International Journal of Innovation and Applied Studies
ISSN 2028-9324 Vol. 13 No. 4 Dec. 2015, pp. 881-889

http://www.ijias.issr-journals.org/

Chemical Characterization and Antibacterial Evaluation of *Juniperus phoenicea* L. Leaves and Fruits' Essential Oils from Eastern High Atlas (Morocco)

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ABSTRACT: Aromatic and medicinal plants are a great source of complex molecules exploited by mankind in many industrial fields. Currently, the increasing use of natural occurring compounds has been observed and this fact justifies the increasing production of certain medicinal and aromatic plants (MAP). In this work, we were interested to valorize Juniperus phoenicea, a native species from Moroccan Eastern High Atlas (Tounfite). This plant is used in traditional medicine for its medicinal properties to treat many infectious diseases. J. phoenicea's leaves and fruits harvested in the flowering period (October 2013) have been subjected to hydrodistillation using a Clevenger-type apparatus. The yields of extracted essential oils (EOs) are about 1.71% and 2.01% respectively for leaves and fruits. Analysis of the chemical composition of both EOs (from leaves and fruits), by gas chromatography coupled with mass spectrometry, has shown their richness in monoterpenes (81.05% and 51.73%) and sesquiterpenes (13.71% and 38.08%). Both EOs are widely dominated by α-pinene (78.11% - 48.18% respectively). Antimicrobial activity of these oils was evaluated against four clinical strains: Escherichia coli, Pseudomonas aeruginosa; Staphylococcus aureus and Klebsiella pneumoniae. The results have shown that P. aeruginosa and S. aureus strains are sensitive to leaves' EO with inhibition areas that reached 23 mm and 26 mm respectively. The same minimum inhibitory concentration of 5.60µl/ml was found. These two strains are also sensitive to fruits' EO with inhibition zones of 19 mm and 11.50 mm and the minimum inhibitory concentrations are 11.20µl/ml and 2.40µl/ml respectively. In conclusion, fruits of J. phoenicea were richer in essential oils than its leaves with very high dominance of α- pinene. For the antimicrobial activity, EO from J. phoenicea's leaves, richer in monoterpenes, has been more effective against P. aeruginosa and S. aureus than the one extracted from the fruits. Nevertheless, both organs of J. phoenicea (leaves and fruits) showed a moderate antibacterial activity against the tested microorganisms.

KEYWORDS: *Juniperus phoenicea*, α-pinene, Essential oils, antibacterial activity, bacterial strains.

1 INTRODUCTION

Medicinal plants remain undeniably a major source of drugs, either because their constituents are valuable active ingredients, or because chemists were able to change the structure of certain molecules they contain in order to make them less toxic, more effective or to give them a better bioavailability. Currently, about 25% of modern drugs are developed from plants [1]. Indeed, the use of essential oils (EOs) is increasingly widespread since they are considered as natural products of high value. They exhibit pharmacological properties both at human and industrial scales; they have important applications in medicine, either by their good smell or their physiological efficacy or their capacity to relieve pain.

Juniperus (*Cupressaceae*) genus contains about seventy-five species grouped into three sections. The *Caryocedrus* section, in which we find only one species, is limited to the Eastern hemisphere [2], [3]. The *Juniperus* section which contains fourteen species: twelve only in the Eastern hemisphere, one endemic in North America and one species (J. *communis*) is circumboreal

[3]. and Sabina section where you can find about sixty species distributed equally between the Eastern and Western hemispheres [2].

Juniperus phoenicea L. is the only juniper with serrated leaves in the Eastern hemisphere. It is generally considered *Juniperus phoenicea* var. *phoenicea* or *Juniperus phoenicea* var. *turbinata* [2] or *Juniperus phoenicea* subsp. [4]. However, Adams and Schwarzbach (2013) [5] have recently shown that *Juniperus phoenicea* L. does not belong to a clad serrated leaves juniper that occurs in the Western Hemisphere, for that, they have designated *J. phoenicea* as "pseudo serrate" juniper to distinguish from serrated leaves juniper in the Western Hemisphere. In addition, they also found that two varieties of *J. phoenicea*. (var. *phoenicea* and var. *turbinata*) differ in their DNA sequences as well as several other recognized species of *Juniperus.*

Juniperus phoenicea L. also called "red cedar", is a shrub or a small tree native to the Northern Mediterranean, from Portugal to Israel [6]. It is also native to North-Africa, Algeria and Morocco and the Canary Islands [6].

