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Chemical Composition of Essential Oils of Ripe and Unripe Berries and Leaves of *Juniperus Phoenicea* L. and determination of their Antimicrobial Activities

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ABSTRACT

In the present study, evaluation of the essential oil (EO) of the aerial parts (leaves, ripe berries and unripe berries) of *Juniperus phoenicea* L. collected from Eastern Morocco, and comparison of their chemical composition and their antibacterial and antifungal activities were carried out. The average yields of EOs obtained was varied; the unripe berries sample was the highest. The EOs components were analyzed and identified chromatographically by using (GC and GC/MS). Forty one compounds were identified in the leaves oil, while 34 and 28 compounds were identified in unripe and ripe berries, respectively. *J. phoenicea* is dominated by the presence of the major compound α-pinene only in leaves and unripe berries with 34.36% and 33.7%, respectively, while the major compound in the ripe berries was β-pinene oxide (18.17%). The antibacterial and antifungal activities of the EOs of *J.phoenicea* were evaluated against four ATTC types of bacterial strains, (*Bacillus subtilis, Escherichia coli, Staphylococcus aureus* and *Micrococcus luteus*) and seven ATCC types of fungal strains in which three are molds (*Asper gillusniger*, *Penicillium digitatum* and *Penicillium expansum*), the others are fungal species (*Gloeophyllum trabeum, Coniophoraputeana, Poria placenta* and *Coriolus versicolor).* The minimum inhibitory concentration was determined and the results obtained led to a significant inhibitory effect against most of studied microorganisms. The results showed that, EOs inhibited the growth of all bacterial strains at highest concentration (1/100 v/v) from all samples, and the most effective EO was obtained from the ripe berries. Additionally, the four wood rot fungi were sensitive to the EO from all samples at highest concentration (1/100 v/v), and only EO from ripe berries has antifungal activity even at low concentration (1/1000 v/v). The sensitivity was appeared also in all molds in case of ripe berries and leaves EOs at high concentration (1/100 v/v), while unripe berries EO inhibited the growth of *Penicillium expansum* only, the other molds were resistant.

Keywords: *Juniperus phoenicea*; juniper; essential oil; antibacterial activity; antifungal activity.

INTRODUCTION

Plant oils and extracts have been used for a wide variety of purposes for many thousands of years [Jones *et al*. 1996]. In particular, the antimicrobial activity of plant oils and extracts has formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies [Reynolds 1996; Lis-Balchin and Deans 1997]. Essential oils (EOs) are products, generally of rather complex composition comprising the volatile principles contained in the plants, and more or less modified during the preparation process [Bruneton, 1995]. Essential oils are valuable natural products used as raw materials in many fields including perfumes, cosmetics, aromatherapy, phytotherapy, spices and nutrition [Buchbauer, 2000].

Juniperus phoenicea L. (Cupressaceae), is an evergreen plant usually growing as a bush or a tree. The tree's EO is especially rich in the tricyclic sesquiterpene thujopsene; the heartwood contains an estimated 2.2% of this hydrocarbon [Ait Quazzou *et al.* 2012; El-Sawi *et al.* 2007; Barrero *et al.* 2004; Angioni *et al.* 2003]. To date, juniper EO has only been used in traditional medicine. The content of juniper EO differs depending on its origin. The amount of some components may significantly vary [Stankovi M. Z. *et al.* 1994]. During the previous studies of the EO of junipers, it was established that the pharmacological features are derived from its constituents. Therefore, its diuretic properties were

ascribed to terpinen-4-ol, and pinene was found to act as a rubefaciens [Damnjanovi B. M. 2000]. All terpene hydrocarbons are antiseptic, anti-inflammatory and antibacterial. They are also pain-killers, sedatives, stimulators and media for the excommunication of excrete mucus [B. Barjaktarevi *et al.* 2005 and L. Janku *et al.* 1957]. In addition, terpenes retard the retention of toxins in human organisms; they increase the abstraction of aggregated toxic material from the veins and liver, and act as antispasmodic agents [Damnjanovi B. M. 2000]. Certainly α-pinene is an acute antiseptic, while cadinene, caryophyllene, terpinene and sabinene have pronounced anti-inflammatory and antibacterial properties [Damnjanovi B. M. 2000]. The mixture of leaves and berries of this plant is used as an oral hypoglycaemic agent [Amer *et al.* 1994], whereas the leaves are used against bronco-pulmonary disease and as a diuretic [Bellakhder, 1997].

