

## Research Article

# Chemical and Antibacterial Polymorphism of *Juniperus oxycedrus* ssp. *oxycedrus* and *Juniperus oxycedrus* ssp. *macrocarpa* (Cupressaceae) Leaf Essential Oils from Tunisia

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Essential oils from *Juniperus oxycedrus* L. have been used since antiquity for fragrance, flavoring, medicinal, antimicrobial, insecticidal, and cosmetic purposes. Several works studied the chemical composition of the essential oils of *Juniperus oxycedrus* leaves. The aim of this study is to investigate the chemotaxonomic relationships and antibacterial activity of two Tunisian subspecies: *Juniperus oxycedrus* ssp. *oxycedrus* (L. K.) Deb. and *Juniperus oxycedrus* ssp. *macrocarpa* (S. & m.) Ball. In addition, and for the first time, we reported the antibacterial activities of Tunisian *J. oxycedrus* ssp. *macrocarpa* and *J. oxycedrus* ssp. *oxycedrus* against four bacteria. Essential oils obtained by hydrodistillation were analysed by GC and GC/MS. Fifty-five constituents were identified. Thirty four major compounds were retained for the study of the chemical variability, and  $\alpha$ -pinene, sylvestrene, *p*-cymene, and 13-*epi*-manoyl oxide were the main ones. The chemical principal components analysis (PCA) identified three chemotypes. The study of the antibacterial activity showed that *Escherichia coli* was found to be extremely resistant (zone diameter 0 mm) to all the oils tested, while *Staphylococcus aureus* was the most sensitive strain (zone diameter 13.5 mm and MIC ranged from 600 to 650  $\mu$ g/mL).

## 1. Introduction

Due to the increasing consumer demand for more natural foods, natural substances isolated from plants are considered as promising sources of food preservatives [1]. Particularly, the study of essential oils as food additives proved to be advantageous, as observed by the increase in foods shelf-life [2]. However, several factors influence the chemical composition of plant essential oils, including the extraction methods, the species, geographical origin, and also the season of harvesting and consequently their bioactive properties [3–5].

*Juniperus oxycedrus* L., one of two species of the genus *Juniperus* growing wild in Tunisia, is subdivided in two subspecies: *J. oxycedrus* ssp. *macrocarpa* (S. & m.) Ball. and *J. oxycedrus* ssp. *oxycedrus* (L. K.) Deb. Aromatic oils from *J. oxycedrus* have been used since antiquity for fragrance, flavoring, medicinal, antibacterial, insecticidal, and cosmetic purposes. Indeed, leaf essential oil from Lebanon [6], Corsica [7], and Croatia [8] has been reported in varying details. Juniper has been recommended as a mouth analgesic and for stomach disorders in folk medicine in Spain [9]. It is used as a spice, particularly in European cuisine, and it also gives gin its

TABLE 1: Geographical coordinates of the sites of collection of *Juniperus* populations.

Sites	Code	Longitude (E)	Latitude (N)	Altitude (m)	Subspecies	Samples	Voucher specimens
Laazib	(La)	9°51'	36°50'	45	<i>J. oxycedrus</i> ssp. <i>macrocarpa</i>	10	JOM1-JOM10
Tabarka	(Tab)	8°45'	36°57'	22		8	JOM11-JOM18
Oued el Bir	(OB)	10°44'	35°53'	52		8	JOM19-JOM26
Hawaria	(Ha)	10°50'	36°57'	48		8	JOM27-JOM34
Dkhila	(Dk)	9°26'	36°11'	502	<i>J. oxycedrus</i> ssp. <i>oxycedrus</i>	10	JOO1-JOO10
Sidi Ameur	(SA)	9°49'	35°88'	250		10	JOO11-JOO20
Kbouche	(Kb)	8°34'	36°13'	349		8	JOO21-JOO28

distinguishing flavor. According to one FAO document, juniper berries are the only spice derived from conifers.

In previous works, we have studied the essential oil composition of the Tunisian *Juniperus* and showed intraspecific variations in the chemical composition of these essential oils according to seasonal, geographical, and extract methods variations [10, 11]. Thus the objectives of this work are first, compiling the chemical components isolated from Tunisian *J. oxycedrus* essential oils, second, performing a comparative analysis between chemical compositions of each subspecies using principal component analysis (PCA), and last, investigating the antibacterial properties of essential oils of each of *J. oxycedrus* ssp. *macrocarpa* and *J. oxycedrus* ssp. *oxycedrus* populations against four food spoilage bacteria models by means of paper disc diffusion method and minimum inhibitory concentration (MIC) assays.

## 2. Results

**2.1. Chemical Composition.** The yields (v/w%) of the essential oils of *J. oxycedrus* ssp. *macrocarpa* leaves from Hawaria, Tabarka, Laazib, and Oued El Bir were comprised between  $0.08 \pm 0.04$  and  $0.28 \pm 0.03$  while the essential oil yields of *J. oxycedrus* ssp. *oxycedrus* leaves from Dkhila, Sidi Ameur, and Kbouche were comprised between  $0.28 \pm 0.19$  and  $0.4 \pm 0.14$ .

