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Catalytic Oxidation and Antioxidant Properties of Thyme Essential Oils (*Thymus vulgarae* L.)

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The antioxidant properties of two chemotypes of thyme essential oils were examined in relation to their catalytic transformation. Catalytic oxidations were performed with potassium hydrogentrioxoperoxosulfate(VI) and the Fe^{III} *meso*-tetraphenylporphyrin catalytic system, after which the chemical compositions of essential oils were changed. The antioxidant activity was investigated by two different methods: the 2,2'-diphenyl-1-picrylhydrazyl radical scavenging method and the Ferric Reducing / Antioxidant Power assay. Both methods have shown that the phenolic chemotype possesses stronger antioxidant properties than the non-phenolic one. After catalytic oxidation, the essential oils of both chemotypes showed stronger antioxidant effects than before oxidation.

Keywords
Thymus vulgaris L.
antioxidants
thymol
thymoquinone

INTRODUCTION

In the last few years, there has been a growing interest in providing natural antioxidants.[1,2] The antioxidant properties of many herbs and spices have been reported effective in this respect.[3,4] Many terpenoids have been identified as potent antioxidants.[5] Tsimidou and Boskou[6] concluded that, among the herbs and spices extensively studied, the plants obtained from the *Lamiaceae* (*Labiatae*) family possess appreciable antioxidant activity. Lagouri *et al.*[7] found that oregano essential oil, rich in thymol and carvacrol, has a considerable antioxidant effect on the process of lard oxidation.

Synthetic porphyrin metal complexes with several oxygen donors can be used to catalyze essential oils oxidation.[8,9] In our research, catalytic oxidation was applied to transform thyme essential oils and to determine the relationship between their antioxidant abilities before and after such transformations. In oxidations, the system of potassium hydrogentrioxoperoxosulfate(VI) ($KHSO_5$) was used as oxygen donor and Fe^{III} *meso*-tetraphenylporphyrin served as a catalyst. Two different methods were applied to evaluate antioxidant activities: determination of ferric reducing/antioxidant power (FRAP) and free radical scavenging using 1,1-diphenyl-2-picrylhydrazyl (DPPH).

EXPERIMENTAL

Chemicals

All chemicals and reagents were of analytical grade and were obtained from Sigma Chemical Co. (St Louis, MQ, USA), Aldrich Chemical Co. (Steineheim, Germany), Merck (Darmstadt, Germany) and Kemika (Zagreb, Croatia). Dithymoquinone and thymohydroquinone were prepared in our laboratory by the methods of Smith and Tess[10] and Stolow *et al.*,[11] respectively.

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Plant Material and Essential Oil Isolation

Both chemotypes of thyme (*Thymus vulgarae* L.), a phenolic chemotype (thymol and carvacrol) and a non-phenolic chemotype (linalool), were collected in central Dalmatia (Croatia). A hundred grams of dried plant material was subjected to a three-hour hydro-distillation using a Clevenger-type apparatus to produce essential oils in yields of 1.2 % (phenolic chemotype) and 0.9 % (non-phenolic chemotype).

Analysis of Essential Oil

Analyses of volatile compounds (Table I) were run on a Hewlett-Packard GC-MS system (GC 5890 series II; MSD 5971A, Hewlett Packard, Vienna, Austria). The fused-silica HP-101 capillary column (dimethylpolysiloxane; 25 m × 0.2 μm i.d.; film thickness 0.2 μm, Hewlett Packard, Vienna, Austria) was directly coupled to the mass spectrometer. Helium (1 mL/min) was used as carrier gas. The program was 4 min isothermal at 70 °C, then the temperature increased by 4 °C/min to 180 °C and 10 min isothermal at 180 °C. The injection port temperature was 250 °C and that of the detector was 280 °C. Ionization of sample components was performed in the EI mode (70 eV). The relative linear retention indices (RRI) for all compounds were determined by co-injecting the sample with a solution containing the homologous series of C_8–C_{22} *n*-alkanes. Particular constituents were identified by their identical retention indices with respect to the compounds known from literature data,[12] and also by comparing their mass spectra either with spectra of known compounds or with those stored in the Wiley mass spectral database.

