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Assesment of Larvicidal Activities of Essential Oil Extracted from Country Onion (*Afrostyrax lepidophyllus*) Seed on Anopheles Mosquito Larvae

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Abstract

Mosquitoes particularly anopheles are the main vectors for the malaria parasites that causes malaria fever diseases which is the highest killer of human beings in the tropics. There has been exploration of various methods over the centuries to control mosquito borne diseases. Larvicidal activities of essential oil extracted from country onion (*Afrostyrax lepidophyllus*) seed, on anopheles mosquito larvae has been determined in this study. Essential oils were extracted from *Afrostyrax lepidophyllus* seed by Hydrodistillation method, with a percentage yield of 4.12% and characterized using gas chromatography-mass spectrometry (GC-MS). Eighteen (18) compounds were identified to be Methanamine (1.07%), Dimethyl trisulfide (16.7%), Furan (0.79%), 1,2-Benzenediol (1.53), Disulfide (52.3%), 1,2,4 trithiolane (0.40%), 2,4-dimethyl-3-nitrobicyclo [3.2.1] (2.85%), 2-pyridinemethanamine (0.66%), Bis (2sulfhydrylethyl (16.7%), Benzenemethanol (0.28%), 2,2-dimethylpropanoic acid (0.50%),1,2 bis (trimethylsily) benzene (0.27%), Cyclotrisiloxane (0.28%), Silane (0.27%), Tetrasiloxane (0.68%), Methyltris (trimethylsiloxane) silane (1.00%), Silicic acid (2.15%), 1,4-phenylenebis[trimethyl (1.53%). The percentage amounts of the components was determined based on their relative abundance and their retention indices. There were significance differences ($P< 0.05$) in concentrations of *Afrostyrax lepidophyllus* essential oil activities against anopheles mosquito's larvae. Results of the larvicidal activity showed that, 200ppm and 400ppm concentration of essential oil induced 100% larval mortality in 24hr. While At lower concentration of 50ppm, 100ppm and 150ppm, induced 45% and 95% and 99% larval mortality within 24hr respectively. This results of larvicidal activity of Essential oil from the *Afrostyrax lepidophyllus* seed shows that the oil is highly reactive against anopheles mosquito larvae indicating that it could be explored as a more potentially effective, economical and cost effective mosquito control agent for industrial and household usage.

Keywords

Afrostyrax lepidophyllus, Essential, Larvicidal and Hydrodistillation, Mosquito

1. Introduction

Mosquito borne diseases are major human and animal health problem in all tropical and subtropical countries [1]. The diseases transmitted include malaria, filariasis, yellow fever, Japanese encephalitis and dengue fever. There has been exploration of various methods over the centuries to control mosquito borne diseases [2]. Anopheles mosquito takes a blood meal as protein source to complete egg development, by injecting the saliva which may contain pathogens into the host animal, the pathogens thus complete an obligatory life cycle phase and multiply in the mosquito's salivary glands. This thereby makes female mosquitoes ideal transmitters of

diverse blood borne pathogens and agents of devastating human diseases [3]. Essential oils being complex mixtures of volatile organic compounds are generally produced as secondary metabolites in plants. The metabolites like the monoterpenes such as citronellal [A], thymol [B], α-pinene [C], cineole, eugenol, terpinolene, and citronellol, are the common constituents in a number of essential oils presenting mosquito repellent activity [4]. Several methods of controlling malaria diseases have been developed but most of the effective methods are based on synthetic chemicals that may be hazardous and pose great danger to living things especially humans. These synthetic chemicals are also causing a lot of environmental problems because most of them just like heavy metals are not biodegradable [5]. There is therefore need to search for alternative environmentally friendly and biodegradable chemicals for the control of mosquitoes. The test of the larvacidal activities of essential oil from *Afrostyrax lepidophyllus* seed on anopheles mosquito larvae will be a welcome initiative.

