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Antioxidative Properties of *Thymus vulgaris* Leaves: Comparison of Different Extracts and Essential Oil Chemotypes

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Thyme (*Thymus vulgaris* L., Lamiaceae) is a subshrub from the Lamiaceae family with plants that are rich in essential oils and antioxidative phenolic substances. Twelve accessions originating from southern France and the variety 'Deutscher Winter' were grown in an experimental field in eastern Austria. Leaf samples from these plants as well as from a commercial thyme rich in thymol were analyzed for their essential oil and the antioxidative potential in various extracts. The assays for antioxidative activity were the total phenolics according to the Folin−Ciocalteu method, DPPH decoloration, and Fe^{3+} reduction (FRAP). Both extraction techniques used, in the water bath at 40 °C and in the ultrasonic bath at room temperature, proved to be efficient. The best results were obtained with 60% ethanol as extractant. In the comparison of the different accessions the less active and the most active of these extracts differed by factors of 2.1 and 2.6 in the total phenolics and FRAP assay, respectively, and by factors 1.5−2.0 in the DPPH assay. Rosmarinic acid accounted for 22−55% of the antioxidant activity in the ethanolic extracts. Essential oils with high proportions of the phenolic components thymol and/or carvacrol showed the highest antioxidant activity. Ethanolic extracts from the residues after distillation were considerably lower in antioxidant activity than the respective extracts from the dried leaves. Extracts with CH_2Cl_2 in the ultrasonic bath contained volatiles in proportions close to the essential oil but displayed very low antioxidant activity.

KEYWORDS: *Thymus vulgaris*; antioxidant activity; DPPH; FRAP; total phenolics; rosmarinic acid; variability; essential oil; chemotypes; extraction; distillation residue; GC; GC-MS

INTRODUCTION

Thyme (*Thymus vulgaris* L., Lamiaceae), a small subshrub native to the western Mediterranean region of Europe, with a long tradition of various uses, is a chemically variable species. In phytopharmaceutical preparations thyme acts as an expectorant and spasmolytic agent for the bronchia, and in folk medicine it is part of herbal teas and infusions. Also, the nonmedicinal uses are important: thyme serves as preservative for foods and as an aromatic ingredient for seasoning various dishes (*1*).

Extracts from sage (*Salvia officinalis*) and rosemary (*Rosmarinus officinalis*), rich in phenolic acids such as rosmarinic acid and carnosic acid with strong antioxidant properties, have been proposed to be used as preservation for certain foods and nutraceutical products to avoid synthetic antioxidants (*2*). Various compounds with antioxidant properties have also been described in thyme. They include components of the essential oil, phenolic acids and flavonoids (*3–5*). Caffeic, syringic, and genistic acid were the main phenolic acids, and luteolin was the main flavonoid in *T. vulgaris* of Greek origin (*6*). Those flavonoids possessing *o*-dihydroxy groups displayed antioxidative activity (*5*).

Regarding the essential oils, various chemotypes have been described within *T. vulgaris* (*7–9*) on the basis of the main oil components and their biosynthesis. These compounds include geraniol, linalool, α-terpineol, sabinene hydrate, 1,8-cineol, carvacrol, and thymol. The latter two are phenolic compounds, and essential oils containing them proved to be antioxidatively active (*10*). The antioxidant properties of *Thymus* species has been reported by various authors and often compared to those of other plant materials. For instance, thyme proved to be less antioxidative than sage, rosemary, or oregan (*11*). However, the variability in antioxidant activity between thyme plants of different origins has not yet been studied in detail; there may be provenances possessing higher activities.

The processing of fruits and vegetables and other plants leaves large amounts of plant wastes, which are ordinarily not further used but may contain valuable bioactive compounds such as antioxidants. In the case of thyme the residues after the distillation of the essential oils may be of interest. The presence of antioxidative active compounds in acetone extracts of the plant material remainig after hydrodistillation has been demonstrated in sage, rosemary, and thyme (*12*). Residues after

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distillation may also be of interest in animal feeding. Although such material did not alter dairy yields when supplied as an additive in goat feeds, the presence of antioxidative polyphenols in the cheese prepared from these animals' milk could be demonstrated (*13*). Various solvents as methanol, ethanol, and acetone in different proportions with water have been used to extract antioxidative substances from plant material.

The purposes of this study were to (i) test the extractability of antioxidant compounds from thyme leaves with a practicable and widely harmless solvent and simple extraction techniques, (ii) assay whether the plant residues left after distillation are suitable for further extraction of antioxidants, and (iii) investigate the variability in antioxidant activity in extracts from different accession of thyme belonging to the various essential oil chemotypes that were grown under the same environmental conditions. The findings may support the further exploitation of thyme as a source of natural antioxidants.

MATERIALS AND METHODS

Plant Material. Small field plots of *T. vulgaris* were established from seeds collected from various locations in France and from seeds of the thyme variety 'Deutscher Winter' at the experimental station in Gross-Enzersdorf near Vienna, Austria (*9*). Voucher specimens from the field-grown accessions were deopsited in the Herbarium of the University of Vienna (WU-Generale, http:\\herbarium.univie.ac.at). The 5-year-old plants were harvested at the end of July after blooming and fruit setting. Little branches were cut from 15−20 individuals of each tested accession, dried at ambient air temperature, and separated into stems and leaves. The leaves were used for the analyses.

Also, a commercial sample of dried thyme leaves was anlayzed, obtained from Dr. Junghanns GmbH, Gross-Schiersted, Germany.

Hydrodistillation. Dry whole leaves (10−15 g) were subjected to hydrodistillation for 2 h in a Clevenger-type apparatus containing 200 mL of double-distilled water. The plant material after distillation was dried and used for further extraction as described below. The water remainig in the distillation unit was discarded. The essential oil was stored at −18 °C until GC-MS analysis. Prior to GC and GC-MS anlysis, 6 μL of the oils was diluted with 600 μL of CH_2Cl_2. For the antioxidant test 20−25 μL of the oils was diluted in 10 mL of methanol.

Extraction. *Ethanolic Extracts.* The dried leaves and the remainings from the hydrodistillation were finely powdered and extracted with three different ethanol concentrations (30, 60, and 96%); 0.4 g of plant material was treated with 25 mL of solvent. Two techniques were applied: an extraction in a shaking water bath at 40 °C that lasted 180 min and an extraction at room temperature in an ultrasonic bath for 30 min. The resulting extracts were filtered and stored at −18 °C until analysis.

