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Original Article

LARVICIDAL ACTIVITY OF ESSENTIAL OILS OF *THYMUS VULGARIS* AND *ORIGANUM MAJORANA* (LAMIACEAE) AGAINST OF THE MALARIA VECTOR *ANOPHELES LABRANCHIAE* (DIPTERA: CULICIDAE)

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ABSTRACT

Objective: We evaluated the properties of larvicidal activity of essential oils of *Thymus vulgaris* and *Origanum majorana* family of Lamiaceae collected at Taounate province in the North East of Morocco, against the larvae of the malaria vector *Anopheles labranchiae* (Diptera: Culicidae). There are no published data on the effect of these plants on this mosquito, formerly responsible for indigenous malaria.

Methods: Biological tests were realized according to a methodology inspired from standard WHO protocol, slightly modified, using the Ethanol as a solvent instead of DMSO. The method consists of making expose the larvae of stages 3 and 4 of the species *An. labranchiae* to the various concentrations of essential oils. After 24 h of contact, we counted living and dead larvae.

Results: The mortality percentages were determined after 24 h. Lethal Concentrations (LC_{50} and LC_{90}) were calculated and measured. They were respectively of the order of 351.63 µg/ml and 621.34 µg/ml for the essential oil of *Thymus vulgari*; whereas *Origanum majorana* were found of the order of LC_{50} = 107.13 µg/ml and LC_{90} = 365.9 µg/ml.

Conclusion: The results could be useful and interesting for the new application in the production of biocides against the larvae of *Anopheles.* Also, this information will be needed for the control program and for improving the vector control practices.

Keywords: *Thymus vulgaris*, *Origanum majorana*, Larvicidal activity, *Anopheles labranchiae*, Biological tests, North Eastern Morocco

INTRODUCTION

Mosquitoes, being vectors for many tropical and subtropical diseases, are the most important single group of insects which are well-known for their public medical importance [1].

Mosquito-borne diseases are prevalent in more than 100 countries across the world, infecting over 700,000,000 people every year globally [2]. They act as a vector for most of the life-menacing diseases like malaria, yellow fever, dengue fever, chikungunya fever, filariasis, encephalitis, West Nile virus infection, etc. [2].

Human malaria caused by an infection with protozoa of the genus Plasmodium continues to be the most important vector-borne disease which is transmitted only by females of the genus *Anopheles* [3].

The statistical data estimates that, the number of malaria episodes recorded per annum is 216 million, 81% of cases are in Africa [4]. The children less than 5 y and pregnant women constitute the portion of the most vulnerable population [4].

The impact of epidemics could be minimized through prediction, as well as prevention by means of regular vector control, and deployment of appropriate control measures [5].

In 2004, Morocco recorded the last case of indigenous malaria [6]. Since then, to prevent a return of the malaria transmission, a vector control program has been implemented [6]. It is based mainly on the fight against the larvae of the main vector *Anopheles labranchiae (An. labranchiae)* (Falleroni 1926) [7].

A research on Culicidae, which was conducted in the region of Fez in the North center of Morocco showed that the species of Anopheles genus are found almost throughout the year, particularly the *An. labranchiae* vector of indigenous malaria [8].

Thus, the presence of these species could be a menace to the population of the region of Fez that has the particularity of combining a number of risk factors notably; having a semi-arid bioclimate and hosting foreign residents coming from many African, Asian and European countries [9], who are affected by many diseases transmitted by species of the genus of Anopheles [10].

To fight against these vectors of diseases, insecticides are the most widely used products, but they have several disadvantages since they can be the source of various environmental problems [11-13]. Indeed, most chemical insecticides utilized cause a major problem, especially, developing mosquitoes' resistance [14-16].

To face this phenomenon of resistance which has become the main obstacle to the prevention and treatment of malaria, a comparison of natural products to synthetic chemicals makes it possible to exploit better the natural bio-insecticides.

Several herbs and various essential oils from plants, that possess potential insecticidal effect, were as well documented to have high toxic activity against microbes and insects. *Thymus vulgaris* (*T. vulgaris*), for example, is widely used as an antitussive, antispasmodic, antibroncholitique, anthelmintic, carminative and diuretic as well as insecticides [17, 18].

