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Research Article
Antifungal Potential and Chemical Composition of Essential Oils Extracted From *Artemisia herba-alba* and *Salvia lavandulifolia* Plants

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Abstract

Background and Objective: In postharvest, citrus fruit are very susceptible to be infected by pathogenic fungi during the period between harvest and consumption. The current study described the antifungal activity and chemical composition of *Artemisia herba-alba* (Asso.) and *Salvia lavandulifolia* (Vahl.) essential oils against *Penicillium digitatum*, *Penicillium italicum* and *Geotrichum citri-aurantii,* major pathogens of citrus fruit. **Materials and Methods:** The essential oils obtained by hydrodistillation from areal parts were characterized by gas chromatography hyphenated with mass spectrometry analysis (GC-MS). **Results:** The major components were α-Thujone 50.5%, Camphor 13.5%, β-Thujone 11.6% and the Camphene 6.1% for *Artemisia herba-alba* and Camphre 31.17%, α –pinene 17.52%, Camphene 11.83% and 1,8-cineole 9.11% for *Salvia lavandulifolia.* An important antifungal effect was observed with total inhibition of mycelial growth and spore germination of the two *Penicillium* by *Artemisia herba-alba* essential oils. *Artemisia herba-alba* essential oils had the lowest Minimum Inhibitory Concentration (MIC) against the three pathogens with 1000 µL L^{-1} for *Penicillium digitatum*, 2000 µL L^{-1} for *Penicillium italicum* and 2000 µL L^{-1} for *Geotrichum citri-aurantii*. **Conclusion:** The results of this study suggest that the essential oil of *Artemisia herba-alba* can be a source of natural antifungal agents.

Key words: *Artemisia herba-alba*, *Salvia lavandulifolia*, antifungal activity, essential oils, *Penicillium* , *Geotrichum citri-aurantii*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The citrus fruit is the most produced fruit for human consumption and it is cultivated in more than 100 citrus countries. In 2019 citrus production exceeds 158 M tons worldwide according to FAO statistics. Postharvest processing in packing houses is intended to commercialize fruit of high quality, prolong their postharvest life and limit postharvest losses[1].

Among the postharvest diseases of citrus fruit, bleu mould, green mould and sour rot, caused by *Penicillium italicum*, *Penicillium digitatum* and *Geotrichum citri-aurantii*, respectively, cause significant losses during storage and marketing in all production countries that, like Morocco, are characterized by a Mediterranean-type climate. These fungi are pathogenic to wounds and infect fruits during harvest, in packing stations, at storage, during distribution and marketing[2,3]. Actual losses due to postharvest diseases are variable and depend on climate and orchard factors, citrus cultivar, the extent of physical injury to the fruit during harvest and subsequent handling, the effectiveness of antifungal treatments and the postharvest environment[4].

Until now, chemical control using synthetic fungicides, such as thiabendazole, imazalil, sodium ortho phenylphenate, fludioxonil, pyrimethanil, guazatine or their combination are the principal methods for the control of the main phytopathogenic fungi of citrus fruits in post-harvest[5-9]. However, most of these fungicides are no longer authorized in Morocco as well as the importing countries. Furthermore, the use of fungicides is increasingly becoming restricted owing to stringent regulation, carcinogenicity, high and acute residual toxicity, long degradation period, environmental pollution and growing public concern about chemical residues in fruits[7,10].

Therefore, the challenge is to develop effective, safe and biological alternatives for the control of citrus postharvest diseases. In these last decades, the biological approach by using natural substances of plant origin (plant extracts, volatile compounds, essential oils) was reported as effective and healthy alternatives for the control of citrus diseases in post-harvest[11,12]. Essential oils and plant extracts, generally known as non-phytotoxic, systemic and biodegradable compounds with an important activity against microorganisms, are very attractive as an alternative or complementary control means[8,9,11-17].

In previous studies, the current research team evaluated the *in vitro* and *in vivo* antifungal activities of aqueous, organic extracts and essential oils from many Moroccan plant species against the main fungal pathogens of citrus[8,9,18].