CH_2Cl_2 Extracts. About 0.5 g of dried whole leaves from each accession was extracted with 20 mL of CH_2Cl_2 for 30 min in an ultrasonic bath at room temperature. The resulting extract was stored at −18 °C until analysis. The CH_2Cl_2 extracts were directly analyzed by GC and GC-MS. For the antioxidant test, portions of the extracts were evaporated to dryness in the nitrogen stream and reconstituted in methanol.

GC. The pattern of volatiles in the CH_2Cl_2 extracts and the distilled oils was recorded using an Agilent Technologies 6890N GC with FID. The separation was done on a DB-5 narrow-bore column 10 m × 0.10 mm with 0.17 μm film thickness. The analytical conditions were as follows: carrier gas, He; initial flow, 0.5 mL/min (42 cm/s); constant pressure, 42.78 psi; injector temperature, 250 °C; split ratio, 100:1; temperature program, 1 min at 60 °C, raised at 6 °C/min to 85 °C, raised at 12 °C/min to 180 °C, then raised at 20 °C/min to 280 °C, and held for 3 min at 280 °C. The detector temperature was set at 280 °C, and the injection volume was 1 μL. The FID was operated at 260 °C with an air flow of 350 mL/min and a hydrogen flow of 35 mL/min. The percentage of the oil and the CH_2Cl_2 extract composition presented in **Table 1** were calculated from the FID response without corrections.

GC-MS. To ensure the identification of the volatile components in the extracts and oils, also a HP 6890 GC was available equipped with a 5972 quadrupole mass selective detector. The separation was done on a 30 m × 0.25 mm column coated with 0.25 μm HP5-MS. The analytical conditions were as follows: carrier gas, He, 1.3 mL/min in the constant flow mode; injector temperature, 250 °C; injection volume, 1 μL; split ratio, 15:1; temperature program, 2 min at 40 °C, raised at 3 °C/min to 180 °C, raised at 10 °C/min to 280 °C; transfer line to MSD, 280 °C; MSD, 170 °C. The ionization energy was 70 eV. The range *m/z* 40−300 was scanned at a rate of 0.52 scans/s.

A mixture of the *n*-alkanes (C_9−C_{30}) was analyzed under the same conditions to calculate the retention indices. The compounds were identified according to their mass spectra and their retention indices (*14, 15*).

Determination of Rosmarinic Acid. Selected ethanolic extracts were filtered. The content of rosmarinic acid was measured using a Waters HPLC system consisting of a 626 pump, a 600S controller, a 717plus autosampler, a column oven operated at 25 °C, and a 996 DAD. The separation was carried out on a Symmetry C18, 5.0 μm particle size, 4.6 × 150 mm column. The mobile phase used was 1% acetic acid/ acetontrile 85:15 (solvent A) and methanol (solvent B). The analysis started with a solvent ratio of A/B of 9:1, and a linear gradient was performed to reach 100% B within 30 min (*16*). The flow rate was 1.5 mL/min and the injection volume, 20 μL. The quantification of rosmarinic acid was done using the external standard method by preparing six calibration standards ranging from 1 to 500 μg/mL and recording the calibration curve at 330 nm. A calibration line with the correlation coefficient $R^2 = 0.99998$ could be established. The intraday and interday presicions were 0.5% RSD (relative standard deviation, $n = 5$) and 2.3% RSD ($n = 4$), respectively.

The following tests for total phenolics and antioxidative substances are based on colorimetric reactions, which were measured with a Hitachi 150-20 double-beam spectrophotometer (Hitachi Ltd., Tokyo, Japan).

Total Phenolics. An estimation of the total phenolics content of the extracts was executed with the Folin−Ciocalteu method. Two hundred microliters of sample and 0.5 mL of Folin−Ciocalteu reagents (Merck) were added to 10 mL of distilled water in a 25 mL flask. After 3 min, 1 mL of saturated Na_2CO_3 was added, and the volume was made up to 25 mL. The samples were left for 1 h in the dark, and then the absorbance was measured at 725 nm against a blank. A calibration curve with caffeic acid was established, where 25−200 μg of caffeic acid in 100 μL of distilled was used instead of the sample, and the total phenolic content was expressed in milligrams of caffeic acid per gram of dried plant material. Each analysis was carried out at least twice, so that coefficients of variation of <3.5% could be achieved.

Antioxidant Activity. *DPPH Radical Scavenging Activity.* The radical scavenging activity was measured using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH; Fluka) and Trolox (2.5 mM in methanol) as reference substance. The presence of antioxidative active substances in the assay leads to the reductive decoloration of the DPPH radical. Depending on the content of antioxidative active substances, 50−400 μL of the ethanolic or methanolic sample was adjusted to 1 mL with 50% methanol and then added to 1 mL of DPPH reagent (7.5 mg in 50 mL of methanol). After 0.5 h in the dark at room temperature, the absorbance was measured against a blank at 515 nm. The blank was a solution where 500 μL of Trolox and 500 μL of methanol reacted with 1 mL of DPPH reagent to obtain the complete decoloration of that radical. For the calibration curve 6−50 μg of Trolox in 1 mL of methanol was used. In the case of the CH_2Cl_2 extracts, 0.5 mL was reduced to dryness in the nitrogen stream and taken up in 1.5 mL of methanol. Each analysis was carried out at least twice, so that coefficients of variation of <3.5% could be achieved. The comparison of the DPPH decoloring ability of four different Trolox and rosmarinic acid concentrations gave for rosmariníc acid a 1.62 times higher activity than for Trolox. With this ratio the contribution of rosmarinic acid to the total DPPH reducing activity in the metanolic extracts can be calculated (**Table 5**).

