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Research Article

Chemical Constituents and Larvicidal Activity of Essential Oil of *Lavandula Stoechas* (Lamiaceae) From Morocco Against the Malaria Vector *Anopheles Labranchiae* (Diptera: Culicidae)

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ABSTRACT
The use of synthetic larvicides to control vector populations is detrimental to human and environmental health and selects for insecticide resistance. Plants can be alternative sources of effective and safe mosquito control agents. In the present work the chemical composition and larvicidal activity, of essential oil of *Lavandula stoechas* (lamiaceae), growing in wilds of Morocco, against the malaria vector *Anopheles labranchiae* (Diptera: Culicidae), were studied. The obtained percent yield of the hydro-distilled volatile oil from aerial parts of *Lavandula stoechas* was 1.74 ± 0.24. The GC/MS analysis of *Lavandula stoechas* essential oil has led to the identification of 20 components. Camphor (36.14%), 1,8-Cineole (25.16%), Camphene (11.44%) and Fenchone (9.08 %), were the major constituents of which. The biological test performed using a methodology that is inspired from WHO standard protocol, revealed that the essential oil of Lavandula has larvicidal properties. The minimal dose required to achieve 100% larvicidal effect on *Anopheles labranchiae* was 500 mg/l for essential oil of *Lavandula stoechas.* The Lethal Concentration (LC_{50} and LC_{90}) values were 112.51 mg/l and 294.51 mg/l respectively. The relationship between the chemical composition and biological activity of essential oil of *Lavandula stoechas* is confirmed by the above-mentioned results. Therefore, the potential for exploiting these essential oils, such as bioinsecticides for vector control, can be taken into account.

Keywords: *Anopheles labranchiae*, *Lavandula stoechas,* Chemical composition, Larvicidal activity, North Eastern Morocco

INTRODUCTION

Genus *Anopheles* mosquitoes, are vectors of the most debilitating and life threatening parasitic diseases including "malaria", which is a major public health problem in Africa. Current estimates suggest that 216 million episodes of malaria occur every year in Africa accounting for 81% of cases[1]. Most cases occur among children less than 5 years and pregnant women[1]. The malarial vectorial system in Africa is very complex. It typically includes several mosquitoes. The major of which are: *Anopheles gambiae*, *Anopheles arabiensis*, and *Anopheles funestus* as primary vectors and a number of complementally vectors especially *Anopheles pharoensis*, *Anopheles coustani* and *Anopheles Rivurolum*[2,3]. In Morocco, studies on mosquitoes (Diptera: Culicidae) show the presence of ten species of *Anopheles*[4]. By its abundance, density, vectorial capacity and wide geographical distribution, *Anopheles labranchiae (An. labranchiae)* (Falleroni 1926) is the main vector of malaria in Morocco[4]. Despite the fact that the last case of indigenous malaria recorded in Morocco was in 2004[5], this disease continues to pose a public health problem in some regions of the Kingdom particularly in Northern center due to some imported cases[6,7]. To prevent a possible return of the transmission of malaria, a vector control program has been established and implemented by health authorities. Thus, Morocco was certified by World Health Organization (WHO) for being free of indigenous malaria in May 2010[8], hence it is the first North African country to obtain such certification. The vector control program of Morocco is mainly based on chemical and/or biological controls against the larvae of the vector *An. labranchiae*[9]. The chemical control is an effective strategy used extensively in vector control. However, the widespread use of synthetic insecticides has caused many damages to the environment and human health[10]. One major drawback with the use of these chemical insecticides is that they are

