

Chemical composition, antioxidant and antibacterial activities of the essential oils of *Juniperus phoenicea*, *Juniperus thurifera* and *Juniperus oxycedrus*

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Abstract: This study aimed to compare the chemical composition, the antioxidant activity and the antibacterial activity of essential oils (EOs) of *Juniperus phoenicea*, *Juniperus thurifera* and *Juniperus oxycedrus*, obtained from Ait Bouguemez region (Province of Azilal, Morocco). The analysis by GC/MS of essential oils led to identify 37, 54 and 38 components for *J. phoenicea*, *J. thurifera*, and *J. oxycedrus*, respectively. Monoterpenic fraction was found predominant in essential oils of the three samples. The DPPH free radical scavenging activity showed that essential oil of *J. thurifera* has the strongest antioxidant activity with an IC₅₀ value of 12.07 µg/mL. The antibacterial activity showed that *S. aureus* was more sensitive than *P. aeruginosa* and *E. coli* for the three EOs tested.

Keywords: *Juniperus*; Essential Oils; GC/MS; Antioxidant activity; Antibacterial activity.

1. Introduction

Juniperus species (genus *Juniperus*, family of *Cupressaceae*) belong to the coniferous plants is an important component of arid and semi-arid ecosystems throughout the northern hemisphere^{1,2}. This genus is the second most diverse genus of the conifers, it is consisting of approximately 67 species and 28 varieties¹.

In Morocco, the genus *Juniperus* is present in several places among which the valley of Ait Bouguemez (Province of Azilal, Morocco) where we find the three species *J. phoenicea*, *J. thurifera* and *J. oxycedrus*. The valley of Ait Bouguemez is located in the central High Atlas limestone (Morocco). It extends at an altitude ranging from 1800 to 2200 m over nearly 30 km³.

Juniperus is one of the most used genus by the traditional healers in central High Atlas of Morocco⁴⁻⁷. *Juniperus* tar, leaves and fruits are employed to heal wounds, abdominal pain and stomachic disorders, metabolic diseases, and against fungal infections⁴⁻⁶. The treatment of infections and health disorders with herbal medicines is usually involves active natural products mostly of low

molecular weight of great structural diversity. These substances traditionally referred to as secondary metabolites, often are differentially distributed among limited taxonomic groups within the plant kingdom^{8,9}. The function or importance of secondary metabolites in plants is ecological. It is for interactions between the plants and their environment^{10,11}. Secondary metabolites include three major classes: terpenoids, alkaloids and phenolics. They contain numerous natural products, some of which have good pharmacological properties especially essential oils (EOs)^{12,13}.

Many studies around the world have been performed on the chemical composition of the EOs and extracts of *Juniperus* species. *Juniperus* Oils and extracts contain various chemotypical compounds: from 2,6-dimethyloctane to sesquiterpene skeletons, and flavonoids and biflavonoids, but the main classes identified in almost all *Juniperus* are mono and sesquiterpenoids and their derivatives¹⁴⁻¹⁸.

Many constituents present in *Juniperus* EOs are responsible for their biological properties⁸. According to Majewska¹⁹, EOs of *Juniperus* species showed antimicrobial activity against Gram-positive

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bacteria: *Bacillus cereus* ATCC 11778, *Bacillus subtilis* NCTC 8236, *Micrococcus flavus* MFBF, *Micrococcus luteus* ATCC 9341, *Staphylococcus aureus* ATCC 6538, *Staphylococcus aureus* MFBF, *Staphylococcus epidermidis* MFBF and *Enterococcus faecalis* MFBF. Gram-negative bacteria were also sensitive to *Juniperus* EOs. Misharina²⁰ reported that EOs of *Juniperus* species have a strong antioxidant activity and it depends on the nature and concentration of EOs components and also the geographical origin of plant.

In this regard, the purpose of this research is both to explore the chemical composition of EOs of the *Juniperus* species growing in valley of Ait Bouguemez in comparison to the EOs from other Mediterranean countries and to evaluate the antioxidant and antibacterial activities of the EOs tested.

Table 1. Characteristics of sites of sampling.