There are many papers report on the chemical composition and antimicrobial activity of leaves and berries EO of *J. phoenicea* grown in north Mediterranean basin but this is the first time to achieve like this study in order to include investigation the composition of unripe berries EO and their antifungal activity against wood rot fungi.

The aim of the present investigation was to identify the chemical composition of oils of *J. phoenicea* obtained from plant growing in eastern Morocco as well as to assess their antibacterial and antifungal activity.

MATERIALS AND METHODS

Plant Materials

The *Juniperus phoenicea* plant samples were collected from Idni forest, the forest known in High Atlas in Eastern Morocco (GPS, 30° 54. 959 N, 008 17. 847 W) in June 2015. The plant materials were kindly identified and classified by the Botanists from Forest Research Center in Rabat, Morocco.

Essential Oils Isolation

The extraction of essential oils (EOs) was performed by hydrodistillation in a Clevenger type apparatus [Clevenger J.F. *et al.* 1928]. Three distillations were carried out by boiling 200g of fresh plant material with 1 liter of water in a 2l flask for two hours surmounted by a column of 60cm in length connected to a condenser. The EO yield is determined from a dry matter, estimated from three samples of 30g dried to constant weight for 48 to 60 hours in an oven at 60 °C. The EO was stored at 4 °C in the dark in the presence of anhydrous sodium sulphate. Then it was diluted in methanol (1/20 v/v) prior to analysis by GC and GC/MS according to AFNOR standard [Anfor, 2000].

Analysis of Essential Oils

Chromatographic analysis of the *juniperus phoenicea* EO samples was performed on a gas chromatograph with electronic pressure control, type Hewlett Packard (HP series 6890) equipped with a capillary column HP-5 (5% diphenyl, 95 % dimethylpolysiloxane) (30 m x 0.25 mm) with a film thickness of 0.25 µm, with an FID detector set at 250 °C and fed by a gas mixture and a H2/Air split-splitless injector set at 250 °C. The volume injected is 1µl. The injection mode was split (split ratio: 1/50 flow: 66 ml/min). The gas used is nitrogen with a flow rate of 1.7 ml/min. The column temperature is programmed to increase from 50 to 200°C at a rate of 4 °C/min and held for 5 minutes at the final temperature. The detection limit is less than 1ppm. The device is controlled by a computer system type "HP ChemStation", managing the operation of the device and monitoring the changes in chromatographic analysis. Identification of components was performed based on their Kovats indices (KI) [Jalali H. M. *et al.* 2000], and on gas chromatography coupled with mass spectrometry electron impact (GC-SMIE) [Kovat E., 1965]; The latter is performed on a gas chromatograph, type Hewlett-Packard (HP series 6890) coupled with a mass spectrometer (HP 5973 series). Fragmentation is performed by electron impact at 70 eV. The column used was a HP-5MS capillary column (30m x 0.25mm); the film thickness is 0.25 µm. The column temperature is programmed from 50 to 200 °C at 4 °C/min. The carrier gas is helium with a flow rate set at 1.5 ml/min. The injection method is the split mode (split ratio: 1/70). The device is connected to a computer system that manages a library of mass spectra NIST 98. Indeed, the index system is based on the concept of relative retention. It compares the retention of any product to that of a linear alkane. This system is applicable in gas chromatography to any compound on any column. By definition, it assigns an index of 800 in the linear alkane C8 (n-octane), 1000 to C10 linear alkane (n-decane), and this, whatever the stationary phase, the length of the column, the temperature or flow rate. KI are determined by injecting a mixture of C9 to C24 alkanes in the same operating conditions [Jalali H. M. *et al.* 2000].

Microorganisms

The antibacterial activity of the oils was evaluated against four ATCC (American Type Culture Collection) types of bacterial strains (*Bacillus subtilis, Escherichia coli, Staphylococcus aureus* and *Micrococcus luteus*). They are maintained by subculture on nutrient agar favorable to their growth during 24 h in obscurity at 37°C.

The antifungal activity of the oils was evaluated against seven ATCC types of fungal strains in which three are molds (*Asper gillusniger*, *Penicillium digitatum* and *Penicillium expansum*); these molds are known by their high degree to contaminate the food stuffs and by their pathogenicities. The others are fungal species (*Gloeophyllum trabeum, Coniophoraputeana, Poria placenta* and *Coriolus versicolor)* in which they are known by their responsibility for brown and white rot wood. They are chosen for their considerable damage they cause to timber and derived products.

