JACEP

Journal of Applied Chemistry and Environment Protection

Chemical Composition, Antioxidant and Antibacterial Activity of Juniperus oxycedrus subsp. oxycedrus L. berry, essential oil from Morocco

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Abstract: The present study was conducted to evaluate in vitro antibacterial and antioxidant properties of essential oil obtained ed from berry of two Morocco natural populations of Juniperus oxycedrus subsp. Oxycedrus L. The essential oils have been extracted by steam distillation and analyzed by GC and GC–MS. The identified components constituting 99.5% and 100% of the Ourika and the Tighadwine populations respectively. The main components of both oils were α-pinene (69.43% and 48.12%), Germacrene D (16.62% and 32,96%), β pinène (3.28% and 2%), Myrcene (4.19% and 2.12%) of the Tighadwine and the Ourika populations respectively. Other components were more presented in the essential oil of the Ourika population (>1%) as β caryophyllene (1.49%), α humulene (1.48%) and δ cadinene (2.38%). The essential oils of both populations exhibited antibacterial activity against Escherichia coli, Pseudomonas aeruginosa and staphylococcus aureus. Moreover, the essential oil of the Tighdwine population efficiently inhibited the growth of Entrococcus hirea. In vitro evaluation of antioxidant activity of essential oil with the DPPH method showed a significant activity of both populations with IC50 values of 31.96 µg /ml for the Tighadwine population and 31.23 µg /ml for the Ourika population. The results revealed that the essential oil of Juniperus oxycedrus subsp. Oxycedrus.L exhibits interesting antioxidant and antibacterial activity, and could be used as a natural preservative in food and /or pharmaceutical industries.

***Keywords*:** *Juniperus soxycedrus subsp. Oxycedrus.L; (Cupressaceae); Essential oil; Chemical composition; Antioxidant activity; Antibacterial activity.*

1. Introduction

Juniperus oxycedrus subsp. oxycedrus (Cupressaceae) is a medicinal plant which has long been used in traditional medicine as antispasmodic, antimicrobial, antidiarrheal, etc. The therapeutic benefits of medicinal plants are often attributed to their antioxidant properties. *Juniperus oxycedrus subsp oxycedrus* L. is a Mediterranean species of growing tree that can grow up to 14 m and whose woodlands represent the mature ecosystem on outer dunes and cliffs of Mediterranean coasts [1]. The oil known as Cade is obtained as a distillate from its berry [2]. Different approaches have been followed in the taxonomy of *Juniperus*. Farjon (2005) considered that *Juniperus oxycedrus* has 4 subspecies: subsp. oxycedrus, ssp. Macrocarpa (Sibth. & Sm.) Ball, subsp. badia (H.Gay) Debeaux, and subsp. Transtaganaranco. Many aromatic, spicy and medicinal plants contain antioxidant compounds. However, information about antioxidant properties of various plants been used in traditional medicine but used presently, are not available. Therefore, the evaluation of such properties is attractive, particularly for finding new sources of natural antioxidants [3].

Butyl-hydroxytoluene (BHT) is one of the major synthetic antioxidants with a wide range usage. Replacement of artificial antioxidants with natural antioxidants is highly considered.

However, interest is growing internationally for herbal products, such as essential oils, to replace the synthetic anti-oxidants based on their emerging deleterious side effects. For example [4], it revealed that when Butyl-hydroxytoluene (BHT) is administered in the diet of rats it induced papilloma and squamous cell carcinomas in their fore-stomach. One of the essential oils that have demonstrated significant potential as a replacement for the synthetic anti-oxidants based on its preservation effects is rosemary [5].

In recent years due to an upsurge in antibiotic-resistant infections, the search for new prototype drugs to combat infections is an absolute necessity and in this regard plant essential oils may offer great potential and hope. Volatile compounds from plants, especially essential oils have antimicrobial, fungicidal and insecticidal activities [6]. These products have frequently been reported to be antimicrobial agents [7-9]. The volatile essential oils released from leaves, flowers and fruits into the atmosphere and from roots into the soil defend herbivores and pathogens [10].

The aim of this study was to evaluated and to compare chemical composition; the antioxidant and the antibacterial activity of essential oils of berry of two populations *Juniperus oxycedrus. subsp. oxycedrus* L. collected in the region of Tensift- Al Haouz, Marrakech in Morocco, where the inhabitants frequently use these plants in traditional medicine.

