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Effectiveness of the mixture of Cannabis sativa L and piper umbellatum leaves essential oils with temephos and permethrin on major vectors Anopheles gambiae S.L and culex quinquefasciatus

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Abstract

Most of Arthropods borne diseases are endemic in tropical regions and especially in Sub-Sahara Africa. Synthetic insecticides use for vector control remain the first line of defence but they are associated with high toxicity, vector resistance, environmental adverse and limited alternative. The mixture of conventional insecticide with plant essential oil can be an alternative. Our study was undertaken to evaluate the efficacy of the mixture of *Cannabis sativa* L and *Piper umbellatum* leaves essential oil with permethrin and temephos on two major vectors *Anopheles gambiae* S.L and *Culex quinquefasciatus* under laboratory conditions.

Cannabis sativa L and *Piper umballatum* Leaves essential oils were extracted by hydro distillation with Clavenger type apparatus. The chemical analysis of essential oils was carried out by GC-FID and GC-MS respectively for *Cannabis sativa* L and *Piper umbellatum*. Based on literature the LC_{50} for permethrin and temephos were found by serial dilution. These constant concentrations were mixed with increasing sublethal concentrations of *Cannabis sativa* L and *Piper umbellatum* leaves essential oil. Bioassays were performed on the larvae and female adults of field population of *Anopheles gambiae* S.L and *Culex quinquefasciatus* according to the WHO and CDC guidelines. Probit regression was used to calculate the LC_{50} and LC_{95} of each mixture. The Kruskal Wallis test was used to assess the significance of difference on the mortality rate between treatments.

The mixture of temephos with essential oils is effective on larvae while the mixture of essential oils with permethrin is toxic for adult female of *Anopheles gambiae* S.L and *Culex quiquefasciatus*. The mortality is essential oil dose–dependent. For larvicidal activity, the LC_{50} and LC_{95} after 24 hours exposure for the mixture of *Cannabis sativa* L leaf essential oil with temephos were: 0. 14%; 0.002% and 0. 35%; 0. 026% respectively for *Anopheles gambiae* S.L and *Culex quinquefasciatus;* while these values for the mixture of *Piper umbellatum* leaf essential oil with temephos were: 0.30%; 0. 0011% and 0. 64%; 0.024% respectively for *Anopheles gambiae* S.L and *Culex quinquefasciatus.* After 48 hours exposure the LC_{50} and LC_{95} for the mixture of *Cannabis sativa* L leaf essential oil with temephos were: 0.011%; 0.0013% and 0.17%; 0.013% while for the mixture of *Piper umbellatum* leaf essential oil with temephos these values were: 0.023%; 0.001% and 0.38%; 0.015% respectively for *Anopheles gambiae* S.L and *Culex quinquefasciatus*. For the knock down activity of the mixture of *Piper umbellatum* leaf essential oil with permethrin, time necessary for knocking down 50% and 95% of exposed mosquito are: 27±0.4min; 14±0.8min and 53±0.6min; 52±0.14min respectively for *Anopheles gambiae* S.L and *Culex quinquefasciatus*. For *Cannabis sativa* L leaf essential oil mixture, time necessary for knocking down 50% and 95% of exposed mosquitoes are: 16±0.75min; 8±0.15min and 53±0.94min; 53±0.6min respectively for *Anopheles gambiae* S.L and *Culex quinquefasciatus*. For adulticidal activity, the LC_{50} and LC_{95} for the mixture of *Piper umbellatum* leaf essential oil with permethrin were: 0.024%; 0.011% and 0.18%; 0.17% respectively for *Anopheles gambiae* S.L and *Culex quinquefasciatus*; while for the mixture of *Cannabis sativa* L leaf essential oil these values were: 0. 027%; 0.011% and 0.18%; 0.15% respectively for *Anopheles gambiae* S.L and *Culex quinquefasciatus.*

Conclusion: The mixtures of *Cannabis sativa* L and *Piper umbellatum* leaves essential oils with temephos and permethrin showed the efficacy with low concentrations against larvae and adult female of field population of *Anopheles gambiae* S.L and *Culex quiquefasciatus*.

Keywords: *Anopheles gambiae* S.L, *Culex quiquefasciatus*, *Cannabis sativa L*, *Piper umballatum*, temephos, permethrin, essential oils mixture, insecticide

Introduction

Arthropod borne diseases and particularly malaria remain a serious public health issue in Africa. The prevention of these vector-borne diseases is based on the use of synthetic insecticides (Zaim *et al*., 2002) [1]. However, all efforts made to eradicate these diseases, although making progress in recent years, are limited. Indeed, the modification of the environment by anthropic activities such as agriculture (Nkya *et al*., 2014; Janko *et al*., 2018) [2, 3] urbanization (Oladepo *et al*., 2010; Pimnon *et al*., 2018) [4, 5.] industries are at the origin of vectors resistance to conventional insecticides. Other factors also contribute to the development of vector resistance: the application of the same insecticides in a locality where resistance to these

insecticides is proven, and the under dosage of insecticides (Diniz *et al*., 2015) [6]. This resistance is expressed in various ways in target insects: avoidance behaviour of the insecticide (Agossa *et al*., 2015; Carasso *et al*., 2019), rapid degradation of the insecticide by detoxification (Ibrahim *et al*., 2016) [9], modification of the site of fixation of the insecticide (Lynd *et al*., 2018; Alout *et al*., 2008) [11, 12], cuticular structures that prevent the penetration of insecticides (Wang *et al*., 2019; Ingham *et al*., 2018) [13, 14]. Based on the findings on the hazardous effects of synthetic insecticides on the environment and resistance of vectors, several authors have proposed alternatives including the use of bio pesticides (Gnankiné *et al*., 2017; Bolzonella *et al*., 2019) [15, 16] because they are non-toxic for non-target organisms, biodegradable and offer the advantage of being composed of several active ingredients. Unfortunately, all the tests proposed in this way remain limited to the tests carried out in the laboratory. Thus, the essential oils although effective are very volatile. A combination of raw plant extracts or essential oils with synthetic insecticides can be an alternative to the vector resistance and the adverse effect of synthetic insecticides.

In the present study, we evaluated the effectiveness of the combination of *Cannabis satiiva* L and *Piper umbellatum* leaves essential oils with temephos and permethrin on two major vectors, *Anopheles gambiae* S.L and *Culex quinquefasciatus* under laboratory conditions. In our previous work, the essential oils from the leaves of the two plants were found to be effective on the larvae and adults of *Anopheles gambiae* S.L and *Culex quinquefasciatus* under laboratory conditions (Abé *et al*., 2018; Abé *et al*., 2019) [17, 18]. Besides, these two plants are used worldwide for several purposes. *Cannabis sativa* L is known not only for its psychotropic effects on human subjects, but also in chemotherapy against nausea, to improve the treatment of HIV / AIDS (Mechoulam *et al*., 2002) [9], the treatment of pain (Manzanares *et al*., 2006) [20], treatment of multiple sclerosis (Hagenbach *et al*., 2001) [21] and Alzheimer's disease (Milton, 2002) [22], the treatment of cancer due to its antiproliferative effect (Galve-Roperh *et al*., 2000). The crude extract of *Piper umbellatum* non-toxic for small mammals (Da Silva *et al*., 2014) [24] appears to be effective against phospholipase of myotoxic venom of snake (Núñez *et al*., 2005) [25], exhibits protective and healing properties against stomach ulcers on an experimental basis in rodents (IF da Silva Junior *et al*., 2016) [26], anti-cancer and anti-inflammatory in Brazil (Hespporte Iwamoto *et al*., 2015); is effective in treating injuries in Cuba (Salehi *et al*., 2019) [27] and in West Africa in several tribes (Setzer *et al*.; 1999) [28]; to treat fever in Peru and onchocerciasis in Cameroon (Cho-Ngwa *et al*., 2016) [29]. *Piper umbellatum* is also used to treat kidney diseases, skin, burns, diarrhea, rheumatism, malaria and intestinal parasites (Roersch, 2010) [30].

