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Insecticidal activity of essential oils of *Cupressus arizonica* greene and *C. sempervirens* L. on *Tortrix viridana* (Lepidotera, Tortricidae)

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ABSTRACT

Tortrix viridana is one of the most important defoliators of oak trees. In 2006 and 2007, this insect caused significant defoliations of oak forests in the Northwestern Tunisia. This work aims at studying the insecticidal activity of essential oils of *Cupressus arizonica* and *C. sempervirens* on 3rd, 4th and 5th instars larvae of *T. viridana* by the contact test. Three different concentrations of essential oils (0.05%, 0.1% and 0.5%) were used to test their contact effectiveness; the Deltamethrin and the ethanol were used as positive and negative controls. Larvicidal activity was determined by measuring the Mean Mortality Time (MMT) to kill 100% of larvae. A total of 28 chemical compounds have been identified from *C. arizonica* and 20 chemical compounds from *C. sempervirens*. The difference in MMT of the 3rd instar larva was highly significant between the 3 concentrations of the two tested essential oils, the negative and positive controls. In fact, the concentration (0.5%) was more efficient than the two other concentrations with a MMT=1h31min12s for *C. arizonica* and 5h11min20s for *C. sempervirens*. The same results were observed for the 4th (MMT=3h23min24s (Ca) and 4h1min (Cs)) and 5th (MMT= 3h23min24s (Ca) and 4h01min (Cs)) instars. The results revealed that the essential oil of *C. arizonica* was more effective than *C. sempervirens*. Therefore, it is recommended to use the essential oil of *C. arizonica* and not that of *C. sempervirens*, given its insecticidal efficiency on the 3rd instar larva of *T. viridana*.

Keywords: *Cupressus arizonica, C. sempervirens,* essential oils, larvicidal activity, *Tortrix viridana, Tunisia*.

INTRODUCTION: The green oak leaf roller moth, *Tortrix viridana* is one of the most important defoliators of oaks in the Western Palaearctic. It attacks cork oak (Mannai *et al.*, 2010), holm oak, pedunculate oak, sessile oak, pubescent oak, hairy oak (Tiberi *et al.*, 2005), afares oak and zeen oak (Mannai *et al.*, 2018). In 2006 and 2007, this insect caused significant defoliations of oak forests in Northwestern Tunisia (Mannai *et al.*, 2010). Pest control is essentially chemical or biological using *Bacillus thuringiensis* (Ruiu *et al.*, 2012). The research for other control alternatives based on natural active compounds, mainly essential oils, seemed to be extended in recent years. In addition to its antifungal (Donato *et al.*, 2020) and antibacterial (Oroojalian *et al.*, 2010) activities, this complex mixture of terpenic compounds has shown insecticidal activity on beetles, mosquitoes and Lepidoptera (Slimane *et al.*, 2014). In this context, our work aims at investigating the chemical composition and larvicidal activity of the leaf essential oil of *Cupressus arizonica* and *C. sempervirens* on larvae of *T. viridana*. The genus *Cupressus* (12 species) is distributed in North America, the Mediterranean regions and subtropical Asia in high altitudes (Rawat *et al.*, 2010). *Cupressus sempervirens* is the only species of this genus native from Tunisia (Boukhris *et al.*, 2012). *Cupressus arizonica* is a native plant from Southwestern United States. *Cupressus* species were subject of study on the biological activities of their essential oils: antifungal (Rguez *et al.*, 2018), antibacterial, antioxidant (Elansary *et al.*, 2012) and insecticidal activities (Rguez *et al.*, 2018).

OBJECTIVES: This study was conducted to: (i) determine the efficacy of the essential oils of *C. sempervirens* and *C. arizonica* against 3rd, 4th and 5th instars larvae of *Tortrix viridana* in laboratory and (ii) identify the chemical composition of the essential oil.

MATERIALS AND METHODS: Plant material: Fresh leaves of *C. arizonica* (Ca) and *C. sempervirens* (Cs) were collected in March 2018 from Ain Draham forest (36 ° 58 ' N, 8 ° 52 'E and alt. 850 m) in Northwestern Tunisia.

Essential oils extraction: From each species Ca and Cs, fresh leaves (100g) were placed in distilled water (400ml) and heated to 250°C (3 hours) for essential oils (EOs) extraction with vapor dragging and water distillation using hydro-distillation Clevenger apparatus method (Riahi *et al.*, 2013) and stored at −4◦C until analysis. The yield of each EO was calculated by the formula:

R% = (Mass of EO / Mass of plant material used) × 100.

