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Research Article

Essential oil composition and antimicrobial activities of some *Cupressaceae* species from Algeria against two phytopathogenic microorganisms

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Abstract: The objective of this work is to determine the chemical composition of essential oils of four *Cupressaceae* species and to bring out some of their antimicrobial activities. For this purpose, the extraction of essential oils was carried out by hydrodistillation and the obtained oils varied from 0.4% to 0.91%, with a better yield for *Cupressus sempervirens* equal to 0.91%. The chromatographic analyses revealed that the chemical composition of *Juniperus phoenicea*, *Juniperus oxycedrus* and *C. sempervirens* is largely dominated by monoterpenes at rates higher than 34% with majority of α-pinene like component. In addition, the results of the antibacterial activity showed a significant inhibitory effect of essential oils, particularly those of *J. phoenicea* was found to be the most active with values of Minimum Inhibitory Concentration (MIC) equal to 50µg/ml. Moreover, the mycelial growth inhibition confirms that essential oils of *J. phoenicea* and *J. oxycedrus* are most effective, with values of MIC equal to 1000µg/ml for both. These antimicrobial properties would be due to the richness of these essences in bioactive compounds such as the terpenoids, polyphenols and alkaloids known for their implication in the plants self-defense. Consequently, the inhibitory effect of these essential oils on the microbial development suggests prospects for their application as natural and safe alternatives to synthetic compounds for plant protection.

Keywords: Biorational pesticides, Chromatography, Hydrodistillation, *Pseudomonas*, *Fusarium*

Introduction

The use of pesticides is one of the components of the fabulous increase of the yield observed during the last decades. This use of chemical product which compromised on safety for the humans and the environment was regarded as inevitable; currently poses problems and their impact was certainly insufficiently estimated. Among the most direct consequences, let us quote the appearance of resistance, the impoverishment of useful auxiliary fauna, causing serious disturbances in the biocenotic equilibrium, and finally the contamination of the environment and the appearance of toxic residues in the collected food products or their transformation products (Descoins, 1991). Faced with the growing concern of consumers and farmers about synthetic pesticides and

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following WHO (2007) recommendations in favour of their elimination and search for harmless and non-polluting alternatives, a renewed interest in the use of biopesticides, formerly relatively marginal in terms of commercial importance (Philogène *et al*., 2008) lets perceive hopes of developments that are much more generalized and better scientifically evaluated.

Plants have by natural selection during evolution developed adaptation mechanisms to various environmental conditions. There are therefore, within each plant a reserve of natural bioactive substances in which new molecules can be found (Deshayes, 1991).

The knowledge of empirically determined traditional remedies, as well as recent discoveries of antimicrobial activities, of essential oils, together with a better knowledge of their action, is currently a very important database for the rigorous scientific development inherent in biological control by the use of these natural substances. Moreover, because of their particular geographical location, the Algerian steppe regions with their characteristic climate (low rainfall and high temperatures), are endowed with a rich plant biodiversity with a panoply of aromatic and medicinal plants, many of which are endemic (Benkhettou *et al*., 2016). The extraction and the production of bioactive molecules from these plants can constitute a means of valorization of these species and exteriorize their potential. With this in mind, the objective of this study is to determine the chemical composition of the essential oils extracted from some *Cupressaceae* species, collected in the region north of Tiaret (northwestern Algeria) bordering with the steppe zones and to study their *in vitro* antifungal and antibacterial activity, on phytopathogens.

Materials and Methods

Plant materials

The plants used come from the area of Tiaret (northwestern Algeria) (Fig. 1), pertaining to the semi-arid bioclimatic area characterized by limited natural resources, a poor ground, vegetal formations consisting of herbaceous or more or less shrubby plants (Benkhettou *et al*., 2016). The choice of the sites was based on edapho-climatic diversity that can influence the yield and the chemical composition of essential oils. They are plants belonging to four spontaneous species of *Cupressaceae*: *Tetraclinis articulata*, *Cupressus sempervirens, Juniperus phoenicea* and *Juniperus oxycedrus*. The leaves were collected in the autumn of 2016 in a random manner.

