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Blocking activity of *Aloysia citrodora* Leaf Extract on the Sporogonic Development of *Plasmodium falciparum* field isolate against *Anopheles gambiae S.L.* (Culicidae: Diptera)

Aminu, M.A.[1*] and [2]Abdullahi, N.
[1]Department of Biology, Al-istiqama University, Sumaila, Kano
[2]Department of Biological Sciences, Bayero University Kano, State

ABSTRACT

Mosquito ingests blood consisting of male and female gametocytes during a blood meal on the human host. The gametocytes fertilize and undergo division and produce infective sporozoites ready for transmission in the human host. This study is aimed at developing simple and effective technique of preventing malaria through spraying of an effective dose of *Aloysia citrodora* leaf extract in an environment in order to prevent the sporozoite development (sporogony) in the mosquito. Using membrane feeding method, female Anopheles mosquito (*Anopheles gambiae*) was directly fed with blood infected with *Plasmodium falciparum.* Transmission was assessed using 150 mosquitoes' samples (test:100; control:50). *A. citrodora* leaf extract concentration was prepared in three-fold dilution ranging from 300, 150, and 75. One hundred and fifty (150) mosquitoes was assigned to each of the four test groups and assayed by one of the three concentrations of the extract. The mosquitoes were dissected after 7-9 days to determine infection (Oocyst carriage). The result for oocyst after treatment with extract, while the control group shows the presence of Oocysts. Large number of sporozoite were present in mosquitoes fed with infected (treated) blood meal with ethyl acetate extract of *A. citrodora* extract 98 (19.7 ±24.9) when compared to control with 45 (8.9±9.6) number of oocyst. This result show ethyl acetate has low blocking effect on oocyst development. 62 number of oocysts presence out of 107 dissected mosquitoes with average mean of 57.9±12.9) when compared with control (untreated) with 28 (24.7±70.0) respectively. In conclusion, the possibility of using *A. citrodora* as bio-insecticide against *Anopheles gambiae* was established. The approach will be of great benefit to the poor populace by reducing the financial burden on the patients as well as reduce exposure and excessive spread of harmful insecticide chemicals in the environment.

Keywords*: Aloysia citrodora*; Sprogonic; *P. falciparum; Anopheles gambiae*

INTRODUCTION

Malaria is a life-threatening disease caused by parasites that are transmitted to people through the bites of infected female Anopheles mosquitoes. Malaria is caused by six species that affect humans, belonging to the genus *Plasmodium*: *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi* and recently *P. stephensi* (Olawale *et al.*, 2021). *P. falciparum* and *P. vivax* are the most important in clinical relevance. Malaria caused by *P. falciparum* is the most deadly form, and it predominates in Africa including Nigeria. *P. falciparum* malaria if left untreated can progress to severe illness and death within a period of 24 hours. The *P. vivax* is widely distributed than *P. falciparum* because it is able to develop in the *Anopheles* mosquito vector at lower temperatures, and survives at higher altitudes and in cooler climates (Panda & Mahapatra, 2022). In 2020, nearly half of the world's population was at risk of malaria.

Some population groups are at considerably higher risk of contracting malaria and developing severe disease: infants, children under five years of age, pregnant women and patients with HIV/AIDS, as well as people with low immunity moving to areas with intense malaria transmission such as migrant workers, mobile populations and travelers. The first symptoms – fever, headache and chills – usually appear 10–15 days after the infective mosquito bite and may be mild and difficult to recognize (ref). Despite malaria being preventable and curable, in 2021, there were estimated 247 million cases in 84 endemic countries (ref). The WHO African Region carries a disproportionately high share of the global malaria burden. The WHO African Region, has estimated 234 million cases in 2021, accounted for about 95% of global cases. About 96% of malaria deaths globally were in 29 countries. Four countries accounted for just over half of all malaria deaths globally in 2021: Nigeria (31%), the Democratic Republic of the Congo (13%), the Niger (4%) and the United Republic of Tanzania (4%). The estimated number of malaria deaths stood at 619 000 in 2021. Children under five accounted for an estimated 80% of all malaria deaths in the region (WHO, 2022).