Materials and Methods

Plant material

The leaves of *Piper umbellatum* were harvested in the morning at the University of Yaounde I campus while the *Cannabis sativa* L leaves were harvested in the village of Salla in the district of Ayos. Before hydro distillation, plants were identified at National Herbarium in comparison with the numbers of the collectors. For *Piper umbellatum*: R. Letouzey 2499 of the specimen number 3614SRFK, the specimen was classified as belonging to *Piperarceae* family and for *Cannabis sativa* L the specimen was identified under the number 3614SRFK and classified as belonging to *Cannabacea* family.

Essential oil extraction and synthetic insecticide collection

The plant leaves essential oil extraction took place in the laboratory of the department of Microbiology of the University of Yaounde I using a Clavenger type apparatus. This extraction was performed in several sessions, each session during 6 hours in order to allow a maximum extraction. The essential oils were collected in a dark bottle, dried using anhydrous magnesium sulphate and stored at 4°C, away from UV rays before use. The mass of plants used and extraction yield are presented in table 2. The synthetic insecticides for our bioassays (permethrin and temephos) were provided by OCEAC (Organisation de Coordination pour la lutte Contre les Endémies en Afrique Centrale) with reference of AL International industry (France).

Chemical analysis of *Piper umbellatum* and *Cannabis sativa* L leaves essential oils

The chemical analysis took place at two different laboratories: At Lorraine University, France, for *P. umbellatum* and at the National Institute of the Analytic Science of Paris for *C. sativa* L. The chemical analysis of *Piper umbellatum* leaf essential oil was performed by Gas Chromatography, coupled with Mass Spectrometry. The analysis was performed with two Perkin Elmer instruments, a Clarus 500 GC gas chromatograph coupled to an MS Clarus 500 mass spectrometer.

The column used in chromatography was an Elite 5MS (5% Diphenyl / 95% DimethylPolysiloxane stationary phase) 30 m long, with an internal diameter of 0.25 mm and a film thickness of the 0.25 µm stationary phase. The carrier gas was helium with a flow rate of 0.75 mL / min.

The injector was brought to 300 ° C, the program begins with a step of 3 min at 60 ° C and the temperature increases from 10 ° C / min to 300 ° C where a step of 3 min is performed.

The acquisition of the mass spectrum was carried out on an m / z range of between 20 and 400 with 0.1 scan / s and an electron ionization energy of 70 eV. The temperature of the source and the transfer line were 250 ° C.

The sample to be analysed was solubilized in a small volume of dichloromethane.

The data processing software was Turbomass 6.1 and the database for the comparison of mass spectra were NIST MS Search 2.0.

For *Cannabis sativa* L leaf essential oil, the determination of retention data and the area percentage of the identified compounds were carried out on a two GC-FID systems:

1- An Agilent 5890 system equipped with HP-1 (ref: 1909 1 Z-115) column (50m x 320µm; 0.5 µm film thickness). GC oven temperature was kept at 80 °C for 8 minutes and programmed to 220 °C at the rate of 2 °C/min;

2- An Agilent 6890 system equipped with HP-Innowax (ref: 1909 1 N-216, Agilent Technologies, Santa Clara, CA 95051, USA) column (60 m x320µm,0.5µm film thickness).GC temperature was kept at 60 °C and programmed to 245 °C at the rate of 2 °C/min, then constant at 250 °C for 20 min.

The split ratio was adjusted to 1/100. The injector

temperature was 250 °C and the FID detector was kept at 250 °C. The carrier gas was Helium (1.3ml/min). Gas chromatography- Mass spectrometry was carried out using the first system Agilent 5890 equipped with HP-1column.The mass spectra was recorded in the electron impact mode at 70 eV using the aforementioned chromatographic conditions. Individual components of *Cannabis sativa* L leaves essential oil were identified by their retention index as described in Adams (2012) and their mass spectra were interpreted using the WILEY L computer library

Mosquito's collection

For our bioassays, we used the larvae and adult female of wild population of *Anopheles gambiae* S.L and *Culex quinquefasciatus*. The aquatic stages of *Anopheles gambiae* S.L were collected in Yaounde down town, near river Ewoé (N $03^0$51'34.9'' and E $011^0$31'3''). *Culex quinquefasciatus* were sampled at Melen pound behind the Faculty of medicine and Biomedical Sciences of the University of Yaounde I (N $03^0$85'63.1'' and E $011^0$48'49.4''). Mosquitoes collected were reared in the insectary of the Higher Teachers' Training College of the University of Yaounde I at 25-27°C and 75-78% relative humidity under photoperiod 12L: 12D. Larvae were fed with Tetramin Baby Fish Food at a rate of 2.5mg for 100 larvae per day (Price *et al*., 2015, Vantaux *et al*., 2016). The pupae were collected in plastic cups and placed in emergence cages. Adults from the pupae were fed using a 10% glucose solution. *Anopheles gambiae* S.L and *Culex quinquefasciatus* third and early four instars larvae and female aged of 2-5 days old were used to carry out bioassays.

Preparation of tests solutions

Base on literature, we have previously determined the discriminatory doses of temephos (WHO, 2016) and permethrin (WHO, 2013) causing less than 98% of larval and adult mortality respectively. Absolute alcohol has been used for a serial dilution of synthetic insecticides and essential oils according to the WHO Protocol, (2005). We then mixed the given concentrations of essential oils with constant concentrations (CL_{50}) of temephos (0.6%) and permethrin (0.375%) according to Pennetier Protocol (Pennetier *et al*., 2008) (table 1).

Table 1: Protocol of mixing essential oils with temephos and permethrin (synthetic insecticides)

Concentration of synthetic insecticide (%)	Essential oil concentration (%)	Volume of synthetic insecticide(ml)	Volume of essential oil(ml)	Final volume(ml)	Final concentration of essential oil (%)
Temephos	*C.sativa* L				
0.6	0.4	3	3	6	0.2
0.6	0.2	3	3	6	0.1
0.6	0.1	3	3	6	0.05
0.6	0.05	3	3	6	0.025
0.6	0.025	3	3	6	0.0125
0.6	0.0125	3	3	6	0.00625
0.6	0.00625	3	3	6	0.003125
0.6	0.003125	3	3	6	0.0015625
Temephos	*P.umbellatum*				
0.6	0.8	3	3	6	0.4
0.6	0.4	3	3	6	0.2
0.6	0.2	3	3	6	0.1
0.6	0.1	3	3	6	0.05
0.6	0.05	3	3	6	0.025
0.6	0.025	3	3	6	0.0125
0.6	0.0125	3	3	6	0.00625
0.6	0.00625	3	3	6	0.003125
0.6	0.003125	3	3	6	0.0015625
Permethrin	*C.sativa* L				
0.375	0.4	3	3	6	0.2
0.375	0.2	3	3	6	0.1
0.375	0.1	3	3	6	0.05
0.375	0.05	3	3	6	0.025
0.375	0.025	3	3	6	0.0125
permethrin	*P.umbellatum*				
0.375	0.4	3	3	6	0.2
0.375	0.2	3	3	6	0.1
0.375	0.1	3	3	6	0.05
0.375	0.05	3	3	6	0.025
0.375	0.025	3	3	6	0.0125

Temephos and permethrin provided by Al International industry (France)

Bioassays

Bioassay with larvae

The larvicidal potential of the essential oil mixture of each plant with temephos was evaluated on field mosquito larvae for 24 hours and 48 hours exposure period.