Identification of terpenic compounds: Assessment of the chemical composition was carried out by gas chromatography/Mass Spectrometry method. The GC/MS was performed in a Hewlett Packard 5972 MSD type apparatus (capillary column HP-5 MS (30m length and film thickness is 0.25mm) coupled to mass spectrometry. Helium was used as the carrier gas at a flow rate of 1.2 mL/min. The oven temperature was programmed as follows: 50 ° C for 1 min; from

50 to 175 ° C at 5°C/min then, 175°C for 10 min and from 175°C to 250°C at 15°C/min followed by isothermal hold for 4 min. The temperature of the injector (250 ° C), that of the detector (280 ° C), injected solution (0.1 mL of 1%) was diluted in hexane in splitless mode; mass spectrometry: e HP5972 at 70 eV, scanning time: 1.5 s; gram mass: 40-300 amu. Spectra and mass chromatograms were managed by ChemStation and the identification of terpenic compounds was carried out by comparison of their mass spectra with those recorded in Wiley 275 library (Amri *et al.*, 2013).

Larvae collection and rearing: Investigations were made in the cork oak forest of El Jouza (36 ° 513 'N, 9 ° 015E and alt. 550 m) in the Northwestern Tunisia. From April to May 2018, every week, two branches from 10 mature trees of *Quercus suber* were monitored, one low-level branch and one from crown height level were carefully cut and bagged in a large plastic bag to avoid losing larvae. In the laboratory, branches were sorted and larvae of *T. viridana* were identified according to works of Mannai *et al.* (2010). Larvae of 3rd, 4th and 5th instars of *T. viridana* were stored in plastic boxes and reared on fresh cork oak leaves from seedlings from the National Research Institute for Rural Engineering, Water and Forests nursery.

Preparation of solution: Each EO was diluted with ethanol (96%) to prepare three concentrations (0.05%, 0.1% and 0.5%). Deltamethrin (Decis ND, Atlas Agro-Tunis, Tunisia) was used as a positive control and as a reference product according to the dose prescribed by the supplier. Ethanol (96%) was used as a negative control (Akkari *et al.*, 2015).

Insecticidal activity: All prepared solutions were tested for their contact effectiveness on the back of larva (Kanat and Alma, 2003). Thus, a quantity of 10µl of each solution (EOs, Deltamethrin and ethanol) was directly deposited on the back of each caterpillar individually placed in a Petri dish; For each concentration, 3 replicates were performed (10 caterpillars / replicate) per larval stage (Slimane *et al.*, 2014). In total 90 caterpillars per EO, 30 per positive control and 30 per negative control. The insecticidal activity evaluation of each test solution was determined by measuring the Mean Mortality Time (MMT) corresponding to the time required to kill all tested larvae (100%) (Akkari *et al.*, 2015).

Statistical analysis: Statistical analysis was performed using the SPSS-17.0 package software for Windows. The Mean Mortality Time of caterpillars of different instars larvae (MMT) by EOs, Deltamethrin and ethanol were calculated and statistically evaluated using analysis of variance (ANOVA) and supplemented by multiple comparisons of means by the SNK test (Student-Newman-Keuls) (Akkari *et al.*, 2015). Results were presented as mean ± standard error of the mean (MES).

RESULTS: Yield and chemical composition of essential oils: The percentage of water content in Ca and Cs were 50.58% and 44% respectively. Yields were respectively 0.35% and 0.27%. A total of 28 chemical compounds were identified from Ca, with 3 major components, Umbellulone (17.93%), α-pinene (10.33%) and Limonene (8.83%) and, 20 chemical compounds in Cs, with 3 major compounds α-pinene (27.5%), α-cedrol (19.3%) and δ-3-carene (7.2%) (table 1).

Insecticidal activity. Action on the 3rd instar larva: The difference of larvae mortality was highly significant between the 3 concentrations of two essential oils, the negative and positive controls with ($F_{(4, 145)}$=41.803; p<0.001) for Ca and ($F_{(4, 145)}$=26.680; p<0.001) for Cs. This difference was also highly significant between the 3 concentrations of each EO with ($F_{(2, 87)}$=72.527; p<0.001) for Ca and ($F_{(2, 87)}$=13.401; p<0.001) for Cs. The MMT of caterpillars obtained after treatments with the 3 concentrations of the two EOs was shorter than that of Deltamethrin and ethanol (figure 1).

