Journal of Scientific Research and Studies Vol. 4(11), pp. 333-339, November, 2017
ISSN 2375-8791

http://www.modernrespub.org/jsrs/index.htm

Full Length Research Paper

Susceptibility of aquatic stages of *Anopheles gambiae* sensu lato to *Capsicum annuum* (red pepper) fresh fruits essential oil

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Accepted 16 November, 2017

The anopheline resistance to synthetic pesticides has led to the use of plant biocides as alternative biological and natural control strategies. The red variety of *Capsicum annuum* (Solanaceae), locally used to fight against insects was tested in this study for its insecticidal effect against aquatic stages of *Anopheles gambiae* s.l. mosquitoes. Fresh ripe fruits were crushed, and essential oil was extracted by hydro distillation in Clevenger-type apparatus. Seven working concentrations (0, 10, 20, 30, 40, 50, and 100 ppm) were tested against eggs, larval and pupal stages of *An. Gambiae* s.l. Overall, the hatching rates were low and decreased with the oil concentrations with median inhibitory concentration (IC_{50}) of 13.735 ppm after 24 h exposure to *C. annuum* fresh fruits essential oil. This oil revealed high larvicidal effect against third instars larvae (L3) of *An. Gambiae* s.l. with median lethal concentration (LC_{50}) of 26.181 ppm and 19.643 ppm, respectively after 30 min and 1 h exposure. A pupicidal effect was also observed against pupal stages of *An. Gambiae* s.l. with LC_{50} of 27.303 ppm and 20.939 ppm, respectively after 30 min and 1 h exposure. Overall, this study revealed and confirmed the biological potentials of *C. annuum* fresh fruits essential oil as a safe tool to fight against eggs, larvae and pupal stages of *An. Gambiae* s.l. in areas where this insect is known as vector borne disease implicated in the transmission of malaria.

Key words: Plant biocides, Insecticidal properties, *Capsicum annuum, Anopheles gambiae,* Mfoundi Market.

INTRODUCTION

Malaria remains an important health concern among children under five years old and pregnant women living in poor endemic regions. It is responsible for up to 40% of outpatient visits and 28% of hospitalizations in Cameroon (MINSANTE, 2013). As in other endemic countries, malaria control programs in Cameroon are based on the application of chemical insecticides through either long lasting insecticide-treated nets (LLINs) or indoor residual spraying (IRS) (WHO, 2016). However, insecticides resistance developed n vector populations could impede the efficiency of these tools and the success of malaria control programs. The resistance of malaria vectors to four mainly used insecticide classes (DDT, Pyrethroids, Carbamates and Organochlorines) available for public health interventions has been reported in the country (Brevault et al., 2003). The phenomenon of insecticides resistance has led to the use of synergists which increase the efficacy of insecticides by inhibiting the activity of the detoxifying enzyme, or the development of new tools such as plant-derived insecticides. Furthermore, the use of synthetic insecticides has been associated with an eco-toxicity effect as well as the toxicity towards non-target animal species (Ombito et al., 2014). Safe and environmentally friendly methods of malaria vector control were therefore envisaged.