Afrostyrax lepidophyllus is a species of plant in the Class; *Mognoliopsida*, Order; *Violates*, and Family; *Huaceae*. It is found in Cameroon, Gabon, and Ghana. It is threatened by habitat loss. It is sometimes known as country onion. The bark extract of *Afrostyrax lepidophyllus* has shown pesticidal activity against nematodes and anthropods, including insecticide-resistant strains of lice and blowflies [6]. The country onion "*Afrostyrax lepidophyllus*" is a spice and medicine of the Cameroon Rain forest. *Afrostyrax lepidophyllus*, known as "country onion " in west African, is a small tree native to rain forest of the west and central Africa. In the Rumpi Hill of south-west Cameroon, country onion grows natural, women and children harvest the seeds and bark and sell them in village markets and the buyers carry them to the major markets in Cameroon where seeds and bark are sold as medicine [7], such as remedy for child`s cough, heart rate, worms, constipation, hermia abscesses, and boils. A. *Lepidophyllus* plant is used throughout west and central African region. This plant parts are used in this region for various purposes, such as medicine, food, spice, and incense. The plant is therefore a source of income where ever it is found in this region. *Afrostyrax lepidophyllus* is also used as a traditional pesticide in controlling weevils in stored grains [8]. Fai *et al.* also reported antifugal activities of essential oil extracted from the bark of *Afrostyrax lepidophyllus* [9] while Toumnou, *et al.* reported pest management of storage using extracts of *afrostyrax lepidophyllus* against *Sitophilus zeamaïs, Tribolium castaneum* and *Rhyzopertha dominica* [8]. The unpredictable availability of seeds creates extreme price variations, which affect village economy and how villagers collect, store, sell and use the seeds. The plant exists much naturally only in the volcanic hills of Cameroon (the Rumpi Hills). The tree information on *Afrostyrax lepidophyllus* is currently being researched and will or may appear shortly [6]. The information is awaiting authentication by a species expert, and will be up dated as soon as possible according to IUCN [6]. The *afrostyrax* dry seeds are sold in almost all markets in Cameroon and are used as species in the traditional African cuisines [7]. At the basis of their aromatic properties are volatile sulphur containing compounds [9] a class of natural products having a very low flavour threshold (e.g 0.1 ppt in water) and for this reason considered key-odorants. Iranshahi *et al*. determine that, molecules play an important role in determining the flavour and odour properties of many food stuffs [4].

Mosquito borne diseases are major human and animal health problem in all tropical and subtropical countries. The diseases transmitted include malaria, filariasis, yellow fever, Japanese encephalitis and dengue fever. There has been exploration of various methods over the centuries to control mosquito borne diseases [2]. Anopheles mosquito takes a blood meal as protein source to complete egg development, by injecting the saliva which may contain into the host animal, the pathogens thus complete an obligatory life cycle phase and multiply in the mosquito's salivary glands. This thereby makes female mosquitoes ideal transmitters of diverse blood borne pathogens and agents of devastating human diseases [3]. Essential oils being complex mixtures of volatile organic compounds are generally produced as secondary metabolites in plants. The metabolites like the monoterpenes such as citronellal [A], thymol [B], α-pinene [C], cineole, eugenol, terpinolene, and citronellol, are the common constituents in a number of essential oils presenting mosquito repellent activity [10]. Many methods of controlling malaria diseases have been developed but most of the effective methods are based on synthetic chemicals which are hazardous to living things including humans. The test of the larvacidal activities of essential oil extracted from *Afrostyrax lepidophyllus* seed on anopheles mosquito larvae has been investigated in this study as an alternative environmentally friendly and biodegradable means for control of mosquitoes.

2. Material and Method

2.1. Sample Collection and Processing

The *Afrostyrax lepedophyllus* (Country onion) seed samples were bought from a market in Cameroon. The seed bought were of good quality. They were sorted and crushed into fine powder with mortar and pestle, sieved and stored in an air tight plastic container for analysis.

2.2. Extraction

Clevenger method of hydrodistillation of essential oils was adopted [11] and modified. 340 grams of *Afrostyrax Lepidophyllus* powdered seeds were weighed and put into 5000 ml distillation flask, using a plastic funnel. Water was added into the flask until it was half full with water and sample. The flask and its content were mounted on a heating mantle coupled with Clevenger type apparatus connected to a condenser. The flask was heated slowly at temperature of 100°C for 30 minutes after which the temperature was reduced to between 30°C -40°C. The extraction was carried

out for the period of three hours (3hrs). The extracted essential oil was collected into pre-weighed 1ml glass specimen bottles and weighed.

2.3. Analysis

2.3.1. Determination of Yield

The percentage yield was determined using the formula;

$$\% \text{ Yield} = \frac{w2-w1}{WO}$$

Where W_0= weight of sample
W_1= weight of empty specimen bottle
W_2= weight of extracted essential oil and specimen bottle

2.3.2. Characterisation of Essential Oil (GC-MS Analysis)

The GC-MS analysis was carried out in Usman Danfodio University Sokoto. The GC-MS analysis was carried out on Agilent Technologies 6890N Network GC System and Agilent technologies 5973 network mass selective detector coupled with 7693B series injection (model number 122-5533 capillary column with specification: 0.25mm*30m*1µm). The carrier gas used was helium at a flow rate of 1.2ml/min with injection volume 1 NL and an inlet temperature was maintained at 230°C. The oven temperature was programmed initially at 50°C for 5 minutes, then programmed to increase to 300°C at a rate of 10°C for 25 minutes. Total run time was 45 minutes. The MS transfer line was maintained at 230°C and MS Quad at 150°C. The ionization mode used was electron ionization mode at 70ev. Total ion count (TIC) was used to evaluate compound identification and quantification. The spectrum of the separated compound was compared with the database of the spectrum of known compound saved in the NIST02 reference spectra library. Data analysis and peak area measurement were carried out using Agilent Chemistation software.