J. phoenicea in Morocco is a circum-Mediterranean species; it is confined in the Middle-Atlas, High Atlas, Rif, dunes of the Mediterranean and Atlantic coasts. It is represented by two subspecies. The subspecies *lycia* extending from Essaouira to the mouth of the Moulouya River, covers also the sandy dunes, semi-arid bioclimate and warm sub-humid to temperate on soft sandy substrate. The *Phoenicea* subspecies that has a continental ecology grows in semi-arid, sub-humid and humid-temperate, fresh, hot and very cold bioclimatic variations [7]. We can distinguish the red cedar from other cedar species by the red color of its fruits, by its branched and less powerful port, the bushy appearance of its cylindrical branches and finally by its absence in high mountains (altitudes higher than 2200 meters) [8].

J. phoenicea L. species is considered as an important medicinal plant widely used in traditional medicine. The leaves are used as a decoction or an infusion to treat diarrhea, rheumatism [9], [10] and diabetes [9], [11], [12]. They can also be taken as powder against bronchopulmonary diseases or as diuretic. Tar of this species is used in the same way as the one of Barbary cedar, it is a substitute [13]; this plant species is considered as emmenagogue, it is also used against intestinal infection in Children [10]. Powder of dried fruits can heal skin ulcers and abscesses [14], and leaves and fruits' mixture is used as an oral hypoglycemic agent [15].

J. phoenicea L. is worth to be appreciated through its essential oils that contain a diversity of substances; they give them a wide variety of roles and biological properties. In the literature, some works have been performed on biological activities of J. *phoenicea* essential oils, they have shown that this species is antibacterial [16], [17], [19], antifungal [18], [20], antioxidant [17], [18] and cytotoxic against cell lines [19], [20].

The aim of this work is to study the bioactive substances extracted from leafy branches and fruits' EOs of J. *phoenicea* in order to use them as a natural source to fight against human pathogenic bacterial strains causing major problems in hospital environments.

2 MATERIAL AND METHODS

2.1 PLANT MATERIAL

Plant material is made up of fruits and leafy branches of *J. phoenicea* L. harvested from Tounfite, Midelt province (Eastern High Atlas) in October 2013. Both samples are dried away from light and moisture at room temperature.

The species has been identified at the Botanical Laboratory of Plant Ecology in the Scientific Institute of Rabat (Morocco) by Professor IBN TATOU.

2.2 EXTRACTION OF ESSENTIAL OILS

Extraction of essential oils was conducted by hydrodistillation using a Clevenger-type apparatus; the extraction was repeated three times to determine EOs' yields. Essential oils collected, measured (ml/100g of the dried plant), were introduced into dark sealed glass bottles and then, they were stored at 4°C to be preserved from heat and light [21].

2.3 ANALYSIS AND IDENTIFICATION OF EOS' CHEMICAL COMPOSITION

Chromatographic analysis of both samples of *J. phoenicea* EOs was performed on a gas chromatograph Thermo Electron type (Trace GC Ultra) coupled with a mass spectrometer type Thermo Electron Trace MS system (Thermo Electron: Trace GC

Ultra, Polaris Q MS), the fragmentation is performed by electronic impact (intensity 70 eV). The chromatograph is equipped with a DB-5 column (5% phenyl-methyl-siloxane) (30m x 0.25mm x 0,25µm film thickness), a flame ionization detector (FID) supplied by a gas mixture of H_2 / Air. The column temperature is programmed at a rate of 4°C / min from 50 to 200 °C for 5 min. The injection mode is split (leakage ratio: 1/70, flow ml / min), the carrier gas used was nitrogen with a flow rate of 1ml / min.

