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Chemical composition and antifungal activity of essential oils of *Haplophyllum tuberculatum* (Forssk.) A. Juss. from South Algerian

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Abstract

Utilization of natural products as control agents reveals a biological preventive approach to reduce significantly the use of pesticides and in particular fungicides.In the present study we determined chemical composition of an essential oil extracted from *Haplophyllum tuberculatum* (Forssk.) A. Juss. Three fungi tomato pathogens are tested to examine antifungal activity of the extracted oil. The essential oil was extracted by hydrodistillation and the chemical composition was determined by gas chromatography coupled with flame ionization detector (GC-FID). Various concentrations (2.5, 5, 10, 20 and 30 mg / ml) of the essential oil were used to investigate *in vitro* the antifungal activity on a solid agar medium against FORL, *B. cinerea and A. solani.* The percent of the fungal inhibition was calculated and the ranges of MICs were determined for each fungal isolate tested. The results indicate that the yield of essential oil obtained by hydrodistillation is 0.101%. The major components identified in the essential oil of H. *tuberculatum* were Piperitone (13.35%), Germacren-B (12.30%) and Beta-Phellandren (5.05%). In addition, the essential oil at 10mg / ml, 20mg / ml and 30mg/ml inhibited completely the growth of *B.cinerea*, *A.solani* and 90% of *FORL.* The two first concentrations were found to be the MIC of *A.solani* and *B.cinerea* respectively. The present study shows clearly the antifungal power of the essential oil of *H. tuberculatum.*

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Introduction

The use of essential oils in biological control becomes *very promising* and considered as one of scientific interest. The works carried out by some authors have noted the antiseptic properties of plants essential oils against clinical pathogens. However, very few studies have been carried out until this day on the antimicrobial effect of essential oil of *H. tuberculatum* against phytopathogenic microorganisms.

Haplophyllum tuberculatum (Forsk.) A. Juss. is a spontaneous perennial herb occurring throughout southern Algeria belonging to the *Rutaceae* family. This genus distributed throughout temperate and subtropical zones of Eurasia and the northern tropical zone of eastern Africa (Somalia) (Alatar *et al.*, 2012). The plant's chemical composition has been shown to vary as a function of geographic location and time of collection. It includes alkaloids, lignans, flavonoids and essential oils (Javidnia *et al.*, 2006).

Very few studies have been carried out until this date on the *Haplophyllum tuberculatum* products. This is why we found it interesting to analyze the essential oil of the cited plant and to study its antifungal activity against a number of plant pathogens belonging to *Fusarium oxysporium*, *Botrytic cinerea* and *Alternaria solani*.

Material and methods

Plant materials

The studied plant was harvested from Adrar during the flowering season in March 2014. The region of harvesting is situated in South Algeria (27 ° 52 '27 ") of the North and (0 ° 17' 37") of the West at an altitude of 257 meters. The botanical identification was made by Dr Sekkal.F / Z; the authenticated specimens were deposited in the herbarium of the plant ecology laboratory at Ahmed Ben Bella University 1 Oran (Algeria). The drying of the plant was carried out under the shade at an ambient temperature to preserve as much as possible the integrity of the molecules. They were then weighed and grinded with a mortar for further studies.

Fungal strains tested

Three fungi belonged to *Fusarium Oxysporum f.* sp. *Radicis lycopersici* (*FORL*), *Botrytis cinerea* and *Alternaria solani* were used in the present study. They were isolated from a diseased tomato plant and characterized in Plant Protection Laboratory of Abd El Hamid Ibn Badis University (Mostaganem, Algeria).The three obtained fungi were activated and purified on PDA medium.

Essential oil extraction and yield calculation

The extraction of the essential oil was carried out by hydrodistillation. 100 g of dry plant material were submitted to hydrodistillation with a Clevenger-type apparatus and extracted with 1000 ml of water for 120 minutes. The flask was surmounted by a column of 60 cm length connected to a refrigerant. The essential oil was obtained from organic phase using separating funnel and stored at 4˚C until used. The yield of essential oil was defined according to the Afnor standard (1988) , it was calculated as the ratio between the mass of the essential oil obtained after extraction and the mass of the plant material used , using the equation: $Y_{EO}\ \% = M_{EO} / M_{PM}$

Y_{EO} %: Extraction yield;

M_{EO}: Mass in grams of the essential oil;

M_{PM}: Mass in grams of plant material.

Chemical analysis of essential oil

The analysis of the essential oil of the aerial part of *H. tuberculatum* was carried out by gas chromatography coupled with a flame ionization detector.

