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Biological activities of four essential oils against *Anopheles gambiae* in Burkina Faso and their *in vitro* inhibition of acetylcholinesterase

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ABSTRACT

The control of malaria is still a challenge partly due to mosquito's resistance to current available insecticides. The aim of this work was to evaluate the ovicidal, larvicidal and repellent activities of *Lantana camara*, *Hyptis suaveolens*, *Hyptis spicigera* and *Ocimum canum* essential oils against *Anopheles gambiae* s.l. according to the World Health Organization standard method. The *in vitro* acetylcholinesterase inhibition activity of these oils was also evaluated. The repellent effect using the method of "separated arms" was evaluated. *Lantana camara* oil was more effective on both eggs and larvae. The LD_{50} and LD_{90} values observed in this oil solution were respectively 53.59 and 170.89 ppm on eggs whereas LD_{50} and LD_{90} were 61 and 125 ppm respectively on larvae. All oils exhibited repellent activities against adult mosquitoes. The most effective repellent was the oil of *Hyptis suaveolens* with a 50% efficacy dose value of 67 ppm. The highest acetylcholinesterase inhibitory activity was observed with *O. canum* and *H. suaveolens* essential oils which IC_{50} was 0.21 and 0.55 µg/ml respectively. Results suggest that these essential oils have a potential for vector control and can be considered as a source of natural and ecofriendly substances for malaria vector control.

Keywords: *Anopheles gambiae* s.l., malaria, essential oil, acetylcholinesterase, insecticidal activity.

INTRODUCTION

Malaria remains a public health problem killing mostly pregnant women and under five years old children. It was estimated to 207 millions of cases with 627 000 deaths the burden of malaria in 2012 (WHO, 2010; WHO, 2013). Burkina Faso is a Malaria endemic country with a total of 3.7 million malaria cases in all age population, 4,264 deaths with 4,438 within under five years old children in 2011 (DGISS, 2012).

Insecticide-treated nets (ITNs) and indoor residual spraying with insecticide (IRS) are the main vector control methods recommended by the World Health Organization for malaria control (WHO,

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2008). The use of ITNs has yielded interesting results, including a reduction in malaria morbidity and mortality correlated with a reduction in vector biting and parasite inoculation rates (Binka et al., 1996; Habluetzel et al., 1997; Ilboudo-Sanogo et al., 2001).

The adverse environmental effects from chemical insecticides, the spread of resistance to insecticide in the main malaria vector species could jeopardize these control tools.

Anopheles gambiae is becoming more and more resistant to synthetic insecticides (Badolo et al., 2012; Toe et al., 2014).

In Burkina Faso, recent studies showed that *Anopheles gambiae* has developed resistance to all the four classes of insecticides (carbamate, pyrethroid, organophosphates, organochlorines) currently available for public health purpose (Dabiré et al., 2009; Ranson et al., 2009; Djogbénou et al., 2009) and the resistance has increased over time and space (Badolo et al., 2012). It has been demonstrated a loss of indoors residual spraying efficacy in highest resistance areas in Equatorial Guinea (Sharp et al., 2007). In Benin, N'guessan et al. (2007) have reported a loss of bednets efficacy in area of high resistance to pyrethroid. The prospection of new compounds as alternative to chemical insecticides is urgently needed.

The potential of plants as insecticide, repellent or fumigant sources against mosquitoes and other pest and vectors insects is well known (Lucia et al., 2012; Regnault-Roger et al., 2012; Dias et al., 2014). Several ethnobotanical studies in East and West Africa have shown that human populations use local plants, mostly aromatic plants, to protect themselves against mosquitoes bites (Palsson and Jaeson, 1999; Seyoum et al., 2002).