Bio insecticides are effective vector control tools, environmentally safe (biodegradable) and generally based on plant extracts and essential oils. The development of insect resistance and side-effects associated with synthetic insecticides, make plant extracts and essential oils the focus of intense research efforts. The use of bio insecticides stands as an effective and easiest approach to control malaria vectors and decrease the incidence of the disease (Madhumathy et al., 2007). Bio insecticides can safely be used as preventive measures to interrupt the mosquito's life cycle and the dissemination of malaria parasites. Various plant organs like fruits in the case of *C. annuum* (Solanaceae) have been reported to possess insecticidal potentials (Jill, 1993). *C. annuum* L. known as red/chili pepper is a fruit plant from tropical and subtropical regions. It contains a range of essential nutrients and bioactive compounds which are known to exhibit a range of bioactivities including free radical scavenging (antioxidant), antimicrobial, antiviral, anti-inflammatory and anticancer (Khan et al., 2014). The different organs of the *Capsicum* plant have been reported to show several uses. Fresh and dried fruits are crushed and added in food as spice to serve as stimulant. The paste derived from fresh fruits is used in folk medicine to heal wounds and have equally been reported to be an effective therapy to treat hemorrhoid locally (Noumi, 1984). The fruit of the *Capsicum* plant contains an active chemical which is capsaicin. Capsaicin seems to reduce pain sensations when applied to the skin. The pungent principle of pepper is due to the presence of a group of five closely related compounds called capsaicinoids with capsaicin and dihydrocapsaicin being responsible for about 90% of the pungency (Madhumathy et al., 2007). Previously, Mathur et al. (2000) showed that crude extract of *C. annuum* contains 12.5% oleoresin, 0.2% capsaicin and 0.6% dihydrocapsaicin. In Cameroon, powder obtained from red and yellow varieties of *C. annuum* have also been reported to possess insecticidal effects against the growth, development and behavior of adults *An. gambiae* s.l. mosquitoes (Foko et al., 2007a). Recently, Foko et al. (2011) described the adulticidal effect of *C. annuum* essential oil against *An. gambiae* s.l., the main malaria vector in Cameroon. However, the effect of this oil against the aquatic stages of these mosquitoes remains unknown. Fighting against aquatic stages of malaria vectors could be better since the larvae are relatively confined to a geographical area and unable to escape the effects of insecticides (Azhari et al., 2012). In addition, it is suitable to identify the most sensitive stage of the vector to fight against this disease of the poor world. This study is aimed at evaluating in laboratory bioassays the ovicidal, larvicidal and pupicidal activities of *C. annuum* fresh fruits essential oil against aquatic stages of *An. gambiae* s.l., the main malaria vector in Cameroon.

MATERIALS AND METHODS

Laboratory rearing of *Anopheles gambiae* s.l.

Immature eggs of *An. gambiae* s.l. were obtained and incubated for hatching in the zoology laboratory of the Higher Teacher Training College of University of Yaoundé I. Briefly, eggs were kept between 2 and 3 days in Petri dishes for maturation and were subsequently divided for ovicidal tests and hatching at 27°C and 70-80% RH. Eggs for 100 offspring were maintained in spring water supplemented with 30 mg TétraMin® baby fish food in basins (40 x 7.5 cm) as described by Foko et al. (2007b). After hatching, larvae and pupae were reared and daily fed in the same conditions, and were conveniently collected for bioassay activities.

Plant harvest and extraction of essential oil

Fresh ripe fruits of *C. annuum* (red pepper) (Figure 1) were purchased from the Mfoundi local market in Yaoundé (latitude: 3°51'47.91''N; longitude: 11°31'15.55''E), capital city of Cameroon. Essential oils were extracted by hydrodistillation in Clevenger-type apparatus for 5 h 25 min as described by Nyegue (2006). Briefly, fresh ripe fruits of *C. annuum* were weighed (300 g) and half crushed inside an electric blender. The mixture was introduced into a steel pot with adequate quantity of water and placed on an electric heater which is connected to a stimulator and a condenser through a triangular glass. After 2 h incubation at ebullition, the stimulator was set at -20°C and maintained for 3 h. The oil yellowish colored and gathered by decantation was filtered, dried on a column of anhydrous Sodium Sulfate, and introduced into dark glass bottles and refrigerated at 4°C prior to analysis.

Laboratory bioassay activities

C. annuum fresh fruits essential oil was prepared in a test tube and diluted in absolute alcohol to constitute a stock solution of 500 ppm. Seven dilutions were then performed to obtain the working concentrations of 0, 10, 20, 30, 40, 50, and 100 ppm. Seven hundred individuals of each aquatic stage of *An. gambiae* s.l. (eggs, L3 and pupae) were used for bioassays. For this purpose, four breeding racks containing 25 individuals were constituted for each test concentration and according to mosquito's developmental stage. Each plastic bowl was filled with 99 ml spring water and 1 ml of the required concentration of

Figure 1. Fresh fruit of *Capsicum annuum*.

essential oil according to WHO protocol (WHO, 2005). The experiment was replicated thrice before validation and the oil was replaced with ethanol in each control tube. The hatching rates were read 24 h post-exposure, while the mortality rates of larvae and pupae were read after 30 min and 1 h exposure with the essential oil. Mosquitoes were considered dead if immobile and unable to reach water surface.

