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Larvicidal Activity of *Clausena Anisata* Oils and Extracts *Anopheles Gambie* Larvea

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Abstract
Clausena anisata extracts are known to show bioactivity against several diseases here we report their larvicidal activity. Dried and ground 600 g of aerial parts of the plant *C. anisata* were sequentially extracted with solvents of increasing polarity, five fractions were obtained. The oils were obtained by hydro-distillation in a modified Clevenger-type of fresh whole plant. The essential oils (4.25% w/w) were dried over anhydrous sodium sulphate. The oils and active nonvolatile fractions were characterized by spectroscopic techniques. The major compounds in the oils were monoterpenes whereas the nonvolatile fractions had sesquiterpenes. The bioassays were performed with third instar larvae of *A. gambiae s.s*. The LC_{50} were 75.96 and 2095.46 mg l^{-1} for the oils and the active ethyl acetate fraction respectively. Their corresponding LC_{99} were 256.80 and 4438.75 mg l^{-1} respectively. Application of these extracts to larval habitats may prove useful in malaria and mosquito management programmes.

Keywords: Larvicide, phytochemicals, plant extracts

1. Introduction

The single most important insect transmitted disease globally is malaria [1, 2] and the greatest challenge in the fight against this pandemic is: resistance of the parasite to anti-malarial drugs, toxicity of the drugs to humans and high treatment cost. Recent WHO estimates are that there are 300 - 500 million cases of clinical malaria per year [3, 4], with 2.6 million deaths, mainly among African children [4]. Malaria is therefore a major cause of infant mortality and is the only insect borne parasitic disease comparable in impact to the world's major killer transmissible diseases: diarrhea, acute respiratory infections, tuberculosis and AIDS. The major problem of parasite drug resistance has necessitated policy change to more expensive drugs which the majority of the afflicted cannot afford. Similarly there is a notable vector resistance to insecticides [5]. This has led to interest in the development of malaria vaccines but the only one, which has been extensively field-tested, only gave a limited degree of protection. The situation is much more grave especially for the third world countries which are mostly infested with *A. gambiae*; a notorious carrier of the parasite [6, 7]. There is no vaccine to prevent infection caused by *A. gambiae* mosquito and the malaria parasite is continually developing resistance to the available drugs, so vector control is the best option [8, 10]. A considerable number of plant derivatives have shown to be effective against mosquitoes[11]. Larval control strategies against the vectors of malaria in sub-Saharan Africa should be prioritized for further development, evaluation and implementation as an integral part of controlling malaria. The ideal method of controlling mosquito infestation is the treatment of its breeding through use of larvicides. Many synthetic larvicides have been used in several countries since the 1960's [12]. However, repeated use of synthetic insecticides for mosquito control has disrupted natural biological control systems and led to resurgences in mosquito populations [13]. It has also resulted in the development of resistance [14]; an undesirable effect on non-target organisms has fostered environmental and human health concern[15]. In addition, the synthetic insecticides are toxic and adversely affect the environment by polluting soil, water and air. Thus, resistance to pesticides has guided research to develop new tools to control mosquitoes. Recent research has focused on natural product alternatives.

Natural product extracts for instance the extract from the leaves of Blumea balsamifera are used for the manufacture of borneol, and Oxalis corymbosa is used as a source of pyrethrin pesticides [16]. This family is a rich source of sesquiterpenoid natural products, especially those with the eudesmane framework. Sesquiterpenoids exhibit a wide range of biological activities, and include compounds that are plant growth regulators, insect anti-feedants, anti-fungals, anti-tumour compounds and anti-bacterial [16]. Secondary metabolites of plants, many of them produced by the plant for its protection against micro-organisms and predator insects are natural candidates for the discovery of new products to combat mosquitoes. Several studies have focused on natural products for controlling *Aedes* mosquitoes as insecticides and larvicides, but with varied results [17]. Controlling the vectors using various methods can interrupt disease transmission. Due to the problem of pollution and vector resistance, safe plant products are being tested around the world as pest control agents. Table 1 provides details of plant products reported for larvicides growth inhibition and repellent activity against mosquito vectors. A survey of literature on larvicidal effects of plant products on mosquitoes indicates that most of the studies include well-known horticultural and commonly grown plants[18]. But, larvicidal activities of wild plants those found in vast areas on plains as well as on hilly regions have not been attempted so far. Although several plants show mosquitocidal activity, only a few botanicals have moved from laboratory to field use, because they are poorly characterized, in most cases, active principals are not

