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Chemical analysis and antimicrobial activity of indigenous medicinal species volatile oils

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Abstract

Phytochemical and antimicrobial screenings of 5 volatile oils of plants (Thymus vulgaris L, Salvia officinalis L, Foeniculum vulgare L., Achillea millefolium L., Carum carvi L.) growing in Southeastern Romanian area were performed. Specific compounds for each species were detected by GC: anethol (80.89% in Foeniculi fructus), carvone (17.37% in Carvi fructus), β-thujone and camphor (30.88% and respectively 21.73% in Salviae herba), thymol (30.86% in Thymi herba) but also other compounds like 1,8-cineol (15.59% in Millefolii flores and 8.55% in Salviae herba), p-cymene (30.53% in Thymi herba and respectively 24.24% in Carvi fructus), borneol (25.79% in Millefolii flores), etc.

In vitro antimicrobial activities of volatile oils were tested using two methods: the dilution method for determination of minimal inhibitory concentration and the cylinder-plate diffusion method. All volatile oils showed different levels of inhibitory activity against microbial strains tested. Thymus vulgaris volatile oil was the most active.

Keywords: thyme, fennel, sage, caraway, yarrow, volatile oil, antimicrobial

Introduction

In recent years, a large number of essential oils and their constituents have been investigated for their antimicrobial properties against bacteria and fungi. Natural antimicrobial agents can be used for a wide variety of purposes, including food preservation, natural therapeutics, cosmetics, etc.

Volatile oils antimicrobial property is due to the capacity of various constituents to defeat infectious germs (bacteria, viruses, fungi, parasites) and to inhibit their proliferation in living organisms or in the environment. Antiseptic and antibacterial properties are proportional to the lipophilicity of the constituents. Phenols (carvacrol, thymol, eugenol) and their methyl ethers have the greatest antiseptic potential, followed by cinnamic aldehyde, monoterpene alcohols (geraniol, linalool, thuyanol-4, myrcenol, terpineol, menthol), monoterpene aldehydes (neral, geranial, citronellal, cumin which defeat bacterial spores), monoterpene ketones (verbenone, thujone, camphor, fenchone, menthone, carvone which are active in mucopurulent infections), ethers (estragole, anethole), oxides monoterpenes (1,8-cineol). Thymol, geraniol, citral and linalool are 20; 7.1; 5.2 and respectively 5 times more active than phenol. [1]

Different chemotypes of the same species may grow in the same place and produce different oils with different activity. Generally, antimicrobial action is determined by more than one component. In such cases, the major component is not only responsible for the antimicrobial activity but a synergistic effect may take place.

Monoterpene R(+) limonene accompanies carvone in *Carum carvi* L (caraway) fruit volatile oil, with the ratio of both main components varying from 3:2 up to 3:1, depending on variety of plant and storage conditions. Many physiologically active constituents identified in *Carum* fruits are low molecular weight monoterpenes with oxygen-containing functional groups.[2]

Salvia officinalis L. (sage) is considered to have the highest volatile oil yield among *Salvia* species. The major components of the volatile oil of *S. officinalis* are α- and ß-thujones; at least two chemotypes of *S. officinalis* exist, one with a low a-thujone content (4–8%) and another with a relatively high content (16–32%). A decrease in the α-thujone content, with a corresponding increase in the relative amount of camphor is related to leaf age. [3]

The most abundant volatile constituents in *Foeniculum vulgare* L (fennel) fruits are trans-anethole, estragole, limonene and fenchone, compounds with well-known antimicrobial potential [4]

The quantitatively most important components of *Achillea millefolium* L. (yarrow) are sabinene, β-pinene, 1,8-cineole, linalool, α- si β-thujone, camphor, borneol, bornyl acetate, (E)-β-caryophyllene, germacrene D, β-bisabolol, δ-cadinol, chamazulene etc. A recent study shows that yarrow samples from Estonia, Hungary, Greece, Moldavia, Latvia, Lithuania and Germany contain high amounts of monoterpenes and chamazulene. The oils from France, Belgium, Russia, Armenia, Spain and Italy are rich in oxygenated monoterpenes and contain a little amount of chamazulene. The vegetal materials from Greece, Estonia, Moldavia and Scotland are rich also in sesquiterpenes. [5] In *A. millefolium* growing in Romania, the main components of the monoterpene fraction of the oils are 1.8 cineol, camphor, and borneol.[6]