Transmission reduction is a global approach to control and eliminate malaria. Over the last two decades, expanded access to WHO-recommended malaria prevention tools and strategies including effective vector control and the use of preventive antimalarial drugs has had a major impact in reducing the global burden of this disease (WHO, 2022. Vector control programs play a major role in malaria prevention in Africa (Okumu & Finda, 2021). Insecticide-treated nets (ITNs) and indoor residual spraying (IRS) in particular have yielded exemplary gains even in areas historically considered endemic. Scale-up of vector control, diagnosis, and treatment approaches averted 663 million clinical cases of malaria between 2000 and 2015 Vector control programs include vaccine development, sporogony blockage. Sporogony is a sexual reproduction process in sporozoan parasites leading to formation of oocysts and sporozoites. In one phylum of Protozoa, the Apicomplexa, a cycle of multiple fission (schizogony), alternates with a period of sexual reproduction (sporogony). The zygote then becomes an oocyst for all of these parasites. Sporozoites are formed within the oocyst by an asexual process of sporogony and when released, penetrate host tissue cells, and begin another asexual cycle as trophozoites. The only phase of this life cycle that is diploid is when the zygote is formed. All other stages in the life cycle are haploid.

MATERIALS AND METHODS

Study Area

The study was carried out in the Department of Medical Laboratory Science, Faculty of Basic Health Science, Al-istiqama University, Sumaila – Kano.

Collection and Identification of *A. gambiae*

Anopheles gambiae larvae were obtained from the insectary unit, Department of Biochemistry, Bayero University Kano. Insects collected were authenticated by an expert in insect taxonomy at the Department of Zoology, Ahmadu Bello University, Zaria.

Rearing of the *A. gambiae*

Healthy emerged adult females and males of *A. gambiae* were reared and remained inside the insectary for at least 5days for mating to take place. Adults were fed with 10% sucrose before then fed with blood meal after 3-5 days (Des *et al.,* 2007). After each blood meal feeding exercises, successfully fed mosquitoes were became engorged with red colorations abdomen (Clements, 1992) and lay eggs immediately overnight. Beaker was placed inside the cage containing water and a piece of filter paper for oviposition.

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Eggs were laid on filter paper over night. Filter paper containing eggs was placed in a plastic tray with 300ml of distilled water and allowed to hatching into larva (Des *et al.,* 2007). Developing larvae were fed with pinch yeast every day and the use of clean water is also important for refreshing the environment after every feed. Separation of pupa from larvae was done daily and placed into a plastic bowl for adult to emerge after 2-3days, inside the insectary (Edillot *et al.,* 2007). Colonies wer maintained and all experiments carried out at a constant temperature of 25 ± 2ºC and 80 ± 10% relative humidity (Clements, 1992).

Collections and identifications of plant materials

Plants materials (*A. citrodora*) were obtained from Goron-maje ward, Dambatta LGA, Kano State. The plants were authenticated by an expert in plant taxonomy at the Department of Plant Biology, Bayero University Kano.

Plant sample preparation

Healthy leaves of *A. citrodora were* washed with tap water, cut into small pieces and air dried. After the plants were completely dried, they were ground into powder (Jeyabalan *et al*., 2003). The powdered leaves was extracted with different solvent using different extraction methods or technique which include soxhlet extraction and maceration methods

Bioassay

Mid-gut dissection and procedure

Mosquitoes were anesthetized by placing in a petri dish kept in a cold ice. Place a drop of phosphate buffer solution (PBS) onto on a glass slide mounted under the light microscope (Coleman, 2007). Transfer mosquito onto the prepared slide by stabbing the mosquito thorax with a need-tip probe. Pull off mosquito legs using finger or forceps, then transfer the mosquito back on the slide. Remove the head of mosquito using forceps while holding down the mosquito thorax with the probe, use the forceps to grasp the second to the last abdominal segment and gently pull off the mosquito abdomen in a single motion. The mid-gut should remain attached to the immobilized thorax. Discard the abdomen. Use the forceps to detach the mid-gut from the thorax (Coleman, 2007).

Infection of female mosquito with treated and untreated blood meal protocol

The mosquito ingests blood consisting of male and female gametocytes during a blood meal on the human host (Coleman, 2007). The gametocytes will fertilize and undergo division and produce infective sporozoite ready for transmission to the human host. Using membrane feeding method, female *Anopheles* mosquito (*Anopheles gambiae*) will be caught and directly fed with blood infected with *Plasmodium falciparum.* Human-to-mosquito transmission were assessed using 150 mosquitoes samples (test:100; control:50). *Aloysia citrodora* leaf extract concentration were prepared in two-fold dilution ranging from 300, 150 and 75 ppm.

