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Chemical Composition and Biological Activity of Aerial Parts of Thymus collinus Bieb. Growing in Georgia

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ABSTRACT

Background: Thymus L is one of the most important genera of the Lamiaceae family. In traditional medicine, leaves and flowers of Thymus species are used as a tonic, antiseptic, analgesic, anti-inflammatory, and antitussive. Complex medicines are prepared from Thymus species, such as Neopertussin and Pertussin.

Objectives: The research aimed to study essential oil's chemical composition and biological from aerial part of Thymus collinus Bieb. endemic species growing in Georgia.

Methods: Thymus collinus aerial parts essential oil were hydro-distilled with a Clevenger-type apparatus. The quantitative content of the dominant compound thymol was determined. In order to develop a rational use of raw material and a resource-saving technology, the study on biologically active, nonvolatile substances in the residual plant material was continued. The aqueous extract was lyophilized, and the dried residual plant material was successfully extracted with chloroform and methanol. The obtained extracts were dried using a rotary evaporator. Essential oil DPPH radical scavenging capacity was evaluated. Also, the antibacterial activity of essential oil was evaluated using five different strains. Essential oil cytotoxic screening was conducted against lung carcinoma A-549, colon carcinoma DLD-1, and human normal fibroblasts WS-1 cell lines. Total phenolic content in aqueous, methanolic, and chloroformic extracts was determined using the Folin-Ciocalteu (FC) reagent. Essential oil, aqueous, methanolic, and chloroformic extracts antioxidant activity was evaluated using ORAC test and tests on human skin fibroblast (WS1). Also, anti-inflammatory activity was evaluated (inhibition of nitric oxide production).

Results: In the essential oil from aerial parts of Thymus collinus, endemic to the Caucasus, the major constituents are thymol (42.62 %) and carvacrol (16.51 %). Among the terpene compounds, oxygenated monoterpenes are dominants - 63.23 %. A high content of phenols (53.2 ± 0.9 %) was detected in aqueous extract. Essential oil's scavenging rate of DPPH free radicals was 94.2 % at a concentration of 2.87 mg/ml. The essential oil showed high antioxidant activity in the ORAC and in vitro tests using a cellular model (WS-1). At the same time, essential oil showed high antibacterial activity against 5 (Escherichia coli, Salmonella enterica, Enterococcus faecium, Staphylococcus epidermidis, and Pseudomonas aeruginosa) strains. Thymus collinus essential oil at 80 µg/ml has inhibited NO production by 63 %; no toxicity was revealed even at 160 µg/ml concentration. Thymus collins aqueous extract showed high antioxidant activity in the ORAC test (3.38 ± 0.05 µmol TE/mg); in the case of methanolic extract, slight anti-inflammatory activity was revealed. Essential oil demonstrated moderate general cytotoxicity (85 ± 16 µg/ml) against lung carcinoma (A-549).

Conclusions: Thymol is the main component in Thymus collinus essential oil; oxygenated monoterpenes are abundant in essential oil. EO exhibited high antioxidant, anti-inflammatory, and antibacterial activities. Based on the results, Thymus collinus Bieb., an endemic species to Caucasus, may be used as a substitute raw material for officinal species.

Keywords: Biological activity; essential oil; phenolic compounds; Thymus collinus.

INTRODUCTION

One of the most important genera of the Lamiaceae family is Thymus L.,[1] which consists of about 350 species spread worldwide, especially in the Mediterranean area.[2,3] Genus Thymus 14 species are spread in Georgia; 9 are endemic to the Caucasus, and two are endemic to Georgia.[4,5] In traditional medicine, leaves and flowers of Thymus species are used as a tonic, antiseptic, analgesic, anti-inflammatory, and antitussive.[6,7]

Thymus species essential oil dominant compounds thymol and carvacrol are characterized by antioxidant, antibacterial, anti-inflammatory, and anticancer activities; also, the official species essential oils antimicrobial, antioxidant, anti-inflammatory, anticarcinogenic, cardioprotective, neuroprotective, fungicidal, hypoglycemic activities, etc. are revealed.[1,7-12]

Among Thymus species, Thymus vulgaris L. and Th. serpyllum L. is the most widespread and is used for medicinal purposes.

