American Journal of Essential Oils and Natural Products

Available online at www.essencejournal.com

AJEONP American Journal of Essential Oils and Natural Products

AkiNik

ISSN: 2321-9114
AJEONP 2018; 6(1): 09-15

Received: 05-11-2017
Accepted: 08-12-2017

Foko Dadji Gisèle Aurelie
Laboratory of Zoology, Higher Teacher Training College, University of Yaoundé I, P.O.Box., 47, Yaoundé, Cameroon

Bobo Ngowe Manuela Pierre
Laboratory of Zoology, Higher Teacher Training College, University of Yaoundé I, P.O.Box., 47, Yaoundé, Cameroon

Nyegue Maximilienne Ascension
Laboratory of Microbiology, Faculty of Science, University of Yaoundé I, P.O. Box, 812, Yaoundé,Cameroon

Tamesse Joseph Lebel
Laboratory of Zoology, Higher Teacher Training College, University of Yaoundé I, P.O.Box., 47, Yaoundé, Cameroon

Correspondence
Foko Dadji Gisèle Aurelie
Laboratory of Zoology, Higher Teacher Training College, University of Yaoundé I, P.O.Box., 47, Yaoundé, Cameroon

Chemical composition and biocide properties of *Clausena anisata* (Rutaceae) essential oil against developmental stages of the malaria vector *Anopheles coluzzii*

Foko Dadji Gisèle Aurelie, Bobo Ngowe Manuela Pierre, Nyegue Maximilienne Ascension and Tamesse Joseph Lebel

Abstract
Mosquito-borne diseases including malaria remain a serious threat for the development of low income countries where the disease is endemic. Although several harmful chemicals have successfully been used to fight against mosquitoes, new effective intervention tools safe for both humans and the environment are prominent for Public Health interventions to stop the spread of malaria vectors and the burden of the disease. We evaluated in this study the biocide activity of *Clausena anisata* leaf essential oil on the aquatic and adult stages of the malaria vector *Anopheles coluzzii*. We further identified the active components of theessential oil. Fresh leaves of *C. anisata* (wild type) were subjected to hydrodistillation in a Clevenger-type apparatus and extracted essential oil was characterized by gas chromatography-flame ionization detector (GC/FID) and GCcoupling mass spectrometry (GC-MS). The toxicological test was performed by immersion of mosquito larvae/eggs in water contaminated with various dilutions of essential oil. The WHO cone tests procedure was performed on adult females *An. coluzzii*and probit analysis was performed on computed data. Overall, *C. anisata* essential oil revealed significant inhibitory potentials on *An. coluzzii* eggs with a median inhibitory concentration (IC_{50}) of 0.147%. Third instar larvae revealed a full sensitivity (100% mortality) 48h post-exposure to *C. anisata* essential oil with LC_{50}of0.44%. The knockdown effect of oil-impregnated nets increased significantly with the exposure time with median knockdown times(kdt_{50}) of 1.93 min, 18.58 min, 51.02 min and 60.15 min, respectively for 2%, 1.5%, 1% and 0.5% of essential oil. The killing effect of the essential oil read 24h post-exposure revealed the LC_{50} of1.215%. Overall, 52compounds were detected with a total yield of 0.242% (w/w). (Z) anethole (37.25%), p-cymene (16.01%), γ-terpinene (4.07%) and linalool (3.80%) constituted the main ingredients of the essential oil.*C. anisate* leaf essential oil showed high insecticidal potentials against *An. coluzzii*. This oil therefore stands as a natural biocide that could be used for public health interventions to control the burden of malaria in endemic areas.

Keywords: *Clausena anisata*, *Anopheles coluzzii*, biocide properties, Bamougoum

Introduction
Mosquito borne diseases are widely distributed and remain the main cases of epidemic worldwide. Malaria which has received the main interest since the 18thcentury remains a serious threat across Africa, the continent with the highest cases of malaria. Malaria is transmitted by *Anopheles* mosquito species and is responsible for 90% disease-related deaths in the African regions [1]. It deeply deprives the economy of endemic countries and appears to be one of the main obstacles to the emergence of countries like Cameroon [2, 3]. Several intervention strategies have been attempted across the country to reduce the burden of malaria including the use of Long Lasting Insecticide Nets (LLINs), the use of new chemicals in indoors and outdoors spraying to respectively reduce the density of adult and larvae mosquito, the free-distribution of chemoprophylaxis tools (LLINs, drugs) among the most vulnerable groups (children under five years old and pregnant women), and the raising in public awareness on the disease [1-3]. In spite of these efforts, the disease is still not completely under control and the scientific community still struggling towards the eradication of malaria. Weaknesses in malaria control strategies has mainly been attributed to the resurgence in *Plasmodium* resistance against traditional antimalaria drugs used to cure the infected patients [1- 4] and the vector's resistance against commonly used public health insecticides [1-3, 5]. These failures have strengthened the search of new prominent compounds such as the use of

biological insecticides to control the spreading of insecticide-resistant mosquitoes. Biological insecticides are new vector-integrated control strategies that have widely been used and include both the plant extracts and microbial agents (bacteria and viruses). Plant extracts with insecticidal properties such as essential oils are very prominent to overcome the environmental and human toxicity caused by synthetic public health insecticides [6].

