

CHEMICAL COMPOSITION AND CANCER CHEMOPREVENTIVE EFFECT OF VOLATILE OIL ISOLATED FROM *THYMUS PULEGIOIDES* L.

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Abstract

Thymus species have been used for centuries as a folk remedy against cancer. The aim of the present study is to highlight some of the molecular mechanisms involved in the anticancer properties of *Thymus pulegioides* L. The experiments were performed on male Wistar rats, using the volatile oil from *Thymus pulegioides* L. The modifications of cytochrome b₅ content, cytochromes P450 content and activity, glutathione S-transferase (GST), DT-diaphorase activity and glutathione (GSH) content were evaluated. GC-MS (gas chromatography - mass spectrometry) analysis showed that the main components of *Thymus* volatile oil were: carvacrol 17.92%, thymol 4.37% and germacrene D 9.84%. The positive effects of *Thymus* volatile oil could be seen in modifications of the tumors volume and the immune response. An increase was determined in the activity of phase II enzymes, GST and DT-diaphorase, with 75% and 120% respectively. The most remarkable result of this study is the difference in the macroscopic aspects of the tumors, following the administration of *Thymus* volatile oil, which could recommend it as a chemopreventive agent.

Rezumat

Speciile de *Thymus* s-au folosit din cele mai vechi timpuri ca remediu popular împotriva cancerului. Scopul acestui studiu este de a sublinia unele dintre mecanismele moleculare implicate în proprietățile anticancerigene ale speciei *Thymus pulegioides* L. Cercetarea s-a efectuat pe șobolani masculi Wistar folosind ulei volatil izolat din specia *Thymus pulegioides* L. Au fost urmărite modificările conținutului de citocrom b₅, citocrom P450 și glutatation (GSH), cât și ale activității citocromului P450, glutatation S-transferazei (GST) și DT-diaforazei. Analiza GC-MS (gaz cromatografie cuplată cu spectrometrie de masă) a arătat că principalele componente ale uleiului volatil de *Thymus* sunt: carvacrol 17,92%, timol 4,37% și germacren D 9,84%. Efectele uleiului volatil de *Thymus* asupra tumorilor canceroase au fost reliefate atât de scăderea volumului tumorilor, cât și de intensitatea răspunsul imun. S-a evidențiat creșterea activității enzimelor de faza II, GST și DT-diaforaza, cu 75% și respectiv 120%. Rezultatul remarcabil al acestui studiu este diferența netă dintre aspectele macroscopice ale tumorilor după administrarea uleiului volatil de *Thymus* față de cele martor. Pe baza acestui rezultat uleiul de *Thymus* poate fi recomandat drept agent chemopreventiv în boala canceroasă.

Keywords: *Thymus pulegioides* L., GST, cytochrome P450, DT-diaphorase

Introduction

Cancer chemoprevention, as first defined by Sporn [22], uses natural, synthetic or biologic chemical agents to reverse, suppress or prevent carcinogenic progression. It is based on the concepts of multifocal field carcinogenesis and multistep carcinogenesis. Chemoprevention trials are based on the hypothesis that interruption of the biological processes involved in carcinogenesis will inhibit this process and, in turn, reduce cancer incidence. This hypothesis provides a framework for the design and evaluation of chemoprevention trials, including the rationale for the selection of agents that are likely to inhibit biological processes and the development of intermediate markers associated with carcinogenesis [11,13,14,17].

The aim of the present study is to elucidate the chemical composition of the volatile oil from *Thymus pulegioides* L. growing in Romania and to evaluate its biological activity. The study was carried on male Wistar rats inoculated with cells from Walker tumor (epithelial carcinoma). The effects were appreciated as changes in: a) tumors volume and morphology, b) immune status, c) contents and activity of cytochrome P450, d) content of cytochrome b₅, e) activity of glutathione-S transpherase (GST), and f) content of glutathione (GSH).