The ethanolic extract obtained from Thymus vulgaris L. aerial parts showed cytostatic activity against the leukemia cell line.[13] Antibacterial, antioxidant, and anthelmintic actions characterize ethanolic extract.[14,15] Leaves ethanolic extract revealed a therapeutic effect on albino mice in treating chronic toxoplasmosis.[16]

Thymus serpyllum methanolic extract revealed significant cytotoxic activity against breast cancer cells (MCF-7 and MDAMB-231).[17] Aqueous extract is characterized by free radical scavenging activity and antihypertensive activity in mouse experiments.[18]

Thymus collins L. is endemic to South and East Transcaucasia. It is spread throughout Georgia.[19,20] Aerial parts contain essential oil, phenolic acids, and flavonoids. In folk medicine, aerial parts extracts are used as an expectorant.[19,20]

Genus Thymus officinal species do not grow wild in Georgia.[4,21] Therefore, to expand the raw material base, studying the aerial parts of the endemic species Thymus collinus growing in Georgia is relevant. Considering the perspective of biologically active substances detection and use in medicine.

METHODS

Cell culture

The human lung carcinoma A-549 (ATCC, Manassas, USA), colon adenocarcinoma DLD-1 (ATCC, Manassas, USA), normal skin fibroblast WS-1 (ATCC® CRL-1502, Manassas, VA, USA) and murine macrophage RAW 264.7 (ATCC Manassas, USA) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, USA).

Plant material

The object of the study was the aerial parts of Thymus collinus collected in the Tbilisi area. The plant was identified at TSMU Iovel Kutateladze Institute of Pharmacochemistry by chief scientist Ketevan Mchedlidze—specimen voucher - TBPH- 22303.

Isolation of the essential oils

Essential oil was hydro-distilled with three replicates for two hours with a glass Clevenger-type apparatus. The yield of essential oils in ml/kg (also in %) was determined by calculation of the absolute dry mass of raw materials. Essential oil was stored at +4^{0}C until use.

Preparation of aqueous, methanol, and chloroform extracts

After obtaining essential oil from the aerial parts of Thymus collinus, the aqueous extract was lyophilized, and the dried residual plant material was extracted with chloroform and methanol. The obtained extracts were dried using a rotary evaporator.

GC-MS analysis

The study of the chemical composition of the essential oil, the quantitative content of the dominant component, and the qualitative analysis of the compounds of terpenic nature were performed by Gas Chromatography (Agilent technologies 7890B) - Mass Spectrometry (Agilent Technologies 5977A MSD) technique. Mass spectra were obtained in scan mode (70 eV).

Total phenolic content

The total phenolic content was detected using the Folin-Ciocalteu (FC) reagent.[22]

Antioxidant activity

Essential oil, aqueous, methanolic, and chloroformic extracts antioxidant activity was evaluated using the oxygen radical absorbing capacity assay (ORAC test)[22] and human skin fibroblast (WS1).[23] The study sample's inhibitory concentration 50 (IC50), which inhibits the oxidation of 2',7'-dichlorofluorescein (DCFH) by 50 %, was determined. The reference drug was etoposide and trolox.

DPPH radical scavenging assay

Thymus collinus essential oil antioxidant activity was evaluated by the spectrophotometric method using a 2,2 – diphenyl-1-picrylhydrazyl (DPPH) reagent. The formula calculated the ability to scavenge free radicals in percentage:

$$x\ \% = \frac{\mathrm{Ac} - \mathrm{As}}{\mathrm{Ac} * 100}$$

Where: Ac – absorption of DPPH methanolic solution;
As – absorption of samples.

The standard thymol solution was prepared at five different concentrations, with an inhibitory concentration of IC50=3.99 mg/ml.

