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GC/MS analysis and antibacterial activity of garden thyme (*Thymus vulgaris* L.) extracts against non-tuberculous mycobacteria strains isolated from diseased ornamental fish

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Summary

This study was carried out to evaluate the antibacterial activity of aqueous and organic extracts of *Thymus vulgaris* L. (Lamiaceae). The hexan (HEX), ethanol (ETH) and water (WAT) extracts from aerial parts of *T. vulgaris* exhibited antimicrobial activity against slow growth mycobacteria – SGM (n = 49) and rapid growth mycobacteria – RGM (n = 50) strains isolated from diseased ornamental fish. The MIC_{AV} values of HEX, ETH, and WAT extracts against RGM strains were found to be at 146.50 µg/mL, 728.00 µg/mL, and > 800 µg/mL, respectively. The MIC_{AV} values of HEX, ETH, and WAT extracts against SGM strains were found to be at 50.77 µg/mL, 253.06 µg/mL, and > 800 µg/mL, respectively. The most active extract against RGM and SGM strains was the hexane extract. Its chemical composition was determined using GC-MS, which allowed the identification of 53 compounds. The major chemical compounds in HEX extracts were found to be thymol (27.35%), tetratetracontane (19.68%), dotriacontane (10.34%), p-cymene (5.92%), stigmast-5-en-3-ol (4.89%), carvacrol (2.64%), thymoquinone (2.00%), (1r)-1,6,6-trimethyl-cis-bicyclo[3.3.0]octan-3-one (2.52%), 5,10-dihexyl-5,10-dihydro indole[3,2b]indole-2,7-dicarbaldehyde (2.15%), and linalyl anthranilate (1.06%), the proportion of other components being below 1%. In conclusion, among the tested extracts, the HEX extract exhibits the strongest properties against nontuberculosis mycobacteria isolated from diseased ornamental fish.

Keywords: mycobacteria, plant extracts, *Thymus vulgaris*

Garden thyme (*Thymus vulgaris* L.), member of the Lamiaceae family, is native to southern Europe, where it is often cultivated as a culinary herb. *T. vulgaris* is widely used in folk medicine for its expectorant, antitussive, anti-inflammatory, antioxidant, and antimicrobial properties (37). Thyme herb contains many different phenolic compounds (e.g., thymol, carvacrol, caffeic acid, quinic acid, ferulic acid, syringic acid, rosmarinic acid, gallic acid), monoterpenes (e.g., p-cymene, α-terpinene, linalool), flavonoids (e.g., luteolin, naringenin, apigenin), polysaccharides (e.g., starch, homogalacturonan, rhamnogalacturonan I, and cellulose), saponins, alkaloids, steroids, tannins, mineral compounds (e.g., sodium, potassium, magnesium, calcium, iron, phosphorus, selenium), and vitamins (e.g., B6, B9, E, A, C) (5, 37).

It has been shown that essential oils obtained from *T. vulgaris* have antiparasitic properties: against trophozoites of *Entamoeba histolytica* (7), *Trypanosoma brucei* (32), *Trypanosoma cruzi* (44), *Giardia lamblia* (9); antifungal properties against *Aspergillus niger* (33); antibacterial properties against *Klebsiella pneumonia*, *Escherichia coli*, *Enterobacter cloacae*, *Salmonella* sp. (8), *Mycobacterium tuberculosis* (39). Hexane extracts obtained from *T. vulgaris* have been shown to have activity against *E. histolytica* (7), *M. tuberculosis* (24). Chloroform extract obtained from *T. vulgaris* has been shown to have activity against *E. coli* and *Shigella dysenteriae* (34) and non-tuberculous mycobacteria (20). Hydroalcoholic extract showed activity against the protozoan *E. histolytica* (7), *Staphylococcus aureus* and *E. coli* (15), bacteria such as *K. pneumoniae*,

S. aureus, *Listeria monocytogenes*, *Enterococcus faecalis* (16), and multiple antibiotic resistant *Vibrio parahaemolyticus* and *Vibrio fluvialis* (36).

Fish mycobacterioses are diseases caused by mycobacteria belonging to species other than the *Mycobacterium* complex (*M. tuberculosis*, *M. bovis*, *M. africanum*) and *Mycobacterium leprae*. These mycobacteria are referred to as nontuberculosis mycobacteria (NTM). Various NTM species, including *M. marinum*, *M. chelonae*, *M. peregrinum*, and *M. fortuitum*, cause diseases in fish (18, 27, 40, 42). Fish mycobacteriosis is a chronic, systemic granulomatous disease that can affect various tissues and organs. The symptoms of the disease are non-specific. Observed signs include lethargy, loss of appetite, weight loss, scale loss, ulcers, spinal deformities, ascites, and general emaciation of the fish. Grey or whitish nodules may be present in internal organs such as the kidneys, liver, and spleen. In the early stages of the disease, nodules may be invisible. Mycobacteria causing diseases in fish can also cause skin and soft tissue infections in humans (45). Furthermore, increasing resistance to many anti-mycobacterial drugs is being observed (19, 21). Therefore, new anti-mycobacterial substances are being sought. Plants and their secondary metabolites are rich sources of anti-mycobacterial compounds (12, 41, 43). Literature reports indicate that *T. vulgaris* contains compounds effective against *M. tuberculosis* (24, 39). To the best of the authors' knowledge, *T. vulgaris* extracts have not been studied for their activity against NTMs isolated from fish. Therefore, in this study the anti-NTM potential of the aerial parts of *T. vulgaris* was examined for the first time *in vitro* using hexane (HEX), ethanol (ETH), and water (WAT) extracts.

