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Hyptis suaveolens Extract Exhibits Larvicidal Activity Against *Anopheles gambiae* Larvae

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ABSTRACT

Hyptis suaveolens (Lamiaceae) is traditionally used to repel insects. The aim of this study was to determine the larvicidal activity of dichloromethane aerial part extract of *H. suaveolens* against 4th instar *Anopheles gambiae* (Diptera: Culicidae) larvae by establishing dose response of the extracts, determining the lethal concentrations (LC_{50} and LC_{90}) of the extracts and the efficacy of the plant as a mosquito larvicide following the World Health Organization guidelines for laboratory testing of mosquito larvicides. Two samples of the aerial parts were collected and used for the test; sample Z1 was collected in the morning and sample Z2 in the afternoon. Concentrations ranging from 10 - 100 mg/mL of the extract were used. The results showed that there is a very high significant difference between the two samples in relation to the concentrations ($df = 4$, $P < 0.0001$). The larvae were resistant at 24th hour and at 48th hour but became susceptible to the extracts at 72nd hour with 99% and 97% mortality rates with both samples. There was significant difference in mortality rates between the two samples ($P < 0.05$). LC_{50} at 72nd hour for Z1 and Z2 was 3.51 mg/mL. LC_{90} at 72nd hour for Z1 was 1.52 mg/mL and 33.47 mg/mL for Z2. The result of this study indicates that *H. suaveolens* possesses larvicidal properties and supports its traditional application as a repellant/insecticide. Thus, the plant extract could be applied to mosquito breeding sites as a measure to control malaria vector, particularly *A. gambiae*.

Keywords: *Hyptis suaveolens*, Mosquito larvicide, *Anopheles gambiae*, Malaria.

Introduction

Mosquitoes spread more diseases than any other arthropod, one of which is malaria.[1] Malaria has been confronting the continent of Africa at an alarming rate for ages. In the year 2016, an estimated 216 million cases of malaria were recorded and 445,000 deaths globally and ninety-one percent (91%) of these deaths occurs in Africa.[2] Although there are several species of anopheles mosquitoes, *Anopheles gambiae* is the most efficient vector of *Plasmodium falciparum* due to its preference for humans as a host and its indoor-feeding behavior.[3] for this reason, *A. gambiae* is named African malaria mosquito.[4] *A. gambiae* is not only an effective vector of human malaria, but also for lymphatic filariasis (elephantiasis).[4] Despite several efforts in controlling this vector, the medical and economic burdens caused by it continue to grow.[4] The failure in current control measures and the growing insecticide resistance is necessitating search for newer control strategies.[5] Warm temperatures and stagnant waters are factors that contribute to the malaria endemicity in tropical and sub-tropical countries.[1]

Studies have shown that *A. gambiae* larvae can develop in man-made structures such as concrete tanks and drainage canals and natural pools such as swamps, hoof prints and marshes.[6, 7] The larvae of *A. gambiae* pupate after the fourth instar acquires an appropriate amount of nourishment.[7] Mosquitoes in the larval stage are attractive targets for pesticides because they breed in water, and thus, are easy to deal with in their habitat.[8] But the use of conventional pesticides in water sources introduces many risks to people and to the environment.[8] Natural pesticides, especially those derived from plants, are a perfect fit in view of environmental concerns.[9] Some plants contain essential oils, natural volatile mixtures of hydrocarbons with a diversity of functional groups, which impart on them the ability to be used as repellants and as insecticide.[10] Mosquito repellent activity has been reported in several plants such as *Azadirachta indica*, *Azadirachta Juss* (neem tree), *Ocimum basilicum* (basil oil), and *Citronella species*.[11] Onions, garlic and *Aloe vera* have also been subjected to larvicidal bioassay.[12] There are over 2000 plants species identified as having insecticidal properties and about 344 plant products that are known to possess mosquito repellant activity.[11] Such facts stimulate research efforts targeted at demonstrating larvicidal activity of various plant extracts which are prepared from different solvents. For example, the hexane extract of dried fruit of *Solanum nigrum* Linn is reported to be more potent than the aqueous extract of the same plant against mosquito larvae.[13] Repellant activity of plants has been linked to the presence of monoterpenes and sesquiterpenes.[11]

