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Research Article

Phytochemical Screening and Larvicidal Activity of Moroccan *Ammi visnaga* Against Larvae West Nile Vector Mosquito *Culex pipiens* (Diptera: Culicidae)

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ABSTRACT
Main method to control insect pest is using synthetic insecticides but the development of insect resistance to this products, the high operational cost, environmental pollution, toxicity to humans and harmful effect on non-target organisms have created the need for developing alternative approaches to control insect pest. *Culex pipiens* was strongly suspected as the vector responsible for transmission of several parasitic and viral diseases. In the North center of Morocco, this species has developed resistance to synthetic insecticides. There is an urgent need to find alternatives to the insecticides as natural biocides. In this work, the phytochemical screening and the insecticidal activity of the hydro-ethanolic extract of the *Ammi visnaga*, which has never been tested before in the North center of Morocco, was studied on larval stages 3 and 4 of *Culex pipiens*. The hydro-ethanolic extract is screened for phytochemical constituents using simple qualitative methods. Biological tests were realized according to a methodology inspired from standard World Health Organization protocol. The mortality values were determined after 24 h of exposure and LC_{50} and LC_{90} values were calculated. The percent yield of the hydro-ethanolic extract from aerial parts of *Ammi visnaga* was 6.4±0.2% and the phytochemical screening of the hydro-ethanolic extract of *Ammi visnaga* indicates the presence of flavonoids, tannins, catechic tannins, sterols, terpenes, coumarins, mucilages and glycosides. However, gallic tannins were not detected. The extract had toxic effects on the larvae of culicid mosquitoes. The hydro-ethanolic extract of *Ammi visnaga* applied against the larvae of *Culex pipiens* has given the lethal concentrations LC_{50} (lower limit-upper limit) and LC_{90} (lower limit-upper limit) in the order of 0.42 (0.14 – 0.52) mg/ml and 0.68 (0.59 – 1.20) mg/ml, respectively. This investigation indicates that *Ammi visnaga* could serve as a potential larvicidal, effective natural biocide against mosquito larvae, particularly *Culex pipiens*.

Keywords: *Culex pipiens*, larvicidal activity, *Ammi visnaga*, phytochemical screening, hydro-ethanolic extract, North East of Morocco.

INTRODUCTION

Mosquitoes are the most important of insects in terms of public health importance which transmit a number of diseases such as dengue, chikungunya, Japanese encephalitis, filariasis and malaria, causing millions of deaths every year[1]. The mosquito *Culex pipiens (C. pipiens)* L. (Diptera: Culicidae) is a common and abundant species in the world. This species serve as bridge vectors of the West Nile virus from birds to humans in the United States[2,3]. The main breeding or larval developmental sites of this species are septic tanks, artificial containers, animal watering basins, water irrigation channels and temporary pools[4]. In the years 1996, 2003, and 2010 and according to a research, outbreaks of West Nile virus infection were reported in Morocco[5,6]. The fight against vector-borne diseases means using chemical insecticides. However, the use of insecticides faces several serious problems today. In addition to the negative effects of synthetic insecticides on the environment and non-target organisms, including man[7,8], the development of resistant mosquito populations in particular is one of the most serious problems[9,10]. These problems have become the main impetus for an expeditious search for new alternatives, which would be acceptable for both the environment and health, for protection against insects. Among the existing alternative strategies aimed at decreasing vector populations, the use of pesticides based on plant extracts is currently one of the most promising[11]**.** The use of botanical insecticides is a plant and vector protection alternative, generally considered safe for the environment and health[12,13]. Therefore, significant efforts are currently being devoted

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to the search for new, highly efficient plant extracts which would be suitable for the development of botanical insecticides[14]. *Ammi visnaga* (*A. visnaga*) has many English common names, including Khella, Visnaga, Bisnaga, and Toothpick weed. It is a member of the Apiaceae (Umbelliferae) family; widely and wildly distributed in Asia, Europe, and North Africa[15,16] especially in Egypt, Morocco and Islamic republic of Iran[17]. Therefore, the purpose of this study was to assess the phytochemical screening of Moroccan *A. visnaga* and to determine of larvicidal activity of hydro-ethanolic extract (HEE) of this plant on *Culex pipiens* (*C. pipiens*). The insecticidal activity of *A. visnaga* plant against *C. pipiens* has never been studied before in the North center of Morocco.