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non-selective and could be harmful and toxic to other organisms in the environment[11]. Currently, this technique generates and faces difficulties, particularly the development of mosquito resistance towards the insecticides used. Indeed, most chemical insecticides (such as organophosphate that contains Temephos, Malathion and Chlorpyrifos) utilized in the world cause a major problem in the development of resistance by certain mosquitoes, especially for *Anopheles* mosquito genus[12,13]. In Morocco, the phenomenon of mosquito resistance to chemical insecticides has recently been reported. Indeed, some authors[14,15] have described the resistance of *Culex pipiens* (Diptera: Culicidae) to Temephos, which is the most widely used insecticide in the country in the control of larvae. Add to this, Faraj *et al.*[4] indicated that the concentration of 0.125 mg/l of Temephos, an effective dose found previously, did not give a 100% mortality in a population of *An. labranchaie* (originally from Sidi Kacem, northwestern region of Morocco), suggesting the emergence of resistance to this species. On the other hand, in order to avoid this kind of resistance, various plant extracts and phytochemicals including essential oils can be considered as potential sources of commercial mosquito control agents[16,17]. Traditional herbal medicine is an important entity of healthcare in Africa[18,19]. This is largely due to poverty, inadequacy of health services and shortage of ealth workers[18]. The WHO estimates that up to 80% of the population in some developing countries use of botanical traditional medicine[20]. It is necessary to note that the plant-derived chemicals, that are used to fight mosquitos, have seen an interesting progress. For example, Maia and Moore[21] reported that in the past few years, a plant derived repellent, PMD (para-methane-3-8-diol) had been proven to be suitably efficacious and safe to compete with DEET (N, Ndiethyl-3-methylbenzamide) in the field of disease prevention. These repellents have been recognised by WHO as a useful disease prevention tool to complement insecticide-based means of vector control. Indeed, PMD (p-menthane-3,8-diol) being safe to human health, is the only plant based repellent advocated by the Centers for Disease Control (CDC) for public use[22]. Hence, the natural insecticides of plant origin have been receiving attention as alternatives for the control of medical importance anthropods[23]. Several plants, in the Mediterranean region, are known to have insecticidal activity especially against mosquitoes (Diptera: Culicidae)[24-28]. Among the types of plants, the genus "Lavandula" (Lamiaceae) is a wild plant in the Mediterranean basin including over 34 species[29] and which is known to have insecticidal activity against various insect species[30]. The species of the "Lavandula" genus are also used in traditional medicine[31] as well as in pharmaceutical and cosmetic industries[32]. One species of this genus is *Lavandula stoechas* (*L. stoechas*), which is one of the most explored and exploited lavenders in the world. Some studies focused on the antibacterial[33,34], antifungal[33,35], and antioxidant[36,37,33] properties of *L. stoechas*. It is in this framework that we were interested in the study of the essential oil (EO) of *L. stoechas*. This study is designed to examine the chemical composition of the essential oil of *L. stoechas* growing in the North center of Morocco, as well as to search its larvicidal activity against the malaria vector *An. labranchiae*. In Morocco, there is no work reported on the biological activity of the EO of *L. stoechas* against mosquitoes *An. labranchiae* larvae.

MATERIALS AND METHODS

Extraction of the essential oil

A sample of 100 g of aerial parts of *L. stoechas* including flowers, branches and leaves was air-dried and subjected to hydrodistillation during 2 hours using a Clevenger apparatus[38]. The obtained EO was dried using anhydrous sodium sulphate; then was stored at 4 °C after filtration until use stage. All experiments were conducted in triplicates and results were expressed on the basis of dry matter weight.

Chemical characterization of essential oil

The essential oil was analyzed using Gas chromatography (GC) coupled to mass spectrometry GC/MS (Polaris Q ion trap MS). Analyses were performed on a Hewlett-Packard (HP 6890) gas chromatograph (FID), equipped with a 5% phenyl methyl silicone HP-5 capillary column (30m x 0.25 mm x film thickness 0.25 µm). The operational temperature was 50°C after 5 min initial hold at 200°C at 4°C/min. The used gas chromatography included: Nitrogen (N_2) such as carrier gas (1.8 ml/min), split mode was used (Flow: 72.1 ml/min, ratio: 1/50), temperature of injector and detector was 250°C and final hold time was 48 min. The machine was driven by 'HP ChemStation' computer system, this allowed managing the running of the machine and to following the chromatographic analyses evolution. Diluted samples (1/20 in methanol) of 1µl were injected manually.

Characteristic of aquatic habitat and collection of *An. labranchiae larvae*

An. labranchiae larval samples were collected in an aquatic habitat located in Ain Boukhnafer urban area of Fez city located in coordinates (1 132 m altitude, 34°01'35" N and 5°11'44" E), the selected area was 22 500 m^2. This site, which is a lake, had high densities of homogenous larvae of *An. labranchiae*. Larvae were collected using a rectangular plastic tray inclined of 45° with respect to the water surface. The collected larvae were maintained in breeding in rectangular trays at 23.42 °C in the Entomology Unit, Regional Laboratory of Epidemiological Diagnostic and Environmental Health within of the Regional Health Directorate of Fez, Morocco.

Larvae were identified using a Moroccan Culicidae identification key[39] and the Mediterranean Africa mosquitoes identification software[40].