The fungal strains and molds belong to the Mycotheque Collection of Microbiology laboratory of the Forest Research Centre (Rabat, Morocco). They are regularly maintained by transplanting on the nutrient environment PDA (Potato Dextrose Agar).

Microbiological Procedure

The Minimum Inhibitory Concentration (MIC) of the EO was determined according to an improved method reported by Remmal [Remmal A. *et al.* 1993] with some modifications done by Satrani [Satrani B. *et al.* 2001]. The EOs are immiscible with water; therefore, emulsification was realized by dispersing EO in to 0.2% agar solution to obtain a homogeneous distribution and make the higher maximum of component/germ contact.

Dilutions are prepared at 1/10e, 1/25e, 1/50e, 1/100e, 1/200e and 1/300e in these agar solutions. In test tubes, containing each 13.5 ml of broth solid media TSA (Tryptic Soy Agar) for bacteria, and PDA for fungi, sterilization in autoclave for 20 min at 121°C and cooled at 45°C were done. Aseptically 1.5 ml of each dilution was added to obtain the final concentrations of 1/100, 1/250, 1/500, 1/1000, 1/2000 and 1/3000 (v/v). By shaking the tubes, the mixtures were all homogenized to disperse the EO in the cultural medium properly before pouring them into Petri dishes. Negative controls containing the cultural medium and agar solution at 0.2% without EO were equally prepared.

The seeding has been done by streaking with a help of calibrated platinum loop to withdraw the same inoculums volume. This latter is presented in the form of culture broth in 24 h for bacteria and in the form of a suspension in physiological water of spores resulting from a culture in 7 days in the PDA for fungi. Later after seeding, Incubation has been done at 37°C during 24 h for bacteria, and at 25°C during 7 days for fungi. Each test was repeated three times.

RESULTS AND DISCUSSION

Chemical Composition of the Essential Oils

Results of constituents of EOs extracted from ripe and unripe berries and leaves of *J. phoenicea* are presented in order of their appearance in Table 1. The EO of unripe berries is characterized by the highest yields rate (1.76%), while the lowest yield is observed in the leaves (0.92%), in the other hand, the ripe berries EO yield was (1.43%).

The analysis led to the identification of fifty two components in all our samples. Forty one components that represent (99.93%) of the total composition of EO from leaves against thirty four constituents (98.71%) extracted from unripe berries and only twenty eight components (99.97%) extracted from ripe berries, were revealed. Data analysis of the chemical composition revealed two main classes of components; monoterpene hydrocarbons (MH) and sesquiterpene hydrocarbons (SH). Monoterpene hydrocarbons were the most abundant fraction in all investigated oils (76.88%, 73.53% and 50.19%) from the leaves, ripe berries and unripe berries respectively, as well as sesquiterpenes were (23.05%, 26.44% and 48.52%) respectively. The ratios between monoterpenes (MH) and sesquiterpenes (SH) were 1:1, 3:1 and 3:1 for the unripe berries, ripe berries and leaves respectively (Fig. 1).

GC-FID & GC-MS analysis of the *Juniperus* EO showed presence of two main components, the major compound α-pinene was in the leaves and unripe berries (34.36%) and (33.7%) respectively, while the major compound of ripe berries EO was β-pinene oxide (18.17%). Additionally, Terpinen-4-ol was identified with (11.52%) in ripe berries only, while β-humullene was the most abundant oxygen-

containing sesquiterpenes in the unripe, ripe and leaves (14.46%, 6.25% and 4.84%) respectively, where α-muurolene was presented with percentages of (12.78%, 1.55% and 4.28%) respectively. Evaluation of the occurrence of the main components that characterized the EO of the Moroccan High Atlas *Juniper* (Fig.2), showed that α-pinene was only found with high amount in the unripe berries and leaves, and was absent in the ripe berries, while Terpinen-4-ol was only appears in the ripe berries. Major differences were found in the essential oils from ripe and unripe berries. This aspect could be due to local environmental conditions or due to oxidation stage of *Juniperus* berries. [Rezzi S. *et al*. 2001; Cavaleiro C. *et al*. 2001; Falchi Delitala 1980 and Caramiello R. *et al*. 1995].

Similarity in the composition of the leaves and berries EOs was obtained by [Barrero *et al*. 2006; Achak *et al*. 2008 and Achak *et al*. 2009] in their studies of Moroccan J. *phoenicea*. They found that the largest group of constituents in the EOs is the monoterpenes (71.1%) with α-pinene constituents (45.5%), (38.2%) and (58%) respectively.