It has been shown that the essential oils of several plants like *Ocimum spp*.(Bassole et al., 2003), *Cymbopogon spp*. (Nonviho et al., 2010), *Lantana camara* (Seyoum et al., 2002), have significant insecticidal and repellent properties against mosquitoes. In addition, it has been demonstrated that *Cyperus giganteus* and *Cyperus rotundus, Mentha piperita*, *Ocimum basilicum*, *Rosemarinus officinalis*, *Cymbopogon nardus*, and *Apium graveolens*, *Ocimum canum*, *Lippia multiflora* have ovicidal properties against mosquitoes (Vivek et al., 2008; Warikoo et al., 2011). Previous studies have also reported the effectiveness of essential oils against mosquito larvae (Tchoumbougnang et al., 2009; Madhu et al., 2010; Kweka et al., 2011; Kalaivani et al., 2012). Through their odour and their composition, essential oils have repellent properties against mosquitoes (Nerio et al., 2010). Some authors have found that essential oils (EOs) of *Ocimum forskolei*, *Ocimum fisheri* (Odalo et al., 2005) and *Lippia ukambensis* (Omolo et al., 2004) exert a strong repulsion on *Anopheles gambiae*. Other authors have found that *Eucalyptus camandulensis*, *Ocimum basilicum* EOs are effective repellent against *Culex pipiens* (Erler et al., 2006) a highly ubiquitous mosquito.

The mode of action of essential oils on mosquito larvae and adults is not well known. However, Nyamador et al. (2010) has previously shown that EO of *Cymbopogon gyganteus* inhibits the activity of Glutathion s-transferase and acetylcholinesterase of *Callosobruchus subinnotatus*.

In this study, we have investigated the ovicidal, larvicidal and repellent properties of EOs of four aromatic plants (*Lantana camara*, *Hyptis suaveolens*, *Hyptis spicigera and ocimum canum*) collected in Burkina Faso against *Anopheles gambiae* s.l. We have also assessed their *in vitro* inhibition of acetylcholinesterase.

MATERIALS AND METHODS

Plant material

Young branches with leaves of *Hyptis suaveolens* Poit. (Lamiaceae) and *Hyptis spicigera* Lam. (Lamiaceae*)* were collected in October 2008 at Gampela (20 Km, East of Ouagadougou, Burkina Faso). Fresh leaves of *Ocimum canum* Sims. (Lamiaceae) and *Lantana camara* L. (Verbenaceae) were collected in August 2008 at Kamboinsé (15 Km, north of Ouagadougou, Burkina Faso).

Taxonomic identification of plant materials was confirmed by Doctor Amadé Ouedraogo of "Laboratoire de Biologie et d'Ecologie Végétales" (University of Ouagadougou, Burkina Faso) where voucher specimens (OUA) have been deposited.

Mosquitoes rearing

The laboratory strain of *Anopheles gambiae s.l*, named "Goden" has been used for all experiments. This strain is composed of *Anopheles gambiae* s.s. and *Anopheles coluzzii*. Mosquitoes were reared in the insectary of "Centre National de Recherche et de Formation sur le Paludisme" (CNRFP). Female mosquitoes were fed on blood meal of a male rabbit and allowed to oviposit on a filter paper. This paper was then placed in plastic jar containing water. The jar was covered with a mesh screen to prevent the escaping of emerging adult mosquitoes. After egg hatching, the larvae were fed with Tetramin and adults were provided with 10% sucrose solution. Mosquitoes were kept at 27±2 °C, 70±10% relative humidity, with a 12-hours light and 12-hours dark photo period.

Extraction of essential oils

Plant materials were dried at room temperature. Dried materials were subjected to hydro distillation for 3 h using a Clevenger-type apparatus. The obtained essential oils were dried over anhydrous sodium sulphate and stored in dark glass bottle at 4 °C until used. The EOs was light yellow and the yields of oils were 0.76, 0.23, 0.27, and 0.26% for *O. canum*, *H. suaveolens*, *L. camara* and *H. spicigera* respectively.

