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Research Article

Chemical Composition and Antibacterial Activity of Berries Essential Oil of Algerian *Juniperus thurifera* (Var. *aurasiaca*)

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ABSTRACT

Background: Over the past decade, most antibiotic research programs have focused on finding new compounds with antimicrobial activity. This study aims to investigate the chemical composition and antibacterial activity of the essential oil (EO) extracted from ripe berries of Algerian *Juniperus thurifera* var. *aurasiaca*.
Methods: The chemical composition of *J. thurifera* EO extracted by hydrodistillation was analyzed by using the GC-MS technique. Antibacterial activity of EO alone and in combination with three conventional antibiotics was assessed by using disc diffusion method against four bacterial strains.
Results: Thirty-five components were identified, representing ~87 % of the oil. The main components were m-mentha-6,8-diene (15.43 %), β-pinene (10.59 %), elemol (8.31 %) and terpinene-4-ol (7.44 %). The essential oil showed strong antibacterial activity against *S. aureus* and *E. coli*, but no activity against *P. aeruginosa* and *B. subtilis*. Synergistic effects were observed because of the combined application of EO with gentamicin against all strains tested, and with amoxicillin against *B. subtilis*. Furthermore, the combination of EO/cefazolin demonstrated an additive effect against *B. subtilis*. In contrast, the combination of EO with amoxicillin and céfazoline revealed antagonistic effects against *S. aureus*, *E. coli*, and *P. aeruginosa.*
Conclusion: This is the first report on the chemical composition and antibacterial activity of Algerian juniper berries' essential oil. The results indicate that the studied EO may be a promising source of antibacterial compounds that could be useful for pharmaceutical applications especially in combination with conventional antibiotics.

Introduction

Nowadays, the rise of bacterial resistance to antibiotics has become a serious public health concern, highlighting the urgent need for new and readily available drugs for novel therapeutic options in both human and veterinary medicines.[1] The use of medicinal and aromatic plants as a source of antimicrobial drugs (e.g., essential oils) is appropriate because plants naturally produce a wide variety of secondary metabolites that can serve an important defensive role against bacteria, viruses, and other microbes. Therefore, the ineffectiveness of conventional therapy may be avoided by combining essential oils (EOs) with antibiotics (ABs).[2] In recent years, interest in the application of EOs in the treatment of infectious diseases has increased,[3] because they appear to exert synergistic interactions with ABs,[4-8] which reduces the minimum effective dose of ABs and prevents their side effects.[9]

Juniperus thurifera L. (*Cupressaceae*) is a medicinal plant used in folk medicine in Algeria and many countries for the treatment of a variety of diseases.[10,11] This species is a dioecious tree or shrub endemic to the south-western Europe and Northern Africa.[11,12] The Algerian population of juniper (var. *aurasiaca*) is genetically closely related to the European population (subsp. *thurifera*), but is phytochemically and morphologically related to the Moroccan population (var. *africana*).[10,13]

Although the chemical composition and biological properties of *J. thurifera* EOs are well documented,[10-22] the chemical composition of EO extracted from *J. thurifera* berries var. *hispanica* has been reported in only one study.[14] Furthermore, among the few studies that have dealt with the Algerian *J. thurifera* var. *aurasiaca*, none were conducted on berries EOs neither for their chemical composition nor their biological activities. Therefore, this study, for the first time, investigated the chemical composition and antibacterial activity of the EO extracted from berries of Algerian *J. thurifera* var. *aurasiaca*. Moreover, since the interaction of EOs with ABs is one of the new ways to overcome bacterial resistance,[4-7] it was

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also decided to test the potential interactions between essential oil of Algerian *J. thurifera* berries and conventional antibiotics.

Materials and Methods

Plant material and essential oil extraction

Samples of ripe berries were collected randomly from individual trees (~ 100 m apart from each other) in late Autumn (2015) in Ain El-baïdha's mountain (1500 m of altitude) province of Batna, at 35.632^{0}N of latitude and 6.213^{0}E of longitude. The samples were then crushed and homogenized with a mortar and pestle. The species was identified by Dr. Zeraib A. Lecturer at Abbes Laghrour University. A voucher specimen has been deposited to the Herbarium of Laboratory of Natural Resources Valorization, Setif 1 University.

The essential oil of *J. thurifera* berries (100 g) was extracted by hydrodistillation with a Clevenger-type apparatus for 3 h.[10] The yield of EO was averaged over three experiments and calculated according to the dry weight of the plant material. The extracted oil was dried over anhydrous sodium sulfate and stored in sealed glass vials at 4-5°C prior to analysis.

