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Adulticidal Activities and Synergistic Effects of *Citrus Aurantiifolia* Peels and *Hyptis spicigera* Leaves Essential Oils against *Anopheles gambiae* s.l. (Diptera: Culicidae)

Damas Dainone Ignareki[1]*, Gisele Aurelie Foko Dadji[2], Elie Njantou Baudelaire[3,4,5], Amadou Dicko[4], Lame Younoussa[1], Dimitri Wendgida Wangrawa[6,7], Antonio Nkondjio[8], Joseph Lebel Tamesse[2], Abraham Fomena[1]

[1]Department of Animal Biology and Physiology, Faculty of Science, University of Yaounde I, Yaounde, Cameroon
[2]Department of Biological Sciences, Higher Teacher's Training College, University of Yaounde I, Yaounde, Cameroon
[3]AGRITECH France, R&D, France
[4]Laboratory of Physics and Chemistry, Jean Baritiot Institute, University of Lorraine, Metz, France
[5]Department of Food Sciences and Nutrition, ENSAI, University of Ngaoundere, Ngaoundere, Cameroon
[6]Fundamental and Applied Entomology Laboratory, Joseph Ki-Zerbo University, Ouagadougou, Burkina Faso
[7]Norbert Zongo University, Koudougou, Burkina Faso
[8]Malaria Research Laboratory, Organization for Coordinating the Fight against Endemics in Central Africa (OCEAC), Yaoundé, Cameroon.

ABSTRACT: Research on new plant-based insecticides meets the need for an alternative to address mosquito resistance to synthetic insecticides. This study assessed the adulticidal activity and synergistic effects of essential oils (EOs) from *Citrus aurantiifolia* peels and *Hyptis spicigera* leaves on female adults of *Anopheles gambiae* s.l. The extraction yields were on the order of 0.17 and 0.11%, respectively, for *C. aurantiifolia* and *H. Spicigera* EOs obtained by hydrodistillation. The phytochemical composition of the EOs was analysed by Gas Chromatography coupled with Mass Spectrometry (GC-MS). GC-MS revealed that the essential oils of *C. aurantiifolia* and *H. spicigera* contained high amounts of monoterpene compounds (100% and 77.45%, respectively). Adulticidal activity was assessed using WHO and CDC bottle bioassays at concentrations of 2.5, 5, 7.5 and 10 mg/ml/btl. The EOs of both plants caused significant concentration-dependent Knock down and adulticidal activities. Individually, *H. spicigera* EOs had more adulticidal effects (LC_{50} = 4.42 mg/ml/btl) than did *C. aurantiifolia* EOs (LC_{50} = 6.79 mg/ml/btl). An EO mixture 25%Ca + 75%Hs from *C. aurantiifolia* and *H. spicigera* had synergistic effects (CI = 142.84; SF = 1.42) on female *Anopheles gambiae* adults. Combinations of the two plant EOs considerably optimize their insecticidal effectiveness.

KEYWORDS: *Anopheles gambiae*, adulticidal, essential oils, Malaria, synergistic effects.

I. INTRODUCTION

Despite significant advances in the fight against malaria, it remains one of the world's leading pandemics and a major public health problem. In 2022, the World Health Organization (WHO) estimated that there will be 249 million cases of malaria worldwide, with 608 000 deaths[1]. The African region continues to bear the heaviest burden, with 94% (233 million) of cases and 580 000 deaths[1]. Malaria is a parasitic disease caused by Hemococcidae of the genus *Plasmodium* and is transmitted to humans by the bite of female mosquitoes of the *Anopheles* genus[2]. In sub-Saharan Africa, *Anopheles gambiae* s.l. is the most widely distributed species. This species is the most efficient vector of malaria, has very high infestation and entomological inoculation rates and is resistant to most molecules used in vector control[3,4,5]. Malaria control has long been based on pest and vector control. The main current approaches for reducing human-vector contact rely on the use of synthetic insecticides in the form of long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS)[6]. However, several problems have arisen, such as the operational failures observed in the interventions deployed, the toxicity of synthetic products affecting ecosystems and the growing resistance of mosquitoes[7,3]. Faced with this situation, other less toxic, affordable, environmentally safe, accessible and natural products, such as plants, would be interesting and exploitable alternatives. *Citrus aurantiifolia* (Rutaceae) and *Hyptis spicigera* (Lamiaceae) are two plants widely used in traditional medicine and are rich in many phytocompounds, such as monoterpenes, sesquiterpenes, aromatic compounds and linear compounds with insecticidal properties[8,9,10,11]. *C. aurantiifolia* is a well-known medicinal and food plant widely cultivated