Material and methods

Plant material and extraction process. Dried *S. hortensis* plants were obtained from the herb wholesaler NANGA (Przemysław Figura, Złotów, Poland). Hexane (HEX), ethanol (ETH), and water (WAT) extracts were prepared following the methods previously outlined by Puk et al. (43).

Study of the antimicrobial activity. The rapid growth mycobacteria (RGM): *Mycobacteroides abscessus* (known previously as *Mycobacterium abscessus*) (n = 1), *Mycobacteroides chelonae* (*Mycobacterium chelonae*) (n = 16), *Mycolicibacterium fortuitum* (*Mycobacterium fortuitum*) (n = 10), *Mycolicibacterium mucogenicum* (*Mycobacterium mucogenicum*) (n = 1), *Mycolicibacterium neoaurum* (*Mycobacterium neoaurum*) (n = 2), *Mycolicibacterium peregrinum* (*Mycobacterium peregrinum*) (n = 12), *Mycobacteroides salmoniphilum* (*Mycobacterium salmoniphilum*) (n = 1), *Mycobacteroides saopaulense* (*Mycobacterium saopaulense*) (n = 1), *Mycolicibacterium senegalense* (*Mycobacterium senegalense*) (n = 4), *Mycolicibacterium septicum* (*Mycobacterium septicum*) (n = 2), and slow growth mycobacteria (SGM): *Mycobacterium gordonae* (n = 15), *Mycobacterium marinum* (n = 33), *Mycobacterium szulgai* (n = 1) were studied. The mycobacterial strains were identified on the basis of molecular characters as described previously (17, 41). *M. fortuitum* ATCC 6841, *M. peregrinum* ATCC 700686, *M. marinum* ATCC 927, and *M. gordonae* ATCC 14470 were used as reference strains. The antimicrobial activity of the extracts was investigated by the method previously described by Puk et al. (43).

Gas chromatography-mass spectrometry (GC-MS). The GC-MS analyses were repeated three times for each sample using a Thermo TRACE GC Ultra gas chromatograph (Thermo Fisher Scientific Inc., Waltham, MA, USA) connected to a Thermo ITQ 1100 ion trap mass spectrometer detector (Thermo Fisher Scientific Inc., Waltham, MA, USA) as previously described by Puk et al. (43).

Results and discussion

The HEX, ETH and WAT extracts from aerial parts of *T. vulgaris* exhibited antimicrobial activity against SGM (n = 49) and RGM (n = 50) strains isolated from diseased ornamental fish. The MIC_{AV} values of HEX, ETH, and WAT extracts against RGM strains were found to be at 146.50 µg/mL, 728.00 µg/mL, and > 800 µg/mL, respectively (Tab. 1). The MIC_{AV} values of HEX, ETH, and WAT extracts against SGM strains were found to be at 50.77 µg/mL, 253.06 µg/mL, and > 800 µg/mL, respectively (Tab. 1 and 2).

The most active extract against RGM and SGM strains was the hexane extract. Its chemical composition was determined using GC-MS, which allowed the identification of 53 compounds (Fig. 1, Tab. 3). The major chemical compounds in HEX extracts were found to be thymol (27.35%), tetratetracontane (19.68%), dotriacontane (10.34%), p-cymene (5.92%), stigmast-5-en-3-ol (4.89%), carvacrol (2.64%), thymoquinone (2.00%), (1r)-1,6,6-trimethyl-cis-bicyclo[3.3.0]octan-3-one (2.52%), 5,10-dihexyl-5,10-dihydro indole[3,2b]indole-2,7-dicarbaldehyde (2.15%), and linalyl anthranilate (1.06%), the proportion of other components being below 1% (Fig. 1, Tab. 3).

In our study, the hexane extract showed the highest activity against the tested SGM and RGM bacteria (MIC_{AV} = 99.12) compared to the ethanol extract (MIC_{AV} = 492.93) and the aqueous extract (MIC_{AV} > 800) (Tab. 1 and 2). The activity of ethanol extracts from *T. vulgaris* has been demonstrated against multiple antibiotic-resistant *V. parahaemolyticus* and *V. fluvialis* (36). The extract studied by Oramadike and Ogunbanwo (36) exhibited high content of flavonoids (89.64 mg/100 g), saponins (54.53 mg/100 g), phenolic compounds (51.76 mg/100 g), anthraquinones (30.35 mg/100 g), alkaloids (26.60 mg/100 g), cardiac glycosides (18.23 mg/100 g), and tannins (6.76 mg/100 g). In our study, the ETH extract exhibited significantly weaker activity against the tested SGM and RGM strains compared to the HEX extract. The WAT extract had the weakest activity, and at the tested concentrations, it did not exhibit any activity (Tab. 1 and 2).

The composition of the HEX extract, analyzed using GC-MS, revealed several chemical compounds

Tab. 1. Sensitivity of the tested NTM strains to HEX, ETH, and WAT extracts of *T. vulgaris*

