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Chemical composition and antioxidant activity of *Thymus vulgaris* L. volatile oil obtained by two different methods

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Abstract

In this paper two extraction methods of the Thymus vulgaris L. volatile oil have been comparatively investigated – steam distillation and extraction with non-polar solvents - reflected in oil quality and in the pharmacological activity. The qualitative analysis was performed by high performance thin layer chromatography (HPTLC) and the quantitative analysis by gas chromatography (GC). The antioxidant potential was determined by phosphomolybdenum reduction assay and DPPH assay. Results show that the Thymus vulgaris used for the present study belongs to thymol chemotype. Volatile oil obtained by steam distillation contains high amounts of thymol and p-cymene. For the sample obtained by non-polar solvent extraction, the above mentioned terpenes are the only volatile compounds detected by GC. Both samples exhibit antioxidant activity, slightly higher for volatile oil obtained by steam distillation. The study proves that the scavenging activity is not entirely due to volatile compounds but also to other liposoluble substances.

Keywords: *Thymus vulgaris* L., volatile oil, antioxidant

Introduction

Thymus vulgaris L. (thyme) is an aromatic plant belonging to the Lamiaceae family, used for medicinal and spice purposes almost everywhere in the world (R. MORALES [1]). In Romania, *Thymus* genus contains one cultivated species as aromatic plant (*Thymus vulgaris* L.) and other 18 wild species (A. MARCULESCU & al. [2]). *Thymus vulgaris* shows a polymorphic variation in monoterpene production, the presence of intraspecific chemotype variation being common in the genus *Thymus*. Each of the six chemotypes, geraniol (G), α-terpineol (A), thuyanol-4 (U), linalool (L), carvacrol (C), and thymol (T), is named after its dominant monoterpene (J. THOMPSON & al. [3]).

Many pharmacological *in vitro* experiments carried out during the last decades revealed well defined pharmacological activities of both, the thyme essential oil and the plant extracts. The non-medicinal use of thyme is worthy of attention, because thyme is used in the food and aroma industries; it is widely used as culinary ingredient and it serves as a preservative for foods especially because of its antioxidant effect. Thyme essential oil constitutes raw material in perfumery and cosmetics due to a special and characteristic aroma (A. ZARZUELO & al. [4]).

Essential oil quality and yield depend on many factors and choosing a suitable extraction method is very important. For example, steam distillation procedure is widely used for essential oil separation; beyond its efficiency, this method gives a greater or lesser compounds instability under the influence of high temperature. Extraction with organic

solvents has many deficiencies – residual solvent in the extracts, insufficient solvent selectivity so that, in addition to the active substances, other compounds are dissolved. This paper aims to show the comparison between two extraction procedures reflected in essential oil quality and in the pharmacological activity.

Materials and methods

Plant material

Thymus vulgaris L. (Thymi herba) dried and milled plant material was obtained from an indigenous crop (Bucharest, Romania).

Chemicals

2,2-Di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH), ascorbic acid, quercetin, p-cymene, thymol, carvacrol, linalool, borneol, cineole, terpinyl acetate, geraniol, terpineol, caryophyllene were purchased from Sigma Aldrich-Fluka. All other chemicals were analytical grade reagent.

Samples preparation

The steam distillation was done using a Neoclevenger system: In 5 L round bottom glass flask were added 300 g of milled Thymi herba and 3 L of distilled water (vegetal material/ extraction solvent rate = 1/10 (m/v). The mixture was left under reflux for 5 hours. The yellow and intense aromatic volatile oil (2,7mL) was collected and stored at 4° C for further analysis. The yield (v/w) of volatile oil was 0.9%.

Liposoluble fraction were obtained by a method based on active principles extraction from Thymi herba with 1500 mL hexane (vegetal material/ solvent rate = 1/10 m/v) under solvent reflux temperature and continuous stirring for 2 hours. After filtration, the hexane solution was concentrated at 30°C under reduced pressure (72-74 mmHg) and 7 mL liposoluble fraction was finnaly obtained and stored at 4° C for further analysis.

