ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Chemical composition and antibacterial activity of essential oils extracted from three species of Moroccan *Juniperus*.

Houda EL HAJJOUJI[1,2]*, Chaimaa ELAGDI[1], Rachid RAHHAL[1], Said GMOUH[1], Mohamed HSAINE[1], Hassan FOUGRACH[1], and Wadi BADRI[1].

[1]University Hassan II of Casablanca, Faculty of Sciences Ben M'Sik, Laboratory of Ecology and Environment, Casablanca, Morocco.
[2]Higher Institute of Nurses Professions and Health Techniques, Béni-Mellal, Morocco.

ABSTRACT

Chemical composition of Moroccan *Juniperus thurifera*, *Juniperus phoenicea* and *Juniperus oxycedrus* were studied using Gas Chromatography-Mass Spectrometry (GC-MS). Antibacterial activity of these essential oils was determinate against three bacterial species: *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* by using paper disc diffusion method. The essential oils of the 3 species were predominantly composed of monoterpenes hydrocarbon (55.53-79.49%), in which α-pinene was dominant. Amongst the three tested bacteria *Escherichia coli* was the most resistant one while *Staphylococcus aureus* was the most sensitive.

Keywords: *Juniperus* ; Essential oil ; GC-MS ; Antibacterial activity.

https://doi.org/10.33887/rjpbcs/2019.10.3.60

Corresponding author

ISSN: 0975-8585

Introduction

In the light of developments made in the scientific field, the medicinal properties of plants have received a great interest because of their low toxicity, pharmacological activities and economic viability [1]. Such studies have focused on the benefits of plant-extracted phytochemicals and their effect on human health. It has been reported that direct addition of aromatic plant essential oils and extracts to foodstuffs exert an antioxidant or antimicrobial effect [2]. Among compounds of natural origin, biological activities have been shown by essential oils from aromatic and medicinal plants [3]. Extensive documentation on the antimicrobial properties of essential oils and their constituents has been carried out by several workers. Although the mechanism of action of a few essential oil components has been elucidated in many pioneering works in the past, detailed knowledge of most of the compounds and their mechanism of action is still lacking [4].

The genus *Juniperus L*. belongs to the Cupressaceae family, representing about 70 species all over the world, and widely distributed throughout the forests of the temperate and cold regions of the northern hemisphere, from the far north to the Mediterranean [5]. The species of *Juniperus* are considered as an important medicinal plant largely used in traditional medicine. They have many uses in traditional medicine in several parts of the world. Juniper berries are used as a spice, particularly in European cuisine, which are the only spice derived from conifers. In Morocco, Cupressaceae tar, leaves, and fruits are widely used to treat different hair and skin problems like dandruff, eczema, itchiness, and fungal infections. Additionally, infusions of Cupressaceae species of dried leaves are used internally to treat rheumatism, diarrhea, and diabetes mellitus [6-7].

Plants produce a wide diversity of secondary metabolites which serve them as defense compounds against herbivores, and other plants and microbes, but also as signal compounds [8]. The main classification system of secondary metabolites includes three major groups: terpenoids, alkaloids and phenolics. Their classification is based on chemical structure, composition, their solubility in various solvents, or the pathway by which they are synthesized. Secondary metabolites, also known as phytochemicals, natural products or plant constituents are responsible for medicinal properties of plants to which they belong. The role they play in the plant is not, to date, well known or understood, but it may be beyond the protection [9].

In this context, the aim of this work is to report chemical composition and antibacterial activity of essential oils extracted from three plants used in Moroccan traditional medicine: *Juniperus thurifera*, *Juniperus phoenicea* and *Juniperus oxycedrus* collected in Tizi n Tichka in the High Atlas Mountains of Morocco.

MATERIALS AND METHODS

Plant material

The aerial parts of *J.thurifera*, *J.phoenicea and J.oxycedrus* were collected in Tizi n Tichka (in the High Atlas Mountains of Morocco) during October 2017. This region is chosen because of the presence of the 3 species at the same altitude. The characteristics of collection site are shown in table 1.

