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ORIGINAL ARTICLES

Antifungal and Chemical Composition of Essential Oils of *Juniperus communis* L. and *Thymus vulgaris* L. against Two Phytopathogenic Fungi

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ABSTRACT

The essential oils of the aerial parts of *Juniperus communis* and flowering tops and leaves of *Thymus vulgaris* were isolated by hydrodistillation with a yield of 0.1 and 0.3 % (wt/wt), respectively. The isolated oils were tested for their *in vitro* antifungal activity against two phytopathogenic fungi, *Rhizoctonia solani* and *Rhizopus stolonifer*. These oils showed a remarkable antifungal effect against both fungi. The essential oil of *T. vulgaris* was more potent as antifungal agent against *R. solani and Rh. stolonifer*, respectively, with EC50 values of 0.385 and 0.491 mg/ml than the oil of *J. communis* (EC_{50}: 0.554 and 0.704 mg/ml). The essential oil of *J. communis* was chemically analyzed by GC-MS. The main components were oxygenated monoterpenes.Therefore, the antifungal activity of the oil can be attributed to its relatively high content of oxygenated monoterpenes.Thymol was isolated from *T. vulgaris* essential oil and was found to be superior as antifungal agent against both fungi than the two oils. The fungistatic test revealed that the essential oils and thymol were fungistatic in their nature. These results suggest the potential use of the above essential oils for the control of fungal diseases.

Key words: Antifungal activity,Fugistatic activity, Essential oils, GC/MS analysis,Thymol

Introduction

Synthetic pesticides are widely used in the control of plant diseases. However, these chemicals may cause human hazards and environmental pollution owing to their biodegradation (Barnard *et al*., 1997) in addition, the risk of developing the resistance by microorganisms is other disadvantage of synthetic pesticide usage (Brent and Hollomon, 1998). Therefore, research for development of new antifungal agent from plants is an urgent need. Alternative natural pesticides are necessary for use in the control of pathogenic fungal diseases in plants. Essential oils and extracts from various parts of plants is one of the most promising groups of natural compounds which may be developed for use as natural fungicides substitute the synthetic pesticides due to the presence of terpene constituents within differing groups found in the oils.

Juniper, *Juniperus communis* L. was used in aromatherapy, through inhalation, massage, bathing, or ingestion to create good health and beauty. Oil of juniper, distilled from the wood and leaves of several species, is used in perfumes. It is noted by German authorities that the tea is diuretic and urinary antiseptic. The diuretic activity is thought to be largely due to the tea's content of terpinen-4-ol, a non-irritatingterpene (Hagar, 1979).

Thyme, *Thymus vulgaris* L. is indigenous to the north coast in Egypt, and is now widely cultivated as a tea, spice, and herbal medicine. Its leaf is listed in the German and British Herbal Pharmacopoeia, and has been used as a stomachic, carminative, diuretic, urinary disinfectant, and vermifuge(Wichtl, 1994). Many essential oils derived from many aromatic plants are known to possess antimicrobial activities (Janssen *et al*., 1987). Therefore, this study was undertaken to investigate the antifungal activities of the essential oils isolated by hydrodistillation from both *Juniperus communis*(L.) *and Thymus vulgaris*(L.) and their chemical compositions.

Materials and Methods

Plant material:

Aerial parts of *Juniperus communis* (L.) as well as flowering tops and leaves of *Thymus vulgaris* (L.) were collected during the flowering stage from Sinai. The plants were identified according to the taxonomic characters of Tackholm (1974). The samples were air- dried at room temperature (26± 1ºC) and then finely powdered.

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Test fungi:

Two plant pathogenic fungi, *Rhizoctonia solani* (Kuhn.) and *Rhizopus stolonifer* (Ehren), were used in this study. The fungi were obtained from department of Plant Pathology, Fac. of Agric. Damanhour University, and maintained during the course of the experiments on Czapek- Dox Agar (CDA) at 25ºC.

Isolation of the essential oils:

Each essential oil (EO) was extracted by hydrodistillation method using Clevenger trap apparatus (Guenther, 1952 and Lamaty *et al*., 1987). The powder of the air dried parts of each plant was placed in 2L. capacity flask, sufficient quantity of water was added to approximately 3/4 of the final volume of plant material. A proper essential oil trap and condenser were attached to the flask and enough water added to fill the trap. The distillation continued until no further increase in the oil layer was observed. The oil was permitted to stand undisturbed so that a good separation from water could be obtained. Each fraction of oil extract was dried over anhydrous sodium sulphate and weighed. Then the dried oil was kept in a refrigerator till the use in different bioassay tests.