MATERIALS AND METHODS

Crop plants and ultrasound-assisted extraction

A sample collection (leaves, stems and roots) of a local plant (*A. visnaga*) was conducted in April (2014) at the mountain of Timezgana falling within the rural community of Timezgana (area of Taounate, North center of Morocco) to an approximate altitude of 800m. In a 500mL beaker, 20 g of a dried plant powder was mixed with 150mL of hexane. 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[18].

Phytochemical screening

The hydro-ethanolic extract is screened for phytochemical constituents (tannins, catechic tannins, gallic tannins, coumarins, flavonoids, sterols, terpens, glycosids and mucilages) using a simple qualitative method as described in the studies of Diallo[19] and Paris et al[20].

Characteristics of larval site

The collection of larvae of *C. pipiens* was performed in a breeding site located in the urban area of the city of Fez, called "Grand Canal" (402 m altitude, 30°03'37" N and 5°08'35"E). This site, originating from a hot spring, is characterized by a very high density of Culicidae larvae. The warm water from a thermal spring called "Ain Lah" promotes the proliferation of larvae of *C. pipiens*.

Collecting larvae of C. pipiens

Larvae were collected using a rectangular plastic tray. The larvae gathered were maintained in breeding in rectangular trays at an average temperature of 21.7°C ± 2°C in the Entomology Unit at the Regional Diagnostic Laboratory Epidemiological and Environmental Health (RDLEH) falling within Regional Health Directorate of Fez.

Identification of larvae

The identification of morphological characters of larvae has been determined using the Moroccan key of identification of Culicidae[21] and the identification software of mosquitoes of the Mediterranean Africa[22].

Protocol of larval susceptibility testing

The susceptibility tests were carried out in accordance with the standard protocol developed by WHO[23]. From the initial extract (0.1 mg/ml stock solution) of plant, concentrations of 0.25, 0.35, 0.45, 0.55, 0.65 and 0.75 mg/ml were prepared. Preliminary experiments were used to select a range of concentrations for the tests previously mentioned. 1mL of each solution prepared was placed in beakers containing 99mL of distilled water in contact with 20 larvae of stages 3 and 4; the same number of larvae was placed in a beaker containing 99mL of distilled water plus 1ml of ethanol. Three replicates were carried out for each dilution and for the control. After 24 hours of contact, we counted the living and dead larvae. The results of susceptibility testing were expressed in the percentage of mortality versus the concentration of plant extract used. If the percentage of mortality in control is greater than 5%, the percentage of mortality in larvae exposed to the extract shall be corrected by using Abbott's formula[24]:

$$\%\ \text{Mortality Corrected} = \left[\frac{(\%\ \text{Mortality Observed} - \%\ \text{Mortality Control})}{(100 - \%\ \text{Mortality Control})}\right] \times 100$$

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

Data processing

For the data processing we used the log-probit analysis (Windl version 2.0) software developed by CIRAD-CA/MABIS[25]. The analysis of the averages and standard deviation was also performed by using the test of analysis of variance ANOVA.

RESULTS

Phytochemical screening and yielding extraction

The percent yield of the HEE from aerial parts of *A. visnaga* was 6.4±0.2%. As it is illustrated in Table 1, the phytochemical screening of extract of *A. visnaga*, grown in North center of Morocco, revealed a presence of tannins, catechic tannins, flavonoids, sterols, terpenes, glycosides, coumarins and mucilages components. However, Gallic tannins were not detected.

Variation in mortality rate

The sensitivity test by Hydro-ethanolic extract of *A. visnaga* on *C. pipiens* has allowed observing a mortality rate ranged between 12.68 % and 100% (Figure 1). The lowest concentration necessary to achieve 100% mortality of larvae of *C. pipiens* was evaluated at 0.75 mg/ml.