2. Materials and method

2.1. Plant Material

The samples of plant of two populations of *Juniperus oxycedrus subsp. Oxycedrus L.*: Ourika and Tighdwine were collected on Tensift El haouz area, in 22/04/2011 and authenticated by Prof. Ahmed Ouhammou, Biology Department, Faculty of Sciences Cadi Ayyad University - Morocco.

2.2. Hydrodistillation.

The essential oil from fresh berry (250g) of two natural populations: Ourika and Tighadwine of *Juniperus oxycedrus subsp oxycedrus* L. were obtained by hydrodistillation for 3 h using a Clevenger apparatus. The essential oils were collected, and dried over anhydrous sodium sulphate to remove trace of moisture and kept under refrigeration in a sealed vial until required [11-12].

2.3 Analysis of the oils

The essential oils from the berries of *Juniperus oxycedrus subspoxycedrusb* L. were analysed by GC and (GC- MS. Samples were analyzed on a fused silica capillary column BP-5 (polydimethylsiloxane) (30 m x 0.25 mm i.d., film thickness 0.25 µm). injector temperature, 220°C; transfer line, 240°C; oven temperature programmed from 60 to 240°C at 3°C/min; carrier gas: helium 1 mL/min; injection volume, 0.1 mL (10 % solution); split ratio 1:10. Ion source temperature, 150°C; EI mass spectra were collected at 70 eV ionization voltages over the mass range 40-450 Da. Quantitation was carried out using a Varian 3400 gas chromatograph equipped with a flame ionization detector (FID), under the following operation conditions: vector gas : helium, flow rate 1 mL/min; injected volume, 0.1 mL (10 % solution); split ratio 1:10. Injector and detector temperature, 240°C; the oven temperature was programmed from 60 to 240°C at a rate of 3°C/min. Samples were analyzed on a fused silica capillary column BP-5 (polydimethylsiloxane) (30 m x 0.25 mm i.d., film thickness 0.25 µm).

The identifications of the components were made by comparison of mass spectra with those existing in the data system library NIST and cited in the [13-14] as well as by comparison of their retention indices (RI) with literature values.

2. 4. Screening for antioxidant activity

The DPPH scavenging activity of essential oil of *Juniperus oxycedrus subsp oxycedrus* .L was measured according to the procedure described by Offord and al 1995 [15] with some modifications. Radical scavenging activity of plant essential oils against the stable DPPH radical was determined spectrophotometrically. The colorimetric changes (from deep-violet to light-yellow), when DPPH is reduced, were measured at 517 nm on a UV/visible lights spectrophotometer. The antioxidant activities of essential oils were measured in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH. Forty microliters of various concentrations (5, 10, 25, 35, 45, 55 and 65µg/ml) of the essential oils in Methanol (MeOH) as well as vitamin C and BHT (as standard antioxidants compounds) were put into appropriate tubes and 4 ml of 0.004% methanolic solution of DPPH was added to each tube. Tests were carried out in triplicate. Absorbance measurements commenced immediately. The decrease in absorbance at 517 nm was determined after 1 h for all samples. Methanol was fixed to zero of the spectrophotometer. Absorbance of the DPPH radical without antioxidant, the control, was measured. Special care was taken to minimize the loss of free radical activity of the DPPH radical stock solution. Radical scavenging activity was expressed as percentage inhibition of DPPH radical and was calculated by following equation [16]:

% inhibition = (Absorbance of control-Absorbance of sample/Absorbance of control) × 100.

The inhibitory concentration 50% (IC_{50}) was calculated by nonlinear regression curve. The dose response curve was obtained by plotting the percentage of inhibition versus the concentrations.

2. 5. Antibacterial activity

The essential oils of two populations of *Juniperus oxycedrus subsp oxycedrus* L. were screened against four bacterial strains: *Escherichia coli* (CIP 54125), *Staphylococcus aureus (*CIP 53154), *Pseudomonas aeruginosa (*CIP A22) and *Enterococcus hirea (*CIP 5855).

2. 5. 1. Disc-diffusion assay

Antimicrobial tests were then carried out by disc-diffusion method [17] using 100 µL of suspension containing 10^5-10^6 CFU/mL of bacteria. The discs (6mm in diameter) were impregnated with essential oils and placed on the inoculated agar. Negative controls were prepared using the solvent employed to dissolve the essential oils, and/or Antibiotic (30 µg/disc) were used as positive reference standards to determine the sensitivity of one strain/isolate in each microbial species tested. The inoculated plates were

incubated at 37◦C for 24 h for bacterial strains. Antimicrobial activity was evaluated by measuring the zone of inhibition with the test organisms. This experiment was three repeated times.