For compound identification, Kovàts Index [22] of each compound was calculated in relation to the retention time of linear alkanes' series (C_7-C_{40}). The calculated index was then compared to those of Adams reference [23]. Mass spectra of compounds were also matched with those stored in National Institute of Standards and Technology (2014).

2.4 MICROBIAL STRAINS

Bacterial strains used in antibacterial tests were: Gram- bacteria: *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* and Gram + bacterium: *Staphylococcus aureus*.

They come from the CHU (University Hospital center) Hassan II-Fez, and were stored at 4 °C in test tubes containing Mueller-Hinton solid medium until use.

2.5 MICROBIOLOGICAL PROCEDURE

A. ANTIBACTERIAL TESTS

Macrodilution method in liquid medium was used to determine minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of essential oils [24].To dimethylsulfoxide solution (DMSO), we added a quantity of essential oil about 30:70 (EO / DMSO: v/v). We vortexed for enough time to obtain a homogeneous stock solution, "S".

From the stock solution S, we selected specific volumes to have final concentrations about 0.18; 0.35; 0.70; 1.40; 2.80; 5.60; 11.20; 22.40 and 44.80 (µl/ml) which were added aseptically in a series of test tubes containing first a volume in ml of sterile Mueller-Hinton Broth. Each tube of the set containing 3.96 ml of the test solution (broth medium + EO), was inoculated with 40 µl of a 10^8 CFU/ml-standardized inoculum. This inoculum was obtained as a suspension in sterile saline solution (0.9% NaCl). The final volume of the solution in each tube is equal to 4 ml. After incubation for 18 to 24 hours at 37°C, MICs of EOs are determined. MIC corresponds to the concentration of the first tube in which there is no growth of the test germ visible to eye. We then seeded the surface of Mueller-Hinton agar (MHA) Petri dishes with 100 µl of the contents of tubes having a concentration greater than or equal to the MIC to determine the MBC. This is the lowest concentration that completely inhibits bacterial growth in 24 hours. Furthermore, the ratio MBC/MIC of each extract was calculated to assess its antibacterial power.

B. DISC DIFFUSION ASSAYS

These tests were performed *in vitro* using 90 mm-Petri dishes, by the agar diffusion method on Mueller-Hinton [25].to determine diameters of inhibition zone. A disc of 6 mm paper (Whatman No.1) was sterilized and then placed gently on the center of the surface of a previously inoculated medium. The disc is impregnated with a volume of 2µl of EO. The inhibition diameters around the disc were measured after incubation at 37 °C for 24 h. the test was repeated three times to minimize experimental errors. The antibiotic discs used as positive control for the disc diffusion assay are amoxicillin and imipenem.

3 RESULTS AND DISCUSSION

3.1 YIELDS AND CHEMICAL COMPOSITION OF *J. PHOENICEA* ESSENTIAL OILS

The yields of essential oils obtained from samples of fruits and leafy branches of J. *phoenicea* are about 2.01% and 1.71% respectively. These yields are higher than those of red juniper from Boulmane, Morocco (1.62%) [26], two subspecies *Turbinata* and *lycia*, from Assif Almal Moroccan region (fruits 1.10%, 1.02%); and Mehdia (Morocco) (leafy branches 0.98%, 0.90%) [27]. They are also higher than those of the red cedar from Tunisia (0.5% leaves) [28], and Algeria (leaves 0.52 %) [29].This variation in yields may depend not only on the studied part of the plant, but also on ecological, environmental and genetic factors.