Materials and methods

Chemicals

Solvents and reagents were purchased from Merck, Darmstadt, Germany: dichloromethane was supraSolv for gas-chromatography; anhydrous Na₂SO₄ granulated for organic trace analysis was used. The n-alkanes C₈ – C₂₀ solution in hexane was used for the determination of Kovats index. 5,5'-dithio-bis-2-nitro-benzoic acid (DTNB) was purchased from Sigma (St. Louis, MO, USA).

Plant material

The aerial plant material of *Thymus pulegioides* L. was harvested during its flowering stage, from mountainous pasture with altitudes between 800 – 900 m (Bușteni, June 2009).

Thyme volatile oil extraction

50 g of dried plant material were hydrodistilled in a Clevenger-type apparatus without organic solvent for 4 h according to the Romanian Pharmacopoeia [20]. The volatile oil was dried over anhydrous Na₂SO₄, stored in a dark glass bottle and kept at 4°C until analysis or biological test. The volatile oil was diluted in dichlorometane (1/200) for GC analysis and 1 µL was injected.

Volatile oil analysis by GC-MS

GC-MS analysis of *Thyme* oil was carried out using a Fisons Instruments GC 8000 with an electron impact quadrupole, MD 800 mass spectrometer detector. The electron ionisation energy was 70eV. A fused silica column 5% phenylpoly (dimethylsiloxane) (SLB – 5 ms, 30 m x 0.32 mm i.d., film thickness = 0.25 µm) was employed. The operating conditions were the following: a split-splitless injector (split ratio, 1/30) at 280°C, ion-source temperature 200°C and the interface temperature 280°C; initial column temperature, 40°C for 3 min, raised at 4°/min to 280°C and finally held isothermally for 20 min; the carrier gas (helium) flow rate was 2 mL/min; sample volume injected, 1 µL. Data acquisition was performed with MassLab Software for the mass range 30-600 u with a scan speed of 1 scan/s.

The identity of volatile oil components was established from their GC Kovats retention indices and from mass spectra by computer matching with mass spectra library (NIST, WILEY and a personal library of 600 spectra). The linear retention indices were determined in relation to a homologous series of n-alkanes (C₈ – C₂₀).

Animals' treatments

Male Wistar rats (weighing between 180 and 200g) were purchased from the Institute of Oncology (Bucharest, Romania). Twenty animals were randomly divided in four groups and fed *ad libitum*, for 3 days with: lot – 0 control, (without tumor), water; lot – I, (tumor), water; lot – II, (tumor), sunflower oil (0.2mL/animal); lot – III, (tumor), *Thymus pulegioides* L. volatile oil and sunflower oil (dilution 1:100), 0.2mL/animal, by gavage. Twentyfour hours after the last administration, rats belonging to lots I – III were inoculated subcutaneously into the flanks of rat back with suspension cells (1 mL) from ascitic fluid of Walker tumor. The animals were inspected daily. Tumors appeared 9 days *postinoculum*. After 14 days, all the animals were sacrificed by exsanguination and the tumor size was measured. Blood and liver were collected for evaluation of the immune status and enzymatic activity. All the animal experiments were performed according to the rules of EU council directive 76/768/EEC.

Flow cytometric analysis

Flow cytometric analyses were performed on a FACSCalibur flow cytometer (Becton-Dickinson), equipped with 5 W argon laser operating at 250 mW and 488 nm. Data were analyzed using CellQuest software. Data were expressed as percentage (%) of labeled cells, or in the form of separate, comparative, contour histograms and dot plots. The rat immune status was expressed as the ratio CD4/CD8.

Preparation of liver microsomes

Microsomal liver fraction was prepared from rat tissue as previously described [21].

Determination of cytochromes P450 content

The content of cytochrome P450 in the microsomal suspension was calculated using the extinction coefficient $91 \text{ mM}^{-1}\text{cm}^{-1}$ from the difference spectrum of sodium dithionite-reduced and CO-saturated P450 as described by Johannesen [5].