Ferric Reducing Antioxidant Power (FRAP). This test system is based on the ability of antioxidants in the sample to reduce Fe^{3+} ions. It measures the appearance of Fe^{2+} ions building a blue complex with

Table 1. Composition of the Essential Oil (EO) and Dichloromethane (DCM) Extracts As Calculated from the FID Response

		Accessions													
		17		9A		27		34		9		1		20	
	RI[a]	EO	DCM	EO	DCM	EO	DCM	EO	DCM	EO	DCM	EO	DCM	EO	DCM
oil yield (% v/w)		3.4		2.5		4.4		3.1		1.9		1.2		3.8	
α-thujene	926	0.7	0.6	1.0	0.6	1.4		1.1	0.3	0.9	0.4	1.0		0.5	
α-pinene	933	0.5	0.4	1.1	0.5	0.9	0.4	1.3	0.3	1.0	0.3	1.0		0.8	
camphene	948	0.4	0.3	0.9		0.8	0.8	1.2	0.3	0.6	0.3	0.7		0.6	
sabinene	972		0.4	0.2		0.3		0.5	0.2	0.4	0.3	0.2			
β-pinene	975	1.3	0.7	1.8	0.7	0.9	0.4	0.7	0.4	0.5	0.3	0.9		1.2	0.7
1-octen-3-ol	987			2.2						0.5	0.2				
myrcene	987	1.7	0.8	0.2	1.2	1.9	1.3	2.0	1.4	1.3	0.4	1.2		2.2	1.2
α-terpinene	1013	1.5	5.2	1.7	0.5	1.8	2.3	1.4	3.2	1.1	2.0	0.7		1.8	6.2
p-cymene	1022	13.3	6.9	12.4	8.6	10.7	7.0	14.6	11.9	15.7	10.2	27.7	34.2	19.0	10.5
limonene	1027	2.6	1.7				0.3	1.4	1.2	2.9		0.7			
1, 8-cineol	1028			2.9	0.2	1.4	1.0	2.8	2.6	4.8	6.3	1.5		1.7	0.4
γ-terpinene	1058	16.5	17.0	18.1	17.2	18.7	14.1	13.1	13.3	11.4	12.0	6.4	3.7	19.6	18.3
cis-sabinenehydrate	1066	1.6		1.3		1.1	0.7	4.4	5.0	3.9	3.7	0.7		2.1	1.1
cis-linalool oxide	1070														
trans-linalool oxide	1083														
terpinolene	1085										0.4				
trans-sabinenehydrate	1092		2.8	0.2	0.6			0.5		0.8		0.2			
linalool	1093	5.5	2.6	1.5	1.5	5.9	2.9	18.8	13.9	21.9	23.1	17.4	9.3	1.8	0.8
octenol acetate	1102														
camphor	1137	0.6	0.6	0.2	0.2	0.4	0.4	0.6	0.5	0.6	0.5	0.5		0.9	
borneol	1162	1.0	0.9	1.6	1.4	1.3	1.1	1.9	1.4	0.8	0.4	0.9	1.1	1.2	
terpinen-4-ol	1173	0.3	0.5	0.3	0.7	0.3	0.5	1.1	0.4	0.8	0.9	0.5		0.4	
α-terpineol	1185	0.2	0.2	0.3				0.7	0.7	0.8	1.1	0.2	0.7		
nerol	1220							0.4	0.3						
thymol-methyl ether	1237											0.7	0.8		
neral	1232							0.2	0.2						
carvacrol-methyl ether	1248											0.7	0.6		
linalyl acetate	1248														
geraniol	1248	1.7	1.5					2.0	3.0						
geranial	1262							0.3	0.3						
bornyl acetate	1278			0.3				0.3	0.2	0.2	0.2				
thymol	1289	9.1	9.6	12.9	13.9	23.1	32.2	20.4	19.5	9.0	9.8	26.3	30.3	36.6	34.2
carvacrol	1299	37.6	35.2	35.4	39.2	26.2	16.7	5.7	7.6	16.5	15.4	2.6	3.7	5.9	6.6
α-terpinyl acetate	1343														
neryl acetate	1354														
geranyl acetate	1372	0.6	3.2						1.9						
β-caryophyllene	1418	1.1	2.0	1.0	2.3	1.5	2.4	0.8	1.8	0.7	2.1	1.4	2.2	1.5	2.3
germacrene D	1480			0.2		0.3		0.2	0.5		0.3	0.1		0.3	
% identified		98.1	93.2	97.7	89.4	98.8	84.5	98.1	92.2	97.3	90.7	94.3	86.7	98.3	82.4