The essential oil were analysed on a AGILENT gas chromatograph Model 7890, coupled to a AGILENT MS model 5975, equipped with a DB5 MS column (20m X 0,18mm, 0,18µm), programming from 50°C (3.2 min) to 300°C at 8°C/mn, 5 min hold. Helium as carrier gas (1, 0 ml/min); injection in split mode (1: 150) at 300°C.

The MS working in electron impact mode at 70 ev; electron multiplier, 1800 V; ion source temperature, 230°C; mass spectra data were acquired in the scan mode in *m/z* range 33-550.

Antifungal effect of the essential oil of H. tuberculatum

Determination of the inhibition rate and minimal inhibitory concentrations (MIC) of the essential oil of H. tuberculatum

Essential oils are generally insoluble in water, making their biological study very difficult. The dimethylsulfoxide (DMSO) has been used as an appropriate solvent of essential oils and it also not show inhibition of the fungal species used (Duraffourd et Lapraz, 2002; Bajpai *et al.*, 2004; Hili, 1997; Kolai *et al.*, 2012).

The method of direct contact was adopted to evaluate the antifungal activity. The essential oil was diluted with DMSO (dimethyl sulfoxide) (%) and the concatenations of 2, 5 mg/ml, 5 mg/ml, 10 mg/ml, 20 mg/ml and 30mg/ml were prepared. 1 ml of each concentration is added to each Petri dish containing 15 ml of potato dextrose agar (PDA) medium, and then stirred for 5min to homogenize the medium PDA with essential oil. After solidification of the medium, explants of 5 mm diameter were taken from a seven-day old culture using a sterile punch. Each explant was deposited in a well dug at the center of the medium.

Three repetitions were realized for each concentration (Kolai *et al.*, 2012; Haddouchi *et al.*, 2013; Kanoun *et al.*, 2014; Amri *et al.*, 2014**).** The DMSO without essential oil was used as a negative control.

The cultures were incubated for seven days at 28 °C. Colony diameter measurements were made for each concentration to evaluate the level of inhibition of each fungal strain.

The antifungal action was evaluated by calculating the growth inhibition rate using the formula described by Leroux and Credet (Leroux and Credet, 1978).

$$\boldsymbol{T}\ (\%) = ([\boldsymbol{L} - \boldsymbol{I}] / \boldsymbol{L})\ \boldsymbol{X100}$$

T: Inhibition rate.

L: mycelial growth of the control.

I: mycelial growth of treated fungi

Minimal Inhibitory Concentrations (MIC)

The Minimal Inhibitory Concentrations (MIC) was determined by direct agar contact method (Mishra and Dubey, 1994; Cakir *et al.*, 2004).

Statistical analysis

Research results were analyzed statistically by statistical software Statbox (version 6.4). All experimental measurements were performed five times and were expressed as (means ± SD). The results are significant when $p < 0.05$.

Results and discussion

Chemical composition and antifungal activity of essential oil of H. tuberculatum

The yield of essential oil obtained by hydrodistillation of *H. tuberculatum* is 0.101%.

The results of essential oil analysis by gas chromatography coupled with a flame ionization detector (CG-FID) are summarized in Table 1 and Fig. 1. The chemical composition shows a quantitative and qualitative variation in the chemical profile were thirty-eight constituents were found. In the studied oil we can report the great richness in ketones, sesquiterpenes and terpenoides with a dominance of Piperitone (13.35%), Germacren-B (12.30%) and Beta-Phellandren (5.05%).

The study carried out on the antifungal activity of essential oil on *FORL*, *Alernari solani* and *Botrytis cinerea* showed remarkable efficacy at different concentrations of the essential oil studied (Fig. 2). Indeed, the doses 2.5mg/ml and 5mg/ml show a lower inhibition compared to the other doses. As for doses of 10mg/ml, 20mg/ml and 30mg/ml, they show complete inhibition against *A.solani* and *B. cinerea*.

The plant essential oil caused significant differences ($p<0.01$) on growth inhibition rates. A proportional relationship is observed between the two factors: inhibition rate and essential oil concentrations, where increasing of concentrations increased the rates of inhibition.

Table 1. Chemical composition of *Haplophyllum tuberculatum* essential oil identified by GC / FID.

