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PAPER

ANTIOXIDANT PROPERTIES OF THYME (*THYMUS VULGARIS* L.) AND WILD THYME (*THYMUS SERPYLLUM* L.) ESSENTIAL OILS

PROPRIETÀ ANTIOSSIDANTI DEGLI OLI ESSENZIALI DEL TIMO (*THYMUS VULGARIS* L.) E DEL TIMO SELVATICO (*THYMUS SERPYLLUM* L.)

T. KULISIC*, A. RADONIC and M. MILOS
Department of Biochemistry and Food Chemistry, Faculty of Chemical Technology, University of Split, Teslina 10/V, 21000 Split, Croatia
* Corresponding author: Fax: + 385 21 384-964, e-mail: tea@ktf-split.hr (T. Kulisic)

ABSTRACT

In this study, the composition of the volatile compounds and the antioxidant activity of thyme (*Thymus vulgaris* L.) and wild thyme (*Thymus serpyllum* L.) essential oils were investigated using three different methods: DPPH radical scavenging method, the thiobarbituric acid reactive species (TBARS) assay and the β-carotene bleaching method. The results obtained for the total essential oils in both plants and their two fractions (hydrocarbon- and oxygen- containing fractions) were compared with

RIASSUNTO

In questo studio sono state esaminate la composizione della frazione volatile e l'attività antiossidante degli oli essenziali ottenuti dal timo (*Thymus vulgaris* L.) e dal timo selvatico (*Thymus serpyllum* L.) utilizzando tre differenti metodologie: il metodo di cattura dei radicali liberi del 2,2′-diphenyl-1-picrylhydrazyl (DPPH), il saggio delle specie reattive dell'acido tiobarbiturico (TBARS) e il metodo della decolorazione del β-carotene. I risultati ottenuti per tutti gli oli essenziali in entrambe le piante e nel-

- Key words: antioxidant activity, β-carotene bleaching, DPPH radical scavenging, essential oil, TBARS, thyme, wild thyme -

those of α-tocopherol, ascorbic acid and the synthetic antioxidants – butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). Thyme and wild thyme essential oils exhibit significant *in vitro* antioxidant activity, while fractions of the essential oils that contain hydrocarbon compounds (CH fractions) only show antioxidant activity in the TBARS assay.

le loro due frazioni (CH e CHO, frazioni contenente idrocarbonili e carbossili, rispettivamente) sono stati confrontati con quelli dell'α-tocoferolo, dell'acido ascorbico e degli antiossidanti sintetici – butil idrossitoluene (BHT) e butil idrossianisolo (BHA). Gli oli essenziali di timo e di timo selvatico mostrano una significante attività antiossidante *in vitro,* mentre le frazioni di questi oli essenziali che contengono composti idrocarbonilici (frazioni CH) mostrano attività antiossidante soltanto nel saggio con metodo TBARS.

INTRODUCTION

Interest in natural antioxidants for the food industry has increased due to speculation about the possible toxic effects of synthetic antioxidants (BRANEN, 1975; NAMIKI, 1990; BARLOW, 1990; POKORNY, 1991). Aromatic plants have been studied extensively because a rich source of natural antioxidants is available in their essential oils or diverse extracts. SHAHIDI *et al.* (1992) reported that the antioxidant effect of aromatic plants is closely related to the presence of hydroxyl groups in phenolic compounds.

Plants belonging to the *Lamiaceae* family are known to be rich in compounds possessing strong antioxidant activity (YOUDIM *et al.*, 1999; LEE and SHIBAMOTO, 2002; MIURA *et al.*, 2002). Thyme and wild thyme are also regarded as medicinal herbs with antispasmodic, expectorant and flatulence-reducing action (GUENTHER, 1949). The essential oils of the genus *Thymus* are mainly monoterpenic (C_{10}), and often contain large amounts of thymol and carvacrol (HEGNAUER, 1962). AESCHBACH *et al.* (1994) reported that thymol and carvacrol inhibit the peroxidation of liposome phospholipids in a concentration-dependent manner. Previous studies (KULISIC *et al.*, 2004) have shown that oregano essential oil that contains a high content of thymol and carvacrol has a strong antioxidant activity.