		Accessions													
		48		2		26		28		51		13		Com[b]	
	RI	EO	DCM	EO	DCM	EO	DCM	EO	DCM	EO	DCM	EO	DCM	EO	DCM
oil yield % (v/w)		1.8		3.0		2.0		1.3		2.5		3.2		1.4	
α-thujene	926	0.4		0.9	0.2	0.7	0.5	0.6	0.4	0.4		0.4			
α-pinene	933	1.0	0.5	1.1	0.5	1.4		0.8	0.4	0.5		0.7	0.4	0.6	
camphene	948	0.6		0.3	0.2	0.7		1.2	0.4	0.2		0.7	0.4	0.2	
sabinene	972	1.3	0.5	1.6	0.9	2.6	1.4	0.2		1.0	0.6	0.3		0.2	
β-pinene	975	0.4		0.4	2.6	0.7		1.3	0.4	0.2	0.5	0.5	0.2	0.5	0.4
1-octen-3-ol	987			0.5				0.2		0.5	0.3	0.7	0.2		
myrcene	987	2.3	0.5	9.4	5.7	4.7	1.1	1.3	0.4	1.1	0.2	0.7		0.7	
α-terpinene	1013	1.3		1.1	1.5	1.5		0.7	1.6	0.4	1.0	0.3		1.1	
p-cymene	1022	1.8		5.6	3.3	6.1	2.2	9.9	4.0	5.5	2.7	3.5	1.0	13.1	11.8
limonene	1027	2.1		1.8	2.4	1.3	1.7	1.6		1.8	0.6			0.4	
1, 8-cineol	1028			1.0		0.5	0.5	1.1		1.3	0.5	3.7	2.4	0.6	
γ-terpinene	1058	2.6	7.4	7.3	5.1	5.3	1.2	4.9	2.3	3.1	1.4	3.8	1.8	5.6	5.0
cis-sabinene hydrate	1066	15.5	17.9	18.5	21.7	33.4	42.7	0.8		0.4		1.4	1.3	0.5	0.4
cis-linalool oxide	1070											0.4	1.0		
trans-linalool oxide	1083											0.4	1.1		
terpinolene	1085	2.9	4.7			0.6		1.0							
trans-sabinene hydrate	1092	0.8		3.1		3.3		1.5	3.9		0.2				
linalool	1093	0.2		0.9	4.1	20.1	24.4	0.3		1.8	0.5	67.2	59.9	2.2	1.9
octenol acetate	1102					0.6				0.7	0.6				
camphor	1137	0.6		0.3	0.2	0.6		1.2	1.6			0.4	0.4	0.3	
borneol	1162	0.7	1.0	0.3	0.4	3.6	0.4	1.4	1.6	0.4	0.6	1.1	1.3		
terpinen-4-ol	1173	4.7	0.8	1.6	0.3		1.3	0.8		0.3		0.3		0.7	
α-terpineol	1185	11.5	14.7	9.9	10.4	1.5	2.3	0.7	1.6	10.1	11.7	0.7	2.3	0.2	
nerol	1220	4.4	5.1					2.3	2.7	0.5	0.9				
thymol-methyl ether	1237														
neral	1232	1.1	1.2					2.5	2.6	0.5	0.7				
carvacrol-methyl ether	1248														
linalyl acetate	1248											3.4	5.5		
geraniol	1248	12.4	13.4					32.0	34.4	6.1	10.1				
geranial	1262	1.5	1.3					3.3	3.8	0.7	1.0				
bornyl acetate	1278	0.3						0.4							
thymol	1289	0.8	0.7	14.5	14.5	5.4	4.7	9.9	6.8	2.9	1.6	3.2	3.3	66.5	60.9
carvacrol	1299	0.2	0.6	0.9	1.5	0.5	0.4	0.9	1.1	20.7	11.6	3.3	2.4	4.2	4.4
α-terpinyl acetate	1343	8.9	9.9	15.0	14.7			0.7		33.4	39.6				
neryl acetate	1354	0.6													
geranyl acetate	1372	11.5	9.5					11.7	12.8	2.2	2.0				
β-caryophyllene	1418	1.4	2.1	2.0	2.3	1.1	1.1	0.9	2.3	1.1	1.9	1.2	2.3	1.2	1.9
germacrene D	1480			0.4	0.6	0.4	0.7		0.6			0.2	0.4		
% identified		93.8	91.7	98.6	93.2	96.4	86.6	96.1	85.6	98.0	90.9	98.6	87.6	98.8	86.6

[a] RI, retention index. [b] Com, commercial sample.

Table 2. Total Phenolics in the Various Extracts (Milligrams of Caffeic Acid per Gram of Plant Dry Matter)

		30% ethanol				60% ethanol				96% ethanol			
		WB[a]		USB[b]		WB		USB		WB		USB	
accession	DCM[c]	leaves	waste	leaves	waste	leaves	waste	leaves	waste	leaves	waste	leaves	waste
17	4.18	39.7	15.1	37.9	13.5	71.5	18.3	65.3	17.5	32.0	10.4	35.4	8.4
9A	5.47	50.0	14.3	39.4	12.0	81.4	16.9	69.8	15.8	29.3		32.1	5.4
27	4.17	41.2	15.4	41.8	12.8	66.6	18.5	71.0	17.6	32.4	10.3	32.3	11.7
34	3.32	43.4	17.1	40.4	13.4	50.7	19.0	51.0	18.5	25.3	7.1	nd[d]	6.4
9	2.69	44.1	17.0	41.1	12.9	48.6	18.7	45.6	16.4	22.2	6.3	nd	4.2
1	2.85	41.9	23.0	39.9	14.1	51.0	20.3	52.5	18.8	24.2	8.0	nd	5.8
20	1.80	50.4	15.7	45.5	13.4	67.1	20.5	64.6	19.9	32.2	11.8	33.2	10.6
48	1.28	31.6	19.5	31.1	16.5	36.8	19.8	37.3	17.3	16.4	4.5	16.9	2.9
2	1.74	33.9	10.9	37.1	7.4	42.8	11.6	41.0	11.0	20.2	4.9	nd	3.3
26	1.42	35.6	14.7	31.9	10.7	39.7	15.9	39.1	15.1	15.7	6.3	15.3	3.7
28	0.77	31.5	16.2	27.7	12.2	34.8	16.4	33.9	14.5	15.0	4.7	nd	3.3
51	0.97	30.4	16.3	24.6	12.7	31.2	17.1	32.2	15.1	15.8	5.2	16.5	3.5
13	1.79	48.2	20.5	45.3	16.6	55.5	21.4	54.5	21.1	22.1	7.7	nd	5.1
Com	3.42	41.7	31.3	35.7	30.8	46.4	35.0	46.5	34.5	20.7	9.6	20.4	5.4
mean	2.56	40.3	17.6	37.1	14.2	51.7	19.2	50.3	18.1	23.1	7.5	25.3	5.7
s	1.4	6.8	4.9	6.3	5.3	15.0	5.2	13.2	5.3	6.4	2.4	8.7	2.8

[a] WB, water bath. [b] USB, ultrasonic bath. [c] DCM, dichloromethane extract. [d] nd, not determined.

2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ) when antioxidants reduce Fe^{3+} (*17*). A working solution was prepared by adding 25 mL of 0.3 M acetate buffer pH 3.6 to 2.5 mL of 10 mM TPTZ in 40 mM HCl and 2.5 mL of freshly prepared 20 mM $FeCl_3 \cdot 6H_2O$. For the assay 1800 μL of working reagent was mixed with 60 μL of sample or calibration standard and 180 μL of distilled water. After a resting period of 4 min, the absorbance was measured at 593 nm. A calibration curve was constructed using calibration standards of Trolox from 50 to 2500 μM (corresponding to 0.75−37.6 μg in 60 μL) in ethanol. Each analysis was carried out at least twice, so that coefficients of variation of <3.5% could be achieved. A calibration curve made with rosmarinic acid gave a 1.38 times higher activity of rosmarinic acid in comparison to Trolox.

In the case of the CH_2Cl_2 extracts 0.5 mL was reduced to dryness in the nitrogen stream and taken up in 0.5 mL of methanol. Sixty microliters of this reconstitued solution was taken for the assay.