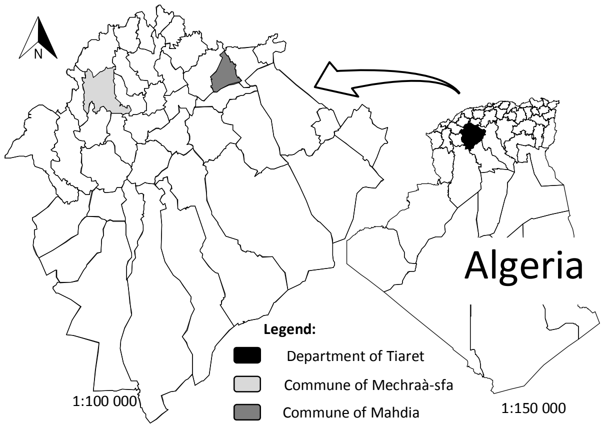

Figure 1 Situation map of Tiaret department and location of the different sampling stations.

Phytopathogenic microorganisms

In order to test the antimicrobial activity of essential oils, we opted for two phytopathogenic microorganisms, namely *Pseudomonas savastanoi* pv. *savastanoi* which is a Gram-negative bacteria, agent of the tuberculosis of the olive-tree causing one of the most serious omnipresent bacterial diseases in the Mediterranean basin (Benjama, 1994) without effective treatment at present and *Fusarium oxysporum* f. sp. *lycopersici* which is a fungal strain responsible for the rot and deterioration of the tomato cultures (Henni *et al*., 1994) treated by chemical fungicides which become in their turn very dangerous for human health and the environment.

The bacteria was obtained from the tumors of infected olive-trees, then isolated and identified, while the fungal strain was obtained from a collection preserved within the laboratory of Microbiology of the Faculty of Science of Nature and life of Ibn Khaldoun University of Tiaret.

Isolation and characterization procedure of the essential oils

The extraction of essential oil was carried out by hydrodistillation of 100 g samples of leaves from each species in Clevenger (1928) apparatus for 3 hours. Essential oils obtained were preserved at + 4 °C in the darkness until their use for the different tests. The yield of extraction expressed as a percentage is the relationship between the weight of essential oil and the weight of the plant parts used, the yield was calculated by the formula of Carre (1938):

Y_{EO} (%) = (W_{EO}/W_L) × 100

W_{EO}: Weight of oil in g; W_L: Weight of the dry leaves in g; Y_{EO} (%): Essential oil yield.

The chromatographic analyses were carried out by gas chromatography (GC) on a Hewlett-Packard (6890) coupled with mass spectrometry (GC/MS) at the Chemistry laboratory, analytical pharmacy department, faculty of medicine, University of Algiers. The apparatus was equipped with a capillary tube HP-5MS (30m × 0.25mm), with 0.25µm film thickness. The temperature of the column was programed from 50 to 250 °C at a rate of 4 °C/min. The carrier gas was the helium whose flow was fixed at 1.5ml/min. The mode of injection was mode Split (leakage ratio: 1/70), fragmentation was carried out by an electronic impact with 70 eV. The apparatus was connected to a computing system managing a library of mass spectrum NIST 98 and controlled by "HP ChemStation" software, making it possible to follow the evolution of the chromatographic analyses.

Biological activities of essential oils

Antibacterial activity

The study of the antibacterial activity of essential oils was carried out *in vitro* according to the aromatogram method (diffusion in agar test) (Remmal *et al.*, 2011) which is based on the migratory capacity of essential oils on the surface of a solid agar medium containing Mueller-Hinton. The bacterial suspension was prepared starting from a young culture (18 hours) in the nutrient broth, diluted in physiological water so as to contain 10^8 CFU/ml, was initially sown on agar medium surface in Petri dishes 9cm in diameter. Then, with the filter paper discs 6mm in diameter were soaked with pure essential oil with increasing concentration (5, 10 and 20µl) of each plant species and placed each one in the middle of the agars. Lastly, the Petri dishes were placed in a drying oven for incubation at 25 ± 2 °C for 72 hours.

