Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

J E Z S Journal of Entomology and Zoology Studies

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2015; 3(4): 334-339

Received: 25-06-2015
Accepted: 27-07-2015

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Larvicidal activity of essential oils from pericarps of ripe Citrus fruits cultivated in Cameroon on pyrethroids sensitive and resistant strains of *Anopheles gambiae* Giles, 1902

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Abstract
Essential oils of pericarps of ripe fruits from *Citrus aurantifolia*, *C. limon*, *C. sinensis* and *C. reticulata* were analyzed and their effectiveness assessed. Pericarps of *C. sinensis* (0.8%) had higher essential oil content than that of other species, mostly monoterpenes (83.60 to 97.29%). The major compound was limonene (46.84%, 76.14%, 91.43% and 94.92%, respectively for *C. aurantifolia*, *C. reticulata*, *C. limon* and *C. sinensis*). These volatile oils induced 100% mortality of larval stages of *An. Gambiae* strains at 400 ppm after 12 hours of exposure, except for *C. aurantifolia*. Volatile oil from *C. limon* is the most effective (LC_{50} = 13.75 ppm and LC_{95} = 54.94 ppm for the susceptible strain; LC_{50}= 32.28 and LC_{95} = 104.7 for the resistant strain). Probably because of higher proportions of limonene or synergistic action of certain minor compounds. Pericarps of *C. limon* ripe fruit could be recommended for the development of natural biocides.

Keywords: *Anopheles gambiae*, *Citrus aurantifolia*, *Citrus limon*, *Citrus sinensis*, *Citrus reticulata*, essential oil, biocide, vector control

1. Introduction
Mosquitoes are main vectors of pathogens transmitted to men and animals. Malaria remains one of the most dangerous diseases transmitted by mosquitoes [16]. It is caused by a Hemococcidae of the genus *Plasmodium* and transmitted to humans by a female mosquito of the genus *Anopheles*. According to the World Health Organization report [46], about 198 million people worldwide are infected annually, among which 82% are found in sub-saharan Africa. Children under five years and pregnant women are the most vulnerable groups [46]. Much has been done in order to reduce the burden of malaria but, results remain unsatisfactory. This may owe to approximate implementation of recommended preventive and curative methods, poor accessibility to prevention tools and drugs, as well as the up rise of resistant strains of the malaria parasite and vector [8, 34]. To date, several studies on the sensitivity of vectors to chemical insecticides have been performed. Most of malaria vectors were found to be resistant to dieldrin, permethrin, deltamethrin and DDT [26]. To overcome this resistance phenomenon which is a major obstacle to malaria prevention and treatment, the use of plant extracts with proven insecticidal effect is highly recommended. Many studies highlighting the insecticidal activity of aqueous extract, powder or essential oil of many plants from the African pharmacopoeia have been carried out [6, 27]. Regarding essential oils, it has been shown that their use on mosquito nets for adult mosquitoes control campaigns is inappropriate because of their volatile and biodegradable features [13, 14]. However, their effectiveness as larvicide in breeding sites of reduced dimensions like those of *An. gambiae* s.l. has been proven [2, 4, 9, 22, 32, 39, 40]. Furthermore, the ability of these oils to act through in tegumentary dissemination and oral way [7] increases their toxic potential, allowing for real hope for the development of an effective essential oil based biological larvicide. Essential oils thus appear as a potential source of molecules on which vector control stakeholders could base to successfully control mosquito larvae while preserving the environment.
Cameroon has an impressive biodiversity from which some plants may yield essential oils likely to be used for the development of biological larvicides. In recent years, many studies

aimed at determining the larvicidal properties of essential oils have been carried out, with some encouraging results. However, none of these studies concerned the pericarps of ripe fruit of plants of the Rutaceae family. The antifungal properties of these plants on *Phaeoramularia angolensis* have been reported [20]. This study aims at evaluating the larvicidal activity of pericarps of ripe fruits of *Citrus reticulata*, *C. limon*, *C. aurantifolia* and *C. sinensis* on *Anopheles gambiae* s.l., a major malaria vector in Cameroon.