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around the world, and it is native to Southeast Asia and is also known as lime. All of its parts are used in traditional medicine as astringents, diuretics, insect repellents, antiseptics and antimicrobial agents for the treatment of gastrointestinal disorders, coughs, colds and sore throats[12]. *C. aurantiifolia* has been found to have insecticidal and repellent effects on *Camponotus nearcticus*[11] and is also effective against mosquitoes, cockroaches and houseflies in aerosol form[13]. *H. spicigera* is an erect hairy aromatic herb in the Lamiaceae family commonly found in the bushlands of southern Sudan, northern Cameroon, Nigeria and western Kenya. The leaf of the plant is commonly used for treating diarrhea, dysentery, colds, headaches and several other illnesses, and its insecticidal activity is also well documented[14,15]. Previous synergistic effects on plants have demonstrated that synergistic combinations of extracts or essential oils (EOs) can overcome the side effects associated with high doses of single extracts and decrease the risk of resistance development[16,17,18,19]. In addition, exposure to various mixtures of biosynthetically different compounds found in plants has been shown to delay resistance[20]. To contribute to this perpetual fight against malaria vectors, the adulticidal activity and synergistic effects of EOs from *Citrus aurantiifolia* peels and *Hyptis spicigera* leaves were evaluated on adult females of *Anopheles gambiae* s.l. in the laboratory.

II. MATERIALS AND METHODS

A. *Plant Material*

1) *Harvest and processing of the plants:* *Hyptis spicigera* L. and *Citrus aurantiifolia* are two unprotected wild medicinal plants available and accessible. This study and collection of plants was carried out with the academic authorization of the head of Department of Animal Biology and Physiology of the Faculty of Science at the University of Yaounde 1, Cameroun. The study complies with all relevant regulations and guidelines.

Green leaves of *Hyptis spicigera* L. were collected from Wack (Adamaoua-Cameroon: latitude 7°40'43''; longitude 13°33'20'') in November 2021 and identified by the head of the National Herbarium of Cameroon in comparison with Leeuwenberg material n°10472 of the Herbarium collection n° 49078/HNC. Lime peels were removed from the ripened fruits of *Citrus aurantiifolia* at "Ngaoundai" (Ngaoundéré; Adamaoua-Cameroon: latitude 7°27'95''; longitude 13°61'10'') during the same period and identified in comparison with the Essombe material n°1 of the Herbarium collection specimen n° 67471/HNC. The plant parts collected were washed with tap water and used to extract essential oils.

2) *Essential oil extraction:* The EOs of *H. spicigera* leaves and *C. aurantiifolia* peels were extracted through hydrodistillation for 4 hours using a Clevenger-type apparatus. After distillation, the EOs collected by decantation were filtered through an 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 according to the following formula in relation to the weight of the plant material before extraction:

$$\text{Extraction yield (\%)} = \frac{\text{weight (g) of EO obtained}}{\text{weight (g) of plant material used}} \text{x}100$$

3) *Chemical analysis of the essential oils*: EOs of *H. spicigera* leaves and *C. aurantiifolia* peels were analysed at the Laboratory of Physics and Chemistry, Jean Baritiot Institute, University of Lorraine, Metz, France by Gas Chromatography (GC) coupled with Mass Spectrometry (MS).

Gas chromatography

The analysis of the EOs was carried out using a Varian CP-3380 type chromatograph equipped with a flame ionization detector and a capillary column (30 m × 0.25 mm) with a stationary apolar phase of the methylsilicone type (DB5, film thickness 0.25 µm). The temperature was increased from 50°C to 200°C with a temperature gradient of 5°C/min. The injector temperature was 200°C, and the detector temperature was set to 200°C. Nitrogen was used as the carrier gas with a flow rate of 1 mL/min. The retention indices of the constituents were determined relative to the retention times of a series of n-alkanes, and their relative percentages were calculated by electronic integration without taking into account their response factors.

Gas Chromatography/Mass Spectrometry (GC/MS)

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

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Identification of Compounds

Identification of various constituents was performed through comparison of their retention indices and mass spectra with references in the NBS75K databank[21,22], stored laboratory mass spectra library[23,24] and data from the literature.

B. *Mosquito strain*

Mature eggs of susceptible strains of *Anopheles gambiae* s.l. were reared in the insectarium of the Zoology Laboratory of Higher Teacher's Training College of Yaoundé under ambient conditions (27 ± 2°C; 74 ± 4% R.H.) according to the standard WHO protocol[25]. The mature eggs were subsequently transferred into a tray containing 1 L of tap water for hatching. After 24 hours, the larvae obtained were divided into several batches to avoid overpopulation and were fed TetraMin® baby food (3 mg per 100 larvae per day) for approximately 5 days until mature larval stages were obtained. After six to seven days, the stage 4 larvae that had transformed into pupae were removed using a 3 ml Pasteur pipette, transferred to plastic cups and subsequently introduced into cubic mosquito cages for emergence. Adult females from this emergence were fed a sugar solution (10%) applied to cotton wool for 2 to 3 days before the adulticide tests[26].

C. *Adulticidal bioassay*

The insecticidal efficacy of *H. spicigera* leaves and *C. aurantiifolia* peels EOs on adults of *An. gambiae* were performed according to the WHO protocol[27] on CDC bottle bioassays[28]. Efficacy was evaluated individually and in binary combinations of 25%A + 75%B, 50%A + 50%B, and 75%A + 25%B, where A and B represent the different EO solutions.