Larvae of third and early four instars were used to assess the larvicidal activity of the mixture of *Piper umbellatum* and *Cannabis sativa* L leaves essential oil with temephos, following WHO Guidelines for Laboratory and Field Testing of Mosquito Larvicides (WHO, 2005). Before running the test, larvae were maintained during one hour in distilled water for observation. Tests concentrations were

prepared by adding 1ml of appropriate concentration of the mixture to disposable test cups containing 99 ml of spring water. Batches of 25 larvae of third and early four instars were transferred from observation cups to test cups. Four replicates were run for each concentration and an equal number of controls were run as well. The positive control was prepared by adding 1ml of temephos (0.6%) to 99 ml of spring water. Maintaining the constant concentration of temephos(0.6%) for the mixture, eight different concentrations of *Cannabis sativa* L leaf essential oils were used (0.2%; 0.1%; 0.05%; 0.025%; 0.0125%; 0.00625%; 0.003125% and 0.00156%). For *Piper umbellatum* leaf essential oil, nine different concentrations (0.4%; 0.2%; 0.1%; 0.05%; 0.025%; 0.0125%; 0.00625%; 0.00325% and 0.00125%) were also used against the larvae of *Anopheles gambiae* S.L and *Culex quinquefasciatus*. Each test was conducted three times on different days. No food was added to cups during the exposure period. Larvae were considered dead, when they were incapable of any movement or not swimming actively when touch. The mortality rate was recorded after 24 and 48 hours of exposure. The lethal concentrations of essential oil killing 50% and 95% of exposed larvae (LC_{50} and LC_{95}) to the mixture were calculated using a log probit approach with STATISTICA software version 6.0.

Bioassay with adult

Adult bioassays were performed with 2-5 days- old non-blood- fed females following the WHO(2013) and CDC(2013) Guidelines with cone using impregnated bed net with the mixture at the following concentrations of essential oils: 0.0125%, 0.025%, 0.05%; 0.1% and 0.2% for *Anopheles gambiae* S.L and *Culex quiquefasciatus*. The concentration of permethrin was constant in the mixture (0.375%). Absolute alcohol was used to dilute the essential oil at the various concentrations before mixing and 3ml of each concentration was used to impregnate a portion of bed net (98.47cm^2). Due to the high volatility of essential oils, pieces of net prepared as indicated above were dried at room temperature away from sun light for 15 minutes before carrying susceptibility assays (Wanng *et al*., 2014, Faraone *et al*.,2015) [39]. Before each test, female mosquitoes were transferred from the cages to the cup for one hour and specimens with broken legs or unable to fly were discarded and replaced. Twenty replicates of batches of 5 female mosquitoes per cone were exposed to each concentration of the mixture for 1hour**.** Five replicates were run as control using a portion of net impregnated only with permethrin at 0.375%. Considering only the higher concentration of each essential oil, the number of knock down (KD) mosquitoes was recorded at 10 minutes intervals during 1 hour exposure period and the time required for knocking down 50% and 95% of the individuals(KDT_{50} and KDT_{95}) estimated with 95% of confident interval. After the exposure period, mosquitoes were transferred back to recovery cups and provided with 10% of glucose solution soaked on cotton pad. Mosquito mortality was recorded 24 hours post-exposure; individuals with broken legs or not able to fly were also considered as dead. After the tests, adult mosquitoes of *Anopheles gambiae* S.L from field population and *Culex quiquefasciarus* were kept in Eppendorf tubes at -4°C for identification.

Adult mosquito identification

Adults female of the field population of *Anopheles gambiae* S.L were morphologically identified using the Gillies and Coetzee key (1987) while *Culex quinquefasciatus* mosquito were identified using Peter Gupp (1996) and Reuben (1994) keys. Genomic DNA of *An.gambiae* S.L was extracted according to the Livak protocol (1984) and molecular identification of females was conducted according to Santolammazza *et al* protocol (2008). As for the adult females of *Culex quinquefasciatus*, their molecular identification was not possible because the TAG MANN device used did not allow it.

Data analysis for Bioassays

The lethal concentrations inducing 50% and 95% (LC_{50} and LC_{95}) larval mortality were calculated using log probit approach with WINDL CIRAD-CA software version 2.0 and STATISTICA software version 6.0 to plot the graphs. The relation between the exposure time, the mortality and the doses was assessed using probit regression.

For the adulticidal activity, the time at which 50% and 95% of adult mosquitoes were knocked down (Knock down time, KTD_{50} and KDT_{95}) and the LC_{50} and LC_{95} were calculated using WINDL CIRAD-CA software version 2.0. The relation between the knock down time, mortality and the doses were assessed using probit regression.

Results

Yield of *Piper umbellatum* and *Cannabis sativa* L Essential oil

The table 2 below provides characteristics of *Piper umbellatum* and *Cannabis sativa* L leaves

Table 2: The oil yield of *Piper umbellatum* and *Cannabis sativa* L

Plant name	Family	Certification number	Leaves weight(g)	Essential oil weight(g)	Extraction yield
Piper umbellatum	*Piperacea*	3614SRFK	7390.8g	0.9g	0.012%
Cannabis sativa L	*Cannabacea*	25967SRF/Cam	3,375g	1.5g	0.044%

The extraction yield of *Piper umbellatum* and *Cannabis sativa* L leaves essential oils were very low and therefore require a huge mass of plant to obtain a volume of essential oils sufficient for biological assays. A better method of extraction, the judicious choice of the period of harvesting and the control of some edaphic factors could allow a good yield.

Chemical composition of *Piper umbellatum* and *Cannabis sativa* L leaves essential oils

The chemical analysis of *Piper umbellatum* leaf essential oil revealed up to 24 different compounds comprising 89.60% of the essential oil. Major compounds were sesquiterpenes: copaene (9.63%) β-elemene (10.56%) and γ-murolene (11.37%). Unidentified components comprise 10.40 % of the essential oil.