In the present study, EOs isolated from two Moroccan aromatic and medicinal plants (*Artemisia herba-alba* (Asso) and *Salvia lavandulifolia* (Vahl)) were characterized by GC-MS and investigated to evaluate their antifungal activity against the principal postharvest fungal pathogens of citrus fruit (*P. digitatum*, *P. italicum* and *G. citri-aurantii*) at different growth stages, for possible use in the organic agriculture and postharvest sector.

MATERIALS AND METHODS

Plant material: *Artemisia herba-alba* and *Salvia lavandulifolia*, two aromatic and/or medicinal plants, are the plant's species investigated in this work. The areal parts of the tested plants were harvested from two locations of the Souss Massa region, Morocco, in April, 2017. The fresh samples of the collected plants were cleaned and dried in the shade at room temperature for about twenty days and stored in the herbarium of the Laboratory of Biotechnology and Natural Resources Valorization (LBVRN), Faculty of Sciences, Ibn Zohr University, Agadir, Morocco.

Extraction of essential oil: The Essential Oils (EOs) were extracted from dried aerial plant materials by hydrodistillation using a Clevenger type apparatus for 4 hrs as recommended by European Pharmacopoeia[19]. The EOs obtained were stored in an amber bottle at -20°C until used. The extraction yield was determined as a percentage by the following Eq[20]:

$$\text{Extraction yield (\%)} = \frac{M}{Ms} \times 100$$

where, M is the masse of essential oil (g) and Ms is the mass of dry matter (g).

Gas chromatography-mass spectrometry analysis: The isolated volatile compounds were analyzed by GC/MS, using an Agilent GC-MSD system (Agilent Technologies 6890/5973) with helium (high purity) as the carrier gas at a constant linear velocity of 37 cm s^{-1}. The transfer, source and quadruple temperatures were 280, 230 and 150°C, respectively, operating at 70 eV ionization energy and scanning the m/z range 41-450. The column used was an Agilent DB5MS capillary column (30.0 m × 0.25 mm ID × 0.25 µm film thickness; Model Number: 122-5532) programmed from 60-246°C at 3°C min^{-1}.

EO samples (60 µL) were diluted with acetone (2 mL). The injection volume was 1.0 µL, the split ratio was 1:50 and the injector temperature was 260°C. Identification of the individual components was based on: comparison with the mass spectra of authentic reference compounds where

possible and by reference to WILEY275, NBS75K and Adams terpenes library[21]; comparison of their Retention Indices (RI) on a DB5 (apolar, 5% phenyl polysilphenylene-siloxane), calculated relative to the retention times of a series of C-9-C-24 n-alkanes, with linear interpolation, with those of authentic compounds or literature data[21]. For semi-quantitative purposes, the normalized peak area of each compound was used without any correction factors to establish abundances.

Fungal cultures: *Penicillium digitatum*, *Penicillium italicum* and *G. citri-aurantii*, were isolated from naturally infected citrus fruits. Single spore strains of these fungi were prepared and maintained on Potato Dextrose Agar (PDA) plates at 4°C. A seven-day-old culture of each fungus was used to inoculate the agar plates. Fungal spores were harvested by flooding PDA plates with 5 mL of sterile distilled water containing 0.05% (v/v) of Tween 80 and passing the suspension through two layers of sterile cheesecloth to remove hyphal fragments. The spore concentration was determined with the aid of a haemocytometer and adjusted to 10^6 spores mL^{-1} with sterile distilled water.

Determination of antifungal effects of the essential oils on mycelial growth: *In vitro* antifungal assays was conducted according to the method of Boubaker *et al.*[8], with slight modifications. Briefly, sterile molten Potato-Dextrose-Agar (PDA) supplemented with EOs, at a final concentration of 1000 µL L^{-1}, was poured into Petri plates (6 mm diameter). All tests were performed in PDA supplemented with 0.05% (v/v) Tween 80 to enhance oil solubility[22]. Afterwards, plates were inoculated with pathogens, using a 5 mm diameter agar disk taken from seven-day-old cultures, mycelia surface facing down. The agar plates were then incubated at 25°C for 7 days. The control consisted of an unamended PDA medium supplemented with 0.05% Tween 80. The antifungal activity was expressed in terms of percentage of mycelial radial growth inhibition and calculated according to the following Eq[8]:

$$\text{MGI (\%)} = \frac{C - T}{C} \times 100$$

where, C and T represent mycelial growth diameter in control and EOs treated Petri plates, respectively.