Chemical analysis of the essential oil

Chemical analysis was carried out using a GC apparatus (Thermo) equipped with a Bruker BR5-MS column (5 % phenyl methyl siloxane, 30 m long and 0.32 mm i.d., with 0.25 µm film thickness), coupled to a mass spectrometer (MS) type DSQII (Thermo) with a detector impact of electrons 70 eV. The carrier gas was helium at a rate of 1.2 mL.min^{-1}; the injection volume was 0.1 µL; injector split mode 1:100. The initial temperature of the column was kept at 70 ºC for 1 min, and programmed up to 300 ºC at a rate of 10 ºC.min^{-1} and then kept constant at 300 ºC (column cleaning step) for 5 min. The mass spectra of each compound were recorded across an m/z range of 40 to 500 m/z.

Identification of compounds was achieved by comparison of their mass spectra and retention times with those of standards (NIST 2008 v2.0/Xcalibur data system) and those in the literature.[23] Retention indices (RI) were calculated by estimating the retention times of the eluting peaks with those of a mixture of n-alkanes.

Antibacterial activity

Antibiotics and strains

The three antibiotics, GEN: gentamicin (10µg/disc), AMX: amoxicillin (25µg/disc) and CFZ: cefazolin (30µg/disc), and the four bacterial strains, two Gram positive bacteria: [*Staphylococcus aureus* (*S. aureus*) ATCC 25923 and *Bacillus subtilis* (*B. subtilis*) ATCC 21332], and two Gram negative bacteria: [*Escherichia coli* (*E. coli*) ATCC 25922, *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 27853], used in this experiment were obtained from Bacteriology Laboratory of Hakim Saâdan Hospital, Biskra-Algeria.

Disc diffusion assay

The antibacterial activity of the *J. thurifera* EO alone (using several concentrations: 1/1, 1/2, 1/4, 1/8 and 1/16 in methanol) and in combination with antibiotics was assessed by disc diffusion method.[24]

The isolated colonies were picked from overnight grown cultures (18-24 h) in nutrient agar, inoculated in sterile saline solution by adjusting the turbidity to match 0.5 McFarland standards. Petri dishes (90 mm of diameter) were prepared with 20 mL of Mueller-Hinton Agar and seeded with 100 µL of the test bacteria (log phase cultures).

In the first experiment, sterile filter paper discs (6 mm in diameter) soaked with 10 µL of different EO concentrations were placed in the center of agar plate seeded with the respective bacteria. In the second experiment, the standard antibiotic discs (gentamicin, amoxicillin, cefazolin) soaked with 10 µL of selected concentrations of the essential oil were placed in the center of the respective plates of the test organisms. Plates were placed at 4 °C for 2 h and then incubated at 37 °C for 24 h. Standard discs of antibiotics (without EO) were used as positive control while discs soaked with methanol were used as negative control. Each experiment was carried out in triplicate and the mean diameter of the inhibition zone was recorded.

The sensitivity to the different antibacterial solutions was classified by the diameter of the inhibition zone as: not sensitive for diameters less than 8 mm, sensitive for diameters of 9-14 mm, very sensitive for diameters of 15-19 mm and extremely sensitive, for diameters larger than 20 mm.[25]

Statistical analysis

All experiments were carried out in triplicate. Data are expressed as mean ± SD. Differences were evaluated by one–way ANOVA test (0.5 %) using Statistica 8.0 software, StatSoft Inc., USA.[26] The combination of EO with ABs can provide synergistic, additive, or antagonistic interactions. If the value of combined EO/ABs is significantly higher ($P < 0.05$) than the sum of individual values (after removing the disc diameters), it is considered to be a synergistic effect; and if it is equal ($P \geq 0.05$), it is an additive effect. However, the antagonistic effect occurs when the value of one or both EO/ABs is significantly higher than the value of their mixture.[27]

Results

Chemical composition of the essential oil

Hydrodistillation of the ripe berries gave a faint green color essential oil with a mean yield of 1.38 % (v/w). Off the fifty components detected, thirty-five were successfully identified representing 86.93 % of the oil (Table 1). Six constituents were monoterpene hydrocarbons accounting for 31.89 % of the EO, fifteen constituents were oxygenated monoterpenes (26.5 %), four constituents were sesquiterpene hydrocarbons (3.4 %), and ten constituents were oxygenated sesquiterpenes (25.05 %). The major constituents identified in the oil were m-mentha-6,8-diene (15.43 %), β-pinene (10.59 %), elemol (8.31 %), terpinene-4-ol (7.44 %), α-cadinol (6.63 %) and linalool (4.64 %).

