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Control of fungal pathogens of *Citrus sinensis* L. by essential oil and hydrosol of *Thymus capitatus* L.

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Summary

Essential oil, hydrosol extract and hydrosol of *Thymus capitatus* L. from Algeria were tested for antifungal activity against four phytopathogenic fungi (*Aspergillus niger*, *Aspergillus oryza*, *Penicillium italicum* and *Fusarium solani*) causing the deterioration of *Citrus sinensis* fruits. Essential oil and hydrosol extract showed strong *in vitro* antifungal activity based on the inhibition zone and minimal inhibitory concentration values against the pathogens. *Citrus sinensis* fruits infected by *Penicillium italicum* were treated *in vivo* with essential oil, hydrosol extract and hydrosol. 0.2 µg/mL of *Thymus* hydrosol was needed for the absence of orange infection and causing 100 % mycelial growth inhibition. This activity can be correlated with chemical composition of extracts which are rich in carvacrol (more than 69 %). Therefore, the preventive and curative effects of *T. capitatus* essential oil and hydrosol could be exploited as an ideal alternative to synthetic fungicides for using in the treatment of many fungal phytopathogens causing severe destruction to oranges.

Introduction

Citrus is an important crop with world production estimated at 115 million tons per year. During 2010-2011, 571 thousand tons were producer in Algeria which is the 19th produced in the world and the 3rd in the Arab Maghreb Union (Lagha-Benamrouche and Madani, 2013). *Citrus* are among the most popular fruits grown in Algeria. Furthermore, *Citrus* production represents an important agricultural and economic activity in the country. Oranges and mandarins are traditionally produced for local consumption and also for export. Citrus is an important crop with world production estimated at 115 million tons per year. During 2010/2011, 571 thousand tons were produced in Algeria which is the 19th producers in the world and the 3rd in the Arab Maghreb Union (FAO, 2012) (Lagha-Benamrouche and Madani, 2013). Oranges, lemons, grapefruits and mandarins represent approximately 98 % of industrial cultures. Oranges are most pertinent with about 82 % of total (Lagha-Benamrouche and Madani, 2013).

Fungal growth on fresh fruits and vegetables is responsible for food spoilage and numerous plant diseases, which lead to significant economic losses. *Penicillium* and *Aspergillus* were responsible for spoilage of many foods and causes decay on stored fruits damaged by insects, animals, early splits, and mechanical harvesting (Tu et al., 2013; Rojas et al., 2005). The industries of food products nowadays are using synthetic chemical preservatives to prevent the growth of pathogens, but these chemicals convert certain ingested materials into toxins and carcinogens (Farag et al., 1989). Alternative control methods are needed because of negative public perceptions about the use of pesticides, development of resistance to fungicides, and high cost for development of new chemicals preservatives (Stojković et al., 2011). Thus, there has been a growing interest on the research of the possible use of plant secondary metabolites for pest and disease control in agriculture (Gikh et al., 2002). The plants have long been recognized to provide a potential source of different class of chemical compounds, known as phytochemicals, such as terpenoids, alkaloids, phenolics, glucosides, etc., which are effective products against pathogenic microorganisms (Feng and Zheng, 2007). Essential oils are of growing interest both in the industry and scientific research because of their antibacterial and antifungal properties which make them useful as natural additives in foods (Pattnaik et al., 1997). Such antimicrobial activity is due to the presence of bioactive substances such as monoterpenes, sesquiterpenes and related alcohols, other hydrocarbons and phenols (Griffin et al., 1999; Kalemba and Kunicka, 2003).