Table 1. Characteristics of the collection site

Collection site	Latitude Longitude	Altitude (m)	Sol	Precipitations (mm)	Bioclimatic stage
Tizi n Tichka	31°15'N 07°23'W	2050	Schists	500-600	Upper semi-arid

Aerial parts were identified in the Ecology and Environment Laboratory, Faculty of Sciences Ben M'Sik, University Hassan II of Casablanca, Morocco. The collected plants were air dried at room temperature (25°C) and kept in a dark, dry and cool place until use.

Essential oil extraction

100g of air dried plant material were crushed and placed in a round-bottom flask with 300 mL (for *J. thurifera* and *J. phoenicea*) and 600 mL (for *J. oxycedrus*) of distilled water. The extraction took 3 hours [10]. The collected essential oils were stored at 4°C until use.

Gas Chromatography-Mass Spectrometry (GC-MS)

The essential oils 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 [11] as well as on comparison of their retention indices either with those of authentic compounds or with literature values.

Antimicrobial activity (Disc diffusion method)

Antibacterial activity of essential oils was determined by the disc diffusion method [12] against the following bacterial strains: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 29213). Microorganisms were maintained on Muller-Hinton agar. The inoculums suspension were prepared by diluting suitably overnight (24 h at 37°C) cultures in Muller Hinton Broth medium with sterilized distilled water. The cell density was standardized with spectrophotometer (620 nm) to contain 1-3×10^8 microorganisms CFU/mL. The inoculum (100 µL) containing 10^6 CFU/mL of each bacterial strain was swabbed on the entire surface of Muller-Hinton agar. Sterile paper discs (6 mm in diameter) were impregnated with 10 µL of oil, extracts and their dilutions and then placed on the surface of inoculated Petri dishes. The plates were left at ambient temperature for 30 min to allow excess prediffusion of extracts prior to incubation at 37°C for 24 h. Diameters of inhibition zones were measured in millimeters. Standard disc of Ampicillin (30 µg) and blank discs (impregnated with DMSO) were used as positive and negative controls, respectively. Tests were carried out in duplicate.

Statistical analysis

Data were expressed as mean Standard Deviation (SD). The data were analyzed using the ANOVA analysis of variance followed by the Turkey test. Differences between means were considered significant at P values of less than 0.05.

RESULTS AND DISCUSSION

Gas Chromatography-Mass Spectrometry (GC-MS)

Table 2 represents the quantitative and qualitative results obtained by using GC-MS. The compounds identified in these oils are presented in order of their appearance.

The yields of essential oils of *J.thurifera*, *J.phoenicea* and *J.oxycedrus* were 0.67%, 0.80% and 0.10%, respectively. The rates provided by *J.thurifera* and *J.oxycedrus* were lower than those obtained by Satrani et al. [13] from the same species (1.32% and 0.15%, respectively), collected in Eastern Middle-Atlas (Morocco). In contrast, the rate provided by *J.phoenicea*, remained higher than that obtained by the same author in the High Atlas (Morocco), which did not exceed 0.48%. Barrero et al., Achak et al. and Derwich et al. [14-16] obtained a yield of 0.70, 0.94 and 1.62% of Moroccan *J.phoenicea* leaves. Algerian *J.thurifera* and *J.phoenicea* had a yield inferior than our samples (0.52% and 0.53%) [17-18]. The yield of *J.oxycedrus* remained higher than that in Turkey (0.02%) [5], and lower than those in Spain (0.20%) and Tunisia (0.15-0.21%) [19-20]. This difference in essential oil content is related to several factors, such as the geographical area of collection, climate, stage of development and the season [21]. According to Zeraib et al. [22], inter-population variation of essential oil yield is quite common phenomenon and encountered earlier in several other plant species. These variations

might be due to climatic conditions of the growing site, pedoclimatic variation or due to difference in the genetic makeup of the populations.