Table 1. Chemical composition of essential oil isolated from ripe berries of *Juniperus thurifera* var. *aurasiaca.*

RT	KI	Components	%
4.16	940	α-pinene	0.41
4.83	977	Sabinene	0.98
4.93	983	**β-pinene**	**10.59**
5.12	994	β-Myrcene	1.29
5.73	1028	O-Cymene	3.28
5.81	1033	**m-Mentha-6,8-diene**	**15.43**
6.48	1071	cis-Sabinene hydrate	1.96
7.04	1104	**Linalool**	**4.64**
7.4	1125	cis-p-Menth-2-en-1-ol	0.79
7.61	1138	cis-Limoneneoxide	0.58
7.72	1145	Camphor	0.74
8.01	1163	Sabina ketone	0.8
8.33	**1182**	**terpinene-4-ol**	**7.44**
8.45	1190	p-Cymen-8-ol	1.1
8.55	1196	α-terpeneol	1.15
9.35	1249	Carvone	0.79
9.5	**1259**	**Phellandral**	**2.37**
10.2	1305	Myrtenylacetate	0.42
10.8	1354	α-terpinylacetate	1.57
11.1	1369	2R,4R-p-Mentha-1(7), 8-diene-2-hydroperoxide	0.71
11.3	**1386**	Nerolidylacetate	1.44
11.5	1399	β-Elemene	0.75
12.8	1505	α-Amorphene	0.55
13.1	1525	γ-muurolène	1.24
13.5	1559	**Elemol**	**8.31**
13.6	1567	E-Nerolidol	0.48
13.9	1598	Caryophylleneoxide	1.37
14.5	1643	(-)-Spathulenol	0.74
14.5	1646	β-Gurjunene	0.86
14.6	1654	t-Muurolol	1.84
14.7	1668	**α-cadinol**	**6.63**
15.4	1727	Junipercamphor	1.3
16.2	1801	8-α-Acetoxyelemol	1.19
16.8	1855	Isoaromadendreneepoxide	**2.44**
17.3	1908	α-Copaen-11-ol	0.75
Total identified			**86.93**
Yield (g/100 g dry weight)			**1.38**
Monoterpene hydrocarbons			**31.98**
Oxygenated monoterpenes			**26.5**
Sesquiterpene hydrocarbons			**3.4**
Oxygenated sesquiterpenes			**14.15**

RT, retention time; KI, Kovats index. The major constituents and the chemical groups of identified compounds with corresponding portion in analyzed essential oil are presented in bold.

Antibacterial activity

As shown in Table 2, the oil tested alone showed notable antibacterial activity against *E. coli* and *S. aureus* at concentrations of 1/1, 1/2, 1/4 and 1/8 (v/v in methanol), with inhibition zone diameters ranging from 8 to 27 mm, while *P. aeruginosa* and *B. subtilis* exhibited resistance to all tested EO concentrations.

The antibiotics sensitivity test revealed that *E. coli*, *S. aureus* and *B. subtilis* were sensitive to all the tested antibiotics, while *P. aeruginosa* was resistant to Amoxicillin and Cefazolin discs (Table 3). The same table also indicates that the combination of *J. thurifera* EO with antibiotics demonstrated synergistic, additive and antagonistic interactions, depending on the combination of EO and ABs. The combination of EO/GEN exhibited a synergistic effect against all bacterial strains tested as evidenced by the significant increase of the inhibition zone diameters. In contrast, the combination of berries EO with the other antibiotics displayed antagonistic interactions on *E. coli*, *S. aureus* and *P. aeruginosa*. However, the combined application of EO/AMX and EO/CFZ demonstrated synergistic and additive effects against *B. subtilis* respectively.

Discussion

Plants have always been a source of inspiration for novel therapeutic drugs, as plant-derived medicines have made a significant contribution to human health.[28,29] The focus of this study was to characterize and evaluate the putative antibacterial and synergistic activities of the EO extracted from the berries of Algerian *Juniperus thurifera* var. *aurasiaca*.

Considering the yield and chemical composition of the obtained EO, our results differ from those reported by Hernandez et al[14] in that they found lower proportion of berries essential oil 0.13 % (v/w), with as main components limonene (84.32 %), β-myrcene (3.82 %), α-pinene (3.48 %) and α-terpinolene (1.39 %). They also differ from those of Zeraib et al,[10,11] who, by analyzing the essential oils extracted from the leaves of the same subspecies (var. *aurasiaca*) obtained respectively 0.40 % to 0.53 % (v/w) of yield with sabinene as the major component. However, our results were consistent with the fact that it is widely accepted that juniper berries contain a higher proportion of essential oils than leaves and wood.[30,31] Generally, the yield and composition of the plant essential oils are influenced by several factors such as the method of extraction, plant part used for sampling, plant age, genetic makeup and environmental condition.[21] Therefore, the disagreement of our results with those previously reported on *J. thurifera* berries,[14] or leaves[10,11] could be explained by any of these factors.[10,18,21]

Table 2. Antibacterial activity expressed as mean ± SD of growth inhibition zone diameters (mm) of *J. thurifera* berries essential oil.