Statistical analysis

The data were analyzed using SPSS software v17.0. Chi-square test was used to calculate the frequency of tested variables while the Wilcoxon Z test was used to compare average mortality rates between individuals. Data were recorded, and dose-mortality regressions were computed by probit analysis using WinLD software (CIRAD, Montpellier, France). Results were expressed as percentage mortalities, corrected for untreated (check) mortalities using Abbott's formula. LC_{50} (Lethal Concentration to kill 50% of insects) as well as median Inhibitory Concentration of eggs (IC_{50}) were recorded to evaluate the exact efficacy of the essential oil toxicity. P-value was set at 5%.

RESULTS

Extraction yield of the essential oil

Overall, 5 g of essential oil were obtained from 300 g of *C. annuum* fresh fruits. The oil yellowish colored was obtained with a total yield of 1.67% (w/w).

Ovicidal effect of *C. annuum* (red pepper) essential oil against *An. Gambiae* s.l.

The effect of *C. annuum* fresh fruits essential oil was assessed against the hatching rate of *An. Gambiae* s.l. eggs in laboratory bioassays. Overall, the hatching rates decreased significantly with the essential oil concentrations with a mean percentage of 96.73±1.63%, 68.00±2.03% and 26.77±0.68% respectively, 24 h post-exposure to 0, 10 and 20 ppm concentrations of the oil (Table 1). Log-probit dose analysis revealed a median inhibitory concentration (IC_{50}) of 13.735±0.0445 ppm (Y = -4.49157 + 3.94741*X; R^2= 0.983; p = 0.107) after 24 h exposure to the essential oil (Figure 2).

Larvicidal effect of *C. annuum* fresh fruits essential oil against *An. Gambiae* s.l.

Third instars larvae of *An. Gambiae* s.l. revealed high susceptibility to *C. annuum* fresh fruits essential oil. Overall, the mortality rates increased significantly with the concentrations of essential oil and ranged from zero to 90.32±3.00% after 30 min exposure to 0, 10, 20, 30, 40 and 50 ppm concentrations of the oil. These mortality rates increased to 100% after 1 h exposure to 50 ppm of the oil. 100% of larvae died 30 min post-exposure to 100 ppm of *C. annuum* fresh fruits essential oil (Table 2). LC_{50} of 26.181±0.0731 ppm (Y = -5.15585 + 3.63601*X; R^2=