Obtaining a clear halo around the disc, measured in millimeters with a digital slide caliper, indicates the inhibition of the bacterial development. The tests were carried out in three repetitions.

This qualitative test gave positive results, the study continued with a second more specific test to quantitatively evaluate the minimum inhibitory concentration (MIC) for each essential oil.

The MIC of essential oils is measured by the dilution method in a liquid medium (Fisher and Phillips, 2009).

A concentration range of each essential oil from 1600µg/ml to 25µg/ml was prepared from stock solution according to the method of dilution in cascades in geometric progression at the rate of ½ by adding 2.5ml of the stock solution to 2.5ml of broth contained in the test tube.

A volume of 15µl of the inoculum was then introduced into each of the tubes of the range. All the tubes were incubated at the same conditions mentioned previously.

A control of bacterial growth, of which 15µL of the standardized inoculum were deposited in the culture medium in the presence of the only solvent used for the solubilization.

The MIC of the essential oil was deduced from the first tube of the range devoid of bacterial growth.

Antifungal activity

The Antifungal activity of essential oils was evaluated *in vitro* by the dilution method in solid medium to determine the inhibition rates. Different concentrations 25, 50 and 100µl of essential oil, emulsified in Tween 80 (5ml) were prepared and incorporated, in the culture medium based on the potato dextrose agar (PDA). Then, the mixture was poured in Petri dishes to be sown by a 5mm disc of fungus mycelium resulting from a 7 days old culture. Lastly, incubation was done in darkness at 25 ± 2 °C. The mycelial growth of the colonies was estimated after 7 days of incubation by the average of two perpendicular diameters. The control was carried out under the same conditions, without addition of essential oil. These tests were repeated three times. The inhibition rate of mycelial growth was calculated according to the formula of Leroux and Credet. (1978):

$$Ti\ (\%) = [(D_0 - D_C)/D_0] \times 100$$

with Ti: inhibition rate in%; D_0: diametrical growth of the control; D_C: diametrical growth of fungi treated by essential oils.

At the end of this preliminary study, all the essential oils having antifungal activity were selected for the determination of the minimum inhibitory concentration (MIC) by the method of dilution in liquid medium according to the method reported by Remmal *et al*. (1993).

The culture of the fungal strain was carried out on Sabouraud medium, the essential oils were used in the form of emulsions, and then they were added to the culture medium at different concentrations ranging from 250µg/ml to 4000µg/ml.

Results

Essential oil yield

According to the essential oils yields extracted by hydrodistillation from the dry leaves of the four species (Fig. 2), we note that *C. sempervirens* gives the best yield with an average of 0.91%, followed by *J. phoenicea* with 0.76%, which agrees with the results of 1% obtained by Tapondjou *et al*. (2005) as well as 0.7% obtained by Barrero *et al*. (2006) respectively for *C. sempervirens* and *J. phoenicea*. However, for Benali Toumi *et al*. (2011) with the same process of extraction of the leaves of *T. articulata*, get 0.76% EO yield higher than our finding 0.6%

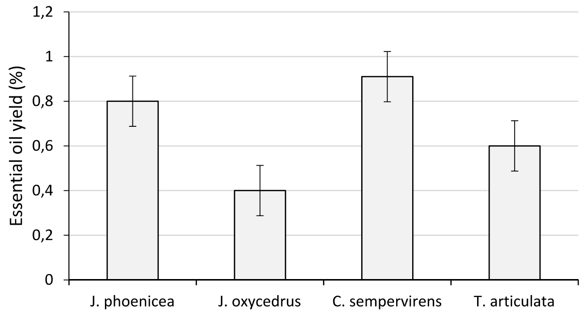

Figure 2 Essential oil yield of different species.

With regard to *J. oxycedrus*, yield slightly lower than our results is 0.3% was obtained by several authors (Alberto *et al*., 2003; Ghanmi *et al*., 2010). These variations in yield of EO can be attributed to the genetic variability of the species, but also to the specific geographical site of each species.

