Trop J Nat Prod Res, February 2025; 9(2): 487 - 494 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Chemical Profiling and Antioxidant Capacity Assessment of Three Endemic *Thymus* Species Distributed in Bulgaria

Denitsa K. Kancheva*, Milena T. Nikolova, Ina Y. Aneva

Department of Plant and Fungal Diversity and Resources, Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia, 1113 Bulgaria

ARTICLE INFO

Article history:
Received 03 July 2024
Revised 13 November 2024
Accepted 02 January 2024
Published online 01 March 2025

ABSTRACT

The genus *Thymus*, a member of the *Lamiaceae* family, encompasses species renowned for their medicinal properties. This study aimed to determine the metabolite composition and free radical scavenging potential of three endangered species with restricted distribution in Bulgaria: *T. perinicus* (Velen.) Jalas, *T. jalasaianus* Stoyanov & Marinov, and one endemic to the Balkans – *T. aznavourii* Velen., all fall under the section *Hyphodromi*. Non-polar (chloroform and diethyl ether) and polar (methanol) extracts were prepared from the studied species. GC/MS and HPTLC analyses were applied to identify the metabolites. Antioxidant capacity was assessed using the DPPH test, while the Folin-Ciocalteu colourimetric assay measured total phenolic content (TPC). Chromatographic analyses revealed a rich array of primary and secondary metabolites, some known for their health-promoting properties. HPTLC identified compounds such as rosmarinic acid, lupeol, uvaol, ursolic, and oleanolic acids, while GC/MS detected terpenes like thymol, caryophyllene, cis–*β*-farnesene, and germacrene D, as well as phenolic acids such as 4-hydroxybenzoic, 4-hydroxycinnamic, vanillic, and caffeic acids, alongside fatty acids, fatty alcohols, and saccharides. Methanol extracts exhibited significant antiradical activity (IC_{50} < 50 µg/mL), with TPC values ranging from 7.44 to 17.97 mg GAE/g extract, aligning with DPPH assay findings. This study represents the first report on the chemical composition of extracts with varying polarity from *T. jalasaianus* and *T. aznavourii*. It offers valuable insights into the phytochemistry of these *Thymus* species, highlighting their potential applications across diverse industries and the need for their conservation.

Keywords: Thyme, Medicinal and Aromatic Plants, Phytochemistry, Natural Antioxidant.

Introduction

The genus *Thymus*, part of the *Lamiaceae* family, has garnered substantial interest worldwide from a phytochemical point of view. Originating from the Mediterranean region, it comprises over 250 species,[1] categorised into eight sections,[2] and is characterised by perennial herbaceous plants, often referred to as subshrubs.[3] These species are renowned for their variability in phenology and chemical composition, contributing to their extensive use since ancient times[4] in various sectors, including pharmaceuticals, food, cosmetics, phytotherapy, and aromatherapy. Their medicinal acclaim, particularly for antimicrobial,[5] antibacterial,[6] antioxidant,[7] and anti-inflammatory[8] properties, is attributed to phenolic compounds and essential oils.[9] While bioactive compounds are concentrated in the aerial parts of these plants, root extracts have also demonstrated biological activities, such as allelopathic potential.[10] Twenty-one species, divided into two sections (*Serpyllum* and *Hyphodromi*), occur naturally in Bulgaria. Despite their medicinal importance, they remain underexplored on a phytochemical and genetic level due to taxonomic complexities and challenges in species identification.[11]

*Corresponding author. Email: kanchevadenitsa@gmail.com
Tel: +00359877115255

Citation: Kancheva DK, Nikolova MT, Aneva IY. Chemical Profiling and Antioxidant Capacity Assessment of Three Endemic *Thymus* Species Distributed in Bulgaria. Trop J Nat Prod Res. 2025; 9(2): 487 – 494 https://doi.org/10.26538/tjnpr/v9i2.11

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

However, recent investigations have unveiled the volatile compounds of sixteen species, both from section *Hyphodromi*[12] and section *Serpyllum*.[13,14] Notably, *T. perinicus* has been studied extensively, revealing its chemical profile, rich in essential oils (characterised as thymol/borneol chemotype)[12] and phenolic compounds.[15,16] Conversely, data regarding the chemical composition of *T. aznavorii* and *T. jalasianus* are scarce, highlighting the need for further exploration. Only one study describing the Turkish region's *T. aznavourii essential oil profile* was found, and 17 sesquiterpenes were abundant. Additionally, *T. jalasianus* is a species relatively new to science,[18] with no previous reports on its phytochemistry. *T. perinicus* and *T. aznavourii* are part of an ongoing scientific inquiry into phylogenetic relationships between *Thymus* species occurring in Bulgaria. The DNA barcoding method serves as a tool, but the authors highlight the need for a more comprehensive investigation of this taxonomic complex group.[19] Thyme is a well-known herb in Bulgarian folk medicine, used as an antiseptic for oral health, aids in treating respiratory ailments, and an anti-inflammatory agent for digestive issues. It is often administered in the form of infusions or decoctions.[20] Considering the worldwide interest in the *Thymus* species, increasing the scientific data for its taxa is imperative. Expanding knowledge about their taxonomy, phytochemistry, genetic relationships, and ethnobotany is necessary to fill data gaps. This research aims to advance the scientific understanding of the phytochemical composition and antioxidant activity of three *Thymus* species with limited distribution: two Bulgarian endemics: *T. perinicus* (Velen.) Jalas and *T. jalasianus* Stoyanov & Marinov, and one Balkan endemic: *T. aznavourii* Velen. *T. perinicus* grows only on the limestone of the alpine belt of Pirin Mts.[21] *T. jalasianus* is found on the dry serpentine rocky slopes in two serpentine areas of the Eastern Rhodope Mts,[18] *and T. aznavourii* grows in the southernmost parts of Sakar Mts in dry calcareous habitats.[22] All three species belong to section *Hyphodromi* (A. Kerner) Halácsy.

Materials and Methods

Materials

The reagents and chemicals used in this study were Folin-Ciocalteu's phenol reagent 2N (Sigma–Aldrich), 2,2-diphenyl-1-picrylhydrazil (DPPH) (Aldrich), N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA, Sigma–Aldrich) p-anisaldehyde (Aldrich), 2-aminoethyldiphenylborat (MERCK) were supplied by FOT. Rosmarinic acid, ursolic acid and oleanolic acid standards were purchased from Extrasynthese (Genay, France). Methanol, chloroform, pyridine (HPLC grade, Alfa Aesar), and sodium carbonate were purchased from Valerus (Bulgaria).