General Catalytic Procedures

Catalytic oxidations of essential oils were carried out at 25 °C in a thermostated reaction tube equipped with a magnetic stirrer. In a typical experiment, the essential oil (75 μL) and potassium hydrogentrioxoperoxosulfate(VI) (0.15 mmol) were dissolved in acetonitrile (3.0 mL). The reaction was initiated by addition of the catalyst Fe^{III} *meso*-tetraphenylporphyrin (5×10^{-3} mmol). Aliquots (10 μL) were withdrawn in the course of the reaction and were poured into pentane (1 mL), from which 2 μL was used for the GC-MS analysis. The reaction was stopped when the product yields remained constant after two successive analyses (approximately one hour).

Free Radical Scavenging Capacity Using the Stable Radical

The antioxidant activity of thyme volatile compounds was measured with respect to hydrogen donating or radical scavenging ability using the stable radical DPPH.[13] The 50 μL methanolic stock solution of 4.5 g/L of antioxidant (0.15 g/L in thymoquinone degradation measurements) was placed in a cuvette, and 1 mL of 6×10^{-5} M methanolic solution of DPPH was added. Absorbance measurements commenced immediately. The decrease in absorbance at 517 nm was determined with a UV-Vis (double-beam) Perkin-Elmer Lambda EZ 201 spectrophotometer. Methanol was used to zero the spectrophotometer. The absorbance of DPPH radical without antioxidant, *i.e.*, the control, was measured on a daily basis. All determinations were done in triplicate.

$$\text{Inhibition / \%} = ((A_{C(0)} - A_{A(t)}) / A_{C(0)}) \times 100$$

where $A_{C(0)}$ is the absorbance of the control at $t = 0$ min and $A_{A(t)}$ is the absorbance of the antioxidant at $t = 1$ h.

Determination of FRAP – Ferric Reducing/Antioxidant Power

Determination of the ferric reducing/antioxidant power FRAP is a simple direct test of antioxidant capacity. This method was initially developed to assay plasma antioxidant capacity, but can be used for plant extracts too. The total antioxidant potential of the sample was determined by means of the ferric reducing ability (FRAP) assay[14] as a measure of the »antioxidant power«. The FRAP assay measures the change in absorbance at 593 nm due to the formation of the blue colored Fe^{II}-tripyridyltriazine compound from the colorless oxidized Fe^{III} form by the action of electron donating antioxidants. The working FRAP reagent was prepared by mixing 10 parts of 300 mmol/ L acetate buffer, pH = 3.6, with 1 part of 10 mmol/L TPTZ (2,4,6-tripyridyl-*s*-triazine) in 40 mmol/L hydrochloride acid and with 1 volume of 20 mmol/L iron(III) chloride. Freshly prepared FRAP reagent (1.5 mL) was warmed to 37 °C and the reagent blank reading was taken at 593 nm (M1 reading). Subsequently, 50 μL of the sample (concentrations of stock solutions were 4.5 g/L (0.15 g/L in thymoquinone degradation measurements) and 150 μL of deionized water was added to the FRAP reagent. The final dilution of the sample in the reaction mixture was 1 : 34. Absorbance readings were taken every 15 s thereafter during the monitoring period (8 minutes). Since there was little decrease in absorbency between 4 and 8 minutes of incubation, its value after 4 minutes was used for further calculations. The sample was incubated at 37 °C throughout the monitoring period. The change in absorbance between the final reading (4-min reading) and the M1 reading was chosen for FRAP values calculation. The standard curve was prepared using different concentrations (0.1–3 mmol/L) of $FeSO_4 \cdot 7H_2O$. All solutions were used on the day of preparation. In the FRAP assay, the antioxidant efficiency of the antioxidant tested was calculated with respect to the reaction signal given by an Fe^{2+} solution of known concentration. The results were corrected for dilution and expressed as $[Fe^{2+}]$ / mmol L^{-1}. All determinations were performed in triplicate.