***In vitro* inhibition of acetylcholinesterase**

The inhibition effect of essential oils on acetylcholinesterase activity was evaluated using adaptation of the spectrophotometric method of Ellman as described by Rhee et al.(2001). Assays were performed on a 96-well microtiter plate microplate spectrophotometer (Epoch, BioTeck instruments). Briefly, 25 µl of essential oil (0.1% in 50 mM Tris-HCl, pH 8 buffer, 10% methanol) was mixed with 25 µl of AChE (acetylcholinestérase) (0.22 U/ml in 50 mM Tris-HCl, pH 8 buffer, 0.1% BSA) and 50 µl of buffer (50 mM Tris-HCl, pH 8, 0.1% BSA). The mixture was incubated for 5 min at 30 °C in a 96-well microtiter plate. Subsequently, 125 µl of DTNB (3 mM in Tris-HCl, pH 8 buffer, 0.1 M NaCl, 0.02 M MgCl2) and 25 µl of ATCI (15 mM in water) were added. A blank was also prepared by replacing AChE with 25 µl of buffer (50 mM Tris-HCl, pH 8 buffer, 0.1% BSA). The reaction was monitored for 5 min at 412 nm and velocity (V_0) recorded. Buffer (0.1 % in 50 mM Tris-HCl, pH 8, 10% methanol) was used as negative control. Antiacetylcholinesterase activity (I %) was calculated using the formula:

$$I(\%) = \frac{V_{0\ Control} - V_{0\ Sample}}{V_{0\ Control}} \times 100$$

$V_{0\ Sample}$ and $V_{0\ Control}$ represent the initial velocities of samples and control respectively. IC_{50} values were obtained through Log-Probit plotting. Galanthamine HBr was used as positive control.

***In vivo* bioassays on mosquitoes**

Preparation of essential oils solutions

Solutions of essential oils were prepared using serial dilution (v/v) of essential oils in absolute ethanol. Biological activity of essential oils on eggs and larvae were evaluated at 20, 40, 50, 100, 150, 200 and 300 ppm to produce a range of mortality from 10% to 100%. From the stock solution (1000 ppm), these concentrations were prepared with dechlorinated tap water for bioassays on eggs and larvae.

Ovicidal activity

The method recommended by WHO guideline (WHO, 1996), slightly modified was used to determine the effect of essential oils on eggs viability. Twenty (20) eggs carefully selected according to their entirety from a paper where mosquitoes have previously

oviposited were introduced into plastic cups containing 49 ml of distilled water supplemented with 1ml of diluted Eos after pipetting for homogenisation. Seven final concentrations comprised between 20 and 300 ppm (part per million) were used for the ovicidal bioassay. The control consisted of distilled water (49 ml) and pure ethanol (1ml). Each experiment was eight times replicated. Eggs were incubated (27 ± 2 °C, 70 ± 10% relative humidity and 12 h photoperiod) for 48 h. Hatched larvae were counted in each plastic cups and the percentage of hatchability calculated as a ratio of the number of hatched larvae to the number of eggs used.

Larvicidal activity

Larvicidal effect of essential oils was evaluated according to WHO guideline (WHO, 1996). Twenty (20) fourth instars larvae were introduced in a Plastic cup containing distilled water which volume is known. After 30 minutes observation, stock solution was added to have appropriate concentration. The final volume was 250 ml. Eight replicates were tested for each concentration; Distilled water (249 ml) and pure ethanol (1 ml) were used as negative control. Dead and moribund larvae were counted in each plastic cup 24 h after treatment. Mortality rate was assessed as the ratio of the number of dead and moribund larvae to the total number of larvae used.

Repellent activity

One hundred (100) females of *Anopheles gambiae* of 3 to 5 days old obtained from the laboratory strain "Goden" have been used for the experiment. Mosquitoes were starved for five hours before the bioassay. The tests were performed in the insectarium where humidity was 70 ±10% and temperature 27±2 °C. The method of the "separated arms" of Curtis et al. (1987) modified by Badolo et al. (2004) was used. A Man of 35 years old was used as volunteer after having signed an informed consent. Five concentrations (10, 50, 100, 500 and 1000 ppm) of essential oils were tested. Five (5) ml of each concentration tested were separately applied on one forearm of the volunteer. The second forearm was treated with ethanol as control. The test started with the lowest concentration and the forearm cleaned before the next concentration was applied to reduce the contamination effect. After all the concentrations were tested, the control forearm was tested again using ethanol only. During the tests the volunteer's hands were protected with gloves to avoid mosquito bites on that part.