One hundred and fifty (150) mosquitoes were assigned to each of the four test groups and assayed by one of the three concentrations of the extract. The mosquitoes were dissected after 7-9 days after blood meal to determine infection (Oocyst carriage) and mosquito mid-guts were examined for oocysts under a light microscope, sporozoite within developing oocyst were also recorded using the relation:

$$\text{Average No. of oocysts} = \frac{\text{Number of mosquitoes with oocysts}}{\text{Total Number of mosquitoes dissected}} \times 100$$

Light and transmission electron microscopy (TEM) analysis

This work was carried out using the facilities provided by the Laboratory of Insects-Toxicants Interactions and the Histology Laboratory, at the department of histopathology, Aminu Kano Teaching Hospital (AKTH), Kano state. *A. citrodora* extracts was used in this study.

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Procedures

Fragments of mid-gut median region were dissected from adult female *A. gambiae* of both treated and non-treated after holding the insects at low temperature (4 °C) for 5 min. The tissue was fixed using 2.5% glutaraldehyde solution in phosphate buffer (0.1 M, pH 7.2), washed three times with phosphate buffer and post-fixed with 2% osmium tetroxide (OsO_4) in phosphate buffer (0.1 M, pH 7.2) for 1 h. The fragments were again washed and dehydrated through an increasing series of acetone (30 min per stage) at room temperature. The fixed tissues were embedded in EMBED812/Araldite resin (Electron Microscopy Sciences, AKTH). Semi-thin sections were stained with methylene-blue for light microscopy, while ultrathin sections were contrasted with uranyl acetate for 1 h, then with lead citrate for 10 min, and examined in electron microscope.

STATISTICAL ANALYSIS

All data analyzed were compute using version 20.0.(SPSS Inc. Chicago, IL, USA). Standard deviation was calculated based on the mean values of the experiments to compare between means treated and untreated blood meal with a control groups. Comparison between means of treatment and control groups were use performed analysis of variance (ANOVA).

RESULTS AND DISCUSSION

The result from the test groups shows the absence of the Oocyst after treatment with leaf extract, while the control group shows the presence of Oocyst (Table 1). The results on Table 1 also shows large number of sporozoite present in mosquitoes fed with infected blood meal treated with ethyl acetate of *A. citrodora* extract with (98) when compared to control (45) number of oocyst. This result show ethyl acetate has low blocking effect on oocyst development when compared to control from (Table 1) between treated and untreated blood meal. Methanol extract of *A. citrodora* show 62 number of oocysts presence out of 107 dissected mosquitoes with average mean of 57.9±12.9 when compared to the control (untreated) with 28 (70.0±14.2) respectively. Earlier studies used different solvents such as ethyl acetate, ethanol and methanol to evaluate the effects of extract on transgenic plants activities against *A. Gambia* (Su and Mulla, 1999). Therefore, the present study investigated the effects of leaf extract on the mid-gut morphophysiology of *An. gambiae* and there have been no previous reports of the effects of *A. citrodora* on the gut of this insect. However, plant such as *C. micrantum* leaf extract has shown high effect of blocking the activity of oocyst and sporozoite development in the mid-gut with 19.0±15.0 number of oocysts detected when compared to control (untreated). Generally, the results of the present study further confirmed that the efficacy of botanical extracts against developmental stages of sporozoite in mosquitoes may be influenced by factors such as extraction solvent. It may therefore, be inferred from the results of the present study that the solvent types (ethanol and water) selected for the extraction processes affected the efficacy of the extract because it has been demonstrated that different phytochemicals of varying volatility usually constitute the final extract (Shaalam *et al*., 2005). Earlier, Su and Mulla (1999) had reported that ethanol extracts of both *Azadiracta indica* and *C. micrantum* (15 and 20 ppm respectively) (24±0.57) were more toxic against *Culex pipiens* on blocking oocyst development compare to result. Histologically, the mid-gut wall of control *A. gambiae* fed with untreated blood meal exhibited similar characteristics to those fed with treated blood with leaf extracts of *A. citrodora* and regenerative cells in the base of the epithelium, with pyramidal morphology, voluminous nucleus were observed (1A).