RESULTS AND DISCUSSION

Chemical Composition and Catalytic Oxidation of Thyme Essential Oil

During typical catalytic oxidations of thyme essential oils, the reactions were followed by GC-MS measurements. The main components were quantified as the peak area

TABLE I. Chemical composition of thyme (*Thymus vulgarae* L.) essential oil. Two chemotypes, phenolic and non-phenolic, before and after catalytic transformation[a]

Compound	RI	Peak area / %			
		A ± (SD)	B ± (SD)	A ± (SD)	B ± (SD)
		Phenolic chemotype		Non-phenolic chemotype	
β-Pinene	968	0.6 (0.1)	2.9 (0.6)	1.1 (0.1)	2.0 (0.3)
p-Cymen	1010	34.7 (2.6)	41.2 (5.4)	13.5 (1.3)	15.5 (0.8)
Terpinene	1042	8.4 (0.2)	6.8 (0.3)	2.5 (0.5)	2.2 (0.3)
Linalool	1304	1.7 (0.3)	4.2 (0.1)	43.0 (2.0)	40.2 (3.3)
Borneol	1158	1.3 (0.1)	4.1 (0.2)	0.7 (0.3)	1.2 (0.2)
1,4-Terpineol	1172	1.6 (0.2)	1.5 (0.1)	4.3 (0.3)	6.8 (0.6)
α-Terpineol	1189	–	0.4 (0.0)	1.5 (0.3)	2.7 (0.8)
Thymoquinone	1221	–	19.0 (3.6)	–	4.1 (0.4)
Linayl acetate	1262	–	–	1.5 (0.3)	3.2 (0.2)
Geraniol	1271	–	–	0.5 (0.2)	0.3 (0.2)
Bornyl acetate	1278	–	1.1 (0.1)	–	0.5 (0.1)
Neryl acetate	1360	–	–	1.1 (0.8)	0.6 (0.2)
Thymol	1378	35.3 (2.5)	0.1	16.3 (0.6)	–
Carvacrol	1391	5.8 (1.3)	–	6.8 (1.7)	–
Geranyl acetate	1404	–	–	1.2 (0.2)	1.1 (0.1)
Caryophyllene	1418	0.5 (0.2)	0.9 (0.1)	0.8 (0.3)	1.4 (0.4)
Thymohydroquinone	1518	–	3.2 (0.3)	–	1.3 (0.3)

[a] RI – retention index, A – values before transformation, B – values after transformation.

of total oil after the oxidation process compared to that obtained before oxidation expressed in percents. As shown in Table I, the chemical composition of essential oils changed after catalytic oxidation. In both chemotypes, thymol and carvacrol were completely transformed after one hour to thymoquinone and a small amount of thymohydroquinone (determined by the GC-MS measurement). Fractions of other constituents remained for the most part unchanged. Without addition of the catalyst (Fe^{III} *meso*-tetraphenylporphyrin) and oxidant ($KHSO_5$), the blank experiment showed no transformation of essential oils and no formation of thymoquinone. These results are in agreement with the previous study, which revealed that pure thymol and carvacrol can be transformed by catalytic transformation to thymoquinone.[8,9]

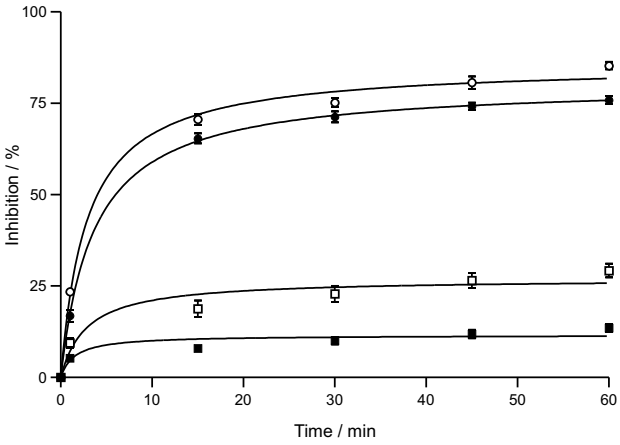


Figure 1. Inhibition, expressed in percents, of the DPPH radical by two thyme essential oil chemotypes. Before catalytic oxidation: phenolic (□) and nonphenolic (■) chemotypes, after catalytic oxidation: phenolic (○) and nonphenolic (●). Reacting concentrations of thyme essential oils were 214 mg/L.