LC_{50} and LC_{90} Lethal Concentrations

Figure 1 confirms the analysis performed to the order of effectiveness of hydro-ethanolic extracts tested. The hydro-ethanolic extract of *A. visnaga* exhibits the lowest LC_{50} of 0.42 mg/ml (equation of the regression line: $Y = 2.32093 + 6.23790 * X$; calculated Chi^2: 11.462) and LC_{90} = 0.68 mg/mL (Table 2).

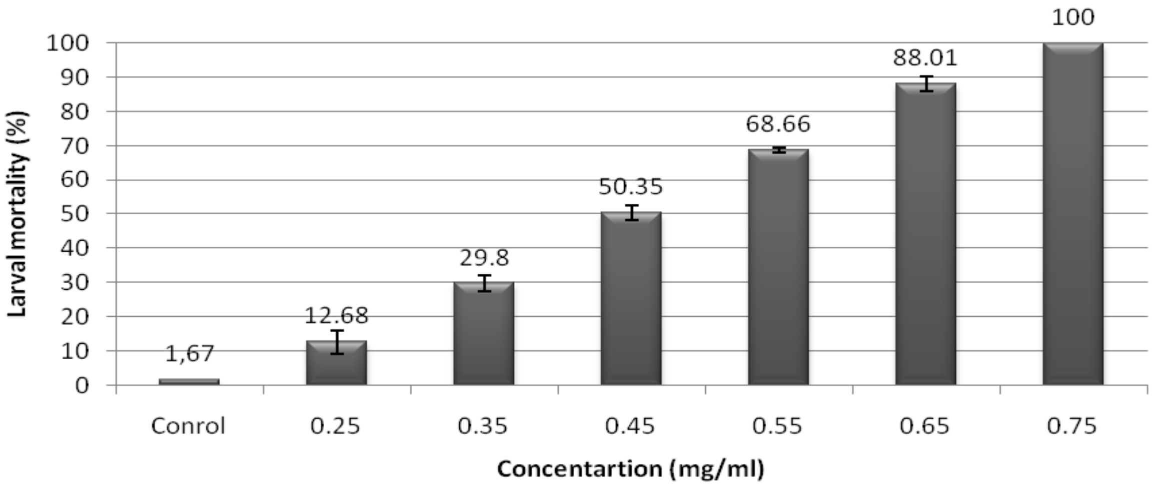

Figure 1: Percentage of mortality recorded in the larvicidal activity of hydro-ethanolic extract of *A. visnaga* on *C. pipiens*.

Table 1: Phytochemical screening of hydro-ethanolic extract of *A. visnaga*.

Phytochemical	Ethanolic extract
Tannins	+
Catechic tannins	+
Gallic tannins	-
Flavonoids	+
Sterols and terpenes	+
Coumarins	+
Glycosids	+
Mucilages	+