After the preliminary tests, a stock solution was prepared at a concentration of 20 mg/mL by dissolving 200 mg of each EO in 10 mL of acetone in a glass flask. Test concentrations of 2.5, 5, 7.5 and 10 mg/mL were then prepared by diluting each appropriate volume of the stock solution with an adequate quantity of acetone.

The bottles were washed with warm soapy water, rinsed thoroughly with tap water at least three times and placed in open air until completely dry. Afterwards, 1 ml of each prepared concentration extract was transferred into a clean and sterile 250 ml CDC bottle, and 1 ml of acetone was also introduced into sterile CDC bottles and used as a negative control. Deltamethrin (DBR) was used as a positive control at the recommended dose of 1 mg/mL/bottle. Each concentration and test was repeated four times. The bottles were shaken for 30 min to allow the products to cover the entire inner surface of the bottles, they were then opened, placed in the shade and covered with aluminum foil until complete evaporation of the solvent.

Twenty-five (25) adult female mosquitoes aged 3-5 days were introduced into CDC bottles previously impregnated with different concentrations of our solutions using a mouth aspirator. After 1 hour of exposure, a knockdown effect was recorded, and the mosquitoes were transferred to cardboard cups covered with unimpregnated mosquito net tiles on which we placed a piece of cotton soaked in a 10% sucrose solution for feeding and mortality was noted after 24 h.

The percentage of mosquito mortality was calculated according to the following formula:

$$\text{Mortality percent (\%)} = \frac{\text{number of mosquitoes dead}}{\text{total number of mosquitoes used}} \times 100$$

The results were corrected using Abbott's formula[29] if the percentage of adulticidal mortality in the negative control was between 5% and 10% according to the following formula:

$$\text{Corrected mortality (\%)} = \frac{\text{\% of Test Dead} - \text{\% of Control Dead}}{100 - \text{\% 0f Control Dead}} \times 100$$

The LC_{50} values of the EOs of all the plants were subsequently determined, as were those of the different binary combinations tested, which allowed us to identify the various interactions between combinations, as described by Lame *et al.*[17]: Interactions between combinations were determined using the cotoxicity index and the synergistic factor.

For the binary combination *Citrus aurantiifolia* (Ca) + *Hyptis spicigera* (Hs), we have: Toxicity index (TI) of Ca = 100 and (TI) of Hs $= \frac{\text{LC50 Ca}}{\text{LC50 Hs}} \times 100$

$$\text{Observed TI of the mixture (Ca + Hs)} = \frac{\text{LC50 Ca}}{\text{LC50 (Ca + Hs)}} \times 100$$

Theoretical TI of the mixture (Ca + Hs) = TI Ca × % Ca in the mixture + TI Hs × % Hs in the mixture.

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$$\text{Cotoxicity index} = \frac{\text{Observed TI of the mixture}}{\text{Théoretical TI of the mixture}} \times 100$$

When one component of the mixture causes a low mortality (< 20%) at all doses tested, the cotoxicity index of the combination was calculated as follows:

$$\text{Cotoxicity index} = \frac{\text{LC50 Ca}}{\text{LC50 (Ca + Hs)}} \times 100$$

According to Sun and Johnson[30]:

- A cotoxicity index less than 80 was considered to indicate an antagonistic effect;
- A cotoxicity index between 80 and 120 was considered to indicate an additive effect;
- A cotoxicity index greater than 120 was considered to indicate synergistic action.

Synergistic factors were also calculated according to the Kalyanasundaram and Das[31] method as follows:

$$\text{Synergistic factor (SF)} = \frac{\text{LC50 of the plant extract alone}}{\text{LC50 of the mixture}}$$

A value of SF > 1 indicates synergistic action, and a value of SF < 1 indicates antagonistic action.

D. *Statistical analysis*

Statistical analysis of the data was performed using SPSS (Statistical Package for the Social Sciences) software version 20. The corrected mortality percentages were subjected to an analysis of variance (ANOVA) to obtain the average mortality percentage and the standard error between these different means. The separation of means was performed using Tukey's comparison test at 5% (P = 0.05). Probit analysis was performed to determine the lethal concentrations that caused 50% (LC_{50}) and 95% (LC_{95}) mortality in adult mosquitoes. Microsoft Excel (2016) was used to code the data and plot the curves and graphs.

III. RESULTS

A. *Extraction yields and chemical composition of the essential oils*

The yields of the EOs from each studied plant are presented in **Table I**. Hydro distillation of fresh *C. aurantiifolia* peels and fresh *H. spicigera* leaves gave extraction yields of 0.17% and 0.11%, respectively.

Table I: Extraction yield of *Citrus aurantiifolia* and *Hyptis spicigera* essential oils.