Thyme (*Thymus)* is a genus containing about 350 species of aromatic perennial herbs and sub-shrubs to 40 cm tall, belong to the family *Lamiaceae*. This family is distributed throughout the arid, temperate and cold regions including Europe, North Africa and Asia. It is in leaf all year, flowering from July to September (Gruenwald et al., 2004). Several studies have assessed the ability of the *Thymus* essential oils and their constituents as fumigants and repellents against a number of insect pests (Clemente et al., 2003; Lee et al., 2001; Hori, 2003, Salama et al., 2012). Effective antifungal activity of *T. propolis* from regions of Algeria was explained by their high content in thymol (49.3 %) and carvacrol (57.7 %) (Melliou et al., 2007). Molluscicidal activity of *T. capitatus* essential oils rich with carvacrol (32.98 %) and thymol (32.82 %) on adult and eggs of *Biomphalaria alexandrina* as well as on different stages of *Culex pipiens* was evaluated for their effectiveness on vector control (Salama et al., 2012). Askarne et al. (2012) showed that aqueous extract of *Thymus leptobotrys* completely inhibited mycelial growth of *Penicillium italicum*, a phytopathogenic fungi of *Citrus*. On the other hand, there is no report on the hydrosol composition from this species. Also, the antifungal activity of this hydrosol was reported for the first time against the development of fungi of *Citrus sinensis* fruit. Therefore, the present study was made (i) to examine the *in vitro* antifungal activity of the essential oil and hydrosol extract obtained from *T. capitatus* against four phytopathogenic fungi (*Aspergillus niger*, *Aspergillus oryza*, *Penicillium italicum* and *Fusarium solani*) and (ii) to test *in vivo* the essential oil, hydrosol extract and hydrosol against *Penicillium italicum* responsible of rotting the oranges.

Materials and methods

Plant material

The aerial parts of *T. capitatus* were collected from Beni Snous forests near Tlemcen, Algeria in May 2011. Voucher specimens were deposited in the herbarium of the Tlemcen University Botanical Laboratory (Voucher number: UTL 05.11).

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Essential oil, hydrosol and hydrosol extract

Essential oil of fresh aerial parts (600 g) was isolated by hydrodistillation (HD) in a Clevenger type apparatus for 5 h, giving clear yellow oil. The yield of the oils was 0.52 %. The obtained essential oil was stored at +4 °C until further tests. The first liter of hydrodistillate is recovered in order to obtain *T. capitatus* hydrosol. Hydrosol was submitted to Liquid Liquid Extraction (LLE). Half a liter of hydrosol was extracted three times with 200 mL of diethyl ether at room temperature. The organic layer dried over Na_2SO_4 and evaporated, so giving an oil yellowish, called hydrosol extract, with yield of 0.0016 %. (w/w). The 500 mL of hydrosol remaining were used to study the in vivo activity.

Gas chromatography

Analyses were carried out using a Perkin Elmer Clarus 600 GC apparatus equipped with a dual flame ionization detection system and 2 fused-silica capillary columns (60 m x 0.22 mm I.D., film thickness 0.25 μm), Rtx-1 (polydimethylsiloxane) and Rtx-Wax (polyethylene glycol). The oven temperature was programmed from 60 °C to 230 °C at 2 °C/min and then held isothermally at 230 °C for 35 min. Injector and detector temperatures were maintained at 280 °C. Essential oil and hydrosol extract were injected in the split mode (1/50), using helium as the carrier gas (1 mL/min); the injection volume was 0.2 μL. Retention indices (RI) of the compounds were determined from Perkin-Elmer software.

Gas chromatography-mass spectrometry

Essential oil and hydrosol extract were analyzed with a Perkin–Elmer TurboMass quadrupole analyzer, coupled to a Perkin-Elmer Autosystem XL, equipped with 2 fused-silica capillary columns and operated with the same GC conditions described above, except for a split of 1/80. Electronic Impact (EI) mass spectra were acquired under the following conditions: Ion source temperature 150 °C, energy ionization 70 eV, mass range 35-350 Da (scan time: 1 s).

Component identification and quantification

Identification of the components of the essential oil obtained by hydrodistillation (HD) and the hydrosol extract obtained by LLE was based (i) on the comparison of their GC retention indices (RI) on non-polar and polar columns, determined relative to the retention time of a series of n-alkanes with linear interpolation, with those of authentic compounds or literature data (Jennings and Shibamoto, 1980; König et al., 2001; National Institute of Standards and Technology, 2008) and (ii) on computer matching with commercial mass spectral libraries (Mc Lafferty and Stauffer, 1994; Mc Lafferty and Stauffer, 1988; National Institute of Standards and Technology, 1999) and comparison of spectra with those of in-house laboratory library. The quantification of essential oil components was carried out using peak normalization including response factors (RFs) with internal standard. The normalized % abundances were calculated, using the methodology reported by (Bicchi et al., 2008) in order to perform the statistical analysis of the oil. Tridecane was introduced in all sample oils at same concentration (0.7 g/100 g) as internal standard.