Chemical constituents of essential oils

The chemical composition of essential oils determined by gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC/MS) revealed the presence of a constant chemical polymorphism and the specificity expressed by each plant species (Fig. 3). Monoterpenes represent the biochemical family most widespread in the essences extracted from *J. phoenicea*, *J. oxycedrus* and *C. sempervirens* with "α-pinene" as the major component and the respective contents of 38.26%, 34.15% and 43%, followed at lower levels by sesquiterpenes, esters and the ketone (Table 1).

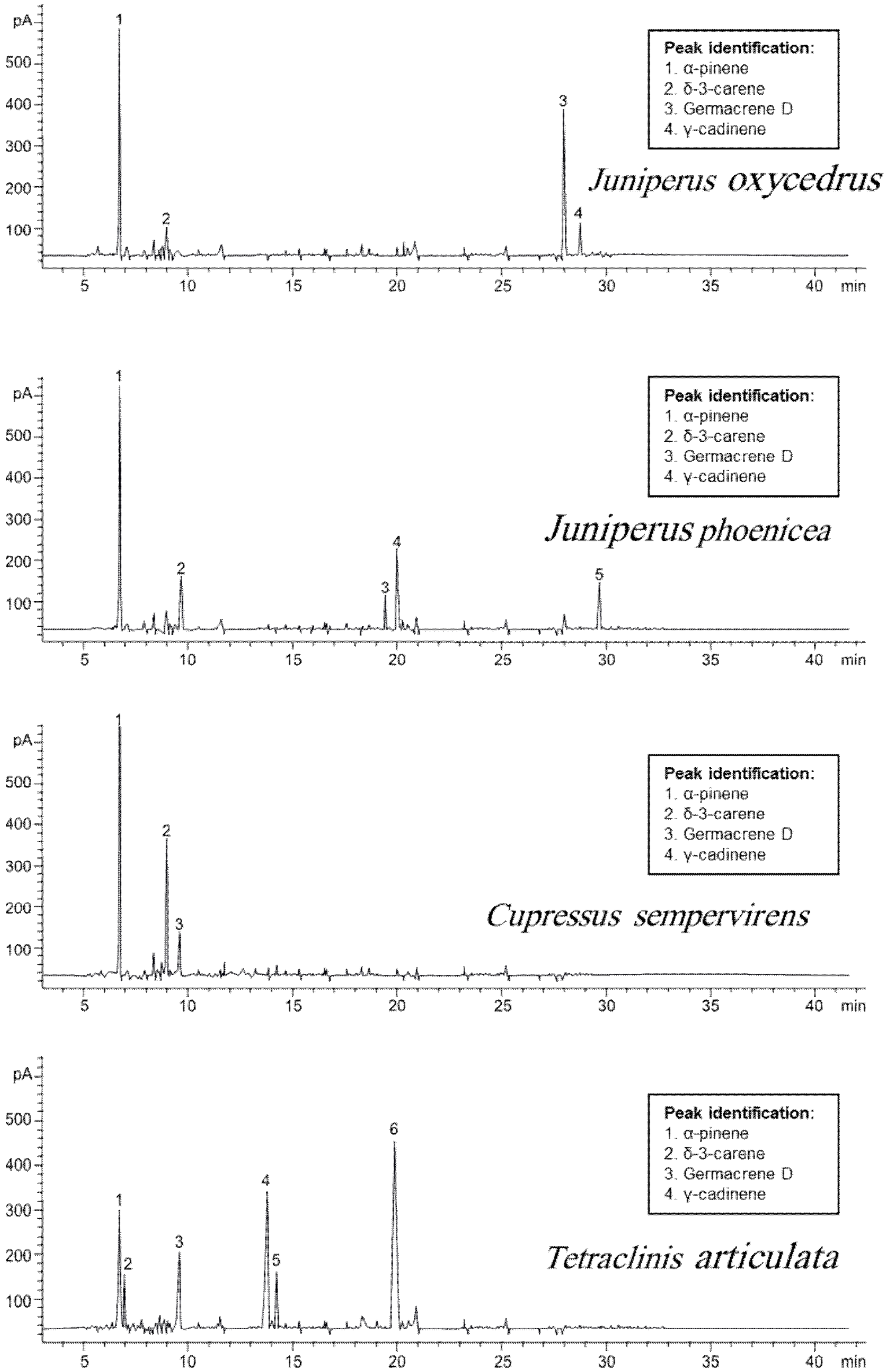

Figure 3 Chromatographic profile of essential oils from leaves of tested plants**.**

Table 1 Chemical constituents (%) of the essential oils of the dry leaves of different species.