Anti-inflammatory activity assay

The anti-inflammatory activity was evaluated by assessing nitric oxide (NO) inhibition in vitro. A positive control, N-l-nitro-L-arginine methyl ester hydrochloride (L-NAME) was used. The presence of nitrite was quantified using a NaNO2 standard curve.[24]

Cytotoxicity by resazurin and Hoechst assay

In vitro, cytotoxicity was assessed using the Hoechst (DNA determination) and Resazurine spectrophotometric methods, and fluorescence was measured using an automated 96-well Fluroskan Ascent FITM plate reader (Labsystems) at 530 nm, 590 nm (Rezaurine) and 365 nm, 460 nm (Hoechst). In the Hoechst test, the fluorescence in each well is proportional to the number of cellular DNA; in the Resazurine test - fluorescence was proportional to the cellular metabolic activity in each well. In vitro, the anticancer activity of samples was evaluated against lung carcinoma A-549, colon carcinoma DLD-1, and human normal fibroblasts WS-1 cell lines. The concentrations of samples were determined, which caused the inhibition of 50 % of cell growth (IC50 µg/ml). Etoposide was used as a positive control.[25,26]

Antibacterial activity assay

Antibacterial activity of essential oil was conducted using Spot test (screening) against five strains: E. coli,

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Pseudomonas aeruginosa, Salmonella enterica, Enterococcus faecium, and Staphylococcus epidermidis.[27]

RESULTS

Determination of the EO chemical composition

In the aerial parts of Thymus collinus essential oil, 26 compounds were detected (Tab.1). The significant constituents were characterized as thymol (42.62 %) and carvacrol (16.51 %). The essential oil's oxygenated monoterpenes content is 63.23 %, monoterpene hydrocarbons - 24.94 %, and sesquiterpene hydrocarbons 6.48 % (Fig.1).

TABLE 1. Terpene composition of essential oil from Thymus collinus

	Compounds	RT	RI (exp)	RI (ref)	Essential oil content, %
1	α-Thujene (MH)	8.71	980	1102	0.78
2	Pinene (MH)	8.94	998.5	974	0.56
3	Camphene (MH)	9.55	1001	946	0.47
4	Myrcene (MH)	11.72	1057	1090	0.85
5	1-Octen-3-ol (O)	11.33	918	972	0.6
6	α - terpinene (MH)	12.87	1025	1014	1.37
7	Cymene (MH)	13.45	1098	1020	14.39
8	Eucalyptol (MH)	13.65	1023	1026	1.61
9	Terpinene (MH)	15.16	1055	1054	4.91
10	Cis-Sabinene hydrate (OM)	15.59	1065	1065	1.3
11	Camphor (OM)	19.58	1141	1141	0.11
12	Endo-borneol (OM)	20.84	1140	1165	2.24
13	Terpinen-4-ol (OM)	21.59	1160	1174	0.45
14	Thymol methyl ether (O)	22.69	1132	1232	0.09
15	Thymol (OM)	26.18	1289	1289	42.62
16	Carvacrol (OM)	30.37	1279	1298	16.51
17	Thymol acetate (O)	34.49	1348	1349	0.08
18	Bourbonene (SH)	36.52	1388	1387	0.1
19	Caryophyllene (SH)	39.40	1418	1408	1.12
20	Aromadendrene (SH)	40.87	1429	1439	0.14
21	Humulene (SH)	41.96	1435	1438	0.06
22	Germacrene D (SH)	43.98	1477	1481	0.88
23	Bicyclogermacrene (SH)	45.01	1492	1500	0.95
24	Bisabolene (SH)	46.22	1500	1505	3.07
25	Cadinene (SH)	46.89	1537	1513	0.16
26	Spathulenol (OS)	53.41	1577	1578	0.05

Abbreviations: RI (exp) Retention index is calculated with the help of a homologous series of n-alkanes; RI (ref) Retention Index of compounds from literature RT: Retention times were obtained from GC/MS analysis.