Three plates were used for each treatment as replications. The experiment was repeated twice and similar results were obtained in each experiment.

Effect of EOs on spore germination: Different concentrations, ranging from 62.5-8000 µL L^{-1}, of essential oils were prepared by dissolving the requisite amounts in 80 µL of Malt Extract Broth (MEB) with Tween 80 (0.2% v/v) and transferred to sterile depression slides[18]. Thereafter, 20 µL of conidial suspensions (10^6 spores mL^{-1}) were individually added to each depression slide. Inoculated slides were placed on moist filter paper in Petri plates, sealed with Parafilm to avoid evaporation and then incubated at 25°C for 24 hrs. Each depression slide was then fixed with acid fuchsine solution to stop further germination. Spore germination was estimated under a microscope using a micrometre. At least 100 spores within each replicate were observed. A spore was scored as germinated if the germ tube length was equal or superior to the length of the spore body at least. In the control, an equal amount of sterilized MEB and Tween 80 was used. The results were expressed as percent spore germination inhibition and calculated by using the following Eq[18]:

$$\text{GI (\%)} = \frac{Gc - Gt}{Gc} \times 100$$

where, Gc and Gt represent the mean number of germinated spores in control and treated slides, respectively. Each treatment included three replicates and the experiment was conducted twice.

Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC): The Minimal Inhibitory Concentrations (MICs) of EOs were determined by the agar dilution method. They were first diluted to the highest concentration to be tested (4000 µL L^{-1}) and then serial twofold dilution was made in a concentration ranging from 4000-250 µL L^{-1}. In 10 mL test tubes containing melted PDA medium with 0.5% (*v*/v) Tween 80. Aliquots (10 µL) of a spore suspension (10^6 spores mL^{-1}) of each fungus were then dropped onto the surface of the agar medium. Hemolysis tubes were incubated at 25°C (±1°C) for 48 hrs. The MICs were recorded by reading the lowest EOs concentration that allowed no visible growth of the pathogen[23]. The MFCs were determined by taking agar plugs from tubes showing no visible mycelial growth and re-inoculating them on an unamended PDA medium. MFC was regarded as the lowest concentration of the EOs that prevented the growth of the pathogen after the period of incubation. There were three replicates for each plant EO at each concentration and the experiment was conducted twice.

Statistical analysis: All data were subjected to statistical analysis of variance (ANOVA) using STATISTICA software, ver. 6 (Stat-Soft, 2001, Créteil, France). Percentage values were subjected to arcsine square root transformation before analysis of variance. Mean separation was performed following the Newman and Keuls test at $p<0.05$.

RESULTS

Extraction yield and chemical composition of the essential oils: The average yields of the essential oils of the air-dried aerial parts of the representative samples of *A. herba-alba* and *S. lavandulifolia*, were 1.006 and 1.91% (v/w, on a dry weight basis), respectively. GC-MS analyses of *A. herba-alba* and *S. lavandulifolia* led to the identification and quantification of 30 and 24 compounds, respectively in Table 1. The most abundant compounds found in *A. herba-alba* EO were α-Thujone (50.5%), Camphor (13.5%), β-Thujone (11.6%) and the Camphene (6.1%). While the major components identified in *S. lavandulifolia* EO was Camphre (31.17%), α-pinene (17.52%), Camphene (11.83%) and 1,8-cineole (9.11%).