Compounds	TR	%
Alpha-thujene	5,25	0,17
Alpha-Pinene	5,39	1,29
Camphene	5,72	0,50
Sabinene	6,19	0,45
Beta-Pinene	6,27	0,67
Myrcene	6,53	1,72
AlphaPhellandrene	6,83	2,76
Delta-3-Carene	6,87	1,86
Alpha-Terpinene	7,03	1,20
1,4-Cineol	7,17	0,20
Para-Cymene	7,17	0,27
Limonene	7,27	1,37
Beta-Phellandrene	7,29	5,05
Eucalyptol	7,33	0,42
(Z) -Beta-Ocimene	7,39	0,13
(E) -Beta-Ocimene	7,58	0,14
Gamma-Terpinene	7,77	0,08
1-Octanol	8,05	0,29
Terpinolene	8,24	0,18
2-nonanone	8,36	0,7
Cis-Thujone	8,62	0,19
Unknown beta-phellendrene	8,93	10,60
Unknown beta-phellendrene	9,22	6,84
Unknown beta-phellendrene	1,05	2,72
Unknown beta-phellendrene	10,24	5,44
Piperitone	10,91	13,35
Bornyl acetate	11,31	1,23
2-Undecanone	11,41	0,30
Unknown ditto Gamma-Terpinene	12,00	6,16
Beta-Bourbonene	12,65	0,32
Beta-Elemene	12,72	0,18
Beta-Caryophyllene	13,12	4,14
Gamma-Elemene	13,22	2,91
Alpha-Humulene	13,57	0,34
Germacrene-D	13,88	1,82
Germacrene-B	14,82	12,30
Total	88,73	

RT : retention time.

Minimal inhibitory concentrations (MIC)

It corresponds to the minimal concentration inhibiting the fungal mycelia growth observed by naked eyes. Its determination was made by observing the total absence of the growth of the strains in the different concentrations of essential oil used. According to the recorded results, 100% inhibitory activity was observed by applying concentrations of 10, 20 and 30 mg/ml for *Alternaria solani* and 20 and 30 mg /ml for *Botrytis cinerea*. The MIC values obtained are summarized in Table 2.

Given the total inhibition of *Botrytis cinerea* and *Alternaria solani,* it was important to know whether the essential oil was fungistatic or fungicidal at these concentrations.

Indeed, the transfer of fungi discs, totally inhibited, to another PDA culture medium was necessary to evaluate the viability of these fungi. The results of the *in vitro* tests showed that mycelial growth reappeared after the disc was exposed to the essential oil for 1, 3, 6 and 12 days. Thus, the results obtained reveal that the effect represented by the essential oil of the three species exerts a fungistatic effect towards the fungi tested.

Table 2. Fungicidal/fungistatic effect and MIC of the essential oil of *H. tuberculatum*.

MIC	*H. tuberculatum*	Fungicidal/fungistatic effect
FORL	-	-
Botrytis cinerea	20mg/ml	Fungistatic effect
Alternaria solani	10mg/ml	Fungistatic effect

The presented results are in agreement with those of Hadouchi *et al.* (2013) who reported that the essential oil of *Haplophyllum tuberculatum* obtained by hydrodistillation from the West of the Algerian Sahara (Béchar: South Algerian) has a yield of 0.11%. The chemical profile of the oil studied is relatively different quantitatively from what has been reported in the literature.

