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Research Article

Study of Phytochemical Screening and Larvicidal Efficacy of Ehtanolic Extract of *Salvia officinalis* (Lamiaceae) from North Center of Morocco Against *Culex pipiens* (Diptera: Culicidae) Vector of Serious Human Diseases

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ABSTRACT
Mosquitos, including *Culex pipiens* (Diptera: Culicidae), are the responsible vectors for the transmission of several deadly disease: Dengue fever, Japanese encephalitis, Filariais, Yellow fever… The use of synthetic larvicidal cause's negative effects on organisms and environment. Most of mosquitoes such as *Culex pipiens*, are becoming resistant to these chemicals. In the present study, the ethanolic extract from *Salvia officinalis* grown in Morocco was analyzed and studied for its insecticidal effect on the *Culex pipiens* as well as has been compared against Temephos, the insecticide which is currently used for larval control. The phytochemical investigation was studied using a simple qualitative analysis method and the biological test was performed using a methodology inspired the WHO standard protocol. The percent yield of the hydro-ethanolic extract from aerial parts of *Salvia officinalis* was 12.3 ± 1.2%. Presence of flavonoids, tannins, catechic tannins, sterols and terpenes, glycosides**,** gallic tannins and mucilage have been observed. The biological test revealed that the extract from *Salvia officinalis* has remarkable larvicidal properties. The minimum levels necessary to achieve 100% larval mortality of *Culex pipiens* was valued at 600 ppm. The LC_{50} and LC_{90} lethal concentration measured for the extracts of *Salvia officinalis* appears to be effective with respective values of about 287 ppm and 487 ppm. The insecticidal activity of ethanolic extract from *Salvia officinalis* plants against *Culex pipiens* has not been studied previously in Morocco. This extract has provided valuable mortality of *Culex pipiens* and can be used as botanical insecticides in integrated management programs of this mosquito vector of diseases.

Keywords: *Salvia officinalis,* Phytochemical screening, *Culex pipiens*, Larvicidal activity, North center Morocco.

INTRODUCTION

Several species belonging to mosquitoes (Diptera: Culicidae), such as species *Culex pipiens* (*C. pipiens*), are vectors for the pathogens of various diseases like Yellow fever, Filariasis, Japanese encephalitis and Dengue haemorrhagic fever. These diseases are continuing to be a major public health problem. WHO has announced the mosquito as "Public enemy number one" because they are responsible for the transmission of various dreadful diseases[1]. Chemical control is an effective strategy used extensively in daily life[2]. However, in the worldwide the widespread use of synthetic insecticides has resulted, the environmental hazards and also extension and development of physiological resistance among vector mosquito species. *C. pipiens*, the species which was strongly suspected in the transmission of epidemics that have affected Morocco in 1996[3,4], 2003[5] and recently in 2010 has developed resistance to synthetic insecticides[6].

In North center of Morocco, the species of *C. pipiens* are among the species inventoried[7] and has also presented recently a resistance to Temephos, the insecticide mostly used in larval control in Morocco[8]. In order to overcome this problem, scientific researchers have explored and examined other products (Entamopathogenic bacteria, Crop products,). Plant products (extracts, oils Essential and powder ...) are known and considered as a potential alternative approach for chemical insecticides because they are environmentally friendly, biodegradable and specific target[9]. Many plants of the genus of *Salvia* (Lamiaceae) are widely used in the traditional medicine for their medicinal properties such as to treat cancer, malaria, loss of memory and to disinfect homes after sickness[10,11]. *Salvia officinalis* (*S. officinalis*) belonging to the genus of *Salvia* (Lamiaceae), is commonly known as sage and is a potent medicinal herb with many activities[12-14]. Some studies have been shown that the extracts of

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several species of this plant could be used as potential insecticides[15,2]. Indeed, some authors have recently reported that this plant possessed a larvicidal effects against the mosquitos (Diptera: Culicidae) especially Culex sp.[16,2,9]. In Morocco, there is no work reported on the larvicidal activity of the *Salvia officinalis* against mosquito's larvae on *C. pipiens.* The aim of our study was to determine the phytochemical screening of the ethanolic extract of *S. officinalis* from North center of Morocco and to evaluate its insecticidal activity against larvae of *C. pipiens* as well as to compare it with that of temephos, the organophosphorus insecticide currently used for larval control. The results of this work may prove helpful in developing effective and ecofriendly larvicides, as favorable alternatives for the management of mosquitoes.