Also, the literature reports that the species of *Origanum majorana* (*O. majorana*) attracted the attention of consumers due to its antimicrobial, antifungal, insecticidal and antioxidative effects on human health [19].

It is within this framework that this work, carried out for the first time in Morocco, aims to study the larvicidal activity of the essential oils of *T. vulagaris* and *O. majorana* (Lamiaceae), cultivated in the North East of the country, on *An. labranchiae*, the vector of indigenous malaria.

MATERIALS AND METHODS

The vegetal matter of *T. vulgaris* and *O. majorana* (Lamiaceae) were collected during the months of February and March 2014 in the town of Taounate (North East of Morocco). The samples of plants studied were identified, and a specimen of each species 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. 200g of the biomass of the vegetal matter of *T. vulgaris* and *O. majorana* were subjected to a hydrodistillation for 3 h, using a modified Clevenger Apparatus. Essential oils obtained from decantation at the end of the distillation were dried over anhydrous sodium sulfate to remove traces of residual water. The obtained gasoline were placed in small vials and stored in an opaque 4 °C container before their use.

Characteristics of breeding site

The collection of the larvae of *An. labranchiae* was performed in a breeding site located in the urban area of the city of Fez, called Ain Boukhnafer (1132 m altitude, 34 °01´35´´N and 5 °11´44´´E), with an area of 22500 m^2. This site is characterized by a high density of larvae belonging to Culicidae. The dominant vegetation in this site is composed of Roseau and Weed that promote the proliferation of larvae of *An. labranchiae*.

Collecting larvae *Anopheles labranchiae*

The larvae, which were collected using a rectangular plastic tray, were maintained therein breeding at an average temperature of 23.4 °C±2 °C in the Entomology Unit at the Regional Diagnostic Laboratory of Epidemiological and Environmental Hygiene (RDLEH) falling within the Regional Directorate of Health in the city of Fez.

Identification of larvae

The identification of morphological characters of the larvae were determined using the identification key of the Moroccan Culicidae [20] and the software of mosquitoes' identification of Mediterranean Africa [21].

Larvicidal bioassays

Biological tests were realized according to a methodology inspired from standard WHO protocol, slightly modified, using the Ethanol as a solvent instead of DMSO. We prepared a stock solution (1% or 1000 µg/ml) of each essential oil in Ethanol and a dilution series: 100, 200, 300, 400, 500 and 600 µg/ml for *T. vulgaris* and 50, 100, 200, 400, 500 and 600 µg/ml for *O. majorana*. Preliminary experiments were used to select a range of concentrations previously tested. 1 ml of each prepared solution was placed in a beaker containing 99 ml of distilled water in contact with 20 larvae at stages 3 and 4. The same number of larvae was placed in a beaker containing 99 ml of distilled water plus 1 ml of Ethanol. Three replicates were carried out for each dilution and similarly for the control. After 24 h of contact, we counted living and dead larvae. The results of susceptibility test were expressed in percentage of mortality in function of the concentration of the essential oil used. If the mortality percentage in control is more than 5%, the percentage of mortality in larvae exposed to the essential oil shall be corrected by using Abbott's formula [22].

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

If the control mortality exceeds 20%, the test is invalid and must be repeated.

Processing of data

For the entry and processing of data, we used the log-probit analysis (Windl version 2.0), a software developed by CIRAD-CA/MABIS [23]. The results were also expressed as mean±SEM (standard deviation) by using the test of variance ANOVA analysis.

RESULTS

Variation of mortality

After exposing the larvae of stages 3 and 4 of the species *An. labranchiae* to the various concentrations of essential oils for 24 h, the mortality rate ranged from 7.14 to 100% for *T. vulgaris* (fig. 1), and from 27.27 to 100% for *O. majorana* (fig. 2). Fig. 1 below shows the larvicidal effect of the essential oil of *T. vulgaris* on *An. Labranchiae.* We found 100% mortality of larvae at 600 µg/ml concentration. With the same concentration for *O. majorana*, we found 100% mortality of larvae of *An. labrancheae* (fig. 2).