No.	Component	IK	*J. oxy*	*J. pho*	*C. sem*	*T. art*
01	Tricyclene	926	0.12	0.85	0.35	-
02	α-pinene	931	34.15	38.26	43.07	16.45
03	Camphene	949	0.85	0.27	0.45	4.87
04	α-fenchene	951	-	-	-	3.86
05	Sabinene	976	0.12	1.37	1.28	0.38
06	β-pinene	981	1.3	4.67	1.37	8.22
07	β-myrcene	991	-	1.87	-	2.28
08	Myrcene	997	1.86	2.14	1.88	0.13
09	α-phellandrene	1005	0.12	3.42	0.6	0.86
10	δ-3-carene	1011	4.12	2.25	18.04	0.1
11	α-terpinene	1018	0.27	0.52	1.45	0.28
12	p-cymene	1026	0.1	0.39	0.21	-
13	Limonene	1031	0.51	8	6.71	12.57
14	β-phellandrene	1036	0.19	1.05	-	-
15	γ-terpinene	1059	-	0.35	0.53	-
16	Terpinolene	1085	0.56	6.1	4.28	2.09
17	Linalol	1103	-	4.12	2.51	0.21
18	Myrcenol	1119	-	0.2	-	1.23
20	Trans-pinocarveol	1140	-	-	-	1.35
21	Camphre	1143	0.21	0.47	-	18.6
22	Trans-pinocarveol	1161	-	0.52	-	0.56
23	Borneol	1165	-	-	1.32	8.41
24	Terpinen-4-ol	1172	0.08	0.8	1.53	2.52
25	α-terpineol	1189	0.04	1.61	0.51	3.11
26	Trans-carveol	1217	-	0.31	-	0.28
27	Citronellol	1228	-	6.8	-	-
28	Piperitone	1250	0.17	0.56	-	-
29	Bornyl acetate	1285	0.12	-	1.52	28.42
30	α-terpenyl acetate	1350	-	9.4	3.1	-
31	α-copaene	1376	-	-	-	1.02
32	β-bourbonene	1386	0.11	1.17	-	3.68
33	β-elemene	1391	2.31	1.55	0.2	-
34	Junipene	1402	0.8	3.22	-	-
35	γ-elemene	1425	0.15	0.25	-	0.75
36	α-humulene	1455	2.38	-	0.3	0.31
37	Germacrene D	1478	25.22	2.84	2.46	0.78
38	γ-muurolene	1497	3.21	-	0.19	-
39	δ-cadinene	1524	2.85	3.77	0.45	-
40	γ-cadinene	1539	4.76		-	-
41	Germacrene B	1556	2.12	7.11	-	0.32
42	Caryophyllene oxyde	1581	-	0.41	0.11	2.15
43	Cedrol	1607	-	-	4.42	-
	Total		95.21	97.67	94.63	92.59

IK: Kováts indices; *J. oxy: J. oxycedrus; J. pho: J. phoenicea; C. sem: C. sempervirens; T. art: T. articulata;* (%): percentage; (–): absence.

These results agree with those of Milos and Radonic (2000) and Tapondjou *et al*. (2005) which confirm that the aromatic essences of the three species have a high monoterpenes rate (α-pinene) and confirm also the presence of sesquiterpenes in appreciable quantity. In addition, the comparative studies carried out on the three species of different provenances by

Mansouri *et al*. (2010) in Morocco, by Velasco-Negueruela *et al*. (2003) in Spain and Bouzouita *et al*. (2008) in Tunisia, revealed the presence of the same identified compounds with, however a heterogeneity of the major compounds with the predominance of α-pinene (from 52.13 to 59.8%).