Bacteria	Extracts	Minimal inhibitory concentration (µg/mL)									
		> 800	800	400	200	100	50	25	12.5	6.25	< 6.25
Rapid growth mycobacteria (n = 50)											
M. abscessus (n = 1)	HEX					1					
	ETH		1								
	WAT	1									
M. chelonae (n = 16)	HEX			1	2	13					
	ETH		15	1							
	WAT	16									
M. fortuitum (n = 10)	HEX				3	4	2	1			
	ETH		10								
	WAT	10									
M. peregrinum (n = 12)	HEX			4	5	2	1				
	ETH		6	6							
	WAT	12									
M. neoaurum (n = 2)	HEX						2				
	ETH		2								
	WAT	2									
M. mucogenicum (n = 1)	HEX				1						
	ETH		1								
	WAT	1									
M. saopaulense (n = 1)	HEX						1				
	ETH		1								
	WAT	1									
M. septicum (n = 2)	HEX				1	1					
	ETH		2								
	WAT	2									
M. salmoniphilum (n = 1)	HEX				1						
	ETH			1							
	WAT	1									
M. senegalense (n = 4)	HEX					2	2				
	ETH		2	2							
	WAT	4									
Slow growth mycobacteria (n = 49)											
M. gordonae (n = 15)	HEX					2	9	4			
	ETH			12	3						
	WAT	15									
M. marinum (n = 33)	HEX					5	20	7	1		
	ETH			3	24	6					
	WAT	33									
M. szulgai (n = 1)	HEX						1				
	ETH			1							
	WAT	1									

Explanations: HEX – hexan extract; ETH – ethanol extract; WAT – water extract

Tab. 2. The MIC values for *T. vulgaris* HEX extract (µg/mL) against isolates of NTMs (n = 99): SGM (n = 49) and RGM (n = 50)

MIC	Hexan			Ethanol			Water		
	RGM	SGM	RGM + SGM	RGM	SGM	RGM + SGM	RGM	SGM	RGM + SGM
MIC_{AV}	146.50	50.77	99.12	728.00	253.06	492.93	> 800	> 800	> 800
MIC_{90}	200	100	200	800	400	800	> 800	> 800	> 800
MIC_{50}	100	50	50	800	200	400	> 800	> 800	> 800

Explanations: RGM – rapid growth mycobacteria; SGM – slow growth mycobacteria; MIC – minimum inhibitory concentration; MIC_{AV} – the mean inhibitory concentration of the tested strains; MIC_{90} – minimum inhibitory concentration required to inhibit the growth of 90% of bacterial strains; MIC_{50} – minimum inhibitory concentration required to inhibit the growth of 50% of bacterial strains

belonging to the terpene group: such as cis-ocimene, camphene, 4-methyl-1-penten-3-ol, α-terpinene, p-cymene, ocimene, eucalyptol, γ-terpinene, sabinene hydrate, linalyl anthranilate, norbornan-2-one, nopol, terpinen-4-ol, p-cymen-8-ol, β-fenchol, cis-limonene-oxide, thymol methyl ether, carvacryl methyl ether, thymoquinone, carvacrol, thymol, α-copaene, β-caryophyllene, germacrene D, calamenene, caryophyllene oxide, alpha-gurjunene, isoledene, guaiazulene, and squalene. Among them, thymol, p-cymene, carvacrol, thymoquinone, and linalyl anthranilate occurred in quantities exceeding 1% (Fig. 1, Tab. 3). The structural similarity of thymol, carvacrol, and p-cymene allows p-cymene to isomerize into the other two. Furthermore, it should be noted that γ-terpinene, p-cymene, carvacrol, and thymol occur in the same biosynthetic pathway (25).

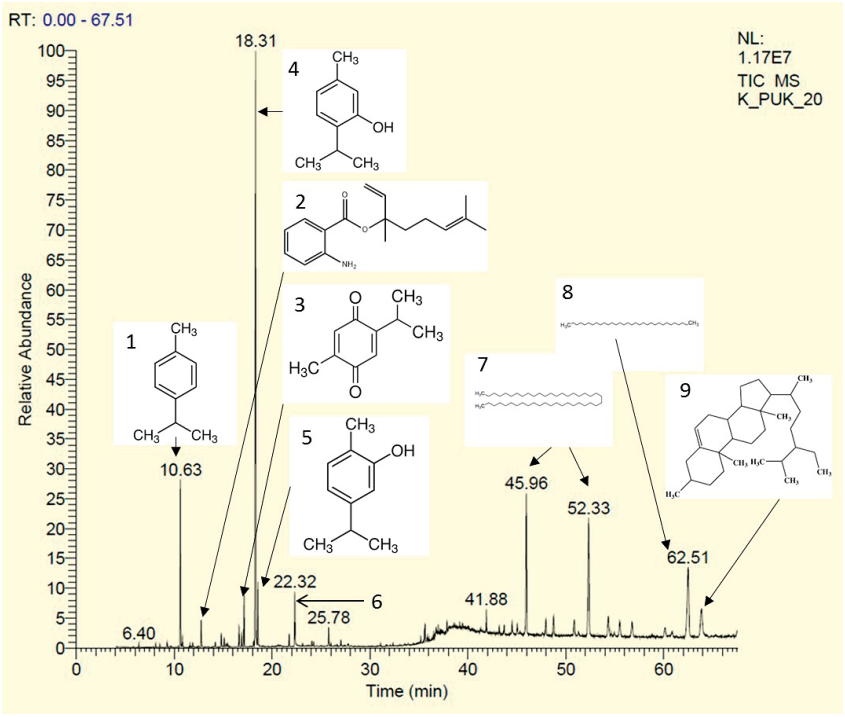

Fig. 1. GC-MS analysis of *T. vulgaris* HEX extract
Explanations: 1) p-cymen (RT = 10.63); 2) linalyl anthranilate (RT = 12.76); 3) thymoquinone (RT = 17.13); 4) thymol (RT = 18.32); 5) carvacrol (RT = 18.54); 6) (1r)-1,6,6-trimethyl-cis-bicyclo[3.3.0]octan-3-one (RT=22.32); 7) tetratetracontane (RT = 45.96 and 62.51); 8) dotriacontane (RT = 62.51); 9) sigma-5-en-3-ol (RT = 63.85)

Thymol [2-isopropyl-5-methylphenol] is one of the main monoterpene phenol occurring *Thymus vulgaris* essential oil (2). Thymol is biosynthesized by the aromatization of γ-terpinene to p-cymene followed by hydroxylation of p-cymene (30, 38). Many studies have described the antibacterial properties of thymol against bacterial species such as *E. coli*, *Streptococcus mutans*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *E. faecalis*, *Yersinia enterocolitica*, and *Bacillus subtilis* (26, 46). Its antibacterial properties have also been demonstrated against *Mycobacterium smegmatis* (26), *M. tuberculosis* (3), and *M. bovis* (3). The antibacterial effect of thymol was attributed to the ability to permeabilize and depolarize the cytoplasmic membrane (49). In the HEX extract examined in this study, thymol constituted 27.35%.