Statisitcal Analysis. The statistical analyses were done with the package SPSS for Windows, version 14.0. A hierarchical cluster analysis using the Euclidian distance was carried out to group the thyme accessions according to the essential oil composition. The main occurring components *p*-cymene, γ-terpinene, *cis*-sabinene hydrate, linalool, α-terpineol, geraniol, thymol, carvacrol, geranyl acetate, and α-terpinyl acetate have been taken into consideration. An anlysis of variance has been performed to assert the differences in total phenolics and antioxidant activity between extraction techniques, plant material, and accessions. The Pearson correlation coefficients were calculated to compare total phenolics and both antioxidant tests.

RESULTS AND DISCUSSION

Volatiles in Essential Oils and CH_2Cl_2 Extracts. The yield and composition of the essential oils of the various accessions are presented in **Table 1**, where the accessions are listed in the rows according to oil composition. The oil yields ranged between 1.2 and 4.4% (v/w) of the air-dried leaves; the highest yield was obtained from accession 27. The European Pharmacopeia (*18*) requires a minimum essential oil content of 1.2% (v/w) for Herba Thymi. A hierarchical cluster analysis was performed to group the various accessions according to their essential oil composition. The resulting dendrogram is displayed in **Figure 1**, and the same order of the accessions has been taken for **Tables 1–4**.

At the bottom of the dendrogram are the accessions with one main component in the essential oil, 61% thymol in the commercial sample and 67% linalool in accession 13. In the discussion of the present chemotypes one has to take into account the biosynthesis of the main oil components. *p*-Cymene and γ-terpinene are precursors of the phenolics thymol and carvacrol (*19*) and occur in variable proportions in these two chemotypes (accessions 1, 9, 17, 27, and 34). Geranyl acetate is derived from geraniol, as in accession 28, and α-terpinyl acetate from α-terpineol, as in accession 51. Accession 26 was dominated by *cis*-sabinene hydrate. Furthermore, as the field plots originated from seeds collected in the wild, more than one chemotype may be present in the same accession. Besides thymol, accessions 1 and 34 displayed also an appreciable portion of linalool. Accessions 48 and 2 were heterogeneous; they contained geraniol/geranyl acetate, α-terpineol/α-terpinyl acetate, and sabinene hydrate or α-terpineol/α-terpinyl acetate, sabinene hydrate, and thymol as main compounds, respectively.

The composition of the volatiles in the CH_2Cl_2 extracts of the dried leaves, as presented in **Table 1**, showed a pattern very similar to that of the essential oils. All of the main monoterpene components were also present in these extracts, indicating that this solvent is well suited to extract the volatiles stored in the plants. Similarly, the plant volatiles extracted by CH_2Cl_2 from rosemary were close to those found in distilled essential oils (*20*).

Total Phenolics and Antioxidant Activity of the Extracts. Acetone or methanol may also be good solvents for the extraction of antioxidants from plants but may lead to unacceptable levels of residues in the resulting extracts. Therefore, ethanol was chosen as extractant in this study.

To characterize the antioxidative potential of the thyme extracts, the total phenolics (TP), DPPH radical scavenging activity, and Fe^{3+} reducing antiradical power (FRAP) of the various extracts was measured, and the results are presented in **Tables 2–4**.

In both antioxidant tests the CH_2Cl_2 extracts showed the lowest activity, suggesting that this solvent is not appropriate to extract the mainly polar antioxidant penolic compounds.

In all cases the extracts from the distillation residues (wastes) exhibited a considerably lower activity than the respective extracts from the ground leaves. During distillation the water-soluble phenolic antioxidants were removed with the wastewater. Also, the concentration of ethanol played a major role: the 60% ethanolic extracts yielded the highest total phenolis contents and highest antioxidant activities and the 96% extracts the lowest. Optimal conditions for the extraction of phenolics from dried sage were achieved with 55−75% ethanol, where total phenolics

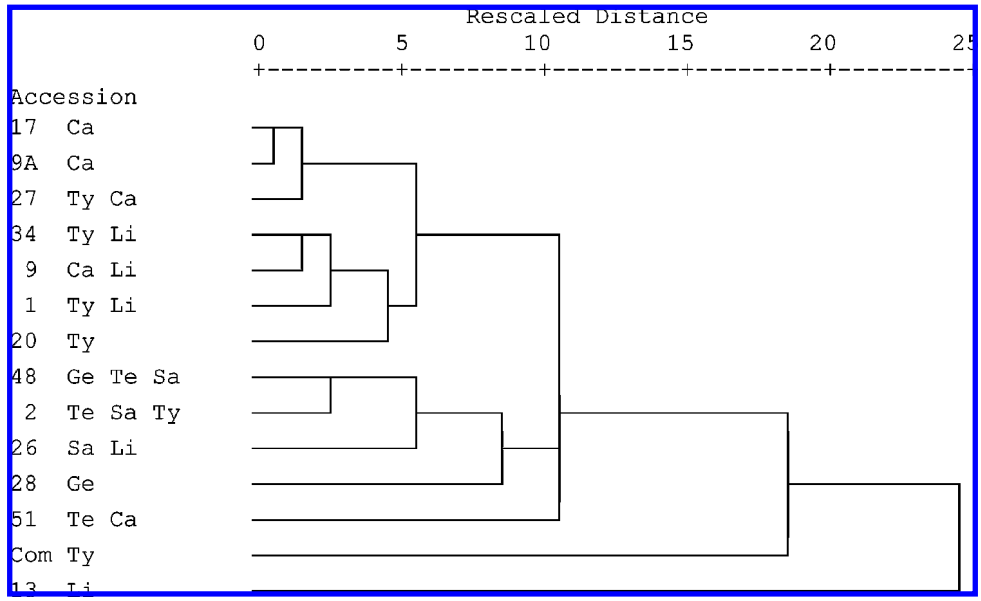

Figure 1. Dendrogram showing the similarities between the accessions according to the essential oil composition. Assigned chemotypes: Ca, carvacrol; Ge, geraniol; Li, linalool; Sa, sabinene hydrate; Te, α-terpineol; Ty, thymol; Com, commercial sample.