Plant material

Aerial parts of the target species were collected from their natural localities in Bulgaria during the flowering period (May-August) 2022. *T. perinicus* was gathered from the alpine belt of the Pirin Mts, near Vihren Peak; *T. jalasianus* from a site east of the village of Golyamo Kamenyane in the Eastern Rhodope Mountain; and *T. aznavourii* from a locality east of the village of Sladun, in the Tundzha Hilly Plain. The GPS coordinates for these collection sites are presented in Table 1. The plant species were identified by Associate Professor Ina Aneva and Denitsa Kancheva from the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences.Voucher specimens (SOM) were deposited in the Herbarium of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia, Bulgaria. The plant samples were dried at room temperature of 25°C and subsequently powdered for further analysis.

Table 1: List of the studied species, their coordinates of gathering, and voucher specimens

Species	GPS – coordinates	Altitude (m a.s.l)	Voucher specimen
T. perinicus	N 41°45'34.6788" E 23°23'52.4616"	2650	SOM 177781
T. jalasianus	N 41°24'06.8" E 25°42'15.2"	422	SOM 176658
T. aznavourii	N 41°51'03.1" E 26°29'08.3"	160	SOM 176478

Preparation of the extracts

Non-polar extracts

Dry plant material (100 mg) of the studied samples was placed in Eppendorf tubes and extracted with 1 mL chloroform in an ultrasonic bath for 15 min. After decanting the aliquots into vials, the extracts were evaporated to obtain *chloroform extracts*. Second, non-polar extracts were prepared with diethyl ether using the same procedure, resulting in diethyl ether extracts.

Polar extracts

Dry plant material (100 mg) of the studied samples was placed in 2 mL Eppendorf tubes and extracted using methanol (1 mL) in an ultrasonic bath for 30 min. The extracts were filtered, placed in a glass vial, and evaporated to obtain methanol extracts.

Derivatisation for GC/MS analysis

All extracted extracts obtained before GC/MS analysis were derivatised. 100 µL of pyridine and 100 µL of N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA, Sigma-Aldrich) were added to the dried extract samples, and the samples were heated at 70 °C for 2 h. After cooling, 300 µL of chloroform was added, and the samples were analysed by GC/MS.

Extracts for DPPH and Folin-Ciocalteu assays

The dry plant material (1 g) from each species was extracted with 5 mL methanol in an ultrasonic bath for 30 minutes. The liquids were filtered and evaporated to dryness. The crude extract was subjected to further analysis using 2,2-diphenyl-1-picrylhydrazil (DPPH) (Aldrich) reagent and Folin-Ciocalteu's phenol reagent 2N (Sigma–Aldrich).

Phytochemical analyses

Gas chromatography-mass spectrometry (GC/-MS)

GC/MS analysis was performed using a Thermo Scientific Focus GC coupled with a Thermo Scientific DSQ mass detector operating in EI mode at 70 eV. A DB-5MS column (30 m x 0.25 mm x 0.25 µm) was used. The temperature program was: 100-180ºC at 15ºC/min, 180-300ºC at 5ºC/min, and 23 min at 300ºC. The injector temperature was 250ºC. The flow rate of the carrier gas (helium) was 0.8 mL/min. The split ratio was 1:10. 1 µL of the solution was injected. The components were identified by comparing their mass spectra and retention indices (RI) with those of authentic standards in the National Institute of Standards and Technology (NIST) spectral library. Values were presented as percent of all compounds' TIC (Total ion current). The mean value of one component from the five replicates was then calculated.

Thin Layer Chromatography analyses

HPTLC analyses were performed on 20 × 10 cm HPTLC silica gel 60 F254 plates (Merck, Darmstadt, Germany). Dry methanol extracts were dissolved in methanol to an equal concentration, and 5 µL of the solution was applied to the TLC plates. The mobile phase chloroform:ethylacetate:formic acid (50:40:10) was used for rosmarinic acid detection. Chromatograms were viewed under UV light at 336 nm before and after spraying with ''Naturstoffreagenz A'', a 1% diphenylboric acid ethanolamine complex solution in methanol. Hexane:ethylacetate (30:10) was used as the mobile phase for terpenoids. Chromatograms were viewed in daylight after spraying with the anisaldehyde–sulphuric acid reagent and heating the plate for 5–10 min at 100°C.

Free radical scavenging activity

Antioxidant activity was evaluated using the DPPH assay.[23]

In this assay, methanol extract solutions of the studied species at different concentrations were prepared (10, 20, 50, 100, and 200 µL), and each extract (2.5 mL) was mixed with 1mL of 0.3 mM methanolic DPPH and stored in the dark for 1h at room temperature. After 30 minutes of incubation, the absorbance values were measured at 517 nm using a Jenway 6320D visible spectrophotometer (Cole-Parmer, United Kingdom) and converted into the percentage antioxidant activity using the following equation:

$$\text{DPPH as free radical scavenging capacity (\%)} = \frac{1-(\text{Ab of sample} - \text{Ab of blank})}{\text{Ab of control}}\ x\ 100$$

All measurements were performed in triplicates. Methanol (1.0 mL) plus plant extract solution (2.5 mL) was used as a blank, whereas DPPH solution (1 mL) plus methanol (2.5 mL) was used as a control. The IC_{50} values were calculated by sigmoid non-linear regression model using plots, where the abscissa represents the concentration of the tested plant extracts and the ordinate, the average percentage of scavenging capacity from three replicates (Software Prizm 3.00). The results are expressed as IC_{50} value.

Total polyphenol content (TPC)

The total phenolic content (TPC) was determined using the Folin–Ciocalteu reagent, with gallic acid as the standard, following the protocol previously described by Petrova *et al*.[24] Methanol extracts were diluted to a concentration of 2 mg/mL, and 0.20 mL were mixed with 2 mL of Folin–Ciocalteu reagent (previously diluted 10-fold with distilled water) and 1.8 mL of Na_2CO_3 (7.5%). After 1 hour at room temperature, the absorbance of the samples was measured at 765 nm using a spectrophotometer and compared with a blank sample. All samples were analysed in triplicates. The results are expressed as gallic acid equivalents (GAE per gram of extract).

The total polyphenol content was calculated using the following formula: $C = \frac{c \cdot V}{m'}$

Where C is the total content of phenolic compounds (mg g^{-1} plant extract) in GAE; c is the concentration of gallic acid established from the calibration curve in mg mL^{-1}; V is the volume of extract in mL; and m is the weight of pure plant methanol extract in grams.