The objective of this research was to evaluate the antioxidant activity of thyme and wild thyme essential oils using a multiple-method approach in relation to their chemical composition. Three different methods were used: the β-carotene bleaching method, the 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method and the thiobarbituric acid reactive species (TBARS) assay (PRATT, 1980; MALLET *et al.*, 1994; CUENDET *et al.*, 1997; YEN and DUH, 1994; RUBERTO and BARATTA, 2000). The results were compared with the effect of α-tocopherol, ascorbic acid and the synthetic antioxidants, BHT and BHA.

MATERIAL AND METHODS

Materials

Thyme (*Thymus vulgaris* L.) and wild thyme (*Thymus serpyllum* L.) were collected in central Dalmatia and Bosnia and Herzegovina during the summer,

2002. After air-drying, the essential oils were extracted from the plant materials (flowered tops and stalks). A hundred grams of dried material from each plant were hydrodistillated for 3 h using a Clevenger-type apparatus. The distillated essential oils were dried over anhydrous sodium sulphate and stored under nitrogen in a sealed vial at -20°C until analysis. The voucher specimens of thyme and wild thyme plant material, as well as their essential oils, are stored in the Department of Biochemistry and Food Chemistry, Faculty of Chemical Technology, Split, Croatia.

The essential oils of thyme and wild thyme (0.5 g) were fractionated using a silica gel (30-60 µm, Mallinckrodt Baker B.V., Deventer, The Netherlands) column (length 20 cm; i.d. 2 cm). Pentane (50 mL) was used to obtain the fraction, which contained only non-polar hydrocarbons (CH fraction), while diethyl ether (50 mL) was used for the fraction containing polar (oxygen-containing, CHO fraction) compounds. These fraction solutions were concentrated to 0.5 mL and subjected to thin layer chromatography (TLC) on silica gel plates in order to check the results of the column chromatographic separation. Different solvents were used as mobile phase: *n*-hexane for the CH fraction and *n*-hexane:ethyl acetate 85:15 (v/v) for the CHO fraction. Two percent vanillin-sulphuric acid was used as a detection reagent. The fractions obtained by column chromatography were also subjected to GC/MS analysis.

GC-MS analysis

Volatile compounds were analysed on a Hewlett-Packard GC-MS system (GC 5890 series II; MSD 5971A, Hewlett Packard, Vienna, Austria). The fused-silica HP-20 M polyethylene glycol column (50 m x 0.2 mm, 0.2 µm thickness, Hewlett-Packard, Vienna, Austria) was directly coupled to the mass spectrometer. The carrier gas was helium (1 mL/min). The program used was 4 min isothermal at 70°C, then 4°C/min to 180°C and 10 min isothermal. The injection port temperature was 250°C and the detector temperature was 280°C. Ionisation of the sample was performed in the EI mode (70 eV).

The linear retention indices for all the compounds were determined by co-injection of the sample with a solution containing the homologous series of C_8-C_{22} *n*-alkanes (VAN DEN DOOL and KRATZ, 1963). The individual constituents were identified by their retention indices with reference to the compounds known from literature data (ADAMS, 1995), and also by comparing their mass spectra with spectra of either, known compounds or with those stored in the Wiley mass spectral database (Hewlett-Packard, Vienna, Austria).

Determination of antioxidant using 2,2´-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging

The antioxidant activity of the thyme and wild thyme volatile compounds was measured in terms of hydrogen-donating or radical-scavenging ability, using the stable radical, DPPH (2,2´-diphenyl-1-picrylhydrazyl) according to the modified method of CUENDET *et al.* (1997). The colour of DPPH changes from purple to yellow as a consequence of radical scavenging by antioxidants through donation of a hydrogen atom to form the stable DPPH-H (VON GADOW *et al.*, 1997). The remaining DPPH, measured after a certain time, corresponds inversely to the radical scavenging activity of the antioxidant (BRAND-WILLIAMS *et al.*, 1995).