Results of chromatographic analysis of essential oils extracted from fruits and leafy branches are shown in Table 1. Thirty-eight components that represent 98.59% of the total composition of EO from fruit against fifty constituents (99.99%) in EO

extracted from leafy branches, were revealed. Essences of both parts of the red cedar have the following compounds, fruits EO: α- pinene (78.11%) as the major one along with other compounds with smaller percentages: germacrene D (5.42%), trans-dauca-4 (11), 7-diene (2.96%) and E-caryophyllene (2.77%) (Figure 1), and leafy branches's EO: α- pinene (48.18%) as major component also accompanied by other components with moderate amounts: δ -cadinene (6.61%), trans-Dauca-4 (11), 7-diene (5.66%), E-caryophyllene (4.06%), germacrene D (3.52%) and α-himachalene (2.27%) (Figure 2).

Essential oils from fruits and leafy branches are rich in monoterpenes (81.05% and 51.73%) and sesquiterpenes (13.71% and 38.08%) respectively. We also note the presence of a small proportion of oxygenated monoterpenes (1.37%, 2.53%), oxygenated sesquiterpenes (1.97%, 6.54%), and oxygenated diterpenes (0.41%, 1.11%) and hydrocarbon diterpenes specific to essential oil from fruits (0.08%). Indeed, 22 of hydrocarbon terpenes from fruit essence represent 94.84% and 30 hydrocarbon terpenes from leafy branches essence represent 89% (Table 2). As for terpene oxides, they represent 3.34% for fruits and 8.98% for the leafy branches.

Table 1. Chemical composition of EOs from fruits and leafy branches of J. phoenicea harvested in Eastern High Atlas

N°	Compound	IK (Adams)	Area %	
			Fruits	Leafy branches
1	Tricyclene	926	0,27	0,21
2	α- pinene	939	**78,11**	**48,18**
3	Camphene	954	0,45	0,55
4	β- pinene	979	0,95	0,41
5	δ- 2 Carene	1002	0,4	0,43
6	δ- 3 Carene	1011	0,1	0,93
7	P- Cymene	1026	0,18	0,29
8	Limonene	1029	0,37	0,49
9	Gama- Terpinene	1059	0,1	0,24
10	Fenchone	1086	-	0,46
11	Terpinolene	1088	0,12	-
12	Linalool	1096	-	0,68
13	Fenchol (endo)	1116	-	0,15
14	α- Campholenal	1126	0,12	-
15	Trans- pinocarveol	1139	0,08	-
16	Camphor	1146	-	0,25
17	Cis- Verbenol	1141	0,48	-
18	Camphene hydrate	1149	-	0,26
19	isoborneol	1160	0,51	0,28
20	Dihydrocarveol (neo-)	1194	0,18	0,27
21	Citronellol	1225	-	0,09
22	Thymol, methyl ether	1235	-	0,09
23	δ – Elemene	1338	-	0,32
24	α- Cubebene	1351	-	0,2
25	α- Copaene	1376	-	0,74
26	Patchoulene	1381	-	0,15
27	β- Elemene	1390	0,16	0,39
28	Sibirene	1400	0,19	0,13
29	**E- Caryophyllene**	1419	**2,77**	**4,06**
30	β- Copaene	1432	-	0,09
31	Gama- Elemene	1436	-	0,16
32	**α- Himachalene**	1451	0,9	**2,27**
33	Cis-Muurolr-4(14),5 diene	1466	-	0,14
34	Trans- Cadina-1(6),4-diene	1476	-	5,42
35	**Germacrene D**	1481	**5,42**	**3,52**
36	Widdra-2,4(14)-diene	1482	-	0,38
37	β- Selinene	1490	-	1,48