Protocol for larval susceptibility testing

The susceptibility tests were carried out in accordance with the standard protocol developed by WHO[41]. A stock solution (10%) of essential oil in ethanol and dilution series of 31.25, 62.5, 125, 250 and 500 mg/l, were prepared. Preliminary experiments were used to select a range of concentrations to be tested. Each solution prepared included 1 ml and was placed in beakers containing 99 ml of distilled water in contact with 20 larvae of stadium 3 (L3) and 4 (L4)[41]. The same number of larvae was placed in a beaker containing 99 ml indicator of distilled water plus 1 ml ethanol. Three repetitions were carried out for each dilution and for the negative control (ethanol). After 24 hours contact, the living and dead larvae were counted. The results of susceptibility testing sites were expressed in percentage of mortality versus concentrations of the used EOs. If the percentage of mortality in control is greater than 5%, the percent mortality in larvae exposed to the essential oil is corrected by using Abbott's formula[42]. % Mortality Corrected = [(% Mortality Observed - % Mortality Control) / (100 - % Mortality Control)] × 100.

If the control mortality exceeds 20%, the test was considered unacceptable and must be repeated.

Temephos, the insecticide mostly used in larval control in Morocco, was considered as the positive control.

Data processing

The log-probit analysis (Windl version 2.0) software developed by CIRAD-CA/MABIS was used[43]. This software enables the determination of threshold doses or lethal concentrations (LC_{10}, LC_{50}, LC_{90}) causing some mortality in the larvae population (10%, 50%, 90%). In biological assay, probit analysis is preferred[44,45].

The obtained results were submitted to a variance analysis (ANOVA) and the percentage values were compared to Chi-square's test ($P = 0.05$) which is calculated by the program Epi Info 3.4.

RESULTS

Percent yield and physical characters of L. stoechas

The percent yield of the hydro-distilled volatile oil from aerial parts of *L. stoechas* was 1.74±0.24. The extracted oil is characterized by light yellow color and typical aromatic odor.

Component analysis of essential oil of L. stoechas

GC/MS analysis of *L. stoechas* EO led to the identification of 20 components. Camphor (36.14%), 1,8-Cineole (25.16%), Camphene (11.44%) and Fenchone (9.08 %) were the major components of which. Details of all components are reported in Table 1.

Variation in mortality

After exposing the larvae *An. labranchiae* to different concentrations of EO of *L. stoechas* for 24 h, the percentage of mortality varied according to concentrations (Figure 1). The minimum concentration of *L. stoechas* EO required to achieve 100% of *An. labranchiae* larvae mortality was 500mg/l.

Figure 1 also shows the different concentrations used with their standard deviations and significant correlations between larvae mortality and the concentration of EO of *L. stoechas*. Indeed, larvicidal properties of *L. stoechas* were demonstrated while using all studied concentrations. The found Chi^2 test was 64 and statistical analysis showed $P < 0.05$. Thus, the difference between the percentages is significant.

Lethal Concentrations LC_{50} and LC_{90}

Table 2 demonstrates that *L. stoechas* EO remains effective while using concentrations of 112.51 mg/l for LC_{50} (which varies between a lower limit 69.46 mg/l and an upper limit of 147.59 mg/l) and 294.51 mg/l for LC_{90} (which also varies between a minimum of 225.98 mg/l and a maximum value of 463.44 mg/l).

Table 2 shows also the regression equation and the Chi^2 analyses results. The regression analysis indicates that the mortality rate is positively correlated with *L. stoechas* concentration. The adjustment model of tested data (Chi^2 Test), which is not significant at 5%, showed a good model fit.