Table 3: Chemical composition of *Piper umbellatum* leaf essential oil (Abé *et al*., 2019)

Elution order	Compounds	Percentage	Kovat retention index	
			Npo.	Po.
1.	α-pinene	6.23	931	1035
2.	camphene	1.83	944	1063
3.	β-pinene	1.83	971	1132
4.	β-myrcene	4.60	981	1161
5.	limonene	1.56	1022	1203
6.	Z-β-ocimene	1.33	1025	1242
7.	E-β-ocimene	2.63	1037	1247
8.	linalool	1.27	1086	1533
9.	L-borneol	2.45	1153	1719
10.	terpinen-4-ol	2.96	1163	1611
11.	p-menth-1-en-8-ol	5.32	1280	-
12.	copaene	9.63	1375	1491
13.	β-elemene	10.56	1386	1577
14.	caryophyllene	1.37	1419	1612
15.	α-caryophyllene	1.18	1453	-
16.	γ-muurolene	11.37	1473	1689
17.	germacrene D	3.92	1480	1708
18.	α-farnesene	1.90	1496	1744
19.	δ-cadinene	2.30	1513	1765
20.	calamenene	1.14	1532	-
21.	z-nerolidol	4.56	1550	2036
22.	caryophyllene oxyde	1.95	1567	2001
23.	α-eudesnol	1.6	1652	2227
24.	phytol	6.1	2116	2613
	Total	89.6%	-	-

Analysis performed by gas chromatography with two Perkin Elmer instruments, a Clarus 500 GC gas chromatograph coupled to an MS Clarus 500 mass spectrometer. Individual components were identified by their retention index as described in Adams (2012) and their mass spectra are interpreted using the WILEY L computer library. Constituents are presented in the order of elution from the columns. Npo: non polar; Po: polar

Among these 24 identified compounds of *P.umbellatum*, α-pinene; β-pinene; β-myrcene; linalool and α-caryophyllene were reported effective on various insects and pest.

The chemical analysis of *Cannabis sativa* L leaf essential oil revealed up to 81 components unlike that of *Piper umbellatum* (table 4). The essential oil of *Cannabis sativa* L is also composed of monoterpenes and sesquiterpenes, the most abundant of which were α-pinene, β-pinene, myrcene, E-β-Ocimene, terpinolene, E-β-Caryophyllene and α-Humulene.

Table 4: The chemical composition of *Cannabis sativa* L leaf essential oil (Abé *et al*., 2018)

Elution order	Compound	Percentage	Kovat indices	
			HP-1	Innowax
1.	Z-3-Hexenol	0.03	842	1380
2.	E-2--Hexenol	0.02	852	1408
3.	Heptanone-2	0.03	864	1182
4.	Hexenol	0.03	869	1351
5.	Heptanal	0.03	880	1185
6.	2-Amyl furan	0.01	884	1124
7.	α-Thujene	0.12	923	1032
8.	α-Pinene	8.12	931	1035
9.	Camphene	0.013	944	1063
10.	Octene-1-ol-3	0.03	961	1427
11.	6-Methyl-5-heptenone-2	0.005	964	1116
12.	Sabinene	0.12	967	1118
13.	β-Pinene	3.64	971	1132
14.	6- Methyl-5-hepten-2-ol	0.02	974	1163
15.	Myrcene	31.75	981	1159
16.	Ϫ-3-Carene	0.92	997	1011
17.	α-Phellandrene	0.42	998	1175
18.	α-Terpinene	0.31	1010	1188
19.	Hexylacetate	0.02	1010	1272
20.	P-Cymene	0.11	1014	1200
21.	Limonene	1.3	1022	1203
22.	1,8-Cineole+β-phellandrene	1.29	1022	1213
23.	Z-β-Ocimene	1.10	1025	1242
24.	E-β-Ocimene	9.65	1037	1247
25.	ϒ-Terpinene	0.23	1049	1255
26.	E-Thujan-4-ol	0.03	1053	1463

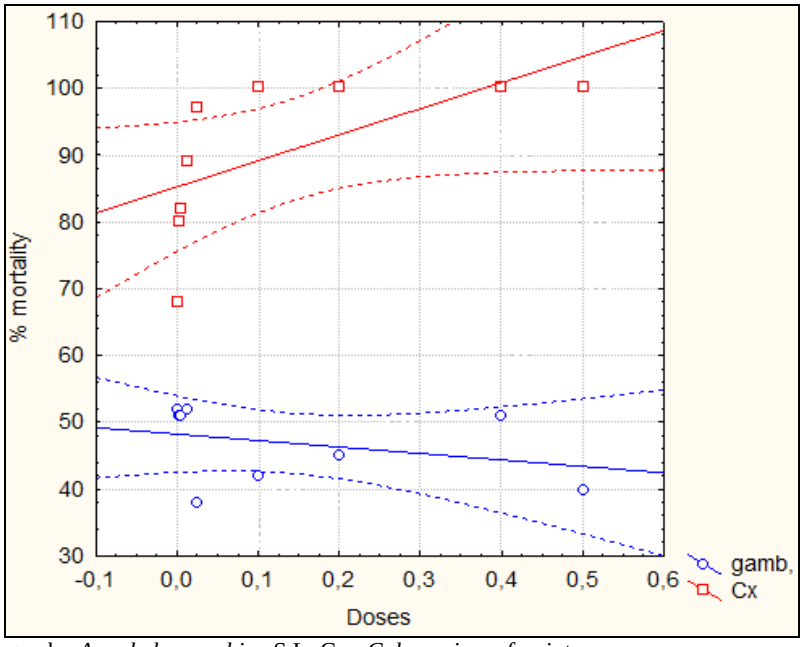
27.	Fenchone+methyl benzoate	0.02	1072	-
28.	p-Cymenene	0.09	1072	1437
29.	Terpinolene	14.76	1079	1290
30.	Nonanal	0.03	1083	1391
31.	Linalool	0.09	1086	1533
32.	Z-Thujan-4-ol	0.03	1092	-
33.	Perillen	0.02	1098	1429
34.	α-Fenchol	0.07	1100	1570
35.	Z-p- Menth-2-en-1-ol	0.03	1114	1570
36.	E-p- Menth-2-en-1-ol	0.07	1114	1571
37.	4E, 6Z- Allo-ocymene	0.01	1119	1375
38.	Ipsdienol	0.03	1125	-
39.	Epoxy terpinolene	0.05	1130	-
40.	Menthone	0.04	1136	1465
41.	Borneol	0.04	1153	1719
42.	P-Cymen-8-ol	0.12	1161	1864
43.	Terpinen-4-ol	0.07	1163	1611
44.	α-Terpineol	0.04	1175	1682
45.	Citronellol	0.02	1212	1764
46.	Hexyl butyrate +methylchavicol	0.06	1224	-
47.	E-Anethole	0.10	1264	1826
48.	α-Ylangene	0.20	1370	1490
49.	α-Copaene	0.01	1375	1491
50.	β-Elemene	0.02	1386	1577
51.	Isocaryophyllene	0.23	1408	-
52.	Z-α-Bergamotene	0.11	1410	1559
53.	α-Santalene	0.14	1415	1582
54.	E-β-Caryophyllene	10.72	1419	1612
55.	E-α-Bergamotene	0.85	1435	1575
56.	Allo-Aromadendrene	0.30	1439	1620
57.	α-Guaiene +unidentified compound	0.04	1442	-
58.	E-β-Farnesene	1.23	1449	1663
59.	ϒ-Elemene+unidentified compound	0.04	1449	-
60.	α-Humulene	3.28	1452	1654
61.	ϒ- Muurolene	0.08	1473	1689
62.	Selina-4,11-diene	0.22	1475	1674
63.	Selina-4,7(11)diene+E-α-Bisabolene	0.36	1475	1688
64.	β-Selinene	0.58	1480	1700
65.	α-Selinene	0.41	1491	1707
66.	E,E-α-Farnesene	0.20	1496	1744
67.	β-Bisabolene	0.21	1499	1727
68.	7-Epi-α-Selinene	0.21	1507	1764
69.	ϒ-Cadinene	0.05	1513	1740
70.	ϰ-Cadinene	0.09	1513	1765
71.	12-Nor-caryophyll-5-en-2-one	0.02	1515	-
72.	ϒ-Selinene	0.24	1526	-
73.	Germacrene B	0.14	1535	1823
74.	Selina-3,7(11)-diene	0.37	1537	1783
75.	E-Nerolidol	0.15	1550	2036
76.	Spathulenol	0.03	1565	2107
77.	Epoxy Caryophyllene	0.08	1563	-
78.	CaryophylleneOxide	1.25	1567	2001
79.	HumuleneOxide	0.30	1597	2047
80.	Not identified compound	0.14	-	-
81.	Not identified compound	0.18	-	-
	TOTAL	98.27		

Analysis performed by gas chromatography with FID detection on 2 columns of different polarity: HP1 and INNOWAX Individual components are identified by their retention index as described in Adams (2012) and their mass spectra are interpreted using the WILEY L computer library. Constituents are presented in the order of elution from the columns.