	Altitude (m)	Latitude-longitude	Soil	Bioclimatic stage
<i>J. phoenicea</i>	1870	31°63'N-6°44'W	Limestone	Semi-arid
<i>J. thurifera</i>	2200	31°65'N-6°41'W	Limestone	Semi-arid
<i>J. oxycedrus</i>	1950	31°62'N-6°46'W	Limestone	Semi-arid

2.2. Extraction of the EO

Dry aerial parts (100 g) of the three species were subjected to the hydrodistillation for three hours, using a cleavenger-type apparatus, according to the method recommended by the European Pharmacopoeia to produce Eos²¹. The obtained EOs were stored at +4 °C until use.

2.3. GC/MS analysis

The EOs were analyzed on a Hewlett-Packard gas chromatograph Model 5890, coupled to a Hewlett-Packard model 5971, equipped with a DB5 MS column (30 m X 0.25 mm; 0.25 μm), programming from 50°C (5 min) to 300°C at 5°C/min, with a 5 min hold. Helium was used as the carrier gas (1.0 mL/min); injection in split mode (1:30); injector and detector temperatures, 250 and 280°C, respectively. The mass spectrometer worked in EI mode at 70 eV; electron multiplier, 2500 V; ion source temperature, 180°C; MS data were acquired in the scan mode in the m/z range 33-450. The identification of the components was based on comparison of their mass spectra with those of NIST mass spectral library (NIST 2011) as well as on comparison of their retention indices either with those of authentic compounds or with literature values¹.

2.4. Antioxidant activity

The slightly modified method of Pothitirat et al.²² was of great help to assess the radical scavenging abilities of EOs. 0.5 mL of various concentrations of EO and standards butylhydroxytoluene (BHT), vitamin C and α-tocopherol separately were added to 1 mL of 100 μM methanol solution of DPPH. The reaction mixture was incubated in the dark at room temperature for 20

2. Materials and methods

2.1. Plant material

To begin with, we selected samples of *J. phoenicea*, *J. thurifera* and *J. oxycedrus* from the wilderness of Ait Bouguemez valley (Province of Azilal, Morocco). This region is chosen because of the presence of the 3 species at the same ecological conditions. Table 1 shows the ecological traits of the samples' collection setting. Authentication and identification of the plants was done at Laboratory of Ecology and Environment Laboratory, Faculty of Sciences Ben M'Sik (University Hassan II of Casablanca, Morocco). After that, samples were air dried, at room temperature (25°C) and put them away from sunlight for 21 days until their weight was stable. Finally, they were packed in bags made of paper and kept in a dark, cool and dry place after which their analysis started.

minutes. The optical density was monitored at 517 nm against blank containing methanol. The decrease in optical density of DPPH on addition of test samples in relation to the control was used to calculate the antioxidant activity as percentage of inhibition (%IP) of DPPH radical:

$$\% \text{ IP} = \{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})/(\text{Abs}_{\text{control}})\} \times 100$$

Abs_{control} is the absorbance of DPPH radical + methanol;

Abs_{sample} is the absorbance of DPPH radical + sample or standard.

The antioxidant activity of samples was expressed as IC₅₀ in μg/mL required inhibiting the formation of DPPH radicals by 50%. A low IC₅₀ value represents a high antioxidant activity. Experiments were carried out in triplicate.

2.5. Antibacterial activity

2.5.1. Bacterial strains

To evaluate the antibacterial properties of the samples, three strains of pathogenic bacteria were used in the study: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853).

2.6. Disc diffusion method

The antibacterial activities of the EOs were determined by using paper disk diffusion method to screen the efficacy of Eos²³. The EOs were diluted with dimethylsulfoxide (DMSO 10%) at the concentration 100 μg/disc. A volume of 10 μL of the concentration was impregnated into the paper disk with 6 mm diameter, and then placed onto Mueller-

Hinton agar plates (90 mm), which were previously inoculated on the surface agar with 100 μ L of 1.5×10^8 CFU/mL (equivalent to 0.5 McFarland) suspension for each tested bacterium. Respectively we used disc impregnated with DMSO 10% and standard disc of Ampicillin as negative and positive controls. In order to trigger excess prediffusion we kept the plates at room temperature for 30 minutes before incubation at 37°C for 24 h. We measured diameters of inhibition zones in mm. The experiments were carried out in triplicate.

2.7. Statistical analysis

Table 2. Chemical composition of the essential oils of the three species of *Juniperus*.