Determination of cytochrome b_5 content

The content of cytochrome b_5 in microsomal suspension was evaluated according to the method of Omura [12] by recording the difference in absorbance between 424 nm and 409 nm, using an absorption coefficient of $185 \text{ mM}^{-1}\text{cm}^{-1}$.

Determination of cytochrome P450 activity

Microsomal cytochrome P450 activity was determined by the method of Moldens [10]. This activity is expressed as nmoles p-nitrophenol formed/min/mg protein.

Determination of Glutathione S-transferase (GST) activity

The cytosolic glutathione S-transferase activity was determined spectrophotometrically at 340 nm following the formation of GSH conjugate with 1-chloro-2,4-dinitrobenzene (CDNB) at 37°C , according to the procedure of Habig [4]. This specific activity of GST is expressed as nmoles of GSH-CDNB conjugate formed / min / mg protein using an extinction coefficient of $9.6 \text{ mM}^{-1}\text{cm}^{-1}$.

Determination of DT-diaphorase activity

The DT-diaphorase activity was measured as described by Ernster [1] with NADPH as the electron donor and 2,6-dichlorophenol-indophenol (DCPIP) as the electron acceptor at 600 nm. The DT-diaphorase activity was calculated by reading the absorbance after 3 min at 600 nm with an extinction coefficient value of $21 \text{ mM}^{-1}\text{cm}^{-1}$. One unit of enzyme activity has been defined as the amount of enzyme required to reduce one nmole of DCPIP/min/mg protein.

Glutathione (GSH) content

The glutathione content was determined by Fiala method [2].

Estimation of protein

The protein content was estimated by measuring the difference in absorbance at 280 nm and 310 nm (optical density - $\text{OD}_{280-310}$) [3].

Statistical analysis

Data are expressed as mean $\pm$ S.E.M. The comparison between the means of treatment (*Thymus* volatile oil and control group) was performed using Student's t-test. P-values ≤ 0.05 were considered statistically significant.

Results and discussion

Chemical composition of volatile oil isolated from Thymus pulegioides L.

The essential oil was obtained from air-dried plant material in a yield of 0.99 – 1.0% v/w. The data concerning the chemical composition of the volatile oil from *Thymus pulegioides* growing wild in Romania have been reported in previous studies [15,18]. The composition of the volatile oil obtained from the aerial parts of *Thymus pulegioides* L. is reported in Table I. The chemical composition of the essential oil obtained and used by us in this study is similar with the results reported in literature on the same species of *Thymus pulegioides* [6,16].

Table I
Composition of the volatile oil of aerial parts of *Thymus pulegioides*

No.	Compound	Area %	No.	Compound	Area %
1	α -thujene	0.33	31	geraniol	0.33
2	α -pinene	1.12	32	E-citral	1.12
3	camphene	1.10	33	endo-bornyl acetate	1.10
4	sabinene	0.17	34	thymol	0.17
5	β -pinene	0.21	35	carvacrol	0.21
6	vinyl amyl ketone	0.10	36	α -terpenyl acetate	0.10
7	1-octen-3-ol	0.42	37	β -bourbonene	0.42
8	β -myrcene	2.92	38	β -elemene	2.92
9	3-octanol	0.10	39	trans-caryophyllene	0.10
10	α -phellandrene	0.04	40	α -humulene	0.04
11	3- δ -carene	0.02	41	alloaromadendrene	0.02
12	α -terpinene	0.53	42	β -cadinene	0.53
13	p-cymene	1.41	43	germacrene D	1.41
14	limonene	0.63	44	bicyclogermacrene	0.63
15	1,8-cineole	0.76	45	α -muurolene	0.76
16	Z- β -ocimene	0.43	46	α -farnesene	0.43
17	phenylacetaldehyde	0.01	47	γ -cadinene	0.01
18	E- β -ocimene	3.68	48	δ -cadinene	3.68
19	γ -terpinene	3.49	49	α -cadinene	3.49
20	α -terpinolene	0.13	50	E- α -bisabolene	0.13
21	linalool	6.61	51	elemol	6.61
22	nonanal	0.04	52	germacrene B	0.04
23	1-octen-3-yl acetate	0.01	53	β -copaen-4- α -ol	0.01
24	(D)-camphor	0.73	54	caryophylla-3,8(13)-dien-5-beta-ol	0.73
25	borneol	0.87	55	γ -eudesmol	0.87
26	1-terpinen-4-ol	0.70	56	t-cadinol	0.70
27	α -terpineol	0.23	57	t-muurolol	0.23
28	nerol	0.16	58	α -muurolol	0.16
29	methyl thymyl ether	0.11	59	α -cadinol	0.11
30	carvacryl methyl ether	1.41	60	agarospirol	1.41
			61	(Z,Z) farnesol	1.65
TOTAL					95.65