Essential oils provide a rich source of biologically active monoterpenes and are well documented for bioactivities against insect pests. To date, numerous studies have highlighted the insecticidal activity of plant species from the Cameroonian pharmacopoeia [7-12]. *Clausena anisata*, Hook (family: Rutaceae) which is the only Clausena tribe found in Africa has been effective against some diseases and disorders including tuberculosis, diabetes, inflammation, worm infections, respiratory ailments, heart disorders and malaria fever [13-17]. *C. anisata* essential oil is considered nontoxic to humans and is widely used in flavoring, pharmaceuticals and confectionary industries [18]. The main active compounds of *C.anisata* with insecticidal properties include anethole, estragole, limonene and linalool [14, 17]. However, the toxicity level of these active ingredients against larvae and adult mosquito species has not been thoroughly documented in Africa and especially in Cameroon. This African indigenous medicinal plant is widely distributed across the continent and whether its insecticidal properties against malaria vectors are confirmed, it could stand as a prominent and accessible tool to fighting against mosquito in countries where the disease is endemic. We attempted here to investigate the insecticidal potentials of *C. anisata* leaf essential oil against developmental stages (eggs, larvae and adults) of the malaria vector *Anopheles coluzzii*. We further identified the main active ingredients of *C. anisata* essential oil.

Material and methods

The mosquito laboratory strain *Anopheles coluzzii*

Anopheles coluzzii immature eggs were kept 24 hours for maturation and incubated for hatching at 26-28°C and 70-80% RH as previously described [11]. Eggs for 100 first instars larvae were maintained in spring water supplemented with Tetramin® Baby Fish food in breeding bowls as described by [7]. After emergence the adults were maintained on 10% honey solution. Mosquito developmental stages (larvae and adults) were collected and exposed to different doses of *C. anisata* leaf essential oil according to WHO protocol [19, 20].

Collection of plant specimens and extraction of essential oil

C. anisate fresh leaves were harvested in December 2015 from Bamougoum (5°30'0''N, 10°19'60''E), in the western region of Cameroon. The plant materials (leaves) were identified at the Cameroon National Herbarium and registered under the number 7599 SRFCAM/ HNC. Identified plant leaves were dried at room temperature in the laboratory environment for four consecutive days, then weighed with a semi-automatic balance (mark SCOUT pro).The leaf specimens were thereafter subjected to essential oil extraction by hydrodistillation with a Clevenger-type apparatus. The oil extraction was performed for 5 h 25 min as previously described [21]. The oil gathered by decantation at the end of the distillation was filtered, dried on a column of anhydrous sodium sulfate, and introduced into dark glass bottles, covered with aluminum foil and stored at 4 °C.

Identification of active ingredients of *Clausena anisata* Essential Oil by Gas chromatography-Flame ionization detector (GC-FID) and Gas Chromatography Coupling Mass-spectrometry (GC-MS)

Gas chromatography-flame ionization detector: GC/FID analyses were performed on a Thermo Scientific gas chromatograph (model TRACE 1300) with a flame ionization detector equipped with two fused silica capillary columns DB-5 (30 m x 0.25 mm, film thickness 0.25µm) (95% methyl 5% phenyl polysiloxane) and DB-Wax (30 m x 0.25 mm, film thickness 0.25 µm). N_2 was the carrier gas at 0.5 ml/min and injection of 2 µl 10/100 diethyl ether solution was performed in split mode 1:20. The injector temperature and the detector temperature were respectively 220 °C and 250 °C. The program was performed at 60-220 °C with 3 °C/min, and kept at 220 °C for 17 min. The linear retention indices (LRI) of the components were determined relative to the retention times of a series of *n*-alkanes with linear interpolation. The percentage composition of the essential oil was computed by the normalization method from the GC/FID peak areas on the DB-5 capillary column, response factors being taken as one for all compounds.

Gas chromatography coupling Mass spectrometry: GC/MS analyses were performed using an Agilent 5977 apparatus MSD series equipped with two silica capillary columns HP-5 MS (5%-phenyl-methylpolysiloxane) (30 m x 250 µm; film thickness 0.25 µm), HP-INNOWAX fused silica column (30 m x 250 µm; film thickness 0.25 µm) interfaced with a quadrupole detector (single quadrupole acquisition Method-MS parameters report), source temperature 230°C, Quadrupole temperature 150 °C; the temperature program was 60 °C for 2 min, 60-240 °C at 3 °C/min, then kept at 240 °C during 8 min; injector temperature, 240 °C; MS transfer line temperature, 250 °C; carrier gas, helium at a flow rate of 0.7 ml/min; injection type, split, 20:1 (1 µl of a 10% dichloromethane solution); ionization voltage, 70 eV; electron multiplier 1000 eV; scan range 33-400 amu; scan rate, 1.56 scan/s. The oil components were identified based on their relative retention indices with either those of authentic samples or with published data in the literature[22], and by matching their mass spectra with those obtained with authentic samples and/or the NIST14, NIST98, FFNSC 2.L. libraries spectra.

Toxicological test of *C. anisata* essential oil against aquatic stages of *An. coluzzii*

The toxicological tests of *C. anisata* essential oil against *An. coluzzii* were performed according to WHO protocol [19, 20]. Prior to laboratory bioassays, *C. anisata* essential oil was diluted in absolute ethanol to constitute a 1% stock solution (w/v). Seven dilutions were thereafter performed from the stock solution to make solutions of0%, 0.278%, 0.334%, 0.482%, 0.579%, 0.694% and 0.833%. One hundred individuals (4 replicates of 25 individuals) of each aquatic stage of *An. coluzzii* (eggs and third instars larvae) were handled for the bioassay tests with each dilution of the oil. Each test tube was filled with spring water and 1 ml of the essential oil to make a total volume of 100 ml in the breeding bowl. Each test was replicated twice, and a control bowl was constituted with water. The hatching and the mortality rates in each test were read after 1 hour and 24 hours of contact with the essential oil. Exposed mosquitos were considered dead or moribund if they were immobile and unable to reach the surface of water in the breeding bowl.

