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Chemical composition and larvicidal activity of Moroccan Atlas Cedar (*Cedrus atlantica* Manetti) against *Culex pipiens* (Diptera: Culicidae)

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ABSTRACT

The aim of this research was to determine the larvicidal activity of essential oil of *Cedrus atlantica* Manetti from Moroccan Middle Atlas against *Culex pipiens,* one of the most widely distributed mosquitoes in the world. The essential oil was obtained from aerial part of plant by hydrodistillation. The biological test was performed using a methodology inspired the WHO standard protocol. The percent yield of the essential oil of *Cedrus atlantica* Manetti was 1.12±0.2%. The GC/MS analysis of essential oil from *Cedrus atlantica* Manetti revealed the presence of twenty five (25) compounds, representing 97.48 % of the total composition. The major group of compounds, the main one being α-himachalene (35.34%) followed by β-himachalene (13.62%), γ-himachalene (12.6%), cedrol (10.32%), isocedranol (5.52%) and α-pinene (5.5%). The Lethal Concentrations LC_{50} and LC_{90} measured for the Moroccan *Cedrus atlantica* Manetti essential oil appears to be effective with respective values of 782.43 ppm and 1253.93 ppm and the minimum levels necessary to achieve 100% larval mortality of *Culex pipiens* was valued at 1500 ppm. The larvicidal activity of essential oil of *Cedrus atlantica against Culex pipiens*, has not been studied previously in Morocco, may prove helpful in developing effective and ecofriendly larvicides, as favorable alternatives for the management of mosquitoes.

INTRODUCTION

Culex pipiens (*C. pipiens*) (Diptera: Culicidae) is one of the most widely distributed mosquitoes in the world. The species commonly referred to as "house mosquito", can be found in urban and suburban areas and lives near people (Bernard *et al*., 2001). Several commercially available insecticides (e.g. temephos, chlorpyrifos-methyl, diflubenzuron) can be effective to control the species at immature stages (Cetin *et al*., 2006a, 2006b). However, many of these chemical insecticides are expensive and harmful to the environment as well as to humans (Huseyin *et al*., 2009). The WHO expert committee (Samuel *et al*., 2013; WHO, 1982) felt the resistance in vectors was probably the "biggest single obstacle in the struggle against vector-borne diseases". Many mosquito species are known to have developed resistance to temephos in many parts of the world and also in Morocco (El-akhal *et al*., 2016; El Ouali Lalami *et al*., 2014; Cui *et al*., 2006; Braga *et al*., 2004; Faraj *et al*. 2002). Natural plant products can be an excellent alternative source of novel insecticidal chemistries. With some exceptions, botanicals are considered to be less toxic to non-target species and more environmentally friendly because of their biodegradable nature (El Ouali Lalami *et al*., 2016; Copping 1996). In Morocco, several studies have been carried out on the larvicidal effect of plants against *Culex pipiens*, for exemple *Origanum majorana* (El-Akhal *et al*., 2014),

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Lavandula stoechas (El Ouali Lalami *et al*., 2016), *Citrus sinensis* and *Citrus aurantium* (El-Akhal *et al*., 2014). Currently, the genus *Cedrus* includes three extant species native to the Mediterranean mountains (*Cedrus atlantica* Manetti from Algeria and Morocco; *Cedrus libani* Rich. in Asia Minor; *Cedrus brevifolia* Henry in Cyprus) and in the Himalaya (*Cedrus deodara* Don) (Farjon, 2008). The Atlas cedar is an endemic species of North Africa Mountains (Morocco, Algeria). In Morocco, the Atlas cedar occupies an area of 132,000 hectares divided into two blocks of unequal importance: the Rif; the Moroccan Middle and High Atlas (Cheddadi et al., 2009; M'hirit *et al*., 1993), presenting an altitudinal ranging from 1500m to 2600m. The Middle Atlas in northern Morocco contains about the 80% of the world's Atlas cedar forest area (Linares *et al*., 2011; Benabid, 1994).

This raw material would be important for essential oil production to be used for its medicinal properties and perfumery (Boudarene *et al*., 2004). Indeed, essential oils are a part of several products such as drugs and perfumes (Adams, 1991). The essential oil of *C. atlantica* Manetti (*C. atlantica*) has been shown to possess antifungal (Bouchra *et al*., 2003), antimicrobial (Zrira and Ghanmi, 2016), antiviral (Monica *et al*., 2008), and anti-inflammatory activities (Sugita *et al*., 2004), but its larvicidal activity is not reported against *C. pipiens* in the literature.