2.4. Determination of Larvicidal Activity

The larvicidal activity of the *Afrostyrax lepidophyllus* essential oil was carried out in Malaria research laboratory of Gombe State University according to the WHO Guide line Laboratory and Field Testing of Mosquitor Larvicides [1].

2.4.1. Materials Required for Testing

One pipette delivering 100–1000 µl, disposable tips (100 µl, 500 µl) for measuring aliquots of dilute solutions, five 1 ml pipettes for insecticides and one for the control, three droppers with rubber suction bulbs, two wire loops, one piece of nylon netting (30 cm^2) and one tube of cement. Two pieces of netting were cut and cemented to opposite sides of the larger end of the wire loops. More cement should then be applied around the edges of the loops to join the two pieces of netting.

2.4.2. Preparation of Stock Solutions or Suspensions and Test Concentrations

Ethanol was used as the solvent for diluting the essential oil. The volume of stock was 20 ml of 1%, obtained by weighing 200 mg of the technical material and adding 20 ml solvent to it. It was kept in a screw-cap vial; with aluminium foil covering the mouth of the vial. The stock solution was serially diluted (ten-fold) in ethanol (2 ml solution to 18 ml solvent). Test concentrations were then obtained by adding 0.1–1.0 ml (100–1000 µl) of the appropriate dilution to 100 ml. For other volumes of test water, aliquot of dilutions added was adjusted according to Test concentrations. Small volumes of dilutions were transferred to test cups by means of pipettes with disposable tips. Distilled water was used in the preparation of the 1% stock solution or suspension and in subsequent serial dilutions, according to the content of the active ingredient.

2.4.3. Bioassays

The mosquito larvae were collected from Malam-Inna in Gombe town and Hinna in Dadin kowa in Gombe State, Nigeria. The mosquito larvae were exposed to a wide range of test concentrations and a control to find out the activity range of the materials under test. The mortality of larvae was determined within a wide range of concentrations, a narrower range (of 4–5 concentrations, yielding between 10% and 95% mortality in 24 hrs) was used to determine LC_{50} and LC_{90} values. Batches of 25 third or fourth instars larvae were transferred by means of strainers, screen loops or droppers to small disposable test vessels, each containing 200 ml of water. Small, unhealthy or damaged larvae were removed and replaced. The depth of the water in the vessels was between 5cm and 10 cm; deeper levels may cause undue mortality. The appropriate volume of dilution was added to 200 ml water contained in the test vessels to obtain the desired target dosage, starting with the lowest concentration. Two replicate set up for each concentration and an equal number of controls was set up simultaneously with distilled water, to which 1 ml alcohol (or the organic solvent used) was added. Each test was performed two times on different days. For long exposures, larval food was added to each test vessels, particularly if high mortality is noted in control. The test containers were held at 25–28°C on a photoperiod of 12 hr light followed by 12 hr dark (12L: 12D). After 24 hr exposure, larval mortality was recorded. Moribund larvae were counted and added to dead larvae for calculating percentage mortality. Dead larvae are those that cannot be induced to move when they are probed with a needle in the siphon or the cervical region. Moribund larvae are those incapable of rising to the surface or not showing the characteristic diving reaction when the water is disturbed. The results was recorded, where the LC_{50}, LC_{90} and LC_{99} values, and slope and heterogeneity analysis was also noted. Larvae that have pupated during the test period will negate the test. The control mortality was between 5% and 20%, the mortalities of treated groups was also corrected according to Abbott's formula.

$$\text{Corrected mortality} = \frac{\text{OM in treatment} - \text{OM in control}}{100 - \text{Control mortality}} \times 100$$

Where OM = Observed mortality in treated sample and

Observed moratality in the control.