H. suaveolens is an aromatic herb commonly known as bush mint. It belongs to the mint family Lamiaceae.[14] In Nigeria, *H. suaveolens* is of high economic and medicinal value used widely for its mosquito-repellent properties.[15] The plant is collected, burnt like grass to release smoke that drives mosquitoes away. Crude ethyl acetate extracts of *H. suaveolens* at 1% concentration exhibited feeding deterrents activity against the larvae of *Helicoverpa armigera*[16] and exhibited growth inhibitory activity against pathogenic bacteria like *Aeromonas formicans*, *Aeromonas hydrophilia*, *Bacillus subtilis* and *Pseudomonas*

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aeruginosa more than double the results with distilled water extract.[17] Diterpenes identified in the plant are suaveolic acid, suaveolol, methyl suaveolate and a wide range of monoterpenes have also been identified in the essential oil.[18] This study is designed to investigate the effects of dichloromethane aerial extracts of *H. suaveolens* on the larvae of *A. gambiae* by establishing dose response of the extract, determining the lethal concentrations (LC_{50} and LC_{90}) of the extract as well as the efficacy of the plant with respect to time of sample collection.

Materials and Methods

Sample Collection

H. suaveolens aerial parts were collected in the month of December, 2016 around the uncultivated farmlands of the Faculty of Agriculture, Department of Agronomy, Nasarawa State University Keffi, Lafia campus, Nigeria. The identity of the plant was confirmed by Mr Namson Tsaku of the Agronomy Laboratory, Nasarawa State University Keffi. The sample labeled as Z1 was collected at 8:00hrs while the sample labeled as Z2 was collected at 13:00hrs. Both samples were dried in the laboratory then crushed and grinded.

Sample Extraction

The dried *H. suaveolens* aerial parts (250 g) was extracted with dichloromethane (770 mL) by maceration at room temperature for 72 hours. The extract was filtered and concentrated using a rotary evaporator at 45˚C to give a dry residue. The crude dichloromethane extract was kept in an air-tight container and stored in a refrigerator at 4˚C until use.

Identification of Mosquito Larvae

Third and fourth instar *A. gambiae* larvae were collected from different breeding sites in Lafia and identified by Mr Akwashiki Ombugadu of the Zoology Department, Federal University Lafia. They were kept in dark containers to avoid sunlight penetration and allowed to acclimatize for 24 hours at room temperature before carrying out the test. The larvae were left in the same water fetch from their breeding site.

Larvicidal Test

Bioassay for the larvicidal test was carried out following the WHO 2013 standard procedures for laboratory testing of mosquito larvicides.[19] The dichloromethane extracts of *H. suaveolens* were evaluated using different concentrations of 10, 20, 50 and 100 mg/mL. Distilled water (100 mL) to which 1 mL acetone was added was used as control. Twenty-five larvae were put into each of four disposable cups (150 mL capacity) containing 100 mL of water from the breeding site to which a measured concentration of the test solution is added. Larval mortality was counted at 24, 48 and 72 hours after treatment. Mortality was calculated at each time interval, replicated three times and the results used to determine the LC_{50} and LC_{90} values for the extracts by Probit analysis. Larvae were considered either alive, if they were clearly moving normally, or dead when there is no movement and no response to gentle probing with a fine glass rod.

The interpretation of the mortality rate of *Anopheles* larvae based on WHO (2013)[20] susceptibility tests is:

Mortality rate between 98 – 100% within the diagnostic time.
Mortality rate between 80 – 97% suggest possible resistance.
Mortality rate < 80% indicates resistance.

