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A Comparative Study of Headspace and Hydro-Distillation Techniques for Volatile Compounds in Thyme Species across Drought Stress and Phenological Stages

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ABSTRACT

Variations in essential oils and phenolic compounds of *T. daenensis*, *T. armeniacus*, and *T. vulgaris* were investigated at two phenological stages: 50 and 100% flowering. The volatile components of these species extracted using headspace and hydrodistillation techniques were analyzed via gas chromatography-mass spectrometry (GC-MS) under two water stress conditions (90 and 50% field capacity). GC-MS analysis identified the major constituents, including thymol (5.175–51.53%), carvacrol (1.77–24.52%), γ-terpinene (3.95–23.66%), and p-cymene (3.35–24.50%), in the three species using the hydrodistillation method. Maximum thymol content of 51.53% and 43.20% was recorded in *T. daenensis* during 100% flowering under 90 and 50% FC conditions, respectively. A decline in thymol content in *T. daenensis* and *T. vulgaris* was recorded as a decrease from 90 to 50% FC, while *T. armeniacus* showed an increase. In the headspace method, different compounds were identified as the primary components. Notably, thymol (7.68–54.34%) was identified as the dominant compound in *T. daenensis*. Drought stress significantly increased thymol composition at the 50% flowering stage but reduced it at the 100% flowering stage. Hierarchical clustering and principal component analysis revealed three distinct groups, separating the compounds identified by the headspace and hydrodistillation methods. The findings suggest that hydrodistillation is more reliable for determining the true concentrations of compounds.

Keywords: Compounds, Drought stress, Headspace, Hydrodistillation, Thyme

Abbreviations
GC-MS: gas chromatography-mass spectrometry, HS: Headspace HCA: Hierarchical cluster analysis, PC: Principal component, FC: Field capacity

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INTRODUCTION

The family Lamiaceae has over 250 genera and is extensively employed in flavoring ingredients, spices, and folk medicine [1]. The essential oils from these plants have remarkable antimicrobial and antioxidant activity as well as other therapeutic properties including choleretic, anthelmintic, antiseptic, scarring, and diuretic activity [2]. The essential oils of medicinal plants are basically divided into two different chemical classes of terpenoids and phenylpropanoids [3]. The medical significance of thyme is due primarily to its phenolic compounds thymol, p-cymene, γ-terpinene, and carvacrol in its essential oil [4]. Plants rich in thymol are widely employed in traditional medicine in different parts of the world. Apart from being applied as food preservatives, these compounds have diverse biological properties, including anti-inflammatory, antispasmodic, anti-carcinogenic, and antimicrobial activity [5, 6] The quality and price of medicinal plant commodities are generally assessed based on the composition of its essential oil [7]. A variety of techniques are applied for extracting essential oils from medicinal plants, including hydrodistillation, steam distillation, and headspace solid-phase microextraction [8]. Among these methods, conventional hydrodistillation is the most widely applied one [9]. Plant material is packed in a fixed vessel in this process and stirred in water and then heated. Steam can also be injected into plant matter directly. Hot water and steam are responsible for desorbing bioactive compounds from plant cells. The vapor of water and oil is condensed through indirect cooling and transferred to a separator from where oil and bioactive compounds get separated from water automatically [10]. The headspace (HS) method involves adsorption in a fiber of volatile components, which are injected into a gas chromatograph for separation. The process is especially helpful in detecting volatile compounds from solid or liquid samples that are not injectable in a direct manner into the instrument, e.g., fresh or dried plant tissues when sample volume is small or limited [11]. The headspace method has several benefits in comparison to other procedures including reduced amounts of plant matter and short processing times. Headspace sampling, a vapor-phase extraction method that involves partitioning analyses between a non-volatile liquid or solid medium and vapour above a sample, is typically considered the fastest and cleanest procedure for analyzing volatile components [12]. This approach provides results similar to those achieved with conventional methods for extracting volatile compounds [13]. On the other hand, hydrodistillation method requires more time and a greater amount of plant material. Despite these limitations, it remains one of the most commonly employed techniques for extracting essential oils and identifying volatile

compounds from plant samples. Conversely, the headspace method is relatively new, efficient, user-friendly, and convenient, making it ideal for the rapid separation of components from plant materials [14]. The HS-SPME/GC/MS method facilitates qualitative and quantitative investigation of active compounds in plants. For instance, Nezhadali *et al*. [15] compared the volatile compounds in *T. vulgaris* using both headspace and hydrodistillation methods, found that thymol was the most abundant compound in all experiments, regardless of the method employed. Thymol and p-cymene were identified as the major compounds with the highest concentrations in the plants. Similarly, a comparison between HS-SPME and hydrodistillation-GC-MS for analyzing the volatiles of *T. daenensis* showed that both methods detected similar major components, although the quantities of these compounds varied depending on the extraction technique [16]. In the essential oil of *T. serpyllum*, hydrodistillation (HD) yielded a predominance of oxygenated monoterpenes, while the headspace (HS) method resulted in a higher concentration of monoterpene hydrocarbons [17]. Abbasi *et al*. [18] identified p-cymene, γ-terpinene, α-pinene, and thymol as the primary components of *Zataria multiflora* through hydrodistillation. They also noted that the highest levels of thymol and p-cymene were observed at the 50% flowering stage under 90% field capacity (FC), along with the maximum γ-terpinene content. The chemical composition of essential oils has been thoroughly investigated various *Thymus* species [3, 19]. In *T. armeniacus*, the most abundant metabolites identified by hydrodistillation were γ-terpinene, p-cymene, and α-pinene [20]. Nikavar *et al*. [21] identified thymol, p-cymene, β-caryophyllene, and methyl carvacrol as the main compounds in *T. daenensis*. Omidbaigi *et al*. [22] detected that *T. vulgaris* from Iran had the highest and lowest essential oil content during the early flowering and seed ripening stages, respectively. Similarly, Nejad-Ebrahimi *et al*. [23] found that *T. caramanicus* Jalas had the highest carvacrol content during the vegetative stage.

Water stress is one of the most significant environmental factors limiting product yield [24]. Thyme plants respond to water stress by limiting growth and allocating more carbohydrates to the roots to enhance their absorption capacity [25]. Alavi-Samani *et al*. [26] found that varying irrigation levels significantly influence the composition of essential oils, with higher levels of p-cymene, γ-terpinene, and carvacrol in plants experiencing drought stress compared to those not under stress. They also observed a greater reduction in thymol content in *T. vulgaris* than in *T. daenensis* under drought conditions. Furthermore, they noted an increase in essential oil yield under moderate drought stress.

This investigation focused on examining how various extraction techniques influence the chemical makeup of volatile compounds found in three species of thyme. The study emphasizes the extraction of volatile compounds from the essential oils of the aerial parts of *T. daenensis* Celak, *T. armeniacus* Klok. et Shost, and *T. vulgaris* L. utilizing headspace and hydrodistillation methods, with analysis conducted via GC/MS during the 50% and 100% flowering stages under conditions of 90% and 50% FC. This study provides a comparative quantitative evaluation under drought stress, which has not been simultaneously reported for three *Thymus* species.

MATERIALS AND METHODS

The plant materials used in this study included three species: *T. daenensis* Celak (voucher no. 49), *T. armeniacus* Klok. et Shost, and *T. vulgaris* L. Seeds of these species were obtained from the Agriculture and Natural Resources Research Center in Isfahan, Iran. A randomized complete block design was implemented with three replicates. seeds went into trays containing a blend of peat moss, sand, and coco peat (4:2:1 ratio), where they developed under greenhouse conditions enhanced with artificial lights. To standardize replicates, stem cuttings were taken from each plant and transplanted into 25 × 30 cm pots. These potted specimens were subsequently relocated outside the Shahrekord University greenhouse (coordinates: 50°49'E, 32°21'N; elevation 2050 m). Two irrigation treatments were implemented: 90% (normal conditions) and 50% FC (drought stress). The aerial parts of the plants were harvested for analysis at two growth stages: 50 and 100% flowerings. After harvesting, the samples were dried in the shade at room temperature (20–25 °C).