Antioxidant Activity of Thyme Essential Oil

Antioxidant activities of both chemotypes of thyme essential oils were determined by the DPPH radical scavenging method and by the ferric reducing/antioxidant power test (FRAP). Both phenolic and non-phenolic chemotypes were able to reduce the stable 2,2'-diphenyl-1-picrylhydrazyl radical (Figure 1). As expected, a stronger effect was recorded for the phenolic chemotype. This observation can be explained by the higher total concentration of thymol and carvacrol in this chemotype. Similar results were obtained in the FRAP test (Figure 2). The phenolic chemotype showed a more expressed antioxidant ability than the non-phenolic one.

After catalytic oxidation, the essential oils of both chemotypes showed stronger antioxidant effects than before oxidation, as measured by both methods, DPPH and FRAP (Figures 1 and 2). These results suggest that thyme essential oil possesses a hidden potential for antioxidant properties. Thymoquinone and the compounds develop-

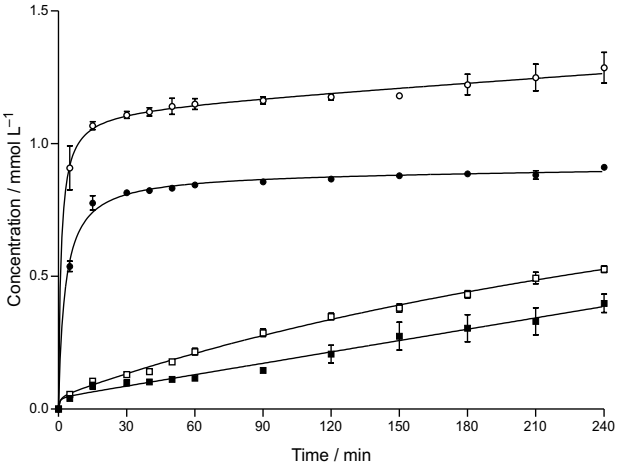


Figure 2. Typical profile of Ferric Reducing/Antioxidant Power (FRAP) of two thyme essential oil chemotypes. Before catalytic oxidation: phenolic (□) and nonphenolic (■) chemotypes, after catalytic oxidation: phenolic (○) and nonphenolic (●). Reacting concentrations of thyme essential oils were 132 mg/L.

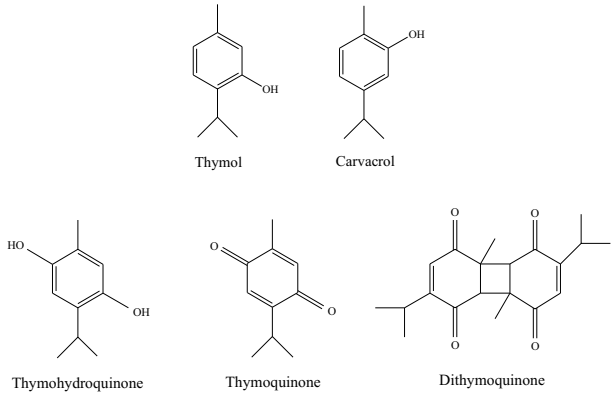
ed during the oxidation process could have remarkable antioxidant abilities and probably play a crucial antioxidative role. These compounds are already known as bioactive substances. Houghton *et al.*[15] demonstrated the role of thymoquinone as an inhibitor of membrane lipid peroxidation. Some studies have also shown the antitumor and hepatoprotective activities of thymoquinone.[16,17]

Thymoquinone Stability and Its Antioxidant Ability

Thymoquinone is a light- and heat-sensitive compound. According to Smith and Tess,[10] when exposed to light for five days, thymoquinone gets gradually converted to dithymoquinone (70–80 %) and some other derivatives. Robbins and Falvey[18] have reported that dithymoquinone can undergo redox-cycling reactions. The univalent reduction of dithymoquinone to semiquinone is followed by its fragmentation to thymoquinone and further to the monomeric quinone or hydroquinone. Kruk *et al.*[19] have shown by two different methods (measurement of the chemiluminescence intensity and bleaching of *p*-nitrosodimethyl vaniline) that thymoquinone and dithymoquinone have significant antioxidant properties.