The percentage mortality was calculated by employing the formula:

$$\%\ Mortality = \frac{Number\ of\ Dead\ Larvae}{Number\ of\ Larvae\ introduced}\ X\ 100$$

Determination of LC_{50} and LC_{90}

Lethal concentrations (LC_{50} and LC_{90}) were determined by Probit analysis as described by Finney (1971) for both samples at the different concentrations and times used in this study. Microsoft Excel regression probit analysis was employed. Percentage mortalities were converted to probits by looking up the percentage in Finney's table. The log of concentrations is calculated. A graph of probits versus the log of concentration is plotted to fit a line of regression. Extrapolating the probit of 5 in the y-axis to the x-axis followed by taking the inverse of log of the extrapolated value on the x-axis gives the LC_{50}. A similar procedure was used to determine the LC_{90}.

Statistical Analysis

Data obtained were analyzed using R Console software (Version 2.9.2). Mortality rate of the *Anopheles* larvae in relation to concentrations of extracts were compared using Pearson's Chi-square test. The P-values < 0.05 were considered statistically significant.

Results and Discussion

Results of mortality rates recorded against *A. gambiae* by both samples at varying concentrations (10 mg/L to 100 mg/mL) are presented in Table 1. LC_{50} and LC_{90} are presented in Tables 2 and 3. The LC_{50} and LC_{90} are the lethal concentrations of the extract that kills 50% and 90%, respectively of the *A. gambiea* larvae population. It is important to note that the lower the LC_{50} and LC_{90}, the more effective the larvicidal activity of the extract.

For sample Z1, at 24 hours, there was a significant ($\chi^2 = 58.679$, df = 4, P < 0.0001) concentration-dependent mortality rate of *A. gambiae* larvae. However, the mortality rates recorded at the 24th hour at the various concentrations tested showed that they are resistant (mortality rate < 80%) to the extract (Figure 1).

At 48 hours, the mortality rate of *A. gambiae* larvae in relation to concentrations showed a very high significant difference ($\chi^2 = 91.58$, df = 4, P < 0.0001). However, the mortality rates recorded at the 48th hour for concentrations 0 to 50 mg/mL showed that the larvae were resistant (mortality < 80%) while at 100 mg/mL they showed possible resistance (mortality rate = 91%) to the extract (Figure 1).

At 72 hours, the mortality rate of *A. gambiae* larvae in relation to concentrations showed a very high significant difference ($\chi^2 = 92.017$, df = 4, P < 0.0001). However, the mortality rates recorded at the 72nd hour for concentrations 0 to 20 mg/mL showed that the larvae were resistant (mortality rate < 80%) while at 50 mg/mL they showed possible resistance (mortality rate = 91%) and at 100 mg/mL they were susceptible (mortality rate = 100%) to the extract (Figure 1).

For Sample Z2, at 24 hours, the mortality rate of *A. gambiae* larvae in relation to concentrations showed a very high significant difference ($\chi^2 = 56.00$, df = 4, P < 0.0001). However, the mortality rates recorded at the 24th hour at the various concentrations tested showed that the larvae were resistant (mortality rate < 80%) to the extract (Figure 2).

At 48 hours, the mortality rate of *A. gambiae* larvae in relation to concentrations showed a very high significant difference ($\chi^2 = 78.705$, df = 4, P < 0.0001). However, the mortality rates recorded at the 48th hour for concentrations 0 to 50 mg/mL showed that the larvae were resistant (mortality rate < 80%) while at 100 mg/mL they showed possible resistance (mortality rate = 85%) to the extract (Figure 2).

At 72 hours, the mortality rate of *A. gambiae* larvae in relation to concentrations showed a very high significant difference ($\chi^2 = 90.618$, df= 4 P < 0.0001). However, the mortality rates recorded at the 72nd hour for concentrations 0 to 20 mg/mL showed that they larvae were resistant (mortality rate < 80%) while at 50 mg/mL they showed possible resistance (mortality rate = 91%) and at 100 mg/mL they were susceptible (mortality rate = 97%) to the extract (Figure 2).