KI	Compound	<i>J. phoenicea</i>	<i>J. thurifera</i>	<i>J. oxycedrus</i>
926	Tricyclene	1.33	-	0.22
930	α -Thujene	-	1.43	-
939	α -Pinene	27.92	20.07	67.33
953	α - Fenchene	-	0.21	tr
953	Camphene	0.53	0.2	0.43
957	Thuja-2.4(10)-diene	-	-	0.21
975	Verbenene	-	-	0.48
976	Sabinene	0.24	19.57	1.02
980	β -Pinene	10.78	0.57	2.33
991	Myrcene	0.83	1.51	0.43
1001	δ -2-Carene	1.23	-	-
1005	α -Phellandrene	3.74	tr	-
1011	δ -3-Carene	0.56	3.49	7.21
1018	α -Terpinene	5.09	0.48	tr
1026	p-Cymene	1.19	2.6	0.74
1031	Limonene	-	2.65	0.66
1031	β -Phellandrene	17.67	-	-
1040	(Z)- β -Ocimene	-	tr	-
1050	(E)- β -Ocimene	-	0.37	-
1062	γ -Terpinene	1.01	0.94	0.26
1068	Cis-sabinene hydrate	tr	tr	-
1088	Terpinolene	2.19	0.84	0.21
1098	Linalool	0.2	2.85	0.76
1102	α -Thujone	-	0.33	-
1103	Cis-4-thujanol	-	0.29	-
1104	Trans-4-thujanol	-	0.21	-
1094	α -Pinene oxide	0.31	-	-
1119	Fenchol	-	-	0.71
1125	α -Campholenal	-	-	0.56
1177	Terpinen-4-ol	0.33	7.77	2.24
1183	p-Cymen-8-ol	-	0.31	0.22
1189	α -Terpineol	0.45	1.12	0.39

In each test, the experimental data represent the mean \pm Standard Deviation (SD). The IC₅₀ was obtained with a computer program GraphPad (Prism 8.0.1). Statistical analyses were performed with XLSTAT V.7.1. The significance of the difference among groups was analyzed using ANOVA followed by the Fisher (LSD test) and Dunnett tests. Differences were considered significant at the level $p < 0.05$.

3. Results and Discussion

3.1. Essential Oils composition

The EOs' chemical composition of the three species of *Juniperus* is presented in Table 2.

1204	Verbenone	1.59	0.25	0.95
1217	Trans-carveol	tr	-	1.1
1228	Citronellol	0.22	-	-
1257	Linalyl acetate	0.4	3.29	0.12
1272	Thujyl neo-3-acetate	-	-	0.3
1282	Verbenyl cis-acetate	-	-	0.72
1312	(2E,4E)-2,4-Decadienal	6.87	0.38	-
1339	δ -Elemene	-	0.7	-
1350	α -Terpinyl acetate	-	0.25	-
1376	α -Copaene	-	0.97	-
1383	β -Bourbonene	0.76	-	-
1391	β -Elemene	0.28	0.84	0.99
1418	β -Caryophyllene	0.33	0.29	1.01
1429	γ -Elemene	-	0.46	-
1454	α -Humulene	0.27	0.51	-
1460	Cis muurola-4(14),5-diene	-	0.96	-
1472	γ -Gurjunene	-	0.38	-
1477	γ -Muurolene	-	0.18	0.98
1480	Germacrene D	0.22	1.01	0.42
1491	Valencen	-	1.99	-
1499	α -Muurolene	0.26	1.17	0.21
1516	β -Curcumene	-	2.79	-
1513	γ -Cadinene	1.47	0.4	0.41
1524	δ -Cadinene	0.32	2.09	0.56
1529	Trans-calamenene	-	0.43	-
1538	α -Cadinene	-	0.5	0.35
1549	Elemol	0.47	0.42	tr
1556	Germacrene B	-	0.75	-
1574	Germacrene D-4-ol	-	0.36	-
1581	Caryophyllene oxide	-	0.47	tr
1596	Cedrol	-	1.61	-
1630	γ -Eudesmol	0.22	0.25	-
1645	α -Muurolol	-	-	tr
1649	β -Eudesmol	1.32	-	-
1652	α -Eudesmol	1.02	-	-
1653	α -Cadinol	0.36	0.69	0.24
1666	Bulnesol	-	1.63	-
1686	Epi- α -bisabolol	-	0.8	-
Total identified (%)		91.58	94.63	94.91
yield (%)		1.2	0.93	0.1
Monoterpenes hydrocarbon (%)		74.31	54.93	81.60
oxygenated monoterpenes(%)		9.97	17.05	8.07
Sesquiterpenes hydrocarbon (%)		3.91	16.42	4.93
Oxygenated sesquiterpenes(%)		3.39	6.23	0.31