2.4.4. Thin Layer Chromatography

The essential oil of *Afrostyrax lepidophyllus* was spotted on TLC plate, the plate was transferred into the development tank containing a mixture of n-hexane and ethyl acetate ratio of 6:1 and 9:1. The solvent ratio, n-hexane to ethyl acetate 6:1 gave two spots and the retention factor (R_f) values were determined (plate 1) and 9:1 gave five spots with (R_f) values which were also determined (plate 2). This showed that due to solvent ratio of hexane to ethyl acetate in the ratio of 9:1 gave a good separation of the component, this implies that lower polarity is good for the determination of the component of *Afrostyrax lepidophyllus.*

3. Result and Discussion

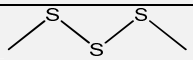
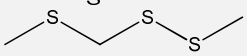
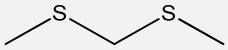
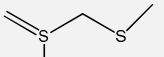
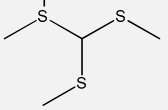
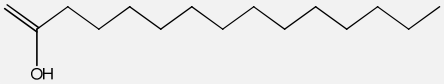
3.1. GC-MS Results

The GC-MS analysis gave a total of 18 volatile compound identified in this research as presented on table 1 and 2. The total of 18 component were identified in the essential oil, representing 99.96%.

Table 1. *GC-MS analysis result showing the chemical composition of afrostyrax lepidophyllus essential oil retention index.*

Constituences	Retention indices	%Area
Methanamine	1040	1.07
Dimethyl trisulfide	1140	16.7
Furan	1155	0.79
1,2-Benzenediol	1231	1.53
Disulfide	1244	52.3
1,2,4-trithiolane	1259	0.40
2,4-dimethyl-3-nitrobicyclo[3.2.1]	1355	2.85
2-pyridinemethanamine	1468	0.66
Bis (2-sulfhydrylethyl)	1474	16.7
Benzenemethanol	1478	0.28
2,2-dimethylpropanoic acid	1608	0.50
1,2-bis (trimethylsily) benzene	1673	0.27
Cyclotrisiloxane	1694	0.28
Silane	1795	0.27
Tetrasiloxane	1931	0.68
Methyltris (trimethylsiloxane) silane	1939	1.00
Silicic acid	2069	2.15
1,4-phenylenebis[trimethyl	2086	1.53
TOTAL	99.96%	

Table 2. *Structural Composition of some sulphur rich compounds from afrostyrax lepidophyllus essential oil detected by GC-MS.*

CONSTITUENT	STRUCTURE
1. Dimethyl trisulfide	
2. Disulfide	
3. 2,4-Dithiapentane	
4. Methane	
5. Methyl thiosulfide	
6. Tetradecanoic acid	
7. 9, 12-Octadecadien-1-ol	

3.2. Larvicidal Activity

The results of the larvicidal activity of *Afrostyrax lepidophyllus* essential oil against Anopheles mosquito larvae is presented on table 2. The result on percentage mortality of larvae of anopheles mosquito, shows that mortality increases with increased in essential oil concentration.

Table 3. *The larvicidal activity of Afrostyrax lepidophyllus essential oil against Anopheles mosquito larvae.*

Test indicator (ppm)	50	100	150	200	400	D_{10}	D_{50}	Negative control
Oil concentration (ml) of solution	1.0	2.0	3.0	4.0	8.0	10	25	0.00
Test No. 1 Mortality in 24hr	15	25	24	25	25	10	25	0
Test No. 2 Mortality in 24hr	13	22	25	25	25	10	25	0
Average Mortality in 24hr	14	25	25	25	25	10	25	0
Percentage of observed mortality $\% \text{ OM} = \frac{\text{No of death larvae}}{\text{No of larvae treated}} \times 100\%$	56	96	99	100	100	40	100	0
Percentage of corrected mortality $= \frac{\% \text{ OM} - \%\text{CM}}{100 - \% \text{ CM}} \times 100\%$	45	95	99	100	100	25	100	0

Where OM = percentage of observed mortality

CM = Percentage of control mortality

D = Commercial Deltamethrin

D_{10} and D_{50} = Positive Control

3.3. Thin Layer Chromatorgraphy

In the essential oil of *Afrostyrax lepidophyllus* that was spotted on TLC plate, the plate that was transferred into the development tank containing a mixture of n-hexane and ethyl acetate in the ratio of 6:1 and 9:1. The solvent ratio, n-hexane to ethyl acetate 6:1 gave two spots with R_f values of 0.5660 and 0.320 (plate 1) and 9:1 gave five spots with R_f values of 0.66, 0.50, 0.304, 0.143 and 0.054 (plate 2).