The ethanolic stock solution (50 µL) of two different concentrations (0.2 and 2 g/L) of antioxidants was placed in a cuvette with 1 mL of 0.004% ethanolic solution of DPPH. Absorbance measurements started immediately. The decrease in absorbance at 517 nm was determined with a Perkin-Elmer spectro-

photometer after 1 h. Ethanol served as a blank. The absorbance of the DPPH radical without antioxidant, i.e. the control, was measured daily and special care was taken to minimize the loss of free radical activity of the DPPH stock solution (storage under a nitrogen atmosphere; the glassware of the storage system was covered with aluminium foil according to BLOIS, 1956). All determinations were performed in triplicate. The amount of sample necessary to decrease the absorbance of DPPH (IC_{50}) by 50% was calculated graphically (% of inhibition was plotted against the sample concentration in reaction system). The percentage inhibition of the DPPH radical was calculated according to the following formula (YEN and DUH, 1994):

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

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 antioxidant activity using thiobarbituric acid reactive species (TBARS)

A modified TBARS assay (RUBERTO and BARATTA, 2000) was used to measure the potential antioxidant capacity using egg yolk homogenate as lipid-rich media. Yolk homogenate, (0.5 mL of 10% water solution w/v) and 0.1 mL of different concentrations of ethanolic stock solutions of the samples (4.0, 20.0 and 40.0 g/L), prepared immediately before use, were added to a test tube and made up to 1.0 mL with distilled water. Subsequently, 0.05 mL of 2,2′-azobis (2-amidinopropane) dihydrochloride solution (0.07 M) in water was added to induce lipid peroxidation and 1.5 mL of 20% acetic acid and 1.5 mL 0.8% (w/v) thiobarbituric acid (TBA) in 1.1% (w/v) sodium-dodecyl sulphate solution were then added. The resulting mixture was vortexed and heated at 95°C for 60 min. After cooling, 5.0 mL of butan-1-ol were added to each tube, vortexed and centrifuged at 1,200 g for 10 min. The absorbance of the organic upper layer was measured using a spectrophotometer (Perkin-Elmer Lambda EZ 201, Roma, Italia), at 532 nm. All determinations were performed in triplicate and the values were calculated as the percentage antioxidant index (AI%) (RUBERTO and BARATTA, 2000):

$$AI\% = (1 - A_T/A_C) \times 100$$

where A_C is the absorbance value of the fully oxidized control and A_T is the absorbance of the test sample.

Determination of antioxidant activity using β-carotene bleaching (BCB)

Antioxidant activity of the thyme and wild thyme volatile compounds was determined according to a slightly modified version of the β-carotene bleaching method (PRATT, 1980). β-carotene (0.1 mg) was added to a boiling flask together with linoleic acid (20 mg) and Tween 40 (100 mg), all dissolved in chloroform. After evaporation to dryness, under vacuum at 50°C with a rotary evaporator, distilled water under oxygen gas flow (50 mL) was added and the mixture was emulsified for 1 min in an ultrasonic bath to form emulsion A. Two different concentrations (0.04 and 0.2 g/L) of ethanolic stock solution of the samples were prepared, of which, 200 µL were mixed with 5 mL of emulsion A in open-capped cuvettes. A control, without antioxidant, consisted of 200 µL of ethanol and 5 mL of emulsion A. Emulsion B consisted of 20 mg of linoleic acid, 100 mg of Tween 40 and 50 mL of distilled water under an oxygen gas flow. The mixture of 200 µL of ethanol and 5 mL of emulsion B served as a blank. Readings of all samples were taken immediately (t=0) and at 15 min intervals for 120 min on a Perkin-Elmer Lambda EZ 201

spectrophotometer at 470 nm. The cuvettes were incubated at 50°C between measurements. All determinations were performed in duplicate. The antioxidant activity coefficient (AAC) was calculated from the data with the following formula (MALLET *et al.*, 1994):

$$AAC = [(A_{A(120)} - A_{C(120)}) / / (A_{C(0)} - A_{C(120)}] \times 1000$$

where $A_{A(120)}$ is the absorbance of the antioxidant at t=120 min, $A_{C(120)}$ is the absorbance of the control at t=120 min, and $A_{C(0)}$ is the absorbance of the control at t=0 min.

Statistical analysis

The results obtained from all the methods used were analyzed by ANOVA, using the computer software Mathematica 5.0.