(+) = present; (−) = absent

DISCUSSION

Botanical phytochemicals with larvicidal potential are now recognized as potent alternative insecticides to replace synthetic insecticides in mosquito control programs due to their excellent larvicidal properties[1]. Our phytochemical results are in accordance with other studies related to *A. visnaga*. A study realized by Jaradat et al[26] in Palestine concluded that *A. visnaga* methanolic extract contains a mixture of phytochemical classes as proteins, tannins, flavonoids, glycosides and steroids. The results of Pavela et al[11], who mentioned that *A. visnaga* contain furanchoromones such as khellin and visnagin, coumarins and flavonoids. Simillarly, Stahl and Schild[27] reported that *A. visnaga* fruits contained γ–pyron coumarins and flavon derivative. Among the γ -pyrones, khellin and visnagin are the major ones. Khellinol, ammiol, visammiol, khellol, khellinin, khellinone, visnaginone are other important γ-pyrones. Coumarin is another important group of major constituents, the main one being the pyranocoumarins/visnagans comprising mainly of visnadin, samidin and dihydrosamidin. Coumarins also include furanocomarines (xanthotoxin and ammoidin) only in trace amount[28,29]. Two flavonols (quercetin and kaempferol) were identified in *A. visnaga* growing in Iraq[30]. Eleven flavonols were isolated from the aerial parts of *A. visnaga*[31]. There were four aglycones, four monoglycosides, two diglycosides and one triglycoside. Among the aglycones flavonoids, one was hydroxylated (quercetin) and three methoxylated (rhamnetin, isorhamnetin and rhamnazin). The monoglycosides were actually modified rhamnetin, isorhamnetin and rhamnazin with 3-O-glucosides and one 7-O-glucoside of isorhamnetin. The two diglycosides were 3-O-rutin of quercetin and isorhamnetin while the single trioside was quercetin 7,3,3'-O-triglucoside[31]. The composition of extracts of *A. visnaga* is generally influenced by several factors such as the harvesting period, the gathering sites, the agricultural practices, the plant age, the part of plant used and the extraction technique. In the present work, realized for the first time in the North center of Morocco, we are interested in examining the chemical composition of the hydro-ethanolic extract of the *A. visnaga* from Morocco, as well as studying its larvicidal activity against the *C. pipiens*. Taking into account the absence of studies on the extracts of *A. visnaga* against *C. pipiens* except a preliminary study realized by Aouinty et al[32]. During this study the researchers have not determined LC_{50} of the extract of *A. visnaga* against *C. pipiens* but they determined the lethal dose of the extract can kill 100% of the larvae (the lethal dose found is 4 %). We tried to compare the action of *A. visnaga* against the family on species of Culex. A study realized by Pavela[33] determined that the methanol extract from *A. visnaga* seeds had an excellent effect against *C. quinquefasciatus* larvae with a calculated LC_{50} of about 180 mg/ml, achieving 50% or 90% larval mortality within approximately 2 or 3h after application, respectively. Based on comparing the LC_{50} values of the khellin and visnagin (majors compounds extracted from *A. visnaga* and represented almost 60% of the extract), larvae of *C. quinquefasciatus,* khellin was found to be significantly more efficient (LC_{50}=10 mg/ml) compared to visnagin (LC_{50}=26mg/ml)[11]. The observed difference between LC_{50} of previous studies could be explained by many factors impacting the effects of larvicidal activity, namely: part of plant used[34], physico-chemical characteristics and chemical composition of the extract[35]. This efficiency could be explained by the action or effect of phenolic component (flavonoids, tannins, catechic

Table 2: Lethal Concentrations LC_{50} and LC_{90} of larvae of *C. pipiens* after 24 hours of exposure.

Plant	LC_{50} (LI-UI)* (mg/ml)	LC_{90} (LI-UI)* (mg/ml)
A. visnaga	0.42 (0.14- 0.52)	0.68 (0.59 – 1.20)

∗Ll-Ul: lower limit-upper limit.

tannins, sterols, terpenes, coumarins, mucilages and glycosides) against the *C. pipiens*. According Harborne et al[36], the flavonoids have a key role in the stress response mechanisms in plants. The role of adaptation flavonoids in self-protection of plants against bacterial, fungal, viral diseases and insects begin to become important in the understanding of plant defense[37]. Several studies have shown that flavonoids act as enzyme inhibitors or antioxidants. They are involved in the process of photosynthesis and transfer of cellular energy and can be used as toxic substances or precursors having a pharmacological activity[36,37]. Comparing our results with the work mentioned previously[11,33] we can deduce that the LC_{50} obtained by the larvicidal action of the *A. visnaga* plant grown in North East of Morocco on the larvae of the *C. pipiens* is very effective. That is why, the extracts of the aqueous phase of the *A. visnaga* grown in North center of Morocco should be subjected to separation to isolate and concentrate the active substances, which certainly can present much lower LC_{50} and would be valued as an alternative insecticide.

CONCLUSION

This study demonstrated that the hydro-ethanolic extract of A. visnaga containing: the flavonoids, the tannins, the catechic tannins, the sterols, the terpenes, the coumarins, the mucilages and the glycosides possesses larvicidal activity against harmful mosquitoes (*C. pipiens*). The Lethal Concentrations (LC50 and LC90) were calculated and measured and they were respectively of the order of 0.42 mg/ml and 0.68 mg/ml. We can recommend application of this potential botanical insecticide as larvicidal product against mosquito larvae. However, further studies on identification of active compounds, toxicity and field trials are needed to recommend the active fraction of these plant extract for development of eco-friendly for control insect vectors.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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