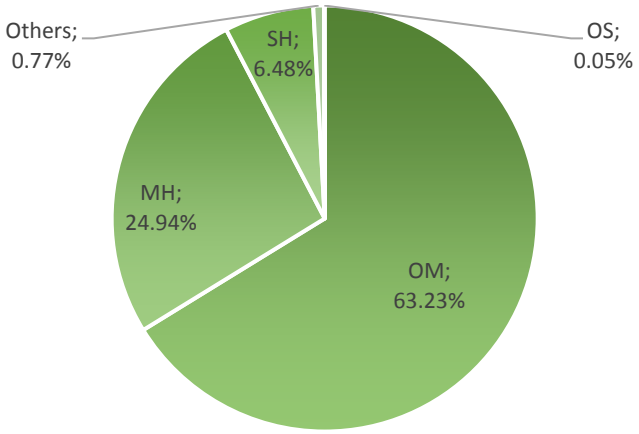
Thymol quantitative analysis

Thymus collinus essential oil dominant compound - thymol quantitative content was determined by gas chromatography with mass spectrometry. A linear dependence was established between 5 concentrations of thymol standard 0.98, 0.49, 0.245, 0.1225, 0.06125 mg/ml, and peak area. The average of 3 parallel trials is 33.69 %.

Total phenolic content

The total phenolic content in the aqueous extract is 53.2 ± 0.9 %, in methanolic 15 ± 2 %, and in chloroformic 2.0 ± 0.3 %; the results were calculated in grams (gallic acid equivalent) per 100 g of extract.

FIGURE 1. Nature of Terpenic composition of essential oil from Thymus collinus (%)



Abbreviations: MH: monoterpene hydrocarbons; OM: oxygenated monoterpenes; OS: oxygenated sesquiterpenes; SH: sesquiterpene hydrocarbons.

Evaluation of cytotoxicity

The results of Thymus collinus essential oil cytotoxic activity assessments are given (Tab.2). The essential oil showed moderate inhibition against lung carcinoma (A-549) in the Hoechst test (85 ± 16 µg/ml). General toxicity was noted against human normal fibroblasts (WS-1) (IC50=150 ± 18 µg/ml).

TABLE 2. Thymus collins essential oil in vitro cytotoxic activity (Inhibitory concentration 50, µg/ml)

	Resazurin			Hoechst		
Samples/ Cell line	A-549	DLD-1	WS-1	A-549	DLD-1	WS-1
Essential oil	173±12	>200	>200	85±16	>200	150±18
Etoposide (µM)	16±3	6.7±1.0	3.4±0.3	<0.391	4±1.0	<0.391

Evaluation of DPPH radical scavenging activity

Essential oil antioxidant activity was determined by the spectrophotometric method using DPPH (2,2 –diphenyl-1-picrylhydrazyl) reagent (Fig.2). By increasing the concentration of the essential oil (0.32 mg/ml - 2.87 mg/ml) the antioxidant activity of increases from 4.94 % up to 94.2 %. The thymol standard increases from 19.75 % (0.7 mg/ml) to 91.11 % (9 mg/ml).

The inhibitory concentration was determined to be IC50 = 1.19 mg/ml. The obtained results confirm the antioxidant activity of the essential oil.

Evaluation of the anti-inflammatory and antioxidant activity

The essential oil showed antioxidant activity 12.2 ± 0.9 µmol TE/mg, both in the ORAC test and in the in vitro test IC50=0.79 ± 0.03 µg/ml using a cellular model (Tab.3). According to the results, the essential oil is characterized by high anti-inflammatory action (63 % inhibition of nitric oxide production) in a low concentration - 80 µg/ml. Toxicity was

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not revealed against murine monocyte-macrophage cells (RAW 264.7), even at 160 µg/ml. Thymus collinus methanolic extract exhibited slight anti-inflammatory activity (36 % inhibition of NO production) without significant toxicity.

FIGURE 2. Essential oil DPPH radical scavenging capacity

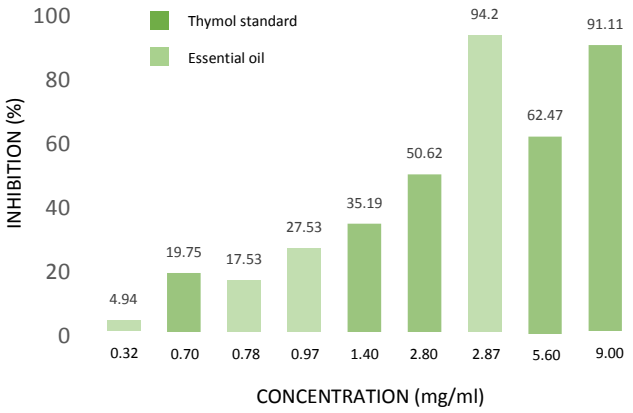

TABLE 3. Antioxidant and anti-inflammatory activity of Essential oil and three extracts from Thymus collinus aerial parts