determined and most of the works are restricted to preliminary screening[19]. Co-evolution has equipped plants with a plethora of chemical defenses against insect predators. Aware of this effect, humanity has used plant parts or extracts to control insects since ancient times. Phytochemicals derived from plant sources acting as larvicides, insect growth regulators, repellent, and ovipositor attractant; have shown different activities observed by many researchers[19]. However, insecticides of plant origin have been extensively used on agricultural pests and to a very limited extent, against insect vectors of public health importance. The selective pressure of conventional insecticides is enhancing resistance of mosquito populations at an alarming rate[20], increasing the demand for new products that are environmentally safe, target-specific and degradable. Consequently, plant derived products have received increased attention from scientists and more than 2000 plant species are already known to have insecticidal properties [21, 1, 2, 3]

Table 1: Plant products reported for bioactivity against mosquito vectors

Plant species (Family)	Plant product	Species tested	Type of activity
Artimisia cina (Compositeae)	Aqueous extract	*Culex pipens*	Larvicidal [22]
Atlantia monophylla (Rutaceae)	Methanol extract	*Cx. Quinquefasciatus Ae. aegypti, Anapheles spp.*	Larvicidal Pupicidal [23]
Azadirachta indica (Meliaceae)	Aqueous extract	*An. gambiae, Cx. quinquefasciatus*	Larvicidal [18]
Azadirachta indica (Meliaceae)	Neem leaves extract + *Malathian*	*Culex fatigans*	Larvicidal [24]
Azadirachta indica (Meliaceae)	Neem oil-Oil water emulsion on wood scrapping	*Cx. quinquefasciatus, An. Stephensi, Ae. aegypti*	Larvicidal, Repellent [22, 24]
Citrus *spp*. (Rutaceae)	Essential oil	*Cx. pipens, Cx. quinquefasciatus*	Adulticidal, Larvicidal [20, 22, 25, 26]
Cleome droserifolia (Capparidaceae)	Aqueous extract	*Culex pipens*	Larvicidal Larvicidal [22]
Citrus *spp*. (*Rutaceae*)	Fruit peel oil	*Cx. pipens, Cx. quinquefasciatus*	Adulticidal, Larvicidal [25] E
Mentha piperita (Labiatae)	Essential oil	*Cx. quinquefasciatus*, An. Stephensi, Ae. aegypti	Larvicidal, Repellent [27]
Mentha piperita (Labiatae)	Fruit peel oil	*Cx. pipens, Cx. quinquefasciatus*	Adulticidal, Larvicidal [28]
Momordica charantia (Cucurbitaceae)	Plant extract	*Cx.quinquefasciatus Ae. aegypti, Anapheles spp*	Larvicidal [29]
Ocimum sanctum (Labiatae)	Steam distillated essential oil	*Cx. quinquefasciatus, An. Stephensi, Ae. aegypti*	Larvicidal [22]
Polyalthia longifolia (Annonaceae)	Leaf extract	*Cx. quinquefasciatus*	Larvicidal Larvicidal, Growth regulator [30]
Tagetes errecta (Compositeae)	Acetone extract, Steam distillated essential oil	*Cx. quinquefasciatus, An. Stephensi, Ae. aegypti*	Larvicidal, [31]
Solanum nigrum Linn. (Solanaceae)	Crude leaf extract	*An. Culicifacies, Cx. quinquefasciatus Ae. aegypti*	Larvicidal [10,32]
Solanum nigrum Linn. (Solanaceae)	Ethanolic leaf extract	*Ae. Caspius, Cx pipiens*	Larvicidal, Growth regulator [27,32]