The number of mosquitoes landing on the forearm of the volunteer were counted and recorded. Exposition time was 30 seconds. This time has been kept to reduce the risks of being bitten by the mosquitoes.

Statistical analysis

Bioassay data were analysed using XLstat software with a significant level of 0.05. All the data were corrected using Abbott (Abbott, 1925) formula when the mortality or inhibition rate in the control was higher than 10%.

A Log-probit model was also fitted to the data from EOs repellent effect to calculate the 50% efficacy dose (ED_{50}) and 90% efficacy dose (ED_{90}), which are defined as the doses to repel 50% and 90% mosquitoes respectively. The confidence limits of these efficacy doses were also determined. A log-probit model was fitted to the data recorded in ovicidal and larvicidal bioassay to assess the 50% lethal dose (LD_{50}), 90% lethal dose (LD_{90}) and their 95% confidence limits.

In the acetylcholinesterase inhibition assay, IC_{50} (Concentration inhibiting 50% of enzymatic activity) values were obtained through Log-Probit plotting with Table curve 2.5 software. IC_{50} were separated by Turkey test. Only probability level $P<0.05$ was used for the significance of differences between IC_{50}, to simplify statistical analysis.

RESULTS

Ovicidal effects

In total 140 eggs were used to test each EO concentration for 20 eggs per test and 7 repetitions. All essential oils exhibited ovicidal activity on eggs of *Anopheles gambiae* with a slope statistically different from zero (P<0.0001) (Table 1). The LD_{50} values range from 41.11 to 81.72 ppm and LD_{90} range from 152.19 ppm to 965 ppm according to plants. The EOs of *H. spicigera* and *L. camara* were most effective against eggs with LD_{50} values of 69.61 ppm (95% Confidence Limits: 42.6 - 47.77) and 53.59 ppm (95% CL: 58.79-64.77) respectively. Their LD_{90} values were respectively 170.89 ppm (95% CL: 152.19-195.76) for *Lantana camara* versus 393.71 ppm (95% CL: 321.19-507.24) for *H. spicigera.*

Larvicidal effects

The results of efficacy of plants' essential oils against *An. gambiae* larvae are summarized in Table 2. A total of 160 larvae were submitted to each EO concentration for 20 larvae per test and 8 repetitions. The slope of the regression of larvae mortality over EOs concentrations was statistically significant (Chi^2>328; P<0.0001). The LD_{50} values range from 45.18 ppm (95% CL: 42.6 - 47.77) for *H. spicigera* to 159.48 (95% CL: 146.41-175.41) for *H. suaveolens*. The 90% lethal concentration values range from 125.32 ppm (95% CL: 116.52 - 136.13) for *L. camara* to 575.85 ppm (CL: 478.88 - 720.73) for *H. suaveolens*. EOs of *L. camara* was the most effective against *An. gambiae* larvae *and H. suaveolens* the least effective regarding LD_{50} and LD_{90} values (Table 2).

Repellent effects

All of the EOs tested exhibited repellent activity against adult *An. gambiae* mosquitoes (Table 3). The slope of the logistic regression of number of mosquitoes repelled by the EOs was statistically different from 0 (Chi-2 >29, P<0.0001). The highest repellent activity was observed with *H. suaveolens* with ED_{50} and ED_{90} values of 67.90 ppm (95% CL: 34.86 – 108.9) *and* 666.70 ppm (95% CL: 455.6 – 1029.81) respectively. The lowest repellent activity was observed with *H. spicigera* with ED_{50} and ED_{90} values of respectively 225.96 ppm (95% CL: 56 – 390.93) and 2655 ppm (CL: 1572.02-9808.07).