Effects of the tested EOs on mycelial growth: EOs of *A. herba-alba* and *S. lavandulifolia* present a very important antifungal activity against *P. digitatum, P. italicum* and *G. citri-aurantii* in Fig. 1. Indeed, the EO extracted from *A. herba-alba* had completely inhibited the mycelial growth (100%) of *P. digitatum* and *P. italicum* after 7 days of incubation at concentration of 1000 µL L^{-1}. In other, the *S. lavandulifolia* EO presents a moderate effect against the three fungi pathogens. The mycelial growth of *P. italicum* had been reduced to 57.3% after the treatment by the EO of *S. lavandulifolia.*

Effect of EOs on spore germination: The *in vitro* effect of *A. herba-alba* and *S. lavandulifolia* essential oils on spore germination of *P. digitatum, P. italicum* and *G. citri-aurantii* is shown in Table 2.

EO of *A. herba-alba* had inhibited the spore germination of *P. digitatum* and *P. italicum* at 4000 µL L^{-1}. For *G. citri-aurantii*, the EO inhibited 87% of spore germination at the same concentration and the inhibition attained 100% at 8000 µL L^{-1}.

Furthermore, *S. lavandulifolia* EO had reduced the spore germination of *P. digitatum*, *P. italicum* and *G. citri-aurantii* by 87.67, 81 and 22%, respectively at 8000 µL L^{-1}.

Table 1: Percentage compositions of essential oils obtained from the aerial part of *A. herba-alba* and *S. lavandulifolia*

Compounds	*Salvia lavandulifolia*	*Artemisia herba alba*
Santolina triene	-	0.2
Tricyclene	1.12	0.2
α-thujene	0.41	-
α-pinene	17.52	1.2
Camphene	11.83	6.1
1-Octen-3-ol	-	0.
Sabinene	-	0.5
Sabinene	1.12	-
β-pinene	3.34	0.3
β-myrcene	1.87	-
Isolyratone	-	tr
α-terpinene	0.27	-
p-cymene	0.42	1.2
Limonene	3.95	-
1,8-cineole	9.12	4.2
Filifolone	-	1.9
α-Thujone	-	50.5
β-Thujone	-	11.6
γ-terpinene	1.24	tr
cis-Sabinene-hydrate	0.48	-
α-Terpinolene	0.61	-
Camphor	-	13.5
Pinocarvone	-	1.6
Trans-sabinene hydrate	0.52	-
Camphre	31.17	-
Borneol	2.23	0.9
Myrtenal	-	tr
Terpinen-4-ol	2.46	0.7
Myrtenol	-	0.3
Trans-Piperitol	-	0.4
α-terpineol	0.63	0.2
cis-Carveol	-	0.1
Carvone	-	0.7
Isopiperitenone	-	tr
cis-Chrysanthenyl acetate	-	0.3
Bornyl acetate	2.97	1.5
δ-Caryophyllene	1.49	-
α-Humulene	0.67	-
Germacrene	-	0.5
Spathulenol	-	0.1
Globulol	-	0.2
Caryophyllene oxide	2.85	-
Humulene oxide II	0.74	-

tr: Traces (<0.05%)

MIC and MFC: According to the results obtained, the bioactivity of the essential oils was different between the two studied species in Table 3.

Penicillium digitatum, P. italicum and *G. citri-aurantii* were completely inhibited at 1000, 2000 and 2000 µL L^{-1} of A. herba-alba EO, respectively. However, the MIC of *S. lavandulifolia* EO was 4000 µL L^{-1} for *G. citri-aurantii* and >4000 µL L^{-1} for the two other pathogens. While the fungicidal effect of two tested EOs against the three pathogens appeared at a higher concentration of 4000 to >4000 µL L^{-1}.