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The mid-gut of the *A. gambiae* fed the treated blood with *A. citrodora* leaf extract of ethyl acetate showed alterations in structure, after ingestion. These changes could be detected in semi-thin sections, and affected both the epithelial lamina and the muscle. Some areas of the epithelium exhibited stratifications while other areas showed only a simple epithelium when compared with control (1A and 2B). These areas of the gut wall presented projections into the lumen conferring on it a folded aspect (Fig. 3C and 2B). Morphological modifications were also observed in the columnar and goblet cells. Columnar cells became more elongated and thin and numerous vacuoles of varied sizes and forms were observed in their cytoplasm (Fig. 4D) treated with *A. citrodora* leaf extract of ethanol. Goblet cells showed deformations in their morphology, acquiring an elongated, spherical as well as the reduction or absence of the plasma membrane projection that delimits the goblet chamber (Fig 4D and Fig. 5E). The muscle layer showing clear degeneration (Fig. 3E) when compared with control was observed.

However, further fractionation and compound isolation studies are needed to identify the active phytochemical components of the extracts. Therefore, the possibility of using *Aloysia citrodora* as bio-insecticide against *Anopheles gambiae* was established. The approach is of great benefit to the poor populace by reducing the financial burden on the patients as well as reduces exposure and excessive spread of harmful insecticide chemicals into the environment.

Table 1: Oocysts count and mosquitoes infected with *Aloysia citrodora* ethyl extract with blood meal

Conc. (ppm)	No. of Mosquitoes tested	No. of Mosquitoes dissected	No. of mosquitoes with oocysts	Average no. of oocysts	Mean no. of treated Mosq.
Ethyl Acetate (300ppm)	150	121	98	24.7	19.9
		30	22	23.2	20.2
		67	51	9.6	8.0
		54	46	18.8	13.4
		35	33	27.7	20.8
Control (Untreated)	50	50	0	0	0
		38	29	3.4	3.8
		52	45	8.9	7.9
		42	33	4.8	4.4
		44	31	4.3	3.7
		45	27	3.4	3.7

Avg.no of oocysts= Number of mosquitoes with oocysts/ total Number of mosquitoes dissected Number of independent mosquito feedings; $p<0.05$ by t test compared with treated/untreated blood

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Table 2: Oocysts count and mosquitoes infected with *Aloysia citrodora* Ethanol extract with blood meal

Conc. (ppm)	No. of Mosquitoes tested	No. of Mosquitoes dissected	No. of mosquitoes with oocysts	Average no. of oocysts	Mean no. of treated Mosq.
	150				
		100	15	15.00	19.9
Ethanol		30	0	0	0
(75 ppm)		67	08	11.9	5.3
		54	01	1.8	0.52
		35	0	0	0
Control	50	50	0	0	0
(Untreated)		38	30	78.9	52.7
		52	36	69.2	21.7
		42	20	47.6	20.6
		44	29	65.9	19.8

Avg.no of oocysts= Number of mosquitoes with oocysts/ total Number of mosquitoes dissected Number of independent mosquito feedings; $p<0.05$ by t test compared with treated/untreated blood meal.

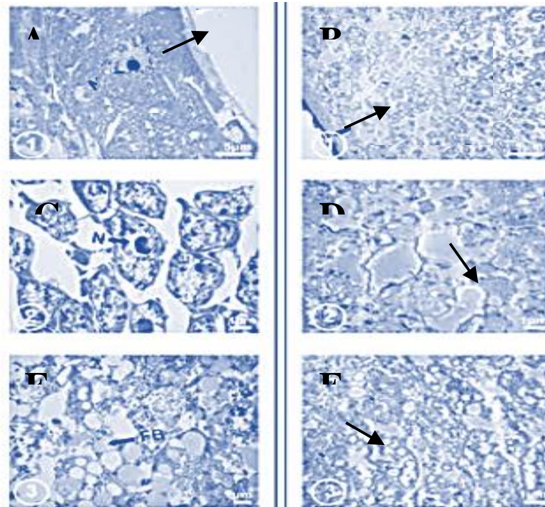

Plate I: Mid-gut of *Anopheles gambiae* untreated (control) and treated with Acetate Extract (Magnification x100 and x400): Key; (A) = Negative control; (B) = Positive control ethyl acetate extract of *A. citrodora*; (C) Negative control; (B) = Positive control methanol extract of *A. citrodora;* (E) = Negative control; (F) = Positive control ethanol extract of *A. citrodora* extracts

CONCLUSION

From the results of this study, it can be concluded that based on the sporogonic development of *p. falciparum* field isolate against *Anopheles gambiae .L.* effects of different plant extracts tested in this study have been ascertained. The approach will be of great benefit to the poor populace by reducing the financial burden on the patients as well as reducing exposure and excessive spread of harmful insecticide chemicals in the environment.

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