Pathogenic fungi

Four fungal isolates causing *Citrus* rot. *Aspergillus niger*, *Aspergillus oryza*, *Penicillium italicum* and *Fusarium solani* were isolated directly from rotten *C. sinensis* fruits harvested from orchards of the El-Fhoul cooperative in Tlemcen (Algeria). All isolated fungal species were transferred to sterilized three replicates 9 cm Petri dishes containing fresh Potato Dextrose agar medium (PDA) in the presence of a quantity of lactic acid (20 %) to stop the growth of bacteria. The plates were incubated at 25 ± 2 °C for 8 days and darkness. The developing fungal colonies were purified and identified up to the species level by microscopic examination through the help of the following references (Barnett and Hunter, 2006).

In vitro antifungal assay

The antifungal activity of *T. capitatus* essential oil and hydrosol extract was tested using radial growth technique (Bajpai et al., 2007). Appropriate volumes of the stock solutions of the natural mixtures (essential oil and hydrosol extract) in dimethyl sulfoxide (DMSO) to 10 %, were added to PDA medium immediately before it was poured into the Petri dishes (9.0 cm diameter) at 40-45 °C to obtain a series of concentrations (0.01 to 0.5 μg/mL). Each concentration was tested in triplicate. Parallel controls were maintained with DMSO mixed with PDA. The discs of mycelial felt (0.5 cm diameter) of the plant pathogenic fungi, taken from 7-day-old cultures on PDA plates, were transferred aseptically to the centre of petri's dishes were incubated. Amphotericin B (5.0 to 126 μg/ml) was used as a positive control for antifungal activity. The treatments were incubated at 27 °C in the dark. Colony growth diameter was measured after the fungal growth in the control treatments had completely covered the Petri dishes. Percentage of mycelial growth inhibition was calculated from the formula (Pandey et al., 1982):

$$(I\%) = [(DC-DT)/DC] \times 100 \text{ (Pandey et al., 1982);}$$

where DC and DT are average diameters of fungal colony of control and treatment, respectively. The measurements were used to determine the minimum inhibitory concentration (MIC) (The minimum concentration causing 100 % mycelial growth inhibition). The fungistatic-fungicidal nature of essential oil and hydrosol extract was tested by observing resumption of growth of the inhibited mycelial disc following its transfer to non-treated PDA. In order to determine fungistatic or fungicidal activity of volatile vapours of essential oils on mycelial growth, plates were further incubated at 26 °C for 7 days. Fungi resuming mycelial growth were considered to be fungistatic.

In vivo antifungal assay

For the *in vivo* antifungal assay, we used method described and developed previously by Dikbas et al. (2008). The selected orange fruits for the experiments were washed in water, dipped in ethanol (70 %) for 2 min, rinsed twice with double distilled sterile water (10 min each) and air-dried. Surface-sterilized oranges were wounded with a flamesterilized nail to a uniform depth of 3 mm. The fungal inoculums containing 10^6 spores/mL was prepared by scraping spore material from the surfaces of the colonies with a wet cotton swab and resuspending the material in distilled water containing 0.5 % Tween 80.

For testing antifungal *in vivo* activity against *P. italicum*, the essential oil and hydrosol extract, were separately mixed vigorously with distilled water to obtain two concentrations 0.1 and 0.2 μg/mL, respectively. However, the hydrosol was applied directly with the concentration of 20 μg/mL. The essential oil, hydrosol extract and hydrosol of *T. capitatus* and fungal inoculum were sprayed separately on wounded orange fruits. The antifungal activity was tested on healthy fresh oranges. These experiments were arranged as three different applications. Fruits inoculated with only pathogen were used as positive control for each experiment. Non-inoculated fruits with pathogen were used as negative control. The fruits were sealed in polyethylene-lined plastic boxes to retain 70 % humidity and incubated at 25 °C storage condition. The diameters of decay on fruits were measured at 3, 6, 9, 12 and 15th days after inoculation.