Acetylcholinesterase inhibition

All tested EOs inhibited acetylcholinesterase activity (Table 4). The highest inhibition was observed with *O. canum and H. suaveolens* EOs with IC_{50} values of 0.21 and 0.55 µg/ml respectively. The activity of these EOs was followed by this of *L. camara* EO with IC_{50} value of 1.75 µg/ml. There was no significant difference between *H. suaveolens* and *O. canum* inhibition activities (p>0.05). The lowest inhibition was observed with EO of *H. spicigera* (IC_{50} = 6.14 µg/ml).

Table 1: Ovicidal activity of essential oils against *Anopheles gambiae* expressed as 50% and 90% lethal doses (LD_{50} and LD_{90}) and their 95% confidence limits.

	Plants			
Regression parameters	***O. canum***	***L. camara***	***H. spicigera***	***H. suaveolens***
Slope	1. 33±0.2	2.54±0.26	1.70±0.21	1.47±0.2
P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Chi^2	161.80	359.68	237.18	190 .80
LD_{50}	71.56	53.59	69.61	61.71
95%CL	(62.14- 81.72)	(41.11-58.18)	(62.10-77.55)	(53.83-69.90)
LD_{90}	648.56	170.89	393.71	456.80
95%CL	(481.17-965)	(152.19-195.76)	(321.19-507.24)	(358.6-624.32)

LD_{50}; LD_{90} = 50% and 90% lethal dose expressed in ppm; CL95%= 95% confident limit.

Table 2: Larvicidal activity (LD_{50} and LD_{90}) of essential oils against *Anopheles gambiae* expressed as 50% and 90% lethal doses and their 95% confidence limits (95%CL).

Regression parameters	Plants			
	O. canum	***L. camara***	***H. spicigera***	***H. suaveolens***
Slope	4.89 ± 0.27	4.16 ± 0.17	2.83 ± 0.11	2.29 ± 0.12
P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Chi^2	328.03	573.54	567.97	350.60
LD_{50} (95% CL)	138.66 (131.83 - 146.18)	61.69 (58.79-64.77)	45.18 (42.6 - 47.77)	159.48 (146.41-175.41)
LD_{90} (95%CL)	253.40 (233.10 - 280.06)	125.32 (116.52 - 136.13)	127.92 (117.77 - 140.55)	575.85 (478.88 - 720.73)

LD_{50}; LD_{90} = 50% and 90% lethal dose expressed in ppm; CL95%= 95% confident limit

Table 3: Repellent activity of essential oils against *Anopheles gambiae* expressed as 50% and 90% effective doses (ED_{50} and ED_{90}) and their 95% confidence limits (95%CL).

Regression parameters	Plants			
	O. canum	***L. camara***	***H. spicigera***	***H. suaveolens***
Slope	1.37±0.22	0.74±0.13	1.41±0.36	1.29±0.36
P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Chi^2	38.20	29.80	15.46	71.84
ED_{50}	70.78	192.00	225.96	67.90
(95%CL)	(26.34 – 124.45)	(45 – 456.97)	(56 – 390.93)	(34.86 – 108.9)
ED_{90}	696.43	9964.20	2655.00	666.70
(95%CL)	(479.32 – 1103.7)	(4183.34-42618.75)	(1572.02-9808.07)	(455.6 – 1029.81)

ED_{50}; ED_{90} = 50% and 90% lethal dose expressed in ppm; CL95%= 95% confident limit

Table 4: Antiacetylcholinesterase inhibitory activities (IC_{50}) of essential oils.

Plants	IC_{50} (µg/ml)
O. americanum	0.21 ± 0.01^a
H. suaveolens	0.55 ± 0.12^a
L. camara	1.75 ± 0.12^b
H. spicigera	6.3 ± 0.43^c
Galanthamine HBr	0.19 ± 0.02^a

Values were expressed as mean ± SD of three replicates, IC50 expressed the concentration of essential oil (µg/ml) inhibiting 50% of acetylcholinesterase activity; Data from the IC50, marked with the same letter do not shown statistical differences by Turkey test (p>0.05).

