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Activity evaluation of botanical essential oils against immature mosquitoes of *Culex pipiens* (Diptera: Culicidae)

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Abstract
The secondary effects emerging from the use of conventional pesticides on environmental and human health were lead scientists to look for other safety products. Natural pesticides are promising in pest control, especially those derived from plants. In this study, the larvicidal activity and their effect on the reproduction potency of the essential oils of *Ruta chalepensis* and *Eucalyptus camaldulensis* were evaluated against the mosquito species *Culex pipiens*. Newly exuviated larvae of third and fourth instar larvae were exposed to various concentrations of *Ruta chalepensis* (150-700 ppm) and *Eucalyptus camaldulensis* (100-600 ppm). The results, for both plants, showed a toxic effect of the treated larval series with dose-response relationship. For *Ruta chalepensis* oil bioassay the lethal concentrations (LC_{50} and LC_{90}) were estimated at 95ppm and 550 ppm for the third-instar larvae, respectively, while these respective values were 245ppm and 830ppm for the fourth-instar larvae of *Culex pipiens*. For *Eucalyptus camaldulensis* oil the LC_{50} and LC_{90} values of were 185 and 580ppm for the third-instar larvae, and 280 and 1050 ppm for the fourth-instar larvae respectively. In other experiments the compound was applied at LC_{50} and LC_{90} against the fourth instars larvae and its effects was investigated on fecundity of female emerged from larval treated series with lethal concentrations. The results showed that the two oils reduced significantly the laying egg number and the percentage of fecundity.

Keywords: Larvicide, Mosquito, *Ruta chalepensis* Toxicity, *Eucalyptus camaldulensis*

1. Introduction

Culex pipiens called the domestic mosquito is known to be the most abundant mosquito species in Algeria [1]. It is present practically during all the year particularly in the urban areas due to the extending number of breeding sites [2]. Mosquitoes are vectors of several diseases like malaria, filariasis, dengue fever, yellow fever, etc., causing serious health problems to human beings. These insects are generally controlled by conventional pesticides that have exhibited secondary effects on nontarget animals. In this context, screening of natural products has received the attention of researchers around the world, but seems to be particularly important for public health in developing countries. Plants have long been used by many societies throughout the world to kill or repel pests and their extracts have offered numerous beneficial uses in applications ranging from pharmaceuticals to insecticides [3, 4]. Plants produce chemicals that function as defense mechanisms to reduce feeding injury caused by phytophagous organisms. These natural chemicals have multiple modes of action, including anti-feedant and repellent activities, molting and respiration inhibition, growth and fecundity reduction and cuticle disruption [5, 6]. The search for insecticides and repellents of botanical origin has shown promising alternatives that are effective, but also safer for the environment and could be cheaper than current used products. In recent decades, research on the interactions between plants and insects has revealed the potential use of plant metabolites for this purpose. Also, it is known that some plant chemical constituents of essential oils have insecticidal properties [7]. The botanical insecticides are generally pest specific and are relatively harmless to non-target organisms, including man, and are biodegradable. Various plant essential oils have been studied for their efficacy in controlling larvae of different mosquito species [8]. Larviciding can reduce overall pesticide use in a control programme by reducing or eliminating the need for the ground or aerial application of chemicals to kill adult mosquitoes. The application of easily degradable plant comp ounds is considered to be one of the safest methods of control of insect pests and vectors [9].

As an example the essential oils of peppermint *Mentha piperita* was examined as a repellent and as a larvicide against the mosquito species *Ae. aegypti*, *Ae. stephensi* and *Cx. quinquefasciatus* [10, 11]. Several studies have documented the anti-oxidant, anti-microbial, anti-viral, anti-ulcerous and anti-carcinogenic properties of plant essential oils. Investigations in some countries confirm that some plant essential oils not only repel insects, but have contact and fumigant insecticidal actions against specific pests, and fungicidal actions against some important plant pathogens [5, 7]. The aim of the present study was to evaluate the potential larvicidal and the action on the potential of reproduction of essential oils of *Ruta chalepensis* and *Eucalyptus camaldulensis* against immature stages of mosquito species Culex pipiens.