N°	Compound	RI	*Cupressus arizonica*	*Cupressus sempervirens*
1	Tricyclene	926	0.05	0.1
2	α-thujene	931	0.73	0.1
3	α-pinene	939	10.33	27.5
4	α-fenchene	950	0.06	0.6
5	Camphene	954	2.42	
6	Sabinene	968	1.57	0.2
7	β-pinene	976	0.19	0,8
8	β-myrcene	991	1.29	1
9	δ-2-carene	1002	1.47	
10	α-phellandrene	1007	0.18	1.4
11	δ-3-carene	1011	1.49	7.2
12	*p*-cymene	1026	1.55	0.2
13	Limonene	1031	8.83	2.2
14	allo-ocimene	1132	0.14	
15	δ-terpinene	1062	1.95	
16	α-terpinolene	1088		1.3
17	δ-terpinolene	1088	1.41	
18	1,8,cineole	1021	0.13	1
19	Linalool	1098	0.07	0.1
20	Camphor	1142	0.21	0.1
21	Borneol	1149	0.85	0.2
22	Umbellulone	1171	17.93	
23	terpinen-4-ol	1179	3.04	1.8
24	Spathunelol	1578	0.21	
25	α-cedrol	1592	2.01	19.3
26	β-oplopenone	1607	0.13	
27	T-cadinol	1616	0.66	0.5
28	T-murrolol	1627		0.6
29	iso-	1641	0.13	
30	α-cadinol	1654	0.13	

Table 1: Chemical composition (%) of essential oils from leaves of *Cupressus arizonica* and *Cupressus sempervirens*.
RI = Retention Index

It seems that C3 (MMT=1h31min12s (Ca) and 5h11min20s (Cs)) was more efficient than C1 and C2 (figure 1). For each EO, the difference between the 3 concentrations was not significant for C1, but was highly significant for C2 ($F_{(1, 58)}$=19.460; p<0.001) and C3 ($F_{(1, 58)}$=69.585; p<0.001). It seems that the EO of Ca is more effective than that of Cs (table 2).

Action on the 4th instar larva: The difference of larvae mortality was highly significant between the 3 concentrations

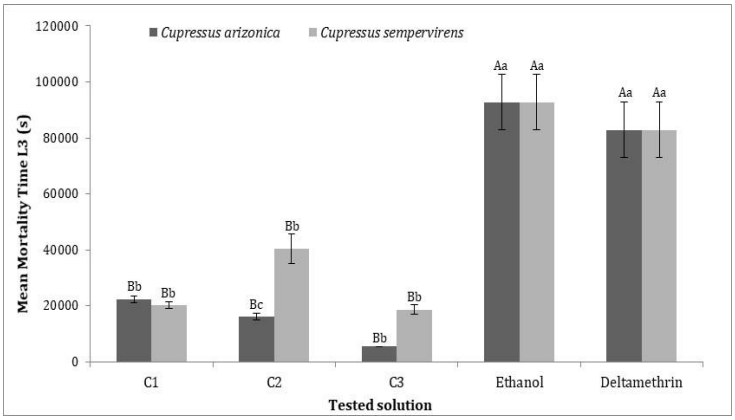

Figure 1: Mean Mortality Time of the 3rd instar larva of *Tortrix viridana* (Mean±SE). C1: concentration 0.05%; C2: concentration 0.1% and C3: concentration 0.5%; Within tested solutions (EOs, Decis and ethanol), values labeled with different uppercase letters are significantly different (SNK test, α=0.05); Within tested solutions values labeled with different lowercase letters are significantly different (SNK test, α=0.05)

Essential oils	3rd instar larve		
	C1	C2	C3
C. sempervirens	20292.56 ± 1192.79	40387.56 ± 5367.97	18683.53 ± 1581.21
C. arizonica	22370.4 ± 1197.53	16069.23 ± 1254.715	5474.56 ± 84.60
	4th instar larvae		
	C1	C2	C3
C. sempervirens	22785.9 ± 2091.7	98811.56 ± 14008.52	14458.26 ± 1254.54
C. arizonica	34820.56 ± 2260.78	12399.73 ± 1320.71	12183.8 ± 1351.11
	5th instar larvae		
	C1	C2	C3
C. sempervirens	21365.63 ± 831.48	31670.33 ± 1566.87	26562.2 ± 1310.77
C. arizonica	24626.56 ± 1828.77	29538.06 ± 1312.81	26779.36 ± 1538.38