Several studies have focused on the antimicrobial activity of the volatile oil of *Thymus vulgaris* L. (thyme) and showed that thymol and carvacrol seem to play an outstanding role. These terpene phenols join to the amine and hydroxylamine groups of the proteins of the bacterial membrane altering their permeability and resulting in the death of the bacteria. Antibacterial activity is also observed for the aliphatic alcohols, especially geraniol, and ester components. In some cases, esters are more active than their corresponding free alcohols, but sometimes less active (7)

The current work presents an evaluation of antimicrobial activity of volatile oils from aromatic and medicinal plants from Romania and their inhibitory effect against various microbial strains (one species of Gram positive and two species of Gram negative bacteria, one species of fungus, one species of yeast).

Materials and methods

Plant material

Thymus vulgaris L. (Thymi herba), *Foeniculum vulgare* L. (Foeniculi fructus), *Salvia officinalis* L. (Salviae herba), *Carum carvi* L. (Carvi fructus) - dried and milled plant material was obtained from indigenous crop (Southeastern area).

Chemicals

Reference compounds - p-cymene, camphene, thymol, carvacrol, linalool, borneol, cineole, terpinyl acetate, geraniol, terpineol, caryophyllene, thujone, anethol were purchased from *Sigma Aldrich-Fluka*. Growth media (Sabouraud, Tryptic soy agar, peptone water) were purchased from *Fluka*. McFarland standard was provided by *BioMerieux, France*. All other chemicals were analytical grade reagents.

Extraction of volatile oil by steam distillation

The steam distillation was done using a Neoclevenger system: In 5 L round bottom glass flask were added 200 g dried thyme/ sage herba or yarrow flowers and 2 L (vegetal material/ extraction solvent rate = 1/10 (m/v). The mixture was left under reflux for 5 hours. Volatile oil was collected and kept in cool and dark place until use.

For fennel and caraway fruits 1kg vegetal material and 7.5L distilled water (vegetal material/ extraction solvent rate = 1/7.5 (m/v) in a 10L glass flask were used. The extraction conditions were the same as for the above mentioned species.

Gas chromatographic analysis (GC)

GC analysis was carried out by using an Agilent 6890N gas chromatograph equipped with a FID detector, 7683B autosampler and a capillary column HP 5 (30m x 0.32mm; film thickness 0.25µm). The injector and detector temperatures were kept at 250°C and 280°C, respectively. Nitrogen was used as carrier gas, a flow rate of 2mL/min; oven temperature programmed was 40-200°C at a rate of 5°C/min. respectively. Identification of the main components was carried out by the comparison of the GC retention times against those of the reference standards.

Test organisms

The organisms used comprised of two Gram-negative organisms (*Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027), a Gram-positive bacterium (*Staphylococcus aureus* ATCC 25923), a fungus (*Candida albicans* ATCC10231) and a yeast (*Aspergillus niger* ATCC 16404). The test organisms were purchased from *Microbiologics, MediMark Europe*.

Inoculum preparation

To achieve a vegetative form of the test microorganism, bacterial strains were activated in the appropriate culture medium and incubated for 18-24 hours at 30-35°C. In the case of microorganisms which were used in a sporulate form (fungus), the strain was activated in the thermostat for 7 days at 20-25°C. The turbidity of actively growing microbial suspension was adjusted to match the turbidity standard of 0.5 McFarland units. This turbidity was equivalent to approximately 10^8 colony-forming units per milliliter (CFU/ml).

Antimicrobial assay

In vitro antimicrobial activities of volatile oils were tested using two methods: the dilution method for determination of minimal inhibitory concentration and the cylinder-plate diffusion method.