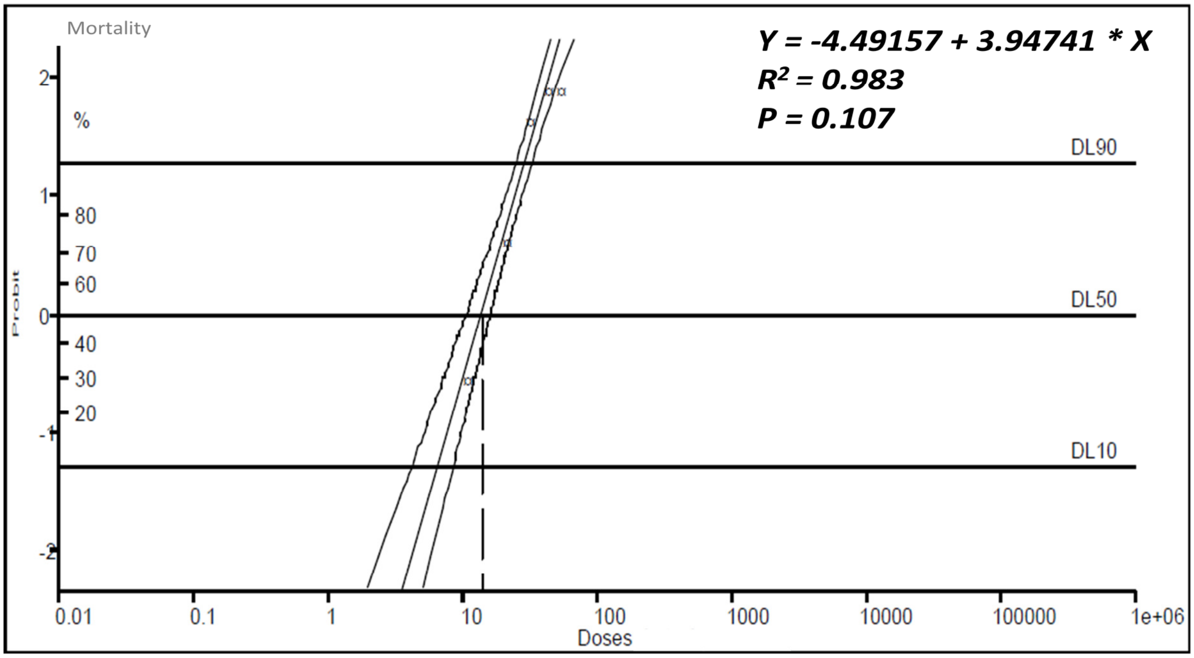

Figure 2. Log-probit dose curve of *An. Gambiae* s.l. eggs after 24 h exposure to *C. annuum* fresh fruits essential oil.