2. Materials and methods

2.1 Collection and rearing of mosquito

The larvae of *Culex pipiens* (Diptera: Culicidae) were obtained from a stock colony of the laboratory. The origin strain was obtained from untreated sites from an urban locality. Each larval stage were kept in a storage jar containing 500 ml of stored tap water and maintained at a temperature between 25-27°C and a photoperiod of 14L:10D. Larvae were daily fed with fish food consisting of a mixture of Biscuit Petit Regal-dried yeast (75:25 by weight), and water was replaced every four days. When pupae appeared the jars are placed in screened cages (20x20x20 cm^3) until the adult emergence. Adults were fed on 10% glucose solution and females are supplied with a periodic blood meal. The laying egg rafts were used for stock-rearing of next generations.

2.2 Extraction of plant essential oils

Fresh leaves of *Ruta chalepensis* and *Eucalyptus camaldulensis* were macerated and steam distilled using a clavenger distillatory with condenser. Distillation carried out up to 3–5h at temperature of 100˚C. The volatile compounds containing the water-soluble fractions were allowed to settle for 30min. The essential oil layer was separated and purified using filter papers. Finally, the obtained essential oils were stored in a refrigerator at 4 ˚C for the coming bioassays.

2.3 Toxicity bioassays

The oils of *Ruta chalepensis* and *Eucalyptus camaldulensis* were tested at various concentrations against third and fourth-instar larvae of *Cx. pipiens* mosquitoes in order to evaluate their toxic effect on mosquito larvae. The extracted oils were prepared by dissolving the suitable amount of oil in tap water using ethanol 100% to produce to produce 600 ml of the final using concentrations; 100, 200 and 600 ppm of *Ruta chalepensis* and *Eucalyptus camaldulensis* oils. The test was carried out with three replicates containing 25 larvae each. Each solution of the different concentrations was poured into three rearing jars (200 ml of the solution for each jar). The control series only 2 ml of ethanol were added to the tap water to make a final quantity of 200ml. Newly ecdysed third and fourth-instars larvae of *Cx. pipiens* were exposed to the different concentrations for 24 hours, according to the World Health Organization [12]. After the exposure time the larvae were removed, and placed in clean breading water.

2.4 Statistical Analysis

The mortality percentage recorded at various developmental stages was corrected [13] and toxicity data were studied by probit analysis [14] and LC_{50} 50% lethal concentration) and LC_{90} the lethal concentration that kills, with 90%, confidence limits (95%). and slope of the concentration-mortality lines were calculated by the method of Swaroop et al. [15]. In addition, statistical analysis was carried out for all the estimated measurements of treatments and compared with the control values by test ANOVA and Student's t-test using the computer program (MINITAB, version 14).

2.5 Reproduction potency

The experiments on the reproduction potency were conducted on the eggs collected from the breeding jars of the mosquito females of *Cx. pipiens* emerged from the fourth instar larvae series treated with the lethal concentrations LC_{50} = 245 and LC_{90} = 830 ppm of *Ruta chalepensis* and LC_{50} = 280 and LC_{90} =1050 ppm *Eucalyptus camaldulensis*. For each concentration 10 females and 10 males were kept in separate breeding cage. The collected laying eggs for each series were counted and transferred to a new jar containing just water and kept for hatching process. The reproduction potency was estimated by the number of laid eggs, hatching rate and the fecundity. The fecundity was evaluated by the number of eggs laid divided by the number of females let to mate (The death of adults in the experiments was also considered). The obtained results were subjected to a statistical analysis using the t test of student with a computer program (MINITAB, version 14). The hatching rate (H) and the fecundity (F) were calculated using the following formulas [15].

$$H = \frac{\text{Number of hatched eggs}}{\text{Total number of eggs}} X\ 100$$

$$F = \frac{\text{Total Number of laid eggs}}{\text{Total Number of females}}$$

3. Results

3.1 Lavicidal activity of *Ruta chalepensis* and *Eucalyptus camaldulensis* oils

Bioassays showed that *Ruta chalepensis* and *Eucalyptus camaldulensis* oils have toxicity against the two larval stages L3 and L4 of the mosquito species, *Culex pipiens*. The mortality (%) varied with concentration for the different larval stages and the two of the plants. Mortality (%) was observed during the treated larval stage and the following ones.