Minimal Inhibitory Concentration (MIC) was determined using the method of progressive dilutions in liquid media containing 0.25% to 2% (D1=2%; D2=1%; D3=0,5 %; D4=0,25%) of the compound being tested. Each solution was inoculated with 0.2 mL of one of the microbial strain cultures previously prepared to achieve a concentration of 10^4-10^5 cfu/ml. The inoculated solutions were incubated for 18-24 h at 30-35°C. After incubation, the microbial turbidity was estimated. 30% ethylic alcohol in distilled water was used as control and growth medium alone was used as blank and tested for each microbial strain.

The antimicrobial activities of the extracts were determined by the cylinder-plate diffusion method according to Romanian Pharmacopoeia X/2000 edition (8). Tryptic soy agar was used for the antibacterial activity test and Sabouraud medium was used for the antifungal activity test. Under aseptic conditions in the biosafety chamber, inoculum was added to culture media to achieve 10^6 CFU/ml and 15 ml of each medium was dispensed into pre-sterilized Petri dishes. Four sterile stainless steel cylinders of 8 mm diameter were put in the

solid medium and completely filled with the test solutions (0.2ml/cylinder, duplicate samples). The plates were incubated for 18 h at 35-37°C. The mean value obtained for the four cylinders was used to calculate the zone of growth inhibition of each sample.

Antimicrobial activity depending on the diameter of inhibition was noted as follows:

<10 mm - no antimicrobial activity

10-15 mm – weak antimicrobial activity

16-20 mm - moderate antimicrobial activity

20mm >- certain antimicrobial activity

Results and discussions

Volatile oil yields obtained by steam distillation were: 0,45% for Millefolii flos; 1% for Salviae herba; 2.64% for Carvi fructus; 1,8% for Foeniculi fructus and 0.8% for Thymi herba.

GC analysis

28 compound were identified in the 5 tested volatile oils. Table 1 shows the GC analysis of the volatile oil obtained by steam distillation (sample dilution: 1% in methanol). *Thymus vulgaris* volatile oil analysis was published previously (9)

Table 1. GC analysis of volatile oils

Nr.	Retention time (min)	Compound	Millefolii herba %	Foeniculi fructus %	Carvi fructus %	Salviae herba %	Thymi herba %
1.	9.550	**α-pinene**	-	1.50	0.72	3.82	1.23
2.	9.999	**camphene**	1.20	-	-	3.94	0.63
3.	10.891	**β-pinene**	-	-	14.26	-	0.32
4.	11.668	**unknown**	10.25	-	0.76	0.74	-
5.	12.264	**α-terpinene**	-	-	-	-	0.8
6.	12.570	**p-cymene**	1.11	-	24.24	1.45	30.53
7.	12.721	**limonene**	-	13.10	-	1.29	0.62
8.	12.827	**1,8-cineole**	15.59	-	-	8.55	1.24
9.	13.927	**unknown**	-	-	21.39	-	-
10.	14.278	**sabinene**	5.88	-	-	-	4.24
11.	14.944	**α-thujone**	3.81	2.98	-	-	-
12.	15.675	**linalool**	-	-	-	-	2.73
13.	15.928	**β-thujone**	14.79	-	-	30.88	-
14.	16.457	**unknown**	-	-	-	16.71	-
15.	17.724	**camphor**	9.82	-	-	21.73	0.83
16.	18.338	**i-borneol**	1.09	-	-	-	-
17.	18.792	**borneol**	25.79	-	-	4.13	3.16
18.	19.353	**unknown**	1.53	-	-	-	-
19.	20.053	**α-terpineol**	2.15	-	-	-	1.24
20.	20.610	**anethole**	-	80.89	-	-	-
21.	22.637	**carvone**	-	-	17.37	-	-
22.	23.429	**geraniol**	2.57		-	-	0.64
23.	24.926	**unknown**	-	0.81		-	-

24.	25.114	**unknown**	-	-	1.57	-	-
25.	25.357	**thymol**	1.30	-	-	-	30.86
26.	27.537	**carvacrol**	-	-	17.13	-	3.37
27.	27.934	**geranyl acetate**	-	-	-	-	-
28.	28.737	**caryophyllene**	-	-	-	-	2.48