Tab. 1: Chemical compositions of *T. capitatus* essential oil (EO) and hydrosol extract (HY).

No.[a]	Components	/RI$_a$ [b]	RI$_a$ [c]	RI$_p$ [d]	EO	HY	Identification [e]
1	α-Thujene	932	924	1028	0.2	-	RI, MS
2	α-Pinene	936	931	1028	0.9	-	RI, MS
3	Camphene	950	945	1071	0.2	-	RI, MS
4	Oct-1-en-3-ol	962	962	1441	0.5	0.2	RI, MS
5	β-Pinene	978	972	1113	0.1	-	RI, MS
6	Myrcene	987	982	1160	2.1	-	RI, MS
7	3-Octanol	981	982	1366	tr	0.1	RI, MS
8	α-Phellandrene	1002	999	1161	0.2	-	RI, MS
9	3-Carene	1005	1006	1149	0.1	-	RI, MS
10	α-Terpinene	1008	1011	1270	1.7	-	RI, MS
11	*p*-Cymene	1015	1015	1270	12.4	-	RI, MS
12	(*Z*)-β-Ocimene	1029	1022	1234	0.6	-	RI, MS
13	γ-Terpinene	1051	1050	1245	4.3	-	RI, MS
14	(*E*)-Sabinene hydrate	1051	1054	1445	0.1	0.6	RI, MS
15	Terpinolene	1082	1079	1281	0.2	-	RI, MS
16	Linalool	1083	1085	1538	1.7	0.5	RI, MS
17	Phenylacetaldehyde	1112	1108	1591	-	0.1	RI, MS
18	Camphor	1123	1124	1506	0.1	-	RI, MS
19	Isoborneol	1143	1144	1670	-	0.5	RI, MS
20	Borneol	1148	1150	1688	0.3	-	RI, MS
21	Terpinen-4-ol	1164	1162	1591	1.1	-	RI, MS
22	α-Terpineol	1176	1176	1690	0.1	0.2	RI, MS
23	*trans*-dihydro Carvone	1180	1182	1607	tr	-	RI, MS
24	*trans*-Myrtanol	1241	1242	1859	tr	-	RI, MS
25	Thymol	1266	1263	2181	0,6	0.1	RI, MS
26	**Carvacrol**	1278	1286	2193	**69.6**	**95.1**	RI, MS
27	Eugenol	1330	1329	2164	0.1	0.2	RI, MS
28	cis-Carvyl acetate	1343	1345	1858	0.1	0.2	RI, MS
29	(*E*)-β-Caryophyllene	1421	1416	1591	1.6	-	RI, MS
30	(*E*)-α-Bergamotene	1434	1435	1573	tr	-	RI, MS
31	α-Humulene	1455	1448	1668	0.1	-	RI, MS
32	γ-Humulene	1483	1480	1702	tr	-	RI, MS
33	β-Bisabolene	1503	1499	1721	0.1	-	RI, MS
34	δ-Cadinene	1520	1511	1760	tr	-	RI, MS
35	(*E*)-α-Bisabolene	1530	1531	1755	0.1	-	RI, MS
36	Spathulenol	1572	1560	2120	-	0,1	RI, MS
37	Caryophyllene oxide	1578	1567	1969	0.1	1.1	RI, MS
38	Humulene epoxide II	1602	1599	2044	-	0.1	RI, MS
	Total identification %				**99.2**	**99.1**	
	% Hydrocarbon compounds				**12.6**	**-**	
	% Monoterpene hydrocarbons				**10.7**	-	
	% Sesquiterpene hydrocarbons				1.9	-	
	% Oxygenated compounds				**86.7**	**99.1**	
	% Oxygenated monoterpenes				3.5	2.2	
	% Oxygenated sesquiterpenes				0.1	1.3	
	% Non terpenic oxygenated compounds				0.5	0.4	
	% Aromatic terpenes				**82.6**	**95.2**	