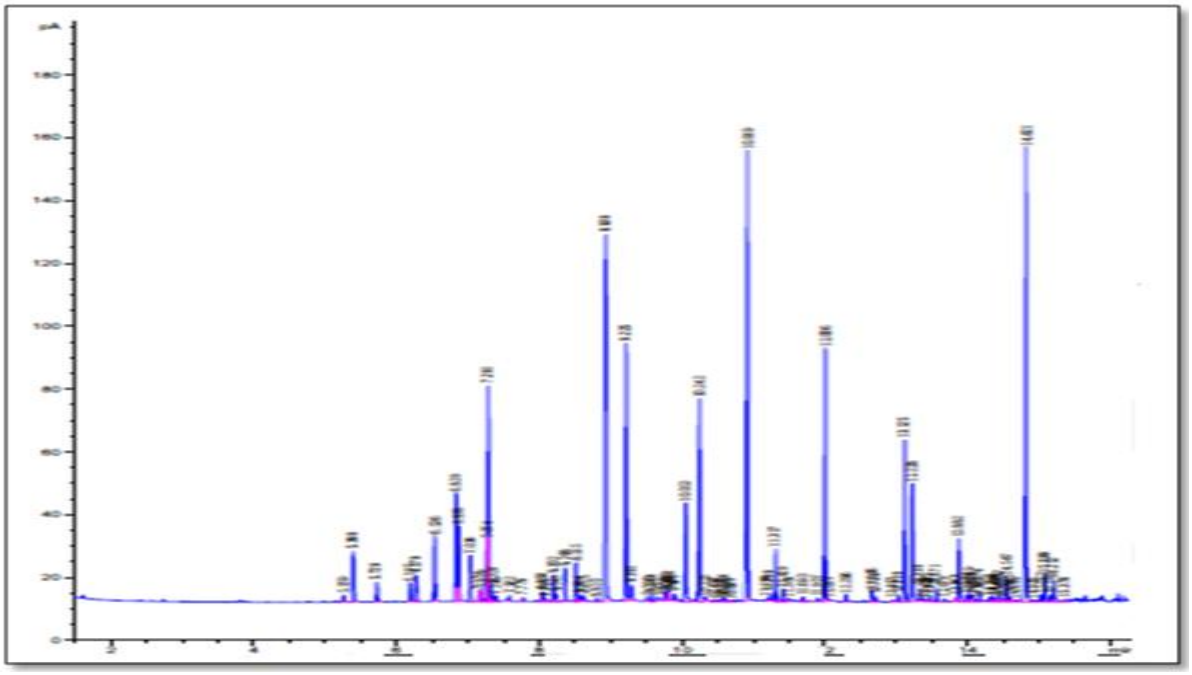

Fig. 1. Gas chromatography coupled with a flame ionization detector of the essential oil of *H. tuberculatum*.

Indeed, other studies have indicated that the essential oil extracted from *H. tuberculatum* originating from Egypt is characterized by the abundance of monoterpenes and sesquiterpenes where Trans-p-Menth-2-ene-1-ol represented (9.7-14.1%) as the most dominant group representing (El-Naggar *et al.*,2014). However, the work of Al-Rehaily *et al.* (2014) on the same plant originally from Saudi Arabia reported two major constituents: trans-p-menth-2-en-1-ol (19.2%), cis-p- menth-2- 1-ol (13.2%), myrcene (10,1%). Gas chromatography/mass spectrometry analysis of extracted essential oil *H. tuberculatum* froum Tunisia revealed 18 indentified components representing 93.46 % of total oil. The major components were hexadecanoic acid (40.21 %) and oleic acid (26.75 %) (Debouba *et al.*, 2014).The quantitative and qualitative difference in the chemical composition of the essential oil of the studied plant could be explained by the impact of many biotic and abiotic factors, such as: origin of samples, geographical

factors such as altitude, Soil type, environmental conditions (temperature, luminosity, rainfall) and the vegetative plant stage (Pandey *et al.*,1982; Bruneton ,1999 ; Panizzi *et al.*,1993; Abu-Darwish and Abu-Dieyeh,2009; Merghache *et al.*,2006; Rodolfo *et al.*,2006). Other factors can also influence chemical composition of the essential oil, like crops conditions (sowing and harvesting dates), phytosanitary treatments, use of fertilizers, and techniques of plant harvesting and extraction as indicated by several authors (Zellagui *et al.*,2007; Ferhat *et al.*,2014; Riahi *et al.*,2015; Ghazghazi *et al.*,2015).