The chemical composition of the leaf essential oils of *J. oxycedrus* ssp. *macrocarpa* gathered in four coastal sites and *J. oxycedrus* ssp. *oxycedrus* collected in three sites in the Tunisian mainland is reported in Table 1.

Fifty-five constituents representing 54.12 to 79.42% and 40.96 to 56.87% of the total oils were identified for *J. oxycedrus* ssp. *macrocarpa* and *J. oxycedrus* ssp. *oxycedrus*, respectively. The essential oil content shows variations in plants of different origins and different subspecies. Thus, monoterpenes made up the highest contribution representing 31.21 to 63.61% in *J. oxycedrus* ssp. *macrocarpa* essential oil and 42.88 to 75.87% in *J. oxycedrus* ssp. *oxycedrus* essential oil. The oxygenated monoterpenes represented only a small portion (2.82 to 9.18% and 0 to 1.92%) of the total oil, for *J. oxycedrus* ssp. *macrocarpa* and *J. oxycedrus* ssp. *oxycedrus*, respectively. However, the largest fraction is attributed to monoterpene hydrocarbons; it varies from 29.36% to 60.24% for *J. oxycedrus* ssp. *macrocarpa* and 40.96 to 56.87% for *J. oxycedrus* ssp. *oxycedrus*. The main compounds of this class are the  $\alpha$ -pinene which is the major

component in all the oils analysed, then sabinene and *p*-cymene, and followed by sesquiterpenes accounting from 16.83 to 20.83% of all the identified compounds. The germacrene D and 13-*epi*-manoyl oxide represented the main components of this fraction.

A great variability was found in the essential oil composition of the subspecies investigated in this work. Thus, the essential oils extracted from *J. oxycedrus* ssp. *oxycedrus* leaves were richer in  $\alpha$ -pinene (31.55 to 49.46%) than those from *J. oxycedrus* ssp. *macrocarpa* (15.97 to 35.52%). The highest level of the major compound ( $\alpha$ -pinene: 49.46%) was observed in the subspecies *J. oxycedrus* ssp. *oxycedrus* of Kbouche, while the lowest content (15.97%) was observed in *J. oxycedrus* ssp. *macrocarpa* collected in Tabarka. Moreover, *J. oxycedrus* ssp. *macrocarpa* oils were relatively richer in sabinene, *p*-cymene, 13-*epi*-manoyl oxide, and abietariene and relatively poor in germacrene D.

Chemical diversity reported in the *J. oxycedrus* subspecies prompted a geographical variation study on the essential oil composition. In order to determine and verify these variations between different populations and subspecies, the composition data were analyzed by principal component analysis (PCA). A graphic representation of the projection of variables and samples onto the two first principal components is given in Figure 1. The horizontal axis explained 41.15% of the total variance while the vertical axis a further 14.39%, generating three distinct chemotypes. The first group (G1) with sabinene/ $\alpha$ -terpineol chemotype, detected in *J. oxycedrus* ssp. *macrocarpa* populations, was collected in coastal sites (Laazib, Tabarka, Oued Bir El, and Hawaria). The second group (G2) with  $\alpha$ -pinene/germacrene D chemotype characterized the *J. oxycedrus* ssp. *oxycedrus* populations of Kbouche and Sidi Ameur. Leaf samples of *J. oxycedrus* ssp. *oxycedrus* harvested in Dkhila are characterized by  $\alpha$ -copaene/ $\delta$ -3-carene chemotype (G3).

The mean chemical compositions of the Tunisian *Juniperus oxycedrus* subspecies differed from those of other countries: (a) Italy (ssp. *oxycedrus*), limonene/ $\alpha$ -terpinyl acetate/ $\alpha$ -pinene/ $\beta$ -caryophyllene (12.3/9.5/8.1/7.1%) [12]; (b) Greece,  $\alpha$ -pinene (2.3–56.6%), accompanied by  $\beta$ -phellandrene (6.8–52.6%) and terpinolene (0.1–22.7%) [13] (subspecies not specified); and (c) Italy, supercritical CO<sub>2</sub> extract, germacrene D (15.9%), and manoyl oxide (10.2%) [14]. The oils of Tunisia differed also from most of the oils reported by Adams, who distinguished the leaf oils of three subspecies of *J. oxycedrus*