a Order of elution is given on apolar column (Rtx-1), b Retention indices on the apolar Rtx-1 column (RIa), c Retention indices on the polar Rtx-Wax column (RIp), d Retention indices on the polar Rtx-Wax column (RIp), e RI: Retention Indices; Normalized % abundances; MS: Mass Spectrometry in Electronic Impact (EI) mode; EO: essential oil from aerial parts obtained by HD; HY: Extract of hydrosol from aerial parts obtained by LLE.

All treatments consisted of three replicates, and experiments were repeated three times and determined the averages of the repeated experimental results.

Taste panel

Sensory evaluation of oranges treated with hydrosol was assessed by a group of 20 untrained panellists. Panellists were selected among students and staff of the laboratory of chemistry (LASNABIO). Orange samples were soaked into the hydrosol during 24 h, before the oranges were given to panellists to eat. The panellists were asked to evaluate flavor and odor of the orange samples on a scale from 5 to 1, where 1 = extremely dislike, 2 = dislike, 3 = neither like nor dislike, 4 = like; 5 = extremely like, according to a previous reports (STOJKOVIĆ et al., 2011). A general taste score was calculated as the average of all grades. Sensory evaluation was accomplished at 1st, 2nd and 3rd day. Results were expressed as average grades given by 20 panellists.

Statistical Analysis

Statistical analysis of variance (ANOVA) was performed using the SAS software and means were separated using the Least Significant Difference (LSD) test at $P \leq 0.05$. Analysis of each test was performed in triplicate.

Results

Chemical analyses

A total of 32 components accounting to 99.2 % of the essential oil (EO) composition of *T. capitatus* were identified by comparison of their EI-mass spectra and their retention indices (RI) with those of our own authentic compound library (Tab. 1). The essential oil was highly dominated by oxygenated compounds (20 components: 74.4 %) with high amount of aromatic terpenic components (82.6 %). However, hydrocarbons appeared also in appreciable proportion (19 components: 25.0 %) with monoterpene hydrocarbons are well represented (23.1 %). Indeed, the main constituents of essential oil were carvacrol (69.6 %), p-cymene (12.4 %) followed by γ-terpinene (4.3 %), myrcene (2.1 %), α-terpinene (1.7 %), linalool (1.7 %) and terpinen-4-ol (1.1 %). Conversely, the analysis of hydrosol extract (HY) obtained by LLE showed only 14 oxygenated compounds (7 monoterpenes, 3 sesquiterpenes, 3 non-terpenic components and 1 phenylpropanoid), no hydrocarbons were reported.

In vitro antifungal activity of T. capitatus extracts against the development of fungi of *C. sinensis*

The results obtained in assays of antifungal activity of *T. capitatus* essential oil and hydrosol extract by radial growth technique are reported in Tab. 2. However, data analysis showed that the antifungal activity of the essential oil and hydrosol extract concentration against the four fungi tested exhibited a significant difference ($P < 0.05$). The results indicate that the inhibition of the mycelial growth of each strain was significantly influenced by the extract concentrations. Essential oil and hydrosol extract inhibited completely all strains. The potent activity was observed against *P. italicum* with the EC_{50} of 0.022 µg/mL followed by *A. niger*, *F. solani* and *A. oryzae* with the EC_{50} of 0.121, 0132 and 0.143 µg/mL, respectively. The minimum concentration causing 100 % mycelial growth inhibition against *P. italicum*, *A. oryzae* and *F. solani* phytopathogens were very effective at 0.1, 0.2 and 0.2 µg/mL, respectively. However, the minimum concentration causing 100% mycelial growth inhibition for *A. niger* strain was 0.5 µg/mL. The essential oil and hydrosol extract tested showed strong activity in comparison to the commercial drug Amphotericin B. The most sensitive species were *P. italicum*, *F. solani* and *A. oryzae* with MIC of 0.1, 0.2 and 0.2 µg/ml, respectively. *A. niger* (MIC of 0.5 µg/ml) was the most resistant species to the essential oil and hydrosol extract tested. While, MIC of Amphotericin B ranged to 46.2 to 126 µg/ml for fungal species isolated. Moreover, it is important to know the fungitoxic nature of the essential oils and hydrosol extract. Indeed, the transfer of a mycelial disk of the plate containing a PDA medium and samples on fresh PDA (without oil and hydrosol) showed that no growth had developed after an incubation period of 7 days, suggesting a fungicidal effect of essential oils and hydrosol extract on *F. solani*, *A. oryza*, *A. niger* at 0.2, 0.2 and 0.5 µg/mL, respectively. On the other hand, essential oils and hydrosol extract was fungistatic on *P. italicum* (Tab. 2).