Statistical analyses

Statistical analyses were performed using Microsoft Excel software. The results are presented as mean with standard deviation (SD). All experiments were carried out in triplicate. The free radical scavenging data were analysed using the Prism 9 software package (Graph Pad Inc., San Diego, USA).

Results and Discussion

The GC/MS analysis results are presented in Table 2. and Figures 1-3. Analysis of chloroform extracts revealed 24, 26, and 42 metabolites in *T. perinicus, T. jalasianus*, and *T. aznavourii*, respectively. Among these, fatty acids were notably abundant, particularly in *T. perinicus* and *T. aznavourii*, with the latter exhibiting a higher quantity (11.5%). Specifically, hexadecanoic acid (C16:0) emerged as both species' most concentrated fatty acid. In *T. aznavourii*, aliphatic alcohols were prominent, with glycerol being the most prevalent at 13.27%.

Table 2: Bioactive compounds identified in thyme samples

Compounds	RI	TP CH	TP DE	TP ME	TJ CH	TJ DE	TJ ME	TA CH	TA DE	TA ME
TERPENES										
Monoterpenes										
Limonene	1032							0.96		
Bicyclic monoterpenes										
Borneol	1224	0.25						0.18		
Oxygenated monoterpenes										
Thymol	1322	0.98	0.21	0.43	0.88	0.34	0.22	1.2	0.33	0.31
Sesquiterpenes										
α-Cubebene	1351								0.14	
β-Bourbonene	1384								0.21	
Caryophyllene	1419	0.17	1.76		0.05	0.96	0.09	2.56	0.59	
cis-β-Farnesene	1444	0.01	0.13		0.91	0.31		0.15	0.1	
Germacrene D	1481					0.23			0.42	
Caryophyllene oxide	1581		0.29		0.1	0.88		1.39		
Terpene alcohols										
α-Terpineol	1323		0.1			0.2				
Nerolidol	1671	0.2	0.3			0.42				
β-Eudesmol	1760		0.33			0.9				
Phytol	2180	0.2	0.2	0.11	0.38	0.2	0.06	0.16	0.7	
Diterpenes										
Neophytadiene	1837	0.68	0.59	0.02	0.88			0.15		
Triterpenoids										
Squalene	2826							0.42		
β –Amyrin	3335			0.01						
POLYPHENOLS										
Phenolic acids										
Benzoic acid	1358			0.01	0.02		0.2	0.02	0.03	0.02
Salicylic	1504						0.52	0.03		1.22
4-Hydroxybenzoic	1635		0.1	0.08		0.18	0.45		0.23	0.29
Vanillic	1753							0.08	0.04	0.1
Protocatechuic	1811							0.1		
Quinic	1843		0.15	2.2			2.8		0.74	2.3
4-Hydroxycinnamic	1934		0.03	0.27		0.06	0.22	0.2	0.06	0.26
Caffeic	2142		0.34	0.82		0.11	0.64		0.7	1.01
Phenol glycosides										

Arbutin	2561						1.27			
ORGANIC ACIDS										
Lactic acid	1066	0.42		1.1	0.1	0.4	0.47	0.04	0.2	
Glycolic acid	1081				0.02	0.01	0.16	0.04		0.02
Succinic acid	1310		0.03	0.37	0.04	1.2	0.46	0.18		0.35
Glyceric acid	1319			0.02			0.4	0.02		0.02
Fumaric acid	1353						0.04			0.26
Malic acid	1498	0.05		0.1	0.1		4.1	0.2	0.8	0.17
Azelaic acid	1806							0.04		
Citric acid	1845								0.35	
FATTY ACIDS										
Hexanoic acid C6:0	1074						0.7	0.01		0.01
Nonanoic acid C9:0	1355			0.01	0.03			0.47	0.12	
Decanoic acid C10:0	1450							0.02		0.2
Tetradecanoic acid C14:0	1850	0.27	0.59		0.71	0.68		0.37	0.71	
Hexadecanoic acid C16:0	2050	2.96	11.8	0.4	2.02	14.76	0.25	6.13	14.5	0.24
9,12-Octadecadienoic acid C18:2	2212	2.37	2.5	0.2	1.22	3.7	0.06	1.26	2.07	
9-Octadecenoic acid C18:1	2218		2.0			1.9		1.72	1.93	0.05
α-Linolenic acid C18:3	2220	2.52	1.8	0.2	1.55	2.9	0.05		0.59	
Octadecanoic acid C18:0	2246	0.85	1.6	0.1	1.71	1.8	0.1	1.42	1.53	0.1
Tetracosanoic acid C24:0	2838							0.1		
FATTY ALCOHOLS										
1-Pentanol	831	0.02			0.02		0.1	0.02		
1-Octanol	1277	0.02		0.03	0.02			0.04		0.01
1-Decanol	1393	0.02						0.02		
1-Dodecanol	1560				1.1			0.4		
1-Tetradecanol	1756	0.06						0.6		
9-Tetradecen-1-ol	1757	0.5		0.03			0.01	0.06		0.03
9-Octadecen-1-ol	2126	0.3			0.04		0.3	0.01		
2-Tridecanol	1510		0.4							
ALIPHATIC ALCOHOLS										
Glycerol	1265	3.61	0.47	6.32	0.87	0.85	2.52	13.27	0.66	10.01
1,2,3-Butanetriol	1286			0.1	0.31		0.2	0.14		
Ribitol	1747	0.2		0.1	1.6		0.3	0.2	0.04	0.1
BENZYL ALCOHOLS										
4-Hydroxybenzyl	1500						0.8	0.02	0.2	0.02
AMINOACIDS										
L-Proline	1305			0.89			0.5			0.71
SACCARIDES										
D-Fructose	1867	0.99		13.61	2.16		25.66	3.14		15.74
D-Glucose	1926			4.86			10.11			10.16
Galactorypanose	1931			6.34			10.21			11.49
Myo-Inositol	2129			1.6			1.16			0.65
Sucrose	2628	1.48	2.2	37.75	2.16	2.31	27.4	3.95	2.86	24.43

CH – chloroform extract; DE – diethyl ether extract; ME - methanol extract; RI - retention indices. Values were presented as percent of all compounds' TIC (Total ion current)