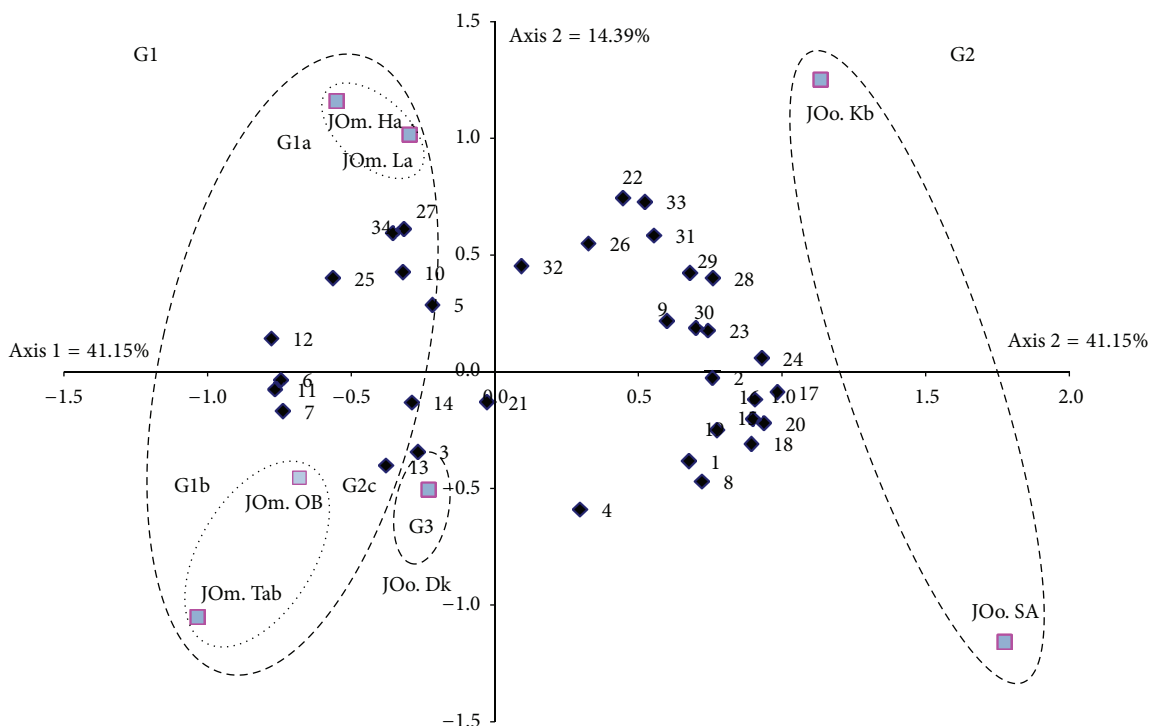


FIGURE 1: PCA of the 34 major components of the leaf essential oils of *J. oxycedrus* ssp. *oxycedrus* and *J. oxycedrus* ssp. *macrocarpa*. JOm: *J. oxycedrus* ssp. *macrocarpa*, JOo: *J. oxycedrus* ssp. *oxycedrus*, Dk: Dkhila, OB: Oued El Bir, Tab: Tabarka, SA: Sidi Ameur, Ha: Hawaria, La: Laazib, Kb: Kbouche, 1:  $\alpha$ -pinene, 2: Sabinene, 3:  $\beta$ -pinene, 4: myrcene, 5:  $\alpha$ -phellandrene, 6:  $\delta$ -3-carene, 7: Limonene, 8: *p*-cymene, 9:  $\beta$ -phellandrene, 10: terpinolene, 11:  $\alpha$ -campholenal, 12:  $\gamma$ -terpinene, 13:  $\alpha$ -terpineol, 14:  $\alpha$ -terpinyl acetate, 15:  $\alpha$ -copaene, 16:  $\alpha$ -humulene, 17:  $\gamma$ -muurolene, 18: Germacrene-D, 19: *trans*- $\gamma$ -cadinene, 20: *cis*-calamenene, 21:  $\alpha$ -cadinene, 22: occidentalol, 23: caryophyllene oxide, 24: humulene epoxide II, 25:  $\beta$ -elemene, 26: 1,10-*di-epi*-cubenol, 27:  $\alpha$ -eudesmol, 28: 5-OH-isobornyl isobutyrate, 29: cadalene, 30: (*Z*)- $\alpha$ -santalol, 31: (*E,E*)-farnesol, 32: 13-*epi*-manoyl oxide, 33: abieta-8,12-diene, and 34: abietariene.

(ssp. *oxycedrus*, *badia* and *macrocarpa*), all dominated by  $\alpha$ -pinene (25–43%), by the presence of limonene (4.5–28%) for the subspecies *oxycedrus* (Spain and Greece), germacrene D (3.4–24.5%) and variable amounts of manoyl oxide (0.2–21%) for the subspecies *badia* (Spain), and sabinene (26.5%) for the subspecies *macrocarpa* (Spain) [15]. Milos and Radonic [8] reported the composition of the essential oils of fresh leaves of *Juniperus oxycedrus* from Croatia. The major compounds were  $\alpha$ -pinene (41.37%), manoyl oxide (12.29%), farnesol (8.60%) a mixture of dodecenyl acetate isomers (6.32%), sesquiterpene hydrocarbon (4.40%), and dihydrofarnesol (3.35%). The composition of the essential oil from the leaves of Tunisian *J. oxycedrus* is quite different from that of essential of Spanish and Croatian *J. oxycedrus* found in the literature.