DISCUSSION

Among the species of the genus lavandula, *Lavandula gibsoni* is known to have insecticidal activity especially against mosquitoes (Diptera: Culicidae)[46]. In the present work, realized for the first time in Morocco, we are interested in examining the chemical composition of the essential oil of one of the genus lavandula from Morocco (*L. stoechas*), as well as studying its larvicidal activity against the malaria vector *An. labranchiae*. Our study showed that, the percent yield obtained from the hydro-distilled volatile oil from aerial parts of *L. stoechas*, was 1.74±0.24. The collected plant in Morocco generates 0.9 to 1.4 percent yield depending on the region of collection. Indeed, *L. stoechas* lavander [Lamiales: lamiaceae (labiatae)], is usually growing in high altitudes of calcareous soils in Morocco. *L. stoechas* is a local plant grown in dry hills, garrigue, maquis, open woodlandson limestone, granite soils or rockroses. The sample from 'Idni' in High Atlas generates a good percentage of EO after distillation (1.4 %) and has very high rate of Camphor (72.8 %)[47,48]. *L. stoechas* collected in Morocco was studied for its essential oil content. Fenchone and Camphor represent 47 to 84.3 % of its components and the dominant of them depends on the sample's origin[47]. The other constituents of *L. stoechas* EO are alpha-pinene, beta-pinene, camphene, eucalyptol, para-cymene, linalol, borneol, borneol acetate, carvacrol, iso-eugenol and iso-eugenol-methylether. According to a study achieved in the Atlas region of Morocco, *L. stoechas* oil is characterized by a high content of Camphor (55.24%), Camphene (15.37%), 1,8-Cineole (15.17%), Terpinolene (12.85%) and β- Pinene (7.04%)[48]. The EO composition is qualitatively similar to that found in other Mediterranean countries[47]. The EO of *L. stoechas* exhibited a LC_{50} and LC_{90} concomitant with 112.51 and 294.51 mg/l respectively (Table 2). This larvicidal activity of *L. stoechas* EO could be explained by the effect of major components including Camphor, 1,8-Cineole, Camphene and Fenchone. Thus, Camphor, which represents the first major component of *L. stoechas* EO, is known to have insecticidal activities[21,49-51] and deliver a fragrant vapor that scares away mosquitoes. 1,8-Cineol represents the second major constituent of *L. stoechas* EO and was reported to be toxic for several insect species. Indeed, Cavalcanti *et al*., mentioned that monoterpenoids eugenol and 1,8-Cineol for *Ocimum*

Table 1: Chemical composition of essential oil of *L. stoechas* analyzed by GC-MS.

No.	Compounds	Retention Time (min)	Concentration (peak area %)
1	Camphene	7.928	11.44
2	Verbene	8.105	0.48
3	β-L-Pine	8.935	0.19
4	ρ-Cymene	10.834	1.08
5	1,8-Cineol	11.160	25.16
6	Gamma-Terpinene	12.150	0.22
7	2-Camphenilone	13.106	0.49
8	Fenchone	13.335	9.08
9	β -Linalool	13.838	0.36
10	Camphor	15.658	36.14
11	Trans-Pinocarveol	16.167	0.18
12	1-Terpinen-4-ol	16.728	0.12
13	α-Terpineol	17.237	0.22
14	Verbenone	17.890	0.51
15	(+)-Carvone	19.143	0.18
16	Bornyl acetate	20.636	1.97
17	Eugenol methyl ether	23.074	0.19
18	Caryophyllene	25.116	0.41
19	β -Selinene	27.222	0.20
20	β -Cubebene	27.399	0.23
Total			90.22

gratissimum showed good larvicidal activity against *Aedes aegypti*[52]. Camphene which represents the third major component of *L. stoechas* EO is also known to have insecticidal activities[49-51] and record a great effect against insects. Concerning the larvicidal activity of *Lavandula* genus essential oils against the mosquito (*Aedes*, *Anopheles* or *Culex*), the bibliography shows that only few reports are available. Thus, studies performed by Traboulsi et al.[24] have shown the insecticidal activity of *L. stoechas* against larvae of *Culex pipiens molestus*, with an LC_{50} in the order of 89 mg/l concentration. This concentration is approximately similar to our value. Conti B et al.[25] have achieved laboratory bioassays on insecticidal activity of EO extracted from six Mediterranean plants including *Lavandula angustifolia*. All plants revealed insecticidal activity against the larvae of *Aedes albopictus* Culicidae mosquito. Furthermore, in 2013 Kulkarni R.R. et al.[46] reported that EO and acetone extracts from *Lavandula gibsoni* were investigated for their mosquito larvicidal activity against the larvae of 4[th] instar (*Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus*). The LC_{50} values of *L. gibsoni* EO against three species were 48.3, 62.8 and 54.7 mg/l. This is a unique study that reported the larvicidal activity of *Lavandula* sp against *Anophele* sp larvae and showed an LC_{50} twice less than that found in this study. Recently, Ben Slimane et al. reported that *L. stoechas* EO has effective high toxicity to the fourth larval instars of *Orgyia trigotephras* (Lepidoptera: Lymantriidae), a larvae of the insect class[30]. However, if we compare the larvicidal activity of *L. stoechas* EO against *An. labranchiae* with that of chemical insecticides, the literature indicates that the limit dose allowing to monitor the susceptibility of *An. labranchiae* to Temephos in Morocco was 0.025 mg/l[4], while the limit dose of WHO is 0.1 mg/l. Our concentration is very high compared to that recorded by the Temephos. The observed difference between LC_{50} of previous studies could be explained by many factors impacting the effects of larvicidal activity, namely: physico-chemical characteristics and chemical composition of EO, the environmental conditions, the used extraction technique, the drying process, the period and growing environment, the cultivation practices and the age of the vegetal material[53-55].