3.1.1 Toxicity of *Ruta chalepensis* oil

The *Ruta chalepensis* oils exhibited a larvicidal effect against the third and fourth-instar larvae. The toxicity of the L3, treated with the lower concentration of 100 ppm, was represented by the mortality, recorded during the stage period and was corresponding to 56.34±13.81%, whereas with the higher concentration of 600 ppm, it was 96±5% (Fig. 1). Using probit regression analysis and a linear correlation between concentration and mortality, the LC_{50} and LC_{90} were calculated and the confidence limits (FL) for all stages were estimated (Table 1). These lethal concentrations were, the LC_{50} = 95 ppm and the LC_{90} = 650 ppm (Table1). The mortality of the treated L4 at with the concentration 150 ppm of *Ruta chalepensis* oils larval mortality was 37.89±4.55%, whereas at the higher concentration of 700 ppm it was 68.42±0.00% (Fig.1). With probit, the LC_{50} and LC_{90} and were 245ppm and 830ppm respectively and the confidence limits (FL) for all stages were estimated (Table 1).

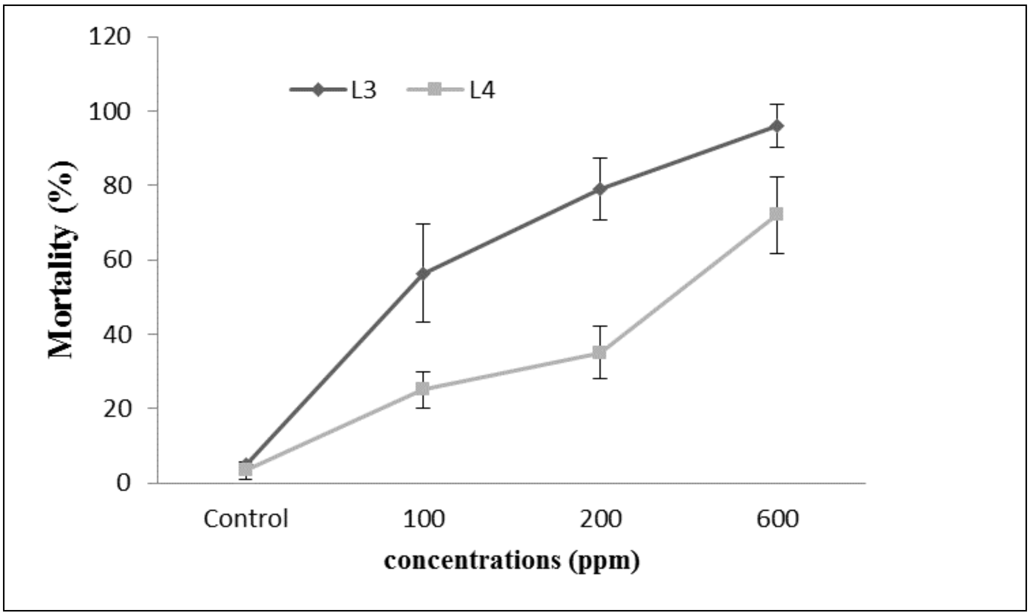

Fig. 1 Larvicidal activity of *Ruta chalepensis* oil against the 3rd and the 4th instars larvae of *Culex pipiens.*

Table 1: Toxicity of *Ruta chalepensis* oil against the 3rd and the 4th instar larvae of *Culex pipiens*.