Sixty-one components of the volatile oil were identified accounting for 95.65% of the oil. The oxygen-containing phenolic monoterpene, carvacrol (17.92% from total content), was found to be the major constituent of the volatile oil while its isomer, thymol, is only 4.37%. The percentage of monoterpene hydrocarbons varies between 0.02% for 3- δ -carene and 3.68% for E- β -ocimene with a total of 16.21% from the amount of volatile oil obtained. The phenolic precursors were present in the volatile oil: 3.49% γ -terpinene and 1.41% p-cymene. Sesquiterpene hydrocarbons had concentration values between 0.9% (E- α -bisabolene) and 9.84% (germacrene D), accounting for 23.82% of the total area. The total content of monoterpene alcohols was 8.87%, the most abundant component of this fraction being linalool (6.61%). The added value of sesquiterpene alcohols reached 15.17%. Thus the following alcohols account for more than 1%: elemol (5.52%), α -cadinol (4.33%), β -copaen-4- α -ol (2.33%), farnesol (1.65%) and t-murolol (1.01%). The esters, terpenyl acetate and bornyl acetate, were found in the volatile oil with a content of 2.04% and 0.27%, respectively.

Effects of the volatile oil on tumor evolution

The clinical status of animals preventively treated was in physiological levels (i.e. temperature, nasal mucosa, appetite, were normal). Aspect of tumors is presented in Table II and Figure 2.

Table II

Differences in tumor evolution

Group ^a	Tv ^b (cm ³)	Tumor aspect
II	2.3	big tumors with many adherence, strong vascularization
III	1.0	nonadherence, dispersation, necrosis

^a see Material and Methods; ^b Tv=tumor volume, medium value, $Tv = 4/3\pi R^3$, R= tumor medium diameter.

Assays for the immune status

The monoclonal antibodies used to discriminate between lymphocytes population and subpopulations are given in Table III.

Table III

Monoclonal antibodies used to identify lymphocytes antigens

Monoclonal antibodies	Receptor	Purchased from
IF4/W3-25	CD3/CD4- Tcells/Thelper	AbD Serotek, UK
IF4/OX8	CD3/CD8+, Tcells/Tsuppressor	AbD Serotek, UK
OX 33	CD45RA+, Bcells	AbD Serotek, UK

There was a remarkable increase in the immune status (CD4/CD8) for the animals with tumor treated with *Thymus* volatile oil. The results obtained are presented in Table IV.

Table IV
Changes in the lymphocytes subpopulations induced by *Thymus* volatile oil

Lot ^a	% CD3	% CD4	% CD8	% CD45RA	CD3 % (CD4+CD8)	CD4/CD8
0	46.82	35.01	10.84	12.50	45.85	3.22
II	55.54	9.98	47.04	4.48	57.02	0.21
III	30.42	15.94	11.20	9.30	27.14	1.42

^a see Material and Methods.

Assays for cytochromes P450 and b₅

The effect of dietary feeding of the rats with *Thymus* volatile oil was evaluated through the activities of cytochromes P450 and b₅ systems. There was, as expected, a moderate increasing in their content and activity due to the sunflower oil (group II, III). Addition of *Thymus* volatile oil (group III) induces a slight but significant reduction of the level and activity in cytochrome P450 correlated with an increase in b₅ content (Table V).