Plant species	Material used (g)	Oil obtained (g)	Extraction yield (%)
Citrus aurantiifolia	2000	3.4	0.17
Hyptis spicigera	2000	2.2	0.11

Table II displays the analytical results for phytochemicals in EOs from *C. aurantiifolia* peels and *H. spicigera* leaves. The results showed that *C. aurantiifolia* and *H. spicigera* EOs are essentially composed of monoterpenes (100% and 87.14%, respectively). Isolimonene (77.93%), sabinene (11.01%), myrcene (3.55%) and gamma-terpinene (3.10%) are the main compounds in *C. aurantiifolia* EO. The principal constituents of *H. spicigera* EO were terpinolene (24.97%), beta-ocimene (24.68%), caryophyllene (11.76%), beta-pinene (11.37%), D-limonene (7.69%), eucalyptol (6.14%) and beta-phellandrene (4.71%).

Table II. Chemical composition (%) of essential oils of *Citrus aurantiifolia* peels and *Hyptis spicigera* leaves

Compounds :	**ret time**	***Hyptis spicigera* (%)**	***Citrus aurantiifolia* (%)**
Hydrocarbon monoterpenes		**76.12**	**98.55**
Isolimonene	14.520	-	**77.93**
.beta.-Ocimene	9.493	**24.68**	-
.beta.-Pinene	11.833	**11.37**	-
beta.-Phellandrene	12.135	**4.71**	-
beta.-Myrcene	13.187	1.36	-
D-Limonene	14.407	**7.69**	-

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alpha.-Pinene	9.381	-	1.43
gamma.-Terpinene	15.744	1.10	**3.10**
.gamma.-Terpinen	18.243	-	1.53
beta-Cymene	16.476	1.34	-
Terpinolene	16.921	**24.97**	-
Sabinene	12.083	-	**11.01**
Myrcene	13.129	-	**3.55**
Oxygenated monoterpenes		**11.02**	**1.45**
Carvacrol	37.814	2.24	-
Eucalyptol	14.812	**6.14**	-
Thymol acetate	31.185	2.64	-
Terpineol	25.412	-	1.45
Hydrocarbon sesquiterpenes		**11.76**	-
Caryophyllene	25.649	**11.76**	-
Oxygenated Sesquiterpenes	-	-	-
TOTAL	-	**99**	**100**

B. ***Adulticidal activity***

1) ***Knock down effect:*** EOs of *C. aurantiifolia* and *H. spicigera* alone and all their combinations show the lowest Knockdown effect **(Figure 1),** ranging from 0% at 2.5 mg/mL/bottle to 39% at 10 mg/mL/bottle against female adults of *An. gambiae* after 1 hour of exposition to the CDC bottle. The knockdown effect of all the solutions tested increased with increasing concentration and exposure period. A knockdown effect of 100% was also detected for the positive control (DBR: Deltamethrin) at the recommended single concentration of 1 mg/mL (1000 ppm).

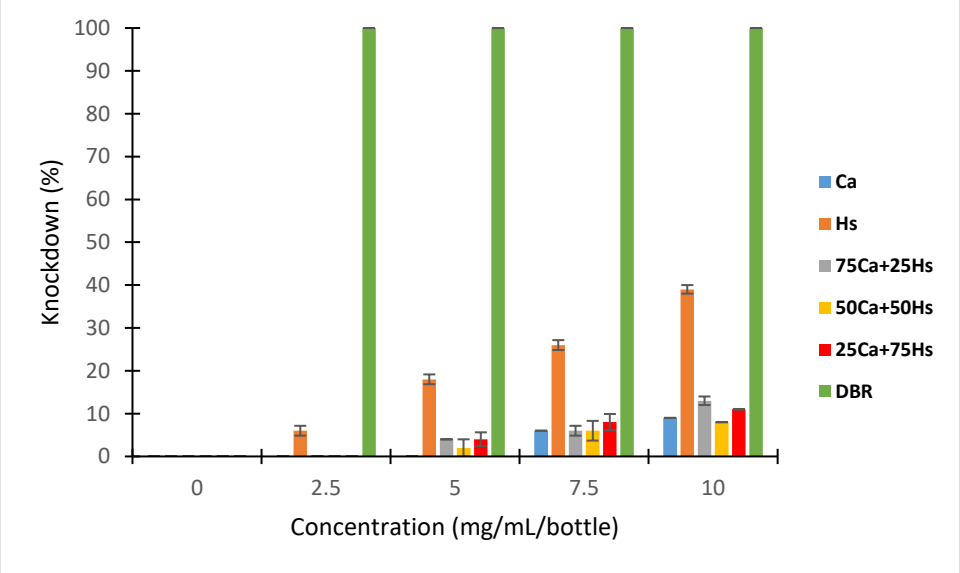

Figure 1. Knock down effects of *Citrus aurantiifolia* and *Hyptis spicigera* essential oils and their combinations against *An. gambiae* adults after 1 hour of exposure.