Strains tested	EO concentration (diluted in methanol)					
	Pure	1/2	1/4	1/8	1/16	ME
E. coli	24.33±0,58	12.67±0.58	**10.33±0.58**	8.0±0.58	6.0±0.0	6.0±0.0
S. aureus	27.0±1.0	24.67±0.58	15.0±1.0	**12.67±0.58**	7.0±1.0	6.0±0.0
P. aeruginosa	**7.33±0.58***	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
B. subtilis	**6.0±0.0**	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0

*The results illustrated in bold correspond to the concentrations selected for the test of the combination of *J. thurifera* berries EO with the ABs. ME, Methanol. When inhibition zone diameters = 6 mm, no inhibition.

Table 3. Antibacterial activity expressed as mean ± SD of growth inhibition zone diameters (mm) of conventional antibiotics alone and in combination with *J. thurifera* berries essential oil.

Test substance	*E. coli*	*S. aureus*	*P. aeruginosa*	*B. subtilis*
GEN	28 ± 1.0	32 ± 1.0	19.67 ± 0.58	22.83 ± 0.29
AMX	26 ± 1.0	29.67 ± 0.58	6.0 ± 0.0	15.33 ± 0.58
CFZ	42 ± 1.0	40.67 ± 0.58	6.0 ± 0.0	28 ± 1.0
GEN + EO	34.67 ± 0.58	46 ± 1.0	24.33 ± 0.58	27 ± 1.0
AMX + EO	14.67 ± 0.58	28 ± 1.0	6.0 ± 0.0	20.33 ± 0.58
CFZ + EO	38.67 ± 0.58	32.67 ± 0.58	6.0 ± 0.0	28.33 ± 0.58
P values				
EO/GEN	0.007**	3.8×10^{-4}***	0.0074 **	0.0022 **
EO/AMX	5×10^{-6}***	2.3×10^{-4}***	0.016*	4.4×10^{-4}***
EO/CFZ	8.4×10^{-5}***	3×10^{-5}***	0.016*	0.643 ns
Combination Effect				
EO/GEN	*Synergistic*	*Synergistic*	*Synergistic*	*Synergistic*
EO/AMX	*Antagonistic*	*Antagonistic*	*Antagonistic*	*Synergistic*
EO/CFZ	*Antagonistic*	*Antagonistic*	*Antagonistic*	*Additive*

ns, no significant; *, significant (P<0.05); **, highly significant (P<0.01); ***; very highly significant (P<0.001). GEN, gentamicin; AMX, amoxicillin; CFZ, cefazolin; EO, essential oil.

Because of their complex bioactive composition, EOs interacts with several targets at the same time, thus preventing pathogens from acquiring resistance.[8]

Therefore, the interaction between EO and antibiotics can produce three types of effects, namely: synergistic, additive or antagonistic effects. Interestingly, the combined application of *J. thurifera* EO with gentamicin demonstrated synergistic effects against all tested bacteria. A synergistic effect can be produced if the constituents of a mixture affect different targets.[36] This could be explained by the fact that EO penetrates the microbial cell, presumably because of its hydrophobic character, alters the cellular functions,[37] increases membrane permeability,[38] which facilitates the penetration of gentamicin.[39] Once inside, gentamicin interrupts protein synthesis by blocking the 30S subunit of the bacterial ribosome. Also, EOs are often more effective against Gram-positive than Gram-negative bacteria due to the fact that the outer lipopolysaccharide layer of gram-negative bacteria limits the diffusion of hydrophobic compounds.[32] Our results are in agreement with these findings in that the combined addition of EO/GEN had greater effects on Gram-positive bacteria (*S. aureus*, *B. subtilis*). In contrast, the combination of products acting on the same target of the microorganism leads to antagonistic or additive effects.[36] Consistent with this, the antagonistic and additive interactions observed respectively between EO/amoxicillin, and EO/cefazolin could be explained by the fact that EO and these two antibiotics act on the same target of the bacterial cells (e.g., cell membrane). Nevertheless, the interpretation of such results requires some caution since growth media and culture conditions can influence the observed effects.

In conclusion, this study provides, for the first time, important data on the chemical composition and biological activity of the essential oil of *J. thurefira* var. *aurasiaca* berries. The studied essential oil (alone or in combination with antibiotics) showed significant activity against almost all the tested bacterial strains. However, further studies should be carried out on its antibacterial activity using other bacterial groups and on its antifungal, antioxidant, cytotoxic and phytotoxic properties as well.

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Conflict of Interests

The authors claim that there is no conflict of interest.

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