2. 5. 2. Microdilution assay

The minimal inhibition concentration (MIC) values were also studied for the microorganisms which were determined as sensitive to the essential oils of *Juniperus oxycedrus subsp oxycedrus*.L in disc-diffusion assay. The inocula of microorganisms were prepared from 12-h broth cultures and suspensions were adjusted to10^5-10^6 CFU/mL. The essential oils of *J. oxycedrus subsp oxycedrus*.L dissolved in 10% Methanol were first diluted to the highest concentrated sample (500 µg/mL) to be tested, and then serial twofold dilutions were made in a concentration range from 7.8–500 µg/mL in 10 mL sterile test tubes containing nutrient broth. MIC values of *Juniperus oxycedrus subsp oxycedrus* .L oils with bacterial strains were determined based on microdilution method according to published procedures [18-20]. The MIC was the lowest concentration of essential oil in which bacteria failed to grow, so no visible changes were detected in the broth medium. The evaluation of MIC has been carried out in three times.

3. Results and discussions

The yield of essential oil of *Juniperus oxycedrus subsp oxycedrus .L* berry on dry weigh of the plants was as follows 1.02% and 0.97% for Tighadwine and Ourika populations respectively.

The chemical composition of the essential oil tested is shown to be a complex mixture of many compounds. Table 1 shows the identified compounds (in total 30 volatile compounds of the Ourika population (JOO) and 19 of the Tighadwine population (JOT).

α-pinene was the most abundant individual compound of both oil (69.43% and 48.12% followed by Germacrene D (16.62% and 32%), β-pinène (3.28% and 2%) and Myrcene (4.19% and 2.12%) for JOT and JOO respectively. Others components were more presented in the essential oil of Ourika population (>1%) such as β caryophyllene (1.49%), α humulene (1.48%) and δ-cadinene (2.38%). Whereas the Tighdwine population (JOT) contained less amounts of these components (< 1%). However, the concentration of α-pinene was more present in JOT than in JOO population. Conversely, the amounts of Germacrene D were significantly lower than those in JOT population.

The mean chemical compositions of the two natural populations of J. Oxycedrus subsp *Oxycedrus* L: Ourika and Tighadwine differed from those of other countries: (a) Italy (ssp. *oxycedrus*), α-pinene and

limonene (26.3% and 30.0%); [21] (b) Italy (subsp. *oxycedrus*), limonene/α-terpinyl acetate/α-pinene/β-caryophyllene (12.3/9.5/8.1/7.1%) [22]; (c) Greece, α-pinene (2.3–56.6%), accompanied by β-phellandrene (6.8–52.6%) and terpinolene (0.1–22.7%) [23] (subspecies not specified); (d) Croatia (*J. oxycedrus*), α-pinene (41.4%) followed by manoyl oxide (12.3%) [24]. Our result was also different from that reported by Adams in Tensift- El haouz area recently, who distinguished the leaf oils of three subspecies of *J.oxycedrus* subsp. *oxycedrus*, *badia* and *macrocarpa*), all dominated by α-pinene (25–43%), by the presence of limonene (4.5–28%). Another result obtained from the *J. Oxycedrus* subsp *badia* and *macrocarpa* in Spain [25] showed that the essential oil contained germacrene D (3.4–24.5%) and variable amounts of manoyl oxide (0.2–21%) for the subspecies *badia* and subsp *macrocarpa* respectively. The sabinene was more present in essential oil of the subspecies *macrocarpa.*

Essential oil of different origin could be differentiated by the presence of other components in appreciable contents. Generally, a great variability and diversity are observed concerning the chemical composition of the essential oils of *Juniperus* species and subspecies of different origin due to climatic and soil variation condition, the vegetative cycle, seasonal variation [27-28].

The essential oils extracted from *Juniperus oxycedrus subsp oxycedrus* .L berry reveal a very important antioxidant activity, confirmed by radical scavenging activity (% inhibition) as shown in Figure 1 and table 2. The dose response curve was obtained by plotting the percentage of inhibition versus the concentration. The obtained data were used to determine the concentration of sample required to scavenge 50% of de DDPH free radicals (IC_{50}). The oil obtained from the both populations: JOO and JOT showed an interesting activity with an IC_{50} value of 31,32 µg/mL and 31, 96 µg/mL respectively. The antioxidant capacities of essential oils of *Juniperus oxycedrus subsp oxycedrus* .L is equal to that of BHT (IC_{50} = 32, 38 µg/mL) and low than that of ascorbic acid (IC_{50} = 20,20µg/mL). In other researches, the radical scavenging activity of *Juniperus oxycedrus subsp Oxycedrus* .L berry and wood oils from Lebanon was also reported [28]. There are a few reports about antioxidant activity of *Juniperus* genus. The methanol extract of *Juniperus chinensis* heartwood revealed strong antioxidant activity determined by DPPH method [29]. In another study, the water and ethanol extracts of *Juniperus communis* exhibit strong total antioxidant [30].