3.4. Discussion

The percentage yield of *Afrostyrax lepidophyllus* extracted essential oil was 4.12%, which was colourless similar to the findings and determined compounds of *Afrostyrax lepidophyllus* by Hervet *et al.* and UNAFAS [13-14]. The essential oil composition was characterized by GC- MS and the compounds identified were sulphur rich as reported by previous study [9, 14]. The major constituents were 2,4,5,7-tetrathiaoctane (1; 52.9%), 2,4,5,7,9-pentathiadecane (2; 11.7%), 2,3,5- trithiahexane (3; 8.1%), and an unknown compound that was tentatively identified as 6-methyl-2,4,5,7,9-pentathiadecane (4; 10.8%). The identification of four (4) compound was based on the comparison of the retention index (RI) with that published in the literature and on its characteristic MS fragments (246 (Mþ), 121, 93, 61) and the isotopic pattern, which were considered diagnostic. The resultant oils devoid of monoterpene hydrocarbons and phenylpropanoids were scarce, being represented by eugenol (1.2%) in comparison to literature [13-14]. The following identified components, found in this study were not identified in previous study by Hervet, *et al*. [13] but identified by Fai *et al*. [9]: Methanamine (1.07%), Dimethyl trisulfide (16.7%), Furan (0.79%),1,2-Benzenediol (1.53), Disulfide (52.3%),1,2,4-trithiolane (0.40%), 2,4-dimethyl-3-nitrobicyclo[3.2.1] (2.85%), 2-pyridinemethanamine (0.66%), Bis (2-sulfhydrylethyl) (16.7%), Benzenemethanol (0.28%), 2,2-dimethylpropanoic acid (0.50%), 1,2-bis (trimethylsily) benzene (0.27%), Cyclotrisiloxane (0.28%), Silane (0.27%), Tetrasiloxane (0.68%), Methyltris (trimethylsiloxane) silane (1.00%), Silicic acid (2.15%) and 1,4-phenylenebis [trimethyl (1.53%). The final result obtained from the GC-MS gave the chemical composition and structures of the extract indicating sulphur rich containing compound (table 2) similar to reports by Fai, *et al*. [9]. The structure of each of them was suggested by the library information in the GC-MS software liberary.

The result obtained from the bioactivity test (larvicidal test) showed that the extract of *afrostyrax lepidophyllus* was very effective than the control used Deltamethrine. From table 2, it could be seen that, at higher concentration of essential oil solution (200 and 400ppm), more larvae died. In the control, the mortality rate observed was 20% within 24hr, the larvae developed into pupae and to adult within 48hr of observation. Furthermore, at low concentration of essential oil solution of (50ppm, 100ppm and 150ppm) there were no pupae or adult emergence observed.

There were significance differences (P> 0.05) in *Afrostyrax lepidophyllus* essential oil activity against anopheles mosdquito larvae. At concentration (200 and 400ppm) essential oil solution induces 100% larval mortality rate within 24hr. While at lower concentration, 50ppm, 100ppm and 150ppm, the larval mortality induce by *Afrostyrax lepidophyllus* essential oil were 45%, 95% and 99% within 24hr respectively. However, the result of the larvicidal activities showed that, the essential oil induced 100% larval mortality in 24hr with the concentration of 200ppm and 400ppm concentration. *Afrostyrax lepidophyllus* was found to be more active and effective against anopheles mosquito larvae because it induced 99% larval mortality even when the concentration was as low as 150ppm. From this result a positive contrast and correlation was made against concentration of the essential oil, at 10ppm and 50ppm concentration of Deltamethrine that induced (36% and 100%) larval mortally, and it was seen that at 10ppm not all of the larvae died and at 50ppm almost all the larvae were dead. Bhupen *et al*. reported plant essential oils as mosquito repellent in a review [2]. Fai *et al.* also reported antifugal activities of essential oil extracted from the bark of *Afrostyrax lepidophyllus* [9] while Toumnou, *et al.* reported pest management of storage using extracts of *afrostyrax lepidophyllus* against *Sitophilus zeamaïs, Tribolium castaneum* and *Rhyzopertha dominic* [8].

The essential oil components obtained from the TLC result shows that it contained five components with the retention factors as presented in the result indicating that at the polarity ratio of 6:1 there is small Rf values in contrast to the polarity ratio of 9:1 which move faster along distance follows by the five spots. This retention factors values gotten due to solvent ratio of n- hexane/ethyl acetate ratio 9:1 gave a good five separation of the component confirming that the result from the GC-MS was more accurate than the TLC result.

4. Conclusion

This study has confirmed reports from previous study that essential oil from *Afrostyrax lepidophyllus* is rich in sulphur compound based of GC-MS analysis and that this essential oil was more active against anopheles mosquito larvae compared to deltamethrine synthetic chemical used for mosquito control in this study. Thus could be explored as a more potentially effective, economical and cost effective mosquito control agent for industrial and household usage.

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