Clausena anisata is in the family of *Rutaceae* and grows in the tropics. It is a shrub or small tree. Leaves are pinnately compound with 10 - 17 alternate or sub-opposite leaflets and a terminal leaflet. The leaves are densely dotted with glands and have a strong scent when crushed. The scent has been likened to aniseed and opinions vary on its pleasantness. Inflorescence, a branched auxiliary spray; flowers small but attractive, white with orange-yellow stamens[33]. It is used as a cure and remedy for epilepsy and convulsions, arthritis, heart ailments, parasitic infections, malaria and diabetes (type 2 *Diabetes mellitus*). There has not been lot phytochemical investigations of this plant reported but four new carbazole alkaloids were isolated from it as inhibitors of Epsteins-Barr virus early antigen activation induced by 12-tetradecanoylphorbol-13-acetate in Raji cells[34]. Chakraborty *et al.* [35] also isolated two carbazole alkaloids, Clausenol and Clausenine from the alcoholic extract of the *C anisata.* In the current work the oils and nonvolatile extracts of *C. anisata* were investigated for their larvicidal activity against *A gambiess.*

2. Materials and methods

2.1 Collection and identification of the plant

Clausena anisata twigs and leaves were collected from Kakamega equatorial forest in Kenya. The plant grows wildly in the mid altitudes in the range 1500 m to 1700 m of the tropical rainforest conditions which receive about 2000 mm of rainfall a year. The average temperatures remain similar throughout between 15 - 28 °C. A taxonomist identified the plant materials and a voucher specimen deposited at the department of biological sciences of Egerton University.

2.2 Extraction

The volatile (essential oils) and non-volatile secondary metabolites were extracted by use of methods reported by Matasyoh *et al.* [20] and Matasyoh *et al.* [36] respectively.

A) Non – volatile compounds: The plant materials were dried under shade to constant weight and ground to a fine powder. A powder weighing 600 g was extracted sequentially with hexane (3 x 1.5 L), ethyl acetate (3 x 1.5 L), chloroform (3 x 1.5 L), acetone (3 x 1.5 L) and methanol (3 x 1.5 L) after soaking the sample in each solvent for 24 hours. The extracts were filtered through a Buchner funnel fitted to a vacuum pump with a thin layer of activated charcoal, and then concentrated using a rotary evaporator and the solvent recovered. All crude extracts were partitioned between equal volumes (250 ml each) of distilled water and chloroform to remove sugars. The chloroform fraction was concentrated under reduced pressure. The dry sample was then subjected to column chromatography using hexane (4 x 200 ml), ethyl acetate (4 x 200 ml), chloroform (4 x 200 ml), acetone (4 x 200 ml), and methanol (4 x 200 ml). The solvents were recovered using rotor evaporator to obtain 12.52 g, 15.69 g, 17.02 g, 16.45 g and 15.91 g of dry hexane, chloroform, ethyl acetate, acetone, and methanol soluble fractions. The extracts were then subjected to larvicidal assays.

B) Essential oils: A weighed amount; 165 g of fresh whole plant of C. anisata was subjected to hydro-distillation in a modified Clevenger-type apparatus for at least four hours according to the British pharmacopoeia. The essential oil obtained was 7 g (4.25% w/w) C. anisata yield after dried over anhydrous sodium sulphate respectively. The oil was

stored in sealed glass vial (Bijoux bottle) at 4 °C. Then the oils were characterized and subjected to larvicidal assays.

C) GC-MS analysis: Samples of essential oils were diluted in methyl-t-butylether (MTBE) (1:100) and analysed on an Agilent GC-MSD apparatus equipped with an Rtx-5SIL MS ('Restek') (30 m x 0.25 mm i.d. 0.25 μm film thickness) fused-silica capillary column. Helium (0.8 mL/min) was used as a carrier gas. Samples were injected in the split mode at a ratio of 1:10 – 1: 100. The injector was kept at 250 °C and the transfer line at 280 °C. The column was maintained at 50 °C for 2 min and then programmed to rise to 260 °C at 5 °C min^{-1} and held for 10 min at 260 °C. The MS was operated in the EI mode at 70 eV, in m/z range 42-350. The identification of the compounds was performed by comparing their retention indices and mass spectra with those found in literature (Adams, 1995) then supplemented by Wiley and QuadLib 1607 GC-MS libraries. The relative proportions of the essential oil constituents were expressed as percentages obtained by peak area normalization, all relative response factors being taken as one.