Larval stage	95% Confidence limit		Regression equation	Slope
	LCL <LC50< UCL (ppm)	LCL <LC90< UCL (ppm)		
L3	165 <95<192.45	667.5 <550 < 812.02	Y=6.10X-8.74	1.53
L4	154.20 <245 <389.85	710.70 < 830< 932.68	Y=1.12+2.30	10.63

3.1.2 Toxicity of *Eucalyptus camaldulensis* oil

The toxic effect of *Eucalyptus camaldulensis* oil against the larval stages of *Cx. pipiens* was traduced by the recorded mortality. The lower concentration caused a mortality of 100ppm exhibits a mortality of 37% and 27% for the L3 and L4 respectively. The highest concentration of 600 ppm caused 94.20 ±6 for L3 and61.59 ±0.99% of mortality for the L4 of the mosquito species. The analysis of variance of the data showed a significant (p<0.001) insecticidal effect with a dose response relationship too (Fig. 2). With probit analysis the lethal concentrations were calculated and were for the stage L3 as following; the LC_{50} = 180 and the LC_{90} = 580 ppm. For the stage L_4 the LC_{50} was estimated at 280 ppm and the LC_{90} was at 1050 ppm (Table 2).

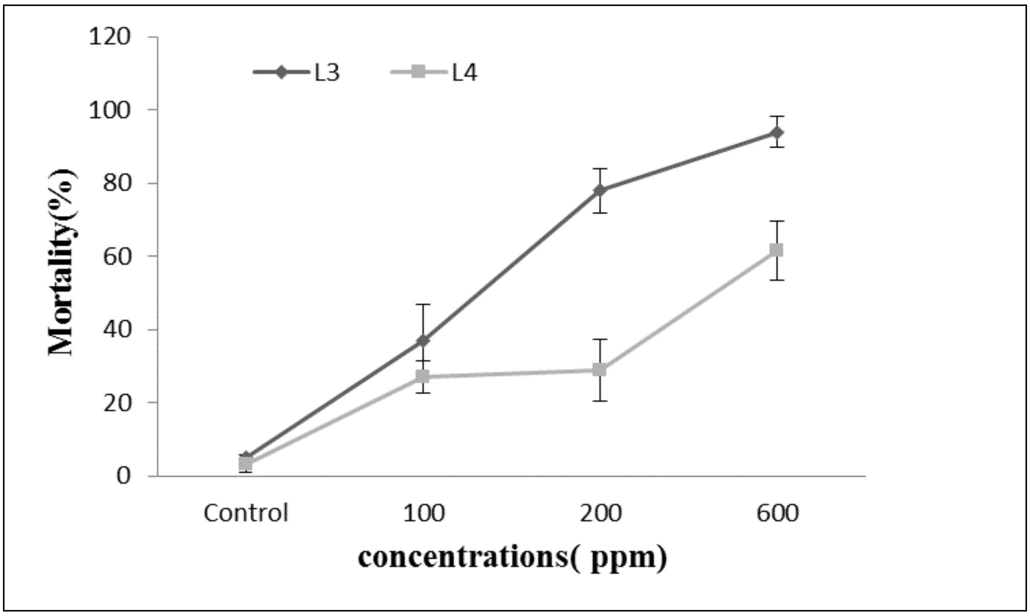

Fig 2: Larvicidal activity of *Eucalyptus camaldulensis* oil against the 3rd and the 4th instars larvae of *Culex pipiens.*

Table 2: Toxicity of *Eucalyptus camaldulensis* oil against the 3rd and the 4th instar larvae of *Culex pipiens*.

Larval stage	95% Confidence limit		Regression equation	Slope
	LCL <LC50< UCL (ppm)	LCL <LC90< UCL (ppm)		
L3	142.03 <185<237.13	504.08<580< 1006.52	Y=2.03X+0.39	3.68
L4	282.56<280 <723.37	925.20< 1050< 1127.32	Y=1.12X+2.01	10.74

The comparison between the two stages and larvicidal activity of two oils showed that the third stage is more sensitive than the fourth stage. The target mosquito species tested were more sensitive to the essential oils of both plants, with the most toxical oil of *Ruta chalepensis* than *Eucalyptus camaldulensis* (Fig. 3).