On the other hand, it should be noted that the major components identified in *T. articulata* essential oils, differ in other oils in particular for monoterpenes (α-pinene 16.45%. limonene 12.57%) and the esters (bornyl acetate 28.42%) which are in relatively identical quantity. In addition, other authors thus revealed this variability of the chemical composition with predominance of the bornyl acetate (30.6%) (Bourkhiss *et al*., 2007) and of camphor (31.60%) (Benali Toumi *et al*., 2011).

Lastly, several studies showed that the major compounds (chemotype) vary quantitatively and qualitatively (Burt, 2004). The reasons for this variability can be mainly due to the difference in the ecological factors and the age of the plants geographical sources, harvest seasons, genotype, climate, plant organ used, the mode of extraction. All of these factors influence the chemical composition and the relative concentration of each component of essential oils and consequently, its biological activity.

Biological activities of essential oils

Antibacterial activity

With the reading of results obtained during this antibacterial test by aromatogram (Fig. 4), it appears clearly that all essential oils exercised an antibacterial activity. This activity was in addition, variable from one essential oil to another, the bacteria *Pseudomonas savastanoi* pv. *savastanoi* proved more sensitive to the inhibitory action of the essential oil of *J. phoenicea* with inhibition zones diameter varying between 25 and 53mm for the three applied concentrations (5, 10 and 20µl), followed by *C. sempervirens* essential oils with 9, 27 and 41mm and *J. oxycedrus* with slightly lower values (12, 22 and 37mm). While for *T. articulata* the antibacterial activity was moderate with only 12 and 18mm for the same concentrations, and an absence of this inhibiting effect for the lowest concentration.

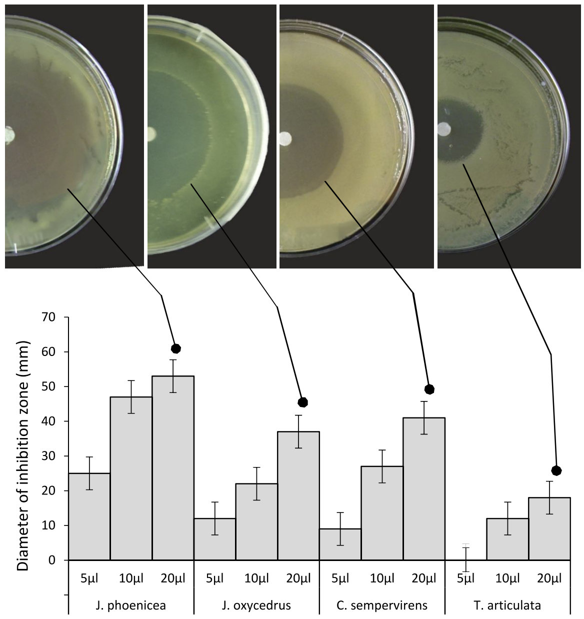

Figure 4 Antibacterial activity of essential oils of the different species.

Our results correspond with most of the studies relating to the action of the essential oils of the species of *Cupressaceae* (Bouzouita *et al*., 2008; Mansouri *et al*., 2010; Amara and Boughérara, 2017) demonstrated an important antibacterial activity against several strains tested despite their morphological diversity (Gram- and Gram +).

This antibacterial effect seems to be the result of the diversity of molecules contained in essential oils, of the secondary metabolites biosynthesized by the plants following millions of years of evolution against the aggression exerted by phytopathogenic agents.

The quantitative evaluation of the antibacterial activity of the oils was carried out by the determination of the MIC

The results confirmed that the essential oil of *J. phoenicea* showed the strongest activity with a minimum inhibitory concentration (MIC) of 50µg/ml, followed by *C. sempervirens* and *J.*

oxycedrus which showed a similar and moderate antibacterial activity (MIC 100µg/ml), while the essential oil of *T. articulata* was the least active with a MIC value of 400µg/ml.