Similar work studying the chemical polymorphism of *J. oxycedrus* ssp. *oxycedrus* leaves was carried out by Boti et al., [16] showing the chemical variability of the oils of *J. oxycedrus* ssp. *oxycedrus* leaves collected from five sites along the Corsican site. The main constituents were terpene hydrocarbons, especially  $\alpha$ -pinene (56.1–73.3%),  $\beta$ -phellandrene (3.3–4.2%), and  $\delta$ -3-carene (0.7–8.2%). The principal component analysis allowed the distinction of two compositions in the leaf oils differentiated by the content of  $\alpha$ -pinene,  $\beta$ -phellandrene, and  $\delta$ -3-carene.

Adams et al. [17] in a comparative study of *J. oxycedrus* ssp. *oxycedrus* chemical profiles of some Mediterranean countries have reported that the oils of western Mediterranean

populations (Morocco, Spain, France, and Portugal) were characterized by relatively high levels of  $\alpha$ -pinene (45.3 to 50.3%). While the oils of *J. oxycedrus* ssp. *oxycedrus* leaves collected in the eastern Mediterranean countries (Italy, Greece and Turkey) were characterized by low concentrations of  $\alpha$ -pinene (19.3 to 32.7%) and moderate levels of  $\alpha$ -phellandrene,  $\alpha$ -terpineol, *p*-cymene,  $\beta$ -phellandrene, limonene, myrcene,  $\alpha$ -terpineol, (*E*)-nerolidol, and manoyl oxide. This result confirms what we found in the chemical composition of *J. oxycedrus* ssp. *oxycedrus* leaf oils of Tunisia (western Mediterranean country) which are characterized by a concentration of  $\alpha$ -pinene ranging from 31.55 to 49.46%. These findings are substantially similar to those reported by Adams et al. [17] in the oils of the western Mediterranean countries (Morocco, Spain, France, and Portugal). Moreover, *J. oxycedrus* ssp. *oxycedrus* oils of Tunisia are characterized by relatively large concentrations of germacrene D and 13-*epi*-manoyl oxide, which reveals their difference and particularity from the same subspecies from other origins.

## 2.2. Antibacterial Activity

**2.2.1. Disc Diffusion Results.** The antimicrobial activities of *J. oxycedrus* ssp. *oxycedrus* and *J. oxycedrus* ssp. *macrocarpa* essential oils were examined in the present study and their potency was qualitatively and quantitatively assessed by the presence and absence of inhibition zones and zone diameter.

The results are given in Table 3 and show that the essential oils of the *J. oxycedrus* ssp. *oxycedrus* have a potential for antibacterial activities against two strains among four, while *J. oxycedrus* ssp. *macrocarpa* have an antibacterial effect against three strains among four. *E. coli* was found to be the most resistant organism (there is no inhibition zone around the disk), whereas *Staphylococcus aureus* was the most sensitive organism to all the essential oils tested. The zone of inhibition is ranging from 6.5 mm (against *Salmonella enteridis*) to 13.5 mm (against *Staphylococcus aureus*). *Salmonella typhimurium* is sensitive only to *J. oxycedrus* ssp. *macrocarpa* (8 mm).

The bioactivity of leaf essential of *J. oxycedrus* subspecies is relatively low to moderate when compared to that of the standard antibiotic gentamicin (13 mm < zone diameter < 25 mm). We also note that the tested oils are more active against Gram<sup>+</sup> bacteria than Gram<sup>-</sup> ones.

**2.2.2. MIC and MBC Results.** MIC and MBC were estimated only for the oils which are active against each strain tested by the disc diffusion method. The results shown in Table 4 revealed that the MIC values accord on the whole with the diameters of inhibition. Essential oils that induced significant inhibition zone had smaller MIC on the corresponding strains. In the different strains tested, various levels of activity were found with minimal inhibitory concentration (MIC) values in the range 600–1500 µg/mL and minimal bactericidal concentration (MBC) values of 1000–>6000 µg/mL. Thus, *Staphylococcus aureus* show the highest sensitivity with MIC values ranging from 600 to 650 µg/mL followed by *Salmonella typhimurium* then *Salmonella enteridis*. Compared to gentamicin used as positive control, the tested essential oils were generally two to three orders of magnitude less active. We compared the MIC and MBC of the oils on the given microorganisms according to Faucher and Avril method [18], for which a substance is bactericidal when the MBC/MIC ratio is ≤2 and bacteriostatic when this ratio is >2. We deduce that *J. oxycedrus* ssp. *oxycedrus* collected from Kbouche are bactericidal against *Salmonella enteridis* and *Staphylococcus aureus* at concentration levels of 1000 µg/mL and 600 µg/mL, respectively. Essential oils of the same species harvested in Sidi Ameur and Dkhila are bactericidal against *Salmonella enteridis* and bacteriostatic against *Staphylococcus aureus* at concentration values of 2000 and 1500 µg/mL, respectively. On the other hand, *J. oxycedrus* ssp. *macrocarpa* essential oils have a bacteriostatic effect in the whole except essential oils extracted from *J. oxycedrus* ssp. *macrocarpa* leaves harvested from Tabarka which were bactericidal against *Salmonella typhimurium* with a MBC/MIC ratio equal to 1.76.