Toxicological test of *C. anisata* essential oil against adult *An. coluzzii*

Serial dilutions (2%, 1.5%, 1% and 0.5%) of *C. anisata* essential oil stock solution (w/v) were exposed to females of adult *An. coluzzii*. The WHO cone tests procedure was carried out according to the protocols previously described by [9, 19, 20].

Impregnation of mosquito nets with *C. anisata* essential oil

Briefly, twenty pieces of non-impregnated mosquito nets (10×10 cm) were soaked with correspondent doses of *C. anisata* essential oil. Four pieces of nets were used for each dose of the oil. Nets impregnation procedure consisted as spreading the net into a small petri dish following by a gently mixture with the essential oil. Impregnated nets were then kept at room temperature until complete evaporation of the diluents (acetone). Only acetone was used for the impregnation of the nets constituting the control group.

WHO cone tests procedure

WHO cone test was performed as previously reported [9]. Briefly, impregnated net fragment with no trace of acetone was adjusted on the cone and maintained using a tape. The cones were introduced into individual holes created in a Plexiglas and maintained with a plexiglass plate thinner. Four cones constituting four repetitions for each dose of the oil were introduced and experimented once. Resulting device was maintained by clamps and placed on wooden stand with 45° slope. Prior to the toxicological test, mosquitos were left to acclimatize for 1-hour in cardboard cups covered with non-oil impregnated nets. Four replicates of 25 mosquitoes each were exposed to each concentration for 1-hour. Knocked down (KD) mosquitoes were recorded at 10-minute intervals during the 1-hour exposure period and KDT_{50} (time required for knocking down 50% of individuals) estimated. Remaining mosquitoes were transferred back into a recovery clean cup after the exposure period and fed with 10% honey solution throughout the toxicological test. The experiment was repeated six times to minimize errors and for data validation.

Statistical analysis

The data were computed in Microsoft office Excel 2013 and analyzed using SPSS v20.0.Kruskal-Wallis H test was performed to evaluate the toxicological effect of the oil on mosquito (larvae and adults). Results were expressed as percentage mortality, corrected for untreated (check) mortality using Abbott's formula. Dose-mortality regressions were computed by probit analysis within Win LD (CIRAD, Montpellier, France). The LC_{50} (lethal concentration to kill 50% of mosquito larvae), median inhibitory concentration (IC_{50}), and the median knockdown time (kdt_{50}, time to knock down 50% of adult mosquito population) were recorded accordingly. The level of significance was set at $p<0.05$.

Results

Chemical composition of *C. anisata* leaf essential oil

Overall, 6.3g of essential oil (yellow color) were obtained from the 2603.5g of *C. anisata* fresh leaves subjected to hydrodistillation, given a total yield of 0.242% (w/w). The chemical characterization of *C. anisata* leaf essential oil revealed the presence of 52compounds representing 97.97% of the oil. (Z) anethole (34.24%) was the main phenylpropanoids in the extracted essential oil. The main abundant hydrocarbon terpenes (mono- and sesquiterpenes) included p-cymene (16.01%), γ-terpinene (4.07%), δ-muurolene (2.63%) and limonene (2.05%).Linalool (3.80%),α-terpineneol (2.85%) and meta-anisyl alcohol(2.63%) were, respectively, the main oxygenated terpenes found in *C. anisata* leaf essential oil. (Table 1)

Table 1: Chemical composition of *Clausena anisata* leaf essential oil.

Elution order	Compounds	RI HP_5[a]	RI (DB_5) [12]	(%)
1	Isobutanone	657	660	0.09
2	1-Hexen-3-ol	776	776	0.08
3	α-Thujene	930	930	0.20
4	α-Pinene	933	939	0.16
5	Sabinene	977	975	0.41
6	β-Pinene	982	979	0.41
7	Myrcene	991	990	0.34
8	α-Phallendrene	1008	1002	0.51
9	δ-3-Carene	1015	1011	1.69
10	p-Cymene	1027	1024	16.01
11	Limonene	1032	1029	2.05
12	β-Ocimene	1046	1050	0.12
13	γ-Terpinene	1059	1059	4.07
14	*cis*- Hydrate de sabinene	1067	1065	1.01
15	Terpinolene	1089	1088	1.69
16	Linalool	1096	1096	3.80
17	*trans* Menta-2;8-dien-1-ol	1121	*1122*	1.45
18	Estragole	1138*	-	1.24
19	Anisaldehyde	1156*	-	0.25
20	(z) Anethole	1178	1182	34.24
21	α-Terpineol	1189	1188	2.85
22	*meta*-Anisaldehyde	1193	1195	0.38
23	Cymen-9-ol	1203	1205	0.65
24	*trans*-Carveol	1214	1216	0.22
25	Anisol	1236	1235	0.34
26	*cis* Carvone oxyde	1264	1263	1.14
27	*meta*-Anisyl alcohol	1281	1282	2.63
28	*meta*-Acatanisole	1295	1298	1.27
29	δ-Terpenyl acetate	1313	1317	1.68
30	δ-Elemene	1332	1338	0.36
31	α-Cubebene	1350	1351	0.70
32	Not identified	1358	-	0.61
33	α-Copaene	1362	1376	0.28
34	7-Episesquithujene	1382	1391	0.92
35	α-*cis*Bergamotene	1417	1412	0.87
36	β-epi-Sentanele	1450	1452	1.65
37	δ-Muurolene	1470	1479	2.63
38	Germacrene D	1476	1485	0.24
39	α-Muurolene	1496	1500	1.19
40	(Z) δ-Bisabolene	1515	1515	0.31
41	(E) δ-Bisabolene	1530	1531	0.18
42	Germacrene B	1548	1561	0.60
43	Spathulenol	1574	1577	0.74
44	Humulene epoxyde II	1596	1608	1.23
45	β-Atlantol	1607	1608	1.96
46	β-Cendreneoxyde	1621	1622	0.71
47	Naphth-1-ol	1636	1641	0.34
48	α-Muurolol	1645	1646	0.65
49	α Bisabololoxyde	1660	1658	0.21
50	α -Epi-bisabolol	1674	1684	0.34
51	(2E, 6E) Farnesoic acid	1811	1817	0.30
52	(2E, 6E) Farnesyl acetate	1859	1846	0.35