In our study, we examined the antioxidant activity of pure thymoquinone in relation to its degradation during five days. Among thymoquinone degradation products, we determined a non-volatile compound dithymoquinone (identified by TLC and NMR) and volatiles thymohydroquinone, a small quantity of thymol and carvacrol (Scheme 1) and some other non-identified compounds in traces (all determined by GC-MS).

Figures 3 and 4 show the DPPH inhibition expressed in percents and FRAP (ferric reducing/antioxidant power) in relation to the degree of thymoquinone degradation. Both methods show that the appearance of even a small



Scheme 1.

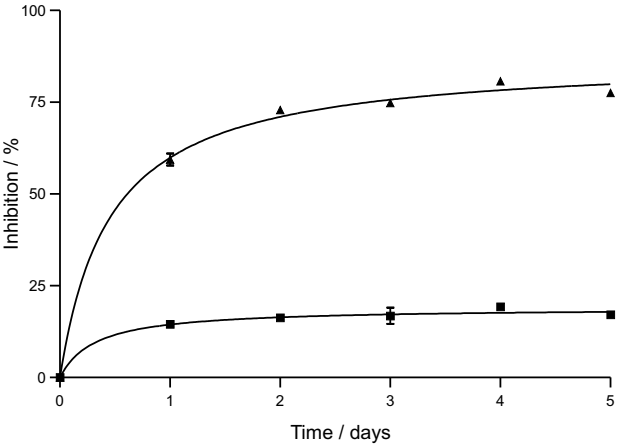


Figure 3. Measurements of the DPPH inhibition, expressed in percents, in relation to thymoquinone degradation on exposure (▲) and without exposure (■) to light. Reacting concentrations of thymoquinone were 7.1 mg/L.

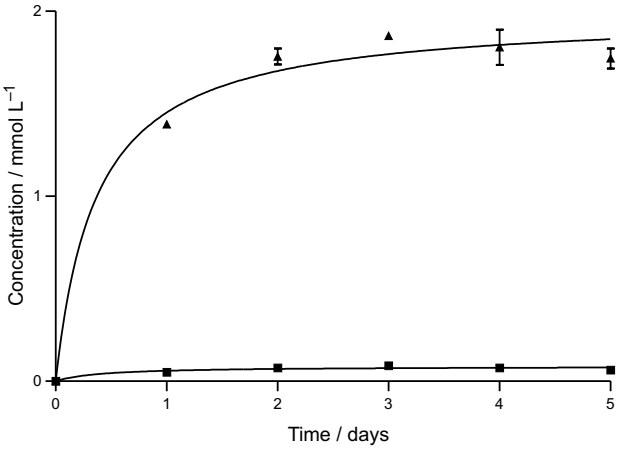


Figure 4. Measurements of Ferric Reducing/Antioxidant Power (FRAP) in relation to thymoquinone degradation on exposure (▲) and without exposure (■) to light. Reacting concentrations of thymoquinone were 4.4 mg/L.

quantity of thymoquinone degradation products substantially changes and strengthens its antioxidant ability. The diagrams show that the process of thymoquinone degra-

dation is completed after two days when exposed to sunlight at room temperature. They also show that the strongest antioxidant effect is achieved during this period and that it remains constant afterwards. Control was performed without exposure to light: immediately after preparation, the vial containing thymoquinone was refrigerated and covered with aluminum foil. Under such conditions, the experiments showed no significant changes of thymoquinone antioxidant ability.

Antioxidant Abilities of Pure Constituents of Thyme Essential Oils

Thyme essential oils and their pure constituents were examined for their ability to act as antioxidant agents by being compared among themselves and with Trolox and vitamin C. As shown in Table II, the antioxidant power decreased in the order: thymohydroquinone > Trolox > vitamin C > essential oil after catalytic oxidation (phenolic) > essential oil after catalytic oxidation (non-phenolic) > essential oil before catalytic oxidation (phenolic) > thymol > carvacrol > oil before catalytic oxidation (non-phenolic) > dithymoquinone > thymoquinone, as measured by both methods. All these measurements show that the antioxidant ability of thyme essential oils is due to the presence of phenolic compounds thymol, carvacrol and thymohydroquinone.