2.3 Larvicidal assays

The extracts were solubilized in dimethyl-sulphoxide (DMSO) an analytical reagent obtained from Lobarchemi and diluted to give 2 mg ml^{-1} of stock solution with DMSO kept at a concentration of 1%. The bioassay experiments were conducted mainly according to standard WHO procedure (1981) with slight modifications. The bioassays were conducted at the Kenya Medical Research Institute (KEMRI), Centre for Disease Control (CDC), Kisumu, Kenya, where the larvae were reared in plastic and enamel trays in spring river water. The larvae were maintained, and all experiments carried out at 26 ± 3 °C and the humidity ranged between 70 to 75%. The bioassays were performed with third in-star larvae of *A. gambiae s.s* and carried out in triplicate using 20 larvae for each replicate assay. The larvae were placed in 50 ml disposable plastic cups containing 15 ml of test solution and fed on tetramin fish feed during all testing. Larvae were considered dead if they were unrousable within a period, even when gently prodded. The dead larvae in the three replicates were combined and expressed as the percentage mortality for each concentration. The negative control was spring river water while the positive control was the pyrethrum-based larvicide, pylarvex.

2.4 Isolation, purification and structure elucidation of larvicidal compounds

To isolate, purify and elucidate the structures of larvicidal compounds from *C. anisata* the following analytical techniques were employed.

A) Thin layer chromatographic technique

The crude extracts were analyzed using the thin layer chromatographic technique (TLC) to establish suitable solvent system (silica gel, 20 x 20 cm, 0.20 mm thick, cut into 5 x 15 cm for use). The main solvents used as the mobile phase were hexane and ethyl acetate. The ratios of the solvent were changed while using the hexane as the main solvent in the following percentages: 0, 10, 20 and 30% of ethyl acetate in hexane. The TLC analysis with the above solvent systems showed that hexane and ethyl acetate in a ratio of 7:3 gave the most pronounced separation with distinct spots. Column chromatography was performed using Merck silica gel 60 (70-230 mesh). The ethyl acetate extracts of *C. anisata* was chromatographed on a silica gel column using gradient elution of hexane - ethyl acetate solvent system to give four fractions (Table 3). The column used was of the dimension 50 cm height by 19 mm i.d. The silica gel used was about 65 g per column to give 45 cm of gel height.

B) Preparative thin layer chromatography analysis

The ethyl acetate extracts showed significant bioactivity and were subjected to preparative thin layer chromatography (PTLC) analysis. This was done on silica gel plates (Merck, 60F254) using the solvent system Hex-EtOAc, 7:3. The solvents were distilled before use. The visualization and identification of spots of the compounds was done using an ultra violet lamp at a wavelength of 254 nm. The retention factor (Rf) values were determined.

C) GC-MS analysis

The isolated fractions with notable activity were subjected to spectroscopic techniques to identify the active agents. Ethyl acetate fractions of *C. anisata* were derivatised using trimethylsilyl ethers in order to increase their volatility to pass through the GC column as well as increasing their stability in gaseous phase. The four fractions were subsequently subjected to GC-MS analysis. The identification of the compounds was performed by comparing their retention indices and mass spectra with those found in literature (Adams, 1995) then supplemented by Wiley and QuadLib1607 GC-MS libraries. Mass spectra were recorded on Finnigan Triple-Stage-quadrupol Spectrometer (TSQ-70) with electro spray ionisation (ESI) Method. GC-MS analyses were done using a Hewlett-Packard model 6890 series GC.

2.5 Statistical analysis

The lethal concentrations were determined using SPSS package version 11.5. The bioassay data was subjected to probit regression analysis according to Finney[37,38]. Probit analysis of concentration-mortality data was conducted to estimate the LC_{50} and LC_{99} values and associated 95% confidence limits as shown in appendices 1 and 2.