HPTLC analysis

Chromatography was performed on silica gel F254 HPTLC pre-coated plates. Samples were applied on the plates as band of 7mm width using a Camag Linomat V sample applicator at the distance of 14mm from the edge of the plates. The mobile phase was constituted of toluene-ethyl acetate 93:7 (v/v). After development, plates were dried and derivatised in anysaldehyde-sulphuric acid reagent. The fingerprints were evaluated in visible mode with a WinCats and VideoScan software after drying the plates at 110°C for 10 min. Reference compounds for HPTLC analysis were thymol, carvacrol, linalool, borneol, cineole, terpinyl acetate, geraniol, terpineol, caryophyllene (1% solutions in dichloromethane).

GC analysis

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.

Antioxidant potential assay

The antioxidant power of the extracts has been assessed with the phosphomolybdenum reduction assay according to Prieto et al. (PRIETO P. et al [5]). The reagent solution contained ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulfuric acid (0.6 M) mixed with the samples diluted in dichloromethane at the concentration of 0.01-10 mg/mL. The samples were incubated for 90 min at 90 °C and the absorbance of the green phosphomolybdenum complex was measured at 695 nm. For reference, the appropriate

solutions (0.2-2mM) of ascorbic acid have been used. The reducing capacity of the extracts has been expressed as the ascorbic acid equivalents (milligrams per gram extract). For absorbance measurements, a Helios Gamma UV/VIS spectrophotometer was used.

Free radical scavenging assay

The samples were diluted in dichloromethane at the concentration of 0.01-10mg/mL. 50 µL aliquots of the extract were mixed with 2950 µl of the methanolic DPPH solution (0.025g/L). The reduction of the DPPH free radical was measured by reading the absorbance at 517nm and related to the absorbance of the control without the herbal drugs. Inhibition ratio (percent) was calculated from the following equation: % inhibition [(absorbance of control – absorbance of sample)/absorbance of control] x 100%. Quercetin was used as positive control. For absorbance measurements, a Helios Gamma UV/VIS spectrophotometer was used.

Results and discussions

HPTLC analysis

The fingerprint of the constituents present in samples was recorded using Camag TLC visualizer and WinCats Software. The chromatograms (Figure 1) showed characteristic spots for volatile oil components: linalool – brown spot at Rf 0.27, caryophyllene – brown spots at Rf 0.39 and 0.98, terpinyl acetate – brown spot at Rf 0.62, terpineol – brown spot at Rf 0.19, geraniol – brown spot at Rf 0.17, cineole – brown spot at Rf 0.37, thymol – red spot at Rf 0.55 and carvacrol – red spot at Rf 0.49. Chromatographic profiles comparison showed that both volatile oil sample and liposoluble fraction have similar content of volatile compounds. There is a difference concerning the amount of these volatile compounds in the two samples, that shows the higher quality of the oil isolated by steam distillation. Thymol is the predominant compound in volatile oil sample, but it contains also high amounts of linalool and caryophyllene.