38	δ- Selinene	1492	0,12	2,35
39	α- Muurolene	1500	0,17	1,34
40	Trans β- Guaiene	1502	0,22	-
41	Cubebol	1515	0,29	1,84
42	**δ- Cadinene**	1523	0,71	**6,61**
43	Gama-Cuprenene	1533	-	0,87
44	α- Cadinene	1538	0,09	0,99
45	Elemol	1549	0,12	0,35
46	**Trans-Dauca-4(11) ,7-diene**	1557	**2,96**	**5,66**
47	Maaliol	1567	0,16	0,19
48	Caryophyllene oxide	1583	0,32	0,25
49	Presilphiperfolan-8-ol	1586	-	0,22
50	Allocedrol	1589	0,18	-
51	β- Atlantol	1608	0,09	-
52	Junenol	1619	-	0,29
53	Cubenol(1-epi-)	1628	-	2,32
54	Muurola-4,10(14)-dien-1- β-ol	1631	0,27	-
55	Muurolol (epi-α)	1642		0,47
56	Germacra-4(15),5,10(14)-trien-1-α-ol	1686	0,25	-
57	Valerianol	1658	-	0,61
58	Longiborneol acetate	1685	-	0,81
59	Shyobunol	1689	0,29	-
60	Manool oxide	1987	0,11	1,11
61	Abietadiene	2087	0,08	-
62	Abietal (4-epi-)	2298	0,3	-
Total			**98,59**	**99,99**

Table 2. Different classes of compounds identified in EOs from fruits and leafy branches of J phoenicea

classes of compounds	**Fruits**		**Leafy branches**	
	Number of identified compounds	%	Number of identified compounds	%
Hydrocarbon monoterpenes	10	81,05%	9	51,73%
Oxygenated Monoterpenes	5	1,37%	8	2,53%
Hydrocarbon sesquiterpenes	11	13,71%	21	38,08%
Oxygenated sesquiterpenes	9	1,97%	9	6,54%
Diterpenes	1	0,08%	0	-
Oxygenated diterpenes	2	0,41%	1	1,11%
Total	**38**	**98,59%**	**50**	**99,99%**