In this work, the chemical quality of the Atlas cedarwood oil obtained from the middle Atlas forest were studied and its insecticidal activity against larvae of *C. pipiens* was determined in the first time in Morocco.

MATERIALS AND METHODS

Plant material and extraction of the Essential Oil (EO)

The aerial parts (leaves, stems and wood) of *C. atlantica* (Fig. 1) are collected in Boulmane region (Middle Atlas Mountains, Morocco), between April and May 2014. The botanical identification and authenticated voucher specimens have been deposited in the Herbarium of National Institute of Medicinal and Aromatic Plants, Sidi Mohamed Ben Abdellah University, Fez, Morocco. Samples of 100g of the fresh aerial parts of *C. atlantica* were subjected to hydrodistillation for 2 hours using a Clevenger apparatus; the obtained Essential Oil (EO) was stored at 4°C so that can be used in the upcoming experiments.

Chemical characterization of essential oil of *C. atlantica*

The chromatographic analysis of essential oil was conducted in ''Centre Universitaire Régionale d'Interface'' (CURI) in Fez city. The gas chromatography (GC) analysis were performed using a Hewlett-Packard (HP 6890) gas chromatograph (flame ionization detector, FID), equipped with a HP-5 capillary column (30m x 0.25mm x 0.25 µm). The temperature was programmed from 50°C after 5 min initial hold to 200°C at 4°C min^{-1}. GC conditions were as follows: N_2 as carrier gass (1.7ml min^{-1}); split mode was used (flow: 66 ml min^{-1}, ratio: 1/50) and the injected volume was about 1 µl. The Gas Chromatography/Mass Spectrometry (GC/MS) analysis were performed by a Hewlett-Packard Gas Chromatographer (HP 6890) coupled with a mass spectrometer (HP 5973). Fragmentation was performed by electron impact at 70 eV. The carrier gas is helium whose flow is fixed at 1.5 ml/min. The injection mode was split (split ratio: 1/70). The apparatus was controlled by a "Chemstation" computer system. The identification of the components is based on the comparison of their mass spectra (GC/MS), respective with spectra of the library (NIST 98), of the bibliography (Adams, 2007), Kovats index for each compound on OPTIMA-5 column was calculated in reference to n-alkanes.

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 is characterized by a very high density of Culicidae larvae.

Collecting larvae of *C. pipiens*

Larvae were collected using a rectangular plastic tray in June 2014. 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 (Himmi *et al*., 1995) and the identification software of mosquitoes of the Mediterranean Africa (Brunhes *et al*., 2000).

Protocol of larval susceptibility testing

The susceptibility tests were carried out in accordance with the standard protocol developed by WHO in 2005 (WHO, 2005). From the initial essential oil (1 mL stock solution) of plant, concentrations of 250, 500, 750, 1000, 1250 and 1500 ppm 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 essential oil shall be corrected by using Abbott's formula (Abbott, 1925):

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

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

Table 1: Percent yield and physical characters of *C. atlantica* from Morocco.

Botanical name	Part used	Physical characteristics			
		Color	**Odor**	**Density (g/ml)**	**Yield(%)**
Cedrus atlantica Manetti	Areal part	Light Golden Yellow	dirty-woody, resinous, urinous odour	0.948	1.12±0.2

Data processing

For the data processing we used the log-probit analysis (Windl version 2.0) software developed by CIRAD-CA/MABIS (Giner *et al*., 1999). The analysis of the averages and standard deviation was also performed by using the test of analysis of variance ANOVA.

RESULTS AND DISCUSSION

Percent yield and physical characters of *C. atlantica*

The percent yield of the hydro-distilled volatile oil from aerial parts of *C. atlantica* and its physical characters are summarized in Table 1. The average yield of the cedar essential oil of Eastern Middle Atlas was 1.12±0.2%. Our result is lower than that obtained for the origin of the High Atlas (Morocco) (2.6%) (Rhafouri *et al*., 2014) and also of that of Djurdjuran region in Algeria (1.7%) (Boudarene *et al*., 2004). On the other hand our result is superior to that found by Mathieu *et al*. (2011) about *C. atlantica* growing in Corsica (0.05 –0.49%).