Table 3. Antioxidant aActivity in the DPPH Assay (Milligrams of Trolox per Gram of Plant Dry Matter)

			30% ethanol				60% ethanol				96% ethanol			
			WB[a]		USB[b]		WB		USB		WB		USB	
accession	EO[c]	DCM[d]	leaves	waste	leaves	waste	leaves	waste	leaves	waste	leaves	waste	leaves	waste
17	47.2	2.94	42.3	18.7	34.3	16.3	55.8	21.3	53.3	21.8	31.9	9.4	35.5	7.9
9A	48.0	0.46	58.5	16.6	50.7	14.9	88.4	20.9	53.6	20.4	29.9	19.3	32.8	7.1
27	34.0	3.52	51.3	16.8	46.9	16.5	53.9	21.6	51.8	21.4	29.5	8.2	28.7	12.9
34	38.7	3.08	43.5	22.8	55.5	17.1	54.1	25.7	72.5	22.9	30.7	10.0	nd[e]	6.3
9	27.7	1.77	58.4	24.9	56.5	18.5	54.4	27.9	66.5	21.6	28.2	7.0	nd	4.5
1	15.5	2.75	58.9	27.0	56.8	20.0	54.5	28.1	64.4	26.2	34.3	9.5	nd	7.4
20	45.0	3.78	51.9	18.1	52.1	18.3	52.5	24.5	53.3	25.1	31.2	13.7	55.6	10.6
48	6.3	0.35	53.4	31.0	44.9	22.4	53.8	30.8	57.2	24.0	21.3	6.6	23.5	4.3
2	11.2	1.01	52.8	13.6	50.6	9.5	54.5	14.9	70.0	14.5	24.8	5.1	nd	4.0
26	8.0	0.60	50.5	20.1	46.1	14.2	54.3	22.1	62.8	19.2	20.8	6.5	21.0	3.7
28	22.3	0.94	52.0	22.8	41.1	16.2	52.8	24.1	51.8	18.9	19.8	5.2	nd	3.8
51	31.7	0.92	40.9	25.5	36.4	17.4	44.6	26.9	47.8	21.7	17.9	7.2	20.9	4.7
13	12.7	0.81	58.4	28.9	56.8	23.5	54.6	29.6	66.5	30.0	28.7	8.1	nd	4.4
Com	68.1	3.18	58.2	44.4	50.3	46.9	54.9	47.7	65.6	47.7	22.0	11.9	22.4	6.0
mean		1.87	52.2	23.7	48.5	19.4	55.9	26.1	59.8	23.9	26.5	9.1	30.0	6.3
s		1.27	6.2	7.8	7.4	8.6	9.7	7.5	7.9	7.7	5.2	3.8	11.7	2.7

[a] WB, water bath. [b] USB, ultrasonic bath. [c] EO, essential oil. [d] DCM, dichloromethane extract. [e] nd, not determined.

and rosmarinic acids gave higher recoveries with lower ethanol proportion, whereas the lipophilic carnosic type antioxidants were better extracted with higher proportions of ethanol (*2*).

Total phenolics, as presented in **Table 2**, expressed as milligrams per gram of caffeic acid, showed significant differences between the accessions. The highest levels were in the 60% ethanol extract of accession 9A, which is rich in carvacrol (**Table 1**) and also in rosmarinic acid (**Table 5**). The lowest total phenolics values were recorded in the geraniol chemotype (accession 28), which was also low in rosmarinic acid (**Table 5**).

The 60% ethanolic water bath extracts from the leaves gave in the DPPH test for most samples very similar values of approximately 52−55 mg/g, so that no significant differences between the accessions could be detected. The lowest activity was recorded in accession 51, which contains α-terpinyl acetate and carvacrol in the essential oil. The FRAP values of the 60% ethanolic extract from the leaves, however, showed significant differences between the accessions, where the highest value was in accession 9A, a carvacrol/thymol chemotype, and the lowest again in accession 51.

Altogether, the less active and the most active 60% ethanolic extracts of the leaves differed by a factor of 2.1−2.6 in the total phenolics and FRAP assay, respectively, and by a factor of 1.5−2.0 in the DPPH assay. Among 12 tomato varieties an almost 4-fold variation in FRAP antioxidant activity could be recorded (*21*). A 3−4-fold variation of total phenolics in methanolic extracts was measured in six *Portulaca oleracea* L. (Portulacaceae) cultivars (*22*).

There were some small differences between both extraction techniques. The relative differences between both techniques are presented in **Figure 2** by means of box plots for the three different assays and the three ethanol concentrations for extraction. The *Y*-axis represents the relative differences in antioxidant activity calculated as (activity of water bath extract − activity of sonication extract)/activity of water bath extract × 100. It can be seen that for the wastes the activity was higher in the water bath extracts than in the sonicated extracts. From the

Table 4. Antioxidant Activity in the Fe^{3+} Reduction Assay (Milligrams of Trolox per Gram of Plant Dry Matter)[a]

			30% ethanol				60% ethanol				60% ethanol			
			WB		USB		WB		USB		WB		USB	
accession	EO	DCM	leaves	waste	leaves	waste	leaves	waste	leaves	waste	leaves	waste	leaves	waste
17	27.3	2.32	27.5	23.0	23.7	14.1	91.3	18.4	85.64	18.2	35.1	10.5	37.01	7.1
9A	15.8	1.68	40.7	15.8	26.0	13.0	93.8	16.3	94.89	17.1	31.5		36.80	5.1
27	30.3	3.18	28.2	14.4	29.0	13.2	84.5	18.6	88.01	17.5	32.3	10.5	27.82	10.9
34	22.0	2.61	43.0	18.7	35.2	15.3	61.7	20.7	68.92	19.3	28.5	7.6	nd	5.4
9	20.4	1.82	59.5	19.5	41.6	15.8	63.2	20.5	65.41	17.9	27.5	6.3	nd	4.2
1	22.7	2.93	74.0	20.2	40.2	17.0	62.9	21.8	80.70	22.0	33.5	9.2	nd	6.2
20	32.4	3.15	51.1	15.8	36.6	14.7	84.1	22.2	70.88	21.0	34.3	13.6	38.90	10.6
48	1.1	0.25	39.4	24.9	31.8	21.7	45.7	23.6	53.61	21.2	19.3	5.1	22.7	2.6
2	11.1	1.01	50.5	10.4	28.2	8.5	57.1	12.1	46.48	11.3	25.0	4.5	nd	3.0
26	5.8	0.46	50.1	15.9	30.0	13.0	51.3	15.6	62.59	16.0	18.9	5.3	18.6	3.0
28	8.7	0.70	36.3	18.0	28.9	14.3	41.9	17.0	47.43	15.9	17.0	4.2	nd	2.8
51	13.1	0.73	30.2	26.4	24.8	15.0	35.7	42.9	39.70	18.0	14.5	5.1	18.6	2.8
13	4.2	0.70	70.2	24.2	42.9	18.0	83.6	23.8	80.74	24.9	30.6	7.3	nd	3.6
Com	39.9	2.95	49.0	43.0	42.2	40.4	56.3	19.4	69.70	45.5	20.8	12.0	22.28	6.2
mean		1.99	46.4	20.7	32.9	16.7	65.2	20.9	68.2	20.4	26.3	7.8	27.8	5.2
s		1.21	14.4	7.8	6.8	7.4	19.1	7.1	16.9	7.9	7.0	3.1	8.6	2.8