It is important to note that the antioxidant activities of the studied essential oils are due essentially to its abundance of the monoterene hydrocarbons and also to the overall chemical constituents contained in this oil. Essential oils of *Juniperus oxycedrus subsp oxycedrus*.L and their active components analyzed

showed good antioxidant capacities compared with vitamin C (Aca) and Butyl-hydroxytoluene (BHT) (standard antioxidant compound). *Juniperus oxycedrus subsp oxycedrus*.L can be used as an easily accessible source of natural antioxidants.

Table 1: Essential oil composition (%) of *J. oxycedrus subsp oxycedrus* in Ourika population (J.O.O) and Tighadwine population (J.O.T)

RI Kovats	RI Van den dool	RI tab	Compound	% J.O.O	J.O.T
926	922	926	Tricyclene	0.12	0.19
937	932	939	α-Pinene	48.12	69.43
953	947	954	Camphene	0.35	0.59
976	972	975	Sabinene	0.20	0.26
980	976	979	β-Pinene	2.00	3.28
992	990	990	Myrcene	2.12	4.19
1012	1010	1011	α-3-Carene	0.11	0.08
1032	1028	1030	Sylvestrene	1.01	1.57
1034	1030	1031	1,8-Cineole	0.10	-
1062	1057	1059	γ-Terpinene	0.06	-
1090	1088	1088	Terpinolene	0.47	0.49
1287	1285	1288	Bornylacetate	0.68	0.42
1351	1349	1351	α Cubebene	0.93	0.09
1376	1375	1376	α-Copaene	0.25	-
1390	1389	1388	β-Cubebene	0.06	-
1419	1418	1419	β-Caryophyllene	1.49	0.62
1454	1452	1454	α-Humulene	1.46	0.65
1477	1476	1479	γ-Muurolene	0.79	0.17
1482	1480	1485	Germacrene D	32.96	16.62
1494	1493	1495	γ-Amorphene	0.38	-
1499	1499	1500	α-Muurolene	0.57	0.12
1513	1513	1513	γ-Cadinene	0.89	0.45
1524	1523	1523	δ-Cadinene	2.38	0.42
1538	1536	1538	α-Cadinene	0.08	-
1556	1554	1561	Germacrene B	0.15	0.12
1590	1589	1592	Viridiflorol	0.14	-
1642	1640	1640	epi-α-Cadinol	0.20	-
1654	1653	1654	α-Cadinol	0.28	-
2013	2013	2022	Abieta-8,12-diene	0.14	-
2076	2075	2080	Abietadiene	1.02	-

(-): trace

Thus the ability to scavenger free radicals is an important property in order to minimize oxidative damage to living cells. Synthetic free radicals available .e.g. BHT have damage promoters of carcinogenesis and general consumer rejection of synthetic food additives hence the need for their replacement or as specific pharmaceutics [31].

The antibacterial activity of the essential oil of juniper berry was examined by Disc diffusion assay and microdilution assay. The results presented in table 3 and 4 reveal that the oil of J.O.O population inhibited the growth of *Escherichia coli, Pseudomonas aeruginos* and *Staphylococcus aureus.*