Table 2: Mean mortality time of larvae (MEAN±SE). C1, C2 and C3 diluted solutions of EOs

of the two tested EOs, negative and positive controls with ($F_{(4, 145)}$= 25.370; p<0.001) for Ca and ($F_{(4, 145)}$= 17.066 ; p<0.001) for Cs. This difference was also highly significant between the 3 concentrations of each EO with ($F_{(2, 87)}$= 58.471; p<0.001) for Ca and ($F_{(2, 87)}$= 32.061; p<0.001) for Cs. The MMT of caterpillars treated with the 3 concentrations of the two tested EOs was shorter than of caterpillars treated with Deltamethrin and ethanol (figure 2). It seems that C3 of the two EOs (MMT=3h23min24s (Ca) and 4h1min (Cs)) was more efficient than the two other concentrations (figure 2). For each EO, the difference between the 3 concentrations was highly significant for C1 ($F_{(1, 58)}$=15.267; p<0.001) and C2 ($F_{(1, 58)}$= 37.715; p<0.001), but not significant for C3. It seems that Ca was more effective than that of Cs (table 2).

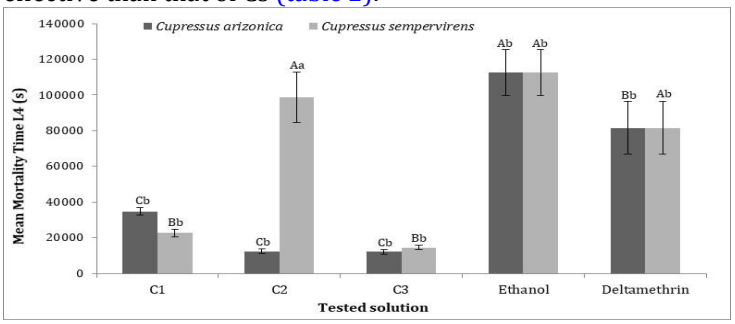

Figure 2: Mean Mortality Time of the 4th instar larva of *Tortrix viridana* (Mean±SE). C1: concentration 0.05%; C2: concentration 0.1% and C3: concentration 0.5%; Within tested solutions (EOs, Decis and ethanol), values labeled with different uppercase letters are significantly different (SNK test, α=0.05); Within tested solutions values labeled with different lowercase letters are significantly different (SNK test, α=0.05).

Action on the 5th instar larva: The difference of larvae mortality was highly significant between the 3 concentrations of the two tested EOs, negative and positive controls with ($F_{(4, 145)}$=143,252; p<0.001) for Ca and ($F_{(4, 145)}$=146.456; p<0.001) for Cs. This difference was also highly significant between the 3 concentrations of each EO with ($F_{(2, 87)}$=2.446; p<0.001) for Ca and ($F_{(2, 87)}$=16.372; p<0.001) for Cs. The MMT of caterpillars treated with the 3 concentrations of the two tested EOs was shorter than that of caterpillars treated with Deltamethrin and ethanol (figure 3). It seems that C3 of the two EOs (MMT= 3h23min24s (Ca) and 4h01min (Cs) was more effective than the two other concentrations (figure 2). For each EO, the difference between the 3 concentrations was not significant. It seems that the two EOs have the same effectiveness on caterpillars of the last instar larvae (table 2).

DISCUSSION: Studies on essential oils of *Cupressus arizonica* and *C. sempervirens* conducted by Chaudhary *et al.* (2012); Elansary *et al.* (2012); Al-Mouhajer *et al.* (2017) showed that Ca and Cs have an antibacterial activity. Other studies revealed an antifungal activity of Cs (Rguez *et al.*, 2018) and an insecticidal activity of Ca (Ali *et al.*, 2013).