Phenolic acids were detected in minor amounts in *T. aznavourii* (0.43%), while only benzoic acid was observed in *T. jalasianus*. The highest quantities of terpenes were found in *T. aznavourii* (7.17%), with thymol and its isomer carvacrol present across all species. In diethyl ether extracts, 23 compounds were detected in both *T. perinicus* and *T. jalasianus* and 27 in *T. aznavourii*, representing a decrease in detected

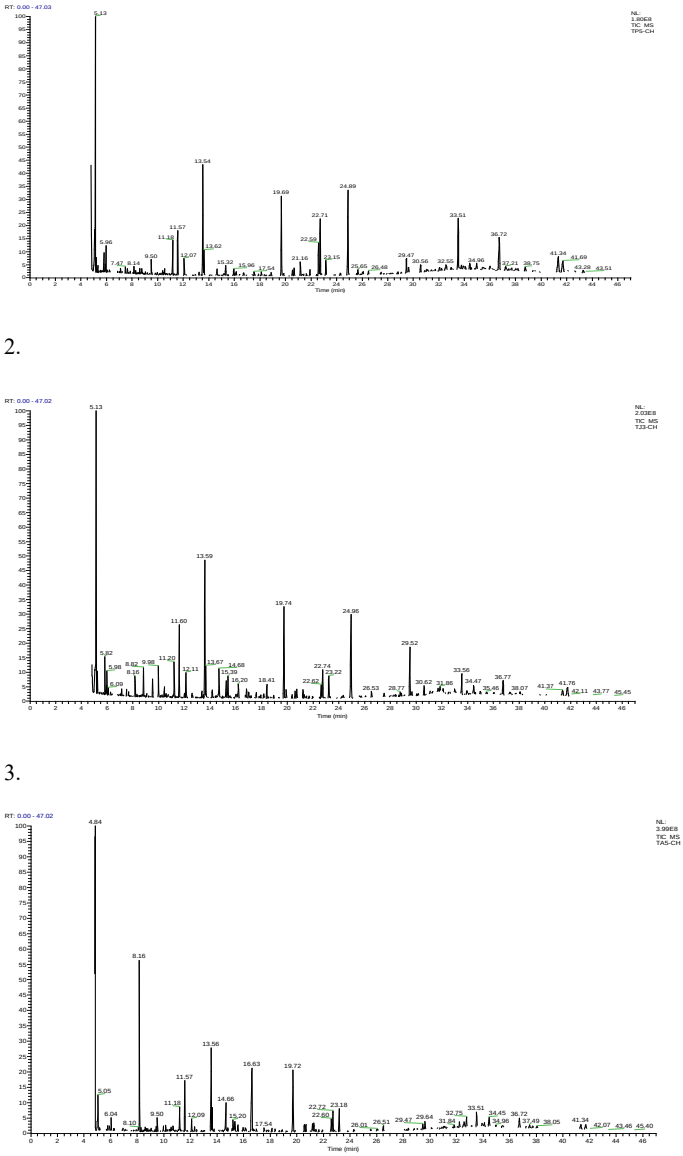


Figure 1: GC/MS chromatograms of chloroform solutions. 1 - *Thymus perinicus*, 2 - *T. jalasianus*, 3 - *T. aznavourii*.

compounds compared to chloroform extracts. Fatty acids again predominated, especially in *T. jalasianus* (25.74%), with hexadecanoic acid (C16:0) most abundant across all samples. Fatty alcohols were detected solely in *T. perinicus*. Phenolic acids were present in all extracts, with 4-hydroxybenzoic, hydroxycinnamic, and caffeic acids observed in all species. Sesquiterpenes were prominently found, with the highest concentration in *T. jalasianus* (2.38%). Overall, saturated fatty acids were dominant in both types of extracts. Saccharides occur in small quantities due to their polar nature, with sucrose present in all species. Diethyl ether samples demonstrated greater efficiency in extracting polyphenols and terpenes. Methanol (polar) extracts identified 29, 34, and 29 metabolites for *T. perinicus, T. jalasianus,* and *T. aznavourii*. These extracts contain mainly polar compounds, composed primarily of saccharides and phenols. Fatty acids and terpenes appear significantly less. *T. aznavourii* was differentiated by a substantial glycerol concentration (10.01%). Phenolic acids were observed in all samples, with the highest content in *T. aznavourii* (5.2%), and quinic acid was present in the highest percentage for all extracts. The only polyphenol that occurred in *T. jalasianus* was arbutin. Among terpenes, thymol was reported for all extracts. HPTLC analysis was conducted on methanol extracts of the studied species to investigate the presence of triterpenes and rosmarinic acid, with the results presented in Figures 4 and 5. Rosmarinic acid was identified in all extracts. Among the triterpenes, oleanolic and ursolic acids were the primary components; however, due to the HPTLC conditions, it was impossible to discern which acid predominated. Lupeol and uvaol were detected at the highest concentrations in the extract of *T. perinicus*. Also, the methanol extracts of the studied species were assessed for antiradical potential using the DPPH method. All studied extracts exhibited significant antiradical activity: *T. perinicus* – 49.79 µg/mL; *T. jalasianus* – 25.08 µg/mL; and *T. aznavourii* – 27.29 µg/mL. In the total phenolic content determination (Table 3), the total phenolic content in the extracts ranged from 7.44 to 17.97 mg GAE/g extract. Based on the study results, the tested samples were arranged in the following order: *T. aznavourii* > *T. jalasianus* > *T. perinicus*. 1.