Extraction of Essential Oil

To assess the content of essential oil, each sample was subjected to hydrodistillation using a Clevenger-type apparatus with 250 mL of distilled water for a duration of 3 hours [27]. Essential oil samples were then dried drying with anhydrous sodium sulfate before being sealed and held at 4°C for GC-MS analysis. The extracted essential oil was kept in dark vials at a temperature of 4°C until it was ready for further analysis. For compounds detection at the 50 and 100% flowering stages using the headspace method, 2 g of fresh aerial plant material was placed in 20 mL vials and sealed with silicone septa. A 100-micrometer thick polymethyl-siloxane fiber was prepared along with a complete SPME set. Temperatures of 100 and 105℃ were chosen as the equilibrium temperatures for the headspace in the incubation oven and the syringe, respectively. Each sample vial was placed in a thermal cabinet and stirred for 30 minutes. As a subsequent step, a volume of 250µl of headspace gas was introduced into the gas chromatograph (GC) for analysis.

Identification Methods of Composition

To carry out gas chromatography-mass spectrometry (GC-MS), a 7890 type gas chromatograph manufactured by Agilent Technologies Inc. at the laboratory of Islamic Azad University Isfahan (Khorasgan) branch, which is based in Santa Clara, United States of America, was utilized. This device was equipped with a detector and an HP-5MS capillary column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness). The mass spectra (MS) were produced using an ionization source voltage of 70 eV in electron ionization (EI) mode, with helium as the carrier gas. The oven temperature was programmed to rise from 70 to 290 °C, where it was maintained isothermally for 10 minutes. The temperatures for the injector and interface were set at 290 °C and 280 °C, respectively. Additionally, the essential oils were analyzed using a Thermoquest-Finnigan apparatus, also equipped with an HP-5MS capillary column (film thickness: 0.25 µm, length: 30 m, internal diameter: 0.25 mm). Helium was used as the carrier gas at a flow rate of 2.1 mL/min, and the oven temperature program mirrored the one previously described. The detector and injector temperatures were adjusted to 290 °C and 250 °C, respectively. Component identification relied on the retention times of n-alkanes (C6–C24) injected into the HP-5MS column, along with comparisons to data from the NIST 08 and WILEY libraries and existing literature [28, 29]. For headspace analysis, the procedure adhered to the methodology outlined in our earlier study [18].

Statistical Analysis

To categorize the *Thymus* species under study, hierarchical cluster analysis (HCA) was performed using SPSS software (version 16) following the Ward method. Furthermore, principal component analysis (PCA) was utilized to identify differences or similarities

among the three *Thymus* species and their main components under two conditions: control and drought stress and the findings are displayed as biplot.

RESULTS AND DISCUSSION

Identified Chemical Compounds by Headspace and Hydrodistillation Methods

T. daenensis

By using the headspace method, it was revealed that the *T. daenensis* samples included a total of 31 different components, the majority of which were monoterpenes, sesquiterpenes, and esters (Table 1). These compounds varied in number and percentage at the plant's two different phenological stages. GC-MS analysis of the headspace revealed 21 and 15 components at 90% FC, and 10 and 21 components at 50% FC for the 50 and 100% flowering stages, respectively. The identified compounds accounted for 79.32–94.94% of the total composition across the two phenological stages. One of the main oxygenated monoterpenes identified was thymol, which constituted 54.34% during the 50% flowering stage and 22.29% during the 100% flowering stage under drought stress (50% FC) and control conditions (90% FC), respectively. Other principal compounds identified in *T. daenensis* included α-thujene, α-pinene, camphene, myrcene, p-cymene, γ-terpinene, carvacrol, and β-caryophyllene. Notably, only four compounds, p-cymene, γ-terpinene, thymol, and β-caryophyllene, were present in both phenological stages and under both moisture conditions (90 and 50% FC). During the 50% flowering stage under drought stress conditions, the compounds of α-thujene, α-pinene, camphene, and myrcene were not found. However, they were detectable at 100% flowering in 90 and 50% FC, respectively. Strangely, when soil moisture reduced, the amounts of these compounds rose. During the 50% flowering stage, their content was higher than at the 100%. Flowering stage when the moisture conditions were managed. Compared to the control conditions, the most important component of the essential oil, thymol, showed a considerable increase with reduced soil moisture at the 50% flowering stage. In contrast, at the 100% flowering stage, thymol levels decreased due to drought stress. Carvacrol was only detected at the 50% flowering stage under low soil moisture conditions. Furthermore, at the 50% flowering stage under drought stress, *T. daenensis* exhibited a significant decrease in the levels of p-cymene and γ-terpinene compared to the control conditions. This reduction coincided with a significant increase in thymol, suggesting that these two compounds are precursors for thymol synthesis and were almost entirely converted into thymol during the 50% flowering stage. A rise in p-cymene and a decrease in γ-terpinene were observed at the 100% flowering stage. Under these conditions, thymol levels were lower in plants subjected to drought stress than in those under control conditions. Using the hydrodistillation technique, the identified compounds accounted for 94.29% to 98.33% of the essential oils under both 50 and 90% FC conditions. FC (Table 1). Based on the results of the GC-MS analysis, it was determined that the main constituents were thymol (41.44–51.53%), β-caryophyllene (9.25–10.07%), carvacrol (4.91–7.90%), p-cymene (3.35–7.61%), γ-terpinene (3.95–6.63%), and borneol (3.65–6.03%). It is possible that the prolonged watering period is responsible for the differences in these compounds that occur under drought stress in comparison to the conditions that serve as the control. Bahreininejad *et al*. (2013) identified thymol as the major compound in the essential oil composition of *T. daenensis*, along with carvacrol, p-cymene, γ-terpinene, β-caryophyllene, and borneol, under drought stress conditions [30]. These findings are consistent with the results of the present study. Similarly, Tohidi *et al*. [31] found that the essential oils of *Thymus* species contained thymol, carvacrol, geraniol, and p-cymene as the main components. This conclusion is in agreement with the findings that have been presented here. Nickavar *et al*. [21] also found thymol, p-cymene, β-caryophyllene, and methyl carvacrol to be the key constituents of *T. daenensis* essential oil, further supporting the results of this study. Thymol, one of the main oxygenated monoterpenes in the samples, reached its highest concentration (51.53%) during the 100% flowering stage under normal conditions. However, its levels significantly decreased at both the 50% and 100% flowering stages under control and drought stress conditions. Askary *et al*. [32] noted that thymol content increased as soil moisture decreased from 100 to 67% of FC, but declined under severe water stress (33% FC). They also identified thymol as the main component of *T. daenensis* essential oil. Among the analyzed compounds, γ-terpinene, thymol, and β-caryophyllene were present at both the 50 and 100% flowering stages under both control and drought conditions. The significant production of β-caryophyllene in plants may be associated with biotic and abiotic stress factors, as its synthesis has been confirmed in response to herbivory [33]. Carvacrol content increased at the 50% flowering stage but decreased at the 100% flowering stage under drought stress. Other studies have also reported a reduction in carvacrol content in *T. daenensis* under moderate and severe stress conditions [30, 34]. In contrast, the levels of p-cymene increased during both blooming stages when the plant was subjected to drought stress. On the other hand, the levels of γ-terpinene increased during the 100% flowering stage after drought stress, but reduced during the 50% flowering stage. This was in comparison to the control conditions. According to Askary *et al.* [32], a decline in p-cymene was detected in *T. daenensis* when the soil moisture decreased from 100 to 33% of the FC. In addition, it was observed by Ghasemi Pirbalouti [35] that there was a decrease in the thymol content, whereas there were no significant changes in the carvacrol and β-caryophyllene content under conditions of 50% FC.