The concentrations at which 50% and 95% of the mosquitoes were knocked out after 1 hour of exposure to the CDC bottle are shown in **Table III.** All the solutions tested produced a nonsignificant (P>0,05) knockdown effect. The efficacy of EOs from *H. spicigera* (KdC_{50}=14.24 and KdC_{95}=88.95 mg/ml/bottle) alone was revealed to cause greater knockdown of *An. gambiae* adults

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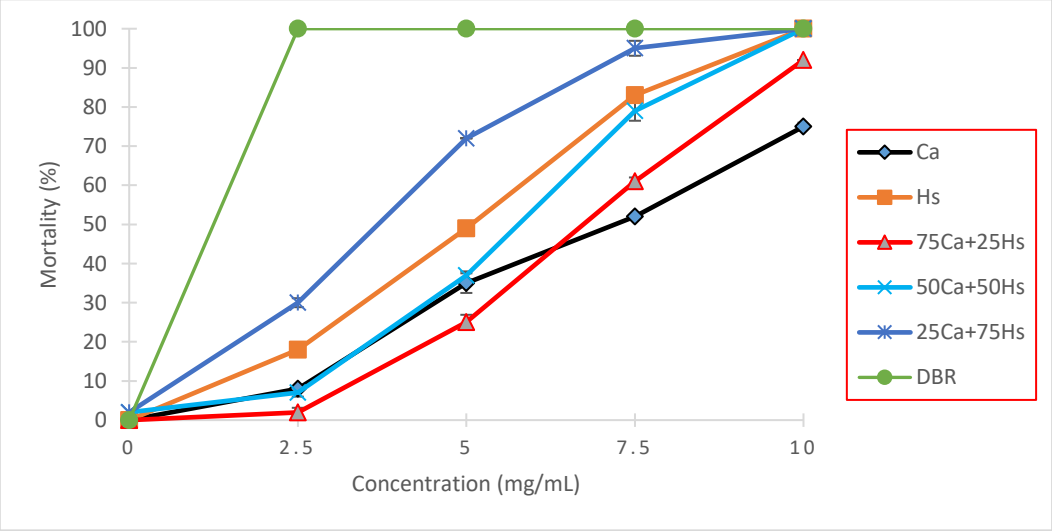
compared to *C. aurantiifolia* alone (KdC_{50}=20.41 and KdC_{95}=51.87 mg/mL/bottle). In combination, the combination of 75%Ca+25%Hs (KdC_{50}=27.20 and KdC_{95}=115.77 mg/mL/bottle) was more effective than the combinations of 25%Ca+75%Hs (KdC_{50}=29.61 and KdC_{95}=121.92 mg/mL/bottle) and 50%Ca+50%Hs (KdC_{50}=36.21 and KdC_{95}=147.82 mg/mL/bottle).

Table III. KdC_{50} and KdC_{95} (mg/mL/bottle) values of *Citrus aurantiifolia* and *Hyptis spicigera* essential oils and their combinations against *An. gambiae* adults at 1 hour postexposure.

Plants	Slope±SE	R^2	KdC_{50} (CI)	KdC_{95} (CI)	χ^2
100%Ca+0%Hs	4,06±0.69	0.66	20.41(15.29-44.17)	51.87(29.07-251.69)	22.64^{ns}
75%Ca+25%Hs	2,61±0.28	0.83	27.20(20.29-45.62)	115.77(63.02-344.01)	8.14^{ns}
50%*Ca*+50%*Hs*	2.69±0.55	0.71	36.21(23.20-102.13)	147.82(63.46-1081.54)	15.62^{ns}
25%*Ca*+75%*Hs*	2.67±0.43	0.65	29.61(19.42-84.92)	121.92(52.40-1044.78)	22.36^{ns}
0%Ca+100%Hs	2.06±0.18	0.97	14.24(12.38-17.22)	88.95(59.67-156.75)	6.76^{ns}

R^2= coefficient of determination; KdC= knockdown concentration; CI= confidence interval; $\chi 2$= chi-square test; Tukey test (P=0.05): ^{ns}P>0,05; *P<0,05; **P<0,01; ***P<0,001; SE= standard error; Hs= *Hyptis spicigera;* Ca= *Citrus aurantiifolia*

2) *Adulticidal efficacy of the combination of Citrus aurantiifolia and Hyptis spicigera against Anopheles gambiae:* **Figure 2** presents the mortality percentage of *An. gambiae* female adults exposed to different concentrations of *C. aurantiifolia* and *H. spicigera* EOs individually and in combination (75% + 25%; 50% + 50%; 25% + 75%) 24 h post treatment. EOs of *H. spicigera* alone and combinations 75%Ca+25%Hs, 50%Ca+50%Hs, 25%Ca+75%Hs caused significant mortality of *An. gambiae* which increased with concentration and time postexposure. The mortality percentages of *H. spicigera* alone and in combination 50%Ca+50%Hs, 25%Ca+75%Hs varied from 18%, 7% and 30%, respectively, at 2.5 mg/mL/bottle to 100% at 10 mg/mL/bottle. The 75%Ca+25%Hs combination had a mortality percentage ranging from 2% at 2.5 mg/mL/bottle to 92% at 10 mg/mL/bottle. EOs of *C. aurantiifolia* alone presented the lowest mortality rate, ranging from 8% at 2.5 mg/mL/bottle to 75% at 10 mg/mL/bottle. A mortality rate of 100% was also registered for the positive control (DBR: Deltamethrin) at the recommended single concentration of 1 mg/mL (1000 ppm), and a mortality rate less than 5% was recorded for the negative control.