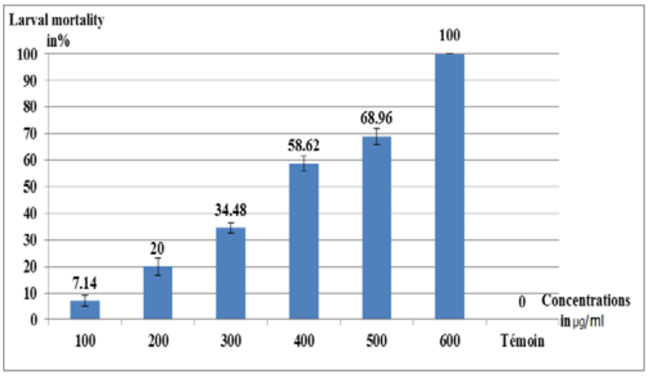

Fig. 1: Mortality (%) of larvae of *An. labranchiae* depending on the plant species *T. vulgaris* essential oil concentration after 24 h of exposure

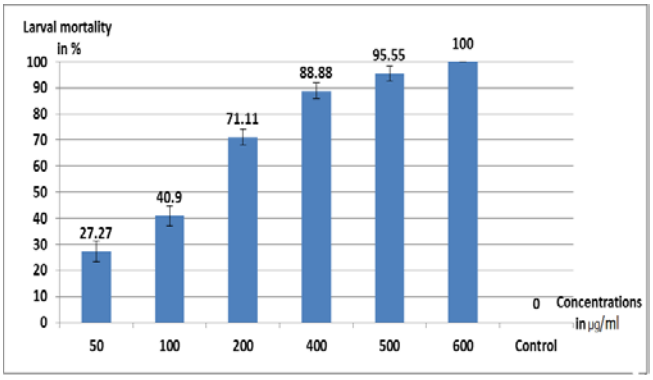

Fig. 2: Mortality (%) of larvae of *An. labranchiae* depending on the plant species *O. majorana* essential oil concentration after 24 h of exposure

LC_{50} and LC_{90} lethal concentrations

According to the table below (table 1), LC_{50} and LC_{90} show the larvicidal effect of the essential oils tested. The essential oil of *O. majorana* has the lowest LC_{50} of 107.13 µg/ml and an LC_{90} = 365.90 µg/ml (Equation of the regression line: Y =-4.87722+2.40268 * X; calculated Chi^2: 5.559). A larvicidal activity is also attributed to *T. vulgaris* with an LC_{50} of the order of 351.63 µg/ml and an LC_{90} = 621.34 µg/ml (Equation of the regression line: Y =-13.19903+5.18404 * X; Chi^2 calculated: 12.842). We noticed that the LC_{50} assigned to the essential oil of *O. majorana* is three times the LC_{50} recorded by *T. vulgaris*.

Table 1: Concentrations LC_{50} and LC_{90} lethal larvae of *An. labranhiae* after 24 h of exposure

Plant species	LC_{50} (µg/ml) (Ll-Ul)*	LC_{90} (µg/ml) (Ll-Ul)*
T. vulgaris	351.63 (36.8533-497.063)	621.34 (454.042-1009.65)
O. majorana	107.13(14.2951-201.679)	365.90(188.102-510.679)

* Ll-Ul: Lower limit-Upper limit, According to the shown LC_{50} (and LC_{90}) values (table 1), there are no significant differences between the tested essential oils (CI95 overlap).

DISCUSSION

The literature reports that several compounds obtained from plants possess insecticidal potential, growth deterrent or repellent characteristics [24, 25].

Essential oils and their volatile constituents are widely used in the prevention and treatment of human illnesses. Various essential oils were also documented to show acute toxic effects against microbes and insects, including mosquitoes [26].

In our study, realized for the first time in Morocco, the two essential oils of *T. vulgaris* and *O. majorana* demonstrate an interesting larvicidal activity. This activity could be due to their chemical composition consisting mainly of chemical substances, known for their larvicidal properties.

Let us note that the analysis, identification and determining the relative percentage of the composition of the various constituents of *T. vulgaris* and *O. majorana*, used and tested on *An. labranchiae*, were performed by gas chromatography coupled with mass spectrometry (GC-MS) [27,28].