RESULTS AND CONCLUSION

Chemical composition of thyme and wild thyme essential oils

GC-MS analyses of thyme and wild thyme essential oils indicate that they are qualitatively similar. The compounds identified in the total essential oils of thyme and wild thyme and the compounds identified after fractionation of the essential oils are reported in Table 1. Four compounds were identified in thyme essential oil without fractionation. The predominant compounds were: γ-terpinene (5.5%), p-cymene (11.6%) and phenolic monoterpenes – thymol (80.4%) and carvacrol (2.1%). In wild thyme essential oil eight compounds were identified without fractionation; p-cymene (5.2%), γ-terpinene (5.3%), thymol (30.0%) and carvacrol (49.4%) being the main compounds. After fractionation, the oils were subjected to a more comprehensive GC-MS analysis and eleven compounds were identified in the thyme hydrocarbon (CH) fraction; γ-terpinene (41.1%), *p*-cymene (26.5%) and *trans*-caryophyllene (23.9%) were the main components. On the other hand, eight compounds were identified in the wild thyme hydrocarbon (CH) fraction; α-terpinene (2.3%), γ-terpinene (29.5%), *p*-cymene (26.1%) and *trans*- caryophyllene (29.3%) were the main components. The oxygen-containing compounds (CHO) in the fractions in both cases were two phenolic compounds; namely thymol (91.8% in thyme, 35.4% in wild thyme) and carvacrol (5.8% in thyme, 62.7% in wild thyme).

Antioxidant activity of thyme and wild thyme essential oils

DPPH radical scavenging method

Table 2 shows the radical scavenging effect of thyme and wild thyme essential oils in comparison with those of the synthetic antioxidants BHT and BHA, α-tocopherol and ascorbic acid. The radical scavenging effect of thyme and wild thyme essential oils and oil fractions was much lower than those of the reference compounds. The concentrations needed for 50% inhibition of the DPPH radical were several times lower than those of thyme and wild thyme essential oils and their fractions.

Thyme essential oil was a slightly better scavenger of the DPPH radical than wild thyme essential oil. The CHO fraction of thyme essential oil was better than the wild thyme CHO fraction. The CH fraction of both essential oils did not show any radical scavenging effect. This is logical because it is known that the radical scavenging effect of the DPPH method is determined by the number and substitution pattern of the -OH groups (BRAND-WILLIAMS *et al.*, 1995; KOLEVA *et al.*, 2002).

Solvent effects are also important for the capability of the phenols to donate a hydrogen-atom (VALGIMIGLI *et al.*, 1995).

Table 1 - The composition of thyme (*Thymus vulgaris* L.) and wild thyme (*Thymus serpyllum* L.) essential oils.

N.	Compound	RI[1]	Area (%)			
			Thyme		Wild thyme	
			In total oil	In fraction	In total oil	In fraction
Hydrocarbons CH fraction (CH)						
1.	α-Pinene	1023	-	-	0.6	1.2
2.	α-Thujene	1031	-	0.7	-	-
3.	Δ-Carene	1131	-	0.3	-	-
4.	β-Myrcene	1148	-	-	0.4	1.0
5.	α-Terpinene	1231	-	0.8	0.7	2.3
6.	γ-Terpinene	1231	5.5	41.1	5.3	29.5
7.	*p*-Cymene	1247	11.6	26.5	5.2	26.1
8.	Terpinolene	1260	-	0.1	-	-
9.	Caryophyllene	1585	-	23.9	3.5	29.3
10.	α-Humulene	1683	-	0.5	-	0.6
11.	β-Cubebene	1694	-	0.7	-	-
12.	D-Germacrene	?[2]	-	1.5	-	-
13.	Δ-Cadinene	1716	-	2.8	-	-
14.	unknown compound		-	-	-	6.1
				Total 98.8		Total 96.3
Oxygen-containing compound fraction (CHO)						
15.	Linalool	1507	-	1.3	-	-
16.	Benzyl alcohol	1919	-	-	-	0.2
17.	Thymol	2115	80.4	91.8	30.0	35.4
18.	Carvacrol	2140	2.1	5.8	49.4	62.7
				Total 98.9		Total 98.3

[1]Retention indices relative to C_8-C_{22} alkanes on polar HP-20M column.
[2]Tentatively identified on the basis of mass spectra (MS) only.

Table 2 - Scavenging activity of thyme and wild thyme essential oils, their fractions, BHT, BHA, α-tocopherol and ascorbic acid on the DPPH radical (mean values ± standard deviation and ANOVA analysis are given).