2. Materials and Methods

2-1. Harvest of plants and extraction of essential oils

The plant material consisted of the pericarps of ripe fruits of *Citrus reticulata*, *C. limon*, *C. aurantifolia* and *C. sinensis* harvested from an orchard in Edéa (Cameroon) in March 2013 (Table I). From the landlord's interview, this plot had not been treated with chemical insecticides, which may influence the chemical composition of volatile oils. These plant species were chosen because of their antifungal activity on *Phaeoramularia angolensis* highlighted as shown by Jazet *et al.* [20]. Identification of fruits was confirmed by the National Herbarium of Yaoundé (Cameroon). The pericarps of ripe fruits of each plant sample were processed through hydrodistillation for 5 hours using a Clevenger type apparatus. After distillation, essential oil collected by decantation was filtered through anhydrous sodium sulfate column to eliminate residual water. The essence thus obtained was stored in dark bottles, at 4°C. The extraction yield was calculated in relation with the weight of the plant material before extraction.

Table 1: Data on essential oil extraction from 4 Citrus species

Plant			Harvest		Essential oil	
Family	Species	Organ	Location	Date	Colour	Yield (%)
Rutaceae	*Citrus aurantifolia*	Pericarp of ripe fruits	Edéa	21/03/13	Light yellow	0.26
Rutaceae	*Citrus limon*	Pericarp of ripe fruits	Edéa	21/03/13	Light yellow	0.15
Rutaceae	*Citrus reticulata*	Pericarp of ripe fruits	Edéa	21/03/13	Light yellow	0.24
Rutaceae	*Citrus sinensis*	Pericarp of ripe fruits	Edéa	21/03/13	Light yellow	0.80

2-2. Chemical Analysis of the Essential Oils

Essential oils were analysed by gas chromatography (GC) coupled with mass spectrometry (MS).

2-2-1. Gas Chromatography

The oil was analysed on a Varian CP-3380 GC with flame ionisation detector fitted with a fused silica capillary column (30 m × 0.25 mm coated with DB5, film thickness 0.25µm); temperature program 50-200 °C at 5 °C/min, injector temperature 200 °C, detector temperature 200 °C, carrier gas N_2 at 1 mL/min.

The linear retention indices of compounds were determined on the basis of the retention times of a series of *n*-alkanes and the percentage compositions were obtained from electronic integration measurements not taking relative response factors into account.

2-2-2. Gas Chromatography/Mass Spectrometry (GC/MS)

GC/MS analyses were performed using a Hewlett-Packard apparatus equipped with an HP1 fused silica column (30 m × 0.25 mm, film thickness 0.25µm) and interfaced with a quadrupole detector (GC-quadrupole MS system, model 5970). Column temperature was programmed from 70-200 °C at 10 °C/min; injector temperature was 200 °C. Helium was used as carrier gas at a flow rate of 0.6mL/min, the MS was operated at 70eV.

2-2-3. Identification of Compounds

Identification of various constituents was performed through comparison of their retention indices and mass spectra with references in the NBS75K databank [5, 42] and stored laboratory mass spectra library [12, 36].

2-3. Origin of *Anopheles gambiae* strains and biological tests

Strains of *An. gambiae* used for biological tests were of two types; pyrethroids resistant strain (Logbessou strain) collected in October 2013 in temporary breeding sites of the Logbessou area (Douala), and Kisumu strain, sensitive to pyrethroids and domesticated in the malaria research laboratory of the Organization for Coordination of the fight against Endemic Diseases in Central Africa (OCEAC). The two strains of *An. gambiae* were separated for breeding in two different insectariums, free of any insecticide, following the Desfontaines *et al.* [11] method. The ambient temperature was 28 ± 3 °C and relative humidity was 78 ± 5%. During breeding, sensitivity testing with permethrin and deltamethrin were regularly performed on adult stages, according to the WHO standard protocol [45], in order to confirm the status of the two strains.