Table V
Effect of *Thymus* oil on liver cytochromes

Cytochromes	Group 0	Group I	Group II	Group III
Level of P450 ^a	0.3214 ± 0.041	0.2856 ± 0.034	0.3035 ± 0.063	0.2432 ± 0.083
Activity ^b of P450 ^a	1.69 ± 0.12	1.1 ± 0.06	2.42 ± 0.478	2.27 ± 0.355
Level of b ₅ ^a	0.1516 ± 0.006	0.1447 ± 0.087	0.2095 ± 0.1	0.2647 ± 0.05

^a expressed as nmols/mg protein; ^b expressed as nmols/mg protein.min.

It is difficult to explain the changes in cytochrome P450 activity. We found a significant increase, at the same level, in the case of rats treated with sunflower oil (group II) and those treated with sunflower oil and *Thymus* volatile oil (group III). The treatment produced a significant change in the total hepatic cytochromes P450 content (a decrease of 11%) and cytochrome b₅ content (an increase of 43%).

Assays for GST and DT-diaphorase enzyme activities and GSH content

The cytosolic GST activity was determined according to the standard procedure of Habig [4]. The obtained results are presented in Figure 1.

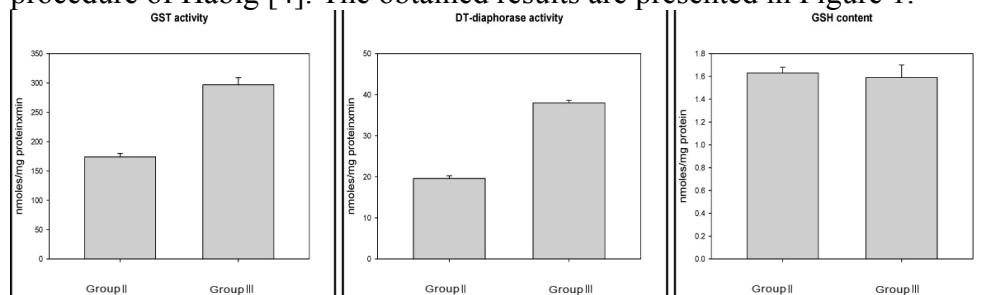


Figure 1

Effect of *Thymus* volatile oil on liver glutathione S-transferase and DT-diaphorase enzyme activities and liver glutathione level.

As expected we found an increase in the activity of these phase II enzymes, GST and DT-diaphorase (compared with controls), 75% and 120% respectively, but the GSH content did not change. Chemopreventive agents influence the immune response through a number of mechanisms and can be evaluated by different parameters as reducing the tumor volume and tumor necrosis, activities of phase I and II enzymes and the immune response.

The modulatory influence of *Thymus* volatile oil on metabolizing enzymes along with the inoculation of cancerous cells (Walker tumor cells) was assessed. The cytochrome P450 is known to play a pivotal role in the oxidative activation of xenobiotics. GST is a critical detoxification enzyme that primarily functions in conjugating the cytochrome P450 metabolites to endogenous ligands (GSH), favoring their elimination from the body. Thus, in practice, both inhibition of cytochrome P450 (phase 1) and induction of electrophile detoxification enzymes (phase 2) can be envisioned to contribute to the chemoprevention performed by drugs. The species differences in P450 enzymes and their modulation by dietary chemicals are also a factor of concern, but the effects observed in animals may be different from those in humans [8,9]. The enhanced level of cytochrome b₅ augments the functional activity of cytochrome P450. Increases in the levels of cytochrome b₅ and P450 may thus lead towards overall protection (Table V).

An enhancement of GST activity and other phase II enzymes due to treatment with aqueous extracts of plants, namely *Camellia sinensis* and *Rosmarinus officinalis* has been reported and related to cancer chemoprevention [7,19,23].