T. armeniacus

When applying the headspace method for *T. armeniacus*, it was observed that key compounds such as α-thujene, α-pinene, camphene, myrcene, and γ-terpinene diminished under drought stress at both the 50 and 100% flowering stages compared to normal moisture conditions (Table 2). Conversely, p-cymene and 1,8-cineole were detected at both the 50% and 100% flowering stages under both control and drought stress conditions. However, under drought stress, the levels of these compounds rose at the 50% flowering stage but decreased at the 100% flowering stage. Notably, 1,8-cineole was only detected in this species, suggesting that its presence may depend on the plant species, environmental conditions, and agronomic practices.

Using the hydrodistillation technique, GC-MS analysis revealed 35 compounds, making up 92.70–96.24% of the essential oil in *T. armeniacus*. The highest concentration of these compounds was found at the 50% flowering stage under normal conditions, while the lowest was at the 100% flowering stage during drought stress (Table 2). The essential oil of *T. armeniacus* was mainly composed of monoterpenes, both hydrocarbons and oxygenated types, such as carvacrol (17.95–24.52%), 1,8-cineole (11.31–13.43%), p-cymene (8.04–11.86%), thymol (5.18–10.32%), γ-terpinene (5.52–8.30%), α-pinene (5.20–5.73%), borneol (3.76–5.97%), and β-caryophyllene (2.70–3.97%). These compounds were present at both phenological stages under both control and drought stress conditions. Thymol reached its highest concentration (10.32%) at the 100% flowering stage and was more abundant at both stages under drought stress compared to control conditions. Conversely, carvacrol levels decreased under drought stress at both growth stages, with its maximum concentration (24.52%) recorded in *T. armeniacus*. Research on *T. caramanicus* indicated that carvacrol was most abundant during the vegetative stage (before flowering) [23]. The highest concentrations of specific compounds were as follows: α-pinene (5.72%) at the 50% flowering stage under drought stress, p-cymene (11.86%) at the 100% flowering stage under control conditions, γ- terpinene (8.30%) at the 50% flowering stage under drought stress, and 1,8-cineole (13.43%),

Table 1 Chemical composition (%) of essential oils of *T. daenensis* extracted by HS and HD methods under drought conditions

Compounds		Hydrodistillation				Headspace			
		50% flowering		100% flowering		50% flowering		100% flowering	
	RI	I_1	I_2	I_1	I_2	I_1	I_2	I_1	I_2
3-Methylbutanal	663					0.15			
Pentanal	663								0.41
2-Methylbutanal	669					0.10			
Furan, 2-ethyl-	696								0.09
Methyl 3-methylbutanoate	766	0.36	0.27	0.30	0.11				
Methyl isovalerate	768					1.53		0.60	1.31
Ethyl 3-methylbutanoate	848	0.06	0.09	0.06	0.06				
α-Thujene	926	1.50	1.58	1.53	1.25	11.14		6.00	12.99
α-pinene	935	1.01	1.29	0.96	0.77	5.57		2.79	6.80
Camphene	954	0.98	1.66	0.81	1.05	3.69		1.89	4.78
Sabinene	975	0.06	0.20		0.10	0.98			0.98
β-Thujene	978							0.64	
β-Pinene	982	0.36	0.48	0.35	0.26	1.34			1.66
Myrcene	990	2.55	2.03	2.81	1.77	10.25		8.12	11.69
α-Phellandrene	1013	0.30	0.26	0.38				0.98	
α-Terpinene	1023	1.06	0.76	1.23	1.20	3.24		3.72	3.98
p-Cymene	1034	4.47	5.60	3.35	7.61	11.83	0.45	10.72	14.82
1,8-Cineole	1038	2.41	3.80	2.06					
Z-Ocimene	1043	0.13	0.10	0.15					
(E)-Ocimene	1052					0.34			0.48
γ-Terpinene	1066	5.70	3.95	6.11	6.63	14.27	1.24	17.09	15.79
trans-Sabinenehydrate	1086	1.43	1.95	1.48	1.20				
Terpinolene	1092			0.28		0.33			0.48
Linalool	1116	1.32	0.57	0.64	0.79	0.47			0.62
cis-β-Terpineol	1131				0.14				
Borneol	1198	3.65	6.03	3.65	5.41	0.98	6.01		1.58
α-Terpineol	1208	0.55	0.22	0.51					
Thymol methyl ether	1236		0.26	0.06	0.08				
Carvacrol methyl ether	1237	0.11							
Isobornyl acetate	1292	0.09							0.21
Bornyl acetate	1295	0.47	0.97	0.47	1.25	0.12	0.83		
Thymol	1311	50.41	41.44	51.53	43.20	7.68	54.34	22.29	8.92
Carvacrol	1317	4.91	7.90	6.13	4.81		5.48		
β-Caryophyllene	1441	9.67	9.25	9.73	10.07	4.70	15.12	11.38	6.14
Aromadendrene	1456	0.53	0.27	0.59	0.33				
α-Humulene	1478	0.47	0.42	0.47	0.44	0.19			
α-Farnesene	1508		0.10					0.96	0.60
Viridiflorene	1509	0.38			0.23				
β-Bisabolene	1517	1.28	1.71	0.97	1.62	0.42	2.68		
δ-Cadinene	1531	0.16							
β-Sesquiphellandrene	1534				0.07				
α-Bisabolene	1548	0.84	0.79	1.09	1.32		1.69	0.58	0.61
Caryophyllene oxide	1615	0.90	2.03	0.63	2.52				
1-Hexacosanol	1653							3.09	
9-Octadecenal	1774						4.55		
Monoterpene hydrocarbons (%)		19.55	19.86	19.44	21.84	62.98	1.69	51.95	74.45
Oxygenated monoterpenes (%)		63.92	61.19	65.05	55.68	9.25	66.66	22.29	11.33
Sesquiterpene hydrocarbons (%)		13.33	12.54	12.85	14.08	5.31	19.49	12.92	7.35
Oxygenated sesquiterpenes (%)		0.90	2.03	0.63	2.52	0	0	0	0
Others		0.42	0.36	0.37	0.17	1.78	4.55	3.69	1.81
Total amount of constituents		98.12	95.98	98.33	94.29	79.32	92.39	90.85	94.94

RI: Retention indices on the HP-5MS column, I_1: irrigation at 90% FC, I_2: irrigation at 50% FC

Table 2 Chemical composition (%) of essential oils of *T. armeniacus* extracted by HS and HD methods under drought conditions