Biological tests were carried out in laboratory conditions as defined by WHO [43]. These tests consisted in evaluating the insecticidal efficacy of different dosages of essential oils vis-à-vis stage 4 larvae of sensitive and resistant strains. The methodology was inspired by the WHO protocol [44]. Twenty five (25) larvae from each strain of *An. gambiae* were taken from their breeding areas and put in 5cm diameter bowls, each containing 99.9 mL of spring water. Preliminary experiments were done in order to select a range of concentrations for the actual tests. For this purpose, stock solutions of essential oils of each sample were prepared by diluting 10,000 µl of essential oil in 10,000 µl of ethanol 70°. After obtaining this stock solution diluted to ½, many other dilutions were prepared from the stock solution, with varying volumes of ethanol 70°. The following concentrations were obtained: 400 ppm, 200 ppm and 100 ppm and 50 ppm. 0.1 mL of each concentration was finally introduced into each bowl with the prior 99.9 mL of spring water and stage 4 larvae. Four repetitions were performed for each concentration. The negative control contained 99.9 ml of spring water, 0.1 ml of 70° alcohol and larvae. Dead larvae were counted every 30 minutes for 12 hours. Death was confirmed when larvae were motionless and showed no reaction to any of our stimuli.

2-4. Data analyses

Analyses were performed using the Staview version 5.0 package (SAS Institute, Inc, USA). The Kruskal Wallis H test was used to compare the mean numbers of death stage 4 larvae of *An. gambiae*. The significance level was set at a probability value lower than 0.05.

Overall mosquito larvae mortality was adjusted according to Abbott's formula [1] when mortality in the control population was between 5% and 20%.

$$\%M = \frac{\%\text{ test mortality} - \%\text{ control mortality}}{100 - \%\text{ control mortality}} \times 100$$

3. Results and discussion

The yields of essential oils from pericarps of ripe fruits varied from 0.15% and 0.80% (v/v). The pericarps of *C. sinensis* (0.80%) had the highest essential oil content, followed by those of *C. aurantifolia* (0.26%), *C. reticulata* (0.24%) and *C. limon* (0.15 %) (Table I). These yields are lower than those obtained by Jazet [19] with pericarps of fruits of same plant species, harvested in Yaoundé. As shown by various studies, these differences of yields within the same plant family may be due to differences in the parts of the plant that was processed, extraction method, climatic conditions, location of the harvest site, period of harvest, state of maturation and pathophysiological state of the plant at harvest time [42, 38, 41]. Each of these factors should thus be taken into account in order to improve the yields of essential oils.

The chemical composition of essential oils is presented in Table II. Monoterpenes were by far the major compounds of these essential oils (79.32% to 95.92%). Limonene overall was the most abundant compound, representing 46.84% to 94.92%.

Table 2: Chemical composition of essential oils from pericarps of *C. limon, C. reticulata, C. sinensis* and *C. aurantifolia*