3. Results and Discussion

3.1 The GC-MS Analysis of *C. anisata* Oils

C. Anisata gave on drying 7 g of neat oil. Approximately 2 g was subjected to total chemical analysis. The chemical composition in Table 2 lists twenty-six compounds identified by GC-MS, which constitute 99.1 % of the total oils components. The oil was dominated by monoterpenoids, which accounted for 56.7 % of the oil. The monoterpenoids fraction was characterized by a high percentage of β-phelandrene (20.1 %), γ-terpinene (13.8%), α–phelandrene (4.6%), (E)-β–ocimene (3.7%), myrcene (3.4%), α-pinene (3.4%) and para-cymene (2.8%). The sesquiterpenoids components were accounting for 42.4% of the total oils constituents. The main sesquiterpenoids constituents were δ-Germacrene (18.8%) and β-germacrene (6.0%). Other sesquiterpenoids present in appreciable amounts were bicyclogermacrene (3.7%), α-humulene (3.6%), (E)-caryophyllene (2.5%), and β-elemene (2.0%).

Table 2: *Clausena anisata* Oils Chemicals Composition

Compounds	R.T.	% of total	RI	ID Method
Monoterpenes				
α-Thujene	5.4	0.5	925	MS, RI
α-Pinene	5.6	3.4	932	MS, RI
Sabinene	6.6	0.9	971	MS, RI
β-Pinene	6.7	1.3	976	MS, RI
Myrcene	7.0	3.4	989	MS, RI
α -Phellandrene	7.5	4.6	1006	MS, RI
α -Terpinene	7.8	0.6	1017	MS, RI
Cymene<para>	8.0	2.8	1024	MS, RI
β -Phelandrene	8.2	20.1	1032	MS, RI
β -Ocimene(E)	8.6	3.7	1046	MS, RI
γ-Terpinene	9.0	13.8	1059	MS, RI
Terpinolene	9.8	1.1	1085	MS, RI
δ-Elemene	17.0	0.5	1335	MS, RI
	Total %	56.7		
Sesquiterpenes	**18.2**	**0.6**		
α -Copaene	18.4	0.6	1376	MS, RI
β -Bourbonene	18.5	2.0	1384	MS, RI
β -Elemene	19.3	2.5	1389	MS, RI
Caryophyllene(E)	19.6	0.8	1420	MS, RI
γ -Elemene	20.3	3.6	1429	MS, RI
α -Humulene	21.0	18.8	1456	MS, RI
Germacrene-D	21.3	3.7	1483	MS, RI
Bicyclogermacrene	21.6	1.4	1496	MS, RI
β -Bisabolene	21.9	0.7	1508	MS, RI
δ -Cadinene	22.7	1.1	1519	MS, RI
Elemol	23.0	6.0	1549	MS, RI
Germacrene-B	23.4	0.6	1560	MS, RI
Spathulenol	Total %	42.4	1578	MS, RI
Unknown	21.9	0.8	1516	

3.2 GC-MS Analysis of C. anisata Extracts

The ethyl acetate fraction showed exceptional larvicidal activity compared to other fractions of *C. anisata*. This was then considered for further analysis to isolate compounds presenting the observed activity. However, on further application of column chromatography Hex-EtOAc with increasing polarity to 7:3, four fractions were obtained. These were then purified using preparative TLC on silica gel but it was discovered that most compounds present were known compounds prompting total compound composition to be done using GC-MS yielding compounds in Tables 3 as major compounds at 90% quality match with Wiley electronic library of organic compounds. This fraction was dominated by sesquiterpenes.

Table 3: Major Compounds from Ethyl acetate Fraction of *C. anisata*.