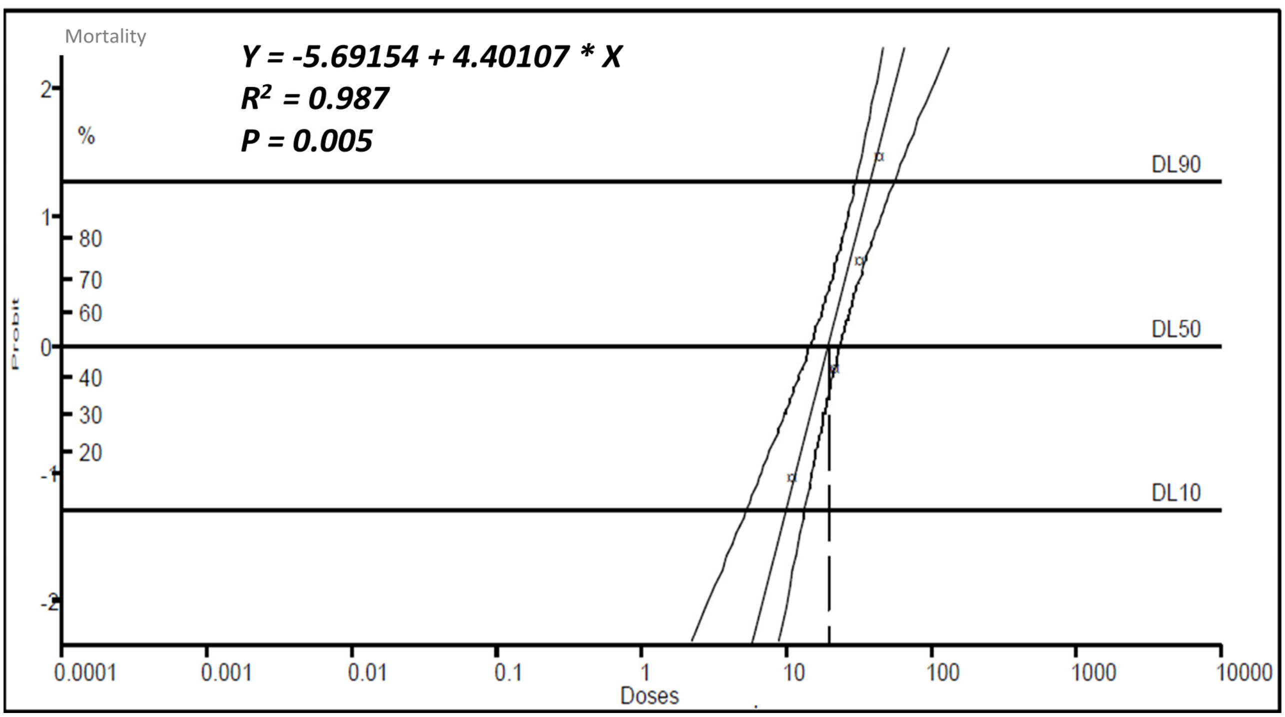

Figure 3. Log-probit dose curve of *An. Gambiae* s.l. third instars larvae after 1 h exposure to *C. annuum* fresh fruits essential oil.

0.994; p = 0.004) and 19.643±0.0337 ppm (Y = -5.69154 + 4.40107*X; R^2= 0.987; p = 0.005) were respectively recorded after 30 min and 1 h exposure of L3 larvae to the oil (Figure 3).

Pupicidal effect of *C. annuum* fresh fruits essential oil against *An. Gambiae* s.l.

The toxic effect of *C. annuum* fresh fruits essential oil

Table 1. Ovicidal effect of *C. annuum* fresh fruits essential oil against *Anopheles gambiae* s.l.

Exposure time		Essential oil concentrations (ppm)							p-value	LC50 (ppm)
		100	50	40	30	20	10	0		
Hatching rates (%)	24 h	0±0	2.68±0	2.95±2.32	4.67±2.00	26.77±0.68	68.00±2.03	96.73±1.63	0.107	13.735±0.0445

Table 2. Larvicidal effect of *C. annuum* fresh fruits essential oil against *Anopheles gambiae* s.l. third instars larvae.

Exposure time		Essential oil concentrations (ppm)							p-value	LC50 (ppm)
		100	50	40	30	20	10	0		
Mortality rates (%)	30 min	100±0.00	90.32±3.00	74.32±1.79	48±2.36	29.78±1.77	12.5±2.6	0	0.004	26.181±0.0731
	1 h	100±0.00	100±0.00	93±0.85	74.00±1.32	43.11±2.47	15.26±1.98	0	0.005	19.643±0.0337

Table 3. Pupicidal effect of *C. annuum* fresh fruits essential oil against *Anopheles gambiae* s,l.

Exposure time		Essential oil concentrations (ppm)							p-value	LC50 (ppm)
		100	50	40	30	20	10	0		
Mortality rates (%)	30 min	100±0.00	88.26±2.07	70.12±1.09	46.00±2.22	31.09±1.00	9.80±3.04	0	0.017	27.303±0.0576
	1 h	100±0.00	98.24±0.00	89±1.14	68.00±0.83	40.55±2.47	14.2±0.43	0	0.035	20.939±0.0744

was less pronounced in pupal stages of *An. Gambiae* s.l. in comparison to larvae. The pupal mortality rates ranged from 0 to 88.26±2.07% after 30 min exposure to 0-50 ppm concentrations of the essential oil, and increased to 98.24% after 1 h exposure. As with the larvae, all pupae died 30 min and 1 h post-exposure to 100 ppm of the essential oil (Table 3). Log-probit dose analysis revealedthe LC_{50} of 27.303±0.0576 ppm (Y = -5.29072 + 3.68379*X; R^2= 0.994; p = 0.017) and 20.939±0.0744 ppm (Y = -5.45145 + 4.12689*X; R^2= 0.995; p = 0.035) respectively after 30 min and 1 h exposure to *C. annuum* fresh fruits essential oil (Figure 4).