Tetratetracontane is a long chain alkane found in the *T. vulgaris* HEX extract examined in the present study, at a concentration of 19.68%. There is limited literature on the antibacterial activity of tetratetracontane. It has been demonstrated that tetratetracontane is one of the antibacterial constituents in the HEX extract of *Quisqualis indica* (1), as well as in the chloroform and ethyl acetate extracts of *Cryptococcus rajasthanensis* (6).

Dotriacontane is an alkane substance occurring in the HEX extract of *T. vulgaris* examined in the present study, at 10.34%, and in the available literature, there is no information on its antibacterial activity.

p-Cymene[1-methyl-4-(1-methylethyl)-benzene, 4-isopropyltoluene, 1-isopropyl-4-methylbenzene, or 1-methyl-4-isopropylbenzene] is a monoterpene phenol found in *T. vulgaris*, and utilized for medicinal purposes. The antimicrobial properties of this compound are well known (29). Previous studies have shown that its antibacterial effects are related to several mechanisms, such as inhibition of biofilm formation, disruption of lipid function in the bacterial membrane, destabilization of the membrane, and a decrease in membrane potential (10, 29, 47). In the HEX extract examined in this study, p-cymene constituted 5.92%.

Tab. 3. Chemical composition of *Thymus vulgaris* HEX extract

No.	CAS number	RT (min)	Compound	Formula	MW (Da)	%
1	995-83-5	5.82	1,1,3,3,5,5,7,7,9,9-decamethylpentasiloxane	C10H32O4Si5	356	0.02
2	108-65-6	6.40	1-methoxy-2-propanol acetate	C6H12O3	132	0.11
3	127744-55-2	7.90	(e)-2-[(n-hydroxy-n-phenyl)-amino]-3-[n(phenylimino)]-indole	C22H19N3O	341	0.01
4	6874-10-08	8.11	cis-ocimene	C10H16	136	0.13
5	79-92-5	8.54	camphene	C10H16	136	0.10
6	4798-45-2	9.28	4-methyl-1-penten-3-ol	C6H12O	100	0.19
7	540-97-6	9.48	dodecamethylcyclohexasiloxane	C12H36O6Si6	444	0.01
8	93772-22-6	9.61	1-methyl-3-(3,4-dimethoxyphenyl)-6,7-dimethoxyisochromene	C20H22O5	342	0.04
9	NA	9.73	3-(4'-methoxyphenyl)-1-acetyl-2-phenylindolizine	C23H19NO2	341	0.03
10	99-86-5	10.40	alpha-terpinene	C10H16	136	0.04
11	99-87-6	10.63	p-cymene	C10H14	134	5.92
12	3779-61-1	10.75	ocimene	C10H16	136	0.05
13	470-82-6	10.84	eucalyptol	C10H18O	154	0.39
14	99-85-4	11.61	gamma-terpinene	C10H16	136	0.13
16	546-79-2	11.88	sabinene hydrate	C10H18O	154	0.17
17	107454-09-1	12.00	2-[à-(p-bromophenyl)-á-mercaptoethenyl]isoquinolin-1-(2h)-one	C17H12BrNOS	357	0.03
19	7149-26-0	12.76	linalyl anthranilate	C17H23NO2	273	1.06
20	107454-09-1	13.47	2-[à-(p-bromophenyl)-á-mercaptoethenyl]isoquinolin-1-(2h)-one	C17H12BrNOS	357	0.02
21	76-22-2	14.20	norbornan-2-one	C10H16O	152	0.19
22	128-50-7	14.81	nopol	C11H18O	166	0.61
23	562-74-3	15.10	terpinen-4-ol	C10H18O	154	0.37
24	1197-01-9	15.32	p-cymen-8-ol	C10H14O	150	0.18
25	470-08-6	15.47	beta-fenchol	C10H18O	154	0.13
26	13837-75-7	15.65	cis-limonene oxide	C10H16O	152	0.05
27	1076-56-8	16.62	thymol methyl ether	C11H16O	164	0.72
28	6379-73-3	16.90	carvacryl methyl ether	C11H16O	164	0.53
29	490-91-5	17.13	thymoquinone	C10H12O2	164	2.00
30	93772-22-6	17.76	1-methyl-3-(3,4-dimethoxyphenyl)-6,7-dimethoxyisochromene	C20H22O5	342	0.05
31	89-83-8	18.30	thymol	C10H14O	150	27.35
32	499-75-2	18.54	carvacrol	C10H14O	150	2.64
33	3856-25-5	20.55	α-copaene	C15H24	204	0.09
34	NA	20.65	2,5,5-trimethyl-2-(butylthio)cycloheptatriene	C14H22S	222	0.08
35	2444-28-2	20.83	2,6-ditert-butyl-1,4-benzenediol	C14H22O2	222	0.09
36	87-44-5	21.75	beta-caryophyllene	C15H24	204	0.48
37	NA	22.32	(1r)-1,6,6-trimethyl-cis-bicyclo[3.3.0]octan-3-one	C11H18O	166	2.52
38	23986-74-5	23.11	germacrene D	C15H24	204	0.12
39	23986-74-5	24.05	germacrene D	C15H24	204	0.29
40	483-77-2	24.25	calamenene	C15H22	202	0.17
41	1139-30-6	25.78	caryophyllene oxide	C15H24O	220	0.78
42	NA	26.00	1,2-dihydroacenaphthylen-3-carbaldehyde	C13H10O	182	0.11
43	489-40-7	26.63	alpha-gurjunene	C15H24	204	0.07
44	95910-36-4	27.02	isoledene	C15H24	204	0.28
45	489-84-9	27.80	guaiazulene	C15H18	198	0.06
46	55401-65-5	31.08	octahydro-1-(2-octyldecyl) pentalene	C26H50	362	0.11
47	84-74-2	31.66	dibutyl phthalate	C16H22O4	278	0.04
48	7683-64-9	32.34	squalene	C30H50	410	0.09
49	7098-22-8	45.96	tetratetracontane	C44H90	618	8.91
50	7098-22-8	52.33	tetratetracontane	C44H90	618	19.68
51	NA	54.34	5,10-dihexyl-5,10-dihydro indole[3,2b]indole-2,7-dicarbaldehyde	C28H34N2O2	430	2.15
52	544-85-4	62.50	dotriacontane	C32H66	450	10.34
53	83-47-6	63.85	stigmast-5-en-3-ol	C29H50O	414	4.89