Elution order is given withapolar column (HP-5); **a**: Linear Retention Index on apolar (HP-5) column; RI (DB_5) [12]: retention index of Adams; Identification methods: GC, identification based on co-injection with authentic sample; RI: Retention Indices;%: relative percentage; (*) identified by mass spectra with NIST14, library.

Ovicidal activity of *C. anisata* leaf essential oil on *Anopheles coluzzii*

Overall, the hatching rate significantly decreased with the concentrations of *C. anisata* leaf essential oil after 24h incubation period (p = 0.0039, H = 0.71). Although 80% of eggs hatched in the control group, only 1% hatching rate was recorded at 0.833% which corresponds to the highest concentration tested for *C. anisata* (Table 2). Probit analysis revealed a median inhibitory concentration (IC_{50}) of 0.147% after 24h incubation of mosquito eggs.

Larvicidal activity of *C. anisata* leaves essential oil on *An.coluzzii* third instars larvae

Essential oil of *C. anisata* leaves showed an important activity against *An. coluzzii* third instars larvae. A complete activity (100% mortality) was observed after 48h exposure to the highest concentration of the oil. Revealing a low potential of *C. anisata* leaves essential oil to kill all the larvae at 24h post-exposure (p = 0.39, H = 0.717). Overall, the mortality rates significantly increased with the oil concentrations after 48h exposure (p = 0.0084, H = 6.926). 100%, 94%, 88%, 61%, 13%, and 9% mortality rates were respectively recorded after 48h post-exposure to 0.833%, 0.694%, 0.579%, 0.482%, 0,334% and 0.278% *C. anisata* leaf essential oil (Table 2). Probit dose analysis revealed the LC_{50} of 0.6588 ± 0.0312% (95% CI, 0.5416-0.7300; Y = 0.7519 + 4.1498*X; p = 0.360) and 0.4433 ± 0.0088% (95% CI, 0.4255-0.4611; Y = 2.7705 + 7.8437*X; p = 0.154) respectively after 24hand 48h exposure to *C. anisata* essential oil.

Table 2: Hatching rates (eggs) and mortality rates (third instars larvae) of *Anopheles coluzzii*after exposure to *Clausena anisata* leaf essential oil.

Essential oil concentrations (%)		0.833	0.694	0.579	0.482	0.334	0.278	0	P-value	H-value
Hatching rates (%)	After 24 hours	1 ± 0.25	2 ± 0.5	6 ± 0.5	5 ± 0.94	12 ± 0.86	20 ± 0.75	80 ± 0.48	0.0039	0.71
Mortality rates (%)	After 24 hours	65.6 ± 4.19	48 ± 1.471	45 ± 0.946	33 ± 0.478	8 ± 0.577	6 ± 0.645	0± 0.0	0.39	0.717
	After 48 hours	100 ± 0.25	94 ± 2.217	88 ± 1.914	61 ± 1.796	13 ± 0.478	9 ± 0.75	0± 0.0	0.0084	6.926

Hatching and mortality rates are explained by mean±SD (standard deviations). p-value<0.005 are statistically significant

Knockdown and killing effect of *C. anisata* leaf essential oil on females *An. coluzzii*

Essential oil of *C. anisata* leaves revealed high potentials against adult females *An. coluzzii* using the WHO cone tests procedure. The knockdown effect significantly increased with the oil concentrations at different intervals of exposure time (5, 10, 15, 20, 30, 40, 50 and 60 mins) (Figure 1). The knockdown rates of 27.5%, 47.5%, 90% and 100% were respectively recorded after 1h exposure to 0.5%, 1%, 1.5%, and 2% of the essential oil (p = 0.0036, H = 13.5) (Table 3). Not the entire mosquitos knocked down during the observation times were killed 24h post-exposure to mosquito nets impregnated with *C. anisata* leaves essential oil. However, the killing effect highly correlated with the knockdown effect. The mortality rates ranged as 1.37%, 25%, 68.75% and 100% after 24h exposure to 0.5%, 1%, 1.5% and 2% concentrations of essential oil respectively (p = 0.0027, H = 14.12) (Table 3). Adult females of *An. coluzzii* were therefore able to resist at low doses (0.5, 1 and 1.5%) of *C. anisata* leaf essential oil. The required dose for 24h public health intervention could be 2% for *C. anisata* essential oil. The time to knock down 50% of mosquito population (median knockdown time, kdt_{50}) 24h post-exposure to essential oil impregnated nets was 60.15 min, 51.02 min, 18.59 min and 1.93 min, respectively for 0.5%, 1%, 1.5% and 2% of *C. anisata* leaf essential oil (Figure 2). Whereas the required dose to kill 50% of mosquito population (LC_{50}) was 1.2157 ± 0.0312% (95% CI, 0.8918-1.6572; Y = -0.6624 + 7.8086*X; p = 0.000).