Specific compounds for each species were detected: anethol (80.89% in Foeniculi fructus), carvone (17.37% in Carvi fructus), β-thujone and camphor (30.88% and respectively 21.73% in Salviae herba), thymol (30.86% in Thymi herba) but also other compounds like 1,8-cineol (15.59% in Millefolii flores and 8.55% in Salviae herba), p-cymene (30.53% in Thymi herba and respectively 24.24% in Carvi fructus), borneol (25.79% in Millefolii flores), etc. The amounts of specific compounds were not always in accordance to European Pharmacopoeia limits for the studied plants, probably due to variables factors like the time and type of harvest and drying conditions.

Sage volatile oil contains higher amount of β-tujone and camphor which are markers for plant maturity, as is presented in the literature. As regards Milleforii flores, previous data concerning the major compounds of Romanian yarrow oil were confirmed.

Antimicrobial assays

Determination of antimicrobial activity for the highest concentration (D1=2% active compound) by diffusion method led to the qualitative evaluation of the activity for each volatile oil. Evaluation of antimicrobial activity by serial dilution method resulted in setting the minimum concentration of active product produced in the plant, but also the type of actions they have.

Control sample tested against *E. coli* was inactive, the growth of colony forming units causing turbulence in the whole volume of liquid at all concentrations of alcohol / distilled water. This indicates that the hydroalcoholic solution, at any concentration, does not influence the inhibitory activity (antimicrobial) that samples of different concentrations might have against this bacterium.

Thymus vulgaris volatile oil exhibits a certain antimicrobial activity on two of the three bacterial strains tested, respectively prior to the test organism *Staphylococcus aureus* and *Pseudomonas aeruginosa* and, but also it is active against *Aspergillus niger* (Table 2). In terms of bacteriostatic action, it is obvious at concentrations of 2% for all three bacteria tested. A weak bactericidal action against *Candida albicans* was found at the lowest concentration of the sample, 0.25%.

Table 2 - Antimicrobial activity of *Thymus vulgaris* volatile oil against various strains

***T. vulgaris* volatile oil (dilutions)**	***E. coli***			***S. aureus***			***P. aeruginosa***			***C. albicans***			***A. niger***		
	Dish Ømm	LM*	SM**	Dish Ømm	LM	SM	Dish Ømm	LM	SM	Dish Ømm	LM	SM	Dish Ømm	LM	SM
D1	<10	+		20	-	-	34	-	+	<10	+		30	-	+
D2	X	+		X	-	-	X	+		X	+		X	+	

D3	X	+	+	X	-	-	X	+		X	+		X	-	+
D4	X	-	+	X	-	+	X	+		X	-	-	X	-	+

*liquid medium
**solid medium

Achillea millefollium volatile oil has a moderate antimicrobial activity against *Staphylococcus aureus*, while on the other bacterial strains shows no antimicrobial activity for any of the tested dilutions (Table 3). 0.25% concentration is the minimum inhibitory concentration against *Escherichia coli* and also at this concentration it has bactericidal action against *Candida albicans*.

Table 3 - Antimicrobial activity of *Achillea millefolium* volatile oil against various strains

***A. millefolium* volatile oil (dilutions)**	***E. coli***			***S. aureus***			***P. aeruginosa***			***C. albicans***			***A. niger***		
	Dish Ømm	LM	**SM**	Dish Ømm	LM	**SM**	Dish Ømm	LM	**SM**	Dish Ømm	LM	**SM**	Dish Ømm	LM	**SM**
D1	<10	+		20	ppt*	-	<10	+		<10	+		<10	+	
D2	X	+		X	+		X	+		X	+		X	+	
D3	X	+		X	-	+	X	+		X	+		X	+	
D4	X	-	+	X	-	+	X	-	+	X	-	-	X	-	+

*precipitate

Salvia officinalis volatile oil shows a moderate antimicrobial action against *Staphylococcus aureus* at the highest dilution D1 (2% sample concentration) which is also the minimum inhibitory concentration (Table 4). A weak growth inhibitory activity on yeast culture was found for D1 dilution; D2 dilution of the sample (1% concentration of volatile oil) was determined as the minimum inhibitory concentration against this strain.