Explanations: RT – retention time, MW – molecular weight

Stigmast-5-en-3-ol [β-sitosterol], a pentacyclic triterpene, is a bioactive phytosterol that is naturally derived from plant cell membranes. Its antibacterial properties against *E. coli*, *S. aureus*, *K. pneumonia*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Corynebacterium ulcerans*, *Pseudomonas auruginosa*, *Salmonella typhi*, *S. dysenteriae*, *Candida albicans* and *Candida virusei* have been demonstrated (4, 28, 52). The antibacterial activity of β-sitosterol involves inhibiting peptidoglycan biosynthesis and preventing cell wall formation (13). In the HEX extract examined in this study, stigmast-5-en-3-ol constituted 4.89%.

Carvacrol [5-isopropyl-2-methylphenol] is a monoterpene phenol found in *T. vulgaris*. Its antibacterial properties have been demonstrated against *M. tuberculosis* (3), *M. bovis* (3), *M. abscessus*, *M. chelonae*, *M. mucogenicum*, *M. smegmatis* (31). The antibacterial effect of carvacrol has been attributed to its ability to permeabilize and depolarize the cytoplasmic membrane (49). Additionally, carvacrol has shown strong inhibition of biofilm formation (31). In the HEX extract examined in this study, carvacrol constituted 2.64%.

Thymoquinone [2-isopropyl-5-methyl-1,4-benzoquinone] (2.00%) is the monoterpene molecule found in *T. vulgaris*. Its activity against *M. tuberculosis* (11) has been demonstrated. The mechanism of action of thymoquinone is related to the disruption of the bacterial cytoplasmic membrane (14) and a decrease in intracellular ATP concentration (48). Jankowski et al. (23) demonstrated that the antimycobacterial activity of thymoquinone is associated with the depletion of NAD and ATP pools and the down regulation of plasma membrane lipids. In the HEX extract examined in this study, thymoquinone constituted 2.00%.

Linalyl anthranilate [3,7-dimethylocta-1,6-dien-3-yl 2-aminobenzoate]is an arene and terpene found in various plants such as *Agastache mexicana*, *Lavandula angustifolia*, *Origanum majorana*, and *T. vulgaris* (22, 35, 51). A study by Yang et al. (50) reported its antimicrobial activity against carbapenemase-producing *K. pneumonia* without affecting the efflux system. Linalyl anthranilate reacts with bacterial membrane compounds, forms ROS, and degrades nucleic acids, lipids, and proteins (50). In our study, linalyl anthranilate was present in the HEX extract of *T. vulgaris* at a concentration of 1.06%.

In this study, the HEX extract also identified two unidentified compounds: (1r)-1,6,6-trimethyl-cis-bicyclo[3.3.0]octan-3-one at 2.52% and 5,10-dihexyl-5,10-dihydro indole[3,2b]indole-2,7-dicarbaldehyde at 2.15%. To the best of the authors' knowledge, there is no literature on their antibacterial properties.

Conclusions. This study demonstrated that the HEX extract of *T. vulgaris* has a notable anti-NTM effect. These effects may be due to the presence of various phytochemicals, primarily terpenes such as thymol, p-cymene, carvacrol, and thymoquinone. GC-MS analysis revealed the presence of thymol in high concentrations. According to the literature, thymol exhibits strong anti-mycobacterial properties. Further studies are needed to determine the *in vivo* anti-NTM effect of thymol and its potential clinical use.

