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Antimicrobial and Antioxidant activities of Algerian *Juniperus phoenicea* and *Salvia officinalis* Essential Oils

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Abstract. In this work, the chemical composition, antioxidant and antifungal activities of *Juniperus phoenicea* and *Salvia officinalis* essential oils (Eos) were evaluated. The Gas Chromatography-Mass spectrometry (GC-MS) identification of *S. officinalis* Eo revealed the predominance of cis-chrysanthenyl acetate (64.82 %), and α- thujone (14.7 %). The main compounds of *J. phoenicea* oil were α-Pinene (64.4 %) and δ-3-Carene (7.02 %). The antibacterial activity was evaluated using the agar well diffusion method. The most susceptible bacteria was *Staphylococcus aureus*. The antifungal activity was tested against *Aspergillus* and *Penicillium* species by the poisoned food method. The two essential oils (Eos) exhibited an antifungal activity, with *S. officinalis* oil being the most potent one (8-82 % of inhibition). The antioxidant activity was characterized by the DPPH free radical scavenging method. *J. phoenicea* and *S.officinalis* Eos had both a moderate antioxidant effect. Additionally, an antagonistic effect was observed between the Eos when used in combination.
Keywords: Antibacterial; antifungal; antioxidant; essential oils; GC-MS.

Resumen. En este trabajo se evaluó la composición química, actividad antioxidante y antifúngica de los aceites esenciales de *Juniperus phoenicea* y *Salvia officinalis* (Eos). La identificación por cromatografía de gases-espectrometría de masas (GC-MS) de los aceites de *S. officinalis* reveló el predominio del *cis*-crisantenil acetato (64.82%) y α-tujona (14.7%). Los principales compuestos del aceite de *J. phoenicea* fueron α-pineno (64.4%) y el δ-3-careno (7,02 %). La actividad antibacteriana se evaluó mediante el método de difusión en pozo de agar. La bacteria más susceptible fue *Staphylococcus aureus*. La actividad antifúngica se probó contra especies de *Aspergillus* y *Penicillium* por el método de alimentos envenenados. Los dos aceites esenciales exhibieron actividad antifúngica, siendo el aceite de *S. officinalis* el más potente (8-82% de inhibición). La actividad antioxidante se caracterizó por el método de captación de radicales libres DPPH. *J. phoenicea* y *S.officinalis* tuvieron un efecto antioxidante moderado. Además, se observó un efecto antagónico entre los Eos cuando se usaban en combinación.
Palabras clave: Antibacteriano; antifúngico; antioxidante; aceites esenciales; GC-MS.

Introduction

Microbial food contamination is a severe public health problem worldwide. Food products may become contaminated with pathogenic microorganisms. Bacteria and fungus are among the most common microorganisms that can cause foodborne diseases. Nowadays, more than 200 foodborne illnesses are known. Food spoilage is caused by microbial enzymes that can also reduce or destroy the nutritive value of foodstuffs [1]. Mold

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contamination of food products may occur at any stage (in the field, during storage and processing). This contamination can be very threatening to human health. Indeed, some microorganisms can synthetize toxins such as aflatoxins, known to be potent carcinogens [2]. The second most common cause of food deterioration is lipid oxidation. Consumption of such food products may trigger several human health disorders, and increase the risk of developing cardiovascular diseases, emphysema, and carcinogenesis [3].

The prevention of food contamination is achieved mainly with synthetic chemical substances. However, the intensive use of these substances enhanced the resistance among microorganisms. Plants include several species used in the pharmaceutical and food industries. Some plants' phytochemicals are considered as potential sources of alternative to food preservatives, with a low risk of environmental contamination that can be exploited to reduce food spoilage [4].