Some authors[20] suggest that the synergy between constituents could be responsible for their antioxidant efficacy. Our results indicate that the antioxidant ability of thyme essential oils after catalytic oxidation is not due to the main oxidation product thymoquinone but, specifically, to its degradation product thymohydroquinone. Thymohydroquinone possesses the antioxidant ability significantly stronger than that of pure thymoquinone or other degradation products. Its antioxidant ability can be compared to those of the well-known antioxidant compounds Trolox and vitamin C.

Monitoring showed that the thyme essential oil, containing thymol as a major compound, can be easily transformed to an oil with thymoquinone as the main component, which can be converted to thymohydroquinone and dithymoquinone. With the appearance of even small quantities of these compounds, the thyme essential oil becomes a more potent antioxidant. As these compounds are interesting for their possible use in medicine,[16,17] the catalytic transformation of phenolic type essential oils from other plant sources merits further investigation.

TABLE II. Antioxidant abilities of thyme essential oils and their pure constituents compared among themselves and with Trolox and vitamin C[a]

Antioxidant compounds[b]	DPPH Inhibition/%	FRAP $[Fe^{2+}]$/mmol L^{-1}
Thyme essential oil A (phenolic)	29.2	0.5
Thyme essential oil B (phenolic)	85.3	1.3
Thyme essential oil A (nonphenolic)	13.6	0.3
Thyme essential oil B (nonphenolic)	75.9	0.9
Thymol	25.3	1.1
Carvacrol	24.0	1.0
Thymoquinone	7.3	8.6×10^{-2}
Dithymoquinone	18.0	0.4
Thymohydroquinone	100.0	> 3.0
Trolox	92.6	2.0
Vitamin C	91.5	2.0

[a] Reacting concentrations of antioxidant compounds were 214 mg/L (DPPH measurements) and 132 mg/L (FRAP measurements).

[b] A – before transformation, B – after transformation.

Dithymoquinone

^{1}H NMR (C_6D_6, 300 MHz) δ/ppm: 0.8187 (q^d, J_{AX} = 4.9 Hz, **12H**, $4CH_3$), 0.9549 (s, **6H**, $2CH_3$), 2.9227 (q^t, J = 6.8 Hz, **2H**, 2CH–$(CH_3)_2$–R), 3.0984 (s, **2H**, $2(R_2CH–CO–R)$), 6.3858 (s, **2H**, 2R=CH–CO–R)

^{13}C NMR (C_6D_6, 75.47 MHz) δ/ppm: 20.4 (**6C**H_3), 27.1 (**2C**$H(R)_3$), 47.1 (**2C**(R_4)), 54.7 (**2C**$H(R_2R=O)$, 134.5 (**2C**$H(R)_2$), 160.4 (**2C**$(R)_3$), 193.6 (**2C**=O), 197.6 (**2C**=O)

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SAŽETAK

Katalitička oksidacija i antioksidacijska svojstva eteričnih ulja timijana (*Thymus vulgaris* L.)

Mila Jukić i Mladen Miloš

Ispitana su antioksidacijska svojstva eteričnih ulja dvaju kemotipova timijana ovisno o njihovoj katalitičkoj transformaciji. Katalitička transformacija je provedena s kalijevim hidrogentrioksoperoksosulfatom(VI) i Fe^{III} *mezo*-tetrafenilporfirin katalitičkim sustavom, poslije čega je kemijski sastav eteričnih ulja izmijenjen. Antioksidacijska aktivnost ispitana je s dvije različite metode (2,2'-diphenyl-1-picrylhydrazyl radical scavenging i Ferric Reducing / Antioxidant Power). Obje su metode pokazale da fenolni kemotip posjeduje jača antioksidacijska svojstva od nefenolnoga tipa. Poslije katalitičke oksidacije, eterična ulja obaju kemotipova timijana pokazivala su jači antioksidacijski učinak.