Compounds	KI*	Mass percentages (%)			
		C. limon	*C. reticulata*	*C. sinensis*	*C. aurantifolia*
Aromatic compounds		0.12	0.07	0.19	3.33
Methylnaphtalene I	1276	0.12	0.07	0.19	3.33
Monoterpenes		97.29	83.60	96.23	90.20
Hydrocarbon monoterpenes		95.49	80.92	95.92	79.32
α-thujene	923	0.03	0.44	-	0.36
α-pinene	930	0.58	1.31	0.43	2.46
Camphene	943	-	-	-	0.14
sabinene	965	2.76	0.16	-	2.83
β-pinene	971	0.19	1.10	0.31	24.88
Myrcene	981	-	1.10	0.03	0.15
β-phellandrene	995	0.14	0.29	0.08	-
Δ^3Carene	1005	0.05	0.06	0.04	-
α-terpinene	1010	0.15	0.32	0.06	0.26
p-cymene	1012	-	-	-	1.37
Limonene	1024	91.43	76.14	94.92	46.84
(*E*)-*β*-Ocimene	1038	0.16	-	-	0.33
γ- terpinene	1050	-	-	0.05	-
Oxygenated monoterpenes		1.80	2.68	0.31	10.88
Hydrated (*E*)-sabinene	1054	0.25	-	0.02	5.44
Linalool-1-oxide	1061	0.08	0.23	0.08	-
Linalool	1083	0.07	0.71	0.02	0.44
α-pinene oxide	1098	0.05	0.05	-	-
Limonene-1-oxide	1116	0.02	-	-	-
Terpinen-4-ol	1163	-	-	-	0.10
α-terpineol	1172	-	-	-	1.35
Myrtenol	1177	0.62	0.44	-	-
γ-terpineol	1184	0.30	0.80	-	-
Carvone	1225	0.14	-	-	-
Carveol	1200	0.03	0.09	0.04	0.21
Neral	1210	0.03	0.12	-	-
Nerol	1220	0.08	-	-	0.36
Geraniol	1235	0.08	-	0.13	2.59
Lynalyl acetate	1245	0.04	-	-	0.39
Bornyl acetate	1276	0.01	0.08	0.02	-
Thymol	1287	-	0.16	-	-
Sesquiterpenes		0.38	1.01	0.03	2.35
Hydrocarbon sesquiterpenes		0.35	1.01	0.03	2.35
Bicyclo-elemene	1380	-	-	-	0.20
β-elemene	1388	0.03	-	-	0.10
Santalene	1410	-	0.80	-	-
β-caryophyllene	1418	0.04	0.06	-	0.51
(*E*-*E*) *α*-bergamotene	1433	0.11	-	-	0.42
Bicyclogermacreme	1490	0.03	-	-	-
β-bisabolene	1500	-	-	0.03	0.63
γ-cadinene	1506	0.14	0.15	-	0.49
Oxygenated Sesquiterpenes		0.03	-	-	-
Globulol	1563	0.03	-	-	-
Linear compound		1.97	15.68	1.97	2.81
Octanal	987	1.97	1.54	1.79	1.08
Octanol	1058	-	14.14	-	-
Decanal	1190	-	-	0.12	1.73
Decenol	1252	-	-	0.06	-

*KI: Kovats indices listed according to their order of elution on a DB-1 type column.

These results corroborate those of Azzous *et al.* [3] and Haro & Faas [17]. These authors worked on pericarps of Citrus aurantifolia fruits from India and found that limonene was the major compound of the essential oil of Citrus aurantifolia. Furthermore Quoc *et al.* [33] and Soumaya *et al.* [35] also found limonene as a major compound in the essential oils of the pericarp of *Citrus sinensis* fruit from Vietnam and Tunisia respectively. In Cameroon, Jazet [19] also reported that limonene is the major compound of the pericarp of fruits of *C. aurantifolia*, *C. sinensis* and *C. grandis* from Yaoundé (Cameroon). Limonene is thus a characteristic compound of essential oils from pericarp of fruits from Citrus species [10, 23] followed by sabinene (2.76%), octanol (14.14%), octanal (1.79%) and β-pinene (24.88%) respectively in essential oils of *C. limon*, *C. reticulata*, *C. sinensis* and *C. aurantifolia*.

Laboratory tests showed that essential oils of *C. aurantifolia*, *C. limon*, *C. reticulata and C. sinensis* present insecticidal properties vis-à-vis stage 4 larvae of pyrethroids susceptible and pyrethroids resistant strains of *An. gambiae* (Figures 1 and 2, Tables III and IV).

Table 3: Larvicidal activity of essential oils from pericarps of ripe fruits from four citrus species on stage 4 larvae of Kisumu and Logbessou strains of *An. gambiae*

Citrus species	Larvicidal activity* (95% CI)			
	Kisumu strain		Logbessou strain	
	LC_{50} ($)	LC_{95} (£)	LC_{50}	LC_{95}
C. limon	13.75 (12.25 - 14.95)	54.94 (52.34 - 57.44)	32.28 (30.58 - 33.88)	104.70 (97.50 - 111.90)
C. reticulata	33.17 (30.87 - 35.77)	88.32 (84.62 - 91.38)	49.64 (46.44 - 53.34)	132.13 (126.23 - 137.33)
C. sinensis	37.45 (35.65 - 39.56)	150.38 (146.18 - 152.4)	56.54 (52.64 - 60.04)	150.38 (141.48 - 157.98)
C. aurantifolia	57.47 (54.27- 60.37)	201.86 (197.76 - 205.46)	73.81 (69.41 - 77.91)	271.89 (254.69 - 285.39)