References

1. *Agarwal A., Prajapati R., Raza S. K., Thakur L. K.*: GC-MS analysis and antibacterial activity of aerial parts of Quisqualis indica plant extracts. Indian J. Pharm. Educ. Res. 2017, 51 (2), 329-336, doi: 10.5530/ijper.51.2.39.
2. *Ahmad A., van Vuuren S., Viljoen A.*: Unravelling the complex antimicrobial interactions of essential oils – The case of Thymus vulgaris (thyme). Molecules 2014, 19, 2896-2910, doi: 10.3390/molecules19032896.
3. *Andrade-Ochoa S, Nevárez-Moorillón G. V., Sánchez-Torres L. E., Villanueva-García M., Sánchez-Ramírez B. E., Rodríguez-Valdez L. M., Rivera-Chavira B. E.*: Quantitative structure-activity relationship of molecules constituent of different essential oils with antimycobacterial activity against Mycobacterium tuberculosis and Mycobacterium bovis. BMC Complem. Altern. Med. 2015, 15, 333, doi: 10.1186/s12906-015-0858-2.
4. *Anwar R., Sukmasari S., Aisyah L. S., Lestari F. P., Ilfani D., Yun Y. F., Prestya P. D.*: Antimicrobial activity of β-sitosterol isolated from Kalanchoe tomentosa leaves against Staphylococcus aureus and Klebsiella pneumonia. Pak. J. Biol. Sci. 2022, 25 (7), 602-607, doi: 10.3923/pjbs.2022.602.607.
5. *Banerjee P., Mukherjee S., Bera K., Ghosh K., Ali I., Khawas S., Ray B., Ray S.*: Polysaccharides from Thymus vulgaris leaf: Structural features, antioxidant activity and interaction with bovine serum albumin. Int. J. Biol. Macromol. 2019, 125, 580-587, doi: 10.1016/j.ijbiomac.2018.11.117.
6. *Barretto D. A., Vootla S. K.*: GC-MS analysis of bioactive compounds and antimicrobial activity of Cryptococcus rajasthanensis KY627764 isolated from Bombyx morigut microflora. Int. J. Adv. Res. 6 (3), 525-538, doi: 10.21474/IJAR01/6700.
7. *Behnia M., Haghighi A., Komeylizadeh H., Tabaei S. J. S., Abadi A.*: Inhibitory effects of Iranian Thymus vulgaris extracts on in vitro growth of Entamoeba histolytica. Korean J. Parasitol. 2008, 46 (3), 153-156, doi: 10.3347/kjp.2008.46.3.153.
8. *Benameur Q., Gervasi T., Pellizzeri V., Pľuchtová M., Tali-Maama H., Assaous F., Guettou B., Rahal K., Gruľová D., Dugo G., Marinog A., Ben-Mahdi M. H.*: Antibacterial activity of Thymus vulgaris essential oil alone and in combination with cefotaxime against blaESBL producing multidrug resistant Enterobacteriaceae isolates. Nat. Prod Res. 2019, 33 (18), 2647-2654, doi: 10.1080/14786419.2018.1466124.
9. *Calzada F., Yepez-Mulia L., Aguilar A.*: In vitro susceptibility of Entamoeba histolytica and Giardia lamblia to plants used in Mexican traditional medicine for the treatment of gastrointestinal disorders. J. Ethnopharmacol. 2006, 108 (3), 367-370, doi: 10.1016/j.jep.2006.05.025.
10. *Cristani M., D'Arrigo M, Mandalari G., Castelli F., Sarpietro M. G., Micieli D., Venuti V., Bisignano G., Saija A., Trombetta D.*: Interaction of four monoterpenes contained in essential oils with model membranes: implications for their antibacterial activity. J. Agric. Food Chem. 2007, 55 (15), 6300-6308, doi: 10.1021/jf070094x.
11. *Dey D.*: Antibacterial and antitubercular activity of selected plant products against multi-drug resistant clinical isolates, Jadavpur University, Jadavpur, India 2015, doi: 10.13140/RG.2.1.1901.9124.
12. *Dey D., Ray R., Hazra B.*: Antitubercular and antibacterial activity of quinonoid natural products against multi-drug resistant clinical isolates. Phytother. Res. 2014, 28 (7), 1014-1021, doi: 10.1002/ptr.5090.
13. *Evangelina I. A., Herdiyati Y., Laviana A., Rikmasari R., Zubaedah C., Anish, Kurnia D.*: Bio-mechanism inhibitory prediction of β-sitosterol from kemangi (Ocimum basilicum L.) as an inhibitor of MurA enzyme of oral bacteria: In vitro and in silico study. Adv. Appl. Bioinforma. Chem. 2021, 14, 103-115, doi: 10.2147/AABC.S301488.
14. *Fan Q., Liu C., Gao Z., Hu Z., Wang Z., Xiao J., Yuan Y., Yue T.*: Inactivation effect of thymoquinone on Alicyclobacillus acidoterrestris vegetative cells, spores, and biofilms. Front. Microbiol. 2021, 12, 679808, doi: 10.3389/fmicb.2021.679808.
15. *Fayad N. K., AL-Obaidi O. H. S., Al-Noor T. H., Ezzat M. O.*: Water and alcohol extraction of thyme plant (Thymus vulgaris) and activity study against bacteria, tumors and used as anti-oxidant in margarine manufacture. ISDE 2013, 4 (1), 41-51.
16. *Gnat S., Majer-Dziedzic B., Nowakiewicz A., Trościańczyk A., Ziółkowska G., Jesionek W., Choma I., Dziedzic R., Zięba P.*: Antimicrobial activity of some plant extracts against bacterial pathogens isolated from faeces of red deer (Cervus elaphus). Pol. J. Vet. Sci. 2017, 20 (4), 697-706, doi: 10.1515/pjvs-2017-0087.