Table 2. Chemical composition of essential oil of *J. thurifera, J. phoenicea* and *J. oxycedrus*

KI	Compounds	*J.thurifera* (%)	*J.phoenicea* (%)	*J.oxycedrus* (%)
926	tricyclene	-	0.06	0.27
930	α-thujene	1.78	-	-
939	α-pinene	22.88	45.67	63.54
953	α-fenchene	0.58	-	-
953	camphene	0.47	0.09	0.76
957	thuja-2.4(10)-diene	-	-	0.36
967	verbenene	-	-	0.44
976	sabinene	11.9	0.12	0.5
980	β-pinene	0.78	0.29	0.7
991	β-myrcene	1.69	0.1	0.91
996	δ-2-carene	-	0.27	-
1006	α-phellandrene	0.34	0.07	-
1011	δ-3-carene	3.54	0.25	9.23
1018	α-terpinene	0.64	1.07	0.08
1026	p-cymene	0.35	8.85	0.86
1031	limonene	5.39	-	0.86
1032	β-phellandrene	-	16	-
1040	(Z)-β-ocimene	2.43	-	-
1050	(E)-β-ocimene	0.52	-	-
1062	γ-terpinene	1.02	0.13	0.21
1068	cis-sabinene hydrate	0.33	-	-
1088	terpinolene	0.89	0.18	0.77
1094	α-pinene oxide	-	0.66	-
1098	linalool	2.24	0.3	0.4
1099	α-thujone	0.35	-	-
1100	cis-4-thujanol	0.33	-	-
1104	trans-4-thujanol	0.41	-	-
1106	hotrienol	0.39	-	-
1119	fenchol	-	-	0.67
1128	α-campholenal	-	-	0.77
1159	pinene oxide-β	-	-	0.48
1166	borneol	0.35	-	-
1177	terpinen-4-ol	5.38	0.38	1.49
1183	p-cymen-8-ol	-	-	0.34
1189	α-terpineol	1.14	2.26	0.51

1204	verbenone	-	0.47	0.51
1219	trans-carveol	-	-	0.91
1226	citronellol	-	0.23	-
1257	linalyl acetate	2.65	0.16	0.16
1272	thujyl neo-3-acetate	-	-	0.35
1282	verbenyl cis-acetate	-	-	0.51
1285	bornyl acetate	-	0.14	-
1312	2.4-decadienal. (2E.4E)-	0.41	3.32	-
1314	terpenyl trans dehydro- α -acetate	-	-	0.33
1321	myrtenyl acetate	3.27	-	-
1338	δ-elemene	0.41	-	-
1343	α-terpinyl acetate	0.44	-	-
1376	α-copaene	0.49	1.93	0.11
1383	β-bourbonene	-	2.75	-
1391	β-elemene	0.88	0.83	0.43
1414	β-caryophyllene	0.33	2.94	1.17
1429	γ-elemene	0.66	-	-
1432	thujopsene	-	0.51	-
1451	α-humulene	0.48	0.33	0.36
1460	cis muurola-4(14).5-diene	0.4	-	-
1472	γ-gurjunene	0.8	-	-
1477	γ-muurolene	0.47	-	0.22
1480	germacrene-D	0.67	0.08	0.93
1491	valencen	0.37	-	-
1499	α-muurolene	1.03	0.24	0.56
1513	γ-cadinene	1.05	0.24	0.29
1515	δ-cadinene	2.36	0.17	0.83
1516	β-curcumene	0.39	-	-
1529	trans-calamenene	0.43	-	-
1539	α-cadinene	0.5	-	0.3
1549	elemol	1.94	0.38	0.55
1550	(E)-nerolidol	-	-	0.54
1556	germacrene B	0.62	-	-
1579	germacrene D-4-ol	0.55	-	-
1581	caryophyllene oxide	0.44	-	0.26
1596	cedrol	0.44	-	-
1630	γ-eudesmol	0.69	0.22	-
1646	α-muurolol	-	-	0.3
1649	β-eudesmol	-	0.06	-
1652	α-eudesmol	-	0.15	-
1653	α-cadinol	1.63	0.11	0.22