Table 2: *In vitro* effect of *A. herba-alba* and *S. lavandulifolia* essential oils on spore germination of *P. digitatum*, *P. italicum* and *G. citri-aurantii*

		Inhibition of spore germination (%)				
		Essential oils concentration (μL L^{-1})				
Species	Pathogens	500	1000	2000	4000	8000
Artemisia herba alba	*Penicillium digitatum*	0^{j}	47.33^{e}	91.67^{b}	100^{a}	100^{a}
	Penicillium italicum	0^{j}	41.67^{f}	96.33^{a}	100^{a}	100^{a}
	Geotrichum citri-aurantii	0^{j}	0^{j}	15.33^{i}	87^{b}	100^{a}
Salvia lavandulifolia	*Penicillium digitatum*	0^{j}	0^{j}	$19^{h,i}$	56.33^{d}	87.67^{b}
	Penicillium italicum	0^{j}	0^{j}	0^{j}	35^{g}	81^{c}
	Geotrichum citri-aurantii	0^{j}	0^{j}	0^{j}	0^{j}	22^{h}

Each value represents the mean of three replicates. Means followed by a different letter(s) in each column are significantly different at $p<0.05$

Table 3: Minimal inhibitory concentrations (MICs) and minimal fungicidal concentrations (MFCs) of the two tested EOs

	Pathogens		
Species	*Penicillium digitatum*	*Penicillium italicum*	*Geotrichum citri-aurantii*
Artemisia herba alba			
MIC (μL L^{-1})	1000	2000	2000
MFC (μL L^{-1})	>4000	>4000	4000
Salvia lavandulifolia			
MIC (μL L^{-1})	>4000	>4000	4000
MFC (μL L^{-1})	>4000	>4000	>4000

MIC: Concentration that was fungistatic, MFC: Concentration that was fungicidal

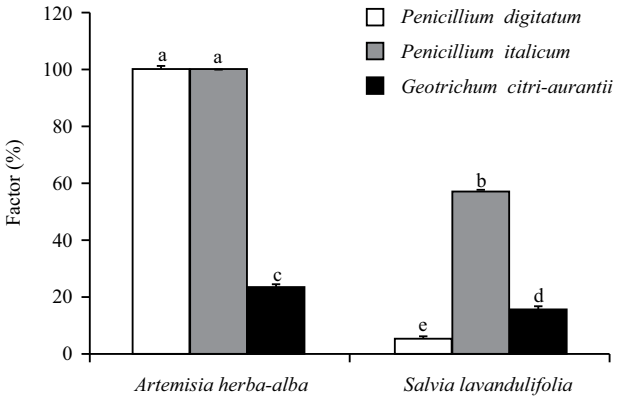

Fig. 1: *In vitro* effects of *A. herba-alba* and *S. lavandulifolia* EOs on mycelial growth of *P. digitatum*, *P. italicum* and *G. citri-aurantii*

Values are Mean of three replicates. Different letters on columns indicate a significant difference ($p<0.05$) according to Newman and Keuls test

DISCUSSION

This is the first trial to evaluate the *in vitro* antifungal activities of the essential oil from the *Artemisia herba-alba* and *Salvia lavandulifolia* to inhibit different growth stages of postharvest citrus fungal pathogens.

Over the last decades, the *A. herba-alba* and *S. lavandulifolia* essential oils had been thoroughly investigated and the diversity in oil composition from plants grown in different countries and even those from different localities in the same country have led to the many oil-dependent chemotypes assigned to the plant[24]. Generally, the *A. herba-alba* EO was largely reported to be composed of monoterpenoids, mainly oxygenated, such as 1,8-cineole, chrysanthenone, chrysanthenol (and its acetate), α/β-thujones and camphor as the major components[25].

For further comparison, the composition of *A. herba-alba* essential oil dominated by thujones was found in Morocco[26], Tunisia[27,28] and Jordan[29]. Camphor-type oils were reported in Morocco[30], Algeria[31] and Tunisia[32]. Chrysanthenone was reported as an important component in essential oil hydro distilled from Morocco[33,34].

In this study, the major compound found in *A. herba-alba* EO were α-Thujone (50.5%), Camphor (13.5%). Current results are following what has been previously reported on the *A. herba-alba* EO from Morocco with the major compound is α-thujone (59.07-65%)[26]. This compound was also found in Algerian and Tunisian *A. herba-alba* EO as a major one 47.1 and 37.9%, respectively[35,36].

Otherwise, different studies have reported the relative composition of *S. lavandulifolia* EO. Camphor is found in our EO with a maximum of 31.17%, similarly as observed in previous studies. Zrira *et al.*[37] detected camphor (16-30%) and 1, 8-cineole (13-19%) as major compounds in wild *S. lavandulifolia* EO from two different locations of Morocco.