17. *Gupta R. S., Lo B., Son J.*: Phylogenomics and comparative genomic studies robustly support division of the genus Mycobacterium into emended genus Mycobacterium and four novel genera. Front. Microbiol. 2018, 9, 67, doi: 10.3389/fmicb.2018.00067.
18. *Guz L., Grądzki Z., Krajewska M., Lipiec M., Zabost A., Augustynowicz-Kopeć E., Zwolska Z., Szulowski K.*: Occurrence and antimicrobial susceptibility of Mycobacterium peregrinum in ornamental fish. Bull. Vet. Inst. Pulawy 2013, 57, 489-492, doi: 10.2478/bvip-2013-0085.
19. *Guz L., Puk K.*: Antibiotic susceptibility of mycobacteria isolated from ornamental fish. J. Vet. Res. 2022, 66, 69-76, doi: 10.2478/jvetres-2022-0011.
20. *Guz L., Puk K., Pastuszka A.*: In vitro activity of garden thyme (Thymus vulgaris L.) chloroform extract against non-tuberculous mycobacteria. Med. Weter. 2025, 81 (2), 93-95.
21. *Guz L., Puk K., Szwajgier D., Pastuszka A.*: Combined antimicrobial action of streptomycin and terpenes against atypical mycobacteria isolated from fish. Pol. J. Vet. Sci. 2024, 27 (1), 135-138, doi: 10.24425/pjvs.2024.149334.
22. *Hashmi L. S., Hossain M. A., Weli A. M., Al-Riyami Q., Al-Sabahi J. N.*: Gas chromatography-mass spectrometry analysis of different organic crude extracts from the local medicinal plant of Thymus vulgaris L. Asian Pac. J. Trop. Biomed. 2013, 3 (1), 69-73, doi: 10.1016/S2221-1691(13)60026-X.
23. *Jankowski G., Sawicki R., Truszkiewicz W., Wolan N., Ziomek M., Hryć B., Sieniawska E.*: Molecular insight into thymoquinone mechanism of action against Mycobacterium tuberculosis. Front. Microbiol. 2024, 15, 1353875, doi: 10.3389/fmicb.2024.1353875.
24. *Jiménez-Arellanes A., Martinez R., García R., León-Díaz R., Luna-Herrera J., Molina-Salinas G., Said-Fernández S.*: Thymus vulgaris as a potencial source of antituberculous compounds. Pharmacologyonline 2006, 3, 569-574.
25. *Kowalski R., Wawrzykowski J.*: Essential oils analysis in dried materials and granulates obtained from Thymus vulgaris L., Salvia officinalis L., Mentha piperita L. and Chamomilla recutita L. Flavour Frag. J. 2009, 24 (1), 31-35, doi: 10.1002/ffj.1914.
26. *Kumar A., Singh S. P., Chhokar S.*: Thymol and its derivatives as antimicrobial agents. Nat. Prod. Comm. 2008, 3 (5), 823-828, doi: 10.1177/1934578X0800300528.
27. *Lescenko P., Maltova L., Dvorska L., Bartos M., Vavra O., Navratil S., Novotny L., Pavlik I.*: Mycobacterial infection in aquarium fish. Vet. Med. – Czech. 2003, 48 (3), 71-78, doi: 10.17221/5752-VETMED.
28. *Luhata L. P., Usuki T.*: Antibacterial activity of β-sitosterol isolated from the leaves of Odontonema strictum (Acanthaceae). Bioorg. Med. Chem. Lett. 2021, 48, 128248, doi: 10.1016/j.bmcl.2021.128248.
29. *Marchese A., Arciola C. R., Barbieri R., Sanches Silva A., Nabavi S. F., Tsetegho Sokeng A. J., Izadi M., Jafari N. J., Suntar I., Daglia M., Nabavi S. M.*: Update on monoterpenes as antimicrobial agents: A particular focus on p-cymene. Materials 2017, 10, 947, doi: 10.3390/ma10080947.
30. *Marchese A., Orhan I. E., Daglia M., Barbieri R., Di Lorenzo A., Nabavi S. F., Gortzi O., Izadi M., Nabavi S. M.*: Antibacterial and antifungal activities of thymol: A brief review of the literature. Food Chem. 2016, 210, 402-404, doi: 10.1016/j.foodchem.2016.04.111.
31. *Marini E., Di Giulio M., Ginestra G., Magi G., Di Lodovico S., Marino A., Facinelli B., Cellini L., Nostro A.*: Efficacy of carvacrol against resistant rapidly growing mycobacteria in the planktonic and biofilm growth mode. PLoS ONE 2019, 14 (7), e0219038, doi: 10.1371/journal.pone.0219038.
32. *Mikus J., Harkenthal M., Steverding D., Reichling J.*: In vitro effect of essential oils and isolated mono- and sesquiterpenes on Leishmania major and Trypanosoma brucei. Planta Med. 2000, 66 (4), 366-368, doi: 10.1055/s-2000-8548.
33. *Moghtader M.*: Antifungal effects of the essential oil from Thymus vulgaris L. and comparison with synthetic thymol on Aspergillus niger. J. Yeast Fungal Res. 2012, 3 (6), 83-88, doi: 10.5897/JYFR12.023.
34. *Mousavi S. M., Kamani H., Bagheri G., Mohammadi L., Dargahi A.*: Determining the efficacy of ethanolic, ethyl acetate and chloroform extracts of Thymus vulgaris and Nepeta binaloudensis medicinal plants on the inhibition and removal of standard bacteria from aquatic environments. J. Health 2023, 14 (3), 258-266, doi: 10.61186/j.health.14.3.258.