Larvicidal activity of the mixture of *Piper umbellatum* and *Cannabis sativa* L leaves essential oil with temephos

Larvicidal activity of the mixture of *Piper umbellatum* with temephos on *Anopheles gambiae s.l* and *Culex quinquefasciatus* in 24 hours

Anopheles gambiae S.L and *Culex quinquefasciatus* larvae were exposed to the mixture of *Piper umbellatum* leaf essential oil with temephos for 48 hours exposure period. Dead larvae were recorded after 24 hours and 48 hours.

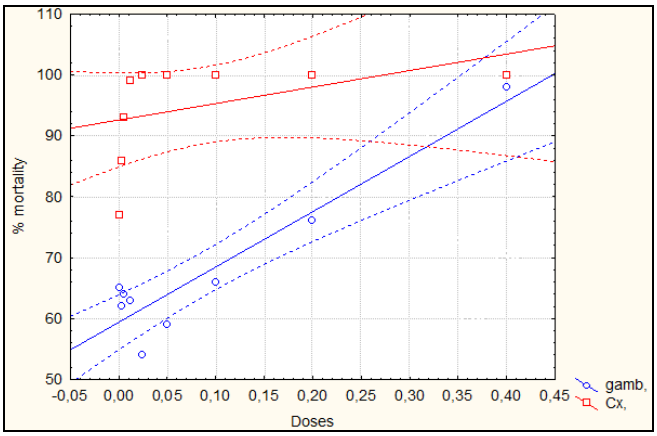


gamb.: *Anopheles gambiae* S.L; Cx.: *Culex quinquefasciatus*

Fig 1: Larvicidal activity of the mixture of *Piper umbellatum* leaf essential oil with temephos on *Anopheles gambiae* S.L and *Culex quinquefasciatus* after 24 h

The different regression line equations of the effect of the mixture of *Piper umbellatum* leaf essential oil with temephos on *Anopheles gambiae s.l* and *Culex quinquefasciatus* for 24 hours exposure are: Y_g=9.91+132.58X (H= 3.12; *p*= 0.014) and Y_{Cx}=54.42+2071.32X(H=23.2; *p*<0.05) respectively. Lethal concentrations for 50% and 95% mortality (LC_{50} and LC_{95}) are: 0.30%; 0. 0011% and 0.64%; 0.024% respectively for *Anopheles gambiae* S.L and *Culex quinquefasciatus*.

Larvicidal activity of *Piper umbellatum* mixture on *Anopheles gambiae* S.L and *Culex quinquefasciatus* in 48hours



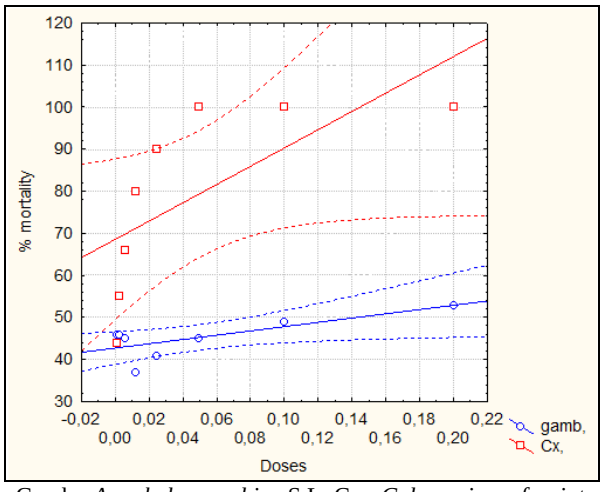
gamb.:*Anopheles gambiae* S.L; Cx.: *Culex quinquefasciatus*

Fig 2: Larvicidal activity of the mixture of *P.umbellatum* leaf essential oil with temephos on *Anopheles gambiae* S.L and *Culex quinquefasciatus* after 48 h

The observation was extended to 48 hours. The efficacy increases with the duration of the exposure and the concentrations of the essential oil. The regression line equations generated are: Y_g=89.39+90.75X (H=9; p=0.0008) and Y_{Cx}=73.75+1412.28X (H=10; p<0.05) respectively for *Anopheles gambiae* S.L and *Culex quinquefasciatus*. Thus, lethal concentrations for killing 50% and 95% of the exposed larvae (CL_{50}) after 48 hours are: 0.023% and 0.001% respectively for *Anopheles gambiae* S.L and for *Culex quinquefasciatus*. While the lethal concentrations for 95% (LC_{95}) mortality are: 0.38% and 0.015% respectively.

Larvicidal activity of *Cannabis sativa* L leaf essential oil mixture on *Anopheles gambiae* S.L and *Culex quinquefasciatus*

Larvicidal activity of *Cannabis sativa* L leaf essential oil mixture on *Anopheles gambiae* S.L and *Culex quinquefasciatus* in 24h



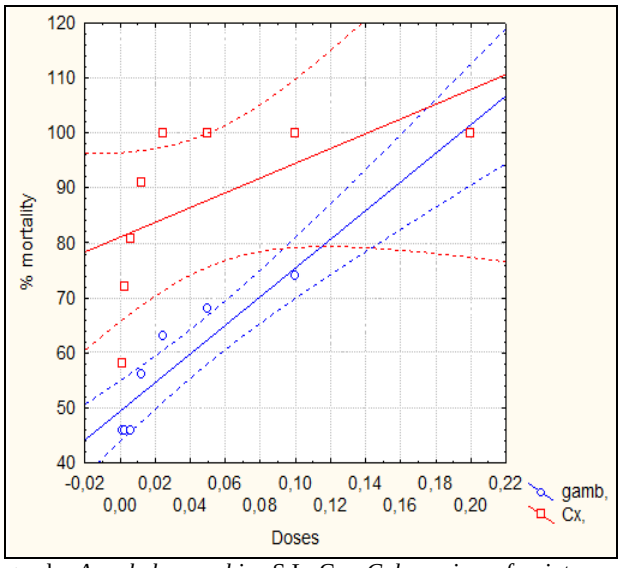
Gamb.: *Anopheles gambiae* S.L; Cx.: *Culex quinquefasciatus*

Fig 3: Larvicidal activity of the mixture of *Cannabis sativa* L leaf essential oil with temephos on *Anopheles gambiae* S.L and *Culex quinquefasciatus* after 24 h

The regression line equations for larvicidal acitivity of the mixture of *Cannabis sativa* L leaf essential oil with temphos generated are: Y_g=42.71+50.91X (H=3.34; p=0.012) and Y_{Cx}=68.55+217.30X (H=10.17; p<0.05) respectively for *Anopheles gambiae* S.L and *Culex quinquefasciatus*. Lethal

concentrations for 50% and 95% mortality of the larvae are: 0.14%; 0.002% and 0.35% and 0.026% respectively for *Anopheles gambiae s.l* and *Culex quinquefasciatus. Culex quinquefasciatus* larvae are more susceptible to the mixture of *Cannabis sativa* L leaf essential oil with temephos than those of *Anopheles gambiae* S.L.