-: not detected; tr: traces (<0.1%); KI: Kovat's Index¹

J. phoenicea provided the highest yield of EOs with approximately 1.2% compared to *J. thurifera* and *J. oxycedrus*. In contrast, the yield provided by *J. phoenicea*, remains higher than those in Greece (0.21%), Spain (0.30%), Portugal (0.41%)²⁴, Egypt (0.36%)²⁵ and Algeria (0.70% to 0.92%)²⁶. The yield of EO of *J. thurifera* is 0.93%. This yield was different from those reported in the literature. Zeraib et al.²⁷ reports values from 0.40 to 0.53 % in different populations of Algerian *J. thurifera*. The rate provided by *J. thurifera* is still higher than that obtained by El Hajjouji et al.²⁸ in Tizi N'tichka (High Atlas Mountains of Morocco) for this species, which did not exceed 0.67%. Finally, *J. oxycedrus* has the lowest yield among the three species (0.1%). This result is lower than that found for the same species in Kosovo (0.4 to 1.8 %) ²⁹, Spain (0.2%)¹⁴ and Tunisia (0.15-0.21 %) ³⁰. EOs yield showed variations from site to another and these variations could be accounted for by pedoclimatic variations of the growing site or by differences in the genetic arsenal of plants ^{1,27}.

The analysis of EO of *J. phoenicea* led to the identification of 37 components that represented 91.58% of the oil. The chemical composition is dominated by the presence of monoterpenes hydrocarbon (74.31%) followed by the oxygenated monoterpenes (9.97%), sesquiterpenes hydrocarbon (3.91%). Oxygenated sesquiterpenes were present in lower quantities with a percentage of 3.39%. Major products were α -pinene (27.92%), β -phellandrene (17.67%) and β -pinene (10.78%). The chemical composition of this species contains other components of a lower rate. The comparison of the chemical components of the EO of our sample with those of other *J. phoenicea* Oils showed that α -pinene is the major product of the Oil. In Tunisia, Ennajar ³¹ reported that monoterpenes (75.9%) represented the main fraction of the EO, α -pinene (55.7%) and δ -3-carene (10.7%) were the major components. The rate of α -pinene in the population of Spain is 53.5% and in Greece, α -pinene represent 41.8 % of the Oil ²⁴. In Algeria, α -pinene is the major product of the EO of *J. phoenicea* with an average of 48.08% ²⁶. For the Moroccan *J. phoenicea*, samples collected in the eastern middle Atlas were dominated by α -pinene, with a percentage of 64.19% ³² and in Oukaimden his rate was 38.2% ¹⁵.

For *J. thurifera* 54 components were identified representing 94.63% of the oil. The monoterpenes hydrocarbon represented the main portion (54.93%) followed by the oxygenated monoterpenes (17.05%) and sesquiterpenes hydrocarbon (16.42%), at last comes oxygenated sesquiterpenes (6.23%). The major components were: α - pinene (20.07%), sabinene (19.57%) and terpinene-4-ol (7.77%). A comparison

of the chemical composition of EOs of *J. thurifera* from Algeria showed some quantitative differences. Monoterpenes hydrocarbon varied from 18.6% to 40.1% in different populations of Algerian *J. thurifera* and the major constituent was sabinene (5.2 - 20%) ²⁷. In Morocco, Achak ¹⁵ reported that the major constituents of EO of Oukaimden (Western high Atlas, Morocco) were sabinene (16.5%) and γ -terpinene (9.3%). Another study showed that the EO of *J. thurifera* collected in eastern middle Atlas Mountains of Morocco are rich in monoterpenes (97.09 %) and the major constituent was β -pinene (36.26%) ³³.