These findings confirm what has been reported by the authors in [19]. They showed that *E. coli* has the same resistance to essential oil of *J. oxycedrus* ssp. *oxycedrus* leaves of Sardinia. While *Staphylococcus aureus* was the most sensitive microorganism.

Slight variability of the antibacterial activity between essential oils of the two subspecies studied was observed. This polymorphism in the antimicrobial activity of essential oils of Tunisian *Juniperus oxycedrus* is mainly due to their chemical

profiles characterized by the diversity of chemotypes. However, a biological activity of a substance is caused by the presence therein of certain molecules. In the case of volatile oils, antibacterial activity is attributed to compounds of which we know the action. These molecules would act most often either in synergy with other compounds or individually. Notably, the essential oils considered are active towards pathologically relevant bacterial strains. This activity is probably associated with the monoterpene  $\alpha$ -pinene which is present in significant amounts and which is known to display antibacterial activities. Indeed, in their work on the species *Croton stellulifer*, the authors in [20] attributed the activity observed against the tested bacteria and fungi especially to the presence of  $\alpha$ -pinene among the major compounds of this essential oil. Also, the bioassay conducted by Aligiannis et al. [21] on *Sideritis sipylea* essential oils showed that it exhibited a high activity against microorganisms tested due to the  $\alpha$ -pinene action.

## 2.3. Experimental

**2.3.1. Plant Material.** The aerial parts of 62 samples of *J. oxycedrus*, used for the study of their chemical variability, were collected in January 2007 from female trees growing wild from seven locations in Tunisia (Table 1): *J. oxycedrus* ssp. *macrocarpa* (S. & m.) Ball. leaves were gathered from Hawaria, Laazib, and *J. oxycedrus* ssp. *oxycedrus* leaves were collected from Dkhila, Sidi Ameur and Kbouche. The Randomly selected samples of the 3 populations of *J. oxycedrus* ssp. *oxycedrus* and 2 populations of *J. oxycedrus* ssp. *macrocarpa* collected from Tabarka and Oued El Bir were used for the antibacterial investigations. Botanical voucher specimens have been deposited in the herbarium of the Pharmacognosy laboratory of the Faculty of Pharmacy, Monastir, Tunisia. The voucher numbers are provided in Table 2.

**2.3.2. Extraction of Essential Oils.** Extraction was carried out by hydrodistillation for 4 h, using a standard apparatus recommended in the European Pharmacopoeia (2009). We repeated this 3 times for each sample of 100 g of dried leaves and for each subspecies. The oil collected from each plant was dehydrated with Na<sub>2</sub>SO<sub>4</sub> and stored at 4°C, until analysis and biological activities testing.

**2.3.3. Chemical Analysis.** Quantitative and qualitative data of all the essential oils were determined by GC and GC/MS, respectively.

A gas chromatograph, GC, AGILENT Technologies Inc. (Santa Clara, CA, USA) model 6890N was employed for analysis of the extracts. It was equipped with a splitless injector, an autosampler AGILENT model 7683, and an AGILENT HP5 fused silica column; 5% phenyl-methylpolysiloxane, 30 m–0.25 mm i.d., film thickness 0.25 mm. GC conditions used were: programmed heating from 60 to 280°C at 3°C min<sup>-1</sup> followed by 30 min under isothermal conditions. The injector was maintained at 250°C. Helium was the carrier gas at 1.0 mL·min<sup>-1</sup>; the detector temperature was 280°C; the sample (1 µL; samples were run in chloroform with a dilution ratio

TABLE 2: Chemical composition of *J. oxycedrus* ssp. *oxycedrus* and *J. oxycedrus* ssp. *macrocarpa* leaf essential oils.