Analysis of essential oils:

Essential oil *J. communis* was diluted by diethyl ether and 1µl from oil solution was injected into a gas chromatography (TRACE GC 2000, THERMO)/ mass spectroscopy (SSQ 7000, FINNIGAN) (GC/ MS) set up. The GC column was a 60 m (0.25m m i.d.) DB-5 (5%-phenyl) methyl polysiloxane capillary column. The GC conditions were as follows: injector temperature, 220 °C; column temperature, isothermal at 40 °C for 2 min, then programmed to 250 °C at 5 °C/2 min and held at this temperature for 2 min; ion source temperature, 200 °C. Helium was used as a carrier gas at the rate of 1ml/ min. The effluent of the GC column was introduced directly into the ion source of the MS. Spectra were obtained in the El made with 70 eV ionization energy. The sector mass analyzer was set to scan from 40 to 400 amu for 5s. The GC/ MS analysis was carried out in the Egyptian Agricultural Research Center, Cairo 2010.

In vitro antifungal assay:

The antifungal activity of the isolated essential oils and thymol was tested using the mycelia radial growth inhibition technique (Kagale *et al*., 2004 and Boyraz and Ozcan 2005). Appropriate volumes of the stock dilutions of the tested oils in Triton-x 100 were added to molten nutrient agar medium (Czapek-Dox Agar; CDA) to obtain a range of concentrations (0.1, 0.3, 0.5, 0.8, and 1 µg/ml before pouring into the Petri dishes (9.0 cm in diameter) at 40–45 ◦C. Each concentration was tested in triplicate. Parallel controls were maintained with Triton-x 100 mixed with CDA medium. The discs of mycelial felt (0.5 cm diameter) of the plant pathogenic fungi, taken from 8-day-old cultures on CDA plates, were transferred aseptically to the centre of Petri dishes. The treatments were incubated at 25 °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: Mycelial growth inhibition = [(DC–DT)/DC]×100 (Pandey *et al.*, 1982), where DC and DT are average diameters of the fungal growth for control and treatment, respectively. The concentration of each essential oil that inhibiting fungal mycelial growth by 50% (EC_{50}) was calculated by a linear regression method (Finney, 1971).

Fungistatic assay:

Mycelial discs (0.7 cm diameter) were cut. Onemycelial disc was placed in the center of solid fungal growth medium in Petri dish; one circle of sterile filter paper was placed inside of the cover of the Petri dish. 50 µL from each oil concentration, thy mol and potassium sorbate to the filter paper.

When the growth of the colony in the control treatment reaches the edge of the plate, the diameters were measured of both treatments and control.

Comparative growth inhibition (CGI) is calculated using the following equation

$CGI = 100[(D_c - D_t)/D_c] - 100$

Where D_c is the mean colony diameter for the control and D_t is the mean colony diameter for the treatment. This formula determines the growth inhibition as a percent of the control. Thus, a CGI value of 0 indicates that the growth of the fungus in the treatment was completely inhibited, while a CGI of -100 indicates that the colonies on the control and treatments were the same size (Schadler and George 2006).

Results and Discussions

Antifungal activity of essential oils:

The essential oils were isolated by hydrodistillation of the dried aerial parts of *Juniperuscommunis* (L.) and the leaves, flowering tops of *Thymus vulgaris* (L.). The oil yields were calculated on a dry weight basis as 0.1% for *J. communis* and 0.3 % for *T. vulgaris*. Antifungal activities of these oils were recorded as inhibition of fungal mycelial growth and conidial germination (Table1). The essential oils of *T. vulgaris* and *J. communis* at 0.1mg/ml significantly reduced the mycelial growth of *R. solani* and *Rh. stolonifer* by 24.07%, 22.22% and 20.37%, 16.67%, respectively. At a concentration of 1mg/ml the mycelial growth of both fungi was reduced by 96.30%, 83.33 % and 83.33%, 75.93% for *T. vulgaris* and *J. communis* oils, respectively. According to the calculated EC_{50} value for each oil, results revealed that the oil of *T. vulgaris*was the most fungitoxic with EC_{50} values of 0.384mg/ml and 0.491mg/ml for *R. solani* and *Rh.stolonifer* respectively. The corresponding EC_{50} values for EO of *J. communis* were 0.554 and 0.703mg/ml respectively. These results revealed that the essential oil of *T. vulgaris* was significantly fungitoxic than the oil of *J. communis*. These results are in agreement with those of Zambonelli *et al*., 1996 and El-Sherbieny *et al*.,2002. The different antifungal activity of both oils may be due to the differences in the content of known antimicrobial compounds in each EO as earlier determined by Lamaty *et al.*, 1987; Farag*et al*., 1989; Amvam Zollo *et al*., 1998 and Tassou *et al*., 2000.