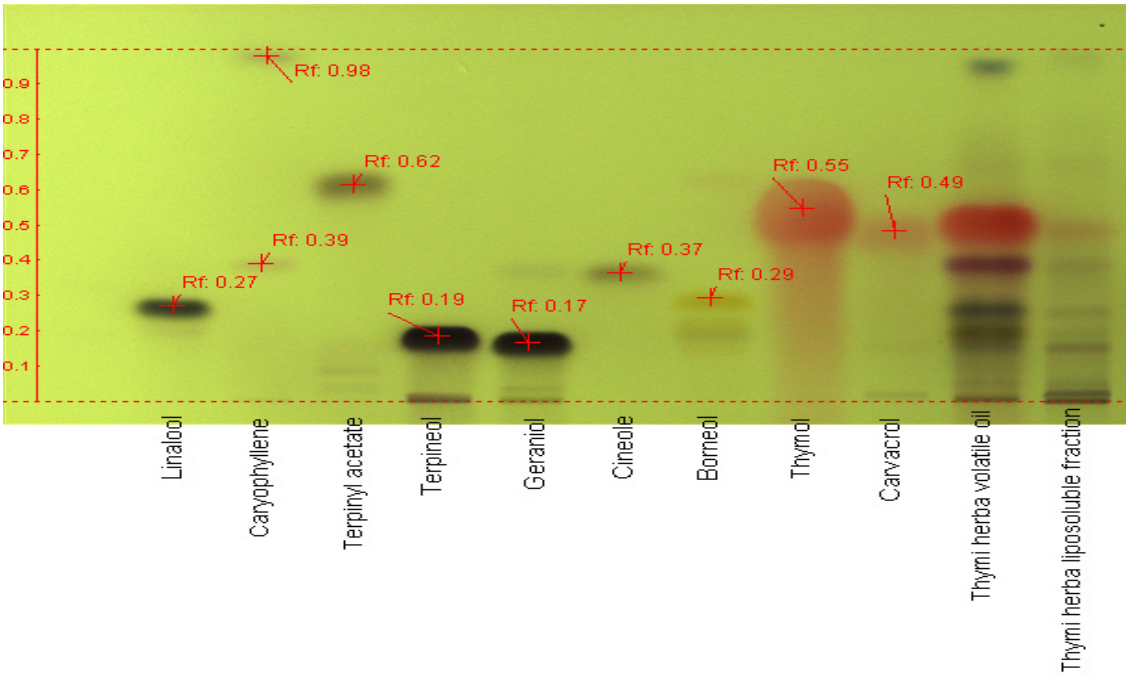

Figure 1. HPTLC fingerprint of Thyme samples

GC analysis

Figures 2 and 3 show the GC analysis of the volatile oil obtained by steam distillation (sample dilution: 1% in n-hexane) and by hexane extraction (sample dilution: 1% in n-hexane).