DISCUSSION

C. annuum (fresh red pepper) fresh fruits essential oil was obtained with an output of 1.67% (w/w). The oil output is related to the combination of several factors such as the origin of the harvested plant (e.g., nature of the ground where the plant was collected), the neighboring vegetable populations and the climate of the localities of harvest (Bruneton, 1999; Mohammedi, 2006). With this great yield, *C. annuum* fresh fruits extracted essential oil showed an important insecticidal property against watery stages of *An. Gambiae* s.l. Overall, the eggs hatching rates decreased significantly with the essential oil concentrations, revealing an ovicidal property with strong inhibitory factor of *C. annuum* essential oil against eggs *of An. gambiae*. These results correlate with those obtained by Foko et al. (2007a) who described that *C. annuum* powder had an impact on the development of the watery stages of *A. gambiae* s.l. In fact, these authors reported that the powder of *C. annuum, Piper nigrum* and *Zingiber officinale* completely inhibit the hatching potentials of *An. Gambiae* s.l. mature eggs. On the same extent, Prajapati et al. (2005) reported an inhibitory potential of *Z. officinale* essential oil against *An. Stephensi* eggs. This

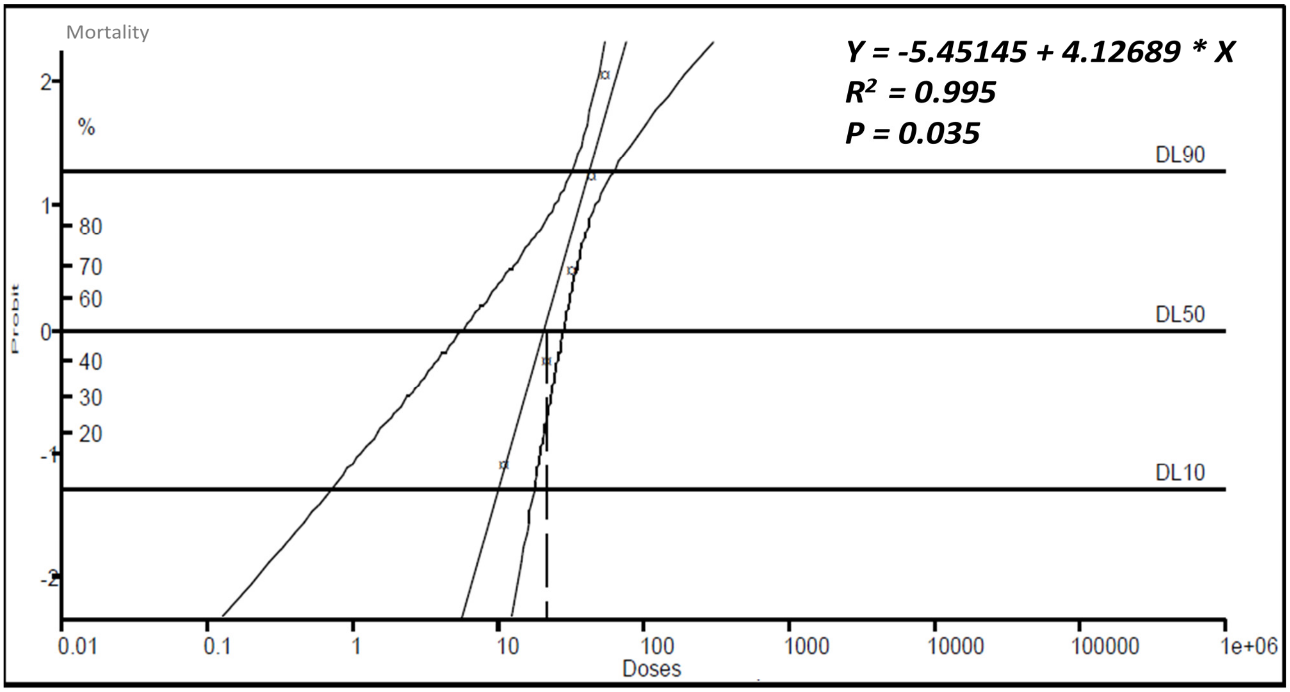

Figure 4. Log-probit dose curve of *An. Gambiae* s.l. pupal stage after 1 h exposure to *C. annuum* fresh fruits essential oil.

ovicidal activity could be attributed to the chemical content of *C. annuum* fresh fruits essential oils. In fact, analysis of the chemical content of *C. annuum* fresh fruits essential oil as performed by Madhumanthy et al. (2007) revealed that they contain active ingredients including 90% capsaicin and capsaicinoids which are efficient potentials against aquatic stages of *An. Gambiae* s.l.