N°	RI	Components	<i>J. oxycedrus</i> ssp. <i>macrocarpa</i>				<i>J. oxycedrus</i> ssp. <i>oxycedrus</i>		
			OB	Ha	La	Tab	DK	SA	Kb
1	926	Tricyclene	0.11	tr	tr	0.14	0.12	tr	tr
2	931	$\alpha$ -Thujene	tr	0.1	tr	tr	tr	tr	tr
3	939	<b><math>\alpha</math>-Pinene</b>	<b>27.98</b>	<b>22.85</b>	<b>15.97</b>	<b>35.52</b>	<b>31.55</b>	<b>38.81</b>	<b>49.46</b>
4	951	$\alpha$ -Fenchene	0.19	0.20	tr	0.26	0.15	—	—
5	957	Thuja-2,4(10)-diene	0.21	0.13	tr	tr	tr	—	—
6	976	<b>Sabinene</b>	<b>10.13</b>	<b>9.11</b>	<b>12.12</b>	<b>11.5</b>	<b>0.11</b>	—	—
7	980	$\beta$ -Pinene	0.74	0.70	0.47	0.19	0.92	1.37	1.36
8	991	Myrcene	1.45	1.23	0.77	2.75	1.13	1.12	1.23
9	1001	$\delta$ -2-Carene	0.25	0.19	0.13	0.35	0.11	—	—
10	1005	$\alpha$ -Phellandrene	0.97	0.70	0.38	1.45	0.26	0.43	1.96
11	1011	<b><math>\delta</math>-3-Carene</b>	0.11	0.33	<b>5.93</b>	1.08	1.74	0.51	—
12	1026	<b><i>p</i>-Cymene</b>	<b>8.53</b>	<b>7.30</b>	<b>3.93</b>	<b>14.50</b>	<b>3.57</b>	0.39	—
13	1027	Limonene	0.45	0.48	0.36	0.34	0.54	—	—
14	1031	<b><math>\beta</math>-Phellandrene</b>	0.24	0.92	0.85	0.66	0.48	—	1.92
15	1062	$\gamma$ -Terpinene	2.15	1.77	1.04	3.10	0.87	0.86	—
16	1088	Terpinolene	0.47	0.47	0.43	0.80	0.35	1.39	0.85
17	1125	$\alpha$ -Campholenal	0.59	0.60	0.32	0.32	0.51	0.86	—
18	1177	Terpinene-4-ol	0.10	0.15	0.20	0.23	0.20	0.50	—
19	1189	$\alpha$ -Terpineol	1.19	1.09	1.88	1.74	0.61	—	—
20	1285	IsoBornyl acetate	tr	tr	tr	tr	0.10	—	—
21	1350	<b><math>\alpha</math>-Terpinyl acetate</b>	<b>7.27</b>	0.99	0.39	1.04	0.50	0.97	0.68
22	1376	$\alpha$ -Copaene	tr	tr	tr	tr	3.35	—	—
23	1384	$\beta$ -Bourbonene	tr	0.14	0.13	tr	0.28	0.67	0.82
24	1390	$\beta$ -Elemene	tr	0.33	0.27	0.27	0.12	—	—
25	1418	(E)-Caryophyllene	0.10	0.58	0.22	0.34	0.52	0.58	1.26
26	1454	$\alpha$ -Humulene	0.11	0.63	0.33	0.50	0.60	0.67	1.22
27	1477	$\delta$ -Muurolene	tr	0.41	0.13	0.34	0.40	1.15	1.56
28	1480	<b>Germacrene-D</b>	0.53	2.73	0.90	2.50	<b>3.95</b>	<b>4.11</b>	<b>8.96</b>
29	1491	Valencene	tr	0.18	tr	0.16	0.16	—	—
30	1497	Epizonarene	0.90	0.24	0.09	0.19	0.26	—	—
31	1513	<i>Trans</i> - $\delta$ -Cadinene	tr	0.36	0.20	0.22	0.77	0.63	1.06
32	1521	<i>Cis</i> -Calamenene	0.16	1.03	0.44	0.72	1.04	1.51	2.98
33	1538	$\alpha$ -Cadinene	tr	tr	tr	tr	0.38	—	—
34	1548	Occidentalol	0.28	0.95	0.76	0.65	0.41	0.99	0.67
35	1564	Nerolidol	tr	0.32	0.35	0.14	0.13	—	—
36	1581	Caryophyllene oxide	0.25	1.01	0.86	0.56	0.41	0.86	1.47
37	1594	<i>cis</i> - $\beta$ -Elemene	0.13	0.43	0.32	0.35	0.21	—	—
38	1606	Humulene epoxide II	0.11	0.44	0.35	0.30	0.20	0.89	1.23
39	1614	1,10-di- <i>epi</i> -Cubenol	tr	0.19	0.14	0.38	0.25	0.98	—
40	1634	$\beta$ -Acorenol	tr	0.38	0.17	0.11	0.21	0.17	0.11
41	1641	<i>Épi</i> - $\alpha$ -muurolol	tr	0.43	0.33	0.39	0.47	0.38	0.45
42	1645	$\alpha$ -Muurolol	tr	0.34	0.31	0.23	0.20	0.23	0.16
43	1649	$\beta$ -Eudesmol	tr	0.23	0.16	0.16	0.33	0.18	0.10
44	1652	$\alpha$ -Eudesmol	0.34	1.31	1.18	1.11	0.61	0.64	0.33
45	1655	5-OH-isoBornyl isobutyrate	tr	0.21	0.31	0.31	0.05	0.99	0.59

TABLE 2: Continued.