Ca: *Citrus aurantiifolia;* Hs: *Hyptis spicigera;* DBR: Deltamethrin

Figure 2: Percentage mortality of *Anopheles gambiae* adults treated with *Citrus aurantiifolia* and *Hyptis spicigera* essential oils and their combinations 24 hours postexposure.

LC_{50} and LC_{95} (mg/mL/bottle) values of *C. aurantiifolia* and *H. spicigera* individually and in combination (75%+25%; 50%+50%; 25%+75%) against *An. gambiae* female adults 24 h post exposure under laboratory conditions are presented in **Table**

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IV. All the solutions tested produced significant (P<0,001) concentration-dependent adulticidal activity, with the exception of *C. aurantiifolia* EOs, which did not significantly differ (P>0,05). Individually, *H. spicigera* EOs (LC_{50}=4.42 and LC_{95}=10.18 mg/mL/bottle) were more effective than *C. aurantiifolia* EOs (LC_{50}=6.79 and LC_{95}=18.44 mg/mL/bottle). For the combinations, the mixture 25%Ca+75%Hs (LC_{50}=3.39 and LC_{95}=7.74 mg/mL/bottle) was more potent than the mixtures 50%Ca+50%Hs (LC_{50}=5.20 and LC_{95}=10.07 mg/mL/bottle) and 75%Ca+25%Hs (LC_{50}=6.36 and LC_{95}=12.09 mg/mL/bottle).

Table IV. LC_{50} and LC_{95} (mg/mL/bottle) values of *Citrus aurantiifolia* and *Hyptis spicigera* essential oils and their combinations against *An. gambiae* adults at 24 hours postexposure.

Plants	Slope±SE	R^2	LC_{50} (CI)	LC_{95} (CI)	χ^2
100%Ca+0%Hs	3,79±0.19	0.95	6.79 (6.51-7.09)	18.44 (16.60-20.92)	12.07^{ns}
75%Ca+25%Hs	5.90±0.26	0.93	6.36 (6.00-6.74)	12.09 (10.92-13.87)	41.69***
50%Ca+50%Hs	5.73±0.23	0.94	5.20 (4.80-5.60)	10.07 (9.00-11.74)	62.29***
25%Ca+75%Hs	5.73±0.23	0.93	3.39 (3.15-3.62)	7.74 (7.07-8.65)	72.29***
0%Ca+100%Hs	4.54±0.19	0.98	4.42(4.02-4.81)	10.18 (8.91-12.21)	58.11***

R^2= coefficient of determination; LC= Letal concentration; CI= Confidence interval; χ2= chi-square test; Tukey test (P=0.05): ^{ns}P>0,05; *P<0,05; **P<0,01; ***P<0,001; SE= standard error; Hs= *Hyptis spicigera;* Ca= *Citrus aurantiifolia*

Table V below shows the cotoxicity index and the synergistic factors of the binary combinations of *C. aurantiifolia* and *H. spicigera* EOs on female adults of *An. gambiae* 24 hours postexposure; the combinations 75%Ca+25%Hs (CI: 94.14; SF: 0.94) and 50%Ca+50%Hs (CI: 102.97; SF: 1.02) each presented an additive action, while the combination 25%Ca+75%Hs (CI: 142.85; SF: 1.42) exhibited a synergistic action on *An. gambiae* female adults 24 hours postexposure.

Table V: Synergistic factor and cotoxicity index of the combination of *Citrus aurantiifolia* and *Hyptis spicigera* against *An. gambiae* mosquitoes at 24 h postexposure.

Combinations	LC_{50} (mg/mL)	SF	CI	Type of action
100%Ca: 0%Hs	6.79	-	-	-
75%Ca: 25%Hs	6.36	0.941	94.14	Additive
50%Ca: 50%Hs	5.20	1.029	102.97	Additive
25%Ca: 75%Hs	3.39	1.428	142.85	Synergistic
100%Hs: 0%Ca	4.42	-	-	-

SF=Synergistic factor; CI=Cotoxicity index; LC=Lethal concentration. SE= standard error; Hs= *Hyptis spicigera*; Ca= *Citrus aurantiifolia*