In vivo orange assay

The results of *in vivo P. italicum Citrus* rot treatment with essential oil, hydrosol extract and hydrosol are presented in Tab. 3. According to the increase of concentration, a decrease of disease incidence was recorded. According to the results of lesion diameters on the fruits, both concentrations (0.1 and 0.2 µg/mL) of the essential oil and hydrosol extract showed strong antifungal activity even at the end of 15th day, there was no significant difference in lesion diameters among those treatments in comparison to the negative control. The concentration of 0.2 µg/mL of essential oil and hydrosol extract were needed for the absence of orange infection and low disease incidence. More, the hydrosol showed a complete absence of orange infection and no disease incidence with a concentration of 0.2 µg/mL. The result obtained from the hydrosol was showed on Fig. 1.

Taste panel

Tab. 4 shows the acceptability scores of orange samples. Results showed that there were no significant differences in the sensory properties between samples treated with hydrosol, essential oil and control (without hydrosol), since the sensory properties of oranges treated with hydrosol (0.2 µg/mL) and essential oil (0.2 µg/mL)

Tab. 2: Antifungal activity of essential oil (EO) and hydrosol extract (HY) of *T. capitatus* against *A. niger, A. oryzae, P. italicum* and *F. solani*.

Extracts (µg/mL)	*A. niger*		*A. oryzae*		*F. solani*		*P. italicum*	
	MIC [a]	EC_{50} [b]	MIC [a]	EC_{50} [b]	MIC [a]	EC_{50} [b]	MIC [a]	EC_{50} [b]
EO	0.5 ± 0.06 [d]	0.121 ± 0.80	0.2 ± 0.01 [d]	0.143 ± 0.80	0.2 ± 0.01 [d]	0.132 ± 0.22	0.1 ± 0.01 [e]	0.022 ± 0.06
HY	0.5 ± 0.01 [d]	0.121 ± 0.81	0.2 ± 0.01 [d]	0.143 ± 0.56	0.2 ± 0.01 [d]	0.132 ± 0.32	0.1 ± 0.01 [e]	0.022 ± 0.06
Am B [c]	46.2 ± 1.81	15.62 ± 1.06	46.2 ± 1.39	15.62 ± 1.50	126 ± 3.56	62.50 ± 1.79	61.2 ± 2.01	31.25 ± 1.11

[a] minimum concentration causing 100 % mycelial growth inhibition; [b] minimum concentration causing 50 % mycelial growth inhibition; [c] Am B : Amphotericin B (µg/ml) was used as reference Antibiotic; [d] fungicidal effect; [e] fungicistatic effect; The results are expressed as mean ± standard deviation.

Tab. 3: Means of decay diameters (mm) measured after 3, 6, 9, 12 and 15 days on orange fruits treated with 0.1 or 0.2 µg/mL of essential oil, hydrosol extract and hydrosol from *T. capitatus*.