Peak	RT	Area%	Compound	RI	Quality Match (%)
1	10.36	1.86	β-.Bourbonene	1384	98
2	10.59	2.48	β-Elemene	1389	99
3	11.30	5.82	*Trans*-(β)-Caryophyllene	1420	98
4	11.69	13.37	γ.-Elemene	1429	99
5	12.28	8.01	α.-Humulene	1456	98
6	13.01	20.96	δ-Germacrene	1483	98
7	3.37	3.37	Cyclohexene	-	94
8	14.09	2.86	δ.-Cadinene	1519	98
9	15.04	2.64	β-Germacrene	1590	98
10	42.22	4.39	Squalene	-	93
11	47.11	34.24	Nonacosane	-	98
15	47.14	63.47	Octacosane	-	99
16	53.42	2.72	Neophytadiene	-	95
17	47.05	100.00	Hentriacontane	-	97

3.3 Hexane, chloroform, acetone and methanol fractions

The hexane, chloroform, acetone and methanol fractions of *C. anisata* did not give 100% mortality at very high concentration of 4000 mg/l. The medicinal properties of plant extracts normally depend upon the presence of active compounds [39] possessing specific functional groups that are soluble only in solvents of particular polarity. The active compounds in these extracts of *C. anisata* were therefore not soluble appreciably in these solvents.

3.4 Larvicidal activity of C. anisata active fraction and oils

C. anisata oils showed much better activity compared to the active fraction (Table 4). The log probit analysis gave LC_{50} and LC_{99} as 75.96 mg l^{-1} and 256.80 mg l^{-1} respectively (Appendix 1). While the corresponding LC_{50} as 2095.46 mg l^{-1} and LC_{99} were 4438.75 mg l^{-1} (Appendix 2) respectively for the most active extract (ethyl acetate fraction).

Table 4: Confidence Limits for Effective concentration

95% Confidence Limits			
Probits	**concentration**	**Lower**	**Upper**
.01	-104.88368	-32.39867	-44.25533
.02	-83.69289	-99.32482	-28.50098
.03	-70.24802	-78.36455	-18.48133
.04	-60.13396	-62.61219	-10.92869
.05	-51.90696	-49.81024	-4.77383
.06	-44.90449	-38.92298	47413
.07	-38.76469	-29.38485	5.08344
.08	-33.26723	-20.85155	9.21748
.09	-28.26751	-13.09716	12.98353
.10	-23.66526	-05.96507	16.45602
.15	-4.61073	-76.50839	30.90515
.20	10.53322	-53.20639	42.49810
.25	23.52537	-33.32529	52.55375
.30	35.19273	-15.59172	61.70432
.35	46.00427	.70133	70.32343
.40	56.26337	15.99137	78.67258
.45	66.18916	30.56779	86.96735
.50	75.95759	44.62698	95.41676
.55	85.72602	58.29751	104.25480
.60	95.65181	71.65208	113.77144
.65	105.91091	84.72090	124.34181
.70	116.72245	97.53054	136.44434
.75	128.38981	110.19215	150.66686
.80	141.38196	123.03288	167.76288
.85	156.52591	136.75859	188.93212
.90	175.58044	152.83567	216.76085
.91	180.18269	156.58831	223.61279
.92	185.18241	160.62288	231.09866
.93	190.67987	165.01552	239.37336
.94	196.81966	169.87552	248.66079
.95	203.82214	175.36873	259.30281
.96	212.04914	181.76677	271.86158
.97	222.16320	189.56581	287.36754
.98	235.60807	199.84514	308.06812
.99	256.79886	215.89934	340.84212

Table 5: Confidence Limits for Effective concentration

95% Confidence Limits			
Probits	**concentration**	**Lower**	**Upper**
.01	-247.82254	-1514.36301	428.03933
.02	26.76098	-1117.78159	640.08870
.03	200.97543	-866.50135	774.96520
.04	332.03016	-677.68910	876.64361
.05	438.63308	-524.26700	959.51325
.06	529.36891	-393.81276	1030.18040
.07	608.92648	-279.54312	1092.25488
.08	680.16079	-177.32879	1147.93566
.09	744.94555	-84.46044	1198.66661
.10	804.58003	.94011	1245.44944
.15	1051.48270	353.46392	1440.20017
.20	1247.71312	632.02036	1596.60018
.25	1416.06134	869.34948	1732.42497
.30	1567.24325	1080.66034	1856.21785
.35	1707.33582	1274.34926	1973.05248
.40	1840.26996	1455.55547	2086.50277
.45	1968.88529	1627.61758	2199.52422
.50	2095.46147	1792.75362	2314.95185
.55	2222.03765	1952.42917	2435.83997
.60	2350.65297	2107.65257	2565.70014
.65	2483.58712	2259.38436	2708.62483
.70	2623.67969	2409.18143	2869.35132
.75	2774.86160	2560.02875	3053.60774
.80	2943.20982	2717.29582	3269.49457
.85	3139.44024	2890.43582	3531.31102
.90	3386.34291	3098.38972	3870.63166
.91	3445.97738	3147.49902	3953.70575
.92	3510.76214	3200.47779	4044.32627
.93	3581.99646	3258.34124	4144.35794
.94	3661.55403	3322.54930	4256.49400
.95	3752.28986	3395.32159	4384.84309
.96	3858.89278	3480.29718	4536.15925
.97	3989.94751	3584.12932	**4722.81778**
.98	4164.16195	3721.29857	4971.80526
.99	4438.74548	3936.02624	5365.70838