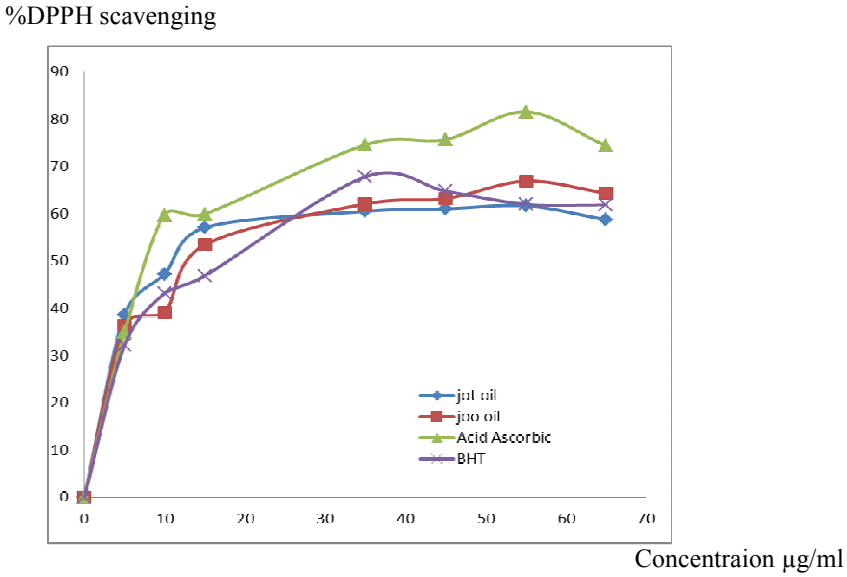

Figure 1: Radical scavenging activity of *J. oxycedrus subsp oxycedrus* .L oils (JOO and JOT), using DPPH assay compared to BHT and Ascorbic acid. Each data represents the mean ± SD n=3)

Table 2: IC 50 of essential oil of *Juniperus. Oxycedrus subsp Oxycedrus* L. (JOO and JOT) compared with BHT and Ascorbic acid (Aca).

	JOO	JOT	BHT	Aca
IC 50	31.32±0.65	31.96±0.58	32.38±0.67	20.20±0.69

Data are given as means ± SD. Viamin C (Aca) and BHT were used as positive controls for antioxidant.

Table 3: Inhibition growth effect of *Juniperus oxycedrus* oil against bacterial strains Inhibition zone (mm)*

	J.O.O	J.O.T	MeOH	Am	Gm
Escherichia coli	11±0.43	18±0.68	-	16	20
Pseudomonas aeruginosa	18±0.2	13±0.31	-	22	16
Staphylococcus aureus	12±0.55	10±0.16	-	26	20
Enterococcushirea	-	16±0.52	-	20	16

J.O.O: *Juniperus oxycedrus* from Ourika ; J.O.T: *Juniperus oxycedrus* from Tighadwine ; (-): Inactive ; Positive control: Gm: Gentamicin (10 µg/disc); Am: Amoxilaine (10 µg/disc)

Table 4: Concentration minimal of inhibition (µL/mL) of *Juniperus oxycedrus* oil (J.O.O and J.O.T)

	J.O.O	J.O.T
Escherichia coli	*0.5±0.2*	*0.25±0.6*
Pseudomonas aeruginosa	*0.25±0.6*	*0.75±0.3*
Staphylococcus aureus	*0.1±0.5*	*0.5±0.2*
Enterococcus hirea	-	*0.5±0.43*

-: no inhibition

However, we observed no effect on *Enterococcus hirea.* The essential oil of J.O.T population inhibited the growth of four bacteria strains tested. In low concentration, both oil exhibited bacteriostatic activity. The lowest values of MIC were the range to 0.1 – 0.5 µL/mL for J.O.O and to 0.25 – 0.75 for J.O.T. This result indicates that the essential oil of J.O.O was more efficient than that of J.O.T particularly against *Staphylococcus aureus* and *Pseudomonas aeruginos.* This result agrees with previous studies on inhibition of bacteria species by essential oils (Eos) extracted from plants [32]. The effectiveness of the essential oil of *Juniperus. Oxycedrus* against susceptible bacteria was higher than reported for methanol extracts of this [33]. The stronger activity of the *Juniperus Oxycedrus subsp Oxycedrus* L. against the bacteria tested may be due to a high concentration of α-Pinene (a monoterpene hydrocarbon) as well as other minor constituents such β caryophyllene. In fact, the synergistic effects of the diversity of major and minor constituents present in the essential oils should be taken in to consideration to account for their biological activity.

3. Conclusion

The chemical analyses, by GC/MS, GC-FID allowed identification of ~99.5% of the total volatile products for *Juniperus oxycedrus subsp oxycedrus* L. and 30-19 volatile compounds of the Tighadouine and the Ourika population respectively. A major constituent in aerial parts was α-pinene (69.43% and 48.12%) and the yield of essential oils was 1.02% and 0.97% for Tighadwine and Ourika populations respectively. This yield of the plants essential oil that has been studied was important. These extracts reveal in vitro antibacterial activity on the studied bacterial, confirmed by MIC ranging from 0.1 to 0.75 µL/mL of oil. The antibacterial activity besides several biological activities can be employed in place of costly antibiotics for effective control of food borne pathogens. Essential oils of *Juniperus oxycedrus subsp oxycedrus* L. and their active components, analyzed showed good antioxidant capacities compared with BHT and ascorbic acid (standards antioxidants compounds). The results suggest that essential oil of *Juniperus oxycedrus subsp oxycedrus* L. can be considered as an alternative to traditional food preservatives.

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