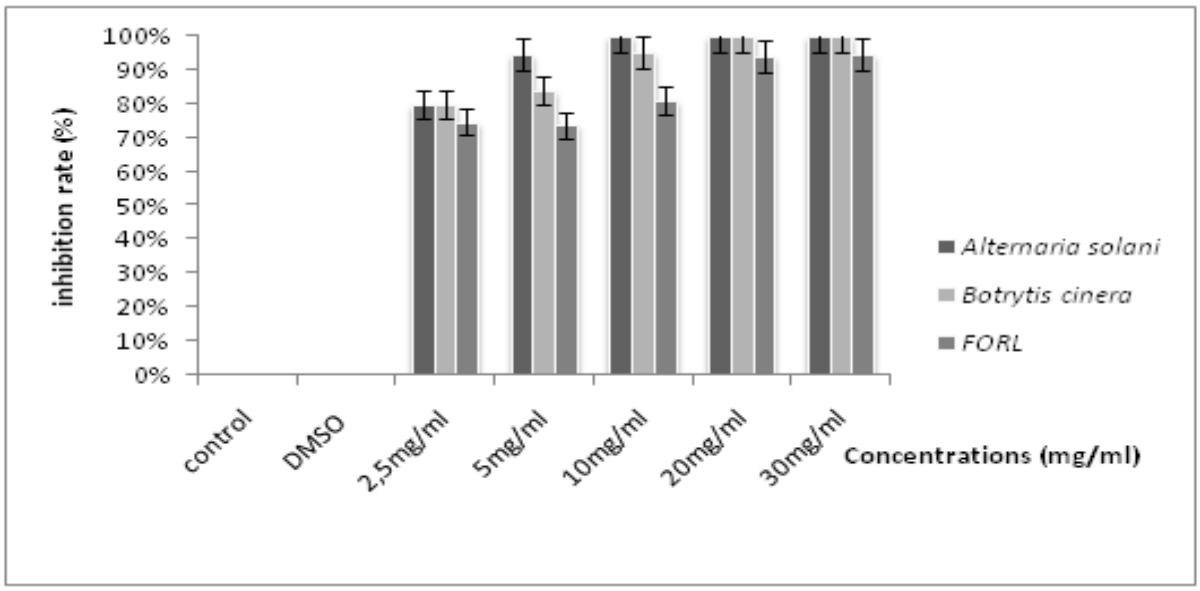

Fig. 2. Inhibition rate of mycelial growth of the three fungal strains by essential oil of *H. tuberculatum*.

The results of antifungal tests activity indicate that the essential oils of *H. tuberculatum* possess a very interesting antifungal power. Similarly, antifungal activity of essential oil of Rutaceae was also confirmed by Sharma and Tripathi (2006) and Al-Burtamani *et al.* (2005).

The richness of the essential oil of the plant in terpenic and ketonic compounds could be responsible for the antifungal effect of this oil on the three fungi tested. Indeed, several studies show the efficacy of oxygenated monoterpenes as antifungal compounds (Salamci *et al.*, 2007). The chemical diversity of essential oil of *H. tuberculatum* in compounds make it affecting several targets simultaneously and rare microbes could be resistant (Bakkali *et al.*, 2008).

The compounds act by hydration and by degradation with enzymes such as chitinase and α and β -glucanases leading to decomposition of the cell wall of the thickened conidia and mycelia. Once this event takes place, there is a balance between the lytic and synthetic systems of enzymes necessary for the normal prolongation of hyphae. As such, the active compounds attack the cell wall and membrane, thereby affecting the permeability and release of intracellular constituents, also interfering with membrane function (Bajpai and Kang, 2010). Perturbation of this matrix may result in a defective wall, which becomes sensitive to osmotic and sensitive to antifungal agents (Yen and Chang, 2008).

Derwich *et al.* (2010) reported that antimicrobial activity is related to the ability of terpenes to affect not only permeability but also other functions of the cell membrane.

The terpenes also act by binding to amine and hydroxylamine groups of membrane proteins causing impaired permeability and leakage of intracellular constituents.

Several authors, notably Mares *et al.* (2004); Rasooli and Abyaneh (2007) and Sharma and Tripath (2004)

found that essential oils can cause morphological changes including inadequate sporulation, loss of pigmentation, abnormal development of conidiophores and deformation of hyphae. It seems that the terpenes exert a dysfunction of the ATPase proton pumps leading to cells death (Viuda-Martos *et al.*,2008).Similarly, it has been shown that some sesquiterpene are antifungal (Mitscer and Hasennhuettl,1975; Alilou *et al.*,2016).

The inhibitory activity may also be due to the different modes of action and synergistic effect of all the components of the essential oil on the fungi. According to Randrianarivelo *et al.* (2008) the minority constituents in studied essential oil such as Alcohols, aldehydes and esters are also known for their antimicrobial activity.

Conclusion

As a conclusion we consider that the present work is interesting and the results obtained are encouraging. The extraction of the essential oil from *H. tuberculatum* by hydrodistillation provided a yield of 0.101% and its identification by GC-MS showed the presence of Piperitone (13.35%), Germacrene-B (12.30%) and Beta-Phellandrene (5.05%) with richness in ketones, sesquiterpenes and terpenoides. The essential oil of the plant proved to be an effective antifungal agent against the three fungi tested with a total inhibition of 100% at a dose of 20 mg/ml for *Botrytis cinerea* and 10mg /ml for *Alternaria solani*. The MIC is estimated to be 20m g /ml for *Botrytis cinerea* and 10mg/ml for *Alternaria solani*.The antifungal activity can be attributed to the chemical composition of the essential oil.

Accordingly, the results of the present work suggest the possibility to use the essential oil of *Haplophyllum tuberculatum* (Forsk.) A. Juss. as a natural fumigant antifungal in open fields.

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