Table 3: Adulcidal activity of *Clausena anisata* leaves essential oil on adult females *Anopheles coluzzii*.

Adulcidal activity (knockdown and killing effects)	Exposure time	Essential oil concentration (%)					P-value	H-value
		2	1.5	1	0.5	0		
Knockdown rate (%)	5 mins	81.25 ± 0.63	5.00 ±0.40	1.25±0.25	0± 0.00	0 ± 0.00	0.015	10.46
	10 mins	90.00 ± 0.00	22.5 ± 2.02	8.75 ± 0.48	2.50 ± 0.58	0 ± 0.00	0.011	10.97
	15 mins	92.50 ± 0.28	48.75 ± 1.88	11.25 ± 0.95	6.25 ± 0.25	0 ± 0.00	0.0049	12.87
	20 mins	95.00 ± 0.40	56.25 ± 1.65	16.25 ± 1.31	15.00 ± 0.00	0 ± 0.00	0.0045	13.06
	30 mins	100 ± 0.00	67.5 ± 1.19	25.00 ± 1.78	20.00 ± 0.71	0 ± 0.00	0.006	12.45
	40 mins	100 ± 0.00	78.75 ± 1.31	30.00 ± 1.68	22.50 ± 0.645	0 ± 0.00	0.0051	12.76
	50 mins	100 ± 0.00	85.00 ± 1.73	36.25 ± 1.25	23.70 ± 0.48	0 ± 0.00	0.0045	13.02
	60 mins	100 ± 0.00	90.00 ± 1.08	47.50 ± 1.32	27.50 ± 0.28	0 ± 0.00	0.0036	13.5
Mortality rate (%)	24 hours	100 ± 0.00	68.75 ± 3.75	25.00 ± 2.40	1.37 ± 0.47	0 ± 0.00	0.0027	14.12

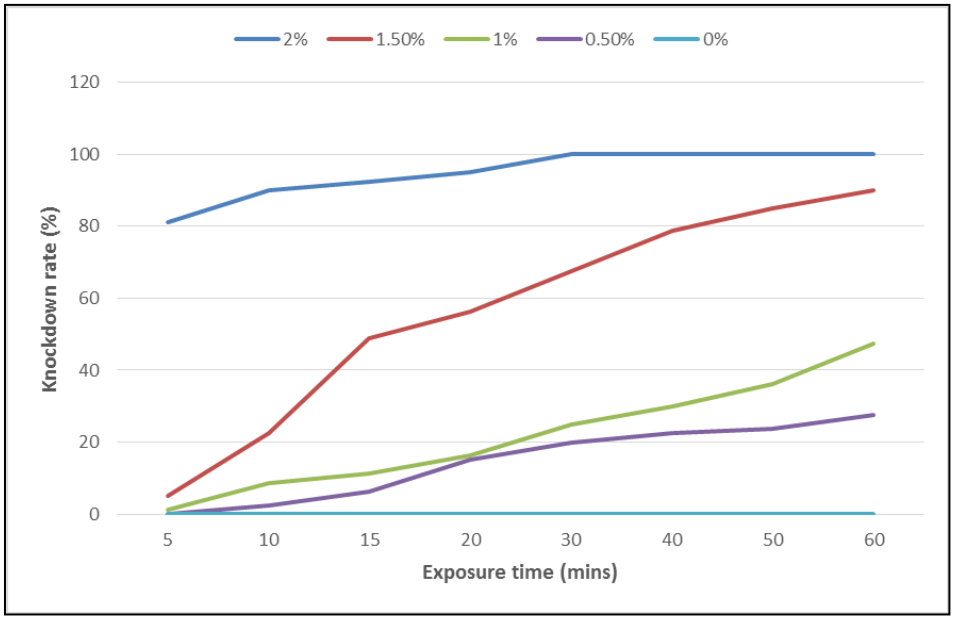

Fig 1: Knock down effect of *Clausena Anisata* leaf essential oil on adult females *Anopheles coluzzii* at different exposure times.