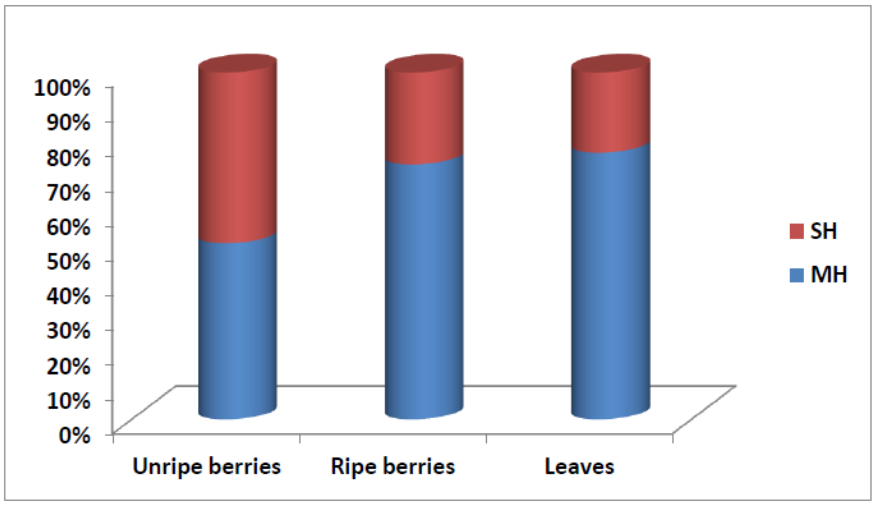

Fig1. *The monoterpenes (MH) / sesquiterpenes (SH) ratio of unripe berries, ripe berries and leaves essential oil from juniperus phoenicea.*

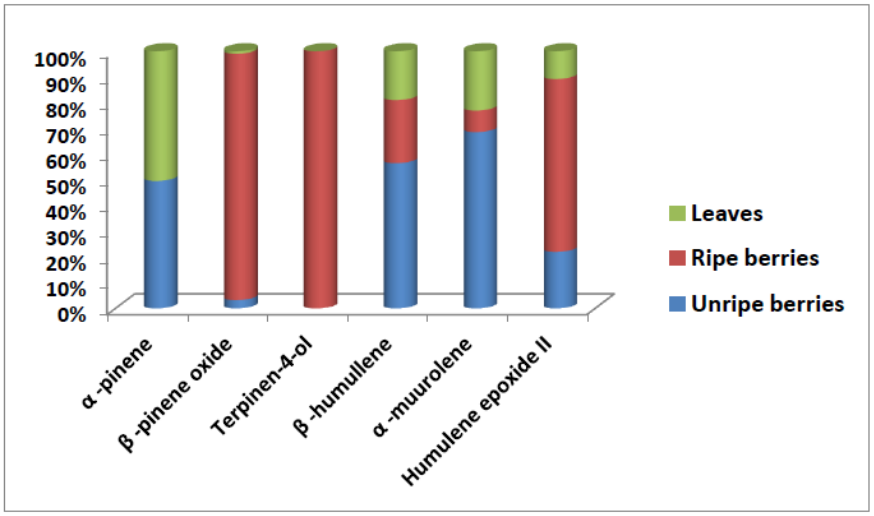

Fig2. *Predominant components in the unripe, ripe and leaves EO from juniperus phoenicea in High Atlas Morocco.*

Table1. *Constituents of essential oils of (Unripe and Ripe berries) and Leaves of Juniperus phoenicea and their percentages of essential oils yield.*

N°	Compound	KI*	Unripe Berries %	Ripe Berries %	Leaves %
1	**α -pinene**	**934**	**33.7**	**-**	**34.36**
2	**Myrcene**	**987**	**0.94**	**-**	**0.08**
3	**Dehydroxy-trans-linalool oxide**	**992**	**1.77**	**-**	**2.71**
4	**α -terpinene**	**1015**	**0.74**	**-**	**8**
5	**β -phellandrene**	**1026**	**0.29**	**-**	**-**