Compounds		Hydrodistillation				Headspace			
		50% flowering		100% flowering		50% flowering		100% flowering	
	RI	I_1	I_2	I_1	I_2	I_1	I_2	I_1	I_2
3-Methylbutanal	663					0.19	0.42	0.15	
Methyl 3-methylbutanoate	766	0.06		0.04					
Methyl isovalerate	768					0.32		0.15	
α-Thujene	926	1.27	1.13	1.29	1.03	7.04	4.66	7.85	0.69
α-pinene	935	5.73	5.60	5.49	5.20	22.88	21.58	23.67	4.82
Thuja-2,4(10)-diene	946			0.07				0.37	
Camphene	954	1.46	1.99	1.73	1.57	3.55	6.16	5.35	1.74
Sabinene	975	0.68	0.53	0.53	0.64	1.75	1.47	1.90	
β-Pinene	982	1.17	0.94	1.00	0.93	2.89	2.39	2.96	
Myrcene	990	2.00	1.75	1.61	1.97	6.82	5.58	8.26	0.88
3-Octanol	1008				0.02				
α-Phellandrene	1013	0.23	0.21		0.20		0.69	0.76	
α-Terpinene	1023	0.81	0.82	0.51	0.57	2.21	1.84	1.95	0.34
p-Cymene	1034	10.87	9.98	11.86	8.04	10.23	11.08	9.94	6.67
γ-Terpinene	1066	7.60	8.30	5.52	5.94	14.99	8.08	7.40	1.59
1,8-Cineole	1038	12.65	12.10	11.31	13.43	13.98	14.36	14.73	8.52
trans-Sabinenehydrate	1086	2.64	2.19	2.39	2.64				
Terpinolene	1092					0.29	0.29	0.36	
Linalool	1116	0.24	0.19	0.23					
cis-β-Terpineol	1131			0.48					
Limonene oxide	1142	0.05	0.12	0.09	0.05				
camphor	1162	3.33	3.86	3.95	3.10	0.57	0.88		1.33
Borneol	1198	3.76	5.58	4.59	5.97		2.42	1.88	3.35
α-Terpineol	1208	2.77	2.13	2.83	2.56				
trans-Dihydrocarvone	1214		0.31						
Thymol methyl ether	1236	0.09	0.77	0.19	0.73	0.60	0.33		0.33
Carvacrol methyl ether	1237	1.65	1.98	1.66	0.98				
bornyl acetate	1292	0.42	0.55	0.40	0.36				
Thymol	1311	5.18	7.51	6.43	10.32		5.32	5.05	4.13
Carvacrol	1317	24.52	17.95	21.73	20.18	2.13			
(E)-2-Octenal	1392							0.27	
β-Bourbonene	1397	0.13		0.15	0.10				
β-Caryophyllene	1441	3.97	3.89	3.64	2.70	1.91	1.55		
Aromadendrene	1456	0.10							
α-Humulene	1478	0.19	0.22	0.19	0.13				
Unknown	1485							0.47	
Germacrene D	1499	0.48	0.57	0.49	0.10				
β-Bisabolene	1517		1.70	1.25	1.66		1.38	1.05	1.31
δ-Cadinene	1531	0.10		0.10					
(E)-2-Tridecenal	1550							0.73	
α-Bisabolene	1547	0.33	0.24	0.22	0.21				
(E)-Nerolidol	1554		0.06	1.81	0.12			0.65	
Caryophyllene oxide	1615	1.76	1.77	2.14	1.26				
Elemol acetate	1670		0.24						
10-Undecenal	2068								61.71
Monoterpene hydrocarbons (%)		34.46	33.43	31.99	28.72	72.64	63.82	70.77	16.74
Oxygenated monoterpenes (%)		54.6	52.67	55.61	57.74	17.27	23.32	22.31	17.66
Sesquiterpene hydrocarbons (%)		5.31	6.61	6.05	4.91	1.90	2.93	1.51	1.31
Oxygenated sesquiterpenes (%)		1.81	1.89	2.23	1.31	0	0	0	0
Others		0.06	0.55	0.04	0.02	0.51	0.41	1.3	61.71
Total amount of constituents		96.24	95.15	95.92	92.70	92.32	90.48	95.89	97.41

RI: Retention indices on the HP-5MS column, I_1: irrigation at 90% FC, I_2: irrigation at 50% FC

Borneol (5.97%), and β-caryophyllene (3.97%) at the 100% flowering stage under drought stress. Ghasemi-Pirbalouti [35] noted that α-pinene content was higher in stressed plants than in non-stressed ones. This compound is often linked to inflorescences and is known to attract pollinators [36]. The levels of p-cymene, α-pinene, and β-caryophyllene decreased at both 50 and 100% flowering stages as soil moisture declined. In contrast, γ-terpinene increased at both phenological stages under drought stress compared to control conditions. γ-terpinene is a significant component of thyme essential oil, making up to 30% of the oil and acting as a precursor to major aromatic monoterpenes like p-cymene (25%) and thymol (40%) [37]. A study on *T. daenensis* also reported an increase in γ-terpinene levels under drought stress [30], which is consistent with the findings of this study.

T. vulgaris

In *T. vulgaris*, the key compounds α-thujene, myrcene, α-terpinene, and γ-terpinene were reduced at the 50% flowering stage under drought stress but increased at the 100% flowering stage as soil moisture decreased (Table 3). Under drought stress, thymol levels rose at the 50% flowering stage but increased at the 100% flowering stage. The highest concentrations of p-cymene (38.53%) and γ-terpinene (34.67%) were recorded at the 100% flowering stage and the 50% flowering stage, respectively, under control conditions. Conversely, α-terpinene levels decreased under drought stress compared to control conditions at both the 50 and 100% flowering stages. Since p-cymene is a major derivative of α-terpinene in essential oils, its presence in this species is due to the rapid spontaneous conversion of α-terpinene to p-cymene, which occurs even fast than its conversion from γ-terpinene. compounds like α-phellandrene, are highly sensitive to moisture levels and are only found in *T. vulgaris* under drought conditions. Soleimani *et al*. [8] identified thymol, p-cymene, γ-terpinene, and carvacrol as the main components of *T. vulgaris* using methods such as microwave distillation and solid-phase microextraction (MD-SPME), headspace solid-phase microextraction (HS-SPME), and conventional hydrodistillation (HD). Wesolowska *et al*. [38] also recognized thymol as a significant component.

Based on GC-MS analysis using the hydrodistillation technique, 33 to 36 chemical constituents were identified in the essential oil of *T. vulgaris*, comprising 94.28% to 97.78% of the total essential oil. The major components were p-cymene (18.05–24.50%), γ-terpinene (18.60–23.66%), thymol (20.22–22.83%), thymol methyl ether (3.17–4.51%), and carvacrol (1.77–3.48%) (Table 3). The highest total compound content (97.78%) was found at the 100% flowering stage under normal conditions, while the minimum (94.37%) was recorded at the 50% flowering stage under drought conditions. Jordan *et al*. [39] in their research on seasonal changes in *T. vulgaris* essential oils, identified 1,8-cineole, terpinyl-acetate, borneol, linalool, β-pinene, α-terpineol, and camphor as the main components of this species. In this study, the highest levels of p-cymene (24.50%) and thymol (20.22%) were noted at the 50% flowering stage under drought stress, whereas γ-terpinene (18.66%) and thymol methyl ether (4.48%) reached their peak at the 100% flowering stage under drought stress. The greatest carvacrol content (3.48%) was observed at the 50% flowering stage under control conditions. The p-cymene content increased as soil moisture decreased from 90 to 50% FC, while γ-terpinene content declined at both phenological stages under these conditions.

The level of thymol methyl ether exhibited a notable variation when subjected to drought stress, showing an increase compared to control conditions. In contrast, the amounts of thymol and carvacrol decreased as the soil moisture content declined from 90 to 50% FC. Sarajuoghi *et al*. [40] in their study on *T. vulgaris* under drought conditions, found that the highest concentrations of thymol (42.37%) and carvacrol (3.14%) were recorded at 80% FC.

McGimpsey *et al*. [41] identified the highest levels of thymol and carvacrol during the flowering phase. They also discovered that the highest percentage of phenolic compounds was present in the summer and after the complete % flowering stage. In the hydrodistilled essential oil of *T. vulgaris*, the predominant compounds were thymol, carvacrol, p-cymene, and γ-terpinene. Furthermore, it was established that the distillation technique had a significant impact on the chemical composition of *T. vulgaris* essential oil, although it did not notably affect the essential oil content [38]. Naghdi-Badi *et al*. [19] reported that the thymol content in *T. vulgaris* was highest during the blooming stage and lowest during the vegetative stage. Studies have indicated that the major components of *T. vulgaris* essential oils are monoterpenes like thymol, carvacrol, γ-terpinene, and p-cymene, and the percentage of these key constituents also rises under drought stress conditions [42].