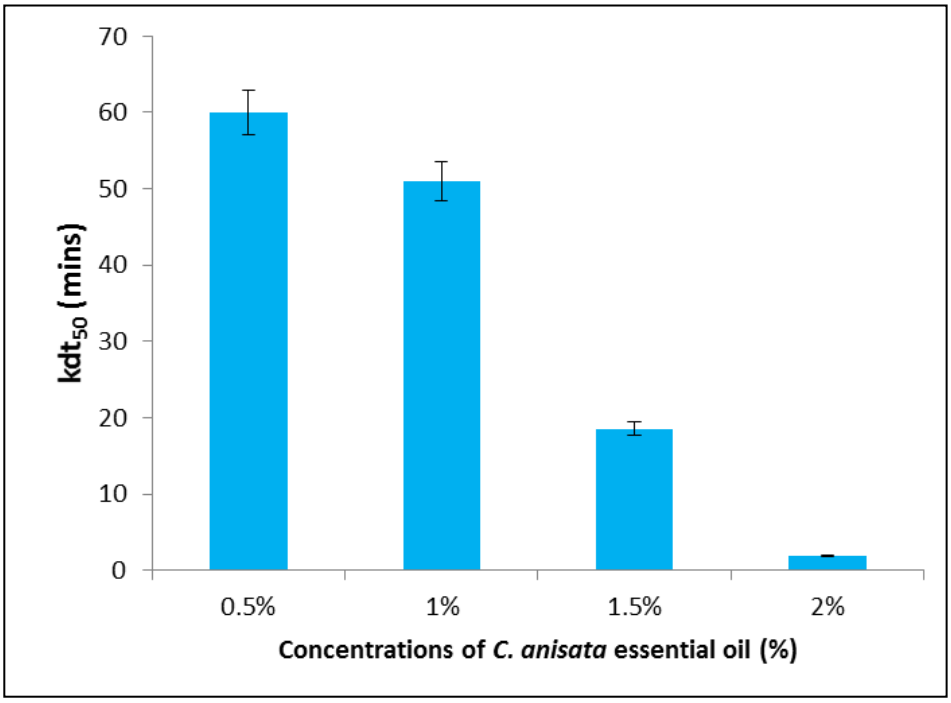

Fig 2: Median knockdown time ($kdt_{50,}$ time to knockdown 50% of mosquito population) of *Clausena anisata* leaf essential oil on adult females *Anopheles Coluzzii* using the WHO cone tests procedure. *Kdt_{50} are given in minutes with error bars at 5%.*

Discussion

Chemical composition of *C. anisata* leaf essential oil

One thing that we can learn from our ancestors is the power of natural extracts to make us feel better. Generations of humans from all cultures, countries and climates have made use of plants and minerals in medicines and for relaxation. This knowledge has not been lost, despite the appearance of extremely effective medicines in modern times; natural extracts are still widely used in essential oils and aromatherapy. Essential oils are more widely used in modern products than one might expect. Usually extracted through distillation, they are used to fragrance bathing products, incense, perfumes and cosmetics, as well as in some types of household cleaner. In terms of alternative medicine and for public health importance, essential oils are most frequently used today in aromatherapy and as alternative insecticides to ineffective commonly used public health insecticides. Now, plant extracts are still being researched by scientists to evaluate how they may help patients and prevent against virus, bacterial and parasitic diseases. We characterized in this study the main active ingredients of *C. anisata* leaf essential oil. We further screened the potentials of this oil against the developmental stages of the malaria vector *An. coluzzii*. The hydrodistilled leaves of *C. anisata* yielded 0.242% (w/w) of essential oil which is low compared to the 0.55% yield (w/w) obtained from Nigeria where *C. anisata* also grows aswell [14]. Although 52compounds were identified from the oil extracted from this study, only 30 compounds were identified from the same oil in Nigeria [14]. Variations in quantitative composition patterns of the oils from the two geographical locations might suggest the existence of different chemotypes of the oil both in Cameroon and Nigeria. Qualitatively, there was no

significant difference in the patterns of the oils extracted from plants harvested in Cameroon, Nigeria and India [5]. As described previously [5, 14], the main oil ingredients included phenylpropanoids (anethol), monoterpenoids (γ-terpinene,p-cymeneand limonene) and sesquiterpenoids). Oxygenated compounds such as meta-anisyl alcohol, terpineol and linalool were found in small quantities. Overall, little has been published regarding the mode of action of plants chemicals in insects [23]. The modes of action of anethol, estragole, ocimene, terpinene, terpineol, cubebene, caryophyllene, limonene, cadinene, and linaloolin insects are not fully understood. However, these chemicals are thought to cause an increase in the spontaneous activity of sensory nerves. This heightened activity sends spurious information to motor nerves and results in twitching, lack of coordination, and convulsions. The central nervous system may also be affected, resulting in additional stimulation of motor nerves [23]. Massive over stimulation of motor nerves leads to rapid knockdown paralysis such as mechanisms observed with synthetic insecticides against mosquito's species.

Ovicidal and larvicidal activities of *C. anisata* leaf essential oil on *An. coluzzii*

Fighting against early stages of disease vectors is seminal to stop both the spread of the vector and the burden of the disease. Early Public Health intervention strategies have been crucial in preventing diseases and controlling epidemics. Vector-borne diseases including malaria can easily be controlled since the aquatic stages (eggs and larvae) of the vectors are relatively confined to a geographical area and unable to escape the effects of insecticides [24]. *C. anisata* leafessential oil demonstrated clear inhibitory potentials against eggs of *An. coluzzii* after 24h incubation in the laboratory environment. The oil concentrations significantly decreased with the hatching rates, revealing an ovicidal effect which could be beneficial to fighting against *An. coluzzii* since the early developmental stages. Ovicidal potentials of essential oils have often been linked to the presence of oxygenated monoterpenes [5]. Although we did not evaluate the ovicidal effect of each active component of the extracted oil, the inhibitory potentials of *C. anisata* essential oil could be inferred to the presence of linalool which constitute the main oxygenated monoterpenoids of the oil. However, this should be confirmed in further studies before implementing the specific use of the oil in Public Health interventions. *C. anisata* leaf essential oil also revealed powerful larvicidal potentials against third instars larvae of *An. coluzzii*as previously shown with *An. stephensis* [5] in India. The larvicidal effect of the oil became significant after 48h incubation with mosquito larvae. These data therefore reveal the low potentials of the essential oil to be used in 24h larvicidal interventions against *An. coluzzii* as previously described by [5] with *An. stephensis*. The efficiency of *C. anisata* essential oil against third instars mosquito larvae might therefore vary from one region to another and could be attributed to oxygenated terpenes such as linalool and estragole. Indeed, according to [5], estragolestands as the main active component of the oil against third instars mosquito larvae. These findings have previously been described with oils extracted from *C. anisata* [17], *Ocimum basilicum* [24], *Callistemon rigidus* and *Callistemon citrinus* [25] and *Ocimum kenyense* [26]; confirming the potentials of *C. anisata* oil as green pesticide to control the spread of mosquito vectors and the burden of malaria in Cameroon.