Appendix 1: Probit analysis of *Clausena anisata* oils Observed and Expected Frequencies

Number of Observed Expected					
concentration	**Subjects**	**Responses**	**Responses**	**Residual**	**Prob**
250.00	20.0	19.7	19.748	-.078	.98742
200.00	20.0	19.7	18.894	.776	.94472
175.00	20.0	17.7	17.974	-.304	.89868
150.00	20.0	15.0	16.591	-1.591	.82957
125.00	20.0	14.7	14.719	-.049	.73594
100.00	20.0	14.3	12.429	1.901	.62145
75.00	20.0	10.0	9.902	.098	49509
50.00	20.0	6.7	7.384	-.714	.36922

Appendix 2: Probit analysis of *Clausena anisata* ethyl acetate fraction 2 extract Observed and Expected Frequencies

Number of Observed Expected					
Concent	**Subjects**	**Responses**	**Responses**	**Residual**	**Prob**
4000.00	20.0	20.0	19.413	.587	.97067
3500.00	20.0	18.7	18.368	.302	.91840
3200.00	20.0	17.3	17.272	.058	.86358
3000.00	20.0	15.0	16.308	-1.308	.81541
2850.00	20.0	14.7	15.462	-.792	.77310
2500.00	20.0	12.0	13.120	-1.120	.65602
2000.00	20.0	11.3	9.245	2.085	.46225
1800.00	20.0	9.0	7.693	1.307	.38464
1500.00	20.0	4.3	5.544	-1.214	.27721

Table 6: Larvicidal assay for *C. anisata* oils and ethyl acetate extract

Larvicidal assay for *C. anisata* oils		Larvicidal assay for *C. anisata* ethyl acetate extract	
Concentration (mg l^{-1})	**(%) mortality**	**Concentration (mg l^{-1})**	**(%) mortality**
250	98.35	4000.00	100
200	98.35	3500.00	93.35
175	88.35	3200.00	86.65
150	75.00	3000.00	75.00
125	73.35	2850.00	73.35
100	71.65	2500.00	60.00
75	50.00	2250.00	55.25
50	33.35	2000.00	49.35

4. Discussion

The plant *C. anisata* grows wildly in the rural parts of Kenya where *A. gambiae* is a serious problem. The chemical components identified in oils have been associated with insecticidal activity. For instance Govindarajan [40]demonstrated that the oils of *C. anisata* containing mainlyα-pinene, sabinene, germacrene-D, estragole, linalool exhibited significant against *Culex quinquefasciatus, Aedes aegypti and Anopheles Stephensi. L* larvae. This author found the LC_{50} for crude neat oil as 140.96, 130.19 and 119.59 mg l^{-1} respectively for the three species. Also Rajkumar and Jebanesan [41]working on *Clausena dentate* essentials oils obtained LC_{50} and LC_{90} of 140.2 and 341.6 mg l^{-1}, respectively against *Aedes aegypti* larvae. In this work we report a much lower LC_{50} and LC_{99} of 75.96 and 256.80 mg l^{-1} respectively against *A. gambiae.* The difference could be attributed to difference in chemical composition arising from the parts of plant considered. The previous authors considered oils from majorly the leaves of the plant while in the current work we considered a whole plant. The chemicals distribution in plant parts depend on their role at the part in question, for instance chlorophyll will be found more in the leaves for photosynthetic purposes. The compounds β-phelandrene, γ-terpinene, α–phelandrene, (E)-β–ocimene, and myrcene may play a major role in the observed activity. These chemical components have not been previously named as major components as in our case. However, further work need to be done to ascertain their individual contribution. Ajaiyeoba, Sama, Essien, Olayemi, Ekundayo, Walker and Setzer [42] also working on essential oils from the leaves and rhizomes of *Curcuma longa L* demonstrated significant larvicidal activity against *A. gambiae* with LC50 of 17 mg l^{-1}. These authors found *α*-turmerone and *β*-turmerome as main components in oils of both leaves and rhizomes and argued that the combination of the two could have enhanced that observed activity. Recently Matasyoh, Wathuta, Kariuki and Chepkorir [26] also showed that essential oils from whole plant of *Piper capense* had significant larvicidal activity against larvae of *A. gambiae.* These authors found LC_{50} and LC_{90} values of 34.9 and 85.0 mg l^{-1}, respectively. It is therefore prudent to consider the entire plant when extracting essential oils for larvicidal application.