N°	RI	Components	<i>J. oxycedrus</i> ssp. <i>macrocarpa</i>				<i>J. oxycedrus</i> ssp. <i>oxycedrus</i>		
			OB	Ha	La	Tab	DK	SA	Kb
46	1674	Cadalene	tr	0.86	0.80	0.76	1.22	2.40	1.14
47	1678	(Z)- $\alpha$ -Santalol	tr	0.18	0.15	0.13	0.60	0.97	0.64
48	1682	$\alpha$ -Bisabolone oxide	0.17	0.85	0.70	0.61	0.46	0.43	0.23
49	1709	14-OH- $\alpha$ -Humulene	tr	0.15	0.14	tr	0.18	tr	tr
50	1722	(E,E)-Farnesol	—	0.36	2.20	—	0.32	2.09	1.37
51	1955	Ni	tr	0.21	0.19	tr	tr	tr	—
52	1989	<b>13-<i>epi</i>-Manoyl oxide</b>	<b>3.47</b>	<b>6.40</b>	<b>3.17</b>	<b>2.46</b>	<b>2.58</b>	<b>5.60</b>	<b>3.62</b>
53	2007	Abieta-8,12-diene	0.11	1.31	0.95	tr	0.16	1.17	0.94
54	2054	Abietariene	0.76	4.52	3.48	0.52	0.65	tr	tr
55	2142	Ni	—	—	tr	tr	0.32	0.34	0.14
56	2209	Ni	tr	0.40	0.46	tr	tr	—	—
57	2288	Ni	tr	tr	tr	tr	tr	—	—
Total			63.96	77.76	65.92	80.96	66.03	76.36	88.63
Monoterpenes hydrocarbons			53.32	45.13	31.39	71.74	40.96	44.92	56.87
Oxygenated monoterpenes			1.88	1.84	2.40	2.29	1.32	1.36	—
Sesquiterpenes hydrocarbons			2.23	7.73	3.71	6.24	14.19	11.72	19
Oxygenated sesquiterpenes			1.86	8.6	9.23	6.04	5.43	8.12	7.36
Diterpenes			4.34	12.23	7.6	3.05	3.39	6.8	4.58
Others			0.33	2.66	0.96	1.6	0.74	3.44	0.82

RI: retention index, tr: trace, OB: Oued El Bir, Mat: Matmata, Ko: Korbous, La: Laazib, SA: Sidi Ameur, JA: J. Abderrahmen, BH: Bouhedma, Dk: Dkhila, Tab: Tabarka, and Ha: Hawaria.

Percentages in bold refer to major components.

TABLE 3: Antimicrobial activity of the investigated essential oils in agar diffusion test\* (zone diameter are expressed in mm).

Species	Site	<i>Escherichia coli</i>	<i>Salmonella enteridis</i>	<i>Salmonella typhimurium</i>	<i>Staphylococcus aureus</i>
<i>J. oxycedrus</i> ssp. <i>oxycedrus</i>	Kb	0	6 $\pm$ 4	—	13.5 $\pm$ 00
	SA	0	6 $\pm$ 1	—	13.5 $\pm$ 00
	Dk	0	6.5 $\pm$ 00	—	13.5 $\pm$ 00
<i>J. oxycedrus</i> ssp. <i>macrocarpa</i>	Tab	0	6 $\pm$ 1	8 $\pm$ 1	13 $\pm$ 1
	OB	0	6 $\pm$ 2	8 $\pm$ 2	13 $\pm$ 1
Gentamicin		20	13	15	25

\* Values are means  $\pm$  SD of triplicate determinations.

Dk: Dkhila, Tab: Tabarka, SA: Sidi Ameur, Kb: Kbouche, and OB: Oued El Bir.

TABLE 4: Minimum inhibitory concentration (MIC) ( $\mu$ g/mL) of *J. oxycedrus* ssp. *macrocarpa* and *J. oxycedrus* ssp. *oxycedrus* essential oils against bacterial strains\*.

Species	Sites	<i>Salmonella enteridis</i>			<i>Salmonella typhimurium</i>			<i>Staphylococcus aureus</i>		
		MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
<i>J. oxycedrus</i> ssp. <i>oxycedrus</i>	Kb	1500 $\pm$ 2.3	2500 $\pm$ 5.3	1.66	NT	NT	—	600 $\pm$ 0.0	1000 $\pm$ 2.3	1.66
	SA	1000 $\pm$ 1.5	2000 $\pm$ 1.5	2	NT	NT	—	600 $\pm$ 2.3	2500 $\pm$ 2.1	4.16
	Dk	1000 $\pm$ 00	1500 $\pm$ 1.1	1.50	NT	NT	—	600 $\pm$ 1.6	3000 $\pm$ 1.4	5
<i>J. oxycedrus</i> ssp. <i>macrocarpa</i>	Tab	1200 $\pm$ 1.8	>6000	—	850 $\pm$ 2.2	1500 $\pm$ 1.8	1.76	600 $\pm$ 3.1	>6000	—
	OB	1200 $\pm$ 2.9	>6000	—	850 $\pm$ 2.1	1800 $\pm$ 1.9	2.11	650 $\pm$ 0.0	>6000	—

\* Values are means  $\pm$  SD of triplicate determinations.