For both samples used at the concentration range of 10 mg/mL – 100 mg/mL for 24 hours the mortality rates recorded is not significant enough to say that the dichloromethane extract can be used as an effective larvicide against *A. gambiae* based on World Health Organization (WHO) standards. Irrespective of the concentration, no significant mortality was achieved with any of the extracts (as shown in Table 1 since P > 0.05) within 24 hours, a higher concentration of Z2 will be required to kill 50% and 90% of the larvae than Z1 as indicated by their LC_{50} and LC_{90} values (Tables 2 and 3). The larvae were resistant to the extracts at the 24th hour. On comparing mortality rate of *A. gambiae* in relation to concentration that was achieved by the two samples, a very high significant difference (P < 0.00001) was observed. The trend in mortality rates of the larvae as observed at the 24th hour of treatment with both extracts continued even at the 48th hour but only with concentrations ranging from 10 mg/mL – 50 mg/mL. An appreciable improvement in mortality from < 80% to 85% was observed at 48th hour when concentration was increased to 100 mg/mL. This still falls below the WHO standard for effective bio-larvicides.

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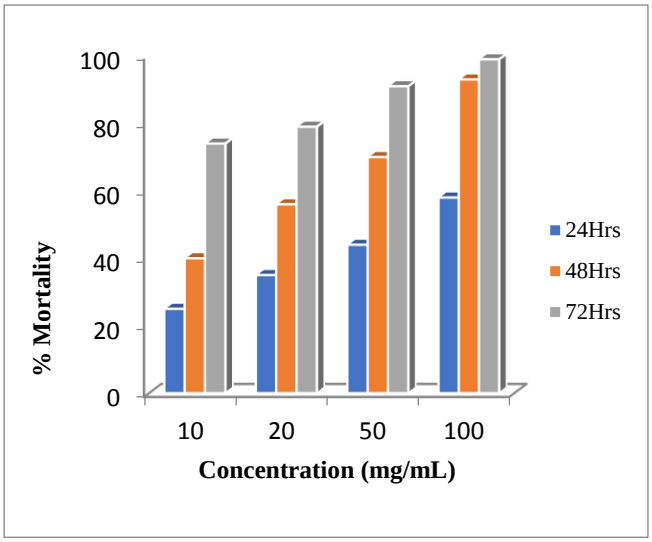

Figure 1: Mortality Rate of *Anopheles gambiae* in Relation to Concentration for Sample Z1.

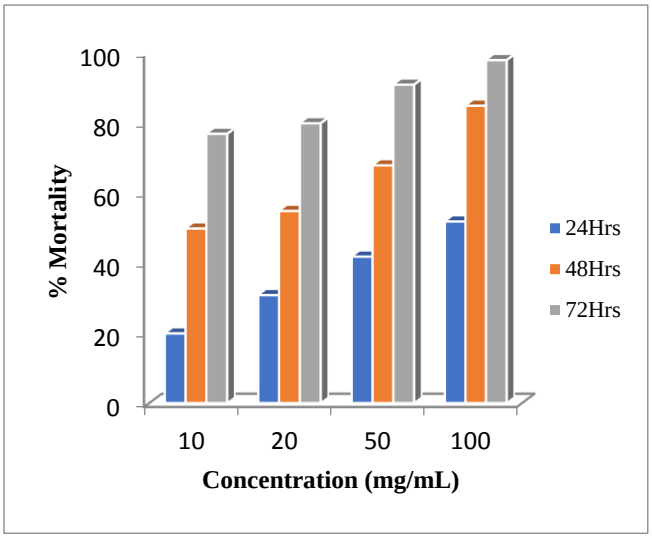

Figure 2: Mortality Rate of *Anopheles gambiae* in Relation to Concentration for Sample Z2.

Table 1: Comparison of Percentage Mortality between Sample Z1 and Sample Z2.