The study of the EO of *J. oxycedrus* showed the presence of 38 components accounting for approximately 94.91%. Monoterpenic hydrocarbon fraction was found predominant in EO composition (81.60%). However, the contents of oxygenated monoterpenes, sesquiterpenes hydrocarbon and oxygenated sesquiterpenes were 8.07%, 4.93% and 0.31%, respectively. α -pinene (67.33%) and δ -3-carene (7.21%) were the major components. In Spain, Llorens-Molina ¹⁴ reported that monoterpenic hydrocarbon fraction was also found predominant in EO composition of leaves both for siliceous and calcicolous locations (72.5-71.4 %). α -pinene was also the main component accounting for 42% to 53.2% ¹⁴. Another study of *J. oxycedrus* in Lebanon showed that EO also contains as majority components α -pinene with a rate of 27.4%, in addition to myrcene (18.9%), α -phellandrene (7.1%) and limonene (6.7%) ³⁴. Monoterpenes, with a percentage ranging between 34.94% and 81.25%, were the main class of constituents in Italy ³⁵. The EOs from leaves collected in Tunisia showed sesquiterpenes as main class of constituents, ranging from 34.78% to 44.16% ³⁵. Identification of the volatile constituents of the EO of *J. oxycedrus* from the north centre region of Morocco showed high contents of α -pinene (31.25%) ³⁶.

The results obtained for the three species are in agreement with those announced by Adams ¹ and Angioni ³⁷ from the analysis of *Juniperus* genus, in which pinenes are generally dominant. Because of the genetic diversity ³⁸, different climatic conditions or of a large geographical diversity¹ the oils compositions of the same plant showed variability in different populations.

3.2. Antioxidant activity

The antioxidant activity of EOs of our samples is showed in Figure 1. The IC50 (half maximal Inhibitory Concentration) values of EOs and those of standards: BHT, vitamin C and α -tocopherol are represented in Table 3.

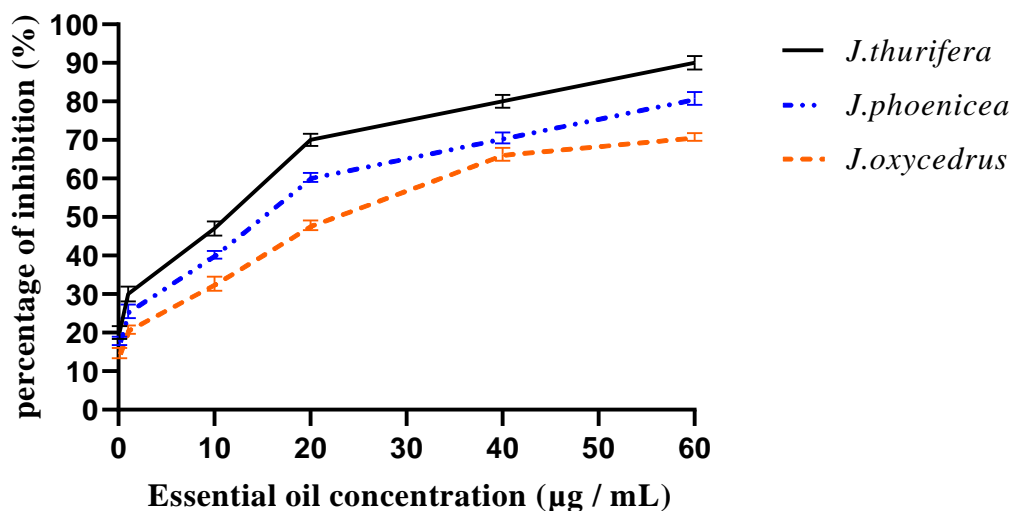


Figure 1. DPPH scavenging activity of EO of *J. phoenicea*, *J. thurifera* and *J. oxycedrus*.

Table 3. IC50 values of the three essential oils and the three standards.

Samples	IC50 (µg/mL)
<i>J. phoenicea</i>	14.39±0.94 ^b
<i>J. thurifera</i>	12.07 ±0.74 ^a
<i>J. oxycedrus</i>	22.14±1.16 ^d
BHT	18.04± 0.49 ^c
Vitamin C	13.45± 0.56 ^b
α-tocopherol	18.82 ±0.87 ^c

Letters superscript denote statistical differences between samples

EO of *J. thurifera* has the strongest antioxidant activity against DPPH radical with an IC50 value of 12.07 µg/mL, followed by EO of *J. phoenicea* with an IC50 of 14.39 µg/mL. At last comes EO of *J. oxycedrus* (22.14 µg/mL). The antioxidant activity of *J. thurifera* is significantly stronger than the three standards antioxidants: BHT, vitamin C and α-tocopherol. *J. phoenicea* has activity similar to vitamin C and higher than BHT and α-tocopherol. The antioxidant activity of *J. oxycedrus* is the lowest in comparison with both EOs and standards.