Treatments	Means ± SD of decay diameters (mm)				
	3 days	6 days	9 days	12 days	15 days
Controls negative	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Controls Positive	1.1 ± 0.2	2.8 ± 0.3	5.7 ± 0.5	8.1 ± 0.8	13.2 ± 0.2
Essential oil					
0.1 µg/mL	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.01	1.5 ± 0.2	2.5 ± 0.5
0.2 µg/mL	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.01	1.0 ± 0.2
Hydrosol extract					
0.1 µg/mL	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.06	1.5 ± 0.2
0.2 µg/mL	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.02
Hydrosol					
0.2 µg/mL	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

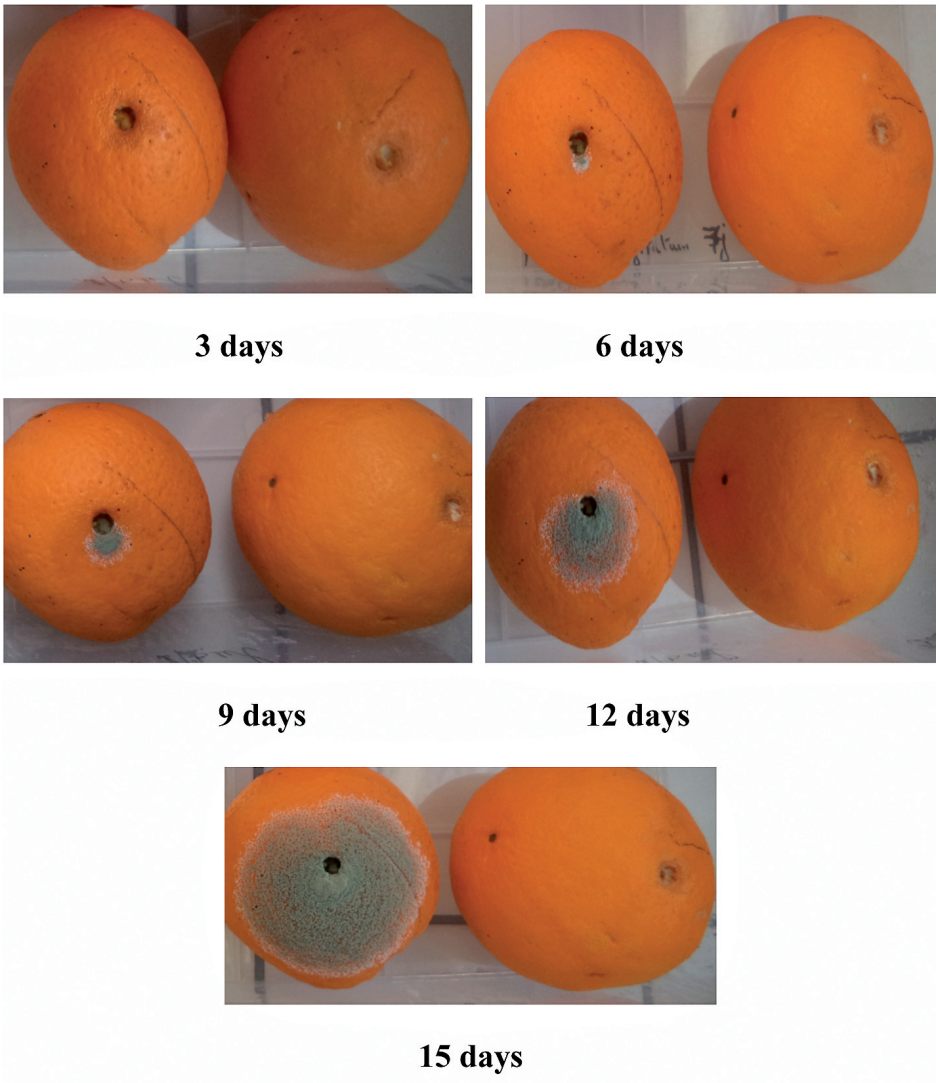

Fig 1: Decayed orange (left), inoculated with only pathogen *P. italicum* (positive control), and not decayed orange (right) onto which hydrosol (0.2 µg/mL) was applied 30 min before pathogen inoculation, kept at 25 °C for the period stated below the respective picture.

Tab. 4: Effect of essential oil (EO) and hydrosol (HY) on acceptability sensory scores of orange stored at 25 °C.