Antifungal activity

The observation of the strain treated by essential oils revealed the presence of an antifungal activity. Thus, the application of the first concentration of 25µl of the different essences tested on the mediums, was accompanied by a decrease in the mycelial growth compared to the control without essential oils. Noting that the inhibition rate is proportional to the essential oils concentration in the medium; with the highest concentration (100µl) the inhibition of mycelial growth was very effective for the two species *J. phoenicea* and *J. oxycedrus* with an inhibition rate higher than 60% followed by *C. sempervirens* with a rate close to 51%. On the other hand, *T. articulata* showed the weakest antifungal activity with 43.3% and this trend remained valid for all of the concentrations tested.

In addition, the results of figure 5 show that the growth of *F. oxysporum* f. sp. *lycopersici* was inhibited by all of the tested essences (inhibition rate ranging between 24% and 68%). This is fully in line with the majority of previous works (Kasmi *et al.*, 2017). Other researchers Bouyahyaoui *et al.* (2017); Amara and Boughérara (2017) have found that these same essences destroy a broad range of fungi. These results allow us to attribute this antifungal activity to essential oils and the secondary metabolites, which confer to those plants their antimicrobial capacity.

The MIC results of the 4 essential oils are in agreement with the results obtained in the preliminary test by the aromatogram method. In other words, the most active essential oils on *F. oxysporum* f. sp. *lycopersici* are those of *Juniperus phoenicea* and *Juniperus oxycedrus* with a MIC of 1000µg/ml, followed by an essential oil of *Cupressus sempervirens* with a MIC of 2000µg/ml, while *Tetraclinis articulata* oil revealed a medium antifungal power with a MIC 4000µg/ml.

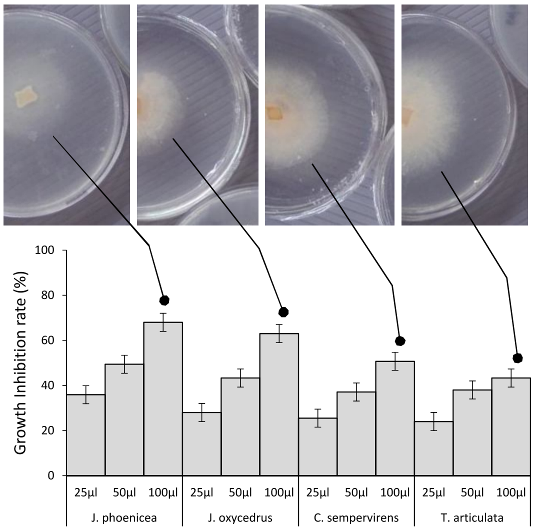

Figure 5 Antifungal activities of the essential oils of different species according to the concentration (µl) applied.

Discussion

The study of biological activities has revealed that the species richest in monoterpene compounds (α-pinene, limonene, δ-3-carene, β-pinene, terpinolene) are the same species that have the most inhibiting efficacy, which suggests that these effects are certainly related to the presence of these compounds.

According to Bekhechi *et al.* (2008) the sensitivity of microorganisms to plant extracts can vary depending on various parameters such as the chemical composition and the relative concentration of each of the constituents of the essential oil.

Similarly, Fernandez and Chemat (2012) cited that many essential oils and their major compounds were defined as antimicrobials. Their spectrum of action is very wide they act against a large range of phytopathogenic microorganisms including those that have already developed resistance to antibiotics.

This finding allows us to conclude that these different properties are related to the chemical composition of the essential oils which remains rather complex to the functional groups of the major compounds (alcohols, phenols, terpenic and ketonic compounds) and for their synergistic effects. Nevertheless, these synergistic effects can also relate to the minority compounds and consequently the value of an essential oil depends on the integrality of its components.