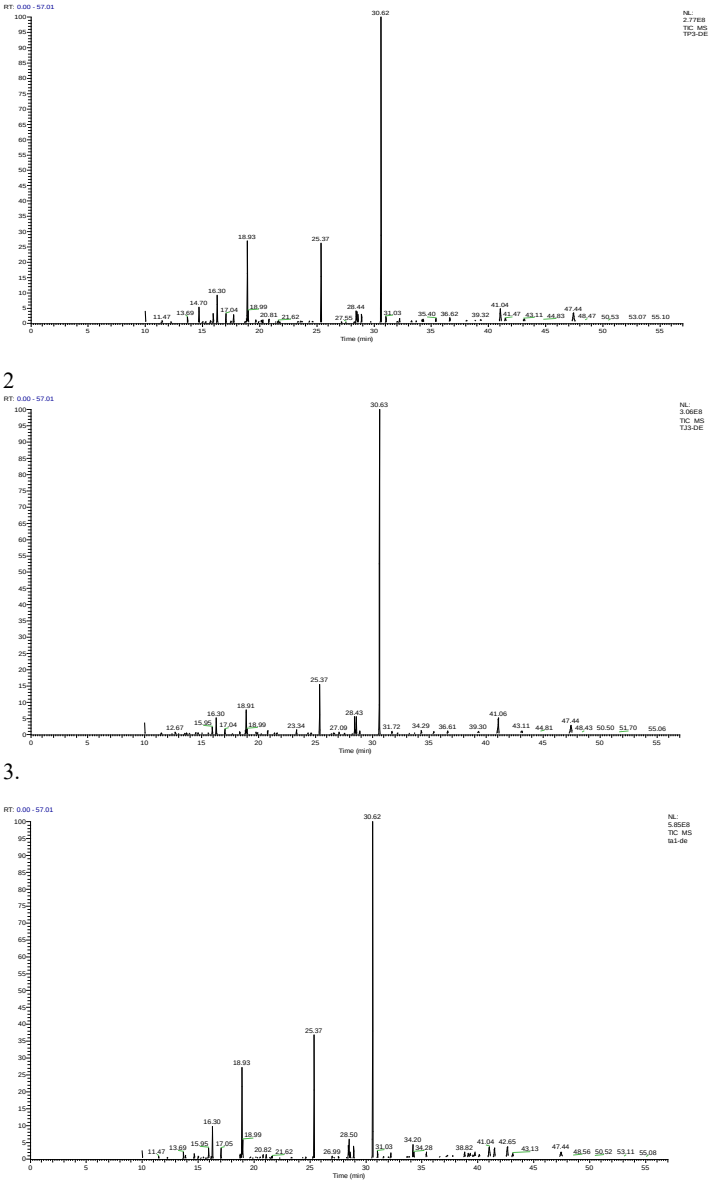
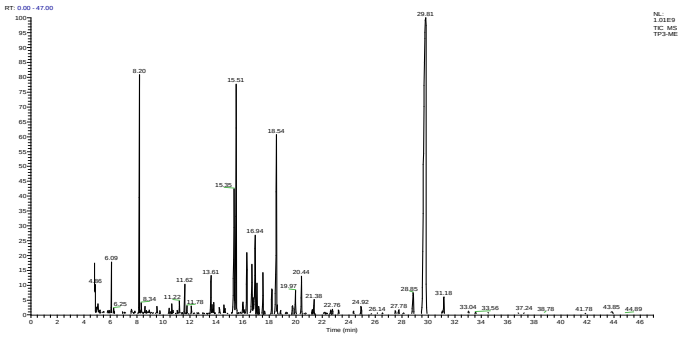


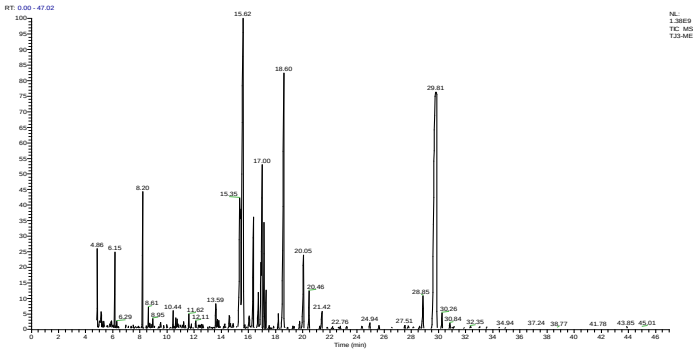
Figure 2: GC/MS chromatograms of diethyl ether solutions. 1 - *Thymus perinicus*, 2 - *T. jalasianus*, 3 - *T. aznavourii*

A linear correlation between the total phenolic content and the radical-scavenging capacity of the extracts was observed in the current experiment. The complexity of the genus *Thymus* and the difficulty in identifying its species result from a lack of phytochemical studies at the species level. In addition, the studied species have a relatively limited distribution in Bulgaria. *T. jalasianus* was reported and described for the first time in 2020.[18]

1.



2.



3.

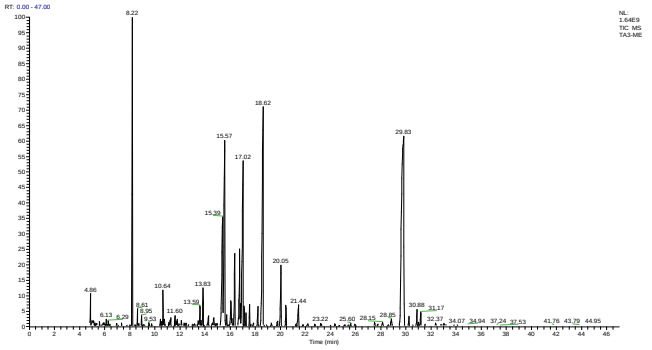


Figure 3: GC/MS chromatograms of methanol solutions. 1 - *Thymus perinicus*, 2 - *T. jalasianus*, 3 - *T. aznavourii*.

T. aznavourii, a globally endangered species found in Turkey and Greece, was reported near the Bulgarian-Turkish border in 2021.[22] *T. perinicus* is a local endemic species for Pirin Mts. and grows at altitudes ranging from 1970 to 2900 meters.[21] Phytochemical studies on *T. perinicus* have confirmed the species' consistency across various studies, identifying its essential oil as a thymol/borneol chemotype,[12] rich in monoterpenoids (37.8%) and aromatic compounds (36.0%).[15,16,25] Conversely, *T. aznavourii*'s essential oil, characterised in 1998 by Tümen *et al*., shows a low yield (0.3%) and is dominated by sesquiterpenes like germacrene-D (22.8%) and β-farnesene (16.1%), with only trace amounts of thymol and carvacrol.[17] To date, no phytochemical data are available for *T. jalasianus*. The GC/MS analysis of methanol extracts from hydroponically propagated *T. zygioides* (section *Hyphodromi*) and *T. longedentatus* (sect. *Serpyllum*), species also found in Bulgaria, identified thymol, carvacrol, triterpene acids (ursolic and oleanolic), and phenolic acids such as rosmarinic, chlorogenic, and quinic acids. Consistent with the findings of Traykova *et al*., our the study revealed both similarities and distinctions. The studied species demonstrated a rich presence of phenolic acids, with the polar extract of *T. jalasianus* exhibiting an exceptionally high concentration of quinic acid (2.8%). However, *T. jalasianus* also contained arbutin, a polyphenol, absent in the hydroponically grown species. These variations highlight the chemical diversity within the genus and the significant influence of environmental and genetic factors on phytochemical profiles, emphasising the need for further comparative research.[26] Analyses revealed a rich array of bioactive compounds across the species, with significant variations depending on the solvent used for extraction. Non-polar extracts were particularly rich in fatty acids, such as tetradecanoic acid, known for its repellent properties.[27] Diethyl ether extracts across all species showed a remarkable abundance of hexadecenoic acid, a natural product used in dermatology due to its anti-inflammatory action.[28] It is reported to possess high antioxidant and antibacterial capacity, applicable in the food and pharmaceutical industries.[29] Furthermore, hexadecenoic acid has been identified as a potential agent in anticancer therapies.[30] *T. perinicus* DE extract had the highest percentage of fatty acids (25.74%). *T. jalasianus* methanol extract revealed the presence of arbutin, a highly valuable polyphenol used in the skincare industry as a whitening agent.[31] Among the phenolic acids, quinic acid in the *T. jalasianus* polar sample had the highest concentration (2.8%). Liu *et al.* (2020) have confirmed its antioxidant, anti-inflammatory, and neuroprotective properties.[32] HPTLC analyses confirmed the high quantity of rosmarinic acid in all studied samples (Fig. 4). The presence of this phenolic acid is distinctive for *Thymus* and has been stated in many scientific studies.[33,34,35] It has a variety of proven biological activities, including antioxidant, antimicrobial, and anti-inflammatory properties,[36] and its presence in the studied extracts highlights their potential beneficial properties. Lupeol, uvaol, ursolic acid, and oleanolic acid were observed in all samples (Fig. 5). All of them are part of the pentacyclic terpenoid group and have been reported to have anti-inflammatory activity.[37,38,39]

