and secondary effect the gastric caeca and the malpighian tubules in mosquito larvae. Characterization and structural elucidation of the isolated compounds are still ongoing.

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Conflict of Interest

No conflict of interest was declared.

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