Table 1: Antifungal activity of essential oils on mycelial growth of the two testedfungi

Conc. (mg/ml)	Inhibition%			
	Thymus vulgaris		*Juniperuscommunis*	
	R. solani	*Rh. stolonifer*	*R. solani*	*Rh. stolonifer*
0.1	24.07	22.22	20.37	16.67
0.3	31.48	25.93	25.93	20.37
0.5	46.30	40.74	40.74	29.63
0.8	62.96	59.26	48.15	48.15
1	96.30	83.33	83.33	75.93
EC50	0.384	0.491	0.554	0.703
Lower	0.356	0.471	0.545	0.685
Upper	0.399	0.505	0.566	0.721
Slope	1.75	1.54	1.47	1.53

$LSD_{0.05}$= 6.58

Chemical composition of essential oil of J. communis L:

GC/MS analysis of essential oil of *J. communis* L. led to the identification of 45 different components, representing 95.35% of the total oil. The volatile components identified by GC/MS, their relative area and retention time are summarized in Table 2. The essential oil contained high percentages of monoterpene hydrocarbons (10.66%), sesqueterpene hydrocarbons (13.36%), oxygenated monoterpenes (34.43%) and oxygenated sesqueterpenes (36.9%). The dominant component was alpha- cedrol (19.44%). On the other hand (Tomaino *et al*., 2005) reported that the major component of thyme essential oil was thymol (45.35%). Since the essential oil of thyme was more antifungal to *R.solani* and *Rh.stolonifer* fungi than the essential oil of *J.communis*, therefore its major constituent, thymol, was evaluated against both fungi.

Antifungal activity:

Results in Table 3 show that thymol at 0.01mg/ml significantly reduced the mycelial growth of *R. solani* and *Rh. Stolonifer* by 29.63% and 22.22%, respectively. Total inhibition of conidial germination of these fungi was observed at 0.5 mg/ml. According to the calculated EC_{50} values of the tested oils, thymol was more potent as fungitoxic against *R. solani* and *Rh.stolonifer* agent with EC_{50} values of 0.059 and 0.088mg/mlrespectively. Thus thymol was more toxic with 22.9 fold than thymus oil.

Fungistatic activities of J.communis, T. vulgaris essential oils and thymol:

Results of the fungi static activities of the thymol, potassium sorbate and essential oils of *J.communis* and *T. vulgaris* against *R.solani* and *Rh.stolonifer* are summarized in (Table 4). After following incubation for five days, any further growth indicated a fungistatic effect; if mycelial development was not noted and the death of fungal cells is confirmed, the effect was appreciated as fungicidal.

Recent reports on the success of essential oils as biodegradable and ecofriendly fungitoxicants have shown the possibilities for their exploitation as natural fungicides (Dixit *et al*., 1995). Thus, the oils of of *J.communis*, *T. vulgaris* and thymol with their strong fungitoxicity were tested for their fungistatic properties against both fungi. Results in Table 4 show that the fungistatic activity varied greatly according to compound and tested

fungus and the compound concentration. Potassium sorbate has fungicidal effect against fungi, *R. solani* and *R.stolonifer* since it didn't activate these fungi. The results also revealed that thymol activate the mycelial growth of both fungiby 27% and 66%, respectively. The activation of mycelial growth of fungi *R. solani*and *Rh. stolonifer* were 44% and 88%, respectively by essential oil of *J. communis* while *T. vulgaris* essential oil caused activation percentages of 49% and 100%, respectively. Thus, these oils and thymol on account of their strong fungitoxicity and fungistatic nature (effect) should prove useful fungitoxic agents for controlling the tested fungi.

Table 2: Main Components in the Essential Oil of *Juniperuscommunis* L.