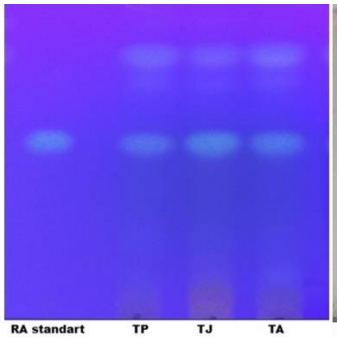


Figure 4: TLC plate for rosmarinic acid

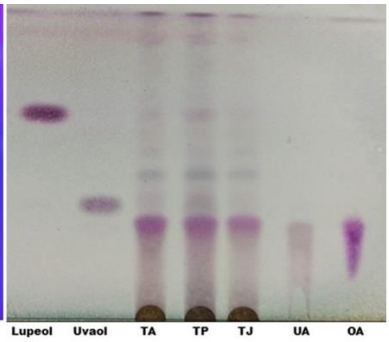


Figure 5. TLC plate for terpenoid content. TA – *T. aznavourii*, TP – *T. perinicus*, TJ – *T. jalasianus*, RA – rosmarinic acid, UA – Ursolic acid, OA – Oleanolic acid.

All studied methanol extracts of species from section *Hyphodromi* showed significant free radical scavenging activity with IC_{50} values under 50 µg/mL. According to Phongpaichit *et al*. report, IC_{50} values between 10 and 50 µg mL^{-1} indicate that extracts possess high antioxidant capacity.[40] Among the studied samples in the current study,

the extracts of *T. perinicus* displayed the lowest antiradical activity. This result is consistent with previously reported data.[16] Methanol extracts of other species (*T. atticus* and *T. comptus*) from section *Hyphodromi* studied by Nikolova *et al*., showed close values compared to the currently studied species (*T. jalasianus* and *T. aznavourii*).[16] Regarding total phenolic content, the lowest value was observed in the methanol extract of *T. perinicus*, which corresponded to the lowest antiradical activity. Many reports confirm the positive relationship between higher phenolic content and antioxidant activity, as found in the present study.[35,16]

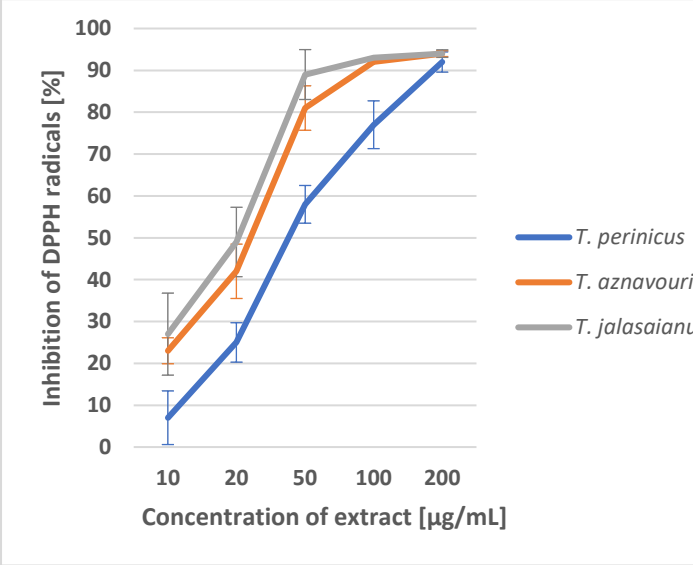


Figure 5: DPPH free radical scavenging activity extracts, expressed as IC_{50} values (µg/mL). Values represent mean ±SD, n=3.

Various analyses from different *Thymus* species present the genus as a potential source of natural antioxidants. Ethanol leaf and flower extracts from *Thymus pseudopulegioides* were reported to possess a high inhibition percentage against DPPH - 5.87 µg/mL and 1.12 µg/mL, respectively.[41] Methanol extract from *Thymus serpyllum* collected from India showed an IC_{50} value of 48.58 µg/mL.[42]

Table 3: TPC of thyme species samples

Plant sample	TPC (mg GAE/g of Extract)
T. perinicus	7.44 ± 0.20
T. jalasianus	15.77 ± 0.44
T. aznavourii	17.97 ± 0.85

Values are the mean ± standard deviation (SD); n=3.

Conclusion

This study represents the first report on the metabolite composition of extracts of *Thymus* species with varying polarities from *T. jalasianus* and *T. aznavourii*, providing foundational data for further research. HPTLC, DPPH, and TPC analyses revealed significant antioxidant capacity of the tested Thymes, reinforcing the need for future phytochemical exploration. Moreover, detailed studies on their mechanisms of action and bioavailability in various formulations could enhance their industrial applications. All studied species demonstrated rich phytochemical diversity and significant antioxidant potential. Considering their ecological and medicinal significance, efforts should also be directed toward the sustainable cultivation and conservation of these *Thymus* species to prevent the loss of genetic diversity. This research lays the groundwork for future investigations into these valuable species' therapeutic potential and industrial applications.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgements

This publication is supported by the National Recovery and Resilience Plan of the Republic of Bulgaria, under project N PVU-66, 16.12.2024 /BG-RRP-2.017-0015-C01/. We gratefully acknowledge the financial and institutional support provided, which has been instrumental in achieving the objectives of this research.