MATERIALS AND METHODS

Plant material, authentication and the Ultrasound-assisted extraction

A sample of *S. officinalis* (leaves, stems and roots) was collected in February and March 2014 from natural habitat in province of Taounate (North east of Morocco). Plant has been identified and a specimen was deposited in the herbarium of The National Institute of Medicinal and Aromatic Plants of the city of Taounate falling within of the Sidi Mohamed Ben Abdellah University, Fez, Morocco. A total of 20 g of a dried plant powder was mixed with 150mL of hexane in a beaker of capacity (500ml). The beaker was set in a Sonicator brand "ELMA" a frequency of 35 kHz for 45 min, with a temperature of 25∘C. The extract was filtered through Whatman paper and the recovered solvent was rejected. Drying the powder in a plant incubator at a temperature of 40∘C for 30mins, the powder was re-extracted again with ethanol at 80% for 45min under the same conditions. The final extract was recuperated from the mixture (ethanol/water) after filtration by Whatman paper and evaporation under vacuum at 40∘C on a rotary evaporator[17].

Phytochemical Screening

The hydro-ethanolic extract was screened for phytochemical constituents (tannins, catechic tannins, gallic tannins, coumarins, flavonoids, sterols and terpens, glycosids and mucilages using a simple qualitative method as described in the studies of Diallo[18] and Paris et al[19].

Characteristic of breeding site

The collection of larvae of *C. pipiens* was performed in a breeding site located in the urban area of the city of Fez, named "Hafat My Driss" (395 m altitude; 34°04'00.76'' latitude and 4°59'28.96'' longitude). This site corresponded to reed beds with a homogeneous population and very high density of *C. pipiens* larvae.

Collecting C. pipiens larvae

Larvae were collected using a rectangular plastic tray inclined 45 °C with respect to the water surface. Larvae harvested were maintained in breeding in rectangular trays at 23 ± 2°C, 70 ± 10% relative humidity in the Entomology Unit at the Regional Diagnostic Laboratory Epidemiological and Environmental Hygiene falling within Regional Health Directorate of Fez.

Morphological identification of larvae

Larvae were determined using a Moroccan Culicidae identification key[20] and the Mediterranean Africa mosquitoes identification software[21].

Protocol for larvicidal efficacy of plant extracts of S. officinalis on C. pipiens.

Biological tests were inspired from standard World Health Organization protocol slightly modified[22]. A stock solution (0.1 mg/mL=100ppm) and dilution series for *S. officinalis* extract, was prepared. Preliminary experiments were used to select a range of concentrations to be tested. About 1 mL of each solution prepared was placed in beakers containing 99 mL of distilled water in contact with 20 larvae of stadium 3 (L3) and 4 (L4)[22], and the same number of larvae was placed in a beaker containing 99 mL indicator of distilled water plus 1 mL of ethanol. Three replicates were carried for each dilution and for the control. After 24 h contact, living and dead larvae were counted. The results of susceptibility testing sites were expressed in percentage of mortality versus concentrations of essential oils used. If the % mortality in controls is greater than 5%, the % mortality in larvae exposed to the essential oil is corrected by using Abbott's formula[23].

% Mortality Corrected = [(% Mortality Observed - % MortalityControl) / (100 - % Mortality Control)] × 100.

If the control mortality exceeds 20%, the test is invalid and must be repeated. Temephos, the insecticide mostly used in larval control in Morocco, was considered as the positive control.

Data processing

Probit analysis of concentration-mortality data (Windl version 2.0) software developed by CIRAD-CA/MABIS was conducted to estimate the LC_{50} values[24] and associated 95% confidence limits for each treatment[25]. The analysis of the averages and standard deviation was also performed by using the test of analysis of variance ANOVA. Mean and standard deviation (± SD) were determined from at least three independent experiments.

RESULTS

Yield and Phytochemical screening of S. officinalis

The percent yield of the hydro-ethanolic extract from aerial parts of *S. officinalis* was 12.3 ± 1.2%.

Table 1 shows the results of the phytochemical screening of extract of *S. officinalis* from North center of Morocco. The outcomes revealed a presence of flavonoids, sterols, terpenes, tannins, catechic tannins, gallic tannins, glycosids and mucilages components. The coumarins were not detected.

Larvicidal activity, LC_{50} and LC_{90} lethal concentrations

The results of the larvicidal activity are presented in Table 2. The ethanol extract of *S. officinalis* was found to be effective with a LC_{50} value of 287 ppm and 487ppm for LC_{90} against the larvae of *C. pipiens.* The minimum levels necessary to achieve 100% larval mortality of *C. pipiens* was valued at 600 ppm. No mortality was observed in controls. However, the concentration of 0.5

Table 1: Phytochemical screening of hydro-ethanolic extract of *S. officinalis*

S. officinalis	Ethanolic extract
Tannins	+
Catechic tannins	+
Gallic tannins	+
Flavonoids	+
Sterols and terpenes	+
Coumarins	-
Glycosids	+
Mucilages	+

(+) = present ; (−) = absent

Table 2: Mean percent mortality ± SE of hydro-ethanolic extract of *S. officinalis* at six concentrations when compared with Temephos against third-fourth instar *C. pipiens*.