Treatment		Days of Storage		
	HY	1	2	3
Acceptability[a]		4.5	4.6	4.2
	EO	3.9	4.0	3.8

The results are expressed as the average of all grades. [a] 1 = extremely dislike, 2 = dislike, 3 = neither like nor dislike, 4 = like; 5 = extremely like.

were deemed acceptable by the panelists at the supplementation levels. More work on the acceptability of both extracts as oranges preservative will be necessary.

Discussion

In the present investigation, a total of 14 and 38 compounds comprising 99.1 and 99.2 % of the hydrosol extract and essential oil were identified from *T. capitatus* respectively, carvacrol being the major component, comprising 95.1 and 69.6 % of the hydrosol and essential oil, respectively. This study agrees with the findings of Ruberto et al. (1992), Amarti et al. (2008) and Tawaha et al. (2012), who reported carvacrol to be the major component of *T. capitatus* essential oil.

In the present investigation, the reduction of the mycelial growth of colonies in presence of essential oil and hydrosol extract of *T. capitatus* showed that it effectively controlled all strains. This efficiency can be explained by the presence of active molecules that inhibited the growth of the phytopathogenic fungi. The antifungal properties of *T. capitatus* essential oil and hydrosol extract are probably associated with the high amount of phenolic terpenes, especially the main component carvacrol. Indeed, carvacrol is used as a disinfectant, fungicide, and fragrance ingredient in cosmetic formulations. In addition, Daferera et al. (2000) reported that the fungitoxic activity of essential oils may have been due to formation of hydrogen bonds between the hydroxyl group of oil phenolics and active sites of target enzymes.

Although the extracts or essential oils of *T. capitatus* have been screened for their antifungal activity under *in vitro* conditions (Melliou et al., 2007; Dikbas et al., 2008; Nychas, 1996), there are no reports on the control of *P. italicum* under *in vivo* conditions by using the essential oil and hydrosol of *T. capitatus*. The essential oil and hydrosol of *T. capitatus* from Algeria was characterized by high content of carvacrol (69.6 % and 95.1 %, respectively). Little literature exists on the effect of carvacrol on these food pathogenic fungi (Morcia et al., 2012), although carvacrol was effective on inhibiting spore germination of *Botrytis cinerea* when applied to the potato dextrose agar (Martinez-Romero et al., 2007). In addition, Markovic et al. (2011) demonstrated that carvacrol has a remarkably antifungal potential against *Aspergillus* spp. and *Penicillium* spp. In a study conducted by Muller-Riebau et al. (1995), carvacrol showed a remarkable antifungal activity by inhibition of the mycelial growth of *Fusarium spp*. Bouddine et al. (2012) revealed that *Aspergillus niger* growth was completely inhibited by carvacrol at concentrations of 0.025 %. Furthermore, this study confirmed the antifungal activity of this component against mycelial growth of *P. italicum* in *vitro* and in *vivo* assays. The World Health Organization (WHO) has stated that carvacrol residues in food are without danger to the consumer as long as they do not exceed 50 mg kg^{-1} (WHO, 2012). It is thus clear that treatment by hydrosol is an easy to prepare and the cost price of extraction is not expensive. However, the benefits of hydrosol (non-toxic at low doses, biodegradable, and no risk for resistance development) and disadvantages of chemical fungicides on health and on the environment make hydrolate of *T. capitatus* more interesting for citrus postharvest treatment.

Conclusion

In conclusion, 38 compounds of *T. capitatus* essential oil and hydrosol extract were identified. This oil was characterized by high content of carvacrol. Furthermore, this study confirmed the antifungal activity of the essential oil and hydrolate against mycelial growth and spore production of *A. niger*, *A. oryzae*, *F. solani* and *P. italicum* from in vitro assay. In vivo inhibitory properties of hydrosol on disease incidence of *P. italicum*-causal agent of *penicillium* rot on oranges were recorded. Therefore, the preventive and curative effects of *T. capitatus* hydrolate could be exploited as an ideal alternative to synthetic fungicides for using in the treatment of many fungal phytopathogens causing severe destruction to oranges.

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