Monoterpene hydrocarbons	Rt	%
1) a-Pinene	4.4	1.95
2) dl- Limonene	6.33	0.96
3) Alpha.- Pinene	10.78	0.8
4) (+)-4-Carene	12.44	3.86
5) Bicyclo[4.1.0]hept-2-ene,3,7,7-trimethyl	12.81	0.71
6) Azulene	18.1	2.13
7) Phenanthrene	20.83	0.25
sesqueterpene hydrocarbons	Rt	%
8) Alpha- cedrene	12.98	0.15
9) Alpha- Cadina-4,9-Diene	13.08	0.93
10) Cedrene	13.64	4.04
11) Gamma. 1-cadinene	14.09	1
12) Alpha.-cadina-4,9-dinene	14.45	1.71
13) 1-Isopropyl-7- methyl-4-methylene gamacadenine	14.84	0.43
14) Alpha.-gurjunene	18	2.9
15) Cyclohexadecane	19.96	0.89
16) Cembrene	24.47	1.31
oxygenated monoterpenes	Rt	%
17) 1- Indanone	7.6	1.15
18) Linalool	7.85	2.34
19) 2,3,3-Trimethyl-3- cyclopentene acetaldehyde	8.35	2.09
20) 3,4-Pentadien-1-ol, 2,2-dimethyl	9.08	0.71
21) Cyclopentanecarboxylic acid	9.22	0.75
22) 3-Cyclohexene-1-methanol	9.69	2.18
23) 7a-Methyl-1,4,5,6,7,7A-hexahydro-2H-inden-2-one	10.05	0.26
24) Trans-(+)- Carveol	10.22	1.58
25) β-Citronellol	10.36	1.13
26) 5- Decene-1-ol	10.89	2.26
27) Cyclohexanol, 2-methyl-5- (1- methyl acetate)	11.18	3.53
28) Bicyclo(3,2,2)non-6-en-3-one	11.38	1.98
29) 2- Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)	12.23	0.5
30) 3,4- Difluoro-4-methoxybiphenyl	18.21	4.96
31) 4-Isopropyl-6-methyl-1,2,3,4-tetrahydronaphthalen-1-one	18.33	2.33
32) 2,4,4-trimethyl-3-[(1E)-3-oxo-1-butenyl]-2-cyclohexen-1-one	18.61	1.52
33) 2-Tridecanone	19.53	1.35
34) 1,2,4-Triazolo(3,4-c)(1,2,4)-benzotriazin-1(5H)-one	24.74	0.53
35) Furo[2,3-H]coumarine	20.6	0.23
36) Neryl linalool isomer	22.17	2.36
37) 1,2-Benzenedicarboxylic acid	27.12	0.69
oxygenated sesqueterpenes	Rt	%
38) Cedrene epoxide	18.94	2.79
39) Cedran-8-ol	20.08	0.19
40) Oxacycloheptadecan-2-one	20.73	0.6
41) 3,4A,7,7,10A-pentamethyl-3- vinyldodecahydro-1H-benzo(F)chromene	21.47	7.86
42) Phytol Isomer	22.63	0.66
43) Pimara-7,15-dien-3-one	24	0.79
44) Alpha.-cedrol	16.80	19.44
45) Cadinol	17.91	4.57

Table 3: Antifungal activity of thymol on the growth of *R. solani and Rh. stolonifer*

Conc.	Inhibition%	
(mg/ml)	*R. solani*	*Rh. stolonifer*
0.01	29.63	22.22
0.05	38.89	31.48
0.1	44.44	38.89
0.3	72.22	62.96
0.4	83.33	79.45
0.5	100	100
EC50	0.059	0.088
Lower	0.046	0.073
Upper	0.073	0.103
Slope	1.12	1.20

Table 4: Fungistatic activity of thymol, potassium sorbate and essential oil *of J.communis* and *T. vulgaris* against of *R.solani* and *Rh.stolonifer*

Compounds	Activation%	
	R. solani	*Rh. stolonifer*
Thymol	27	66
potassium sorbate	0	0
Essential oilof *J.communis*	44	88
Essential oilof*T. vulgaris*	49	100

Conclusion:

GC/MS analysis of essential oil of *Juniperus communis* L. led to the identification of 45 different components, representing 95.35% of the total oil. The essential oil of *J. communis* contained high percentages of monoterpene hydrocarbons (10.66%), sesqueterpene hydrocarbons (13.36%), oxygenated monoterpenes (34.43%) and oxygenated sesqueterpenes (36.9%). The dominant component was alpha- cedrol (19.44%).

The results of this study revealed that *J.communis* and Thymus vulgaris are potent antifungal activity. *J.communis* oil can be attributed to its relatively high content of oxygenated monoterpenes. Thymol was found to be superior as antifungal agent against both fungi than the two oils. These results suggest the potential use of the above essential oils for the control of fungal diseases.

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