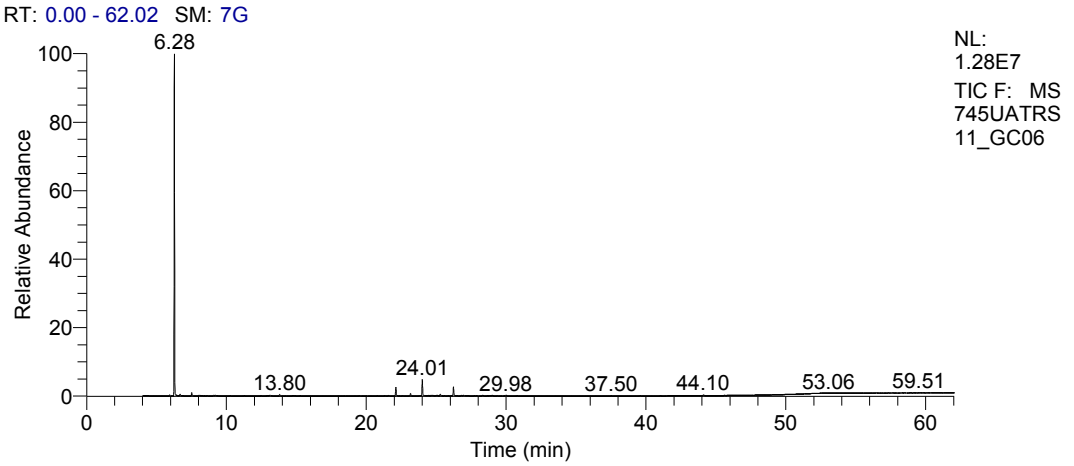

Fig. 1. Chromatogram of Juniperus phoenicea fruits'essential oil

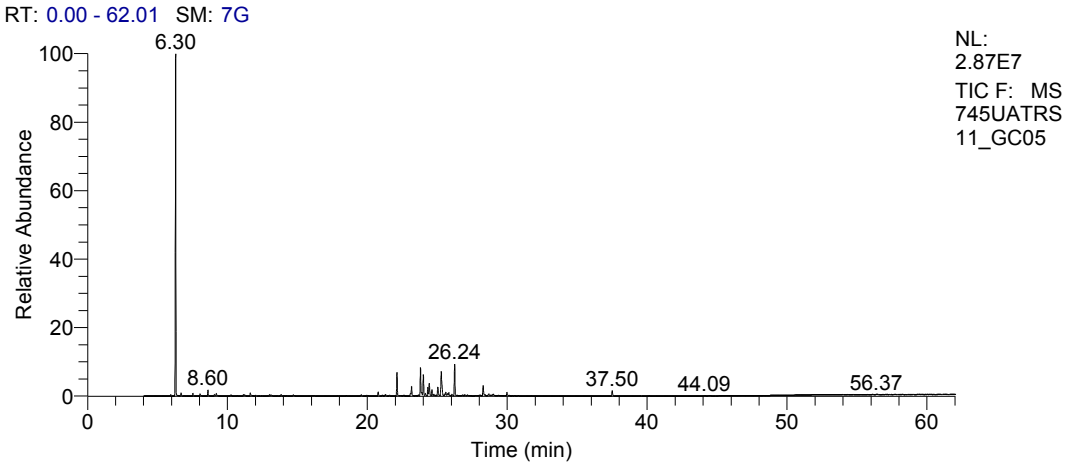

Fig. 2. Chromatogram of Juniperus phoenicea leaves' essential oil

3.2 SENSITIVITY OF BACTERIA TO EOS FROM LEAFY BRANCHES AND FRUITS OF J. PHOENICEA L.

Disc diffusion method allowed highlighting the antibacterial action of *J. phoenicea* leafy branches and fruits' essential oils towards the four bacterial strains. Results of the preliminary tests of antibacterial activity are summarized in Table 3.

Table 3. Diameters of inhibition zone against four pathogenic strains by disc diffusion method on the Mueller-Hinton solid medium after 24 hours-incubation at 37°C

Bacterial strains	Diameter of inhibition zone (mm)			
	Leafy branches	Fruits	Imipenem	Amoxicilline
Escherichia coli	8,30	7,00	26,00	6,00
Pseudomonas aeruginosa	21,00	19,00	25,00	6,00
Staphylococcus aureus	18,00	11,50	61,00	19,50
Klebsiella pneumoniae	15,50	8,00	28,00	6,00

J. phoenicea L. leafy branches and fruits' EOs have shown a relatively good activity compared to the applied concentration (2µl) against bacterial strains tested on solid medium.

Despite its high resistance to certain antibiotics, *P. aeruginosa* strain had a sensitivity to essential oils from leafy branches with an inhibition zone of 21.00 mm, followed by *S. aureus* strain that was considered a highly pathogenic species of the

genus *Staphyloccocus. S. aureus* inhibition zone was 18.00 mm, and *K. pneumonia* inhibition zone was 15.50 mm. However, this EO showed a weak antimicrobial activity against *E. coli* (8.30 mm).

As for fruits' EO, a strong antimicrobial activity was observed against *P. aeruginosa* (19.00 mm) followed by *S. aureus* with 11,50 mm as inhibition diameter, while this EO showed a low antimicrobial activity against *E. coli* and *K. pneumoniae* with 7.00 and 8.00 mm of inhibition respectively.

Among all these tested bacterial strains and relatively to all tested bacteria, *P. aeruginosa* has shown a marked sensitivity towards fruits and leafy branches' EOs, whereas E. *coli* was considered to be the most resistant strain to the tested EOs. However, EO from fruits has a relatively lower antibacterial activity compared to EO from leafy branches against all strains.

Inhibition diameters generated by these EOs, are very significantly lower than those produced by imipenem and higher than those of amoxicillin except for S. *aureus*.