Adulticidal activity of *C. anisata* leaf essential oil on *An. coluzzii*

Further investigations in this study also confirmed the insecticidal potentials of *C. anisata* leaf essential oil against the adult stages of *An. coluzzii* mosquito. The adulticidal potentials of the oil was significant 24h post-exposure to adult females of *An. coluzzii* using the WHO cone tests; with a required dose of 2% to kill all exposed mosquito. This concentration might be suitable for Public Health interventions with *C. anisata* essential oil against malaria vectors. However, the insecticidal property of the oil was more pronounced in mosquito larvae (LC_{50} = 0.44%) than in adult mosquito (LC_{50} = 1.27%). It is therefore suitable to quickly act against the first developmental stages of malaria vectors and avoid the wasting of effective materials. Using low quantity of insecticide (biological or synthetic) is also seminal to overcome the phenomenon of resistance which is attributed to the huge use of insecticides. These data correlate with findings published with commonly used insecticides in Public Health interventions against malaria vectors [27]. Overall, the knockdown effect of the oil was comparable to the one caused by pyrethroid and carbamate chemical insecticides and could be attributed to the inhibitory effect of monoterpenoids and 1,8-cineoleon acetylcholinesterase [28]. In fact, acetylcholinesterase is the main enzyme used by insecticide-resistant mosquito species to bypass the activity of the carbamate family of insecticides. Its inhibition therefore favors the activity of the insecticide and the dead of the mosquito. The high composition of *C. anisata*leaf essential oil in monoterpenoids makes this oil an efficient fighting tool to overcome the resistance observed with synthetic pesticides on malaria *Anopheles* vectors. The active component 1,8-cineole found in the oil has also shown antifungal, antioxidant and repulsive activitiesagainst weevils of foodstuffs [24, 25, 29]. Additional molecules such as caryophyllene and caryophyllene oxide have also been confirmed with insecticidal properties against arthropod insects [23]. Overall, this study demonstrated the potentials of *C. anisata* leaf essential oil as efficient biocide that can be used as alternative to ineffective synthetic insecticides in fighting *An. coluzzii* in malaria endemic areas. Further studies investigating the efficacy of individual ingredient of this essential oil, the mode of action and the synergism with the biocides under field conditions are needed to further validate its great importance for Public Health interventions.

Acknowledgement

We express our gratitude to Francis Zeukeng (Department of Biochemistry and the Biotechnology Center, University of Yaoundé I) for reviewing this manuscript. We also extend our gratitude to OCEAC for providing us with *Anopheles coluzzii*eggs used in this study, and the Ecole Nationale Supérieure de Chimie de Montpellier for analyzing the chemical composition of our essential oil.

Consent

Not applicable.

Ethical approval

Not applicable.

Competing interests

Authors have declared that they have no competing interests.