DISCUSSION

Plants are known to be rich in biocide components, which can be used to develop products for vector control (Mittal, 2003). The main objective of this study was to evaluate the insecticidal and repellent properties of EOs extracted from four local plants and secondarily to test their inhibiting activity of acetylcholinesterase (AChE). Our results showed that the EOs of *L. Camara, H. spicigera, H. suaveolens and O. canum* had significant ovicidal, larvicidal and repellent properties against *An. gambiae* s.l., the main vector of malaria in West Africa (Muriu et al., 2013).

Previous studies with the *O. canum* oil on *An. gambiae* showed their ovicidal and larvicidal properties (Bassole et al., 2003). The hatchability rate decrease when the EO concentration increases and we observed the maximum in the control trays. The potential of ovicidal activity in our study with the four EOs was similar to the results obtained by Warikoo et al. (2011) and to those of Vivek et al. (2008). The oils of *H. spicigera* and *L. camara* are the most active against the eggs of *An. gambiae*. This could be an object of further investigation seeing that it is the first report of the ovicidal activity of *H. spicigera, L. camara and H. suaveolens* on *An. gambiae.*

All the EOs have shown a larvicidal activity increasing from the lowest to the highest concentrations. The EO of *H. spicigera* exhibited a strong larvicidal activity followed by Lantana *camara*. The EOs of *O. canum* and *H. suaveolens* presented an interesting larvicidal activity but less active than the two previous ones. These results are similar to those of Kweka et al. (2011) who also evaluated the larvicidal activity of the EOs of *Schinus terebinthifolia on An. gambiae s.s.* The Biological activity of EOs observed in our study could be linked to active ingredients such as hydrocarbons terpenoids, as demonstrated using *Ocimum sp.* Essential oil (Ntonga et al., 2012; Dias et al., 2014).

The EOs of the plants of this study were active against the larvae of *An. gambiae s.l.* These plants could play a key role in the search of new plant-based pesticides to control the aquatic stages of *An. gambiae s. l.*

The EOs of our study were also evaluated for their repellent activity. Two of the plants with the strongest odor exhibited the most repellent activities against *An. gambiae* adults. It mainly concerns *O. canum* and *H. suaveolens*, which are more repellent than *L. camara* and *H. spicigera*. Abagli et al. (2012) found that 10% *H. suaveolens* EO induced also the maximal repellency rate in the field.

These EOs we studied have shown an *in vitro* inhibition of AChE. *O. canum* and *H. suaveolens* have been the most active oil against this enzyme. These results are in accordance with those of Ferreira et al. (2006) who evaluated the inhibition activity of EOs of some medicinal plants on AChE . Several studies have shown that EOs inhibited the activities of AChE (Lopez et al., 2010; Nyamador et al., 2010) . This indicates the presence of some components in the EOs which have inhibited properties of AChE (Kiendrebeogo et al., 2011; Regnault-Roger et al., 2012). These observations could be explained by the insecticidal activity observed on the larvae because of their neurotoxic activity (Huignard et al., 2008) The repellent and toxic activities could be explained by the inhibition of other types of neuromodulator enzymes.

The quality and the activities of the EOs depend on the part of the plant used, the weather and geographical conditions, the harvest season and the method used for extraction of the EOs (Tawatsin et al., 2006). Many parameters may influence the final activity of EOs. Based on our findings, we can say as Shaalan et al. (2005) that botanical insecticides may serve as suitable stand alone alternatives to synthetic insecticides in future as they are relatively safe, degradable and are readily available in many areas of the world.

Conclusion

The repellent and insecticidal properties of local plants can play an important role in malaria vectors control. The Eos tested have shown high insecticidal and ovicidal properties and strong repellency against malaria vector and then, may be promoted. Further evaluations are ongoing for determination of compounds of these EOs and residual effect on substratum.

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