The *Cupressaceae* and *Lamiaceae* families contain many valuable medicinal plants with biological activities and therapeutic potential. Species belonging to these families produce a wide variety of secondary metabolites, including essential oils [5,6]. Essential oils are mixtures of volatile, lipophilic, and odoriferous compounds with various pharmacological effects such as spasmolytic, carminative, anti-inflammatory, hepatoprotective, antiviral, antimicrobial, and anticancerogenic [7]. Their effectiveness against a wide range of microorganisms has been demonstrated. Among these essential oils, *Juniperus phoenicea* (Cupressaceae) and *Salvia officinalis* (*Lamiaceae*) Eos are of importance to the pharmaceutical and food industries. The aqueous extracts of these species are widely used in traditional medicine in Algeria to treat several diseases. Nowadays, Eos can be easily purchased from several stores in our country. Besides, in order to enhance the desired effect or to achieve a better or a long lasting fragrance, many people apply Eos in combination. However, in some cases, Eos can lose their biological effects when combined together. Therefore, the objectives of the present work were the determination of the chemical composition of commercial *Juniperus phoenicea* and *Salvia officinalis* Eos produced and sold in Algeria and evaluate their effectiveness as antibacterial and antifungal agents, as well as to study the antioxidant effect of these Eos when used separately and in combination.

Experimental

Chemical and reagents

All the media components and chemicals were purchased from Sigma Aldrich. Solvents were of analytical grade and were from Merck (Germany).

Essential oils

The Eos used in this study were received from a local producer in Algeria (Aromabioil) and stored at +4 °C in amber glass bottles until analysis. Eos were obtained by hydrodistillation from the aerial parts (leaves and stems) of wild *Salvia officinalis* (sage), and *Juniperus phoenicea* (Phoenician juniper), growing in northern Algeria.

GC-MS analysis

The GC-MS analysis was performed on a Hewlett Packard Agilent 6890 plus (Agilent Technologies, USA). The column used was an HP-5MS column (30 m × 0.25 mm i.d. × 0.25 μm film thickness). The injector temperature was maintained at 260 °C. The column oven temperature was initially held at 40 °C for 10 min and then increased to 280 °C at 5 °C/min. The debit of the gas vector (helium) was fixed to 0.5 mL/min. Essential oils were dissolved in hexane at a concentration of (10 %), and a volume of 2 μL of the diluted Eos was injected in split mode (1:80). The ionization energy was 70 eV. The retention indices of individual components were calculated using a series of n-alkanes (C8-C28). The components were identified by comparing their retention indices and mass spectra with those reported in the literature [8], and with data on the MS library NIST (National Institute of Standards and Technology). The relative percentage of each compound in the Eos were obtained as percentages of a peak area from the total chromatogram.

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Antioxidant property

DPPH free radical scavenging assay

Antioxidant activity was evaluated using the method described by Sahin *et al*. [9]. 0.5 mL of Eos at different concentrations in ethanol were mixed with 1.5 mL of DPPH ethanolic solutions (0.004 %). The mixtures were vortexed and kept in the dark for 30 min. The DPPH solution served as a blank. The absorbance was measured at 517 nm with a spectrophotometer against a blank, and compared with a standard (Ascorbic acid). The percentage of inhibition was calculated according to the following formula:

$$\%\ \text{free radical scavenging} = (\text{A. blank} - \text{A. sample})/\text{Ablank} \times 100$$

where A.sample and A.blank are the absorbances of DPPH solution after the addition of Eos and the absorbance of DPPH solution with ethanol, respectively. The IC_{50} value (concentration providing 50 % inhibition) was obtained from the graph by plotting the percentage of inhibition against Eos concentration.

Determination of antioxidant combination index (CI)

The classical isobologram-combination index equation, based on the IC50 values was used to determine the presence of synergy or antagonism between the Eos [10].

$$\text{CI} = (\text{D})1/(\text{Dx})1 + (\text{D})2/(\text{Dx})2$$

where (D)1 and (D)2 are the doses (IC_{50} values) of two active Eos in combination; (Dx)1 and (Dx)2 are the doses (IC_{50} values) of two active Eos individually. The type of antioxidant interactions was interpreted as follows: CI < 1: synergistic; CI = 1: additive; CI > 1: antagonistic. In this work, extracts were paired at 1:1 ratio.

Antibacterial assay

The antibacterial activity of *Juniperus phoenicea* and *Salvia officinalis* Eos was evaluated against four bacterial isolates (*Pseudomonas aeruginosa* ATCC27853, *Proteus vulgaris* ATCC13315, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC25923) using agar well diffusion method. Plates containing Mueller-Hinton Agar (MHA) were inoculated with 100 µL of the standardized suspension (10^6 CFU/mL) of pathogens. A sterilized cork borer was used to bore wells in the solid culture medium. Different concentrations (10 and 30 mg) of the tested Eos were loaded on the wells. After 24 h of incubation at 37 °C, inhibition zone diameters were determined in millimeters. Gentamicin and ampicillin were used as positive controls.