Chemical composition of the essential oil

The GC/MS analysis of essential oil extracted from *C. atlantica* revealed the presence of twenty five (25) compounds, representing 97.48 % of the total composition (Table 2). The major group of compounds, the main one being α-himachalene (35.34%) followed by β-himachalene (13.62%), γ-himachalene (12.6%), cedrol (10.32%), isocedranol (5.52%) and α-pinene (5.5%). The chemical composition of our oil revealed that was relatively similar to those of other *C. atlantica* essential oils analyzed by Teisseire and plattier, (1974), which the major compounds was himachalene and it's relatively similar to the composition of essential oil of leaves of *C. atlantica* study in Lebanon, which the major constituents were himachalol (22.50%), β-himachalene (21.90%) and α-himachalene (10.50%) (Monica *et al*., 2008). Contrary to the composition of essential oils of leaves of *C. atlantica* study in Algeria, which main constituents were: α-pinene (37.1-5.5%), β-pinene (8.6-1.9%), myrcene (3.6-0.6%), limonene (2.5-0.6%), bornyl acetate (5.4-4.0%), (E)-β-farnesene (6.8-1.9%) and manool (8.3-20.7%) (Boudarene *et al*., 2004). In Morocco, a recent study realized by Zrira and Ghanmi (2016), about essential oil of *C. atlantica* from Azrou Province (89 km south of Fez city), found that the main compounds identified are as follows: α-(E)-atlantone (19.3 %), β-himachalene (15.1 %), 8-cedren-13-ol, (13.1 %), α-himachalene (5.1 %), cedroxyde (4.6 %) and deodarone (4.6 %). The wingless seeds essential oil of *C. atlantica* from Morocco (Regional Station of Azrou City Forest), isolated by Rachid *et al*. (2014), was characterized by high contents of the monoterpene hydrocarbons such as α-pinene (46.16 %), manool (25.47 %), bornyl acetate (10.18%), β-pinene (5.95%) and α-terpinene (2.71%). In our study, there was an absence of some major constituents like himachalol, α-(E)-atlantone, deodarone and β-pinene, previously reported by Zrira and Ghanmi (2016), Monica *et al*. (2008) and Aberchane *et al.* (2003). This observed difference qualitative and quantitative between the chemical composition of the *C. atlantica* essential oils, could be explained by climatic conditions, the specific geographical factors to each region (Mansouri *et al*., 2010), genetics (degree of hybridization), part of plant extract and harvest period (Muñoz *et al*., 2007; Marcum *et al*., 2006).

Table 2: Chemical composition of areal part of essential oil of *C. atlantica* from Morocco.

Peak	KI	Compounds	% Area
1	924	α-thujene	*tr**
2	930	α-pinene	5.5
3	969	sabinene	0.13
4	1127	Rose oxide	0.25
5	1447	α-himachalene	35.34
6	1476	γ-himachalene	12.6
7	1480	γ -curcumene	1.1
8	1499	β-himachalene	13.62
9	1526	β -sesquiphellandrene	0.72
10	1542	α -calacorene	0.1
11	1573	oxydohimachalene	0.9
12	1591	Caryophyllene oxide	0.1
13	1594	longiborneol	1.18
14	1611	cedrol	10.32
15	1620	cedranone	1.62
16	1628	1-epi-cubenol	0.71
17	1661	isocedranol	5.52
18	1669	5-isocedrol	1.47
19	1693	Z-trans bergamotol	0.12
20	1694	deodarone	0.1
21	1704	cedroxyde	1.95
22	1717	Z- α -atlantone	1.3
23	1736	khusimol	1.08
24	1773	E- α -atlantone	1.65
25	1775	14-hydroxy-muurolene	0.1
Total identified constituents			**97.48 %**

******tr:* Trace for percentages ≤ 0.07%. KI: Kovats Index determined on OPTIMA-5 non-polar column in reference to n-alkanes.