The changes in cytochromes P450 content and activity due to the treatment with *Thymus pulegioides* volatile oil are consistent with these explanations. It may be speculated that an increase of GST activity will detoxify hydrophobic electrophiles by binding them covalently or by establishing their conjugation with GSH. Induction of DT-diaphorase has been evaluated as a mean of determining the potency of many chemopreventive substances. Consistent with these facts, we observed a significant induction in both GST and DT-diaphorase in *Thymus* volatile oil treated rats (Fig. 1).

DT-diaphorase induction facilitates bioreductive activation metabolism of quinones by two electron reduction to hydroquinone, obliterating semi-quinone and oxygen radical formation. Hydroquinone formed *via* two electron reduction by DT-diaphorase can be conjugated by glucuronide or sulfate and excreted. It may thus be inferred that induction of DT-diaphorase in the liver by the *Thymus* oil, provides *in vivo* protection (Figure 1).

Thymus pulegioides L. volatile oil pretreatment decreased cytochrome P450 content and its activity but increased the activity of antioxidant enzymes GST and DT-diaphorase in the rat liver. The present findings demonstrate that the potential cancer chemoprevention of *Thymus* could be attributed to its modulatory effect on the xenobiotic bioactivation and detoxification processes. These results suggest that the inhibition of cytochrome P450 and the induction of phase II enzymes, could be responsible for the cancer chemopreventive effect of *T. pulegioides* volatile oil.

The most important result of this study is the difference in the macroscopic aspect of the tumors following the administration of *Thymus* volatile oil, which could recommend it as a chemopreventing agent. The tumors developed in rats treated with *Thymus* volatile oil are smaller, less adherating than those in rats that received only water or sunflower oil (Fig. 2).

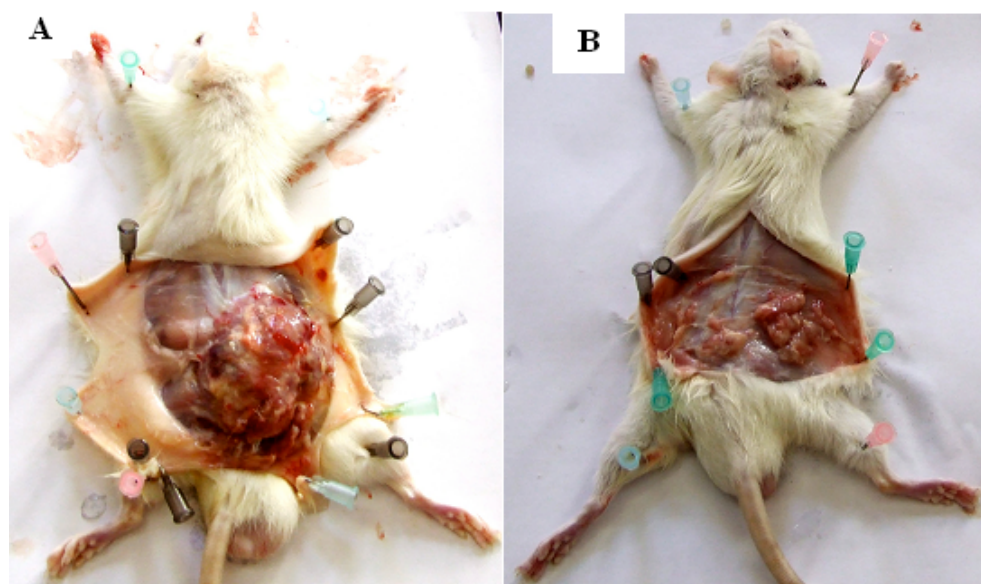


Figure 2

The animals from groups II(A)-III(B), inoculated subcutaneously with suspension cells from ascitic fluid of Walker tumor.

Conclusions

The most important result of this study is the difference in the macroscopic aspect of tumors, which could recommend *Thymus* volatile oil as a cancer chemopreventive agent. The key factor in these results can be the complex composition of *T. pulegioides* volatile oil. The results obtained in this experiment are encouraging and justify further research on larger numbers of subjects.

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