The biosynthesis of all terpenoids in plant cells is governed by terpene synthases (*TPSs*). Among these, the *TPS2* gene plays a critical role in producing the key essential oil components, Thymol and Carvacrol, in *Thymus* species [43]. In leaves of *T. daenensis* at the 50% flowering stage, *TPS2* expression levels remained statistically unchanged across the two moisture regimes. A significant difference was observed exclusively in the trichomes under control conditions. The *TPS2* gene expression pattern in *T. daenensis* leaves was significantly different compared to its expression in both the leaves and trichomes of *T. vulgaris* under control and drought conditions, across both the vegetative and 50% flowering stages. At the 50% flowering stage, transcript levels of the *TPS4* gene were elevated under well-watered conditions compared to drought in both species. However, this pattern was reversed in *T. vulgaris* at the vegetative stage. Furthermore, GC analysis revealed that thymol production mirrored the expression profile of *TPS4* [44].

Comparison of Two Methods of Headspace and Hydrodistillation to Determine Compounds

The major constituents of essential oils in the species examined, as determined by both using both headspace and hydrodistillation methods, include chemical compounds such as thymol, p-cymene, γ-terpinene, carvacrol, myrcene, and β-caryophyllene (Fig. 1). Consequently, the analysis concentrated on these substances during the phenological phases of 50 and 100% flowering. According to Fig. 1, thymol exhibited no notable variation between the 50 and 100% flowering stages when using the hydrodistillation method, whereas a significant difference was noted with the headspace method.

Table 3 Chemical composition (%) of essential oils of *T. vulgaris* extracted by HS and HD methods under drought conditions

Compounds		Hydrodistillation				Headspace			
		50% flowering		100% flowering		50% flowering		100% flowering	
	RI	I1	I2	I1	I2	I_1	I_2	I_1	I_2
Methyl 2-methylbutanoate	766	0.58		0.51	0.47				
(E)-2-Hexenal	859	0.19	0.18	0.16					
α-Thujene	926	2.08	1.54	1.72	1.50	6.08	5.37	5.38	5.82
α-pinene	935	1.00	0.94	0.93	0.94	3.29	3.09	2.85	3.24
Camphene	954	0.45	0.50	0.38	0.66	1.10	1.16	1.04	1.26
β-Thujene	968			0.32					
Sabinene	975	0.58	0.27		0.26	0.50	0.43	0.38	0.41
β-Pinene	982	0.34	0.33	0.33	0.48	0.75	0.77	0.79	0.94
7-Octen-4-ol	983		0.83						
1-Octen-3-ol	993	0.88		1.13	0.67				
Myrcene	990	3.00	2.53	2.86	2.43	6.68	5.95	5.70	6.01
α-Phellandrene	1013		0.36	0.45	0.34		0.62		
α-Terpinene	1023	2.30	1.76	2.00	1.61	4.22	3.81	2.87	3.21
p-Cymene	1034	18.05	24.50	20.39	23.47	22.17	32.34	38.53	34.28
β-Phellandrene	1042		0.69	0.61					
β-Ocimene	1050	0.18	0.20	0.18		0.23	0.43		
γ-Terpinene	1066	23.66	20.14	23.65	18.60	34.67	34.60	29.30	29.35
trans-Sabinenehydrate	1086	2.22	2.53	2.11	2.66				
Isoterpinolene	1091			0.22					
Hexyl-propionate	1100				0.11				
p-Cymenene	1103		0.05		0.07				
Linalool	1116	3.41	3.14	3.73	3.13	1.17	1.18		0.98
cis-3-Hexenyl iso-butyrate	1137	0.05	0.04	0.04	0.04				
cis-p-Menth-2-en-1-ol	1142	0.69	0.13	0.61	0.09				
camphor	1161	0.37	0.60	0.27	0.70				
(Z)-3-hexenyl butyrate	1182	0.05	0.04		0.05				
Terpinen-4-ol	1197			1.45		0.34	0.52		0.53
Borneol	1198				2.02				
α-Terpineol	1208	0.38	0.35						
Thymol methyl ether	1236	3.17	4.48	3.24	4.51	1.22	1.68	1.48	1.73
Carvacrol methyl ether	1237	1.96	2.23	1.89	2.34		0.82	0.74	0.86
Bornyl acetate	1295	0.27	0.39	0.22	0.39				
Thymol	1311	22.83	20.22	22.59	22.24	1.09	1.76	1.67	1.63
Carvacrol	1317	3.48	1.77	2.23	2.14				
β-Bourbonene	1397		0.14		0.14				
β-Caryophyllene	1441	1.76	1.63	1.40	2.19	0.41	0.96		0.82
Geranyl propionate	1464	0.29		0.37	0.09				
α-Humulene	1478	0.12	0.07	0.09	0.09				
γ-Muurolene	1488	0.04							
α-Farnesene	1508			0.29	0.36				
δ-Cadinene	1531	0.17	0.16	0.15	0.19				
Geranyl butyrate	1543	0.04			0.06				
(E)-Nerolidol	1554			0.05					
Germacrene D-4-ol	1603		0.13	0.16	0.15				
Caryophyllene oxide	1615	1.01	1.34	0.98	1.54				
α-Cadinol	1684	0.08	0.09	0.09	0.10				
Monoterpene hydrocarbons (%)		53.86	56.34	55.93	53.03	79.67	88.58	86.84	84.52
Oxygenated monoterpenes (%)		36.64	33.44	36.57	37.68	3.82	5.95	3.88	5.73
Sesquiterpene hydrocarbons (%)		2.38	1.99	2.31	3.07	0.41	0.96	0	0.82
Oxygenated sesquiterpenes (%)		1.01	1.34	0.98	1.54	0	0	0	0
Others		1.78	1.18	1.99	1.55	0	0	0	0
Total amount of constituents		95.67	94.28	97.78	96.87	83.91	95.49	90.72	91.07

RI: Retention indices on the HP-5MS column, I_1: irrigation at 90% FC, I_2: irrigation at 50% FC