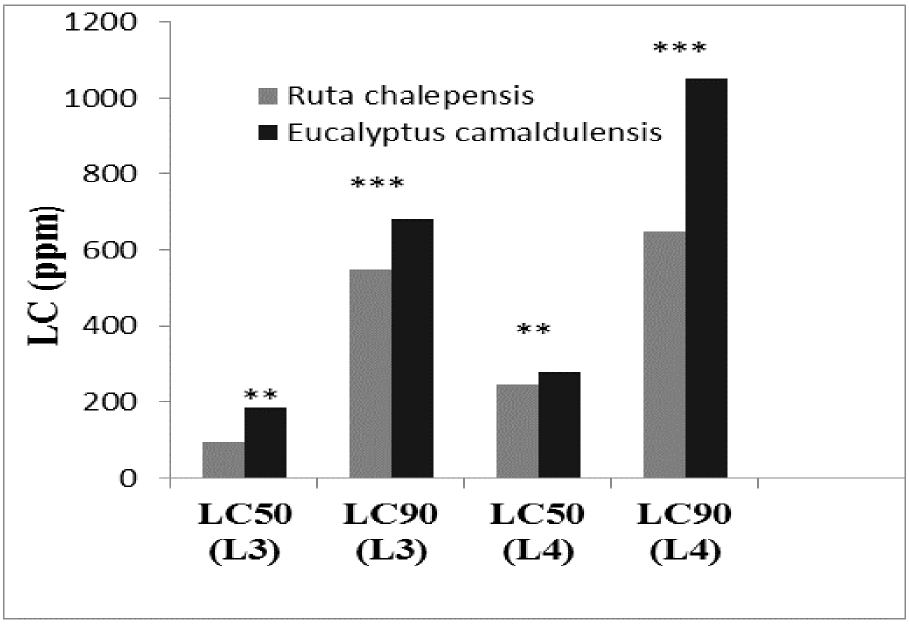

Fig. 3 The comparison of the toxicity of the tested *Ruta chalepensis* and *Eucalyptus camaldulensis* oils agaist the 3rd and the 4th instar-larvae of *Culex pipiens*.

3.2. Effects of *Ruta chalepensis* and *Eucalyptus camaldulensis* oils on reproduction

The effect of *Ruta chalepensis* and *Eucalyptus camaldulensis* oils on reproduction was evaluated on different parameters, of the females emerged from the treated fourth instar larvae of *Cx. pipiens* and presented in table 3. The number of eggs laid was inversely proportional to the concentration in the treatment. For female treated *Ruta chalepensis* oil the number of eggs laid was reduced from 176 to 124 and 96 as the concentration was increased, from CL_{50} to CL_{90}. The same observation was recorded for treated ones with *Eucalyptus camaldulensis* oil, where the egg number decreased from 176 to 153 and 166 under treatment effect of CL_{50} and CL_{90} respectively (Table 3). For the same series of experiments the hatching rates was calculated and showed a significant decrease according to the treatment with *Ruta chalepensis* and *Eucalyptus camaldulensis* oils (Table 3). The fecundity was also highly reduced after the treatment with the lethal concentrations ($CL_{50}$and CL_{90}) of both oils (Table 3).

Table 3: Effect of *Ruta chalepensis* and *Eucalyptus camaldulensis* on the reproduction of the females emerged from the treated 4th instar larvae of *Culex pipiens* (n= 10 females). Means followed by the same letter indicate a significant difference (*P*<0.005).