Antifungal activity

Poisoned food technique

The antifungal potential was investigated on CYA medium by the poisoned food technique. In this work, five fungal strains (*Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus fumigatus*, *Aspergillus carbonarius*, *Penicillium* sp.) were tested. All strains were obtained from the microbial culture collection of the faculty of natural sciences, Algiers (Algeria). Plates containing CYA medium with the tested Eos at a concentration of 2 mg/mL were prepared. After solidification of the medium, mycelial discs (6 mm diameter) from 10-days-old cultures were placed in the center of the Petri dishes [11]. Plates were sealed and incubated for 7 days at 25 °C. Thiophanate methyl (0.1 mg/mL) was used as positive control. The antifungal activity was recorded in terms of percentage of inhibition of mycelial growth and calculated using the following formula [12].

$$\text{Inhibition}\ (\%) = (1 - \text{Da/Db}) \times 100$$

where: Da = Average diameter of the fungal colony in treatment, and Db = Average diameter of the fungal colony in control.

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Determination of minimum inhibitory concentration (MIC)

The broth macrodilution method previously described by Shukla *et al*. [13] has been used for the determination of the minimum inhibitory concentration (MIC). First, fungal suspensions were prepared by transferring spores from 10 days-old cultures in 10 mL sterile distilled water, and by adjusting the final concentrations to 10^6 spores/mL. Then, Two-fold serial dilutions of Eos were made with acetone and mixed with sterile Potato Dextrose Broth (PDB). The tubes with various concentrations of Eos (4 to 0.03 µL/mL) were inoculated with 10 µL of spore suspension of each test strain and incubated for 7 days at 25 °C. In control tubes, only the fungal suspension was added to the medium. The MIC was defined as the lowest concentration required to prevent visible growth.

Statistical analysis

The experiments were conducted in triplicate, and the results are expressed as the mean ± standard deviation of the experiments.

Results and discussion

Chemical composition of essential oils

In this study, the chemical composition of the Eos was determined by GC-MS. The identified constituents and their amounts are listed in Table 1. Oxygenated monoterpene and monoterpene hydrocarbons were the most abundant compounds found in *S. officinalis* and *J. phoenicea,* respectively. The Eo of *S. officinalis* consisted predominantly of *cis*-chrysanthenyl acetate (64.82 %) and α-thujone (14.7 %). In *J. phoenicea* Eo, α-Pinene (64.44 %) was found in a high percentage, followed by δ-3-carene (7.02 %).

Our results are comparable to those reported in Algeria and Tunisia by other reserchers, who described α-pinene as a dominant component of *J. phoenicea* L. Eos [14,15]. In another study, 1,8-cineol, camphor, borneol, α-pinene, β-pinene, camphene, β-myrcene, and caryophyllene were described as the major components of the Eos of *S. officinalis* collected in Syria [16]. In a different investigation conducted in Tunisia, camphor, α-thujone, 1,8-cineole, viridiflorol, β-thujone, and β-caryophyllene were reported as the major components in the Eo of *S. officinalis* [17]. Monoterpenes are a widespread group of plants secondary metabolites that are commonly found in essential oils. The variation in Eos composition is usually associated with differences in its geographical location or any other abiotic factors [18].

Table 1. Chemical constituents (%) of the investigated essential oils detected by GC–MS.

No.	Compounds	RI	*S. officinalis*	*J. phoenicea*
1	Tricyclene	922	-	0.46
2	α-Pinene	931	-	64.44
3	Camphene	952	-	1.08
4	β-Myrcene	991	0.26	1.55
5	δ-3-Carene	1009	0.07	7.02
6	p-Cymene	1025	0.16	0.79
7	Limonene	1028	-	3.22
8	(E)-β-Ocimene	1050	0.14	-
9	γ-Terpinene	1059	-	0.1
10	α-Terpinolene	1087	-	0.38
11	Linalool	1103	-	0.25
12	α-Thujone	1105	14.7	-
13	*Cis*-β-Terpineol	1142	-	0.32
14	Terpinene-4-ol	1176	0.09	0.1
15	α-Terpineol	1189	1.24	0.14
16	*Cis*-Piperitol	1194	-	0.06