Larvicidal activity of *Cannabis sativa* L leaf essential oil mixture with temephos on *Anopheles gambiae* S.L and *Culex quinquefasciatus* after 48h



gamb.: *Anopheles gambiae* S.L; Cx.: *Culex quinquefasciatus*

Fig 4: Larvicidal activity of the mixture of *C.sativa* L leaf essential oil with temephos on *An.gambiae* S.L and *Cx.quinquefasciatus* after 48h

Larvicidal activity of the mixture increases with dose and the duration of exposure. The regression line equations of the adjuvant effect of *Cannabis sativa* Leaf essential oil for effectiveness of temephos on the larvae of *Anopheles gambiae* S.L and *Culex quinquefasciatus* after 48 hours exposure are: Y_g=49.39+260.55X (H=3.2; p=0.014) and Y_{Cx}=81.06+134.27X (H=15.52; p<0.05) respectively for *Anopheles gambiae* S.L and *Culex quinquefasciatus*. The lethal concentrations for 50% and 95% mortality of the exposed larvae are: 0, 011%; 0.0013% and 0.17%; 0.013% respectively for *Anopheles gambiae* S.L and for *Culex quiquaefasciatus*.

Lethal concentrations of the mixture efficient after 48 hours exposure are tenfold lower than those necessary for 24 hours exposure, both for the larvae of *Anopheles gambiae* S.L and *Culex quinquefasciatus*.

Adjuvant property of *Cannabis sativa* L and *Piper umbellatum* leaves essential oil for effectiveness of permethrin on adult female of *Anopheles gambiae* S.Land *Culex quinquefasciatus*

Knock down activity of the mixture of *Piper umbellatum* leaf essential oil with permethrin on *Anopheles gambiae* S.L and *Culex quinquefasciatus*

According to WHO(20013) and CDC(2013) procedures, adult female of *Anopheles gambiae* S.L and *Culex quinquefasciatus* were exposed to the impregnated material for 1 hour; at 10 minutes interval, individuals feld on their back or on the side were recorded.

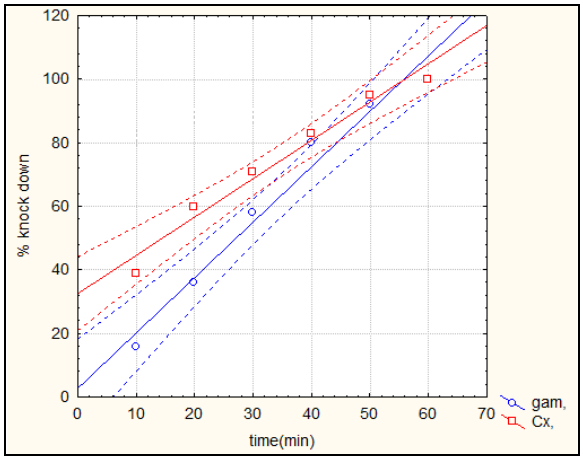


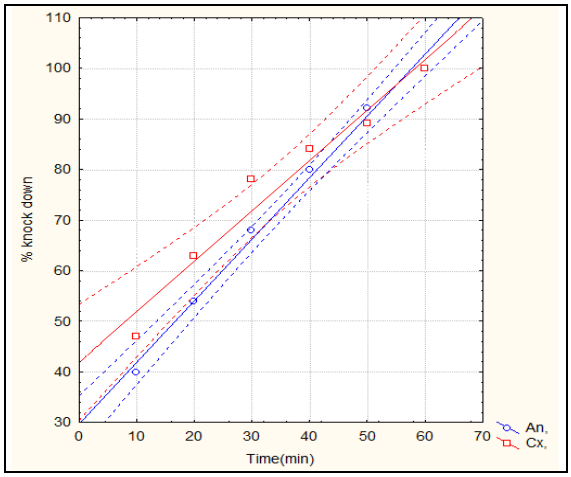
Fig 5: Knock down activity of the mixture of *Piper umbellatum* leaf essential oil with permethrin on *Anopheles gambiae* S.L and *Culex quinquefasciatus*

For 1 hour exposure, the mixture of *Piper umbellatum* leaf essential oil with permethrine knock down activity on adult female of mosquito was assessed with 95% confident interval. The regression equations generated for each mosquito are:

Yg= 2.66+1.74X (H=5.051; p=0.003) and Ycx= 32.46+1.20X (H=7.98; p=0.0005**)** respectively for *Anopheles gambiae* S.L and *Culex quinquefaciastus*. Time necessary for knocking down 50% and 95% (KDT_{50} and KDT_{95}) of exposed mosquito are: 27±0.4min; 14±0.8min and 53±0.6min; 52±0.11min respectively for *Anopheles gambiae* S.L and *Culex quinquefasciatus*. The females of *Culex quinquefasciatus* succumb more quickly to the toxic effect of the mixture compared to those of *Anopheles gambiae* S.L.

Knock down activity of the mixture of *Cannabis sativa* L leaf essential oil with permethrin on *Anopheles gambiae* S.L and *Culex quinquefasciatus*

Considering only the higher concentration of the mixture, adult female of *Anopheles gambiae* S.L and *Culex quinquefasciatus* were exposed to the mixture of *Cannabis sativa* L leaf essential oil with permethrin for 1 hour. At 10 minutes interval, individuals knocked down were recorded.



gamb.:*Anophles gambiae* S.L Cx.: *Culex quinquefasciatus*

Fig 6: Knock down activity of *Cannabis sativa* L leaf essential oil mixture with permethrin on adult female of *Anopheles gambiae* S.L and *Culex quinquefasciatus*

The regression line equations generated are: Yg=29. 73+1.21X (H=8.22; p=0.0004) and Y_{Cx}=41. 93+0.99X (H=9.52; p=0.0002) respectively for *Anopheles gambiae* S.L and *Culex quinquefasciatus*. Thus, time necessary for knocking down 50% and 95% of exposed mosquitoes are: 16±0.75min; 8±0.15min and 53±0.94min; 53±0.6 min respectively for *Anopheles gambiae* S.L and *Culex quinquefasciatus*. After one hour of contact no difference of susceptibility appears between the two mosquitoes species (H=1.88; p=0.82).

Adulticidal activity of the mixture of *Piper umbellatum* leaf essential oil with permethrin on *Anopheles gambiae* S.L and *Culex quinquefasciatus*

After the exposure, 24h late, dead mosquito were recorded and the curve below was plotted.