Dose (ppm)	*S. officilinis*
Control	0 ± 0.0
100	11.66 ± 2.22
200	30 ± 3.33
300	48.33 ± 2.22
400	72.89 ± 1.92
500	94.82 ± 0.12
600	100 ± 0.0
0.5 (Temephos)	100 ± 0.0

ppm of chemical insecticide (Temephos) has resulted to 100% mortality of *Culex pipiens*. Table 3 demonstrates that *S. officinalis* hydro-ethanolic extract has recorded an LC_{50} of order of 287 ppm for (which varies between a lower limit 117 ppm and an upper limit of 374 ppm) and an LC_{90} of 487 ppm (which also varies between a minimum of 374 ppm and a maximum value of 1321 ppm). Table 3 also shows the weighted regression equation of the line and Chi^2. The regression analysis indicates that the mortality rate is positively correlated with *S. officinalis* concentration. The adjustment model of tested data (Chi^2 Test), which is not significant at 5%, showed a good model fit.

DISCUSSION

In Morocco, the chemical insecticides utilized, especially Temephos, were caused a major problem in development of resistance by certain mosquitoes (Diptera: Culicidae) such as *C. pipiens*. Recently and in order to palliate this resistance phenomenon, we have conducted research works and have examined alternatives methods in use of chemical insecticides based on natural products. Various studies conducted on plant extracts originating in central Morocco, have showed larvicidal effects on mosquitoes (Diptera: Culicidae) in particular on *C. pipiens*[26,27] and on *Anopheles labranchiae*[28-31]. This work is part of this series of studies looking for alternative natural products: Bio insecticides. Throughout human history, plants were considered to have secondary metabolites that give them an important role in the defense purposes. Thus, various plant extracts and phytochemicals have been considered as potential sources of commercial mosquito control agents. They are biodegradable and due to their multi-component nature are less prone to the development of resistance[32]. Morocco is one of the Mediterranean countries with a long tradition based on medicinal and aromatic plants[33]. It presents a rather important floristic richness through to changes in climate and ecological conditions. Among the existing 4500 plant species, over 280 plants are currently valued[34]. The Lamiaceae in Morocco are an important botanical family that includes about 226 species growing in Morocco[35]. Among the species of this family, we find *S. officinalis* L. which is a perennial plant (subshrub), native to the Mediterranean region and is now grown all over the world. In addition, it has been reported that *S. officinalis* exhibits CNS (Central Nervous System) acetylcholine receptor activity[36]. Let's remember also that, the genus *Salvia* (Lamiaceae) is one of the largest genera of flowering plants, with nearly 1000 species in the World, largely cultivated for aromatic and culinary usage[37]. About the yield of hydro-ethanol extract of the aerial parts of *S. officinalis* found in this study, it is in the range of 12.3 ± 1.2%. In accordance with our result, Balouiri et al., in 2014[38] have reported that they had found the methanol extract yield, for *S. officinalis* collected from the same region that our plant, of the order of 13.88 ± 1.24. Contrary to these results, a study conducted recently by Bouharb et al., 2014[39], has found the yield of aqueous and ethanol extracts of dry plants per 100g for *S. officinalis*, from the massif of Zerhoun in central Morocco distant about 60 km from the city of Fes, in the order of 2.33% and 0.4%. Those yields are much lower than the our. According to the same study[39], these authors also concluded that water was the best solvent in the concentration of plant active ingredients and for all plants studied the aqueous extract is better than the ethanol extract. Concerning the phytochemical screening of *S. officinalis*, the literature reports that the family of (Lamiaceae) is an important source of essential oils, phenols and flavonoids[40]. Several kinds of secondary metabolites have been isolated from *Salvia* species. However, most characteristic secondary metabolite constituents of *Salvia* species are terpenoids and flavonoids[11]. Our outcomes of phytochemical screening of the extract of *S. officinalis*, are agree with the findings of these studies. Our results are also in accordance with other studies related to *S. officinalis*. Indeed, we cite for example: a phytochemical screening by chemical simple test of hydro methanolic extract of *S. officinalis* leaves realized recently in Algeria by Mekhaldi et al[41] showed a presence of flavonoids (caffeic acid, quercetin and luteolin), triterpenoids and steroids (β-sitosterol, β-amirin) and tannins. The proceedings of Balouiri et al[38], reported that the aerial parts of *S. officinalis* contained only the flavonoid. Simillarly, Ramu et al[42] concluded that methanolic extract of *S. officinalis* (species from India Flora) contains a mixture of phytochemical classes as flavonoids, tannins, terpenoids and steroids. This difference recorded in the results of yields found previously and in phytochemical screening, could be attributed to the age of the plant, the extraction technique (Maceration, Sonication, Soxhlet and classical method)[43], the nature of solvent (as has been observed in the

Table 3: Lethal concentrations LC_{50} and LC_{90} of larvae of *C. pipiens* after 24h.