References

1. Jaouadi R, Boussaid M, Zaouali Y. Variation in essential oil composition within and among Tunisian *Thymus algeriensis* Boiss et Reut. (Lamiaceae) populations: Effect of ecological factors and incidence on antiacetylcholinesterase and antioxidant activities. Biochem Syst Ecol. 2023; 106:104543. https://doi.org/10.1016/j.bse.2022.104543.
2. Morales R. Synopsis of the genus *Thymus* L. in the Mediterranean area. Lagascalia. 1997; 19(1-2):249-262.
3. Javed H, Erum S, Tabassum S, Ameen F. An overview on medicinal importance of *Thymus vulgaris*. J Asian Sci Res. 2013; 3(10):974-982.
4. Taher M, Salloom Y, Al-Asadi RAU, Al-Mousswi Z, Alamrani H. The medicinal importance of Thyme plant (*Thymus vulgaris*). Biomedicine. 2021; 41(3):531-534.
5. Azaz AD, Irtem HA, Kurkcuoglu M, Baser KHC. Composition and the *in vitro* antimicrobial activities of the essential oils of some *Thymus* species. Z Naturforsch C J Biosci. 2004; 59(1-2):75-80. https://doi.org/10.1515/znc-2004-1-216.
6. Marino M, Bersani C, Comi G. Antimicrobial activity of the essential oils of *Thymus vulgaris* L. measured using a Bioimpedometric method. J Food Prot. 1999; 62(9):1017-1023. https://doi.org/10.4315/0362-028x-62.9.1017.
7. Serra JJ, Mura J, Fagoaga C, Castellano G. Oxidative stability of margarine is improved by adding natural antioxidants from herbs and spices. Preprints. 2023; 2023051445. https://doi.org/10.4315/0362-028x-62.9.1017.
8. Oubihi A, Ballaoui FZ, Imtara H, Jaber H, Ettouil A, Haida S, Ouhssine M, Noman OM, Mothana RA, Tarayrah M, & Guessous Z. Phytochemical compounds, acute toxicity, anti-inflammatory and antioxidant activities of *Thymus leptobotrys* Murb essential oil. Molecules. 2023; 28(3). https://doi.org/10.3390/molecules28031355.
9. Rodrigues V, Cabral C, Évora L, Ferreira I, Cavaleiro C, Cruz MT, Salgueiro L. Chemical composition, anti-inflammatory activity and cytotoxicity of *Thymus zygis* L. subsp. *sylvestris* (Hoffmanns. & Link) Cout. Essential oil and its main compounds. Arab J Chem. 2019; 12:3236-3243. https://doi.org/10.1016/j.arabjc.2015.08.026.
10. ElHadj Ali IB, Bahri R, Chaouachi M, Boussaïd M, Harzallah-Skhiri F. Phenolic content, antioxidant and allelopathic activities of various extracts of *Thymus numidicus* Poir. Organs. Ind Crops Prod. 2014; 62:188-195. https://doi.org/10.1016/j.indcrop.2014.08.021.