6	**(Z)- β -ocimene**	**1033**	**0.93**	**-**	**0.14**
7	**Cis linalool oxide**	**1067**	**-**	**-**	**0.07**
8	**linalool**	**1094**	**-**	**0.46**	**3.55**
9	**α -pinene oxide**	**1101**	**-**	**-**	**2.23**
10	**6-camphenol**	**1111**	**-**	**-**	**1.5**
11	**Trans-pinene hydrate**	**1121**	**1.22**	**0.85**	**3.38**
12	**Linalool dihydro**	**1131**	**-**	**-**	**0.79**
13	**Dehydro-linalool**	**1133**	**-**	**6.2**	**-**
14	**β -pinene oxide**	**1153**	**0.61**	**18.17**	**0.18**
15	**Cis-dehydro-b-terpineol**	**1155**	**1.83**	**8.91**	**0.1**
16	**Trans- β -terpineol**	**1158**	**-**	**3.39**	**-**
17	**Cis linalool oxide**	**1172**	**-**	**-**	**0.18**
18	**Terpinen-4-ol**	**1175**	**-**	**11.52**	**-**
19	**α -terpineol**	**1186**	**0.65**	**2.03**	**1.06**
20	**Dehydro-carveol**	**1191**	**0.84**	**2.89**	**-**
21	**γ -terpineol**	**1199**	**1.51**	**3.85**	**0.23**
22	**Trans-piperitol**	**1207**	**0.5**	**4.02**	**0.32**
23	**Cis-sabinene hydrate acetate**	**1220**	**0.39**	**4.96**	**0.08**
24	**Cis carveol**	**1227**	**-**	**4.28**	**7.9**
25	**Linalool acetate**	**1255**	**-**	**-**	**0.48**
26	**Dihydro-linalool acetate**	**1273**	**0.78**	**-**	**1.91**
27	**Trans linalool oxide acetate**	**1288**	**-**	**-**	**0.87**
28	**γ -terpinen-7-ol**	**1291**	**1.89**	**2**	**2.39**
29	**α -longipinene**	**1350**	**0.39**	**-**	**-**
30	**Neoiso-dihydro carveol acetate**	**1356**	**1.21**	**1.88**	**2.72**
31	**(Z) caryophyllene**	**1407**	**3.57**	**1.75**	**1.65**
32	**(E) caryophyllene**	**1420**	**1.26**	**0.2**	**-**
33	**β -humullene**	**1437**	**14.46**	**6.25**	**4.84**
34	**Cis-muurola-3,5-diene**	**1447**	**2.93**	**-**	**2.53**
35	**Cis muurola-4(14),5-dine**	**1466**	**-**	**-**	**0.1**
36	**γ -muurolene**	**1477**	**5.14**	**2.31**	**0.19**
37	**Germacrene D**	**1486**	**-**	**-**	**1.87**
38	**α -muurolene**	**1501**	**12.78**	**1.55**	**4.28**
39	**γ -cadinene**	**1513**	**-**	**-**	**0.32**
40	**Trans-cadina 1,4-diene**	**1532**	**0.88**	**0.24**	**0.25**
41	**α -cadinene**	**1538**	**1.03**	**0.65**	**5.11**
42	**Cis-cadinene ether**	**1553**	**-**	**-**	**tr**
43	**(z) isoeugenol acetate**	**1565**	**0.24**	**0.51**	**0.34**
44	**Caryophyllene oxide**	**1583**	**1.24**	**-**	**0.9**
45	**Trans- β -elemenone**	**1602**	**0.22**	**1.23**	**-**
46	**Humulene epoxide II**	**1609**	**2.55**	**7.82**	**1.25**
47	**Epi-α-cardinol**	**1637**	**0.54**	**0.39**	**-**
48	**α -muurolol**	**1645**	**0.47**	**-**	**-**
49	**α -cadinol**	**1651**	**0.97**	**1.32**	**0.47**
50	**Eudesmol dihydro**	**1662**	**-**	**-**	**0.13**
51	**Elemol acetate**	**1679**	**-**	**0.34**	**0.43**
52	**Junicedranol**	**1690**	**0.24**	**-**	**-**
	Total identified		**98.71 %**	**99.97 %**	**99.93 %**
	Yield %		**1.76 ±0.02**	**1.43 ±0.02**	**0.92 ±0.02**
	Monoterpene hydrocarbons (MH)		**50.19 %**	**73.53 %**	**76.88 %**
	Sesquiterpene hydrocarbons (SH)		**48.52 %**	**26.44 %**	**23.05 %**