References

1. OMS. Stratégie Technique de luttecontre le Paludisme 2016-2030.Genève, Suisse, 2015, 33.
2. MINSANTE. Plan stratégique national de luttecontre le paludisme. Santé publique du Cameroun, Yaoundé, 2011-2015. 2011, 82.
3. Programme National de Luttecontre le Paludisme (PNLP). Rapport d'activité 2011, Yaoundé-Cameroun, 2012, 82.
4. Ombito O, Japheth, Matasyoh C, Josphat, Vuvule M. Chemical composition and larvicidal activity of *Zanthoxyllum gilletii* essential oil against *Anopheles gambiae*. Afr. J. Biotechnol. 2014; 13:2175-2180.
5. Marimithu G. Chemical composition and larvicidal activity of leaf essential oil from *Clausena anisata* (Willd.) Hook. J. Asia-PacEntomol. 2010, 874-877.
6. Ndomo A, Tapondjou L, Tendonkeng F, Mbiopo T. Evaluation des propriétés insecticides des feuilles de *Callistemon viminalis* (Myrtaceae) contre les adultesd' *Acanthoscelides obtectus* (Say) (Coleoptera; Bruchidae). Tropicultura. 2009; 27(3):137-143.
7. Foko DGA, Messi J, Tamesse JL. Influence of water type and commercial diets on the production of *Anopheles gambiaegille* under laboratory conditions. Pak.J. Biol. Sci. 2007a; 102:280-286.
8. Tchoumbougnang F, Dongmo PMJ, Sameza ML, Nkouaya MEG, Tiako FGB, AmvamZollo PH *et al*. Activitélarvicidesur *Anopheles gambiae* Giles et composition chimique des huiles essentielles extraites de quatreplantescultivées au Cameroun. Biotechnol. Agron. Soc. Environ. 2008; 13(1):77-84.
9. Foko DGA, Tamesse JL, Feukam BF. Adulticidal Effects of essential oils Extracts from *Capsicum annuum* (Solanaceae) *Piper nigrum* (Piperaceae) and *Zingiber officinale* (Zingiberaceae) on *Anopheles gambiae* (Diptera: Culicidea), vector of malaria. J Med. Entomol. 2011; 8(2):152-163.
10. Akono N, Belong P, Tchoumbougnang F, Bakwo F, Fankem H. Composition chimique et effets insecticides des huilesessentielles des feuilles fraichesd' *Ocimum canum* etd' *Ocimum basilicumsur* les adultesd '*Anopheles funestuss.s*., vecteur du paludisme au Cameroun. J. App. Biosci. 2012; 59:4340-4348.
11. Foko DGA, Nyegue MA, Tsila HG, Awono APH, Ndong MP, Tamesse JL. Chemical Composition and Ovicidal, Larvicidal and Pupicidal Activity of *Ocimum basilicum* Essential Oil against *Anopheles gambiae*. (Diptera: Culicidae). European J. Med. Plants. 2016a; 16(3):1-13.
12. Foko DGA, Nyegue MA, Tamesse JL. Effet insecticide de l'huileessentielle des graines de *Aframomum sulcatum* (Oliv. & D. Hanb. Ex Baker) K. Schum. sur les stadesaquatiques de *Anopheles gambiae* Giles 1902, vecteur du paludisme. Syllabus Review. 2016b; (6):69-80.
13. Hamza OV, Van den bout-Van den beukel CJ, Matee MI. Antifungal activity of some Tanzanian plants for the treatment of fungal infections. J Ethnopharmacol. 2006; 108(1):124-132.
14. Usman LA, Hamid AA, Olawore NO, Fakunle CO, Oladosu IA, Zubair MF. Chemical Composition of Leaf Essential Oil of *Clausena anisata* Growing in north-Central Nigeria. JASR. 2010; 6(6):891-894.
15. Teugwa MC, Piam G, Tane O, Amvam ZPH. Activité insecticide des extraitsd' *Ageratum houstonianum*, de *Clausena anisata* et de *Croton macrostachyus* surle bruche du niébé (*Vigna unguiculata* Walp). Food Africa 2002, 1- 4.
16. Lawal IO, Grierson DS, Afolayan AJ. Phytotherapeutic Information on Plants Used for the Treatment of Tuberculosis in Eastern Cape Province, South Africa. Evid. Based Complementary Altern. Med, 2014, 11.
17. Lawal IO, Grierson DS, Adesanya AA, Afolayan AJ. Brine shrimp toxicity evaluation of the acetone crude extracts and identified compounds from *Clausena anisata* (Willd.) Hook. F. Ex Benth Growing In South Africa. J. For. Res.Manag. 2016; 13:95-106.
18. Bagvan A, Rahuman AA, Kamraj C, Geetha K. Larvicidal activity of saponin from Achyranthes aspera against *Aedes aegypti* and *Culex quinquefasciatus* (Dipterra: Culicidae). Parasitol Res. 2008; 103:223-9.
19. WHO. Guidelines for laboratory and field testing of mosquito larvicides. Communicable Disease Control, Prevention and Eradication, WHO Pesticide Evaluation Scheme, 2005; WHO/CDS/WHOPES/GCDPP/1.3.
20. WHO. Test procedures for insecticide resistance in malaria vector Mosquitoes. Geneva: World Health Organization, 2013, 1-40.
21. Nyegue M. Propriétéschimiques et biologiques des huilesessentielles de quelquesplantesaromatiques et/oumédicinales du Cameroun : Evaluation de leursactivités anti-radicalaires, antiinflammatoires et anti-microbiennes. Thèse de doctorat de troisième cycle. Université de Monpéllier II. 2006, 194.
22. Adams RP. Identification of essential oils by gas chromatography quadrupolemass spectroscopy. Allured Publishing Corporation, 4th Edition, Carol Stream 9. 2012, 698.
23. Opender K, Suresh W, Dhaliwal G. Essential Oils as Green Pesticides: Potential and Constraints. Biopest. Inter. 2008; 4(1):63-84.
24. Azhari HN, Abdurahman HN, Mashitah MY, Jessinta DOS. Bioactive compoundsfrom Basil (*Ocimum basilicum*) essentialoils with larvicidal activity against *Aedes aegypti* Larvae. IPCBEE. 2012; 46(5):21-24.
25. Jazet DP, Tatsadjieu N, Ndongso D, Kuate J, Amvam Z, Menut C. Correlation between chemical composition and antifungal properties of essential oils of *Callistemon rigidus* and *Callistemon citrinus* of Cameroon against *Phaeoramularia angolensis*. J Med. Plants Res. 2009; 3(1):009-015.
26. Obeng-Ofori D, Reichmuth C, Bekele J, Hassanali A. Biological activity of 1,8-cineole, a major component of essential oil of *Ocimum kenyense* (Ayobangira) against stored product beetles. J App. Entomol. 1997; 121:237-243.
27. Foko DGA, Tamesse JL, Messi J. Insecticidal effects of *Capsicum annuum* on *Anopheles gambiae* Gilles under laboratory conditions. J. Entomol. 2007b; 4:299-307.
28. Houghton PJ, Ren Y, Howes MJ. Acetylcholinesterase inhibitors from plants and fungical. National Production. 2006; 23(2):181-199.
29. Byug-Ho L, Peter C, Fa'ale T, Won-sik C. Fumigant toxicity of essential oils from the Myrtaceae family and 1,8-cineole against 3 major stored-grain insects. J Stored Prod. Res. 2004; 40:553-564.