11. Aneva I, Zhelev P, Stoyanov S, Marinov Y, Georgieva K. Survey on the distribution, diversity and phytochemistry of genus *Thymus* in Bulgaria. Ecol Balk. 2018; 10:101-110.
12. Trendafilova A, Todorova M, Ivanova V, Zhelev P, Aneva I. Essential oil composition of five *Thymus* species from Bulgaria. Chem Biodivers. 2021; 18(10). https://doi.org/10.1002/cbdv.202100498.
13. Aneva I, Trendafilova A, Nikolova M, Todorova M, Georgieva K. Essential oil composition of the Balkan endemic *Thymus longedentatus* (Degen & Urum.) Ronniger. Boletín Latinoamericano Y Del Caribe De Plantas Medicinales Y Aromáticas. 2019; 18(2),197-203. https://doi.org/10.37360/blacpma.19.18.2.13
14. Trendafilova A, Todorova M, Ivanova V, Zhelev P, Aneva I. Essential oil composition of ten species from sect. Serpyllum of genus *Thymus* growing in Bulgaria. Diversity. 2023; 15:759. https://doi.org/10.3390/d15060759.
15. Alipieva K, Kamenova-Nacheva M, Staleva P, Popova M, Aneva I. LC-MS phenolic profile and quantification of rosmarinic acid in methanolic extracts from Bulgarian *Thymus* species [Poster presentation]. GA – 70th Annual Meeting 2022, Thesaloniki, Greece. 2022. DOI: 10.1055/s-0042-1759049.
16. Nikolova M, Aneva I, Zhelev P, Berkov S. Phenolic acid and free radical scavenging activity of endemic *Thymus* species. Biol Bull. 2023; 50(5):895-900. http://dx.doi.org/10.1134/S1062359023601969.
17. Tümen G, Başer K, Demirc B, Ermin N. The essential oils of *Satureja coerulea* Janka and *Thymus aznavourii* Velen. Flavour Fragr J. 1998; 13:65-67. https://doi.org/10.1002/(SICI)1099-1026(199801/02)13:1%3C65::AID-FFJ695%3E3.0.CO;2-Q.
18. Stoyanov S, Marinov Y. *Thymus jalasianus* (Lamiaceae), a new species from the serpentine area of the Eastern Rhodope Mountains, Bulgaria. Ann Bot Fenn. 2020; 57:163-172. https://doi.org/10.5735/085.057.0122.
19. Aneva I, Zhelev P, Bonchev G, Boycheva I, Simeonova S, Kancheva D. DNA barcoding study of representative *Thymus* species in Bulgaria. Plants. 2022; 11:270. https://doi.org/10.3390/plants11030270.
20. Ivancheva S, Stantcheva B. Ethnobotanical inventory of medicinal plants in Bulgaria. J Ethnopharmacol. 2000; 69(2):165-172. https://doi.org/10.1016/s0378-8741(99)00129-4.
21. Markova M. *Thymus* L. In: Velčev V, editor. Flora Reipublicae Popularis Bulgaricae 9: 295. Aedibus Academiae Scientiarum Bulgaricae, Serdicae [Sofia]; 1989. 9: 295p., [In Bulgarian].
22. Stoyanov S, Marinov Y. *Thymus aznavourii* Velen. (Lamiaceae): First records for Bulgarian and Greek flora. C R Acad Bulg Sci. 2021; 74(3):352-362.
23. Ijoma K, Ajiwe VI, Odinma S. The organic extracts from the leaves of *Ficus thonningii* Blume, *Jatropha tanjorensis* J.L. Ellis & Saroja, and *Justicia carnea* Lindley as potential nutraceutical antioxidants and functional foods. Trends Phytochem Res. 2023;7(1):76–85. https://doi.org/10.30495/TPR.2023.1977670.1318.
24. Petrova M, Nikolova M, Dimitrova L, Dimitrova M, Sergiev I. *In vitro* multiplication and GC/MS-based metabolic profiles of *Cichorium intybus* L. J Microbiol Biotechnol Food Sci. 2023;13(2):e9688. https://doi.org/10.55251/jmbfs.9688.
25. Nikolova M, Aneva I, Zhelev P, Berkov S. Analysis of exudate compounds of *Thymus* species. Plant Biosyst. 2024. https://doi.org/10.1080/11263504.2023.2293034.
26. Traykova B, Nikolova M, Stanilova M, Aneva I. Growth and GC/MS-based metabolic profile of the hydroponically propagated species of genus *Thymus* (Lamiaceae). Phytologia Balcanica. 2024;30(1):85-94.
27. Sivakumar R, Jebanesan A, Govindarajan M, Rajasekar P. Larvicidal and repellent activity of tetradecanoic acid against *Aedes aegypti* (Linn.) and *Culex quinquefasciatus* (Say.) (Diptera: Culicidae). Asian Pac J Trop Med. 2011; 4(9):706-710. https://doi.org/10.1016/s1995-7645(11)60178-8.
28. Fatima Z, Shahidulla SM. Formulation, optimisation, and evaluation of hexadecanoic acid phytosomal gel for anti-fungal activity. Int J Pharm Sci Res. 2022; 14(1):519-529. http://dx.doi.org/10.13040/IJPSR.0975-8232.14(1).519-29.
29. Ganesan T, Subban M, Christopher Leslee DB, et al. Structural characterisation of n-hexadecanoic acid from the leaves of *Ipomoea eriocarpa* and its antioxidant and antibacterial activities. Biomass Conv Bioref. 2022. http://dx.doi.org/10.1007/s13399-022-03576-w.
30. Bharath B, Perinbam K, Devanesan S, AlSalhi MS, Saravanan M. Evaluation of the anticancer potential of hexadecanoic acid from brown algae *Turbinaria ornata* on HT–29 colon cancer cells. J Mol Struct. 2021; 1235:130229. http://dx.doi.org/10.1016/j.molstruc.2021.130229.
31. Saeedi M, Khezri K, Zakaryae A. A comprehensive review of the therapeutic potential of α-arbutin. Phytother Res. 2021; 35(8):4136-4154. https://doi.org/10.1002/ptr.7076.
32. Liu L, Liu Y, Zhao J, Xing X, Zhang C, Meng H. Neuroprotective effects of D-(-)-quinic acid on aluminum chloride-induced dementia in rats. Evid Based Complement Alternat Med. 2020. https://doi.org/10.1155/2020/5602597.
33. Ivasenko S, Shakarimova K, Bokayeva A, Marchenko A, Lavrinenko A, Kolesnichenko S. Study of the phenolic compounds of the dry extract of *Thymus crebrifolius* using a combined HPLC–UV and HPLC-ESI-MS/MS method. Bull Univ Karaganda Chem. 2021; 102(2):18-23. https://doi.org/10.31489/2021ch2%2F18-23.
34. Kontogiorgis C, Ntella M, Mpompou L, Karallaki F, Papadopoulos A, Hadjipavlou-Litina D, Lazari D. Study of the antioxidant activity of *Thymus sibthorpii* Bentham (Lamiaceae). J Enzyme Inhib Med Chem. 2016; 31(S4):154-159. https://doi.org/10.1080/14756366.2016.1222583.
35. Afonso A, Pereira O, Neto R, Silva A, Cardoso S. Health-promoting effects of *Thymus herba-barona*, *Thymus pseudolanuginosus*, and *Thymus caespititius* decoctions. Int J Mol Sci. 2017; 18(9):1879. https://doi.org/10.3390/ijms18091879.
36. Park S, Uddin M, Xu H, Kim Y, Lee S. Biotechnological applications for rosmarinic acid production in plants. Afr J Biotechnol. 2008; 7(25):4959-4965.
37. Kashyap D, Sharma A, Tuli H, Punia S, Sharma AK. Ursolic acid and oleanolic acid: Pentacyclic terpenoids with promising anti-inflammatory activities. Recent Pat Inflamm Allergy Drug Discov. 2016; 10(1). https://doi.org/10.2174/1872213x10666160711143904.
38. Park JS, Rehman IU, Choe K, Ahmad R, Lee HJ, Kim MO. A triterpenoid lupeol as an antioxidant and anti-neuroinflammatory agent: Impacts on oxidative stress in Alzheimer's disease. Nutrients. 2023; 15:3059. https://doi.org/10.3390/nu15133059.
39. Carmo J, Cavalcante-Araújo P, Silva J, Ferro J, Correia AC, et al. Uvaol improves the functioning of fibroblasts and endothelial cells and accelerates the healing of cutaneous wounds in mice. Molecules. 2020; 25(21). https://doi.org/10.3390/molecules25214982.
40. Phongpaichit S, Nikom J, Rungjindamai N, Sakayaroj J, Hutadilok-Towatana N, Rukachaisirikul V, Kirtikara K. Biological activities of extracts from endophytic fungi isolated from Garcinia plants. FEMS Immunol Med Microbiol. 2007; 51(3):517-525. https://doi.org/10.1111/j.1574-695X.2007.00331.x.
41. Gül L, Özdemir N, Gul O, Çon A. Evaluation of *Thymus pseudopulegioides* plant extracts for total phenolic contents, antioxidant and antimicrobial properties. Eur Food Sci Eng. 2022; 3. https://doi.org/10.55147/efse.1091864.
42. Shahar B, Indira A, Santosh O, Dolma N, Chongtham N. Nutritional composition, antioxidant activity and characterisation of bioactive compounds from *Thymus serpyllum* L.: An underexploited wild aromatic plant. Meas Food. 2023; 10. https://doi.org/10.1016/j.meafoo.2023.100092.