Antioxidant	IC_{50}[1]	% inhibition of DPPH concentration in reacting system	
		0.2 g/L	2 g/L
Thyme essential oil	0.30±0.06	38.80±0.10	91.30±0.30
Wild thyme essential oil	0.40±0.05	30.50±0.04	82.00±0.07
Thyme CH fraction[2]	-	5.70±0.10	11.70±0.05
Wild thyme CH fraction[2]	-	5.30±0.08	12.60±0.50
Thyme CHO fraction	0.40±0.05	37.20±0.06	93.20±0.80
Wild thyme CHO fraction	0.70±0.06	28.60±1.00	82.20±0.70
BHT[3]	$1.80x10^{-2}$±$0.20x10^{-2}$		
BHA[3]	$5.40x10^{-2}$±$0.20x10^{-2}$		
α-Tocopherol[3]	$8.60x10^{-3}$±$0.30x10^{-4}$		
Ascorbic acid[3]	$4.40x10^{-3}$±$0.30x10^{-4}$		

[1]Concentration (g/L) for 50% inhibition. [2]CH fractions could not inhibit 50% of DPPH under test conditions. [3]At these concentrations (0.2 and 2 g/L), kinetic behaviour of BHT, BHA, α-tocopherol and ascorbic acid is so fast that they immediately achieve 100% DPPH inhibition. [4]ANOVA analysis gave 116.15 for F-ratio and $1.64x10^{-12}$ for null-hypothesis P-value.

"Water-like" solvents induce a dramatic decrease in the hydrogen-atom donating capability in comparison to chlorobenzene, methanol, ethanol (used in this method) or other alcohols.

TBARS

The thiobarbituric acid assay (TBA) involves the spectrophotometric detection of thiobarbituric acid reactive substances (TBARS), namely malonaldehyde (MDA), one of the secondary lipid peroxidation products, the quantity of which indicates the extent of lipid degradation (RUBERTO and BARATTA, 2000). Table 3 shows the antioxidant activity of thyme and wild thyme essential oils before and after fractionation, in comparison with those of α-tocopherol, ascorbic acid and the synthetic antioxidants, BHT and BHA. α-Tocopherol and BHA showed the highest antioxidant activities. α-Tocopherol had a high antioxidant activity even at the lowest concentration (AI of 72.6% at 100 ppm). Ascorbic acid did not show any antioxidant activity. The antioxidant activity of thyme essential oil was higher than that of wild thyme essential oil. In contrast, the CHO fraction of wild thyme essential oil was slightly better than that of the thyme essential oil. Non-polar CH fractions exhibited good antioxidant activities. RUBERTO and BARATTA (2000) reported similar results for non-polar compounds from essential oils. The authors tested about 100 pure components of essential oils and demonstrated that the monoterpene hydrocarbons γ-terpinene, *p*-cymene and α-terpinene had significant antioxidant activities. These compounds were representative of the CH fractions of thyme and wild thyme essential oils.

Egg yolk water emulsion was used in this method. Many antioxidants in emulsions will partition into both the lipid and water phase of emulsions. This portioning behaviour is critical in the activity of the antioxidant (DECKER *et al.*, 2005).

It has been already observed that compared to lipid-soluble antioxidants, water-soluble antioxidants like ascorbate yield better protection to strongly lipophilic food systems like pure oils. In contrast, antioxidants soluble in lip-

Table 3 - Antioxidant activity of thyme and wild thyme essential oils, their fractions, BHT, BHA, α-tocopherol and ascorbic acid measured by the thiobarbituric acid method (mean values ± standard deviation and ANOVA analysis are given).

Antioxidants	AI (%)[1]		
	100 ppm[2]	500 ppm	1,000 ppm
Thyme essential oil	27.3±4.5	57.0±3.3	75.3±2.7
Wild thyme essential oil	27.4±3.1	36.6±1.7	55.3±2.4
Thyme CH fraction	10.0±1.7	42.3±3.6	60.7±4.3
Wild thyme CH fraction	45.2±3.7	66.2±7.5	77.0±3.5
Thyme CHO fraction	38.2±6.2	50.6±2.4	66.0±5.0
Wild thyme CHO fraction	51.8±2.9	65.3±4.2	73.5±3.7
BHT	37.5±1.9	64.0±2.5	68.6±1.1
BHA	38.5±3.2	78.9±5.1	88.7±2.9
α-Tocopherol	72.6±3.6	79.5±2.3	90.0±1.8
Ascorbic acid	12.5±1.2	5.3±2.4	2.7±1.6