1666	bulnesol	2.38	-	-
1686	epi-α-bisabolol	0.33	-	-
1990	manoyl oxide	-	-	0.4
Total identified (%)		93.63	92.01	94.39
Yield (%)		0.67	0.8	0.1
Monoterpenes hydrocarbon (%)		55.53	73.15	79.49
Oxygenated monoterpenes (%)		17.36	7.92	7.43
Sesquiterpenes (%)		12.34	10.02	5.2
Oxygenated sesquiterpenes (%)		8.4	0.92	1.87
Diterpenoids (%)		-	-	0.4

KI = Kovat's Index

Total 79 compounds were characterized and identified by Gas Chromatography-Mass Spectrometry (GC-MS), representing 92.01-94.39% of the total oils composition. The oils of the 3 species were predominantly composed of monoterpenes hydrocarbon (55.53-79.49%), followed by the oxygenated monoterpenes (7.43-17.36%) and the sesquiterpenes (5.2-12.34%). Compared to the literature, monoterpenes contents were similar to that obtained in Morocco by Akkad et al. [23] (approximately 60%) and lower than that obtained by Satrani et al. [13] (97.09%). Medini et al. [24] reported that for essential oil of Tunisian *J.phoenicea* leaves and berries, the largest group of constituents in the essential oil was the monoterpenes. Similar results were obtained for Moroccan and Algerian *J.phoenicea* [14-15;17].

The chemical composition of the essential oil of *J.thurifera* is dominated by the presence of a major product, α-pinene with an average 22.88%, followed by sabinene (11.9%), limonene (5.39%) and terpinen-4-ol (5.38%). *J.thurifera* contained other components of lower rates: δ-3-carene, myrtenyl acetate and linalyl acetate. For *J.phoenicea*, the major compound of essential oil is also the α-pinene (45.67%), followed by β-phellandrene (16%) and p-cymene (8.85%). We note the presence of 2.4-decadienal. (2E.4E)-, β-caryophyllene and β-bourbonene in lower rates. *J. oxycedrus* is dominated also by α-pinene (63.54%) as a major compound, followed by δ-3-carene (9.23%). It contained other components of a lower rate: terpinen-4-ol and β-caryophyllene. The results obtained are in agreement with those announced by Adams [25] from the analysis of *Juniperus* genus, in which pinenes are generally dominant. These compounds can constitute important molecules discriminating various species of juniper [25]. Another study realized by Satrani et al. [13] in Morocco showed that the majority components obtained are pinenes and especially β-pinene (36.3%) for the essential oils from the branches of *Juniperus thurifera* and α-pinene for those of *Juniperus oxycedrus* (52.1%) and *Juniperus phoenicea* (64.2%). In contrast, Alan et al. [5] reported manoyl oxide (32.8%) and caryophyllene oxide (11.9%) as main constituents in leaf oil of *J. oxycedrus* subsp. *oxycedrus* from Turkey.

According to Skoula et al. [26], variability of the oil composition in different populations of the same plant species might be attributed mainly to genetic diversity. This variability could be also explained by the influence of environmental factors in the chemical composition of essential oils in the genus *Juniperus* [27-31].

In summary, data obtained in the present study show typical essential oil profiles for *J.thurifera*, *J.phoenicea* and *J.oxycedrus*, which can be related to other ones reported from different countries. Our investigation allows us to support that the species of *Juniperus* of Hight Atlas of Morocco had several variability quantitative and qualitative.

Antibacterial activity results

The essential oils of *J. thurifera, J. phoenicea* and *J. oxycedrus* were tested for their antibacterial activity against three selected bacterial strains, results are summarized in table 3.