KI*: *Kovats Index; (-): Absent; (%): Percentage; (tr): ≤ 0.05.*

Antimicrobial Activity

Antimicrobial screening of the essential oils was made by broth dilution method against four ATTC types of bacterial strains, (*Bacillus subtilis, Escherichia coli, Staphylococcus aureus* and *Micrococcus luteus*) and seven ATCC types of fungal strains in which three are molds (*Asper gillusniger, Penicillium digitatum* and *Penicillium expansum*), and the others are fungal species (*Gloeophyllum trabeum, Coniophoraputeana, Poria placenta* and *Coriolus versicolor*). (Table2). The results showed

that the oils inhibited the growth of all bacterial strains tested at the highest concentrations of the oils used (1/100 v/v) from all samples, while the inhibition decreased with dilution the concentrations 1/1000, 1/2000 and 1/3000 v/v. At the same time, in case of antifungal activity results, the sensitivity to EOs was appeared with all molds tested only in case of ripe berries and leaves; the sensitivity to the EO was in the highest concentration (1/100 v/v) too. The EO from unripe berries inhibited the growth of *Penicillium expansum* and has no effect on *Aspergillus niger* and *Penicillium digitatum.* Additionally, the four wood rot fungi used showed a sensitive to the EO from all samples at highest concentration (1/100 v/v), while they become resistance to the EO with dilution. It was shown that the EO from ripe berries has antifungal activity even at low concentration (1/1000 v/v).

The antimicrobial activity of the EO of J. *phoenicea* associated with their major constituents such as α-pinene, β-pinene oxide, β-humullene and α-muurolene. These components have been reported to display antimicrobial effects [Cosentino *et al*. 1999; Alessandra *et al*. 2005; Yang *et al*. 2007 and Demirci *et al*. 2007]. The essential oils containing terpenes are also reported to possess antimicrobial activity [Dorman and Deans, 2000], which are consistent with our present study. In addition, the components in lower amount may also contribute to antimicrobial activity of the essential oils, involving probably some type of synergism with other active compounds [Marino *et al*. 2001].

The results showed that, EO from *J. phoenicea* was rich with terpenic alcohols; it was observed 51.30% in ripe berries, 18.38 % in leaves and 11.21 % in unripe berries.

Table2. *Antibacterial and antifungal activities results from Juniperus phoenicea essential oil samples determined by the dilution method*

Conc. v/v	**1/100**			**1/250**			**1/500**			**1/1000**			**1/2000**			**1/3000**			**NC**
***J. phoenicea* samples**	**UB**	**RB**	**L**	**UB**	**RB**	**L**	**UB**	**RB**	**L**	**UB**	**RB**	**L**	**UB**	**RB**	**L**	**UB**	**RB**	**L**	
Bacterial Strains																			
Staphylococcus aureus	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Bacillus subtillis	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Escherichia coli	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+
Micrococcus luteus	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+
Molds																			
Aspergillus niger	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Penicillium expansum	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Penicillium digitatum	+	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Wood rot fungi																			
Coniophora puteana	-	-	-	-	-	-	+	-	-	+	-	-	+	-	+	+	+	+	+
Poria placenta	-	-	-	-	-	-	+	-	-	+	-	-	+	+	+	+	+	+	+
Gloeophyllum trabeum	-	-	-	+	-	-	+	-	-	+	-	+	+	+	+	+	+	+	+
Coriolus versiocolor	-	-	-	+	-	-	+	-	-	+	-	+	+	+	+	+	+	+	+

UB: *Unripe Berries; RB: Ripe Berries; L: Leaves; NC: Negative Control; (-): Sensitive; (+): Resistant.*

CONCLUSION

The present study determined the chemical composition of the essential oil of *Juniperus phoenicea* L. from east part of Morocco and evaluated their antibacterial and antifungal activities. The juniper ripe berries and leaves oil from High Atlas region contains more monoterpene (73.53% and 76.88%) and less sesquiterpene (26.44% and 23.05%) respectively. α-pinene was the major compound of the monoterpene hydrocarbons in leaves and unripe berries, while the major compound in the ripe berries was β-pinene oxide. This study demonstrates that, at high concentrations of the EOs used (1/100 v/v); the results showed inhibition the growth of all bacterial strains tested, while in case of molds, the sensitivity was appeared only from ripe berries and leaves EO. At the same time, the four wood rot fungi

used showed a sensitive to the EO from all samples at highest concentration (1/100 v/v), and only the ripe berries EO has antifungal activity even at low concentration (1/1000 v/v). The activity of the essential oil varies with its concentration, dilution affected its efficiency.

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