NT: not tested, Dk: Dkhila, Tab: Tabarka, SA: Sidi Ameur, Kb: Kbouche, and OB: Oued El Bir.

of 1:100) was injected. The GC was fitted with a quadrupole mass spectrometer, MS, AGILENT model 5973 detector. MS conditions were as follows: ionisation energy 70 eV, electronic impact ion source temperature 200°C, quadrupole temperature 100°C, scan rate 1.6 scan s<sup>-1</sup>, and mass range 50–500  $\mu$ . Software adopted to handle mass spectra and chromatograms was a ChemStation. NIST98 (NIST/EPA/NIH, 1998), FLAVOUR, and LIBR (TP) Adams mass Spectra Libraries were used as references [22]. Moreover, whenever possible, identification has been confirmed by injection of authentic sample of the compound. Compounds were identified by matching their mass spectra and retention indexes (RI) with those reported in the literature or those of analytical standard solution;  $\alpha$ -pinene,  $\beta$ -pinene,  $\alpha$ -phellandrene, *p*-cymene,  $\alpha$ -terpinyl acetate, (E)-caryophyllene, and  $\alpha$ -humulene were obtained from Extrasynthèse (Lyon, France) and myrcene and caryophyllene oxide from Fluka (Milan, Italy).

**2.3.4. Antibacterial Testing.** The *in vitro* antibacterial activity of mixed essential oils of each of *J. oxycedrus* populations (Kbouche, Sidi Ameer, Dkhila Tabarka, and Oued El Bir) was tested against four laboratory control strains, two Gram positive bacteria: *Staphylococcus aureus* ATCC 25923 and *Salmonella enteridis* ATCC 13076, and Gram negative bacteria, *Escherichia coli* ATCC 35214 and *Salmonella typhimurium* NRRLB 4420 [23].

**2.3.5. Disc Diffusion Assay.** Antibacterial activity testing was done according to Bert and Lambert-Zechovsky [24] method with slight modifications. Briefly, bacterial suspensions were adjusted to  $1 \times 10^7$  CFU mL<sup>-1</sup> and spread in Muller Hinton broth. Subsequently, filter paper discs (6 mm Ø, wattman number 1) were placed on the surface of Petri dishes and impregnated with 10  $\mu$ L of essential oil. All petri dishes were incubated at 37°C (24 h). All determinations were performed in triplicate. Antibacterial activity was evaluated by measuring the radius of the inhibition zone to the nearest millimeter.

**2.3.6. Microwell Determination of MIC and MBC.** The MIC of essential oils determined by the disc diffusion method was also tested for antibacterial activity, using liquid analysis. A 96-well microliter-plate was used. In each well, 100  $\mu$ L of microbial suspension was added to 100  $\mu$ L of the tested oil dilution with a concentration range from 600 to 6000  $\mu$ g/mL. The last row, containing only agar and microbial suspension, served as a growth control. The plate was incubated at 37°C for 4 h, and the minimal inhibition concentration (MIC) was determined by the help of a microplate reader as the lowest concentration of compound whose UV/VIS absorbance at 570 nm was comparable to that of the negative-control wells. The MIC value was defined as the lowest concentration of the test sample resulting in complete inhibition of visible growth in the broth medium. The minimal bactericidal concentration (MBC) was determined by subculturing 20  $\mu$ L from each negative well without visible growth and from the positive control of MIC determination, onto substance-free Muller Hinton agar plates. The plates were incubated at 37°C for 24 h. the MBC values are defined as the lowest concentration of

the essential oil killing 99.99% of the test bacteria [24]. The standard antibiotic gentamicin was used as a positive control to check the sensitivity of the test organisms.

**2.3.7. Statistical Analysis.** To evaluate if the essential oils components can be useful in reflecting chemotaxonomic relationships, 34 compounds detected in the oil samples at an average concentration greater than 1% of the total oil were selected and used for this purpose. These components were subjected to principal components analysis (PCA) using SPSS 12.0 software (SPSS Inc. Chicago, IL, USA). The data related to the diameters of the growth inhibition bacteria were given as means  $\pm$  standard deviation.

### 3. Conclusion

The PCA of the chemical composition of the leaf essential oil of 62 individuals of two *J. oxycedrus* subspecies, *J. oxycedrus* ssp. *macrocarpa* and *J. oxycedrus* ssp. *oxycedrus*, separated them in three groups; each group constituted a chemotype. The second group formed by the essential oils of *J. oxycedrus* ssp. *oxycedrus* of Sidi Ameer and Kbouche is characterized by the highest level of the major component ( $\alpha$ -pinene) and therefore by the highest antibacterial activity. In general not only the strong activity was related to a high content of one major but the presence of the moderate and minor compounds is indispensable. The *J. oxycedrus* essential oils activity varied significantly within species and within strains. In general, the Gram positive bacteria *Staphylococcus aureus* was the most sensitive. However, the Gram negative bacteria *E. coli* is the most resistant. *J. oxycedrus* ssp. *oxycedrus* essential oils showed a bactericidal effect against *Salmonella enteridis* while *J. oxycedrus* ssp. *macrocarpa* essential oils from Tabarka were bactericidal against *Salmonella typhimurium*. However, the antibacterial activity was not very impressive for the therapeutic use; it could have potential applications in food products.

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