[1]Antioxidant effectiveness expressed as antioxidant index (as described in Materials and methods). Values are the average of triplicates ± standard deviation. [2]Antioxidant concentration in reacting system. [3]ANOVA analysis gave 427.87 for F-ratio and 0.00 for null-hypothesis P-value.

ids like the tocopherols yield better protection to oil-in-water emulsions when compared to water-soluble antioxidants (PORTER, 1993). Polar antioxidants are more effective in bulk oils because they can accumulate at the air-oil interface, the location where lipid oxidation reactions would be the greatest due to high concentrations of oxygen and prooxidants. In contrast, non-polar antioxidants are more effective in emulsions because they are retained in the oil droplets and/or may accumulate at the oil-water interface. In emulsions polar antioxidants would tend to partition into the aqueous phase and they would not be able to protect the lipid (DECKER *et al.*, 2005). This could explain the good antioxidant activity of the non-polar CH fractions and the lack of the antioxidant activity of ascorbic acid in this method.

β-Carotene bleaching method

The antioxidant activites of compounds from thyme and wild thyme essential oils, BHT, BHA, α-tocopherol and ascorbic acid assessed with the β-carotene bleaching method, expressed as the antioxidant activity coefficient, are presented in Table 4. The synthetic antioxidants, BHA and BHT, and α-tocopherol were the strongest antioxidants. In spite of its already documented antioxidant nature, ascorbic acid did not show any antioxidant effect with this method. This could be due to the previously described polar paradox. The polar antioxidants, such as ascorbic acid that remain in the aqueous phase of the emulsion are more diluted in the lipid phase and are thus less effective in protecting the linoleic acid (KOLEVA *et al.*, 2002; PORTER, 1993). Using this method, the thyme and wild thyme essential oils generally showed significant antioxidant activities. The antioxidant activity of thyme essential oil was slightly higher than that of wild thyme essential oil. The effect of the CHO fractions was almost the same as that of the total oils, probably because of the elimination of hydrocarbon fractions. The CH fractions of the thyme and wild thyme essential oils had low antioxidant activities.

Table 4 - The antioxidant activity of thyme and wild thyme essential oils, BHT, BHA, α-tocopherol and ascorbic acid assessed with the β-carotene bleaching method (mean values ± standard deviation and ANOVA analysis are given).

Antioxidant	AAC[1] concentration in reacting system	
	0.04 g/L	0.2 g/L
Thyme essential oil	376±160	657±52
Wild thyme essential oil	400±24	545±181
Thyme CH fraction	82±3.6	314±53
Wild thyme CH fraction	5.1±3.1	168±28
Thyme CHO fraction	483±35	669±38
Wild thyme CHO fraction	299±12	555±34
BHT	693±59	852±37
BHA	980±18	992±14
α-Tocopherol	613±23	813±65
Ascorbic acid[2]	/	/

[1]AAC – the antioxidant activity coefficient (calculated as described in Materials and methods). [2]Using this method ascorbic acid did not show any antioxidant activity. [3]ANOVA analysis gave 25.50 for F-ratio and 1.10×10^{-7} for null-hypothesis P-value.

The behaviour of different antioxidants in emulsion is not well understood (SCHWARZ *et al.*, 2000). Partitioning effects in emulsions may be more important than the antioxidative structure, the chemical properties of the emulsion components are also important and finally the unusual antioxidative properties of β-carotene is an additional important factor in the presentation of the results obtained with this method (KOLEVA *et al.*, 2002). Therefore, this method can only be used in combination with other methods to evaluate the antioxidant capacity of different compounds, especially in the case of complex samples such as plant extracts or essential oils.

CONCLUSION

The evaluation of the antioxidant activity of thyme and wild thyme essential oils based on the results of three different methods shows that these oils have an antioxidant activity (thyme is slightly better than wild thyme). However, the term antioxidant is limited by the specificity of the method, the chemical structure of the putative antioxidant, its polarity and concentration in the reaction medium and the possible interactions in complex systems like essential oils.

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