(*): Unit is ppm ($): Dosage that causes 50% mortality rate of larvae
(£): Dosage that causes 95% mortality rate of larvae

The LC_{50} and LC_{95} determined from the simplified table of Henry that transforms cumulative mortality into probit [15] were used to classify these essential oils according to their toxicity for pyrethroids sensitive and pyrethroids resistant strains of *An. gambiae* s.l. Essential oil from *C. limon* was the most effective, followed by that from *C. reticulata*, *C. sinensis* and *C. aurantifolia* (Table III).

Table 4: Mortality of stage 4 larvae of 2 strains of *Anopheles gambiae* exposed for 12 hours to various concentrations of essential oils from 4 Citrus species fruits.

Essential oils dosages (ppm)		Stage 4 larvae		H$	P-value
		Kisumu strain	Logbessou strain		
Rutaceae					
	C. limon				
	400	25.00±0.0*	25.00±0.0	-	-
	200	25.00±0.0	24.75±0.5	1.0	0.31
	100	24.50±0.5	22.75±2.0	4.6	0.19
	50	23.50±1.2	20.00±1.6	5.2	0.38
	C. reticulata				
	400	25.00±0.0	25.00±0.0	-	-
	200	25.00±0.0	24.50±0.6	2.3	0.12
	100	24.00±0.0	21.25±1.3	7.0	0.07
	50	17.50±0.5	14.50±2.5	4.6	0.19
	C. sinensis				
	400	25.00±0.0	25.00±0.0	-	-
	200	24.50±0.5	23.50±1.2	2.3	0.50
	100	22.75±0.5	19.25±4.2	7.0	0.22
	50	18.25±1.5	17.75±7.2	7.0	0.32
	C. aurantifolia				
	400	24.75±0.5	24.50±0.5	0.4	0.49
	200	24.50±0.5	23.00±1.4	3.5	0.32
	100	17.00±0.8	15.25±2.8	7.0	0.22
	50	11.00±1.2	8.00±1.40	5.2	0.26

(*)= n ± standard deviation; mean number of deaths after 4 tests on 25 larvae ($): Kruskal-Wallis H test

The potential toxicity of these essential oils may owe to their high content in limonene (Table II). Many studies have demonstrated the insecticidal activity of pure forms of this molecule in all stages of certain mosquito species [18, 21, 25, 28]. However, our results show that the essential oil of *C. limon* is more effective despite its somewhat lower content in limonene (91.43%) compared with that of *C. sinensis* (94.92%) (Table II). This suggests that the effectiveness of essential oils from pericarp of *C. limon*, *C. reticulata* and *C. sinensis* is not related to limonene only. This compound could act synergistically with other minor compounds. Some previous studies have highlighted the important insecticidal role of minor compounds of essential oils from *Plectranthus marrubioides*, *Ocimum fischeri* or *Ocimum forskolei* [29, 31]. Compounds such as α-pinene and β-pinene seem to play an important role in increasing the potential toxicity of essential oils from pericarp of *C. limon*, *C. reticulata* and *C. sinensis*. The insecticidal activity of these molecules has been demonstrated by Ojimelukwe & Alder [30] and Lucia *et al.* [24] who evaluated the insecticidal activity of α-pinene vis-à-vis *Tribolium confusum* and the insecticidal activity of the β-pinene on *Aedes aegypti* respectively. Furthermore, Jazet [19] in his evaluation of the antifungal effects of essential oils of *C. latifolia*, *C. limon*, *C. sinensis*, *C. paradisi*, *C. grandis* and *C. unshiu* found that the most active extracts had higher concentrations of citral and β-pinene. The higher potential toxicity of essential oils from pericarp of fruits of *C. limon*, *C. sinensis* and *C. reticulata* may result from a synergistic action of limonene with other compounds such as α-pinene and β-pinene. However, additional studies to test the efficacy of α-pinene and β-pinène- / limonene combinations should be conducted to assess the possible synergistic action.