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17	*Trans*-Carveol	1217	0.41	-
18	Citronellol	1218	-	0.09
19	*Cis*-q-Mentha-1(7),8-dien-2-ol	1232	0.18	-
20	*Cis*-Chrysanthenyl acetate	1266	64.82	-
21	Bornyl acetate	1285		1.52
22	Carvacrol	1303	0.14	0.02
23	Myrthenyl acetate	1327	-	0.23
24	*Trans*-Carvyl acetate	1342	0.13	-
25	Terpinyl acetate	1349		1.02
26	Eugnol	1362	0.54	-
27	α-Copaene	1374	1.12	0.1
28	β-Bourbonene	1386	1.76	0.11
29	β-Elemene	1390	-	0.31
30	β-Caryophyllene	1418	3.78	0.77
31	α-Humulene	1445	0.16	0.53
32	Germacrene D	1480	2.39	0.52
33	Viridiflorene	1494	0.71	-
34	γ-Cadinene	1514	0.15	0.34
35	δ-Cadinene	1523	0.24	1.99
36	Germacrene B	1553	-	0.89
37	Caryophyllene oxide	1580	1.08	0.62
38	Guaiol	1600	0.95	-
39	*epi*-Cubenol	1627	-	1.12
40	α-Cadinol	1653	-	1.16
	Total identified (%)		95.22	91.25

RI: values of calculated retention indices

Antioxidant activity

Free radical scavenging activity of Eos was determined through DPPH assay. The DPPH scavenging index and the half-maximal inhibitory concentration (IC50) values are summarized in Table 2. As can be seen from our data, *J. phoenicea* and *S. officinalis* Eos had very low-antioxidant activities, especially when compared with ascorbic acid (positive control), which exhibited an IC50 value of 15 µg/mL in the same conditions. It is known from the literature that the antioxidant activity is related to the chemical composition of the Eos. The major compounds obtained from the investigated samples were monoterpenes. Terpenes are important components of Eos from medicinal plants that may contribute to the antioxidant properties [19]. In this work, the low antioxidant activities found might be explained by the absence of phenolic compounds. In fact, molecules like thymol and carvacrol play a notable role in inactivating free radicals and are responsible for the antioxidant activity of many Eos (20). Besides the dominant components, many constituents may contribute to the antioxidant activity due to the synergy of components' action. In our case, *J. phoenicea* and *S. officinalis* Eos have shown to interact with each other as antagonistic agents (Table 2). Most studies attributed additive and synergistic effects to phenolic and alcohol compounds [4]. The predominance of monoterpenes and differences in the chemical composition of the investigated Eos could explain the observed result.

Table 2. Antioxidant potential of *S. officinalis*, *J. phoenicea,* and their mixture.

	IC_{50} (µg/mL)	**CI1= D1/DX1**	**CI2= D2/DX2**	**CI= CI1+CI2**	**Effect**
S. officinalis	311.4 ± 4.5	-	-	-	-
J. phoenicea	271.2 ± 1.79	-	-	-	-
J. phoenicea + *S.officinalis*	435 ± 1.98	1.6	1.39	2.99	Antagonistic
Ascorbic acid	15± 0.12	-	-	-	-

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Antibacterial activity

Essential oils are known to possess antimicrobial properties against a wide range of organisms. In our work, the Eos were tested in vitro against four strains. The obtained data demonstrated that, Gram-negative bacteria were more resistant to the Eos compared to the Gram-positive strain (*S. aureus*) (Table 3). We noticed that *S. officinalis* was inactive against Gram-negative bacteria even at a concentration of 30 mg. *Escherichia coli* and *Pseudomonas aeruginosa* showed the highest levels of resistance to the tested Eos. In contrast, *J. phoenicea* was the only Eo active against the Gram-negative organism *Proteus vulgaris*.

Table 3. Mean diameters (mm) of inhibition zones after treatment with *S. officinalis* and *J. phoenicea* Eos.