C. annuum fresh fruits essential oil also revealed an insecticidal property against third instars larvae (L3) and pupae of *An. Gambiae* s.l. However, L3 larvae were more susceptible to the oil and stand as the most promising stage for vector control strategies directed against *An. Gambiae* s.l. mosquitoes. These data further confirmed that young L3 larvae are more sensitive to active ingredients of *C. annuum* essential oils as previously described with essential oils from various plant species (Azhari et al., 2012; Ombito et al., 2014). The high mortality rate of L3 larvae as found in this study could be associated to capsaicin, an important active ingredient of *C. annuum* fresh fruits essential oil (Madhumathy et al., 2007). According to Dray (1992) and Madhumathy et al. (2007), active ingredients of essential oils might diffuse into the digestive system of exposed mosquitoes (larvae or pupae) and cause neurotoxicity which finally leads to the death of the organism. Beside capsaicin and capsaicinoids, the death of the aquatic stages could also be associated to the anoxic condition, as the oil forms a film on the water surface, which prevents oxygen from dissolving into the water; hence, they suffocate and die (Wigglesworth, 1953). Foko et al. (2011) previously reported that capsaicin is more efficient against aquatic stages of *An. Gambiae* s.l. than isobutylamine, an important active component found in *P. nigrum.* This makes *C. annuum*, one of the most promising plants which could be used to fight against insect populations such as malaria vectors. The toxicity and virulent nature of an active ingredient in an essential oil on a target insect depends on the presence of specific receptors in the target insect as well as the speed of detoxification of the active ingredient. The higher the speed of detoxification and absence of active side, the lesser is the toxicity of the active ingredient. This phenomenon has clearly been observed with piperine (principal component of *P. nigrum* crude extract), which showed low activity against *An. Gambiae* s.l. developmental stages (Foko et al., 2011). Old developmental stages of *An. Gambiae* s.l. as previously described are less susceptible to plant extracts and essential oils than young stages as found in this study (Tchoumbougnang et al., 2008). The resistance of pupae to plant biocides could be attributed to the presence of thick and tough cuticle that surrounds their cell wall. The cuticle is made up of chitin that forms the exoskeleton and generally protects the aquatic stages against external hostile environmental stresses such as extreme temperature, salinity or acidity (Kouninki et al., 2007). Overall, this study demonstrated the potentials of *C. annuum* fresh fruits essential oil as efficient biocide that can be used as alternative to ineffective synthetic insecticides in fighting *An. Gambiae* s.l. mosquitoes in malaria endemic areas.

Conclusion

This study provided clear information on the potentials of *C. annuum* fresh fruits essential oil against aquatic developmental stages of *An. Gambiae* s.l. in laboratory bioassays. Although the active ingredients of the oil were not assessed, this oil highly inhibited the hatching of *An. Gambiae* s.l. eggs and revealed an important insecticidal property against third instars larvae (L3) and pupae of this mosquito's species. However, the insecticidal potential was greater with L3 larvae, which stands as the most suitable stage to investigate the toxic effect of biological insecticides. Further studies, including the chemical assessment of *C. annuum* fresh fruits essential oil, as well as the efficacy of each individual ingredient against developmental stages of *An. Gambiae* s.l. would be helpful to specifically validate the utility of this plant essential oil as a promising biocide to fight against malaria vectors and to decrease the incidence of malaria in endemic regions.

Acknowledgements

The study received financial support from the Laboratory of Zoology, Higher Teacher Training College of University of Yaounde I. We express our gratitude to OCEAC for providing us with *Anopheles gambiae* s.l. strains used in this study.

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