However, in other studies results are quite different. In addition, the main compounds of *S. lavandulifolia* EO detected by Herraiz-Peñalver *et al.*[38] were α-pinene (23.2%), β-pinene (19.2%), 1,8-cineole (34.5%) and camphor (15.4%). Contrary, four *S. lavandulifolia* EO studied by Cutillas *et al.*[39] contain camphor (30.8-37.2%), 1,8-cineole (21.7-25.7%) and camphene (7.2-9.4%) as the main compounds.

Where, 1, 8-cineole (36.7%) was the major compound in *S. lavandulifolia* EO located in Central Spain[40].

Moreover, the Spanish standard UNE 84310:2013 and International ISO 3526:2005 are dedicated to the quality of

S. lavandulifolia essential oil and consist of a chemical profile of 11 main compounds. This profile does not take into account Camphene; the presence of this component in our sample supports the hypothesis of a Moroccan specificity.

Prior reports described antifungal activities of plants EOs and several of their components against some of the pathogens examined in current work and demonstrated that the mechanisms involved in the control of these pathogens by plant EOs include restriction of their conidial germination and hyphal growth[41-45].

In the present work, the results of the antifungal screening showed that EOs from *A. herba-alba* and *S. lavandulifolia* possess antifungal activity against *P. digitatum*, *P. italicum* and *G. citri-aurantii* (Fig. 1). *A. herba-alba* produced the highest antifungal activity against the three fungi. The EO extracted from a different genus of *Artemisia* and *Salvia* had shown antifungal activity against several plant pathogens[43,46-50]. For example, The EO of *Artemisia nilagirica* had inhibited 100% of mycelial growth of *Aspergillus flavus*, *A. niger* and *A. ochraceus*[25]. In another work, Kordali *et al.*[51], reported the antifungal proprieties of *Artemisia santonicum, A. spicigera* and *A. absinthium* EOs against 11 plant fungi. This study demonstrates the high sensitivity of *Penicillium* spp. to essential oils of plants tested.

The strong and poor antifungal effect of essential oil can be attributed to its chemical composition. Besides, the antimicrobial activity of essential oils has been mainly attributed to the presence of 1, 8- cineole, thujone, camphor, borneol and pcymene[52]. Also, Umpiérrez *et al.*[14] found that species-rich in thujone showed potent fungicidal activity against *Alternaria* sp. and *Botrytis cinerea*. But it is difficult to attribute the antifungal activity of a complex mixture to a single or particular constituent, as possible synergistic and/or antagonistic effects of compounds in the EO should also be given consideration.

Scientists suggested that the high antifungal propriety may be due to the deterioration of fungal hyphae by chemical compounds of essential oils. They are absorbed in membranes, increased the permeability of the cell membrane, causing membrane dilatation and reduction of membrane function[53]. Furthermore, because of their lipophilic properties, essential oils enter the cell walls of fungi, affecting the enzymes related to cell wall synthesis reactions, causing morphological alterations in the pathogen, which eventually leads to the lysis of the fungal cell wall[54].

CONCLUSION

Artemisia herba-alba essential oil exhibited an important antifungal potential, against most prevalent postharvest citrus fungal pathogens, suggesting that it can be considered as an eco-friendly alternative to synthetic fungicides for the control of post-harvest citrus diseases. The GC–MS analyses of *A. herba-alba* essential oil led to the identification and quantification of 30 compounds. The most abundant compound found in *A. herba-alba* EO is α-Thujone (50.5%).

SIGNIFICANCE STATEMENT

The findings of this study will serve as a starting point for the discovery of new natural compounds with an important antifungal activity than currently available chemical fungicides against the most common postharvest citrus fungal infections. More experimental researches are needed to evaluate the commercial use of EOs as postharvest botanical fungicides in the citrus sector respecting problems associated with potential phytotoxicity, organoleptic features and compatibility with standard postharvest practices.

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