[a] See **Table 3** footnotes.

Table 5. Rosmarinic Acid in 60% Ethanolic Water Bath Extracts of the Leaves and Wastes of the Various Thyme Accessions (Milligrams per Gram of Plant Dry Matter)

	leaves			wastes		
accession	mg/g	% of DPPH activity	% FRAP activity	mg/g	% of DPPH activity	% FRAP activity
Com	10.1	29.6	24.6	5.8	19.8	41.6
1	16.1	47.8	35.3	2.4	13.6	15.0
20	15.6	48.2	25.6	2.2	14.3	13.5
34	10.0	29.9	22.3	2.5	15.4	16.3
27	17.3	52.0	28.2	2.5	18.8	18.5
17	19.1	55.4	28.8	2.1	15.6	15.3
9A	20.9	38.2	30.6	2.1	16.1	17.5
9	15.7	46.6	34.2	3.4	19.9	23.1
13	18.9	55.9	31.1	4.2	23.0	24.3
28	8.8	26.8	28.8	2.2	14.7	17.7
51	6.3	22.7	24.2	1.7	10.0	5.3
48	13.6	40.8	41.0	3.5	18.6	20.6
26	11.5	34.1	30.7	2.1	15.7	18.9
2	11.6	34.3	27.9	1.0	11.2	11.8

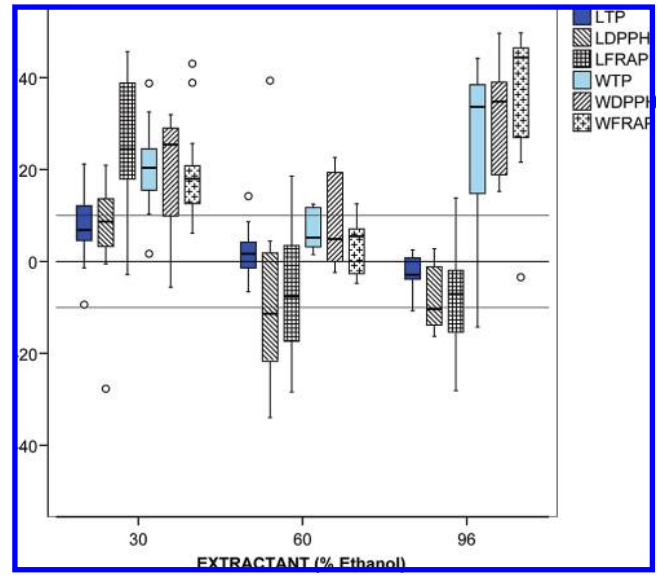

Figure 2. Comparison of water bath extracts and sonocation extracts. Relative differences in antioxidant activity with different test systems and extractants calculated as % = (activity of water bath extract − activity of sonication extract)/activity of water bath extract × 100. LTP, leaves total phenolics; LDPPH, leaves DPPH activity; LFRAP, leaves FRAP; WTP, wastes total phenolics; WDPPH, wastes DPPH activity; WFRAP, wastes FRAP.

whole leaves, however, in most cases more total phenolics and DPPH and FRAP reactive substances were extracted with 60 and 96% ethanol than in the hot water bath. As for most samples the differences did not exceed 10%, and it can be concluded that both extraction techniques with 60% ethanol were suitable to extract the main antioxidant active compounds. The extraction of total phenolics and chlorogenic acid from eggplant (*Solanum melongena* L. Solanaceae) in a shaking water bath gave slightly higher values than the extraction by sonication (*23*).

Rosmarinic Acid. Rosmarinic acid was measured in the 60% ethanolic water bath extracts (**Table 5**) of the leaves and the residues after distillation. In the leaves the contents varied from 6.3 to 20.9 mg/g and in the residues, from 1.0 to 5.8 mg/g. The highest amount in the residues was found in the commercial sample and in accession 13, a linalool chemotype. The lower content in the residues indicates that during distillation a considerable portion of rosmarinic acid was removed by the wastewater as rosmarinic acid was also the main phenolic compound in water extracts from thyme analyzed by Dorman et al. (*11*).

The antioxidative activity of rosmarinic acid has also been determined, and the contribution of rosmarinic acid in the present ethanolic extracts to the DPPH activity and FRAP could be calculated. In the leaves this contribution to the DPPH activity ranged from 22 to 55%, and in the wastes it ranged from 10 to 23%. The contribution to FRAP was similar, with 22−41% in the leaves and 5−24 (41) % in the wastes. Flavonoids, rosmarinic acid derivatives, and *p*-cymene-2,3-diol as described by Dapkevicius et al. (*12*) may account for the remaining antioxidant activity.

Table 6 presents the rosmarinic acid content in the various ethanolic water bath extracts from three selected accessions and the commercial sample. The highest contents were extracted with 60% ethanol from the leaves. In the wastes from the field-grown samples remained less rosmarinic acid than in those from the commercial sample. Leaves extracted with 30 or 96% ethanol gave comparable yields of rosmarinic acid, whereas with

Table 6. Rosmarinic Acid (Milligrams per Gram of Plant Dry Matter) in the 30, 60, and 96% Ethanolic Extracts of Four Selected Accessions

		extractant ethanol		
	accession	30%	60%	96%
leaves	Com[a]	6.9	10.1	3.9
	1	8.2	16.1	8.0
	26	3.7	11.5	4.8
	2	4.1	11.6	5.7
waste	Com	5.7	5.8	1.3
	1	2.2	2.4	0.4
	26	1.9	2.1	0.5
	2	1.0	1.0	0.2

[a] Com, commercial sample.