	Antioxidant IC50 (µg/mL)	ORAC Values (µmol TE/mg)	Anti-Inflammatory IC50 (µg /mL)	Inhibition maximum nontoxic concentration (%)	Toxicity (>20% mortality) (µg/ml)
Quercetin	0.027±0.004	21.1±0.6	-	-	-
Trolox	-	5.1±0.20	-	-	-
L-NAME 250 µM	-	-	-	44±10.0	-
L-NAME 1 mM	-	-	-	67±8.00	-
Essential oil	0.79±0.03	12.2±0.9	69 ± 1.0	63	160
Aqueous extract	4.8± 0.70	3.38±0.05	>80	<0	160
Methanolic extract	11.0±5.0	1.41±0.03	>160	36	ND
Chloroformed extract	>100	0.074±0.01	>2.5	<0	5

Abbreviation: ND: not determined.

Antibacterial activity

The high antibacterial activity of Thymus collinus 0.1 % aqueous essential oil solution was revealed (4+) against five different strains: (E. coli, Salmonella enterica, Enterococcus faecium, Staphylococcus epidermidis, Pseudomonas aeruginosa).

DISCUSSION

A study on the chemical composition of the essential oil obtained from the aerial parts of Thymus collinus Bieb., endemic to Caucasus, was carried out. The percentage of dominant components was determined by integration. The dominant component in the essential oil was thymol - 42.62 % and carvacrol - 16.51 %, which are also the dominant monoterpenes in the essential oil of the officinal species.[6,28] The Quantitative content of the dominant compound thymol, in Thymus collinus essential oil, was determined using a standard solution. From terpene compounds, oxygenated monoterpenes are dominants (63.23 %). The content of phenolic compounds was determined by using the Folin-Ciocalteu reagent in the aqueous, methanolic, and chloroformic extracts of the residual plant material. In the aqueous extract, the total phenolic content is 53.2 ± 0.9 %. In methanolic extract, 15 ± 2 %, chloroformic - 2.0 ± 0.3 %. Essential oil from areal parts of Thymus collinus revealed DPPH free radical scavenging activity. The 2.87 mg/ml concentration exhibited inhibition of DPPH radical at 94.2 %, IC50=1.19 mg/ml. Essential oil showed antioxidant activity in the ORAC test (12.2 ± 0.9 µmol TE/mg), also in the in vitro test IC50=0.79 ± 0.03 µg/ml using a cellular model. The essential oil showed different intensities of antibacterial activity against five strains (Escherichia coli, Salmonella enterica, Enterococcus faecium, Staphylococcus epidermidis, and Pseudomonas aeruginosa). Thymus collinus essential oil at 80 µg/ml concentration inhibits 63 % of NO production, and toxicity was not revealed against murine monocyte-macrophage cells (RAW 264.7) even at 160 µg/ml. Thymus collinus aqueous extract showed significant antioxidant activity (3.38 ± 0.05 µmol TE/mg) in the ORAC test. In the case of methanolic extract, the anti-inflammatory effect was weak. The essential oils exhibited moderate cytotoxic activity against (85 ± 16 µg/ml) lung carcinoma (A-549).

Our research revealed essential oil's high anti-inflammatory and antioxidant activity from Caucasus endemic Thymus collinus. A screening test showed a significant antibacterial activity of the essential oil against tested pathogenic bacteria. Based on the preliminary biological activity data, the research study should be orientated toward isolating and identifying biological markers from active extracts.

CONCLUSIONS

Thymol is the main component in Thymus collinus essential oil; oxygenated monoterpenes are abundant in essential oil. EO exhibited high antioxidant, anti-inflammatory, and antibacterial activities. Based on the results, Thymus collinus Bieb., an endemic species to Caucasus, may be used as a substitute raw material for officinal species.

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