Conc. (mg/mL)	Time of Exposure	%mortality Z1	%mortality Z2	χ^2	Df	P Value
0	24Hrs.	0	0	0	1	1
	48 Hrs.	0	0	0	1	1
	72 Hrs.	0	0	0	1	1
10	24Hrs.	25	20	0.5556	1	0.4561
	48Hrs.	40	50	1.1111	1	0.2918
	72Hrs.	74	77	0.0596	1	0.8071
20	24Hrs.	35	31	0.2424	1	0.6225
	48Hrs.	56	55	0.009	1	0.9244
	72Hrs.	79	77	0.256	1	0.8728
50	24Hrs.	44	42	0.04561	1	0.8292
	48Hrs.	70	68	0.0289	1	0.8648
	72Hrs.	91	91	0	1	1
100	24Hrs.	58	52	0.3272	1	0.5672
	48Hrs.	91	85	0.2046	1	0.6511
	72Hrs.	99	97	0.0204	1	0.8864

Table 2: LC_{50} and LC_{90} of dichloromethane extract of *Hyptis suaveolens* for sample Z1.

Time of exposure (hours)	LC_{50} (mg/mL)	LC_{90} (mg/mL)
24	63.50	218.72
48	16.09	114.86
72	3.51	1.52

Table 3: LC_{50} and LC_{90} of dichloromethane extract of *Hyptis suaveolens* for sample Z2.

Time of exposure (hours)	LC_{50} (mg/mL)	LC_{90} (mg/mL)
24	84.60	249.57
48	12.47	2.36
72	3.51	33.79

However, lower concentrations of the extracts of both samples will be able to kill more *A. gambiae* larvae at the 48th hour than at 24th hour. Similar to the observation at the 48th hour, at the 72nd hour, two trends were also observed. The first trend was at concentrations less than or equal to 20 mg/mL and the second trend was observed above 20 mg/mL. In the former, mortality rate of *A. gambiae* larvae was < 80% while in the later mortality rate recorded was between 91% - 100%. Concentration of 100 mg/mL is the most effective concentration that is required to kill 99% of the larvea. The least concentration that is able to kill 99% of the *A. gambiae* larvae population is 1.52 mg/mL of sample Z1 and the least concentration that is required to kill 50% of the *A. gambiae* larvae population is 3.51 mg/mL of any of the two samples.

Of the twenty-four tests carried using the concentrations ranging from 10 mg/mL – 100 mg/mL, the bioactivity of the samples against the larvea followed a regular pattern except when 10 mg/mL was used. Twenty-three out of the twenty-four tests carried out showed a dose-dependent and a time-dependent relationship in the mortality rates.

Time and concentration dependency of plants extracts as potential effective bio-larvicide has also been noted by Ebuka *et al* (2017).[20] The literatures report different solvents employed in extracting *H. suaveolens* to give varying bioactivity on different malaria vectors. Solvents commonly used in extraction of *H. suaveolens* are polar solvents like acetone, methanol and ethanol. This informed the choice of solvent (dichloromethane) used in this study. Most of the studies observed mortality rates at 24 hours after administering extracts. Because of the variations, particularly in terms of duration of exposure of tested organisms, between this study and those already reported, it is difficult to compare and say which extract is most effective. Bobbo *et al* (2016)[21] reported a mortality rate of 53.33±1.16 mg/mL and LC_{50} 1.78 ± 0.97 mg/mL with acetone leaf extract of *H. suaveolens* at 24th hour which gives an LC_{50} lower than what this study achieved using dichloromethane. Ayange-kaa *et al* (2015)[22] reported mortality rate of 99.2% and 51.3% respectively with 80% ethanol of *H. suaveolens* 500 mg/mL leaf extract on eggs and adults of *A. gambiea*. Dichloromethane extracts in this study were tested on fourth instar larvae and not on eggs or adults of the anopheles. The study by Ayange-kaa *et al* (2015)[22] did not mention the specie of mosquito egg or adult that the extract was tested against. Efficacy of plants extracts on organisms are time dependent.[23]

Conclusion

The result of this study has indicated that *H. suaveolens* possesses larvicidal properties and supports its traditional application as a repellant/insecticide. Concentration as low as 2.36 mg/mL of Z2 can be used to achieve a mortality rate of 90% in 48 hours. A lower concentration of 1.52 mg/L of Z1 can be used to kill 90% of *A. gambiae* population in 72 hours. Extract of the *H. suaveolens* aerial parts can be applied to mosquito breeding sites as a measure to control malaria vector, particularly *A. gambiae*.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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