The nonvolatile fractions have also shown activity larvicidal activity against other species of mosquito larvae for instance Chalannavar, Hurinanthan, Singh, Venugopala, Gleiser, Baijnath and Odhav [43] recently showed that aqueous and methanolic extracts of twelve plant species exhibited insecticidal activity, including larvicidal against *Anopheles arabiensis.* Another report by Bagavan, Rahuman, Kamaraj and Geetha [44] indicate that ethyl acetate fraction of *Achyranthes aspera* exhibit larvicidal activity larvae of *Aedes aegypti L* and *Culex quinquefasciatus.*[44] In this work the crude ethyl acetate extracts of *C. anisata* gave much higher LC_{50} and LC_{99} values of 2095.46 mg l^{-1} and 4438.75 mg l^{-1} respectively. The high values indicate low concentration of active components that may have evaporated with time hence low activity. The major components; nonacosane (34.24%) and octacosane (63.47%) have no reported larvicidal activity. However, a long term larvicidal activity is exhibited nonetheless and could promise longer shelf life and could be useful in mosquito control as eco-friendly larvicides. From our results, (LC_{99} 4438.75 mg l^{-1}) these extracts have shown that up to 99% of the larvae population targeted can be killed with application of a few grams of dry powdered extract on a pond of stagnant water. These stagnant water points are the main breading sites of mosquitoes. Application of longer lasting nonvolatile extract may help to reduce the mosquito population drastically.

Considering that a large proportion of the human population living in malaria prone areas suffer from varying degrees of poverty, the discovery of plant extracts that could control the mosquito population is of great value. There is no reported adverse arising from use of the oils and extracts of C anisate indicating that active ingredients may have not be toxic to humans. However, caution needs to be exercised as over application may results to undesirable effects in the long term. In this case, however, *C. anisata* plant from which the extracts were obtained has been used as traditional medicine for centuries without any reported illness or side effects resulting from its use[45]. The oils of *C. anisata* showed markedly enhanced activity against *A. gambiae s.s*, larvae with an LC_{50} value of 75.96 mg l^{-1} much better than LC_{50} (2095.46 mg l^{-1}) of C. anisata nonvolatile extracts. However, the oils formulations require immediate use due to volatility of the constituent compounds, which imply the extracts could prove to be better larvicides than the oils. It can be fairly put that both the essential oil and non-volatile extracts of *C.anisata* contains compounds with remarkable larvicidal properties, and consequently a promising candidate for the manufacture of eco-friendly larvicides.

5. Conclusions

The essential oil and non-volatile extracts of the plant *C. anisata* contains bioactive chemical components in varied proportions. Consequently the oil demonstrated much higher activity compared to the non-volatile extracts. Application of these extracts to larval habitats may lead to promising results in malaria and mosquito management programmes. However,

in this work the larvicidal assays were carried out on laboratory bred larvae of *A. gambiae s.s*, field study application on *A. gambiae s.l* larvae is necessary.· There is also need to explore ways of stabilizing the oils so as to enhance their shelf life.

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