Conclusion

The present study relates to the valorization of natural bioactive compounds in this case essential oils for their application in the crop protection which is a new process in full mutation and which arouses more and more the interest of the researchers. For this purpose and for targeting the molecules responsible for the antimicrobial properties (antifungal and antibacterial potentiality) essential oils of four plant species namely *Tetraclinis articulata*, *Cupressus sempervirens*, *Juniperus phoenicea* and *Juniperus oxycedrus* were subjected to chromatographic analyses and a study of their antimicrobial activity. The results of the extraction of essential oils by hydrodistillation, allowed us to note a clear difference in yield between the four species. The study of the chemical composition of the species having been the subject of this study, confirms well the specificity expressed by each botanical species. The essences produced by *J. phoenicea*. *J. oxycedrus* and *C. sempervirens* are largely dominated by monoterpenes with α-pinene like majority component has rates higher than 34% whereas *T. articulata* contains relatively identical quantities of monoterpenes (α-pinene 16.45%. limonene 12.57%) and the esters (bornyl 28.42% acetate). These essential oils contain other compounds, but in less quantities. In addition, the tests of the antibacterial activity for the different ranges of essential oil concentrations, revealed that all essential oils exert an antibacterial activity against *P. savastanoi* pv. *savastanoi* in particular *J. phoenicea* essential oils. *C. sempervirens* and *J. oxycedrus*, also exhibit a very important antibacterial activity. The results of the experiments carried out on the fungus species *F. oxysporum* f. sp. *lycopersici* reveals that the application of the essences tested at different concentrations, is accompanied by a reduction in the mycelial growth. Most effective of the essences remain those of *J. phoenicea* and *J. oxycedrus*. On the other hand, the least effective are those of *C. sempervirens* and *T. articulate.* The sensitivity of the microorganisms to tested essential oils would be due to the molecular diversity which they contain, molecules that can be exploited and valorized for their use in the biological control strategies. These conclusions make it possible to suggest the possibility of using the essential oils of these plants as an interesting alternative to synthetic compounds, as well in the field of crop protection as in other very varied roles. Although of natural origin, nevertheless it remains that study on their possible toxicity must be undertaken not to fall back into the same problems caused by the synthetic pesticides.

Author's contributions

Alem Aicha Somia conducted the antimicrobial activities experiment. Boufares Khaled conducted the experiment, analyzed the results, discussed the results, made figures and drafted the manuscript. Hassani Abdelkrim supervised and designed the research and reviewed the manuscript.

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ترکیبات اسانس و فعالیت ضدمیکروبی برخی از گونه‌های Cupressaceae از الجزایر روی دو میکروارگانیسم فیتوپاتوژنیک

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چکیده: هدف از این پژوهش تعیین ترکیب شیمیایی اسانس چهار گونه گیاهی از تیره Cupressaceae و برخی از فعالیت‌های ضدمیکروبی آن است. برای این‌منظور، استخراج اسانس با استفاده از روش تقطیر انجام شد و روغن‌های حاصل از ۰/۴ تا ۰/۹۱ درصد متغیر بودند به‌طوری که گونه *Cupressus sempervirens* با ۰/۹۱ درصد عملکرد بالاتری داشت. تجزیه‌وتحلیل کروماتوگرافی نشان داد که ترکیب شیمیایی گونه‌های *Juniperus phoenicea*، *Juniperus oxycedrus* و *C. sempervirens* عمدتاً از مونوترپن آلفاپینین با نرخ بالاتر از ۳۴ درصد است. به‌علاوه، اسانس‌های مورد مطالعه فعالیت ضدباکتریایی قابل‌توجهی نشان دادند. به‌ویژه گونه *J. phoenicea* که بیش‌ترین فعالیت را با مقادیر حداقل غلظت مهاری (MIC) برابر با ۵۰ میکروگرم در میلی‌لیتر داشت. هم‌چنین، مهار رشد قارچی تأیید می‌کند که اسانس‌های J. phoenicea و J. oxycedrus با MIC برابر µg/ml۱۰۰۰ مؤثرتر بودند. این خواص ضدمیکروبی می‌تواند به علت غنی بودن اسانس‌ها از ترکیبات زیست فعال ترپنوئیدی، پلی‌فنل‌ها و آلکالوئیدها باشد. در نتیجه، اثر مهاری این اسانس‌ها بر رشد میکروبی، نویدبخش چشم‌انداز کاربرد آن‌ها را به‌عنوان جایگزین‌های طبیعی و ایمن به‌جای ترکیبات شیمیایی برای حفاظت از گیاهان می‌باشد.

واژگان کلیدی: آفت‌کش‌های سالم، کرماتوگرافی، تقطیر با آب، سودوموناس، فوزاریوم