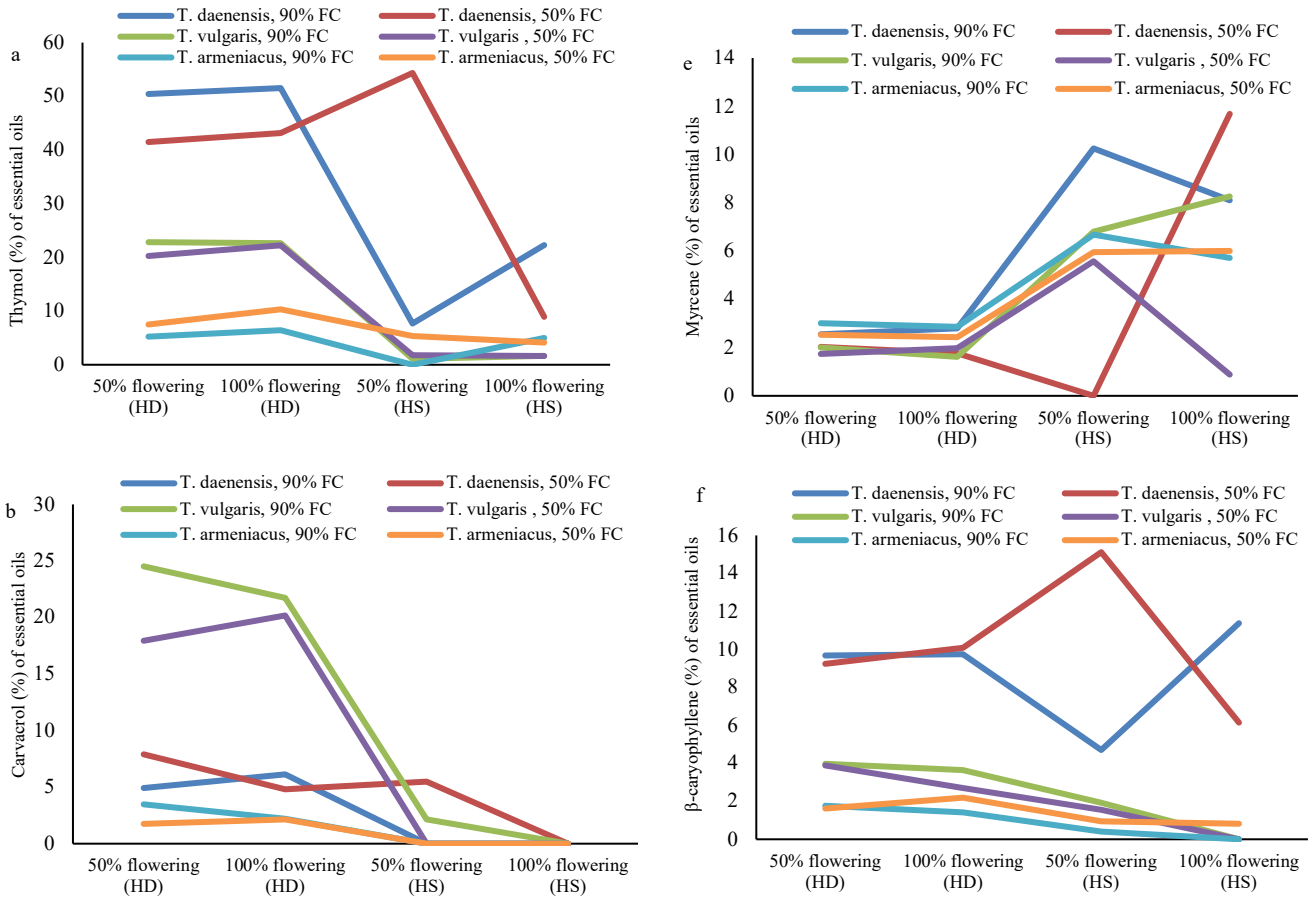

Fig. 1 Amounts of Thymol (A), Carvacrol (B), p-cymene (C), γ-Terpinene (D), (E) Myrcene and β-Caryophyllene (F) components in studied spicies at phenological stages of 50 and 100% flowering under 90 and 50% FC.

Furthermore, the highest concentration of carvacrol was found in *T. vulgaris* through hydrodistillation at both 50 and 90% FC. In the headspace method, p-cymene and γ-terpinene displayed significant differences between the 50 and 100% flowering stages under drought stress conditions. The heights levels of p-cymene and γ-terpinene were recorded in *T. armeniacus* at the 100 and 50% flowering stages under control conditions, respectively. The findings are detailed in Tables 1, 2, and 3. A notable difference was observed between the hydrodistillation and headspace methods for the compounds myrcene and β-caryophyllene. β-Caryophyllene, a sesquiterpene with the formula C15H24, was found in higher quantities using the hydrodistillation method compared to the headspace method, with the highest concentration detected in *T. daenensis*. This compound is known for its anti-cancer, antibiotic, anti-inflammatory, antioxidant, and local anesthetic properties [45]. Overall, based on the varied results of volatile compounds obtained through the headspace method, it can be inferred that this method is less effective for analyzing volatile compounds compared to the extraction and analysis of essential oil compounds. The number of chemical compounds in a plant can vary significantly across different developmental and growth stages [46]. Consequently, identifying the best time for harvesting is essential for the successful cultivation of medicinal plants. Research indicates that environmental conditions, including water availability, temperature, herbivore presence, altitude, daily cycles, seasonal variations, and light intensity, can impact both the yield and chemical compounds of essential oils, as well as the density of glandular trichomes [47, 48]. While the type and structure of trichomes remain unchanged across different developmental phases, their density is influenced by the stage of development. The

highest number of leaf peltate glandular trichomes was recorded during the vegetative phase and at the beginning of blooming under 50 and 90% FC, respectively [18]. The species type and water content play a significant role in determining the composition an quantity of essential oils that plants produce [44, 49, 50]. Several studies have identified the beginning of blooming and the 100% flowering stages as the ideal times for harvesting to achieve the highest concentrations of thymol and carvacrol in various species, populations, and genotypes [19, 41]. It appears that genetic differences and their responses to varying environmental conditions play a crucial role in wide range of plant breeding and cultivation results. More comprehensive studies are needed to better understand these variations and their underlying causes, which is essential for the successful breeding and cultivation of these plants. Therefore, the harvesting time should be determined based on the specific region and its environmental conditions [19]. Additionally, Yarmoohammadi *et al*. [51] observed that variations in glandular structures might explain the differences in essential oil composition among *Nepeta* species.

In the species examined, essential oils predominantly consist of chemical components such as thymol, p-cymene, γ-terpinene, carvacrol, myrcene, and β-caryophyllene, as identified by both methods (Fig. 2). Consequently, the study concentrated on these substances during the phenological phases of 50 and 100% flowering. According to Fig. 2, thymol showed no significant variation between the 50 and 100% flowering stages when using the hydrodistillation method, whereas the headspace method revealed a notable difference. Furthermore, the hydrodistillation method detected the highest levels of carvacrol in *T. vulgaris* at both 50 and 90% FC. In contrast, the headspace method showed significant differences in p-cymene and γ-terpinene between the 50 and 100% flowering stages under drought stress. The highest concentrations of p-cymene and γ-terpinene were found in *T. armeniacus* at the 100% flowering stage under control conditions and the 50% flowering stage under control conditions, respectively. These findings are detailed in Tables 1, 2, and 3. A significant difference was noted between the hydrodistillation and headspace methods for the myrcene and β-caryophyllene compounds. The sesquiterpene β-caryophyllene, with the formula C15H24, was found in higher amounts using the hydrodistillation method compared to the headspace method, with the highest concentration in *T. daenensis*. This compound is known for its anti-cancer, antibiotic, anti-inflammatory, antioxidant, and local anesthetic properties [45]. Overall, given the varied results of volatile compounds obtained through the headspace method, it can be inferred that this method is less effective for analyzing volatile compounds compared to the extraction and analysis of essential oil compounds. In the headspace method, a minimal sample size was employed, leading to the identification of compounds such as thymol, a key biologically active element of thyme [15], in smaller amounts compared to other techniques.

For instance, *T. daenensis* exhibited the highest thymol concentration in essential oil analysis relative to other species. Although most components were similar across both methods, the main difference lay in their amounts. Notably, the headspace method revealed lower thymol levels than other methods. Furthermore, carvacrol was detected in *T. daenensis* (at the 50% flowering stage under drought stress) and *T. armeniacus* (at the 50% flowering stage under normal conditions) using the headspace method, but only at one stage. Conversely, the hydrodistillation method identified carvacrol at both phenological stages under both moisture conditions (Fig. 2).