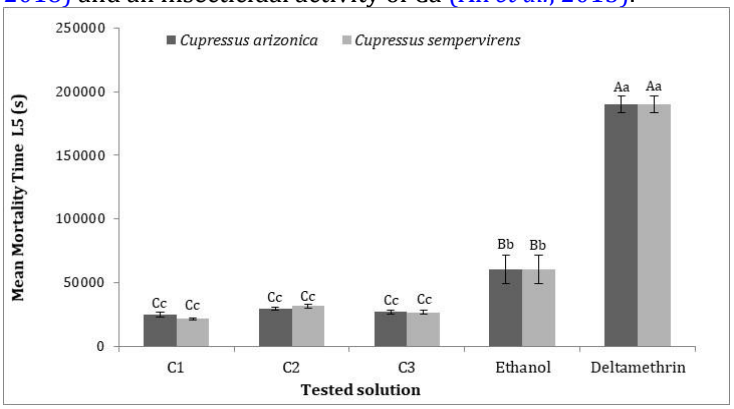

Figure 3: Mean Mortality Time of the 5th instar larva of *Tortrix viridana* (Mean±SE). C1: concentration 0.05%; C2: concentration 0.1% and C3: concentration 0.5%; Within tested solutions (EOs, Decis and ethanol), values labeled with different uppercase letters are significantly different (SNK test, α=0.05); Within tested solutions values labeled with different lowercase letters are significantly different (SNK test, α=0.05).

Our results showed that EOs of Cs and Ca have a contact insecticidal activity on larvae of the 3rd, 4th and 5th instars of *T. viridana*. Study of Slimane *et al.* (2014) on the insecticidal activity of EOs of two *Eucalyptus* species on caterpillars of the Erebidae, *Orgyia trigotephras*, pest of kermes oak and maquis Ezzine *et al.* (2015) showed that the MMT of the 3rd instar larva was 2 min 38 s (*E. lehmannii*), greater than *E. globulus* (MMT=1 min 39s) for a concentration of 0.5%. On the other hand, the activity of the EO of *Ruta chalepensis* carried out by Akkari *et al*., (2015) on the same instar larva and the same concentration (0.5%) revealed a MMT of 42.53 min. In our work, C3 (0.5%) seemed to be the most effective concentration against the 3rd instar larva of *T. viridana*, with MMT of 1h31min12s for Ca and 5h11min20s for Cs that seemed to be less effective.

This could be attributed to the limited distribution of this EO (Cs) over the larval skin and its permeability unlike the other tested EOs which spread quickly and easily on the back of the insect. This toxic action on the nerves leads to disruption of the vital system of insects (Enan, 2001). Studies conducted by Sedaghat *et al.* (2011) on EO of *C. arizonica* showed their insecticidal effect on larvae of *Anopheles stephensi* (LC90 = 79.30). Among terpenes: δ-3-carene, α-humulene, α-pinene, β-phellandrene and α-cedrol showed antibacterial activity (Elansary *et al.*, 2012). In this present work, we found that α-pinene content is quite high in both species, with the highest level in *C. sempervirens* (27.5%) and the lowest in *C. arizonica* (10.33%). Three major compounds (Umbellulone, α-pinene and Limonene) of Ca were reported to have an insecticidal effect and their contents varied based on the origins of *C. arizonica* (Sedaghat *et al.*, 2011). Indeed, Umbellulone contents were 45.1%, 37.3%, 16.5%, 13.2% and 5.4% in *C. arizonica* EOs originating from Italy, Algeria, Argentina, Iran and Texas, respectively (Sedaghat *et al*., 2011). In our present work, Umbellulone content was 17.93, close to that of Ca EO from Argentina (Sedaghat *et al*., 2011). Contents of α-pinene and Limonene were moderate (10.33% and 8.83% respectively), and close to those of Ca EO from Algeria (% α-pinene = 10.5), France (% Limonene = 8.7). Study conducted by Malizia *et al*. (2000) in Argentina on EO of *C. arizonica* showed that the main constituents were α-pinene (22.9%), Limonene (8.5%), Umbellulone (16.5%), terpinen-4-ol (5.5%) and cis-muurola-4(14), 5-diene (90%) that of Afsharypuor and Tavakoli (2005) in Iran showed that the main constituents were α-pinene (19.2%), cis-muurola-4(14),5-diene (10.0%), β-phellandrene (9.6%) and sabinene (8.1%). A study conducted by Selim *et al*. (2014) in Saudi Arabia on EO of leaves of *C. sempervirens* showed that α-pinene (48.6%), δ-3-carene (22.1%), limonene (4.6%) and α-terpinolene (4.5%) were the main components that of Rguez *et al.* (2018) showed that this species was characterized by the highest level of α-pinene (28.9%).

CONCLUSION: In the light of these results and, to control pest attack and more particularly against leaf rolls moths, it is recommended to use the essential oil of *C. arizonica* and not *C. sempervirens*, given its insecticidal efficacy on caterpillars of the 3rd instar larva. Thus, more researches are needed in this area in order to identify the active compounds responsible for this insecticidal effect.

CONFLICT OF INTEREST: Authors have no conflict of interest.

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