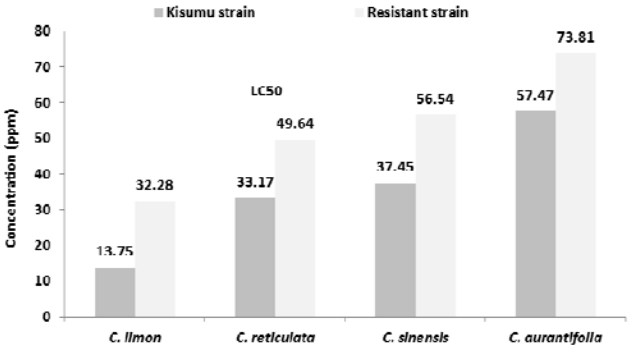

Fig 1: Sensitivity of stage 4 larvae of Kisumu and Logbessou strains of *An. gambiae* exposed to essential oils from 4 Citrus species, based on the LC_{50}.

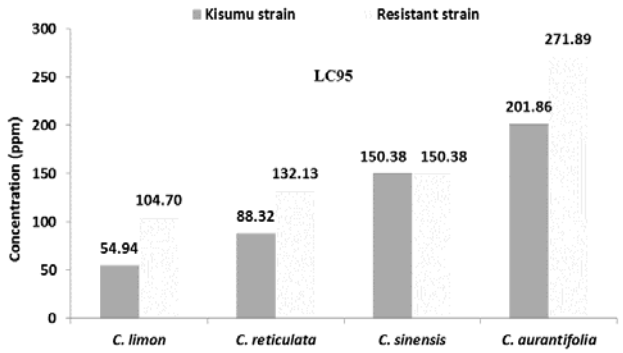

Fig 2: Sensitivity of stage 4 larvae of Kisumu and Logbessou strains of *An. gambiae* exposed to essential oils from 4 Citrus species, based on the LC_{95}.

LC_{50} and LC_{95} are higher for pyrethroids resistant than for sensitive strain as shown in figure 1 and figure 2. However, the Kruskal Wallis H test showed that the difference was not significant (Table IV). This suggests a kind of tolerance of the Logbessou strain to lethal doses of essential oils from Citrus species. This tolerance might be due to the presence of some trees of the citrus species at the collection site of *An. gambiae* (Logbessou strain) larvae. The permanent exposition to citrus volatile compounds has probably led to the selection of resistant individual within this anopheline population. Few studies have reported and explained mosquito resistance to essential oils. Additional studies should be carried out in order to better understand the genetic mechanisms behind this resistance. Despite the resistance observed, essential oil from pericarps of ripe fruits of *C. limon* has however showed interesting insecticidal activity vis-à-vis the larvae of pyrethroids resistant strain (Logbessou strain; LC_{50}=32.28 ppm). The essential oil of *C. limon* has the advantage of being biodegradable, safe for the environment, extracted from left over from plants (pericarp) and has interesting insecticidal activity vis-à-vis pyrethroids susceptible and resistant strains of *An. gambiae*. This volatile substance offers interesting possibilities in the search for substitutes to synthetic insecticides. These synthetic insecticides are expensive, destroy untargeted organisms; they are faced with the up rise of resistant strain in treated mosquito populations and are insufficient for solving the problem of malaria in sub-Saharan Africa.

Essential oils of ripe fruits from *Citrus aurantifolia*, *C. limon*, *C. sinensis* and *C. reticulate* have remarkable insecticidal properties on larval stages of both pyrethroids susceptible and resistant strains of *Anopheles gambiae*, mostly that of *C. limon*. This efficiency might be related to higher proportion of limonene and/or to the synergistic action of certain minor compounds. Endemic countries should thus promote the use of pericarps of *C. limon* ripe fruits as natural biocides in the implementation of national policies for effective control of malaria vectors.

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