Reported literature and inhere reported results demonstrate that *Lavandula* genus shows significant larvicidal activity against mosquito. The EO from these

Table 2: Lethal Concentrations LC_{50} and LC_{90} of larvae of *An. labranchiae* after 24h.

Plant species	LC_{50} (mg/l) (Ll-Ul)*	LC_{90} (mg/l) (Ll-Ul)*	Equation of the regression line	Calculated (χ2)	*Chi*-square
Lavandula stoechas	112.51(69.46-147.59)	294.51(225.98-463.44)	*Y = -6.29123 + 3.06708 * X*	1.979	

* Ll-Ul: Lower limit-Upper limit.

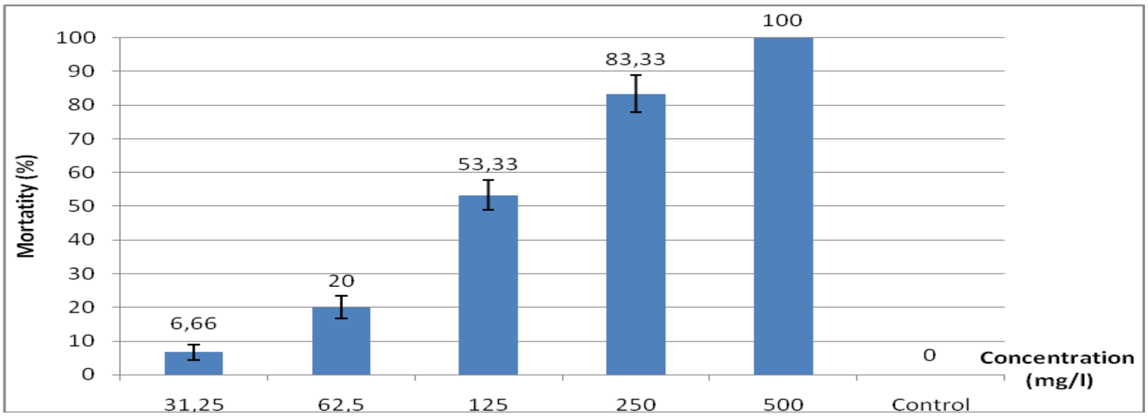

Figure 1: Mortality (%) of larvae of *An. labranchiae* depending on the concentration of essential oil of the plant species *L. stoechas* after 24 h exposure.

aromatic plants are generally rich of monoterpenoids, which are components with strong insecticidal activity against various arthropods[21,49-51]. For this reason, we attempt to valorize this essential oil. Thus, further studies are required to clarify the components obtained from the fractionation operation in parallel with biological tests on larvae mosquitoes. Each single constituent of EO or synergized mixture might record an LC_{50} lower compared to recorded results in this work. Inhere results are the first in Morocco; this might allow developing an effective low cost and eco-friendly larvicides. It can serve as a promising alternative for the management of mosquitoes.

CONCLUSION

A few studies have reported that the species of the genus lavandula had insecticidal activity against various mosquito species (Diptera: Culicidae). This study has examined the chemical composition of *L. stoechas* growing in wilds of Morocco and has demonstrated its larvicidal effect on *An. labranchiae* mosquito larvae in stages 3 and 4. The GC/MS analysis of *L. stoechas* essential oil allowed identifying major components including Camphor (36.14%), 1,8-Cineole (25.16%), Camphene (11.44%) and Fenchone (9.08 %). The study of larvicidal effect of *L. stoechas* essential oil on *An. labranchiae* has presented an LC_{50} and LC_{90} concomitant with 112.51 and 294.51 mg/l concentrations respectively. This larvicidal activity could be due to the major constituents. Thereby, *L. stoechas* presents an important potential to control against Anopheles larvae especially *An. labranchiae* which constitutes a serious threat to public health in Morocco. Therefore, this EO of *L. stoechas* and/or its fractions could be a promising alternative against mosquitoes.

CONFLICT OF INTERESTS

We declare that we have no conflict of interests.

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