96% ethanol only small amounts of this acid could be recovered from the wastes.

Thyme (*T. vulgaris* L.) and wild thyme (*T. serpyllum* L.) infusions, which are hot water extracts, showed antioxidant properties but were less active than oregano [*Origanum vulgare* L. ssp. *hirtum* (Link) Ietsw.] infusions. Phenolic substances such as rosmarinic acid and flavonoids were present in these infusions (*24*).

The deodorized water extract of various aromatic plants, which is the water fraction in a hydrodistillation, exhibited no or only a weak antioxidant activity in the carotene bleaching test (*25*). The essential oil and an acetone extract of the remaining plant material after hydrodistillation of thyme showed moderate antioxidant activity in the carotene bleaching test (*26*).

In ground sage leaves rosmarinic acid was better extracted with ethanol concentrations below 70% (*2*). Deodorized extracts of *Thymus pulegioides* had considerably lower rosmarinic acid contents than nondeodorized extracts (*26*).

Antioxidative Activity of the Essential Oils. The antioxidant activity in the distilled oils as measured by the DPPH and FRAP tests can be seen in **Tables 3** and **4**, respectively. The highest activities in both tests were found in the oils from the commercial sample, which is rich in thymol and the phenolic chemotypes (accessions 20, 34, 17, and 9A). The lowest activity was found in the oil from accession 48, containing mainly α-terpineol, geraniol, and sabinene hydrate.

Ruberto and Barratta (*10*), who tested the antioxidant activity of about 100 pure components of essential oils, pointed out that the phenolic compounds such as thymol and carvacrol showed the highest activity.

Kulisic et al. (*27*) fractionated the essential oils of *T. vulgaris* and *T. serpyllum* into a thymol- and carvacrol-rich fraction and a hydrocarbon fraction with γ-terpinene, *p*-cymene, and caryophyllene as main compounds. Antioxidant activity, as tested with three different methods, was mainly found in the first, phenolics-containing fraction. In the same way, the essential oil of a phenolic chemotype of *T. vulgaris* had a stronger antioxidant potential than a nonphenolic chemotype. Oxidation products of thymol such as thymoquinone and thymohydroquinone are a more potent antioxidant than thymol.

Oxygenated phenolic compounds of the essential oil of *T. vulgaris* were the most effective compounds in neutralizing DPPH (*28*). In several test systems carvacrol had a higher antioxidant activity than thymol (*10, 28*).

As reported by many authors, the phenolic monterpenes thymol and carvacrol are strong antioxidants. Depending upon the test system used, other oil components may also present some antioxidant activity as in TBARS assay for antioxidant activity also γ-terpinene showed a high activity, whereas α-terpineol and geraniol gave only little activity. In the same system linalool exhibited prooxidant activity; linalyl acetate, however, exhibited low antioxidant activity (*10*). Of several tested pure oil components γ-terpinene, terpinolene, and geraniol showed considerable DPPH reducing activity, whereas geranyl acetate did not (*29*). The essential oil of *Thymus caespitosus*, dominated by α-terpineol, demonstrated a considerable antioxidant capacity in contrast to the oils from *Thymus mastichina* and *Thymus camphoratus*, all species from the Iberian peninsula, where 1,8-cineol and linalool, linalyl acetate, and 1,8-cineol chemotypes prevailed (*30*).

Table 7. Pearson Correlation Coefficients between the Individual Antioxidative Tests for the Different Extraction Methods

		extractant[a]			
correlated test system	essential oils	DCM[b]	30% ethanol	60% ethanol	96% ethanol
N	14	14	56	56	49
DPPH − TotPhen		0.398	0.949**	0.857**	0.946**
DPPH − Fe^{3+}Red	0.825**	0.673**	0.843**	0.862**	0.971**
Fe^{3+}Red − TotPhen		0.440	0.797**	0.967**	0.980**

[a] *, $P < 0.5$; **, $P < 0.01$. [b] DCM, dichloromethane.

Correlation between Antioxidant Test Systems. As oxidative processes in biological matrices are complex, involving many different substances and reactions, it is often stated that one test system alone cannot be representative for an overall antioxidant capacity. Various plant-derived antioxidants may therefore be differently effective in the various test systems. Nevertheless, it is of interest to compare the antioxidative activities measured with different tests.

The Pearson correlation coefficients between the tests for antioxidants and total phenolics were calculated and are presented in **Table 7**. In all ethanolic extracts and in the essential oils good correlations between DPPH, FRAP, and total phenolics could be obtained. The close correlation between total phenolics and antioxidant activity indicates that mainly phenolic antioxidants are present in the respective extracts. Also, the DPPH activity and FRAP of the distilled oils were well correlated. This might be due to the fact that only extracts from thyme leaves were compared, where similar constituents are present. When extracts from quite different plant sources were examined, the different antioxidant tests do usually not give such close correlations. However, also in the comparison of 11 Algerian medicinal plants from 8 different plant families the content of total phenolics was well correlated with the Trolox equivalent antioxidant capacity measured by the decoloration of ABTS (*31*). Also, a linear relationship was established between total phenolics and the oxygen radical absorbance capacity for various medicinal plants and culinary herbs (*32*). The failing correlation between total phenolics and DPPH or Fe^{3+} reduction in the CH_2Cl_2 extracts may reflect the fact that this solvent is not suitable to extract phenolic constitutents.

Using the data from the 14 essential oils analyzed, the correlation coefficients between antioxidant activity and the sum of the phenolic compounds thymol and carvacrol were 0.917 and 0.914 for DPPH and FRAP, respectively.

The Pearson correlation coefficients between rosmarinic acid content of ethanolic extracts and DPPH reducing activity, Fe^{3+} reducing activity, and total phenolic content were 0.839, 0.912, and 0.937 ($N = 44$), respectively, indicating that rosmarinic acid is a major antioxidative component in thyme. Also in *Salvia* species a strong correlation between rosmarinic acid content and antioxidant activity could be demonstrated (*33*).

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