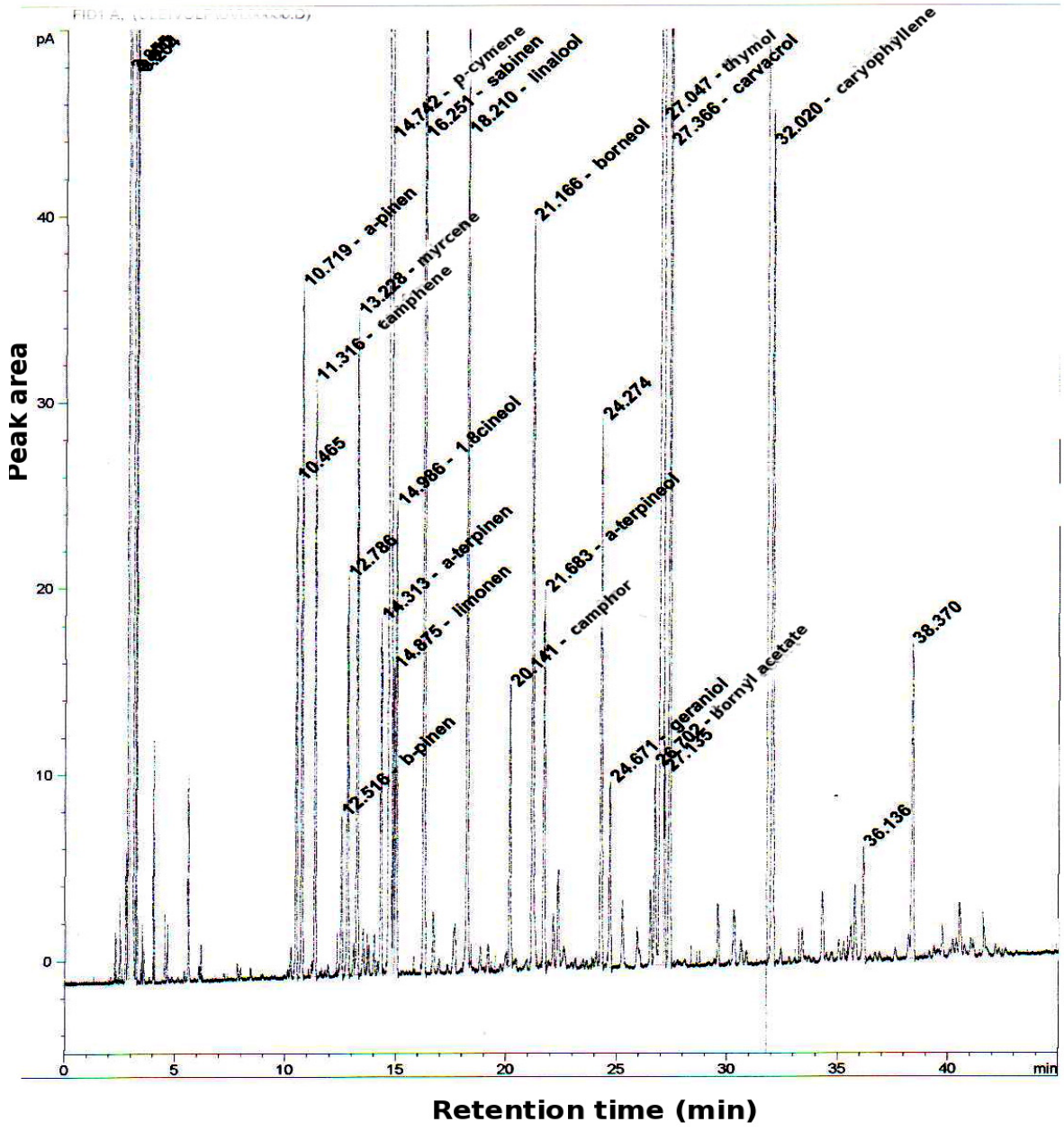

Figure 2. GC chromatogram of thyme volatile oil

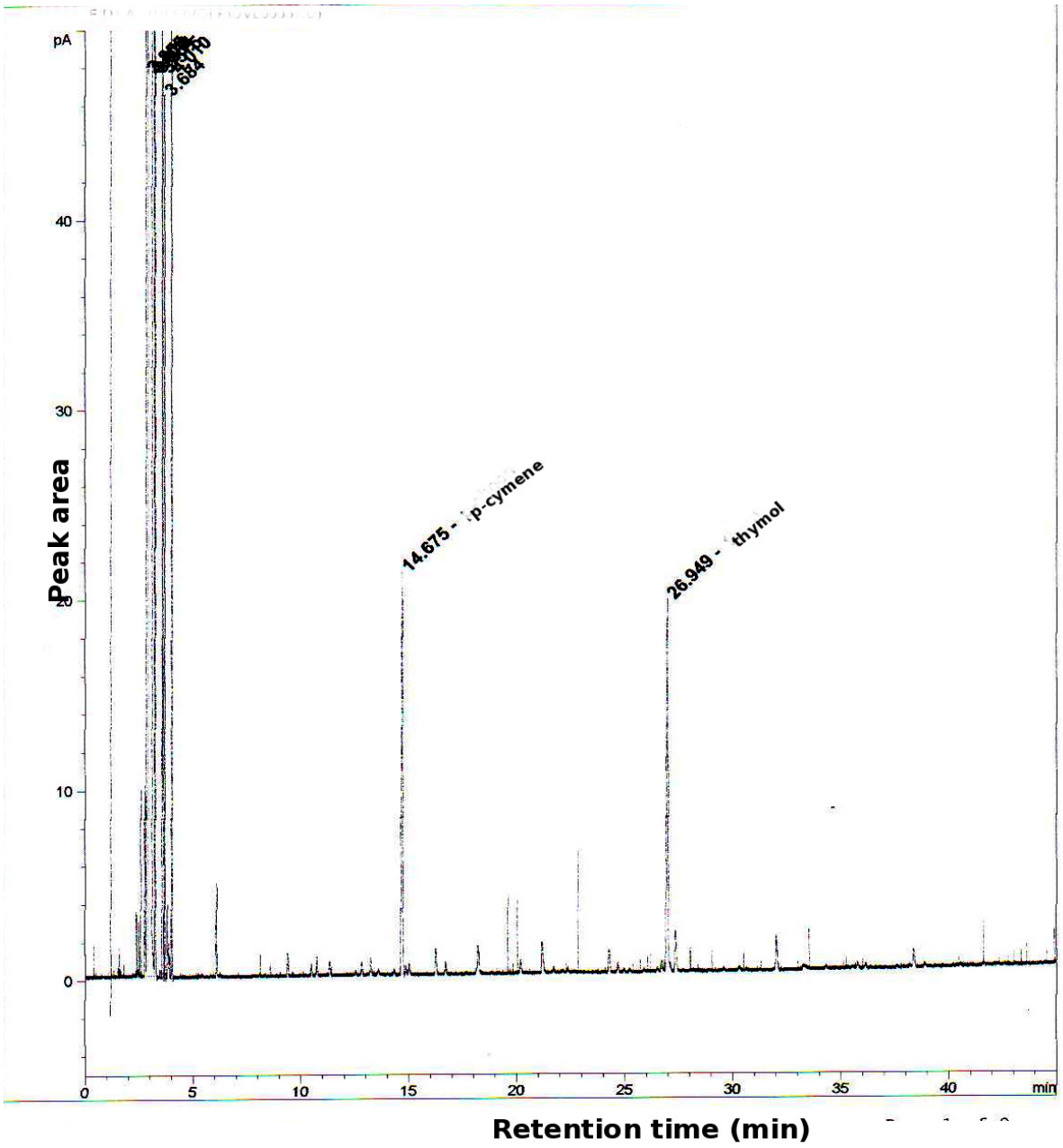

Figure 3. GC chromatogram of thyme liposoluble fraction

The percentages shown in Table 1 refer to the area of the peaks detected by the GC. It can be observed that the thyme volatile oil contains high amounts of p-cymene (30.53%) and its monoterpene phenol derivative - thymol (30.86%) which suggests that the chosen herb belongs to thymol chemotype. Also, there are detected other minor compounds in ranges between 0.32-4.24%.

The liposoluble fraction reveals a scarce content of volatile compounds, only thymol and p-cymene (1.01% and 0.81%, respectively).

Table 1. Volatile components identified in thyme samples

Compound	Thyme volatile oil (%)	Thyme liposoluble fraction (%)
Monoterpene hydrocarbons		
α-pinene	1.23	-
camphene	0.63	-
β-pinene	0.32	-
myrcene	1.63	-
α-terpinene	0.8	-
p-cymene	30.53	0.81
limonene	0.62	-
sabinene	4.24	-
Monoterpene eters		
1,8-cineole	1.24	-
Monoterpene alcohols		
linalool	2.73	-
α-terpineol	1.24	-
geraniol	0.64	-
borneol	3.16	-
Monoterpene ketones		
camphor	0.83	-
Monoterpenic esters		
bornyl acetate	0.7	-
Terpenoidic phenols		
thymol	30.86	1.01
carvacrol	3.37	-
Sesquiterpene hydrocarbons		
caryophyllene	2.48	-

Antioxidant potential assay

Total antioxidant capacity of the thyme samples is presented in Figure 4. Total antioxidant capacity of the extracts is expressed as the number of equivalents of ascorbic acid. The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo (V) complex with a maximal absorption at 695 nm. Both samples show a dose-dependent antioxidant capacity, higher for volatile oil fraction. At low doses (0.1-0.03 mg/mL) the activity is weak and detectable for volatile oil, and at 0.01mg/mL is absent.