Table 4. Minimum inhibitory concentrations (MIC) and Minimum bactericidal concentrations (MBC) of EOs from leaves and fruits of J. phoenicea (µl/ml)

Bacterial strains	**Leafy branches' EO**			**Fruits'EO**		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
Escherichia coli	2,80	11.20	4	>44,80	>44,80	-
Pseudomonas aeruginosa	5,60	22,40	4	11,20	22,40	2
Staphylococcus aureus	5,60	11,20	2	22,40	44,80	2
Klebsiella pneumoniae	2,80	11,20	4	11,20	22,40	2

Table 4 summarizes the values of minimum inhibitory and bactericidal concentrations of J. *phoenicea* EOs determined in liquid medium for the four tested bacterial strains. Overall, EOs of both samples of *J. phoenicea* have proved its effectiveness against all bacteria. Based on these results, we can conclude that *E. coli*, P. *aeruginosa*, S. *aureus* and K. *pneumoniae* had sensitivity to EO from leafy branches of *J. phoenicea*. These four strains were also inhibited by the following concentrations: 2,80 µl/ml, 5,60 µl/ml, 5,60 µl/ml and 2,8µl/ ml respectively.

Essential oils extracted from fruits of this plant showed inhibitory activity against three bacterial strains P. *aeruginosa*, S. *aureus* and *K. pneumonia* at the following concentrations: 11,20 µl/ml, 22,40 µl/ ml and 11,20 µl/ ml respectively. However, *E. coli* strain was the most resistant against this extract.

MIC values of leafy branches and fruits EOs are not consistent with those of inhibition diameters except for fruits' EO against *E. coli* strain. This extract from fruits has proved its inactivity against this strain, since its minimal inhibitory concentration is greater than the final concentration which is equal to 44.80 µl /ml.

According to the mismatched results of *J. phoenicea*'s oils activity tested by both methods (Aromatogram and direct contact), it appears that extracts of this plant either badly diffuses onto agar or the contact compounds/germs in the liquid medium changes.

Comparison of MBC / MIC ratio has allowed to define the bacteriostatic or bactericidal nature of these EOs. These latter are considered bactericidal when this ratio is equal to 2, and bacteriostatic if it is greater than 2 [30].So, fruits' EO seems therefore to exert a bactericidal action against three bacterial strains: *P. aeruginosa, S. aureus and K. pneumoniae*. While leafy branches' EO exerts bacteriostatic action against all tested bacteria except *S. aureus*.

Generally, antimicrobial activity of EOs is linked to their chemical compositions and synergistic effects of its components. In our case, the antimicrobial activity of *J. phoenicea'* s EOs can be attributed mainly to monoterpene hydrocarbons, including α- pinene which has several biological activities: antimicrobial [31], anti-inflammatory, antiviral, expectorant, sedative, herbicide and insect repellent [32], [33]. Also, this activity may be due to the synergistic role of its constituents and not to its major compounds [34]

4 CONCLUSION

In our research, we studied and compared the chemical and biological profiles of essential oils extracted from leafy branches and fruits of *J. phoenicea* from Tounfite (East High Atlas). The best essential oil yield was obtained from fruits (2.01%) compared to that of the leafy branches (1.71%).

Qualitative and quantitative analyses of essential oils have permitted to identify α- pinene as the main component of leafy branches and fruits (48% - 78.11% respectively). Fruits' EO also contains δ- cadinene (6.61%) and Trans-Dauca-4 (11), 7-diene (5.66%) with a relatively large content, whereas leafy branches' EO presents germacrene D (5.42%) with less amount.

The difference in yield and chemical composition between EOs from fruits and leaves in the studied plant can be attributed either to genetic factors or to the plant organs chosen to be extracted.

All the tested microorganisms presented, on solid medium, a growth inhibition by contact with essential oils from leaves and fruits, except *E. coli* which showed resistance to fruits' essential oils.

This work also highlighted a weak antimicrobial activity of both studied essential oils compared to imipenem (antibiotic). However, the results of antibacterial activity of both EOs in a liquid medium are different depending on the tested strains. Fruits' essential oil exhibits a greater bactericidal activity compared to leafy branches' oil, probably due to the difference in chemical composition and especially to the high dominance of α- pinene.

ACKNOWLEDGEMENT

We are sincerely grateful towards Doctor Nabil Boucetta from laboratoire Saiss des analyses médicales (Fès) for bacterial strains providing. Special thanks are addressed to Mr. Mohammed Ibn Tatou, Professor at the scientific institute of Rabat for plant species identification.

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