Plant species	LC_{50} (ppm)	(Ll-Ul)*	LC_{90} (ppm)	(Ll-Ul)*	Equation of the regression line	Calculated *Chi*-square (χ2)
S. officinalis	287 (117- 374)		487 (374 -1321)		$Y = -13.75489 + 5.59398 * X$	19.518

* Ll-Ul: Lower limit-Upper limit.

previous study) and other factors such as climate change and ecological conditions. Regarding the larvicidal activity of plant extracts of *S. officinalis* on *C. pipiens*, we wish to remind that after exposing larvae of the species *C. pipiens* to different concentrations of ethanolic extract for 24 hours, we have found that the mortality rate varies according to the concentrations (Table 2) and the larval mortality rate reached 100% at a concentration of 600ppm. The value of LC_{50} (287ppm) that we found in this study is higher than that found by some studies. Indeed, Roman Pavela[16] has shown that the extract of *S. officinalis* possesses larvicidal activity against *Culex quinquefasciatus* (LC_{50} :159ppm) whereas in other work, Cetin et al. in 2006[44], have studied the larvicidal activity of ethanol extracts of the aerial parts from five Labiatae (Lamiaceae) whose species *Salvia sclarea*, obtained from Turkey, against *C. pipiens* and have found a very low lethal concentration (LC_{50}) of order of 62.7 ppm. According to studies conducted in our laboratory[8] the sensitivity of the species *Culex pipiens* towards the chemical insecticide (Temephos), this latter remains largely effective with an LC_{50} recorded between 0.0065 and 0.0094 ppm. Note that there is practically a few study describing the larvicidal activity of hydro-ethanolic extract of *S. officinalis* on *C. pipiens.* In Morocco, this is the first study to be conducted. By cons, several studies have reported the insecticidal activity of essential oils of *S. officinalis* on *Culex pipiens* larvae (Diptera: Culicidae)[45-47]. Most of these studies have described that the essential oil of *S. officinalis* is effective against this species of mosquitos. The difference on the larvicidal activity observed in the previously cited works compared to our results could be explained and linked to a number of factors like : the time and gathering sites, the environmental conditions, the plant age, the extraction technique, the concentration of the extract, the concentration of its active components, or even factors regarding the mosquito can influence the performance of the extract[26]. On the other hand, the efficiency of ethanol extract of *S. officinalis* against the *C. pipiens* could be explained by the action or effect of phytochemical components : flavonoids, tannins sterols, terpenes and mucilage. Indeed, flavonoids have a key role in stress response mechanisms in plants. The adaptive role of flavonoids in plant self protection against bacterial, fungal, and viral diseases as well as insects starts to gain importance in the understanding of plant defense[26]. Recently, Keerti et al[48] have reported that flavonoid extracts from different parts of two selected plants possess larvicidal activity against two selected mosquito species (*Aedes aegypti* and *Anopheles stephensi*) and could be utilized for developing flavonoid-based, ecofriendly insecticide as an alternative to synthetic insecticides. Abu Raihan[49] in his paper, indicated that many of the plant toxins are flavonoids and that they are generally responsible for resistance of plants to insect attack. In contrast, some authors[50,51] have confirmed that, tannins, a toxic component present dominantly in plants and vegetables, and their by-products are well known in degrading aquatic habitat and inflict mortalities in aquatic organisms. Others works[52,53], long before, have reported an interesting background regarding the use of tannins in the leather industry and also demonstrated the toxicity of the tannery effluent to the larvae of the mosquito. For treated insects several reports indicate that monoterpenoids causing their deaths by inhibiting the enzyme acetylcholinesterase[54,55]. It is ultimately the mixture of all these compounds (in our case : flavonoids, tannins, sterols and terpenes) and/or their synergy which is responsible for the activity of the plant. Further investigations to validate these observations and to complete the results are necessary.

CONCLUSION

The hydro-ethanolic extract of *S. officinalis* examined in this study showed an interesting activity on larvae of mosquito (Diptera: Culicidae) in stage 3 and 4. Indeed, it was found effective, with an LC_{50} of 287ppm and LC_{90} of 487ppm. It offer great potential as new control agents against Culex larvae especially *C. pipiens*, which is considered as a serious threat to human health in World and in Morocco. The aromatic plant extracts tested in our study demonstrated potent larvicidal properties that have potential to be developed further as natural insecticides. Nevertheless, more studies are required to be done before the *S. officinalis* of extract could be exploited at commercial scale. We plan to continue this study to clarify the nature of the compounds responsible for the activity in parallel with biological tests.

CONFLICT OF INTERESTS

We declare that we have no conflict of interests.

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