ISSN: 0975-8585

Table 3. Antibacterial activity of the essential oils of *J. thurifera, J. phoenicea and J. oxycedrus*. Diameters of inhibition zones are expressed in mm.

	J. thurifera	*J. phoenicea*	*J. oxycedrus*
Escherichia coli	7 ±0.88[c]	6 ±1.85[c]	7 ±1.13[c]
Pseudomonas aeruginosa	15 ±1.14[b]	16 ±0.99[b]	15 ±1.57[b]
Staphylococcus aureus	23 ±0.00[a]	20 ±0.42[a]	22 ±0.70[a]

Values followed by the same letter are not significantly different.

The results showed that essential oils of *J. thurifera, J. phoenicea and J. oxycedrus* exhibited moderate to appreciable antibacterial activity against two bacteria (*P. aeruginosa* and *S. aureus*), with diameter of inhibition zones ranging from 15 to 23 mm. *E. coli* was the most resistant with diameter of inhibition zones ranging from 6 to 7 mm. *S. aureus* was reported as the most sensitive one with a diameter of inhibition zones of 23 mm for the *J. thurifera*. Comparing to literature data, similarities and differences could be noticed. Several authors proved that these Gram (-) bacteria appeared to be less sensitive to the action of many other plant essential oils [21;24;32-33]. Ennajar et al. [34] have tested the antibacterial activity of essential oils of *J. phoenicea* leaves and buds against Gram (+) and Gram (-) bacteria. They approved that these oils inhibited the growth of *E. coli*. These results are in disagreement with our findings. The study of the antimicrobial activity of leaf essential oil of *J. oxycedrus* from Tunisia showed that *E. coli* was extremely resistant to this oil while *S. aureus* was the most sensitive strain [20]. Essential oils of many *Juniperus* species were known to exhibit antimicrobial activity against Gram (+) and Gram (-) bacteria [21;24;35]. Indeed, antimicrobial activity of *Juniperus* essential oils was previously investigated and literature data pointed out wide range of activity from no antimicrobial effects to some antimicrobial activity against various tested microbial strains [35]. According to Medini et al. [24], the activity of the essential oil varies with its concentration and the bacteria. These differences in the susceptibility of the test organisms to the essential oil could be attributed to variation on the level of the essential oil penetration through the cell wall and cell membrane structure.

The antibacterial activity of the essential oil of *J. thurifera, J. phoenicea* and *J. oxycedrus* could, in part, be associated with the major constituent: α-pinene (22.88-63.54%). Djoukeng et al. [36] reported that essential oils containing terpenoids are more active against Gram (+) bacteria, which is in agreement with our findings. According to Ramdani et al. [21], the antimicrobial activity is likely to be associated with the high concentration of α-pinene. Additionally, α-pinene has been considered as antimicrobial active component responsible for the activity of *J. excelsa* essential oils [37]. In our case, even if α-pinene concentrations were different, antibacterial activity of the three species were statistically not different, which could be explained by the complexity of the secondary metabolites action and the synergy between some essential oils compounds. Cakir et al. [38] reported that the inhibitory action of the essential oil could be attributed to the occurrence of high proportions of monoterpenes and sesquiterpenes in the oil. Antimicrobial properties of action might be related to these compounds which have a high potential in strongly inhibiting microorganism pathogens [39].

CONCLUSION

In conclusion, the essential oils of *J. thurifera*, *J. phoenicea* and *J. oxycedrus*, collected in Tizi n Tichka in the High Atlas Mountains of Morocco, were rich in monoterpenes, with α-pinene as the major compound. The antibacterial activity showed that essential oils of *J. thurifera, J. phoenicea and J. oxycedrus* exhibited moderate to appreciable antibacterial activity against two bacteria (*P. aeruginosa* and *S. aureus*) while *E. coli* was the most resistant.

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