IV. DISCUSSION

The management of insecticide resistance remains a major challenge for achieving effective malaria elimination. In insect pest control agent research, the insecticide combination approach is encouraged not only to optimize the efficacy of insecticide products but also to solve the problem of insect resistance, as this approach might apparently preserve the efficacy of the insecticide product for many years[17]. The main goal of this study was to evaluate the insecticidal effects of EOs from *C. aurantiifolia*, *H. spicigera* and their combinations on female adults of *An. gambiae* 24 h postexposure. The EO yield of *C. aurantiifolia* fruit peels (0.17%) obtained in our work was lower than the 0.26% and 0.32% yields obtained by Akono-Ntonga *et al*. in 2015 and 2016[4,32], respectively, with pericarps of fruits of the same plant species harvested in Edea and Yabassi (Littoral-Cameroon). The yield of EOs from *H. spicigera* (0.11%) that we obtained was lower than the 0.26% yield obtained by Wangrawa *et al*.[33]. Djibo's work in Burkina Faso in 2000[34] showed that the yield of EOs from *H. spicigera* varies from 0.28% to 0.52% depending on the part of the plant used, the harvesting period and the site. These differences in yields within the same plant family may be due to differences in the parts of the plant that were processed, extraction methods, climatic conditions, locations of the harvest sites, periods of harvest, maturation states and pathophysiological states of the plant at harvest[35,5].

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The EOs of the two plants used in this study contained a mixture of major and minor compounds. Also some monoterpenes, such as isolimonene, sabinene, myrcene and gamma-terpinene, are the main compounds in the peels of *Citrus aurantiifolia* EOs, but no sesquiterpenes have been identified. These results corroborate those of Shagun *et al.*[9] in the "Review on *Citrus aurantifolia* essential oil", who also identified monoterpenes (94.6%), such as limonene (39.3%) and p-pinene (28.4%), as the major components of *C. aurantiifolia* peel EOs. The authors also revealed that the chemical composition of *C. aurantifolia* peel oil is very similar to that of *Citrus hystrix* and comprises monoterpenes (97.2%), p-pinene (39.3%), limonene (14.2%), citronellal (11.7%) and terpinen-4-ol (8.9%) as the major components and sesquiterpenoids in small quantities (2.6%)[9]. In 2015 and 2016, Akono-Ntonga *et al.*[5,32] identified 90.20%, 2.35%, 3.3%, 2.81% and 88.16%, 2.4%, 3.3%, 2.4% of monoterpenes, sesquiterpenes, aromatic compounds and linear compounds, respectively, in EOs from the pericarps of *C. aurantifolia*. The phytochemical composition of *H. spicigera* leaf EOs is similar to that obtained by Conti *et al.*[36], who identified sixty compounds in the EOs of *H. spicigera* and revealed that monoterpene hydrocarbons were the most represented class of volatiles (70.4%), followed by sesquiterpene hydrocarbons (22.6%). Other previous work revealed that the major constituents of these EOs were β-aryophyllene (25.7%), caryophyllene oxide (11.56%), sabinene (9.60%), 2-carene (8.78%), α-pinene (6.52%) and 1-octen-3-ol (4.91%)[10]. The phytochemical components present within plant species vary qualitatively and quantitatively. These variations can be attributed to seasonal and maturing variations, geographical origins, genetic variations, growth stages, plant utilization and postharvest drying and storage[37,38].

Knockdown and adulticidal activity are the keys to preventing mosquito bites. All the EOs and their combinations tested exhibited a moderate but nonsignificant knockdown effect on female adults of *An. gambiae* after 1 hour of exposition to the CDC bottle. These results may be because the concentrations used were not high enough to knock out a significant number of mosquitoes during this time interval. Several authors noted that the knockdown effect increased with increasing concentration and exposure time[39,40]. Treatment with natural compounds such as EOs or pure compounds such as pyrethroids may cause neurotoxic symptoms, including hyperactivity, seizures and tremors, followed by paralysis (knock down) and death of insect[41].