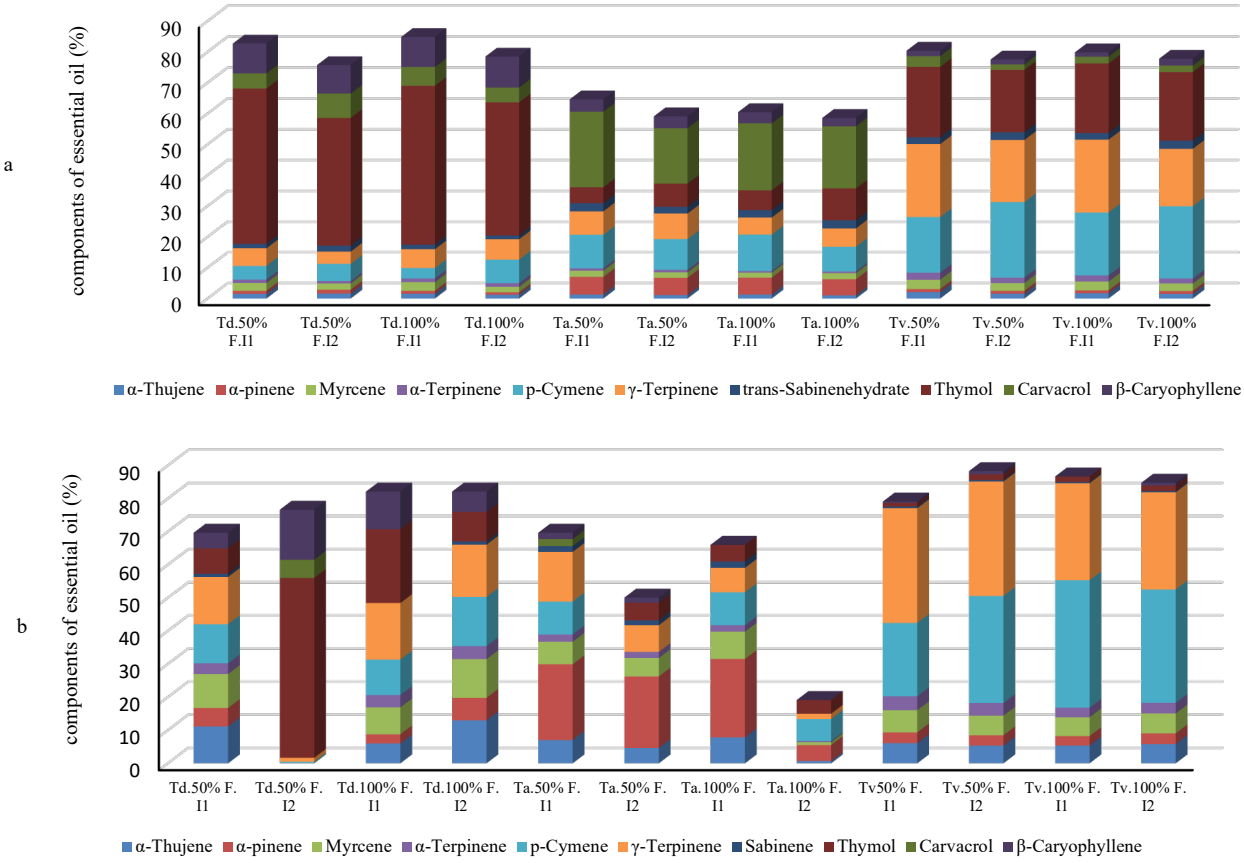

Fig. 2 Subdivision of main components among three species by hydrodistillation (A) and headspace (B) at phenological stages of 50 and 100% flowering under 90 and 50% FC.

Hierarchical Cluster Analysis (CA) and Principal Component Analysis (PCA)

To assess the similarities and connections among *T. daenensis*, *T. armeniacus*, and *T. vulgaris*, as well as their phenolic compounds, hierarchical cluster analysis and principal component analysis (PCA) were conducted using the 12 most abundant compounds (Fig. 3 and 4). The HCA results are presented as dendrogram in Figure 3, showing a maximum composition distance of about 25 km. This analysis offered additional insights into the distribution of volatiles in *Thymus* species through the headspace and hydrodistillation methods. The first cluster included *T. daenensis* with a high thymol content (41.44–51.53%) at both the 50 and 100% flowering stages under control and drought stress conditions at the hydrodistillation method. and 50% flowering stage (54.34% thymol) using headspace methods.

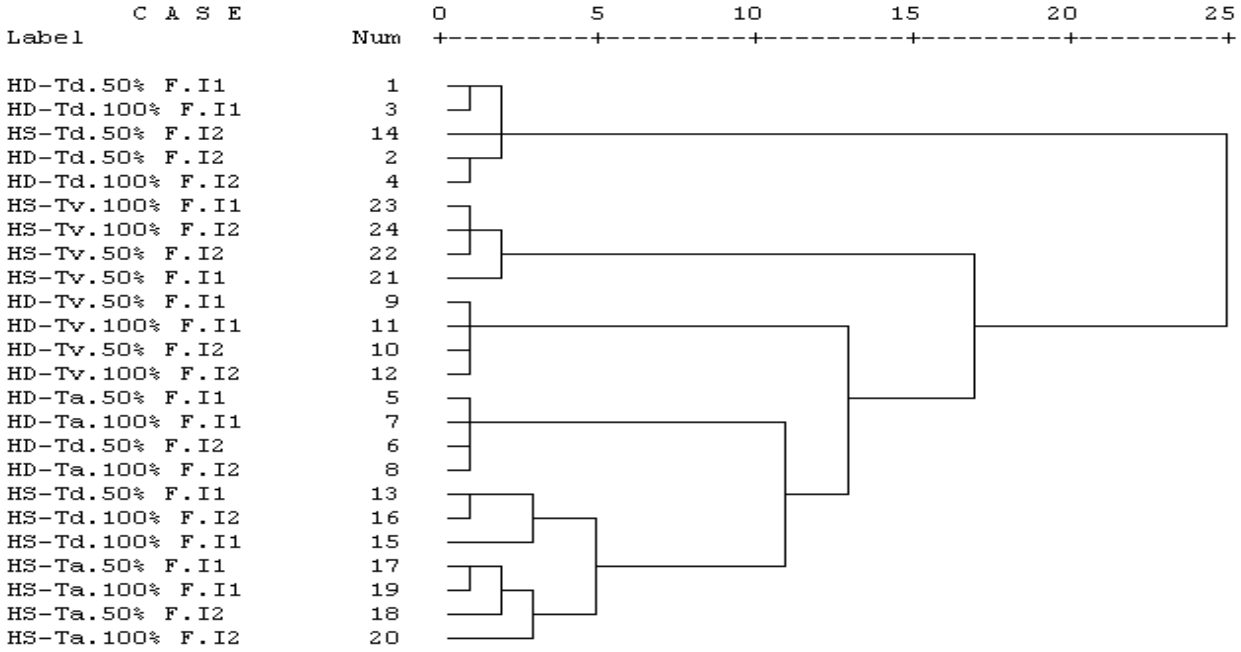

Fig. 3 Dendrogram of Td (*T. daenensis*), Ta (*T. armeniacus*) and Tv (*T. vulgaris*) species based of main components such as α-Thujene, α-pinene, Myrcene, α-Terpinene, p-Cymene, γ-Terpinene, trans-Sabinenehydrate, Thymol, Carvacrol and β-Caryophyllene using Ward clustering method in 50% F (50% flowering) and 100% F (100% flowering) stages at 90 (I1) and 50% (I2) FC (by HS: headspace and HD: hydrodistillation methods).

The third cluster was classified as *T. vulgaris* at the 50 and 100% flowering stages under control and drought stress conditions at the hydrodistillation method, characterized by high levels of p-cymene (18.05–24.50%) and γ-terpinene (18.60–23.66%), and moderate levels of thymol (20.22–22.83%). The fourth cluster included *T. armeniacus* plants rich in carvacrol (17.95–24.52%) at the 50 and 100% flowering stages under both control and drought stress conditions, with the highest carvacrol content using hydrodistillation method. The fifth cluster by headspace method represented *T. daenensis* with a high thymol content (54.34%) at the 50% flowering stage at control condition (I1) and 50 and 100% flowering under control and drought stress (I2) conditions. In the headspace method, sixth clusters were formed due to the its lower thymol and carvacrol content compared to the hydrodistillation method in *T. armeniacus* at the 50 and 100% flowering stage under control and stress conditions. Cluster analysis clearly differentiated.

Thymus species based on dominant volatiles and extraction method. *T. daenensis* consistently formed thymol-rich clusters, while *T. vulgaris* clustered by high γ-terpinene and p-cymene levels its grouping depended on extraction method (headspace vs. hydrodistillation). *T. armeniacus* grouped by carvacrol content, notably higher in hydrodistillation. Flowering stages and drought stress caused variations within clusters but didn't redefine them. Crucially, headspace method generally revealed lower thymol and carvacrol in *T. armeniacus* than hydrodistillation.