This analysis revealed that the essential oil of *T.* vulgaris contains the thymol with 41.4%, the dominant monoterpene fraction with 97.35 %, consisting of 46.5 % in the oil form and 50.85% in oxygenated compounds form; hydrocarbons sesqui-tepéniques represent only a small percentage of 1.7% [27]. Whereas, the oil of *Origanum majorana* was found composed mainly of monoterpene and sesquiterpenes. The major components are 4-terpinene (28.96%), γ-terpinene (18.57%), α-terpinene (12.72%) and sabinene (8.02%) [28].

Thus, the important larvicidal activity observed among the essential oil of *T. vulgaris* might be explained by the action or the effect of the major components. Indeed, the oil of *T. vulgaris*, is characterized by a high quantity of thymol [27], while in the case of oil *O. majorana*, the dominant constituent is 4-terpinene, γ-terpinene and α-terpinene [28].

It is noteworthy that, the species of plants has a number of very different chemo types, and sometimes, the share of the principal constituent may be only minor in the essential oil. Although until the present, major substances have been considered as responsible, the synergism of multiple substances may actually be very important in terms of biological activity, including substances in minor quantities.

Szczepanik and al. [18], reported that essential oils and their major components, mainly the monoterpenoids, are potential sources of ecologically safe botanical insecticides.

On the other hand, Bakkali and al. [29], indicated that the essential oils are very complex mixtures as they contain about 20-60 components in different concentrations. Moreover, they (oils) typically consist of two or three major components present in higher concentrations (20-70%) than other components present in trace amounts. Generally, the main components determine the biological properties of the essential oil they were isolated from.

Thymus is a taxonomic group of plants which contains different species. In fact, it should be noted that 350 species were reported from this genus [30]. The antibacterial, insecticidal and antifungal properties of *thymus* species were reported in various studies [31, 17, 18]. *Thymus vulgaris* is an aromatic plant which is native to the north central regions of Morocco. It was illustrated that thymol (41.4%), γ-terpinène (22.25%), p-cymène (15.59%) and carvacrol (2.06%) are the major constituents of this plant [17, 28].

To our knowledge, the study of the insecticidal activity of the essential oil of *T. vulgaris* against the larvae of *An. labranchiae* has never been studied.

The insecticidal activity of the oil of *T. vulgaris* can be considered acceptable when we consider that more than 90% of *An. labranchiae* larvae were killed using 621.34 µg/ml of the essential oil (LC_{50}: 351.63 µg/ml).

In the literature, it has been shown that *Thymus* species have strong insecticidal activity against various insects such as *Aedes albopictus* [32], *Aedes aegypti* (L) [33], *Tribolium castaneum* and *Callosobruchus maculates* [34], and *Sitophilus oryzae* [35].

Similar studies by Tchoumbougnang *et al.* [36], demonstrated the insecticidal activity of popular *Thymus* collected in Cameroon on larvae *Anopheles gambiae*. The LC_{50} obtained was 119±1.5 µg/ml, and CL_{90} was 147±2.4 µg/ml. Recently, Dargahi and al. [37] studied the larvicidal activity of *Thymus transcaspicus*, an aromatic plant of *Thymus* genus, against *An. stephensis*. The highest toxicity was observed at 250 µg/l of essential oil with the LC_{50} values of 134.1µg/l after 24 h.

Pavela and al. [26], conducted a study about mosquitocidal activities of thymus oils (*Thymus vulgaris* L.) on *Culex quinquefasciatus* (Diptera: Culicidae). It is apparent from the results of this research, as well as that of other authors, that the essential oils made from *T. vulgaris*, especially chemo type T, have strong potential in the production of new larvicides and adulticides that are safe for the environment and for human health.

On the other side, an insecticidal activity of *T. vulgaris* essential oil and its components (thymol, γ-terpinène, p-cymène, and carvacrol) was conducted but against larvae of another insect family of (Coleoptera: Tenebrionidae) [18]. The results of this study showed that the components of *T. vulgaris* essential oil (thymol, carvacrol, and thyme) were more active against the larvae of this insect.