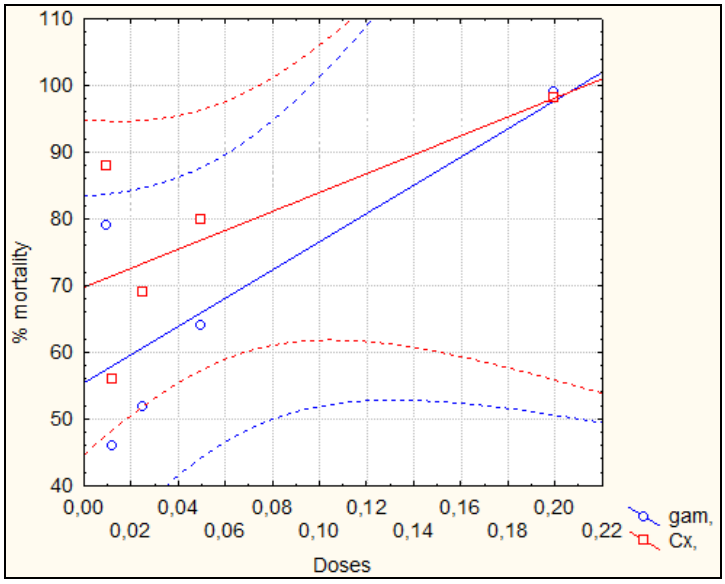
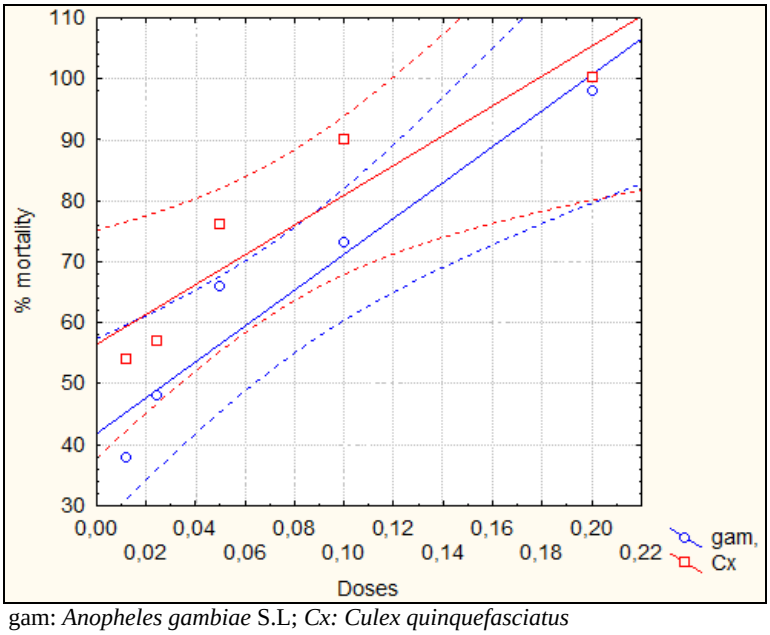


Fig 7: Adulticidal property of the mixture of *Piper umbellatum* leaf essential oil with permethrin on *Anopheles gambiae* S.L and *Culex quiquefasciatus*

The regression line equations generated are: Yg=55.40+211.64X (H=8.14; p=0.0012) and Y_{Cx}=69.76+141.76X (H=11.22; p=0.0003). From these equations, lethal doses for 50% and 95% mortality (LC_{50} and LC_{95}) are: 0. 024%; 0.011% and 0.18%; 0.17% respectively for *Anopheles gambiae* S.L and *Culex quinquefasciatus*. Adult of *Anopheles gambiae* S.L seem to be less susceptible to the toxicity of the mixture of permethrin with *Piper umbellatum* leaf essential oil than those of *Culex quinquefasciatus* (H=4; p=0.03).

Adulticidal activity of the mixture of *Cannabis sativa* L leaf essential oil with permethrin on *Anopheles gambiae* S.L and *Culex quinquefasciatus*

After the contact of adult mosquitoes with increasing concentrations of the mixture and 24 hours post exposure, dead individuals were counted and the curve below was plotted with 95% confident interval.



gam: *Anopheles gambiae* S.L; *Cx: Culex quinquefasciatus*

Fig 8: Adulticidal activity of the mixture of *Cannabis sativa* L leaf essential oil with permethrin on *Anopheles gambiae* S.L and *Culex quinqueafsciatus*

The different regression line equations of the activity of the mixture of permethrin and *Cannabis sativa* L leaf essential oil are: Y_{An}=41.79+294.3X(H=6.2; p=0.003) and Y_{Cx}=56.45+244.4X (H=8.4; p=0.001) respectively for *Anopheles gambiae* S.L and *Culex quinquefasciatus*. Lethal concentrations for 50% and 95% (LC_{50} and LC_{95}) mortality are: 0.027%; 0.01% and 0.18%; 0.15% respectively for *Anopheles agmbiae* S.L and *Culex quinquefasciatus*. Adult of *Anopheles gambiae* S.L seem to be less susceptible the toxicity of the mixture of *Cannabis sativa* L leaf essential oil than those of *Culex quinquefasciuatus* (H=3.9; p=0.016).

Identification of adult mosquitoes

Molecular analysis carried out with DNA extracted from 71 adult individuals of the *gambiae* complex collected near the Ewoé River and the morphmogical determination of individuals collected from Melen pond revealed the results given in the table 5 below.

Table 5: Specific composition of adult mosquitoes collected at Melen pond and on the banks of the Ewoé River (Abé *et al*., 2019)

Harvest site	gender	Species	Number	%
Melen Pond	*Anopheles*	*An. gambiae s.l*	4	2%
	Culex	*Cx.perfuscus*	14	7%
		Cx.quinquefasciatus	182	91%
	Total	-	200	100%
Ewoe river	*Anopheles*	*An. gambiae s.s*	3	4,23%
		An. coluzzii	68	95,77%
	Total	-	71	100%

Discussion

The aim of our study was to evaluate the effectiveness of the combination of *Piper umbellatum* and *Cannabis sativa* L leaves with temephos and permethrin on *Anopheles gambiae* S.L and *Culex quinquefascaitus*, two major vectors under Laboratory conditions.

The chemical analysis of essential oils revealed 81 compounds for *Cannabis sativa* L and 24 compounds for *Piper umbellatum*. These compounds constitute 98.27% and 89.6% of the mass of essential oil analysed (respectively for *Cannabis sativa* L and for *Piper umbellatum*) and are mostly terpenes. In addition, during this chemical analysis of essential oils, several compounds remained undetermined. More sophisticated equipment and software will allow their subsequent determination

Comparing the results of chemical analysis, there are more compounds in *Cannabis sativa* L than in *Piper umbellatum* leaf essential oil. This difference would depend on the specificity of each plant and also on the type of equipment used for the chemical analysis of essential oils. Terpenic compounds such as pinenes, terpinolene, 1-8 cineol found in these plants have been reported to have insecticidal, bactericidal, acaricidal, antiviral and fungicidal activity by severals authors (Brochot *et al*., 2017).

The morphological and molecular identification of adult mosquitoes from wild individuals of *Anopheles gambiae* S.L collected near Ewoe River revealed a composition of 95.77% of *Anopheles coluzzii* and 4.23% of *Anopheles gambiae* S.L; while the only morphological identification of adult mosquitoes from Melen's pond revealed: 2% of *Anopheles coluzzii*; 7% *Culex perfuscus* and 91% *Culex quinquefasciatus*. Thus, according to the CDC (2013), the results of biological assays carried out on mosquitoes from Ewoé River and Melen pond are attributed to *Anopheles coluzzii* (*Anopheles gambiae* S.L) and *Culex quinquefasciatus* respectively.