López³⁹ identified an important antioxidant effect in several species of the *Juniperus* genus originating from Spain. Setrani³² noted that EO of *J. thurifera* collected in eastern middle Atlas (Morocco) have the strongest antioxidant activity in comparison with those of *J. phoenicea* and *J. oxycedrus*. This result can be explained by the chemical composition of each EO.

Our results are in agreement with studies of Amorati⁴⁰ which showed that the antioxidant properties are in close relationship to the presence of oxygenated compounds (oxygenated monoterpenes and oxygenated sesquiterpenes) (23.28% for *J. thurifera* and 8.38% for *J. oxycedrus*). According to Amorati⁴⁰, oxygenated monoterpenes have been found to have good antioxidant activity because they undergo autoxidation, characterized by very fast termination process thereby reducing overall rate of oxidation. However, it is difficult to assign this activity to the only oxygenated compounds because of the chemical complexity of EOs which can generate a synergy effect between the various compounds⁴¹.

3.3. Antibacterial activity

Results of antibacterial activity of EOs of our samples are shown in Table 4.

Table 4. Diameters of inhibition zones of the three species of *Juniperus*.

	<i>J. phoenicea</i>	<i>J. thurifera</i>	<i>J. oxycedrus</i>
<i>S. aureus</i>	30.55±2.65 ^a	31.12±3.11 ^a	22.89±2.48 ^{ab}
<i>E. coli</i>	11.32±2.98 ^c	13.23±2.59 ^c	11.20±2.10 ^c
<i>P. aeruginosa</i>	17.20±3.54 ^b	18.27±2.29 ^b	20.22±3.30 ^b

Letters superscript denote statistical differences between samples

Ours results showed that *S. aureus* was very sensitive to the EOs of the three species with diameters of

inhibition zones of 31.12 mm, 30.55 mm and 22.89 mm respectively of *J. thurifera*, *J. phoenicea*

and *J. oxycedrus*. *P. aeruginosa* and *E. coli* were more resistant. Our results are in agreement with several works. As shown in studies of the antibacterial activity of EOs of *J. oxycedrus* from Morocco and Tunisia *E. coli* was highly resistant to this Oil whereas *S. aureus* was the most sensitive strain^{28,42}. *S. aureus* was very sensitive to the EO of Algerian *J. thurifera* and two *Pseudomonas* strains proved resistant²⁷. Bonsignore⁴³ confirmed that the EOs of Sardinian *J. oxycedrus* are active on Gram(+) bacteria.

Although, there were many differences in the chemical composition of the three species' EOs, their antibacterial activity was still the same. We also noticed that Gram(+) bacteria (*S. aureus*) are more sensitive to EOs of the three samples than Gram(-) bacteria (*E. coli* and *P. aeruginosa*). This is mainly due to the fact that Gram(-) bacteria possess an outer membrane that is more complex, rigid and rich in lipopolysaccharide (LPS), this outer membrane limits the diffusion of hydrophobic compounds. Yet, Gram(+) bacteria lacks such a membrane and possess instead peptidoglycan wall which is not thick enough to fight small antimicrobial molecules, thus making the cell membrane easily accessible^{44,45}. Besides, Gram(+) bacteria can facilitate the penetration of hydrophobic compounds of EOs owing to the lipophilic ends of lipoteichoic acid which exists in cell membrane⁴⁶. Furthermore, some constituents of our EOs such as α -pinene, α -terpineol, β -phellandrene, δ -3-carene, δ -2-carene and terpinen-4-ol should also be taken into account when dealing with the antibacterial activity of *Juniperus* species Eos^{47,48}.

Conclusion

The EOs of *J. phoenicea*, *J. thurifera* and *J. oxycedrus*, obtained from Ait Bouguemez region (Province of Azilal, Morocco), showed a chemical composition rich in monoterpenes hydrocarbon and oxygenated monoterpenes. They showed an important antioxidant activity and a good antibacterial activity against *S. aureus*, which can justify the multiple uses of the three *Juniperus* in the traditional medicine of Ait Bouguemez region. Thanks to their antioxidant and antibacterial activities, EOs of *J. thurifera*, *J. phoenicea* and *J. oxycedrus* have to be valorized via several domains. However, numerous investigations should be carried out on their mode of action and their probable toxicological effects in order to optimize their potential uses.

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