Table 4 - Antimicrobial activity of *Salvia officinalis* volatile oil against various strains

***S. officinalis* volatile oil (dilutions)**	***E. coli***			***S. aureus***			***P. aeruginosa***			***C. albicans***			***A. niger***		
	Dish Ømm	LM	**SM**	Dish Ømm	LM	**SM**	Dish Ømm	LM	**SM**	Dish Ømm	LM	**SM**	Dish Ømm	LM	**SM**
D1	<10	ppt	+	20	-	+	<10	+		13	-	-	<10	+	
D2	X	+		X	+		X	+		X	-	+	X	+	
D3	X	+		X	+		X	+		X	+		X	+	
D4	X	+		X	+		X	+		X	+		X	+	

Foeniculum vulgare volatile oil shows a weak antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* (Table 5). Against *Aspergillus niger*, the sample has no antimicrobial activity. The bacteriostatic activity was observed for *Escherichia coli* at D2 dilution and for *Staphylococcus aureus* at D3 dilution.

Table 5. Antimicrobial activity of *Foeniculum vulgare* volatile oil against various strains

***F. vulgare* volatile oil (dilutions)**	***E. coli***			***S. aureus***			***P. aeruginosa***			***C. albicans***			***A. niger***		
	Dish Ømm	LM	**SM**	Dish Ømm	LM	**SM**	Dish Ømm	LM	**SM**	Dish Ømm	LM	**SM**	Dish Ømm	LM	**SM**
D1	14	ppt*	+	12	-	-	14	ppt		12	-	-	<10	+	
D2	X	-	+	X	-	-	X	-	+	X	-	-	X	+	
D3	X	-		X	-	+	X	-	+	X	-	+	X	+	
D4	X	-		X	+		X	-	+	X	-	+	X	+	

*precipitate

Carum carvi volatile oil shows a weak antimicrobial activity against *Pseudomonas aeruginosa* and *Candida albicans* at D1 dilution (2% sample concentration) (Table 6). 1% concentration of the tested product is the minimum inhibitory against *Escherichia coli* and 0.5% concentration (dilution D3) against *Pseudomonas aeruginosa*. Against *Candida albicans*, caraway volatile oil exhibits antimicrobial activity at any tested dilution.

Table 6. Antimicrobial activity of *Carum carvi* volatile oil against various strains

***C. carvi* volatile oil (dilutions)**	***E. coli***			***S. aureus***			***P. aeruginosa***			***C. albicans***			***A. niger***		
	Dish Ømm	LM	**SM**	Dish Ømm	LM	**SM**	Dish Ømm	LM	**SM**	Dish Ømm	LM	**SM**	Dish Ømm	LM	**SM**
D1	<10	ppt*	+	<10	-	-	13	ppt	+	15	-	-	<10	+	
D2	X	-	+	X	-	-	X	-	+	X	-	-	X	+	
D3	X	+	-	X	-	+	X	-	+	X	-	-	X	+	
D4	X	+	-	X	+	+	X	-	+	X	-	-	X	+	

*precipitate

Conclusions

Volatile oils were obtained by steam distillation from 5 indigenous Romanian species - *Thymus vulgaris* L, *Salvia officinalis* L, *Achillea millefolium* L, *Foeniculum vulgare* L and *Carum carvi* L.

The main compounds of each volatile oil detected by GC were: p-cymene and thymol in Thymi herba, anethole in Foeniculi fructus, p-cymene and carvone in Carvi fructus, borneol, 1,8-cineol and borneol in Millefolii herba, β-tujone and camphor in Salviae herba.

Antimicrobial activities of volatile oils were tested using two methods: the dilution method for determination of minimal inhibitory concentration and the cylinder-plate diffusion method.

Results confirmed that phenolic compounds like thymol and monoterpenic alcohols like linalool (constituents of thyme volatile oil) exhibit a strong antimicrobial effect on Gram-positive and negative bacteria, yeasts and fungi.

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