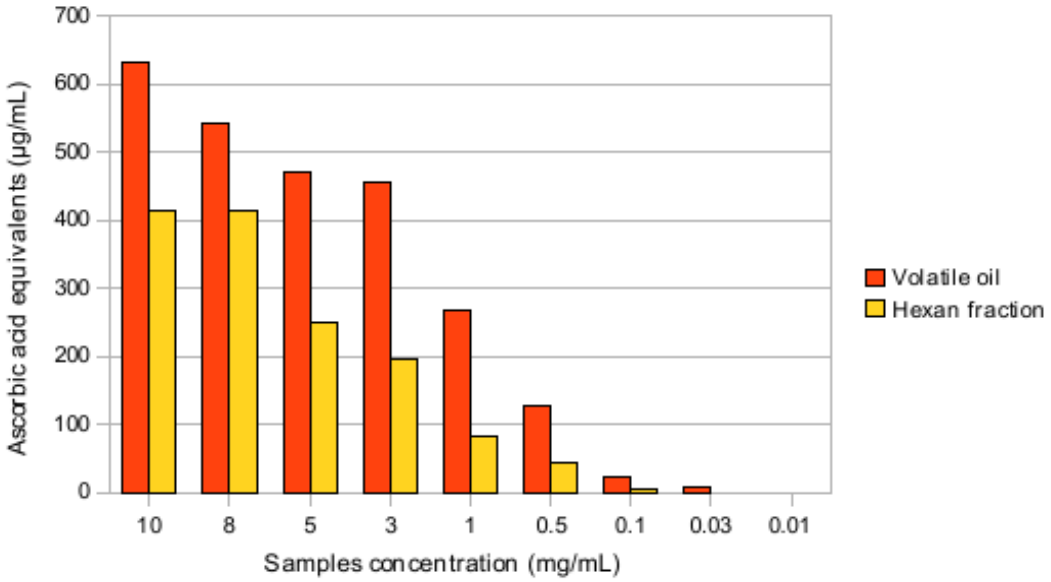

Figure 4. Total antioxidant capacity of the thyme fractions

Free radical scavenging assay

The DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to discolor in the presence of antioxidants. Comparison of the antioxidant activity of the two samples (at doses ranging of 0.01-10mg/mL) and a reference standard - quercetin (at a dose of 0.2mg/mL) is shown in Figure 5. At doses higher than 3mg/mL both extracts exhibit over 50% inhibition on DPPH free radical. Reference standard quercetin showed significant inhibition activity – 89%.

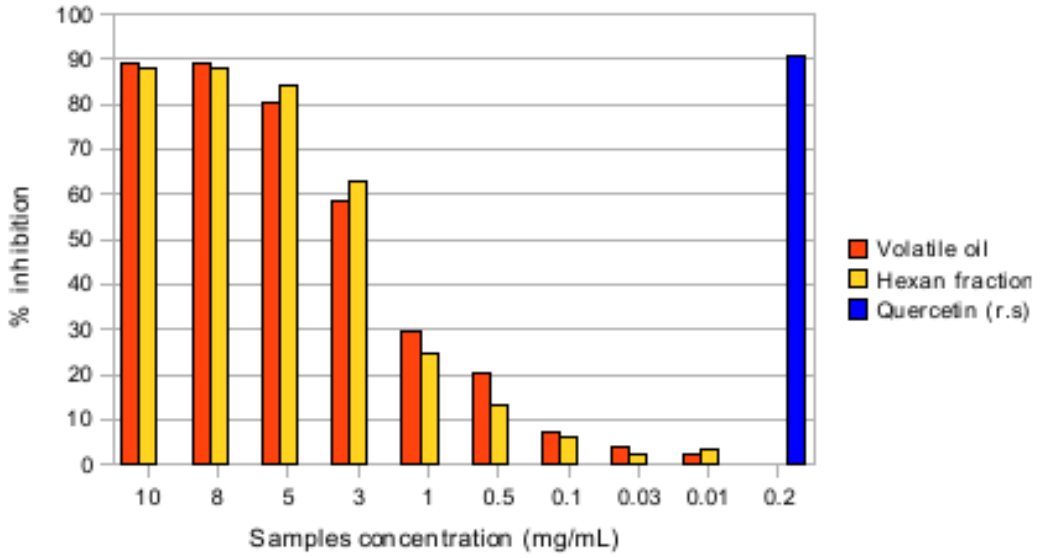

Figure 5. DPPH scavenging activity of the Thyme samples at different doses

Conclusions

As it is described in literature (J. THOMPSON & al. [3]), the present study confirmed that in the oil of the thymol chemotype (which occur at the end of the biosynthetic chain), the dominant monoterpene co-occurs with relatively high levels (up to 30% of the oil) of two

precursors, γ -terpinene and para-cymene.

Thyme volatile oil was obtained by two different methods – steam distillation in a Neoclevenger system which gave a higher quality oil and extraction with organic solvents which gave a mixture of volatile oil and other non-polar compounds.

Although the HPTLC fingerprint of the thyme volatile oil samples obtained by different methods shows the chemical composition similarity, GC analysis revealed the presence of only two monoterpenes in the liposoluble fraction, the same that are found in higher amounts in volatile oil sample. Predominant compounds are thymol (30.86% w/w and 1.01% w/w, respectively) and p-cymene (30.53% w/w and 0.81% w/w, respectively).

The antioxidant potential was investigated by two methods: the phosphomolybdenum reduction assay and DPPH assay. Both samples show a good antioxidant capacity, dose-dependent, slightly higher for volatile oil fraction.

The results show that the activity is not necessarily correlated with volatile oil content; other non-volatile compounds from lipophilic fraction exhibit a good scavenging activity on free radicals.

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