Principal component (PC) classification (Fig. 4) confirmed the findings of the cluster analysis (Fig. 3). The two principal components (PC1 and PC2) accounted for 73.93% of the overall variation (Fig. 4.A). *T. daenensis* at both 50 and 100% flowering stages, under control and drought stress conditions, along with *T. armeniacus* at the 50% flowering stage under both 90% and 50% FC and at the 100% flowering stage under control conditions, clustered together, characterized by myrcene, sabinene, α-thujene, and α-pinene. *T. vulgaris*, which exhibited the highest concentrations of p-cymene, γ-terpinene, and α-terpinene, formed a separate distinct group. Furthermore, under both drought stress and control conditions, *T. daenensis* at the 50 and 100% flowering stages and *T. armeniacus* at the 100% flowering stage grouped together by thymol, carvacrol, and β-caryophyllene.

PC analysis indicated that PC1 accounted for 46.93% of the variance, showing a negative association with thymol (-0.39), carvacrol (-0.38), and β-caryophyllene (-0.3), while it was positively linked to p-cymene (0.28) and γ-terpinene (0.31). PC2 contributed to 27% of the overall variance and had positive correlations with the concentrations of myrcene, sabinene, α-pinene, and α-thujene. In a separate PC analysis (Fig. 4.B), PC1 and PC2 together explained 90.14% of the data's total variation. PC1 alone accounted for 51.98% of the variance, positively correlating with thymol (0.14), p-cymene (0.26), and γ-terpinene (0.37). PC2 explained 27.4% of the total variability, showing positive correlations with carvacrol (0.19), p-cymene (0.36), and γ-terpinene (0.26), but negative correlations with thymol (-0.47) and β-caryophyllene (-0.49). *T. daenensis* formed a distinct group, characterized by elevated thymol levels at both the 50% and 100% flowering stages under both control and drought stress conditions.

T. vulgaris, noted for the highest p-cymene and γ-terpinene levels, and *T. armeniacus*, with the highest carvacrol content, each formed separate groups across both phenological stages under control and drought stress conditions.

The second cluster included *T. vulgaris* under both control (I1) and drought stress (I2) conditions at the 50 and 100% flowering stages using headspace method, characterized by high levels of γ-terpinene (29.30–34.67%) and p-cymene (22.17–38.53%).

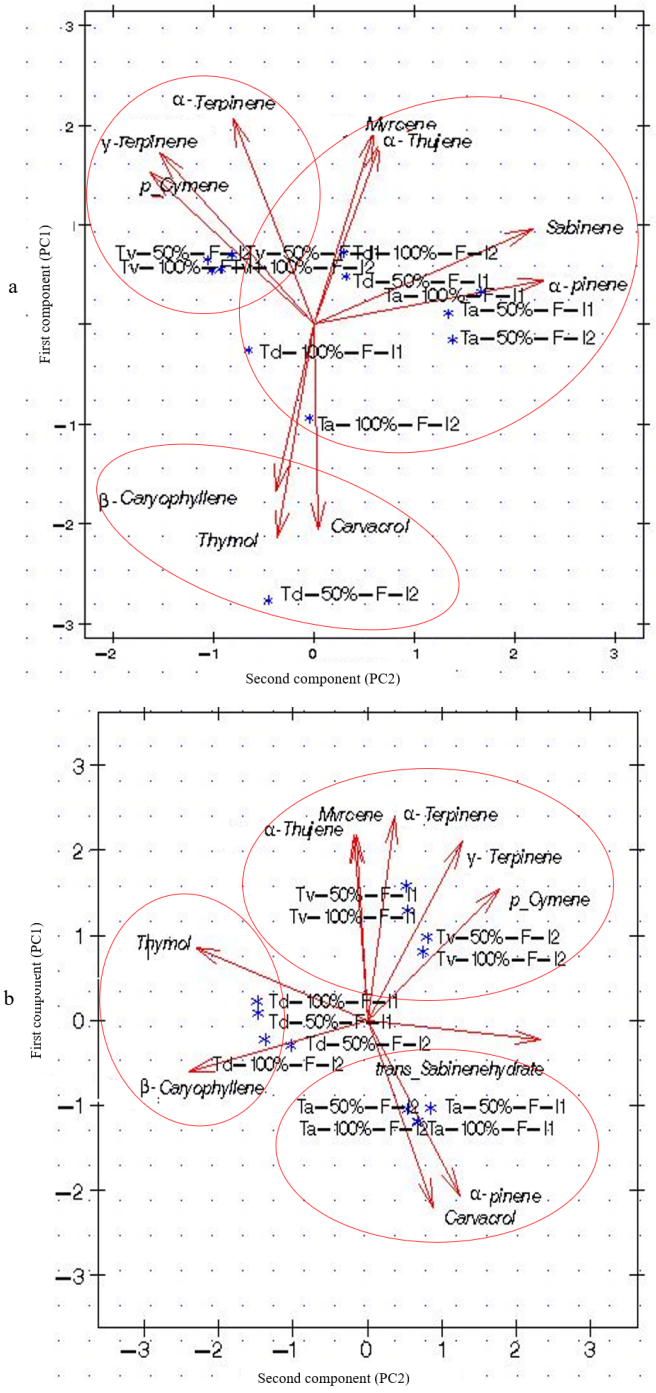

Fig. 4 Biplot of principal component (PC) analysis of essential oil constituents in aerial parts such as α-Thujene, α-pinene, Myrcene, α-Terpinene, p-Cymene, γ-Terpinene, trans-Sabinenehydrate, Thymol, Carvacrol and β-Caryophyllene of *T. daenensis*, *T. vulgaris* and *T. armeniacus* species under well-watered (I1) and drought stress (I2) conditions in the development stages of 50% and 100% flowering (a: headspace and b: hydrodistillation).

CONCLUSION

Timing the harvest is a crucial element that significantly impacts both the quantity and quality of essential oils derived from plants, as it has a direct effect on plant growth and production. Consequently, investigating the effects of harvest timing and identifying the best period to maximize biomass and achieve the desired plant composition are vital objectives in the cultivation of medicinal plants. The findings indicated that the headspace method is advantageous in terms of energy efficiency, extraction duration, and the amount of plant material required. While both methods identified similar primary components, their quantities differed. The study highlights that the essential oil compositions of different *Thymus* species are greatly affected by species type, phenological stage, and moisture conditions. Hydrodistillation method showed a higher thymol content (51.53%) in *T.* daenensis and carvacrol (24.52%) in *T. armeniacus*.

Genetic variations among species have resulted in differing responses to environmental conditions, emphasizing the importance of determining optimal harvesting times based on specific regions and species. Notably, the synthesis pathways of thymol and carvacrol were closely related, with species high in thymol showing lower γ-terpinene levels. The hydrodistillation method consistently detected key compounds across growth stages more effectively than the headspace method, which had limitations in quantifying bioactive components like thymol. These results underscore the significance of choosing the appropriate extraction method and considering environmental factors to optimize essential oil quality for medicinal and agricultural uses. Further research is necessary to resolve these conflicting results and improve cultivation strategies under different climatic conditions. Utilizing both headspace and hydrodistillation methods provided valuable insights into how environmental stress affects essential oil composition. Both hydrodistillation and headspace methods demonstrated that drought stress significantly changes the volatile composition of *Thymus* species, potentially affecting their medicinal and aromatic properties. The combination of these techniques offers a robust framework for understanding the adaptive mechanisms of *Thymus* species under varying environmental conditions and their potential applications in agriculture, pharmacology, and the essential oil industry. This study provides a comparative quantitative evaluation under drought stress, which has not been simultaneously reported for three *Thymus* species.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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