Variation in mortality rate and Lethal Concentrations (LC_{50} and LC_{90})

After exposing the larvae *C. pipiens* to different concentrations of EO of *C. atlantica* for 24 h, the percentage of mortality varied according to concentrations (Figure 2). The minimum concentration of *C. atlantica* EO required achieving 100% of *C. pipiens* larvae mortality was 1500 ppm. Figure 1 also shows the different concentrations used with their standard deviations and their larvicidal activity, we have found that the mortality rate varies according to the concentrations (Figure 2) and the larval mortality rate reached 100% at a concentration of 1500 ppm

Fig. 1: *Cedrus atlantica* Manetti of Morocco.

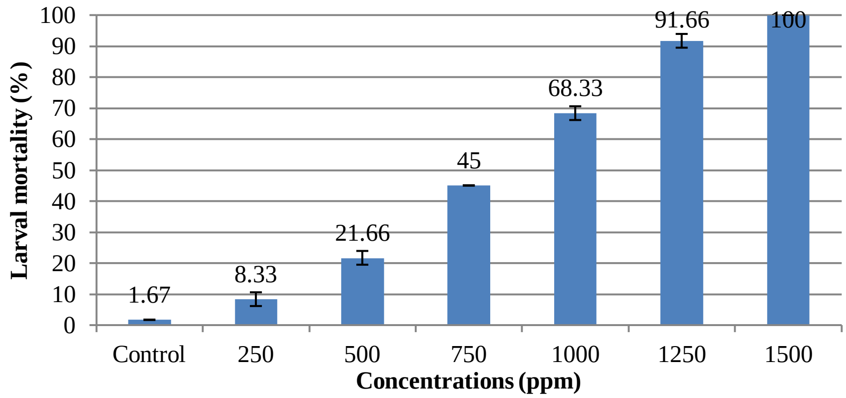

Fig. 2: Percentage of mortality recorded in the test sensitivity by essential oil of *C. atlantica* on *C. pipiens*.

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

Plant	LC_{50} (ppm) (LI-UI)*	LC_{90} (ppm) (LI-UI)*	Equation of the regression line	Calculated *Chi*-square (χ2)
C. atlantica	782.43 (554.77-934.84)	1253.93 (1042.80- 1900.82)	*Y = -18.10651 + 6.25776 * X*	11.063

∗Ll-Ul: Lower Limit-Upper Limit.

Table 3 demonstrates that *C. pipiens* EO remains effective while using concentrations of 782.43 ppm for LC_{50} (which varies between a lower limit 554.77 ppm and an upper limit of 934.84 ppm) and 1253.93 ppm for LC_{90} (which also varies between a minimum of 1042.8 ppm and a maximum value of 1900.82 ppm). Table 3 shows also the regression equation and the Chi-square (χ2) analyses results. The regression analysis indicates that the mortality rate is positively correlated with *C. pipiens* concentration.

Taking into account the absence of studies on *C. atlantica* essential oil against *C. pipiens*, we tried to compare the action of *Cedrus* family against the *C. pipiens*. A study realized by Huseyin *et al*. (2009) demonstrated that the larvicidal activity of *Cedrus libani* from Antalya (southwestern Turkey) on *C. pipiens* essential oil, with LC_{50} values ranging from 47.8 to 116.0 ppm. The larvicidal activity observed in the essential oil of *C. atlantica* against *C. pipiens* could be explained by the chemical composition of this oil and the action or effect of compound majority. Indeed, Naples *et al*. (1992) reported that cedrol, in particular, a principal component of cedar wood oil, seems to have a high toxicity to cercariae (*Schistosoma mansoni*), a parasite for humans. Himachalenes and atlantones fractions essential oil of Himalayan cedar (*C. deodara*) showed insecticidal activity against *Plutella xylostella* (Chaudhary *et al*., 2011).

CONCLUSION

The essential oil obtained from cedar wood had a wide variety of volatile constituents, which made up 97.48 % of the total essential oil. The essential oil yield was 1.12±0.2% and the major constituents were α-himachalene (35.34%), β-himachalene (13.62%), γ-himachalene (12.6%), cedrol (10.32%), isocedranol (5.52%) and α-pinene (5.5%). The oil also showed, an interesting larvicidal activity against *C. pipiens* with values in the order of LC_{50} = 782.43 ppm and LC_{90} = 1253.93 ppm.

Our results suggest that essential oil of *C. atlantica* has potential to be used in the search for chemical components as new larvicides. Further studies are needed to determine and isolate major oil components that are most effective for larvicidal activity.

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