35. *Navarrete A., Avila-Rosas N., Majín-León M., Balderas-López J. L., Alfaro-Romero A., Tavares-Carvalho J. C.*: Mechanism of action of relaxant effect of Agastache mexicana ssp. mexicana essential oil in guinea-pig trachea smooth muscle. Pharm. Biol. 2017, 55 (1), 96-100, doi: 10.1080/13880209.2016.1230140.
36. *Oramadike C. E., Ogunbanwo S. T.*: Antagonistic activity of Thymus vulgaris extracts against Vibrio species isolated from seafoods. J. Food Sci. Technol. 2017, 54 (5), 1199-1205, doi: 10.1007/s13197-017-2543-6.
37. *Patil S. M., Ramu R., Shirahatti P. S., Shivamallu C., Amachawadi R. G.*: A systematic review on ethnopharmacology, phytochemistry and pharmacological aspects of Thymus vulgaris Linn. Heliyon 2021, 7, e07054, doi: 10.1016/j.heliyon.2021.e07054.
38. *Poulose J. J., Croteau R.*: Biosynthesis of aromatic monoterpenes: Conversion of γ-terpinene to p-cymene and thymol in Thymus vulgaris L. Arch. Biochem Biophys. 1978, 187 (2), 307-314, doi: 10.1016/0003-9861(78)90039-5.
39. *Pourazar Dizaji Sh., Soleimani N., Afrugh P., Saedi S.*: In vitro antibacterial activity of Thymus vulgaris essential oil against Mycobacterium tuberculosis. Infect. Epidemiol. Microbiol. 2018, 4 (2), 47-51, doi: 10.29252/modares.iem.4.2.47.
40. *Puk K., Banach T., Wawrzyniak A., Adaszek Ł., Ziętek J., Winiarczyk S., Guz L.*: Detection of Mycobacterium marinum, M. peregrinum, M. fortuitum and M. abscessus in aquarium fish. J. Fish Dis. 2018, 41 (1), 153-156, doi: 10.1111/jfd.12666.
41. *Puk K., Guz L.*: Effect of alkaloid berberine on the susceptibility of nontuberculous mycobacteria to antibiotics. Pol. J. Vet. Sci. 2022, 25, 479-481, doi: 10.24425/pjvs.2022.142034.
42. *Puk K., Guz L.*: Occurrence of Mycobacterium spp. in ornamental fish, Ann. Agric. Environ. Med. 2020, 27, 535-539, doi: 10.26444/aaem/114913.
43. *Puk K., Wawrzykowski J., Guz L.*: Evaluation of the anti-mycobacterial activity and composition of Carlina acaulis L. root extracts, Pol. J. Vet. Sci. 2023, 26, 57-63, doi: 10.24425/pjvs.2023.145007.
44. *Santoro G. F., das Gracas Cardoso M., Guimaraes L. G., Salgado A. P., Menna-Barreto R. F., Soares M. J.*: Effect of oregano (Origanum vulgare L.) and thyme (Thymus vulgaris L.) essential oils on Trypanosoma cruzi (Protozoa: Kinetoplastida) growth and ultrastructure. Parasitol. Res. 2007, 100 (4), 783-790, doi: 10.1007/s00436-006-0326-5.
45. *Szmygin-Milanowska K., Grzywa-Celińska A., Zwolska Z, Krawczyk P., Guz L., Milanowski J.*: 'TB or not TB?' Problems of differential diagnosis of cutaneous mycobacteriosis and tuberculosis – A Case Study and interdisciplinary discussion. Ann. Agric. Environ. Med. 2016, 23 (1), 97-102, doi: 10.5604/12321966.1196861.
46. *Trombetta D., Castelli F., Sarpietro M. G., Venuti V., Cristani M., Daniele C., Saija A., Mazzanti G., Bisignano G.*: Mechanisms of antibacterial action of three monoterpenes. Antimicrob. Agents Ch. 2005, 49 (6), 2474-2478, doi: 10.1128/AAC.49.6.2474–2478.2005.
47. *Ultee A., Bennik M. H. J., Moezelaar R.*: The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen Bacillus cereus. Appl. Environ. Microbiol. 2002, 68 (4), 1561-1568, doi: 10.1128/AEM.68.4.1561–1568.2002.
48. *Wang S., Deng H., Wang Y., Rui W., Zhao P., Yong Q., Guo D., Liu J., Guo X., Wang Y., Schi C.*: Antimicrobial activity and action mechanism of thymoquinone against Bacillus cereus and its spores. Food Secur. 2021, 10, 3048, doi: 10.3390/foods10123048.
49. *Xu J., Zhou F., Ji B. P., Pei R. S., Xu N.*: The antibacterial mechanism of carvacrol and thymol against Escherichia coli. Lett. Appl. Microbiol. 2008, 47 (3), 174-179, doi: 10.1111/j.1472-765X.2008.02407.x.
50. *Yang S. K., Yusoff K., Ajat M., Yap W. S., Lim S. H. E., Lai K. S.*: Antimicrobial activity and mode of action of terpene linalyl anthranilate against carbapenemase-producing Klebsiella pneumoniae. J. Pharm. Anal. 2021, 11 (2), 210-219, doi: 10.1016/j.jpha.2020.05.014.
51. *Yap P. S. X., Krishnan T., Yiap B. C., Hu C. P., Chan K. G., Lim S. H. E.*: Membrane disruption and anti-quorum sensing effects of synergistic interaction between Lavandula angustifolia (lavender oil) in combination with antibiotic against plasmid-conferred multi-drug-resistant Escherichia coli. J. Appl. Microbiol. 2014, 116 (5), 1119-1128, doi: 10.1111/jam.12444.
52. *Yinusa I., George N. I., Ayo R. G., Rufai Y.*: Evaluation of the pharmacological activities of beta-sitosterol isolated from the bark of Sarcocephalus latifolius (Smith Bruce). GJPACR 2015, 3(3), 7-14.

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