Overall, all the EOs tested showed an adulticidal effect on female adults of *An. gambiae* after 24 hours of postprocessing. However, individually, the EOs from *H. spicigera* leaves had significant adulticidal activity, while the EOs from *C. aurantiifolia* peels produced nonsignificant activity. Previous work has also demonstrated the adulticidal efficacy of *H. spicigera* EOs on the sensitive strain (Kisumu) and the local strain (Goden) of *An. gambiae* s.l., with LC_{50s} of 1.04% and 1.45%, respectively, at 24 h postexposure; these activities were greater than those of the EOs from *Hyptis suaveolens* and *Ocimum canum*[33]. These EOs also have repellent effects on *Sitophilus granarius* (L.) (Coleoptera: Dryophthoridae) adults at the lowest dose (2 × 10−4 µl oil per cm2)[36]. The insecticidal potential of *C. aurantiifolia* peel EOs has been documented. Laboratory tests showed that the EOs of *C. aurantifolia* pericarps presented insecticidal properties lower than those of *C. reticulata and C. sinensis* against larvae of pyrethroid-susceptible (LC_{50}: 57.47 ppm) and pyrethroid-resistant strains (LC_{50}: 73.81 ppm) of *An. gambiae* s.l.[5]. However, for *Culex pipiens*, *C. aurantifolia* pericarp EOs exhibited significant insecticidal activity compared with *Citrus sinensis* and *Citrus grandis* EOs, with an LC_{50} of 348 ppm against larvae and 273 ppm against nymphs[32]. The adulticidal activity of *H. spicigera* and *C. aurantiifolia* EOs could be due to differences in their complex chemical compositions. *H. spicigera* contains more groups of chemical compounds, such as hydrocarbon monoterpenes, oxygenated monoterpenes and hydrocarbon sesquiterpenes, while *C. aurantiifolia* EO is composed mainly of monoterpenes. Indeed, EOs are mixtures of volatile chemical compounds of different natures and functions[42]. Insecticides act on insects by inhibiting either acetylcholinesterase or esterase or by binding to sodium channels and gamma-aminobutyric acid receptors, thus impairing synaptic transmission and killing susceptible insects[43]. Some related research has focused on the mechanism of action of certain phytochemical compounds[44], but other avenues remain to be explored. Some monoterpenes, such as eugénol, thymol, α-terpineol, terpinen-4-ol, 1,8-cinéole and linalool, in EOs interfere with synaptic transmission[44,45]. Previous work has shown that, monoterpenes such as 1,8-Cinéole, α-pinène, β-pinène, α-Phellandrène, Sabinène, and sesquiterpenes such as trans-caryophyllène, Germacrène, caryophyllène, α-humulène and nerolidol oxide contained in some EOs are thought to have biological effects on mosquitoes[35,46,47]. Many studies have demonstrated that the insecticidal activity of EOs from the pericarp of *C. aurantiifolia* is due to their high content of limonene combined with other minor compounds[32,48,49].

All the EO combinations showed significant adulticidal activity and are beneficial because they optimized the efficacy of the EOs by considerably decreasing the LC_{50} value. The best combination was the synergistic combination 25%Ca+75%Hs, followed by the additive combinations 75%Ca+25%Hs and 50%Ca+50%Hs. The efficacy of plant extract mixtures depends on the type of mixture produced (proportional or balanced) and the species considered[18]. Ngatanko and Ngamo[19] showed that the proportional mixture of 190 ppm *H. spicigera* EO and 680 ppm *Vepris heterophylla* EO had synergistic effects and caused 100%

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mortality from *Sitophilus oryzae*, while a balanced mixture had antagonistic effects. Ayiki *et al.*[18] also showed that the various combinations of *Hyptis spicigera* + *Azadirachta indica*, *Hyptis spicigera* + *Vepris heterophylla* and *Azadirachta indica* + *Vepris heterophylla* had significant toxic effects on *Tribolium castaneum*. The combined effect of *C. aurantiifolia* peel EO has not yet been explored, so Adina *et al.*[30,51] demonstrated that the combination of ethanolic extracts of *C. aurantiifolia* peel and doxorubin had synergistic effects on the inhibition of the growth of MCF-7 cancer cells. The insecticidal activities of the plant EOs individually and in combination might be due to the composition and chemical nature of the active compounds acting jointly or independently on mosquitoes[52]. The adulticidal effects of diallyldisulfide, eugenol, methyl eugenol, limonene, carvone, α-pinene, eucalyptol, eudesmol and their combinations have been reported previously against *Aedes aegypti*[53]. Simeon de Buochberg[54] showed that phenolic compounds such as eugenol, linalool and thymol increase the toxic potential of plants when combined with terpinene. Some authors have shown that the synergistic effect of EOs is likely due to the formation of additional new molecules from the phytocompounds found in the mixture, which may act synergistically as neurotoxic insecticides, interfering with the ligand-gated chloride channel of the mosquito nervous system by blocking octopamine or cholinergic receptors, which are important target sites for insect pest control[55]. The increased activity may also be due to the simultaneous effects on different targets and the appearance of new compounds due to chemical interactions[20], increasing the insecticidal effect of the tested extracts up to tenfold, severely impacting the survival of the insect[56].

V. CONCLUSION

This work is the first to report the use of combined EOs from *C. aurantiifolia* peels and *H. spicigera* leaves against female adults of *An. gambiae* under laboratory conditions. Our results showed that all the EOs tested had adulticidal effects. However, individually, the EO of *H. spicigera* leaves had significant adulticidal activity, while the EO of *C. aurantiifolia* peels produced nonsignificant activity. The best combination was the synergistic combination 25%Ca+75%Hs, followed by the additive combinations 75%Ca+25%Hs and 50%Ca+50%Hs. This will lead to a long way in the development of new insecticides that will replace toxic and nonbiodegradable synthetic insecticides to rid communities of malarial infections.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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AUTHORS' CONTRIBUTIONS

Foko Dadji G.A., Fomena A., and Dainone I.D. designed the study. Dainone I.D., Foko Dadji G.A. and Baudelaire E.N. conducted the experiments. Dainone I.D., Baudelaire E.N and Lame Y. analysed the data and wrote the first draft of the manuscript. All the authors read, corrected, and approved the final version of the manuscript.

COMPETING INTERESTS

The authors declare that no competing interests exist.

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ADDITIONAL INFORMATION

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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