	S. officinalis		***J. phoenicea***		**Gentamicin**	**Ampicillin**
Concentration	10 mg	30 mg	10 mg	30 mg	10 mg	10 mg
Escherichia coli	-	-	-	-	22±0	-
Staphylococcus aureus	12 ± 0.8	14 ± 0.6	11 ± 0	15 ± 1.1	28±0	15±0
Proteus vulgaris	-	-	9.1± 0.7	12± 0.5	26±0	-
Pseudomonas aeruginosa	-	-	-	-	23±0	-

(-): Resistant strain

Our observations are in concordance with those of previous experiences which reported an antibacterial activity of *S. officinalis* and *J. phoenicea* against Gram-negative and Gram-positive bacteria [21,22]. In our study, the Gram-positive strain was more sensitive than the Gram-negative bacteria which is probably due to a difference in the cell wall composition. In fact, the lipopolysaccharides of the outer membrane in the Gram-negative strains are responsible for protecting the bacteria against the external environment [23,24]. Previous reports stated that, the antimicrobial activity depends on the chemical composition of the Eos. The mechanism of action of these compounds against bacteria lies mainly in their capacity to induce toxic effects on the membrane structure and functions [25].

Antifungal activity

The obtained results indicated that all Eos had an inhibitory effect on the spore germination. Complete inhibition of all fungal strains was observed with thiophanate methyl at a concentration of 0.1 mg/mL (Table 4). The tested Eos showed percentages of inhibition ranging between 3.4% and 82.6%. The Eo of *S. officinalis* was characterized by a strong inhibitory effect on the mycelial growth of *A. fumigatus* (82.6%). In addition, *J. phoenicea* was less effective against the investigated fungi with a maximum inhibition rate of 14.5% in *Penicillium* sp. This antifungal activity could be due to the presence of bioactive chemical constituents. It has been previously reported that Eos of *S. officinalis* and *J. phoenicea* can suppress several plant pathogenic fungi [26,27].

Table 4. Effect of the tested essential oils on the mycelial growth inhibition (%) at a concentration of 2 mg/mL.

	A. flavus	***A. parasiticus***	***A. fumigatus***	***A. carbonarius***	***Penicillium* sp.**
S. officinalis	26 ± 1.2	7.8 ± 1.1	82.6 ± 1.5	9.3 ± 1.2	8 ± 1.7
J. phoenicea	6.25 ± 0.9	3.4 ± 0	0 ± 0	0 ± 0	14.5 ± 1.5
Thiophanate methyl (0.1 mg/mL)	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0

Minimum inhibitory concentration (MIC) was determined by the broth macrodilution method. The obtained results are shown in Table 5. Our data demonstrated that all Eos had an antifungal activity against the tested strains. The growth of *A. flavus* and *A. parasiticus* was uniformly inhibited by the tested oils. *A.*

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carbonarius showed a strong sensitivity to *S. officinalis* Eo. However, *Penicillium* sp. was more sensitive to the Eos treatments than the *Aspergillus* strains. This weak antifungal activity obtained with *J. phoenicea* and *S. officinalis* Eos in our work can be attributed to the predominance of α-pinene, β-pinene, and cis-chrysanthenyl acetate. These compounds are known for their moderate antimicrobial activity compared to alcoholic and phenolic monoterpenes [25]. Several studies have shown that Eos can affect the fungal cell permeability and functions as well, through direct interaction with the cytomembrane [28, 29]. However, molecules like α-pinene, and β-pinene are characterized by a hydrophobicity that enables them to penetrate the cell membrane and exert their toxic effect [30].

Table 5. Minimum inhibitory concentration (μL/mL) of the tested essential oils against the selected food spoilage fungi.

	A. flavus	***A. parasiticus***	***A. fumigatus***	***A. carbonarius***	***Penicillium* sp.**
S. officinalis	0.125	0.125	<0.03	0.03	0.25
J .phoenicea	0.06	0.06	1	0.25	0.06

Conclusions

This study showed that the composition of the Eos *S. officinalis* and *J. phoenicea* were characterized by high amounts of *cis*-chrysanthenyl acetate and α-Pinene, respectively. The Eos exhibit moderate antimicrobial activities against the tested pathogens. The Eo of S. *officinalis* was found to be the most potent antifungal agent against *Aspergillus* species. From the antibacterial activity results, it has been observed that *Staphylococcus aureus* was the most sensitive bacteria. The studied oils showed a weak antioxidant activity. A decrease in free radical scavenging activity was also found after a combination of the two Eos. Thus, these essential oils should be used separately. However, further investigations are needed to study the biological effects of other commercial Eos and their interactions in order to prevent their misuse in our country.

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