Regarding the adjuvant effect of the essential oil of the leaves of *Cannabis sativa* L for the effectiveness of temephos on the larvae of the wild strain of *Anopheles gambiae* S.L after 24 hours, the mortality rate (46 ± 0.2%) is almost constant between essential oil concentrations from 0.00156% to 0.0062% and similar to the larval mortality rate for temephos acting alone. But from a concentration of 0.0125% of *Cannabis sativa* L leaf essential oil in the mixture, there is a drop in larval mortality from 46 ± 0.2% to 37 ± 0.2%. Unlike the larvae of *Anopheles gambiae* S.L, this drop in larval motility in *Culex quinquefasciatus* (44 ± 0.4%) is immediately observed from a concentration of 0.00125% from Cannabis sativa L leaf essential oil in the mixture with temephos. Larvae mortality then increases for each of the species with the concentration of Cannabis sativa Lleaf essential oil in the mixture with teemphos. Likewise, mixing temephos with *Piper umbellatum* laef essential oil gives almost similar larval mortality rates for *Anopheles gambiae* S.L (52 ± 1.2%) between the essential oil concentrations of 0.00125% to 0.0125% in the mixture with temephos. But from a concentration of 0.025% of P.umbellatum leaf essential oil, there is a drop in larval mortality of the field strain of *Anopheles gambiae* S.L from 52 ± 1.2% to 38 ± 1%. Subsequently, the effectiveness of the combination against the larvae of the field strain of *Anopheles gambiae* S.L increases with the doses of *Piper umbellatum* leaf essential oil in the mixture with teemphos. For *Culex quinquefasciatus* larvae, the mortality rate (68 ± 0.7%) immediately increases with the concentration of essential oil in the mixture. It thus appears, an effect of partial neutralization of the toxicity of temephos by the essential oils of *Cannabis sativa* L and *Piper umbellatum* leaves essential oil.

As for the activity of mixing the essential oils each plant leaf, *Cannabis sativa* L and *Piper umbellatum* with permethrin on wild adults of *Anopheles gambiae* S.L and *Culex quinquefasciatus*, the comparison of the average knock down rates on the effect of the mixture of *Cannabis sativa* L leaf with permethrin does not show a significant difference between adults of the two species (H = 1.88; p = 0.12). For the permethrin-essential oil combination of *Piper umbellatum* leaf essential oil on the contrary, the comparison of the means of the Knock down rates of the two species shows a significant difference (H = 4; p = 0.042). *Culex quinquefasciatus* succumbing faster than *Anopheles gambiae* S.L Knock down although being a preliminary phase of mortality does not lead to mortality with the same proportions for the mixture permethrin-essential of *Cannabis sativa* L leaf. Thus, comparison of the average mortality rates of the two species shows that adults of *Culex quinquefasciatus* are more susceptible to the mixture than those of the field strain of *Anopheles gambiae* S.L Comparison of average mortality rates of adults of the field strain of *Anopheles gambiae* S.L and of *Culex quinquefasciatus* shows that the individuals of *Culex quinquefasciatus* are more sensitive as well for the mixture permetrin-essential oil of *Cannabis sativa* L leaf (H = 3.9; p = 0.016) than for the permethrin – essential oil mixture of *Piper umbellatum* leaf (H = 4; p = 0.03). This behavior would be linked to the specificity of each of the two species. There is also a drop in the mortality rate of adults of the field strain of *Anopheles gambiae* S.L compared to the

control in the lowest concentrations of essential oil in the permethrin-essential oil mixtures. These rates thus go from 49 ± 0.5% for permethrin acting alone to 38 ± 0.6% in the mixture of permepthine with 0.0125% from *Cannabis sativa* L leaf essential oil and from 51 ± 0, 7% for permethrin acting alone to 46 ± 1% in the mixture of permethrin with a concentration of 0.0125% from *Piper umbellatum* leaf essential oil. This drop in the mortality rate, on the contrary, is not observed in *Culex quinquefasciatus*; their mortality rate immediately increases with the concentrations of essential oil in the mixture.

Indeed, whether for the larvicidal or adulticidal activity of mixtures of synthetic insecticides with essential oils on *Anopheles gambiae* S.L and *Culex quinquefasciatus*, there appears to be a partial attenuation of the toxicity of synthetic insecticides used in this work (temephos and permethrin). This partial neutralization or attenuation of the toxicity of temephos and permethrin by *Cannabis sativa* L and of *Piper umbellatum* leaves essential oil could be due to a blocking or partial inhibition of the active principles of temephos and permethrin. This partial antagonism has been observed by some authors when combining linalol-thymol with spirotetramat against *Myzus persicae* (Faraone *et al*., 2015) [39] or when combining several essential oils with permethrin against adults of *Aedes aegypti* and of *Anopheles gambiae* (Gross *et al*., 2017) [46]. This activity of partial inhibition of some of the active ingredients of temephos and permethrin without permanently altering their effectiveness, would offer an advantage in the fight against vectors and other harmful insects. Because the impact of reference insecticides on non-target organisms could thus be limited. In addition, the rebound effect observed for the activity of temephos and permethrin thereafter with increasing doses of essential oils would allow their effectiveness to be improved compared to resistant insects. The work carried out by several authors to assess the effectiveness of the combination of essential oils with reference insecticides has shown the effectiveness of the essential oil-permethrin combinations against adults of *Aedes aegypti* (Chansang *et al*., 2018) [47] and flies domestic (Joffe *et al*., 2011) [48]. In the work of these authors, by mixing with essential oils, the concentrations of synthetic insecticides are reduced while preserving their effectiveness due to the low concentrations of essential oils. This is the case in this work; the essential oils from *Cannabis sativa* L and *Piper umbellatum* leaves increase the effectiveness of temephos and permethrin, just like studies carried out in Ghana on a strain of *Anopheles gambie* S.L resistant to deltametrin and permethrin (Dadzie *et al*., 2017) [49] and in Iran on the effectiveness of the combination of the essential oils of *Eucalyptus globulus* and *Rosmarinus officinalis* on cockroaches, *Culex pipiens*, *Anopheles stephensi* and house flies (Zibaee and Khorram;, 2015) [50].

Conclusion

From this study, it appears that low concentrations of essential oil from *Cannabis sativa* L and *Piper umbellatum* partially inhibits the effectiveness of temephos and permethrin on adult and larvae of *Anopheles gambiae* S.L and *Culex quinquefasciatus*. The effectiveness of the mixture then increases with the concentrations of essential oil.

To properly establish the effectiveness of the mixture of *Cannabis sativa* L and *Piper umbellatum* leaves essential oil with synthetic insecticides on *Anopheles gambiae* S.L and *Culex quinquefasciatus*, trials must be carried out in the field. More appropriate apparatus and methods will allow an improvement in the extraction yields of essential oils and the identification of the compounds which have hitherto been unknown. Other in-depth studies may explore how to resolve the problem of the extreme volatility of essential oils; determine the essential oil responsible for the partial inhibition of the toxicity of temephos and permethrin and the mechanisms involved. The exploration of the adjuvant property of local plants essential oil must continue, in view of the fact that the combination of essential oil with synthetic insecticides would reduce their hazardous effect to non-target organisms and enhance their effectiveness against resistant insects.

Authors Declaration

The authors declare no competing interests.

Authors' participation

All authors participated equality in this work.

Acknowledgments

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