About the Origanum plant, it was reported that the dried Origanum species were used as stimulants, analgesics, antitussives, expectorants, sedatives antiparasitics, antihelminthics, antirheumatics and gastrointestinal troubles in folk medicine [38, 39, 19]. The genus Origanum is around 900 different species, and many species were extensively used for the flavoring of alcoholic beverages, food products, and in perfumery [40, 41].

O. majorana, like many other species of the genus Origanum, is an evergreen herbaceous plant belonging to the Lamiaceae family [41], and is found as a wild plant in the Mediterranean areas. Its oil consists mainly of monoterpene and sesquiterpenes. Indeed, the major components are 4-terpinene (28.96%), γ-terpinene (18.57%), α-terpinene (12.72%) and sabinene (8.02%) [28].

We have not found any work concerning the larvicidal activity of *O. majorana* on the species of *An. labranchaie*. The insecticidal activity of the oil of *O. majorana*, obtained in our study, is very significant if we consider that more than 90% of *An. labranchiae* larvae were killed due to their exposure to 365.90 µg/ml of the essential oil (LC_{50}: 107.13 µg/ml).

The very important larvicidal activity observed in the essential oil of *O. majorana* could be explained by the chemical composition of this oil and the action or the effect of the major components.

Recently, a work realized by El-Akhal *et al.* [28], demonstrated the insecticidal activity of *O. majorana* against the *Culex pipiens*, the West Nile virus vector. The LC_{50} and LC_{90} obtained were 258.71µg/ml and 580.49 µg/ml respectively.

The LC_{50} (107.13 µg/ml) and LC_{90} (365.90 µg/ml) of the essential oil of *O. majorana*, found in our study on the *An. labranchiae*, are significant when compared to those found from *T. vulgaris*. They are also very important if compared to those found from *O. majorana* on *Culex pipiens* in research works conducted in our laboratory [28].

The non-significant difference between the tested essential oils: *T. vulgaris* and *O. majorana* (CI95 overlapping), could be explained by their similar chemical composition [27, 28]. Indeed, the major components of the two essential oils, which belong to the same family (Lamiaceae), are the monoterpenes. In this context, the literature reports the larvicidal effect of monoterpene against mosquitos [18, 42].

The isolation of these major components and the study of their biological activity against mosquitos, especially Anopheles, could explain the overlap observed between these two essential oils: *T. vulgaris* and *O. majorana*.

The results found in this study are encouraging and highly interesting if we consider that it reported the case of crossresistance in *Anopheles* mosquito genera towards organophosphate, such as: Temephos, Malathion and Chlorpyrifos [43, 44].

In the central area of Morocco, the resistance of the *An. labranchiae* against synthetic insecticides has not been reported [6], but in another area of the country (Sidi Kacem–West of Morocco) the same authors [6] reported that the concentration of 0.125 mg/l to Temephos didn't give the 100% mortality in the population of Sbikh (Sidi Kacem), suggesting the emergence of the beginning of resistance.

It should also be noted, that the widespread resistance from other Culicidae species like *Culex pipeins* has been signaled, in central Morocco, against synthetic insecticides including Temephos, Malathion and other organophosphorus [16].

So, this study could contribute to assessing the possibility of using medicinal plants as potential insecticides. These data will undoubtedly help to find control alternatives based on plants in the city of Fez and at the national level, in the case of confirmed resistance of the anopheles of mosquitoes towards the chemical insecticides.

CONCLUSION

In this study, we evaluated the potential larvicide of the essential oils from two aromatic and medicinal plants (*T. vulgaris* and *O. majorana.*) cultivated in the North East of Morocco on the malaria vector *An. labranchiae*. The two samples showed a larvicidal activity against larvae in stages 3 and 4. The *O. majorana* essential oil is the most effective sample, with an LC_{50} of 107.13 µg/ml and LC_{90} of 365.90 µg/ml; (compared to the essential oil of *T. vulgaris* with an LC_{50} of the order of 351.63 µg/ml and an LC_{90} = 621.34 µg/ml). This result opens interesting perspectives for the application of these oils (*T. vulgaris* and *O. majorana.*) in the production of biocides.

We are planning to continue this work to determine the nature of the constituents which are responsible for the activity by fractioning as well as testing their biological performance.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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