Oil plant	Treatment	N° egg laid	Fecundity	Hatching rate (%)
Ruta chalepensis	Control	176^{a}	19.6^{a}	98.84^{a}
	CL_{50} = 245 ppm	124^{ab}	17.71^{ab}	75.40^{ab}
	CL_{90} = 830 ppm	96^{ab}	24^{ab}	55.02^{ab}
Eucalyptus camaldulensis	Control	176^{a}	19.6^{a}	98.84^{a}
	CL_{50} = 180 ppm	153^{ab}	19.1^{ab}	88.65^{a}
	CL_{90} = 580 ppm	166^{ab}	21.8^{a}	72.25^{a}

4. Discussion

The mosquito vector control, using chemical insecticides, is not encouraged due to rapid increase in mosquito resistance and growing public concern over environmental pollution and the alternative uses of effective and eco-friendly alternatives is promoted. A program for biological control of mosquitoes the evaluation of new isolate plant products is one of the most important steps taken to determine their effect on target populations, and thereby selecting the most promising ones for producing biological insecticides [2]. In the present study, bioassays were carried out in order to evaluate the effect of *Ruta chalepensis* and *Eucalyptus camaldulensis* oils at different concentrations as natural pesticides against the larval stages of the mosquito species *Cx. pipiens.* The bioassays of both plant oil extracts showed a larvicidal activity and the younger stages were more sensitive than the old ones and the mortality exhibits a concentration-response relationship. These confirm a previous work when it was reported that the use of plant extracts have the same toxic effects; such as plant aqueous extracts of *Calotropis procera* and *Ricinus communis* [17] against mosquitoes and the 3rd instar larvae were more susceptible than 4th instar larvae. In addition, the essential oil obtained from *Ipomoea cairica* showed a high toxicity with the LC_{50} values of 58.9 and 14.9 µg/l, when treatment was done against the larvae of *Cx. quinquefasciatus* and *As. stephensi*s respectively [18]. A larvicidal activity of *Ruta chalepensis* and *Eucalyptus camaldulensis* oils was in concordance with earlier findings when the essential oils of neem clove, pine and rosemary were used against *Cx. quinquefasciatus* and *Ae. aegypti* [19]. The ethanolic extract of *Derris urucu* roots showed toxicity too against *Ae. aegypti* [20]. Also, the methanolic extract of *A. indica* leaves shows 90% of mortality against *Aedes aegypti* larvae of third and fourth instar with the concentration of 200mg [21]. The saponin extracted from the fruit of *Balanites aegyptiaca* showed 100% larvicidal activity against *Ae. aegypti* mosquito larvae [22]. In addition, the essential oil of *Ocimum americanum* was found to be effective against *Ae. albopictus* larvae of 4th instar by causing 100% of mortality with the concentration of 6.6μg/ml at 24 hours of observation [23]. In a another study, it was found that the hexane extract of *M. koenigii* leaves used against 3rd instar of *Cx. quinquefasciatus* caused mortality over 24 hours [23]. A previous study using Azadirachtin which is an active constituent of *Azadirachta indica* was found to be effective against the fourth instar of *Cx. pipiens* [24].

The emerged adult from treated the fourth instar larvae with *Ruta chalepensis* and *Eucalyptus camaldulensis* oils, were morphologically normal but showed a great reduction in fecundity. The same results were mentioned when the *Curcuma zedoaria* essential oil was tested against dengue vector mosquito, *Aedes aegypti* [25]. The treatment with essential oil from *Boesenbergia rotunda* and *Cinnamomum zeylanicum* presented oviposition deterrent and repellent activities and the essential oils of *Zingiber officinale* and *Rosmarinus officinalis* also showed both ovicidal and repellent activities against *As. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* [26].

5. Conclusion

From the present study we conclude that *Ruta chalepensis* and *Eucalyptus camaldulensis* oils proved a promising larvicidal agent against *Cx. pipiens* larvae in the laboratory and also reduced (egg productions and fecundity. A comparative study of the oils of both the plants, conducted in the laboratory, revealed that *Ruta chalepensis* oil was more toxic than *Eucalyptus camaldulensis*.

This revealed the need for development of new insecticides from those plants that have larvicidal activity of controlling mosquito species.

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